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DOUTORAMENTO EM CIÊNCIAS FARMACÊUTICAS
ESPECIALIDADE DE NUTRIÇÃO E QUÍMICA DOS ALIMENTOS

AllPumpkin – Development of a high-quality pumpkin pulp enriched and preserved by added-value molecules from pumpkin by-products, an integrative and sustainable strategy

Maria Gabriela Leichtweis

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AllPumpkin – Development of a high-quality pumpkin pulp enriched and preserved by added-value molecules from pumpkin by-products, an integrative and sustainable strategy

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This work was supervised by Doctor Lillian Bouçada de Barros (CIMO-IPB) and co-supervised by Doctor Carla Susana Correia Pereira (CIMO-IPB) and Professor Doctor Maria Beatriz Prior Pinto Oliveira (Faculty of Pharmacy, University of Porto).

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"We ourselves feel that what we are doing is just a drop in the ocean. But the ocean would be less because of that missing drop."

— Mother Teresa

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"If you want to go fast, go alone. If you want to go far, go together"

— African Proverb

Abstract

The limitation of natural resources, combined with current consumer trends and needs, makes it essential to adopt sustainable food systems capable of producing healthier products and meeting the principles of the circular economy. Simultaneously, the food industry faces a growing demand for healthy, practical and ready-to-eat foods that meet daily nutritional needs. In addition, consumers are looking for products with varied organoleptic characteristics and free of artificial additives. On the other hand, the food industry still depends on the use of artificial additives to preserve and enhance the qualities of food. Therefore, it is essential to develop sustainable processing and natural preservation methods that guarantee food quality and safety.

Thus, this work presents a large characterization of different 17 pumpkin genotypes, including three Portuguese and three Algerian obtained from local markets, and eleven local landraces cultivated in Greece. In turn, some of the Greek genotypes were subdivided according to their morphology (round, cylindrical, and flattened), resulting in 16 Greek samples. In this way, 16 fruit pulps cultivated in Greece were evaluated for their quality in terms of nutritional, following the AOAC procedures for the total fat, crude protein, carbohydrates, dietary fiber content, ash and total energy, and chemical composition, including free sugar profile (by HPLC-RID) and fatty acids (by GC-FID) composition. Overall, a profile rich in carbohydrates was observed, followed by proteins and fibers, and low fat content. The main free sugars detected were glucose, sucrose and fructose, followed by trehalose and raffinose. While the main fatty acids were palmitic, oleic, linoleic, and linolenic acids. It was evidenced the presence of high innate variability in terms of crop performance, chemical composition, and bioactive properties not only among the different genotypes, but also at the intra-population level.

The pumpkin by-products, divided into peels, seeds and fibrous strands, totaling 60 samples, were screened for their bioactive compound profiles and their preservative potential. For that, samples were assessed for organic acids (by UFLC-PDA), tocopherols (by HPLC-FL), and phenolic compounds (by HPLC-DAD-ESI/MS). (-)-Epicatechin was the most commonly found phenolic compound, followed by significant phenolic acids and other flavonoids. Important profiles of organic acids, such as oxalic, quinic, malic, shikimic, and fumaric acids, were also found, in addition to tocopherol isomers (*alpha*-, *beta*-, *gamma*-, and *delta*-tocopherols). Moreover, all samples showed significant bioactive activity in terms of antioxidant, antibacterial, and antifungal in vitro assays, revealing their potential as a source of natural preservative for food application. Through this rich data set, together with interpretation of economic relevance, the peels of three Greek genotypes and of the commercial butternut squash (*Cucurbita moschata* Duch.) variety were selected to be assessed in the next steps.

These selected samples were further evaluated for the optimized recovery of their phenolic content and bioactive properties to produce extracts with the highest preservative potential to be used as natural ingredients in foods. The extraction process was optimized using response surface methodology (RSM) based on a Box–Behnken experimental design. Two extraction techniques were employed: heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE). The independent variables considered were time, solvent concentration, and temperature/power (for HAE and UAE, respectively), while the dependent variables included dry residue (DR), reducing power (RP), and total phenolic content (TP). Optimization by RSM allowed us to find the conditions that led to a higher extraction yield of compounds of interest and preservative power, with a reduction in time, energy and solvent parameters, for the four selected samples. Specifically, the low energy demand (30 °C) and the elimination of ethanol (using only water as a solvent) achieved with butternut squash peels extracted by HAE were key factors in selecting the extract of this variety, under these conditions, among the optimized samples and methods, to be incorporated into the pumpkin pulp formulations.

These peel extract obtained from butternut squash variety by HAE was then incorporated into a pumpkin pulp product at various concentrations, and it was compared with a formulation containing seed extract. The incorporation tests were carried out at the company Decorgel - Produtos Alimentares, S.A (Porto-Portugal). Controlled experiments were conducted to determine the extract's ability to maintain the microbiological and physicochemical quality of the pulp during storage. Two pumpkin pulp formulations preserved with natural ingredients from pumpkin peels and seeds were developed, capable of retaining the nutritional profile, color and microbiological quality for up to 45 days (extended shelf life beyond the company standard of 30 days), both with partial and complete replacement of the artificial preservative. Pumpkin pulp formulations were also processed using non-thermal high-pressure technology. The combination of HPP with the peel extract showed great potential for extending the shelf life of pumpkin pulp. Since no contamination was observed, the use of potassium sorbate in this product could be 100% replaced with this natural ingredient, ensuring extended shelf life of up to 45 days.

These findings highlight the potential for agro-industrial by-products to be effectively valorized, promoting sustainability through the adoption of environmentally friendly extraction methods. Furthermore, this approach aligns with the principles of a circular economy, promoting resource efficiency and contributing to the development of sustainable and healthier food systems.

Keywords: pumpkin (*Cucurbita* spp.); natural preservatives; agro-industrial by-products; eco-friendly extraction; food sustainability; circular economy.

Resumo

A limitação dos recursos naturais, aliada às tendências e necessidades atuais de consumo, torna essencial a adoção de sistemas alimentares sustentáveis, capazes de produzir produtos mais saudáveis e em conformidade com os princípios da economia circular. Simultaneamente, a indústria alimentar enfrenta uma crescente procura por alimentos saudáveis, práticos e prontos a consumir, que satisfaçam as necessidades nutricionais diárias. Além disso, os consumidores procuram produtos com características organoléticas variadas e isentos de aditivos artificiais. Por outro lado, a indústria alimentar ainda depende do uso de aditivos artificiais para preservar e melhorar as qualidades dos alimentos. Assim, é essencial desenvolver métodos de processamento sustentáveis e de preservação natural que garantam a qualidade e a segurança dos alimentos.

Assim, este trabalho apresenta uma ampla caracterização de 17 genótipos diferentes de abóbora, incluindo três portugueses e três argelinos obtidos em mercados locais, e onze variedades locais cultivadas na Grécia. Por sua vez, alguns dos genótipos gregos foram subdivididos de acordo com a sua morfologia (redondos, cilíndricos e achatados), resultando em 16 amostras gregas. Desta forma, foram avaliadas 16 polpas de frutos cultivados na Grécia quanto à sua qualidade nutricional, seguindo os procedimentos da AOAC para gordura total, proteína bruta, hidratos de carbono, teor de fibra dietética, cinzas e energia total, bem como a composição química, incluindo os perfis de açúcares livres (por HPLC-RID) e de ácidos gordos (por GC-FID). No geral, foi observado um perfil rico em hidratos de carbono, seguido de proteínas e fibras, com baixo teor de gordura. Os principais açúcares livres detetados foram glucose, sacarose e frutose, seguidos por trealose e rafinose. Os principais ácidos gordos foram ácidos palmítico, oleico, linoleico e linolénico. Evidenciou-se a presença de elevada variabilidade inata em termos de desempenho das culturas, composição química e propriedades bioativas, não só entre os diferentes genótipos, mas também ao nível intra-populacional.

Os subprodutos da abóbora, divididos em cascas, sementes e fibras, totalizando 60 amostras, foram analisados quanto aos perfis de compostos bioativos e ao seu potencial de preservação. Para tal, as amostras foram avaliadas quanto a ácidos orgânicos (por UFLC-PDA), tocoferóis (por HPLC-FL) e compostos fenólicos (por HPLC-DAD-ESI/MS). A (-)-Epicatequina foi o composto fenólico mais comummente encontrado, seguido por ácidos fenólicos significativos e outros flavonoides. Também foram identificados perfis importantes de ácidos orgânicos, como oxálico, quínico, málico, chiquímico e fumárico, além de isómeros de tocoferol (*alfa*-, *beta*-, *gama*- e *delta*-tocoferóis). Todas as amostras demonstraram atividade bioativa significativa em ensaios *in vitro* de atividade antioxidante, antibacteriana e antifúngica, revelando o seu potencial como fonte de

conservante natural para aplicação alimentar. Através deste conjunto de dados robusto e da interpretação da relevância económica, as cascas de três genótipos gregos e da variedade comercial de abóbora-manteiga (*Cucurbita moschata* Duch.) foram selecionadas para serem avaliadas nas etapas seguintes.

Estas amostras selecionadas foram posteriormente avaliadas para a recuperação otimizada do seu teor fenólico e propriedades bioativas, com o objetivo de produzir extratos com maior potencial conservante para serem utilizados como ingrediente natural em alimentos. O processo de extração foi otimizado utilizando a metodologia de superfície de resposta (RSM) baseada num desenho experimental de Box–Behnken. Foram empregues duas técnicas de extração: extração assistida por calor (HAE) e extração assistida por ultrassons (UAE). As variáveis independentes consideradas foram tempo, concentração de solvente e temperatura/potência (para HAE e UAE, respetivamente), enquanto as variáveis dependentes incluíram resíduo seco (DR), poder redutor (RP) e conteúdo fenólico total (TP). A otimização por RSM permitiu identificar as condições que levaram a um maior rendimento de extração dos compostos de interesse e ao poder conservante, com redução de tempo, energia e parâmetros de solvente, para as quatro amostras selecionadas. Especificamente, a baixa demanda energética (30 °C) e a eliminação do etanol (utilizando apenas água como solvente) alcançadas com as cascas de abóbora-manteiga extraídas por HAE foram fatores-chave para selecionar o extrato desta variedade, sob estas condições, entre as amostras e métodos otimizados, para incorporação nas formulações de polpa de abóbora.

Este extrato de cascas obtido da variedade de abóbora-manteiga por HAE foi então incorporado num produto de polpa de abóbora em várias concentrações, sendo comparado com uma formulação contendo extrato de sementes. Os testes de incorporação foram realizados na empresa Decorgel - Produtos Alimentares, S.A (Porto-Portugal). Experiências controladas foram conduzidas para determinar a capacidade do extrato de manter a qualidade microbiológica e físico-química da polpa durante o armazenamento. Foram desenvolvidas duas formulações de polpa de abóbora preservadas com ingredientes naturais provenientes de cascas e sementes de abóbora, capazes de manter o perfil nutricional, cor e qualidade microbiológica por até 45 dias (prazo de validade alargado para além do padrão da empresa de 30 dias), tanto com substituição parcial quanto completa do conservante artificial. As formulações de polpa de abóbora também foram processadas utilizando tecnologia de alta pressão não térmica. A combinação de HPP com o extrato de casca mostrou grande potencial para prolongar a vida útil da polpa de abóbora. Como não foi observada contaminação, o uso de sorbato de potássio neste produto pôde ser 100% substituído pelo ingrediente natural, garantindo uma vida útil estendida de até 45 dias.

Estes resultados destacam o potencial para a valorização eficaz de subprodutos agroindustriais, promovendo a sustentabilidade através da adoção de métodos de extração ecologicamente corretos. Além disso, esta abordagem está alinhada com os princípios da economia circular, promovendo a eficiência dos recursos e contribuindo para o desenvolvimento de sistemas alimentares sustentáveis e mais saudáveis.

Palavras-chave: abóbora (*Cucurbita* spp.); conservantes naturais; subprodutos agroindustriais; extração ecoeficiente; sustentabilidade alimentar; economia circular.

List of publications

This section presents all publications published within the scope of the PhD thesis.

Patent

Barros, L., Leichtweis, M.G., Pereira, C., Ferreira, I.C.F.R., Pires, T.C.S., Carocho, M.; Molina, A.K., Dias, M.I. (2024). Processo para a obtenção de um ingrediente conservante a partir das cascas de abóbora (Butternut squash) para aplicação alimentar. 119241. Portuguese Institute of Industrial Property (INPI).

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1. Leichtweis, M.G., Oliveira, M.B.P., Ferreira, I.C.F.R., Pereira, C., Barros, L. Sustainable recovery of preservative and bioactive compounds from food industry bioresidues (2021). *Antioxidants*, *10*(11), 1827. https://doi.org/10.3390/antiox10111827 (IF: 6.0; Q1: Chemistry, Medicinal).

2. Leichtweis, M.G., Molina, A.K., Pires, T.C.S., Dias, M.I., Calhelha, R., Bachari, K., Ziani, B.E.C., Oliveira, M.B.P.P., Pereira, C., Barros, L. (2022). Biological activity of pumpkin byproducts: Antimicrobial and antioxidant properties. *Molecules*, *27*(23), 8366. http://dx.doi.org/10.3390/molecules27238366 (IF: 4.2; Q2: Chemistry, Multidisciplinary).

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4. Leichtweis, M.G., Molina, A.K., Dias, M.I., Calhelha, R.C., Pires, T.C.S., Ourania, P., Oliveira, M.B.P.P., Petropoulos, S. A., Barros, L., Pereira, C. (2023) Variability in chemical profile and bioactivities of the flesh of Greek pumpkin landraces. *Horticulturae*, *9*(11), 1232. https://doi.org/10.3390/horticulturae9111232 (IF: 3.1; Q1: Horticulture).

5. Leichtweis, M.G., Molina, A.K., Dias, M.I., Calhelha, R.C., Pires, T.C.S., Pavli, O., Oliveira, M.B.P., Ferreira, I.C.F.R., Petropoulos, S., Barros, L., Pereira, C. (2025). Valorisation of pumpkin by-products: Chemical composition and bioactive properties of pumpkin seeds, peels, and fibrous strands from different local landraces of Greece. *Food Chemistry*, 475, 143306. https://doi.org/10.1016/j.foodchem.2025.143306 (IF: 4.2; Q2: Chemistry, Multidisciplinary).

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7. Leichtweis, M.G., Molina, A.K., Dias, M.I., Carocho, M., Pires, T.C.S., Rodrigues, P., Azevedo, M., Silva, B., Oliveira, M.B.P., Petropoulos, S., Pintado, M., Pereira, C., Barros, L. Novel all-pumpkin product: Optimized by-products extraction and

development of pulp formulations preserved with natural ingredients. Submitted to Food Research International.

8. Ribeiro, L.R., Leichtweis, M.G., Landgräber, E., Carocho, M., Pereira, C., Barros, L., Schlüter, O. Enhancing shelf life of pumpkin pulp with natural-based preservative and high pressure processing. Submitted to Food Chemistry.

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1. Leichtweis, M.G., Molina, A.K., Pereira, C., Pires, T.C.S., Bachari, K., Borhane E.C.Z., Ferreira, I.C.F.R., Barros, L. Bioactive properties of bio-residues from food industry: pumpkin seeds, peels, and fibrous strands. 2nd Meeting of the Group of Portuguese Chemists Abroad of the Portuguese Chemical Society. Lisbon, Portugal. 10 June 2021.

2. Leichtweis, M.G., Molina, A.K., Pereira, C., Chaski, C., Polyzos, N., Petropoulos, S.A., Ferreira, I.C.F.R., Barros, L. Food industry by-products applied as pumpkin-based foodstuff preservatives. Chromatography helps in times of crisis, Webinar. Lisbon, Portugal. 17 December 2021.

3. Leichtweis, M.G., Molina, A.K., Pereira, C., Pires, T.C.S.P., Calhelha, R.C., Oliveira, Maria B., Ferreira, I.C.F.R., Barros, L. Pumpkin by-products as a source of preservative compounds for food application: valorization of industrial bioresidues towards a sustainable system. XVI Encontro de química dos alimentos. Castelo Branco, Portugal. 23-26 October 2022.

4. Leichtweis, M.G., Molina, A.K., Pereira, C., Pires, T.C.S., Calhelha, R.C., Neji, T., Oliveira, M.B.P.P., Ferreira, I.C.F.R., Barros, L. Subprodutos de abóbora como fonte de conservantes naturais para aplicação alimentar. IV Congresso Nacional das Escolas Superiores Agrárias. Santarém, Portugal. 03-04 November 2022.

5. Leichtweis, M.G., Molina, A.K., Pires, T.C., Dias M.I., Calhelha, R.C., Bachari, K., Ziani, B.E.C., Oliveira, M.B.P.P., Pereira, C., Barros, L. Evaluation of the phenolic profile and bioactive potential of Portuguese and Algerian pumpkin by-products. 8th Portuguese Young Chemists Meeting (8PYCheM). Vila Real, Portugal. 17-19 May 2023.

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1. Leichtweis, M.G., Molina, A.K., Pereira, C., Pires, T.C.S.P., Ferreira, I.C.F.R., Barros, L. Pumpkin bioresidues as sources of bioactive compounds for food application. 7th Portuguese Young Chemists Meeting (7PYCheM), Online edition. Bragança, Portugal. 19-21 May 2021.

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9. Leichtweis, M.G., Molina, A.K., Pereira, C., Dias, M.I., Carocho, M., Oliveira, M.B.P.P., Ferreira, I.C.F.R., Barros, L. Identificação e otimização da extração de compostos fenólicos de cascas de abóbora 'Butternut squash'. XXVI Encontro Galego-Portugués de química. Santiago de Compostela, Spain. 16-18 November 2022.

10. Molina, A.K., Leichtweis, M.G., Pereira, C., Pires, T.C.S., Calhelha, R.C., Bachari, K., Ziani, B.E.C., Ferreira, I.C.F.R., Barros, L. Food preservative extracts from pumpkin by-products. 1st International Congress on Food, Nutrition & Public Health - Towards a sustainable future (ICFNH 2022). Lisbon, Portugal. 17 November 2022.

11. Leichtweis, M.G., Molina, A.K., Pereira, C., Pires, T.C.S., Calhelha, R.C., Mohamed, M.H., Oliveira, M.B., Ferreira, I.C.F.R., Barros, L. Egyptian pumpkin by-product extracts as natural food preservatives. 1st International Congress on Food, Nutrition & Public Health - Towards a sustainable future (ICFNH 2022). Lisbon, Portugal. 17 November 2022.

12. Leichtweis, M.G., Molina, A.K., Pereira, C., Dias, M.I., Oliveira, M.B.P.P., Ferreira, I.C.F.R., Barros, L. Caracterização cromatográfica de compostos bioativos obtidos a partir de casca, sementes e fibras remanescentes do aproveitamento industrial da polpa de abóbora. 12º Encontro Nacional de Cromatografia. Aveiro, Portugal. 06-08 December 2022.

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List of abbreviations

A

ANOVA – Analysis of variance

3D – Three-dimensional

4-pCoQA – 4-*O*-*p*-coumaroylquinic acid

a* – Greenness-redness

AAE – Ascorbic acid equivalent

ACE2 – Angiotensin-converting enzyme 2

AOAC – Association of official analytical chemists

ATCC – American type culture collection

B

b* – Blueness-yellowness

b_o – Constant coefficient

BBD – Box–Behnken design

b_i – Coefficient of linear effect

b_{ii} – Coefficient of quadratic effect

b_{ij} – Coefficient of interaction effect

C

C – Cylindrical

C – Control formulation

C. – *Cucurbita*

CFU – Colony-forming unit

COVID-19 – Coronavirus disease 2019

D

D% – Degradation percentage

DAD – Diode array detector

DES – Deep eutectic solvents

DPPH – 2,2-Diphenyl-1-picrylhydrazyl

DR – Dry residue

DRBC – Dichloran-rose bengal chloramphenicol agar

dw – Dry weight

E

e.g. – For example

EAE – Enzyme-assisted extraction

EFSA – European Food Safety Authority

EMM – Estimated marginal means

ES – Eutectic solvent

ESI – Electrospray ionization

EU – European Union

F

F – Flattened

FAME – Fatty acids methyl ester

FAO – Food and Agriculture Organization

F-C – Folin–Ciocalteau

FID – Flame ionization detector

FL – Fluorescence

FRAP – Ferric reducing antioxidant power

FUSIONS – Food Use for Social Innovation by Optimizing Waste Prevention Strategies

fw – Fresh weight

G

GAE – Gallic acid equivalents

GC – Gas-chromatography

H

HAE – Heat-assisted extraction

HPLC – High-performance liquid chromatography

HPP – High pressure processing

HSD – Tukey's honestly significant difference

HSP – Hansen solubility parameters

Ht_{50} – 50% hemolytic time

I

i.e. – That is

IC_{50} – Extract concentration that causes 50% of the respective bioactive effect

IL – Ionic liquids

INT – p-Iodonitrotetrazolium chloride

IS – Internal standard

ISO – International organization for standardization

J

K

k – Number of factors

L

L* – Lightness

LOD – Limit of detection

LOQ – Limit of quantification

LSR – Liquid–solid ratio

M

m/z – Mass-to-charge ratio

MAE – Microwave-assisted extraction

MBC – Minimum bactericidal concentration

ME – Maceration

MFC – Minimum fungicidal concentration

MIC – Minimum inhibitory concentration

MS – Mass spectrometry

MS^2 – Second stage of mass spectrometry

MUFA – Monounsaturated fatty acids

N

n – The number of measurements or elements in a sample

n.a. – Not applicable

n.d. – Not detected

n.t. – Not tested

n-6/n-3 – Omega-6 to omega-3 ration

NI – Natural ingredient

O

ORAC – Oxygen radical absorbance capacity

OS – Oxidative stress

OxHLIA – Oxidative hemolysis inhibition assay

P

p – Significance level

P1 – Landrace from the region of Trikala sample

P2 – Voutirato sample

P3 – Leuka Melitis (round) sample

PAS – Pasteurization without preservative

PC – Principal component

PCA – Principal component analysis

PCA – Plate Count Agar

PDA – Photodiode array detector

PE – Pumpkin peel extract rich in preservative compounds

PES – Partial eta squared

pH – Hydrogen potential

PLE – Pressurized liquid extraction

PLP2 – Primary culture of non-tumor porcine liver cells

PS – Traditional formulation

PS – Potassium sorbate

PT – Preservative type

PUFA – Polyunsaturated fatty acids

Q

R

R – Round

R_1 or DR – Response variable in dry residue

R^2 – Coefficient of determination

R_2 or RP – Response variable in reducing power

R_3 or TP – Response variable in total phenolic content

RGB – Red-green-blue color system

RID – Refractive index detector

RP – Reducing power

RSM – Response surface methodology

Rt – Retention time

RT – Room temperature

RW – Raw weight

S

SARS Cov-2 – Severe Acute Respiratory Syndrome Coronavirus 2

SC – Supercritical

SCF – Supercritical fluid

SD – Standard deviation

SE – Soxhlet Extraction

SFA – Saturated fatty acids

SFE – Supercritical fluid extraction

SI – Sweetness index

sp. – Species (a single unspecified species within a genus)

spp. – Species (multiple species within a genus)

SPSS – Statistical package for the social sciences

SRB – Sulphorhodamine B

ST – Storage time

SubC – Subcritical

T

T – Turbinate

T0 – The production day

T2 – Tamhane's T2 test

T21 – 21 days of storage

T45 – 45 days of storage

TBARS – Thiobarbituric Acid Reactive Substances

TL – Landrace from the region of Trikala

TP – Total phenolic content

TPA – Profile texture analysis

tr. – Traces

Trolox – 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

TT – Tukey's test

U

UAE – Ultrasound-assisted extraction

UFLC – Ultra-fast liquid chromatography

UN – United Nations

UPLC – Ultra performance liquid chromatography

USA – United States of America

UV – Ultraviolet radiation

V

υ/υ – Volumetric percentage

vis – Visible

VRBLA – Violet red bile glucose agar

vs. – Versus

W

X

X_1 or t – Time variable

X_2 or T/P – Temperature variable, for HAE, or power variable, for UAE

X_3 or EtOH – Ethanol percentage in the solvent variable

X_i – Independent variables

X_j – Independent variables

Y

Z

ZAR – South African Rand

λ

λmax – Wavelength of maximum absorption

Thesis layout

The thesis structure was organized as shown in **Figure 1** and described below.

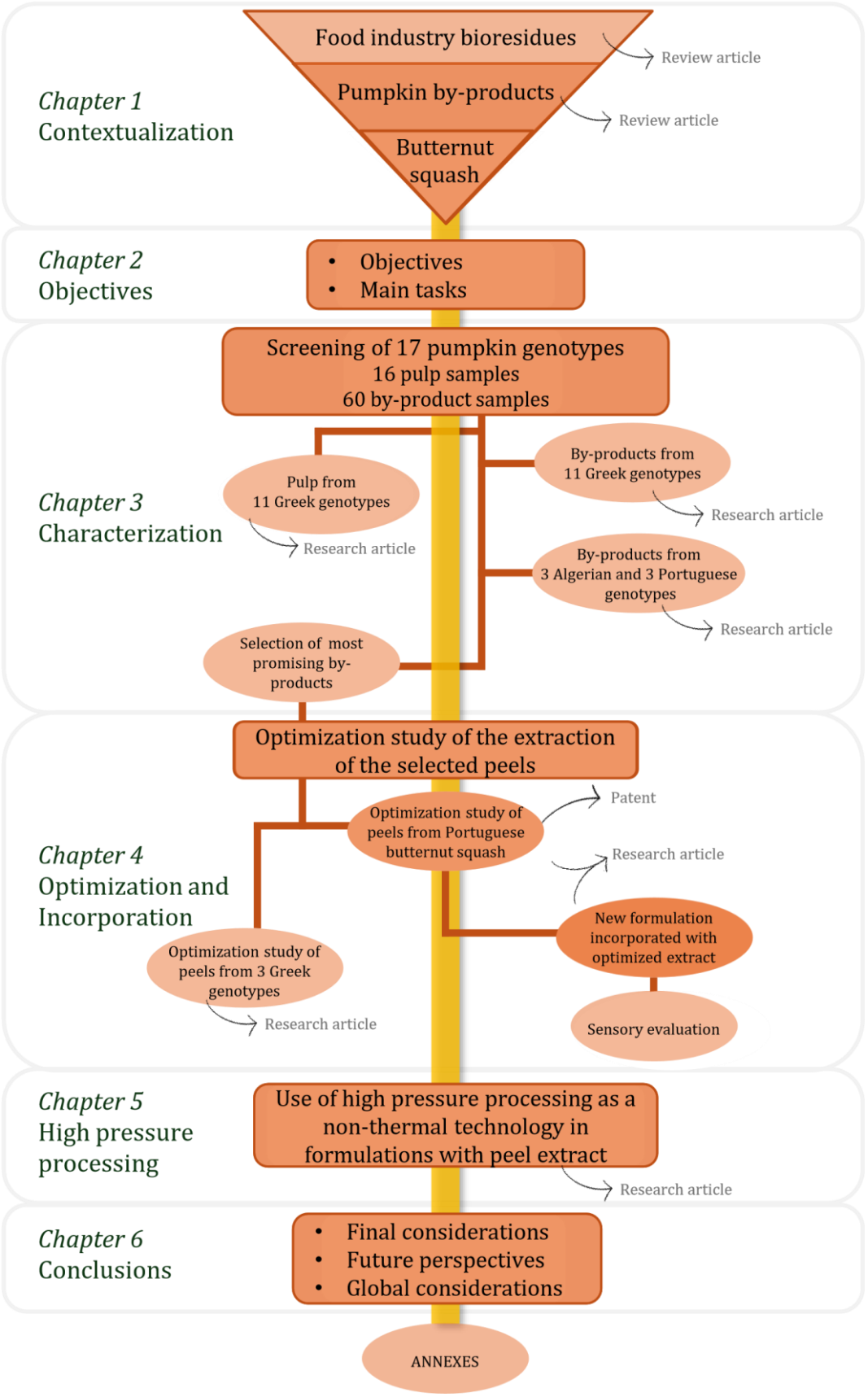

Figure 1. Thesis structure scheme.

Chapter 1 consists of an in-depth analysis of the current status of industrial food biowaste, bioactive compounds obtained from them, extraction techniques and industrial applications, provided through two comprehensive review articles. It is followed by a contextualization that highlights the motivations behind the present research.

Chapter 2 provides the objectives and organization of the tasks developed in the thesis research.

Chapter 3 consists of three research articles. Two of them present the phytochemical and bioactivity characterization of pumpkin by-products, while the other article concerns the nutritional and chemical profiles, as well as the functional properties, of Greek pumpkin pulps.

Chapter 4 comprises the development and application of natural ingredients. It is composed of two research articles, which address extraction optimization studies and development of pumpkin pulp formulations, followed by a discussion on the sensory acceptance of the pulps.

Chapter 5 covers the use of high pressure processing technology to reduce or eliminate the need for artificial preservatives in pumpkin pulp formulations.

Chapter 6 presents the general conclusions of the research, including the main contributions achieved through this doctoral thesis and future perspectives

CHAPTER 1

Contextualization

This chapter consists of two comprehensive review articles that provide an in-depth analysis of the current status of bioresidues, and the bioactive compounds obtained from them. It is followed by a contextualization that highlights the motivations behind the present research.

1.1. Sustainable recovery of preservative and bioactive compounds from food industry bioresidues

antioxidants

MDPI

Review

Sustainable Recovery of Preservative and Bioactive Compounds from Food Industry Bioresidues

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Abstract: With the increasing demand for convenient and ready-to-eat foods, the use of antioxidants and preservative additives in foodstuff formulation is essential. In addition to their technological functions in food, bio-based additives confer beneficial properties for human health for having antioxidant capacity and acting as antimicrobial, antitumor, and anti-inflammatory agents, among others. The replacement of preservatives and other additives from synthetic origin, usually related to adverse effects on human health, faces some challenges such as availability and cost. An opportunity to obtain these compounds lies in the food industry itself, as a great variety of food waste has been identified as an excellent source of high value-added compounds. Large amounts of seeds, fibrous strands, peel, bagasse, among other parts of fruits and vegetables are lost or wasted during industrial processing, despite being rich sources of bioactive compounds. From a circular economy perspective, this work reviewed the main advances on the recovery of value-added compounds from food industry bioresidues for food application. Bioactive compounds, mainly phenolic compounds, have been largely obtained, mostly from seeds and peels, and have been successfully incorporated into foods. Additionally, alternative and eco-friendly extraction techniques, as ultrasound and microwave, have showed advantages in extracting antioxidant and preservatives compounds.

Keywords: bioresidues; value-added compounds; antioxidant molecules; green extraction methods; food applications

1.1.1. Introduction

According to a survey carried out between 2010 and 2011, it is estimated that about one-third of the food produced for human consumption in the world is lost or wasted, which represents ~1.3 billion tons per year (FAO, 2011). The interest in estimating these values is not new; in 2007, Mahro & Timm (2007) carried out a study about the possibilities of using food processing residues as a biomass resource and concluded that, despite the well-established state of food industry, reliable data on the amounts of the generated waste along the distinct processing stages were difficult to obtain.

More recently, this topic has been gaining interest and, in 2018, Corrado & Sala (2018) published a review of existing studies on the generation of food waste on a global and European scale. Through this study, variations in food waste were estimated from 194 to 389 kg per person per year worldwide and from 158 to 298 kg per person per year in the EU. Among the reported works, the project FUSIONS (Food Use for Social Innovation by Optimizing Waste Prevention Strategies) stands out, with the estimation of food waste generated at an European level (Stenmarck et al., 2016). This project was carried out between 2012 and 2016 through the 7th Framework Program of the European Community, and represented a milestone in the accounting of food waste, with the generation of a manual on food waste quantification (Tostivint et al., 2016).

The interest in estimating these values is in line with the concern for the reduction of food waste generated worldwide. In the EU, as summarized on the EU business portal (EUbusiness, 2011), the Waste Framework Directive (Directive 2006/12/EC) has been revised through the Directive 2008/98/EC, to encourage the reuse and the recycling of waste materials and to simplify existing legislation, establishing measures to protect the environment and human health. Another action was through the United Nations Sustainable Development Goal 12.3 (UN General Assembly, 2015), where member states have pledged to halve per capita global food waste, in retail and consumer levels, by reducing food waste along production and supply chains by 2030.

Achieving reduced food waste and losses has been the focus of several recent studies, which involve identifying the dynamics of bioresidues generation and developing mechanisms to avoid this generation, as also managing unavoidable losses through strategies such as reuse. In fact, a great variety of bioresidues has proven to be a valuable source for the recovery of high value-added compounds, mainly with antioxidant capacity (Brasil et al., 2016; Buratto et al., 2021; Carpiné et al., 2020; Gaudino et al., 2020; Kļava et al., 2018; Leichtweis et al., 2019; Martinez-Fernandez et al., 2021; Soquetta et al., 2016).

In this context, this review aims to explore the potential of using these bioresidues and by-products, through the concept of a circular economy, for the extraction of bioactive compounds. It also addresses the application of green and environmentally-friendly

extraction methods and the incorporation of the recovered compounds into foodstuff.

Characterization

In addition to governmental interests in decreasing the volume of generated waste, views of characterization and ways of using these residues have also been gaining traction in the scientific circles (Cádiz-Gurrea et al., 2020; Carpiné et al., 2020; Gaudino et al., 2020; Leichtweis et al., 2019). The food waste data, discussed by Stenmarck et al. (2016), were divided into the following sectors: primary production, processing, wholesale and logistics combined with retail and market, food services, and household; according to food waste quantification manual (Tostivint et al., 2016). Through the results obtained, it is possible to verify that the biggest impact on the generation of waste comes from household and processing in the food manufacturing, in more detail 53% and 19%, respectively, considering food and inedible parts of food. The beginning of the food supply chain (primary production and processing) resulted in an estimated loss of approximately 26 million tons of food wasted in 2012 by the EU-28 (Stenmarck et al., 2016). Some cases of food waste are reported by the author Tristram Stuart in his famous book entitled "Waste: discovering the global waste scandal" (Stuart, 2009), with the outstanding example of bread processing, which generates the waste of 4 slices from each loaf, the crust, and the first slice at either end, amounting to 13,000 slices of fresh bread wasted every day. However, the most impactful pictures are related to the waste generated at the harvest level, where thousands of fruits and vegetables are discarded for not meeting the sales standard, in terms of size and overall appearance.

FAO data (FAO, 2011) reports that in low-income countries, the generation of food waste is bigger during the initial and intermediate stages of the food supply chain. In industrialized countries, on the other hand, more than 40% of food losses occur at retail and consumer levels, despite also occurring at the beginning of the food supply chain in significant quantities. Analyzing data, it is also verified that, in Europe, more than 50% of the production of roots and tubers and ~46% of the production of fruits and vegetables are lost or wasted, considering the edible parts of food products produced for human consumption. The sub-Saharan Africa region has the lowest production volume of fruit and vegetable group, along with North America and Oceania, compared to other five groups of regions in the world (Europe, Industrialized Asia, North Africa, West and Central Asia, South and Southeast Asia, and Latin America), and ~20% of the amount is lost during industrial processing.

In another study (Alexander et al., 2017), it was found that, due to cumulative losses, the proportion of global agricultural dry biomass consumed as food is only 6% and 24.8% of the harvested biomass, i.e., only a small fraction of agricultural production is

consumed as food. While losses during processing are also considerable (15–59% of processed crops), they widely vary between dry matter, energy, protein, and wet mass.

This brief information reveals a huge lack in the use of food. The potential of waste in commercial exploitation and circular use will be discussed below, focusing on by-products generated at industrial levels. **Table 1** summarizes recent works in terms of the characterization of the main components of nutritional and/or commercial value that can be recovered from food industry biowaste and explored for different purposes.

It is possible to highlight the fruit and vegetable group as the food raw materials that generate bioresidues with greater potential and interest for scientific study and commercial exploitation. This is because residues from fruit and vegetable processing are rich in organic matter, phytochemicals, and compounds with nutraceutical properties (R. Sharma et al., 2016). However, in general, diverse food residues are important sources of value-added compounds, with considerable levels of phenolic compounds, dietary fibers, polysaccharides, vitamins, carotenoids, pigments, oils, and others (Dueñas & Garciá-Estévez, 2020), justifying their recovery (Khan et al., 2019; Vuong et al., 2010).

The works performed by Buratto et al. (2021) and Cádiz-Gurrea et al. (2020) are recent examples of that, as summarized in **Table 1**. In the first study, it was found that about 5–15% of the weight of açaí fruit is edible, being almost 80% of the weight of the total fruit composed of seeds and lost during processing. In the extract of these seeds, significant amounts of polyphenols and total flavonoids were identified, among other compounds, with high ORAC (oxygen radical absorbance capacity) value. In the second study, a value of ~60% for the generation of by-products from the processing of tropical fruits was accounted, where the bioresidues, composed mostly by peels and seeds, revealed to be rich in photochemical compounds.

Table 1. Biocompounds found in bioresidues from food industry.

Industrialization process	By-product(s)/ Bioresidue(s)	Biocompounds	References
Processed potatoes	Peel	Polyphenols, antioxidants, proteins, polysaccharides, vitamins, and minerals	(Gaudino et al., 2020; Martinez-Fernandez et al., 2021)
Depulping of juçara fruit	Pomace and seeds – about 74%	Antioxidants compounds and unconventional starch	(Carpiné et al., 2020)
Pumpkin processing	Peel, seeds, and the flesh between seeds	Phenolic compounds and carotenes	(Ķļava et al., 2018)
Kiwi fruit processing	Skin and bagasse	Dietary fiber and bioactive compounds with antioxidant activity	(Soquetta et al., 2016)
Blackthorn fruit processing	Epicarp	Anthocyanins	(Leichtweis et al., 2019)
Açaí fruit processing	Seeds, slurry, and pulp residue –	Antioxidant compounds, polyphenols and flavonoids,	(Buratto et al., 2021)

Industrialization process	By-product(s)/ Bioresidue(s)	Biocompounds	References
	about 80 to 95%	cellulose, hemicellulose, and lignin	
Passion fruit processing	Rinds and bagasse – about 40 to 60%	Tocopherols and tocotrienols, fatty acids, carotenoid, and phenolic compounds (mostly piceatannol)	(Viganó et al., 2020)
Wine production	Bagasse (grape skins and seeds), stalks, and sludge - about 30%	Organic matter, phytochemicals, and compounds with nutraceutical properties	(Brasil et al., 2016)
Cashew nut processing	Shell liquid, testa, cashew apple, and cashew apple bagasse	Bioactive compounds, polymers, and potent lignocellulosic material	(P. Sharma et al., 2020)
Tamarind processing	Peel, fiber, and seeds – about 50 to 70%	Phytochemicals, mainly polyphenols, fatty acids, and polysaccharides	(Martins et al., 2020)
Sugar cane industry	Sugarcane bagasse	Cellulose, hemicellulose, and lignin	(Pandey et al., 2000)
Cereal grinding	Cereal bran	Minerals, phenolic acids, amino acids, and vitamins	(Bartkiene et al., 2020)
Beer production	Spent grain	Oligosaccharides, for example arabino-xylooligosaccharides (prebiotic nutraceutical)	(Amorim et al., 2019; Steinmacher et al., 2012)
Tomato processing	Peels or mixture of peels, seeds, and a small amount of pulp	Carotenoids, mainly lycopene	(Pataro et al., 2020)
Vanilla extract processing	Bagasse of the pod	Water- and ethanol-insoluble aromatic compounds	(Baqueiro-Peña & Guerrero-Beltrán, 2017)
Tropical fruit processing	Seeds, peels, and leaves – up to 60%	Phenolic compounds, carotenoids, proteins, vitamins, or dietary fibers	(Cádiz-Gurrea et al., 2020)
Sardine processing	Waste from canning facility – about 20 to 75%	Proteins, peptides, amino acids, lipids (omega-3 polyunsaturated fatty acids, PUFAs), enzymes (pepsin, trypsin), vitamins (A, D, E) and biopolymer	(Melgosa et al., 2020)
Jabuticaba processing	Epicarp	Antioxidant compounds, tocopherols, anthocyanins, and ellagitannins	(Albuquerque, Pereira, et al., 2020)
Melon croá processing	Epicarp	Anthocyanins and tocopherols	(Albuquerque, Dias, et al., 2021)

PUFA: polyunsaturated fatty acids.

1.1.2. Value-added compounds

The bioactive compounds (e.g., antioxidants, polyphenols, tocopherols, carotenoids, and vitamins) naturally present in food are important for human nutrition (Galanakis, 2017; Hooper & Cassidy, 2006). Found in cereals, fruits, vegetables, grains,

and several other foods and their subproducts, these compounds are secondary plant metabolites (Verma & Shukla, 2015; Vuong, 2017) that can cause a significant impact in human and animal body functionality, with pharmacological or toxicological effects (M. Goyal & Ayeleso, 2018). The primary metabolites are responsible for the growth and development of the plant, e.g., proteins, lipids, and carbohydrates (Galanakis, 2017; Santos, 2015). On the other hand, and despite being considered as by-products of metabolic and biosynthetic pathways of those molecules, bioactive compounds exert significant functionality in plants. They exert, for example, protective support, acting as free radical scavengers; they are able to act as signalers, appealing to pollinating insects or seed dispersers; and can also provide defense, repelling insects, parasites or competing plants, among other diverse functions, through the many varieties of compounds (Khan et al., 2019; Vuong, 2017).

When these bioactive compounds are incorporated into the diet, by food or medicinal bases, they can provide benefits to human health, developing positive effects on body functionality. Studies have linked the consumption of foods rich in bioactive compounds with a reduction in the risk of developing chronic diseases such as cancer, diabetes, obesity, and cardiovascular diseases (Hooper & Cassidy, 2006; Khan et al., 2019). The beneficial health effects exhibited by these compounds are due to their ability to modulate metabolic processes, such as antioxidant activity, enzyme inhibition or induction, inhibition of receptor activities, and induction and inhibition of gene expression (Galanakis, 2017). In fact, the antioxidant activity is often highlighted among their functions (Backes et al., 2020; da Silva Veloso et al., 2020; López et al., 2019), once they protect the body's cells against oxidative damage, reducing the oxidative stress and preventing cancer, arteriosclerosis, and aging processes. These properties have been reported as highly suitable for preservative, fortifier, and stabilizer additives development (Galanakis, 2017).

Many different illnesses, such as cancer, cardiovascular and pulmonary diseases, neurological disorders, diabetes, arthritis, ageing process, and other neurological and endocrinological disorders, result from oxidative stress (OS). In turn, the OS is the result of an imbalance between the free radicals and the antioxidants on the metabolism, originated from environmental causes, inflammation processes, exposure to radiation, drugs, and others unfavorable conditions (M. Goyal & Ayeleso, 2018). In this case, when the antioxidant defense enzymes are overwhelmed, the nutrients with antioxidant capacity, found in natural food, are important to help the body combating these free radicals. Thus, when significant amounts of bioactive compounds are part of the diet regularly, they are capable of exerting antioxidant activities (Carocho & Ferreira, 2013; M. Goyal & Ayeleso, 2018).

There are several therapeutic properties and mechanisms of modulation of metabolic processes reported in the literature. In a recent review (Bhushan et al., 2021), the antiviral capacity of bioactive compounds focused on the COVID-19 management was highlighted. A two-way strategy to combat SARS Cov-2 infection using bioactive compounds as blockers has been reported: blocking S protein of the virus and blocking ACE2 receptor of the cell. In addition to nutraceutical properties, as previously measured, bioactive compounds are also known for their functional properties (antioxidant activity, solubility, absorption, coloring, stabilization, flavor, preserving, etc.). Natural pigments such as anthocyanins, carotenoids, and betalains are proposed as natural colorant additives in foods, being also linked to other benefits, such as antioxidant effects (Rodriguez-Amaya, 2019). Carotenoids, e.g., lycopene, are also used in cosmetic products, due to its photo-protection capacity, protecting the skin from the sun (Bin-Jumah et al., 2021). In another study, Rodriguez-Garcia et al. (2016) proposed the essential oil obtained from oregano as antimicrobial and antioxidant food additive. In the food industry, there is a growing interest in isolating these compounds from natural sources for further application in processed food, as alternatives to the use of synthetic additives (preservatives, nutritional additives, flavoring agents, coloring agents, texturizing agents, or miscellaneous additives).

In general, numerous studies have been reporting that a great variety of food wastes are rich sources of bioactive compounds, with great potential for recovery and application in food, cosmetic, and pharmaceutical industries (Vuong, 2017). Vitamin D_2, e.g., was recovered from the biological surplus remaining from the mushroom cultivation industry (Cardoso, Fernandes, Barreira, et al., 2021); phenolic acids and flavonoids were obtained from tomato crop remains (pruning and end-of-cycle plant materials) (Añibarro-Ortega et al., 2020), and organic acids, phenolic compounds, and high concentrations of anthocyanins were extracted from *Sicana odorifera* fruit epicarp (Albuquerque, Dias, et al., 2021). The recovery of value-added compounds from bioresidues and their applicability in food will be further discussed**.**

There are approximately more than 200,000 chemicals isolated and identified, considering primary and secondary metabolites, from higher plants worldwide (Campos, 2019) – such as proteins and amino acids, phenolic compounds, and other molecules with antioxidant activity, vitamins, dietary fiber and functional polysaccharides, minerals, fatty acids, enzymes, aromatic compounds, etc. – that can have added value by recovery from undervalued sources in agriculture and industry (Bonilla-Hermosa et al., 2014; Rico et al., 2020). Note that the recovery of these compounds from natural sources is normally affected by some factors as the matrix properties of the plant materials and the extraction method conditions (e.g., solvent, temperature, pressure, and time) (Ben Ayache et al.,

2021; Dias et al., 2020).

Bioactive compounds can be classified considering their solubility, polarity, and distribution in nature (M. Goyal & Ayeleso, 2018). They are also commonly classified based on the biosynthetic route, the structural features, the basis of clinical function related to their pharmacological effect, and the botanical approach considering their families (Vuong, 2017). This classification results in a vast and heterogeneous number of classes of these compounds, e.g., phenolic compounds, carotenoids, tocopherols, alkaloids, and vitamins (Verma & Shukla, 2015). More details are presented through the diagram in **Figure 2**.

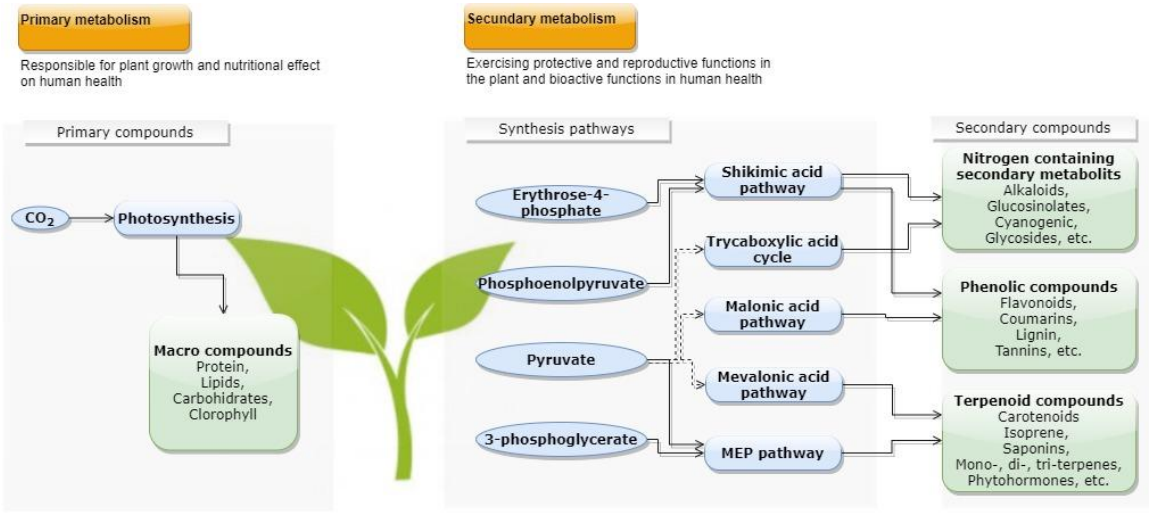

Figure 2. Simplified diagram of the synthesis pathways of secondary metabolites in plants and their groups of compounds. The different formation routes originate compounds with different characteristics and functionalities. Adapted from (Santos, 2015; Verma & Shukla, 2015; Vuong, 2017).

Phenolic compounds are one of the most widely found groups of secondary metabolites in plants (Tungmunnithum et al., 2018). They have a great structural diversity that, based on the number of constitutive carbon atoms conjugated with the structure of the basic phenolic skeleton, result in different subclasses: flavonoids, phenolic acids, stilbenes, lignans, tannins, and oxidized polyphenols (Ferreira et al., 2009; M. Goyal & Ayeleso, 2018). These compounds are final products of the shikimate and acetate pathways, and their structure comprises aromatic hydroxylated compounds, having one or more aromatic rings with one or more hydroxyl groups. They can range from relatively simple phenolic molecules to highly polymerized compounds, most commonly being conjugated to mono- and polysaccharides, associated with one or more phenolic groups, and can also be linked to methyl esters and esters (Campos, 2019; Ferreira et al., 2009).

Among the more than 8,000 known phenolic compounds, approximately 6,000 constitute the group of flavonoids (Campos, 2019; Tungmunnithum et al., 2018).

Flavonoid compounds are often stored in the plant linked to one or more sugars, being this form more stable than the free flavonoid (Campos, 2019). These compounds generally have a yellow to red color and, when added to food products, they contribute to the color and flavor of food, in addition to being able to prevent oxidation of fats and protect vitamins and enzymes (Campos, 2019; da Silva Veloso et al., 2020). Anthocyanins, on the other hand, present red, blue, and purple colors, with a structure consisting of two aromatic rings linked by a three carbon heterocyclic ring that contains oxygen (Rodriguez-Amaya, 2019), while tannins, or tannic acids, exert antimicrobial action and are responsible for the astringency of many fruits, containing in its structure a large number of hydroxyl or other functional groups (Campos, 2019).

In turn, alkaloids and glycol sides are the categories, pointed by Vuong (2017), which are currently attracting increasing attention for research regarding their recovery from industrial food bioresidues, once these natural nitrogen-containing organic compounds present great biological activities. For example, steroidal alkaloids were extracted from potato peel waste (Hossain et al., 2014); alkaloids were obtained from cocoa bean shell (Okiyama et al., 2018); glycosides were extracted from pineapple waste (Sepúlveda et al., 2018), and quercetin glycosides from onion solid waste (Nile et al., 2017). These groups of compounds, and others molecules, e.g., terpenoids and coumarins, were studied for their potential to protect against liver fibrosis (Ma et al., 2020). The terpenoids are generally insoluble in water and based on five carbon units (Verma & Shukla, 2015). Carotenoids are an example of terpenoid compounds (tetraterpenes) widely explored from plant material.

1.1.3. Green extraction methods

The extraction of bioactive compounds from natural sources is an extensive topic of discussion. On the one hand, the seek to increase yield, optimize the parameters of extraction processes, and minimize the influences that affect the extraction and degradation of target compounds, in addition to reducing costs and process times. On the other hand, the challenge remains of obtaining the best results through methods that do not impact the environment nor the consumers' health, reducing energy costs and using safe solvents.

The term green method is used to classify extraction methods that have certain advantages over conventional ones. While conventional methods generally require long extraction times to obtain greater performance and involve large amounts of solvent, which are sometimes toxic, alternative methods are more sustainable, using green solvents and more ecological techniques with high extraction yield and compounds preservation.

Extraction solvents

Green solvents, in general, have non-toxic characteristics, are biodegradable, and obtained from renewable sources. In the data book of green solvents (Wypych & Wypych, 2014), it is possible to find more than 300 green solvents, with detailed information about usage considerations, physical properties, health and safety issues, potential substitutes, and for which products the solvent is recommended.

Among the green solvents, water is a great option with greenness characteristic as non-toxicity to health and the environment, safety, bioavailability, and price. In addition, it is possible to tune the properties of water by changing the temperature: at high temperatures, coupled to high pressure to keep the water in a liquid state (subcritical condition), the dielectric constant is decreased and the penetration of water into the sample matrix is favored, along with the decrease of the surface tension and viscosity, and the improvement of the analyte diffusion and mass-transfer kinetics (Castro-Puyana et al., 2017). Another well-known solvent is ethanol, a cheap and renewable solvent, produced by the fermentation of biological material, recognized as non-toxic, although flammable, but a final purification step is required in some processes. The equilibrium constant of this alcohol is strongly influenced by temperature, as the extractability of the material increases with temperature (Baümler et al., 2016). It has been presenting great selectivity to extract oil (Baümler et al., 2016) and has been employed in many researches on the extraction of phenolic compounds, in hydroalcoholic solutions (see **Table 2**).

Recently, the large application of organic solvents such as benzene, toluene, xylene, methanol, and ethanol in many laboratories and industrial chemical processes has generated an environmental concern due to its high volatility, which contributes to global climatic changes, air pollution, and human health-related issues (Mohammad & Inamuddin, 2012). With this, new alternatives, such as supercritical fluids (SCFs), eutectic solvents (ESs), fluorous solvents, and ionic liquids (ILs), have been widely proposed (González-Miquel & Díaz, 2021; Jiang et al., 2019; Mohammad & Inamuddin, 2012).

The challenge of choosing the solvent involves considerations such as: being suitable for the method and efficient for the target compound and its matrix, economically viable, safe for health, and environmentally friendly. Some methods can help predict solvent performance through its physicochemical and thermodynamic properties. Mokashi et al. (2021) used a mathematical model equation to estimate the extraction efficiency of various solvents for the recovery of pyruvic acid. The model relates Hansen Solubility Parameters (HSPs) with distribution coefficient, where HSPs is an equation of solubility parameters with different types of energy including polar, diffusivity, and hydrogen bonding interactions. In another work (González-Miquel & Díaz, 2021), a review of computational methods for screening green solvents is presented. The quantum

chemistry (QC) methods based on continuum solvation models (CSM), used for solvent selection and design, and methods based on the Conductor-like Screening Model (COSMO) are discussed, which guides solvent selection from thermodynamic performance indicators.

Extraction methods

Principles

The solid–liquid extraction process has been used for many decades, from home use to prepare tonics to a popular way of obtaining essential oils and bioactive compounds. The process consists of extracting the target compound by mixing the solid raw material with a solvent for a certain time. Thereafter, the solid–liquid phases are separated and the extract is purified. This technique is the principle of traditional extractions and many new enhanced extraction methods (Chemat & Strube, 2015; Wang & Weller, 2006). **Figure 3** shows a simplified scheme of the main extraction methods employed in natural matrices.

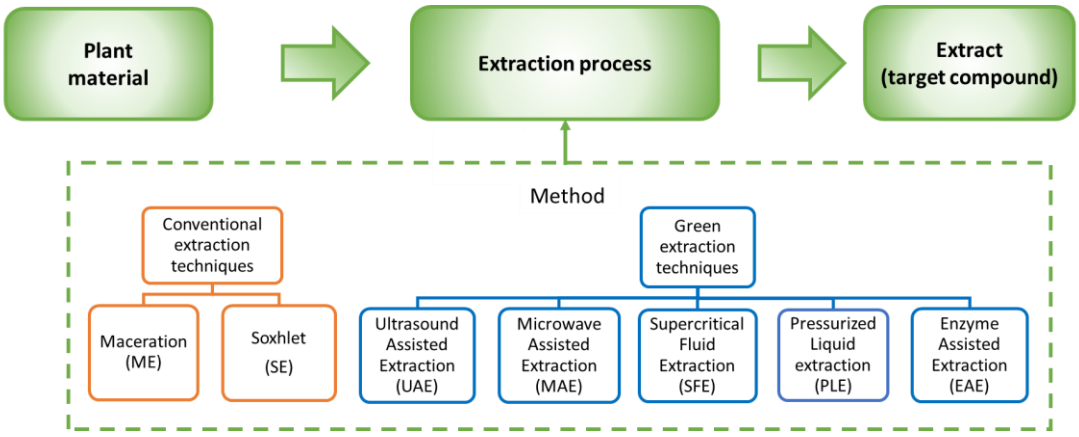

Figure 3. Simplified scheme of the extraction techniques employed to obtain target compounds from natural matrices.

The dynamic of extraction is influenced by parameters such as the choice of the solvent, pH of the medium, solid–liquid ratio, process temperature, and contact area between the solid and the solvent. In turn, these variables affect the energy consumption, the quantity of solvent used and its recovery capacity, the extraction yield, and other factors, which are increasingly studied through the optimization of parameters and comparison between techniques for different target compounds and their matrix (Chemat & Strube, 2015; Leichtweis et al., 2019).

Main methods explored in the literature

Maceration (ME) consists of mixing the plant material with an appropriate solvent

in a vessel and staying in contact for a certain (and usually long) time (Albuquerque et al., 2018). This process can be assisted by heating and/or stirring, e.g., thermostatic water bath, electro-magnetic stirring, and bain-marie with agitation. Generally, the solid matrix is ground to increase the surface area. It involves isolation and purification steps, usually by filtration and solvent evaporation (Vuong, 2017). Advantages and limitations: it is a simple and low-cost method, but requires long times of extraction and high quantities of solvent (Rasul, 2018).

Soxhlet (SE) was originally developed for the extraction of lipids from a solid material, but it has also been adapted for bioactive compounds from various natural sources. In the Soxhlet equipment, the plant material is placed in a thimble of the apparatus and accoupled in a distillation flask containing the solvent, where the sample is washed by the solvent through intermittent reflux. When the solvent heats and condenses to an overflow level, a siphon system arrangement drains out the solution (solvent plus extracted solute) into the flask. There, the solution is heated until the solvent vaporizes and the process runs repeatedly for exhaustive extraction (Aravind et al., 2021; Lama-Muñoz et al., 2020). Advantages and limitations: it is a simple technique that demands less quantity of solvent, when compared to maceration, due to solvent recirculation. However, the extraction time is long and heat-labile compounds can be affected (Rasul, 2018).

Ultrasound-assisted extraction (UAE) is based on the dispersion of sound waves in the liquid medium (solvent) that contains the sample to be extracted. Upon the waves reaching sufficient intensity of successive compression and distension in the medium, cavitation bubbles are formed. These bubbles, when collapsing, cause the rupture of cellular structures, facilitating the penetration of the solvent and increasing the mass transfer (Picot-Allain et al., 2021). Advantages and limitations: it is an inexpensive and simple method, capable of improving extraction yield and faster kinetics (Wang & Weller, 2006), in addition to less consumption of energy, solvent, and extraction time. Nevertheless, the heat generated can affect heat-labile compounds and the reproducibility can be reduced by the aging of the instrument (Picot-Allain et al., 2021).

Microwave-assisted extraction (MAE) is a method that uses microwave radiation, i.e., non-ionizing electromagnetic waves (300 MHz to 300 GHz). Through this technique, an electric field is generated by the simultaneous effects of ionic conduction and dipolar rotation. The larger the dielectric constant of the solvent, the higher the heating and dipolar rotation. Through this mechanism, pressure is generated inside the plant cell and its consequent rupture, exposing the cell and then facilitating solvent penetration (Nabet et al., 2019). Advantages and limitations: it is a low-cost equipment, which requires reduced extraction time and quantity of solvent, with improved extraction yield. It also

allows processing without using solvent. On the other hand, the heat generated can affect heat-labile compounds and it loses efficiency in scaling up (Picot-Allain et al., 2021; Wang & Weller, 2006).

Supercritical fluid extraction (SFE) has as principle the use of the solvent fluid in its supercritical state. For this, the temperature and pressure parameters of the process are controlled to achieve the conditions in which the fluid is between the gas and liquid states, presenting similar liquid density and gas viscosity (Vuong, 2017). Carbon dioxide (CO_2) is an attractive solvent largely used in this method (Sun et al., 2021). The supercritical solvent flows through the raw material, placed in an extractor vessel, transports the dissolved solute to the separator, and then can be regenerated and returned to the process (Vuong, 2017). Advantages and limitations: high selectivity for non-polar compounds, non-degradation of heat sensitive compounds, and non-residues of toxic solvents. However, it demands high costs and complex operation and training (Picot-Allain et al., 2021; Sun et al., 2021).

Pressurized liquid extraction (PLE) uses pressurized solvents at high temperatures and pressures, but without reaching the critical point values. Through this mechanism, it is possible to ensure rapid extraction rate, by decreasing the dielectric constant of the solvent (Lama-Muñoz et al., 2020). Advantages and limitations: the use of pressure allows a faster extraction, with less solvents and higher yields, but, the high temperatures can damage thermolabile compounds (Vuong, 2017).

Enzyme-assisted extraction (EAE) utilizes enzymes, as cellulase, xylanase, and pectinase, for example, capable to degrade the cell wall structure, facilitating the extraction of many bioactive compounds, to which the access is often hindered because they are linked to the constituents of the cell wall (Gligor et al., 2019). Advantages and limitations: it presents high selectivity and improves yield, however, the enzymes are expensive and demand rigorous control of medium pH and temperature for optimal enzyme action (Picot-Allain et al., 2021).

Extraction parameters

Despite the summarized contextualization of the principles of the most common extraction methods, there is a great deal of processes and combinations of these in the literature. **Table 2** presents some recent extraction studies and the used parameters, as well as the conditions optimized and/or highlighted by the authors.

There are several factors that can influence the success and yield of the extraction. Parameters as solvent type, liquid–solid ratio (LSR), particle size, temperature, time of exposure, power, and pressure, are commonly studied. To outline the best conditions of the extraction procedure for different plant materials and target compounds, optimization

studies are very important. For this, the most relevant independent parameters are evaluated, in a predetermined range of values, combined (or not) with the comparison of different extraction methods.

For instance, anthocyanin-rich extracts were obtained in optimization and comparison studies of heat- and ultrasound-assisted extraction techniques, using different fruits as sources: *Arbutus unedo* L. fruits (López et al., 2018), *Prunus spinosa* L. fruit epicarp (Leichtweis et al., 2019), and *Ficus carica* L. fruit peel (Backes et al., 2018). These works followed the same evaluation structure, however, the alternative UAE method proved to be more efficient for *F. carica* and *P. spinosa*, while for *A. unedo* the conventional method (heat-assisted extraction) was the most effective in the evaluated responses. These results demonstrate the influence of the matrix on the method's performance. In another study (Lama-Muñoz et al., 2020), the conventional method (Soxhlet) also stood out when compared to the emerging technology pressurized liquid extraction (PLE), for the extraction of phenolic compounds and mannitol from olive leaves. However, although the Soxhlet extraction revealed a better performance in most of the analyzed variables, the authors highlighted shorter extraction times (5 min vs. 4 h), and lower solvents consumption and energy costs as advantages achieved by PLE method, which is important to be considered in industrial scale application.

Reduction of extraction time and energy consumption was also reported by Jesus et al. (2019), by using the microwave-assisted extraction method in comparison to the heat-assisted one. In addition to these advantages, through the alternative method, the authors achieved higher yields and concentrations of ellagic acid. The conventional maceration technique was compared to the heat and ultrasound assisted extraction processes to obtain polyphenols from *Thymus serpyllum* L. herb (Jovanović et al., 2017). In this work, the UAE was the most efficient, followed by the HAE (heat-assisted extraction) and, last, the ME. The authors discussed the influence of the process time and, in the preliminary screening, the high time of exposure caused a slight decline in the polyphenol content. On the other hand, in the results obtained in the optimization study (running in low temperatures), time did not significantly affect the extraction, with the relevant factors being the particle size, solid-to-solvent ratio, solvent type, and extraction procedure.

Other studies reported enzymes being used as pretreatment for supercritical carbon dioxide extraction of lycopene from tomato (Lenucci et al., 2015). The use of plant cell wall glycosidases led to a significant increase in the concentrations of lycopene and total lipids in the matrices, when compared to the control. On the other hand, Jiang et al. (2019) defended the use of deep eutectic solvents (DESs) as an alternative to organic solvents for more efficient and green extraction. DESs are a mixture of two or more

hydrogen bond acceptor and donor, bound together by strong intermolecular interactions, which are being proved as new high-efficiency extraction solvents. In this study, the authors optimized an efficient DESs extraction method to different types of bioactive alkaloids. Moreover, Babova et al. (2016) combined the supercritical (SC) and subcritical (SubC) CO_2 extraction methods in a multistage process to improve the extraction of antioxidant compounds (anthocyanins, other flavonoids, other phenolics, and proanthocyanidins) from bilberry (*Vaccinium myrtillus* L.). This methodology proved to be efficient showing great performance of selectivity through the stepwise extraction procedure. Other high-pressure methods (SFE and PLE) were compared to low-pressure methods (SE and UAE) to obtain an ethanolic extract with high antioxidant potential from black pepper (Andrade et al., 2017). The authors pointed out that each extraction procedure presented particular characteristics, but, in general, SE and UAE extracts presented higher yields when compared to SFE method; PLE has shown good results to the extraction of bioactive compounds; and SFE showed effectiveness for the recovery of extracts rich in piperine, providing a high value-added and solvent-free extract.

Table 2. Optimized green methods for the extraction of natural compounds

Method	Source	Compound	Solvent	Extraction Conditions	Reference
Maceration extraction (ME)	*Eucalyptus globulus* L. leaves	Phenolic compounds (mostly flavonoids)	Ethanol	LSR 20 L/Kg, 2 Hz, 50 °C, 225 min, 56% ethanol	(Gullón et al., 2017)
	Arbutus unedo L. fruits	Anthocyanins	Ethanol (pH 4, 0.05% of hydrochloric acid)	LSR 5–40 L/Kg, 8.33 Hz, 5 min, 90 °C, 80% ethanol	(López et al., 2018)
Microwave-assisted extraction (MAE)	Lamiaceae (*Origanum glandulosum* Desf.)	Phenolic compounds (mostly gallocatechin)	Ethanol	LSR 15 L/Kg, 850 W/2455 MHz, 42 °C, 2 min, 0% ethanol	(Nabet et al., 2019)
	Lamiaceae (*Thymus fontanesii* Boiss. et Reut.)	Phenolic compounds (mostly rosmarinic acid)	Ethanol	LSR 15 L/Kg, 850 W/2.455 × 10^9 Hz, 150 °C, 9.5 min, 50% ethanol	
	Coriolus versicolor (L. ex Fr.) Quél. mushroom	Phenolic compounds	Ethanol	LSR 10 L/Kg, 125 W, 3.8 min, 40% ethanol	(Maeng et al., 2017)
	Vine pruning residue	Polyphenols (mostly ellagic acid and apigenin)	Ethanol	LSR 40 L/Kg, 120 °C, 5 min, 60% ethanol	(Jesus et al., 2019)
	Morus nigra L. fruits	Phenolic compounds (mostly anthocyanins)	Ethanol	LSR 50 L/Kg, 500 W, 35 °C, 10 min, 35% ethanol	(Koyu et al., 2018)
	Arbutus unedo L. fruits	Flavonoids	Ethanol	LSR 20 L/Kg, 400 W, 120 °C, 1.5 min, 0% ethanol	(Albuquerque et al., 2018)
Ultrasound assisted extraction (UAE)	Goji berry fruit	Carbohydrates and phenolic compounds	Water	LSR 28 L/Kg, 283 W, 64.29 °C, 39.7 min	(Skenderidis et al., 2017)
	Thymus serpyllum L. herb	Phenolic compounds	Ethanol	LSR 30 Kg/L, 25 °C, 15 min, 50% ethanol (750 W output with a 2 × 10^4 Hz converter)	(Jovanović et al., 2017)
	Prunus spinosa L. fruit epicarp	Anthocyanins	Ethanol (pH 3, citric acid)	LSR 20 L/Kg, 400 W, 5 min, 47.98% ethanol	(Leichtweis et al., 2019)
	Ficus carica L. peel	Anthocyanins	Ethanol	LSR 6.66 L/Kg, 310 W, 21 min, 100% ethanol	(Backes et al., 2018)

Method	Source	Compound	Solvent	Extraction Conditions	Reference
	Tarchonanthus camphoratus L. leaves	Parthenolide	Ethanol	LSR 20.4 L/Kg, 38.8 °C, 50 min, 100% ethanol	(Siddiqui et al., 2021)
	Agro-industrial acerola (*Malpighia emarginata* DC) residue	Anthocyanins, other flavonoids, other phenolic compounds, carotenoids, and ascorbic acid	Ethanol (pH 2, hydrochloric acid 2 M)	LSR 8.66 L/Kg, 50 kHz and 250 VA, 30 °C, 49.30 min, 46.49% ethanol	(Rezende et al., 2017)
Subcritical fluid extraction (SubFE)	Black mulberry, wall germander, wild geranium, and comfrey	Phenolic compounds (mostly gallic acid)	Water	LSR 40 L/Kg, 160 °C, 1×10^6 Pa, 3 Hz, 30 min	(Nastić et al., 2018)
	Chestnut shells	Phenolic antioxidants (mostly caffeoylquinic acid isomers)	Water	LSR 10 L/Kg, 220 °C, 4×10^6 Pa, 30 min	(Pinto et al., 2021)
Supercritical fluid extraction (SFE)	Canola seeds	Tocopherol-rich oil	Carbon dioxide (CO_2)	70 °C, 8×10^6 Pa, 30 min, 1.67×10^{-5} L/s	(Sun et al., 2021)
	Raspberry seeds	Oil	Carbon dioxide (CO_2)	40 °C, 3.5×10^7 Pa, 240 min, 1.11×10^{-4} Kg/s	(Pavlić et al., 2020)
	Apple seeds	Fatty acids rich oil (mostly linoleic acid)	Carbon dioxide (CO_2)	40 °C, 4×10^6 Pa, 140 min, 2.78×10^{-4} L/s	(Ferrentino et al., 2020)
Pressurized liquid extraction (PLE)	Olive leaves	Phenolic compounds and mannitol	Ethanol	LSR 3×10^{-3} Kg dw sample into 2.2×10^{-2} L stainless steel cells, 190 °C, 5 min, 60% ethanol	(Lama-Muñoz et al., 2020)
	Pomegranate peels	Condensed tannins, anthocyanins, other flavonoids, other phenolic compounds	Ethanol	LSR 60 L/Kg, 4.92×10^8 Pa, 20–38 °C, 30 min, 37% ethanol	(Alexandre et al., 2017)
	Truffles *Tuber melanosporum* Vittad.	Sterols and *beta*-glucans	Ethanol and water	5×10^{-4} Kg, 1.67×10^7 Pa, 180 °C, 30 min, 100% ethanol or water	(Tejedor-Calvo et al., 2020)

LSR: liquid–solid ratio.

1.1.4. Value-added compounds in the development of innovative food products

The recovery and valorization of compounds through green methodologies and from bioresidues, adding value to what would be wasted, corroborates the principles of a circular economy (Usmani et al., 2021). The circular economy is the concept related to the reduction, reuse, and recycling of food losses and wastes along the food supply chain (de Oliveira et al., 2021). The use of bioresidues to obtain value-added compounds is a management strategy for waste and food loss, which can contribute to reducing its environmental impacts, minimizing the use of virgin materials, in addition to promoting opportunities for savings, as innovation of products and methods, competitiveness and productivity (de Oliveira et al., 2021).

Innovation in the food industry through the use of biocompounds not only adds to the circular economy sustainability concept but is also an opportunity to attend consumers' expectations. In fact, consumers' concern for safer and healthier foods is increasing, and food industry is under pressure to offer healthy, convenient, and ready-to-eat foods, able to meet daily nutritional needs, provide pleasure and satiety, and attend to consumers' expectations and safety issues (Pereira et al., 2020; Subiria-Cueto et al., 2021). In this scenario, the replacement of synthetic compounds, generally associated with toxicity and allergenic problems, with healthy natural alternatives is increasingly evident (Pereira et al., 2020). Alongside, the enrichment of products by using compounds with nutritional value is also a growing tendency. The use of agro-industrial residues, rich in bioactive compounds, has been the focus of studies that propose the use of these by-products in the formulation of functional foods (Subiria-Cueto et al., 2021). As synthetized in **Table 3**, value-added compounds were recovered from industrial processes wastewater, from commercially unexplored fruits, and industrial processing by-products, and their applicability was verified. Below, some relevant works available in the literature on the valorization of these compounds and practical applications in foodstuff are discussed.

Table 3. Value-added compounds recovery from food waste and its applicability.

Compound(s) of interest	Source(s)	Benefit for health	Applicability	Reference
Vitamin D_2	Surplus mushrooms	Antitumoral	Food industry	(Cardoso, Fernandes, Barreira, et al., 2021)
Anthocyanins	Fig peel and blackthorn fruit epicarp	Antioxidant and antimicrobial activities	Natural purple colorant in pastry products	(Backes et al., 2020)
Dietary fiber	Pumpkin seeds and rinds	Nutritional value	High fiber bakery product	(Nyam et al., 2013)
Phenolic compounds	Peel of camu-camu fruit	Antimicrobial potential	Yogurt fortification	(Conceição et al., 2020)
Anthocyanins	Strawberry tree fruit	Antioxidant and antifungal activities	Natural colorant in wafers	(López et al., 2019)
Phenols	Olive mill wastewater	UVA and UVB filter potential	UV booster in cosmetics	(Galanakis et al., 2018)
Sugar	Coffee silverskin and spent coffee grounds	n.a. [1]	Ethanol production by fermentation	(Mussatto et al., 2012)
Phenolic acids, hydrolysable tannins, flavonoids, and anthocyanins	Pomegranate epicarp	Antioxidant and antibacterial activities	Natural colorant and antioxidant in pastry products	(da Silva Veloso et al., 2020)
Phenolic and carotenoid compounds	Pumpkin peel	Antioxidant activity	Retard canola oil oxidation	(Salami et al., 2020)
Anthocyanins	Jabuticaba epicarp	Antioxidant, antimicrobial, antitumor and anti-inflammatory activities	Natural colorant in macarons	(Albuquerque, Pinela, et al., 2020)

[1] Not Applicable.

For example, potato peel wastes were valorized as a source of protein and dietary fiber through their addition to cake (Ben Jeddou et al., 2017). The protein and soluble and insoluble fiber contents of the potato peel powders were about 15%, 19%, and 10%, respectively. The cakes enriched with 10% of potato peel flour achieved a percentage of protein improvement of ~17%. Regarding dietary fiber, the soluble fiber content increased from 3.3% in control cake to almost 5% in enriched cakes, and the insoluble fiber content significantly increased from 15.9% (control) to ~22% for cakes with potato peel flour. In addition to improving the nutritional value, the authors reported technological effects: the incorporation of potato peel powder at 5% increased the dough strength and elasticity-to-extensibility ratio.

Grape seeds and apple peels were also valorized as sources of natural antioxidants, especially phenolic compounds, through the fortification of yogurts with these bioresidues powders (Brahmi et al., 2021). In this line, the authors also optimized the extraction

conditions, using green solvents, to obtain extracts with high phenolic compounds content from these by-products. In another study, Chen et al. (2020) discussed the applications of grape seed extract in food industry as preservative. They proposed the use of the extract as raw material to develop healthy foods as it improves the nutritional value and promotes benefits such as enhancing the body immunity, prevent hyperlipidemia, hypertension, and diabetes; as natural antioxidant and preservative in food, due to its antioxidant and antimicrobial activity; as food film/coating in food packaging, to improve certain functional properties; and as substitute of nitrite and nitrate in meat products, and sulfur dioxide (SO_2) and animal protein in wine making.

Moreover, the ethanolic extracts of apple peels were fractionated and their use for inhibition of fish oil oxidation was studied using the thiobarbituric acid reactive substances (TBARS) assay (Sekhon-Loodu et al., 2013). The crude and fractionated extracts presented inhibitory effect on fish oil oxidation, where the greatest antioxidant capacity was verified with the fractions containing quercetin glycosides and epicatechin in combination with other polyphenols, such as phloridzin and cyanidin-3-galactoside. The apple peel was also successfully used as prebiotic in yoghurt (Ahmad et al., 2020). Through this study, a probiotic yoghurt fortified with apple peel polyphenol extract was obtained, which can act as natural high-quality antioxidant and bioactive compound.

In another study (da Rosa et al., 2020), a green extraction method was used to obtain phenolic compound-rich extracts from olive leaves. The extracts were obtained by solvent-free MAE and presented high antioxidant activity, so they were proposed as having a great potential as functional ingredients for food packaging. In fact, the developed biodegradable films based on carrageenan containing olive leaf extract showed good barrier and mechanical properties, and the total phenolic compounds and antioxidant activity of the films significantly increased with increased concentrations of the olive leaf extract.

Promising antioxidant extracts were also obtained from the peel of eggplant. Horincar et al. (2019) used the green method of UAE to obtain a methanolic extract of this by-product. Six anthocyanins were identified in the extract: delphinidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-rutinoside-5-glucoside, malvidin-3-rutinoside-5-glucoside, and petunidin-3-rutinoside. In a subsequent study (Horincar, Enachi, Barbu, et al., 2020), the extract was microencapsulated with whey protein and acacia gum, resulting in a purple colored powder. The addition of the eggplant peel powder in a pastry cream allowed a significant increase of total phenolic content and antioxidant activity, which were rather stable over 72 h of storage under refrigeration conditions. The ethanolic extract of eggplant peel was, then, proposed as supplement in beer (Horincar, Enachi, Bolea, et al., 2020), with the supplemented beer presenting high

functional potential and good sensory characteristics, being stable without the incorporation of artificial preservatives.

Anthocyanin-rich extracts from blackthorn epicarp (Leichtweis et al., 2019) and fig peel (Backes et al., 2018), obtained by a green optimized extraction method (previously discussed), were proposed as alternative natural colorants. The extracts were incorporated in confectionery products, more specifically "beijinhos" (a typical Brazilian pastry) and doughnut icing. The obtained purple colorant extracts conferred attractive color to the products, improved the texture properties, and significantly increased the antioxidant and antimicrobial activities. In fact, anthocyanins are widely found in many fruits. As reviewed by Albuquerque, Oliveira, et al. (2021), fruits and their bioresidues are an excellent source of natural compounds, including a wide range of coloring, in addition to bioactive properties and with great potential to be implemented in the food industry as alternative to the use of synthetic additives. Moreover, the bioresidues from food industry of *Morus nigra* L. and *Rubus fruticosus* L., for not presenting adequate size or properties to be marketed, were also studied as sources of anthocyanins (Vega et al., 2021). The juices from these fruits were used in the preparation of solid colorants using the spray-drying technique, which resulted in colorants with a great and stable coloring capacity over time and safe for application in the food industry. In another study (Sampaio et al., 2021), an anthocyanin-rich extract was obtained from purple and red potatoes and evaluated as natural colorant in a soft drink formulation in comparison with the commercial colorant E163. The extracts showed suitable profiles in the sensory and shelf-life assessments, with high color stability during a 30-day shelf-life. Despite their multiple health benefits, some of these fruits are not used for consumption for not presenting the suitable size or properties to be included in the market, constituting a food industry residue.

Furthermore, as alternative for the use of synthetic additives in food industry, sage (*Salvia officinalis* L.) and basil (*Ocimum basilicum* L.) were exploited for their preservative purposes (Ueda et al., 2021). For that, extracts were obtained and incorporated into yogurt. The results were very satisfactory, with the extracts presenting antioxidant and antimicrobial activity, without changing the physicochemical and nutritional characteristics of the yogurts and the growth of lactic acid bacteria.

However, there is a wide range of sources to be explored and valued. In the literature, several biowastes were characterized and identified as having great potential for the recovery of value-added compounds, which could be applied in the food industry. Grape (*Vitis vinifera* L. var. Albariño) and mulberry (*Morus nigra* L.) seed pomace was characterized and the first presented high contents of organic acids and phenolic compounds, mainly catechins, while the mulberry seeds revealed to be rich in tocopherols and ellagic acid derivatives. The extracts containing these compounds showed antioxidant

and antimicrobial activity and no cytotoxicity on PLP2 cells (a primary culture of porcine liver non-tumor cells), being their use proposed as natural preservative in the food industry. The epicarp of the eggplant fruit (*Solanum melongena* L.) was highlighted by the authors (Silva et al., 2021) as a potential natural source of coloring compounds for food application, once it is rich in anthocyanins. Cereal by-products from the flour milling industry, more specifically wheat germ, maize bran–germ mixture, rye bran, and wheat bran, were reported (Cardoso, Fernandes, Pinela, et al., 2021) as underexploited alternative sources of nutrients and bioactive compound, such as protein and vitamin E.

1.1.5. Conclusions

Bioresidues from food industry have a great potential for the recovery of many value-added compounds. In the inedible parts as seeds, peel, and bagasse, as well as edible but rejected raw materials, many nutritional and bioactive compounds with high antioxidant capacity have been identified. The valorization of by-products and bioresidues, and the recovery of these compounds through green and environmentally friendly methods goes towards a circular economy. In the literature, several reports of the successful application of compounds obtained from food waste can be increasingly found. However, the transition to a circular food system with an efficient use of resources and food distribution is still a long challenge. There is a huge quantity of residues and unexploited plants to be characterized and valorized, and extraction processes to be optimized, leading to energy, solvent, and time reduction, among other relevant parameters. Studies on the stability of the recovered compounds, their stabilization through innovative techniques, and application in different matrices, as well as the scale-up process from laboratory to industrial level are also of great importance and increasingly needed. In general, this review aimed to contribute with new perspectives on underexploited and wasted biomaterials, aiming at the sustainability in industrial processes with the consequent increase in the use of natural compounds compared to artificial ones, meeting the emerging expectations of consumers, and promoting a circular food economy.

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1.1.6. References

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1.2. Sustainable approach of valorizing pumpkin fruit and bioproducts within the circular economy context

A Sustainable approach of valorizing pumpkin fruit and bioproducts within the circular economy context

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Abstract: *Cucurbita* spp. were reviewed to enhance both its pulp and by-products for use in a circular economy context. The discussion focused on the varieties of greatest economic relevance; however, aspects of fruit cultivation were reported from historical facts that help to understand the genetic diversity among the various cultivars, as well as the genetic heterogeneity at an intrapopulation level due to cross-fertility between species. Adaptability to various climate and soil conditions makes this fruit very promising in the face of climate change, but the best cultivation conditions and obtaining of the varieties of interest were also discussed. The nutritional and chemical composition of pumpkin pulp and its by-products (seeds and peels) were also highlighted, with emphasis on its essential nutrients and the variability of chemical composition considering species, country, and cultivation method. Subsequently, its health benefits were associated with a comprehensive discussion of the bioactive effects demonstrated by this fruit in the literature, as well as its applications in different types of products, such as food, cosmetics, and textiles, with promising results. Finally, high-pressure processing technologies and pulsed electric fields have been explored as innovative methods to expand the nutritional and functional properties of pumpkin products.

