

**Potential valorization of olive oil pomace:
extraction, encapsulation, particles
characterization, *in vitro* release and oxidative
stability studies**

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“It always seems impossible until it’s done”
Nelson Mandela

*I dedicate this thesis to my parents Orlanda Teixeira and Antero Paulo,
and my godmother Patricia Ramalho*

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"If you want to go fast go alone, if you want to go far go together"
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Preface

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Abstract

In this work, a potential valorization strategy of olive mill pomace was investigated – an agricultural waste considered to be phytotoxic but owning bioactive compounds with proven benefits on human health. Olive mill pomace is an exceptional source of phenethyl alcohols, namely hydroxytyrosol and tyrosol, which bewitch remarkable antioxidant activity.

This thesis aimed to investigate the protection of phenolic compounds present in the olive mill pomace extract through their encapsulation. Additionally, it was intended to use the obtained microparticles for *in vitro* gastrointestinal release studies as well as their incorporation in oxidative sensitive food matrices. For this purpose, commercial olive oils were selected as food matrices selected for the fortification studies.

In the first stage of the project, the water-in-oil-in-water double emulsion solvent evaporation technique was exploited to encapsulate standards of hydroxytyrosol and tyrosol. During this study, the influence of the theoretical loading content on the physicochemical characteristics of loaded microparticles was assessed. To evaluate the release behavior of these bioactive compounds during the *in vitro* assays simulating the gastrointestinal digestion conditions, an *in vitro* gastrointestinal model was employed.

Afterward, it was performed the extraction of phenolic compounds present in olive mill pomace, through a two-step solid-liquid extraction procedure considering a central composite rotatable response surface design. It was evaluated the influence of the process variables on the total phenolic content of olive mill pomace extract was examined. The optimal extraction conditions were obtained using the Derringer's desired function methodology. Subsequently, the obtained extract was encapsulated, applying the encapsulation technique used to encapsulate hydroxytyrosol and tyrosol, being assessed the effect of the loading content in phenolic compounds on the main physicochemical characteristics of loaded microparticles. Lastly, were conducted *in vitro* studies of: gastrointestinal release and bioaccessibility of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol.

Furthermore, the oxidative stability of three commercial types of olive oils - extra-virgin olive oil, virgin olive oil, and a blend of virgin and refined olive oil - in the presence of olive mill pomace extract loaded ethylcellulose microparticles was appraised.

The phenolic compounds were efficiently extracted from an olive mill pomace sample through a two-step solid-liquid extraction procedure. The application of the Derringer's desirability function granted the optimization of the procedure: a duration of 3.2 hours for the primary extraction, solvent-to-sample ratio during the primary extraction of 10.0 mL/g, and 1.3 hours for the secondary extraction and a solvent-to-sample ratio during the secondary extraction of 3.0 mL/g. The obtained extract under optimized extraction conditions exhibited a total phenolic content of $50.5 \pm 1.5 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$, a radical scavenging activity of $79.1 \pm 7.9\%$, a content of hydroxytyrosol and tyrosol of $2870.2 \pm 1.2 \text{ mg}/\text{kg}_{\text{OMP}}$, and $1622.1 \pm 2.2 \text{ mg}/\text{kg}_{\text{OMP}}$, respectively.

The selected encapsulation technique was efficiently applied to encapsulate hydroxytyrosol, tyrosol, and an olive mill pomace extract. The encapsulation efficiencies varied from $83.7 \pm 2.0\%$ in the case of antioxidants present in the encapsulated olive mill pomace extract, considering a theoretical loading content in phenolic compounds of 10% w/w and $99.3 \pm 0.5\%$ for tyrosol encapsulated solely considering a theoretical loading content in tyrosol of 5% w/w. The yield of production ranged between $71.2 \pm 3.7\%$ in the case of microparticles containing hydroxytyrosol and $85.0 \pm 1.7\%$ for microparticles loaded with tyrosol. The obtained mean particle sizes ranged from $304.0 \pm 16.0 \text{ }\mu\text{m}$ in the case of hydroxytyrosol-loaded ethylcellulose microparticles formulated considering a theoretical loading content of 10% w/w and $2.3 \pm 0.1 \text{ }\mu\text{m}$ in the case of microparticles loaded with 5% w/w of tyrosol.

The data from the *in vitro* release gastrointestinal studies indicate that selected compounds in the present work were efficiently protected during the salivary and gastric digestions. The highest amount of bioactive compounds released was observed in the intestinal tract.

During olive oil samples fortification, it was verified efficient retardation of the peroxidation processes as olive oils enriched with microparticles exhibited lower peroxide values than plain olive oils and even enriched olive oils with synthetic antioxidants. Moreover, loaded microparticles efficiently lowered the content of secondary oxidation products in the tested olive oil samples. It was also verified that the incorporation of only olive mill pomace extract and even microparticles containing olive mill pomace extract effectively slowed down hydrolysis processes.

In summary, this work brings new insights regarding the obtainment of phenolic compounds-rich extracts from olive mill pomace samples. These can be valorized through encapsulation processes to be subsequently incorporated in food oxidative sensitive matrices as olive oils. This work demonstrated that ethylcellulose microparticles loaded with olive mill pomace extracts can be efficiently designed as gastrointestinal delivery systems and used to fortify food matrices, specifically, lipophilic food matrices sensitive to oxidation.

Resumo

Neste trabalho foi investigada uma potencial estratégia de valorização do bagaço do azeite – um resíduo agrícola considerado fitotóxico mas que possui compostos bioativos com comprovados benefícios para a saúde humana. O bagaço do azeite é uma fonte excepcional de compostos fenólicos nomeadamente o hidroxitirosol e o tirosol que possuem uma notável atividade antioxidante.

Esta tese teve como objetivo, investigar a proteção dos compostos fenólicos presentes no extrato de bagaço do azeite, através da sua encapsulação. Adicionalmente, pretendeu-se utilizar as micropartículas obtidas para estudos *in vitro* de libertação gastrointestinal, bem como a sua incorporação em matrizes alimentares sensíveis à oxidação. Para tal, nos estudos de fortificação foram escolhidos azeites comerciais como matrizes alimentares.

Numa primeira fase do projeto, a técnica de dupla emulsão água-em-óleo-em-água associada à evaporação do solvente foi explorada para encapsular padrões de hidroxitirosol e o tirosol. Durante este estudo, foi avaliada a influência do carregamento teórico nas propriedades físico-químicas das micropartículas carregadas. Para avaliar o comportamento de libertação destes compostos bioativos durante os ensaios *in vitro* de simulação das condições digestão gastrointestinal, foi utilizado um modelo gastrointestinal *in vitro*.

Posteriormente, procedeu-se à realização de extração dos compostos fenólicos existentes no bagaço do azeite, através de um procedimento de extração sólido-líquido em duas etapas, considerando um desenho de superfície de resposta rotativo do composto central.

Foi avaliada a influência das variáveis de processo no teor fenólico total do extrato do bagaço do azeite. As condições ótimas de extração foram obtidas utilizando a metodologia de função desejada de Derringer. Posteriormente, o extrato obtido foi encapsulado aplicando a técnica utilizada para encapsular o hidroxitirosol e o tirosol, tendo sido avaliado o efeito do carregamento em compostos fenólicos nas principais características físico-químicas das micropartículas carregadas. Por fim, foram ainda conduzidos estudos *in vitro* de: libertação gastrointestinal, de bio acessibilidade de

antioxidantes, de compostos fenólicos, do hidroxitirosol e do tirosol.

Foi ainda avaliada a estabilidade oxidativa de três tipos comerciais de azeites – azeite virgem-extra, azeite virgem e uma mistura de azeites virgem e refinados – na presença de micropartículas de etilcelulose contendo extrato do bagaço do azeite.

Os compostos fenólicos foram eficientemente extraídos de uma amostra de bagaço do azeite através de um procedimento de extração sólido-líquido em duas etapas. A aplicação da função desejada de Derringer permitiu a otimização do procedimento: duração de 3,2 horas para a extração primária, razão solvente-amostra de 10,0 mL/g durante a extração primária; 1,3 horas para a extração secundária e uma razão solvente-amostra durante a extração secundária de 3,0 mL/g. O extrato obtido sob condições de extração otimizadas exibiu um teor fenólico total de $50,5 \pm 1,5 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$, uma atividade de eliminação de radicais de $79,1 \pm 7,9\%$ e teores de hidroxitirosol e tirosol de $2\,870,2 \pm 1,2 \text{ mg}/\text{kg}_{\text{OMP}}$ e $1\,622,1 \pm 2,2 \text{ mg}/\text{kg}_{\text{OMP}}$, respectivamente.

A técnica de encapsulação selecionada foi eficientemente aplicada à encapsulação do hidroxitirosol, do tirosol e do extrato do bagaço do azeite. As eficiências de encapsulação variaram entre $83,7 \pm 2,0\%$ no caso dos antioxidantes presentes no extrato encapsulado considerando um carregamento teórico em compostos fenólicos de 10% m/m, e $99,3 \pm 0,5\%$ para o tirosol encapsulado individualmente, considerando um carregamento teórico de 5% m/m. O rendimento de produção variou entre $71,2 \pm 3,7\%$ no caso de micropartículas contendo hidroxitirosol e $85,0 \pm 1,7\%$ para as micropartículas carregadas com tirosol. Os tamanhos médios de partícula obtidos variaram entre $304,0 \pm 16,0 \text{ }\mu\text{m}$ no caso de micropartículas de etilcelulose carregadas com hidroxitirosol formuladas considerando um carregamento teórico de 10% m/m e $2,3 \pm 0,1 \text{ }\mu\text{m}$ no caso de micropartículas carregadas com 5% m/m de tirosol.

Os dados dos estudos *in vitro* de libertação gastrointestinal indicam que os compostos selecionados no presente trabalho, foram eficientemente protegidos durante as digestões salivares e gástricas. A maior quantidade de compostos bioativos foi libertada no trato intestinal simulado.

Durante os estudos de fortificação das amostras de azeites, verificou-se uma eficiente retardação dos processos de peroxidação, uma vez que os azeites enriquecidos

com micropartículas exibiram valores de peróxido mais baixos do que os azeites não enriquecidos, bem como em relação e aos azeites enriquecidos com antioxidantes sintéticos. Acresce que, as micropartículas carregadas reduziram de forma eficiente o teor de produtos de oxidação secundária nas amostras de azeite testadas. Verificou-se ainda, que a incorporação em azeites só do extrato do bagaço do azeite, e inclusive de micropartículas contendo extrato do bagaço do azeite, abrandou eficientemente os processos de hidrólise.

Em suma, este trabalho traz novas perspetivas relativamente à obtenção de extratos ricos em compostos fenólicos a partir de amostras de bagaço do azeite. Estas, podem ser valorizadas através de processos de encapsulação para serem posteriormente incorporadas em matrizes alimentares sensíveis à oxidação, como os azeites comerciais. Este estudo demonstrou que micropartículas de etilcellulose carregadas com extratos de bagaço de azeite, podem ser eficientemente desenhadas como sistemas de entrega gastrointestinal e utilizadas para fortificar matrizes alimentares, mais concretamente matrizes lipofílicas sensíveis à oxidação.

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Nomenclature

Abbreviations

2FI	Two-Factor Interaction
2-PhP	Two-Phase Process
3,4-DHPEA	3,4-dihydroxyphenolethanol
3,4-DHPEA-EA	Oleuropein aglycon mono-aldehyde
3,4-DHPEA-EDA	Oleuropein aglycon di-aldehyde
3D	Three Dimensional
3-PhP	Three-Phase Process
AA	Antioxidant Activity
Abs	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ACD	Advanced Chemistry Development
ALC	Actual Loading Content; Actual Loading Content of hydroxytyrosol
ALC _{AO}	Actual Loading Content of Antioxidants
ALC _H	Actual Loadig Content of Hydroxytyrosol
ALC _{PC}	Actual Loadig Content of Phenolic Compounds
ALC _T	Actual Loadig Content of Tyrosol
BA	Bioaccessibility
BA _i	Bioaccessibility of the bioactive compound(s) i
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
AO	Antioxidant
ATR	Attenuated Total Reflectance
AU	Arbitrary Units
BAC	Bioactive Compound
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
BOD	Biological Oxygen Demand
BP	Boiling Point
BPC	Bonded Phenolic Compounds
CAS	Chemical Abstracts Service
CCD	Central Composite Design
CCRD	Central Composite Rotatable Response Surface Design
COD	Chemical Oxygen Demand
Conc.	Concentration
COST	European Cooperation in Science and Technology
CV	Coefficient of Variance
D1E	Duration of the Primary Emulsification

D2E	Duration of the Secondary Emulsification
DAD	Diode Array Detector
DCM	Dichloromethane
DEP	Duration of the Evaporation Process
DF	Degrees of Freedom
DFDP	Duration of Freeze-Drying Process
DNA	Deoxyribonucleic Acid
DoE	Design of Experiments
DOPET	3,4-Dihydroxyphenylethanol
DP	Dried Powder
DPPH	Radical 2,2-diphenyl-2-picrylhydrazyl
DS	Dissolution Medium
DSC	Dynamic Scanning Calorimetry
DTG	Derivative Thermogravimetry
dw	dry weight
%E	Percentage of Error
EC	Ethyl Cellulose
EE	Encapsulation Efficiency
EE _{AO}	Encapsulation Efficiency of Antioxidants
EE _{HT}	Encapsulation Efficiency of Hydroxytyrosol
EE _i	Encapsulation Efficiency of bioactive compound(s) i
EE _{PC}	Phenolic Compounds Encapsulation Efficiency of Phenolic Compounds
EE _T	Encapsulation Efficiency of Tyrosol (% w/w)
EFSA	European Food Safety Authority
EMA	European Medicine Agency
ES	Extraction Solvent
ESI	Electrospray Ionization
EU	European Union
EVOO	Extra Virgin Olive Oil
EXT	Extract
F-C	Folin–Ciocalteu
FDA	Food and Drug Administration
FFA	Free Fatty Acids
FL	Fluorescence
For.	Formulation
FPA	Fingerprint Area
FPC	Free Phenolic Compounds
FR	Fat Removal
FRAP	Ferric Reducing Antioxidant Potential
FTIR	Fourier Transform Infrared Spectroscopy

GAE	Gallic Acid Equivalents
GC	Gas-Chromatography
GI	Gastrointestinal
GID	Gastrointestinal Digestion
GRAS	Generally recognized as safe
HEE	Hydroxytyrosol Encapsulation Efficiency
HPLC	High-Performance Liquid Chromatography
HPLES	High-Performance Liquid Emulsification Speed
HS	Hydrolyzed Extracted Sample
HT	Hydroxytyrosol
HV	High Value
HXT	Hydroxytyrosol
I%	Inhibitory Percentage of DPPH•
K _{ow}	Octanol-water partition coefficient
KP	Korsmeyer-Peppas
LC	Liquid Chromatography
LDL	Low-density lipoproteins
Lio-OOWW	Lyophilizate of Olive Oil Wastewater
LLE	Liquid-liquid Extraction
L-MP	Loaded-Microparticles
LOD	Limit of Detection
LOQ	Limit of Quantification
LV	Low Value
MC	Moisture Content
MS	Mass Spectrometry
MUFA	Monounsaturated Free Fatty Acids
MW	Molecular Weight
N.F.	Not Found
NE-1	Non-Encapsulated pattern of F-1 formulation
NE-2	Non-Encapsulated pattern of F-1 formulation
NHS	Non-Hydrolyzed extracted Sample
NP	Nanoparticle
NS	Not Significant
o	Organic Phase
o/w/o	Oil-in-water-in-oil
OFAT	One-factor-at-time
OLE	Olive Leaf Extract
OMP	Olive Mill Pomace
OMPE	Olive Mill Pomace Extract
OMW	Olive Mill Waste
OMWW	Olive Mill Wastewater

OO	Olive Oil
OOBP	Olive Oil By-product
OOP	Olive Oil Pomace
OOR	Olive Oil Residue
OOS	Olive Oil Sample
OOW	Olive Oil Waste
OOWW	Olive Oil Wastewater
ORAC	Oxygen Radical Absorbance Capacity
OV	Observed Value
OVAT	One-variable-at-time
OWR	Oil-to-water ratio
P1E	Primary Extraction
P2E	Secondary Extraction
<i>p</i> -AV	<i>para</i> -anisidine Value
PC	Phenolic Compound; Percentage Contribution
PCL	Polycaprolactone
PDI	Polydispersity Index
<i>p</i> -HPEA-EDA	Ligstroside aglycone di-aldehyde
PI	Predicted Interval
PLGA	poly(D,L-lactide- <i>co</i> -glycolide)
PMA	Phorbol 12-myristate 13-acetate
PO	Polymer Only
PP	Polypropylene
PRESS	Predicted Residual Error Sum of Squares
PUFA	Polyunsaturated Fatty Acid
PV	Predicted Value; Peroxide Value
PVA	Polyvinyl Alcohol
PY	Product Yield
R%	Recovery Percentage
RE	Residual Error
Ref.	Reference
ROO	Blend of Virgin and Refined Olive Oils
ROS	Reactive Oxygen Species
RP	Reverse Phase
rpm	Rotations per minute
RRLC	Rapid Resolution Liquid Chromatography
RSA	Radical Scavenging Ability
RSM	Response Surface Methodology
S	Significant
SC	Swelling Capacity
SEM	Scanning Light Microscopy

SEPC	Sample containing extracted phenolic compounds
SEPC-FR	Sample containing extracted phenolic compounds after the fat removal
SEPC-P1E	Sample containing extracted phenolic compounds after the primary extraction
SEPC-P2E	Sample containing extracted phenolic compounds after the secondary extraction
SGD	Simulated Gastric Digestion
SGDF	Simulated Gastric Digestion Fluid
SGF	Simulated Gastric Fluid
SID	Simulated Intestinal Digestion
SIDF	Simulated Intestinal Digestion Fluid
SIF	Simulated Intestinal Fluid
SOD	Superoxide Dismutase
SPAN	Polydispersity of particle size distributions
SSD	Simulated Salivary Digestion
SSDF	Simulated Salivary Digestion Fluid
SSF	Simulated Salivary Fluid
SSR	Solvent-to-sample ratio
SSSE	Stirring Speed during Solvent Evaporation
ST	Storage Time
Std. Dev.	Standard Deviation
TAA	Total Antioxidant Activity
TAC	Total non-enzymatic Antioxidant Capacity
TALC	Tyrosol Actual Content
TAPC	Total Amount of Phenolic Compounds
TBA	Tyrosol Bioaccessibility
TBHQ	<i>tert</i> -butyl hydroquinone
TE	Trolox Equivalents
TEE	Tyrosol Encapsulation Efficiency
TLC	Theoretical Loading Content
TLC-1	Theoretical Loading Content of Formulation F-1
TLC-2	Theoretical Loading Content of Formulation F-2
TOF	Time-of-flight
TOTOX	Total Oxidation
TPC	Total Phenolic Content
TPOMW	Two-Phase Olive Mill Waste
TPOOW	Two-Phase Olive Oil Waste
TPY	Tyrosol Product Yield
TYP	Tyrosol Product Yield
TYR	Tyrosol

UNESCO	United Nations Educational, Scientific, and Cultural Organization
UPLC	Ultra Performance Liquid Chromatography
UPW	Ultra Pure Water
UV-Vis	Ultraviolet-Visible
Vis	Visible
Vol.	Volume
VOO	Virgin Olive Oil
VP	Vapour Pressure
w/o/w	Water-in-oil-in-water
w ₁	Internal Aqueous Phase
w ₁ /o	Dispersed Phase; Primary Emulsion
w ₁ /o/w ₂	Water-in-oil-in-water
w ₂	External Aqueous Phase
WAI	Water Absorption Index
WET	Wettability
WF	Weight Factor
WS	Water Solubility
WSI	Water Solubility Index
YP	Yield of Production

Notations

2K	Number of axial points on the axis of each design factor (design of experiments)
2 ^K	Number of factorial points in the design (design of experiments)
A	Area
a	Slope
A ₀	Absorbance of the control (DPPH radical in methanol) (AU)
A ₁	Absorbance of the DPPH radical plus tested sample in methanol (AU)
Abs	Absorbance (AU)
C	Concentration (e.g., mg/L, g/L, mg/kg)
C ₀	Concentration in Trolox Equivalent of the sample well of the hydroxytyrosol standard solution encapsulated (nmol/μL)
C _{Di}	Concentration of bioactive compound(s) <i>i</i> in the <i>digestas</i>
C _p	Concentration of tyrosol in a second supernatant in Trolox Equivalents (nmol/μL); Number of replicates at the center point (design of experiments)
C _s	Concentration in Trolox Equivalents of the unknown sample well containing a standard of hydroxytyrosol (nmol/μL); Equivalent

	concentration of tyrosol present in the supernatant in Trolox Equivalents (nmol/ μ L)
C_{TD}	Concentration of tyrosol in the <i>digestas</i> (mg/L)
C_{Ti}	Theoretical concentration of bioactive compound <i>i</i> (mg/L)
C_{TT}	Theoretical concentration of tyrosol (mg/L)
d_i	Dimensionless individual desirability function of the variable <i>i</i>
$D_{v,10}$	Maximum particle diameter below which 10% of the sample volume exists (μ m)
$D_{v,50}$	Maximum particle diameter below which 50% of the sample volume exists (μ m)
$D_{v,90}$	Maximum particle diameter below which 90% of the sample volume exists (μ m)
DW_{part}	Initial dry weight of microparticles (mg)
DW_{sup}	Dry weight of microparticles' supernatant (mg)
EC_{50}	Concentration of sample necessary to decrease the initial DPPH radical concentration by 50% (ppm)
e_i	Error of the model (design of experiments)
f_1	Difference factor
f_2	Similarly factor
G	Overall desirability function
H_0	Null hypothesis
<i>i</i>	Bioactive compound(s); Variable under study (design of experiments)
IC_{50}	Half-inhibitory concentration (ppm)
K	Number of process variables (design of experiments);
K_0	Zero-order release of tyrosol constant (mg/min)
$K_{0,i}$	Zero-order release of bioactive compound(s) <i>i</i> constant (mg/min)
K_1	First-order release of tyrosol constant (min^{-1})
$K_{1,i}$	First-order release of bioactive compound(s) <i>i</i> constant (min^{-1})
K_{232}	Extinction coefficient to assess olive oil stability (AU)
K_{270}	Extinction coefficient to assess olive oil stability (AU)
K_H	Higuchi release of tyrosol constant ($\text{mg} \cdot \text{min}^{-0.5}$)
$K_{H,i}$	Higuchi release of bioactive compound(s) constant ($\text{mg} \cdot \text{min}^{-0.5}$)
K_K	Korsmeyer constant related to tyrosol release (min^{-n}) (Korsmeyer-Peppas Model)
$K_{K,i}$	Korsmeyer constant related to bioactive compound(s) <i>i</i> release (min^{-n}) (Korsmeyer-Peppas Model)
m/z	Mass-charge ratio
m_a	Mass of tyrosol to be analyzed (mg)
$m_{a,i}$	Mass of bioactive compound(s) <i>i</i> to be analyzed (mg)
m_f	Mass of tyrosol analyzed after fortification (mg)
$m_{f,i}$	Mass of bioactive compound(s) <i>i</i> analyzed after fortification (mg)

m_i	Mass weighted of tyrosol before fortification (mg)
$m_{i,i}$	Mass weighted of bioactive compound(s) i before fortification (mg)
m_{is}	Mass of the specific antioxidant i in the spiked sample (mg)
m_{ia}	Mass added of the antioxidant i to the sample (mg)
m_{ius}	Mass of the antioxidant i in the unspiked sample (mg)
M_t	Release of tyrosol (%) at time t (min.)
$M_{t,i}$	Release of bioactive compound(s) i (%) at time t (min.)
M_∞	Release of tyrosol (%) at infinite time
$M_{\infty,i}$	Release of bioactive compound(s) i (%) at infinite time
n	Release exponent related to the release of tyrosol (Korsmeyer-Peppas model); number of time points (similarly factor)
n_i	Release exponent related to the release of tyrosol (Korsmeyer-Peppas model)
p	Probability
PW	Weight of microparticles' pellet after centrifugation
Q_0	Initial amount of tyrosol (mg)
$Q_{0,i}$	Initial amount of bioactive compound i (mg)
Q_t	Cumulative release of tyrosol at time t
Q_t/Q_∞	Fraction of tyrosol released until time t
$Q_{t,i}/Q_{\infty,i}$	Fraction of bioactive compound(s) released until time t
R	Correlation coefficient
R^2	Determination coefficient
R_a^2	Adjusted determination coefficient
R_p^2	Predicted determination coefficient
R_t	Release value of the reference (pre-change batch) at time t (similarly factor)
s_a	Error associated to the slope
t	Time (min., hr., s)
t_0	Lag-time of the release of tyrosol (min.) (Weibull Model)
$t_{0,i}$	Lag-time of the release of bioactive compound(s) i (min.) (Weibull model)
T_c	Critical Temperature (°C)
T_d	Degradation Temperature (°C)
T_{DH}	Dehydration temperature (°C)
T_g	Transition temperature (°C)
T_s	Concentration in Trolox Equivalents in the supernatant (nmol/μL)
T_t	Concentration in Trolox Equivalents in the pellet (nmol/μL); Release value of the test (post-change batch) at time t (similarly factor)
W	Weight of oil (g)

W_0	Weight of hydroxytyrosol present in the w/o/w double emulsion after 3 hours of microparticles hardening (mg); Weight of tyrosol non-encapsulated (mg)
$W_{0,i}$	Weight of non-encapsulated bioactive compound(s) i (mg)
W_a	Sum of the weight of hydroxytyrosol initially added for the microencapsulation and Weight of the polymer used for the microencapsulation (mg); Sum of the weight of tyrosol initially added for the microencapsulation and Weight of the polymer used for the microencapsulation (mg)
W_{AO}	Weight of antioxidants in microparticles (mg)
W_{AO0}	Weight of antioxidants present in the w_2 after 3 hours of microparticles hardening (mg)
W_{AOi}	Weight of antioxidants initially added for microencapsulation (mg)
W_H	Weight of hydroxytyrosol in microparticles (mg)
W_{H0}	Weight of hydroxytyrosol present in the w_2 after 3 hours of microparticles hardening (mg)
W_{Hi}	Weight of hydroxytyrosol initially added for microencapsulation (mg)
W_{PC}	Weight of phenolic compounds in microparticles (mg)
W_{PC}	Weight of phenolic compounds present in the w_2 after 3 hours of microparticles hardening (mg)
W_{PC}	Weight of phenolic compounds initially added for microencapsulation (mg)
W_T	Weight of tyrosol in microparticles (mg)
W_T	Weight of tyrosol present in the w_2 after 3 hours of microparticles hardening (mg)
W_T	Weight of tyrosol initially added for microencapsulation (mg)
W_H	Weight of hydroxytyrosol in the microparticles (mg)
W_i	Weight of hydroxytyrosol initially added for the microencapsulation (mg); Weight of tyrosol initially added (mg); weight of bioactive compound(s) i in the microparticles (mg)
$W_{l,i}$	Weight of bioactive compound(s) i initially added for microparticles formulation (mg)
W_m	Weight of microparticles recovered after the freeze-drying process (mg); Weight of tyrosol-loaded microparticles recovered after the freeze-drying process (mg)
$W_{p,i}$	Weight of bioactive compound(s) i adsorbed at the surface of microparticles
W_p	Weight of the polymer used for the microencapsulation (mg)
W_S	Weight of tyrosol present in the supernatant of 3 mL of the $w_1/o/w_2$ double emulsion after 3 hours of microparticles hardening (mg)

$W_{S,i}$	Weight of recovered supernatant of a sample $w_1/o/w_2$ emulsion after 3 hours of microparticles hardening
W_T	Weight of tyrosol in the microparticles (mg)
x_i	Dimensionless coded value of the independent variable i
X_i	Actual value of the independent variable i
X_z	Real value of the independent variable i at the center point
Y	Response of the model (dependente variable)

Greek symbols

α	Distance of axial points on the axis of each design factor (design of experiments)
α_i	Importance of the response Y_i
β	Parameter relative to the shape of the curve (Weibull Model)
β_0	Model intercept coefficient (design of experiments)
β_j	Interaction coefficients of the linear term (design of experiments)
β_{jj}	Interaction coefficients of quadratic terms (design of experiments)
β_{ji}	Interaction coefficients of second-order terms (design of experiments)
ΔX_i	Step change of the real value of the variable i
λ	Wavelength
$\lambda_{\max}$	Maximum wavelength
τ_d	Time when 63.2% of M has been released (min.) (Weibull Model)
τ_d^{exp}	Time when 63.2% of M has been released determined experimentally (min.) (Weibull Model)

Part I: Framework

In this section a general overview of the project is presented including the project relevance and the motivation background. Moreover, the main objectives of the project are defined. Lastly in Part I, the structure of this thesis is presented and explained, chapter by chapter.

Relevance and Motivation

Over the past few decades, it has been acknowledged the lower prevalence of certain diseases as the breast and the colon cancers and the coronary heart disease in the countries around the Mediterranean basin when compared with the northern European countries (Bosetti et al., 2003; Buckland et al., 2013; Couto et al., 2011; Dilis et al., 2012; Giacosa et al., 2013; Keys, 1995; Mente et al., 2009; Misirli et al., 2012; Panagiotakos et al., 2002; Pelucchi et al., 2011; Schwingshackl et al., 2015; Sofi et al., 2010; Trichopoulou et al., 1995; Verberne et al., 2010). Epidemiological studies have been shown that these observations are related to the safer and protective dietary patterns in the southern countries where olive oil is the primary source of fat (Keys, 1995; Lipworth et al., 1997; Schwingshackl et al., 2017; Trichopoulou et al., 1995).

The worldwide recognition of the health benefits regarding the consumption of olive oil has stimulated an intensive plantation of olive trees and production of olive oil, fostering the economic relevance of the olive oil sector, especially among the producing countries (Patumi et al., 2002; Stillitano et al., 2017). In this context, the olive oil production industry is recognized as one of the most significant agro-food business sectors, in the Mediterranean area (Bartolini et al., 1994; Cavalheiro et al., 2015; Foscolou et al., 2018; Nunes et al., 2016; Rossi, 2017; Servili et al., 2009; Vossen, 2007). Accordingly, olive oil is one of the most edible oils consumed worldwide which consumption volume has been rising steadily since 2005 (Cavalheiro et al., 2015; Dermeche et al., 2013; Lalas et al., 2011; Nunes et al., 2016).

The olive oil can be processed through one of the following extraction processes: (i) traditional press olive oil extraction, (ii) three-phase centrifugal olive oil extraction, or (iii) two-phase centrifugal olive oil extraction. Alternative extraction techniques to obtain olive oil from olives are reported in the literature, such as the continuous combined percolation-centrifugation process also known as the Sinoela process. The wastes generated during the processing of olive oil are designated as olive oil by-products (OOBPs) or olive mill wastes (OMWs). The traditional press and the three-phase olive oil extraction processes generate more liquid effluents commonly referred as olive oil wastewaters (OOWWs) or alternatively as olive mill wastewaters (OMWWs).

In the case of the two-phase centrifugal olive oil extraction large amounts of a semi-solid waste generally recognized as olive oil pomace (OOP) or olive mill pomace (OMP) (Artajo et al., 2007; Azbar et al., 2004; Dermeche et al., 2013; Dubos et al., 2011; Kapellakis et al., 2008; Nunes et al., 2016; Obied, 2013; Qdais & Alshraideh, 2016; Roig et al., 2006; Rubio-Senent et al., 2015; Sampedro et al., 2004). The main compounds found in each olive oil waste and their typical applications are schematically presented in Figure I.1. Additionally, a schematic representation of the most commonly employed olive oil extraction processes is presented in Figure I.2.

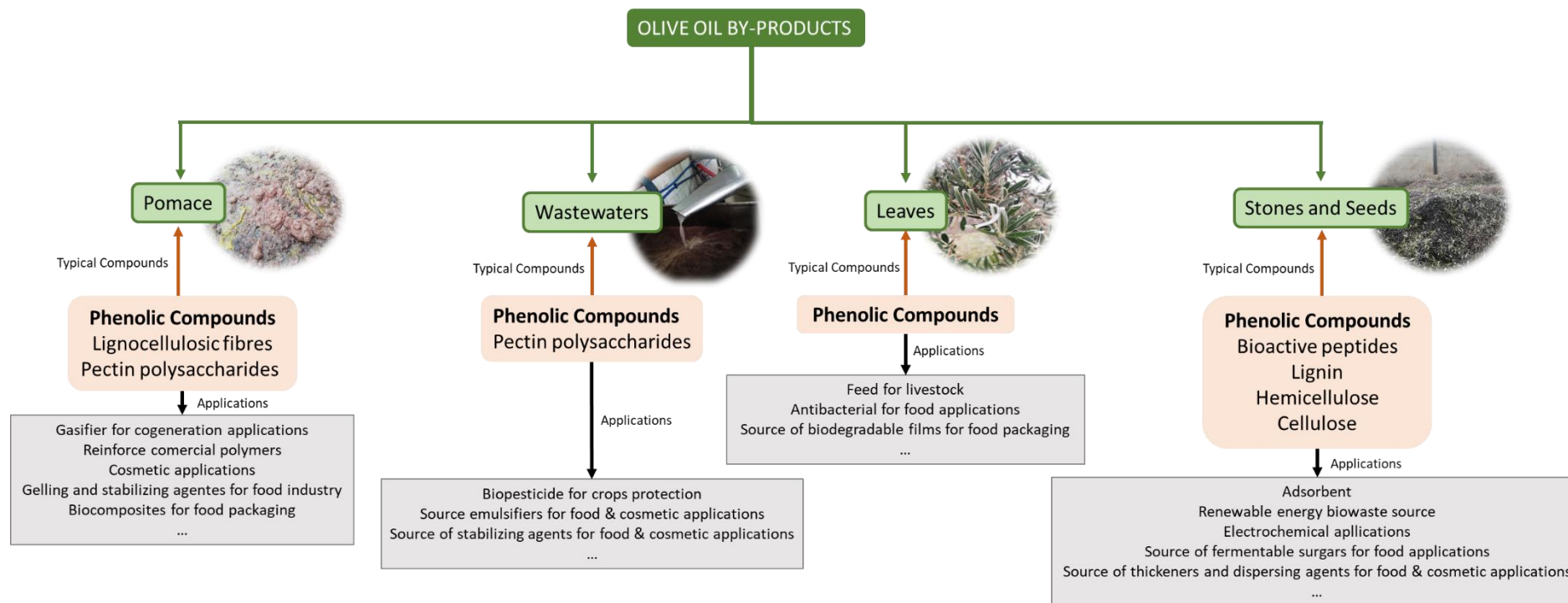


Figure I.1 - Main olive oil by-products, their principal bioactive compounds and their mostly common applications

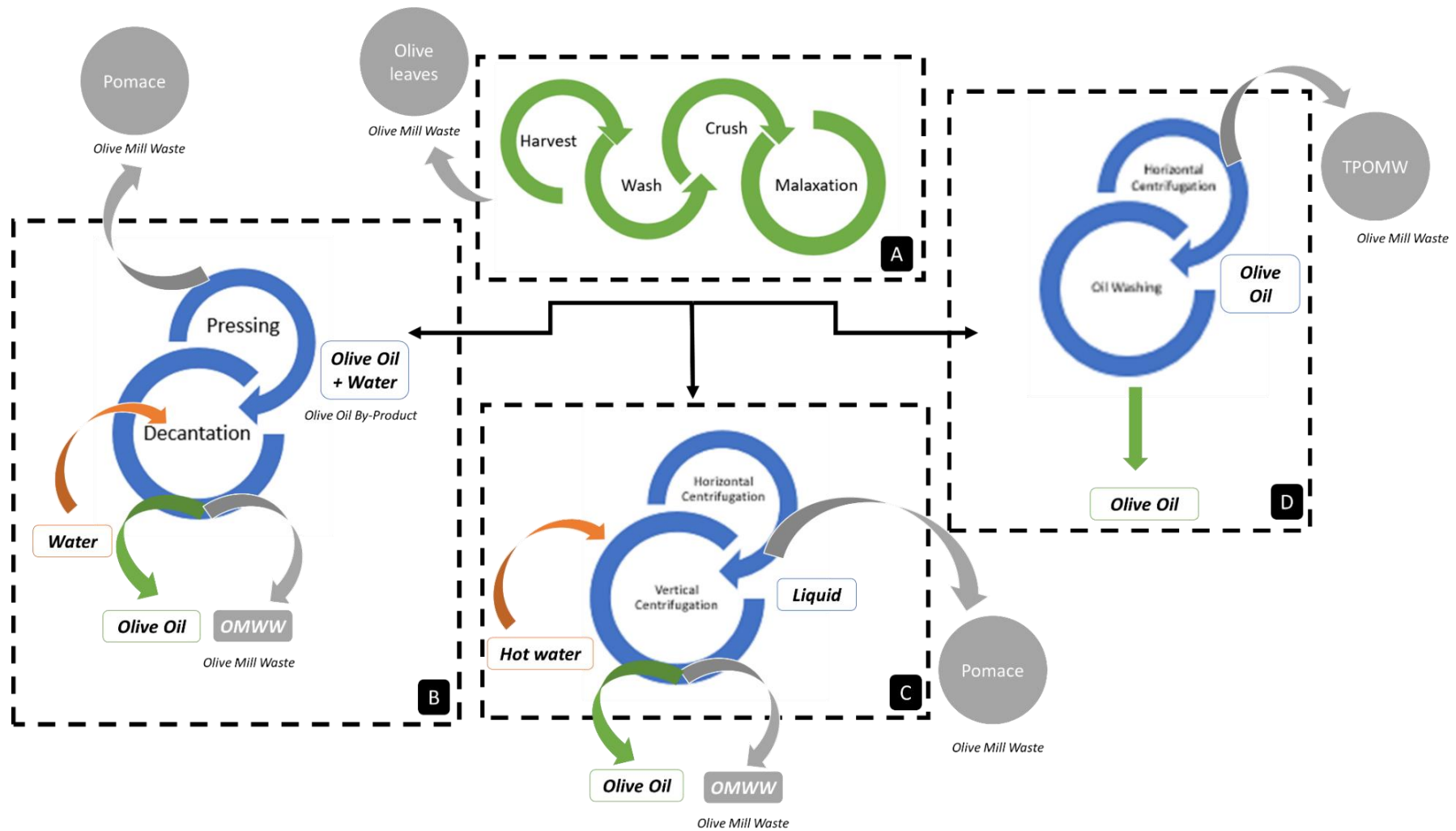


Figure I.2 - Main extraction processes of olive oil from olives (**A** – Pre-treatment; common to all processes; **B** – Discontinuous system; **C** - Three-phase system; **D** – Two-phase system; OMWW – Olive Mill Wastewater; TPOMW – Two-phase Olive Mill Waste)

The worldwide production and consumption of olive oil were fostered by the advent of the health benefits of olive oil consumption alongside with its unique organoleptic properties (Foscolou et al., 2018; Servili et al., 2009; Vossen, 2007).

Epidemiological studies support the assumption that the positive health benefits of the Mediterranean diet are related to the consumption of olive oil (Alonso et al., 2006; Assy et al., 2009; Buckland & Gonzalez, 2015; Estruch et al., 2006; Owen et al., 2004; Perona & Botham, 2013; Psaltopoulou et al., 2004; Ruiz-Canela & Martínez-González, 2011; Sotiroudis & Kyrtopoulos, 2008; Visioli & Galli, 2001).

The intake of olive oil is associated with the prevention of cardiovascular diseases, the lowering of blood pressure. Moreover, this oily matrix may act as a cancer therapy coadjuvant, which may reduce DNA and lipid oxidation. It also improves the lipid profile in blood and insulin-resistance. Additionally, it is described its positive effects on inflammation processes and as well as in endothelial dysfunction (Buckland & Gonzalez, 2015; D'Amore et al., 2016; López-Miranda et al., 2010; Owen et al., 2000; Pelucchi et al., 2011; Perona et al., 2006; Psaltopoulou et al., 2011; Violi et al., 2015). The consumption of olive oil as a key component of the Mediterranean diet is strongly correlated to the postponing of chronic diseases as well as in the reduction of morbidity and mortality rates (Glade, 1999; Nudler & Gottesman, 2002; Wagner & Brath, 2012).

The rising popularity of olive oil is mainly attributed to its content of phenolic compounds (PCs), which act as natural antioxidants and therefore, prevent many diseases (Dermeche et al., 2013).

Epidemiological studies have been demonstrated the correlation between the presence of reactive oxygen species (ROS) (e.g., the hydroxyl radical - $\text{HO}^\bullet$; the hydrogen peroxide - H_2O_2 ; the peroxynitrite (ONOO^-); the superoxide anion radical ($\text{O}_2^{\bullet -}$), the peroxy radical - $\text{ROO}^\bullet$; the singlet oxygen - $^1\text{O}_2$) and human pathophysiologies (Prasad et al., 2017; Volpe et al., 2018). The ROS are extraordinarily reactive, being responsible for biological damage in vital cell molecules (e.g., proteins, DNA, lipids, carbohydrates) (Ginter et al., 2014; Ighodaro & Akinloye, 2018).

The imbalance between the generation and the removal of ROS may cause homeostatic disruption, leading to cell damage and, consequently to the appearance of chronic diseases (Fang et al., 2015; Ighodaro & Akinloye, 2018; Lobo et al., 2010; Scandalios, 2005).

Generally, the physiological damages resulting from the excess of ROS are attenuated through the action of endogenous enzymatic and non-enzymatic action of antioxidants (Fridovich, 1997; Sies, 1993). In this context, typical natural antioxidants as olive PCs may protect against the development of oxidation-linked chronic diseases, especially in the cases of the imbalance between the generation and the removal of ROS or even in the cases of inefficacy of the endogenous antioxidant defense system.

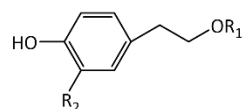
The health benefits of the Mediterranean diet are partially ascribed to the dietary consumption of olive oil; nevertheless, the olive oil processing industries generate a large amount of OOBPs (Mirabella et al., 2014; Naziri et al., 2014). For instance, it is reported that 1 m³ of OMWW is equivalent to 200 m³ of domestic sewage, and the phenolic content of OMWW is about 1000 times higher than in domestic sewage (El-Abbassi et al., 2012; Meullenet & Kennedy, 2005; Shilev & Naydenov, 2007).

Several studies have been demonstrated the adverse effect of OOBPs disposal on aquatic ecosystems, soil microbial populations and, air through sulfur dioxide emissions. Studies have been suggested that OOBPs are biotoxic and phytotoxic (Albahari et al., 2018; Cereti et al., 2004; DellaGreca et al., 2001; Ferri et al., 2011; Khoufi et al., 2007; Linares et al., 2003; Mekki et al., 2007, 2008; Meullenet & Kennedy, 2005; Morillo et al., 2009; Oller et al., 2011; Paredes et al., 1987; Rinaldi et al., 2003; Sammouda, 2009; Sampedro et al., 2009; Sierra et al., 2001; Young et al., 2019).

Olive oil PCs are considered the major contributors for the polluting load of OOBPs as the octanol/water partition coefficients of the majority of olive PCs range between 6.0×10^{-4} and 1.5 and thereby, they present more affinity to water systems rather than oily systems. Therefore, the majority of PCs - around 98% - are retained in OOBPs (approximately 53% in OOWWs and 45% in OOPs depending on the olive oil extraction process) (Dermeche et al., 2013; El-Abbassi et al., 2014). The main PCs found in OOBPs are presented in Figure I.3.

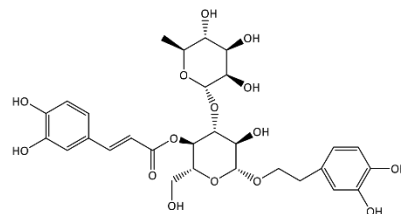
PHENOLIC COMPOUNDS FOUND IN OLIVE OILS & WASTES

Phenyl Alcohols

 $R_1=H$; $R_2=OH$ **Hydroxytyrosol** $R_1=H$; $R_2=H$ **Tyrosol** $R_1=glucose$; $R_2=OH$

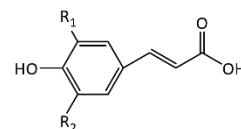
Hydroxytyrosol glucoside

Phenylethanoid glycoside



Verbascoside

Phenolic Acids/Aldehydes

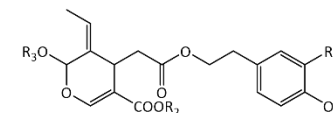
 $R_1=OH$; $R_2=H$

Caffeic acid

 $R_1=H$; $R_2=H$ *p*-Coumaric acid $R_1=H$; $R_2=OCH_3$

Ferrulic acid

Secoiridoids

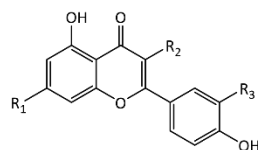
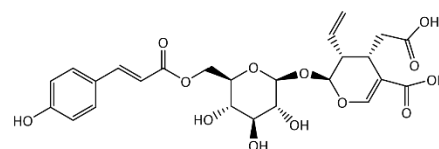
 $R_1=OH$; $R_2=CH_3$; $R_3=glucose$ **Oleuropein** $R_1=OH$; $R_2=CH_3$; $R_3=H$

3,2-DHPEA-EA

 $R_1=H$; $R_2=CH_3$; $R_3=glucose$

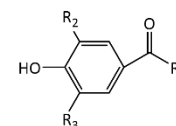
Ligstroside

Flavonoids

 $R_1=OH$; $R_2=O$ -rutinose; $R_3=OH$ **Rutin** $R_1=OH$; $R_2=H$; $R_3=OH$ **Luteolin** $R_1=OH$; $R_2=O$ -glucose; $R_3=OH$ Luteolin-7-*o*-glucoside $R_1=OH$; $R_2=H$; $R_3=H$ **Apigenin** $R_1=OH$; $R_2=O$ -glucose; $R_3=H$ Apigenin-7-*o*-glucoside*p*-Coumaric acid derivative

Comselogoside

Phenolic Acids/Aldehydes

 $R_1=OH$; $R_2=OH$; $R_3=OH$

Gallic acid

 $R_1=OH$; $R_2=OCH_3$; $R_3=H$

Vanillic acid

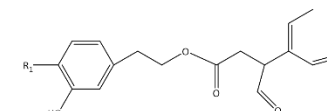
 $R_1=OH$; $R_2=OH$; $R_3=H$

Protocatechuic acid

 $R_1=H$; $R_2=OCH_3$; $R_3=H$

Vanillin

Secoiridoids

 $R_1=OH$

3,4-DHPEA-EDA

 $R_1=H$ *p*-HPEA-EDA

Figure I.3 - Main phenolic compounds found in olive oil by-products (3,4 – DHPEA-EA - oleuropein aglycone mono-aldehyde; 3,4-DHPEA-EDA – oleuropein aglycone di-aldehyde; *p*-HPEA-EDA – ligstroside aglycone di-aldehyde) (adapted from Araújo et al. (2015))

The most abundant PCs occurring in OOBPs are tyrosol (TYR) and, hydroxytyrosol (HXT) (Takaç & Karakaya, 2009). The schematic representation of TYR and HXT formation during olive oil production is presented in Figure I.4. The main physicochemical properties of HXT and TYR are presented in Table I.1.

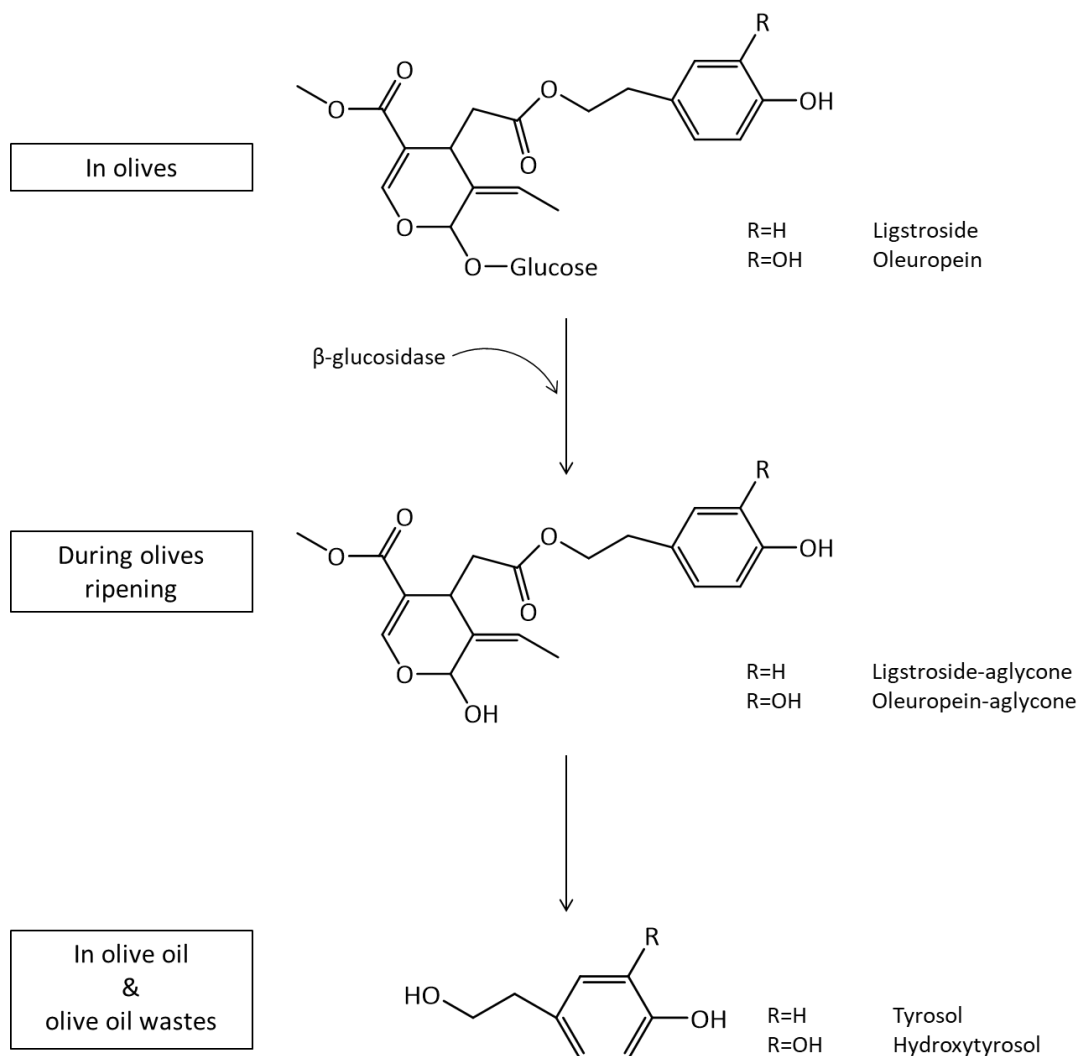
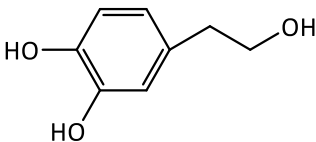
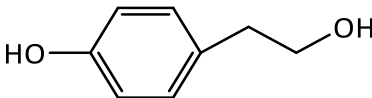


Figure I.4 – Chemical conversion of ligstroside and oleuropein into tyrosol and hydroxytyrosol during olive oil production (adapted from Visser et al. (2001))

Table I.1 – Physicochemical properties of the main phenolic compounds found in olive oil by-product (hydroxytyrosol and tyrosol)

Designation	<i>Hydroxytyrosol</i>	<i>Tyrosol</i>
Chemical Structure		
CAS ^a	10597-60-1	501-94-0
Molecular Formula	C ₈ H ₁₀ O ₃	C ₈ H ₁₀ O ₂
MW (g/mol) ^b	154.16	138.16
WS at 25 °C (g/L) ^b	271.6	123.8
BP (°C) ^b	355.4	287.8
VP (mmHg) ^b	5.7 x 10 ⁻⁶	1.37 x 10 ⁻⁴
Log K _{ow} ^b	0.61	1.09

^a Source: PubChem database (2020)^b Source: ChemSpider database (2020)

BP – Boiling Point; CAS – Chemical Abstracts Service; K_{ow} – Octanol-water partition coefficient; MW – Molecular Weight; VP – Vapor Pressure; WS – Water Solubility;

In the viewpoint of the problematic of disposal and waste management of OOBPs, many strategies have been exploited for the valorization of PCs. One alternative valorization strategy of OOBPs is the combination of (i) extraction of PCs from OOBPs and (ii) encapsulation of the obtained extracts to address the demands of added value products for food, cosmetic, pharmaceutical and nutraceutical industries (Paulo & Santos, 2017).

Recently, antioxidant-rich foods have been received considerable attention as they may prevent the oxidation of lipidic foods as well as they may deliver natural antioxidants to the human body, reinforcing the endogenous antioxidant defense system and re-establish the homeostasis between the generation and the removal of ROS on the human body (Lee et al., 2009). Therefore, PCs extracted from OOBPs, especially the PCs - HXT and TYR - are now recognized as unique bioactive compounds and singular templates for development of added-value food products.

Objectives

Considering the problematic regarding the disposing and management of agricultural wastes, the renewed interest in PCs, in natural products over the synthetic ones, and the demand of innovative added-value food products containing functional ingredients, microencapsulation and extraction arise as combined potential technological strategies to overpower the challenges concerning the management of agricultural wastes and to accomplish the goals regarding the obtainment of functional ingredients for added-value products aimed by both food researchers, producers and the general public.

There are available several alternative encapsulation techniques that allow the entrapment, protection, and release of PCs as the spray-drying, the freeze-cooling, and the spray-chilling techniques, emulsification procedures, coacervation, inclusion complexation, cocrystallization, fluidized-bed coating, extrusion among others (Ballesteros et al., 2017; Fang, 2010; Nori et al., 2011; Wilkowska et al., 2016; Buckland et al., 2013; Pang et al., 2014; Xu et al., 2010; Motilva et al., 2016; Rocha-Parra et al., 2016; Rosa et al., 2014; Mohammadi et al., 2016; Cilek et al., 2012; Paulo & Santos 2018a, 2018b, 2020b).

However, the majority of these techniques allow the entrapment of PCs in edible, water-soluble or hydratable polymeric matrices as maltodextrins, modified chitosan, corn syrup, dextran, modified starch, cyclodextrins, carboxymethylcellulose, gum acacia, agar, sodium alginate, gluten, casein, gelatin, albumin among others (Desai & Park, 2005). Nevertheless, the microparticles (MPs) coated with hydratable or water-soluble polymers present limited applicability. These MPs are not compatible with many food matrices as they are hydrophilic, contrasting with the majority of food matrices, which generally are from lipophilic nature. Therefore, hydratable or water-soluble MPs when embedded in food matrices do not allow PCs to be controlled released, not grating shield to other vital molecules. In the case of fatty-foods, the embedment of these MPs does not limit the oxidative processes during the storage these food products, and consequently, not affecting favorably, the shelf-life of these products.

Additionally, due to their hydrophilic nature, during the gastrointestinal (GI) digestion, PCs shielded in hydratable or water-soluble MPs, are promptly released, turning prone to degradation not allowing them to strain their human health-protective effects.

Alternatively, PCs can be microencapsulated using water-insoluble polymeric matrices as ethylcellulose (EC). Specifically, the water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation technique empowers the protection of water-soluble PCs as HXT and TYR – the main PCs present of OOBPs. These water-soluble PCs (HXT and TYR) are susceptible to environment conditions (e.g., light, temperature, pH), to food matrices compounds and formulation conditions (e.g., pH and enzymes, presence of other antioxidants) and to GI tract environment (e.g., enzymes and pH) (Covas et al., 2010; Paulo & Santos, 2018a, 2020b).

Differently to hydratable and water-soluble MPs, the water-insoluble MPs loaded with PCs are generally compatible with the most of food products due to their both lipophilic nature. When embedded in food matrices, these MPs may release the PCs, allowing the effective protection of other vital molecules. In the case of fatty-foods, supplementation with MPs may improve the oxidative status of these matrices, extending the shelf-life of these products. Additionally, due to the lipophilic nature of MPs, the PCs can be controlled released during the GI tract digestion, allowing the PCs to reach the absorption sites on their original form and thereby uphold their beneficial impact on human health.

Therefore, the main goals of this project were (i) to encapsulate the PCs HXT and TYR individually and assess their release behavior under *in vitro* GI tract conditions, (ii) to obtain PCs rich extracts from a OOBP with a goal (iii) to encapsulate these PCs rich extracts and assess their release behavior under *in vitro* GI tract conditions. Moreover, it was also intended (iv) to embed PCs rich extracts loaded MPs in fatty matrices as olive oils (OOs) evaluating their oxidative status during storage.

Specific goals of this project included (i) the development of analytical methodologies to identify and quantify the PCs selected – HXT and TYR, (ii) the development of specific analytical methodologies to quantify the total phenolic content (TPC) and the total antioxidant activity (TAA) of loaded MPs, (iii) the deployment of *in vitro* gastrointestinal tract release studies, (iv) the implementation of MPs characterization procedures, and (v) the evaluation of the bioavailability of the PCs. It was also intended (vi) to perform the mathematical modeling of the *in vitro* release profiles of PCs from loaded MPs in order (vii) to evaluate the process and formulation conditions on loaded MPs final characteristics as well as in their release behavior in order (viii) to optimize simultaneously the formulation and process conditions of PCs extract rich loaded MPs, so as (ix) to optimize the extraction conditions of PCs from the selected OOBPs through the implementation of a statistical design – a response surface methodology. Moreover, it was also intended (x) to evaluate the protective effect given by microencapsulation to the PCs, (xi) to investigate some chemical indices (e.g., peroxide value – PV, *p*-anisidine value, total oxidation status, free fatty acids content) in OOs submitted to accelerated oxidation and (xii) to evaluate the possibility to reduce oxidation processes in selected OOs using PCs rich extracts loaded MPs as an alternative to synthetic antioxidants.

An overview of this project is presented in Figure I.5.

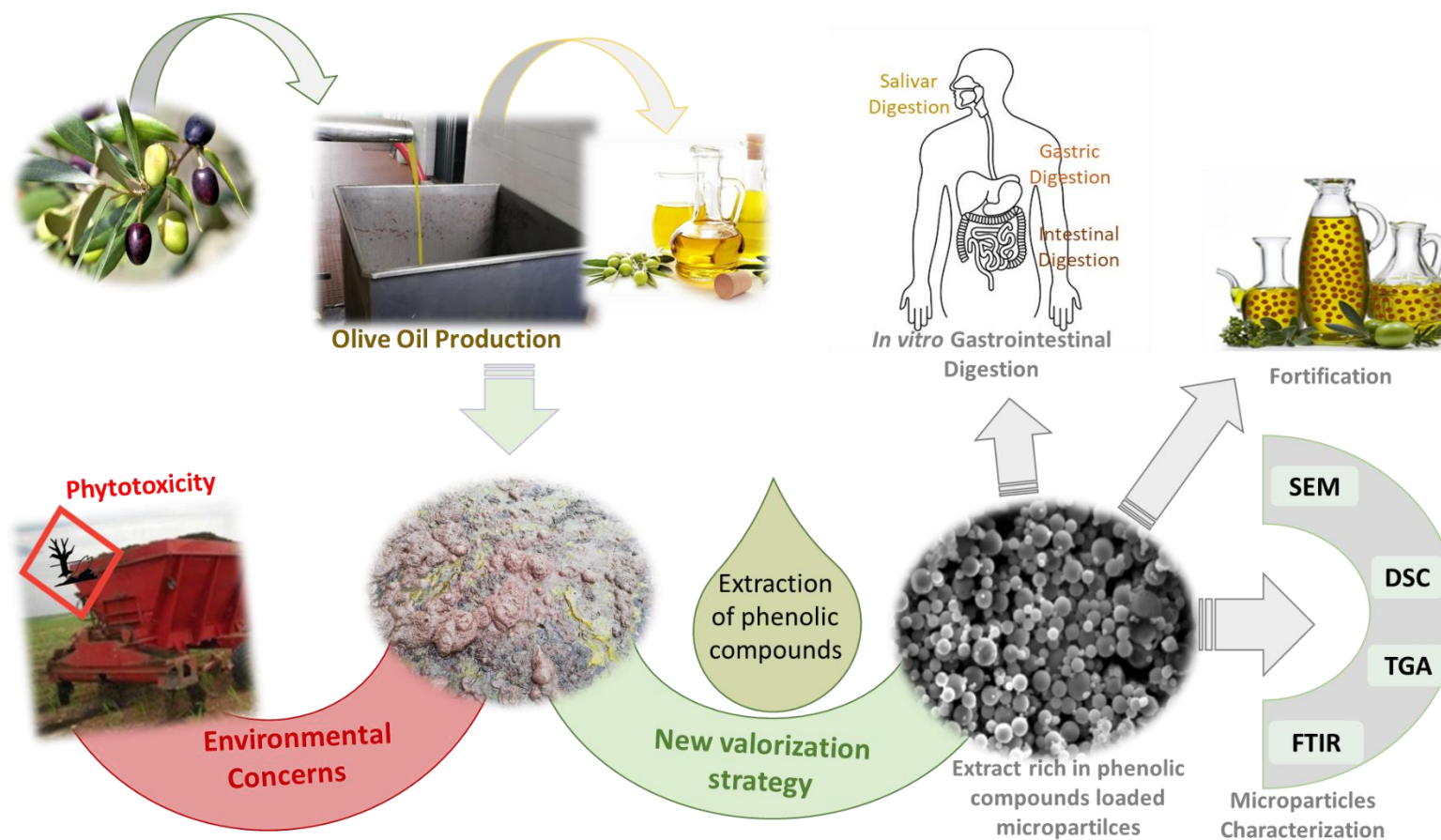


Figure I.5 - Schematic representation for the project overview (SEM – Scanning electron microscopy; DSC – Differential scanning calorimetry; TGA – Thermogravimetric analysis; FTIR – Fourier Transform Infrared Spectroscopy)

Outline

This thesis is divided into VI parts. The first part (Part I) corresponds to the Framework, where a general overview, including the relevance and motivation, the main objectives, and the thesis organization, is presented.

The Part II corresponds to the introduction and the state-of-the-art section and comprises a chapter (Chapter 1) that explores the problematic of OOBPs, an overview of the valorization approaches of OOBPs as the extraction of PCs and encapsulation of extracts rich in PCs obtained from OOBPs.

Part III includes three chapters (Chapter 2 to Chapter 4) regarding the encapsulation and *in vitro* release studies of the individual PCs selected for this study – HXT and TYR. Chapter 2 is devoted to the encapsulation of HXT into ethylcellulose MPs, the characterization of the obtained microparticles, and the *in vitro* release studies under simulated GI tract conditions. Chapter 3 includes the results regarding the preparation and characterization of microparticles loaded with TYR and formulated considering alternative encapsulating materials, namely ethylcellulose, poly(D,L-lactide-co-glycolide), and polycaprolactone. Chapter 4 is devoted to assessing the *in vitro* release behavior of TYR during simulated GI tract digestion.

Part IV encompasses three chapters (Chapter 5, Chapter 6 and, Chapter 7) and corresponds to the extraction of PCs from an OOBP – olive mill pomace (OMP), encapsulation of the extract obtained from a OMP sample, and the performance of *in vitro* release studies. In Chapter 5, the extraction of PCs from OMP was optimized through response surface modeling. Chapter 6 includes the results regarding the encapsulation of PCs-rich extract from OMP into ethylcellulose microparticles and particles' characterization. Finally, in Part IV, Chapter 7 is devoted to the *in vitro* release studies of PCs, antioxidants, HXT, and TYR from OMP extract-loaded ethylcellulose MPs.

Part V comprises one chapter (Chapter 8) corresponding to the oxidative stability studies of OOs enriched with OMP extract-loaded ethylcellulose MPs.

Part VI is the final section where a general discussion is developed, and the main conclusions presented (Chapter 9) and finally, the future work is discussed (Chapter 10).

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Part II: Introduction and State of the Art

In this section, a comprehensive review of olive oil extraction processes, the most updated data regarding the generation and characterization of olive oil by-products, as well as the environmental impact of olive oil by-products, is presented in Chapter 1. Moreover, in this chapter is also presented relevant data regarding the valorization approaches of phenolic compounds namely through extraction and encapsulation.

Chapter 1. Deriving valorization of phenolic compounds from olive oil by-products for food applications through microencapsulation approaches: a comprehensive review ¹

Filipa Paulo, Lúcia Santos

Abstract:

Nowadays, olive oil consumption is correlated to many health benefits, essentially due to the presence of antioxidants, especially phenolic compounds, which fostered its intensive production worldwide.

During olive oil extraction, through continuous or discontinuous processes, many olive oil by-products are generated.

These by-products constitute an environmental problem regarding its management and disposal. They are phytotoxic and biotoxic due to their high content of phenolic compounds, presenting contrastingly relevant health benefits due to their potent radical scavenging activities.

In the framework of the disposal and management of olive oil by-products, treatment, and valorization approaches are found. As currently, the majority of the valorization techniques applied have a null market value, alternative strategies for the obtainment of innovative products as fortified foods are being investigated.

The recovery and valorization strategies of olive oil by-products may comprise extraction and further encapsulation of bioactive compounds, as an innovative valorization blueprint of phenolic compounds present in these by-products.

The majority of phenolic compounds present in olive oil by-products possess limited

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application on the food industry since they are promptly amended by environmental factors like temperature, pH, and light. Consequently, they must be protected previously ending in the final formulation. Prior to foods fortification with phenolic-rich extracts obtained from olive oil by-products, they should be protected through microencapsulation approaches, allowing a sustained release of phenolic compounds in the fortified foods, without losing their physicochemical properties.

The combined strategies of extraction and microencapsulation will contribute to promoting the sustainability of the olive oil sector and aid the food industry to obtain reinvented added-value products.

Keywords: olive oil by-products; olive pomace; olive oil wastewaters; olive leaves; microencapsulation; phenolic compounds; extraction; valorization;

1.1 Introduction

The Mediterranean Diet is a cornerstone on the prevention of cardiovascular diseases and some types of cancer, the lowering of the blood pressure and the positive effects on inflammatory processes as well as in the treatment of endothelial dysfunction (Buckland & Gonzalez, 2015; D'Amore et al., 2016; López-Miranda et al., 2010; Pelucchi et al., 2011; Psaltopoulou et al., 2011; Violi et al., 2015). These positive health benefits associated with the Mediterranean dietary pattern are, in part related to the consumption of a specific source of fat – the olive oil (Buckland & Gonzalez, 2015; D'Amore et al., 2016; López-Miranda et al., 2010; Pelucchi et al., 2011; Psaltopoulou et al., 2011; Violi et al., 2015).

The worldwide acknowledgment of the health benefits associated with the consumption of olive oil integrated into the context of the Mediterranean Diet has been stimulated an intensive production of olive oil, fostering the economic importance of the olive oil sector, especially among the producing countries (Patumi et al., 2002; Stillitano et al., 2017).

However, the olive oil sector generates a large amount of undesired olive oil by-products (OOBP) as olive leaves, olive stones, olive oil pomace (OOP), and olive oil wastewaters (OOWW). In the spectrum of OOBP, the olive leaves are the first olive oil by-product obtained during the pre-treatment of olives. Considering the other typical OOBP, the olive pomace corresponds to the solid residue obtained from olive oil production. Contrastingly, olive oil wastewaters are the effluents obtained during the extraction of olive oil from olive drupes (Gogus & Maskan, 2006; Rodríguez et al., 2008).

The majority of the compounds present in olives drupes are phenolic compounds which the octanol/water partition coefficient ranges between 6×10^{-4} and 1.5, and therefore they can present more affinity to aqueous systems rather than oily phases. A considerable amount of phenolic compounds - around 98% - are retained in olive oil by-products, and only 2% of these bioactive compounds are transferred to olive oil (El-Abbassi et al., 2014).

The high loading of phenolic compounds in OOBP has been rising attention of the scientific community due to their pollutant load. Moreover, olive oil by-products retain potent phytotoxic and biotoxic compounds effectively resistant to degradation, which constitutes a problem regarding by-products management and wastes disposal (Dermeche et al., 2013; Ghanbari et al., 2012; Rodrigues et al., 2015).

Considering the polluting load of phenolic compounds and simultaneously the outstanding protective effect of these compounds for human health mainly due to their potent radical scavenging activities, alternative and innovative valorization approaches of OOBP are currently being considered. One alternative that started to be explored is the extraction of these valuable compounds followed by its microencapsulation for further incorporation in complex matrices as foods considering the achievement of two leading goals: (i) the valorization of phenolic compounds present in olive oil by-products, and (ii) the address of the demand of added value products for food industry.

The scope of the present review is focused on (i) the compilation of data regarding OOBP, (ii) the presentation of the most commonly used extraction processes of olive oil from olives, (iii) the explanation of the environmental impact of olive oil by-products. Moreover, (iv) it is intended to introduce relevant data regarding valorization approaches of phenolic compounds present in olive oil by-products, (v) the display of relevant information regarding valorization of phenolic compounds present in olive oil by-products by extraction followed by the encapsulation of phenolic-rich extracts obtained, (vi) the critical exhibition of scientific studies regarding the phenolic content of OOBP and (vii) the review of studies regarding the valorization of OOBP extracts using encapsulation techniques exploring its main advantages.

1.2 Olive Oil by-products composition

There are available different types of techniques that can be used to extract the olive oil from the olives, such as: (i) the traditional pressing mill process mostly used by small producers, (ii) the two-phase process and (iii) the three-phase process (Azbar et al., 2004; Dermeche et al., 2013; Obied et al., 2005; Qdais & Alshraideh, 2016; Roig et al., 2006; Sampedro et al., 2004). The olive oil extraction can be accomplished in traditional or modernized mills through discontinuous (pressing) or continuous (centrifuging) processes (Dermeche et al., 2013; Roig et al., 2006).

Several steps employed to extract olive oil from the olives are common to the three extraction processes, as the harvesting of the olives, followed by its subsequently washing, crushing of the olives, and the malaxation of the obtained paste. As olive leaves are firstly separated during the harvesting process, they may be considered the first obtained olive oil by-product (Dermeche et al., 2013; Roig et al., 2006).

The oldest and most outspread technique used to obtain olive oil from olives is the discontinuous pressing process (Roig et al., 2006). The obtained malaxated olive paste is spread in the top fiber disks. These fiber disks containing the olive paste are placed at the top of the previous one. Then, pressure is applied in the fiber disks to compact the solid phase of the olive paste, allowing the percolation of the liquid phases (oil and water). In this step, a small amount of water is added to aid the separation of the oily phase from the other phases (aqueous and solid paste) (Azbar et al., 2004). Then after the press subprocess, a solid fraction is obtained. This new-obtained solid product is commonly known as olive oil pomace or olive cake and is made of olive skins, pulp, stones, and water. The liquid phases are then separated by decantation, resulting in the final of the extraction process, the olive oil, and olive oil wastewater. Some water may be added in the last step of the extraction process to promote the separation phase between olive oil wastewater and olive oil during decantation (Dermeche et al., 2013).

Until approximately the 1980's, the discontinuous pressing extraction technique was the most popular and employed technique to extract olive oil from olives.

However, during the 1970's, this process was gradually replaced by innovative approaches (the two-phase process and the three-phase process) in order to reduce the labor force intense and dependence. The continuous processes (the two-phase process and the three-phase process) share a characteristic feature: all the phases (aqueous, oily, and solid) are separated by centrifugation using industrial decanters. Some decanters can be operated alternately either for the three-phase or the two-phase processes (Pedro et al., 2006).

The three-phase continuous centrifugation process requires the addition of warm water in the centrifugation step. This consequently leads to a higher production of OOWW (around 80 to 120 L per 100 kg of olives). The designation of the process as the three-phase is due to the fact that at the end of the continuous centrifugation process three different phases are obtained as a liquid oily phase (the olive oil), an aqueous phase (OOWW) and a solid residue, the olive oil residue (OOR) also known as olive oil pomace (Roig et al., 2006). Even though its requirement of high-water consumption, the three-phase process is still employed in some countries as Portugal, Greece, and Italy to obtain olive oil from olives, especially in mills where is mandatory the production of olive oil in a short period of time (Dermeche et al., 2013). Nevertheless, the three-phase process has been replaced by the two-phase process in leading olive oil-producing countries like Spain – where around 90% of olive-mills operate using the two-phase process - as it allows to reduce the energy and water consumption (Food and Agriculture Organization of the United Nations, 2019; Ruiz et al., 2017; Albuquerque et al., 2004).

In this context, the two-phase process was developed in the 1990's to minimize the water consumption that was used with the three-phase process. Using this technology, only two phases are produced in the end of the centrifugal process such as an oily phase (the olive oil) and a semi-solid phase – the wet pomace also known as the two-phase olive oil waste (TPOOW) also known as “alperujo” or even as “alpeorujo” (Azbar et al., 2004; Benitez et al., 1997; Roig et al., 2006; Tortosa et al., 2012). This semi-solid by-product obtained can be described as a combination of olive husk and olive oil wastewater. The TPOOW is usually further re-processed through second centrifugation to increase the olive oil extraction yield (Roig et al., 2006; Albuquerque et al., 2004).

Alternative extraction techniques to obtain olive oil from olives are reported in the literature, such as the continuous combined percolation-centrifugation process, also known as Sinoela process. In this process, against the three and the two-phase processes in which are employed, a combination of pressure and centrifugation, in this process, is used a combination of filtration and centrifugation to obtain olive oil from the olive paste. The extraction technique is based on the difference of interfacial tension between the oil and the water that gets in contact with the steel plate. When the steel plate gets in contact with the olive paste, it is coated with oil due to the lower oil interfacial tension over water interfacial tension. However, the extraction efficiencies of this process are relatively low compared to the other ones, and additionally, it requires high operational and maintenance costs and high energy input. Generally, the olive paste obtained with the Sinolea process is re-processed using the traditional centrifugal apparatus in order to increase the extraction efficiency (Dermeche et al., 2013; Kapellakis et al., 2008).

During the olive oil production, and regardless, the olive oil production process (continuous or discontinuous), two types of residues are produced: (i) a liquid one known as olive oil wastewater also known as black water in the case of the continuous three-phase process and the TPOOW in the case of the two-phase process and (ii) a solid residue, commonly known as olive oil pomace or even as olive oil residues or “prina” (Dermeche et al., 2013; Mirabella et al., 2014; Naziri et al., 2014). Additionally, olives leaves can be considered the first residue that is obtained during olive oil production during the pre-treatment phase, common to all olive oil extraction processes.

Examples of studies performed regarding the quantification of the total phenolic content and the antioxidant capacity of extracts obtained from alternative types of olive oil by-products (olive leaves, OOP, and OOWW) are presented in Table II.1.1.

1.2.1 Olive Leaves

During the pre-treatment of olives, olive leaves are the first OOBP obtained. The chemical composition of olive leaves is widely dependent on several factors such as the geographical origin, the proportion of branches in the olive tree, the climatic conditions, storage conditions, moisture content and degree of contamination with soil and oils (Delgado-Pertíñez et al., 2000; Sabry, 2014). The olive leaves, even though they account only 5% of olive weight, own many potential health benefits (Xynos et al., 2012).

For centuries, the leaves of olives and their extracts have been widely used in folk medicine for the treatment of diabetes, hyperglycemia, and infectious diseases (Komaki et al., 2003). Animal and *in vitro* studies pointed out that leaves and its extracts may have positive health effects on a wide range of diseases such as hypercholesteremia, hypertension, also possessing a vasodilator and antiallergic properties (Komaki et al. 2003; Gonzalez et al. 1992; Zarzuelo et al. 1991; Bock et al. 2013).

The authors Benavente-García et al. (2000) (Table II.1.1) determined the total antioxidant capacity of extracts obtained from olive leaves through the ability to scavenge the radical cation 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS•+) (1.58 ± 0.06 mM of Trolox Equivalents). They also assessed the presence of specific phenolic compounds and their abundance. They found out that the major constituent of the olive leaf extracts obtained was oleuropein (24.54%), followed by hydroxytyrosol (1.46%), luteolin-7-glucoside (1.38%), apigenin-7-glucoside (1.37%), verbascoside (1.11%) and tyrosol (0.71%) Residual amounts of other phenolic compounds were also found (vanillic acid, dosmetin-7-glucoside, caffeic acid, luteolin, rutin, diosmetin, vanillin, and catechin; for all, the relative abundance was lower than 1%). Nevertheless, the authors' findings are significative: they identified that the relative amount of each phenolic compound was not directly related to its antioxidant capacity. Even though oleuropein was the compound mostly abundant in the obtained olive leaf extracts, it demonstrated a slight ability to scavenge radicals (total antioxidant capacity of 0.88 ± 0.09 mM of Trolox Equivalents). Alternatively, rutin which relative abundance was determined to be 0.05%, demonstrated the highest scavenging capacity (2.75 ± 0.05 mM of Trolox Equivalents).

Nevertheless, it is generally recognized that the main health properties of olive leaves and their extracts are associated to their high content of phenolic compounds, particularly, oleuropein and hydroxytyrosol as these two phenolic compounds demonstrate a strong radical scavenging capacity (El & Karakaya, 2009; Souilem et al., 2017). Hydroxytyrosol is considered a novel safe food pursuant according to the last report of the European Food Safety Authority (EFSA) published in 2017 (Turck et al., 2017).

Moreover, it is believed that the antioxidant capacity of extracts obtained from olive leaves is strongly affected by several factors as (i) the maturation status of olive leaves - mature olive leaves present more antioxidant capacity than young ones, (ii) the olive variety, (iii) the geographical localization of olives cultivation, (iv) the cultivation conditions, (v) the olive oil extraction processes – the content of water used for the extraction of the olive oil sets the antioxidants recovered and consequently the antioxidant activity of extracts as during the extraction many high molecular weight antioxidants are converted to low molecular weight antioxidants through hydrolysis - among others (De Marco et al., 2007; Şahin & Bilgin, 2018).

1.2.2 Olive Pomace

The olive pomace, also known as olive oil pomace (OOP), is semi-solid to a semi-liquid residue obtained through the olive oil extraction from olives. It presents low pH, high salinity, high organic loads, and high phenolic content. Due to its high phenolic content, olive pomaces are phytotoxic and biotoxic, nevertheless they present antioxidant and antimicrobial properties (Albuquerque et al., 2006; Bertin et al., 2011; Bleve et al., 2011; Khoufi et al., 2007; Linares et al., 2003; Morillo et al., 2009; Piotrowska et al., 2011; Sampedro et al., 2009).

Depending on the olive oil extraction process used, approximately 35 to 40 kg of OOP is produced for 100 kg of olives processed. It consists of a complex matrix of pits (42 to 54% w/w), pulp fragments (21 to 33% w/w), and olive skins (10 to 11% w/w) (Akay et al., 2015; Chanioti & Tzia, 2019).

Subsequently, the OOP can be refined, submitting to a centrifugation process to extract residual olive oil and the resulting by-product can be dried and further extracted using solvents (e.g., hexane) to obtain edible oil. The amount of edible oil obtained after refining OOP is dependent on the moisture content, however, it is pointed out that the amount of OOP obtained can reach 25-30%, 45% and 70% w/w on a dry basis, using the pressing, the three-phase, and the two-phase process, respectively (Meziane & Kadi 2008; Akar et al., 2009; Albuquerque et al., 2004; Roig et al., 2006).

Even though the physicochemical composition of the olive pomace is dependent on the olive variety, geographical localization of olives cultivation, olive oil extraction processes, and cultivation conditions, the final chemical composition is quite similar among different samples of OOP. However, olive oil pomaces are rich in pectic polysaccharides, cellulose, and hemicellulosic polymers. Regarding the mineralogical content of OOP, it is mostly constituted by potassium (P), calcium (Ca), and sodium (Na). Additionally, it contains high quantities of water (70 to 80%), low olive oil content (2 to 3%), and many phytochemicals as antioxidants (especially phenolic compounds) and peptides (Dermeche et al., 2013; Rodríguez-Gutiérrez et al., 2014).

The authors Lesage-Meessen et al. (2001) studied the effects of using the three-phase centrifugation process and the two-phase centrifugation process on the phenolic profile of olive pomaces and the antioxidant capacity of extracts obtained from the pomaces (Table II.1.1). Regarding the total phenolic content of OOPs, they found out that the amount of phenolic compounds was higher in the OOP obtained through the three-phase system (24.0 % w/w on a dry basis) rather than the olive pomace obtained through the two-phase system (20.4% w/w on a dry basis). During the three-phase process are produced pomaces with higher content of phenolic compounds when compared to the two-phase olive oil production processes.

Interestingly, when the authors submitted the olive oil pomaces to the same extraction procedure, they verified that the total amount of phenols recovered was significantly higher in the extract recovered from the pomace obtained from the two-phase process (42.2% w/w in the dry residue) rather than in the three-phase process (28.8% w/w in the dry residue).

It can be concluded that the amount of phenolic compounds recovered from OOP is significantly dependent on the extraction technique – even though the pomace obtained during the three-phase system presented a higher amount of phenolic compounds, the respective extract presented a lower content in phenolic compounds. Additionally, the authors Lesage-Meessen et al. (2001) measured the antiradical activity of the extracts by the concentration of the sample (EC_{50}) required for the decrease of the initial amount of the radical 2,2-diphenyl-2-picrylhydrazyl (DPPH) concentration by 50%, being the obtained value inversely proportional to the extract antioxidant activity. According to the results obtained for the total phenolic content, the highest antiradical activity was obtained with the extract processed after the recovery of olive oil through the two-phase centrifugation process (IC_{50} =2.61 mg of gallic acid equivalents/L of reaction medium) rather than the three-phase process (IC_{50} =5.27 mg of gallic acid equivalents/L of reaction medium).

The authors, Obied et al. (2007), quantified the total antioxidant capacity and the total phenolic content of the OOP extracts obtained from different cultivars. Even though the authors do not specify the differences among the two cultivars, the highest antiradical activity of extracts was associated to the lowest phenolic compounds content (OOP extract obtained using the Mission cultivar: IC_{50} of 60.6 ± 2.3 ppm and total phenolic content of 17.7 ± 0.9 mg of gallic acid equivalents/g of dry weight; OOP extract obtained using the Frantoio cultivar: IC_{50} of 79.9 ± 2.0 ppm and total phenolic content of 21.1 mg of gallic acid equivalents/g of dry weight). These findings are in agreement with the ones obtained by Benavente-García et al. (2000) when they were determining the total antioxidant capacity of extracts obtained from olive leaves through the ability to scavenge the radical cation $ABTS^{\bullet+}$. Nevertheless, this tendency should be examined thoroughly as other researches obtained opposite results – the highest value for the total antioxidant capacity can be correlated to the highest amount of phenolic compounds present in the evaluated extract. The results obtained by Obied et al. (2007) are significant as they prove the total antioxidant capacity and the total phenolic content dependence of the olive cultivar.

1.2.3 Olive Oil Wastewater

Generically, the olive oil wastewater is an organic residue with a high content of phenolic compounds, and other valuable nutrients. This high conductivity liquid is also rich in nitrogen (N), phosphorus (P), potassium (K), and magnesium (Mg). Alongside its water high content (around 83 to 92%), minerals, and phenolic compounds, it also may contain sugars and organic acids (Dermeche et al., 2013; Mekki et al., 2007; Piotrowska et al., 2011).

The octanol/water partition coefficients of the majority of olive phenolic compounds range between 6.0×10^{-4} and 1.5, and therefore, they may present more affinity to water systems rather the oily systems. This consideration agrees with the reported data regarding the amount of phenolic compounds in olive oil (around 2%), in OOWW (approximately 53%) and OOP (around 45%). Therefore, the majority of phenolic compounds are present in the olive oil wastewaters (El-Abbassi et al., 2014).

The OOWW physicochemical composition varies according to the olive variety, cultivation features, olives storage time, and extraction process applied (Dermeche et al. 2013).

The high organic load of OOWW is associated with its high content in phenolic compounds and low pH. These physicochemical features of olive oil wastewaters contribute significantly to environmental pollution, especially in the olive oil production areas.

Even though olive oil wastewater represents a threat to the environment, it also owns relevant biological properties, and it is a potent radical-scavenging and metal-chelating matrix (Khoufi et al., 2008).

The recovery of phenolic compounds-rich extracts from OOWW by liquid-liquid extraction using ethyl acetate as the extraction solvent was described by Khoufi et al. (2008). The extraction was applied as a pre-treatment of electrocoagulation of the exhausted fraction of wastewater to reduce its toxicity on anaerobic digestion. The analytical results of the OOWW extract by gas-chromatography mass-spectrometry (GC-MS) analysis pointed out that the major compound present in the extract was hydroxytyrosol (66.5%).

Minor concentrations of tyrosol, caffeic acid, *p*-coumaric acid, and ferulic acid were also detected. The authors concluded that olive oil wastewater is a valuable source of natural antioxidants. Additionally, other authors concluded that the antimicrobial and phytotoxicity potential of extracts obtained from olive oil wastewater is mainly attributed to its high content of minor phenolic compounds and phenolic acids (Casa et al., 2003; Ghanbari et al., 2012; González et al., 1990; MacCì et al., 2010).

The positive effect of extracts recovered from OOWW as an antiatherogenic matrix was highlighted by Leger et al. (2000), reinforcing the olive oil wastewaters potential as a matrix source of phenolic compounds-rich extracts to be incorporated into foods.

The OOWW presents not only oxidative scavenging potential but also antimicrobial activity, which makes this olive oil by-product an excellent source of valuable natural antioxidants after their extraction from the wastewater complex matrix.

The valorization of OOWW through the extraction of phenolic compounds may have a positive impact regarding the disposal and the by-product management of this complex matrix.

Regarding the studies of the total antioxidant capacity and the phenolic content of extracts obtained from OOWW (Table II.1.1), Visioli et al. (1999) evaluated different extraction methods for the recovery of bioactive compounds for OOWW. They concluded that the extract obtained from OOWW submitted to a freeze-drying process and afterward fractioned, presented the lowest antiradical activity ($EC_{50}=9.42$ ppm), followed by the extract obtained by liquid-liquid extraction ($EC_{50}=3.12$ ppm), being the liquid-liquid extraction followed by the fractionation of the extract in a Sephadex LH-20 column, the best extraction approach for the recovery of antioxidants (obtained extracts presented an EC_{50} value of 1.84 ppm). The research presented by Visioli et al. (1999) supports the thesis of the significant influence of the extraction approach on the total antioxidant capacity of extracts obtained from the same initial sample.

The study on the dependence on the modernization degree of olive oil production bases both operating in a three-phase process of the total antioxidant capacity and the total phenolic content of extracts obtained from OOWW was performed by El-Abbassi et al. (2012).

The degree of modernization of olive oil production bases seems to affect the antiradical activity and the total phenolic content of the extract – the most modernized olive oil production plant presented the lowest antiradical activity (IC_{50} of extracts obtained of OOWW from a semi-modernized base of $263 \pm 2.5 \mu\text{g/mL}$ *versus* IC_{50} of extracts obtained of OOWW from a modernized plant of $169 \pm 5.2 \mu\text{g/mL}$). Similarly to the results obtained Obied et al. (2007), in the case of extracts obtained from olive oil pomace and Benavente-García et al. (2000), in the case of extracts obtained from olive leaf extracts, the highest recovery of phenolic compounds was associated to the lowest antiradical activity – the semi-modernized olive oil production plant presented the highest total phenolic content ($9.82 \pm 0.13 \text{ g of tyrosol/L}$) but simultaneously the lowest antiradical activity.

Accordingly, from the comprehensive compilation of studies presented in Table II.1.1, can be concluded that the type of OOBP (olive leaves, olive oil pomace or olive oil wastewater), the extraction technique applied, the olive oil production process (three-phase process *versus* two-phase process), the type of cultivar and the degree of modernization of the olive oil production process plant from where the olive oil by-products are obtained influence considerably the total antioxidant capacity of the extracts as well as their phenolic content.

Table II.1.1 - Examples of studies performed regarding the quantification of the total antioxidant and phenolic content of different olive oil by-products

Olive Oil By-Product	Study	Extract origin	Extracts Type/Origin	TAC	TPC	References
Olive Leaves	-Identification of phenolic compounds present OLE -Determination of the TAC of each compound and the extract	Alcantarilla, Spain	OLE	1.58 ± 0.06^a	N.F.	Benavente-García et al. (2000)
Olive Oil Pomace	-Study the effects of the 3-PhP and the 2-PhP on the phenolic composition of olive pomaces -Quantification of simple phenolic compounds regarding their antioxidant potentiality	Alpes-Côte-d'Azur, France	OOP (3-PhP)	N.F.	24.0^c	Lesage-Meessen et al. (2001)
			OOP (2-PhP)		20.4^c	
			Extract (3-PhP)	5.27^b	28.8^g	
	-Quantification of the TAC and the TPC of the OOP extracts obtained from different cultivars -Evaluation of the antimicrobial activity of the obtained extracts	Wagga Wagga, Australia	Extract (2-PhP)	2.61^b	42.2^g	Obied et al. (2007)
			Mission OOP extract	60.6 ± 2.3^c	17.7 ± 0.9^d	
			Frantoio OOP extract	79.9 ± 2.0^c	21.1 ± 0.9^d	
	-Evaluation of TAC of hydroalcoholic extracts in cell-free assays and in cell systems exposed to free radical attack	Northern Barese, Italy	Coratina OOP	26.96 ± 1.56^d	19.7^c	Aldini et al. (2006)

Table II.1.1 - Examples of studies performed regarding the quantification of the total antioxidant and phenolic content of different olive oil by-products (cont.)

Olive Oil By-Product	Study	Extract origin	Extracts Type/Origin	TAC	TPC	References
Olive Oil Wastewater	-Evaluation of different extraction methods for the recovery of bioactive compounds for OOWWs -Evaluation of the <i>in vitro</i> TAC and anti-inflammatory activities of the obtained extracts	Italy, Spain, and France	Extract obtained by Lio-OOWW fractionation	9.42 ^d	N.F.	Visioli et al. (1999)
			Extract obtained by LLE	3.12 ^d		
			Extract obtained LLE fractioned in a Sephadex LH-20 column	1.83 ^d		
Olive Oil Wastewater	-Determination of the TPC, flavonoids, flavanols, and proanthocyanidins of two different OOWWs -Evaluation of TAC and radical scavenging activity of phenolic extracts and microfiltered samples using different tests (iron(II) chelating activity, total antioxidant capacity, DPPH assays, and lipid peroxidation test).	Marrakech, Marroco	OOWW 1 Semi-modern 3-PhP	263 ± 2.5 ^e	9.82 ± 0.53 ^h	El-Abbassi et al. (2012)
			OOWW 2 Modern 3-PhP	169 ± 5.2 ^e	6.11 ± 0.2 ^h	

Table II.1.1 - Examples of studies performed regarding the quantification of the total antioxidant and phenolic content of different olive oil by-products (cont.)

Olive Oil By-Product	Study	Extract origin	Extracts Type/Origin	TAC	TPC	References
Olive Oil Wastewater	-Determination of the TPC, flavonoids, flavanols, and proanthocyanidins of two different OOWWs	Marrakech, Marroco	OOWW 1 Semi-modern 3-PhP	N.F.	N.F.	De Marco et al. (2007)
	-Evaluation of TAC and radical scavenging activity of phenolic extracts and microfiltered samples using different tests (iron(II) chelating activity, total antioxidant capacity, DPPH assays, and lipid peroxidation test).		OOWW 2 Modern 3-PhP	55.8 ^f	25.2 ⁱ	

^a Expressed in Trolox Equivalent Antioxidant Capacity (mM of Trolox Equivalents); Data is expressed as mean $\pm$ standard deviation; ^b IC₅₀ values; Antiradical activity was defined as the concentration of sample (EC₅₀) necessary to decrease the initial DPPH radical concentration by 50%; Data expressed in mg of gallic acid equivalents/L of reaction medium; ^c % (w/w) dry basis; Determined as gallic acid equivalents by Folin–Ciocalteu method; ^d mg/g of dry matter; EC₅₀ (ppm); ^e IC₅₀ values; Determined for 0.1 g/L of DPPH initial concentration; Expressed in μ g/mL. ^f mmol of Trolox equivalents/L OOWW; ^g % (w/w) of the total phenols present in the dry residue; ^h g of tyrosol equivalent/L; ⁱ mmol tyrosol/L OOW;

2-PhP – Two-Phase Process; 3-PhP – Three-Phase Process; ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay; DPPH- 2,2-diphenyl-2-picrylhydrazyl Radical Scavenging Test; Lio-OOWW – Lyophilizate of Olive Oil Wastewater; LLE – Liquid-liquid Extraction; N.F. – Not Found; OLE – Olive Leaf Extract; OOP – Olive Oil Pomace; OOW – Olive Oil Waste; OOWW – Olive Oil Wastewater; TAC – Total Antioxidant Capacity; TPC – Total Phenolic Content;

1.3 Phytotoxicity and biotoxicity of olive oil by-products – an environmental concern

The olive oil production industry generates a large amount of undesired liquid and semi-solid by-products.

Regarding the organic load, the amount of olive oil by-products produced by the classical (discontinuous) process and by the continuous process are 1.18 and 1.68 m³/tonne of olives processed, respectively. The chemical oxygen demand (COD) and the biological oxygen demand (BOD) of the residues obtained through the discontinuous process can be found at maximum values of 100 and 79.2 g/L, respectively. In the case when the olive oil is produced by continuous processes, the BOD and COD levels may reach values of 100 and 200 g/L (maximum concentrations reported) (Ergüder et al., 2000; Eroğlu et al., 2004, 2006).

The disposal of untreated olive oil residues is considered an environmental issue mainly due to the highly toxic organic loads and also due to its low pH and the presence of sugars, nitrogenous compounds, volatile acids, polyalcohols, pectins, fats and most importantly, polyphenols (Dermeche et al., 2013; Ghanbari et al., 2012). Regarding the disposal and management of olive oil by-products, the high loading of phenolic compounds in these by-products has a relevant and negative effect on the environment.

The main effluents of olive oil production are OOWW. It is estimated that the worldwide OOWW generation accounts approximately between 10 to more than 30 millions m³, corresponding approximately to 0.55 – 2 L of OOWW/kg of olives processed (El-Abbassi et al., 2012; Ergüder et al., 2000; Eroğlu et al., 2004, 2006).

Even though the amount of residues produced is much lower than compared to residues from other agro-food industries, its impact on the environment is considered to be significant. As regards environmental pollution, is reported that 1 m³ of OOWW is equivalent to 200 m³ of domestic sewage (El-Abbassi et al., 2012; Shilev & Naydenov, 2007).

During the production of olive oil through the two-phase process, the recycling of olive oil wastewater can be performed during the processing of the olives in order to reduce the wastewater effluent and environmental disposal problems (Lesage-Meessen et al., 2001; La Casa & Castro 2014). Considering the OOWW disposal, a pre-treatment is essential as its disposal in water systems (surface water, groundwater systems, seashores, and sea) without previous pre-treatment leads to a significant impact on the ecosystems. Many studies regarding the impact of olive oil wastewater as soil, and water bodies polluter as well as a cause of undesired underground seepage and odors, are available (Panagiota et al., 2006; Rinaldi et al., 2003).

The OOWW are also a toxic waste for some microorganisms and plants as their total phenolic content, organic load, total tannins content are considerably high (El-Abbassi et al., 2012; Rinaldi et al., 2003). Moreover, the presence of long-chain fatty acids increases olive oil wastewater pollutant load (Boari et al., 1984; Panagiota et al., 2006).

Although different authors report alternative chemical explanations for the OOWW environmental impact, it is generally recognized that the phytotoxicity and biotoxicity of these wastewaters are mainly due to the high phenolic content of this organic by-product.

Notwithstanding, contradictory studies are found in the literature. Some authors claim that both soils and plants may benefit from the application of OOWW and even olive oil pomaces as fertilizers, as others report that it may have a phytotoxicity effect as it may inhibit seeds germination and plants growth (Panagiota et al., 2006; Rinaldi et al., 2003; Saviozzi et al., 2001).

The authors Mekki et al. (2007) studied the phytotoxicity of phenolic compounds in a soil amended with olive oil wastewater. They verified that the infiltration of olive oil wastewater in the soil caused a significative modification of its physicochemical characteristics. Phenolic compounds were detected at a depth of 1.2 meters after four months of the final application of the olive oil wastewater in the soil. They verified the presence of a moderate phytotoxic phenolic residual fraction after one year of the olive oil wastewater application.

The phenolic phytotoxicity level was comparable to a 25 times-fold diluted olive oil wastewater. The negative impact on the amended soil properties was also discussed by Mekki et al. (2006) and Paredes et al. (1999).

The toxicological effect of OOWW and its phenolic content against seeds, shellfishes, and freshwater animals was exhaustively studied by Aggelis et al. (2003). They found that some specific organisms can minimize the phenolic toxicity of olive mill wastewaters in bioreactor cultures. After the biotreatment, the OMWW were used as water for the irrigation of lettuces and tomatoes. Even though the biotreated olive oil wastewaters did not affect the uptake the majority of the cultivated plants' nutrients, it resulted in a decrease in the plant yields. The decrease in the plant yields was minimized when diluted biotreated olive oil wastewater was used for irrigation purposes. The authors acknowledged that high olive oil wastewater dilutions after biotreatment are required and/or additional treatments should be considered before the use of OOWW for water irrigation. Moreover, the authors clarified that research in this field should be transferred to real/industrial conditions.

The possibility of using OOWW for the irrigation of plants and its phytotoxicity was evaluated by Sassi et al. (2006). The authors found out that the use of non-diluted olive oil wastewater blocked the germination of the *Ordeum vulgare* seeds. The germination of the seeds was only possible after dilution of 1:16 of olive oil wastewater. Additionally, this research paper describes that the high concentration of heavy metals and phenolic compounds may be the explanatory reason for the toxicity of olive oil wastewater.

Regarding the biotoxicity effects of OOWW, it is reported that the river fish *Gambusia affinis* and the crustacean *Daphnia magna* were intoxicated when exposed to phenolic compounds derivatives at a concentration of 40 mg/L for only 15 minutes (Angus, 1983). Immediate toxic effects were verified on the fish and the ray-finned fish *Cyprinus carpio* and *Chondrostoma polylepis* at concentrations of 6.8% v/v and 8.8% v/v and on the goldfish *Carassius auratus* at a concentration level of 10% v/v (Mekki et al., 2007).

Further to the environmental impact of OOWW, the olive oil pomace is also in the highlight of recent studies of its phytotoxicity and biotoxicity (Salomone & Ioppolo, 2012). Due to its high moisture content, several drawbacks are faced during its disposal and by-product management as it is required high energy input for the drying process, which is inevitably associated with disposal high costs (Salomone & Ioppolo, 2012).

Nevertheless, it was verified a lack of studies regarding the phytotoxicity and biotoxicity of olive oil pomaces.

Alongside olive oil wastewaters and pomaces, olive stones are becoming in the highlight of environmental concerns due to problems of its disposal. Even though olive stones do not present significant phytotoxicity and biotoxicity as no large amounts of this olive residue are generated, its disposal has been studied to diminish its environmental impact. Most of the olive stones are used as biomass for fuel and active carbon production. However, a significant impact in air pollution may arise from the direct combustion of this olive oil residue (Nieto et al., 2010).

Ultimately, olive oil wastewater, the major effluent of olive oil production, is under intensive research as it has been demonstrated to be phytotoxic and biotoxic, mainly due to presence in high quantities of phenolic compounds. Even though biotreatments can be applied to reduce the polluting load of OOWW, in the majority of the studies, it only minimized the phytotoxicity after several OOWW dilutions. Therefore, the disposal and application of OOWW are considered to be a relevant environmental concern. The olive oil pomace and olive stones are also in the highlight regarding the phytotoxicity and the biotoxicity in the context of the disposal and management of olive oil by-products. Specifically, in the case of OOP, its disposal and by-product management are still challenging ascribable to the high content of phenolic compounds (Dermeche et al., 2013).

1.4 Deriving valorization from olive oil by-products

In the framework of the disposal and management of olive oil by-products, two complementary approaches are found: (i) its treatment and (ii) its valorization.

Olive oil production generates a large amount of undesired by-products. Therefore, a balanced address of both technologies (treatment and valorization) has been intensively investigated as a direct result of the problematics regarding the olive oil residues disposal and management and the phytotoxicity and biotoxicity of these residues.

The most commonly applied OOR treatments are based on physicochemical and biological methods. Moreover, olive oil residues can be treated using oxidation and advanced oxidation processes or even combined processes.

The physicochemical processes can be distinguished in chemical processes (e.g., coagulation/flocculation, electro flocculation, adsorption), thermal processes (e.g., evaporation, combustion, pyrolysis) and membrane processes (microfiltration, nanofiltration, ultrafiltration, reverse osmosis). The biological processes can be from anaerobic or aerobic nature. The oxidation and advanced oxidation processes include ozonation, Fenton advanced oxidation, and wet oxidation (Rharrabti & Yamani, 2018). The authors Panagiota et al. (2006) reported that the adsorption chemical process for the treatment of olive oil wastewater is effective in the removal of the total phenolic content by a maximum of 81% using activated clay as an adsorbent.

Alternatively, phenolic compounds present in olive oil residues can be valorized through the obtainment of phenolic compounds rich extracts instead of being treated through their passage from OOBP to adsorbents (e.g., activated clay). When phenolic compounds are treated by an adsorption process, a mass transference is observed, and phenols present in the activated clay should be further treated – and the problem persists.

The valorization of olive oil residues includes (i) land application, (ii) biogas production, (iii) composting, (iv) extraction of valuable compounds, and (v) alternative and innovative uses as enzymes, bioplastics and even proteins production (Rharrabti & Yamani, 2018).

A schematic representation of the most relevant processes and methods for olive oil by-products valorization is shown in Figure II.1.1.

Regarding the potentially harmful impact of OOBP, combustion processes for fuel production have been applied for the withdrawal of the phytotoxicity and biotoxicity due to olive oil by-products high content in phenolic compounds and simultaneously as volume waste-reducing strategy (Miranda et al., 2012; Rubio-Senent et al., 2015).

Alternatively, olive oil by-products management can be performed through the controlled disposal in soils. However, only a minor fraction of OOBP is used as a natural fertilizer or as an additive in animal food feeding. None of these olive oil by-products management strategies is surprisingly considered to have a significant economic impact (Akar et al., 2009).

Therefore, considering the null market value of olive oil by-products controlled disposal on soils and its use as a natural fertilizer or even as an additive in animal food feeding, research in this scientific field has been focused on the finding of innovative strategies to olive oil by-products valorization, especially, its phenolic compounds as they the leading cause of olive oil by-products phytotoxicity and biotoxicity. These innovative valorization strategies of phenolic compounds from OOBP, generally encompass extraction techniques as the ultrasound-assisted, microwave-assisted, pressurized liquid, liquid-solid extractions, and extraction through the application of pulsed electric fields high voltage electrical discharges (Dermeche et al., 2013). These extraction techniques allow not only to recover phenolic compounds but even proteins from OOBP. Supercritical fluid extraction has been also studied as valorization technique of olive oil by-products, aiming the recovery of tocopherols and squalene (Barba et al., 2015; Galanakis et al., 2018; Koubaa et al., 2017; Puértolas & Barba, 2016; Putnik et al., 2017; Roselló-Soto et al., 2015; Şahin et al., 2017). Therefore, OOBP can be a natural source of antioxidants, especially of phenolic compounds that may be used by several industries as food, cosmetic, pharmaceutical, and nutraceutical industries.

The research in field of bio adsorbents and dye removals have making use of OOBP as adsorbent of heavy metals and dye removals (Akar et al., 2009; Al-Anber & Matouq, 2008; Banat et al., 2007; Doyurum & Çelik, 2006; Konstantinou et al., 2007; Malkoc et al., 2006; Martín-Lara et al., 2008; Vegliò et al., 2003).

Even though some signs of progress have been seen regarding olive oil by-products management and their valorization, there is no EU regulation regarding the disposal of olive oil by-products, so far. Therefore, each country is responsible for its disposal in nearby lands, lakes, seas, and storage or evaporation in lagoons (Panagiota et al., 2006).

Hence, at the olive oil production sector, a set of inclusive practices, considering the negative polluting load of olive oil by-products, the disposal, management, and valorization of these by-products, should be implemented.

