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starting from vegetable waste, using classical and innovative
biotechnological techniques of food enrichment”***

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*To my grandfather,
who taught me the beauty and the hardness of the world,
my source of inspiration,
always.*

*To my family,
my support and shelter.*

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LIST OF ABBREVIATIONS:

ABTS	2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACAE	Acarbose Equivalent
AChE	Acetylcholinesterase
ACN	Acetonitrile
ANOVA	Analysis of Variance
AR	Androgen Receptor
ASE	Accelerated Solvent Extraction
BChE	Butyrylcholinesterase
CUPRAC	CUpric Reducing Antioxidant Capacity
DC	Decoction
DE	Dried extract
DES	Deep Eutectic Solvent
DPPH	2,2-diphenyl-1-picrylhydrazyl
DS	Dielectric Spectroscopy
DSC	Differential Scanning Colorimetry
DTNB	5,5-dithio-bis(2-nitrobenzoic)
EAE	Enzyme-Assisted Extraction
ECACC	European Collection of Authenticated Cell Cultures
EDTA	Disodium Edetate
EDTAE	Disodium Edetate Equivalents
EtOH	Ethanol
EVO	Extra Virgin Olive
EVOO	Extra Virgin Olive Oil

FBS	Fetal Bovine Serum
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalent
GALAE	Galanthamine Equivalent
HPLC-ESI-Q-TOF-MS	High Performance Liquid Chromatography – Electrospray Ionization- Quadrupole- Time Of Flight – Mass Spectrometry
HPLC-UV	High-Performance Liquid Chromatography-Ultraviolet
IL-1β	Interleukin-1 beta
IL-6β	Interleukin-6 beta
KAE	Kojic Acid Equivalent
MAE	Microwave-Assisted Extraction
MCA	Metal Chelating Activity
MeOH	Methanol
mRNA	Messenger RNA
NADES	Natural Deep Eutectic Solvent
NF-κB p65	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PBD	Phosphomolybdenum
PBS	Phosphate Buffered Saline
PHWE	Pressurized Hot Water Extraction
PLE	Pressurized-Liquid Extraction
PNPG	4-N-trophenyl- α -D-glucopyranoside
ppm	Part Per Million
PSA	Prostate-Specific Antigen
PTFE	Polytetrafluoroethylene
r.t.	Room Temperature

RE	Rutin Equivalent
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
rpm	Repetition per minute
SCW	Subcritical Water Extraction
SCW	Subcritical Water Extraction
SD	Standard Deviation
SE	Solvent Extraction
SEM	Standard Error of the Mean
SFE	Supercritical Fluid Extraction
TE	Trolox equivalents
TEA	Triethylamine
TFC	Total Flavonoid Content
THF	Tetrahydrofuran
TIPC	Total Individual Phenolic Content
TLR4	Toll Like Receptor 4
TNF-α	Tumour Necrosis Factor alpha
TPC	Total Phenolic Content
TPTZ	2,4,6-tris(2-pyridyl)-S-triazine
t_R	Retention Time
u.a.	Under Agitation
UAE	Ultrasound-Assisted Extraction

Chapter 1:
Introduction

1.1 Aim of the thesis

Industrialization and globalization are the bases of the modern society. Food industries, specifically, the fruit and vegetable sectors, are growing globally¹. The market growth involves, for example, fresh-cut fruit and vegetables^{1,2}.

In September 2015, United Nations leaders had a Summit establishing 17 goals and 169 targets for sustainable development to accomplish by 2030 (Figure 1). On 1 January 2016, the 17 Sustainable Development Goals (SDGs) of the 2030 Agenda for Sustainable Development became effective³. Nutrition, food production and waste generation are important topics covered by the 2030 Agenda. Ending poverty, erasing extreme poverty conditions and guaranteeing food security are the purposes of Goal 2 (Figure 1)³.



Figure 1. 17 Sustainable Development Goals of the 2030 Agenda for Sustainable Development.

Despite the advantages of food industrial development, including the increase in employment and economic incomes, the huge production of waste and by-products represents an important drawback.

Currently, food production sustainability is a strongly debated topic. Indeed, the reduction in the generation of food waste is another essential aim of Agenda 2030 (Figure 1). Specifically, the 12th goal is entitled “Responsible Consumption and Production”, which includes target 12.3 that states “By 2030, halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses”.³ This statement clearly shows the importance of managing food waste production and the strong correlation to environmental sustainability. Indeed, waste generation is one of the main issues food industries go through and it represents an important global challenge from different points of view, including ethical, social, economic and environmental^{4,5}. Nowadays, scientific research is focused on finding new ways for the reuse and valorization of food by-products, *e.g.* their use to produce functional and enriched food or food packaging materials.^{4,6,7}

In this context, the main aim of this thesis is to emphasize the potential of food waste as a source of biologically active compounds with beneficial effects on human health. The concomitant application of sustainable and green approaches and reagents is the key point of this dissertation. In the following pages, I’m going to provide an overview of the main research I conducted during these three years. Specifically, I worked on two types of food waste which are highly produced in Italy, tomato skins and grape pomace. The valorization of these matrices was achieved by the extraction of valuable natural compounds opening new perspectives in their application for the production of nutraceuticals and enriched food.

1.2 Main topics

In the next pages, I provide an overview of the traditional and innovative extraction procedures applied in food chemistry, an introduction to the concept of deep eutectic solvents (DESs) and the types of natural compounds found in food by-products.

In Chapter 2, I discuss the use of the DES menthol-thymol for the extraction of lycopene from tomato skins waste and the comparison in terms of lycopene yields with α -pinene and *n*-hexane applying ultrasound-assisted extraction (UAE) as extraction method.

Chapter 3 is focused on the extraction of tomato skins waste using extra-virgin olive oil as an extraction solvent and its consequent enrichment with lycopene. *In vitro* studies have been conducted on the obtained enriched oil to demonstrate the improvement in its antioxidant activity and to evaluate its enzyme inhibition ability. The assessment of its safety has been determined on the PNT1A normal human prostate cell line.

Chapter 4 describes the polyphenolic extraction from grape pomace using different techniques and the comparison among grape pomace extracts and elderberry, cranberry, rose hip berry, goji berry and raisin extracts in terms of phytochemical profile and biological activity. Through this comparative study, we aimed to demonstrate the higher potential of this waste derived from the wine-making process against commonly available berries and fruit, promoting the use of grape pomace extracts as the main ingredients of novel nutraceuticals or enriched food.

Chapter 5 summarises the key points of my dissertation and the future perspectives.

1.3 Overview of the main extraction techniques

The conventional extraction techniques applied on plant and food matrices include maceration, digestion, decoction, Soxhlet extraction, infusion and percolation. In the last decades, modern and innovative extraction procedures have been introduced, in particular, accelerated solvent extraction (ASE), pressurized hot water extraction (PHWE), cold plasma-assisted extraction, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), supercritical fluid extraction (SFE) and subcritical water extraction (SCW)⁸⁻¹¹. A rapid overview of these techniques is provided below.

Maceration is an easy and inexpensive extraction method extensively used for the recovery of bioactive compounds and essential oils; the main disadvantage is the length of the process^{12,13}. The plant matrix used for maceration is ground increasing the surface area which will be in contact with the extraction solvent. The solvent is put into a locked vessel that can be occasionally shaken to improve the diffusion; the saturated solvent can be removed and a new rate of solvent added to increase the extraction yield. At the end of the extraction, the solvent is recovered along with that obtained by pressing the solid plant matrix¹³. Digestion is a kind of maceration that is used when the application of high temperature is not allowed and includes a soft heat¹⁴.

Decoction and infusion follow the same principles of maceration. Decoction is a simple method consisting of boiling the plant matrix in water. It is suitable for tough plant matrices, for example, barks or roots, but it's not suggested for the extraction of thermosensitive compounds and the potential presence of water-soluble impurities could represent a problem^{12,14,15}. Sometimes, glycerol or aqueous ethanol may be used for decoction preparation¹⁶. Decoction is different from infusion because of the lack of active boiling in infusion¹⁴.

Percolation is preferred in the case of thermolabile compounds. It's performed using a specific instrument, the percolator, and it results in being more effective than maceration due to the continuous replacement of the saturated solvent by a fresh one^{12,14}.

For Soxhlet extraction, a small quantity of the sample is put in a thimble located in the distillation flask containing the extraction solvent. When the overflow status is achieved, a siphon aspirates the solution contained in the thimble-holder and empties the solution into the distillation flask again transporting the extracted compounds in the bulk liquid. The

solvent returns to the solid matrix and this process is repeated until the end of the extraction¹³. The main drawbacks of this method are the high amount of solvent and time required.

In the last decades, innovative techniques have been developed for the extraction of biologically active compounds. The advancement of green methods is due to their speed, diminished thermal effects related to the generation of hazardous residual volatiles and a reduced waste-water production¹⁷.

The ultrasound-assisted extraction is based on a phenomenon called cavitation that produces powerful shear forces into the plant matrix during the extraction leading to the production of micro-jetting on the surface. This phenomenon induces particle disruption, surface peeling or/and erosion¹⁸.

Microwave-extraction method differs from the conventional techniques because it induces modifications in the cell structure due to the electromagnetic waves¹⁹. Indeed, microwaves can penetrate the biomaterials and the interaction between these waves and the polar molecules (*e.g.* water) found inside the material generates heat²⁰. The absorption of microwave energy from the water contained in the plant matrix facilitates cell disruption and consequently the desorption and recovery of nutraceutical substances from the matrix^{20,21}.

Accelerated solvent extraction is a method that exploits organic solvents under conditions of high pressure and high temperature allowing the decrease in the consumption of extraction solvent and the increase in the speed of extraction^{22,23}.

The SFE is an extraction method in which a supercritical fluid is exploited, namely a substance that, at the critical point, is characterized by the physical properties of both liquid and gas phases. The critical point is achieved over the critical temperature and pressure; at this point, the gas and liquid phases aren't distinguishable. Thus, during the extraction, this solvent solubilises the compounds in the matrix while flowing across the packed bed. After the solvent has left the extractor, the extract is freed from the solvent thanks to the increase in temperature and the decrease in pressure¹¹.

The EAE is useful for the extraction of compounds bound to the lignin network which is stabilized through H-bonds and hydrophobic interactions. These compounds, dispersed in the cytoplasm, can't be extracted using solvent-based procedures. The pre-treatment of the matrix with particular enzymes, *e.g.* amylase, cellulase or pectinase, and facilitates the

release of the bound compounds by disrupting the cell walls and hydrolysing the carbohydrates^{11,24}.

PHWE is a method in which water at elevated pressure is exploited as extraction solvent resulting extremely sustainable and, thus, a valid alternative to conventional extraction techniques due to its good yields. The principle this method is based on is that water is maintained in the liquid state under pressure; a pressure of 5MPa assures that water remains a liquid at 100-250°C^{11,25}.

SCW is a technique in which water, heated between 100°C and 374°C and maintained at the liquid state applying high pressure (> 5 MPa), is used as an extractive solvent. This method needs less pressure than the extraction with supercritical CO₂. This type of extraction results in being more sustainable than that in which organic solvents are applied^{26,27}.

Cold plasma-assisted extraction exploits plasma, which occurs in particular conditions, and has encouraging applications in food processing. With the increasing in the energy of the molecules of a system, the solids convert to liquids and the liquids to gases altering the intermolecular interactions. A further boost in the gases energies leads to disappearance of all the interactions, the release of negative and positive ions and the ionization of some atoms and molecules forming the plasma²⁸⁻³¹.

1.4 Green extraction solvents: in-depth on Deep Eutectic Solvents

Besides the proper extraction technique, the choice of a green solvent is essential to promote the sustainability of the extraction procedure. The replacement of organic solvents by green alternatives opens new challenges and restrictions related to their diverse physicochemical properties. Green solvents can be divided into four categories comprising supercritical fluids (water, CO₂), neoteric (ionic liquids and Deep Eutectic Solvents (DES)), Bio-based solvents (terpenes, alcohols, esters, ethers) and supramolecular solvents (temperature-, salt-, acid- and poor solvent-induced). These alternatives have been evaluated for the valorization of food waste in numerous studies taking into account their advantages and limitations³².

In Chapter 2, I'm going to discuss a research work focused on the use of a Natural DES as extractive solvent composed of menthol and thymol.

The concept of DES was born with the studies of Abbott et al.^{33,34} DESs are generally composed of a hydrogen bond donor and a hydrogen bond acceptor that, when mixed, have a melting point lower than the single components of the mixture³⁴. DES can be classified into 5 types^{35,36}:

- Type I: Mixtures of metal chlorides and quaternary ammonium salts.
- Type II: Based on the combination of metal hydrates and quaternary ammonium salts.
- Type III: Given by the mixture of quaternary ammonium salts and organic compounds (*e.g.* amides, carboxylic acids or alcohols).
- Type IV: Based on the combination of metal halides and hydrogen bond donors.
- Type V: Given from the combination of non-ionic compounds.

Moreover, Choi et al. proposed the concept of NADES, namely DES formed by plant metabolites³⁷.