Keywords: *Cucurbita pepo*; *C. maxima*; *C. moschata*; Pumpkin valorization; Sustainability; Nutritional composition; By-product applications; Non-thermal processing

1.2.1. Agronomic aspects

The genus Cucurbita is an assemblage of 20-27 species of American origin (Castellanos-Morales et al., 2018; Yoo et al., 2023). It belongs to squash family (e.g. *Cucurbitaceae*) and consists of a group of varieties also known as summer and winter squash, gourd, zucchini, courgetti and vegetable marrow with fruit that are consumed throughout the world (Paris, 2001). The main domesticated and commercially cultivated species of the genus include *Cucurbita pepo* L., *C. maxima* Duch., *C. moschata* Duch., *C. argyrosperma* Huber, and *C. ficifolia* Bouché, all of which showing great differences not only in plant morphology and growth habit but also in fruit visual parameters, such as shape, size, flesh and rind color etc. (Rajasree et al., 2016). Moreover, the differences in visual appearance of fruit are also accompanied by significant differences in the chemical composition and bioactive profile of the edible parts of plants, mostly the fruit and seeds (Tzortzakis et al., 2018).

Among cucurbits, *Cucurbita* is considered a genus with considerable genetic variability in terms of plant growth habit, fruit morphology and disease resistance as well as nutritional and phytochemical profile of fruit, which is mainly attributed to its complex domestication trajectories, the selective breeding that enforced genetic variability ancient allotetraploidization between the diverged diploid progenitors *C. maxima* and *C. moschata* (Chomicki et al., 2020; H. Sun et al., 2017; UPOV, 2007). Adding to the complex domestication and evolutionary pathways, the genetic variability in *Cucurbita* species was increased over the long cultivation history, enabling farmers' selection and introduction of cultivars with unique traits (Yoo et al., 2023). The genus presents a pre-Holocene distribution, originally in the form of bitter fruits (Kistler et al., 2015), while its domestication took place in northern South America and Central America around 10,000 B.P. through the use of *Lagenaria* sp. and wild cucurbits fruit as vessels and cooking and drinking containers, thus being included among the earliest crop domestication records (Domic et al., 2024). Later on, the domestication of *C. moschata* was evidenced (approximately 4,000 B.P.) followed by *C. pepo (*approximately 2,000 B.P.) both of the species being used for their edible fruit (Erickson et al., 2005; Piperno, 2009). Archaeological evidence shows that the relationships between wild and domesticated species are not well elucidated (Kates et al., 2017), while wild cucurbits were utilized for very long before domestication took place involving the selection of genotypes based on fruit quality traits e.g. fruit size and taste (Chomicki et al., 2020; Piperno, 2009; Savage et al., 2015).

The genetic diversity in cultivated species has been widely studied, using various approaches and marker systems, allowing for species differentiation and estimations of phylogenies between and within *Cucurbita* species (Formisano et al., 2012; K Kaźmińska

et al., 2016; Wang et al., 2021; Yoo et al., 2023; Zhu et al., 2021), yet the relationships between cultivated and wild species remain partly elucidated (Zhu et al., 2021). In recent years, as the genomic resources become more available they provide valuable insights into the genetics of important traits, such as growth habit, fruit quality and disease resistance, thus providing novel tools to be introduced into breeding activities aimed at cucurbit improvement of yield and fruit quality parameters (He et al., 2024; Hernandez et al., 2020, 2023; Karolina Kaźmińska et al., 2018; Montero-Pau et al., 2017; Wu et al., 2019; Xanthopoulou et al., 2019; Zhong et al., 2017).

Among the cultivated cucurbits, three of them are of economic interest and are currently commercially cultivated throughout the world, e.g. *C. maxima*, *C. moschata* and *C. pepo*, owing to their use either for consumption, in the form of mature or immature fruit, seeds and flowers, or for decorational and industrial purposes (Ferriol & Picó, 2020).

The cultivated *Cucurbita* species share common plant growth requirements, while they are characterized by wide adaptability to soil and climatic conditions which make them good candidate crops to address the negative impact of climate change on vegetable crops (Salehi et al., 2019). Considering that the progenitors of the species are native to Latin America, cultivation of cucurbits should takes place in warm (air temperatures between 20 and 35 °C and soil temperatures above 16 °C) and humid climates with no frost incidences (Singh, 1990). Plants can be grown in various soils, although they prefer fertile ones with good drainage; the soil pH should be within the range of 6.0 to 6.5, although slight variations do significantly impact plant growth and development (Buwalda & Freeman, 1986). Plant propagation is performed with direct sowing in the soil, although in some occasions, e.g. earliness of production, untimely frosts or low temperatures late in the growing season, seedlings can firstly be prepared in sowing trays and then transplanted to the soil (Salehi et al., 2019). Plant density varies among the cropping systems (e.g. field vs greenhouse cultivation), the growth habit of plants (erect or prostrate), as well as among the various parts of the world, while it may affect the fruit size depending on market needs; therefore, in field conditions lower plant densities are usually considered compared to greenhouse conditions (e.g. 2,000-4,000 plants/ha and 20,000 – 30,000 plants/ha, respectively) (Bannayan et al., 2017). Crop rotation with other crops is suggested to reduce the risk of soil-borne pathogens by avoiding the successive cultivation of pumpkins or other Cucurbitaceae species in the same land for consecutive years (Napier, 2009). In terms of irrigation, the regular application of water is essential to ensure high productivity and fruit quality, since plants are susceptible to water deficit conditions with severe implications on plant growth, fruit setting and eventually on crop yield (Yavuz et al., 2015). The required amounts of water range between 4,000 and 8,000 m^3/ha, depending on the growing conditions and the genotype (Napier, 2009). Fertilizer

inputs usually consist of 150 kg/ha of N, 95 kg/ha of P and 80 kg/ha of K, although these amounts vary depending on the fertilization method (soil application or fertigation), the expected yield and the quality of soil (Salehi et al., 2019; Swiader et al., 1994).

The edible parts of the fruit of *Cucurbita* species are rich in fibers, carotenoids, polyphenols, tocopherols, minerals, vitamins, whereas they contain low amounts of fat and present low glycemic index, owing to their high pectin content (Fissore et al., 2007; Kostecka-Gugała et al., 2020; Zdunić et al., 2016). However, a great variability in the chemical profile and the bioactive compounds content should be expected due to the high impact of genetic background, growing conditions, pre-harvest and post-harvest practices, as well as the diverse domestication pathways (Chomicki et al., 2020; Tzortzakis et al., 2018). Therefore, apart from genetic diversity among the various cultivars, the cultivated species also present considerable genetic heterogeneity at the intra-populational level, due to out-crossing cross-fertility among the species, as for example is the case of *C. moschata* with *C. pepo* and *C. maxima* (H. Sun et al., 2017).

In terms of fostering genetic variation through breeding activities, *C. moschata* is highly appreciated as a valuable genetic pool for selecting genotypes resistant to various biotic and abiotic stressors, whereas *C. maxima* has been suggested for its fruit quality traits (Darrudi et al., 2018). On the other hand, *C. pepo* is characterized by high variability in fruit morphology, and is positioned among the most polymorphic species in the plant kingdom (Sajid et al., 2022). Given the economic importance of *Cucurbita* species, an increasing number of studies focus on estimation and maintenance of genetic diversity as well as development of genomic resources to be directed towards advancing breeding efficiency. In this context, as genome-wide markers become available they will be progressively integrated into various breeding approaches, including those of marker-assisted selection and marker-assisted breeding as well as breeding by design, thus offering possibilities of enhancing crop improvement potential by optimizing the use of available genetic resources (Cobb et al., 2019; Peleman & Van Der Voort, 2003).

Given the economic importance of cucurbits, there is increasing interest on characterizing and filling the knowledge gap in the existing genetic diversity within each species aiming to further explore their potential valorization in breeding programs (Yu et al., 2023).

1.2.2. Nutritional aspects of fruit pulp

The nutritional value of plants and vegetables can be influenced by several factors, including the species and variety, the growing environment, the agricultural practices employed, and even the processing and storage methods (Al-Ghamdi et al., 2020; Amin et al., 2019; Bannayan et al., 2017; Pereira et al., 2020). **Table 4** presents the nutritional

composition of pumpkin pulp from different species (e.g. *C. pepo*, *C. moschata*, and *C. maxima*), varieties and countries of cultivation, based on data obtained from different studies, highlighting relevant differences between their nutritional values, as well as their nutritional richness.

Moisture content is a factor that directly influences nutritional composition, with moister samples presenting lower concentrations of dry nutrients. The pulp is generally the moistest part of the fruit, with most of the samples presented in **Table 4** having at least 80% moisture, with up to 96.77% found in the case of *C. pepo* from Korea (Kim et al., 2012). This difference is reflected in the higher density of the other ingredients in the less moist varieties, such as the Kab23 variety from Uganda, which presented 13.69% of fiber content (Nakazibwe et al., 2020), or a Nigerian variety, which presented around 10% of protein (Blessing et al., 2011).

Considering the different species, *C. maxima* presented a higher carbohydrate content when compared to *C. pepo*, as observed in the Brazilian *C. maxima* variety C347, which reached 10.2% (Pereira et al., 2020), whereas *C. pepo* from Korea presented only 2.62% of this nutrient (Kim et al., 2012). This data reflects differences in the energy potential of the species, with different applications depending on the food purpose. The impact of geographic origin is also important when comparing nutritional values between countries. For example, fruit of *C. pepo* cultivated in China (Li et al., 2022) showed a protein content (1.30%) very close to that of the same species cultivated in Egypt (1.10%) (Adnan et al., 2017), although both had a much higher value when compared to this specie cultivated in Korea (0.21%) (Kim et al., 2012). This phenomenon can be explained by differences in climatic conditions, soil type and agricultural practices, which directly affect the nutritional profile (Armesto et al., 2020; Bannayan et al., 2017).

Through the study of Armesto et al. (2020), it is suggested that organic management can positively influence the concentration of functional nutrients when compared to conventional cultivation. In this study, the Ariel variety of *C. moschata*, cultivated organically, presented higher levels of fiber (2.68%) and carbohydrates (8.04%) compared to the same variety cultivated conventionally, which presented 2.47% of fiber and 6.47% of carbohydrates.

Differences are also observed in the caloric energy of the samples. The highest energy content was recorded in the Brazilian *C. maxima* variety C347, with 55 kcal/100 g, due to its high carbohydrate (10.2%) and fat (0.97%) content (Pereira et al., 2020). On the other hand, samples with a higher moisture content, such as *C. pepo* from Korea, have a lower energy density (Kim et al., 2012). It is also important to highlight the variability in protein content, which ranged from 0.21% in *C. pepo* from Korea to 10% in a sample from Uganda (Kan22) (Kim et al., 2012; Nakazibwe et al., 2020). The reported values indicate

that certain varieties can be explored as complementary dietary sources of protein, especially in regions with limited access to traditional protein sources. Likewise, the fiber content, which is particularly high in the Kab23 variety from Uganda (13.69%) (Nakazibwe et al., 2020), highlights these varieties as potential functional ingredients in food formulations aiming at digestive health.

In summary, **Table 4** highlights the nutritional richness of pumpkin pulp and its variability across the species, variety, geographic origin and cultivation system. These results indicate the potential use of pumpkin as a functional food and reinforce the need to choose varieties and crop management that maximize its nutritional value in specific nutrients.

Table 4. Nutritional composition of *Cucurbita* sp. fruit pulp.

Cucurbita sample		Country sample	Nutritional Composition (% fw)						Energy (kcal)	Reference
			Moisture	Ash	Fibers	Proteins	Fat	Carbohydrates		
C. pepo		Egypt	92.93±1.01	0.47	0.80	1.10	00.27	3.42	-	(Adnan et al., 2017)
C. pepo		China	95.9±0.386	-	0.630±0.020	1.30±0.014	0.330±0.020	3.45±0.001	-	(Li et al., 2022)
C. pepo		Korea	96.77±0.02	0.344±0.004	0.372±0.002	0.21±0.01	0.06±0.01	2.62±0.02	-	(Kim et al., 2012)
C. moschata			94.231±0.008	1.036±0.001	0.741±0.007	0.31±0.07	0.09±0.01	4.34±0.08	-	
C. maxima			84.04±0.02	1.05±0.01	1.09±0.04	1.1±0.1	0.42±0.02	13.4±0.1	-	
C. maxima	Indigenous variety	Bangladesh	92.5±0.7	0.42±0.06	0.12±0.06	0.79±0.03	0.11±0.04	0.64±0.05	6.00±0.05	(Amin et al., 2019)
	Hybrid variety		91.6±0.6	0.30±0.04	0.29±0.07	0.89±0.04	0.16±0.04	0.47±0.06	6.36±0.07	
C. maxima	C8	Brazil	91.3±0.3	0.62±0.01	1.7±0.1	0.9±0.1	0.17±0.01	5.3±0.2	26±6	(Pereira et al., 2020)
	C347		84.6±0.3	0.96±0.07	1.9±0.3	1.4±0.1	0.97±0.02	10.2±0.4	55±1	
	C269		95.1±0.6	0.50±0.02	1.01±0.08	0.4±0.02	0.074±0.000	2.9±0.1	13.9±0.4	
	C38		95.5±0.3	0.381±0.005	0.67±0.09	0.38±0.03	0.074±0.000	2.99±0.04	14.1±0.3	
C. maxima		-	91.6	0.8	0.5	1.0	0.1	6.5	26.0516	(Lucan & Mitroi, 2024)
C. maxima		Malaysia	92.24±0.01	0.76±0.13	0.56±0.08	0.98±0.06	0.15±0.03	5.31±0.04	-	(See et al., 2007)
C. maxima		Kenya	88.2±0.4	0.767±0.1	-	0.4248±0.001	-	-	-	(Fedha et al., 2010)
C. moschata			89.5±0.3	0.6195±0.1	-	0.4±0.1	-	-	-	
C. moschata		Colombia	85.34±0.265	0.70±0.155	-	1.32±0.098	-	11.94±2.54	-	(Quintana et al., 2018)
C. moschata	Variety Ariel	Spain	91.58	1.03	2.20	0.76	0.12	6.50	30.18	(Armesto et al., 2020)
	Variety Pluton		88.90	1.31	2.97	1.45	0.17	8.18	40.00	
	Conventional farming		91.20	1.10	2.47	1.07	0.17	6.47	31.66	
	Organic farming		89.47	1.22	2.68	1.14	0.13	8.04	37.83	
C. moschata		India	86.2±0.07	0.52±0.003	0.66±0.003	-	-	-	-	(El Khatib & Muhieddine, 2020)
Cucurbita spp.	Mean of 10 genotypes	Nigeria	73±2	6.8±0.3	4.4±0.9	10±2	2.0±0.5	4±1	81±8	(Blessing et al., 2011)

Cucurbita sample		Country sample	Nutritional Composition (% fw)						Energy (kcal)	Reference
			Moisture	Ash	Fibers	Proteins	Fat	Carbohydrates		
C. maxima		Colombia	92.70±0.34	0.64±0.01	2.00±0.03	0.68±0.02	0.173±0.02	3.81±0.12	-	(Márquez Cardozo et al., 2021)
Cucurbita spp.	Kan22 (Anderina)	Uganda	82±1	2.7±0.2	10.61±0.05	1.63±0.03	0.094±0.002	-	-	(Nakazibwe et al., 2020)
	Kan3 (Kihaza/Wujju)		88.5±0.3	0.086±0.003	2.55±0.01	0.85±0.01	0.06±0.01	-	-	
	Kan4 (Enjogotte/Dulu)		89.6±0.6	0.112±0.001	4.172±0.003	1.07±0.02	0.164±0.009	-	-	
	Kan2 (Anderina)		81.26±0.07	0.39±0.01	5.858±0.002	1.66±0.04	0.290±0.002	-	-	
	Kan6 (Enjogotte/Dulu)		92±4	0.3114±0.0008	1.5422±0.0008	0.50±0.02	0.047±0.003	-	-	
	Kab10 (Ekihaza/Wujju)		83±2	0.283±0.002	6.59±0.02	1.33±0.04	0.217±0.000	-	-	
	Kab5 (Rwamabondo/Sweety pumpkin)		77±1	0.58±0.03	9.0±0.1	3.5±0.2	0.105±0.002	-	-	
	Kab23 (Mulembe/Dulu)		80.6±0.4	0.86±0.03	13.69±0.01	3.21±0.07	0.122±0.002	-	-	
	Mas16 (Oziga)		88±2	0.42±0.04	4.7±0.1	1.57±0.04	0.080±0.002	-	-	
	Mas14		90.22±0.03	0.124±0.000	2.177±0.001	1.09±0.03	0.08±0.01	-	-	
	Mit11 (Oziga)		79.5±0.9	0.666±0.006	7.54±0.01	2.01±0.04	0.111±0.002	-	-	
	Mit9 (Rwamabondo/Sweety pumpkin)		84±8	1.00±0.02	8.36±0.02	1.63±0.04	0.13±0.02	-	-	
	Fort18 (Anderina)		90.7±0.7	0.22±0.01	6.91±0.05	0.97±0.03	0.093±0.006	-	-	
	Fort19 (Bala)		82.2±0.7	0.44±0.04	9.38±0.04	2.20±0.03	0.213±0.007	-	-	

"-": data not available by the authors.

1.2.3. Chemical compositions of pumpkin by-products

Among the most popular by-products of pumpkin are its seeds. Consumption of pumpkin seeds has become widespread, both in the form of seed oil and whole seeds in various preparations or as a snack. Consequently, it has garnered increased attention and interest from the scientific community. Pumpkin seeds are indeed regarded as one of the foods of the future, owing to their rich nutritional profile consisting of nutrients, minerals, oils, essential amino acids, and high-quality fiber. Additionally, the extraction and utilization of pumpkin seed oil have been the subjects of study in the medical and food industries. However, driven by sustainability, another important by-product, pumpkin peels, have also begun to receive attention regarding its use to obtain bioactive compounds and high-value-added molecules. The peels have potential for applications in both the food industry and the pharmaceutical-cosmetic industry.

Below, we discuss the nutritional and chemical compositions of pumpkin by-products. In particular, the discussion on seeds and seed oil is divided according to the three species of the greatest commercial interest (*C. pepo*, *C. moschata*, and *C. maxima*; see **Topic 1**), while the peels are compared across these species.

Seeds

Cucurbita pepo seeds

Among the large family of Cucurbitaceae, *C. pepo* is the oldest known cultivated species, and it is widely consumed in different forms, both as an immature vegetable or as mature fruit, in various dishes such as cooked or in confectionery (Ratnam et al., 2017). The seeds of the species are reported in different studies for being a great source of nutritional compounds and chemical constituents with pharmacological capacity. **Table 5** shows different studies and chemical composition of seeds and treated seeds from *C. pepo* cultivated in distinct countries, including whole seeds, seed oils, and their variations involving seeds with or without the shell.

Regarding the proximate composition, it is noticeable that the great nutritional value presented by the pumpkin seeds. Whole seeds presented high contents of protein (around 20 to 30% dw) and fat (about 40% dw) (Ardabili et al., 2011; Elinge et al., 2012; Kwiri et al., 2014; Ouattara et al., 2015; Srbinoska et al., 2012). High levels of proteins were also presented by the seed kernels (39.54% dw) and the shell of the seeds (21.16%) (Srbinoska et al., 2012). In the oil obtained from the seeds, the main nutritional relevant components were proteins (about 20% dw) and fatty acids (Adeel et al., 2014; Ouattara et al., 2015). In addition, some authors (Adeel et al., 2014; Ardabili et al., 2011; Elinge et al., 2012; Kwiri et al., 2014) have reported significant contents of carbohydrates and crude fibers, suggesting the seeds of *C. pepo* as a highly nutritious food.

The fatty acid profile of seed oil is rich in unsaturated fatty acids, with high amounts of linoleic, oleic, palmitic, and stearic acids (Adeel et al., 2014; Ardabili et al., 2011; Ouattara et al., 2015). In three studies reported in **Table 5**, the main fatty acids were linoleic acid followed by oleic acid, comprising about 80% of the total amount of fatty acids. On the other hand, in seed oil from Iran (Ardabili et al., 2011), the amounts of these acids (e.g. linoleic and oleic acids) were almost the same (39.84±0.08 and 38.42±0.37%, respectively), while in seed oil from West Africa the content of linoleic acid was significantly higher than oleic acid (62.97±0.62 and 17.22±1.12 %, respectively). This difference is probably explained by variations in cultivation methods, environmental conditions, genotypes, among other factors (Zhou et al., 2007). Ouattara et al. (2015) compared the oil from pumpkin seeds and shea nut, where the second one presented a ratio unsaturated/saturated of 1.13, lower than the ratio of 4.15 obtained from *C. pepo*, which was suggested as a potential source of omega 6.

In *C. pepo* seeds, important mineral contents were also reported. Sample from Zimbabwe presented significant contents of sodium (Na), iron (Fe), phosphorous (P), calcium (Ca), zinc (Zn), and magnesium (Mg), especially P which was the major mineral (1040.8±0.663 mg/100g), followed by Mg (344.6±0.245 mg/100g), and Ca (141±0.316 mg/kg) (Kwiri et al., 2014). On the other hand, in seeds from Nigeria the most abundant mineral was Potassium (K; 237.24±0.09 mg/100g), followed by Na (170.35±0.08 mg/100g), and Mg (67.41±0.05 mg/100g), while Manganese (Mn) and Cobalt (Co) were also detected. These variations of mineral composition were highlighted by Dalda-Şekerci et al. (2020a), who evaluated the mineral composition of seeds of 36 genotypes of *C. pepo*. The range of content was 2,869.6–1,390.2 mg/100g for P; 2,179.8–849.0 mg/100g for K; 769.2–362.6 mg/100g for Mg; and 801.0–345.7 mg/100g for S, regarding macro minerals; and 15.740–6.534 mg/100g for Fe; 9.612–3.186 mg/100g for Zn; 5.328–2.304 mg/100g for Mn; 8.538–0.654 mg/100g for Na; 4.230–1.704 mg/100g for Cu; and 1.326–0.648 mg/100g for B, regarding microelements.

In addition, Elinge et al. (2012) evaluated the antinutritional composition of the seeds, focusing on phytate, oxalate, hydrocyanic acid, and nitrate contents, and they suggested values within the acceptable daily intake range, according to WHO.

Table 5. Chemical composition of *C. pepo* seeds and their by-products.

Pumpkin sample		**Seeds**	**Seed oil**	**Seeds**	**Seed oil**	**Seeds**	**Seeds**	**Seed oil**	***C. pepo* seeds***	**Seeds**	**Seeds kernels**	**Shell seeds**
Country sample		Zimbabwe	West Africa	Pakistan		Nigeria	Iran		Turkey	Republic of Macedonia		
Proximate Composition	Moisture (% fw of oil)	5.662±0.016	4.6±0.11	6.0±1.45	6.0±1.20	5.00	5.20±0.28	-	-	6.0	4.5	5.5
	Crude Ash (% dw of oil)	3.324±0.010	-	6.0±0.13	6.0±0.42	5.50	5.34±0.04	-	-	4.69	6.35	3.21
	Crude Fiber (% dw of oil)	2.578±0.007	-	2.5±0.83	10.0±0.64	1.00	2.49±0.11	-	-	12.88	3.45	5.54
	Crude Protein (% dw of oil)	32.860±0.103	18.87±1.50	22.0±1.06	20.0±1.90	27.48	25.40±0.61	-	-	24.48	39.54	21.16
	Crude Fat (% dw of oil)	43.460±0.098	41.07±0.73	-	-	38.00	41.59±2.71	-	-	-	-	-
	Carbohydrates (% dw of oil)	12.160±0.142	-	25.0±0.61	24.0±0.42	28.03	25.19±3.3	-	-	-	-	-
	Total sugar (mg/100g dw of oil)	-	-	-	-	-	-	-	-	2.67	3.21	0.50
Mineral Composition (mg/100 g)	Sodium (Na)	67.956±0.037	-	-	-	170.35±0.08	-	-	3.574	-	-	-
	Iron (Fe)	11.980±0.086	-	-	-	3.75±0.02	-	-	11.61	-	-	-
	Phosphorous (P)	1040.8±0.663	-	-	-	47.68±0.04	-	-	2220.07	-	-	-
	Calcium (Ca)	141±0.316	-	-	-	9.78±0.03	-	-	-	-	-	-
	Zinc (Zn)	1.24±0.01	-	-	-	14.14±0.02	-	-	6.421	-	-	-
	Magnesium (Mg)	344.6±0.245	-	-	-	67.41±0.05	-	-	594.65	-	-	-
	Potassium (K)	-	-	-	-	237.24±0.09	-	-	1536.47	-	-	-
	Manganese (Mn)	-	-	-	-	0.06±0.01	-	-	4.212	-	-	-
	Cobalt (Co)	-	-	-	-	2.17±0.02	-	-	-	-	-	-
	Boron (B)	-	-	-	-	-	-	-	0.961	-	-	-
	Copper (Cu)	-	-	-	-	-	-	-	2.51	-	-	-
	Sulfur (S)	-	-	-	-	-	-	-	571.36	-	-	-
Physiochemical Properties	Acid value (mg KOH/g oil)	-	-	-	0.834±0.005	-	-	0.78±0.02	-	-	-	-
	Saponification value (mg KOH/g oil)	-	-	-	194.606±0.579	-	-	190.69±1.40	-	-	-	-
	Unsaponifiable matter (%)	-	-	-	-	-	-	5.73±0.82	-	-	-	-
	Specific gravity (30 °C)	-	-	-	0.8087±0.0006	-	-	0.9151±0.0002	-	-	-	-
	Iodine value (g I_2/100 g oil)	-	-	-	97.98±0.06	-	-	104.36±0.04	-	-	-	-
	Peroxide value (meq O_2/kg oil)	-	-	-	6.74±0.4849	-	-	10.85±0.62	-	-	-	-
	Total sterol content (%)	-	-	-	-	-	-	1.86±0.10	-	-	-	-
	Total phenolics content (mg gallic acid/kg oil)	-	-	-	-	-	-	66.27±3.69	-	-	-	-
	Total tocopherols content (mg/kg)	-	-	-	-	-	-	882.65±18.32	-	-	-	-

Pumpkin sample		Seeds	Seed oil	Seeds	Seed oil	Seeds	Seeds	Seed oil	*C. pepo* seeds*	Seeds	Seeds kernels	Shell seeds
Fatty acid profile (%) (%)	Palmitic acid C16:0	-	-	-	16.36	-	-	10.68±0.42	-	-	-	-
	Palmitoleic acid C16:1	-	-	-	--	-	-	0.58±0.14	-	-	-	-
	Stearic acid C18:0	-	-	-	9.2	-	-	8.67±0.27	-	-	-	-
	Oleic acid C18:1	-	-	-	34.27	-	-	38.42±0.37	-	-	-	-
	Linoleic acid C18:2	-	-	-	48.02	-	-	39.84±0.08	-	-	-	-
	Linolenic acid C18:3	-	-	-	0.17	-	-	0.68±0.14	-	-	-	-
	Gadoleic (C20:1)	-	-	-	-	-	-	1.14±0.00	-	-	-	-
	Total saturated fatty acids	-	-	-	-	-	-	19.35±0.16	-	-	-	-
	Total unsaturated fatty acids	-	-	-	-	-	-	80.65±0.16	-	-	-	-
	Ratio unsaturated/ saturated	-	4.15	-	-	-	-	-	-	-	-	-
Antinutritive factors (mg/100 g)	Cyanide	-	-	-	-	0.22±0.02	-	-	-	-	-	-
	Nitrate	-	-	-	-	2.27±0.03	-	-	-	-	-	-
	Phytate	-	-	-	-	35.06±1.10	-	-	-	-	-	-
	Oxalate	-	-	-	-	0.023±0.02	-	-	-	-	-	-
References		(Kwiri et al., 2014)	(Ouattara et al., 2015)	(Adeel et al., 2014)		(Elinge et al., 2012)	(Ardabili et al., 2011)		(Dalda-Şekerci et al., 2020)	(Srbinoska et al., 2012)		

"-": data not available by the authors; *: Average of 36 genotypes

Cucurbita moschata seeds

C. moschata, also called winter squash or butternut squash, is the most widely cultivated of the *Cucurbita* species (Lebeda & Paris, 2004; Lia Barbieri Rosa & Tempel Stumpf Elisabeth Regina, 2008). Its seeds are considered the diet of the future, due to their high content of nutrients, oils, and fiber, which makes them an excellent appetizer with innumerable beneficial properties for health, including the prevention of diseases associated with the cardiovascular, nervous, and immune systems, the strengthening of vision, the prevention of kidney stones formation and the improvement of urethral and bladder function (Gomes et al., 2020; Marie-Magdeleine et al., 2011).

The evaluation of *C. moschata* seeds is of great interest due to their chemical and nutritional value, as cited in **Table 6**; they have a high protein content of approximately 20-30% and a lipid content of up to 49% (Ardabili et al., 2011; Gomes et al., 2020).

Regarding the seed oil, it is rich in bioactive compounds such as vitamin E and carotenoids (Veronezi & Jorge, 2015). It has about 70% of unsaturated fatty acids, mostly polyunsaturated fatty acids such as linoleic acid (C18:2n6c) and monounsaturated fatty acids such as oleic acid (C18:1n9c), as well as saturated fatty acids comprising mainly palmitic acid (C16:0) and stearic acid (C18:0). It is worth mentioning that the composition and quantity of fatty acids depends on several factors such as variety, location, climate conditions, and degree of ripening (Jarret et al., 2013; Veronezi & Jorge, 2015).

A study in Bulgaria evaluated the chemical and nutritional composition of seeds and seed oil of *Cucurbita moschata* at different stages of seed development. Regarding the seeds, these showed a higher content of oil, proteins and fibers and a lower content of minerals (ash) and carbohydrates in the final stage of maturation than in the first and second stages; the amount of sterols and tocopherols was higher in the third stage of maturation, unlike the phospholipids which were higher in the first stage; as for the seed oil, this presented better results in the first stage. The fatty acid composition determined by gas chromatography detected linoleic (40.8 to 50.2%), followed by palmitic (21.5 to 25.9%) and oleic (20.5 to 21.0%) as the main fatty acid of the oils in all maturation stages (Petkova & Antova, 2015).

Another study compared the seeds of three varieties of *C. moschata* pumpkin (Nova Caravela, Mini Paulista, and Menina Brasileira), and one variety of *C. maxima* (Moranga de Mesa), and it was found that unsaturated fatty acids ranged between 70 to 78% of the total fatty acids obtained, with linoleic acid and oleic acid standing out, while among the saturated fatty acids, palmitic acid and stearic acid were the major ones. Regarding vitamin content, two of the three varieties of *C. moschata* showed a high content of vitamin A (223 and 217 retinol/100 g), and vitamin E (14.6 to 60.2 α-TEs), which are responsible for important biological processes in the human body, especially in

the process of vision (Veronezi & Jorge, 2015). A study conducted in Cameroon compared the chemical composition of the seeds of five different varieties of pumpkin, where *C. moschata* had the highest fiber content compared to the other varieties, and a similar protein and lipids content among the studied varieties (Edith et al., 2017).

It is worth mentioning that there are not many studies concerning the evaluation of the chemical composition of seeds and seed oils of *C. moschata*, as shown in **Table 6**.

Table 6. Chemical composition of *C. moschata* seeds and their by-products.

Pumpkin sample		Seeds	Seed oil Nova Caravela	Seed oil Mini Paulista	Seed oil Menina Brasileira	Seeds	Seed oil	Seeds	Growing period		
									30 Seeds/oil	60 Seeds/oil	90 Seeds/oil
Country sample		Cameroon		Brazil		Kenya	USA	Korea	Bulgaria		
Proximate Composition	Moisture	7.47±0.41	-	-	-	5.7±0.4	-	5.18±0.60	-	-	-
	Crude Ash (% dw)	3.68±0.07	-	-	-	4.0±0.1	-	5.61±0.02	7.2±0.1	4.7±0.2	4.5±0.1
	Crude Fiber (% dw)	2.93±0.02	-	-	-	-	-	11.44±0.88	4.0±0.1	6.9±0.2	10.0±0.1
	Crude Protein (% dw)	28.94±0.30	-	-	-	35.4±0.3	-	31.44±1.56	26.0±0.3	35.9±0.2	38.2±0.1
	Crude Fat (% dw)	49.17±0.16	-	-	-	-	-	48.17±1.23	10.7±0.2	41.1±0.1	47.1±0.3
	Carbohydrates (% dw)	7.81±0.10	-	-	-	-	-	14.78±0.80	25.8±0.9	8.6±0.2	3.6±0.3
Physiochemical Properties	Acid value (mg NaOH/g)	-	0.32±0.09	2.79±0.00	3.40±0.06	-	0.722	-	-	-	-
	Index of refraction	-	1.462±0.000	1.461±0.000	1.461±0.000	-	-	-	-	-	-
	Peroxide value (meq O_2/kg oil)	-	3.13±0.27	3.09±0.13	3.85±0.16	-	-	-	-	-	-
	Iodine (g I_2/100 g	-	104±0	104±0	95.5±0.1	-	-	-	-	-	-
	Saponification value (mg KOH/g oil)	-	218±2	233±1	241±1	-	-	-	-	-	-
	Unsaponifiable matters %	-	1.35±0.08	2.27±0.11	6.22±0.22	-	-	-	1.8±0.04 / 16.5±0.2	1.0±0.01 / 2.4±0.1	1.0±0.1 / 2.2±0.3
	Alfa-tocopherol (mg/Kg)	-	-	-	-	-	-	25.74±0.73	- / 894.5±21.2	- / 75.8±6.3	- / 20.0±2.6
	Gamma-tocopherol (mg/Kg)	-	-	-	-	-	-	66.85±4.90	- / 944.7±23.7	- / 383±18.5	- / 53.3±4.3
	Gamma-tocotrienol (mg/Kg)	-	-	-	-	-	-	-	- / 120.6±12.4	- / 52.7±4.8	- / 53.3±4.3
	Delta-tocopherol (mg/Kg)	-	-	-	-	-	-	-	- / 50.2±5.6	tr.	tr.
	Beta-carotene (mg/Kg)	-	-	-	-	-	-	7.15±1.50	-	-	-
	Beta-cryptoxanthin (mg/Kg)	-	-	-	-	-	-	-	-	-	-
	Beta-sitosterol (mg/Kg)	-	-	-	-	-	-	277.58±23.48	-	-	-

	Sample	-	Seeds			-	Seeds	Seeds	Seed oil		
Fatty acid profile	Caprylic acid (C8:0)	-	-	-	-	-	-	-	0.1±0.02	0.1±0.04	-
	Lauric acid (C12:0)	-	-	-	-	-	-	-	0.1±0.01	-	-
	Myristic acid (C14:0)	-	0.07±0.01	0.09±0.01	0.11±0.01	-	-	-	0.3±0.1	0.2±0.05	0.2±0.05
	Pentadecanoic acid (C15:0)	-	-	-	-	-	-	-	0.1±0.02	-	-
	Palmitic acid (C16:0)	-	13.9±0.0	14.9±0.1	17.9±0.1	-	20.74±2.52	12.78±0.11	25.9±0.4	24.7±0.2	21.5±0.5
	Palmitoleic acid (C16:1)	-	0.04±0.01	0.06±0.01	0.07±0.01	-	-	-	-	-	-
	Heptadecanoic (C17:0)	-	0.12±0.01	0.06±0.01	0.09±0.01	-	-	-	0.1±0.01	0.1±0.02	0.1±0.01
	Stearic acid (C18:0)	-	9.99±0.01	8.54±0.14	10.6±0.0	-	7.47±1.43	7.33±0.20	9.3±0.4	9.2±0.2	6.7±0.5
	Oleic acid (C18:1n9c)	-	28.4±0.0	30.3±0.1	29.8±0.0	-	22.66±8.94	31.34±0.12	20.5±0.2	21.0±0.3	21.0±0.1
	Linoleic acid (C18:2n6c)	-	46.1±0.0	44.6±0.1	40.1±0.0	-	48.52±8.37	35.72±0.25	40.8±0.5	43.6±0.2	50.2±0.2
	Alfa-linoleic acid (C18:3n3)	-	0.12±0.01	0.19±0.01	0.13±0.01	-	-	-	1.9±0.2	0.3±0.1	0.2±0.05
	Arachidic (C20:0)	-	0.53±0.01	0.55±0.01	0.67±0.01	-	-	-	0.4±0.1	0.4±0.1	0.1±0.02
	Gadoleic acid (C20:1)	-	-	-	-	-	-	-	0.1±0.02	0.1±0.02	-
	Behenic acid (C22:0)	-	-	-	-	-	-	-	0.4±0.1	0.3±0.1	-
	Lignoceric (C24:0)	-	0.42±0.01	0.52±0.01	0.40±0.03	-	-	-	-	-	-
	Total saturated fatty acids	-	25.1±0.0	24.7±0.2	29.7±0.0	-	-	20.11±0.11	36.7	35.0	28.6
	Total monounsaturated	-	28.5±0.0	30.4±0.1	29.8±0.0	-	-	31.34±0.12	20.6	21.1	21.0
	Total polyunsaturated	-	46.3±0.0	44.8±0.1	40.3±0.0	-	-	35.72±0.25	42.7	43.9	50.4
	Ratio unsaturated/ saturated	-	2.98	3.04	2.36	-	-	-	-	-	-
Vitamin	Vitamin A (µg retinol/100 g)	-	63.7±9.6	223±7	217±14	-	-	-	-	-	-
	Vitamin E (α-TE)	-	32.5±0.4	14.6±0.2	60.2±0.2	-	-	-	-	-	-
Reference		(Edith et al., 2017)	(Veronezi & Jorge, 2015)			(Fedha et al., 2010)	(Jarret et al., 2013)	(Kim et al., 2012)	(Petkova & Antova, 2015)		

"-": data not available by the authors.

Cucurbita maxima seeds

Native of South America, *C. maxima* fruit are berries, indehiscent, of various size and coloration, and their seeds are large, flat, oval-shaped and end in a tip (Lemus-Mondaca et al., 2019). As the others *Cucurbita* fruits mentioned above, *C. maxima* is also known by its great nutritional value and industrial potential, especially the seeds and its by-products which will be further discussed.

Regarding the proximate composition, the seeds of *C. maxima* showed high nutritional value, presenting important contents of all macronutrients. Through **Table 7**, it is possible to notice that crude fat, protein, and fibers are the major compounds of *C. maxima* seeds. More than 50% of the dry weight of seeds from Korea is crude oil (Kim et al., 2012), while both varieties from Bangladesh, indigenous and hybrid, are rich in crude fiber, 46.65±0.845% dw and 52.38±0.61% dw, respectively (Amin et al., 2019). The partially defatted seeds from Romania, the by-product obtained during the process of seed oil production, are good source of protein, with 42.75±0.22% dw content (Apostol et al., 2018). The results obtained from Tagwi Williams et al. (2020), show that the crude oil is concentrated mostly in the kernel, and the crude fibers in the coat of the seeds. High contents of oil and protein in the kernel are also reported by Alfawaz (2004). The kernels of seeds from Saudi Arabia also presented a rich mineral composition, with major compounds being P, 1036±4.72 mg/100 g, and K, 753.11±33.29 mg/100 g. In fact, all the sample reviewed in **Table 7** showed high value of K, ranging from 434.71±0.57 to 1290±247 mg/100 g. On the other hand, P content was high in the seeds of Nigeria (Tagwi Williams et al., 2020) and Tunisia (Rezig et al., 2012), 875.63±0.01 and 824.53±21.38 mg/100 g, respectively. High contents of Na, Fe, Ca, Zn, Mg, K, Mg and Cu were also reported, revealing that seeds as important source of micronutrients.

The nutritional value of seed oil was also highlighted by various authors. Rezig et al. (2012) reported the rich content of tocopherols, with a predominance of δ-tocopherol (177±14.17 mg/100 g), as well as the presence of six phenolic acids, being syringic acid the predominant one (15.91±0.39 mg/100 g), and a total sterol content of 127.88±2.97 mg/100 g. The sterols composition was also studied in the oil seeds from Italy (Montesano, Blasi, et al., 2018), where 15.7 mg/100 g of Δ^5-sterols and 279.3 mg/100 g by Δ^7-sterols were identified.

Similarly to *C. pepo* and *C. moschata*, the fatty acid profile of *C. maxima* is mostly constituted by oleic (C18:1) and linoleic acids (C18:2). In two varieties of the species, the amount of linoleic acid was greater than 50% (Alfawaz, 2004; Kim et al., 2012). Besides the predominance of unsaturated fatty acids, palmitic acid (C16:0) was also present in great amounts, ranging from 10.84±0.12% to 22.84±0.50%.

Tagwi Williams et al. (2020) also reported the presence of anti-nutrients, e.g.

phytate, tannin, and oxalate which were found in seeds and seed kernels, while cyanide was detected only in the seeds in very low amounts. The same authors concluded that the level of these compounds is within the acceptable range for safe human consumption.

Table 7. Chemical composition of *C. maxima* seeds and their by-products.

Pumpkin sample		Seeds		Seeds	Kernel	Seeds	Oil seed	Seeds	Seed partially defatted	Oil seed	Kernel
		Indigenous variety	Hybrid variety								
Country sample		Bangladesh		Nigeria		Tunisia		Korea	Romania	Italy	Saudi Arabia
Proximate Composition	Moisture (%)	56.74±0.70	41.63±0.38	10.5±0.0	10.30±0.00	8.46±0.62	-	2.75 ± 0.02	-	-	6.27±1.36
	Crude Ash (% dw)	3.54±0.68	3.79±0.60	4.49±0.01	5.25±0.01	4.34±0.02	-	4.55 ± 0.04	7.50±0.11	-	5.14±1.23
	Crude Fiber (% dw)	46.65±0.84	52.38±0.61	17.63±0.01	1.86±0.01	24.00±4.72	-	16.61 ± 0.70	26.64±0.45	-	2.13±0.57
	Crude Protein (% dw)	21.31±0.50	20.68±0.61	35.46±0.01	33.80±0.01	37.05±3.45	-	28.26 ± 1.03	42.75±0.22	-	39.22±2.46
	Crude Fat (% dw)	23.45±0.72	17.89±0.55	2.20±0.00	43.15±0.00	34.49±4.05	-	53.92 ± 0.14	12.28±0.19	-	43.69±3.92
	Carbohydrates (% dw)	5.18±0.67	14.54±0.48	6.08±0.02	5.65±0.03	0.12±0.09	-	13.27 ± 0.85	37.40±0.05	-	9.82±2.70
	Total sugar (mg/g dw)	9.73±0.54	8.06±0.61	-	-	-	-	-	-	-	-
	Energy (kcal/100 g)	311.54±0.56	227.64±0.75	-	-	-	-	-	-	-	-
Mineral Composition (mg/100 g)	Sodium (Na)	1.35±0.31	0.98±0.01	68.26±0.02	51.23±0.01	356.75±18.43	-	-	19.50±4.4	-	68.58±16.85
	Iron (Fe)	6.02±0.58	5.51±0.57	10.49±0.02	13.59±0.01	15.37±0.87	-	-	87.80±1.80	-	13.66±1.60
	Phosphorous (P)	0.74±0.01	0.68±0.01	875.63±0.01	124.35±0.00	824.53±21.38	-	-	-	-	1036±4.72
	Calcium (Ca)	4.00±0.58	3.76±0.54	128.67±0.02	145.30±0.002	271.89±24.81	-	-	127±1.92	-	139.70±4.19
	Zinc (Zn)	18.77±0.50	16.43±0.56	6.44±0.02	1.76±0.01	25.19±0.63	-	-	11.5±2.5	-	1.09±0.06
	Magnesium (Mg)	4.34±0.51	3.69±0.60	284.54±0.01	351.63±0.01	146.13±9.91	-	-	697±115	-	364. 43±32.88
	Potassium (K)	434.71±0.57	557.64±0.44	511.43±0.01	752.64±0.02	886.56±16.34	-	-	1290±247	-	753.11±33.29
	Manganese (Mn)	1.35±0.33	0.98±0.01	-	-	3.42±0.76	-	-	8.20±1.89	-	-
	Copper (Cu)	0.31±0.01	0.26±0.01	-	-	36.66±4.93	-	-	2.30±0.41	-	0.30±0.05
Phenolic acid Composition (mg/100 g)	Protocatechuic acid	-	-	-	-	-	1.81±0.26	-	-	-	-
	Caffeic acid	-	-	-	-	-	3.88±0.03	-	-	-	-
	Syringic acid	-	-	-	-	-	7.96±0.13	-	-	-	-
	Vanillic acid	-	-	-	-	-	2.46±0.37	-	-	-	-
	p-coumaric acid	-	-	-	-	-	2.50±0.95	-	-	-	-
	Ferulic acid	-	-	-	-	-	4.99±0.29	-	-	-	-

Pumpkin sample		Seeds		Seeds	Kernel	Seeds	Oil seed	Seeds	Seed partially defatted	Oil seed	Kernel
		Indigenous variety	Hybrid variety								
Physiochemical Properties	Acid value (mg KOH/g oil)	-	-	-	-	-	7.54±0.02	-	-	-	0.53±0.25
	Saponification value (mg KOH/g oil)	-	-	-	-	-	175 ±1.30	-	-	-	185.20±5.84
	Unsaponifiable matter (%)	-	-	-	-	-	1.25±0.15	-	-	-	-
	Specific gravity (25 °C)	-	-	-	-	-	0.91	-	-	-	0.913±0.004
	Iodine value (g I_2/100 g oil)	-	-	-	-	-	153.66±0.65	-	-	-	105.12±5.83
	Peroxide value (meq O_2/kg oil)	-	-	-	-	-	2.33±0.65	-	-	-	0.85±0.46
Sterol composition (mg/100 g oil or fw)	Cholesterol	-	-	-	-	-	25.35±0.56	-	-	0.9 ±0.1	-
	Cholestanol	-	-	-	-	-	3.03±0.07	-	-	-	-
	Campesterol	-	-	-	-	-	-	-	-	10.3±1.0	-
	24-Methylenecholesterol	-	-	-	-	-	3.62±0.11	-	-	-	-
	Stigmasterol	-	-	-	-	-	3.17±0.13	-	-	4.5±0.8	-
	Spigmasterol	-	-	-	-	-	-	-	-	61.8±4.2	-
	Sitosterol	-	-	-	-	-	50.64±1.96	23.516±1.44	-	-	-
	Δ5,24-Stigmastadienol	-	-	-	-	-	27.25±0.45	-	-	-	-
	Δ7,25-Stigmastadienol	-	-	-	-	-	-	-	-	78.4±5.5	-
	Δ7,22,25-Stigmastadienol	-	-	-	-	-	-	-	-	91.2±6.8	-
	Δ7-Stigmastenol	-	-	-	-	-	-	-	-	15.1±1.5	-
	(24R)-24-Ethyl cholesta-7,25 (27)-dien-3β-ol	-	-	-	-	-	8.52±0.57	-	-	-	-
	Δ7-Avenasterol	-	-	-	-	-	6.3±0.30	-	-	32.8±2.8	-
	Δ5-Sterol	-	-	-	-	-	-	-	-	15.7	-
	Δ7-Sterol	-	-	-	-	-	-	-	-	279.3	-

Pumpkin sample		Seeds		Seeds	Kernel	Seeds	Oil seed	Seeds	Seed partially defatted	Oil seed	Kernel
		Indigenous variety	Hybrid variety								
Tocopherol and Carotenoid composition	*Alfa*-tocopherol (mg/100 g)	-	-	-	-	-	128±14.42	2.08±0.13	-	-	-
	Gamma-tocopherol (mg/100 g)	-	-	-	-	-	113.66±1.52	2.87±0.21	-	-	-
	Delta- tocopherol (mg/100 g)	-	-	-	-	-	177±14.17	-	-	-	-
	Beta-carotene (mg/kg)	-	-	-	-	-	-	31.40±3.02	-	-	-
	Beta-cryptoxanthin (mg/kg)	-	-	-	-	-	-	0.21±0.06	-	-	-
Fatty acid profile (relative %)	Capricacid (C10:0)	0.45±0.01	0.63±0.01	-	-	-	-	-	-	-	-
	Lauric acid (C12:0)	1.34±0.33	2.28±0.38	-	-	-	-	-	-	-	-
	Myristic acid (C14:0)	0.01±0.00	0.18±0.01	-	-	-	-	0.16±0.01	-	0.2±0.0	0.18±0.03
	Palmitic acid (C16:0)	20.79±0.50	22.84±0.50	-	-	-	15.97±0.39	10.84±0.12	-	14.2±0.4	16.41±0.95
	Palmitoleic acid (C16:1)	-	-	-	-	-	trace	-	-	0.2±0.0	0.16±0.04
	Margaric acid (C17:0)	-	-	-	-	-	-	-	-	0.2±0.0	-
	Heptadecenoic acid (C17:1)	-	-	-	-	-	-	-	-	0.1±0.0	-
	Stearic acid (C18:0)	4.52±0.50	6.60±0.54	-	-	-	4.68±0.56	5.84±0.03	-	5.8±0.2	11.14±1.03
	Oleic acid (C18:1)	25.42±0.51	23.82±0.67	-	-	-	44.11±0.63	14.83±0.05	-	41.4±0.7	18.14±0.60
	Linoleic acid (C18:2)	49.42±0.68	46.511±0.52	-	-	-	trace	56.60±0.29	-	37.0±0.5	52.69±0.92
	Linolenic acid (C18:3)	2.25±0.49	0.36±0.06	-	-	-	-	0.24±0.01	-	0.2±0.0	1.27±0.22
	Arachidic acid (C20:0)	-	-	-	-	-	0.41±0.40	0.36±0.02	-	0.5±0.0	-
	Eicosenoic acid (C20:1)	-	-	-	-	-	-	0.07±0.00	-	0.1±0.0	-
	Behenic acid (C22:0)	-	-	-	-	-	-	0.09±0.01	-	0.1±0.0	-
	Cis-cetoleic acid (C22:1)	-	-	-	-	-	-	-	-	-	-
	Erucic acid (C22:1)	-	-	-	-	-	-	-	-	-	0.76±0.13
	Lignoceric acid (C24:0)	-	-	-	-	-	-	-	-	0.1±0.0	-
	Total saturated fatty acids	-	-	-	-	-	21.07±1.19	17.47±0.13	19.28	21.1	27.73±1.80
	Total unsaturated fatty acids	-	-	-	-	-	78.9±0.7	71.74±0.165	80.72	78.9	73.03±0.78

Pumpkin sample		Seeds		Seeds	Kernel	Seeds	Oil seed	Seeds	Seed partially defatted	Oil seed	Kernel
		Indigenous variety	**Hybrid variety**								
Antinutritive factors (%)	Cyanide	-	-	0.062±0.00	-	-	-	-	-	-	-
	Phytate	-	-	4.54±0.02	0.86±0.01	-	-	-	-	-	-
	Tannin	-	-	3.14±0.02	0.86±0.01	-	-	-	-	-	-
	Oxalate	-	-	2.92±0.01	1.96±0.01	-	-	-	-	-	-
Reference		(Amin et al., 2019)		(Tagwi Williams et al., 2020)		(Rezig et al., 2012)		(Kim et al., 2012)	(Apostol et al., 2018)	(Montesano, Blasi, et al., 2018)	(Alfawaz, 2004)

"-": data not available by the authors.

Peels

Apart from seeds, chemical characterization of pumpkin peels has also been found in the literature. When comparing different varieties (**Table 8**) a wide range of content of significant compounds emerges, highlighting their distinctive nutritional characteristics. For example, the peels of *C. pepo* cultivated in Korea presented the highest moisture content, reaching 93.598±0.027%, while those of *C. maxima* from the same country presented 75.679±0.044% of water content (Kim et al., 2012). On the other hand, the peel of an indigenous variety of *C. maxima* showed a notable crude protein content of 14.7±0.6% dw, followed by a Korean *C. pepo* with 14.449% (Amin et al., 2019; Kim et al., 2012). Kim et al. (2012) also analyzed the amino acids content, in which the peel of *C. maxima* presented higher contents than *C. pepo* and *C. moschata*, especially that of glutamic acid 4.10±0.67 mg/kg raw weight (RW), while *C. moschata* had a high amount of aspartic acid 2.82±0.06 mg/kg RW and *C. pepo* had the highest amount of arginine among the 3 varieties (1.12±0.05 mg/kg RW). Samples also presented important fiber content, as shown in **Table 8**. The presence of significant mineral content was also observed, with potassium levels notably ranging from 458±7 (Hussain, Kausar, Din, Murtaza, Jamil, Noreen, Rehman, et al., 2021) to 3085±1097 mg/100 g (Martínez-Valdivieso et al., 2014). Phosphorus was present in great quantities in the peels of Indian *Cucurbita* sp. and Spanish *C. pepo* (319.33±0.05 mg/100g and 704±211 mg/100 g, respectively) (Mala & Kurian, 2016; Martínez-Valdivieso et al., 2014), while sodium, iron, calcium, and zinc were also detected in significant amounts.

The chemical characterization of peels proved to be very relevant, revealing an important value of bioactive compounds. The substantial presence of β-carotene in *Cucurbita* sp., with 11.9±0.1 mg/100 g, and in Korean *C. maxima*, with 12.319±3.061 mg/100 g, stands out for its beneficial effects on vision and skin health (Kim et al., 2012; Mala & Kurian, 2016). On the other hand, significant amounts of total phenols and flavonoids were detected in Pakistani *C. maxima* peel (93.4±0.7 mg GAE/100 g and 45±0.6 mg CE/100 g, respectively) (Hussain, Kausar, Din, Murtaza, Jamil, Noreen, Rehman, et al., 2021). There are also reported important contents of tocopherols and β-Cryptoxanthin (Kim et al., 2012), which are forms of vitamin E and precursors to vitamin A, respectively, and contribute to the nutritional value and antioxidant properties of pumpkin fruit. In another study (Rolnik et al., 2020), the phytochemical composition of extracts from five types of cucurbit vegetables was determined using UPLC-ESI-QTOF-MS. A total of 36 phytochemicals were characterized, and their antioxidant effects were also evaluated. However, the abundance of bioactive compounds and their associated benefits still remain largely unexplored.

The reported content of phytochemicals highlights the nutritional diversity and

benefits of different pumpkin varieties, emphasizing their importance in a balanced and healthy diet, and their relevance to obtain valuable compounds.

Table 8. Chemical composition of *Cucurbita* sp. peels.

Pumpkin sample		*C. maxima* peel		*Cucurbita* sp. peel powder	Fruit with rind		*C. pepo* peel	*C. maxima* peel flour	Peel		
		Indigenous variety	Hybrid variety		*C. moschata*	*C. maxima*			*C. pepo*	*C. moschata*	*C. maxima*
Country sample		Bangladesh		India	Kenya		Spain	Pakistan	Korea	Korea	Korea
Proximate Composition	Moisture (%)	89.5±0.7	88.5±0.7	7.23±0.07	87.9±0.0	87.1±0.2	-	10.79±0.03	93.598±0.027	87.186±0.009	75.679±0.044
	Crude Ash (% dw)	7.3±0.4	5.5±0.8	6.04±0.05	6.7±0.1	6.9±0.3	-	3.61±0.04	9.841	10.894	4.605
	Crude Fiber (% dw)	13.4±0.6	12.3±0.7	13.91±0.04	-	-	-	3.14±0.03	19.182	26.752	9.190
	Dietary Fiber (% dw)	-	-	28.81±0.05	-	-	-	-	-	-	-
	Crude Protein (% dw)	14.7±0.6	11.6±0.7	12.5±0.08	4.9±0.2	3.9±0.1	-	1.90±0.03	14.449	8.818	6.801
	Crude Fat (% dw)	1.7±0.4	2.7±0.6	-	-	-	-	1.77±0.04	7.357	5.143	3.573
	Carbohydrates (% dw)	12.4±0.5	6.7±0.6	-	-	-	-	-	68.354	75.144	85.021
	Total sugar (mg/g dw)	7.6±0.5	10.8±0.7	-	-	-	-	-	-	-	-
	Energy (kcal/100 g)	124.5±0.6	92.1±0.6	-	-	-	-	-	-	-	-
Mineral Composition (mg/100 g)	Sodium (Na)	9.7±0.6	60.6±0.5	-	-	-	9.6±3.9	8.96±0.08	-	-	-
	Iron (Fe)	4±0.6	15.7±0.5	43±0.1	-	-	6±2	4.05±0.06	-	-	-
	Phosphorous (P)	1.4±0.4	0.74±0.01	319.33±0.05	-	-	704±211	-	-	-	-
	Calcium (Ca)	1.4±0.4	0.96±0.01	-	-	-	317±130	4.58±0.06	-	-	-
	Zinc (Zn)	0.15±0.01	18.8±0.5	-	-	-	4.2±1.6	0.3±0	-	-	-
	Magnesium (Mg)	3.4±0.3	3.6±0.7	-	-	-	315±116	-	-	-	-
	Potassium (K)	687.5±0.6	1232.7±0.6	-	-	-	3085±1097	458±7	-	-	-
	Manganese (Mn)	0.36±0.01	0.38±0.01	-	-	-	2.4±1.0	-	-	-	-
	Copper (Cu)	0.025±0.000	0.023±0.000	-	-	-	0.4±0.3	-	-	-	-

Pumpkin sample		*C. maxima* peel		*Cucurbita* sp. peel powder	Fruit with rind		*C. pepo* peel	*C. maxima* peel flour	Peel		
		Indigenous variety	Hybrid variety		*C. moschata*	*C. maxima*			*C. pepo*	*C. moschata*	*C. maxima*
Phenolic acid Composition (mg/100 g)	*Beta*-carotene (mg/100 g)	-	-	11.9±0.1	-	-	-	4.6±0.05	3.948±0.024	6.83±0.202	12.319±3.061
	Ascorbic acid (mg/100 g)	-	-	18.9±0.1	-	-	-	-	-	-	-
	Total Phenols (mg GAE/g)	-	-	5.19±0.05	-	-	-	-	-	-	-
	Total Phenols (mg GAE/100 g)	-	-	-	-	-	-	93.4±0.7	-	-	-
	Total carotenoids (mg/100 g)	-	-	-	-	-	-	23.7±0.2	-	-	-
	Total flavonoids (mg CE/100 g)	-	-	-	-	-	-	45±0.6	-	-	-
	Alfa-tocopherol (mg/100 g)	-	-	-	-	-	-	-	0.449±0.072	0.617±0.219	0.962±0.079
	Gamma-tocopherol (mg/100 g)	-	-	-	-	-	-	-	0.066±0.009	-	0.355±0.017
	Beta-cryptoxanthin (mg/kg)	-	-	-	-	-	-	-	0.15±0.02	0.13±0.03	6.57±1.87
Reference		(Amin et al., 2019)		(Mala & Kurian, 2016)	(Fedha et al., 2010)		(Martínez-Valdivieso et al., 2014)	(Hussain, Kausar, Din, Murtaza, Jamil, Noreen, Rehman, et al., 2021; Hussain, Kausar, Murtaza, et al., 2023)	(Kim et al., 2012)		

"-": data not available by the authors.

1.2.4. Health benefits

Cucurbita sp. possesses a wide range of health benefits due to its rich nutrients and phytochemicals content. Pumpkin is an excellent source of vitamins A, C, and E, which are powerful antioxidants. Vitamin A is particularly important for vision health, while vitamins C and E boost the immune system. Additionally, pumpkin contains important minerals such as potassium, magnesium, and iron, which help maintain cardiovascular health, muscle function, and red blood cell formation (U.S. Department of Agriculture, 2021).

Antioxidant properties

Pumpkin contains a high content of vitamins A, C, and E which are highly appreciated for their ability to protect cells from oxidative damage caused by free radicals. Vitamin A, present in the form of beta-carotene, is particularly effective in promoting eye health and preventing age-related macular degeneration (Hussain, Kausar, Sehar, et al., 2023; Zujko & Witkowska, 2023). Vitamin C is another powerful antioxidant found in high abundance in pumpkin fruit. It helps protect the body's cells from oxidative damage, supports the immune system, and improves skin health by promoting collagen production. Research has shown that a diet rich in vitamin C can reduce the risk of chronic diseases such as heart disease and cancer (U.S. Department of Agriculture, 2021). Pumpkin seeds are also a good source of vitamin E which helps maintain skin health and mitigates oxidative damage of cells. Vitamin E presents anti-inflammatory properties and also contribute to cardiovascular health (U.S. Department of Agriculture, 2021). A study by Bharti et al. (2013) demonstrated the significant antioxidant properties of pumpkin seeds and highlighted that the tocopherol fraction significantly reduced oxidative markers in diabetic rats, showcasing the antioxidant potential of this fruit by-product.

Beyond vitamins, pumpkin contains various polyphenols, which also are compounds known for their antioxidant activity, since they help in reducing inflammation and protecting against certain types of chronic diseases (Grzybek et al., 2016).

Pumpkin also contains minerals such as selenium and zinc, which have antioxidant properties. These minerals contribute to the protection of cells from oxidative damage and support the immune system (Feitosa et al., 2012). Another study by Grzybek et al. (2016) evaluated the antioxidant activity of various pumpkin seed extracts and found that they exhibited significant antioxidant properties. This study confirmed that pumpkin seed extracts could effectively neutralize free radicals and reduce oxidative stress (Grzybek et al., 2016).

Anthelmintic properties

Pumpkin possesses various anthelmintic (anti-worm) properties mainly due to certain compounds found in the seeds and pulp of the fruit. Among the active compounds are cucurbitacins and certain amino acids like cucurbitin, commonly found in seeds. Cucurbitacins have toxic properties for intestinal parasites such as worms, while cucurbitin has a paralyzing effect on parasites (Alhawiti et al., 2019). The mechanism of action of these compounds involves interfering with the metabolism of the worms, leading to their paralysis and subsequent discarding through feces. Additionally, these compounds can damage the cell membrane of worms, eventually causing the death of these parasites. Pumpkin seeds are particularly effective against parasites such as tapeworms and roundworms, and studies have shown that regular consumption of pumpkin seeds can significantly reduce parasitic load (Alhawiti et al., 2019).

Pumpkin seeds can be consumed raw, roasted, or ground into powder. They can be added to smoothies, salads, or eaten as a healthy snack. Research by Grzybek et al. (2016) evaluated the *in vitro* and *in vivo* anthelmintic efficiency of different pumpkin seed extracts, including hot water extract (HWE), cold water extract (CWE), and ethanol extract (ETE). The study used two model nematodes: *Caenorhabditis elegans* and *Heligmosoides bakeri*. All pumpkin seed extracts showed nematodicidal potential *in vitro*, significantly affecting the survival of L1 and L2 larvae of *H. bakeri*. Among these, the ethanol extract (ETE) demonstrated the strongest effect, showing positive results on egg hatching and inhibitory properties against worm motility. The *in vivo* studies revealed that the ethanol extract of pumpkin seeds exhibited significant anthelmintic properties against *H. bakeri* fecal egg counts and adult worm burdens. A decrease in fecal egg counts was accompanied by a significant reduction in worm burden in treated mice compared to the control group (Grzybek et al., 2016). Additionally, a study by Feitosa et al. (2012) verified the effectiveness of pumpkin seeds in controlling gastrointestinal helminths in naturally infected ostriches. The study divided the ostriches into four groups and administered treatments for three consecutive days. The group treated with pumpkin seeds (1 g/kg body weight) showed a significant decrease in egg counts per gram of feces (EPG) compared to the control and drug-treated groups. The results demonstrated that pumpkin seeds were effective in controlling gastrointestinal helminths in naturally infected ostriches. It is notable that no significant effects of pumpkin seed extracts on the integrity or motility of *C. elegans* were found, indicating a specificity in the action of the extracts against the parasitic nematodes without harming non-target organisms (Feitosa et al., 2012).

Anti-hyperglycemic properties

Pumpkin has garnered significant attention for its anti-hyperglycemic properties, which are primarily attributed to its rich composition of bioactive compounds. These compounds include polysaccharides, amino acids, antioxidants, and dietary fiber, all of which contribute to its potential in managing blood glucose levels (Alfahel et al., 2023; L. Huang et al., 2023). Recent studies have highlighted several mechanisms through which pumpkin exerts its anti-hyperglycemic effects. Polysaccharides extracted from pumpkin have been shown to enhance insulin secretion and improve insulin sensitivity. For instance, research by Huang et al. (2023) demonstrated that pumpkin polysaccharides significantly reduced blood glucose levels in diabetic rats, suggesting their potential utility in diabetes management. Pumpkin seeds also play a crucial role due to their high content of amino acids like tryptophan, which aids in regulating blood sugar levels. The seeds are particularly rich in antioxidants such as beta-carotene, vitamin C, and vitamin E, which help reduce oxidative stress—a common issue in diabetic conditions. A study by Alfahel et al. (2023) confirmed that the antioxidant properties of pumpkin contribute to better blood glucose control by mitigating oxidative damage (Alfahel et al., 2023). Additionally, phenolic compounds of pumpkin have been identified to inhibit enzymes like alpha-amylase and alpha-glucosidase, which are involved in carbohydrate digestion, thereby reducing glucose absorption. This enzyme inhibition property further supports the anti-hyperglycemic potential of pumpkin (Hussain, Kausar, Sehar, et al., 2023). Therefore, incorporation of pumpkin into human diet, whether through its flesh, seeds, or extracts, can be an effective natural strategy for managing blood sugar levels and supporting overall metabolic health.

Anti-inflammatory properties

Pumpkin exhibits significant anti-inflammatory properties due to its rich content in polysaccharides, vitamins, minerals, and essential fatty acids. These compounds contribute to reducing inflammation through various mechanisms. Pumpkin polysaccharides suppress inflammation and oxidative stress by inhibiting inflammatory pathways and by reducing the production of pro-inflammatory cytokines (Ji et al., 2021). The high levels of vitamins A, C, and E in pumpkin act as antioxidants, scavenging free radicals and protecting cells from inflammatory damage (Hussain, Kausar, Sehar, et al., 2023). Pumpkin seed oil also contains omega-3 and omega-6 fatty acids, which reduce inflammatory markers in the body. Additionally, pumpkin contains phenolic compounds that inhibit enzymes involved in the inflammatory process, contributing to its anti-inflammatory effects (Andjelkovic et al., 2010). These properties make pumpkin a valuable natural anti-inflammatory agent, supporting its use in traditional medicine and modern

dietary interventions.

Antimicrobial and antiviral activities

Various parts of the pumpkin plant contain compounds that have antimicrobial properties, being effective against bacterial and fungal infections (Ji et al., 2021). Pumpkin exhibits notable antimicrobial and antiviral activities due to its rich content of bioactive compounds, including polysaccharides, phenolic acids, flavonoids, and essential oils. Pumpkin extracts have demonstrated effectiveness against a variety of bacterial pathogens. Compounds such as phenolic acids and flavonoids present in pumpkin seeds and flesh inhibit the growth of both Gram-positive and Gram-negative bacteria. For example, a study by Hussain et al. (2021) reported that pumpkin seed extracts exhibited significant antibacterial activity against common pathogens like *Escherichia coli* and *Staphylococcus aureus* (Hussain, Kausar, Din, Murtaza, Jamil, Noreen, & Iqbal, 2021). The antifungal properties of pumpkin are also well-documented. Pumpkin seed oil contains essential fatty acids and other bioactive compounds that inhibit fungal growth. A study by Petropoulos et al. (2021) found that pumpkin seed oil was effective against *Aspergillus versicolor*, *A. niger*, and *Penicillium verrucosum* var. *cyclopium,* common fungal pathogens. Pumpkin polysaccharides and other bioactive compounds have shown potential in inhibiting viral replication. These compounds enhance the immune response and inhibit the lifecycle of various viruses. The research conducted by Ji et al. (2021) highlighted that pumpkin polysaccharides exhibited antiviral activity against several viruses, including influenza virus and enterovirus, by enhancing the host's immune response and directly inhibiting viral replication (Ji et al., 2021). Recent studies have explored the potential of pumpkin compounds in managing viral infections, including COVID-19. Pumpkin extracts, rich in antioxidants and immunomodulatory compounds, can potentially reduce the severity of viral infections by boosting the immune system. Ebrahimi et al. (2022) evaluated the efficacy of squalene extracted from pumpkin seed oil in a clinical study for treating COVID-19, showing promising results in reducing viral load and inflammation (Ebrahimi et al., 2022).

Cardiovascular health

Pumpkin is beneficial for heart health due to its ability to lower triglycerides and cholesterol levels. The presence of essential fatty acids and antioxidants in pumpkin seeds and seed oil contributes to its cardioprotective effects (El-Mosallamy et al., 2012; Hussain, Kausar, Sehar, et al., 2023). Pumpkin contributes positively to cardiovascular health due to its nutrient-rich profile, which includes antioxidants, vitamins, minerals, and essential fatty acids. These components collectively aid in reducing risk factors associated with

cardiovascular diseases through the neutralization of free radicals and the consequent reduction of oxidative stress, which is a key factor in the development of cardiovascular diseases. The antioxidant properties of pumpkin also help protect the heart and blood vessels from damage caused by oxidative stress (Seybold et al., 2004). The consumption of pumpkin seeds and seed oil has been shown to improve lipid profiles. They help lower levels of low-density lipoprotein (LDL) cholesterol and triglycerides while increasing high-density lipoprotein (HDL) cholesterol. This lipid-modulating effect helps reduce the risk of atherosclerosis and other cardiovascular conditions. A study by Caili et al. (2006) demonstrated that pumpkin seeds could lower serum cholesterol levels and enhance lipid metabolism in humans. Moreover, pumpkin contains compounds that exhibit anti-inflammatory properties, which are beneficial in reducing chronic inflammation associated with heart disease. The anti-inflammatory effects help in maintaining the health of blood vessels and preventing the development of atherosclerosis. The research of Fahim et al. (1995) indicated that pumpkin seed oil inhibited inflammation in rats, suggesting its potential for cardiovascular health. Pumpkin seeds are a good source of magnesium, potassium, and other minerals that are essential for maintaining healthy blood pressure levels. These minerals help in relaxing blood vessels, improving blood flow, and reducing the risk of hypertension (Ghodeshwar et al., 2023). Finally, pumpkin is low in calories but high in dietary fiber, which aids in weight management, a crucial factor in cardiovascular health. The high fiber content helps regulate blood sugar levels, reduce cholesterol absorption, and promote satiety, all of which contribute to a healthy heart (Aziz et al., 2023).

Anticancer potential

The anticancer potential of pumpkin can be attributed to its rich composition of carotenoids, polysaccharides, and various phytochemicals. Pumpkin is an excellent source of carotenoids, such as beta-carotene, lutein, and zeaxanthin. Beta-carotene, a precursor of vitamin A, has been extensively studied for its role in reducing the risk of various cancers. It functions as an antioxidant, neutralizing free radicals that can damage cellular DNA and lead to cancerous transformations. Studies have shown that a diet high in beta-carotene is associated with a lower risk of lung, prostate, and breast cancers (Ebadi et al., 2023). Pumpkin polysaccharides have demonstrated significant antitumor activities in both *in vitro* and *in vivo* studies. These complex carbohydrates can enhance the immune response, stimulate the production of interferons, and activate natural killer cells and macrophages, which are crucial in targeting and eliminating cancer cells. Research indicates that pumpkin polysaccharides may inhibit the growth of melanoma and hepatoma cells (Gan et al., 2020; Shen et al., 2017).

Beyond carotenoids and polysaccharides, pumpkins contain various phytochemicals that contribute to their anticancer effects. These include flavonoids, phenolic acids, and saponins, which possess strong antioxidant properties, can induce apoptosis (programmed cell death) in cancer cells, inhibit angiogenesis (the formation of new blood vessels that supply tumors), and prevent metastasis (the spread of cancer cells to other parts of the body) (Elekofehinti et al., 2021; Montesano, Rocchetti, et al., 2018).

In conclusion, the comprehensive health benefits of pumpkin, from antioxidant, anthelmintic, anti-hyperglycemic, anti-inflammatory, antimicrobial and antiviral properties, and cardiovascular effects, underscore its value as a functional food.

1.2.5. Application of pumpkin by-products

Given its previously demonstrated notable benefits, the pumpkin by-products, such as seeds and peels, have gained significant attention for their nutritional value and potential applications in both the food and pharmaceutical industries. Traditionally, the focus has been on consuming pumpkin pulp, but recent research and innovation has shifted attention to fruit processing by-products (e.g. fibrous strands, peels and seeds). In a recent study, Gavril (Rațu) et al. (2024) proposed using pumpkin peel powder as a natural additive to develop a yogurt with added value and superior overall quality. Incorporating the peel powder into yogurt improved its nutritional composition, mainly through increasing β-carotene and bioactive compounds content. This addition also improved the physicochemical and phytochemical properties, texture, color, sensory qualities, and antioxidant activity of yogurt. The authors highlighted the importance of using food processing by-products, such as pumpkin peel, both in contributing to sustainable food production, but also offering consumers more diversified food options with improved quality characteristics.

In another study, pumpkin seed flour was tested in porridge supplementary flour blends in order to obtain nutritional value from locally sourced foods as a potential solution to mitigate infant malnutrition and adequately complement breastfeeding. By adding the pumpkin seed flour in amounts between 15 to 30% together with the other tested ingredients, composite mixture had up to 76.92% compliance level with recommended standards, which ensures adequate nutrient complementation to breastfeeding. The authors emphasized the nutritional contributions of pumpkin seeds to porridge in terms of protein and fat content, as well as important minerals, mainly phosphorus and magnesium (Marcel et al., 2022). Furthermore, pumpkin seeds have been evaluated as a natural ingredient to improve the quality of chicken burgers (Longato et al., 2017). Powdered pumpkin seeds were incorporated into the burgers at concentrations ranging from 1% to 2%, and the burgers were evaluated over a 4-day storage period. The

addition of pumpkin seeds resulted in desirable changes in the cooking characteristics of the chicken burgers, suggesting potential improvements in texture and juiciness. In addition, the inclusion of pumpkin seeds increased the resistance of chicken burger meat to lipid oxidation during heat treatment and storage. This was evidenced by similar or slightly higher TBARS values compared to raw burgers on day 1, and significantly lower levels of malondialdehyde at the end of the storage period compared to control burgers. Also, the inclusion of pumpkin seeds did not significantly interfere with the sensory quality attributes of the chicken burgers (Longato et al., 2017). The comparison of pumpkin peel, pulp, and seed powders, being used as substitutes of white flour in biscuits at levels of up to 15% has also been evaluated (Hussain, Kausar, Murtaza, et al., 2023). The authors reported that increasing the percentage of pumpkin powder resulted in greater cookie thickness, reduced width, and lowered spread factors. The ash, fiber, and fat content of the cookies significantly increased by adding more pumpkin powder. Pumpkin seed powder notably enhanced the protein quality of the cookies, comparable to casein-based formulations. Substitutions of 5-10% for all three pumpkin powders also yielded good scores in color, taste, texture, aroma, and overall acceptability (Hussain, Kausar, Murtaza, et al., 2023).

Apart from their use in flour and powders and their direct application in foods, pumpkin peels, seeds, and fibrous materials are also being explored for their ability to provide high-value bioactive compounds. These compounds, including polyphenols, carotenoids, and flavonoids, have demonstrated antioxidant, anti-inflammatory, and antimicrobial properties. In fact, Hussain et al. (2022) discussed the use of pumpkin to promote health in the post-COVID-19 period, proposing pumpkin and its by-products, in various forms such as powders, extracts, isolates, purified bioactives, and functional food products, in pharma foods as a key strategy to counter the impact of the disease. The authors described the functional constituents of pumpkin, such as vitamins C, E, and D, β-carotene, zinc, iron, selenium, polysaccharides, proteins (including peptides, amino acids, and enzymes), lipids (including fats, oils, fatty acids, and sterols), and phenolic acids, that have been linked to diverse pharmacological activities (e.g. antioxidant, immunomodulatory, anti-inflammatory, antihyperlipidemic, antiviral, antihyperglycemic, antihypertensive, and antimicrobial mechanisms).

In this sense, bioactive compounds obtained from pumpkin peels have been proposed as a treatment for the anthracnose caused by *Colletotrichum musae*, a significant post-harvest disease that leads to banana losses in Thailand. In addition to its important antioxidant activity, the hydromethanolic extract from pumpkin peels was able to delay the radial growth of *C. musae* during a 6-day incubation period compared to the control (dimethyl sulfoxide: DMSO). The authors suggested the use of this peel extract in

combination with fungicides as a strategy to reduce dependence on chemical treatments (Jarungjitaree & Naradisorn, 2019). In another study, pumpkin peel extract, combined with oregano essential oil, was incorporated into a cassava starch-based film to evaluate its physical, barrier, antioxidant, and antimicrobial properties for application in ground meat. The results were promising, maintaining low pH levels in the meat up to the sixth day of storage. Moreover, the developed films demonstrated both antioxidant and antimicrobial activities, the latter being effective against coliforms and *S. enteritidis* SE86 when applied to ground meat (Caetano et al., 2017). In addition, the hull-less pumpkin oil cake was tested in the production of edible film pouches to protect cold-pressed flaxseed oil. Although improvements are needed to address the interference with the sensory characteristics of the stored oil, the films demonstrated high quality. Compared to oil packed in PET/PE pouches and oil stored in control bottles, oil packed in pouches made with pumpkin oil cake showed lower peroxide values and lower levels of conjugated dienes and trienes, indicating a better protective influence of the biopolymer packaging materials. Films based on pumpkin oil cake also ensured good oxidative stability for the oil storage (Hromiš et al., 2022).

The recent emphasis on sustainable practices has also further encouraged the use of pumpkin by-products. These by-products are being explored not only for their nutritional contributions and bioactive properties but also as environmental solutions within the industry. Pumpkin peels have proven to be an excellent and economically viable substrate for the production of oxidative enzymes from *Aspergillus iizukae* EAN605 (Noman et al., 2020). In this study, the production of these enzymes was successfully optimized using a submerged fermentation process with pumpkin peels as the substrate. The enzymes produced were then effectively utilized for the decolorization of azo dyes, which are significant environmental pollutants. So far, azo dyes are widely used in various industries, including textiles, food, cosmetics, printing, and plastics, despite being significant environmental pollutants and associated with health impacts, such as allergic reactions, toxicity, and carcinogenicity (Noman et al., 2020). Following the issue of dyes, Che et al. (2023) proposed the extraction of dyes from pumpkin peel to be used as vegetable dyes in silk and cotton fabrics. In this research, carotenoid dyes extracted from pumpkin peels showed excellent thermal stability under normal dyeing conditions, as well as good stability in acidic and neutral environments. Moreover, Lima et al. (2021) produced a rich-carotenoid colorant from pumpkin peels with potential use in food, pharmaceuticals, and cosmetics.

Pumpkin by-products are also gaining traction in the field of cosmeceuticals and pharmacology. Bioactive compounds extracted from pumpkin seeds and peels can be used in skin care products, hair care formulations, and other cosmetic applications. These

compounds can help protect the skin from oxidative damage, promote wound healing, and improve overall skin health. In pharmacology, the antioxidant and anti-inflammatory properties of pumpkin extracts are being studied for their potential in the prevention and treatment of various chronic diseases. Regarding the last strand, pumpkin fruits with and without seeds were discussed for their use in the prophylaxis and treatment of diseases involving oxidative stress, including CVD (cardiovascular diseases), through the various bioactive compounds with antioxidant activities found in these matrices (Rolnik et al., 2020). Moreover, Ghahremanloo et al. (2018) observed that pumpkin extract ameliorates oxidative stress in obese rats, leading to a decreased risk of cardiovascular disease associated with obesity, when treating groups of obese rats with a daily dose of 100, 200 or 400 mg of hydroalcoholic pumpkin extract. On the other hand, Ahmed (2022) used pumpkin aqueous extract in the preparation of iron nanoparticles in the treatment of burns.

Targeting cosmetic industries, pumpkin seed oil has been reviewed for its use in anti-aging, protective, and hair care products, owing to its bioactive compounds such as tocopherols, phytosterols, and phenolic acids, which act as potent antioxidants (Wargala et al., 2023). The authors also highlighted its sunscreen effect, as it absorbs approximately 20% of harmful UVB rays. The pumpkin seeds oil was also used in a pumpkin seed oil-based vitamin E cream formulation (Ong et al., 2020). This incorporation showed synergistic effect with great antioxidant activity. Moreover, pumpkin seed oil was investigated for clinical efficacy in treating female pattern hair loss, where results were promising when compared to the standard treatment of minoxidil 5% foam (Ibrahim et al., 2021). Furthermore, the hydro-ethanolic extract of red pumpkin seed has been proposed to reduce melanosome transfer to keratinocytes by activating Nrf2 signalling (Endo et al., 2019). This extract has demonstrated benefits for skin care formulations aimed at lightening skin tone or reducing pigmentation. It leveraged the Nrf2 pathway to inhibit the transfer of pigment-producing organelles to skin cells, particularly by suppressing melanosome transfer stimulated by reactive oxygen species (ROS) generated following UVB exposure (Endo et al., 2019).

The use of pumpkin by-products aligns with the principles of a circular economy, integrating what would previously be discarded into the production cycle and thus minimizing the environmental impact. Ongoing research continues to discover new benefits and applications for these by-products, with scientists perfecting techniques such as the extraction and concentration of specific bioactive compounds to maximize their effectiveness and applicability. The development of innovative products incorporating these compounds is expected to grow, driven by consumer demand for natural ingredients that promote health. In conclusion, the exploitation of pumpkin seeds, peels and fibrous

materials for their nutritional and bioactive properties presents a promising path for sustainable development and innovation in the food and pharmaceutical industries. Using these by-products can reduce waste, increase product value, and contribute to better health outcomes for consumers.

1.2.6. Non-thermal processes

Thermal technologies for preserving food safely have been well established and widely used for centuries and these methods are recognized for their high level of microbiological safety. However, an additional aspect that has gained increasing significance among today's consumers is the impact of thermal processes on nutritional quality, appearance, and flavor (freshness), alongside their microbiological safety. In response to this demand, the new concepts of non-thermal technologies arised, bringing new ideas / methods for processing food that not only ensure the microbiological safety of products but also increase their nutritional value and improve other quality characteristics, including color, texture, and maximum preservation of freshness.

There are different non-thermal technologies that are nowadays applied for food processing, some of them already well-established in the food industry and other sectors, as high pressure processing (HPP), pulsed electric fields (PEF), among others, or others as ultrasound, ozone, ohmic heating, supercritical carbon dioxide technology (SC-CO_2), UV light, cold plasma (CP) etc. which are well established in and/or largely studied by the scientific community with a great potential for food applications. All these non-thermal technologies have in common the possibility to treat food without the application of high levels of temperature, which consequently brings advantages in the maintenance of food quality attributes.

Due to the characteristics of fresh products, i.e. a high water content, good source of nutrients and an attractive environment for the development of contaminating and pathogenic microorganisms, fruits and vegetables and their derivatives, such as juices, pulps, smoothies, are greatly benefited from the use of non-thermal technologies, which will be the main topic of the following section, with a focus on high pressure processing (HPP) and pulsed electric fields (PEF).