The obtainment of extracts rich in phenolic compounds that may have protective effects on human health for further incorporation in complex matrices as foods is a research field area that must be explored in the framework of valorization, disposal, and management of olive oil by-products.

Phenolic compounds are widely distributed in the plant kingdom in the form of by-products produced during plants metabolism (Renes & Arci, 2004). However, the majority of these compounds have a restricted application in the food industry, as they may be easily modified by external/environmental factors like pH, temperature, and light. After the attainment of the phenolic-rich extracts from OOBP, in order to shield the integrity of extracts, they must be protected from the external environment through coating/encapsulation approaches, finishing in the final formulation (e.g., food matrix) with the capacity of a sustained release of phenolic compounds, without losing their physicochemical properties (Fang, 2010; Munin & Edwards-lévy, 2011).

From this perspective, the valorization of OOBP should address the challenge regarding the obtainment of valorized, innovative, and even reinvented products through extraction and microencapsulation approaches.

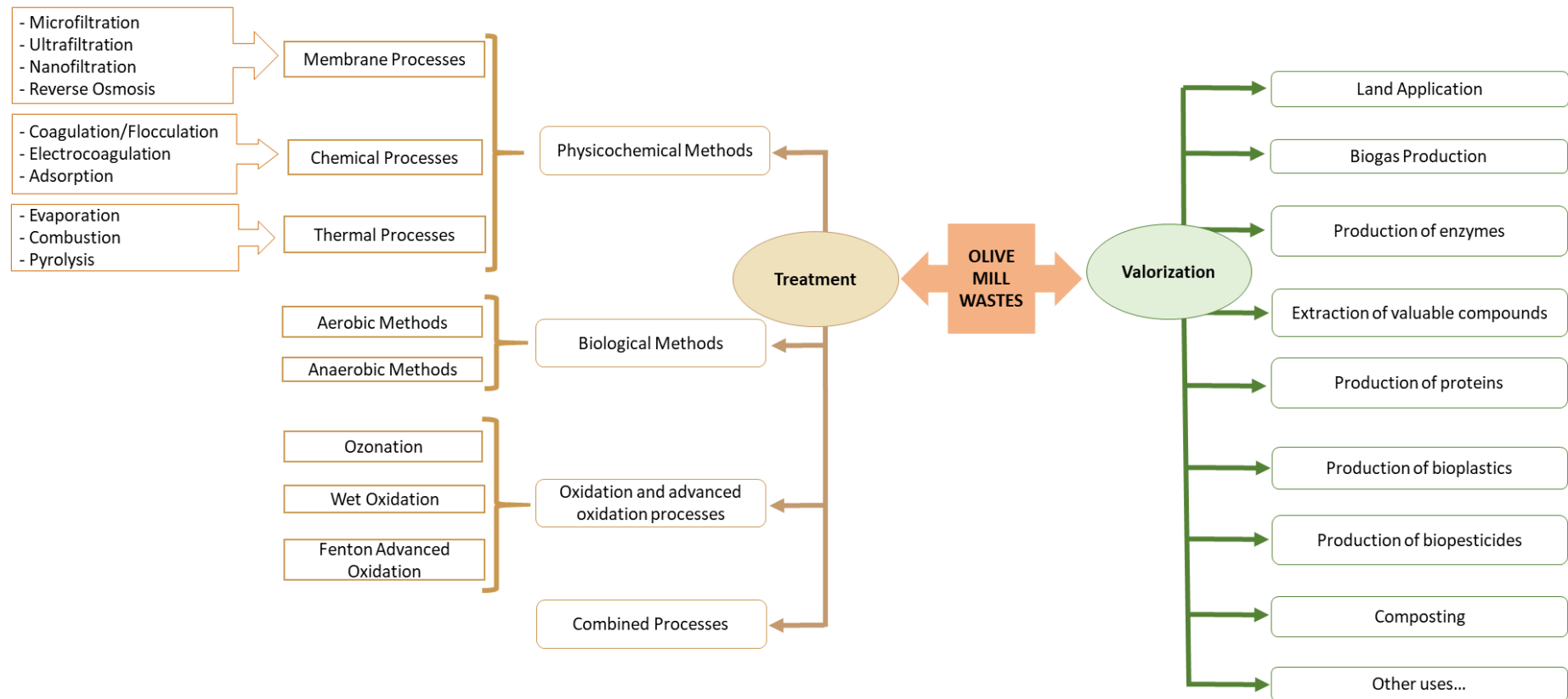


Figure II.1.1 - Olive oil by-products valorization and treatment approaches

1.4.1 Valorisation of phenolic compounds present in olive oil by-products through extraction and microencapsulation approaches

Regarding the potential bio and phytotoxic impact of the presence of phenolic compounds in olive oil by-products, alternative and innovative approaches have been proposed for the recovery of these valuable compounds from OOBP. Among them, extraction and microencapsulation approaches can be combined to obtain a phenolic-rich extract that can be coated using encapsulating approaches to shield the integrity of the extracts, and be further incorporated in the final food formulation/matrix, allowing the release of the phenolic compounds without any defeat on extracts physicochemical composition, yielding the crude olive oil by-product with a low content in phenolic compounds that can be further used for water irrigation, in the case of OOWW and soil fertilizer in the case of OOP, or even for other alternative applications.

1.4.1.1 Extraction of phenolic compounds from olive oil by-products

Over the years, the research on extraction techniques allowed to select the commendable extraction approach to be applied to OOBP and optimized them. The extraction technique that should be implemented depends on the type of OOBP – pomaces, wastewaters, and olive leaves - require alternative extraction approaches to obtain phenolic compound rich extracts.

Three key considerations should be reviewed prior to the extraction: (i) the procedures regarding the sample preparation, (ii) the selection of the most appropriate solvent, and (iii) the selection of the most suitable extraction method itself.

The procedures regarding the sample preparation (i) are considered to be an utmost important step as it is envisaged physicochemical modifications on the chemical structure of phenolic compounds. After collection, OOBP samples should be conserved in a liquid nitrogen environment or freeze-dried and storage in moisture-free environments, a desiccator (De Marco et al., 2007; El-Abbassi et al., 2012; Araújo et al., 2015).

As an alternative, the olive oil by-product sample can be acidified until pH of 2-3 as alkaline mediums promote oxidation and polymerization reactions. Nevertheless, the low pH may promote the precipitation of proteins, which may increase the amount of free phenolic compounds in the solvent used for the extraction (De Marco et al., 2007; El-Abbassi et al., 2012).

During the OOP sample preparation and due to the high content of water of the OOP (*circa* 60%), it can be submitted to a centrifugation process in order to separate the solid residue from the vegetative waters. Vegetative waters are composed of the water used for olive oil production and the water present in the olive drupes (Suárez et al., 2009a).

Prior to extraction, it is typical to remove the lipidic fraction of the OOBP samples. The non-polar compounds as fatty acids, triacylglycerols, fat-soluble pigments, and vitamins, among others may hinder the quantification of phenolic compounds present in the obtained extract. Thereof, non-polar compounds are extracted using the solvent *n*-hexane or petroleum ether (Cioffi et al., 2010; El-Abbassi et al., 2012). Nevertheless, the lipidic fraction can be extracted after the extraction of phenolic compounds. In this case, the use of the solvent mixture methanol/water is commonly applied. However, in the case of the extraction of phenolic compounds from a non-defatted olive oil by-product sample, the phenolic profile of extract may differ from the phenolic profile from a defatted olive oil by-product sample. Generally, non-defatted samples present high quantities of phenolic compounds, probably due to the occurrence of reactions between the fats present in olive oil by-product sample and phenolic compounds (Obied et al., 2007; Alu'datt et al., 2010). The obtainment of a defatted sample prior to the extraction of phenolic compounds is highly recommended.

1.4.1.1.1 Extraction techniques of phenolic compounds from olive oil by-products

In the literature review were found many scientific articles regarding the extraction techniques of phenolic compounds from OOBP. As a comprehensive scientific revisions on this topic can be found on the literature (Caporaso et al., 2018; Araújo et al., 2015; Dermeche et al., 2013; Nguyen, 2017), only the more relevant subjects regarding the extraction of phenolic compounds present in OOBP are going to be briefly discussed. An exhaustive description of the extraction procedures of phenolic compounds from olive oil by-products is out of the scope of this paper.

Several authors have proposed optimized extraction procedures of phenolic compounds from OOBP. Among the extraction procedures commonly applied stands out (i) the solid-liquid extraction, (ii) the ultrasound-assisted extraction, (iii) supercritical fluid extraction, (iv) the pressurized liquid extraction and (v) the superheated liquid extraction.

The solid-liquid extraction (i) is usually performed, starting on the homogenization or alternatively maceration of the OOBP sample. The homogenization of the OOBP samples is commonly achieved through vortexing, magnetic stirring, or using high-performance liquid homogenizers as the Ultra-Turrax®. The homogenization step is followed by centrifugation or sedimentation. Then, two phases are obtained (a solid one and a liquid one), and the analytes are distributed according to their polarity (or alternatively to their affinity to the organic solvent added). Afterward, the two phases are separated, and the analytes can be recovered. A critical design step is the choice of the solvent. It must be selected according to the analytes intended to be recovered, the extraction efficiencies, and the final application desired for the extract. This is especially critical when the extract is intended to be earmarked for food applications. An unyielding control of the contamination of extract with solvent is required. Generally, solvent evaporation is performed, and the trace solvent may be removed by freeze-drying, and the control of the presence of trace solvent can be performed through thermogravimetric analysis (Araújo et al., 2015).

For the extraction of phenolic compounds from OOBP, generally recognized as safe (GRAS) solvents are usually employed. Solvents as methanol, ethanol, or even hydro-alcoholic mixtures are frequently used in this context due to their high selectivity to phenolic compounds. The most used one for the recovery of phenolic compounds from olive oil by-products is methanol (Alu'datt et al., 2010; Klen & Mozeti, 2011, 2012). Nevertheless, its concentration in food matrices is limited by the EU regulation 2009/32/EC to a maximum concentration of 10 mg/kg (European Commission, 2009). Still, according to the EU regulation 2009/32/EC, ethanol is considered an extraction solvent in compliance with the good manufacturing practices. Therefore, ethanol is a safer alternative to methanol regarding the extraction of phenolic compounds from OOBP.

Nevertheless, during the choice of solvent, it should be considered that ethanol is more lipophilic than methanol, and therefore, the analytes recovered with ethanol can be as well more lipophilic and structurally complex. To increase the hydrophilicity of ethanol in order to achieve higher recovery rates of phenolic compounds, ethanol can be used at higher temperatures and pressurized as performed by Lozano-Sánchez et al. (2014), Omero et al. (2009) and Peralbo-Molina et al. (2012).

Unexpectedly, Lafka et al. (2011) obtained similar recoveries of phenolic compounds using methanol, ethanol, ethanol/water 1/1, and propanol, when they were studying the extraction variables – solvent, pH, time and solvent concentration – on the recovery of phenolic compounds from OOBP.

Therefore, it is recommended that further studies in order to analyze the effect of choosing alternatively ethanol instead of methanol for the recovery of phenolic compounds. The meaningful results obtained by Lafka et al. (2011) should be corroborated by further studies.

An alternative solvent that can be used for the recovery of low to medium molecular weight phenolic compounds is ethyl acetate. This solvent is also in compliance with the good manufacturing practices according to the EU regulation 2009/32/EC (European Commission, 2009). Notwithstanding, this solvent presents a pellicular odor that can be considered unpleasant if the extract is directly incorporated in foods.

To circumscribe that, encapsulation purposes may protect the extract and mask the unpleasant odor of ethyl acetate. The authors De Marco et al. (2007), El-Abbassi et al. (2012), and Omero et al. (2009) studied the application of ethyl acetate in the recovery of phenolic compounds from OOBP.

Conclusively, the solid-liquid extraction of phenolic compounds from olive oil by-products should be accomplished, not only considering the recoveries rates but also the toxicity of the selected solvent. For food applications, the solvent chose for the extraction should be classified as GRAS and recognized as an extraction solvent in compliance with the good manufacturing practices by EU directives.

Generally, lipophilic structures or cell wall polysaccharides with a phenolic moiety are not recovered by solid-liquid extraction using a mixture of solvents (ethanol/water or methanol/water); therefore, it commonly employed hydrolysis to breaking the ester or glycosidic bonds of phenols to the olive oil by-product matrix. The breakage of these bonds in order to improve the recovery in phenols can be performed by acidic, basic, or even enzymatic hydrolysis (Watson, 2019). The acidic hydrolysis allows to break both the ester and the glycosidic bonds, and it is usually achieved through the addition of a hydrochloric acid solution. In the case of basic hydrolysis, it can be added sodium or potassium hydroxide solutions. However, it only allows breaking essentially the ester bonds. In the case of enzymatic hydrolysis, specific enzymes should be used – esterases to break ester bonds and glycosidases for the breakage of glycosidic bonds (Watson, 2019).

During the research regarding the optimization, characterization, and quantification of phenolic compounds present in OOBP, Alu'datt et al. (2010) recovered the free phenols by solid-liquid extract with methanol and the bonded phenols through alkaline followed by acid hydrolysis later to the extraction of free phenolic compounds. For the recovery of bonded phenols, the authors promoted the ester and glycosidic bounds breakage using firstly a sodium hydroxide solution (pH of 12) followed by acidic hydrolysis using a hydrochloric acid solution (pH 2). The authors determined that the amount of bonded phenols corresponded from 13% to 25% of the total amount of phenols recovered.

A straightforward alternative for the recovery of phenolic compounds from OOBP is the (ii) ultra-sound assisted extraction. It comprises the application of high-frequency waves (≥ 2 MHz). These high-frequency waves are responsible for the generation of a negative pressure that changes the physicochemical properties of the medium, allowing the development of small cavitation bubbles (Chen et al., 2012). The formation of these cavitation bubbles allows to disrupt cell membranes and therefore favor the contact between the extraction solvent and the matrix. It can be performed using an ultrasonic bath or a probe. In the case of using an ultrasonic bath, the high-frequency waves reach the sample through the wall of the sample container, while in the case of using a probe, the high-frequency waves reach the sample directly as they are immersed in the extraction solution. Beyond being a swift and easy to handle extraction method, it does not require high temperatures, which makes this method suitable for the extraction of temperature-sensitive compounds as phenolic compounds from OOBP.

The authors Klen & Mozeti (2011) performed an ultrasonic extraction of phenolic compounds from OOWW and compared with the conventional methods. The authors accomplished five alternative extraction procedures: filtration, solid-phase extraction, liquid-liquid extraction, and ultrasonic-assisted extraction of the liquid OOWW and the freeze-dried OOWW. They verified that the use of ultrasonication allowed to obtain higher phenolic recovery yields. Comparing the extraction in an organic solvent of phenolic compounds from OOWW without previously ultrasonication was less efficient than the ultrasound-assisted counterpart. However, the authors did not obtain a representative phenol chromatogram probably due to the use of ethyl acetate as the extraction solvent – ethyl acetate probably induced modifications. Contrasting, the ultrasound-assisted extraction of the freeze-dried OOWW using methanol as the extraction solvent allowed to obtain high recovery yields of phenolic compounds without significant ultrasonic-assisted extraction modifications. Additionally, as described in section II.1.4.1., the freeze-drying of OOWW prior to extraction, prevents significant physicochemical modifications of the sample.

A recent alternative to the reported extraction procedures is (iii) the supercritical fluid extraction with carbon dioxide.

The application of the technique is based on the fact that the solvating properties of a fluid can be improved, exceeding the fluid critical pressure and temperature. When in the form a supercritical fluid, it displays a higher diffusion through the sample matrix, allowing the obtainment of higher recovery rates (Souza et al., 2013). The most commonly employed supercritical fluid is carbon dioxide. According to the EU 2009/32/EC regulation, carbon dioxide can be used as an extraction solvent in compliance with manufacturing practices, which can be used during the processing of raw materials, foodstuffs, food components, or ingredients (European Commission, 2009).

The supercritical fluid extraction with carbon dioxide presents many advantages as the critical temperature is considerably low (31 °C), and it is associated with low toxicity and reactivity. Nevertheless, as phenolic compounds are relatively polar, it is required the addition of a polar modifier (e.g., methanol, ethanol).

The study performed by Lafka et al. (2011) regarding the quantification of phenolic compounds and the antioxidant activity of OOWW suggests that recovery of phenolic compounds is lower when the supercritical fluid extraction with carbon dioxide is applied when compared to the traditional liquid-liquid extraction. The amount of phenolic compounds recovered from olive oil wastewaters using the supercritical fluid extraction was only higher to the amount of phenolic compounds recovered by conventional liquid-liquid extraction using ethyl acetate as an organic solvent.

The authors Taamalli et al. (2012) investigated the use of different extraction techniques of phenolic compounds from olive leaves (liquid-solid extraction, microwave-assisted extraction, and pressurized liquid extraction) in order to identify the phenolic profile of obtained extracts and their activity against human breast cancer cells. Similarly to the results obtained by Lafka et al. (2011), the supercritical fluid extraction shown to be the extraction technique with the lowest recovery rates in phenolic compounds. Moreover, the authors verified that applying the supercritical fluid extraction allowed to obtain high recovery rates of lipophilic phenolic compounds as luteolin and apigenin.

The leading innovative extraction procedure of phenolic compounds from OOBPs are (iv) the pressurized liquid extraction and the related (v) superheated liquid extraction. These extraction techniques dwell on the employment of organic solvent combined with temperature and pressure that are controlled externally and electronically selected. The pressurized/heated liquid extraction system is composed by a stainless-steel chamber or cell where the sample is placed, an oven, a pump used for the inlet of the solvent into the system and a set of valves that allows preventing temperature or pressure drops (Carabias-Martínez et al., 2005). Both techniques can operate in three alternative operating modes: (i) the dynamic mode in which fresh solvent is continuously pumped to the system/extraction medium, (ii) the static mode in which the fresh solvent is only pumped between extraction cycles and (iii) the semi-dynamic operating mode which is a combination of the dynamic and static operating modes.

The solvents used in this technique for the extraction of phenolic compounds are generally mixtures of methanol/water or ethanol/water, even though other solvents as acetonitrile, ethanol, methanol, and ethyl acetate are frequently employed in the extraction of hydrophilic compounds (Carabias-Martínez et al., 2005; Herrero et al., 2011; Lozano-Sánchez et al., 2014; Omero et al., 2009; Paper, 2008). Pressure and temperature contribute differentially to the extraction efficiency. The pressure on the system does not contribute significantly for the efficiency of extraction as it is mostly used to maintain the solvents in the liquid phase and improve the flow of the solvents, and to promote the contact between the solvent and the extract (Lozano-Sánchez et al., 2014; Taamalli et al., 2012). Contrastingly, the temperature is considered an essential factor that may significantly influence the efficiency of extraction. When water is used as a solvent or more commonly, in a solvent mixture generally, temperatures above 100 °C and below the critical temperature of water (*circa* 374 °C) are employed for the extraction of phenolic compounds. Temperatures below 100 °C are not recommended as water is too polar for the extraction of organic compounds. Above 100 °C, water presents a lower dielectric constant and therefore acquires a less polar character, more suitable for the extraction of organic compounds.

Temperatures above the critical temperature of water are also not recommended as many compounds are degraded at this temperature. The employment of high temperatures contributes to the dissociation of the more lipophilic phenolic compounds from the matrix, allowing, therefore, to extract them. Other factors as the flowrate of solvents and time are considered to be significant for the extraction efficiency (Kronholm et al., 2007).

There are many advantages associated with the employment of these innovative extraction techniques (pressurized liquid extraction and superheated liquid extraction) for the recovery of phenolic compounds from OOBP intended to further be coating and inclusion on food matrices. Compared to the conventional solid-liquid and liquid-liquid extractions, the pressurized liquid and superheated liquid extractions present a higher degree of automation, require less solvent, and they are less time-consuming approaches. Moreover, it is generally possible to achieve efficient recoveries of phenolic compounds using GRAS solvents like ethanol and water.

The authors Taamalli et al. (2012) verified when they were studying the use of advanced techniques of the extraction of phenolic compounds from Tunisian olive leaves, that amount of phenolic compounds recovered was higher using the pressurized liquid extraction technique with a solvent mixture of methanol and water when compared to the employment of a supercritical fluid and microwave-assisted extractions. Nevertheless, the results obtained by Suárez et al. (2009) indicate that a higher recovery of phenolic compounds was achieved using similarly a mixture of methanol and water but applying the solid-liquid extraction.

Nowadays, are available many extraction techniques that allow the recovery of phenolic compounds from OOBP. During the selection of the extraction technique of phenolic compounds intended to be coated and included in food matrices, should be considered the recovery of phenolic compounds, if it is desired the recovery of lipophilic or/and hydrophilic phenolic compounds, if the solvent or the solvent mixture is GRAS, the maximum concentration of solvent allowed in food matrices by the EU regulation 2009/32/EC, the amount of solvent required for the extraction, the time-consumption of the extraction procedure as well as its energy consumption.

1.4.1.2 Microencapsulation of phenolic-rich extracts obtained from olive oil by-products

Considering that the majority of phenolic compounds present in olive oil by-products possess limited application on the food industry since they are promptly amended by environmental factors like temperature, pH, and light, they must be protected previously ending in the final formulation. In the specific case of foods, prior to the fortification of them with phenolic-rich extracts obtained from OOBP, they should be protected from the surrounding environment.

Moreover, it is generally intended a sustained release of phenolic compounds in fortified foods, without losing their physicochemical properties. Therefore, microencapsulation arises a potential technological strategy to protect phenolic-rich extracts obtained from OOBP to be further incorporated in food matrices.

Microencapsulation is a general term that encompasses a set of technological techniques that allows the protection and coating of micro-sized substances (solid, liquid, or gases) from the surrounding external environment. Moreover, this set of techniques allows a delayed or sustained release of the incorporated bioactive compounds (Benita, 2006; Ghosh, 2006; Kaur & Kakkar, 2014; Paulo & Santos, 2017). The bioactive compound or extract is termed as core material, or encapsulated material, internal phase, or fill, and the material used for the coating purposes is termed as encapsulating material, shell, wall material or membrane (Fang, 2010). The encapsulated material can be temporary or permanently protected from the external environment (Carvalho et al., 2015; Casanova et al., 2016). The resulting products of the encapsulation techniques are designed microparticles. Particles can be distinguished according to their internal morphology in microcapsules and microspheres. Microcapsules and microspheres are differentiated in reservoir systems (the core is surrounded by an outside layer) or matrix systems (the core is homogeneously dispersed within the encapsulating material), respectively (Jyothi et al., 2010; Paulo & Santos, 2017).

The microencapsulation technology was first presented by Green & Schleicher in the 1950's by means of a patent registration regarding the preparation of capsules containing dyes intended to be incorporated into paper for copying purposes (Ghosh, 2006; Paulo & Santos, 2017).

Microencapsulation technological strategies have been investigated by pharmaceutical (68%), food (13%), cosmetic (8%), textile (5%), biomedical (3%), agricultural (2%) and electronic (1%) industries (Casanova & Santos, 2015; Ghosh, 2006; Kim et al., 2007; Paulo & Santos, 2017).

The second driving force of significant signs of progress in microencapsulation is the food industry. The high-demand of fortified and functional products by many consumers is the major motivation for the intensive investigation in this research field.

Additionally, to their sensitivity to the external environment (light, pH, and temperature), phenolic compounds-rich extracts are prone to oxidation processes and gastrointestinal degradation. Moreover, they may present an unpleasant flavor. Therefore, microencapsulation may foster the resolution these issues related to the direct incorporation of phenolic-rich extracts in foods. The incorporation of phenolic-rich extract loaded microparticles and the delayed or sustained release of phenolic compounds may aid to prevent the oxidative degradation of other vital molecules present in the food matrix; therefore, phenolic-rich extracts loaded microparticles can be added to foods as a high-tech preservatives, extending foodstuff shelf-life. Moreover, the protection of phenolic compounds from the external environment may increase the bioavailability of these compounds (Chen et al., 2019).

One of the main advantages of microencapsulation approaches is the possibility to control the release of bioactive compounds. The sustained release of phenolic compounds from microparticles incorporated in foods may reduce the amount of additives added to preserve them and extend foods shelf-life (Azeredo, 2005). The release can be classified as (i) delayed – when the release is intended to occur in a specific moment or place - or (ii) sustained – when is intended to control the release rate of bioactive compound(s). Generally, the actual release is a combination of both patterns (Aguilar et al., 2016).

The release can occur by mass-diffusion through water-filled pores or throughout the polymer, osmotic pumping or even through erosion processes of the coating material which do not require transport of the bioactive compound(s) contrarily to the other release mechanisms (Fredenberg et al., 2011).

There are many properties of the release pattern and the external environment that may influence the actual release.

The properties affecting the delivery system can be divided in five alternative categories: (i) the coating material (molecular weight of the polymer, the chemical end groups of the polymer, the crystallinity state of the polymer), (ii) the encapsulated agent (the physicochemical characteristics of the bioactive compound(s), loading, location inside of microparticles, the presence of additives as salts, surfactants or even plasticizing agents), (iii) the delivery system itself (size, porosity, density and shape), (iv) *in vitro* release conditions (temperature, stirring, composition of the release medium, pH and osmolality) and (v) *in vivo* release conditions (presence of enzymes, lipids, physicochemical characteristics of the matrix) (Fredenberg et al., 2011).

The most common release mechanism of bioactive compounds, including the case of phenolic-rich extracts is by osmotic pumping, generally by a solvent-activation approach. Nevertheless, the use of surfactants, the control of the pH, the induce of enzymatic release, and even light may trigger the release of bioactives from microparticles in a food matrix (Aguiar et al., 2017).

The design of fortified foods with microencapsulated phenolic-rich extracts must consider several constraints as the manufacturing and storage properties of foods, economic feasibility, and consumer adherence (Aguiar et al., 2016; Paulo & Santos, 2017). Additionally, particle size is considered to exert a significant influence on microparticles design for food applications as the presence of particles larger than 30 µm may irk a gritty mouthfeel (Merkus & Meesters, 2014).

1.4.1.2.1 Microencapsulation techniques for the entrapment of phenolic-rich extracts obtained from olive oil by-products

Over the years, new and innovative microencapsulation techniques have been studied and optimized considering the utmost important factors as (i) physicochemical properties of the core material (ii) physicochemical properties of the wall material and (iii) the final intended application of obtained microparticles (Poshadri & Kuna, 2010).

There are available a significant variety of microencapsulation techniques; however, no single encapsulation technique is adjustable to all core materials, polymers, and applications (Wilson & Shah, 2007).

Some of the most used encapsulation techniques for food applications are the spray-drying, the freeze-drying, the coacervation, the emulsification, the inclusion complexation, the liposome entrapment, and the fluid bed coating.

Mostly, the encapsulation techniques applied for the entrapment of phenolic-rich extracts from OOBP are the spray-drying, the freeze-drying, and emulsification techniques. Among the emulsification techniques/solvent evaporation approaches, the double emulsions water-in-oil-in-water and the oil-in-water-in-oil are described for the encapsulation of olive leaf extracts. The supercritical assisted atomization is an innovative alternative also described for the protection of OOP extracts. In Table II.1.2 are presented the main advantages and disadvantages of each technique found in the literature for the microencapsulation of phenolic-rich extracts obtained from olive oil by-products for food applications.

Comprehensive reviews of microencapsulation techniques are available in the literature (Aguiar et al., 2016; Fang, 2010; Ozkan et al., 2019). Therefore, only a brief description of the microencapsulation techniques used for the entrapment of phenolic-rich extracts obtained from OOBP is presented.

The spray-drying technique is the most outspreading technique for the encapsulation of phenolic-rich extracts obtained from olive oil by-products intended for food applications. The spray-drying can be defined as an atomization process of a liquid in a dry powder using an injector and a hot drying chamber (Rattes & Oliveira, 2007).

During the spray-drying, occurs the homogenization of the feed solution using an atomizer, followed by the drying of feed solution in a chamber passing a hot carrier gas to promote the evaporation of the solvent and finally, microparticles are recovered in a cyclone or a filter (Ozkan et al., 2019; Schafröth et al., 2012). The feed solution contains the core and the wall materials in a solution, emulsion, or a suspension form (Gharsallaoui et al., 2007).

The feature characteristics of the powder obtained by the spray-drying technique depend largely on operating conditions as the feed solution flow rate, the drying temperature, the drying air flow rate, the speed on the atomizer, and the polymer carrier (type and concentration) (Schoubben et al., 2010).

The carriers used for the encapsulation by spray-drying are generally water-soluble polymers as polysaccharides (e.g., Arabic gum, maltodextrins, cyclodextrins), proteins (e.g., sodium caseinate, whey protein, soybean protein), starch, gelatin, chitosan and gellan gum (Lee & Wong, 2014).

Additionally to the application for the coating and protection of phenolic-rich extracts obtained from OOBP, the spray-drying has been used for the microencapsulation of a broad range of bioactive compounds for food industry as colors, vitamins, flavors, fats, oils and antioxidant-rich extracts (Aguiar et al., 2016; Ferris et al., 1984; Nedovic et al., 2011; Pillai et al., 2012).

An alternative microencapsulation technique that has been widely employed for the encapsulation of phenolic-rich extracts from OOBP is the freeze-drying technique, also termed as lyophilization. This is a multi-stage process in which the mixture of the wall and the core materials is submitted to a freezing process followed by a sublimation known as primary drying. Then, it occurs the desorption (secondary drying) and the storage of the dry powder (Laokuldilok & Kanha, 2015). It is described that wall material characteristics - structure and composition - seize an impact on both adequate protection and release (Young et al., 1993).

The freeze-drying technique has been used for the encapsulation of several bioactive compounds for food industry as antioxidants (Galmarini et al., 2013; Wilkowska et al., 2016), food flavors (Kaushik & Roos, 2008), enzymes (Anjani et al., 2007; Kawai & Suzuki, 2007; Liu et al., 2011; Mehrnoush et al., 2011; Santagapita et al., 2011) and probiotics (Morgan et al., 2006; Song et al., 2008; Saarela et al., 2006; Carvalho et al., 2004).

The microencapsulation through emulsification and solvent removal processes can be generally defined as a set of procedures in which an organic solvent is removed from an emulsion dispersed in another solvent, generally water.

The emulsion contains embedded the core material, and the wall material dissolved in an organic solvent (Poncelet, 2005). According to this technique, firstly an emulsion, suspension or a solution of the core material and the wall material is formed. The coating polymer is previously dissolved in an organic solvent. Afterward, occurs the emulsification of the dispersed phase (core and wall material) in a continuous phase (aqueous solution) generally by stirring, even though it can be accomplished by static mixing, dripping, or extrusion. After that, the solvent removal is promoted through evaporation and liquid extraction. The solid powder obtained after the solvent removal is recovery by filtration or centrifugation from the dispersive phase. The obtained microparticles are subsequently dried (Hwisa et al., 2013; Paulo & Santos, 2018a, 2018b; 2019).

There are many formulation and operating conditions that affect the microencapsulation through emulsification followed by solvent removal procedures as the nature of the solvent, the volume of solvent, the physicochemical properties of the polymer, the polymer concentration, the emulsification type, the concentration of emulsifiers, the rate of solvent removal, the addition of salts or buffers to the internal phase and/or external phase, the continuous to dispersive phase volume ratio, the temperature, the stirring speed among others (Tiwari & Verma, 2011).

Some authors claim that there is a lack of studies regarding the impact of this set of encapsulation approaches intended for food applications, even though they have been widely investigated for pharmaceutical applications (Ozkan et al., 2019). However, according to Lu et al. (2016), during their scientific review on emulsion-based encapsulation and delivery systems of phenolic compounds, there is a vast number of studies regarding the encapsulation of phenolic compounds by emulsification approaches intended to obtain microparticles to be further incorporated in foods. Nevertheless, the authors reported that they found very little information regarding the *in vivo* absorption, transportation, and release of these compounds enclosed in emulsions. Therefore, agreeing with Lu et al. (2016), a systematic and comprehensive research on the metabolism and effects of encapsulated phenolic compounds in emulsions is imperative.

The innovative and alternative microencapsulation technique supercritical assisted atomization is based on the use as a solvent, a supercritical fluid at temperature (T_c), and pressure (P_c) above its critical point.

Above its critical point, the selected solvent acquires properties between liquids and gases such as the density and the solvating capacity of liquids and the typical low viscosity, high diffusivities, and high mass transfer rates of gases. The most commonly used solvent as a supercritical fluid is carbon dioxide due to its mild critical conditions ($T_c = 31.1\text{ }^{\circ}\text{C}$ and $P_c = 7.38\text{ MPa}$). Nevertheless, examples of other solvents that can be used as supercritical fluids are water, nitrogen, and propane (Gouin, 2004). The supercritical assisted-atomization processes can be classified as (i) rapid expansion of supercritical solutions and related processes, (ii) supercritical antisolvent precipitation and derived processes, and (iii) particles from gas saturated solutions and the related processes (Munin & Edwards-lévy, 2011).

In the case of a rapid expansion of supercritical solutions (i), the supercritical fluid acts as a solvent. The bioactive compound(s), other solutes, and the polymer are dissolved in the supercritical fluid. Afterward, the solution is expanded using a small nozzle into a region with a lower pressure, which allows the precipitation of the solutes (Debenedetti et al., 1993).

In the case of supercritical antisolvent precipitation, the supercritical fluid acts as anti-solvent. The technique is based on the contact between the supercritical fluid with a solution containing the organic solvent and the bioactive compound(s) and other solutes. The contact between the solution containing the bioactive compound and the supercritical fluid is promoted through the injection of the solution through a nozzle into a pressurized chamber. When the supercritical fluids contact with the solution, the solubility of bioactive compound(s) and other solutions in the atomized particles is reduced, resulting in supersaturation, nucleation, and formation of microparticles. The organic solvent is then eliminated through a continuous flow of the supercritical fluid (Mattea et al., 2009; Sosa et al., 2011; Visentin et al., 2012).

In the case of particles from gas saturated solutions procedure (iii), the supercritical fluids act as a solute. The bioactive compound(s) and other solutes are saturated with the supercritical fluid. Then, occurs the expansion of the gas-saturated solution through an atomization nozzle, which allows the formation of microparticles. During the release of the supercritical fluid, a cooling effect is verified. The occurrence of this cooling effect is directly responsible for the formation of microparticles (Mattea et al., 2009; Ozkan et al., 2019).

Table II.1.2 - Advantages and disadvantages of microencapsulation techniques of phenolic-rich extracts obtained from olive oil by-products

Method	Advantages	Disadvantages	References
Freeze-drying	-Capacity of retaining high amount of antioxidants	-Multi-stage process	Ozkan et al. (2019)
	-Simple process	-Long-time process	Chen et al. (2015)
	-Low operating temperature	-High operating costs	Haryani & Kunz, (2011)
	-Drying in the absence of an oxidation atmosphere (e.g., air)	-Presence of porous structures during the sublimation of ice	Desai & Park (2007)
	-Suitable technique for the coating of heat-sensitive and/or water-soluble compounds	-Difficult control of particle size distribution	Nandharamakrishnan et al. (2010)

Table II.1.2 - Advantages and disadvantages of microencapsulation techniques of phenolic-rich extracts obtained from olive oil by-products (cont.)

Method	Advantages	Disadvantages	References
Spray-drying		-Not suitable for the coating of thermosensitive compounds	
	-Scalable	-High energy demand and sophisticated equipment	Aguiar et al. (2016)
	-Fast procedure	-High possibility of compounds degradation	Bhandari et al. (2007)
	-Allows continuous operation	-Low yields of production due to loss of particle on the drying vessel	Dalmoro et al. (2012)
	-Low moisture content of the final powder		Zhu et al. (2014)
	-Economic	-Wide particle size distribution	Ozkan et al. (2019)
	-Reproducible	-Difficult control of particle size and shape	
		-Selection of wall materials is limited	
		-Wall materials with a low glass transition temperature and stickiness are challenging to process	

Table II.1.2 - Advantages and disadvantages of microencapsulation techniques of phenolic-rich extracts obtained from olive oil by-products (cont.)

Method	Advantages	Disadvantages	References
Emulsification/Solvent Evaporation	-Includes a broad range of approaches as water-in-oil-in-water, oil-in-water-in-oil, water-in-oil, water-in-oil, and multiple emulsions. -Easy to scale up -Accessible to scale-down (e.g., production of nanoparticles) -Use of mild conditions -Lower residual solvent content -No change on the activity of the bioactive compounds coated	-Use of organic solvents -Time-consuming technique	Hwisa et al. (2013) Aguiar et al. (2016) Ozkan et al. (2019)

Table II.1.2 - Advantages and disadvantages of microencapsulation techniques of phenolic-rich extracts obtained from olive oil by-products (cont.)

Method	Advantages	Disadvantages	References
Supercritical Assisted Atomization	-High-performance technique -Continuous operating mode -High selectivity due to the possibility to adjust both temperature and pressure -Mild conditions operation -Solventless or amount of solvent significantly reduced -Easy to scale-up	-Generally, carbon dioxide is used as the solvent; however, it is not a suitable solvent for polar compounds -Difficult to coat bioactive compounds using polymers with a low glass transition temperature -Adjustable for the incorporation of hydrophobic compounds. Prior modifications on hydrophilic compounds are required in order to be coated (e.g., water-soluble proteins)	- Kesente et al. (2017) - Parhi & Suresh (2013) - Chimica et al. (2005) - Davies et al. (2008) - Adami et al. (2011)

1.4.1.2.2 Valorization of phenolic-rich extracts from olive oil by-products through microencapsulation

To the authors best knowledge, this is the first review focused on the main studies regarding the encapsulation of olive oil by-products such as olive leaves, olive oil pomace extracts, olive oil wastewater extracts, and even olive kernel (search criteria: all databases; keywords: olive leaves, olive stones; olive kernel; olive oil wastewater; encapsulation; microencapsulation; August 2019; studies regarding the encapsulation of phenolic-rich extracts obtained from OOBP cosmetic and pharmaceutical applications were not considered for this literature review). Studies regarding the encapsulation of extracts from OOBP are presented in Table II.1.3.

An incomparable number of studies regarding the encapsulation of olive leaves extracts were found compared to the ones found concerning the encapsulation of extracts from other olive oil residues (pomaces, wastewaters and olive kernel). A thesis that allows supporting this observation is proposed now on by the authors of this review article. This is probably related to the fact of olive leaf extracts are commercially available, while in the case of extracts from olive oil pomace, olive oil wastewater and olive kernel are always necessary the prior obtainment of the raw material for encapsulation – the extract. Moreover, some studies reported the use of a spray-dryer to obtain extract-loaded microparticles, which is straightforward in the case of extracts of lipophilic nature, as is the case of olive leaves, since the process consists in the formulation of an oil-in-water emulsion which is then dried in an atomizer chamber. Therefore, lipophilic extracts are easily emulsified in aqueous phases containing water-soluble polymers.

As regards, the majority of phenolic compounds present in the olive drupes are retained in aqueous phases as the octanol/water partition coefficients of these compounds are, generally, favorable to the aqueous phase. Therefore, a considerable amount of phenolic compounds (around 98%) are retained in OOBP, such as in OOWW (around 53%) and OOP (approximately 45%) (El-Abbassi et al., 2014).

Dissimilarly, the incorporation of hydrophilic extracts is not favored by the spray-drying technique. Therefore, alternative approaches as emulsification techniques should be applied to obtain extracts-loaded microparticles from OOWW and OOP.

Contrary to extracts obtained through the spray-drying technique, these extracts should be incorporated in polymers soluble in organic solvents.

Concerning the encapsulation of olive leaf extracts, in the eight studies found, three of them presented results regarding the encapsulation of extracts through the spray-drying process. Out of three studies found had employed emulsification strategies to encapsulate olive leaf extracts. The application of nanoprecipitation was proposed by Kesente et al. (2017) as an alternative encapsulation approach for the incorporation of extracts in a polymeric shell intended for cosmetic applications; therefore, this study was not included in the revision presented in Table II.1.3. Additionally, two studies regarding the encapsulation of olive leaf extracts by freeze-drying were also found in the literature review.

A considerable variety of polymers from natural to synthetic origin were used as encapsulating materials of olive leaf extracts as insulin, soybean oil, chitosan, maltodextrin, sodium caseinate, and Arabic gum. The authors Taghvaei et al. (2014) used a blend of soybean oil with maltodextrin to study the storage stability of the encapsulated extract. On their turn, Mohammadi et al. (2016b) used a complex of whey protein concentrate and pectin to study the release behavior during mid-term storage conditions.

Regarding the encapsulation of OOP extracts, all of the three studies found in the literature used maltodextrin as the encapsulating agent, being the spray-drying technique the most applied one. Supercritical assisted atomization was proposed as an alternative encapsulation technique for the inclusion of OOP extracts in maltodextrin polymeric matrix by Aliakbarian et al. (2017).

About the encapsulation of OOWW extracts, only two studies were found. They evaluated the possibility of incorporation of OOWW extracts in polymeric matrices by spray-drying and freeze-drying encapsulation processes.

The inclusion of olive leaf extract in a β -cyclodextrin matrix was studied by Ourtzinos et al. (2007). Results demonstrate some of the main advantages of using inclusion/encapsulation techniques: the extract was efficiently protected against oxidation. Complexes were stable at high temperatures, unlike the pure extract.

Additionally, the olive leaf extract in β -cyclodextrin increased the aqueous solubility of phenolic compounds residues.

The encapsulation of olive leaf extracts in insulin was performed by Urzúa et al. (2017). Results pointed out that the total phenolic content of particles was considerably lower than the extract (around 21% of the initial phenolic content). The authors tested the incorporation of loaded microparticles in starch–gluten matrices. Like previously, essential features of encapsulation approaches were verified: the microencapsulation of the extract had a protective role in the degradation of phenolic compounds when atmospheric frying was applied. In the case of using the vacuum frying approach, the protective effect was not observed due to lower frying temperature.

In two complementary studies, Mohammadi et al. (2016), evaluated the particle size distribution alongside time when particles were produced by emulsification processes. In both studies, the authors concluded that the encapsulation of olive leaf extract in whey protein concentrate - pectin complex improved antioxidant capacity and allowed a sustained release of bioactive compounds.

In the early times of encapsulation progress, when the research of encapsulation and characterization techniques had started, Kosaraju et al. (2006) efficiently incorporated olive leaf extracts in chitosan microparticles. Even though the results were quite far from expected (encapsulation efficiency around 27%), in fact, these authors made use of some relevant characterization techniques in encapsulation processes as the Fourier Transform Infrared Spectroscopy (FT-IR) and the dynamic scanning calorimetry (DSC). Later, these authors encapsulated olive leaf extracts using sodium caseinate as the encapsulating material. They evaluated the retention of antioxidant activity of phenolic compounds after the encapsulation process, and they found the maximum radical scavenging activity around 60%. Therefore, they concluded that, is possible significant retention of antioxidant activity of the initial extract, after encapsulation procedures.

The antioxidant activity of olive leaf extract encapsulated was evaluated by Taghvaei et al. (2014), separately, in Arabic gum and maltodextrin. Moreover, particles storage stability was evaluated for 20 days.

They verified that only a small amount of phenolic compounds were released during the 20 days at 55 °C. The encapsulation approach employed was able to protect the extract from decomposition during storage at 55 °C.

The comparison of the encapsulation of two different OOBP extracts (olive leaf extract and olive kernel extract) was performed by Chanioti et al. (2016). After the optimization of extraction conditions, they evaluated a vast number of distinguishing features of the obtained powder through microparticles characterization techniques. It can be concluded that even though the encapsulation efficiency of olive leaf extracts was slightly higher (around 1%), the antioxidant capacity of encapsulated olive kernel extract was surprisingly high.

The studies found regarding the encapsulation of OOP extracts are based on the optimization of operational encapsulation conditions. However, the results presented by Aliakbarian et al. (2017), revealed that the total phenolic content of OOP extract-loaded particles was higher using the supercritical assisted atomization in optimized conditions when compared to the results obtained by Paini et al. (2015) and Aliakbarian et al. (2018) when they encapsulated OOP extracts by spray-drying using maltodextrin in optimized conditions. In the study conducted by Paini et al. (2015), microcapsules were stable under storage conditions for 70 days at 5 °C in darkness. It was only verified a degradation of 21% after increasing the storage temperature up to 25 °C. The results from light exposure of microparticles revealed a 66% loss in phenolic compounds after 48 hours.

Regarding the studies of the encapsulation of OOWW extracts, the results obtained by Petrotos et al. (2012) demonstrated once again the protective effect of encapsulated phenolic compounds: when they incorporated loaded particles in yogurt, they verified a protective effect against undesirable pH drop during yogurt storage.

The results presented in this literature review are outstanding significant as in all the studies, the authors revealed the adequate protection of phenolic compounds present in different OOBP as olive leaves, kernel, olive oil wastewater, and olive oil pomace.

Encapsulation approaches allowed the adequate protection of extracts from oxidation, high temperatures, also demonstrating the protective effect of the phenolic compounds at atmospheric frying when leaf-extract loaded particles were incorporated in a starch matrix for frying applications.

Accordingly, new insights in the research areas of (i) OOBP management and disposal, in (ii) microencapsulation techniques and (iii) fortification of foods with phenolic-rich extract loaded microparticles may unlock innovative applications of OOBP in the future. Alternative scientific areas will benefit from the microencapsulation of phenolic compounds rich-extracts obtained from olive oil by-products. Firstly, it will aid the OOBP management and disposal. Afterward, the food industry may benefit from the extraction and incorporation of OOBP extracts in particles for the obtainment of added-value products, resistant of a wide range of environmental conditions and simultaneously allowing a delayed or sustained release of phenolic compounds that have been demonstrated positive health benefits, increasing food products shelf-life.

1.4.2 Identification and quantification of phenolic compounds

For the valorization from olive oil by-products is crucial the quantification of the total phenolic content of the obtained extracts from OOBP, the quantification of the amount of phenolic-rich extracts encapsulated and the amount of phenolic compounds released over time. Moreover, the quantification of the total amount of phenolic compounds released over time is paramount for the evaluation of phenolic compounds release pattern both in the fortified foods as well as during the gastrointestinal release. Simultaneously, it could be intended for the evaluation of the radical scavenging potential of extracts, loaded microparticles, and the phenolic compounds released.

Additionally, to the quantification of the total phenolic content and the total antioxidant capacity, it may be required the identification of specific phenolic compounds during the different instars of the valorization of phenolic compounds present in OOBP.

The evaluation of the total phenolic content is generally performed employing the Folin-Ciocalteu method.

This spectrophotometer method is reported to be widely used for the estimation of the total amount of phenolic compounds present in extracts obtained from olive oil by-products (Cioffi et al., 2010). This colorimetric method is based on the electron transference between the Folin-Ciocalteu reagent and phenolic compounds. Alternatively, it can be defined as a method that detects calorimetrically a product obtained from the reduction of phosphotungstic-phosphomolibdic complex. Even though is easy to implement, this method presents several drawbacks as the possibility of the interference of other reducing agents as ascorbic acid, vitamin E, vitamin C, carotenoids, reducing sugars, and even certain aminoacids. Moreover, an additional limitation is the impossibility of the detection and quantification of individual compounds (Sánchez-Rangel et al., 2013; Singleton et al., 1999).

For the quantification of radical scavenging activity/capacity of extracts obtained from OOBP, the encapsulated amount of antioxidants in polymeric shells and the amount of antioxidants released from microparticles over time can be accomplished determining the presence of alternative radicals as (i) the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and (ii) the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical. Standardized assays can also be used for the quantification of the radical scavenging activity as (i) the oxygen radical absorbance capacity (ORAC) assay, (ii) the superoxide dismutase (SOD) assay, (iii) the ferric reducing antioxidant potential (FRAP) assay, among others (Dudonné et al., 2009). The radical scavenging activity can be alternatively evaluated through the formation of Cu^{2+} -antioxidants complexes, as described by Jabeen et al. (2017).

For the identification and quantification of specific phenolic compounds present in OOBP, it is commonly applied a chromatographic technique as the reverse-phase (RP) high-performance liquid chromatography (HPLC).

The detection of phenolic compounds using a RP-HPLC can be performed using two detectors – the diode array detector (DAD) and the fluorescence (FL) detector – that can be used individually or simultaneously. The detection and quantification of phenolic compounds extracted from OOBP are based on the ultra-violet absorption spectra in the case of using the DAD and on the maximum fluorescence excitation and emission wavelengths in the case of using the FL detector.

Different compounds present alternative maximum absorption (in the case of the DAD) wavelength ($\lambda_{\max}$) and differing maximum excitation and emission wavelengths in the case of using the FL detector (Alu'datt et al., 2010; El-Abbassi et al., 2012). The compounds found in high amounts in olive oil by-products present alternative maximum absorption wavelengths (e.g. $\lambda_{\max}$ of hydroxytyrosol: 236 nm and 280 nm; $\lambda_{\max}$ of tyrosol: 234 nm and 275 nm; $\lambda_{\max}$ of oleuropein: 244 nm and 275 nm) (Klen, 2012). Some compounds found on OOBP (e.g., tyrosol, hydroxytyrosol, vanillic acid, apigenin-7-O-glucoside) present a maximum fluorescence wavelength of excitation and emission at 280 nm and 330 nm, respectively (Obied et al., 2007). However, even though when both detectors are combined, the identification of complex or unknown phenolic compounds can be a challenging task.

Additionally, when occurs, the co-elution of compounds that present similar spectra and/or are verified low limits of detection, and quantification of phenolic compounds is compromised (Suárez et al., 2009a). In these cases, is widely recommended to use a mass spectrometer (MS), which allows a specific analysis based on the molecular ion and fragmentation patterns for the identification of dubious compounds. The confirmation of the presence of specific phenolic compounds in OOBP can be performed associating an electrospray ionization (ESI) interface with liquid chromatography (LC)-MS through the fragmentation of the molecular ion and the identification of the respective molecular mass (De Marco et al., 2007). The authors Cardoso et al. (2011) identified seven new isomers of oleuropein and ligstroside derivates using ESI-MS and ESI-MSⁿ.

Other alternatives have been exploited to confirm the presence of specific phenolic compounds or identify them as the time-of-flight mass spectrometry (TOF-MS), which allows the determination of the m/z through time measurement. The authors Lozano-Sánchez et al. (2014) identified several phenolic compounds and their related derivates present in OOP as hydroxytyrosol, oxidized hydroxytyrosol, decarboxymethyl-oleuropein aglycone, decarboxymethyl-ligstroside aglycone, luteolin, and 10-hydroxy-oleuropein aglycone. It is also described the use of rapid resolution liquid chromatography (RRLC) and ultra-performance liquid chromatography (UPLC) associated with MS (Herrero et al., 2011; Lozano-s et al., 2011).

The reduction of the particle size in the column results in the increase of the linear velocity and the peak capacity – the number of separated peaks per unit of time – also increases. The RRLC and the UPLC systems use smaller particles ($< 2\ \mu\text{m}$) than the typical HPLC system (particle size $\geq 3\ \mu\text{m}$), which contributes to an increase of sensibility and the speed of samples running. The authors Suárez et al. (2009) compared the chromatographic separation of phenolic compounds extracted from olive cake by HPLC and UPLC. They successfully separated phenolic compounds in 24 minutes using the UPLC and in 60 minutes, using the HPLC apparatus.

From this perspective, during the choice of the analytical method to employ should be considered which are the main purposes of the study (if it is to identify specific compounds increasing the complexity of the process; if it is only necessary to quantify the total amount of phenolic compounds), the availability of analytical apparatus, the feasibility of the study, the time of analysis, the consumption of solvents among others.

A combination of chromatographic and spectrophotometric quantification methods should be carefully considered as both methods' present limitations.

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts

Core	Shell	Method	Objectives	Results	Ref.
Olive leaf extract	β -cyclodextrin	Freeze-drying	-Study the inclusion of OLE through DSC and nuclear magnetic resonance spectroscopy -Determination of OLE solubility in the presence of β-cyclodextrin by a phase solubility study	-Oxidative DSC studies indicated that the complex of OLE with β-cyclodextrin was protected against oxidation since it remained intact at temperatures where the free OLE was oxidized -Phase solubility studies demonstrated that inclusion OLE in β-cyclodextrin increased the aqueous solubility of the polyphenolic residue from the OLE by more than 150%	Mourtzinou et al. (2007)
	Sodium caseinate-soy	Spray-drying	-Evaluation of the retention of AA	-Maximum radical scavenging activity ~ 60% (OLE concentration of 0.5 mg/mL)	Kosaraju et al. (2008)

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts (cont.)

Core	Shell	Method	Objectives	Results	Ref.
Olive leaf extract	Insulin	Spray-drying	-Optimization of encapsulation conditions using a DoE (CCD) -Determination of EE of oleuropein, a compound present in the OLE -Study the effect of microencapsulation and the frying method on the TPC and AA, fat content, and crispness, after adding MPs to starch–gluten fried matrices	-TPC of OLE: 64.3 mg of gallic acid equivalents/g of dry leaves -OLE PY: 90.1% -EE of oleuropein: 87.1% -TPC in MPs: 13.2 mg of gallic acid equivalents/g of MPs -The frying method affects the content of polyphenols -Vacuum fried matrices show high phenolic content than atmospheric fried matrices	Urzúa et al. (2017)

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts (cont.)

Core	Shell	Method	Objectives	Results	Ref.
Olive leaf extract	Poly(lactic acid)	Nanoprecipitation	-Encapsulate OLE in poly(lactic acid) NPs -Characterization the NPs and define the experimental parameters that affect the encapsulation procedure. -Incorporation of NPs in a cosmetic formulation -Study the stability of the formulation for 3 months	-NPs exhibited anionic ζ-potential -Burst release at the first 2 hours -Increased stability of loaded NPs compared to the extract regarding viscosity, pH among others	Kesente et al. (2017)
	Complex of whey	Water-in-oil-in-water emulsification	-Evaluation of AA of OLE encapsulated through a nano-emulsification approach	-TPC: 206.81 ± 0.02 mg of gallic acid equivalents/g of sample -Nanoencapsulation of OLE in whey protein concentrate - pectin complex improved AA and allowed a sustained release of bioactive compounds	Mohammadi et al. (2016b)

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts (cont.)

Core	Shell	Method	Objectives	Results	Ref.
Olive leaf extract	Arabic gum and maltodextrin	Oil-in-water-in-oil emulsification	-Evaluation of the AA of OLE encapsulated by Arabic gum and maltodextrin, in soybean oil at OLE concentration of 70 mg/kg -Evaluation of storage stability of OLE-Arabic gum and maltodextrin-soybean oil for 20 days	-Slow-release of phenolic compounds: 20 to 30 mg/kg of phenolics was not released after 20 days storage at 55 °C (initial inclusion amount of 70 mg/kg) -Encapsulation can protect phenolic compounds OLE from decomposition during storage at 55 °C -The AA of encapsulated extracts was lower than the corresponding non-encapsulated contrapeneur	Taghvaei et al. (2014)
	Complex of whey protein concentrate	Water-in-oil-in-water emulsification	-Study the behavior of water-in-oil-in-water double emulsion encapsulating OLE in a complex of whey protein concentrate and pectin	-EE (day 1): In whey protein concentrate only: 93.34% In whey protein concentrate-pectin complex: 96.64% -EE (day 22): In whey protein concentrate only: 72.73% In whey protein concentrate-pectin complex: 88.81% -Total amount released (20 days, 30 °C): Using only whey protein concentrate: 22% Using whey protein concentrate-pectin complex: 8.1%	Mohammadi et al. (2016a)

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts (cont.)

Core	Shell	Method	Objectives	Results	Ref.
Olive leaf extract	Chitosan	Spray-drying	-Preparation of chitosan OLE loaded MPs -Understand the structural interactions caused by the inclusion of the extract in chitosan MPs by FTIR and DSC -Estimation of the encapsulation loading of phenolic compounds	-From FTIR studies was verified that occurred interaction between the hydroxyl/carboxyl/aldehyde groups of the OLE and the amine - the functionality of the chitosan molecules -From DSC studies was verified a shift in T_g from PO-MP to L-MP supporting the efficient encapsulation of polyphenolic compounds in chitosan-based matrices -Loading content of 27% in gallic acid equivalents	Kosaraju et al. (2006)
	Maltodextrin	Freeze-drying	-Choose the best extraction technique comparing conventional <i>versus</i> alternative extraction techniques -Perform the encapsulation of the extracts -Evaluation of MPs hygroscopicity, solubility, MC, and EE	-MPs obtained by conventional extraction (1 h, 60 °C) showed the best encapsulation features: EE: $91.06 \pm 1.40\%$ MC: $0.78 \pm 0.04\%$ Water activity: 0.16 ± 0.02 (at 25 °C) Hygroscopicity: 26.59 ± 0.07 g H₂O/100 g of dry weight Solubility: $98.19 \pm 0.12\%$ AA: 0.61 ± 0.05 mg Trolox equivalents/g of raw material	Chanioti et al. (2016)

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts (cont.)

Core	Shell	Method	Objectives	Results	Ref.
Kernel/stones extract	Maltodextrin	Freeze-drying	-Choose the best extraction technique comparing conventional <i>versus</i> alternative extraction techniques -Perform the encapsulation of the extracts -Evaluation of MPs hygroscopicity, solubility, MC, and EE	-MPs obtained by conventional extraction (1 h, 60 °C) showed the best encapsulation features: - EE $90.12 \pm 1.87\%$ - MC: $0.99 \pm 0.01\%$ - Water activity: 0.16 ± 0.00 (at 25 °C) - Hygroscopicity: 22.88 ± 0.13 g H₂O/100 g of dry weight - Solubility: $97.41 \pm 0.11\%$ - AA: 1.25 ± 0.05 mg Trolox equivalents/g of raw material	Chanioti et al. (2016)

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts (cont.)

Core	Shell	Method	Objectives	Results	Ref.
Olive pomace extract	Maltodextrin	Supercritical Assisted Atomization	-Study the effect of the ratio of maltodextrin content to the total solid content of the extract and drying temperature on physical characteristics, TPC and AA of the powdered product	-TPC: 105.0 ± 0.1 mg of caffeic acid equivalents/g of dry powder -AA: 98.8 ± 3.0 mg of DPPH/mL of extract -WSIs: 53.0 ± 0.7 - $67.0 \pm 1.0\%$ -WAI: $0.28\text{--}0.58$ g/g of dry powder -PY: 46.0 and 65.3% -SC higher than 2.4 ± 0.0 g/g of dry powder, with the maximum SC obtained of 3.2 ± 0.0 g/g of dry powder when the extract was encapsulated using 30% of encapsulating material and treated at 75 °C -MC lower than 6% w/w	Aliakbarian et al. (2017)

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts (cont.)

Core	Shell	Method	Objectives	Results	Ref.
Olive pomace extract	Maltodextrin	Spray-Drying	-Study different concentrations of maltodextrin as a coating material -Study the effects of inlet temperature (130 °C and 160 °C) and feed flow (5 mL/min and 10 mL/min) in the spray-dryer -Evaluation of AA and stability of MPs	-The high inlet temperature was associated with lower MC and bulk density without affecting AA -The increase of maltodextrin concentration caused lower bulk density and higher MPs sizes, while higher feed flow leads to an increased MC -Fixing the operational conditions at an inlet temperature of 130 °C, maltodextrin concentration at 100 g/L and a feed flow of 10 mL/min resulted in: PY: $94 \pm 0.4\%$ EE: $76 \pm 3.3\%$ TPC: 39.5 ± 4.9 mg of caffeic acid equivalents/g of dry powder AA: 33.8 ± 4.3 mmol of DPPH/L of extract -MPs were stable at 5 °C in dark condition for 70 days, and only 21% were degraded, increasing storage temperature up to 25 °C. Light exposure resulted in a 66% loss in polyphenols after 48 hours	Paini et al. (2015)

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts (cont.)

Core	Shell	Method	Objectives	Results	Ref.
Olive pomace extract	Maltodextrin	Spray-Drying	-Optimize spray-drying conditions for the MPs of olive pomace extract, stabilizing its phenolic compounds. -Optimization approaches study using a RSM or ANN -Optimization variables considered were: Maltodextrin concentration: 100 – 500 g/L Inlet-drying temperatures: 130 – 160 °C Drying compressed flowrates: 20 – 32 m³/h	-At optimized conditions: PY: 65 – 82% MC: 9 – 14 µg/100 g of dry powder WSI: 64 – 65% Specific TPC: 38 – 52 mg of caffeic acid equivalents/g of dry powder Specific AA: 230 – 487 µg of Trolox equivalents/ g of dry powder EE: 85 – 92%	Aliakbarian et al. (2018)

Table II.1.3 - Studies regarding the encapsulation of olive oil by-products extracts (cont.)

Core	Shell	Method	Objectives	Results	Ref.
Olive Oil Wastewater extract	Whey protein isolate and xanthan gum	Spray-drying	-Study the effects of OMWW extract concentration, whey protein isolate concentration, xanthan gum concentration on physical stability, viscosity, emulsion droplet size, particle size distribution primary and secondary oxidation products, under accelerated storage conditions. -Application of a RSM as a DoE for process optimization	-The effects of OMWW phenolic extracts, the concentration of both whey protein isolate and xanthan gum were statistically significant on emulsion creaming rate, viscosity among other responses studied -The performance of the RSM applied was considered accurate for creaming rate and viscosity	Caporaso et al. (2016)
	Modified starch	Freeze-drying	-Enrichment of non-encapsulated and encapsulated OMWW extracts of yogurt	-TPC: 20% w/w -A protective effect against undesirable pH drop during yogurt storage was verified	Petrotos et al. (2012)

AA – Antioxidant Activity; ANN -Artificial Neural Network; CCD – Central Composite Design; DoE – Design of Experiments; EE – Encapsulation Efficiency; FTIR – Fourier Transform Infrared Spectroscopy; L-MPs – Loaded-Microparticles; MC – Moisture Content; MPs – Microparticles; NPs – Nanoparticles; OLE – Olive Leaf Extract; PO-MPs – Polymer-only Microparticles; PY – Product Yield; Ref. – References; RSM – Response Surface Methodology; SC – Swelling Capacity; T_g – Glass Transition Temperature; TPC – Total Phenolic Content; WAI – Water Absorption Index; WSI – Water Solubility Index;

1.5 Perspectives and future challenges

Olive oil has been produced since ancient times; however, the awareness for the problematic of olive oil by-products management and disposal is considerably recent.

In the regard of the conscious awareness of the negative environmental impact of phenolic compounds present in olive oil by-products (olive oil pomace, olive leaves, olive oil wastewater and olive kernel) combined with the finding of innovating alternative valorization strategies for extraction and encapsulation approaches, arise a potential pathway for the obtainment of added-value food products fortified with encapsulated phenolic-rich extracts.

It is expected that encapsulation approaches associated with extraction techniques of phenolic compounds with solvent generally recognized as safe will contribute positively to the environmental problem as well as improve or redesign added value products for the food industry.

In the best expectations, research will be focused on the combination of extraction and encapsulation techniques for the recovery of phenolic compounds from olive oil by-products, addressing the environmental problem associated to the presence of phenolic compounds in olive oil by-products, answering the market demand of innovative products. Moreover, it is expected that comprehensive studies should be conducted in the scope of the fortification of foods with phenolic-rich extracts loaded microparticles. An integrated approach regarding the fortification of foods with loaded microparticles and *in vivo* absorption, transportation, and release of phenolic compounds enclosed in emulsions should be considered and evaluated.

1.6 Conclusions

The olive oil is one of the most consumed edible oils around the world, mostly due to the success of the Mediterranean diet in which olive oil intake is associated to many health benefits as some compounds present in olive oil may prevent cardiovascular diseases, may lower the blood pressure, may have a positive effect on inflammation processes and in endothelial dysfunction.

The worldwide recognition of the importance of the consumption of olive oil fostered its production intensively. Olive oil can be extracted from olive drupes through continuous and discontinuous processes. During olive oil production, different olive oil by-products are generated as olive leaves, olive oil wastewaters, olive oil pomace, and olive stones/kernel.

These olive oil by-products constitute, nowadays, an environmental problem regarding its disposal and management. Moreover, the majority of phenolic compounds remain in the olive oil by-products (around 98%) due to their higher affinity for aqueous systems rather than oily phases. Therefore, another problem arises: the high content of phenolic compounds in olive oil by-products is responsible for the phytotoxic and biotoxic of these by-products.

One of a potentially innovative strategy to valorize olive oil by-products is to recovery phenolic compounds which have many health benefits from these by-products through the obtainment of extracts that can be encapsulated. Encapsulation strategies may protect the extracted phenolic compounds from olive oil by-products for the external environment, improve extracts antioxidant capacity and stability to mid to long term storage, to high temperatures, oxidation conditions, and undesired pH drops, allowing a delayed or sustained release of these valuable compounds.

Olive oil by-products extracts encapsulated in polymeric matrices for further incorporation in complex matrices as foods, is expected to aid the olive oil sector regarding the problematic of the disposal and management of olive oil by-products and also aid food industry to reinvent through the obtainment of added-value products as fortified foods with phenolic-rich extract loaded microparticles that may exhibit outstanding radical scavenging activities.

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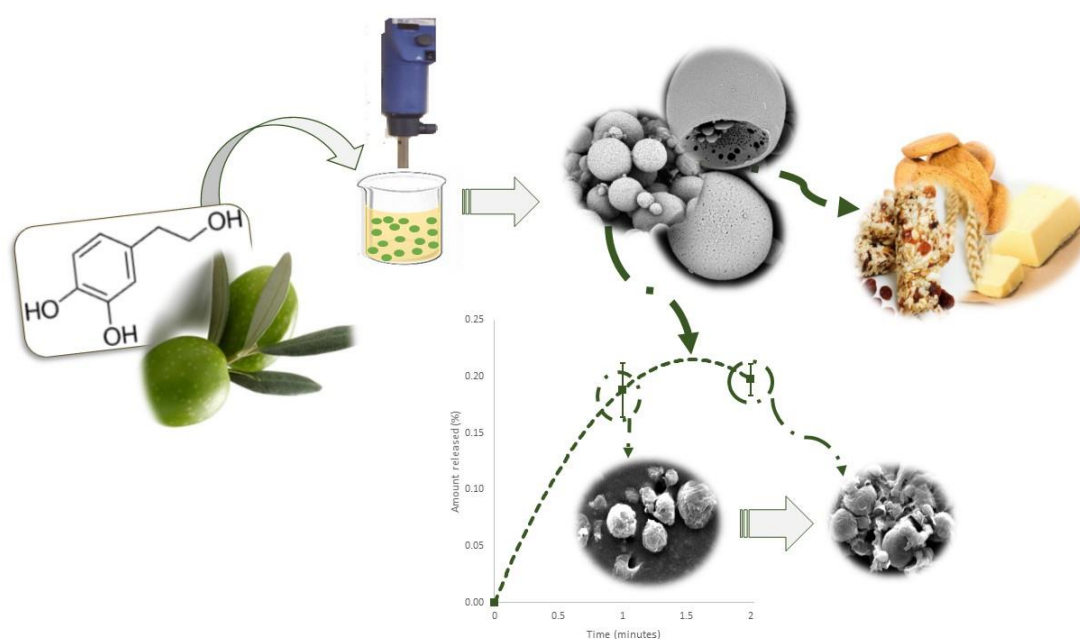
Part III: New insights in the individual encapsulation of phenolic compounds present in olive mill residues – preparation, characterization and *in vitro* release studies of loaded microparticles

In this section, the experimental procedures, and results regarding the individual encapsulation of the most abundant phenolic compounds present in the olive mill pomace namely hydroxytyrosol and tyrosol are presented. Chapter 2 and 3 are devoted to the assessment of the efficient embedment of hydroxytyrosol and tyrosol into microparticles, respectively. In Chapter 4 are presented the principal results regarding the *in vitro* gastrointestinal release studies of tyrosol from loaded microparticles.

Chapter 2. Inclusion of hydroxytyrosol in ethyl cellulose microparticles: *in vitro* release studies under digestion conditions ²

Filipa Paulo, Lúcia Santos

Graphical Abstract:



² This chapter was adapted from: Paulo, F., and Santos L. 2018. Inclusion of hydroxytyrosol in ethyl cellulose microparticles: *in vitro* release studies under digestion conditions. *Food Hydrocolloids*, 84, 104-116.

Abstract:

Hydroxytyrosol, existing at high concentrations in olive oil, presents remarkable health benefits mainly due to its antioxidant properties. Nevertheless, the inclusion of hydroxytyrosol in functional foods and pharmaceutical compounds is restricted due to its high hydrophilicity. This study aimed to obtain hydroxytyrosol-loaded microparticles using ethyl cellulose as a carrier. Microparticles were produced by double emulsion solvent evaporation technique considering two theoretical loadings (5 and 10%). Microparticles were obtained and characterized concerning encapsulation efficiency, product yield, wettability, thermal transitions, particle size distribution, morphology and total antioxidant capacity. *In vitro* release studies were performed using simulated gastrointestinal fluids. Additionally, a morphological degradation analysis was accomplished. The maximum encapsulation efficiency and product yield were $88.1 \pm 0.1\%$ and $82.4 \pm 2.1\%$, respectively, using the formulation with the lowest loading. Hydroxytyrosol-loaded ethyl cellulose microparticles were nearly stable in a temperature range of 30 °C to 280 °C. The mean particle size ranged from $156.6 \pm 6.9 \mu\text{m}$ to $304.0 \pm 16.0 \mu\text{m}$. The antioxidant capacity retainment remained above 50%. Results demonstrated the effectiveness of gastro-resistance of the produced microparticles. The morphological degradation analysis suggested a bulk-to-surface erosion controlling process. The findings of this research indicate the possible application of microencapsulated hydroxytyrosol in foods, drugs, and nutraceuticals.

Highlights:

1. Hydroxytyrosol (HT) presents health benefits due to its antioxidant properties.
2. The inclusion of HT in foods is restricted due to its poor bioavailability.
3. Double emulsion solvent evaporation technique was applied for HT encapsulation.
4. The antioxidant capacity was kept above 50% in gastrointestinal simulated fluids.
5. HT-loaded ethyl cellulose microparticles shown to be the gastro-resistant.

Keywords: hydroxytyrosol; microencapsulation; ethyl cellulose; antioxidant; release studies; simulated gastrointestinal fluids; double emulsion;

2.1 Introduction

The growing interest of some food industries to develop functional food products enhanced with natural food additives has driven research in the food industry area for the obtainment of bioactive ingredients from natural raw sources or food production by-products (Da Silva et al., 2013).

Remarkably, olive oil, the olive tree (*Olea europaea* L.) and their plant components have been identified as sources of many natural compounds associated to an improvement of human health (Caporaso et al., 2016). These products present many monosaturated fatty acids as well as antioxidants, specifically, polyphenols. The polyphenols existing in these products are responsible for the antioxidant stability and organoleptic properties of olive oil and its derivatives (Buckland & Gonzalez, 2015; González-Alonso et al., 2015; López-Miranda et al., 2010; Robles-Almazan et al., 2017).

Hydroxytyrosol also known as 3,4-dihydroxyphenylethanol (DOPET), 4-(2-hydroxyethyl)-1,2-benzenediol or 3,4-dihydroxyphenolethanol (3,4-DHPEA) is an amphiphilic phenol present at high concentrations in olive oil. Additionally, this compound is also present in the soluble fraction of the extra-virgin olive oil, being also one of the major components present in the leaves of the olive tree. Many studies reported that hydroxytyrosol presents many biological activities as an antioxidant, antimicrobial, antiatherogenic, anti-inflammatory and even as anti-carcinogenic compound (Angelo et al., 2005; Bernini et al., 2017; Buckland & Gonzalez, 2015; Bulotta et al., 2014; Covas et al., 2015; López-Miranda et al., 2010; Pérez-Bonilla et al., 2014; Robles-Almazan et al., 2017; Rodrí, 2015; Tasioula-Margari & Tsabolatidou, 2015).

The primary source of hydroxytyrosol is the hydrolysis of the phenolic compound oleuropein during the ripening of olives, the processing and storage of table olives (Charoenprasert & Mitchell, 2012; Gambacorta et al., 2007). The content of hydroxytyrosol in olive oil depends on the type of olive tree, plantation, location and the olive oil processing (Romero et al., 2004). During olives processing to obtain olive oil from crushing, three types of products are obtained, such as the i) olive mill wastewater, ii) olive pomace and iii) olive oil. These three types of products have polyphenol-enriched compounds.

Considering the physicochemical amphiphilic property of hydroxytyrosol, this compound may be found in the three types of products aforementioned in its free form, in the acetate form or more complex forms as verbascoside, oleacein and oleuropein (Boskou, 2008; Robles-Almazan et al., 2017).

Nevertheless, some authors outline that the hydroxytyrosol and its by-products from mechanical oil extraction are not commonly found in their free form but generally as derivatives of oleuropein and demethyloleuropein (Fabiani et al., 2006; Robles-Almazan et al., 2017; Servili et al., 2004).

Hydroxytyrosol may also be found in red wines (3 mg/L), aged red wines (20 mg/L) and white wines (2 mg/L). However, the concentration of hydroxytyrosol in these beverages is reported to be lower than in olive tree leaves (12 mg/g) (Di Tommaso et al., 1998; Fernández-Mar et al., 2012; Minuti et al., 2006; Quirantes-Piné et al., 2013; Ramírez-Tortose et al., 2014).

Many studies have been carried out to associate the hydroxytyrosol absorption, distribution, metabolism and excretion processes to its health benefits (Bernini et al., 2013, 2015; Mateos et al., 2011). It is described that hydroxytyrosol is widely metabolized in the bowel and the liver, suffering a dose-dependent absorption in the gut. At the intestinal absorptive cells, also known as enterocytes, hydroxytyrosol is hydrolyzed and afterward is transformed into glucuronide, methylated and sulfate by-products. When hydroxytyrosol is administrated in the olive oil form, the majority of hydroxytyrosol is detected in the plasma and urine in the form of glucuronide. Only 2% of the hydroxytyrosol unmetabolized form is detected in these fluids (Miro-Casas et al., 2003; Suárez et al., 2011). De La Torre (2008) postulates that there is clear evidence of proper absorption of polyphenols as hydroxytyrosol. However, this compound presents high hydrophilicity which constitutes the major barrier to the potential application of hydroxytyrosol in foods to increase and improve hydroxytyrosol health benefits.

In this context, microencapsulation arises as a potential technological strategy to increase the fraction of unchanged hydroxytyrosol that may reach the systemic circulation, its stability, protect this compound against oxidation and enhance its benefits for health.

Additionally, due to its antioxidant and antimicrobial properties, the microencapsulation of hydroxytyrosol seems to have a positive impact in the food industry due to its ability to retard lipid oxidation, demonstrating to have a beneficial effect on food products shelf-life and stability.

Microencapsulation is a set of technological approaches that comprises several techniques by which active compounds are coated with a polymeric shell or embedded homogeneously or heterogeneously in a polymeric matrix. The final products of microencapsulation processes are known as microparticles, microspheres, and microcapsules (Aguar et al., 2016, 2017; Casanova et al., 2016; Paulo & Santos, 2017). Several microencapsulation techniques have been applied in order to protect and controlled deliver bioactive ingredients as follow: double emulsion, emulsification-solvent evaporation, spray-drying, coacervation, milling techniques and even encapsulation techniques using supercritical fluids as described by Baena-Aristizábal et al. (2016).

The double emulsion solvent evaporation microencapsulation technique is a simple and straight forward method widely described in the literature (Ahmed et al., 2012; Baena-Aristizábal et al., 2016; Ibraheem et al., 2014; Iqbal et al., 2015a; Miladi et al., 2015a, 2015b). This technique is mostly employed by the pharmaceutical industry as described by Iqbal et al. (2015b), however, the food industry is increasingly applying the double emulsion solvent evaporation technique (Bonnet et al., 2009; Garti & Bisperink, 1998; Li et al., 2014; Sapei et al., 2012). This technique allows entrapping both hydrophilic and lipophilic molecules, according to the choice of the water-in-oil-water double emulsion (w/o/w) or the oil-in-water-in-oil double emulsion (o/w/o). The water-in-oil-in-water (w/o/w) is the most commonly used approach, in which the active ingredient is dissolved in an internal aqueous phase. The internal aqueous phase is then admixed with an organic phase containing the selected wall material dissolved in an organic solvent. Afterward, the double emulsion is formed when the primary emulsion is added and emulsified to an aqueous phase (external aqueous phase) containing a hydrophilic emulsifier. Then, the evaporation of the solvent is promoted for the microparticles formation and hardening (Raval et al., 2014).

Many biocompatible polymers are commonly used for the formulation of the organic phase as ethylcellulose, carboxymethylcellulose, casein, gelatin, whey protein, acacia gum, xanthan gum, polylactide, polycaprolactone, polyglycolide among others (Aguilar et al., 2017; Dickinson, 2011; Park et al., 2005). Ethyl cellulose is a biocompatible cellulose-derived polymer, where ethyl ether groups replaced some of the hydroxyl groups, being an attracting polymer for microencapsulation. This compound is soluble in organic solvents as ketones, alcohols, esters, and ethers but not in aqueous solutions. As ethyl cellulose only has some free hydroxyl groups in its backbone structure, can only uptake water through hydrogen bond formation between those free hydroxyl groups and water and also between the remaining oxygen atoms present in the backbone structure of ethyl cellulose and water (Murtaza, 2012). Therefore, ethyl cellulose is attractive for the formulation of the organic phase in the w/o/w double emulsion solvent evaporation technique.

This work aimed to microencapsulate hydroxytyrosol by w/o/w double emulsion solvent evaporation technique. Ethyl cellulose was employed as the wall material dissolved in dichloromethane for the organic phase preparation. The effect of hydroxytyrosol loading on the hydroxytyrosol encapsulation efficiency and the yield of production was evaluated. Additionally, hydroxytyrosol-loaded ethyl cellulose microparticles were characterized regarding wettability, thermal transitions by differential scanning calorimetry (DSC), particle size distribution and morphology by scanning light microscopy (SEM). The inclusion of hydroxytyrosol in ethyl cellulose polymer-based matrices was confirmed by Fourier Transform Infrared Spectroscopy (FTIR). Moreover, the total antioxidant capacity over time was evaluated as well as *in vitro* release studies were performed using simulated gastrointestinal fluids. A morphological degradation analysis was also performed.

2.2 Experimental procedures

2.2.1 Materials

2.2.1.1 Chemicals

The antioxidant of this study, hydroxytyrosol standard (Ref. 91404, $C_8H_{10}O_3$, CAS 10597-60-1), polyvinyl alcohol used as surfactant of the double emulsion (Ref: P8136-250G, 87-90% hydrolyzed, average molecular weight of 30,000-70,000, CAS 9002-89-5) and ethyl cellulose, used a carrier for the delivery of hydroxytyrosol (Ref. 433837-250G, viscosity of 46 cP, CAS 9004-57-3) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). Dichloromethane (Ref. ACRO433991000, CH_2Cl_2 , CAS 1665-00-5) used as a solvent was purchased from VWR International (Fontenay-sous-Bois, France). Salts were used for the production of electrolyte solutions. The salts potassium dihydrogen phosphate (Ref. 104871001, KH_2PO_4 , CAS 7778-77-0), potassium chloride (Ref. 1049380050, KCl, CAS 7447-40-7), sodium hydrogen carbonate (Ref. 1063290500, $NaHCO_3$, CAS 144-55-8), sodium chloride (Ref. 1370170001, NaCl, 7647-14-5), magnesium chloride hexahydrate (Ref. 1023675000, $MgCl_2 \cdot 6H_2O$, CAS 7791-18-6) and ammonium carbonate (Ref. 1011369051, $(NH_4)_2CO_3$, CAS 10361-29-2) were obtained from Merck kGaA (Darmstadt, Germany). A total antioxidant capacity assay kit (Ref. MAK187) was purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). The water used in this work was de-ionized and double-distilled using a Millipore™ water purification system (Massachusetts, USA) having 18.2 Ω electrical resistivity. All the reagents were either chromatographic or analytical grade and used as received.

2.2.2 Methods

2.2.2.1 Analytical methods validation

The ultraviolet-visible (UV-Vis) spectrophotometer method was employed on a UV-Vis spectrophotometer V-530 (Jasco, OK, USA) at 280 nm using quartz cells of 10 mm of a light path. The SPECTA MANAGER software was used for all the absorbance measurements.

2.2.2.2 Preparation of the hydroxytyrosol-loaded microparticles

A water-in-oil-water double emulsion solvent evaporation technique was employed to formulate hydroxytyrosol-loaded ethyl cellulose microparticles. For this purpose, two hydroxytyrosol standard solutions (500 mg/L for F-1 formulation and 1 100 mg/L for F-2 formulation) were previously prepared using double distilled water to formulate the internal aqueous phase. For each formulation, a mass of 50 mg of ethyl cellulose was dissolved in 5 mL of dichloromethane to form the polymer solution used as the oily phase. The obtained polymer solutions for F-1 and F-2 formulations were sonicated during 15 minutes in an ultrasonic bath (P Selecta, Barcelona, Spain). To the previous polymer solutions were added 5 mL of the internal aqueous phase and emulsified using a vortex shaker (IKA VORTEX GENIUS 3, Staufen, Germany) for 3 minutes to form a stable o/w primary emulsion. These o/w emulsions were added to 100 mL of polyvinyl alcohol solutions of 1 w/w% and emulsified using a high-performance homogenizer (IKA T18 Digital ULTRA-TURRAX®, Staufen, Germany), at 5 000 rpm for 5 minutes. The evaporation of the solvent and the hardening of microparticles were performed by continuously stirring the w/o/w double emulsion in a stirring plate (AREX Digital, VELP Scientifica, Monza, Italy) at 650 rpm for 3 hours in the fume hood at room temperature. Hydroxytyrosol-loaded ethyl cellulose microparticles were recovered by filtration using a 0.2 μ m quantitative paper filter and washed with 500 mL of distilled water to remove polyvinyl alcohol residues. The recovered microparticles were frozen for 24 hours at -4 °C and freeze-dried for 72 hours on a bench top freeze-dryer (SP Scientific, NY, USA).

Further considerations are presented in Table III.2.1 as the oil-water ratio (OWR) in which is considered the ratio between the volume of hydroxytyrosol solution and the polymer solution used to form the primary emulsion. Additionally, is considered the theoretical loading content (TLC %) as the ratio between the weight of hydroxytyrosol added and the weight of polymer and hydroxytyrosol added. The weight measurements were performed on an analytical scale Mettler Toledo AG245 balance (Columbus, OH, USA). The TLC (%) values chosen for this study are widely found as typical TLC (%) values in the literature. All the experiments were performed in triplicate.

Table III.2.1 - Composition of the formulations coded (F-1 and F-2)

Formulation ^a	Volume DCM (mL)	HT Conc. (mg/L)	Polymer Conc. (g/L)	OWR (v/v)	PVA Conc. (%w/w)	Volume PVA (mL)	TLC (%w/ w)
F-1	5	500	10	1	1	100	5
F-2		1 100					10

^a Each formulation was performed in triplicate

Conc. – Concentration; DCM – Dichloromethane; HT – Hydroxytyrosol; OWR – Oil-in-Water Ratio; PVA – Polyvinyl Alcohol; TLC – Theoretical Loading Content

2.2.3 Characterization of hydroxytyrosol-loaded ethyl cellulose microparticles

2.2.3.1 Determination of the hydroxytyrosol encapsulation efficiency, loading content, and yield of production

The obtained hydroxytyrosol-loaded ethyl cellulose microparticles were characterized regarding encapsulation efficiency, loading, and yield of production.

The calculation of the hydroxytyrosol encapsulation efficiency (HEE) was performed using Equation (III.2.1) and as presented by Calderón-Oliver et al. (2017) when they conducted a microencapsulation study of nisin in combination with an antioxidant avocado extract by complex coacervation.

$$HEE (\%) = \frac{W_H}{W_I} = \frac{W_I - W_O}{W_I} \quad (\text{III.2.1})$$

where W_H is the weight of hydroxytyrosol in the microparticles and W_I is the weight of hydroxytyrosol initially added for the microencapsulation. The W_H was evaluated considering the difference between the weight of hydroxytyrosol initially added (W_I) and the weight of hydroxytyrosol present in the w/o/w double emulsion after 3 hours of microparticles hardening (W_O). The W_O was evaluated after the filtration of 1.5 mL of the w/o/w double emulsion using a 0.2 μm syringe filter (Ref: 514-0070, VWR International, Fontenay-sous-Bois, France) A volume of 1 mL of the aliquot was transferred to a quartz cell, and the weight of hydroxytyrosol non-microencapsulated was calculated considering the absorbance measurement at 280 nm in UV-Vis spectrophotometer and using ultra-pure water as a blank.

The actual loading content (ALC) was calculated through Equation (III.2.2).

$$ALC (\%) = \frac{W_H}{W_M} \quad (\text{III.2.2})$$

where W_M is the weight of microparticles recovered after the freeze-drying process.

The yield of production (YP) was calculated considering Equation (III.2.3).

$$YP (\%) = \frac{W_M}{W_A} = \frac{W_M}{W_I + W_P} \quad (\text{III.2.3})$$

where W_A is the weight of hydroxytyrosol initially added for the microencapsulation (W_I) and the weight of the polymer used for the microencapsulation (W_P).

2.2.3.2 Wettability analysis

The wettability of the formulations was evaluated as described by De Barros et al. (2014). About 1 g of each formulation was sprinkled over the surface of distilled water without agitation at 20 °C. The time required for the microparticles to sink, disappear from the water's surface, to become sediment or to be submerged was recorded.

2.2.3.3 Differential Scanning Calorimetry (DSC) analysis

The Differential Scanning Calorimetry (DSC) technique was selected to assess the thermal behavior of hydroxytyrosol-loaded ethyl cellulose microparticles, pure hydroxytyrosol, polymer-only microparticles and ethyl cellulose as described by Piletti et al. (2017) with some modifications to the procedure proposed by these authors. The analyses were performed using a NETZSCH DSC 214 Polyma calorimeter (NETZSCH DSC 214 Polyma, Selb, Germany) with a temperature range of 30 °C to 270 °C at a heating rate of 10 K.min⁻¹, under a nitrogen atmosphere (flow rate of 40 mL.min⁻¹).

2.2.3.4 Fourier transform infrared spectroscopy (FTIR) studies

To evaluate if the inclusion of hydroxytyrosol in ethyl cellulose polymer-based systems occurred, the infrared spectra were recorded using a VERTEX 70 FTIR spectrometer (BRUKER) in transmittance mode with a high sensitivity DLaTGS detector at room temperature. Samples were measured in attenuated total reflectance (ATR) mode, with an A225/Q PLATINUM ATR Diamond crystal with single reflection accessory. The spectra were recorded from 4000 to 500 cm⁻¹ with a resolution of 4 cm⁻¹.

2.2.3.5 Evaluation of the morphology

A PHENOM XL scanning light microscope (Eindhoven, The Netherlands) at an accelerating voltage of 10 kV was used to evaluate the hydroxytyrosol-loaded ethyl cellulose microparticles external morphology and polydispersity. Freeze-dried microparticles were placed on an aluminum stub with a carbon double-sided adhesive tape.

The samples were sputter-coated with gold for 20 seconds using a vacuum-sputtering coater (Leica, EM SCD 500, Wetzlar, Germany) as described by Negrão-Murakami et al. (2017).

2.2.3.6 Evaluation of the particle size distribution

The particle size distribution of hydroxytyrosol-loaded ethyl cellulose microparticles was evaluated by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyzer (Miami, FL, USA) as described by Aguiar et al. (2017). The particle size distribution was recorded considering the differential volume distribution. Each formulation was analyzed in triplicate. Average values were considered in data analysis. The polydispersity of particle size distributions was analyzed regarding the SPAN Equation (Equation (III.2.4)).

$$SPAN = \frac{D_{v,90} - D_{v,10}}{D_{v,50}} \quad (\text{III.2.4})$$

where the $D_{v,10}$, $D_{v,50}$ and $D_{v,90}$ represent respectively, the maximum particle diameter below which 10% of the sample volume exists, the maximum particle diameter below which 50% of the sample volume exists and the maximum particle diameter below which 90% of the sample volume exists.

2.2.3.7 Evaluation of the total antioxidant capacity

The antioxidant activity of the hydroxytyrosol-loaded ethyl cellulose microparticles was measured using the total non-enzymatic antioxidant capacity (TAC) kit (Ref. MAK187, Sigma-Aldrich Chemical, St. Louis, MO, USA). Briefly, 100 μL of filtrated samples at different moments during the release of hydroxytyrosol from microparticles or the hydroxytyrosol standard solutions were transferred to a 96 well plate. Afterward, 100 μL of Cu^{2+} working solution was added to all sample wells. The content was mixed by pipetting and incubated for 90 minutes at room temperature. The plate was protected from light during the incubation. Afterward, the absorbance was read at 570 nm in a microtiter plate reader (Synergy HT, Biotek, USA).

Previously, a standard Trolox calibration curve was obtained adding 0, 4, 8, 12, 16 and 20 μL of the 1 mM of the Trolox Standard Solution into the 96 well plate. Ultra-pure water was added to each well to bring to volume to 100 μL . The 0, 4, 8, 12, 16 and 20 nmol/well standards were generated. All the measurements were performed in triplicate.

The TAC (%) activity was calculated considering Equation (III.2.5).

$$TAC (\%) = \frac{C_S - C_0}{C_0} \times 100 \quad (\text{III.2.5})$$

where C_S is the Trolox equivalent of the unknown sample well (nmol/ μL) from the standard curve and C_0 is the Trolox equivalent of the sample well (nmol/ μL) of the hydroxytyrosol standard solution encapsulated.

2.2.3.8 *In vitro* release studies

The *in vitro* release of hydroxytyrosol from the different formulations at different moments was evaluated in a simulated salivary fluid (SSF), in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). To all simulated gastrointestinal fluids were added enzymatic solutions. Briefly, 10 mg of microparticles were suspended in 1.5 mL of selected solution in a flask. Each suspension was placed in the incubator (Lovibond incubator, Amesbury, United Kingdom) at 37 °C and stirred in a horizontal shaker (Orbital IKA KS 130 basic, Germany) at 170 rpm. The amount of hydroxytyrosol released was measured after filtration of each sample with a 0.2 μm syringe filter (Ref: 514-0070, VWR International, Fontenay-sous-Bois, France). From each sample, a volume of 500 μL was transferred to a flask, and 500 μL of the respective simulated fluid was added to the container. The amount released was evaluated considering the absorbance read of the sample using the respective simulated fluid as a blank. The composition and the concentration of each salt used in each electrolyte solution are presented in Table III.2.2. All electrolyte solutions, enzymes used, procedures, duration of the release and typical pH values were used as recommended by Minekus et al. (2014). Further considerations are presented in Table III.2.3.

Table III.2.2 - Concentrations of electrolytes of each simulated gastrointestinal fluid

Salt	Stock Conc. (g/L)	SSF		SGF		SIF	
		Vol. stock (mL)	Conc. (mmol/L)	Vol. stock (mL)	Conc. (mmol/L)	Vol. stock (mL)	Conc. (mmol/L)
KCl	37.3	15.1	15.1	6.9	6.9	6.8	6.8
KH ₂ PO ₄	68	3.7	3.7	0.9	0.9	0.8	0.8
NaHCO ₃	84	6.8	13.6	12.5	25	42.5	85
NaCl	117	-	-	11.8	47.2	9.6	38.4
MgCl ₂ (H ₂ O) ₆	30.5	0.5	0.15	0.4	0.1	1.1	0.33
(NH ₄) ₂ CO ₃	48	0.06	0.06	0.5	0.5	-	-

Conc. – Concentration; SGF – Simulated Gastric Fluid; SIF – Simulated Intestinal Fluid;
 SSF – Simulated Salivary Fluid; Vol. – Volume;

Table III.2.3 - Experimental procedure for the *in vitro* release studies

Procedure	SSF	SGF	SIF
	Mixture 1:1 (v:v) of SSF and enzymes	Mixture 1:1 (v:v) of SGF and enzymes	Mixture 1:1 (v:v) of SIF and enzymes
Enzymes	Salivary amylase (75 U/mL)	Pepsin (2 000 U/mL)	Pancreatin (based on trypsin activity at 100 U/mL) Bile salts (10 mM)
Duration of the release	2 minutes	2 hours	2 minutes
pH	7	3	7

SGF – Simulated Gastric Fluid; SIF – Simulated Intestinal Fluid; SSF – Simulated Salivary Fluid;

2.2.3.9 Morphological hydroxytyrosol-loaded ethyl cellulose degradation analysis

Hydroxytyrosol-loaded ethyl cellulose microparticles degradation was evaluated through morphological inspection. During the release of hydroxytyrosol in simulated gastrointestinal fluids, powder samples were recovered and freeze-dried for 24 hours. Afterward, the morphological changes during the controlled release studies were evaluated using a PHENOM XL scanning light microscope (Eindhoven, The Netherlands).

2.2.4 Statistic analysis

Data analysis was performed using the MINITAB 18 software (Minitab Inc., State College, PA, USA). The analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$).

2.3 Results

2.3.1 Analytical methods validation

The selection of the UV-Vis spectrophotometer method allowed a simple, quick and economic quantification of hydroxytyrosol in ultra-pure water as well as in each release medium. The simplicity, quickness, and robustness of the UV-Vis spectrophotometer method for the detection of bioactive compounds were described by Mendez et al. (2003). The maximum absorption wavelength of hydroxytyrosol in ultra-pure water, in the SSF, in the SGF, and in the SIF, was found to be 280 nm. This wavelength was used in all measurements. To the authors best knowledge, this is the first time that is reported the maximum absorption wavelength of hydroxytyrosol in water as well as in simulated gastrointestinal fluids.

For each gastrointestinal fluid, a calibration curve was drawn, and a linear range was observed in all cases. Calibration curves were constructed in the range of 0.25 mg/L to 20 mg/L in the case of ultra-pure water. However, the linearity range for the quantification of hydroxytyrosol in the SSF, SGF, and SIF ranged between 0.5-20 mg/L, 1.0-20 mg/L, and 4-20 mg/L, respectively. In the cases of the SSF, SGF and the SIF, the linearity range was tested using different starting concentrations so that it could be verified that the limit of quantification (LOQ) was lower than the lowest concentration analyzed in the controlled release studies. The minimum amount released of hydroxytyrosol from microparticles in SSF, SGF, and SIF was corresponding to the concentrations of 2.086 mg/L, 5.051 mg/L, and 23.481 mg/L, respectively and the LOQ values of 1.67 mg/L, 2.22 mg/L, and 2.28 mg/L.

Both the LOD and the LOQ values were satisfactorily low, which demonstrate the possibility of application of the proposed analytical methods to the quantification of hydroxytyrosol associated with microencapsulation purposes by UV-Vis method. The results of the regression analysis of hydroxytyrosol quantification by UV-Vis spectrophotometric method are presented in Table III.2.4.

Table III.2.4 - Results from the regression analysis for hydroxytyrosol quantification in ultra-pure water and simulated gastrointestinal fluids

HT detection fluid	Regression equation ^{a,b}	Correlation coefficient	Relative standard error of slope (%)	Conc. range (mg/L)	LOD (mg/L)	LOQ (mg/L)
UPW	$Abs=(0.017\pm0.001) C + (-0.004\pm0.009)$	0.995	2.119	0.25-20	0.69	2.31
SSF	$Abs=(0.015\pm0.001) C + (0.015\pm0.005)$	0.997	1.589	0.5-20	0.50	1.66
SGF	$Abs=(0.012\pm0.001) C + (0.018\pm0.006)$	0.995	2.142	1.0-20	0.67	2.24
SIF	$Abs=(0.015\pm0.001) C + (0.014\pm0.008)$	0.996	2.014	4-20	0.68	2.28

^aAbs is the absorbance (UV-Vis spectrophotometer method), and C is the hydroxytyrosol concentration (mg/L)

^bCalibration curve based on three absorbances read

Conc. – Concentration; HT – Hydroxytyrosol; LOD – Limit of Detection; LOQ – Limit of Quantification; SGF – Simulated Gastric Fluid; SIF – Simulated Intestinal Fluid; SSF – Simulated Salivary Fluid; UPW – Ultra-Pure Water;

2.3.2 Characterization of hydroxytyrosol-loaded microparticles

2.3.2.1 Hydroxytyrosol encapsulation efficiency, loading content, and yield of production

In this study, hydroxytyrosol was successfully microencapsulated into ethyl cellulose polymer matrix using the double emulsion solvent evaporation method. Two different formulations were prepared, F-1 and F-2, corresponding to different theoretical loadings: 5% and 10% respectively. All the formulations were run in triplicate.

The HEE ranged from 79.1% to 88.2%. The mean HEE of F-1 and F-2 formulations were 88.1 ± 0.1 and 79.7 ± 0.7 , respectively. There is a significant difference ($P < 0.05$) between F-1 and F-2 formulations. The highest HEE was observed using F-1 formulation (lowest theoretical loading). An increase in the theoretical loading led to a decrease of encapsulation efficiency. A similar trend was observed by Freytag et al. (2000) when these authors microencapsulated an oligonucleotide into poly(lactide) microparticles. This observation is also in accordance with the definition of encapsulation efficiency as it can be seen as a ratio between the actual loading and the theoretical loading. Additionally, the decrease of the HEE with the increase in hydroxytyrosol loading can be explained by the significant diffusion of hydroxytyrosol to the external aqueous phase due to a higher flux at high hydroxytyrosol content during the formation of microparticles (Gabor et al., 1999; Krishnamachari et al., 2007).

The actual loading content varied from 2.1% to 5.2%. The mean actual loading content of F-1 formulation was found to be 2.2 ± 0.04 and 4.9 ± 0.2 , in the case of formulation F-2. Therefore, the actual amount of hydroxytyrosol per gram of dried dispersion was 22 ± 1.0 mg of hydroxytyrosol/g of dried dispersion, in the case of F-1 formulation and 49 ± 2.2 mg of hydroxytyrosol/g of dried dispersion. The highest actual loading content ($P < 0.05$) was observed with F-2 formulation (theoretical loading content of 10%). Among the two formulations was found a decrease of 56% and 51% from the theoretical loading to the actual loading for the F-1 and F-2 formulations, respectively. The non-proportionality between the actual and the theoretical loading was also considered by Alex & Bodmeier (1990) in the case of the microencapsulation of pseudoephedrine HCl, a highly water-soluble drug.

The product yield ranged from 67.3% to 84.9%. The mean product yield was 82.4 ± 2.1 for F-1 formulation and 71.2 ± 3.7 for F-2 formulation. A significant ($P < 0.05$) effect of the loading content on the product yield was observed. The increase in hydroxytyrosol load while the weight of ethyl cellulose remained constant, is probably the main explanatory fact for the insufficient amount of ethyl cellulose for the microencapsulation which resulted in a decrease of the product yield. Similar conclusions were drawn by Freytag et al. (2000). Schematic results of the HEE, ALC, and YP are presented in Figure III.2.1.

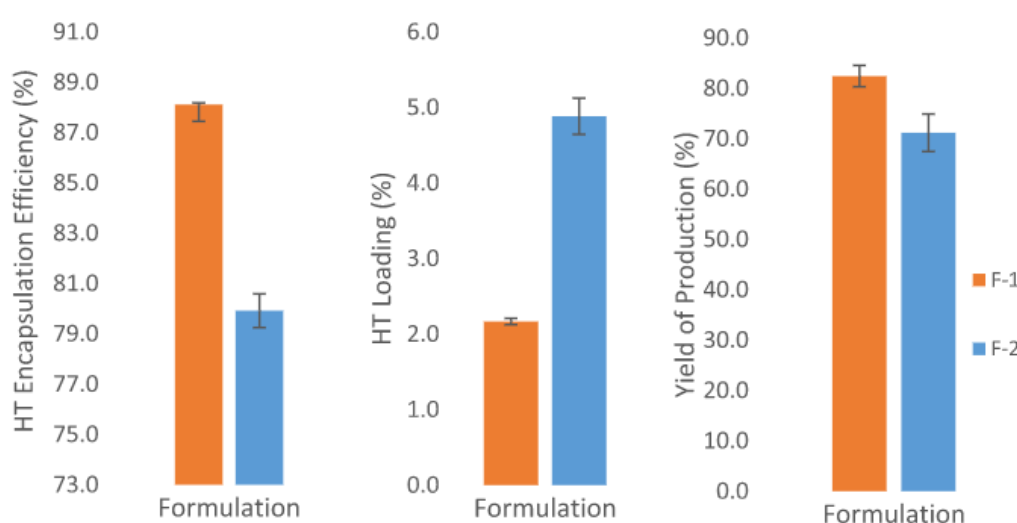


Figure III.2.1 - Results of the hydroxytyrosol (HT) encapsulation efficiency, loading, and yield of production

2.3.2.2 Microparticles wettability

The wettability represents the ability of the obtained microparticles to absorb water. This physical property is related to the molecular interaction between the solid phase and the liquid phase as described by different authors (Bae & Lee, 2008; Cuq et al., 2011; De Barros Fernandes et al., 2014). As presented by Kim et al. (2002) the wettability depends on several parameters as the particle size, porosity, microparticles density and the presence of amphiphilic molecules on the microparticles surface. The time required for the powders become entirely wet ranged between 615 and 1690 s. The mean time needed for the powders become wet were 1647 ± 60 s and 635 ± 25 for F-1 and F-2 formulations, respectively.

There is a significant difference between the wettability values obtained in the F-1 and F-2 formulations ($P < 0.05$). The obtained values agree with the observation of other authors. The increase in agglomeration degree (expressed through the SPAN value, see section III.2.2.3.6), resulted in the improvement of wettability of microparticles (formulation F-2). The agglomeration may increase the wettability as water may penetrate among pores more easily (Bae & Lee, 2008; Buffo et al., 2002).

2.3.2.3 Differential Scanning Calorimetry analysis

The DSC thermograms of pure hydroxytyrosol, powders of F-1 and F-2 formulations, polymer-only microparticles, and ethyl cellulose are presented in Figure III.2.2.

Hydroxytyrosol DSC thermogram revealed a sharp endothermic peak corresponding to the melting point of 56.9 °C which is related to its crystallinity. The crystallinity of a material can be detected by the melting point depression on DSC thermogram. If the active ingredient does not present a crystalline structure, the melting point depression could not be distinguished in the DSC thermogram (Salonen et al., 2005; Zhang et al., 2010).

Polymer-only microparticles and hydroxytyrosol-loaded microparticles present a broad endothermic in the range of 36 °C to 82 °C with a peak at 60.5 °C. These endotherms are widely reported to be present in microstructures and are referred as dehydration temperature (T_{DH}). The T_{DH} is related to the evaporation of volatile portion of molecules or bounded water (Luo et al., 2012; Müller et al., 2011; Neo et al., 2013).

The absence of melting peaks in DSC thermograms of F-1 and F-2 formulations powders reveals that the incorporated hydroxytyrosol lost its original crystalline structure and confirms that hydroxytyrosol was efficiently microencapsulated in ethyl cellulose-polymer based systems.

From the observation of Figure III.2.2, can be concluded that the transition temperature (T_g) of ethyl cellulose is modified when polymer-only microparticles and loaded microparticles (F-1 and F-2 formulations) are prepared.

There is a subtle baseline change at *circa* 100 °C of ethyl cellulose that became more predominant using polymer-only microparticles and loaded-microparticles. Ethyl cellulose is considered to be a highly or even fully amorphous material (Lai et al., 2010; Sakellariou et al., 1985). Sakellariou et al. (1985) determined that the T_g of ethyl cellulose is in the range of 130 °C and 150 °C. Additionally, these authors reported that the determination of T_g is dependent on the type of ethyl cellulose as well as the measurement method used. Therefore, results of the present study indicate that ethyl cellulose is strong glass former, presenting a narrow heat capacity and rheological change during the glass transition. The aggregation of ethyl cellulose in microparticles, this is, in polymer-only microparticles favored heat capacity and rheological changes during the glass transition.

The degradation of hydroxytyrosol-loaded ethyl cellulose microparticles began around 220 °C. The T_g values obtained for F-1 and F-2 formulations powders were 131.1 °C and 132 °C. It was observed an increase on the T_g value from ethyl cellulose, polymer-only microparticles to loaded-microparticles.