The advantages of using NADESs are their general biodegradability, no toxicity, low expensiveness and eco-friendliness; moreover, their physicochemical properties can be modulated by the choice of the components^{38–40}.

1.5 Biologically active compounds extracted from food waste

Each phase of the food life cycle, from agricultural production to industrial processes and distribution, leads to the production of food waste⁴¹. Agro-industrial wastes are commonly used as fertilizers and animal feed. In the last years, numerous research works have been conducted to develop new methods to exploit fruit and vegetables wastes for therapeutic purposes⁸. Vegetables waste can comprise peels, stems, seeds, shells, bran and other residues⁴².

Food waste contains a huge array of biologically active compounds. The biomolecules recovered from food waste can be sugars, proteins and their derivatives, polysaccharides, EtOH, fibres, flavour compounds and phytochemicals⁴².

The class of molecules extracted from waste materials depend on the type of matrix and the extraction methodology applied, including the technique and the eventual solvent used.

Being this dissertation focused mainly on two kinds of food waste (tomato waste and grape pomace), I'm going to provide a brief overview of the compounds of interest extracted from them and an introduction on carotenoids and polyphenols, which are the classes of compounds of major interest for our studies.

Tomato processing leads to the generation of different types of waste; numerous studies have been conducted on these by-products. For instance, the phytochemical composition of the leaf extracts of two varieties of Italian tomatoes (Datterino and Piccadilly) was determined by observing the presence of alkaloids, fatty acids, terpenes, lipids and flavonoids⁴³. Schiavi et al. evaluated the potential extraction of cellulose nanocrystals from tomato harvesting waste through enzymatic and chemical protocols. Both the procedures gave good results⁴⁴. Tomato wastes were also used for the extraction of pectin obtaining interesting results using ultrasound-assisted extraction which shortened a lot the extraction procedure⁴⁵. The recovery of carotenoids and phenolic compounds was also investigated starting from tomato wastes^{46,47}.

Grape pomace has been widely studied to define its phytochemical profile, especially in terms of polyphenol content. Below, are reported some examples of previous research works on this matrix.

Onache et al. evaluated the total phenolic and flavonoid contents, and the total catechin and tannins contents of different grape pomace by spectrophotometric assays, while the polyphenolic profile was determined by ultra-high-performance liquid chromatography-high resolution mass spectrometry analysis⁴⁸. The skins of distinct grape pomace were analysed to evaluate their phenolic composition and dietary fibre⁴⁹. In another study, ten varieties of grape pomace including seeds and skins were analysed for the determination of the polyphenol profile observing a prevalence of protocatechin, chlorogenic acid, synergic acid and ferulic acid in all the varieties. Furthermore, the analyses of minerals and fatty acids composition showed the presence of P, Fe, Zn and K as the main minerals and linoleic, palmitic and oleic acids as the main fatty acids⁵⁰.

1.5.1 Carotenoids

Carotenoids can't be considered only a simple group of natural pigments; indeed, they are compounds characterized by numerous impressive properties and biological functions. Carotenoids are found in plants as well as in animals and microorganisms. Carotenoids are fundamental in photosynthetic organisms. The different actions of carotenoids depend on their physicochemical properties which are related to their structures, e.g. the molecular geometry and the conjugated double bonds presence⁵¹. About 600 carotenoids have been isolated, but few were found in the human tissues⁵².

Structurally, these compounds are isoprenoids which are biosynthesized through the tail-to-tail linkage of 2 molecules of geranylgeranyl diphosphate (C₂₀) forming the parent compound (C₄₀) from which derives all the variations. The basic skeleton can be mutated by end cyclization (at just one end or both) forming seven different types of end groups, by modifications in the hydrogenation level and by adding functional groups containing oxygen⁵¹. A scheme elucidating the carotenoid biosynthetic pathway in plants is reported in Figure 2⁵³. Xanthophylls are the carotenoids containing oxygen functions, while the parent hydrocarbons are called carotenes⁵¹. The conjugated polyene chromophore is responsible for the colour and the photoprotective activities of the carotenoids^{53,54}.

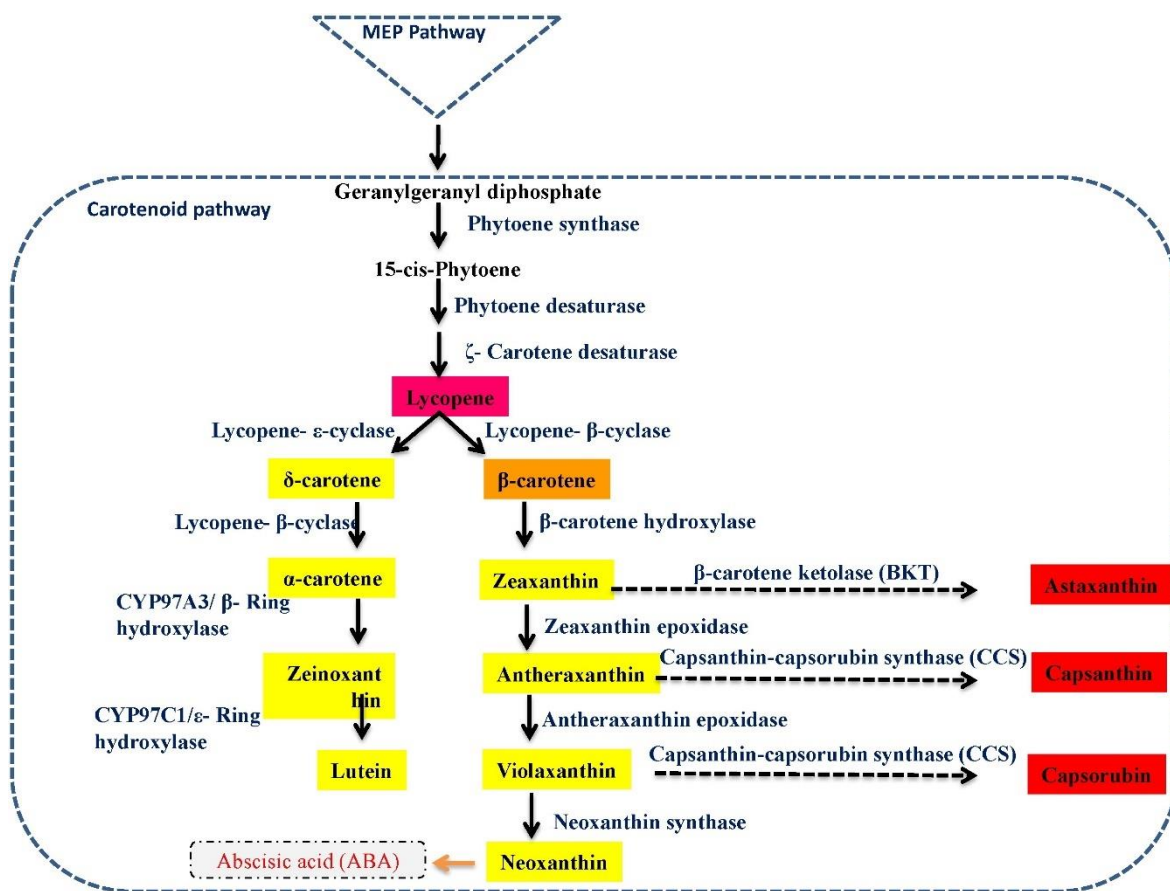


Figure 2. Carotenoids biosynthetic pathway in plants⁵³.

From a biological point of view, carotenoids have different roles in animals, being involved in bone metabolism, embryonic development and reproduction, immune modulation, gap junctions communication, cell differentiation, anti-inflammation, haematopoiesis etc.⁵³

1.5.2 Polyphenols

Polyphenols are a group of compounds widely distributed in nature and comprise more than 8000 different molecules⁵⁵. These compounds are secondary metabolites found in plants that show numerous biological effects, *e.g.* antioxidant, anticancer and anti-inflammatory⁵⁶.

Polyphenol is a term attributed to compounds containing more than two phenolic -OH groups; flavonoids, tannins and phenylpropanoids are comprised in this class of compounds⁵⁷.

A generic classification of polyphenols, considering the synthetic ones and those of natural origins, is reported in Figure 3⁵⁸.

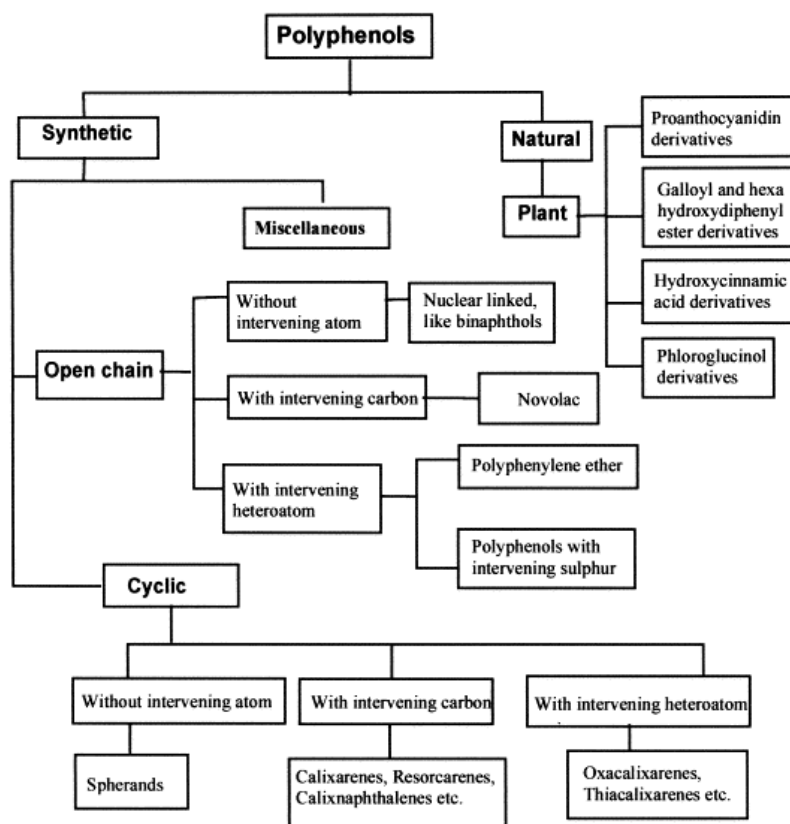


Figure 3. Classification of polyphenolics⁵⁸.

Polyphenols are present also in vegetable and fruit wastes, for instance, in fruit by-products like grape and wine, olive and oil, orange and lemon, pomegranate and apple by-products⁵⁹.

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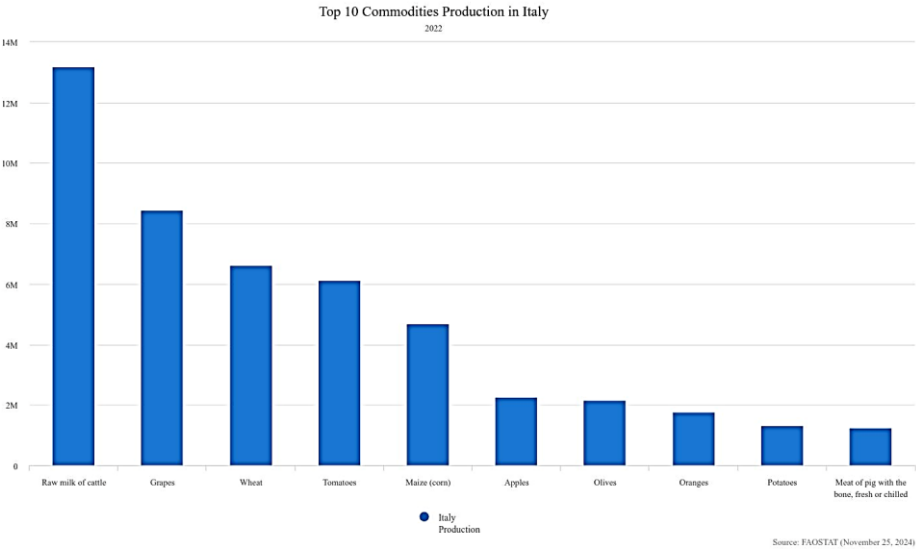
Chapter 2:

*Ultrasound-assisted extraction of lycopene using DES menthol-thymol,
 α -pinene and n-hexane*

2.1 Introduction

Tomatoes represent one of the main cultivations in Italy. Indeed, this crop was the fourth in the list of the top 10 Commodities Production in Italy (2022) according to FAOSTAT (Food and Agriculture Organization of the United Nations), accounting for 6136380 tons (Figure 1a). Moreover, in accordance with the available data, Italy was the sixth Country in the world for the production of tomatoes in 2022 (Figure 1b)¹.

a)



b)

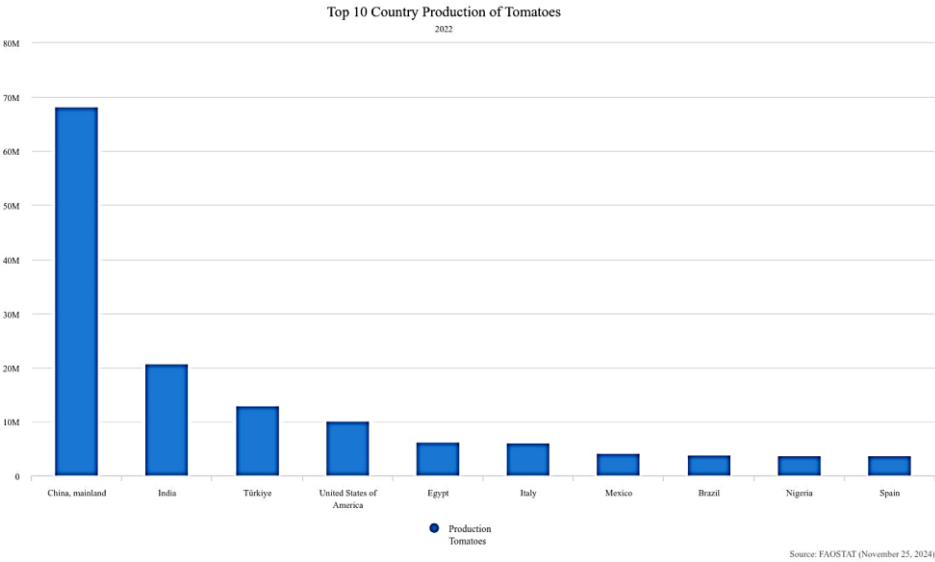


Figure 1. a) Top 10 Commodities Production in Italy (2022). Resource: FAOSTAT (Food and Agriculture Organization of the United Nations); **b)** Top 10 Country Production of Tomatoes (2022). Resource: FAOSTAT (Food and Agriculture Organization of the United Nations)¹.