High pressure processing (HPP)

HPP is a method for food preservation that uses pressure ranging from 100 to 1000 MPa for few minutes, offering safety benefits, process uniformity, energy efficiency and low waste (Zheng et al., 2023). Pressure, temperature, and time are the main process parameters controlled, and the most common pressure medium transmitter is water. Pressure is transmitted in a hermetically closed vessel, and due to the isostatic principle,

equal pressure is received to all product parts, independently of size/shape of the food (Huang et al., 2020). During the pressurization, the adiabatic heating takes place with an increase in the water/product temperature in 3 °C for each 100 MPa (for water), while when considering the application of 600 MPa, it can be assumed that in this process conditions the maximal increase in temperature will be circa 18 °C; besides that, the pressure transfer medium can be recycled after processing (Rastogi et al., 2007).

Another important aspect for HPP processing is the packaging used, since the material characteristics need to possess good heat sealability integrity, barrier properties, resistance to pressure and flexibility to withstand volume changes (compression up to 15%), being able to return to its original shape after decompression without leaching undesirable packaging chemicals into the product (Hoque et al., 2022). Materials such as polyethylene terephthalate (PET), polyethylene (PE), polypropylene (PP), and EVOH can be used in isolation or in combination, that is, LDPE/PA/LDPE, LDPE/EVOH/LDPE, and PET/Al/PA/PP, respectively (Marangoni Júnior et al., 2020). Soft polymeric bags, cans, trays, and bottles are commonly used packaging materials for HPP in the food industry (Huang et al., 2020).

Due to the non-thermal conditions and the microbial inactivation capability, HPP is recognized as a cold pasteurization technique, since it inactivates contaminant and pathogens microorganisms, as well as some undesirable enzymes related to browning. Due to its success in food application, HPP is a well stablished technique in the food industry and has been approved as a non-thermal pasteurization technology by governmental authorities in the United States, Europe and Canada for reducing pathogens in fruit juices and vegetable products and extending their shelf-life (Huang et al., 2017).

Fruits and vegetables represent an important group of foods for HPP application due to their high water content and thermosensitive nutritional constituents, which have great potential for HPP treatment. The sensorial properties and nutritional qualities of the food product may remain the closest to the natural product after the processing as compared to the conventional thermal techniques (Rostamabadi et al., 2023). Besides that, HPP offers a low contamination risk due to its in-pack technique, i.e. the food is packed in the final package before treatment, which does not offer direct contact with the processing devices, during or after treatment, thus preventing the secondary and or/ cross contamination after treatment (Hoque et al., 2022).

In terms of quality attributes, HPP also has a small effect on low molecular weight compounds such as vitamins and pigments compared to thermal. Thus, the quality of the HPP treated foods are comparable to the fresh non-treated foods (Muntean et al., 2016), offering a reliable and stable source of nutrients, vitamins, minerals and health components (Houška et al., 2022).

HPP shows a significant effect on the bacterial inactivation due to its ability to induce changes in the cell membrane permeability and morphology as well as protein and enzyme denaturation (partial or complete), inhibition of protein or key enzymes synthesis and disruption of genetic mechanisms (Podolak et al., 2020; Sehrawat et al., 2021). However, several factors related to the process application i.e., pressure, temperature, pH, time, and medium characteristics (aw, salt and sugar content) (Podolak et al., 2020) will direct influence the effectiveness of the treatment for inactivation purposes (Georget et al., 2015). Besides that, at ambient temperature, HPP has minimal effects on bacterial spores (Ribeiro & Cristianini, 2021), thus, the application of high pressure for sterilization proposes is limited, therefore an alternative has been developed which involves the combination of high pressure and temperature, known as high pressure assisted thermal processing (HPTP) and or/ pressure assisted thermal sterilization (PATS), offering the advantage of short processing time due to the adiabatic compression heat, which is generated under pressure (Al-Ghamdi et al., 2020; Sevenich et al., 2015).

Pumpkin (*Cucurbita maxima* Duch) was investigated under different high-pressure conditions and time (350, 450 and 550 MPa for 0.5 – 30 min) at room temperature (Zhou et al., 2014). The authors found out that the higher the pressure treatment the higher was the inactivation ($p < 0.05$), with 3.16, 3.85 and 4.16 $\log_{10}$N reduction at 350, 450 and 550 MPa/5 min, respectively. After 45 days, the total plate count in pumpkin treated with 400 MPa and 500 MPa increased to 2.57 and 1.69 log10 CFU/g, respectively. The TPC in pumpkin treated with 550 MPa increased to 1.63 log10 CFU/g after 15 days; however, TPC increased slowly during 60 days, being lower than 2 log10 CFU/g. Besides the good microbial growth control, HPP shows a great performance in preserving the quality characteristics with retention of color, texture (hardness), vitamin C, antioxidant activity, and elevated total phenols being recorded immediately after processing (Zhou et al., 2014).

The effect of HPP on the physico-chemical and structural properties of plant tissues has been widely investigated, and pressure appears to carry out an important role on the cell, inducing microstructural changes and damage pressure depending on the vegetable tissues. Once the cell is damaged, cell components can be released, including enzymes such as pectin methyl esterase (PME) (Rinaldi et al., 2023).

The effect of HPP on the physico-chemical and structural properties of two pumpkin species (*C. maxima L.* var. Delica and *C. moschata Duchesne* var. Butternut) were also investigated up to two months. Regarding the microstructure modifications in comparison to control samples, at pressures of 200 and 400 MPa no difference was observed; however, 600 MPa affected the internal parenchyma cells (mesocarp), showing detachment of the cell membrane from the cell wall and a significant decrease in the

quantity of starch granules (Paciulli et al., 2019).

Pumpkin cubes (cv. *Violina rugose*) were investigated under pressure ranging from 100 to 600 MPa for 3 min. In comparison with the control samples, no significant differences were observed for moisture, total soluble solids, and pH. However, pumpkin tissues showed great structural modifications such as changes in cell size and shape, cell wall damage, increased cell wall thickness, cell detachment and dehydration, and calcium ions deposition when pressure of 300 to 600 MPa was applied. At 400 MPa, histological analysis indicated evident damage in the parenchyma when compared to less intense treatments (i.e. 200 and 300 MPa). At 500 MPa the parenchyma cells showed plasmolysis, gaps, and increased cell wall thickness (Rinaldi et al., 2023).

High pressure has an important effect on enzymatic profile of vegetable products, since pressure can alter quaternary and tertiary structures of enzymes (Huang et al., 2020; Terefe et al., 2014), which may directly affect its active site configuration (Leite Júnior et al., 2017). However, enzyme inactivation through HPP represents a challenge, since enzymes can exhibit higher resistance to high pressure conditions (Terefe et al., 2014), especially under ambient temperature. During pressurization, water can infiltrate the protein's interior, and pressure can induce conformational changes and lead to protein unfolding; therefore, the stability of proteins under high pressure is primarily influenced by their conformational flexibility, which allows them to adjust to the loss of non-covalent bonds resulting from the migration of water molecules (Eisenmenger & Reyes-De-Corcuera, 2009). Thus, it's crucial to study individually the enzymes to better comprehend its effects, since the inactivation/activation is strongly dependent on the pressure, temperature, pH, and matrix conditions in which the enzyme is present (Knorr et al., 2006). In pumpkin puree (cv. *Butternut*), the percentage of residual activity of polyphenoloxidase (PPO) was strongly dependent on the pressure applied, showing activation, inactivation or no effects. At 300 MPa/70 °C the activity of the enzyme increased by 12% compared to the control, while 300, 600 and 900 MPa treatments significantly reduced the PPO activity, and no difference was observed between 600 and 900 MPa (García-Parra et al., 2016).

Both technologies, HPP and PEF, have already been deeply investigated and continue to be studied in order to improve their benefits and overcome challenges. As non-thermal technologies in food processing, HPP and PEF stand out for allowing producing safe food products from a microbiological point of view and with minimal effects on quality attributes, such as freshness, color and sensorial properties. HPP has a consolidate market around the world, with hundreds of companies using the technology, mainly represented in North America and Europe, but also in Asia and South America showing a significant potential (Hiperbaric, 2023). A high initial investment may still

present a considerable challenge for the implementation of these technologies, especially for SME companies (small and medium-sized enterprises), since machinery costs, installation, training, and maintenance can be quite expensive. Besides that, dry products (powdered, spices, dried nuts / fruits) are not adequate for HPP processing, once isostatic pressure requires water in the food product for its homogeneous, uniform, and efficient transfer and, consequently, the desired inactivation of the microorganisms present in the product.

Pulsed electric field (PEF)

Pulsed electric field is a non-thermal technology which is based on the application of electric pulses of high voltage (0.1 – 100 kV/cm) and short duration (μs–ms) to the food through two electrodes in batch or in a continuous treatment chamber (Mahnič-Kalamiza et al., 2014). Pulsed electric field processing is considered a non-thermal technology, since it usually does not increase the temperature of food during processing or the temperature elevation is much lower when compared to thermal treatment (Yogesh, 2016). The intensity of the treatment is dependent on the objectives to be achieved; usually, for microbial inactivation proposes voltages of 15–80 kV/cm with several pulses of 1–5 μs are applied and 0.1–5 kV/cm with pulses of 10–1000 μs for electroporation of plant cells and non-thermal extraction from solid foods are recommended (Arshad et al., 2020).

The mechanism responsible for PEF efficiency effects on plant tissue or microbial inactivation is commonly attributed to the electroporation, a mechanism strongly dependent on the set parameters (i.e., electric field strength, energy input, pulse duration, treatment time) and the specific plant cell characteristics (i.e., cell size, thickness of membrane; shape, composition, orientation in the electric field). Additionally, the cell electroporation can be reversible or irreversible (Yogesh, 2016). Intensities of 0.5–1.5 kV/cm can be used for the induction of stress responses and reversible electroporation; 1.0–3.0 kV/cm are used for irreversible permeabilization in plant or animal tissues; and 15–40 kV/cm are applied for microbial inactivation (Raso et al., 2016). Although the mechanism of electroporation is not fully understood, it is strongly believed that, due to membrane permeabilization, it will lead to an increase in the permeability of the cell membrane to ions and molecules (Kotnik et al., 2012). In terms of microbial inactivation, electroporation affects the biological function of the membrane, which acts as a semi-permeable barrier responsible for the transfer of biological molecules; besides that, it also affects the synthesis pathways of RNA, DNA, proteins and other essential cell components (Yogesh, 2016).

PEF has been extensively studied to increase the yield of extractions of bioactive compounds of interest, such as carotenoids (García-Parra et al., 2018), anthocyanins

(Pataro et al., 2017), polyphenols (Carullo et al., 2024), vitamins and proteins (Ramaswamy et al., 2024). PEF has the advantage of allowing an increase in the extraction yield with reduced energy costs, the preservation of heat-sensitive substances, since the electroporation of plant tissue with its non-thermal characteristics enables the preservation of natural quality, color and vitamin composition of food products, thus being considered a "green" extraction method (Chemat et al., 2017), while additionally it avoids the use of harsh chemicals, heavy solvents, acids, alkalis. In PEF-treated (pulse width of 3.0 ms, treatment time of 144.6 ms, voltage of 25 kV/cm) carrot juice, greater levels of α-carotene, i.e. ≤ 37.40 mg/mL were noted compared to the thermal process (TP; 90 °C/1 min) i.e. 12.99 mg/mL. The same tendency was observed for ascorbic acid content, where PEF samples had 14.5 mg/100mL compared to 4.4 mg/mL in TP. On the other hand, no difference ($p < 0.05$) was observed for microbial inactivation (total aerobic count, molds and yeasts) between TP and PEF treatments (Xiang et al., 2014).

The use of PEF technology has also been investigated as a promising approach for preserving the fresh characteristics of juice through inactivation of microorganisms and enzymes, i.e. pectinolytic enzymes, which play a fundamental role in the fruit juice industry (Roobab et al., 2022). Depending on the particular enzyme, the medium where it is suspended and the PEF conditions (electric field strength, treatment time, number of pulses, pulse width, field polarity, frequency and treatment temperature), most enzymes are greatly inactivated, although some may exhibit resistance to PEF processing (Martín-Belloso & Elez-Martínez, 2005).

PEF (2 kV/cm, 20 monopolar pulses of 4 μs at a frequency of 0.1 Hz) effects were studied in pumpkin cv. *Butternut* slices. The process condition showed no effect on the PPO inactivation and no color change compared to control samples. On the other hand, PEF treated samples showed a higher content of carotenoids (lutein, α- and β-carotene) compared to the non-treated ones, e.g. 4.73 mg/100 g and 3.44 mg/100 g, respectively for total carotenoids (García-Parra et al., 2018). According to the authors, the PEF conditions applied induced an abiotic stress, thus increased the production of some bioactive compounds. Similarly, PEF has been proposed as a promising tool to induce stress in horticultural crops, thus triggering the accumulation of phenolic compounds during postharvest life, as well reported by López-Gámez et al. (2020), where PEF (5 pulses of 350 kV m^{-1}/580 ± 80 J kg^{-1}, frequency of 0.1 Hz) influenced phenolic compounds content, respiration rate, volatile organic compounds production and enzyme activities of carrots, 24 h after treatment, with total phenolic content being enhanced by 80.2%. After 12 h of storage, PME activity increased by 164% compared to untreated carrots, whereas for PG (polygalacturonase) a decrease of activity by 31% was observed compared to the untreated ones.

In coconut water, PEF (22.5 kV/cm, 119 kJ/L, 40 °C) showed promising results not only for microbial inactivation but also for quality attributes. An inactivation of *Escherichia coli* K12 and *Listeria innocua* by a 6.60 and 5.90 log CFU/mL, respectively, was noticed, resulting in a 35 days extension of shelf life. Due to PEF application, a disruption in the in cell membrane was exhibited on the bacterial surface as well as changes in the form and rough cell surfaces of both *E. coli* K12 and *L. innocua*. Besides that, the pink color development was delayed by 7 days compared to thermal treatment (85°C/10 min) (Tongdonyod et al., 2023).

Pulsed electric field (PEF) has also been investigated as a pretreatment to reduce the duration of drying process and consequentially reducing its energy demands. Different products have been studied for this propose, including pumpkin (*C. pepo* L.) in pieces of rectangular parallelepiped shape (2.0×2.0×0.5 cm). According to the results, 1500 pulses at electric field strength intensity of 2.0 kV/cm (processing time of 0.01 s), with a pulse width of 15 μs and a frequency of 20 Hz, caused 90% cell rupture (or Zp = 0.9), which allowed the assessment of tissue damage degree, evaluating intercellular changes and effectiveness of PEF as a pre-treatment. PEF reduced the drying time by 12% ($p < 0.05$), and when combined with osmotic dry (OD) the maximum reduction of processing time by 27% was observed (compared to untreated sample). When PEF pretreatment was combined with OD prior to air drying, the energy was 50% lower than the respective energy required for non-processed samples, showing the great potential of PEF as a pretreatment of processing (Paraskevopoulou et al., 2022).

PEF is a promising non-thermal food preservation technology, which faces some challenges and limitation concerns related to regulatory considerations, consumer acceptance, potential safety concerns, optimization, treatment reproducibility, poor description of the operating protocols, in addition to the lack of a standardized way of reporting treatment conditions (Raso et al., 2016). Besides that, the electrochemical reactions and corrosion that occur at the electrode-food interface appear as another important limitation of the technology, as a result of the current that flows through the PEF treatment chamber which may allow metal release from electrodes (Pataro & Ferrari, 2020) and may interfere with the chemical and sensorial properties of food-treated products (Zhao et al., 2012).

1.2.7. Conclusions

The sustainable valorization of pumpkins and their by-products offers significant opportunities to improve food systems within the circular economy. This review highlights the importance of addressing several interconnected dimensions – agronomic optimization, nutritional profile, chemical composition, health benefits, innovative

applications and advanced processing techniques. This knowledge is of huge importance for further advances to fully realize the potential of pumpkin valorization.

A key challenge lies in standardizing agronomic practices to maximize yield and nutrients content across different regions and environmental conditions. In addition to the traditionally consolidated cultivars, future research should focus on developing cultivars with greater resilience to climate variability and higher nutrient density, with a focus on local cultivars. Furthermore, sustainable cultivation practices should be encouraged to minimize environmental impacts and align with circular economy principles.

The nutritional profile of pumpkin pulp and by-products showed promising results; however, comprehensive analyses are needed to better understand the bioavailability of nutrients and their interactions. These insights may pave the way for the development of functional foods and nutraceuticals tailored to specific health needs. Although pumpkin seeds have already received commercial interest, mainly related to their oil content, seeds and peels remain underutilized in the food industry, despite their nutritional and bioactive potential. The application of pumpkin by-products has been reviewed, but research studies and technological advances should focus on cost-effective methods to extract compounds of interest and establish robust value chains for these by-products thus reducing waste and increasing economic viability.

Non-thermal processing techniques, especially HPP and PEF, have shown potential in preserving the nutritional and functional qualities of pumpkin products. Future studies should aim to optimize these technologies in terms of scalability and cost-efficiency. Furthermore, exploring synergistic effects between non-thermal methods and other preservation techniques can further improve product quality and shelf life.

In addition to their nutritional and functional roles, pumpkins and their by-products can contribute to environmental sustainability by reducing food waste and promoting resource efficiency. Integrating pumpkin valorization into local and global food systems can support sustainable development goals, particularly those related to food security, health safety, and environmental protection. In conclusion, the comprehensive utilization of pumpkins within the context of circular economy requires a multidisciplinary approach involving agronomy, food science, technology, and policy. Collaborative efforts among researchers, industry stakeholders, and policymakers are essential to overcome existing barriers and unlock the full potential of pumpkin valorization. By addressing these challenges and leveraging emerging opportunities, pumpkins can become a cornerstone of sustainable food systems worldwide.

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1.2.8. References

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1.3. Real case of butternut squash pulp product in Porto-Portugal

1.3.1. Real case of butternut squash pulp product in Porto-Portugal

Butternut squash (*Cucurbita moschata* Duch.), a member of the Cucurbitaceae family, is a widely cultivated and economically significant crop known for its adaptability to diverse climatic conditions. Although the production of the specific pumpkin variety of this study is not estimated individually, FAO data reports approximately 24 million tons harvested from 1.5 million hectares globally in 2021, from the aggregate of pumpkins, squash and gourds (FAO, 2021). However, the significant impact of butternut squash within this category is highlighted by data from South Africa: in 2018, a total of 191,000 tons of *C. moschata* were sold, generating revenue of 303 million South African Rand (ZAR). This figure is notably higher than the combined 78,000 tons of *Cucurbita maxima* (pumpkin and hubbard squash), which accounted for 186 million ZAR (Swanepoel, 2021). Furthermore, Ugrinović et al. (2021) reviewed that the yield of butternut squash varied from 19.4 to 34.9 t/ha for different cropping densities, from 18.9 to 31.3 t/ha for different chemical and organic fertilizers in the agro-ecological conditions of Iraq, and from 59.4 to 79.8 t/ha for different irrigation and mulching regimes in the agro-ecological conditions of Northern India.

According to Centre for the Promotion of Imports from developing countries (CDI), in Europe, Spain leads the production of pumpkins, squash, and gourds, with an annual production of approximately 700,000 to 800,000 tons. This significant production caters to both the domestic and European markets, positioning Spain as a key player in the pumpkin and squash sector. Additionally, Spain is the continent's seventh-largest importer of pumpkins, squash, and gourds from developing countries, ranking just behind Belgium (CDI, 2024). Given its economic relevance in Portugal and Spain, a seed company, together with 200 Portuguese and Spanish producers, joined forces to create a brand to promote squash, called 'Love Butternut'. According to the initiative (Love Butternut, 2021), Portugal is the third largest producer of pumpkin in Europe, just behind Spain and France, with a per capita consumption of 0.85 kilos per year and a production volume of 60 thousand tons. Of this, 90% of the butternut squash produced in Portugal is destined for export. In addition, they claim that the number of new pumpkin-based products is increasing in Europe, with more than 500 new innovations launched since 2016, and that the project has contributed to an increase in pumpkin sales of around 20% in Portugal since its launch in 2021 (Agroportal, 2024; Love Butternut, 2021). These numbers underscore the economic relevance of butternut squash within local and global agricultural markets.

From an agronomic perspective, this variety, as well as other *Cucurbita* species, that typically share similar growth requirements, is characterized by its wide adaptability to climatic and soil conditions, making it an excellent candidate to face the negative

impacts of climate change on agricultural crops (Salehi et al., 2019). This has increased the interest of the academic community in optimizing fruit growing conditions in different contexts, e.g. understanding the effect of soil regimes subject to water and saline stress (R. A. R. Restrepo et al., 2023; Tarchoun et al., 2022), as well as in the implementation of genetic tools to combat infections in the plant (Fu et al., 2024), in addition to assessing their adaptability to organic farming systems, aligning with the increasing demand for sustainable agricultural practices (Armesto et al., 2020).

This interest is combined with the potential of butternut squash as a nutrient-rich food source with a significant role in combating malnutrition and promoting food security worldwide. The nutritional and chemical composition as well as the medicinal properties discussed above (see **Section 1.2**) make butternut squash an important component of healthy diets (Armesto et al., 2020; El Khatib & Muhieddine, 2020; Fedha et al., 2010; Jarret et al., 2013; Kim et al., 2012; Petkova & Antova, 2015; Quintana et al., 2018; Veronezi & Jorge, 2015). Furthermore, pumpkin's versatility in culinary applications, ranging from soups and purees to baked goods and beverages, has positioned it as a staple in both traditional and modern cuisines. Its nutritional properties, combined with its affordability and long shelf life, contribute to its widespread acceptance and integration into global food systems (Dhiman et al., 2020; Mala et al., 2018; Marcel et al., 2022; M. V. Restrepo et al., 2023).

In Portugal, pumpkin is widely used in the production of a variety of food products, both traditional and processed. Data from commercial sources and local markets indicate that available products include pumpkin jams and sweets, traditional pumpkin cakes, and pumpkin sausage, a typical delicacy from Montalegre. In addition, pumpkin seeds are sold as snacks or for culinary use, while pumpkin puree, available in organic versions, is used both in baby food and in various recipes. Specifically, a product made from butternut squash pulp is marketed for use as a ready-to-use filling in sweets and other products. Produced in Porto, this product is primarily made from processed pulp and starch, with its safety ensured through pasteurization and preservation using the artificial preservative potassium sorbate (E202). This gel-textured product is then marketed to secondary customers in airtight packaging, offering a shelf life of up to 30 days.

Motivated by this product, the present study identified two key areas for improvement aimed at enhancing sustainability and promoting health: the valorization of butternut squash by-products derived from pulp processing, and the replacement of the artificial preservative with a natural ingredient and/or industrial processing methods that eliminate the need for preservatives. Both aspects are discussed in detail below.

Valorization of butternut squash by-products

Industrial processing of butternut squash for applications such as canning, pureeing, and baby food generates substantial amounts of bioresidues, including peels, seeds, and fibrous strands. Despite the lack of concrete data on the generation of industrial by-products from butternut squash, in their review, Rico et al. (2020) estimated that pumpkin processing results in approximately 2–16% of peels and 3.1–4.4% of seeds, with 72–76% of the fruit being used as pulp. This means that approximately 25% of the fruit is typically discarded, representing food waste, economic losses, and a challenge to sustainability efforts. However, these bioresidues represent an underutilized source of value-added compounds with significant potential for valorization (see **Section 1.1** and **1.2**).

The sustainable recovery of bioactive compounds from butternut squash residues aligns with the principles of the circular economy by transforming biowaste into valuable resources. Despite its potential, both the recovery of these compounds and their application to other matrices are still very scarce. Few studies focus on *C. moschata* when compared to *C. maxima* and *C. pepo*, as can be seen in the studies above (**see Section 1.2**), despite their demonstrated commercial relevance.

Replacement of the artificial preservative potassium sorbate

Replacing artificial preservatives, such as potassium sorbate, with healthier natural alternatives is a growing trend in the food industry, driven by the demand for safer and more sustainable products. Potassium sorbate, widely used for its efficacy against microorganisms, has been associated with potential adverse reactions in sensitive individuals, in addition to contributing to consumers' negative perception of artificial additives (Carocho et al., 2014; Mamur et al., 2010; Martínez-Pineda et al., 2021). Natural alternatives, such as plant extracts rich in bioactive compounds offer antimicrobial and antioxidant properties while aligning products with consumers' health and well-being expectations (Chauhan & Rao, 2024; Fink & Filip, 2021; Karimpour et al., 2021). In addition, the use of innovative industrial methods, such as barrier technologies and non-thermal technologies treatments (such as high pressure processing), can reduce or eliminate the need for artificial preservatives (Huang et al., 2017; Roobab et al., 2022), promoting not only public health but also more sustainable practices in food production.

1.3.2. Concluding remarks

In this sense, the work of this thesis seeks to enhance the value of pumpkin and its by-products in a collaboration between academia and industry to establish a more sustainable value chain for this fruit, which supports the obtaining and commercialization

of products derived from this biowaste. By fully utilizing its bioresidues, it's possible to increase its economic value, reduce food waste and contribute to the development of sustainable food systems. This approach not only addresses environmental and resource efficiency goals, but also supports the creation of innovative products that meet the growing demand for sustainability in the food sectors as well as the consumption of healthier products.

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CHAPTER 2

Objectives and Organization

This chapter presents the main general and specific objectives of the research, as well as details of the fundamental tasks that guided the development of the study.

2.1. Objectives

The main objective of this PhD work was to stimulate a value chain with innovative processes that cover all stages of development of the pumpkin fruit pulp formulation functionalized with a natural preservative extracted from pumpkin by-products. The overall process is aligned with sustainability principles and new processes/technologies throughout all stages: pumpkin cultivation; extraction of active ingredients; evaluation of preservation capacity, processing and long-term stability of the final product. The development of the proposed project was achieved through research activities in the following specific objectives:

I. Characterization of the chemical and nutritional profile of the pulp of different pumpkin genotypes;
II. Chromatographic characterization of the phytochemical composition of the pumpkin by-products;
III. Evaluation of the bioactive potential of the by-products extracts, in terms of antioxidant and antimicrobial capacity, and cytotoxic effects;
IV. Optimization of the extraction condition to obtain preservative compounds using two different techniques, namely heat- and ultrasound-assisted extraction;
V. Development of different pumpkin pulp formulations preserved with natural ingredients;
VI. Use of high pressure processing as a non-thermal technology in the formulations;
VII. Evaluation of the capacity of natural ingredients to maintain the microbiological and physical-chemical quality of the pulp during storage;
VIII. Test the acceptance of the final pumpkin pulp formulation by consumers.

2.2. Tasks plan

Main task 1: The nutritional and chemical profiles, as well as the functional properties of the fruit pulp, were investigated in different local varieties and commercially available genotypes, grown in Greece under the same condition.

Main task 2: The by-products (seeds, peels and fiber strands) of different pumpkin genotypes were evaluated for their phytochemical characterization, revealing their chromatographic profiles of phenolic compounds, organic acids and tocopherol, as well as their bioactivities, in terms of antimicrobial capacity, antioxidants and cytotoxic effects.

Main task 3: The peels of three selected pumpkin genotypes grown in Greece and of the butternut squash commercialized in Portugal were subjected to an extraction optimization study for obtaining preservative compounds. For this purpose, a conventional technique (heat-assisted extraction) and an alternative technique (ultrasonic-assisted extraction) were used, employing different extraction times, temperatures/power and ethanol percentages, and designed by a response surface methodology (RSM).

Main task 4: Different pumpkin pulp formulations were obtained with total or partial replacement of the artificial preservative potassium sorbate per natural ingredient obtained from pumpkin by-products. The formulations were evaluated during the extended shelf life of 45 days for maintenance of the microbiological and physicochemical quality of the pulp during storage.

Main task 5: The application of high pressure processing at ambient temperature, combined with natural ingredient extracted from the pumpkin peel, was performed to increase the shelf life of pumpkin pulp without the use of chemical preservatives (potassium sorbate) or elevated temperatures (pasteurization or sterilization). The formulations were evaluated during the extended shelf life of 45 days for maintenance of the microbiological and physicochemical quality of the pulp during storage.

Main task 6: The final pumpkin formulation from Task 4 was tested for consumer acceptance, for the attributes of color, taste, aroma, texture pleasantness, and the overall acceptance, alongside the traditional formulation containing potassium sorbate as a preservative.

CHAPTER 3

Cucurbita spp. Characterization

This chapter consists of three articles that present the results of the characterization of different pumpkin varieties (*Cucurbita* spp.). It includes the phytochemical and bioactive characterizations of pumpkin by-products, together with the nutritional and chemical profiles and functional properties of different pumpkin cultivated genotypes.

3.1. Biological activity of pumpkin byproducts: Antimicrobial and antioxidant properties

molecules

MDPI

Article

Biological Activity of Pumpkin Byproducts: Antimicrobial and Antioxidant Properties

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Abstract: Pumpkin fruits are widely appreciated and consumed worldwide. In addition to their balanced nutritional profile, pumpkin species also present valuable bioactive compounds that confer biological and pharmacological properties to them. However, the seeds, peels, and fibrous strands resulting from pumpkin processing are still poorly explored by the food industry. The current study used those fruit components from the genotypes of pumpkin that are economically significant in Portugal and Algeria to produce bioactive extracts. In order to support their usage as preservatives, their phenolic content (HPLC-DAD-ESI/MS) and antioxidants (OxHLIA and TBARS) and antimicrobial properties (against eight bacterial and two fungal strains) were assessed. In terms of phenolic profile, the peel of the Portuguese 'Common Pumpkin' showed the most diversified profile and also the highest concentration of total phenolic compounds, with considerable concentrations of (-)-epicatechin. Regarding the antioxidant capacity, the seeds of 'Butternut Squash' from both countries stood out, while the fibrous strands of Portuguese 'Butternut Squash' and the seeds of Algerian 'Gold Nugget Pumpkin' revealed the strongest antimicrobial activity. The bioactive compounds identified in the pumpkin byproducts may validate their enormous potential as a source of bio-based preservatives that may enhance consumers' health and promote a circular economy.

Keywords: biologically active compounds; phenolic profile; antimicrobial activity; antioxidant activity; pumpkin byproducts; bio-based food preservatives.

3.1.1. Introduction

Natural matrices have been increasingly investigated as sources of bioactive molecules, not only for their benefits for human health, but also for their technological functionalities in food and cosmetic products (Besrour et al., 2022; Ceccanti et al., 2022; Jesus et al., 2019; Rocchetti et al., 2020; Takwa et al., 2018). Their wealth in such compounds has been widely demonstrated over the last decades, as have their bioactive properties. Nevertheless, with the current lifestyle of modern societies, vegetable-based meals are often limited to practical solutions as ready-to-use foodstuffs. With the increasing demand for these products, a considerable amount of byproducts are generated in the food industry, where distinct parts of plants, vegetables, and fruits are simply discarded along the process.

To promote the sustainability of these processes, recent studies have been focusing on the recovery of distinct byproducts for the extraction of high value-added compounds (Nguyen et al., 2021; Roriz et al., 2022). As examples, a great profile of bioactive compounds was reported for the extracts of sweet potato leaves, which were mainly composed of phenolic compounds with related antioxidants, anti-mutagenic, antidiabetic, anticancer, and anti-inflammatory activity, as well as heart and hepatoprotection properties (Nguyen et al., 2021). In another study, antibacterial and antioxidant activities were shown by the betacyanin-rich extracts of red pitaya peels (Roriz et al., 2022).

A common process in the food industry is the production of pumpkin pulp formulations, which generates high volumes of byproducts such as peels, seeds, and fibrous strands. This fruit is appreciated worldwide for its pleasant taste and nutritional properties, and is a source of carbohydrates, protein, fat, vitamins, and minerals. Additionally, it also presents diuretic, antirheumatic, stimulant, anti-inflammatory, antidiabetic, antidepressant, and antioxidant properties, among many other beneficial effects well-reported in the literature (Adnan et al., 2017; Dhiman et al., 2009; Kaur et al., 2020; Ratnam et al., 2017; Sharma et al., 2020). Despite the fact that distinct parts of the fruit can be consumed, the pulp is more appreciated, while the byproducts are often discarded or underutilized. However, these fruit parts can present important contents of value-added compounds such as minerals, polyunsaturated fatty acids, tocopherols, polyphenols, carotenoids, and phytosterols (Amin et al., 2020; Mala & Kurian, 2016; Ratnam et al., 2017; Rico et al., 2020; Villamil et al., 2022). For instance, *Cucurbita pepo* species seed oil from Pakistan revealed high nutritional components, including proteins, minerals, and unsaturated fatty acids, mainly linoleic and oleic acids, in addition to effective inhibitory activity against the gram-positive bacterium *Staphylococcus aureus* (Adeel et al., 2014). In another study, *Cucurbita maxima* seed oil compounds were associated with high protection against oxidative stress, among which six phenolic

compounds were detected, with the prevalence of syringic acid, in addition to tocopherols and sterols, especially *delta*-tocopherol and *beta*-sisosterol, respectively (Rezig et al., 2012). Pumpkin skin and seeds were also reported as presenting high contents of total phenolic compounds and a strong antioxidant potential, evaluated through different chemical assays (Yang et al., 2022). Moreover, pumpkin rinds and seeds were applied to bakery products to increase their antioxidant capacity and total phenolic concentration (Nyam et al., 2013). Given the chemical composition of these byproducts and their important antioxidants and antimicrobial capacities, they could find useful application in the development of natural food preservatives.

In order to scientifically demonstrate this potential, the current study evaluated the byproducts (peels, fibrous strands, and seeds) from three Portuguese ('Butternut Squash', 'Common Pumpkin', and 'Kabocha Squash') and three Algerian ('Butternut Squash', 'Gold Nugget Pumpkin', and 'Musquée de Provence') genotypes of pumpkins. The HPLC-DAD/ESI-MS was used to investigate the hydroethanolic extracts' phenolic content, while further bioactivities, namely the antioxidant, antibacterial, antifungal, and cytotoxic properties, were assessed in order to determine their potential to be used as natural food preservatives

3.1.2. Materials and methods

Sample preparation

The 'Butternut Squash', 'Common Pumpkin', and 'Kabocha Squash' genotypes of pumpkin fruits cultivated in Portugal and 'Butternut Squash', 'Gold Nugget Pumpkin', and 'Musquée de Provence' cultivated in Algeria were obtained in local markets of both countries at the end of the summer season. The samples were prepared by separating the pulp from the by-products, which were divided into peels (thickness < 150 mm), fibrous strands, and seeds. The samples were then lyophilized (FreeZone 4.5, Labconco), crushed, and extracted by maceration. Briefly, 2 g of powdered sample was extracted with 60 mL of an ethanol solution (ethanol:water, 80:20) at room temperature, with magnetic stirring, for 60 min. The extracts were filtered, and this procedure was repeated with the residue. For the combined extracts the ethanol was vacuum evaporated at 45 °C, and the residual water was lyophilized to dryness for subsequent analyses.

Characterization of the phenolic compounds profile

The phenolic composition was assessed by high performance liquid chromatography coupled to a diode array detector and electrospray ionization—mass spectrometry (HPLC-DAD-ESI/MS), following the methodology described by Barros et al. (2013). The chromatographic data were acquired using a Dionex Ultimate 3000 UPLC

(Thermo Scientific, San Jose, CA, USA), coupled to a diode array detector (280 and 370 nm) and an electrospray ionization mass detector (Linear Ion Trap LTQ XL, Thermo Finnigan, San Jose, CA, USA), working in the negative mode. The chromatographic separation was performed using aWaters Spherisorb S3 ODS-2 C18 (3 m, 4.6 mm 150 mm, Waters, Milford, MA, USA) column at 35 °C. The identification was performed by comparison with available standards or literature data; the quantification was achieved using the equations presented in the table footnotes. The results are presented in mg/mL.

Bioactive performance

To evaluate the antioxidant potential, two cell-based assays were applied, namely OxHLIA in sheep erythrocytes (Takebayashi et al., 2012) and TBARS in porcine brain homogenates (Barros et al., 2010). The extracts' capacity to inhibit the oxidative hemolysis was assessed in sheep blood erythrocytes and the extract concentration able to promote a delay of 60 min on the hemolysis was calculated based on the Ht_{50} values of the hemolytic curves for each concentration of extract. The results were expressed as IC_{50} values (µg/mL), which give the extract concentration required to keep 50% of the erythrocyte population intact for 60 min. On the other hand, the capacity to inhibit the TBARS formation was tested using porcine brain cells as oxidizable biological substrates and the results were expressed as IC_{50} values (µg/mL), meaning the extract concentration responsible for 50% of antioxidant activity. Trolox was used as a positive control in both assays. The antimicrobial activity was tested against two fungi and eight bacteria of interest in food contamination, following the methodology described by Heleno et al. (2013), using the p-iodonitrotetrazolium chloride (INT) method Kuete et al. (2011). The microrganisms (Frilabo, Porto, Portugal) assessed were *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), *Staphylococcus aureus* (ATCC 25923), *Aspergillus fumigatus* (ATCC 204305), and *Aspergillus brasiliensis* (ATCC 16404). The results are presented as IC_{50} values, in mg/mL.

Furthermore, the cytotoxicity was tested in a primary culture of non-tumor porcine liver cells (PLP2) obtained from a freshly harvested porcine liver (purchased from a local slaughterhouse), by the Sulforhodamine B (SRB) colorimetric assay (Guimarães et al., 2013). Ellipticine was used as positive control and the results were presented as IC_{50} values (extract concentration inhibiting 50% of the net cell growth), in µg/mL.

Statistical analysis

All samples were analyzed in triplicate and the results were expressed as mean ± standard deviation. For the comparison of only two groups of data, a student's *t*-test was

applied, while for more groups, the one-way analysis of variance (ANOVA) was used. For that purpose, the normal distribution and the homogeneity of variance of data were evaluated by the Shapiro Wilk's and Levene's tests, respectively. The Tukey's honestly significant difference (HSD) test was applied for homoscedastic data ($p > 0.05$) and Tamhane's T2 multiple comparison test was for the heteroscedastic data. The tests were performed at a 5% significance level using SPSS Statistics software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA: IBM Corp).

3.1.3. Results and discussion

Phenolic compounds profile

The chromatographic characterization of phenolic compounds, regarding UV-vis at the maximum absorption, deprotonated ion, mass fragmentation, and tentative identification of the hydroethanolic extracts of Portuguese and Algerian pumpkin byproducts are described in **Table 9**. Eight compounds were found, belonging to the phenolic acids (peak 3), flavan-3-ols (peak 1), and flavonoids (peaks 2, 4, 5, 6, 7, and 8) families. As examples, **Figure 4** and **Figure 5** show the phenolic profile obtained for Portuguese 'Common Pumpkin' seeds and Algerian 'Gold Nugget Pumpkin' peel, respectively.

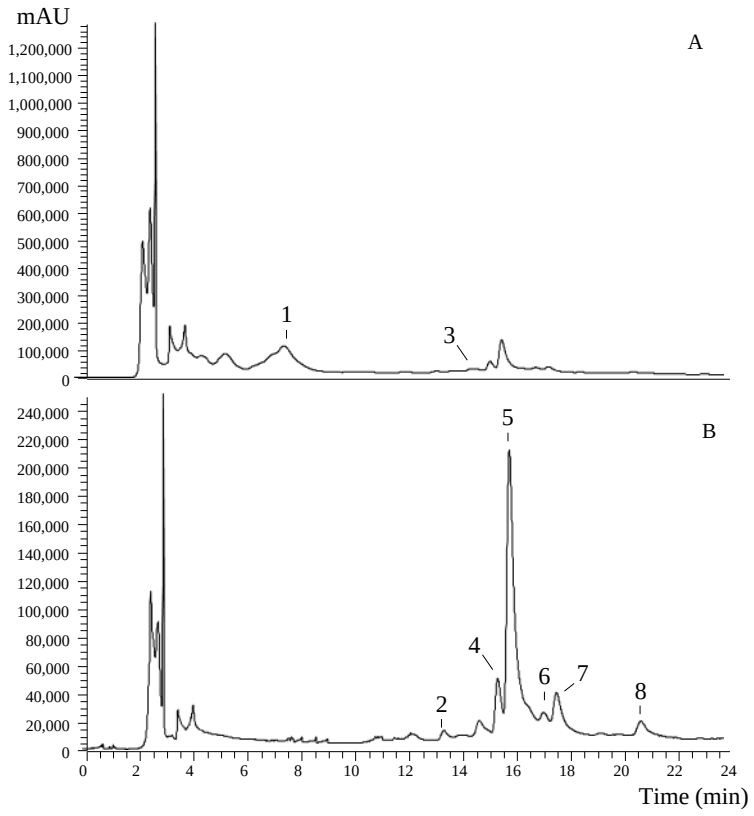

Figure 4. Portuguese 'Common Pumpkin' seeds chromatogram, recorded at 280 nm (**A**) and 370 nm (**B**).

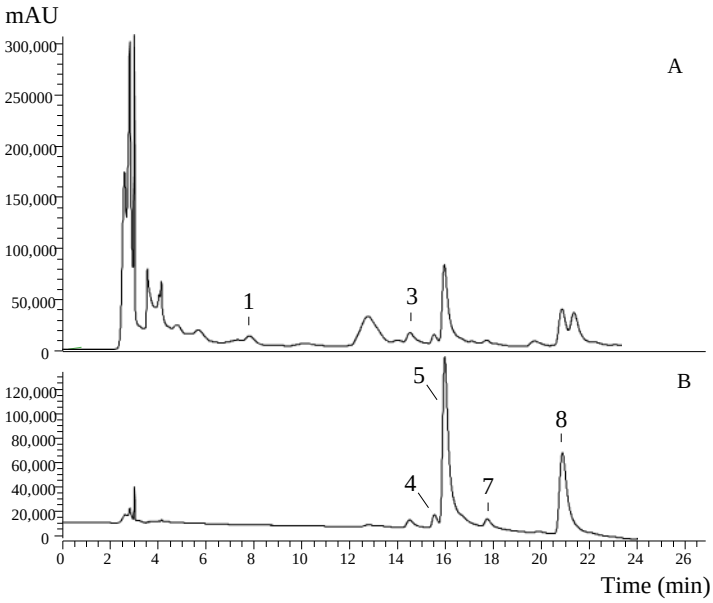

Figure 5. Algerian 'Gold Nugget Pumpkin' peel chromatogram, recorded at 280 nm (**A**) and 370 nm (**B**).

Table 9. Phenolic compounds characterized by HPLC-DAD-ESI/MS in the different samples of pumpkin.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative Identification
1	7.71	280	289	245 (100), 205 (45)	(-)-Epicatechin
2	13.42	345	775	301 (100)	Quercetin-*O*-dideoxyhexosyl-hexoside
3	14.73	263	405	281 (100), 137 (12), 93 (5)	7 4-*O*-(6′-*O*-Glucosyl-4″-hydroxybenzoyl)-4-hydroxybenzyl alcohol
4	15.42	344	739	285 (100)	Kaempferol-*O*-dideoxyhexosyl-hexoside
5	15.85	354	769	315 (100)	Isorhamnetin-*O*-dideoxyhexosyl-hexoside
6	17.12	348	593	285 (100)	Kaempferol-*O*-deoxyhexosyl-hexoside
7	17.6	365	623	315 (100)	Isorhamnetin-*O*-deoxyhexosyl-hexoside
8	20.73	365	623	315 (100)	Isorhamnetin-*O*-deoxyhexosyl-hexoside

Peak 3 presented a deprotonated ion $[M-H]^-$ at *m/z* 405 and a major MS^2 fragment at *m/z* 281 that corresponded to the loss of the 4-hydroxybenzyl alcohol moiety (124 Da); it also produced MS^2 fragments at *m/z* 137 (hydroxybenzoic acid) and *m/z* 93 (loss of glucosyl residue and CO_2). These chromatographic responses were in accordance with those previously described by Jaiswal & Kuhnert (2014) and the peak was tentatively identified as 7 4-*O*-(6′-*O*-glucosyl-4"-hydroxybenzoyl)-4-hydroxybenzyl alcohol. It is also important to state that this compound was found in *Lagenaria siceraria* Stand. (Bottle Gourd) (Jaiswal & Kuhnert, 2014) that belong to the *Cucurbitaceae* family, as pumpkins.

Peak 1 ($[M-H]^-$ at *m/z* 289) was identified as (-)-epicatechin by comparing the retention time, UV-vis at the maximum absorption ($\lambda_{máx}$ 280 nm), and mass spectra with the available standard compound. These compounds were previously described in *Cucurbita moschata* samples from Australia (Yang et al., 2022).

The family of flavonoids was the most abundant in terms of the number of compounds detected, mainly *O*-glycosylated derivatives of quercetin, kaempferol, and

isorhamnetin, as previously described by Iswaldi et al. (2013) in *Cucurbita pepo* L. The detected compounds could be divided into two groups, the first one presented two sugar moieties linked to the flavonoid aglycone (peaks 6, 7, and 8), and the second one presented three sugar moieties (peaks 2, 4, and 5). Peaks 6 ($[M-H]^-$ at *m/z* 593) and 7/8 ($[M-H]^-$ at *m/z* 623) presented only one MS^2 fragment at *m/z* 285 (kaempherol aglycone) and *m/z* 315 (isorhamnetin aglycone), respectively, corresponding to the jointed loss of a deoxyhexosyl and hexosyl moiety ($[M-H-146-162]^-$), being tentatively identified as kaempferol-*O*-deoxyhexosyl-hexoside and isorhamnetin-*O*-deoxyhexosyl-hexoside, respectively. Finally, peaks 2 ($[M-H]^-$ at *m/z* 775), 4 ($[M-H]^-$ at *m/z* 739), and 5 ($[M-H]^-$ at *m/z* 769) also presented a unique MS^2 fragment at *m/z* 301 (quercetin aglycone), *m/z* 285, and *m/z* 315, respectively, that corresponded to the loss of two deoxyhexosyl moieties and one hexosyl moiety ($[M-H-146-146-162]^-$), being tentatively identified as quercetin-*O*-dideoxyhexosyl-hexoside, kaempferol-*O*-dideoxyhexosyl-hexoside, and isorhamnetin-*O*-dideoxyhexosyl-hexoside, respectively.

As shown in **Table 10**, concerning the Portuguese samples, the 'Common Pumpkin' peel presented the statistically higher ($p < 0.05$) total of phenolic compounds (9.4 ± 0.3 mg/g of extract), followed by the fiber of 'Kabocha Squash' (4.8 ± 0.1 mg/g of extract) and the peel of 'Butternut Squash' (4.73 ± 0.01 mg/g of extract), the values of which did not differ significantly ($p > 0.05$). These totals are mainly comprised of the flavan-3-ols and flavonoids families, while phenolic acids are not representative or were not detected. The (-)-epicatechin (Peak 1) was the most abundant compound of all the samples evaluated. Epicatechin was also reported to be the major constituent in *Momordica caranthia* (bitter melon) (Busuioc et al., 2020), which belongs to the same family as pumpkins.

Different profiles were seen in the samples from Algeria (**Table 11**), where more expressive contents of phenolic acids were found in the 'Gold Nugget Pumpkin' fibrous strands (2.27 ± 0.02 mg/g of extract), while flavonoids are the most representative compounds of the total phenolic compounds were found in all the peels and in the seeds of 'Musquée de Provence'. High levels of phenolic acids and flavonoids were also reported by Mokhtar et al. (2021), in mature pumpkins (*Cucurbita moschata* Duchesne). The 'Gold Nugget Pumpkin' peel presented the highest value of total phenolic compounds, followed by the fibrous strands of this genotype (4.1 ± 0.1 and 3.93 ± 0.05 mg/g of extract, respectively), being statistically different ($p < 0.05$) from each other. Furthermore, peaks 2 and 6 were not found in the Algerian extracts and no peak was identified in the extracts of 'Gold Nugget Pumpkin' and 'Butternut Squash' seeds. To the best of our knowledge, this is the first report on the phenolic composition of these pumpkin genotype byproducts generated in the food industry.

Table 10. Quantification of the phenolic compounds found in the pumpkin samples from Portugal (mg/g of extract).

Peak	Common Pumpkin			Butternut Squash			Kabocha Squash		
	Peel	Fibrous Strands	Seeds	Peel	Fibrous Strands	Seeds	Peel	Fibrous Strands	Seeds
1	4.58 ± 0.08 ^{a}	3.04 ± 0.05 ^{b}	1.74 ± 0.03 ^{f}	2.56 ± 0.03 ^{d,e}	2.47 ± 0.07 ^{e}	2.63 ± 0.02 ^{c,d}	1.50 ± 0.07 ^{g}	2.7 ± 0.1 ^{c}	1.29 ± 0.05 ^{h}
2	0.50 ± 0.02 ^{b}	0.484 ± 0.006 ^{b}	0.49 ± 0.02 ^{b}	n.d.	n.d.	n.d.	n.d.	0.533 ± 0.003 ^{a}	0.474 ± 0.001 ^{b}
3	0.214 ± 0.009 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	0.116 ± 0.004 ^{b}	n.d.	n.d.
4	0.65 ± 0.03 ^{a}	0.487 ± 0.007 ^{e}	0.458 ± 0.008 ^{f}	0.6096 ± 0.0002 ^{b}	n.d.	0.457 ± 0.008 ^{f}	0.58 ± 0.02 ^{c}	0.5218 ± 0.0004 ^{d}	0.461 ± 0.003 ^{e,f}
5	1.60 ± 0.08 ^{a}	n.d.	n.d.	0.543 ± 0.004 ^{c}	n.d.	0.454 ± 0.007 ^{d}	0.62 ± 0.03 ^{b}	0.494 ± 0.002 ^{c,d}	n.d.
6	0.59 ± 0.03 ^{a}	n.d.	n.d.	0.519 ± 0.005 ^{b}	n.d.	n.d.	0.58 ± 0.03 ^{a}	0.493 ± 0.003 ^{b}	n.d.
7	0.69 ± 0.03 ^{a}	n.d.	n.d.	0.496 ± 0.005 ^{c}	n.d.	n.d.	0.56 ± 0.02 ^{b}	n.d.	n.d.
8	0.55 ± 0.03 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	0.54 ± 0.03 ^{a}	n.d.	n.d.
Total flavan-3-ols	4.58 ± 0.08 ^{a}	3.04 ± 0.05 ^{b}	1.74 ± 0.03 ^{f}	2.56 ± 0.03 ^{d,e}	2.47 ± 0.07 ^{e}	2.63 ± 0.02 ^{c,d}	1.50 ± 0.07 ^{g}	2.7 ± 0.1 ^{c}	1.29 ± 0.05 ^{h}
Total phenolic acids	0.214 ± 0.009 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	0.116 ± 0.004 ^{b}	n.d.	n.d.
Total flavonoids	4.6 ± 0.2 ^{a}	0.97 ± 0.01 ^{d}	0.95 ± 0.03 ^{d}	2.17 ± 0.01 ^{c}	n.d.	0.91 ± 0.02 ^{d}	2.9 ± 0.1 ^{b}	2.042 ± 0.003 ^{c}	0.934 ± 0.002 ^{d}
Total phenolic compounds	9.4 ± 0.3 ^{a}	4.01 ± 0.06 ^{d}	2.693 ± 0.004 ^{f}	4.73 ± 0.01 ^{b,c}	2.47 ± 0.07 ^{f}	3.538 ± 0.007 ^{e}	4.50 ± 0.06 ^{c}	4.8 ± 0.1 ^{b}	2.23 ± 0.04 ^{g}

n.d. – not detected. Calibration curves used for quantification: (-)-catequin (y = 84,950x - 23,200, R^2 = 0.999, LOD = 0.17 g/mL; LOQ = 0.68 g/mL, peak 1); p-hydroxybenzoic acid (y = 208,604x + 173056, R^2 = 0.9995, LOD = 1.37 g/mL; LOQ = 4.15 g/mL, peak 3); and quercetin 3-*O*-glucoside (y = 34,843x - 160,173; R^2 = 0.9998; LOD = 0.21 g/mL; LOQ = 0.71 g/mL, peaks 2, 4, 5, 6, 7 and 8). ANOVA analysis – In each row different letters mean significant differences ($p < 0.05$).

Table 11. Quantification of the phenolic compounds found in the pumpkin samples from Algeria (mg/g of extract).

Peak	Gold Nugget Pumpkin			Butternut Squash			Musquée de Provence		
	Fibrous Strands	Seeds	Peel	Fibrous Strands	Seeds	Peel	Fibrous Strands	Seeds	Peel
1	1.66 ± 0.07 [a]	n.d.	0.51 ± 0.02 [e]	0.97 ± 0.03 [c]	n.d.	0.346 ± 0.009 [f]	1.13 ± 0.01 [b]	n.d.	0.577 ± 0.008 [d]
3	2.27 ± 0.02 [a]	n.d.	0.134 ± 0.006 [d]	0.176 ± 0.006 [c]	n.d.	tr.	0.207 ± 0.007 [b]	0.0762 ± 0.0002 [e]	n.d.
4	n.d.	n.d.	0.94 ± 0.05 [a]	0.462 ± 0.002 [c]	n.d.	0.4526 ± 0.0003 [c]	0.4790 ± 0.0007 [b]	n.d.	0.476 ± 0.003 [b]
5	n.d.	n.d.	1.65 ± 0.07 [a]	n.d.	n.d.	0.44904 ± 0.00006 [b]	n.d.	0.460 ± 0.005 [b]	0.474 ± 0.002 [b]
7	n.d.	n.d.	n.d.	n.d.	n.d.	0.4418 ± 0.0001	n.d.	n.d.	n.d.
8	n.d.	n.d.	0.85 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total flavan-3-ols	1.66 ± 0.07 [a]	n.d.	0.51 ± 0.02 [e]	0.97 ± 0.03 [c]	n.d.	0.346 ± 0.009 [f]	1.13 ± 0.01 [b]	n.d.	0.577 ± 0.008 [d]
Total phenolic acids	2.27 ± 0.02 [a]	n.d.	0.134 ± 0.006 [d]	0.176 ± 0.006 [c]	n.d.	tr.	0.207 ± 0.007 [b]	0.0762 ± 0.0002 [e]	n.d.
Total flavonoids	n.d.	n.d.	3.4 ± 0.1 [a]	0.462 ± 0.002 [d]	n.d.	1.3434 ± 0.0004 [b]	0.4790 ± 0.0007 [d]	0.460 ± 0.005 [d]	0.951 ± 0.005 [c]
Total phenolic compounds	3.93 ± 0.05 [b]	n.d.	4.1 ± 0.1 [a]	1.61 ± 0.03 [d,e]	n.d.	1.689 ± 0.008 [d]	1.818 ± 0.007 [c]	0.536 ± 0.005 [f]	1.53 ± 0.01 [e]

n.d. – not detected. tr. – traces. Calibration curves used for quantification: (-)-catequin (y = 84,950x - 23,200, R^2 = 0.999, LOD = 0.17 g/mL; LOQ = 0.68 g/mL, peak 1); p-hydroxybenzoic acid (y = 208,604x + 173056, R^2 = 0.9995, LOD = 1.37 g/mL; LOQ = 4.15 g/mL, peak 3); and quercetin 3-*O*-glucoside (y = 34,843x - 160,173; R^2 = 0.9998; LOD = 0.21 g/mL; LOQ = 0.71 g/mL, peaks 2, 4, 5, 6, 7 and 8). ANOVA analysis – In each row different letters mean significant differences ($p < 0.05$).

Antioxidant activity

The bioactive capacity of the pumpkin byproducts was assessed in order to evaluate the preservative potential of their hydroethanolic extracts. The antioxidant capacity was analyzed through two cell-based assays, which present the advantage of evaluating oxidizable biological targets. The samples presented great antioxidant results, shown in **Table 12** and **Table 13**, in the two mechanisms evaluated: the inhibition of oxidative hemolysis (OxHLIA) in sheep erythrocytes suspension and the inhibition of lipid peroxidation (TBARS) in porcine brain homogenates.

Table 12. Antioxidant activity of the byproducts of three pumpkin genotypes from Portugal, obtained through cell-based assays.

Pumpkin Genotype from Portugal	Part	OxHLIA 60 min IC_{50} [1], µg/mL	TBARS IC_{50} [1], µg/mL
Butternut Squash	Peel	88 ± 3 ^{c}	7461 ± 315 ^{b}
	Seeds	59 ± 6 ^{d}	185 ± 7 ^{h}
	Fibrous strands	44 ± 4 ^{d}	6887 ± 53 ^{c}
Common Pumpkin	Peel	90 ± 3 ^{c}	3921 ± 33 ^{e}
	Seeds	43 ± 3 ^{d}	756 ± 27 ^{g}
	Fibrous strands	365 ± 13 ^{a}	6375 ± 68 ^{d}
Kabocha Squash	Peel	209 ± 10 ^{b}	7765 ± 31 ^{a}
	Seeds	46 ± 2 ^{d}	164 ± 8 ^{h}
	Fibrous strands	96 ± 2 ^{c}	1568 ± 53 ^{f}
Trolox		21.8 ± 0.2 ^{e}	139 ± 5 ^{h}

[1] IC_{50}: Extract concentration that inhibits lipid peroxidation by 50%. ANOVA analysis—In each column, different letters mean significant differences ($p < 0.05$).

Table 13. Antioxidant activity of the byproducts of three pumpkin genotypes from Algeria, obtained through cell-based assays.

Pumpkin Genotype from Algeria	Part	OxHLIA 60 min IC_{50}, µg/mL	TBARS IC_{50}, µg/mL
Butternut Squash	Peel	588 ± 18 ^{a}	4569 ± 277 ^{a}
	Seeds	115 ± 6 ^{f}	573 ± 31 ^{e}
	Fibrous strands	257 ± 13 ^{d}	3508 ± 91 ^{b}
Gold Nugget Pumpkin	Peel	362 ± 8 ^{b,c}	3123 ± 136 ^{c}
	Seeds	n.d.	91 ± 4 ^{f}
	Fibrous strands	566 ± 13 ^{a}	3659 ± 199 ^{b}
Musquée de Provence	Peel	335 ± 4 ^{c}	2123 ± 101 ^{d}
	Seeds	400 ± 34 ^{b}	549 ± 27 ^{e}
	Fibrous strands	188 ± 2 ^{e}	4385 ± 242 ^{a}
Trolox		21.8 ± 0.2 ^{g}	139 ± 5 ^{f}

IC_{50}: Extract concentration that inhibits oxidative hemolysis by 50%. n.d.: Not detected. ANOVA analysis: In each column, different letters mean significant differences ($p < 0.05$).

Regarding the Portuguese pumpkin genotype extracts (**Table 12**), the seeds presented the best results in the TBARS assay, especially the 'Kabocha Squash' (IC_{50}: 164 ± 8 µg/mL) and the 'Butternut Squash' (IC_{50}: 185 ± 7 µg/mL) genotypes. These results did not differ significantly ($p > 0.05$) from the positive control Trolox, which represents great results for a natural extract. In the OxHLIA assay, the results were quite similar among the samples. The IC_{50} values ranged from 43 to 96 µg/mL, which represent about 2 to 4.5-fold

higher concentrations than that of Trolox (21.8 µg/mL), except for the 'Common Pumpkin' fibrous strands and the 'Kabocha Squash' peel, which presented higher ($p < 0.05$) IC_{50} values (365 ± 13 µg/mL and 209 ± 10 µg/mL, respectively). In fact, samples from Portugal showed greater anti-hemolytic capacity than those from Algeria (**Table 13**), which presented IC_{50} values from 115 ± 6 µg/mL to 588 ± 18 µg/mL. Interestingly, despite not presenting anti-hemolytic properties, the seeds of 'Gold Nugget Pumpkin' revealed the strongest lipid peroxidation inhibition capacity (91 ± 4 µg/mL), which can possibly be explained by the interference of other compounds, such as lipids, that have a great influence in the OxHLIA assay (Takebayashi et al., 2010).

These results are in agreement with the literature, where some authors also reported the antioxidant capacity of different pumpkin parts, mostly the seeds, through different methods. Akomolafe et al. (2016) evaluated the antioxidant potential of pumpkin (*Cucurbita pepo* L.) seeds and reported that their methanolic extract caused a notable reduction in the TBARS produced in albino rat's testicular tissue. In another study (Saavedra et al., 2015), pumpkin seeds and shells extracted with different solvents presented a great capacity for DPPH radical scavenging. The most efficient solvent was the 70% ethanol, and the shell samples presented the higher inhibition percentage, reaching up to 71.0 ± 0.97% of DPPH radicals inhibition. Furthermore, in a study assessing the incorporation of pumpkin seeds into chicken burgers, the lipid stability during storage and the antioxidant properties were improved when compared to the raw burgers (Longato et al., 2017). The results found in the literature were not comparable to the ones presented herein given the difference in the parts of the plant used and the different methods employed. In the present study, only cell-based methods were applied in order to better mimic the mechanisms involved in *vivo* systems.

Antimicrobial and antifungal activity

The microorganisms used in this assay are important food contaminants that can affect the quality of foodstuffs by deterioration and organoleptic damage and/or also affect the consumers' health, causing intoxication and infection with serious related complications. The extracts obtained from pumpkin byproducts were capable of inhibiting the growth of at least two of the eight bacterial strains and one of the two fungal strains assessed.

Table 14 to **Table 17** present the antimicrobial (antibacterial and antifungal) capacity of the Portuguese and Algerian samples. All of the samples from Portugal exhibited inhibition capacity against *Yersinia enterocolitica*, while the ones from Algeria inhibited *Staphylococcus aureus*. In terms of food preservation, these are important results because, according to the EFSA Journal, yersiniosis is recognized as the third most

common zoonotic disease in the EU and, on the other hand, coagulase-positive *Staphylococcus* spp. was found in a considerable number of food samples reported by Bulgaria, Italy, and Spain (European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2018). More specifically, *Y. enterocolitica* was found in 2.33% of retail food samples from China (Wang et al., 2021), being responsible for diarrhea, abdominal pain, and fever in consumers. In turn, *S. aureus* is the main representative of coagulase-positive *Staphylococcus* spp. in food and, recently, its presence was reported in cold meals, mostly in salads served in the university canteens of northern Portugal (Soares et al., 2020). Regarding fungi, the pumpkin byproduct extracts were tested against *Aspergillus*, which is an important fungus genus in food for causing its deterioration and producing mycotoxins. In fact, *Aspergillus brasiliensis* is a target microorganism in the validation of food packaging sterilization and *Aspergillus fumigatus* is considered as the most important filamentous fungal human pathogens (Paterson & Lima, 2017; Racchi et al., 2021). All samples revealed the capacity to inhibit *A. brasiliensis* growth and the fibrous strands of Algerian pumpkins also protected against *A. fumigatus*, as well as the 'Gold Nugget Pumpkin' peel.

Furthermore, both samples of 'Butternut Squash' fibrous strands from Portugal and Algeria presented activity against the eight tested bacterial strains and six of the eighteen samples inhibited seven bacterial strains growth. The samples presented inhibition capacity in concentrations ranging from 2.5 to 10 mg/mL, and none of the samples revealed bactericidal nor fungicidal capacity.

These results do not agree with those obtained by Saavedra et al. (2015), where pumpkin shells and seeds extracted with different solvents (70% ethanol, 70% methanol, 70% acetone, water, and dichloromethane) did not present antibacterial capacity against *Pseudomonas aeruginosa*, *Escherichia coli*, *S. aureus*, and *Listeria monocytogenes* up to the maximum tested concentration of 10 mg/mL. These differences could be explained by the different extraction solvents employed, and in the case of shells, also for being a different part of the plants. Regarding the seeds, in the present study, the hydroethanolic extract of those from Portuguese 'Kabocha Squash' were able to inhibit *P. aeruginosa*, *E. coli*, and *S. aureus* in a concentration of 10 mg/mL. *S. aureus* was also inhibited by the same concentration of Algerian 'Musquée de Provence' seed extracts. Moreover, the seeds of 'Gold Nugget Pumpkin' from Algeria inhibited the growth of *E. coli* (MIC = 10 mg/mL), *L. monocytogenes* (MIC = 2.5 mg/mL), and *S. aureus* (MIC = 2.5 mg/mL). *S. aureus* was also inhibited by the Portuguese 'Butternut Squash' seeds (MIC = 10 mg/mL).

Similar results were also found in a study performed with pumpkin leaves (Kim et al., 2013), where their hydroethanolic extracts did not inhibit *E. coli*, *Shigella flexneri*, *Salmonella enterica*, *L. monocytogenes*, *S. aureus*, and *Bacillus subtilis* growth, until 10

mg/mL was reached. Despite the fact that the authors tested the same extract concentration, these results are not directly comparable to the ones obtained in the present study given the differences in the parts of the plant used. More recently (Amin et al., 2020), the oil obtained from indigenous pumpkin seeds (*Cucurbita maxima* Linn.) presented antibacterial activity against eight strains of *E. coli* and *Shigella sonnei*, with inhibition zones ranging from 10.66 ± 0.57 to 18 ± 1.0 mm, by using the disc diffusion method. Moreover, polysaccharides extracted from pumpkin pulp presented antimicrobial activity against three bacteria and three fungi. The highest inhibition zone was found against *E. coli*, followed by *S. aureus*, also inhibiting *P. aeruginosa*, *Aspergillus flavus*, *A. fumigatus*, and *Aspergillus niger*. It was not possible to compare these results with the ones obtained herein because different methods were employed, as were different microorganisms, but it is interesting to observe that seed-related products also presented antimicrobial properties similar to the seeds assessed in this study.

Table 14. Antibacterial activity of the byproducts of three pumpkin genotypes from Portugal.

	Butternut Squash						Common Pumpkin						Kabocha Squash						Streptomicin 1 mg/mL		Methicilin 1 mg/mL		Ampicillin 10 mg/mL	
	Seeds		Peel		Fibrous Strands		Seeds		Peel		Fibrous Strands		Seeds		Peel		Fibrous Strands							
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria																								
Enterobacter cloacae	10	>10	10	>10	2.5	>10	10	>10	10	>10	>10	>10	10	>10	10	>10	10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	>10	>10	10	>10	10	>10	10	>10	10	>10	>10	>10	10	>10	10	>10	10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	10	>10	>10	>10	>10	>10	>10	>10	10	>10	10	>10	10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterica	>10	>10	10	>10	10	>10	10	>10	10	>10	>10	>10	10	>10	10	>10	10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	10	>10	5	>10	5	>10	10	>10	10	>10	10	>10	10	>10	10	>10	5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria																								
Bacillus cereus	>10	>10	>10	>10	5	>10	10	>10	2.5	>10	10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	>10	>10	>10	>10	5	>10	10	>10	10	>10	10	>10	>10	>10	10	>10	10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	10	>10	>10	>10	10	>10	10	>10	5	>10	10	>10	10	>10	10	>10	10	>10	0.007	0.007	0.007	0.007	0.15	0.15

MIC: Minimum inhibitory concentration (mg/mL); MBC: Minimal bactericidal concentration (mg/mL). n.t.: not tested.

Table 15. Antifungal activity of the byproducts of three pumpkin genotypes from Portugal.

	Butternut Squash						Common Pumpkin						Kabocha Squash						Ketoconazole	
	Seeds		Peel		Fibrous Strands		Seeds		Peel		Fibrous Strands		Seeds		Peel		Fibrous Strands			
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus brasiliensis	10	>10	10	>10	5	>10	5	>10	10	>10	10	>10	10	>10	10	>10	10	>10	0.06	0.125
Aspergillus fumigatus	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.5	1

MIC: Minimum inhibitory concentration (mg/mL); MFC: Minimal fungicidal concentration (mg/mL).

Table 16. Antibacterial activity of the byproducts of three pumpkin genotypes from Algeria.

	Gold Nugget Pumpkin						Butternut Squash						Musquée de Provence						Streptomicin 1 mg/mL		Methicilin 1 mg/mL		Ampicillin 10 mg/mL	
	Seeds		Peel		Fibrous Strands		Seeds		Peel		Fibrous Strands		Seeds		Peel		Fibrous Strands							
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria																								
Enterobacter Cloacae	5	>10	5	>10	>10	>10	5	>10	10	>10	10	>10	10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	10	>10	10	>10	10	>10	10	>10	10	>10	10	>10	>10	>10	10	>10	>10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterica	10	>10	10	>10	>10	>10	10	>10	10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	5	>10	5	>10	10	>10	5	>10	10	>10	>10	>10	10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria																								
Bacillus cereus	5	>10	>10	>10	>10	>10	2.5	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	2.5	>10	5	>10	10	>10	10	>10	>10	>10	5	>10	>10	>10	>10	>10	5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	2.5	>10	5	>10	10	>10	5	>10	5	>10	10	>10	10	>10	5	>10	10	>10	0.007	0.007	0.007	0.007	0.15	0.15

MIC: Minimum inhibitory concentration (mg/mL); MBC: Minimal bactericidal concentration (mg/mL). n.t.: not tested.

Table 17. Antifungal activity of the byproducts of three pumpkin genotypes from Algeria.

	Gold Nugget Pumpkin						Butternut Squash						Musquée de Provence						Ketoconazole	
	Seeds		Peel		Fibrous Strands		Seeds		Peel		Fibrous Strands		Seeds		Peel		Fibrous Strands			
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus brasiliensis	5	>10	5	>10	10	>10	5	>10	10	>10	10	>10	5	>10	10	>10	10	>10	0.06	0.125
Aspergillus fumigatus	>10	>10	10	>10	10	>10	>10	>10	>10	>10	10	>10	>10	>10	>10	>10	10	>10	0.5	1

MIC: Minimum inhibitory concentration (mg/mL); MFC: Minimal fungicidal concentration (mg/mL).

Cytotoxic potential

The potential safety of the extracts obtained from the pumpkin byproducts was verified by assessing their toxicity in a primary culture of non-tumor porcine liver cells (PLP2). None of the samples revealed cytotoxic properties up to the maximum concentration of 400 µg/mL tested, which is commonly used for the assessment of possible toxic effects of natural extracts in non-tumor cells. This is an important first validation as, so far, no studies have been found regarding the toxic effects of pumpkin extracts. On the contrary, many studies revealed their cytoprotective and anticarcinogenic effects (Dotto & Chacha, 2020). In addition to the non-hepatotoxic effect of the pumpkin extracts reported in this study, Abou Seif (2014) demonstrated the capacity of pumpkin oil to protect the liver against alcohol-induced hepatotoxicity and oxidative stress in albino rats.

3.1.4. Conclusions

The byproducts of different pumpkin genotypes were evaluated in terms of biologically active compounds. The phenolic profile was analyzed, with a tentative identification followed by quantification. It was also possible to verify that, despite the influence of the different genotypes of pumpkin, environmental and agronomic conditions between countries, and major and secondary metabolites composition, all samples presented bioactive properties, with the genotype 'Butternut squash' from both countries presenting the strongest antioxidant properties. In turn, Portuguese 'Butternut squash' fibrous strands and Algerian 'Gold Nugget Pumpkin' seeds presented a highest antimicrobial activity than the remaining byproducts. Regarding the phenolic profile, Portuguese 'Common Pumpkin' peel was the sample presenting the most diversified profile and the highest total content of phenolic compounds, among which (-)-epicatechin stood out. Along with the assessed phenolic compounds, many other compounds could be responsible for the bioactivities reported in this study; as also other bioactive properties could possibly be presented by the samples; however, this is an important first screening that corroborates the importance of reusing and recycling this kind of byproduct to be reintroduced in other steps of the production chain or even in other fields, such as pharmaceutics or cosmetics, for instance.

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3.2. Variability in chemical profile and bioactivities of the flesh of Greek pumpkin landraces

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Article

Variability in Chemical Profile and Bioactivities of the Flesh of Greek Pumpkin Landraces

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Abstract: The aim of this study was to evaluate the chemical profile of the flesh and yield parameters of Greek pumpkin genotypes, including nine local landraces and two commercially available cultivars, focusing on valorizing the genetic pool of *Cucurbita* sp. with high added value products. Yield parameters (mean fruit weight and total fruit yield) recorded high variability with genotypes V8 and V2 showing the highest fruit yield. Moreover, genotype V11 was the most abundant in glucose and total sugars and scored the highest sweetness index suggesting good taste and promising marketing attributes. The highest antioxidant activity (OxHLIA assay) was assessed in the V8 genotype, while the V2 genotype showed the highest *alpha*-, *beta*- and total tocopherols content. Oxalic acid was the main organic acid, followed by malic and citric acids, while organic acid composition varied among the tested genotypes. Moreover, the flesh extracts showed varied antimicrobial activity against several bacteria and fungi, while no toxicity against non-tumor cells was recorded. In conclusion, our results make evident the presence of high innate variability in terms of crop performance, chemical composition and bioactive properties not only between the different genotypes but also at the intra-populational level. This finding is of high importance for the valorization of the local genetic pool of *Cucurbita* species through the selection of elite genotypes with high yield and quality of fruit, contributing to the conservation of valuable genetic material and limitation of the risk of genetic erosion due to neglect of local landraces.

Keywords: *Cucurbita* sp.; antimicrobial activity; local landraces; bioactive compounds; antioxidant activity; cytotoxicity; fruit quality

3.2.1. Introduction

Among cucurbits, Cucurbita is considered a genus with considerable diversity in terms of the plant growth habits, fruit morphology and disease resistance, as well as the nutritional and phytochemical profile of fruit, which is mainly attributed to its complex domestication trajectories (Chomicki et al., 2020). The genus presents a pre-Holocene distribution, originally in the form of bitter fruits (Kistler et al., 2015), while its domestication took place in northern South and Central America ca. 10,000 B.P. (Smith, 1997), being included among the earliest crop domestication records. Although the relationships between wild and domesticated species are not well elucidated (Kates et al., 2017), it is known that domestication involved traits related to fruit quality, primarily focusing on the loss of bitterness and fruit size (Chomicki et al., 2020). Indeed, among all plant species cultivated for their fruit, squashes and pumpkins produce the most profoundly sizable fruit in comparison to their non-domesticated wild forms (Savage et al., 2015).

Pumpkin is the fruit of *Cucurbita* species that belongs to the Cucurbitaceae family, and it is traditionally consumed all over the world. Pumpkins belong to the genus *Cucurbita* L., which encompasses 9 species – *C. maxima*, *C. pepo*, *C. moschata*, *C. argyrosperma*, *C. digitata*, *C. ficifolia*, *C. foetidissima*, *C. okeechobeensis* and *C. palmata*—the first three being the most common around the world (Integrated Taxonomic Information System, 2021). Although these three species have common requirements for optimum growth and development, due to their wide adaptation to soil and climatic conditions, they vary remarkably in fruit morphology (e.g., fruit size, shape, skin and flesh color and flesh structure), as well as in nutritional value (Saavedra et al., 2015). Moreover, interspecies hybridization results in considerable heterogeneity at the intra-populational level with a large number of subspecies and cultivars that vary in size, shapes, colors and taste (Sun et al., 2017). The vernacular term of pumpkin is commonly used to describe the fruit of *Cucurbita pepo* L., *C. moschata* Duchesne ex Poir, *C. maxima* Duchesne, *C. ficifolia* Bouché and *C. argyrosperma* K.Koch species, while it is interchangeably used with squash or winter squash terms (Kulczyński & Gramza-Michałowska, 2019). In addition to the pleasant flavor and versatility, the known nutritional value and functional properties of pumpkin have gathered significant interest in scientific studies and health- conscious consumers (Petropoulos et al., 2018). As global consumer trends increasingly lean towards healthier and sustainable food choices, the diverse nutraceutical properties of pumpkin fruit have earned it a place of importance in modern diets (Hussain, Kausar, Sehar, Sarwar, Ashraf, Jamil, Noreen, Rafique, Iftikhar, Quddoos, et al., 2022; Montesano et al., 2018; Weaver, 2014).

The flesh (pulp) is the most appreciated part of the fruit, which represents more

than 70% of the whole fruit (Rico et al., 2020); it is a good source of carbohydrates, dietary fibers (especially pectins) and proteins, while it contains significant amounts of fat and minerals, varying among species and genotypes (Fedha et al., 2010; Mohaammed et al., 2015; Nwofia et al., 2012). In nineteen accessions of *Cucurbita* ssp evaluated by Nwofia et al. (2012), the crude fibers varied from 0.55% to 1.04% in fresh weight (moisture from 78.46 to 91.97%). Considering an adequate intake of 25 g/day of fibre by ESFA (Kistler et al., 2015), this fruit contributes to a balanced diet. The high mineral content of iron, potassium, phosphorus, magnesium, selenium, calcium and copper has been well documented so far in different pumpkin varieties (Kaur et al., 2020; Ratnam et al., 2017). In the pulp of *Cucurbita maxima*, 184.34 ± 1.24 mg/100 g dw of potassium and 53.67 ± 0.19 mg/100 g dw of iron was found (Nwofia et al., 2012), while the "Waïgoré dollugo" dish, which consists of boiled pumpkin (78.2% of pumpkin fruit and 21.8% of water), presented 2753.0 ± 3.2 mg/100 g dw of potassium and 4.3 ± 0.2 mg/100 g dw of iron (Ponka et al., 2015), which adequate intake and average requirement is, according to EFSA (Euro-pean Food Safety Authority), about 3500 and 6 mg/day, respectively (EFSA). In addition to macronutrients, pumpkin is also a rich source of essential micronutrients such as vitamins, antioxidant compounds and other phytochemicals. Several studies have shown that pump- kins are a notable source of vitamins A, C and E, which play crucial roles in supporting immune function and acting as potent antioxidants to combat oxidative stress, in addition to the presence of alkaloids, flavonoids, polyphenols, tannins, phytosterols and cucurbitacins (Adnan et al., 2017; Dhiman et al., 2009; Kaur et al., 2020; Ratnam et al., 2017). Kaur et al. (2020) also highlighted the great amounts of pro-vitamin A carotenoids in pumpkin and their importance in developing important antioxidant effects, reducing the risk of certain diseases including cancer. These compounds are known as antioxidant and antimicrobial agents for their capacity to neutralize harmful free radicals in the body, preventing oxidative damage in cells and tissues, and inhibiting the growth or killing of microorganisms, such as bacteria or fungi, combating infections or preventing their spread, respectively. Contents of 171.9 µg/g of carotenoids, 2–10 mg/100 g of vitamin C, and 9–10 mg/100 g of vitamin E are reported in pumpkin flesh, which also contributes to a healthy diet (average requirement of 9 g/day of carotenoids with provitamin A activity, and 110 mg/day of vitamin C, of which adequate intake is 13 mg/day) (EFSA, n.d.).

Beyond its nutrients, pumpkins possess functional properties that are of particular interest to the food industry. Sharma et al. (2020) recently reviewed the health-promoting potential of pumpkin in terms of antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-diabetes, antimicrobial and other bioactivities, as well

as its application in the food industry, as a cooked, powdered or pureed ingredient in different food products. Their attractive taste and vibrant color make them suitable for the formulation of health-promoting food products, including soups and snacks (Bochnak & Świeca, 2020; Caili et al., 2006; Lemus-Mondaca et al., 2019).

Pumpkin cultivation is widely practiced across the globe, being generally a warm-season crop that thrives in well-drained, nutrient-rich soils with good sunlight exposure. Its cultivation and appreciation may vary according to the wide variety of pumpkin species and cross-pollinations between cultivated species and/or wide relatives, which results in high genetic diversity and inherent polymorphism (Abdein, 2018; Hamdi et al., 2017; Kaźmińska et al., 2017). According to data from FAO (Food and Agriculture Organization of the United Nations), in 2021 the pumpkin production (the aggregate of pumpkins, squash and gourds) was approximately 24 million tons harvested from 1.5 million hectares, while the world's leading producers were China (approximately 7.4 million tons), followed by Ukraine (approximately 1.43 million tons), Russian Federation (approximately 1.2 million tons) and USA (approximately 1.1 million tons) (FAO, n.d.). Within the European Union, pumpkin is cultivated on 84,270 hectares with a total production of 2.7 million tons, while the main producers are Spain (789,780 tons), Italy (601,660 tons), France (410,360 tons) and Poland (316,300 tons) (FAO, n.d.). In Greece, although pumpkin does not occupy many hectares of agricultural land (2170 hectares with annual production of 61,120 tons (FAO, n.d.)), there is wide genetic diversity with several local landraces being cultivated all through the country. The annual world production over the last ten years fluctuated between 21.9 and 26.6 million tons, whereas the harvested area showed decreasing trends dropping from almost 2.0 million hectares in 2015 to 1.5 mil- lion hectares in 2021 (FAO, n.d.). On the other hand, in Greece, there has been a great increase in harvested area and annual production over the last ten years, since harvests have in- creased from 536 ha to 2170 ha and annual production from 673 tons to 61120 tons (FAO, n.d.). Fruit yield may also vary with values that range between 37 to 98 tons/ha (Swiader et al., 1994), 25.2 to 55.7 tons/ha (Biesiada et al., 2009) or 7.3 to 41.8 tons/ha (Yavuz et al., 2015), depending on fertilization rate and irrigation level.

However, despite the rising pumpkin production during the last few years, the cultivation of this valuable crop faces mounting challenges due to the intensifying impacts of abiotic stress factors. The climate changes observed in the world have changed patterns of temperature and rainfall, thus increasing the occurrence of extreme weather events, affecting the yield of several crop species, including pumpkins (Habib-ur-Rahman et al., 2022; Kumari et al., 2022). Moreover, the intense exploitation of the soil without proper management also has negative effects through land degradation, drought and salinization of irrigation water and agricultural land

(Petropoulos et al., 2017). These effects have challenged the academic community in search of alternatives that aim to provide high-yield and quality crops, through the understanding of the effects of these adversities on traditional crops as well in the search and promotion of new, more promising crops (Petropoulos et al., 2017, 2020; Polyzos et al., 2022).

Considering the high fruit polymorphism, more and more studies of the characterization in terms of composition and functional properties of different pumpkin genotypes are being disseminated (Kim et al., 2012; Leichtweis et al., 2022; Stryjecka et al., 2023). However, the genetic pool of Greek pumpkin germplasm is not adequately characterized so far, especially when considering the high genetic variability of the species which could be a valuable tool for the adaptation of modern horticulture in the changing climate conditions. Therefore, the present study aimed to provide useful information regarding the biochemical and nutritional diversity in pumpkin genotypes cultivated in Greece. For this purpose, eleven local landraces and commercially available genotypes were cultivated under the same conditions and nutritional and chemical profiles, and the functional properties of fruit flesh were investigated.