When an active ingredient is microencapsulated in a polymer-based system, three alternatives relative to an active ingredient-polymer interaction may occur: the active ingredient and the polymer form a solid solution and in this case is observed a shift in the position of the T_g of the polymer due to plasticizing/anti-plasticizing effects of the active compound and the endotherm associated to the melting of the active ingredient is not present in microparticles; the active ingredient is dispersed in the polymer in its amorphous form and therefore the T_g of the polymer is not changed and the melting endothermic of the active compound is not present in microparticles. Lastly, the active ingredient can be dispersed in the polymer in its crystalline form and consequently, the T_g of the polymer or the melting endothermic of the active compound are not changed (Mandal et al., 2002; Pamujula et al., 2008, 2004). In this study was observed a shift of the T_g of ethyl cellulose to higher temperatures when hydroxytyrosol was microencapsulated and the, melting endotherm of hydroxytyrosol is absence on hydroxytyrosol-loaded ethyl cellulose microparticles DSC curves (Figure III.2.2). The incorporation of hydroxytyrosol in the ethylcellulose polymer-based system resulted in the formation of solid solution due to anti-plasticizing effects.

The results of this study suggest molecular interactions between hydroxytyrosol and ethyl cellulose. The presence of bigger molecules in ethyl cellulose-based microparticles had restricted the molecular rearrangement of the polymer when the temperature was increased. However, the comparison of F-1 formulation powder (or alternatively F-2 formulation powder) with polymer-only microparticles suggested that did not occur chemical interactions between hydroxytyrosol and the coating material, ethyl cellulose.

Due to the anti-plasticizing effect observed, can be concluded that if small molecules are required to be encapsulated in ethyl cellulose polymer-based microsystems, it will be necessary a suitable amount of moisture content to increase the shelf-life of foods enhanced with loaded ethyl-cellulose microparticles. Similar conclusions were drawn by Ismail et al. (2012) when they were studying the thermal behaviour of getominic-loaded poly-lactide-co-glycolide microparticles.

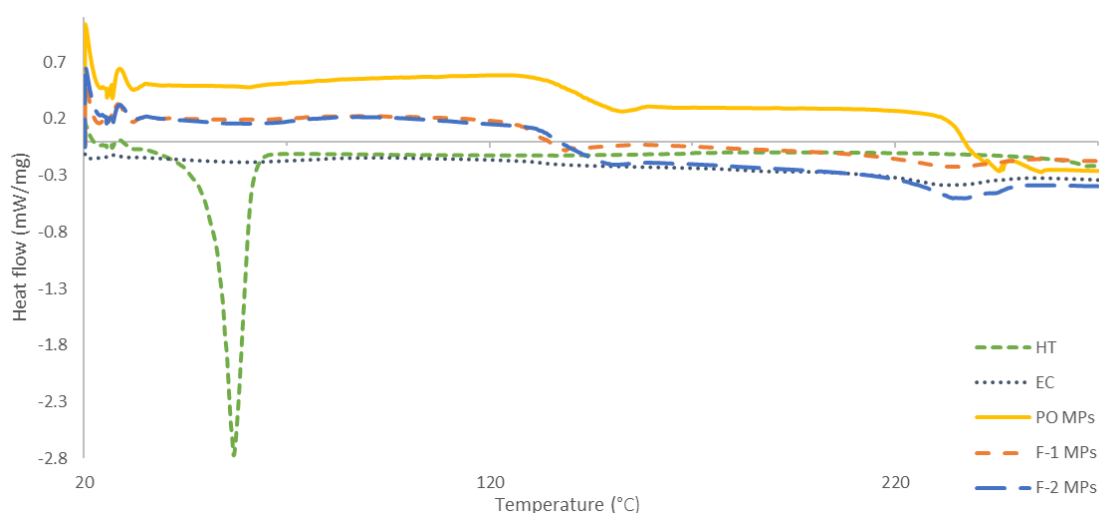


Figure III.2.2 - Differential Scanning Calorimetry thermograms of different samples: hydroxytyrosol (--- HT); ethyl cellulose (..... EC); polymer-only microparticles (— PO MPs); F-1 (5% TLC) microparticles (--- F-1 MPs) and F-2 (10% TLC) microparticles (— F-2 MPs)

2.3.2.4 Fourier transform infrared spectroscopy analysis

The Fourier Transform Infrared Spectroscopy (FTIR) was used to evaluate if the inclusion of hydroxytyrosol in ethyl cellulose polymer-based systems occurred, confirm the presence of specific functional groups in hydroxytyrosol-loaded ethyl cellulose microparticles and to assess if the encapsulation process affected the chemical integrity of ethyl cellulose microparticles. The ATR-FTIR spectra of hydroxytyrosol, ethyl cellulose, polymer only microparticles, F-1 formulation and F-2 formulation are presented in Figure III.2.3 for the wavenumber interval from 500 - 4000 cm^{-1} .

The FTIR spectrum of polymer-only microparticles shows characteristic bands around 4000 cm^{-1} and 2500 cm^{-1} which include a weak stretching band at 3464 cm^{-1} and medium intensity stretching bands at 2974 cm^{-1} and 2872 cm^{-1} . The weak stretching band at 3464 cm^{-1} corresponds to the vibrational mode of the stretching band of the O-H bond present in about 53% of ethyl cellulose molecules (ethylation degree of 47% of ethyl cellulose). The medium intensity stretching bands correspond to the vibrational modes from the stretching of the C-H bond present in alkane groups of ethyl cellulose. Typical stretching bands from 1500 cm^{-1} to 1300 cm^{-1} were found in the FTIR spectrum of polymer-only microparticles which include the bands at 1443 cm^{-1} and 1375 cm^{-1} which correspond to the bending vibrational mode of $-\text{CH}_3$ groups and the band at 1483 cm^{-1} corresponding to the vibrational mode of the stretching band of $-\text{CH}_2$ groups of ethyl cellulose molecules. Another characteristic band was found at 1053 cm^{-1} corresponding to the vibrational mode of the stretching of C-O bonds in ethyl cellulose molecules. The set of bands presented in this study were also found by other authors when they were analyzing the FTIR spectra of ethyl cellulose (Gavini et al., 2015; Patel et al., 2011).

The spectrum obtained for hydroxytyrosol molecules shows typical vibrational bands from 3500 cm^{-1} to 2000 cm^{-1} which include the band at 2972 cm^{-1} associated to the vibrational mode of the stretching of aromatic C-H bonds. The bands at 3390 cm^{-1} and 3215 cm^{-1} are related to the stretching of O-H groups of hydroxytyrosol. The bands associated with the double bond between the aromatic C=C are shown at 1607 cm^{-1} and 1441 cm^{-1} . The C-H out-of-plane bending vibration of the substituted benzenoid compound (at 1,2 and 4 positions) at 849 cm^{-1} and 808 cm^{-1} are characteristic band bonds of hydroxytyrosol molecules.

The F-1 and F-2 formulation spectra, when hydroxytyrosol was encapsulated, there were not observed significant differences compared to the polymer-only FTIR spectrum. A total disappearance of the characteristics bands of hydroxytyrosol was observed in the spectra of hydroxytyrosol-loaded ethyl cellulose microparticles which may be partially attributed to the low loading content of hydroxytyrosol. The overall results, especially, the non-observation of chemical modification on polymer-only microparticles when hydroxytyrosol was loaded is a reliable indicator of the incorporation of hydroxytyrosol into ethyl cellulose polymer based microparticles. Similar conclusions regarding the efficient incorporation of other bioactive ingredients in polymer-based microsystems through the study of FTIR spectra were drawn by many authors (Gavini et al., 2015; Müller et al., 2011; Neo et al., 2013; Nuchuchua et al., 2009; Piletti et al., 2017; Sajomsang et al., 2012; Sutaphanit & Chitprasert, 2014; Woranuch & Yoksan, 2013).

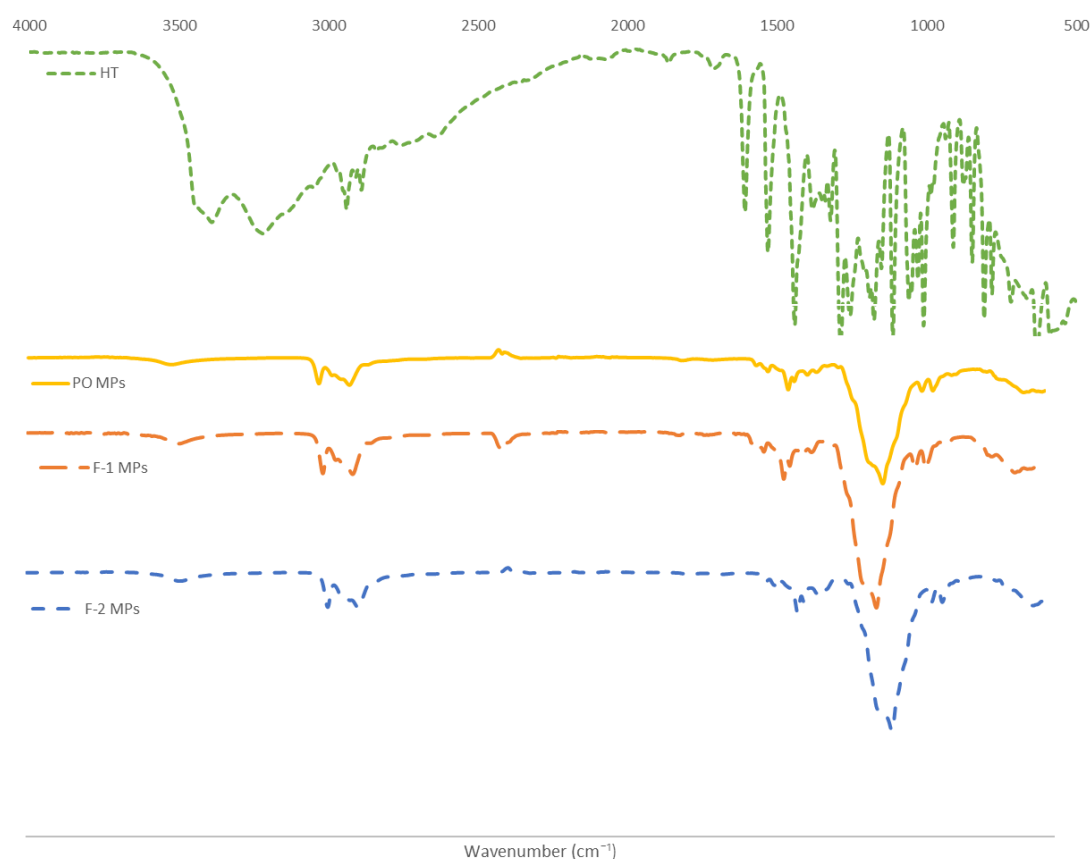


Figure III.2.3 - ATR-FTIR of different samples: hydroxytyrosol (--- HT); polymer-only microparticles (— PO MPs); F-1 (5% TLC) microparticles (--- F-1 MPs) and F-2 (10% TLC) microparticles (--- F-2 MPs)

2.3.2.5 Evaluation of the morphology

Microphotographs of samples from both formulations (F-1 and F-2 formulations) at two different magnifications (5 000 times and 10 000 times) are presented in Figure III.2.4.

Independently of the hydroxytyrosol loading, microparticles obtained present similar appearances. The microphotographs showed that microparticles are spherical presenting polymeric walls practically with no pores, fissures or cracks. This type of morphology was also observed in other research studies as follows: when polymer only microparticles were produced using polycaprolactone as wall material and the w/o/w double emulsion as microencapsulation technique (Ibraheem et al., 2014); when bovine serum albumin was microencapsulated with w/o/w double emulsion solvent evaporation technique (Yang et al., 2000).

At both magnifications, it was observed that samples formulated with higher antioxidant loading (formulation F-2; Figure III.2.4-B and Figure III.2.4-D) present the biggest microparticles' sizes. A low loading content (formulation F-1) led to the formation of the smallest microparticles (Figure III.2.4-A and III.2.4-C). It can also be concluded that an increase in the loading, resulted in the obtainment of more polydisperse microparticles. Similar trends were also observed in another study regarding the microencapsulation of tetracosactide using the w/o/w double emulsion solvent evaporation technique (Witschi & Doelker, 1998). In the lowest magnification microphotograph of the F-2 formulation (Figure III.2.4-B) is displayed a disrupted round polymer cavity containing many smaller microparticles. The presence of this structure may be attributed to the disruption of a big microparticle during the re-emulsification process in the high-performance liquid homogenizer. Smaller microparticles arranged themselves inside this polymer network.

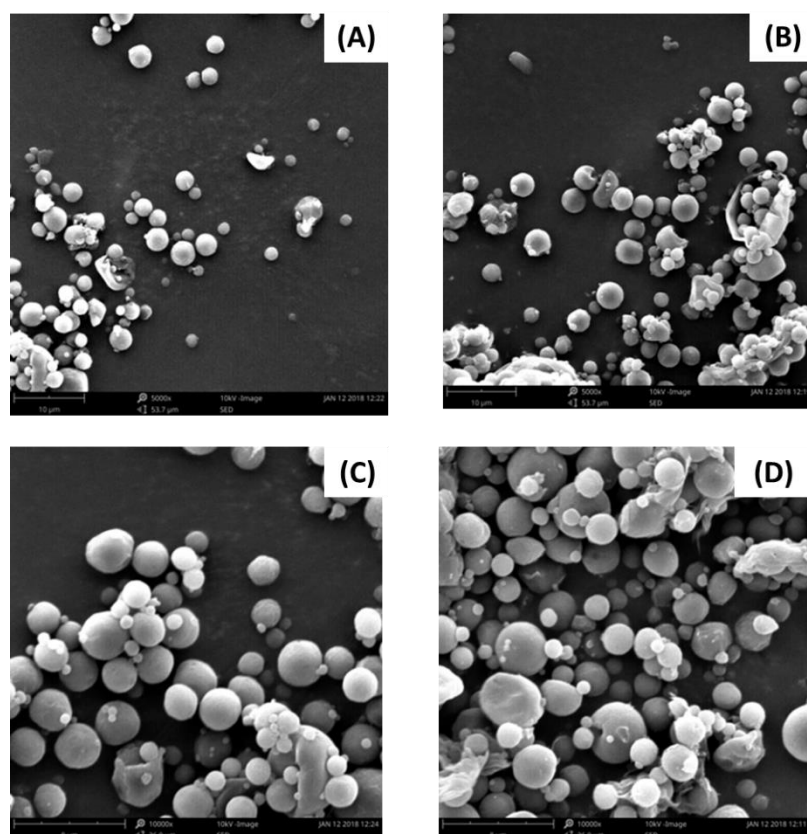


Figure III.2.4 - Microphotographs of F-1 ((A) and (C)) and F-2 ((B) and (D)) formulations (amplification of 5 000 times is presented in (A) and (C) micrographs and 10 000 times is shown in (B) and (D); beam intensity of 10.00 kV)

2.3.2.6 Evaluation of the particle size distribution

The mean particle sizes observed were $156.6 \pm 6.9 \mu\text{m}$ and $304.0 \pm 16.0 \mu\text{m}$ for the F-1 and F-2 formulations, respectively. The mean SPAN values obtained were $2.9 \pm 0.1 \mu\text{m}$ and $5.5 \pm 0.3 \mu\text{m}$. These results are in accordance with the conclusions depicted from the morphological analysis. The F-2 formulation was loaded with higher content of hydroxytyrosol, which resulted in the obtainment, in mean, bigger microparticles and almost twice more polydisperse. The increase in the mean particle size can be attributed to the higher amount of hydroxytyrosol present in the microparticles. These results are in agreement with results described by other authors using the w/o/w double emulsion solvent evaporation method (Krishnamachari et al., 2007; Sharma et al., 2016).

Results regarding the particle size distribution (differential volume *versus* particle size) of the two formulations are displayed in Figure III.2.5. A bi-modal particle size distribution was obtained in the case of 10% of theoretical loading (F-1 formulation) while the distribution is unimodal in the case of a theoretical loading of 5% (F-2 formulation).

Several processes and formulation and process parameters are described to affect the particle size distribution as the stirring speed, the loading, polymer concentration, polymer molecular weight, surfactant concentration in the external aqueous phase among others (Krishnamachari et al., 2007). The obtained curves support the hypothesis of the complexity of the w/o/w double emulsion solvent evaporation method affects the particle size distribution.

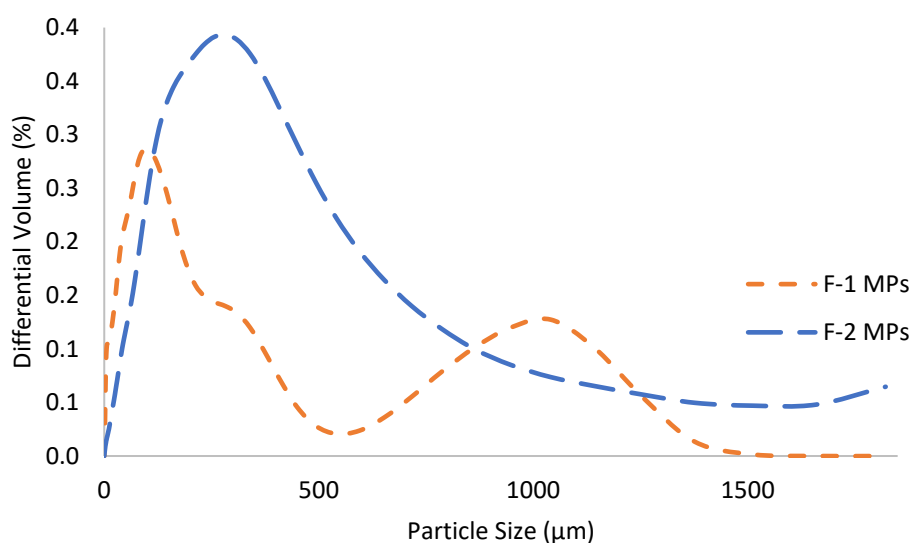


Figure III.2.5 - Particle size distribution of the microparticles of the formulations F-1

(— F-1 MPs) and F-2 (— F-2 MPs)

2.3.2.7 Evaluation of the total antioxidant capacity

The results regarding the total antioxidant retainment of microparticles are presented in Figure III.2.6. The non-encapsulated hydroxytyrosol was used as a positive control of the assay.

In the simulated salivary fluid (SSF) there were no significant differences between the antioxidant preservation capacity ($P=0.46>0.05$) of the F-1 and F-2 formulations. The antioxidant capacity retainment remained above 80%. For the other release media, the simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF), the powders had not also shown significant difference among samples ($P>0.05$). The retainment capacity was above 50% and 60% in the SGF and the SIF respectively during the 2 hours of the study. These results suggested that the loading content not show to be significant in the antioxidant retainment by microparticles. Additionally, there were no significant differences ($P>0.05$) among the antioxidant capacity retainment among the three release mediums. Calvo et al. (2010) studied the antioxidant capacity of different types of olive oil. The antioxidant activity ranged from 1.212 ± 200 to 2.798 ± 451 mmol Trolox/kg of oil. The results obtained in this study demonstrate that the total amount of antioxidant released after 2 hours in the SIF corresponds to 36.5 ± 3.3 and 34.5 ± 0.2 mmol Trolox/L, respectively. This study is remarkable important as the obtained results of the amount of hydroxytyrosol released in the SIF indicate that the total antioxidant released in the gut it was higher than the content of hydroxytyrosol present in the olive oil considering the results obtained by Calvo et al. (2010).

The effective concentration range of the *in vitro* inhibition of low-density lipoproteins (LDL) oxidation is between 5 and 10 μM (Poppel et al., 1999; Visioli et al., 1995). The total antioxidant concentration released in the SIF after 2 hours of the study was 36.5 ± 3.3 and 34.5 ± 0.2 μM , for the F-1 and F-2 formulations, respectively. Therefore, the amount released is enough to excrete effects of inhibition of LDL oxidation. Other biological effects as the inhibition of platelet and leukocytes arachidonate lipoxygenases and phorbol 12-myristate 13- acetate (PMA) induced respiratory burst in human neutrophils are reported to range from 10 to 100 μM and therefore the amount released is enough to achieve the desired effects (De La Puerta et al., 1999; Kohyama et al., 1997; Poppel et al., 1999; Visioli et al., 1998).

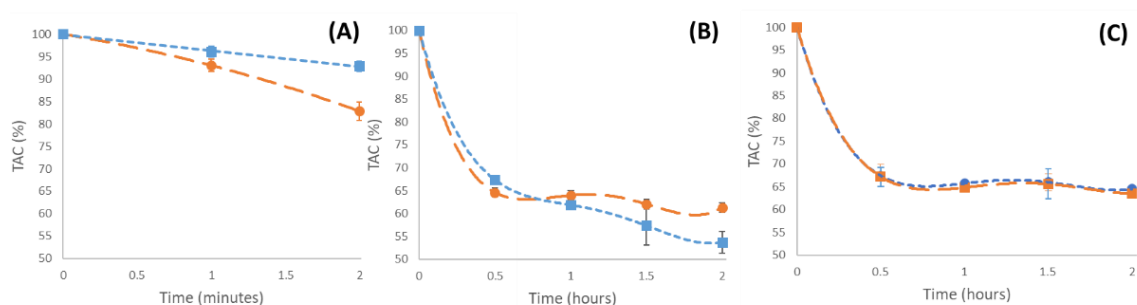


Figure III.2.6 - Total antioxidant capacity (TAC) of microparticles of the formulations F-1 (— F-1 MPs) and F-2 (— F-2 MPs) during the controlled release studies ((A) – SSF; (B) – SGF; (C)-SIF)

2.3.2.8 *In vitro* release studies

The release profiles of the hydroxytyrosol from microparticles in the simulated gastrointestinal fluids are presented in Figure III.2.7.

After 2 minutes of the beginning of the study, only a little amount of hydroxytyrosol was released in the SSF (about $0.38 \pm 0.02\%$ of F-1 formulation and about $0.20 \pm 0.01\%$ of the F-2 formulation, figure III.2.7 (A)). Hydroxytyrosol was efficiently protected in the SSF, resulting in a lower amount of hydroxytyrosol released before the site of its absorption is desired. After 2 hours, the total amount released of hydroxytyrosol was $0.73 \pm 0.09\%$ and $2.35 \pm 0.11\%$ from F-1 and F-2 formulations. The higher amount of hydroxytyrosol released associated to the F-2 formulation can be related to its higher loading content. These results prove the gastro-resistance of hydroxytyrosol-loaded ethyl cellulose microparticles.

Regarding the release in the SIF, the total amount released was $3.26 \pm 0.15\%$ and $3.00 \pm 0.11\%$ from F-1 and F-2 formulations, respectively. The amount of hydroxytyrosol released after 2 hours in the SIF was similar in both gastrointestinal release environments.

Interestingly, a substantial increase of the hydroxytyrosol total amount released was observed using the F-1 formulation (lower loading content) after 2 hours and in the SIF.

An erosion-controlled release and water-hydroxytyrosol interaction can be pointed out as some reasons for the release improvement in the SIF of hydroxytyrosol release from F-1 formulation powder.

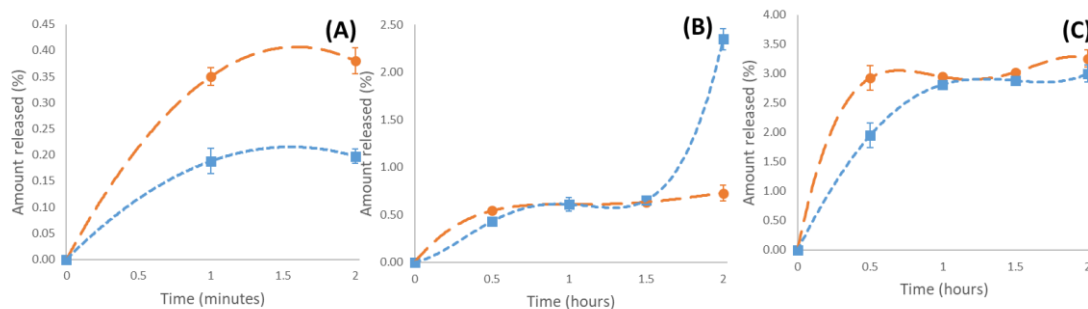


Figure III.2.7 - Release profiles of hydroxytyrosol from microparticles of the formulations F-1 (— F-1 MPs) and F-2 (— F-2 MPs) during the controlled releasestudies ((A) –SSF; (B) –SGF; (C) -SIF)

2.3.3 Morphological hydroxytyrosol-loaded ethyl cellulose degradation analysis

The degradation behavior of microparticles was assessed using formulation F-2 due to its higher loading content as a model for this study.

Considering the morphological changes, in the SSF, the microparticles underwent through an erosion process. From the original microparticles (Figure III.2.4-C and III.2.4-D), there is an increase in the surface roughness once they were incubated in the SSF. The surface roughness increased from the sample taken 1 minute and 2 minutes after (Figure III.2.8). This observation may be explained due to the reorganization of the polymer chains as the water contacts with the surface of microparticles.

From Figure III.2.8 to III.2.10, the formation of external pores non-existing previously is evident. From 1 hour to 2 hours of the study in the SGF, the pore size significantly increased. Probably a bulk-to-surface erosion occurred as this type of final morphology is typical of bulk-to-surface erosion processes as presented in similar observations by Feng et al. (2015) when they observed the morphological changes in unloaded microparticles obtained by single and double emulsion methods.

Microparticles lost their original (spherical) structural morphology after 1 hour of the study in the SIF (Figure III.2.10).

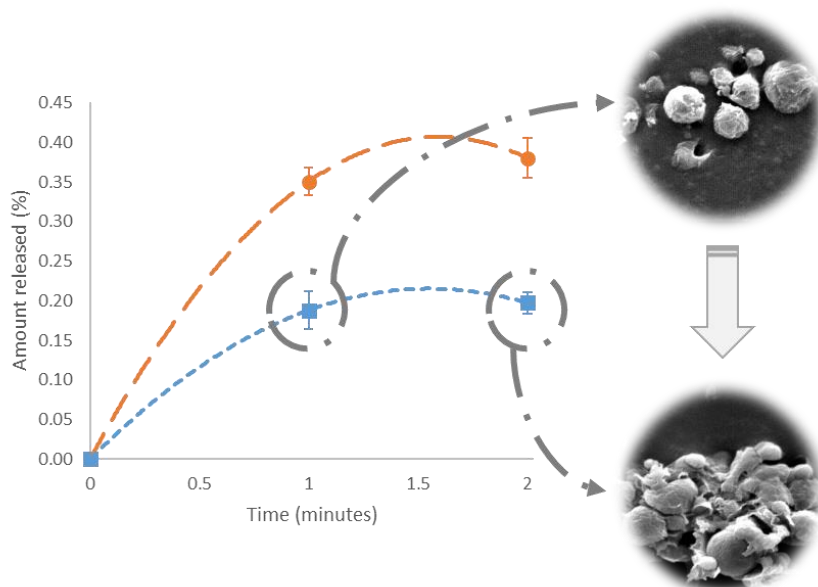


Figure III.2.8 - Release profile and micrographs of hydroxytyrosol-loaded ethyl cellulose microparticles under morphological changes in the SSF (amplification of 10 000 times is presented) of formulations F-1 (— — F-1 MPs) and F-2 (— — F-2 MPs)

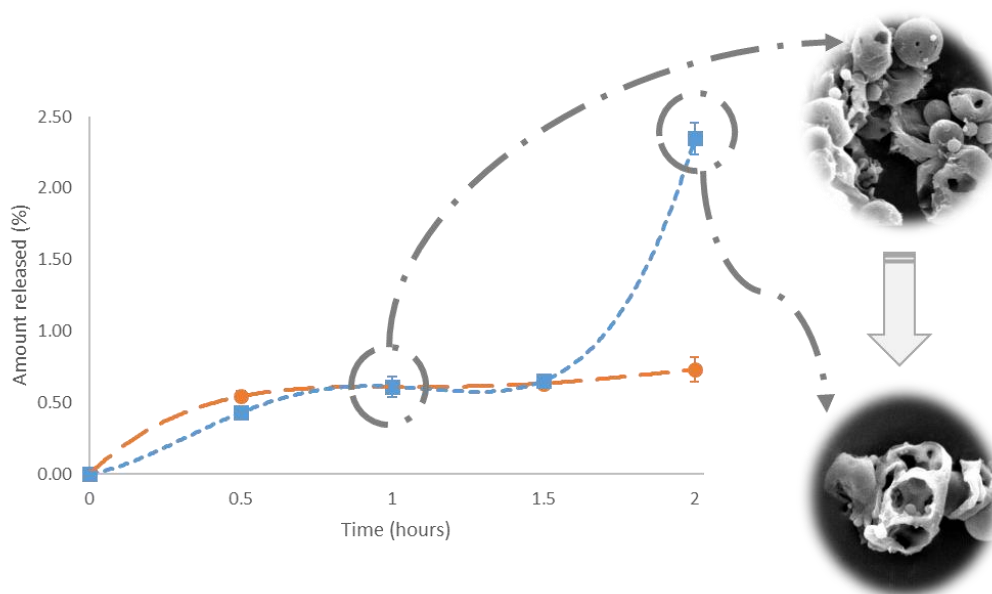


Figure III.2.9 - Release profile and micrographs of hydroxytyrosol-loaded ethyl cellulose microparticles under morphological changes in the SGF (amplification of 10 000 times is presented) of formulations F-1 (— — F-1 MPs) and F-2 (— — F-2 MPs)

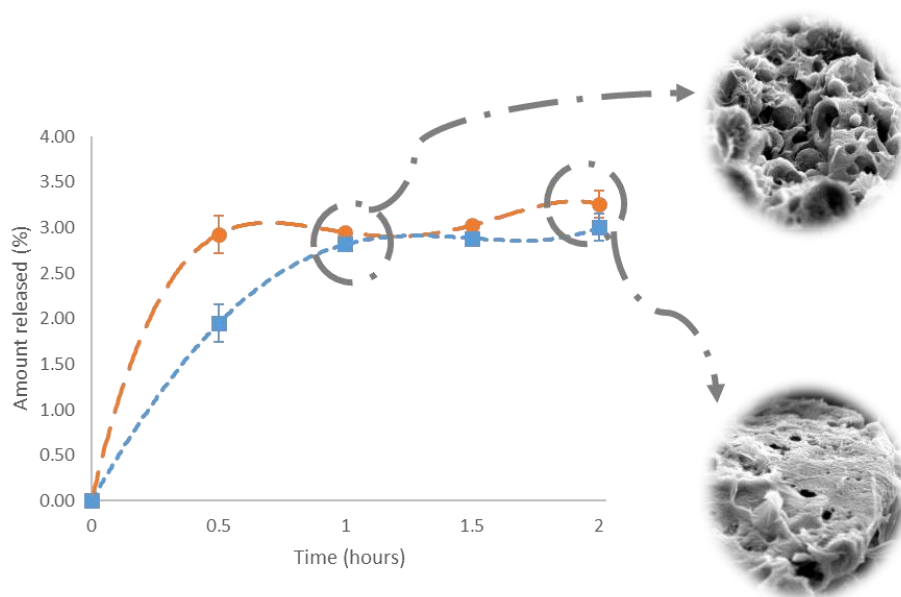


Figure III.2.10 - Release profile and micrographs of hydroxytyrosol-loaded ethyl cellulose microparticles under morphological changes in the SIF (amplification of 10 000 times is presented) of formulations F-1 (— F-1 MPs) and F-2 (— F-2 MPs)

2.4 Conclusions

The double emulsion solvent evaporation technique was successfully applied to the microencapsulation of hydroxytyrosol, an antioxidant present in high concentrations in olive oil. Two different theoretical loadings (5 and 10%) were tested. The results showed that the lower theoretical loading content was associated with, the higher encapsulation efficiency and product yield. The results of this research reveal that the total antioxidant capacity was kept above 50% in all simulated gastrointestinal fluids. The total antioxidant concentration was within the effective concentration range of many biological positive effects. Hydroxytyrosol-loaded ethyl cellulose microparticles shown to be the gastro-resistant. The morphological degradation analysis during the *in vitro* release studies demonstrated that an increase of the surface roughness in the simulated salivary fluid is probably due to the rearrangement of polymer chains as water contacts with microparticles. Additionally, the morphological appearance demonstrated that probably a bulk-to-surface erosion is the main driving force for microparticles degradation. Moreover, hydroxytyrosol-loaded ethyl cellulose microparticles were nearly stable in a wide temperature range.

To the authors best knowledge this is the first research regarding the microencapsulation of hydroxytyrosol even though the positive health impact has been studied for a long time.

The results of this research are promising for the inclusion of microencapsulated hydroxytyrosol in functional foods, pharmaceuticals, and nutraceuticals mainly regarding the antioxidant capacity and gastrointestinal resistance of hydroxytyrosol-loaded ethyl cellulose microparticles.

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Chapter 3. Encapsulation of the antioxidant tyrosol and characterization of loaded microparticles: an integrative approach on the study of the polymer-carriers and loading contents ³

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Abstract:

Tyrosol, a powerful antioxidant present in high amounts in olive oil and mill wastes, possesses relevant health benefits due to its antioxidant properties. Therefore, tyrosol is a promising compound for further incorporation in functional foods. However, its incorporation in food matrices is limited due to its hydrophilicity. Therewith, encapsulation by double emulsion solvent evaporation emerges as an alternative for its protection and incorporation in lipophilic matrices, as some foods.

This work aimed to encapsulate tyrosol in alternative carriers - poly(D,L-lactide-co-glycolide), ethylcellulose, and polycaprolactone, using two theoretical loading contents (TLC) (5% and 10% w/w).

Tyrosol was efficiently incorporated in the selected polymer-carriers. Significant effects of the TLC were found on the product yield, encapsulation efficiency, thermal stability, morphology, and on the particle size distribution.

This is the first study regarding the encapsulation of tyrosol and brings new insights concerning the encapsulation of antioxidants for further incorporation in food matrices.

Keywords: tyrosol; encapsulation; antioxidant; loading; polymer-carriers; olive oil;

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3.1 Introduction

The worldwide recognition of the positive health effects of the Mediterranean diet has fostered the consumption of its typical foods. This dietary pattern is based on the consumption of olive oil as the primary source of fat; it also considers the high to moderate consumption of fruits, vegetables and fish, and low consumption of wine and meat (Castro-Quezada et al., 2014; Malheiro et al., 2012; Vlachogianni et al., 2015).

The positive health effects associated to this dietary pattern include the general reduction of the morbidity and the mortality rates, the prevention of cardiovascular diseases and the lower incidence of diabetes type 2 (Castro-Quezada et al., 2014; Gómez-Gracia et al., 2018; Mitrou et al., 2015; Ruiz-Gutie et al., 2017; Sofi et al., 2010, 2013). It may also have positive effects in the prevention and treatment of certain types of cancer and neurodegenerative diseases (Castro-Quezada et al., 2014; Couto et al., 2011; Sofi et al., 2010, 2014).

The Mediterranean diet was considered Intangible Cultural Heritage of Humanity since December 4, 2013, by United Nations Educational, Scientific, and Cultural Organization (UNESCO) (United Nations Educational, Scientific and Cultural Organization, 2013).

It has been suggested by the scientific community that the positive health benefits of the Mediterranean Diet are strongly correlated to the presence of specific micro-constituents as phenolic compounds (Fernández-Mar et al., 2012; Nadtochiy et al., 2011). In this dietary pattern, the majority of the phenolic compounds are found in olives, extra virgin olive oil, and red wine (Cicerale et al., 2012; Fernández-Mar et al., 2012).

Moreover, during the processing of olive oil, many olive mill residues are produced. Among them, it is claimed that olive mill wastewaters (De Marco et al., 2007; El-Abbassi et al., 2012; Visioli et al., 1999), olive leaves (Benavente-García et al., 2000; El & Karakaya, 2009; Pereira et al., 2007) and olive pomace (Aldini et al., 2006; Lesage-Meessen et al., 2001; Obied et al., 2007) present high content of the phenolic compounds that have strong radical scavenging capacity, responsible for many health benefits associated to the Mediterranean Diet.

Noticeably, tyrosol, also known as 2-(4-hydroxyphenyl)ethanol or alternatively, 4-(2-hydroxyethyl)phenol, is one of the most simple phenols found in olive oil and olive mill wastes. It well documented its unique biological properties acting as an effective and powerful antioxidant (Giovannini et al., 1999; Lee et al., 2016). Recent studies pointed out the outstanding proinflammatory activity of tyrosol (Chang et al., 2019; Sato et al., 2016; Serra et al., 2017). Moreover, it is described its potential antigenotoxic activity, and it is also described its effectiveness in the prevention of the apoptosis of keratinocytes (Lee et al., 2016; Salucci et al., 2015). Additionally, it was demonstrated that it prevents endothelial dysfunction, and it may inhibit platelet-induced aggregation (Carluccio et al., 2003; Petroni et al., 1995). Furthermore, it is documented that tyrosol may ameliorate hyperglycemia in rats (Chandramohan et al., 2015; Lee et al., 2016).

Even though tyrosol presents outstanding properties is not widely reported its employment in dietary supplements or as an additive, bioactive ingredient or even as a stabilizer in foods, pharmaceutical formulations, cosmetics, and other industrial formulations or recipes, probably as a consequence of its hydrophilic chemical nature. Moreover, tyrosol solubilization or even incorporation in lipid matrices, as many foods, cosmetics, and pharmaceutical formulations, is still challenging.

Moreover, it is reported that tyrosol presents low bioavailability as a consequence of its low absorption and high metabolism in the human liver and small intestine (Vlachogianni et al., 2015).

Therefore, microencapsulation arises a potential strategy for the incorporation of tyrosol in polymer-based systems compatible with lipophilic matrices (e.g., the majority of foods). The incorporation of tyrosol in lipophilic carriers compatible with food matrices (food grade polymers) allows not only to protect tyrosol from oxidative food matrices environments but also allows its controlled/sustained release thus allowing other vital molecules present in food matrices to be protected (Aguiar et al., 2016, 2017; Zhou et al., 2017). Moreover, the inclusion of tyrosol-loaded microparticles in food matrices may boost the bioavailability of tyrosol. Additionally, tyrosol is an antioxidant, and therefore, it is susceptible to auto-oxidation processes. In this context, microencapsulation may potentiate the protection of this powerful antioxidant from auto-oxidation processes.

Microencapsulation has been highly studied for the incorporation of water-soluble phenolic compounds (e.g., hydroxytyrosol) and even phenolic compounds that present low bioavailability as resveratrol (Paulo & Santos, 2018a; Veiga & Cla, 2011; Vlachogianni et al., 2015).

Regarding the case of the incorporation of water-soluble compounds in lipophilic matrices, the most commonly used microencapsulation approach is the water-in-oil-water ($w_1/o/w_2$) double emulsion solvent evaporation technique. Accordingly, the internal aqueous phase (w_1) that contains the water-soluble compound is emulsified in an organic phase (o) containing the polymer matrix that will coat the active ingredient. Afterward, the primary emulsion obtained (w_1/o) is then re-emulsified in an external aqueous phase (w_2). Then after the evaporation of the solvent and the hardening of microparticles are promoted (Paulo & Santos, 2018a, 2018b, 2019).

Many biocompatible polymers can be used as lipophilic coating materials that can be from a natural source or can be semi-synthetic or even totally synthesized. Among them, poly(D,L-lactide-co-glycolide) (PLGA) is one of the most commonly used polymers worldwide. It is a food and drug administration (FDA), and a European Medicine Agency (EMA) approved family of copolymers of lactic acid and glycolic acid (Danhier et al., 2012). Moreover, it is classified as a group of biocompatible and biodegradable polymers that presents versatile and adjustable mechanical properties and displays a wide range of erosion times (Makadia & Siegel, 2012).

Other alternative water-insoluble polymers that can be applied for coating purposes of water-soluble compounds are polycaprolactone (PCL) and ethylcellulose (EC). The synthetic polymer PCL is an aliphatic polyester that combines repeated units of hexanoate (Labet & Thielemans, 2009). It is a biocompatible and a biodegradable polymer that presents remarkable permeability and compatibility for many bioactive compounds (Chen et al., 2000).

As an alternative, EC is a semi-synthetic cellulose-derived polymer widely used for microencapsulation proposes. Even though it is not a biodegradable polymer, it is a biocompatible one, suitable for the coating and protection of bioactive ingredients.

It is soluble in many organic solvents like ketones, alcohols, esters among others; however, as it holds some hydroxyl groups on its backbone structure, it can uptake water through hydrogen bond formation between the free hydroxyl groups in EC and water (Murtaza, 2012; Paulo & Santos, 2018a). The availability of EC to uptake water depends on the ethylation degree of the polymer. A low ethylation degree indicates that the polymer possesses more hydroxyl groups on its backbone structure, and therefore, it can uptake water more efficiently. Therefore, this polymer-carrier is not only suitable for the protection of bioactive compounds, but also allows the permeation and release of bioactive compounds in aqueous systems.

This work aimed to incorporate tyrosol in alternative polymer-carriers as poly(D,L-lactide-co-glycolide), ethylcellulose, and polycaprolactone by $w_1/o/w_2$ double emulsion solvent evaporation approach. A comprehensive assessment of the effect of the theoretical loading content (TLC) chosen (5% w/w or 10% w/w) on relevant physicochemical properties of tyrosol-loaded microparticles was performed. Specifically, the effect of the theoretical loading content on the production yield, the actual loading content, the encapsulation efficiency, the thermal and thermogravimetric stability, the outer morphology and the particle size distribution was evaluated.

Additionally, a qualitative assessment of the efficient incorporation of tyrosol in the selected polymer-carriers was accomplished by Fourier Transform Infrared Spectroscopy (FTIR). Moreover, an evaluation of the total antioxidant capacity of tyrosol-loaded microparticles was carried out through the analysis of the Cu^{2+} chelating activity of tyrosol incorporated in microparticles.

In this work, an in-depth study of the physicochemical properties of tyrosol-loaded microparticles was carried out. Considering the possibility of the future incorporation of tyrosol-loaded microparticles as food additives in functional foods, a prior and relevant study of the physicochemical properties of tyrosol-loaded microparticles, that will affect the microparticles behavior in the fortified foods, is presented in this study.

3.2 Experimental procedures

3.2.1 Materials

3.2.1.1 Chemicals

Tyrosol standard (Ref. 188255-5G, $C_8H_{10}O_2$, CAS 501-94-0), polyvinyl alcohol used as the surfactant of the double emulsion (Ref: P8136-250G, 87-90% hydrolyzed, an average molecular weight of 30,000-70,000, CAS 9002-89-5) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). Moreover, the polymers used for the encapsulation of tyrosol, ethylcellulose (Ref: 433837-250G, viscosity of 46 cP, ethylation degree of 48%, CAS 9004-57-3), poly(D,L-lactide-*co*-glycolide (Resomer® RG 504, Ref: 739944-5G, viscosity of 0.45-0.6 dL/g, CAS 26780-50-7, lactide/glycolide 50/50) and polycaprolactone (Ref. 440744-250G, presentation in pellets ~ 3 mm, CAS 24980-41-4) were also obtained from Sigma Aldrich Chemical (St. Louis, MO, USA).

Dichloromethane (Ref. ACRO433991000, CH_2Cl_2 , CAS 1665-00-5) used as a solvent was purchased from VWR International (Fontenay-sous-Bois, France). The eluents used in the high-performance liquid chromatograph (HPLC) apparatus for the quantification of tyrosol, namely methanol (HiPerSolv CHROMANORM® Reagent Ph. Eur. Grade, HPLC grade, purity $\geq 99.8\%$, Ref: 20864.320, CH_4O , CAS 67-56-1), acetonitrile (LiChrosolv® Reagent Ph. Eur. grade, Supelco®, supplier Merck, VWR Ref: 1.00030.2500, C_2NH_3 , CAS 75-05-8), as well as the pH regulator, acetic acid standardized solution 0.1 N (supplier Alfa-Aesar, Ref: 35567.K7, $C_2H_4O_2$, CAS 64-19-7), were obtained from VWR International (Fontenay-sous-Bois, France).

A total antioxidant capacity assay kit (Ref. MAK187) was purchased from Sigma Aldrich Chemical (St. Louis, MO, USA).

The water used in this work was de-ionized and double-distilled using a Millipore™ water purification system (Massachusetts, USA) having 18.2 Ω electrical resistivity.

Specifically, in the case of ultrapure water (Ref. 83645.32, H_2O , CAS 7732-18-5) used in the eluent composition of the detection and quantification of tyrosol in the high-performance liquid chromatography (HPLC) apparatus was purchased from VWR International (Fontenay-sous-Bois, France).

All the reagents were either chromatographic or analytical grade and used as received.

3.2.2 Methods

3.2.2.1 Preparation of the tyrosol-loaded microparticles

The encapsulation of tyrosol into alternative coating materials as poly(D,L-lactide-co-glycolide) (PLGA), ethylcellulose (EC), and polycaprolactone (PCL) was performed by $w_1/o/w_2$ water-in-oil-in-water double emulsion solvent evaporation microencapsulation technique as described by Paulo & Santos (2018a, 2018b, 2019).

Regarding its hydrophilicity degree, tyrosol was dissolved in ultrapure water. A stock solution (concentration of 15 g/L) was prepared by rigorous weighing of the standard in analytical scale Mettler Toledo AG245 balance (Columbus, OH, USA). From this stock, the working solution was prepared by dilution of different standard solutions corresponding to different loading contents of tyrosol in the internal aqueous phase (w_1). For the volume measurements of the standard solutions were used 10-100 μ L, 100-1000 μ L, 500-5000 μ L micropipettes (Eppendorf, Hamburg, Germany). A standard control solution in ultrapure water of 0.5 g/L was prepared weekly from the stock solution. All solutions were stored at 4 °C in amber glass vials during the study.

Distinct polymers, namely PLGA, EC, and PCL, were considered in the present study regarding their hydrophilic properties. The polymer PLGA present higher hydrophilicity, followed by EC, being PCL, the most hydrophobic polymer investigated in this study. The polymers were dissolved in dichloromethane at a polymer concentration of 10 g/L, constituting the organic phase (o). The obtained polymer solutions were sonicated for 5 minutes in an ultrasonic bath (P Selecta, Barcelona, Spain).

The external aqueous phase (w_2) was constituted by 100 g of polyvinyl alcohol (PVA) solution at a concentration of 1% w/w. The external aqueous phase was previously prepared the day before the encapsulation experiment. For that, 1 g of PVA polymer was dissolved in 99 g of ultrapure water, under magnetic stirring (stirring plate from AREX Digital, VELP Scientifica, Monza, Italy) at around 120 °C.

The process was performed until the total clearance of the polymer solution. The polymer solution was left equilibrate to room temperature overnight. The exact amount of ultrapure water lost during the dissolution of the polymer was added before the encapsulation process.

The volume of 1 mL of the internal aqueous phase (w_1) was admixed with 10 mL of the organic phase (o) forming the primary emulsion – water-in-oil (w_1/o) and vortexed during 3 minutes. The obtained emulsions were re-emulsified in the external aqueous phase (w_2) using a high-performance homogenizer at 5 000 rpm (IKA T18 Digital ULTRA-TURRAX®, Staufen, Germany), for 5 minutes. The evaporation of the solvent and the hardening of microparticles were performed by continuously stirring the $w_1/o/w_2$ double emulsion in a stirring plate (AREX Digital, VELP Scientifica, Monza, Italy) at 700 rpm for 3 hours in the fume hood at room temperature.

Tyrosol-loaded microparticles were recovered by filtration using a 0.2 μm quantitative paper filter and washed with 500 mL of distilled water to remove polyvinyl alcohol residues. The recovered microparticles were frozen for 24 hours at $-4\text{ }^{\circ}\text{C}$ and freeze-dried for 72 hours in a benchtop freeze-dryer (SP Scientific, NY, USA).

Two theoretical loading contents (TLC) of tyrosol (5% w/w and 10% w/w) were evaluated for each polymer-carrier. The theoretical loading content (% w/w) was appraised as the ratio between the weight of tyrosol added, and the sum of the weight of polymer and tyrosol added. The TLC (% w/w) values chosen for this study are widely found as typical values in the literature. Therefore, in this study, the effectiveness of the encapsulation of tyrosol was evaluated using three polymers, each at two different TLCs, as presented in Table III.3.1. All the experiments were performed in triplicate in a total formulation runs of 18 experiments. Further considerations are presented in Table III.3.1.

Table III.3.1 - Experimental conditions of the study

Entry	Polymer	TLC (% w/w)	Polymer Conc. (g/L)	PVA Conc. (% w/w)
1	PLGA	5	10	1
2		10		
3	EC	5	10	1
4		10		
5	PCL	5	10	1
6		10		

Conc. – Concentration; EC – Ethyl Cellulose; PCL – Polycaprolactone; PLGA - Poly(D,L-lactide-co-glycolide) PVA – Polyvinyl Alcohol; TLC – Theoretical Loading Content;

3.2.2.2 Physicochemical characterization of tyrosol-loaded microparticles

3.2.2.2.1 Determination of the tyrosol loading content, the encapsulation efficiency, and the product yield

The tyrosol-loaded microparticles production yield (TPY) and the tyrosol actual loading content (TALC) were examined.

The tyrosol-loaded microparticles production yield was assessed through Equation (III.3.1) as described by Jansen-Alves et al. (2019) when they encapsulated propolis extract by a spray-drying technique using pea protein as a wall material.

$$TYP (\%) = \frac{W_M}{W_A} \times 100 = \frac{W_M}{W_I + W_P} \times 100 \quad (\text{III.3.1})$$

where W_M is the weight of tyrosol-loaded microparticles recovered after the freeze-drying process, W_A is the weight of tyrosol initially added for the encapsulation (W_I) and the weight of the polymer used for the encapsulation (W_P).

The actual loading content of tyrosol-loaded microparticles was evaluated considering Equation (III.3.2).

$$TALC (\%) = \frac{W_T}{W_M} \times 100 = \frac{W_I - W_O}{W_M} \times 100 = \frac{W_I - (W_S + W_L)}{W_M} \times 100 \quad (\text{III.3.2})$$

where W_T is the weight of tyrosol in the microparticles. The weight of tyrosol in the microparticles (W_T) was evaluated considering the difference between the weight of tyrosol initially added (W_I) and the weight of tyrosol non-encapsulated (W_O).

The weight of tyrosol non-encapsulated (W_O) was computed considering the weight of tyrosol present in the supernatant of 3 mL of the $w_1/o/w_2$ double emulsion after 3 hours of microparticles hardening (W_S), when a sample of the $w_1/o/w_2$ double emulsion was submitted to a low-intensity centrifugation process (4 000 rpm corresponding to 2 670 G, using a centrifuge Rotofix 32A, Hettich) for 15 minutes. The supernatant (W_S) was collected and stored at $-20\text{ }^{\circ}\text{C}$, prior to analysis. The pellet containing microparticles was reconstructed in ultrapure water – the dissolution solvent of tyrosol – and again centrifuge for 15 minutes (low-intensity centrifugation process; 4 000 rpm corresponding to 2 670 G, using a Rotofix 32A centrifuge). The supernatant after this second centrifugation (W_L) was collected and stored at $-20\text{ }^{\circ}\text{C}$ prior to analysis.

Instrumental analysis was performed using a Merck Hitachi Elite LaChrom (Tokyo, Japan) high-performance liquid chromatography (HPLC) equipped with a Hitachi L-7100 pump and L-7250 autosampler and coupled to a L-7450A diode array detector. The HPLC analysis was conducted by using a Purospher® STAR RP-18 end-capped LiChroCART® column (250 mm x 4.0 mm, 5.0 μm) (Merck KGaA), attached to a guard column (4.0 mm x 4.0 mm, 5.0 μm) of the same type. The elution was performed as described by Tasioula-Margari & Tsabolatidou (2015) at a flow rate of 1 mL/min, using a mobile phase composed by a mixture of ultrapure water/acetic acid (0.1 N) (97.5/2.5 v/v) (A) and methanol/acetonitrile (50/50 v/v) (B). The following elution gradient was adapted from the protocol presented by Tasioula-Margari & Tsabolatidou (2015): 95% of A and 5% of B as initial conditions kept during 5 minutes, 70% of A and 30% of B during 15 minutes (from minute 5 to minute 20) and then again, for the last 5 minutes, the initial conditions were reestablished (95% of A and 5% of B from the minute 20 to 25). The detection of tyrosol was performed at 280 nm. The quantification of tyrosol was performed after the computation of the obtained areas in a calibration curve previously obtained using different concentrations of the commercial standard of tyrosol.

The quantitative assessment of tyrosol encapsulation efficiency (TEE) was performed using Equation (III.3.3), as previously described by Paulo and Santos (2018b).

$$TEE (\% \text{ w/w}) = \frac{W_T}{W_I} \times 100 = \frac{W_I - W_O}{W_I} \times 100 \quad (\text{III.3.3})$$

The chromatographic analyses required for the assessment of tyrosol loading content and encapsulation efficiency were performed based on three independent assays.

3.2.2.2.2 Fourier Transform Infrared Spectroscopy (FTIR) analysis

The qualitative assessment of the encapsulation efficiency of tyrosol-loaded microparticles was evaluated by Fourier Transform Infrared Spectroscopy (FTIR) method.

Samples were evaluated using the attenuated total reflectance (ATR) mode, with an A225/Q PLATINUM ATR Diamond crystal with a single reflection accessory. The spectra were recorded from 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1} .

The absence of on the FTIR-ATR spectra of specific functional groups of tyrosol indicates the efficient incorporation of tyrosol in the selected polymer-carriers.

Specifically, bands associated with the benzene ring present in the chemical structure of tyrosol should be carefully considered, such as: (i) two bands relative to the C=C stretch (in-ring) from 1600 cm^{-1} to 1400 cm^{-1} and (ii) out of the plane bending C-H, from 900 cm^{-1} to 675 cm^{-1} .

Moreover, the comparison of FTIR spectra of unloaded microparticles, polymers, and tyrosol allows supporting the conclusions previously taken by the study of FTIR spectra of tyrosol-loaded microparticles. Each FTIR result presented and discussed in this work is based on the mean of three independent spectra obtained.

3.2.2.2.3 Differential Scanning Calorimetry (DSC) analysis

The Differential Scanning Calorimetry (DSC) technique was selected to evaluate the thermal behavior of tyrosol-loaded microparticles, pure tyrosol, and the coating materials used in the present study (PLGA, EC, and PCL) as described by Paulo & Santos (2018b).

The analyses were performed in triplicate using a NETZSCH DSC 214 Polyma calorimeter (NETZSCH DSC 214 Polyma, Selb, Germany) with a temperature range of 30 °C to 270 °C at a heating rate of 10 K.min⁻¹, under a nitrogen atmosphere (flow rate of 40 mL.min⁻¹).

3.2.2.2.4 Thermogravimetric Analysis (TGA)

The thermogravimetric analyses (TGA) were employed to assess the thermal stability of tyrosol-loaded microparticles, pure tyrosol, and the polymers used as carriers of tyrosol as described by Piletti et al. (2017) and Paulo & Santos, (2018b). Slight modifications were employed to the procedure proposed by Piletti et al. (2017).

The analyses were performed in triplicate using a Netzsch STA449 F3 Jupiter thermogravimetric analyzer (Netzsch STA449 F3 Jupiter, Selb, Germany) with a temperature range of 30 °C to 810 °C at a heating rate of 10 K.min⁻¹ under inert atmosphere (N₂, a flow rate of 30 mL.min⁻¹).

3.2.2.2.5 Evaluation of the morphology

The external morphology of tyrosol-loaded microparticles was evaluated according to the procedure described by Paulo & Santos (2018a, 2018b, 2019).

Accordingly, the main external morphological characteristics of tyrosol-loaded microparticles, as well as microparticles polydispersity, were assessed using a PHENOM XL scanning light microscope (Eindhoven, The Netherlands) at an accelerating voltage of 10 kV. Freeze-dried microparticles were placed on an aluminum stub with a carbon double-sided adhesive tape. The samples were sputter-coated with gold for 20 seconds using a vacuum-sputtering coater (Leica, EM SCD 500, Wetzlar, Germany).

3.2.2.2.6 Evaluation of the particle size distribution

The particle size distributions of tyrosol-loaded PLGA microparticles, tyrosol-loaded EC microparticles, and tyrosol-loaded PCL microparticles were evaluated using the laser granulometry technique. The particle size distribution and polydispersity were evaluated for each formulation in triplicate using a Coulter Counter-LS 230 Particle Size Analyzer (Miami, FL, USA) equipment. Average values were considered in data analysis.

The polydispersity of particle size distributions was analyzed considering the polydispersity index (PDI) as presented in Equation (III.3.4) as previously reported by Paulo and Santos (2018a, 2018b, 2019).

$$PDI = \frac{D_{v,90} - D_{v,10}}{D_{v,50}} \quad (\text{III.3.4})$$

where the $D_{v,90}$, $D_{v,10}$ and $D_{v,50}$ represent the maximum particle diameter below which 90% of the sample volume exists, the maximum particle diameter below which 10% of the sample volume exists, and the maximum particle diameter below which 50% of the sample volume exists, respectively.

3.2.2.3 Evaluation of the total antioxidant capacity of tyrosol-loaded microparticles

The antioxidant capacity of tyrosol-loaded PLGA microparticles, tyrosol-loaded EC microparticles, tyrosol-loaded PCL microparticles, and pure tyrosol were evaluated using the total non-enzymatic antioxidant capacity (TAC) kit (Ref. MAK187, Sigma-Aldrich Chemical, St. Louis, MO, USA).

Similarly to the quantitative determination of tyrosol encapsulation efficiency into different polymer matrices, two concentrations were taken into account for the computing and calculation of the total antioxidant capacity of tyrosol-loaded microparticles – the equivalent concentration (C_S) of tyrosol present in the supernatant (T_S) in Trolox Equivalents (TE) of the $w_1/o/w_2$ double emulsion after 3 hours of microparticles hardening, when the sample of the $w_1/o/w_2$ double emulsion was submitted to a low-intensity centrifugation process (4 000 rpm corresponding to 2 670 G, using a centrifuge Rotofix 32A, Hettich) for 15 minutes and the corresponding concentration of tyrosol in a second supernatant (C_P) in TE, after the reconstruction of the pellet from the previous sample (T_P) in ultrapure water and its submission to a second centrifugation process for 15 minutes (low-intensity centrifugation process; 4 000 rpm corresponding to 2 670 G, using a Rotofix 32A centrifuge).

In brief, 100 μ L of each sample were transferred to a 96 well plate. Afterward, 100 μ L of Cu^{2+} working solution were added to all sample wells. The content was mixed by pipetting and incubated for 90 minutes at room temperature. The plate was protected from light during the incubation. Afterward, the absorbance was read at 570 nm in a microplate reader (Synergy HT, Biotek, USA) as described by Paulo & Santos (2018b). The authors applied the same technical approach for the determination of the antioxidant activity of hydroxytyrosol-loaded EC microparticles using two different theoretical loadings (5% w/w and 10% w/w) by double emulsion solvent evaporation technique.

A standard Trolox calibration curve was obtained, adding 0, 4, 8, 12, 16, and 20 μ L of the 1 mM of the Trolox Standard Solution into the 96 well plate. Ultrapure water was added to each well to bring to volume to 100 μ L. The 0, 4, 8, 12, 16, and 20 nmol/well standards were generated. All the measurements were performed in triplicate.

The results were obtained in TE of the unknown sample well. Results are expressed in mM of TE in the case of the working solutions prepared considering a TLC of 5% w/w and 10% w/w and in mg of TE/g of dried dispersion in the case of loaded microparticles.

3.2.3 Statistical analysis

Data analyses were performed using the MINITAB 18 software (Minitab Inc., State College, PA, USA). The analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$).

The quantification of tyrosol was performed using the EZChrom Elite Chromatography Data System – Agilent (California, United States of America). The quantification of the antioxidant activity of tyrosol and tyrosol-loaded microparticles was executed using the Omega multiplate reader software.

3.3 Results

Even though it is well described that tyrosol presents remarkable radical scavenging activity, its potential incorporation in food matrices is still limited. To circumvent that, it is proposed, in the present study, its incorporation in polymer-carriers compatible with food matrices. Tyrosol-loaded microparticles were obtained by $w_1/o/w_2$ double emulsion solvent evaporation technique using three alternative polymer-carriers (PLGA, EC, and PCL). Moreover, for each polymer-carrier, two different TLCs were studied (5% w/w and 10% w/w). Additionally, the encapsulation efficiency and the radical scavenging potential of tyrosol and tyrosol-loaded microparticles were also evaluated as these parameters are considered to be relevant when the incorporation of loaded microparticles in functional foods is contemplated.

Furthermore, unloaded microparticles were produced and characterized similarly to tyrosol-loaded microparticles. The physicochemical characterization of polymer-only (PO) microparticles allowed to appraise some of the obtained results as well as it favored the interpretation of results and assumptions related to physicochemical properties of tyrosol-loaded microparticles. In the section III.3.3.1, during the qualitative analysis of tyrosol encapsulation by FTIR-ATR, the comparison of the FTIR spectra of loaded microparticles with PO microparticles allows to prove that tyrosol was efficiently incorporated in the selected polymeric system as the presence of functional groups at similar wavenumbers of PO microparticles allows to infer about the surface' similarity of the loaded and unloaded microparticles.

3.3.1 Analytical methods validation

The validation of the analytical methods was performed considering two complementary quantifications: (i) the quantification of tyrosol and (ii) the quantification of the total antioxidant capacity of loaded microparticles.

The methods were validated regarding the correlation coefficient, the relative standard error of the slope, the concentration range, the limit of detection (LOD) and the limit of quantification (LOQ) (part of the data is presented in Table III.3.2).

In the case of the HPLC method, tyrosol was detected in ultrapure water and in the case of UV-Vis spectrophotometer method, the total amount of antioxidant encapsulated was evaluated considering the amount of antioxidants present in the external aqueous phase (polyvinyl alcohol solution at a concentration level of 1% w/w) after the hardening of microparticles (w_2).

For each method, a mean calibration curve was drawn based on the three independent calibration curves obtained. In the case of the HPLC method, a linear range was observed in the range of 1.0 mg/L to 15.0 mg/L, and in the case of the UV-Vis spectrophotometer method, a linear range was observed in the range of 2.0 nmol/ μ L to 20.0 nmol/ μ L.

The LOD and LOQ obtained values for both calibration curves were acceptably low, demonstrating the possibility of the application of the obtained regression equations for the quantification of tyrosol (HPLC method) and antioxidants (UV-Vis method).

Table III.3.2 - Results from the regression analysis for the quantification of tyrosol and the total amount of antioxidants encapsulated

Analytical methods validation parameters	HPLC Method	UV-Vis Method
Regression Equation ^{a,b}	$A = (2.4 \pm 0.2) \times 10^5 C$ (mg/L) + $(-1.9 \pm 3.4) \times 10^4$	$Abs = (3.0 \pm 0.3) \times 10^{-2} C$ (nmol/ μ L) + $(2.1 \pm 3.6) \times 10^{-2}$
Correlation Coefficient	0.999	0.995
Concentration range	1.0-15.0 (mg/L)	2.0-20.0 (nmol/ μ L)

^a A is the area of the peak (HPLC method), and C is the concentration of tyrosol (μ g/L) in the case of the HPLC method, Abs is the absorbance (UV-Vis spectrophotometer method), and C is the Trolox Equivalents (TE) concentration (nmol/ μ L)

^b Mean calibration curve based on three independent calibration curves

A – Area; Abs – Absorbance; C – Concentration; HPLC – High-Performance Liquid Chromatography; TE – Trolox Equivalents; UV-Vis – Ultraviolet-Visible;

3.3.2 Determination of the tyrosol loading content, and the product yield

The actual loading content (% w/w) and the product yield (% w/w) by referenced to the TLC (% w/w) of the different powders obtained are presented in Figure III.3.1 (A – actual loading content (% w/w) *versus* theoretical loading content; B – product yield (% w/w) *versus* theoretical loading content).

Considering the TLC of 5% w/w, the mean actual loading content of tyrosol-loaded PLGA, EC and PCL microparticles were $4.9 \pm 0.1\%$ w/w, $4.9 \pm 0.1\%$ w/w and $4.7 \pm 0.1\%$ w/w, respectively. The amount of tyrosol per gram of dried dispersion was 49 ± 1 mg of tyrosol/g of dried dispersion, 49 ± 1 mg of tyrosol/g of dried dispersion, 49 ± 2 mg of tyrosol/g of dried dispersion, in the case of tyrosol-loaded PLGA, EC and PCL microparticles, respectively.

The results of the actual loading content, considering a TLC of 10% w/w of tyrosol-loaded microparticles formulated using PLGA, EC, and PCL as polymer-carriers were $9.9 \pm 0.1\%$ w/w, $9.9 \pm 0.1\%$ w/w and $9.5 \pm 0.4\%$ w/w, respectively.

The amount of tyrosol per gram of dried dispersion was 99 ± 1 mg of tyrosol/g of dried dispersion using PLGA as the polymer-carrier, 99 ± 1 mg of tyrosol/g of dried dispersion in the case of using EC as the coating material and 95 ± 4 mg of tyrosol/g of dried dispersion, in the case of tyrosol-loaded PCL microparticles.

Regarding the statistical analysis, the actual loading content (% w/w) of tyrosol-loaded microparticles seemed not to be affected by the polymer chosen as the carrier for the encapsulation of tyrosol, as there were no observed statistically significant differences between the experimental groups (grouped by the polymer type) since the P-values obtained were 0.58 and 0.15 for a tyrosol load of 5% w/w and 10% w/w, respectively (considering a significance level of 95%). Not surprisingly, when the results are grouped by the TLC (5% w/w and 10% w/w), for each polymer was observed that actual loading content was dependent on the TLC ($P=1.48 \times 10^{-8}$ in the case of using PLGA, 2.73×10^{-8} in the case of tyrosol-loaded EC microparticles and 4.08×10^{-5} when PCL was used as a coating material; for all cases, $P<0.05$).

The results regarding the actual loading content support the efficient incorporation of tyrosol in different polymer-carriers.

Moreover, microparticles can be tailored-made with alternative loadings, being the actual loading content of microparticles similar to the designed loading (the TLC). A similar trend was also observed by Yekdane et al. (2019). The authors performed the encapsulation of pomegranate seed oil into alternative polymer-carriers (Arabic gum and xanthan gum). Accordingly, to the obtained results of the present study, the actual loading contents of pomegranate seed oil (ranged between 13.7% and 13.9%) were found to be similar to the theoretical loading content considered by the authors (theoretical loading content of pomegranate seed oil of 15%)

The phenolic compound, hydroxytyrosol, also present in high quantities in both olive oil and olive mill wastes was encapsulated in EC polymer-based systems in similar conditions to the ones reported in the present study (Paulo & Santos, 2018b).

Similarly, two alternative TLCs (5% and 10% w/w) were studied. However, in the case of the encapsulation of hydroxytyrosol using EC as a coating material, the actual loading content was 2.2 ± 0.1 % w/w (corresponding to 22 ± 1 mg of hydroxytyrosol encapsulated/g of dried dispersion) in the case of the TLC of 5% w/w and 4.9 ± 0.2 % w/w (corresponding to 49 ± 2 mg of hydroxytyrosol encapsulated/g of dried dispersion). A decrease of around 50% was observed from the TLC to the actual loading content when hydroxytyrosol-loaded EC microparticles were produced. In the present study, the actual loading content was quite similar to the TLC against the results obtained by Paulo & Santos (2018b).

Tyrosol is structurally related to hydroxytyrosol, as their chemical structures are identical except that hydroxytyrosol holds an extra hydroxy group in meta position (Figure III.3.2). The fact that hydroxytyrosol holds on the benzene moiety an extra hydroxyl group next to other hydroxyl group provides to hydroxytyrosol an ortho diphenol or catechol structure (Tuck & Hayball, 2002). It is reported that the absence of this *o*-diphenol structure, in tyrosol, entails considerable differences in the compound susceptibility to oxidation as well as its antioxidant capacity and potency as chemopreventive compounds under oxidative stress conditions (Preedy & Watson, 2017).

The absence of the extra hydroxy group in tyrosol is responsible for its lower aqueous solubility character when compared to hydroxytyrosol. Due to its slightly lower solubility in an aqueous system, tyrosol has a less tendency to diffuse from the internal aqueous phase (w_1) to the external aqueous phase (w_2), and therefore a higher amount of tyrosol was entrapped in the polymer matrix. In the case of hydroxytyrosol, due to its slightly higher hydrophilicity, its loss into the external aqueous phase (w_2) was promoted while the dispersed phase (w_1/o) stayed in the semi-solid/transitional state (Jyothi et al., 2010).

Moreover, another relevant factor that should be considered in the present analysis is tyrosol-polymers interaction. The slightly higher lipophilicity of tyrosol, when compared to hydroxytyrosol, is probably responsible for the higher amount of tyrosol retained in the polymers. Relative stronger interaction forces between tyrosol and the polymers probably had promoted its retention in the polymer matrices.

The yield of production was evaluated for 6 different formulations corresponding to three polymers, and two alternative TLC, for each polymer tested. All the formulations were run in triplicate in a total formulation number of 18. Only average results are presented.

The mean production yields of tyrosol-loaded PLGA microparticles with a TLC of 5% w/w and 10% w/w were $80.3 \pm 1.9\%$ w/w and $73.2 \pm 2.2\%$ w/w, respectively.

Regarding the product yield results of tyrosol-loaded EC microparticles with a TLC of 5% w/w and 10% w/w were $95.0 \pm 1.7\%$ and $78.8 \pm 3.7\%$, respectively.

In the case of tyrosol-loaded PCL microparticles formulated considering a TLC of 5% and 10% w/w, the mean production yields were $79.4 \pm 4.2\%$ and $82.0 \pm 1.6\%$, respectively.

The results of this study demonstrate that both in the case of tyrosol-loaded PLGA microparticles and tyrosol-loaded EC microparticles, the TLC affected the production yield as was observed a statistically significant difference between the mean production yield among the TLC tested ($P = 0.01 < 0.05$, in the case of using PLGA and $P = 1.71 \times 10^{-4} < 0.05$ using EC as a coating material; significance level considered: 95%).

For both polymers was verified that an increase in the TLC from 5% w/w to 10% w/w led a decrease of the production yield as displayed in Figure III.3.3.

To the authors' best knowledge, this is the first time that is reported the encapsulation of tyrosol. Therefore, there is a lack of published data suitable for the direct comparison regarding the production yield. However, regarding the results of the encapsulation of a related compound of tyrosol – hydroxytyrosol – into EC microparticles, was also observed a significant ($P < 0.05$) decrease in the production yield from the microparticles formulated with a TLC of 5% w/w to the ones formulated with 10% w/w (Paulo & Santos, 2018b).

Regarding the results of the production yield of tyrosol-loaded PCL microparticles, the production yield seemed not to be affected by the TLC as there were no observed differences between the experimental groups (grouped by the TLC) at the production yield level as the P-value obtained was 0.38 ($P > 0.05$; confidence level of 95%).

During the study of the effects of wall materials and operating conditions on physicochemical properties of encapsulated banana passionfruit pulp by spray-drying, the authors Troya et al. (2018) found that the production yield was dependent of the inlet temperature on the spray-drier and the core to encapsulating material ratio (physicochemical feature that can be related to the theoretical loading content as an increase of the core to the encapsulation material ratio imply an increase of the theoretical loading content). Similarly to the results obtained in the present study regarding the inclusion of tyrosol into PLGA and EC polymeric systems – an increase in the loading content led to a decrease in the product yield. The obtained values by Troya et al. (2018) for the production yield (production yield ranged between 29.35% and 60.84%) differing from those obtained in the present study. Even though the chemical composition of the selected polymers and core materials, microencapsulation technique and even operating conditions, the use of the spray-dryer may limit the production yield– the operational temperature conditions restrained the mass of microparticles obtained after the microencapsulation process: when the temperature in the spray-drying chamber is too low, microparticles tend to adhere to the drying chamber and the cyclone filter wall due to the high moisture content; when the temperature is too high, microparticles tend to crack and degrade Troya et al. (2018).

The results of this study are significant as was found a lack of published data regarding the comparison of the theoretical loading content with the production yield.

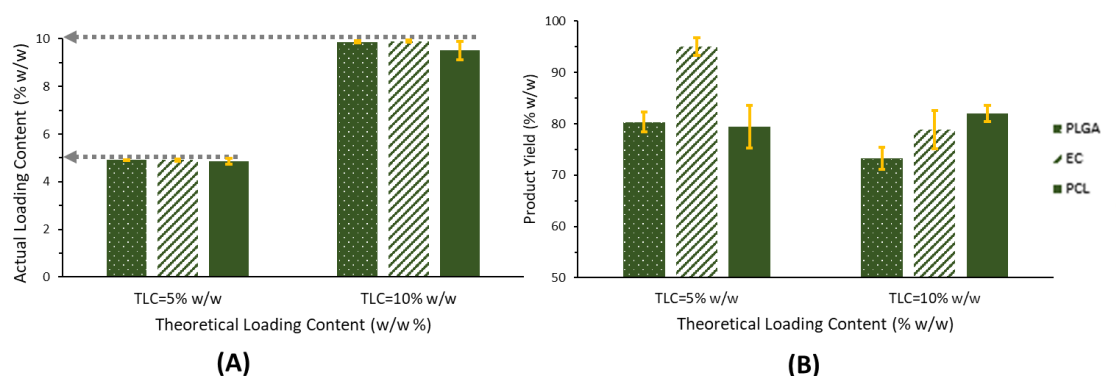


Figure III.3.1 - Actual loading content (% w/w) (A) and product yield (% w/w) (B) of tyrosol-loaded microparticles (PLGA - poly(D,L-lactide-co-glycolide); EC - ethylcellulose; PCL - polycaprolactone;

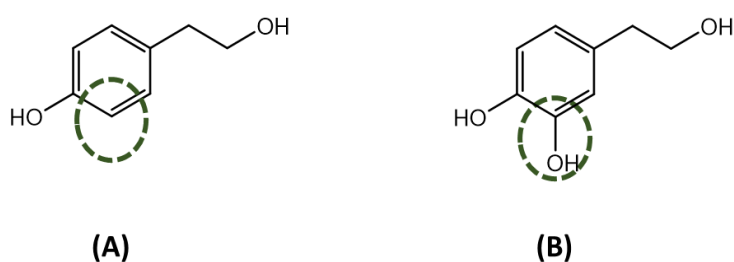


Figure III.3.2 - Chemical structure of tyrosol (A) and hydroxytyrosol (B)

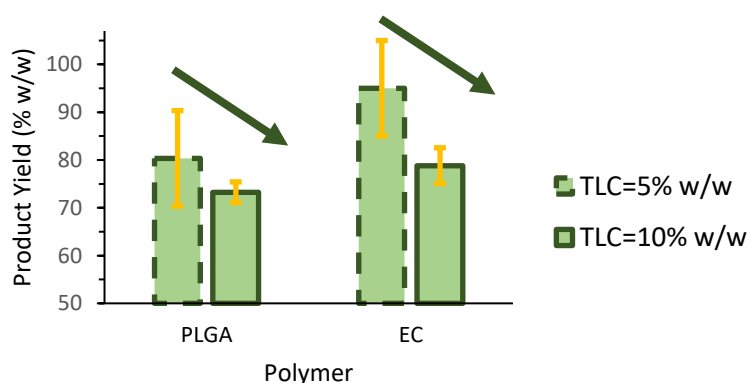


Figure III.3.3 - Results regarding the production yield of tyrosol-loaded PLGA and EC microparticles; Two alternative TLC are presented (5% w/w and 10% w/w) (PLGA – poly(D,L-lactide-co-glycolic) acid; EC – Ethylcellulose; TLC – Theoretical Loading Content); The yellow bars correspond to the error bars based on the standard deviation of three independent assays

3.3.3 Assessment of tyrosol encapsulation efficiency

The encapsulation efficiency of tyrosol-loaded microparticles was evaluated through qualitative and quantitative methods. For the qualitative evaluation of the efficient incorporation of tyrosol into polymer matrices selected for this study, the Fourier Transform Infrared Spectroscopy (FTIR) method was applied. The Attenuated Total Reflectance (ATR) mode of FTIR was used. It allows evaluating surface properties of the materials rather than their bulk properties. The quantitative assessment of tyrosol encapsulation efficiency into microparticles was performed as described in section III.3.2.2.1 and accordingly to Equation (III.3.3).

3.3.3.1 Qualitative assessment of tyrosol encapsulation efficiency by Fourier Transform Infrared Spectroscopy (FTIR)

In the present study, the FTIR-ATR was employed in order to identify specific functional groups present in the surface of tyrosol-loaded microparticles to evaluate the effectiveness of the incorporation of this bioactive compound in different polymeric matrices.

The presence of characteristic functional groups of tyrosol which are not present in the coating materials used indicates the possibility of the adsorption of tyrosol in the surface of microparticles. If tyrosol was not efficiently incorporated in microparticles, specific FTIR bands associated with the benzene ring present in the chemical structure of tyrosol (Figure III.3.2 – A) should be observed in FTIR spectra. The characteristic FTIR bands of tyrosol are: (i) the C-H stretch from 3100 cm^{-1} to 3000 cm^{-1} , (ii) two bands related to the C=C stretch (in-ring) from 1600 cm^{-1} to 1400 cm^{-1} and (iii) the out of the plane bending C-H aromatic disubstituted in *para* position, from 900 cm^{-1} to 800 cm^{-1} .

The wavenumbers (cm^{-1}) and the assignments of the bands observed in the FTIR spectra of tyrosol (TYR), PLGA, PO microparticles formulated with PLGA and tyrosol-loaded PLGA microparticles formulated considering a TLC of 5% w/w and 10% w/w are presented in Table III.3.3. Similarly, the corresponding wavenumbers (cm^{-1}) and assignments of TYR, EC, loaded (TYR-EC TLC=5% and TYR-EC TLC=10%) and PO microparticles (PO-EC MPs) are presented in Table III.3.4. Similar assignments using PCL as the polymer-carrier are presented in Table III.3.5.

The signature peaks of tyrosol, related to the presence of an aromatic structure, are highlighted in grey from Table III.3.3 to Table III.3.5. These signature peaks include the ones related to (i) the C-H aromatic, stretching vibration mode at 3130 cm^{-1} , (ii) the C-H aromatic disubstituted in *para* position bending out of the plane at 815 cm^{-1} , (iii) two bands related to the C=C aromatic stretching vibration at 1510 cm^{-1} and at 1450 cm^{-1} . The characteristic peaks of tyrosol were not observed in loaded microparticles. The wavenumber corresponding to the C-O stretching vibrational mode of tyrosol is present in the fingerprint area (FPA), and more importantly, it is a characteristic band for all the polymers under study, so it was not considered for the quantitative detection of tyrosol at the surface of loaded microparticles.

The signature peaks of PLGA, PO microparticles formulated with PLGA and tyrosol-loaded PLGA microparticles formulated considering two alternative TLC (5% w/w and 10% w/w) are similar (Table III.3.3) and do not present any relevant similarity with the signature peaks of tyrosol. Analogous results were obtained with EC (Table III.3.4) and PCL (Table III.3.5) when they were used as coating materials of tyrosol. For all the polymers studied, significant band shiftings were not observed in the FTIR spectra of loaded microparticles when compared to the spectra of PO microparticles. These observations confirm the efficient incorporation in PLGA, EC, and PCL micro-systems as were not found any of the signature peaks of tyrosol in loaded microparticles.

To the authors' best knowledge, this is the first time that is reported the encapsulation of tyrosol in alternative polymer-carriers. Therefore, there is a lack of published data regarding the FTIR studies of tyrosol encapsulated in polymeric matrices suitable for direct comparison.

However, it is reported that using the same encapsulation technique ($w_1/o/w_2$ solvent evaporation technique), similar formulation and process conditions, is possible to achieve efficient incorporation of the selected bioactive compound. According to Paulo and Santos (2018a), hydroxytyrosol, a similar compound to tyrosol, was efficiently encapsulated using EC as the polymer coating material.

Table III.3.3 - Wavenumbers (cm^{-1}) and assignments for the bands observed in the FTIR spectra of TYR, PLGA, PO-PLGA microparticles and loaded microparticles considering a TLC of 5% w/w (TYR-PLGA MPs TLC=5%) and a TLC of 10% w/w (TYR-PLGA MPs TLC=10%)

Bond type	Chemical functional group	Vibration mode	Wavenumber (cm^{-1})				
			TYR	PLGA	PO-PLGA MPs	TYR-PLGA MPs (TLC=5%)	TYR-PLGA MPs (TLC=10%)
O-H	-	Stretching	3382	3496	NF	NF	NF
C-H	Alkanes	Stretching	2879	2997	2997	2997	2297
				2950	2951	2951	2945
	-CH ₂ -	Bending	1450	1384	1384	1385	1392
	-CH ₃		-	1452	1429	1429	1429
	Aromatic	Stretching	3130	-	-	-	-
	Aromatic, disubstituted, <i>para</i> position	Bending out-of-plane	815	-	-	-	-
C=C	Aromatic	Stretching	1510 1450	-	-	-	-
C-O	Esters, alcohols and carboxylic acids	Stretching	FPA	1166 1085	1162 1086	1167 1085	1167 1085
C=O	Esters and carboxylic acids	Stretching	-	1751	1747	1749	1749

FPA -Fingerprint Area; MP – Microparticle; NF – Not Found; PLGA – Poly(D,L-lactide-co-glycolic) acid; PO – Polymer Only; TLC -Theoretical Loading Content (% w/w); TYR – Tyrosol;

Table III.3.4 - Wavenumbers (cm⁻¹) and assignments for the bands observed in the FTIR spectra of TYR, EC, PO-EC and loaded microparticles considering a TLC of 5% w/w (TYR-EC MPs TLC=5%) and a TLC of 10% (TYR-EC MPs TLC=10%)

Bond type	Chemical functional group	Vibration mode	Wavenumber (cm ⁻¹)				
			TYR	EC	PO-EC MPs	TYR-EC MPs (TLC=5%)	TYR-EC MPs (TLC=10%)
O-H	-	Stretching	3382	3460	3470	3470	3470
C-H	Alkanes	Stretching	2879	2867	2867	2868	2867
	-CH ₂ -	Bending	1450	FPA	FPA	FPA	FPA
	-CH ₃		-	1375	1375	1375	1375
	Aromatic	Stretching	3130	-	-	-	-
	Aromatic, disubstituted, <i>para</i> position	Bending out-of-plane	815	-	-	-	-
C=C	Aromatic	Stretching	1510 1450	-	-	-	-
C-O-C	Aliphatic ether	Stretching	FPA	1051	1055	1055	1055

EC – Ethyl Cellulose; FPA – Fingerprint Area; MP – Microparticle; PO – Polymer Only; TLC -Theoretical Loading Content (% w/w); TYR – Tyrosol;

Table III.3.5 - Wavenumbers (cm^{-1}) and assignments for the bands observed in the FTIR spectra of TYR, PCL, PO-PCL and loaded microparticles considering a TLC of 5% w/w (TYR-PCL MPs TLC=5%) and a TLC of 10% (TYR-PCL MPs TLC=10%)

Bond type	Chemical functional group	Vibration mode	Wavenumber (cm^{-1})				
			TYR	PCL	PO-PCL MPs	TYR-PCL MPs (TLC=5%)	TYR-PCL MPs (TLC=10%)
C-H	Alkanes	Stretching	2879	2943 2868	2943 2868	2943 2868	2943 2868
	-CH ₂ -	Bending	1450	FPA	FPA	FPA	FPA
	-CH ₃		-	1361	1361	1361	1361
	Aromatic	Stretching	3130	-	-	-	-
	Aromatic, disubstituted, <i>para</i> position	Bending out-of-plane	815	-	-	-	-
	Aromatic	Stretching	1510 1450	-	-	-	-
C=O	Aliphatic ether	Stretching	FPA	1107	1106	1107	1107
C=O	Ester	Stretching	-	1726	1726	1726	1726

FPA – Fingerprint Area; MP – Microparticle; PCL - Polycaprolactone; PO – Polymer Only; TLC -Theoretical Loading Content (% w/w); TYR – Tyrosol;

3.3.3.2 Quantitative assessment of tyrosol encapsulation efficiency

The quantitative assessment of tyrosol encapsulation efficiency was evaluated considering Equation (III.3.3) and as described in section III.3.2.2.2.1. The graphical representations of tyrosol encapsulation efficiency for each polymer-carrier studied (PLGA, EC, and PCL) and for each TLC are presented in Figure III.3.4.

The mean tyrosol encapsulation efficiency of loaded microparticles formulated considering a TLC of 5% w/w were $99.1 \pm 0.3\%$ w/w for tyrosol-loaded PLGA microparticles, $99.3 \pm 0.5\%$ w/w for tyrosol-loaded EC microparticles and $99.1 \pm 0.1\%$ w/w in the case of for tyrosol-loaded PCL microparticles.

The mean tyrosol encapsulation efficiency of tyrosol-loaded PLGA, EC and PCL microparticles, considering a TLC of 10% w/w were $98.7 \pm 0.5\%$ w/w, $98.5 \pm 1.0\%$ w/w and $98.9 \pm 0.1\%$ w/w, respectively.

In the case of tyrosol-loaded PLGA and EC microparticles, the tyrosol encapsulation efficiency was not affected by the TLC as was not observed statistically significant differences among the two experimental groups (TLC of 5% w/w and TLC of 10% w/w) for each polymer, considered individually as the P-value was higher than 0.05, considering a significance level of 95% ($P=0.32$ in the case of tyrosol-loaded PLGA microparticles and $P=0.26$ in the case of loaded EC microparticles). The tyrosol loss during the encapsulation was only $0.9 \pm 0.3\%$ w/w and $1.3 \pm 0.5\%$ w/w in the case of PLGA loaded microparticles formulated considering 5% and 10% w/w of the TLC, respectively. The tyrosol loss during the microencapsulation procedure of tyrosol-loaded EC microparticles was $0.7 \pm 0.5\%$ w/w and $1.5 \pm 1.0\%$ w/w, for a TLC of 5% w/w and 10% w/w, respectively.

The results regarding the tyrosol encapsulation efficiency of tyrosol-loaded PCL microparticles were dissimilar to the ones obtained for tyrosol-loaded PLGA and EC microparticles. The encapsulation efficiency in the biocompatible polymer PCL was affected by the theoretical loading content as, considering a confidence level of 95%, the null hypothesis (H_0 : tyrosol encapsulation efficiency is not affected by the TLC value) was not verified ($P=0.01<0.05$).

The total amount of tyrosol lost during the encapsulation process was 9 ± 1 mg of tyrosol/g of tyrosol used for the formulation in the case of a TLC of 5% w/w and 11 ± 1 mg tyrosol/g of tyrosol used for the formulation in the case of a TLC of 10% w/w.

In this case, the increase of the TLC led to a decrease of tyrosol encapsulation efficiency. Similar trends were also observed by other authors as Freytag et al. (2000) and Paulo and Santos (2018a).

Comparing the results obtained when hydroxytyrosol was encapsulated in EC polymer-based system considering two alternative TLC (5% w/w and 10% w/w) (Paulo & Santos, 2018a), a related pattern was observed, in the present study – an increase in TLC from 5% w/w to 10% w/w, resulted in a decrease in the encapsulation efficiency.

Similarly to the results obtained by Troya et al. (2018) and even though that the encapsulating and core materials, and microencapsulation technique applied were dissimilar, the authors verified that an increase of the core to the encapsulating material ratio and therefore the theoretical loading content led to a decrease of the encapsulation efficiency.

The reported observation can be explained by the fact that when microparticles are loaded with higher amount of tyrosol, probably a higher flux of tyrosol may occur when microparticles are in the semi-solid state (Gabor et al., 1999; Krishnamachari et al., 2007; Paulo & Santos, 2018b).

The results regarding the quantitative assessment of the encapsulation efficiency are fairly significant. For all polymer-carriers was observed high encapsulation efficiency values. In the case of using PLGA or even EC as polymer-carriers, the encapsulation efficiency was independent of the TLC. Differently, when PCL is used as the coating material, the TLC seemed to affect the encapsulation efficiency. This observation should be carefully considered when is intended the coating of tyrosol in a PCL polymer-based system, and its incorporation in food matrices – a compromise between the encapsulation efficiency and a load of tyrosol should be considered.

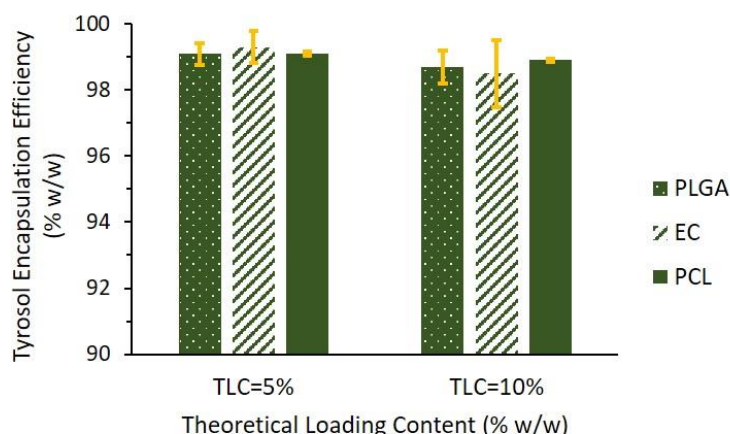


Figure III.3.4 - Tyrosol Encapsulation Efficiency (% w/w) of tyrosol-loaded microparticles (PLGA - Poly(D,L-lactide-co-glycolide); EC - Ethylcellulose; PCL – Polycaprolactone; TLC – Theoretical Loading Content (% w/w)); Data are expressed as mean $\pm$ standard deviation

3.3.4 Differential Scanning Calorimetry (DSC) analysis

The efficient incorporation of tyrosol in PLGA, EC, and PCL microsystems can be indirectly confirmed by differential scanning calorimetry (DSC) analysis. The comparison of the thermal stability of the encapsulated forms of tyrosol (tyrosol-loaded microparticles) with the free form (tyrosol standard) allows to indirectly confirm the adequate incorporation of this bioactive compound in the selected polymer matrices (Hill et al., 2013; Karathanos et al., 2007). Moreover, the analyses of DSC thermograms allow characterizing the physicochemical status of the bioactive compound formulated into microparticles (Dubernet, 1995; Zhang & Gao, 2007).

From the DSC thermograms analysis (Figure III.3.5 – A), pure tyrosol presents a sharp endotherm peak at 95 °C (correspondent area of 173.8 J/g) corresponding to the melting point of the pure compound. In a research, regarding the coupling of tyrosol, quercetin or ferulic acid and electron beam irradiation to cross-link chitosan-gelatin films, Benbettaieb et al. (2015) reported that the melting point of tyrosol is ranged between 89 °C and 92 °C, as predicted values from ACD/Labs software from Chemspider and PubChem.

However, to the authors' best knowledge, this is the first time that is performed a DSC analysis of tyrosol. The obtained value is relatively close to the range presented by Benbettaieb et al. (2020); nevertheless, the purity degree may contribute significantly to the melting point value obtained - 98% of the analyzed solid corresponded to pure tyrosol. Impurities (about 2%) present in the sample may be the explanatory reason for the obtained melting point value slightly above the predicted range values.

Concerning the DSC results of tyrosol-loaded PLGA microparticles, the absence of the melting endotherm peak of tyrosol at 95 °C yield some evidence of the efficient incorporation of tyrosol in PLGA microparticles as the melting points tends to shift or disappear when during the incorporation of a bioactive compound in a polymer-based system. Any of the crystalline tyrosol material in microparticles was not detected in the DSC study of tyrosol-loaded PLGA microparticles. Therefore, it can be concluded that tyrosol was incorporated in PLGA shells in an amorphous or disordered-crystalline phase of a molecular dispersion or a solid solution state in PLGA matrices after microparticles production. Similar results were obtained by Zhang & Gao (2007) when they encapsulated temozolomide drug into PLGA microparticles intended for antitumor activity studies against Glioma C6 cancer cells.

According to Friess and Schlapp (2002), the glass-transition temperature (T_g) of PLGA polymers ranges between 40 °C and 65 °C, increasing with the increase on the molecular weight. In the present study, the T_g value of PLGA was found to be 53 °C (Resomer® RG 504, lactide/glycolide 50/50) corresponding to an area of 8 J/g. The DSC thermograms of tyrosol-loaded PLGA microparticles formulated considering a TLC of 5% w/w and 10% w/w present an endotherm sharp peak at 53 °C (area of 11 J/g) and 54 °C (correspondent area of 8 J/g), respectively. As no significant changes were found on the T_g value of tyrosol-loaded PLGA microparticles compared to PLGA, it corroborates the hypothesis of the incorporation of tyrosol into PLGA microparticles on its amorphous form (Mandal et al., 2002; Pamujula et al., 2008, 2004; Paulo & Santos, 2018a).

Regarding the DSC results of tyrosol-loaded EC microparticles, it can be stated that the absence of a sharp endotherm peak between 70 °C and 120 °C, relative to the tyrosol melting point at 95 °C indicates the efficient incorporation of tyrosol in EC polymer matrices. The mid T_g value of EC was found to be 122 °C, whereas the T_g values of tyrosol loaded EC microparticles formulated considering a TLC of 5% w/w and 10% w/w were found to be 150 °C and 138 °C. There was verified a shift on the T_g value from EC to higher values when tyrosol was encapsulated using EC as a shell material. In the case of tyrosol-loaded microparticles were observed shifts of the T_g value position of the polymer due to anti-plasticizing effects of the active ingredient. Moreover, the endotherm peak of tyrosol is not present in the DSC thermograms; therefore, tyrosol and EC formed a solid solution. A similar result was obtained by Paulo & Santos (2018a): solid solutions due to anti-plasticizing effects were obtained when hydroxytyrosol was encapsulated using EC as a coating material.

Respecting the DSC analysis of tyrosol-loaded PCL microparticles as well as only PCL, it was verified that the melting endotherm peak of PCL (found to be 64 °C) was the absence of tyrosol-loaded PCL microparticles. Tyrosol-loaded PCL microparticles endotherm peaks were found to be 60 °C in the case of microparticles formulated considering a TLC of 5% w/w and 95 °C, in the case of microparticles formulated considering a TLC of 10% w/w. In the case of tyrosol-loaded PCL microparticles, alternative thermal behaviors were found between the samples prepared considering a TLC of 5% w/w and 10% w/w. In the case of tyrosol-loaded microparticles formulated considering 5% w/w of TLC, the endotherm peak of tyrosol is not present in the DSC thermogram of tyrosol-loaded PCL microparticles (TLC of 5% w/w; Figure III.3.5-D) and was observed a shift on the T_g of PCL to lower temperatures indicating that tyrosol formed a solid solution with PCL due to plasticizing effects. In the case of tyrosol-loaded microparticles formulated considering a TLC of 10% w/w, tyrosol was encapsulated on its crystalline form as the T_g of tyrosol-loaded PCL microparticles (TLC=10% w/w) was found to be close to the T_g value of tyrosol (actual value found for loaded microparticles of 95 °C). The obtained results are similar to some found by other authors.

The authors Natarajan et al. (2011) found a similar value for the T_g of PCL (61 °C) when they were formulating microspheres containing quercetin for the treatment of rheumatoid arthritis. A similar value (69 °C) for the melting point of PCL was also presented by Nagy et al. (1999) when they were characterizing blends of PCL with polyvinyl alcohol. Also, Dasaratha & Sathyamoorthy (2018) found the melting temperature of PCL at 63 °C when they were preparing etoposide-loaded PCL microspheres by solvent evaporation technique. The results found in the literature are in agreement with the ones obtained in the present study.

The typical events that may occur when a bioactive ingredient is encapsulated in a polymer matrix are of 3 different types as follow: (i) the bioactive ingredient form with the polymer a solid solution and consequently the melting endotherm peak of the encapsulated material is not present in the thermogram of loaded microparticles, and there is verified a shift of the T_g of the polymer to higher (anti-plasticizing effects) or to lower (due to plasticizing effects) temperatures; (ii) the bioactive ingredient is dispersed in the polymer matrix in its amorphous form, therefore, the T_g of the polymer is not changed, and the melting endotherm peak of the bioactive compound is not shown on the thermogram of loaded microparticles; and (iii) the bioactive compound is incorporated in the polymer matrix in its crystalline form and, in this case, neither the T_g value of the polymer and the bioactive compound are changed (Mandal et al., 2002; Pamujula et al., 2004, 2008; Paulo & Santos, 2018a). However, in case (ii) the absence of the melting peak of the bioactive ingredient maybe not detected due to the dispersion of a low amount of bioactive compound in the polymeric matrix as described by Natarajan et al. (2011).

The DSC results of tyrosol-loaded microparticles are significant. The pure compound presents its melting point at 95 °C. In the case of tyrosol-loaded PLGA microparticles for both TLC, considered tyrosol was incorporated in PLGA on its amorphous form, contrasting with tyrosol-loaded EC microparticles as tyrosol formed a solid solution with EC at both TLCs considered.

Distinctively, tyrosol-loaded PCL microparticles formulated considering alternative TLCs presented different thermal behaviors: when a TLC of 5% w/w was considered, tyrosol formed a solid solution (due to plasticizing effects) with PCL, however, when it was considered a TLC of 10% w/w, tyrosol was incorporated in PCL matrix on its crystalline form, indicating, therefore, that tyrosol kept its crystallinity until the degradation of tyrosol-loaded PCL microparticles (TLC=10% w/w).

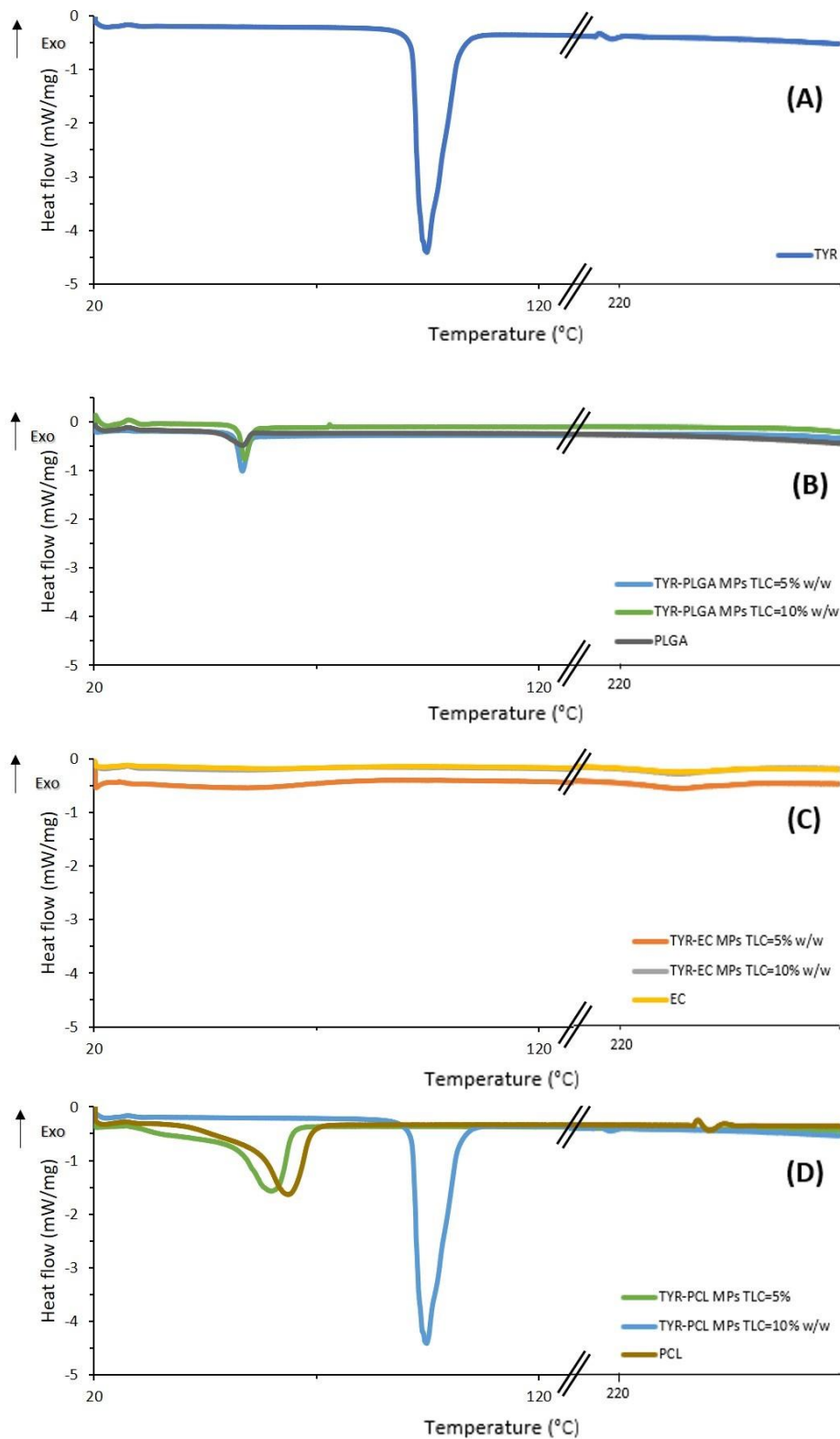


Figure III.3.5 - Differential Scanning Calorimetry thermograms of pure tyrosol **(A)**, tyrosol-loaded PLGA microparticles **(B)**, tyrosol-loaded EC microparticles **(C)** and tyrosol-loaded PCL microparticles **(D)** (EC – Ethylcellulose; MPs – Microparticles; PCL – Polycaprolactone; PLGA – Poly(D,L-lactide-co-glycolide); TYR - Tyrosol)

3.3.5 Thermogravimetric Analysis (TGA)

The TGA thermograms and the derivative thermogravimetry (DTG) thermograms from 30 °C to 810 °C of tyrosol, powders of loaded microparticles (tyrosol-loaded PLGA microparticles, tyrosol-loaded EC microparticles, and tyrosol-loaded PCL microparticles at two different TLCs – 5% w/w and 10% w/w) and polymers (PLGA, EC and PCL) are presented in Figure III.3.6-A and III.3.6-B, respectively.

These thermogravimetric analyses were performed to evaluate the weight change of microparticles with temperature. The weight loss extent of formulations was similar in similar temperature ranges, excluding the TGA thermogram of pure tyrosol. Both pure tyrosol, polymers (PLGA, EC, and PCL) and the powders of loaded microparticles presented only one level of weight loss.

According to Yoksan et al. (2010), the peaks on the DTG thermogram (Figure III.3.6-B) corresponds to the highest rate of weight loss and therefore to the degradation temperature (T_d) of the component in the analyzed material. The weight loss of tyrosol happened in the range of 132 °C to 237 °C (Figure III.3.6-A). The analysis of the DTG thermogram of tyrosol revealed that tyrosol was degraded at 230 °C (Figure III.3.6-B).

The interpretation of TGA and DTG thermograms of tyrosol-loaded PLGA indicate that the weight loss change occurred in the range of 265 °C and 381 °C, corresponding to a T_d of 330 °C in the case of tyrosol-loaded PLGA microparticles formulated considering a TLC of 5% w/w and the weight loss occurred in the range of 261 °C to 381 (T_d of 343 °C), in the case of microparticles formulated considering a TLC of 10% w/w. It was observed a shift to higher temperatures of the T_d when PLGA was loaded with 5% w/w to 10% w/w. These results are significant as tyrosol-loaded PLGA microparticles formulated with a higher loading presented higher thermogravimetric stability than microparticles formulated considering a TLC of 5% w/w.

When EC was used as a polymer-carrier, the weight loss change occurred in the range of 287 °C and 401 °C associated with a T_d of 306 °C in the case of loaded EC microparticles formulated with a TLC of 5% w/w; in the case of tyrosol-loaded EC microparticles associated with TLC of 10% w/w, the weight loss occurred between 303 °C and 397 °C (T_d at 363 °C).

It was verified a shift in the T_d value from 306 °C to 363 °C when the TLC was increasing from 5% w/w to 10% w/w. An increase of the loading content led to an increase in the thermogravimetric stability of microparticles. A similar conclusion was drawn when hydroxytyrosol was encapsulated using the same polymer-carriers (EC) and considering the TLC of 5% w/w and 10% w/w (Paulo & Santos, 2018a).

