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Skin and seed grape extract as an antioxidant for mechanically deboned chicken meat, during frozen storage

Hernán Horacio Tournour

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Dissertation Thesis

Skin and seed grape extract as an antioxidant for mechanically deboned chicken meat, during frozen storage

Dissertation presented to Faculty of Nutrition and Food Science to obtain the degree of

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Supervisor: Dr. Luís Miguel Cunha, Associated Professor, FCUP

Co-supervisors: Dr. Marcela Alves Segundo, Assistant Professor, FFUP

Dr Jorge Bernardo Lacerda de Queiroz, Assistant Professor, FCUP

Hernán Horacio Tournour

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Author's Declaration

Under the terms of the "Decreto-lei nº 216/92, de 13 de Outubro" is hereby declared the following original articles were prepared in the scope of this thesis.

Under the terms of the referred "Decreto-lei", the author declares that he afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in the thesis.

This dissertation is constituted by the following papers submitted for publications:

I. Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Luísa Barreiros, Jorge Queiroz, Luís M. Cunha. Valorization of grape pomace: extraction of bioactive phenolics with antioxidant properties. (Submitted for publication).

II. Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Jorge Queiroz, M. Beatriz P. P. Oliveira, Luís M. Cunha. Single and successive oxidative stress factors applied to mechanically deboned chicken meat (MDM): protective effect of grape pomace extract. (Submitted for publication).

III. Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Telmo J. R. Fernandes, M. Beatriz P. P. Oliveira, Luís M. Cunha. Influence of Portuguese grape pomace extracts on the oxidative stability, nutritional and color characteristics of mechanically deboned chicken meat. (Submitted for publication).

IV. Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Anabela S. G. Costa, Luís M. Cunha. Effect of *"Touriga nacional"* grape extract on quality characteristics of mechanically deboned chicken meat kept under frozen storage. (Submitted for publication).

V. Hernán H. Tournour, Luís M. Cunha, Luís M. Magalhães, Rui Costa Lima, Marcela A. Segundo. Evaluation of the joint effect of the incorporation of mechanically deboned meat and grape extract on the formulation of chicken nuggets. (Submitted for publication).

Moreover, the author declares that he has actively participated in the preparation and writing of each paper, being actively engaged in the stages of experimental design, sample preparation and evaluation, data collection, analysis and interpretation of results.

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Dedication

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Abstract

Grape pomace, composed of seeds, skins (also known as peels), stalks and pulps, is a valuable by-product from the winemaking industries, recognized due to its relevant polyphenolic compounds content.

Mechanically deboned chicken meat (MDM) is a raw material highly affected by degradation reactions associated to lipid oxidation. Synthetic antioxidants commonly used at industrial level in order to minimize peroxidation are currently suspected of causing toxic affect in consumer health.

Therefore, characterization of antioxidant properties of grape pomace extract (GPE) from different Portuguese varieties (Vitis vinifera L., varieties "*Touriga nacional"* –TNac-*, "Touriga franca"* –TF- and "*Tinta roriz*" –TR-), through the evaluation of total phenolic content (TPC), scavenging capacity against DPPH$^{\bullet}$**,** oxygen reactive absorbance capacity (ORAC) and iron(II) chelating ability (ICA) assays, was performed. Additionally, GPE from TF was evaluated regarding its protection against lipid oxidation, conducted through an accelerated degradation experience using a meat model exposed to single and successive oxidative factors, and in comparison with the use butylated hydroxytoluene (BHT). Overall quality characteristics of MDM supplemented with different GPEs, including nutritional composition, pH, color variables and oxidative stability throughout storage time, were analyzed. Finally, consumer evaluation of chicken nuggets incorporating different amounts of MDM and GPE from TNac was also performed.

Results indicated that GPE obtained using an environmentally friendly extractive mixture (80 % v/v ethanol/water) presented high antioxidant properties. GPE from TNac presented the highest TPC (142.4 mg GAE g^{-1}residue), DPPH$^{\bullet}$ (1.12 mmol TE g^{-1}residue) and ORAC (1579 µmol TE g^{-1}residue) values, including also the highest individual phenols assessed by HPLC, with all values significantly ($p < 0.05$) differing from the other GPEs.
Oxidative stability of MDM samples exposed to degradation factors was probed to be successfully monitored by FCR, ORAC and ICA assays. The antioxidant effectiveness was dependent on the combination of stress-factors and type of

antioxidant (GPE from TF or BHT). Additionally, successive exposure to stress conditions affected the final antioxidant performance.

Experiences regarding duration of storage under freezing conditions indicated that MDM supplemented with different GPEs (60 mg/kg) kept stable up to 30 days, according to FCR, ORAC and ICA assay. GPE supplementation resulted in significant ($p < 0.05$) changes regarding color variables (CIE - *L*a*b**). On the other hand, after 365D of frozen storage, all MDM samples with added GPE did not differ significantly ($p > 0.05$) from control at 1D, regarding the oxidation of fatty acids aggregated in n-*3*, whilst samples with added BHT-BHA (butylated hydroxytoluene-butylated hydroxyanisole, 200 mg/kg) have undergone oxidation.

Sensory evaluation of chicken nuggets formulated with MDM and GPE, was conducted with 75 naïve assessors, using a 9-point hedonic scale, complemented with open comments. Conclusions from the evaluation of overall acceptance indicated that addition of GPE up to 120 mg/kg and MDM up to 15 % guarantee a satisfactory acceptance level (acceptance score > 7.0) for the perceived appearance of chicken nuggets. Analysis of open comments, through Correspondence analysis allowed the construction of a perceptual map yielding additional insights into the effect of varying the compositions of chicken nuggets. MDM and GPE can be used successfully in the elaboration of novel products aiming at the exploitation of by-products from winemaking.

Resumen

El bagazo de la uva, compuesto por pepitas, cáscaras (también conocidas como pieles), tallos y pulpas, es un valioso subproducto de las industrias vinícolas, reconocido por su importante contenido de compuestos polifenólicos.

La carne de pollo deshuesada mecánicamente ("*mechanically deboned chicken meat"*, MDM) es una materia prima muy afectada por las reacciones de degradación asociados a la oxidación de lípidos. Los antioxidantes sintéticos comúnmente utilizados a nivel industrial con el fin de minimizar la peroxidación están actualmente sospechados de causar tóxicos que afectan la salud del consumidor.

En este contexto, fue realizada una caracterización de las propiedades antioxidantes de los extractos del bagazo de la uva (*"grape pomace extract"*, GPE) de diferentes variedades portuguesas (Vitis vinifera L. variedades *"Touriga nacional"* –TNac-, *"Touriga franca"* –TF- y *"Tinta roriz"* –TR-), a través de los ensayos del contenido fenólico total (TPC), la capacidad de captación contra $DPPH^{\bullet}$, la capacidad de absorción de radicales del oxigeno (ORAC) y la capacidad quelante del hierro(II). Además, GPE de TF fue evaluado en cuanto a su protección frente a la oxidación lipídica llevada a cabo a través de una experiencia de degradación acelerada utilizando un modelo cárnico expuesto a simple o sucesivos factores oxidantes, y en comparación con el uso de butil hidroxi tolueno (BHT). Se analizaron también, las características globales de calidad de MDM adicionada con diferentes GPEs, incluyendo composición nutricional, pH, variables de color y estabilidad oxidativa durante todo el tiempo de almacenamiento. Finalmente, fue realizada una evaluación por consumidores, de *nuggets* de pollo elaborados con diferentes proporciones de MDM y de GPE de TNac.

Los resultados indicaron que GPE obtenidos utilizando una mezcla extractiva amigable con el medio ambiente (80 % v/v etanol/agua) presentaron altas propiedades antioxidantes. GPE proveniente de TNac presentó los valores significativamente más elevados de TPC (142,4 mg GAE g^{-1}residuo), de DPPH• (1,12 mmol TE g^{-1}residuo) y de ORAC (1,579 µmol TE g^{-1}residuo), incluyendo

también los más altos fenoles individuales evaluadas por HPLC, siendo todos los valores significativamente ($p < 0,05$) diferentes de los otros GPEs.

Los ensayos de FCR, ORAC e ICA probaron ser efectivos para monitorear la estabilidad oxidativa de muestras MDM frente a factores de degradación. La eficacia antioxidante fue dependiente del factor de estrés aplicado y del ensayo de antioxidante (GPE de TF o BHT). Además, una exposición sucesiva a condiciones de estrés afecta la capacidad antioxidante final.

Experiencias sobre el tiempo de almacenamiento en condiciones de congelación indicaron que MDM adicionada con diferentes GPEs (60 mg/kg) se mantuvieron estables hasta 30 días, según ensayos de FCR, ORAC e ICA. La adición de GPE resulto en cambios significativo ($p < 0,05$) con respecto a variables de color (CIE-*L* a* b**). Por otro lado, luego de 365D de almacenamiento congelado, todas las muestras de MDM adicionadas con GPE no difirieron significativamente ($p > 0,05$) del control al día 1 (1D), con respecto a la oxidación de los ácidos grasos agregados en n-*3*, mientras que las muestras adicionadas con BHT-BHA (hidroxitolueno butilado-hidroxianisol butilado, 200 mg/kg) sufrieron oxidación.

Se realizó una evaluación sensorial de los *nuggets* de pollo formulados con MDM y GPE a través de 75 probadores *naive* utilizando una escala hedónica de 9 puntos complementadas con comentarios libres. Las conclusiones de la evaluación de la aceptación general indicaron que la adición de GPE hasta 120 mg/kg y de MDM hasta un 15 % garantizo un nivel satisfactorio de aceptación (valores de aceptación > 7,0) para la apariencia percibida los en los *nuggets* de pollo. El análisis de los comentarios libres a través del análisis de correspondencia, permitió la construcción de un mapa perceptual resaltado los efectos de la variación de la composición de los nuggets de pollo. MDM y GPE pueden ser utilizados con éxito en la elaboración de nuevos productos tendientes al aprovechamiento de los subproductos de la vinificación.

Resumo

O bagaço de uva, composto de grainhas, películas (também conhecidas como peles), talos e polpas, é um valioso subproduto da indústria de vinificação, reconhecido devido ao seu relevante teor de compostos polifenólicos.

A carne de frango mecanicamente desossada (*"mechanically deboned chicken meat"*, MDM) é uma matéria-prima altamente afetada por reações de degradação associadas à oxidação lipídica. Os antioxidantes sintéticos habitualmente utilizados a nível industrial, a fim de minimizar a peroxidação, são atualmente suspeitos de causar efeitos tóxicos que afetam a saúde do consumidor.

Neste contexto, foi realizada uma caracterização das propriedades antioxidantes dos extratos de bagaço (*"grape pomace extract"*, GPE) de diferentes variedades portuguesas de variedades portuguesas (Vitis vinifera L. variedade "Touriga nacional" –TNac-, "Touriga franca" –TF- e "Tinta roriz" –TR-), através dos ensaios do conteúdo de compostos fenólicos totais (TPC), capacidade de captação do radical $DPPH^{\bullet}$, capacidade de absorção de radical peroxilo (ORAC) e capacidade quelante do ferro (II). Além disso, estimada a proteção oferecida pelo GPE de TF em face à oxidação lipídica em condições de degradação acelerada utilizando um modelo de carne sob um único fator de stress ou sob uma exposição sucessiva de fatores de stress, e em comparação com o uso de hidroxitolueno butilado (BHT). Foram analisadas as características gerais de qualidade da MDM suplementadas com diferentes GPEs, incluindo composição nutricional, pH, variáveis de cor e estabilidade oxidativa ao longo do tempo de armazenamento. Finalmente, foi conduzida uma avaliação sensorial por consumidores *naive* quanto à aparência percebida de nuggets de frango elaborados com diferentes quantidades de MDM e GPE de TNac.

Os resultados indicaram que os GPE obtidos, utilizando uma mistura de extracção amiga do ambiente (80 % v/v etanol/água), apresentaram uma elevada propriedade antioxidante. O GPE de TNac apresentou valores significativamente mais altos de TPC (142,4 mg GAE g^{-1} resíduo), de captação do DPPH• (1,12 mmol TE g^{-1} resíduo) e de ORAC (1579 µmol TE g^{-1} resíduo),

incluindo também os teores mais altos de compostos fenólicos totais avaliados por HPLC, com todos os valores significativamente ($p < 0,05$) diferentes dos outros GPEs. Os ensaios de FCR, ORAC e ICA foram efetivos para monitorizar a estabilidade oxidativa de amostras de MDM perante fatores de degradação. A eficácia antioxidante foi dependente da combinação dos factor de stress e do antioxidante (GPE de TF ou BHT). Além disso, verificou-se que a exposição sucessiva às condições de stress afetou o desempenho final do antioxidante.

As experiências de o armazenamento congelado indicaram que amostras de MDM suplementadas com diferentes GPEs (60 mg/kg) manteve-se estável até 30 dias sob de acordo com os ensaios de FCR, ORAC e ICA. A suplementação com GPE resultou numa mudança significativa ($p < 0,05$) em relação às variáveis de cor (CIE-*L* a* b**). Por outro lado, após 365D de armazenamento congelado, todas as amostras de MDM suplementadas com GPE não foram significativamente ($p > 0,05$) diferentes do controlo para 1D, relativamente à oxidação de ácidos gordos agregados em n-*3*, enquanto que, as amostras com BHT - BHA (hidroxitolueno butilado - hidroxianisole butilado, 200 mg/kg) sofreram oxidação.

Foi realizada uma avaliação sensorial de nuggets de frango formulados com MDM e GPE, conduzida por 75 avaliadores *naive* utilizando uma escada hedônica de 9 pontos complementada com comentários abertos. As conclusões da avaliação de aceitação geral indicam que a adição de GPE até 120 mg/kg e de MDM até 15 %, garante um nível satisfatório de aceitação (valores de aceitação > 7,0) relativamente à aparência percebida em nuggets de frango. Analise dos comentários abertos através da analise de correspondência permitiu a construção de um mapeamento perceptual visando o efeito da variação da composição dos nuggets de frango. MDM e GPE podem ser utilizados com sucesso na elaboração de novos produtos, fomentando a exploração de subprodutos da vinificação.

Scope and Aims

Portugal is the eighteenth grape global producer with an important wine production spread throughout the whole territory of the country. This production is channeled for the creation of high quality wine, generally yielding relevant amounts of seeds, skins, stalks and pulps, conjointly known as grape pomace. There is a vast amount of literature regarding polyphenolic compounds in grape and their healthy impact in human diet. For several fields, including chemical, pharmacological and food industries this fact represents an inexpensive source of bioactive compounds to be used as target in extractive procedures.

On the other hand, the existence of a growing interest by consumers regarding wellbeing and health encourages industries to develop new applications concerning properties of polyphenolic compounds in foods. Moreover, studies related to the antioxidant protection in food matrices, including consumer issues associated to sensory impact of supplemented food are also needed, in order to define a comprehensive understanding and assessment.

The work depicted in the present dissertation thesis has the following overall aim:

To characterize skin and seed grape (pomace) extracts from Portuguese varieties towards the prevention of the lipid oxidation of mechanically deboned chicken meat (MDM), including the assessment of the final physico-chemical characteristics and consumer acceptance of nuggets containing MDM and supplemented with grape extracts.

According to the general work chronogram the results belonging to the present thesis are displayed in four sections in accordance to the specific objectives.

Section A: To characterize Portuguese grape pomace extracts (GPE) regarding their antioxidant properties.

A1. "Valorization of grape pomace: extraction of bioactive phenolics with antioxidant properties".

- To perform a complete study regarding polyphenolic content and antioxidant capacity of Portuguese GPE.

- To compare the influence of the choice of solvent on the final antioxidant properties of GPE.
- To select the most suitable Portuguese grape variety, under study, towards future recovery of bioactives products and application in other industries.

Section B: To evaluate the effectiveness of GPE against lipid oxidation of MDM.

B1. "Single and successive oxidative stress factors applied to mechanically deboned chicken meat (MDM): protective effect of grape pomace extract".

- To evaluate methodologies to determine the oxidative stability in a meat model.
- To evaluate the performance of GPE against the application of a single stress factor, compared with BHT.
- To study the performance of GPE against successive exposure to degradation factors, compared with BHT.

Section C: To evaluate the effect of GPE on overall characteristics of MDM through a shelf life real-time analysis, under frozen storage conditions.

C1. "Influence of Portuguese grape pomace extracts on the oxidative stability, nutritional and color characteristics of mechanically deboned chicken meat".

- To investigate the effect of GPE supplementation on MDM, regarding oxidative stability and nutritional characteristics.
- To understand the contribution of GPE on the changes of color on MDM supplemented samples.
- To study the protection conferred by GPE against fatty acids oxidation throughout frozen storage.

C2. “Effect of *“Touriga nacional”* grape extract on quality characteristics of mechanically deboned chicken meat kept under frozen storage”.

- To understand the influence of the initial MDM composition on the antioxidant performance of GPE.
- To evaluate the effect of GPE concentration on the proximate composition, pH, and oxidative stability of frozen MDM.
- To study the effect of GPE supplementation on color changes, aiming at possible implications on a finished product.

Section D: To evaluate the effect of the implementation of GPE on a real finished product.

D1. “Evaluation of the joint effect of the incorporation of mechanically deboned meat and grape extract on the formulation of chicken nuggets”.

- To optimize nugget formulation concerning GPE concentration and MDM content.
- To study the influence of MDM addition on the nutritional composition of chicken nuggets.
- To understand the joint contribution of GPE and MDM on consumers acceptance and perceptual description of chicken nuggets.

List of Publications in International Peer-Reviewed Journals

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Luísa Barreiros, Jorge Queiroz, Luís M. Cunha. Valorization of grape pomace: extraction of bioactive phenolics with antioxidant properties. (Submitted for publication).

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Jorge Queiroz, M. Beatriz P. P. Oliveira, Luís M. Cunha. Single and successive oxidative stress factors applied to mechanically deboned chicken meat (MDM): protective effect of grape pomace extract. (Submitted for publication).

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Telmo J. R. Fernandes, M. Beatriz P. P. Oliveira, Luís M. Cunha. Influence of Portuguese grape extracts on the oxidative stability, nutritional, and color characteristics of mechanically deboned chicken meat. (Submitted for publication).

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Anabela S. G. Costa, Luís M. Cunha. Effect of “Touriga nacional” grape extract on quality characteristics of mechanically deboned chicken meat kept under frozen storage. (Submitted for publication).

Hernán H. Tournour, Luís M. Cunha, Luís M. Magalhães, Rui Costa Lima, Marcela A. Segundo. Evaluation of the joint effect of the incorporation of mechanically deboned meat and grape extract on the formulation of chicken nuggets. (Submitted for publication).

Poster in Conferences

Hernán Tournour, Marcela Segundo, Luís M. Magalhães, Jorge Queiroz, Luís M. Cunha, Estudio comparativo de la capacidad antioxidante de extractos del bagazo de uvas tintas portuguesas en un medio de grado alimenticio, CIBIA9 Congreso Iberoamericano de Ingeniería de Alimentos, Universidad Politécnica de Valencia, España (13-16 de Janeiro de 2014).

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Jorge Queiroz, Luís M. Cunha, Solvent effect on the antioxidant properties in terms of total phenolic content (TPC) and oxygen radical absorbance capacity (ORAC) in grape pomace extracts from a Portuguese red grape cultivar ("Touriga nacional"), 3rd International ISEKI_Food Conference to be held in Athens, Greece (May 21-23, 2014).

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Anabela S. G. Costa, Jorge Queiroz, Luís M. Cunha, INFLUENCE of grape pomace extract on the quality characteristics of the mechanically deboned chicken meat (MDM): Towards functional foods, 12º ENCONTRO DE QUÍMICA DOS ALIMENTOS, Instituto Superior de Agronomia, Lisboa, Portugal (10-12 de Setembro de 2014).

Abbreviations

The abbreviations list includes acronyms from the dissertation thesis, except from the results section.

AAPH	2,2′-azobis(2-amidino-propane) dihydrochloride
ABTS	2,2′-Azinobis-(3-ethylbenzothiazole-6-sulphonate)
ABTS•+	2,2′-Azinobis-(3-ethylbenzothiazole-6-sulphonate) radical
AOAC	Association of American Analytical Chemists
AUC	Area under curve
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
DAD	Diode array detection
DPPH•	2,2′-Diphenyl-1-picrylhydrazyl radical
EC	European Commission
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
FCR	Total Phenols Assay by Folin-Ciocalteu Reagent
GPE	Grape pomace extract
HIC	Haem iron content
HPLC	High Performance Liquid Chromatography
ICA	Iron(II) chelating ability
INE	*Instituto Nacional de Estatistica*
ISO	International Organization for Standardization
IVDP	*Instituto dos Vinhos do Douro e Porto*
MDM	Mechanically deboned meat
MhL	Millions of hectoliter
MRM	Mechanically recovered meat
MS	Mass spectrometry
MSM	Mechanically separated meat
MT	Millions of tons
NCC	National Chicken Council
OIV	*Organisation Internationale de la Vigne et du Vin*

ORAC	Oxygen Radical Absorbance Capacity
psi	Pound per square inch
TBARS	Thiobarbituric Acid Reactive Substances
TBHQ	*tert*-butylhydroquinone
TE	Trolox equivalent
TEAC	Trolox equivalent Antioxidant Capacity
TPC	Total Phenolic Content
UNESCO	United Nations, Educational, Scientific and Cultural Organization
USA	United States of America
UV-vis	Ultraviolet and visible

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1.

Introduction

1.1 Wine production: global and Douro wine region

Wine is one of the most well-known alcoholic beverages distributed around the world, with 271 million of hectoliters (MhL) produced in 2014 according to the last bulletin emitted by *Organisation Internationale de la Vigne et du Vin* (OIV) (OIV, 2014).

Wine has existed since a long time as part of ancient civilizations culture. Roman and Greek cultures have begun the winemaking process some 8,000 years ago (McGovern, 2013). Certain chemical residues associate to wine, like tartaric acid, were discovered on 8,000-year-old pottery fragments in Greece (Dougherty, 2012).

Throughout time, grape exploitation has represented major advancements and important profits in ancient and current cultures.

These days three main species of grapes are distributed in the world: European grapes (*Vitis vinifera*), North American grapes (*Vitis labrusca* and *Vitis rotundifolia*) and French hybrids (En-Qin, Gui-Fang, Ya-Jun, & Hua-Bin, 2010). However, in some Central and Eastern European countries, *Vitis rupestris, Vitis berlandieri* and *Vitis amurensis* species can be found, but because of their low quality- grape they are not suitable for the winemaking process (FAO, 2013).

The OIV is an intergovernmental organization of a scientific and technical nature of recognized competence for its works concerning vines, wine, wine-based beverages, table grapes, raisins and other vine-based products. According to its statistics databases, the world wine production reached 271 MhL in 2014, including the following top five wine global producers: France (46.2 MhL), Italy (44.4 MhL), Spain (37 MhL), USA (22.5 MhL) and finally Argentine (15.2 MhL) (OIV, 2014).

The global viticulture stage, as well as other crops, is under constant variations, mainly related to climatic factors and in other cases, due to economical policies implemented by each country. Poor weather conditions, namely mild winter, excessive humidity in spring and summer, and decreased land destined for vineyards are largely responsible for the production drops. Thus, a trend for a

particular year cannot be the same for the next one. On one hand, the international scene until 2012 for countries such as China, Chile, Australia and South Africa was greatly positive once they have experimented an increasing (from 41 % for China to over 88 % for Chile) in the total amount of wine produced between 2000 and 2012 (OIV, 2012).

On the other hand, countries which were commonly recognized as references because of the high quality of their wines and the quantities produced annually, seems to have decreasing perspectives for the future. This is the case of France, Italy and Spain, which have experimented a declining tendency (28, 22, and 27 %, respectively), considering the same period of time (200-2012) in terms of wine production (OIV, 2012).

The OIV also shared the ranking for the countries which actively participate in the global wine production (Table 1).

Table 1

Wine production (1000 hL excluding juice and musts) [a]

Country	2010	2011	2012	2013	2014	Ranking 2014
France	44,381	50,757	41,548	42,004	46,151	1
Italy	48,525	42,772	45,616	52,429	44,424	2
Spain	35,353	33,397	31,123	45,650	37,000	3
United States	20,890	19,140	21,740	23,500	22,500	4
Argentina	16,250	15,473	11,780	14,984	15,200	5
Australia	11,420	11,180	12,260	12,310	12,560	6
China	13,000	13,200	13,810	11,780	11,178	7
South Africa	9,327	9,725	10,568	10,980	11,420	8
Chile	8,844	10,646	12,554	12,846	10,029	9
Germany	6,906	9,132	9,012	8,409	9,725	10
Portugal	7,148	5,622	6,327	6,238	5,886	11
Romania	3,287	4,058	3,311	5,113	4,093	12
New Zeeland	1,900	2,350	1,940	2,480	3,200	13
Greece	2,950	2,750	3,115	3,343	2,900	14
Brazil	2,459	3,460	2,967	2,710	2,810	15

[a] Adapted from OIV (2014)

Portugal is the country with the highest variety of wine vinery. It has also the highest global vine biodiversity, including 342 grape varieties (OIV, 2014) and 258 grape varieties of Portuguese origin. Besides, Portugal presents the highest world genetic heritage and varietal density per sq. km (2.7 grape varieties / 1000 km^2). Hence, thanks to these characteristics, Portugal reached 5.9 MhL of wine production in 2014 (Table 1). This fact positioned Portugal as the eleventh in the ranking of the world's largest wine producers.

Different demarcated regions are displayed for the winemaking in the Portuguese territory. The top five of Portuguese wine productive areas includes *Douro, Alentejo, Beiras, Lisboa* and *Minho*, participating with 25, 18, 14, 14 and 13 %, respectively, based on the total wine production. The general characteristics of their vines vary according to the localization, region and also climatic conditions.

Regarding to the Douro region, the vineyards were settled down in 1756 thanks to Marquês de Pombal and it was declared a World Heritage region by United Nations, Educational, Scientific and Cultural Organization (UNESCO) in 2001. It is located in Northeast of Portugal, within the Douro River basin, surrounded by craggy mountains that give it very particular soil and climacteric characteristics. This region spreads over a total area of approximately 250,000 hectares and is divided into three sub-regions that differ greatly from each other not only regarding weather aspects but also for socio-economical reasons. The three sub-regions include Lower Corgo, Upper Corgo and Upper Douro, and they differ in terms of area under vines, number of farmers, chemical composition of the soils and climatic characteristics, namely rainfall and temperature.

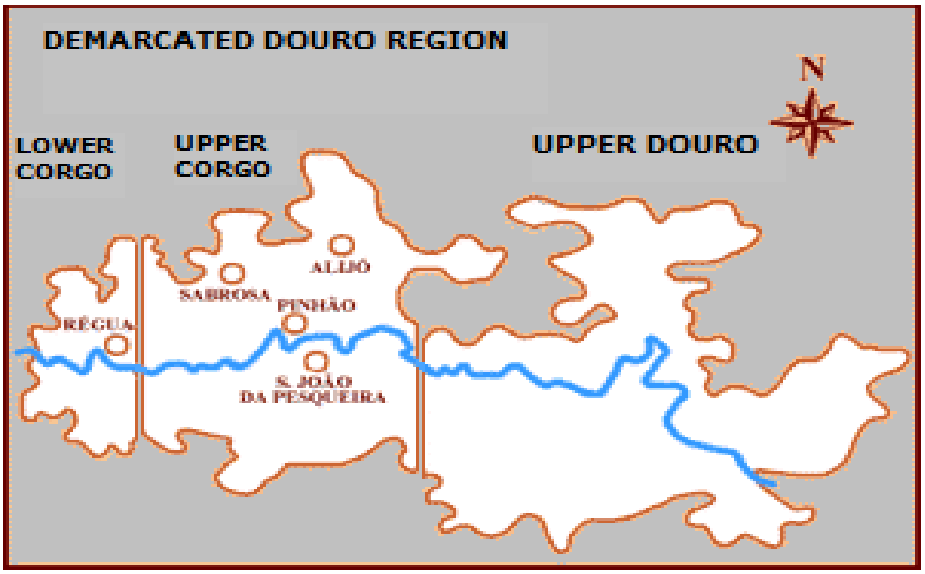


Figure 1. Demarcated Douro river region. Source: IVDP (2014).

Located in deep valleys, protected by mountains, the climate in the region is characterized by very cold winters and hot, dry summers. Thus, Douro region has been worldwide recognized as a reference for the high quality of its wines.

Different ways to vinify wine "Douro" were developed through time. The traditional prepared in mill using large shallow rock containers, generated wines which exhibited the highest extraction of color and tannins, that gives them a good aging potential. On the other hand, there is a more modern production system, which has recorded a substantial increase, and uses stainless steel tanks with temperature control, improving the enological characteristics in terms of aromas and colors. It has also been an evolution in the maturation of the wine before it is bottled. Large wooden casks were used traditionally as containers that have been gradually being replaced by new oak barrels with lower volume, or by stainless steel vats (IVDP, 2014).

Farmers in the Douro region, exhibit a wide range of products, having red, white, rosé and special wines. Red wines are elaborated from indigenous grape varieties such as "*Touriga nacional", "Touriga franca", "Tinta roriz" (Aragonez), "Tinta barroca"* and "*Tinto cão"*. Basically, several grape varieties are blended with each other in order to increase the richness and complexity of the Douro profile wines. Nevertheless, there are still mono-varietal wines; this means wines produced with only one variety, especially the first three listed before.

Moreover, Douro region presents strong white wines, because of their dryness resulting by blending several grape varieties such as "*Malvasia fina"*, "*Viosinho",* "*Gouveio"* and "*Rabigato"*. Finally, rosé wines are produced in response to global trends in wine consumption, especially among young people. They are elaborated through specific changes during the winemaking processes, reducing the maceration time, which gives the final characteristic pink color (IVDP, 2014).

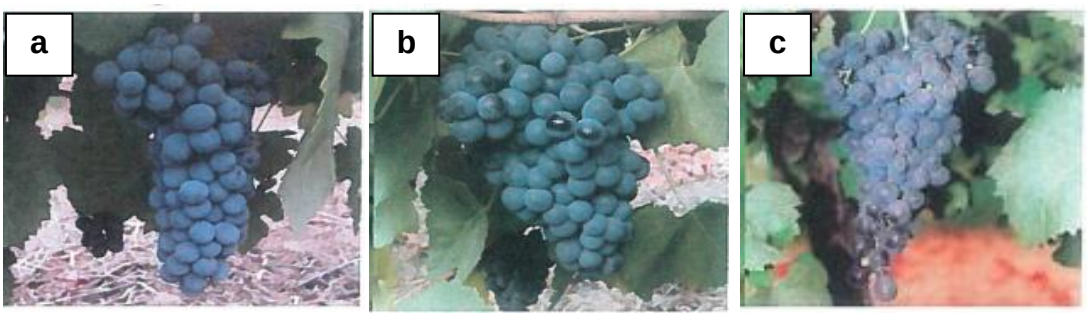


Figure 2. Portuguese grape varieties from Douro, región: a) "*Touriga nacional";* b) "*Touriga franca"* and c) "*Touriga roriz".* Source: Eiras-Dias et al. (2011).

1.2 Grape pomace: Applications

During winemaking steps, significant amounts of waste, in their majority solids are also generated. Residues of the wine industry, including seeds, peels or skins, stalks and pulps are denominated in its whole as grape pomace.

Based on a traditional winemaking process it is estimated that per six liters of wine is generated one kg of solid waste. Thus, taking in consideration a global wine production around 271 MhL (OIV, 2014) over 4.5 million of tons of solid waste would be generated worldwide. Environmental concerns about the production and accumulation of waste worldwide are increasing. Meanwhile the European Commission, early in 2006, issued a series of regulations towards a sustainable European wine sector aimed the inclusion of minimum environmental requirements for the wine sector covering the main pressures

from the sector (notably, soil erosion and contamination, the use of plant protection products, and waste management).

The current destinies for the grape pomace comprise the disposal or fertilization/compost (Laufenberg, Kunz, & Nystroem, 2003; Reeve, Carpenter-Boggs, Reganold, York, & Brinton, 2010), cattle feeding (Lu & Yeap Foo, 1999), landfills, fermentation/distillation industry either for the extraction of food natural colorants and bioactive compounds (Lapornik, Prošek, & Golc Wondra, 2005; Mendes, Prozil, Evtuguin, & Lopes, 2013). In certain cases, grape pomace (mainly the seeds) are used in wood adhesives extractive processes (Ping, Pizzi, Guo, & Brosse, 2011). Some authors refer disadvantages in using grape pomace without any pre-treatment cattle feeding or in post winemaking fermentation/distillation process, due to its high polyphenols content with implications in animal nutrition and inhibition of yeasts germination (Mendes et al., 2013; Ping et al., 2011).

The most recent and innovate application is associated to a new pesticide, namely “phytosanitary bioproducts” used for the control of the incidence of diseases in some crops (Benouaret et al., 2014; Goupil et al., 2012).

The composition of the residue of the grape has significant variations depending on grape variety and technology applied during the winemaking steps. Generally, it consists largely in seeds and skins (or peels), and the rest is represented by stems or stalks. After fermentation step, considerable contents of polyphenols (over 10% on dry bases) are retain in grape pomace, depending on the type of grape (white or red), the part of the tissue (skins, seeds, etc.), as well as the processing conditions (e.g., contact time between skins and must) (Guendez, Kallithraka, Makris, & Kefalas, 2005; Makris, Boskou, & Andrikopoulos, 2007). Regarding to its chemical composition, lignans, cellulose and tannins have been assessed previously by several authors, providing indication for content range as shown in Table 2 (Mendes et al., 2013; Prozil, Evtuguin, & Lopes, 2012; Yu & Ahmedna, 2013).

Table 2

Chemical composition of grape pomace [a]

Components	% dry weight
Ashes	7.0 – 7.8
Extractives	
Dichloromethane	1.0 – 5.5
Water	23.7 – 26.4
Proteins	6.1 – 18.8
Tannins	13.8 – 15.9
Cellulose	20.8 – 30.3
Hemicelluloses	12.5 – 21.0

[a] Data from Mendes et al. (2013); Prozil et al. (2012); Yu and Ahmedna (2013)

Many authors highlight the importance in research and development of emerging recovering technologies in order to get advantages from the bioactives compounds in by-products. In this context, numerous systems have been developed in order to extract such bioactives compounds, which as described above have important properties that can be exploited in fields of pharmaceutical, and food industries and also nutrition sciences, cosmetic and medicine (Fontana, Antoniolli, & Bottini, 2013).

1.3. Polyphenolic compounds: antioxidant properties

Phenolic compounds (or just polyphenols) represent a wide family of compounds, including various groups of molecules classified as plant secondary metabolites. Phenolics have been considered the most important, numerous and ubiquitous groups of compounds in the plant kingdom (Naczk & Shahidi, 2004). More than 8,000 different compounds have been identified and the number is still growing (Ignat, Volf, & Popa, 2011), including complex chemical structures which exert diverse biological functions.

In order to simplify the understanding, a first classification can be made based on their solubility. The water-soluble polyphenols comprise compounds such as phenolic acids, phenylpropanoids, flavonoids and quinones; whilst those which are water-insoluble include: condensed tannins, lignins and cell-wall bound hydroxycinnamic acids (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012).

Vermerris & Nocholson classified these bioactive compounds according to the number of phenol rings they contain in: phenolic acids, stilbenes, flavonoids, lignins and tannins (Vermerris & Nicholson, 2006). All these groups present one or more hydroxyl groups directly attached to an aromatic ring, conferring the phenolic characteristics.

Flavonoids, the most important single group of polyphenols, include 13 subclasses with more than 5,000 different compounds present mainly in fruits and plants (Bravo, 1998; Haminiuk et al., 2012). Among these subclasses, compounds namely, chalcones, dihydrochalcones, aurones, flavones, flavonols, dihydroflavonol, flavanones, flavanols, flavandiol, anthocyanidins, isoflavoinids, bioflavonoids, and proanthocyanidins or condensed tannins, can be found in food sources. The flavonoids basic structure consists in a common diphenylpropanes (C_6-C_3-C_6) skeleton with an essential structure consisting in two aromatic rings, A and B joined by a 3-carbons bridge, usually in the form of an oxygenated heterocyclic ring C, as shown in Figure 3. Variations in the substituent groups in the ring C give the major flavonoid aforementioned subclasses. Moreover, flavonoids can be found in a non-glycosylated form (aglycone) as occasionally occur in plants, or most commonly attached to a sugar molecule (glycoside) (Bravo, 1998; Ignat et al., 2011).

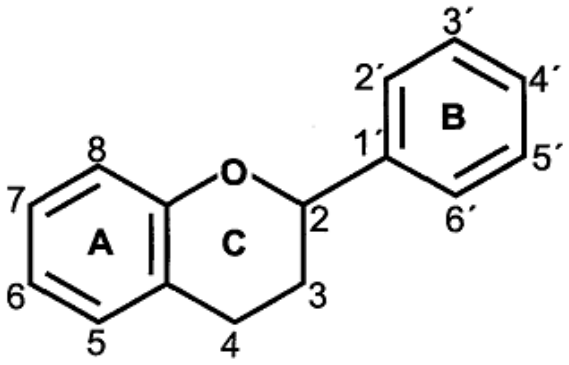


Figure 3. Basic structure and numbering of the flavonoid nucleus. Source: Bravo (1998).

Considering the importance of flavonoids compounds, a brief description, highlighting the most remarkable characteristics of main subclasses, is given bellow.

Flavonols, the most ubiquitous flavonoids in foods, include as main representative compounds, kaempferol and quercetin. They present strong antioxidant properties, mainly quercetin, through the free radical scavenging activity. Quercetin (Figure 4) presents the three fundamental criteria to be considered a strong free radical scavenger as follow:

- The *O*-dihydroxy structure in the B ring, which confers higher stability to the radical form and participates in electron delocalization;
- The 2,3 double bond in conjugation with 4-oxo function in the C ring is responsible for the electron delocalization from the B ring, in other words, the antioxidant tendency is associated to this structure regarding the resonance effect of the aromatic nucleus;
- The 3- and 5-OH groups with 4-oxo function in A and C rings are required for maximum radical scavenging potential (Rice-Evans et al., 1996).

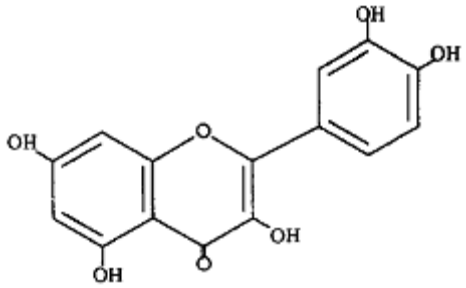


Figure 4. Chemical structure of quercetin. Source: Rice-Evans, Miller, and Paganga (1996).

Good flavonols sources are onions, curly lake, blueberries, passion fruit, pomegranate and broccoli. Red wine and tea also contain up to 45 mg flavonols per portion (El Gharras, 2009; Haminiuk et al., 2012).

Flavones are much less common than flavonols in fruit and vegetables. Significant quantities are found in the polymethoxylated form as tangeretin, nobiletin and sinensetin in the skin of fruit citrus (essential oil of mandarin, for example). The only important edible sources of flavones identified till these days are parsley and celery. These polymethoxylated flavones are the most hydrophobic flavonoids (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004).

Flavanones are found in tomatoes and certain aromatic plants such as mint, but they are present in high concentrations only in citrus fruit. They can appear in the aglycone form like naringenin in grapefruits, hesperidin in oranges, and eriodictyol in lemons. Nevertheless, the most common forms appear generally as glycosylated (*O*- or *C*-glycosides) by a disaccharide in certain cases or by a rutinose in others. High flavanones concentrations are found in the solid parts of citrus fruit, particularly the albedo (the white spongy portion) and the membranes separating the segments (Bravo, 1998; El Gharras, 2009).

Isoflavones, such as daidzein and genistein, with ring B of the flavone molecule attached to the carbon 3 of the heterocycle, especially occur in legumes (Bravo, 1998). According to El Gharras isoflavones are provided only by soybean-derived products. They can be present as aglycones or glycosides, depending on the soy preparation. Soya and its processed products are the main source of isoflavones in the human diet (El Gharras, 2009). The interest in this group of

compounds lies in the fact that certain physiological effects are attributed to their similar structure to estrogens like ß-estradiols. Additionally, for this reason they are sometimes described in the literature as "phytoestrogens" (Ignat et al., 2011).

Flavanols comprise two types of associations that may exist between compounds. On one hand, it may exist in the form of monomers like catechins and On the other hand, it is also possible to find them in the polymer form reveling a more complex structure (proanthocyanidins). Some sources for catechins are many types of fruits like apricots and sweet cherry, some beverages such as red wine, although green tea and chocolate are the richest sources (Manach et al., 2004).

Catechin, epicatechin and gallocatechin are the monomeric constituents of the condensed tannins, although they are also commonly found as free monomers (Bravo, 1998). Catechin and epicatechin are the main flavanols in fruit, whereas gallocatechin, epigallocatechin, and epigallocatechin gallate are found in certain seeds of leguminous plants, in grapes, and more importantly in tea (Manach et al., 2004). Likewise, most of the polyphenolic compounds, catechins and their esters, particularly epigallatocatechin gallate present in the green tea, have shown anticarcinogenic actions in human and animal tissues (Rice-Evans et al., 1996). According to a research work by Arts *et al*. (2002) the intake of catechin originating from fruits, but not from tea, was associated to a lower risk of cancer of the upper-digestive tract (Arts, Jacobs Jr, Gross, Harnack, & Folsom, 2002).

Finally the last groups of compounds belonging to flavonoids are the water-soluble vacuolar pigments that may appear as red, purple, or blue depending on the pH, are the anthocyanins (Ignat et al., 2011).

The term anthocyanin refers to the glycoside of anthocyanidins (Bravo, 1998). The anthocyanidins consist of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen, which is also linked by carbon-carbon bond to a third aromatic ring B (Konczak & Zhang, 2004). Up to now there are reports of more than 500 different anthocyanins and 23 anthocyanidins (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009), although the most frequently reported in the plant kingdom are the following six

anthocyanins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Among the sugars linked to the anthocyanidins forms are the monosaccharides: glucose, galactose, rhamnose and arabinose (Ignat et al., 2011). The color of the anthocyanins is largely conditioned by the substitution pattern of the ring B of the anthocyanidins, the pattern of glucosylation and the degree and nature of esterification of the sugar with aliphatic or aromatic acids and by the pH, temperature, type of solvent and presence of co-pigments (Shipp & Abdel-Aal, 2010).

The major antioxidant activity of the anthocyanins can be ascribed to the reducing power of the *O*-dihydroxy structure in the B ring (Rice-Evans et al., 1996).

This significant property plays a vital role in the prevention of neuronal and cardiovascular illnesses, cancer and diabetes, among others. For example, previous study have showed that anthocyanins from wine and grape skin inhibited phosphodiesterase-5 activity, which reduced the risk of cardiovascular diseases by vasorelaxation (En-Qin et al., 2010).

In fact, the group of flavonoids was found to be very effective scavengers of free radical concerning *in vitro* assays, showing important antioxidant activity due to their high redox potential which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. Metal chelating properties were also described for flavonoid compounds (Haminiuk et al., 2012; Ignat et al., 2011; Rice-Evans et al., 1996).

Regarding their importance, the second most remarkable polyphenolic group is the phenolic acids, which represent one- third of the polyphenols present in the human diet. They can be found not only in bound forms but also in the free form in plant (Ignat et al., 2011). Nevertheless, their methyl and ethyl esters, also with their glycosides occur very commonly as bound forms (Bravo, 1998). There are two subclasses for the phenolic acids: hydroxycinnamic and hydroxybenzoic acids. The presence of one carboxylic group in the structure confers their acidic character. Phenolics with C_6-C_1 skeleton such as gallic, vanillic, syringic and *p*-hydroxybenzoic acids, and their aldehydes are quite common inn higher plants and ferns. The most important phenylpropanoids (C_6-C_3 skeleton) are the

hydroxycinnamic acids like *p*-coumaric, caffeic, ferulic and sinapic and their derivatives. Both these groups (phenylpropanoids and more simple phenols) are usually linked by covalent bonds to cell wall polysaccharides or to the so-called lignin core (Bravo, 1998; Haminiuk et al., 2012; Ignat et al., 2011).

Tannins represent polyphenols of intermediate to high molecular weight compounds. They are mostly present in fruits in the polymeric form and they are responsible for the astringency of tannin-rich foods, due to their ability to precipitate the proteins present in the saliva (Bravo, 1998). Tannin group could be divided into two sub-classes: hydrolysable and condensed tannins (also called proanthocyanidins).

Hydrolysable tannins are gallic acid and its dimeric condensation product, hexahydroxydiphenic acid, esterified to polyol, which is mainly glucose. Further esterification or cross linked oxidation reactions take place to yield more complex hydrolysable tannins (Hagerman, 2002). They are found in fruits and as their name indicates, these compounds are easily hydrolyzed in acid or alkali medium, for hot water or enzymatic action, giving as result polyhydric alcohol and phenylcarboxylic acid. One of the most representative compound belonging to hydrolysable tannins is the tannic acid (Bravo, 1998).

On the other hand, condensed tannin or proanthocyanidins, pertaining to the family of flavonoids, consist in monomeric units of flavan-3-ol (catechin, epicatechin, etc) with a flavan-3,4-diol as its precursor. Pathways involved in their biosyntheses although are well understood, the steps leading to condensation and polymerization have not been elucidated yet (Ignat et al., 2011). For this reason, most of the published literature refers to oligomeric proanthocyanidins like dimmers, trimers. However, proanthocyanidins can reach high polymerization degrees over 50 (Bravo, 1998). The properties behind the chemical structure for the tannins are mainly linked to potential metal chelators, protein precipitating agents and biological antioxidants (Ignat et al., 2011; Rice-Evans et al., 1996). One important source for the condensed tannins is grapes, where they are mainly localized in hard part of the fruit, like seeds (El Gharras, 2009).

Since the chemical point of view, stilbenes are phenylpropanoid-derived compounds characterized by a 1,2-diphenylethylene skeleton (C_6-C_2-C_6). They are not abundant in the human diet. Resveratrol is probably the most representative compound belonging to this group and exists in red skin grape, peanuts and berries (Ignat et al., 2011). It can be found in both *cis*- and *trans*-resveratrol (3,5,4'-trihydroxystilbene) isomers, and also as resveratrol-3-*O*-β-D-glucopyranoside (piceid), piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) and resveratrol dimmers in grapes (Flamini, Mattivi, De Rosso, Arapitsas, & Bavaresco, 2013; Ignat et al., 2011). It has been intensively studied all over the world due to its beneficial health properties, linked to circulatory system, prevention the development of degenerative diseases like arteriosclerosis and also anticarcinogenesis (El Gharras, 2009; Gülçin, 2010; Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). Because resveratrol is synthesized as response to insect attack, injury and fungal infection (particularly *Botrytis cinerea*), it represents a phytoalexin substance (Atanacković et al., 2012). Stilbenes can also occur in oligomeric and polymeric forms, so-called *viniferins*. They are induced by oxidative polymerization of the monomer resveratrol through the activity of a peroxidase (Moreno-Arribas, Polo, & Carmen, 2009).

Lignans or phytoestrogens represent one of the major groups of polyphenolic compounds with a chemical behavior which allow oestrogen-like activities. In other words, they are converted into certain compounds (enterodiol and enterolactone) in the intestinal lumen which exhibit both oestrogenic and anti-oestrogenic properties. Lignans are generated by oxidative dimerisation of two phenylpropane units. In most of the cases they are present in nature in free form and very few can be seen as glycoside derivatives (Ignat et al., 2011). Flaxseed, sunflower and certain cereals such as rye, oats and barley are the major source of lignans in the human diet (Meagher & Beecher, 2000; Scalbert et al., 2005). Particularly, the scientific areas have been expressed interest in lignans research due to potential applications in cancer therapies and because it seems to exist a relationship between the consumption of whole-grain cereals, the major source of lignans, and the risk reduction of various cancers (Scalbert et al., 2005).

A literature search was performed on the **Scopus** using “polyphenolic compounds” as key word. It revealed that the number of publications has increased about 1,741% in the past decade (just 263 until 1994, whilst between 1994 and 2014 around 4,589 papers were published), demonstrating the increasing interest by this topic as investigation subject.

1.4. Extraction of polyphenols from grape pomace

In a context where the world production of wine each year generates tons of waste liable to be used in obtaining valuable bioactive compounds, a large number of works have been published in relation to the utilization of by-products (Fontana et al., 2013; Laufenberg et al., 2003; Pinelo, Sineiro, & Núñez, 2006; Schieber, Stintzing, & Carle, 2001; Wijngaard, Hossain, Rai, & Brunton, 2012).

Usually, all the extractives procedures start with a sample pre-treatment including oven or freeze drying, ground to finer powder or crushing fresh tissues (Fontana et al., 2013). Extraction procedures existing in the literature range from conventional methods of solvent mediated solid-liquid extraction, through methodologies based on supercritical properties of fluids (supercritical liquid and supercritical fluid extraction) to the most emerging technologies such as enzymatic hydrolysis treatments, ultrahigh pressure systems, high voltages electric discharges and pulsed ohmic heating.

Regarding conventional methodologies, there are extractive solvent systems based on solid-liquid system transfer phenomena which are used during separation operations. After applications of these methodologies, phenolic-rich crude extracts are obtained (not individual or compounds families). Thus, the extraction efficiency can be improved by changes in concentration gradients, diffusion coefficients, solvent type, particle size, temperature, and extraction time as well as the presence of interfering substances in the matrix. The solvent type (polar or hydrophobic nature) has been pointed out as one of the most important variable in the extraction efficiency of the process (Fontana et al., 2013). The extraction yields in terms of the total polyphenol content can be

enhanced with the assistance from as a simple and economic ultrasound technology technique (Luque de Castro & Priego-Capote, 2007).

The use of supercritical fluids resulted in advances in the extractive process as it takes advantage of the ease with which manages to penetrate the matrices of solid waste under conditions that avoid the presence of light and oxygen, also improving process efficiency (Wells, 2003). Among the most common solvents used in supercritical fluid extraction, supercritical carbon dioxide presents advantages due to its relatively low temperature during the extraction steps, avoiding the degradation of the valuable bioactive compounds. The application of supercritical CO_2 is enhanced with the addition of small amounts of solvents (co-solvents or modifiers) such as methanol and ethanol, thus improving contact with more hydrophilic compounds (Wang & Weller, 2006). The choice of a particular modifier is restricted to the subsequent use of the extracted compounds. Although this technique still represents a promising extractive methodology, further studies regarding to costs involved should be done.

Accelerated solvent extraction, also known as pressurized fluid extraction or pressurized liquid extraction, uses solvent at high temperature (100-180 °C) and pressure (1500-2000 psi) in order to improve the extraction of bioactives compounds from solids matrixes (Fontana et al., 2013). Recently Rockenbach *et al*. proposed a promising new approach (at 25 °C) once the properties of the polyphenols are influenced by high temperatures (Rockenbach et al., 2012).

In other research areas, enzymatic hydrolysis procedures have been performed in order to improve the extraction of bioactive compounds. Mixtures of pectinolytic and cell-wall polysaccharide degrading enzymes in aqueous medium (Kammerer, Claus, Schieber, & Carle, 2005), carbohydrases (cellulolytic and pectinolytic activities) and tannase (Chamorro, Viveros, Alvarez, Vega, & Brenes, 2012) and more recently, pectinase, cellulase and tannase (single and blended treatments) (Fernández, Vega, & Aspé, 2015) were successfully exploited. Nevertheless, further studies are needed to identify more specific enzymes with potential use in the releasing of polyphenols from grape pomace. This is of particular relevance since several *in vitro* antioxidant assays showed that the bound phenolic fraction demonstrated a significantly higher

antioxidant capacity than free and esterified phenolics (Liyana-Pathirana & Shahidi, 2006).

In the same context of new trends for the extraction of bioactive compounds, other technologies like high voltage electric discharge and pulsed ohmic heating are under study. High voltage electric discharge, combining temperature, different solvents, energy and exposition time, was demonstrated to be useful for the particle fragmentation and cell structure damage accelerating the extraction of intracellular compounds. Still, studies regarding to the associated costs and design at a commercial level are required (Boussetta & Vorobiev, 2014). Pulsed ohmic heating is an emerging technology which allows high cell membrane permeabilization of the materials under study, with low energy consumption combining electrical and thermal treatments. El Darra *et al.* believe that this methodology is promising for future application in the valorization of pomace from fruits and vegetables without hydroalcoholic solvent use (El Darra, Grimi, Vorobiev, Louka, & Maroun, 2013).

1.5. Polyphenolic compounds from grape pomace: separation, *in vitro* characterization and evaluation of antioxidant properties

Emerging attention to trends related to the grape pomace bioactives recovery processes lead to the exploration of accurate techniques to assess their antioxidant properties. Thus, there is an increasing interest in high-throughput techniques, automatic and rapid assessment methodologies to evaluate the antioxidant properties in complex matrixes like grape pomace.

Different efforts were made in order to study, classify and propose a general guideline about the current involved antioxidant methodologies and assays (Fontana et al., 2013; Gülçin, 2012; Huang, Boxin, & Prior, 2005; Magalhães, Segundo, Reis, & Lima, 2008; Prior, Wu, & Schaich, 2005). Nevertheless, the specific conditions for an adequate separation and then, identification and quantification of individual phenolics still represents a challenge for the scientific community due mainly to the complexity involved in this by-product matrix.

Generally speaking, the quantification of the polyphenols in grape pomace starts by evaluating the Total Phenolic Content (TPC), with forward steps consisting in an evaluation of the antioxidant capacity (through more than one single methodology) and a complementary identification and quantification of the individual phenols.

The simplest method for a fast estimation of TPC is the measurement of absorption at 280 nm (in a suitably diluted sample). The second method most commonly used for TPC assessment is the Folin−Ciocalteu assay (Fontana et al., 2013) also named Folin-Ciocalteu reducing assay (FCR). The FCR actually measures the sample's reducing capacity, but this is not reflected in the name "total phenolic assay" (Huang et al., 2005).

It has been strongly recommended to use at least two methods for the assessment of antioxidant properties when working with complex matrixes (Schlesier, Harwat, Böhm, & Bitsch, 2002). It is also advantageous to select methods that are commonly accepted, validated and standardized, with a large body of comparable data available in the literature (Magalhães et al., 2008). According to Prior *et al*., there are certain requirements or criteria which must be followed in order to select and accurately standardize an "ideal" methodology for the antioxidant capacity assessment: (i) measures chemistry actually occurring in potential application(s); (ii) utilizes a biologically relevant radical source; (iii) simple; (iv) uses a method with a defined endpoint and chemical mechanism; (v) instrumentation is readily available; (vi) good within-run and between-day reproducibility; (vii) adaptable for assay of both hydrophilic and lipophilic antioxidants and use of different radical sources; (viii) adaptable to "high-throughput" analysis for routine quality control analyses (Prior et al., 2005).

Particularly when working with grape pomace many spectrophotometric assays have been proposed: DPPH$^{\bullet}$ (2,2′-diphenyl-1-picrylhydrazyl) assay, ORAC (Oxygen Reactive Absorbance Capacity) assay, TEAC (Trolox Equivalent Antioxidant Capacity) and TBARS (Thiobarbituric Acid Reactive Substances) assay. Besides, as some polyphenols are also effective as chelators of transition metal ions (which may induce Fenton-type oxidation reactions in their

free states (Rice-Evans et al., 1996)), assays based on this antioxidant property like iron(II) chelating ability (ICA) assay, have been applied.