3.2.2. Materials and methods

Plant material and growing conditions

The experiment was conducted at the experimental farm of the University of Thessaly, Greece during the growing period of 2021 (e.g., May–November 2021). Seeds from eleven genotypes (V1–V11) were directly sown in the soil on 19 May 2021. The description of genotypes is presented in **Table 18**. The genotypes included 9 local landraces collected from the respective regions, while two commercial genotypes were also included (e.g., F1 Fytro FS-243, Fytro seeds S.A., Athens, Greece and Big Max, Geniki Fytotechniki S.A., Athens, Greece). Seeds were sown in rows at distances of 1.6 m within each row and 2.0 m between the rows. Each row consisted of 32 plants, with 3 rows for each genotype being used (96 plants for each genotype in total), while rows were laid out according to the completely randomized design (n = 3). Before sowing, a complex fertilizer (18-9-18, N-P-K; Complex Haifa Turbo K + Mg + S + Fe and Zn; Haifa Group, Israel) was applied as a base dressing at a rate of 250 kg/ha and was evenly distributed with a rototiller. Irrigation was applied via a drip irrigation system (one emitter per plant with a water supply of 6 L/h) at regular intervals based on the environmental conditions during the experimental period. Irrigation was scheduled based on the recordings of soil moisture content taken at regular intervals aiming to retain 100% of field capacity. Soil moisture content was recorded with PR2 Profile Probe (Delta T PR2/4 +HH2; Delta-T

devices Ltd., Burwell, UK) using access tubes 40 cm long, while measurements were taken at soil depths of 10, 20, 30 and 40 cm. Access tubes were established with Delta-T augering and extraction kit (PR-ASK1-S, Delta-T Devices Ltd., Cambridge, UK), using one access tube per genotype (15 tubes in total).

Table 18. Genotype name of pumpkin cultivated in Greece according to the fruit type.

Pumpkin Genotypes	Sample Code	Harvest Date
Fytro FS-243	V1	26 October–19 November 2021
Landrace from the region of Trikala (Turbinate)	V2 T	25 August–19 October 2021
Landrace from the region of Trikala (Cylindrical)	V2 C	25 August–19 October 2021
Big Max	V3	25 August–19 October 2021
Local landrace "Nychaki" (Cylindrical)	V4 C	23 September 2021
Local landrace "Nychaki" (Round)	V4 R	23 September 2021
Local landrace "Leuka Melitis" (Flattened)	V5 F	5 October–19 November 2021
Local landrace "Leuka Melitis" (Round)	V5 R	5 October–19 November 2021
Local landrace from the region of Lakonia	V6	25 August 2021
Local landrace from the region of Lakonia (Pyriform)	V7 P	24 September–19 November 2021
Local landrace from the region of Lakonia (Flattened)	V7 F	24 September–19 November 2021
Local landrace from the region of Lakonia	V8	22 November 2021
Local landrace "Makedonika prasina" (Cylindrical)	V9 C	22 November 2021
Local landrace "Makedonika prasina" (Round)	V9 R	22 November 2021
Local landrace from the region of Laconia	V10	22 November 2021
Local landrace ("Voutirato")	V11	22 November 2021

After crop establishment, fertilization was applied via the drip irrigation system (fertigation). In particular, on 16 July 2023 plants received ammonium nitrate (34.5% nitrogen; 40 kg/ha), Solusop (0-0-52, N-P-K; Agri.fe.m Ltd., Athens, Greece; 20 kg/ha) and Disper bloom (Disper Bloom; Agrofarm S.A., Athens, Greece; 1.5 kg/ha). On 26 July 2023, plants were foliar sprayed with Disper Bloom (100 g/100 L of water) and Root and Leaf (20-202-20, N-P-K +TE; 200 g/100 L of water). On 28 July 2023, plants were fertigated with 40 kg of ammonium nitrate and 20 kg of Solusop. On 12 August 2023, plants were treated with 25 kg of ammonium nitrate, 25 kg/ha of Solusop and 75 kg/ha of Mannitol 3 GR (Agrofarm S.A., Greece). Weeds were controlled chemically with pre-emergence herbicides, as well as manually with a hand hoe during the growing period and until crop establishment. Pests and pathogens were chemically controlled based on the recommended practices of the crop.

Harvest took place when fruit reached maturity from 25 August 2021 to 22

November 2021, depending on the genotype. Due to polymorphism that some genotypes showed (e.g., V2, V4, V5, V7 and V9), two types of fruit that differed in their form were harvested, as described in **Table 18**. After harvest, fruit yield was calculated as the sum of the weight of all the fruit from each cultivar extrapolated to the harvested area of one hectare.

Samples preparation

After collection, fruits were weighted and 15 randomly selected fruits from each genotype and fruit form were cut in half; after removing seeds and pericarp flesh samples were collected, put in an air-sealed bag and stored under freezing conditions (−19 °C). Then, the frozen samples were lyophilized, ground to powder and stored at deep-freezing conditions until further analysis. To obtain the flesh samples, the fruits were peeled, and we removed the seeds and fibrous strands. The fleshes were then lyophilized (Sublimator model EKS, manufactured by Christian Zirbus Co. in Osterode am Harz, Germany), crushed (~20 mesh) and stored until subsequent analysis. The applied lyophilization program for drying the plant biomass was the following:

Step 1: -35 °C for 2 h at atmospheric pressure (1000 mbar);

Step 2: from -35 °C to -20 °C in 6 h under vacuum 0.150 mbar;

Step 3: from -20 °C to 0 °C in 12 h under vacuum 0.150 mbar;

Step 4: from 0 °C to 10 °C in 12 h under vacuum 0150 mbar;

Step 5: from 10 °C to 25 °C in 12 h under vacuum 0.150 mbar.

For the bioactivities, the hydroethanolic (80 ethanol: 20 water) flesh extracts were obtained by maceration for 1 h, twice, with a solid/liquid ratio of 1 g/30 mL, following the standard methodology of the group, previously described in Leichtweis et al. (2022). The extracts were then lyophilized as described above and stored until subsequent analysis.

Nutritional characterization

The AOAC procedures (AOAC, 2016) were followed to obtain the nutritional composition of flesh samples, including the total fat, crude protein, carbohydrates, dietary fibre content, ash and total energy. The fat content was extracted with petroleum ether in a Soxhlet apparatus; for the crude protein, we used the macro-Kjeldahl method (equipment Pro-Nitro A, Selecta, Barcelona, Spain) and a nitrogen-to-protein conversion factor of 6.25; the total dietary fiber content was determined using a combination of enzymatic and gravimetric method; and, the ash content was assessed by incinerating the samples at 550 ± 10 °C.

Total carbohydrates were calculated by difference, and the total energy was calculated using the following equation:

energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat) + 2 × (g total dietary fiber)

The results were expressed in g/100 dry weight (dw).

HPLC assays

Tocopherol content was determined by the HPLC system described above coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA), following the methodology described by Pereira et al. (2011). The detector was configured to excite at 290 nm and measure emission at 330 nm and the chromatographic separation was achieved with a Polyamide II normal-phase column (250 × 4.6 mm; YMC Waters) operating at 30 °C. Tocol was used as an internal standard (IS) and the compound isoforms (*alpha*-, *beta*-, *gamma*-, and *delta*) were identified by comparisons with authentic standards. The quantities were obtained using the following calibrations curves: *alpha*-tocopherol, $y = 1.295x$, $R^2 = 0.991$, LOD = 18.06 ng/mL, LOQ = 60.20 ng/mL); *beta*-tocopherol, $y = 0.396x$, $R^2 = 0.992$, LOD = 25.82 ng/mL, LOQ = 86.07 ng/mL); and *gamma*- tocopherol, $y = 0.567x$, $R^2 = 0.991$, LOD = 14.79 ng/mL, LOQ = 49.32 ng/mL. The results were expressed in mg/100 g dw.

The organic acid characterization was performed by ultra-fast liquid chromatography (UFLC, Shimadzu Corporation, Kyoto, Japan) coupled to a photodiode array detector (PDA), using 215 and 245 nm (for ascorbic acid) as preferred wavelengths (Brown & Summers, 1985). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 µm, 250 mm × 4.6 mm i.d.) thermostatted at 35 °C. Identification and quantification were achieved by comparison with commercial standards. The calibration curves were as follows: oxalic acid, $y = 8 \times 106x + 331{,}789$; $R^2 = 0.9912$; malic acid, $y = 942{,}562x + 38{,}506$, $R^2 = 0.9987$; ascorbic acid, $y = 5 \times 107x + 449{,}262$; $R^2 = 0.9813$; shikimic acid, $y = 8 \times 107x + 567{,}119$; $R^2 = 0.9903$; citric acid, $y = 968{,}367x - 12{,}295$; $R^2 = 0.9974$; and fumaric acid, $y = 9 \times 10^7x - 100{,}894$, $R^2 = 0.9986$. The results were expressed in g/100 g dw, except for fumaric acid (mg/100g dw).

The analysis of free sugars was carried out using HPLC coupled to a refractive index detector, more specifically an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD, USA) and an RI detector (Knauer Smartline 2300), following a previously described method (Barros et al., 2013). A Eurospher 100-5 NH2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 °C (7971 R Grace oven) was used. For this analysis, melezitose was used as IS for the quantification method

and the compounds were identified by chromatographic comparisons with authentic standards D(-)-fructose, D(+)-sucrose, D(+)-glucose, D(+)- trehalose and D(+)-raffinose pentahydrate. The results were expressed in g/100 g dw. The content of individual free sugars was used to calculate the sweetness index (SI) of fruit through the formula (Brown & Summers, 1985):

SI = fructose content × 157.5 + glucose content × 67.5 + sucrose content × 100

Fatty acids were determined by gas–liquid chromatography with flame ionization detection (GC-FID), a DANI model GC 1000 instrument and a Macherey–Nagel column (30 m × 0.32 mm ID × 0.25 μm df), following the methodology published by Pereira et al. (2011). The identification and quantification were performed by comparing the retention times of peaks detected to the ones of FAME peaks of commercial standards (FAME reference standard mixture, standard 47885-U, Sigma-Aldrich, St. Louis, MO, USA). The contents were presented in relative percentages.

Bioactivities

The antioxidant capacity of the flesh extracts was evaluated using two in vitro cell-based assays. The first screening was obtained using porcine (*Sus scrofa*) brain homogenates, to observe the ability of the extract to inhibit lipid peroxidation by the thiobarbituric acid reactive substances inhibition (TBARS) assay, as described by Pereira et al. (2014), and the second one using a red cell suspension of healthy sheep blood, investigating the extracts capacity to inhibit the oxidative hemolysis (OxHLIA), as described by Backes et al. (2020). The results were expressed in IC_{50} values (μg/mL), meaning the extract concentration that inhibits 50% of oxidation and that delays 50% of erythrocyte hemolysis for 60 min, respectively. Trolox was used as a positive control in both assays.

For the antimicrobial activity, the extracts were tested against eight bacteria and two fungi related to food contamination, more specifically, *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19111), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 49741), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica subsp* (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610), *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404). The microdilution and *p*-iodonitrotetrazolium chloride (INT) methods were used (Kuete et al., 2011). Streptomycin and ampicillin were used as positive controls for bacteria and ketoconazole and bifonazole for fungi. The results were expressed in mg/mL.

Additionally, the cytotoxic effects were assessed in a primary culture of non-tumor porcine liver cells (PLP2) derived from porcine liver obtained from a local slaughterhouse.

The evaluation was conducted using the Sulforhodamine B (SRB) colorimetric assay (C. Pereira et al., 2013). The IC_{50} values (inhibiting 50% of the net cell growth) for extract concentration, presented in µg/mL, were compared to the positive control, Ellipticine.

Statistical analysis

For chemical analysis, fruit samples were analyzed in triplicate, and the results were expressed as mean ± standard deviation. For comparing two groups of data, a Student's *t*-test was used, while for three or more groups, the one-way analysis of variance (ANOVA) was applied. Prior to analysis, normal distribution and homogeneity of variance were assessed using the Shapiro–Wilk and Levene tests, respectively. For homoscedastic data with $p > 0.05$, Tukey's honestly significant difference (HSD) test was employed and, for heteroscedastic data, Tamhane's T2 multiple comparison test was used. All analyses were conducted at a 5% significance level using IBM SPSS Statistics software (Version 22.0, IBM Corp, Armonk, NY, USA).

In addition, principal component analysis (PCA) was performed to identify the contribution of each variable to the total genetic diversity and classify the studied pumpkin landraces based on their chemical composition and antioxidant activities of fruit flesh. All statistical analyses were carried out using the StatGraphics Centurion-XVII statistical package (StatPoint Technologies Inc., Warrenton, VA, USA).

3.2.3. Results and discussion

Crop performance

The results of crop performance (e.g., total fresh yield of fruit, number of fruits per plant and mean fruit weight) are presented in **Table 19** where significant differences among the studied genotypes were recorded. In particular, the highest fruit yield was recorded for the V8 genotype (34,069 kg/ha), followed by V2 and V4 genotypes (30,695 kg/ha and 27,997 kg/ha, respectively), whereas the lowest overall yield was recorded for V5 genotype with a production of only 7618 kg/ha. Moreover, significant differences were recorded in the number of fruits per plant where V1 recorded the highest mean value (6.1 fruit per plant) with an average fruit weight of 1.0 kg per fruit. On the other hand, the lowest number of fruits per plant was recorded in V5 with an average weight of 3.6 kg. Significant differences were also recorded in mean fruit weight where V3 recorded the highest mean value (8.1 kg) followed by V2, V8 and V11 (6.3, 6.1 and 5.6 kg, respectively). These results indicate that the high number of fruits is accompanied by smaller fruit weight, as in the case of V2, V3, V8 and V11. The high variability recorded in the tested genotypes is mainly attributed to the differences in their genetic background, especially regarding the local landraces which are acclimatized in specific

edaphoclimatic conditions through the cultivation in specific regions with particular microclimates. According to the literature, genotypic diversity in *Cucurbita* species is expected to result in differences in crop performance(Jamal Uddin et al., 2014) and quality features (Gomes et al., 2020; Soltani et al., 2017), while differences observed in multi-site experiments can justify the acclimatization of pumpkin genotypes to specific environmental conditions and agronomic practices (El-Hamed & Elwan, 2011; Ghimire et al., 2018). Moreover, the differences in plant morphology and phenology (e.g., vine length, fruit maturation, number of branches, etc.) may influence crop performance (Ahamed et al., 2012), especially when plants are grown under the same conditions and agronomic practices as those in our study.

Table 19. Crop performance (total fruit weight in kg/ha) of the tested genotypes.

Genotype	Total Fruit Weight (Tons/ha)	Numbers of Fruit/Plant	Mean Fruit Weight (kg)
V1	15.5 ± 6.6 ^{g}	6.1 ± 0.5 ^{a}	1.0 ± 0.1 ^{g}
V2	30.7 ± 5.6 ^{b}	0.9 ± 0.1 ^{e}	6.3 ± 0.6 ^{b}
V3	19.6 ± 7.8 ^{e}	1.0 ± 0.1 ^{d,e}	8.1 ± 0.7 ^{a}
V4	28.0 ± 3.6 ^{c}	3.9 ± 0.4 ^{b}	2.9 ± 0.3 ^{f}
V5	7.6 ± 2.4 ^{j}	0.4 ± 0.1 ^{g}	3.6 ± 0.4 ^{e}
V6	10.9 ± 4.5 ^{i}	1.2 ± 0.2 ^{d}	3.8 ± 0.4 ^{e}
V7	24.0 ± 9.6 ^{d}	2.9 ± 0.3 ^{c}	1.7 ± 0.2 ^{g}
V8	34.1 ± 5.4 ^{a}	1.1 ± 0.1 ^{d}	6.1 ± 0.5 ^{b}
V9	16.7 ± 2.2 ^{f}	0.7 ± 0.1 ^{f}	4.8 ± 0.4 ^{d}
V10	27.7 ± 6.3 ^{c}	4.1 ± 0.3 ^{b}	2.7 ± 0.3 ^{f}
V11	11.8 ± 3.9 ^{h}	0.8 ± 0.1 ^{e,f}	5.6 ± 0.5 ^{c}

Means of the same column followed by different Latin letters are statistically different according to Tukey's honestly significant difference (HSD) test at $p < 0.05$.

Nutritional profile

The proximate composition of the studied genotypes is presented in **Table 20**, including two samples of the same genotype that varied in fruit morphology as indicated in **Table 18** (e.g., V2, V4, V5, V7 and V9). A variable content was recorded with significant differences not only among the studied genotypes but also between the different fruit types of the same genotype. In particular, fat content ranged between 0.38 and 1.17 g/100 g dw with the highest content being recorded in V2 T, V6 and V11, whereas the lowest content was found in the flesh of V4 R genotype. Protein and ash content ranged between 8.0–21.4 g/100 g dw and 3.5–10.95 g/100 g dw, respectively, with V9 C genotype having the highest content (21.4 g/100 g and 10.95 g/100 g dw, respectively), whereas the lowest overall content of protein and ash was recorded in V4 C and V5 R genotypes (8.6 g/100 g dw and 3.5 g/100 g dw), respectively. V1 showed the highest energy content (360.1 Kcal/100 g dw), V2 had the highest content in

carbohydrates (72.2 g/100 g dw), while V9 C was the richest in fibres (27.4 g/100 g dw). The detected amounts of macronutrients were within the same range of literature reports regarding the flesh of *Cucurbita* species (Amin et al., 2019; Badr et al., 2011; Jacobo-Valenzuela et al., 2011; Kim et al., 2012), although protein and fat content was higher and lower than other studies, respectively (Salehi et al., 2019). The high variability in macronutrients presented in our study indicates the genetic inter- and intra-population diversity of the studied germplasm, which could be due to breeding through the farmer's seed system which facilitates heterogeneity, especially in the case of local landraces as in our study and can justify the recorded differences with other reports in the literature (Louwaars, 2018; A. M. Pereira et al., 2020).

The main free sugars detected in the flesh of the studied genotypes were glucose, sucrose and fructose, followed by trehalose and raffinose (**Table 21**), while typical chromatographs of samples rich in fructose and sucrose are presented in **Figure 6**. The highest amounts of the main sugars were recorded in V11 (26.9 g/100 g dw) and in both fruit types of V2 (9.7 and 10.0 g/100 g dw for T and C fruit types, respectively) and V7 (14.0 g/100 g dw for both fruit types), while trehalose was the highest in V2 C (0.67 g/100 g dw) and raffinose in V2 T, V6 and V8 genotypes (and 0.29, 0.27 and 0.25 g/100 g dw, respectively). The highest total free sugar content was recorded for the local landrace V11 due to the increased content of glucose. Moreover, the same genotype (V11) presented the highest sweetness index (3367) due to the overall composition of individual and total free sugars which indicates a sweeter taste and is highly associated with better acceptability from consumers (Marek et al., 2008). Similarly to our study, Seroczynska et al. (2014) evaluated twelve forms of *C. maxima* fruit and identified sucrose, fructose and glucose in variable amounts depending on the genotype while they detected mannose in specific genotypes. The same sugars were detected by Dhenge et al. (2022) in *C. moschata* fruit although a high fraction of total free sugars was not tentatively identified. Moreover, Kostecka-Gugała et al. (2020) highlighted the intra-species variability between different cultivars of *C. pepo*, *C. maxima*, C. *moschata* and *C. ficifolia* in terms of soluble sugar content which explains the differences recorded in our study, while similar results were reported for indigenous and hybrid varieties of (*Cucurbita maxima* Linn) by Amin et al. (2019).

Table 20. Proximate composition of the flesh of the tested pumpkin genotypes on a dry weight (dw) basis.

Genotype	Fat (g/100 g dw)	Protein (g/100 g dw)	Ash (g/100 g dw)	Energy (Kcal/100 g dw)	Carbohydrates (g/100 g dw)	Fibres (g/100 g dw)
V1	0.83 ± 0.04 ^{c}	12.6 ± 0.6 ^{g,h}	4.511 ± 0.009 ^{l}	360.1 ± 0.4 ^{a}	69.1 ± 0.7 ^{b}	13.0 ± 0.1 ^{j}
V2 T	1.12 ± 0.06 ^{a}	13.7 ± 0.4 ^{d,e}	6.07 ± 0.04 ^{j}	332.8 ± 0.3 ^{h}	54.9 ± 0.7 ^{h}	24.27 ± 0.02 ^{c}
V2 C	0.645 ± 0.002 ^{e}	12.9 ± 0.1 ^{f,g}	7.1 ± 0.1 ^{h}	334.6 ± 1.2 ^{g}	59.2 ± 0.8 ^{f,g}	20.2 ± 0.8 ^{f}
V3	0.45 ± 0.02 ^{h}	18.07 ± 0.03 ^{c}	7.7 ± 0.2 ^{e}	321.85 ± 0.02 ^{j}	49.0 ± 0.2 ^{j}	24.7 ± 0.5 ^{c}
V4 C	0.64 ± 0.03 ^{e,f}	8.6 ± 0.1 ^{k}	7.1 ± 0.1 ^{g,h}	332.5 ± 0.1 ^{h}	62.6 ± 0.2 ^{e}	21.1 ± 0.2 ^{e,f}
V4 R	0.38 ± 0.02 ^{h}	17.46 ± 0.04 ^{c}	9.08 ± 0.07 ^{d}	321.7 ± 0.6 ^{j}	51.1 ± 0.3 ^{i}	21.9 ± 0.4 ^{d,e}
V5 F	0.58 ± 0.03 ^{f,g}	12.61 ± 0.05 ^{g}	5.126 ± 0.006 ^{k}	353.70 ± 0.07 ^{b}	67.3 ± 0.1 ^{c}	14.34 ± 0.04 ^{i}
V5 R	0.55 ± 0.03 ^{g}	9.9 ± 0.3 ^{j}	3.5 ± 0.1 ^{m}	361.0 ± 0.1 ^{a}	72.2 ± 0.6 ^{a}	13.8 ± 0.4 ^{i,j}
V6	1.17 ± 0.02 ^{a}	13.4 ± 0.3 ^{e,f}	6.98 ± 0.05 ^{h}	341.5 ± 0.8 ^{e}	60.27 ± 0.03 ^{f}	18.2 ± 0.3 ^{g}
V7 P	0.92 ± 0.04 ^{b}	14.1 ± 0.1 ^{d}	7.49 ± 0.03 ^{f}	343.6 ± 0.6 ^{d}	61.9 ± 0.6 ^{e}	15.5 ± 0.5 ^{h}
V7 F	0.70 ± 0.03 ^{d,e}	11.9 ± 0.1 ^{h,i}	7.27 ± 0.06 ^{g}	348.76 ± 0.05 ^{c}	67.26 ± 0.6 ^{c}	12.8 ± 0.2 ^{j}
V8	0.76 ± 0.03 ^{c,d}	11.3 ± 0.4 ^{i}	7.29 ± 0.02 ^{g}	329.4 ± 1.6 ^{i}	58.0 ± 1.3 ^{g}	22.7 ± 0.9 ^{d}
V9 C	0.73 ± 0.03 ^{d}	21.4 ± 0.6 ^{a}	10.95 ± 0.05 ^{a}	315.05 ± 0.96 ^{k}	44.5 ± 0.2 ^{k}	22.4 ± 0.5 ^{d}
V9 R	0.65 ± 0.03 ^{e}	20.3 ± 0.3 ^{b}	10.19 ± 0.07 ^{b}	307.7 ± 1.5 ^{l}	41.4 ± 0.4 ^{l}	27.4 ± 0.8 ^{a}
V10	0.73 ± 0.03 ^{d}	9.9 ± 0.2 ^{j}	9.3 ± 0.1 ^{c}	313.8 ± 0.1 ^{k}	53.7 ± 0.2 ^{h}	26.3 ± 0.1 ^{b}
V11	1.12 ± 0.02 ^{a}	8.0 ± 0.3 ^{k}	6.71 ± 0.04 ^{i}	338.52 ± 0.34 ^{f}	64.0 ± 0.4 ^{d}	20.1 ± 0.2 ^{f}

Means of the same column followed by different Latin letters are statistically different according to Tukey's honestly significant difference (HSD) test at $p < 0.05$; T: turbinate; C: cylindrical; R: round; F: flattened.

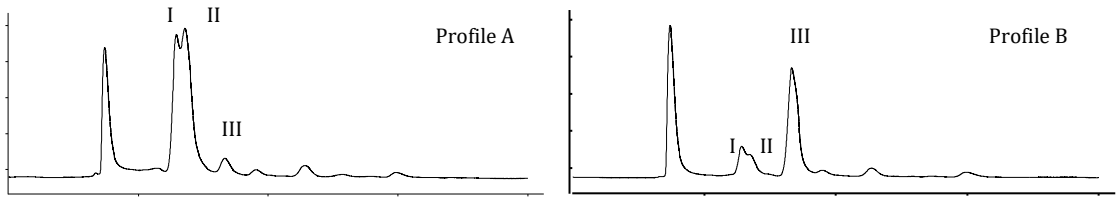

Figure 6. Profile A (from V2 T sample), rich in fructose (I) and glucose (II); Profile B (from V1 sample), rich in sucrose (III).

Table 21. Free sugar content of the flesh of the tested pumpkin genotypes (g/100 g dry weight (dw)).

Genotype	Fructose	Glucose	Sucrose	Trehalose	Raffinose	Total
V1	2.42 ± 0.03 ^{i}	2.50 ± 0.08 ^{j}	12.2 ± 0.4 ^{b}	0.46 ± 0.03 ^{c,d,e}	0.0579 ± 0.0003 ^{h,i}	17.7 ± 0.5 ^{g}
V2 T	9.7 ± 0.9 ^{a}	21.5 ± 0.3 ^{b,c}	1.4 ± 0.2 ^{i}	0.45 ± 0.02 ^{c,d,e}	0.29 ± 0.03 ^{a}	33 ± 1 ^{b}
V2 C	10.0 ± 0.3 ^{a}	22 ± 1 ^{b}	0.9 ± 0.2 ^{i}	0.67 ± 0.02 ^{a}	0.09 ± 0.02 ^{g,h,i}	34 ± 1 ^{b}
V3	6.57 ± 0.02 ^{e}	9.6 ± 0.4 ^{g}	2.6 ± 0.3 ^{g}	0.23 ± 0.02 ^{i}	0.23 ± 0.03 ^{b,c,d}	19.2 ± 0.8 ^{f,g}
V4 C	7.854 ± 0.001 ^{c,d}	18.6 ± 0.8 ^{e}	6.4 ± 0.7 ^{d}	0.51 ± 0.06 ^{b,c}	0.13 ± 0.04 ^{f,g}	33 ± 2 ^{b}
V4 R	6.6 ± 0.3 ^{e}	10.2 ± 0.6 ^{g}	3.47 ± 0.01 ^{f}	0.47 ± 0.03 ^{c,d}	0.24 ± 0.03 ^{b,c}	20.9 ± 0.9 ^{e,f}
V5 F	3.790 ± 0.002 ^{g}	4.8 ± 0.1 ^{h}	3.6 ± 0.2 ^{f}	0.408 ± 0.005 ^{d,e,f}	0.18 ± 0.01 ^{d,e,f}	12.8 ± 0.4 ^{h}
V5 R	2.55 ± 0.07 ^{i}	4.66 ± 0.05 ^{h}	4.91 ± 0.05 ^{e}	0.46 ± 0.01 ^{c,d,e}	0.06 ± 0.05 ^{h,i}	12.6 ± 0.1 ^{h}
V6	8.18 ± 0.04 ^{c}	20.33 ± 0.06 ^{c,d}	1.12 ± 0.06 ^{i}	0.50 ± 0.03 ^{b,c}	0.27 ± 0.02 ^{a,b}	30.41 ± 0.09 ^{c}
V7 P	2.8 ± 0.2 ^{h,i}	3.1 ± 0.2 ^{i,j}	14.0 ± 0.5 ^{a}	0.56 ± 0.02 ^{b}	0.05 ± 0.01 ^{i}	20.4 ± 0.8 ^{f}
V7 F	3.3 ± 0.2 ^{g,h}	4.7 ± 0.2 ^{h}	14.0 ± 0.7 ^{a}	0.50 ± 0.02 ^{b,c}	0.057 ± 0.008 ^{h,i}	23 ± 1 ^{d,e}
V8	7.4 ± 0.4 ^{d}	19 ± 2 ^{d,e}	4.0 ± 0.2 ^{f}	0.37 ± 0.02 ^{f,g}	0.25 ± 0.02 ^{a,b}	31 ± 1 ^{c}
V9 C	8.23 ± 0.04 ^{c}	12.2 ± 0.5 ^{f}	2.2 ± 0.1 ^{g,h}	0.26 ± 0.04 ^{h,i}	0.16 ± 0.02 ^{e,f}	23.1 ± 0.3 ^{d}
V9 R	4.9 ± 0.1 ^{f}	4.3 ± 0.4 ^{h,i}	10.4 ± 0.3 ^{c}	0.40 ± 0.06 ^{e,f}	0.16 ± 0.03 ^{e,f}	20.2 ± 0.9 ^{f}
V10	6.7 ± 0.4 ^{e}	19.3 ± 0.8 ^{d,e}	3.48 ± 0.08 ^{f}	0.3273 ± 0.0007 ^{g,h}	0.108 ± 0.004 ^{g,h}	30 ± 1 ^{c}
V11	8.9 ± 0.4 ^{b}	26.9 ± 0.9 ^{a}	1.50 ± 0.06 ^{h,i}	0.48 ± 0.04 ^{c}	0.20 ± 0.03 ^{c,d,e}	38.0 ± 0.5 ^{a}

Means of the same column followed by different Latin letters are statistically different according to Tukey's honestly significant difference (HSD) test at $p < 0.05$. T: turbinate; C: cylindrical; R: round; F: flattened.

The fatty acids composition of the flesh of the studied genotypes is presented in **Table 22**. The main fatty acids were palmitic (19.6–63.4%), oleic (1.98–14.1%), linoleic (5.1–35.8%) and *gamma*-linolenic acids (0.5–38.8%). So far, most of the reports in the literature focused on the fatty acids composition of seeds and seed oils where palmitic, oleic and linoleic acids are highlighted as the main compounds (Amin et al., 2019; Salehi et al., 2019), while the edible flowers contain mostly myristic, oleic, stearic and heneicosanoic acid (Ghosh & Rana, 2021) and the leaves palmitic, linoleic and linolenic acids (Iosypenko et al., 2019). To the best of our knowledge, there is a lack of scientific literature regarding the composition of fatty acids in the flesh of pumpkin fruit. The majority of fatty acids were classified as saturated and polyunsaturated fatty acids which accounted for approximately 85 to 95% of total fatty acids, while the rest of the identified compounds were monounsaturated fatty acids SFA and PUFA, respectively. The only exceptions were genotypes V2 C and V4 C where SFA was the predominant class of fatty acids accounting for approximately 85% due to the high content of palmitic acid, whereas V6 and V11 genotypes had the highest content of PUFA (approximately 65%). Moreover, the PUFA/SFA and n-6/n-3 values were higher than 0.45 and lower than 4.0, respectively, for all the tested genotypes indicating high nutritional value of pumpkin pulp (Guil et al., 1996), except for genotypes V2 C and V4 C where high amounts of PUFA and very low amounts of n-3 fatty acids were recorded.

Table 22. Fatty acids composition of the flesh of the tested pumpkin genotypes (relative %).

Compound	V1	V2 T	V2 C	V3	V4 C	V4 R	V5 F	V5 R	V6	V7 P	V7 F	V8	V9 C	V9 R	V10	V11
C8:0	n.d.	n.d.	0.264 ± 0.006 ^{a}	n.d.	0.24 ± 0.01 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.141 ± 0.001 ^{c}	n.d.	n.d.	n.d.
C10:0	0.137 ± 0.001 ^{c}	n.d.	0.1775 ± 0.0007 ^{a}	0.0830 ± 0.0000 ^{g}	0.143 ± 0.002 ^{b}	n.d.	n.d.	0.0720 ± 0.0000 ^{h}	n.d.	0.120 ± 0.001 ^{d}	0.070 ± 0.002 ^{i}	0.0680 ± 0.0000 ^{i}	0.100 ± 0.001 ^{f}	0.1125 ± 0.0007 ^{e}	n.d.	n.d.
C12:0	1.893 ± 0.004 ^{a}	0.219 ± 0.03 ^{j,k}	0.49 ± 0.02 ^{e}	0.500 ± 0.004 ^{e}	0.61 ± 0.01 ^{d}	0.3050 ± 0.0000 ^{g,h}	0.25 ± 0.01 ^{i,j,k}	0.330 ± 0.001 ^{f,g}	0.2135 ± 0.0007 ^{k}	1.49 ± 0.05 ^{b}	1.21 ± 0.04 ^{c}	0.259 ± 0.003 ^{i,j}	0.342 ± 0.004 ^{f,g}	0.36 ± 0.01 ^{f}	0·2755 ± 0·0007 ^{h,i}	0.125 ± 0.004 ^{l}
C13:0	n.d.	n.d.	n.d.	0.1020 ± 0.0000 ^{b}	n.d.	n.d.	0.058 ± 0.001 ^{d}	0.0935 ± 0.0007 ^{c}	0.0940 ± 0.0000 ^{c}	n.d.	n.d.	n.d.	n.d.	0.120 ± 0.006 ^{a}	n.d.	n.d.
C14:0	1.898 ± 0.007 ^{a}	1.22 ± 0.03 ^{e}	1.84 ± 0.03 ^{b}	1.0505 ± 0.0007 ^{g}	1.5980 ± 0.0000 ^{c}	0.7960 ± 0.0000 ^{j,k}	0.90 ± 0.01 ^{h}	1.053 ± 0.006 ^{g}	0.866 ± 0.001 ^{h,i}	1.55 ± 0.04 ^{c}	1.13 ± 0.05 ^{f}	0.83 ± 0.01 ^{i,j}	1.29 ± 0.02 ^{d}	1.21 ± 0.01 ^{e}	0.748 ± 0.008 ^{k}	0.83 ± 0.04 ^{i,j}
C15:0	0.079 ± 0.004 ^{k}	0.117 ± 0.004 ^{i}	0.53 ± 0.01 ^{b}	0.1690 ± 0.0000 ^{g}	0.648 ± 0.007 ^{a}	0.190 ± 0.001 ^{f}	n.d.	0.0895 ± 0.0007 ^{j}	0.1350 ± 0.0000 ^{h}	0.1095 ± 0.0007 ^{i}	0.0775 ± 0.0007 ^{k}	0.290 ± 0.003 ^{e}	0.445 ± 0.005 ^{c}	0.1945 ± 0.0007 ^{f}	0.3455 ± 0.0007 ^{d}	0.133 ± 0.006 ^{h}
C16:0	28.9 ± 0.2 ^{e}	20.9 ± 0.6 ^{k}	61.4 ± 0.5 ^{b}	36.46 ± 0.04 ^{c}	63.4 ± 0.2 ^{a}	29.8 ± 0.2 ^{d}	26.97 ± 0.03 ^{f,g}	26.3 ± 0.1 ^{h}	20.88 ± 0.03 ^{k}	25.67 ± 0.01 ^{i}	22.3 ± 0.2 ^{j}	30.2 ± 0.1 ^{d}	26.49 ± 0.05 ^{g,h}	27.3 ± 0.1 ^{f}	25.982 ± 0.007 ^{h,i}	19.6 ± 0.5 ^{l}
C16:1	0.42 ± 0.02 ^{g}	0.269 ± 0.006 ^{k}	0.586 ± 0.006 ^{b}	0.295 ± 0.004 ^{j}	0.53 ± 0.01 ^{c,d}	0.50 ± 0.02 ^{e}	0.45 ± 0.01 ^{f}	0.388 ± 0.002 ^{h}	0.61 ± 0.02 ^{a}	0.283 ± 0.009 ^{j,k}	0.36 ± 0.02 ^{i}	0.50 ± 0.01 ^{e}	0.520 ± 0.004 ^{d,e}	0.231 ± 0.008 ^{l}	0.37 ± 0.01 ^{h,i}	0.55 ± 0.02 ^{c}
C17:0	0.390 ± 0.002 ^{f,g}	0.224 ± 0.002 ^{i,j}	6.39 ± 0.05 ^{b}	1.23 ± 0.04 ^{c}	7.01 ± 0.04 ^{a}	0.969 ± 0.007 ^{d}	0.222 ± 0.001 ^{i,j}	0.237 ± 0.001 ^{i,j}	0.1520 ± 0.0000 ^{k}	0.602 ± 0.004 ^{e}	0.349 ± 0.001 ^{h}	0.93 ± 0.01 ^{d}	0.350 ± 0.004 ^{g,h}	0.26 ± 0.01 ^{i}	0.423 ± 0.001 ^{f}	0.199 ± 0.009 ^{j}
C17:1	0.182 ± 0.006 ^{d}	0.107 ± 0.002 ^{h}	0.84 ± 0.01 ^{a}	0.3770 ± 0.0000 ^{c}	0.65 ± 0.01 ^{b}	n.d.	0.132 ± 0.001 ^{g}	0.1465 ± 0.0007 ^{e,f}	0.1250 ± 0.0000 ^{g}	0.377 ± 0.009 ^{c}	n.d.	0.1355 ± 0.0007 ^{f,g}	0.0945 ± 0.0007 ^{i}	n.d.	0.1805 ± 0.0007 ^{d}	0.151 ± 0.006 ^{e}
C18:0	2.5 ± 0.1 ^{e,f,g}	1.85 ± 0.05 ^{j}	4.89 ± 0.07 ^{b}	2.45 ± 0.08 ^{f,g}	5.30 ± 0.02 ^{a}	2.614 ± 0.008 ^{e}	2.25 ± 0.06 ^{h}	2.02 ± 0.01 ^{i}	1.568 ± 0.005 ^{k}	4.2 ± 0.1 ^{c}	3.0 ± 0.1 ^{d}	2.53 ± 0.05 ^{e,f}	2.3 ± 0.1 ^{h}	1.64 ± 0.04 ^{k}	2.37 ± 0.04 ^{g,h}	1.54 ± 0.06 ^{k}
C18:1n9c	12.1 ± 0.3 ^{c}	14.1 ± 0.1 ^{a}	8.4 ± 0.2 ^{f}	6.0 ± 0.2 ^{i}	4.95 ± 0.05 ^{j}	7.6 ± 0.2 ^{g}	8.6 ± 0.2 ^{e,f}	6.53 ± 0.03 ^{h}	9.57 ± 0.04 ^{d}	13.30 ± 0.02 ^{b}	8.9 ± 0.4 ^{e}	4.720 ± 0.007 ^{j}	5.86 ± 0.01 ^{i}	1.980 ± 0.006 ^{l}	3.919 ± 0.006 ^{k}	9.46 ± 0.03 ^{d}

Compound	V1	V2 T	V2 C	V3	V4 C	V4 R	V5 F	V5 R	V6	V7 P	V7 F	V8	V9 C	V9 R	V10	V11
C18:2n6c	22.9 ± 0.2 ^{k}	34.0 ± 0.8 ^{c}	5.1 ± 0.1 ^{m}	25.8 ± 0.2 ^{i}	5.9 ± 0.2 ^{l}	28.71 ± 0.08 ^{g}	27.0 ± 0.1 ^{h}	32.2 ± 0.2 ^{e}	30.12 ± 0.03 ^{f}	24.8 ± 0.1 ^{j}	29.8 ± 0.2 ^{f}	35.78 ± 0.03 ^{a}	31.97 ± 0.04 ^{e}	33.4 ± 0.1 ^{d}	34.65 ± 0.07 ^{b}	25.09 ± 0.07 ^{j}
C18:3n6	0.162 ± 0.006 ^{c}	0.080 ± 0.002 ^{d}	n.d.	n.d.	0.54 ± 0.03 ^{a}	n.d.	0.216 ± 0.006 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C18:3n3	25.9 ± 0.3 ^{f}	24.9 ± 0.4 ^{g}	0.512 ± 0.001 ^{l}	20.4 ± 0.1 ^{j}	0.60 ± 0.02 ^{l}	24.48 ± 0.02 ^{h}	29.81 ± 0.02 ^{c}	27.3 ± 0.4 ^{e}	32.87 ± 0.04 ^{b}	23.42 ± 0.07 ^{i}	29.53 ± 0.08 ^{c}	19.003 ± 0.0007 ^{k}	25.73 ± 0.07 ^{f}	29.59 ± 0.09 ^{c}	28.5 ± 0.1 ^{d}	38.8 ± 0.3 ^{a}
C20:0	n.d.	n.d.	1.03 ± 0.03 ^{a}	0.3790 ± 0.0000 ^{c}	0.75 ± 0.03 ^{b}	n.d.	n.d.	0.213 ± 0.001 ^{g}	0.1840 ± 0.0000 ^{h}	0.311 ± 0.003 ^{d,e}	0.25 ± 0.01 ^{f}	0.289 ± 0.001 ^{e}	0.335 ± 0.001 ^{d}	0.37 ± 0.01 ^{c}	0.2315 ± 0.0007 ^{f,g}	0.235 ± 0.008 ^{f,g}
C20:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2015 ± 0.0007 ^{b}	n.d.	n.d.	n.d.	0.542 ± 0.002 ^{a}	n.d.	n.d.	n.d.
C21:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1345 ± 0.0007 ^{f}	0.1455 ± 0.0007 ^{e}	0.184 ± 0.002 ^{c}	n.d.	0.241 ± 0.001 ^{a}	0.1555 ± 0.0007 ^{d}	0..2135 ± 0.0007 ^{b}	n.d.	n.d.
C20:3n6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1355 ± 0.0007	n.d.	n.d.	n.d.
C22:0	0.68 ± 0.02 ^{j}	0.811 ± 0.004 ^{i}	3.23 ± 0.01 ^{a}	1.11 ± 0.03 ^{e,f}	2.02 ± 0.02 ^{b}	1.26 ± 0.04 ^{c}	1.16 ± 0.04 ^{d,e}	1.089 ± 0.006 ^{f}	0.876 ± 0.009 ^{h}	1.16 ± 0.01 ^{d,e}	1.13 ± 0.05 ^{d,e,f}	1.18 ± 0.02 ^{d}	0.838 ± 0.003 ^{h,i}	0.973 ± 0.005 ^{g}	0.653 ± 0.002 ^{j}	1.26 ± 0.05 ^{c}
C20:5n3	0.099 ± 0.003	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C22:1	n.d.	n.d.	n.d.	0.166 ± 0.006 ^{c}	n.d.	0.602 ± 0.009 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.435 ± 0.002 ^{b}	n.d.	n.d.	n.d.
C22:2	0.264 ± 0.004 ^{d}	0.163 ± 0.004 ^{h}	n.d.	0.223 ± 0.002 ^{f}	0.246 ± 0.008 ^{e}	n.d.	0.152 ± 0.006 ^{i}	0.28 ± 0.01 ^{c}	0.141 ± 0.005 ^{j}	0.536 ± 0.006 ^{a}	0.240 ± 0.007 ^{e}	0.334 ± 0.002 ^{b}	0.225 ± 0.001 ^{f}	0.1840 ± 0.0000 ^{g}	0.1775 ± 0.0007 ^{g}	0.150 ± 0.004 ^{i,j}
C22:6n3					0·291 ± 0·006											
C23:0	0.368 ± 0.001 ^{g,h}	0.33 ± 0.01 ^{i}	0.98 ± 0.04 ^{a}	0.519 ± 0.009 ^{d}	0.87 ± 0.03 ^{b}	0.427 ± 0.006 ^{f}	0.385 ± 0.008 ^{g,h}	0.37 ± 0.01 ^{g,h}	0.39 ± 0.01 ^{g}	0.539 ± 0.006 ^{d}	0.47 ± 0.02 ^{e}	0.438 ± 0.002 ^{e,f}	0.537 ± 0.002 ^{d}	0.615 ± 0.001 ^{c}	0.360 ± 0.001 ^{h,i}	0.598 ± 0.006 ^{c}
C24:0	1.14 ± 0.01 ^{h}	0.78 ± 0.03 ^{i}	3.4 ± 0.2 ^{b}	2.63 ± 0.06 ^{c}	3.696 ± 0.008 ^{a}	1.67 ± 0.03 ^{e}	1.38 ± 0.01 ^{f}	1.141 ± 0.006 ^{h}	0.854 ± 0.008 ^{i}	1.36 ± 0.01 ^{f}	1.25 ± 0.05 ^{g,h}	2.3 ± 0.1 ^{d}	1.136 ± 0.004 ^{h}	1.1905 ± 0.0007 ^{g,h}	0.811 ± 0.003 ^{i}	1.28 ± 0.03 ^{f,g}
C24:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0875 ± 0.0007	n.d.	n.d.	n.d.

Compound	V1	V2 T	V2 C	V3	V4 C	V4 R	V5 F	V5 R	V6	V7 P	V7 F	V8	V9 C	V9 R	V10	V11
SFA	38.0 ± 0.3 ^{e}	26.4 ± 0.5 ^{k}	84.6 ± 0.4 ^{b}	46.7 ± 0.1 ^{c}	86.3 ± 0.2 ^{a}	38.8 ± 0.09 ^{e}	33.58 ± 0.02 ^{h}	33.1 ± 0.2 ^{h}	26.36 ± 0.03 ^{k}	37.3 ± 0.2 ^{d}	31.2 ± 0.3 ^{j}	39.53 ± 0.02 ^{d}	34.41 ± 0.03 ^{g}	34.6 ± 0.2 ^{g}	32.20 ± 0.02 ^{i}	25.8 ± 0.4 ^{l}
MUFA	12.7 ± 0.2 ^{c}	14.5 ± 0.1 ^{a}	9.8 ± 0.3 ^{f}	6.9 ± 0.2 ^{j}	6.13 ± 0.07 ^{k}	8.7 ± 0.2 ^{h}	9.2 ± 0.2 ^{g}	7.07 ± 0.04 ^{j}	10.51 ± 0.05 ^{d}	13.96 ± 0.00 ^{b}	9.2 ± 0.4 ^{g}	5.36 ± 0.02 ^{l}	7.533 ± 0.006 ^{i}	2.211 ± 0.003 ^{n}	4.46 ± 0.01 ^{m}	10.157 ± 0.008 ^{e}
PUFA	49.3 ± 0.5 ^{i}	59.1 ± 0.4 ^{d}	5.6 ± 0.1 ^{m}	46.4 ± 0.3 ^{k}	7.6 ± 0.3 ^{l}	53.2 ± 0.1 ^{h}	57.2 ± 0.2 ^{f}	59.8 ± 0.2 ^{c}	63.13 ± 0.02 ^{b}	48.8 ± 0.2 ^{j}	59.59 ± 0.07 ^{c,d}	55.12 ± 0.03 ^{g}	58.05 ± 0.04 ^{e}	63.2 ± 0.2 ^{b}	63.34 ± 0.03 ^{b}	64.0 ± 0.4 ^{a}

Means of the same row followed by different Latin letters are statistically different according to Tukey's honestly significa nt difference (HSD) test at $p < 0.05$; n.d.: not detected. T: turbinate; C: cylindrical; R: round; F: flattened; Caprylic acid (C8:0); Capric acid (C10:0); lauric acid (C12:0); tridecylic acid (C13:0); myristic acid (C14:0); pentadecylic acid (C15:0); palmitic acid (C16:0); palmitoleic acid (C16:1); m argaric acid (C17:0); heptadecenoic acid (C17:1); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6c); -linolenic acid (C18:3n6); _-linolenic acid (C18:3n3); arachidic acid (C20:0); gondoic acid (C20:1); heneicosylic acid (C21:0); arachidonic acid (C20:3n6); behenic acid (C22:0); ei cosatrienoic acid (C20:5n3); erucic acid (C22:1); docosadienoic acid (C22:2); (C20:6n3); tricosylic acid (C23:0); lignoceric acid (C24:0); nerv onic acid (C24:1); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Bioactivities

Antioxidant capacity

Varied lipid peroxidation and oxidative hemolysis inhibition capacity were recorded among the tested genotypes (**Table 23**). In particular, V4 C and V7 P genotypes recorded the highest antioxidant activity for the TBARS assay (lowest IC_{50} values), while the V8 genotype was the most effective in the case of the OxHLIA assay. In any case, none of the tested extracts was more effective than Trolox which was used as the positive control for both assays. Recently, Leichtweis et al. (2022, 2023) determined the antioxidant activity in different parts of *Cucurbita* fruit cultivated in Portugal, Algeria and Greece suggested a variable response to TBARS and OxHLIA assays depending on the fruit type and the growing location, while seeds recorded the highest antioxidant activity compared to peels and endocarp. On the other hand, Jahan et al. (2023) suggested that flowers showed the highest antioxidant activity, followed by flesh, leaves, seeds and peels, which indicates the effect of the implemented assay on the obtained results. Moreover, the addition of powder from pumpkin fruit and flowers improved the capacity of lipid peroxidation inhibition in cooked sausages and chicken (Bulambaeva et al., 2014; Santos et al., 2022). In the studies of Rolnik et al. (2020, 2021), where the inhibition of lipid peroxidation was determined via the TBARS assay, it was reported a significant variability among cucurbit vegetables, including pumpkin, zucchini and squash which highlights the importance of the genotypic impact on the antioxidant capacity of *Cucurbita* fruit. In contrast, Tarwadi & Agte (2005) who compared fruit and root vegetables classified pumpkin fruit among the species with poor performance in terms of the TBARS assay.

Table 23. Lipid peroxidation inhibition capacity (TBARS) and oxidative hemolysis inhibition capacity (OxHLIA) of the flesh of the tested pumpkin genotypes (inhibition capacity (IC_{50}), µg/mL).

Genotype	TBARS (IC_{50} [1], µg/mL)	$OxHLIA_{60min}$ (IC_{50} [1], µg/mL)
V1	2877 ± 79 ^{i}	674 ± 21 ^{c}
V2 T	3000 ± 120 ^{g,h}	302 ± 9 ^{h,i}
V2 C	5339 ± 151 ^{b}	980 ± 44 ^{a}
V3	3328 ± 164 ^{f}	236 ± 11 ^{j,k}
V4 C	1864 ± 76 ^{m}	114 ± 7 ^{l}
V4 R	2310 ± 47 ^{k}	187 ± 7 ^{k}
V5 F	2556 ± 121 ^{j}	350 ± 13 ^{g,h}
V5 R	2218 ± 111 ^{l}	599 ± 10 ^{d}
V6	3022 ± 103 ^{g}	284 ± 8 ^{i,j}
V7 P	1816 ± 91 ^{m}	385 ± 30 ^{g}
V7 F	4035 ± 157 ^{d}	452 ± 12 ^{f}
V8	2915 ± 133 ^{h,i}	35 ± 3 ^{m}
V9 C	4149 ± 192 ^{c}	254 ± 13 ^{i,j}
V9 R	2981 ± 84 ^{g,h}	520 ± 17 ^{e}
V10	5694 ± 129 ^{a}	255 ± 8 ^{i,j}
V11	3561 ± 161 ^{e}	792 ± 31 ^{b}
Trolox	139 ± 5	21.8 ± 0.2

[1] IC_{50}: extract concentration that inhibits oxidation by 50%; means of the same column followed by different Latin letters are statistically different according to Tukey's honestly significant difference (HSD) test at $p < 0.05$. T: turbinate; C: cylindrical; R: round; F: flattened.

Antimicrobial activity

The antimicrobial properties of the extracts obtained from the fruit of the tested genotypes are presented in **Table 24**. In general, despite some slight differences among the tested extracts in terms of MIC. MBC and MFC values, none of them showed higher activity against various Gram– and Gram+ bacterial strains and two species of *Aspergillus* compared to streptomycin, ampicillin and ketoconazole. However, almost all the tested extracts showed the ability to inhibit the growth of at least one bacterial strain (MIC values up to 10 mg/mL), except for the case of V9 R where no effects were recorded. Moreover, the lowest overall MIC values were recorded for V2 T against *Salmonella enterica* (5 mg/mL); V6 against *Salmonella enterica* (5 mg/mL for both bacteria); V7 F against *Yersinia enterocolitica* (2.5 mg/mL); V8 against *Escherichia coli* (5 mg/mL); V9 C against *Enterobacter cloacae* (5 mg/mL); and V10 against *Enterobacter cloacae* and *Yersinia enterocolitica* (5 mg/mL). Similarly, most of the tested extracts showed efficacy against at least one of the studied *Aspergillus* species, except for V1, V2, V4 C and V5 genotypes. Similar to our study, Leichtweis et al. (2022, 2023) suggested significant antimicrobial properties from fruit parts (peels, endocarp and seeds) of different pumpkin genotypes. Moreover, Mokhtar et al. (2021) reported varied antimicrobial properties of *C. moschata* fruit depending on their

ripeness with extracts from mature fruit being more effective against various bacterial strains compared to young and ripe fruit. According to the same authors, this finding was mainly attributed to the highest content of polyphenols detected in this particular stage (Mokhtar et al., 2021). Badr et al. (2011) also recorded moderate efficacy of rind and flesh extracts against *Bacillus subtilis* and *B cereus*. Hussain et al. (2021) suggested seed extracts showed higher activity against *Candida albicans*, *Fusarium oxysporum*, *Mucor miehei* and *Trichoderma* spp. compared to peels and flesh, whereas flesh extracts recorded greater activity against *Salmonella typhi*, *Escherichia coli*, *Bacillus subtilis* and *Streptococcus aureus* compared to the other two extracts. The beneficial effects of different pumpkin powders, extracts, isolates, purified bioactives and pumpkin-based functional food products are well reported in the review performed by Hussain, Kausar, Sehar, Sarwar, Ashraf, Jamil, Noreen, Rafique, Iftikhar, Aslam, et al. (2022), which corroborates its health promotion properties. In contrast, Saavedra et al. (2015) did not record any antimicrobial effects for peel and seed extracts obtained from *C. pepo* byproducts (shells and seeds).

The reported antimicrobial activity of the pumpkin pulp extracts assessed in the present study, together with the antioxidant activity described above, supports the importance of including this nutritious and bioactive fruit in healthy diets.

None of the tested samples presented cytotoxic activity against the non-tumor porcine liver cells (up to 400 μg/mL), which indicates the high potential of using pumpkin flesh as a natural antioxidant agent in the food industry. This finding is in agreement with the previous reports of Leichtweis et al. (2022, 2023), who did not record any cytotoxic effect on non-tumor cells for the extracts from different parts of pumpkin fruit. Moreover, according to Gaweł-Bęben et al. (2022), peel extracts did not show any toxic effects against human keratinocytes up to the concentration of 1000 μg/mL suggesting its safe use in the cosmeceutical industry. Similarly, the extracts of zucchini fruit showed a high potential for use in cosmetic emulsions since they did not show toxic effects against HaCaT cell lines or reconstructed human epidermis (Piccolella et al., 2019). This satisfactory result was expected since pumpkin pulp has been used for thousands of years for animal and human consumption. However, considering mainly local varieties, which are not yet widely cultivated and consumed, these results add new safety insights, since varieties of the same species can considerably vary in terms of chemical composition. In fact, the presence of bioactive compounds with cytotoxic activity against tumor cell lines is widely reported in pumpkins, such as, for example, cucurbitin (Massironi et al., 2023; Montesano et al., 2018), and in the present study we aimed to confirm their safety, testing the obtained extracts against non-tumor cells.

Table 24. Antimicrobial and antifungal activity of the flesh of different pumpkin varieties from Greece (mg/mL).

	V1		V2 T		V2 C		V3		V4 C		V4 R		V5 F		V5 R		V 6		V7 P	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria																				
Enterobacter cloacae	>10	>10	10	>10	>10	>10	>10	>10	>10	>10	10	>10	10	>10	>10	>10	10	>10	>10	>10
Escherichia coli	>10	>10	10	>10	10	>10	10	>10	10	>10	10	>10	10	>10	10	>10	5	>10	>10	>10
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Salmonella enterica	>10	>10	5	>10	10	>10	10	>10	10	>10	10	>10	10	>10	10	>10	5	>10	>10	>10
Yersinia enterocolitica	10	>10	>10	>10	10	>10	>10	>10	10	>10	>10	>10	>10	>10	>10	>10	>10	>10	10	>10
Gram-positive bacteria																				
Bacillus cereus	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Listeria monocytogenes	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Staphylococcus aureus	10	>10	10	>10	10	>10	>10	>10	10	>10	>10	>10	10	>10	10	>10	>10	>10	10	>10

	V7 F		V8		V9 C		V9 R		V10		V11		Streptomicin		Methicilin		Ampicillin	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MIC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria																		
Enterobacter cloacae	10	>10	>10	>10	5	>10	>10	>10	5	>10	10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	>10	>10	5	>10	10	>10	>10	>10	>10	>10	10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	10	>10	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterica	10	>10	10	>10	>10	>10	>10	>10	>10	>10	10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	2.5	>10	>10	>10	>10	>10	>10	>10	5	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria																		
Bacillus cereus	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	10	>10	10	>10	>10	>10	>10	>10	10	>10	10	>10	0.007	0.007	0.007	0.007	0.15	0.15

	V1		V2 T		V2 C		V3		V4 C		V4 R		V5 F		V5 R		V 6	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Aspergillus brasiliensis	>10	>10	>10	>10	>10	>10	10	>10	>10	>10	10	>10	>10	>10	>10	>10	10	>10
Aspergillus fumigatus	>10	>10	>10	>10	>10	>10	10	>10	>10	>10	10	>10	>10	>10	>10	>10	>10	>10

	V7 P		V7 F		V8		V9 C		V9 R		V10		V11		Ketoconazole	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Aspergillus brasiliensis	10	>10	10	>10	10	>10	10	>10	10	>10	10	>10	10	>10	0.06	0.125
Aspergillus fumigatus	>10	>10	>10	>10	10	>10	>10	>10	>10	>10	10	>10	>10	>10	0.5	1

MIC: minimum inhibitory concentration; MBC: minimal bactericidal concentration; MFC: minimal fungicidal concentration; n.t.: not tested; T: turbinate; C: cylindrical; R: round; F: flattened.3.3.3.

Chemical characterization

Organic acids

The main detected organic acids were oxalic and malic acid, followed by citric and fumaric acid, whereas ascorbic and shikimic acid were either detected in traces or not detected (**Table 25**). The V9 C genotype recorded the highest content of oxalic and citric acids (6.4 and 3.2 g/100 g dw, respectively), while malic acid was the highest in V4 R and V10 genotypes (5.38 and 5.2 g/100 g dw, respectively) and fumaric acid in V6 genotype (0.0943 mg/100 g dw). According to Priecina & Karklina (2015) oxalic acid was the major detected compound in fresh pumpkin fruit followed by malic and citric acid, while the amounts of oxalic acid were considerably higher than those of our study (approximately 15 g/100 g dw). Moreover, Iswaldi et al. (2013) tentatively identified eight organic acids and derivatives in three zucchini cultivars, although the authors did not quantify the detected compounds. Abbas et al. (2020) suggested that organic acid composition and content in pumpkins may be affected by the growing conditions, the genotype and the developmental stage of fruits. In particular, they indicated a significant decrease in organic acids content throughout the maturation process, while they suggested oxalic and fumaric acids as the prevalent ones at all the developmental stages (Nawirska-Olszańska et al., 2014). In contrast, Nawirska-Olszańska et al. (2014) and Zhou et al. (2017) reported the presence of only malic, citric and fumaric acid with varied content among the different genotypes of pumpkin species (*C. pepo*, *C. maxima* and *C. moschata*). Therefore, any contradictions among reports from the literature could be associated with differences in the genotype, the growing conditions and the harvesting stage of fruit.

Table 25. Quantification of the organic acids in the flesh of the studied pumpkin genotypes.

Genotype	Oxalic g/100 g dw	Malic g/100 g dw	Ascorbic g/100 g dw	Shikimic g/100 g dw	Citric g/100 g dw	Fumaric mg/100 g dw
V1	3.61 ± 0.04 ^{f}	2.7 ± 0.1 ^{j}	tr..	tr.	1.08 ± 0.02 ^{d}	0.0091 ± 0.0001 ^{m}
V2 T	4.45 ± 0.04 ^{e}	3.96 ± 0.06 ^{f,g}	n.d.	tr.	n.d.	0.02825 ± 0.00007 ^{g}
V2 C	4.8 ± 0.2 ^{d}	4.1 ± 0.2 ^{d,e,f}	n.d.	tr.	n.d.	0.034 ± 0.001 ^{e}
V3	5.50 ± 0.01 ^{b}	4.0 ± 0.2 ^{e,f,g}	n.d.	n.d.	n.d.	0.0169 ± 0.0007 ^{k,l}
V4 C	4.5 ± 0.1 ^{e}	4.9 ± 0.2 ^{b}	n.d.	n.d.	n.d.	0.0255 ± 0.0008 ^{h}
V4 R	5.17 ± 0.03 ^{c}	5.38 ± 0.07 ^{a}	n.d.	n.d.	n.d.	0.0193 ± 0.0009 ^{i,j}
V5 F	5.301 ± 0.006 ^{c}	4.28 ± 0.03 ^{c,d,e}	n.d.	tr.	3.16 ± 0.09 ^{a}	0.0155 ± 0.0002 ^{l}
V5 R	1.80 ± 0.03 ^{h}	3.14 ± 0.07 ^{i}	tr.	tr.	n.d.	0.031 ± 0.002 ^{f}
V6	3.641 ± 0.006 ^{f}	5.16 ± 0.06 ^{a,b}	n.d.	n.d.	n.d.	0.0943 ± 0.0002 ^{a}
V7 P	3.75 ± 0.07 ^{f}	4.2 ± 0.1 ^{c,d,e,f}	tr.	tr.	1.38 ± 0.06 ^{c}	0.021 ± 0.001 ^{i}
V7 F	3.64 ± 0.02 ^{f}	4.3 ± 0.2 ^{c,d,e,f}	tr.	tr.	1.62 ± 0.01 ^{b}	0.0161 ± 0.0003 ^{l}
V8	4.77 ± 0.01 ^{d}	3.48 ± 0.05 ^{h}	n.d.	tr.	n.d.	0.069 ± 0.003 ^{b}
V9 C	6.4 ± 0.1 ^{a}	3.7 ± 0.2 ^{g,h}	n.d.	n.d.	3.2 ± 0.1 ^{a}	n.d.
V9 R	3.40 ± 0.04 ^{g}	4.3 ± 0.2 ^{c,d}	tr.	n.d.	n.d.	0.0188 ± 0.0009 ^{j,k}
V10	4.79 ± 0.05 ^{d}	5.2 ± 0.2 ^{a,b}	n.d.	n.d.	n.d.	0.0393 ± 0.0004 ^{d}
V11	4.36 ± 0.09 ^{e}	4.50 ± 0.06 ^{c}	n.d.	n.d.	n.d.	0.061 ± 0.001 ^{c}

n.d.: not detected. tr.: traces. Means of the same column followed by different Latin letters are statistically different according to Tukey's honestly significant difference (HSD) test at $p < 0.05$. T: turbinate; C: cylindrical; R: round; F: flattened.

Tocopherols

The main detected vitamin E isoform was *alpha*-tocopherol which recorded in amounts that ranged between 0.218 (V7 F) and 4.90 mg/100 g dw (V2 T), followed by *beta*- and *gamma*- tocopherols (**Table 26**). So far, there is scarce literature regarding the tocopherol composition in the flesh of pumpkin fruit since most of the studies refer to seeds and seed oils. Similarly to our study, Kim et al. (2012) suggested *alpha*-tocopherol as the main compound in the flesh of three pumpkin species (*C. pepo*, *C. moschata* and *C. maxima*), while *gamma*-tocopherol was detected only in low amounts in *C. moschata* fruit. Kulczyński & Gramza-Michałowska (2019) detected only *alpha*- and *gamma*-tocopherols in the pulp of *C. pepo* and *C. moschata* fruit in amounts that were towards the highest range of our study, whereas the other two vitamin E isoforms (*beta*- and *delta*-tocopherols) were not detected. Therefore, the results of our study along with the literature reports suggest that pumpkin fruit flesh can be a rich source of tocopherols (especially *alpha*-tocopherol), although a high genotypic variability should be expected at the intra- or inter-species level.

Table 26. Tocopherol content of the extracts of the studied pumpkin genotypes of pumpkin flesh (mg/100 g dw).

Genotype	*alpha*-tocopherol	*beta*-tocopherol	*gamma*-tocopherol	Total tocopherols
V1	1.8 ± 0.1 ^{g}	0.1496 ± 0.0005 ^{d}	n.d.	1.9 ± 0.1 ^{h}
V2 T	4.90 ± 0.08 ^{a}	6.59 ± 0.08 ^{a}	n.d.	11.5 ± 0.2 ^{a}
V2 C	0.81 ± 0.03 ^{k}	0.238 ± 0.003 ^{c}	n.d.	1.05 ± 0.03 ^{k}
V3	1.57 ± 0.02 ^{h}	n.d.	1.05 ± 0.02 ^{d}	2.62 ± 0.04 ^{f}
V4 C	1.15 ± 0.03 ^{i,j}	n.d.	0.1998 ± 0.0002 ^{g,h}	1.35 ± 0.03 ^{j}
V4 R	2.68 ± 0.06 ^{c}	n.d.	1.619 ± 0.005 ^{b}	4.30 ± 0.06 ^{d}
VP F	2.3 ± 0.1 ^{e}	0.85 ± 0.01 ^{b}	n.d.	3.19 ± 0.08 ^{e}
V5 R	1.03 ± 0.02 ^{j}	n.d.	1.02 ± 0.03 ^{d}	2.05 ± 0.04 ^{h}
V6	2.47 ± 0.01 ^{d}	n.d.	4.5 ± 0.1 ^{a}	7.0 ± 0.1 ^{b}
V7 P	0.70 ± 0.04 ^{k}	0.043 ± 0.001 ^{e}	0.185 ± 0.004 ^{h}	0.93 ± 0.04 ^{k}
V7 F	0.218 ± 0.008 ^{m}	n.d.	0.2685 ± 0.0007 ^{f,g}	0.486 ± 0.009 ^{l}
V8	1.16 ± 0.04 ^{j}	n.d.	0.58 ± 0.02 ^{e}	1.73 ± 0.02 ^{i}
V9 C	1.70 ± 0.09 ^{g}	n.d.	0.572 ± 0.003 ^{e}	2.3 ± 0.1 ^{g}
V9 R	2.91 ± 0.06 ^{b}	n.d.	1.24 ± 0.03 ^{c}	4.15 ± 0.04 ^{d}
V10	1.97 ± 0.09 ^{f}	n.d.	0.28 ± 0.01 ^{f}	2.3 ± 0.1 ^{g}
V11	0.55 ± 0.01 ^{l}	n.d.	4.52 ± 0.07 ^{a}	5.07 ± 0.08 ^{c}

n.d.: not detected. Means of the same column followed by different Latin letters are statistically different according to Tukey's honestly significant difference (HSD) test at $p < 0.05$. T: turbinate; C: cylindrical; R: round; F: flattened.

Principal component analysis

The principal component analysis (PCA) was implemented to reveal groups and highlight similarities and differences based on multivariate data. The processing of our data showed that the first eleven principal components (PCs) were associated with Eigen values higher than 1, explaining 95.9% of the cumulative variance, with PC1 accounting for 24.6%, PC2 for 18.4% and PC3 for 13.3%; and PC4 for 8.8%, PC% for

7.9%, PC6 for 6.3%, PC7 for 4.7%, PC8 for 3.9%, PC9 for 3.6%, PC10 for 2.4% and PC11 for 2.1%. In particular, PC1 showed a positive correlation with C18:2n6c and PUFA and a negative correlation with C8:0, C10:0, C15:0, C16:0, C17:0, C20:0, C22:0, C23:0, C24:0 and SFA. On the other hand, PC2 showed a positive correlation with ash, fibres, fructose, oxalic acid and raffinose content, as well as with TBARS, C22:1 and C24:1, whereas a negative correlation with carbohydrates and energy content was recorded. Finally, PC3 showed a positive correlation with fat, fumaric acid, glucose, total sugars and total tocopherols content, whereas a negative correlation was observed for C20:3n6, C22:1, C24:1 and citric acid. Therefore, PCA may help to discriminate the studied landraces as depicted in the following scatterplots and loading plots. The scatterplot in **Figure 7** shows four distinct groups of the studied landraces based on their chemical composition and bioactive properties of fruit flesh.

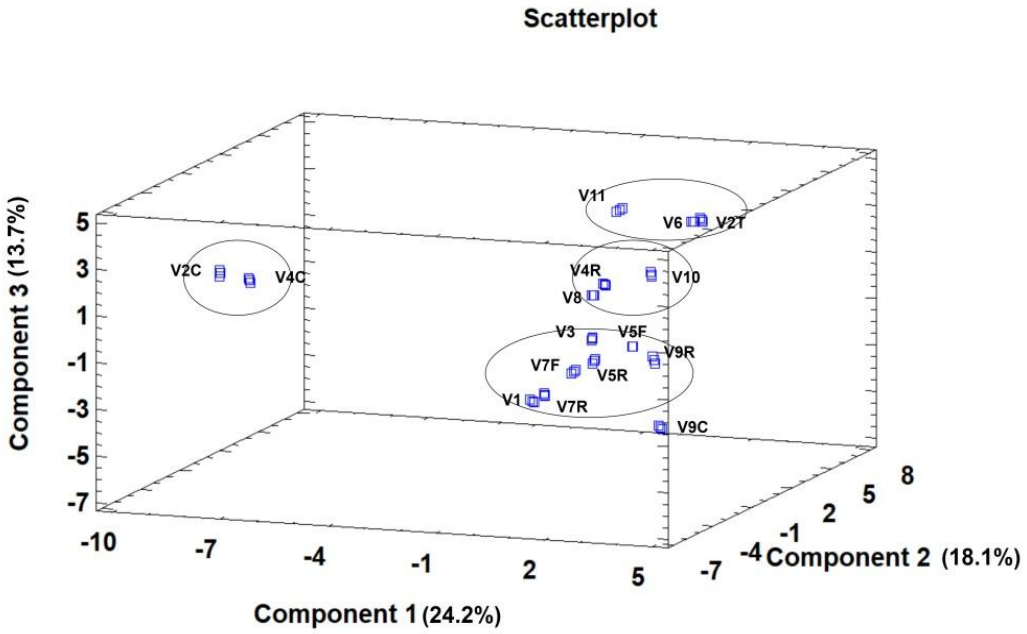

Figure 7. Three-dimensional scatterplot of principal components 1, 2 and 3 for pumpkin fruit flesh.

The loading plot of PC1 and PC2 presents the following correlations: the upper left quadrant included fibers, ash, oxalic acid, fructose, glucose, total sugars, total organic acids, malic acid, TBARS, C8:0, C15:0, C16:1, C17:1, C20:0 and C23:0; the lower left quadrant included SFA, C10:0, C12:0, C14:0, C16:0, C17:0, C18:0, C22:0, C22:6n3, C24:0, trehalose, OxHLIA and carbohydrates; the upper right quadrant included C13:0, C18:2n6, C18:3n6, C20:1, C20:3n6, C21:0, C22:1, C24:1, *alpha*-tocopherol, *beta*-tocopherol, *gamma*-tocopherol, total tocopherols, raffinose, fumaric acid, protein, fat and PUFA; the lower right quadrant included C18:1n9c, C18:3n3, C20:5n3, C22:2, citric acid,

MUFA, sucrose and energy content (**Figure 8**).

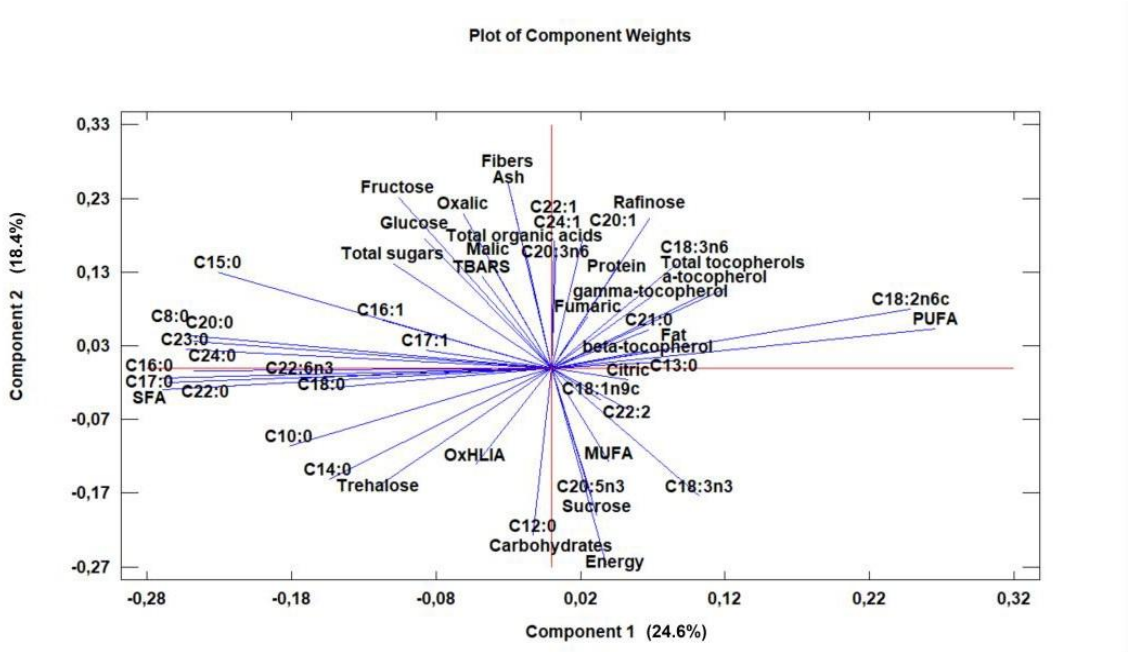

Figure 8. The loading plot of principal components 1 and 2 for pumpkin fruit flesh.

Moreover, the loading plot of PC1 and PC3 also revealed groups of positively correlated variables (**Figure 9**). The upper left quadrant included glucose, fructose, trehalose, total sugars, malic acid, carbohydrates, fibers, TBARS, OxHLIA, C16:1, C17:0, C17:1, C22:0 and C22:6n3; the lower left quadrant included C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C18:0, C20:0, C23:0, C24:0, SFA, oxalic acid, total organic acids and ash; the upper right quadrant included fumaric acid, fat, *alpha*-tocopherol, *beta*-tocopherol, *gamma*-tocopherol, total tocopherols, fat, raffinose, MUFA, energy content, C13:0, C18:1n9c and C18:3n3; the lower right quadrant included C18:3n6, C18:2n6c, C20:3n6, C20:1, C20:5n3, C21:0, C22:1, C22:2, C24:1, PUFA, sucrose, protein and citric acid.

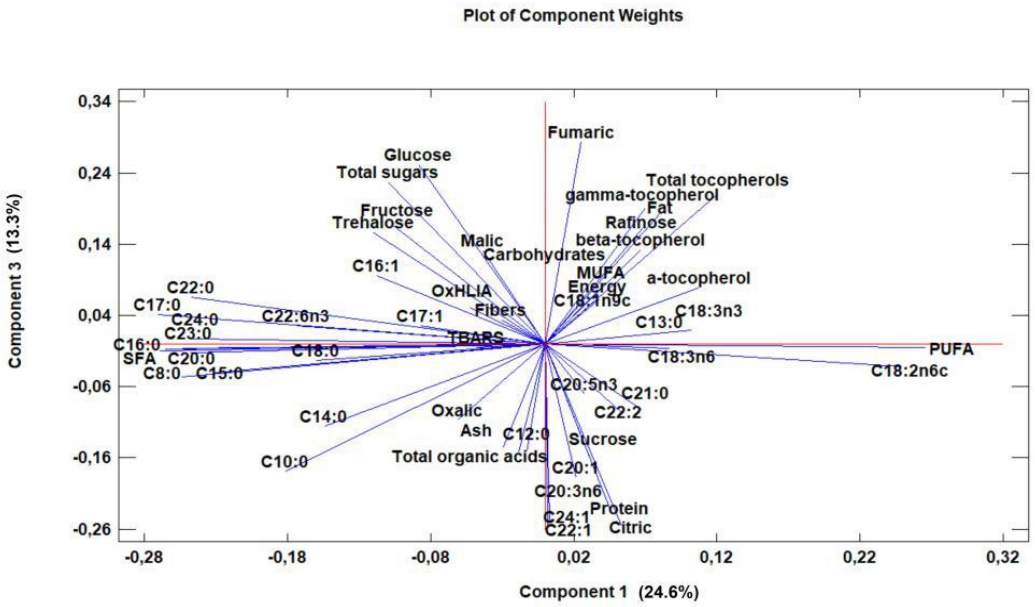

Figure 9. The loading plot of principal components 1 and 3 for pumpkin fruit flesh.

3.2.4. Conclusions

The genetic pool of Greek pumpkin germplasm is not adequately characterized so far, especially when considering the high genetic variability of the species which could be a valuable tool for the adaptation of modern horticulture in the changing climate conditions. The heterogeneity in terms of agronomic performance and chemical profile of fruit in pumpkin germplasm makes it necessary to evaluate local landraces aiming to identify promising genotypes with high yield and quality of fruit. Our results indicate that the local landraces and commercial genotypes studied had a high variability not only in fruit yield but also in all the tested parameters related to the chemical composition and bioactive properties of fruit flesh. The local landraces V2 and V8 (from "Trikala" and "Laconia", respectively) recorded the highest fruit yield, while the V2 landrace also had the highest tocopherols (*alpha*-, *beta*- and total tocopherols) and fructose, trehalose and raffinose content. On the other hand, V8 landrace was the richest in linoleic acid and also presented the highest antioxidant activity for the OxHLIA assay. However, the rest of the tested landraces presented interesting features such as V11 ("Voutirato") which had the highest sweetness index. Finally, the flesh extracts of most of the tested genotypes showed promising antimicrobial properties, while none of them was toxic against non-tumor cells. Nevertheless, our results support the importance of the conservation and valorization of local genetic material for the selection of elite genotypes with improved nutritional value and quality. However, further research is needed in order to reveal the genetic heterogeneity of the material studied that would help to introduce local landraces in

breeding programs.

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3.2.5. References

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3.3. Valorisation of pumpkin by-products: Chemical composition and bioactive properties of pumpkin seeds, peels, and fibrous strands from different local landraces of Greece

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Valorisation of pumpkin by-products: Chemical composition and bioactive properties of pumpkin seeds, peels, and fibrous strands from different local landraces of Greece

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Abstract: This study evaluated the fruit by-products (peels, seeds, and fibrous strands) from 11 pumpkin genotypes cultivated in Greece aiming to valorize them as natural sources of bioactive compounds. Five compounds, including (-)-epicatechin and chicoric acid isomers, were identified in peels, while seeds and fibrous strands mainly contained (-)-epicatechin. Organic acids and tocopherols varied significantly among genotypes, with oxalic, quinic, and malic acids being predominant. Total tocopherols content (mg/100 g) ranged up to 7.38 ± 0.03 in fibrous strands, 30.7 ± 0.2 in peels, and 14.58 ± 0.09 in seeds. Extracts exhibited potent antioxidant activity, particularly the seeds of genotypes "V5" and "V6", and strong antimicrobial effects, notably the peels of "V2 T" and "V11", which showed significant inhibition of microbial strains. These findings contribute to the advancement of sustainable practices in the agro-industrial waste management, as well as to the production of functional natural ingredients for various industrial applications.

Keywords: Antioxidant activity; Antimicrobial activity; Phenolic compounds; Organic acids; Tocopherols; Cytotoxicity; Pumpkin by-products

3.3.1. Introduction

Pumpkin (*Cucurbita* sp. L.) is a widely cultivated vegetable crop known for the versatility and nutritional value of its fruit. Beyond its culinary applications, the most commonly used part of the pumpkin fruit is the pulp, yielding valuable by-products such as peels, seeds, and fibrous materials. These by-products have drawn increasing attention due to their rich content of bioactive compounds, including phenolic compounds, tocopherols, and carotenoids, which possess antioxidant, antimicrobial, and other beneficial properties (Chonoko & Rufai, 2011; Medjakovic et al., 2016; Nawirska-Olszańska et al., 2014; Yang et al., 2022). Utilizing these by-products for the extraction of bioactive compounds holds significant promise for various industrial applications, particularly for the development of natural additives and preservatives for the food and pharmaceutical industries (Dotto & Chacha, 2020; Leichtweis et al., 2021; Oliveira et al., 2023; Salehi et al., 2019).

So far, the pumpkin's most explored part in the literature is the seeds, especially pumpkin seed oil (Dotto & Chacha, 2020; Dowidar et al., 2020). Whole seeds are widely consumed in many countries as snacks, while they are used as toppings for salads and other dishes, in addition to consumption in seed oil and seed flour forms. They are a rich source of bioactive components that can benefit human health, such as antioxidant phenolic compounds, tocopherols, triterpenes, saponins, phytosterols, lignans and carotenoids (Dowidar et al., 2020). The seed oils from different pumpkin species were evaluated in terms of their chemical composition and antioxidant activity, where the richness in polyunsaturated acids (>50%), phenolic compounds (27.52 mg gallic acid equivalents/g of methanolic extract), and tocopherols (633.51 mg/kg of total tocopherols) were verified, with consequent high antioxidant potency being demonstrated (Boujemaa et al., 2020). Moreover, in another study the flour from pumpkin seed kernels was tested as a fat substitute and functional ingredient in beef meatballs due to its high protein (31.96%), oil (49.87%), oleic acid (44.78%), and linoleic acid (39.40%) contents (Öztürk & Turhan, 2020). However, the peels and fibrous strands have also been shown to be a rich source of bioactive compounds. In our previous study (Leichtweis et al., 2022), the peel, fibrous strands, and seeds of different varieties of commercial pumpkins grown in Portugal and Algeria revealed important antioxidant and antimicrobial capacity, supported by the profile of phenolic compounds, with 8 individual compounds being tentatively identified (up to 3.93 ± 0.05 mg/g of extract). In fact, pumpkin peel extract has been proven to be a natural alternative to effectively prevent oxidation of lipids in edible oil (Salami et al., 2020).

However, it is important to recognize that the composition and bioactivity of these pumpkin by-products can vary significantly depending on pre- and post-harvest factors

such as the genotype, growing conditions, and processing methods (Kulczyński et al., 2020; Leichtweis et al., 2023; Polyzos et al., 2022). Different pumpkin genotypes may exhibit distinct profiles of bioactive compounds and associated activities (Abbas et al., 2020; Boujemaa et al., 2020; Kostecka-Gugała et al., 2020). For example, Kostecka-Gugała et al. (2020) found high intra-species variability when compared eighteen cultivars of the *Cucurbita* species, where some cultivars stood out as extraordinary sources of phenolic compounds, including syringic and protocatechuic acids, catechin and kaempferol, or minerals such as zinc and copper, while other cultivars exhibited more significant antioxidant and antiradical capacities than the others. In addition, when comparing *C. pepo* from different cultivation systems, a higher sugar content was found in the fruits from organic system compared to the conventional system, while higher polyphenol contents, including phenolic acids and flavonoids were recorded when plants were fertilized with manure. Therefore, comprehensive studies evaluating the bioactive potential of different pumpkin parts and cultivars are crucial for maximizing the valorization of pumpkin by-products.