Tomatoes contain a plethora of bioactive compounds, *e.g.* carotenoids, ascorbic acid, carbohydrates, phenolics, flavonoids and volatiles²⁻⁴. Regarding the carotenoids in tomatoes, lycopene accounts for 80-90% of the total amount and β -carotene for the 2-3%^{5,6}. Carotenoids are usually located in fruits; however, they can be produced by microorganisms, plants and fish. Approximately 600 carotenoids have been reported, but only a few are present in human tissues including lycopene⁷. Lycopene of natural origin is available on the market, but it's particularly expensive and sold in small lots⁸.

Tomato-based products, *e.g.* tomato sauces, pastes and peeled tomatoes, are daily consumed. Their high production and consumption lead to the generation of amounts of waste. The research works focusing on the management and reuse of tomato waste are numerous and varied. For instance, Almeida et al. evaluated the potential recovery through solid-liquid extraction of value-added compounds and the biochemical methane potential using anaerobic digestion of the tomato residues, in particular branches and rotten and green tomatoes⁹. Durmuş et al. evaluated the application of tomato compost, obtained microbiologically from tomato waste produced in the greenhouse, for the improvement of tomato yields and the biological properties of the soil¹⁰. Bugatti et al. used tomato peels and natural halloysite nanotubes to prepare green composites with encapsulated carvacrol¹¹.

Tomato skins, a waste produced during tomato processing, are a rich source of lycopene which is five times more abundant here than in tomato pulp⁸. A schematic representation of our research project is reported in Figure 2.

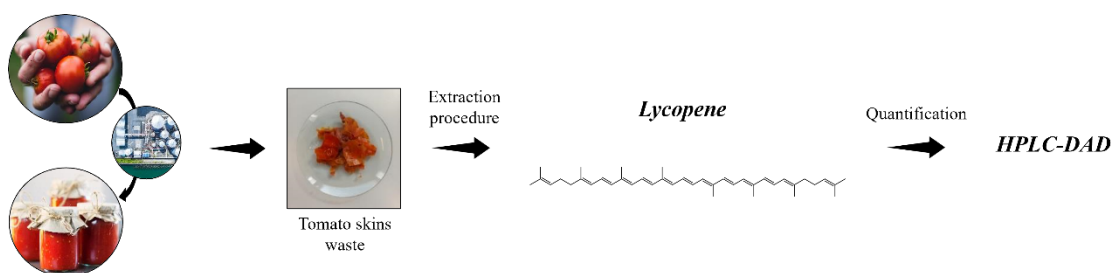


Figure 2. Schematic representation of the workflow.

2.1.1 Lycopene structure and biological activities

Lycopene ($C_{40}H_{56}$) is a carotenoid placed in the chloroplasts which determines the red colour of the fruit (Figure 3). The acyclic terpenoid structure presents 11 carbon-carbon double bonds (11 linearly conjugated) and consists of 8 isoprene units. This feature contributes to making it a powerful antioxidant^{8,12}.

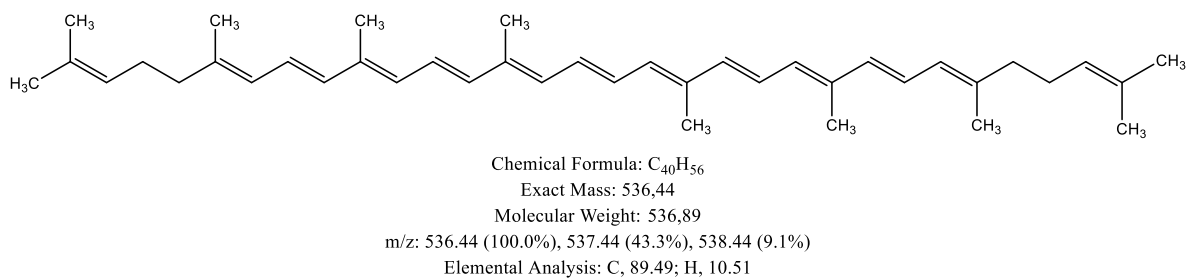


Figure 3. Lycopene structure.

Lycopene exists in nature in different cis- and trans-configurations. The 90% of lycopene found in nature is represented by the all-trans-isomer (all-E-lycopene) and the remaining 10% comprises cis/Z-isomeric form, specifically 5Z, 9Z and 13Z. Approximately 72 lycopene configurations are structurally favoured, among these important isomers are all-E-lycopene, 5Z-lycopene, 6-cis-lycopene (neolycopene A), 1-, 3-, 5-, 7-, 9-, 11- cis-lycopene (prolycopene), and 1-, 3-, 5-, 6-, 7-, 9-, 11-cis-lycopene (cis-lycopene). In terms of thermodynamic stability, 5-cis > all-trans > 9-cis > 13-cis > 15-cis > 7-cis > 11-cis¹³. Some factors may promote the cis isomerization of lycopene, in particular light irradiation and strong thermal energy, improving its bioavailability and antioxidant activity¹⁴.

Many research projects were conducted to evaluate the potential biological effects of lycopene^{7,15}. One of the activities investigated was its anticancer effect against prostate cancer. Previous studies highlighted lycopene's ability to inhibit cell proliferation, stimulate apoptosis, arrest the cell cycle and reduce DNA damages^{16–18}. The antioxidant effect of lycopene is due to its ability to neutralize some reactive species (e.g. hydroxy radicals, hydrogen peroxide and NO_2)¹⁹. Lycopene could also be involved in the reduction of inflammatory processes; being atherosclerosis an inflammatory process, lycopene could have a role in affecting this disorder^{19,20}. A previous study reported the neuroprotective effect of lycopene on rats with bisphenol A intoxication, a compound widely used for the production of epoxy resins and polycarbonate plastic with reported neurotoxic effect. The

neuroprotective effect was related to the antioxidant properties of lycopene and its ability to activate the MAPK/ERK pathway and inhibit the neuronal apoptosis²¹. Lycopene has been also studied for its anti-inflammatory effects¹⁹. Liu et al. investigated the protective role of lycopene in inflammatory responses caused by β -amyloid in the early phase of Alzheimer's disease. The study, conducted on rats, was focused on the determination of cytokine serum profiles (TNF- α , IL-1 β and IL-6 β), learning and memory capabilities and Toll-like receptor 4 (TLR4), NF- κ B p65 mRNA and protein expressions at the level of choroid plexus. This study showed that the learning and memory deficits were improved in the groups treated with lycopene and the levels of cytokines in the serum (TNF- α , IL-1 β and IL-6 β) were increased. Furthermore, the study revealed the role of lycopene in stopping the activation of the expression of NF- κ B p65 and TLR4 (Figure 4) and the cytokines production causing the reduction of inflammatory injuries and confirming its potential in reducing the deposition of the β -amyloid in the hippocampus²².

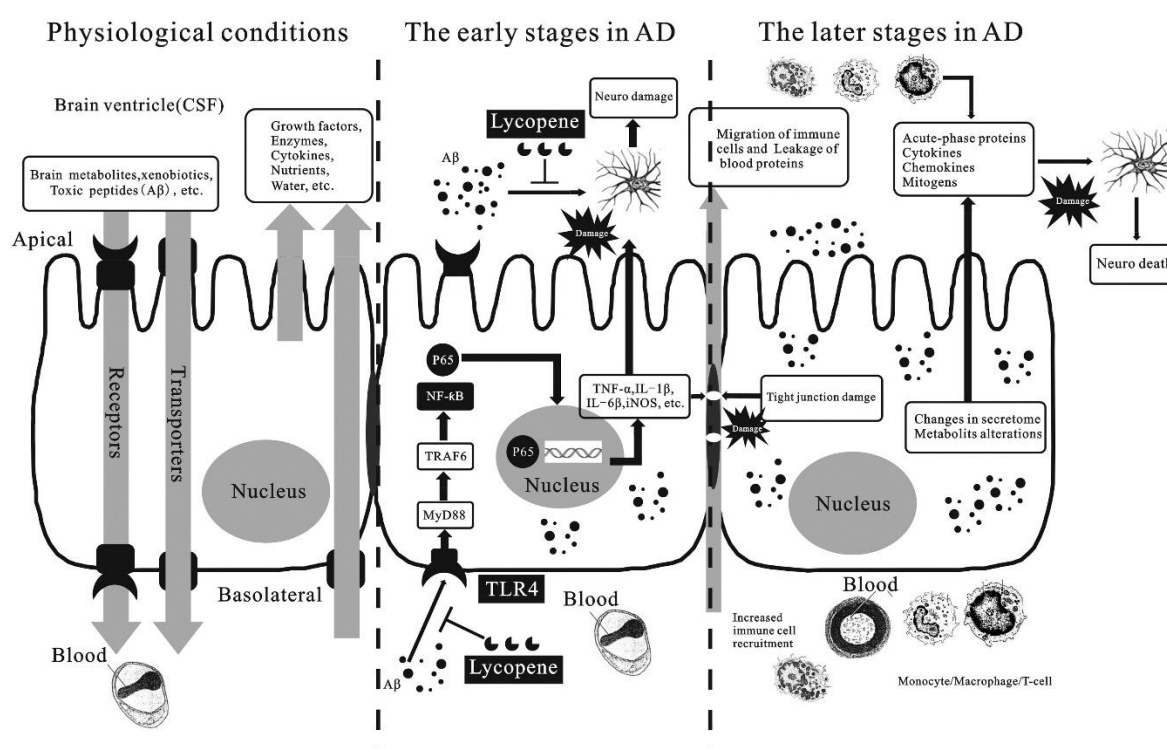


Figure 4. Schematic representation of lycopene anti-inflammatory mechanisms of action through TLR4-mediated NF- κ B signaling cascade in the choroid plexus²².

2.1.2 Lycopene extraction

The extraction of lycopene has been performed using numerous conventional and innovative techniques and exploiting different types of solvents^{6,23–25}. The extraction of lycopene based on ultrasound-assisted technique was extensively reported^{6,23,26,27}. Among the more innovative and green techniques, microwave-assisted extraction was also applied for the extraction of lycopene from tomato peels^{28,29}. Papaioannou & Karabelas exploited an enzymatic pre-treatment for the hydrolyzation of the pectin in which the pigment is accumulated and performed a subsequent extraction with non-ionic surfactants⁸. Cuccolini et al. extracted lycopene from tomato peels using a technique based on pH modification and enzymatic hydrolysis³⁰. Chen et al. recently made a theoretical and experimental study considering the subcritical extraction of lycopene as a case study³¹.

The extraction solvent used for carotenoids' extraction from a food matrix depends on the chemical properties of the specific type of carotenoid considered. For instance, while xanthophylls have more affinity for hydrophilic solvents, carotenes are better extracted using non-polar solvents. The main issue in using organic solvents, like *n*-hexane, is their toxicity⁶. For this reason, in this research work the use of green solvents like the DES menthol-thymol and α -pinene were evaluated as an alternative to the commonly used *n*-hexane.