In DPPH$^{\bullet}$ assay, the purple chromogen radical 2,2′-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine, following the decrease of absorption at 517 nm. Results are typically expressed in Trolox equivalents (TE).

In ORAC assay a peroxyl radical is thermally generated *in situ* from AAPH (2,2′-azobis(2-amidino-propane) dihydrochloride) which reacts later with a fluorescent probe (typically fluorescein or phycoerythrin). The antioxidant presence avoids the fluorescent probe degradation, prolonging its emission upon time. The quantification is performed measuring the area under curve (AUC) that represents the oxidation of the probe along time. The protective effect of antioxidants is evaluated from the net integrated area under the fluorescence decay curves ($AUC_{sample} - AUC_{blank}$) and results are expressed as µM of TE (Magalhães et al., 2008).

TEAC assay, also called ABTS$^{\bullet+}$ (2,2′-azinobis-(3-ethylbenzothiazole-6-sulphonate) cation radical assay, have been used for antioxidant quantification of grape pomace extracts (González-Paramás, Esteban-Ruano, Santos-Buelga, de Pascual-Teresa, & Rivas-Gonzalo, 2004). It consists in the measurement of the capacity of a given antioxidant to reduce the stable ABTS$^{\bullet+}$ radical cation (green/blue specie) into the non-radical and colorless species (ABTS), generally in an aqueous media.

Separation and analysis of phenols from grape pomace

HPLC (High Performance Liquid Chromatography) techniques are broadly used for the polyphenolic separation and later quantification due to their polar nature. Grape pomace dry powders are accurately dissolved in solvent and filtered before analysis. The columns are almost exclusively of the reverse phase type, with C18 as stationary phase (Lorrain, Ky, Pechamat, & Teissedre, 2013).

Combination of columns, solvent systems, and conditions has been successfully applied for the separation of families of phenolics such as

anthocyanins, procyanidins, flavanols, isoflavones, flavonols, phenolic acids, flavanones, and stilbenes (Fontana et al., 2013).

Among the detection methods, UV-vis (ultra violet visible), photodiode array detector (DAD), fluorescence and mass detectors were implemented, although UV detection remains the most commonly applied. Improvements in the structural information and the possibility of analysis of high complexity matrixes were brought by to more efficient techniques based on mass spectrometry (MS), such as coupling to liquid chromatography or multiple quadruple MS detectors.

1.6. Poultry meat: global and Portuguese production

Poultry is defined as any type of domesticated fowl raised for meat and/or eggs according to National Chicken Council (NCC, 2007)**,** including mainly chicken and turkey. Poultry has been and still is a major animal product in diets. Poultry meat and products are consumed broadly around the world due to different reasons, with no intake restriction associated to religion compared with other kind of meat (pork and beef). There are specialized slaughtering industries with *Kosher* and *Halal* slaughter procedures (poultry products with certification on meeting Jewish or Islamic dietary laws and standards regarding slaughter and processing, respectively). Additionally, poultry is recognized as a healthy meat because when consumed skinless, the muscle of birds rarely has higher values than 1 % fat and even less saturated fat than in beef. Its proteins are easily digestible and assimilable. Nutritionally, people eat poultry meat for its high content of high-quality protein with all the essential amino acids. Besides, poultry meat has a great potential to be industrialized offering a wide range of food choices for consumers. In addition, with the advances in preservation techniques for fresh poultry and processed products, consumer preferences for poultry and poultry products are higher than ever (Guerrero-Legarreta, 2010).

In terms of global production, five countries concentrate the production, ranking as follows: United States of America (USA) (18 %), China (14 %), Brazil (12 %), Russian Federation (4 %) and Mexico (3 %) with a total world production of 92,730,419 MT (millions of tons) of chicken meat, according to FAOSTAT (FAOSTAT, 2012). In Figure 5 is presented the ranking for the ten major producers of poultry meat.

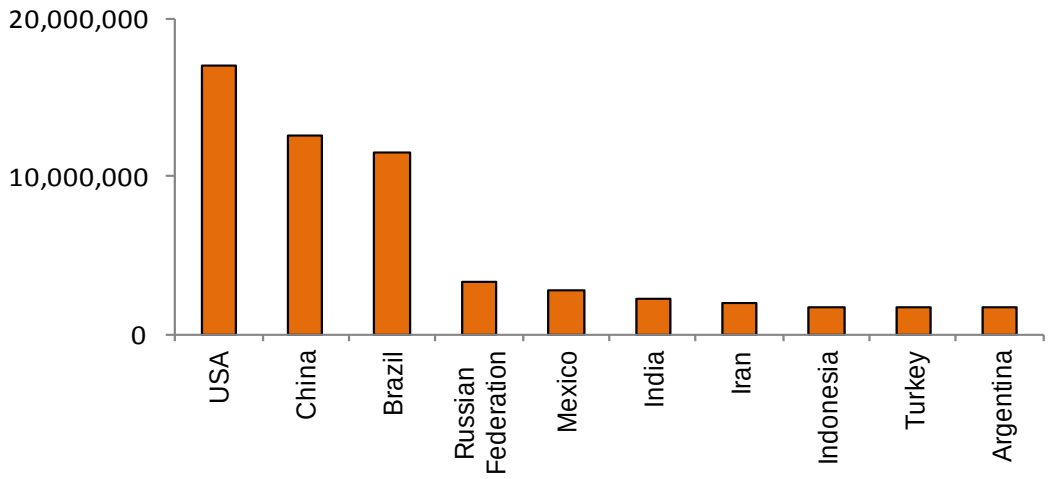


Figure 5. World production (MT) of chicken meat. Source: FAOSTAT (2012).

European countries represent almost 17 % of the total world production (15,435,698 MT). The leading countries in poultry meat production are France, closely followed by UK, Spain, Germany and Poland. These five member states account for 60 % of total EU production of poultry meat (EUROSTAT, 2012).

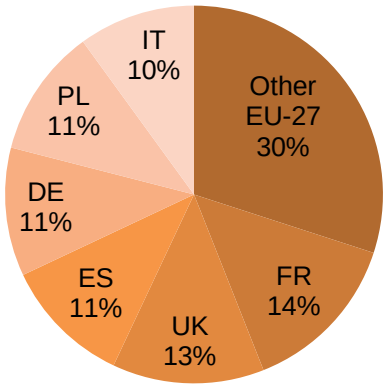


Figure 6. European production of poultry meat. Source: EUROSTAT (2012).

Globally speaking, according to Henchion *et al.*, it is sharply established an increasing trend in meat consumption in kg per capita between 1990 and 2009 (Henchion, McCarthy, Resconi, & Troy, 2014), accompanied by a downward

trend for red meat and an important upward trend for white ones, mainly in poultry meat (see Table 3). Possible relative prices of different types of meat can be highlighted as the main reason, as the real price of beef is higher than poultry and pig meat in most countries (Guerrero-Legarreta, 2010).

Table 3
Global meat consumption, 1990-2009, kg/capita, adapted from Henchion et al. (2014)

	1990	2009	%change
Bovine meat	10.4	9.6	-7.7
Mutton and goat meat	1.7	1.9	11.8
Pig meat	13.2	15.8	19.7
Poultry meat	7.7	13.6	76.6
Other meats	0.7	0.9	28.6
Aggregate	33.7	41.9	24.3

According to the perspective established by the European Commission, poultry meat is expected to overtake pig meat as the most consumed meat in the world by 2022. In the other side, a similar analysis was assumed by Kearney from Dublin Institute of Technology, who projected that by 2050 the consumption of meat will increase moderately, and this will largely mirror increases in pork and particularly in poultry (Kearney, 2010).

In the European scenario, the trends in meat consumption are exactly the same where white meat is projected to replace the red meat in Europe as well as globally (European-Commission, 2012).

Particularly, in Portugal the chicken meat production is ranked in the sixth place of the total commodities produced in this country ahead of the meat pig

production (millions of tons, MT) (283,999 and 282,951 MT, respectively) (FAOSTAT, 2012) (see Figure 7).

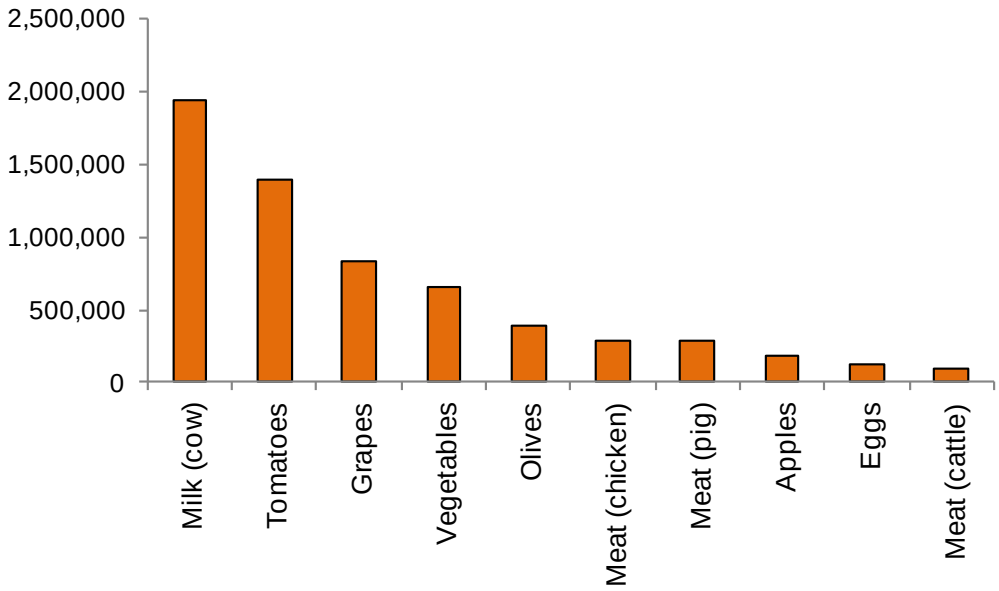


Figure 7. Portuguese production (MT) for main commodities. Source: FAOSTAT (2012).

In terms of consumption in Portugal among 2008 and 2013, a downward trend for cow and pig meat (-14.3% and - 9.1% respectively) was registered by *Instituto Nacional de Estatistica* (INE), whilst an upward trend (+ 8%) was observed for poultry meat by the same period (INE, 2013).

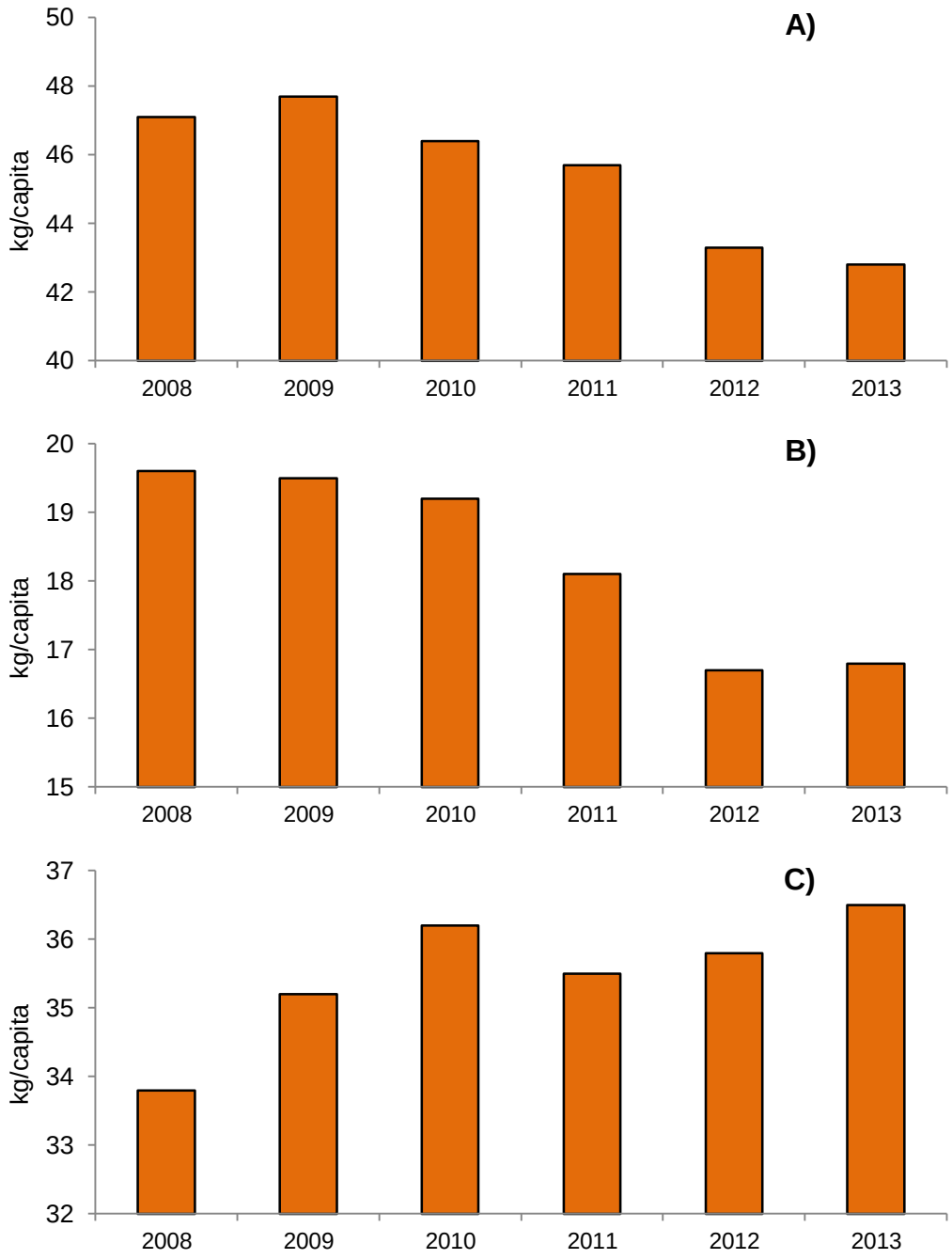


Figure 8. Evolution of consumption for different types of meat **A)** pork; **B)** beef and **C)** poultry (chicken), in Portugal. Source: INE (2013).

Once perspectives are exposed, it can be deduced that both locally (Portugal), at European and even at worldwide level, projections regarding poultry sector are highly favorable leading due to changes in relative meat prices, to concepts like “nutrition transition” (Hawkesworth et al., 2010) associated to dietary patterns and lifestyle trends, positioning the chicken in a place of privileged.

1.7. Mechanically chicken meat: different uses

According to **Point 1(14) of Annex I to Regulation (EC) Nº 853/2004 and Article 3 (1) (n) to Regulation (EC) Nº 999/2001** "mechanically separated meat or MSM means the product obtained by removing meat from flesh-bearing bones after boning or from poultry carcasses, using mechanical means resulting in the loss or modification of muscle fibre structure". It is noteworthy that MSM from bovine, caprine and ovine animals are currently prohibited by the European regulations.

Mechanically deboned meat (MDM), mechanically recovered meat (MRM) or MSM are synonyms used to designate the same product (Püssa, Pällin, Raudsepp, Soidla, & Rei, 2008).

Increasing amount of poultry pieces resulted from the industrialization of chicken, turkey and poultry in general, are generated in processing industries these days. Meat attached to the soft bones can be manually or mechanically separated. Although the original aim of MDM technology application was to reduce the rate of repetitive strain injury of workers caused by short cyclic boning work in cutting rooms of meat operations, the procedure became into a profitable way to generate low-price raw material. Thus, mechanical recovery of poultry from necks, backs and other bones with attached flesh started in the late 1950s. Removal of beef and pork from irregularly shaped bones began in the 1970s (Field, 2004).

The texture of the resulting meat product is a finely ground material that has a paste-like consistency in which the myofibrils are heavily fragmented (Barbut, 2002). The overall characteristics and therefore the latter destiny of the MDM depend on the specific part of the animal used, conditions (temperature, aeration, pressure, and contact with metal) during the extraction. Although its use in meat sector represents a low-cost source of animal protein with satisfactory binding capacity, strict regulations concerning to risks associated to the use of MDM are mandatory.

In term of proximate composition (calcium content, moisture, protein, ashes and fat) the values can vary broadly depending on the type of machine, anatomical location of bones, animal species, temperature, and amounts of lean meat (Field, 1988).

Depending on the pressure applied (or equipment) during the extractive steps, the final product can be classified into low and high pressure MDM, according to European Legislation. The final characteristics of MDM, in terms of overall appearance, consistency, nutritional composition, and even microbiological loading content, depend on the raw materials used and on the strict procedures followed during the extraction itself. In general, the low pressure MDM or also called “Baader meat”, “3 mm meat” or ”desinewed meat” in the meat sector and it has a similar consistency and appearance to a ground meat, whilst the high pressure MDM consists in a fine-consistency product that even macroscopically can clearly distinguishable from the low pressure MDM as a product with a characteristic and particularly pasty texture resulting from the loss or modification of the muscle fibre structure.

The mechanical process of removing meat from the bones causes cell breakage, protein changes and increases fat and haem contents, thus, the final product is subjected to strict regulations concerning its use in food preparations.

Currently there are three methodologies for the MDM process: 1) belt-drum system, 2) auger type, and 3) hydraulically powered presses (Barbut, 2002).

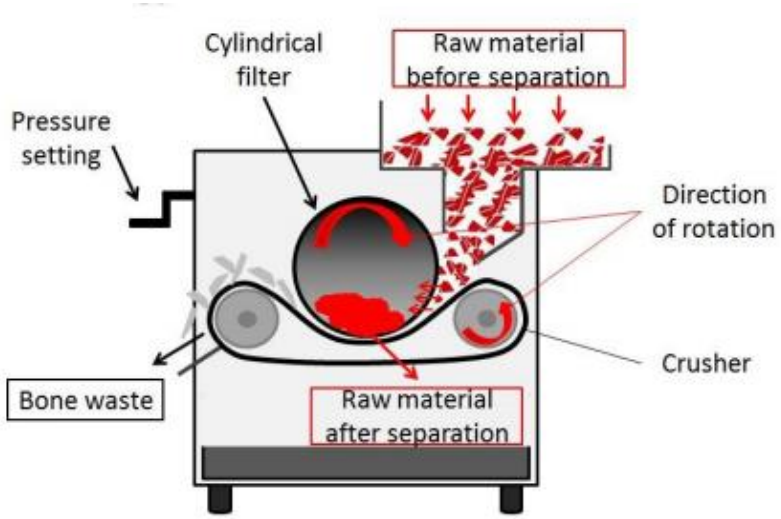


Figure 9. Belt-drum meat deboner system. Source: EFSA (2013).

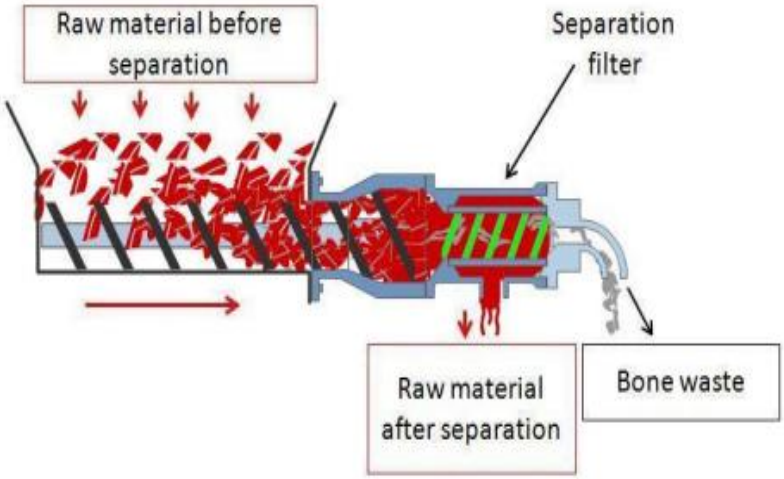


Figure 10. Endless screw meat deboner technology. Source: EFSA (2013).

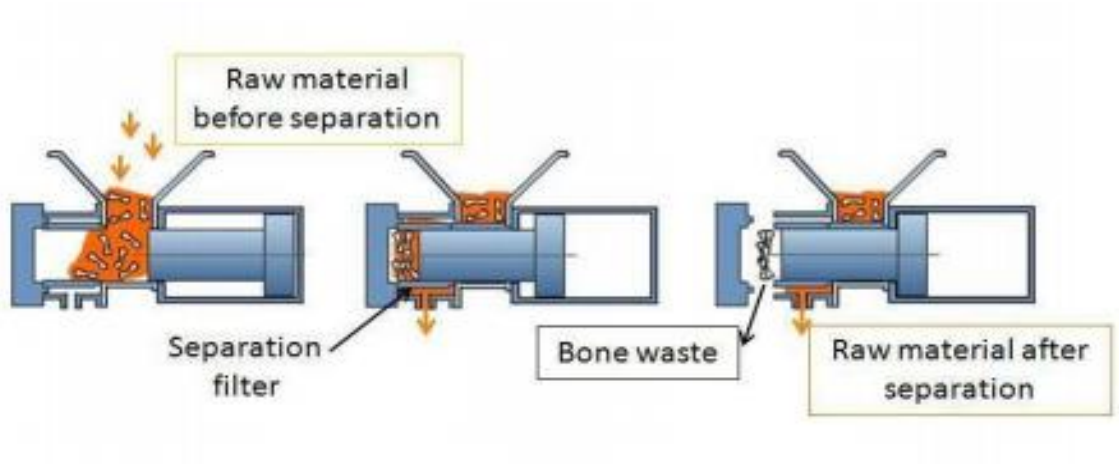


Figure 11. Linear meat deboner system. Source: EFSA (2013).

During the recovery process, under regulated safety conditions, the raw material (mainly poultry carcasses) is exposed to the action of different mechanisms at high pressure. Crushing and compressing of the initial material foster the mechanical separation, producing first mechanically recovered meat and afterward the bones, which are generally processed latter in the by-product industries.

Production

The total production of MDM in Europe is about 700,000 tons per year; in 2007, the high pressure MDM accounted for 77 % and low pressure MDM 23 %. Regarding to species used during the deboning processes, 88 % of MDM is derived from poultry, and 12 % from pigs. Information related to MDM production is quite limited due to the lack of cooperation from European countries to share their production numbers. Some member states do not have approved extractive installations. Nevertheless an estimated evaluation about production of MDM in European territory is presented in Table 4.

Table 4

Production of MDM during a period 2006-2007 adapted from European report on the future necessity and use of mechanically separated meat in the European Union, including the information policy towards consumers (Brussels, February 2010)

Types of MDM	Species reported				Total
	Poultry	Pigs	Rabbits	Not specified	
High pressure	255,867	13,574	0	200,564	470,005
Low pressure	87,347	18,827	0	30,979	137,153
Not specified	65,000	25,000	73	1,170	91,243
Total	408,214	57,401	73	232,713	698,401

Technological drawbacks associated with the MDM production

The increased fat content after extraction, contact with iron haem group of the internal parts of the bones (bone narrow) (Froning, 1981), the cellular disruption, aeration, microbiological development (Trindade, Contreras Castillo, & De Felício, 2006) and, if not adequately controlled, the temperature elevation during the mechanical efforts that are put bones in the extraction MDM (Yuste, Pla, Capellas, & Mor-Mur, 2002) makes this product a susceptible material to the development of lipid oxidation and general degradation.

In the literature there are several examples of research work regarding ways to minimize lipid oxidation in MDM and high fat content products. The oxygen partial exclusion by vacuum packaging (Jantawat & Dawson, 1980; M. K. Pettersen, 2004), the action of endogenous antioxidant from the enriched poultry diets (Cortinas et al., 2005; Sáyago-Ayerdi, Brenes, & Goñi, 2009; Tang, Kerry, Sheehan, & Buckley, 2002) and/or the addition of synthetic antioxidants (Ozer & Sariçoban, 2010) are some of the alternatives studied.

Regarding to the use of synthetic antioxidants, nowadays there are certain restrictions about those which were traditionally used in the food industry (butylated hydroxyanisole BHA; butylated hydroxytoluene BHT and *tert*-butyl hydroquinone TBHQ) for food applications due to their potential carcinogenetic effects (Juntachote, Berghofer, Bauer, & Siebenhandl, 2006). In this context, many efforts were evaluated in order to analyze the potential application of bioactive compounds extracted from vegetables sources in the prevention of the lipid oxidation in fatty food matrices like MDM.

Concerning "*natural*" food additives with preservatives properties certain vegetable and fruits sources were investigated: dried sea buckthorn (*Hippophae rhamnoides*) berry powder residues (Püssa et al., 2008); commercial rosemary antioxidants (Mielnik, Aaby, & Skrede, 2003); cranberry press cake (Raghavan & Richards, 2006); cranberry powder (Lee, Reed, & Richards, 2006): grape seed extract (Mielnik, Olsen, Vogt, Adeline, & Skrede, 2006); cocoa leaf extracts (Hassan, 2005); oil essential from some spices (sage, rosemary, thyme, oregano, and clove) (Viuda-Martos, Ruiz Navajas, Sánchez Zapata, Fernández-López, & Pérez-Álvarez, 2010); oils of marjoram (*Origanum marjorana* L.) and rosemary (*Rosmarinus officinalis* L.) (Mohamed & Mansour, 2012).

European requirements

In 2013 the European Commission conducted a study on the scientific opinion regarding to public health risks linked to MDM types from pork and poultry. In the same context, the establishment of objective measurement methods and values for parameters to distinguish MDM types and compare them with fresh meat, minced meat and meat preparations (non-MDM) was also proposed (EFSA, 2013).

Once the vast amount of published works were analyzed by European Commission in order to determine a parameter able to distinguish between high and lower pressure MDM, the calcium content was chosen as the most appropriated property. Table 5 summarizes the specific requirements regarding to MDM production and destiny.

Table 5
Specific requirements related to microbiological and calcium level in MDM according to the Official Journal of the European Union. European-Commission (2010)

Regulation	Microorganism	Sampling plan and Limits	Analytical reference method
Regulation (EC) Nº 2073/2005 Microbiological criteria for Food stuffs	*Salmonella*	n = 5, c = 0 Absence in 10 g	(EN/ISO 6579)
	Aerobic colony count	n = 5, c = 2 m = $5x10^5$ cfu/g M = $5x10^6$ cfu/g	(ISO 4833)
	E. coli	n = 5, c = 2 m = 50 cfu/g M = 500 cfu/g	(ISO 16649-1 or 2)
Regulation (EC) Nº 2074/2005 Maximum calcium content	Calcium content of MDM $\leq$ 0.1 % (100 mg/100 g or 1000 ppm) and determined by the standardized method is not considered significantly higher than that of minced meat.		(AOAC 983.19)

ISO: International Organization for Standardization. AOAC: Association of American Analytical Chemists.

Besides, there are European requirements related to the raw material with latter MDM recovery destiny. The meat can quickly deteriorate if the product is not

handled properly. According to European Commission (EFSA, 2013) there are different recommendations for the raw material for the low or high pressure MDM, summarized in Table 6 and Table 7.

Table 6
Hygiene requirements of raw materials for MDM according to (EC) Nº 853/2004 and 2074/2005. European-Commission (2010)

Raw material	**Low pressure MDM**	**High pressure MDM**
Poultry carcasses	Maximum 3 days old	Maximum 3 days old
Other raw material from on-site slaughterhouse	Maximum 7 days old	Maximum 7 days old
Other raw material from other site	Maximum 5 days old	Maximum 5 days old
Mechanical separation	Immediately after de-boning	If not immediately after deboning storage and transport at < 2 °C or freezing at < - 18 °C of the bones

Table 7

Hygiene requirements of MDM after production. European-Commission (2010)

	Low pressure MDM	**High pressure MDM**
Storage if not immediately used	Wrapped and packaged, chilling at max 2 °C or frozen at an internal T of < - 18 °C	Wrapped and packaged, chilling at max 2 °C if processed within 1 to 24 h; if not, frozen within 12 h after production, reaching at an internal T of < - 18 °C within 6 h. Maximal storage of frozen MDM of 3 months at < - 18 °C
Use	In meat preparations which are clearly not intended to be consumed without heat treatment; In meat products	Only for heat-treated meat products produced in approved establishments

Applications of mechanically deboned meat (MDM)

As mentioned in previous sections, there is a global trend regarding to meat consumption consisting in the replacement of the red meat (pig or beef) for “healthier white meat”, mainly poultry (Henchion et al., 2014). Therefore, the use of MDM, principally poultry meat, has increased in the food industry based on this consumption trend in industrialized countries and also due its lower price compared with other kinds of meat (Daros, Lucia Masson, & Amico, 2005). The main applications of MDM are in products which do not require a fibrous texture but demand emulsion, stability, natural color, and relatively low cost (Barbut, 2002).

If all the legal requirements described above for the production of MDM are properly complied and controlled, this product can be used as a satisfactory agent for binding structure in product prepared with minced meat and comminuted meat products namely, frankfurter sausages, meatballs, nuggets, and meat emulsions, including “chicken pate”.

The incorporation of MDM into emulsified meat product (10-35 %) and in lower proportions into nonemulsified meat product (1-20 %) has opened up additional markets for this type of meat (Mielnik, Aaby, Rolfsen, Ellekjær, & Nilsson, 2002). Most of these meat products, formulated primarily to suit the local palate, not only target the changing needs of consumers in terms of convenience, nutrition, quality and variety, but also allow a broad marketing of new alternatives (Guerrero-Legarreta, 2010). Therefore, many exotic recipes and ready-to-cook marinated products are presented as dietary convenient options not only for households holding a single individual but also for large family nucleus.

Overall aim

Once established the state-of-art concerning necessary fundamentals which indicate that implementation of GPE in order to reduce lipid oxidation of MDM still have not been explored, it is pretended as overall aim of the thesis:

To characterize skin and seed grape (pomace) extracts from Portuguese varieties towards the prevention of the lipid oxidation of mechanically deboned chicken meat (MDM), including the assessment of the final physico-chemical characteristics and consumer acceptance of nuggets containing MDM and supplemented with grape extracts.

References

Arts, I. C., Jacobs Jr, D. R., Gross, M., Harnack, L. J., & Folsom, A. R. (2002). Dietary catechins and cancer incidence among postmenopausal women: the Iowa Women's Health Study (United States). *Cancer Causes & Control, 13*(4), 373-382.

Atanacković, M., Petrović, A., Jović, S., Bukarica, L. G., Bursać, M., & Cvejić, J. (2012). Influence of winemaking techniques on the resveratrol content, total phenolic content and antioxidant potential of red wines. *Food Chemistry, 131*(2), 513-518.

Barbut, S. (2002). *Poultry Products Processing. An Industry Guide*: CRC Press.

Benouaret, R., Goujon, E., Trivella, A., Richard, C., Ledoigt, G., Joubert, J. M., Mery-Bernardon, A., & Goupil, P. (2014). Water extracts from winery by-products as tobacco defense inducers. *Ecotoxicology, 23*(8), 1574-1581.

Boussetta, N., & Vorobiev, E. (2014). Extraction of valuable biocompounds assisted by high voltage electrical discharges: A review. *Comptes Rendus Chimie, 17*(3), 197-203.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews, 56*(11), 317-333.

Castañeda-Ovando, A., Pacheco-Hernández, M. d. L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry, 113*(4), 859-871.

Cortinas, L., Barroeta, A., Villaverde, C., Galobart, J., Guardiola, F., & Baucells, M. (2005). Influence of the dietary polyunsaturation level on chicken meat quality: lipid oxidation. *Poultry Science, 84*(1), 48-55.

Chamorro, S., Viveros, A., Alvarez, I., Vega, E., & Brenes, A. (2012). Changes in polyphenol and polysaccharide content of grape seed extract and grape pomace after enzymatic treatment. *Food Chemistry, 133*(2), 308-314.

Daros, F. G., Lucia Masson, M., & Amico, S. C. (2005). The influence of the addition of mechanically deboned poultry meat on the rheological properties of sausage. *Journal of Food Engineering, 68*(2), 185-189.

Dougherty, P. H. (2012). *The geography of wine: regions, terroir and techniques*: Springer.

EFSA. (2013). Scientific Opinion on the public health risks related to mechanically separated meat (MSM) derived from poultry and swine. EFSA Journal (Vol. 11, pp. 1-78).

Eiras-Dias, J., Faustino, R., Clímaco, P., Fernandes, P., Cruz, A., Cunha, J., Veloso, M., & Castro, R. (2011). Catálogo das castas para vinho cultivadas em Portugal. *Instituto da Vinha e do Vinho, Lisboa, 1*.

El Darra, N., Grimi, N., Vorobiev, E., Louka, N., & Maroun, R. (2013). Extraction of polyphenols from red grape pomace assisted by pulsed ohmic heating. *Food and Bioprocess Technology, 6*(5), 1281-1289.

El Gharras, H. (2009). Polyphenols: food sources, properties and applications–a review. *International Journal of Food Science & Technology, 44*(12), 2512-2518.

En-Qin, X., Gui-Fang, D., Ya-Jun, G., & Hua-Bin, L. (2010). Biological Activities of Polyphenols from Grapes. *International Journal of Molecular Sciences, 11*(2), 622-646.

European-Commission. (2010). Communication from the Commission to the European Parliament and the Council on the future necessity and use of

mechanically separated meat in the European Union, including the information policy towards consumers. . Retrieved 2014 November, from http://ec.europa.eu/agriculture/index_en.htm

European-Commission. (2012). European Commission Agriculture and Rural Development. Retrieved 2014 October, from http://ec.europa.eu/agriculture/index_en.htm

EUROSTAT. (2012). European Union Statistical Office. Retrieved 2014 November, from http://ec.europa.eu/eurostat

FAO. (2013). Agribusiness Handbook, Grapes wines. Retrieved 2014 November, from http://www.fao.org/

FAOSTAT. (2012). FAO statistical database. Retrieved 2014 December, from http://faostat3.fao.org/browse/rankings/commodities_by_country/E

Fernández, K., Vega, M., & Aspé, E. (2015). An enzymatic extraction of proanthocyanidins from *País* grape seeds and skins. *Food Chemistry, 168*, 7-13.

Field, R. (1988). Mechanically separated meat, poultry and fish. *Advances in meat research (USA)*.

Field, R. (2004). Mechanically recovered meat. *Encyclopedia of Meat Sciences*.

Flamini, R., Mattivi, F., De Rosso, M., Arapitsas, P., & Bavaresco, L. (2013). Advanced Knowledge of Three Important Classes of Grape Phenolics: Anthocyanins, Stilbenes and Flavonols. *International Journal of Molecular Sciences, 14*(10), 19651-19669.

Fontana, A. R., Antoniolli, A., & Bottini, R. (2013). Grape Pomace as a Sustainable Source of Bioactive Compounds: Extraction, Characterization, and Biotechnological Applications of Phenolics. *Journal of Agriculture and Food Chemistry, 61*(38), 8987-9003.

Froning, G. W. (1981). Mechanical Deboning of Poultry and Fish (Vol. 27, pp. 109-147).

González-Paramás, A. M., Esteban-Ruano, S., Santos-Buelga, C., de Pascual-Teresa, S., & Rivas-Gonzalo, J. C. (2004). Flavanol content and antioxidant activity in winery byproducts. *Journal of Agriculture and Food Chemistry, 52*(2), 234-238.

Goupil, P., Benouaret, R., Charrier, O., Ter Halle, A., Richard, C., Eyheraguibel, B., Thiery, D., & Ledoigt, G. (2012). Grape marc extract acts as elicitor of plant defence responses. *Ecotoxicology, 21*(5), 1541-1549.

Guendez, R., Kallithraka, S., Makris, D. P., & Kefalas, P. (2005). Determination of low molecular weight polyphenolic constituents in grape (Vitis vinifera sp.) seed extracts: Correlation with antiradical activity. *Food Chemistry, 89*(1), 1-9.

Guerrero-Legarreta, I. (2010). *Handbook of Poultry Science and Technology: Volume 1: Primary processing*: John Wiley & Sons, Inc.

Gülçin, I. (2012). Antioxidant activity of food constituents: An overview. *Archives of Toxicology, 86*(3), 345-391.

Gülçin, İ. (2010). Antioxidant properties of resveratrol: A structure–activity insight. *Innovative Food Science & Emerging Technologies, 11*(1), 210-218.

Hagerman, A. E. (2002). *Tannin handbook* O. O. Miami University (Ed.) Retrieved from http://chemistry.muohio.edu/hagerman/index.php/handbook/15-handbook-intro

Haminiuk, C. W. I., Maciel, G. M., Plata-Oviedo, M. S. V., & Peralta, R. M. (2012). Phenolic compounds in fruits - an overview. *International Journal of Food Science and Technology, 47*(10), 2023-2044.

Hassan, O., Swet Fan, Lam. (2005). The anti-oxidation potential of polyphenol extract from cocoa leaves on mechanically deboned chicken meat (MDCM). *LWT - Food Science and Technology, 38*(4), 315-321.

Hawkesworth, S., Dangour, A. D., Johnston, D., Lock, K., Poole, N., Rushton, J., Uauy, R., & Waage, J. (2010). Feeding the world healthily: the challenge of measuring the effects of agriculture on health. *Philosophical transactions of the royal society B: biological sciences, 365*(1554), 3083-3097.

Henchion, M., McCarthy, M., Resconi, V. C., & Troy, D. (2014). Meat consumption: Trends and quality matters. *Meat Science, 98*(3), 561-568.

Huang, D., Boxin, O. U., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agriculture and Food Chemistry, 53*(6), 1841-1856.

Ignat, I., Volf, I., & Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry, 126*(4), 1821-1835.

INE. (2013). Instituto Nacional de Estatistica. Statistics Portugal. Retrieved 2014 December, from http://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_base_dados

IVDP. (2014). Instituto dos Vinhos do Douro e do Porto. Retrieved 2014 October, from http://www.ivdp.pt/

Jantawat, P., & Dawson, L. E. (1980). Effects of Air at Various Tension Levels on Storage Stability of Mechanically Deboned Poultry Meats. *Poultry Science, 59*(8), 1788-1794.

Juntachote, T., Berghofer, E., Bauer, F., & Siebenhandl, S. (2006). The application of response surface methodology to the production of phenolic extracts of lemon grass, galangal, holy basil and rosemary. *International Journal of Food Science and Technology, 41*(2), 121-133.

Kammerer, D., Claus, A., Schieber, A., & Carle, R. (2005). A Novel Process for the Recovery of Polyphenols from Grape (Vitis vinifera L.) Pomace. *Journal of Food Science, 70*(2), C157-C163.

Kearney, J. (2010). Food consumption trends and drivers. *Philosophical transactions of the royal society B: biological sciences, 365*(1554), 2793-2807.

Konczak, I., & Zhang, W. (2004). Anthocyanins—more than nature's colours. *BioMed Research International, 2004*(5), 239-240.

Lapornik, B., Prošek, M., & Golc Wondra, A. (2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. *Journal of Food Engineering, 71*(2), 214-222.

Laufenberg, G., Kunz, B., & Nystroem, M. (2003). Transformation of vegetable waste into value added products:: (A) the upgrading concept; (B) practical implementations. *Bioresource Technology, 87*(2), 167-198.

Lee, C., Reed, J. D., & Richards, M. P. (2006). Ability of various polyphenolic classes from cranberry to inhibit lipid oxidation in mechanically separated turkey and cooked ground pork. *Journal of Muscle Foods, 17*(3), 248-266.

Liyana-Pathirana, C. M., & Shahidi, F. (2006). Importance of insoluble-bound phenolics to antioxidant properties of wheat. *Journal of Agricultural and Food Chemistry, 54*(4), 1256-1264.

Lorrain, B., Ky, I., Pechamat, L., & Teissedre, P.-L. (2013). Evolution of Analysis of Polyhenols from Grapes, Wines, and Extracts. *Molecules, 18*(1), 1076-1100.

Lu, Y., & Yeap Foo, L. (1999). The polyphenol constituents of grape pomace. *Food Chemistry, 65*(1), 1-8.

Luque de Castro, M., & Priego-Capote, F. (2007). Ultrasound assistance to liquid–liquid extraction: a debatable analytical tool. *Analytica Chimica Acta, 583*(1), 2-9.

M. K. Pettersen, M. B. M., T. Eie, G. Skrede and A. Nilsson. (2004). Lipid Oxidation in Frozen, Mechanically Deboned Turkey Meat as Affected by Packaging Parameters and Storage Conditions.

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Anal Chim Acta, 613*(1), 1-19.

Makris, D. P., Boskou, G., & Andrikopoulos, N. K. (2007). Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. *Journal of Food Composition and Analysis, 20*(2), 125-132.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition, 79*(5), 727-747.

McGovern, P. E. (2013). *Ancient wine: the search for the origins of viniculture*: Princeton University Press.

Meagher, L. P., & Beecher, G. R. (2000). Assessment of data on the lignan content of foods. *Journal of Food Composition and Analysis, 13*(6), 935-947.

Mendes, J. A. S., Prozil, S. O., Evtuguin, D. V., & Lopes, L. P. C. (2013). Towards comprehensive utilization of winemaking residues: Characterization of grape skins from red grape pomaces of variety Touriga Nacional. *Industrial Crops and Products, 43*(0), 25-32.

Mielnik, M. B., Aaby, K., Rolfsen, K., Ellekjær, M. R., & Nilsson, A. (2002). Quality of comminuted sausages formulated from mechanically deboned poultry meat. *Meat Science, 61*(1), 73-84.

Mielnik, M. B., Aaby, K., & Skrede, G. (2003). Commercial antioxidants control lipid oxidation in mechanically deboned turkey meat. *Meat Science, 65*(3), 1147-1155.

Mielnik, M. B., Olsen, E., Vogt, G., Adeline, D., & Skrede, G. (2006). Grape seed extract as antioxidant in cooked, cold stored turkey meat. *LWT - Food Science and Technology, 39*(3), 191-198.

Mohamed, H. M. H., & Mansour, H. A. (2012). Incorporating essential oils of marjoram and rosemary in the formulation of beef patties manufactured with mechanically deboned poultry meat to improve the lipid stability and sensory attributes. *LWT - Food Science and Technology, 45*(1), 79-87.

Moreno-Arribas, M. V., Polo, M. C., & Carmen, M. (2009). *Wine chemistry and biochemistry* (Vol. 223): Springer.

Naczk, M., & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A, 1054*(1), 95-111.

NCC. (2007). National Chicken Council. Statistics and Research. Retrieved 2014 September, from http://www.nationalchickencouncil.org/

OIV. (2012). Organisation Internationale de la Vigne et du Vin. . Retrieved 2014 September, from http://www.oiv.int/oiv/info/es

OIV. (2014). Organisation Internationale de la Vigne et du Vin. Retrieved 2014 December, from http://www.oiv.int/oiv/info/es_OIV_Press_Conference_23_October_2014

Ozer, O., & Sariçoban, C. (2010). The effects of butylated hydroxyanisole, ascorbic acid, and α-tocopherol on some quality characteristics of mechanically deboned chicken patty during freeze storage. *Czech Journal of Food Sciences, 28*(2), 150-160.

Pinelo, M., Sineiro, J., & Núñez, M. a. J. (2006). Mass transfer during continuous solid–liquid extraction of antioxidants from grape byproducts. *Journal of Food Engineering, 77*(1), 57-63.

Ping, L., Pizzi, A., Guo, Z. D., & Brosse, N. (2011). Condensed tannins extraction from grape pomace: Characterization and utilization as wood adhesives for wood particleboard. *Industrial Crops and Products, 34*(1), 907-914.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agriculture and Food Chemistry, 53*(10), 4290-4302.

Prozil, S. O., Evtuguin, D. V., & Lopes, L. P. C. (2012). Chemical composition of grape stalks of Vitis vinifera L. from red grape pomaces. *Industrial Crops and Products, 35*(1), 178-184.

Püssa, T., Pällin, R., Raudsepp, P., Soidla, R., & Rei, M. (2008). Inhibition of lipid oxidation and dynamics of polyphenol content in mechanically deboned meat supplemented with sea buckthorn (Hippophae rhamnoides) berry residues. *Food Chemistry, 107*(2), 714-721.

Raghavan, S., & Richards, M. P. (2006). Partitioning and inhibition of lipid oxidation in mechanically separated turkey by components of cranberry press cake. *Journal of Agriculture and Food Chemistry, 54*(17), 6403-6408.

Reeve, J. R., Carpenter-Boggs, L., Reganold, J. P., York, A. L., & Brinton, W. F. (2010). Influence of biodynamic preparations on compost development and resultant compost extracts on wheat seedling growth. *Bioresource Technology, 101*(14), 5658-5666.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*(7), 933-956.

Rockenbach, I. I., Jungfer, E., Ritter, C., Santiago-Schübel, B., Thiele, B., Fett, R., & Galensa, R. (2012). Characterization of flavan-3-ols in seeds of grape pomace by CE, HPLC-DAD-MS^n and LC-ESI-FTICR-MS. *Food Research International, 48*(2), 848-855.

Sáyago-Ayerdi, S. G., Brenes, A., & Goñi, I. (2009). Effect of grape antioxidant dietary fiber on the lipid oxidation of raw and cooked chicken hamburgers. *LWT - Food Science and Technology, 42*(5), 971-976.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., & Jiménez, L. (2005). Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition, 45*(4), 287-306.

Schieber, A., Stintzing, F. C., & Carle, R. (2001). By-products of plant food processing as a source of functional compounds — recent developments. *Trends in Food Science & Technology, 12*(11), 401-413.

Schlesier, K., Harwat, M., Böhm, V., & Bitsch, R. (2002). Assessment of antioxidant activity by using different in vitro methods. *Free Radical Research, 36*(2), 177-187.

Shipp, J., & Abdel-Aal, E.-S. M. (2010). Food applications and physiological effects of anthocyanins as functional food ingredients. *The Open Food Science Journal, 4*, 7-22.

Tang, S. Z., Kerry, J. P., Sheehan, D., & Buckley, D. J. (2002). Antioxidative mechanisms of tea catechins in chicken meat systems. *Food Chemistry, 76*(1), 45-51.

Trindade, M. A., Contreras Castillo, C. J., & De Felício, P. E. (2006). Mortadella sausage formulations with mechanically separated layer hen meat preblended with antioxidants. *Scientia Agricola, 63*(3), 240-245.

Vermerris, W., & Nicholson, R. (2006). Families of phenolic compounds and means of classification *Phenolic Compound Biochemistry* (pp. 1-34): Springer.

Viuda-Martos, M., Ruiz Navajas, Y., Sánchez Zapata, E., Fernández-López, J., & Pérez-Álvarez, J. A. (2010). Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. *Flavour and Fragrance Journal, 25*(1), 13-19.

Wang, L., & Weller, C. L. (2006). Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science & Technology, 17*(6), 300-312.

Wells, M. J. (2003). Principles of extraction and the extraction of semivolatile organics from liquids. *Sample Preparation Techniques in Analytical Chemistry*, 37.

Wijngaard, H., Hossain, M. B., Rai, D. K., & Brunton, N. (2012). Techniques to extract bioactive compounds from food by-products of plant origin. *Food Research International, 46*(2), 505-513.

Yu, J. M., & Ahmedna, M. (2013). Functional components of grape pomace: their composition, biological properties and potential applications. *International Journal of Food Science and Technology, 48*(2), 221-237.

Yuste, J., Pla, R., Capellas, M., & Mor-Mur, M. (2002). Application of high-pressure processing and nisin to mechanically recovered poultry meat for microbial decontamination. *Food Control, 13*(6), 451-455.

2.

General materials and methods

2.1. Introduction

In the present chapter, reagents, standards and solutions used throughout all the experiments are described. Furthermore, aspects regarding to general steps for the sample preparation and latter analysis, are also explained.

2.1.1. Reagents, standards and samples

Analytical grade reagents were purchased from different suppliers and used for all experiments. They were stored according to the supplier specifications. For the preparation of all solutions, water from Sartorius AG system (resistivity > 18 MΩ cm) and absolute ethanol pro analysis (p.a.) were used throughout the work. Standard stock solutions were prepared by rigorous weighing the respective reagent in a Mettler Toledo analytical balance (model AG 285), followed by dissolution in the appropriate solvent (water, buffer solution, ethanol, acetone, or mixture of the previous solvents). All working solutions were freshly obtained through rigorous dilution of standard stock solutions with Gilson micropipettes with disposable tips, using volumetric flasks (class A) of different volumes. Micropipettes were regularly calibrated with deionized water. The pH of all buffer solutions was adjusted using a glass pH electrode (Crison 52-02).

Red grape pomace was kindly supplied by a local wine farm, located in Gouvinhas, Sabrosa, Portugal. Approximately 2 kg of each grape variety, including skins, peels and stalks or stems, was separated after the last alcoholic fermentation step and packaged into dark polyethylene bags in smaller portions. Grape pomace was frozen and transported to laboratory till extraction procedure. Grape pomace extracts **(Papers I to V)** were obtained according to the general scheme in Figure 14. The extraction procedure was performed based on previous work by Shirahigue *et al*. (Shirahigue et al., 2010). For MDM experiments **(Papers II to V)**, meat samples were fully homogenised in food processor (KenWood) before further processing.

In the other hand, mechanically deboned chicken meat (MDM) samples were supplied by a poultry industry, located in São Pedro do Sul, Viseu, Portugal. MDM samples were obtained from refrigerated carcasses (backs and chests)

from males and females poultries belonging to the same batch (each year), slaughtered the same day of sample collection. Stork Proteton was the deboner equipment used during the meat recovering processes. After MDM separation, samples of approximately 2.5 – 3.0 kg were vacuum packaged in the poultry industry. Finally, meat samples were transported under refrigerated conditions till laboratory in which they were storage under frozen conditions (- 23.0 °C ± 1°C) till experiences development.

Figure 12. General scheme for preparation of grape pomace extracts (from top left to right: grape skins and seeds (grape pomace); drying step; extraction step under orbital agitation, vacuum filtration step, concentration step in a rotary evaporator, final grape pomace extracts re-suspended in water).

Meat extracts from MDM used in **Papers II to IV** were obtained according to the methodology described by Qwele *et al*. (Qwele et al., 2013) with some modifications. Briefly, 1 g of each MDM sample was rigorously weighed into a 15 mL Falcon tube where 10 mL of 0.05 M phosphate buffer (pH 7.0) was added. The extraction steps consisted in alternating ultra sound (30 s, 3 times) and vortex cycles (2 min, 3 times, 3,000 rpm). Before the last cycle, samples

were left to stand 10 min in order to improve the tissue hydration and the consequent extraction. After that, samples were centrifuged at 5,580 × g for 30 min at 4 °C and the supernatant was displayed in Eppendorf tubes with one drop of concentrated HCl (38% w/w).

For nuggets elaboration **(Paper V)**, all food ingredients were purchased at local supermarket. Before use or quantifications, all grape pomace and/or meat extracts were rigorously diluted with Gilson micropipettes with disposable tips, using the appropriate solvent.

2.1.2. Spectrophotometric measurements

In the present work, several methodologies for antioxidant capacity assessment, namely Folin-Ciocalteu, DPPH$^{\bullet}$, ORAC and ICA assays were performed for both grape pomace extracts (in ethanol/water solvent and in aqueous suspension) **(Papers I to V**, including ethanolic extracts only in **Paper I)** and for meat extracts **(Papers II to IV)**. Additionally, the evaluation of the haem iron content (HIC) was also performed for meat extracts **(Papers III and IV)**. All spectrophotometric procedures were performed using a microplate reader, model Synergy HT, from Bio-Tek Instruments Inc. where diluted standards and samples were displayed in disposable 96 wells microplates (Orange Scientific) with exception of the HIC protocol, which was performed in a conventional spectrophotometer (Jasco V-660 Spectrophotometer), using acid acetone as blank. Room temperature (25.0 °C ± 0.1°C) was kept for all measurements, with exception of the ORAC assay, which was performed at 37 °C, condition required for the thermal decomposition of AAPH in 75 mM phosphate buffer, pH 7.4. Further specifications for all assays were previously mentioned in **Introduction section**.

2.1.2.1. Folin-Ciocalteu assay

The Folin-Ciocalteu assay (Magalhães, Santos, Segundo, Reis, & Lima, 2010; Singleton, Orthofer, & Lamuela-Raventos, 1999) was performed to assess the total phenolic content (TPC) in the case of grape pomace extracts, and the Folin-Ciocalteu reducing substances in the case of meat extracts. This assay is conventionally used for the total phenolic content measurement and it is based

on the ability of certain substances in alkaline medium to reduce the phosphomolybdic / phosphotungstic acid reagent to complexes spectrophotometrically detected at 760 nm.

2.1.2.2. DPPH• assay

In the DPPH• method, the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) is reduced due to the presence of antioxidant compounds causing the decrease in the absorbance values monitored at 517 nm (Brand-Williams, Cuvelier, & Berset, 1995; Magalhães, Barreiros, Maia, Reis, & Segundo, 2012).

2.1.2.3. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002; Wang, Jónsdóttir, & Ólafsdóttir, 2009) is based on the evaluation of the protection conferred by certain antioxidants to probe fluorescence (fluorescein) thus preventing the decay of its fluorescent intensity along time. The area under the curve (AUC), obtained after integrating the relative fluorescence curve over the reaction time, is used to evaluate the antioxidant protection. In the present work the fluorimetric measurements were performed using a tungsten halogen lamp registering the fluorescence at excitation and emission of 485 and 525 nm, respectively.

2.1.2.4. Iron(II) chelating ability (ICA) assay

The ICA assay is used to evaluate the presence of compounds in the sample that are able to disrupt the complex formed between iron(II) and ferrozine. The colour decreasing in the iron(II)-ferrozine complex was monitored al 562 nm. The procedure was carried out according to Wang *et al*. (Wang et al., 2009).

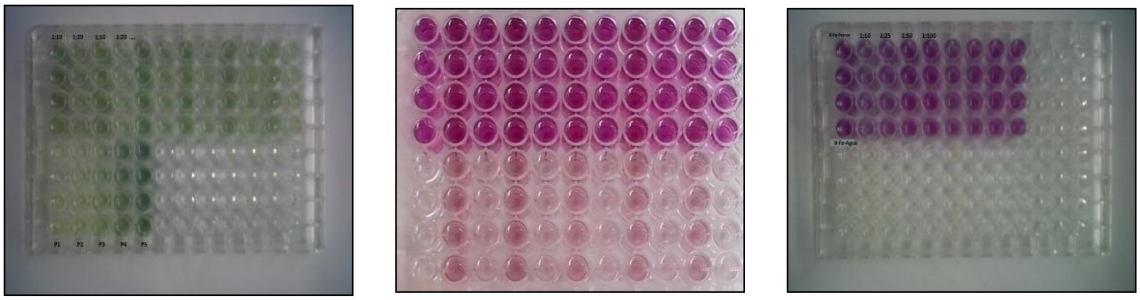


Figure 13. Examples of microplates used for Folin-Ciocalteu, DPPH$^{\bullet}$ and ICA assay (from left to right).

2.1.2.5. Haem iron content (HIC)

For HIC assay, minced meat samples (2 g) were mixed with 9 mL of acidified acetone and macerated using a glass rod to be left during 1 h at room temperature in order to extract the total pigments. Afterwards, centrifugation (2,200 × g, 10 min) and filtration steps, the filtrate absorbance at 640 nm was measured. Results were expressed as HIC (µg g meat^{-1}) = (A_{640} x 680) x 8.82 / 100 (Clark, Mahoney, & Carpenter, 1997).