Regarding the analysis of TGA and DTG thermograms of tyrosol-loaded PCL microparticles, the weight loss change of loaded PCL microparticles considering a TLC of 5% w/w occurred between 330 °C and 467 °C, associated to a T_d at 412 °C. Regarding the weight loss change of tyrosol-loaded PCL microparticles formulated considering a TLC of 10% w/w, it was observed between 304 °C and 437 °C (T_d of 412 °C). Even though, as opposed to obtained results for tyrosol-loaded PLGA and EC microparticles, with PCL was not verified a shift in the T_d to higher values with the increase of the loading content. However, PCL seemed to be the polymer to better guaranty the thermogravimetric stability of tyrosol-loaded microparticles as with PCL the weight loss change started to occur at higher temperatures (specifically in the case of tyrosol-loaded PCL microparticles formulated considering a TLC of 5% w/w) and the T_d value was found to be the highest. Therefore, it can be concluded that PCL is an excellent polymer-carrier for tyrosol regarding the thermogravimetric stability of microparticles. Nevertheless, it can be stated that generally, tyrosol-loaded microparticles were nearly thermogravimetrically stable in the temperature range of 30 °C to 261 °C (the minimum value temperature found for the onset temperature – the case of tyrosol-loaded PLGA microparticles formulated considering a TLC of 10% w/w).

Comparing the DTG (Figure III.3.6-B) thermograms were not detected significant differences in the mass loss at the beginning of the study (lower temperatures).

Therefore, it can be concluded that free tyrosol molecules did not arrange on the surface of the microparticles; otherwise, a significant weight loss should have occurred in the initial stages of the process (around the degradation temperature of tyrosol), and differences in the TGA thermograms would be detectable. Moreover, it can be stated that no residual solvents were present in all the powders, as no weight loss (corresponding to ramps in the TGA thermograms or peaks in DTG thermograms) was distinguishable at low temperatures.

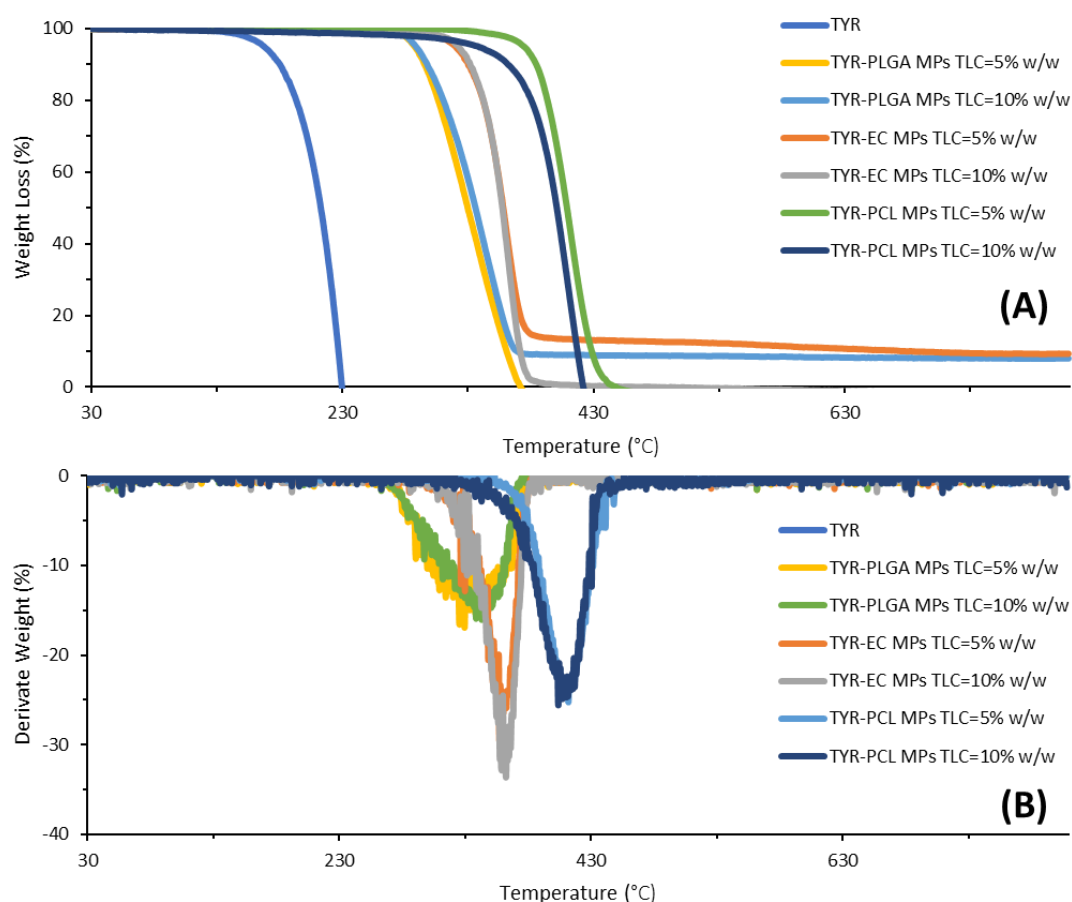


Figure III.3.6 - Thermogravimetric analysis thermograms of different samples pure tyrosol, tyrosol-loaded PLGA microparticles, tyrosol-loaded EC microparticles and tyrosol-loaded PCL microparticles (TGA *versus* temperature **(A)** and DTG *versus* temperature **(B)**) (EC – ethylcellulose; MPs – Microparticles; PCL – Polycaprolactone; PLGA – Poly(D,L-co-glycolide; TYR – Tyrosol)

3.3.6 Evaluation of the morphology

The scanning electron microscopy (SEM) was used to evaluate the main morphological characteristics of the powders obtained.

Microphotographs of tyrosol-loaded PLGA, EC and PCL microparticles are displayed in Figure III.3.7 (A – tyrosol-loaded PLGA microparticles considering a TLC of 5% w/w; B - tyrosol-loaded EC microparticles considering a TLC of 5% w/w; C - tyrosol-loaded PCL microparticles considering a TLC of 5% w/w; D - tyrosol-loaded PLGA microparticles considering a TLC of 10% w/w; tyrosol-loaded EC microparticles considering a TLC of 10% w/w; tyrosol-loaded PCL microparticles considering a TLC of 10% w/w). Microphotographs are presented with a magnification of 5 000 times.

Regardless of the loading content and the polymer-carrier, generally, the obtained microparticles present a smooth surface with almost no pores, fissures, or cracks. Moreover, the obtained microparticles present a spherical shape.

This type of outer topography seems to be characteristic of microparticles obtained by $w_1/o/w_2$ double emulsion solvent evaporation technique.

Similar microphotographs of loaded PLGA microparticles and PO microparticles using PLGA are displayed in the study presented by Ling et al. (2008) when they encapsulated bovine serum albumin in PLGA polymer matrices by double emulsion solvent evaporation technique. The outer morphological characteristics of tyrosol-loaded PLGA microparticles exhibited in the present study (Figure III.3.7-A and Figure III.3.7-B) are almost indistinguishable from those presented by Ling et al. (2008) at the 0 weeks of degradation morphological analysis.

Identical outer morphological characteristics were found between the tyrosol-loaded EC microparticles (Figure III.3.7-B and Figure III.3.7-E) obtained in this study and the ones presented by Paulo & Santos (2018b) during the encapsulation of hydroxytyrosol into EC polymer matrices using similar formulation and operational conditions to the ones reported in the present study.

Polymer only microparticles using PCL as the coating material were produced by $w_1/o/w_2$ (Ibraheem et al., 2014). The obtained PCL coated microparticles obtained by Ibraheem et al. (2014) present a similar outer morphology to the obtained ones in the present study (tyrosol-loaded PCL microparticles; Figure III.3.7-C and Figure III.3.7-F).

Comparing the microphotographs obtained for all the polymers, an increase in the loading content led to an increase in the agglomeration degree of microparticles. From Figure III.3.7-A (tyrosol-loaded PLGA microparticles formulated considering a TLC of 5% w/w) to Figure III.3.7-B (tyrosol-loaded PLGA microparticles formulated considering a TLC of 10% w/w) is detectable an increase on the agglomeration degree. Similar conclusions can be drawn for both tyrosol-loaded EC and PCL microparticles.

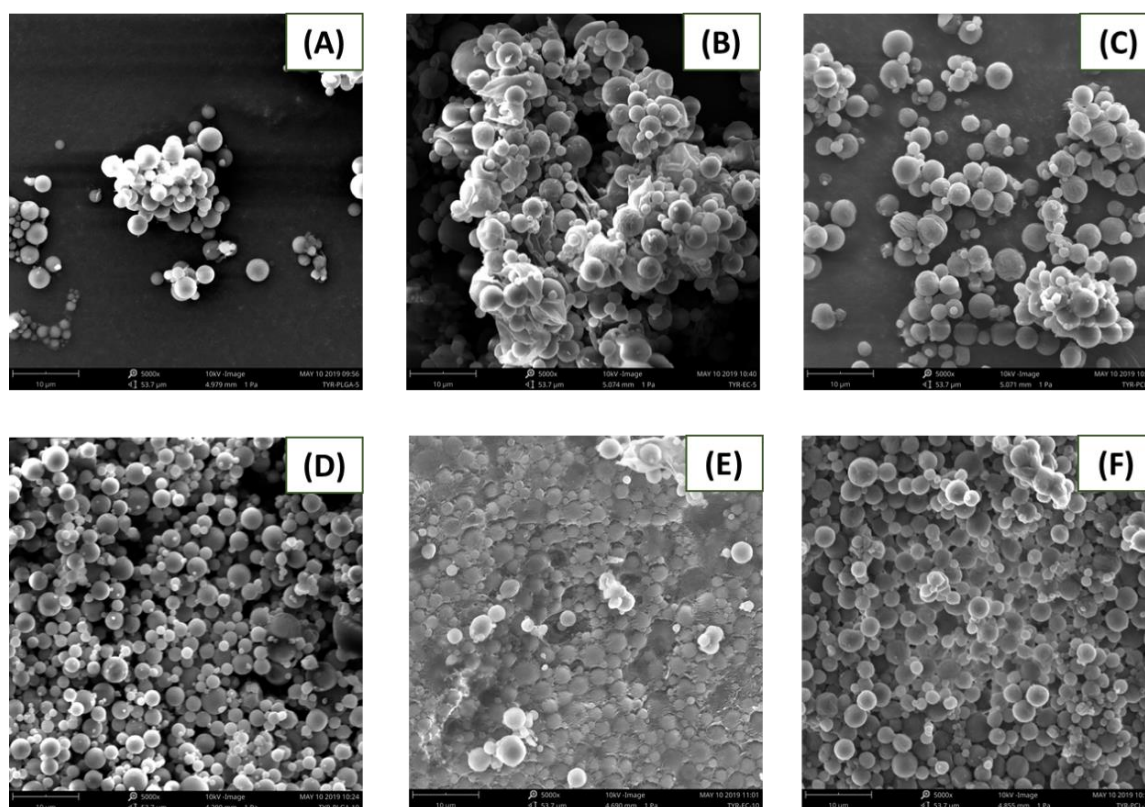


Figure III.3.7 - Microphotographs of tyrosol-loaded PLGA microparticles considering a TLC of 5% w/w **(A)** and 10% w/w **(D)**, tyrosol-loaded EC microparticles formulated considering a theoretical loading content of 5% w/w **(B)** and 10% w/w **(E)** and tyrosol-loaded PCL microparticles considering a theoretical of 5% w/w **(C)** and 10% w/w **(F)** (amplification of 5000 times is presented in all microphotographs; beam intensity of 10.00 kV)

3.3.7 Evaluation of the particle size distribution

The particle size distribution of tyrosol-loaded PLGA, EC, and PCL microparticles are presented in Figure III.3.8 (differential volume (%) *versus* mean particle size (μm)). Considering a TLC of 5% w/w, the mean particle size was $2.3 \pm 0.1 \mu\text{m}$, $2.3 \pm 0.1 \mu\text{m}$ and $7.5 \pm 1.5 \mu\text{m}$ for tyrosol-loaded PLGA, EC and PCL microparticles, respectively. The mean particle size of tyrosol-loaded microparticles formulated considering a TLC of 10% w/w were $3.4 \pm 0.1 \mu\text{m}$ for tyrosol-loaded PLGA microparticles, $8.8 \pm 0.7 \mu\text{m}$ for tyrosol-loaded EC microparticles and $7.8 \pm 1.9 \mu\text{m}$, in the case of tyrosol-loaded PCL microparticles.

Regarding the results of the mean particle size of tyrosol-loaded PLGA microparticles and tyrosol-loaded EC microparticles, the mean particle size seemed to be affected the loading content chosen as were observed statically significant differences between the experimental groups (for each polymer, grouped by the TLC) as the P-values obtained were $P=2.62 \times 10^{-4}$ and $P=7.81 \times 10^{-5}$, in the case of tyrosol-loaded PLGA microparticles and tyrosol-loaded EC microparticles, respectively (confidence level considered of 95%).

Considering the results of the mean particle size of tyrosol-loaded PCL microparticles, the mean particle size seemed not to be affected by the loading content as were not observed significant differences between the two TLCs tested for each polymer as the P-value obtained was higher than 0.05 ($P=0.84>0.05$; confidence level of 95%).

In the case of the encapsulation of tyrosol using PLGA or EC as coating materials, an increase of the loading content led to an increase of the mean particle size. Considering a TLC of 10% w/w, more amount of tyrosol was encapsulated, which resulted in the recovery of larger microparticles. Microparticute systems consisted of budesonide-loaded PLGA microparticles were produced by Krishnamachari et al. (2007). The authors of this research also observed that an increase in the loading content led to an increase in the mean particle size.

A similar trend was also observed by Paulo & Santos (2018a) during the encapsulation of tyrosol in EC microparticulate system: an increase of the TLC from 5% w/w to 10% w/w led to increasing of the mean particle size from $156.6 \pm 6.9 \mu\text{m}$ to $304.0 \pm 16.0 \mu\text{m}$.

Regarding the graphical representation of differential volume *versus* the mean particle size (μm), tyrosol-loaded PLGA microparticles formulated with a TLC of 5% w/w presented a unimodal particle size distribution (Figure III.3.8 – A) whereas tyrosol-loaded PLGA microparticles present a bimodal particle size distribution. Similar considerations can be drawn from tyrosol-loaded EC microparticles: microparticles formulated with a TLC of 5% w/w present a unimodal particle distribution whereas microparticles formulated considering a TLC of 10% w/w present a multimodal particle size distribution.

However, the results regarding the particle size distribution of tyrosol-loaded PCL microparticles, for both TLCs, the particle size distributions were multimodal. These results are in agreement to the ones obtained for the statistical significance of the influence of the TLC in the mean particle size: both tyrosol-loaded PLGA and EC microparticles mean particle sizes are affected by the TLC, and they present a unimodal particle size distribution in the case of TLC of 5% w/w and a bimodal or multimodal particle size distribution in the case of a TLC of 10% w/w. Contrastingly, tyrosol-loaded PCL microparticles mean particle size is shown to be independent of the TLC chose ($P=0.84>0.05$), and it was observed for both TLCs, a multimodal particle size distribution (Figure III.3.8-C).

Comparative results were obtained by Paulo & Santos (2018a) as was noticed a unimodal particle size distribution when was considering a TLC of hydroxytyrosol of 5% w/w and a bimodal particle size distribution when a TLC of 10% w/w was considered.

Regarding the results of polydispersity, the mean polydispersity values were obtained according to Equation (III.3.4) and as described in III.3.2.2.2.6 section.

Tyrosol-loaded PLGA microparticles formulated with a TLC of 5% w/w and 10% w/w presented a mean PDI of 1.3 ± 0.1 and 1.8 ± 0.3 , respectively. In the case of microparticles coated with EC, the mean PDI values obtained were 1.3 ± 0.1 and 3.0 ± 0.2 for the TLC of 5% w/w and 10% w/w, respectively. Tyrosol-loaded PCL microparticles presented mean PDI values of 2.7 ± 0.2 and 2.9 ± 0.3 for a TLC of 5% w/w and 10% w/w, respectively.

Only tyrosol-loaded EC microparticles PDI seemed to be affected by the TLC as the P-value obtained was $P=4.00 \times 10^{-5}<0.05$. In the case of microparticles produced using PLGA and PCL as the shell material, the PDI seemed not to be affected by the TLC as the P-values obtained were higher than 0.05 ($P=0.06$ in the case of tyrosol-loaded PLGA microparticles and $P=0.84$ in the case of tyrosol-loaded PCL microparticles).

In the case of tyrosol-loaded EC microparticles, an increase of the TLC led to an increase of the polydispersity.

This observation is also in agreement with the conclusions drawn when hydroxytyrosol was encapsulated in EC microparticles: the PDI increased from 2.9 ± 0.1 to 5.5 ± 0.3 when the theoretical loading content was shifted from 5% w/w to 10% w/w (Paulo & Santos, 2018a).

However, it should be considered that a considerable number of factors (e.g., formulation and process factors) may affect the mean particle size, particle size distribution as well as the polydispersity of microparticles (Krishnamachari et al., 2007; Paulo & Santos, 2018a).

The obtained results regarding the evaluation of the particle size distribution are significative. The mean particle size of both tyrosol-loaded PLGA and EC microparticles was dependent on the TLC: an increase of the loading content led to an increase of the mean particle size. Dissimilarly, the mean particle size of tyrosol-loaded PCL microparticles was not affected by the TLC. Moreover, in the case of using PLGA and EC as coating materials, microparticles formulated with a TLC of 5% w/w present a unimodal particle size distribution whereas microparticles formulated with TLC of 10% w/w present a multimodal particle size distribution.

Considering the polydispersity of the obtained microparticles, it can be stated that only for tyrosol-loaded EC microparticles, the PDI was dependent on the loading content. In this case, an increase in the loading content led to an increase in the PDI of the obtained powder.

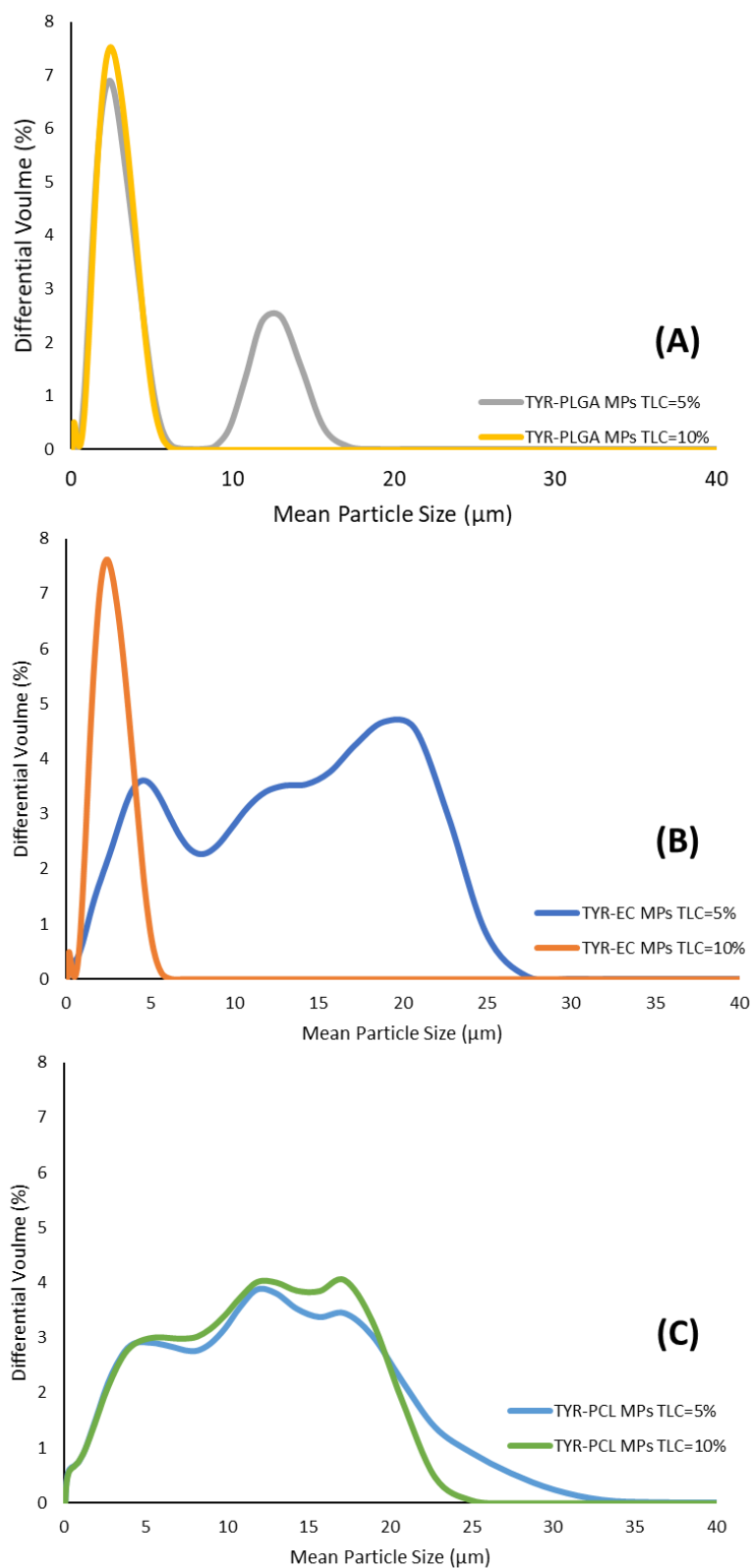


Figure III.3.8 - Mean particle size distribution of tyrosol-loaded PLGA microparticles **(A)**, tyrosol-loaded EC microparticles **(B)** and tyrosol-loaded PCL microparticles **(C)** ((EC – Ethylcellulose; MPs – Microparticles; PCL – Polycaprolactone; PLGA - Poly(D,L-lactide-co-glycolide; TYR – Tyrosol)

3.3.8 Evaluation of the total antioxidant capacity

The Cu^{2+} chelating activity was evaluated for free tyrosol (tyrosol aqueous solutions with a concentration corresponding to the TLC chosen for the present study) and encapsulated tyrosol using three alternative polymer-carriers. The main goal was to evaluate the free radical scavenging capacity of tyrosol and tyrosol-loaded microparticles.

Alternative methodologies can be employed to quantify the radical scavenging capacity of pure compounds and the corresponding microparticles as the (i) the application of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methodology, (ii) the use of the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging methodology, (iii) using the oxygen radical absorbance capacity (ORAC) assay, (iv) applying the superoxide dismutase (SOD) assay or even (v) using the ferric reducing antioxidant potential (FRAP) assay, among others (Dudonné et al., 2009).

The authors Jabeen et al. (2017) reported the application of Cu^{2+} -antioxidants complexes formation for the radical scavenging determination. In the present study, this alternative method was employed in order to assess the radical scavenging capacity of tyrosol and tyrosol-loaded microparticles.

The total antioxidant capacity of tyrosol-loaded microparticles (expressed as mg of TE/g of tyrosol) *versus* the theoretical loading content (% w/w) is presented in Figure III.3.9.

For the TLC of 5% w/w, the working solution presented a total antioxidant capacity of 106 ± 4 mg of TE/L (0.4 ± 0.1 mM of TE) and the working solution used for the encapsulation of tyrosol considering a TLC of 10% w/w showed an antioxidant capacity of 126 ± 18 mg of TE/L (0.5 ± 0.1 mM of TE). It was verified that the Cu^{2+} chelating activity of tyrosol was not proportional to the amount of tyrosol dissolved in ultrapure water – the amount of tyrosol dissolved in ultrapure water was doubled from the working solution used for the obtainment of tyrosol-loaded microparticles with a TLC of 5% w/w to 10% w/w; however, the Cu^{2+} chelating activity of tyrosol only increased 18.3% from the theoretical loading content of 5% w/w to 10% w/w.

In the case of tyrosol-loaded PLGA microparticles formulated considering a TLC of 5% w/w and 10% w/w the mean antioxidant capacity (Cu^{2+} chelating capacity) was 26 ± 4 mg of TE/g of dried dispersion and 82 ± 2 mg of TE/g of dried dispersion, respectively.

Regarding the results of the Cu^{2+} chelating capacity of tyrosol-loaded EC microparticles, the mean antioxidant capacity encapsulated was 28 ± 1 mg of TE/g of dried dispersion in the case of TLC of 5% w/w and 46 ± 3 mg of TE/g of dried powder when considered the TLC of 10% w/w.

When PCL was used as the polymer-carrier of tyrosol, the Cu^{2+} chelating capacity of loaded microparticles was 30 ± 1 mg of TE/g of dried microparticles in the case of the TLC of 5% w/w and 61 ± 2 mg of TE/g of microparticles (dry basis) when the TLC of 10% w/w was considered.

Regarding the statistical analysis, it can be stated that, when the results are grouped by TLC, for all the polymer-carriers studied, the TLC seemed to affect the Cu^{2+} chelating capacity of loaded microparticles as the P-values were found to be lower than 0.05 for all the polymers ($P=2.00 \times 10^{-5}$ in the case of tyrosol-loaded PLGA microparticles, $P=2.70 \times 10^{-4}$ respecting to tyrosol-loaded EC microparticles and $P=4.61 \times 10^{-5}$ in the case of loaded microparticles formulated with PCL). When the results were grouped by the polymer, for the two TLC studied, it was verified that in the case of a TLC of 5% w/w, the polymer chosen did not affect the Cu^{2+} chelating capacity of loaded microparticles ($P=0.19>0.05$). In the case of microparticles formulated considering a TLC of 10% w/w, the Cu^{2+} chelating capacity of loaded microparticles was significantly affected by the polymer chosen ($P=4.40 \times 10^{-6}<0.05$).

The presented results are significant as not only they prove that tyrosol was efficiently incorporated in distinctive polymer-carriers as it shows that the mean antioxidant capacity encapsulated was dependent of the theoretical loading content for the polymers studied, independent of the polymer chosen when selected powders formulated with a TLC of 5% w/w and dependent of the polymer-carrier in the case of considering a TLC of 10% w/w.

These results bring new insights for the encapsulation of antioxidants in polymer matrices intended for food, pharmaceutical, cosmetic and nutraceutical applications – in the specific case of the powerful antioxidant tyrosol, the antioxidant capacity of loaded microparticles can be tailored-made for the matrices reported.

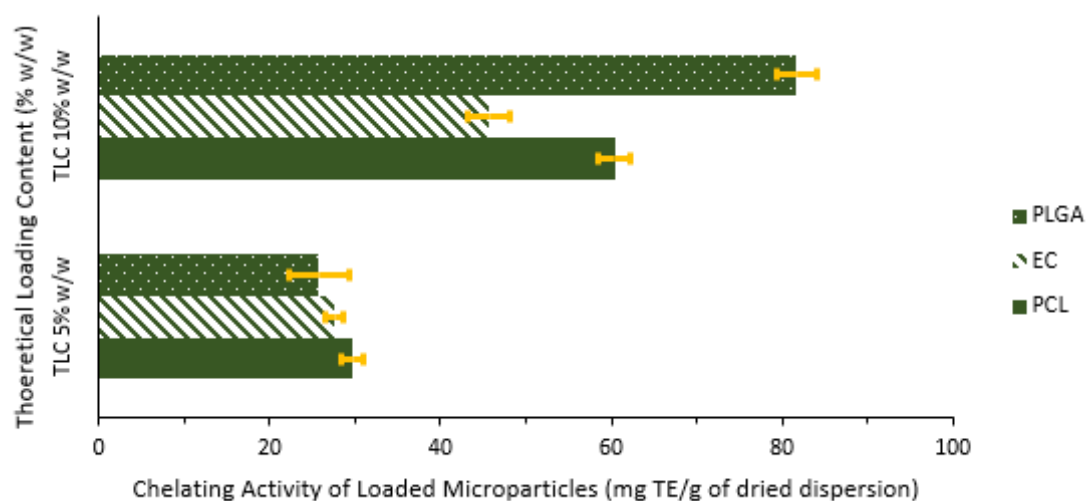


Figure III.3.9 - Cu^{2+} Chelating activity of tyrosol-loaded microparticles (mg TE/g of tyrosol) (EC – Ethylcellulose; PCL – Polycaprolactone; PLGA - Poly(D,L-lactide-co-glycolide; TE – Trolox Equivalents; TLC – Theoretical Loading Content). Data are expressed as mean $\pm$ standard deviation of three independent assays. The yellow bars correspond to the error bars based on the standard deviation of three independent assays

3.4 Conclusions

The worldwide recognition of the health benefits of the Mediterranean dietary pattern has fostered the consumption of olive oil as the preferential fat source. Tyrosol is a powerful antioxidant present in high quantities in olive oil and olive mill wastes. Even though it presents beneficial properties, its incorporation as a free compound in foods and other matrices is still limited. Therefore, water-in-oil-in-water double emulsion solvent evaporation technique may be applied to encapsulate this compound for further incorporation in foods and other alternative matrices.

The results of this study demonstrate that the Fourier Transform Infrared Spectroscopy confirmed the efficient incorporation of tyrosol in the polymer matrices selected for this study. Tyrosol-loaded microparticles were thermogravimetrically stable in the temperature range of 30 °C to 261 °C, and polycaprolactone was considered to be an exceptional carrier regarding the thermogravimetric stability of microparticles. It was observed that an increase in the theoretical loading content led to an increase in the agglomeration degree of microparticles.

The production yield was dependent on the loading content for both poly(D,L-lactide-co-glycolide) and ethylcellulose. The encapsulation efficiency was dependent on the loading content in the case of using polycaprolactone as a coating material. In the case of using poly(D,L-lactide-co-glycolide) and ethylcellulose as polymer-carriers of tyrosol, it was verified that an increase of the theoretical loading content from 5% w/w to 10% w/w led to a decrease of production yield. It was also verified that an increase of the loading content led to a decrease in the production yield when polycaprolactone was used as a polymer-carrier for tyrosol.

The results of this study on the encapsulation of tyrosol using alternative polymer-carriers bring new insights regarding the encapsulation of natural antioxidants and antioxidant-rich extracts obtained from olives, olive oil and olive mill wastes intended for further incorporation in food, pharmaceutical, cosmetic and nutraceutical matrices.

Acknowledgments

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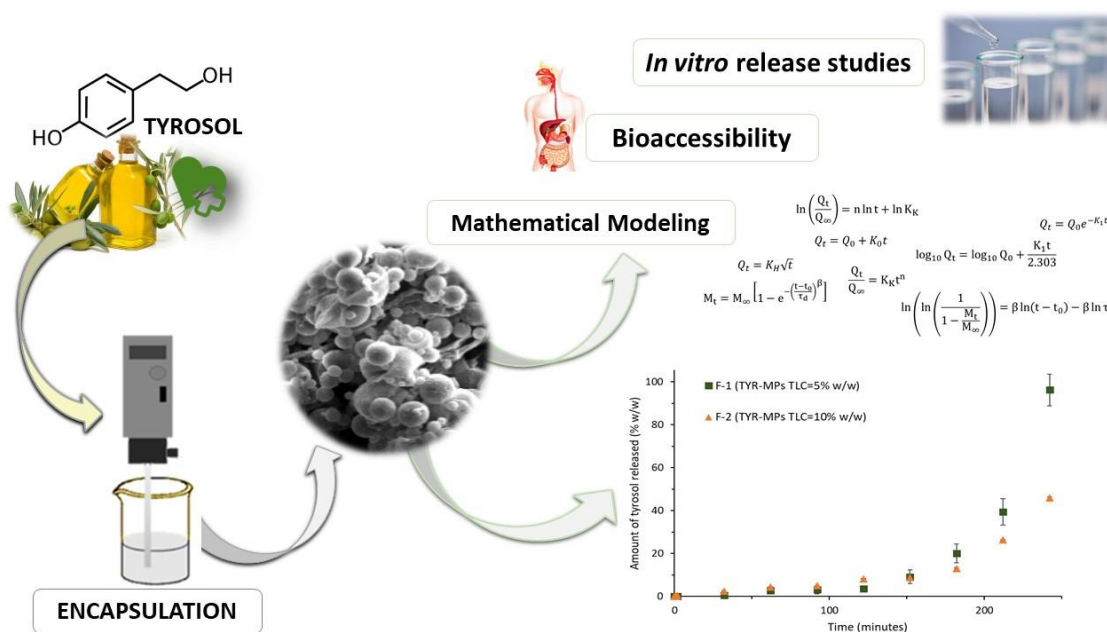
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Chapter 4. New insights in the *in vitro* release of phenolic antioxidants: the case study of the release behavior of tyrosol from tyrosol-loaded ethylcellulose microparticles during the *in vitro* gastrointestinal digestion ⁴

Filipa Paulo, Lúcia Santos

Graphical Abstract:



⁴ This chapter was adapted from: Paulo, F. & Santos, L. (2020). New insights in the *in vitro* release of phenolic antioxidants: the case study of the release behavior of tyrosol from tyrosol-loaded ethylcellulose microparticles during the *in vitro* gastrointestinal digestion. *Colloids and Surfaces B: Biointerfaces*. <https://doi.org/10.1016/j.colsurfb.2020.111339>

Abstract:

In this study, tyrosol – a phenolic antioxidant that present in olive oil and olive mill wastes – was embedded in ethylcellulose microparticles by double emulsion solvent evaporation technique. The effect of loading content (5% w/w and 10% w/w) on the release behavior and bioaccessibility of tyrosol was evaluated.

The polymer endowed efficient protection to tyrosol during the *in vitro* gastrointestinal digestion of loaded microparticles as the maximum release of tyrosol was observed during the simulated intestinal digestion, and the releases were kept outstanding low during the simulated salivary and gastric digestions. The bioaccessibility of tyrosol was improved when encapsulated. The best-fitting models of the release profiles of tyrosol were the first, and the zero-order models for formulations considering a loading of 5% w/w and 10% w/w, respectively.

The results of this study bring new perspectives for the design of loaded microparticles that will be further submitted to gastrointestinal digestion.

Highlights

1. Tyrosol(TYR) was efficiently encapsulated by double emulsion solvent evaporation.
2. The effect of TYR loading was evaluated on its release pattern and bioaccessibility.
3. Loaded microparticles were resistant to simulated salivary and gastric digestions.
4. TYR bioaccessibility was improved 1.8 times fold when it was encapsulated.
5. TYR release patterns were similar for both theoretical loading contents studied.

Keywords: tyrosol; encapsulation; *in vitro* release studies; bioaccessibility; release mechanisms; mathematical modeling;

4.1 Introduction

Olive oil is considered to be the major fat element of the Mediterranean diet (Miró-Casas et al., 2003). The consumption of olive oil is associated with many health benefits as a lower incidence of coronary heart disease, prostate, colon, and breast cancers (Miró-Casas et al., 2003; Tuck & Hayball, 2002; Visioli & Galli, 2001). Other relevant health benefits associated with the consumption of olive oil are widely described in the literature (Cicerale et al., 2009; Covas et al., 2006; Paulo & Santos, 2020b; Tegou & Konstantinidou, 2010; Visioli & Galli, 1998).

Olive oil presents high quantities of a huge variety of phenolic compounds – over 30 phenolic compounds have been identified in olive oil (Cicerale et al., 2010; Tegou & Konstantinidou, 2010).

Particularly, tyrosol (TYR) is a characteristic phenolic compound present in high concentrations in olive oil (Tuck & Hayball, 2002). Moreover, TYR can also be found in high concentrations in olive mill wastes (De Marco et al., 2007; Visioli et al., 1999).

Tyrosol, also known as *p*-hydroxyphenethyl alcohol, is a phenolic compound that possesses many positive health properties, mostly associated with its radical scavenging potential/antioxidant activity. It is postulated its effectiveness in oxidation inhibition of cholesterol in low-density lipoproteins, in the inhibition of leukocyte 5-lipoxygenase, also exhibiting a protective role against cytostatic effects produced by oxidized low-density lipoproteins in the Caco-2 intestinal mucosa cells (Giovannini et al., 1999; Tuck & Hayball, 2002).

As a drawback, TYR, like many other phenolic compounds, is sensible to several environmental conditions as oxygen, light, and heat. Furthermore, TYR presents low bioavailability as a direct consequence of an intense first-pass metabolism in the liver and the small intestine even though it bears a noticeably proper absorption (Bonechi et al., 2019; Rodríguez-Morató et al., 2016; Vlachogianni et al., 2015).

Several microencapsulation approaches allow obtaining microsystems that may effectively protect sensitive bioactive compounds as TYR, such as the spray-drying, the spray chilling or cooling, the fluid coating, the coextrusion, the inclusion complexation, the freeze or vacuum drying, the melt injection or extrusion, the ionotropic gelation and the preparation of liposomes (Mirafzali et al., 2014; Sobel et al., 2014). The encapsulation and co-precipitation processes with supercritical fluids for food and nutraceutical industries have been exploited in the last few years (Silva & Meireles, 2014; Priamo et al., 2010; Visentin et al., 2012). The majority of the encapsulating processes mentioned are based on the production of droplets of the bioactive compound(s) that are subsequently coated/surrounded by a membrane. The exceptions of this bioactive compound(s) protective microsystems based on droplet and membrane arrangement listed are the liposomes, the preparation of melt extrudates, the inclusion complexation approaches, and the employment of natural encapsulates such as yeasts (Mirafzali et al., 2014; Sobel et al., 2014).

The spray-drying technique has been extensively used for the encapsulation of food ingredients such as vitamins, minerals, fats, oils, flavors, and even pigments, to extend food product shelf life through the protection of these specific bioactives from the environment. Nevertheless, the high drying temperatures applied during the assembly of microparticles may damage the bioactive compound(s) (Ozkan et al., 2019). Moreover, it is reported that the spray-drying technique is associated with low microparticles product yield, and it is virtually impossible the control of particle size (Dalmoro et al., 2012; Ozkan et al., 2019). Additionally, challenges arise regarding the selection of the wall material that should be water-soluble in an acceptable range. The most widely employed wall materials that include polysaccharides (e.g., gum acacia, maltodextrins, and modified starch) and proteins (e.g., sodium caseinate, soy proteins, and whey proteins) present limited solubility in water and therefore the amount of water that needs to be evaporated is much larger due to the lower dry matter content which requires, accordingly, the reduction of the bioactive compound(s) in the feed (Desai & Park, 2005; Ozkan et al., 2019).

In this context, alternative techniques to spray-drying have been developed, such as the ionic gelation, the preparation of liposomes and the multiple emulsification-evaporation approaches (e.g., water-in-oil-in-water double emulsion solvent evaporation technique).

The ionotropic microsystem production technique, also known as ionic gelation, is based on the ability to crosslink polyelectrolytes in the presence of multivalent ions such as Al^{3+} , Ca^{2+} , and Ba^{2+} that can be employed through extrusion or by gelation/emulsification (Lupo et al., 2015; Ozkan et al., 2019). Moreover, the production of gel microparticles through ionic gelation can be classified as external or internal. The process is termed as external in the case of ions diffuse from an external source into the polymer solution and internal in the case of the salt embedded in a liquid solution is added dropwise into the polymer solution (Davarcı et al., 2017; Funami et al., 2009). The wall materials generally employed for gelling purposes during ionic gelation microencapsulation are soluble in aqueous systems (e.g., alginate, chitosan, gellan gum, pectin, konjac, carboxymethylcellulose) (Leong et al., 2016; Ozkan et al., 2019). Even though encapsulation through ionic gelation can be performed under safe and mild conditions, the use of water-soluble polymers limits the application of this technique as the protected bioactive compounds are promptly released during the gastrointestinal digestion. Therefore, the adequate protection given by the gel can be lost.

In this regard, liposomes are alternative carriers of bioactive compounds for foods. Liposomes are vesicular structures consisting of one or several bilayer phospholipids membranes enclosing a defined volume of an aqueous solution (Bonechi et al., 2019; Lopez-Polo et al., 2020; McClements, 2015; Umagiliyage et al., 2017). The physicochemical characteristics of the phospholipid coat make liposomes attractive bioactive compounds delivery systems – due to the amphiphilicity of phospholipids, these systems allow the entrapment of hydrophilic, lipophilic, or even amphiphilic compounds (Bonechi et al., 2019; De Leeuw et al., 2009; Lopez-Polo et al., 2020; Yokota et al., 2012). Even though liposomes are unique microsystems regarding their biodegradability and biocompatibility, they are not generally chemically stable at room temperature (e.g., they are prone to hydrolysis and/or to oxidation). Moreover, these vesicles are susceptible to aggregation and fusion over time (Mirafzali et al., 2014).

Another interesting alternative that started to be exploited for food products is the emulsification-evaporation microencapsulation approaches. This set of techniques can be distinguished according to the type of the inner layer where the bioactive compound(s) is/are dispersed (aqueous phase *versus* oily-like phase) and the number of emulsion interfaces (e.g., in the single emulsion there only one interface, in the double emulsion there are two emulsion interfaces). Among emulsification-evaporation encapsulation techniques, the water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation approach has been on the highlight for the coating of water-soluble bioactive compounds intended for food applications (Paulo & Santos, 2018a, 2019, 2020a). The principals of this technique comprise (i) the solubilization of the bioactive(s) compound(s) in the internal aqueous phase (w_1), followed by the dispersion of the internal aqueous phase in an organic phase (o) containing the dissolved polymer, (iii) succeeded by the re-emulsification of the w_1/o emulsion in an external aqueous phase (w_2) containing an aqueous stabilizer. The last steps (iv) of the production of microparticles by double emulsion solvent evaporation include the evaporation of the solvent and the hardening of microparticles (Paulo & Santos, 2018a, 2018b, 2019).

Therefore, the encapsulation of TYR in a gastro-resistant polymer, delivering TYR directly into the intestine, allows protecting TYR effectively from the first-pass metabolism. Moreover, the combined selection of the encapsulation technique and the polymer may assist the stabilization and the improvement of TYR shelf-life. Also, the release rate of TYR and its solubility in the intestinal fluid can be modulated through the proper choice of the polymer (Manach et al., 2005; Sansone et al., 2011).

Among the encapsulating agents, ethylcellulose has been studied as a potential gastrointestinal resistant polymer for the protection of bioactive ingredients in functional foods as hydroxytyrosol (another relevant phenolic compound present in olive oil and olive mill wastes) and other dietary compounds (Paulo & Santos, 2018a; Shi & Tan, 2003; Zheng et al., 2011). Ethylcellulose is approved by the European Union as a carrier in food additives by the European Commission Regulation 1130/2011 (European Commission, 2011).

Food grade ethylcellulose polymers do not present almost combustion residues (ash content up to 0.4%) (Wustenberg, 2015). Furthermore, ethylcellulose polymers have been gaining attention for the coating of bioactive compounds to be further incorporated in foods due to their versatility: they are odorless and tasteless polymers, they are stable to many exposure conditions as light, heat, humidity, and oxygen and they are non-toxic and non-irritant (Murtaza, 2012).

Among the encapsulation techniques, water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation approach has been used for the coating of water-soluble bioactive compounds intended for food applications (Paulo & Santos, 2018a, 2019, 2020a). The principals of this technique comprise (i) the solubilization of the bioactive(s) compound(s) in the internal aqueous phase (w_1), followed by the dispersion of the internal aqueous phase in an organic phase (o) containing the dissolved polymer, (iii) succeeded by the re-emulsification of the w_1/o emulsion in an external aqueous phase (w_2) containing an aqueous stabilizer. The last steps (iv) of the production of microparticles by double emulsion solvent evaporation include the evaporation of the solvent and the hardening of microparticles (Paulo & Santos, 2018c, 2018b, 2019).

Regarding the complexity of the digestion of foods in the human digestive tract, many *in vivo* and *in vitro* procedures have been developed to understand the physiological response to specific food additives or even to possible allergenic food components (Brodkorb et al., 2019; Dupont & Mackie, 2015). The evaluation of *in vivo* digestion processes includes invasive procedures (e.g., aspiration from the stomach or the small intestinal) and less invasive procedures (e.g., imaging approaches including magnetic resonance imaging and wireless telemetric approaches) (Koziolek et al., 2015; Sullivan et al., 2014). *In vivo* animal studies are still widely employed; however, they entangle the death of the animal or surgical procedures to place cannulas in the digestion organ to obtain samples from gastrointestinal fluids. Not surprisingly, the use of animal models is doubted based on the ethical ground (Brodkorb et al., 2019). Therefore, *in vitro* gastrointestinal models have been employed to simulate food digestion to understand the complexity of digestion.

In the light of this, the present study aimed to evaluate the release performance of TYR from TYR-loaded ethylcellulose microparticles in alternative dissolution media, stimulating *in vitro* the conditions of the whole gastrointestinal tract (salivary tract, gastric tract and proximal intestine tract).

Tyrosol-loaded ethylcellulose microparticles were obtained by $w_1/o/w_2$ double emulsion solvent evaporation technique, considering two alternative theoretical loading contents: 5% w/w and 10% w/w. The influence of the loading content on the release profile of TYR from loaded microparticles was evaluated. The similarity among the release profiles (release profile of TYR from microparticles loaded considering a theoretical loading content of 5% w/w *versus* the release profile of TYR from microparticles loaded considering a theoretical loading content of 10% w/w) was assessed. Moreover, in the present study, mathematical modeling of the release profiles was performed through simple model-dependent methods as the zero-order and the first-order release kinetic models and more complex model-dependent methods as the Higuchi, Korsmeyer-Peppas and Weibull models. The bioaccessibility of TYR released in the gastrointestinal tract from loaded microparticles was evaluated, and the influence of the loading content on this parameter was assessed.

Comprehensively, the release behavior of TYR-loaded ethylcellulose microparticles was assessed, in perspective to design an optimized TYR-loaded ethylcellulose microparticles delivery system for further incorporation in food matrices. The present study intends to provide new insights on the release behavior of phenolic compounds as TYR to be incorporated in foods that will further suffer digestion on the gastrointestinal tract.

Notably, this research aimed to bring new insights on delivery systems that effectively protect bioactive compounds, increasing their bioaccessibility.

4.2 Experimental procedures

4.2.1 Materials

4.2.1.1 Chemicals

The antioxidant selected for the present study, tyrosol (Ref. 188255, $C_8H_{10}O_2$, CAS 501-94-0), chemicals used during microencapsulation namely the double emulsion-stabilizer/surfactant polyvinyl alcohol (Ref: P8136-250G, 87-90% hydrolyzed, average molecular weight of 30,000-70,000, CAS 9002-89-5) and the polymer/coating material ethylcellulose, (Ref. 433837-250G, viscosity of 46 cP, CAS 9004-57-3) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA).

The solvent used for microencapsulation purposes, dichloromethane (Ref. ACRO433991000, CH_2Cl_2 , CAS 1665-00-5) and ethyl acetate (SupraSolv[®] for GC-MS, purity $\geq 99.8\%$, Ref: 1.00789.2500, $C_4H_8O_2$, CAS 141-78-6) used during TYR extraction procedures were obtained from VWR International (Fontenay-sous-Bois, France). The solvents used in the high-performance liquid chromatography (HPLC) apparatus for the quantification of TYR, namely acetonitrile (LiChrosolv[®] Reagent Ph. Eur. grade, Supelco[®], supplier Merck, VWR Ref: 1.00030.2500, C_2NH_3 , CAS 75-05-8), methanol (HiPerSolv CHROMANORM[®] Reagent Ph. Eur. Grade, HPLC grade, purity $\geq 99.8\%$, Ref: 20864.320, CH_4O , CAS 67-56-1) and acetic acid standardized solution 0.1 N (supplier Alfa-Aesar, Ref: 35567.K7, $C_2H_4O_2$, CAS 64-19-7) were also obtained from VWR International (Fontenay-sous-Bois, France).

The salts magnesium chloride hexahydrate (supplier Merck, VWR Ref: 1.05833.0250, $MgCl_2 \cdot 6H_2O$, CAS 7791-18-6), potassium dihydrogen phosphate (supplier Merck, VWR Ref: 1.04873.0250, KH_2PO_4 , CAS 7778-77-0), potassium chloride (supplier Merck, VWR Ref: 1.04936.0250, KCl, CAS 7447-40-7), ammonium carbonate (supplier Merck, VWR Ref: 5.43835.0100, $(NH_4)_2CO_3$, CAS 10361-29-2) and sodium hydrogen carbonate (supplier Merck, VWR Ref: 1.06329.0500, $NaHCO_3$, CAS 144-55-8), sodium chloride (supplier Merck, VWR Ref: 1.06404.0500, NaCl, 7647-14-5) were purchased from VWR International (Fontenay-sous-Bois, France).

The enzymes used during the *in vitro* release studies namely α -amylase from human saliva (Ref: A0521, Type IX-A, 1000-3000 units/mg of protein, CAS 9000-90-2), pancreatin from porcine pancreas (Ref: P7545, 8 x USP specifications, CAS 8099-47-6), pepsin from porcine gastric mucosa (Ref: P7000, ≥ 250 units/mg of solid, CAS 9001-75-6) and bile extract from porcine (Ref: B8631, CAS 8008-63-7) were obtained from Sigma Aldrich Chemical (St. Louis, MO, USA).

The water used in this work was de-ionized and double-distilled using a MilliporeTM water purification system (Massachusetts, USA) having 18.2 Ω electrical resistivity. All the reagents were either chromatographic or analytical grade and used as received.

4.2.2 Methods

4.2.2.1 Preparation and characterization of tyrosol-loaded ethylcellulose microparticles

The microparticles considered in this study were previously obtained by $w_1/o/w_2$ double emulsion solvent evaporation technique, using ethylcellulose as the encapsulating material. Two theoretical TYR loading contents (5% w/w and 10% w/w) were considered.

In our previous study (Paulo & Santos, 2020a), TYR loaded ethylcellulose microparticles were formulated and characterized regarding (i) the actual loading content, (ii) the encapsulation efficiency, (iii) the yield of production, (iv) the thermal stability through differential scanning calorimetry, (v) the thermogravimetric stability, (vi) the morphology, (vii) the particle size distribution and (viii) the total antioxidant activity.

In the present study, a detailed description of the preparation of loaded microparticles is presented in section III.4.2.2.1.1 and the most relevant characterization methods are described in section III.4.2.2.1.2.

4.2.2.1.1 Preparation of tyrosol-loaded ethylcellulose microparticles

As previously mentioned, TYR was encapsulated by $w_1/o/w_2$ water-in-oil-in-water double emulsion solvent evaporation microencapsulation technique using ethylcellulose as a coating material. Considering this, a stock solution of TYR in UPW (15 g/L) was previously prepared through the rigorous weighing of the standard in analytical scale Mettler Toledo AG245 balance (Columbus, OH, USA). From this stock, the working solutions were prepared by dilution different standard solutions corresponding to different loading contents of TYR in the internal aqueous phase (w_1): 5.3 mg/L for F-1 formulation corresponding to the TLC of 5% w/w and 11.1 mg/L for F-2 formulation corresponding to a TLC of 10% w/w. A standard control solution in ultrapure water of 500 mg/L was prepared weekly from the stock solutions. All solutions were stored at 4 °C in amber glass vials during the study.

For each experiment, the oily phase (o) was prepared dissolving ethylcellulose in dichloromethane at a polymer concentration of 10 g/L. Dichloromethane was chosen as the solvent for the dissolution of ethylcellulose as it promotes a fast solidification of microparticles essentially due to (i) the fact that this polymer is almost freely soluble in the selected solvent, (iii) dichloromethane solubility in water is negligible and (iii) it is greatly volatile which results in a fast solvent removal rate. The polymer solution was sonicated for 5 minutes in an ultrasonic bath (P Selecta, Barcelona, Spain).

The external aqueous phase (w_2) was constituted by polyvinyl alcohol (PVA) solution at a concentration of 1% w/w. The PVA acts as a double emulsion stabilizing surfactant, preventing aggregation of the recently formed microparticles. The w_2 was previously prepared the day before of the encapsulation experiments. For that, 1 g of PVA polymer was dissolved in 99 g of ultrapure water, under magnetic stirring (stirring plate from AREX Digital, VELP Scientifica, Monza, Italy) at around 120 °C. The process was performed until the total clearance of the polymer solution. The polymer solution was left equilibrate to room temperature overnight. The amount of ultrapure water lost during the dissolution of the polymer was added before encapsulation.

Afterward, 1 mL of w_1 was admixed with 10 mL, the organic phase forming the primary emulsion – water-in-oil (w_1/o).

The obtained emulsion was re-emulsified in the external aqueous phase (w_2) using a high-performance homogenizer at 5 000 rpm (IKA T18 Digital ULTRA-TURRAX®, Staufen, Germany), for 5 minutes.

The evaporation of the solvent and the hardening of microparticles were performed by continuously stirring the $w_1/o/w_2$ double emulsion in a stirring plate (AREX Digital, VELP Scientifica, Monza, Italy) at 700 rpm for 3 hours in the fume hood at room temperature.

Tyrosol-loaded microparticles were then recovered by filtration using a 0.2 μm quantitative paper filter and washed with 500 mL of distilled water. The recovered microparticles were frozen for 24 hours at $-4\text{ }^{\circ}\text{C}$ and freeze-dried for 72 hours, as previously described by Paulo & Santos (Paulo & Santos, 2018a, 2018b 2020a).

Two theoretical loading contents (TLC) of tyrosol (5% w/w and 10% w/w) were considered. The TLC (% w/w) was appraised as the ratio between the weight of tyrosol added, and the weight of polymer and tyrosol added. The TLC (% w/w) values chosen for this study are widely found as typical values in the literature. Each formulation (F-1 formulation corresponding to a TLC of 5% w/w and F-2 formulation corresponding to a TLC of 10% w/w) was produced in triplicate. Further considerations are presented in Table III.4.1.

Table III.4.1 - Formulation and operating conditions of the batches coded (F-1 – TLC of 5% w/w and F-2 – TLC of 10% w/w)

Formulation Conditions					Operating conditions					
Formulation ^a	TLC (% w/w)	TYR Conc. (g/L)	Polymer Conc. (g/L)	PVA Conc. (% w/w)	D1E (min.)	D2E (min.)	HPLES (rpm)	SSSE (rpm)	DEP (hr.)	DFDP (hr.)
F-1	5	5.3	10	1	3	5	5 000	700	3	72
F-2	10	11.1								

^aEach formulation was performed in triplicate

Conc. – Concentration; D1E – Duration of the Primary Emulsification; D2E – Duration of the Secondary Emulsification; DEP – Duration of the Evaporation Process; DFDP – Duration of Freeze-Drying Process; HPLES – High-Performance Liquid Emulsification Speed; hr. – hours; min. – minutes; PVA – Polyvinyl Alcohol; SSSE – Stirring Speed during Solvent Evaporation; TLC – Theoretical Loading Content; TYR – Tyrosol;

4.2.2.1.2 Characterization of tyrosol-loaded ethylcellulose microparticles

4.2.2.1.2.1 Determination of the encapsulation efficiency and the loading content of tyrosol-loaded ethylcellulose microparticles

The determination of the encapsulation efficiency of tyrosol-loaded ethylcellulose microparticles (TEE) was carried out according to Equation (III.4.1), as described by Paulo & Santos (2018a).

$$TEE (\% w/w) = \frac{W_T}{W_I} \times 100 = \frac{W_I - W_0}{W_I} \times 100 = \frac{W_I - (W_S + W_P)}{W_I} \times 100 \quad (\text{III.4.1})$$

where, W_T is the weight of tyrosol in the microparticles. It was evaluated considering the weight of tyrosol initially added for microparticles formulation (W_I) and the weight of tyrosol non-encapsulated (W_0). The W_0 was evaluated considering the weight of tyrosol present in the recovered supernatant of a sample of the $w_1/o/w_2$ emulsion after 3 hours of the hardening of microparticles (W_S), submitted to a low-intensity centrifugation process (4 000 rpm corresponding to 2 670 G, using a centrifuge Rotofix 32A, Hettich) for 15 minutes. The W_S was collected and stored at $-20\text{ }^{\circ}\text{C}$, prior to high-performance liquid chromatography (HPLC) analysis. By contrast, the W_P corresponds to the weight of tyrosol adsorbed at the surface of microparticles. To quantify the W_P , a sample of the $w_1/o/w_2$ emulsion after 3 hours of the hardening of microparticles was submitted to a centrifugation process as previously described. Afterward, the supernatant was forsaken, and the pellet was reconstituted in ultrapure water and further submitted to a centrifugation process for 15 minutes. The W_P corresponded to the supernatant after the washing of microparticles to remove adsorbed tyrosol molecules on the surface of microparticles. The W_P was collected and stored at $-20\text{ }^{\circ}\text{C}$, prior to the HPLC analysis. For each sample, TEE was evaluated in triplicate, and the mean $\pm$ standard deviation was computed.

The tyrosol actual loading content in microparticles (TALC) was assessed considering Equation (III.4.2).

$$TALC (\% w/w) = \frac{W_T}{W_M} \times 100 = \frac{W_I - W_0}{W_M} \times 100 = \frac{W_I - (W_S + W_L)}{W_M} \times 100 \quad (III.4.2)$$

where W_M is the weight of tyrosol-loaded microparticles recovered after freeze-drying.

Similarly to the TEE, the TALC was evaluated for each sample in triplicate. Results are presented as the mean $\pm$ standard deviation.

4.2.2.2 *In vitro* gastrointestinal release studies

In vitro gastrointestinal digestion models are classified as static models (constant ratio of food to enzymes and electrolytes, at a constant pH at each phase of the digestion) and dynamic models in which the physicochemical conditions are changed in each phase of the digestion. Static *in vitro* gastrointestinal digestion models are reported to be valuable regarding the prediction of the *in vivo* digestion (Bohn et al., 2018; Sanchón et al., 2018).

There are available multiple static *in vitro* digestion models that vary in terms of complexity (Bohn et al., 2018; Sanchón et al., 2018). The European Cooperation in Science and Technology (COST) developed a standardized procedure for the *in vitro* digestion of dietary compounds in multiple food matrices, known as the INFOGEST method (Rodrigues et al., 2017). A template for the harmonized *in vitro* digestion method from COST INFOGEST is accessible from <https://www.cost-infogest.eu> (European Union, 2018).

In the present study, the INFOGEST model was adapted to stimulate the *in vitro* digestion of TYR released from ethylcellulose loaded microparticles, as described in Table III.4.2.

The *in vitro* gastrointestinal digestion of TYR-loaded ethylcellulose microparticles was divided into three parts: (i) preparation, (ii) digestion procedure, and (iii) treatment and analysis of the samples.

The (i) preparation of the reagents and the digestion tubes included a time-lagged determination of enzyme activities. During this stage, the simulated salivary fluid (SSF), the simulated gastric fluid (SGF), and the simulated intestinal fluid (SIF) stock solutions were pre-warmed at 37 °C. The enzymatic solutions were prepared immediately before the digestion experiments, keeping all the enzyme solutions on ice.

Similarly, the (ii) digestion procedure was divided into three different parts: (a) oral phase digestion, (b) gastric phase digestion, and (c) small intestinal phase digestion.

During the (a) oral phase digestion, and for each sample – representative of each formulation – 1 g of microparticles was admixed with 0.8 mL of SSF and vortexed to obtain a paste-like consistency mixture similar to a swallowable bolus. Then, a volume of 5 µL of 0.3 M CaCl₂ solution was added being added afterward, 0.1 mL of α-amylase (prepared in UPW to achieve an activity of 75 U/mL in the final mixture) and 0.0095 mL of UPW in order to achieve the desired concentration of the SSF in the simulated salivary digestion fluid (SSDF). Afterward, the mixture (SSDF plus microparticles) was incubated at 37 °C, for 2 minutes, while was continuously mixed on an orbital shaker (IKA® KS 130 basic, IKA Works GmbH & Co. KG, Staufen im Breisgau, Germany).

Afterward, during the (b) gastric phase digestion, a volume of 1.6 mL of the pre-warmed at 37 °C SGF electrolyte solution was added to the previous mixture. Then, the pH was adjusted to 3.0 by adding 0.1 mL of a 15.6 mM HCl solution (previously determined during the pH-test adjustment experiment). A volume of 1 µL of 0.3 M CaCl₂ aqueous solutions was also added to the mixture. After, a volume of 0.1 mL of porcine pepsin solution prepared in UPW was added (corresponding to an activity of 2 000 U/mL). Finally, a volume of 0.199 mL of UPW was added to the mixture in order to achieve a final ratio of 1/1 v/v of the simulated gastric digestion fluid (SGDF) and the SSDF plus microparticles. Samples were incubated at 37 °C mixing continuously in an orbital shaker for 2 hours.

The (c) small intestinal phase digestion was performed, mixing 1.7 mL of the pre-warmed at 37 °C of the SIF to the gastric chyme. A volume of 0.2 mL of 8.4 mM NaOH aqueous solution was added to adjust the pH to 7.0. Then, 0.5 mL of a bile solution was added to the SIF and gastric chyme solution (final concentration of 10 mM).

The bile solution was previously placed in an orbital shaker at 37 °C until complete solubilization of bile. Afterward, 0.5 mL of a 0.3 M CaCl₂ aqueous solutions was added to the mixture. The final steps consisted in the addition of 1 mL of pancreatin solution prepared in the SIF, based on trypsin activity of 100 U/mL in the final mixture. Finally, 0.592 mL of UPW was added to achieve the 1/1 v/v final ratio of the SIF electrolyte solution and the gastric chyme. The samples were incubated at 37 °C, being promoted the continuous mixing using an orbital shaker for 2 hours.

The (iii) treatment and analysis of the samples began after the gastrointestinal digestion through snap freezing of the samples to inhibit different enzymatic activities of the digested samples. Samples were kept at least 5 hours at -20 °C before the subsequent instrumental analysis of TYR. Prior to the instrumental analysis, TYR dissolved in simulated gastrointestinal digestion fluids was submitted to solvent extraction using ethyl acetate as the extraction solvent as the simulated media presented dissolved inorganic salts that may precipitate when in contact with some organic solvents as acetonitrile and methanol used as eluents for instrumental analysis. The extraction procedure conducted in the present study is presented in detail in the III.4.2.2.2.1 section. Afterward, the amount of TYR released was evaluated using a high-performance liquid chromatograph (HPLC) as described in the section III.4.2.2.2.2.

The release of TYR was evaluated at different time points during digestion – 2-time points during the simulated salivary digestion and 4-time points during both simulated gastric and intestinal digestions, corresponding to a total number of 10-time points. One tube per time point in triplicate was considered for each formulation (F-1 and F-2 formulations). Triplicates of individual test tubes were considered due to sampling reproducibility. The experiments were performed in sealed containers with limited headspace, as in open vessels or containers with large headspace, the CO₂ would be released, and consequently, the pH would progressively be increasing with time.

Additionally, stability test tubes were also prepared to evaluate microparticles stability during the exposure to the simulated digestion fluids without enzymes and bile and after each digestion phase (e.g., oral phase, gastric phase, and small intestinal phase). Further considerations are presented in Table III.4.2.

Table III.4.2 - Digestion parameters considered for the *in vitro* release of tyrosol from tyrosol-loaded ethylcellulose microparticles

Digestion parameters		Digestion conditions
Digestion mass of microparticles ^a		1.0 g
Incubation conditions		37 °C, 85 rpm
Simulated Digestion Salivary Fluid (SSDF) ^b	Duration	2 minutes
	pH	7
	Electrolyte solution (SSF)	15.1 mM of KCl; 3.7 mM of KH ₂ PO ₄ ; 13.6 mM of NaHCO ₃ ; 0.15 mM of MgCl ₂ (H ₂ O) ₆ ; 0.06 mM of (NH ₄) ₂ CO ₃
	Volume of SSF	0.8 mL
	Enzyme	α-amylase (75 U/mL)
	Volume of enzyme	0.1 mL
	Volume of 0.3 M of CaCl ₂	5 µL
	Volume of added UPW	0.095 mL
Simulated Gastric Digestion Fluid (SGDF) ^b	Duration	120 minutes
	pH	3
	Electrolyte solution (SGF)	6.9 mM of KCl; 0.9 mM of KH ₂ PO ₄ ; 25.0 mM of NaHCO ₃ ; 47.2 mM of NaCl; 0.1 mM of MgCl ₂ (H ₂ O) ₆ ; 0.5 mM of (NH ₄) ₂ CO ₃
	Volume of SGF	1.6 mL
	Enzyme	Pepsin (2 000 U/mL)
	Volume of enzyme	0.1 mL

Table III.4.2 - Digestion parameters considered for the *in vitro* release of tyrosol from tyrosol-loaded ethylcellulose microparticles (cont.)

Simulated Gastric Digestion Fluid (SGDF) ^b	Volume of 0.3 M of CaCl ₂	1 µL
	Volume of HCl solution to reach pH of 3	0.1 mL (15.6 mM)
	Volume of added UPW	0.199 mL
Simulated Intestinal Digestion Fluid (SIDF) ^b	Duration	120 minutes
	pH	7
	Electrolyte solution (SIF)	6.8 mM of KCl; 0.8 mM of KH ₂ PO ₄ ; 85 mM of NaHCO ₃ ; 38.4 mM of NaCl; 0.33 mM of MgCl ₂ (H ₂ O) ₆
	Volume of SIF	1.7 mL
	Enzymes	Pancreatin (based on trypsin activity at 100 U/mL)
		Bile (10 mM; 8.5 mg/mL)
	Volume of pancreatin solution	1 mL
	Volume of bile solution	0.5 mL
	Volume of 0.3 M of CaCl ₂	8 µL
	Volume of NaOH solution to reach pH of 7	0.2 mL (8.4 mM)
	Volume of added UPW	0.592 mL

^a In the adapted method, used in the present study, microparticles to simulated fluids/enzymes/water and other electrolytes used in the INFOGEST method were followed;

^b Simulated Digestion Fluids (SSDF, SGDF and SIDF) differ from the Simulated Fluids (SSF, SGF, and SIF) as simulated fluids (SSF, SGF, and SIF) are only the electrolyte solutions added to the simulated digestion fluids (SSDF, SGDF and SIDF);

rpm – rotations per minute; SGDF – Simulated Gastric Digestion Fluid; SGF – Simulated Gastric Fluid; SIDF – Simulated Intestinal Digestion Fluid; SIF – Simulated Intestinal Fluid; SSDF – Simulated Salivary Digestion Fluid; SSF – Simulated Salivary Fluid; U – Enzymatic Units; UPW – Ultrapure Water;

4.2.2.2.1 Solvent extraction of tyrosol

Prior to the HPLC analysis, TYR dissolved in simulated gastrointestinal digestion fluids was submitted to solvent extraction. The simulated media present dissolved inorganic salts may precipitate in the HPLC apparatus when in contact with organic solvents as acetonitrile and methanol. Therefore, prior solvent extraction of TYR was performed using ethyl acetate as the extraction solvent. Alternative solvents and extraction procedures (e.g., one-step liquid-liquid extraction; multiple liquid-liquid extractions) were tested (data not showed). The one-step liquid-liquid extraction methodology was chosen for the extraction of TYR. Extractions were performed in triplicate.

Briefly, for each tube corresponding to alternative time points, 1 mL of the simulated digestion fluid containing TYR was placed in a 15 mL Falcon tube (polypropylene tube; conical bottom), and 6 mL of ethyl acetate was added. Afterward, the mixture was vigorously vortexed for 1 minute. Then, the mixture was ultrasonicated for 15 minutes at room temperature in a 420 W ultrasonic bath (J.P. Selecta, Barcelona, Spain). After this, the organic phase (containing TYR) was separated by centrifugation at 2670 g for 15 minutes. A volume corresponding to 90% of the initial volume) of the organic phase (supernatant) was carefully removed to a 12 mL amber vial and evaporated to dryness under a gentle N₂ stream. After dryness, the sample was solubilized in 1 mL of UPW. The final step included the transference of the solubilized TYR in ultrapure water to an amber vial for instrumental analysis.

The amount of TYR recovered after the *in vitro* release studies, and the solvent extraction procedure was evaluated through the calculation of the recovery (%R) percentage considering the standard addition method as presented in Equation (III.4.3).

$$\%R = \frac{m_f - m_i}{m_a} \times 100 \quad (\text{III.4.3})$$

where, m_f is the mass of TYR analyzed after fortification, m_i is the weighted mass of TYR before fortification and m_a is the mass of TYR to be analyzed. For each formulation, the recovery experiments were performed in triplicate.

In the case of F-1 formulation, the %R was $97.5 \pm 0.2\%$, and in the case of F-2 formulation, the %R was $98.2 \pm 0.9\%$. The results presented regarding the *in vitro* release studies account for the recovery percentages obtained during these studies (data not shown).

4.2.2.2.2 HPLC analysis

The detection and quantification of TYR was performed using a Merck Hitachi Elite LaChrom (Tokyo, Japan) high-performance liquid chromatograph equipped with a Hitachi L-7100 pump and L-7250 autosampler and coupled to a L-7450A diode array detector. The HPLC analyses were conducted by using a Purospher® STAR RP-18 end-capped LiChroCART® column (250 mm x 4.0 mm, 5.0 µm) (Merck KGaA), attached to a guard column (4.0 mm x 4.0 mm, 5.0 µm) of the same type.

The elution method applied for the detection and quantification of TYR was adapted from Tasioula-Margari & Tsabolatidou (2015) at a flowrate of 1 mL/min. The mobile phase consisted in a mixture of ultrapure water/acetic acid (0.1 N) (97.5/2.5 v/v) (A) and methanol/acetonitrile (50/50 v/v) (B). The injection volume was 99 µL, and the detection wavelength was defined at 280 nm. The elution gradient was adapted from the protocol presented by Tasioula-Margari & Tsabolatidou (2015) (Tasioula-Margari & Tsabolatidou, 2015) as follow: 95% of A and 5% as initial conditions that were kept for 5 minutes, 70% of A and 30% of B during 10 minutes, returning to the initial conditions of 95% of A and 5% of B during 5 minutes (from the minute 15 to the minute 20).

4.2.2.3 Assessment of the bioaccessibility of tyrosol released from tyrosol-loaded ethylcellulose microparticles

The total amount of TYR released from TYR-loaded ethylcellulose microparticles, corresponding to the concentration of TYR in *digestas*, was determined in order to assess the bioaccessibility. The bioaccessibility was evaluated according to the methodology described by Gómez-mascaraque et al. (2017) with some modifications.

The *digestas* was submitted to a low-intensity centrifugation process (4 000 rpm corresponding to 2 670 G, using a centrifuge Rotofix 32A, Hettich) for 30 minutes. Afterward, the supernatant was collected, and TYR was extracted by solvent extraction using ethyl acetate as the extraction solvent.

The concentration of TYR in the *digestas* was determined by HPLC. The bioaccessibility of TYR was estimated according to Equation (III.3.4):

$$TBA = \frac{C_{TD}}{C_{TT} \times TEE} \times 100 \quad (\text{III.3.4})$$

where TBA is the TYR bioaccessibility, C_{TD} is the concentration of TYR in the *digestas*, C_{TT} is the theoretical concentration of TYR, and TEE is the TYR encapsulation efficiency. Each sample was analyzed, considering six replicates. Results are presented as mean $\pm$ standard deviation.

4.2.2.4 Mathematical modeling of the release profile of tyrosol-loaded ethylcellulose microparticles

Currently, are available several release models that allow evaluating the release of bioactive compounds from loaded microparticles. These release models can be categorized in (i) statistical methods, (ii) model-dependent methods, and (iii) model-independent methods. The (i) statistical methods include the multivariate analysis of variance, exploratory data analysis, and repeated-measures designs. Contrasting, in the (ii) model-dependent methods are included the commonly known zero-order, first-order release kinetics models, the Korsmeyer-Peppas, the Higuchi, the Weibull models, among others. The (iii) model-independent methods include the difference factor (f_1) and the similarity factor (f_2) (Dash et al., 2010). In the present study, the assessment of the release of TYR from TYR-loaded ethylcellulose microparticles was accomplished through (ii) model-dependent methods and a (iii) model-independent method.

4.2.2.4.1 Assessment of the release behavior of tyrosol from tyrosol-loaded ethylcellulose microparticles through model-dependent methods

Data obtained from the *in vitro* release studies were fitted to alternative kinetic release models (model-dependent methods), specifically, the zero-order model, the first-order model, the Higuchi model, the Korsmeyer-Peppas release model and the Weibull model having regard the considerations presented in Table III.4.3.

Table III.4.3 - Model-dependent methods used in the assessment of the release behavior of bioactive compounds

Release model	Initial Equation	Linearized Equation Parameters description	Observations
Zero-order	$Q_t = Q_0 + K_0 t$	(III.4.5) Q_t - Cumulative release of TYR at the time t Q_0 - Initial amount of TYR (in this study, it was assumed that $Q_0 = 0$) K_0 - Zero-order release constant	The zero-order release model (constant release rate) describes the cases when do not occur the disintegration of microparticles and the release occurs slowly (Dash et al., 2010).
First-order	$Q_t = Q_0 e^{-K_1 t}$	(III.4.6) Q_t - Cumulative release of TYR at the time t Q_0 - Initial amount of TYR (in this study, it was assumed that $Q_0 = 0$) K_1 - First-order release constant	The first-order release model is useful to describe the dissolution of water-soluble bioactive compounds from porous matrices.
Higuchi	$Q_t = K_H \sqrt{t}$	(III.4.8) Q_t - Cumulative release of TYR at the time t K_H - Higuchi release constant	Mathematical model proposed by Higuchi, in 1963, to describe the release of a bioactive compound from a matrix system.

Table III.4.3 - Model-dependent methods used in the assessment of the release behavior of bioactive compounds (cont.)

Release model	Initial Equation	Linearized Equation Parameters description	Observations
Korsmeyer-Peppas	$\frac{Q_t}{Q_\infty} = K_K t^n \quad (\text{III.4.9})$	$\ln \left(\frac{Q_t}{Q_\infty} \right) = n \ln t + \ln K_K \quad (\text{III.4.10})$ Q_t/Q_∞ - Fraction of TYR released until time t K_K - Korsmeyer constant n - Release exponent	Simple derived equation that described the release of bioactive compounds from a polymeric system equation. This model allows to identify the release mechanism through the fitting of the first 60% of the release data in the Korsmeyer-Peppas model (Dash et al., 2010; Korsmeyer et al., 1983). The Korsmeyer constant embodies the geometrical features of the released form (e.g., spheres, film, cylinder), and the release exponent establishes the release mechanism.

Table III.4.3 - Model-dependent methods used in the assessment of the release behavior of bioactive compounds (cont.)

Release model	Initial Equation	Linearized Equation Parameters description	Observations
Weibull	$M_t = M_\infty \left[1 - e^{-\left(\frac{t-t_0}{\tau_d}\right)^\beta} \right] \quad (\text{III.4.11})$	$\ln \left(\ln \left(\frac{1}{1 - \frac{M_t}{M_\infty}} \right) \right) = \beta \ln(t - t_0) - \beta \ln \tau_d \quad (\text{III.4.12})$ M_t - release/dissolution of TYR (%) at time t (minutes) M_∞ - release/dissolution of TYR (%) at infinite time t_0 - lag-time (minutes) of the release/dissolution (in this study it was considered $t_0 = 0$) β - parameter relative to the shape of the curve τ_d - time (minutes) when 63.2% of M has been released/dissolved	The Weibull model is considered to be a relevant model when is intended the comparison release profiles of bioactive compounds from matrix-type delivery system

4.2.2.4.2 Comparison of release profiles of tyrosol from tyrosol-loaded ethylcellulose microparticles through model-independent methods

The release profiles of TYR-loaded ethylcellulose microparticles formulated considering a TLC of 5% w/w (F-1 formulation) and 10% w/w (F-2 formulation) were compared through a model-independent method - the similarity factor (f_2). The f_2 is the logarithmic reciprocal square root transformation of the sum of squared error. It is a measure of the similarity in the dissolution/release percent among the two curves evaluated (Dash et al., 2010; Demirturk & Oner, 2005; Diaz et al., 2016; Shah et al., 1998).

$$f_2 = 50 \times \log \left\{ 1 + \left[\frac{1}{n} \times \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \times 100 \right\} \quad (\text{III.4.13})$$

where n is the number of time points, R_t is the dissolution/release value of the reference (pre-change batch) at time t , T_t is the dissolution/release value of the test (post-change batch) at time t and $\log$ is the logarithm based on 10.

The f_2 ranges between 0 and 100. High values of f_2 indicate similarity among the release curves evaluated. As a result of batch-to-batch variations, the f_2 is not expected to be anywhere near to 0 and 100, respectively, to ensure similarity between dissolution/release curves. Therefore, an empirical consideration has been considered: two dissolution/release profiles are considered to be similar if f_2 is higher than 50 (Demirturk & Oner, 2005).

4.3 Results and Discussion

4.3.1 HPLC analysis

Each sample was submitted to the extraction of TYR, from the respective release medium, using a water-immiscible solvent (ethyl acetate) and reconstructed in UPW as described in Section of Experimental Procedures.

Calibration curves were obtained concerning each release medium (Table III.4.4). Each presented calibration curves in Table III.4.4 were drawn based on three independent calibration curves. In the case of the detection of TYR in the SSD fluid and the SGD fluid, a linear range was observed between 1.0 mg/L and 20.0 mg/L. Regarding the detection and quantification of TYR in the SID fluid, a linear range was observed between 0.5 mg/L and 20 mg/L. The determination coefficient resulted in $R \geq 0.995$.

For all the calibration curves, the relative standard error of the slope was considerably low (<5%) and the LOD and LOQ values for each calibration curve were satisfactorily low, ascertaining the possibility of the application of the obtained regression equations for the detection and quantification of TYR in distinctive release media.

Table III.4.4 - Results from the regression analysis for the quantification of tyrosol in alternative gastrointestinal release media

DS	Regression Equation ^{a,b}	R	S _a /a (%)	Conc. range (mg/L)	LOD (mg/L)	LOQ (mg/L)
SSDF	$A = (1.7 \times 10^4 \pm 1.4 \times 10^2) C \text{ (mg/L)} + (5.6 \times 10^3 \pm 8.0 \times 10^2)$	0.997	3.5	5.0-20.0	1.1	3.6
SGDF	$A = (1.3 \times 10^4 \pm 1.0 \times 10^2) C \text{ (mg/L)} + (4.9 \times 10^4 \pm 1.2 \times 10^2)$	0.995	3.5	5.0-20.0	1.1	4.0
SIDF	$A = (4.5 \times 10^4 \pm 1.6 \times 10^2) C \text{ (mg/L)} + (-1.7 \times 10^4 \pm 9.2 \times 10^2)$	0.997	2.6	5.0-20.0	1.0	3.1

^a A is the area of the peak (HPLC method), and C is the concentration of TYR (mg/L)

^b Mean calibration curve based on three independent calibration curves

A – Area; C – Concentration; DS – Dissolution Medium; HPLC – High-Performance Liquid Chromatography; LOD – Limit of Detection; LOQ – Limit of Quantification; R – Determination Coefficient; S_a/a – Relative standard error of slope; SGDF – Simulate Gastric Digestion Fluid; SIDF – Simulated Intestinal Digestion Fluid; SSDF – Simulated Salivary Digestion Fluid; TYR – Tyrosol;

4.3.2 Characterization of tyrosol-loaded ethylcellulose microparticles

Tyrosol-loaded ethylcellulose microparticles, considering two alternative TLCs (5% w/w and 10% w/w), were successfully obtained by $w_1/o/w_2$ double emulsion solvent evaporation microencapsulation technique.

The encapsulation efficiencies were about $99 \pm 1\%$ in the case of both F-1 formulation (TLC of 5% w/w) and the case of F-2 formulation (TLC of 10% w/w).

The actual loading content of F-1 formulation (TLC of 5% w/w) was $5 \pm 1\%$ w/w, corresponding to a 49 ± 1 mg of tyrosol/g of dried dispersion. Regarding the results of F-2 formulation (TLC of 10% w/w), an actual loading content of $10 \pm 1\%$ w/w corresponding to a 99 ± 1 mg of tyrosol/g of dried dispersion, was achieved.

The obtained values for both characterization parameters (encapsulation efficiency and actual loading content) were considered appropriate for the main goal of the present work.

4.3.3 *In vitro* release studies of tyrosol for tyrosol-loaded ethylcellulose microparticles

The release profiles of TYR from TYR-loaded ethylcellulose microparticles are presented in Figure III.4.1.

Examining the release profile of TYR from TYR-loaded ethylcellulose microparticles, the total amount of TYR released in the SSDF was lower than 0.2% for a TLC of 5% w/w (F-1 formulation) and 0.5% when a TLC of 10% w/w (F-2 formulation) was considered. For both TLCs, the amount of TYR released in the SSDF was remarkably low, which indicates the adequate salivary protection of TYR, through microencapsulation.

As a matter of fact, the SSDF contains several electrolytes (K^+ , Na^+ , Cl^- , $H_2PO_4^-$, HCO_3^- , Mg^{2+} , NH_4^+ , Ca^{2+}) and α -amylase from human saliva as described in section III.4.2.2.2. The enzyme α -amylase catalyzes the hydrolysis of the glycosidic bonds α -D-(1 $\rightarrow$ 4), which are absent ethylcellulose – the polymeric matrix that was used to encapsulate TYR. Probably, some TYR molecules adsorbed to the surface of microparticles were released when the SSDF was admixed to microparticles.

The water present in the SSDF, possibly, promoted the hydrolysis of hydrogen bonds between the ethylcellulose and adsorbed TYR molecules at the surface of microparticles. Nevertheless, as for both formulations were not observed in an initial burst release, it can be stated that the amount of TYR adsorbed at the surface of microparticles was presumably low.

After two hours from the commencement of the release studies, the amount of TYR released from F-1 (TLC of 5% w/w) and F-2 (TLC of 10% w/w) was $4 \pm 1\%$ and $8 \pm 1\%$, respectively. During the SGD, the amount of tyrosol released was lower than 10% for both experiments (F-1 and F-2 formulations). Therefore, it can be concluded that ethylcellulose confers adequate gastric protection to tyrosol.

Even though it is reported that ethylcellulose presents excellent pH stability (pH 2 to pH 13) as it is a non-ionic substance, in the present study was observed that ethylcellulose allowed a slight release of TYR in the SGDF (Wustenberg, 2015). A similar trend was also observed by Zheng et al. (2011) when they encapsulated bayberry polyphenolic extracts into ethylcellulose microparticles - they concluded that ethylcellulose promotes a slight release of bayberry polyphenols in the SGDF.

After 2 hours from the beginning of the study, TYR-loaded ethylcellulose microparticles (F-1 and F-2 formulations) were incubated in the SIDF in order to assess the *in vitro* digestion of TYR from TYR-loaded ethylcellulose microparticles in this release medium. The maximum release of TYR from TYR-loaded ethylcellulose microparticles was observed in the SIDF (observed percentage increases of more than 400%).

At the end of the study, it was verified that the total amount of TYR released from microparticles was $96 \pm 7\%$ in the case of F-1 formulation (TLC of 5% w/w) and $46 \pm 1\%$ in the case of the F-2 formulation (TLC of 10% w/w).

Over time, the conditions of the *in vitro* release studies as the stirring speed, the pH and the osmolality were changed which might be combined trigger the release mechanisms: (i) diffusion through water-filled pores, (ii) diffusion through the polymer, (iii) osmotic pumping and (iv) erosion (Fredenberg et al., 2011).

With time, in the SIDF, the external and internal polymeric pores assisted the water-polymer contact and therefore promoted (i) the increase on the diffusion of TYR through water-filled pores and the (iv) polymer erosion which exposed TYR molecules entrapped in the polymer matrix, increasing the amount of TYR released. Moreover, presumably, the time of *in vitro* digestion promoted (ii) the diffusion of TYR through the polymer. These combined events may have promoted the highest release of TYR in the SIDF.

In brief, these results suggest that TYR can be efficiently protected during the gastrointestinal tract.

The obtained results in the present study are in agreement with the ones presented by Bonechi et al. (2019). In a research study when the authors were chemically characterizing liposomes containing tyrosol, hydroxytyrosol, and oleuropein. The authors verified that zwitterionic liposomes containing tyrosol were biocompatible and empowered an optimized release of this nutraceutical ingredient.

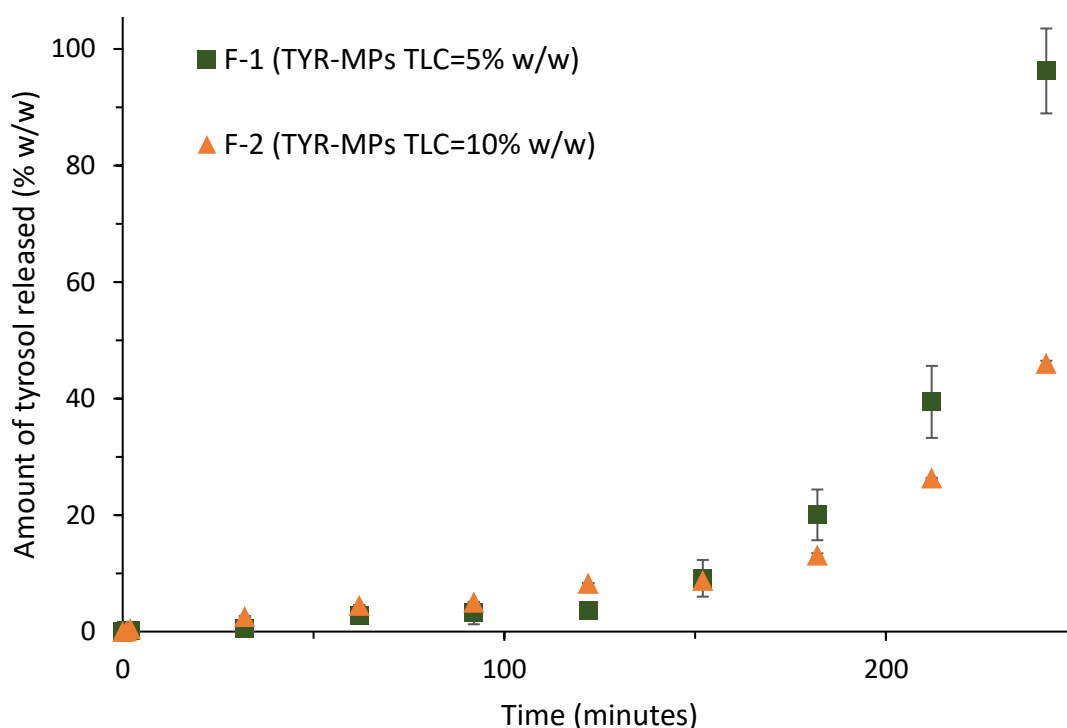


Figure III.4.1 - Tyrosol release profiles from ethylcellulose microparticles prepared using alternative theoretical loading contents (■ – theoretical loading content of 5% w/w; ▲ – theoretical loading content of 10% w/w)

4.3.4 Assessment of the bioaccessibility of tyrosol released from tyrosol-loaded ethylcellulose microparticles

According to Gómez-Mascaraque et al. (2017), the term bioaccessibility can be defined as the fraction of the compound soluble in the gastrointestinal tract and available for absorption.

In this context, for the assessment of the bioaccessibility of TYR, it was considered the amount of dissolved TYR present at the end of the *in vitro* gastrointestinal digestion. The bioaccessibility of pure TYR was $10 \pm 1\%$ w/w, whereas the bioaccessibility of TYR released from ethylcellulose microparticles was $29 \pm 1\%$ w/w, formulated considering both a TLC of 5% w/w (F-1 formulation) and 10% w/w (F-2 formulation).

The bioaccessibility of pure TYR was low, contrasting with the obtained values for TYR released from microparticles. Microencapsulation efficiently protected TYR from the gastrointestinal environment, increasing its bioaccessibility as it was observed an increase in 1.9 times fold in the bioaccessibility from TYR to TYR-loaded ethylcellulose microparticles. Therefore, it can be concluded that the coating material – ethylcellulose – efficiently protected TYR during the intense first-pass metabolism in the small intestine.

The bioaccessibility of TYR released from microparticles prepared considering different loadings led to similar results – the bioaccessibility of TYR released from TYR-loaded ethylcellulose microparticles formulated considering a TLC of 5% w/w was similar to the bioaccessibility value obtained for TYR released from loaded microparticles when a TLC of 10% w/w was considered. Increasing the loading content did not lead to an increase in TYR bioaccessibility – most likely, the maximum fraction of TYR soluble in the gastrointestinal tract and available for absorption was achieved considering the minimum theoretical loading content (5% w/w).

Therefore, it can be concluded that load ethylcellulose microparticles with TYR considering a TLC of 10% w/w is not desirable as the increase in the loading (from 5% w/w to 10% w/w) did not improve the bioaccessibility of TYR. The highest loading content is associated with the requirement of more raw materials for encapsulation (polymer and bioactive compound) and to a low bioaccessibility.

Moreover, TYR-loaded ethylcellulose microparticles formulated considering a TLC of 10% w/w presented a lower encapsulation efficiency (Paulo & Santos, 2020a). Similar results were obtained by Gómez-Mascaraque et al. (2017) when they were preparing protein-coated liposomes through electrospraying for the stabilization and improvement of the bioaccessibility of curcumin.

4.3.5 Mathematical modeling of the release profile of tyrosol-loaded ethylcellulose microparticles

4.3.5.1 Assessment of the release behavior of tyrosol from tyrosol-loaded ethylcellulose microparticles through model-dependent methods

The experimental profiles (Figure III.4.1) were fitted in the zero-order, first-order, Higuchi, Korsmeyer-Peppas, and Weibull release models in order to evaluate the release behavior of TYR from TYR-loaded ethylcellulose microparticles and specify the most suitable release model to the experimental release profiles. The results regarding the parameters and the correlation coefficients of the alternative release kinetic models applied to experimental profiles are presented in Table III.4.5.

In the present work, the experimental data adjusted to the zero-order release kinetic model presented, correlation coefficients ranging from 0.850, in the case F-1 formulation (TLC of 5% w/w) and 0.974 in the case of F-2 formulation (TLC of 10% w/w). The K_0 increased with the increase of the loading content.

The first order release model, even though it is difficult to define using a basic theory, describes the change in the concentration concerning the change on time as only dependent on the concentration (Bruschi, 2015). The K_1 were similar for both formulations, however, only the experimental data adjusted from F-1 formulation (TLC of 5% w/w) presented an acceptable correlation coefficient (0.902).

The experimental profiles did not fit properly to the Higuchi release model as the correlation coefficients varied from 0.683, in the case of F-2 formulation (TLC of 10% w/w) to 0.875 in the case of F-1 formulation (TLC of 5% w/w).

However, it was expected that K_H varied similarly to K_0 : the highest values of K_0 are generally associated with the highest values of K_H . This tendency was observed, in the present study: the highest K_0 (0.0014) value and the highest K_H value (0.0047) was observed for the same formulation (F-2, highest loading content).

The correlation coefficients obtained related to the application of the Korsmeyer-Peppas model were 0.880 and 0.817, in the case of F-1 and F-2 formulations, respectively. The values obtained for n give some insights into the release mechanism. It is reported that for $n > 0.85$, as observed for the two data release profiles (Table III.4.5), the type of mass transport is defined as super-case II transport and the rate of release is a function of time (t^{n-1}). Therefore, the release of TYR from TYR-loaded ethylcellulose microparticles in the gastrointestinal tract followed a zero-order release influenced by the swelling of ethylcellulose (Bruschi, 2015). Additionally, it was expected the increase of K_K for faster releases, presenting a similar trend as the obtained K_0 (first-order release model) and K_H (Higuchi release model). This tendency was observed in the present work: the fastest release applying the Korsmeyer-Peppas model was observed for the same formulation that presented the fastest zero-order and Higuchi release (F-2 formulation).

The application of the Weibull model resulted in the obtainment of correlation coefficients ranging from 0.844 (F-1 formulation) and 0.947 (F-2 formulation). The parameter τ_d was determined experimentally (τ_d^{exp}) corresponding to the time when 63.2% of M was released. In the case of F-1 formulation (TLC of 5% w/w) the calculated τ_d (1667 minutes) was dissimilar from the experimental value obtained for this parameter (223 minutes). Closer values for the τ_d parameters (experimental *versus* computed values) were obtained for F-2 formulation (τ_d^{exp} =215 minutes *versus* τ_d = 665 minutes). The parameter β was, in both formulations, lower than 1 (β =0.9888, for F-1 formulation and β =0.8808, in the case of the F-2 formulation); therefore, the release of TYR from TYR-loaded ethylcellulose microparticles is represented by a parable, exhibiting a high initial slope and then a consistent exponential profile (Bruschi, 2015).

The best-fitting release model for experimental data from F-1 formulation was the first order release model (correlation coefficient of 0.902).

It is reported in the literature that the first-order release model is suitable to describe the release of hydrophilic bioactive compounds from porous matrices, as happened in the present case (Bruschi, 2015; Dash et al., 2010).

In the case of F-2 formulation (TLC of 10% w/w), the best fitting release model was the zero-order release model (correlation coefficient of 0.974). Therefore, the release of TYR is best described by only a function of time in which the release occurs at a constant rate independent of the concentration of tyrosol inside of loaded microparticles.

Table III.4.5 - Parameters and correlation coefficients of different release kinetics models applied to experimental data of release profiles

Release model	Parameter	F-1 (TYR-MPs TLC=5% w/w)	F-2 (TYR-MPs TLC=10% w/w)
Zero-order ^a	K_0 (mg.min ⁻¹)	0.0005	0.0014
	Q_0 (mg)	-0.0039 ^b	0.0021
	R^2	0.850	0.974
First-order ^a	K_1 (min ⁻¹)	0.0281	0.0210
	Q_1 (mg)	-2.7421 ^b	-1.9623 ^b
	R^2	0.902	0.757
Higuchi ^a	K_H (mg.min ^{-0.5})	0.0032	0.0047
	R^2	0.875	0.683
Korsmeyer-Peppas ^b	K_K (min ⁻ⁿ)	0.0002	0.1562
	n	2.1705	1.0747
	R^2	0.880	0.817
Weibull	τ_d^{exp} (min)	223	215
	τ_d (min)	1667	665
	β	0.9888	0.8808
	R^2	0.844	0.947

^a applied to the linear part of the experimental curve

^b Q_0 should be ≥ 0

4.3.5.2 Comparison of release profiles of tyrosol from tyrosol-loaded ethylcellulose microparticles through a model-independent method

According to the Food and Drug Administration (FDA) guides for dissolution profiles comparison – similarly factor (f_2) – greater than 50 ensure the equivalence of two dissolution curves (Food and Drug Administration, 1997). The release profiles of TYR from TYR-loaded ethylcellulose microparticles formulated considering a TLC of 5% w/w and a TLC of 10% w/w were similar as the similarly factor (f_2) obtained was higher than 50 (obtained value: 55). The f_2 values are listed in Table III.4.6. In this context, it can be concluded that the higher loading content did not lead to a higher dissolution rate of TYR. Therefore, when it is intended a high-volume production of TYR-loaded ethylcellulose microparticles (e.g., in industrial applications as a supplement intended for the fortification of foodstuff), it is economically advantageous to produce only microparticles considering a TLC of 5% w/w as less raw materials – specifically the bioactive compound – is required, turning the process economically more efficient.

Additionally, the similarly factor for the two formulations was evaluated in each release medium (e.g., SSDF, SGDF, and SIDF).

The release profiles of F-1 and F-2 formulations were similar as the f_2 obtained was closer to 100 ($f_2 = 91$). The release of TYR from ethylcellulose microparticles in the SSDF was independent of the loading content. This result was expected as the release of TYR in the SSDF occurred during only 2 minutes, not favoring a significative dissolution of the bioactive compound in the release medium. Moreover, the SSDF (see details Table III.4.2) contains α -amylase that only catalyzes the hydrolysis of the glycosidic bonds α -D-(1 $\rightarrow$ 4), which are absent in ethylcellulose. Therefore, the release of TYR during the SSDF was limited through the dissolution of TYR molecules present at the surface of microparticles or TYR clustered in superficial pores.

The similarity between the F-1 and F-2 formulation TYR dissolution profiles decreased during the SGD ($f_2 = 54$) and even more during the SID ($f_2 = 52$). Probably, the duration of the release of TYR from loaded ethylcellulose microparticles accentuated the effect of the dependence of dissolution profiles from the loading content of microparticles. The unique characteristics of the TYR dissolution profiles from formulations considering different loadings become more evident over time.

It can be hypothesized that the loading content has a more significant impact on the dissolution of TYR over time.

From the pair-wise comparison of the dissolution media (Section 2, Table III.4.6), for both formulations, it was observed that the TYR dissolution was dissimilar during the SSD *versus* SID and also when comparing the SGD and SID. It can be concluded that the electrolyte concentration and the presence of specific enzymes significantly affected the dissolution pattern of TYR from loaded microparticles. Nevertheless, it was observed for both formulations that the release pattern of TYR was similar during the SSD and in the SGD. This observation can be explained, considering the time required for each digestion. In the case of TYR dissolution during the SSD and during SGD, the time-lagging between the dissolution in these release media is lower when compared to the time-lagging between the dissolution of TYR in the SSDF *versus* SIDF (around 4 hours) and also between the bioactive compound dissolution in the SGDF and SIDF (around 2 hours).

It can be concluded that the loading content tempted significative effects on the dissolution pattern of TYR, in time. Moreover, it can be stated that for large scale applications as industrial applications, the F-1 formulation (lower loading content) should be chosen considering the economic feasibility of the process as it requires a lower amount of bioactive compound when compared to F-2 formulation, being associated to an overall gastrointestinal dissolution pattern similar to F-2 formulation (higher loading content). Additionally, it was observed that the electrolyte concentration and the presence of enzymes influenced the dissolution pattern of TYR in the release media.

Table III.4.6 - Computed similarly factors (f_2): SECTION 1 – Effect of the *in vitro* digestion media: pair-wise comparison of dissolution media; SECTION 2 – Effect of loading (formulation type): pair-wise comparison of F-1 and F-2 formulations in each release medium

SECTION 1				SECTION 2			
Formulations (F-1 versus F-2) f_2 comparison				Digestion media f_2 comparison			
<i>In vitro</i> GID	SSD	SGD	SID	Formulation	SSDF vs. SGDF	SSDF vs. SIDF	SGDF vs. SIDF
55	91	54	52	F-1	77	25	23
				F-2	53	26	24

GID – Gastrointestinal Digestion; SGD – Simulated Gastric Digestion; SGDF – Simulated Gastric Digestion Fluid; SID – Simulated Intestinal Digestion; SIDF – Simulated Intestinal Digestion Fluid; SSD – Simulated Salivary Digestion; SSDF – Simulated Salivary Digestion Fluid;

4.4 Conclusions

The aim of this study was to encapsulate tyrosol, a phenolic antioxidant present in high concentrations in olive oil and olive mill wastes by water-in-oil-in-water double emulsion solvent evaporation technique, using ethylcellulose as the encapsulating material and considering two alternative theoretical loading contents of tyrosol: 5% w/w and 10% w/w. It was intended to study the effect of the loading content on the release behavior of tyrosol from tyrosol-loaded ethylcellulose microparticles and the bioaccessibility of tyrosol. The release behavior of tyrosol was assessed through model-dependent and model-independent methods.

Tyrosol was efficiently incorporated in ethylcellulose microparticles by water-in-oil-in-water double emulsion solvent evaporation technique. The encapsulation efficiency was $99 \pm 1\%$ in the case of both formulations (theoretical loading content of 5% w/w and 10% w/w).

The maximum amount of tyrosol released corresponded to $96 \pm 7\%$ of the initial amount incorporated in the case of formulation considering a theoretical loading content of 5% w/w and $46 \pm 1\%$ of the initial amount of tyrosol entrapped into ethylcellulose microparticles in the case when a theoretical loading content of 10% w/w was considered. For both theoretical loading contents considered, the maximum release of tyrosol during the simulated salivary digestion and the simulated gastric digestion was kept below 1% w/w and 9% w/w, respectively. The *in vitro* release studies confirmed the *in vitro* salivary and gastric protection of tyrosol by the encapsulating material – ethylcellulose.

The bioaccessibilities observed were $10 \pm 1\%$ and $29 \pm 1\%$ in the case of pure tyrosol and encapsulated tyrosol. The bioaccessibility of tyrosol was improved 1.9 times fold when tyrosol was encapsulated. The bioaccessibility of tyrosol was largely improved when encapsulated probably due to protection conferred by microparticles to the intense first-pass metabolism of tyrosol in the small intestine. As tyrosol bioaccessibility values were similar for both theoretical loading contents, it can be concluded that the maximum amount of soluble tyrosol in the gastrointestinal tract and available for absorption was reached considering a minimal theoretical loading content of 5% w/w.

Loading microparticles with further tyrosol did not lead to improvements in tyrosol bioaccessibility.

The best-fitting release model for tyrosol released from microparticles formulated considering a theoretical loading content of 5% w/w was the first order release model – typical dissolution profile of water-soluble bioactive compounds from porous matrices – whereas in the case when a theoretical loading content of 10% w/w was considered, the best fitting model was the zero-order - tyrosol was released as a pure material from tyrosol-loaded ethylcellulose microparticles delivery system.

The analysis of the release pattern of tyrosol was also assessed using the similarity factor – a model-independent method. The release patterns of tyrosol were similar for both theoretical loading contents studied as the similarity factor was higher than 50. Nevertheless, it was observed that the loading content exhibited significant effects on the release pattern, in time.

Moreover, it was observed that the concentration of electrolytes and the presence of specific enzymes on the release media affected the dissolution pattern of tyrosol.

In summary, this study comprehensively evaluated the release behavior of tyrosol from tyrosol-loaded ethylcellulose microparticles prepared by double emulsion solvent evaporation technique in *in vitro* simulated gastrointestinal tract. These results are remarkable regarding the design of loaded microparticles that can be further incorporated in fortified foods, suffering then after gastrointestinal digestion.

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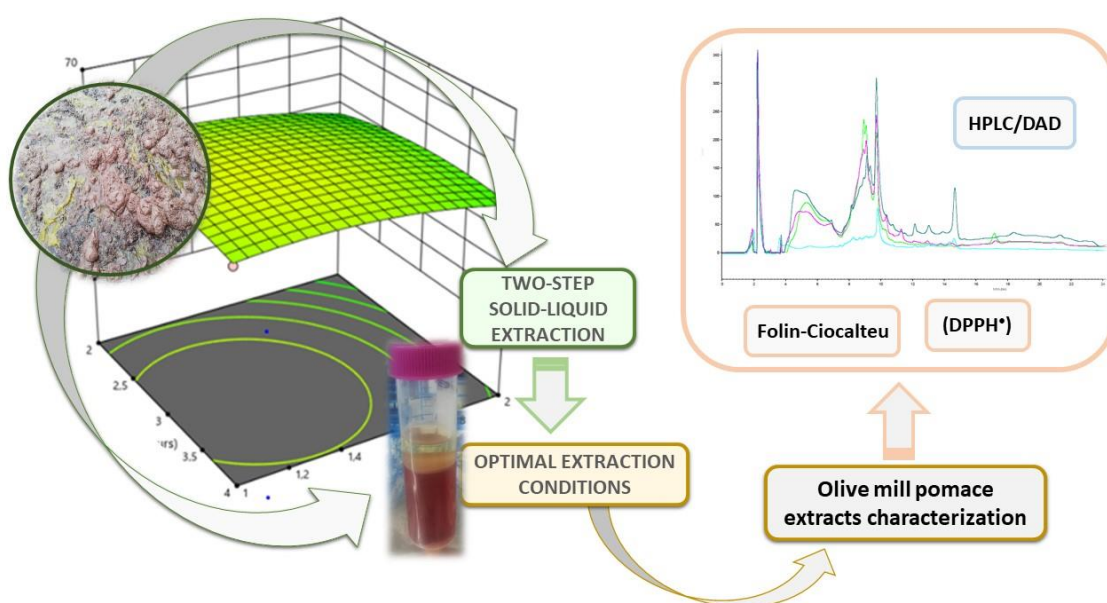
Part IV: New insights in the encapsulation of extract rich in phenolic compounds obtained from olive mill pomace – preparation, characterization and *in vitro* release studies of loaded microparticles

In this part, is proposed an optimized extraction procedure of phenolic compounds from olive mill pomace (Chapter 5). In Chapter 6, the extract rich in phenolic compounds obtained through the optimized extraction procedure described in Chapter 5, was encapsulated. In this chapter (Chapter 6), are presented the main results regarding the physicochemical characteristics of phenolic rich extract of olive mill pomace loaded ethylcellulose microparticles. Finally, in Chapter 7, are presented the main results regarding the *in vitro* release studies of bioactive compounds from loaded microparticles.