2.1.3 Deep Eutectic Solvent Menthol-Thymol

Menthol and thymol are two natural compounds classified as monoterpenes^{32,33}. Menthol is a natural and non-toxic hydrophobic compound ($\log P \approx 3.4$), low cost and with low reactivity. It has a low melting point ($T_m \approx 43^\circ\text{C}$) and it's stable in water^{34,35}. Thymol is one of the most common monoterpenes found in plants and it is biosynthesized through the aromatization of γ -terpinene to form *p*-cymene that successively goes through hydroxylation³³. The use of terpenes as extraction solvents is a greener approach than the common organic solvents. In comparison with *n*-hexane, terpenes are bio-solvents which result in being environmentally safer, slightly more polar and less flammable and hazardous³⁶. On the other hand, terpenes have higher viscosity and density, and strongly consume energy during the evaporation processes^{37,38}. Being both natural compounds, menthol-thymol DES can be defined as a NADES (Natural Deep Eutectic Solvent).

Menthol-Thymol DES belongs to type V DESs', non-ionic DES with lower viscosity than the other types (I-IV), chloride free and often hydrophobic³⁹⁻⁴². Abranches et al. measured the solid-liquid equilibrium phase diagram confirming that the system menthol-thymol represents a true DES, indeed, although these two compounds are structurally similar (Figure 5a), there is an intense negative deviation to the ideal behaviour when they are mixed. The non-ideal system is due to the H-bonds formed between menthol and thymol being much stronger than that in the pure compounds. The resonance effects may be involved in the interpretation of the severe negative deviations of the system menthol-thymol from ideality. As shown in Figure 5b, the lone pairs of the oxygen present in the structure of thymol are delocalized in the aromatic ring making the positivity of the hydroxyl group higher than usual. The oxygen results in a worse H-bond acceptor, thus, when thymol is in its liquid phase, it forms H-bonds between good H-bond donor and poor H-bond acceptor. In thymol-menthol mixtures, the hydrogen of thymol preferentially interacts with the oxygen of menthol, which has a normal -OH group due to the lack of aromaticity. This results in the formation of an H-bond stronger than that in the pure constituents leading to a strong negative deviation from ideality³⁹.

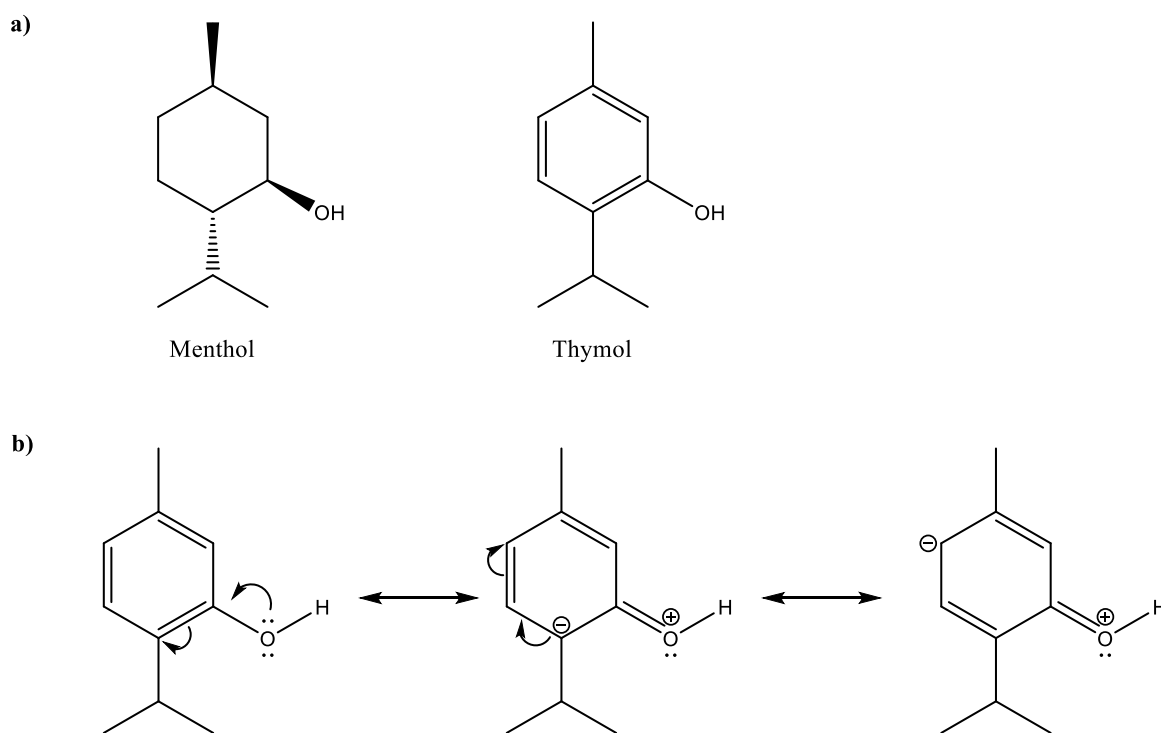


Figure 5. a) Menthol and thymol structures; b) Resonance structures of the thymol.

D'Hondt & Morineau described the dynamics of eleven compositions of menthol/thymol mixtures which included the pure compounds by using Differential Scanning Colorimetry (DSC) and Dielectric Spectroscopy (DS) in a timescale ranging from 10^{-6} to 10^2 seconds. By DSC, they classified the mixtures into four groups based on the thermal behaviour observing that in the mixtures at about the equimolar compositions (x_{Thymol} comprised from 0.4 to 0.6) the crystallization never happened⁴³.

Recently, different studies on menthol/thymol mixtures have been conducted to enhance the understanding of their properties. Dorosh et al. recently evaluated the solvation of hydroquinone, 1,4-benzo-quinone, catechol and resorcinol in different thymol-menthol mixtures, while Srivastava et al. evaluated the solute dynamics of Coumarin 153 in menthol-thymol mixtures^{44,45}. Strzemiński et al. evaluated the potential application of Volatile Natural Deep Eutectic Solvents, specifically, menthol/camphor and thymol/menthol mixtures, for the extraction and recovery of alkaloids present in the roots and herb of the Chelidonium plant⁴⁶. The hydrophobic nature of this DES allows its exploitation as an extraction solvent for hydrophobic compounds, this is the reason why we tested it for lycopene recovery.

2.2 Materials and Methods

2.2.1 Chemicals

Crystal solids menthol (99.0%) and thymol (98.5%) were purchased by Sigma-Aldrich, USA (currently Millipore Sigma) along with analytical grade solvents *n*-hexane (99.0%) and α -pinene (98.0%). ACN, MeOH and tetrahydrofuran were $\geq 99.9\%$ for HPLC. Lycopene analytical standard for HPLC ($\geq 85.0\%$) was purchased by Sigma-Aldrich (Milano).

2.2.2 Instruments

The sample preparation and the extraction procedures were performed using: Büchi Lyovapor L-200 apparatus; Lab-scale ultrasound sonicator (Cole-Parmer, Illinois, USA) (frequency 20 kHz, power 400W and amplitude 70%); Eppendorf centrifuge 5702; rotavapor Büchi Vac V-513; vacuum vortex evaporator (Buchler Instrument).

2.2.3 Sample preparation

Ice-dried tomato skins waste (furnished by S.A.L.P.A. - Società Abruzzese Lavorazione Prodotti Agricoli, Roseto Degli Abruzzi, Teramo, IT) were collected after the production of sauces and stored at -4°C until the preparation of the sample. Tomato skins were lyophilized (48 h) and ground in a blender; the obtained powder was sieved to 1.08 ± 0.10 mm. The sample was stored at -20°C until the extraction.

2.2.4 Preparation of the menthol-thymol DES

An equimolar solution was obtained by adding 10.0 g of menthol to 9.6 g of thymol in a round bottom flask. The mixture was put under agitation at room temperature for 1 hour. A total of 20 mL of the solution was obtained (Figure 6).

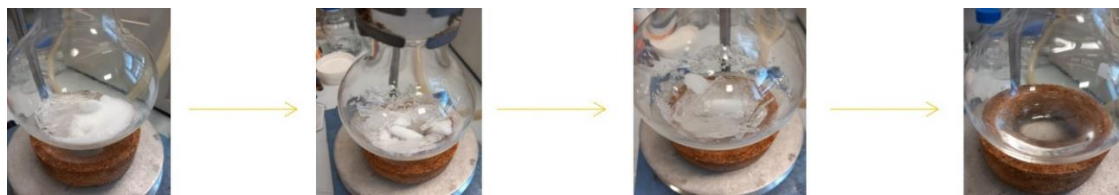


Figure 6. Preparation of the Deep Eutectic Solvent Menthol:Thymol 1:1.

2.2.5 Extraction procedure

Ultrasound-assisted extraction with n -hexane

To 1.0156 g of tomato skins powder, 20 mL of n -hexane was added. The ultrasound-assisted extraction was performed at 36°C for 20 minutes. After centrifugation (4400 rpm; 20 minutes) and filtration on a mesh filter, the supernatant was evaporated by a rotary evaporator followed by high vacuum obtaining 12.1 mg of dried extract (DE).

Ultrasound-assisted extraction with α -pinene

The sample (1.0057 g) was extracted as above (UAE at 36°C for 20 minutes, centrifugation (4400 rpm, 20 minutes) and filtration). Further centrifugation of 5 minutes at 4400 rpm was necessary due to the higher viscosity of this solvent in comparison with n -hexane. The evaporation of α -pinene required stronger conditions than n -hexane, thus the rotary evaporator was connected to the high vacuum obtaining 28.5 mg of dried extract.

Ultrasound-assisted extraction with menthol-thymol DES

Tomato skins waste (1.0112 g) was extracted as above (UAE at 36°C for 20 minutes, centrifugation (4400 rpm, 20 minutes), filtration and further centrifugation for 5 minutes at 4400 rpm). For the evaporation of this DES, we had to apply very strong conditions. 1 mL of the collected supernatant was evaporated by a vacuum vortex evaporator working at 80°C and 1 mBar under agitation for 3 hours. The weight of the obtained dried extract was 1.1 mg.

To reduce the temperature and the time of evaporation, was utilized an azeotrope. 1.0140 g of tomato skins powder was extracted with DES menthol:thymol 1:1 as described before. Before the evaporation, 2.5 mL of water was added to 1 mL of extract for the formation of the azeotropic mixture. The evaporation was performed by a vacuum vortex evaporator working at 60°C and 1 mBar under agitation for 1.5 hours obtaining 2.5 mg of dried extract.

2.2.6 HPLC-DAD analysis

A RP-HPLC (Reversed-Phase High-Performance Liquid Chromatography) (Agilent Technologies, 1100 Infinity, Germany) equipped with a binary pump, degasser, automatic injector, and a DAD (diode array detector) G1315D 1260 DAD VL was used for the quantification of lycopene in each dried extract. The column was a ZORBAX Eclipse Plus (C18 3.5 μm , 4.6×100 mm, USUXR42236, USA). The analyses were conducted following the method described by Olives Barba et al. with slight modifications⁴⁷. The temperature was set at 30°C and the injection volume was 100 μL . The mobile phase MeOH/ACN (9:1) + 0.125% TEA was filtered on 0.2 μm PTFE filters and sonicated (15 minutes) before the use. The analyses were performed in isocratic mode at a flow rate of 1.2 mL/min for 15 minutes. Lycopene was detected at 475 nm and identified by comparison with the analytical standard at a retention time of 8.1-8.4 minutes. For its quantification, the calibration curve was prepared in a range of 0.5-500 mg L^{-1} ($y = 28.80x + 75.65$; $R^2=1$). For each dried extract, approximately 1 mg was solubilized in 1 mL of the mixture THF/ACN/MeOH (1.5:3:5.5) and then filtered using 0.45 μm filters. The same mixture was used for the solubilization of the analytical standard. Each analysis was performed in triplicate and the results expressed as the media of the experiment $\pm$ the standard deviation (SD).

2.3 Results and Discussion

In this work, the extraction efficiency of a conventional organic solvent and innovative green solvents was put under comparison⁴⁸. Different factors influence the extraction yield from a vegetable matrix. The type of solvent is one of the main factors, indeed its physical properties (*e.g.* density and surface tension) play a crucial role⁴⁶. Another important feature is the pre-treatment of the matrix, like drying and grinding, which can influence the physical properties of the raw material. Generally, the decrease in the particle size led to an increase in the extraction yield due to a lowering in the mass transfer resistance. The extraction technique applied is an essential part of the extraction process. In this study, ultrasound-assisted extraction was chosen because it's a green technique that can accelerate the extraction kinetics and the mass transfer rate. The reduction of the particle size induced by UAE facilitates the interactions between the selected extraction solvent and the plant matrix leading to an improved solubilization of the pigments⁴⁹. Considering the thermal and chemical stability of lycopene, mild extraction conditions were chosen, namely a temperature of 36°C and a time extraction of 20 minutes. Given the low stability of lycopene in this deep eutectic solvent and to be more accurate in comparing the extraction yield, the evaporation of the solvent was performed. The concomitant application of high temperature (60-80°C), high vacuum (1mBar) and mechanical agitation was necessary to remove the deep eutectic solvent menthol-thymol. Lycopene is sensible to heat, however, Gheonea (Dima) et al. reported that lycopene can be degraded above 100°C⁵⁰. The preparation of an azeotropic mixture was performed to reduce the boiling point and improve lycopene extraction yield. Even though the time of evaporation was reduced from 3 hours to 1.5 hours, there wasn't an improvement in the yield. The quantification of lycopene was conducted by HPLC-DAD, the chromatograms are reported in Figure 7. The results are reported in Table 1 and are expressed in mg of lycopene per gram of dried extract.