2.1.3. Color and pH determination

For color assessment in **Papers III to V**, CIELab space color, including C standard illuminant and 2 ° as observer, were used. A CR-400 spectro-colorimeter (Konica Minolta Sensing) was used and *L** (luminosity), *a** (redness), *b** (yellowness), chroma and Hue angle as color variables, were determined. Once samples packaging was opened, meat was left to stand 30 min at room temperature and in contact to the air before the color measurement. For nuggets samples, after pre-frying step when pieces reached the room temperature, they were longitudinally cut and the internal color was registered on both sides of the product. Concerning pH determination **(Papers III to V)**, it was carried out according to Ozer and Sariçoban (Ozer & Sariçoban, 2010), by weighing 10 g of the samples, homogenized in 10 mL of water for 1 min using a blender. Then, pH was measured using a glass electrode (Hanna Instruments) with magnetic agitation and adjustment by temperature.

References

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology, 28*(1), 25-30.

Clark, E. M., Mahoney, A. W., & Carpenter, C. E. (1997). Heme and total iron in ready-to-eat chicken. *Journal of Agriculture and Food Chemistry, 45*(1), 124-126.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *Journal of Agriculture and Food Chemistry, 50*(16), 4437-4444.

Magalhães, L. M., Barreiros, L., Maia, M. A., Reis, S., & Segundo, M. A. (2012). Rapid assessment of endpoint antioxidant capacity of red wines through microchemical methods using a kinetic matching approach. *Talanta, 97*, 473-483.

Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S., & Lima, J. L. (2010). Rapid microplate high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity. *Talanta, 83*(2), 441-447.

Ozer, O., & Sariçoban, C. (2010). The effects of butylated hydroxyanisole, ascorbic acid, and α-tocopherol on some quality characteristics of mechanically deboned chicken patty during freeze storage. *Czech Journal of Food Sciences, 28*(2), 150-160.

Qwele, K., Hugo, A., Oyedemi, S. O., Moyo, B., Masika, P. J., & Muchenje, V. (2013). Chemical composition, fatty acid content and antioxidant potential of meat from goats supplemented with Moringa (Moringa oleifera) leaves, sunflower cake and grass hay. *Meat Science, 93*(3), 455-462.

Shirahigue, L. D., Plata-Oviedo, M., de Alencar, S. M., d'Arce, M. A. B. R., de Souza Vieira, T. M. F., Oldoni, T. L. C., & Contreras-Castillo, C. J. (2010). Wine industry residue as antioxidant in cooked chicken meat. *International Journal of Food Science & Technology, 45*(5), 863-870.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods In Enzymology, 299*, 152-178.

Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chemistry, 116*(1), 240-248.

3.

Results and discussion

Paper I

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Luísa Barreiros, Jorge Queiroz, Luís M. Cunha. Valorization of grape pomace: extraction of bioactive phenolics with antioxidant properties. [Submitted for publication].

Valorization of grape pomace: extraction of bioactive phenolics with antioxidant properties

Hernán H. Tournour[1,2], Marcela A. Segundo[3], Luís M. Magalhães[3], Luisa Barreiros[3], Jorge Queiroz[1], Luís M. Cunha[1,*]

[1] REQUIMTE/DGAOT Faculdade de Ciências, Universidade do Porto, Rua Padre Armando Quintas, 7, 4486-661 Vairão, Portugal
[2]Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

[3] UCIBIO, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

**Corresponding author: lmcunha@fc.up.pt*
Tel.: (+351)252660400 / Fax: (+(351)252661780

ABSTRACT:

Grape pomace can be regarded as an excellent and affordable source of polyphenolic compounds. Hence, the main objective of this work was to conduct a comparative study of different Portuguese grape varieties, using an extraction methodology with possible applications in sustainable agriculture and pest management. Scavenging capacity against DPPH$^{\bullet}$, oxygen radical absorbance capacity (ORAC), iron(II) chelating ability (ICA) and Folin-Ciocalteu assays were performed in order to evaluate the antioxidant capacity profile and total phenolic content (TPC) in ethanol/water extracts and aqueous grape pomace suspensions. Strong significant correlation between TPC and DPPH$^{\bullet}$ (R = 0.944), and a low correlation between ORAC and the other assays was obtained ($R \leq 0.632$). ICA was not correlated with any of the other assays ($R \leq$ 0.263). All grape pomace extracts have presented high antioxidant properties (ORAC) and chelating ability, ranging from 906 to 2337 μmol TE g^{-1} residue and from 55 to 104 % inhib. mg^{-1} residue, respectively. Results from HPLC analysis showed the presence of gallic acid, caffeic acid, syringic acid, (+)-catechin and (-)-epicatechin being syringic acid and (+)-catechin the major compounds. Although further studies are required, "*Touriga Nacional*" was the most promising grape variety regarding its highest values for TPC (142.4 ± 1.1 mg

GAE g^{-1} dry residue), $DPPH^{\bullet}$ (1.12 ± 0.04 mmol TE g^{-1} dry residue) and ORAC (1579 ± 244 μmol TE g^{-1} dry residue) assays. Since Portugal is a major wine producer, utilization of pomace generated during the wine elaboration steps opens a new trend towards compounds extraction with high antioxidant activity in order to contribute to emerging industrial applications and sustainable agriculture.

Keywords: antioxidant capacity, food products, Portuguese varieties, red grape pomace, environmental-friendly extraction.

Research highlights

- Grape pomace from Portuguese varieties was targeted as source of polyphenolic compounds.
- An environmentally friendly extract with polyphenols was obtained.
- Extracts showed bioactive properties in radical scavenging and iron(II) chelating assays.
- *"Touriga Nacional" grape variety* showed the highest potential for industrial applications.

1. Introduction

Portugal is now the eighteenth largest global grape producer, with a production close to 839,000 million tons (FAOSTAT, 2012). Winemaking process generates significant amount of wastes (steams, seeds, skins and marcs). It is estimated that for each 6 liters of wine, 1 kg of grape pomace is produced which is mainly destined to animal feed and for compost elaboration (Mendes, Prozil, Evtuguin, & Lopes, 2013). Nevertheless, large amounts of the residual quantities of bioactive substances are maintained into the vegetable tissues (Lapornik, Prošek, & Golc Wondra, 2005). Due to the chemical composition in the final grape waste (high content of sugars, tannins, polyphenols, polyalcohols, pectins and lipids), effluent treatment considerably increase in the chemical oxygen demand (COD) and the biochemical oxygen demand (BOD_5). Considering the above, for the industrial sector this situation represents a low cost source of usable polyphenolic compounds. Furthermore, considering the current governmental and legislative pressures, this fact tends to eliminate or reduce the costs associated with effluent treatments.

In this context, phenolic compounds present in grape pomace represent bioactive substances with many applications related to healthy benefits: scavenging activity against free radicals, anti-inflammatory properties (Terra et al., 2007), anti-proliferation and cancer therapy (Nandakumar, Singh, & Katiyar, 2008). A literature search revealed that the number of publications on the key words: “antioxidant” and “grape pomace” has strongly increased in the last ten years emphasizing the growing interest in the topic. Currently the available information on the polyphenolic compounds from Portuguese grape pomace is limited. Most of the published work are focused on the composition and antioxidant activity of Portuguese grape (whole or parts of the grape) (Cosme, Ricardo-Da-Silva, & Laureano, 2009; Dopico-García et al., 2008; Matias et al., 2010; Paixao, Perestrelo, Marques, & Camara, 2007) others have centered their work on the pomace chemical composition (Mendes et al., 2013; Prozil, Evtuguin, & Lopes, 2012), but mainly connected to wines (Baptista, Tavares, & Carvalho, 2001; Jordao et al., 2010; María Monagas, Gómez-Cordovés, Bartolomé, Laureano, & Ricardo da Silva, 2003).

Grape pomace extracts represent widespread uses in pharmaceutical, cosmetic, and the most recent is linked to the new class of “phytosanitary bioproducts” able to control the incidence of diseases in some crops (Benouaret et al., 2014), Thus, data concerning the polyphenolic content and antioxidant capacity from Portuguese grape pomaces aiming its valorization, are still scarce. Hence, the present study aims to determine the antioxidant profile and the total phenolic content of Portuguese grape pomace extracts. When dealing with complex matrixes coming from vegetables tissues, in order to appropriately assess the antioxidant capacity, experiments should be done through more than one assay, and at least two methods have been recommended (De Nisco et al., 2013). Considering this, two antioxidant assays (DPPH$^{\bullet}$ and ORAC) were established. Moreover, the ability of grape pomace extracts to chelate iron(II) was also performed. Since, extraction is a primordial step because it influences the further application of the phenolic compounds, ethanol was chosen as extractive liquid. Firstly, it is the natural solvent present in wines (Spigno, Tramelli, & De Faveri, 2007), it is safe (Shi et al., 2005), due to its relatively low boiling point (volatile) which facilities the elimination and recovering steps, and finally it has an environmentally friendly behavior (Corrales, García, Butz, & Tauscher, 2009), compared to other organic solvents, namely methanol. We also compared the final antioxidant properties in ethanol/water extracts and aqueous re-suspensions of their dry residue in order to enhance the approach towards the grape pomace emerging applications (sustainable pest management, biopesticides and controlled animal diet). It is also intended to select the Portuguese grape variety under study that would be most suitable for future polyphenolic extraction processing towards bioactive products recovery. To our knowledge, this paper represents one of the few attempts to assess the polyphenolic content and the antioxidant profile of the pomace coming from the most representative red grape varieties in Douro, Portugal with perspectives towards a sustainable agriculture and an environmentally friendly pest management approach.

2. Material and methods

2.1. Chemicals

All chemicals used were of analytical reagent grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulfonic acid sodium salt (ferrozine) and 2,2-azobis(2-methylpropionamide) dihydrochloride (AAPH) were purchased for Aldrich (Milwaukee, WI). Folin-Ciocalteu (F-C) reagent and fluorescein sodium salt were obtained from Sigma (St. Louis, MO), while iron(II) chloride tetrahydrate, gallic acid, and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were obtained from Fluka (Buchs, Switzerland). HPLC standards (Gallic, *p*-hydroxybenzoic, caffeic, syringic, *p*-coumaric and o-coumaric, sinapic, ferulic acids employed for Phenolic Acids *(PA method),* whilst (+)-Catechin, (-)-Epicatechin, (-)-Epicatechin gallate, *trans*-resveratrol, quercetin, kaempferol and chlorogenic acid, used for Anthoxanthins and Stilbenes (*AX method*), were purchased from Sigma. Water from Sartorius AG system (resistivity > 18 MΩ cm) (Göttingen, Deutschland) and absolute ethanol p. a. (Panreac Química, Spain) were used in the preparation of all solutions.

2.2. Solutions

For assessment of total phenolic content (TPC), the commercial F-C reagent was diluted 3:10 (v/v) in water. A solution of $Na_2CO_3 \cdot 10\ H_2O$ 24.3% (w/v) was prepared, corresponding to 9% (w/v) of sodium carbonate, and also gallic acid standard solutions (1.0 - 15.0 mg L^{-1}) for calibration purposes. For the DPPH$^{\bullet}$ assay, a stock solution of DPPH$^{\bullet}$ in ethanol (600 μM) was prepared and kept in dark at room temperature. Three dilutions from the stock DPPH$^{\bullet}$ solution (between 75 and 225 μM) were prepared in ethanolic solution 50% (v/v) in order to determine the dilution factor needed to provide an absorbance value of 0.900 ± 0.020 at 517 nm, after dilution in the microplate well. For DPPH$^{\bullet}$ assay, all Trolox standard solutions (5.0 - 50.0 μM) were prepared in ethanolic solution 50% (v/v). For iron(II) chelating ability (ICA) assay, all iron(II) solutions were freshly prepared including the stock solution (6 mM) at pH 3.0 and the iron(II) solution (0.12 mM) added to microplate. The ferrozine solution (0.6 mM) and a solution of acetate buffer (50 mM) were also prepared. For oxygen radical absorbance capacity (ORAC) assay, AAPH (40 mM) and fluorescein stock

155 solutions (0.5 mM) were prepared in a 75 mM phosphate buffer (pH 7.4). Stock
156 standard solutions for HPLC analysis, were prepared accurately weighing each
157 compound and by dissolving them in an appropriate solvent (ethanol or water)
158 to a final concentration of 1000 mg L^{-1}. Working solutions were prepared from
159 stock standard solutions in mobile phase (5 and 2.5 mg L^{-1}; 15 standards
160 mixture). HPLC grade acetic acid and acetonitrile (Aldrich, Milwaukee, WI) were
161 used. All solutions were filtered through a 0.45 µm membrane and degassed in
162 ultrasound.
163
164 2.3. *Red grape pomace samples*
165 Douro´s region is located in the northeast area of Portugal and classified by
166 UNESCO as World Heritage. Red grape pomace was collected in a wine farm
167 situated at average altitude of 150 m, coordinates 41º 10' 10" North latitude and
168 7º 38' 14" West longitude. Grapes were harvested upon ripening in 2012
169 vintage. The present research includes three autochthonous red grape varieties
170 (*Vitis vinifera* L. grape variety): 1) *"Touriga Nacional"* (TNac) among red grape
171 varieties studied is one the noblest, its bunch has tiny berries, rounded, non-
172 uniform size, with blue-black skin coated strong bloom, while the pulp is stiff and
173 not colored; 2) *"Touriga Franca"* (TF) is a very qualitative variety and the most
174 planted grape variety in the Douro and Trás-os-Montes regions (Portugal); 3)
175 *"Tinta Roriz"* (TR) (Syn. *"Tempranillo"* in Spain; Syn. *"Aragonéz"* in South of
176 Portugal), is the most important grape variety planted in all the Iberia Peninsula
177 and is used in the production of quality wines. A mixture (Mix) composed of
178 1:1:1 proportion of each variety was also analyzed.
179
180 *2.4. Preparation of the grape pomace extracts (GPE)*
181 Seeds and skins of red grape and a given amount of stems (5-6% of the whole
182 bunch that comprised the pomace) from the above grape varieties were
183 obtained after the last alcoholic fermentation step, packaged into a dark-
184 polyethylene-bag, labeled, frozen immediately and transported to the laboratory.
185 Samples were defrosted at room temperature prior efficiently mixture to
186 guarantee a representative proportion of seeds and skins. Most of the stems
187 were removed manually. Then, a portion of each variety (500 g) was placed on

188 a tray and dried in an oven (Thermo Scientific ^{TM}, Pittsburgh PA). Oven
189 operating conditions were 55 ºC with no forced air.
190 The final point of the drying was assessed by sampling and evaluating the
191 moisture content by weighing differences till reaching less than 5% (w/w) (in
192 triplicate). Finally, dried material was stored in dark-packaged polyethylene at -
193 18 ºC and grinding in the following day, was performed. A grinder for grains
194 (food processor, KenWood, New Lane, UK) was applied to provide a particle
195 size of 2-3 mm within intervals of a few seconds to prevent thermal stress of the
196 material. The entire procedure was performed protecting the material from the
197 light. The finely ground material was vacuum packaged in an oxygen barrier
198 bag (Vacuum Packaging Machine, Sammic, Guipúzcoa Spain) covered with foil
199 and stored at -18 ºC until further use.
200 Extraction was carried out as described by Shirahigue *et al*. (Shirahigue et al.,
201 2010) with a few modifications (schematic representation shown in Figure 1).
202 Briefly, grinded grape pomace (20 g) was thawed and placed on a glass flask
203 where 80% (v/v) of ethanol/water mixture (100 ml) was added. Next, the mixture
204 was placed under orbital agitation at 300 rpm for 48 h, at room temperature and
205 in darkness. The liquid phase was then separated from the solid by vacuum
206 filtration through a glass filter and a 45 µm Millipore (Billerica, MA)
207 polyvinylidene fluoride (PVDF) membrane filter. The filtrate was placed on a 100
208 ml amber glass volumetric flask and the volume was completed to 100 ml with
209 ethanol/water solvent. Samples were taken before concentration step to assess
210 the TPC, antioxidant capacity and iron(II) chelating ability (ICA). Finally, the
211 liquid was concentrated in a vacuum rotary evaporator (Büchi, Flawil,
212 Switzerland) at 65 ºC aided with a nitrogen stream, until dryness. The dry
213 residue obtained was weighed and redissolved in 50 ml of water, separated in
214 smaller portions and reserved in an ultra high deep freezer at -80 ºC in amber
215 recipients until further analysis. Extraction, filtration, concentration, weighing
216 and re-dissolution steps were performed in duplicate (n = 2) for each grape
217 variety.
218
219 *2.5. Determination of dry weight*

Dry weight in red grape pomace was assessed by drying a sample (5 g) of grinded residue at 98-100 ºC until a constant weight was achieved and then the dried residue was transferred to desiccators for cooling (AOAC, 2002).

2.6. Equipment

All antioxidant assays were performed in a microplate format (Synergy HT, Bio-Tek Instruments, Winooski, VT) using spectrophotometry or fluorimetry as detection system. The microplate reader was controlled by Gen5 software (Bio-Tek Instruments). ORAC assay was carried out at 37 ºC, while the other three assays were carried out at room temperature. All samples were analyzed in quadruplicate (or triplicate in ORAC assay) using at least two dilution factors.

2.7. Total phenolic content (TPC)

The TPC was assessed employing a 96-well microplate Folin-Ciocalteu procedure, with carbonate buffer as alkaline reagent (Luís M Magalhães, Santos, Segundo, Reis, & Lima, 2010; Singleton, Orthofer, & Lamuela-Raventos, 1999). Hence, 150 µl of gallic acid standard solution (1.0 - 15.0 mg L^{-1}) or diluted red grape pomace extracts (1:200 v/v) and 50 µl of F-C reagent (3:10, v/v) were placed in each well. After that, 100 µl of carbonate solution (9%, w/v) was added. The reduction at alkaline pH of phosphotungstate-phosphomolybdate complexes was monitored at 760 nm during 120 min. The reagent blank was performed by the addition of 150 µl of water instead of sample. The TPC, expressed as mg of gallic acid equivalents per gram of dry residue (obtained from the solid material after the concentration step) was calculated by interpolation of absorbance values after 120 min of reaction in the gallic acid standard curve ($Abs_{760\ nm}$ = 0.0510 × [gallic acid, (mg L^{-1})] + 0.065, R>0.9996).

2.8. Radical scavenging assessment

2.8.1. DPPH• assay

For microplate DPPH• methodology (Brand-Williams, Cuvelier, & Berset, 1995; L. M. Magalhães, Barreiros, Maia, Reis, & Segundo, 2012), 150 µl of Trolox standard solution (5.0 - 50.0 µM) or diluted red grape pomace extracts (1:400 v/v) and 150 µl of DPPH• ethanolic solution (50%, v/v) were placed in each well.

The DPPH$^{\bullet}$ scavenging capacity was monitored at 517 nm during 120 min. The absorbance of DPPH$^{\bullet}$ in the absence of antioxidant species (control) was monitored after the addition of 150 µl of ethanolic solution (50%, v/v) instead of standard solution, in order to evaluate the stability of the radical upon reaction time. To evaluate the intrinsic absorption of samples, 150 µl of ethanolic solution (50%, v/v) was added to 150 µl of sample. The net absorbance, calculated by the difference of DPPH$^{\bullet}$ absorbance in the absence and in the presence of sample after 120 min, was calculated. Results were expressed as mmol of Trolox equivalent (TE) per gram of dry residue by interpolation in Trolox standard curve ($\Delta Abs_{517\,nm}$ = 7.40 × [Trolox, (mM)] + 0.028, R>0.9957).

2.8.2. ORAC assay

The oxygen radical absorbance capacity (ORAC) assay is based on the scavenging of peroxyl radicals generated AAPH, which prevents the degradation of the fluorescein probe and, consequently, prevents the loss of fluorescence. For ORAC assay (Dejian Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002; Wang, Jónsdóttir, & Ólafsdóttir, 2009), 100 µl of Trolox standard solution (1.0 – 7.5 µM) or diluted red grape pomace extracts (1:600, 1:800, 1:1000, 1:1200 and 1:1500 v/v) and 100 µl of fluorescein (117 nM) were placed in each well, and the microplate was brought to preincubation for 15 min at 37 ºC. Following this, 100 µl of AAPH solution (40 mM) was added and the fluorescence intensity (λ_{exc} 485 nm, λ_{em} 520 nm) was monitored every minute during 240 min. The reaction milieu was 75 mM phosphate buffer (pH 7.4) at 37 ºC. Control signal profile (absence of sample) was assessed by adding 100 µl of buffer solution instead of sample. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve over the reaction time. The net AUC of the sample was calculated by subtracting this value to the AUC of the control (absence of sample). The regression equation between net AUC and Trolox concentration was determined, and the results were expressed as µmol of Trolox equivalents (TE) per gram of residue by interpolation (Net AUC (%) = 10.6 × [Trolox, (µM)] + 10.5, R>0.9998). Results for ORAC assay were also expressed as µmol of Trolox equivalents per gram of dry pomace, by multiplying the previous value by the ratio between the mass of

dry residue and the mass of initial dry pomace, all expressed as μmol Trolox equivalents (TE) per gram.

2.9. Iron(II) chelating ability assay (ICA)

For iron(II) chelating ability assay (ICA) (Wang et al., 2009), 100 μl of diluted red grape pomace extracts (1:10, 1:25, 1:50 and 1:100, v/v) in acetate buffer (50 mM, pH 4.6) were mixed with 100 μl $FeCl_2 \cdot 4H_2O$ (120 μM) and placed in each well. After 5 min, 100 μl of ferrozine solution (600 μM) was added to each well. Solutions were left standing 10 min at room temperature, after which the absorbance was measured at 562 nm. Control assay was performed by adding 100 μl of water instead of sample, while the blank of the sample was performed by adding 100 μl of water instead of ferrozine solution. The percentage of inhibition of ferrozine-iron(II) complex formation of each sample was calculated using the formula: ICA (%) = $[A_0 – (A_1 – A_2)] / A_0 \times 100$, where A_0, A_1 and A_2 correspond to absorbance of the control, sample and blank of the sample, respectively. In A_0 the intrinsic absorbance of iron(II) was subtracted from the initial absorbance. As the reaction proceeds the resulting red colour from the ferrozine-iron(II) complex decreases in the presence of chelating substances. Hence, ICA (%) values represent the reduction in absorbance values relative to the control due to the chelating effect of sample components. Results were expressed as % inhibition obtained per mg of dry residue. Before performing each assay procedure, 200 μl of sample were mixed to 200 μl absolute ethanol p. a. in order to guarantee the total polyphenolic compounds dissolution.

2.10. HPLC analysis

The phenolic profile for ethanol/water extracts and aqueous suspensions were obtained using an analytical HPLC unit (Jasco, Easton, USA) comprising: pump, automatic injector, DAD, equipped with a Kinetex (250 × 4.6 mm; 5 μm particle size; C18; 100 Å) core-shell column, controlled by Chrom-Nav software. The HPLC characterization was performed according to Kammerer *et al.* (Kammerer, Claus, Carle, & Schieber, 2004) as following:

Phenolic acid *(PA)* method: the mobile phase consisted of 2% (v/v) aqueous acetic acid (eluent A) and 0.5% (v/v) aqueous acetic acid and

acetonitrile (50:50, v/v; eluent B) using the following gradient program: from 10 to 15% B (10 min), 15% B isocratic (3 min), from 15 to 25% B (7 min), from 25 to 55% B (30 min), from 55 to 100% B (1 min), 100% B isocratic (5 min), from 100 to 10% B (10 min), with total run time of 67 min.

Anthoxanthins and Stilbenes (*AX*) method: the mobile phase consisted of the same eluents as described above using instead the following gradient program: from 10 to 24% B (20 min), from 24 to 30% B (20 min), from 30 to 55% B (20 min), from 55 to 100% B (15 min), 100% B isocratic (8 min), from 100 to 10% B (2 min), with a total run time of 95 min. For both methods, the injection volume was 10 µL and the absorbance was monitored at three monitoring channels (280, 320 and 370 nm). The flow rate was 1.0 mL min^{-1}. The peaks detected in the samples were first compared with respect to retention time and the spectral data with those in the standards mixture. Quantification was performed based on the molar absorptivity (ε, L mol^{-1}) values for each compound, according to chromatography peak area, molar mass and standard concentrations. Each sample was injected in duplicate. Results were expressed as means in milligrams GAE per gram of residue (mg GAE g residue $^{-1}$).

2.11. Statistical analysis

Values were reported means ± standard deviation (S.D.) for each antioxidant assay. Two dilution factors (*n* = 16) for ethanol/water extracts and one dilution factor (*n* = 8) for aqueous suspension were performed for TPC assay. One dilution factor (*n* = 8) for ethanol/water extracts and aqueous suspension step were performed in the case of $DPPH^{\bullet}$ assay and five dilution factors (*n* = 30) for ethanol/water extracts and aqueous suspensions were assessed for ORAC method. One dilution factor (*n* = 8) for ethanol/water extracts and two dilution factors (*n* = 16) in the case of aqueous suspensions were performed for ICA assay. All methodologies aforementioned were conducted in duplicate for each variety. Significant differences between means were separated by a two-factor MANOVA with TPC, $DPPH^{\bullet}$, ORAC and ICA values as dependent variables and solvent and grape varieties as fixed factors with sampling point nested into grape varieties. Following the identification of significant differences, univariate

353 ANOVA models were applied for each assay. Moreover multiple comparisons
354 between varieties were performed using Tukey test. Except if referred, all tests
355 were applied with a 95% confidence level. Statistical data analysis was
356 performed with IBM SPSS Statistics for Windows version 21.0 (IBM SPSS
357 Statistics, New York)
358
359 **3. Results and Discussion**
360 Different assays were performed in order to evaluate the total phenolic content
361 (TPC), scavenging capacity ($DPPH^{\bullet}$ and ORAC) and iron(II) chelating ability
362 through ICA assay in the ethanol/water extract obtained from the grape pomace
363 and in the aqueous suspension obtained from the dry residue of the previous
364 extract, according to the extraction scheme depicted in Figure 1.
365

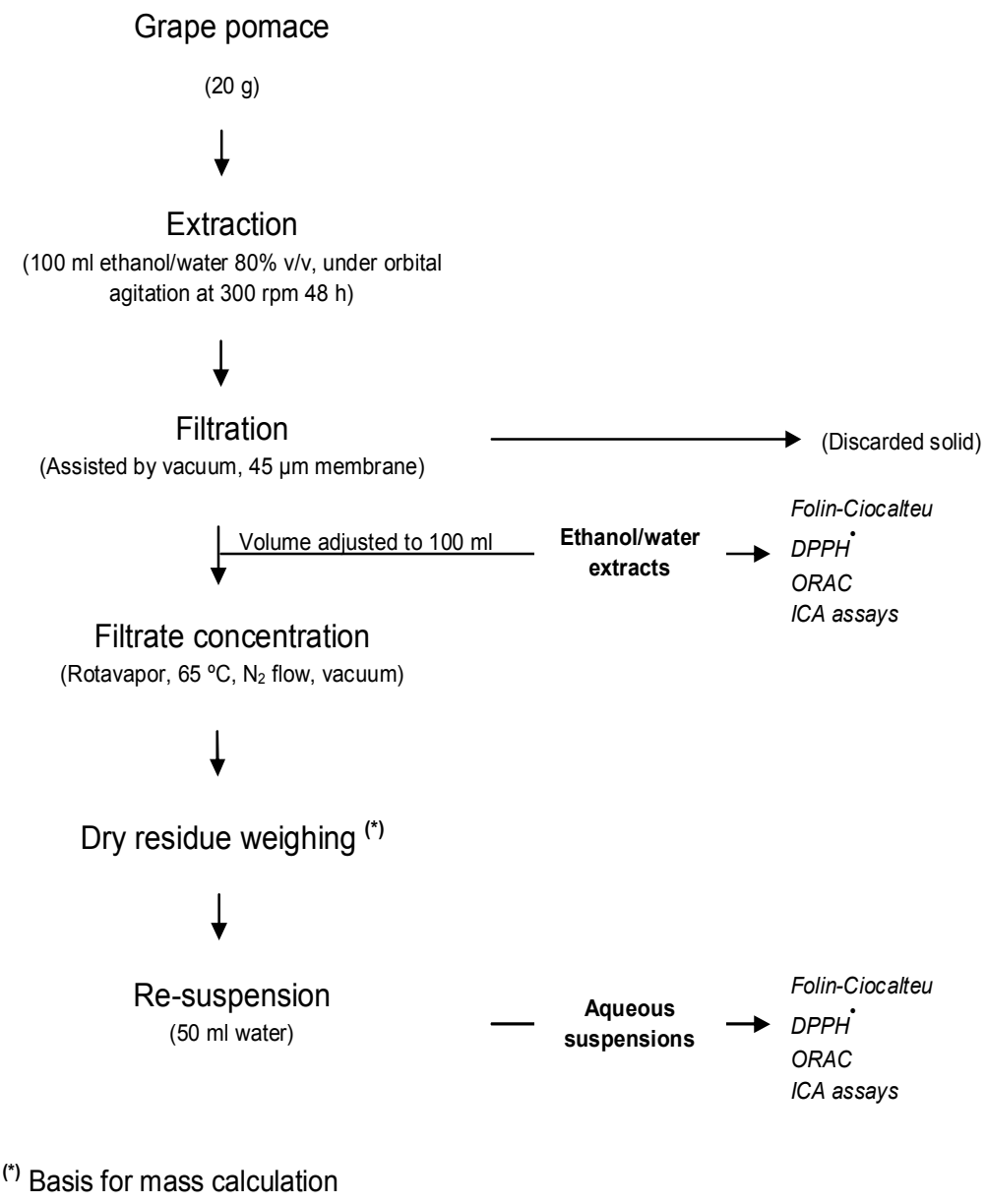


Figure 1. Schematic representation of the extraction of polyphenolic compounds present in samples of *"Tinta Roriz"*, *"Touriga Franca"*, *"Touriga Nacional"* and Mix red grape pomace. The whole procedure was performed in duplicate for each grape variety. DPPH•: 2,2-diphenyl-1-picrylhydrazyl radical scavenging; ORAC: oxygen radical absorbance capacity; ICA: iron(II) chelating ability.

The Folin-Ciocalteu assay is a method commonly used for the total phenolic content measurement and it is based on the ability of certain compounds (phenolic and nonphenolic) in alkaline medium to reduce the phos-

400 phomolybdic/phosphotungstic acid reagent to complexes, which is
401 spectrophotometrically detected (Luís M. Magalhães, Segundo, Reis, & Lima,
402 2008). In the DPPH$^{\bullet}$ assay, DPPH$^{\bullet}$ radical is reduced due to the presence of
403 antioxidant compounds causing decrease in the absorbance values at 515 nm.
404 Upon reduction, the color of the solution fades (D. Huang, Boxin, & Prior, 2005).
405 The principle for ORAC assay is based on the intensity of fluorescence
406 decrease of the target/probe along time under constant flux of peroxyl radicals
407 (due the thermal decomposition of AAPH) in aqueous buffer. When a sample is
408 analyzed due to the presence of chain-breaking antioxidants the decay of
409 fluorescence is inhibited (Luís M. Magalhães et al., 2008).
410 In the ICA assay, the presence of chelating compounds in the sample, disrupt
411 the complex formed between ion(II) and ferrozine. The colour decreasing on the
412 ion(II)-ferrozine complex monitored at 562 nm is taken as an estimation of the
413 chelating activity.
414 Firstly an extract was obtained from an ethanol/water (80% v/v) solvent, after
415 filtration step. Aqueous suspensions were obtained once the previous solvent
416 was evaporated and the obtained dry extract was re-suspended in water.
417 Before the re-suspension, dry residues obtained were weighted and the
418 percentage of residue recovered per g of initial dry pomace (% extraction yield)
419 was calculated. The percentages for extraction yield were: 3.8 % (*"Touriga*
420 *Nacional"*); 6.5 % (*"Touriga Franca"*); 3.8 % (*"Tinta Roriz"*); and 6.1 % (Mix) in %
421 g of dry residue per g of dry pomace. Due to these differences and in order to
422 carry an appropriate assessment, results of all analyses were expressed per
423 gram of dry residue (Table 1).

424

Table Nº1
TPC, antioxidant capacity determined by DPPH$^{\bullet}$ and ORAC assays and iron(II) chelating ability for ethanol/water extracts and aqueous suspensions.

Grape varieties	TPC†,§ (mg GAE g^{-1} residue)		DPPH$^{\bullet\dagger,\S}$ (mmol TE g^{-1} residue)		ORAC‡,§ (µmol TE g^{-1} residue)		ICA†,§ (%inhibition mg^{-1} residue)	
	Ethanol/water extract	Aqueous suspension	Ethanol/water extract	Aqueous suspension	Ethanol/water extract	Aqueous suspension	Ethanol/water extract	Aqueous suspension
TR	$69.3^{c} \pm 1.9$	$75.8^{d} \pm 4.0$	$0.52^{c} \pm 0.15$	$0.59^{d} \pm 0.02$	$1054^{c} \pm 199$	$1230^{b} \pm 91$	$76^{b} \pm 5$	$45^{c} \pm 6$
TF	$100.1^{b} \pm 7.4$	$106.1^{b} \pm 1.9$	$0.87^{b} \pm 0.04$	$0.90^{b} \pm 0.02$	$1343^{c} \pm 102$	$1325^{b} \pm 147$	$63^{c} \pm 10$	$55^{b,c} \pm 14$
TNac	$131.7^{a} \pm 8.1$	$142.4^{a} \pm 1.1$	$1.09^{a} \pm 0.13$	$1.12^{a} \pm 0.04$	$2337^{a} \pm 368$	$1579^{a} \pm 244$	$70^{b,c} \pm 12$	$66^{a,b} \pm 9$
Mix	$104.1^{b} \pm 5.5$	$102.5^{c} \pm 1.8$	$0.81^{b} \pm 0.09$	$0.86^{c} \pm 0.02$	$1649^{b} \pm 164$	$906^{c} \pm 66$	$109^{a} \pm 17$	$73^{a} \pm 10$

† Values represent means of quadruplicate ± S.D. ‡ and triplicate ± S.D.

§ Data were analyzed by MANOVA and within each column different letters indicate statistically differences according to the Tukey multiple comparison test at 95% confidence level (TPC: total phenolic content; DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical assay; ORAC: oxygen radical absorbance capacity; ICA: iron(II) chelating ability; GAE: gallic acid equivalents; TE: Trolox equivalents).

425 *3.1. Total phenolic content and antioxidant capacity*

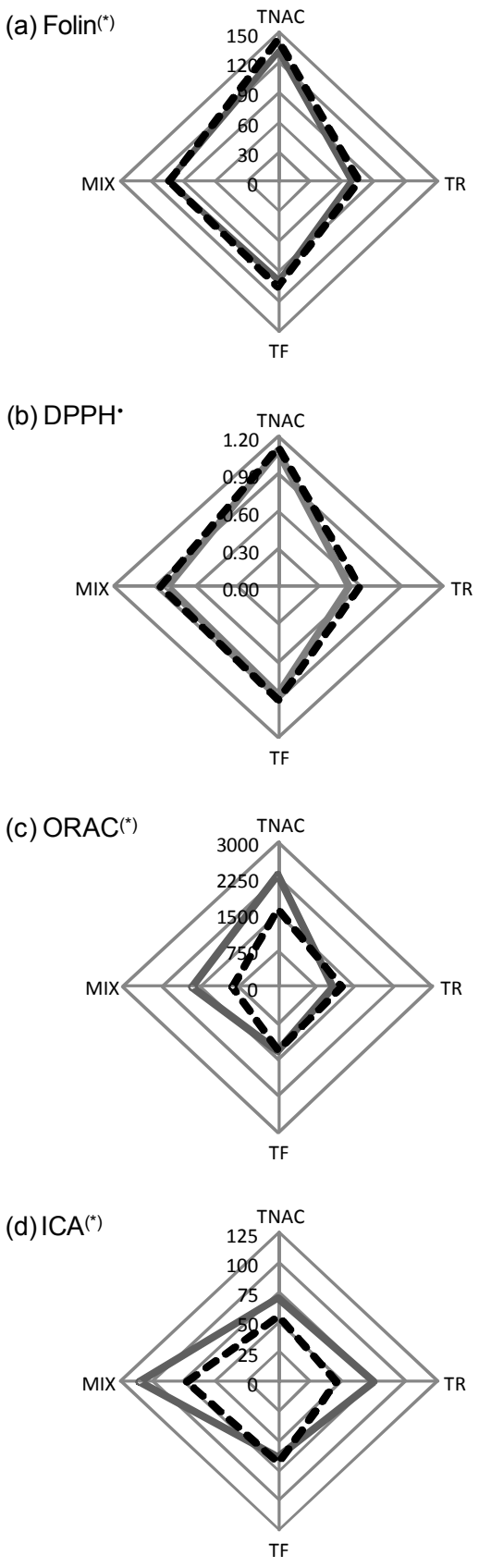
426 For ethanol/water extracts, TPC assay values for all samples from single
427 varieties have shown significant differences (P = 0.05) as presented in Table 1.
428 Values for TNac sample were 1.9-fold higher (131.7 ± 8.1 mg GAE g^{-1} residue)
429 than the average for TR (69.3 ± 1.9 mg GAE g^{-1} residue). The same result was
430 observed for anti-radical activity assessed through the DPPH$^{\bullet}$ assay, as 1.09 ±
431 0.13 mmol TE g^{-1} residue and 0.52 ± 0.15 mmol TE g^{-1} residue were the highest
432 and the lowest values obtained for the same samples. For ORAC assay, TNac
433 sample exhibited the strongest peroxyl scavenging capacity with an average of
434 2337 ± 368 µmol TE g^{-1} residue, while values for TR and TF were 1054 ± 199
435 µmol TE g^{-1} residue and 1343 ± 102 µmol TE g^{-1} residue, respectively. Metal
436 chelating ability represents an important aspect of the antioxidant properties of
437 the polyphenolic compounds. Most of the main strategies to avoid reactive
438 oxygen species (ROS) formation involves ion chelation (Ebrahimzadeh,
439 Pourmorad, & Bekhradnia, 2008). Any compound which exhibits the ability of
440 complexing ions, avoiding or reducing damages caused due to the pro-oxidant
441 effect of transition metals, can be recognized as a potential antioxidant. All the
442 samples have presented important metal binding capabilities, measured
443 through ICA assay. Nevertheless, related to the chelating ability to iron(II),
444 samples did not follow the previous trend. In the ICA assay, Mix sample
445 quelated all available iron (109 ± 17 % inhibition mg^{-1} residue) and on the other
446 hand, values for TF and TNac samples were the lowest (63 ± 10 %inhibition mg^{-1}
447 residue and 70 ± 12 %inhibition mg^{-1} residue). The chelating potential is
448 strongly dependent on the arrangement of hydroxyls and carbonyl group around
449 the molecules (Gülçin, 2012), therefore it depends on specific polyphenolic
450 compounds present in the extract. In this case, there seems to be a synergetic
451 combination of the compounds within Mix sample which increased its ICA
452 values in comparison with those from single variety extracts.

453 When aqueous suspensions are compared, results are similar to those obtained
454 for ethanol/water extracts for TPC and DPPH$^{\bullet}$ assays, where TNac and TR
455 samples showed the highest (142.4 ± 1.1 mg GAE g^{-1} residue; 1.12 ± 0.04
456 mmol TE g^{-1} residue) and the lowest (75.8 ± 4.0 mg GAE g^{-1} residue; 0.59 ±
457 0.02 mmol TE g^{-1} residue) results, respectively. On the other hand, when
458 evaluating ORAC values among the samples analyzed TNac exhibited the

strongest peroxyl scavenging capacity (1579 ± 244 µmol TE g^{-1} residue), and Mix sample corresponded to the lowest value (906 ± 66 µmol TE g^{-1} residue) in terms of ORAC. Concerning to chelating ability, TR exhibited the lowest ICA values, whilst Mix with 73 ± 9.7 %inhibition mg^{-1} residue, was 1.6-folds higher than TR regarding to ICA values. Data presented suggests that Mix interfered better than other extracts evaluated, in the iron(II)-ferrozine complex formation by chelating more iron(II) before ferrozine addition.

Published data related to extracts from the grape varieties present in this study has not been found in the literature for comparison purposes. Nevertheless, Negro *et al.* (Negro, Tommasi, & Miceli, 2003) has obtained similar results, 1.40 g GAE L^{-1} extract for TPC values, our results ranged from 1.17 to 2.79 g GAE L^{-1} extract, (see Table S1 and S2 supplementary data), having worked with marc pomace extract from *"Negro amaro"* variety under comparable extraction conditions (80% v/v ethanol/water). Jordão *et al.* (Jordao, Simoes, Correia, & Goncalves, 2012) have recently presented data about wines from TR and TNac winemaking process. Their TPC values were in average 2771 ± 32 mg GAE L^{-1} wine and 3216 ± 105 mg GAE L^{-1} wine for TR and TNac, respectively. It is important to note that during the traditional winemaking process (maceration), due to the contact between must-seeds and skins and the mass transfer phenomena, once this step is completed, seeds and skins will contain less quantity of polyphenolic compounds. Additionally, Lapornik *et al*. (Lapornik et al., 2005) have compared extracts prepared from plant by-products using different conditions (solvents and extraction time) and extracted 5790 mg GAE L^{-1} from grape pomace. Different extraction condition may be the origin of the variance between the values. Cristino *et al.* (Cristino, Costa, Cosme, & Jordao, 2013) have recently published lower values related to total antioxidant capacity for red wines from two Portuguese Appellations of Origin measured by $DPPH^{\bullet}$ with values ranging between 8.0 ± 1.7 and 23.3 ± 0.5 TE mM having worked with 0.1 mL sample. On the other hand, concerning the solvent used in the extraction step, Rockenbach *et al.* (Rockenbach et al., 2011) have worked with pomace (skins or seeds) from different Brazilian red grapes extracted by contact with an acidified mixture using methanol instead of ethanol. Their values were in average 2076 and 8517 µmol TE 100 g^{-1} of dry pomace for skins or seed extracts, respectively. Our results (ranged 2188 - 5688 µmol TE 100 g^{-1} of

493 dry pomace, see Table S2 supplementary data) are comparable having worked
494 with different extractive mixture, and moreover under extraction conditions safer
495 and environmentally friendly as methanol is replaced by ethanol in our case.
496 Regarding ORAC results, our values (906 - 1579 μmol TE g^{-1} residue, see
497 Table S2 supplementary data) are 3.3-folds lower than those presented by
498 Hogan *et al.* (Hogan, Canning, Sun, Sun, & Zhou, 2010). Hogan *et al*. have
499 worked with Norton GPE and they obtained 4133 ± 94 μmol TE g^{-1} GPE using
500 ethanol 80% (v/v) as extractive solvent under overnight shaking. Yilmaz and
501 Toledo (Yilmaz & Toledo, 2006) have worked with grape/wine industry
502 byproducts and studied the antioxidant properties of the grape and seed
503 extracts through ORAC assay. Their ORAC values ranged from 311 to 638
504 μmol TE g^{-1} dry seed, and 70 to 103 μmol TE g^{-1} dry skin. It is important to note,
505 when comparisons were carried on, most of the published literature have
506 presented the results having evaluated parts of the grape separately, not whole
507 grape or pomace.
508 According to previous work published by Ebrahimzadeh *et al.* our findings are
509 comparable in terms of iron(II) chelating ability. They have worked in the
510 assessment of the iron chelating activity of some medicinal plants from Iran and
511 published values 18.20% inhib. (*Feijoa sellowiana*, aqueous extract) and 20.8
512 %inhibition (*Sambucus ebulus*, aqueous extract), both extracts at 3.2 mg ml^{-1}
513 (Ebrahimzadeh et al., 2008).
514
515 3.2. Evaluation of solvent change
516 Results for TPC, $DPPH^{\bullet}$, ORAC and ICA assays for ethanol/water extracts and
517 aqueous suspensions were shown in Figure 2.
518



519

520 **Figure 2.** (a) Folin: total phenolic content (TPC) (mg GAE g^{-1} residue); (b)
521 $DPPH^{\bullet}$: scavenging capacity against $DPPH^{\bullet}$ (mmol TE g^{-1} residue); (c) ORAC:

oxygen radical absorbance capacity (μmol TE g^{-1} residue) and (d) ICA: iron(II) chelating ability (%inhib. mg^{-1} residue) for TR (*"Tinta Roriz"*), TF (*"Touriga Franca"*), TNac (*"Touriga Nacional"*) and Mix. (Ethanol/water extracts (solid grey line), aqueous suspensions (dotted line), GAE: gallic acid equivalents; TE: Trolox equivalents). Homogenous groups according to the Tukey multiple comparison test at 95% confidence level.

Therefore, comparing results for ethanol/water extracts and aqueous suspensions for all assays, and according to a two-factor MANOVA for variety and solvent, with solvent nested into variety, significant differences ($P < 0.05$; Lambda de Wilks < 0.01) were found for all methods for grape varieties and significant differences were also found for TPC, ORAC and ICA assays ($P <$ 0.01; Lambda de Wilks < 0.01), excepting for $DPPH^{\bullet}$ assay results.

As the same assay protocol was applied to all samples, the differences found must be ascribed to the solvent change or other factors (temperature, light, and oxygen exposure) that took place during the concentration and ethanol/water solvent removal. In fact, TPC average values significantly ($P < 0.01$) increased and for ORAC and ICA assay values decreased in average for aqueous suspensions compared with ethanol/water extract. Concerning $DPPH^{\bullet}$ values, there was not any significant difference ($P = 0.35$) between ethanol/water extract and aqueous suspensions.

Published data suggests that temperature has an important effect on structure and biological properties of the polyphenolic compounds. The main consequence is the increase of the polymerization degree which turns one monomer into a compound with higher possibilities of radical stabilization in the aromatic ring. It has been described that this improvement reaches its maximum with four monomers as larger molecules may not be so efficient due to steric interaction between functional groups (Pinelo, Rubilar, Jerez, Sineiro, & Nunez, 2005). Since no significant differences were obtained for $DPPH^{\bullet}$ assay, it seems that the combination time-temperature (1 h at 65 ºC) applied during the concentration step was not significant enough to change the polymerization degree and consequently, enhance the extract scavenging capacity against $DPPH^{\bullet}$ radical. It was reported that at higher temperature (1 h at 150 ºC)

555 increased four times DPPH$^{\bullet}$ values for aqueous extracts citrus skins (Jeong et
556 al., 2004).
557 Results for ORAC and ICA assays were significantly (P = 0.01) lower after
558 concentration and solvent change (16% and 20%, respectively). The key for
559 binding ion ability is in the chemical structure (functional groups and their
560 number). It was reported that compounds containing C-OH and C=O functional
561 groups can chelate metal ions (Gülçin, 2012). While the temperature effect
562 tends to increase the number of polymers in the final compounds and
563 consequently improved the chelating ability to iron(II), therefore a reduction in
564 the values for ICA may be due to the influence of solvent change rather than
565 chemical differences arisen from temperature exposition.
566 Published data related to the chemical behavior of antioxidant species in ORAC
567 assay is contradictory. Some authors state that as ORAC is based on hydrogen
568 atom transfer (HAT) it should be solvent and pH independent (Gülçin, 2012).
569 Nevertheless, it has been reported that when changes in solvent take place,
570 changes on oxygen radical absorbance capacity were observed. In this context
571 extracts obtained by aqueous solvents exhibited lower antioxidant capacity in
572 comparison to ethanolic mixtures, according to Pérez-Jimenez *et al. (Pérez-*
573 *Jiménez & Saura-Calixto, 2006).* Moreover, regarding the solvent effect on
574 DPPH$^{\bullet}$ values, the same authors observed that, among the different antioxidant
575 capacity assays evaluated for the effect of the solvent, DPPH$^{\bullet}$ was the assay in
576 which the influence of the solvent was weakest in comparison with ORAC,
577 ABTS$^{\bullet+}$ and FRAP (Ferric Reducing Ability of Plasma) (Pérez-Jiménez & Saura-
578 Calixto, 2006).
579 In order to assess the possible interactions from having mixed TNac, TR and TF
580 the contribution of each grape variety was calculated to the final Mix sample
581 (Mix estimated, Table 2). Standard deviations were estimated according to the
582 equation of propagation of the errors. Finally, Mix experimental and Mix
583 estimated values were compared in a one sample t-test (P = 0.05). Our findings
584 suggest that when grape varieties are mixed their potential antioxidant
585 properties in terms of TPC and ORAC values were significantly reduced (P =
586 0.05). On the other hand, ICA values for experimental Mix was 1.3 folds-higher
587 (P < 0.01) than ICA values for estimated Mix. DPPH$^{\bullet}$ values did not differ
588 statistically (P = 0.05). Differences between Mix experimental and Mix estimated

may be ascribed to the composition of each extract and the chemical interactions between the compounds which may take place.

Table Nº2

Total phenolic content (TPC), antioxidant capacity determined by DPPH$^{\bullet}$ and ORAC assays and iron(II) chelating ability for experimental and estimated Mix sample in aqueous suspensions.

Grape variety	TPC†,§ (mg GAE g^{-1} residue)	DPPH$^{\bullet\dagger,\S}$ (mmol TE g^{-1} residue)	ORAC‡,§ (µmol TE g^{-1} residue)	ICA†,§ (%inhibition mg^{-1} residue)
Mix (experimental)	103^{b} ± 1.8	0.86^{a} ± 0.02	906^{b} ± 66	73^{a} ± 10
Mix (estimated)$^{¤}$	108^{a} ± 1.5	0.87^{a} ± 0.02	1378^{a} ± 99	55^{b} ± 6

† Values represent means of quadruplicate ± S.D. ‡ and triplicate ± S.D.

§ Data were analyzed by one sample T-test and within each column different letters indicate statistically differences at 95% confidence level.

$^{¤}$: standard deviation estimated according to the equation of propagation of errors.

(TPC: total phenolic content; DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical assay; ORAC: oxygen radical absorbance capacity; ICA: iron(II) chelating ability; GAE: gallic acid equivalents; TE: Trolox equivalents).

Since extracts from vegetable tissues represent a highly complex matrix, many possibilities of reaction take place in the media, turning the endogenous polyphenols into new compounds, usually with higher molecular weight as mentioned before, and some compounds combinations or chemical associations can result in a mixture with lower antioxidant properties. In this fact, Pinelo *et al.* have compared the antioxidant behavior for individual polyphenols and mixture of them through DPPH$^{\bullet}$ assay, and their results suggested that the total values of antioxidant capacity reached for the mixture was always lower than those from single compounds (Pinelo, Manzocco, Nuñez, & Nicoli, 2004). It is also expected that as systems under study become more complex, the greater the chances of interaction of the compounds, and this fact may contribute to a trend difficult to predict.

605 Linear correlations (Pearson´s coefficients) between TPC and values for ORAC,
606 DPPH$^{\bullet}$ and ICA assays based on dry residue for aqueous suspensions are
607 presented in Table 3**.** Significant and strong correlation was found between TPC
608 and scavenging antioxidant capacity for DPPH$^{\bullet}$ assay (R = 0.944, P < 0.01).
609 The positive correlation indicates that the higher total phenolic content resulted
610 in a higher scavenging antioxidant activity. This strong correlation has also been
611 previously reported for twenty Chinese grape varieties (Xu, Zhang, Cao, & Lu,
612 2010) and for the same grape varieties (TR and TNac) in this study (Jordao et
613 al., 2012). The ORAC values did not correlate as well as the previous assays,
614 focused on scavenging of a single biologically relevant radical (peroxyl) (-0.356
615 ≤ R ≤ 0.632). This fact has been previously reported (Maria Monagas et al.,
616 2005). On the other hand, ORAC assay represents a hydrogen atom transfer
617 assay and its measurement is more linked to the reactivity of the groups than
618 the number of phenol compounds (Perez, Leighton, Aspee, Aliaga, & Lissi,
619 2000). Hence, a significant correlation, yet lower, was found between TPC and
620 DPPH$^{\bullet}$ results.
621 Concerning the iron(II) chelating ability, values were not significantly correlated
622 with those from the other assays (R ≤ 0.263). Those results were expected,
623 considering the chemical nature of each assay as discussed previously.
624

Table Nº3

Pearson´s correlation coefficients between antioxidant capacity values (DPPH$^{\bullet}$ and ORAC assays); iron(II) chelating ability (ICA assay) and TPC values for aqueous suspensions.

	TPC [a]	DPPH$^{\bullet}$ [b]	ICA [c]	ORAC [d]
TPC	1	0.944**	0.250^{ns}	0.632**
DPPH$^{\bullet}$		1	0.263 ^{ns}	0.557*
ICA			1	-0.356 ^{ns}
ORAC				1

* significant correlation at 0.05 level (2-tailed)

** significant correlation at 0.01 level (2-tailed)

^{ns} non-significant. [a] mg GAE g^{-1} residue; [b] mmol TE g^{-1} residue; [c] % Inhib. mg^{-1} residue; [d] µmol TE g^{-1} residue. (TPC: total phenolic content; DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical assay; ORAC: oxygen radical absorbance capacity; ICA: iron(II) chelating ability; GAE: gallic acid equivalents; TE: Trolox equivalents).

625

626 3.3. Individual phenols by HPLC

627 Table 4 represents the individual phenols indentified for ethanol/water extracts
628 and aqueous suspensions. No significant ($P < 0.05$) differences among varieties
629 were obtained for the gallic acid and between the both solvent types. It can be
630 stated that, syringic acid (0.23 to 1.72 mg g^{-1} residue) and (+)-Catechin (0.34 to
631 2.37 mg g^{-1} residue) were the most abundant individual phenols found in all
632 extracts (ethanol/water or aqueous suspensions). Lafka *et al.* have previously
633 the presence of the phenols identified in this research (Lafka, Sinanoglou, &
634 Lazos, 2007). The HPLC results are consistent with the TPC and the
635 antioxidant properties described above. Hence, the relative abundance of each
636 compound was also affected by the solvent, being detected in higher
637 concentration in aqueous suspensions through the HPLC analysis. In
638 concordance with our finding regarding to the antioxidant properties ($DPPH^{\bullet}$,
639 ORAC and ICA assays), TNac showed the highest individual phenols values
640 (0.14 to 0.59 mg g^{-1} residue gallic acid; 0.56 to 2.37 mg g^{-1} residue (+)-
641 Catechin; 0.16 to 0.54 mg g^{-1} residue caffeic acid; 0.58 to 1.72 mg g^{-1} residue
642 syringic acid and 0.49 to 1.40 mg g^{-1} residue (-)-Epicatechin for ethanol/water
643 extract and aqueous suspensions, respectively). Regarding to differences in
644 TPC values between Folin-Ciocalteu assay and HPLC assessment, can be
645 ascribed to fundamentals of each method itself. Folin-Ciocalteu assay is based
646 on the absorbance of forming blue complexes between Folin-Ciocalteu reagent
647 and reducing species, spectrophotometrically determined, whilst HPLC
648 methodology is based on the molar absorptivity of molecules. A significant
649 strong correlation (Pearson´s coefficient $R = 0.973$, $p < 0.01$) between TPC by
650 Folin-Ciocalteu assay and TPC by HPLC methodology, was obtained.

Table Nº4
Total phenolic content (TPC) and individual phenols evaluated by HPLC for ethanol/water extracts and aqueous suspensions.