Chapter 5. Response surface modeling and optimization of the extraction of phenolic compounds from olive mill pomace⁵

Filipa Paulo, Lúcia Santos

Graphical Abstract:



⁵ This chapter was adapted from: Paulo, F. & Santos, L. (2020). Response surface modeling and optimization of the extraction of phenolic compounds from olive mill pomace. *Submitted*.

Abstract:

In this study, phenolic compounds from olive mill pomace (OMP) were extracted through a two-step solid-liquid extraction procedure considering four factors at five levels central composite rotatable response surface design. The influence of the process variables time of the primary extraction (2.0 – 4.0 hours), the solvent-to-sample ratio during the primary extraction (5.0 – 10.0 mL/g), the time of the secondary extraction step (1.0 – 2.0 hours) and the solvent-to-sample ratio during the secondary extraction (3.0 – 5.0 mL/g) on the total phenolic content of OMP extracts, were examined.

The factors considered influenced the extraction of phenolic compounds. Data were fitted in a second-order polynomial equation. The optimal extraction conditions obtained using the Derringer's desired function methodology were: 3.2 hours for the primary extraction, solvent-to-sample ratio during the primary extraction of 10.0 mL/g, 1.3 hours for the secondary extraction and a solvent-to-sample ratio during the secondary extraction of 3.0 mL/g associated to a predicted maximum total phenolic content of 50 mg_{GAE}/g_{OMP}. The final extract exhibited a total phenolic content of 50.5 ± 1.5 mg_{GAE}/g_{OMP}, and it retained mostly polar antioxidants and phenolic compounds. The acidic hydrolysis pre-treatment revealed paramount importance on the extraction of bonded phenolic compounds.

Highlights

1. Olive mill pomace (OMP) is a significant source of phenolic compounds (PCs).
2. OMP samples were submitted to a solid-liquid extraction to recover PCs.
3. Response surface methodology was applied to maximize process variables (PV).
4. A quadratic regression model described the total phenolic content of OMP from PV.
5. Hydrolysis and fat removal pretreatments favored the recovery of PCs from OMP.

Keywords: olive mill pomace; central composite rotatable response design; phenolic compounds; solid-liquid extraction; tyrosol; hydroxytyrosol;

5.1 Introduction

Over the past few decades, it has been observed a growing interest in the role of reactive oxygen species (ROS) such as the superoxide anion radical ($O_2^{\bullet-}$), the hydroxyl radical ($HO^{\bullet}$), the peroxy radical ($ROO^{\bullet}$), the hydrogen peroxide (H_2O_2), the singlet oxygen ($^1O_2$) and the peroxynitrite ($ONOO^-$) on human pathophysiology from health and food science researchers as well as medical and nutritional experts, as it becomes widely evident the physiologic relationship between the presence of ROS in the human body and the appearance of several diseases including atherosclerosis, cancer, rheumatoid arthritis, diabetes, neurodegenerative and even cardiovascular diseases (Abe & Berk, 1998; Forrester et al., 2018; Incalza et al., 2018; Papaharalambus & Griendling, 2007; Prasad et al., 2017; Terrone et al., 2019; Volpe et al., 2018).

The ROS are extremely reactive and may stimulate biological damage in vital molecules such as proteins, DNA, lipids, and even carbohydrates (Ginter et al., 2014; Ighodaro & Akinloye, 2018). An imbalance between the generation and the removal of ROS may cause oxidative stress which can drive free radicals to attack vital human body cell macromolecules, causing cell damage and homeostatic disruption leading consequently to the appearance of chronic diseases (Fang et al., 2015; Ighodaro & Akinloye, 2018; Lobo et al., 2010; Scandalios, 2005). The physiological damages caused by the excess of ROS are generally minimized through enzymatic and non-enzymatic endogenous action of antioxidants (Fridovich, 1997; Sies, 1993). However, in the cases of imbalance between generation and removal of ROS or ineffective antioxidant defense system, the reinforcement of endogenous antioxidants may be essential to reduce the effects of oxidatively damaged molecules (Beckhauser et al., 2016; Briante et al., 2003).

Epidemiological studies have been shown that the incidence of certain cancers as the breast and colon cancers, as well as coronary heart disease, is lower in the countries around the Mediterranean basin when compared with the northern European countries (Keys, 1995; Schwingshackl et al., 2017). This may be related to the safer and protective dietary patterns in the southern countries where olive oil is the primary source of fat (Keys, 1995; Lipworth et al., 1997; Paulo & Santos, 2020b; Schwingshackl et al., 2017; Trichopoulou et al., 1995).

Both *in vitro* and animal studies suggest that the high concentration of phenolic antioxidants in extra virgin olive oil widely contribute to the healthy pattern of the Mediterranean diet (de Pablos et al., 2019; García-Gavilán et al., 2018; Tuck & Hayball, 2002; Visioli et al., 2002). Nevertheless, the majority of phenolic compounds – around 98% - are retained in olive oil by-products during olive oil processing (Araújo et al., 2015; Dermeche et al., 2013). From this perspective, olive mill pomace (OMP), generated in large quantities during olive oil processing, may be considered a powerful source of natural phenolic antioxidants as tyrosol, hydroxytyrosol, and oleuropein (Paulo & Santos, 2020a, 2020b).

Furthermore, it has been observed an increasing interest in the replacement of synthetic food antioxidants by the natural ones which conversely has fostered research on vegetable sources and the screening of raw materials regarding the identification of new antioxidants (Qadir et al., 2017; Moure et al., 2001). As a matter of fact, the oxidative deterioration of oils and fats in food matrices is responsible for the appearance of compounds associated with rancid odors and off-flavors, which are responsible for the decrease in the nutritional safety and quality of food products. Therefore, the incorporation of antioxidants in food matrices is required to retain the original color, odor, flavor and to avoid the damage of other vital macromolecules (e.g., vitamins) (Qadir et al., 2017).

Several techniques have been proposed to efficiently extract phenolic compounds from OMP that can be further used for the replacement of synthetic antioxidants in oxidative sensitive food matrices. Among these techniques are included solvent extraction, membrane separation, centrifugation, chromatographic procedures, and even enzymatic treatments (Dermeche et al., 2013).

Remarkably, environmentally friendly assisted extraction methods (e.g., homogenization, microwave heating, pulsed electric field, ultrasound, infrared radiation, high hydrostatic pressure, high-voltage electrical discharge, accelerated conditions of temperature and pressure) have been investigated for the efficient extraction of phenolic compounds from olive mill pomace (Andreou et al., 2020; Chanioti & Tzia, 2018; Goldsmith et al., 2018; Habibi & Mohammadi, 2018; Xie et al., 2019).

Generically, these methods are claimed not only as environmentally friendly, but also as being less time and energy consumption methods as well as they commonly require simplified manipulation and reduced solvent consumption (Chanioti & Tzia, 2018; Guo et al., 2017; Li et al., 2010; Liu et al., 2013; Zhu et al., 2014).

Nevertheless, solvent extraction approach is still the most widely described technique for the extraction of phenolic compounds from OMP even though it is generically classified as time and solvent consuming method (Chanioti & Tzia, 2018; Guo et al., 2017; Li et al., 2010; Liu et al., 2013; Zhu et al., 2014). Even so, the non-requirement of specific and cutting-edge technological equipments, protocols simplicity, and the use of mild conditions (e.g., high temperatures would compromise the stability of phenolic compounds) engender the widespread use of this method.

Previous findings have reported the influence of independent variable such as the extraction procedure, the solvent composition, the pH, the temperature, the extraction time, the solid-to-liquid ratio and the inclusion of pre-treatment steps as fat removal and acidic and/or alkaline hydrolysis on the solvent extraction of phenolic compounds from OMPs (Alu'datt et al., 2010; Cioffi et al., 2010; Japón-Luján & De Castro, 2007; Klen & Mozeti, 2012; Lafka et al., 2011; Obied et al., 2005b, 2007a, 2007b, 2008; Peralbo-Molina et al., 2012b; Rubio-Senent et al., 2012, 2015; Suárez et al., 2010, 2009; Uribe et al., 2015). Nevertheless, to the authors' best knowledge, there is no published research regarding the analysis of the dependence of process variables on the extraction of phenolic compounds from OMP through solid-liquid extraction.

Among the available statistic and mathematical models for the multivariate analysis, the response surface methodology (RSM) is one of the most commonly used (Paulo & Santos, 2017). The study of one variable while the others remain constant – known as one-variable-at-time (OVAT) or interchangeably one-factor-at-time (OFAT) methodology - generally leads to non-optimized responses/outputs as the OVAT methodology does not consider the fact that different variables can interact and have an impact on the response and therefore on the optimization process (Frey et al., 2003; Kharia & Singhai, 2013; Paulo & Santos, 2017).

The RSM encloses a set of mathematical and statistical approaches to express relationships between factors and responses. Dissimilarly to the OVAT methodology, the RSM outlines the effect of factors alone or combined, considering the goal of response(s) optimization (Bas, 2007; Paulo & Santos, 2017). Even though the RSM methodology has been applied for the optimization of extraction conditions of phenolic compounds from different sources, the application of this methodology is not, to the authors best knowledge, reported for the optimization of solid-liquid extraction of phenolic compounds from OMP (Hayouni et al., 2007; Pinelo et al., 2005; Pompeu et al., 2009; Yang et al., 2010).

Hence, the goal of this study was to investigate the individual and interactive effect of two-step solid-liquid extraction process variables such as the time of the primary extraction (hours), the solvent-to-sample ratio (SSR) during the primary extraction step (mL/g), the time of the secondary extraction (hours) and the SSR during the secondary extraction step (mL/g) on the total phenolic content (TPC) of OMP extracts.

It was also intended to optimize the process variables of the solid-liquid extraction procedure aiming the maximization of the phenolic content of OMP extracts using a central composite rotatable response surface design associated with the Derringer's desired function methodology. Additionally, the extract obtained under optimized extraction conditions was characterized regarding its TPC, total antioxidant activity (TAA), and the total amount of specific biophenols (hydroxytyrosol, tyrosol, and oleuropein).

The distribution of phenolic compounds and antioxidants among OMP extract fractions was evaluated. Moreover, the TPC and the TAA of the extract obtained considering the optimized extraction conditions was compared to the TPC and the TAA of the extract obtained not considering the pre-treatment of the OMP samples, evaluating, therefore, the extent effect of the acidic hydrolysis on the extraction of phenolic compounds from OMP samples through a two-step solid-liquid extraction procedure.

5.2 Experimental procedures

5.2.1 Samples

The OMP samples were obtained from a continuous two-phase centrifugation system by a local olive oil mill in Portugal (Vilas Boas, Vila Flor, Bragança, 41359822,-7123743). Samples were collected in January 2019. The OMP samples were stored at -22 °C before prior analysis. The samples were freeze-dried for 72 hours on a benchtop freeze-dryer (SP Scientific, NY, USA) (moisture content of 71.0 ± 5.5 % w/w) and grounded on an electric mill (Qilive Q5321 Grinder) to an average particle size of 142.2 ± 9.6 μm , as described by Uribe et al. (2015) with slight modifications. The mean particle size of OMP was evaluated by laser granulometry technique using a Coulter Counter-LS 230 Particle Size Analyzer (Miami, FL, USA) equipment, as described by Paulo & Santos (2018a).

5.2.2 Reagents and Standards

Reagents used for the estimation of the TAA and the TPC such as the Folin-Ciocalteu reagent (Ref: 47641-500ML-F, 2M with respect to acid, density of 1.3 g/mL at 20 °C), gallic acid standard (Ref: 91215, $\text{C}_6\text{H}_6\text{O}_5$, CAS 149-91-7), Trolox standard (purity $\geq 97\%$, Ref: 238813, $\text{C}_{14}\text{H}_{18}\text{O}_4$, CAS 53188-07-1), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Ref: D9132, $\text{C}_{18}\text{H}_{12}\text{N}_5\text{O}_6$, CAS 1898-66-4), sodium carbonate anhydrous (Ref: 1613757, Na_2CO_3 , CAS 497-19-8) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA).

The standards used for chromatographic analysis namely hydroxytyrosol (purity $\geq 98\%$, Ref: H4291, $\text{C}_8\text{H}_{10}\text{O}_3$, CAS 10597-60-1), tyrosol (purity $\geq 98\%$, Ref: 188255, $\text{C}_8\text{H}_{10}\text{O}_2$, CAS 501-97-0) and oleuropein (purity $\geq 98\%$, Ref: 12247, $\text{C}_{25}\text{H}_{32}\text{O}_{13}$, CAS 32619-42-4) were also obtained from Sigma Aldrich Chemical (St. Louis, MO, USA).

Solvents such as hexane (HiPerSolv CHROMANORM® for HPLC, Ref: 83992.320, C₆H₁₄, CAS 110-54-3), ethanol (purity ≥ 99.8%, PESTINORM®, Ref: 85933-320, C₂H₆O, CAS 64-17-5), ethyl acetate (purity ≥ 99.8%, SupraSolv®, Ref: 1.00789.2500, C₄H₈O₂, CAS 141-78-6), acetonitrile (purity ≥ 99.9%, LiChrosolv®, Ref: 1.00029.2500, C₂H₃N, CAS 75-05-8) and methanol (purity ≥ 99.9%, LiChrosolv®, Ref: 1.03726.2002, CH₄O, CAS 67-56-1) were obtained from VWR International (Fontenay-sous-Bois, France). Additionally, hydrochloric acid solution at 37% v/v (EMSURE® ACS, Supelco®, Ref: 1.00317.1011, HCl, CAS 7647-01-0), acetic acid standardized solution 0.1 N (supplier Alfa-Aesar, Ref: 35567.K7, C₂H₄O₂, CAS 64-19-7) were also obtained from VWR International (Fontenay-sous-Bois, France).

The water used in this work was de-ionized and double-distilled using a Millipore™ water purification system (Massachusetts, USA) having 18.2 Ω electrical resistivity. All the reagents were either chromatographic or analytical grade and used as received.

5.2.3 Extraction of phenolic compounds

Grounded freeze-dried OMP samples (1 g/sample) were submitted to pre-treatment procedures prior to the extraction of phenolic compounds such as (i) acidic hydrolysis and (ii) fat removal.

5.2.3.1 Acidic hydrolysis

It is described that solvent mixtures of ethanol/water or methanol/water do not allow the complete extraction of phenolic compounds present in OMPs as many of these compounds are linked to OMP matrix components (e.g., cell wall polysaccharides, lipophilic structures) by glycosidic or ester bonds. Higher recoveries of phenolic compounds may be achieved through the breaking of these specific bonds through acid, basic, and/or enzymatic hydrolysis (Araújo et al., 2015). In the present study, an optimized acidic hydrolysis pre-treatment was employed (data not shown).

For that, grounded freeze-dried OMP samples (1 g/sample) were admixed with 25 mL of an aqueous solution of HCl (0.1 M, pH 2) in 50 mL Erlenmeyer flasks.

The hydrolyses were performed continuously in an orbital shaker (IKA® KS 130 basic, IKA Works GmbH & Co. KG, Staufen im Breisgau, Germany) for 12 hours.

In the present study, only the acidic hydrolysis was considered as HCl allows breaking simultaneously ester and glycosylic bounds. Moreover, the low pH promotes the precipitation of proteins and consequently increases the content of free phenolic compounds (FPCs) in the extraction solvent (De Marco et al., 2007).

5.2.3.2 Fat Removal

Afterward, the acidic hydrolyses, acidified grounded OMP samples were submitted to a fat removal procedure using hexane, as described by Lafka et al. (2011). The fats were extracted, adding 5 mL/g of hexane to the hydrolyzed and grounded OMP samples. The flasks were shaken continuously in an orbital shaker for 1 hour (SSR of 5 mL/g) at room temperature (20 ± 2 °C).

Then, samples were transferred into 50 mL polypropylene (PP) centrifuge tubes (Ref: 525-0402, VWR International, Fontenay-sous-Bois, France) and centrifuged at 2 670 g for 15 minutes (Rotofix 32A centrifuge, Hettich). After the centrifugation process, the supernatants obtained corresponded to the organic phase containing hexane, while the pellets corresponded to the aqueous phase containing, at higher concentrations, the compounds of interest (phenolic compounds).

The pellets – containing the compounds of interest – were submitted to the solid-liquid extraction whereas, the supernatants were submitted to liquid-liquid extractions using UPW as the extraction solvent.

The organic supernatants containing hexane, fats, among other non-relevant organic compounds were admixed with 15 mL of UPW. The emulsions were vortexed (IKA VORTEX GENIUS 3, Staufen, Germany) for 5 minutes and ultrasonicated (P Selecta, Barcelona, Spain) for 15 minutes. Samples were then submitted to a centrifugation process (2 670 g, 15 minutes).

The organic phases were discarded while the aqueous phases were filtered under vacuum using Whatman™ nylon membrane filters (Ref. 7404-004, VWR International, Fontenay-sous-Bois, France) prior to further analyses. Samples containing the phenolic compounds recovered from the fat removal procedure were termed as samples containing extracted phenolic compounds from fat removal procedure (SEPC-FR) were stored in amber flasks under dark conditions at room temperature (20 ± 2 °C) before being admixed with the final filtrates (Figure IV.5.S1; Annex I).

5.2.3.3 Solid-liquid extraction of phenolic compounds

Subsequently to the fat removal, phenolic compounds present in the acidified and defatted OMP samples were extracted in a two-step procedure using ethanol as the extraction solvent. Distinctive defatted OMP samples were processed considering alternative conditions such as the time of each extraction step and the SSRs (mL/g). The alternative process conditions tested are presented in Table IV.5.1.

The hydrolyzed and defatted OMP samples were transferred for 50 mL Erlenmeyer flasks prior to the primary extractions. During the primary extractions, pre-defined volumes of the extraction solvent (corresponding to the SSR values for the primary extraction; Table IV.5.1) were added to the respective OMP sample. The obtained solutions were continuously shaken in an orbital shaker at room temperature (20 ± 2 °C) for a pre-defined interval of time (Table IV.5.1).

Afterward, the solid and liquid phases were separated through centrifugation (2 670 g, 15 minutes). The supernatants recovered from the primary extractions (P1E) were filtered under vacuum using Whatman™ nylon membrane filter and stored in an amber flask under dark conditions at room temperature (20 ± 2 °C) before being admixed with the final filtrates. The samples containing extracted phenolic compounds from the primary extractions were named as samples containing extracted phenolic compounds from primary extraction (SEPC-P1E) (Figure IV.5.S1; Annex I).

The pellet samples were reconstituted in a pre-defined volume of the extraction solvent (corresponding to the SSR values for the secondary extraction) and transferred to 50 mL Erlenmeyer flasks for the subsequent secondary extraction processes.

The secondary extraction processes were performed similarly to the described processes for the extraction of phenolic compounds during the primary extractions. The supernatants recovered from the secondary extraction (P2E) were filtered under vacuum using Whatman™ nylon membrane. The phenolic compounds extracted from the secondary extractions procedures were designated as samples containing extracted phenolic compounds after the secondary extraction (SEPC-P2E) (Figure IV.5.S1; Annex I).

For each extraction, the SEPC-FR, SEPC-P1E, and SEPC-P2E were combined, and the final filtrates were submitted to a liquid-liquid extraction using ethyl acetate as the extraction solvent. A volume of ethyl acetate corresponding to three times the volume of each final filtrate samples was admixed with the final filtrate sample, vigorously shaken, vortexed for 5 minutes, ultrasonicated for 15 minutes and centrifuged (2 670 g, 15 minutes). Afterward, the phases were separated, and for each sample, ethyl acetate was removed at 50 °C using a rotary evaporator (BUCHI R-210, Buchi Laboratotiums Tchnik AG, Flawil, Switzerland). Solvent traces were removed by a gentle nitrogen stream. The final extracts were stored at -22 °C before prior analyses.

A schematic representation of the extraction procedure employed is presented in Annex I (Figure IV.5.S1).

5.2.4 Characterization of the extracts

The obtained extracts were allowed to come to room temperature (20 ± 2 °C) before use. Afterward, the extracts were reconstituted in 3 mL of UPW and stored light protected before prior analyses.

5.2.4.1 Determination of the total phenolic content

The TPC of OMP extracts was determined according to the Folin-Ciocalteu procedure, as described by Singleton et al. (1999) with slight modifications considering the scale-down of the colorimetric assay for microplates as described by Barroso et al. (2016).

To each microplate well (Ref: 10062-900, VWR International, Fontenay-sous-Bois, France), it was added 25 µL of the sample or standard solution.

Then, 75 μL of UPW and 25 μL of the Folin Ciocalteu reagent (diluted 1/1 v/v in UPW) were admixed with the sample/standard, in each well. The reaction mixtures were homogenized for 1 minute at 400 rpm on an orbital shaker and incubated for 5 minutes, protected from light at room temperature ($20 \pm 2^\circ\text{C}$). Afterward, to each well was added 100 μL of sodium carbonate solution (75.0 g/L). The solutions were homogenized for 1 minute at 400 rpm on an orbital shaker, incubated for 2 hours protected from light at room temperature. Subsequently, the absorbance was read at 760 nm using a microplate reader (Synergy HT, Biotek, USA).

The calibration curve obtained was based on the mean of three independent calibration curves prepared using gallic acid as the standard: $\text{Abs} = (6.7 \pm 0.6) \times 10^{-3} (\text{L/mg}) C (\text{mg/L}) + (2.3 \pm 1.9) \times 10^{-4}$; correlation coefficient – $R = 0.997$; limit of detection – $\text{LOD} = 0.5 \text{ mg/L}$ limit of quantification – $\text{LOQ} = 1.7 \text{ mg/L}$; linear range: $0.5 \text{ mg/L} - 20.0 \text{ mg/L}$. Each analysis was performed in triplicate. The results were expressed as mg of gallic acid equivalents (mg_{GAE}) per g of dry weight (dw) of OMP ($\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$).

5.2.4.2 Determination of the total antioxidant activity

The total antioxidant activity (TAA) of OMP extracts was estimated through the determination of the free radical-scavenging ability (RSA) of extracts using the stable 2,2-diphenyl-2-picrylhydrazyl radical (DPPH $^\bullet$) (El-Abbassi et al., 2012).

The RSA was evaluated as described by Prieto (2012) in the protocol named ‘Prieto’s DPPH microplate assay’ with slight modifications. A solution of 0.2 mM of DPPH $^\bullet$ in methanol was prepared, lightly protected at room temperature.

The obtained DPPH $^\bullet$ solution was diluted several times to obtain a negative calibration curve (1.1 mg/L, 3.1 mg/L, 6.3 mg/L, 12.5 mg/L, 25.0 mg/L, 50.0 mg/L, 75.0 mg/L, 100.0 mg/L, 125.0 mg/L, 150.0 mg/L, 175.0 mg/L, 200.0 mg/L). A volume of 100 μL of each extract sample was added to the respective matching well in the microplate and diluted 8 times. Then, to each well was added 100 μL of the DPPH $^\bullet$ solution.

Then, the microplates were covered with the lid to minimize evaporation and then wrapped in aluminum foil in order to protect the DPPH $^\bullet$ radical from degradation by light and homogenized for 5 minutes on an orbital shaker.

Afterward, the reaction mixtures were incubated for 45 minutes (time observed for the obtainment of stable Vis absorbance values). The absorbance was then read at 515 nm using a microplate reader.

The scavenging activity of extracts was extrapolated in the inhibitory percentage of DPPH• (%) (Equation (IV.5.1)):

$$\% I = \frac{A_0 - A_1}{A_0} \times 100 \quad (\text{IV.5.1})$$

where A_0 is the absorbance of the control (DPPH radical in methanol) and A_1 is the absorbance of the DPPH radical plus tested sample in methanol.

The calibration curve obtained was based on the mean of three independent calibration curves: $\text{Abs} = (3.9 \pm 0.1) \times 10^{-3} C \text{ (mg/L)} + (3.8 \pm 0.3) \times 10^{-2}$; $R = 0.998$; $\text{LOD} = 3.0 \text{ mg/L}$; $\text{LOQ} = 10.0 \text{ mg/L}$; linear range: $3.1 \text{ mg/L} - 200.0 \text{ mg/L}$.

5.2.4.3 Analysis of individual phenolic compounds by reserved-phase high-performance liquid chromatography (RP-HPLC)

The qualitative and quantitative assessment of hydroxytyrosol, tyrosol, and oleuropein in the OMP extracts obtained under optimized extraction conditions, was performed by RP-HPLC analysis using gradient elution. Analyses were performed using a Merck Hitachi Elite LaChrom (Tokyo, Japan) high-performance liquid chromatograph equipped with a Hitachi L-7100 pump and L-7250 autosampler and coupled to a L-7450A diode array detector. Samples and standards were injected in a Purospher® STAR RP-18 end-capped LiChroCART® column (250 mm x 4.0 mm, 5.0 µm) (Merck KGaA), attached to a guard column (4.0 mm x 4.0 mm, 5.0 µm) of the same type.

The elution method applied was adapted from Tasioula-Margari & Tsabolatidou (2015) and was carried out at a flow rate of 1 mL/min, considering the following two-buffer gradient system: (A) UPW/acetic acid (0.1 N) (97.5/2.5 v/v); (B) acetonitrile/methanol (50/50 v/v). The following gradient elution was applied: 95% A and 5% B as initial conditions that were kept for 5 minutes, 70% A and 30% B during 30 minutes, being re-established the initial conditions (95% A and 5% B) during for 5 minutes (from minute 35 to minute 40). The absorbance of the eluate at 280 nm was recorded at 1 s intervals. The linearity was evaluated by the direct injection of calibration standards prepared in UPW, containing the target phenolic compounds.

For each phenolic antioxidant, the calibration curve was obtained based on the mean of three independent calibration curves. In the case of hydroxytyrosol: $\text{Abs} = (5.5 \pm 0.2) \times 10^5 C \text{ (mg/L)} + (4.6 \pm 0.8) \times 10^5$; $R = 0.997$; LOD = 0.7 mg/L; LOQ = 2.2 mg/L; linear range: 5.0 mg/L – 20.0 mg/L. In the case of tyrosol: $\text{Abs} = (8.4 \pm 0.7) \times 10^5 C \text{ (mg/L)} + (-6.8 \pm 0.3) \times 10^3$; $R = 0.995$; LOD = 1.0 mg/L; LOQ = 3.2 mg/L; linear range: 5.0 mg/L – 20.0 mg/L. During the identification and quantification of oleuropein, the obtained oleuropein concentrations were below of both LOD and LOQ, therefore, oleuropein calibration curve is not presented.

The results were expressed as mg of each phenolic antioxidant per g of dry weight (dw) of OMP (mg/g_{OMP}).

The amount of each specific phenolic antioxidant (hydroxytyrosol or tyrosol) recovered after the optimized extraction procedure was evaluated through the calculation of the recovery (%R) percentage considering the standard addition method as presented in Equation (IV.5.2).

$$\%R = \frac{m_{is} - m_{ius}}{m_{ia}} \times 100 \quad (\text{IV.5.2})$$

where, m_{is} is the mass of the specific antioxidant i in the spiked sample, m_{ius} is the mass of the antioxidant i in the unspiked sample and m_{ia} is the added mass of the antioxidant i to the sample.

5.2.5 Design of experiments for the optimization of the extraction procedure

5.2.5.1 Experimental Design

In this work, a central composite rotatable response surface design (CCRD) was employed as a mathematical and statistical design to optimize the effect of selected process variables such as (i) X_1 – the time of the primary extraction (hours), (ii) X_2 - the SSR (mL/g) during the primary extraction step, (iii) X_3 – the time of the secondary extraction step (hours) and (iv) X_4 –the SSR during the second extraction (mL/g) on the response Y – total phenolic content ($\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$). The four process variables were coded in five levels (-2,-1,0,1,2).

The coding of the variables was performed as described by Paulo & Santos (2017) and Prakash et al. (2013) as follow (Equation (IV.5.3)):

$$x_i = \frac{X_i - X_z}{\Delta X_i}, \quad i=1,2,\dots,k \quad (\text{IV.5.3})$$

where, x_i corresponds to the dimensionless coded value of an independent variable, X_i is the actual value of the independent variable i , X_z is the real value of the independent variable at the center point and ΔX_i corresponds to the step change of the real value of the variable under study, i .

The CCRD consisted of 30 experiments, containing 16 factorial points, 8 axial points, 6 center points considering one block run. Considering that the center point was multiply replicated to estimate the experimental error, the replication of the entire CCRD was not required, as described by Prakash et al. (2013).

The total number of experiments, N , was evaluated considering Equation (IV.5.4) (Prakash et al., 2013):

$$N = 2^K + 2.K + C_p \quad (\text{IV.5.4})$$

where K is the number of process variables (in the present study, $K = 4$), 2^K corresponds to the number of factorial points in the design ($2^K = 2^4 = 16$), $2.K$ the number of axial points on the axis of each design factor at a distance of $\pm\alpha$ (for $K = 4$, $\alpha = 2^{K/4} = 2$, $2.K = 8$) and C_p is the number of replicates at the center point ($C_p = 6$).

The design was randomized in order to reduce the errors from the experimental process relative to extraneous factors. The experimental data were fitted in a second-order polynomial (Equation (IV.5.5)) using a non-linear regression method, expressing, therefore, the mathematical relationship between the process variables (from X_1 to X_4) and the response Y .

$$Y = \beta_0 + \sum_{j=1}^K \beta_j x_j + \sum_{j=1}^K \beta_{jj} x_j^2 + \sum_i \sum_{j=2}^K \beta_{ij} x_i x_j + e_i \quad (\text{IV.5.5})$$

where Y corresponds to the response, β_0 is the model intercept coefficient, β_j , β_{jj} and β_{ij} are the interaction coefficients of the linear, quadratic and second-order terms, respectively, K is the number of independent variables ($K = 4$) and e_i is the error. A $2K$ design was selected for this study and variables were coded at 5 levels (-2, -1, 0, +1 and +2).

5.2.5.2 Statistical Analysis

The statistical software Design Expert 12 (Stat-Ease Inc, MN, USA) was employed for the analysis of the obtained experimental data. Experimental data were evaluated using multiple regressions analysis and the Pareto analysis of variance. The linear, quadratic, and interaction terms for the responses were found through analysis of variance (ANOVA), and the significance of terms was evaluated considering the F-statistical value obtained. Descriptive statistical analysis including the calculation of (i) the p-value, (ii) the F-value, (iii) the degrees of freedom (DF), (iv) the sum of squares, (v) the coefficient of variation, (vi) the determination coefficient (R^2), (vii) the adjusted determination coefficient (R_a^2) and the (viii) correlation coefficient (R) were calculated in order to evaluate the statistical significance of the model. Experimental data were fitted into a quadratic model that was used to sketch the three-dimensional surface plots.

5.2.5.3 Contributions of process variables

The percentage contribution (PC) of each process variables was assessed based on the regression coefficients from the ANOVA and evaluated as described by Khataee et al. (2010) and Prakash et al. (2013) (Equation (IV.5.6)):

$$PC_i = \left(\frac{\beta_i^2}{\sum \beta_i^2} \right) \times 100 \quad (i \neq 0) \quad (\text{IV.5.6})$$

where β_i corresponds to the regression coefficient of the individual process variable.

5.2.5.4 Determination of the optimized extraction conditions

The Derringer's desired function methodology was considered to assess the optimal extraction conditions to maximize simultaneously the responses of the study (Derringer & Suich, 1980).

The generic assessment of the desirability function is based firstly on the transformation of a specific response into a dimensionless individual desirability function (d_i) that ranges between 0 (lowest desirability) to 1 (highest desirability). The overall desirability function (G) was obtained considering the geometric means of each d_i as follows (Equation (IV.5.7)):

$$G = (d_1 \times d_2 \times d_3 \times \dots \times d_n)^{1/n} \quad (\text{IV.5.7})$$

where d_i denotes the desirability of the response i and n represent the number of responses of the study. If any of the responses are beyond the desirability, the G turns into zero.

The G can be extended, considering the importance of the responses (Equation (IV.5.8)):

$$G = (d_1^{\alpha_1} \times d_2^{\alpha_2} \times d_3^{\alpha_3} \times \dots \times d_n^{\frac{1}{n}}), \quad (IV.5.8)$$

$$0 \leq \alpha_i \leq 1 \ (i = 1, 2, 3, \dots, n), \alpha_1 + \alpha_2 + \alpha_3 + \dots + \alpha_n = 1$$

where α_i represents the importance of the response Y_i ($i = 1, 2, 3, \dots, n$) (Mourabet et al., 2012). According to this optimization approach, response goals should be assigned to a low (LV) and a high value (HV).

In the present study, a desirability function criterion (Equation (IV.5.9)) was employed to maximize the response.

$$d_i = 0 \text{ if } Y_i < LV$$

$$0 \leq d_i \leq 1 \text{ if } LV < Y_i < HV \quad (IV.5.9)$$

$$d_i = 1 \text{ if } Y_i > HV$$

The shape of the specific desirability response can be modeled through the weight factor (WF). A WF of 1 creates a linear function between the HV and the LV and the goal or between the HV and the goal. Increasing the WF (up to 10) switch the result towards the goal and reducing the WF (down to 0.1) generates the opposite effect (Prakash et al., 2013). In the present study, a default WF of 1 was chosen. The default importance of 3 was chosen, representing that the goals should be considered of equal importance.

5.2.5.5 Verification of the predicted optimized extraction conditions

The applicability and the accuracy of the generated model were assessed through triplicate experiments under the optimized conditions predicted by the model. The adequacy of the optimized model was evaluated, comparing the average values obtained for the response at optimized conditions and the predicted values by the model.

Table IV.5.1 - Experimental design for the optimization of the extraction of antioxidants and phenolic compounds from olive mill pomace

Run no.	Process Variables (Coded Variables)				Response		
	X_1 : Time P1E (hours) ^a	X_2 : P1E-SSR (mL/g) ^a	X_3 : Time P2E (hours) ^a	X_4 : P2E-SSR (mL/g) ^a	Y: TPC (mg _{GAE} /g _{OMP})		
					OV	PV	RE
1	3.0 (0)	7.5 (0)	0.5 (-2)	4.0 (0)	31.3	29.0	2.3
2	3.0 (0)	7.5 (0)	1.5 (0)	4.0 (0)	36.9	36.6	0.4
3	3.0 (0)	12.5 (2)	1.5 (0)	4.0 (0)	37.0	34.1	2.9
4	2.0 (-1)	5.0 (-1)	1.0 (-1)	5.0 (1)	43.1	45.0	-2.0
5	4.0 (1)	5.0 (-1)	1.0 (-1)	5.0 (1)	39.1	39.4	-0.3
6	3.0 (0)	7.5 (0)	1.5 (0)	4.0 (0)	39.9	36.6	3.3
7	2.0 (-1)	5.0 (-1)	1.0 (-1)	3.0 (-1)	40.4	40.5	-0.1
8	2.0 (-1)	10.0 (1)	2.0 (1)	5.0 (1)	41.8	4.0	-0.3
9	4.0 (1)	10.0 (1)	1.0 (-1)	3.0 (-1)	45.4	46.2	-0.8
10	4.0 (1)	5.0 (-1)	1.0 (-1)	3.0 (-1)	44.7	43.0	1.7
11	3.0 (0)	7.5 (0)	1.5 (0)	6.0 (2)	66.1	62.4	3.7
12	1.0 (-2)	7.5 (0)	1.5 (0)	4.0 (0)	25.3	23.5	1.7
13	3.0 (0)	7.5 (0)	1.5 (0)	4.0 (0)	37.4	36.6	0.8
14	4.0 (1)	10.0 (1)	2.0 (1)	3.0 (-1)	43.8	40.4	3.4
15	2.0 (-1)	10.0 (1)	1.0 (-1)	3.0 (-1)	43.7	44.3	-0.6
16	4.0 (1)	5.0 (-1)	2.0 (1)	5.0 (1)	34.3	32.3	2.0
17	3.0 (0)	7.5 (0)	1.5 (0)	4.0 (0)	35.1	36.6	-1.5
18	3.0 (0)	7.5 (0)	1.5 (0)	2.0 (-2)	58.1	60.6	-2.5

Table IV.5.1 - Experimental design for the optimization of the extraction of antioxidants and phenolic compounds from olive mill pomace (cont.)

Run no.	Process Variables (Coded Variables)				Response		
	X ₁ : Time P1E (hours) ^a	X ₂ : P1E-SSR (mL/g) ^a	X ₃ : Time P2E (hours) ^a	X ₄ : P2E-SSR (mL/g) ^a	Y: TPC (mg _{GAE} /g _{OMP})		
					OV	PV	RE
19	2.0 (-1)	5.0 (-1)	2.0 (1)	5.0 (1)	34.3	36.1	-1.9
20	4.0 (1)	10.0 (1)	2.0 (1)	5.0 (1)	35.0	37.6	-2.6
21	2.0 (-1)	5.0 (-1)	2.0 (1)	3.0 (-1)	9.7	5.9	3.9
22	2.0 (-1)	10.0 (1)	2.0 (1)	3.0 (-1)	34.2	36.7	-2.4
23	3.0 (0)	7.5 (0)	1.5 (0)	4.0 (0)	34.2	36.6	-2.3
24	4.0 (1)	5.0 (-1)	2.0 (1)	3.0 (-1)	9.0	10.3	-1.3
25	3.0 (0)	7.5 (0)	2.5 (2)	4.0 (0)	13.3	14.3	-1.0
26	4.0 (1)	10.0 (1)	1.0 (-1)	5.0 (1)	15.2	17.7	-2.4
27	5.0 (2)	7.5 (0)	1.5 (0)	4.0 (0)	21.2	21.7	-0.5
28	2.0 (-1)	10.0 (1)	1.0 (-1)	5.0 (1)	22.5	23.9	-1.4
29	3.0 (0)	2.5 (-2)	1.5 (0)	4.0 (0)	23.4	25.0	-1.7
30	3.0 (0)	7.5 (0)	1.5 (0)	4.0 (0)	35.9	36.6	-0.7

^a The values presented between brackets represent the coded values for the variables of the study

%E – Percentage of Error; GAE – Gallic Acid Equivalents; OMP – Olive Mill Pomace; OV – Observed Value; P1E – Primary Extraction; P2E – Secondary Extraction; PV – Predicted Value; RE – Residual Error; SSR – Solvent-to-Sample Ratio; TPC – Total Phenolic Content;

5.3 Results

Designed 30 two-step solid-liquid extraction batch experiments were performed to study and optimize the individual and combined effects of the selected variables (time of primary extraction, the sample-to-solvent ratio during the primary extraction, time of the secondary extraction and the sample-to-solvent ratio during the secondary extraction) on the total phenolic content of OMP extracts. In Table IV.5.1 are presented the experimental design (variables), the experimental/observed values (OV), and the predicted values (PV) for the response, the residual error (RE) and the percentage of the error of each experiment on the response.

5.3.1 Central composite rotatable design analysis

The regression equation that describes the dependence of the response (TPC) of the variables (time of the primary extraction, SSR during the primary extraction, time of the secondary extraction, SSR during the secondary extraction) was obtained fitting the experimental data into alternative models like the (i) the linear model, (ii) the two-factor interaction (2FI) model, (iii) the quadratic model and (iv) the cubic model.

For the evaluation of the adequacy of the models, three different tests were conducted as (i) the sequential model sum of squares, (ii) the lack-of-fit test, and (iii) the model summary statistics. The results are presented in Table IV.5.2.

Accordingly, for the TPC of OMP extracts, the sequential model sum of squares presented a p-value lower than 0.0001 for the quadratic model, and therefore this model is suggested to describe the dependence of the TPC of the process variables.

Regarding the results of the lack-of-fit test, the highest lack-of-fit p-values were obtained for the quadratic and cubic models (the lack-of-fit should be insignificant, and lack-of-fit p-values > 0.10 are desirable). Accordingly, the quadratic model is suggested by the lack-of-fit test as the cubic model is aliased (the RSM model chosen was too small to estimate a cubic model).

The model summary statistics lists other relevant statistics used to compare models. This model focuses on the model, maximizing the adjusted R^2 and the predicted R^2 . The maximization of both the adjusted R^2 and the predicted R^2 was possible using the quadratic model. Moreover, it was observed the lowest value for the predicted residual error sum of squares (PRESS) for the quadratic model. The PRESS statistic is a form of cross-validation. Generally, the lowest values of PRESS indicate more robust regression models.

The fit summary for the TPC response indicates that the quadratic model should be selected as for this model was observed the lowest sequential p-value (p-value<0.00001), a robust lack-of-fit p-value (lack-of-fit p-value>0.1) and reasonable high values for the adjusted and the predicted R^2 .

Therefore, the quadratic model incorporating linear, the 2FI and the quadratic terms, was chosen to describe the effect of the time of the primary extraction, the SSR during the primary extraction, the time of the secondary extraction and the SSR during the secondary extraction on the two-step extraction procedure of phenolic compounds from OMP.

5.3.2 Fitting of the design model

A second-order polynomial equation was generated, applying multiple regression analyses on the experimental data. The final equation obtained in terms of uncoded variables (real values) is presented in Equation (IV.5.10) as follow:

$$\begin{aligned} \text{TPC} = & 100.9 + 27.7 X_1 + 7.2 X_2 - 57.3 X_3 - 43.9 X_4 - 0.1 X_1 X_2 + 0.9 X_1 X_3 - 2.1 X_1 X_4 + 5.4 \\ & X_2 X_3 - 2.5 X_2 X_4 + 12.9 X_3 X_4 - 3.5 X_1^2 - 0.3 X_2^2 - 14.9 X_3^2 + 6.2 X_4^2 \end{aligned} \quad (\text{IV.5.10})$$

where TPC is the total phenolic content (expressed in $\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$) and X_1 , X_2 , X_3 and X_4 are the uncoded variables of the time of the primary extraction (hours), the SSR during the primary extraction (mL/g), the time of the secondary extraction (hours), and the SSR during the secondary extraction (mL/g), respectively.

Table IV.5.2 - Sequential model fitting for the extraction of phenolic compounds from olive mill pomace extracts

Y: TPC (mg _{GAE} /gOMP)	Model	Source	Sum of squares	DF	Mean Square	F-value	p-value	Remarks
	Sequential model sum of squares	Mean	3544.7	1	35447.7	-	-	-
		Linear	454.5	4	113.6	0.65	0.6300	-
		2FI	2081.8	6	347.0	2.90	0.0400	-
		Quadratic	2151.2	4	537.8	64.51	<0.0001	Suggested
		Cubic	83.3	8	10.4	1.75	0.2400	Aliased
		Residual	41.8	7	6.0	-	-	-
		Total	40260.2	30	1342.0	-	-	-
	Model	Source	Sum of squares	DF	Mean Square	F-value	p-value	Remarks
	Lack-of-fit Tests	Linear	4338.0	20	216.9	54.27	0.0002	-
		2FI	2256.3	14	161.1	40.33	0.0003	-
		Quadratic	105.1	10	10.5	2.63	0.1488	Suggested
		Cubic	21.8	2	10.9	2.73	0.1583	Aliased
		Pure Error	20.0	5	4.0	-	-	-
	Model	Source	Std. dev.	R²	Adjusted R²	Predicted R²	PRESS	Remarks
	Summary Statistics	Linear	13.2	0.094	-0.050	-0.410	6785.6	-
		2FI	11.0	0.527	0.278	0.205	3827.5	-
		Quadratic	2.9	0.074	0.950	0.868	634.0	Suggested
		Cubic	2.4	0.991	0.964	0.342	3165.4	Aliased

Table IV.5.2 - Sequential model fitting for the extraction of phenolic compounds from olive mill pomace extracts (cont.)

Y: TPC (mg _{GAE} /g _{OMP})	Fit Summary	Source	Sequential p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2	Remarks
		Linear	0.6310	0.0002	-0.054	-0.410	-
		2FI	0.0354	0.0003	0.278	0.201	-
		Quadratic	<0.0001	0.1488	0.950	0.868	Suggested
		Cubic	0.2387	0.1583	0.964	0.342	Aliased

2FI – Two Factors Interaction; DF – Degrees of Freedom; OMP – Olive Mill Pomace; PRESS – Predicted Residual Error Sum of Squares; R^2 – Determination Coefficient; Std. Dev. – Standard Deviation; TPC – Total Phenolic Content;

5.3.3 Statistical analysis

The statistics analysis of variance (ANOVA) and the multiple regression analysis were considered for the evaluation of the fitness of the quadratic model chosen to the experimental data. The results of these analyses are presented in Table IV.5.3. The statistical significance of the regression equation was evaluated, considering models F and p-values.

The quadratic model presented a F-value of 40.16, which implies that the model is significant. There was only 0.01% chance that an F-value this larger could occur due to noise. The obtained p-value of the quadratic regression model was lower than 0.0001. The quadratic model was highly statistically significant for the TPC.

The lack-of-fit test was employed to analyze the fitness of the model. The quadratic regression model presented a lack-of-fit F-value of 2.63 and a lack-of-fit p-value of 0.1488, which indicated that the selected model is suitable to predict variations. The obtained lack-of-fit F-value implied that the lack-of-fit is not significant in relation to the pure error. There was only 14.9% chance that a lack-of-fit F-value this large could occur due to noise.

The goodness of fit of the model was evaluated considering the statistics (i) determination coefficient (R^2), (ii) the predicted determination coefficient (R_a^2), (iii) the predicted determination coefficient (R_p^2), (iv) the adequate precision, and (v) the coefficient of variance (CV).

It was observed R^2 and R_a^2 values of 0.974 and 0.950, respectively. According to Yetilmezsoy et al. (2009), if the sample size is not large enough or if there are many terms in the model, the R_a^2 maybe significantly smaller than the R^2 . In this study, the difference between R^2 and R_a^2 values was low (0.024) for the quadratic regression - a high degree of correlation between the experimental and the predicted values was found. The R_a^2 and the R_p^2 values should be reasonably close – a difference of less than 0.2 is desired. The quadratic regression for the TPC presented R_a^2 (0.950) and R_p^2 (0.868) values in reasonable agreement ($R_a^2 - R_p^2 = 0.082 < 0.2$).

The adequate precision, also known as the signal-to-noise ratio, allows the comparison between the range of the predicted values at the design points and the average prediction error. A ratio greater than 4 indicates adequate model discrimination. It was observed an adequate precision of 27.7 highly than 4 for the model adequacy for the TPC, indicating an adequate precision of the evaluated model.

The CV reflects the ratio of the standard error of the estimate to the average value of the observed response, establishing the reproducibility of the model. The low CV value (8.4%) observed, indicate that the deviations between the experimental and the predicted values were reasonably low, and the model demonstrated a high degree of precision, also indicating a satisfactory reliability of the experiments performed.

The PRESS value was found to be the lowest (PRESS=634.0) when compared to the PRESS values obtained for the other regressions (e.g., mean model PRESS=5150.1, linear model PRESS=6785.6, 2FI model PRESS=3827.5). Therefore, it can be concluded that the quadratic model fitted each point in the design better than the other models considered (mean, linear, and 2FI models).

A quadratic regression model was selected to establish the relationship between the factors and the response. The model F-value and the associated p-values confirmed the model significance. Moreover, the R_p^2 and R_a^2 were in reasonable agreement (within 0.2), the CV was remarkably low, and the model exhibited adequate precision sufficiently high (>4). Therefore, it can be concluded that the model chosen afforded a systematic explanation of the relationship between the factors (X_1 : time of primary extraction, X_2 : SSR during the primary extraction, X_3 : time of the secondary extraction and X_4 : SSR during the secondary extraction) and the response (TPC expressed as mg_{GAE}/g_{GOMP}).

Table IV.5.3 - Analysis of variance for the total phenolic content of olive mill pomace extracts

	Source	Coefficient estimate	Standard Error	Sum of Squares	DF	Mean Square	F-value	p-value	Remarks
Y: TPC (mg _{GAE} /g _{OMP})	Model	-	-	4687.5	14	334.8	40.16	<0.0001	S
	Intercept	36.6	1.2	-	1	-	-	-	-
	X ₁	-0.5	0.6	5.1	1	5.1	0.62	0.4459	-
	X ₂	2.3	0.6	122.7	1	122.7	14.71	0.0016	-
	X ₃	-3.7	0.6	322.3	1	322.3	38.66	<0.0001	-
	X ₄	0.4	0.6	4.4	1	4.4	0.53	0.4781	-
	X ₁ X ₂	-0.2	0.7	0.4	1	0.4	0.05	0.8313	-
	X ₁ X ₃	0.5	0.7	3.4	1	3.4	0.41	0.5313	-
	X ₁ X ₄	-2.1	0.7	67.0	1	67.0	8.03	0.0126	-
	X ₂ X ₃	6.8	0.7	729.8	1	729.8	87.54	<0.0001	-
	X ₂ X ₄	-6.2	0.7	620.7	1	620.7	74.45	<0.0001	-
	X ₃ X ₄	6.4	0.7	660.5	1	660.5	79.22	<0.0001	-
	X ₁ ²	-3.5	0.6	332.7	1	332.7	39.90	<0.0001	-
	X ₂ ²	-1.6	0.6	83.8	1	83.8	10.05	0.0063	-
	X ₃ ²	-3.7	0.6	381.3	1	381.3	45.73	<0.0001	-
	X ₄ ²	6.2	0.6	1066.5	1	1066.5	127.93	<0.0001	-
	Residual	-	-	125.06	15	8.34	-	-	-
	Lack of Fit	-	-	105.07	10	10.51	2.63	0.1488	NS
	Pure Error	-	-	19.98	5	4.00	-	-	-
	Cor Error	-	-	4812.52	29	-	-	-	-

Table IV.5.3 - Analysis of variance for the total phenolic content of olive mill pomace extracts (cont.)

y	Std. Dev.	Mean	CV (%)	R^2	Adjusted R^2	Predicted R^2	Adeq. precision	PRESS	-
	2.9	34.4	8.4	0.974	0.950	0.868	27.7	634.0	-

CV(%) – Coefficient of Variance; DF – Degrees of Freedom; GAE – Gallic Acid Equivalents; NS – Not Significant; OMP – Olive Mill Pomace; PRESS – Predicted Residual Error Sum of Squares; R^2 – Determination Coefficient; S – Significant; Std. Dev. – Standard Deviation; TPC – Total Phenolic Content;

5.3.4 Diagnostics of the adequacy of the models

It is crucial to confirm if the fitted model provides enough similarity between the predicted and the experimental values. Therefore, in addition to the ANOVA and the multiple regression analysis, the adequacy of the selected model was evaluated through the analysis of the residuals using graphical methods.

Normal probability plots are appropriated graphical methods to evaluate the residuals normality. Residuals, defined as the difference between the observed values and the predicted response values, are elements of variation that cannot be explained by the fitted model, which are expected to follow a normal distribution. The observed residuals expressed in terms of externally studentized residuals were plotted against the normal probability (%) (Figure IV.5.1-A).

The design point values lay reasonably close on the straight line. Some scatter was found – which is not considered an unusual occurrence. Nevertheless, if a defined pattern is observed (e.g., an ‘S-shaped’ curve), a transformation of the response may provide better analysis. It was not observed a defined pattern. This assumption was validated through the analysis of the Box-Cox Plot for Power Transforms (Figure IV.5.1-B). The best lambda value ($\lambda=0.683$) – found at the minimum point of the curve generated by the natural logarithm of the sum of squares of the residuals – considering a 95% of confidence interval ($0.157 \leq \lambda \leq 1.126$) included the lambda of 1, and therefore, no power transformation was considered. Therefore, it can be concluded that there was observed a normal distribution of the data for the response, supported by the Box-Cox Plot for Power Transformations (Figure IV.5.1-B).

The diagnostic plot predicted *versus* actual design values is presented in Figure IV.5.1-C. There was observed a strong correlation between model predictions and their actual values as the data points lay close to the straight line.

The Cook’s distance plot (Figure IV.5.1-D) displays that all the data points were within the desired limits, and no outliers were found in the design since the Cook’s distance values were below the defined limit (Cook’s distance of 1).

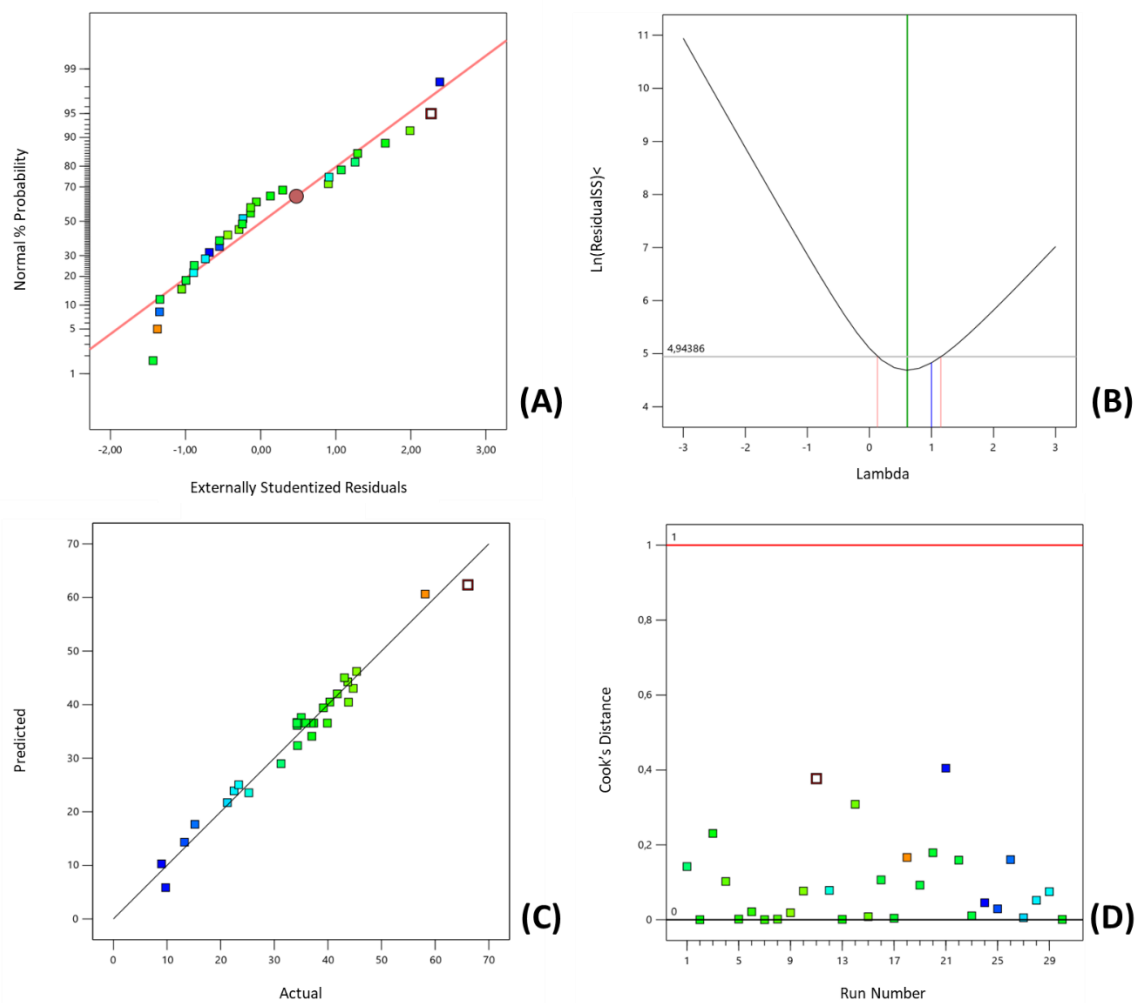


Figure IV.5.1 - Diagnostic plots for the quadratic model adequacy to the total phenolic content of olive mil pomace extracts ((A) – Normal probability plot; (B) – Box-Cox plot for power transforms; (C) – Predicted *versus* Actual plot; (D) – Cook's Distance plot)

5.3.5 Percentage contribution of process variables

The PC of each process variable of the linear, the 2FI, and the quadratic effect was evaluated (Figure IV.5.2). The PCs were calculated as described in section IV.5.2.5.3 and considering the coded regression coefficients from ANOVA.

The 2FI between the SSR during the primary extraction and the time of the secondary extraction (X_2X_3) (21.0%) followed by the 2FI between the time of the secondary extraction and the SSR during the secondary extraction (X_3X_4) (19.0%) exhibited the highest effect on the extraction of phenolic compounds from OMP.

On the opposite, the 2FI between the time of the primary extraction and the SSR during the primary extraction (X_1X_2) (0.01%) followed by the SSR during the secondary extraction (X_4) (0.08%) showed to excrete the lowest impact on the extraction procedure (Figure IV.5.2).

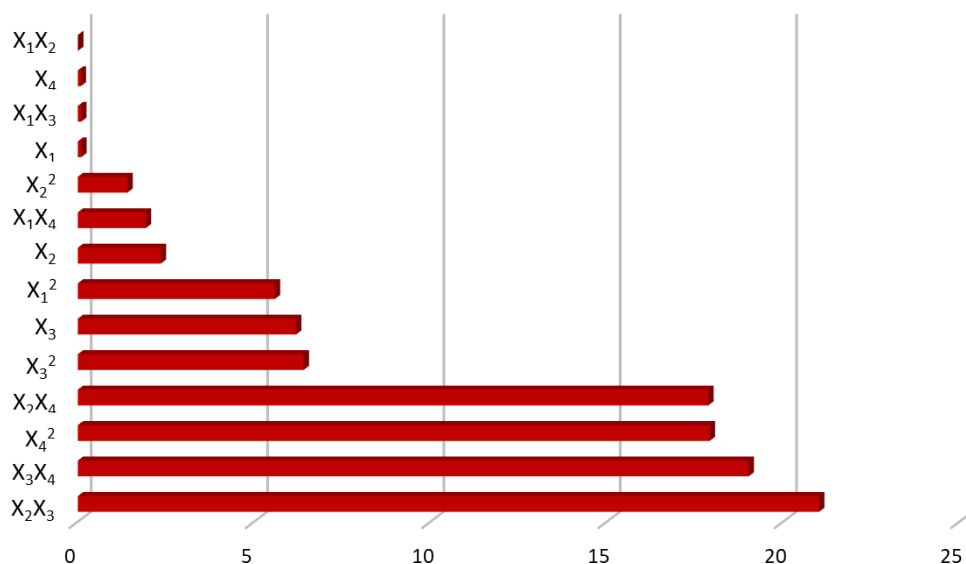


Figure IV.5.2 - Detailed diagram of the percentage contribution of the linear, the two-factor interaction and the quadratic effects on the total phenolic content of olive mill pomace extracts

5.3.6 Assessment of the influence of process variables

A CCRD considering four factors coded at five levels was selected to examine the influence of the time of the primary extraction (X_1 , hours), the SSR during the primary extraction (X_2 , mL/g), the time of the secondary extraction (X_3 , hours) and the SSR during the secondary extraction (X_4 , mL/g) on the two-step solid-liquid extraction of phenolic compounds.

One-variable plots and three-dimensional (3D) response surface plots were plotted to evaluate the effect of each factor considered individually and the interaction among the independent variables, respectively, on total phenolic of OMP extracts. The 3D response surface plots were obtained considering two factors at a constant level while the other two factors were changed.

5.3.6.1 Assessment of the influence of individual process variables on the total phenolic content of olive mill pomace extracts

The effect of each individual variable on the extraction of phenolic compounds estimated through the total phenolic content of OMP extracts was evaluated through the analysis of one-variable plots (Figure IV.5.3).

The time of the primary extraction influenced non-linearly the extraction of phenolic compounds from OMP extracts (Figure IV.5.3-A). The highest phenolic content was observed in the experiments conducted considering the lowest and the highest values of the process variable time of the primary extraction. The lowest phenolic content was observed for experiments in which were considered median values of the variable X_1 . A study regarding the optimization of a solid-liquid extraction procedure was performed by Alu'datt et al. (2010). The authors considered alternative conditions to the ones exposed in the present research during an OVAT optimization as the solvent, the number of extraction steps, temperature, among others. Contrarily to the results obtained in the present study, the authors inferred that increasing the time of extraction lead to an increase in the total phenolic content of the OMP extracts obtained. Even though a direct comparison should be performed carefully as many variables were different among the two studies, it can be stated that probably considering the extraction conditions proposed by the authors, the required extraction time to observe a decrease in the phenolic content of OMP extracts probably was not reached.

Moreover, it is expected a decrease in the total phenolic content of OMP extracts with the extraction time due to degradation processes that may occur, which was not observed by Alu'datt et al. (2010). The results of the present study are supported by the results obtained by Rubio-Senent et al. (2012). The authors performed the extraction of phenolic compounds through a hydrothermal treatment and observed that the amount of phenolic compounds extracted increased not linearly with the increase of the extraction time.

A similar trend to the process variable X_1 , was observed with the process variable SSR during the primary extraction (X_2). It was observed the highest phenolic content in the experiments associated with the lowest and the highest values of the SSR during the primary extraction.

The minimum phenolic content was observed in the experiments, which were considered the median values of SSR (X_2) (Figure IV.5.3-B). The phenolic and antioxidant potential of olive mill wastes was evaluated by Lafka et al. (2011). The authors observed an increase in the TPC of olive mill waste extracts with the increase of the SSR value.

Nevertheless, the authors demonstrated that for SSR values higher than 5 v/w, it was almost impossible to extract more phenolic compounds (a plateau on the total phenolic content of extracts *versus* the SSR value was reached). In contrast, the results of this study demonstrated that a plateau is not reached: an increase of the total phenolic content was observed from the median to the highest SSR values. The addition of more extraction solvent favors the non-saturation of the extraction solvent, and therefore more phenolic compounds can be extracted from OMP to the extraction solvent.

The time of the secondary extraction influenced the extraction of phenolic compounds in an opposite trend to the process variable X_1 (time of the primary extraction). In the case of the dependence of the total phenolic content of OMP extracts from the time of the secondary extraction (X_3), it was observed the maximization for the median values of the process variable X_3 . The lowest phenolic content was observed in experiments in which were considered the lowest and the highest values considered for the time of the secondary extraction (Figure IV.5.3-C).

Contrasting with the non-linear pattern dependence of the TPC of OMPs from the X_1 , X_2 , and X_3 variables, the X_4 variable – SSR during the secondary extraction - did not affect largely the efficient extraction of phenolic compounds when considered individually. It was observed a non-dependence of the single factor SSR during the secondary extraction corresponding to a slope of the line equal to zero (Figure IV.5.3-D).

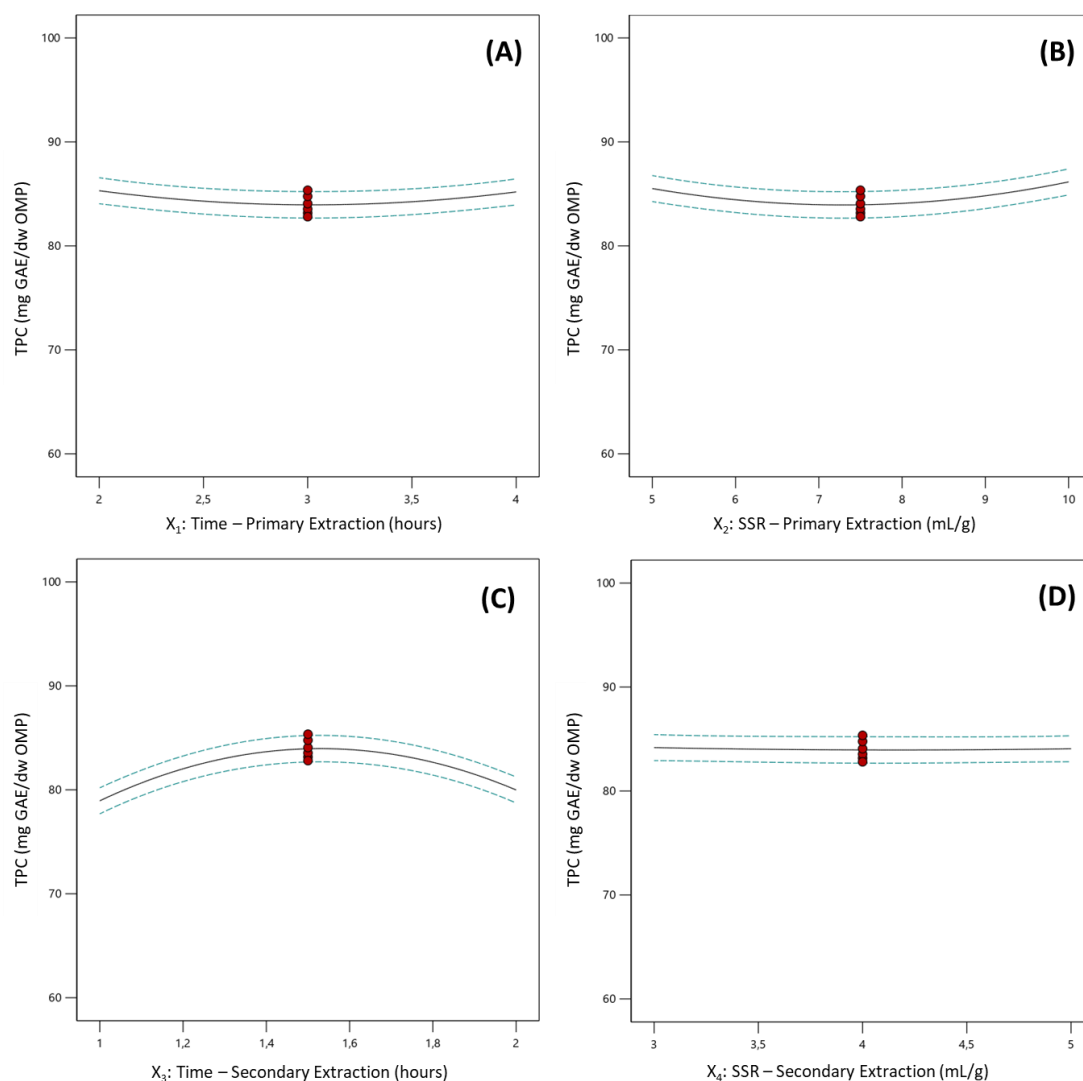


Figure IV.5.3 - One variable plots portraying the effect of each process variable on the total phenolic content of the extracts obtained from olive mill pomace samples ((A) – effect of the time of the primary extraction; (B) – effect of the solvent-to-sample ratio during the primary extraction; (C) – effect of the time of the secondary extraction; (D) – effect of the solvent-to-sample ratio during the secondary extraction (● represent design points; --- represent 95% confident interval bands

5.3.6.2 Assessment of the influence of combined process variables on the total phenolic content of olive mill pomace extracts

Experiences were conducted to observe the effect of the time and the SSR during both the primary and secondary extractions on the recovery of phenolic compounds estimated through the TPC of OMP extracts.

It was observed that for the lowest SSR during the primary extraction (5.0 mL of ethanol/g_{OMP}), the time of primary extraction did not excrete influence on the TPC. Nevertheless, when the SSR was increased, it was also observed an increase of the TPC alongside the time of the primary extraction. For a SSR of 10.0 mL/g the and 2 hours for the primary extraction, the extracts exhibited a TPC of 34.4 mg_{GAE}/g_{OMP}. However, when the extraction time was doubled, keeping the SSR constant (10.0 mL/g), the TPC was increased (Figure IV.5.4-A).

When the 3D response surface plot was plotted exhibiting the effects on the TPC of the contributions of the time of the primary extraction and the time of the secondary extraction, it was observed a compromise among the time of each extraction step. The longest time of the secondary extraction seemed not to influence the extraction of phenolic compounds positively. However, around median times for the first extraction stage (around 3.0 to 3.5 hours) associated with reduced times during the second extraction stage, seemed to favor the extraction of phenolic compounds (Figure IV.5.4-B).

The relationship between the time of the primary extraction and the SSR during the secondary extraction was challenged to infer. It was observed that from the median to low SSRs, the recovery of phenolic compounds was increased, especially for the lowest and the highest values for the time of the primary extraction. From the median to higher SSR for the secondary extraction, the recovery of phenolic compounds was almost independent of the time of the primary extraction (Figure IV.5.4-C).

In Figure IV.5.4-D is depicted the influence of the SSR during the primary extraction and the time of the secondary extraction. It was observed that the combination of both low values for these two variables did not favor the extraction of phenolic compounds. The TPC of OMP extracts was increased with the simultaneous increase of the SSR during the primary extraction of the decrease of time of the secondary extraction.

When the SSRs were plotted to infer their influence on the TPC, it was observed that the maximum total phenolic content was achieved when considered the highest SSR value during the primary extraction combined with the lowest SSR value during the secondary extraction (Figure IV.5.4-E).

The relationship between the time and SSR value during secondary extraction was not linear. The combination of the low time of extraction and high SSR value during the secondary extraction negatively affected the extraction of phenolic compounds. The highest recovery of phenolic compounds was observed to low SSR values combined with median times for the secondary extraction (Figure IV.5.4-F). No data was found in the literature for the direct comparison of the obtained results in the present study.

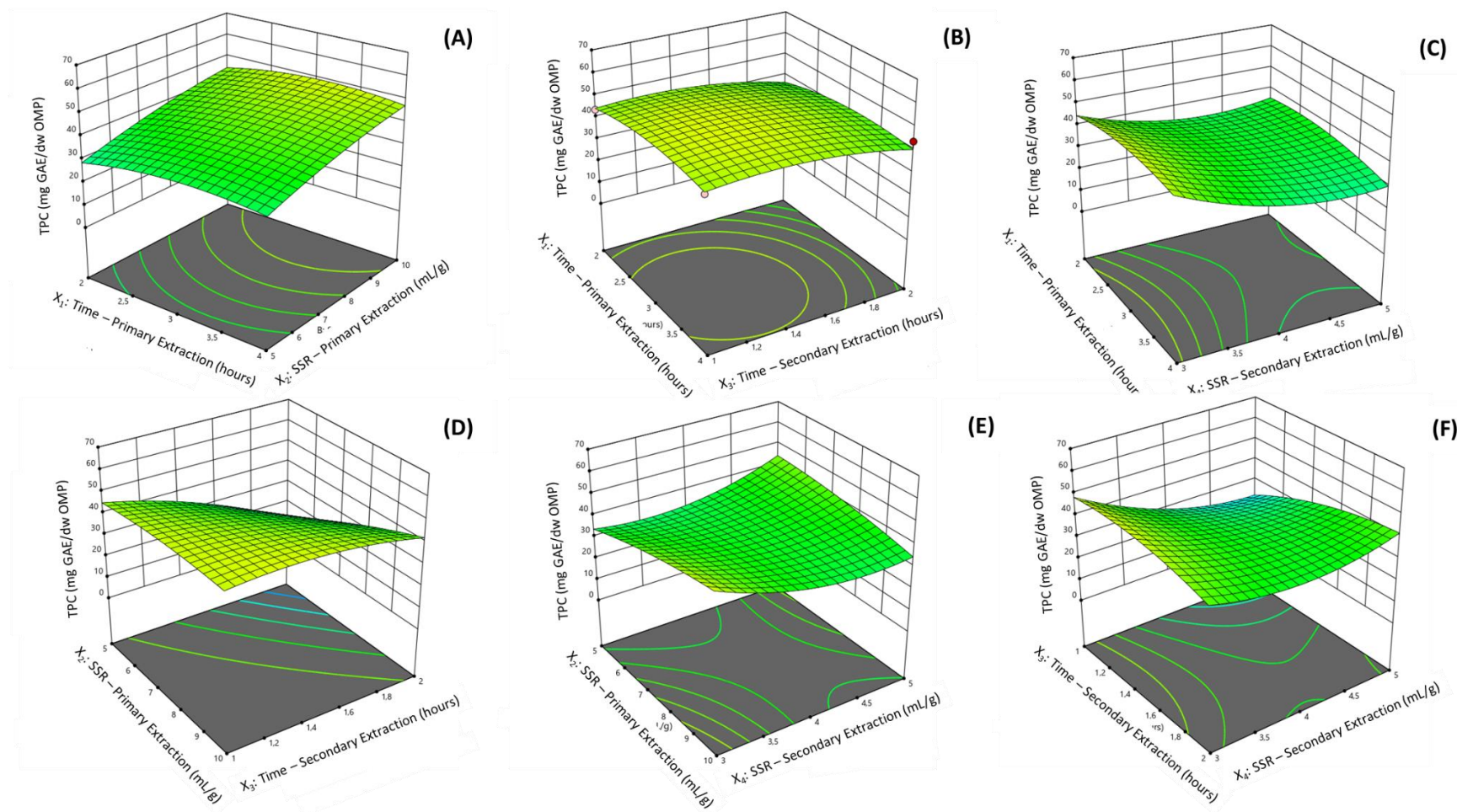


Figure IV.5.4 - Response surface plots representing the effect of each process variable on the total phenolic content of the extracts obtained from OMP samples (● represents a design point)

5.3.7 Optimization of process variables and validation of the optimized conditions

The Derringer's desired function methodology was applied to optimize the extraction conditions with the purpose of the maximization of the extraction of phenolic compounds estimated through the TPC of OMP extracts. According, the optimal extraction conditions were found to be: time of the primary extraction of 3.2 hours (X_1), SSR during the primary extraction of 10.0 mL/g (X_2), time of the secondary extraction of 1.3 hours (X_3) and SSR during the secondary extraction of 3.0 mL/g (X_4). Considering the optimized conditions, the predicted TPC was 50.0 mg_{GAE}/g_{OMP}, associated with a desirability value of 0.717. A desirability ramp was generated from the optimal points through a numerical optimization approach (Figure IV.5.5).

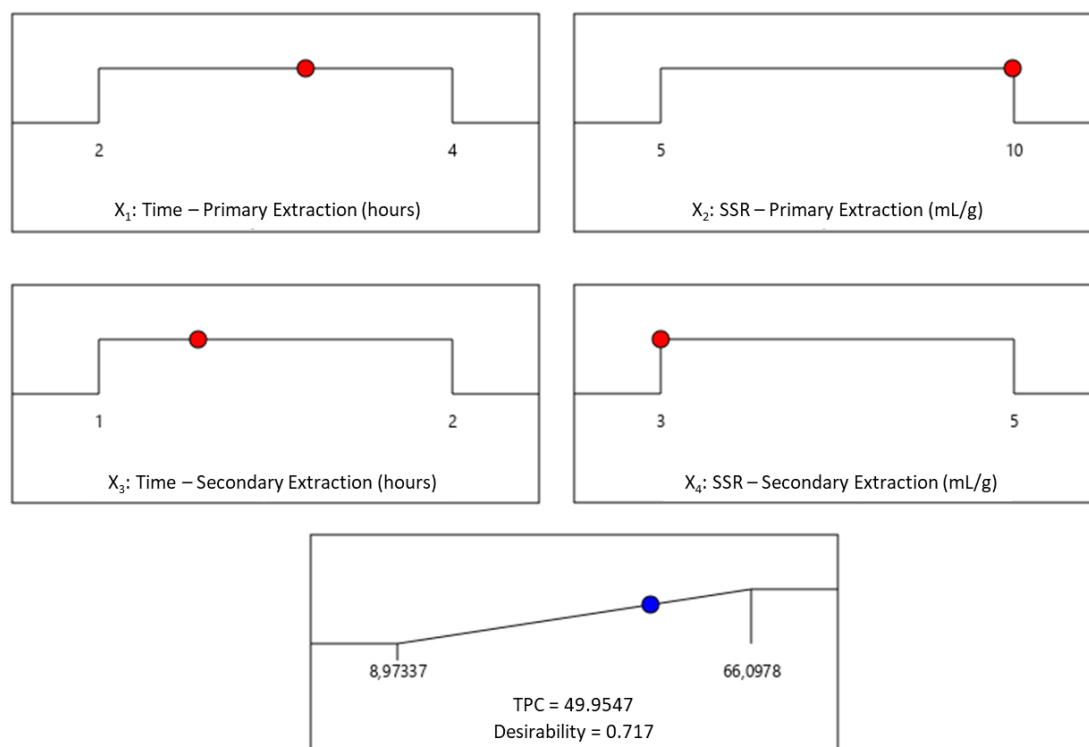


Figure IV.5.5 - Desirability ramp for the optimization of the two-step extraction of phenolic antioxidants from olive mill pomace (● represent design points; ● represents a predicted point)

The accuracy of the prediction of the optimum response values, which indicates the suitability of the optimized extraction conditions, was evaluated comparing the obtained experimental results with the post-analysis of the optimization procedure. As presented in Table IV.5.4, the observed TPC was 50.5 ± 1.5 ($n=3$) which is within the predicted interval (95% confidence interval) of the model (lowest predicted value by the model = $45.2 < \text{obtained value} = 50.5 < \text{highest predicted value by the model} < 54.7$). Therefore, it can be concluded that the model accurately predicted the optimum TPC considering optimized extraction conditions of phenolic compounds from OMP samples.

Table IV.5.4 - Experimental and Post analysis of the response

Post analysis	
Predicted ($\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$)	50.0
95% PI low ($\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$)	45.2
95% PI high ($\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$)	54.7
Experimental ($\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$) ^a	50.5 ± 1.5
Residual Error	0.5
%Error	1.0

^a Data expressed as mean $\pm$ standard deviation of three independent extractions considering the optimized extraction conditions

GAE – Gallic Acid Equivalents; OMP – Olive Mill Pomace; PI – Predicted Interval;

5.3.8 Characterization of the optimized olive mill pomace extracts

The extract ($n=3$) obtained considering the optimized extraction conditions (time of the primary extraction of 3.2 hours; SSR during the primary extraction of 10 mL/g; time of the secondary extraction of 1.3 hours; SSR during the secondary extraction of 3.0 mL) was characterized regarding its TAA and TPC. Moreover, the contribution of each fraction – phenolic compounds extracted from the fat removal step, phenolic compounds recovered during the primary extraction, and the phenolic compounds extracted during the secondary extraction – for the TAA and TPC was evaluated.

The phenolic profile and the quantification of specific phenolic compounds were assessed by RP-HPLC analysis of the final extract.

Furthermore, The effect of the application of an acidic hydrolysis step prior to the solid-liquid extraction of phenolic compounds from OMP samples was evaluated generically for antioxidants and specifically for phenolic compounds, considering the total, the bonded and the free phenolic compounds distribution in hydrolyzed extracted samples and non-hydrolyzed extracted samples.

5.3.8.1 Contribution of alternative extract fractions on the total phenolic content and the total antioxidant activity

The contribution of each fraction (SEPC-FR, SEPC-P1E, and SEPC-P2E) on the (i) the total phenolic content and (ii) the total antioxidant activity was evaluated (Figure IV.5.6).

The amount of phenolic compounds extracted from the SEPC-FR was 0.6 ± 0.1 mg_{GAE}/g_{OMP} corresponding only to $1.2 \pm 0.2\%$ of the total phenolic content of OMP extracts obtained considering optimized extraction conditions. There were not observed significant differences ($p=0.83>0.05$) on the TPC among the SEPC-P1E and SEPC-P2E fractions. The TPC of the fractions SEPC-P1E and SEPC-P2E were 26.1 ± 1.5 mg_{GAE}/g_{OMP} (percentage proportion of $51.7 \pm 3.0\%$ of the TPC) and 23.8 ± 0.7 mg_{GAE}/g_{OMP} (percentage proportion of $47.1 \pm 1.4\%$ of the TPC), respectively. The total amount of polar phenolic compounds (fractions SEPC-P1E and SEPC-P2E) was 49.9 ± 1.3 mg_{GAE}/g_{OMP}, corresponding to a proportion of $98.8 \pm 2.3\%$ of the TPC. The high amount of phenolic compounds present in SEPC-P1E and SEPC-P2E fractions and the low amount of phenolic compounds recovered from the fat removal step (SEPC-FR fraction) reveals the majority of the extracted phenolic compounds from OMP samples are from hydrophilic nature. Furthermore, the present results demonstrate that ethanol can be safely used as an extraction solvent of phenolic compounds from OMP samples, unraveling new perspectives for the replacement of toxic solvents commonly on solid-liquid extraction of phenolic compounds from OMP samples (e.g., methanol).

A similar pattern was found for the TAA distribution between the fractions SEPC-FR, SEPC-P1E, and SEPC-P2E. The fraction SEPC-FR exhibited only $2.4 \pm 0.5\%$ of TAA, corresponding to a percentage contribution to the TAA of $3.0 \pm 0.6\%$. It was not observed a statistically significant difference in the TAA between the fractions SEPC-P1E and SEPC-P2E ($p=0.78>0.05$).

The TAA of SEPC-P1E and SEPC-P2E fractions were $45.7 \pm 4.1\%$ (percentage contribution to TAA of $57.8 \pm 5.2\%$) and $30.9 \pm 3.6\%$ (percentage contribution to TAA of $39.1 \pm 4.6\%$), respectively. The two-step extraction (SEPC-P1E and SEPC-P2E fractions) using ethanol as the extraction solvent (combined TAA of SEPC-P1E and SEPC-P2E of $76.7 \pm 7.7\%$) contributed to $97.0 \pm 9.7\%$ for the TAA of the final extract. These results reinforce the hypothesis that the majority of the antioxidants extracted were phenolic compounds. From the statistical analysis, as it was not observed significant differences in the TPC and TAA of both SEPC-P1E and SEPC-P2E fractions, it can be confirmed that the secondary extraction is essential for the recovery of phenolic compounds from OMP samples. In this context, a multi-step extraction should be considered when it is intended for the recovery of phenolic compounds from OMP samples for their further valorization. To the authors' best knowledge, there was a lack of published data suitable for the direct comparison regarding the obtained results.

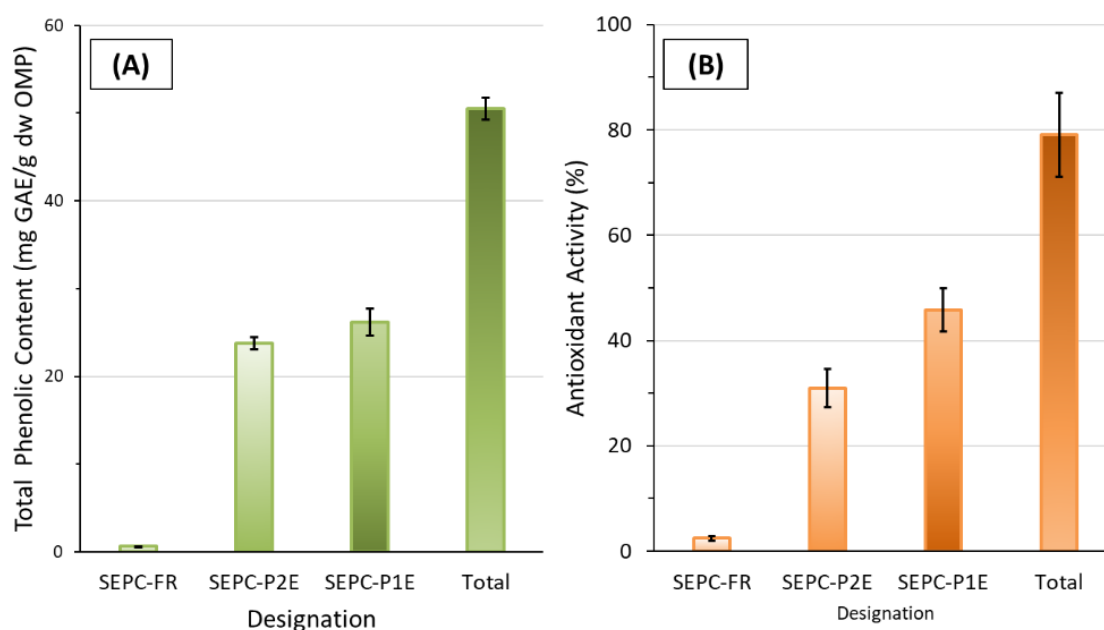


Figure IV.5.6 - Distribution of the total phenolic content **(A)** and the total antioxidant activity **(B)** among different fractions (SEPC-FR, SEPC-P2E, and SEPC-P1E) of OMP extracted samples obtained considering extraction optimized conditions (SEPC-FR – Extracted phenolic compounds during the fat removal process; SEPC-P1E – Extracted phenolic compounds during the primary extraction; SEPC-P2E – Extracted phenolic compounds during the secondary extraction procedure)

5.3.8.2 RP-HPLC analysis of phenolic compounds

The RP-HPLC analysis of the present study was focused on identification, detection, and quantification of two OMP phenyl alcohols - hydroxytyrosol and tyrosol - and a glycosylated seco-iridoid compound – oleuropein. Oleuropein is the major biophenol present in olives and olive leaves (Goldsmith et al., 2018). Oleuropein is a heterosidic ester of elenolic acid and 3,4-dihydroxyphenylethanol, which hydrolysis yields hydroxytyrosol – one of the most potent phenyl alcohols present in olive oil, olive products, and by-products – and elenolic acid glucoside (Del et al., 2003). Nevertheless, oleuropein is often hydrolyzed to hydroxytyrosol during olive oil processing as a high amount of water is required during the olive oil extraction process (Paulo & Santos, 2020b). Hydroxytyrosol is considered one of the most potent antioxidants present in olive oil and olive oil by-products (Goldsmith et al., 2018a; Paulo & Santos, 2018).

In contrast, tyrosol can be found on olive oil, olive oil products, and olive oil by-products in as a free phenol or on its oleoside form (tyrosol glucoside) (Soler-Rivas et al., 2000). As complex chemical transformations occur during olive fruit maturation and olive oil processing, the antioxidants hydroxytyrosol, tyrosol, and oleuropein may be found in OMP samples in their free forms along with several other analogs (Malapert et al., 2018). Moreover, the phenolic profile of OMP extracts is widely affected by the olive fruit variety, climatic conditions, agronomic practices, OMP storage and handling, phenolic compounds extraction procedures, among others (Peralbo-Molina et al. 2012b). The RP-HPLC identification of the compounds hydroxytyrosol, tyrosol, and oleuropein was performed by comparison with the respective analytical standards. The hydroxytyrosol and tyrosol concentrations on the OMP extract obtained considering optimized extraction conditions were $2\,870.2 \pm 1.2$ mg/kg_{OMP} and $1\,622.1 \pm 2.2$ mg/kg_{OMP}, respectively. The obtained concentrations for oleuropein were below of both LOD and LOQ; therefore, it can be concluded that oleuropein was converted into hydroxytyrosol and/or other analogs during phenolic compounds extraction. Comparable results were described by a previous investigation of Japón-Luján & Luque de Castro (2007). The authors described the extraction of hydroxytyrosol, tyrosol, and other olive biophenols using static-dynamic superheated liquids.

Similarly to the present study, a multivariate methodology was employed to optimize the extraction conditions. In that investigation, the authors reported it was possible to achieve a total amount of hydroxytyrosol and tyrosol of 2 872 mg/kg_{OMP} and 1 565 mg/kg_{OMP}, respectively, in the extract obtained considering the optimized extraction conditions. Moreover, it was observed a higher amount of hydroxytyrosol rather than tyrosol which is in line with the majority of the published investigations on the extraction of phenolic compounds from OMP (Obied et al., 2005a, 2007a; Rubio-Senent et al., 2012; Suárez et al., 2009, 2010; Uribe et al., 2015).

5.3.8.3 Effect of acidic hydrolysis pre-treatment on the total phenolic content and the total antioxidant activity

The effect of the application of an acidic hydrolysis step prior to the solid-liquid extraction of phenolic compounds from OMP samples was evaluated generically for antioxidants and specifically for phenolic compounds, considering the total, the bonded and the free phenolic compounds distribution in both hydrolyzed extracted samples (HS) and non-hydrolyzed extracted samples (NHS).

Considering both HS and NHS, in each type of extract, it was observed a significant difference ($p < 0.05$) in the distribution of bonded and free phenolic compounds. Similarly, it was also noticed statistically significant differences ($p < 0.05$) between bonded and free phenolic compounds concentration between HS and NHS.

The highest phenolic content was observed in hydrolyzed samples (50.5 ± 1.5 mg_{GAE}/g_{OMP}) (Table IV.5.5). The TPC of extracts obtained considering the optimized extraction conditions, but in which was not considered the HCl hydrolysis step, exhibited only $43.2 \pm 1.4\%$ of the phenolic content of the samples obtained under optimized extraction conditions (21.7 ± 0.7 mg_{GAE}/g_{OMP}). This suggests that the acidic hydrolysis step is crucial for the recovery of phenolic compounds. The obtained results are in agreement with the ones obtained by Lafka et al. (2011) when the authors were describing the phenolic and antioxidant potential of OMP samples submitted to a solid-liquid extraction. The authors submitted the OMP samples to hydrolysis at different pH verifying higher recovery of phenolic compounds at the lowest pH selected (pH 2).

Likely, the acidic hydrolysis had promoted the solubilization of phenolic compounds in the extraction solvent through two alternative mechanisms: (i) the detachment of phenolic compounds linked to OMP cell walls by ester or glycoside bonds and through the (ii) promoting of OMP cell walls disintegration. In the case (i) of phenolic compounds detachment from OMP cell walls, the acidic environment promoted the cleavage of the bonds in which cell wall phenolic compounds were linked (ester and glycosidic bonds) which has promoted the solubilization of cell wall phenolic compounds in the extraction solvent. Additionally, the acidic hydrolysis (ii) promoted OMP cell walls disintegration, enabling the diffusion and the solubilization of these compounds in the extraction solvent, increasing their stability.

In the case of extracts obtained considering the optimized extraction conditions, the contribution of bonded and free phenolic compounds for the total phenolic content of the extracts was $26.9 \pm 0.5\%$ ($13.6 \pm 1.1 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$) and $73.1 \pm 0.8\%$ ($36.9 \pm 0.4 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$), respectively (Table IV.5.5). It was observed an increase of the amount of bonded phenolic compounds extracted when the 12-hr hydrolysis was considered (bonded phenolic compounds in NHS: $2.0 \pm 0.1 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$; bonded phenolic compounds in HS: $13.6 \pm 1.1 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$), leading to an increase of bonded phenolic compounds contribution from $9.2 \pm 0.5\%$ - in the case of non-hydrolyzed samples – to $26.9 \pm 2.2\%$ - in the case of hydrolyzed samples. Therefore, it can be concluded that the acidic hydrolysis favored the recovery of both bonded and free phenolic compounds. The highest recovery of phenolic compounds when the acidic hydrolysis was considered can be explained considering (i) the stability of free phenolic compounds in the extraction solvent, (ii) the detachment of OMP cell wall bonded phenolic compounds and (iii) the disruption of OMP cell walls and the consequent diffusion and solubilization of phenolic compounds in the extraction solvent. A similar pattern was observed regarding the TAA of HS and NHS. In both types of extracts (HS and NHS), it was observed significant differences ($p < 0.05$) on the TAA between the free and the bonded fractions. Similarly, it was verified significant differences ($p < 0.05$) on the TAA between the HS and NHS. The HS exhibited higher TAA than NHS (TAA of HS: $79.1 \pm 7.9\%$; TAA of NHS: $53.5 \pm 3.9\%$) (Table IV.5.5). The TAA of NHS corresponded to $67.6 \pm 4.9\%$ of the TAA of HS.

The percentage contribution of the bonded and the free extract fractions were respectively $36.8 \pm 4.9\%$ and $63.1 \pm 5.9\%$ in the case of HS and $35.1 \pm 7.3\%$ and $64.9 \pm 7.1\%$ in the case of NHS. Roughly, the percentage contributions of each fraction (free and bonded antioxidants) were similar in both HS and NHS (about 60% of proportion contribution of free antioxidants and 40% of proportion contribution of bonded antioxidants) even though, overall, HS samples present higher TAA. The hydrolysis prior to the solid-liquid extraction promotes the dissolution of bonded phenolic compounds in the extraction solvent (proportion of bonded phenolic compounds extracted from HS was *circa* 70%; the proportion of free phenolic compounds extracted from NHS was around 10% of the total amount of extracted phenolic compounds); nevertheless, as the proportions of extracted antioxidants from free and bonded fractions were respectively around 60% and 40% for both HS and NHS, it can be hypothesized that the TAA of extracts submitted to hydrolysis was positively affected by the dissolution of bonded phenolic compounds.

To the authors' best knowledge, this is the first time that is reported the extraction of phenolic compounds from OMP using two-step solid-liquid extraction, including pre-treatment steps of the samples as hydrolysis and fat removal procedures. Therefore, there is a lack of published data suitable for the direct comparison regarding the obtained results. Nevertheless, some bibliographic data regarding the total phenolic content of extracts obtained from OMPs considering alternative extraction conditions were found on literature, allowing an outward comparative analysis. The authors Obied et al. (2007b) performed the extraction of phenolic compounds from Frantoio olive fruit (90% black skin coloration), processed in a two-phase olive oil extraction system in WaggaWagga, Australia. The extraction was performed considering a solvent mixture of methanol and water at a ratio of 80/20 v/v, at pH 2; it was also described as a lipid removal step with *n*-hexane. The authors reported that the phenolic content of the obtained extracts was $17.7 \pm 0.9 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$. The results obtained in the present study allowed a higher recovery of phenolic compounds, considering the optimized extraction conditions ($50.5 \pm 1.5 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$). Nevertheless, it should be taken into consideration that the total phenolic content of OMP extracts is widely dependent on the geographic origin of olives, cultivar factors (e.g., olives type, climatic conditions), phenolic compounds extraction techniques among others (Paulo & Santos, 2020b).

Table IV.5.5 - Total phenolic content and their proportion of different factions of olive mill pomace extracted full-fat samples and extracted defatted samples

Extract fraction	Hydrolyzed extracted samples				Non-hydrolyzed extracted samples			
	TPC (mg _{GAE} /g _{OMP})	TPC Proportion (%)	TAA (%)	TAA Proportion (%)	TPC (mg _{GAE} /g _{OMP})	TPC Proportion (%)	TAA (%)	TAA Proportion (%)
TAPCs	50.5 ± 1.5	100	79.1 ± 7.9	100	21.8 ± 0.7	100	53.5 ± 3.9	100
BPCs	13.6 ± 1.1	26.9 ± 2.2	29.1 ± 3.9	36.8 ± 4.9	2.0 ± 0.1	9.2 ± 0.5	18.8 ± 4.6	35.1 ± 7.3
FPCs	36.9 ± 0.4	73.1 ± 0.8	49.9 ± 4.7	63.1 ± 5.9	19.8 ± 0.6	91.2 ± 2.8	34.7 ± 3.8	64.9 ± 7.1

BPCs – Bonded Phenolic Compounds; FPCs – Free Phenolic Compounds; GAE – Gallic Acid Equivalents; OMP – Olive Mill Pomace; TAA – Total Antioxidant Activity; TAPCs – Total Amount of Phenolic Compounds; TPC – Total Phenolic Content;

5.4 Conclusions

Recent studies have been suggested that olive mill pomace – an olive oil by-product generated in large quantities during olive oil processing – is a significant source of phenolic compounds. Several techniques have been investigated to extract phenolic compounds from olive mill pomace. Among them, the solid-liquid extraction is the most widespread one regarding its simplicity, the non-requirement of cutting-edge and specific technological equipments and it can be performed under mild conditions.

In this study, a central composite rotatable response surface design was employed to a two-step solid-liquid extraction to investigate and optimize the influence of the individual and the interactive effect of process variables as the time of the primary extraction, the solvent-to-sample ratio during the primary extraction, the time of the secondary extraction and the solvent-to-sample ratio during the secondary extraction on the total phenolic content of olive mill pomace extracts. Hydrolysis and fat removal procedures were considered prior to the extraction, as in preliminary studies was verified that the combination of these pretreatments favored the recovery of phenolic compounds. Moreover, the extraction of phenolic compounds was processed in two steps as it was verified that one-step solid-liquid extraction was inefficient in the extraction of these bioactive compounds.

The results suggested that the process variables had a significant effect on the recovery of phenolic compounds estimated through the total phenolic content of olive mill pomace extracts. From the analysis of alternative model statistics, a quadratic model incorporating the linear, the two-factor interactive, and the quadratic terms, was chosen to describe the effect of the process variables on the total phenolic content of olive mill pomace extracts. The quadratic model F-value and the associated p-values confirmed the significance of the selection of the quadratic regression model. The Derringer's desired function methodology allowed to optimize the extraction conditions. In this context, the optimum conditions were found to be 3.2 hours for the primary extraction, 10.0 mL/g for the solvent-to-sample ratio during the primary extraction, 1.3 hours for the secondary extraction associated to a solvent-to-sample ratio of 3.0 mL/g leading to a predicted maximum total phenolic content of olive mill pomace extracts of 50.0 mg_{GAE}/g_{OMP}.

The extract obtained considering the optimized extraction conditions exhibited a total phenolic content of $50.5 \pm 1.5 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$. Therefore, the accuracy of the optimization function prediction was verified.

The fractioning of antioxidants and phenolic compounds was evaluated in the extract obtained under optimized extraction conditions. The majority of antioxidants (percentage proportion of $78.7 \pm 7.7\%$) and phenolic compounds (percentage proportion of $98.8 \pm 2.3\%$) were detected in the polar fractions of the olive mill pomace final extract sample. The greater part of the antioxidants extracted were phenolic compounds, and the majority of phenolic compounds were from hydrophilic nature. The total amount of OMP phenyl alcohols hydroxytyrosol and tyrosol were $2870.2 \pm 1.2 \text{ mg}/\text{kg}_{\text{OMP}}$ and $1622.1 \pm 2.2 \text{ mg}/\text{kg}_{\text{OMP}}$, respectively. The amount of the glycosylated seco-iridoid oleuropein was below its limit of detection and quantification. Additionally, the effect of the hydrolysis pre-treatment on the extraction of phenolic compounds was assessed. The acidic hydrolysis presented an utmost importance for the extraction of phenolic compounds as this step allowed the efficient extraction of phenolic compounds linked to olive mill pomace cells by ester and glycosidic bonds and the dissolution of phenolic compounds retained inside of olive mill pomace cells.

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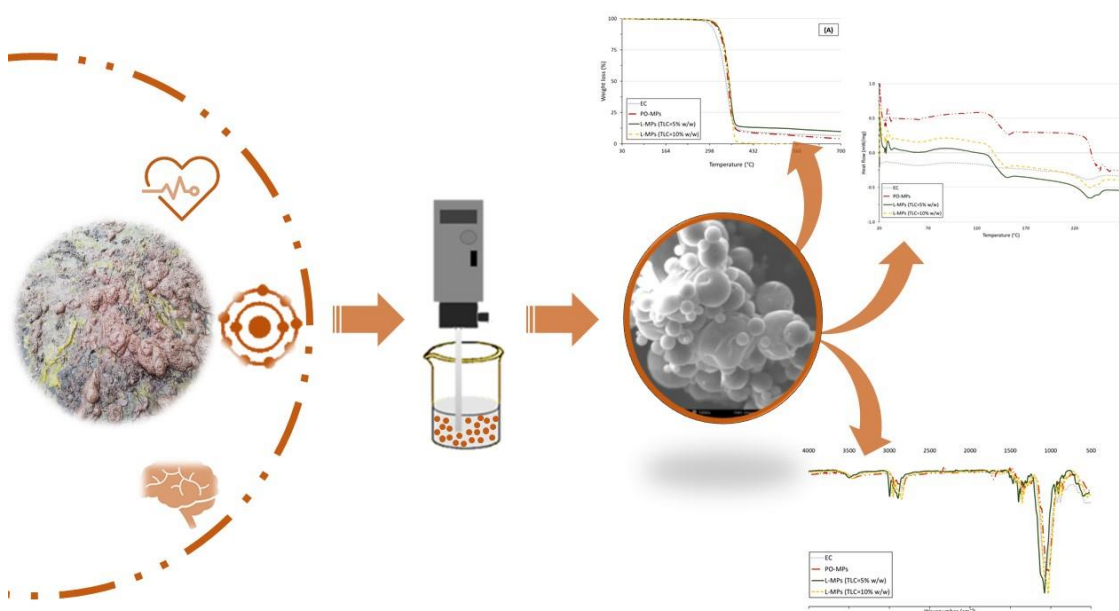
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Chapter 6. Encapsulation of an olive mill pomace extract by double emulsion solvent evaporation technique: evaluation of the effect of the loading content on physicochemical and morphological properties of microparticles ⁶

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Graphical Abstract:



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Abstract:

Olive mill pomace is an outstanding source of bioactive compounds as phenolics. The extraction and encapsulation techniques can be combined to reduce the environmental impact of these wastes due to their high content on phenolic compounds and simultaneously protect added-value compounds.

In this study, the water-in-oil-in-water double emulsion solvent evaporation technique was employed for the encapsulation into ethylcellulose carrier phenolics extracted from olive mill pomace by an optimized two-step solid-liquid extraction. The effect of the loading content (phenolic compounds based) on the main physicochemical characteristics of loaded microparticles was assessed.

The results confirmed the efficient embedment of bioactive compounds in ethylcellulose polymer carrier, especially the phenethyl alcohols hydroxytyrosol and tyrosol. The encapsulation of the olive mill pomace extract at two alternative loading contents resulted in high encapsulation yields (above 75%) and high encapsulation efficiencies (above 80%) with great content of phenolic compounds and antiradical power. Microparticles were thermogravimetrically stable in the range of 30 °C to 280 °C, exhibiting outstanding granular stability.

The results of this study bring new perspectives regarding the incorporation of extracts obtained from agricultural residues into microparticles that can be latter embedded in added-value food, pharmaceutical, cosmetic, and nutraceutical matrices.

Highlights

1. Olive mill pomace (OMP) is a valuable waste source of phenolic compounds.
2. OMP extract microparticles were obtained by double emulsion solvent evaporation.
3. Phenolic compounds loading on microparticles characteristics was evaluated.
4. Bioactives were efficiently embedded into ethylcellulose microparticles.
5. Loaded microparticles presented outstanding thermogravimetric stability.

Keywords: antioxidants; phenolics; olive mill pomace; extraction; encapsulation; hydration properties;

6.1 Introduction

The worldwide recognition of the health benefits associated with olive oil consumption has been foster olive oil massive and intensive production. Therefore, nowadays, olive oil is one of the most edible oils being consumed worldwide. It has been described that olive oil consumption has been rising steadily since 2005. Accordingly, the olive oil-producing industry has been noteworthy, along with the agro-food business (Balias et al., 2016; Dermeche et al., 2013; Nunes et al., 2016).

Nowadays, olive oil is essentially obtained through two alternative processes: (i) the three-phase process and (ii) the two-phase process. The (i) three-phase process has been replaced by (ii) the two-phase process as this process is associated with a reduction in energy and water consumption. At the end of (ii) two-phase process, two products are obtained: olive oil and a wet semi-solid pomace commonly known as olive mill pomace (OMP) (Albuquerque et al., 2004; Azbar et al., 2004; Dermeche et al., 2013; Obied et al., 2005; Qdais & Alshraideh, 2016; Roig et al., 2006; Ruiz et al., 2017; Sampedro et al., 2004, Nunes et al., 2016).

Olive mill pomace is considered one of the most significant by-products of olive oil production, and even though it is obtained from an eco-friendly olive oil extraction procedure, it is a phytotoxic by-product, essentially due to its high content of phenolic compounds (PCs) (Aliakbarian et al., 2011, 2012; Cioffi et al., 2010; Malapert et al., 2018; Paini et al., 2015), nevertheless, phenolics present in olive oil and olive mill wastes present relevant health benefits. It is described their neuroprotective, cancer chemopreventive, and cardioprotective activities (El-Abbassi et al., 2012; Tsagaraki et al., 2007). Therefore, OMP is an appealing source of phenolics.

In the last few decades, the potential application of recovered phenolics from OMP in food, pharmaceutical, nutraceutical, and even in cosmetic industries has been extensively investigated by the scientific community (Aliakbarian et al., 2011; 2017; Paini et al., 2015).

Nevertheless, the application of recovered phenolics from OMP is still limited as these compounds are sensitive to adverse environmental conditions as high temperatures, light, oxygen, and even to moisture, which may result in the degradation of these valuable bioactive compounds and the loss of their beneficial bioactivities (Aliakbarian et al., 2017; de Vos et al., 2010; Fang & Bhandari, 2012; Paini et al., 2015). Moreover, the direct application of phenolics in formulations that will suffer salivary digestion is limited due to their bitterness and astringency. In the specific case of PCs, they may promote the astringency – the precipitation of mucopolysaccharides and salivary glycoproteins in the mouth – decreasing consumers adherence when recovered phenolics are incorporated in food, pharmaceutical, or even in nutraceutical formulations (Aliakbarian et al., 2017; Kosaraju et al., 2008).