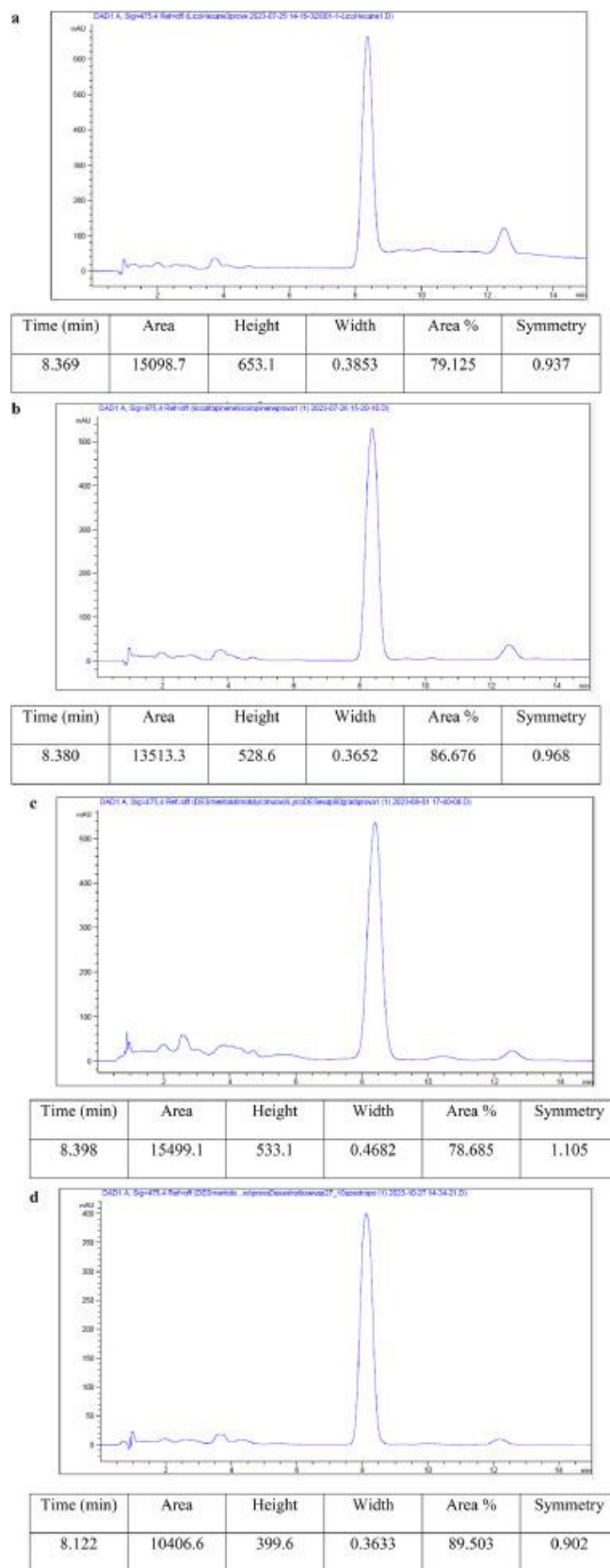


Figure 7. Chromatograms of *n*-hexane extract (a), α -pinene extract (b), DES menthol-thymol extract (c) and DES menthol-thymol (azeotropic mixture) extract (d) obtained by HPLC-DAD.

UAE <i>n</i> - hexane (mg/g DE $\pm$ SD)	UAE α -pinene (mg/g DE $\pm$ SD)	UAE DES menthol:thymol (mg/g DE $\pm$ SD)	UAE DES menthol:thymol (azeotropic mixture) (mg/g DE $\pm$ SD)
735.9 $\pm$ 8.8	468.1 $\pm$ 1.4	484.2 $\pm$ 2.4	358.7 $\pm$ 1.2

Table 1. Lycopene yield in each extract. The results represent the media of three experiments $\pm$ the standard deviation (SD).

The organic solvent *n*-hexane gave the best result due to its lower viscosity; however, the extraction yield of the Deep Eutectic Solvent was sensibly high, followed by α -pinene. The formation of the azeotrope didn't determine an improvement in terms of lycopene content, but a faster recovery of lycopene was achieved. Considering the numerous advantages in terms of ecological and environmental sustainability of terpene-based solvents, the application of this DES is recommended despite the slight decrease in extraction yield compared to organic solvents. A comparison between the lycopene content before and after the evaporation was performed. For DES extract, lycopene content was 1.19 ± 0.01 mg/mL before the evaporation which turned to 0.533 ± 0.002 mg/mL after the evaporation, thus a decrease of about 55% in lycopene extraction yield was determined. For the DES-azeotrope extract, the lycopene content was 2.32 ± 0.01 mg/mL before evaporation and 0.9 ± 0.01 mg/mL after the evaporation (approximately 61% of reduction). It is noteworthy that despite the reduction during the evaporation, probably due to the low stability of lycopene given to its chemical structure, the lycopene content in the dried extract was high.

2.4 Conclusion

A sustainable and green extraction method was adopted for the recovery of lycopene by performing an ultrasound-assisted extraction using DES menthol-thymol and α -pinene as green extraction solvents. To the best of our knowledge, this is the first study on the extraction efficiency of DES menthol:thymol 1:1 for lycopene recovery. This type of VNADES, after the optimization of the extraction procedure, resulted in a good extraction efficiency despite the partial degradation. The application of this DES could be extremely useful for the extraction of lipophilic compounds less sensible to heat being a good alternative for waste management with greener technologies.

An important aspect that has not been investigated by us and that should be evaluated in future is the industrial scalability of this extraction process. In general, the evaluation of the total cost of the process is one of the main concerns for industries. This calculation includes the costs of raw materials, energy consumption, generation and treatment of the produced waste. More specifically, an industrial extraction procedure should consider all the treatments and activities related to the plant materials as well as the recovery of the liquid extract and eventually the purification steps for the recovery of the extracted compounds. The extraction parameters should be optimized during the scale-up. The main limitation in the scaling up of extraction using DESs is the viscosity of these types of solvents which makes it more difficult to mix, filter or transport compared to the conventional solvents. Hence, an industrial implementation related to the equipment design should be required and the energy consumption would be higher for mixing and pumping⁵¹. These are the main limitations in terms of industrial DES extractions, but the great benefits in sustainability lead scientific research to move forward in the study of novel DES extraction procedures which can combine a high extraction efficiency with sustainability and industrial scalability of the processes.

To conclude, “green” is the keyword of my entire PhD project, the extraction of a biologically active compound from a food by-product in a completely sustainable way fits perfectly with the main aim of my research.

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Chapter 3:

Lycopene-enriched Extra Virgin Olive Oil

3.1 Introduction

As discussed in Chapter 2, tomato skins are an abundant by-product in Italy. Due to the high content of lycopene in tomato skins, which is approximately five times higher than the pulp¹, a study about the possibility of enriching a food with lycopene from tomato skins was conducted. The discussion about lycopene structure, bioactivity and extraction procedures was reported in Chapter 2, sections 2.1.1 and 2.1.2.

The use of vegetable oils as an alternative to conventional solvents to extract beneficial compounds for human health is growing interest due to the environmental safety of the procedure². Some examples of extraction using vegetable oils are described in literature. Tiwari et al. performed the UAE to recover carotenoids from carrot pomace using linseed oil as extraction solvents³. Linseed oil was also used to extract carotenoids from sea buckthorn pomace⁴. Civan and Kumcuoglu extracted carotenoids and capsaicin from red Jalapeño with olive oil⁵. Chutia and Mahanta used the UAE combined with olive oil for the extraction of carotenoids from passion fruit peels⁶. Bendini et al. evaluated the production of lycopene enriched olive oil by co-milling olives and tomato waste⁷.

This research project aimed to determine a simple and eco-friendly procedure for the obtainment of an enriched Extra Virgin Olive Oil (EVOO) starting from tomato skins waste. The choice of enriching an EVO oil is due to its high Italian production and export, further valorizing a territorial product. Furthermore, enrich a daily consumed food or beverage is a smart approach to increase the assumption of a peculiar beneficial compound among the population. The determination of the antioxidant properties and enzyme inhibition activity of the enriched EVOO was performed through *in vitro* assays and compared to that of the simple EVOO. Moreover, the safety assessment of the enriched oil was performed on PNT1A normal prostate cell line⁸.

3.2 Materials and Methods

3.2.1 Chemicals

The solvents used were acetonitrile, methanol and tetrahydrofuran ($\geq 99.9\%$) for HPLC. Lycopene analytical standard ($\geq 85.0\%$) for HPLC was purchased by Sigma-Aldrich (Milano).

3.2.2 Instruments

The lyophilisation of the sample was performed using a Büchi Lyovapor L-200 apparatus. For the extraction procedure were used a Lab-scale ultrasound sonicator (Cole-Parmer, Illinois, USA) with a frequency of 20 kHz, power of 400 W and amplitude of 70 % and a Eppendorf centrifuge 5702.

3.2.3 Sample preparation and extraction

Tomato skins were gently furnished by S.A.L.P.A. (Società Abruzzese Lavorazione Prodotti Agricoli) and conserved at -20°C until use. Before the extraction, tomato skins were lyophilized for 48 hours and ground in a blender obtaining a powder.

Approximately 1 g of the powdered sample was extracted using 20 mL of EVO oil by ultrasound-assisted extraction (20 minutes, 36°C). After the centrifugation (4400 rpm, 20 minutes, $25 \pm 2^{\circ}\text{C}$) and filtration, the red-coloured lycopene enriched oil was obtained.

3.2.4 HPLC-DAD analyses

An Agilent HPLC-DAD 1100 was used with a column Agilent Eclipse Plus, C18 3.5 μm , 4.6×100 mm (USUXR16700, USA). The column temperature was set at 30°C , the injection volume was 100 μL , the time run was 15 minutes, and the flow rate of 1.2 mL/min. The mobile phase was composed of MeOH/ACN (9:1) + 0.125% TEA, the analyses were performed in isocratic mode.

The analytical standard and the oil were solubilized in a mixture of THF/ACN/MeOH (1.5:3:5.5). For the detection and quantification of lycopene the wavelength was set to 475 nm. The calibration curve was built in the range of concentrations 0.5–500 mg/L. The results were expressed as the mean of three different experiments $\pm$ the standard deviation.

3.2.5 Antioxidant assays

To determine the total content of phenols and flavonoids, spectrophotometric assays were performed as described by Slinkard & Singleton⁹. The antioxidant properties of the EVO oil and the lycopene-enriched EVO oil were assessed as previously reported in literature by Grochowski et al. through DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), CUPRAC (Cupric Reducing Antioxidant Capacity), FRAP (Ferric Reducing Antioxidant Power), PBD (Phosphomolybdenum assay) and MCA (Metal Chelating Activity)¹⁰. The results of DPPH, ABTS, CUPRAC and FRAP assays were reported as milligrams of Trolox equivalents (TE) per gram of sample. The results of PBD were expressed as millimoles of Trolox equivalents (TE) per gram of sample, while the results of MCA were in milligrams of EDTAE (Disodium Edetate Equivalents) per gram of extract. For all the assays, the sample solutions were prepared in ethanol (2 mg/mL).

3.2.6 Enzyme inhibition assays

The protocols for the enzyme inhibition assays were described by Grochowski et al.¹⁰ The AChE and BChE inhibition activity was expressed as equivalents of galantamine per gram of the sample using Ellmann's method. The tyrosinase inhibition was reported as equivalents of kojic acid per gram of the sample using the dopachrome method. The α -amylase and α -glucosidase inhibition of the samples was expressed as equivalents of acarbose per gram of the sample using the iodine/potassium iodide method and the chromogenic PNPG method, respectively. Each sample was tested using a concentration of 2 mg/mL.

3.2.7 Cell culture

For the cell proliferation and cellular morphology assays, the PNT1A cell line was used which are normal human prostatic cells without androgen receptor (AR) and prostate-specific antigen (PSA) expression. PNT1A cells (catalogue no: 95012614) were retrieved from the European Collection of Authenticated Cell Cultures (ECACC, Wiltshire, England). They were cultured in RPMI 1640 Medium (catalogue no: A1049101) (Gibco, Waltham, MA, USA) supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin in polystyrene cell culture flasks and the incubation was done in a humidified standard incubator with 5% CO₂ at 37°C¹¹. When the cells' density became 80%, they were subcultured with trypsin-EDTA 0.25% (Gibco, Waltham, MA, USA). Before performing each experiment, the cell counting was done through a hemocytometer and the cell viability was determined using the Trypan Blue (Gibco, Waltham, MA, USA) exclusion method¹².