Measurement[†]	ethanol/water extracts				aqueous suspensions			
	TR	TF	TNac	Mix	TR	TF	TNac	Mix
Gallic acid	$0.15^{a} \pm 0.01$	$0.13^{a} \pm 0.00$	$0.14^{a} \pm 0.01$	$0.15^{a} \pm 0.01$	$0.57^{a} \pm 0.01$	$0.53^{a} \pm 0.04$	$0.59^{a} \pm 0.01$	$0.50^{a} \pm 0.03$
(+)-Catechin	$0.43^{b} \pm 0.01$	$0.34^{c} \pm 0.00$	$0.56^{a} \pm 0.01$	$0.56^{a} \pm 0.01$	$0.56^{c} \pm 0.03$	nd	$2.37^{a} \pm 0.06$	$1.55^{b} \pm 0.08$
Caffeic acid	nd	$0.12^{b} \pm 0.00$	$0.16^{a} \pm 0.00$	$0.11^{c} \pm 0.00$	$0.21^{d} \pm 0.00$	$0.46^{b} \pm 0.00$	$0.54^{a} \pm 0.01$	$0.37^{c} \pm 0.01$
Syringic acid	$0.23^{c} \pm 0.01$	$0.44^{b} \pm 0.01$	$0.58^{a} \pm 0.00$	$0.40^{b} \pm 0.01$	$0.72^{d} \pm 0.01$	$1.44^{b} \pm 0.02$	$1.72^{a} \pm 0.01$	$1.16^{c} \pm 0.01$
(-)-Epicatechin	nd	$0.35^{b} \pm 0.06$	$0.49^{a} \pm 0.00$	$0.33^{b} \pm 0.02$	$0.41^{c} \pm 0.01$	$0.89^{b} \pm 0.07$	$1.40^{a} \pm 0.01$	$0.95^{b} \pm 0.01$
TPC	$1.08^{c} \pm 0.08$	$2.80^{b} \pm 0.11$	$2.77^{b} \pm 0.04$	$3.92^{a} \pm 0.20$	$5.72^{c} \pm 0.33$	$9.58^{b} \pm 0.42$	$12.81^{a} \pm 0.66$	$8.03^{b,c} \pm 0.81$

[†]Values represent means of duplicate ± S.D. and expressed as mg g^{-1}residue; values for *p*-hydroxybenzoic, *p*-coumaric and o-coumaric, sinapic, ferulic acids and (-)-Epicatechin gallate, *trans*-resveratrol, quercetin, kaempferol and chlorogenic acid, were below the detection limit, nd: not detected. Data were analyzed by MANOVA and within each row different letters indicate statistically differences according to the Tukey multiple comparison test at 95% confidence level (TPC: total phenolic content).

651

3.4. Choice of pomace extract for future applications

After analyzing the results presented in Table 1, in order to select the Portuguese grape varieties among those studied with more potential for the preparation of an antioxidant extract concerning TPC (142.4 ± 1.1 mg GAE g^{-1} residue), and results from antioxidant methods ($DPPH^{\bullet}$: 1.12 ± 0.04 mmol TE g^{-1} residue; ORAC: 1579 ± 244 μmol TE g^{-1} residue), *"Touriga Nacional"* seems to be the most promising variety. Further studies, evaluating a larger number of samples in different vintages, are required. It noteworthy that "*Touriga Nacional"* is recognized as the most notable variety from the Douro's region, due to the analytical and sensorial characteristics of its wines (Pinto-Sintra, 2007), broadly extended beyond European territory, namely Brazil and Australia (Jordao et al., 2012). Additionally, Douro´s region is a important area for the high quality wine production, with more than 40,000,000 liters of wine produced for 2012. Douro red wines with 45% of that production included the red varieties presents in this study, registered earnings over 72,000,000 Euros L^{-1} wine for 2014 (IVDP, 2014). These features in conjunction with our results show for "*Touriga Nacional*" promising scenery for potential applications may in IPM, biopesticides fields, and animal controlled diet.

4. Conclusions

Data presented in this paper showed that ethanol/water extracts obtained from Portuguese red grape pomace exhibited satisfactory and comparable antioxidant properties with other published data in terms of TPC and antioxidant capacity ($DPPH^{\bullet}$ and ORAC assays), even though having extracted the compounds with a mixture less suitable from the standpoint of yield with a more environmentally friendly behavior. The results presented are aimed at the broad utility that possesses local grape varieties in terms of the development of industrial derivates towards a more sustainable agriculture. The information showed should be taken as a start; further studies evaluating a larger number of grape pomace are needed. The real use of these extracts should be appropriately evaluated in the crops fields against certain plant diseases protecting as "phytosanitary bioproducts". Portuguese grape pomace is definitely an under-exploited potential source of polyphenolic compounds. Knowledge about polyphenolic contents and their antioxidant properties

686 contribute to a more comprehensive of the transcendental aptitude and potential
687 destiny of the Portuguese grapevine pomace.
688
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References

AOAC. (2002). Official methods of analysis (17 th ed.). Gaithersburg, MD: Association of Official Analytical Chemists, USA.

Baptista, J. A., Tavares, J. F. d. P., & Carvalho, R. C. (2001). Comparison of polyphenols and aroma in red wines from Portuguese mainland versus Azores Islands. *Food Res. Int., 34*(4), 345-355.

Benouaret, R., Goujon, E., Trivella, A., Richard, C., Ledoigt, G., Joubert, J.-M., et al. (2014). Water extracts from winery by-products as tobacco defense inducers. *Ecotoxicology, 23*(8), 1574-1581.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol., 28*(1), 25-30.

Corrales, M., García, A. F., Butz, P., & Tauscher, B. (2009). Extraction of anthocyanins from grape skins assisted by high hydrostatic pressure. *J. Food Eng., 90*(4), 415-421.

Cosme, F., Ricardo-Da-Silva, J. M., & Laureano, O. (2009). Tannin profiles of Vitis vinifera L. cv. red grapes growing in Lisbon and from their monovarietal wines. *Food Chem., 112*(1), 197-204.

Cristino, R., Costa, E., Cosme, F., & Jordao, A. M. (2013). General phenolic characterisation, individual anthocyanin and antioxidant capacity of matured red wines from two Portuguese Appellations of Origins. *J. Sci. Food Agric., 93*(10), 2486-2493.

De Nisco, M., Manfra, M., Bolognese, A., Sofo, A., Scopa, A., Tenore, G. C., et al. (2013). Nutraceutical properties and polyphenolic profile of berry skin and wine of Vitis vinifera L. (cv. Aglianico). *Food Chem., 140*(4), 623-629.

Dopico-García, M. S., Fique, A., Guerra, L., Afonso, J. M., Pereira, O., Valentão, P., et al. (2008). Principal components of phenolics to characterize red Vinho Verde grapes: Anthocyanins or non-coloured compounds? *Talanta, 75*(5), 1190-1202.

Ebrahimzadeh, M. A., Pourmorad, F., & Bekhradnia, A. R. (2008). Iron chelating activity, phenol and flavonoid content of some medicinal plants from Iran. *Afr. J. Biotech., 7*(18).

FAOSTAT. (2012). FAO statistical database., from http://faostat.fao.org/site/291/default.aspx

Gülçin, I. (2012). Antioxidant activity of food constituents: An overview. *Arch. Toxicol., 86*(3), 345-391.

Hogan, S., Canning, C., Sun, S., Sun, X. X., & Zhou, K. Q. (2010). Effects of Grape Pomace Antioxidant Extract on Oxidative Stress and Inflammation in Diet Induced Obese Mice. *J. Agric. Food Chem., 58*(21), 11250-11256.

Huang, D., Boxin, O. U., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem., 53*(6), 1841-1856.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *J. Agric. Food Chem., 50*(16), 4437-4444.

IVDP. (2014). Instituto dos Vinhos do Douro e do Porto. Base de dados Estatisticos. Retrieved 10/02/2014

Jeong, S.-M., Kim, S.-Y., Kim, D.-R., Jo, S.-C., Nam, K., Ahn, D., et al. (2004). Effect of heat treatment on the antioxidant activity of extracts from citrus peels. *J. Agric. Food Chem., 52*(11), 3389-3393.

Jordao, A. M., Goncalves, F. J., Correia, A. C., Cantao, J., Rivero-Perez, M. D., & SanJose, M. L. G. (2010). Proanthocyanidin content, antioxidant capacity and scavenger activity of Portuguese sparkling wines (Bairrada Appellation of Origin). [Article]. *J. Sci. Food Agric., 90*(12), 2144-2152.

Jordao, A. M., Simoes, S., Correia, A. C., & Goncalves, F. J. (2012). Antioxidant activity evolution during Portuguese red wine vinification and their relation with the proanthocyanidin and anthocyanidin composition. [Article]. *J. Food Process Preserv., 36*(4), 298-309.

Kammerer, D., Claus, A., Carle, R., & Schieber, A. (2004). Polyphenol screening of pomace from red and white grape varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS. *J. Agric. Food Chem., 52*(14), 4360-4367.

Lafka, T.-I., Sinanoglou, V., & Lazos, E. S. (2007). On the extraction and antioxidant activity of phenolic compounds from winery wastes. *Food Chem., 104*(3), 1206-1214.

Lapornik, B., Prošek, M., & Golc Wondra, A. (2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. *J. Food Eng., 71*(2), 214-222.

Magalhães, L. M., Barreiros, L., Maia, M. A., Reis, S., & Segundo, M. A. (2012). Rapid assessment of endpoint antioxidant capacity of red wines through microchemical methods using a kinetic matching approach. *Talanta, 97*, 473-483.

Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S., & Lima, J. L. (2010). Rapid microplate high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity. *Talanta, 83*(2), 441-447.

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Anal. Chim. Acta, 613*(1), 1-19.

Matias, A. A., Serra, A. T., Silva, A. C., Perdigao, R., Ferreira, T. B., Marcelino, I., et al. (2010). Portuguese winemaking residues as a potential source of natural anti-adenoviral agents. [Article]. *Int. J. Food Sci. Nutr., 61*(4), 357-368.

Mendes, J. A. S., Prozil, S. O., Evtuguin, D. V., & Lopes, L. P. C. (2013). Towards comprehensive utilization of winemaking residues: Characterization of grape skins from red grape pomaces of variety Touriga Nacional. *Ind. Crop. Prod., 43*(0), 25-32.

Monagas, M., Gómez-Cordovés, C., Bartolomé, B., Laureano, O., & Ricardo da Silva, J. M. (2003). Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from Vitis vinifera L. cv. Graciano, Tempranillo, and Cabernet Sauvignon. *J. Agric. Food Chem., 51*(22), 6475-6481.

Monagas, M., Hernandez-Ledesma, B., Garrido, I., Martin-Alvarez, P. J., Gomez-Cordoves, C., & Bartolome, B. (2005). Quality assessment of commercial dietary antioxidant products from Vitis vinifera L. grape seeds. *Nutr. Cancer, 53*(2), 244-254.

Nandakumar, V., Singh, T., & Katiyar, S. K. (2008). Multi-targeted prevention and therapy of cancer by proanthocyanidins. *Cancer Lett., 269*(2), 378-387.

Negro, C., Tommasi, L., & Miceli, A. (2003). Phenolic compounds and antioxidant activity from red grape marc extracts. *Bioresour. Technol., 87*(1), 41-44.

Paixao, N., Perestrelo, R., Marques, J. C., & Camara, J. S. (2007). Relationship between antioxidant capacity and total phenolic content of red, rose and white wines. [Article]. *Food Chem., 105*(1), 204-214.

Pérez-Jiménez, J., & Saura-Calixto, F. (2006). Effect of solvent and certain food constituents on different antioxidant capacity assays. *Food Res. Int., 39*(7), 791-800.

Perez, D. D., Leighton, F., Aspee, A., Aliaga, C., & Lissi, E. (2000). A comparison of methods employed to evaluate antioxidant capabilities. *Biol. Res., 33*(2), 71-77.

Pinelo, M., Manzocco, L., Nuñez, M. J., & Nicoli, M. C. (2004). Interaction among Phenols in Food Fortification: Negative Synergism on Antioxidant Capacity. *J. Agric. Food Chem., 52*(5), 1177-1180.

Pinelo, M., Rubilar, M., Jerez, M., Sineiro, J., & Nunez, M. J. (2005). Effect of solvent, temperature, and solvent-to-solid ratio on the total phenolic content and antiradical activity of extracts from different components of grape pomace. *J. Agric. Food Chem., 53*(6), 2111-2117.

Pinto-Sintra, A. L. (2007). Establishment of embryogenic cultures and plant regeneration in the Portuguese cultivar 'Touriga Nacional' of Vitis vinifera L. *Plant Cell Tissue Organ Cult., 88*(3), 253-265.

Prozil, S. O., Evtuguin, D. V., & Lopes, L. P. C. (2012). Chemical composition of grape stalks of Vitis vinifera L. from red grape pomaces. *Ind. Crop. Prod., 35*(1), 178-184.

Rockenbach, I. I., Gonzaga, L. V., Rizelio, V. M., Gonçalves, A. E. d. S. S., Genovese, M. I., & Fett, R. (2011). Phenolic compounds and antioxidant activity of seed and skin extracts of red grape (Vitis vinifera and Vitis labrusca) pomace from Brazilian winemaking. *Food Res. Int., 44*(4), 897-901.

Shi, J., Nawaz, H., Pohorly, J., Mittal, G., Kakuda, Y., & Jiang, Y. M. (2005). Extraction of polyphenolics from plant material for functional foods - Engineering and technology. *Food Rev. Int., 21*(1), 139-166.

Shirahigue, L. D., Plata-Oviedo, M., de Alencar, S. M., d'Arce, M. A. B. R., de Souza Vieira, T. M. F., Oldoni, T. L. C., et al. (2010). Wine industry residue as antioxidant in cooked chicken meat. *Int. J. Food Sci. Technol., 45*(5), 863-870.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol., 299*, 152-178.

Spigno, G., Tramelli, L., & De Faveri, D. M. (2007). Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *J. Food Eng., 81*(1), 200-208.

Terra, X., Valls, J., Vitrac, X., Mérrillon, J.-M., Arola, L., Ardèvol, A., et al. (2007). Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. *J. Agric. Food Chem., 55*(11), 4357-4365.

Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chem., 116*(1), 240-248.

Xu, C., Zhang, Y., Cao, L., & Lu, J. (2010). Phenolic compounds and antioxidant properties of different grape cultivars grown in China. *Food Chem., 119*(4), 1557-1565.

Yilmaz, Y., & Toledo, R. T. (2006). Oxygen radical absorbance capacities of grape/wine industry byproducts and effect of solvent type on extraction of grape seed polyphenols. *J. Food Compos. Anal., 19*(1), 41-48.

Supplementary data

Valorization of grape pomace: extraction of bioactive phenolics with antioxidant properties

Hernán H. Tournour[1,2], Marcela A. Segundo[3], Luís M. Magalhães[3], Luisa Barreiros[3], Jorge Queiroz[1], Luís M. Cunha[1,*]

[1] REQUIMTE/DGAOT Faculdade de Ciências, Universidade do Porto, Rua Padre Armando Quintas, 7, 4486-661 Vairão, Portugal

[2] Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

[3] UCIBIO, REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

*Corresponding author: lmcunha@fc.up.pt
Tel.: (+351)252660400 / Fax: (+(351)252661780

Table S1

TPC, antioxidant capacity determined by DPPH$^{\bullet}$ and ORAC assays and iron(II) chelating ability (ICA) for ethanol/water extracts.

Grape varieties	TPC †,§ (mg GAE L^{-1} extract)	DPPH$^{\bullet\dagger,\S}$		ORAC‡,§		ICA†,§ (%inhibition (g L^{-1} extract)$^{-1}$)
		(mmol TE L^{-1} extract)	(mmol TE $100g^{-1}$ dry pomace)	(µmol TE g^{-1} residue)	(µmol TE g^{-1} dry pomace)	
TR	534^{d} ± 16	4.1^{c} ± 1.2	1.9^{c} ± 0.6	1054^{c} ± 199	40^{c} ± 7.4	22.8^{b} ± 1.5
TF	1313^{a} ± 80	11.9^{a} ± 1.5	5.9^{a} ± 0.7	1343^{c} ± 102	88^{a} ± 8.2	18.4^{c} ± 2.3
TNac	1000^{c} ± 22	8.4^{b} ± 1.5	4.2^{b} ± 0.7	2337^{a} ± 368	88^{a} ± 12.2	21.0^{b} ± 3.6
Mix	1272^{b} ± 71	9.9^{b} ± 1.4	5.0^{b} ± 0.7	1649^{b} ± 164	101^{a} ± 11.6	32.8^{a} ± 1.3

† Values represent means of quadruplicate ± S.D. ‡ and triplicate ± S.D.

§ Data were analyzed by MANOVA and within each column different letters indicate statistically differences according to the Tukey multiple comparison test at 95% confidence level (TPC: total phenolic content; DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical assay; ORAC: oxygen radical absorbance capacity; ICA: iron(II) chelating ability; GAE: gallic acid equivalents; TE: Trolox equivalents).

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Table S2

TPC, antioxidant capacity determined by DPPH$^{\bullet}$ and ORAC assays and iron(II) chelating ability (ICA) for aqueous suspensions.

Grape varieties	TPC †,§ (mg GAE L^{-1} extract)	DPPH$^{\bullet\dagger,\S}$		ORAC‡,§		ICA†,§ (%inhibition (g L^{-1} extract)$^{-1}$)
		(mmol TE L^{-1} extract)	(mmol TE $100g^{-1}$ dry pomace)	(µmol TE g^{-1} residue)	(µmol TE g^{-1} dry pomace)	
TR	$1166^{d} \pm 65$	$9.0^{d} \pm 0.3$	$2.2^{d} \pm 0.1$	$1230^{b} \pm 91$	$46^{c} \pm 3.3$	$13.5^{c} \pm 1.9$
TF	$2790^{a} \pm 133$	$22.9^{a} \pm 1.6$	$5.7^{a} \pm 0.4$	$1325^{b} \pm 147$	$88^{a} \pm 11.5$	$16.7^{b,c} \pm 4.3$
TNac	$2170^{c} \pm 144$	$17.0^{c} \pm 1.6$	$4.3^{c} \pm 0.4$	$1579^{a} \pm 244$	$60^{b} \pm 12.3$	$19.6^{a,b} \pm 2.8$
Mix	$2504^{b} \pm 122$	$20.9^{b} \pm 1.1$	$5.2^{b} \pm 0.3$	$906^{c} \pm 66$	$55^{b} \pm 5.2$	$21.7^{a} \pm 3.0$

† Values represent means of quadruplicate ± S.D. ‡ and triplicate ± S.D.

§ Data were analyzed by MANOVA and within each column different letters indicate statistically differences according to the Tukey multiple comparison test at 95% confidence level (TPC: total phenolic content; DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical assay; ORAC: oxygen radical absorbance capacity; ICA: iron(II) chelating ability; GAE: gallic acid equivalents; TE: Trolox equivalents).

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TPC (mg GAE L^{-1} extract): (A_{sample} -intercept) / slope (mg GAE L^{-1}).DF

DPPH$^{\bullet}$ (mmol TE L^{-1} extract): (A_{sample} -intercept) / slope (µM TE).DF. (0.05 L g^{-1} residue)

DPPH$^{\bullet}$ (mmol TE $100g^{-1}$ dry pomace): [(A_{sample} -intercept) / slope (µM TE).DF. (0.05 L g^{-1} residue). (g residue g^{-1} dry pomace)].100

ORAC (µmol TE g^{-1} residue): (relative AUC - intercept) / slope (µM TE). DF. (0.05 L g^{-1} residue)

ORAC (µmol TE g^{-1} dry pomace): (relative AUC - intercept) / slope (µM TE). DF. (0.05 L g^{-1} residue) . (g residue g^{-1} dry pomace)

ICA (%inh. (g L^{-1} extract) $^{-1}$);

Where: %ICA = [(A1-A2)/A1]. 100;

A1= A ($_{Fe}^{2+}$$_{-Ferrozine}$);

A2= A ($_{sample}$) - A($_{sample\ blank}$), then

%ICA= [%ICA/ (g residue / 0.05 L). DF] / 3 (3 because it were used 100 µl each solution in the well)

Paper II

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Jorge Queiroz, M. Beatriz P. P. Oliveira, Luís M. Cunha. Single and successive oxidative stress factors applied to mechanically deboned chicken meat: protective effect of grape pomace extract. [Submitted for publication].

Single and successive oxidative stress factors applied to mechanically deboned chicken meat: protective effect of grape pomace extract

Hernán H. Tournour[1,2], Marcela A. Segundo[3], Luís M. Magalhães[3], Jorge Queiroz[1], M. Beatriz P.P. Oliveira[4], Luís M. Cunha[1,*].

[1] *LAQV,REQUIMTE/DGAOT, Faculty of Sciences, University of Porto, Porto, Portugal.*

[2] *Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal.*

[3] *UCIBIO,REQUIMTE, Faculty of Pharmacy, Department of Chemistry, University of Porto, Porto, Portugal.*

[4] *LAQV,REQUIMTE, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal.*

**Corresponding author: lmcunha@fc.up.pt*
Campus Agrário de Vairão
Rua Padre Armando Quintas, 7
4486-661 Vairão
Portugal
Tel.: (+351)252660400 / Fax: (+351)252661780

ABSTRACT

The protective effect of grape pomace extract (GPE) added to mechanically deboned chicken meat (MDM) subjected to different oxidative factors: iron(II); UV-C ; modified atmosphere packaging and temperature, was assessed following single and successive exposure. Experimental design followed a four-factor nested ANOVA and compared with the effect of butylated hydroxytoluene (BHT). The effectiveness of Folin-Ciocalteu reducing (FCR), scavenging capacity against 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), oxygen radical absorbance capacity (ORAC) and iron(II) chelating ability (ICA) assays to evaluate changes in MDM was examined. All assays evaluated meat oxidation, exception for $DPPH^{\bullet}$, due to meat pigments interference at λ=517 nm. GPE (150 mg/kg) had a consistent protective effect, although lower than BHT (100 mg/kg). The antioxidant protection depended on the stress factor applied. Successive stress exposure affected antioxidants performance with similar behavior concerning ORAC. FCR,

ORAC and ICA assays were effective to monitor the oxidative stability in a meat model, highlighting the potential use of polyphenols from GPE as food preservative.

Keywords: antioxidant protection; degradation factors; meat model; polyphenols.

Highlights

- Mechanically deboned chicken meat was targeted as model for oxidative stability assessment.
- Different approaches were applied to monitor changes in MDM exposed to oxidative stress factors.
- $DPPH^{\cdot}$ assay is not adequate, as meat color pigments interfere in measurements.
- Antioxidant protection was stress-factor dependent.
- Grape pomace extract showed potential as food preservative.

1. Introduction

In the last decade, concern by consumers and researchers regarding food composition and the role diet plays in the prevention and treatment of health related issues has widely increased (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2010). The addition of natural antioxidants to foodstuffs represents a suitable alternative to synthetic additives, including butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). Moreover, chronic consumption of food supplemented with these compounds has been associated with toxic effects, especially cancer (Juntachote, Berghofer, Siebenhandl, & Bauer, 2006; Naveena, Sen, Vaithiyanathan, Babji, & Kondaiah, 2008). On the other hand, antioxidants obtained from natural sources such as vegetable and fruits tissues have lower toxicity and have been used to prevent the development of off-flavors, off-odor and off-color as consequences of lipid oxidation in meat and meat products (Karre, Lopez, & Getty, 2013; Shah, Bosco, & Mir, 2014).

Among the natural antioxidants, grape pomace -which is the solid waste remaining after grape pressing and fermentation composed by grape skins, seeds and stems-, contains a larger quantity of polyphenols when compared with other agro-food solid wastes (Makris, Boskou, & Andrikopoulos, 2007). In fact, according to "*Organisation Internationale de la Vigne et du Vin"* (OIV, 2011), over 260,000,000 hL of wine are produced worldwide. Additionally, it is estimated that for each 6 L of wine produced, 1 kg of pomace is generated, then, over 4.4 millions of tons (MT) of this by-product would be obtained. For this reason, these residues represent a low-cost natural source of bioactive compounds for the pharmaceutical and cosmetic industries, and particularly for food industry (Fontana, Antoniolli, & Bottini, 2013; Perumalla & Hettiarachchy, 2011).

Regarding to application of grape pomace extract (GPE) as food antioxidant, several studies have been performed in many types of meat and meat products, including raw beef and pork patties (Rojas & Brewer, 2008), cooked beef patties (Banon, Díaz, Rodríguez, Garrido, & Price, 2007), raw and cooked pork (Carpenter, O'Grady, O'Callaghan, O'Brien, & Kerry, 2007; Sasse, Colindres, & Brewer, 2009), cooked turkey meat (Mielnik, Olsen, Vogt, Adeline, & Skrede, 2006), raw and cooked chicken meat (Brannan, 2009; Lau & King, 2003; Selani et al., 2011; Shirahigue et al., 2010), and beef sausage (Kulkarni, DeSantos, Kattamuri, Rossi, & Brewer, 2011). This

research field has already provided some patents (Guzman Nieves, Antonio Eldar, & Carlos Antonio Eldar; Sharma, Srivastava, Gupta, & Prakash) and also commercial antioxidant products are available (ActiVin^{TM} and Gravinol Super^{TM}).

For almost all these works, the protective effects of natural antioxidants incorporated in food matrixes is mainly evaluated through the assessment of lipid oxidation (primary or secondary) markers such as thiobarbituric acid reactive substances (TBARS assay) (Carpenter et al., 2007; Lau & King, 2003), conjugated dienes/trienes (Choe et al., 2011), and volatile compounds (such as hexanal, pentanal, octanal) (Mielnik et al., 2006). On the other hand, the oxidation process of food products have been monitored through shell-life studies (Sasse et al., 2009) or applying stress conditions using different oxidative stress factors, such as iron (II), temperature and radiation (Brito et al., 2011; Chen & Ahn, 1998; Elhamirad & Zamanipoor, 2012). However, these studies did not evaluate the influence of these stress factors towards total antioxidant capacity of food samples and the activity of several stress factors in successive exposure was not investigated yet. Moreover, the food models used for induced lipid oxidation studies, including vegetable oils (Chen & Ahn, 1998; Erkan, Ayranci, & Ayranci, 2012) and fish oils (Luther et al., 2007), represent simple matrices where the influence of other food components were not considered as a whole.

Therefore, the aim of this work was to evaluate the impact of different oxidative stress factors (iron (II), UV-radiation, modified atmosphere packaging, temperature increase) following single and successive exposure on food oxidation process. As food model mechanically deboned chicken meat (MDM) was used, because it represents a complex matrix used in a wide variety of meat products and it is a perishable raw material susceptive to oxidative reactions. Moreover, the application of MDM as an oxidisable substrate is used here for the first time.

The oxidative stability of MDM supplemented with GPE obtained from "*Touriga franca*", Portuguese *Vitis vinifera* L. variety, or with synthetic antioxidant (BHT) was assessed through total antioxidant profile under several stress factors. For this, several antioxidant assays covering different antioxidant mechanisms including reducing capacity (Folin-Ciocalteu assay), synthetic radical scavenging capacity (DPPH$^{\bullet}$ assay), peroxyl radical scavenging capacity (ORAC assay) and iron(II) chelating ability assay were applied (Fontana et al., 2013; Gülçin, 2012; L. M. Magalhães, Ramos, Reis, &

Segundo, 2014). All these methodologies were performed in a high-throughput microplate format.

2. Material and Methods

2.1. Preparation of the grape pomace extract

Grape pomace comprising, seeds and skins from *Vitis vinifera* L. grape variety "*Touriga franca*", were used. A sample of 500 g was dried in an oven (Thermo Scientific) at 55 ºC, until reaching final moisture content lower than 5 g/100g. A grain grinder (Kenwood, New Lane, UK) was used to achieve a particle size of 2-3 mm. Extraction was performed as described by Shirahigue *et al*. (Shirahigue et al., 2010). After the extraction and filtration steps the liquid was concentrated in a vacuum rotary evaporator (Büchi, Flawil, Switzerland) at 65 ºC, until dryness. The dried remaining residue, once the solvent was completely evaporated, was redissolved in 50 mL of water and reserved under -80 ºC for further analysis.

2.2. Exposure of meat to stress factors

Mechanically deboned chicken meat (MDM) was chosen as meat system. The MDM was divided in three portions and two of them were supplemented with antioxidants (Table 1): Control: (MDM without addition of antioxidant); GPE (MDM + 150 mg/kg of GPE) and BHT (MDM + 100 mg/kg of BHT).

Each portion was submitted to four oxidative stress factors: iron(II), UV-C radiation, modified atmosphere packaging (MAP) and temperature, successively applied following a fully Nested experimental design (Zar, 1999). The development of the experiment was carried out as depicted in Fig. 1. First, a portion (30 g) of MDM was divided into two portions of 15 g each: one was reserved and the other was mixed with an iron(II) solution, in order to obtain a final concentration of 20 mg/kg. Next, half of each previous portions (7.5 g each of treated and non-treated) were exposed to UV-C (UV radiation system, Vilber Lourmat, Australia) consisting in 0.500 J/cm^2, λ = 254 nm, at a distance of 12 cm during 5 min (Marquenie et al., 2002). Those samples which did not receive UV-C radiation were exposed to regular laboratory fluorescent lights. Once UV exposition finished, meat samples were placed into Falcon tubes. Reproduction of the MAP was carried out by exposing meat to a stream of 40:60 % v/v

144 (O_2:N_2) gas mixture during 2 min, using a gas mixer (Gas Mixer, MAP mix 9000,
145 Dansensor, Denmark) connected into a glass chamber. Those samples to which oxygen
146 was not applied, were immediately packaged in oxygen barrier bags (polyethylene,
147 140×200 cm, 90 μm thickness, IdeaPack, Viseu, Portugal) and stored in vacuum
148 (Vacuum Packaging Machine, Sammic, Guipúzcoa, Spain) sealed packages. Finally,
149 meat samples were placed in two controlled temperature chambers at different
150 temperatures (4 ºC and 25 ºC) during 2 h. Once all treatments were performed, the
151 experiment was interrupted by freezing meat samples to -24 ºC. The same sequence was
152 exactly performed for each supplemented sample (Control, GPE and BHT).
153

Table 1
Stress factors applied to MDM samples.

Factor name	(+)	(-)
Addition of iron(II)	20 mg/kg	none
UV-C radiation	0.500 J/cm^2	none
MAP	40 % (v/v) O_2	Vacuum
Temperature	25 ºC	4 ºC

MDM: mechanically deboned chicken meat, MAP: modified atmosphere packaging

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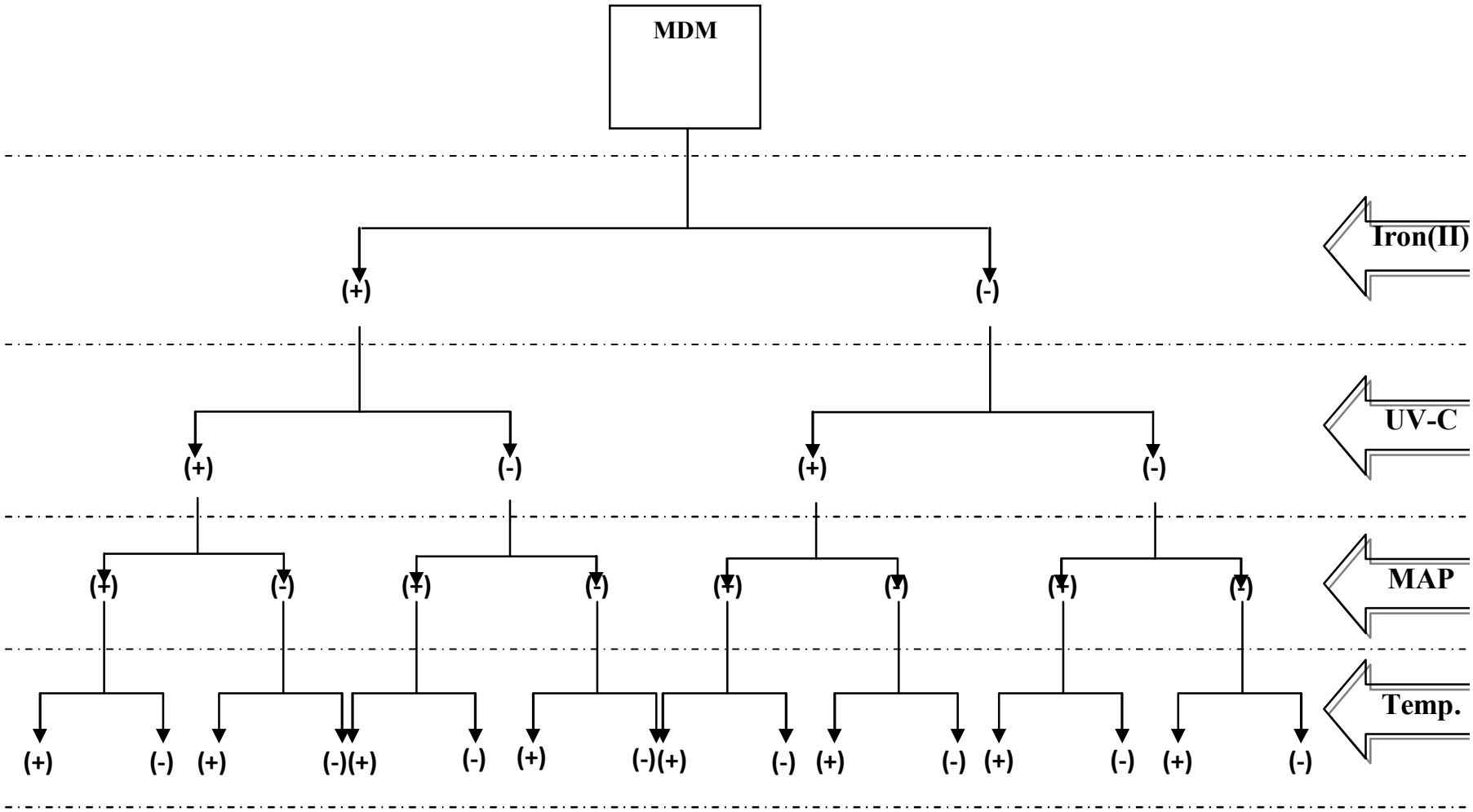

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Figure 1: Nested scheme applied for the assessment of the oxidative stability against iron(II), UV-radiation (UV-C), modified atmosphere packaging (MAP) and increase in temperature (T ºC). Symbols for oxidative stress factors (+) when applied and (-) when not applied, were used. Samples: Control (MDM without addition of antioxidant); GPE (MDM + 150 mg/kg) and BHT (MDM + 100 mg/kg); MDM: mechanically deboned chicken meat.

2.3. Meat extracts for analysis

Extracts for FCR, DPPH•, ORAC and ICA assays were prepared according to Qwele *et al.* 2013 (Qwele et al., 2013) with some modifications. One gram of each sample was homogenized with 10 mL of 0.05 M KH_2PO_4 phosphate buffer (pH 7). The extraction step was carried out alternating ultrasound (30 s, 3 times) and vortex cycles (2 min, 3 times at 3000 rpm). Before the last cycle, meat samples were left for 10 min to stand in order to assist in tissue hydration and improve the extraction. Finally, extracted samples were centrifuged at 5,580 x g for 30 min at 4 ºC. Supernatant aliquots (1 mL) were disposed in Eppendorf tubes. Extracts were frozen at -80 ºC until analysis by FCR, DPPH•, ORAC and ICA assays. Before performing each assay procedure, analyses were started mixing 200 µL of each extract and 200 µL absolute ethanol p. a. in order to guarantee the total dissolution of polyphenolic compounds.

2.3.1. Meat extracts

FCR reducing capacity (FCR)

The FCR was assessed employing a 96-well microplate Folin-Ciocalteu procedure (L. M. Magalhães, Santos, Segundo, Reis, & Lima, 2010; Singleton, Orthofer, & Lamuela-Raventos, 1999). Hence, 150 µL of gallic acid standard solution (1.0 - 15.0 mg/L) or diluted meat extracts (1:50 - 1:100, v/v) and 50 µL of F-C reagent (3:10, v/v) were placed in each well. After that, 100 µL of carbonate solution (9 g/100mL) was added and the reaction was monitored at 760 nm during 120 min. The FCR was expressed as mg of gallic acid equivalents per gram of meat ($Abs_{760\ nm} = 0.0504 \times$[gallic acid, (mg/L)] + 0.058, R>0.9997, $n = 6$).

Antioxidant capacity assessment

DPPH• assay

For microplate DPPH• methodology (Brand-Williams, Cuvelier, & Berset, 1995; L. M. Magalhães, Barreiros, Maia, Reis, & Segundo, 2012), 150 µL of Trolox standard solution (5.0 - 50.0 µmol/L) or diluted meat extracts (1:20 – 1:40, v/v) and 150 µL of DPPH• ethanolic solution (50 mL/100mL) were placed in each well. The DPPH• scavenging capacity was monitored at 517 nm during 120 min. The results were

expressed as mmol of Trolox equivalent (TE) per gram of meat ($\Delta Abs_{517\ nm}$ = 7.20×[Trolox, (mmol/L)] -0.056, R>0.9926, *n* = 5).

ORAC assay

For ORAC assay (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002; Wang, Jónsdóttir, & Ólafsdóttir, 2009), 100 µL of Trolox standard solution (1.0 – 7.5 µmol/L) or diluted meat extracts (1:125 – 1:250, v/v) and 100 µL of fluorescein (117 nmol/L) were placed in each well, the microplate was brought to under preincubation for 15 min at 37 ºC. Followed, 100 µL of AAPH solution (40 mmol/L) was added by rapidly using multichannel pipet and fluorescence intensity (λ_{exc} 485 nm, λ_{em} 520 nm) was monitored every minute during 240 min. Reaction carried out in 75 mmol/L phosphate buffer (pH 7.4) at 37 ºC. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve over the reaction time. The net AUC of the sample was calculated subtracting the AUC of the control. The regression equation between net AUC and Trolox concentration was determined, and the results were expressed as µmol of Trolox equivalents (TE) per gram of meat by interpolation (Net AUC (%) = 15.1×[Trolox, (µmol/L)] + 21.3, R>0.9983, *n* = 8).

Iron(II) chelating ability assay (ICA)

For iron(II) chelating ability assay (ICA) (Wang et al., 2009), 100 µL of diluted meat extracts (1:5 - 1:10, v/v) in acetate buffer (50 mmol/L, pH 4.6) were mixed with 100 µL Fe(II) solution (120 µmol/L) and placed in each well. After 5 min, 100 µL of ferrozine solution (600 µmol/L) was added to each well. Solutions were left standing 10 min at room temperature. Thereafter, the absorbance was monitored at 562 nm. The percentage of inhibition of ferrozine-iron(II) complex formation of each sample was calculated according to: ICA (%) = $[A_0 – (A_1 – A_2)] / A_0$ x 100; where A_0, A_1 and A_2 correspond to absorbance of the control, absorbance of sample and blank of the sample, respectively. In A_0 the intrinsic absorbance of iron(II) was subtracted from control absorbance. Results were expressed as % inhibition obtained per mg of meat.

2.4. **Statistical analysis**

Values for all possible combinations (4 factors at 2 levels each) for Control, GPE and BHT experiments (48 samples in total, 16 for each added antioxidant) were reported as

mean ± standard deviation (S.D.) for each antioxidant assay. Data regarding the effects of the different oxidative stress factors were assessed using a fully Nested (Hierarchical) ANOVA (Zar, 1999) with four factors organized as follows: iron(II), UV-C radiation (nested within iron(II)), MAP (nested within UV-C radiation), and temperature (nested within MAP). Statistical data analysis was performed with STATISTICA for Windows version 12.0 (STATISTICA 12 Software, StatSoft, Tulsa, OK). Moreover, multiple comparisons were performed using Tukey test at 95% confidence level, in order to identify significant differences between experiments.

3. Result and Discussion

3.1. GPE characterization

Information available in literature about pomace extracts from the grape cultivar used in this study is scarce. Nevertheless, our results (Table 2) are in agreement with the total phenolic content (TPC) and antioxidant properties of Portuguese wines (Cristino, Costa, Cosme, & Jordao, 2013; Jordao, Simoes, Correia, & Goncalves, 2012).

Table 2
Antioxidant capacity for GPE from "*Touriga franca*" (vintage 2012) Portuguese cultivar, used during the experiments

Antioxidant capacity	Mean ± S.D.
TPC (mg GAE g extract^{-1})	106.1 ± 1.9
DPPH$^{\cdot}$ (mmol TE g extract^{-1})	0.90 ± 0.02
ORAC (μmol TE g extract^{-1})	1325 ± 147
ICA (%Inhib. mg extract^{-1})	55 ± 14

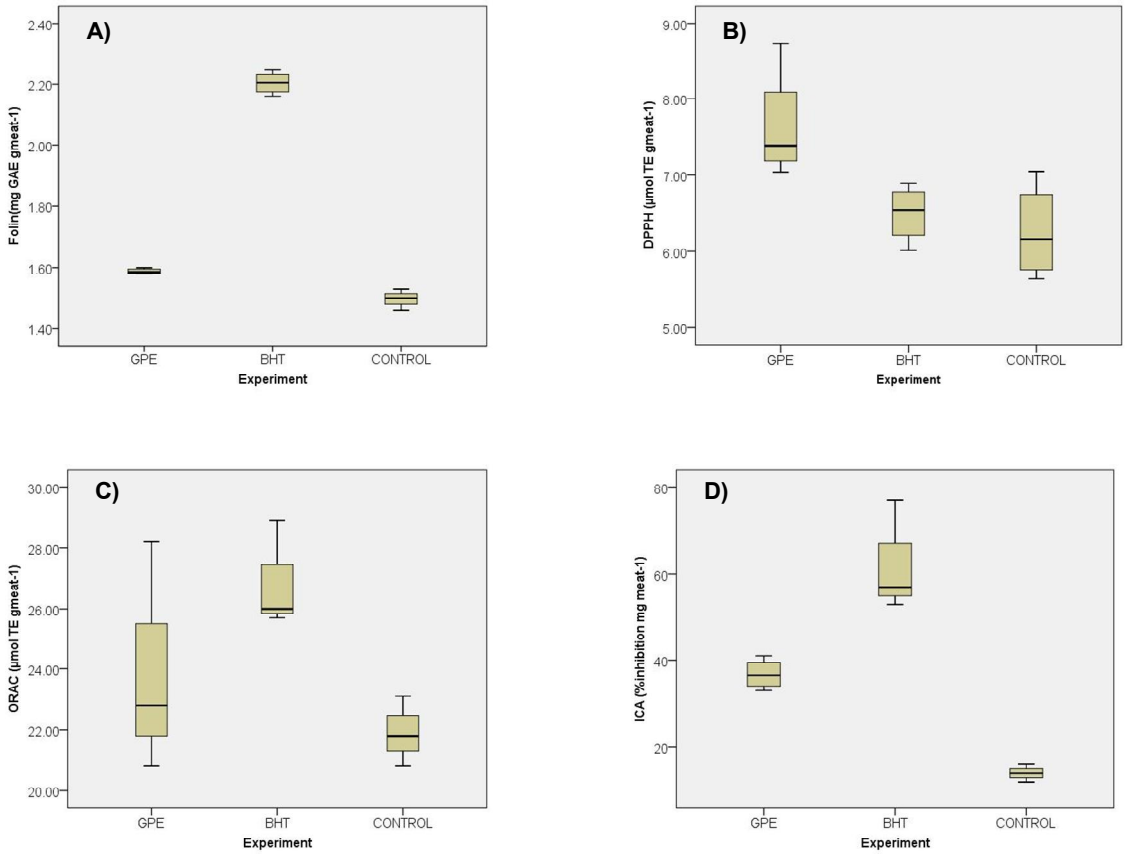
Values represent means ± standard deviation (S.D.) of triplicate (n = 3). TPC: Total phenolic content; DPPH$^{\cdot}$: 2,2-diphenyl-1-picrylhydrazyl; ORAC: oxygen reactive absorbance capacity; ICA: Iron(II) chelating ability; GAE: Gallic acid equivalents; TE: trolox equivalents.

3.2. *Nutritional and fatty acids composition for MDM raw material*

The composition of meat is dependent on many factors, namely, type, sex, age, diet of the animals, animal part -with/without skin-for the deboning process and also on operations settings (Hald & Baggesen, 2013; Püssa, Pällin, Raudsepp, Soidla, & Rei, 2008). Nutritional and fatty acids composition of MDM is shown in **Table S1**. The MDM proximate composition indicated 61.2%, 13.4%, 1.4% and 24.8% for moisture, protein, ashes and total fat, respectively and in agreement to previous reports (Henckel, Vyberg, Thode, & Hermansen, 2004; Püssa et al., 2009). Fatty acids profile is shown in **Table S1**. Palmitic acid was the main saturated fatty acid (SFA) in MDM raw material (24.0 ± 0.1%). In the case of monounsaturated fatty acids (MUFAs) oleic acid was detected in the highest concentration (41.37 ± 0.02%). Linoleic acid was the most predominant polyunsaturated fatty acid (PUFA) with a concentration of 15.2 ± 0.2%. Our results are in agreement to those by Trindade *et al.* (Trindade, Felício, & Castillo, 2004).

3.3. *Evaluation of methodologies to determine the oxidative stability in a meat model*

Data were obtained from different antioxidant assays (FCR, $DPPH^{\bullet}$, ORAC and ICA assays) for all possible combinations of meat and oxidative stress factors. Univariate tests of significance, effect sizes, and powers are presented in **Table S2.**



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257 **Figure 2**: Boxplots for **A)** FCR; **B)** DPPH; **C)** ORAC and **D)** ICA assays. FCR:
Folin-Ciocalteu reducing content; DPPH•: 2,2-diphenyl-1-picrylhydrazyl radical
258 assay, ORAC: oxygen radical absorbance capacity and ICA: iron(II) chelating
259 ability. MDM: mechanically deboned chicken meat; GPE: grape pomace extract.

260 According to results obtained through the DPPH• assay, it was not possible to evaluate
261 the oxidative stability once this assay was not able to distinguish possible differences
262 among experiments ($p = 0.59$, Table S2). Pigments present in MDM, mainly myoglobin
263 (Mb), can exist in any of the four redox states, namely: deoxymyoglobin (DeoxyMb),
264 oxymyoglobin (OxyMb), carboxymyoglobin (COMb), and metmyoglobin (MetMb),
265 depending on the ligands bound to haem iron and also on the iron redox state
266 (ferrous/Fe^{2+} or ferric/Fe^{3+}). Additionally, as MDM is not a fresh meat, DoexyMb is not
267 expectable in the samples. These pigments have an absorbance spectrum ranging from
268 500 to 600 nm. OxyMb has double peaks at 542 and 582 nm, whilst MetMb exhibits its
269 maximum peak at 503 nm (Tang, Faustman, & Hoagland, 2004). We hypothesize that
270 the lack of accuracy of DPPH• assay to distinguish differences among experiments may
271 be due to the existence of a spectra/absorption overlapping in the signal registered by
272 the method, once DPPH• assay is spectrophotometrically monitored at 517 nm, as well

as, the absorbance spectrum of the own colored phenolic compounds from GPE itself. Tang *et al.* observed an absorbance for Mb species (namely, DeoMb, MetMb and OxyMb) over 0.60 at 517 nm for a concetration of 0.11 mmol/L (Tang et al., 2004). Additionally, $DPPH^{\bullet}$ assay involves reactions based in a electron transfer mechanism, then, is highly influenced by solvent and pH of the reaction.The $DPPH^{\bullet}$ assay was performed in a 50mL/100mL ethanolic media which may lead to micro protein precipitations with latter interferences in the signal as previously described for samples like plasma by Magalhães *et al.* (L. M. Magalhães, Segundo, Reis, & Lima, 2008). The Folin-Ciocalteu assay is a method used for the measurement of the total phenolic content and it is based on the ability of certain compounds in alkaline medium to reduce the phosphomolybdic/phosphotungstic acid reagent to complexes, which are spectrophotometrically detected. Despite its non-specificity, it is operationally simple, reproducible, a rather standardized procedure and the absorption of the reaction product at a long-wavelength minimizes interferences from most sample matrixes (L. M. Magalhães et al., 2008). The ORAC assay is based on the intensity of fluorescence decrease of the target/probe along time under constant flux of peroxyl radicals in aqueous buffer. When a sample is analyzed in presence of chain-breaking antioxidants, the decay of fluorescence is inhibited (L. M. Magalhães et al., 2008). In the ICA assay, chelating compounds in the sample disrupt the complex formed between iron(II) and ferrozine. Therefore, the color decreasing on the iron(II)-ferrozine complex monitored at 562 nm is taken as an estimation of the chelating activity. FCR, ORAC and ICA assays showed a similar behavior as depicted in Fig. 2, registering lower protection conferred by GPE face to BHT, but consistently present in the assays. Moreover, based in these findings and with supporting statistical analysis about the effectivesness of the FCR, ORAC and ICA assays to identify significant differences among experiments ($p < 0.01$); three methodologies were applied to monitor the antioxidant performance upon exposure of MDM to stress factors, as showed in the following section.

3.4. Evaluation of GPE and BHT performance for protection of MDM against stress factors

Results presented in Table 3 allow the comparison upon the addition of GPE and BHT against a single stress factor. No significant differences ($p > 0.05$) were obtained against the addition of iron(II) as stress factor in ORAC values, whereas FCR and ICA values were significantly different. Iron and copper, are both considered as an important

306 catalyst for lipid oxidation reactions. As such, substances capable of chelating ions can
307 act as fat antioxidants (Hogan, Zhang, Li, Wang, & Zhou, 2009). Although less
308 pronounced than for Control samples, both antioxidants were affected by the presence
309 of iron, in FCR values. Chen & Ahn, reported trends of inhibition with increasing final
310 phenolic concentrations in an iron(II)-lipid oxidation (LO) system working with six
311 different polyphenols (Chen & Ahn, 1998). They also showed that caffeic acid (present
312 in our GPE, Table S3) was effective inhibitor of the iron(II)-LO. Additionally, they
313 experimented with BHT showing that the ability of polyphenolic compounds in
314 chelating Fe was not the most critical for phenolics to inhibit iron(II)-LO, as BHT (not
315 chelating agent) had low IC_{50} (TEAC) (Chen & Ahn, 1998). Concerning UV-C,
316 significant differences were obtained for all methodologies when samples were exposed
317 to UV-C as stress factor. Based on the results, we hypothesize that UV-C originate
318 reactive species with unsaturated fatty acids as target, although in the presence of
319 phenolic compounds from GPE. These bioactive compounds are significantly reduced,
320 affecting the protection they confer to the fluorescent probe oxidation in ORAC assay.

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Table 3
Influence of a single oxidative stress factor on MDM experiments, measurement through FCR, ORAC and ICA assays

Factor	Experiment	FCR (mg GAE g meat^{-1})		ORAC (μmol TE g meat^{-1})		ICA (% inhibition mg meat^{-1})	
		Mean	SD	Mean	SD	Mean	SD
Fe	Control	1.50^{d}	0.03	21.9^{a}	1.2	14^{c}	2
	Control+Fe	1.31^{e}	0.02	18.9^{a}	2.2	33^{b}	6
	GPE	1.59^{c}	0.01	23.9^{a}	3.8	37^{b}	4
	GPE+Fe	1.48^{d}	0.02	25.7^{a}	2.2	58^{a}	3
	BHT	2.21^{a}	0.04	26.9^{a}	1.8	62^{a}	13
	BHT+Fe	1.80^{b}	0.03	23.3^{a}	5.9	29^{b,c}	6
UV	Control	1.50^{c}	0.03	21.9^{a,b}	1.2	14^{c}	2
	Control+UV	1.43^{d}	0.02	12.8^{c}	1.9	32^{b}	3
	GPE	1.59^{b}	0.01	23.9^{a,b}	3.8	37^{b}	4
	GPE+UV	1.61^{b}	0.03	15.6^{b,c}	3.0	56^{a}	4
	BHT	2.21^{a}	0.04	26.9^{a}	1.8	62^{a}	13
	BHT+UV	1.49^{c,d}	0.01	22.7^{a,b}	5.3	31^{b}	6
MAP	Control	1.50^{c}	0.03	21.9^{a,b}	1.2	14^{c}	2
	Control+MAP	1.39^{d}	0.02	20.7^{a,b}	2.9	34^{b}	3
	GPE	1.59^{b}	0.01	23.9^{a,b}	3.8	37^{b}	4
	GPE+ MAP	1.46^{c}	0.03	13.7^{c}	1.1	35^{b}	2
	BHT	2.21^{a}	0.04	26.9^{a}	1.8	62^{a}	13
	BHT+ MAP	1.38^{d}	0.01	17.8^{b,c}	1.4	34^{b}	6
Temperature	Control	1.50^{d}	0.03	21.9^{a,b}	1.2	14^{c}	2
	Control+T	1.64^{c}	0.04	26.2^{a}	1.2	17^{c}	4
	GPE	1.59^{c}	0.01	23.9^{a,b}	3.8	37^{b}	4
	GPE+T	1.48^{d}	0.03	18.9^{b,c}	2.5	41^{b}	4
	BHT	2.21^{a}	0.04	26.9^{a}	1.8	62^{a}	13
	BHT+T	1.77^{b}	0.03	15.1^{c}	0.8	36^{b}	4

Data were analyzed using a fully nested ANOVA with four factors; within each column for each factor tested, different letters indicate statistically significant differences according to the Tukey multiple comparison test at 95% confidence level (FCR: Folin-Ciocalteu reducing content; ORAC: oxygen radical absorbance capacity; ICA: iron(II) chelating ability; GAE: gallic acid equivalents). Mean ± S.D. (n = 8).

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Therefore, under advanced scission and degradation processes, proteins from MDM may be exposed to further spoilage, generating smaller peptides with chelating properties (Storcksdieck, Bonsmann, & Hurrell, 2007), as it was verified by the increase of ICA values for Control (14 to 32 % inhibition mg meat^{-1}) and GPE (37 to 56 % inhibition mg meat^{-1}) added samples. MAP is a common operation applied to meat in order to protect its pigments from the discoloration, although it increases the oxidative processes (Faustman, Sun, Mancini, & Suman, 2010). Significant differences were observed for all assays, with the Control exhibiting a similar behavior when UV-C was applied, regarding ICA assay. GPE and BHT added samples showed a stronger reduction when exposed to MAP, in comparison with UV-C exposure results, registered by FCR and ORAC values. Additionally, GPE reduction (1.59 to 1.46 mg GAE g meat^{-1}), although significant when compared to the Control, was less strong than BHT (2.21 to 1.38 mg GAE g meat^{-1}) for the previous factor. GPE is composed by anthocyanins, flavonoids, phenolic acids and resveratrol (Teixeira et al., 2014). The antioxidant efficiency of phenols is structure-dependent on their hydrogen-donating ability, which is directly related to the number of phenolic hydroxyl moieties present. Intermediate radicals generated during the lipid oxidation can be stabilized by the resonance delocalization of the electron within the aromatic ring(s) (Gheldof & Engeseth, 2002; Rice-Evans, Miller, & Paganga, 1996). This fact explains the differences between GPE and BHT which has only one aromatic ring not providing the stabilization effects accomplished by more complex structures as presented by polyphenols. Temperature increase leads to the acceleration of reactions, as radical-mediated chain reaction of lipid oxidation was reported to progress in a temperature-dependent fashion (Gatellier, Sante-Lhoutellier, Portanguen, & Kondjoyan, 2009). ICA assay registered significant strong reduction for BHT (62 to 36% inhibition mg meat^{-1}), whilst no significant differences observed for both Control and GPE. Stronger reductions were registered for BHT added samples, compared to GPE, in FCR and ORAC values. Temperature acts differently on polyphenolic compounds depending on their concentrations with other components of studied system (Brewer, 2011).