In this context, the valorization of phenolics present in OMP through combined extraction and encapsulation processes demonstrate a great potential since the combination of these techniques may increase phenolics bioavailability, extend the shelf-life of products incorporating these encapsulated bioactive compounds, and also increasing the consumers' adherence, as encapsulation may reduce the bitterness and astringency taste of PCs (Paulo & Santos, 2020a).

There are available many extraction and encapsulation techniques that can be combined in order to recover, protect, and deliver phenolic ingredients from OMP. Nevertheless, some extraction and encapsulation techniques apply non-mild conditions, which may cause the degradation of these compounds during processing, decreasing their bioavailability. Even though it is considered traditional and even obsolete extraction, the solvent extraction can be performed under mild conditions, and its know-how is widespread.

Among encapsulation techniques, the water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation technique prompts the efficient protection of the PCs. According to the principles of this encapsulation technique, the bioactive compound(s) should be dissolved in an internal aqueous phase (w_1).

Since most phenolics present in olives have more affinity for aqueous phase rather than oily phases (the partition coefficient of most of these compounds ranges between 6.0×10^{-4} and 1.5), these valuable bioactive compounds can be efficiently dissolved in a w_1 and entrapped in a polymer shell. Moreover, the coating material is generally insoluble in water and, therefore, compatible with most food, pharmaceutical, nutraceutical, and cosmetic formulations, generally from lipophilic nature. The compatibility of the coating material with most matrices contributes to the efficient protection of the PCs as the shell is not instantly degraded when microparticles contact the formulation. The incorporation of encapsulated phenolic ingredients by $w_1/o/w_2$ double emulsion solvent evaporation in fatty-foods grant a controlled release of these bioactive compounds in lipidic matrices, protecting fatty-foods from undesired changes in taste, odor, texture, flavor, among others (El-Abbassi et al., 2014; Paulo & Santos, 2018a, 2020a, 2020b)

Some studies have reported the extraction of phenolic compounds from OMP, followed by encapsulation. Nevertheless, the number of studies regarding the extraction and encapsulation of extracts obtained from OMP is still limited: not only regarding the number of studies as well as the encapsulation procedures applied – to the authors best knowledge, only the spray-drying and supercritical assisted atomization encapsulation techniques are described for the encapsulation of OMP extracts (Aliakbarian et al., 2017, 2018; Paini et al., 2015). This is the first time – to the authors best knowledge – that is reported the encapsulation of OMP extracts by $w_1/o/w_2$ double emulsion solvent evaporation technique – a remarkable technique for the protection of PCs extracted from OMP, considering not only their hydrophilic nature as well as their sensibility to environmental conditions.

As a part of a comprehensive study on the valorization of phenolics present in OMP through the combination of extraction and encapsulation procedures, this study aimed to extract phenolics from OMP samples through an optimized two-step solid-liquid extraction process and encapsulating the obtained extracts by $w_1/o/w_2$ double emulsion solvent evaporation technique.

Additionally, it was intended to study the influence of phenolics loading content (5% w/w or alternatively 10% w/w) on the main physicochemical characteristics of loaded microparticles: encapsulation efficiencies, recovery, actual loading contents, morphologies, particle size distributions, thermal stabilities, and microparticles rehydration properties.

6.2 Experimental procedures

6.2.1 Materials

6.2.1.1 Reagents and Standards

The standards, tyrosol (TYR) (Ref: 188255, $C_8H_{10}O_2$, CAS 501-94-0), hydroxytyrosol (HXT) (Ref: 91404, $C_8H_{10}O_3$, CAS 10597-60-1) and gallic acid (GAE) (Ref: 91215, $C_6H_6O_5$, CAS 149-91-7) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA).

Specific reagents used for the estimation of the total antioxidant activity and the total phenolic content such as the Folin-Ciocalteu reagent (Ref: 47641-500ML-F, 2M with respect to acid, density of 1.3 g/mL at 20 °C), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Ref: D9132, $C_{18}H_{12}N_5O_6$, CAS 1898-66-4), sodium carbonate anhydrous (Ref: 1613757, Na_2CO_3 , CAS 497-19-8) were obtained from Sigma Aldrich Chemical (St. Louis, MO, USA). Additionally, the polymers ethylcellulose (EC) (Ref: 433837-250G, viscosity of 46 cP, ethylation degree of 48%, CAS 9004-57-3) and polyvinyl alcohol (PVA) (Ref: P8136-250G, 87-90% hydrolyzed, average molecular weight of 30,000-70,000, CAS 9002-89-5) were also purchased from Sigma Aldrich Chemical (St. Louis, MO, USA).

Solvents such as hexane (HiPerSolv CHROMANORM® for HPLC, Ref: 83992.320, C_6H_{14} , CAS 110-54-3), ethanol (purity $\geq$ 99.8%, PESTINORM®, Ref: 85933-320, C_2H_6O , CAS 64-17-5), ethyl acetate (purity $\geq$ 99.8%, SupraSolv®, Ref: 1.00789.2500, $C_4H_8O_2$, CAS 141-78-6), acetonitrile (purity $\geq$ 99.9%, LiChrosolv®, Ref: 1.00029.2500, C_2H_3N , CAS 75-05-8) and methanol (purity $\geq$ 99.9%, LiChrosolv®, Ref: 1.03726.2002, CH_4O , CAS 67-56-1) and dichloromethane (Ref. ACRO433991000, CH_2Cl_2 , CAS 1665-00-5) were obtained from VWR International (Fontenay-sous-Bois, France). Additionally, hydrochloric acid solution at 37% v/v (EMSURE® ACS, Supelco®, Ref: 1.00317.1011, HCl, CAS 7647-01-0), acetic acid standardized solution 0.1 N (supplier Alfa-Aesar, Ref: 35567.K7, $C_2H_4O_2$, CAS 64-19-7) were also obtained from VWR International (Fontenay-sous-Bois, France).

The water used in this work was de-ionized and double-distilled using a Millipore™ water purification system (Massachusetts, USA) having 18.2 Ω electrical resistivity. All the reagents were either chromatographic or analytical grade and used as received.

6.2.1.2 Samples

The OMP samples were obtained from a continuous two-phase centrifugation system by a local olive oil mill in Portugal (Vilas Boas, Vila Flor, Bragança, 41359822,-7123743). Samples were collected in January 2019. The OMP samples were stored at -22 °C (moisture content of 71.0 ± 5.5 % w/w) before prior analysis and then submitted to a freeze-drying process (72 hours on benchtop freeze-dryer; SP Scientific, NY, USA). Then, samples were grounded on an electric mill (Qilive Q5321 Grinder) to an average particle size of 142.2 ± 9.6 μm . The mean particle size of OMP was determined, as described by Paulo & Santos (2020a).

6.2.2 Methods

6.2.2.1 Analytical methodologies and instrumental analyses

Throughout this study, it required the development of analytical methodologies to evaluate the total phenolic content, the total antioxidant activity, and to identify and quantify individual phenolic compounds in the extract and microparticles.

6.2.2.1.1 Determination of the total phenolic content

The total phenolic content (TPC) was evaluated as described by Barroso et al. (2016) based on the original method described by Singleton et al. (1999) considering a scale-down for microplates. The TPC values were obtained based on the Folin-Ciocalteu reagent reaction with reduced phenols, producing a stable blue product at the end of the colorimetric reaction.

To each well was added 25 μL of sample or standard solution. Afterward, 75 μL of UPW and 25 μL of the Folin-Ciocalteu reagent (diluted 1/1 v/v in UPW) were added. The reaction mixtures, in each well, were homogenized for 1 minute at 400 rpm on an orbital shaker (IKA® KS 130 basic, IKA Works GmbH & Co. KG, Staufen im Breisgau, Germany) and incubated for 5 minutes, protected from light, and at room temperature (20 ± 2 °C). Then, 100 μL of sodium carbonate solution (75 g/L) was added to each well. Again, the reaction mixtures were homogenized for 1 minute at 400 rpm on the orbital shaker and incubated for 2 hours, protected from light at room temperature (20 ± 2 °C).

Later, the absorbance was read using a microplate reader (Synergy HT, Biotek, USA) at 760 nm.

Each analysis was performed in triplicate. Results are presented as mean $\pm$ standard deviation and expressed as mg of gallic acid equivalents (mg_{GAE}) per g of dry weight of OMP ($\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$) or alternatively per gram of dried powder of microparticles ($\text{mg}_{\text{GAE}}/\text{g}_{\text{MP}}$) or even as g of gallic acid equivalents (g_{GAE}) per liter (L) of extract ($\text{g}_{\text{GAE}}/\text{L}$).

6.2.2.1.2 Determination of the total antioxidant activity

The total antioxidant activity (TAA) was estimated through the determination of the free radical-scavenging ability (RSA) of extracts using the 2,2-diphenyl-2-picrylhydrazyl radical (DPPH $^{\bullet}$) (El-Abbassi et al., 2012).

The RSA was evaluated as described by Prieto (2012), with slight modifications. Previously, a negative calibration curve (1.1 mg/L – 200.0 mg/L; number of points: 12) was obtained by diluting a 0.2 mM of DPPH $^{\bullet}$ solution in methanol. To each well was added 100 μL of methanol, followed by 100 μL of each sample (positive control). The content of each well was diluted 8 times. Afterward, to each well was added 100 μL of the DPPH $^{\bullet}$ methanolic solution.

Subsequently, the plates were covered with the lid to minimize evaporation and wrapped in aluminum foil to protect the DPPH $^{\bullet}$ radical from degradation by light and homogenized for 5 minutes on an orbital shaker. The reaction mixtures were incubated for 45 minutes (time observed to obtain stable Vis absorbance values). Afterward, the absorbance was read at 515 nm using a microplate reader.

The scavenging activity of extracts was extrapolated in the inhibitory percentage of DPPH $^{\bullet}$ (%) (Equation (IV.6.1)) as follow:

$$\% I = \frac{A_0 - A_1}{A_0} \times 100 \quad (\text{IV.6.1})$$

where A_0 is the absorbance of the control reaction (DPPH radical in methanol) and A_1 is the absorbance of the DPPH radical plus tested sample in methanol.

The computation of the %I against the logarithmical concentration permitted the half-inhibitory concentration (IC_{50}) calculation by linear regression analysis, as described by Ahsan et al. (2010).

6.2.2.1.3 Analysis of individual phenolics by reserved-phase high-performance liquid chromatography (RP-HPLC)

The identification and quantification of specific phenolics (tyrosol, hydroxytyrosol) were performed by RP-HPLC analyses using a Merck Hitachi Elite LaChrom (Tokyo, Japan) high-performance liquid chromatograph equipped with a Hitachi L-7100 pump and L-7250 autosampler and coupled to a L-7450A diode array detector. Samples and standards were injected in a Purospher® STAR RP-18 end-capped LiChroCART® column (250 mm x 4.0 mm, 5.0 μ m) (Merck KGaA), attached to a guard column (4.0 mm x 4.0 mm, 5.0 μ m) of the same type.

The elution method applied was adapted from Paulo & Santos (2020a) and Tasioula-Margari & Tsabolatidou (2015) and conducted at a flow rate of 1 mL/minute, considering the following two-buffer gradient system: UPW/acetic acid (0.1 N) (97.5/2.5 v/v) (A); acetonitrile/methanol (50/50 v/v) (B). An elution gradient consisting of (i) 95% A and 5% B as initial conditions kept during 5 minutes, (ii) 70% A and 30% B for 30 minutes, (iii) being re-established the initial conditions during 5 minutes was applied. The absorbance of the eluate was recorded at 1 s intervals, at a wavelength of 280 nm.

Each analysis was performed in triplicate. Results are presented as mean $\pm$ standard deviation and expressed as mg of HXT or TYR per kilogram of dry weight of OMP (mg/kg_{OMP}) or alternatively per gram of dried powder of microparticles (mg/g_{MP}) or g per liter of extract (g/L extract).

6.2.2.2 Extraction of phenolics from olive mill pomace

Prior to phenolics extraction, the OMP sample (1 g) was submitted to a pre-treatment that included (i) an acidic hydrolysis handling and (ii) a fat removal step.

The (i) acidic hydrolysis was performed admixing the sample with 25 mL of an aqueous solution of HCl (0.1 M, pH 2) in 50 mL Erlenmeyer flask, continuously shaken in an orbital shaker for 12 hours. The acidic hydrolysis prompted the break of both ester and glycosylic bonds. Additionally, the low pH favored the precipitation of proteins, increasing the free phenolic compounds (FPCs) content in the extraction solvent (De Marco et al., 2007).

The (ii) fat removal step was performed as described by Lafka et al. (2011). The fats were removed, adding 5 mL/g of hexane to the hydrolyzed and grounded OMP sample in 50 mL Erlenmeyer flask. The flask was continuously shaken in an orbital shaker for 1 hour at room temperature (20 ± 2 °C). Afterward, the sample was transferred to a 50 mL polypropylene (PP) centrifuge tube (Ref: 525-0402, VWR International, Fontenay-sous-Bois, France) and centrifuged at 2 670 g for 15 minutes in a Rotofix 32A centrifuge (Hettich, Germany). The supernatant – containing hexane and fats – was admixed with 15 mL of UPW in a 50 mL PP centrifuge tube, vortexed (IKA VORTEX GENIUS 3, Staufen, Germany) for 5 minutes, and ultrasonicated (P Selecta, Barcelona, Spain) for 15 minutes. Then, the sample was submitted to centrifugation (2 670 g, 15 minutes). The aqueous phase was removed and filtered using sterile and disposable syringes without needle (Ref: 613-1591, VWR International, Fontenay-sous-Bois, France) and 0.2 μ m nylon syringe filters (Ref: 514-0070, VWR International, Fontenay-sous-Bois, France). The obtained sample was termed as sample containing extracted phenolic compounds from the fat removal procedure (SEPC-FR) was admixed with the final filtrate after the extraction.

The PCs were recovered through a two-step solid-liquid extraction procedure. The primary extraction was performed, adding 10 mL of ethanol to the pellet recovered from the fat removal procedure. The mixture was continuously shaken for 3.2 hours in a 50 mL Erlenmeyer flask, being afterward centrifuged (2 670 g, 15 minutes).

The supernatant termed as sample containing extracted phenolic compounds after the primary extraction (SEPC-P1E) was filtered under vacuum using a Whatman™ nylon membrane filter (Ref. 7404-004, VWR International, Fontenay-sous-Bois, France) and stored in an amber flask under dark conditions at room temperature (20 ± 2 °C) before being admixed with the final filtrate.

The pellet was reconstituted in 3 mL of ethanol and transferred to a 50 mL Erlenmeyer flask. The mixture was continuously shaken for 1.3 hours. Afterward, the supernatant was separated from the pellet through centrifugation (2 670 g, 15 minutes) and vacuum filtrated using Whatman™ nylon membrane filter. The filtrate was designated as sample containing extracted phenolic compounds after the secondary extraction (SEPC-P2E). The filtrates (SEPC-FR, SEPC-P1E, and SEPC-P2E) were admixed, constituting the final filtrate.

After that, the final filtrate was submitted to a liquid-liquid extraction using ethyl acetate as the extraction solvent. The filtrate was admixed ethyl acetate in a 50 mL PP centrifuge tube (extraction solvent volume corresponding to three times the final filtrate volume), vigorously shaken, and vortexed for 5 minutes. The mixture was then ultrasonicated for 15 minutes and centrifuged (2 670 g, 15 minutes). Afterward, the supernatant was submitted to solvent evaporation using a rotary evaporator (BUCHI R-210, Buchi Laboratoriums Technik AG, Flawil, Switzerland) at 50 °C. Solvent traces were removed by a gentle nitrogen stream. The final extract was stored at -22 °C before prior analyses.

The extraction experiments were performed in triplicate.

6.2.2.3 Encapsulation of olive mill pomace extracts

The microencapsulation of OMP extract was performed by water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation, as described by Paulo & Santos (2018a, 2018b, 2019, 2020a, 2020c) with slight modifications. Two alternative theoretical loadings (TLCs) of phenolics were considered in the present study: 5% w/w in the case of F-1 formulation and 10% w/w in the case of F-2 formulation.

For this purpose, the extract was reconstituted in 3 mL of UPW. The extract solution was diluted according to the TLC. The obtained diluted extract solutions constituted the internal aqueous phases (w_1). Similar encapsulation procedures were considered during both experiments (F-1 and F-2 formulations).

In this context, for each formulation, a mass of 100 mg of ethylcellulose was dissolved in 10 mL of dichloromethane to form the polymer solution used as the oily phase (o). The obtained polymer solution was sonicated for 15 minutes in an ultrasonic bath. Then, a volume of 1 mL of w_1 was added to the polymer solution. To obtain a stable w_1/o stable primary emulsion, the mixture was vortexed for 5 minutes. Afterward, the w_1/o solution was poured into 100 g of PVA solution (concentration of 1% w/w) and emulsified using a high-performance homogenizer at 5 000 rpm for 5 minutes.

The final mixture ($w_1/o/w_2$ double emulsion) was continuously mixed in a stirring plate at 700 rpm for 3 hours in the fume hood at room temperature (20 ± 2 °C) to promote the solvent evaporation and, therefore, the hardening of microparticles.

Microparticles were then recovered by filtration using a 0.2 μm quantitative paper filter and washed with 500 mL of distilled water. The recovered microparticles were frozen for 24 hours at -22 °C and freeze-dried for 72 hours.

Unloaded microparticles, also termed as polymer-only microparticles (PO-MPs), were formulated in a similar formulation and operative conditions as reported for OMP extracts' encapsulation to evaluate the effect of encapsulation of the main physicochemical characteristics of microparticles. Further considerations are presented in Table IV.6.1. All the experiments were performed in triplicate.

Table IV.6.1 - Composition of the formulations coded (F-1 and F-2)

<i>Formulation</i> ^a	TLC ^b (% w/w)	OMPE Concentration ^b (g _{GAE} /L)	Polymer Concentration (g/L)	PVA Concentration (% w/w)
F-1	5	5.3	10	1
F-2	10	11.1	10	1

^a Each formulation was performed in triplicate

^b Theoretical loading content on phenolics

^c Reported Olive Mill Pomace Extract (OMPE) concentrations based on the concentration of phenolics

GAE – Gallic Acid Equivalents; OMPE – Olive Mill Pomace Extract; PVA - Polyvinyl Alcohol; TLC – Theoretical Loading Content;

6.2.2.4 Characterization of olive mill pomace extract-loaded ethylcellulose microparticles

6.2.2.4.1 Determination of encapsulation efficiencies, loading contents, and recovery of powders

The encapsulation efficiency (EE), the actual loading content (ALC) were evaluated in relation to (i) the amount of encapsulated bioactive compounds exhibiting antioxidant activity, (ii) the amount of phenolic compounds encapsulated, and to (iii) the amount of specific phenolic compounds encapsulated namely hydroxytyrosol and tyrosol. The calculation of the EEs and ALCs through Equations IV.6.2 to IV.6.9 are described in Table IV.6.2, similar to described in our previous studies (Paulo & Santos, 2018a, 2019, 2020a). The recovery of powdered products was determined by weighing the total amount of particles recovered after the freeze-drying and comparing it to the total amount of solids use for encapsulation.

Table IV.6.2 - Computed equations (IV.6.2 – IV-6.9) for the determination of the encapsulation efficiency (EE) and the actual loading content (ALC) of antioxidants (AO), phenolic compounds (PC), hydroxytyrosol (HXT), and tyrosol (TYR)

BAC(s)	EE (% w/w)	ALC (% w/w)	Description
Antioxidants	$EE_{AO} (\%) = \frac{W_{AO}}{W_{AOI}} = \frac{W_{AOI} - W_{AOO}}{W_{AOI}} \times 100$ (IV.6.2)	$ALC_{AO} (\%) = \frac{W_{AO}}{W_M} \times 100$ (IV.6.3)	EE_{AO} – Encapsulation Efficiency of AO (% w/w) ALC_{AO} – Actual Loading Content of AO (% w/w) W_{AO} – Weight of AO in the microparticles (mg) W_{AOI} – Weight of AO initially added for the microencapsulation (mg) W_{AOO} – Weight of AO present in the w_2 after 3 hours of microparticles hardening (mg) W_M – Weight of microparticles (mg)
Phenolic compounds	$EE_{PC} (\%) = \frac{W_{PC}}{W_{PCI}} = \frac{W_{PCI} - W_{PCO}}{W_{PCI}} \times 100$ (IV.6.4)	$ALC_{PC} (\%) = \frac{W_{PC}}{W_M} \times 100$ (IV.6.5)	EE_{PC} – Encapsulation Efficiency of PC (% w/w) ALC_{PC} – Actual Loading Content of PC (% w/w) W_{PC} – Weight of PC in the microparticles (mg) W_{PCI} – Weight of PC initially added for the microencapsulation (mg) W_{PCO} – Weight of PC present in the w_2 after 3 hours of microparticles hardening (mg) W_M – Weight of microparticles (mg)

Table IV.6.2 - Computed equations (IV.6.2 –IV-6. 9) for the determination of the encapsulation efficiency (EE) and the actual loading content (ALC) of antioxidants (AO), phenolic compounds (PC), hydroxytyrosol (HXT), and tyrosol (TYR) (cont.)

BAC(s)	EE (% w/w)	ALC (% w/w)	Description
Hydroxytyrosol	$EE_H (\%) = \frac{W_H}{W_{HI}} = \frac{W_{HI} - W_{HO}}{W_{HI}} \times 100$ (IV.6.6)	$ALC_H (\%) = \frac{W_H}{W_M} \times 100$ (IV.6.7)	EE_H – Encapsulation Efficiency of HXT (% w/w) ALC_H – Actual Loading Content of HXT (% w/w) W_H – Weight of HXT in the microparticles (mg) W_{HI} – Weight of HXT initially added for the microencapsulation (mg) W_{HO} – Weight of HXT present in the w_2 after 3 hours of microparticles hardening (mg) W_M – Weight of microparticles (mg)
Tyrosol	$EE_T (\%) = \frac{W_T}{W_{TI}} = \frac{W_{TI} - W_{TO}}{W_{TI}} \times 100$ (IV.6.8)	$ALC_T (\%) = \frac{W_T}{W_M} \times 100$ (IV.6.9)	EE_T – Encapsulation Efficiency of TYR (% w/w) ALC_T – Actual Loading Content of TYR (% w/w) W_T – Weight of TYR in the microparticles (mg) W_{TI} – Weight of TYR initially added for the microencapsulation (mg) W_{TO} – Weight of TYR present in the w_2 after 3 hours of microparticles hardening (mg) W_M – Weight of microparticles (mg)

ALC – Actual Loading Content; AO – Antioxidants; BAC – Bioactive Compound; EE – Encapsulation Efficiency; HXT – Hydroxytyrosol; PC – Phenolic Compounds; TYR – Tyrosol;

6.2.2.4.2 Fourier transform infrared spectroscopy (FTIR) studies

The qualitative evaluation of the inclusion of phenolics in ethylcellulose microparticles was performed as described in our previous studies (Paulo & Santos, 2018a, 2020a), recording the infrared spectra of F-1 and F-2 formulations, polymer-only microparticles, and ethylcellulose using a VERTEX 70 FTIR spectrometer (BRUKER) in transmittance mode with a high sensitivity DLaTGS detector at room temperature. Samples were measured in attenuated total reflectance (ATR) mode, with an A225/Q PLATINUM ATR Diamond crystal with a single reflection accessory. The spectra were recorded from 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1} .

6.2.2.4.3 Differential scanning calorimetry (DSC) analysis

The assessment of the thermal behavior of loaded microparticles (F-1 and F-2 formulations), polymer-only microparticles, and ethylcellulose was performed as described by Piletti et al. (2017) and Paulo & Santos (2018a) with slight modifications. The analyses were performed using a NETZSCH DSC 214 Polyma calorimeter (NETZSCH DSC 214 Polyma, Selb, Germany) with a temperature range of 30 °C to 270 °C at a heating rate of 10 $\text{K}\cdot\text{min}^{-1}$, under a nitrogen atmosphere (flow rate of 40 $\text{mL}\cdot\text{min}^{-1}$).

6.2.2.4.4 Thermogravimetric analyses (TGA)

The evaluation of the thermal stability of loaded microparticles, polymer-only microparticles, and ethylcellulose was performed through thermogravimetric analyses (TGA), as described by Piletti et al. (2017) with slight modifications. The analyses were performed using a Netzsch STA449 F3 Jupiter thermogravimetric analyzer (Netzsch STA449 F3 Jupiter, Selb, Germany) with a temperature range of 30 °C to 810 °C at a heating rate of 10 $\text{K}\cdot\text{min}^{-1}$ under inert atmosphere (N_2 , a flow rate of 30 $\text{mL}\cdot\text{min}^{-1}$).

6.2.2.4.5 Evaluation of microparticles morphology by scanning light microscopy (SEM)

The characterization of the external morphology and the qualitative assessment of mean particle size and polydispersity of F-1 and F-2 formulations were performed by scanning light microscopy (SEM) using a PHENOM XL scanning light microscope (Eindhoven, The Netherlands) at an accelerating voltage of 10 kV as described by Negrão-Murakami et al. (2017) and Paulo & Santos (2018a). Freeze-dried microparticles were placed on an aluminum stub with a carbon double-sided adhesive tape. Samples were sputter-coated with gold for 20 seconds using a vacuum-sputtering coater (Leica, EM SCD 500, Wetzlar, Germany).

6.2.2.4.6 Evaluation of particle size distribution

The evaluation of the particle size distribution and the polydispersity of powders of F-1 and F-2 formulations and unloaded microparticles (polymer-only microparticles) was performed by laser granulometry using a Coulter Counter-LS 230 Particle Size Analyzer (Miami, FL, USA) equipment. Each experiment was performed in triplicate. Average values are presented in data analysis.

The polydispersity of particle size distributions was analyzed according to Paulo and Santos (2018a, 2018b, 2019, 2020a), considering the polydispersity index (PDI) as presented in Equation (IV.6.10):

$$PDI = \frac{D_{v,90} - D_{v,10}}{D_{v,50}} \quad (IV.6.10)$$

where the $D_{v,90}$, $D_{v,10}$ and $D_{v,50}$ represent the maximum particle diameter below which 90% of the sample volume exists, the maximum particle diameter below which 10% of the sample volume exists, and the maximum particle diameter below which 50% of the sample volume exists, respectively.

6.2.2.4.7 Assessment of water absorption index (WAI), water solubility index (WSI), and swelling capacity (SC)

The water absorption index (WAI) reflects microparticles' ability to absorb water, being a measure of the amount of fully or partially degradation of the polymer shell. The water solubility index (WSI) is an indicator of the polymeric shell's total degradation, suggesting the high content of low molecular weight molecules. These two indexes are related, and they are useful in the estimation of a material behavior if further processed (e.g., as a binder, as a stabilizer, or even as a source of bioactive compounds in processed foods) (Gutkoski & El-Dash, 1999).

The WAI and WSI were determined according to the method described by Ahmed et al. (2010) with slight modifications. About 2.5 g of powder samples (F-1 and F-2 formulations and polymer-only microparticles) were admixed with 30 mL of distilled water in a 50 mL centrifuge tube and vigorously vortexed. Afterward, the mixture was incubated in a water bath at 30 °C for 30 minutes being then centrifuged (2 670 g) for 15 minutes. The supernatant was collected and disposed in a pre-weighted Petri dish, and the residue was weighed after oven-drying at 105 °C for 12 hours. The WAI and the WSI were calculated according to Equations (IV.6.11) and (IV.6.12).

$$WAI = \frac{PW}{DW_{part}} \quad (IV.6.11)$$

$$WSI = \frac{DW_{sup}}{DW_{part}} \times 100 \quad (IV.6.12)$$

where PW is the weight of the pellet after centrifugation, DW_{part} is the initial dry weight of microparticles (on a dry basis) and DW_{sup} is the dry weight of the supernatant. The WAI results are presented in grams per gram of dried powder (g/g_{DP}).

The swelling capacity (SC) was calculated according to Lai & Cheng (2004) using Equation (IV.6.13):

$$SC = \frac{DW_{sup}}{DW_{part} \times (100 - WSI)} \quad (IV.6.13)$$

Similarly to WAI, SC results are presented in grams per gram of dried powder (g/g_{DP}). All the experiments were performed in triplicate, and results are presented as mean $\pm$ standard deviation.

6.2.2.4.8 Evaluation of the wettability

The wettability of the powders (F-1 formulation, F-2 formulation, and polymer-only microparticles) was evaluated as described by Barros Fernandes et al. (2014). About 1 g of each powder was sprinkled over the surface of distilled water without agitation at 20 °C. The time required for the microparticles to sink, disappear from the water's surface, become sediment, or be submerged was recorded. The values regarding the wettability of the powders were expressed as a function of time (seconds). All the experiments were performed in triplicate, and results are presented as mean $\pm$ standard deviation.

6.2.3 Statistic Analysis

All the experiments were performed in triplicate. Results are present as mean $\pm$ standard deviation. The analysis of variance (ANOVA) was used to determine significant differences ($p < 0.05$).

6.3 Results and Discussion

6.3.1 Instrumental analyses

Analytical methodologies were developed to evaluate the amount of antioxidants (AOs), phenolic compounds (PCs), and selected phenethyl alcohols – hydroxytyrosol (HXT) and tyrosol (TYR) - on the obtained extract (n=3) and encapsulated in microparticles (n=6).

The amount of AOs was assessed by a spectrophotometric method using the 2,2-diphenyl-2-picrylhydrazyl radical (DPPH[•]). The amount of PCs was determined considering the Folin-Ciocalteu method (spectrophotometric method), whereas the phenolic profiles were appraised by HPLC.

The main results regarding the instrumental analyses are presented in Table IV.6.S1 of the Annex II. Calibration curves were drawn based on three independent calibration curves. The limit of detection (LOD) and the limit of quantification (LOQ) were determined to assess the sensibility of the methods. The obtained values for all LOD and LOQ were satisfactory low, demonstrating the adequacy of the obtained regression equations for the quantification of the selected bioactives. High correlation coefficients ($R \geq 0.995$) and low relative standard error of the slopes ($s_a/a < 5\%$) were obtained for all the four calibration curves, indicating a good linearity response of the methods proposed.

6.3.2 Olive mill pomace extract characterization

Olive mill pomace (OMP) extract (n=3) obtained by a sequential two-step solid-liquid extraction followed by a liquid-liquid extraction was characterized in terms of TAA, TPC, and the amount of HXT and TYR. This extract (n=3) resulted in having a TPC of $50.5 \pm 1.5 \text{ mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$, a TAA of $79.1 \pm 7.9\%$, the total content of HXT of $2\,870.2 \text{ mg}/\text{kg}_{\text{OMP}}$, and a total content of TYR of $1\,622.1 \pm 2.2 \text{ mg}/\text{kg}_{\text{OMP}}$.

The phenolics, HXT, and TYR, were identified by comparing the UV-Vis spectra of the respective analytical standards by HPLC.

The quantitative determination of HXT and TYR in OMP extract was achieved with the retention time of 7.7 minutes and 9.3 minutes, respectively. The major phenolic antioxidant identified was HXT, followed by TYR.

The obtained results regarding the content of HXT and TYR in the extract were similar to the ones obtained by Japón-Luján & Luque de Castro (2007) during the study of the potential application of a static–dynamic superheated liquid extraction of HXT, TYR, and other phenolics from OMP (reported amount of HXT: 2872 mg_{GAE}/g_{OMP}; reported amount of TYR: 1565 mg_{GAE}/g_{OMP})

To the authors' best knowledge, there was a lack of published data suitable for the direct comparison regarding the obtained results regarding the TPC and TAA.

6.3.3 Characterization of olive mill pomace extract-loaded ethylcellulose microparticles

Many studies have been described the health benefits associated with the consumption of olive oil. Nevertheless, during olive oil extraction, the physicochemical characteristics of water-soluble phenolics shepherd to their differential partitioning between oil and water. Thereby, the majority (about 98%) of water-soluble phenolics – that present outstanding radical scavenging activities – are shifted from olive pulp to wastes. Remarkably, phenolics-rich extracts can be obtained and embed in microparticles for further incorporation in oxidative-sensitive food matrices (Dermeche et al., 2013; El-Abbassi et al., 2014; Paulo & Santos, 2020b).

In this context, a phenolics-rich extract was obtained from OMP by an optimized two-step solid-liquid extraction followed by liquid-liquid extraction. Afterward, the extract was encapsulated into ethylcellulose microparticles by w₁/o/w₂ double emulsion solvent evaporation technique. Two alternative TLC in phenolic compounds (5% w/w and 10% w/w) were appraised to study the effect of loading content on physicochemical characteristics of loaded microparticles.

Unloaded microparticles (polymer-only microparticles – PO MPs) were formulated and characterized to evaluate the effect of loading on encapsulation properties of microparticles (e.g., the FTIR-ATR spectra comparison between loaded and unloaded microparticles allow to infer about the efficient incorporation of bioactive(s) in the polymeric matrix systems).

In this context, experiments were conducted considering two alternative TLC on phenolic compounds, namely 5% w/w (F-1 formulation) and 10% w/w (F-2 formulation). The obtained extract from the extraction procedure was diluted to obtain two alternative internal aqueous phases (w_1) with the desired concentrations of PCs (Table IV.6.1). The TAA, the TPC, and the amount of each phenethyl alcohol considered in the present study (HXT and TYR) are presented in Table IV.6.3.

Table IV.6.3 - Antioxidant activity, phenolic, hydroxytyrosol (HXT) and tyrosol (TYR) content of extract and the solutions used for encapsulation (F-1 and F-2 formulations)

	FORMULATION		
	EXT ^{a,b}	F-1 ^{a,c}	F-2 ^{a,d}
<i>Polyphenols content</i>			
mg_{GAE}/g_{OMP}	50.5 ± 1.5	15.8 ± 0.4	33.4 ± 0.2
g_{GAE}/L extract	16.8 ± 0.5	5.3 ± 0.7	11.1 ± 0.6
<i>Antioxidant Activity</i>			
IC₅₀ mg/g_{OMP}	223.9 ± 1.2	70.1 ± 0.4	148.1 ± 0.2
g/L extract	74.6 ± 0.4	23.3 ± 0.1	49.3 ± 0.1
<i>HXT content</i>			
mg/g_{OMP}	2.9 ± 0.2	0.9 ± 0.1	1.9 ± 0.1
g/L extract	1.0 ± 0.1	0.3 ± 0.1	0.6 ± 0.0

Table IV.6.3 - Antioxidant activity, phenolic, hydroxytyrosol (HXT) and tyrosol (TYR) content of extract and the solutions used for encapsulation (F-1 and F-2 formulations) (cont.)

	FORMULATION		
	EXT ^{a,b}	F-1 ^{a,c}	F-2 ^{a,d}
<i>TYR content</i>			
mg/g_{OMP}	1.6 ± 0.4	0.5 ± 0.0	1.1 ± 0.1
g/L extract	5.3 ± 0.1	1.7 ± 0.2	3.5 ± 0.0

^a Results are presented as mean ± standard deviation (n=3)

^b Data relative to the obtained extract after extraction

^c Data relative to the F-1 formulation (theoretical loading content in phenolic compounds of 5% w/w)

^d Data relative to the F-2 formulation (theoretical loading content in phenolic compounds of 10% w/w)

EXT – Extract; HXT – Hydroxytyrosol; IC₅₀ - Half-inhibitory Concentration; OMP – Olive Mill Pomace; TYR – Tyrosol;

6.3.3.1 Actual loading contents and encapsulation efficiencies of bioactive compounds entrapped in ethylcellulose microparticles, and recovery of powders products

The actual loading content (% w/w) by referenced to the TLC (% w/w) in PCs of the two formulations considered in the present study are presented in Figure IV.6.1 (Figure IV.6.1 – A: actual loading content of PCs and AOs; Figure IV.6.1-B: actual loading content of HXT and TYR). Bars with the same pattern but in yellow represent the TLC. For all bioactive(s), it was not observed differences ($p > 0.05$) between the ALC observed and the TLC considered.

The F-1 formulation was processed considering a TLC in PCs of 5% w/w. The ALC of PCs was $4.8 \pm 1.2\%$. However, the higher weight of bioactives in F-1 microparticles was devoted to antioxidants – $17.5 \pm 2.9\%$ of the weight of microparticles corresponded to antioxidants' weight. Only $0.3 \pm 0.1\%$ of the weight of microparticles (F-1 formulation; n=3) corresponded to the weight of HXT. The weight of TYR was 5 times higher than the weight of HXT in microparticles (TYR ALC of $1.5 \pm 0.3\%$).

The F-2 formulation (n=3) was obtained considering a TLC in PCs of 10% w/w.

The ALC of these valuable bioactives was $9.4 \pm 0.8\%$ - around 9% of the weight of microparticles corresponded to PCs.

Most of the weight of bioactive compounds in microparticles was devoted to AOs: $27.6 \pm 2.1\%$ of microparticles' weight corresponded to the weight of AOs (AOs TLC of $33 \pm 2\%$). Similarly to obtained results regarding the ALC of HXT and TYR considering the F-1 formulation, in the experiments regarding the F-2 formulation, it was observed that the ALC of TYR (ALC of $3.0 \pm 0.1\%$; TLC of $3.4 \pm 0.2\%$) was about 5 times fold higher than ALC of HXT (ALC of $0.5 \pm 0.2\%$; TLC of $0.6 \pm 0.2\%$).

These results are in agreement with the ones obtained previously in our study regarding the impact of the loading content on the main physicochemical characteristics of tyrosol-loaded ethylcellulose microparticles: the ALC observed of F-1 and F-2 formulations were statistically different ($p > 0.05$). The ALC was dependent on the TLC chosen.

The authors Yekdane et al. (2019) performed the encapsulation of pomegranate seed oil by the spray-drying technique verifying that the loading capacity decreased with the increase of pomegranate seed oil concentration. Contrary, in the present study, it was observed that increasing the extract concentration (from F-1 and F-2 formulation) led to an increase in the loading capacity of microparticles. Probably, there is a maximum extract concentration, for which is observed a linear relationship between the increase of the actual loading with the increase of the theoretical loading. Nevertheless, these comparisons should be considered carefully as not only the encapsulating approach was different as well as the encapsulating material was distinctive.

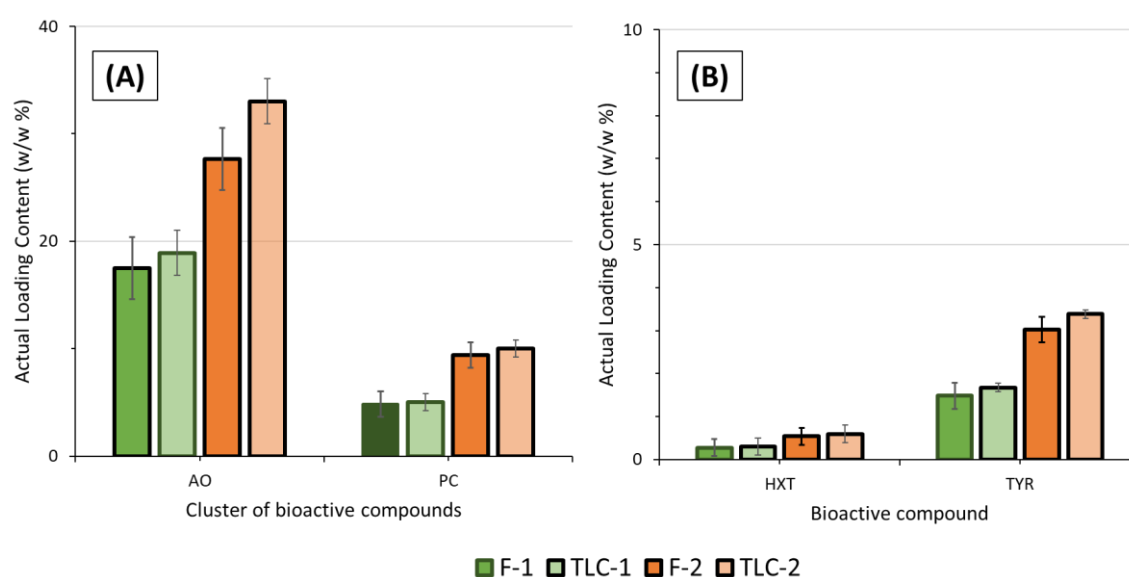


Figure IV.6.1 - Actual loading content of phenolics-rich extract loaded ethylcellulose microparticles ((A) – actual loading content on antioxidants and phenolic compounds; (B) – actual loading content on hydroxytyrosol and tyrosol). Data are presented as mean $\pm$ standard deviation of three independent assays. The vertical bars, in black, correspond to the error bars based on the standard deviation of three independent assays. Bars with toned colors indicate the theoretical loading content considered (F-1 – formulation considering a theoretical loading content in phenolic compounds of 5% w/w; F-2 – formulation considering a theoretical loading content in phenolic compounds of 10% w/w; HXT – Hydroxytyrosol; PC – Phenolic Compounds; TLC-1 – Theoretical Loading Content of 5% w/w in phenolic compounds; TLC-2 – Theoretical Loading Content of 10% w/w in phenolic compounds; TYR – Tyrosol)

The encapsulation efficiency and the recovery by referenced to the TLC of the two powders examined in the present study are presented in Figure IV.6.2. It was observed differences ($p < 0.05$) between the EE of AOs and PCs from F-1 and F-2 formulations. Alternatively, differences in the EE of encapsulated phenethyl alcohols considering two alternative TLC were not observed ($p > 0.05$).

The EE varied from $83.7 \pm 2.0\%$ (AOs; F-2 formulation) and $96.0 \pm 0.3\%$ (PCs; F-1 formulation). The EE of AOs was 92.6 ± 2.1 and 83.7 ± 2.0 in the case of F-1 and F-2 formulations, respectively.

The increase by 111% of the loading content on antioxidants (from IC₅₀ of 70.1 ± 0.4 mg/g_{OMP} to an IC₅₀ of 148.1 ± 0.2 mg/g_{OMP}) led to a decrease around 10% on the EE of AOs. The loss of antioxidants during the formulation of microparticles was 5.2 ± 0.1 mg/g_{MP}, in the case of F-1 formulation and 24.1 ± 0.3 mg/g_{MP}, in the case of F-2 formulation. The total amount of AOs entrapped in microparticles was 21.6 ± 0.3 mg/g_{MP} in the case of F-1 formulation, and 49.3 ± 0.6 mg/g_{MP} in the case of F-2 formulation.

The majority of AOs were efficiently entrapped into ethylcellulose microparticles. Increasing the loading content led to a decrease in encapsulation efficiency. The load of microparticles with higher amounts of antioxidants led to a higher flux of water-soluble antioxidants from the internal aqueous phase to the external aqueous phase during microparticles formation (semi-solid state). During microparticles production, preferential paths are usually formed within semisolid microparticles – responsible for the appearance of channels in hardened microparticles - may prompt the release of water-soluble bioactives as antioxidants from the dispersed internal aqueous phase and the external aqueous phase during microparticles' formulation (Gabor et al., 1999; Krishnamachari et al., 2007; Paulo & Santos, 2020a).

A similar trend was also observed by Freytag et al. (2000) when the authors were encapsulating an oligonucleotide into poly(lactide) microparticles. Similar results were obtained in our previous studies regarding the individual encapsulation of specific antioxidants – HXT and TYR: in the case of both antioxidants, the increase of the loading content from 5% w/w to 10% w/w led to a decrease on the encapsulation efficiency. Similar conclusions were drawn by Troya et al. (2018).

Even though the authors applied an alternative encapsulation technique and used different encapsulating and core materials, they concluded the increase of the core to the encapsulating material ratio (i.e., loading content) led to a decrease in the encapsulation efficiency.

The EE of PCs was 96.0 ± 0.3 when the F-1 formulation was considered and 94.1 ± 3.1, in the case of F-2 formulation. Differences were found between the EE of PCs from the two loadings considered (p<0.05). Similarly to the obtained results regarding the EE of AOs, it was verified a decrease in EE with the increase in the loading content.

The amount of PCs was $5.1 \pm 0.2 \text{ mg}_{\text{GAE}}/\text{g}_{\text{MP}}$ in the case of F-1 formulation being associated with a PCs loss of $4.0 \pm 0.3\%$. The formulation F-2 accounted for $10.4 \text{ mg}_{\text{GAE}}/\text{g}_{\text{MP}}$ being verified a loss of PCs of $5.9 \pm 0.2\%$ during encapsulation.

Phenolic compounds are a broad group heterogeneous group of plants' secondary metabolites presenting outstanding radical scavenging activities (San Miguel-Chávez, 2017). In this context, as similar trends regarding the encapsulation efficiency of AOs and PCs, it can be stated that many of the encapsulated antioxidants were phenolic compounds.

In the present study, the combination of optimized extraction and encapsulation procedures granted the improvement of the EE of PCs compared with the results obtained by Paini et al. (2015). These authors performed a study on the effect of operative parameters on the encapsulation of an OMP extract rich in PCs by spray-drying, obtaining the highest EE ($77 \pm 3.5\%$ on phenolic compounds) considering an inlet temperature of 130°C , a polymer concentration of 100 g/L and a feed flow of 5 mL/min . Potentially, the high temperature on the spray-drying chamber promoted the degradation of some PCs as these compounds are prone to light, temperature, pH, and oxygen degradation. Both extraction and encapsulation of PCs-rich extracts should be performed under mild conditions to avoid the undesired loss of these valuable bioactive compounds.

In the case of simple phenethyl alcohols – HXT and TYR – it was not verified differences ($p>0.05$) between the two loading contents.

The EE of HXT was 92.7 ± 2.3 when the F-1 formulation was considered and 90.8 ± 5.9 , in the case of F-2 formulation. The amount of HXT was $0.3 \pm 0.1 \text{ mg}/\text{g}_{\text{MP}}$ in the case of F-1 formulation being verified a loss on HXT of $7.3 \pm 0.2\%$. In the case of F-2 formulation, $0.6 \pm 0.0 \text{ mg}/\text{g}_{\text{MP}}$ of HXT was incorporated in ethylcellulose microparticles (EE of $90.8 \pm 5.9\%$) associated with a loss of $9.2 \pm 5.8\%$ of this bioactive compound. The EE of HXT was improved compared with our previous results reporting the encapsulation of HXT considering two alternative loading contents ($5\% \text{ w/w}$ and $10\% \text{ w/w}$ on hydroxytyrosol) (Paulo & Santos, 2018a). According to our previous results, the encapsulation of only HXT resulted in EE ranging from 79.1% and 88.2% .

The water content in the internal aqueous phase (w_1) was reduced, comparing the formulation of only HXT microparticles (previous study) and the formulation of HXT containing extract microparticles (present study). The decrease of water content on the w_1 led to an increase in the EE of HXT. This could be related to an increasing proportion of aqueous droplets present at the surface of emulsion drops, in the case of higher water content in the w_1 , prompting the HXT loss rate to the external aqueous phase. These results are in accordance with the ones reported by Freytag et al. (2000). Similarly, these authors verified a decrease on the EE of an oligonucleotide with the increase of the water content in the w_1 during the preparation of microspheres by w/o/w double emulsion solvent evaporation technique.

The EE of TYR varied from $88.7 \pm 0.6\%$ (F-1 formulation) and $89.3 \pm 4.8\%$ (F-2 formulation). The amount of encapsulated TYR was 1.0 ± 0.1 mg/g_{MP} in the case of F-1 formulation and 3.2 ± 4.3 mg/g_{MP}, in the case of F-2 formulation. The loss of TYR was about 11% for both formulations (11.3 ± 0.5 and 10.7 ± 4.7 in the case of F-1 and F-2 formulations, respectively). In our previous study, it was performed the encapsulation of TYR by $w_1/o/w_2$ double emulsion solvent evaporation technique into ethylcellulose microparticles in order to compare the behavior of TYR encapsulated solely or incorporated in an extract. During this study it was verified encapsulation efficiencies ranging from $98.5 \pm 1.0\%$ (TLC on TYR of 10% w/w) and $99.3 \pm 0.5\%$ (TLC of 5% w/w) (Paulo & Santos, 2020a).

Opposing to the observed trend of HXT, in the case of TYR, it was observed a decrease of the EE with the decrease of the water content on w_1 . Probably in a similar fashion, the encapsulation of only TYR – associated with higher water content on the w_1 – led to the increase of the proportion of aqueous droplets at the surface of emulsion drops. Nevertheless, due to the higher lipophilic tendency of TYR over HXT, molecular interactions between EC and TYR were favored, and the loss rate of TYR was diminished, and the EE was improved.

The results regarding the recovery of powders produced considering a TLC in PCs of 5% w/w (F-1 formulation) and 10% w/w (F-2 formulation) are presented in Figure IV.6.2-B. It was not observed differences ($p>0.05$) between the mean product yield among the TLC tested.

The mean yield of production was $77.9 \pm 6.5\%$ and $80.5 \pm 3.5\%$ in the case of F-1 and F-2 formulations, respectively. The powders' recovery was independent of the loading content selected. The yield of the production of polymer-only microparticles was $79.6 \pm 3.1\%$. The comparison of the recoveries of loaded and unloaded microparticles reinforced previous conclusions regarding the independence of the powders' recovery from the loading content of microparticles. The contribution of the increased concentration on bioactive compounds from F-1 to F-2 formulations had a negligible impact on microparticles weight and, consequently, the production yield.

The powders' recovery values were in the range of the obtained values for the yield of production when HXT and TYR were individually encapsulated into ethylcellulose microparticles considering similar encapsulation operative parameters (Paulo & Santos, 2018a, 2020a).

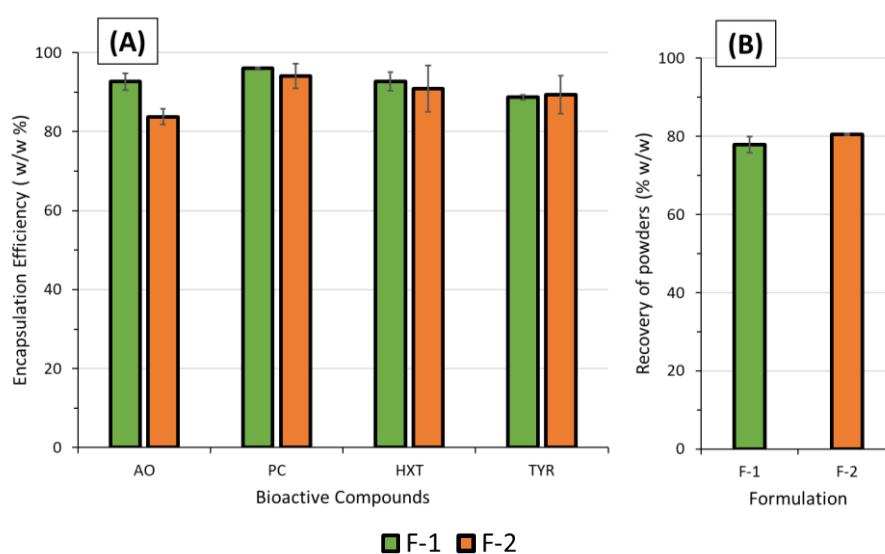


Figure IV.6.2 - Encapsulation efficiency **(A)** of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol; and product yield **(B)** of F-1 (theoretical loading content in phenolic compounds of 5% w/w) and F-2 (theoretical loading content in phenolic compounds of 10%) formulations. Data are expressed as mean $\pm$ standard deviation of three independent assays. Black bars correspond to the error bars based on the standard deviation of three independent assays (AO – Antioxidants; F-1 formulation considering a theoretical loading content in phenolic compounds of 5% w/w; F-2 formulation considering a theoretical loading content in phenolic compounds of 10% w/w; HXT – Hydroxytyrosol; PC -Phenolic Compounds; TYR – Tyrosol)

6.3.3.2 Fourier Transform Infrared Spectroscopy (FTIR) spectra analyses

A qualitative assessment of the efficient incorporation of phenolics into ethylcellulose microparticles was performed by FTIR spectra comparison of the raw material (ethylcellulose), unloaded microparticles (polymer-only microparticles), microparticles formulated considering a TLC in phenolic compounds of 5% w/w (F-1 formulation) and microparticles formulated considering a TLC in phenolic compounds of 10% w/w (F-2 formulation).

The investigation of chemical functional groups at the surface of microparticles empowers the reasoning about the adequate protection of these valuable bioactive compounds in the polymeric matrices and/or their adsorption at the surface of microparticles. The ATR-FTIR spectra of ethylcellulose, polymer only microparticles, and powders of F-1 and F-2 formulations are presented in Figure IV.6.3 for the wavenumber interval from 500 cm^{-1} to 4000 cm^{-1} . The analyzed FTIR-ATR spectra were similar.

The FTIR spectrum of EC includes a weak stretching band at 3466 cm^{-1} and medium intensity stretching bands at 2972 cm^{-1} and 2868 cm^{-1} . The weak stretching band at 3466 cm^{-1} corresponded to the vibrational mode of the stretching band of the O-H bond. The hydroxyl bond is present in about 53% of ethylcellulose molecules (EC ethylation degree of 47%). The medium intensity stretching bands (bands at wavenumbers of 2972 cm^{-1} and 2868 cm^{-1}) corresponded to the vibrational modes from the stretching of the C-H present in alkyl groups of EC. Other typical stretching bands were found from 1500 cm^{-1} to 1000 cm^{-1} , which included the bands at 1424 cm^{-1} and 1373 cm^{-1} corresponding to the vibrational bending mode of $-\text{CH}_3$ groups in EC.

An intense band was found at 1053 cm^{-1} , corresponding to the vibrational mode of the stretching of C-O bonds in EC molecules. The assignments were similar to those described by other authors (Gavini et al., 2015; Patel et al., 2011).

The signature peaks of PO-MPs were similar to the ones of EC. A difference of 28 cm^{-1} was found between the stretching band of EC and PO-MPs (signature peak of PO-MPs at 3438 cm^{-1}) – corresponding to the vibrational mode from the stretching band of the O-H bond in about 50% of EC molecules.

Differences of 36 cm^{-1} and 35 cm^{-1} of the signature peaks of EC and PO-MPs corresponding to the vibrational bending mode of $-\text{CH}_3$ groups and $-\text{CH}_2$ groups in EC were found (signature peaks in PO-MPs at 2937 cm^{-1} and 2833 cm^{-1}). In the FTIR-ATR spectrum of PO-MPs are depicted three signature peaks at 1407 cm^{-1} , 1340 cm^{-1} , and 1018 cm^{-1} corresponding to the vibration mode of $-\text{CH}_3$ groups in EC, $-\text{CH}_2$ groups in EC backbone, and of the stretching of C-O bonds in EC, respectively. These signature peaks differ about 33 cm^{-1} , 35 cm^{-1} , and 17 cm^{-1} from the corresponding pattern in EC. Similar assignments were proposed in our study regarding TYR encapsulation into ethylcellulose microparticles (Paulo & Santos, 2020a).

The powders of F-1 and F-2 formulations exhibited similar signature peaks to EC and PO-MPs. No significant shiftings between the FTIR-ATR spectra of F-1 and F-2 formulations were found. The signature peaks included the ones related to (i) the O-H stretching vibration at 3493 cm^{-1} in the case of F-1 formulation and 3447 cm^{-1} in the case of F-2 formulation, (ii) the C-H alkane stretching vibrations at 2995 cm^{-1} and 2893 cm^{-1} in the case of F-1 formulation and 2893 cm^{-1} and 2849 cm^{-1} in the case of F-2 formulation, (iii) the bending vibration mode of $-\text{CH}_3$ at 1466 cm^{-1} in the case of F-1 formulation and at 1424 cm^{-1} in the case of F-2 formulation, (iv) the bending vibration mode of $-\text{CH}_2$ at 1396 cm^{-1} and 1354 cm^{-1} in the case of F-1 and F-2 formulations, respectively and (v) vibrational mode of the stretching of C-O bonds at 1076 cm^{-1} in the case of F-1 formulation and 1032 cm^{-1} in the case of F-2 formulation. The characteristic bands of loaded powders were similar to the ones of EC and PO-MPs. These observations confirm the efficient incorporation of phenolic compounds in ethylcellulose microparticles: no signature peaks of the incorporated bioactive compounds were found at the surface of microparticles.

The encapsulation technique – $w_1/o/w_2$ double emulsion solvent evaporation – associated with the selection of a proper polymer-carrier, ensured the efficient incorporation of phenolics. Similar conclusions were drawn in our studies regarding the encapsulation of individual bioactive compounds found in OMP as HXT and TYR (Paulo & Santos, 2018a, 2020a). Moreover, results are in accordance with FTIR analyses of phenolics-rich extracts loaded microparticles reported elsewhere (Aliakbarian et al., 2017; Tang et al., 2013; Wu et al., 2008).

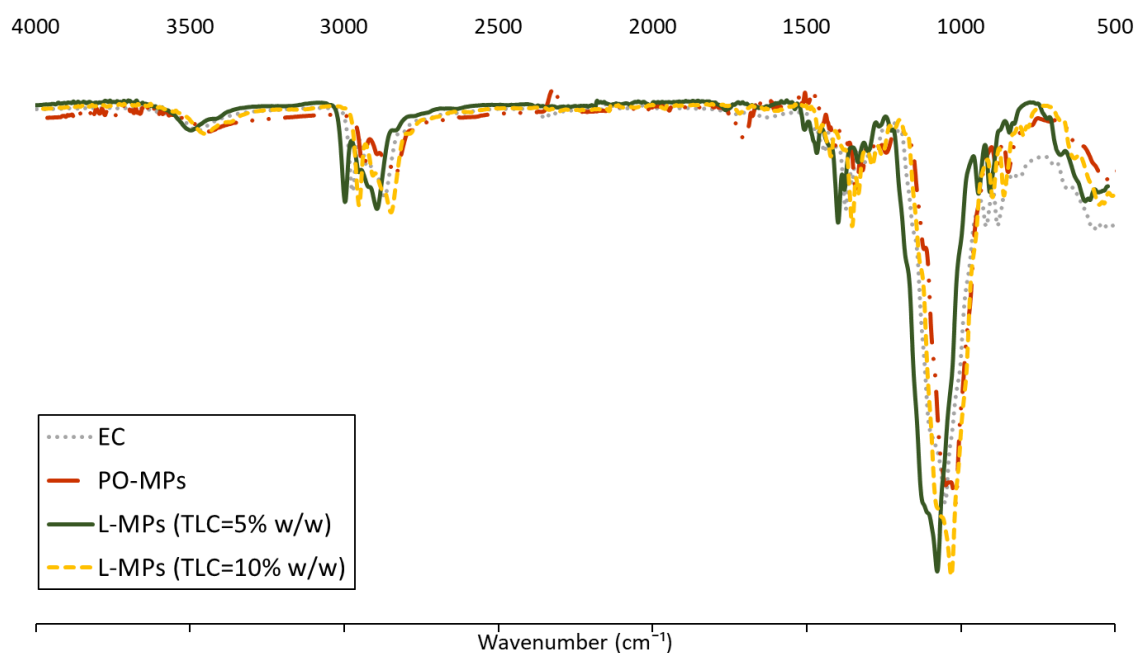


Figure IV.6.3 - ATR-FTIR spectra of ethylcellulose (.....EC), polymer-only microparticles (— PO-MPs), loaded microparticles formulated considering a theoretical loading content of phenolic compounds of 5% w/w (— L-MPs (TLC=5% w/w)), and loaded microparticles formulated considering a theoretical loading content of 10% w/w (--- L-MPs (TLC=10% w/w))

6.3.3.3 Differential Scanning Calorimetry (DSC) analyses

The DSC thermograms of ethylcellulose, polymer-only microparticles, and of the powders of F-1 and F-2 formulations are presented in Figure IV.6.4.

The thermograms of powders corresponding to loaded microparticles present a broad endothermic in the range of 34 °C to 83 °C with a peak at *circa* 61 °C.

On each thermogram, these endotherms are related to the evaporation of the volatile fraction of molecules or bonded water. The temperature at it occurs is known as dehydration temperature (T_{DH}) (Luo et al., 2012; Neo et al., 2013; Paulo & Santos, 2018a; Stuart et al., 2010). The appearance of these broad endotherms was previously described during the DSC characterization of powders of loaded microparticles with alternatively HXT or TYR (Paulo & Santos, 2018a, 2020a).

The occurrence of these endotherms is probably related to the encapsulation technique – the usage of the $w_1/o/w_2$ double emulsion solvent evaporation technique favored the entrapment of bonded water and the volatile fraction of molecules. The encapsulation by water-in-oil-in-water $w_1/o/w_2$ double emulsion solvent evaporation technique was performed under mild conditions. Therefore, as no high temperatures were required during encapsulation, most of the volatile fractions of embedded bioactive compounds were retained in the polymer-carrier.

The DSC thermograms of F-1 and F-2 formulations do not exhibit melting peaks indicating that presumably, the bioactive compounds embed into ethylcellulose microparticles lost their original structure. The absence of melting peaks indicates that the extract's bioactive compounds were efficiently encapsulated in ethylcellulose microparticles.

A subtle baseline change was observed at *circa* 100 °C of EC being more predominant in the DSC thermograms of loaded and unloaded microparticles. The glass transition temperature (T_g) of EC was modified when this polymer was assembled in polymer-only microparticles and also when it was formulated encapsulating an extract (F-1 and F-2 formulations). The observed T_g was in the range of the typical values presented elsewhere (Lai et al., 2010; Paulo & Santos, 2018a; Sakellariou et al., 1985). The results of this study reinforce other studies in the field: EC is a strong glass former, having a narrow heat capacity and rheological change during the glass transition. The formulation of EC in microparticles (loaded and unloaded) favored its heat capacity and rheological changes during the glass transition.

The powders of F-1 and F-2 formulated began to be degraded at *circa* 200 °C. The T_g values of PO-MPs, F-1, and F-2 formulations were similar: 154 °C, 150 °C, and 149 °C in the case of PO-MPs, F-1, and F-2 formulations, respectively.

When a bioactive compound is embedded in a polymeric matrix, three alternative polymer-bioactive interactions may occur: (i) the bioactive compound and the polymer form a solid solution being verified a shift in the T_g of the polymer due to plasticizing/anti-plasticizing effects of the bioactive compound and the endotherm regarding the melting of the bioactive compound is absent in the DSC thermogram of microparticles; (ii) the bioactive compound is dispersed in the polymer in its amorphous form, therefore the T_g of the polymer is not changed and the melting endothermic of the bioactive is not present in the thermogram of microparticles; and (iii) the bioactive ingredient is dispersed in the polymer matrix in its crystalline form and the T_g values of both polymer and bioactive are not changed (Mandal et al., 2002; Pamujula et al., 2004, 2008; Paulo & Santos, 2018a, 2020a). Even though the polymer-active ingredient interactions are described for the case of encapsulation of a single compound, the conceptualization can be extended for the encapsulation of multiple bioactive compounds (e.g., bioactive ingredients present in an extract).

It was observed a shift in the T_g of EC when microparticles were loaded from 137 °C to *circa* 150 °C; moreover, melting endotherms were not observed in both DSC thermograms of loaded microparticles. Therefore, it can be concluded that phenolic compounds present in the optimized OMP extract that was further encapsulated formed with ethylcellulose a solid solution due to anti-plasticizing effects. Molecular interactions between the entrapped bioactive compounds and EC occurred. Additionally, the presence of bigger molecules in EC microparticles had somehow limited the molecular rearrangement of EC when it was heated. Considering the polymer-bioactives interaction due to anti-plasticizing effects, it can be stated that if small molecules of bioactives are required to be encapsulated into EC polymeric matrix, a suitable amount of moisture content should be considered to prevent the excessive brittleness of microparticles and, therefore, to ensure longer shelf-life (Ismail et al., 2012).

A similar trend was previously observed during the encapsulation of HXT in ethylcellulose microparticles: similarly to the present study, HXT and EC formed a solid solution due to anti-plasticizing effects (Paulo & Santos, 2018a).

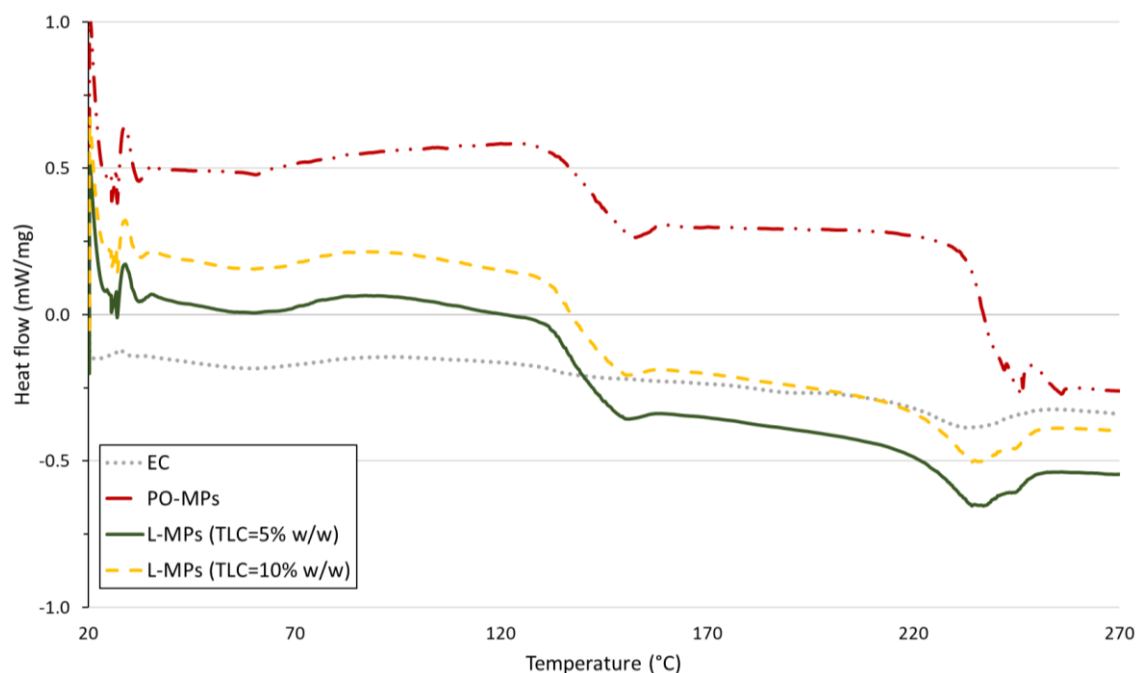


Figure IV.6.4 - Differential Scanning Calorimetry thermograms of ethylcellulose (.....EC), polymer-only microparticles (— PO-MPs), microparticles formulated considering a theoretical loading content in phenolic compounds of 5% w/w (— L-MPs (TLC=5% w/w)), and microparticles formulated considering a theoretical loading content of 10% w/w (— L-MPs (TLC=10% w/w))

6.3.3.4 Thermogravimetric analyses (TGA)

The TGA thermograms and the derivative thermogravimetry (DTG) thermograms from 30 °C to 810 °C of ethylcellulose, polymer-only microparticles, and powders of F-1 and F-2 formulations are presented in Figure IV.6.5-A and Figure IV.6.5-B, respectively.

The TGA were performed to evaluate the microparticles' weight change with temperature. It was observed similar weight loss extent of EC, polymer only microparticles, F-1, and F-2 formulations, in a similar temperature range. The obtained thermograms presented only one level of weight loss.

The analysis of the DTG *versus* temperature is paramount to assess the degradation temperature (T_d) of the analyzed material as peaks on the DTG thermogram correspond to the highest rate of weight loss (Yoksan et al., 2010).

The weight loss change of EC occurred in the range of 268 °C to 425 °C (Figure IV.6.5) being degraded at 352 °C. The interpretation of TGA and DTG thermograms of polymer-only microparticles indicate that the weight loss change occurred in the range of 291 °C and 432 °C, corresponding to a T_d of 357 °C. The weight loss change of powders from F-1 and F-2 formulations occurred in the range of 281 °C to 406 °C, in the case of F-1 formulation and 281 °C and 404 °C, in the case of the powder of F-2 formulation. The determined T_d was 360 °C and 363 °C, in the case of F-1 and F-2 formulations, respectively. Significant mass losses were not found at lower temperatures. Free bioactives molecules did not arrange on the surface of the microparticles; otherwise, a significant weight loss would be observed in the DTG thermograms at low temperatures. Therefore, it can be concluded that loaded ethylcellulose microparticles were nearly stable in the temperature range of most employed temperatures during food processing, handling, storage, and cooking.

There were no significant shifts on the DTG thermograms of F-1 and F-2 formulations around the peak area of 360 °C. At this temperature, a slight increase in the peak area was found from F-1 to F-2 formulations: the peak area increased with the increase of the loading content.

It was observed a shift on the T_d from ethylcellulose (T_d of 346 °C) and PO-MPs (T_d of 356 °C) to higher temperatures when the extract was encapsulated (T_d of 359 °C in the case of F-1 formulation and T_d of 361 °C in the case of F-2 formulation). The thermal decomposition of loaded microparticles started at slightly higher temperatures: the embedment of extracts into ethylcellulose microparticles improved the thermal stability of the polymer-carrier.

The results of this study are in agreement with the ones reported by Lai et al. (2010). Similarly, these authors reported a T_d value of EC close to 350 °C after the analysis of DTG thermograms. Moreover, these results were similar to ones previously obtained during the encapsulation of individual PCs – hydroxytyrosol and tyrosol (Paulo & Santos, 2018a, 2020a). The thermal behavior of loaded microparticles was most likely dependent on the encapsulation technique and the polymer-carrier, rather than the bioactive(s) incorporated. These results support the thesis of the efficient incorporation of phenolic compounds in EC polymer-based microsystems.

The results of the present study are significant: the thermogravimetric stability of loaded microparticles was improved compared to EC and PO-MPs patterns.

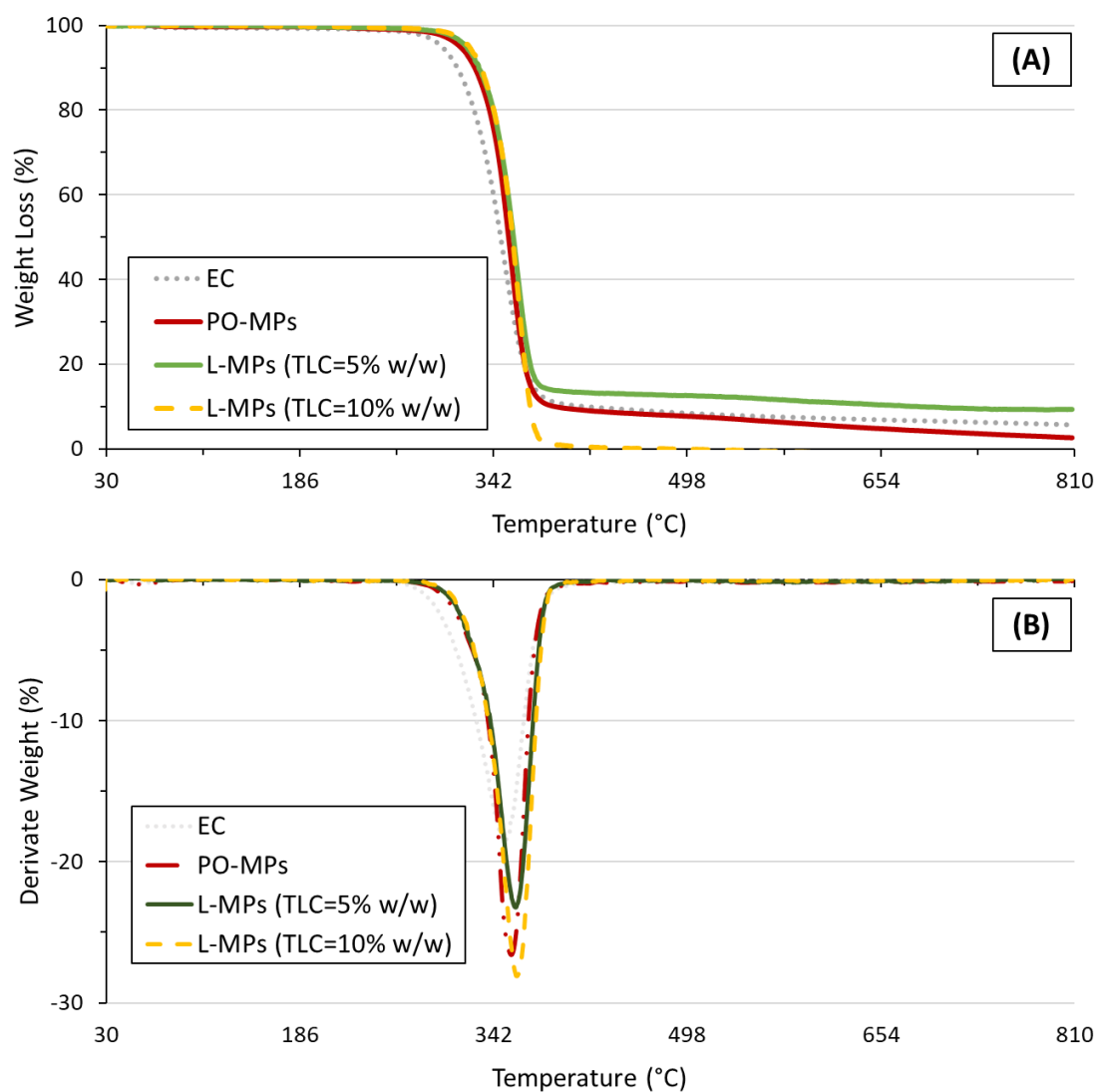


Figure IV.6.5 - Thermogravimetric analysis thermograms of ethylcellulose ($\cdots$ EC), polymer-only microparticles ($-$ PO-MPs), microparticles formulated considering a theoretical loading content on phenolic compounds of 5% w/w ($-$ L-MPs (TLC=5% w/w)), and microparticles formulated considering a theoretical loading content in phenolic compounds of 10% w/w ($-$ L-MPs (TLC=10% w/w)) ((A) – TGA *versus* temperature; (B) – DTG *versus* temperature

6.3.3.5 Evaluation of microparticles morphology and particle size distributions

Microphotographs of powders of F-1 and F-2 formulations at two alternative magnifications (5 000 times and 10 000 times) are presented in Figure IV.6.6. The morphological studies were performed by emission-scanning electron microscopy, as described in Section IV.6.2.2.4.5.

Microparticles formulated considering alternative loading contents presented similar outer morphologies. The water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation technique countenanced the production of particles with spherical shape for both the two loading contents considered. It was not observed significant surface morphological amendments as pores, cracks, and/or fissures. Similar morphological characteristics were obtained in our previous studies: hydroxytyrosol-loaded ethylcellulose microparticles and tyrosol-loaded ethylcellulose microparticles presented smooth outer surfaces with no pores, cracks, or fissures (Paulo & Santos, 2018a, 2020a). Similar morphological characteristics were also observed by other authors when using the $w_1/o/w_2$ double emulsion solvent evaporation technique and alternative encapsulating materials (e.g., polycaprolactone) and encapsulated materials (e.g., bovine serum albumin) (Ibraheem et al., 2014; Yang et al., 2000). Therefore, it can be concluded that microparticles' outer morphology was essentially dependent on the encapsulation technique considered, while the operative conditions were kept constant.

Microparticles formulated considering the higher loading content (Figure IV.6.6-C and Figure IV.6.6-D) presented the biggest particles' sizes. Therefore, it can be deduced that an increase in the loading content led to an increase in particles' sizes. Similar observations were reported by Witschi & Doelker (1998) during the encapsulation of tetracosactide using the $w_1/o/w_2$ double emulsion solvent evaporation technique.

The microphotographs of F-1 and F-2 formulations (Figure IV.6.6-A and Figure IV.6.6-C; lowest magnification) depict disrupted round polymer cavities containing small particles. These observations are attributed to the disruption of bigger microparticles during the re-emulsification of the double emulsion in the high-performance liquid homogenizer: probably, the recently formed microparticles were cut off during their recirculation in the homogenizer. Then, smaller microparticles arranged themselves inside the cracked polymer network.

The scanning light microscopy analyses empowered the acknowledge of: (i) the independence of the outer morphology of microparticles of the loading content; (ii) the dependence of the outer morphology of the encapsulation technique considered; and (iii) the particle size dependence of the loading content: higher loading content led to the production of bigger particles.

The particle size distributions of the powders of loaded and unloaded microparticles are presented in Figure IV.6.7.

The observed mean particle sizes were $5.8 \pm 1.3 \mu\text{m}$ and $19.3 \pm 0.1 \mu\text{m}$, in the case of F-1 and F-2 formulations, respectively. The mean particle size of the polymer-only microparticles was $4.2 \pm 0.9 \mu\text{m}$. The mean PDI values ascertained were 2.1 ± 0.6 , 6.1 ± 0.1 , and 2.2 ± 1.3 , in the case of F-1 formulation, F-2 formulation, and polymer-only microparticles. Significant differences ($p < 0.05$) were found between the two loading contents considered. Polymer-only microparticles presented a lower mean particle size ($4.2 \pm 0.9 \mu\text{m}$) and a similar polydispersity (2.2 ± 1.3) compared to loaded ethylcellulose microparticles formulated considering a theoretical loading content in phenolic compounds of 5% w/w (F-1 formulation).

These results agree with the findings during the morphological analyses: the increase in the loading content led to the appearance of bigger microparticles and more polydisperse (about 2.9 times more polydisperse). These observations are in accordance with the ones reported by Krishnamachari et al. (2007). During the development of pH- and time-dependent oral microparticles to optimize budesonide delivery to ileum and colon, prepared by $w_1/o/w_2$ double emulsion solvent evaporation technique, the authors verified an increase in the mean particle size with the increase of bioactive ingredient concentration. Similar conclusions were drawn by Sharma et al. (2016) during the assessment of process and formulation variables on the preparation of parenteral paclitaxel-loaded biodegradable polymeric particles prepared by $w_1/o/w_2$ double emulsion solvent evaporation technique. Moreover, the mean particle sizes were similar to the reported values by Aliakbarian et al. (2017) when the authors produced particles containing an extract obtained from OMP by supercritical assisted atomization.

The $w_1/o/w_2$ double emulsion solvent evaporation technique was previously applied to the formulation of hydroxytyrosol-loaded ethylcellulose microparticles and tyrosol-loaded ethylcellulose microparticles (Paulo & Santos, 2018a, 2020a). In these studies, it was also verified an increase of the mean particle size with the increase of the loading content. The mean particle size of hydroxytyrosol-loaded ethylcellulose microparticles was about $156.6 \pm 6.9 \mu\text{m}$ in the case of the formulation considering a theoretical loading content on hydroxytyrosol of 5% w/w and $304.0 \pm 16.0 \mu\text{m}$ in the case of the formulation considering a theoretical loading content of 10% w/w on hydroxytyrosol. The encapsulation of TYR into ethylcellulose microparticles resulted in the obtainment of particles exhibiting a mean particle size of $2.3 \pm 0.1 \mu\text{m}$ in the case when a theoretical loading content of 5% on tyrosol was considered and $8.8 \pm 0.7 \mu\text{m}$, in the case of the formulation considering a theoretical loading content of 10% w/w. The obtained mean particle sizes in the present study were amid the mean particle size of hydroxytyrosol-loaded ethylcellulose microparticles and the mean particle size of tyrosol-loaded ethylcellulose microparticles.

The phenethyl alcohols – hydroxytyrosol and tyrosol - are structurally related: HXT holds on the benzene moiety an extra hydroxyl group next to other hydroxyl group providing to HXT an ortho diphenol or catechol structure (Paulo & Santos, 2020a; Tuck & Hayball, 2002). The absence of the extra hydroxyl group entails significant chemical and bioactive differences among them excel the TYR lower solubility in water compared to HXT. Therefore, TYR presents higher compatibility with the polymer-carrier rather than HXT. Presumably, the stronger interactions between TYR and ethylcellulose favored the obtainment of smaller microparticles. On account of the lower solubility of HXT in organic systems, it tended to be cluster in bigger water droplets inside of microparticles, which resulted in the obtainment of bigger particles. In this regard, as the mean sizes of loaded ethylcellulose microparticles were betwixt the mean sizes of tyrosol-loaded ethylcellulose microparticles and the mean sizes of hydroxytyrosol-loaded ethylcellulose microparticles, probably, the majority of the encapsulated antioxidants present an intermediate solubility between hydroxytyrosol and tyrosol.

The F-1 and F-2 formulations, as well as PO-MPs, presented multi-modal particle size distributions. It was verified a dependence on the mean particle size from the type of bioactive(s) incorporated and its/their concentration. From this perspective, as a huge variety of bioactive compounds were encapsulated, the particle size distributions were customarily affected – multi-modal distributions superseded the unimodal and bimodal particle size distributions.

Moreover, many studies have been reporting the effect of process and formulation parameters on the particle size distributions (e.g., stirring speed during emulsification steps, loading, polymer concentration, polymer molecular weight, surfactant concentration) (Krishnamachari et al., 2007; Paulo & Santos, 2018b, 2020a; Sharma et al., 2016).

The mean particle size, the polydispersity, and the particle size distribution were dependent on the physicochemical characteristics of bioactive compound(s) embedded in the polymer matrix and the loading content. The polymer-bioactive(s) physicochemical interactions play an essential role in the particle size and particle size distribution. The obtained results support the hypothesis of the complexity of process and formulation parameters affecting the mean size and particle size distribution.

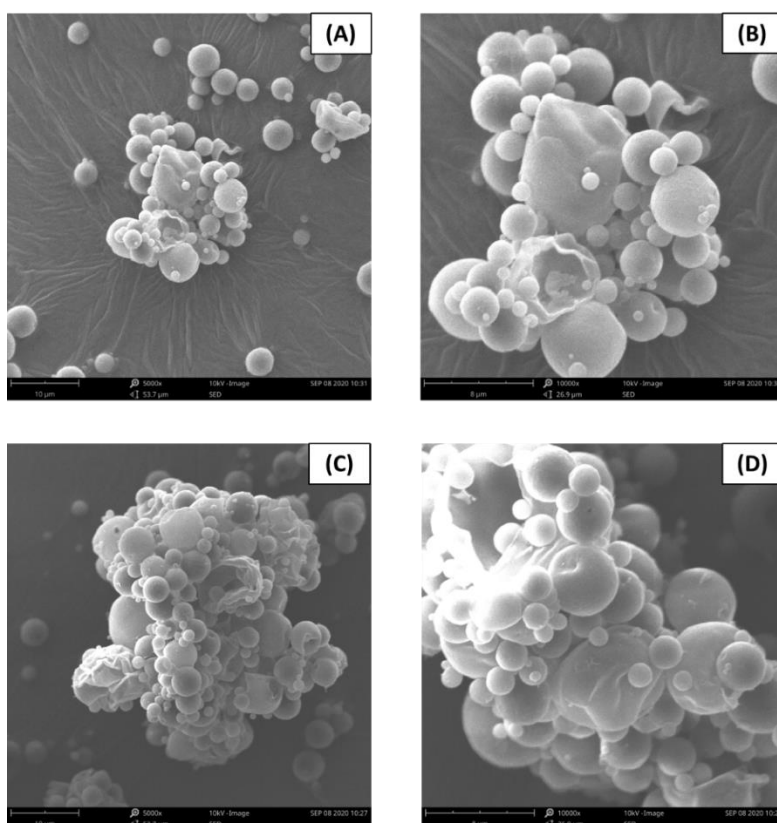


Figure IV.6.6 - Microphotographs of F-1 ((A) and (B)) and F-2 ((C) and (D)) formulations (amplification of 5 000 times is presented in (A) and (C) micrographs and 10 000 times in (B) and (D); beam intensity of 10.00 kV)

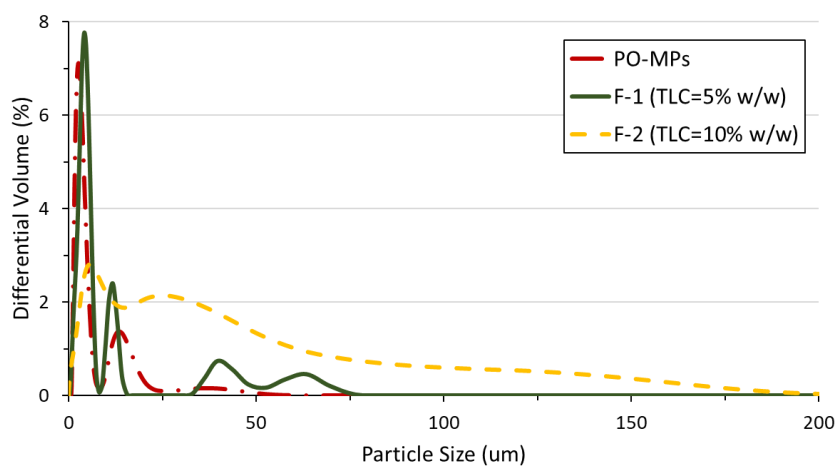


Figure IV.6.7 - Particle size distribution of the powders of polymer-only microparticles (— PO-MPs), microparticles formulated considering a theoretical loading content in phenolic compounds of 5% w/w (— F-1 (TLC=5% w/w)), and microparticles formulated considering a theoretical loading content in phenolic compounds of 10% w/w (— F-2 (TLC=10% w/w))

6.3.3.6 Assessment of rehydration properties of the particles

The rehydration properties, namely the water absorption index (WAI), the water solubility index (WSI) and the swelling capacity (SC); and the wettability of the powders of olive mill pomace extract-loaded ethylcellulose microparticles (F-1 and F-2 formulations) and unloaded microparticles (polymer-only microparticles; PO-MPs) are presented in Table IV.6.4. It was not observed differences ($p>0.05$) between samples (PO-MPs, F-1 formulation, and F-2 formulation).

The WAI reflects the ability of microparticles to adsorb water molecules, and it is a measure of the degree of degradation of the polymer shell, whereas the WSI expresses the percentage of degradation that the polymer carrier experienced. It is also described that high values of the WSI indicate a high content of low molecular weight molecules. These indices are related, as described in Section IV.6.2.2.4.7. The estimation of WAI and WSI is considered to be crucial to estimate microparticles behavior if further processed to be used as a binder, stabilizer, or even as a source of bioactive compounds in foods and beverages (Gutkoski & El-Dash, 1999; Jozinovi, 2017; Zhu et al., 2010).

The WAI was comprised in the range of 0.43 ± 0.08 g/g_{DP} (F-2 formulation) and 0.60 ± 0.21 g/g_{DP} (F-1 formulation). To the authors' best knowledge, this is the first time that is reported the WAI of loaded ethylcellulose microparticles. Therefore, there is a lack of studies for direct comparison. Nevertheless, this parameter has been studied regarding the encapsulation of olive mill pomace extracts. During a study of the operative parameters affecting the encapsulation of PCs from olive pomace using maltodextrin as the encapsulating material, the authors Paini et al. (2015) reported WAI values ranging from 0.28 ± 0.02 g/g_{DP} and 0.58 ± 0.03 g/g_{DP}. The obtained results are in accordance with the ones presented by Paini et al. (2015), indicating that the engagement of hydroxyl groups to form hydrogen bonds were similar in both polymer-carriers.

Moreover, similar losses on the crystalline structures were observed. The authors Aliakbarian et al. (2017) reported WAI values in the range of 0.9 ± 0.03 g/g_{DP} to 1.2 ± 0.01 g/g_{DP} of olive mill pomace extract loaded maltodextrin microparticles formulated by supercritical assisted atomization. The obtained WAI values in the present study were lower than the reported ones by Aliakbarian et al. (2017).

Olive mill pomace extract loaded microparticles obtained in the present study kept a greater percentage of the polymer carrier original structure when compared to the obtained microparticles by Aliakbarian et al. (2017).

The mean obtained values of WSI ranged from $75 \pm 3\%$ (PO-MPs) and $82 \pm 2\%$ (F-2 formulation). These results were similar to those calculated by Paini et al. (2015) during the encapsulation of olive mill pomace extract into maltodextrin polymer shell by spray-drying (WSI values ranging from $79 \pm 2.2\%$ and $87 \pm 1.6\%$) but higher than those obtained by Aliakbarian et al. (2017) ($53.0 \pm 0.7\% - 67.0 \pm 1.0\%$) during the formulation of olive mill pomace extract loaded maltodextrin microparticles by supercritical assisted atomization. In the present study, was observed a lower content of liposoluble compounds in the raw material, which led to the encapsulation of higher levels of hydrophilic compounds (Kha et al., 2010); and consequently to higher WSI values comparing with the results presented by Aliakbarian et al. (2017).

The SC is a measure of the extension of granular stability and the presence of crystallites. Low values of SC indicate the presence of many crystallines. Moreover, low SC values are associated with good granular stability and a reduction of the extent of granular swelling (Ahmed et al., 2010; Gunaratne & Hoover, 2002).

The SC values ranged from $0.03 \pm 0.00 \text{ g/g}_{\text{DP}}$ (PO-MPs) and $0.05 \pm 0.01 \text{ g/g}_{\text{DP}}$ (F-2 formulation). The observed values were lower than ones presented by Aliakbarian et al. (2017) (SC values ranging from $2.4 \pm 0.0 \text{ g/g}_{\text{DP}}$ to $3.2 \pm 0.1 \text{ g/g}_{\text{DP}}$) and by Paini et al. (2015) ($2.10 \pm 0.49 \text{ g/g}_{\text{DP}} \leq \text{SC} \leq 3.12 \pm 0.14 \text{ g/g}_{\text{DP}}$). These results indicate that powders presented excellent granular stability, many crystallines, and the extent of the granular swelling was minimal. These results corroborate the data regarding the non-swell ability of EC (Murtaza, 2012).

The wettability, also known as sinkability, is an alternative measure of microparticles' ability to rehydrate in water; alternatively, it can be seen as the ability of a bulk powder to absorb water (Bae & Lee, 2008; Gaiani et al., 2007). The wetting times varied from $1\,003 \pm 72 \text{ s}$ (F-1 formulation) and $1\,042 \pm 78 \text{ s}$ (PO-MPs). It was not observed differences ($p > 0.05$) between samples.

These results were within the range of the observed wetting times of hydroxytyrosol-loaded ethylcellulose microparticles (Paulo & Santos, 2018a).

In this regard, it can be concluded that olive mill pomace extract loaded ethylcellulose microparticles kept a significant percentage of ethylcellulose original structure, which is a crucial consideration during the incorporation of loaded microparticles in foods or beverages. Moreover, microparticles were formulated with a low content of lipophilic compounds. Microparticles obtained in the present study exhibited outstanding granular stability.

Table IV.6.4 - Water absorption index (WAI), water solubility index (WSI), swelling capacity (SC), and wettability (WET) of olive mill pomace extract-loaded ethylcellulose microparticles

Formulation	WAI (g/g _{DP})	WSI (%)	SC (g/g _{DP})	WET (s)
PO-MPs ^{a,b}	0.60 ± 0.21	77 ± 5	0.04 ± 0.01	1003 ± 72
F-1 ^{a,c}	0.43 ± 0.08	82 ± 2	0.05 ± 0.01	1016 ± 128
F-2 ^{a,d}	0.58 ± 0.07	75 ± 3	0.03 ± 0.01	1042 ± 78

^a Results are presented as mean ± standard deviation (n=3)

^b Data relative to the unloaded microparticles (polymer-only microparticles)

^c Data relative to the F-1 formulation (theoretical loading content in phenolic compounds of 5% w/w)

^d Data relative to the F-2 formulation (theoretical loading content in phenolic compounds of 10% w/w)

DP – Dried Powder; MPs – Microparticles; PO – Polymer-Only; SC – Swelling Capacity; WAI – Water Absorption Index; WET – Wettability; WSI – Water Solubility Index;

6.4 Conclusions

Olive mill pomace is one of the most significant olive oil by-products. Even though olive oil now obtained from an eco-friendly extraction procedure, olive mill pomace constitutes a phytotoxic olive oil by-product due to its high content of phenolic compounds. Nevertheless, phenolic compounds present in olive oil by-product possess relevant health benefits as a result of their neuroprotective, cancer chemopreventive, and cardioprotective activities. In this context, olive mill pomace is an outstanding source of bioactive compounds presenting remarkably antioxidant activity as hydroxytyrosol and tyrosol.

This study demonstrated that the water-in-oil-in-water double emulsion solvent evaporation technique could be efficiently used to encapsulate natural bioactive compounds such as phenolics extracted from olive pomace working at mild conditions. Olive mill pomace extract rich extract was obtained through an optimized two-step solid-liquid extraction procedure. Then, the obtained extract considering the optimized extraction conditions was embedded into ethylcellulose microparticles. The effect of phenolics loading content on the main physicochemical characteristics of loaded microparticles was assessed.

The results of this study reveal that the bioactive compounds were efficiently embedded in ethylcellulose polymer carrier as encapsulation efficiencies were kept above 80% w/w, which was confirmed by the Fourier Transform Infrared spectroscopy analyses. Loaded microparticles were thermogravimetrically stable in the temperature range of 30 °C to about 280 °C. From the differential scanning calorimetry analyses, it was concluded that ethylcellulose and bioactive compounds formed a solid solution during encapsulation due to anti-plasticizing effects. The laser granulometry analyses and the evaluation of particles' microphotographs empowered the acknowledge of the independence of the particles' external morphology of the loading content and the particle size dependence of the loading content: bigger microparticles were obtained with the increase on the loading content. From the rehydration studies, it was observed that olive mill pomace extract loaded ethylcellulose microparticles exhibited outstanding granular stability.

The results of this study are remarkable regarding the obtainment of phenolics rich extracts from olive mill pomace that can be further valorized through encapsulation to be subsequently incorporated in food, pharmaceutical, cosmetic, and even in nutraceutical matrices.

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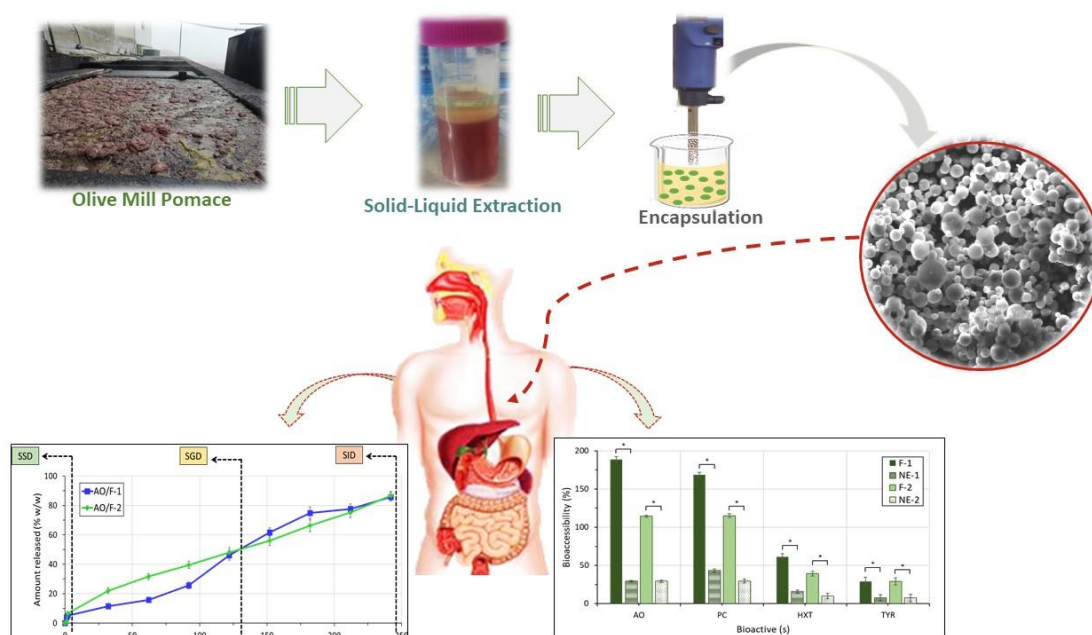
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Chapter 7. *In vitro* evaluation of the release of phenolic compounds of encapsulated olive mill pomace extract by double emulsion solvent evaporation technique ⁷

Filipa Paulo, Lúcia Santos

Graphical Abstract:



⁷ This chapter was adapted from: Paulo, F. & Santos L. (2020). *In vitro* evaluation of the release of phenolic compounds of encapsulated olive mill pomace extract by double emulsion solvent evaporation technique. *Submitted*.

Abstract:

In recent years, an increasing interest in phenolic compounds present in olive mill wastes has been observed due to their health benefits. Particularly, hydroxytyrosol and tyrosol are phenolic compounds present in high quantities in olive mill pomace with outstanding radical scavenging activities.

In this study, phenolic compounds were efficiently protected using a gastro-resistant polymer – ethylcellulose. Loaded ethylcellulose microparticles were formulated by double emulsion solvent evaporation technique. Two theoretical loading contents in phenolic compounds were considered (5% w/w and 10% w/w). The effect of loading content on the release behavior and the bioaccessibility of bioactive compounds was assessed.

The encapsulation efficiencies remained above 83% w/w, and the actual loading contents were similar to the theoretical loading contents considered. The *in vitro* release studies confirmed the salivary and gastric protection of phenolic compounds. The majority of bioactive compounds were released in the simulated intestinal tract.

The compounds' bioaccessibilities were significantly improved when encapsulated. Nevertheless, it was not verified improvements on bioaccessibilities with the increase in the loading content.

From the analysis of the similarity factors, no substantial differences were observed between the release profiles for both loading contents considered.

The results of this study are remarkable, bringing afresh perspectives regarding the design of loaded microparticles that will encounter gastrointestinal digestion.

Highlights

1. Olive mill pomace (OMP) extracts present outstanding radical scavenging capacity.
2. OMP extract microparticles were obtained by double emulsion solvent evaporation.
3. The encapsulation efficiencies remained above 83% w/w.
4. Release studies confirmed the salivary and gastric protection of phenolics.
5. Compounds' bioaccessibilities were significantly improved when encapsulated.

Keywords: phenolic compounds; *in vitro* release studies; gastrointestinal digestion; antioxidants; hydroxytyrosol; tyrosol; mathematical modeling;

7.1 Introduction

Remarkably, food applications of naturally-occurring bioactive compounds presenting nutraceutical properties have been intensively studied in the last few years (Aditya et al., 2017; Atef & Ojagh, 2017; Drosou et al., 2017; Fradinho et al., 2020; Mwaurah et al., 2020; Nirmala et al., 2018; Rezaei et al., 2019; Saucedo-Pompa et al., 2018; Zakaria & Kamal, 2016; Zhang et al., 2018).

Within this framework, it is reported that olive (*Olea europaea* L.), olive oil, and olive oil by-products are outstanding sources of bioactive compounds as phenolic compounds (Aldini et al., 2006; Alu'datt et al., 2010; Benavente-García et al., 2000; De Marco et al., 2007; El-Abbassi et al., 2012; El & Karakaya, 2009; Lesage-Meessen et al., 2001; Obied et al., 2007; Paulo & Santos, 2020a, 2020b; Pereira et al., 2007; Visioli et al., 1999). There are available distinctive olive oil extraction procedures from olives such as the (i) traditional pressing mill process, the (ii) three-phase extraction process, and the (iii) two-phase extraction process. The oldest and most outspread approach used to obtain olive oil from olives is the (i) traditional pressing mill process. However, the advent of the manufacture of hydraulic presses promoted progress in the obtainment of olive oil from olives. Consequently, the (i) traditional pressing mill process was gradually replaced by innovative approaches to reduce the labor force intense and dependence, being widely replaced by the (ii) three-phase extraction process in many olive mills, mainly among those which had high olives processing demand. The (iii) two-phase olive oil extraction process was developed in the '90s to minimize the water consumption used with the three-phase extraction process. It is the most commonly used olive oil extraction approach, at present. Two phases are obtained at the end of the extraction process: an oily phase - the olive oil - and a semi-solid phase – the wet pomace, also known as the two-phase olive mill waste (TPOMW), also known as “alperujo” or even as “alpeorujo” or simply as olive mill pomace (OMP) (Azbar et al., 2004a; Benitez et al., 1997; Dermeche et al., 2013; Kapellakis et al., 2008; Paulo & Santos, 2020b; Roig et al., 2006; Tortosa et al., 2012). The processing of 1 tone of olives using the (iii) two-phase extraction process generates around 200 kg of olive oil and 800-950 kg of OMP (Azbar et al., 2004b).

In this context, the OMP is considered to be inexpensive biomass of phenolic compounds available at large amounts among olive oil-producing countries. Moreover, it is documented the environmental issues regarding the disposal of OMP (Alu'datt et al., 2010; Clemente et al., 1997; Dermeche et al., 2013; Ergüder et al., 2000; Ghanbari et al., 2012; Lesage-Meessen et al., 2001; Paulo & Santos, 2020b; Salomone & Ioppolo, 2012). In this context, intensive research on OMP treatment and valorization has been performed, aiming to reduce the environmental impact of OMP disposal, seizing the potential economic valorization of this waste (Paulo & Santos, 2020b).

In the last few decades, scientific efforts have been devoted to the valorization of OMP, namely through the extraction of valuable bioactive compounds as phenolic compounds due to their potential application in food, cosmetic, pharmaceutical, and nutraceutical industries (Aliakbarian et al., 2011, 2017; Fang & Bhandari, 2010). However, the valorization of natural-occurring bioactive compounds from OMP is still limited as these bioactive compounds are sensitive to light exposure, temperature, moisture, and the presence of oxygen, being prone to degradation during processing and storage (Fang & Bhandari, 2011). Other limitations include the low solubility of some phenolic compounds in water, which curb the amount of these bioactives that can be embedded in hydrophilic food matrices, cosmetic, pharmaceutical, and nutraceutical formulations; and the astringent and bitter taste of phenolic compounds – generally engender by the precipitation of mucopolysaccharides and salivary glycoproteins in the tongue (Kosaraju et al., 2008; Xiao et al., 2011).

In this context, encapsulation arises as a potential technological strategy to protect efficiently the extracted OMP phenolic compounds from the external environment, enhance phenolic compounds solubility in matrix systems (e.g., foods and beverages) and mask unpleasant phenolic compounds taste, increasing, therefore, the bioaccessibility and the astringency of these valuable compounds. The spray-drying and the supercritical assisted atomization encapsulation techniques have been exploited to obtain capsules embedding phenolics-rich extracts obtained from OMP samples (Aliakbarian et al., 2015, 2018). Other alternative encapsulation techniques as emulsification approaches, ionic gelation, and liposomes preparation have been also investigated (Paulo & Santos, 2020c).

Among them, the emulsification technique - water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation - is a suitable method to protect efficiently water-soluble phenolic compounds. Moreover, this approach allows the shield of phenolics-rich extracts from the environmental conditions (e.g., moisture, temperature, light), retaining the bioaccessibility of these valuable compounds and enhancing their compatibility with low-water content foods and beverages.

Accordingly, to the $w_1/o/w_2$ double emulsion solvent evaporation technique, the extract is dissolved in an aqueous phase fashioning the internal aqueous phase (w_1). Then, the w_1 – containing water-soluble bioactive compounds – is emulsified in an organic phase (o) containing dissolved the coating material, forming the primary emulsion (w_1/o). Afterward, the primary emulsion is re-emulsified in an external aqueous phase (w_2) containing an emulsion stabilizer (e.g., polyvinyl alcohol, polyoxyethylene sorbitan monooleate, caseinate, gelatin, whey protein isolate) (Dickinson, 2011; Paulo & Santos, 2018b, 2020a). After that, the evaporation of the solvent and the hardening of microparticles are promoted, and then microparticles are recovered by filtration and/or centrifugation processes (Paulo & Santos, 2018a, 2018b, 2019, 2020a, 2020c). There are commercially available a wide range of alternative encapsulating materials to incorporate bioactive compounds - as phenolic compounds. Among them, ethylcellulose – a cellulose-derivate group of polymers - are outstanding biocompatible polymers commonly used in foods, beverages, and in food-additives processing (Paulo & Santos, 2018a, 2018b, 2019, 2020a, 2020c). They are generally recognized as safe (GRAS) polymers and approved as carriers in food additives by the European Union in the European Commission Regulation 1130/2011 (European Commission, 2011). Alternative ethylcellulose polymer grades are available depending on the relative percentage of hydroxyl groups of cellulose replaced by ethyl ether groups. Ethylcellulose polymers present remarkable properties – they are odorless and tasteless polymers; they are stable to light, heat, moisture, and oxygen exposure being resistant to mechanical stress – which makes ethylcellulose polymers unique for encapsulation proposes (Kamel et al., 2008; Murtaza, 2012; Paulo & Santos, 2018a).