3.2.8 iCELLigence cell proliferation assay

iCELLigence cell analysis system (Agilent Technologies (formerly ACEA Biosciences, San Diego, CA, USA) was exploited for the determination of the effects of EVOO and lycopene-enriched EVOO on cell proliferation. It monitors modifications in cell behaviour in real-time without any labelling, such as cell proliferation, migration and invasion^{13,14}. Before the experiment, the instrument was put in the incubator at 37°C with 5% CO₂. Firstly, the background was measured with 200 µL of growth medium, and then PNT1A cells were seeded in E-Plate L8 wells at a density of 2.0×10^4 . The incubation occurred for 30 minutes in a laminar flow cabinet. Later, the E-plates were put in the iCELLigence system and incubated for 24 hours allowing the cell growth¹⁵. After 24 hours, the cells underwent the treatment with different concentrations of the samples (EVOO and EVOO + Lycopene), specifically, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL. The experiment was conducted in duplicate. The positive and negative controls were, respectively, Docetaxel (Taxotere®) and the growth medium. The cell index was registered every 15 minutes for 72 hours and the data were analysed by using the RTCA Data Analysis Software 1.0 (Agilent Technologies, formerly ACEA Biosciences).

3.2.9 Cell morphology assays

Inverted microscopy

The effects of EVOO and EVOO + lycopene at different concentrations were evaluated on PNT1A cell morphology using an inverted microscope Leica DM IL LED equipped with a DFC-290 camera (Leica, Wetzlar, Germany)^{16,17}. The cells were seeded into 6-well cell culture plates, specifically 2.0×10^5 cells/well in 1 mL of growth medium. PNT1A cells were incubated for 24 hours and then treated with increasing concentrations of each sample (62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL) for 72 hours. The growth medium was exploited as a negative control. After the incubation, the medium was removed, and the wells were washed with PBS. Finally, the cells were observed under an inverted microscope to evaluate their morphological changes. The experiment was conducted in duplicate.

Crystal Violet Staining

This assay was conducted to further evaluate the effects of EVOO and EVOO + lycopene on PNT1A cellular morphology¹⁸. The cells were seeded in 6-well cell culture plates with a density of 2.0×10^5 cells/well. The cells were incubated overnight (37°C and 5% CO₂) and the next day underwent the treatment with various concentrations (125-1000 µg/mL) of EVOO and EVOO + lycopene for 72 hours. The media was removed, and the cells were washed two times using ice-cold PBS. A treatment using 100% methanol for 10 minutes at room temperature was performed to fix the cells. After the aspiration of methanol, PNT1A cells were stained with 0.5% crystal violet solution (15 minutes, 25 ± 2 °C), then washed with distilled water and air dried. After this treatment, the cells were photographed under the inverted microscope (Leica DM IL LED) equipped with a DFC-290 camera (Leica, Wetzlar, Germany).

3.2.10 Statistical analyses

Except for the cell assays, which were performed in duplicates, the other experiments were conducted in triplicates and the results were expressed as their media $\pm$ the standard deviation (SD). The variances between the results were determined through a student t-test ($\alpha = 0.05$) conducted with GraphPad v. 9.0 program.

3.3 Results and Discussion

The valuableness of lycopene in terms of human beneficial effects and its high cost on the market, made its recovery from natural sources an interesting topic, especially if it can be extracted from food waste. Due to the chemical structure of lycopene, it's necessary to use a lipophilic solvent for its extraction. To avoid the drawbacks of organic solvents, the combined use of ultrasound-assisted extraction and edible oil as extraction solvent may represent a green solution. In this case, the extraction of lycopene corresponds to the concomitant enrichment of the food matrix. The lipophilic nature of the vegetable oils as well as their regular consumption, high production in some regions, good stability and bioavailability make them perfect vehicles for carotenoid enrichment¹⁹.

After the extraction procedure (section 3.2.3), the EVOO colour turned red because of lycopene enrichment (Figure 1). The lycopene contained in the EVO oil was quantified by HPLC-DAD and resulted in 0.9 ± 0.2 mg/g of EVO oil. Given the high lipophilicity of lycopene, its solubilisation in solvents like methanol is difficult, thus the analytical methods commonly used for the quantification of carotenoids are not applicable^{20,21}. We applied the HPLC method developed by Olives Barba et al. (2006) with slight modifications. Important was the choice of the mobile phase, the excess of methanol may lead to the precipitation of lycopene while a surplus of non-polar solvent may cause a deformity of the chromatographic peaks due to the difference of polarity²⁰. To avoid or diminish the degradation of carotenoids on-column is useful the addition of TEA to the mobile phase^{20,22,23}. The temperature of the column was set to 30°C to prevent the separation of the pigments²⁰.

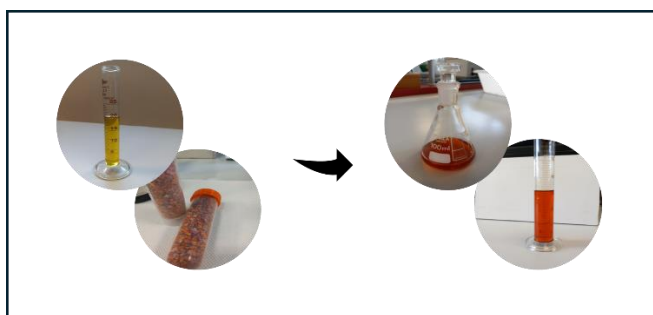


Figure 1. Obtainment of the lycopene-enriched EVO oil.

The total phenolic and flavonoid content (TPC and TPF) and the antioxidant properties of the enriched oil were then compared with the baseline TPC, TPF and antioxidant activity of the EVO oil (Tables 1 and 2).

	<i>TPC</i> (mg GAE/g)	<i>TFC</i> (mg RE/g)
<i>EVO oil</i>	24.72 ± 1.12 ^b	0.25 ± 0.03 ^a
<i>Lycopene-enriched EVO oil</i>	30.95 ± 0.50 ^a	0.07 ± 0.01 ^b

Table 1. Total phenolic and flavonoid content of EVO oil and lycopene-enriched EVO oil. Values are the results of three experiments ± SD. TPC: Total phenolic content, TFC: Total flavonoid content, GAE: Gallic acid equivalent; RE: Rutin equivalent.

	<i>DPPH</i> (mg TE/g)	<i>ABTS</i> (mg TE/g)	<i>CUPRAC</i> (mg TE/g)	<i>FRAP</i> (mg TE/g)	<i>MCA</i> (mg EDTAE/g)	<i>PBD</i> (mmol TE/g)
<i>EVO oil</i>	0.76 ± 0.05 ^b	11.66 ± 0.38 ^a	42.59 ± 1.06 ^b	24.40 ± 1.13 ^b	3.31 ± 0.41 ^b	0.96 ± 0.03 ^a
<i>Lycopene-enriched EVO oil</i>	7.88 ± 0.74 ^a	10.60 ± 0.36 ^b	49.74 ± 1.07 ^a	27.38 ± 1.66 ^a	3.81 ± 0.24 ^a	0.97 ± 0.08 ^a

Table 2. Antioxidant activities of EVO oil and lycopene-enriched EVO oil assessed by DPPH, ABTS, CUPRAC, FRAP, MCA and PBD assays. TE: Trolox equivalent; EDTAE: EDTA equivalent.

As shown in Table 1, the amount of phenolic compounds was higher in the lycopene-enriched EVOO (30.95 mg GAE/g) than in the EVOO (24.72 mg GAE/g), while the total flavonoid content was 3.5 times lower. This could be due to the possible formation of a complex involving lycopene and flavonoids²⁴, while the increasing TPC value after lycopene enrichment is consistent with the work of Montesano et al.²⁵. Given the possibility of a false positive due to the interaction between lycopene and Folin reagent, other *in vitro* assays have been performed to determine the outcomes of lycopene enrichment.

DPPH and ABTS are based on the hydrogen donation ability of the tested antioxidants²⁶. DPPH assay performed on the enriched oil showed an improved result than the EVO oil, in contrast to the ABTS assay. This discrepancy may be related to the different natures of ABTS and DPPH radicals. While ABTS assay allows to test both hydrophilic and

hydrophobic antioxidants, the DPPH method involves a radical dissolved in an organic media, which is proper for hydrophobic compounds.

CUPRAC and FRAP were performed to determine the reducing abilities of the tested samples, indeed a high reducing ability corresponds to a high antioxidant potential. In both cases, the enriched oil showed higher results than EVOO. Müller et al. observed a higher activity of lycopene in FRAP assay than β -carotene and zeaxanthin; the conjugated double bonds of lycopene may be the explanation for this behaviour²⁷.

Phosphomolybdenum assay is based on the reduction of Mo(VI) to Mo(V) under acid conditions performed by the antioxidants. The results of this assay depend on both the phenolic and non-phenolic antioxidants; thus, it measures the total antioxidant activity. In this test, the results obtained for the lycopene-enriched EVOO and EVOO were similar.

Metal chelation ability was assessed because it can be useful in regulating the production of hydroxyl radicals in the Fenton reaction. The fortified EVOO showed a higher metal chelating ability than EVOO. Some structural characteristics of lycopene may justify its metal-chelating ability; indeed, it could chelate metal ions through pi electrons in the polyene chain. Overall, the enrichment of the Extra Virgine Olive Oil with lycopene had a positive impact on its antioxidant abilities.

The enzyme inhibition can be a powerful tool for reducing the symptoms of some diseases. Other previous studies reported the interesting enzyme inhibition effects of tomato-fortified products or lycopene. The inhibitory effect against amylase and glucosidase of tomato-fortified rice was investigated by Ronanki et al. showing higher effectiveness for tomato-fortified rice than non-fortified sample²⁸. Morais et al. mentioned a correlation between lycopene and the inhibition of tyrosinase of *Dalbergia ecastaphyllum*²⁹. Moreover, Bão et al. reported an impressive tyrosinase inhibitory effect of the nano-form of lycopene³⁰. Another paper investigated the tyrosinase inhibition effect of lycopene loaded to solid lipid nanoparticles, which could mitigate melanogenesis in melanoma cells³¹. The inhibition of amylase and glucosidase allows the control of blood sugar levels after a diet rich in carbohydrates³². Given this interesting scenario, were investigated the enzyme inhibition abilities of the lycopene-enriched EVOO and EVOO against cholinesterase, amylase, glucosidase and tyrosinase (Table 3).

While the inhibition of AChE was higher for EVOO than lycopene-enriched EVOO, their inhibition of BChE was similar. For the inhibition of amylase, glucosidase and tyrosinase, lycopene-enriched oil gave the best results.

	<i>AChE</i> (mg GALAE/g)	<i>BChE</i> (mg GALAE/g)	<i>Tyrosinase</i> (mg KAE/g)	<i>Amylase</i> (mmol ACAE/g)	<i>Glucosidase</i> (mmol ACAE/g)
<i>EVO oil</i>	2.22 ± 0.01 ^a	4.42 ± 0.30 ^a	10.17 ± 1.56 ^b	1.17 ± 0.03 ^b	1.43 ± 0.12 ^b
<i>Lycopene-enriched EVO oil</i>	1.93 ± 0.09 ^b	4.41 ± 0.23 ^a	12.36 ± 1.56 ^a	1.42 ± 0.01 ^a	1.59 ± 0.08 ^a

Table 3. Enzyme inhibition activities of EVO oil and lycopene-enriched EVO oil. The data are reported as the mean of three experiments ± SD. GALAE: Galanthamine equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent.

Finally, cytotoxicity and morphological assays were performed to evaluate the effects of the treatment with the lycopene-enriched EVOO on PNT1A cells. The results have been compared to that of the treatment with EVOO. Indeed, the determination of their effects on cell viability in normal cells is essential to determine the safety and therapeutic potential of the enriched product. The iCELLigence real-time analysis system was applied for the determination of EVOO and EVOO + lycopene effects on cell proliferation, establishing their cytotoxicity on PNT1A cells. The PNT1A normal prostate cells were seeded into the iCELLigence E-plates that contain, at the base of each well, gold-plated sensor electrodes. These plates permit to monitor in real-time the cell viability through impedance measurements^{33–36}. The measurements were registered every 15 minutes for 96 hours; 72 hours of treatments were conducted with EVOO and EVOO + lycopene. Tables 4 and 5 showed the cell viability percentages corresponding to each dosage at specific time points for EVOO and EVOO + lycopene, respectively. The cell viability percentages were calculated as previously reported by Bender et al.¹³. The normalized cell index graphs are represented in Figure 2 (a and b).