Regarding other assays, FCR indicated presence of reducing species, lower for GPE when compared to BHT (Fig. 2 A). ORAC methodology showed that protection was conferred by both GPE and BHT against ROO$^{\bullet}$ oxidation, but it was lower for GPE compared with BHT. The ratio between pro-oxidant ions (iron(II)) and antioxidant

357 concentrations may explain the differences of BHT and GPE performances.
358 Additionally, during the chain reactions taking place upon lipid oxidation, the presence
359 of metal ions can participate as strong pro-oxidant agent, generating radical species
360 from Fenton-type reaction, attacking the fluorescent probe used in ORAC assay or
361 degrading polyphenolic compounds. So, the ability of a compound to inhibit fluorescein
362 oxidation could be influenced by its interactions with pro-oxidants or other antioxidants
363 (Nkhili & Brat, 2011).

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Table 4
Influence of successive exposure to oxidative stress factors on MDM experiments, measurement through FCR, ORAC and ICA assays

Experiment	FCR (mg GAE g meat^{-1})		ORAC (μmol TE g meat^{-1})		ICA (% inhibition mg meat^{-1})	
	Mean	SD	Mean	SD	Mean	SD
Control	1.50^{b}	0.03	21.9^{a}	1.2	14^{c}	2
Control+Fe	1.31^{d}	0.02	18.9^{a}	2.2	33^{b}	6
Control+Fe+UV	1.36^{c}	0.02	27.2^{a}	6.7	32^{b}	5
Control+Fe+UV+MAP	1.39^{c,d}	0.02	20.7^{a}	2.9	34^{b}	3
Control+Fe+UV+ MAP +T	1.72^{a}	0.03	24.9^{a}	2.7	65^{a}	3
GPE	1.59^{b}	0.01	23.9^{a}	3.8	37^{c}	4
GPE+Fe	1.48^{c}	0.02	25.7^{a}	2.2	58^{b}	3
GPE+Fe+UV	1.29^{c}	0.01	25.6^{a}	3.4	33^{c}	3
GPE+Fe+UV+ MAP	1.75^{a}	0.03	29.3^{a}	3.5	70^{a}	5
GPE+Fe+UV+ MAP +T	1.41^{d}	0.02	13.2^{b}	0.8	14^{d}	1
BHT	2.21^{a}	0.04	26.9^{a,b}	1.8	62^{a}	13
BHT+Fe	1.80^{b}	0.03	23.3^{a,b}	5.9	29^{b}	6
BHT+Fe+UV	1.71^{c,d}	0.02	29.8^{a}	1.5	34^{b}	5
BHT+Fe+UV+ MAP	1.68^{d}	0.03	19.6^{b,c}	2.5	32^{b}	3
BHT+Fe+UV+ MAP +T	1.77^{b,c}	0.02	14.6^{c}	1.4	50^{a}	4

Data were analyzed using a fully nested ANOVA with four factors; within each column different letters indicate statistically significant differences according to the Tukey multiple comparison test at 95% confidence level (FCR: Folin-Ciocalteu reducing content; ORAC: oxygen radical absorbance capacity; ICA: iron(II) chelating ability; GAE: gallic acid equivalents). Mean ± S.D. (n = 8).

365 The successive exposure to the oxidative stress factors causes changes depending on the
366 components of the matrix and severity of the conditions applied (e.g., exposure time,
367 radiation energy) as shown in Table 4. For Control, although no significant changes

were registered in ORAC values, a significant increase in the final values for FCR (1.50 to 1.72 mg GAE g meat^{-1}) and ICA (14 to 65 % inhibition mg meat^{-1}) assays was observed. Increased ICA values recorded for Control in two situations can ascribed to an "artificial" incorporation of iron. Therefore, in the ICA assay; ferrozine can complex with the “extra” iron, increasing the signal in the determination (Wang et al., 2009). Furthermore, when all stress factors were applied, the higher value found for ICA may be due to protein degradation, generating peptide compounds capable of chelating iron, amplifying the signal for ICA assay. Regarding GPE added samples, although an increase in ICA values was also recorded after the addition of iron (37 to 58% inhibition mg meat^{-1}), this increase was lower than the one observed for Control sample. In ICA assay, ferrozine complexes with iron through a competitive reaction with chelating substances present in the sample under evaluation. Hence, polyphenols from GPE compete with ferrozine in the formation of the colored complex (Gulcin, 2010). Samples with added BHT had a similar behavior when compared with Control, regarding ICA values. BHT addition to MDM samples presents an exception, exerting high initial chelating ability (62% inhibition mg meat^{-1}). It is important to note the similar behavior of the natural antioxidant present in GPE (23.9 to 13.2 µmol TE g meat^{-1}) and BHT (26.9 to 14.6 µmol TE g meat^{-1}), in the reduction of ORAC values, when successive factors stress were applied. It is noteworthy that reactions like Fenton´s reaction could also take place in the medium, even more when iron is added before exposure to UV-C radiation. Alnaizy & Akgerman (Alnaizy & Akgerman, 2000), have worked in the advanced oxidation of phenolic compounds using hydrogen peroxide in presence of UV-radiation. They found that 40 mg/kg of initial phenolics concentration, was reduced to 90 % at 27 ± 2 º C, after 20 min. Thus, under experimental conditions, the remaining polyphenolic concentration in the MDM samples supplemented with GPE after 5 min of exposure to UV-C may be not enough to face further stress.

4. Conclusions

It was possible to monitor the oxidative stability in a meat model by FCR, ORAC and ICA assays. Valuable information from each of the methodologies was obtained, covering total reducing content, scavenging properties against peroxyl radical and also ion binding ability, often underestimated in lipid oxidation studies. Good correlation in

food systems between FCR and ORAC can be determined highlighting the importance of these assays. The antioxidant effectiveness was stress-factor and antioxidant dependent, for instance, BHT added samples exerted better protection than GPE against UV as stress factor, measured through ORAC values; whilst protection conferred by GPE added samples was higher than BHT against the temperature increase, as depicted by ICA values. Additionally, it seems that a successive exposure to stress conditions affects the final antioxidant performance, causing similar behavior in ORAC values under GPE (23.9 to 13.2 μmol TE g meat^{-1}), and BHT (26.9 to 14.6 μmol TE g meat^{-1}) protection effect. Further studies conducted with different GPE levels would provide information about concentration issues. Finally, because real setting conditions for food preservation are expected to be less severe than those used in the present research, it is expected that grape pomace extracts will even exhibit higher antioxidant protection under conventional conditions of food storage. This highlights the potential of polyphenolic and phenolic acid compounds as preservatives.

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References

Alnaizy, R., & Akgerman, A. (2000). Advanced oxidation of phenolic compounds. *Advances in Environmental Research, 4*(3), 233-244.

Banon, S., Díaz, P., Rodríguez, M., Garrido, M. D., & Price, A. (2007). Ascorbate, green tea and grape seed extracts increase the shelf life of low sulphite beef patties. *Meat Science, 77*(4), 626-633.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology, 28*(1), 25-30.

Brannan, R. G. (2009). Effect of grape seed extract on descriptive sensory analysis of ground chicken during refrigerated storage. *Meat Science, 81*(4), 589-595.

Brewer, M. S. (2011). Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Comprehensive Reviews in Food Science and Food Safety, 10*(4), 221-247.

Brito, P. P., Azevedo, H., Cipolli, K. M. V. A. B., Fukuma, H. T., Mourão, G. B., Roque, C. V., et al. (2011). Effect of the Gamma Radiation Dose Rate on Psychrotrophic Bacteria, Thiobarbituric Acid Reactive Substances, and Sensory Characteristics of Mechanically Deboned Chicken Meat. *Journal of Food Science, 76*(2), S133-S138.

Carpenter, R., O'Grady, M. N., O'Callaghan, Y. C., O'Brien, N. M., & Kerry, J. P. (2007). Evaluation of the antioxidant potential of grape seed and bearberry extracts in raw and cooked pork. *Meat Science, 76*(4), 604-610.

Cristino, R., Costa, E., Cosme, F., & Jordao, A. M. (2013). General phenolic characterisation, individual anthocyanin and antioxidant capacity of matured red wines from two Portuguese Appellations of Origins. *Journal of the Science of Food and Agriculture, 93*(10), 2486-2493.

Chen, X., & Ahn, D. U. (1998). Antioxidant activities of six natural phenolics against lipid oxidation induced by Fe2+ or ultraviolet light. *JAOCS, Journal of the American Oil Chemists' Society, 75*(12), 1717-1721.

Choe, J.-H., Jang, A., Lee, E.-S., Choi, J.-H., Choi, Y.-S., Han, D.-J., et al. (2011). Oxidative and color stability of cooked ground pork containing lotus leaf (Nelumbo nucifera) and barley leaf (Hordeum vulgare) powder during refrigerated storage. *Meat Science, 87*(1), 12-18.

Elhamirad, A. H., & Zamanipoor, M. H. (2012). Thermal stability of some flavonoids and phenolic acids in sheep tallow olein. *European Journal of Lipid Science and Technology, 114*(5), 602-606.

Erkan, N., Ayranci, G., & Ayranci, E. (2012). Lipid oxidation inhibiting capacities of blackseed essential oil and rosemary extract. *European Journal of Lipid Science and Technology, 114*(2), 175-184.

Faustman, C., Sun, Q., Mancini, R., & Suman, S. P. (2010). Myoglobin and lipid oxidation interactions: Mechanistic bases and control. *Meat Science, 86*(1), 86-94.

Fontana, A. R., Antoniolli, A., & Bottini, R. (2013). Grape Pomace as a Sustainable Source of Bioactive Compounds: Extraction, Characterization, and Biotechnological Applications of Phenolics. *Journal of Agriculture and Food Chemistry, 61*(38), 8987-9003.

Gatellier, P., Sante-Lhoutellier, V., Portanguen, S., & Kondjoyan, A. (2009). Use of meat fluorescence emission as a marker of oxidation promoted by cooking. *Meat Science, 83*(4), 651-656.

Gheldof, N., & Engeseth, N. J. (2002). Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *Journal of Agricultural and Food Chemistry, 50*(10), 3050-3055.

Gulcin, I. (2010). Antioxidant properties of resveratrol: A structure-activity insight. [Article]. *Innovative Food Science & Emerging Technologies, 11*(1), 210-218.

Gülçin, I. (2012). Antioxidant activity of food constituents: An overview. *Archives of Toxicology, 86*(3), 345-391.

Guzman Nieves, C. A., Antonio Eldar, G. N. C., & Carlos Antonio Eldar, G. N. WO2011062468-A2; MX2009012494-A1; WO2011062468-A3; MX318099-B.

Hald, T., & Baggesen, D. L. (2013). EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the public health risks related to mechanically separated meat (MSM) derived from poultry and swine: European Food Safety Authority.

Henckel, P., Vyberg, M., Thode, S., & Hermansen, S. (2004). Assessing the quality of mechanically and manually recovered chicken meat. *LWT - Food Science and Technology, 37*(6), 593-601.

Hogan, S., Zhang, L., Li, J., Wang, H., & Zhou, K. (2009). Development of antioxidant rich peptides from milk protein by microbial proteases and analysis of their effects on lipid peroxidation in cooked beef. *Food Chemistry, 117*(3), 438-443.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *Journal of Agricultural and Food Chemistry, 50*(16), 4437-4444.

Jordao, A. M., Simoes, S., Correia, A. C., & Goncalves, F. J. (2012). Antioxidant activity evolution during Portuguese red wine vinification and their relation with the proanthocyanidin and anthocyanidin composition. *Journal of Food Processing and Preservation, 36*(4), 298-309.

Juntachote, T., Berghofer, E., Siebenhandl, S., & Bauer, F. (2006). The antioxidative properties of Holy basil and Galangal in cooked ground pork. *Meat Science, 72*(3), 446-456.

Karre, L., Lopez, K., & Getty, K. J. K. (2013). Natural antioxidants in meat and poultry products. *Meat Science, 94*(2), 220-227.

Kulkarni, S., DeSantos, F. A., Kattamuri, S., Rossi, S. J., & Brewer, M. S. (2011). Effect of grape seed extract on oxidative, color and sensory stability of a pre-cooked, frozen, re-heated beef sausage model system. *Meat Science, 88*(1), 139-144.

Lau, D. W., & King, A. J. (2003). Pre- and Post-Mortem Use of Grape Seed Extract in Dark Poultry Meat To Inhibit Development of Thiobarbituric Acid Reactive Substances. *Journal of Agriculture and Food Chemistry, 51*(6), 1602-1607.

Luther, M., Parry, J., Moore, J., Meng, J., Zhang, Y., Cheng, Z., et al. (2007). Inhibitory effect of Chardonnay and black raspberry seed extracts on lipid oxidation in fish oil and their radical scavenging and antimicrobial properties. *Food Chemistry, 104*(3), 1065-1073.

Magalhães, L. M., Barreiros, L., Maia, M. A., Reis, S., & Segundo, M. A. (2012). Rapid assessment of endpoint antioxidant capacity of red wines through microchemical methods using a kinetic matching approach. *Talanta, 97*, 473-483.

Magalhães, L. M., Ramos, I. I., Reis, S., & Segundo, M. A. (2014). Antioxidant profile of commercial oenological tannins determined by multiple chemical assays. *Australian Journal of Grape and Wine Research, 20*(1), 72-79.

Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S., & Lima, J. L. (2010). Rapid microplate high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity. *Talanta, 83*(2), 441-447.

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Analytical Chimica Acta, 613*(1), 1-19.

Makris, D. P., Boskou, G., & Andrikopoulos, N. K. (2007). Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. *Journal of Food Composition and Analysis, 20*(2), 125-132.

Marquenie, D., Michiels, C., Geeraerd, A., Schenk, A., Soontjens, C., Van Impe, J., et al. (2002). Using survival analysis to investigate the effect of UV-C and heat treatment on storage rot of strawberry and sweet cherry. *International Journal of Food Microbiology, 73*(2), 187-196.

Mielnik, M. B., Olsen, E., Vogt, G., Adeline, D., & Skrede, G. (2006). Grape seed extract as antioxidant in cooked, cold stored turkey meat. *LWT - Food Science and Technology, 39*(3), 191-198.

Naveena, B., Sen, A., Vaithiyanathan, S., Babji, Y., & Kondaiah, N. (2008). Comparative efficacy of pomegranate juice, pomegranate rind powder extract and BHT as antioxidants in cooked chicken patties. *Meat Science, 80*(4), 1304-1308.

Nkhili, E., & Brat, P. (2011). Reexamination of the ORAC assay: effect of metal ions. *Analytical and Bioanalytical Chemistry, 400*(5), 1451-1458.

Perumalla, A. V. S., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts - Potential applications in food safety and quality. *Food Research International, 44*(4), 827-839.

Püssa, T., Pällin, R., Raudsepp, P., Soidla, R., & Rei, M. (2008). Inhibition of lipid oxidation and dynamics of polyphenol content in mechanically deboned meat supplemented with sea buckthorn (Hippophae rhamnoides) berry residues. *Food Chemistry, 107*(2), 714-721.

Püssa, T., Raudsepp, P., Toomik, P., Pällin, R., Mäeorg, U., Kuusik, S., et al. (2009). A study of oxidation products of free polyunsaturated fatty acids in mechanically deboned meat. *Journal of Food Composition and Analysis, 22*(4), 307-314.

Qwele, K., Hugo, A., Oyedemi, S. O., Moyo, B., Masika, P. J., & Muchenje, V. (2013). Chemical composition, fatty acid content and antioxidant potential of meat from goats supplemented with Moringa (Moringa oleifera) leaves, sunflower cake and grass hay. *Meat Science, 93*(3), 455-462.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*(7), 933-956.

Rojas, M. C., & Brewer, M. S. (2008). Effect of natural antioxidants on oxidative stability of frozen, vacuum-packaged beef and pork. *Journal of Food Quality, 31*(2), 173-188.

Sasse, A., Colindres, P., & Brewer, M. S. (2009). Effect of Natural and Synthetic Antioxidants on the Oxidative Stability of Cooked, Frozen Pork Patties. [Article]. *Journal of Food Science, 74*(1), S30-S35.

Selani, M. M., Contreras-Castillo, C. J., Shirahigue, L. D., Gallo, C. R., Plata-Oviedo, M., & Montes-Villanueva, N. D. (2011). Wine industry residues extracts as natural antioxidants in raw and cooked chicken meat during frozen storage. *Meat Science, 88*(3), 397-403.

572 Shah, M. A., Bosco, S. J. D., & Mir, S. A. (2014). Plant extracts as natural antioxidants
573 in meat and meat products. *Meat Science, 98*(1), 21-33.
574 Sharma, G., Srivastava, A. K., Gupta, C., & Prakash, D. IN201102860-I1.
575 Shirahigue, L. D., Plata-Oviedo, M., de Alencar, S. M., d'Arce, M. A. B. R., de Souza
576 Vieira, T. M. F., Oldoni, T. L. C., et al. (2010). Wine industry residue as
577 antioxidant in cooked chicken meat. *International Journal of Food Science &*
578 *Technology, 45*(5), 863-870.
579 Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total
580 phenols and other oxidation substrates and antioxidants by means of Folin-
581 Ciocalteu reagent. *Methods In Enzymology, 299*, 152-178.
582 Storcksdieck, S., Bonsmann, G., & Hurrell, R. (2007). Iron-Binding Properties, Amino
583 Acid Composition, and Structure of Muscle Tissue Peptides from in vitro
584 Digestion of Different Meat Sources. *Journal of Food Science, 72*(1), S019-
585 S029.
586 Suman, S. P., & Joseph, P. (2013). Myoglobin Chemistry and Meat Color. *Annual*
587 *Review of Food Science and Technology, 4*(1), 79-99.
588 Tang, J., Faustman, C., & Hoagland, T. (2004). Krzywicki revisited: Equations for
589 spectrophotometric determination of myoglobin redox forms in aqueous meat
590 extracts. *Journal of Food Science, 69*(9), C717-C720.
591 Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., et
592 al. (2014). Natural Bioactive Compounds from Winery By-Products as Health
593 Promoters: A Review. *International Journal of Molecular Sciences, 15*(9),
594 15638-15678.
595 Trindade, M. A., Felício, P. E. d., & Castillo, C. J. C. (2004). Mechanically separated
596 meat of broiler breeder and white layer spent hens. *Scientia Agricola, 61*, 234-
597 239.
598 Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., & Pérez-Álvarez, J. A.
599 (2010). Spices as Functional Foods. *Critical Reviews in Food Science and*
600 *Nutrition, 51*(1), 13-28.
601 Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical
602 scavenging and metal chelation of extracts from Icelandic seaweeds. *Food*
603 *Chemistry, 116*(1), 240-248.
604 Zar, J. H. (1999). *Biostatistical analysis* (4th ed.): Pearson Education India.

Supplementary information

Single and successive oxidative stress factors applied to mechanically deboned chicken meat: protective effect of grape pomace extract

Hernán H. Tournour[1,2], Marcela A. Segundo[3], Luís M. Magalhães[3], Jorge Queiroz[1], M. Beatriz P.P. Oliveira[4], Luís M. Cunha[1,*].

[1]LAQV,REQUIMTE/DGAOT, Faculty of Sciences, University of Porto, Porto, Portugal.

[2]Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal.

[3]UCIBIO,REQUIMTE, Faculty of Pharmacy, Department of Chemistry, University of Porto, Porto, Portugal.

[4]LAQV,REQUIMTE, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal.

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*Corresponding author: lmcunha@fc.up.pt. Tel.: (+351)252660400

Reagent and solutions

Stock standard solutions for HPLC analysis, were prepared accurately weighing each compound and by dissolving them in an appropriate solvent (ethanol or water) to a final concentration of 1000 mg/L. Working solutions were prepared from stock standard solutions in mobile phase (5 and 2.5 mg/L; 15 standards mixture). HPLC grade acetic acid and acetonitrile (Aldrich, Milwaukee, WI) were used. All solutions were filtered through a 0.45 μm membrane and degassed in ultrasound. Fatty acid methyl esters (FAME) mixture 37 patterns (Supelco, Bellefonte, PA) were used for the fatty acids profile determination. Boron trifluoride (BF_3), *n*-heptane (C_7H_{16}) and anhydrous sodium sulfate (Na_2SO_4) were purchased in Sigma Aldrich (St. Louis, MO, USA). Water from Sartorius (Goettingen, Germany) (resistivity > 18 MΩ cm) and absolute ethanol p. a. (Panreac Química, Spain) were used in the preparation of all solutions.

Chemical assays

Characterization of MDM raw material

Nutritional components were determined as percentages of moisture, protein, ashes and total fat according to Association of Official Analytical Chemists methodologies (AOAC methods: 985.14; 928.08; 920.153; 991.36, respectively) (AOAC, 1990) in MDM raw material.

The fatty acids proportion and the level of un-saturations are determinant for the oxidation degree in fat. Therefore, the assessment of the fatty acids composition was carried out using gas chromatography with flame ionization detector (GC/FID). The fatty fraction was firstly extracted according to Folch method (Folch, Lees, & Sloane-Stanley, 1957), with dichloromethane instead of chloroform. For this, sample was weighed (1 g) into a Falcon tube, followed by addition of 10 mL dichloromethane: methanol (2:1) solution. After homogenization in vortex, the sample was placed in an ultrasound bath for 10 min and centrifuged (3000 rpm, 5 min). Supernatant was displayed in a second Falcon tube where 1 g/100mL NaCl aqueous solution was added in a 1:5 proportion related to the supernatant volume. Sample was homogenized in a vortex and centrifuged again (3000 rpm, 5 min), rejecting the upper portion (aqueous phase) in this case. The same procedure was repeated after addition of anhydrous sodium sulfate. The total fat (organic phase) was placed into a vial tube with 100 μL of

0.01 g/100mL BHT solution. The fatty acids derivatization into fatty acid methyl esters (FAME) was performed according to Shantha & Ackman (Shantha & Ackman, 1990), with few modifications. The organic phase obtained above was mixed with 500 µL of 0.5 mol/L KOH in methanol solution. The sample was homogenized and heated (100 ºC, 10 min), followed by cooling into ice, and where 2.5 mL of boron trifluoride (14 mL/100mL in methanol) was added. Sample was again homogenized, heated (100 ºC, 30 min), cooled in ice, and 2 mL *n*-heptane added by. The last steps of the derivatization consisted in a separation of the upper phase into a new vial tube with subsequent agitation and centrifugation. Excess water was removed with anhydrous sodium sulfate. Results were expressed as mass percentages of each fatty acid of its methyl esters or area under the curve (AUC) due to the AUC obtained are equivalents to FAME mass, based on: $\%_A = [(AUC_A \times 100)]^{-1}\Sigma$ (AUC peaks), $_A$ represents a FAME compound.

For the data analises Maitre software (JMBS Developments, Grenoble, France) was used.

HPLC analysis

The phenolic profile for ethanol/water extracts and aqueous suspensions were obtained using an analytical HPLC unit (Jasco, Easton, USA) comprising: pump, automatic injector, DAD, equipped with a Kinetex (250 × 4.6 mm; 5 µm particle size; C18; 100 Å) core-shell column, controlled by Chrom-Nav software. The HPLC characterization was performed according to Kammerer *et al.* (Kammerer, Claus, Carle, & Schieber, 2004), as following:

Phenolic acid *(PA)* method: the mobile phase consisted of 2% (v/v) aqueous acetic acid (eluent A) and 0.5% (v/v) aqueous acetic acid and acetonitrile (50:50, v/v; eluent B) using the following gradient program: from 10 to 15% B (10 min), 15% B isocratic (3 min), from 15 to 25% B (7 min), from 25 to 55% B (30 min), from 55 to 100% B (1 min), 100% B isocratic (5 min), from 100 to 10% B (10 min), with total run time of 67 min.

Anthoxanthins and Stilbenes (*AX*) method: the mobile phase consisted of the same eluents as described above using instead the following gradient program: from 10 to 24% B (20 min), from 24 to 30% B (20 min), from 30 to 55% B (20 min), from 55 to 100% B (15 min), 100% B isocratic (8 min), from 100 to 10% B (2 min), with a total run time of 95 min. For both methods, the injection volume was 10 µL and the

92 absorbance was monitored at three monitoring channels (280, 320 and 370 nm). The
93 flow rate was 1.0 mL/min. The peaks detected in the samples were first compared with
94 respect to retention time and the spectral data with those in the standards mixture.
95 Quantification was performed based on the molar absorptivity (ε, L/mol) values for
96 each compound, according to chromatography peak area, molar mass and standard
97 concentrations. Each sample was injected in duplicate. Results were expressed as means
98 in milligrams GAE per gram of residue (mg GAE g residue $^{-1}$).

99

Table S1
Proximate composition and fatty acids profile of MDM raw material

Measurement		% (w/w)	
Moisture		61.2 ± 1.0	
Protein		13.4 ± 0.2	
Ashes		1.4 ± 0.1	
Total fat		24.8 ± 1.3	
Nomenclature	**Fatty acids**	**% Total fatty acid content**	**mg 100g fat^{-1}**
Myristic acid	C14:0	0.60 ± 0.02	5.96
Palmitic acid	C16:0	24.0 ± 0.1	240.22
Cis-7 hexadecenoic acid	C16:1 n-*9*	0.58 ± 0.001	5.79
Palmitoleic acid	C16:1 n-*7*	6.17 ± 0.01	61.73
Steáric acid	C18:0	6.38 ± 0.09	63.79
Oleic acid	C18:1 n-*9* c	41.37 ± 0.02	413.70
Cis-Vaccenic acid	C18:1 n-*7*	2.09 ± 0.003	20.92
Linoleic acid[e]	C18:2 n-*6* c	15.2 ± 0.2	151.09
α-Linolenic acid[e]	C18:3 n-*3*	0.34 ± 0.01	3.42
Cis-11 eicosenoic acid	C20:1 n-*9*	0.66 ± 0.09	6.63
Arachidonic acid	C20:4 n-*6*	0.53 ± 0.06	5.29
SFA[†]		31.8 ± 0.2	318
MUFA[‡]		51.4 ± 0.04	514
PUFA[§]		16.7 ± 0.08	167
n-*3*		0.61 ± 0.02	6.08
n-*6*		15.1 ± 0.2	152
n-*3* / n-*6*		0.04 ± 0.002	0.40

Values represent means ± S.D. (n = 2).
[e] Essential fatty acid
[†] Saturated fatty acids
[‡] Monounsaturated fatty acids
[§] Polyunsaturated fatty acids

Table S2
Univariate Tests of Significance, Effect Sizes, and Powers for FCR, $DPPH^{\cdot}$, ORAC and ICA assays, Over-parameterized model Type III decomposition

Effect	FCR (mg GAE g $meat^{-1}$)		$DPPH^{\cdot}$ (µg TE g $meat^{-1}$)		ORAC (µmol TE g $meat^{-1}$)		ICA (% inhib. mg $meat^{-1}$)	
	F-value	p	F-value	p	F-value	p	F-value	p
Intercept	170859.5	<0.01	7304.097	<0.01	11943.83	<0.01	6123.177	<0.01
Experiment	114.7	<0.01	0.524	0.59	22.16	<0.01	29.374	<0.01
Iron (Experiment)	207.8	<0.01	17.524	<0.01	22.27	<0.01	16.206	<0.01
UV (Experiment *Iron)	102.2	<0.01	6.916	<0.01	15.53	<0.01	10.629	<0.01
MAP (Experiment *Iron*UV)	72.7	<0.01	14.659	<0.01	24.1	<0.01	10.967	<0.01
Temp (Experiment *Iron*UV*MAP)	67.3	<0.01	14.9	<0.01	21.67	<0.01	11.369	<0.01

Mean ± S.D. (n = 8). Data were analyzed using hierarchical (Nested) ANOVA at 95% confidence level. (FCR: total reducing content, $DPPH^{\cdot}$: 2,2-diphenyl-1-picrylhydrazyl; ORAC: oxygen radical absorbance capacity ; ICA: iron quelating ability; GAE: gallic acid equivalents; TE: Trolox equivalents).

101

Table S3

Individual phenols by HPLC, for GPE from "*Touriga franca"* (vintage 2012) Portuguese cultivar, used during the experiments

Individual phenolics by HPLC[a]	Mean ± S.D.
Gallic acid (mg g extract^{-1})	0.53 ± 0.04
Syringic acid (mg g extract^{-1})	1.43 ± 0.02
(-)-Epicatechin (mg g extrac^{-1})	0.89 ± 0.07
Caffeic acid (mg g extract^{-1})	0.46 ± 0.0002

Values represent means ± standard deviation (S.D.) of triplicate (n = 3). [a]: Values for *p*-hydroxybenzoic, *p*-coumaric and o-coumaric, sinapic, ferulic acids and (+)-Catechin, (-)-Epicatechin gallate, *trans*-resveratrol, quercetin, kaempferol and chlorogenic acid, were below the detection limit.

References

AOAC. (1990). Food Composition additives, Natural contaminants *Official methods of analysis*. Washington D.C. (Reprinted from: 15th Edition).

Folch, J., Lees, M., & Sloane-Stanley, G. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry, 226*(1), 497-509.

Kammerer, D., Claus, A., Carle, R., & Schieber, A. (2004). Polyphenol screening of pomace from red and white grape varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS. *Journal of Agricultural and Food Chemistry, 52*(14), 4360-4367.

Shantha, N., & Ackman, R. (1990). Nervonic acid "versus" tricosanoic acid as internal standards in quantitative gas chromatographic analyses of fish oil longer-chain n-3 polyunsaturated fatty acid methyl esters. *Journal of Chromatography B: Biomedical Sciences and Applications, 533*, 1-10.

Paper III

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Telmo J. R. Fernandes, M. Beatriz P. P. Oliveira, Luís M. Cunha. Influence of Portuguese grape extracts on the oxidative stability, nutritional, and color characteristics of mechanically deboned chicken meat. [Submitted for publication].

Influence of Portuguese grape extracts on the oxidative stability, nutritional, and color characteristics of mechanically deboned chicken meat

Hernán H. Tournour[1,2], Marcela A. Segundo[3], Luís M. Magalhães[3], Telmo J.R. Fernandes[4], M. Beatriz P. P. Oliveira[4], Luís M. Cunha[1,*].

[1]LAQV,REQUIMTE/DGAOT, Faculty of Sciences, University of Porto, Porto, Portugal.

[2]Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal.

[3]UCIBIO,REQUIMTE,Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal.

[4] LAQV,REQUIMTE, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal.

**Corresponding author: lmcunha@fc.up.pt*
Campus Agrário de Vairão
Rua Padre Armando Quintas, 7
4486-661 Vairão
Portugal
Tel.: (+351)252660400 / Fax: (+351)252661780

ABSTRACT

The influence of grape pomace extracts (GPE) from "*Touriga nacional*" (TNac), "*Touriga franca*" (TF) and "*Tinta roriz*" (TR) on oxidative stability, nutritional composition, and physical characteristics of mechanically deboned chicken meat (MDM) under frozen storage was evaluated. MDM nutritional composition did not suffer significant ($p > 0.05$) changes over storage time, although MDM samples with added GPE showed significant ($p < 0.05$) changes in fat content. pH values were not significantly ($p > 0.05$) affected over time neither by the supplementation with GPE. Color variables were significantly ($p < 0.05$) decreased over time and by the addition of GPE (60 mg/kg) including TR and TF. A significant ($p < 0.05$) breakdown point in the MDM oxidative stability was determined after 30 days of storage by FCR and ORAC assays, being lower for

MDM supplemented samples than control, regarding to ICA values. TR and TNac were as effective as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) against the saturated and monounsaturated fatty acid (SFA and MUFA) oxidation. Interestingly, all GPEs were significantly ($p < 0.05$) more effective against *n-3* fatty acids group oxidation than BHT-BHA. These results corroborate Portuguese grape pomace represents an important source of affordable bioactive compounds, with potential applications toward food industry.

Keywords: grape pomace extracts; meat color; oxidative stability; polyphenols; fatty acid profile.

Highlights

- Mechanically deboned chicken meat was targeted for the grape pomace extract supplementation.
- Breakdown point in MDM oxidative stability was observed in antioxidant added samples after 30 days.
- Grape pomace extracts were more effective against n-*3* fatty acid oxidation than BHT-BHA.
- Portuguese grape pomace represents an affordable source of bioactive compounds.

1. Introduction

The increasing demand of convenient and ready-to-eat products based on chicken formulations (Kearney, 2010) originates large amounts of pieces with remaining meat in considerable quantities, which undergo recovery industrial processes in order to improve the yields and originating then the mechanically deboned chicken meat (MDM). The final composition of MDM depends on endogenous factors namely poultry species, sex and edge (Field, 1988), and also on exogenous factors such as extractive procedure mainly conditioned by equipment and pressure. MDM presents overall increased lipid content mainly from bone marrow and bone tissues (Trindade, Felício, & Castillo, 2004), consisted primarily by mono and polyunsaturated fatty acids, which are easily oxidized through air contact and due to pasty consistence and final haem iron content (Froning, 1981; Hui, 2012; Püssa, Pällin, Raudsepp, Soidla, & Rei, 2008). Therefore, lipid oxidation with latter off-flavor and off-color development even under frozen storage constitutes the major issue threatening MDM preservation.

Furthermore, MDM represents a low-cost source of animal protein with satisfactory technological properties, namely its binding capacity, enabling its action as a natural emulsifying agent in meat products (Álvarez et al., 2007). Additionally, MDM exhibits good water-holding capacity within the food matrix (Navarro-Rodríguez de Vera, Sánchez-Zapata, Viuda-Martos, & Pérez-Alvarez, 2010), which is a property highly valuated and broadly extended in comminuted and emulsified meat products. In this context, Portugal is a worldwide recognized wine producer reaching about 5.9 millions hector liters in 2014 according to the *Organisation Internationale de la Vigne et du Vin* (OIV), generating also important quantities of grape pomace (seed and skin) that contains valuable bioactive compounds, namely polyphenols (Lapornik, Prošek, & Golc Wondra, 2005) with proven antioxidant properties in food systems (Yu & Ahmedna, 2013).

Synthetic antioxidants (butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA)) are commonly used against lipid oxidation. Nevertheless their use is currently restricted due to their potential toxicological effects

(Raghavan & Richards, 2007). Natural antioxidants from rosemary, marjoram (Mielnik, Aaby, & Skrede, 2003; Mohamed & Mansour, 2012), spices (Karpinska, Borowski, & Danowska-Oziewicz, 2001), fruits (Lee, Reed, & Richards, 2006; Püssa et al., 2008; Raghavan & Richards, 2007), and also cocoa (Hassan, 2005) and sage leaves (Hac-Szymanczuk, Cegielka, Lipinska, & Ilczuk, 2014) have been tried as antioxidant in mechanically deboned meat from different animal species. Thus, this research aims to investigate the influence of Portuguese grape pomace extracts and synthetic antioxidants (butylated hydroxytoluene and butylated hydroxyanisole, BHT and BHA) on the oxidative stability, nutritional composition, and color characteristics of MDM samples. Furthermore, a complementary fatty acids assessment was also performed. To the best of our knowledge, this is the first report focused on the properties of Portuguese grape pomace extracts towards meat applications.

2. **Material and methods**

2.1. Chemicals

All chemicals used were of analytical reagent grade. 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulfonic acid sodium salt (ferrozine) and 2,2-azobis(2-methylpropionamide) dihydrochloride (AAPH) were purchased from Aldrich (Milwaukee, WI). Folin-Ciocalteu (F-C) reagent and fluorescein sodium salt were obtained from Sigma (St. Louis, MO), whilst iron(II) chloride tetrahydrate, gallic acid, and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were obtained from Fluka (Buchs, Switzerland). Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) Kosher grade (Sigma) were used as synthetic antioxidant. Water from Arium Sartorius (Goettingen, Germany) equipment (resistivity > 18 MΩ cm) and absolute ethanol p. a. (Panreac Química, Spain) were used in the preparation of all solutions. Fatty acid methyl esters (FAME) mixture 37 patterns (Supelco, Bellefonte, PA) were used for the fatty acids profile determination. Boron trifluoride, *n*-heptane and anhydrous sodium sulfate were purchased from Sigma-Aldrich.

2.2. Solutions

For assessment of total phenolic content (TPC), the commercial F-C reagent was diluted 3:10 (*v/v*) in water. A solution of $Na_2CO_3 \cdot 10\ H_2O$ 24.3% (w/v) was prepared, corresponding to 9% (w/v) of sodium carbonate, and also gallic acid standard solutions (1.0 - 15.0 mg L^{-1}) for calibration purposes. For iron(II) chelating ability (ICA) assay, all iron(II) solutions were freshly prepared including the stock solution (6 mM) at pH 3.0 and the iron(II) solution (0.12 mM) added to microplate. The ferrozine solution (0.6 mM) and acetate buffer (50 mM, pH 4.6) were also prepared. For oxygen radical absorbance capacity (ORAC) assay, AAPH (40 mM) and fluorescein stock solutions (0.5 mM) were prepared in a 75 mM phosphate buffer (pH 7.4). For the assessment of fatty acids composition, NaCl (1% (w/v) in water), KOH (0.5 M in methanol) and boron trifluoride (14 % *(v/v)*) in methanol) were prepared. Acidified acetone (90% *(v/v)* acetone, 8% *(v/v)* ultrapure water, and 2% *(v/v)* HCl) was prepared for the determination of haem iron content.

2.3. Preparation of grape pomace extracts

Red grape pomaces from demarcated Douro River region (*Vitis vinifera* L. grape variety), including "*Tinta roriz", "Touriga nacional"* and *"Touriga franca"* , vintage 2012 (TR, TNac and TF, respectively) were used in this study. Grape pomace obtained after the last winemaking fermentation step was dried in oven (Thermo Scientific ^{TM}, Pittsburgh PA) at 55 ºC till reaching a final moisture content lower than 5% (w/w). Dried material was grinded in a food processor (KenWood, New Lane, UK) before the extraction step under orbital agitation (300 rpm) in an Erlenmeyer flask (20 g dried material, 100 mL 80 % (*(v/v)*) ethanol/water, 48 h) at room temperature (Shirahigue et al., 2010). After the extraction step was finished, the resulting extract was vacuum filtered through a glass filter holding a 45 µm Millipore (Billerica, MA) polyvinylidene fluoride membrane filter. The filtrate was then concentrated in a vacuum rotary evaporator (Büchi, Flawil, Switzerland) at 65 ºC aided by nitrogen stream until dryness. The dry residue obtained was finally weighed and resuspended in 50 mL of water. Smaller portions were separated and kept under - 80 ºC until

151 further analysis or application to MDM. Extraction, filtration, concentration,
152 weighing and resuspension steps were performed in duplicate (*n* = 2) for each
153 grape variety.

154 *2.4. Equipment*

155 Antioxidant assays were performed under a microplate format (Synergy HT,
156 Bio-Tek Instruments, Winooski, VT) using spectrophotometric or fluorimetric as
157 detection. The microplate reader was controlled by Gen5 software (Bio-Tek
158 Instruments). ORAC assay was carried out at 37 ºC, while the other two assays
159 were carried out at room temperature. All samples were analyzed in
160 quadruplicate (or triplicate in ORAC assay) using at least two dilution factors.
161 For the assessment of the percentage of moisture a SMO 01 moisture balance
162 (Scaltec, Goettingen, Germany) was used. For protein assessment a Kjeldahl
163 compact equipment was used, consisting in a digester automat K-438 and
164 distillation unit K-360 (Büchi). The percentage of ashes was determined in a
165 Thermolyne 4800 Furnace muffle (Thermo Scientific, USA) and the percentage
166 for the total fat was obtained using a Soxhlet equipment. For determination of
167 haem iron content, a centrifuge (Thermo Scientific ^{TM}, Osterode, Germany) and
168 a UV-vis Spectrophotometer (Jasco V-660 Spectrophotometer, Tokyo, Japan)
169 were used. For pH determination a pH meter (Hanna Instruments, Michigan,
170 USA) was used. The fatty acid methyl esters from MDM samples were
171 quantified through external calibration, by gas chromatography using a
172 Chrompack CP 9001 Chromatograph (Chrompack, Middleburg, The
173 Netherlands), equipped with a split-splitless injector and a flame ionization
174 detector. Fatty acid methyl esters were separated in a fused silica capillary
175 column CP-SIL 88 (0.19 µm, 50 m x 0.25 mm i.d., Chrompack) at 140 ºC for 5
176 min, followed by temperature increase of 5 ºC/min till reach 220 ºC, maintained
177 for more 15 min. For the data analysis, Maitre software (JMBS Developments,
178 Grenoble, France) was applied. Instrumental color was determined using a
179 Minolta CR-300 colorimeter (Minolta Camera Co. Osaka, Japan) with Illuminant
180 C as standard light source and 2º observer.

181 *2.5. Chemical analysis*

182 *2.5.1. Grape pomace extracts (GPE)*

183 *Total phenolic content (TPC)*

184 The TPC was assessed employing a 96-well microplate Folin-Ciocalteu
185 procedure, with carbonate buffer as alkaline reagent (Magalhães, Santos,
186 Segundo, Reis, & Lima, 2010; Singleton, Orthofer, & Lamuela-Raventos, 1999).
187 Hence, 150 µL of gallic acid standard solution (1.0 - 15.0 mg/L) or diluted red
188 grape pomace extracts (1:200 *(v/v)*) and 50 µL of F-C reagent (3:10, *(v/v)*) were
189 placed in each well. After that, 100 µL of carbonate solution (9%, (w/v)) was
190 added. The reduction at alkaline pH of phosphotungstate-phosphomolybdate
191 complexes was monitored at 760 nm during 120 min. The TPC, expressed as
192 mg of gallic acid equivalents (GAE) per gram of dry residue (obtained from the
193 solid material after concentration step) was calculated by interpolation of
194 absorbance values after 120 min of reaction in the gallic acid standard curve
195 ($Abs_{760\ nm}$ = 0.0510 × [gallic acid, (mg/L)] + 0.065, R>0.9996). The Folin-
196 Ciocalteu reducing content in diluted meat extracts (1:10 and 1:20 *(v/v)*) was
197 analyzed as described above. Results were expressed as mg GAE per gram of
198 meat in dry basis.

199 *2.5.2. Mechanically deboned chicken meat (MDM) samples*

200 MDM was obtained at a local slaughter industry, vacuum packaged and
201 immediately transported under refrigerated conditions. MDM was divided in 5
202 portions and supplemented with antioxidants as following: i) Control (MDM
203 without addition of antioxidant); ii) MDM + TNac; iii) MDM + TF; iv) MDM + TR;
204 and v) MDM + BHT-BHA. All grape pomace extracts used in this experience
205 were obtained from grape variety vintage 2012 and added at 60 mg of dry
206 residue/kg, whilst synthetic antioxidants were added at 100 mg/kg each. After
207 mixture step all samples were separated in smaller portions (approximate 30 g),
208 vacuum packaged (Sammic, Guipúzcoa, Spain) in oxygen barrier bags and
209 stored under frozen conditions (at - 23 ± 1 ºC) for seven months. Analyses were
210 performed along frozen storage after 1 (1D), 30 (30D), 60 (60D) and 210 (210D)
211 days after GPE or BHT-BHA addition.

212 *2.5.2.1. MDM nutritional composition*

Moisture, protein, total fat and ash contents were determinate for MDM samples, according to the methods recommended by the Association of Official Analytical Chemists (AOAC, 2002). Results were expressed as % (w/w) in dry basis.

2.5.2.2. Instrumental color and pH

For the color assessment, *L** (luminosity), *a** (redness), *b** (yellowness), chroma ($(a^{*2} + b^{*2})^{1/2}$), and the *Hue angle* as arctg (*b*/a**) in radians (rad.), which indicates the degree of departure from the true redness on the CIE color scale, were determined. For pH determination (Ozer & Sariçoban, 2010), 10 g of each sample was homogenised in 100 mL of ultrapure water for 1 min before pH measurement.

2.5.2.3. Haem iron content (HIC)

The haem iron content (HIC) was determinate according to Clark *et al.* (Clark, Mahoney, & Carpenter, 1997). Briefly, 2 g of each MDM sample were placed into a centrifuge tube and macerated with acidified acetone (9 mL) during 1 h, at room temperature. After centrifugation step (2,200 x g, 10 min), liquid was filtered through Whatman #42 filter paper and the absorbance was measured at 640 nm, using acidified acetone as blank. The HIC was calculated in dry basis as: HIC (µg/gmeat db) = (A_{640} x 680) x 8.82 / 100.

2.5.2.4. Assessment of the oxidative stability

Meat extracts used for the oxidative stability were prepared according to Qwele *et al.* (Qwele et al., 2013) with some modifications. Briefly, 1 g of MDM samples was homogenized with 10 mL of 0.05 M KH_2PO_4 phosphate buffer (pH 7.0). The extraction step was carried out alternating ultrasound (30 s, 3 times) and vortex cycles (2 min, 3 times at 3000 rpm). Before the last cycle, meat samples were left for 10 min to stand in order to allow the tissue hydration and improve the extraction. Finally, extracted samples were centrifuged at 5,580 x g for 30 min at 4 °C. Supernatant aliquots (1 mL) were disposed in Eppendorf tubes and one drop of concentrated HCl was added in order to decrease the pH and circumvent autooxidation of phenolic compounds. Finally, extracts were frozen at - 80 ºC till analysis by FCR, ORAC and ICA assays. Before performing each

assay procedure, analyses were started mixing 200 µL of each extract and 200 µL absolute ethanol p. a. in order to guarantee the total dissolution of phenolic compounds. The oxidative stability of MDM samples was monitored through Folin-Ciocalteu reducing (FCR) as described in section 2.5.1. and through ORAC and ICA assays.

The oxygen radical absorbance capacity (ORAC) assay is based on the scavenging of peroxyl radicals generated by AAPH, which prevents the degradation of the fluorescein probe and, consequently, avoids the loss of fluorescence. For ORAC assay (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002; Wang, Jónsdóttir, & Ólafsdóttir, 2009), 100 µL of Trolox standard solution (1.0 – 7.5 µM) or diluted meat extracts (1:125 and 1:250 *(v/v)*) and 100 µL of fluorescein (117 nM) were placed in each well, and the microplate was pre-incubated for 15 min at 37 ºC. Following this, 100 µL of AAPH solution (40 mM) was added and the fluorescence intensity (λ_{exc} 485 nm, λ_{em} 520 nm) was monitored every minute during 240 min. The reaction milieu was 75 mM phosphate buffer (pH 7.4) at 37 ºC. Control signal profile (absence of sample) was assessed by adding 100 µL of buffer solution instead of sample. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve over the reaction time. The net AUC of the sample was calculated by subtracting this value to the AUC of the control (absence of sample). The regression equation between net AUC and Trolox concentration was determined, and the results were expressed as µmol of Trolox equivalents (TE) per gram of meat in dry basis by interpolation (Net AUC (%) = 10.6 × [Trolox, (µM)] + 10.5, R>0.9998).

For iron(II) chelating ability assay (ICA) (Wang et al., 2009), 100 µL of diluted meat extracts (1:5 and 1:10, *(v/v)*) in acetate buffer (50 mM, pH 4.6) were mixed with 100 µL $FeCl_2 \cdot 4\ H_2O$ (120 µM) and placed in each well. After 5 min, 100 µL of ferrozine solution (600 µM) was added to each well. Solutions were left standing 10 min at room temperature, after which the absorbance was measured at 562 nm. Control assay was performed by adding 100 µL of water instead of sample, while the blank of the sample was performed by adding 100 µL of water instead of ferrozine solution. The percentage of inhibition of ferrozine-iron(II) complex formation of each sample was calculated using the

277 equation: ICA (%) = [A_0 – (A_1 – A_2)] / A_0 x 100, where A_0, A_1 and A_2 correspond
278 to absorbance of the control, sample and blank of the sample, respectively. In
279 A_0 the intrinsic absorbance of iron(II) was subtracted from the initial
280 absorbance. As the reaction proceeds the resulting red colour from the
281 ferrozine-iron(II) complex decreases in the presence of chelating substances.
282 Hence, ICA (%) values represent the reduction in absorbance values relative to
283 the control due to the chelating effect of sample components. Results were
284 expressed as % inhibition obtained per mg of meat in dry basis.

285 *2.5.2.5. Fatty acids profile of MDM samples by gas chromatography*

286 The fatty acids proportion and the level of unsaturations are of utmost
287 importance of the oxidation degree in fat. Therefore, the assessment of the fatty
288 acids composition was carried out using gas chromatography with flame
289 ionization detector. The fatty fraction was firstly extracted according to Folch
290 method (Folch, Lees, & Sloane-Stanley, 1957), with dichloromethane instead of
291 chloroform. For this, sample was weighed (1 g) into a Falcon tube, followed by
292 addition of 10 mL dichloromethane: methanol (2:1) solution. Samples were then
293 ground with an Ultra-turrax T25 Basic® (Jenke & Kunkel Ika, Stanfen,
294 Germany) and centrifuged (3000 rpm, 5 min). The supernatant was displayed in
295 a second Falcon tube where 1 % (w/v) NaCl aqueous solution was added in a
296 1:5 proportion related to the supernatant volume. Sample was homogenized in
297 a vortex and centrifuged again (3000 rpm, 5 min), rejecting the upper portion
298 (aqueous phase). The same procedure was repeated after addition of
299 anhydrous sodium sulfate. Samples were concentrated in a vacuum Rotary
300 evaporator. The total fat (organic phase) was placed into a vial tube with 100 µL
301 of 0.01 % (w/v) BHT solution. The fatty acid derivatization into fatty acid methyl
302 esters (FAME) was performed according to Shantha & Ackman (Shantha &
303 Ackman, 1990), with some modifications. The organic phase obtained above
304 was mixed with 500 µL of 0.5 M KOH in methanol solution. The sample was
305 homogenized and heated (100 ºC, 10 min), followed by cooling into ice, where
306 2.5 mL of boron trifluoride (14 % *(v/v)* in methanol) was added to sample.
307 Sample was again homogenized, heated (100 ºC, 30 min), cooled in ice, and 2
308 mL of *n*-heptane was added. The last steps of the derivatization consisted in a
309 separation of the upper phase into a new vial tube with subsequent agitation

and centrifugation. Excess water was removed with anhydrous sodium sulfate. Results were expressed as mass percentage for each fatty acid of its methyl esters or area under the curve (AUC) as the AUC obtained are equivalents to FAME mass, based on: $\%_A = [(AUC_A \times 100)]^{-1}\Sigma$ (AUC peaks) where $_A$ represents a FAME compound.

2.6. Statistical analysis

Results were expressed as mean ± standard deviation (S.D.) or ± standard error of the mean (SEM). Univariate or multivariate analysis of variance (ANOVA or MANOVA) were performed to evaluate the influence of the antioxidant supplementation of the final MDM characteristics. Principal Component Analysis (PCA) with *Varimax* rotation was carried out in order to detect clustering formation. Except when referred, all tests were applied at a 95% confidence level. Statistical data analysis was performed with IBM SPSS Statistics version 21.0 (IBM SPSS Statistics, New York, USA) and XLStat version 2014 for Windows (Addinsoft, New York, USA).

3. Results and Discussion

3.1. Total phenolic content (TPC) of GPE

GPEs were analyzed regarding to total phenolic content through Folin-Ciocalteu assay, to guarantee 60 mg/kg in the supplementation of MDM samples. Results indicated the ranking order TNac > TF > TR with 142.4 mg GAE/g residue, 106.1 mg GAE/g residue and 75.8 mg GAE/g residue, respectively.

3.2. Evolution of nutritional composition of MDM

Results for nutritional composition of MDM samples under frozen storage were given in Table 1.

Table 1
Nutritional composition evolution for MDM samples under frozen storage.

Variable	Samples	Storage time				
		1D	30D	60D	210D	**Total**
Moisture%	Control	61.4 (±1.1)	63.1 (±0.2)	63.8 (±0.5)	62.4 (±0.3)	**62.9^{a}(±0.4)**
	BHTBHA	63.8 (±0.6)	63.2 (±0.4)	64.1 (±0.3)	64.4 (±0.1)	**63.9^{a}(±0.2)**
	TR	62.8 (±0.7)	62.5 (±0.4)	63.1 (±0.3)	63.0 (±0.7)	**62.8^{a}(±0.2)**
	TNAC	63.0 (±0.3)	63.2 (±0.01)	62.5 (±0.2)	63.4 (±0.2)	**63.0^{a}(±0.2)**
	TF	62.9 (±0.1)	62.9 (±0.6)	62.9 (±0.7)	62.8 (±0.2)	**62.9^{a}(±0.2)**
	Total	**62.8^{A}(±0.3)**	**62.9^{A}(±0.2)**	**63.3^{A}(±0.3)**	**63.2^{A}(±0.3)**	
Protein%	Control	31.5 (±0.2)	32.8 (±0.2)	33.4 (±0.6)	32.8 (±0.5)	**32.9^{a}(±0.2)**
	BHTBHA	34.0 (±0.8)	34.9 (±0.04)	33.4 (±0.1)	34.8 (±1.2)	**34.7^{a}(±0.4)**
	TR	35.6 (±1.6)	33.4 (±0.7)	32.7 (±0.2)	33.9 (±1.8)	**33.9^{a}(±0.6)**
	TNAC	35.3 (±0.7)	33.9 (±0.4)	33.1 (±0.7)	33.3 (±1.7)	**33.9^{a}(±0.5)**
	TF	34.0 (±0.6)	32.7 (±0.3)	32.1 (±0.02)	33.9 (±1.2)	**33.2^{a}(±0.4)**
	Total	**34.1^{A}(±0.6)**	**33.5^{A}(±0.3)**	**32.9^{A}(±0.2)**	**33.8^{A}(±0.5)**	
Fat%	Control	66.8 (±0.1)	63.8 (±2.9)	65.6 (±0.1)	59.8 (±0.2)	**64.5^{a}(±1.3)**
	BHTBHA	59.0 (±0.5)	59.0 (±0.2)	59.4 (±0.8)	61.6 (±0.3)	**59.7^{c}(±0.4)**
	TR	63.2 (±3.3)	63.3 (±1.3)	60.4 (±0.3)	59.7 (±0.6)	**61.6^{a,b,c}(±0.9)**
	TNAC	62.2 (±2.1)	61.3 (±3.7)	58.4 (±1.6)	60.3 (±0.5)	**60.6^{b,c}(±1.0)**
	TF	64.7 (±1.6)	63.0 (±2.1)	63.0 (±1.5)	62.8 (±0.7)	**63.4^{a,b}(±0.7)**
	Total	**63.2^{A}(±1.1)**	**62.1^{A}(±1.0)**	**61.4^{A}(±0.9)**	**60.8^{A}(±0.4)**	
Ashes%	Control	n.a.	2.58 (±0.01)	2.73 (±0.01)	2.50 (±0.03)	**2.88^{a,b}(±0.18)**
	BHTBHA	n.a.	2.70 (±0.03)	2.87 (±0.01)	2.71 (±0.04)	**2.79^{b}(±0.04)**
	TR	n.a.	2.74 (±0.07)	2.72 (±0.03)	2.63 (±0.02)	**2.98^{a}(±0.19)**
	TNAC	n.a.	2.79 (±0.06)	2.74 (±0.07)	2.63 (±0.08)	**2.96^{a}(±0.16)**
	TF	n.a.	2.54 (±0.04)	2.77 (±0.22)	2.54 (±0.03)	**2.90^{a,b}(±0.19)**
	Total		**2.67^{A}(±0.04)**	**2.77^{A}(±0.04)**	**2.60^{A}(±0.03)**	

Values represent mean ± SEM (g/100 g meat db, n = 2). Data were analyzed by ANOVA and within each column (^{a,b}) and each row ($^{A, B}$) different superscripts indicate statistically differences according to the Tukey multiple comparison test at 95% confidence level. MDM: mechanically deboned chicken meat; n.a.: not available.