Recently, encapsulation technology for food and nutraceutical industries poured to exploit microsystems as tailored-made delivery systems of bioactive compounds as phenolic compounds. Accordingly, delivery systems for food and nutraceuticals applications design the obtainment of time-controlled and programmable microparticulated systems in which particles are straight designed in order to bioactive compound(s) is/are released at the desired time, site, and rate (e.g., bioactives released only on the small intestine where the majority of the absorption processes occur after 2 hours of the beginning of the gastrointestinal digestion).

Generically, bioactive compounds delivery systems are designed to protect these valuable compounds during processing, storage, and handling. The majority of bioactive compound present in OMP are prone to salivary and gastric degradations and generally suffer an intense first-pass metabolism in the liver and in the small intestine, which hinders proper absorption of these valuable bioactive compounds (Bonechi et al., 2019; Paulo & Santos, 2020a, 2020c; Rodríguez-Morató et al., 2016; Vlachogianni et al., 2015). Therefore, the design of a delivery system should consider the complexity of food, food carriers, and bioactive compound(s) digestion in the human digestive tract.

In this context, many *in vitro* and *in vivo* procedures have been developed to determine the physiological responses to specific food additives (e.g., phenolic compounds, phenolics-loaded microparticles) and allergenic compounds. While the *in vivo* procedures are doubted on ethical ground, the *in vitro* gastrointestinal digestion models have been widely accepted to simulate foods and food additives digestion to decipher the complexity of human tract digestion (Brodkorb et al., 2019; Dupont & Mackie, 2015; Paulo & Santos, 2020c).

In this framework, many models have been proposed to describe the release of bioactive compounds. These models are classified into three principal categories: (i) statistical models (multivariate analysis of variance, repeated-measures design, exploratory data analysis), (ii) model-dependent methods (e.g., zero-order, first-order, Korsmeyer-Peppas, Higuchi, Baker-Lonsdale, Weibull, Hixson-Crowell models) and (iii) model-independent methods (the difference factor, f_1 and the similarity factor, f_2) (Dash et al., 2010; Paulo & Santos, 2020c).

Thereupon, this study aimed to assess the release behavior of antioxidants (AOs), phenolic compounds (PCs), and specific phenolic compounds as hydroxytyrosol (HXT) and tyrosol (TYR) of an OMP extract extract-loaded ethylcellulose microparticles in an *in vitro* simulated gastrointestinal tract.

Olive mill pomace extract-loaded ethylcellulose microparticles were formulated by $w_1/o/w_2$ double emulsion solvent evaporation technique, considering two loading contents on phenolic compounds: 5% w/w and 10% w/w. The influence of loading content – amount of bioactive compound(s) loaded per unit weight of microparticles - on the release profiles of bioactive(s) was assessed. Moreover, it was evaluated the similarity between the release profiles of bioactive compounds. Furthermore, mathematical modeling of the release profiles was performed through model-dependent methods as the zero-order, the first order, the Higuchi, the Korsmeyer-Peppas, and the Weibull models. The bioaccessibility of bioactive(s) released during the simulated gastrointestinal tract was assessed, being evaluated the influence of the loading content on this parameter.

The release behavior of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol was evaluated regarding the design of OMP extract delivery system for further incorporation in food matrices. This study aims to bring new perspectives regarding the release behavior of phenolic compounds from olive mill pomace extract loaded microparticles. Remarkably, this study provides new insights on bioactive(s) delivery systems that can effectively protect the natural-occurring bioactive compounds, increasing their bioaccessibility.

7.2 Experimental procedures

7.2.1 Materials

7.2.1.1 Reagents and Standards

The extraction of phenolic compounds from OMP samples (n=3) was performed using the solvents hexane (HiPerSolv CHROMANORM[®] for HPLC, Ref: 83992.320, C₆H₁₈, CAS 110-54-3), ethanol (purity ≥ 99.8%, PESTINORM[®], Ref: 85933-320, C₂H₆O, CAS 64-17-5) and ethyl acetate (purity ≥ 99.8%, SupraSolv[®], Ref: 1.00789.2500, C₄H₈O₂, CAS 141-78-6) which were obtained from VWR International (Fontenay-sous-Bois, France). Additionally, hydrochloric acid solution at 37% v/v (EMSURE[®] ACS, Supelco[®], Ref: 1.00317.1011, HCl, CAS 7647-01-0) used during the extraction was also obtained from VWR International (Fontenay-sous-Bois, France).

The reagents used for encapsulation purposes namely ethylcellulose (EC) (Ref: 433837-250G, viscosity of 46 cP, ethylation degree of 48%, CAS 9004-57-3) and polyvinyl alcohol (PVA) (Ref: P8136-250G, 87-90% hydrolyzed, an average molecular weight of 30 000-70 000, CAS 9002-89-5) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). Dichloromethane (DCM) (Ref. ACRO433991000, CH₂Cl₂, CAS 1665-00-5) was obtained from VWR International (Fontenay-sous-Bois, France).

Reagents used for the estimation of the TAA and the TPC such as the Folin-Ciocalteu reagent (Ref: 47641-500ML-F, 2M with respect to acid, density of 1.3 g/mL at 20 °C), gallic acid standard (Ref: 91215, C₆H₆O₅, CAS 149-91-7), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Ref: D9132, C₁₈H₁₂N₅O₆, CAS 1898-66-4), sodium carbonate anhydrous (Ref: 1613757, Na₂CO₃, CAS 497-19-8) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA).

The standards used for chromatographic analysis, namely hydroxytyrosol (HXT) (purity ≥ 98%, Ref: H4291, C₈H₁₀O₃, CAS 10597-60-1) and tyrosol (TYR) (purity ≥ 98%, Ref: 188255, C₈H₁₀O₂, CAS 501-97-0), were also obtained from Sigma Aldrich Chemical (St. Louis, MO, USA).

The eluents for chromatographic analyses such as acetonitrile (purity $\geq 99.9\%$, LiChrosolv®, Ref: 1.00029.2500, C_2H_3N , CAS 75-05-8) and methanol (purity $\geq 99.9\%$, LiChrosolv®, Ref: 1.03726.2002, CH_4O , CAS 67-56-1) were obtained from VWR International (Fontenay-sous-Bois, France). Additionally, acetic acid standardized solution 0.1 N (supplier Alfa-Aesar, Ref: 35567.K7, $C_2H_4O_2$, CAS 64-19-7) was also obtained from VWR International (Fontenay-sous-Bois, France).

The salts used during the *in vitro* release studies namely magnesium chloride hexahydrate (supplier Merck, VWR Ref: 1.05833.0250, $MgCl_2 \cdot 6H_2O$, CAS 7791-18-6), potassium dihydrogen phosphate (supplier Merck, VWR Ref: 1.04873.0250, KH_2PO_4 , CAS 7778-77-0), potassium chloride (supplier Merck, VWR Ref: 1.04936.0250, KCl , CAS 7447-40-7), ammonium carbonate (supplier Merck, VWR Ref: 5.43835.0100, $(NH_4)_2CO_3$, CAS 10361-29-2) and sodium hydrogen carbonate (supplier Merck, VWR Ref: 1.06329.0500, $NaHCO_3$, CAS 144-55-8), sodium chloride (supplier Merck, VWR Ref: 1.06404.0500, $NaCl$, 7647-14-5) were purchased from VWR International (Fontenay-sous-Bois, France).

The water used in this work was de-ionized and double-distilled using a Millipore™ water purification system (Massachusetts, USA) having 18.2 Ω electrical resistivity. All the reagents were either chromatographic or analytical grade and used as received.

7.2.1.2 Samples

The OMP samples were obtained from a local olive oil mill in Portugal (Vilas Boas, Vila Flor, Bragança, 41359822,-7123743) in January 2019. Samples were stored at $-22\text{ }^{\circ}\text{C}$ (moisture content of $71.0 \pm 5.5\%$ w/w) before prior analyses. Frozen OMP samples were freeze-dried for 72 hours on a benchtop freeze-dryer (SP Scientific, NY, USA) and then grounded on an electric mill (Qilive Q5321 Grinder) to an average particle size of $142.2 \pm 9.6\text{ }\mu\text{m}$, as described by Uribe et al. (2015) with slight modifications.

7.2.2 Methods

7.2.2.1 Extraction of phenolic compounds from olive mill pomace

The recovery of phenolic compounds was performed in three stages: (i) sample pretreatment and bonded phenolic compounds extraction, (ii) two-step solid-liquid extraction of free phenolic compounds, and (iii) liquid-liquid extraction of these valuable bioactive compounds. The detailed description of procedures is presented in the following sections.

7.2.2.1.1 Olive mill pomace pretreatment and bonded phenolic compounds extraction

The freeze-dried OMP sample (1 g) was submitted to a pretreatment process prior to the extraction of phenolic compounds that included (i) an acidic hydrolysis step and (ii) a fat removal step. The (i) acidic hydrolysis promotes the breakage of both glycosidic and ester bonds. Moreover, the low pH favors the precipitation of proteins, increasing, therefore, the amount of free phenolic compounds (FPCs) in the extraction solvent as described by De Marco et al. (2007). The (ii) fat removal procedure was considered, as lipids – due to their lipophilic nature – are interfering agents in obtaining hydroalcoholic extracts.

The (i) acidic hydrolysis was performed, adding to 1 g of OMP sample, 25 mL of an aqueous solution of HCl (0.1 M, pH 2). The mixture was continuously shaken in an orbital shaker (IKA® KS 130 basic, IKA Works GmbH & Co. KG, Staufen im Breisgau, Germany) for 12 hours.

The lipids removal procedure was performed as described by Lafka et al. (2011). To the hydrolyzed OMP sample was added 5 mL of hexane (5 mL/g). The lipid removal was processed, promoting the continuously shaken of the mixture in an orbital shaker (400 rpm) for 1 hour at room temperature (20 ± 2 °C). The mixture was then centrifuged at 2 670 g for 15 minutes in a Rotofix 32A centrifuge (Hettich, Germany). Afterward, the aqueous and organic phases were separated. The phenolic compounds present in the organic phase were further recovery by liquid-liquid extraction.

For that, this organic layer was admixed with 15 mL of UPW, and the mixture was vortexed (IKA VORTEX GENIUS 3, Staufen, Germany) for 5 minutes and ultrasonicated (P Selecta, Barcelona, Spain) for 15 minutes. Then, the sample was centrifuged at 2 670 g for 15 minutes. The obtained aqueous layer from the centrifugal process was filtered using sterile and disposable syringes without needle (Ref: 613-1591, VWR International, Fontenay-sous-Bois, France). The final sample – aqueous solution containing phenolic compounds extracted from the lipid layer – was designated as sample containing extracted phenolic compounds after the fat removal procedure (SEPC-FR).

7.2.2.1.2 Solid-liquid extraction of free phenolic compounds

The recovery of free phenolic compounds in the OMP sample was performed in a two-step solid-liquid extraction process. The defatted sample obtained after the fat removal procedure was admixed with 10 mL of the ethanol. The mixture was continuously shaken for 3.2 hours on an orbital shaker (400 rpm; room temperature) being afterward centrifuged (2 670 g, 15 minutes). The supernatant termed as sample containing extracted phenolic compounds from the primary extraction (SEPC-P1E) was collected, filtered under vacuum using a Whatman™ nylon membrane filter and stored in an amber flask under dark conditions at room temperature (20 ± 2 °C) before being admixed with the final filtrate.

The secondary extraction was performed similarity to the primary extraction procedure. The pellet was admixed with 3 mL of ethanol. The mixture was continuously shaken for 1.3 hours. The sample was then submitted to a low-intensity centrifugation process (2 670 g, 15 minutes). Afterward, the supernatant was collected and vacuum filtered using Whatman™ nylon membrane filter. The filtrate was designated as sample containing extracted phenolic compounds from secondary extraction (SEPC-P2E). The filtrates EP-FR, EP-P1E, and EP-P2E, were then admixed, constituting the final filtrate.

7.2.2.1.3 Liquid-liquid extraction of phenolic compounds

Afterward, the final filtrate was submitted to a liquid-liquid extraction using ethyl acetate as the extraction solvent. Thus, the final filtrate was admixed with ethyl acetate (extraction solvent volume corresponding to three times the final filtrate volume), and the mixture was then vigorously shaken and vortex for 5 minutes. Subsequently, the mixture was ultrasonicated (15 minutes) and centrifugated (low-intensity centrifugation process, 2 670 g, 15 minutes). The supernatant was then collected, and the solvent was evaporated at 50 °C using a rotary evaporator (BUCHI R-210, Buchi Laboratoriums Technik AG, Flawil, Switzerland). Solvent traces were removed under a gentle nitrogen stream. The dried extract was stored at -22 °C before prior analyses. The extraction experiments were performed in triplicate, and results are presented as mean $\pm$ standard deviation.

7.2.2.2 Encapsulation of phenolics-rich extracts obtained from olive mill pomace

7.2.2.2.1 Encapsulation of the obtainment of phenolics-rich extract-loaded ethylcellulose microparticles

The encapsulation of phenolics-rich extracts obtained from OMP was carried out by water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation technique as described by Paulo & Santos (2018a, 2018b, 2019, 2020a, 2020c) with slight modifications.

The effect of the theoretical loading content (TLC) of phenolic compounds (F-1 formulation: 5% w/w; and F-2 formulation: 10% w/w) on the release behavior of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol was assessed.

In this regard, the dried extract obtained from OMP was reconstituted in 3 mL of UPW. Successive dilutions were performed to achieve the desired concentrations of phenolic compounds (F-1 formulation: TLC of 5% w/w corresponding to phenolic compounds concentration of 5.3 g_{GAE}/L; F-2 formulation: TLC of 10% w/w corresponding to phenolic compounds concentration of 11.1 g_{GAE}/L). Diluted samples composed the internal aqueous phase (w_1). For each formulation, the organic phase (o) was prepared by admixing 100 mg of the coating material – ethylcellulose – with 10 mL of the dissolution solvent – dichloromethane (polymer concentration of 10 g/L).

The polymer solutions were sonicated for 15 minutes in an ultrasonic bath.

Afterward, to each polymer solution was added 1 mL of the internal aqueous phase (w_1). The w_1/o emulsion was vigorously shaken and vortexed for 5 minutes. The w_1/o emulsion was immediately poured into 100 g of a PVA solution (1 % w/w), and the mixture was emulsified using a high-performance liquid homogenizer at 5 000 rpm for 5 minutes. The evaporation of the solvent that enables the hardening of the particles was fostered by the continuous stirring of the $w_1/o/w_2$ double emulsion in a stirring plate at 700 rpm for 3 hours, in the fume hood at room temperature (20 ± 2 °C). The hardened microparticles were recovered by vacuum filtration using a 0.2 μm Whatman™ nylon membrane filter and washed using 500 mL of distilled water. Afterward, microparticles were collected, frozen for 24 hours at -22 °C, and freeze-dried for 72 hours. Experiments (for each formulation) were performed in triplicate.

7.2.2.2.2 Characterization of phenolics-rich extract-loaded ethylcellulose microparticles

The obtained extract-loaded ethylcellulose microparticles were characterized regarding the (i) encapsulation efficiency of (a) antioxidants, (b) phenolic compounds, and (c) specific phenolic compounds (hydroxytyrosol and tyrosol); and the (ii) actual loading content of loaded microparticles.

The encapsulation efficiencies were determined respectively according to Equation (IV.7.1) similarly to described by Paulo & Santos (2018a):

$$EE_i (\% \text{ w/w}) = \frac{w_i}{w_{l,i}} \times 100 = \frac{w_{l,i} - w_{o,i}}{w_{l,i}} \times 100 = \frac{w_{l,i} - (w_{s,i} + w_{p,i})}{w_{l,i}} \times 100 \quad (\text{IV.7.1})$$

where i denotes the bioactive(s) (e.g., hydroxytyrosol, tyrosol, phenolic compounds, or antioxidants). For each bioactive and cluster of bioactives, the w_i corresponds to the weight of bioactive(s) in the microparticles, which was assessed considering the weight of bioactive(s) initially added for microparticles formulation ($w_{l,i}$) and the weight of non-encapsulated bioactive(s) ($w_{o,i}$).

The $w_{0,i}$ was evaluated considering the weight of bioactive(s) in the recovered supernatant of a sample of a $w_1/o/w_2$ emulsion after 3 hours of microparticles hardening (w_s) after the sample being submitted to a low-intensity centrifugation process (2 670 g, 15 minutes).

Conversely, the w_p corresponds to the weight of bioactive(s) adsorbed at the surface of microparticles. The w_p was evaluated after submitting the $w_1/o/w_2$ emulsion sample to a low-intensity centrifugation process. The pellet recovered from the centrifugation process was reconstituted in UPW, being resubmitted to a centrifugation process for 15 minutes. The w_p corresponded to the weight of bioactive(s) present in the supernatant after the two-step centrifugation process. For each formulation and for each compound or cluster of compounds, the EE was evaluated in triplicate. Results are presented as mean $\pm$ standard deviation.

The actual loading content (ALC) of specific phenolic compounds (hydroxytyrosol and tyrosol), phenolic compounds, and antioxidants was evaluated according to Equation (IV.7.2) as presented by Paulo & Santos (2020b):

$$ALC_i (\% w/w) = \frac{w_i}{w_M} \times 100 = \frac{w_{i,i} - w_{0,i}}{w_M} \times 100 = \frac{w_{i,i} - (w_{s,i} + w_{p,i})}{w_M} \times 100 \quad (\text{IV.7.2})$$

where w_M is the weight of extract loaded ethylcellulose microparticles recovered after freeze-drying. The ALC was evaluated in triplicate for each formulation and for each bioactive or cluster of bioactives. Results were computed as mean $\pm$ standard deviation. The phenolic compounds' weight measurements were processed considering the total phenolic content (TPC) quantification, determined through an UV-Vis spectrophotometric method (detailed description in section IV.7.2.2.8.2.1). The weight of antioxidants was evaluated as described in section IV.7.2.2.8.2.2 by UV-Vis spectrophotometry considering the 2,2-diphenyl-2-picrylhydrazyl radical (DPPH*) scavenging ability. The EE and ALC of hydroxytyrosol and tyrosol were assessed through HPLC analyses.

7.2.2.3 *In vitro* gastrointestinal release studies

In the present study, the INGOGEST model - a standardized procedure for the *in vitro* digestion of dietary compounds in multiple food matrices - developed by the European Cooperation in Science and Technology (COST), was adapted to evaluate the *in vitro* digestion of antioxidants, phenolic compounds and specific phenethyl alcohols (hydroxytyrosol and tyrosol) released from loaded ethylcellulose microparticles (European Union, 2018). The *in vitro* gastrointestinal digestion conditions selected for this study are presented in Table IV.7.1.

The *in vitro* gastrointestinal digestion of natural-occurring bioactive compounds-rich extract-loaded ethylcellulose microparticles was performed in three sequential steps: (i) preparation of the reagents and the digestion tubes, (ii) *in vitro* gastrointestinal digestion procedure, and (iii) treatment and analysis of samples. Detailed descriptions of the methods employed in each step are presented in the following sections.

7.2.2.3.1 Preparation of reagents and digestion tubes

The primary step included the preparation of the simulated salivary fluid (SSF), the simulated gastric fluid (SGF), and the simulated intestinal fluid (SIF), which were obtained diluting electrolyte stock solutions. The electrolytes concentration in each release medium (SSF, SGF, and SIF) was based on human *in vivo* data (Minekus et al., 2014). The electrolyte stock solutions were produced 1.25 times concentrated and store at –22 °C. When required, electrolyte stock solutions were prewarmed at 37 °C, and proper dilutions were then performed.

Additionally, during the (i) preparation of reagents and digestion tubes, time-lagged enzyme activities were determined. For simplicity, enzyme activities are presented in units per mL of final digestion mixture solution rather than their secretion activity. The enzymatic solutions were prepared immediately before the *in vitro* gastrointestinal digestion experiments, preserving enzyme solutions on ice until their admixing with the digestion mixtures.

7.2.2.3.2 *In vitro* gastrointestinal digestion procedure

The *in vitro* gastrointestinal digestion procedure was segmented into three parts: (i) simulated salivary digestion (SSD), (ii) simulated gastric digestion (SGD), and (iii) simulated intestinal digestion (SID).

During the (i) oral phase digestion, each sample (1 g of microparticles) was admixed with 0.8 mL of the SSF and vortexed to obtain a paste-like consistency mixture, similar to a swallowable bolus. Afterward, to this mixture, 5 μ L of a 0.3 M CaCl_2 solution was added, being then added 0.1 mL of α -amylase (concentration of 75 U/mL in the final mixture) solution and 9.5 μ L of UPW. The obtained mixture (microparticles plus simulated salivary digestion fluid [SSFD]) was incubated at 37 °C (Lovibond incubator, Amesbury, United Kingdom) for 2 minutes, while was continuously mixed on an orbital shaker (IKA® KS 130 basic, IKA Works GmbH & Co. KG, Staufen im Breisgau, Germany).

The swallowable mixture obtained after the SSD was admixed with 1.6 mL of the pre-warmed SGF electrolyte solution for the (ii) SGD. Afterward, the pH was adjusted to 3.0, adding 0.1 mL of a 15.6 mM HCl solution.

A 0.3 M CaCl_2 aqueous solution (1 μ L) was added to the previous mixture being followed by the addition of 0.1 mL of porcine pepsin solution (2 000 U/mL in the final mixture). A volume of 199 μ L of UPW was added to achieve a final ratio of 1/1 v/v of the simulated gastric digestion fluid (SGDF) and the SSDF plus microparticles. Samples were incubated at 37 °C, being mixed continuously in an orbital shaker for 2 hours.

The mixture that passed through the gastric digestion was submitted to a SID. The gastric chyme was admixed with 1.7 mL of a pre-warmed SIF. The pH was adjusted to 7.0, adding 0.2 mL of 8.4 mM NaOH aqueous solution. Afterward, 0.5 mL of a bile solution was added (final concentration of 10 mM) followed by the addition of 0.5 mL of a 0.3 M of a CaCl_2 aqueous solution to the previous mixture. Then, 1 mL of pancreatin solution (100 U/mL based on trypsin activity) was added to the mixture. The final step consisted in the addition of 592 μ L of UPW to achieve a 1/1 v/v final ratio of the SIF electrolyte solution and the gastric chyme. The samples were incubated at 37 °C, being promoted the continuous mixing using an orbital shaker for 2 hours.

7.2.2.3.3 Treatment and analyses of samples

Following the gastrointestinal digestion, each digested sample was immediately snap freeze to inhibit the activity of enzymes, which may interfere with the identification and quantification of bioactive compounds. Prior to instrumental analyses, the digested sample was submitted to a liquid-liquid extraction using ethyl acetate as the extraction solvent. The dissolution media present electrolytes that may interfere during the instrumental analyses (e.g. the dissolved inorganic salts present in the dissolution media may precipitate when in contact with organic solvents as acetonitrile and methanol).

The extraction procedure is presented in detail in section IV.7.2.2.8.1 (Solvent extraction of phenolic compounds). The amount of phenolic compounds, antioxidants, and specific phenolic compounds (hydroxytyrosol and tyrosol) were evaluated, as described in section IV.7.2.2.8.2 (Analytical methodologies and instrumental analyses).

The release of each bioactive compound or cluster of compounds was evaluated at 2-time points during the SSD and 4-time points during SGD and SID, in a total run number of 10-time points. One tube per time point in triplicate was considered for each formulation (F-1 and F-2 formulations). Triplicates of individual test tubes were considered due to sampling reproducibility. The experiments were performed in sealed containers with limited headspace, as CO₂ could be released in open containers or vessels with large headspace that would prompt the increase of the pH with time. Stability test tubes were also prepared to evaluate microparticles stability during the exposure to the simulated digestion fluids without enzymes and bile and after each digestion phase. Further considerations are presented in Table IV.7.1.

Table IV.7.1 - Experimental conditions considered during the *in vitro* simulated gastrointestinal digestion of phenolic compounds released from loaded-ethylcellulose microparticles

Digestion mass of microparticles ^a		1.0 g	
Incubation conditions		37 °C, 85 rpm	
Digestion Conditions	Simulated Salivary Digestion Fluid (SSDF) ^b	Simulated Gastric Digestion Fluid (SGDF) ^b	Simulated Intestinal Digestion Fluid (SIDF) ^b
Duration	2 minutes	120 minutes	120 minutes
pH	7	3	7
Electrolyte solution	15.1 mM of KCl; 3.7 mM of KH ₂ PO ₄ ; 13.6 mM of NaHCO ₃ ; 0.15 mM of MgCl ₂ (H ₂ O) ₆ ; 0.06 mM of (NH ₄) ₂ CO ₃	6.9 mM of KCl; 0.9 mM of KH ₂ PO ₄ ; 25.0 mM of NaHCO ₃ ; 47.2 mM of NaCl; 0.1 mM of MgCl ₂ (H ₂ O) ₆ ; 0.5 mM of (NH ₄) ₂ CO ₃	6.8 mM of KCl; 0.8 mM of KH ₂ PO ₄ ; 85 mM of NaHCO ₃ ; 38.4 mM of NaCl; 0.33 mM of MgCl ₂ (H ₂ O) ₆
Volume of electrolyte solution	0.8 mL	1.6 mL	1.7 mL
Enzyme(s); Enzymatic Solution Volume	α -amylase (75 U/mL); 0.1 mL	Pepsin (2 000 U/mL); 0.1 mL	Pancreatin (based on trypsin activity at 100 U/mL); 1 mL
			Bile (10 mM; 8.5 mg/mL); 0.5 mL

Table IV.7.1 - Experimental conditions considered during the *in vitro* simulated gastrointestinal digestion of phenolic compounds released from loaded-ethylcellulose microparticles (cont.)

Digestion Conditions	Simulated Salivary Digestion Fluid (SSDF) ^b	Simulated Gastric Digestion Fluid (SGDF) ^b	Simulated Intestinal Digestion Fluid (SIDF) ^b
Volume of 0.3 M of CaCl ₂	5 µL	1 µL	8 µL
Volume of HCl solution	-	0.1 mL (15.6 mM)	-
Volume of NaOH solution	-	-	0.2 mL (8.4 mM)
Volume of added UPW	0.095 mL	0.199 mL	0.592 mL

^a The digestion conditions presented were adapted from the INFOGEST model;

^b Simulated Digestion Fluids (SSDF, SGDF, and SIDF) differ from the Simulated Fluids (SSF, SGF, and SIF) as simulated fluids (SSF, SGF, and SIF) are only the electrolyte solutions added to the simulated digestion fluids (SSDF, SGDF and SIDF);

rpm – rotations per minute; SGDF – Simulated Gastric Digestion Fluid; SIDF – Simulated Intestinal Digestion Fluid; SSDF – Simulated Salivary Digestion Fluid; U – Enzymatic Units; UPW – Ultrapure Water;

7.2.2.4 Assessment of the bioaccessibility of phenolic compounds released from loaded ethylcellulose microparticles

The bioaccessibility corresponded to the fraction of phenolic compounds released from loaded ethylcellulose microparticles in the gastrointestinal tract that becomes available for absorption. It corresponded to the concentration of each compound (hydroxytyrosol) or cluster of compounds (phenolic compounds and antioxidants) in the *digestas*. It was calculated as described by Gómez-Mascaraque et al. (2017) and Paulo & Santos (2020c) with slight modifications and as presented in Equation (IV.7.3):

$$BA_i (\% \text{ w/w}) = \frac{C_{D,i}}{C_{T,i} \times EE_i} \times 100 \quad (\text{IV.7.3})$$

where i denotes the bioactive(s) (e.g., hydroxytyrosol, tyrosol, phenolic compounds or antioxidants), BA_i is the bioaccessibility of the compound(s) i , $C_{D,i}$ is the concentration of i in the *digestas*, $C_{T,i}$ is the theoretical concentration of i , and EE_i is the encapsulation efficiency of i .

The *digestas* was submitted to a low-intensity centrifugation process (4 000 rpm corresponding to 2 670 g, using a centrifuge Rotofix 32A, Hettich) for 30 minutes. The supernatant was then collected, and bioactives were extracted by solvent extraction using ethyl acetate as the extraction solvent. The concentration of HXT and TYR in the *digestas* was determined by HPLC, and the concentration of phenolic compounds and antioxidants were determined by UV-Vis spectrophotometry methods (Section IV.7.2.2.8.2.2). Each sample was analyzed, considering six replicates. Results are presented as mean $\pm$ standard deviation.

7.2.2.5 Mathematical modeling of the release profile of phenolic compounds from loaded ethylcellulose microparticles

The release behavior of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol from loaded ethylcellulose microparticles was evaluated through (i) model-dependent methods (zero-order, first-order release kinetics models, the Korsmeyer-Peppas, the Higuchi, the Weibull models) and a model-independent method – the similarity factor – as described in the following sections.

7.2.2.5.1 Assessment of the release behavior of phenolic compounds from loaded ethylcellulose microparticles through model-dependent methods

Mathematical models for drug release, namely the zero, first, Higuchi, Korsmeyer-Peppas, and Weibull models were applied to describe and evaluate the release profiles of bioactive compounds.

The delivery systems in which microparticles do not disintegrate and the release(s) occur(s) slowly can be described by the zero-order release model as presented in Equation (IV.7.4):

$$Q_{t,i} = Q_{0,i} + K_{0,i} t \quad (\text{IV.7.4})$$

where i denotes the bioactive(s) (e.g., hydroxytyrosol, tyrosol, phenolic compounds or antioxidants), $Q_{t,i}$ is the cumulative release of i at the time t , $Q_{0,i}$, the initial amount of i (in this study, it was assumed for all bioactives that $Q_0 = 0$) and $K_{0,i}$ represents the zero-order release constant of the compound(s) i . This relationship describes the dissolution profile of low soluble bioactive(s) in the coating material (Dash et al., 2010).

The release behavior of phenolic compounds which followed a first release kinetics can be expressed as by Equation (IV.7.5):

$$\frac{dC_i}{dt} = -K_{1,i} C_i \quad (\text{IV.7.5})$$

where $K_{1,i}$ represents the first-order rate constant of i expressed in units of time^{-1} .

The Equation (IV.7.5) can be presented alternatively as depicted in Equations (IV.7.6) and (IV.7.7):

$$Q_{t,i} = Q_{0,i} e^{-K_{1,i} t} \quad (\text{IV.7.6})$$

$$\log_{10} Q_{t,i} = \log_{10} Q_{0,i} - \frac{K_{1,i} t}{2.303} \quad (\text{IV.7.7})$$

For each compound or cluster of compounds, the *in vitro* gastrointestinal release data was plotted as cumulative log percentage of bioactive(s) remaining *versus* time, which yield a straight line with a slope of $\frac{K_{1,i}}{2.303}$. The first-order release kinetic model is useful to describe the release behavior of water-soluble compounds from porous microparticles.

The mathematical model that describes the bioactive compound release from a matrix system was first developed by Higuchi in 1961.

The Higuchi release model is based on the following hypotheses: (i) the initial concentration of the bioactive compound in the matrix is much higher than its solubility in the release medium; (ii) the diffusion occurs only in one dimension (edge effect must be negligible); (iii) the particles are much smaller than the release system thickness; (iv) the dissolution and swelling of the matrix are considered to be negligible; (v) the bioactive compound diffusivity is constant; and (vi) sink conditions are reached in the release environment.

Hence, the Higuchi model can be expressed as presented in Equation (IV.7.8):

$$Q_{t,i} = K_{H,i} \sqrt{t} \quad (\text{IV.7.8})$$

where $K_{H,i}$ represents the Higuchi release constant of the compound(s) i .

A simple relationship that allows the description of bioactive(s) release, in the polynomial equation system, was presented by Korsmeyer et al. (1983) (Equation (IV.7.9)).

$$\frac{Q_{t,i}}{Q_{\infty,i}} = K_{K,i} t^{n_i} \quad (\text{IV.7.9})$$

where $Q_{t,i}/Q_{\infty,i}$ is the fraction of i released at time t , $K_{K,i}$ denotes the release constant and n_i represents the release exponent. The Korsmeyer-Peppas model allows determining the release mechanism. For that, the first 60% of the release data were fitted in the model presented in Equation (IV.7.9), being the n_i value, an indicator of the release mechanism: $n_i = 0.5$ corresponds to a Fickian diffusion at a rate of $t^{0.5}$; in the case of $0.45 < n_i < 0.89$, a non-Fickian transport of i at a rate of t^{n-1} is observed; when $n_i = 0.89$, a Case II (relaxational) transport phenomenon (zero-order release rate) is noted; in the cases $n_i > 0.89$, the transport mechanism is described as Super Case II transport at a release rate of t^{n-1} .

Data obtained from the *in vitro* release studies were plotted as log cumulative percentage release *versus* log time as presented in Equation (IV.7.10):

$$\ln \left(\frac{Q_{t,i}}{Q_{\infty,i}} \right) = n_i \ln t + \ln K_{K,i} \quad (\text{IV.7.10})$$

The Weibull model, presented in Equation (IV.7.11), has been widely employed to described different dissolution processes being described its uniqueness in the model comparison release profiles of bioactive(s) from matrix-type delivery systems.

$$M_{t,i} = M_{\infty,i} \left[1 - e^{-\left(\frac{t-t_0}{\tau_{d,i}}\right)^{\beta_i}} \right] \quad (\text{IV.7.11})$$

where, $M_{t,i}$ (%) represents the release/dissolution of i at time t (minutes), $M_{\infty,i}$ (%) the release/dissolution of i at infinite time. The t_0 denotes lag-time (minutes) of the release/dissolution (in this study, for all the i , it was considered $t_0 = 0$), the β_i is the parameter related to the shape of the curve and τ_d corresponds to the time (minutes) when 63.2% of M_i has been released/dissolved. For $\beta_i = 1$, the shape of the release curve corresponds to the shape of an exponential profile, if $\beta_i > 1$ the release curve presents a sigmoidal shape with a turning point, whereas $\beta_i < 1$, the curve shows a steeper increase than the corresponding curve for $\beta_i = 1$. Data from release studies were plotted, as presented in Equation (IV.7.12):

$$\ln \left(\ln \left(\frac{1}{\frac{1}{M_{t,i}} - \frac{1}{M_{\infty,i}}}} \right) \right) = \beta_i \ln(t-t_0) - \beta_i \ln \tau_{d,i} \quad (\text{IV.7.12})$$

7.2.2.5.2 Comparison of release profiles of phenolic compounds from loaded ethylcellulose microparticles through a model-independent method

The similarity factor (f_2) - the logarithmic reciprocal square root transformation of the sum of squared error – was chosen as a model-independent method to evaluate the similarity between release profiles (Dash et al., 2010; Demirturk & Oner, 2005; Diaz et al., 2016; Shah et al., 1998).

The f_2 values were computed considering Equation (IV.7.13) as follow:

$$f_2 = 50 \times \log \left\{ 1 + \left[\frac{1}{n} \times \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \times 100 \right\} \quad (\text{IV.7.13})$$

where n is the number of time points, R_t is the dissolution/release value of the reference (pre-change batch) at time t , T_t is the dissolution/release value of the test (post-change batch) at time t , and $\log$ is the logarithm based on 10. The f_2 values range between 0 and 100. High values of f_2 indicate similarity among the release curves evaluated. As a consequence of batch-to-batch variations, it is not expected that f_2 is close anywhere to 0 and 100.

Therefore, to ascertain similarity between release profiles, the empirical consideration of - higher values of f_2 than 50 guarantee similarity between dissolution profiles – was considered (Demirturk & Oner, 2005; Paulo & Santos, 2020c).

7.2.2.8 Analytical methodologies and instrumental analyses

The analytical methodologies and instrumental analyses considered for the identification and quantification of antioxidants, phenolic compounds, and specific phenolic compounds such as hydroxytyrosol and tyrosol, are presented in the following sections. Moreover, the description of the pretreatment procedure employed to samples after the *in vitro* release studies and prior to instrumental analyses is also presented, detailly in section IV.7.2.2.3.3.

7.2.2.8.1 Solvent extraction of phenolic compounds

The samples of the *in vitro* release studies were submitted to a liquid-liquid extraction prior to instrumental analysis as the dissolution media present inorganic salts that may precipitate in contact with organic solvents (e.g., acetonitrile, methanol), which would constitute interferences to the identification and quantification of compound(s). Moreover, compounds precipitation would trigger challenges at an equipment level (e.g., clogging of columns).

Previous experiments were performed to evaluate the most suitable solvent and the number of extraction steps employed. In this context, the one-step liquid-liquid extraction using ethyl acetate was chosen to extract bioactives dissolved in the release media. Extractions were performed in triplicate.

In brief, 1 mL of each tube – corresponding to alternative *in vitro* time points – was transferred to a 15 mL Falcon tube (polypropylene tube; conical bottom) being admixed with 6 mL of ethyl acetate. The mixture was then vortexed for 1 minute and ultrasonicated for 15 minutes in an ultrasonic bath at room temperature (20 ± 2 °C). Afterward, the aqueous and organic phases were separated by centrifugation (2 670 g, 15 minutes).

Subsequently, a volume of 5.4 mL of the organic phase – corresponding to 90% of the extraction solvent initial volume - was withdrawn to a 12 mL amber and evaporated to dryness under a gentle nitrogen stream. After dryness, the sample was solubilized in 1 mL of UPW. The final step included the transference of the solubilized sample in UPW to an amber vial for instrumental analysis.

The amount of *i* recovered after the *in vitro* release studies, and the solvent extraction procedure was evaluated through the calculation of the recovery (% R_i) percentage considering the standard addition method as presented in Equation (IV.7.14).

$$\%R_i = \frac{m_{f,i} - m_{i,i}}{m_{a,i}} \times 100 \quad (\text{IV.7.14})$$

where *i* denotes the bioactive(s), $m_{f,i}$ is the mass of *i* analyzed after fortification, $m_{i,i}$ is the weighted mass of *i* before fortification and $m_{a,i}$ is the mass of *i* to be analyzed. For each formulation, the recovery experiments were performed in triplicate. The results from the recovery procedures are presented in the Annex III (IV.7.S1). The presented results regarding the *in vitro* release studies consider the recovery percentages obtained Annex III.

7.2.2.8.2 Analytical methodologies and instrumental analyses

Throughout this study, analytical methodologies were developed to evaluate the total phenolic content, the total antioxidant activity, and to identify individual phenolic compounds, (i) on the extracts, (ii) on microparticles, and (iii) during the *in vitro* release studies.

7.2.2.8.2.1 Determination of the total phenolic content

The total phenolic content (TPC) was determined by the Folin-Ciocalteu micro-method, as described by Barroso et al. (2016), based on a scale-down procedure of the original method proposed by Singleton et al. (1999).

Briefly, in each well, a volume of 25 μL of the sample or standard solution was admixed with 75 μL of UPW and 25 μL of the Folin-Ciocalteu reagent (diluted 1/1 v/v in UPW). The homogenization of the reaction mixture was promoted through continuous shaking on an orbital shaker (400 rpm; room temperature) for 1 minute, followed by incubation for 5 minutes in the dark, at room temperature. Afterward, to the previous reaction mixture was added 100 μL of a sodium carbonate solution (75 g/L). Subsequently, the mixture was homogenized for 1 minute at 400 rpm on an orbital shaker and incubated for 2 hours, protected from light at room temperature ($20 \pm 2^\circ\text{C}$). The absorbance was read using a microplate reader (Synergy HT, Biotek, USA) at 760 nm.

Gallic acid was selected as a standard for the calibration curve. The obtained calibration curve – mean calibration curve - was based on three independent calibration curves.

Spectrophotometric analyses were performed in triplicate. The TPC values were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry OMP ($\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$) or as milligrams of GAE per gram of dry weight of microparticles ($\text{mg}_{\text{GAE}}/\text{g}_{\text{MP}}$) or even as grams of GAE per liter of extract ($\text{g}_{\text{GAE}}/\text{L}$).

7.2.2.8.2.2 Determination of the total antioxidant activity

The total antioxidant activity (TAA) was determined through the estimation of the free radical-scavenging ability (RSA) of samples (e.g., extracts, microparticles, time-points during the *in vitro* release studies) using the 2,2-diphenyl-2-picrylhydrazyl radical (DPPH[•]) (El-Abbassi et al., 2012).

The RSA was evaluated considering a microplate method described by Prieto (2012), with slight modifications. Accordingly, a 0.2 mM DPPH[•] stock solution in methanol was prepared and a negative calibration curve – based on three independent calibration curves – was obtained considering multiple dilutions of the DPPH[•] stock solution.

To each well was added 100 µL of methanol followed by the addition of the same volume of sample/standard. The content of each well was diluted 8 times, being added, afterward, 100 µL of the DPPH[•] methanolic solution.

Reaction mixtures were covered with microplate lid to minimize methanol evaporation and wrapped in aluminum foil to protect the sensitive radical to light degradation. The reaction mixture was homogenized for 5 minutes and incubated for 45 minutes. The absorbance was read using a microplate reader at 515 nm.

The inhibition percentage of DPPH[•] discoloration (%I) was calculated based on Equation (IV.7.15) as follow:

$$\% I = \frac{A_0 - A_1}{A_0} \times 100 \quad (\text{IV.7.15})$$

where A_0 is the absorbance of the control reaction (DPPH[•] in methanol) and A_1 is the absorbance of the DPPH radical plus tested sample in methanol. The computation of the %I against the logarithmical concentration permitted the half-inhibitory concentration (IC₅₀) calculation by linear regression analysis, as described by Ahsan et al. (2010). Spectrophotometric analyses were performed in triplicate, and results are presented as mean ± standard deviation.

7.2.2.8.2.3 Analysis of individual phenolic compounds by reserved-phase high-performance liquid chromatography (RP-HPLC)

The selected phenolic compounds – hydroxytyrosol and tyrosol – were identified and quantified by following the HPLC procedure proposed by Tasioula-Margari & Tsabolatidou (2015) with slight modifications. The analyses were performed on a Merck Hitachi Elite LaChrom (Tokyo, Japan) high-performance liquid chromatograph equipped with a Hitachi L-7100 pump and L-7250 autosampler and coupled to a L-7450A diode array detector. Samples and standards were injected in a Purospher® STAR RP-18 end-capped LiChroCART® column (250 mm x 4.0 mm, 5.0 µm) (Merck KGaA), attached to a guard column (4.0 mm x 4.0 mm, 5.0 µm) of the same type.

The mobile phase was composed of solvent (A) UPW/acetic acid (97.5/2.5 v/v, pH 2) and solvent (B) acetonitrile/methanol (50/50 v/v). The solvent gradient was as follows: 0-5% B in 5 minutes, 5-30% B in 30 minutes, and 30-5% B in 5 minutes. A flow rate of 1 mL/min was considered, and 99 µL of the sample was injected.

Phenolic compounds were identified and quantified by comparing their retention times and UV-Vis spectral data to known previously injected standards. The absorbance of the eluate at 280 was monitored at 1 s intervals. HPLC analyses were performed in triplicate, and results are presented as mean ± standard deviation. Results were presented as milligrams of HXT or TYR per kilogram of dry weight of microparticles (mg/kg_{MP}) or grams per liter of extract (g/L extract).

7.2.3 Statistical Analysis

All the experiments were performed in triplicate minus the experiments required for the bioaccessibility determinations that were carried out considering six replicates. Thus, the data were presented as the mean (n=3 or n=6) ± standard deviation. One-way analysis of variance (one-way ANOVA) was used to determine significant differences. Values of $p < 0.05$ were considered statistically significant.

7.3 Results and Discussion

7.3.1 Instrumental Analysis

Analytical methodologies were developed to assess the amount of antioxidants, phenolic compounds, and specific phenolic compounds, namely hydroxytyrosol and tyrosol, on extracts and encapsulated in microparticles. Moreover, the development of analytical methodologies was required to evaluate the amount of these bioactive compounds released during the *in vitro* gastrointestinal studies.

Alternative calibration curves are presented in Table IV.7.S2 of the Annex III. Calibration curves were drawn based on three independent calibration curves. All calibration curves presented a significantly low value (<5%) of the relative standard error of the slope (S_a/a), and the obtained values of the limit of detection (LOD) and the limit of quantification (LOQ) were satisfactorily low, empowering the application of the obtained regression equations for the quantification of the selected bioactive compounds.

7.3.2 Olive mill pomace extract characterization

The obtained extract (n=3) from the two-step solid-liquid extraction procedure exhibited a TPC of 50.5 ± 1.5 mg/g_{OMP} and a TAA of $79.1 \pm 7.9\%$. To the authors' best knowledge, there is a lack of published data suitable for the direct comparison regarding the obtained results.

The total content of hydroxytyrosol and tyrosol in the obtained extract was evaluated through HPLC analyses. The amount of hydroxytyrosol and tyrosol was $2\,870.2 \pm 1.2$ mg/kg_{OMP} and $1\,622.1 \pm 2.2$ mg/kg_{OMP}, respectively. The presented results are in line with the ones obtained by Japón-Luján & De Castro (2007) in which the authors performed the extraction of hydroxytyrosol, tyrosol, and other olive biophenols using static-dynamic superheated liquids.

It was observed a higher amount of hydroxytyrosol rather than tyrosol in the final extract, which is in agreement with several previous investigations on the extraction of PCs from OMP samples (Obied et al., 2005, 2007; Rubio-Senent et al., 2012; Suárez et al., 2009, 2010; Uribe et al., 2015).

Considerations regarding the amount of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol present in the extract and the solutions used for encapsulation (F-1 and F-2 formulations) are presented in Table IV.7.2.

Table IV.7.2 - Characterization the antioxidant activity, phenolic, hydroxytyrosol (HXT) and tyrosol (TYR) content of the obtained extract (EXT) and solutions used during encapsulation (F-1 and F-2 formulations)

<i>For.</i>	<i>Polyphenols content</i>		<i>Antioxidant Activity</i>		<i>HXT content</i>		<i>TYR content</i>	
	<i>mg_{GAE}/g_{OMP}</i>	<i>g_{GAE}/L extract</i>	<i>IC₅₀ mg/g_{OMP}</i>	<i>g/L extract</i>	<i>mg/g_{OMP}</i>	<i>g/L extract</i>	<i>mg/g_{OMP}</i>	<i>g/L extract</i>
EXT ^{a,b}	50.5 ± 1.5	16.8 ± 0.5	223.9 ± 1.2	74.6 ± 0.4	2.9 ± 0.2	1.0 ± 0.1	1.6 ± 0.4	5.3 ± 0.1
F-1 ^{a,c}	15.8 ± 0.4	5.3 ± 0.7	70.1 ± 0.4	23.3 ± 0.1	0.9 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	1.7 ± 0.2
F-2 ^{a,d}	33.4 ± 0.2	11.1 ± 0.6	148.1 ± 0.2	49.3 ± 0.1	1.9 ± 0.1	0.6 ± 0.1	1.1 ± 0.1	3.5 ± 0.1

^a Results are presented as mean ± standard deviation (n=3)

^b Data relative to the obtained extract after extraction

^c Data relative to the F-1 formulation (theoretical loading content in phenolic compounds of 5% w/w)

^d Data relative to the F-2 formulation (theoretical loading content in phenolic compounds of 10% w/w)

EXT – Extract; For. – Formulation; HXT – Hydroxytyrosol; IC₅₀ - Half-inhibitory Concentration; OMP – Olive Mill Pomace; TYR – Tyrosol;

7.3.3 Olive Mill Pomace Extract Loaded Ethylcellulose Microparticles Characterization

The $w_1/o/w_2$ double emulsion solvent evaporation microencapsulation technique was successfully applied to obtain olive mill pomace extract loaded ethylcellulose microparticles were formulated considering two alternative TLC on phenolic compounds, namely 5% w/w and 10% w/w.

Results regarding the encapsulation efficiencies of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol into ethylcellulose microparticles considering two loading contents in phenolic compounds are presented in Table IV.7.3. The encapsulation efficiencies varied from $83.7 \pm 2.0\%$ (antioxidants; F-2 formulation) to $96.0 \pm 0.3\%$ (phenolic compounds; F-1 formulation). Most antioxidants were efficiently entrapped into ethylcellulose microparticles. Increasing the loading content led to a decrease in encapsulation efficiency ($p < 0.05$). Differences were found between the EE of PCs from the two loadings considered ($p < 0.05$). Similarly to the obtained results regarding the EE of AOs, it was verified a decrease in EE with the increase in the loading content. In the case of simple phenethyl alcohols – HXT and TYR – it was not verified differences ($p > 0.05$) between the two loading contents.

Results regarding the ALC and the TLC of selected bioactives in the present study are presented in Table IV.7.4. The actual loading contents were similar to the TLC considered, reinforcing the efficient incorporation of phenolic compounds into ethylcellulose microparticles.

Microparticles formulated considering a TLC in phenolic compounds of 5% w/w presented an ALC of $4.8 \pm 1.2\%$. The higher weight of bioactive compounds incorporated in ethylcellulose microparticles (F-1 formulation) were antioxidants - $17.5 \pm 2.9\%$ of the weight of microparticles corresponded to antioxidants' weight. Considering the ALC of phenethyl alcohols – hydroxytyrosol and tyrosol – it was verified an increase of 5 times fold on the ALC of TYR compared to HXT ($ALC_{HXT} = 0.3 \pm 0.1\%$; $ALC_{TYR} = 1.5 \pm 0.3\%$; F-1 formulation).

The F-2 formulation – prepared considering a TLC in phenolic compounds of 10% w/w – contained a high content antioxidants ($ALC_{AO} = 27.6 \pm 2.1\%$).

About 9% of microparticles' weight was phenolic compounds ($ALC_{PC}=9.4 \pm 0.8\%$). Similarly to F-1 formulation, during F-2 experiments, it was observed that the ALC of TYR was about 6 times higher than the ALC of HXT ($ALC_{HXT}=0.5 \pm 0.2\%$; $ALC_{TYR}=3.0 \pm 0.1\%$; F-2 formulation).

The obtained results regarding the encapsulation efficiencies and the actual loading contents were considered to be appropriate considering the aims of the present study.

Table IV.7.3 - Results regarding the encapsulation efficiency (EE) of antioxidants (AO), phenolic compounds (PC), hydroxytyrosol (HXT), and tyrosol (TYR) in F-1 and F-2 formulations

BAC(s)	EE (% w/w) ^a	
	F-1 formulation ^b	F-2 formulation ^b
AO	92.6 $\pm$ 2.1	83.7 $\pm$ 2.0
PC	96.0 $\pm$ 0.3	94.1 $\pm$ 3.1
HXT	92.7 $\pm$ 2.3	90.8 $\pm$ 5.9
TYR	88.7 $\pm$ 0.6	89.3 $\pm$ 4.8

^a Results are presented as mean $\pm$ standard deviation (n=3)

^b Formulations differ on the loading content: a theoretical loading content in phenolic compounds of 5% w/w was considered during F-1 experiments, whereas during F-2 experiments, theoretical loading content in phenolic compounds of 10% w/w was appraised

AO – Antioxidants; BAC – Bioactive Compound; EE – Encapsulation Efficiency; HXT – Hydroxytyrosol; PC – Phenolic Compounds; TYR – Tyrosol;

Table IV.7.4 - Results regarding the actual loading content (ALC) compared to the theoretical loading content (TLC) of antioxidants (AO), phenolic compounds (PC), hydroxytyrosol (HXT), and tyrosol (TYR) in F-1 and F-2 formulations

<i>BAC(s)</i>	ALC (% w/w) ^a	TLC % w/w)	ALC (% w/w) ^a	TLC % w/w)
	F-1 formulation ^b		F-2 formulation ^b	
AO	17.5 ± 1.6	18.9 ± 2.7	27.9 ± 2.1	33.0 ± 2.4
PC	4.8 ± 0.9	5.0 ± 0.4	9.4 ± 1.0	10.0 ± 1.3
HXT	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.6 ± 0.0
TYR	1.5 ± 0.6	1.7 ± 0.3	3.0 ± 0.5	3.4 ± 1.2

^a Results are presented as mean ± standard deviation (n=3)

^b Formulations differ on the loading content: a theoretical loading content in phenolic compounds of 5% w/w was considered during F-1 experiments, whereas during F-2 experiments, theoretical loading content in phenolic compounds of 10% w/w was appraised

ALC – Actual Loading Content; AO – Antioxidants; BAC – Bioactive Compound; HXT – Hydroxytyrosol; PC – Phenolic Compounds; TLC – Theoretical Loading Content; TYR – Tyrosol

7.3.4 *In vitro* release studies of phenolic compounds for loaded ethylcellulose microparticles

The gastrointestinal digestion precedes the adsorption and metabolism of bioactive(s). Therefore, it modulates the bioaccessibility of bioactive compounds by affecting their digestive stability and bioaccessibility (Saura-Calixto et al., 2007; Ydjedd et al., 2017).

The *in vitro* release profiles of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol during the SSD, SGD, and SID, are presented in Figure IV.7.1. Additionally, data regarding the amount released (in % w/w) of bioactive compound(s), after each simulated digestion step (salivary, gastric, and intestinal) are presented in Table IV.7.5.

It was not observed differences between the amount of bioactives released during the SSD ($p > 0.05$), comparing the results presented in Table IV.7.5 and the slope of the release curves (Figure IV.7.1). For all bioactives, it was observed a low release amount of all bioactive compounds during the SSD.

The amount of bioactives released was lower than 10% during the *in vitro* SSD. Therefore, it can be concluded that through microencapsulation, antioxidants, especially the phenethyl alcohols – hydroxytyrosol and tyrosol - were efficiently protected during the SSD. These results reinforce the salivary protection of phenolic compounds exhibiting outstanding radical scavenging activity – hydroxytyrosol and tyrosol. From the loading content study, in each study group (individual bioactives such as hydroxytyrosol and tyrosol; and a cluster of bioactives such as PCs and AOs), it was not observed differences ($p>0.05$) among alternative loading contents of bioactive compounds. These results can be explained considering the reduced SSD duration - samples were processed for only 2 minutes. Moreover, the enzyme α -amylase was added to the SSDF, as described in Section IV.7.2.2.3. Accordingly, the α -amylase enzyme catalyzes the hydrolysis of the glycosidic bonds α -D-(1 $\rightarrow$ 4), which are absent ethylcellulose – the coating material used to encapsulate extracts obtained from OMP samples. Nonetheless, it was observed that a residual release phenolic compounds during the SSD. Probably, some molecules of phenolic compounds adsorbed at the surface of microparticles were released into the SSDF. Likely, the water present in the SSDF promoted the hydrolysis of hydrogen bonds between the ethylcellulose, and the phenolic compounds adsorbed at the surface of microparticles. The obtained results are in agreement with our previous studies regarding the release of the individual compounds – hydroxytyrosol and tyrosol: the amount of both phenolic compounds released was remarkably low (Paulo & Santos, 2018a, 2020c).

Examining the results from the *in vitro* SGD, statistical differences were found between groups (AO, PCs, and phenolic acids – HXT and TYR) ($p<0.05$), but there was not observed differences within each group among the alternative loading contents ($p>0.05$).

The amount of HXT released was kept incredibly low – for both formulations, the maximum release was below 3%. To assess whether the observed HXT release corresponded to the actual release value or if the HXT was released and immediately degraded - which would explain the low observed value – spiked extract samples ($n=3$) were submitted to the *in vitro* gastrointestinal digestion.

The recovery of HXT was $96.6 \pm 0.3\%$ in the case of the extract sample spiked with the same amount of HXT encapsulated during F-1 formulation experiments (ALC_{HXT} of $29.0 \pm 0.4 \text{ mg}_{HXT}/\text{g}_{MP}$) and $97.2 \pm 0.5\%$ when the sample was spiked, considering an ALC_{HXT} of $63.2 \pm 0.8 \text{ mg}_{HXT}/\text{g}_{MP}$ corresponding to the encapsulated amount of HXT during F-2 formulation experiments. Therefore, it can be concluded that the gastric digestion did not lead to chemical modifications of HXT. A similar conclusion was drawn by Pereira-Caro et al. (2012) when the authors studied the digestive stability of hydroxytyrosol and some related compounds. The authors reported that the SSD did not affect the antioxidant activity of HXT – similarly to observed in the present study.

Tyrosol exhibited a similar release pattern to HXT during the SGD. Even though the amount of TYR released from loaded microparticles was about 6 times and 4 times fold than HXT, considering the F-1 formulation and the F-2 formulation, respectively, the amount of TYR released was kept below 17%. Similarly to HXT, the recovery studies prove that TYR did not suffer relevant chemical transformations during the *in vitro* SGD.

It was not observed differences ($p>0.05$) between the amount of PCs released from F-1 and F-2 formulations. The amount of PCs released was about 50% of the amount encapsulated. The TPC determined as the sum of individuals (hydroxytyrosol and tyrosol) and by the F-C method did not show significant correlation ($R=0.367$, $p>0.05$), which suggest that other compounds different than hydroxytyrosol and tyrosol were present in the encapsulated extract and react with the Folin–Ciocalteu reagent and also contribute to their antioxidant properties.

The amount of AOs released during the SGD was about 31% and 46%, considering F-1 and F-2 formulations. No differences ($p>0.05$) were found between the loadings considered.

The F-C reagent is able to interact with a variety of non-PCs as amino acids, sugars, vitamin C, and even with other organic acids, which may result in the overestimation of the TPC. The higher TAA cannot be invariably attributed to the higher TPC. In this context, the correlation between the TPC and the TAA was evaluated for both F-1, F-2, and non-encapsulated samples (Table IV.7.6).

It was verified strong relationships between the TPC and TAA during the SGD ($R=0.996$ in the case of F-1 formulation and $R=0.997$ in the case of F-2 formulation). Therefore, it can be concluded that essentially, the PCs were the class of bioactive compounds that highly contributed to the antioxidant activity during SGD. The antioxidant activity of extracts released during digestion was mainly linked to microparticles' phenolic content. These results are in accordance with the findings of Rodríguez-Solana et al. (2019) and Ydjedd et al. (2017).

Examining the *in vitro* release study results after the SID, it can be concluded that the majority of the encapsulated phenolic compounds were released - about or more than 80% of encapsulated bioactive(s) or cluster of bioactives were released in the SIDF and prompt to suffer the adsorption, absorption and metabolism processes. It was not observed differences among the loading content for PCs, AOs, and HXT. However, the release of TYR from F-1 and F-2 formulation was different ($p<0.05$) – the amount of tyrosol released during the *in vitro* SID was dependent on the loading content of PCs being verified a higher release of TYR when it was considered the lowest loading content (TLC of PCs of 5% w/w corresponding a ALC_{TYR} of $1.5 \pm 0.6\%$). This observed phenomenon can be explained accounting that microparticles encapsulated higher loading contents – present generally bigger mean particle size and a slower release rate (Gaspar et al., 1998). The results are in agreement with the ones obtained in our previous study regarding the encapsulation of tyrosol into ethylcellulose microparticles: the amount of TYR released from TYR-loaded ethylcellulose was higher considering a lower TLC (Paulo & Santos, 2020c).

In the present study, the release rate of HXT was improved about 30 times fold, comparing to a similar study regarding the incorporation of HXT into ethylcellulose microparticles (Paulo & Santos, 2018a). The formulation in a more complex matrix – the OMP extract – prompted a higher release of HXT.

Considering the all gastrointestinal digestion, it was not verified differences ($p>0.05$) between the two alternative loadings considered within each group. Therefore, it can be concluded that microparticles should be formulated considering the lowest loading content (5% w/w on phenolic compounds).

The formulation of microparticles considering a TLC of 5% w/w will reduce the procedure costs, comparing to the formulation of microparticles considering a TLC of 10% w/w, no compromising the amount of phenolic compounds released during the *in vitro* gastrointestinal digestion.

It was verified a higher release of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol during the simulated intestinal phase and lower during the gastric phase digestion compared to the non-encapsulated extracts (data not shown). Therefore, phenolic compounds were efficiently protected during the *in vitro* gastrointestinal digestion, especially against gastrointestinal environmental conditions such as pH variations and enzymes. The results of this study are in accordance with the ones obtained by Flores et al. (2015) when the authors were evaluating the *in vitro* release properties of encapsulated blueberry (*Vaccinium ashei*) extracts. Similarly to the present study, Flores et al. (2015) verified an increased amount of PCs released throughout gastric to intestinal digestion.

Table IV.7.5 - Amount (% w/w) of antioxidants (AO), phenolic compounds (PC), hydroxytyrosol (HXT), and tyrosol (TYR) released after each *in vitro* gastrointestinal digestion step

BAC(s)	Formulation ^a	Amount Released ^b (% w/w)		
		SSD	SGD	SID
AO ^c	F-1	1.6 ± 0.3	30.7 ± 4.6	92.6 ± 3.9
	F-2	1.5 ± 0.6	45.6 ± 4.8	82.2 ± 5.7
PC ^d	F-1	3.8 ± 0.8	47.0 ± 2.9	84.5 ± 2.7
	F-2	4.8 ± 0.9	47.6 ± 2.4	79.5 ± 2.4
HXT ^e	F-1	0.03 ± 0.02	2.7 ± 0.1	96.9 ± 3.9
	F-2	0.03 ± 0.01	2.6 ± 0.1	84.0 ± 4.5
TYR ^e	F-1	0.07 ± 0.01	16.4 ± 0.6	93.1 ± 3.2
	F-2	0.09 ± 0.02	11.3 ± 0.7	65.8 ± 2.4

^a Formulations differ on TLC of PC (F1 – TLC of 5% w/w; F-2 – TLC of 10% w/w)

^b Mean released amounts are based on three measurements

^c Determined by means of DPPH radical-scavenging ability method

^d Determined considering the F-C spectrophotometric method

^e Determined by means of a HPLC method

AO – Antioxidants; BAC – Bioactive Compound; HXT – Hydroxytyrosol; TYR – Tyrosol; SSD – Simulated Salivary Digestion; SID – Simulated Intestinal Digestion; TLC – Theoretical Loading Content; TYR – Tyrosol;

Table IV.7.6 - Correlation coefficients between the total phenolic content and the total antioxidant activity of F-1 and F-2 formulations during *in vitro* release studies

Designation		TPC-DPPH correlation
Raw Material		0.997 ^a
F-1	Non-encapsulated ^a	0.998 ^a
	SSD	0.989 ^a
	SGD	0.996 ^a
	SID	0.997 ^a
F-2	Non-encapsulated ^a	0.998 ^a
	SSD	0.991
	SGD	0.997 ^a
	SID	0.998 ^a

^a The correlation between the TPC and the TAA was evaluated for the extracts samples that were considered for encapsulation purposes, differing on the loading content of phenolic compounds

^b The correlation coefficients are significant at $p < 0.05$

DPPH – 2,2-diphenyl-2-picrylhydrazyl Radical; F-1 – Formulation considering a theoretical loading content in phenolic compounds of 5% w/w; F-2 – Formulation considering a theoretical loading content in phenolic compounds of 10% w/w; SGD – Simulated Gastric Digestion; SID – Simulated Intestinal Digestion; SSD – Simulated Salivary Digestion; TAA – Total Antioxidant Activity; TPC – Total Phenolic Content;

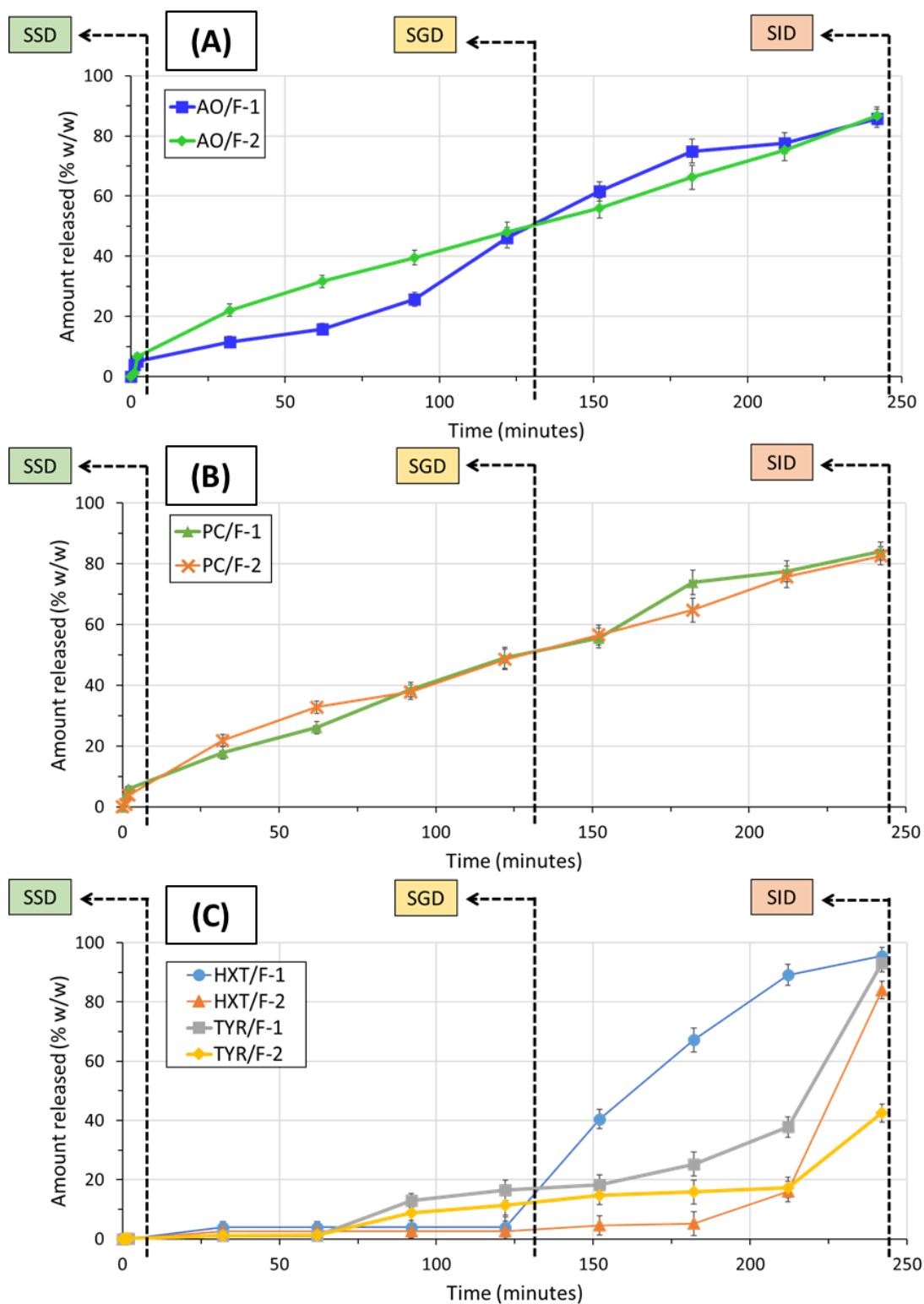


Figure IV.7.1 - Release profiles of antioxidants **(A)**, phenolic compounds **(B)**, hydroxytyrosol **(C)** and tyrosol **(C)** from ethylcellulose microparticles prepared using alternative theoretical loading contents (theoretical loading content of 5% w/w on phenolic compounds; theoretical loading content of 10% w/w on phenolic compounds) (AO – Antioxidants; HXT – Hydroxytyrosol; PC – Phenolic Compounds; SGD – Simulated Gastric Digestion; SID – Simulated Intestinal Digestion; SSD – Simulated Salivary Digestion; TYR – Tyrosol)

7.3.5 Assessment of the bioaccessibility of phenolic compounds released from loaded ethylcellulose microparticles

The bioaccessibility is defined as the fraction of the compound released from the initial matrix (e.g., food matrix, oral formulation) in the gastrointestinal lumen and available for intestinal absorption. It is a crucial step to ensure the bioefficacy (alternatively, the bioavailability) of bioactive compounds. The amount of bioactives soluble in the gastrointestinal tract affect their absorption, distribution, metabolism, and elimination (Gómez-Mascaraque et al., 2017; Paulo & Santos, 2020c; Rein et al., 2013).

The bioaccessibility was evaluated for encapsulated (F-1 and F-2 formulations) and non-encapsulated patterns (NE-1, NE-2 experiments). The NE-1 is the non-encapsulated pattern of F-1 formulation (TLC in PCs of 5% w/w), and NE-2 is the non-encapsulated form of F-2 formulation. Results of phenolic compounds from encapsulated and non-encapsulated extracts obtained from OMP after the intestinal digestion are presented in Figure IV.7.2.

The bioaccessibility of the encapsulated HXT was improved around 4 times fold (in the case of F-1 formulation, the BA_{HXT} was 63.9 ± 3.5 ; in the case of F-2 formulation, the BA_{HXT} was 39.2 ± 1.6) compared to the non-encapsulated pattern (NE-1 $BA=15.8 \pm 1.2\%$; NE-2 $BA=10.1 \pm 1.9\%$). Therefore, it can be concluded that the encapsulating material – ethylcellulose – had a significant influence on the bioaccessibility of HXT. No differences ($p>0.05$) between the BA_{HXT} of F-1 and F-2 formulations were found. The authors Ribeiro et al. (2020) recovered the soluble fraction of OMP samples to formulate a liquid-enriched powder that was submitted to the *in vitro* gastrointestinal digestion. The authors reported a BA_{HXT} of $82.10 \pm 2.59\%$. The BA values of HXT obtained in the present study were lower than the ones reported by Ribeiro et al. (2020); nevertheless, the results are satisfactory as the BA of HXT was improved compared to the non-encapsulated pattern.

A similar trend was observed in the case of BA of TYR. No differences were found between the two batch formulations differing on the loading content. The BA_{TYR} was $27.0 \pm 2.9\%$ in the case of F-1 formulation and $32.0 \pm 2.6\%$ in F-2 formulation.

The non-encapsulated patterns exhibited a BA of $6.9 \pm 1.1\%$ in the case of extract considering a TLC of 5% w/w in PCs and $7.6 \pm 1.7\%$ in the case of NE-2 experiments ($n=3$).

The encapsulation efficiently protected TYR during the *in vitro* gastrointestinal digestion. In our previous study, it was found that the BA of pure TYR was $10 \pm 1\%$ w/w, whereas the bioaccessibility of TYR released from ethylcellulose microparticles was $29 \pm 1\%$ w/w (Paulo & Santos, 2020c). The presence of TYR in a more complex matrix – an extract – did not favor the protection of this bioactive compound (the BA of non-encapsulated pattern decreased from 10 % to 6%).

Interestingly, the BA of PCs considering a TLC of 5% w/w (formulation F-1) was $188.7 \pm 5.7\%$ contrasting with the low BA of its non-encapsulated pattern ($48.6 \pm 2.3\%$). The BA of encapsulated PCs considering a TLC of 10% w/w was $168.9 \pm 3.9\%$ and $43.4 \pm 3.7\%$ when the BA of the non-encapsulated pattern was evaluated. It was not verified differences between the encapsulated release forms (F-1 and F-2 formulations) and among the non-encapsulated patterns ($p>0.05$). However, for each loading considered, it was ascertained differences ($p<0.05$) between the encapsulated and the non-encapsulated patterns (e.g., between F-1 and NE-1 and between F-2 and NE-2 formulations). Therefore, it can be concluded that when encapsulated PCs were efficiently protected during the *in vitro* gastrointestinal tract. The obtained results were similar to the ones obtained by Jilani et al. (2016). The authors investigated the potential application of *Saccharomyces cerevisiae* as a carrier of phenolic compounds extracted from olive leaves. Similarly to the present study, the authors verified the efficient protection of polyphenols bioactivity during the *in vitro* simulated gastrointestinal digestion.

The obtained BA values were over 100%. It is reported BA values ranging from 0% to 380% (Bouayed et al., 2012). Bioaccessibility values higher than 100% indicate that higher amounts of PCs were released into the simulated intestinal fluid. It is hypothesized that most PCs are released during the gastric digestion being time and structure-dependent (Corona et al., 2013). Notwithstanding, the transition from the gastric to the intestinal release environment (changes on the electrolytes concentration, pH, and the activity of enzymes) prompt changes on the bioaccessibility of bioactive compounds, especially of PCs (Jilani et al., 2016).

Most likely, the bioconversions of unknown PCs during the *in vitro* gastrointestinal digestion were related to the increase of PCs in the *digestas* (Bermúdez-Soto et al., 2007; Schulz et al., 2017).

The authors Malapert et al. (2018) performed an investigation regarding the inclusion of extract samples from OMP into β -cyclodextrin, verifying that the inclusion of the extracts in a matrix of β -cyclodextrin improved the BA of PCs and efficiently protected these valuable compounds during the gastrointestinal digestion.

The observed values regarding the BA of AOs were in accordance with the obtained ones for PCs ($R=0.997$ considering F-1 formulation and $R=0.998$ regarding F-2 formulation experiments). These results support the hypothesis considered in section IV.7.3.4 – the majority of bioaccessible AOs were phenolic compounds. Antioxidants were efficiently protected into ethylcellulose microparticles as BA values were improved from non-encapsulated to encapsulated patterns. Similarly to the previously obtained results, the loading content did not affect ($p>0.05$) the BA of AOs.

For all bioactive(s), the loading content did not excrete effect on the bioaccessibility. These results corroborate the obtained results in Section IV.7.3.4. In this context, microparticles should be formulated considering the lowest loading content studied (5% w/w in PCs) as it is associated with a procedure cost reduction, comparing to F-2 formulation (formulation of microparticles considering a TLC of 10% w/w in PCs).

Hence, the encapsulation of PC-rich extracts obtained from OMP samples significantly influenced the bioaccessibility of phenolic compounds. The combined extraction and encapsulation techniques efficiently protect PC-rich extracts and its at specific-site delivery (intestinal lumen) for potential uptake by intestinal epithelial cells.

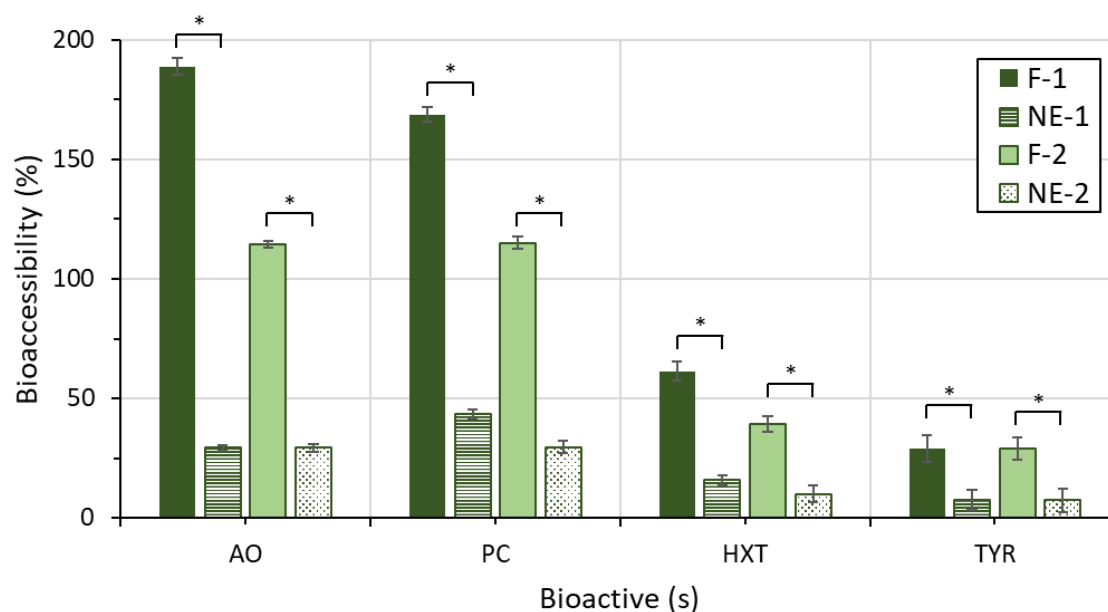


Figure IV.7.2 - Bioaccessibility (%) of antioxidants (AO), phenolic compounds (PC), hydroxytyrosol (HXT), and tyrosol (TYR) during the *in vitro* gastrointestinal digestion of phenolics-rich extract loaded ethylcellulose microparticles. Bars with an asterisk indicate significant differences ($p \leq 0.05$) between non-encapsulated and encapsulated phenolic compounds (F-1 – formulation processed considering a theoretical loading content of 5% w/w in phenolic compounds; NE-1 – the non-encapsulated pattern of F-1 formulation; F-2 – formulation processed considering a theoretical loading content of 10% w/w in phenolic compounds; NE-2 the non-encapsulated pattern of F-2 formulation)

7.3.6 Mathematical modeling of the release profile of phenolic compounds from loaded ethylcellulose microparticles

According to the results presented in section IV.7.3.4 (*In vitro* release studies of phenolic compounds from loaded ethylcellulose microparticles) and in section IV.7.3.5 (Assessment of the bioaccessibility of phenolic compounds from loaded ethylcellulose microparticles), there was not observed differences on bioaccessibility and between release profiles among the two loading contents, within each group. Therefore, the mathematical modeling studies are presented only for PCs-rich loaded ethylcellulose microparticles formulated considering a theoretical loading content of 5% w/w in phenolic compounds as these loaded microparticles are able to efficiently release the bioactive(s) being associated to the lowest production cost, comparing to loaded microparticles formulated considering a TLC of 10% w/w in PCs.

7.3.6.1 Assessment of the release behavior of phenolic compounds from loaded ethylcellulose microparticles through model-dependent methods

The experimental release data from F-1 formulation (TLC in PCs of 5% w/w) were adjusted to the release kinetics models presented in Section IV.7.2.2.5.1 (zero-order, first-order, Higuchi, Korsmeyer-Peppas, and Weibull models) to identify the best model(s) to describe the experimental results as well as to assess the release mechanism of bioactive(s) from loaded ethylcellulose microparticles. The results regarding mathematical modeling are presented in Table IV.7.7.

In this study, the experimental data adjusted to the zero-order kinetic model presented a good correlation ($R \geq 0.970$), excluding the case of HXT ($R = 0.490$). The zero-order release model did not adjust properly to the experimental results of HXT release. The best correlation was observed in the case of the release of AOs. According to this release model principles, the bioactive(s) is/are released as pure substance(s). Antioxidants were the most comprehensive group considered in the present study; additionally, the extraction procedure was optimized to obtain an antioxidants-rich extract. Antioxidants were the major class of compounds present in the final extract, and they were released similarly to pure substances.

The first-order release model is useful to describe the dissolution of substance(s) in dosage forms such as those containing water-soluble bioactive(s) in porous matrices (Dash et al., 2010). The determination coefficient ranged from 0.663, in the case of PCs and 0.903, in the case of TYR. This kinetic release model allows efficiently describes the release behavior of TYR from loaded ethylcellulose microparticles: TYR is a water-soluble compound that was embedded in porous microparticles. Nevertheless, the release behavior of TYR was better described by the zero-order and the Higuchi models.

The experimental results adjusted to the Higuchi model presented a good correlation coefficient (R ranged from 0.704, in the case of the release of TYR and 0.982, in the case of the release of AOs). It was the model that described better the release behavior of AOs ($R=0.982$) and HXT ($R=0.853$). Comparing the mathematical modeling results of AOs and HXT, it was observed a higher release rate of AOs ($0.0504 \text{ mg.min}^{-0.5}$) rather than HXT ($0.0038 \text{ mg.min}^{-0.5}$) – which was released slower. This mathematical model aims to describe the release of bioactives from a matrix system (Dash et al., 2010).

This proposition was in accordance with the proposed design of microparticles – the PCs-rich extract was embedded in a matrix microsystem (microspheres). Moreover, it is described that is a suitable model to describe the release of water-soluble compounds; the majority of encapsulated PCs were water-soluble compounds, which corroborates the model adequacy to describe the release of PCs, particularly in the case of hydroxytyrosol.

The Korsmeyer-Peppas (KP) model assists the identification of bioactive(s) release mechanism. The KP equation (Equation (IV.7.9); Section IV.2.2.5.1) was applied to experimental results for $Q_{t,i}/Q_{f,i} < 0.6$ (where, i denotes the bioactive(s) [e.g., hydroxytyrosol, tyrosol, phenolic compounds or antioxidants], $Q_{t,i}$ is the cumulative release of i at the time t , $Q_{f,i}$ is the cumulative amount of bioactive(s) released at the end of the experiments) and the n values were obtained plotting the $Q_{t,i}/Q_{f,i}$ values against time.

In the case of the KP model, the correlation coefficient of the fit to the experimental data ranged from 0.638, in the case of HXT and 0.983, in the case of PCs. This was the best fit model of PCs. The obtained n value for the release of PCs was 0.7585. According, as the release exponent lays down between 0.45 and 0.89, the transport mechanism of PCs was anomalous (non-Fickian transportation), being these compounds released at a rate of $t^{-0.2415}$. The non-Fickian release of bioactive(s) from microparticles generally results from the combination of diffusion mechanisms and surface/volume anomalous processes as swelling. The encapsulating material used in the present study – ethylcellulose – does not swell. Ethylcellulose is a cellulose-derived polymer in which ethyl ether groups were replaced by hydroxyl groups. Even though ethylcellulose cannot swell, it can uptake surface water through hydrogen bonds forming between free hydroxyl groups and water and between remaining oxygen atoms present in ethylcellulose backbone structure and water (Murtaza, 2012; Paulo & Santos, 2018b). The diffusional mechanisms of PCs associated to surface rearrangements due to water-polymer interactions were paramount in the release of these valuable bioactives.

The experimental results were fitted on the Weibull model – which is the most adequate release kinetic model of bioactive(s) from a matrix-type delivery system.

In the present study, microparticles were obtained by water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation technique. According to this encapsulation technique principles, the encapsulated material(s) is/are dispersed in the encapsulating material. Therefore, the Weibull model should adequately fit the obtained results as a matricial delivery system (microspheres) was produced. As a matter of fact, the experimental data adjusted to the Weibull kinetic model presented excellent correlation coefficients ($0.843 \leq R \leq 0.970$), supporting the fitness of release data to the matrix-type release kinetic model. Nevertheless, it was verified the most appropriate release, kinetic models, to the experimental results (Higuchi model in the case of AOs and HXT; KP model in the case of PCs; zero-order model in the case of TYR). Even though the Weibull model presented overall adequacy, the release of bioactive(s) was/were better described using alternative release kinetic models.

In our previous study, the release kinetics of TYR from loaded ethylcellulose microparticles was assessed (Paulo & Santos, 2020c). Comparing the results presented in that study and the ones obtained in the present study, it can be concluded that the presence of other bioactives in the encapsulating matrix affected the release of TYR and, consequently, the best-fitting kinetic release model as well as fitting parameters (e.g., K_0 , Q_0).

Table IV.7.7 - Parameters and correlation coefficients of different release kinetics models applied to experimental data of release profiles of antioxidants (AO), phenolic compounds (PC), hydroxytyrosol (HXT), and tyrosol (TYR)

Release model ^a	Parameter	F-1 formulation (TLC of 5% w/w)			
		AO	PC	HXT	TYR
Zero-order ^b	K_0 (mg.min ⁻¹)	0.0025	0.0044	0.0017	0.0023
	Q_0 (mg) ^c	0.0199	0.0416	-0.0315	-0.0224
	R	0.977	0.970	0.490	0.922
First-order ^b	K_1 (min ⁻¹)	0.0101	0.0107	0.0169	0.0156
	Q_1 (mg) ^c	-3.0608	-2.5025	-1.3581	-1.6748
	R	0.733	0.663	0.764	0.903
Higuchi ^b	K_H (mg.min ^{-0.5})	0.0504	0.0546	0.0038	0.0207
	R	0.982	0.980	0.853	0.704
Korsmeyer-Peppas ^b	K_K (min ⁻ⁿ)	0.1808	0.0110	0.0110	0.0008
	n	1.0856	0.7585	1.2359	1.9294
	R	0.929	0.983	0.638	0.814
Weibull	τ_d^{exp} (min)	195	152	78	224
	τ_d (min)	269	123	375	596
	β	-4.9487	-4.5718	-8.7852	-7.9080
	R	0.970	0.957	0.843	0.920

Table IV.7.7 - Parameters and correlation coefficients of different release kinetics models applied to experimental data of release profiles of antioxidants (AO), phenolic compounds (PC), hydroxytyrosol (HXT), and tyrosol (TYR) (cont.)

	AO	PC	HXT	TYR
<i>Adjusted Kinetic Model</i>	Higuchi	Korsmeyer-Peppas	Higuchi	Zero-order

^a The parameters and correlation coefficients were obtained for F-1 formulation (theoretical loading content in phenolic compounds of 5% w/w)

^b applied to the linear part of the experimental curve;

^c Q_0 should be ≥ 0

β – Parameter related to the shape of the curve (Weibull model); τ_d – Predicted time (minutes) when 63.2% of bioactive(s) has been released (Weibull model); τ_d^{exp} – Experimental time (minutes) when 63.2% of bioactive(s) has been released (Weibull model); K_0 – Zero-order release constant (Zero-order model); K_1 – First-order release constant (First-order model); K_H – Release constant (Higuchi model); K_K – Release constant (Korsmeyer-Peppas model);

n – Release exponent (Korsmeyer-Peppas model); Q_0 – Initial amount of bioactive(s) (Zero-order model); Q_1 – Initial amount of bioactive(s) (First-order model); R – Correlation coefficient; AO – Antioxidants; HXT – Hydroxytyrosol; PC – Phenolic Compounds; TLC – Theoretical Loading Content; TYR – Tyrosol;

7.3.6.2 Comparison of release profiles of phenolic compounds from loaded ethylcellulose microparticles through a model-independent method

The similarity factor (f_2) empowers assessing the closeness of dissolution between two release profiles (Albertini et al., 2019). According to the Food and Drug Administration (FDA) guidelines, f_2 values higher than 50 ensure the equivalence between two dissolution profiles (Paulo & Santos, 2020c).

While the statistical analysis through the evaluation of the p-value, countenances the comparison of the experimental values at each point of the *in vitro* release studies, the f_2 value incorporates the sum of squared errors during the *in vitro* release studies. In this context, the comparison of release profiles through the analysis of f_2 values is the most accurate method to ensure similarity between two alternative dissolution profiles.

The results regarding a pair-wise comparison of F-1 and F-2 formulations (alternative loading contents) in each release medium are presented in Table IV.7.8 - Section 1. Additionally, for each formulation, the effect of *in vitro* digestion media was performed through pair-wise comparisons. Results are listed in Table IV.7.8 - Section 2.

The overall release profiles (*in vitro* gastrointestinal digestion) were similar for both loading contents considered. Considering the entire gastrointestinal digestion (SSD, SGD, and SID) and for all bioactive(s) considered in the present study (AOs, PCs, HXT, and TYR), the observed f_2 values were higher than 50. Therefore, load microparticles with a higher amount of phenolic compounds (e.g., encapsulating a more concentrated PC-rich extract) did not improve the dissolution of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol. For industrial applications, the loading of microparticles considering a higher concentrate extract is not recommended as the release profiles are similar for both highly concentrate or a more diluted extract. Regarding the economic feasibility, microparticles should be loaded considering a low loading content (e.g., 5% w/w on phenolic compounds) as the release profile(s) of bioactive(s) is/are similar to the release profile(s) from highly concentrated encapsulated extract, being similar the bioaccessibility of the released compound(s) considering alternative formulations differing on the loading content.

The release profiles of bioactive(s) differing on the loading content (F-1 *versus* F-2 formulations) during the SSD were similar ($f_2 > 50$; Table IV.7.8; Section 1). These results may draw from that salivary release studies were carried during just 2 minutes. Probably, the short interval of time of the simulated salivary digestion did not prompt significative physicochemical modifications to ensure noticeable changes in the release profiles.

Regarding the results from the pair-wise comparison of the dissolution media (Table IV.7.8; Section 2), it was observed similar release profiles of antioxidants and phenolic compounds between all simulated release media. Even though the duration of each stage, electrolyte concentration, and the presence of enzymes varied during the simulated salivary, gastric, and intestinal digestions, the overall dissolution patterns of antioxidants and phenolic compounds were not affected by these variables. Contrarily, it was observed that hydroxytyrosol and tyrosol release profiles were dissimilar during the simulated salivary and gastric fluids. In the case of specific phenethyl alcohols – hydroxytyrosol and tyrosol – the duration of each step, the presence of specific enzymes and electrolytes concentration affected the release behavior of these valuable bioactive compounds.

Table IV.7.8 - Calculated similarity factors (f_2): SECTION 1 – Effect of the of loading content (formulation type): pair-wise comparison of F-1 and F-2 formulations in each release medium; SECTION 2 – Effect of *in vitro* digestion media: pair-wise comparison of dissolution media

SECTION 1 – Effect of formulations (F-1 versus F-2)				
Dissolution media	Bioactive(s)			
	AO	PC	HXT	TYR
<i>In Vitro</i> GID	54 *	69 *	51 *	61 *
SSD	79 *	83 *	81 *	78 *
SGD	39	63 *	95 *	63 *
SID	43	70 *	23	53 *

Table IV.7.8 - Calculated similarity factors (f_2): SECTION 1 – Effect of the of loading content (formulation type): pair-wise comparison of F-1 and F-2 formulations in each release medium; SECTION 2 – Effect of *in vitro* digestion media: pair-wise comparison of dissolution media (cont.)

SECTION 2 – Effect of release media

Bioactive(s)	Formulation	SSDF vs. SGDF	SSDF vs. SIDF	SGDF vs. SIDF
AO	F-1	38	18	24
	F-2	27	18	28
PC	F-1	27	16	26
	F-2	26	19	26
HXT	F-1	79 *	45	18
	F-2	76 *	46	24
TYR	F-1	52 *	27	25
	F-2	54 *	28	26

* The asterisk indicates significantly different profiles

AO – Antioxidants; GID – Gastrointestinal Digestion; HXT – Hydroxytyrosol; PC – Phenolic Compounds; SGD – Simulated Gastric Digestion; SGDF – Simulated Gastric Digestion fluid; SID – Simulated Intestinal Digestion; SIDF – Simulated Intestinal Digestion Fluid; SSD – Simulated Salivary Digestion; SSDF – Simulated Salivary Digestion Fluid; TYR – Tyrosol;

7.4 Conclusions

A phenolics-rich extract was obtained by an optimized two-step solid-liquid extraction procedure from olive mill pomace. Further, the obtained extract was efficiently encapsulated in ethylcellulose microparticles by water-in-oil-in-water double emulsion solvent evaporation technique.

In the present study, the effect of the loading content on the release behavior of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol from microparticles was appraised. Model-dependent methods were applied to assist the mathematical modeling of the release profiles of selected bioactive compounds. Additionally, a model-independent method – the similarity factor – was considered to compare the similarity of release profiles.

Moreover, bioaccessibility studies were performed to assess the efficient protection given the encapsulating material to the bioactive compounds during the *in vitro* gastrointestinal digestion.

Natural-occurring bioactive compounds in olive mill pomace were efficiently embedded into ethylcellulose microparticles – the encapsulation efficiencies remained above 83%. The actual loading contents were close to the theoretical loading contents considered.

The maximum release of antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol was verified during the simulated intestinal digestion. The amount of bioactive compounds released in the simulated salivary fluid was neglectable. *In vitro* release studies confirmed the salivary and gastric protection of phenolic compounds being the highest amount of bioactive compounds released in the simulated intestinal tract.

The encapsulation of olive mill pomace extracts significantly influenced the bioaccessibility of phenolic compounds. The bioaccessibilities of encapsulated antioxidants, phenolic compounds, hydroxytyrosol, and tyrosol were improved compared to their respectively non-encapsulated patterns. The combined extraction and encapsulation techniques may grant the efficient protection of bioactive compounds present in olive mill pomace extracts and their at specific-site delivery - intestinal lumen - for potential uptake by intestinal epithelial cells.

From the analysis of the similarity factors, it was observed that overall release profiles were similar for both loading contents considered.

The results of this study are indubitable regarding the effect of the loading content in phenolic compounds on the release behavior of bioactive compounds from olive mill pomace extract encapsulated into ethylcellulose microparticles: no significant differences were found on the release of bioactives independently of the loading content. Therefore, regarding the economic feasibility of the project, it is suggested the formulation of microparticles considering a low loading content (5% w/w in phenolic compounds).

Briefly, in this study, the release behavior of antioxidants, phenolic compounds, and specific phenethyl alcohols (hydroxytyrosol and tyrosol) from olive mill pomace extract loaded ethylcellulose microparticles were comprehensively evaluated. This study brings new insights regarding the design of loaded microparticles that can be further incorporated in fortified foods, suffering subsequently gastrointestinal digestion.

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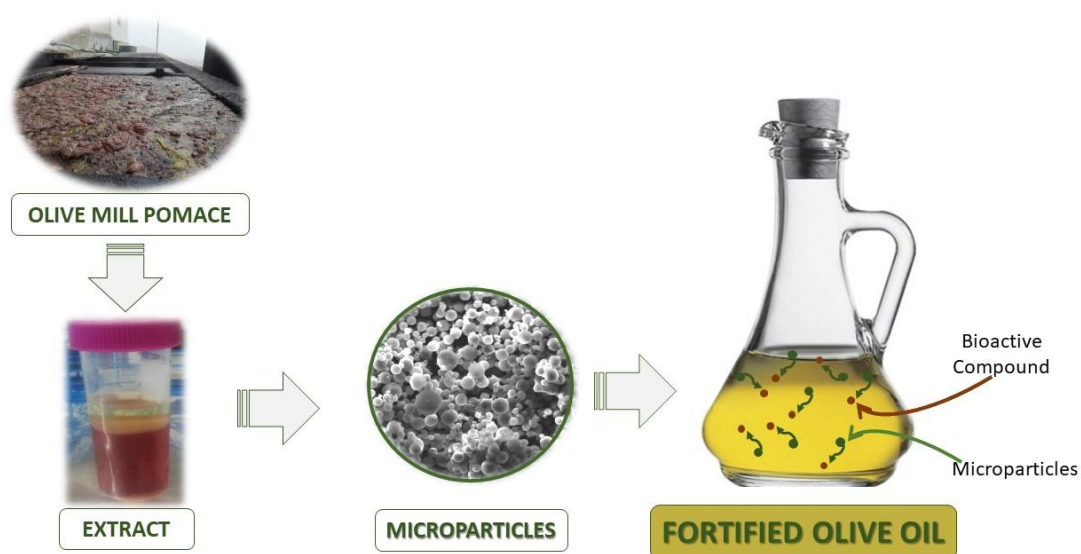
Part V: Incorporation of olive mill pomace extract loaded microparticles to improve olive oils oxidative stability

In this section, the effectiveness of ethylcellulose microparticles loaded with olive mill pomace extract rich in phenolic compounds in the retardation of lipidic oxidation of an extra-virgin olive oil, a virgin olive oil and, a blend of refined and virgin olive oil was assessed (Chapter 8). Moreover, the oxidative stability of olive oils incorporating microparticles was compared to the oxidative stability of olive oils enriched with olive mill pomace extract and two synthetic antioxidants (solely and in a blend) under accelerated storage conditions.

Chapter 8. Olive mill pomace extract loaded ethylcellulose microparticles as a delivery system to improve olive oils oxidative stability⁸

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Graphical Abstract:



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Abstract:

In the present study, the protective effect of olive mill pomace (OMP) loaded ethylcellulose microparticles as an alternative to synthetic antioxidants against oxidation of olive oils was assessed. OMP extract was obtained by an optimized two-step solid-liquid extraction; encapsulation was performed by double emulsion solvent evaporation technique considering a theoretical loading content in phenolic compounds of 5% w/w.

The effect of loaded microparticles on improving the oxidative stability of olive oils was appraised considering the Schaal oven test. Results were compared with plain oils, oils enriched with synthetic antioxidants, and oils fortified with OMP extract. The changes in the peroxide values, the *p*-anisidine values, the total oxidation values, the free fatty acids content, the total antioxidant activity, and the total phenolic content were synchronized under storage at 62 °C.

The fortification of olive oils with microparticles retarded the appearance of peroxides, reduced the content of secondary oxidation products, and slowed down hydrolysis processes. Loaded ethylcellulose microparticles were more efficient in the retarding the free fatty acids formation rather than synthetic antioxidants. The total antioxidant and phenolic content were kept nearly constant during the study. Therefore, it can be concluded that OMP loaded ethylcellulose microparticles were efficiently designed to sustained release of antioxidants to control the oxidative status of oil samples.

The results of this study bring new perspectives regarding the potential use of encapsulated extracts rich in antioxidants as an alternative to synthetic antioxidants to improve oils oxidative stability.

Highlights

1. Olive mill pomace(OMP) loaded ethylcellulose microparticles (MPs) were produced.
2. The protective effect of OMP-MPs in olive oils oxidative stability was evaluated.
3. Improving the oxidative stability was appraised considering the Schaal oven test.
4. OMP-MPs efficiently improved the oxidative status of oil samples.
5. Fortification of oils with OMP-MPs retarded the appearance of oxidation products.

Keywords: olive oil; fortification; oxidative stability; microparticles; peroxides; phenolic compounds; antioxidants; olive mill pomace;

8.1 Introduction

Olive oil from olive fruit (*Olea europaea* L.; *Oleaceae*) is an outstanding source of a great variety of bioactive compounds such as monounsaturated free fatty acids (MUFA) (e.g., oleic acid), hydrocarbon squalene, aroma compounds, tocopherols, and phenolic compounds. A hallmark of the Mediterranean diet is the consumption of olive oil as essential fat, covering up to 17% to 25% of the typical Mediterranean diet calories per day, which has been associated with a low incidence and prevalence of cardiovascular diseases, diabetes type 2, some neurodegenerative diseases (stroke, Alzheimer's and Parkinson's diseases) and certain types of cancer (e.g., prostate, colon and breast cancers) (Angeloni et al., 2017; Castro-Quezada et al., 2014; Couto et al., 2011; Fundación Dieta Mediterránea, 2020; Gómez-Gracia et al., 2018; Hashim et al., 2005; Miró-Casas et al., 2003; Mitrou et al., 2015; Paulo & Santos, 2020a, 2020b; Ruiz-Gutie et al., 2017; Sofi et al., 2010, 2013; Tuck & Hayball, 2002; Visioli & Galli, 2001). Other benefits associated with olive oil consumption include its swift digestibility, anti-ulcer and anti-aging properties, and lowering the capacity of stress and plasma cholesterol. It has been described as the potential application of olive oil in skincare treatments (Assmann et al., 1997; Owen et al., 2000; Sun-Waterhouse et al., 2011).

Even though olive oils contain high amounts of MUFAs, it is also composed of a small fraction of polyunsaturated fatty acids (PUFAs), resulting in poor storage stability (O'Brien, 2009).

It has been acknowledged that the most significant factors affecting oil quality attributes and its shelf-life are lipid oxidation, which drives undesirable changes in texture, flavor, odor, and taste (Da Silva & Jorge, 2014). Moreover, the lipid oxidation may prompt the degradation of bioactive compounds presenting remarkable benefits (e.g., fat-soluble vitamins) (Gallego et al., 2013; Yang et al., 2016; Zeb & Murkovic, 2013). Moreover, lipid oxidation is associated with biological membranes, proteins, and enzymes degradation, which may negatively impact human health (Malheiro et al., 2013).

The nutritional losses associated with lipidic oxidation are associated with reducing consumers' compliance and industrial economic losses. In this context, the olive oil industry has been devoted to diminishing lipid oxidation and improving olive oil stability (Iqbal & Bhanger, 2007; Wu & Nawar, 1986).

Synthetic antioxidants, namely butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and even the *tert*-butyl hydroquinone (TBHQ), have been extensively used as food additives to overcome issues regarding the stability of oils and fats. However, these compounds have been implicated in several human health risks being described their impressive role in carcinogenesis (Hou, 2003; Iqbal & Bhanger, 2007; Prior, 2004; Taghvaei & Jafari, 2015). Even though these synthetic antioxidants are generally recognized as safe (GRAS), their consumption is not recommended by chronically ill patients. Moreover, prolonged consumption is associated with the onset of certain degenerative diseases (Cordeiro, et al., 2013; Xu et al., 2001).

Therefore, regarding safety concerns, food expert investigations have been focusing on the replacement of these synthetic antioxidants by natural ones. Natural-occurring antioxidants may retard the oxidative rancidity (i) through the scavenging of oxygen molecules, (ii) by capturing free radicals, or (iii) deoxidizing/decomposing peroxides (Sun et al., 2010, 2011; Zheng et al., 2012). Among natural-occurring antioxidants, plant phenolic antioxidants have been on the highlight of lipid oxidation prevention in recent years. Alongside their potential activity in rancidity retardment, they also present health-promoting properties.

Olive mill pomace (OMP) is considered a relevant source of phenolic compounds. Most of these compounds - presenting radical scavenging activities - are retained in OMP waste rather than in olive oil during olive oil processing (El-Abbassi et al., 2014; Paulo & Santos, 2020b).

However, many phenolic compounds are sensitive to environmental conditions such as pH, light, temperature, oxygen, moisture content, etc.

Therefore, their encapsulation is a straightforward technological strategy to protect these valuable compounds to be latter embedded in food matrices prone to rancidity as oils (e.g., olive oil). OMP is a natural source of phenolic compounds to effectively stabilize oils as olive oil through their encapsulation and controlled release. Encapsulation can protect and increase phenolic compounds properties (e.g., stability, solubility, bioavailability, and antioxidativity), and simultaneously control the release of these bioactives compounds to efficiently retard lipidic oxidation in rancidity prone food matrices as the vegetable oils.

Among with encapsulation techniques, the $w_1/o/w_2$ water-in-oil-in-water double emulsion solvent evaporation technique is an attractive method to protect water-soluble compounds susceptible to environmental degradation as phenolic compounds. For instance, these multicompartamental microsystems are obtained through the preparation of a primary emulsion (w_1/o), which is re-emulsified in an external aqueous phase (w_2) containing appropriate emulsifiers (e.g., polyvinyl alcohol – PVA) (Gaitzsch & Kraume, 2011; Garti & Bisperink, 1998; Paulo & Santos, 2018a, 2018b, 2019, 2020a; Rodrí, 2015; Schuch et al., 2013). Many food biopolymers can be used to embed water-soluble compounds, among which stands out the gelatin, casein, whey protein, acacia gum, xanthan gum, carboxymethylcellulose, among others (Aguiar et al., 2017; Desai & Park, 2005; Dickinson, 2011; Paulo & Santos, 2018a).

The encapsulation by double emulsion solvent evaporation technique is based on the reasoning of the following considerations: (i) it grants the protection of sensitive bioactive compounds such as phenolic compounds extracted from OMP; (ii) delivery of the encapsulated nutrients in a controlled rate similar to lipidic oxidation rate balancing the amount of antioxidants delivered in the food sensitive matrix and the amount of antioxidants degraded during lipidic oxidation; (iii) prompt a controlled delivery of these bioactive compounds during eating and digestion (Dickinson, 2011; Le Révérend et al., 2010; Norton & Norton, 2010; Sagalowicz & Leser, 2010).

Among coating materials used for encapsulation proposes by the $w_1/o/w_2$ double emulsion solvent evaporation technique, ethylcellulose is a biocompatible cellulose-derived class of polymers attractive to food applications. Their application as carriers in food additives is approved by the European Union (European Commission Regulation 1130/2011) (European Commission, 2011). Ethylcellulose polymers (differing on the ethylation degree) have been receiving attention as they are gastrointestinal resistant polymer-carriers, are tasteless, odorless, non-toxic, non-irritant, and also, they are stable to many environmental conditions are heat, moisture, light, and oxygen. Moreover, they present outstanding resistance to mechanical stress (Kamel et al., 2008; Murtaza, 2012; Paulo & Santos, 2018a). These unique properties render ethylcellulose polymers attractive carriers in food applications.

The purpose of this study was to evaluate the effectiveness of encapsulated olive mill pomace extract on the retarding lipidic oxidation of three types of olive oil – extra-virgin, virgin, and a blend of refined and virgin olive oils – compared to embedment in lipidic matrices of only olive mill pomace extract and synthetic antioxidants (BHA and BHT considered alone or in a blend) using the Schaal oven test. Over a 24-day study at 62 °C, olive oils quality and nutritional values were evaluated by monitoring their peroxide values, *p*-anisidine values, total oxidation values, and free fatty acids content, total antioxidant activity, and total phenolic content changes over storage time.