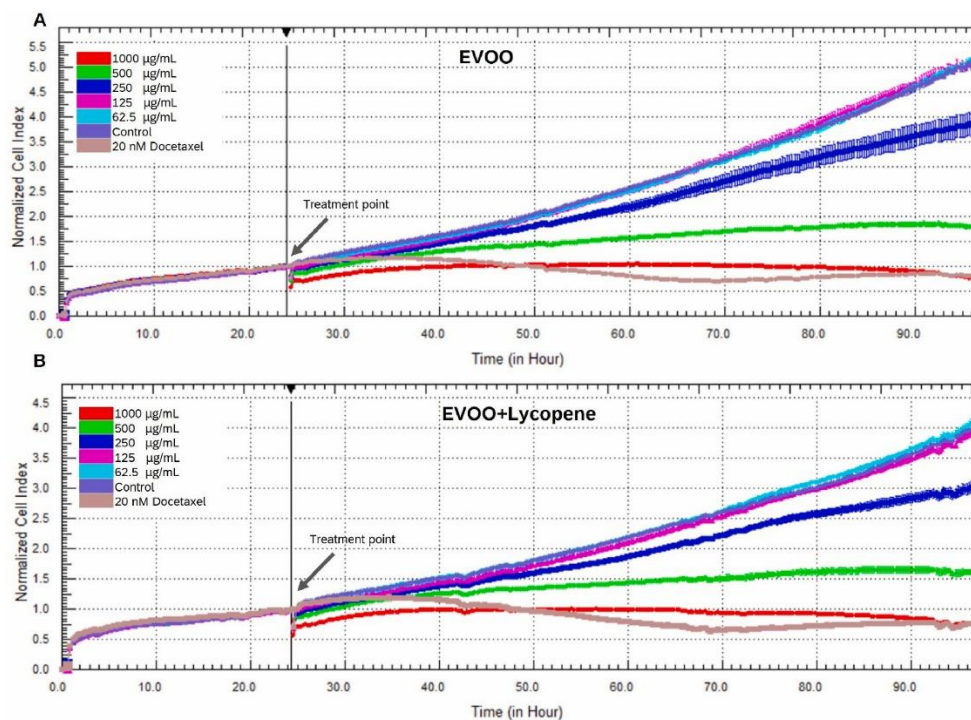


Figure 2. Determination of the cytotoxicity of EVOO (A) and EVOO + lycopene (B) through RTCA assay.

Time point	Cell Viability (% $\pm$ SEM)						
	Control	EVO oil ($\mu\text{g/mL}$)					Docetaxel
		1000	500	250	125	62.5	
24h	99.27 $\pm$ 0.00	51.54 $\pm$ 0.01	72.23 $\pm$ 0.00	87.66 $\pm$ 0.01	92.07 $\pm$ 0.00	95.01 $\pm$ 0.00	51.33 $\pm$ 0.00
48h	99.22 $\pm$ 0.00	30.34 $\pm$ 0.01	52.27 $\pm$ 0.01	84.53 $\pm$ 0.04	92.07 $\pm$ 0.00	95.01 $\pm$ 0.00	21.72 $\pm$ 0.00
72h	98.90 $\pm$ 0.01	14.75 $\pm$ 0.00	34.97 $\pm$ 0.01	75.83 $\pm$ 0.05	92.07 $\pm$ 0.00	95.01 $\pm$ 0.00	15.67 $\pm$ 0.00

Table 4. PNT1A cell viability rates (%) determined at 24h, 48h and 72h after the treatments with EVO oil or Docetaxel (Min %Control).

Time point	Cell Viability (% $\pm$ SEM)						
	Control	EVO oil + lycopene ($\mu\text{g/mL}$)					Docetaxel
		1000	500	250	125	62.5	
24h	97.80 $\pm$ 0.00	54.67 $\pm$ 0.01	75.04 $\pm$ 0.01	87.58 $\pm$ 0.01	93.02 $\pm$ 0.01	97.38 $\pm$ 0.03	55.32 $\pm$ 0.00
48h	94.46 $\pm$ 0.00	32.07 $\pm$ 0.00	53.35 $\pm$ 0.02	81.06 $\pm$ 0.01	91.87 $\pm$ 0.00	94.96 $\pm$ 0.01	23.03 $\pm$ 0.00
72h	94.18 $\pm$ 0.01	18.10 $\pm$ 0.00	38.54 $\pm$ 0.01	71.59 $\pm$ 0.02	91.31 $\pm$ 0.01	94.71 $\pm$ 0.01	17.34 $\pm$ 0.00

Table 5. PNT1A cell viability rates (%) determined at 24h, 48h and 72h after the treatments with EVO oil + lycopene or Docetaxel (Min %Control).

As shown in Figure 2, the cells had a comparable cell growth dynamic when treated with the EVOO or the lycopene-enriched EVOO in a time-dependent manner. In comparison with the control group, both treatments didn't affect in a significant manner the cell viability; nevertheless, after 48 and 72 hours of incubation at a concentration of 1000 $\mu\text{g/mL}$, the highest concentrations tested, the viability rates were respectively $30.34\% \pm 0.01$ and $14.75\% \pm 0.00$ for EVOO and $32.07\% \pm 0.00$ and $18.10\% \pm 0.00$ for EVOO + lycopene, which resulted in being the lowest cell viability. Thus, the PNT1A cell exposure to EVOO or EVOO + lycopene for 48 or 72 hours slightly reduced the cell viability compared to the control. Moreover, the enriched oil didn't show a cytotoxic effect above the only EVO oil. The treatment with the two samples at 1000 $\mu\text{g/mL}$ exhibited a viability profile close to that of 20 nM of docetaxel for all the time points. Observing the results, doses from 1000 $\mu\text{g/mL}$ to 250 $\mu\text{g/mL}$ of EVOO and EVOO + lycopene induced a slight reduction in cell viability in comparison with the control group, but, even at these concentrations, the cell viability rates were still quite high at 24 hours. More specifically, 250 $\mu\text{g/mL}$ and below showed higher cell viability during all time periods. Overall, EVOO + lycopene was not toxic for PNT1A normal prostatic cell line at concentrations below 500 $\mu\text{g/mL}$ throughout all the time periods. At 250 $\mu\text{g/mL}$ and below, it showed higher cell viability during all exposure times.

These findings were further validated through other assays performed to assess any morphological modifications on PNT1A cells after the treatment with EVOO or EVOO + lycopene. These assays allowed us to understand the effects of the two samples on cellular morphology. Figure 3 displayed the PNT1A photographed under an inverted microscope. Figure 4 showed the crystal violet staining, a colorimetric assay that dyes cellular nuclei, on PNT1A cells photographed after 72 hours of treatments. The top of Figures 3 and 4 showed the untreated PNT1A cells (control group), characterized by a polygonal shape and intense adhesion to the bottom of the plate, as expected given their epithelial nature. These cells showed also elongated cytoplasmatic regions and sustained membrane integrity leading to a larger cell volume. Figure 3 showed a considerably decreased viability compared to the control at 1000 $\mu\text{g/mL}$ of EVOO or EVOO + lycopene. Considering a 400x magnification (Figure 4), with the treatment with EVOO or EVOO + lycopene at 1000 $\mu\text{g/mL}$, fewer cells were observed, which appeared more rounded than the initial polygonal membrane structures. At 500 $\mu\text{g/mL}$ in both cases the cell viability was slightly reduced (Figure 3), but the structure of the cells was more intact than that at 1000 $\mu\text{g/mL}$ (Figure 4). As shown in Figure 3, EVOO and EVOO + lycopene from 250 $\mu\text{g/mL}$ to 62.5 $\mu\text{g/mL}$ gave higher cell viability. In accordance with this, Figure 4 showed that the polygonal shape of the cells was

maintained similar to the control group along with a higher cell volume at these concentrations. Overall, the treatments with EVOO or EVOO + lycopene didn't significantly modify the morphology of this cell line at concentrations below 500 $\mu\text{g/mL}$. In particular, for both treatments at 250 $\mu\text{g/mL}$ and below, the cell viability was higher and minimal morphological changes were determined; the integrity and the properties of the cells were maintained. Thus, the morphological evaluations are consistent with the results of the cell proliferation assays. Moreover, there weren't relevant differences in cellular morphology between the treatment using EVOO or EVOO + lycopene.

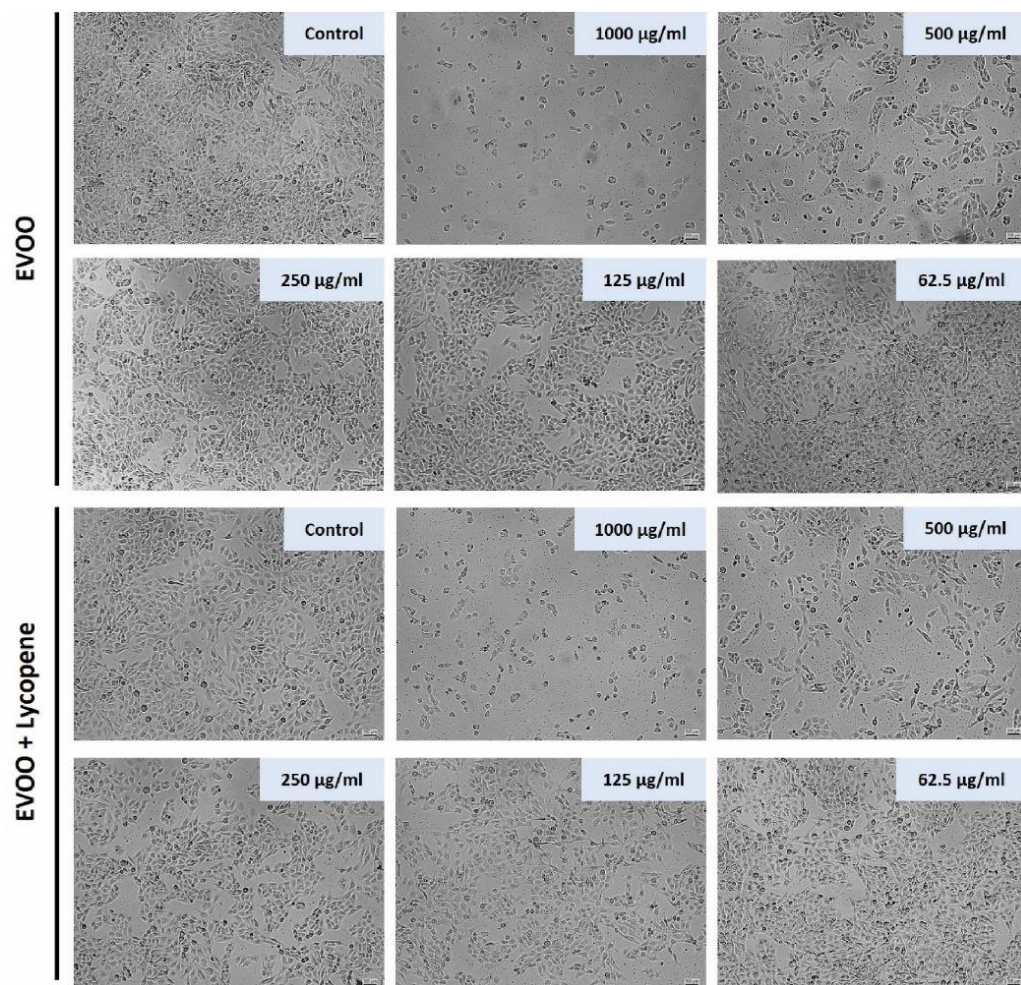


Figure 3. Morphological evaluations on PNT1A cells after the treatments with EVOO and EVOO + Lycopene.

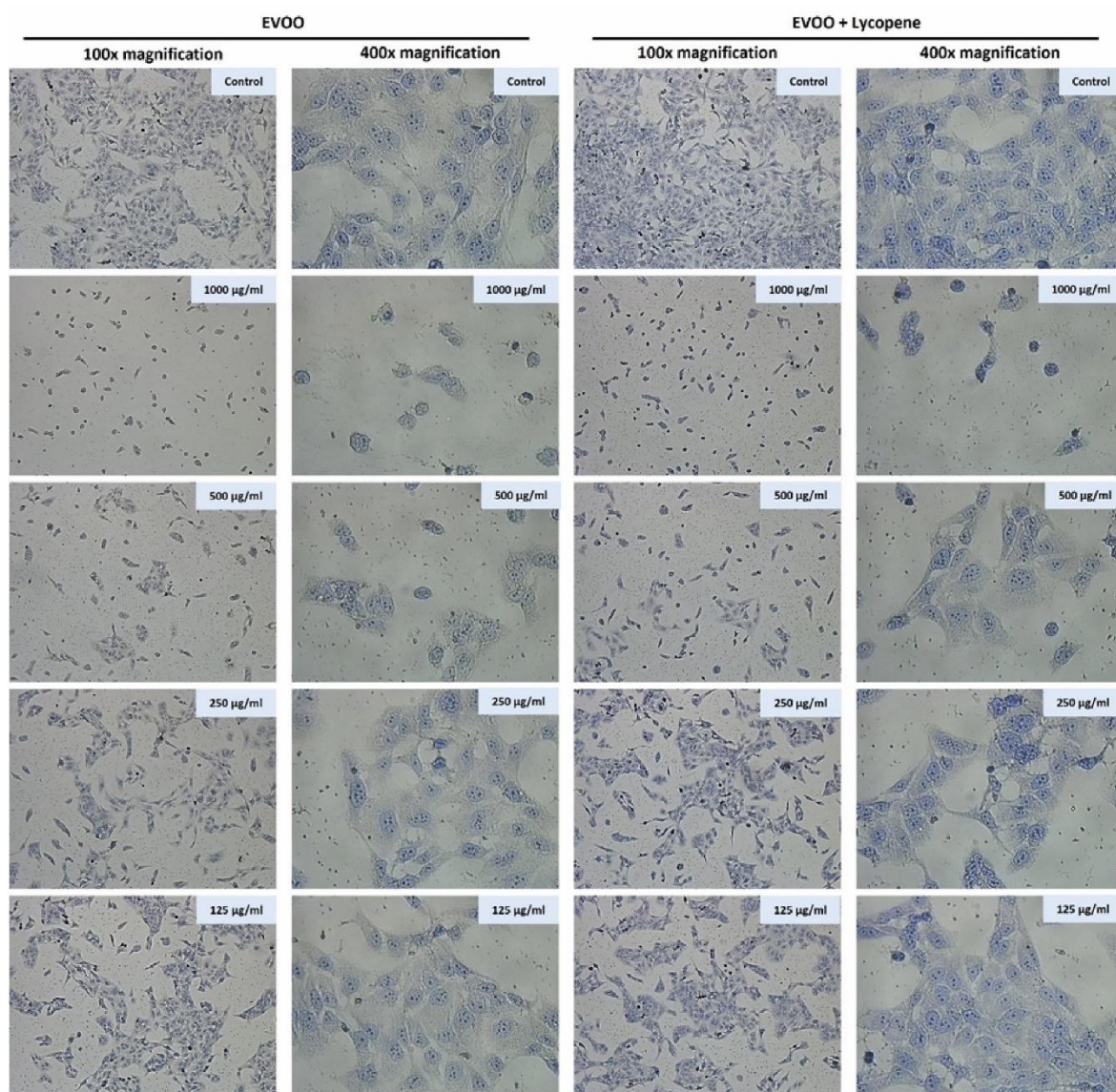


Figure 4. Crystal Violet Staining performed on PNT1A cells after the treatment with EVOO and EVOO + Lycopene.