In this sense, this work aims to explore the potential of utilizing pumpkin by-products, specifically peels, seeds and fibrous strands, as sources of bioactive compounds for industrial applications. Through an investigation of the bioactive profiles of different fruit parts of 11 pumpkin genotypes grown in Greece, this research seeks to reveal the intra-species variability in terms of chemical composition and bioactive properties, as well as to discover new opportunities to harness pumpkin by-products to the development of natural additives and preservatives. Furthermore, it aims to valorize local landraces, thereby fostering sustainability and promoting circular economy through the exploitation of valuable genetic material.

3.3.2. Materials and methods

Samples

Different genotypes of pumpkin, including two commercially available cultivars and 9 local landraces, were cultivated in the experimental farm of the University of Thessaly (Greece), during the growing period of 2021 (for detailed data on specific pumpkin genotypes and harvest dates, refer to Table A in the supplementary material). In the case of local landraces, some genotypes (e.g. V2, V4, V5, V7 and V9) showed high variability in terms of fruit morphology due to interspecific hybridization among the various *Cucurbita* species. Therefore, for each genotype any different types of fruit were sampled and analyzed separately, resulting in 16 distinct samples. Cultivation conditions are described in our previous study (Leichtweis et al., 2023). Fruit from each genotype and form were cut in half and the seeds, peels and fibrous strands samples were collected, after

removing the pulp. Samples were then lyophilized (Sublimator model EKS, manufactured by Christian Zirbus Co. in Osterode am Harz, Germany), grounded to powder (~20 mesh), and stored until further analysis.

To evaluate bioactivities and phenolic composition, hydroethanolic extracts from the samples were obtained using standard methodology as described in the following sections. In the case of the antioxidant activity assays, the fat content of the seeds was removed before the extraction process using a Soxhlet apparatus, following AOAC procedures (AOAC, 2016). These extracts were obtained by macerating 2 g of sample in ethanol solution (80% v/v, 60 mL) at room temperature. The mixture was magnetically stirred for 60 minutes, the extracts were then filtered, and the procedure was iterated with the residue. The ethanol from the combined extracts was rotary evaporated. Then, the extracts were freeze-dried (FreeZone 4.5, Labconco) and stored for later analysis. The extraction yield was calculated by the difference in mass before and after freeze-drying and results are presented in Table A in the supplementary material.

Individual phytochemical characterization

For the phenolic composition, the extracts were redissolved (10 mg/mL) with ethanol solution (20% v/v) and assessed by high performance liquid chromatography coupled to a diode array detector, electrospray ionization and mass spectrometry (HPLC-DAD-ESI/MS), as described by Barros et al. (2013). The identification of the compounds was carried out based on multiple criteria, including retention time, UV-Vis and mass spectra, and comparison with known commercial standards and existing literature. Quantification was performed by measuring the peak areas and comparing them to the calibration curves of the most closely matching commercially available standards. The results were expressed in mg/g of extract.

The characterization of organic acids was conducted using ultra-fast liquid chromatography (UFLC, Shimadzu Corporation, Kyoto, Japan) coupled with a photodiode array detector (PDA), with wavelengths of 215 nm and 245 nm for ascorbic acid (Barros et al., 2013). Separation was performed on a SphereClone reverse phase C18 column (5 µm, 250 mm × 4.6 mm i.d.) maintained at 35°C. Identification and quantification were accomplished by comparing the samples with commercial standards. Calibration curves were established as follows: oxalic acid, $y = 8 \times 10^6 x + 331{,}789$, $R^2 = 0.9912$; quinic acid, $y = 6.9 \times 10^5 x + 11{,}551$, $R^2 = 0.9983$; malic acid, $y = 942{,}562x + 38{,}506$, $R^2 = 0.9987$; ascorbic acid, $y = 5 \times 10^7 x + 449{,}262$, $R^2 = 0.9813$; shikimic acid, $y = 8 \times 10^7 x + 567{,}119$, $R^2 = 0.9903$; citric acid, $y = 968{,}367x - 12{,}295$, $R^2 = 0.9974$; and fumaric acid, $y = 9 \times 10^7 x - 100{,}894$, $R^2 = 0.9986$. The results were expressed in g/100 g dw, except for fumaric acid (mg/100 g dw).

For tocopherol content the samples were mixed with BHT and tocol solutions and the mixture was homogenized with methanol and hexane. A saturated NaCl solution was added, and the mixture was centrifuged. The supernatant was collected, and the extraction process was repeated with hexane. The extracts were dried and redissolved in *n*-hexane before being filtered for the analysis. The HPLC system was coupled with a fluorescence detector (FP-2020; Jasco, Easton, MD, USA), configured to excite at 290 nm and measure emission at 330 nm, and using a Polyamide II normal-phase column (250 × 4.6 mm; YMCWaters) at a temperature of 30 °C, as described by Pereira et al. (2011). Acting as an internal standard (SI), tocol facilitated the identification of isoforms (*alpha*, *beta*, *gamma* and *delta*) through comparisons with authentic standards. The results were expressed in mg/100 g dw.

Bioactivities

To evaluate the antioxidant potential of the extracts, two cell-based assays were employed. The first assay used sheep erythrocytes to assess the inhibition of oxidative hemolysis (OxHLIA) (Palmeira et al., 2019), while the second assay used porcine brain homogenates to measure inhibition of lipid peroxidation (TBARS) (Pereira et al., 2011). In the OxHLIA assay, the extract concentration required to delay 50% of erythrocyte hemolysis for 60 minutes was determined and expressed as IC_{50} values (µg/mL). Likewise, in the TBARS assay, the concentration of the extract responsible for inhibiting 50% of lipid peroxidation was determined and expressed as IC_{50} values (µg/mL). Trolox was used as a positive control in both assays to compare the antioxidant activity of the extracts.

Regarding the antimicrobial activity, the extracts were tested against eight bacteria and two fungi commonly associated with food contamination. The bacteria included *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Yersinia enterocolitica*, while the fungi were *Aspergillus fumigatus* and *Aspergillus brasiliensis*. The microdilution methods and p-iodonitrotetrazolium chloride (INT) were employed for testing, with streptomycin and ampicillin used as positive controls for bacteria and ketoconazole for fungi (Heleno et al., 2013). The results were expressed in mg/mL.

The cytotoxic effect of the extracts was tested on three human tumor cell lines, more specifically the gastric (AGS), colorectal (CaCo2), and breast (MCF-7) adenocarcinoma cells, and on two non-tumor cells, being a primary culture from porcine liver cells (PLP2) and a kidney epithelial cell line of an African green monkey (VERO). The analysis was performed by the sulforhodamine B (SRB) assay (Corrêa et al., 2015), with ellipticine as positive control. The results were expressed as the concentration of extract that inhibited 50% of cell proliferation (GI_{50} in µg/ml).

Statistical analysis

The chemical analysis was conducted in triplicate, with results presented as mean ± standard deviation. Statistical analysis involved one-way analysis of variance (ANOVA) using Student's t-test for the comparison of two means and Tukey's honestly significant difference (HSD) test for the comparison of three or more means. Before analysis, normal distribution and homogeneity of variance were assessed using Shapiro-Wilk and Levene tests, respectively. For homoscedastic data ($p > 0.05$), Tukey's honestly significant difference (HSD) test was employed, while for heteroscedastic data, Tamhane's T2 multiple comparison test was used. All analyses were conducted at a 5% significance level using IBM SPSS Statistics software (Version 22.0, IBM Corp, Armonk, NY, USA).

A principal component analysis (PCA) was carried out to evaluate the contribution of each variable to the total diversity, aiming to classify the studied samples based on their chemical profile and bioactive properties. For this analysis, we did not consider the different forms of fruit for the genotypes that show polymorphism in fruit shape, while only ten genotypes were considered (we excluded genotype V11 because there were not enough seeds available to perform the analysis of chemical profile and bioactive properties). The analysis was performed with the statistical software Statgraphics 5.1.plus (Statpoint Technologies, Inc., Warrenton, VA, USA). Data was also analyzed using IBM SPSS Statistics software (Version 22.0, IBM Corp, Armonk, NY, USA) to determine whether there were any correlations among different compounds and bioactive properties using Pearson correlation.

3.3.3. Results and Discussion

Phytochemical characterization

Phenolic compounds

In all the seeds and fibrous strands samples under investigation, the sole phenolic compound that was identified was (-)-epicatechin (peak 1). This compound was identified by HPLC-DAD-ESI/MS with a retention time of 7.69 minutes and a maximum absorption wavelength at 280 nm, exhibiting a mass-to-charge ratio of 289 m/z and major fragment ions at 245 (100%) and 205 (45%) m/z, by comparison with the available standard compound (**Table 27**). As for the peels, a more diverse profile was found, with five phenolic compounds being tentatively identified, where apart from (-)-epicatechin, two chicoric acids (*cis* and *trans*; peaks 2 and 3) and two flavonoids were also detected (peaks 4 and 5). The chicoric acids compounds were tentatively identified with a retention time of 14.93 and 15.01 minutes, for *cis* and *trans* respectively, and a maximum absorption wavelength at 328 nm, exhibiting a mass-to-charge ratio of 473 m/z and major fragment

ion at 311 (100%) m/z. Peak 5 (with $[M-H]^-$ at m/z 593) displayed a sole MS^2 fragment at *m/z* 285 (kaempferol aglycone), that indicate the simultaneous loss of a deoxyhexosyl and hexosyl moiety ($[M-H-146-162]^-$). For peak 4 (with $[M-H]^-$ at *m/z* 769) the fragment at *m/z* 315 (100%) corresponds to an isorhamnetin aglycone and the jointed loss of a dideoxyhexosyl and hexosyl moiety. Chicoric acid and a diversified profile of flavonoids were previously reported by Iswaldi et al. (2013) in zucchini (*Cucurbita pepo* L.). **Figure 10** shows the chromatographic representation of the profile of phenolic compounds obtained, while **Table 28** presents the quantification of the phenolic compounds and the total phenolic compounds in peels.

In the case of seeds, the (-)-epicatechin ranged from 6.3 ± 0.3 mg/g extract ("V2" genotype) to 1.00 ± 0.05 mg/g extract ("V4" genotype), whereas this particular compound was not detected in "V8" and "V10" genotypes (**Table 28**). In fibrous strands, the highest amounts of (-)-epicatechin were recorded for "V2 T" and "V5 R" genotypes (7.6 ± 0.3 and 7.30 ± 0.05 mg/g extract respectively), whereas the lowest content was found in "V11" (1.53 ± 0.01 mg/g extract) (**Table 28)**. Meanwhile, in the peels, these compounds showed a narrower range, from 1.922±0.002 to 0.49 ± 0.01 mg/g extract, in "V9 R" and "V7 F", respectively. Moreover, for peel extracts the total flavonoids content was higher than the content of (-)-epicatechin (total flavan-3-ols) in genotypes "V4 C", "V7 P", and "V11", while for all the samples, phenolic acids were present in less amounts. The presence of (-)-epicatechin in almost all the samples studied highlights its importance as the predominant phenolic compound. This compound is a potent flavan-3-ol which is highly recognized for its antioxidant properties. Epicatechin stands out for its well-documented health benefits, contributing to improved cardiovascular health by enhancing blood flow and potentially mitigating certain heart-related risks. Moreover, its association with enhanced exercise performance and muscle recovery underscores its relevance in physiological well-being (Slavova-Kazakova et al., 2021). Epicatechin was also detected in higher concentrations in samples of pumpkin seeds and peels, reported by Yang et al. (2022), as well as in peels and in seed and pulp of *Momordica caranthia* (Busuioc et al., 2020), known as bitter melon, a species that also belongs to Cucurbitaceae family.

So far, literature reports a high variability in terms of total phenolic compounds content between various pumpkin (*C. maxima*) cultivars (Kulczyński et al., 2020), while the extraction solvent may also affect the extraction efficiency of phenolic compounds (Singh et al., 2016). In fact, in a previous study of our team (Leichtweis et al., 2023) the extraction protocol was optimized for three pumpkin genotypes, resulting in significant differences in the extraction yield depending on the extraction parameters (extraction temperature, solvent concentration and extraction time) or the extraction technique (ultrasound-assisted or heat-assisted extraction). In contrast to the present study,

Stryjecka et al. (2023) detected ten phenolic compounds in the pulp of five *Cucurbita* species; mostly phenolic acids such as syringic, protocatechuic and salicylic acid, while a great variability in individual compounds content was recorded among the species. Moreover, in the same study the amount of catechin was quite low compared to the most abundant phenolic acids. Similar results were reported in two cultivars of *C. pepo* fruit, where the main phenolic compounds were phenolic acids (gallic, p-coumaric, chlorogenic, ferulic and caffeic acid), whereas the main flavonoids (quercetin and kaempferol derivatives) were detected in lower amounts (Kopczyńska et al., 2021). Interestingly, these authors did not observe any differences in phenolic compounds content between the two cultivars, although they suggested that the cropping system (use of organic fertilizers, manure, or mineral fertilizers) had a significant impact on the content of individual compounds. Similarly, Dragovic-Uzelac et al. (2005) who studied raw pumpkin (*C. pepo*, *C. maxima* and *C. moschata*) fruit and their puree detected only chlorogenic and syringic acid and low amounts or caffeic and p-coumaric acids. Kulczynski & Gramza-Michałowska (2019) evaluated the chemical composition of the pulp of several pumpkin cultivars belonging to *C. pepo* and *C. moschata* and they detected several phenolic acids and flavonoids in amounts that varied greatly among the cultivars. Salehi et al. (2019) suggested the presence of various phenolic acids and flavonoids, while several studies have reported the phenolic profile of seeds (Eleiwa et al., 2014; Fa-Sheng et al., 2009). In the same line, Iswaldi et al. (2013) detected nine phenolic acids, nine hydroxybenzoic acids and fifteen flavonoids in the fruit of three zucchini (*C. pepo*) cultivars. Moreover, in a preliminary study by our group (Leichtweis et al., 2022) the extraction of phenolic compounds in the fruit by-products (peels, seeds and fibrous strands) of three pumpkin genotypes resulted in the identification of eight phenolic compounds in quantities that varied depending on the genotype and the part of the fruit. These contrasting results in the literature indicate the variability in phenolic compounds composition which can be influenced by pre- and post-harvest factors such as species, cultivar, agronomic practices, growing conditions and extraction methods. These findings underscore the diverse and valuable composition of compounds found in various parts of pumpkins, as well as the importance of valorizing valuable genetic material through the utilization of local landraces.

Table 27. Phenolic compounds characterized by HPLC-DAD-ESI/MS in the samples of pumpkin by-products (peels, seeds and fibrous strands).

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	7.69	280	289	245(100), 205(45)	(-)-epicatechin
2	14.93	328	473	311(100), 293(76), 179(5), 149(5)	*cis*-Chicoric acid
3	15.01	328	473	311(100), 293(89), 179(6), 149(7)	*trans*-Chicoric acid
4	15.85	354	769	315(100)	Isorhamnetin-*O*-dideoxyhexosyl-hexoside
5	17.12	348	593	285(100)	Kaempferol-*O*-deoxyhexosyl-hexoside

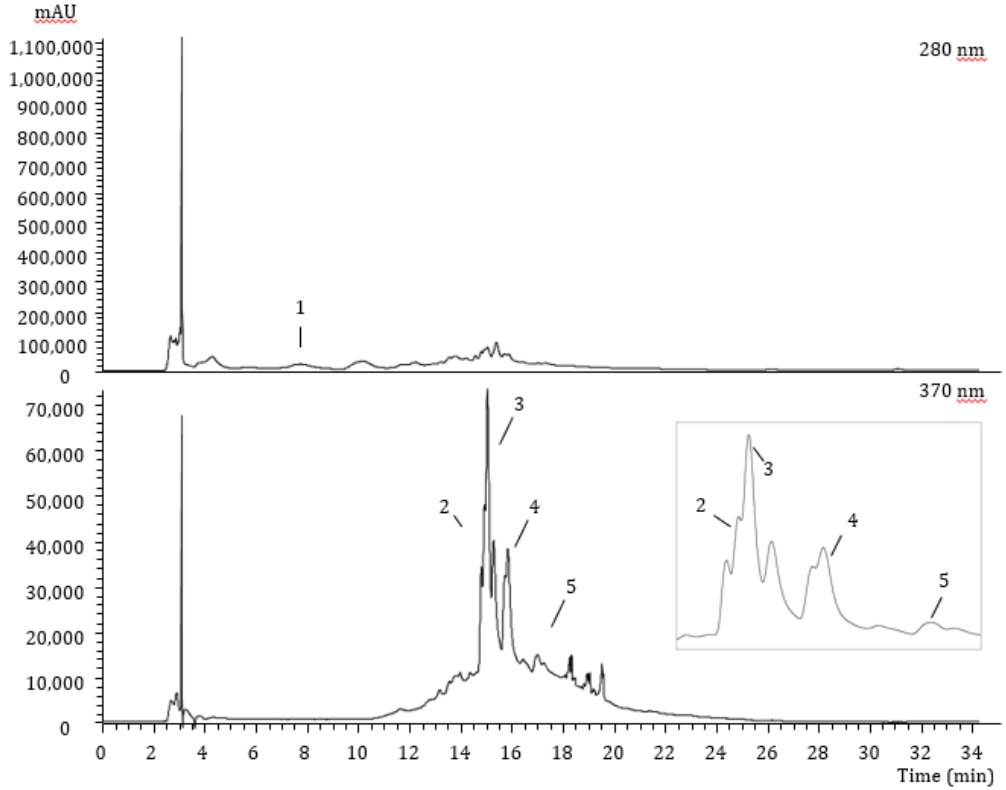

Figure 10. Chromatographic representation of the phenolic compounds profile obtained by HPLC-DAD-ESI/MS for the peels of the "V10" genotype.

Table 28. Quantification of phenolic compounds found in the extracts of pumpkin by-products (seeds, fibrous strands and peels) expressed in mg/g of extract.

Pumpkin by-product	Genotype	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Total Flavan-3-ols	Total Phenolic Acids	Total Flavonoids	Total Phenolic Compounds
Seeds	V1	1.54 ± 0.07 ^{d}	n.d.	n.d.	n.d.	n.d.	1.54 ± 0.07 ^{d}	n.d.	n.d.	1.54 ± 0.07 ^{d}
	V2	6.3 ± 0.3 ^{a}	n.d.	n.d.	n.d.	n.d.	6.3 ± 0.3 ^{a}	n.d.	n.d.	6.3 ± 0.3 ^{a}
	V3	3.7 ± 0.2 ^{c}	n.d.	n.d.	n.d.	n.d.	3.7 ± 0.2 ^{c}	n.d.	n.d.	3.7 ± 0.2 ^{c}
	V4	1.00 ± 0.05 ^{f}	n.d.	n.d.	n.d.	n.d.	1.00 ± 0.05 ^{f}	n.d.	n.d.	1.00 ± 0.05 ^{f}
	V5	1.12 ± 0.06 ^{e,f}	n.d.	n.d.	n.d.	n.d.	1.12 ± 0.06 ^{e,f}	n.d.	n.d.	1.12 ± 0.06 ^{e,f}
	V6	4.1 ± 0.2 ^{b}	n.d.	n.d.	n.d.	n.d.	4.1 ± 0.2 ^{b}	n.d.	n.d.	4.1 ± 0.2 ^{b}
	V7	1.52 ± 0.02 ^{d}	n.d.	n.d.	n.d.	n.d.	1.52 ± 0.02 ^{d}	n.d.	n.d.	1.52 ± 0.02 ^{d}
	V8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	V9	1.4 ± 0.1 ^{d,e}	n.d.	n.d.	n.d.	n.d.	1.4 ± 0.1 ^{d,e}	n.d.	n.d.	1.4 ± 0.1 ^{d,e}
	V10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fibers	V1	3.7 ± 0.1 ^{h}	n.d.	n.d.	n.d.	n.d.	3.7 ± 0.1 ^{h}	n.d.	n.d.	3.7 ± 0.1 ^{h}
	V2 T	7.6 ± 0.3 ^{a}	n.d.	n.d.	n.d.	n.d.	7.6 ± 0.3 ^{a}	n.d.	n.d.	7.6 ± 0.3 ^{a}
	V2 C	4.39 ± 0.03 ^{e}	n.d.	n.d.	n.d.	n.d.	4.39 ± 0.03 ^{e}	n.d.	n.d.	4.39 ± 0.03 ^{e}
	V3	3.97 ± 0.01 ^{g}	n.d.	n.d.	n.d.	n.d.	3.97 ± 0.01 ^{g}	n.d.	n.d.	3.97 ± 0.01 ^{g}
	V4 C	3.14 ± 0.02 ^{i,j}	n.d.	n.d.	n.d.	n.d.	3.14 ± 0.02 ^{i,j}	n.d.	n.d.	3.14 ± 0.02 ^{i,j}
	V4 R	4.25 ± 0.07 ^{e,f}	n.d.	n.d.	n.d.	n.d.	4.25 ± 0.07 ^{e,f}	n.d.	n.d.	4.25 ± 0.07 ^{e,f}
	V5 F	5.42 ± 0.06 ^{c}	n.d.	n.d.	n.d.	n.d.	5.42 ± 0.06 ^{c}	n.d.	n.d.	5.42 ± 0.06 ^{c}
	V5 R	7.30 ± 0.05 ^{b}	n.d.	n.d.	n.d.	n.d.	7.30 ± 0.05 ^{b}	n.d.	n.d.	7.30 ± 0.05 ^{b}
	V6	4.46 ± 0.07 ^{e}	n.d.	n.d.	n.d.	n.d.	4.46 ± 0.07 ^{e}	n.d.	n.d.	4.46 ± 0.07 ^{e}
	V7 P	3.33 ± 0.09 ^{i}	n.d.	n.d.	n.d.	n.d.	3.33 ± 0.09 ^{i}	n.d.	n.d.	3.33 ± 0.09 ^{i}
	V7 F	3.0 ± 0.1 ^{j}	n.d.	n.d.	n.d.	n.d.	3.0 ± 0.1 ^{j}	n.d.	n.d.	3.0 ± 0.1 ^{j}
	V8	4.09 ± 0.07 ^{f,g}	n.d.	n.d.	n.d.	n.d.	4.09 ± 0.07 ^{f,g}	n.d.	n.d.	4.09 ± 0.07 ^{f,g}
	V9 C	3.7 ± 0.1 ^{h}	n.d.	n.d.	n.d.	n.d.	3.7 ± 0.1 ^{h}	n.d.	n.d.	3.7 ± 0.1 ^{h}
	V9 R	4.87 ± 0.01 ^{d}	n.d.	n.d.	n.d.	n.d.	4.87 ± 0.01 ^{d}	n.d.	n.d.	4.87 ± 0.01 ^{d}
	V10	4.11 ± 0.04 ^{f,g}	n.d.	n.d.	n.d.	n.d.	4.11 ± 0.04 ^{f,g}	n.d.	n.d.	4.11 ± 0.04 ^{f,g}
	V11	1.53 ± 0.01 ^{k}	n.d.	n.d.	n.d.	n.d.	1.53 ± 0.01 ^{k}	n.d.	n.d.	1.53 ± 0.01 ^{k}

Pumpkin by-product	Genotype	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Total Flavan-3-ols	Total Phenolic Acids	Total Flavonoids	Total Phenolic Compounds
Peels	V1	n.d.	tr.	0.0032 ± 0.0001 ^{f}	0.4801±0.0001 ^{d}	0.4695±0.0003 ^{e}	n.d.	0.0032 ± 0.0001 ^{f}	0.9496±0.0002 ^{f}	0.9528±0.0003 ^{j}
	V2 T	1.65 ± 0.06 ^{c}	tr.	0.0236 ± 0.0002 ^{c}	0.4712±0.0003 ^{e}	0.507±0.001 ^{c}	1.65±0.06 ^{c}	0.0236 ± 0.0002 ^{c}	0.979±0.001 ^{d}	2.65±0.06 ^{b}
	V2 C	1.79 ± 0.04 ^{b}	tr.	0.0109 ± 0.0004 ^{e}	0.48 ± 0.01 ^{d}	n.d.	1.79±0.04 ^{b}	0.0109 ± 0.0004 ^{e}	0.48±0.01 ^{h}	2.29±0.05 ^{d}
	V3	1.37 ± 0.04 ^{d}	tr.	tr.	0.577±0.004 ^{a}	0.472±0.001 ^{e}	1.37±0.04 ^{d}	tr.	1.048±0.003 ^{c}	2.41±0.04 ^{c}
	V4 C	n.d.	tr.	tr.	0.4597 ± 0.0001 ^{f}	n.d.	n.d.	tr.	0.4597±0.0001 ^{i}	0.4597±0.0001 ^{k}
	V4 R	1.26 ± 0.01 ^{e}	tr.	tr.	n.d.	n.d.	1.260±0.006 ^{e}	tr.	n.d.	1.26±0.01 ^{h}
	V5 F	1.14 ± 0.01 ^{f}	n.d.	tr.	n.d.	0.456 ± 0.001 ^{f}	1.141±0.007 ^{f}	tr.	0.456±0.001 ^{i}	1.60±0.01 ^{g}
	V5 R	1.23 ± 0.05 ^{e}	n.d.	n.d.	n.d.	n.d.	1.23±0.05 ^{e}	n.d.	n.d.	1.23±0.05 ^{h}
	V6	1.40 ± 0.06 ^{d}	tr.	0.015±0.001 ^{d}	0.485 ± 0.001 ^{d}	0.4964±0.0003 ^{d}	1.40±0.06 ^{d}	0.015±0.001 ^{d}	0.982±0.002 ^{d}	2.40±0.06 ^{c}
	V7 P	n.d.	0.052±0.002 ^{a}	0.012±0.001 ^{d,e}	0.520 ± 0.003 ^{b}	0.539±0.003 ^{a}	n.d.	0.064±0.002 ^{c}	1.0591±0.0001 ^{b}	1.123±0.003 ^{i}
	V7 F	0.49 ± 0.01 ^{h}	tr.	tr.	n.d.	0.4589±0.0002 ^{f}	0.49±0.01 ^{h}	tr.	0.4589±0.0002 ^{i}	0.95±0.01 ^{j}
	V8	1.82 ± 0.02 ^{b}	0.011±0.001 ^{c}	0.086±0.003 ^{b}	n.d.	n.d.	1.82±0.02 ^{b}	0.097±0.003 ^{b}	n.d.	1.92±0.02 ^{e}
	V9 C	1.11 ± 0.04 ^{f}	tr.	0.0057±0.0002 ^{f}	0.5008 ± 0.0001 ^{c}	n.d.	1.11±0.04 ^{f}	0.0057±0.0002 ^{f}	0.5008±0.0001 ^{g}	1.62±0.04 ^{g}
	V9 R	1.922 ± 0.002 ^{a}	tr.	tr.	n.d.	n.d.	1.922±0.002 ^{a}	tr.	n.d.	1.922±0.002 ^{e}
	V10	1.71 ± 0.08 ^{c}	0.017±0.001 ^{b}	0.101±0.005 ^{a}	0.58±0.01 ^{a}	0.519±0.002 ^{b}	1.71±0.08 ^{c}	0.12±0.01 ^{a}	1.10±0.01 ^{a}	2.92±0.07 ^{a}
	V11	0.86 ± 0.01 ^{g}	n.d.	n.d.	0.468 ± 0.002 ^{e,f}	0.493±0.006 ^{d}	0.861±0.009 ^{g}	n.d.	0.962±0.007 ^{e}	1.823±0.002 ^{f}

n.d. – not detected. tr. – traces. Calibration curves used for quantification: (-)-catechin (y = 84,950x - 23,200; R^2 = 0.999; LOD = 0.17 g/mL; LOQ = 0.68 g/mL; peak 1); caffeic acid (y = 388,345x + 406,369; R^2 = 0.994; LOD = 0.78 g/mL; LOQ = 1.97 g/mL; peaks 2 and 3) and quercetin 3-*O*-glucoside (y = 34,843x - 160,173; R^2 = 0.9998; LOD = 0.21 g/mL; LOQ = 0.71 g/mL; peaks 4 and 5). ANOVA analysis – In each column and for the same fruit part, different letters mean significant differences among the tested genotypes according to Tukey's HSD test ($p < 0.05$).

Organic acids

The quantification of organic acids in the studied pumpkin by-product is shown in **Table 29**. It is notable that the samples studied showed considerable variation in the composition of the organic acids analyzed. On average, pumpkin seeds presented malic acid concentrations around 1.00 g/100 g dw, while fibrous strands showed concentrations around 9.46 g/100 g dw of this compound. Meanwhile, oxalic acid ranged from approximately 0.07 g/100 g dw in seeds to 4.97 g/100 g dw in peels. Malic and quinic acids were the most abundant in the seeds and fibrous strands, with concentrations varying between 0.34 ± 0.01 to 2.4 ± 0.1 g/100 g dw and 0. 33 ± 0.01 to 1.76 ± 0.08 g/100 g dw, respectively, in the seeds, and between 5.8 ± 0.3 to 15.2 ± 0.6 and 4.9 ± 0.2 to 12.2 ± 0.6, respectively, in fibrous strands. On the other hand, in the peels quinic acid was detected in just 3 samples at an average amount of approximately 1.928 g/100 g dw. Among these samples, malic acid reached concentrations of up to 7.1 ± 0.2 g/100 g dw in sample "V9 R", followed by oxalic acid, which exhibited concentrations of up to 5.9 ± 0.2 g/100 g dw in sample "V3".

The high content of malic and quinic acids in pumpkin by-products is a significant aspect to consider due to their nutritional implications and potential health benefits. Malic acid is recognized for its detoxifying properties and its crucial involvement in the energy production processes of all cells in the body. Supporting this, Yousefi et al. (2023) conducted a study where they found that supplementing diets of *Oncorhynchus mykiss* with acidic malic acid led to notable improvements in the antioxidant status and immune responses of the fish, without adverse effects on its growth performance. On the other hand, quinic acid, responsible for the characteristic bitter taste of certain foods, has antioxidants, antidiabetic, and anticancer properties, along with potential positive effects on liver health and blood sugar regulation (Benali et al., 2022). Thus, the presence of these compounds in the studied pumpkin by-products in high amounts not only contributes to their organoleptic characteristics but also adds nutritional value and health beneficial properties.

Regarding citric acid, although it was only detected in the seeds of "V8", and the fibrous strands of "V2 C", it was also found in 10 out of the 16 peel samples, ranging from 0.38 ± 0.02 to 2.2 ± 0.1 g/100 g dw (**Table 29**). According to the literature, evaluating the organic acid content in eight pumpkin cultivars, Nawirska-Olszańska et al. (2014) found that at post-harvest the average citric acid content was 1.62 g/kg fresh weight (fw), while malic acid had a higher concentration, averaging 3.34 g/kg fw. In contrast, fumaric acid was present in comparatively lower amounts, averaging 0.22 g/kg fw. Furthermore, in melon (*Cucumis melo* L.) cultivars, malic acid was found at an average of 1.31 mg/g

without significant variation, while citric acid ranged from 5.15 to 1.19 mg/g, in the melon cultivar *Yuniang* grafted onto different pumpkin rootstocks (Kaleem et al., 2022).

To a lesser extent, the presence of fumaric acid was found in average amounts of 0.012 mg/100 g dw for seeds, 0.479 mg/100 g dw for fibrous strands and 0.05 mg/100 g dw for peels (**Table 29**). Shikimic acid was detected in very low or only trace amounts in some samples, while ascorbic acid was identified only in trace amounts in some samples (**Table 29**).

Table 29. Quantification of the organic acids in pumpkin by-products (seeds, fibrous strands and peels) of the studied genotypes (mean ± SD).

Pumpkin by-product	Genotype	Oxalic g/100g dw	Quinic g/100g dw	Malic g/100g dw	Ascorbic g/100g dw	Shikinic g/100g dw	Citric g/100g dw	Fumaric mg/100g dw
Seeds	V1	0.051 ± 0.002 ^{f}	1.76 ± 0.08 ^{a}	0.34 ± 0.01 ^{f}	n.d.	n.d.	n.d.	0.017 ± 0.001 ^{b}
	V2	0.029 ± 0.001 ^{g,h}	0.33 ± 0.01 ^{h}	n.d.	n.d.	tr.	n.d.	0.0110 ± 0.0001 ^{d,e}
	V3	0.117 ± 0.002 ^{c}	1.13 ± 0.05 ^{d}	0.97 ± 0.05 ^{d}	n.d.	tr.	n.d.	0.010 ± 0.001 ^{e}
	V4	0.017 ± 0.001 ^{i}	n.d.	n.d.	n.d.	tr.	n.d.	0.0093 ± 0.0003 ^{f}
	V5	0.025 ± 0.001 ^{h}	0.40 ± 0.02 ^{g}	0.99 ± 0.05 ^{d}	n.d.	n.d.	n.d.	0.00649 ± 0.00003 ^{g}
	V6	0.093 ± 0.003 ^{d}	0.68 ± 0.03 ^{e}	2.4 ± 0.1 ^{a}	n.d.	0.063 ± 0.003	n.d.	0.014 ± 0.001 ^{c}
	V7	0.13 ± 0.01 ^{b}	1.32 ± 0.05 ^{b}	1.08 ± 0.05 ^{c}	n.d.	tr.	n.d.	0.0132 ± 0.0001 ^{c}
	V8	0.032 ± 0.001 ^{g}	0.53 ± 0.02 ^{f}	0.64 ± 0.02 ^{e}	n.d.	tr.	0.18 ± 0.01	0.0096 ± 0.0004 ^{f}
	V9	0.14 ± 0.01 ^{a}	1.20 ± 0.06 ^{c}	1.185 ± 0.002 ^{b}	n.d.	n.d.	n.d.	0.01846 ± 0.00003 ^{a}
	V10	0.060 ± 0.002 ^{e}	0.66 ± 0.03 ^{e}	0.37 ± 0.01 ^{f}	n.d.	tr.	n.d.	0.01125 ± 0.00001 ^{d}
Fibrous strands	V1	2.9 ± 0.1 ^{g,h}	8.2 ± 0.4 ^{c}	5.8 ± 0.3 ^{f}	n.d.	n.d.	n.d.	0.220 ± 0.003 ^{k}
	V2 T	2.628 ± 0.004 ^{i,j}	n.d.	15.2 ± 0.6 ^{a}	n.d.	n.d.	n.d.	0.72 ± 0.01 ^{a}
	V2 C	4.44 ± 0.09 ^{c}	n.d.	12.2 ± 0.6 ^{b}	n.d.	n.d.	2.8 ± 0.1	0.44 ± 0.01 ^{h}
	V3	4.75 ± 0.08 ^{b}	9.3 ± 0.3 ^{b}	8.6 ± 0.4 ^{d}	n.d.	n.d.	n.d.	0.394 ± 0.004 ^{i}
	V4 C	3.95 ± 0.09 ^{f}	7.8 ± 0.4 ^{c,d}	10.4 ± 0.4 ^{c}	n.d.	n.d.	n.d.	0.612 ± 0.002 ^{c}
	V4 R	4.455 ± 0.001 ^{c}	9.6 ± 0.5 ^{b}	11.83 ± 0.07 ^{b}	n.d.	n.d.	n.d.	0.61 ± 0.01 ^{c}
	V5 F	3.12 ± 0.09 ^{g}	7.4 ± 0.1 ^{d,e}	7.9 ± 0.4 ^{e}	n.d.	n.d.	n.d.	0.47 ± 0.01 ^{f}
	V5 R	2.519 ± 0.001 ^{j}	7.5 ± 0.4 ^{d,e}	9.2 ± 0.4 ^{d}	n.d.	n.d.	n.d.	0.488 ± 0.003 ^{e}
	V6	2.73 ± 0.04 ^{i}	4.9 ± 0.2 ^{f}	7.8 ± 0.4 ^{e}	n.d.	n.d.	n.d.	0.551 ± 0.003 ^{d}
	V7 P	2.79 ± 0.04 ^{h,i}	7.0 ± 0.3 ^{e}	6.40 ± 0.07 ^{f}	n.d.	0.025 ± 0.001 ^{a}	n.d.	0.284 ± 0.001 ^{j}
	V7 F	4.16 ± 0.08 ^{d,e}	9.7 ± 0.3 ^{b}	8.99 ± 0.09 ^{d}	n.d.	n.d.	n.d.	0.48 ± 0.01 ^{e,f}
	V8	3.93 ± 0.07 ^{f}	n.d.	7.6 ± 0.1 ^{e}	n.d.	0.021 ± 0.001 ^{b}	n.d.	0.479 ± 0.002 ^{e}
	V9 C	5.0 ± 0.2 ^{a}	12.2 ± 0.6 ^{a}	11.6 ± 0.2 ^{b}	n.d.	n.d.	n.d.	0.457 ± 0.004 ^{g}
	V9 R	4.01 ± 0.01 ^{e,f}	9.1 ± 0.4 ^{b}	10.7 ± 0.2 ^{c}	n.d.	n.d.	n.d.	0.608 ± 0.002 ^{c}
	V10	4.3 ± 0.1 ^{c,d}	9.28 ± 0.03 ^{b}	11.5 ± 0.5 ^{b}	n.d.	n.d.	n.d.	0.433 ± 0.004 ^{h}
	V11	1.00 ± 0.02 ^{k}	n.d.	9.00 ± 0.04 ^{d}	n.d.	n.d.	n.d.	0.631 ± 0.003 ^{b}

Pumpkin by-product	Genotype	Oxalic g/100g dw	Quinic g/100g dw	Malic g/100g dw	Ascorbic g/100g dw	Shikinic g/100g dw	Citric g/100g dw	Fumaric mg/100g dw
Peels	V1	4.89 ± 0.07 ^{d,e}	n.d.	4.5 ± 0.2 ^{d}	tr.	n.d.	n.d.	n.d.
	V2 T	5.08 ± 0.02 ^{d}	n.d.	2.61 ± 0.03 ^{j,k}	tr.	tr.	0.92 ± 0.04 ^{e}	0.012 ± 0.001 ^{e,f}
	V2 C	4.9 ± 0.1 ^{d}	n.d.	2.4 ± 0.1 ^{k}	n.d.	n.d.	n.d.	0.012 ± 0.001 ^{e,f}
	V3	5.9 ± 0.2 ^{a}	n.d.	3.0 ± 0.1 ^{i}	tr.	n.d.	1.03 ± 0.05 ^{d}	0.013 ± 0.001 ^{d,e,f}
	V4 C	5.76 ± 0.05 ^{a}	n.d.	3.3 ± 0.2 ^{g,h}	tr.	n.d.	1.13 ± 0.05 ^{c}	n.d.
	V4 R	5.7 ± 0.3 ^{a,b}	n.d.	4.07 ± 0.04 ^{e}	tr.	tr.	n.d.	0.026 ± 0.001 ^{c}
	V5 F	4.06 ± 0.02 ^{f}	2.15 ± 0.09 ^{a}	3.6 ± 0.2 ^{f}	tr.	n.d.	0.571 ± 0.005 ^{g}	0.01211 ± 0.00001 ^{e,f}
	V5 R	2.93 ± 0.01 ^{g}	n.d.	2.8 ± 0.1 ^{i,j}	n.d.	n.d.	0.38 ± 0.02 ^{h}	0.014 ± 0.001 ^{d,e,f}
	V6	5.43 ± 0.04 ^{c}	n.d.	3.10 ± 0.02 ^{h,i}	n.d.	tr.	1.06 ± 0.05 ^{c,d}	0.0106 ± 0.0001 ^{f}
	V7 P	4.93 ± 0.09 ^{d}	n.d.	5.5 ± 0.2 ^{b}	tr.	n.d.	0.62 ± 0.03 ^{f,g}	0.017 ± 0.001 ^{d}
	V7 F	4.7 ± 0.2 ^{e}	1.65 ± 0.01 ^{c}	5.0 ± 0.2 ^{c}	tr.	tr.	1.81 ± 0.03 ^{b}	0.016 ± 0.001 ^{d,e}
	V8	5.51 ± 0.06 ^{b,c}	n.d.	3.53 ± 0.06 ^{f,g}	tr.	0.0016 ± 0.0001	n.d.	0.014 ± 0.001 ^{d,e,f}
	V9 C	5.48 ± 0.04 ^{c}	n.d.	5.1 ± 0.3 ^{c}	n.d.	n.d.	n.d.	0.38 ± 0.01 ^{a}
	V9 R	4.99 ± 0.02 ^{d}	n.d.	7.1 ± 0.2 ^{a}	n.d.	n.d.	0.66 ± 0.03 ^{f}	0.17 ± 0.01 ^{b}
	V10	5.39 ± 0.04 ^{c}	n.d.	2.40 ± 0.09 ^{k}	tr.	tr.	n.d.	0.012 ± 0.001 ^{e,f}
	V11	4.26 ± 0.03 ^{f}	1.98 ± 0.09 ^{b}	4.49 ± 0.02 ^{d}	n.d.	tr.	2.2 ± 0.1 ^{a}	0.015 ± 0.001 ^{d,e,f}

n.d. – not detected. tr. – traces. ANOVA analysis – In each column and for the same fruit part, different letters mean significant differences among the tested genotypes according to Tukey's HSD test ($p < 0.05$).

Tocopherols

In **Table 30**, the tocopherol content in various pumpkin by-products is presented. The profiles of these compounds were more similar between the seeds and peels, with *gamma*-tocopherol being the most prominent vitamin E isoform for almost all the samples, followed by *alpha*-tocopherol, while *beta*- and *delta*-tocopherols were found only in a few samples. In fibrous strands, *gamma*-tocopherol was detected in just 5 out of the 16 studied samples (e.g. "V4 C", "V6", "V7 P", "V8", and "V10") in low concentrations, whereas *alpha*-tocopherol was the most prevalent compound in these samples.

Regarding the seeds, the total tocopherol content ranged from 4.35 ± 0.03 mg/100 g dw in "V10" to 14.58 ± 0.09 mg/100 g dw in "V8", with an average amount of *gamma*-tocopherol of 6.76 mg/100 g dw and an average amount of *alpha*-tocopherol of 1.17 mg/100 g dw. Concerning the peels, the average content of *gamma*-tocopherol was 9.73 mg/100 g dw, varying from 24.1 in "V2 T" and "V10" (with no significant difference, $p > 0.05$) to 0.82 ± 0.02 mg/100 g dw in "V9" and 0.70 ± 0.03 mg/100 g dw in "V4 C" ($p > 0.05$). The *alpha*- and total tocopherol average content in these samples was, 1.43 and 11.28 mg/100 g dw, respectively. Meanwhile, in the fibrous strands, the *alpha*-tocopherol average content was 2.95 mg/100 g dw, and the average total tocopherol content was 3.20 mg/100 g dw, ranging from 0.077 ± 0.001 to 7.38 ± 0.03 mg/100 g dw in the samples "V5 F" and "V7 P", respectively.

In our previous study (Leichtweis et al., 2023), we observed that *alpha*-tocopherol was the predominant form of vitamin E in the pulp of these pumpkin genotypes, ranging from 0.218 ("V7 F") to 4.90 mg/100 g dw ("V2 T"), followed by *beta*- and *gamma*-tocopherols. In their review, Dotto & Chacha (2020) summarized several studies on pumpkin seed oil and reported tocopherols content in the following proportions: *alpha*-tocopherol at 0.204 – 35.300 mg/100 g; *beta*-tocopherol at 0.54 mg/100 g; *gamma*-tocopherol at 9.715 – 89.300 mg/100 g, and *delta*-tocopherol at 0.232 – 2.25 mg/100 g of oil. In the exocarp of summer squash fruit of four genotypes, tocopherols were found in two forms, *alpha*- and *gamma*-tocopherol, with the latter being the predominant one (Rodov et al., 2020). Additionally, in pumpkin fruit from five cultivars of *C. moschata* Duch., the seeds presented *alpha*-tocopherol content which ranged from 3.76±0.48 to 5.65±0.25 mg/100 g, while *beta*- and *gamma*-tocopherol content ranged from 35.83±0.45 to 47.69±0.43 mg/100 g. Moreover, the seed kernels exhibited *alpha*-tocopherol content ranging from 2.39±0.56 to 4.19±0.2 mg/100 g, and *beta*- and *gamma*-tocopherols content ranging from 28.82±0.29 to 33.37±0.39 mg/100 g (A. Singh & Kumar, 2022b).

This analysis of the tocopherol profile and content in different parts of pumpkin fruit by-products is important to characterize their nutritional profile and bioactive properties. Tocopherol, especially *alpha*-tocopherol, is known for its antioxidant

properties, which play a crucial role in protecting against oxidative stress and combating cellular damage caused by free radicals (Rozanowska et al., 2019). The predominant presence of the *gamma*-tocopherol isoform, followed by *alpha*-tocopherol, suggests significant potential for the health benefits of these pumpkin by-products, as these forms of tocopherol have demonstrated significant antioxidant and anti-inflammatory benefits in previous studies (Polyzos et al., 2022; Rozanowska et al., 2019; Singh & Kumar, 2022a). Furthermore, variation in tocopherol contents between different parts of pumpkin fruit may influence the formulation of food products and nutritional supplements that provide these bioactive compounds aiming to improve health and prevent diseases related to oxidative stress.

Table 30. Quantification of tocopherol content in pumpkin by-products (seeds, fibrous strands and peels) of the studied genotypes expressed in mg/100 g dw (mean ± SD).

Pumpkin by-product	Genotype	*alpha*-tocopherol	*beta*-tocopherol	*gamma*-tocopherol	*delta*-tocopherol	Total tocopherols
Seeds	V1	0.1028 ± 0.0003 ^{g}	n.d.	6.5 ± 0.1 ^{f}	n.d.	6.6 ± 0.1 ^{f}
	V2	2.03 ± 0.04 ^{d}	n.d.	3.02 ± 0.06 ^{g}	n.d.	5.0 ± 0.1 ^{g}
	V3	0.371 ± 0.004 ^{e}	n.d.	12.3 ± 0.2 ^{b}	n.d.	12.7 ± 0.2 ^{b}
	V4	2.10 ± 0.06 ^{c}	n.d.	9.0 ± 0.2 ^{d}	n.d.	11.0 ± 0.3 ^{c}
	V5	2.98 ± 0.09 ^{a}	n.d.	0.86 ± 0.02 ^{j}	n.d.	3.8 ± 0.1 ^{i}
	V6	0.116 ± 0.002 ^{g}	n.d.	8.1 ± 0.2 ^{e}	n.d.	8.2 ± 0.2 ^{e}
	V7	0.37 ± 0.02 ^{e}	n.d.	9.89 ± 0.07 ^{c}	n.d.	10.26 ± 0.06 ^{d}
	V8	0.21 ± 0.01 ^{f}	n.d.	14.20 ± 0.08 ^{a}	0.169 ± 0.006	14.58 ± 0.09 ^{a}
	V9	n.d.	n.d.	1.75 ± 0.03 ^{i}	n.d.	1.75 ± 0.03 ^{j}
	V10	2.29 ± 0.03 ^{b}	n.d.	2.057 ± 0.004 ^{h}	n.d.	4.35 ± 0.03 ^{h}
Fibrous strands	V1	4.29 ± 0.06 ^{e}	0.303 ± 0.006 ^{b}	n.d.	n.d.	4.59 ± 0.07 ^{d}
	V2 T	4.37 ± 0.01 ^{d}	n.d.	n.d.	n.d.	4.37 ± 0.01 ^{e}
	V2 C	0.529 ± 0.003 ^{m}	0.62 ± 0.03 ^{a}	n.d.	n.d.	1.15 ± 0.02 ^{k}
	V3	6.03 ± 0.01 ^{b}	n.d.	n.d.	n.d.	6.03 ± 0.01 ^{b}
	V4 C	1.61 ± 0.01 ^{k}	n.d.	0.254 ± 0.003 ^{e}	n.d.	1.86 ± 0.01 ^{j}
	V4 R	2.18 ± 0.01 ^{i}	n.d.	n.d.	n.d.	2.18 ± 0.001 ^{i}
	V5 F	0.077 ± 0.001 ^{o}	n.d.	n.d.	n.d.	0.077 ± 0.001 ^{m}
	V5 R	0.391 ± 0.001 ^{n}	n.d.	n.d.	n.d.	0.391 ± 0.001 ^{l}
	V6	1.91 ± 0.02 ^{i}	n.d.	0.76 ± 0.01 ^{b}	n.d.	2.67 ± 0.01 ^{h}
	V7 P	6.83 ± 0.03 ^{a}	n.d.	0.554 ± 0.001 ^{d}	n.d.	7.38 ± 0.03 ^{a}
	V7 F	5.56 ± 0.06 ^{c}	n.d.	n.d.	n.d.	5.56 ± 0.06 ^{c}
	V8	2.30 ± 0.03 ^{h}	n.d.	1.18 ± 0.04 ^{a}	n.d.	3.48 ± 0.01 ^{g}
	V9 C	1.87 ± 0.06 ^{j}	n.d.	n.d.	n.d.	1.87 ± 0.06 ^{j}
	V9 R	2.70 ± 0.01 ^{g}	n.d.	n.d.	n.d.	2.70 ± 0.01 ^{h}
	V10	1.25 ± 0.01 ^{l}	n.d.	0.589 ± 0.003 ^{c}	n.d.	1.83 ± 0.01 ^{j}
	V11	4.20 ± 0.06 ^{f}	n.d.	n.d.	n.d.	4.20 ± 0.06 ^{f}

Pumpkin by-product	Genotype	*alpha*-tocopherol	*beta*-tocopherol	*gamma*-tocopherol	*delta*-tocopherol	Total tocopherols
Peels	V1	0.110 ± 0.001 [l]	n.d.	4.44 ± 0.09 [j]	0.343 ± 0.005 [c]	4.89 ± 0.09 [k]
	V2 T	0.55 ± 0.02 [h]	n.d.	24.1 ± 0.2 [a]	n.d.	24.6 ± 0.2 [b]
	V2 C	1.486 ± 0.001 [e]	n.d.	13.78 ± 0.04 [d]	n.d.	15.27 ± 0.04 [f]
	V3	1.35 ± 0.02 [f]	0.228 ± 0.008 [a]	5.49 ± 0.01 [h]	0.56 ± 0.08 [b]	7.62 ± 0.06 [h]
	V4 C	0.24 ± 0.01 [k]	n.d.	0.70 ± 0.03 [m]	n.d.	0.94 ± 0.04 [n]
	V4 R	1.663 ± 0.001 [d]	n.d.	3.40 ± 0.03 [l]	n.d.	5.07 ± 0.03 [k]
	V5 F	0.389 ± 0.003 [i]	n.d.	3.96 ± 0.01 [k]	n.d.	4.35 ± 0.01 [l]
	V5 R	1.002 ± 0.004 [g]	n.d.	5.03 ± 0.1 [i]	n.d.	6.3 ± 0.1 [i]
	V6	0.61 ± 0.02 [h]	n.d.	19.0 ± 0.2 [c]	n.d.	19.6 ± 0.2 [d]
	V7 P	0.19 ± 0.01 [k]	n.d.	5.98 ± 0.05 [g]	n.d.	6.17 ± 0.04 [i]
	V7 F	0.227 ± 0.001 [k]	n.d.	5.59 ± 0.02 [h]	n.d.	5.81 ± 0.03 [j]
	V8	1.02 ± 0.02 [g]	n.d.	0.82 ± 0.02 [m]	n.d.	1.83 ± 0.03 [m]
	V9 C	4.178 ± 0.004 [c]	0.116 ± 0.002 [b]	13.20 ± 0.01 [e]	0.26 ± 0.02 [d]	17.76 ± 0.02 [e]
	V9 R	4.71 ± 0.02 [b]	n.d.	8.97 ± 0.01 [f]	n.d.	13.68 ± 0.03 [g]
	V10	5.9 ± 0.1 [a]	n.d.	24.1 ± 0.1 [a]	0.682 ± 0.001 [a]	30.7 ± 0.2 [a]
	V11	0.362 ± 0.001 [i,j]	n.d.	20.55 ± 0.09 [b]	n.d.	20.91 ± 0.09 [c]

n.d. – not detected. ANOVA analysis – In each column and for the same fruit part, different letters mean significant differences among the tested genotypes according to Tukey's HSD test ($p < 0.05$).

Bioactivities

Antioxidant capacity

The antioxidant activity of the hydroethanolic extracts of the peels, seeds, and fibrous strands of the studied genotypes was determined by two *in vitro* assays, namely the lipid peroxidation inhibition assay (TBARS) and the oxidative hemolysis inhibition assay (OxHLIA), and the results are presented in **Table 31**. In the TBARS assay, all samples revealed important antioxidant activity, especially the seeds of genotypes "V5" and "V6" which presented activity (IC_{50}: 208 ± 9 and 125 ± 1 µg/mL, respectively) similar ($p > 0.05$) to the positive control Trolox (IC_{50}: 139 ± 5 µg/mL). In addition, the seeds of the rest of the genotypes also stood out compared to the peels and fibrous strands of the respective genotypes, with IC_{50} values varying from approximately 1 to 10 times higher than the positive control. On the other hand, the samples the peels of genotypes "V2 T" and V2 C" recorded higher antioxidant activity compared to the other fruit parts (e.g. IC_{50}: 825 ± 25 µg/mL and 850 ± 40 µg/mL, respectively). Finally, fibrous strands and peels showed IC_{50} values ranging from 5 to 28 times higher than Trolox, thus demonstrating that the studied by-products, especially the seeds, possess a significant capacity to inhibit lipid peroxidation.

In the OxHLIA assay, a varied response was recorded depending on the fruit part and the genotype tested. The highest and lowest antihemolytic activity was recorded for the peels of genotypes "V7 F" (IC_{50}: 41 ± 2 µg/mL) and "V2 T" (IC_{50}: 822 ± 34 µg/mL), respectively. Moreover, fibrous strands recorded higher antioxidant activity than the other two by-products in 7 out of 16 samples, followed by seeds (4 out of 10 samples) and peels (6 out of 16 samples).

The bioactive properties, particularly the antioxidant activity, of pumpkins have been well documented in the literature (Abbas et al., 2020; Diop et al., 2020; Martínez et al., 2021). Salehi et al. (2021) conducted a comprehensive review focusing on the antioxidant potential of the Cucurbitaceae family, with special attention to the genus *Cucurbita*. In addition to discussing the anti-inflammatory, antidiabetic, anticarcinogenic and hepatoprotective activities, the authors compiled several studies demonstrating antioxidant activity of *Cucurbita* spp. through *in vitro* and *in vivo* assays. It has to be noted that most *in vitro* analyses used chemical methodologies, notably DPPH (2,2-diphenyl-1-picrylhydrazyl), which makes the direct comparison with the results presented here cumbersome. Furthermore, the utilization of cell-based methods in the current study proves to be highly valuable for pumpkin research, as these assays offer a significant complementary approach for assessing the antioxidant activity of vegetable extracts in a biologically relevant context. Moreover, they are still relatively underexplored for this particular matrix which highlights the innovative aspect of the present study.

Apart from the variable response to the antioxidant activity observed through different methodologies, the variation in antioxidant capacity between different pumpkin genotypes has also been reported by other authors. When evaluating 18 pumpkin cultivars belonging to the species *C. maxima* Duch., *C. moschata* Duch., *C. pepo* L., and *C. ficifolia* Bouché, using DPPH, FRAP (ferric reducing antioxidant power) and CUPRAC (cupric ion reducing antioxidant capacity) assays, Kostecka-Gugała et al. (2020) reported a high intra-species variability. In particular, the results (expressed in μmol Trolox per 100 g of fresh weight) ranged from 1.01 ± 0.03 to 32.46 ± 1.06 for DPPH, from 11.7 ± 1.7 to 139.9 ± 13 for FRAP, and from 64.3 ± 3.6 to 256.6 ± 2.6 for CUPRAC assay. Moreover, the differences observed among the different parts of the pumpkin in this study are consistent with findings reported by Hussain et al. (2021). The authors compared the DPPH radical scavenging activity of peel, flesh, and seed powders extracted from a commercially available variety from Sargodha, Pakistan. The results indicated antioxidant capacities of 13.00 ± 0.08, 10.58 ± 0.06, and 16.53 ± 0.09 mg of ascorbic acid equivalent (AAE) per 100 g for peel, flesh, and seeds, respectively, thus indicating that fruit part may also affect the antioxidant capacity due to the variability in the content and the profile of bioactive compounds.

Table 31. Lipid peroxidation inhibition capacity (TBARS) and oxidative hemolysis inhibition capacity (OxHLIA) of pumpkin by-products (seeds, fibrous strands and peels) of the studied genotypes expressed in IC_{50}; μg/mL; Δt = 60 min for OxHLIA; (mean ± SD).

Genotype	TBARS			OxHLIA		
	Deffated seeds	Fibrous strands	Peels	Deffated seeds	Fibrous strands	Peels
V1	1133 ± 54 ^{b}	1312 ± 51 ^{j}	1589 ± 63 ^{h}	52±2 ^{g,h}	210 ± 9 ^{f}	475 ± 24 ^{e}
V2	640 ± 28 ^{d}	n.a.	n.a.	101±3 ^{f}	n.a.	n.a.
V2 T	n.a.	1998 ± 97 ^{e}	825 ± 25 ^{l}	n.a.	168 ± 6 ^{g}	822 ± 34 ^{a}
V2 C	n.a.	3054 ± 142 ^{c}	850 ± 40 ^{k,l}	n.a.	390 ± 5 ^{d}	555 ± 8 ^{d}
V3	434 ± 7 ^{f}	3900 ± 165 ^{a}	885 ± 18 ^{j,k,l}	171±5 ^{e}	638 ± 12 ^{a}	294 ± 11 ^{f}
V4	468 ± 12 ^{e}	n.a.	n.a.	431±9 ^{b}	n.a.	n.a.
V4 C	n.a.	1723 ± 62 ^{h}	1285 ± 13 ^{i}	n.a.	128 ± 5 ^{h,i}	755 ± 33 ^{b}
V4 R	n.a.	1825 ± 84 ^{f,g}	4984 ± 221 ^{b}	n.a.	106 ± 6 ^{i}	197 ± 12 ^{g,h}
V5	208 ± 9 ^{h}	n.a.	n.a.	84±3 ^{f,g}	n.a.	n.a.
V5 F	n.a.	3023 ± 126 ^{c}	1963 ± 94 ^{f}	n.a.	132 ± 9 ^{h}	219 ± 8 ^{g}
V5 R	n.a.	1739 ± 83 ^{g,h}	985 ± 11 ^{j}	n.a.	123 ± 2 ^{h,i}	98 ± 4 ^{j}
V6	125 ± 1 ^{i}	1911 ± 62 ^{e,f}	4973 ± 84 ^{b}	315±14 ^{c}	393 ± 18 ^{d}	243 ± 17 ^{g}
V7	1007 ± 51 ^{c}	n.a.	n.a.	795±31 ^{a}	n.a.	n.a.
V7 P	n.a.	630 ± 25 ^{l}	953 ± 21 ^{j,k}	n.a.	104 ± 6 ^{i}	612 ± 25 ^{c}
V7 F	n.a.	2267 ± 90 ^{d}	2749 ± 127 ^{e}	n.a.	348 ± 8 ^{e}	41 ± 2 ^{k}
V8	405 ± 19 ^{g}	3059 ± 141 ^{c}	5733 ± 260 ^{a}	289±14 ^{c}	140 ± 5 ^{h}	427 ± 8 ^{e}
V9	1771 ± 59 ^{a}	n.a.	n.a.	409±23 ^{b}	n.a.	n.a.
V9 C	n.a.	1645 ± 39 ^{h,i}	1796 ± 62 ^{g}	n.a.	359 ± 11 ^{e}	122 ± 2 ^{i,j}
V9 R	n.a.	1610 ± 59 ^{i}	1710 ± 84 ^{g}	n.a.	444 ± 16 ^{c}	157 ± 7 ^{h,i}
V10	640 ± 31 ^{d}	1188 ± 53 ^{k}	4216 ± 204 ^{c}	241±10 ^{d}	371 ± 4 ^{d,e}	441 ± 18 ^{e}
V11	n.a.	3768 ± 190 ^{b}	3085 ± 135 ^{d}	n.a.	485 ± 7 ^{b}	139 ± 13 ^{i,j}
Trolox	139 ± 5 ^{h,i}	139 ± 5 ^{m}	139 ± 5 ^{m}	21.8 ± 0.2 ^{h}	21.8 ± 0.2 ^{j}	21.8 ± 0.2 ^{k}

n.a. – not applicable. ANOVA analysis – In each column and for the same fruit part, different letters mean significant differences among the tested genotypes according to Tukey's HSD test ($p < 0.05$).

Antimicrobial capacity

Extracts obtained from vegetable by-products such as seeds, leaves, peels, etc., have been of great interest as a defense against microorganisms in different industries (Añibarro-Ortega et al., 2019; Oliveira et al., 2023). In this study, antibacterial and antifungal analysis were developed using 8 bacterial and 2 fungal strains of importance in the food industry, and the detailed results are provided in the supplementary material (Tables B, C and D). None of the samples showed bactericidal nor fungicidal capacity, meaning that none of the samples could kill the fungi nor the bacteria tested; therefore, all the activities discussed below refer to growth inhibition.

Among all the 42 samples (16 samples of peels and fibrous strands and 10 samples of seeds), the most affected bacteria were *Escherichia coli* and *Salmonella enterica*, with 31 samples showing activity against each of these strains, followed by *Staphylococcus aureus*, that was inhibited by 30 samples, *Enterobacter cloacae* (25 samples), and *Yersinia enterocolitica* (24 samples), whereas *Pseudomonas aeruginosa* was the most resistant bacteria against pumpkin extracts, where only fibrous strands of genotype "V10" and peels of genotypes "V2 T", "V2 C", and "V3" were able to inhibit them.

Regarding the different types of pumpkin by-products, all the seed samples were effective against the *E. Cloacae*, *S. enterica*, *Y. enterocolitica*, and *Listeria monocytogenes* growth, showing microbial inhibition concentrations (MIC) of 5 mg/mL, 10 mg/mL and 5 – 10 mg/mL, respectively. It is also worth to highlight the seeds of "V1", "V2", "V3" and "V4" genotypes, which presented the lowest MIC value of 2.5 mg/mL, against *Y. enterocolitica*. Concerning the peels, all samples, except those of "V7 P" genotype, were capable of inhibiting the *E. coli* growth, 12 samples inhibited *S. aureus*, and 9 were effective against *E. cloacae* growth. Regarding the peel samples, the "V2 T" and "V11" genotypes stood out, presenting an MIC of 2.5 mg/mL against 4 and 5 of the 8 strains evaluated, respectively. Meanwhile, none of the fibrous strands samples exhibited significant antibacterial activity, with the lowest MIC value (5 mg/mL) being observed in genotype "V11" against the growth of *E. coli*. In fact, fibrous strands were, in general, less effective against the microorganisms tested, where none of the samples was able to inhibit the growth of *Bacillus cereus* and *L. monocytogenes*. On the other hand, these by-products showed greater effectiveness against *S. enterica* compared to the peels, and against *E. coli* compared to the seeds.

Regarding fungal growth inhibition, the peels have also exhibited the greatest overall effectiveness compared to the other by-products. While all peels and all fibrous strands samples inhibited the growth of at least one fungus, 14 peel samples were effective against both fungi tested, compared to only 5 fibrous strands samples. With the exception of the peels of "V9 C", "V7 P" and "V5 R" genotypes, all the rest of the peel samples were

effective against *Aspergillus brasiliensis* with MIC values of 5 mg/mL, especially the peels of genotype "V11", which inhibited both *A. brasiliensis* and *A. fumigatus* at this concentration. On the other hand, only the seeds from "V7", "V8", "V9" and "V10" genotypes showed antifungal activity, against *A. brasiliensis* at MIC of 10 mg/mL.

These results corroborate other studies available in the literature where different pumpkin extracts also showed antimicrobial activity. The antimicrobial potential of pumpkin (*C. pepo*) was explored through the extraction of its fruit body and leaves using aqueous and methanol solutions. In the fruit body extract, antimicrobial activity was observed against 6 bacteria and 4 fungi using the agar well diffusion method. Notably, all extracts exhibited antimicrobial effects, with significant inhibition zones of 23 mm observed against *Escherichia coli*, 22 mm against *S. aureus*, and 20 mm against *Klebsiella pneumoniae* (Dubey et al., 2010). Similarly, the methanolic extract of *C. pepo* leaves was tested against 29 bacterial strains, revealing inhibitory effects against 28 bacteria with MIC values ranging from 128 to 1024 µg/ml. Additionally, bactericidal activity was observed against 17 bacterial strains, with MBC values ranging from 256 to 1024 µg/ml (Noumedem et al., 2013). Moreover, the peel and seeds of *C. pepo* also exhibited antimicrobial activity. In particular, ethanolic extracts from both seeds and peels showed activity against *S. aureus* and *Salmonella typhi*, whereas methanolic extracts were effective against *S. aureus* at a concentration of 500 µg/disc (Chonoko & Rufai, 2011). Furthermore, pumpkin seeds oil demonstrated inhibitory effects against the growth of 5 bacteria and 4 fungi, with MIC values ranging from 0.5 to 3.0 mg/mL (EI-Aziz & EI-Kalek, 2011). Although these results are not directly comparable, it is possible to conclude the remarkable activity presented by extracts obtained from pumpkin fruit parts.

Cytotoxic activities

None of the tested samples of peel and fibrous strands showed cytotoxic activities up to 400 µg/mL. Among the seed samples, those of the genotypes "V1", "V9", and "V10" exhibited activity against all the cell lines tested, while "V2", "V3", and "V8" genotypes affected all cell lines except VERO (**Table 32**). Therefore, it could be suggested that these samples were capable to inhibit the proliferation of tumor cells without affecting normal liver cells. The best activities were presented by seeds of "V2" genotype against AGS, and "V9" and "V10" genotypes against MCF-7, at concentrations of 59.2 ± 0.8, 59 ± 5, and 58 ± 2 µg/mL respectively, thus highlighting that hepatotoxicity was detected at higher concentrations. Also "V4" seeds recorded GI_{50} values of 140 ± 1 µg/mL against MCF-7, without presenting hepatotoxicity up to 400 µg/mL against both VERO and PLP2 cell lines.

The lack of toxicity against normal cells and the toxic effects against tumor cells for different parts of pumpkin fruit such as peels, fibrous strands, seeds, and pulp, has been described in the literature (Gaweł-Bęben et al., 2022; Leichtweis et al., 2022; Leichtweis et al., 2023; Piccolella et al., 2019). For example, Shokrzadeh et al. (2010) detected activity of *C. pepo* leaves against two cancer cell lines, e.g. human hepatocarcinoma (HepG2) and human colon carcinoma (CT26), and two normal cell lines, e.g. Chinese hamster ovary (CHO) and hamster fibroblast, in concentrations between 132.6 ± 4.3 to 241.4 ± 9.6 µg/ml. The methanolic extracts of pulp of *Cucurbita maxima* fruit showed a moderate cytotoxic effect against human cervical carcinoma (HeLa) cell line (Krstić et al., 2023). In another study, pumpkin seeds extracts were assessed for the treatment of benign prostatic hyperplasia, being verified the cell growth inhibition of prostate-, breast- and colon cancer cells (Medjakovic et al., 2016). These results highlight the importance of conducting thorough studies on the toxicity of different pumpkin varieties and their fruit parts. They also emphasize the significance of integrating the findings from this research aiming to understand how pumpkin by-product extracts can be valorized through industrial applications.

Table 32. Toxicity of different pumpkin seeds expressed in µg/mL.

Genotype	AGS	CaCo2	MCF-7	PLP2	VERO
V1	229 ± 23 ^{a}	173 ± 9 ^{b}	203 ± 18 ^{b}	215 ± 1 ^{b}	233 ± 23 ^{a}
V2	59.2 ± 0.8 ^{e}	148 ± 7 ^{c}	232 ± 3 ^{a}	209 ± 15 ^{b}	n.d.
V3	168 ± 16 ^{d}	161 ± 13 ^{b,c}	240 ± 7 ^{a}	285 ± 8 ^{a}	n.d.
V4	n.d.	n.d.	140 ± 1 ^{c}	n.d.	n.d.
V5	n.d.	n.d.	n.d.	n.d.	n.d.
V6	n.d.	n.d.	n.d.	n.d.	n.d.
V7	n.d.	n.d.	n.d.	n.d.	n.d.
V8	204 ± 18 ^{b,c}	208 ± 9 ^{a}	129 ± 11 ^{c}	191 ± 16 ^{c}	n.d.
V9	223 ± 4 ^{a,b}	90 ± 6 ^{d}	59 ± 5 ^{d}	125 ± 6 ^{d}	206 ± 11 ^{b}
V10	187 ± 16 ^{c,d}	63 ± 1 ^{e}	58 ± 2 ^{d}	72 ± 7 ^{e}	235 ± 6 ^{a}

n.d. – not detected. ANOVA analysis – In each column and for the same fruit part, different letters mean significant differences among the tested genotypes according to Tukey's HSD test ($p < 0.05$).

Correlation analysis

Correlation analyses indicated statistically significant contributions ($p < 0.05$). In the peel, the compounds *cis*-Chicoric acid (peak 2) and *beta*-tocopherol demonstrated a strong negative correlation with TBARS (r = -0.9703 and -0.9969, respectively), indicating that their higher concentration is associated with greater antioxidant activity, reducing lipid peroxidation, while they presented a strong positive correlation with OxHLIA (r = 0.9805 and 0.9931, respectively), which suggests specific characteristics in the antioxidant mechanisms of action of these compounds. Quinic acid showed a strong and significant positive correlation with OxHLIA (r = 0.977), while, despite being negative, it presented a low correlation with TBARS (r = -0.5182). *Beta*-tocopherol was also a highlight in fiber

samples, showing a strong positive correlation, in this case with both TBARS (r = 0.9987) and OxHLIA (r = 0.9946). On the other hand, shikimic acid showed a very strong negative correlation with TBARS (r = -0.9715) and OxHLIA (r = -0.9102), suggesting an important antioxidant role in this extract. Finally, in seeds, (-)-epicatechin (peak 1) correlated strongly and negatively with cytotoxicity in AGS cells (r = -0.9809), indicating an important influence on the cytotoxic effect of the extract. While the other compounds had low correlation values for the bioactivities of this matrix. Complete correlation values are presented in Table E of the supplemental material.

Principal components analysis

Principal component analysis (PCA) is widely used to reduce the complexity of multivariate data, as well as to identify patterns and express data in ways that highlight similarities and differences among the evaluated treatments. In our study, the aim was to identify groups of samples with similarities in terms of chemical profile and bioactive properties. The first seven principal components (PCs) were associated with Eigen values higher than 1 and explained 82.9% of the cumulative variance, with PC1 accounting for 32.7%, PC2 for 16.0%, PC3 for 9.1%, PC4 for 8.1%, PC5 for 6.8%, PC6 for 5.6% and PC7 for 4.7%.

PC1 was positively correlated with *gamma*-tocopherol, total tocopherols, isorhamnetin-*O*-dideoxyhexosyl-hexoside, kaempferol-*O*-deoxyhexosyl-hexoside, total flaval-3-ols, total flavonoids, and total phenolic compounds content, and negatively correlated with malic acid and (-)-epicatechin content. PC2 was positively correlated to fumaric acid, malic acid, oxalic acid, quinic acid, total organic acids, TBARS, and (-)-epicatechin content. Finally, PC3 was positively correlated to α-tocopherol and *trans*-chicoric acid and negatively correlated to citric acid, isorhamnetin-O-dideoxyhexosyl-hexoside, kaempferol-*O*-deoxyhexosyl-hexoside, and total flavonoids content. These results indicate the correct implementation of the PCA, allowing differentiation between the tested samples depending on genotype and the fruit by-product (e.g. peels, fibrous strands, and seeds). The corresponding scatterplots and loading plots are shown in Figure A, B, and C of the supplementary material.

The scatterplot (Figure A, of the supplementary material) shows a clear discrimination of the tested samples based on the fruit part where all the seed samples form a distinct group (group A). Moreover, five more distinct groups were identified, namely group B which included only fibrous strands (F) from genotypes “V1”, “V2” Turbinate fruit, “V3”, “V4” (both Round and Cylindrical fruit), “V5” (both Flattened and Round fruit), “V6”, “V7” Flattened fruit, “V8”, “V9” (both Round and Cylindrical fruit) and “V10”; group C which included only peel samples (P) from genotypes “V4” Round and

"V9" Round; group D which included only peel samples from genotypes "V2" Cylindrical and "V9" Cylindrical; group E which included only peel samples from genotypes "V2" Turbinate, "V3", "V6" and "V7" Pyriform; and group F which included peel samples from genotypes "V1", "V4" Cylindrical, "V5" Flattened, "V7" Flattened and fibrous strands samples from genotype "V2" Cylindrical. Finally, peel samples from genotypes "V5" Round, "V8" and "V10" and fibrous strands samples from genotype "V7" Pyriform were clearly separated from the rest of the samples. These particular samples are distinguished for its high content of TPC (peel samples of "V5" R) and total tocopherols (peel samples of "V10" and fibrous strands samples of "V7" P), as well as the low content of total tocopherols and low antioxidant activity for TBARS assay (peel samples of "V8") and high antihemolytic activity (fibrous strands of "V7" P). It also should be noted that samples from different fruit parts (except for group F) are clearly differentiated from each other (e.g. group A included only seeds samples, group B included only fibrous strands samples and groups C, D, E included only peel samples).

Moreover, the loading plot of the first two components also revealed groups of positively correlated variables (Figure B, of the supplementary material). In particular, the upper left quadrant included quinic acid, malic acid, fumaric acid, shikimic acid, total organic acids, *alfa*-tocopherol, *beta*-tocopherol and peak 1 ((-)-epicatechin); the lower left quadrant included no variables; the upper right quadrant included citric acid, oxalic acid, TBARS, OxHLIA, *delta*-tocopherol, peak 2 (*cis*-chicoric acid), peak 3 (*trans*-chicoric acid), peak 4 (isorhamnetin-*O*-dideoxyhexosyl-hexoside), peak 5 (kaempferol-*O*-deoxyhexosyl-hexoside), total phenolic acids, total flavonoids, total flavan-3-ols, and total phenolic compounds; the lower right quadrant included *gamma*-tocopherol and total tocopherols. Similarly, the loading plot of PC1 and PC3 (Figure C, of the supplementary material) revealed groups of positively correlated variables, namely the upper left quadrant included peak 1, quinic acid, shikimic acid, *alfa*-tocopherol and total organic acids; the lower left quadrant comprised malic acid, fumaric acid, and *beta*-tocopherol; the upper right quadrant included TBARS, *delta*-tocopherol, *gamma*-tocopherol, total tocopherols, peak 2, peak 3, total phenolic acids, total flavan-3-ols, and total phenolic compounds; and the lower right quadrant included oxalic acid, OxHLIA, peak 4, peak 5, and total flavonoids

3.3.4. Conclusions

The characterization of the studied pumpkin fruit by-products revealed their potential as valuable sources of bioactive compounds. These by-products exhibited significant profiles of phenolic compounds, tocopherols, and organic acids, which not only may contribute to the organoleptic properties of the fruit but also hold important nutraceutical properties. Overall, our findings underscore the promising potential of using

various pumpkin parts, including seeds, peels, and fibrous strands, as rich sources of bioactive compounds with significant industrial applications, while a significant genotypic variation was also recorded. The valorization of these by-products not only offers economic benefits but also contributes to the appreciation of local landraces and sustainable agricultural practices. By capitalizing on these valuable genetic resources, we may not only promote sustainability but also foster a circular economy model, wherein waste is minimized, and natural resources are maximally utilized. Ultimately, our research highlights the importance of recognizing and harnessing the diverse benefits of pumpkin by-products, paving the way for innovative industrial applications and sustainable agricultural practices. However, further research is needed, especially regarding the evaluation of toxic effects of certain seed extracts, to ensure their safe utilization in food products.

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CHAPTER 4

Development and Application of Natural Preservative

This chapter is composed of two research articles, which address studies on optimizing the extraction process of selected pumpkin peels and development of pumpkin pulp formulations with natural preservatives replacing the artificial ones. A discussion on the sensory acceptance of the pulps is then presented.

4.1. Valorization of pumpkin peel as a source of bioactive compounds: Optimization of heat- and ultrasound-assisted extraction

molecules

MDPI

Article

Valorization of Pumpkin Peel as a Source of Bioactive Compounds: Optimization of Heat- and Ultrasound-Assisted Extraction

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Abstract: The peels from three pumpkin genotypes cultivated in Greece were assessed for their phenolic content and bioactive properties to obtain extracts with a high preservative capacity. The optimization of the extraction was performed through response surface methodology (RSM) based on a Box–Behnken experimental design after applying two extraction techniques: heat-assisted (HAE) and ultrasound-assisted (UAE) extraction. The implemented independent variables were time, solvent concentration, and temperature/power (for HAE/UAE), while as dependent variables the dry residue (DR), reducing power (RP), and total phenolic content (TP) were considered. In general, HAE was the most effective technique for 'TL' (75 min; 30 °C; 24% ethanol) and 'Voutirato' (15 min; 30 °C; 10% ethanol), while UAE was more effective for 'Leuka Melitis' (5 min; 400 W; 0% ethanol). The extracts obtained in the global optimum conditions for each genotype peel were then assessed for their phenolic profile, by HPLC-DAD-ESI/MS, and bioactive potential. Seven phenolic compounds were detected, including four flavonoids, two phenolic acids, and one flavan-3-ol. The extracts presented high antioxidant, antibacterial, and antifungal potential, with no cytotoxicity for non-tumor cells. The optimized conditions for the extraction of preservative compounds from bioresidues were defined, allowing the acquisition of antioxidant and antimicrobial extracts and proving their potential for food application.

Keywords: *Cucurbita maxima* Duchesne; natural food preservatives; process optimization; phenolic compounds; pumpkin peel; bioactivity; RSM

4.1.1. Introduction

The proper management of biowaste is essential in industrial processes, since its use can generate environmental and economic benefits and increase the added value in various industries. In particular, the by-products generated in the fruit and vegetable processing and juice and jam manufacturing industries, among others, represent a valuable source of bioactive compounds and could be used to obtain natural preservatives for food applications. Studies have shown that the non-edible parts of fruits, such as the peel and seeds, contain high amounts of phenolic compounds (Brusselaers & Van Der Linden, 2020; Munekata et al., 2023). For example, pumpkin peels are a rich source of these compounds, and, beyond its antioxidant properties, it could also act as a natural preservative due to its high content of bioactive molecules. Recent research has shown that pumpkin peel extracts can inhibit the growth of bacteria and fungi, making them a natural alternative to chemical preservatives (Hussain et al., 2022; Leichtweis et al., 2022). In fact, one study on the evaluation of the antimicrobial activity of pumpkin peel extract using the agar disc method demonstrated a remarkable broad-spectrum antimicrobial potential against the Gram-positive bacterium *Streptomyces viridochromogenes* (Badr et al., 2011), whereas Asif et al. reported the satisfactory antibacterial activity of pumpkin peel extracts against four bacterial strains (*Escherichia coli*, *Pasteurella multocida*, *Staphylococcus aureus*, and *Bacillus subtilis*) (Asif et al., 2017). Moreover, pumpkin peels are rich in antioxidants such as ascorbic acid and tocopherols, which can contribute to the overall antioxidant capacity of this by-product (Daiuto et al., 2012), while other important bioactive compounds are carotenoids and *beta*-carotene (Kim et al., 2012).

Numerous techniques have been investigated for the extraction, quantification, and identification of bioactive compounds, with the aim of discovering the optimal conditions for sustainable, cost-effective, and environmentally friendly processes. The key factors that can affect both the yield and bioactivity of these compounds are the extraction time, temperature, solvent type, and solid/liquid ratio. Therefore, it is critical to evaluate the impact of these factors to determine the ideal conditions that minimize losses and increase extraction yield (Lama-Muñoz & Contreras, 2022). Conventional methods such as heat-assisted extraction (HAE) expedite the transfer of compounds from the matrix to the solution and increase the solubility of bioactive compounds, generally resulting in higher extraction yields. However, these methods are limited because they may degrade bioactive compounds due to prolonged exposure to heat and result in the extraction of unwanted compounds, which can further impact the purity of the obtained extract. Thus, other methods such as ultrasound-assisted extraction (UAE) are being explored as an alternative to obtaining these compounds. Ultrasound waves favor the release of

compounds by breaking the matrix membranes through cavitation. This mechanism can reduce time and solvent consumption while increasing extraction yield and decreasing the likelihood of extracting unwanted compounds at the same time (Jovanović et al., 2017; Leichtweis et al., 2021). However, the comparison between two or more methods is necessary to verify the real effectiveness of each method on different matrices (Backes et al., 2018; López et al., 2018). RSM is used to reduce the number of runs with different experimental conditions, thus reducing the time and money spent and maximizing the performance of the output. It uses statistical models to represent the relationship between the input variables and the outputs (the optimal conditions). It works by iteration, with each experiment (run) being used to refine the model and improve its predictions until an optimal solution (condition) is found. The Box–Behnken design is a type of RSM that uses a limited number of experimental runs to estimate a specific response. Its main advantages reside in the fact that it needs fewer runs than other designs as it uses evenly spaced levels for each variable. It includes linear, quadratic, and interaction term. In this work, an RSM was implemented for both extraction techniques, although this did not constitute a literal comparison between the two due to the specific variables of each method (Khuri & Mukhopadhyay, 2010). The main objective was to detail the best conditions to obtain the highest antioxidant and antimicrobial activity for both extraction techniques.

Despite the abovementioned bioactivity of these bioresidues and the high volume generated by the food industry, to the best of our knowledge, few studies have been conducted on the extraction of bioactive compounds from pumpkin peels (Leichtweis et al., 2022; Salami et al., 2021). Therefore, this study was carried out with the aim of optimizing the extraction of preservative compounds from the peel of three pumpkin genotypes cultivated in Greece. For this purpose, a conventional technique (heat-assisted extraction) and an alternative technique (ultrasound-assisted extraction) were used, employing different extraction times, temperatures/power, and ethanol percentages. The process was designed by a response surface methodology (RSM), with the validation of the predictive models, in order to select those extracts that contained higher levels of total phenolic compounds while reducing the power requirements and solid residues. The extracts obtained through the optimized conditions were also assessed in terms of their phenolic compound profile, by HPLC-DAD-ESI/MS, and bioactivity, namely their antioxidant (cell-based assays), antimicrobial, and cytotoxic potential.

4.1.2. Materials and methods

Sample preparation

During the 2020 growing season, three pumpkin genotypes were cultivated at the

experimental field of the University of Thessaly in Velestino, central Greece (22.756E, 39.396 N), namely 'Landrace from the region of Trikala' (TL) (P1), 'Voutirato' (P2), and 'Leuka Melitis' (round) (P3). Fruits were harvested on October 2020, and the peels from 15 fruits from each genotype were separated; subjected to a freeze-drying process using a Sublimator model EKS, manufactured by Christian Zirbus Co. in Osterode am Harz, Germany; and then reduced to a fine powder (~20 mesh) by crushing for subsequent analysis.