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Non-significant differences ($p > 0.05$) were registered throughout the storage period for all parameters, with moisture% ranging from 62.9 to 63.3%; protein% from 32.9 to 34.1%; fat% from 60.8 to 63.2% and ashes% from 2.60 to 2.77 %. Preservation conditions of vacuum and frozen storage, were appropriated to avoid MDM spoilage in agreement with previous research (Ozkececi, Karakaya, Yilmaz, Saricoban, & Ockerman, 2008). Results showed that the degradation of MDM samples was not intense during frozen storage, regarding to nutritional composition. Concerning to differences among MDM with added antioxidants, MDM samples supplemented with BHT-BHA and TNac were significant different ($p < 0.05$) from control for fat %. Similarly, BHT-BHA added samples showed the lowest ashes values (2.80%). Therefore, our results suggest that the nutritional composition of MDM was partially affected by the antioxidants addition concerning fat% and ashes%, although with a similar behavior compared to control samples for moisture and protein values. These differences although significant, may represent a minor practical impact.

3.3. Instrumental color and pH assessment

Results of instrumental color parameters (*L*, a*, b**, chroma and Hue angle) were given in Table 2.

Table 2
Color variables (*L*, a*, b*,* Chroma and Hue angle) and pH evolution for MDM samples under frozen storage.

Variable	Samples	Storage time				
		1D	30D	60D	210D	**Total**
*L**	Control	61.33 (±1.89)	54.69 (±0.55)	49.90 (±1.56)	56.93 (±0.56)	**55.71^{a}(±1.05)**
	BHTBHA	66.52 (±0.83)	52.40 (±0.61)	50.70 (±0.55)	52.31 (±1.80)	**$55.48^{a,b}$(±1.43)**
	TR	67.23 (±0.85)	51.07 (±0.87)	45.44 (±1.94)	47.10 (±2.08)	**52.71^{c}(±1.94)**
	TNAC	66.24 (±0.90)	49.32 (±0.54)	47.84 (±0.68)	48.61 (±3.39)	**$53.00^{b,c}$(±1.81)**
	TF	65.39 (±0.94)	48.12 (±0.62)	49.75 (±0.56)	53.79 (±0.58)	**$54.26^{a,b,c}$(±1.44)**
	Total	**65.34^{A}(±0.62)**	**$51.12^{B,C}$(±0.51)**	**48.73^{C}(±0.61)**	**51.75^{B}(±1.05)**	
*a**	Control	24.28 (±0.64)	26.26 (±0.32)	27.28 (±0.89)	19.22 (±0.51)	**24.26^{a}(±0.71)**
	BHTBHA	23.44 (±0.29)	26.19 (±0.37)	25.22 (±0.91)	20.79 (±0.51)	**23.91^{a}(±0.50)**
	TR	19.99 (±0.25)	24.93 (±0.45)	24.77 (±0.95)	18.12 (±0.37)	**21.95^{b}(±0.67)**
	TNAC	20.30 (±0.48)	24.67 (±0.31)	21.98 (±0.18)	18.69 (±0.56)	**21.41^{b}(±0.50)**
	TF	19.77 (±0.29)	24.77 (±0.34)	22.64 (±0.42)	19.79 (±0.42)	**21.74^{b}(±0.47)**
	Total	**21.56^{B}(±0.39)**	**25.36^{A}(±0.20)**	**24.38^{A}(±0.47)**	**19.32^{C}(±0.26)**	
*b**	Control	17.04 (±0.25)	16.91 (±0.21)	17.06 (±0.56)	14.24 (±0.47)	**16.31^{a}(±0.31)**
	BHTBHA	17.38 (±0.10)	16.36 (±0.13)	16.61 (±0.40)	15.15 (±0.29)	**16.37^{a}(±0.21)**
	TR	15.71 (±0.09)	15.63 (±0.31)	15.76 (±0.33)	13.67 (±0.38)	**15.19^{b}(±0.23)**
	TNAC	15.59 (±0.14)	14.97 (±0.25)	13.33 (±0.39)	13.70 (±0.22)	**14.40^{c}(±0.23)**
	TF	15.17 (±0.11)	15.52 (±0.26)	14.89 (±0.48)	13.13 (±0.65)	**$14.67^{b,c}$(±0.28)**
	Total	**16.18^{A}(±0.17)**	**15.88^{A}(±0.16)**	**15.53^{A}(±0.31)**	**13.98^{B}(±0.22)**	

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Table 2-continuation

		1D	30D	60D	210D	**Total**
Chroma	Control	29.67 (±0.60)	31.24 (±0.28)	32.18 (±1.03)	23.95 (±0.46)	**29.26^{a}(±0.73)**
	BHTBHA	29.18 (±0.28)	30.88 (±0.37)	30.20 (±0.98)	25.74 (±0.49)	**29.00^{a}(±0.50)**
	TR	25.43 (±0.21)	29.42 (±0.53)	29.38 (±0.87)	22.71 (±0.43)	**26.74^{b} (±0.65)**
	TNAC	25.60 (±0.45)	28.86 (±0.34)	25.71 (±0.26)	23.18 (±0.51)	**25.84^{b} (±0.46)**
	TF	24.92 (±0.24)	29.23 (±0.42)	27.10 (±0.56)	23.79 (±0.45)	**26.26^{b} (±0.48)**
	Total	**26.96^{B}(±0.41)**	**29.93^{A}(±0.24)**	**28.92^{A}(±0.54)**	**23.87^{C}(±0.27)**	
Hue angle	Control	0.61 (±0.01)	0.57 (±0.01)	0.56 (±0.01)	0.64 (±0.02)	**0.60^{a}(±0.01)**
	BHTBHA	0.64 (±0.00)	0.56 (±0.00)	0.58 (±0.01)	0.63 (±0.01)	**0.60^{a}(±0.01)**
	TR	0.67 (±0.01)	0.56 (±0.00)	0.57 (±0.02)	0.65 (±0.01)	**0.61^{a}(±0.01)**
	TNAC	0.66 (±0.01)	0.55 (±0.01)	0.54 (±0.01)	0.63 (±0.01)	**0.59^{a}(±0.01)**
	TF	0.65 (±0.01)	0.56 (±0.00)	0.58 (±0.01)	0.58 (±0.03)	**0.60^{a}(±0.01)**
	Total	**0.65^{A}(±0.00)**	**0.56^{B}(±0.00)**	**0.57^{B}(±0.01)**	**0.63^{A}(±0.01)**	
pH	Control	6.73 (±0.01)	6.86 (±0.02)	n.a.	6.75 (±0.02)	**6.57^{a}(±0.15)**
	BHTBHA	6.71 (±0.10)	6.56 (±0.04)	n.a.	6.85 (±0.04)	**6.51^{a}(±0.14)**
	TR	6.37 (±0.16)	6.79 (±0.06)	n.a.	6.72 (±0.06)	**6.42^{a}(±0.17)**
	TNAC	6.84 (±0.08)	6.86 (±0.03)	n.a.	6.99 (±0.03)	**6.68^{a}(±0.15)**
	TF	6.57 (±0.15)	6.97 (±0.16)	n.a.	6.98 (±0.16)	**6.65^{a}(±0.15)**
	Total	**6.64^{A}(±0.07)**	**6.81^{A}(±0.05)**		**6.87^{A}(±0.05)**	

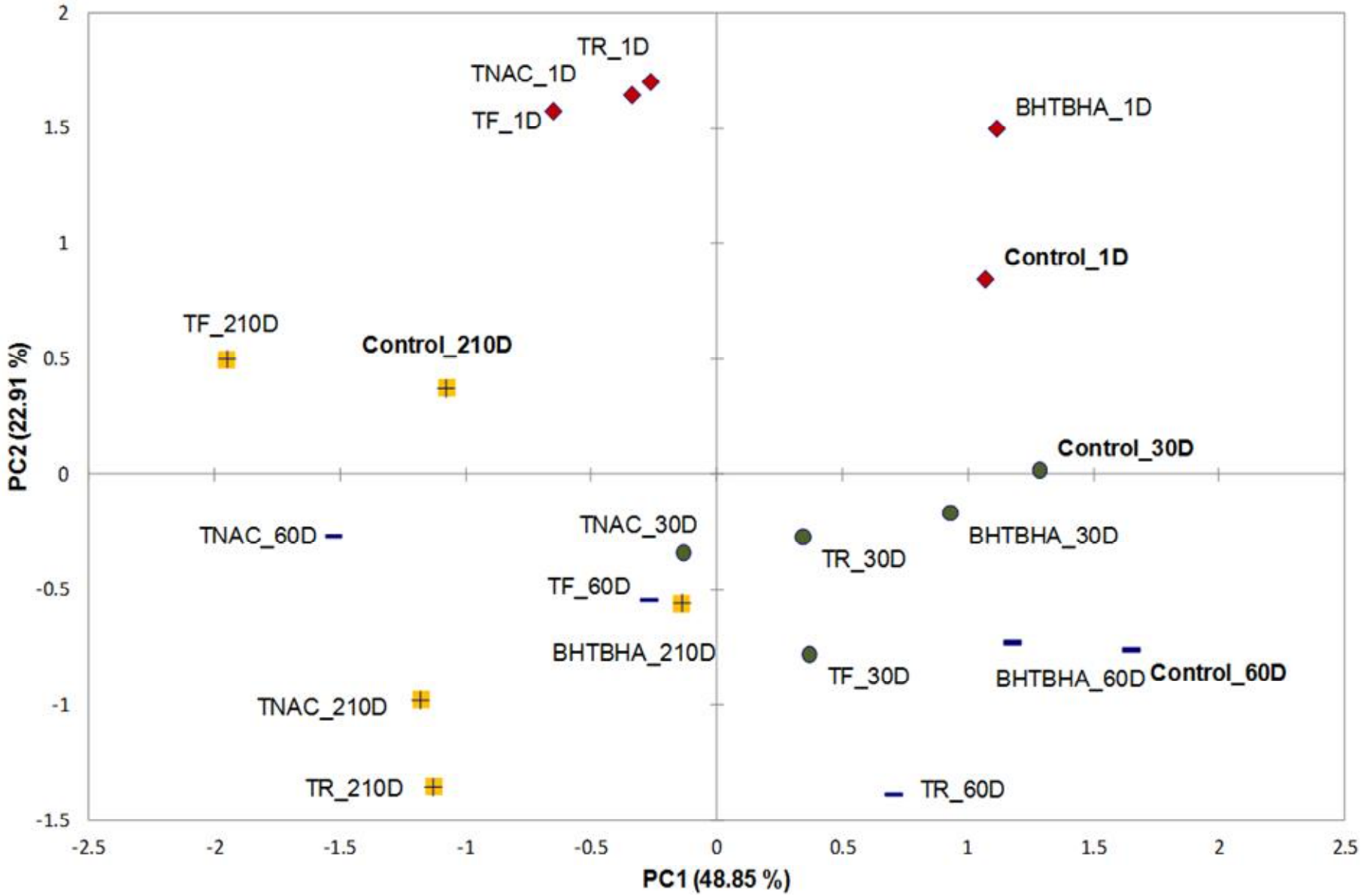
Values represent means (± SEM) (Color variables n = 6, pH n = 2). Data were analyzed by ANOVA and within each column (^{a,b}) and row ($^{A, B}$) different superscripts indicate statistically differences according to the Tukey multiple comparison test at 95% confidence level. MDM: mechanically deboned chicken meat. n.a.: not available.

The *L** value indicates the level of light or dark, the *a** value redness or greenness and finally the *b** value indicates the yellowness or blueness of a given sample. The *L** variable significantly decreased ($p < 0.05$) throughout storage time till 60D, reaching then, an average value of 51.75 ± 1.05. The *a** and *b** values showed a similar decreasing trend, with final values 19.32 and 13.98, respectively, which are significantly different ($p < 0.05$) from the initial ones. Mielnik *et al.* have worked with meat products formulated from mechanically deboned poultry meat. They verified that storage time of raw material resulted in decreased colour parameters (*L,*a**, and *b**) (Mielnik, Aaby, Rolfsen, Ellekjær, & Nilsson, 2002). Besides, the decrease of *a** value during storage is probably due to oxymyoglobin oxidation to metmyoglobin (Ozer & Sariçoban, 2010). In contrast, Selani *et al.* working with raw chicken meat under vacuum frozen storage during nine months, verified non-significant differences concerning *L** and *b**, though slight but still significant reduction in *a** values at 60D was observed (Selani et al., 2011).

With regards to GPE added samples, instrumental color was affected by the addition of GPE at 60 mg/kg concentration. Control and BHT-BHA added samples were lighter, redder and yellower than MDM samples supplemented with any GPE. These effects were also verified for chroma values, which is a measure of the intensity of color, existing a clear separation from control and BHT-BHA added samples, and GPE supplemented samples, likewise described above for *a** values. TR added sample registered the lowest *L** (52.71), whilst TNac samples showed the lowest *a** (21.41) and *b** (14.40) values. In previous work, Rojas and Brewer used a grape seed extract at a concentration of 200 mg/kg and observed non-significant differences between supplemented and non-supplemented meat samples (Rojas & Brewer, 2007). They used a commercial grape seed extract composed by 89% proanthocyanidins, whilst GPEs used in the present work were prepared with skin and seed, which increase the color of the final extract (mainly dark purple and blue color) due to the anthocyanins from grape skins (Teixeira, Eiras-Dias, Castellarin, & Gerós, 2013). Hence, the color differences noted in this study may be ascribed to color of GPE itself.

Final color in meat is dependent not only on the redox state of myoglobin (Mb) but also on its ability to avoid the dissociation of haem from its structure,

393 otherwise meat color deterioration will be observed. The discoloration
394 phenomenon in meat (observed in the oxymyoglobin oxidation to
395 metmyoglobin) is associated with lipid oxidation. One possible mechanism
396 proposed is related to the high reactivity of the primary and secondary oxidation
397 products derived from the unsaturated lipids (Faustman, Sun, Mancini, &
398 Suman, 2010). The linoleic acid present in MDM samples, once oxidized,
399 originates secondary oxidation products (such 4-hydroxynonenal) that
400 accelerate oxymyoglobin oxidation by binding covalently to specific histidine
401 residues in the protein's primary sequence. This fact has been shown for
402 chicken and turkey meat (Naveena et al., 2010).Furthermore, certain molecules,
403 namely Co, NO, H_2O and antioxidants bounded to haem group also influence
404 on the final color in meat samples.
405 The packaging conditions (mainly the partial oxygen pressure) have an effect
406 on the relationship between lipid oxidation and discoloration in meat. In
407 packaged fresh meat, Mb can exists in four redox states, namely
408 deoxymyoglobin (DeoxyMb), oxymyoglobin (OxyMb), carboxymyoglobin (CMb)
409 and metmyoglobin (MetMb), including the last one in the ferric state. Hence, the
410 resulting color can vary from purple-red (DeoxyMb), bright cherry-red (OxyMb
411 and CMb) or brown final color (MetMb) (Suman & Joseph, 2013). Nevertheless,
412 atmospheres containing extremely high or low concentrations of oxygen provide
413 conditions in which the oxidative interaction between lipid and myoglobin is not
414 tightly associated (Faustman et al., 2010). The pH assessment indicated non-
415 significant ($p > 0.05$) differences among samples and throughout the storage
416 time.
417
418 3.3.1 Principal component analysis
419 Concerning to the large number of color variables, a principal component
420 analysis (PCA) was performed to provide a better understanding of the
421 relationship between these variables. Results are depicted in Figure 1.



422 **Figure 1.** Color variables (*L*, a*, b**, chroma and Hue angle) projection on the PC1 and PC2 plane after *Varimax* rotation. Loading and score factors for each observation, are also given in each axis.

PCA explained over 70% of the total variability by two main principal components. Factor loadings for each color variable obtained after *Varimax* rotation were given in Table S1. PC1 was the most important variable regarding to the total variability explained by itself (48.85%) and was positively correlated with *a**, *b** and chroma. Beside, PC1 was inversely correlated with Hue angle. On the other hand, PC2 explained 22.91% of the total variability and was positively correlated with *L**, and inversely with *a** and chroma. As depicted in Figure 1, control and BHT-BHA added samples at 1D are in the positive side of PC1 and PC2, whilst in the PC2 positive side were randomly displayed samples with low or high time values under frozen storage, namely 1D and 210D. Hence, regarding to PC1, this principal component grouped samples under the two first months of frozen storage. Additionally, because PC1 was correlated with *a**, *b** and chroma variables, it is possible to observe the effect of the grape extract addition on color samples. PC2 grouped samples that have higher luminosity (*L**) as occurred with all samples at 1D under frozen storage. The storage time effect on color of samples can be also seen through PC2. In conclusion, PC1 distinguished the reddest and highest yellowness and chroma values samples, namely control and BHT-BHA from GPE supplemented samples.

3.4. Haem iron content and oxidative stability

MDM samples have an increased iron content resulting by the pressure applied in the extractive processes, and hemoglobin meat pigment is the main source of this increment (Froning, 1981). As shown in Table 3, HIC values significantly ($p < 0.05$) changed, showing a decreasing trend with storage time. Concerning to samples with addition of GPE or BHT-BHA, significant differences ($p < 0.05$) were also observed, where control exhibited the highest HIC values (460 µg Fe/g meat db) and TNac added sample presented the lowest value (352 µg Fe/g meat db). The effect of the storage time on the HIC is still not conclusive. HIC values for all GPEs significantly ($p < 0.05$) decreased after 60D, whilst control and BHT-BHA samples did not showed this behavior. Lipolysis occurring in meat pigment over storage time increases the levels of free fatty acids (Gil et al., 2001), then, metmyoglobin can undergo denaturation, with consequent exposure or release of the haem group (Luciano et al., 2009). This fact seems

457 to be associated with the lipid oxidation according to Zakys *et al.,* once they
458 determined a strong correlation between changes in oxymyoglobin and the lipid
459 degradation measured through TBARS (Zakrys, Hogan, O'sullivan, Allen, &
460 Kerry, 2008).

Table 3
Assessment of the oxidative stability for MDM samples under frozen storage.

Assay	Samples	Storage time				
		1D	30D	60D	210D	**Total**
FCR	Control	3.58 (±0.06)	3.61 (±0.11)	3.74 (±0.07)	3.86 (±0.05)	**$3.69^{a,b}$(±0.05)**
	BHTBHA	3.90 (±0.05)	3.36 (±0.09)	3.58 (±0.22)	3.92 (±0.04)	**$3.70^{a,b}$(±0.08)**
	TR	3.66 (±0.08)	3.12 (±0.12)	3.52 (±0.06)	3.90 (±0.03)	**3.55^{c}(±0.08)**
	TNAC	3.70 (±0.07)	3.46 (±0.07)	3.44 (±0.06)	4.22 (±0.04)	**3.70^{a}(±0.09)**
	TF	4.06 (±0.03)	3.44 (±0.09)	3.46 (±0.07)	4.25 (±0.03)	**3.80^{a}(±0.10)**
	Total	**3.78^{B}(±0.05)**	**3.40^{C}(±0.05)**	**3.54^{C}(±0.05)**	**4.03^{A}(±0.04)**	
ORAC	Control	58.6 (±5.2)	39.5 (±2.9)	59.6 (±4.8)	48.4 (±6.2)	**52.6^{a}(±3.3)**
	BHTBHA	64.0 (±4.4)	37.5 (±9.6)	60.3 (±3.7)	52.4 (±5.7)	**55.3^{a}(±3.9)**
	TR	69.4 (±2.8)	49.4 (±1.2)	62.1 (±2.1)	50.9 (±3.6)	**57.9^{a}(±2.7)**
	TNAC	59.2 (±1.5)	44.0 (±3.1)	56.9 (±1.2)	45.4 (±5.1)	**51.4^{a}(±2.4)**
	TF	69.7 (±1.6)	46.1 (±1.7)	60.4 (±1.9)	34.7 (±2.2)	**52.7^{a}(±4.1)**
	Total	**64.2^{A}(±1.8)**	**44.0^{B}(±1.8)**	**59.9^{A}(±1.2)**	**45.9^{B}(±2.5)**	
ICA	Control	105 (±3)	58 (±4)	122 (±4)	9 (±4)	**85^{a}(±12)**
	BHTBHA	101 (±2)	77 (±3)	72 (±4)	18 (±0)	**$79^{a,b}$(±7)**
	TR	103 (±3)	34 (±1)	110 (±2)	21 (±18)	**$77^{a,b}$(±11)**
	TNAC	76 (±3)	46 (±2)	93 (±5)	14 (±2)	**58^{c}(±8)**
	TF	87 (±9)	61 (±4)	120 (±6)	4 (±1)	**73^{b}(±12)**
	Total	**94^{A}(±3)**	**57^{B}(±4)**	**105^{A}(±5)**	**12^{C}(±3)**	

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Table 3-continuation

		1D	30D	60D	210D	**Total**
HIC	Control	n.d.	478 (±2)	565 (±1)	337 (±7)	**460^{a}(±33)**
	BHTBHA	n.d.	446 (±1)	526 (±0)	275 (±1)	**416^{b}(±37)**
	TR	n.d.	416 (±1)	360 (±1)	434 (±2)	**403^{c}(±11)**
	TNAC	n.d.	450 (±2)	298 (±1)	310 (±1)	**352^{d}(±24)**
	TF	n.d.	548 (±2)	334 (±0)	336 (±2)	**406^{c}(±36)**
	Total		**468^{A}(±12)**	**416^{A,B}(±29)**	**338^{C}(±14)**	

Values represent means (± SEM) (n = 4) in dry bases. Data were analyzed by MANOVA and within each column (^{a,b}) and row ($^{A, B}$) different superscripts indicate statistically differences according to the Tukey multiple comparison test at 95% confidence level. n.d.: not determined. FCR: Folin-Ciocalteu reducing (mg GAE/g meat db); ORAC: oxygen reactive absorbance capacity (µmol TE/g meat db); ICA: Iron(II) chelating ability (% inhibit./mg meat db); HIC: haem iron content (µg Fe/g meat db); MDM: mechanically deboned chicken meat; GAE: gallic acid equivalents; TE: trolox equivalents.

The oxidative stability of MDM samples supplemented with antioxidant was evaluated through FCR, ORAC and ICA assays and results showed in Table 3. A significant ($p < 0.05$) decrease from 1D to 30D was observed for all assays, including ICA values that were the most affected over time (94 to 57 % inhibition/mg meat db). After 30D, MDM samples showed significant changes, dependent on the assay. FCR values registered a significant ($p < 0.05$) increase at the end of storage, whilst concerning to ORAC and ICA values a decrease was observed. In addition, chelating ability presented random changes, including a significant ($p < 0.05$) strong increase at 60D, mainly in control sample, followed by a decrease, reaching 12 % inhibition/mg meat db, at 210D. We hypothesize that over storage time, MDM samples experimented degradation reactions with lipids and proteins as targets (Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008). Hence, secondary reaction products with iron binding ability such as peptides and oxidized amino acids (Storcksdieck, Bonsmann, & Hurrell, 2007), would be generated. Therefore, by the end of storage (210D), these compounds would undergo further degradation, including decrease of ICA values. Besides, secondary reaction products can also interfere in the FCR determination, and this can explain the results observed at 210D. It is important to note that when the effect of storage is evaluated, there is a difference in the frequency of sampling after the first two months. FCR assay registered non significant differences ($p > 0.05$) among the MDM samples, with TR as exception, exhibiting 3.55 mg GAE/g meat db, significantly lower compared with the other samples. Concerning to ICA values, TNac showed the lowest iron binding ability (58 % inhibition/mg meat db), whilst TR among the GPE added samples, exhibited the highest ICA values (77 % inhibition/mg meat db). GPE is mainly composed by anthocyanins, flavonols such as catechin and quercetin, flavanols, phenolic acids and resveratrol (Teixeira et al., 2014). The effectiveness of a given antioxidant depends on the relative solubility, in the food matrix. For example, some polyphenols such as catechin are water-soluble, whilst quercetin and synthetic antioxidants (BHT or BHA) are poorly water-soluble (Rice-Evans, Miller, & Paganga, 1996). The MDM composition (high fat content) and the relative solubility of polyphenols from GPE would explain differences in our results.

497 3.5 Fatty acid profile evolution

498 MDM samples were analyzed in order to compare fatty acids (FA) profile at 1D
499 and in advanced storage conditions (365 days, 365D); results are presented in
500 Table S2. Control sample presented the following profile: saturated fatty acids
501 (SFA): 31.7 ± 0.02%; monounsaturated fatty acids (MUFA): 51.5 ± 0.06% and
502 polyunsaturated fatty acids (PUFA): 16.7 ± 0.01%, at 1D. Palmitic acid (16:0)
503 with 24.0 ± 0.10 %; oleic acid (18:1 *n*-9) with 41.4 ± 0.08% and linoleic acid
504 (18:2 *n*-6) with 15.1 ± 0.14%, including the most representative FA for each
505 group, respectively. Our results indicated an overall control sample composition
506 consistent with previous reports (Kolsarıcı, Candoğan, & Akoğlu, 2010;
507 Trindade et al., 2004). The fatty composition significantly ($p < 0.05$) changed
508 over storage time. Under advanced storage (365D), significant ($p < 0.05$)
509 decrease was verified in SFA and MUFA, but not in the case of PUFA, in
510 contrast partway to Püssa *et al.* (Püssa et al., 2008). In their studies,
511 arachidonic acid and linoleic acid (both *n*-6 PUFA) were the main target of
512 oxidation, including a weighed contribution to the summary peroxidation
513 process of PUFA. Ours findings indicated that certain essential FA, namely α-
514 linolenic acid (18:3 *n*-3) and arachidonic acid (20:4 *n*-6) were detected only for
515 control sample after 1D in small quantities (0.34 ± 0.02% and 0.50 ± 0.01%,
516 respectively), whilst linoleic acid (also essential) did not suffered significant ($p >$
517 0.05) decrease over time. Differences in the results may be ascribed to the fatty
518 acids proportion, once composition of MDM samples used in our studies had
519 higher MUFA and lower PUFA concentration than Püssa *et al*. reported.
520 Regarding the protection against SFA degradation, it was observed a significant
521 ($p < 0.05$) reduction comparing with control sample. Nevertheless, TR and TF
522 were as effective as BHT-BHA even despite the synergism that exhibit these
523 two antioxidants (Lorenzo, González-Rodríguez, Sánchez, Amado, & Franco,
524 2013). Likewise, TR and TF did not significantly ($p > 0.05$) differ from BHT-BHA,
525 exhibiting antioxidant activity against the MUFA lipid oxidation. Additionally,
526 BHT-BHA was non-significantly ($p > 0.05$) different from control concerning to
527 MUFA at 365D. Interestingly, all MDM samples supplemented with GPE were
528 non-significantly ($p > 0.05$) different from control for the fatty acids aggregated

529 in n-3, whilst BHT-BHA and, as expectable, control sample after 365D exhibited
530 a significant ($p < 0.05$) decrease over advanced storage. .

531

532 ## 4. Conclusions

533 Briefly, the influence of Portuguese grape extracts on the oxidative stability,
534 nutritional, and color characteristics of MDM samples was investigated. Our
535 results showed that even under vacuum packaging and with antioxidant
536 supplementation, consequences of the MDM lipid oxidation were observed.
537 Nutritional composition of MDM samples were partially affected, mainly in fat
538 content, by the GPE supplementation. Concerning to TR and TNac with
539 important total phenolic content, they also influenced *L*, a** and *b** components
540 of color feature. Nevertheless, GPE, including TR and TF, were able to reduce
541 the consequences of the oxidation in highly perishable samples, proving
542 effectiveness in the oxidation prevention groups of FA, namely SFA and MUFA
543 at low concentration of GPE (60 mg/kg). It is noteworthy that nowadays
544 consumers are more concerned about the food composition, even more when
545 synthetic antioxidant commonly used in industry are suspected of cause
546 carcinogenesis. Further studies testing different GPE levels are needed, in
547 order to guarantee the preservation of MDM over long storage, although at the
548 same time, minimizing the effects regarding to color variables with latter impact
549 of finished products by consumers.

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References

Álvarez, D., Castillo, M., Payne, F., Garrido, M., Bañón, S., & Xiong, Y. (2007). Prediction of meat emulsion stability using reflection photometry. *Journal of Food Engineering, 82*(3), 310-315.

AOAC. (2002). Official methods of analysis (17 th ed.). Gaithersburg, MD: Association of Official Analytical Chemists, USA.

Clark, E. M., Mahoney, A. W., & Carpenter, C. E. (1997). Heme and Total Iron in Ready-to-Eat Chicken. *Journal of Agriculture and Food Chemistry, 45*(1), 124-126.

Estévez, M., Kylli, P., Puolanne, E., Kivikari, R., & Heinonen, M. (2008). Oxidation of Skeletal Muscle Myofibrillar Proteins in Oil-in-Water Emulsions: Interaction with Lipids and Effect of Selected Phenolic Compounds. *Journal of Agriculture and Food Chemistry, 56*(22), 10933-10940.

Faustman, C., Sun, Q., Mancini, R., & Suman, S. P. (2010). Myoglobin and lipid oxidation interactions: Mechanistic bases and control. *Meat Science, 86*(1), 86-94.

Field, R. (1988). Mechanically separated meat, poultry and fish. *Advances in Meat Research (USA), 5*, 83-126.

Folch, J., Lees, M., & Sloane-Stanley, G. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry, 226*(1), 497-509.

Froning, G. (1981). Mechanical deboning of poultry and fish. *Advances in Food Research, 27*, 109-147.

Gil, M., Serra, X., Gispert, M., Oliver, M. À., Sañudo, C., Panea, B., . . . Osoro, K. (2001). The effect of breed-production systems on the myosin heavy chain 1, the biochemical characteristics and the colour variables of Longissimus thoracis from seven Spanish beef cattle breeds. *Meat Science, 58*(2), 181-188.

Hac-Szymanczuk, E., Cegielka, A., Lipinska, E., & Ilczuk, P. (2014). Effect of sage on the microbial quality and TBARS value of mechanically deboned poultry meat. *Medycyna Weterynaryjna-Veterinary Medicine-Science and Practice, 70*(11), 704-708.

Hassan, O., Swet Fan, Lam. (2005). The anti-oxidation potential of polyphenol extract from cocoa leaves on mechanically deboned chicken meat (MDCM). *LWT - Food Science and Technology, 38*(4), 315-321.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *Journal of Agriculture and Food Chemistry, 50*(16), 4437-4444.

Hui, Y. H. (2012). *Handbook of meat and meat processing*: CRC press.

Karpinska, M., Borowski, J., & Danowska-Oziewicz, M. (2001). The use of natural antioxidants in ready-to-serve food. *Food Chemistry, 72*(1), 5-9.

Kearney, J. (2010). Food consumption trends and drivers. *Philosophical transactions of the royal society B: biological sciences, 365*(1554), 2793-2807.

Kolsarıcı, N., Candoğan, K., & Akoğlu, İ. T. (2010). Effect of frozen storage on alterations in lipids of mechanically deboned chicken meats. *GIDA/The Journal of Food, 35*(6), 403-410.

Lapornik, B., Prošek, M., & Golc Wondra, A. (2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. *Journal of Food Engineering, 71*(2), 214-222.

Lee, C.-H., Reed, J. D., & Richards, M. P. (2006). Ability of various polyphenolic classes from cranberry to inhibit lipid oxidation in mechanically separated turkey and cooked ground pork. *Journal of Muscle Foods, 17*(3), 248-266.

Lorenzo, J. M., González-Rodríguez, R. M., Sánchez, M., Amado, I. R., & Franco, D. (2013). Effects of natural (grape seed and chestnut extract) and synthetic antioxidants

(buthylatedhydroxytoluene, BHT) on the physical, chemical, microbiological and sensory characteristics of dry cured sausage “chorizo”. *Food Research International, 54*(1), 611-620.

Luciano, G., Monahan, F., Vasta, V., Pennisi, P., Bella, M., & Priolo, A. (2009). Lipid and colour stability of meat from lambs fed fresh herbage or concentrate. *Meat Science, 82*(2), 193-199.

Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S., & Lima, J. L. (2010). Rapid microplate high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity. *Talanta, 83*(2), 441-447.

Mielnik, M. B., Aaby, K., Rolfsen, K., Ellekjær, M. R., & Nilsson, A. (2002). Quality of comminuted sausages formulated from mechanically deboned poultry meat. *Meat Science, 61*(1), 73-84.

Mielnik, M. B., Aaby, K., & Skrede, G. (2003). Commercial antioxidants control lipid oxidation in mechanically deboned turkey meat. *Meat Science, 65*(3), 1147-1155.

Mohamed, H. M. H., & Mansour, H. A. (2012). Incorporating essential oils of marjoram and rosemary in the formulation of beef patties manufactured with mechanically deboned poultry meat to improve the lipid stability and sensory attributes. *LWT-Food Science and Technology, 45*(1), 79-87.

Navarro-Rodríguez de Vera, C., Sánchez-Zapata, E. J., Viuda-Martos, M., & Pérez-Alvarez, J. A. (2010). Handbook of Poultry Science and Technology. *2*, 73-80.

Naveena, B. M., Faustman, C., Tatiyaborworntham, N., Yin, S., Ramanathan, R., & Mancini, R. A. (2010). Detection of 4-hydroxy-2-nonenal adducts of turkey and chicken myoglobins using mass spectrometry. *Food Chemistry, 122*(3), 836-840.

Ozer, O., & Sariçoban, C. (2010). The effects of butylated hydroxyanisole, ascorbic acid, and α-tocopherol on some quality characteristics of mechanically deboned chicken patty during freeze storage. *Czech Journal of Food Sciences, 28*(2), 150-160.

Ozkececi, R., Karakaya, M., Yilmaz, M., Saricoban, C., & Ockerman, H. (2008). The effect of carcass part and packaging method on the storage stability of mechanically deboned chicken meat. *Journal of Muscle Foods, 19*(3), 288-301.

Püssa, T., Pällin, R., Raudsepp, P., Soidla, R., & Rei, M. (2008). Inhibition of lipid oxidation and dynamics of polyphenol content in mechanically deboned meat supplemented with sea buckthorn (Hippophae rhamnoides) berry residues. *Food Chemistry, 107*(2), 714-721.

Qwele, K., Hugo, A., Oyedemi, S. O., Moyo, B., Masika, P. J., & Muchenje, V. (2013). Chemical composition, fatty acid content and antioxidant potential of meat from goats supplemented with Moringa (Moringa oleifera) leaves, sunflower cake and grass hay. *Meat Science, 93*(3), 455-462.

Raghavan, S., & Richards, M. P. (2007). Comparison of solvent and microwave extracts of cranberry press cake on the inhibition of lipid oxidation in mechanically separated turkey. *Food Chemistry, 102*(3), 818-826.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*(7), 933-956.

Rojas, M. C., & Brewer, M. S. (2007). Effect of Natural Antioxidants on Oxidative Stability of Cooked, Refrigerated Beef and Pork. *Journal of Food Science, 72*(4), S282-S288.

Selani, M. M., Contreras-Castillo, C. J., Shirahigue, L. D., Gallo, C. R., Plata-Oviedo, M., & Montes-Villanueva, N. D. (2011). Wine industry residues extracts as natural antioxidants in raw and cooked chicken meat during frozen storage. *Meat Science, 88*(3), 397-403.

Shantha, N., & Ackman, R. (1990). Nervonic acid "versus" tricosanoic acid as internal standards in quantitative gas chromatographic analyses of fish oil longer-chain n-3 polyunsaturated fatty acid methyl esters. *Journal of Chromatography B: Biomedical Sciences and Applications, 533*, 1-10.

669 Shirahigue, L. D., Plata-Oviedo, M., de Alencar, S. M., d'Arce, M. A. B. R., de Souza Vieira, T. M.
670 F., Oldoni, T. L. C., & Contreras-Castillo, C. J. (2010). Wine industry residue as
671 antioxidant in cooked chicken meat. *International Journal of Food Science &*
672 *Technology, 45*(5), 863-870.
673 Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and
674 other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent.
675 *Methods In Enzymology, 299*, 152-178.
676 Storcksdieck, S., Bonsmann, G., & Hurrell, R. (2007). Iron-Binding Properties, Amino Acid
677 Composition, and Structure of Muscle Tissue Peptides from in vitro Digestion of
678 Different Meat Sources. *Journal of Food Science, 72*(1), S019-S029.
679 Suman, S. P., & Joseph, P. (2013). Myoglobin Chemistry and Meat Color. *Annual Review of*
680 *Food Science and Technology, 4*(1), 79-99.
681 Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., & Garcia-
682 Viguera, C. (2014). Natural Bioactive Compounds from Winery By-Products as Health
683 Promoters: A Review. *International Journal of Molecular Sciences, 15*(9), 15638-15678.
684 Teixeira, A., Eiras-Dias, J., Castellarin, S. D., & Gerós, H. (2013). Berry phenolics of grapevine
685 under challenging environments. *International Journal of Molecular Sciences, 14*(9),
686 18711-18739.
687 Trindade, M. A., Felício, P. E. d., & Castillo, C. J. C. (2004). Mechanically separated meat of
688 broiler breeder and white layer spent hens. *Scientia Agricola, 61*, 234-239.
689 Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging
690 and metal chelation of extracts from Icelandic seaweeds. *Food Chemistry, 116*(1), 240-
691 248.
692 Yu, J. M., & Ahmedna, M. (2013). Functional components of grape pomace: their composition,
693 biological properties and potential applications. *International Journal of Food Science*
694 *and Technology, 48*(2), 221-237.
695 Zakrys, P., Hogan, S., O'sullivan, M., Allen, P., & Kerry, J. (2008). Effects of oxygen
696 concentration on the sensory evaluation and quality indicators of beef muscle packed
697 under modified atmosphere. *Meat Science, 79*(4), 648-655.

698

Supplementary information

Influence of Portuguese grape extracts on the oxidative stability, nutritional, and color characteristics of mechanically deboned chicken meat

Hernán H. Tournour[1,2], Marcela A. Segundo[3], Luís M. Magalhães[3], Telmo J.R. Fernandes[4], M. Beatriz P. P. Oliveira[4], Luís M. Cunha[1,*].

[1]LAQV,REQUIMTE/DGAOT, Faculty of Sciences, University of Porto, Porto, Portugal.

[2]Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal.

[3]UCIBIO,REQUIMTE,Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal.

[4] LAQV,REQUIMTE, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal.

Number of Tables: 2

*Corresponding author: lmcunha@fc.up.pt. Tel.: (+351)252660400

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Table S1

Factor loadings for each color variable studied on the first two principal components obtained after Varimax rotation

Variables	PC1	PC2
*L**	0.127	0.957
*a**	0.822	-0.086
*b**	0.956	0.253
Chroma	0.887	-0.011
Hue angle	-0.220	0.398
Total variance explained	48.85%	22.91%
Acumulative variance	48.85%	71.76%

Table S2
Fatty acids profile of MDM samples after frozen storage. Values represent means ± standard deviation (S.D.) (g /100g, n = 4).

	Control1D		Control365D		BHT-BHA		TR		TNac		TF	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
14:0	0.6^{a}	± 0.03	0.5^{b}	± 0.00	0.5^{b}	± 0.01	0.5^{b}	± 0.03	0.5^{b}	± 0.02	0.5^{b}	± 0.01
16:0	24.0^{a}	± 0.10	21.1^{c,d}	± 0.09	22.3^{b}	± 0.08	21.1^{c,d}	± 0.74	20.7^{d}	± 0.24	21.0^{c,d}	± 0.14
16:1 *n*-9	0.6^{a}	± 0.003	0.5^{b}	± 0.01	0.5^{b}	± 0.01	0.5^{b}	± 0.01	0.5^{b}	± 0.02	0.5^{b}	± 0.00
19:1 *n*-7	6.2^{a}	± 0.01	5.4^{b,c}	± 0.03	5.7^{b}	± 0.03	5.4^{b,c}	± 0.22	5.3^{c}	± 0.04	5.5^{b,c}	± 0.07
17:1	0.2^{b}	± 0.01	0.9^{a}	± 0.01	0.6^{a}	± 0.04	0.8^{a}	± 0.14	0.9^{a}	± 0.25	0.7^{a}	± 0.24
18:0	6.4^{a}	± 0.07	5.8^{c}	± 0.04	6.2^{a,b}	± 0.16	6.1^{a,b,c}	± 0.03	6.0^{b,c}	± 0.02	6.2^{a,b,c}	± 0.19
18:1 *n*-9	41.4^{a}	± 0.08	36.3^{b,c,d}	± 0.14	38.3^{b}	± 0.29	35.6^{c,d}	± 1.56	35.2^{d}	± 0.57	36.2^{b,c,d}	± 0.07
18:1 *n*-7	2.1^{a,b,c}	± 0.02	2.1^{a,b,c}	± 0.01	2.2^{a}	± 0.03	2.1^{b,c}	± 0.06	2.3^{c}	± 0.04	2.1^{a,b}	± 0.03
18:2 *n*-6	15.1^{a}	± 0.14	14.9^{a}	± 0.07	14.6^{a}	± 0.05	14.2^{a}	± 0.22	15.2^{a}	± 1.41	14.5^{a}	± 0.13
20:1 *n*-9	0.7^{a}	± 0.08	0.8^{a}	± 0.02	0.7^{a}	± 0.01	0.7^{a}	± 0.01	0.9^{a}	± 0.18	0.7^{a}	± 0.01
20:3 *n*-6	n.d.		0.9^{c}	± 0.00	1.0^{b,c}	± 0.08	1.3^{a,b}	± 0.20	1.2^{a,b,c}	± 0.14	1.3^{a}	± 0.13
SFA	31.7^{a}	±0.02	28.6^{c,d}	±0.22	30.2^{b}	±0.29	28.9^{b,c,d}	±0.49	28.2^{d}	±0.24	28.9^{b,c,d}	±0.37
MUFA	51.5^{a}	±0.06	46.5^{b,c}	±0.04	48.7^{a,b}	±0.35	45.9^{b,c}	±1.7	45.5^{c}	±0.96	46.5^{b,c}	±0.33
PUFA	16.7^{a}	±0.01	16.2^{a}	±0.21	16.4^{a}	±0.15	16.3^{a}	±0.02	17.3^{a}	±1.24	16.7^{a}	±0.33
***n*-3**	0.6^{a}	±0.02	0.3^{c}	±0.01	0.5^{b}	±0.04	0.6^{a,b}	±0.09	0.6^{a,b}	±0.02	0.6^{a,b}	±0.07
***n*-6**	15.2^{a}	±0.04	15.9^{a}	±0.11	15.8^{a}	±0.11	15.6^{a}	±0.08	16.6^{a}	±1.27	16.0^{a}	±0.26

Data was analyzed by MANOVA and within each row different superscripts (^{a,b}), indicate statistically differences according to Tukey post-hoc test at 95% confidence level. Control1D and Control365D: MDM without antioxidant added; BHT-BHA: butylated hydroxytoluene-butylated hydroxyanisole (100 mg/kg each): TR: Tinta roriz, TNac: Touriga nacional, and TF: Touriga franca (60 mg/kg each). MDM: mechanically deboned chicken meat; n.d.: not detected.

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Paper IV

Hernán H. Tournour, Marcela A. Segundo, Luís M. Magalhães, Anabela S. G. Costa, Luís M. Cunha. Effect of *"Touriga nacional"* grape extract on quality characteristics of mechanically deboned chicken meat kept under frozen storage. [Submitted for publication].

Effect of *"Touriga nacional"* grape extract on quality characteristics of mechanically deboned chicken meat kept under frozen storage

Hernán H. Tournour[1,2], Marcela A. Segundo[3], Luís M. Magalhães[3], Anabela S. G. Costa[4], Luís M. Cunha[2,*]

[1]LAQV,REQUIMTE/DGAOT, Faculty of Sciences, University of Porto, Porto, Portugal,

[2] Faculty of Nutrition and Food Sciences, University of Porto, Porto,Portugal,

[3] UCIBIO,REQUIMTE, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal,

[4] LAQV,,REQUIMTE, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal.

**Corresponding author: lmcunha@fc.up.pt*

Campus Agrário de Vairão

Rua Padre Armando Quintas, 7

4486-661 Vairão

Portugal

Tel.: (+351)252660400 / Fax: (+351)252661780

ABSTRACT

The effect of *"Touriga nacional"* grape pomace extract (GPE) on quality characteristics (composition, pH, colour variables and oxidative stability) of mechanically deboned chicken meat (MDM) under frozen storage was evaluated. Two MDM samples with different compositions, fortified with two levels of GPE (60 and 120 mg/kg) were tested. Colour variables (*L**, *b** and hue angle) and oxidative stability of MDM samples was composition-dependent. Fat content showed a strong influence on the oxidative stability regarding oxygen reactive absorbance capacity and iron(II) chelating ability assays. Although both MDM batches significantly ($p < 0.05$) changed, high-fat-content samples became easily less red (from 24.28 to 19.22, control) during storage. Butylated hydroxytoluene - Butylated hydroxyanisole (BHT-BHA) and GPE added samples exhibited a similar behaviour regarding oxidative stability. The GPE supplementation did not significantly contribute to oxidative stability; reducing the colour attributes associated to fresh MDM. Thoughtfulness about MDM composition would be considered towards GPE applications.

Keywords: antioxidant; meat colour; mechanically deboned chicken meat; quality characteristics; wine grape pomace.

Highlights

- Portuguese grape pomace from "*Touriga nacional*" variety was target for the extraction of bioactives compounds,
- Different proximate compositions of MDM and two levels of GPE were evaluated,
- Proximate composition was proved as an influential factor for the antioxidant performance,
- The GPE supplementation had important influence on colour parameters.

1. Introduction

Prevention of lipid oxidation represents an important issue for pharmaceutical and industrial areas. Secondary oxidation products, namely aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers (Barriuso, Astiasarán, & Ansorena, 2013), resulted from lipid oxidation reactions can alter the physicochemical characteristics, shelf life and also functional properties of meat products (Faustman, Sun, Mancini, & Suman, 2010; G. W. Froning & McKee, 2001).

Mechanically deboned chicken meat (MDM) is a soft texture meat product in which lipid oxidation is the main preservation target. Conditions for development of oxidative processes have bases on its high fat content, unsaturated nature of the fatty acids and contact between fat and iron from co-extracted marrow bones during recovery procedure (G. Froning, 1981; Trindade, Felício, & Castillo, 2004). However, the increasing demand of comminuted and meat batter and reconstructed products, namely, bologna and frankfurters type sausages, breakfast sausages, mortadella, nuggets and roasts (G. W. Froning & McKee, 2001; Hui, 2012; Mielnik, Aaby, Rolfsen, Ellekjær, & Nilsson, 2002) in which MDM is commonly incorporated in the formulation due its great functional properties as natural emulsifier and water holding agent (Navarro-Rodríguez de Vera, Sánchez-Zapata, Viuda-Martos, & Pérez-Alvarez, 2010), fosters the search of alternatives to overcome MDM degradation upon storage, improving its quality concomitantly.

In this context, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ) have been used for over 50 years.

Nevertheless, from the consumers´ point of view, they suffer from a negative image due to their artificial nature (Robert G. Brannan & Mah, 2007). Hence, there is a trend on exploiting alternatives to avoid or to reduce the consequences of the lipid oxidation in meat and meat products gave by natural antioxidants from vegetable, herbs and spices tissues, including therapeutic properties, which has been recently reviewed (Shah, Bosco, & Mir, 2014).

Portugal, worldwide-known as high quality wine producer, with outputs of over 835.000 million tons of wine (FAOSTAT, 2012) generates important quantities of valuable grape pomace after winemaking procedure. Grape pomace has been reported as having remarkable concentration of bioactives, namely polyphenolic compounds with potential applications in food and pharmaceutical fields (Guendez, Kallithraka, Makris, & Kefalas, 2005; Rababah, Hettiarachchy, & Horax, 2004; Serra, Matias, Nunes, Leitão, Brito, Bronze, et al., 2008).

A recent literature search on the ISI Web of Knowledge search engine containing “mechanically deboned meat” and “grape extract” as keywords, revealed no research reporting on the putative effect grape pomace extract (GPE) on MDM, during frozen storage. Therefore, the main aim of this work was to evaluate the effect of pomace extract from Portuguese “*Touriga nacional*” variety on the overall characteristics of MDM. Hence, two approaches were exploited covering the influence of the initial MDM composition on the antioxidant performance of the GPE; and also evaluating the effect of GPE concentration on the quality of MDM through the analysis of changes in proximate composition, pH, colour variables and oxidative stability during frozen storage.

2. **Material and methods**

2.1. Materials

BHT and BHA (Sigma-Aldrich, MO) Kocher grade were used as synthetic antioxidants. Ultrapure water (resistivity > 18 Ω) and absolute ethanol p. a. were obtained from Sartorius Goettingen, Germany and Panreac Química, Spain, respectively. Wine pomace from "*Touriga nacional*" (*Vitis vinifera* L. grape variety) was used in this study. Grape skins and seeds were dried in an oven (Thermo Scientific, Pittsburg, PA) until reaching a final moisture lower than 5% (w/w). Oven operating conditions were 55 ºC with no forced air. Dried material was grinded (KenWood, New Lane, UK) until achieving a particle size of 2-3 mm in order to improve polyphenols´ extraction. The extraction step was performed according to Shirahigue *et al*. (Shirahigue, Plata-Oviedo, de Alencar, d'Arce, de Souza Vieira, Oldoni, et al., 2010). Briefly, 20 g of dried material was displayed into an Erlenmeyer flask using 100 ml of 80% (v/v) ethanol/water mixture as solvent. The mixture was allowed to stand under orbital agitation at 300 rpm for 48 h at room temperature and in darkness. After the extraction step, the liquid phase was separated from solid by vacuum filtration through a 45 µm Millipore (Billerica, USA) polyvinylidene fluoride membrane filter. The liquid filtrated was then concentrated in a vacuum rotary evaporator (Büchi, Switzerland) at 65 ºC aided by a nitrogen stream until dryness. The dried residue, once the solvent was completely evaporated, was redissolved in 50 mL of water to form the final grape pomace extract (GPE). Smaller portions were separated and reserved under -80 ºC in amber recipients. At the analysis time, GPE were thawed to room temperature. All steps were performed in duplicate.

2.2. Experimental design

Two studies were conducted as depicted in Fig.1. In the first study, two different MDM batches were tested regarding to assess the influence of MDM composition on the antioxidant effectiveness of GPE as antioxidant. The second study was carried out to evaluate the GPE concentration response on its performance as antioxidant using a fixed MDM composition. Both studies were conducted under frozen storage.

Samples preparation of Study 1

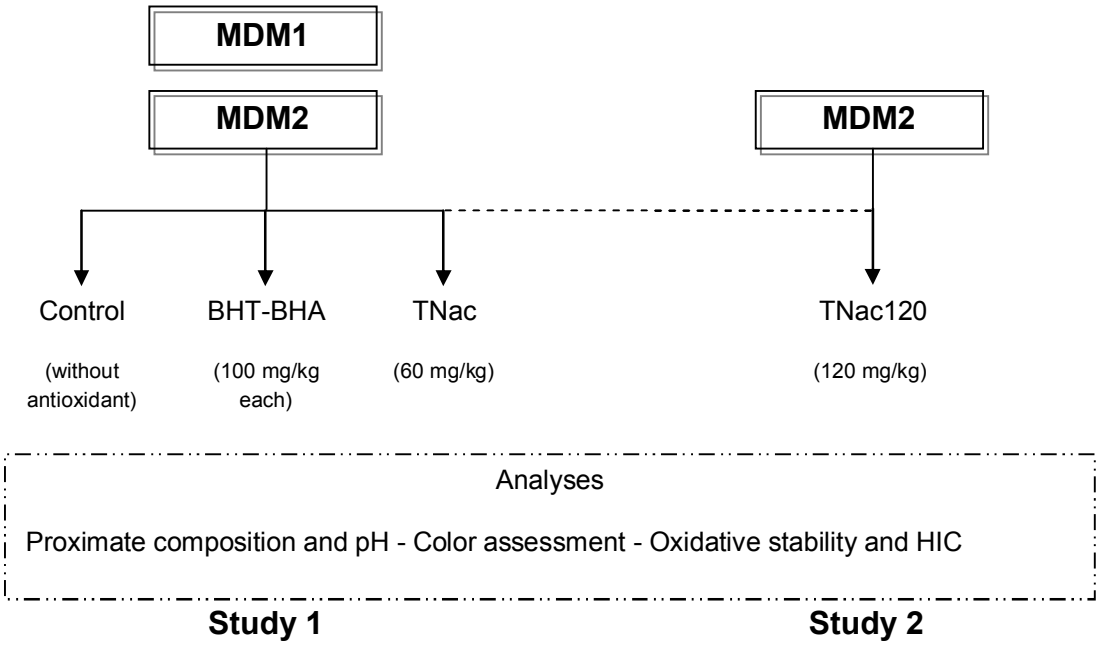

Fig. 1. Experimental design diagram. MDM: mechanically deboned chicken meat; MDM1; MDM2: samples from batch number 1 and batch number 2, respectively.

129 MDM samples from two different production batches (MDM1 and MDM2) were
130 supplied from a local slaughter industry. GPE vintage 2012 was used in
131 supplementation of MDM1, whilst GPE vintage 2013 was used in MDM2. Each
132 MDM batch were divided to the following formulations: GPE from TNac was
133 added at 60 mg/kg (TNac); BHT-BHA was added at 100 mg/kg each (BHT-
134 BHA) and control composed by MDM without antioxidant addition (control).
135 MDM samples were homogenized in a food processor (KenWood, UK). The
136 homogenized samples were separated in smaller portions displayed in a layer
137 form, vacuum packaged (Sammic, Spain) in oxygen barrier bags and stored
138 under frozen conditions (-23 ± 1 ºC) for seven months. Analyses were
139 performed at the beginning (1D) and at the end (210D) of storage time.

140 *Samples preparation of Study 2*

141 GPE (vintage 2013) was also supplemented at 120 mg/kg for MDM2
142 (TNac120). Analyses were also performed at the beginning (1D) and at the end
143 (210D) of storage time.

144 *2.3. MDM sample analysis*

145 The following measurements were performed in MDM samples:

146 *Proximate composition and pH determination*

147 Moisture, protein and total fat were determined for MDM batch according to the
148 methods recommended by the Association of Official Analytical Chemists
149 (AOAC, 2002). Results were expressed as percentages in dry basis. For the pH
150 determination (Ozer & Sariçoban, 2010) a pH meter (Hanna Instruments,
151 Michigan, USA) was used.

Colour variables analysis

For the colour determination, *L** (luminosity), *a** (redness), *b** (yellowness), *chroma* ($(a^{*2} + b^{*2})^{1/2}$), which is the colour saturation index, and the *Hue angle* as arc tan (*b**/*a**) in radians (rad.), which indicates the degree of departure from the true redness on the CIE colour scale, were assessed using a Minolta CR-400 colorimeter (Minolta Camera Co. Osaka, Japan). Illuminant C was used as standard light source with 2º observer.

Oxidative stability assessment

Meat extracts for the oxidative stability assessment were prepared according to Qwele *et al.* (Qwele, Hugo, Oyedemi, Moyo, Masika, & Muchenje, 2013) with some modifications. Briefly, 1 g of each MDM sample was homogenized with 10 ml of 0.05 M KH_2PO_4 phosphate buffer (pH 7.0). The extraction step was carried out alternating ultrasound and vortex cycles (2 min, 3 times at 3000 rpm). Before the last cycle, meat samples were left for 10 min to stand in order to assist in the tissue hydration and improve the extraction. Finally, extracted samples were centrifuged at 5,580 x g for 30 min at 4 °C. Supernatant aliquots (1 ml) were disposed in Eppendorf tubes and one drop of concentrated HCl was added in order to decrease the pH and circumvent autoxidation of phenolic compounds. Finally, extracts were frozen at -80 ºC till analysis by Folin-Ciocalteu reducing content (FCR), Oxygen reactive substances capacity assay (ORAC) and iron(II) chelating ability assay (ICA) as referred in supplementary information using diluted meat extracts instead of GPE.