8.2 Experimental procedures

8.2.1 Materials

8.2.1.1 Reagents and Standards

Reagents used during the extraction procedure namely hydrochloric acid solution at 37% v/v (EMSURE® ACS, Supelco®, Ref: 1.00317.1011, HCl, CAS 7647-01-0), hexane (HiPerSolv CHROMANORM® for HPLC, Ref: 83992.320, C₆H₁₈, CAS 110-54-3), ethanol (purity ≥ 99.8%, PESTINORM®, Ref: 85933-320, C₂H₆O, CAS 64-17-5) and ethyl acetate (purity ≥ 99.8%, SupraSolv®, Ref: 1.00789.2500, C₄H₈O₂, CAS 141-78-6) were obtained from VWR International (Fontenay-sous-Bois, France).

The solvent used for encapsulation purposes – dichloromethane (Ref. ACRO433991000, CH₂Cl₂, CAS 1665-00-5) was obtained from VWR International (Fontenay-sous-Bois, France). The other reagents used during encapsulation namely ethylcellulose (Ref: 433837-250G, viscosity of 46 cP, ethylation degree of 48%, CAS 9004-57-3) and polyvinyl alcohol (PVA) (Ref: P8136-250G, 87-90% hydrolyzed, average molecular weight of 30,000-70,000, CAS 9002-89-5) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA).

Standards used throughout this study as gallic acid standard (Ref: 91215, C₆H₆O₅, CAS 149-91-7), Trolox standard (purity ≥ 97%, Ref: 238813, C₁₄H₁₈O₄, CAS 53188-07-1), butylated hydroxyanisole (BHA) standard (purity ≥ 98.5%, Ref: B1253, C₁₁H₁₆O₂, CAS 25013-16-5) and butylated hydroxytoluene standard (BHT) (purity ≥ 99%, Ref: W218405, C₁₅H₂₄O, CAS 128-37-0) were obtained from Sigma Aldrich Chemical (St. Louis, MO, USA).

Reagents used for the peroxide values determination as the glacial acetic acid (Ref: 1.01830.2500, C₂H₄O₂, CAS 64-19-7) and chloroform (Supelco®, Ref: 02487, CHCl₃, CAS 67-66-3) were purchased from VWR International (Fontenay-sous-Bois, France).

Other reagents used from the determination of peroxide values as potassium iodide (purity ≥ 99%, Ref: 221945, KI, CAS 7681-11-0), sodium thiosulphate (purity= 99%, Ref: 217263, Na₂S₂O₃, CAS 7772-98-7), and starch (Ref: 33615, (C₆H₁₀O₅)_n, CAS 9005-84-9) were obtained from Sigma Aldrich Chemical (St. Louis, MO, USA).

During the determination of the *para*-anisidine value, the reagents isooctane (Ref: PHR1915, C₈H₁₈, CAS 540-84-1) and the *p*-anisidine reagent (purity ≥ 99%, Ref: A88255, C₇H₉NO, CAS 104-94-9) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA).

During the free fatty acids content determination, the reagent phenolphthalein (Ref: 1.07233, C₂₀H₁₄O₄, CAS 77-09-8) was obtained from Sigma Aldrich Chemical (St. Louis, MO, USA), and the reagent sodium hydroxide (Ref: 1.06467.9010, NaOH, CAS 1310-73-2) was purchased from VWR International (Fontenay-sous-Bois, France).

Reagents used for the estimation of the total antioxidant activity and the total phenolic content such as the Folin-Ciocalteu reagent (Ref: 47641-500ML-F, 2M with respect to acid, density of 1.3 g/mL at 20 °C), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Ref: D9132, C₁₈H₁₂N₅O₆, CAS 1898-66-4), sodium carbonate anhydrous (Ref: 1613757, Na₂CO₃, CAS 497-19-8) were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA) and the solvent methanol (purity ≥ 99.9%, LiChrosolv®, Ref: 1.03726.2002, CH₄O, CAS 67-56-1) was obtained from VWR International (Fontenay-sous-Bois, France).

The water used in this work was de-ionized and double-distilled using a Millipore™ water purification system (Massachusetts, USA) having 18.2 Ω electrical resistivity. All the reagents were either chromatographic or analytical grade and used as received.

8.2.1.2 Samples

The OMP samples were obtained from a local olive oil mill in Portugal (Vilas Boas, Vila Flor, Bragança, 41359822,-7123743) in January 2019. Samples were stored at -22 °C (moisture content of 71.0 ± 5.5 % w/w) prior analyses. The OMP samples were freeze-dried on a benchtop freeze-dryer (SP Scientific, NY, USA) for 72 hours. Dried samples were then grounded on an electric mill (Qilive Q5321 Grinder) to an average particle size of 142.2 ± 9.6 µm, as described by Uribe et al. (2015).

Three alternative olive oils were considered in the present study: (i) a commercial extra-virgin olive oil (EVOO), (ii) a virgin olive oil (VOO), and (iii) a commercial blend of refined olive oil and virgin oil (ROO).

Even though the pure refined olive oil is recognized as a commercial type of olive oil by the European Commission (EC) regulation 2568/91 updated in 2019, its commercialization is not regulated in Portugal (European Commission, 2019; República Portuguesa, 2004). In this regard, pure refined oil was not considered in the present study.

8.2.2 Methods

8.2.2.1 Extraction of phenolic compounds from olive mill pomace

The extraction of phenolic compounds was performed by submitting the OMP sample (n=3) to (i) a pretreatment being afterward performed to extract bonded phenolic compounds. The OMP samples were then submitted to a (ii) solid-liquid extraction and subsequently to a (iii) liquid-liquid extraction.

The (i) pretreatment of the OMP sample (n=3) and the extraction of bonded phenolic compounds was performed, submitting the freeze-dried OMP sample (about 1 g) to acidic hydrolysis and fat removal steps.

The acidic hydrolysis was considered as a low pH that favors the precipitation of proteins, increasing the amount of free phenolic compounds (FPCs) in the extraction solvent.

Moreover, the low pH prompts the breakage of both glycosidic and ester bonds, which increases the amount of bonded phenolic compounds in the extraction solvent (De Marco et al., 2007). The acidic hydrolysis was performed, adding to the freeze-dried OMP sample (1 g; n=3), 25 mL of an aqueous solution of HCl (0.1 M, pH 2). The mixture was continuously shaken in an orbital shaker (400 rpm; IKA® KS 130 basic, IKA Works GmbH & Co. KG, Staufen im Breisgau, Germany) for 12 hours.

Afterward, the hydrolyzed OMP sample (n=3) was submitted to a fat removal step similarly to described by Lafka et al. (2011). Accordingly, 5 mL of hexane (5 mL/g) was added to the hydrolyzed OMP sample. The fats removal was processed, promoting the continuously shaken of the mixture in an orbital shaker (400 rpm) for 1 hour at room temperature (20 ± 2 °C).

The mixture was submitted to a centrifugation process at 2 670 g for 15 minutes in a Rotofix 32A centrifuge (Hettich, Germany). Then, the aqueous and organic phases were separated.

The phenolic compounds present in the organic phase were further recovery by liquid-liquid extraction. The lipidic layer was admixed with 15 mL of UPW, and the mixture was vortexed (5 minutes; IKA VORTEX GENIUS 3, Staufen, Germany) ultrasonicated (P Selecta, Barcelona, Spain) for 15 minutes. Then, the sample was centrifuged at 2 670 g for 15 minutes. After the centrifugation step, the recently-obtained aqueous layer was filtered using sterile and disposable syringes without needle (Ref: 613-1591, VWR International, Fontenay-sous-Bois, France). The final sample – aqueous solution containing phenolic compounds extracted from the organic layer – was designated as sample containing extracted phenolic compounds after the fat removal procedure (SEPC-FR).

The hydrolyzed and defatted OMP sample (n=3) was then submitted to a (ii) two-step solid-liquid extraction using ethanol as the extraction solvent. The sample was admixed with 10 mL of ethanol and continuously shaken for 3.2 hours on an orbital shaker (400 rpm; room temperature), being afterward centrifuged (2 670 g, 15 minutes). The obtained supernatant as termed as sample containing extracted phenolic compounds from the primary extraction (SEPC-P1E). The SEPC-P1E was collected, filtered under vacuum using a Whatman™ nylon membrane filter and stored in an amber flask under dark conditions at room temperature (20 ± 2 °C) before being admixed with the final filtrate.

The second step (secondary extraction) was performed similarly to the primary extraction procedure. A volume of 3 mL of ethanol was added to the pellet from the primary extraction. The mixture was continuously shaken for 1.3 hours. Afterward, the sample was submitted to a low-intensity centrifugation process (2 670 g, 15 minutes), and the supernatant was collected and vacuum filtered using Whatman™ nylon membrane filter. The filtrate was designated sample containing extracted phenolic compounds from secondary extraction (SEPC-P2E).

Afterward, the filtrates SEPC-FR, SEPC-P1E, and SEPC-P2E were admixed, constituting the final filtrate.

The ultimate step of the extraction of phenolic compounds from the OMP sample (n=3) was the (iii) liquid-liquid extraction of the final filtrate from the (i) solid-liquid extraction procedure. Ethyl acetate was the extraction solvent selected as the extraction solvent during these experiments. Accordingly, the final filtrate from the solid-liquid extraction procedure was admixed the extraction solvent using a volume corresponding to the final filtrate volume three times. Then, the mixture was vigorously shaken and vortexed for 5 minutes. Afterward, the mixture was ultrasonicated (15 minutes) and centrifugated (low-intensity centrifugation process, 2 670 g) for 15 minutes. The supernatant was then collected, and the solvent was evaporated at 50 °C using a rotary evaporator (BUCHI R-210, Buchi Laborototiums Tchnik AG, Flawil, Switzerland). Solvent traces were removed under a gentle nitrogen stream. The dried extract was stored at - 22 °C prior analyses.

The obtained extract was characterized by its total antioxidant activity (TAA) and total phenolic content (TPC) described in the following sections.

The extraction experiments were performed in triplicate.

8.2.2.2 Preparation of olive mill pomace extract-loaded ethylcellulose microparticles by double emulsion solvent evaporation

The obtained extract from the OMP sample was embedded into ethylcellulose microparticles by water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation technique as described by Paulo & Santos (2018a, 2018b, 2019, 2020a). In the present study, a theoretical loading content (TLC) of 5% w/w in phenolic compounds was considered.

The dried OMP extract was reconstituted in 3 mL of UPW. Successive dilutions were conducted to achieve the desired loading content in phenolic compounds (theoretical loading content of 5% w/w corresponding to a concentration of 5.3 g of phenolic compounds/L of extract).

The diluted sample from the OMP final extract constituted the internal aqueous phase (w_1).

The organic phase (o) was formulated admixing 100 mg of the polymer carrier – ethylcellulose – with 10 mL of dichloromethane to achieve a 10 g/L polymer concentration. The polymer solution was ultrasonicated for 15 minutes in an ultrasonic bath.

Subsequently to the polymer solution, 1 mL of the w_1 was added. The dispersed phase (w_1/o emulsion) was vigorously shaken and vortexed for 5 minutes.

Then w_1/o emulsion was poured into 100 g of a PVA solution (1 % w/w), and the mixture was emulsified using a high-performance liquid homogenizer at 5 000 rpm for 5 minutes.

The solvent evaporation from the mixture $w_1/o/w_2$ double emulsion was promoted through the continuous stirring of the $w_1/o/w_2$ double emulsion in a stirring plate at 700 rpm for 3 hours, in the fume hood at room temperature (20 ± 2 °C). Subsequently, microparticles were recovered by vacuum filtration using a 0.2 µm Whatman™ nylon membrane filter and washed using 500 mL of distilled water. Then, particles were collected, frozen for 24 hours at -22 °C, and freeze-dried for 72 hours. Encapsulation experiments were performed in triplicate.

8.2.2.2.1 Characterization of olive mill pomace extract-loaded ethylcellulose microparticles

Olive mill pomace extract-loaded ethylcellulose microparticles were characterized by encapsulation efficiency and the actual loading content on (i) antioxidants and (ii) phenolic compounds.

The encapsulation efficiencies were determined according to Equation (V.8.1) similarly to described by Paulo & Santos (2018a):

$$EE_i (\% \text{ w/w}) = \frac{w_i}{w_{l,i}} \times 100 = \frac{w_{l,i} - w_{o,i}}{w_{l,i}} \times 100 = \frac{w_{l,i} - (w_{s,i} + w_{p,i})}{w_{l,i}} \times 100 \quad (\text{V.8.1})$$

where i denotes the cluster of compounds (e.g., antioxidants, phenolic compounds). The w_i corresponds to the weight of compounds in the microparticles, which was assessed considering the weight of bioactive compounds initially added for microparticles formulation ($w_{i,i}$) and the weight of non-encapsulated bioactives ($w_{0,i}$). The $w_{0,i}$ was assessed considering the weight of bioactives in the recovered supernatant of a sample of the double emulsion after 3 hours of microparticles hardening (w_s) after the sample being centrifuged (2 670 g, 15 minutes). The w_p corresponds to the weight of bioactive compounds adsorbed at the surface of microparticles. The w_p was evaluated after submitting the multiple emulsion sample to low-intensity centrifugation. The pellet recovered from the centrifugation process was reconstituted in UPW, being resubmitted to a centrifugation process for 15 minutes. The w_p corresponded to the weight of compounds in the supernatant after the two-step centrifugation process. The EEs were evaluated in triplicate. Results are presented as mean $\pm$ standard deviation.

The actual loading content (ALC) was evaluated according to Equation (V.8.2) as presented by Paulo & Santos (2020a):

$$ALC_i (\% \text{ w/w}) = \frac{w_i}{w_M} \times 100 = \frac{w_{i,i} - w_{0,i}}{w_M} \times 100 = \frac{w_{i,i} - (w_{s,i} + w_{p,i})}{w_M} \times 100 \quad (\text{V.8.2})$$

where w_M is the weight of microparticles recovered after freeze-drying. The ALCs were evaluated in triplicate. Results were computed as mean $\pm$ standard deviation.

The weight measurements of phenolic compounds were processed by determining the total phenolic content (TPC) by an UV-Vis spectrophotometric method (detailed description in section V.8.2.2.4.6.2). Antioxidants weight measurements were performed considering the total antioxidant activity (TAA) of samples assessed, as described in Section V.8.2.2.4.6.1 by UV-Vis spectrophotometry.

8.2.2.3 Schaal oven storage stability test

The oil shelf-life – the period of time until the oil develops rancidity – is considered an utmost quality factor during the processing and the marketing of vegetable oils. The oil shelf-life is assessed by its oxidative stability. Therefore, oxidative stability evaluation methods have been developed to evaluate oils shelf-life. Among the available oxidative stability evaluation methods, the Schaal oven test is a straightforward method that allows assessing the rancidity state of oils. According, during Schaal oven test experiments, elevated storage temperatures are employed to intentionally destroy the original physicochemical characteristics of oils (Antolovich et al., 2002; Yang et al., 2016).

In the present study, the Schaal oven test was considered to evaluate OMP extract loaded ethylcellulose microparticles in retarding the oxidative deterioration of three types of extra-virgin olive oil, virgin olive oil, and refined olive oil. The enrichment of olive oil with BHA and BHT was performed up to the legal limit of 200 mg/kg of oil (European Commission, 2011). The legal limit of 200 mg/kg of oil - based on the amount of antioxidants - was also considered during the addition of the extract and microparticles to olive oils. Experiments were performed considering (i) blank controls (oils without added antioxidants), (ii) positive controls (oils enriched with BHA, or BHT or even a mixture 50% BHA and 50% BHT to their legal limit of 200 mg/kg) and enriched samples (oils enriched with extract up to 200 mg/kg of oil and oils enriched with microparticles to their limit of 200 mg of antioxidants/kg of oil).

Prior to the Schaal oven test experiments, compounds or samples (BHA, BHT, BHA+BHT, extract, and microparticles) were precisely weighed in a glass beaker and solubilized in ethanol. The amount of ethanol used was limited, so not surpassed 4% of the final oil weight, similarly to described by Michotte et al. (2011).

The Schaal oven tests were performed as described by Yang et al. (2016) with slight modifications. The controls (blank oils), the positive controls (oils plus BHA and BHT, individually or in combination), and olive oils incorporated with OMP extract and loaded microparticles were accurately weighted (50 ± 0.01 g) into flasks with limited headspace. Prior to the beginning of the studies, samples were vortexed for 10 minutes and flushed with nitrogen for 3 minutes, as described by Michotte et al. (2011).

Samples were stored in an oven at 62 ± 2 °C for 24 days. Samples were taken every 6 days for the determination of TAA, TPC, peroxide value (PV), *para*-anisidine value (*p*-AV), total oxidation (TOTOX) status, free fatty acids content, and quantification of the K_{232} and K_{270} extinction coefficients, which are indicators of olive oils stability. After oven removal, samples were flushed with nitrogen and stored at -20 °C until analysis.

One flask per time point in triplicate was considered for each sample (controls, positive controls, oils fortified with extract, and oils enriched with microparticles).

8.2.2.4 Determination of oxidative stability indices

8.2.2.4.1 Determination of peroxide value (PV)

The PV that measures hydroperoxide concentration is the most commonly employed chemical method to evaluate oils' oxidative deterioration. The PV measurements are based on the redox reaction between hydroperoxides and the excess of KI in an acidic medium, which results in the stoichiometric release of molecular iodine that is titrated against thiosulphate solution (Wai et al., 2009). Even though the accuracy of PV is doubted as hydroperoxides decompose into a mixture of volatile and non-volatile products, they also react further to endoperoxides and other similar products.

Therefore, the PV is still a useful tool to monitor the oxidative deterioration of oils (Pokorny et al., 2003).

The PV of oil samples (blank controls, positive controls, oils enriched with OMP extract, and oils incorporating microparticles) were determined as described by Sun-Waterhouse et al. (2011).

In each experiment, 0.5 g of oil was dissolved in 3 mL of a solution of acetic acid/chloroform (3/2 v/v). Then, 50 μ L of a saturated potassium iodide (KI) solution was added, and the mixture was left to equilibrate for 1 minute. Afterward, a volume of 3 mL of UPW was added. The mixture was then titrated with a 0.01 N standardized sodium thiosulphate solution until the yellow iodine color disappeared. Subsequently, 0.2 mL of starch indicator solution was added (10 g/L).

The titration experiment was continuously processed until the blue color from the iodine disappeared. It was performed a PV experiment of the blank sample as a reagent control. Results are expressed as peroxide milliequivalents per kg oil (mEq/kg oil). Results are presented as mean $\pm$ standard deviation.

8.2.2.4.2 Determination of *p*-anisidine value (*p*-AV)

The *p*-AV measures the α - and β -alkenals as secondary oxidation products (Wai et al., 2009). The *para*-anisidine is a compound that strongly reacts with aldehydes forming products that absorb at a wavelength of 350 nm. It corresponds to the absorbance of a solution resulting from the reaction of the fat or oil with *p*-anisidine in an isooctane solution. The products formed by reaction with unsaturated aldehydes, namely 2-alkenals, absorb intensely at 350 nm. Therefore, the *p*-AV tests are exceptionally sensitive to these oxidation products.

The *p*-AVs were determined as described by Sun-Waterhouse et al. (2011) with slight modifications. In each experiment, 0.5 grams of oil were dissolved in 12.5 mL of isooctane. Iso-octane was selected as the reference (blank). The oil mixture (or blank) (5 mL) was admixed with *p*-anisidine reagent (1 mL at a concentration of 2.5 g/L in glacial acetic acid). The obtained mixture was vortex for 10 minutes.

An aliquot of the mixture (200 μ L) was withdrawal to a 96-well microplate. The absorbance was then read using a microplate reader (Synergy HT, Biotek, USA) at 350 nm.

8.2.2.4.3 Determination of the total oxidation (TOTOX) value

It has been described that a high rate of hydroperoxide generation is not necessarily associated with a high rate of generation of secondary oxidation products – high PVs do not imply that high *p*-AVs are observed (Guillén & Cabo, 2002; Wai et al., 2009). In this regard, the total oxidation (TOTOX) value is a useful parameter that provides an overall picture of the oxidative status of the oil.

The TOTOX values were calculated as described by Wai et al. (2009) as presented in Equation (V.8.3):

$$TOTOX \text{ value} = 2 PV + AV \quad (V.8.3)$$

where *PV* is the peroxide value of the oil, and the *AV* is the *p*-anisidine value of the oil. The *PV* and the *p*-*AV* values were obtained as described in Sections V.8.2.2.4.1 and V.8.2.2.4.2, respectively.

8.2.2.4.4 Determination of free fatty acids (FFA) content

Most of the fatty acids in olive oil are long-chain fatty acids; indeed, it is described that olive oils present high content on mono-saturated fatty acids (MUFA) and low content on polyunsaturated fatty acids (PUFA) (Maggio et al., 2009). Nevertheless, long-chain fatty acids can be converted into short-chain fatty acids that may release free fatty acids (FFA) with time. The liberated fatty acids may suffer β -oxidation, resulting in the formation of methyl ketones and aliphatic alcohols, in a process known as ketonic rancidity (Pokorny et al., 2003).

Therefore, the FFA quantification is considered a relevant quality control parameter of the oxidative stability of olive oils.

The FFAs content was determined through a direct titration. Accordingly, to a flask, an aquilot (1.25 mL) of ethanol, two drops of oil, and 50 μ L of the phenolphthalein indicator were admixed, constituting the neutralized alcohol. The flask was then placed in a water bath at 60 °C.

A solution of NaOH (0.01 N) was added to the neutralized alcohol until the appearance of a permanent faint pink color. Then, 1.41 g of the sample (oil) was added to the neutralized alcohol and titrated with NaOH until the appearance of the faint pink color corresponding to the endpoint of the phenolphthalein indicator.

The FFA content was expressed as oleic acid, as a percentage (Equation (V.8.4)):

$$\% \text{ FFA (as oleic acid)} = \frac{V_{\text{NaOH}} \times N_{\text{NaOH}} \times 282.46}{W} \times 100 \quad (\text{V.8.4})$$

where, V_{NaOH} is the volume of NaOH titrant (mL), N_{NaOH} is the normality of NaOH titrant (mol/1000 mL); the W is the mass of the oil (g), and the value 282.46 corresponded to the molecular weight of oleic acid. Results are presented as mean $\pm$ standard deviation.

8.2.2.4.5 Changes on the K_{232} and K_{270} extinction coefficients

The extinction coefficients K_{232} and K_{270} are excellent indicators of the oxidative status of oil. The extinction coefficients values give some insights on the quality, preservation, and changes that occurred in the oily matrix by technological processes (Casal et al., 2010). The K_{232} and K_{270} extinction coefficients were measured using a UV-Vis spectrophotometer (UV-VIS V-530 Jasco spectrophotometer, OK, USA). The absorption values were obtained for a concentration of 1 % w/v in cyclohexane in a 10 mm cell (European Commission, 2019).

8.2.2.4.6 Evaluation of the total antioxidant activity and the total phenolic content on olive oil samples

Prior to determining the total antioxidant activity and the total phenolic content, oil samples were submitted to an extraction similar to the method described by Bail et al. (2008) and Parry et al. (2005).

Briefly, in each experiment, 1 g of oil was extracted three times with 5 mL of a methanol/water (90/10 v/v) solution. The mixture was vortexed for 10 minutes and centrifuged for 10 minutes (2 670 g). The methanolic extract was concentrated through the flush with nitrogen and stored at -20 °C until analysis. Before analyzing the TAA and TPC, the dried extract was dissolved in a 10/90 methanol/water solution (1 mL). During instrumental analyses, recovery studies were conducted.

The obtained recovered values were kept above 83% (data not shown). Recovery results were taken into consideration in the present study.

8.2.2.4.6.1 Determination of the antioxidant activity (TAA)

The total antioxidant activity (TAA) was determined through the estimation of the free radical-scavenging ability (RSA) of samples using the 2,2-diphenyl-2-picrylhydrazyl radical (DPPH[•]) (El-Abbassi et al., 2012).

The RSA was evaluated considering a microplate method described by Prieto (2012), with slight modifications. A 0.2 mM DPPH[•] stock solution in methanol was prepared; successive dilutions of the DPPH[•] stock solution allowed to obtain a negative calibration curve – based on three independent calibration curves.

In each microplate well, 100 µL of the sample was admixed with the same volume of methanol. The content of each well was diluted 8 times, is then added the DPPH[•] methanolic solution (100 µL). The reaction mixture was homogenized for 5 minutes and incubated for 45 minutes. The absorbance was read using a microplate reader at 515 nm.

The inhibition percentage of DPPH[•] discoloration (%I) was calculated based on Equation (V.8.5) as follow:

$$\% I = \frac{A_0 - A_1}{A_0} \times 100 \quad (\text{V.8.5})$$

where A_0 is the absorbance of the control reaction (DPPH[•] in methanol) and A_1 is the absorbance of the DPPH radical plus tested sample in methanol. The computation of the %I against the logarithmical concentration permitted the half-inhibitory concentration (IC₅₀) calculation by linear regression analysis, as described by Ahsan et al. (2010). Spectrophotometric analyses were performed in triplicate, and results are presented as mean ± standard deviation. The TAAs were expressed as milligrams per gram of dry OMP (mg/g_{OMP}) or as grams per liter of extract (g/L extract) or even as milligrams of Trolox equivalents (TE) per 100 mL of oil (mg_{TE}/100 mL oil).

Data regarding instrumental analysis are not shown.

8.2.2.4.6.2 Determination of the total phenolic content (TPC)

The total phenolic content (TPC) was determined by the Folin-Ciocalteu micro-method, as described by Barroso et al. (2016), based on a scale-down procedure of the original method proposed by Singleton et al. (1999).

In brief, in each microplate well, sample or standard solution (25 μ L) was admixed with UPW (75 μ L) and with the Folin-Ciocalteu reagent (diluted 1/1 v/v in UPW; 25 μ L). The mixture was homogenized by continuously shaking on an orbital shaker (400 rpm; 1 minute). Then, the reaction mixture was incubated for 5 minutes in the dark at room temperature.

Afterward, to the previous reaction mixture was added a sodium carbonate solution (75 g/L; 100 μ L). The mixture was then homogenized for 1 minute at 400 rpm on an orbital shaker and incubated for 2 hours, protected from light at room temperature. The absorbance was read using a microplate reader at 760 nm.

Gallic acid was selected as a standard for the calibration curve. The obtained calibration curve – mean calibration curve - was based on three independent calibration curves. Spectrophotometric analyses were performed in triplicate. The TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry OMP ($\text{mg}_{\text{GAE}}/\text{g}_{\text{OMP}}$) or as grams of gallic acid equivalents (GAE) per liter of extract ($\text{g}_{\text{GAE}}/\text{L extract}$) or even as milligrams of gallic acid equivalents per kilogram of oil ($\text{mg}_{\text{GAE}}/\text{kg oil}$). Data regarding instrumental analysis are not shown.

8.2.3 Statistical Analysis

The experiments were performed in triplicate. Therefore, results are presented as mean ($n=3$) $\pm$ standard deviation. One—way analysis of variance (one-way ANOVA) was used to determine significant differences. Values of $p<0.05$ were considered statistically significant.

8.3 Results and Discussion

8.3.1 Olive mill pomace extract characterization

The extract (n=3) obtained after the extraction procedure described in Section V.8.2.2.1 presented a TAA of $79.1 \pm 7.9\%$ and a TPC of 50.5 ± 1.5 mg/g_{OMP}. Data suitable for direct comparison of the obtained results was not found. Further considerations are presented in Table V.8.1.

Table V.8.1 - Antioxidant activity and phenolic content of the olive mill pomace extract

<i>Antioxidant Activity^a</i>	
IC ₅₀ mg/g _{OMP}	223.9 ± 1.2
g/L extract	74.6 ± 0.4
<i>Phenols content^a</i>	
mg _{GAE} /g _{OMP}	50.5 ± 1.5
g _{GAE} /L extract	16.8 ± 0.5

^a Results are presented as mean $\pm$ standard deviation (n=3)

GAE – Gallic Acid Equivalents; IC₅₀ – Half-inhibitory Concentration; OMP – Olive Mill Pomace;

8.3.2 Olive mill pomace extract loaded ethylcellulose microparticles characterization

Olive mill pomace loaded ethylcellulose microparticles were successfully produced by water-in-oil-in-water (w₁/o/w₂) double emulsion solvent evaporation. Microparticles were formulated considering a TLC in phenolic compounds of 5% w/w (ALC of $4.8 \pm 0.9\%$). Results regarding the encapsulation efficiency of antioxidants and phenolic compounds are presented in Table V.8.2. Moreover, in Table V.8.2, the results concerning the actual loading contents are presented compared to the theoretical loading contents considered in the present study.

The encapsulation efficiencies varied from 92.6 ± 2.1 in the case of antioxidants and $96.0 \pm 0.3\%$ in the case of phenolic compounds.

The majority of antioxidants and phenolic compounds present in the OMP extract was efficiently embedded into ethylcellulose microparticles.

The actual loading contents were similar to the TLC considered, indicating the efficient incorporation of phenolic compounds into ethylcellulose microparticles. Microparticles were designed considering a TLC in phenolic compounds of 5% w/w. The obtained loading content in phenolic compounds was $4.8 \pm 1.2\%$. About $17.5 \pm 2.9\%$ of the weight of microparticles corresponded to antioxidants' weight.

These outstanding regarding the design of tailored made microparticles encapsulating bioactive compounds extracted from agricultural wastes.

Table V.8.2 - Main physicochemical characteristics of olive mill pomace loaded ethylcellulose microparticles

BAC(s)	EE (% w/w) ^a	TLC % w/w) ^a	ALC (% w/w) ^a
AO	92.6 ± 2.1	18.9 ± 2.7	17.5 ± 1.6
PC	96.0 ± 0.3	5.0 ± 0.4	4.8 ± 0.9

^a Results are presented as mean $\pm$ standard deviation (n=3)

ALC – Actual Loading Content; AO – Antioxidants; BAC – Bioactive Compound; EE – Encapsulation Efficiency; PC – Phenolic Compounds; TLC – Theoretical Loading Content

8.3.3 Changes in the peroxide values (PVs)

The PV is commonly employed to assess the formation of hydroperoxides – the main initial products from oil oxidation (Malheiro et al., 2013). Hydroperoxides are related to fatty acids susceptibility to oxidation and oil oxidative status (Casal et al., 2010). It is a mandatory quality parameter that must be evaluated when intended olive oil commercialization (European Commission, 2019; República Portuguesa, 2004).

The results regarding the PV levels of EVOO, VOO, and ROO during the 24-day storage are presented in Table V.8.3.

At the beginning of the study, both simple (blank), BHA-rich, BHT-rich, BHA+BHT-rich, extract-rich, and MPs-loaded extra-virgin olive oil, virgin olive oil, and blended olive oil presented similar PVs (ranged between 11 and 12 mEq/kg) which were within the legal limits: below 20 mEq/kg in the case of EVOOs and VOOs; and 15 mEq/kg in the case of ROOs (European Commission, 2019). Similar PVs were found at the beginning of the study by Casal et al. (2010) when the authors compared the oxidative stability of EVOOs, VOOs, and ROOs under deep-frying conditions. Similarity was verified using ANOVA.

Along with the study, the PVs of all the oils (EVOO, VOO, and ROO) with or without additives (e.g., BHA, BHT, BHA+BHT, OMP extract, or OMP-loaded ethylcellulose microparticles) increased sharply, excepting in the case of ROO. In the case of ROO, it was observed a decrease in the PV from Day 12 to Day 18. The decrease in the PV can be attributed to the appearance of hydroperoxides susceptible to decomposition, leading to the formation of carbonyl compounds (Fereidoon & Ying, 2010). Therefore, it can be concluded that the blend of virgin olive oil with refined olive oil presents poor oxidative stability. Similar results were obtained by Yang et al. (2016) during the analysis of the substitution of synthetic antioxidants by rosemary extract to improve the oxidative stability of soybean oil, cottonseed oil, and rice bran oil. The authors verified a decrease in the PV of blank cottonseed oil associated with the formation of transient chemical compounds, namely hydroperoxides. Analyses were performed in triplicate for each flask, corresponding to total number of PV analyses of 9.

On the Day 24, the blank of all oils presented PVs (20.4 ± 0.6 mEq/kg in the case of EVOO; 21.7 ± 0.5 mEq/kg in the case of VOO; and 15.8 ± 0.2 mEq/kg in the case of ROO) higher than the maximum legal limit (20 mEq/kg in the case of EVOOs and VOOs; and 15 mEq/kg in the case of ROOs). The incorporation of synthetic antioxidants (BHA, BHT, or a blend of BHA and BHT) or natural antioxidants (OMP extract or OMP-loaded ethylcellulose microparticles) prevented oils from excessive oxidation; the embedment favored the oxidative stability of olive oils. On day 24, the blank EVOO, VOO, and ROO exhibited ($p < 0.05$) higher PVs compared to EVOO, VOO and ROO enriched with BHA, BHT, a blend of BHA and BHT, extract, or microparticles. Among the OOs enriched with synthetic antioxidants, the PVs were not significantly different ($p > 0.05$).

The PVs of oils enriched with extract and microparticles at the end of the study (24th day) were statistically significant ($p < 0.05$).

Oils incorporating OMP-loaded ethylcellulose microparticles demonstrated significantly lower PVs than the enriched oils patterns (olive oils enriched with synthetic antioxidants and OMP extract). The OMP-loaded ethylcellulose microparticles were more efficient in the retardation of peroxidation phenomena.

The observed PVs of oils enriched with microparticles were below the maximum legal limit for PVs of olive oils. Olive oils enriched with OMP-extract loaded ethylcellulose microparticles exhibited a great potential to retard the formation of hydroperoxides.

Table V.8.3 - Changes in the peroxide value of olive oils during storage

OOS	ST ^a	Peroxide value (mEq/kg) ^b					
		Blank	BHA	BHT	BHA+BHT	OMP Extract	MPs
EVOO	0	11.5 ± 0.2	11.3 ± 0.2	11.5 ± 0.3	11.2 ± 0.4	11.6 ± 0.1	11.5 ± 0.2
	6	15.5 ± 0.1	13.4 ± 0.2	14.5 ± 0.2	13.9 ± 0.2	13.1 ± 0.1	12.1 ± 0.1
	12	17.7 ± 0.1	15.8 ± 0.2	15.7 ± 0.1	15.4 ± 0.2	14.9 ± 0.2	15.8 ± 0.2
	18	18.6 ± 0.1	17.8 ± 0.1	18.6 ± 0.2	17.9 ± 0.5	17.7 ± 0.3	16.6 ± 0.1
	24	20.4 ± 0.6	19.5 ± 0.3	19.4 ± 0.6	19.1 ± 0.3	18.0 ± 0.2	17.9 ± 0.6
VOO	0	11.4 ± 0.1	11.4 ± 0.1	11.4 ± 0.1	11.3 ± 0.1	11.4 ± 0.1	11.5 ± 0.1
	6	13.5 ± 0.1	12.2 ± 0.2	15.5 ± 0.1	13.5 ± 0.3	12.4 ± 0.2	12.3 ± 0.2
	12	15.9 ± 0.1	13.3 ± 0.3	16.9 ± 0.2	15.4 ± 0.6	14.6 ± 0.2	13.3 ± 0.1
	18	19.7 ± 0.2	15.4 ± 0.2	18.7 ± 0.2	16.7 ± 0.3	16.1 ± 0.1	14.1 ± 0.2
	24	21.7 ± 0.5	18.6 ± 0.4	19.5 ± 0.5	19.1 ± 0.2	18.3 ± 0.4	16.2 ± 0.5
ROO	0	11.7 ± 0.1	11.6 ± 0.2	11.8 ± 0.1	11.6 ± 0.2	11.4 ± 0.1	11.4 ± 0.1
	6	12.6 ± 0.1	12.1 ± 0.1	12.3 ± 0.3	12.3 ± 0.1	12.3 ± 0.2	12.2 ± 0.1
	12	23.4 ± 0.2	13.5 ± 0.5	13.8 ± 0.2	12.7 ± 0.6	12.5 ± 0.1	12.4 ± 0.2
	18	14.7 ± 0.1	13.8 ± 0.3	14.6 ± 0.1	14.3 ± 0.1	13.6 ± 0.1	12.5 ± 0.6
	24	15.8 ± 0.2	14.2 ± 0.7	14.8 ± 0.2	14.9 ± 0.4	14.8 ± 0.2	13.4 ± 0.4

^a Measured in days (Day 0, 6, 12, 18 and 24)

^b Results are presented as mean ± standard deviation

BHA – Butylated Hydroxyanisole; BHT – Butylated Hydroxytoluene; EVOO – Extra-Virgin Olive Oil; mEq – milliequivalents; MPs – Microparticles; OMP – Olive Mill Pomace; OOS – Olive Oil Sample; ROO – Blend of virgin olive oil and refined olive oil; VOO – Virgin Olive Oil; ST – Storage Time;

8.3.4 Changes on the *p*-anisidine values (*p*-AVs)

The *p*-AV is a measure of the presence of secondary oxidation products, namely ketones, aldehydes, and carboxyl acids (Asensio et al., 2011; Sun-Waterhouse et al., 2011). Results regarding the *p*-AV levels of EVOO, VOO, and ROO during the 24-day storage are shown in Figure V.8.1.

After 24 days of the beginning of the study, each oil – blank and enriched – had a higher *p*-AV than their initial values on Day 0. All the olive oil samples (EVOO, VOO, and ROO) exhibited identical evolution on the *p*-AVs during the study, independently of the initial PVs, *p*-AVs, and their variations. The formation of stable secondary oxidation species occurred at similar rates in EVOO, VOO, and ROO samples. A similar observation was drawn by Casal et al. (2010). During the study of olive oils stability under deep-frying conditions, the authors concluded that extra-virgin olive oils, virgin olive oils, and blends of refined olive oils and virgin oils; the *p*-AVs evolution was independent of the initial PVs and *p*-AVs as well as their variations during the study.

The *p*-AVs of blank oils and enriched oils were statistically significant ($p < 0.05$). No differences ($p > 0.05$) were found between the *p*-AVs of oils enriched with synthetic antioxidants (BHA and BHT), considering solely or in a blend. Notwithstanding, the embedment of OMP-extract microparticles led to the obtainment of significantly ($p < 0.05$) lower *p*-AVs, which is related to a lower content of secondary oxidation products in EVOO, VOO, and ROO selected in the present study. In the present study, for all oils, it was observed *p*-AVs and *p*-AV change rates higher than the reported values by Asensio et al. (2011) when the authors were evaluating the effect of adding oregano essential oil to extra-virgin olive oil during long-term storage conditions (maximum *p*-AV observed was about 9.3, after 28 days of storage). Nevertheless, the obtained *p*-AVs in the present study were lower than the ones presented by Casal et al. (2010) (maximum *p*-AV of 66 in an EVOO, after 15 hours of frying). This current study supports the thesis that encapsulated antioxidants are sustainly released from microparticles embedded in olive oils, effectively reducing the presence of stable secondary oxidation products through antioxidants act as hydrogen-donating substituents.

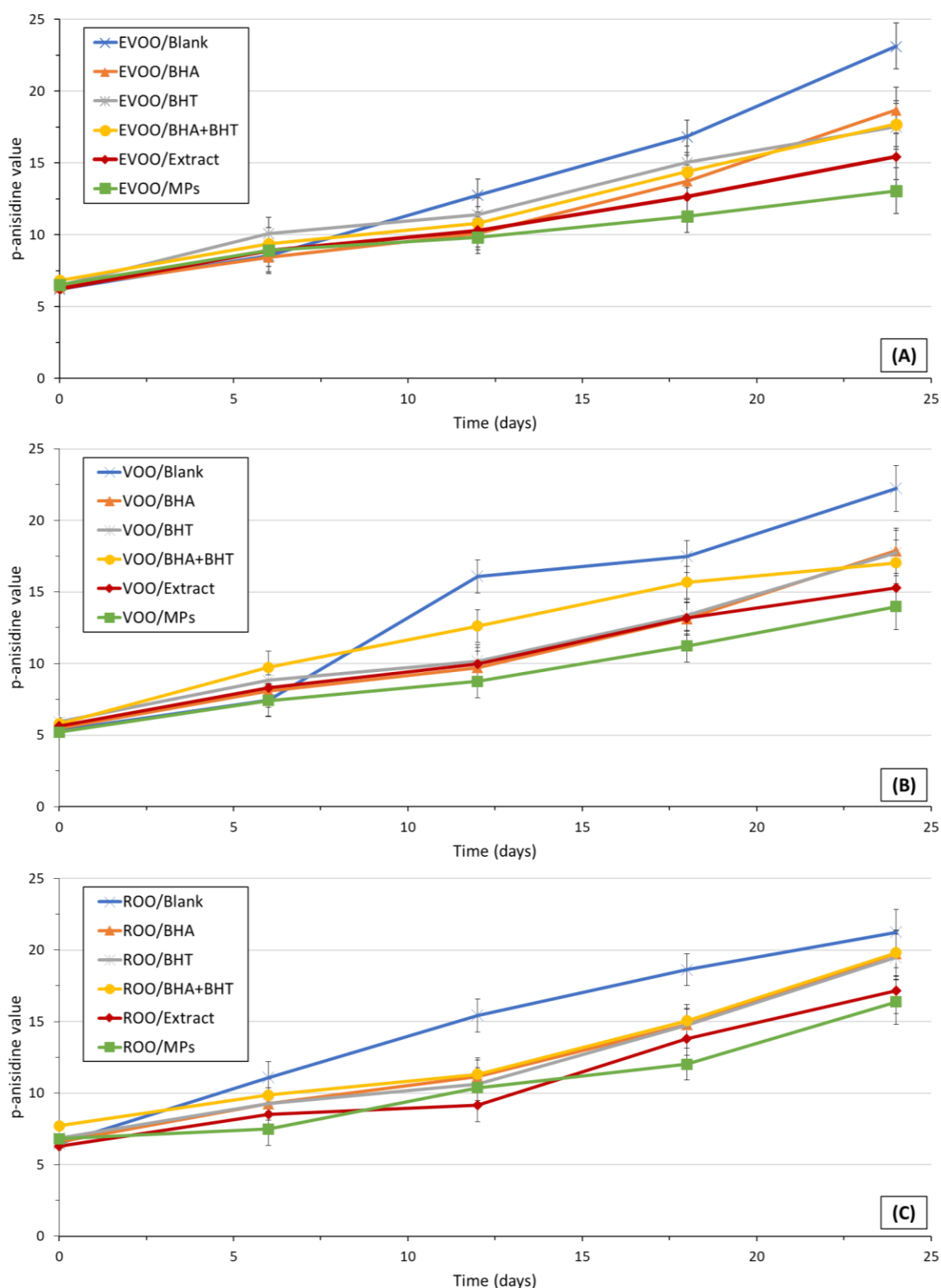


Figure V.8.1 - Changes in the p-anisidine value of plain () and incorporating BHA (), BHT (), BHA+BHT (), extract () and microparticles () extra-virgin olive oil (A), virgin olive oil (B), and a blend of refined and virgin olive oil (C) during storage (BHA – Butylated Hydroxyanisole; BHT - Butylated Hydroxytoluene; EVOO – Extra-Virgin Olive Oil; MPs – Microparticles; ROO – Blend of refined and virgin olive oils; VOO – Virgin Olive Oil)

8.3.5 Changes in the total oxidation (TOTOX) values

The TOTOX value is an indicator of the overall oxidation status of an oil (Pokorny et al., 2003). It is generally acknowledged that the empirical maximum acceptable level of TOTOX value is 30 (Sun-Waterhouse et al., 2011). Results regarding the obtained TOTOX values based on PVs and *p*-AVs are presented in Table V.8.4. Generically, the TOTOX values increased with the increase of storage time of all olive oil samples.

At Day 24 and in the case of EVOO, the TOTOX values decreased in the order of blank > BHA > BHT > BHA+BHT > OMP extract > microparticles. In the case of both VOO and ROO, on Day 24, it was observed that the TOTOX values decreased in the order of blank > BHA > BHT > BHA+BHT > OMP extract > microparticles. In all oil samples, the lowest TOTOX values were observed in the case of the enrichment with microparticles, followed by oils incorporation with OMP extracts.

The TOTOX values exceeding the maximum acceptable level (30) are marked in grey background in Table V.8.4. At the end of the storage period, it was observed that the quality of oils embedding encapsulated antioxidants was acceptable. The quality of oils embedding OMP extract was guaranteed until Day 18 of the study. Nevertheless, the quality of plain oils and oils fortified with BHA, BHT, or with a blend of BHA and BHT were not acceptable from the Day 18 onwards (TOTOX values > 30).

The obtained results in the current study are in agreement with the ones obtained by Sun-Waterhouse et al. (2011) during the study of the stability of an extra-virgin oil during fortified with encapsulated caffeic acid. The authors verified an increase of oil quality (reduction of the TOTOX values) from the blank to enriched oil with encapsulated caffeic acid.

The low TOTOX values are correlated with the high quality of oils. In the present study, it was verified that the quality of extra-virgin olive oil, virgin olive oil, and a blend of virgin oil and refined olive oil was improved when fortified with encapsulated antioxidants obtained from olive mill pomace.

Table V.8.4 - Total oxidation values of olive oils during storage

OOS	ST ^a	TOTOX Value ^b					
		Blank	BHA	BHT	BHA+BHT	OMP Extract	MPs
EVOO	0	17.7 ± 0.4	17.6 ± 0.4	18.0 ± 0.6	18.0 ± 0.6	17.8 ± 0.2	18.0 ± 0.5
	6	24.0 ± 0.3	21.8 ± 0.4	24.6 ± 0.4	23.3 ± 0.3	22.0 ± 0.2	21.0 ± 1.2
	12	30.4 ± 0.4	25.9 ± 0.4	27.1 ± 0.3	26.2 ± 0.8	25.2 ± 0.3	25.6 ± 1.4
	18	35.4 ± 0.2	31.5 ± 0.3	33.7 ± 0.4	32.3 ± 0.8	30.3 ± 0.5	27.9 ± 1.2
	24	43.5 ± 0.9	38.2 ± 0.6	36.9 ± 1.2	36.8 ± 0.7	33.5 ± 0.4	31.0 ± 1.2
VOO	0	16.7 ± 0.2	16.9 ± 0.2	17.3 ± 0.2	17.1 ± 0.4	17.0 ± 0.2	16.7 ± 0.2
	6	20.9 ± 0.3	20.3 ± 0.4	24.3 ± 0.2	23.2 ± 0.4	20.7 ± 0.3	19.7 ± 0.3
	12	32.0 ± 0.4	23.0 ± 0.5	27.1 ± 0.4	28.0 ± 1.5	24.6 ± 0.3	22.1 ± 0.2
	18	37.2 ± 0.4	28.5 ± 0.4	32.1 ± 0.4	32.4 ± 0.6	29.3 ± 0.3	25.3 ± 0.2
	24	43.9 ± 0.9	36.5 ± 0.6	37.2 ± 0.1	36.1 ± 0.8	33.6 ± 0.5	30.2 ± 0.4
ROO	0	18.2 ± 0.3	18.2 ± 0.4	18.7 ± 0.2	19.3 ± 0.3	17.7 ± 0.2	18.2 ± 0.1
	6	23.7 ± 0.2	21.4 ± 0.2	21.6 ± 0.5	22.2 ± 0.2	20.8 ± 0.4	19.7 ± 0.2
	12	38.8 ± 0.6	24.7 ± 0.7	24.4 ± 0.4	24.0 ± 1.0	21.6 ± 0.2	22.8 ± 0.2
	18	33.3 ± 0.4	28.6 ± 0.5	29.3 ± 0.2	29.4 ± 0.2	27.4 ± 0.2	24.5 ± 0.7
	24	37.0 ± 0.9	33.9 ± 1.4	34.3 ± 0.4	34.7 ± 0.4	31.9 ± 0.4	29.8 ± 0.6

^a Measured in days (Day 0, 6, 12, 18 and 24)

^b Results are presented as mean ± standard deviation

BHA – Butylated Hydroxyanisole; BHT – Butylated Hydroxytoluene; EVOO – Extra-Virgin Olive Oil; MPs – Microparticles; OMP – Olive Mill Pomace; OOS – Olive Oil Sample; ROO – Blend of virgin olive oil and refined olive oil; TOTOX – Total Oxidation; VOO – Virgin Olive Oil; ST – Storage Time;

8.3.6 Changes in the free fatty acids (FFAs) content

The monitorization of changes in free fatty acids (FFAs) content is considered to be relevant to measure rancidity in foods, especially in oils and fats. The FFAs are obtained through the hydrolysis of triglycerides; the reaction of oils with moisture prompts the formation of these compounds (Frega et al., 1999). In the present study, the FFA content was evaluated by conventional acidity measurement, as described in Section V.8.2.2.4.4. Results regarding the acidity values are presented in Figure V.8.2.

For all olive oil samples – plain and enriched – it was observed the increase in the FFA content with the increase of the storage period.

Nevertheless, in the present study, a regular pattern of the increase was not observed. Similar conclusions were drawn by Iqbal & Bhanger (2007): similarly, the authors verified, during their study regarding the stabilization of sunflower oil using an extract of garlic acid, that the FFA content increased with the increase of the storage period, not identifying an increasing pattern.

During the current study, the FFA content was below 0.62% in the case of EVOO, 0.67% in the case of VOO, and below 0.83% in the case of ROO. The obtained acidity values were in agreement with the respective labeling category – the FFA (%) should be lower than 0.80%, 2.00%, and 1.00%, in the case of extra-virgin olive oils, virgin olive oils, and blends of refined and virgin olive oils, respectively (European Commission, 2019; República Portuguesa, 2004).

In all oil samples, it was observed higher ($p < 0.05$) acidity of the plain oils compared to the enriched ones. In both EVOO, VOO, and ROO, the acidity of oils enriched with microparticles was significantly lower than those enriched with synthetic antioxidants (BHA, BHT, or a blend of these antioxidants). Nevertheless, the acidity of oils enriched with OMP-loaded ethylcellulose microparticles and OMP extract only were similar ($p > 0.05$) for all time-points during the storage study. Therefore, it can be concluded that the incorporation of olive mill pomace extract plain or formulated in microparticles favored the hydrolytic stability of the oils, namely through the control of the acidity of the oils (lower values of oils acidity enriched with extract and microparticles rather than plain oils and oils incorporating synthetic antioxidants). Similarly, the authors Sun-Waterhouse et al. (2011) verified that encapsulated and non-encapsulated caffeic acid was effective in slowing down FFA production in an extra-virgin oil.

The ROOs presented at all time points higher acidities rather than EVOO and VOO. This observation can be explained considering that (i) the ROO is a blend of refined and virgin olive oils and (ii) olive oils refining prompts the removal of free fatty acids by saponification. In this context, only trace amounts of FFA are expected in refined olive oils. However, as it was observed slighter higher acidity values for the ROOs, it can be hypothesized that the virgin olive oil present in the blend presented higher acidity than the VOO considered solely in the study.

The results of this study indicate the incorporation of olive mill pomace extract and olive mill pomace extract encapsulated into microparticles in olive oils (extra-virgin olive oil, virgin olive oil, and a blend of refined and virgin olive oil) effectively slowed down the hydrolysis, ensuring the acidity values keeping below the rejection points even during accelerated storage conditions. Moreover, in this study, it is proven that natural-occurring antioxidants presented in an extract or formulated in microparticles are more effective in retarding the free fatty acids formation than synthetic antioxidants. To retard hydrolysis processes in oil samples, it is recommended to add natural-occurring antioxidants rather than the synthetic ones (e.g., OMP or encapsulated OMP extract over BHA and BHT).

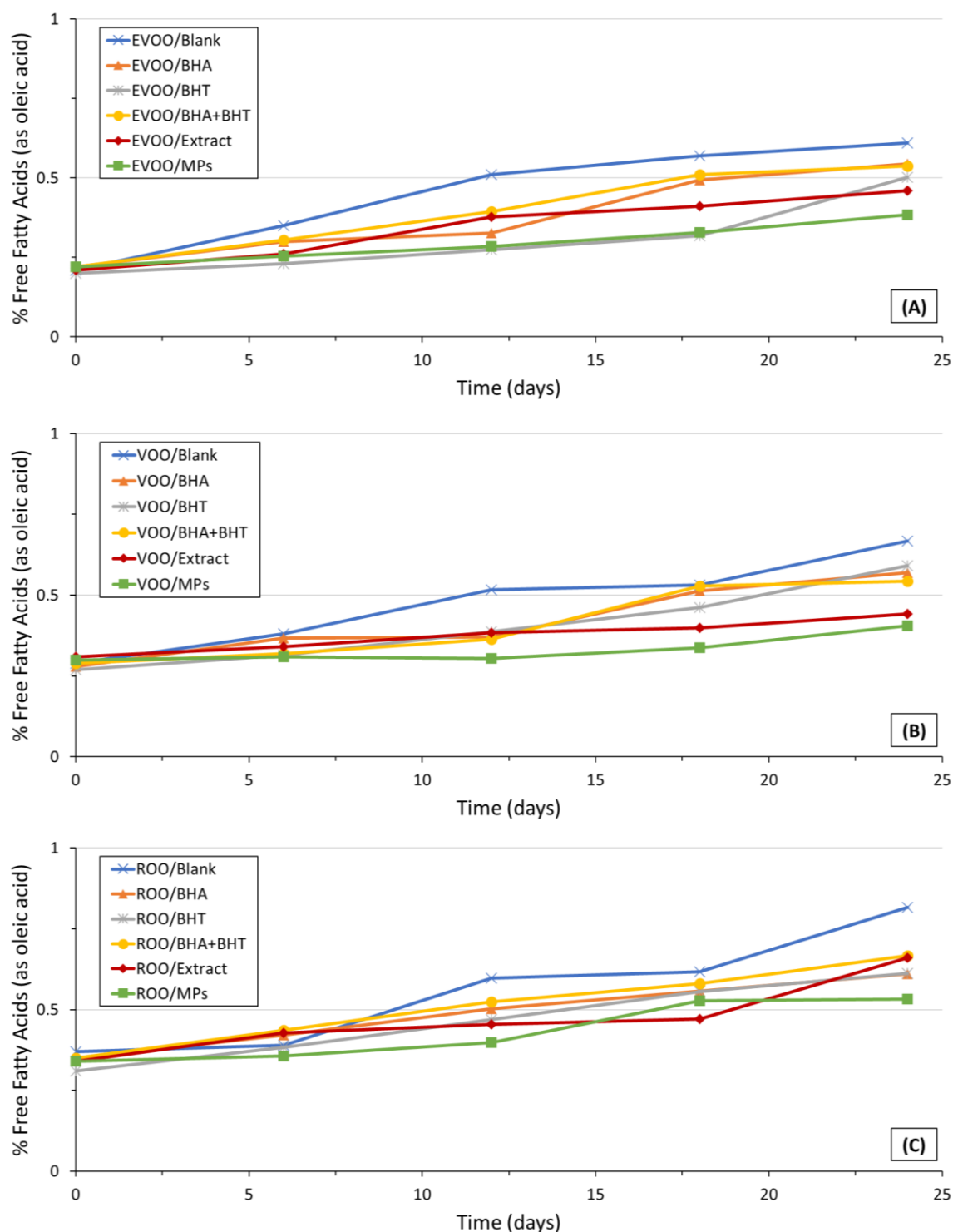


Figure V.8.2 - Changes in free acidity values (% free fatty acids as oleic acid) of plain ($\times$) and incorporating BHA ($\triangle$), BHT ($\ast$), BHA+BHT ($\diamond$), extract ($\blacklozenge$) and microparticles ($\blacksquare$) extra-virgin olive oil **(A)**, virgin olive oil **(B)**, and a blend of refined and virgin oil **(C)** during storage. (BHA – Butylated Hydroxyanisole; BHT – Butylated Hydroxytoluene; EVOO – Extra-Virgin Olive Oil; MPs – Microparticles; ROO – Blend of refined and virgin olive oils; VOO – Virgin Olive Oil)

8.3.7 Changes on the K_{232} and K_{270} extinction coefficients

The ultraviolet spectrophotometric analysis through the specific extinction coefficients' measure provides some useful insights on the olive oil oxidation status. The K_{232} extinction coefficient is related to the formation of conjugated dienes of polyunsaturated fatty acids (PUFA), whereas the K_{270} extinction coefficient value indicates the presence of both primary and secondary oxidation products, namely carbonyl compounds and conjugated trienes (Casal et al., 2010).

The maximum admissible of the K_{232} extinction coefficient is 2.5 in the case of EVOOs, 2.6 in the case of VOOs. Nevertheless, in the case of blends of virgin and refined olive oils, the legal limit of K_{232} is not defined. The legal limit of K_{270} is 0.22, 0.25, and 1.15 in the case of EVOOs, VOOs, and ROOs, respectively (European Commission, 2019).

All olive oil samples presented K_{232} extinction coefficients within the legislation limits at all-time points during the study (Figure V.8.3). In the case of plain EVOO and VOO, it was observed significant differences ($p < 0.05$) in the K_{232} values comparing to their enriched with natural and synthetic antioxidants patterns. It was not observed differences between oil samples enriched with BHA, BHT, a blend of BHA and BHT, extract, and OMP-loaded ethylcellulose microparticles in all oil samples (EVOO, VOO, and ROO). A plateau on the K_{232} values in the case of fortified EVOO during storage was observed. Therefore, it can be concluded that the incorporation of both natural and synthetic antioxidants favored olive oils stability during storage. Probably, only a minor amount of conjugated dienes of PUFA were produced during the 24-days storage under accelerated conditions.

The evolution of the K_{270} extinction coefficients during storage is presented in Figure V.8.4. In the case of the EVOO and the VOO, it was not observed differences ($p > 0.05$) among oil samples (fortified and non-fortified). In the case of the EVOO, the K_{270} values were kept below the legal limit (< 0.22 ; maximum observed K_{270} value of 0.22 ± 0.42 in the case EVOO fortified with a blend of BHA and BHT), excepting in the case of the plain EVOO at Day 24 which was observed a K_{270} value slightly above the legal limit (0.61 ± 0.23). In the case of the EVOO, the K_{270} extinction coefficient was independent of the incorporation of natural or even synthetic antioxidants.

The fortification of extra-virgin olive oil samples with antioxidants (natural; natural embedded in microparticles; synthetic) did not impact the K_{270} extinction coefficient.

Dissimilarly, the K_{270} extinction coefficients of the plain ROO and the ROO fortified with OMP-loaded ethylcellulose microparticles were significantly different. On Day 24, the K_{270} of ROO incorporating OMP-loaded ethylcellulose microparticles (K_{270} observed value of 0.24 ± 0.07) was lower ($p < 0.05$) than the plain ROO (K_{270} observed value of 0.66 ± 0.09), the ROO fortified with synthetic antioxidants (K_{270} observed value of 0.42 ± 0.08 in the case of fortification with BHA; K_{270} of 0.50 ± 0.02 in the case of BHT and 0.44 ± 0.01 in the case of the fortification with a blend of BHA and BHT) and the ROO incorporating an extract of OMP (K_{270} observed value of 0.47 ± 0.03). In all ROO oil samples, the K_{270} extinction coefficients were kept below the legal limit (1.15) throughout the 24-day study.

The present study results are partially in accordance with the ones obtained by Casal et al. (2010) during their study of olive oils stability under deep-frying conditions. The authors also observed an increase in the K_{232} parameter with time, not verifying, similarly to the present study, K_{232} values exceeding the legal limit. Similarly, the authors did not observe clear differences between oil samples.

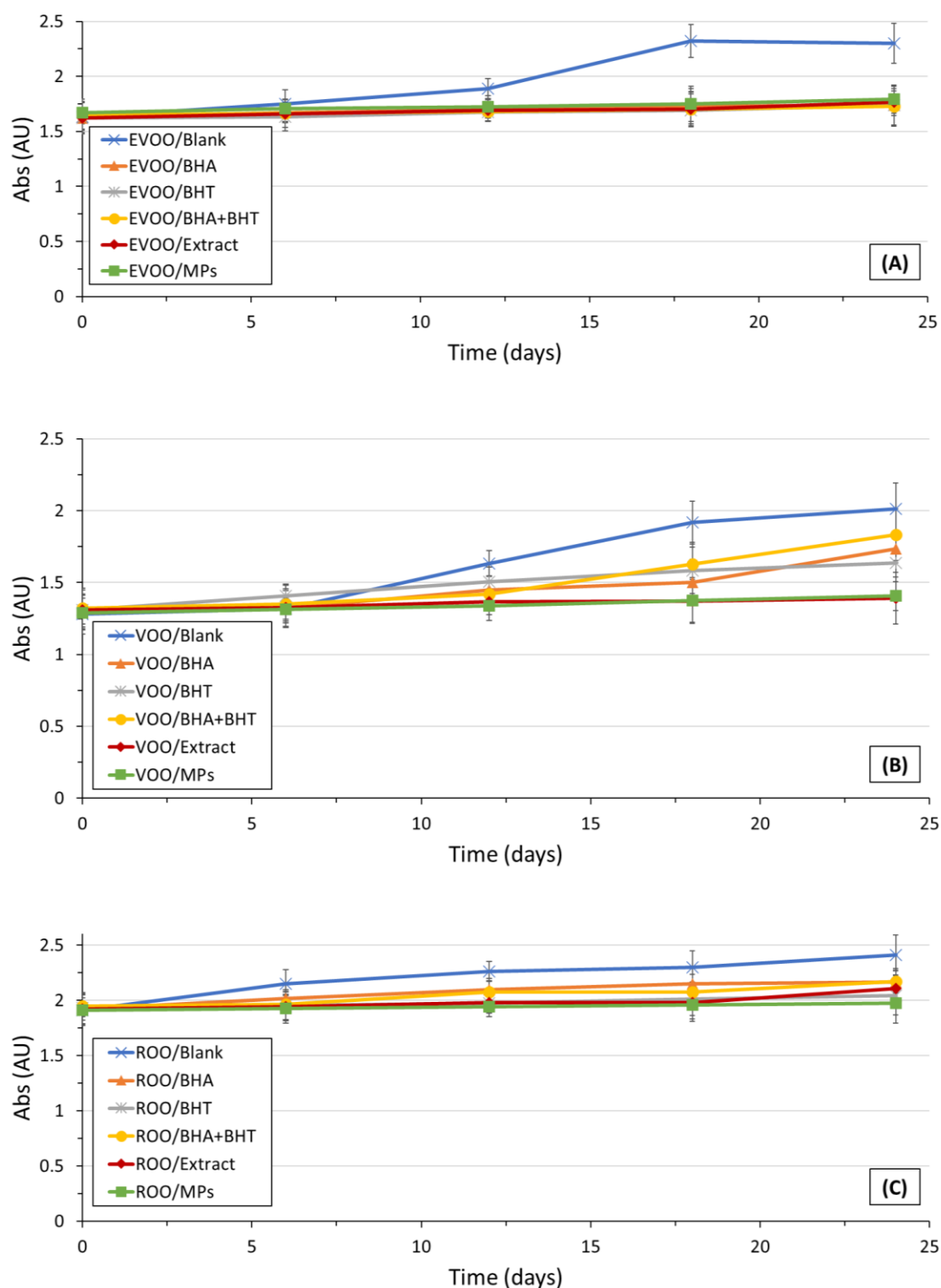


Figure V.8.3 - Changes on the K_{232} extinction coefficient (Abs; AU) of plain (—) and incorporating BHA (—), BHT (—), BHA+BHT(—), extract (—) and microparticles (—) extra-virgin olive oil **(A)**, virgin olive oil **(B)**, and a blend of refined and virgin oil **(C)** during storage. (BHA – Butylated Hydroxyanisole; BHT – Butylated Hydroxytoluene; EVOO – Extra-Virgin Olive Oil; MPs – Microparticles; ROO – Blend of refined and virgin Olive Oils; VOO – Virgin Olive Oil)

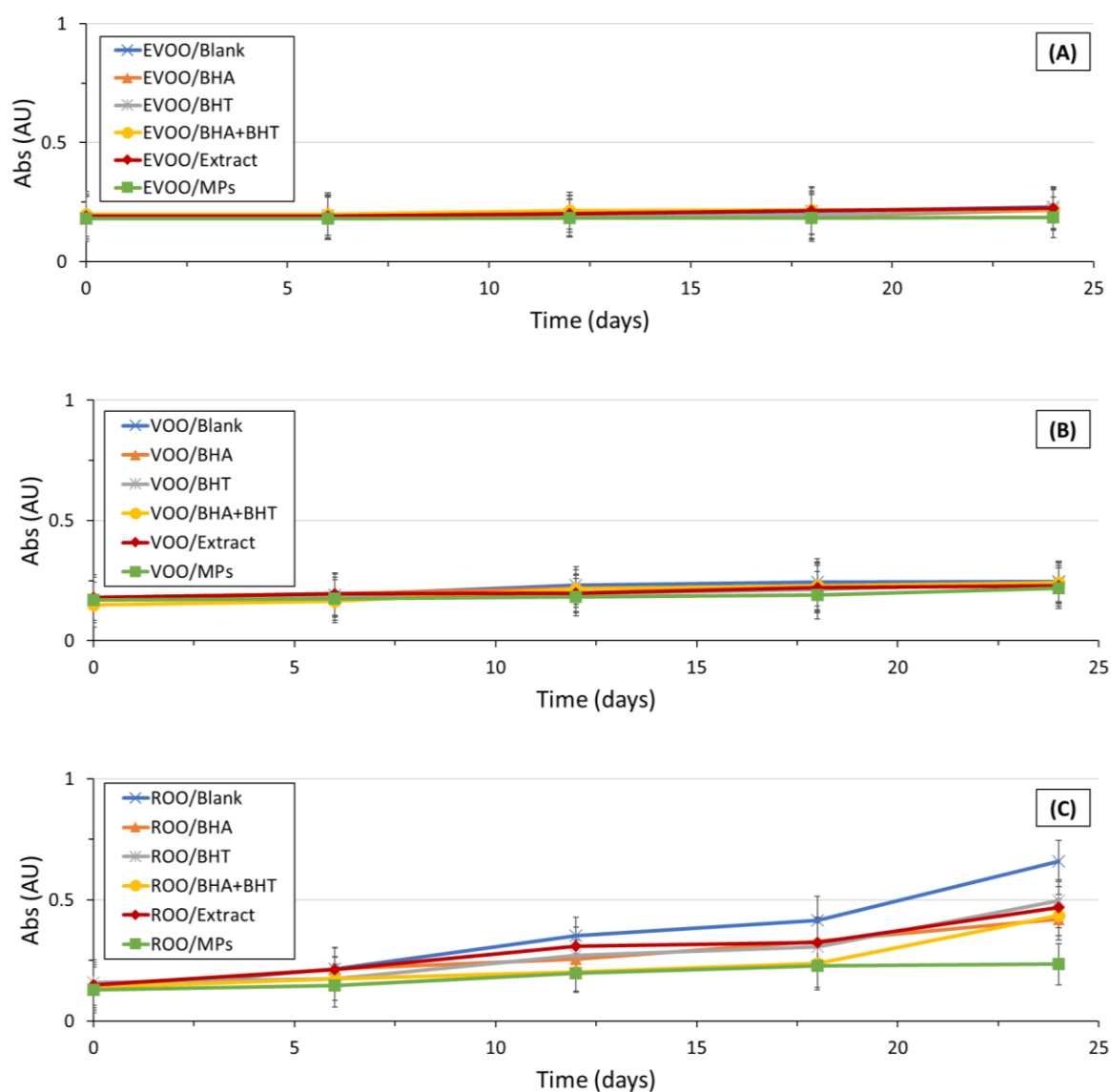


Figure V.8.4 - Changes on the K_{270} extinction coefficient (Abs; AU) of plain (—) and incorporating BHA (—), BHT (—), BHA+BHT (—), extract (—) and microparticles (—) of extra-virgin olive oil **(A)**, virgin olive oil **(B)**, and a blend of refined and virgin oil **(C)** during storage. (BHA – Butylated Hydroxyanisole; BHT – Butylated Hydroxytoluene; EVOO – Extra-Virgin Olive Oil; MPs – Microparticles; ROO – Blend of refined and virgin Olive Oils; VOO – Virgin Olive Oil)

8.3.8 Changes in the total antioxidant activity (TAA) and the total phenolic content (TPC)

The evaluation of the total antioxidant activity (TAA) of oils is commonly employed to determine the stability of oils (Yang et al., 2016; Zeb & Murkovic, 2013). The changes in the TAA were measured by the DPPH assay, as described in Section V.8.2.2.4.6.1.

Results are presented in Figure V.8.5 – A, B, and C. From the ANOVA analysis, no significant differences ($p > 0.05$) were found between the TAA values at days 6, 12, and 18 for all oil samples. A similar observation was drawn by Yang et al. (2016) during their study of the stability of a vegetable oil enriched with rosemary extract during storage (Schaal oven test; 24-day study). Similarly, the authors did not verify differences in the TAA of oils (plain and fortified) from Day 6 to Day 18.

The highest TAA, at Day 0, was observed in the case of oils enriched with OMP extract (TAA of EVOO enriched with extract of 21.1 ± 0.2 mg TE/100 mL of oil; TAA of VOO fortified with OMP extract of 18.3 ± 0.1 mg TE/100 mL of oil and TAA of ROO incorporating OMP extract of 21.2 ± 0.9 mg TE/100 mL of oil). At the beginning of the study, oils fortified with OMP extract demonstrated higher antioxidant activity than blank oils, oils fortified with synthetic antioxidants (BHA, BHT, and a blend of BHA and BHT), and oils enriched with OMP extract-loaded ethylcellulose microparticles.

The TAA of oils incorporating OMP extract loaded ethylcellulose microparticles was similar ($p < 0.05$) of plain oils. This was an expected result as the coating material of microparticles – ethylcellulose – does not exhibit antioxidant activity, and the majority of antioxidants were efficiently embedded inside of microparticles and not adsorbed at the surface of microparticles, which explains the TAA values of oils incorporating microparticles similar to the TAA values of plain oils.

During the 24-day study, the TAA of plain oils, oils fortified with synthetic antioxidants (BHA, BHT, and a blend of BHA and BHT), and oils enriched with OMP extract significantly decreased ($p < 0.05$), excepting the case of oils incorporating microparticles.

In the case of olive oils fortified with OMP-loaded ethylcellulose microparticles, the TAA was kept barely constant during the 24-day storage study (e.g., TAA of EVOO/MPs at Day 0 of 12.2 ± 0.1 mg TE/100 mL oil; TAA of EVOO/MPs at Day 24 of 11.3 ± 0.2 mg TE/100 mL oil). Therefore, it can be concluded that olive mill pomace loaded ethylcellulose microparticles proven to be an efficient delivery system of antioxidants in olive oils to increase the oil oxidative stability during storage.

During the current study, it was not observed differences between the TAA of oils fortified with synthetic antioxidants (BHA, BHT, or a blend of them).

The higher TAA of oils with OMP extract compared with oils enriched with synthetic antioxidants can be explained considering that hydroxytyrosol is the major bioactive compound in the OMP extract exhibiting outstanding antioxidant activity (Angelo et al., 2001; Colica et al., 2017; Mart et al., 2018). Hydroxytyrosol presents two *o*-phenolic hydroxyl groups bonded to the benzene ring, while BHA and BHT present only one hydroxyl group attached to the benzene ring. Even though the TAA is affected by the relative position of *o*-phenolic hydroxyl groups, the presence of specific groups in the molecular structure, it is generally recognized that the TAA is mainly affected by the number of *o*-phenolic hydroxyl groups in the molecular structure (Yang et al., 2016). Therefore, the increase of the number of *o*-phenolic hydroxyl groups from BHA and BHT to hydroxytyrosol led to an increase of the TAA of oils incorporating OMP extract.

The olive oils are receiving particular attention compared to other vegetable oils due to their high content of phenolic compounds, which bestow for olive oils antioxidant and even antimicrobial properties being described their noteworthy role in the maintenance of olive oils oxidative status (Gómez-Alonso et al., 2003; Ocakoglu et al., 2009).

The TPC of olive oils was monotonized through 24 days of accelerated storage conditions. Results are presented in Figure V.8.5-D, E, and F. From the ANOVA, data from days 8, 12, and 18 are not presented as no significant differences ($p > 0.05$) were found between them for all oil samples.

In comparison, blank EVOO presented higher TPC (198.3 ± 12.1 mg_{GAE}/ kg oil) than ROO (124.5 ± 6.8 mg_{GAE}/kg oil) and VOO (87.2 ± 10.5 mg_{GAE}/kg oil).

These results are in agreement with the ones obtained by Casal et al. (2010). Similarly, the authors observed higher TPC values on EVOO, followed by ROO, detecting the lowest TPC values in VOOs.

At the beginning of the study (Day 0), all oil samples (EVOO, VOO, and ROO) incorporating OMP extract exhibited significantly ($p < 0.05$) higher TPC than their blank, enriched with synthetic (BHA, BHT and a blend of BHA and BHT) and microparticles oils patterns.

The total phenolic content of olive oil samples significantly decreased ($p < 0.05$) during storage, excepting in the case of oils enriched with OMP-extract loaded ethylcellulose microparticles. The TPC of oil samples with extract and microparticles on Day 24 was similar ($p > 0.05$). The observed decrease in the TPC probably was due to oxidation and decomposition of phenolic compounds present in the oil matrices, which experience both qualitative and quantitative physicochemical modifications during storage (Gargouri et al., 2015). Moreover, in oils, phenolic compounds act as antioxidants by donating H-atom(s) to free radicals, contributing to the decrease in the TPC (Sánchez et al., 2007). However, in the case of oils with microparticles, the phenolic compounds are controlled dripping. This prompts a balance between the decomposition and the release of phenolic compounds, which allows maintaining the oxidative stability of olive oils.

These results support the thesis that olive mill pomace loaded ethylcellulose microparticles were efficiently designed to sustained release phenolic compounds to control the oxidative status of oil samples. The incorporation of OMP extract loaded ethylcellulose microparticles improved the oxidative resistance of olive oils.

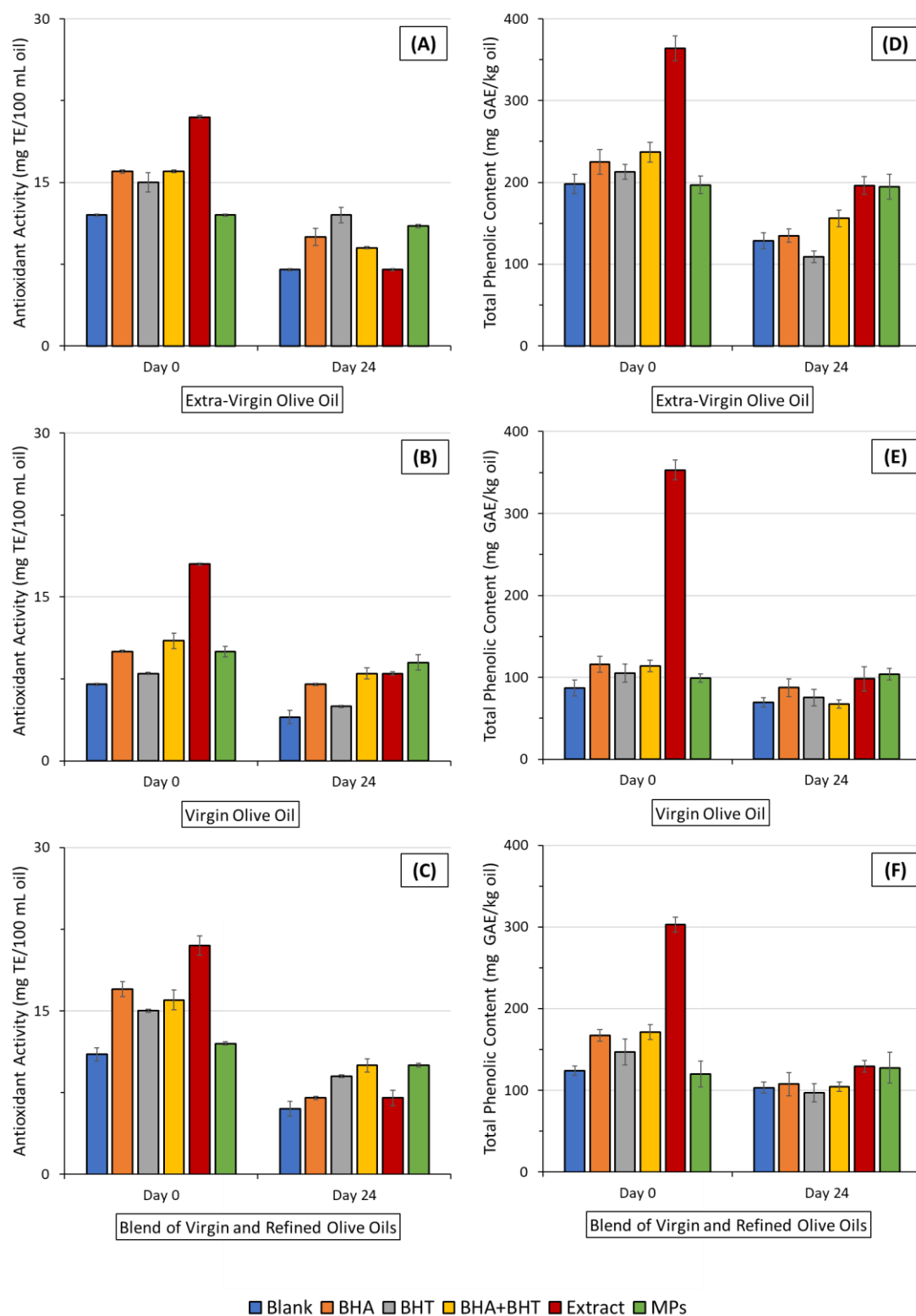


Figure V.8.5 - Changes in the antioxidant capacity measured by the DPPH of an extra-virgin olive oil (A), a virgin olive oil (B) and a blend of virgin and refined olive oils (C); and total phenolic content of an extra-virgin olive oil (D), a virgin olive oil (E) and a blend of virgin and refined olive oils (F) during storage. (BHA – Butylated Hydroxyanisole; BHT – Butylated Hydroxytoluene; GAE – Gallic Acid Equivalents; MPs – Microparticles; TE – Trolox Equivalents)

8.4 Conclusions

In the present study, the oxidation stability of olive oils (extra-virgin, virgin, and a blend of virgin and refined) in the presence of olive mill pomace ethylcellulose microparticles was assessed.

An olive mill pomace extract was obtained through an optimized two-step solid-liquid extraction. The obtained extract was then efficiently encapsulated into ethylcellulose microparticles by water-in-oil-in-water double emulsion solvent evaporation encapsulation technique. Microparticles were formulated considering a theoretical loading content in phenolic compounds of 5% w/w. Afterward, microparticles were incorporated in olive oils. The oxidative stability indices, namely the peroxide value, the *p*-anisidine value, the total oxidation value, the free fatty acids content, the K_{232} and K_{270} extinction coefficients, the total antioxidant activity, and the total phenolic content of plain olive oils, olive oils enriched with synthetic antioxidants (butylated hydroxyanisole, butylated hydroxytoluene and a blend of them), olive oils with olive mill pomace extract and oils fortified with olive mill pomace loaded ethylcellulose microparticles, were appraised.

Olive oil samples fortified with microparticles exhibited lower peroxide values than plain and enriched respective patterns. Olive mill pomace-loaded ethylcellulose microparticles showed to be efficient in the retardation of peroxidation processes. From the analysis of the evolution of *p*-anisidine values, it was possible to conclude that the embedment of olive mill pomace extract loaded microparticles led to the obtainment of lower *p*-anisidine values; therefore, the rate of hydroperoxide generation was lower compared to plain and enriched (synthetic antioxidants and olive mill pomace extract) olive oils. Therefore, it can be concluded that loaded microparticles efficiently lower the content of secondary oxidation products in extra-virgin olive oils, virgin olive oils, and blends of refined and virgin olive oils. The overall quality of extra-virgin olive oil, virgin olive oil, and a blend of virgin oil and refined olive oil was improved when fortified with encapsulated antioxidants obtained from olive mill pomace proven by the total oxidation values obtained.

The incorporation of olive mill pomace extract and olive mill pomace extract encapsulated into microparticles in olive oils effectively slowed down hydrolysis processes, ensuring the acidity values keeping below the rejection points even during accelerated storage conditions.

Moreover, in this study, it is proven that natural-occurring antioxidants presented in an extract or formulated in microparticles are more effective in retarding the free fatty acids formation than synthetic antioxidants. The total antioxidant activity and the total phenolic content of oils incorporating microparticles remained barely constant during storage time. Therefore, it can be concluded that olive mill pomace loaded ethylcellulose microparticles were efficiently designed to sustained release phenolic compounds to control the oxidative status of olive oil samples.

Briefly, the current study brings new insights into the design of natural antioxidants-rich loaded microparticles that be further incorporated in oils to retard oxidation processes occurring in the olive oil matrices. The design of antioxidants-rich loaded microparticles may efficiently improve oils oxidative stability.

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Part VI: Final Remarks

In this section, a general discussion and the main conclusions are presented (Chapter 9). Additionally, in this section, suggestions for future work, are presented in Chapter 10.

*“There only two ways to live your life. One is as though nothing is a miracle.
The other is as though everything is a miracle.”*

Albert Einstein

Chapter 9. General Discussion and Conclusions

In this thesis, the preservation of the antioxidant and phenolic activities of compounds found in an olive oil by-product – the olive mill pomace - was attained by microparticles loaded with olive mill pomace extract, using the water-in-oil-in-water double emulsion solvent evaporation technique. It was investigated the potential combination of the extraction of the phenolic compounds present in olive mill pomace - that are generally considered phytotoxic but presenting simultaneously outstanding health benefits - with their encapsulation to be thereafter embedded in liquid lipophilic oxidative sensitive food matrices - olive oils.

The Mediterranean Diet has been acknowledged as a cornerstone for the prevention of many pathologies. The health benefits associated with the Mediterranean dietary pattern are partially related to the consumption of olive oil as the primary source of fat. However, during olive oil production, phenolic compounds - the majority of compounds present in olive drupes – are retained in olive oil by-products. In this context, olive mill pomace is an exceptional source of bioactive compounds, namely phenolic compounds; among them stands out the phenethyl alcohols – hydroxytyrosol and tyrosol. The phenolic compounds present in olive mill pomace that confer phytotoxicity to this waste but presenting simultaneously outstanding human health benefits can be valorized through the combination of extraction and encapsulation techniques.

The water-in-oil-in-water double emulsion solvent evaporation technique is an encapsulation technique commonly employed to obtain powders for the pharmaceutical industry.

Nevertheless, in this work, this encapsulation technique was applied in the context of the food industry, namely to formulate microparticles containing hydroxytyrosol, tyrosol and olive mill pomace extract in the viewpoint (i) to protect valuable bioactive compounds present in olive mill pomace as hydroxytyrosol and tyrosol from degradation, increasing their stability and bioavailability; (ii) to obtain delivery systems protecting bioactive compounds during the salivary and gastric digestion, prompting a specific-site delivery namely at the intestinal lumen - for potential uptake by intestinal epithelial cells; (iii) and to assess the effect of the addition of encapsulated and non-encapsulated olive mill pomace extracts on olive oils oxidative stability.

Regarding the extraction of phenolic compounds exhibiting radical scavenging activity from olive mill pomace, the novelty of this part of the work was to optimize the most commonly used extraction technique of phenolic compounds from olive mill pomace – the solid-liquid extraction. In this context, the influence of variables namely the solvent-to-sample ratio and the duration of each step during the solid-liquid extraction procedure - that is associated with non-requirement of specific and cutting-edge technological equipments and the use of mild conditions – was assessed through the application of a multivariate analysis, namely a response surface methodology. The optimization was processed, considering Derringer's desired function methodology. The optimized extraction conditions were: 3.2 hours for the primary extraction, solvent-to-sample ratio during the primary extraction of 10.0 mL/g, 1.3 hours for the secondary extraction, and a solvent-to-sample ratio during the secondary extraction of 3.0 mL/g associated with a predicted maximum total phenolic content of 50 mg_{GAE}/g_{OMP}. The obtained olive mill pomace extract exhibited a total phenolic content of 50.5 ± 1.5 mg_{GAE}/g_{OMP}, a radical scavenging activity of $79.1 \pm 7.9\%$ and a content of hydroxytyrosol and tyrosol of $2\,870.2 \pm 1.2$ mg/kg_{OMP} and $1\,622.1 \pm 2.2$ mg/kg_{OMP}, respectively. Moreover, the effect of the application of an acidic hydrolysis step prior to the solid-liquid extraction was evaluated. The application of a hydrolysis pretreatment step improved the total antioxidant activity (increase from $53.5 \pm 3.9\%$ to $79.1 \pm 7.9\%$) and total phenolic content (increase from 21.8 ± 0.7 mg_{GAE}/g_{OMP} to 50.5 ± 1.5 mg_{GAE}/g_{OMP}).

The innovation regarding the encapsulation of bioactive compounds was based on applying alternative encapsulation techniques not commonly exploited by the food industry. Nevertheless, it is an outstanding alternative encapsulation approach of water-soluble compounds like phenolic compounds present in olive mill pomace. Additionally, in this work, ethylcellulose – a food-grade polymer – was selected as a cheap coating material to protect these valuable compounds.

Therefore, regarding the hydrophilic chemical nature of the majority of bioactive compounds present in olive mill pomace and the lipophilic nature of ethylcellulose, the encapsulation by water-in-oil-in-water using ethylcellulose as the coating material allowed to overcome the main problems regarding the employment of extracts rich in phenolic compounds from olive mill pomace in dietary supplements or as an additive, bioactive ingredient or even as a stabilizer in foods. The effect of the loading content was assessed on the main physicochemical properties of microparticles, release behaviors, and bioaccessibilities.

The overall encapsulation efficiencies were higher 80% w/w (minimum encapsulation efficiency of $83.7 \pm 2.0\%$ in the case of antioxidants when the extract was encapsulated considering a theoretical loading content in phenolic compounds of 10% w/w and maximum encapsulation efficiency observed of $99.3 \pm 0.5\%$ observed in the case of tyrosol encapsulated solely considering a theoretical loading content of 5% w/w. The production yields ranged between $71.2 \pm 3.7\%$ in the case of hydroxytyrosol-loaded ethylcellulose microparticles and $85.0 \pm 1.7\%$ in the case of the microparticles containing only tyrosol. The mean particle sizes ranged from $304.0 \pm 16.0 \mu\text{m}$ in the case of microparticles with hydroxytyrosol formulated considering a theoretical loading content of 10% w/w, and $2.3 \pm 0.1 \mu\text{m}$ in the case of the powder of tyrosol-loaded ethylcellulose microparticles formulated considering a theoretical loading content of 5% w/w. In all cases (hydroxytyrosol, tyrosol, and extract loaded ethylcellulose microparticles), an increase in the polydispersity of microparticles with the increase of loading content was observed. Generically, the Fourier Transform Infrared Spectroscopy studies revealed that no superficial chemical modifications occurred comparing loaded, polymer-only, and ethylcellulose samples, which is a reliable indicator of the efficient incorporation of the selected bioactive compounds and extract into microparticles.

The obtained microparticles were spherical with practically no pores, fissures, and cracks. The differential scanning calorimetry analyses indicated that bioactive compounds and the coating material formed solid solutions due to anti-plasticizing effects.

Complementarily to the characterization studies, the *in vitro* release rate of bioactive compounds from ethylcellulose microparticles was evaluated.

The amount of bioactive compounds released in the simulated salivary and gastric digestion fluid was remarkably low, which indicates the efficient protection of compounds during the simulated salivary and gastric digestions - as desired. In all cases, it was observed the highest release in the simulated intestinal tract, which proves that the designed, loaded ethylcellulose microparticles are efficient site-target delivery systems, especially at a simulated intestinal lumen. Generically, the loading content did not exert significant effects on the physicochemical properties of microparticles and the release characteristics of bioactive compounds from loaded microparticles.

The olive mill pomace extract loaded ethylcellulose microparticles were embedded in three types of olive oil namely in extra-virgin olive oil, in virgin olive oil, and in a blend of virgin and refined olive oil to evaluate the possibility of the replacement of synthetic antioxidants. The effectiveness in the oxidative stability of olive oils enriched with microparticles was assessed through the comparison of multiple oxidation indices of plain olive oils, olive oils enriched with synthetic antioxidants, namely butylated hydroxyanisole and butylated hydroxytoluene considered individually or in a blend, and olive oils enriched with olive mill pomace extracts. The evolution of the oxidation processes in olive oils under accelerated storage conditions - using the Schaal oven storage stability test – was monotonized.

Olive oils incorporating loaded microparticles revealed a delay in the commencement of the peroxidation processes as oils enriched with microparticles exhibited lower peroxide values than their related patterns (plain olive oils, olive oils fortified with synthetic antioxidants, and olive oils incorporating olive mill pomace extract only).

Furthermore, the release of bioactive compounds from microparticles into the olive oils efficiently lowered the content of secondary oxidation products and retarded hydrolysis processes in these food matrices.

In summary, the combination of optimized extraction and encapsulation techniques prompted the obtainment of handling and stable powders with improved and controlled release rates in the gastrointestinal tract, being specific in the site-delivering of bioactive compounds, increasing their bioaccessibility. These powders can find applications in the food field, namely in stabilizing and extending oils shelf-life. Thus, this thesis contributes with scientific advances towards conceptualizing the development delivery systems of bioactive compounds - extracted from agricultural wastes –to obtain added-value innovative food products.

Chapter 10. Future Work

Considering the complementation and extension of the results obtained in this work, it is recommended:

- Identify other phenolic compounds that have been described their presence in olive oil by-products, namely in olive mill pomace as caffeic acid, ligstroside, *p*-coumaric acid, ferulic acid, gallic acid, vanillic acid, protocatechuic acid, vanillin, luteolin, apigenin, verbascoside, comselogoside among others.
- Monitoring chemical changes in bioactive compounds found in the olive mill pomace extract, namely the identification and quantification of hydroxytyrosol-glucoside, oleuropein aglycon mono-aldehyde, oleuropein-aglycone di-aldehyde, ligstroside-aglycone di-aldehyde, luteolin-7-glucoside, apigenin-7-*o*-glucoside, among others, and comparing their relative abundance with their related chemical pattern associated to the development of analytical methodologies based on a more accurate analytical instrument as the liquid chromatography (LC) associated with a mass spectrometry (MS) detector.
- Perform the extraction of phenolic compounds from other olive oil by-products, namely olive oil wastewaters and olive leaves, followed by the encapsulation of the obtained extracts, characterization of loaded microparticles, and *in vitro* release studies to be further incorporated in oxidative sensitive food matrices.
- Production of alternative delivery systems as coacervates and Pickling emulsions and compare the main physicochemical characteristics of particles/emulsion with the ones obtained by water-in-oil-in-water double emulsion solvent evaporation technique.
- Production of microparticles for large scale applications (e.g., to be embedded on added-value food products) in a semi-automatic apparatus consisting of a closed vessel with an inlet of a gentle N₂ stream to promote the evaporation of the solvent through an exhaustion valve.

The contact of the primary emulsion (an emulsion of the internal aqueous phase and the organic phase) with the external aqueous phase could be performed in a T-junction microfluidic device containing a particle size control membrane coupled to the closed vessel. In the T-junction microfluid device, the primary emulsion would pass the particle size control membrane at a constant pressure downright to be dispersed in the external aqueous phase that passes in a tangential flow feed to the closed vessel.

- Assess bioactive compounds distribution inside of microparticles using a confocal laser scanning microscope or alternatively obtain slice microparticles using a cryo-microtome and visualize them through a scanning light microscope.
- Perform the analysis of microparticles degradation behavior by effects of mass loss, molecular weight changes – by gel permeation chromatography - and mean particles size changes with time.
- Compare the results regarding the overall oxidative status of olive oils under non-accelerated storage conditions and using an alternative accelerated aging test known as the Rancimat method.
- Perform oxidative stability studies under frying conditions.

Annex

In this section, is presented the supplementary material of Chapter 5 (Annex I), Chapter 6 (Annex II) and Chapter 7 (Annex III).

Annex I. Supplementary Material - Chapter 5

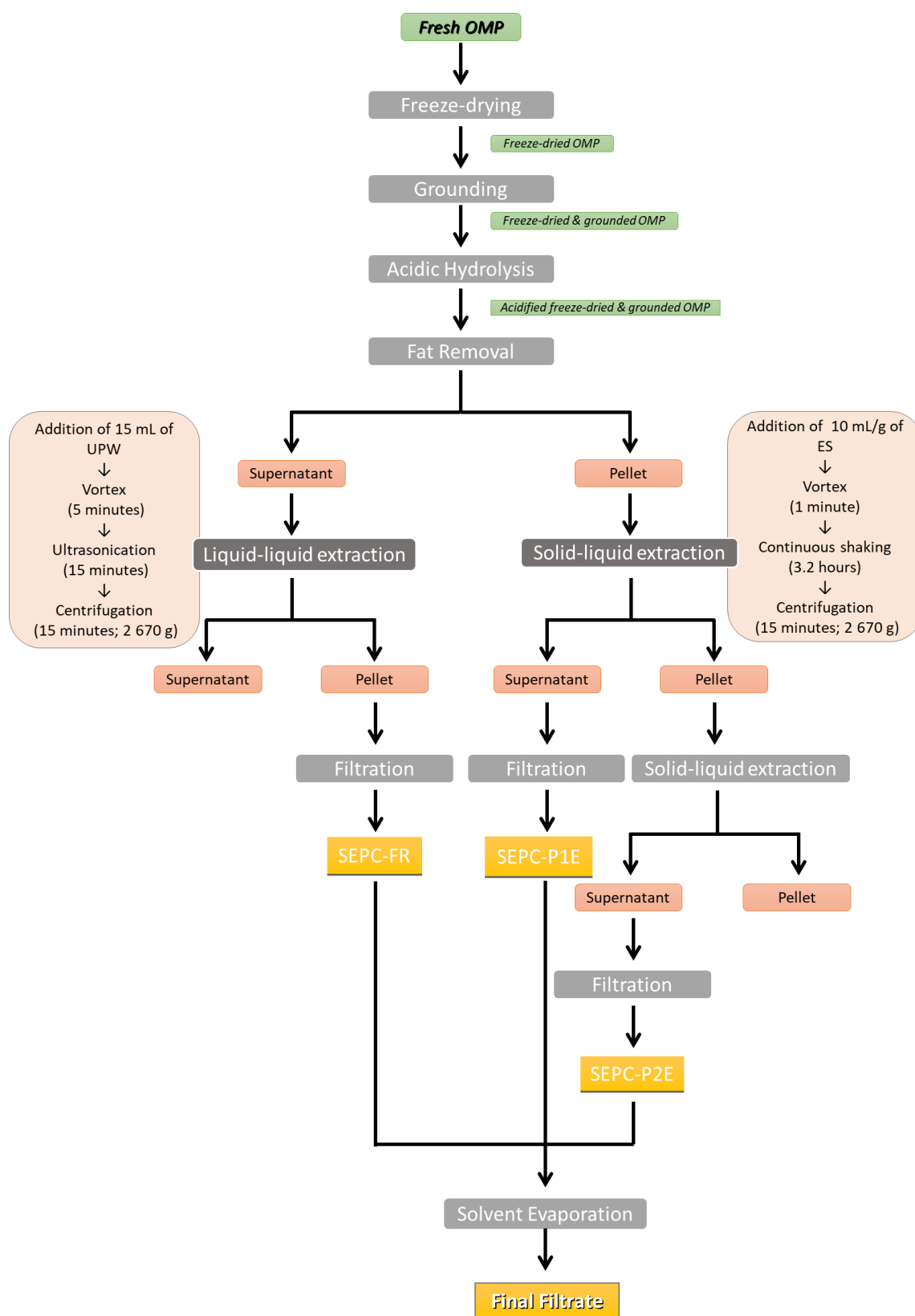


Figure IV.5.S1 - Optimized two-step solvent extraction for the recovery of phenolic antioxidants from olive mill pomace samples

Annex II. Supplementary Material - Chapter 6

Table IV.6.S1 - Results from the regression analysis for the quantification of antioxidants (AO), phenolic compounds (PC), hydroxytyrosol (HXT) and tyrosol (TYR)

<i>Parameters</i>	<i>Bioactive(s)</i>			
	AO	PC	HXT	TYR
Conc. range (mg/L)	50.0-200.0	5.0-20.0	5.0-20.0	5.0-20.0
Regression Equation^{a,b}	$%I = (4.4 \pm 0.4) \times 10^1 \log C + (-2.4 \pm 0.9) \times 10^1$	$Abs = (7.2 \pm 1.6) \times 10^{-3} C (mg/L) + (-1.7 \pm 0.7) \times 10^{-3}$	$A = (5.6 \pm 0.2) \times 10^5 C (mg/L) + (4.6 \pm 0.8) \times 10^4$	$A = (8.4 \pm 0.7) \times 10^5 C (mg/L) + (-6.8 \pm 0.2) \times 10^3$
<i>R</i>	0.995	0.999	0.999	0.997
S_a/a (%)	1.8	1.5	2.8	2.9
LOD (mg/L)	6.9	0.5	0.9	1.0
LOQ (mg/L)	23.1	1.5	3.1	3.2

^a Abs is the absorbance (UV-Vis methods), and C is the concentration of PC or AO (mg/L); alternatively, A is the area of the peak (HPLC method), and C is the concentration of HXT or TYR (mg/L)

^b Mean calibration curve based on three independent calibration curves

A – Area; Abs – Absorbance; AO – Antioxidants; C- Concentration; Conc. – Concentration; HXT – Hydroxytyrosol; LOD – Limit of Detection; LOQ – Limit of Quantification; PC – Phenolic Compounds; *R* – Correlation Coefficient; S_a/a – Relative standard error of slope; TYR – Tyrosol;

Annex III. Supplementary Material - Chapter 7

Table IV.7.S1 - Recovery percentages of phenolic compounds, antioxidants, hydroxytyrosol and tyrosol

BAC(s)	% R (w/w)	
	F-1 formulation ^a (TLC of 5% w/w)	F-2 formulation ^a (TLC of 10% w/w)
Phenolic Compounds ^b	91.6 ± 1.3	92.1 ± 1.5
Antioxidants ^c	88.0 ± 0.6	87.8 ± 1.0
Hydroxytyrosol ^d	85.8 ± 5.4	86.0 ± 5.6
Tyrosol ^d	95.1 ± 1.4	95.2 ± 1.7

^a Each experiment was performed in triplicate. Results are presented as mean ± standard deviation;

^b Phenolic Compounds were quantified considering the Folin-Ciocalteu spectrophotometer method;

^c The total antioxidant activity was estimated through the determination of the free radical-scavenging ability (RSA) using the 2,2-diphenyl-2-picrylhydrazyl radical (DPPH*);

^d Specific phenolics were quantified by HPLC analyses

BAC – Bioactive Compound; GAE – Gallic Acid Equivalents; OMP – Olive Mill Pomace; R – Recovery (% w/w); TLC – Theoretical loading content (expressed in mg_{GAE}/g_{OMP});

Table IV.7.S2 - Results from the regression analysis for the quantification of phenolic compounds, antioxidants, and specific phenolic antioxidants (hydroxytyrosol and tyrosol) in extracts, microparticles, and in the alternative gastrointestinal release media

BAC(s)	DS	Regression Equation ^{a,b}	R	S _a /a (%)	Conc. range (mg/L)	LOD (mg/L)	LOQ (mg/L)
PC	UPW	$Abs = (7.2 \pm 1.6) \times 10^{-3} C \text{ (mg/L)} + (-1.7 \pm 0.7) \times 10^{-3}$	0.999	1.5	5.0-20.0	0.5	1.5
	SSDF	$Abs = (1.0 \pm 0.3) \times 10^{-2} C \text{ (mg/L)} + (-3.4 \pm 0.4) \times 10^{-3}$	0.998	2.1	5.0-20.0	0.7	2.4
	SGDF	$Abs = (1.4 \pm 0.1) \times 10^{-2} C \text{ (mg/L)} + (-1.2 \pm 0.6) \times 10^{-2}$	0.995	2.3	5.0-20.0	0.8	2.6
	SIDF	$Abs = (1.3 \pm 0.2) \times 10^{-2} C \text{ (mg/L)} + (1.2 \pm 0.4) \times 10^{-3}$	0.998	2.5	5.0-20.0	0.9	3.0
AO	UPW	$Abs = (4.6 \pm 0.4) \times 10^{-3} C \text{ (mg/L)} + (1.5 \pm 0.9) \times 10^{-1}$	0.995	1.8	50.0-200.0	6.9	23.1
	SSDF	$Abs = (4.5 \pm 0.6) \times 10^{-3} C \text{ (mg/L)} + (2.5 \pm 1.0) \times 10^{-2}$	0.995	1.6	50.0-200.0	6.1	20.2
	SGDF	$Abs = (4.2 \pm 0.6) \times 10^{-3} C \text{ (mg/L)} + (3.0 \pm 0.3) \times 10^{-2}$	0.996	2.4	50.0-200.0	9.2	30.7
	SIDF	$Abs = (3.6 \pm 0.7) \times 10^{-3} C \text{ (mg/L)} + (4.0 \pm 1.2) \times 10^{-1}$	0.997	1.1	50.0-200.0	4.3	14.4
HXT	UPW	$A = (5.6 \pm 0.2) \times 10^5 C \text{ (mg/L)} + (4.6 \pm 0.8) \times 10^4$	0.999	2.8	5.0-20.0	0.9	3.1
	SSDF	$A = (1.3 \pm 0.7) \times 10^7 C \text{ (mg/L)} + (-1.5 \pm 0.9) \times 10^7$	0.997	2.9	5.0-20.0	0.9	3.2
	SGDF	$A = (8.3 \pm 0.3) \times 10^5 C \text{ (mg/L)} + (-5.6 \pm 1.0) \times 10^5$	0.998	4.2	5.0-20.0	1.6	5.2
	SIDF	$A = (1.6 \pm 0.3) \times 10^6 C \text{ (mg/L)} + (5.8 \pm 1.9) \times 10^5$	0.999	1.6	5.0-20.0	0.5	1.7

Table IV.7.S2 - Results from the regression analysis for the quantification of phenolic compounds, antioxidants, and specific phenolic antioxidants (hydroxytyrosol and tyrosol) in extracts, microparticles, and in the alternative gastrointestinal release media (cont.)

BAC(s)	DS	Regression Equation ^{a,b}	<i>R</i>	S _a /a (%)	Conc. range (mg/L)	LOD (mg/L)	LOQ (mg/L)
TYR	UPW	$A = (8.4 \pm 0.7) \times 10^5 C \text{ (mg/L)} + (-6.8 \pm 0.2) \times 10^3$	0.997	2.9	1.0-20.0	1.0	3.2
	SSDF	$A = (8.2 \pm 0.3) \times 10^6 C \text{ (mg/L)} + (6.1 \pm 1.7) \times 10^6$	0.997	4.4	2.5-20.0	1.6	5.4
	SGDF	$A = (1.3 \pm 0.1) \times 10^6 C \text{ (mg/L)} + (-1.0 \pm 0.2) \times 10^6$	0.997	4.0	2.5-20.0	1.4	4.5
	SIDF	$A = (2.4 \pm 0.2) \times 10^6 C \text{ (mg/L)} + (-1.3 \pm 1.1) \times 10^6$	0.997	4.6	2.5-20.0	1.7	5.6

^a Abs is the absorbance (UV-Vis methods), and C is the concentration of PC or AO (mg/L); alternatively, A is the area of the peak (HPLC method), and C is the concentration of HXT or TYR (mg/L)

^b Mean calibration curve based on three independent calibration curves

A – Area; Abs – Absorbance; AO – Antioxidants; BAC- Bioactive Compound; C- Concentration; DS – Dissolution Media; HXT – Hydroxytyrosol; LOD – Limit of Detection; LOQ – Limit of Quantification; PC – Phenolic Compounds; *R* – Determination Coefficient; S_a/a – Relative standard error of slope; SGDF – Simulated Gastric Digestion Fluid; SIDF – Simulated Intestinal Digestion Fluid; SSDF – Simulated Salivary Digestion Fluid; TYR – Tyrosol; UPW – Ultrapure Water