Pumpkin peel extraction procedures

Heat-assisted extraction (HAE)

To perform the heat-assisted extraction of the powdered pumpkin peel samples, an internally stirred water reactor equipped with a CimarecTM magnetic stirrer (Thermo Scientific, San Jose, CA, USA) running at a constant speed of approximately 500 rpm was used. The extraction process followed a previously described procedure (Roriz et al., 2017), whereby 0.5 g of sample was mixed with 20 mL of solvent, according to the conditions defined by the Box–Behnken experimental design as described in **Table 33**. The experimental parameters of the design varied within the following ranges: time (t or X_1) from 15 to 120 min, temperature (T or X_2) from 30 to 80 °C, and ethanol content (EtOH or X_3) from 0% (total water) to 100% (total ethanol). The solid-to-liquid ratio was kept constant at 25 g/L under all extraction conditions, based on previous optimization studies (Leichtweis et al., 2019). At the end of the extraction, the sample was filtered through Whatman No. 4 filter paper, and the supernatant was directly evaluated.

Ultrasound-assisted extraction (UAE)

An ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA) equipped with a fixed water reactor at a frequency of 40 kHz was used for ultrasound-assisted extraction. The variables were programmed according to the Box–Behnken experimental design, as described in **Table 33**, following a previously described procedure (Backes et al., 2018). Samples of pumpkin peels from each genotype (0.75 g) were placed in a reactor with 30 mL of solvent and were extracted under the planned conditions, keeping the solid/liquid ratio constant at 25 g/L. The experimental design ranges were set as follows: time (t or X_1) from 5 to 60 min; ultrasonic power (P or X_2) from 100 (20% of the total equipment power rating) to 400 W (80%); and ethanol ratio (EtOH or X_3) from 0% to 100%. At the end of the extraction, the extract was filtered through Whatman No. 4 filter paper, and the supernatant was directly used for analysis.

Response value formats for result presentation

The optimized responses for dry residue (DR or R_1), reducing power (RP or R_2), and total phenolic content (TP or R_3) were determined.

The DR (R_1) was obtained by drying 3 mL of extract at 105 °C for 48 h and was expressed in g/100 g.

The RP (R_2) was evaluated considering the ability of the extracts to reduce Fe^{3+}. For this purpose, 0.5 mL of the extract, in serial dilutions using the respective extraction solvent, and the same volume of sodium phosphate buffer solution (0.2 M, pH 6.6) and potassium ferricyanide (1%) were mixed and incubated at 50 °C for 20 min; then, the reaction was stopped with 0.5 mL of trichloroacetic acid (10%). Next, 0.8 mL was mixed with 0.8 mL of distilled water and 160 µL of ferric chloride (0,1%) in a 48-well microplate, and the absorbance was measured at 690 nm (Añibarro-Ortega et al., 2020). The results were expressed as IC_{50} values, referring to the extract concentration necessary to inhibit the iron reduction by 50%, expressed in µg/mL.

Finally, the TP (R_3) was assessed by the Folin–Ciocalteau (F–C) methodology, where 0.5 mL of extract was mixed with 2.5 mL of the F–C reagent (1:10 *v*/*v*) and 2 mL of sodium carbonate (75 g/L). After incubation in a water bath for 30 min at 40 °C, the absorbance was measured at 765 nm (Añibarro-Ortega et al., 2020). The results were expressed in gallic acid equivalents, in milligrams per gram of extract (mg/g).

The objective function used for DR and TP was "maximize" to obtain the highest possible combination of factors, while for RP, the objective function used was "minimize", since for IC_{50} values, lower concentrations represent better results.

Experimental design, model analysis, and statistical evaluation

The study was conducted using an independent quadratic Box–Behnken design (BBD). The BBD is a three-level-three-factor system, through which it was possible to determine the best combination of extraction variables that led to extracts with greater responses (DR, RP, and TP) (Gunst et al., 1996). The experimental design, as well as the response values, are recorded in **Table 33**. The predictive mathematical models were derived from the quadratic **Equation 1**, in which R_n are the responses and b_n are the interception, linear, quadratic, and interaction terms.

$$R_n = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad \mathbf{1}$$

The coefficient of determination (R^2) of the model equations was obtained using analysis of variance (ANOVA), and the significances (p = 0.05) were verified by an F-test. Design Expert software 8.0.6 (State-Ease Inc., Minneapolis, MN, USA) was used for the experimental design and analysis.

Phenolic compounds analysis by HPLC-DAD-ESI/MS

The pumpkin peel extracts were dissolved in an ethanol–water solution (20:80, *v*/*v*) to obtain solutions with a concentration of 10 mg/mL and then filtered through a 0.22 µm nylon syringe filter. The phenolic compounds profile was determined by HPLC coupled to a diode array detector and electrospray ionization-mass spectrometry (HPLC-DAD-ESI/MS) (Bessada et al., 2016). The tentative identification of the compounds was based on the obtained information, including retention times and UV-Vis and mass spectra, as well as through comparison with commercial standards and available bibliographic information. Quantification was carried out by measuring the area of the peaks obtained and comparing them with the calibration curves of the most similar commercially available standards. The final results were expressed in mg/g of extract.

Evaluation of bioactive properties

The antioxidant, antimicrobial, and cytotoxic activity of the three extracts obtained under optimal extraction conditions was evaluated.

Antioxidant activity

The ability to inhibit lipid peroxidation was assessed via the thiobarbituric acid reactive substances inhibition (TBARS) assay, using porcine (*Sus scrofa*) brain homogenates. The antioxidant potential was measured by observing the reduction in the level of TBARS, as outlined by Pereira et al. (2014). The IC_{50} value, which represents the sample concentration providing 50% antioxidant activity, was used to express the results in µg/mL. In addition, the anti-hemolytic activity of the extracts was evaluated using the oxidative hemolysis inhibition assay (OxHLIA), described by Lockowandt et al. (2019), and the results were expressed in µg/mL as the IC_{50} value. This value indicated the concentration of the sample that could cause a delay of 60 min in oxidative hemolysis. To serve as a positive control, Trolox was used in both assays.

Antimicrobial activity

To determine the antimicrobial potential of the samples, a variety of microorganisms were examined, including different types of bacteria and fungi. The bacterial strains comprised both Gram-positive (*Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), and *Listeria monocytogenes* (ATCC 19111)) and Gram-negative strains (*Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 49741), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp (ATCC 13076), and *Yersinia enterocolitica* (ATCC 8610)). The fungi analyzed were *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404). The antibacterial

and antifungal activity were determined using the method outlined by Heleno et al. (2013). Both the minimum inhibitory concentration (MIC) and the minimum bactericidal (MBC) or minimum fungicidal (MFC) concentrations were determined for the bacteria and fungi. The positive controls used for the bacteria were streptomycin and ampicillin, and for the fungi, ketoconazole and bifonazole. The results were expressed in mg/mL.

Cytotoxic activity

In order to assess the cytotoxicity, a primary culture of non-tumorigenic porcine liver cells (PLP2) was used, obtained from freshly harvested porcine liver purchased from a local slaughterhouse. The sulforhodamine B (SRB) colorimetric assay was carried out with ellipticine as a positive control (Guimarães et al., 2013). The IC_{50} values (the concentration of extract inhibiting 50% of net cell growth) were expressed in µg/mL.

Statistical analysis

The samples were analyzed in triplicate, and the results were expressed as mean ± standard deviation. For only two groups of data, a Student's t-test was used for comparison, and one-way analysis of variance (ANOVA) was used for three or more groups. The normal distribution and homogeneity of variance were evaluated using Shapiro–Wilk and Levene tests, respectively. Homoscedastic data with $p > 0.05$ were analyzed using a Tukey's honestly significant difference (HSD) test. All tests were conducted at a significance level of 5% using IBM SPSS Statistics software (Version 22.0, IBM Corp, Armonk, NY, USA).

4.1.3. Results and discussion

Optimized responses and conditions by RSM

The extraction of bioactive compounds from the peels of three different genotypes of pumpkin (P1, P2, and P3) was optimized in order to obtain preservative solutions. For this purpose, two different methods were compared: heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE). For each technique, an optimization procedure was performed through seventeen individual extractions, considering three independent variables. The response surface methodology (RSM) based on a Box–Behnken experimental design was applied. The independent variables, as well their ranges, were sourced from the literature (Leichtweis et al., 2021). The tested conditions determined by the Box–Behnken experimental design for time (X_1 or t), temperature/power (X_2 or T/P), and ethanol percentage (X_3 or EtOH) are presented in **Table 33**.

Table 33. Time, temperature (for the heat-assisted extraction; HAE) or power (for the ultrasound-assisted extraction; UAE), and percentage of ethanol (EtOH) in the solvent, obtained from Box–Behnken experimental design for the extraction optimization.

Run	HAE			UAE		
	X_1 – t (min)	X_2 – T (°C)	X_3 – EtOH (%)	X_1 – t (min)	X_2 – P (% of W) [1]	X_3 – EtOH (%)
1	67.5	30	0	32.5	20	0
2	120	55	100	60	20	50
3	67.5	55	50	5	80	50
4	67.5	55	50	5	50	0
5	67.5	80	0	60	50	0
6	67.5	80	100	32.5	50	50
7	15	80	50	32.5	50	50
8	15	55	100	32.5	80	0
9	67.5	55	50	32.5	50	50
10	15	30	50	32.5	50	50
11	67.5	30	100	60	80	50
12	120	55	0	5	50	100
13	67.5	55	50	32.5	20	100
14	120	30	50	5	20	50
15	120	80	50	32.5	50	50
16	67.5	55	50	60	50	100
17	15	55	0	32.5	80	100

[1] The ultrasonic power variable was designed considering the maximum power rating of the equipment (500 W) as 100%.

The maximize function was chosen to obtain the highest values for dry residue (R_1 or DR) and total phenolic content (R_3 or TP), while the minimize function was used for the reducing power (R_2 or RP). **Table 34** shows the results derived from the Box–Behnken experimental design used to optimize these dependent variables for each extraction technique. In some cases, not all the responses could be optimized, due to a significant lack of fit ($p > 0.05$). These results were analyzed using a quadratic **Equation 1**, applying the response surface methodology.

Table 34. Results of the extraction runs of the independent variables of time, temperature/power, and ethanol percentage in the solvent for the two extraction techniques applied (heat-assisted extraction: HAE; and ultrasound-assisted extraction: UAE) and for the three dependent variables evaluated (R_1, g/100g; R_2, µg/mL; and R_3, mg/g).

Run	P1						P2						P3					
	HAE			UAE			HAE			UAE			HAE			UAE		
	R_1	R_2	R_3	R_1	R_2	R_3	R_1	R_2	R_3	R_1	R_2	R_3	R_1	R_2	R_3	R_1	R_2	R_3
	DR	RP	TP	DR	RP	TP	DR	RP	TP	DR	RP	TP	DR	RP	TP	DR	RP	TP
1	1.3	118	132	1.1	25	163	1.4	244	120	1.4	147	131	0.9	34	105	0.9	215	91
2	0.7	169	126	0.9	38	178	0.7	101	77	1.3	100	90	0.4	202	107	0.8	157	100
3	1.3	329	142	1.6	206	142	1.3	304	131	1.9	495	107	0.9	1333	85	1.2	423	70
4	1.3	313	112	1.2	319	170	1.4	405	116	1.4	337	132	0.9	664	80	1.0	703	111
5	1.3	401	98	1.2	304	127	1.9	612	91	0.6	295	86	1.0	1013	94	0.9	607	64
6	0.8	830	163	1.1	191	127	0.9	351	127	1.3	341	97	0.4	561	125	0.9	461	81
7	1.3	924	124	1.0	856	139	1.4	345	88	1.2	420	96	0.9	608	115	0.8	484	81
8	0.6	274	108	1.5	323	143	0.7	348	99	1.5	340	97	0.3	455	107	1.0	762	108
9	1.2	345	103	1.1	262	133	1.3	416	96	1.3	316	99	0.9	550	78	0.9	736	75
10	1.1	85	98	1.1	93	144	1.3	128	113	1.2	159	86	0.8	148	83	0.7	235	75
11	0.5	131	128	1.2	35	133	0.6	143	124	1.4	103	99	0.2	82	134	0.9	173	73
12	1.3	401	104	0.2	317	180	1.4	504	101	0.5	346	117	0.9	551	87	0.2	348	113
13	1.3	334	107	0.3	316	144	1.4	388	89	0.4	369	147	0.9	498	68	0.1	-	113
14	1.1	261	138	0.9	197	134	1.3	263	113	1.2	377	97	1.0	477	64	0.6	621	274
15	1.3	416	121	1.4	575	106	1.5	470	97	1.3	408	57	1.1	741	76	0.7	628	74
16	1.3	366	117	0.3	267	153	1.3	359	104	0.7	502	126	1.0	597	70	0.1	-	181
17	1.3	332	48	0.5	117	96	1.3	-	132	0.7	267	100	0.9	1066	65	0.7	1528	37

For the construction of the mathematical models, a confidence interval of $p = 0.05$ was used, excluding non-significant values. The final equations obtained to describe the evaluated responses using significant terms are presented in **Table 35**. The parametric values are presented as a function of the codification criteria of the experimental design, making it possible to compare the values between each other and interpret the weight of the influence of the numerical values on the responses.

Table 35. Mathematical models derived from the second-order polynomial model with interaction described in **Equation 1** in terms of encoded values for the two extraction techniques (heat-assisted extraction: HAE; and ultrasound-assisted extraction: UAE) and the three responses, when available (R_1 or DR; R_2 or RP; and R_3 or TP).

				Equation
P1	HAE			
	Dry residue (DR)	$R_1 =$	$1.27 + 0.0714X_1 + 0.0171X_2 - 0.3232X_3 + 0.0027X_1X_2 - 0.0666X_1X_3 + 0.0169X_2X_3 - 0.0396X_1^2 - 0.0245X_2^2 - 0.2882X_3^2$	**2**
	Reducing power (RP)	$R_2 =$	$337.4 + 247X_1 - 83X_2 + 110.5X_3 - 171X_1X_2 + 104X_1X_3 - 226.5X_2X_3 + 43.05X_1^2 + 41.05X_2^2 - 10.45X_3^2$	**3**
	Total phenols (TP)	$R_3 =$	$111.24 + 1.21X_1 + 13.861X_2 + 17.97X_3 + 10.83X_1X_2 + 17.25X_1X_3 + 9.69X_2X_3 + 18.86X_1^2 - 14.93X_2^2 - 4.85X_3^2$	**4**
	UAE			
	Dry residue (DR)	$R_1 =$	$1.14 - 0.0537X_1 - 0.2023X_2 - 0.4659X_3 - 0.0954X_1X_2 + 0.0077X_1X_3 - 0.0401X_2X_3 - 0.0343X_1^2 - 0.0716X_2^2 - 0.3612X_3^2$	**5**
P2	HAE			
	Dry residue (DR)	$R_1 =$	$1.34 + 0.0675X_1 + 0.0255X_2 - 0.33147X_3 + 0.0179X_1X_2 + 0.0811X_1X_3 - 0.0129X_2X_3 - 0.01273X_1^2 + 0.0232X_2^2 - 0.3211X_3^2$	**6**
	Reducing power (RP)	$R_2 =$	$332.03 + 125X_1 + 31.68X_2 - 85.07X_3 - 2.5X_1X_2 - 40X_1X_3 - 121.86X_2X_3$	**7**
	UAE			
	Dry residue (DR)	$R_1 =$	$1.25 - 0.1139X_1 - 0.1471X_2 - 0.3450X_3 - 0.1515X_1X_2 + 0.2518X_1X_3 - 0.0328X_2X_3 - 0.0114X_1^2 + 0.1861X_2^2 - 0.4336X_3^2$	**8**
P3	HAE			
	Dry residue (DR)	$R_1 =$	$0.9179 + 0.0555X_1 + 0.0473X_2 - 0.3145X_3 - 0.0138X_1X_2 + 0.0462X_1X_3 + 0.0145X_2X_3 - 0.0140X_1^2 + 0.0308X_2^2 - 0.3241X_3^2$	**9**
	Total phenols (TP)	$R_3 =$	$76.08 - 6.04X_1 + 4.46X_2 + 15.20X_3 + 12.93X_1X_2 + 0.5X_1X_3 - 5.4X_2X_3 + 6.68X_1^2 - 16.33X_2^2 + 31.75X_3^2$	**10**
	UAE			
	Dry residue (DR)	$R_1 =$	$0.7940 - 0.0405X_1 + 0.1595X_2 - 0.3463X_3 - 0.1440X_1X_2 - 0.0025X_1X_3 + 0.1160X_2X_3 - 0.02X_1^2 + 0.1240X_2^2 - 0.2350X_3^2$	**11**
	Total phenols (TP)	$R_3 =$	$4.35 - 0.2536X_1 - 0.4320X_2 + 0.0063X_3 + 0.2625X_1X_2 + 0.0095X_1X_3 - 0.5298X_2X_3 + 0.1029X_1^2 + 0.2490X_2^2 + 0.0042X_3^2$	**12**

Through a global analysis, it was possible to verify that the variable percentage of ethanol was more expressive in terms of both the linear effect and the quadratic effect, with a negative effect in most cases. As for the interactive effect, the interaction between ethanol and time, generally, had a higher effect than the interaction between time and temperature/power or ethanol and temperature/power.

To fulfill the objective of maximizing the extraction yield in terms of dry residue and total phenols, as well as obtaining extracts with a greater antioxidant capacity (minimizing the IC_{50} values, i.e., the sample concentration that provided 50% antioxidant activity), the values of the independent variables were adjusted to lead to an optimal response for each of the assessed extraction methods. It was possible to obtain

optimal extraction conditions leading to optimal individual and global response values. These data are presented in **Table 36** and subsequently discussed considering each pumpkin genotype.

Table 36. Optimized conditions of the independent variables, presented as real values, with which the optimal values for the individual and global responses were obtained.

Sample	Criteria	Heat-assisted extraction (HAE)					Ultrasound-assisted extraction (UAE)				
		Optimal Variable Conditions			Optimum Response		Optimal Variable Conditions			Optimum Response	
		X_1: t (min)	X_2: T (°C)	X_3: EtOH (%)			X_1: t (min)	X_2: P (W)	X_3: EtOH (%)		
P1	*Individual optimal variable conditions*										
	R_1	40	70	19	1.4	g/100 g dw	18	400	16	1.6	g/100 g dw
	R_2	30	31	29	27	µg/mL	-	-	-	-	µg/mL
	R_3	62	80	97	165	mg/g dw	-	-	-	-	mg/g dw
	Global optimal variable conditions										
	R_1				1.3	g/100 g dw				-	g/100 g dw
	R_2	75	30	24	158	µg/mL	-	-	-	-	µg/mL
	R_3				136	mg/g dw				-	mg/g dw
P2	*Individual optimal variable conditions*										
	R_1	120	73	24	1.5	g/100 g dw	7	380	17	1.9	g/100 g dw
	R_2	15	30	2	100	µg/mL	-	-	-	-	µg/mL
	R_3	-	-	-	-	mg/g dw	-	-	-	-	mg/g dw
	Global optimal variable conditions										
	R_1				1.4	g/100 g dw				-	g/100 g dw
	R_2	15	30	10	112	µg/mL	-	-	-	-	µg/mL
	R_3				-	mg/g dw				-	mg/g dw
P3	*Individual optimal variable conditions*										
	R_1	98	79	27	1.1	g/100 g dw	9	395	31	1.3	g/100 g dw
	R_2	-	-	-	-	µg/mL	-	-	-	-	µg/mL
	R_3	68	30	100	135	mg/g dw	29	100	100	307	mg/g dw
	Global optimal variable conditions										
	R_1				0.9	g/100 g dw				1.1	g/100 g dw
	R_2	67	30	0	-	µg/mL	5	400	0	-	µg/mL
	R_3				106	mg/g dw				120	mg/g dw

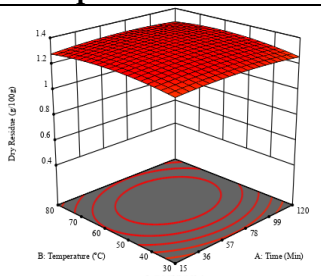
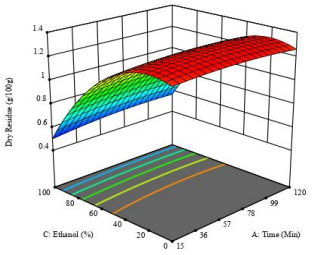
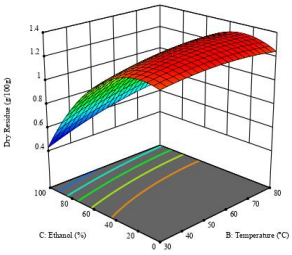
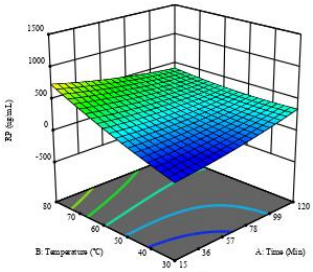
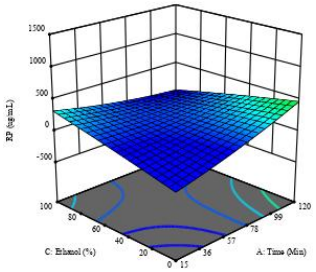
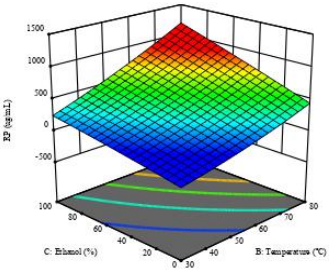
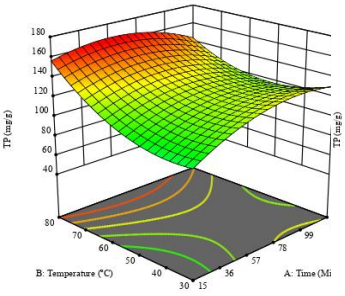
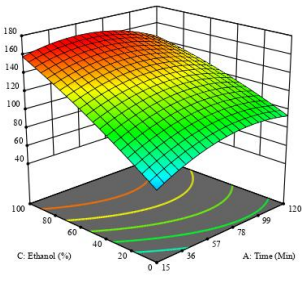
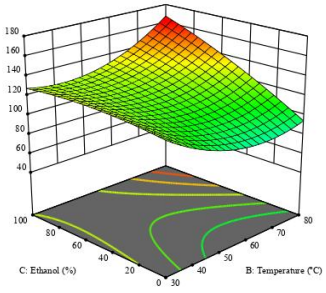
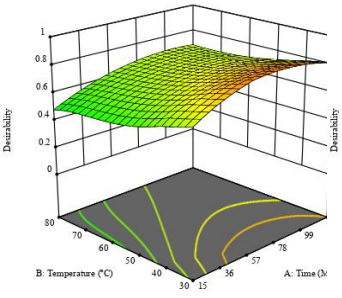
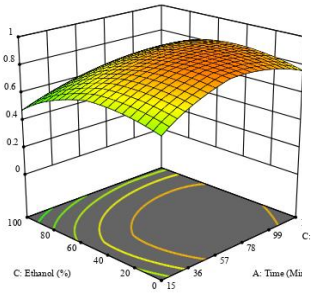
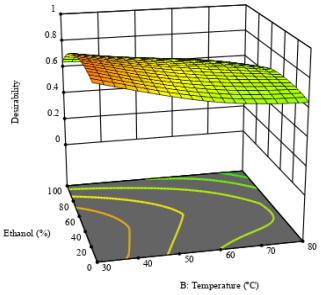
In addition, 3D surface graphs are presented for a better interpretation of the impact of the parametric values on the responses. In each figure, it is possible to observe the interaction between two of the three independent variables, keeping the third variable at a fixed level.

Optimization of the extraction for 'TL' peel (P1)

For the R_1 (DR) of sample P1, extracted through HAE, the analysis of the 17 runs rendered a quadratic function with a significant model, a non-significant lack of fit, an

adjusted R^2 of 0.984, and the coded **Equation 2**. Thus, the optimal values that maximized the amount of dry residue were set at 40 min, 70 °C, and 19% ethanol, which rendered 1.4 g/100g. The first row of **Table 37** shows the 3D response charts for R_1 at the optimal points. Overall, temperature and time did not have much influence on the optimization of the response, causing very low variation, while concentrations of ethanol beyond 40% seemed to reduce the dry residue yield.

Table 37. 3D response charts of the heat-assisted extraction (HAE) of 'TL' peel (P1) at the optimal values.

	Sample P1 by HAE		
	Temperature vs. Time	**Solvent vs. Time**	**Solvent vs. Temperature**
R_1 – DR			
R_2 – RP			
R_3 – TP			
Desirability			

Regarding R_2 (RP), a quadratic function was obtained, although two runs were eliminated for being outliers, which allowed a non-significant lack of fit and an adequate fit of the model, with an adjusted R^2 of 0.997, described by **Equation 3**. For this

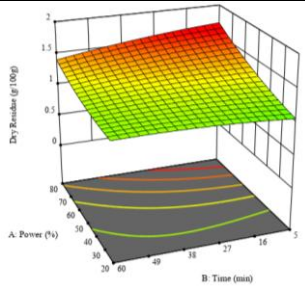
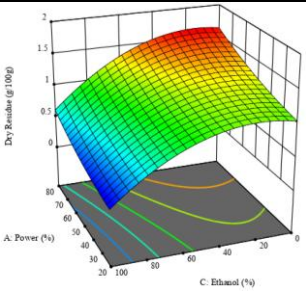
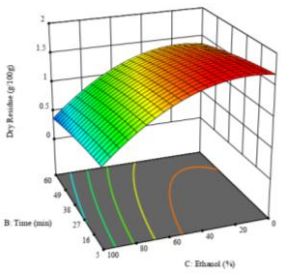
response, the minimize function was chosen, thus obtaining optimal values of 30 min, 31 °C, and 29% ethanol, rendering an estimated IC_{50} of 27 µg/g. The RSM charts can be found in the second row of **Table 37**, with the lowest values colored in dark blue. The two most important factors were extraction time and solvent percentage.

Finally, the R_3 (TP) rendered a quadratic function with an adjusted R^2 of 0.7203 and the coded **Equation 4**. The point that maximized the concentration of total phenols was under the conditions of 62 min, 80 °C, and 97% ethanol, which rendered 165 mg/g. The optimal values are shown in the third row of **Table 37**. Overall, the most important factors were temperature and ethanol percentage, although higher values for temperature should be considered due to the estimation of higher yields at temperatures over 80 °C.

The final row of **Table 37** shows the charts of the desirability function, in which all three responses were considered, allowing for the determination of the optimal point of all responses. For this point, the parameters were set at 75 min, 30 °C, and 24% ethanol, rendering a DR of 1.28 g/100 g, an IC_{50} value of 158 µg/g for RP, and 136 mg/g of TP. This function allowed for an equilibrium of each response that could be individually reduced, but considering all the responses together, the conditions would be suitable and optimized for all.

For the UAE technique applied to this genotype, it was only possible to optimize R_1. This response is detailed in **Equation 5** with an adjusted R^2 of 0.899. The optimal values for R_1 were set at 400 W, 18 min, and 16% ethanol, which were expected to render 1.6 g/100g of dry residue. The 3D charts are shown on **Table 38**, in which it is clear that ethanol content showed a higher influence on the yield, while the ultrasonic power had only a slight influence.

Table 38. 3D response charts of the ultrasound-assisted extraction (UAE) of 'TL' peel (P1) at the optimal values.

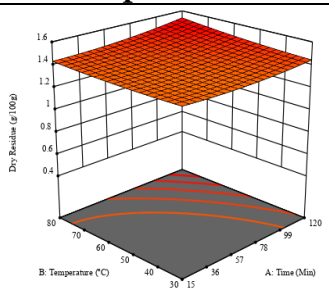
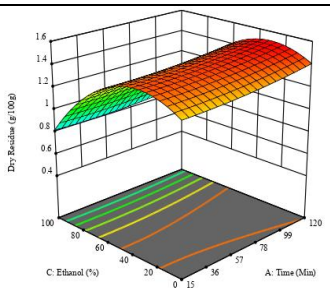
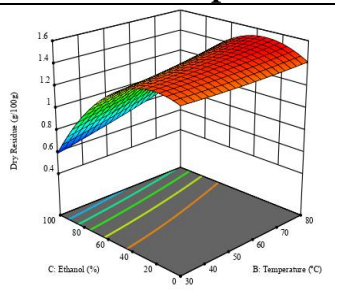
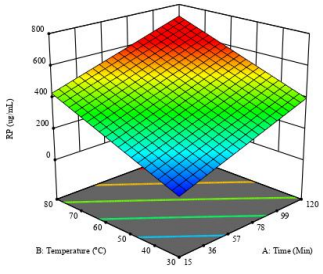
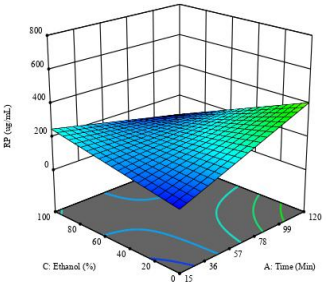
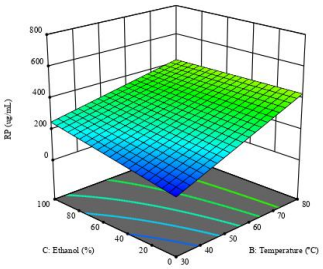
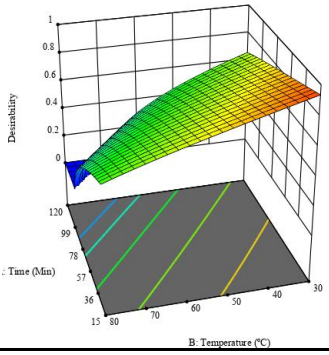
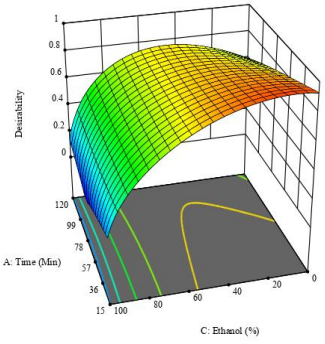
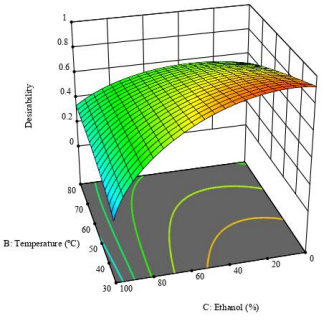
Sample P1 by UAE			
	Power vs. Time	**Power vs. Solvent**	**Time vs. Solvent**
R_1 – DR			

Optimization of the extraction for 'Voutirato' peel (P2)

For the R_1 of sample P2, extracted through HAE, the analysis of the 17 runs rendered the quadratic **Equation 6**, with a significant model, a non-significant lack of fit, and an adjusted R^2 of 0.975, although one run had to be eliminated for being an outlier.

Thus, the optimal values that maximized the amount of dry residue were set at 120 min, 73 °C, and 24% ethanol, which rendered 1.515 g/100g. The first row of **Table 39** shows the 3D response charts at the optimal values, in which it is possible to see that the temperature and extraction time had a weak effect on the variation of dry residue, and, once again, the ethanol content was the determinant, as for sample P1.

Table 39. 3D response charts of the heat-assisted extraction (HAE) of 'Voutirato' peel (P2) at the optimal values.

Sample P2 by HAE			
	Temp. vs. Time	**Solvent vs. Time**	**Solvent vs. Temperature**
R_1 – DR			
R_2 – RP			
Desirability			

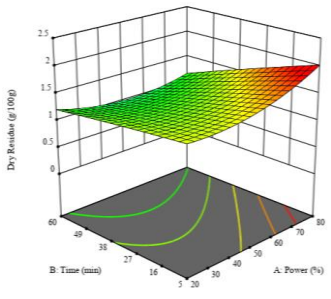
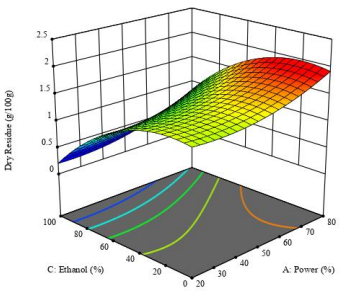
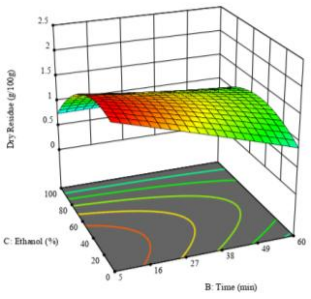
Regarding R_2, the model chosen was a two-factor interaction, and the adjusted R^2 of 0.7900 and the non-significant lack of fit for the 16 runs (one run did not show any antioxidant activity) are shown in the coded **Equation 7**. The minimize function resulted in an optimal point of 15 min, 30 °C, and only 2% ethanol, with an estimated IC_{50} value of 100 µg/g. The second row of **Table 39** shows the 3D charts for this response. Considering the coded equation, the factor with the highest influence was temperature, followed by time, revealing that lower IC_{50} values were favored by lower temperatures and shorter extraction times.

An adequate modeling was not possible for the R_3 of sample P2; thus, the third row

of **Table 39** represents the desirability of R_1 and R_2, which rendered an estimated 1.4 g/100 g of dry residue and 112 µg/g of reducing power at 15 min, 30 °C, and 10% ethanol.

As for the P1 variety, in the extraction of P2 by UAE, it was only possible to optimize R_1. R_2 analysis only rendered a linear model, and thus it was not considered for optimization studies, while R_3 did not produce satisfactory results due to a lack of fit. In this technique, R_1 rendered a quadratic function with a significant model, a non-significant lack of fit, and an adjusted R^2 of 0.8748, described by the coded **Equation 8**. The model placed the optimal points at 380 W of power, 7 min, and 17% ethanol, while predicting 1.9 g/100 g of dry residue. **Table 40** shows the 3D charts for sample P2, revealing that, to some extent, all the parameters influenced the optimal conditions for dry residue. A higher power, lower ethanol content, and shorter extraction time seemed to improve the dry residue quantity.

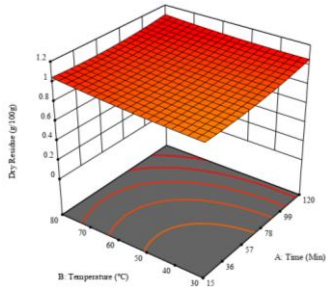
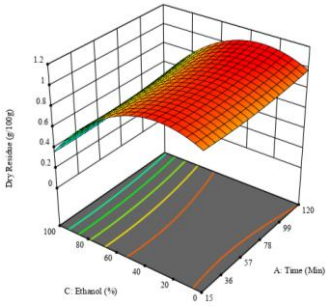
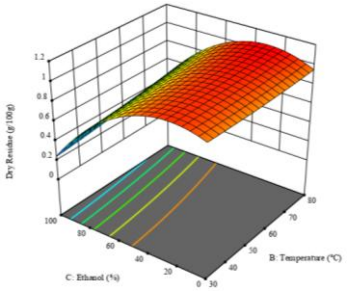
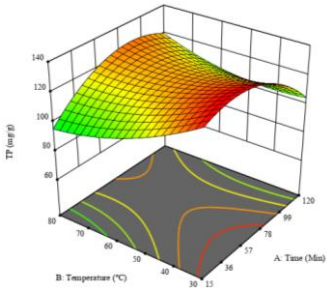
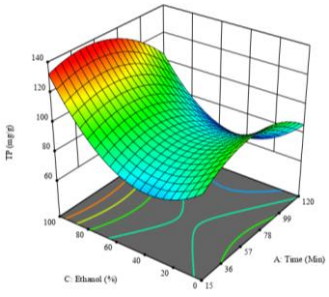
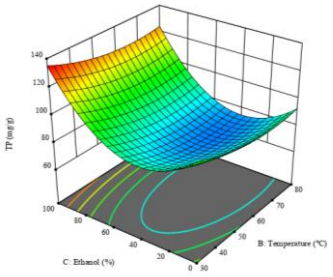
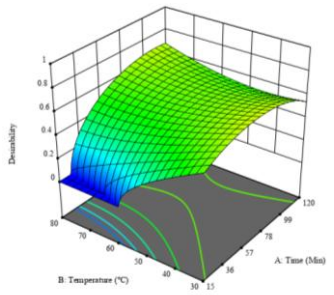
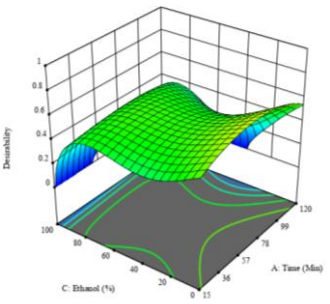
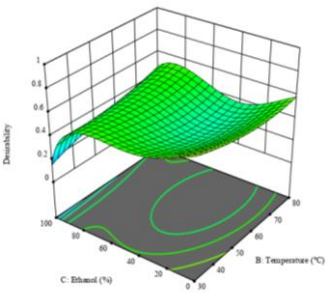
Table 40. 3D response charts of the ultrasound-assisted extraction (UAE) of 'Voutirato' peel (P2) at the optimal values.

	Sample P2 by UAE		
	Power vs. Time	**Solvent vs. Power**	**Solvent vs. Time**
R_1 – DR			

Optimization of the Extraction for 'Leuka Melitis' Peel (P3)

For the R_1 of sample P3 extracted by HAE, the analysis of the 17 runs rendered a quadratic function with a significant model, a non-significant lack of fit, and an adjusted R^2 of 0.9660, as shown in the coded **Equation 9**. Thus, the optimal values that maximized the amount of dry residue were set at 98 min, 79 °C, and 27% ethanol, which were predicted to render 1.06 g/100 g of dry residue. In **Table 41**, the first row shows the 3D charts of R_1 for this sample, indicating that, as in the previous samples, the temperature and time had a low influence on improving the yield, with the concentration of ethanol being the factor that markedly affected this response. The values obtained for the reducing power (R_2) did not allow for a satisfactory model, so it was not included.

Table 41. 3D response charts of the heat-assisted extraction (HAE) of 'Leuka melitis' peel (P3) at the optimal values.

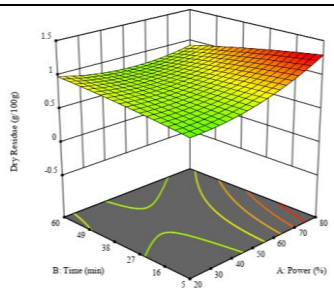
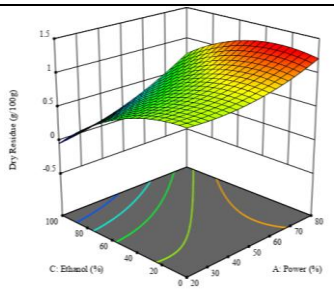
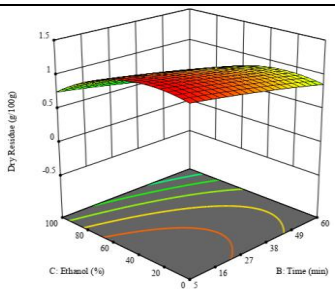
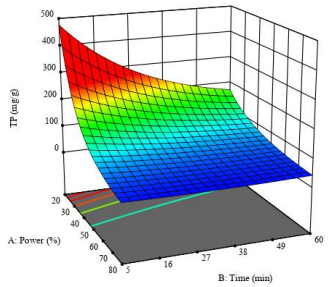
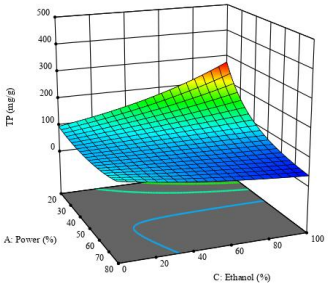
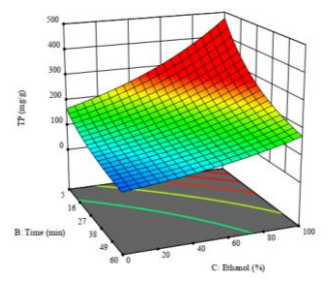
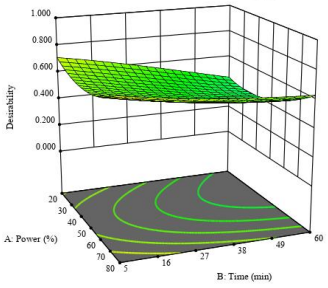
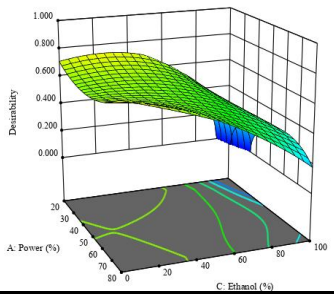
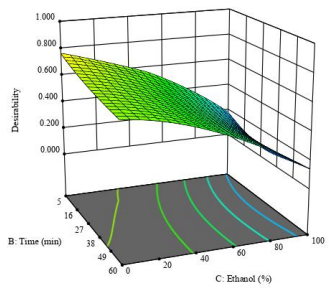
Sample P3 by HAE			
	Temp. vs. Time	**Solvent vs. Time**	**Solvent vs. Temperature**
R_1 – DR			
R_3 – TP			
Desirability			

Considering R_3, the values allowed for a significant model and non-significant lack of fit after removing one outlier. The coded equation is presented in **Equation 10**, and the adjusted R^2 was 0.9161. For this response, the factor with the highest influence was also the percentage of ethanol, which can be seen graphically in the second row of **Table 41**. The optimal values after applying the maximize function were set at 68 min, 30 °C, and 100% ethanol, which rendered 135 mg/g of total phenols. Considering the desirability function for these two responses, the optimal values were set at 67 min, 30 °C, and 0% ethanol, for which 0.9 g/100 g of dry residue and 106 mg/g of total phenols were expected. The 3D charts of the desirability function are presented in the third row of **Table 41**.

Regarding the extraction of P3 by UAE, the analysis of the 17 runs rendered for R_1 a quadratic function with a significant model, a non-significant lack of fit, and an adjusted R^2 of 0.8970, as shown in the coded **Equation 11**. Through the interpretation of the coded equation, the factor with the highest influence seemed to be the power of the ultrasonic waves, as shown by the high values of the optimal point, which were set at 395

W of ultrasonic power, 9 min, and 31% ethanol, producing 1.25 g/100 g of dry residue. The 3D charts, displayed in the first row of **Table 42**, show a similar trend, with higher power intensities rendering higher yields of dry residue, while lower extraction times also seemed to favor this response. As with HAE, the R_2 values did not allow for an optimization procedure.

Table 42. 3D response charts of the UAE of 'Leuka melitis' peel (P3) at the optimal values.

Sample P3 by UAE			
	Power vs. Time	**Solvent vs. Power**	**Time vs. Solvent**
R_1 – DR			
R_3 – TP			
Desirability			

Regarding R_3, the values allowed for a quadratic model with a natural log transformation after ignoring two outliers. The model showed a significant fit and non-significant lack with an adjusted R^2 of 0.9870, as shown in **Equation 12**. In the case of R_3, the factor with the highest influence was the percentage of ethanol, as indicated by the coded values of the equation. The optimal values were set at 100 W, 29 min, and 100% ethanol, which were expected to yield 307 mg/g of total phenols, beyond what was achieved in the variation intervals of the factors. The second row of **Table 42** shows the 3D charts for this response, highlighting the lower ultrasonic power requirements to promote higher total phenols, while high yields of ethanol and a longer extraction time seemed to promote the extraction yield of these bioactive molecules. The desirability function pointed towards optimum values of 80% ultrasound power, a 5 min extraction

time, and 0% ethanol, which would render 1.12 g/100 g of dry residue and 120 mg/g of total phenols. The corresponding 3D charts are shown in the final row of **Table 42**.

General considerations

Overall, the HAE method seemed to be the best candidate for this optimization study, revealing the most robust results. It was clear that the concentration of ethanol had a higher influence on the dry residue yields for HAE, while temperature and time also showed moderate effects on the IC_{50} results for RP. Considering TP, the influence on this response was case-specific. According to the literature, a significant correlation was also recorded between the extraction conditions and the total phenolic compounds content and antioxidant activity in the case of *Berberis asiatica* fruit, which indicated the importance of optimizing the extraction protocol in order to achieve the highest extraction efficiency for bioactive compounds (Belwal et al., 2016).

Regarding the UAE protocol, obtaining satisfactory values for the optimization was difficult, although the impact of the ethanol percentage on the dry yield variable was also determinant. Similarly to our study, Chen et al. (2018) also suggested the importance of optimizing the solvent content in order to obtain the highest amounts of total phenolic compounds from *Lycium ruthenicum* Murr. (LR) fruit. Moreover, Asif et al. (2017) suggested that the methanol content in the extraction solvent may also affect the antioxidant activity of squash peels, while different solvents may result in varied results in terms of the antioxidant capacity of squash fruit parts (Singh et al., 2016).

Considering all of the above, it was logical to select the HAE method as the most appropriate for obtaining extracts with a high residue content and antioxidant potential from pumpkin peels of genotypes 1 and 2, whereas for genotype 3, UAE led to better results. Although the UAE method for P3 demanded the highest power value tested (400 W), it resulted in a fast extraction time of only 5 min, which was the shortest time recorded, and required only water as solvent (0% ethanol). Low requirements in terms of extraction time (15 min) and ethanol content (10%) were also achieved by HAE for P2, with the advantage of also using the lowest temperature tested (30 °C). This low temperature was also the best for P1. According to the literature, the recovery of polyphenols from various bioresidues is highly dependent on the extraction protocol, with several studies suggesting that the ultrasound-assisted method is the most efficient compared to the solid-to-liquid extraction and microwave-assisted extraction protocols (Da Porto & Natolino, 2018).

Despite the fact that some optimal values were found at the limits of the tested variables, it was unfeasible to try to overcome them. However, it is possible to affirm that the optimization process led to an efficient extraction protocol, saving both time and

energy.

Many studies on the optimization of the extraction of bioactive compounds from pumpkins can be found in the literature (Altemimi et al., 2015; Kulczyński et al., 2020; Quanhong & Caili, 2005; Sun et al., 2011). However, the use of pumpkin by-products as a source of such compounds and their use as food preservatives has still been scarcely explored. This work is an important contribution, being, to the best of our knowledge, the first study to optimize preservative compound extraction from pumpkin peels. Motivated by a real need within the pumpkin processing industry, these promising results encourage the replacement of synthetic preservatives, known to be related to adverse health effects, with a natural alternative obtained through sustainable processes. Furthermore, the evaluation of different pumpkin varieties, as well as the comparison of two extraction methodologies, made these results feasible and highly promising for upscaling. Through the predictive mathematical models obtained, it is also possible to simulate the best extraction conditions considering limitations of time, energy, and ethanol use.

Evaluation of the extracts obtained at the optimum conditions

In order to validate the predictive mathematical model of the studied process, experimental validation was performed with the estimated optimal extraction conditions, through which it was possible to verify the consistency within the predicted and real results obtained for the three responses considered in the optimization study (DR, RP, and TP; data not shown). Moreover, the optimal extracts were assessed for their phenolic compound profiles and antioxidant, antimicrobial, and cytotoxic properties in order to validate their potential for application as food preservatives.

Phenolic compound profiles

The pumpkin peels were subjected to extraction under the global optimal conditions by HAE for P1 and P2, and by UAE for P3. These extracts were then evaluated by HPLC-DAD-ESI/MS to obtain their phenolic compound profiles. **Table 43** presents the information of the UV-Vis at the maximum absorption, the deprotonated ion, the mass fragmentation, and the respective tentative identification of the compounds found in the extracts. The extracts from P1 and P2 showed the same profile with seven compounds detected, as presented in **Figure 11**, while sample P3 did not contain peak 6.

Table 43. Characterization of the phenolic compounds detected under the optimal conditions for each extract by HPLC-DAD-ESI/MS.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	7.69	280	289	245(100),205(45)	(-)-Epicatechin
2	14.11	324	337	191(5),173(100),135(5)	*cis*-4-*O*-*p*-Coumaroylquinic acid
3	14.49	325	337	191(5),173(100),135(5)	*trans*-4-*O*-*p*-Coumaroylquinic acid
4	16.51	354	739	285(100)	Kaempferol-*O*-dideoxyhexosyl-hexoside
5	16.93	354	769	315(100)	Isorhamnetin-*O*-dideoxyhexosyl-hexoside
6	21.03	348	593	285(100)	Kaempferol-*O*-deoxyhexosyl-hexoside
7	21.98	365	623	315(100)	Isorhamnetin-*O*-deoxyhexosyl-hexoside

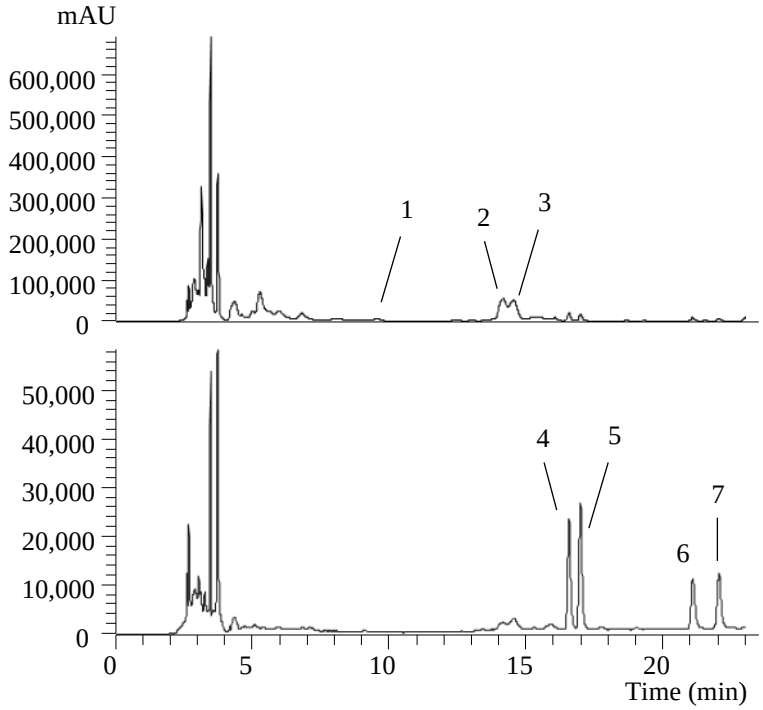


Figure 11. Chromatographic representation of the phenolic compounds profile obtained by HPLC-DAD under the optimal conditions for each extract.

Peaks 1, 4, 5, 6, and 7 were tentatively identified considering the previous characterization of the extracts obtained from Portuguese and Algerian pumpkin peels (Leichtweis et al., 2022). (-)-Epicatechin ($[M-H]^-$ at *m/z* 289, peak 1) was tentatively identified by comparison with the available standard, while the flavonoid compounds (peaks 4, 5, 6, and 7) were compared with the literature (Iswaldi et al., 2013). Regarding these flavonoids, peaks 4 ($[M-H]^-$ at *m/z* 739) and 6 ($[M-H]^-$ at *m/z* 593) presented only one MS^2 fragment at *m/z* 285 (kaempherol aglycone), while peaks 5 ($[M-H]^-$ at *m/z* 769) and 7 ($[M-H]^-$ at *m/z* 623) presented the only MS^2 fragment at *m/z* 315 (isorhamnetin aglycone). Thus, peaks 6 and 7, corresponded to the loss of two sugar moieties: deoxyhexosyl and hexoside ($[M-H-146-162]^-$), which were tentatively identified as kaempferol-*O*-deoxyhexosyl-hexoside and isorhamnetin-*O*-deoxyhexosyl-hexoside,

respectively. On the other hand, peaks 4 and 5, corresponded to three sugar moieties linked to the flavonoid aglycone ([M-H-146-146-162]⁻), which were tentatively identified as kaempferol-*O*-dideoxyhexosyl-hexoside and isorhamnetin-*O*-dideoxyhexosyl-hexoside, respectively.

In addition to the flavan-3-ol (peak 1) and the abovementioned flavonoids, the extracts also presented two phenolic acids (peaks 2 and 3). These peaks were tentatively identified as the *cis* and *trans* isomers of 4-*O*-*p*-coumaroylquinic acid, also called 4-pCoQA, presenting the pseudomolecular ion $[M-H]^-$ at m/z 337 and the fragment ion MS^2 as the base peak at m/z 173; 5% of the base peak at m/z 191 and m/z 135; and a maximum UV-Vis absorbance at 324/325 nm. These chromatographic responses were in accordance with those previously described in the literature (Ncube et al., 2014), which have also been reported in African pumpkin leaves (Mashiane et al., 2021).

As shown in **Table 44**, for the three samples, the flavonoids group was the most abundant, with a total concentration of 2.05548 ± 0.00001 mg/g for P1, 1.7384 ± 0.0004 mg/g for P2, and 1.3388 ± 0.0002 mg/g for P3; however, for P2, the major compound was *cis*-4-*O*-*p*-coumaroylquinic acid (peak 2).

Table 44. Quantification of the phenolic compounds detected under the optimal conditions for each extract (mg/g of extract).

Peak	P1	P2	P3
1	0.244 ± 0.007 [a]	0.210 ± 0.005 [b]	0.091 ± 0.004 [c]
2	0.355 ± 0.003 [b]	0.52 ± 0.02 [a]	0.0583 ± 0.0003 [c]
3	0.371 ± 0.004 [a]	0.36 ± 0.02 [a]	0.0367 ± 0.0003 [b]
4	0.5204 ± 0.0006 [a]	0.42085 ± 0.00007 [c]	0.444589 ± 0.000009 [b]
5	0.5302 ± 0.0003 [a]	0.4800 ± 0.0003 [b]	0.45000 ± 0.00006 [c]
6	0.5005 ± 0.0005 [a]	0.41053 ± 0.00004 [b]	n.d.
7	0.5044 ± 0.0002 [a]	0.42703 ± 0.00009 [c]	0.4442 ± 0.0002 [b]
Total flavan-3-ols	0.244 ± 0.007 [a]	0.210 ± 0.005 [b]	0.091 ± 0.004 [c]
Total phenolic acids	0.7261 ± 0.0006 [b]	0.876 ± 0.005 [a]	0.09495 ± 0.00009 [c]
Total flavonoids	2.05548 ± 0.00001 [a]	1.7384 ± 0.0004 [b]	1.3388 ± 0.0002 [c]
Total phenolic compounds	3.026 ± 0.008 [a]	2.824 ± 0.001 [b]	1.525 ± 0.004 [c]

n.d. – not detected. Calibration curves used for quantification: (-)-catequin (y = 84,950x – 23,200, R^2 = 0.999, LOD = 0.17 µg/mL, LOQ = 0.68 µg/mL, peak 1); *p*-coumaric acid (y = 301,950x + 6966.7, R^2 = 0.9995, LOD = 0.71 µg/mL, LOQ = 2.38 µg/mL, peaks 2 and 3); and quercetin 3-*O*-glucoside (y = 34,843x – 160,173; R^2 = 0.9998; LOD = 0.21 µg/mL, LOQ = 0.71 µg/mL, peaks 4, 5, 6, and 7). ANOVA analysis—in each column, different letters indicate significant differences (p < 0.05).

(-)-Epicatechin (peak 1) was the least representative compound in samples P1 and P2, contrarily to the results reported for the Portuguese pumpkin extracts, in which this was the most abundant compound (Leichtweis et al., 2022). The extracts from P1 and P2 showed higher contents of total phenolic compounds, approximately 3 mg/g each, while P3 presented almost half of the total concentration found in P1. These differences between the tested genotypes were in agreement with the report of Kiat et al. (2014), who also

recorded a variable content of polyphenols in the seeds of different pumpkin genotypes. Considering that in our study all the plants were grown under the same conditions, the effect of cultivation practices could be eliminated, and the genotypic effect could be suggested as the determinant factor.

Antioxidant activity

The optimized extracts were assessed by two biological assays, TBARS and OxHLIA, using homogenates of porcine brain and sheep erythrocytes, respectively, as oxidizable targets. These methods are considered more representative than colorimetric assays as they are cell-based and, thus, more similar to the reactions that occur in biological systems. The results obtained in both assays are shown in **Table 45**.

Table 45. Antioxidant activity at the optimal conditions for each extract (μg/mL).

Sample	TBARS (IC_{50})	OxHLIA (IC_{50}) Δt = 60 min
P 1	850 ± 40 ^{c}	61 ± 1 ^{b}
P 2	1600 ± 88 ^{b}	62 ± 1 ^{b}
P 3	2510 ± 147 ^{a}	540 ± 15 ^{a}
Trolox*	139 ± 5 ^{d}	21.8 ± 0.2 ^{c}

IC_{50}: extract concentration that inhibited oxidation by 50%. * Positive control. One-way analysis of variance (ANOVA) – in each column, different letters indicate significant differences ($p < 0.05$).

It is possible to notice the strong antioxidant capacity of the optimized extracts, especially for sample P1, which presented IC_{50} values of 850 ± 40 μg/mL for TBARS and 61 ± 1 μg/mL for OxHLIA, in agreement with its higher phenolic compounds content. The P2 extract provided an anti-hemolytic activity as strong as that presented by P1 and a lipid peroxidation inhibition capacity of 1600 μg/mL. P3 was the sample presenting the lowest antioxidant potential, in accordance with its lower phenolic compounds content. The IC_{50} values obtained in the present study were comparable or better than those reported for Portuguese and Algerian pumpkins (Leichtweis et al., 2022), which presented IC_{50} values ranging from 88 to 209 μg/mL and 335 to 588 μg/mL for the OxHLIA assay and from 3921 to 7765 μg/mL and 2123 and 4569 μg/mL for the TBARS assay, respectively. These findings indicate the high antioxidant capacity of pumpkin fruit waste and highlight the potential of its further valorization in the food industry, since a significant correlation between the polyphenol extraction efficiency and the antiradical potential of the extracts has been well-confirmed (Rice-Evans et al., 1997).

Antibacterial and antifungal activity

The activity presented by the optimum extracts tested against eight bacterial strains and two fungal strains is shown in **Table 46**. All the extracts were capable of inhibiting the growth of *Aspergillus brasiliensis*, *Listeria monocytogenes*, and *Staphylococcus aureus*. The P2 sample stood out and, in addition to these microorganisms, also presented bacteriostatic activity against the other five bacteria, whereas no samples were effective against *Bacillus cereus* at the maximum tested concentration (10 mg/mL). The best results were presented by the P2 sample against *Salmonella enterica* (MIC of 2.5 mg/mL) and by the P3 sample against *Yersinia enterocolitica* (MIC of 2.5 mg/mL). Moreover, P3 was also effective against *Pseudomonas aeruginosa* growth (MIC of 10 mg/mL) and P1 against *Enterobacter cloacae* and *Escherichia coli* (MIC of 10 and 5 mg/mL, respectively).

Research on the antimicrobial activity of pumpkin peels is very limited. In a study conducted in Pakistan in 2021, pumpkin peel, pulp, and seeds were tested and revealed significant antifungal activity against four strains: *Candida albicans*, *Fusarium oxysporum*, *Mucor miehei*, and *Trichoderma* spp. (Hussain et al., 2021). The authors also found antibacterial activity against *Salmonella typhi*, *Escherichia coli*, *Bacillus subtilis*, and *Streptococcus aureus* (Hussain et al., 2021). In a similar study, the antimicrobial activity of the peel, seeds, and fibers of pumpkin genotypes from Portugal and Algeria were assessed, and all Portuguese pumpkin samples demonstrated inhibitory activity against *Y. enterocolitica*, while those from Algeria inhibited *S. aureus*. In both studies, all samples inhibited the growth of *A. brasiliensis*, as in the present study (Leichtweis et al., 2022).

Table 46. Antimicrobial activity under the optimal conditions for each extract (mg/mL).

	P1		P2		P3		Streptomycin*		Methicilin*		Ampicillin*		Ketoconazole*	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
Enterobacter cloacae	10	>10	10	>10	>10	>10	0.007	0.007	n.t.	n.t	0.15	0.15	n.t.	n.t.
Escherichia coli	5	>10	5	>10	>10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15	n.t.	n.t.
Pseudomonas aeruginosa	>10	>10	10	>10	10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63	n.t.	n.t.
Salmonella enterica	5	>10	2.5	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15	n.t.	n.t.
Yersinia enterocolitica	10	>10	5	>10	2.5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15	n.t.	n.t.
Bacillus cereus	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	10	>10	5	>10	5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15	n.t.	n.t.
Staphylococcus aureus	10	>10	10	>10	10	>10	0.007	0.007	0.007	0.007	0.15	0.15	n.t.	n.t.
Aspergillus brasiliensis	10	>10	10	>10	10	>10	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0.06	0.125
Aspergillus fumigatus	>10	>10	>10	>10	>10	>10	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0.5	1

MIC: minimum inhibitory concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL); MFC: minimum fungicidal concentration (mg/mL); n.t.: not tested. * Positive control.

Cytotoxicity

The optimized extracts were assessed regarding their cytotoxicity in a primary culture of porcine liver cells (PLP2). None of the extracts presented cytotoxicity up to 400 µg/mL, which was an important first step in the verification of their safety when considering their use in the food industry. To the best of our knowledge, no other studies have reported potential toxic effects of pumpkin extracts.

4.1.4. Conclusions

Two extraction methodologies, heat- (HAE) and ultrasound-assisted extraction (UAE), were compared through an RSM optimization study to obtain pumpkin peel extracts rich in food preservatives. The HAE methodology proved to be more efficient for the 'TL' genotype, making it possible to reduce the extraction temperature (to 30 °C) and using low concentrations of ethanol in water (24%). For the 'Voutirato' genotype, apart from requiring low temperatures and ethanol concentrations (10%), this technology (HAE) also allowed a low extraction time (15 min). On the other hand, for the 'Leuka Melitis' genotype, UAE proved to be more efficient, making it possible to save time (5 min of extraction time) and use only water (0% ethanol) as the extraction solvent. The optimization by RSM allowed us to find the conditions that led to a higher extraction yield, reducing power, and total phenols concentration with reduced time, energy, and solvent parameters. The use of the Box–Behnken experimental design allowed the simultaneous evaluation of three independent variables in a wide range through 17 experimental runs.

The extracts obtained under the global optimal conditions were characterized regarding their phenolic profiles by HPLC-DAD-ESI/MS, where the 'TL' and 'Voutirato' genotypes presented the highest contents of phenolic compounds (3.026 ± 0.008 and 2.824 ± 0.001 mg/g, respectively). Moreover, the obtained optimal extracts demonstrated a high antioxidant potential in the TBARS and OxHLIA assays and the capacity to inhibit the growth of at least four of the eight bacterial strains tested and the fungus *Aspergillus brasiliensis* without revealing cytotoxicity in non-tumor liver cells. These bioactive properties corroborated the potential of the obtained extracts to act as food preservatives, while the optimization of the extraction protocols may allow an improvement in extraction efficiency and increase the added value of the crop through the valorization of peel waste for food preservation purposes.

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Conflicts of interest: The authors declare that they have no conflict of interest.

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4.2. Novel all-pumpkin product: Optimized by-products extraction and development of pulp formulations preserved with natural ingredients

Novel all-pumpkin product: Optimized by-products extraction and development of pulp formulations preserved with natural ingredients

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Abstract: The promotion of environmentally friendly extraction methods and the reduction of the use of synthetic additives is aligned with the context of sustainable food production. In this study we optimized the extraction conditions of pumpkin peels (e.g. Heat-Assisted Extraction (HAE) and Ultrasound-Assisted Extraction (UAE)) and further developed two types of pumpkin pulp formulations preserved with natural pumpkin ingredients (formulations using both natural extracts and synthetic additives and ones using only natural extracts). The optimal conditions for UAE were 400 W, 5 min, and 27% ethanol, yielding 2.29 g/100 g of dry residue, while HAE was optimized at 84 minutes, 30 ºC, and 0% ethanol, rendering 1.49 g/100 g of dry residue and 169 mg/g of total phenols. Both formulations retained nutritional profile, color and microbiological quality for up to 45 days. These findings highlight the valorization of agro-industrial by-products and contribute to sustainability through eco-friendly extraction methods and circular economy.

Keywords: Incorporation; Bioactivities; Shelf-life; Eco-friendly extraction; Sustainability; Natural preservatives; Circular economy

4.2.1. Introduction

Pumpkin *(Cucurbita* spp.) is a widely cultivated and consumed vegetable in various parts of the world due to its high nutritional value and culinary versatility (Gavril, Stoica, et al., 2024; Liu et al., 2024). Rich in vitamins, minerals, fibers, and antioxidants, pumpkin plays a significant role in human nutrition, contributing to a balanced and healthy diet (Gavril, Stoica, et al., 2024; Shajan et al., 2024). Among its parts, pumpkin pulp is the main edible portion of the fruit which stands out for its diverse applications in soups, purées, cakes, and other processed food products (e.g. minimally processed or ready-to eat food products) (Kulcu et al., 2024).

However, as with other perishable products, the preservation of ready-to-eat pumpkin pulp products requires the use of preservative additives, which are essential for ensuring microbiological safety and extending shelf life in retail shops (Atilgan & Bayraktar, 2022; Petcu et al., 2023; Venkatesan & Muniyan, 2024). Traditionally, synthetic preservatives such as potassium sorbate (E202) have been widely used due to their effectiveness in inhibiting the growth of a broad range of pathogenic and spoilage microorganisms, offering excellent cost-effectiveness at industrial level. Nevertheless, the continuous use of synthetic preservatives has raised concerns about potential adverse health effects, including allergic reactions and genotoxicity, as well as the environmental burden that these compounds may have (Carocho et al., 2014; Mamur et al., 2010; Martínez-Pineda et al., 2021).

In this context, allied with a growing consumer trend for more natural and healthier foods, prioritizing minimal processing and reducing chemical additives, the use of natural alternatives to synthetic preservatives has gained interest (Chauhan & Rao, 2024; Fink & Filip, 2021; Karimpour et al., 2021). With great impetus in the scientific community, plant and vegetable extracts have been in focus for obtaining molecules with antimicrobial and antioxidant properties, important for food preservation, while several studies have explored the potential use of plant extracts as natural preservatives in food products (Leichtweis et al., 2021; Petcu et al., 2023). However, there is limited research on the optimized extraction and application of pumpkin by-products extracts as natural preservatives, especially in pumpkin-based food products.

Both the peel and the seeds of pumpkins, which are normally discarded, have a significant value in terms of bioactive compounds (Hussain, Kausar, Din, Murtaza, Jamil, Noreen, & Iqbal, 2021; Kulczyński et al., 2020; Mala & Kurian, 2016). However, the optimization of extraction methods is crucial to maximize the yield of bioactive compounds while minimizing energy consumption and the use of harmful solvents, especially when aiming to valorize these by-products at industrial scale. By comparing different extraction techniques with varying processing variables such as the extraction

time, temperature, power, solid-liquid ratio, and solvent type, through the use of mathematical models (as RSM), it is possible to identify the optimal extraction conditions for each specific matrix. This approach makes the process more sustainable and economical in terms of energy, labor, and solvent consumption (Gavril, Constantin, et al., 2024; Leichtweis, Molina, Petropoulos, et al., 2023; Mansour, Falleh, Hammami, et al., 2023; Oufighou et al., 2024).

In light of this, the present study proposed the use of pumpkin peels, a by-product of pumpkin pulp processing, to obtain an extract rich in preservative compounds. In a preliminary study of our research group (Leichtweis et al., 2022), the hydroethanolic extracts of peels, seeds, and fibrous strands of pumpkin showed important *in vitro* antioxidant and antimicrobial activity, attributed to the phenolic composition which consisted of phenolic acids and flavonoids, revealing their potential use as a food preservative. Therefore, this study aimed to investigate the efficacy of pumpkin peel extracts as natural substitutes for potassium sorbate in the preservation of a pumpkin pulp product, with the goal of achieving a more sustainable production process and a healthier end product. For this purpose, butternut squash fruit peels were subjected to an extraction optimization study, comparing two techniques: heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE). The obtained peel extract was then incorporated into a pumpkin pulp product at various concentrations, and it was compared with a formulation containing seed extract which has been evaluated in a previous work of our team. Controlled experiments were conducted to determine the extract's ability to maintain the microbiological and physicochemical quality of the pulp during storage. This work is driven by the need to develop pumpkin pulp formulations that not only retain the product's shelf life but also meet consumer expectations for more natural and health-conscious food options.

4.2.2. Materials and methods

Sample preparation

Mature butternut squash fruit (*Cucurbita moschata*) were purchased locally in Bragança, Portugal. The pumpkins were properly cleaned and peeled. The peels were then freeze-dried (FreeZone 4.5, Labconco) and ground into powder (~20 mesh) for subsequent analysis.

Optimization procedure

Pumpkin peel extraction procedures

The optimization procedure followed the parameters and conditions of a previous study (Leichtweis, Molina, Petropoulos, et al., 2023). Fruit peels were extracted through

two different techniques (heat- and ultrasound-assisted extraction) under conditions defined by an individual Box-Behnken experimental design for each technique, as described below. The solid-liquid ratio was maintained at 25 g/L for both techniques.

- The heat-assisted extraction was done using an internally stirred water reactor equipped with a Cimarec™ magnetic stirrer (Thermo Scientific, San Jose, CA, USA) at a constant speed of approximately 500 rpm. Pumpkin peel sample (0.5 g) was mixed with 20 mL of solvent and the experimental parameters varied within these ranges: time (15-120 min), temperature (30-80 °C), and ethanol content (0-100%).
- For ultrasound-assisted extraction, an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA) equipped with a fixed water reactor operating at a frequency of 40 kHz was used. 0.75 g of sample was placed in the reactor with 30 mL of solvent and extracted under planned conditions. The experimental design ranges were set as follows: time (5-60 min), ultrasonic power (100-400 W), and ethanol ratio (0%-100%).

After extraction, extracts were filtered through Whatman No. 4 filter paper, and the supernatant was directly used for analysis.

Response values

The dry residue (DR or R_1) and the antioxidant activity of reducing power (RP or R_2), and total phenolic content (TP or R_3) were used as responses in each applied technique, following the protocol of a previous study (Leichtweis, Molina, Petropoulos, et al., 2023). Briefly, for DR, 3 mL of the extract were dried at 105 °C until constant weight; RP was assessed by evaluating the extract's ability to reduce Fe^{3+} and results were expressed as IC_{50} values, which indicate the extract concentration needed to inhibit iron reduction by 50%, in μg/mL; TP was measured using the Folin–Ciocalteau (F-C) method and expressed in gallic acid equivalents (GAE), in mg/g.

For the optimization process, the objective function for DR and TP was set to "maximize" to achieve the highest possible combination of factors. Conversely, for RP, the objective function was set to "minimize" because lower IC_{50} values indicate lower concentrations needed for inhibition, representing better results.

Experimental design, model analysis, and statistical evaluation

The study used an independent quadratic Box–Behnken design (BBD), a three-level-three-factor system that enabled the identification of the optimal combination of extraction variables to achieve the highest responses. The experimental design and response values are detailed in Table A of the supplementary material.

Predictive mathematical models were derived from the quadratic **Equation 13**, where Rn is the response or dependent variable, b_o is the intercept coefficient, b_i are the linear

coefficients, b_{ii} are the quadratic coefficients, b_{ij} are the interaction coefficients, X_i and X_j are the independent variables, and k is the number of factors.

$$Rn = b_0 + \Sigma_{i=1}^{k} b_i X_i + \Sigma_{i=1}^{k} b_{ii} X_i^2 + \Sigma_{i<j}^{k} b_{ij} X_i X_j \quad \textbf{13}$$

The coefficient of determination (R^2) for the model equations was obtained through analysis of variance (ANOVA), with significance (p = 0.05) being verified by an F-test. Design Expert software 8.0.6 (State-Ease Inc., Minneapolis, MN, USA) was used for the experimental design and analysis.

Characterization of the final extract

The extract obtained under optimal extraction conditions for HAE and UAE were characterized in terms of their phenolic compounds profile and its bioactivities, more specifically the antioxidant and antimicrobial activities.

- The antioxidant activity was assessed through two assays: the ability of the extracts to inhibit oxidative haemolysis (OxHLIA) in sheep erythrocytes (Takebayashi et al., 2012), as well as their ability to inhibit lipid peroxidation (TBARS) in porcine brain homogenates (Barros et al., 2010). For the first one, the extract concentration needed to delay haemolysis by 60 minutes was calculated using the Ht_{50} values derived from the haemolytic curves at varying extract concentrations. The outcomes were represented as IC_{50} values (µg/mL), indicating the extract concentration necessary to maintain 50% of the erythrocyte population intact for 60 minutes. Moreover, the ability to prevent TBARS formation was evaluated using porcine brain cells as oxidizable substrates, with results reported as IC_{50} values (µg/mL), reflecting the extract concentration required to achieve 50% antioxidant activity. Trolox was utilized as a positive control in both assays.
- The antimicrobial activity was evaluated against two fungal and eight bacterial strains relevant to food contamination. The bacterial strains included Gram-positive (*Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*) and Gram-negative (*Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Yersinia enterocolitica*). The fungal strains tested were *Aspergillus fumigatus* and *Aspergillus brasiliensis*. Antimicrobial activity was evaluated according to the method described by Heleno et al. (2013), with both minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) or fungicidal (MFC) concentrations being determined. Streptomycin and ampicillin served as positive controls for bacteria, while ketoconazole and bifonazole were used as positive controls for fungi. Results were expressed in mg/mL.
- The phenolic compounds profile was analyzed using high-performance liquid chromatography coupled with a diode array detector and electrospray ionization mass spectrometry (HPLC-DAD-ESI/MS), following the protocol outlined by Barros et al. (2013). Chromatographic data were obtained with a Dionex Ultimate 3000 UPLC system

(Thermo Scientific, San Jose, CA, USA), equipped with a diode array detector (set at 280 and 370 nm) and an electrospray ionization mass spectrometer (Linear Ion Trap LTQ XL, Thermo Finnigan, San Jose, CA, USA), operating in negative ion mode. Separation was carried out on a Waters Spherisorb S3 ODS-2 C18 column (3 µm, 4.6 mm x 150 mm, Waters, Milford, MA, USA) maintained at 35 °C. Compound identification was performed by comparing retention times and spectra with reference standards or literature values, while quantification was based on equations provided in the table footnotes. Results were expressed in mg/g of extract.

Pumpkin peel extract incorporation in pumpkin pulp formulations

Pumpkin peel extract rich in preservative compounds (PE), obtained under optimal extraction conditions, was evaluated as an alternative to the artificial preservative potassium sorbate in pumpkin pulp formulation. Formulations with different (and without) preservative ingredients were evaluated and compared to test the efficiency of the peel extract. The comparison between the formulations was carried out by evaluating their quality over their shelf life. Evaluations were made at 0 (day of pulp preparation), 7, 14, 21, 30 (currently the "best before" date of the traditional formulation), and 45 days of storage to assess the efficacy of the different preservatives in preserving the pumpkin pulp.

Natural preservative extracts preparation

Two different natural extracts were tested as alternatives to the artificial preservative potassium sorbate, obtained as described below:

- Pumpkin peel extract (PE): It was obtained under optimal extraction conditions achieved in the previously described optimization study (see topic *Optimization study*), that is, by macerating the peels in water at a ratio of 1 g/40 mL for 84 minutes at 30 °C with constant agitation. The mixture was filtered through filter paper and dried using a spray dryer (BUCHI, B-191, Laboratory-Techniques LTD, Flawil, Switzerland) with maltodextrin as the carrier agent (Freixo et al., 2016). The drying conditions and characterization of the extract after drying are described in the supplementary material (Supplementary Data 2).
- Pumpkin seed extract: A mixture of pumpkin seeds from three local varieties cultivated at the University of Thessaly in Greece, previously freeze-dried and ground into powder, was extracted by maceration for 1 hour, twice, with a solid/liquid ratio of 1 g/30 mL, following the standard methodology of the group, previously described by Leichtweis et al. (2022). These three varieties used, more specifically the local varieties of "Nychaki", "Leuka Melitis" and the "Lakonia" region, were mixed in the respective proportion of

1.8/1/1.3. The characterization in terms of bioactivities of the extract is presented in the supplementary material (Supplementary Data 3).

Preparation of pulp formulations

All pulp formulations were prepared using mashed pumpkin pulp. The additional components included corn starch, beta-carotene colorant, citric acid, sodium citrate, and a preservative. The concentrations of each additional component were kept constant, except for the type of preservative used, which varied as described below.

For each formulation, the ingredients were carefully mixed using a mixer and then pasteurized in a double-jacketed heating tank with an agitation paddle. The mixture was heated to 90 °C, held for 3 minutes, and then packaged at > 76 °C in a sterile airtight container. Each batch was stored in triplicate and under refrigeration at 5 ± 2 °C, for subsequent analysis.

To establish the favorable concentration for incorporating PE into the pumpkin pulp formulation, two sequential tests were conducted. First, in Test I, pumpkin peel extracts were evaluated at two different concentrations within a predetermined range from small-scale preliminary tests: the minimum and maximum possible concentrations. Test II included the possibly best concentration of PE, based on Test I, while pumpkin seed extract was also incorporated for comparison purposes. The specific procedures for these tests were as follows:

Test I:

- Control formulation (C): pumpkin pulp (2737.5 g) + mix of ingredients (natural beta-carotene, starch, citric acid and citrate; 262.5 g).
- Traditional formulation (PS): pumpkin pulp (2736.0 g) + mix of ingredients (natural beta-carotene, starch, citric acid, citrate and potassium sorbate; 264.0 g).
- Pumpkin peel extract at 5 g/kg (Peel 5): pumpkin pulp (2722.5 g) + mix of ingredients (natural beta-carotene, starch, citric acid and citrate; 262.5 g) + peel ingredient (15.0 g).
- Pumpkin peel extract at 15 g/kg (Peel 15): pumpkin pulp (2692.5 g) + mix of ingredients (natural beta-carotene, starch, citric acid and citrate; 262.5 g) + peel ingredient (45.0 g).

Test II:

- Control formulation (C): pumpkin pulp (2737.5 g) + mix of ingredients (natural beta-carotene, starch, citric acid and citrate; 262.5 g).
- Traditional formulation (PS): pumpkin pulp (2736.0 g) + mix of ingredients (natural beta-carotene, starch, citric acid, citrate and potassium sorbate; 264.0 g).

- Pumpkin peel extract at 10 g/kg (Peel 10): pumpkin pulp (2706.8 g) + mix of ingredients (natural beta-carotene, starch, citric acid, citrate and 50% of potassium sorbate; 263.2 g) + peel ingredient (30.0 g).
- Pumpkin seed extract at 10g/kg (Seeds 10): pumpkin pulp (2707.5 g) + mix of ingredients (natural beta-carotene, starch, citric acid and citrate; 262.5 g) + seeds extract (30.0 g).

Proximate composition

The nutritional evaluation of the studied formulations was conducted following the official methods of AOAC (AOAC, 2016). The analysis included the determination of moisture, ash, protein, lipid, carbohydrate contents, and energetic value. Moisture content was determined by drying samples in an oven at 105 °C until a constant weight was achieved. Ash content was quantified by incineration at 550 °C. Protein content was measured using the Kjeldahl method, while lipids were extracted and quantified using the Soxhlet method, with petroleum ether as the solvent. Carbohydrate content was calculated by difference, subtracting the sum of the other components from the total. The energetic value (kcal) was calculated using the Atwater conversion factors, applying 4 kcal/g for protein and carbohydrates and 9 kcal/g for lipids. All analyses were performed in triplicate at both the initial (0 days, T0) and final (45 days, T45) storage time and expressed in g/100 g of fresh weight (fw).

Chemical composition

The analysis of soluble sugars was conducted using high-performance liquid chromatography (HPLC) coupled with a refractive index detector. Specifically, an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD, USA), and an RI detector (Knauer Smartline 2300) was utilized, following a previously established method (Barros et al., 2013). A Eurospher 100-5 NH2 column (4.6 x 250 mm, 5 µm, Knauer) operating at 30 °C (7971 R Grace oven) was employed. Melezitose was used as an internal standard for the quantification procedure, and compounds were identified by chromatographic comparison with authentic standards of D(-)-fructose, D(+)-sucrose, D(+)-glucose, D(+)-trehalose, and D(+)-raffinose pentahydrate. Analyses were performed in triplicate at 0 and 45 days of storage and results were expressed in g/100 g dry weight (dw).

Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID), using a DANI model GC 1000 instrument and a Macherey-Nagel column (30 m x 0.32 mm ID x 0.25 µm df), following the methodology described by

Pereira et al. (2011). Identification and quantification were performed by comparing the retention times of the detected peaks with those of the commercial FAME standard mixture (FAME reference standard mixture, standard 47885-U, Sigma-Aldrich, St. Louis, MO, USA). Analyses were performed in triplicate at 0 and 45 days of storage and fatty acids content was presented as relative percentage (%).

Color evaluation

To evaluate the color of the pumpkin pulp formulations at the day of preparation and during storage, color measurements were performed using a portable colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The color values were recorded as L* (lightness), a* (green to red), and b* (blue to yellow). The CIE values were converted into RGB values using a conversion program (e.g. <http://www.easyrgb.com/en/convert.php>), for better color visualization. All measurements were conducted in triplicate for each assay (n = 9) at 0, 7, 14, 21, 30, and 45 days of storage.

Microbial load

The microbial load was evaluated for aerobic plate count (total viable count; ISO 4833-1:2013; ISO, 2013) in PCA, for coliforms (ISO 4832:2006; ISO, 2006) in VRBLA and for Yeasts and Moulds (ISO 21527-1:2008; ISO, 2008) in DRBC as previously described by Molina et al. (2019) and detailed in supplementary material (Supplementary Data 4).
Samples were evaluated at 0, 21, and 45 days of storage, in triplicate for each assay. For Test II, the samples were also evaluated after 7 and 14 days of opening. For this, the sample was briefly handled, simulating the handling of the product in domestic use, stored under refrigeration (5 ± 2 °C) for 7 days, evaluated, and the process was repeated for another 7 days.

Statistical analysis

All samples were tested in triplicate and the results were expressed as mean ± standard deviation. For the PE characterization, a Student's t-test was employed for comparisons between two groups, while one-way analysis of variance (ANOVA) was used for comparisons involving more than two groups. Prior to these analyses, the normality of data distribution and homogeneity of variances were assessed using the Shapiro-Wilk test and Levene's test, respectively. For datasets meeting homoscedasticity ($p > 0.05$), Tukey's honestly significant difference (HSD) test was used, whereas Tamhane's T2 multiple comparison test was used for heteroscedastic data.