Haem iron content (HIC)

175 The haem iron content was determinate by the modified methodology of Clark
176 *et al.* (Clark, Mahoney, & Carpenter, 1997). The HIC is calculated in dry basis,
177 as: HIC (µg/g meat db) = (A_{640} x 680) x 0.0882, with the factor 0.0882 µg/µg
178 hematin.

179 *2.4. Statistical analysis*

180 Means and standard deviation (S.D.) were given for all parameters calculated.
181 Student´s t-test was performed to distinguish significant differences between
182 vintages. A 3-factor or 2-factor ANOVA fully-nested was carried out, including,
183 batch, samples and storage time as fixed factors. Data analysis was performed
184 with STATISTICA for Windows version 12.0 (STATISTICA 12 Software,
185 StatSoft, Tulsa, OK). Moreover, multiple comparisons using Tukey test in order
186 to identified significant differences between experiments were also obtained.
187 Principal Component Analysis (PCA) with *Varimax* rotation was carried out in
188 order to reduce information on colour variables, composition and antioxidant
189 capacity. Except when referred, all tests were applied at 95% confidence level.

190 3. **Results**

191 3.1. GPE characterization

192 Non-significant differences ($p > 0.05$) were observed between vintages for all
193 assays, with only TPC assay as exception, including vintage 2012 (142 mg
194 GAE/g extract) with significantly ($p < 0.05$) higher TPC values than vintage 2013
195 (135 mg GAE/g extract), see Table S1, supplementary information. Results are
196 in agreement with previous works (Jordao, Simoes, Correia, & Goncalves,
197 2012; Negro, Tommasi, & Miceli, 2003).

198 3.2. Evaluation of MDM initial composition

199 *3.2.1. Proximate composition and pH*

200 Results for moisture, protein, fat contents, and pH values were given in Table 1.

201

Table 1
Mean values (± S.D.; n = 2) for proximate composition and pH values of MDM samples, during frozen storage.

	Batch	Sample	Storage time	
			1D	210D
%Moisture	MDM1	Control	61.4 (±1.5)	62.4 (±0.4)
		BHTBHA	63.8 (±0.9)	64.4 (±0.1)
		TNac	63.0 (±0.4)	63.4 (±0.2)
	MDM2	Control	66.8 (±1.0)	66.4 (±0.2)
		BHTBHA	66.8 (±0.7)	66.4 (±0.4)
		TNac	66.5 (±1.0)	65.7 (±0.4)
p-value	< 0.001	0.027	0.766	
%Protein db	MDM1	Control	31.5 (±0.2)	32.8 (±0.6)
		BHTBHA	34.0 (±1.2)	34.8 (±1.7)
		TNac	35.3 (±1.0)	33.3 (±2.3)
	MDM2	Control	40.9 (±1.6)	39.5 (±1.0)
		BHTBHA	40.3 (±1.0)	41.9 (±1.0)
		TNac	41.1 (±0.7)	40.0 (±0.7)
p-value	< 0.001	0.141	0.407	
%Fat db	MDM1	Control	65.8 (±0.1)	59.8 (±0.3)
		BHTBHA	59.0 (±0.7)	61.3 (±0.5)
		TNac	62.2 (±3.0)	60.3 (±0.8)
	MDM2	Control	57.4 (±1.3)	54.2 (±1.7)
		BHTBHA	51.4 (±1.1)	53.8 (±2.2)
		TNac	52.8 (±0.2)	49.0 (±2.5)
p-value	< 0.001	0.062	0.011	
pH	MDM1	Control	6.73 (±0.01)	6.75 (±0.00)
		BHTBHA	6.71 (±0.14)	6.85 (±0.01)
		TNac	6.84 (±0.11)	6.99 (±0.01)
	MDM2	Control	6.71 (±0.09)	6.78 (±0.14)
		BHTBHA	6.86 (±0.08)	6.85 (±0.01)
		TNac	6.81 (±0.08)	6.77 (±0.05)
p-value	0.649	0.071	0.362	

Data were analyzed by 3-factor fully-nested ANOVA: batch, sample and storage time.

202

203 A 3-factor fully nested ANOVA design indicated significant differences ($p <$
204 0.001) between MDM batches regarding to proximate composition. Concerning
205 pH values, non-significant differences were observed between batches,
206 including control samples with pH 6.72 in average. These pH values favour the

207 water holding capacity, a valuable functional property of MDM. Additionally,
208 when batches were compared among samples nested into then, non-significant
209 ($p > 0.05$) differences were observed for proximate composition parameters and
210 pH values. Moisture content was an as exception, being significantly ($p < 0.05$)
211 for MDM2 samples than MDM1. Hence, as expected, the antioxidant
212 supplementation did not strongly affect the nutritional characteristics of MDM
213 samples. Regarding storage time, the fully-nested design showed non-
214 significant differences between 1D and 210D for most of parameters above,
215 including significant ($p < 0.05$) changes in fat content. Overall, MDM1 samples
216 showed lower values of moisture and protein, although higher fat content in
217 average than MDM2 samples.

218 *3.2.2. Colour variables*

219 Results for colour *L*, a*, b**, chroma and Hue angle were presented in Table 2.

220

Table 2
Mean values (± S.D.; n = 6) for colour variables of MDM samples, during frozen storage.

	Batch	Sample	Storage time	
			1D	210D
*L**	MDM1	Control	61.33 (±4.62)	56.93 (±1.36)
		BHTBHA	66.52 (±2.04)	52.31 (±4.40)
		TNac	66.24 (±2.20)	48.61 (±8.29)
	MDM2	Control	52.10 (±0.70)	49.36 (±0.61)
		BHTBHA	52.36 (±1.01)	50.57 (±0.52)
		TNac	51.51 (±0.41)	48.24 (±0.45)
p-value	< 0.001	0.257	0.089	
*a**	MDM1	Control	24.28 (±1.57)	19.22 (±1.24)
		BHTBHA	23.44 (±0.70)	20.79 (±1.25)
		TNac	20.30 (±1.18)	18.69 (±1.37)
	MDM2	Control	22.57 (±0.54)	18.72 (±0.67)
		BHTBHA	20.36 (±1.09)	18.93 (±0.56)
		TNac	18.53 (±0.81)	15.85 (±0.18)
p-value	0.107	< 0.01	< 0.05	
*b**	MDM1	Control	17.04 (±0.60)	14.24 (±1.16)
		BHTBHA	17.38 (±0.24)	15.15 (±0.71)
		TNac	15.59 (±0.35)	13.70 (±0.54)
	MDM2	Control	11.62 (±0.75)	11.55 (±0.28)
		BHTBHA	11.11 (±0.44)	12.33 (±0.49)
		TNac	10.41 (±0.31)	10.82 (±0.38)
p-value	< 0.01	0.247	0.617	
Chroma	MDM1	Control	29.67 (±1.47)	23.95 (±1.13)
		BHTBHA	29.18 (±0.69)	25.74 (±1.21)
		TNac	25.60 (±1.11)	23.18 (±1.25)
	MDM2	Control	25.40 (±0.44)	21.99 (±0.70)
		BHTBHA	23.20 (±0.97)	22.60 (±0.56)
		TNac	21.26 (±0.81)	19.19 (±0.33)
p-value	0.880	< 0.01	< 0.05	
Hue angle	MDM1	Control	0.61 (±0.03)	0.64 (±0.05)
		BHTBHA	0.64 (±0.01)	0.63 (±0.03)
		TNac	0.66 (±0.02)	0.63 (±0.03)
	MDM2	Control	0.48 (±0.03)	0.55 (±0.01)
		BHTBHA	0.50 (±0.03)	0.58 (±0.02)
		TNac	0.51 (±0.01)	0.60 (±0.01)
p-value	< 0.01	0.269	< 0.05	

Data were analyzed by 3-factor fully-nested ANOVA: batch, sample and storage time.

221

222 A 3-factor fully nested ANOVA design indicated significant differences between
223 MDM batches regarding to *L** ($p < 0.001$), *b** ($p < 0.01$) and Hue angle ($p <$
224 0.01). Overall, MDM1 samples were lighter, and yellower in average, than
225 MDM2 samples. When batches are compared among samples, significant
226 differences ($p < 0.01$) were observed regarding *a** (redness) and chroma
227 values. Hence, it seems that antioxidant supplementation influenced the final
228 colour of MDM samples, including a decrease in redness from 24.28 to 20.30
229 for MDM1 samples and from 22.57 to 18.53 for MDM2 samples. Besides, this
230 trend was also observed regarding chroma *v*alues, including changes from
231 29.67 to 25.60 and from 25.40 to 21.26, for MDM1 and MDM2 samples,
232 respectively, with TNac and BHT-BHA added samples as an exception.
233 Concerning the influence of storage time on colour variables, significant ($p <$
234 0.05) changes were observed in *a**, chroma and Hue angle, including the
235 highest values after 1D for *a** and chroma, and an opposite behaviour for Hue
236 angle.

237 *3.2.3. Oxidative stability and HIC*

238 The results of the oxidative stability assessment were given in Table 3.
239 Significant differences were observed concerning batch and storage time for
240 FCR, ORAC, ICA and HIC values.

241

Table 3
Mean values (± S.D.) for the oxidative stability analyzed through FCR (n = 4), ORAC (n = 3) and ICA (n = 4) assays and HIC (n = 3) of MDM samples, during frozen storage.

	Batch	Sample	Storage time	
			1D	210D
FCR (mg GAE/g meatdb)	MDM1	Control	3.58 (±0.12)	3.86 (±0.11)
		BHTBHA	3.90 (±0.11)	3.92 (±0.08)
		TNac	3.70 (±0.14)	4.22 (±0.09)
	MDM2	Control	4.18 (±0.01)	4.64 (±0.07)
		BHTBHA	4.13 (±0.08)	4.29 (±0.08)
		TNac	4.34 (±0.16)	4.45 (±0.04)
p-value	< 0.01	< 0.01	< 0.01	
ORAC (µmol TE/g meatdb)	MDM1	Control	58.6 (±9.0)	48.4 (±10.7)
		BHTBHA	64.0 (±7.6)	52.4 (±8.1)
		TNac	59.2 (±2.7)	45.4 (±8.8)
	MDM2	Control	51.2 (±7.7)	126.7 (±9.9)
		BHTBHA	53.0 (±7.9)	134.2 (±11.7)
		TNac	58.1 (±2.7)	145.0 (±6.4)
p-value	< 0.001	0.240	< 0.001	
ICA (%inihib./mg meatdb)	MDM1	Control	105 (±7)	9 (±6)
		BHTBHA	101(±4)	18 (±0)
		TNac	76 (±7)	14 (±4)
	MDM2	Control	95 (±8)	44 (±0)
		BHTBHA	91 (±8)	49 (±0)
		TNac	78 (±17)	49 (±7)
p-value	< 0.001	0.640	< 0.01	
HIC (µg/g meatdb)	MDM1	Control	n.a.	337 (±11)
		BHTBHA	n.a.	275 (±2)
		TNac	n.a.	310 (±1)
	MDM2	Control	480 (±2)	595 (±3)
		BHTBHA	486 (±4)	320 (±2)
		TNac	757 (±4)	540 (±4)
p-value	< 0.0001	< 0.0001	< 0.0001	

Data were analyzed by 3-factor fully-nested ANOVA: batch, sample and storage time. FCR: Folin-Ciocalteu reducing assay; ORAC: oxygen radical absorbance capacity assay and ICA: iron(II) chelating ability assay. HIC: haem iron content. GAE: gallic acid equivalents; TE: trolox equivalents. n.a.: not available.

The Folin-Ciocalteu reducing content of MDM2 samples was significantly ($p < 0.01$) higher in average than MDM1 samples, including control samples 4.18 and 3.58 mg GAE/g meatdb for MDM1 and MDM2, respectively. In contrast, ORAC values were significantly higher ($p < 0.001$) for MDM1 samples in average than MDM2. In the same context, MDM1 samples showed significantly

248 ($p < 0.001$) higher ICA values than MDM2 samples, including control samples
249 with iron-binding ability over 90% of the total iron available. Besides, no
250 changes were observed regarding antioxidant addition (BHT-BHA or TNac)
251 through FCR, ORAC and ICA assays.

252 The storage time significantly affected the oxidative stability of MDM samples
253 belonging to both batches, including a strong decrease of ICA values upon
254 210D mainly for control samples in MDM1 and MDM2 samples. FCR values for
255 MDM1 ranged 4.22 to 3.86 mg GAE/g meatdb, whilst for MDM2 ranged 4.29 to
256 4.64 mg GAE/g meatdb, after 210D. Concerning ORAC values, MDM1 samples
257 showed lower final values, ranging 45.4 to 52.4 μmol TE/g meatdb, than MDM2
258 (126.7 to 145.0 μmol TE/g meatdb) upon the end of storage time. The same
259 trend was observed regarding ICA values, including higher values for MDM2
260 (44 to 49 %inhibition/mg meatdb) than MDM1 (9 to 18 %inhibition/mg meatdb).

261 The haem iron content (MDM2) also suffered significant ($p < 0.0001$) changes
262 along the storage time, including an increase in HIC values for control samples,
263 and an opposite behaviour for antioxidant samples added.

264 *3.3.* Evaluation of GPE concentration

265 *3.3.1. Composition and pH*

266 Results for all variables assessed for TNac120 sample were given in Table 4
267 and *p*-values according to a 2-factor fully nested ANOVA including sample
268 (TNac60 and TNac120) and storage (1D and 210D) as factors, were also
269 presented in Table 4.

270

Table 4
Mean values (± S.D.) of all variables assessed for TNac120 sample, during frozen storage, accompanied by (***p*-value)** resulted from comparison between TNac60 and TNac120 samples.

Variable	Storage time	
	1D	210D
%Moisture **(< 0.05)**	68.8 (±0.9)	67.4 (±3.9)
	0.460	
%Protein db **(0.753)**	41.8 (±0.7)	39.9 (±2.2)
	0.297	
%Fat db **(< 0.05)**	54.8 (±0.1)	49.6 (±0.8)
	< 0.05	
pH **(1.000)**	6.53 (±0.06)	6.65 (±0.22)
	0.640	
*L** **(0.102)**	50.27 (±0.34)	47.86 (±0.42)
	< 0.01	
*a** **(0.840)**	17.92 (±0.62)	15.36 (±0.72)
	< 0.05	
*b** **(< 0.01)**	8.83 (±0.28)	9.16 (±0.31)
	0.389	
Chroma **(0.167)**	19.98 (±0.53)	17.89 (±0.69)
	0.059	
Hue angle **(< 0.05)**	0.46 (±0.02)	0.54 (±0.02)
	< 0.05	
FCR (mg GAE/g meatdb) **(0.374)**	4.29 (±0.05)	4.69 (±0.05)
	< 0.05	
ORAC (µmol TE/g meatdb) **(< 0.01)**	50.1 (±7.9)	93.1 (±3.8)
	< 0.001	
ICA (%inhib./mg meatdb) **(0.315)**	79 (±17)	60 (±7)
	0.229	
HIC (µg/g meatdb) **(< 0.001)**	430 (±3)	510 (±4)
	< 0.001	

Proximate composition and pH (n = 2); colour variables (n = 6); Data were analyzed by 2-factor fully-nested ANOVA: sample and storage time. FCR: Folin-Ciocalteu reducing assay (n = 4); ORAC: oxygen radical absorbance capacity assay (n = 3) and ICA: iron(II) chelating ability assay (n = 4). HIC: haem iron content (n = 3). GAE: gallic acid equivalents; TE: trolox equivalents.

271

272

273 Antioxidant supplementation at 120 mg/kg of GPE significantly ($p < 0.05$)
274 increased moisture content, reaching 68.8% as final value. On the other hand,
275 after 210D a significant decrease ($p < 0.05$) concerning fat content was
276 observed, whilst non-significant changes were observed for protein, moisture or
277 pH.

278 *3.3.2. Colour*

279 As depicted in Table 4, doubled GPE concentration significantly ($p < 0.01$)
280 affected *b** values and Hue angle in comparison to GPE at 60 mg/kg. Hence,
281 MDM added samples at 120 mg/kg of GPE exerted a final colour less yellow
282 than at 60 mg/kg and with lower Hue angle. Besides, the effect of storage time
283 was verified in *L*, a** and Hue angle.

284 *3.3.3. Antioxidant and HIC*

285 ORAC assay and HIC of MDM samples supplemented with 120 mg/kg of GPE
286 showed significantly ($p < 0.01$) lower oxygen reactive substances values than
287 MDM 60 mg/kg added samples. Regarding FCR and ICA assays, non-
288 significant differences were observed. Additionally, significant changes were
289 observed after 210D of storage time concerning FCR assay ($p < 0.05$), ORAC
290 assay ($p < 0.001$) and HIC values ($p < 0.001$).

291 *3.4. Principal component analysis (PCA)*

292 Fig. 2 shows a final PCA undertaken on relevant variables of MDM1 and MDM2
293 samples from the evaluation of the effect of MDM composition. The first two
294 principal components of the PCA were able to explain about 84% of the total
295 variability. Factor loading were given in Table S2.The first principal component

296 was the most important regarding its percentage of the total explained variability
297 (PC1 47.94%). PC1 was positively correlated with *a** (redness), chroma and
298 negatively correlated with antioxidant properties, including FCR and ORAC
299 values. The second principal component, PC2, that explains 36.73% of total
300 variability, was positively correlated with *b** (yellowness) and Hue angle, and
301 likewise PC1 negatively correlated with antioxidant properties. Additionally, *L**
302 (luminosity) and fat content showed an intermediate correlation with PC1 and
303 PC2.

304

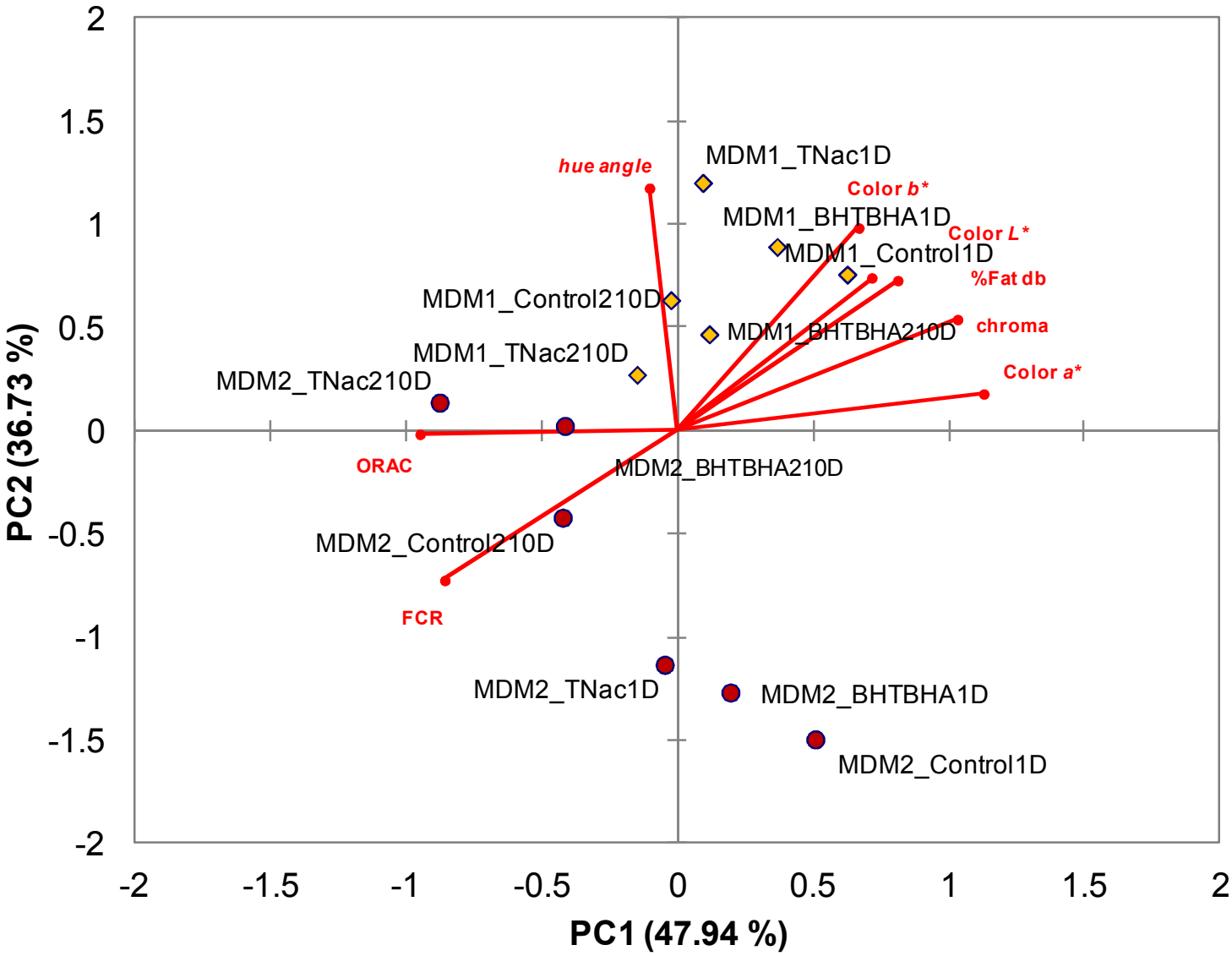

Fig. 2. Relationships among significant variables of MDM samples measured during frozen storage according to PCA. Projections of the variables in the plane defined by the first three principal components explaining 86.67% of total variability.

4. **Discussion**

Recovery processes applied during the MDM extraction deal with considerable shearing action on the frame, drumsticks, backs, and necks, commonly used for this purpose, including marked cellular disruption. Besides, factors, namely, bone-to-meat ratio, age of the bird, skin content, cutting methods, deboner settings, and species influence the final MDM composition (G. Froning, 1981; G. W. Froning & McKee, 2001). In this work, MDM1 samples showed higher fat content than MDM2 (over 16% higher comparing both control samples). In contrast, MDM1 showed lower protein content than MDM2, may be due to the dilution effect of the increased fat content. In this context, Principal component analysis clearly separated MDM samples based mainly on their composition, as depicted in Fig. 2. Hence, MDM1 samples (highest values for fat content and chroma) were projected on positive side of PC1, whilst MDM2 had an opposite projection.

Supplementation of MDM samples with GPE did not significantly affected the proximate composition, although samples containing GPE at 120 mg/kg presented increased moisture content. Differences between %moisture may be ascribed to extra water added during antioxidant supplementation until reach 120 mg/kg of GPE. Although non-significant, when added double GPE concentration, pH value decreased from 6.81 to 6.53. This fact is associated to GPE composition, including gallic acid, syringic acid, and caffeic acid as main phenolic acids present, bearing pKa values for the carboxylic group between 3.6 and 4.0 and therefore contributing to overall pH decrease.

328 The colour in meat is the result of the interaction between incident radiation
329 (illuminant) and meat surface. MDM samples exhibited significant differences
330 regarding colour variables. Colour variable *L** is a measurement of the
331 luminosity on the surface of a given material, ranging from 0 (black) to 100
332 (white). Additionally, a positive *a** value indicates red with a higher value
333 denoting more red whilst, a negative *a** value indicates green. The positive and
334 negative *b** values indicate yellow and blue, respectively. Hence, MDM1
335 samples were significantly lighter, and yellower than MDM2. Previously, it was
336 reported that the additional fat from skin, prior to deboning, decreases the *L**
337 values of MDM (G. W. Froning & McKee, 2001). Hence, samples composition
338 may represent the main source of differences observed in colour variables. In
339 the same context, myoglobin (Mb), heme pigment, influences meat colour,
340 being affected by redox state of its central iron atom and also by the presence
341 of molecules bounded to the heme group (Faustman, Sun, Mancini, & Suman,
342 2010). Previous works (R. G. Brannan, 2009; Lau & King, 2003) suggested that
343 GPE supplementation turn meat samples darker compared to control (without
344 antioxidant).

345 MDM samples became less red with undergoing of storage time as shown in
346 Fig. 2. During frozen storage, Mb can suffer discoloration due to its conversion
347 to metmyoglobin, exhibiting brownish and less red colour upon storage time
348 end. Metmyoglobin presents a ferric heme group with a water molecule bound
349 incapable of binding oxygen (Suman & Joseph, 2013). Brannan *et al.*, working
350 with raw ground chicken thigh evidenced that GSE (1000 mg/kg) caused darker,
351 redder and less yellow colour in samples. Although in contrast with our results,

352 *a** values for Brannan *et al.*, increased during storage time (R. G. Brannan,
353 2009). Different storage condition may explain these differences.

354 Phenolic compounds in complex matrix as wine grape pomace can exert
355 antioxidant capacity as primary or secondary antioxidants, or also contribute as
356 strong agents capable of chelating transition metal ions.

357 Regarding oxidative stability, FCR assay actually measures a sample's
358 reducing capacity, based on the transfer of electrons in alkaline medium from
359 phenolic compounds and other reducing species to molybdenum, forming blue
360 complexes which are spectrophotometrically monitored at 760 nm (Huang,
361 Boxin, & Prior, 2005; Magalhães, Segundo, Reis, & Lima, 2008). The presence
362 of Folin-Ciocalteu reducing capacity was significantly ($p < 0.01$) higher in
363 average, in MDM2 than MDM1. This assay, being a non-specific method,
364 measures not only the polyphenolic compounds, but also other non-phenolic
365 compounds which contributed to FCR values. Proteins, amino-acids, creatinine,
366 certain nitrogenous bases, were reported as reactive species with the FC
367 reagent (Gülçin, 2012). Hence, higher protein content observed in MDM2
368 sample than MDM1 may explain differences in our results concerning FCR
369 assay. On the other hand, ORAC assay measures the inhibition of peroxyl
370 radical-induced oxidations. It is broadly applied in food and supplement industry
371 to quantify antioxidant capacity in complex matrixes such as, plasma (Huang,
372 Boxin, & Prior, 2005). MDM1 samples showed significantly ($p < 0.001$) higher
373 content of oxygen reactive substances which exerted higher protection against
374 the loss fluorescence of the assay probe than MDM2. Meat recovered from
375 chicken skeleton was reported containing high polyunsaturated fatty acids
376 (PUFA) quantities, namely linoleic acid, linolenic acid and arachidonic acid

(Püssa, Raudsepp, Toomik, Pällin, Mäeorg, Kuusik, et al., 2009). Hence, presence of PUFA in MDM1 samples can explain higher ORAC values than MDM2 sample. Besides, MDM1 was a stronger iron-binder than MDM2, regarding ICA assay.

Regarding the GPE supplementation at 120 mg/kg, overall, 2-factor fully nested ANOVA showed minor but significant differences respect to visual appearance (*b** and chroma). These differences can be ascribed to own colour of polyphenolic compounds present in GPE, as strong natural colorant with health-benefiting properties (Carrizzo, Forte, Damato, Trimarco, Salzano, Bartolo, et al., 2013).

As referred above no research until now reports the putative effect of GPE on MDM under frozen storage. Nevertheless, Brannan *et al*. worked with grape seed extract (GSE) in ground "dark" chicken under frozen storage (Robert G. Brannan & Mah, 2007). They found significant TBARS inhibition by GSE added samples compared to control (without antioxidant), after six months under frozen storage. It is noteworthy that the existence of differences may be explained by the much higher concentration applied, including 1 and 0.1 % (10000 and 1000 mg/kg), far higher than those used in our work. In the same context, because colour represents one of the first acceptance attributes evaluated by consumer, it is crucial to consider the possible impact on sensorial attributes on the final product, regarding certain association between red-pink colour and "under cooked" product.

Concerning the influence of storage time, overall, it was observed changes associated to colour variables, though mainly related to oxidative stability.

401 Reports about changes in colour due to the meat pigment discoloration, lipid
402 oxidation and a potential association between these reactions are not
403 conclusive. In one hand, Rojas and Brewer, worked with ground pork
404 supplemented by GSE (200 mg/kg), reported the decrease of TBARS values by
405 about 12% compared with the control though with loss of red colour (Rojas &
406 Brewer, 2008). On the other hand, Ahn *et al.* worked with cooked ground beef
407 treated with commercial GSE (10000 mg/kg) and observed protection against
408 lipid oxidation and colour changes due to Mb oxidation (Ahn, Grün, & Mustapha,
409 2007).
410 Therefore, we hypothesize that even under frozen storage and with antioxidant
411 supplementation, MDM samples undergo lipid oxidation. MDM is a matrix with
412 increased fat content rich in PUFA as referred above. The bone marrow
413 content, rich in iron from meat pigments, is also squeezed and put in contact
414 with fat (Trindade, Felício, & Castillo, 2004). Hence, PUFA as target of lipid
415 oxidation, generates highly reactive unsaturated aldehyde, such as 4-
416 hydroxynonenal (HNE), as secondary compounds which accelerate latter meat
417 pigment oxidation (Naveena, Faustman, Tatiyaborworntham, Yin, Ramanathan,
418 & Mancini, 2010). Additionally, it was reported that an environment at low
419 oxygen partial pressure (about 6 mmHg O_2) favours met-haem formation
420 (Ledward, 1970) and subsequently reaction with PUFA.
421
422 ## 5. **Conclusions**
423 The effect of pomace extract from Portuguese "*Touriga nacional*" variety on the
424 overall characteristics of MDM was investigated. Our results suggested that
425 proximate composition of MDM has certain influence on oxidative stability

426 throughout storage. Colour variables, including *a** and chroma were the most
427 affected by GPE supplementation. MDM samples became less red, less yellow
428 and darker by the GPE addition. Different levels of GPE supplementation (60
429 and 120 mg/kg) showed, although significant, minor alteration regarding colour
430 variables, mainly in *b** and Hue angle. Storage time significantly influenced the
431 oxidative stability after 210D under frozen storage even in BHT-BHA added
432 samples, regarding ORAC and ICA assays. Besides, this behaviour was MDM
433 batch-dependent. Hence, given factors, namely chemical nature and
434 composition, interaction with other components within MDM matrix may have
435 contributed to the lack of protection of GPE in MDM samples against lipid
436 oxidation. Studies on lipid oxidation using HNE as marker in GPE added
437 samples represent a challenge. Higher levels of GPE would be undergo further
438 experiences, although the use of samples rich in increased concentration of
439 natural extracts could result in adverse sensorial changes in the final meat
440 products. Hence, increased supplementation must also be accompanied by the
441 sensorial evaluation conducted by a trained panel.

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455

456 ***Abbreviations:*** AAPH, 2,2-azobis(2-methylpropionamide) dihydrochloride;
457 BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; $DPPH^{\bullet}$, 2,2-
458 diphenyl-1-picrylhydrazyl radical; ferrozine, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-
459 triazine-4,4-disulfonic acid sodium salt; GAE, gallic acid equivalents; TBARS:
460 thiobarbituric acid-reactive substances; Trolox, (±)-6-hydroxy-2,5,7,8-
461 tetramethylchromane-2-carboxylic acid.

References

Ahn, J., Grün, I. U., & Mustapha, A. (2007). Effects of plant extracts on microbial growth, color change, and lipid oxidation in cooked beef. *Food Microbiology, 24*(1), 7-14.

AOAC. (2002). Official methods of analysis. In 17 ed.). Gaithersburg, MD: Association of Official Analytical Chemists, USA.

Barriuso, B., Astiasarán, I., & Ansorena, D. (2013). A review of analytical methods measuring lipid oxidation status in foods: a challenging task. *European Food Research and Technology, 236*(1), 1-15.

Brannan, R. G. (2009). Effect of grape seed extract on descriptive sensory analysis of ground chicken during refrigerated storage. *Meat Science, 81*(4), 589-595.

Brannan, R. G., & Mah, E. (2007). Grape seed extract inhibits lipid oxidation in muscle from different species during refrigerated and frozen storage and oxidation catalyzed by peroxynitrite and iron/ascorbate in a pyrogallol red model system. *Meat Science, 77*(4), 540-546.

Carrizzo, A., Forte, M., Damato, A., Trimarco, V., Salzano, F., Bartolo, M., Maciag, A., Puca, A. A., & Vecchione, C. (2013). Antioxidant effects of resveratrol in cardiovascular, cerebral and metabolic diseases. *Food and Chemical Toxicology, 61*(0), 215-226.

Clark, E. M., Mahoney, A. W., & Carpenter, C. E. (1997). Heme and total iron in ready-to-eat chicken. *Journal of Agriculture and Food Chemistry, 45*(1), 124-126.

FAOSTAT. (2012). FAO statistical database. In).

Faustman, C., Sun, Q., Mancini, R., & Suman, S. P. (2010). Myoglobin and lipid oxidation interactions: Mechanistic bases and control. *Meat Science, 86*(1), 86-94.

Froning, G. (1981). Mechanical deboning of poultry and fish. *Advances in Food Reseach, 27*, 109-147.

Froning, G. W., & McKee, S. R. (2001). Mechanical separation of poultry meat and its use in products. *Poultry Meat Processing*, 243.

Guendez, R., Kallithraka, S., Makris, D. P., & Kefalas, P. (2005). Determination of low molecular weight polyphenolic constituents in grape (Vitis vinifera sp.) seed extracts: Correlation with antiradical activity. *Food Chemistry, 89*(1), 1-9.

Gülçin, I. (2012). Antioxidant activity of food constituents: An overview. *Archives of Toxicology, 86*(3), 345-391.

Huang, D., Boxin, O. U., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agriculture and Food Chemistry, 53*(6), 1841-1856.

Hui, Y. H. (2012). *Handbook of meat and meat processing*: CRC press.

Jordao, A. M., Simoes, S., Correia, A. C., & Goncalves, F. J. (2012). Antioxidant activity evolution during Portuguese red wine vinification and their relation with the proanthocyanidin and anthocyanidin composition. *Journal of Food Processing and Preservation, 36*(4), 298-309.

Lau, D. W., & King, A. J. (2003). Pre- and Post-Mortem Use of Grape Seed Extract in Dark Poultry Meat To Inhibit Development of Thiobarbituric Acid Reactive Substances. *Journal of Agriculture and Food Chemistry, 51*(6), 1602-1607.

Ledward, D. A. (1970). Metmyoglobin formation in beef stored in carbon dioxide enriched and oxygen depleted atmospheres. *Journal of Food Science, 35*(1), 33-37.

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica Acta, 613*(1), 1-19.

Mielnik, M. B., Aaby, K., Rolfsen, K., Ellekjær, M. R., & Nilsson, A. (2002). Quality of comminuted sausages formulated from mechanically deboned poultry meat. *Meat Science, 61*(1), 73-84.

Navarro-Rodríguez de Vera, C., Sánchez-Zapata, E. J., Viuda-Martos, M., & Pérez-Alvarez, J. A. (2010). Handbook of Poultry Science and Technology. In, vol. 2 (pp. 73-80).

513 Naveena, B. M., Faustman, C., Tatiyaborworntham, N., Yin, S., Ramanathan, R., & Mancini, R.
514 A. (2010). Detection of 4-hydroxy-2-nonenal adducts of turkey and chicken myoglobins
515 using mass spectrometry. *Food Chemistry, 122*(3), 836-840.
516 Negro, C., Tommasi, L., & Miceli, A. (2003). Phenolic compounds and antioxidant activity from
517 red grape marc extracts. *Bioresource Technology, 87*(1), 41-44.
518 Ozer, O., & Sariçoban, C. (2010). The effects of butylated hydroxyanisole, ascorbic acid, and α-
519 tocopherol on some quality characteristics of mechanically deboned chicken patty
520 during freeze storage. *Czech Journal of Food Sciences, 28*(2), 150-160.
521 Püssa, T., Raudsepp, P., Toomik, P., Pällin, R., Mäeorg, U., Kuusik, S., Soidla, R., & Rei, M.
522 (2009). A study of oxidation products of free polyunsaturated fatty acids in
523 mechanically deboned meat. *Journal of Food Composition and Analysis, 22*(4), 307-
524 314.
525 Qwele, K., Hugo, A., Oyedemi, S. O., Moyo, B., Masika, P. J., & Muchenje, V. (2013). Chemical
526 composition, fatty acid content and antioxidant potential of meat from goats
527 supplemented with Moringa (Moringa oleifera) leaves, sunflower cake and grass hay.
528 *Meat Science, 93*(3), 455-462.
529 Rababah, T. M., Hettiarachchy, N. S., & Horax, R. (2004). Total Phenolics and Antioxidant
530 Activities of Fenugreek, Green Tea, Black Tea, Grape Seed, Ginger, Rosemary, Gotu
531 Kola, and Ginkgo Extracts, Vitamin E, and *tert*-Butylhydroquinone. *Journal of*
532 *Agriculture and Food Chemistry, 52*(16), 5183-5186.
533 Rojas, M. C., & Brewer, M. S. (2008). Effect of natural antioxidants on oxidative stability of
534 frozen, vacuum-packaged beef and pork. *Journal of Food Quality, 31*(2), 173-188.
535 Serra, A. T., Matias, A. A., Nunes, A. V. M., Leitão, M. C., Brito, D., Bronze, R., Silva, S., Pires, A.,
536 Crespo, M. T., San Romão, M. V., & Duarte, C. M. (2008). In vitro evaluation of olive-
537 and grape-based natural extracts as potential preservatives for food. *Innovative Food*
538 *Science and Emerging Technologies, 9*(3), 311-319.
539 Shah, M. A., Bosco, S. J. D., & Mir, S. A. (2014). Plant extracts as natural antioxidants in meat
540 and meat products. *Meat Science, 98*(1), 21-33.
541 Shirahigue, L. D., Plata-Oviedo, M., de Alencar, S. M., d'Arce, M. A. B. R., de Souza Vieira, T. M.
542 F., Oldoni, T. L. C., & Contreras-Castillo, C. J. (2010). Wine industry residue as
543 antioxidant in cooked chicken meat. *International Journal of Food Science &*
544 *Technology, 45*(5), 863-870.
545 Suman, S. P., & Joseph, P. (2013). Myoglobin Chemistry and Meat Color. *Annual Review of*
546 *Food Science and Technology, 4*(1), 79-99.
547 Trindade, M. A., Felício, P. E. d., & Castillo, C. J. C. (2004). Mechanically separated meat of
548 broiler breeder and white layer spent hens. *Scientia Agricola, 61*, 234-239.

Supplementary information

Effect of *"Touriga nacional"* grape extract on quality characteristics of mechanically deboned chicken meat kept under frozen storage

Hernán H. Tournour[1,2], Marcela A. Segundo[3], Luís M. Magalhães[3], Anabela S. G. Costa[4], Luís M. Cunha[2,*]

[1]LAQV,REQUIMTE/DGAOT, Faculty of Sciences, University of Porto, Porto, Portugal,

[2]Faculty of Nutrition and Food Sciences, University of Porto, Porto,Portugal,

[3]UCIBIO,REQUIMTE, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal,

[4]LAQV,REQUIMTE, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal.

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*Corresponding author: lmcunha@fc.up.pt. Tel.: (+351)252660400

1. Material and methods

1.1. Materials

Folin-Ciocalteu reagent (Sigma-Aldrich, MO) at 3:10 (v/v) in ultrapure water was used for the determination of total phenolic content (TPC). Gallic acid (1.0 - 15.0 mg/l) and Trolox (5.0 – 50.0 µM) in ethanolic solution 50% (v/v)) (Fluka, Switzerland) were used as standards for TPC and antioxidant capacity assays, respectively. Stock solution of DPPH$^{\bullet}$ (Sigma-Aldrich, WI) in absolute ethanol p.a. (600 µM) was diluted in ethanolic solution 50% (v/v) to prepare work DPPH$^{\bullet}$ solutions (27 – 225 µM). Stock solution of fluorescein sodium salt (Sigma-Aldrich, MO) at 0.5 mM and AAPH (Sigma-Aldrich, WI) at 40 mM in a 75 mM phosphate buffer (pH 7.4) were used for oxygen radical absorbance capacity (ORAC) assay. Iron(II) chloride tetrahydrate (Fluka, Switzerland) solutions, including the stock solution (6 mM, in ultrapure water (pH 3.0)) and iron(II) solution (0.12 mM) added to wells, were daily prepared for the determination of iron(II) chelating ability. Ferrozine solution at 0.6mM and a solution of acetate buffer (50 mM, pH 4.6) in ultrapure water were also prepared. All chemicals above were of analytical reagent grade. Ultrapure water (resistivity > 18 Ω) and absolute ethanol p. a. were obtained from Arium Sartorius Goettingen, Germany and Panreac Química, Spain, respectively.

1.2. Total phenolic content and antioxidant capacity of GPE

Total phenolic content (TPC)

TPC was assessed employing a 96-well microplate Folin-Ciocalteu procedure (Luís M Magalhães, Santos, Segundo, Reis, & Lima, 2010; Singleton, Orthofer, & Lamuela-Raventos, 1999). The photometric measurement was carried out at

760 nm for 120 min. Solutions containing 1.0-15.0 mg/l gallic acid were used for calibration purpose. Only sample dilutions with extinctions within the calibration range were used. Results (n = 16) were expressed as mg of gallic acid equivalents per gram of dry residue (obtained from the solid material once concentration step was finished).

DPPH$^{\bullet}$ assay

For microplate DPPH$^{\bullet}$ methodology (Brand-Williams, Cuvelier, & Berset, 1995; L. M. Magalhães, Barreiros, Maia, Reis, & Segundo, 2012), 150 µl of Trolox standard solution (5.0 - 50.0 µM) or diluted red GPE (1:400 and 1:800 v/v) and 150 µl of DPPH$^{\bullet}$ ethanolic solution (50% (v/v)) were placed in each well. The DPPH$^{\bullet}$ scavenging capacity was monitored at 517 nm during 120 min. The absorbance of DPPH$^{\bullet}$ in the absence of antioxidant species (control) was monitored after the addition of 150 µl of ethanolic solution (50% (v/v)) instead of standard solution, in order to evaluate the stability of the radical upon reaction time. To evaluate the intrinsic absorption of samples, 150 µl of ethanolic solution (50%, (v/v)) was added to 150 µl of sample. The net absorbance, calculated by the difference of DPPH$^{\bullet}$ absorbance in the absence and in the presence of sample after 120 min, was calculated. Results were expressed as mmol of Trolox equivalent (TE) per gram of dry residue by interpolation in Trolox standard curve ($\Delta Abs_{517\ nm}$ = 7.40 × [Trolox, (mM)] + 0.028, R>0.9957, n = 16).

ORAC assay

For ORAC assay (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002; Wang, Jónsdóttir, & Ólafsdóttir, 2009), 100 µL of Trolox standard solution (1.0 – 7.5 µM) or diluted red GPE extracts (1:600, 1:800, and 1:1000 v/v) and 100 µL

of fluorescein (117 nM) were placed in each well, and the microplate was brought to preincubation for 15 min at 37 ºC. Following this, 100 µL of AAPH solution (40 mM) was added and the fluorescence intensity (λ_{exc} 485 nm, λ_{em} 520 nm) was monitored every minute during 240 min. The reaction milieu was 75 mM phosphate buffer (pH 7.4) at 37 ºC. Control signal profile (absence of sample) was assessed by adding 100 µL of buffer solution instead of sample. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve over the reaction time. The net AUC of the sample was calculated by subtracting this value to the AUC of the control (absence of sample). The regression equation between net AUC and Trolox concentration was determined, and the results were expressed as µmol of Trolox equivalents (TE) per gram of dry residue by interpolation (Net AUC (%) = 10.6 × [Trolox, (µM)] + 10.5, R>0.9998, n = 18).

Iron(II) chelatin ability assay ICA

For iron(II) chelating ability assay (ICA) (Wang et al., 2009), 100 µL of diluted red GPE (1:10, and 1:25 v/v) in acetate buffer (50 mM, pH 4.6) were mixed with 100 µL $FeCl_2 \cdot 4H_2O$ (120 µM) and placed in each well. After 5 min, 100 µL of ferrozine solution (600 µM) was added to each well. Solutions were left standing 10 min at room temperature, after which the absorbance was measured at 562 nm. Control assay was performed by adding 100 µL of water instead of sample, while the blank of the sample was performed by adding 100 µL of water instead of ferrozine solution. The percentage of inhibition of ferrozine-iron(II) complex formation of each sample was calculated using the equation: ICA (%) = $[A_0 - (A_1 - A_2)] / A_0 \times 100$, where A_0, A_1 and A_2 correspond to absorbance of the control, sample and blank of the sample, respectively. In A_0 the intrinsic

absorbance of iron(II) was subtracted from the initial absorbance. As the reaction proceeds the resulting red colour from the ferrozine-iron(II) complex decreases in the presence of chelating substances. Hence, ICA (%) values represent the reduction in absorbance values relative to the control due to the chelating effect of sample components. Results were expressed as % inhibition obtained per mg of dry residue, n = 8. Previous assays were begun mixing equal parts of absolute ethanol p.a. and GPE, in order to ensure that the phenolic compounds dissolve properly.

Table S1

Mean values (± S.D.) for TPC, antioxidant capacity determined by $DPPH^{\cdot}$ and ORAC assays, and ICA assay for *"Touriga nacional"* aqueous suspensions for different vintages.

Vintage	TPC* (mg GAE/g residue)	$DPPH^{\cdot}$ (mmol TE/g residue)	ORAC (µmol TE/g residue)	ICA (%Inhib./mg residue)
2012	142 (±1)	1.12 (±0.04)	1579 (±244)	66 (±9)
2013	135 (±4)	1.10 (±0.10)	1499 (±211)	62 (±11)

*Significant differences between vintages according to the Student´s t-test at 95% confidence level. TPC: total phenolic content; $DPPH^{\cdot}$: 2,2-diphenyl-1-picrylhydrazyl radical assay; ORAC: oxygen radical absorbance capacity; ICA: iron(II) chelating ability; GAE: gallic acid equivalents; TE: trolox equivalents.

Table S2

Factor loadings for MDM samples significant variables measured during frozen storage on the first two principal components obtained after *Varimax* rotation.

Variables	PC1	PC2
*L**	0.592	0.615
*a**	0.934	0.148
*b**	0.553	0.815
Chroma	0.853	0.448
Hue angle	-0.086	0.974
Folin-Ciocalteu reducing (FCR)	-0.710	-0.600
Oxygen reactive absorbance capacity (ORAC)	-0.786	-0.014
Fat% db	0.671	0.604
Variance explained	47.94%	36.73%
Total variance explained	84.67%	

105 ## References

106

107 Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to
108 evaluate antioxidant activity. *LWT - Food Science and Technology, 28*(1), 25-30.
109 Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-Throughput
110 Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid
111 Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format.
112 *Journal of Agriculture and Food Chemistry, 50*(16), 4437-4444.
113 Magalhães, L. M., Barreiros, L., Maia, M. A., Reis, S., & Segundo, M. A. (2012). Rapid
114 assessment of endpoint antioxidant capacity of red wines through microchemical
115 methods using a kinetic matching approach. *Talanta, 97*, 473-483.
116 Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S., & Lima, J. L. (2010). Rapid microplate
117 high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity.
118 *Talanta, 83*(2), 441-447.
119 Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and
120 other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent.
121 *Methods In Enzymology, 299*, 152-178.
122 Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging
123 and metal chelation of extracts from Icelandic seaweeds. *Food Chemistry, 116*(1), 240-
124 248.

Paper V

Hernán H. Tournour, Luís M. Cunha, Luís M. Magalhães, Rui Costa Lima, Marcela A. Segundo. Evaluation of the joint effect of the incorporation of mechanically deboned meat and grape extract on the formulation of chicken nuggets. [Submitted for publication].

Evaluation of the joint effect of the incorporation of mechanically deboned meat and grape extract on the formulation of chicken nuggets

Hernán H. Tournour[1,2], Luís M. Cunha[1,*], Luís M. Magalhães[3], Rui Costa Lima[4], Marcela A. Segundo[3]

[1] *LAQV,REQUIMTE/DGAOT, Faculty of Sciences, University of Porto, Porto, Portugal.*

[2] *Faculty of Nutrition and Food Sciences, University of Porto, Porto, Portugal.*

[3] *UCIBIO, REQUIMTE, Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal.*

[4] *Sense Test, Lda., Vila Nova de Gaia, Portugal*

**Corresponding author: lmcunha@fc.up.pt*
Campus Agrário de Vairão
Rua Padre Armando Quintas, 7
4486-661 Vairão
Portugal
Tel.: (+351)252660400 / Fax: (+351)252661780

ABSTRACT

The proximate composition, instrumental and perceived appearance of chicken nuggets formulated with varying content mechanically deboned chicken meat (MDM) and varying concentrations of grape pomace extract (GPE) were evaluated, with the choice of formulations following a central composite design. Significant differences ($p < 0.05$) in fat content were mainly associated to the extent of MDM incorporation. Colour variables (*CIE a** and *b**, and Whiteness index) varied significantly ($p < 0.05$), with redness (a*) being the variable most influenced by the incorporation of MDM. Whiteness index decreased with added MDM and GPE. Response surface was applied to identify formulations with higher acceptance scores. Correspondence analysis of open-ended comments complemented information obtained from overall acceptance, adding valuable descriptive attributes of nuggets samples. Thus, addition of GPE up to 120 mg/kg and MDM up to 15 % did not adversely affect the perceived appearance of chicken nuggets. MDM and GPE can be successfully used for the elaboration

of novel products, for different market segments, with healthy connotations highlighted by antioxidant properties retained by the grape pomace extract.

Keywords: appearance; colour; consumer acceptance; open-ended comments; sensory evaluation.

Highlights

- Chicken nuggets were prepared with varying amounts of MDM and GPE,
- Internal colour changed with increase on MDM content and on GPE concentration,
- Highly accepted formulations were identified through response surface methodology,
- CA gave valuable information to a better understanding of consumer demands.

1. Introduction

There is a general scientific agreement that diet and nutrition are important factors in the promotion and maintenance of good health through the entire life (WHO, 2003) In recent years, an increasing concern by consumers regarding to the benefits and burdens from food consumed daily regarding its implications in diet and health has been reported (Falguera, Aliguer, & Falguera, 2012). Moreover, consumers are more informed and interested about benefits associated to food intake (Moura & Cunha, 2005).

On the other hand, innovation processes and new product development gave origin to a novel class of products: functional foods (Arihara, 2006), then a wide offer was originated, making consumers closely linked with their food preferences (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2010). In this context, for meat and meat products, global appearance and organoleptic properties significantly contribute to the perception of quality, with a particular emphasis on meat colour (Faustman, Sun, Mancini, & Suman, 2010).

Regarding global perspectives on meat consumption (mainly beef) a decreasing trend is predicted by 2050, based primarily on ecological issues, global food crisis and outbreaks, and also due to nutritional aspects. However, such data contrasts with previsions for chicken consumption, displaying an optimistic and growing trend for this sector (Henchion, McCarthy, Resconi, & Troy, 2014; Kearney, 2010).

Innovation in the meat sector is based on the production of healthier products with mainly three different approaches: improvements in animal production (changes in meat constituents such as protein, lipid content, fatty acid composition, and vitamin E level) through feed control; handling of raw meat components (procedures to separate and/or extract visible fat) and reformulation of meat derivatives (development of meat product with custom-designed composition and properties, including products with reduced content of specific ingredients - e.g., salt, fat or cholesterol-; lower energy density, and/or with the addition of functional ingredients, such as food fibre, antioxidants, probiotics and prebiotics agents) (Arihara, 2006; Jiménez-

Colmenero, Carballo, & Cofrades, 2001). Therefore, different natural ingredients, such as broccoli, ganghwayakssuk, banana, soybean hulls and mustard (Banerjee, et al., 2012; Hwang, et al., 2011; D. Kumar & Tanwar, 2011; V. Kumar, Biswas, Chatli, & Sahoo, 2011), and also grape pomace extract (Sayago-Ayerdi, Brenes, Viveros, & Goni, 2009) have been used in the elaboration of chicken products. Nevertheless, more information is required to evaluate the sensorial impact of the addition of grape pomace extracts in chicken products as consumers may find products unacceptable due to changes in colour (Karre, Lopez, & Getty, 2013). Moreover, it is worthy to point out the positive contribution of bioactive compounds present in grape extracts, through their widely recognized antioxidant and preservative properties (Lau & King, 2003; Mielnik, Olsen, Vogt, Adeline, & Skrede, 2006; Nandakumar, Singh, & Katiyar, 2008). Moreover, from the literature search on ISI Web of Science and on Elsevier's Scopus databases, no research on the combined effect of varying both MDM content and GPE concentration was found.

Regarding sensory characterization, different fast profiling techniques have been recently developed and evaluated. Novel techniques, such as sensory profiling based on open-ended questions, have been applied for an accurate product description by consumers, obtaining valuable information from the analysis of their comments (Moussaoui & Varela, 2010; Varela & Ares, 2014). Moreover, recent quantitative approaches, including the use of chi-square statistics per cell, allowed a more reliable analysis of the contingency tables built for this purpose (Symoneaux, Galmarini, & Mehinagic, 2012). Nevertheless, it is noteworthy that between 50 and 100 assessors are required to perform a sensory characterization using the methodology described above (Varela & Ares, 2014).

Therefore, the aim of this paper was to evaluate the effect of the addition of GPE and MDM on the nutritional and sensory characteristics of chicken nuggets as an enriched meat product, towards a better understanding of consumer demands regarding sensory and nutritional properties. The analysis of instrumental appearance and also the assessment of perceived appearance through sensory evaluation based on the open-ended-question analysis combined with Contingence Analysis (CA) were performed.

2. **Material and methods**

2.1. *Chemicals*

Folin-Ciocalteu (F-C) reagent was obtained from Sigma (St. Louis, MO), whilst gallic acid, was obtained from Fluka (Buchs, Switzerland). All chemicals used were of analytical reagent grade. Water from Arium Sartorius (Goettingen, Germany) (resistivity > 18 MΩ cm) and absolute ethanol p. a. (Panreac Química, Spain) were used in the preparation of all solutions.

2.2. *Solutions*

For assessment of total phenolic content (TPC), the commercial F-C reagent was diluted 3:10 in water. A solution of $Na_2CO_3 \cdot 10\ H_2O$ 24.3% was prepared, corresponding to 9% of sodium carbonate, and also gallic acid standard solutions (1.0 - 15.0 mg/l) for calibration purposes.

2.3. *Equipment*

All antioxidant assays were performed in a microplate format (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA) using spectrophotometry as detection system. The microplate reader was controlled by Gen5 software (Bio-Tek Instruments) and operated at room temperature. GPE was analyzed in quadruplicate using two dilution factors (n = 8). For colour determination, a spectro-colorimeter CR-400 (Konica Minolta Sensing, Osaka, Japan) with standard C illuminant and 2 º observer in CIE-*L*a*b** space colour was used. For pH determination a pH meter (Hanna Instruments, Michigan, USA) was used.

2.4. *Grape pomace extract preparation*

Skins and seeds (grape pomace) from a red Portuguese cultivar (*Vitis vinifera* L., variety "*Touriga nacional*") were oven dried, grinded, and submitted to extraction (Shirahigue, et al., 2010) under orbital agitation. After extractive step, the sample was filtered and the filtrate was concentrated in a Rotavapor (Büchi, Flawil, Switzerland) till dryness. Finally, the remaining dried residue was re-suspended in water forming the grape pomace extract (GPE) that was kept under refrigeration till further characterization.

2.4.1. *Total phenolic content (TPC)*

TPC was assessed employing a 96-well microplate Folin-Ciocalteu procedure, with carbonate buffer as alkaline reagent (Magalhães, Santos, Segundo, Reis, & Lima, 2010; Singleton, Orthofer, & Lamuela-Raventos, 1999). Hence, 150 µl of gallic acid standard solution (1.0 - 15.0 mg/l) or diluted red grape pomace extracts (1:200 and 1:400) and 50 µl of F-C reagent (3:10) were placed in each well. After, 100 µl of carbonate solution (9% m/v) was added. The reduction at alkaline pH of phosphotungstate-phosphomolybdate complexes was monitored at 760 nm during 120 min. The reagent blank was performed by the addition of 150 µl of water in replacement of the sample. TPC, expressed as mg of gallic acid equivalents per liter of extract was calculated by interpolation of absorbance values after 120 min of reaction in the gallic acid standard curve ($Abs_{760\ nm}$ = 0.0491 × [gallic acid, (mg/l)] + 0.065, R > 0.9996).

2.5. *Nugget elaboration*

Chicken nuggets were chosen as the food matrix because it is one of the most predominant breaded products globally consumed and represent a tasty and convenient food (Guerrero-Legarreta, 2010b). During the product development, different formulations, formats and cooking procedures were evaluated in order to define the most appropriate conditions to be used during the elaboration of such comminuted product, nugget. As a result, ten different formulations were used, following a Central Composite Design (CCD –(Box, Hunter, & Hunter, 2005)), combining different levels of GPE (60 - 180 mg/kg) and of mechanically deboned chicken meat (MDM, 0-30 %), with 66.8 % moisture, 57.4 % fat - dry base and 40.9 % protein - dry base (Tournour, 2014). Besides, breast chicken meat (35 - 65 %), deionized water (ice flakes), wheat flour, boiled rice, egg yolk, corn starch, salt and spices were used during elaboration, according to the general scheme in Fig. 1, where the overall amount of chicken meat (minced breast and MDM) was fixed on 65 %. Firstly, chicken meats were mixed in a food processor (KenWood, New Lane, UK), followed by addition of the remaining ingredients. After this, the resulting mixture was disposed on a constant thickness layer and stored at - 24 ºC for 1 hour. The frozen mixture was cut in individual pieces, battered and breaded. The final product (app. 3.0 ×

182 1.5 × 1.2 cm, 18 ± 2 g), was kept under frozen storage (- 24 °C) until the
183 cooking step. Frozen nuggets were pre-fried in sunflower oil at 190 °C during 30
184 s in a domestic fryer (Model HD6163, Philips, Eindhoven, The Netherlands) and
185 stored under - 24 °C. At the time of analysis, pre-fried frozen nuggets were oven
186 cooked in a forced convection oven (Rational Combi-Master CM61, Rational
187 AG, Germany) at 200 °C during 6 min (Albert, Varela, Salvador, Hough, &
188 Fiszman, 2011), after a 20 min preheating of the oven at 200 °C. Approximately
189 200 nuggets per formulation (n = 10 formulations) were produced for sensory
190 and physical-chemical (colour CIE-*L*a*b** and pH) determinations.

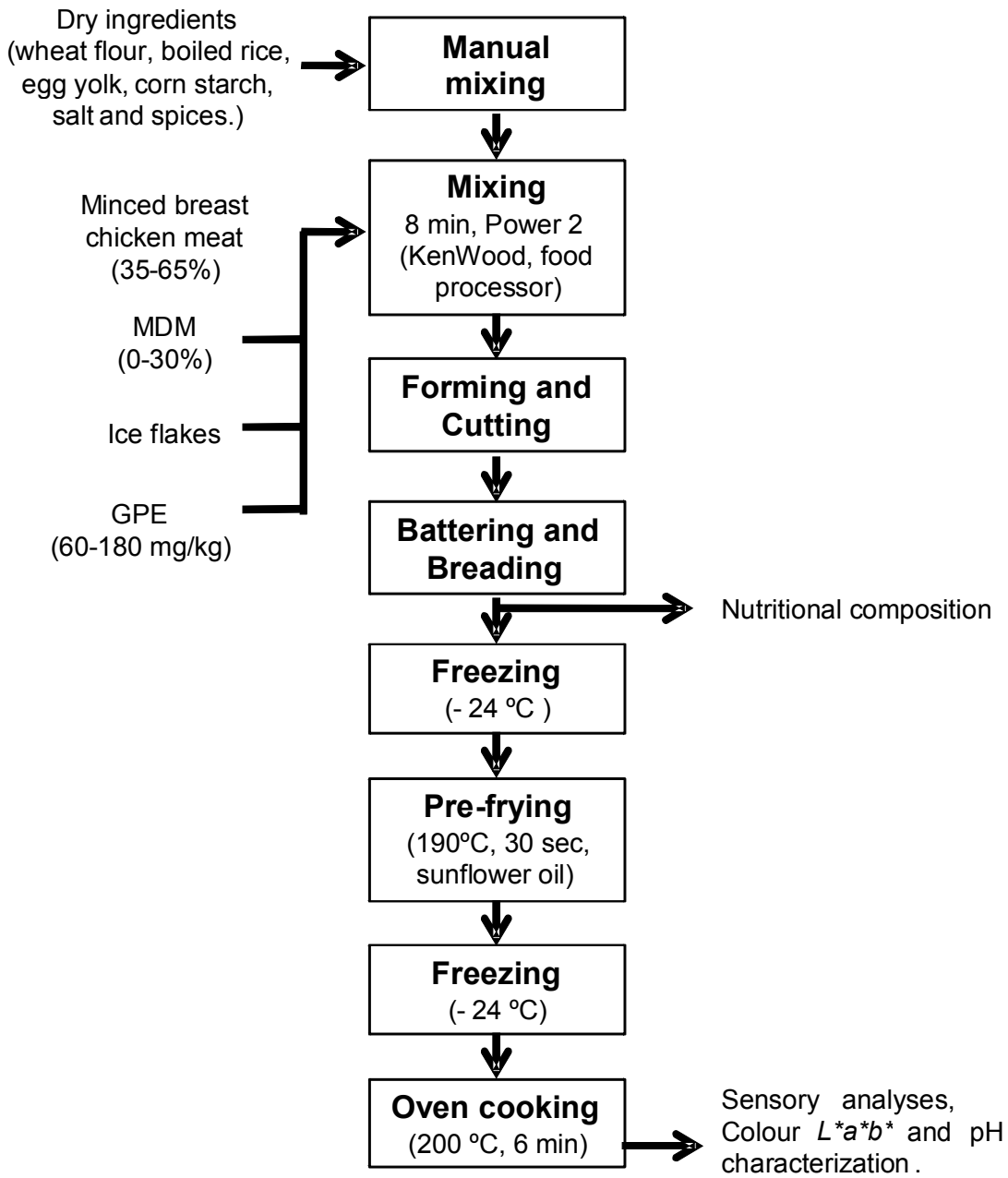


Fig. 1. General block diagram used for the nugget elaboration, using grape pomace extract (GPE) and mechanically deboned chicken meat (MDM) at different levels.

2.6. *Chemical analysis*

2.6.1. *Nutritional composition*

Fat, protein, ash, and moisture content (% w/w) were determined for samples with different levels of MDM incorporation, according to standardized

198 procedures recommended by the Association of Official Analytical Chemists
199 (AOAC, 2002).

200 2.6.2. *Determination of colour variables and pH*

201 Colour variables, namely CIE L^* (luminosity), a^* (redness) and b^* (yellowness)
202 and Whiteness index (WI), with: $WI = 100 - ((100-L^*)^2 + a^{*2} + b^{*2})^{1/2}$ were
203 determined (Hunt & Pointer, 2011). A total of 6 measurements (n = 6) were
204 collected on cooked products (longitudinally cut, on both sides) after reaching
205 room temperature (app. 20 °C). Samples were compared in terms of a^*, b^* and
206 WI. For pH determination, 10 g of each sample were homogenised in 100 mL of
207 ultrapure water during 1 min before pH measurement (Ozer & Sariçoban, 2010).

208 2.7. *Sensory evaluation through overall acceptance assessment*

209 Sensory evaluation was carried out in a room equipped with individual booths in
210 accordance with ISO standard 8589:2007 *Sensory analysis - General guidance*
211 *for the design of test rooms*, with personnel and panel leader following ISO
212 standards 13300-1:2006 *Sensory analysis - General guidance for the staff of a*
213 *sensory evaluation laboratory - Part 1: Staff responsibilities* and 13300-2:2006
214 *Sensory analysis - General guidance for the staff of a sensory evaluation*
215 *laboratory - Part 2: Recruitment and training of panel leaders*, hired from an
216 independent Sensory Analysis Laboratory (Sense Test, Lda.) also responsible
217 for panel recruiting.

218 The recruited panel had 75 naïve assessors (67 % female), between 18 and 49
219 years old, and were familiarized with the consumption of chicken products.

220 Nuggets were presented to each assessor on white porcelain dishes identified
221 by a three-digit random number, in the individual booths, under normal white
222 lighting. All nuggets were presented at consumption temperature. Assessors
223 were asked about the overall acceptance, on a 9-point scale (Peryam & Pilgrim,
224 1957), going from 1 - “dislike extremely” , to 9 – “like extremely”. Assessors
225 were also asked to write down a general comment for each sample. Within a
226 session each assessor received monadically the full set of samples, following a
227 unique order, according to a Latin Square Design, in order to balance serving

order and to compensate possible carry-over effects (MacFie, Bratchell, Greenhoff, & Vallis, 1989).

3. **Data analysis**

Variation of physical-chemical data between samples was evaluated through 2-way ANOVA, followed by the Tukey multiple comparison test and quadratic response surfaces were fitted to aggregated data, with STATISTICA for Windows v. 12.0 (STATISTICA 12 Software, StatSoft, Tulsa, OK). Regarding overall acceptance, differences where evaluated following the Friedman non-parametric test for dependant samples (data evaluated across each assessor), followed by the Wilcoxon test. Additionally, a quadratic response surface was also fitted to aggregated data. Qualitative information obtained from sensory evaluation was analysed through open-ended comments, according to the general procedure described by Symoneaux *et al*. (Symoneaux, et al., 2012). Briefly, this technique consists in a comment analysis, including pre-processing of the data collected, construction of the contingency tables and final statistical analysis. Regarding data pre-processing, it consists in a re-codification of all semantic expressions used by assessors into final descriptive attributes, after excluding hedonic non-descriptive terms. Moreover, terms with lower citations were also excluded to reduce noise and to avoid losing a large amount of information (Guerrero, et al., 2010; Vidal, Ares, & Giménez, 2013). Frequencies of mention of terms were calculated without considering if those were provided by the same participant or by different participants (Ares, et al., 2015; Guerrero, et al., 2010; Schmitt, 1998). Before reporting, all resulting descriptive terms were translated by the researchers from Portuguese to English. A back-translation process (Brislin, 1970) was applied for the terms that were difficult to translate. This procedure was used to provide homogeneity in the coding process. Following, frequency of mention of those terms was used to construct contingency tables of samples by attributes and chi-square per cell analysis was performed. All attributes with non-significant cells for the full set of samples were excluded. Resulting information was analyzed following statistical correspondence analysis (CA) using Xlstat software 2014 (Addinsoft, Paris, France). Except when referred, all tests were applied with a 95 % confidence level.

4. **Results and Discussion**

4.1. *GPE characterization and proximate composition of nuggets*

TPC assay indicated the presence of 2444 ± 268 mg GAE/l in the extract of GPE from *"Touriga nacional"* variety in agreement with previous report (Negro, Tommasi, & Miceli, 2003).

Nuggets were elaborated according to the general block diagram in Fig. 1. Nutritional composition, in terms of fat, protein, ash and moisture content, for the different formulations with varying initial composition are shown in Table 1. The effect of the addition of GPE on those results was considered as negligible.

271

Table 1
Proximate composition of chicken nuggets with different content of mechanically deboned chicken meat (MDM) and grape pomace extract (GPE).

MDM (%)	GPE (mg/kg)	Fat* (%)	Protein* (%)	Ash* (%)	Moisture* (%)
0	120	1.5^{d} (± 0.0)	$13.6^{a,b}$ (± 0.4)	$2.23^{a,b}$ (± 0.01)	61.7^{a} (± 0.4)
4	78 162	2.1^{c} (± 0.1)	13.7^{a} (± 0.4)	2.19^{b} (± 0.01)	61.1^{a} (± 0.6)
15	60 120 120 180	3.5^{b} (± 0.1)	$13.2^{a,b,c}$ (± 0.0)	$2.25^{a,b}$ (± 0.03)	58.3^{b} (± 0.2)
26	78 162	3.6^{b} (± 0.1)	$11.9^{b,c}$ (± 0.3)	2.30^{a} (± 0.02)	57.9^{b} (± 1.2)
30	120	5.5^{a} (± 0.0)	11.7^{c} (± 0.8)	$2.25^{a,b}$ (± 0.02)	$59.5^{a,b}$ (± 0.5)

*Values represent mean (± standard deviation) of duplicates.
^{a,b,c} – homogeneous groups according to the Tukey multiple comparison test, at a 95 % confidence level.

MDM is a meat product characterized by high fat content due to the presence of skin and abdominal fat. Additionally, pressure exerted during the extractive procedures where bone marrow content escapes, contributes to increased lipid content (Froning, 1981). Formulation with high MDM content (30 %) resulted in significantly ($p < 0.001$) higher fat content (5.5 ± 0.0 %), with an opposite effect on the protein content, representing the lowest values (11.7 ± 0.8 %) compared to all formulations ($p < 0.05$). Previous works by Perlo *et al.* with chicken nuggets also reported fat increase when washed MDM was incorporated into nugget formulation (Perlo, Bonato, Teira, Fabre, & Kueider, 2006). Nevertheless, the use of MDM is advantageous, as even at the highest MDM content in the formulation, the final product resulted in a low-fat food, with lower lipid content than standard nuggets (12.3 g lipid/100 g food) (Gibbs, Rymer, & Givens, 2013).

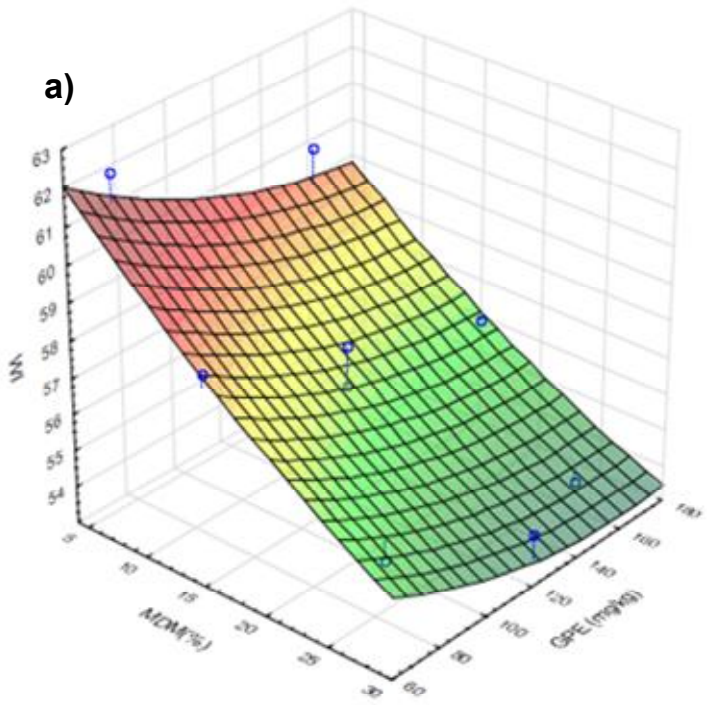
Formulations containing 26 % and 4 % of MDM exhibited the highest and lowest (2.30 ± 0.02 % and 2.19 ± 0.01 % ash values, respectively ($p < 0.05$). While formulations with the highest content of breast meat (65 and 61 %) presented the highest moisture content (61.7 ± 0.4 % and 61.1 ± 0.6 %, respectively). Chicken meat containing over 50 % of moisture in its composition was previously reported (Hui, 2012).

4.2. *Colour (*CIE-*L*a*b*)*

Internal colour (variables *a* b** and Whiteness index, WI) for all cooked samples is shown in Fig. 2.

Colour and overall appearance of a given product highly influences consumer´s choice. Consumers usually relate whiteness of a chicken product with chicken meat (Guerrero-Legarreta, 2010a), particularly with breast meat colour, which is generally associated with “healthy white meat”. MDM, due to its high meat pigments concentration - mainly myoglobin, which can exist in both metmyoglobin or oxymyoglobin forms, depending on the oxygen access and on haem iron oxidation state - may affect the inner colour in cooked meat products. Moreover, GPE solutions presented a deep red/purple colour due to the presence of polyphenolic compounds, including anthocyanins (Bravo, 1998).

303 Regarding WI values, there was a significant, almost linear, decrease with the
304 increase of MDM content and with the addition of more GPE extract (see Fig.
305 2.a).



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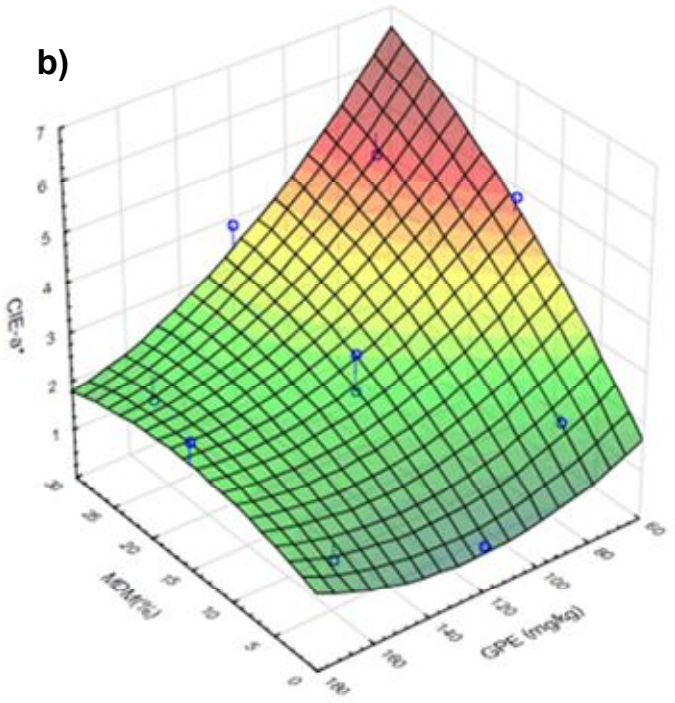

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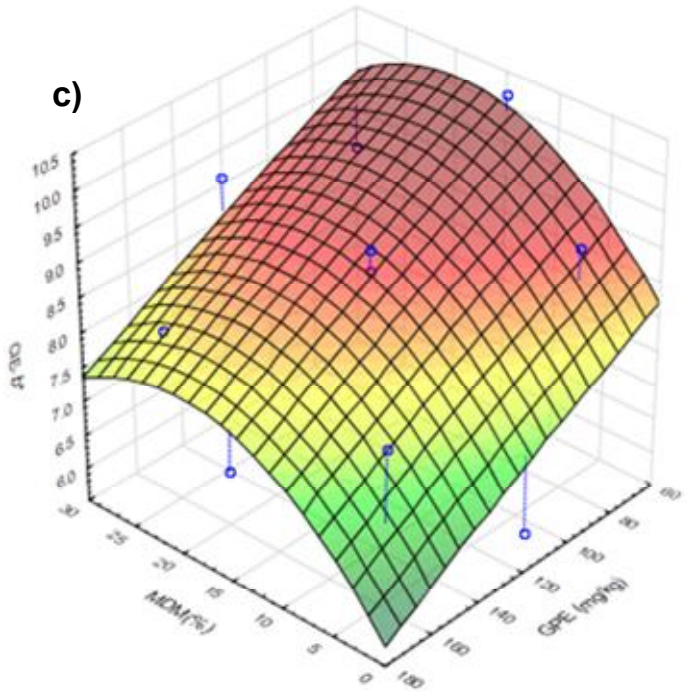

Fig. 2. Plot of mean values (dots) and fitted quadratic response surface obtained for the internal colour variables of chicken nuggets formulated with different concentration of mechanically deboned chicken meat (MDM, % w/w) and grape pomace extract (GPE, mg/kg): a) Whiteness index; b) CIE - *a**, and c) CIE - *b**.

Redness (*a**) also varied significantly w between samples (see Fig. 2.b). For low MDM content, GPE has a reduced impact on redness, with a clear interaction effect for higher values of MDM content, were redness markedly decreased with the increase of GPE (from app. 6.5 to 1.8, for 30 % MDM, going from 60 to 180 mg/kg GPE, respectively).

This effect is complemented with the evolution of *b** values (see Fig. 2.c) as it clearly diminish with the addition of GPE, going for a more “bluish” colour tone, due to the increase concentration of polyphenolic compounds from GPE.

MDM final composition is influenced by endogenous factors (anatomical location of bones, animal species, temperature, and amounts of lean meat, animal sex and wedges) and also by exogenous ones (recovery system,

pressure exerted during the recovery processes, temperature) (Field, 1988; Trindade, Felício, & Castillo, 2004). Regarding pH, values ranged from 7.13 to 7.97, with no particular effect of the joint variation of MDM content and GPE concentration.

4.3. Sensory analysis

4.3.1. Overall acceptance

Results on overall acceptance, based on appearance (exterior and inner, as depicted on Fig. 3) and odour of the different samples are present in Table 2.

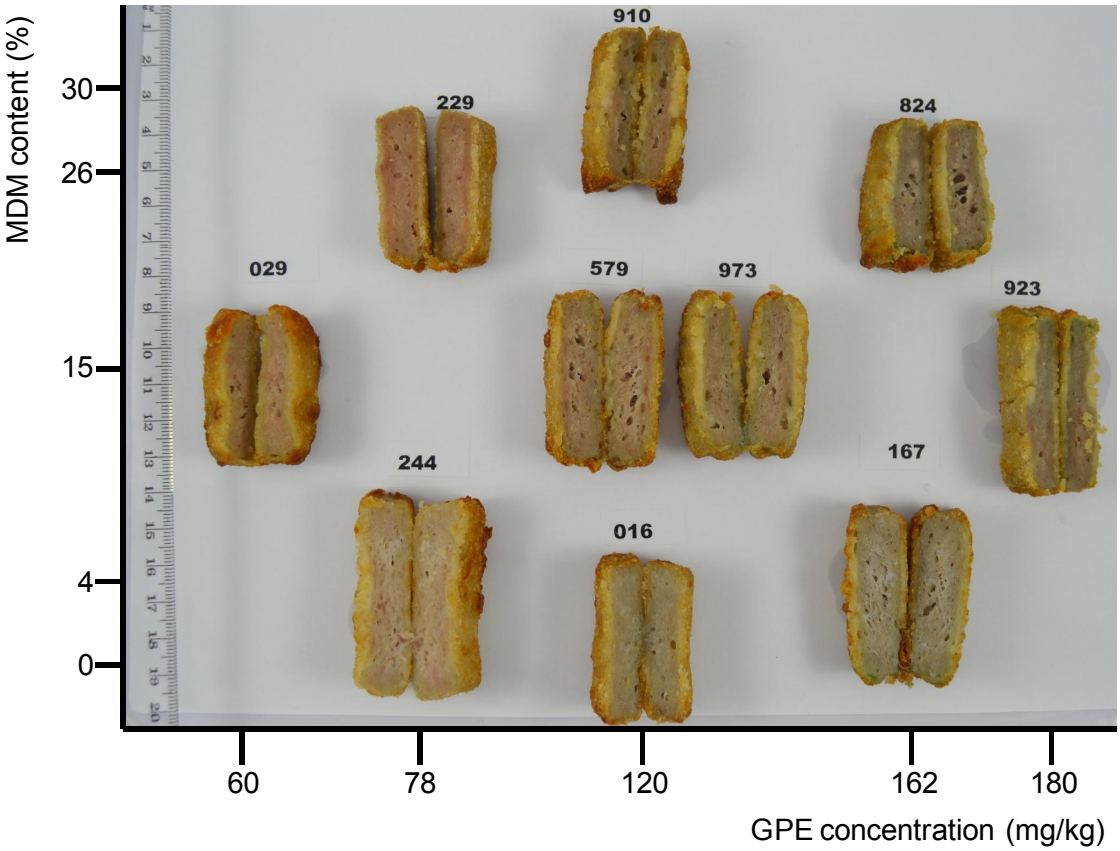

Fig. 3. External and internal appearance of chicken nuggets formulated with different concentration of mechanically deboned chicken meat (MDM, % w/w) and grape pomace extract (GPE, mg/kg). Three-digits random codes used to identify samples during sensory evaluation.

337 Results indicated significant differences ($p < 0.05$) among samples, including
338 nuggets elaborated with 15 % of MDM and 60 mg/kg of GPE as the most
339 accepted formulation (7.2 score). Moreover, nuggets containing low MDM
340 content (4 %) were also positively scored with mean acceptance score of 7.0.

Table 2
Mean (± standard deviation) of overall acceptance values (n = 75) for all nugget formulations.

Formulations (MDM content, GPE concentration)	Overall acceptance*
0 %, 120 mg/kg	$6.4^{a,b,c}$(± 1.9)
15 %, 60 mg/kg	7.2^{a}(± 1.2)
4 %, 162 mg/kg	$5.5^{c,d}$(± 2.1)
26 %, 78 mg/kg	$6.3^{b,c}$(± 1.7)
4 %, 78 mg/kg	$7.0^{a,b}$(± 1.5)
15 %, 120 mg/kg	$5.9^{c,d}$(± 1.9)
26 %, 162 mg/kg	5.2^{d}(± 2.1)
30 %, 120 mg/kg	$5.9^{c,d}$(± 2.0)
15 %, 180 mg/kg	5.3^{d}(± 1.9)
15 %, 120 mg/kg	$5.7^{c,d}$(± 2.0)

*a,b,c,d – homogeneous groups according to the non-parametric Wilcoxon test, at 95 % confidence level.

341

342 There is a particular interest regarding the optimum formulation combining
343 MDM, GPE and taking in consideration the variation of overall acceptance of
344 finished product, plotted as a response surface given in Fig. 4.

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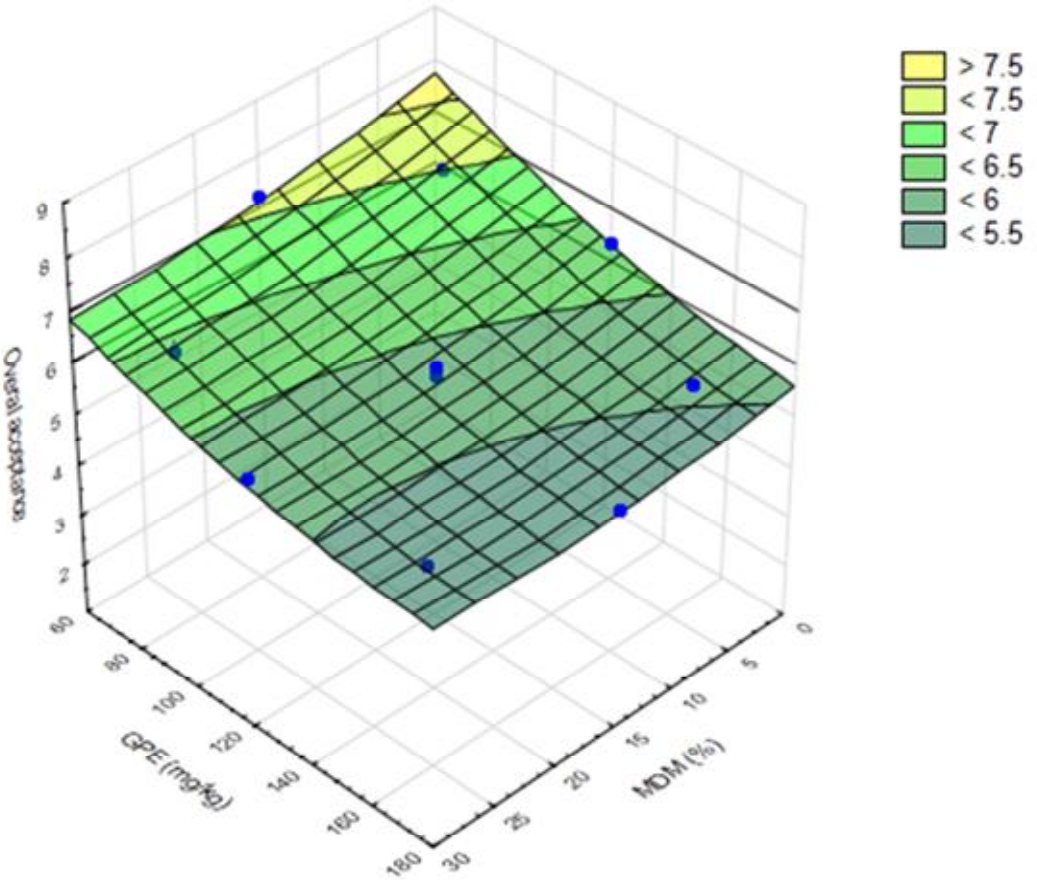

Fig. 4. Plot of mean values (dots) and fitted quadratic response surface obtained for the overall acceptance of chicken nuggets formulated with different concentration of mechanically deboned chicken meat (MDM, % w/w) and grape pomace extract (GPE, mg/kg).

The following full quadratic model was fitted, following a minimal residual sums of squares procedure: Acceptance = 9.83 - 0.0649 × MDM - 0.0404 × GPE + 7.91×10^{-4} × MDM^2 + 1.80×10^{-4} × MDM × GPE + 9.29×10^{-5} × GPE^2, with, Acceptance expressed on the 9-point scale – going from 1 to 9, MDM expressing MDM content (%) and GPE expressing GPE concentration (mg/kg). The fitted model had a R^2 of 0.971. Moreover, GPE concentration has a greater impact on acceptance scores than MDM content. This is somehow in agreement with results obtained by Perlo, et al. (2006), were no significant

differences were found on the sensory description and on preference of nuggets with incorporation of 0, 10, 20, 30 and 40 % of washed mechanically deboned chicken meat. In fact, it is predicted that a GPE increase from 30 to 60 mg/kg at a fixed MDM content of 10 %, decreased acceptance scores from 8.19 to 7.29. While a decrease on the MDM content, from 10 to 5 % at a fixed GPE concentration of 60 mg/kg, indicated a lower increase of the final acceptance scores (from 7.29 to 7.50).

According to predicted results (Figure 3), for a fixed overall acceptance of 7 or more points, possible formulations could go up to a MDM content of 20 %, with a fixed concentration of 60 mg/kg of GPE, or up to 90 mg/kg of GPE, in the absence of MDM. If aiming on an acceptance limit of 6 points (for instance, if developing a more economic product), possible formulations could go up to a MDM content of 30 %, with a concentration of up to 105 mg/kg of GPE, or up to 140 mg/kg of GPE, in the absence of MDM.

4.3.2. Analysis of the open-ended comments

A total of 2527 initial term count, regarding the full set of samples, was filtered, to remove hedonic -non-descriptive- terms. Synonyms were merged and lower frequency (less than 10 citations for the overall set of samples) terms were dropped. A contingency table of samples vs. descriptive terms was drawn and analysed following Correspondence Analysis (CA). Attributes with no significant chi-square per cell results, across the full set of samples, were dropped Resulting set was re-coded into a final set of 13 positive (total count of 303) and 13 negative (total count of 591) descriptive terms. Plotted results after CA are given in Fig. 5. where a “perceptual map” of the data generated from the contingence table (Varela & Ares, 2014) is depicted, which is useful for interpretation.

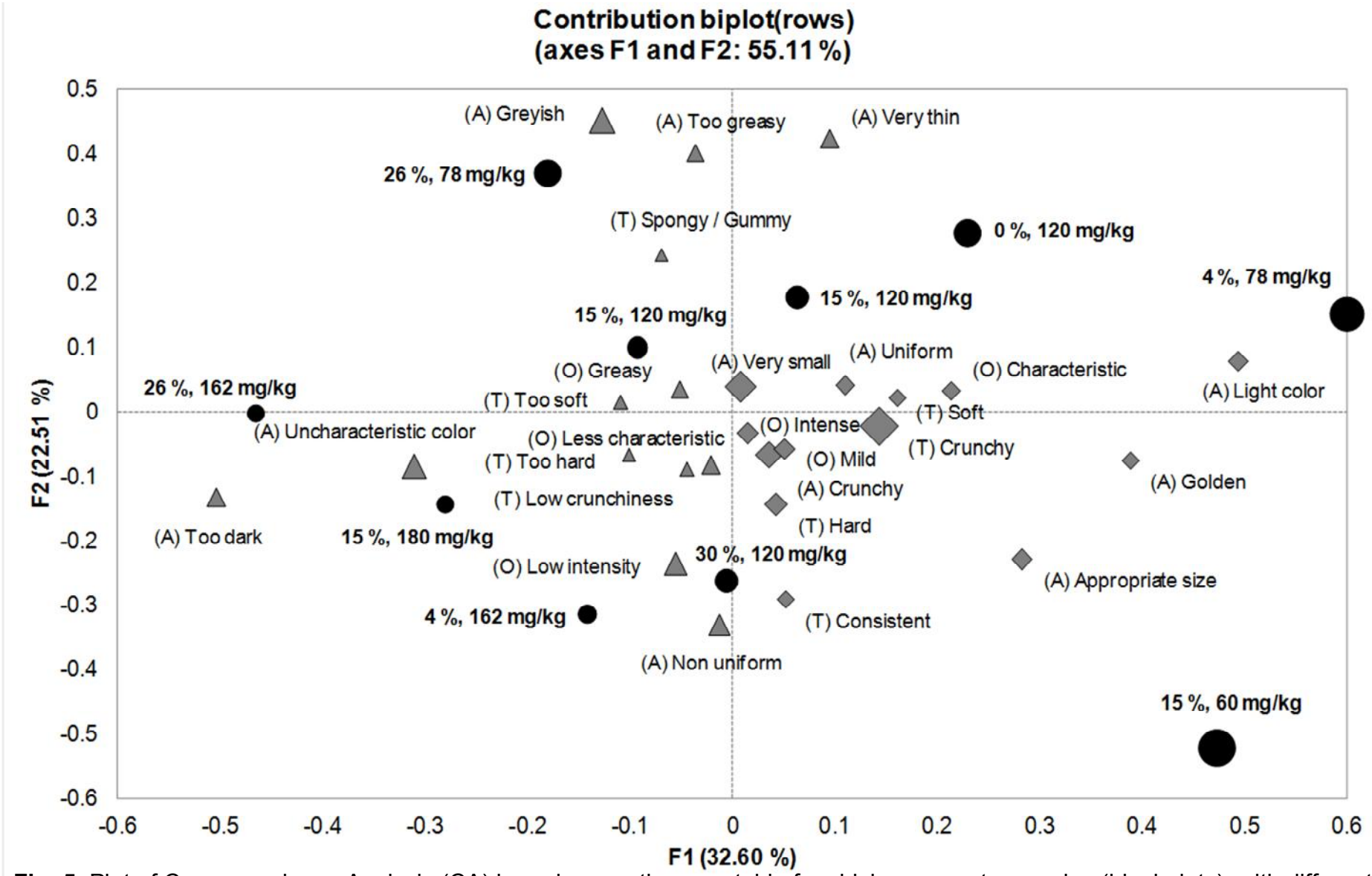

Fig. 5. Plot of Correspondence Analysis (CA) based on contingency table for chicken nuggets samples (black dots), with different concentration of mechanically deboned chicken meat (MDM, % w/w) and grape pomace extract (GPE, mg/kg), and attributes used by each assessor (grey triangles and rhombus used for negative and positive attributes, respectively). Size of symbols is directly proportional to overall acceptance, for samples, and to number of citations, for attributes. A: Appearance; O: Odour and T: Texture.

In general, CA resulted in two first main factors explaining over 55 % of the total variance. Factor 1 (F1) was the most important variable, accounting for over 32 % of the total variance. As depicted in Fig. 5, F1 was directly correlated to positive attributes (rhombus symbols plotted on the right side) whilst indirectly correlated to negative attributes (triangle symbols plotted on the left side). On the other hand, the second main factor, F2, explained about 22 % of the total variance. Generally, positive attributes were displayed around the plot centre, thus, it can be said they were less discriminative than negative ones, which were located more distant from centre. Exception for positive attributes “light colour”, “golden appearance” and “appropriate size”, associated with the most accepted samples: 4 % MDM, 78 mg/kg GPE and 15 % MDM, 60 mg/kg GPE. It is relevant to note that those samples were the ones yielding higher values of Whiteness Index.

On the other hand, formulations containing 15 % MDM, 180 mg/kg GPE or 26 % MDM 162 mg/kg GPE, were strongly associated to “uncharacteristic colour” and “too dark” by assessors. Additionally, these formulations received the lowest acceptance scores during sensory evaluation. The selection of the overall acceptance target, hence the more appropriate combinations, may depend on the interest of the industrial sector.

5. **Conclusion**

Findings indicate that MDM incorporation into nugget formulation resulted mainly in an increase of fat content. However, final fat content values (5.5 g fat / 100 g product) were lower than published lipid content of nuggets commercially available (12.3 g fat / 100 g product). Combination of the effect of varying GPE concentration and MDM content significantly ($p < 0.05$) influenced colour. Redness (*a**) and yellowness (*b**) were predicted as minimum in the absence of MDM and a maximum concentration of 180 mg/kg of GPE. Whiteness Index was maximized by reducing both the MDM content and the GPE concentration. Response surface model of overall acceptance indicated the existence of a range of combinations of MDM content and of GPE concentrations, which could be adjusted in accordance to the desired threshold level of acceptance. These, can be used as a reference indicator for industrial elaboration of chicken nuggets for different quality categories. Additionally, through application of CA, these findings were confirmed, increasing information regarding positive attributes such as “light colour” associated to previous formulations. Hence, it can be concluded that addition of GPE up to 120 mg/kg and MDM up to 15 % did not adversely affect the perceived appearance of chicken nuggets. These findings definitely contribute to a better understanding of current consumer demands regarding meat products.

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References

Albert, A., Varela, P., Salvador, A., Hough, G., & Fiszman, S. (2011). Overcoming the issues in the sensory description of hot served food with a complex texture. Application of QDA®, flash profiling and projective mapping using panels with different degrees of training. *Food Quality and Preference, 22*(5), 463-473.

AOAC. (2002). Official methods of analysis. In (17 th ed.). Gaithersburg, MD: Association of Official Analytical Chemists, USA.

Ares, G., de Saldamando, L., Giménez, A., Claret, A., Cunha, L. M., Guerrero, L., de Moura, A. P., Oliveira, D. C. R., Symoneaux, R., & Deliza, R. (2015). Consumers' associations with wellbeing in a food-related context: A cross-cultural study. *Food Quality and Preference, 40, Part B*(0), 304-315.

Arihara, K. (2006). Strategies for designing novel functional meat products. *Meat Science, 74*(1), 219-229.

Banerjee, R., Verma, A. K., Das, A. K., Rajkumar, V., Shewalkar, A. A., & Narkhede, H. P. (2012). Antioxidant effects of broccoli powder extract in goat meat nuggets. *Meat Science, 91*(2), 179-184.

Box, G. E., Hunter, J. S., & Hunter, W. G. (2005). Statistics for experimenters: design, innovation, and discovery. In (2nd ed., pp. 450-456): Wiley.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews, 56*(11), 317-333.

Brislin, R. W. (1970). Back-translation for cross-cultural research. *Journal of cross-cultural psychology, 1*(3), 185-216.

Falguera, V., Aliguer, N., & Falguera, M. (2012). An integrated approach to current trends in food consumption: Moving toward functional and organic products? *Food Control, 26*(2), 274-281.

Faustman, C., Sun, Q., Mancini, R., & Suman, S. P. (2010). Myoglobin and lipid oxidation interactions: Mechanistic bases and control. *Meat Science, 86*(1), 86-94.

Field, R. (1988). Mechanically separated meat, poultry and fish. *Advances in meat research (USA)*.

Froning, G. (1981). Mechanical deboning of poultry and fish. *Advances in food research, 27*, 109-147.

Gibbs, R. A., Rymer, C., & Givens, D. I. (2013). Fatty acid composition of cooked chicken meat and chicken meat products as influenced by price range at retail. *Food Chemistry, 138*(2–3), 1749-1756.

Guerrero-Legarreta, I. (2010a). *Handbook of Poultry Science and Technology: Volume 1: Primary processing*: John Wiley & Sons, Inc.

Guerrero-Legarreta, I. (2010b). *Handbook of Poultry Science and Technology: Volume 2: Secondary processing*: John Wiley & Sons, Inc.

Guerrero, L., Claret, A., Verbeke, W., Enderli, G., Zakowska-Biemans, S., Vanhonacker, F., Issanchou, S., Sajdakowska, M., Granli, B. S., & Scalvedi, L. (2010). Perception of traditional food products in six European regions using free word association. *Food Quality and Preference, 21*(2), 225-233.

Henchion, M., McCarthy, M., Resconi, V. C., & Troy, D. (2014). Meat consumption: Trends and quality matters. *Meat Science, 98*(3), 561-568.

Hui, Y. H. (2012). *Handbook of meat and meat processing*: CRC press.

Hunt, R. W. G., & Pointer, M. R. (2011). *Measuring colour*: John Wiley & Sons.

Hwang, K. E., Choi, Y. S., Choi, J. H., Kim, H. Y., Kim, H. W., Lee, M. A., Chung, H. K., & Kim, C. J. (2011). Effect of Ganghwayakssuk (Artemisia princeps Pamp.) on Oxidative Stability of Deep Fried Chicken Nuggets. *Food Science and Biotechnology, 20*(5), 1381-1388.

Jiménez-Colmenero, F., Carballo, J., & Cofrades, S. (2001). Healthier meat and meat products: their role as functional foods. *Meat Science, 59*(1), 5-13.

492 Karre, L., Lopez, K., & Getty, K. J. K. (2013). Natural antioxidants in meat and poultry products.
493 *Meat Science, 94*(2), 220-227.
494 Kearney, J. (2010). Food consumption trends and drivers. *Philosophical transactions of the*
495 *royal society B: biological sciences, 365*(1554), 2793-2807.
496 Kumar, D., & Tanwar, V. (2011). Effects of incorporation of ground mustard on quality
497 attributes of chicken nuggets. *Journal of food science and technology, 48*(6), 759-762.
498 Kumar, V., Biswas, A. K., Chatli, M. K., & Sahoo, J. (2011). Effect of banana and soybean hull
499 flours on vacuum-packaged chicken nuggets during refrigeration storage. *International*
500 *Journal of Food Science and Technology, 46*(1), 122-129.
501 Lau, D. W., & King, A. J. (2003). Pre- and Post-Mortem Use of Grape Seed Extract in Dark
502 Poultry Meat To Inhibit Development of Thiobarbituric Acid Reactive Substances.
503 *Journal of Agriculture and Food Chemistry, 51*(6), 1602-1607.
504 MacFie, H. J., Bratchell, N., Greenhoff, K., & Vallis, L. V. (1989). Designs to balance the effect of
505 order of presentation and first-order carry-over effects in hall tests. *Journal of Sensory*
506 *Studies, 4*(2), 129-148.
507 Magalhães, L. M., Santos, F., Segundo, M. A., Reis, S., & Lima, J. L. (2010). Rapid microplate
508 high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity.
509 *Talanta, 83*(2), 441-447.
510 Mielnik, M. B., Olsen, E., Vogt, G., Adeline, D., & Skrede, G. (2006). Grape seed extract as
511 antioxidant in cooked, cold stored turkey meat. *LWT - Food Science and Technology,*
512 *39*(3), 191-198.
513 Moura, A. P. d., & Cunha, L. M. (2005). Why consumers eat what they do: an approach to
514 improve nutrition education and promote healthy eating. In *Taking responsibility* (pp.
515 144-156).
516 Moussaoui, K. A., & Varela, P. (2010). Exploring consumer product profiling techniques and
517 their linkage to a quantitative descriptive analysis. *Food Quality and Preference, 21*(8),
518 1088-1099.
519 Nandakumar, V., Singh, T., & Katiyar, S. K. (2008). Multi-targeted prevention and therapy of
520 cancer by proanthocyanidins. *Cancer letters, 269*(2), 378-387.
521 Negro, C., Tommasi, L., & Miceli, A. (2003). Phenolic compounds and antioxidant activity from
522 red grape marc extracts. *Bioresource Technology, 87*(1), 41-44.
523 Ozer, O., & Sariçoban, C. (2010). The effects of butylated hydroxyanisole, ascorbic acid, and α-
524 tocopherol on some quality characteristics of mechanically deboned chicken patty
525 during freeze storage. *Czech Journal of Food Sciences, 28*(2), 150-160.
526 Perlo, F., Bonato, P., Teira, G., Fabre, R., & Kueider, S. (2006). Physicochemical and sensory
527 properties of chicken nuggets with washed mechanically deboned chicken meat:
528 Research note. *Meat Science, 72*(4), 785-788.
529 Peryam, D. R., & Pilgrim, F. J. (1957). Hedonic scale method of measuring food preferences.
530 *Food technology*.
531 Sayago-Ayerdi, S. G., Brenes, A., Viveros, A., & Goni, I. (2009). Antioxidative effect of dietary
532 grape pomace concentrate on lipid oxidation of chilled and long-term frozen stored
533 chicken patties. *Meat Science, 83*(3), 528-533.
534 Schmitt, N. (1998). Quantifying word association responses: what is native-like? *System, 26*(3),
535 389-401.
536 Shirahigue, L. D., Plata-Oviedo, M., de Alencar, S. M., d'Arce, M. A. B. R., de Souza Vieira, T. M.
537 F., Oldoni, T. L. C., & Contreras-Castillo, C. J. (2010). Wine industry residue as
538 antioxidant in cooked chicken meat. *International Journal of Food Science &*
539 *Technology, 45*(5), 863-870.
540 Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and
541 other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent.
542 *Methods In Enzymology, 299*, 152-178.

543 Symoneaux, R., Galmarini, M., & Mehinagic, E. (2012). Comment analysis of consumer's likes
544 and dislikes as an alternative tool to preference mapping. A case study on apples. *Food*
545 *Quality and Preference, 24*(1), 59-66.
546 Tournour, H. H. (2014). *Skin and seed grape extract as an antioxidant for mechanically*
547 *deboned chicken meat, during frozen storage.* University of Porto, Porto.
548 Trindade, M. A., Felício, P. E. d., & Castillo, C. J. C. (2004). Mechanically separated meat of
549 broiler breeder and white layer spent hens. *Scientia Agricola, 61*, 234-239.
550 Varela, P., & Ares, G. (2014). *Novel Techniques in Sensory Characterization and Consumer*
551 *Profiling*: CRC Press.
552 Vidal, L., Ares, G., & Giménez, A. (2013). Projective techniques to uncover consumer
553 perception: Application of three methodologies to ready-to-eat salads. *Food Quality*
554 *and Preference, 28*(1), 1-7.
555 Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., & Pérez-Álvarez, J. (2010). Effect of
556 orange dietary fibre, oregano essential oil and packaging conditions on shelf-life of
557 bologna sausages. *Food Control, 21*(4), 436-443.
558 WHO. (2003). Diet, nutrition and the prevention of chronic diseases: report of a Joint
559 WHO/FAO Expert Consultation. In: World Health Organization.

4.

Conclusions

4.1. Main conclusions

Data depicted in the present thesis provide valuable information regarding the characterization of certain Portuguese grape varieties residues which are still under-exploited as a source of polyphenolic compounds.

Results from **Paper I** contribute with a characterization of GPE in two solvents (ethanol/water extract and aqueous suspensions) regarding total phenolic content and antioxidants capacity. Findings indicated that Portuguese GPE presented TPC, $DPPH^{\bullet}$ and ORAC values comparable with previous data, giving a positive perspective on the use of a less efficient extractive mixture (80:20 % v/v absolute ethanol: water) having a food grade classification and being environmentally friendly. Hence, considering that Portugal eleventh largest wine producer in the world, and therefore generating large amounts of grape pomace, these results contribute to the valorization of these by-products, highlighting a variety of industrial applications.

Different behaviors were observed regarding the effect of solvent in GPE composition as a dependent response on antioxidant assay. ORAC and ICA values significantly ($p < 0.01$) decreased when ethanolic/water and aqueous suspensions were compared. However, a significant ($p < 0.01$) increase in TPC values was determined when the effect of solvent was compared as above. $DPPH^{\bullet}$ values were not significantly ($p = 0.350$) affected.

Concerning the experiments presented in **Paper I**, although the three most representative Portuguese red grape varieties, from Douro´s region were analyzed, a large number of varieties, and their annual variation were not considered. A broader perspective on those would provide a stronger evidence of our observations. Nevertheless, taking into account these considerations, it was observed that extracts from "*Touriga nacional*" grape pomace yielded the highest TPC and antioxidant capacity according to $DPPH^{\bullet}$ and ORAC assays. Additionally, TNac exhibited the highest total phenolic content according to HPLC analysis, including mainly gallic acid, (+)-catechin, caffeic acid, syringic

acid and (-)-epicatechin. These results showed a promising scenario for future applications of this grape variety.

In what concerns to the antioxidant protection effect of GPE from "*Touriga franca*" in food systems, such as a meat model (**Paper II**), FCR, DPPH$^{\bullet}$, ORAC and ICA assays were proposed and evaluated as indicators of oxidative meat degradation.

Results indicated that FCR, ORAC and ICA assays were suitable and consistent methodologies to monitor the consequences of induced degradation using MDM as food model. In contrast, DPPH$^{\bullet}$ assay was not suitable to distinguish significant differences ($p = 0.59$) among samples with added antioxidant and control ones. Spectra/absorption overlapping from polyphenols and meat system compounds, along with micro precipitations in 50 % v/v ethanolic medium, were depicted as possible reasons for the assay's lack of specificity.

The antioxidant effectiveness of GPE compared to BHT was evaluated against the application of several stress factors, namely presence of iron(II), UV-C radiation, MAP and temperature abuse, in two experimental approaches: single and successive exposure, combined in a single fully hierarchical design of experiments. Nutritional composition and fatty acid profile of MDM revealed high fat content (over 24 %), including MUFA and PUFA, and with oleic acid (41.37 ± 0.02%) and linoleic acid (15.2 ± 0.2%) as main fatty acids found in MDM composition. Concerning to protection conferred by GPE (150 mg/kg) although it was less effective than that the one conferred by BHT (100 mg/kg), results indicated an antioxidant activity of GPE consistently present in all assays.

The antioxidant effectiveness was stress-factor and antioxidant dependent, for instance, this can be depicted if we consider that BHT added samples exerted better protection than GPE against UV, as measured with ORAC; whilst protection conferred by GPE added samples was higher than BHT, regarding temperature abuse, as evaluated with ICA values. Additionally, it yields that a successive exposure to stress conditions affects the final antioxidant performance, causing similar behavior in ORAC values under the effect of both GPE (23.9 to 13.2 µmol TE g^{-1} meat), and BHT (26.9 to 14.6 µmol TE g^{-1} meat).

Information regarding influence of GPE from different Portuguese varieties on oxidative stability, nutritional and physical characteristics of MDM under frozen storage, was provided on **Paper III** and **Paper IV**. MDM samples were supplemented with BHT-BHA (200 mg/kg) and GPE at two different levels (60 and 120 mg/kg). The effect of MDM initial composition on the protection of GPE was also evaluated. MDM samples even under vacuum packaging and with antioxidant supplementation showed signs of lipid oxidation as evaluated by FCR, ORAC and ICA assays.

Significant changes in meat color, measured through CIE - *L*a*b*,* were observed with GPE supplementation, mainly with extracts from "*Tinta roriz*" (TR) and "*Touriga franca*" (TF). Control and BHT-BHA added samples were lighter, redder and yellower than MDM samples supplemented with any GPE. Protection against lipid oxidation of certain groups of fatty acids in MDM samples supplemented with low concentration of GPE (60 mg/kg), from TR and TF grape varieties, was observed throughout storage. On a relevant note, all MDM samples supplemented with GPE exhibited effective protection against oxidation of n-*3* aggregated fatty acids , whilst BHT-BHA and control samples showed a significant ($p < 0.05$) decrease after advanced storage (365D) (**Paper III**). From a nutritional point of view, this fact is of valuable importance, given that n-*3* fatty acids, including α-Linolenic acid (C18:3 n-*3*) correspond to essential fatty acids for human diet.

Regarding the influence of the initial composition of MDM samples (**Paper IV**) our findings suggested that MDM composition affected its oxidative stability throughout storage, according to ORAC and ICA methodologies. Additionally, different levels of GPE supplementation (60 and 120 mg/kg) showed minor significant alterations, regarding color variables -mainly *b** and Hue angle-. Results from **Paper III** and **IV** corroborate that Portuguese grape pomace exerted antioxidant protection, with this protective performance dependent on GPE concentration and on MDM initial composition.

In this context, implications of supplementation of MDM with higher GPE levels, from the consumer perspective were analyzed in **Paper V**. Optimum combinations of MDM content and GPE concentrations for nugget elaboration

and latter sensory evaluation of overall acceptance were obtained. Naïve assessors, regular consumers of poultry meat products, perceived high acceptance scores: for a fixed overall acceptance of 7 or more points, possible formulations could go up to a MDM content of 20 %, with a fixed concentration of 60 mg/kg of GPE, or up to 90 mg/kg of GPE, in the absence of MDM. If aiming from an acceptance limit of 6 points (for instance, if developing a more economic product), possible formulations could go up to a MDM content of 30 %, with a concentration of up to 105 mg/kg of GPE, or up to 140 mg/kg of GPE, in the absence of MDM. Additionally, through application of Correspondence analysis, these findings were confirmed, increasing information regarding positive attributes such as “light colour” (internal appearance descriptor) associated to previous formulations. Hence, it can be concluded that addition of GPE up to 120 mg/kg and MDM up to 15 % did not adversely affect the perceived appearance of chicken nuggets.

These results add up to the valorization of Portuguese grape pomace as an affordable and underestimated source of polyphenolic compounds with relevant properties as a food preservation agent. This study focused on recovery strategies and on the valorization of these agro-food by-products, providing valuable information on their potential industrial applications. Based on this, a new variety of functional products can be offered towards the satisfaction of current consumer demands.

4.2. Perspectives and future trends

Findings presented in this thesis highlighted possible interactions undergone by phenolic compounds from GPE, when incorporated in a food matrix, during storage, by exerting protection against its lipid oxidation.

Considering that several reports indicate relevant beneficial properties for consumers' health, associated to intake of polyphenolic compounds, an additional change in the industrial sector, towards natural and functional ingredients and foods, is anticipated to face consumers´ demands.

Considering the relevance that nonextractable polyphenols fraction represent from the research perspective, experiments covering the study of extractive conditions and procedures, compatible with food incorporation should be considered in future work. This should cover the analysis and characterization of those polyphenols fractions.

In future, *in vitro* experiments may be conducted with simpler food matrixes in order to isolate the effects of the application of this grape pomace extract, comprising a deeper study of its chemical mechanisms behind as protective agents, understanding the potential impacts of their antioxidant compounds on the final product characteristics, namely on its lipid content and fatty acids profile.

From the consumer point of view, it would be interested to improve nugget – or other meat product- formulation and proceed with a larger evaluation of the impact of different processing conditions (e.g., frying, baking or microwave heating). With this being evaluated from a joint metabolomics, nutrigenomics, and consumer perception and acceptance points of view. Such study could be complemented with the evaluation of the impact of different claims, regarding the incorporation of grape pomace extract, on consumers' willingness to pay for such products.