For the evaluation of the different pumpkin formulations, samples were analyzed

through a two-way analysis of variance (ANOVA) with type III sums of squares, after verifying homoscedasticity through Levene's test. In terms of the statistical tools used, for analyses where only two storage times were tested, the post hoc tests of the preservative type (PT) were Tukey's test (TT) (for homoscedastic samples) or Tamhane's T2 (T2) test (for heteroscedastic samples), while for storage time (ST) the Student's t-test was implemented. For color analyses (where six storage times were used), only TT and T2 tests were used in post-hoc, when the samples were either homoscedastic or heteroscedastic. By employing a two-way ANOVA, it was possible to verify the influence of each factor (ST and PT) independently. If a significant (p <0.05) interaction (ST × PT) was detected, the potential tendencies were extracted from the plotted estimated marginal means (EMM). On the other hand, if there was no significant interaction (p > 0.05), each factor was classified independently using the post hoc tests described above.

All statistical analyses were performed using a p-value of 0.05 and IBM SPSS software, version 29 (IBM Corp, Armonk, NY, USA).

4.2.3. Results and discussion

Optimization study

Optimized responses and conditions by RSM

The extraction of bioactive compounds from fruit peels was optimized by comparing the two different methods of heat-assisted and ultrasound-assisted extraction (UAE and HAE, respectively). For each technique, the optimization process was conducted through seventeen individual extractions, taking into account three independent variables. The tested conditions determined by the Box–Behnken experimental design for time (X_1 or t), temperature/power (X_2 or T/P), and ethanol percentage (X_3 or EtOH), as well as the respective results used to optimize these dependent variables for each extraction technique, following the ranges for the independent variables determined in a previous study (Leichtweis, Molina, Petropoulos, et al., 2023), are detailed in Table A of the supplementary material.

Results were analyzed using a quadratic **Equation 13**, after applying the response surface methodology. To construct the mathematical models, a confidence interval of p = 0.05 was employed, with outliers being excluded, and the final equations describing the evaluated responses incorporated only significant terms which are presented in respective **Equations 14** to **16**. The parametric values are expressed according to the codification criteria of the experimental design, allowing for comparative analysis and interpretation of the numerical values' influence on the responses. To achieve the objective of maximizing extraction yield in terms of dry residue and total phenols, the independent variables were adjusted to optimize the response for each extraction method assessed. Optimal extraction

conditions, resulting in the best individual and overall response values, were identified and discussed in the context of each technique. Additionally, 3D surface graphs are plotted to enhance the understanding of the parametric values' impact on the responses. Each figure illustrates the interaction between two of the three independent variables, with the third variable held constant (**Table 47** and **Table 48**).

Heat-assisted extraction

The R_1 response of fruit peels extracted by HAE, rendered a quadratic function with a significant model, a non-significant lack of fit, and an adjusted R^2 of 0.758, as shown in the coded **Equation 14**. Thus, the optimal values that maximized the amount of dry residue were set at 114 min, 76 ºC and 20% of ethanol, which are previewed to render 1.60 g/100 g of dry residue. In **Table 47**, the first row shows the 3D charts of R_1, with ethanol percentage being the defining factor. These findings suggest that prolonged extraction times and higher temperatures may enhance the solubility and extraction of the desired components. However, the ethanol concentration needs to be carefully controlled, as excessively high percentages can negatively impact the yield. Low amounts of ethanol seemed to promote extractability. These three parameters and their interactions also caused significant variability in the extraction of bioactive compounds from the peel, endocarp and seeds of *Cucurbita maxima* (Mansour, Falleh, Hammami, et al., 2023). While the R_2 (reducing power) response could not be optimized due to a significant lack of fit ($p > 0.05$).

Regarding R_3, optimization achieved a significant two-factor interaction (2FI) model after the removal of 2 outliers and an R^2 of 0.775, being the coded equation as described in **Equation 15**. In terms of the optimal points to maximize TF, they are identified at 19 minutes, 77 ºC and 72% of ethanol, rendering 187.5 mg/g of total phenols. In the second row of **Table 47**, the 3D charts of *R3* show that time seemed to be the most important factor for this response, meaning that lower extraction times seemed to show higher extraction yield of total phenolics. This can be attributed to the fact that shorter times may reduce the thermal degradation of heat-sensitive polyphenols, thereby facilitating more efficient extraction of antioxidant compounds (Antony & Farid, 2022).

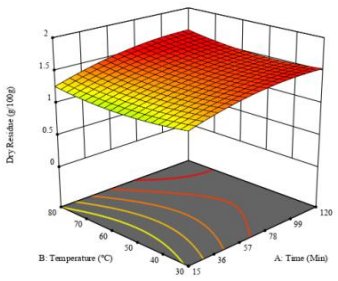
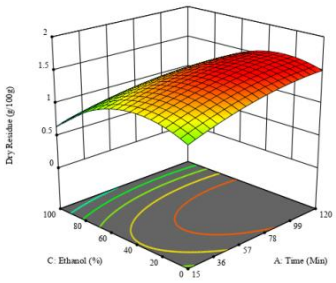
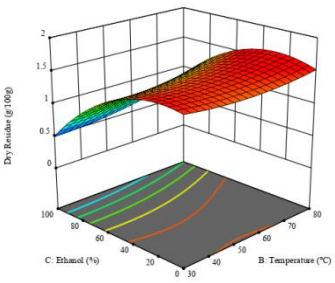
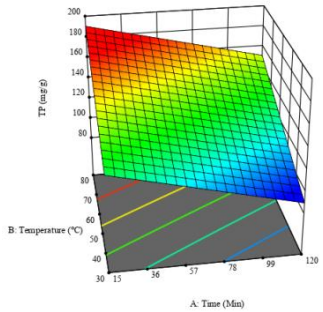
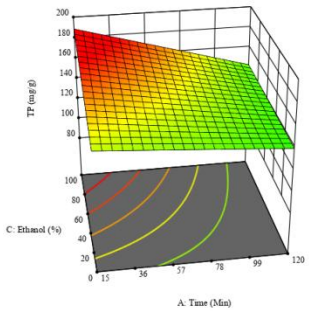
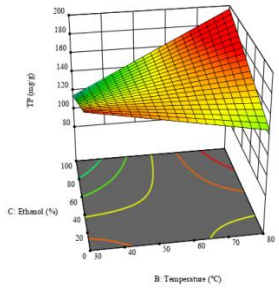
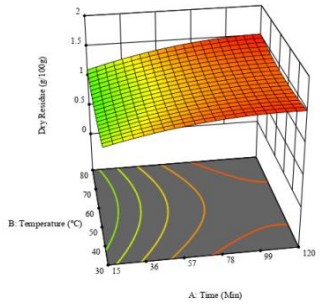
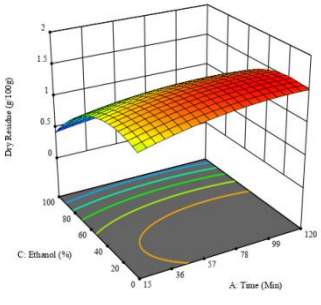
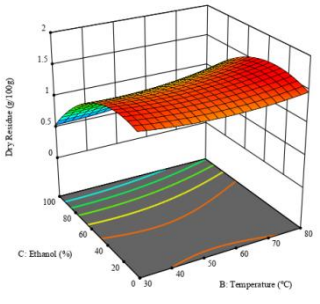
The desirability 3D charts are shown in the last row of **Table 47**, being the optimal point at 84 minutes, 30 ºC and 0% ethanol, rendering 1.49 g/100 g of dry residue and 169 mg/g of total phenols. Desirability is an optimal measure that shows the points in which both responses are maximized. These values represent a balance between time, temperature, and ethanol concentration, promoting effective solubilization of polyphenols while minimizing degradation. It is noteworthy that the use very low ethanol, implying extraction only with water, represents a significant advancement. Although this finding is

in contrast with the fact that pure water does not facilitate the efficient extraction of phenolic compounds (Mansour, Falleh, Nefzi, et al., 2023), other studies corroborate this effect. For example, Kulczyński et al. (2020) obtained higher total phenolic contents from *C. maxima*, in the aqueous and methanolic-aqueous extracts, compared to other solvents such as acetone, ethyl acetate, ethanol and their mixture. In the study of Añibarro-Ortega et al. (2021) and Kim et al. (2014), it was also found that pure water was the most suitable solvent for the extraction of aloesin. Employing water as the exclusive solvent not only reduces the environmental footprint of the extraction process, making it more sustainable and environmentally friendly, but also offers specific benefits for food applications. This approach enhances the sustainability of the process and may increase acceptance of the final product by both consumers and the food industry.

Dry residue $(R_1) = 1.36 + 0.1203X_1 + 0.0748X_2 - 0.3590X_3 + 0.0267X_1X_2 - 0.0635X_1X_3 + 0.0878X_2X_3 - 0.0946X_1^2 + 0.0817X_2^2 - 0.37021X_3^2$ **14**

Total phenolics $(R_3) = 164.64 - 14.75X_1 + 13.29X_2 - 9.71X_3 + 0.5X_1X_2 - 8.5X_1X_3 + 28.07X_2X_3$ **15**

Table 47. 3D response plots of the heat-assisted extraction (HAE) of fruit peels at their optimal values.

	Temp. vs. Time	**Solvent vs. Time**	**Solvent vs. Temperature**
R_1 – DR			
R_3 – TP			
Desirability			

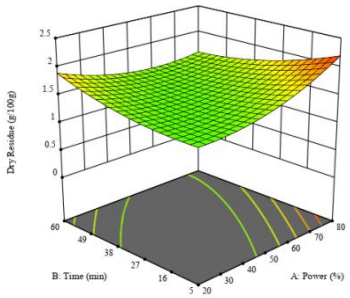
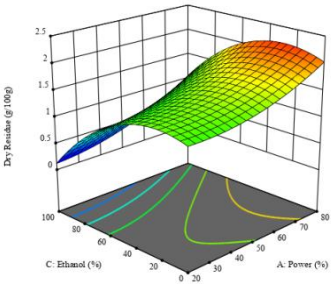
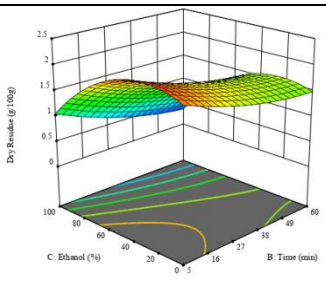
Ultrasound-assisted extraction

For R_1, the residue of fruit peels extracted through UAE, the analysis of the seventeen runs rendered a quadratic function with a significant model, a non-significant lack of fit, and an adjusted R^2 of 0.6745. The coded equation is described in **Equation 16**. The interactions and quadratic terms indicate a complex optimization process, with ethanol percentage demonstrating significant importance, while ultrasonic power emerged as a critical factor. This led to an optimal point at 400 W, 5 minutes and 27% of ethanol, rendering 2.29 g/100 g of dry residue. The 3D charts can be seen in **Table 48**, confirming the higher influence of ethanol percentage in the total yield of dry residue. The concentration of ethanol in the solvent also affected the extraction of total phenolics from *Cucurbita pepo* L. pulp and peel (Oufighou et al., 2024) and *C. maxima* by-products (Mansour, Falleh, Hammami, et al., 2023).

Concerning R_2 and R_3, the results supported only linear models, resulting in unsatisfactory R^2 values, and thus these responses were not optimized. The challenge in optimizing these responses suggests that they may be less influenced by the experimental factors examined than R_1. This could be attributed to the complex nature of the extraction mechanisms and the interactions between experimental factors and the bioactive compounds in pumpkin peels. This highlights the potential need for further adjustments to the extraction protocol or the consideration of additional factors not included in this study. For example, in the study of the extraction of phenolic compounds from pumpkins and peaches, the parameters of extraction temperatures, ultrasonic power levels and extraction times were considered (Altemimi et al., 2016), while to obtain pumpkin extract rich in carotenoids, the parameters of solid-solvent ratio, temperature, and ultrasonic time were considered (Sebdani & Abbasi, 2023). Moreover, in a more in-depth study four parameters were simultaneously optimized using a four-factor, three-level Box-Behnken design (BBD) to enhance phenolic compound content and antioxidant activity in the (UAE) from *Lycium ruthenicum* Murr. fruit (Chen et al., 2018). Therefore, future research could benefit from exploring alternative extraction techniques or incorporating other variables that might more effectively influence PR and TP.

$$\text{Dry residue } (R_1) = 1.31 + 0.1435X_1 - 0.0576X_2 - 0.5979X_3 - 0.2568X_1X_2 + 0.0667X_1X_3 - 0.0430X_2X_3 + 0.1913X_1^2 - 0.1241X_2^2 - 0.5410X_3^2 \quad \textbf{16}$$

Table 48. 3D response plots of the ultrasound-assisted extraction (UAE) of fruit peels at optimal values.

	Time vs. Power	Solvent vs. Power	Solvent vs. Time
R_1 – DR			

Characterization of the extract obtained at the optimum conditions

To validate the predictive mathematical model of the studied process, experimental validation was conducted using the estimated optimal extraction conditions. This allowed for the verification of consistency between the predicted and actual results for the responses considered in the optimization study (DR and TP; data not shown).

Furthermore, the extract obtained under globally optimal conditions by HAE (PE), due to its advantages discussed previously, was selected for the continuation of the present study. Therefore, it was subsequently characterized for its phenolic compound profile, antioxidant activity, and antimicrobial properties to evaluate its potential use as a food preservative.

Extract bioactivities

Regarding the antioxidant activity of the extract, it was evaluated through two cell-based assays: the peroxidation of porcine brain homogenates by TBARS and the anti-haemolytic activity using sheep erythrocytes (OxHLIA). In both methods, the extracts exhibited significant antioxidant activity, although lower ($p < 0.05$) than the positive control Trolox. In TBARS assay, the IC_{50} value was 1152 ± 43 µg/mL (Trolox IC_{50} = 139 ± 5 µg/mL), while for the OxHLIA assay, the respective value at 60 min ($IC_{50(60\ min)}$) was 423 ± 29µg/mL (Trolox IC_{50} = 21.8 ± 0.2 µg/mL).

The antimicrobial activity of the extract was tested against three Gram-positive and five Gram-negative bacteria strains, as well as against two fungi, all of interest in food contamination. Although the extract did not demonstrate efficacy in killing any of the tested microorganisms (as indicated by MBC/MIC values) up to the tested concentration of 10 mg/mL, it significantly inhibited the growth of 7 out of the 10 strains evaluated. Particularly noteworthy, the extract showed promising results by inhibiting the growth of *Salmonella enterica* and *Yersinia enterocolitica* at a concentration of 2.5 mg/mL; *Escherichia coli*, *Listeria monocytogenes*, and *Aspergillus fumigatus* at 5 mg/mL; and *Pseudomonas aeruginosa* and *Aspergillus brasiliensis* at 10 mg/mL (for more detailed

results, please refer to Table F in the supplementary material).

Both the antioxidant capacity and antimicrobial activity results were aligned with literature reports, which suggested significant bioactivities for the peels and other parts of various pumpkin species (Asif et al., 2017; Hussain et al., 2022; Hussain, Kausar, Din, Murtaza, Jamil, Noreen, & Iqbal, 2021; Hussain, Kausar, Din, Murtaza, Jamil, Noreen, Rehman, et al., 2021; Mala & Kurian, 2016; Mansour, Falleh, Nefzi, et al., 2023; Mohsen & Abas, 2024; Pinna et al., 2023). In particular, the present results demonstrated an increase in the bioactivities of the optimized extract compared to those obtained by standard extraction in the previous study of our group (Leichtweis et al., 2022). This enhancement of bioactive properties of the extract, combined with the elimination of ethanol solvent use and the reduction in energy and processing time during the extraction, highlights the significance and efficiency of optimizing extraction conditions. The bioactivities of pumpkin peel were also recently enhanced in the studies of Gavril et al. (2024) and Oufighou et al. (2024), who optimized extraction parameters using UAE and microwave-assisted extraction (MAE), respectively. Moreover, Mansour, Falleh, Hammami, et al. (2023) also reported that pumpkin peels stood out among other by-products due to their higher levels of TP and better antiradical capacity, reaching 73% DPPH inhibition.

Phenolic profile of the extract

The compounds detected by HPLC-DAD-ESI/MS (**Table 49**) were tentatively identified based on previous characterizations of extracts from Portuguese, Algerian, and Greek pumpkin by-products (Leichtweis et al., 2022; Leichtweis, Molina, Petropoulos, et al., 2023). Briefly, the flavonoid compounds (peaks 2 to 11) were tentatively identified by comparison with data from the literature, while (-)-Epicatechin ($[M-H]^-$ at m/z 289, peak 1) was tentatively identified through comparison with an available standard. **Table 49** presents the information regarding the compounds identified in the analyzed extract is presented, including retention times (Rt), the maximum wavelengths (λmax) recorded in nanometres, the molecular mass values of the deprotonated ions ($[M-H]^-$), as well as the fragment ions observed in tandem mass spectrometry (MS^2). The tentative identification of the compounds is also listed in the same table, along with the detected amounts (Leichtweis, Molina, Dias, et al., 2023).

The phenolic profile found in PE, represented in Figure A of the supplementary material, indicates a high antioxidant potential, with a predominance of flavonoids, particularly kaempferol and isorhamnetin glycosides, which are known for their antioxidant and antimicrobial activities (Yuan et al., 2024). These compounds are present in concentrations ranging from 0.4432 mg/g to 0.551 mg/g, representing a significant

fraction of total phenolic compounds, suggesting a high bioactive potential of the obtained extract. Although epicatechin, a flavan-3-ol from a subclass of flavonoids, was found at a relatively low concentration (0.31 mg/g), it may still contribute significantly to the overall antioxidant properties of the extract. Moreover, the low total flavan-3-ols content suggests that this is not the predominant class of flavonoids; however, their well-known efficiency in scavenging free radicals underscores their important role in the extract's bioactivity (Slavova-Kazakova et al., 2021; Yuan et al., 2024).

In a previous study where the extracts were obtained using a standard extraction protocol with 80% ethanol (Leichtweis et al., 2022), the fruit peels of butternut squash showed epicatechin as the major compound, representing half of the total phenolic compounds content, similar to the peels of the kabocha squash fruit. In contrast, the optimized extracts obtained from Greek landraces of pumpkin presented a phenolic profile similar to the present study, with a lower content of epicatechin and a higher prevalence of other flavonoids, also formed by kaempferol and isorhamnetin glycosides (Leichtweis, Molina, Petropoulos, et al., 2023). This suggests that the optimization process, with reduced ethanol in the solution, influences the selective extraction of phenolic compounds, favoring the extraction of compounds with different polarities (Kulczyński et al., 2020; Slavova-Kazakova et al., 2021).

Several studies report the wide presence of flavonoids in pumpkin (Altemimi et al., 2015; Hussain et al., 2022; Hussain, Kausar, Din, Murtaza, Jamil, Noreen, Rehman, et al., 2021; Iswaldi et al., 2013; Mansour, Falleh, Hammami, et al., 2023; Mansour, Falleh, Nefzi, et al., 2023). In one of these studies, nine phenolic compounds were identified in the peels of *C. maxima*, with epicatechin being the most prevalent at 4.243 ± 0.51 mg/g (Mansour, Falleh, Hammami, et al., 2023). This compound was also found in the peel extract of Bejaoui landrace at a concentration of 5.00 ± 0.32 mg/g, alongside kaempferol at 5.01 ± 0.12 mg/g (Mansour, Falleh, Nefzi, et al., 2023). These authors explained that in addition to extraction conditions, composition of the extract is also influenced by factors such as the genotype and growing conditions (e.g., soil, climate, and agricultural practices), since these variables can significantly affect the concentration and presence of bioactive compounds in pumpkin fruit parts (Leichtweis, Molina, Dias, et al., 2023).

Table 49. Characterization and quantification of phenolic compounds detected by HPLC-DAD-ESI/MS under the optimal extraction conditions.

Peak	Rt (min)	λmax (nm)	[M-H]⁻ (*m/z*)	MS² (*m/z*)	Tentative identification	Quantification (mg/g of extract)
1	9.51	280	289	245(100), 205(45)	(-)-Epicatechin	0.31 ± 0.01 [f]
2	16.23	344	739	285(100)	Kaempferol-*O*-dideoxyhexosyl-hexoside	0.4837 ± 0.0006 [b,c]
3	16.51	344	739	285(100)	Kaempferol-*O*-dideoxyhexosyl-hexoside	0.551 ± 0.001 [a]
4	16.92	354	769	315(100)	Isorhamnetin-*O*-dideoxyhexosyl-hexoside	0.5475 ± 0.0002 [a]
5	17.29	354	769	315(100)	Isorhamnetin-*O*-dideoxyhexosyl-hexoside	0.4602 ± 0.0003 [d]
6	17.99	348	593	285(100)	Kaempferol-*O*-deoxyhexosyl-hexoside	0.4438 ± 0.0006 [e]
7	18.49	348	593	285(100)	Kaempferol-*O*-deoxyhexosyl-hexoside	0.4854 ± 0.0002 [b]
8	18.97	365	623	315(100)	Isorhamnetin-*O*-deoxyhexosyl-hexoside	0.4783 ± 0.0003 [b,c]
9	19.66	348	593	285(100)	Kaempferol-*O*-deoxyhexosyl-hexoside	0.4433 ± 0.0004 [e]
10	21.02	348	593	285(100)	Kaempferol-*O*-deoxyhexosyl-hexoside	0.4432 ± 0.0008 [e]
11	21.97	365	623	315(100)	Isorhamnetin-*O*-deoxyhexosyl-hexoside	0.47591 ± 0.00006 [c]
Total Flavan-3-ols						0.31 ± 0.01 [c]
Total Flavonoids						4.812 ± 0.002 [b]
Total Phenolic Compounds						5.12 ± 0.01 [a]

Calibration curves used for quantification: (-)-catequin (y = 84,950x - 23,200; R^2 = 0.999; LOD = 0.17 g/mL; LOQ = 0.68 g/mL; peak 1) and quercetin 3-*O*-glucoside (y = 34,843x - 160,173; R^2 = 0.9998; LOD = 0.21 g/mL; LOQ = 0.71 g/mL; peaks 2 to 11). ANOVA analysis – In each column, different letters mean significant differences ($p < 0.05$).

Pumpkin pulp formulations

Results regarding Test I

The optimized extract (PE) obtained in the present study was tested as a natural preservative ingredient to replace traditional potassium sorbate in a pumpkin pulp-based formulation. Initially, it was necessary to determine a feasible concentration range for PE incorporation. This step was carried out empirically on a small laboratory scale (100 g batches of fruit pulp). Based on changes in parameters such as color, texture, and visual preservation (data not shown), it was proposed to incorporate PE at a minimum concentration of 5 g/kg and a maximum concentration of 15 g/kg (g of PE per kg of final formulation). Test I was conducted on a pilot industrial scale with 3 kg batches, involving four formulations: two with the respective concentrations of PE, one traditional with potassium sorbate, and a control without any preservative.

The formulations were then evaluated and compared based on their proximate composition, color, and microbial load over a 45-day storage period. The traditionally marketed product has a shelf life of 30 days, which was proposed to be extended for more 15 days in this study. For the proximate composition, formulations were evaluated at the production day (T0) and at the end of the extended shelf life (T45), in terms of moisture, crude proteins, crude fat, ash, carbohydrates and energetic value. Due to its susceptibility to degradation, color was meticulously evaluated at six specific time points: on days 0, 7, 14, 21, 30, and 45 of storage.

The data was subjected to a two-factor statistical analysis to assess the effects of

storage time, as well as the influence of preservative replacement in the formulations. The following tables were divided into two sections, the upper section refers to the Storage Time (ST), while the lower section corresponds to the Preservative Type (PT). The sectioning of the tables results from the two-way ANOVA was used to understand the influence of each factor individually, so the upper parts contain all the ST values within the average intervals and the lower part contains all the PT values. This analysis allows a much more reliable interpretation of the results, as each factor can be analyzed independently of the influence of the other. The interaction between the two factors, if present, is demonstrated by ST × FT < 0.05 allowing only general conclusions to be drawn from the Estimated Marginal Means plots (EMM) and the partial eta squared (PES) that allows, in some cases, to understand which factor contributed the most to the final outcome; however, if ST × FT > 0.05, each factor can, in some cases, be independently classified using Tukey's test (TT), Tamhane's T2 test (T2) or Student's t-test (if only two levels are tested). In this statistical analysis, the standard deviation should not be considered as a measure of precision, but rather as a range of values.

Table 50 shows the proximate composition of the different pumpkin formulations, with moisture and carbohydrates being the most abundant components, in agreement with the findings of previous studies (Adnan et al., 2017; Leichtweis, Molina, Dias, et al., 2023). Comparing the different formulations through the two-way ANOVA, moisture, proteins, carbohydrates, and energetic value, showed a significant interaction among ST and PT, not allowing an independent classification. From the EMM, no tendencies could be extracted for these assays. Still, through PES, specific influences for each of the assays with significant interaction could be observed, namely that for moisture, protein, carbohydrates, and energy values and their variation, the biggest contribution for the outcomes was the incorporation of different preservatives and not storage time. Crude fat and ash contents could be classified independently, and thus, for the former, the lowest amounts of fat were recorded in the samples incorporated with PE at 5 g/kg, whereas no significant differences were found between the other samples. No differences were found in fat during the 45 days of storage time. Regarding ash, no differences were also found during storage time while the sample with PE incorporation at 15 g/kg was the one with the highest amount of ash, followed by the incorporation of PE at 5 g/kg, with no significant differences found between the rest of samples. Overall, very few differences were found in the proximate composition of the pumpkin formulations, especially during the 45 days of storage, whereas the incorporation of PE was the factor that slightly affected proximate composition of the tested formulations.

Table 50. Proximate composition (in g/100g fw) and energetic value (in kcal) of the different pumpkin pulp formulations, referring to Test I.

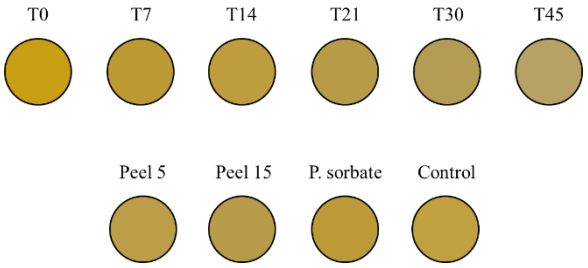
		Moisture	Proteins	Crude Fat	Carbohydrates	Ash	kcal
Storage Time (ST)	0 Days	82.3±0.4	1.3±0.1	0.10±0.03	15.7±0.2	0.7±0.1	69±1
	45 Days	82.1±0.6	1.26±0.03	0.10±0.04	15.7±0.5	0.7±0.1	69±2
***p*-value (n=12)**	Student's t-test	0.042	0.744	0.766	0.363	0.462	0.388
Preservative Type (PT)	Peel 5	82.5±0.3	1.10±0.04	0.06±0.01 ^{b}	15.5±0.3	0.69±0.01 ^{b}	67±1
	Peel 15	81.4±0.3	1.39±0.05	0.13±0.03 ^{a}	16.2±0.3	0.88±0.02 ^{a}	71±1
	Potassium Sorbate	82.4±0.2	1.28±0.07	0.11±0.02 ^{a}	15.6±0.3	0.62±0.01 ^{c}	68.4±0.9
	Control	82.46±0.05	1.24±0.04	0.11±0.03 ^{a}	15.58±0.04	0.61±0.02 ^{c}	68.3±0.3
***p*-value (n=6)**	Tukey's test	<0.001	<0.001	0.002	<0.001	<0.001	<0.001
ST×PT (n=24)	*p*-value	0.006	0.004	0.611	0.032	0.895	0.020

If present, each letter in each column represents a significant difference among samples, obtained from Tukey's or Tamhane's T2 test. The asterisk (*) represents a statistically significant difference by means of Student's t-test. All assays were performed using a significance of 0.05.

Table 51 shows the color coordinates of the different pumpkin formulations, including lightness (L*), red-greenness (a*) and blue-yellowness (b*). Additionally, the coordinates converted to RGB colors are shown in the lower section of the table for better visualization. As can be seen for all three-color coordinates, there was a significant interaction among storage time and the different preservatives, not allowing a classification of each factor individually. Thus, both factors contributed to the final outcome, while some tendencies could be extracted from the estimated marginal means (EMM) and partial eta squared (PES). Therefore, although lightness (L*) did not render any conclusions, in terms of the red-greenness and blue-yellowness, the factor with the highest contribution to the obtained color was storage duration. This can also be seen in **Figure 12**.

Table 51. Color of the different pumpkin pulp formulations, referring to Test I.

		L*	a*	b*
Storage Time (ST)	0 Days	67±2	4±2	68±9
	7 Days	65±3	2.5±0.8	57±4
	14 Days	66±2	1.6±0.8	53±4
	21 Days	65±2	0.8±0.7	47±5
	30 Days	65±3	-0.2±0.8	40±8
	45 Days	67±2	-1.1±0.7	35±6
***p*-value (n=36)**	Tukey's test	<0.001	<0.001	<0.001
Preservative Type (PT)	Peel 5	66±3	2±2	48±11
	Peel 15	65±3	1±2	46±14
	Potassium Sorbate	65±2	2±2	54±10
	Control	67±2	1±2	53±13
***p*-value (n=54)**	Tukey's test	<0.001	<0.001	<0.001
ST×PT (n=216)	*p*-value	<0.001	0.001	<0.001



If present, each letter in each column represents a significant difference among samples, obtained from Tukey's or Tamhane's T2 test. The asterisk (*) represents a statistically significant difference by means of Student's t-test. All assays were performed using a significance of 0.05.

Figure 12 shows the EMM plots for a* and b* coordinates of the samples studied regarding the effect of storage time. Each color in the plots represents one storage time, showing that when storage time increases, the values decrease, meaning there is a slight shift towards green in a* and blue in b* coordinates. The comparison of the different samples regarding the incorporation of preservatives also shows that there is a neglectable difference among each sample in both EMM plots, with the variation being smaller than the standard deviation in most cases.

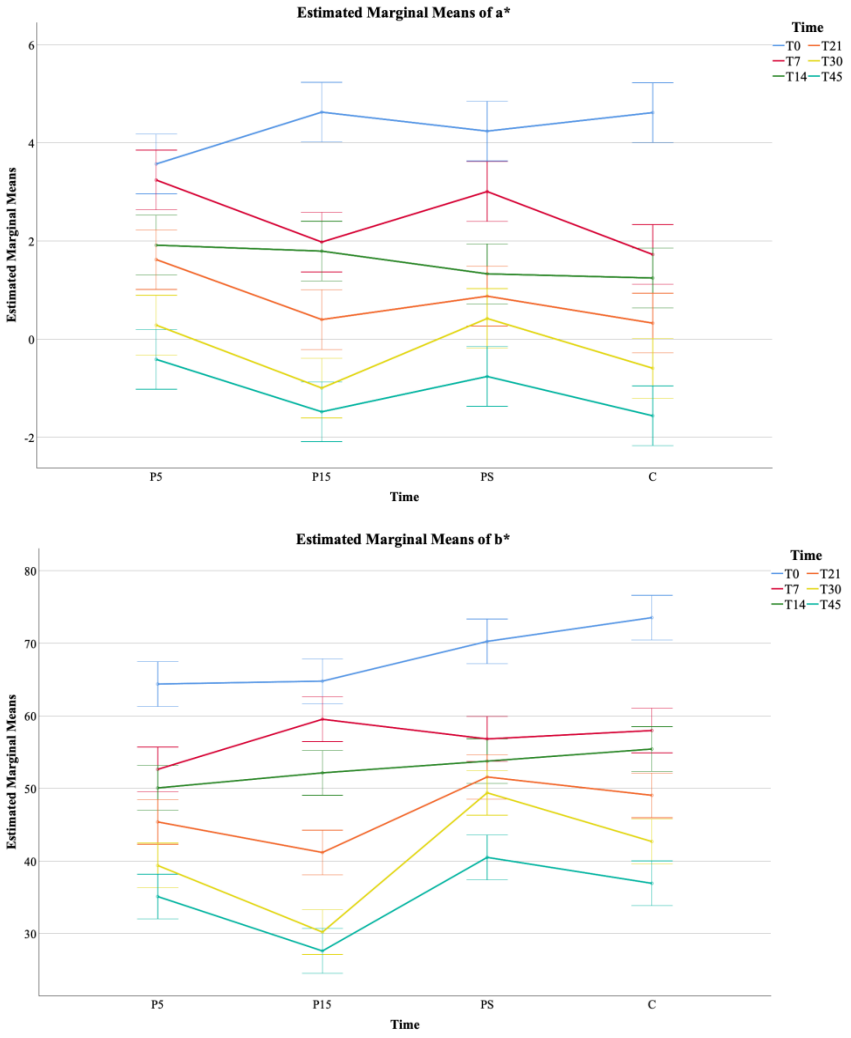


Figure 12. EMM plots of a* and b* coordinates over storage time. P5 – PE at 5g/kg; P15 – PE at 15g/kg; PS – potassium sorbate; and C – control.

This finding indicates that the use of the natural ingredient has a subtle and negligible impact on the color of formulations, without significantly altering their nutritional value. Moreover, the loss of color during storage time can be attributed to the sensitivity of beta-carotene, the primary pigment of pumpkin, which is highly susceptible to oxidation and degradation (Gavril, Constantin, et al., 2024; Norshazila et al., 2017; Sebdani & Abbasi, 2023). Although PE showed significant antioxidant capacity, it may not have been sufficient to protect beta-carotene from degradation throughout the storage period. Natural antioxidants, although beneficial for their bioactive properties, generally have lower stability compared to synthetic preservatives, which can lead to decreased protection against oxidative processes during long storage periods (Fink & Filip, 2021; Petcu et al., 2023; Venkatesan & Muniyan, 2024). As a result, the formulations experienced a reduction in visual appeal due to the degradation of the bright orange color associated with the content of beta-carotene. Therefore, new formulations were proposed, in a new test (Test II), in order to attenuate the effects on the color of formulations, in

search of obtaining more attractive shades.

Regarding the microbial load, as no counts were detected (within the quantification limits, see Supplementary Material 4) in any of the studied formulations at T0, T21, and T45. Given that the traditionally marketed product is intended for consumers who typically use the product all at once as an ingredient in other formulations, samples were evaluated only after the opening of the food package, on the respective storage days. The lack of detectable microbial load, even in the control without preservatives, indicates that the pasteurization thermal processing applied, as well as the packaging conditions, were adequate for the microbiological control of the formulations. Given these promising results, in Test II it was proposed to evaluate the formulations also after opening time.

Results regarding Test II

Based on the color results on Test I and given the challenge of preserving the color of pulp formulation, as in the traditionally marketed pumpkin, where potassium sorbate is used as preservative, the partial substitution of the artificial preservative with the natural extract obtained from the peels (PE) was evaluated. Thus, PE was added at a concentration of 10 g/kg along with 50% of the amount of potassium sorbate commonly used in the traditional formulation.

Additionally, to explore the complete replacement of potassium sorbate and for comparison purposes, a treatment where 100% of a natural pumpkin seed extract was incorporated was also included. The chosen seed landraces demonstrated their significant antioxidant and antimicrobial properties, as shown in the supplementary material. Despite the great potential of using these seeds as a preservative ingredient, the optimization of their extraction was not prioritized over the pumpkin peel. The key reasons for that decision include the lower extraction yields of seeds compared to the peels, the already established use of the seeds in food products (such as snacks or condiments), as well as the higher amounts of peel by-products compared to the seeds, making the seeds not suitable for the sustainable objectives of our work.

Table 52 shows the proximate composition of the second experiment (Test II). Considering moisture, there were no significant differences among the samples at the beginning of the analysis and after 45 days, although the control sample and the one with potassium sorbate showed statistically significant higher moisture content than the samples incorporated with the seeds extract and PE. A similar behavior was recorded for all the other macronutrients, where storage time did not show significant differences, even when it could be classified independently (Student's t-test < 0.05), as was the case for fat, carbohydrates, ash, and energetic value. Considering the effects of the preservative type on proteins content, the sample incorporated with PE and potassium sorbate showed higher

values, being significantly higher than the control sample, which was also superior to the sample incorporated with the seeds extract. The same tendency was recorded for carbohydrates, where no differences were found between the PE and the seeds extract formulations, which were both superior to the samples preserved with potassium sorbate and the control treatment. Ash was found in higher amounts in the sample preserved with PE, followed by the sample preserved with seeds extract, while there were no differences between the control and potassium sorbate samples. Finally, for the energetic value (kcal), a similar trend as in the case of ash was recorded.

Table 52. Proximate composition (in g/100g fw) and energetic value (in kcal) of the different pumpkin pulp formulations, referring to Test II.

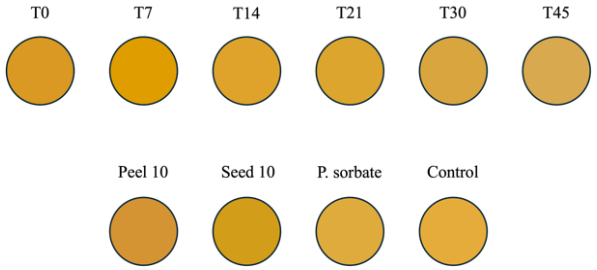
		Moisture	Proteins	Crude Fat	Carbohydrates	Ash	Energy
Storage Time (ST)	0 Days	83.7±0.9	1.16±0.08	0.06±0.02	14.4±0.8	0.67±0.08	63±3
	45 Days	83.3±0.8	1.18±0.08	0.07±0.02	14.7±0.8	0.72±0.08	64±3
***p*-value (n=12)**	Student's t-test	0.005	0.055	0.122	0.019	<0.001	0.010
Preservative Type (PT)	Peel 10	82.4±0.3^{c}	1.23±0.03^{a}	0.078±0.005^{a}	15.5±0.3^{a}	0.80±0.05^{a}	68±1^{a}
	Seeds 10	83.2±0.4^{b}	1.06±0.03^{c}	0.05±0.01^{b}	15.0±0.4^{a}	0.72±0.02^{b}	65±1^{b}
	Potassium Sorbate	84.2±0.3^{a}	1.24±0.03^{a}	0.08±0.01^{a}	13.8±0.3^{b}	0.61±0.04^{c}	61±1^{c}
	Control	84.3±0.5^{a}	1.16±0.03^{b}	0.044±0.004^{b}	13.9±0.4^{b}	0.64±0.04^{c}	61±2^{c}
***p*-value (n=6)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×PT (n=24)	*p*-value	0.294	0.645	0.717	0.258	0.313	0.242

If present, each letter in each column represents a significant difference among samples, obtained from Tukey's or Tamhane's T2 test. The asterisk (*) represents a statistically significant difference by means of Student's t-test. All assays were performed using a significance of 0.05.

Table 53 shows the color coordinates of the formulations studied. For all the samples, a significant interaction was observed for the effect of storage time (ST) and extract incorporation (PT). This interaction was so strong that neither the EMM, nor the PES gave evidence of one factor showing a higher influence over the other. This could be due to having six different sampling times throughout the storage period. Thus, the bottom section of **Table 53** shows the converted L*, a* and b* coordinates to RGB colors, showing the variations of each formulation. Overall, a very homogenous color was recorded for all the formulations studied. The variation over time was not significant, although the formulation incorporated with the PE seemed slightly darker, which could be an appealing attribute to consumers. Moreover, the analysis of the external colors showed that none of the proposed natural extracts had a statistically significant influence.

Table 53. Color of the different pumpkin pulp formulations, referring to Test II.

		L*	a*	b*
Storage Time (ST)	0 Days	68±4	15±5	66±3
	7 Days	69±4	13±5	68±3
	14 Days	71±3	12±3	66±4
	21 Days	71±3	10±3	65±3
	30 Days	71±3	9±2	58±7
	45 Days	72±4	8±3	52±10
***p*-value (n=36)**	Tukey's test	<0.001	<0.001	<0.001
Preservative Type (PT)	Peel 10	66±2	16±4	58±6
	Seeds 10	68±1	9±1	68±2
	Potassium Sorbate	73±3	9±4	61±11
	Control	74±2	11±3	63±6
***p*-value (n=54)**	Tukey's test	<0.001	<0.001	<0.001
ST×PT (n=216)	*p*-value	<0.001	<0.001	<0.001



If present, each letter in each column represents a significant difference among samples, obtained from Tukey's or Tamhane's T2 test. The asterisk (*) represents a statistically significant difference by means of Student's t-test. All assays were performed using a significance of 0.05.

Table 54 shows the soluble sugars found in the samples over 45 days, using only the day of production and the final day of storage as sampling points. The incorporation treatments were the same as in previous tables. The identified soluble sugars were fructose, glucose, sucrose and trehalose, being sucrose the most abundant compound and trehalose the least. Once again, in terms of statistical analysis, a significant interaction was found for ST x PT, not allowing to detect statistical differences and confirming the contributions of both factors for the outcome. Neither the EMM nor the PES showed values that could provide information regarding the most significant factor. This could be due to the absence of microbial contamination in all samples, which would have reduced the monosaccharides and alter the soluble sugars profile (Shankar et al., 2021).

Table 54 also presents the fatty acid profile, including the six most abundant fatty acids found in the samples, namely three saturated, one monounsaturated and two polyunsaturated. Among the detected compounds, C16:0 (palmitic acid) was the most abundant, while C18:3 (linolenic acid) was the least abundant. Considering the statistical analysis, a significant interaction was found for the two tested factors, e.g. storage time and preservative type. Thus, general tendencies could be extracted from the EMM plots, as shown in **Figure 13**. A change in C18:3 (linolenic acid), seen in **Figure 2**, could be sought for most samples, except the control sample where there was no change in the amount of this fatty acid. At T0, for the potassium sorbate incorporated sample, the amount is the highest and tends to reduce after 45 days, while the same tendency was recorded for the sample incorporated with the seed extracts although to a much lower extent. This could be due to the break of the unsaturated links in the aliphatic chain. Inversely, the sample incorporated with the peel extract showed a similar value of this fatty acid as the one incorporated with the seed extract at T0 which tended to increase over the 45 days storage period.

Table 54. Soluble sugars (in g/100g dw) and fatty acids (in %) variation of the different pumpkin pulp formulation, according to Test II.

		Soluble sugars					**Fatty acids**					
		Fructose	**Glucose**	**Sucrose**	**Trehalose**	**Total**	**C14:0**	**C16:0**	**C18:0**	**C18:1n9**	**C18:2n6**	**C18:3n3**
Storage Time (ST)	0 Days	1.2±0.1	1.0±0.3	5.5±0.6	0.23±0.05	8±1	7±4	38±11	13±2	19±4	19±11	3±3
	45 Days	1.0±0.3	0.8±0.5	5±1	0.21±0.05	7±2	4±2	36±13	15±5	21±8	20±14	3±3
***p*-value**	Student's t-test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.316
Preservative Type (PT)	Peel 10	1.4±0.2	1.5±0.2	6.0±0.3	0.22±0.03	9.1±0.7	7±2	43±6	15.2±0.4	18±4	13±3	4±2
	Seeds 10	1.0±0.2	0.7±0.1	5±1	0.21±0.06	7±1	2.1±0.9	28±3	9±2	21±2	38±5	2.1±0.2
	Potassium	0.9±0.1	0.5±0.1	4.2±0.4	0.16±0.01	5.8±0.6	10±4	52±4	18±3	13±4	6.7±0.3	n.d.
	Control	1.2±0.2	0.8±0.3	5.5±0.7	0.28±0.02	8±1	3±1	26.2±0.9	14±3	28±4	20±5	8±1
***p*-value**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×PT	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

n.d.: not detected.

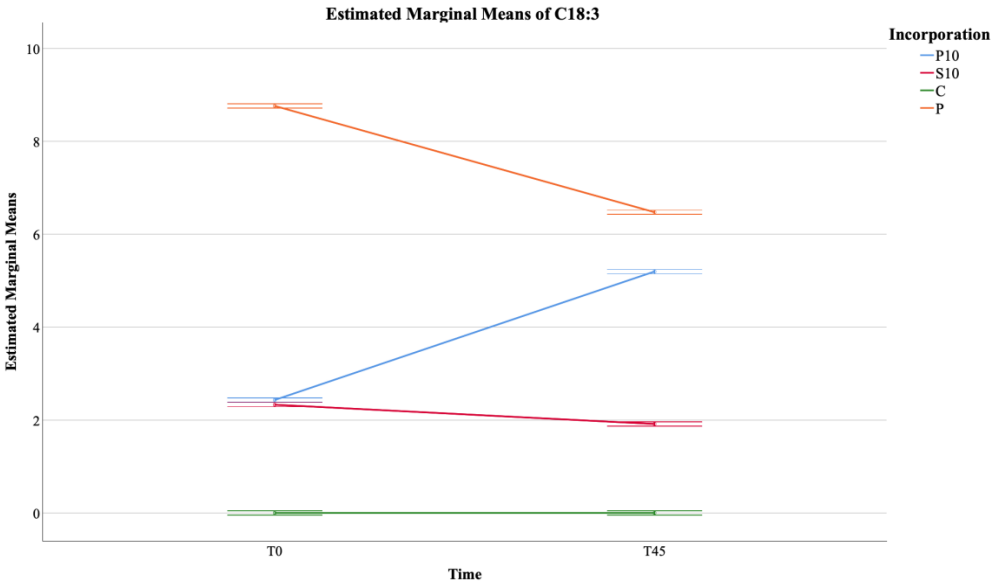


Figure 13. EMM plots for C18:3 (linolenic acid) for the different incorporations and storage time. P10 – PE at 10 g/kg with 50% of the potassium sorbate; S10 – seeds extract at 10 g/kg; P – potassium sorbate; and C – control.

The results were highly satisfactory in terms of microbial growth, as no counts were detected in any formulation, both throughout the storage period (T0, T21, and T45) and after the opening of the package (7 and 14 days post-opening).

The successful formulations achieved in this study are aligned with previous reports for the use of pumpkin in food products, highlighting its potential as a valuable ingredient (Caetano et al., 2017; Dhiman et al., 2020; Ghendov-Mosanu et al., 2023; Longato et al., 2017). Notably, this study is the first to evaluate the shelf life of an optimized extract derived from pumpkin peels, contributing new insights into its stability and preservation potential.

4.2.4. Conclusions

Two major achievements were reached in this work: optimized extraction conditions were obtained for butternut squash fruit peels and two pumpkin pulp formulations preserved with natural ingredients from pumpkin peels and seeds were developed. The optimized extraction conditions were determined for obtaining a natural preservative ingredient from fruit peels using two extraction methods: Heat-Assisted Extraction (HAE) and Ultrasound-Assisted Extraction (UAE). For the UAE method, the optimal conditions were found to be 400 W, 5 min, and 27% ethanol, resulting in 2.29 g/100 g of dry residue. In contrast, the HAE method was more successfully optimized, allowing for the determination of ideal conditions for two variables: dry residue and total phenol content. Thus, the global optimal conditions for this technique were established at

84 min, 30 ºC, and 0% ethanol, yielding 1.49 g/100 g of dry residue and 169 mg/g of total phenols. The low energy demand and the elimination of ethanol were key factors in selecting HAE for obtaining the peel extract that was incorporated into pumpkin pulp formulations. Overall, the evaluation of pumpkin pulp formulations using natural extracts revealed significant advancements in natural preservation and sustainability. Even partial replacement of potassium sorbate with pumpkin by-product extracts not only reduces reliance on artificial preservatives but also adds value to agro-industrial by-products, contributing to more sustainable practices and the circular economy concept. Our results confirm that the formulation with pumpkin peel extract provides effective preservation, meeting the expectations of consumers seeking more natural and healthy products. Additionally, the valorization of pumpkin by-products and the use of eco-friendly extraction methods reinforce the commitment to sustainability. These innovations represent a significant step forward in the development of more sustainable and efficient food products, establishing a solid foundation for future research and commercial applications in the food sector. As future perspectives, it is suggested that this new formulation undergo a feasibility and commercial acceptance analysis for this new color/shelf life standard. Alternatively, an adjustment to the formulation, such as increasing the addition of the already present natural ingredient, beta-carotene, could be considered to help sustain the color for a longer period.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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4.3. Consumers' preference of the new pumpkin fruit pulp formulations

4.3.1. Introduction

In recent years, there has been a growing interest in new food products that meet consumer demands for convenience, health and quality. In particular, the demand for ready-to-eat foods that are practical and at the same time free of artificial preservatives has gained prominence in the market. In this context, food companies are investing in the development of new formulations using natural ingredients, particularly pumpkin-based products, which not only offer significant nutritional benefits but also have the potential to appeal to a broad consumer base.

Sensory evaluation plays a crucial role in this process, as it allows us to understand consumers' preferences and perceptions regarding a product's attributes, such as flavor, aroma, texture, and appearance (McArthur-Floyd & Brako, 2024; Scarton et al., 2021). This data is essential to optimize the development of products that meet the expectations of the target audience. Sensory acceptance, especially in relation to attributes such as color, taste, and texture, plays a key role in determining the market success of new food products. Therefore, carrying out sensory tests with untrained consumers, who represent the general public, is essential to ensure that newly launched products align with the preferences and needs of today's consumers.

In this context, the final pumpkin formulation from **Section 4.2** was tested for consumer acceptance, alongside the traditional formulation containing potassium sorbate as a preservative. Both formulations were evaluated for their color, taste, aroma, and texture pleasantness on a scale of 1 to 5.

4.3.2. Material and methods

Formulations

Both formulations, the pumpkin pulp formulation containing 10 g/kg of the natural ingredient (butternut squash peel extract obtained under optimized conditions), referred to as Peel 10, and the traditional formulation, used as the control, were prepared as outlined in **Section 4.2.2**. The technical data sheet of the formulation is presented in the supplementary material.

Consumer testing

The test involved 106 untrained participants at the premises of the Polytechnic Institute of Bragança, Bragança, Portugal. Participants were asked to evaluate color, taste, aroma, texture pleasantness, and the overall acceptance, on a scale from 1 to 5 (1-very dissatisfactory, 2-dissatisfactory, 3-neutral, 4-satisfactory, 5-very satisfactory). Color was also assessed for its intensity from more yellow to more orange on a 5-point scale. Each participant received portions of both samples, coded discreetly, as shown in **Figure 14**.

Figure 14. Presentation of pumpkin pulp formulations for acceptance assessment testing.

Consumers were also surveyed regarding their awareness and preferences for natural foods, as well as the factors influencing these choices, with multiple-choice answers provided in the evaluation form available in the supplementary material.

Statistical analysis

The data obtained from sensory evaluations were analyzed by Analysis of Variance (ANOVA), and the difference between the means evaluated by Student's t-test, using the IBM SPSS software, version 29 (IBM Corp, Armonk, NY, USA). The radar-chart was created using Microsoft® Excel® for Microsoft 365 MSO (Version 2412 Build 16.0.18324.20092, 64-bit).

4.3.3. Results and discussion

The pumpkin pulp formulation, containing 10 g/kg of the natural ingredient extracted from pumpkin peel, developed in this PhD work, was evaluated in relation to consumer preference, compared to the traditional formulation that uses potassium sorbate as a preservative. The attributes of color, aroma, taste, texture and overall acceptance were evaluated, with a total of 106 responses for each attribute. The sensory analysis data is shown in **Table 55**, and in the radar-chart in **Figure 15**.

Table 55. Acceptability in the consumer test of the pumpkin pulp formulations.

Attribute	Formulation		
	Peel 10	**SP**	***p-value****
Color-Intensity	2.93 ± 1.21	3.02 ± 1.37	0.633
Color-Preference	3.70 ± 1.05	4.20 ± 1.17	0.001
Aroma	3.50 ± 1.01	3.68 ± 1.07	0.209
Taste	3.17 ± 1.11	3.47 ± 1.14	0.052
Texture	3.71 ± 1.08	3.72 ± 1.11	0.950
Overall acceptance	3.47 ± 1.11	3.71 ± 1.12	0.125

Analytical results correspond to the mean ± Standard Deviation (SD). *ANOVA analysis – All assays were performed using a significance of 0.05.

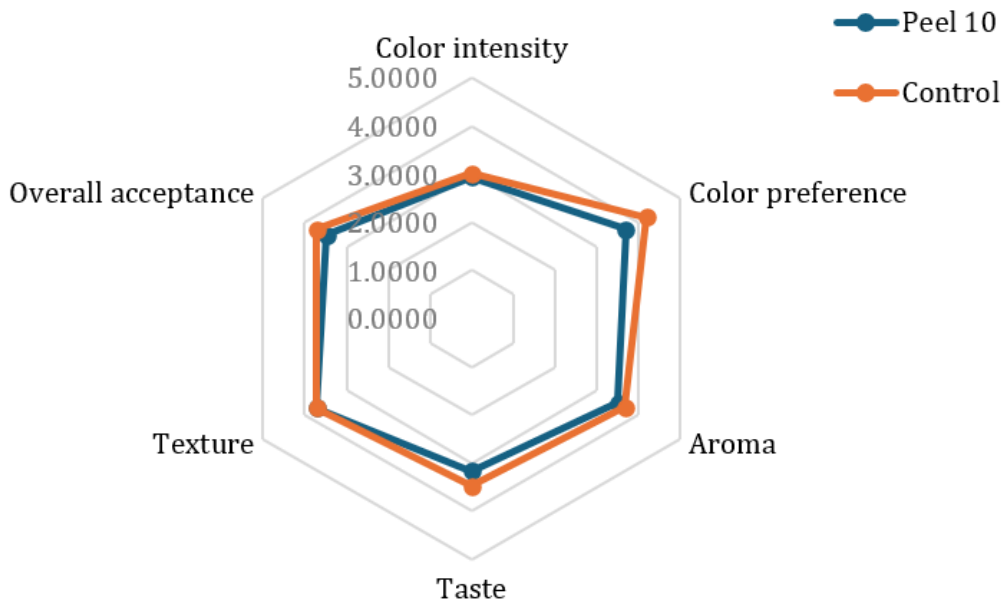

Figure 15. Radar-chart for the acceptability in the consumer test of the pumpkin pulp formulations.

Very promising results were verified, since the attributes of aroma, taste, texture, and overall acceptance did not present statistically significant differences ($p > 0.05$) between the formulations. Although consumers showed a significant preference ($p < 0.05$) for the color of the control formulation compared to the new formulation developed, the perception of color intensity was similar ($p = 0.633$) between the two formulations, indicating that the differences in preference are not related to a perceptible variation in color. It is noteworthy that 49 out of the 106 participants gave the same rating for both formulations for color, which demonstrates similarity in the perception of the formulations. The new formulation, with the innovative ingredient, may still be in a phase of consumer adaptation, and additional adjustments may be necessary to achieve acceptance as strong as that of the traditional formulation, especially considering that the color perception does not present significant differences in terms of intensity.

In fact, consumers are open to this adaptation. This is what the promising results of the survey on consumer awareness and preference for natural foods suggest. When asked about their preference for consuming natural products free from artificial preservatives, the responses were overwhelmingly positive, as shown in **Table 56**.

Table 56. Consumer preference for natural products

Preference	Multiple choice responses
Yes, I prefer natural products without artificial preservatives	66
Yes, but it is not a determining factor in my choice	40
No, I have no preference for natural products	3
I have no formed opinion on the matter	3

The survey results indicate a clear consumer preference for natural products free from artificial preservatives, with 66 respondents strongly favoring these products. Although 40 consumers also prefer natural products, they consider other factors in their purchasing decisions. Only a small fraction of consumers has no preference or opinion on the subject. These insights are valuable for guiding product development, marketing strategies, and consumer education efforts to align with prevailing consumer trends and preferences.

Following consumer preferences for natural products free of artificial preservatives, the next survey then investigated the possible factors that influence these choices. **Table 57** shows the number of consumers who agree with each influencing factor.

Table 57. Consumer decision factors regarding preference for natural food options without artificial preservatives

Factors	Multiple choice responses
Concern for health and food safety	81
Concern for the environment and sustainability	39
Better taste and quality of natural products	33
Influence of information and advertising campaigns	4
I have no formed opinion on the matter	7
Price-quality ratio	1

The survey results highlight several important consumer preferences regarding food choices. A significant majority of respondents (81) expressed strong concerns about health and food safety, indicating a clear priority for safe and nutritious food options. Furthermore, a notable proportion (39) showed interest in environmental sustainability, suggesting that consumers are increasingly aware of the ecological impact of their food choices. The taste and quality of natural products were also valued, with 33 interviewees

emphasizing the importance of authentic flavors and high product standards. In contrast, only 4 interviewees cited the influence of information and advertising campaigns as a decisive factor, indicating that direct benefits for health and personal values can outweigh marketing appeals. A small minority did not indicate any opinion on the matter, possibly reflecting the need for more education or involvement in these issues. Interestingly, only one respondent considered price quality as a significant determinant, suggesting that while cost remains a consideration, it is less influential compared to factors such as health, sustainability and taste quality. These findings highlight the diverse and nuanced considerations that influence consumers' food choices, providing valuable insights for industry stakeholders looking to align products with evolving consumer preferences and values.

4.3.4. Conclusions

Analysis of the survey results reveals that there is a significant demand for natural products among consumers, especially those concerned about health, environmental sustainability and the sensorial quality of food. The high number of respondents who prioritize health and food safety suggest that a natural product free of artificial preservatives may find a receptive market among those seeking safer and more nutritious food options. Furthermore, growing concern about environmental sustainability and the preference for foods with better flavor and natural quality indicate a promising opportunity for the success of products that meet these criteria.

Combined with these concepts and preferences, together with the excellent results between formulations for the attributes evaluated in the research, the great potential of the pumpkin pulp formulation developed here is notable. The replacement, even if partial, of the artificial preservative potassium sorbate with natural pumpkin peel extract, aligns the major aspects of food safety and environmental sustainability.

4.3.5. References

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CHAPTER 5

High Pressure Processing Technology

This chapter presents the research article of the use of high pressure processing technology to reduce or eliminate the need for artificial preservatives in pumpkin pulp formulations.

5.1. Enhancing shelf life of pumpkin pulp with natural-based preservative and high pressure processing

Enhancing shelf life of pumpkin pulp with natural-based preservative and high pressure processing

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Abstract: Formulated pumpkin pulp fruit was incorporated with natural ingredient (NI) extracted from pumpkin peels (butternut squash), industrial mix (with and without potassium sorbate), processed by high pressure (600 MPa / 4 min) and stored at 5 °C for 45 days. The shelf life was evaluated by microbiological analysis (total mesophilic aerobic, Enterobacteria and molds & yeasts), pH, color, texture (TPA) and oxidative stability via changes in beta-carotene, *gamma*-tocopherol, total phenolic content (TP) and antioxidant potential (FRAP). No microbial growing was observed during 45 days of shelf life for all samples conditions, this can be attributed not only for the treatments, but its combination with the industrial mix and the NI (samples 4 and 5) incorporated to the pulp. Besides that, samples added with NI retained the color, textural properties (firmness) and exhibited significantly higher TP compared to other samples. In terms of quality attributes, samples processed by thermal treatment showed the most difference in terms of firmness, stickiness and chewiness as well as for changes in beta-carotene, lutein and *gamma*-tocopherol. The pulp was also analyzed for their nutritional quality, focusing on centesimal composition, fatty acids, and soluble sugars, at the beginning and end of storage (0 and 45 days). The combination of HPP with NI showed a great potential for the shelf life of pumpkin pulp, since no contamination was observed, the use of potassium sorbate in this product could be reduced by 50% or even completely replaced by NI.

Keywords: Pumpkin pulp, high pressure processing, natural-based preservative, microbial inactivation, food quality

5.1.1. Introduction

Pumpkin (*Cucurbita* spp.) is an extraordinary vegetable with great potential for use as both a nutritious and medicinal food. Its pulp and peel contain essential minerals, as well as valuable phytochemicals such as beta-carotene, total flavonoids and phenolic compounds, which can contribute to anti-aging and support the immune system (Hosen et al., 2021). Despite their beneficial properties, pumpkins are one of the most neglected and underutilized food and medicinal plants (Nyabera et al, 2021). Their production is limited by the lack of genetically improved seeds, which compromises their cultivation on a larger scale. Therefore, research exploring the functionalities of pumpkin is essential to promote its wider use and disseminate its benefits.

When pumpkin is consumed as pulp, i.e. baby food, puree, soups, and others, the main processing step typically involves the application of high temperatures (e.g., pasteurization, sterilization) to ensure safety, widely recognized for their negative impact, as they can cause undesirable changes in both the nutritional content and sensory quality of the food (Terefe et al., 2013). In this scenario, non-thermal technologies gain space due to their ability to offer simultaneously safety and high-quality products, since less thermal degradation of quality attributes and nutritional compounds. One if this technologies, successfully implemented to the food industry is the high pressure processing (HPP), a technology that uses pressure for few minutes instead of high temperature, bringing several advantages, including safe and minimally-processed products with extended shelf-life, prepack technique, i.e. the product is packed in their final package (reducing chances of cross contamination), food waste reduction, clean labels, eco-friendliness and a wide range of applications (Houška et al., 2022). However, HPP is a cold pasteurization and alone does not inactivate microbial spores and some enzymes.

Several studies have already demonstrated the potential of the application of HPP in different food products, such as pumpkin puree (Garcia-Parra et al., 2016; Rinaldi et al., 2023), pumpkin cubes (Contador et al, 2014), mango pulp (Ribeiro et al., 2021), açaí (*Euterpe oleracea Martius*) (Jesus et al., 2020), mostly demonstrating a better performance of HPP over thermal treatments.

Besides that, HHP can be combined with other hurdle techniques, such as natural-based preservatives extracted from food side streams, essential oils, to investigate the potential of synergistic effects (Zou et al., 2023; Melhem et al., 2022). Combining HPP with these natural ingredients, allows not only an increase in the microbial inactivation, but also allows milder HPP conditions and consequently reducing processing costs (Balasubamanian & Farkas, 2018). Thus, the objective of this study involves the application of high pressure processing at ambient temperature in combination with natural ingredient extracted from the pumpkin peel in order to increase shelf life of

pumpkin pulp without the application of chemical preservative (potassium sorbate) or elevated temperatures (pasteurization or sterilization).

5.1.2. Material and methods

Chemicals

Lutein (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), beta-carotene and *gamma*-tocopherol (Merck KGaA, Darmstadt, Germany), ethanol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), isopropanol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), ethyl acetate ≥ 99,8 %, LiChrosolv, hypergrade for LC-MS (VWR Chemicals, VWR International, Leuven, Belgium), methanol ≥ 99,8 %, LiChrosolv, hypergrade for LC-MS (VWR Chemicals, VWR International, Leuven, Belgium), formic acid ≥ 99 % (VWR Chemicals, VWR International, Leuven, Belgium), Folin-Ciocalteau reagent (Sigma Aldrich), sodium hydroxide (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), gallic acid monohydrate (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), ascorbic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 2,4,6-Tri-(2-pyridyl)-1,3,5-triazin, 98 % (TPTZ, Thermo Fisher Scientific, Darmstadt, Germany), hydrochloric acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), Iron-(III)-chloride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), acetic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Pumpkin pulp fruit production

Using a blender (Retsch GM 200, Germany) the pumpkin pulp (100% pulp from butternut variety) was produced, showing a final yield of 67 % (**Table 58**). After the production, the pulp was divided into five different sample conditions:

1. Pumpkin Pulp + mix of ingredients (natural beta-carotene, starch, citric acid and citrate) + HPP treatment.

2. Pumpkin Pulp + mix of ingredients (natural beta-carotene, starch, citric acid, citrate and potassium sorbate) + Pasteurization treatment.

3. Pumpkin Pulp + mix of ingredients (natural beta-carotene, starch, citric acid, citrate and potassium sorbate) + HPP treatment.

4. Pumpkin Pulp + mix of ingredients (natural beta-carotene, starch, citric acid and citrate) + Natural ingredient + HPP treatment.

5. Pumpkin Pulp + mix of ingredients (natural beta-carotene, starch, citric acid, citrate and 50% potassium sorbate) + Natural ingredient + HPP treatment.

Each one of the samples (1 to 5) was mixed with its proper mix of ingredients, as previously described. Natural ingredient (NI) was extracted from pumpkin pulp peel and incorporated to Samples 4 and 5. Additionally, sample 4 was not added of potassium sorbate, while sample 5 had a 50% reduction of potassium sorbate to evaluate the

efficiency of the natural ingredient. The samples were vacuum-packed in appropriate packages for high pressure processing (Whirl-Pak® Sample Bag). For pasteurization, 200 g of each pulp was placed into glass jars. Samples were then stored at 5°C for 45 days, protected from the light. The total amount of pumpkin pulp produced was estimated for duplicate of process and triplicate of analysis.

Table 58. Pumpkin (Butternut squash) pulp yield production.

Pumpkin pulp yield	g	%
Total weight	7111.3	100
Seeds	360.5	5.1
Peel	1927.1	27.1
Pulp	4823.7	67.8
By-products (Seeds and peel)	2287.6	32.2

High pressure processing and thermal treatment

The high pressure process (HPP) conditions were 600 MPa for 4 minutes at ambient temperature. These process parameters were previously studied and showed a good potential in microbial inactivation, besides that this range of pressure and time is also in accordance to what is industrially applied for cold pasteurization (600 MPa/ few minutes). The thermal treatment was applied by retort (90 °C / 3 minutes – total time of process was 15 minutes considering the heat up time). For HPP, a U4000 high pressure system (Unipress, Warsaw, Poland) was used. The processed samples were stored at refrigerated condition (5 °C). The shelf life was conducted up to 45 days with 3 points of analysis (day 0, 21 and 45).

Microbiology assays

For the microbiology assays, total aerobic mesophilic were analyzed in plate count agar (Roth, Carl Roth GmbH & Co. KG, Germany) incubated at 37 °C for 48 hours. For *Enterobacteriaceae*, was used Endo agar (Roth, Carl Roth GmbH & Co. KG, Germany), added with fuchsine solution (10 % in 95 % ethanol) which is also a selective agar for *Escherichia coli*, incubated at 37 °C for 48 hours. For molds the Maltodextrose agar (Roth, Carl Roth GmbH & Co. KG, Germany) was used and incubated at 30 °C for 72 hours.

Color and pH measurement

The color measurement was performed using a white light (6000K) reflector from left and right side to guarantee similar light conditions. Prior to taking the pictures the samples were spread (10 g) evenly in a petri dish. The photos were taken with a Nikon camera (COOLPIXP7700) from the top (20 cm). A simple image processing routine implemented in GNU Octave (Version 4.2.1) was used to determine the L*, a*, b*

parameters of the thermal treated and high pressure samples. Besides that, the nearest standard color was also measured through the RGB parameters.

The pH was measured by benchtop pH-meter (SI Analytics Lab 850 pH-Meter, SI Analytics GmbH, Mainz, Germany).

Texture profile analysis (TPA)

The texture profile analysis of the treated and non-treated samples was conducted using a calibrated texture analyzer (TA XT Plus, Stable Micro Systems, UK). Texture parameters such as firmness, adhesiveness, cohesiveness, springiness, and gumminess were measured in triplicate. The following instrumental test parameters were used: mode was forced in compression; load cell value was 1 kN; trigger type was 0.1 N; and a cylindrical aluminum probe (25 mm diameter, 40 mm height) was used. An aliquot of each sample (~50 g) was used. The entry depth of the probe was set to 10 mm using a test speed of 50 mm/min and samples were analyzed at room temperature. The force–time curve was further evaluated to determine the texture parameters using an Octave code that had been programmed according to the interpretation rules for textural profile analysis showed in **Table 59**.

Table 59. Measurement conditions for the texture analyzer.

Measurement		**Condition**
Test Mode		Compression
Speed		50 mm/min
Trigger		0.1 N
Entry depth		10 mm
Beaker		Ø50 mm, Height 105 mm
Sample height		50 mm
Adaptor		Ø25 mm, Height 40 mm
Load cell		1 kN
Textural properties	**Parameter**	**Sensory feeling**
Firmness	F2	Hard/ Firm/ Soft
Elasticity	t4:5 / t1:2	Plastic / Elastic
Cohesion	A4:6 / A1:3	Brittle / Crumble
Gumminess	Firmness (F2) x Cohesion	-
Chewiness	Gumminess x Elasticity	-
Stickiness	A3:4	Sticky / Adhesive

Natural ingredient extraction (NI)

NI was obtained under optimal extraction conditions, previously determined in the optimization study by Leichtweis, et al. (2024). Briefly, butternut squash peels were freeze-dried, reduced to a powder (~20 mesh) and extracted by heat-assisted extraction using just water as solvent, at a ratio of 1 g/40 mL, for 84 min at 30°C with constant stirring. The mixture was filtered through filter paper and dried using a spray dryer (BUCHI, B-191, Laboratory-Techniques LTD, Flawil, Switzerland) with maltodextrin as a

carrier agent.

Oxidative stability evaluation

To evaluate oxidative stability of the pumpkin pulp, changes in beta-carotene, lutein and *gamma*-tocopherol content, phenolic content, and changes in antioxidant capacity were analyzed.

Extraction of carotenoids and gamma-tocopherol

Before vitamin extraction, the pumpkin pulp samples were freeze-dried. The carotenoid and *gamma*-tocopherol extraction were performed according to the method of Guzman et al. (2012) with slight modifications. The extraction was carried out in duplicate for each sample. Briefly, a sample of 0.01 g of freeze-dried pumpkin pulp was extracted using 500 μL of pure ethanol. The samples were shaken for 20 min in the dark (80 rpm, room temperature (RT)) and then centrifuged (9000 g, 15 min, RT). The supernatant was collected entirely and transferred to a new 2-mL-reaction tube. The extraction was performed another two times until the pellet was colorless. The solvent was then wholly evaporated using a centrifugal evaporator (no heating, no pulse ventilation). After complete evaporation of ethanol, the residue was taken up in 15 mL isopropanol each for the analysis of beta-carotene and 100 μL isopropanol each for the analysis of lutein and *gamma*-tocopherol. The extracts were placed in an ultrasonic bath for 5 min to dissolve the residue completely. The two identical samples from one treatment and one day were pooled, and the vitamin content was determined by HPLC-MS/MS.

HPLC-MS/MS

The determination of carotenoid and *gamma*-tocopherol content was performed using an Agilent Infinity 1260 system composed of a binary pump, multicolumn thermostat, and autosampler interfaced with an Agilent G6470A Series triple quadrupole mass spectrometer (Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn, Germany) coupled with an electrospray (ESI) source in positive ionization mode. MS/MS was operated in the multiple reaction monitoring mode. The separation was achieved on a Multospher 120-RP18AQ column (250 x 4.0 mm; 3 μm), and the column temperature was set to 30 °C. The injection volume was 5 μL, and the flow rate was 0.8 mL/min. The sheath gas temperature was set at 275 °C, the sheath gas flow was 11 L/min, the nebulizer pressure was 35 psi, and the collision gas used was nitrogen. The mobile phases were methanol (with 0.1 % formic acid) (A) and ethyl acetate (with 0.1% formic acid) B. The elution gradient was as follows: 96 % A from 0 to 2 min, 96-20 % A from 2 to 5 min, 20 % A from 5 to 10 min and re-equilibrated (post-run) to 96 % A for 5 min. The transitions

used for quantification, fragmentor voltage, collision energy, and dwell time are listed in **Table 60**.

Table 60. Mass spectrometric conditions for the analysis of the selected vitamins.

Compound Name	Precursor Ion	Product Ion	Dwell	Fragmentor	Collision Energy
Lutein	568.5	476.4	20	80	20
		430.2	20	80	10
		338.2	20	80	20
		237.2	20	80	20
		145.2	20	80	20
		430.2	20	80	20
Beta-carotene	536.6	444.6	20	70	20
		269.2	20	70	20
		177	20	70	20
		133.1	20	70	5
Gamma-tocopherol	416.5	191.1	20	90	26
		164.2	20	90	25
		150.9	20	90	27
		149.8	20	90	25

The vitamin content was quantified using an external calibration curve. Stock solutions of beta-carotene (100 µg/mL), lutein (100 µg/mL) and *gamma*-tocopherol (1000 µg/mL) were prepared in absolute ethanol. The concentration of the working standard solutions was checked photometrically before each HPLC-MS/MS measurement and calculated according to their absorbance values E (1 cm/1%) (beta-carotene 233 at 450 nm in acetone, lutein 215 at 450 nm in ethanol (Krajewska et al. 2017), *gamma*-tocopherol 91.4 at 298 nm in ethanol (Chen und Bergman 2005). A mixture of beta-carotene (0.063 to 4 µg/mL), lutein (1 to 8 µg/mL) and *gamma*-tocopherol (0.063 to 1 µg/mL) was prepared. Calibration curves with their corresponding equations were calculated for each standard to determine the concentrations of the freeze-dried pumpkin pulp samples. The analytical sensitivity was determined by calculating the limit of detection (LOD) and quantification (LOQ) (**Table 61**).

Table 61. Limit of detection (LOD) and quantification (LOQ) for lutein, beta-carotene and *gamma*-tocopherol.

Compound Name	LOD (ng/mL)	LOQ (ng/mL)
Lutein	19.10	58.00
Beta-carotene	34.70	105.10
Gamma-tocopherol	7.70	23.40

Preparation of pumpkin pulp extracts

The preparation of pumpkin pulp extracts to determine total phenolic contents and antioxidant capacity was according to the method by Mala & Kurian (2016) with slight modifications. Briefly, duplicate freeze-dried pumpkin pulp samples weighing 0.01 g each

were extracted with 200 µL of a methanol/water mixture (80/20, *v/v*). The extraction was carried out by constant shaking (Vitelli mixer, b4-mode, 80 rpm) for 3 hours in the dark at RT. After extraction, the samples were centrifuged (10000 x g, RT, 10 min), and the supernatants from the duplicated pumpkin pulp samples were combined.

Determination of total phenolic content: Folin-Ciocalteau assay

Total phenolic content (TP) was estimated using the Folin-Ciocalteau method. 20 µL of a blank with methanol/water (80/20, *v/v*) and 20 µL of pumpkin pulp extracts were pipetted onto a 96-well plate. A premixed solution of 150 µL distilled water, 10 µL Folin-Ciocalteau reagent, and 20 µL sodium hydroxide (1 M) was added to each well, and the samples were incubated for 30 min in the dark at RT. The photometric measurement was performed at a wavelength of 765 nm with a Tecan Infinite 200 Pro plate reader. A five-point standard curve with gallic acid (10 µg/mL-100 µg/mL) was used to determine the TP in all pumpkin pulp samples. The results were expressed as mg gallic acid equivalents (GAE)/100 g dry weight (dw).

Determination of antioxidative capacity: ferric reducing antioxidant power-assay

The antioxidative capacity of pumpkin pulp extracts was determined by ferric reducing antioxidant power (FRAP) assay. The FRAP solution was prepared by mixing 10 mM TPTZ (in 40 mM HCl) with 20 mM $FeCl_3$ (in 0.25 M acetic acid, pH 3.6) in a ratio of 1:1. Then, 10 µL of a blank with methanol/water (80/20, v:v) or the pumpkin pulp extracts were pipetted into a 96-well-plate and mixed with 150 µL of the FRAP solution. The samples were incubated for 6 min at RT before measuring the absorbance at 595 nm with a Tecan Infinite 200 Pro plate reader. An external calibration with ascorbic acid (50 µM-1000 µM) was used to determine the antioxidative capacity of the pumpkin pulp samples. The results were expressed as mg ascorbic acid equivalents (AAE)/100 g dw.

Determination of proximate composition

To determine the proximate composition of the pumpkin pulp formulations, were adhered to the Association of Official Analytical Chemists (AOAC, 2016). Ash content was determined by incinerating the samples in a muffle at 550°C. Crude protein content was analyzed using the Kjeldahl method, and crude fat was measured by Soxhlet extraction using petroleum ether. The carbohydrate content was calculated by difference, subtracting the sum of ash, protein, and fat content from 100%. The energetic value (kcal) was calculated using the Atwater conversion factors (4 kcal/g for protein and carbohydrates and 9 kcal/g for lipids). The results were expressed in g/100 dry weight (dw).

Determination of chemical composition

The profiles of free sugars and fatty acids were analyzed by chromatography, more specifically using a HPLC coupled to a refractive index detector and by gas–liquid chromatography with flame ionization detection (GC-FID), respectively.

The HPLC-RID analysis was conducted using an integrated system that included a pump (Knauer, Smartline system 1000, Berlin, Germany), a degasser (Smartline manager 5000), an auto-sampler (AS-2057 Jasco, Easton, MD, USA), and a refractive index detector (Knauer Smartline 2300). This setup followed a previously established method (Barros et al., 2013). A Eurospher 100-5 NH2 column (4.6 x 250 mm, 5 µm, Knauer) was employed, maintained at a temperature of 30°C using a 7971 R Grace oven. Melezitose was utilized as the internal standard (IS) for quantification, and the identification of compounds was achieved through chromatographic comparison with authentic standards. Results were reported as grams per 100 grams of dry weight (g/100 g dw).

For GC-FID, a DANI model GC 1000 equipped with a Macherey–Nagel column (30 m x 0.32 mm ID x 0.25 µm film thickness) was employed. The procedure followed the methodology outlined by Pereira et al. (2011). Fatty acid identification and quantification were accomplished by comparing the retention times of the detected peaks to those of fatty acid methyl ester (FAME) peaks from a commercial standard mixture (FAME reference standard mixture, standard 47885-U, Sigma-Aldrich, St. Louis, MO, USA). Results were expressed as relative percentages.

Statistical evaluation

The experiments were performed in triplicate and quantitative results were expressed as mean ± standard deviation. Data were analyzed using analysis of variance (two-way ANOVA) followed by Tukey's post hoc test for comparisons between samples with 95% confidence (XLSTAT software, version 2015.2.02, Microsoft, Inc., USA). For the analysis of proximate and chemical composition, each test was performed in triplicate, with results presented as mean ± standard deviation. A two-way analysis of variance (ANOVA) with Type III sums of squares was employed, following the assessment of homogeneity of variances using Levene's test. Statistical evaluations included Tukey's test (TT) for homoscedastic samples and Tamhane's T2 (T2) test for heteroscedastic ones when comparing different preservative types (PT), while Student's t-test was applied to examine differences in storage time (ST). The two-way ANOVA facilitated the assessment of the independent effects of ST and PT. When a significant interaction ($p < 0.05$) between ST and PT was identified, trends were analyzed using the plotted estimated marginal means (EMM) and the partial eta squared (PES) that allows, in some cases, to understand which factor most contributed to the outcome. In cases where no significant interaction

was observed ($p > 0.05$), each factor was analyzed separately using the aforementioned post hoc tests. All statistical calculations were conducted with a significance level set at p = 0.05, using IBM SPSS software, version 29 (IBM Corp.).

5.1.3. Results and discussion

Microbiology

During the 45 days of the predictive shelf-life, no growing of mesophilic bacteria (total plate count), Enterobacterias, molds and yeast was observed. This can be attributed not only to the process treatments, but also for the ingredient mix and the natural ingredient (samples 4 and 5) added to the pulp. All five samples studied performed well during their 45 days of shelf life, demonstrating the successful application of the technologies in combination with the mix and the natural ingredient extracted from pumpkin residue.

Sample 1 was processed using High Pressure Processing (HPP) without the preservative potassium sorbate (PS), while sample 2 was treated with potassium sorbate and pasteurized. Both conditions resulted in no microbial growth, demonstrating the efficacy of HPP alone in inhibiting microbial activity without the need for additional preservatives. To further investigate, sample 3 was processed using HPP and also included PS. As with the other samples, no microbial growth was observed. This indicates that the addition of potassium sorbate could be avoided, as sample 1, processed solely with HPP, and showed excellent performance during its shelf life without any microbial growth.

It is important to highlight that sample 4 was added with the natural ingredient (NI) and not added with potassium sorbate, a common preservative used in the food industry for prolonging shelf life of food products. Sample 4 had shown the same good results in keeping the microbial quality of the pulp during 45 days of shelf life with no growing of the microorganisms studied (under the limit of detection), this shows positively the impact of this ingredient as a natural preservative in combination with HPP. Previous study (data not shown) showed that HPP alone (100 % pulp, with no ingredients) could not inactivate all the contaminants, bringing a shorter shelf life when compared to the actual study, 15 days and 45 days, respectively.

Sample 5 was added with NI in combination with sorbate potassium reduced by 50 % compared with the samples 2 and 3. As it can be observed by the results, no growing was observed, showing that in this product the use of potassium sorbate either could be reduced by 50 % or even totally replaced by the natural ingredient extracted from the pumpkin peel, since in sample 4 no microbial growing was observed.

Prior to the shelf-life determination, the raw pumpkin pulp (100 % pulp, with no industrial mix or natural ingredient) had its natural microbial flora evaluated (**Figure**

16), and showed an initial contamination of 2.9 log/g for mesophilic bacteria and 2.3 log/g for Enterobacteria. Thus, the inactivation as well as the inhibition of microbial growth during the 45 days of shelf life could be attributed to the processes as its combinations with the ingredients used. Besides that, it is important to highlight that in previous study (data not shown), raw pumpkin pulp (not added with any ingredient) treated by same conditions HPP showed a considerable contamination at day 20 and at day 34, HPP samples showed a contamination of 4.7 log/g, 3.12 log/g for total aerobic mesophilic and Enterobacteria, respectively. On the other hand, thermal process (same conditions as for the sample A2) at day 34 showed 4.2, 4.3 and 4.5 log/g of total aerobic mesophilic, Enterobacteria and molds and yeast, respectively. This result clearly shows a positive correlation between the process applied and the ingredients used, since a predictive shelf-life of 45 days was achieved with no microbial growth. This result clearly shows a positive correlation between the process applied and the ingredients used, since a predictive shelf-life of 45 days was achieved with no microbial growth.

High pressure is recognized as a cold pasteurization technique, with great ability to inactivate non spores bacteria at ambient temperature (Inanoglu et al., 2022; Silva & Evelyn, 2023). Besides HPP, the industrial mix used contained acid citric and citrate, both ingredients with an antimicrobial properties, thus, it is believed that the great effect in the microbial inactivation is due to a synergistic effect in between HPP, natural ingredient and industrial mix (with and without potassium sorbate). Besides the great browning control (Zou et al., 2023), acid citric also prevent contamination due to the pH decay, metal chelating, so combining acid citric with other decontamination methods can significantly impact the microbial inactivation in fresh foods (Książek, 2024). Similarly to the present study, Zou et al. (2023) showed that the combination of citric acid (CA&PEN) and (HPP 500 or 600 MPa, 5 min) also promoted a synergistic effect and inhibited the total aerobic bacteria in banana puree for up to 3 months. The natural ingredient used has also demonstrated great antimicrobial properties against 5 strains of bacteria and 2 of fungi of interest in food contamination, in concentrations from 10 to 2.5 mg/mL (Leichtweis et al., 2025, submitted for publication).

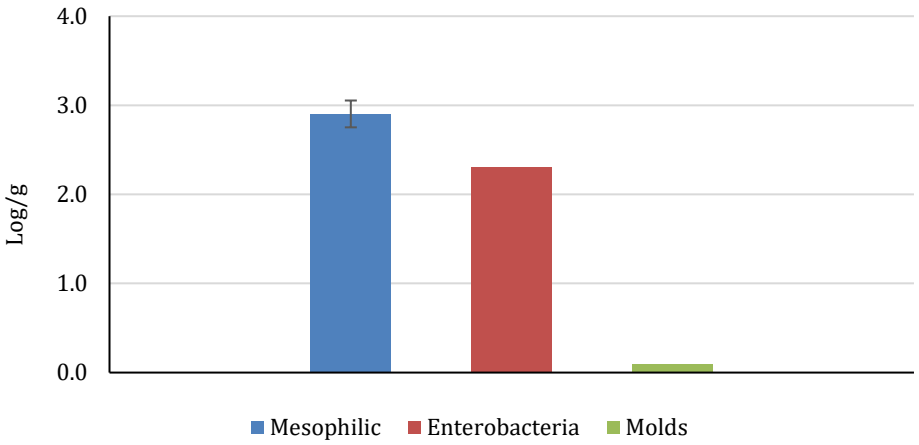

Figure 16. Natural microbial flora from raw pumpkin pulp.

This appears as a positive result, mainly by the ones where the potassium sorbate could be avoided or replaced by the natural ingredient (samples 1, 3 and 5 respectively). The use of potassium sorbate is controlled by different international laws. The *Codex Alimentarius* and European legislation on food additives has established a maximum level of 1,000 ppm for benzoate and sorbate in various products, such as sauces, exceeding this amount not only infringes regulatory guidelines but also represents a danger to human health (Yazdanfar et al., 2023).

pH and color evaluation

Table 62 shows the pH values measured during days 1, 21 and 45 of shelf life. As it can be observed the pH results are in accordance with the microbiological results, showing any or very small variation, and being stable during 45 days of shelf life. Besides the significant difference showed by the statistic evaluation, those differences are possibly negligible since less than ≤ 1.9% of variation was observed in between the samples, representing in general for all samples a very similar pH ~5 (≤ 5.03 and ≥ 4.93). The pH of the pumpkin untreated pulp was 6.5, and after addition of the mix, NI and process application (HPP and pasteurization) an initial (day 1) and final (day 45) value of pH 5 was observed and this may be explained by the ingredients added to the pulp (natural ingredient, potassium sorbate, citric acid and citrate), which was able to reduce the contamination, and consequently not promoting any alteration in pH values. Additionally, the mix contains citrate, a common salt used as pH controller in the food industry, due to its buffering properties (Behera et al., 2021). These results are in agreement with the findings of Rinaldi et al. (2023), with no changes in pH for pumpkin (cv. *Violina rugose*) processed by HPP (100 to 600 MPa).

Table 62. pH variations during 45 days of pumpkin pulp shelf life.

Sample	Days		
	1	21	45
1	4.96 ± 0.01 ^{Aa}	4.94 ± 0.01 ^{Cb}	4.97 ± 0.01 ^{Ca}
2	4.98 ± 0.01 ^{Ba}	5.01 ± 0.00 ^{ABb}	5.04 ± 0.01 ^{Ac}
3	5.02 ± 0.01 ^{Ca}	5.02 ± 0.01 ^{Aa}	4.98 ± 0.01 ^{Cb}
4	4.93 ± 0.01 ^{Da}	5.00 ± 0.01 ^{Bb}	5.03 ± 0.01 ^{Ac}
5	5.01 ± 0.01 ^{Ea}	5.02 ± 0.02 ^{ABa}	5.01 ± 0.01 ^{Ba}

Different capital letters in the same column means diff statistic ($p < 0.05$) in the same day of shelf life for different samples. Different small letters in the same line means diff statistic ($p < 0.05$) for the same sample in different days of shelf life.

Table 63 shows the color evaluation of pumpkin pulp, in general no significant color changes were observed. Usually, samples processed by pasteurization (sample 2) presented a darker color, as it can be observed by the lower value in L* parameter. For the other samples (samples 1, 3, 4 and 5), the same trend was observed over the 45 days of shelf life, a slight increase in the L* value, representing a lighter color for the pulps. As expected, HPP did not change the color of the samples. Same results were previously reported by Contador et al. (2014) in pumpkin puree (*Cucurbita moschata* var. Butternut) treated by HPP (400 – 600 MPa), according to the authors the HPP treatment presented advantage compared with thermal treatment, with less influence in color modifications. Pumpkin cubes (*Cucurbita moschata* var. Butternut) showed reductions in their L*, a*, and b* values after thermal treatments, consistent with this study's findings (except for b*). According to Pimpaporn et al. (2007), the reduction in L* may be influenced by starch gelatinization due to its clarity-like characteristic, what could also apply for this study once in the industrial mix added to the pumpkin pulp, starch was one of the ingredients.

Table 63. Color evaluation during 45 days of shelf life of pumpkin pulp.

Days	Sample	L*	a*	b*	RGB
1	1	47.09 ± 0.58 ^{Aa}	42.70 ± 0.31 ^{Aa}	53.80 ± 0.15 ^{ABa}	
	2	43.82 ± 0.81 ^{Da}	42.80 ± 0.52 ^{Aa}	54.10 ± 0.74 ^{BCa}	
	3	47.05 ± 0.33 ^{Ca}	41.31 ± 0.06 ^{Ba}	56.05 ± 0.38 ^{Aa}	
	4	48.75 ± 0.77 ^{BCa}	40.45 ± 0.86 ^{Ba}	54.06 ± 0.36 ^{ABa}	
	5	49.38 ± 1.31 ^{Ab}	39.03 ± 0.85 ^{Ca}	51.03 ± 1.34 ^{Ca}	
21	1	50.6 ± 0.95 ^{Bb}	39.86 ± 0.34 ^{Bb}	52.25 ± 0.65 ^{Cb}	
	2	48.88 ± 0.77 ^{Cb}	43.49 ± 1.18 ^{Aa}	58.12 ± 0.65 ^{Aa}	
	3	49.67 ± 1.09 ^{BCb}	39.90 ± 0.32 ^{Ba}	54.78 ± 0.62 ^{Ba}	
	4	50.83 ± 0.52 ^{Bb}	37.26 ± 0.27 ^{Cb}	51.72 ± 0.53 ^{Cb}	
	5	55.10 ± 0.76 ^{Aa}	37.04 ± 0.36 ^{Cb}	48.87 ± 0.63 ^{Db}	
45	1	52.26 ± 0.78 ^{Ab}	37.3 ± 0.34 ^{Cc}	49.12 ± 0.43 ^{Cc}	
	2	48.22 ± 0.51 ^{Cb}	39.2 ± 0.45 ^{Ab}	56.75 ± 0.61 ^{Ab}	
	3	51.52 ± 0.57 ^{ABb}	38.12 ± 0.25 ^{Bb}	52.47 ± 0.41 ^{Bb}	
	4	51.26 ± 0.55 ^{Bb}	35.00 ± 0.31 ^{Dc}	49.00 ± 0.54 ^{Cc}	
	5	50.52 ± 0.57 ^{Bb}	34.78 ± 0.39 ^{Dc}	47.57 ± 0.60 ^{Db}	

Different capital letters in the same day of shelf life in between different samples means difference statistic ($p < 0.05$). Different small letters in different days of shelf life and for the same samples means difference statistics ($p < 0.05$).

Texture profile analysis (TPA)

Table 64 shows the TPA for the pumpkin pulp during shelf life, represented by the parameters firmness, elasticity, cohesion, gumminess, chewiness and stickiness. The sample 2 presented the most different characteristics in terms of firmness when compared with all the other samples (≤ 361 % higher than the other samples), showing a significant impact of thermal treatment compared to HPP treatment. This could be attributed to the effect of elevated temperature on starch properties, since starch gelatinization begins at 60 °C (Rittenauer et al., 2021), and it will bring consequently considerable food texture modifications resulted from physicochemical changes in cell-wall materials, as the gelatinization of starch (Kadam et al., 2015). On the other hand, samples 1, 3, 4 and 5 processed by HPP were not exposed to elevated temperature (≤ 30 °C), showing the lower firmness properties. High pressure can in fact reduce the firmness/hardness of plant tissue, led by cell wall breakdown and rupture, degradation of pectin and loss of turgor (Dhenge et al., 2023). Similarly, Rinaldi et al. (2023) found out that pressure levels at 200 - 600 MPa affected the texture of pumpkin slices, the firmness was reduced significantly ($p < 0.05$) in levels of 19 %, 29 %, 45 %, 51 % and 55 %, respectively at 200, 300, 400, 500 and 600 MPa.

It is widely known that during heating of food starch, the granules undergo of gelatinization, a process when starch granules swell up in the presence of water and heat (Balakrishna et al., 2020) and this process will strongly influence not only the properties and structural organization of the starch (Yan et al., 2024) but consequently the texture properties (firmness, stickiness, gumminess) of food that contains starch.

Another important parameter in texture properties of food is the chewiness, which quantifies the energy required for chewing a sample until it can be swallowed, and it is strongly influenced by firmness (Xu et al., 2020). Similarly for firmness, gumminess and chewiness of Samples 2 (pasteurized) showed greater values compared with the HPP samples, being ≤ 360% and 493% higher, respectively. For cohesion and elasticity, no difference was observed during shelf life and between the different samples. Stickiness is referred to the force required to peel off the fraction of product adhering to the interior of the oral cavity (Kouassi et al., 2021). Similarly, for firmness and chewiness, samples processed thermally (sample 2) presented a very much higher value when compared with the HPP samples, showing values ≤ 670 % higher.

In general, for all TPA parameters tested samples processed by HPP showed less influence when compared with pasteurization (sample 2). HPP has influence in starch gelatinization but in very different pathways compared to heat, what can result in different gel texture properties. For wheat starch, for example, pressure gelatinization showed a smaller degree of granule swelling when compared with heat treatment (Liu et al., 2020),

what can result in different functional properties as well as suggested in this present study for TPA characteristics.

Table 64. Texture profile parameters from pumpkin pulp during 45 days of shelf life.

	Sample				
Day 1	**1**	**2**	**3**	**4**	**5**
Firmness	2.59 ± 0.4 ^{Cab}	8.78 ± 0.73 ^{Aa}	4.53 ± 1.28 ^{Ba}	2.3 ± 0.11 ^{Ca}	2.3 ± 0.14 ^{Ca}
Elasticity	0.98 ± 0.06 ^{Aa}	0.93 ± 0.24 ^{Aa}	1.1 ± 0.12 ^{Aa}	1.04 ± 0.04 ^{Aa}	1.04 ± 0.05 ^{Aa}
Cohesion	0.23 ± 0.26 ^{Ab}	0.38 ± 0.14 ^{Aa}	0.14 ± 0.55 ^{Aa}	0.48 ± 0.19 ^{Aab}	0.48 ± 0.25 ^{Aab}
Gumminess	0.60 ± 0.64 ^{Ba}	3.38 ± 1.46 ^{Aa}	0.67 ± 0.93 ^{Ba}	1.09 ± 0.4 ^{ABa}	1.09 ± 0.48 ^{ABa}
Chewiness	0.58 ± 0.73 ^{Aa}	3.27 ± 1.93 ^{Aa}	0.71 ± 1.05 ^{Aa}	1.14 ± 0.43 ^{Aa}	1.14 ± 0.52 ^{Aa}
Stickiness	4.28 ± 1.38 ^{Ba}	29.57 ± 1.45 ^{Aa}	4.50 ± 2.00 ^{Ba}	3.79 ± 0.74 ^{Ba}	3.79 ± 1.08 ^{Ba}
Day 21	**1**	**2**	**3**	**4**	**5**
Firmness	2.82 ± 0.3 ^{Cb}	9.33 ± 0.58 ^{Aa}	4.21 ± 0.41 ^{Ba}	2.47 ± 0.2 ^{Ca}	3.06 ± 0.19 ^{BCa}
Elasticity	1.06 ± 0.04 ^{Aa}	1.11 ± 0.12 ^{Aa}	1.01 ± 0.11 ^{Aa}	1.01 ± 0.09 ^{Aa}	0.88 ± 0.15 ^{Aa}
Cohesion	0.27 ± 0.15 ^{Ab}	0.38 ± 0.10 ^{Aa}	0.29 ± 0.08 ^{Aa}	0.35 ± 0.13 ^{Ab}	0.19 ± 0.09 ^{Ab}
Gumminess	0.76 ± 0.41 ^{Ba}	3.49 ± 1.05 ^{Aa}	1.23 ± 0.3 ^{Ba}	0.89 ± 0.39 ^{Ba}	0.58 ± 0.30 ^{Ba}
Chewiness	0.80 ± 0.40 ^{Ba}	3.90 ± 1.38 ^{Aa}	1.24 ± 0.33 ^{Ba}	0.98 ± 0.42 ^{Ba}	1.96 ± 0.38 ^{Ba}
Stickiness	3.15 ± 0.58 ^{Ba}	22.63 ± 4.8 ^{Ab}	3.85 ± 0.56 ^{Ba}	3.03 ± 0.88 ^{Ba}	3.2 ± 1.47 ^{Ba}
Day 45	**1**	**2**	**3**	**4**	**5**
Firmness	3.76 ± 0.42 ^{Cb}	9.81 ± 0.24 ^{Aa}	4.68 ± 0.28 ^{Ba}	2.71 ± 0.14 ^{Da}	2.13 ± 0.09 ^{Da}
Elasticity	1.34 ± 0.19 ^{Aa}	1.10 ± 0.00 ^{Ba}	1.03 ± 0.03 ^{Ba}	0.98 ± 0.05 ^{Ba}	1.06 ± 0.05 ^{Ba}
Cohesion	0.75 ± 0.17 ^{ABa}	0.59 ± 0.25 ^{ABCa}	0.24 ± 0.08 ^{Ca}	0.36 ± 0.21 ^{BCa}	0.87 ± 0.18 ^{Aa}
Gumminess	2.81 ± 0.72 ^{ABa}	5.87 ± 2.54 ^{Aa}	1.17 ± 0.42 ^{Ba}	0.99 ± 0.66 ^{Ba}	1.85 ± 0.39 ^{Ba}
Chewiness	3.76 ± 1.1 ^{ABa}	6.42 ± 2.78 ^{Aa}	1.22 ± 0.46 ^{Ba}	0.91 ± 0.69 ^{Ba}	0.52 ± 0.40 ^{Ba}
Stickiness	5.22 ± 2.52 ^{Ba}	20.53 ± 3.97 ^{Ab}	4.89 ± 0.51 ^{Ba}	3.64 ± 1.02 ^{Ba}	3.57 ± 0.66 ^{Ba}

Different capital letters in the same line means statistical difference ($p < 0.05$) in between the different samples for each parameter evaluated. Different small letters in the column means statistical difference ($p < 0.05$) for the same sample and same parameter evaluated.

Oxidative analysis

The mass spectrometric analysis of the extracted pumpkin pulp samples revealed a decrease in vitamin content over time. Sample A5 showed the lowest reduction in carotenoid and *gamma*-tocopherol content. However, sample 3 exhibited the highest decrease in lutein and *gamma*-tocopherol contents at 71.98% and 84.42%, respectively. The 45-day storage period significantly affected the *gamma*-tocopherol content, with losses ranging from 62.26% to 84.42%. The decrease in beta-carotene content ranged from 42.73% to 66.16%, with the lowest values found in sample 2, which was lower than all other groups, even at the initial content level (54.98 ± 7.95 mg/100 g dw) (**Table 65**). Sample 2 has a significantly lower carotenoid content than samples 1 and 3. These samples differ mainly in their treatment (HPP vs pasteurization). While sample 2 was pasteurized,

samples 1 and 3 were treated with high pressure. These results suggest that pasteurization itself has a strong influence on the degradation of carotenoids.

The TP in the pumpkin pulp samples at the beginning of the experiment were between 38.01 ± 2.61 and 52.55 ± 1.69 mg GAE/100 g (**Table 66**). These values were lower than those reported in the literature for *Cucurbita moschata* (Piepiórka-Stepuk et al. 2023; Armesto et al. 2020). The extraction method, fruit maturity, agricultural processes, environmental conditions during storage (e.g. temperature, light, oxygen) or the time between harvest and processing may explain these differences (Kamiloglu et al. 2024; Kulczyński et al. 2020). Samples 4 and 5 exhibit significantly higher TP compared to the other samples, probably due to the addition of natural ingredients extracted from peels and seeds, which are rich sources of phenolic compounds (Li, 2020). A non-significant decrease in TP after 45 days was observed in samples 2, 3 and 5, with sample 5 showing the lowest decrease (3.92 %). The phenolic compounds, therefore, appear to be stable over this period. In samples 1 and 4, however, significantly lower values were found after 21 days of storage, although the further decrease in TP by the end of the experiment was no longer significant. Applying high pressure or pasteurization to pumpkin pulp does not seem to have a detrimental effect on the phenolic compounds. González-Cebrino et al. (2016) investigated the impact of high-pressure processing (400, 500, and 600 MPa) on various parameters in pumpkin purée, such as color or bioactive compounds. They concluded that there was no significant difference in carotenoid and phenolic content after the treatment, which resulted in a consistent nutritional quality (González-Cebrino et al., 2016). The slight decrease in TP may also indicate low enzyme activity in the pumpkin pulp samples, which can influence the phenolic compounds. Pretreatment processes like high-pressure treatments or natural food additives (e.g. citric acid) may affect the activity of polyphenol oxidase, which causes browning or discoloration of fruits and vegetables and thus has a direct influence on the shelf life or product quality of food Jukanti 2017; González-Cebrino et al., 2016; Li et al., 2024).

Table 65. Changes in beta-carotene, lutein and *gamma*-tocopherol content in freeze-dried pumpkin pulp samples after different treatments.

Compound	Storage time (days)	Sample 1	2	3	4	5
Beta-carotene (mg/100 g dw)	0	150.01 ± 7.45^{a}	54.98 ± 7.95^{b}	138.01 ± 10.05^{ac}	123.32 ± 5.33^{c}	91.1 ± 5.33^{d}
	21	111.08 ± 27.65^{a}	48.88 ± 9.38^{b}	100.58 ± 5.70^{ac}	102.47 ± 4.39^{ac}	84.46 ± 3.49^{c}
	45	52.06 ± 1.65^{a}	n.d.	47.28 ± 6.75^{a}	41.74 ± 9.68^{a}	52.17 ± 17.32^{a}
	D%	65.30		65.74	66.16	42.73
Lutein (mg/100 g dw)	0	1.57 ± 0.07^{a}	1.21 ± 0.32^{b}	3.74 ± 0.11^{c}	1.46 ± 0.01^{ab}	0.87 ± 0.10^{d}
	21	1.17 ± 0.12^{ad}	1.53 ± 0.11^{b}	2.52 ± 0.16^{c}	1.40 ± 0.31^{ab}	0.99 ± 0.15^{d}
	45	0.54 ± 0.05^{a}	0.58 ± 0.09^{a}	1.05 ± 0.02^{b}	0.90 ± 0.07^{c}	0.63 ± 0.07^{a}
	D%	65.81	51.87	71.98	38.09	27.35
Gamma-tocopherol (mg/100 g dw)	0	0.131 ± 0.008^{a}	0.132 ± 0.003^{a}	0.186 ± 0.002^{b}	0.137 ± 0.02^{a}	0.080 ± 0.020^{c}
	21	0.084 ± 0.004^{a}	0.093 ± 0.003^{b}	0.077 ± 0.003^{c}	0.062 ± 0.005^{d}	0.064 ± 0.002^{d}
	45	0.031 ± 0.002^{a}	0.037 ± 0.001^{b}	0.029 ± 0.001^{c}	0.030 ± 0.002^{ac}	0.030 ± 0.001^{ac}
	D%	76.13	72.27	84.42	78.10	62.26

n.d.: not detected. dw: dry weight, D%: degradation percentage. Different letters indicate significant differences within a row (ANOVA, $p < 0.05$, Tukey-HSD).

Table 66. Changes in total phenolic content (TP, mg GAE/100g dw) in freeze-dried pumpkin pulp samples after different treatments**.**

	Storage time (days)	Sample 1	2	3	4	5
TP (mg GAE/100g dw)	0	48.86 ± 1.13^{Aa}	42.52 ± 2.02^{Aa}	38.01 ± 2.61^{Ab}	52.49 ± 4.20^{Ac}	52.55 ± 1.69^{Ac}
	21	42.89 ± 1.78^{Ba}	40.54 ± 2.66^{Aa}	35.48 ± 1.12^{Ab}	48.75 ± 2.58^{ABc}	52.48 ± 1.87^{Ad}
	45	41.95 ± 1.55^{Bac}	38.91 ± 3.00^{Aab}	35.05 ± 0.77^{Ab}	45.87 ± 2.97^{Bc}	50.49 ± 2.73^{Ad}
	D%	14.14	8.49	7.79	12.61	3.92

TP: Total phenolic content, GAE: Gallic acid equivalent, dw: dry weight, D%: degradation percentage. Different small letters indicate significant differences within a row, different capital letters indicate significant differences within a column (ANOVA, $p < 0.05$, Tukey-HSD).

The highest FRAP values over the entire storage period were found for samples 4 and 5 (**Figure 17**). The higher TP could explain the higher antioxidant capacity of these samples. Studies have shown that antioxidant capacity is positively correlated with phenolic content (Mokhtar et al., 2021; Wu et al., 2010; Li, 2020). While the antioxidant capacity of sample 5 increased significantly after 21 days, no significant difference was observed in the other pumpkin pulp samples after this time. A major difference between the treatment groups is that samples 4 and 5 were not significantly different from the initial value after 45 days of storage. In contrast, the pumpkin pulp samples 1, 2 and 3 showed a significant increase in antioxidant capacity after this storage period. Corleto et al. (2018) also reported a higher antioxidant capacity when storing arugula juice at different temperatures for 32 days. According to the authors, it may be attributed to the degradation of higher phenolic or flavonoid glucosides to lower phenolic components or aglycone moieties, respectively. The present results suggest that the antioxidant capacity and TP do not deteriorate after 45 days at 5 °C. This indicates that the two different treatment processes (HPP, pasteurization) and the added substances (natural ingredient and industrial mix) contribute to maintaining the oxidative stability of pumpkin pulp.

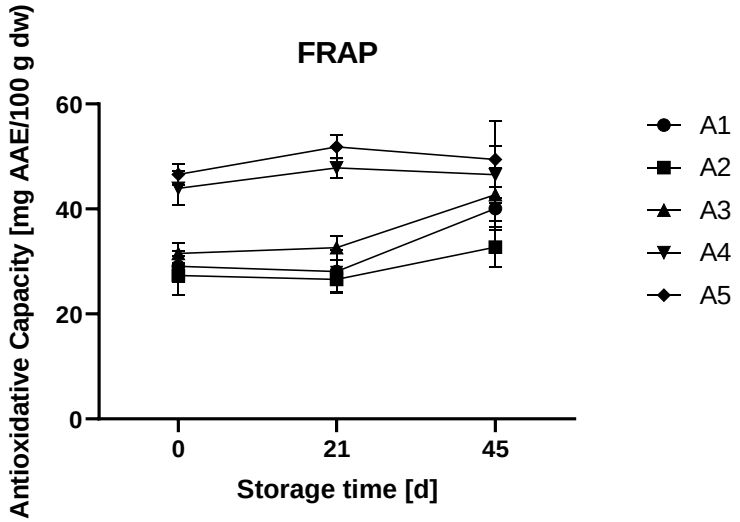

Figure 17. Changes of antioxidative capacity in freeze-dried pumpkin pulp samples after different treatments.
Dw: dry weight, AAE: Ascorbic Acid Equivalent

Proximate and chemical composition

Table 67 shows the proximate composition, in which two storage times were analyzed, namely 0 and 45 days, and five different treatments, namely high-pressure treatment without preservative (HPP) considered one control treatment, pasteurization without preservative (PAS) considered the second control, HPP with potassium sorbate

(HPP+PS), HPP with peel extract at 10 g/kg (HPP+Peel) and finally HPP with a combination of PS and peel (HPP+PS+Peel). Considering the nutritional value, the carbohydrates were the most abundant nutrient, followed by proteins, as expected by a previous study (Leichtweis, et al., 2023). A significant interaction was sought for proteins, crude fat and carbohydrates. Thus, only general tendencies could be extracted from the EMM for crude fat, shown in **Figure 18**. In the figure, it is clear that there was a higher amount of fat at T0 (blue line), then 45 days (red line), except for the sample incorporated with HPP+PS, where there were no differences among the samples. The lowest amount of fat was sought for HPP+PS+Peel at 45 days. This variation can be explained by the very low fat content in the pulp.

Table 67. Proximate composition (in g/100g fw) and energetic value (in kcal) of the formulations, corresponding to different treatments and preservatives.

		Proteins	**Crude Fat**	**Carbohydrates**	**Ash**	**Energy**
Storage Time (ST)	0 Days	5.1 ± 0.7	0.41 ± 0.03	90.4 ± 0.9	4.2 ± 0.4	385 ± 2
	45 Days	5.0 ± 0.8	0.35 ± 0.04	90.5 ± 0.8	4.2 ± 0.4	385 ± 2
***p*-value (n=15)**	Student's t-test	0.014	<0.001	0.117	0.871	0.168
Preservative Type (PT)	HPP	4.8 ± 0.2	0.38 ± 0.05	91.2 ± 0.2	3.6 ± 0.1^{d}	387.6 ± 0.7^{a}
	PAS	4.7 ± 0.3	0.36 ± 0.02	91.0 ± 0.4	4.0 ± 0.1^{c}	385.9 ± 0.5^{b}
	HPP+PS	3.97 ± 0.08	0.43 ± 0.01	90.9 ± 0.2	4.7 ± 0.2^{a}	383.3 ± 0.6^{d}
	HPP+Peel	5.5 ± 0.1	0.37 ± 0.03	89.7 ± 0.3	$4.4 \pm 0.2^{a,b}$	$384.2 \pm 0.8^{c,d}$
	HPP+PS+Peel	6.07 ± 0.03	0.38 ± 0.09	89.4 ± 0.2	$4.1 \pm 0.2^{b,c}$	$385.3 \pm 0.7^{b,c}$
***p*-value (n=6)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001
ST×PT (n=30)	*p*-value	<0.001	<0.001	0.014	0.623	0.878

Each letter in each column represents a significant difference among samples, obtained from Tukey's or Tamhane's T2 test. The asterisk (*) represents a statistically significant difference by means of Student's t-test. All assays were performed using a significance of 0.05.

Figure 18. EMM plots for crude fat. HPP – high pressure preservative; PAS – pasteurization; HPP+PS – high pressure preservative and potassium sorbate; HPP+Peel – high pressure preservative and peel extract; HPP+PS+Peel – high pressure preservative with potassium sorbate and peel extract.

Table 68 contains the different soluble sugars found in the samples, totalling four individual sugars, in which the most abundant was sucrose and the least prevalent, trehalose. In terms of the statistical analysis, a significant interaction was found for all sugars, including the sum of these carbohydrates. Neither the EMM and the PES allowed for any tendencies to be extracts, meaning that both storage time and the incorporation of the preservatives and processing methods highly influenced the results.

Table 68. Soluble sugars detected in the different formulations (in g/100g dw).

		Fructose	Glucose	Sucrose	Trehalose	Total
Storage Time (ST)	0 Days	10 ± 4	9 ± 4	20 ± 9	1.6 ± 0.5	40 ± 3
	45 Days	11 ± 3	10 ± 3	21 ± 3	2.4 ± 0.5	45 ± 5
***p*-value (n=15)**	Student's t-test	<0.001	<0.001	<0.001	<0.001	<0.001
Preservative Type (PT)	HPP	12 ± 4	11 ± 3	22 ± 3	2.2 ± 0.7	48 ± 4
	PAS	8 ± 2	8 ± 2	23 ± 3	2.0 ± 0.5	41 ± 1
	HPP+PS	9 ± 3	9 ± 4	20 ± 3	1.9 ± 0.8	40 ± 4
	HPP+Peel	9 ± 2	8 ± 2	25 ± 1	2.2 ± 0.7	44 ± 4
	HPP+PS+Peel	12 ± 4	13 ± 4	13 ± 10	1.6 ± 0.5	39 ± 2
***p*-value (n=6)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001
ST×PT (n=30)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001

Table 69 shows the individual fatty acids found in the third analysis, totaling 13. Of these, nine were saturated fatty acids, while two were monounsaturated and another

two were polyunsaturated fatty acids. The most abundant was C16:0 (palmitic acid), followed by C18:1 (linoleic acid). C10:0 (capric acid), was the least abundant fatty acid. Overall, as for **Table 68**, in **Table 69**, a significant interaction was sought between the two factors, not allowing for individual classifications. Neither the EMM nor the PES allowed for tendencies to be obtained.

Although statistical differences were observed, it is possible to verify that neither the change in preservatives nor the storage time substantially impacted the nutritional quality of the formulations. It is possible to verify that the quantities of nutrients are very similar and, in general, the composition profiles are not altered. This is a condition that supports the NI use as a food additive, as the Codex Alimentarius specifies that an additive should not alter the nature or qualities of the product (Laganà, P. et al., 2017). In contrast, pumpkin powders, rather than extracts, do alter the nutritional and sensory characteristics of products, even if these changes are positive (Mala et al., 2018; Minarovicova et al., 2017).

Table 69. Individual fatty acids detected in the formulations (in relative %).

		C10:0	C12:0	C14:0	C15:0	C16:0	C18:0	C18:1n9t	C18:1n9c	C18:2n6c	C18:3n3	C20:0	C22:0	C24:0
Storage Time (ST)	0 Days	0.2 ± 0.3	1.3 ± 0.3	1.9 ± 0.4	0.1 ± 0.3	43 ± 4	7 ± 3	8 ± 2	14 ± 7	6 ± 4	3.3 ± 0.7	0.7 ± 0.9	2.5 ± 0.9	3 ± 2
	45 Days	n.d.	1.0 ± 0.3	1.4 ± 0.3	0.1 ± 0. 2	46 ± 4	7 ± 3	7 ± 2	19 ± 3	7 ± 4	3.4 ± 0.8	0.8 ± 0.7	2.0 ± 0.4	3 ± 2
***p*-value (n=15)**	Student's t-test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.265	<0.001	<0.001	<0.001	<0.001
Preservative Type (PT)	HPP	0.4 ± 0.4	1.2 ± 0.4	2.0 ± 0.9	0.5 ± 0.1	43.6 ± 0.1	8.8 ± 0.1	6.0 ± 0.7	15 ± 10	11 ± 1	3.9 ± 0.6	0.6 ± 0.6	2.0 ± 0.4	n.d.
	PAS	n.d.	1.37 ± 0.06	1.88 ± 0.09	n.d.	42 ± 18	1.5 ± 0.2	9 ± 1	15 ± 1	7 ± 1	3.2 ± 0.4	n.d.	1.72 ± 0.03	1.71 ± 0.06
	HPP+PS	n.d.	1.2 ± 0.3	1.6 ± 0.3	n.d.	43 ± 3	8.0 ± 0.7	6.2 ± 0.5	20 ± 1	1.6 ± 0.2	4.2 ± 0.2	n.d.	1.9 ± 0.5	4 ± 3
	HPP+Peel	n.d.	0.79 ± 0.05	1.3 ± 0.2	n.d.	42 ± 4	8.2 ± 0.4	10 ± 2	18.9 ± 0.6	8.8 ± 0.6	2.7 ± 0.5	1.21 ± 0.02	2.9 ± 0.8	4 ± 2
	HPP+PS+Peel	n.d.	1.3 ± 0.2	1.7 ± 0.1	n.d.	43 ± 5	8.6 ± 0.2	6.6 ± 0.5	13 ± 8	7 ± 4	2.6 ± 0.3	1.9 ± 0.4	2.9 ± 0.6	3 ± 1
***p*-value (n=6)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×PT (n=30)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

n.d.: not detected.

5.1.4. Conclusions

Throughout the 45 days, all five samples demonstrated excellent microbial stability, with no detectable growth of microorganisms for all samples conditions. The achievement of the shelf-life extension is attributed to the process of treatments and its combinations with including the natural ingredient and the industrial mix. Notably, sample 4, which contained the NI but no potassium sorbate, showed no microbial growth, proving the natural ingredient's effectiveness as a preservative when combined with high-pressure processing (HPP) as well as the industrial mix, enhancing the shelf life. This result is significant because previous studies showed that HPP alone could not achieve the same level of microbial inactivation, resulting in a shorter shelf life of 15 days compared to 45 days in this study. Besides that, sample 5, which contained the natural ingredient and a 50% reduction in potassium sorbate, also exhibited no microbial growth. This suggests that potassium sorbate could potentially be reduced by 50 % or fully replaced by the natural ingredient (sample 4), without compromising the product's microbial stability. Besides that, quality attributes also positively demonstrated that the combinations of HPP as a non-thermal technique have a potential synergistic effect when combined with natural-based preservative and industrial mix, color kept stable during shelf life, oxidative stability throughout antioxidant capacity showed that samples added with the natural ingredient (sample 4 and 5) had significant higher FRAP values when compared to the ones without NI. Additionally, the presence of the natural ingredient in the samples, along with the varying thermal processes, did not compromise their nutritional quality. The two-factor analysis did not explain a significant influence of either the natural ingredient or the storage time on the evaluated quality parameters.

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CHAPTER 6

Final Considerations and Future Perspectives

This chapter includes the general conclusions of the research, embracing the main contributions achieved through this doctoral thesis and future perspectives

6.1. Final considerations

The development and application of pumpkin by-products as natural food preservatives achieved in this work point to several promising directions. First, the optimization of extraction methods demonstrates the potential to maximize the yield and bioactivity of phenolic compounds and other bioactive constituents, paving the way for more efficient and sustainable production processes. Expanding research on the genetic diversity and biochemical profiles of local and commercial pumpkin genotypes reveals important sources of high-value bioactive compounds, further enhancing their industrial applications. Furthermore, the integration of these natural ingredients into preservation strategies, particularly in combination with innovative non-thermal techniques such as high-pressure processing (HPP), offers a synergistic approach to reduce or eliminate artificial preservatives such as potassium sorbate. This not only aligns with consumer demand for natural and healthy products but also contributes to sustainability through biowaste valorization and the circular economy promotion.

*Characterization of different pumpkin varieties (*Cucurbita *spp.)*

Pumpkin fruit by-products, encompassing various genotypes and geographic origins, demonstrated significant potential as valuable sources of bioactive compounds with diverse industrial applications. Despite variability in environmental conditions, agronomic practices, and genotypic differences, the pulps exhibited high nutritional value, while all analyzed pumpkin byproducts showed promising bioactivities, including antioxidant and antimicrobial properties. Notably, when comparing commercial varieties obtained in Portugal and Algeria, the 'Butternut squash' presented the strongest antioxidant properties across countries, while the peel of the Portuguese 'Common Pumpkin' stood out for its diverse and abundant phenolic profile, and certain fibrous strands and seeds exhibited superior antimicrobial activity. Similarly, Greek local landraces revealed high variability in agronomic performance and chemical composition, emphasizing the potential of local landraces from 'Trikala' and 'Laconia' for high fruit yield and bioactive compound content, including tocopherols and linoleic acid.

In addition, the characterization of pumpkin by-products highlighted their richness in phenolic compounds, tocopherols, and organic acids, which not only enhance their nutraceutical value but also underscore their potential as valuable sources of high value-added compounds. Moreover, the characterization of locally cultivated genotypes provides useful insights into the biochemical and nutritional diversity among pumpkin genotypes, thus contributing to the valorization of local genetic resources and promoting more effective use of their nutritional diversity while supporting initiatives aimed at food security and the development of more sustainable crops.

Optimization of heat- and ultrasound-assisted extraction

The comparative study of heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE) methodologies demonstrated their potential to efficiently obtain bioactive extracts from pumpkin peels for use as natural food preservatives. HAE proved more effective for 'TL', 'Voutirato,' and Portuguese 'Butternut squash', enabling optimized conditions at low temperatures, minimal ethanol concentrations, and reduced extraction times, while UAE was more efficient for the 'Leuka Melitis' genotype, utilizing only water and shorter processing times. Optimization using response surface methodology (RSM) allowed precise determination of conditions for maximum extraction yield and phenolic content, reducing power, minimizing time, energy consumption, and solvent use.

The extracts obtained under optimal conditions, particularly from the 'TL' and 'Voutirato' landraces exhibited high phenolic content and demonstrated significant antioxidant and antimicrobial activities, effectively inhibiting bacterial and fungal growth without cytotoxic effects on non-tumor liver cells. The main milestone achieved in this task was obtaining the extract from butternut squash peel by HAE, whose optimal global conditions were established at 84 minutes, 30 °C, and 0% ethanol, rendering 1.49 g/100 g of dry residue and 169 mg/g of total phenols. The elimination of ethanol as a solvent and the low temperature demand, together with the high relevance of the butternut variety in the world and especially in Portugal, meant that this extract was chosen for continued work in the following steps.

These findings underscore the potential of pumpkin peel extracts as natural food preservatives. Furthermore, the efficient optimization of extraction protocols, particularly the elimination of ethanol in some cases, enhances the sustainability and economic value of pumpkin by-products, paving the way for their valorization in food preservation and other industrial applications.

Development of pumpkin pulp formulations with natural preservatives

The incorporation of natural extracts derived from butternut squash peels, obtained under global optimum conditions by HAE, and Greek pumpkin seeds, obtained under standard extraction conditions, into pumpkin pulp formulations demonstrated significant advancements in natural preservation, sustainability, and reduced reliance on artificial preservatives like potassium sorbate. In the traditional formulation of pasteurized pumpkin pulp, replacing 50% of potassium sorbate with optimized peel extract demonstrated effectiveness in preserving nutritional quality, color and microbiological stability for up to 45 days, extending shelf life beyond the company standard of 30 days. In addition, when comparing the final formulation partially

preserved by the peel extract with the traditional formulation, very promising results were observed, since the aroma, taste, texture and overall acceptance attributes did not present statistically significant differences between the formulations. This approach meets consumer demands for natural and healthy products, while adding value to agro-industrial byproducts. This also aligns sustainability goals and supports the concept of circular economy concept, valuing bioresidues and utilizing eco-friendly extraction methods.

In formulations using high-pressure processing (HPP) as a non-thermal preservation technique, the combination of natural ingredients with HPP proved even more effective. Formulations containing the natural extract maintained excellent microbial stability for 45 days (extended shelf life), in addition to demonstrated higher antioxidant capacity (FRAP values), stable color, and no compromise to nutritional quality throughout the shelf life. With the use of this technology, it was possible to completely eliminate the use of potassium sorbate.

Together, these findings confirm the feasibility of replacing or significantly reducing potassium sorbate in pumpkin pulp formulations through the integration of natural pumpkin byproduct extracts, both in traditional pasteurized and HPP-treated products. This innovation not only enhances food quality and shelf life but also represents a critical step toward sustainable and efficient food production, paving the way for future research and commercial applications in natural food preservation.

6.2. Future perspectives and global conclusions

Future perspectives in the development and application of pumpkin byproducts as natural food preservatives point toward several promising directions. First, the optimization of extraction methods, such as heat-assisted and ultrasound-assisted extraction, has demonstrated the potential for maximizing the yield and bioactivity of phenolic compounds and other bioactive constituents, paving the way for more efficient and sustainable production processes. Expanding research into the genetic diversity and biochemical profiles of local and commercial pumpkin genotypes could uncover additional sources of high-value bioactive compounds, further enhancing their industrial applications. Moreover, integrating these natural ingredients into preservation strategies, particularly in combination with innovative non-thermal techniques like high pressure processing (HPP), offers a synergistic approach to reducing or eliminating artificial preservatives such as potassium sorbate. This not only aligns with consumer demand for natural and health-conscious products but also contributes to sustainability through waste valorization and the circular economy. Future studies should focus on scaling up these findings for industrial applications, exploring the safety and regulatory aspects of these natural ingredients, and investigating their potential in other fields, such as

pharmaceuticals and cosmetics, to fully leverage their versatility and value.

Although the results obtained in this research are promising, further studies are needed to evaluate the use of pumpkin by-product extracts rich in bioactive compounds as natural preservatives. It is essential to investigate the impact of the addition of these extracts on the sensory characteristics of the product and on consumer acceptance, by trained panel and market research. In the technological field, the stability of these preservatives during the marketing process needs to be further investigated. Additionally, a more profound research on the use of stabilization technologies, such as a controlled release systems, may be relevant to improve the stability of natural preservatives in different types of processing, making them more resistant to possible degradation that may occur during the distribution and storage of products. These technologies could protect the extracted bioactive compounds, ensuring greater efficacy and durability in the formulations.

In addition, it is important to study the application of these preservatives in other food matrices that undergo different preservation processes, such as fermentation, freezing and packaging, to better understand the stability parameters of these compounds. The application of these natural preservatives can be expanded beyond the food industry, and not only in pumpkin-based products, with potential for use in cosmetics and pharmaceuticals, given their antioxidant and antimicrobial activity.

Another relevant point is to advance in vitro bioactivity studies to in vivo, to understand more deeply the effects of these compounds on living organisms and evaluate their safety and efficacy in different contexts. With special attention to toxicity studies, which, although conducted *in vitro* in this work, genotoxicity and mutagenic assays, as well as *in vivo* studies, may also be necessary to determine their average daily intake. In the same sense, studies on the presence of pesticides and contaminants in biowaste should be carried out to determine the safety of these natural preservatives.

This PhD research successfully achieved its objectives, focusing on the valorization of the pumpkin value chain. The project developed a new pumpkin pulp formulation, incorporating a natural preservative extracted from the peels of the fruit.

Key achievements of this work include:

- Comprehensive screening of the chemical composition, antioxidant, and antimicrobial properties of pumpkin fruit pulp and by-products.
- Optimized protocols for the extraction of preservative compounds from pumpkin by-products.
- Nutritional analysis data of the developed pumpkin fruit pulp formulation.
- Submission of a national patent for an innovative method of extraction of preservatives from pumpkin by-products (peel).

- Development of a novel, shelf-stable pumpkin fruit pulp, preserved with a natural ingredient (able to reduce 50% of the artificial ingredient) and meeting high-quality and safety standards.
- Advanced preservation technologies (HPP) with natural ingredient maintaining the sensory and nutritional properties of the pulp for 45 days of shelf life (normally 30 days).
- Development of a market-ready, sustainable pumpkin pulp product with consumer appeal with a reduction of 50% artificial preservatives.

Finally, this work has a significant social, economic and environmental impact, which tends to benefit Mediterranean farmers, the food industry and other industrial sectors. The project also involved market stakeholders, including consumers, and the scientific community. Also contributing to technological transfers of research to local producers in Europe, promoting circular economy, sustainability and natural preservation markets. This approach can open new opportunities to explore the applications of these extracts, promoting a more sustainable and ecological use of fruit biowaste, in line with the demands for more natural and environmentally responsible solutions. To this end, collaborative efforts between researchers, industry stakeholders and policy makers are essential to overcome existing barriers and unlock the full potential of valorizing by-products and improving natural additives.

ANNEXES

Supplementary material of research paper 3.3

Table A. Genotypes of pumpkin cultivated in Greece.

Sample Code	Pumpkin Genotypes	Harvest Date	Extraction Yield (g/100g dw)			
			Seeds	Deffated seeds	Fibrous strands	Peels
V1	Fytro FS-243	26 October–19 November 2021	8.7 ± 0.3 ^{c}	4.38 ± 0.09 ^{f}	51 ± 2 ^{f,g,h}	31 ± 2 ^{g}
V2	Landrace from the region of Trikala	25 August–19 October 2021	7.3 ± 0.4 ^{e,f}	6.4 ± 0.2 ^{d}	n.a.	n.a.
V2 T	Landrace from the region of Trikala (Turbinate)	25 August–19 October 2021	n.a.	n.a.	52.7 ± 0.5 ^{f,g}	42 ± 1 ^{c,d}
V2 C	Landrace from the region of Trikala (Cylindrical)	25 August–19 October 2021	n.a.	n.a.	59.7 ± 0.4 ^{c,d}	36.9 ± 0.6 ^{e}
V3	Big Max	25 August–19 October 2021	16.2 ± 0.8 ^{a}	6.8 ± 0.3 ^{c}	47.2 ± 0.6 ^{h}	44 ± 2 ^{b,c}
V4	Local landrace “Nychaki”	23 September 2021	9.84 ± 0.03 ^{b}	4.5 ± 0.1 ^{f}	n.a.	n.a.
V4 C	Local landrace “Nychaki” (Cylindrical)	23 September 2021	n.a.	n.a.	62 ± 1 ^{b,c}	33 ± 2 ^{f}
V4 R	Local landrace “Nychaki” (Round)	23 September 2021	n.a.	n.a.	50.1 ± 0.8 ^{g,h}	40 ± 1 ^{d}
V5	Local landrace “Leuka Melitis”	5 October–19 November 2021	7.4 ± 0.2 ^{e}	5.3 ± 0.1 ^{e}	n.a.	n.a.
V5 F	Local landrace “Leuka Melitis” (Flattened)	5 October–19 November 2021	n.a.	n.a.	36.1 ± 0.3 ^{j}	29.2 ± 1 ^{g}
V5 R	Local landrace “Leuka Melitis” (Round)	5 October–19 November 2021	n.a.	n.a.	42.5 ± 0.9 ^{i}	26.2 ± 1 ^{h}
V6	Local landrace from the region of Lakonia	25 August 2021	9.7 ± 0.2 ^{b}	8.15 ± 0.06 ^{a}	62 ± 1 ^{b,c}	53.1 ± 0.8 ^{a}
V7	Local landrace from the region of Lakonia	25 August 2021	7.8 ± 0.4 ^{d,e}	8.4 ± 0.3 ^{a}	n.a.	n.a.
V7 P	Local landrace from the region of Lakonia (Pyriform)	24 September–19 November 2021	n.a.	n.a.	59 ± 2 ^{c,d}	34 ± 1 ^{f}
V7 F	Local landrace from the region of Lakonia (Flattened)	24 September–19 November 2021	n.a.	n.a.	83 ± 4 ^{a}	35 ± 1 ^{f}
V8	Local landrace from the region of Lakonia	22 November 2021	6.7 ± 0.3 ^{f}	6.3 ± 0.1 ^{d}	64 ± 3 ^{b}	35.5 ± 0.3 ^{e,f}
V9	Local landrace “Makedonika prasina”	22 November 2021	9.0 ± 0.3 ^{c}	7.7 ± 0.3 ^{b}	n.a.	n.a.
V9 C	Local landrace “Makedonika prasina” (Cylindrical)	22 November 2021	n.a.	n.a.	58 ± 3 ^{d,e}	22.4 ± 0.6 ^{i}
V9 R	Local landrace “Makedonika prasina” (Round)	22 November 2021	n.a.	n.a.	55 ± 1 ^{e,f}	44.9 ± 0.9 ^{b}
V10	Local landrace from the region of Laconia	22 November 2021	8.1 ± 0.4 ^{d}	6.6 ± 0.2 ^{c,d}	54 ± 3 ^{e,f}	35 ± 1 ^{e,f}
V11	Local landrace “Voutirato”	22 November 2021	n.a.	n.a.	61 ± 3 ^{b,c,d}	41 ± 1 ^{d}

n.a. – not applicable. ANOVA analysis – In each column and for the same fruit part, different letters mean significant differences among the tested genotypes according to Tukey’s HSD test ($p < 0.05$).

Table B. Antibacterial and antifungal activity of the seeds of different pumpkin genotypes expressed in mg/mL.

SEEDS	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	Streptomicin 1 mg/mL		Methicilin 1 mg/mL		Ampicillin 10 mg/mL	
	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria																
Enterobacter Cloacae	5	5	5	5	5	5	5	5	5	5	0.007	0.007	n.t	n.t	0.15	0.15
Escherichia coli	>10	>10	>10	>10	>10	>10	10	10	10	10	0.01	0.01	n.t	n.t	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.06	0.06	n.t	n.t	0.63	0.63
Salmonella enterica	10	10	10	10	10	10	10	10	10	10	0.007	0.007	n.t	n.t	0.15	0.15
Yersinia enterocolitica	2.5	2.5	2.5	2.5	5	10	5	10	10	10	0.007	0.007	n.t	n.t	0.15	0.15
Gram-positive bacteria																
Bacillus cereus	10	>10	10	10	10	>10	>10	10	10	10	0.007	0.007	n.t	n.t	n.t.	n.t.
Listeria monocytogenes	5	10	10	5	10	5	10	5	5	5	0.007	0.007	n.t	n.t	0.15	0.15
Staphylococcus aureus	10	10	10	10	>10	>10	>10	10	10	10	0.007	0.007	0.007	0.007	0.15	0.15

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	Ketoconazole	
Fungi	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MFC
Aspergillus brasiliensis	>10	>10	>10	>10	>10	>10	10	10	10	10	0.06	0.125
Aspergillus fumigatus	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.5	1

MIC: Minimum inhibitory concentration; MBC: Minimal bactericidal concentration; MFC: Minimal fungicidal concentration; n.t: not tested.

Table C. Antibacterial and antifungal activity of the peels of different pumpkin genotypes expressed in mg/mL.

PEELS	V1	V2 T	V2 C	V3	V4 C	V4 R	V5 F	V5 R	V6	V7 P	V7 F
	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC
Gram-negative bacteria											
Enterobacter Cloacae	>10	2.5	>10	>10	>10	>10	>10	10	5	>10	10
Escherichia coli	10	2.5	10	10	10	10	10	10	5	10	10
Pseudomonas aeruginosa	10	10	10	10	>10	>10	>10	>10	>10	10	>10
Salmonella enterica	>10	10	>10	>10	>10	>10	>10	>10	10	>10	>10
Yersinia enterocolitica	>10	5	>10	>10	>10	>10	>10	5	10	>10	10
Gram-positive bacteria											
Bacillus cereus	>10	10	>10	>10	>10	>10	>10	>10	10	>10	>10
Listeria monocytogenes	5	2.5	>10	>10	>10	>10	>10	>10	5	>10	>10
Staphylococcus aureus	10	2.5	>10	>10	>10	10	10	10	5	>10	10

	V8	V9 C	V9 R	V10	V11	Streptomicin 1 mg/mL		Methicilin 1 mg/mL		Ampicillin 10 mg/mL	
	MIC	MIC	MIC	MIC	MIC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria											
Enterobacter Cloacae	10	2.5	>10	>10	5	0.007	0.007	n.t	n.t	0.15	0.15
Escherichia coli	10	2.5	10	10	5	0.01	0.01	n.t	n.t	0.15	0.15
Pseudomonas aeruginosa	>10	10	>10	10	>10	0.06	0.06	n.t	n.t	0.63	0.63
Salmonella enterica	>10	10	>10	>10	10	0.007	0.007	n.t	n.t	0.15	0.15
Yersinia enterocolitica	5	5	>10	>10	10	0.007	0.007	n.t	n.t	0.15	0.15
Gram-positive bacteria											
Bacillus cereus	>10	10	>10	>10	10	0.007	0.007	n.t	n.t	n.t	n.t
Listeria monocytogenes	>10	2.5	>10	>10	5	0.007	0.007	n.t	n.t	0.15	0.15
Staphylococcus aureus	10	2.5	>10	>10	5	0.007	0.007	0.007	0.007	0.15	0.15

Fungi	V1	V2 T	V2 C	V3	V4 C	V4 R	V5 F	V5 R	V6
	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC
Aspergillus brasiliensis	5	5	5	5	5	5	5	10	5
Aspergillus fumigatus	10	10	>10	10	10	10	10	10	10

Fungi	V7 P	V7 F	V8	V9 C	V9 R	V10	V11	Ketoconazole	
	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MFC
Aspergillus brasiliensis	10	5	5	>10	5	5	5	0.06	0.125
Aspergillus fumigatus	10	10	10	10	10	10	5	0.5	1

MIC: Minimum inhibitory concentration; MBC: Minimal bactericidal concentration; MFC: Minimal fungicidal concentration; n.t: not tested.

Table D. Antibacterial and antifungal activity of the fibrous strands of different pumpkin genotypes expressed in mg/mL.

FIBROUS STRANDS	V1	V2 T	V2 C	V3	V4 C	V4 R	V5 F	V5 R	V6	V7 P	V7 F
	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC
Gram-negative bacteria											
Enterobacter Cloacae	10	10	>10	>10	>10	>10	>10	>10	10	10	10
Escherichia coli	>10	>10	10	10	10	10	10	10	10	10	10
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Salmonella enterica	>10	10	10	10	10	10	>10	10	10	10	10
Yersinia enterocolitica	10	>10	10	10	>10	10	>10	>10	>10	>10	>10
Gram-positive bacteria											
Bacillus cereus	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Listeria monocytogenes	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Staphylococcus aureus	>10	>10	10	10	10	10	10	10	10	10	10

	V8	V9 C	V9 R	V10	V11	Streptomicin 1 mg/mL		Methicilin 1 mg/mL		Ampicillin 10 mg/mL	
	MIC	MIC	MIC	MIC	MIC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria											
Enterobacter Cloacae	>10	>10	>10	10	>10	0.007	0.007	n.t	n.t	0.15	0.15
Escherichia coli	10	>10	>10	>10	5	0.01	0.01	n.t	n.t	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	10	>10	0.06	0.06	n.t	n.t	0.63	0.63
Salmonella enterica	10	10	>10	10	10	0.007	0.007	n.t	n.t	0.15	0.15
Yersinia enterocolitica	10	>10	>10	10	10	0.007	0.007	n.t	n.t	0.15	0.15
Gram-positive bacteria											
Bacillus cereus	>10	>10	>10	>10	>10	0.007	0.007	n.t	n.t	n.t	n.t
Listeria monocytogenes	>10	>10	>10	>10	>10	0.007	0.007	n.t	n.t	0.15	0.15
Staphylococcus aureus	>10	10	>10	10	10	0.007	0.007	0.007	0.007	0.15	0.15

Fungi	V1	V2 T	V2 C	V3	V4 C	V4 R	V5 F	V5 R	V6
	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC
Aspergillus brasiliensis	10	10	10	10	10	10	10	10	10
Aspergillus fumigatus	>10	>10	10	10	>10	>10	>10	>10	10

Fungi	V7 P	V7 F	V8	V9 C	V9 R	V10	V11	Ketoconazole	
	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MFC
Aspergillus brasiliensis	10	10	10	10	10	10	10	0.06	0.125
Aspergillus fumigatus	10	10	>10	>10	>10	>10	>10	0.5	1

MIC: Minimum inhibitory concentration; MBC: Minimal bactericidal concentration; MFC: Minimal fungicidal concentration; n.t: not tested.

Table E. Correlation analysis.

Compound	General		Peel		Fibers strands		Seeds						
	TBARS	**OxHLIA**	**TBARS**	**OxHLIA**	**TBARS**	**OxHLIA**	**TBARS**	**OxHLIA**	**AGS**	**CaCo2**	**MCF-7**	**PLP2**	**VERO**
(-)-epicatechin	-0.0910	-0.1971	0.1962	0.1757	-0.1623	-0.3472	-0.3161	-0.3438	**-0.9809**	0.2789	0.6621	0.3983	0.2712
cis-Chicoric acid	**-0.9703**	**0.9805**	**-0.9703**	**0.9805**	-	-	-	-	-	-	-	-	-
trans-Chicoric acid	0.6869	0.0166	0.6869	0.0166	-	-	-	-	-	-	-	-	-
Isorhamnetin-*O*-dideoxyhexosyl-hexoside	0.1445	-0.2304	0.1445	-0.2304	-	-	-	-	-	-	-	-	-
Kaempferol-*O*-deoxyhexosyl-hexoside	0.0620	0.6201	0.0620	0.6201	-	-	-	-	-	-	-	-	-
Total Flavan-3-ols	0.0281	0.6299	0.0281	0.6299	-	-	-	-	-	-	-	-	-
Total phenolic acids	0.5519	0.1193	0.5519	0.1193	-	-	-	-	-	-	-	-	-
Total flavonoids	0.2341	0.1923	0.2341	0.1923	-	-	-	-	-	-	-	-	-
Total phenolic compounds	0.1911	0.0859	0.1911	0.0859	-	-	-	-	-	-	-	-	-
Oxalic	0.5042	0.1762	0.3000	0.3500	-0.0596	0.1681	0.5220	0.5127	0.3714	-0.4240	-0.2009	-0.0179	-0.8240
Quinic	0.4284	0.1575	-0.5182	**0.9770**	0.0724	0.2703	0.6393	0.2586	0.6539	0.0474	0.0934	0.2114	-0.0514
Malic	0.2056	-0.1049	-0.0757	-0.3734	-0.0131	0.0577	-0.2836	0.2703	-0.1062	-0.0954	-0.0585	0.1954	-0.8324
Citric	0.5002	-0.1062	0.4673	-0.2091	-0.7194	0.4539	-	-	-	-	-	-	-
Fumaric	0.1599	-0.0879	-0.1627	-0.3120	0.2199	0.0087	0.8033	0.1928	0.5089	-0.2976	-0.2458	-0.2421	-0.6144
Shikinic	-0.8603	0.1771	-	-	**-0.9715**	**-0.9102**	-	-	-	-	-	-	-
Total organic acids	0.5179	0.0434	0.0440	-0.3549	-0.3398	0.1262	0.2486	0.2402	0.7335	-0.0564	-0.0640	0.1486	-0.6292
alpha-tocopherol	0.1593	-0.0674	0.1504	-0.2084	0.0493	0.2415	-0.2673	-0.2519	-0.6002	-0.8119	-0.4059	-0.6510	0.0715
beta-tocopherol	0.7703	0.8668	**-0.9969**	**0.9931**	**0.9987**	**0.9946**	-	-	-	-	-	-	-
gama-tocopherol	0.1101	0.2294	0.0703	0.1152	0.2233	0.0655	-0.2887	0.3063	0.1763	0.8091	0.3827	0.6547	0.4356
delta-tocopherol	0.5559	0.4783	0.5559	0.4783	-	-	-	-	-	-	-	-	-
Total tocopherols	0.0627	0.1629	0.0916	0.0611	0.0478	0.2184	-0.4361	0.2611	0.0581	0.7806	0.3811	0.6233	0.7294

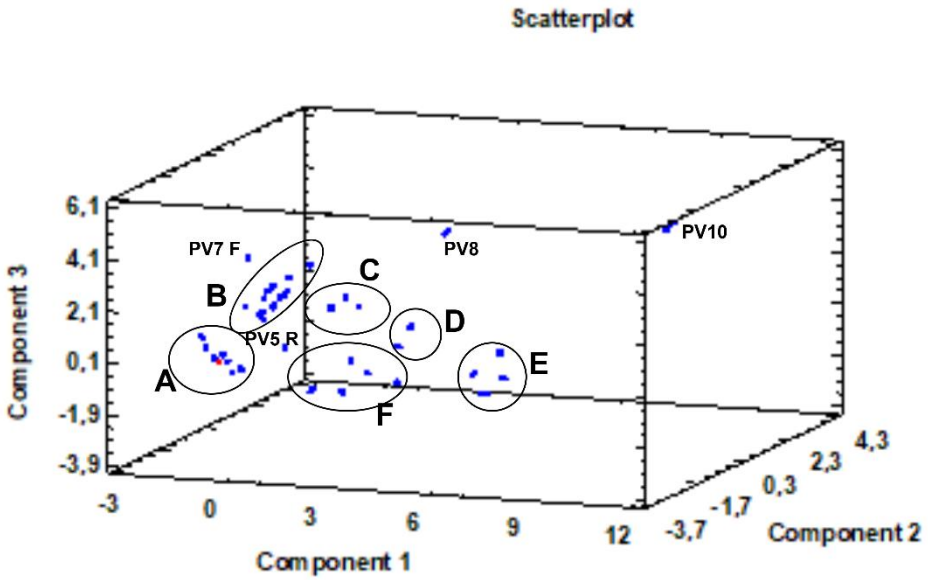

Figure A. Three-dimensional scatterplot of principal components 1, 2 and 3 for the tested samples.

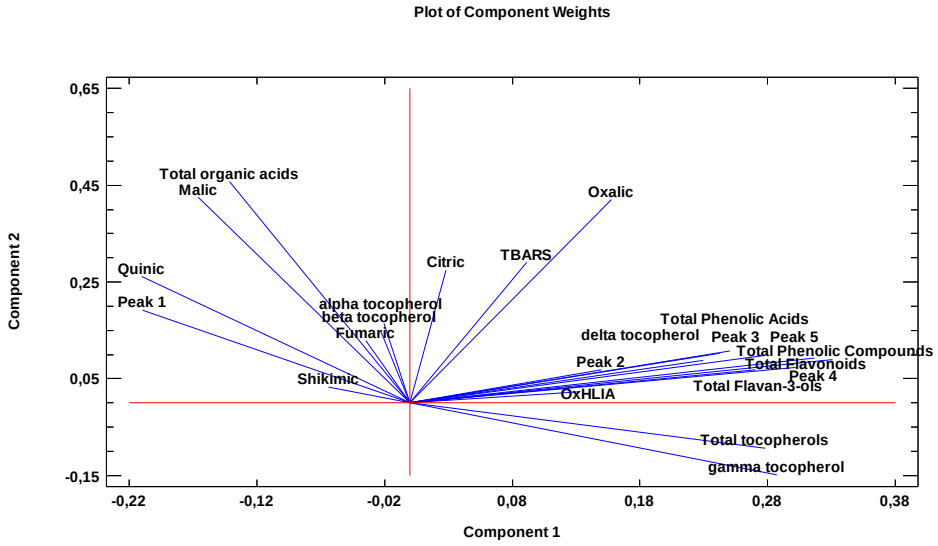

Figure B. The loading plot of principal components 1 and 2 for the tested samples.

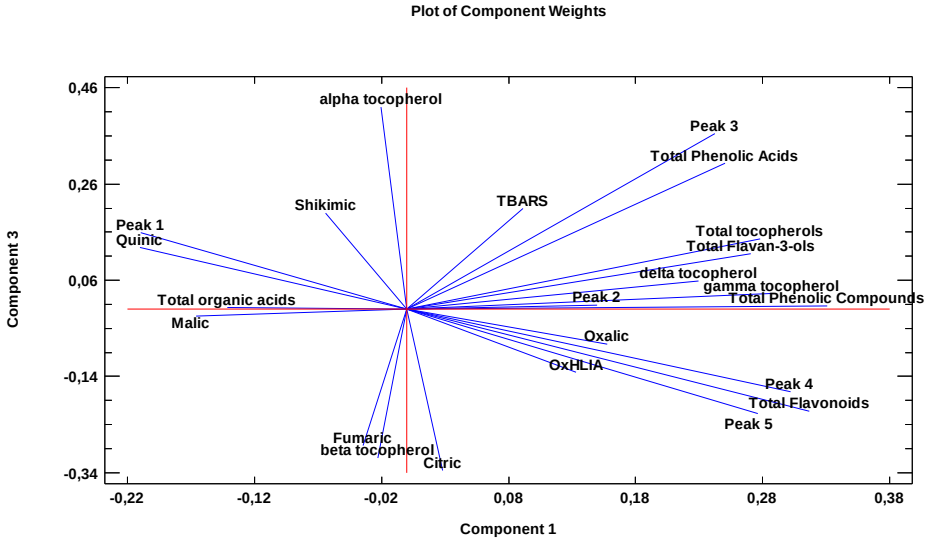

Figure C. The loading plot of principal components 1 and 3 for the tested samples.

Supplementary material of research paper 4.2

Supplementary Data 1

Table A. Extraction conditions of time (t), temperature (T, for HAE) or power (P, for UAE), and ethanol percentage in the solvent (EtOH), determined by the Box–Behnken experimental design for the extraction optimization and experimental results used for the RSM. The results were evaluated across three response metrics: dry residue (R_1), reduction power (R_2), and total phenols content (R_3).

Run	HAE						UAE					
	X_1 – t (min)	X_2 – T (°C)	X_3 – EtHO (%)	R_1 – DR (g/100 g)	R_2 – PR (µg/mL)	R_3 – TPC (mg/g)	X_1 – t (min)	X_2 – P (% of W) [1]	X_3 – EtHO (%)	R_1 – DR (g/100 g)	R_2 – PR (µg/mL)	R_3 – TPC (mg/g)
1	67.5	30	0	14.3	76	167	32.5	20	0	-	114	398
2	120	55	100	0.8	196	112	60	20	50	1.5	95	323
3	67.5	55	50	14.2	1417	161	5	80	50	2.3	208	672
4	67.5	55	50	14.3	355	146	5	50	0	1.4	247	400
5	67.5	80	0	15.5	327	138	60	50	0	1.7	230	246
6	67.5	80	100	0.9	542	70	32.5	50	50	1.7	211	339
7	15	80	50	13.1	330	177	32.5	50	50	0.7	199	569
8	15	55	100	0.4	443	160	32.5	80	0	1.5	290	127
9	67.5	55	50	13.9	243	147	32.5	50	50	1.3	372	170
10	15	30	50	13.5	78	152	32.5	50	50	1.4	199	166
11	67.5	30	100	0.4	178	95	60	80	50	1.4	116	139
12	120	55	0	14.8	271	152	5	50	100	0.2	0	79
13	67.5	55	50	11.4	519	122	32.5	20	100	0.3	523	122
14	120	30	50	13.7	247	123	5	20	50	1.3	589	191
15	120	80	50	13.9	1354	150	32.5	50	50	1.4	388	40
16	67.5	55	50	14.2	419	195	60	50	100	0.3	506	119
17	15	55	0	0.9	668	166	32.5	80	100	0.6	738	94

[1] The ultrasonic power variable was defined by treating the equipment's maximum power capacity (500 W) as 100%.

Supplementary Data 2

Atomization conditions of the pumpkin peel extract (PE): the liquid extract was concentrated by vacuum evaporation at 40 °C to reach a concentration of 2-5 °Brix (approximately 50 g of extract per liter of solution). The solution was dried using maltodextrin as the carrier agent (0.2% w/L) prepared immediately before atomization. Atomization was performed using a mini spray dryer (BUCHI, B-191, Laboratory-Techniques LTD, Flawil, Switzerland) that operated simultaneously and featured a spray nozzle with a diameter of 1 mm. The inlet air temperature was set at 130 °C with an inlet air pressure of 6.5 bars, and the outlet air temperature was maintained at 85 °C. The liquid feeding rate of the dryer was approximately 3 mL/min with a feed temperature of 40 °C. This process was performed under constant conditions.

Characterization of the extract after atomization: PE was characterized to verify possible loss of bioactive quality after the atomization process. No loss of bioactive capacity was detected, as shown in **Table B** and **C**.

Table B. Antioxidant capacity of the atomized PE (IC_{50} in µg/mL).

Antioxidant assay	PE	Trolox
TBARS	1035 ± 46	139 ± 5
PR	2846 ± 84	2937 ± 98
DPPH	1665.4 ± 81.4	42 ± 1

Table C. Antimicrobial activity of the atomized PE (in mg/mL).

	PE		Positive Control							
			Streptomicin		Methicilin		Ampicillin		Ketoconazole	
	MIC	MBC/MIF	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MIF
Enterobacter Cloacae	1.25	>10	0.007	0.007	n.t.	n.t	0.15	0.15	n.t	n.t
Escherichia coli	2.5	>10	0.01	0.01	n.t.	n.t.	0.15	0.15	n.t	n.t
Pseudomonas aeruginosa	2.5	>10	0.06	0.06	n.t.	n.t.	0.63	0.63	n.t	n.t
Salmonella enterica	1.25	>10	0.007	0.007	n.t.	n.t.	0.15	0.15	n.t	n.t
Yersinia enterocolitica	2.5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15	n.t	n.t
Bacillus cereus	5	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.	n.t	n.t
Listeria monocytogenes	2.5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15	n.t	n.t
Staphylococcus aureus	2.5	>10	0.007	0.007	0.007	0.007	0.15	0.15	n.t	n.t
Aspergillus brasiliensis	10	>10	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0.06	0.125
Aspergillus fumigatus	10	>10	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0.5	1

MIC: minimum inhibitory concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL); MFC: minimum fungicidal concentration (mg/mL); n.t.: not tested.

Supplementary Data 3

Characterization of the seeds extract are present in **Table D** and **E**.

Table D. Antioxidant capacity of the seeds extract (IC_{50} in µg/mL).

Antioxidant assay	Seed extract	Trolox
TBARS	110 ± 5	139 ± 5
PR	531 ± 30	43 ± 1
DPPH	4603 ± 290	42 ± 1

Table E. Antimicrobial activity of the seed extract (in mg/mL).

	Seeds extract		Positive Control							
			Streptomicin		Methicilin		Ampicillin		Ketoconazole	
	MIC	MBC/MIF	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MIF
Enterobacter Cloacae	>10	>10	0.007	0.007	n.t	n.t	0.15	0.15	n.t	n.t
Escherichia coli	>10	>10	0.01	0.01	n.t	n.t	0.15	0.15	n.t	n.t
Pseudomonas aeruginosa	>10	>10	0.06	0.06	n.t	n.t	0.63	0.63	n.t	n.t
Salmonella enterica	10	>10	0.007	0.007	n.t	n.t	0.15	0.15	n.t	n.t
Yersinia enterocolitica	10	>10	0.007	0.007	n.t	n.t	0.15	0.15	n.t	n.t
Bacillus cereus	10	>10	0.007	0.007	n.t	n.t	n.t	n.t	n.t	n.t
Listeria monocytogenes	10	>10	0.007	0.007	n.t	n.t	0.15	0.15	n.t	n.t
Staphylococcus aureus	10	>10	0.007	0.007	0.007	0.007	0.15	0.15	n.t	n.t
Aspergillus brasiliensis	5	>10	n.t	n.t	n.t	n.t	n.t	n.t	0.06	0.125
Aspergillus fumigatus	>10	>10	n.t	n.t	n.t	n.t	n.t	n.t	0.5	1

MIC: minimum inhibitory concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL); MFC: minimum fungicidal concentration (mg/mL); n.t.: not tested.

Supplementary Data 4

For aerobic plate count (total viable count; ISO 4833-1:2013; ISO, 2013), the dilutions were inoculated in PCA (Plate Count Agar; Liofilchem, Italy) by the pour plate technique, incubated at 30 °C for 72 h, and counting was performed on plates containing between 15 and 300 colonies (Limit of Quantification (LOQ) = 1 $\log^{10}$ Colony Forming Units per g (CFU/g)); for coliforms (ISO 4832:2006; ISO, 2006), the dilutions were inoculated in VRBLA (Violet, Red Bile Lactose Agar; Liofilchem, Italy) using the pour plate technique, incubated at 30 °C for 48 h, and counting was performed on plates containing 10 to 150 colonies (LOQ = 1 log10 CFU/g); for Yeasts and Moulds (ISO 21527-1:2008; ISO, 2008), the dilutions were inoculated in DRBC (Dichloran Rose Bengal Chloramphenicol; Liofilchem, Italy) using the spread plate technique and incubated at 25 °C for 5 days, and counting was performed on plates containing less than 150 colonies (LOQ = 1.7 log10 CFU/g).

References:

ISO, I. O. for S. (2006). *ISO 4832:2006 - Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms — Colony-count technique*. https://www.iso.org/standard/38282.html

ISO, I. O. for S. (2007). *ISO 7218:2007 - Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*. https://www.iso.org/standard/45109.html

ISO, I. O. for S. (2008). *ISO 21527-1:2008 - Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds — Part 1: Colony count technique in products with water activity greater than 0.95*. https://www.iso.org/standard/38275.html

ISO, I. O. for S. (2013). *ISO 4833-1:2013 - Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 1: Colony count at 30 degrees C by the pour plate technique*. https://www.iso.org/standard/59509.html

ISO, I. O. for S. (2017). *ISO 6887-1:2017 - Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*. https://www.iso.org/standard/63336.html

Supplementary Data 5

Table F. Antimicrobial activity of the extract obtained in the optimal conditions by HAE (in mg/mL).

	PE		Positive Control							
			Streptomicin		Methicilin		Ampicillin		Ketoconazole	
	MIC	MBC/MIF	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MIF
Enterobacter Cloacae	>10	>10	0.007	0.007	n.t	n.t	0.15	0.15	n.t	n.t
Escherichia coli	5	>10	0.01	0.01	n.t	n.t	0.15	0.15	n.t	n.t
Pseudomonas aeruginosa	10	>10	0.06	0.06	n.t	n.t	0.63	0.63	n.t	n.t
Salmonella enterica	2.5	>10	0.007	0.007	n.t	n.t	0.15	0.15	n.t	n.t
Yersinia enterocolitica	2.5	>10	0.007	0.007	n.t	n.t	0.15	0.15	n.t	n.t
Bacillus cereus	>10	>10	0.007	0.007	n.t	n.t	n.t	n.t	n.t	n.t
Listeria monocytogenes	5	>10	0.007	0.007	n.t	n.t	0.15	0.15	n.t	n.t
Staphylococcus aureus	>10	>10	0.007	0.007	0.007	0.007	0.15	0.15	n.t	n.t
Aspergillus brasiliensis	10	>10	n.t	n.t	n.t	n.t	n.t	n.t	0.06	0.125
Aspergillus fumigatus	5	>10	n.t	n.t	n.t	n.t	n.t	n.t	0.5	1

MIC: minimum inhibitory concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL); MFC: minimum fungicidal concentration (mg/mL); n.t: not tested. PE: Butternut Squash peel extract obtained under optimal extraction conditions by HAE.

Supplementary Data 6

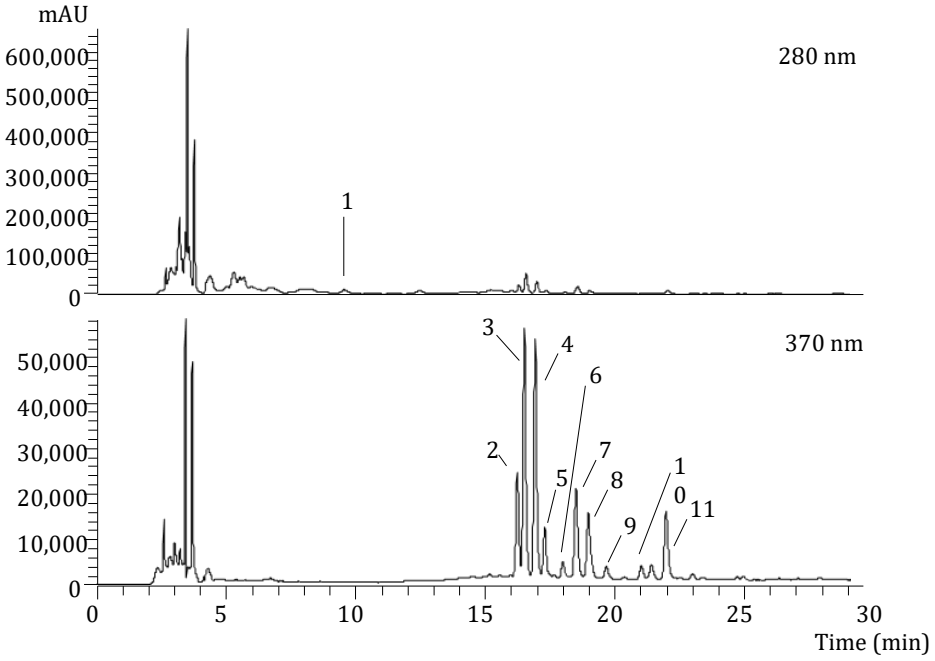

Figure A. Chromatogram of phenolic compounds of the butternut squash peel extract (PE) obtained by HPLC-DAD-ESI/MS under the optimal conditions.

Supplementary material of Section 4.3

Technical data sheet of the final formulation

Technical data sheet - Prototype
PUMPKIN FRUIT PULP

Description:

Pumpkin preparation

Ingredients:

Pumpkin (91%), modified maize starch, acidity regulators (citric acid, sodium citrate), preservative, colour (natural beta-carotene).

Labelling:

Producer/distributer name and address; Commercial designation; Ingredients ; Allergens ; Expiration ("Best before end ..."); Conservation; Method of use; Lot number; Product reference; Net weight.

Characteristics

Organoleptics

Colour	Orange	**Texture**	Compact
Flavour	Pumpkin		

Physical-Chemical

Brix	min: 10 max: 16
pH	min: 4,3 max: 4,7
Exogenous Foreign bodies	Should tend to zero
Endogenous Foreign bodies	<10un/10kg

Microbiological Criteria (Limits)

Microorganisms a 30 ºC	$< 10^3$	Ufc/g	
Coliforms a 30 ºC	$< 10^2$	Ufc/g	
Yeast and Moulds	$< 5x10^2$	Ufc/g	
E. Coli	< 10	Ufc/g	
Listeria Monocytogenes	< 100	Ufc/g	Reg. (CE) n.º 2073/2005
Salmonella	Not detected in 25g		

GMO´S and irradiation

This product does not contain ingredients that require additional specific labeling under Regulation (CE) No. 1829/2003 and Regulation (CE) No. 1830/2003 and its amendments. The product does not make use of irradiation in its production process according to directives 1999/2/EC and 1999/3/EC and their amendments.

Conservation:

Keep in a cool and dry place, since protected from direct sunlight. Once opened, store in a cool temperature.

Instructions for use:

This product can be used directly or diluted into cream, ice-cream, milk or pastry products in order to obtain flavoured compounds.
Apply enough quantity for the wanted final effect.

This product can be consumed by the general public, except allergic people, to any marked substance with (+) on the allergens table in this document.

Type and packaging materials used:

Membrane film with barrier protection

Bucket of polypropylene

Appropriated polypropylene cap.

Package:	**Expiration date:**
Net weight 5,50 kg	30 Days

Allergens:

Celery and products thereof	-
Cereals containing gluten	-
Crustaceans and products thereof	-
Eggs and products thereof	-
Fish and products thereof	-
Lupin and products thereof	-
Milk and products thereof (including lactose)	-
Molluscs and products thereof	-
Mustard and products thereof	-
Nuts and products thereof	-
Peanuts and products thereof	-
Sesame seeds and products thereof	-
Soybeans and products thereof	-
Sulphur dioxide and sulphites	-

(+) Yes (-) No

Decorgel has implemented procedures to maintain the maximum safety of the products, however, it is not possible to exclude the risks of contamination inside the premises. In the facilities there are the following allergens: Gluten, Soy, Eggs, Milk including Lactose, Nuts and Sulphur Dioxide and Sulfites.

Nutrition Information (per 100 g)

Energy	**236 kJ / 56 kcal**
Fat	0,13 g
of which saturates	0,00 g
Carbohydrate	12 g
of which sugars	4,2 g
Protein	1,0 g
Salt	0,00 g

Calculation based on product formulation and the information provided by the supplier of materials. Due to the nature of these products, the nutritional composition may vary, consequently, these data represent typical average values only.

Packaging requirements

Standard palletization		**Primary packaging (PP)**
Dimension (LxWxH)		Dimension (LxWxH)
1200*1000*1100 mm		227*227*180 mm
Net weight	688 kg	
Estimated gross weight	732 kg	
Units base	25	
Units height	5	

Transport can be done at room temperature, since protected from direct sunlight

Observations:

This document is representative of the prototype developed; therefore, final values are dependent of industrial studies.
For industrial use and HORECA channel.
Due to the natural characteristics of the raw materials, this product may undergo color changes over time. According to the natural characteristics of the raw materials, there may be slight differences between batches.
Industrial adjustments can be made to ensure the product specifications.

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Evaluation form

Prova Sensorial - PulpIng

Prova sensorial para avaliação de polpa de abóbora com conservante natural de casca de abóbora em comparação com a formulação tradicional com sorbato de potássio .

Instruções:
Estão a ser entregues duas amostras de formulação de polpa de abóbora para si. Por favor, avalie-as atentamente conforme indicado em cada questão.

Observações:
Por favor, limpe seu paladar entre as amostras com água;
Não discuta suas opiniões com outros participantes durante a avaliação.

* Indica uma pergunta obrigatória

1. Nome *

2. Idade *

Exemplo: 7 de janeiro de 2019

3. Sexo *

Marcar apenas uma oval.

- ◯ Feminino
- ◯ Masculino
- ◯ Prefiro não dizer

4. **Cor** *

Avalie a cor com relação a sua intensidade amarelo/laranja

Marcar apenas uma oval por linha.

	Amarelo	Amarelo-alaranjado	Intermédio	Alaranjado-amarelo	Laranja
Amostra 256	◯	◯	◯	◯	◯
Amostra 875	◯	◯	◯	◯	◯

5. **Cor** *

Avaliae a cor conforme a atratividade visual

Marcar apenas uma oval por linha.

	Muito insatisfatório	Insatisfatório	Indiferente	Satisfatório	Muito satisfatório
Amostra 256	◯	◯	◯	◯	◯
Amostra 875	◯	◯	◯	◯	◯

6. **Aroma** *

Avalie a atratividade do aroma, ou seja, o quanto este é agradável e característico.

Marcar apenas uma oval por linha.

	Muito insatisfatório	Insatisfatório	Indiferente	Satisfatório	Muito satisfatório
Amostra 256	◯	◯	◯	◯	◯
Amostra 875	◯	◯	◯	◯	◯

7. **Sabor** *

Avalie a agradabilidade do sabor, ou seja, o quanto é atrativo e característico.

Marcar apenas uma oval por linha.

	Muito insatisfatório	Insatisfatório	Indiferente	Satisfatório	Muito satisfatório
Amostra 256	◯	◯	◯	◯	◯
Amostra 875	◯	◯	◯	◯	◯

8. **Textura** *

Avalie a agradabilidade da textura dos produtos

Marcar apenas uma oval por linha.

	Muito insatisfatório	Insatisfatório	Indiferente	Satisfatório	Muito satisfatório
Amostra 256	◯	◯	◯	◯	◯
Amostra 875	◯	◯	◯	◯	◯

9. **Avaliação Global** *

Avalie os produtos considerando o aspecto global

Marcar apenas uma oval por linha.

	Muito insatisfatório	Insatisfatório	Indiferente	Satisfatório	Muito satisfatório
Amostra 256	◯	◯	◯	◯	◯
Amostra 875	◯	◯	◯	◯	◯

10. **Comentários adicionais**
Deixo seu comentário sobre o que achou dos produtos

11. **Você prefere consumir produtos naturais e livres de conservantes artificiais?** *

Marque todas que se aplicam.

- [ ] Sim, prefiro produtos naturais sem conservantes artificiais
- [ ] Sim, mas não é um fator determinante na minha escolha
- [ ] Não, não tenho preferência por produtos naturais
- [ ] Não tenho opinião formada sobre o assunto
- [ ] Outro: _____

12. **Qual a principal razão para sua escolha acima?** *
Por favor, selecione a opção que mais se aplica a você

Marque todas que se aplicam.

- [ ] Preocupação com a saúde e segurança alimentar
- [ ] Preocupação com o meio ambiente e sustentabilidade
- [ ] Melhor sabor e qualidade dos produtos naturais
- [ ] Influência de informações e campanhas publicitárias
- [ ] Não tenho opnião formada sobre o assunto
- [ ] Outro: _____