3.4 Conclusions

In the European Union, lycopene is already authorised as a food additive (E 160d) in a variety of food categories. The acceptable daily intake is 0.5 mg/kg of body weight per day considering the assumption from all sources of lycopene³⁷. Given the importance of carotenoids assumption in human diet due to their benefits on health³⁸, the consumption of a daily spoon of an extra virgin olive oil enriched with lycopene could be an interesting alternative, especially for diets poor in red fruits like tomatoes. The use of tomato skins as a source of lycopene enhances the importance of this research which involves the concept of circular economy and re-evaluation of food industry waste for the production of valuable fortified and enriched foods.

The process to produce the enriched oil was completely green, applying an ultrasound-assisted extraction with mild conditions and obtaining a final fortified product safe *in vitro* on PNT1A cells with improved antioxidant and ameliorated enzyme inhibition activities against glucosidase, amylase and tyrosinase.

Future studies to assess lycopene stability in the EVO oil for the prediction of an expiration date and further safety evaluations are required, but these results suggest the potential of this enriched EVO oil as a novel food or food supplement opening new perspectives on the valorization of food waste. Moreover, in a future perspective, a significant aspect that could be investigated is the industrial production of this enriched EVO oil. Achat et al. conducted a study on the enrichment of virgin olive oil in phenolic compounds from olive leaves comparing a classical maceration with an ultrasound-assisted maceration followed by industrial trials of the UAE procedure. Not only the UAE considerably simplified the oil enrichment compared to maceration, but the polyphenols yield obtained in the pilot scale was equal to the laboratory scale. The UAE pilot study lowered the operating costs due to the shorter extraction time, lower operating temperature and high final yields. Already existing big scale ultrasound extraction reactors could be used to produce large quantities of enriched oil, considering volumes from 100 to 1000 L. These types of reactors can be applied to extract 15-150 kg of dry plant matrices per time³⁹. This leads us to think that the production of EVO oil enriched with lycopene could have a future industrial scale-up.

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Chapter 4:

Valorization of grape pomace extracts against commercially available berries and fruit extracts: Phytochemical investigation and biological evaluations

4.1 Introduction

Wine industry development leads to an increased production of waste, in particular waste-water and solid waste. Consequently, the investments in waste-water treatments and the management and valorization of the solid by-products have increased^{1,2}. Grape pomace, which includes grape seeds and peels deriving from the press process, is generated every year accounting for $\approx 20\%$ of the processed grapes^{2,3}. Grape pomace has been studied for its significant content of phenolics and flavonoids, antioxidant compounds which can act in different modes, *e.g.*, as radical scavengers, hydrogen donors, metal chelating agents or singlet oxygen quenchers. This is the reason why grape pomace should be considered for the production of nutraceuticals, cosmetics and fortified foods based on food waste⁴⁻⁷.

It's widely known that antioxidant compounds are abundantly found in natural sources, including berries⁸. Nowadays, berries extracts are prepared using different innovative methods based on ultrasound (UAE), microwave (MAE), pressurized-liquid extraction (PLE) and enzyme-assisted extraction which can be applied in association or not with conventional methods to enhance the extraction efficiency and the sustainability of the procedures⁹. A research work conducted on healthy subjects and people presenting metabolic and cardiovascular risk factors showed the benefits of the assumption of fresh berries on lipidic and carbohydrate metabolism and the antioxidant capacity for both groups¹⁰. Berries are rich in polyphenolic compounds; indeed, a large variety of studies have been conducted on the identification of the phytochemical profiles of these plant matrices and quantification of the main compounds. For instance, the phenolic, flavonoid and anthocyanin contents of cranberry were investigated and reported in literature¹¹⁻¹³. Cranberries and raspberries contain ellagic acid which represents approximately 50% of the total amount of phenolics^{14,15}. Rose hip was studied and 21 phenolics were identified including quercetin, eriodictyol and taxifolin glycosides¹⁶.

Due to the global high production of grape pomace, this research project aims to valorize it through a comparison between its extracts and those of commercial berries and fruits highlighting the nutraceutical potential of this by-product. The determination of equal or better properties of grape pomace compared to commercial products makes it a valuable potential ingredient to enrich food or produce nutraceuticals fitting the perspective of circular economy.

Considering the importance of applying green methods, three types of extraction procedures have been performed, in particular, the decoction and the more innovative

ultrasound and microwave-assisted extractions. For each extract, the phytochemical profile, phenolics and flavonoid content and *in vitro* biological properties have been determined¹⁷.

4.2 Materials and Methods

4.2.1 Plant matrices and reagents

Grape pomace (1 Kg) from the production of the local Montepulciano wine was furnished by Tenuta del Priore, Collecervino (PE), Italy (2019). *Vaccinium Vitis Idaea* L. (dried cranberry berries) (1 g), *Sambucus Nigra* (dried elderberry berries) (1 g) and *Rosa Canina* (dried rose hip berries) (1 g) were bought from a Bulgarian local vendor. *Lycium barbarum* (dried goji berries) (1 g) and raisins (1 g) (*Vitis vinifera* L.) were purchased from an Italian local market. The solvents and reagents were purchased from Sigma Aldrich, St. Louis, Missouri, USA, specifically EtOH puriss., MeOH gradient grade ($\geq 99.9\%$), ACN gradient grade ($\geq 99.9\%$) and formic acid ($\geq 95\%$) reagent grade. The analytical standards, all $\geq 99\%$ for HPLC, benzoic acid, catechin hydrate, citric acid, chlorogenic acid, ellagic acid, gallic acid monohydrate, isorhamnetin, myricetin, p-coumaric acid, quercetin anhydrous, rutin trihydrate and trans-ferulic acid were provided by Sigma Aldrich (Sigma Aldrich, St. Louis, Missouri, USA) along with kojic acid ($\geq 98.5\%$ for HPLC). Phosphomolybdenum, trolox and disodium edentate were purchased from Sinopharm Chemical Reagent Co., Ltd. Shanghai, China and were all of analytical grade. DTNB (5,5-dithio-bis(2-nitrobenzoic)) acid was purchased from Sigma, St. Louis, MO, USA. Acetylcholinesterase from electric eel Type-VI-S (EC 3.1.1.7), horse serum butyrylcholinesterase (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, L-DOPA, tyrosinase α -amylase ex-porcine pancreas (EC 3.2.1.1), α -glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20) and PNPG (4-N-trophenyl- α -D-glucopyranoside) were purchased from Sigma.

4.2.2 Instruments

The ultrasonic bath (Bandelin, Berlino, Germany), centrifuge (Eppendorf centrifuge 5702), Biotage Initiator™ 2.0 (Biotage Sweden AB, Uppsala, Sweden) - high-frequency microwaves 2.45 GHz, 0–300 W (power range) were used for the extractions. An Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA) supplied with an Agilent 6530B Q-TOF MS was used for the analysis of the phytochemical profile of each extract.

4.2.3 Sample preparation and extractions

The plant matrices were lyophilized and then ground through a blender. The plant materials were stored at -20°C until their extractions. The extraction procedures, decoction, ultrasound-assisted extraction and microwave-assisted extraction have been performed according to previously established protocols^{18,19}. The percentage extraction yields of each extract was determined using the formula:

$$\% \text{ yield} = [(\text{weight of dried extract})/(\text{weight of dried plant matrix})] \times 100$$

Decoction (DC)

Approximately 1g of the lyophilized and ground plant matrix was weighted and 20 mL of the mixture EtOH/H₂O (7:3) was added. After heating at reflux (u.a.), the mixture was kept boiling for 10 minutes, centrifuged at 4400 rpm for 3 minutes and filtered through a mesh filter. The solvent was evaporated by rotary evaporation to obtain the dried extract.

Ultrasound-assisted extraction

20 mL of EtOH/H₂O (7:3) was added to about 1 g of the stored sample and heated at 36°C. After the sonication (30 minutes, 36°C), the sample was centrifuged at 4400 rpm for 15 minutes and filtered through a mesh filter. The dried extract was obtained after the removal of the solvent by rotary evaporation.

Microwave-assisted extraction

Approximately 1 g of the plant matrix was weighed and dissolved in the mixture EtOH/H₂O (7:3) (20 mL) in a glass and sealed vial through the automatic Biotage Initiator™ 2.0 setting the temperature at 60°C, 10W for 10 minutes. After centrifugation at 4400 rpm for 20 minutes and filtration (mesh filter), the solvent was evaporated by rotary evaporation obtaining the dried extract.

4.2.4 Total phenolic and flavonoid contents

The procedures followed for the determination of the total phenolic content (TPC) and the total flavonoid content (TFC) were previously reported in literature^{20–22}. Specifically, TPC was determined by Folin-Ciocalteu assay and TFC by AlCl_3 method expressing the results, respectively, as gallic acid equivalents (mg GAE/g DE) and rutin equivalents (mg RE/g DE). For the Folin-Ciocalteu assay, a diluted solution of Folin-Ciocalteu reagent (1 mL, 1:9, v/v) was added to 0.25 mL of the sample solution and shaken. A solution of Na_2CO_3 (0.75 mL, 1%) was added to each sample after 3 minutes; the spectrophotometric read was done at 760 nm after 2 hours of incubation (r.t.). The AlCl_3 method was performed by mixing 1 mL of the sample solution with 1 mL aluminum trichloride (2%) in methanol. The sample and the prepared blank (1 mL of the sample mixed with 1 mL of MeOH without AlCl_3) were read spectrophotometrically after 10 minutes of incubation (r.t.) at 415 nm, subtracting the blank absorbance to that of the extract.

4.2.5 HPLC-ESI-Q-TOF-MS analysis

From 5 to 10 mg of each dried extract was dissolved in 1 mL of the mixture MeOH:H₂O (7:3), sonicated and filtered (0.45 μm). The obtained samples were analysed by HPLC-ESI-Q-TOF-MS (see 4.2.2). A Luna Omega Polar C18 (150 $\times$ 3.0 mm, 5 μm) (Phenomenex, Torrance, CA, USA) was used along with the Polar C18 Security Guard cartridge (4 $\times$ 3.0 mm). The eluents used were A (H₂O + 0.1% HCOOH) and B (ACN + 0.1% HCOOH) with the following gradient elution: 0–25 min 10–25% B, 25–30 min 25% B and 30–35 min 25–100% B; B returned to 10% in 7 min at the end of the run. The flow rate was set at 0.4 mL min⁻¹. The instrument was set with the following specifications: 3500 V (capillary voltage), 45 psi (nebulizer pressure), the flow rate of the drying gas 10 L/min, 325°C (gas temperature), 60 V and 140 V of the skimmer voltage and fragmentor voltage, respectively. The analyses were performed in positive and negative ion modes and the internal calibration was conducted continuously using the signals at $m/z = 112.9855$ and $m/z = 1033.9881$. “MS” and “Auto MS/MS” modes at collision energies equal to 10 V, 20 V and 40V were used to acquire values of m/z in the range of 50–1200 with a scan rate respectively of 2–3 spectra/sec. The data processing was performed with Agilent Mass Hunter Qualitative analysis software (B.06.00).

The main compounds found in the extracts were quantified using the HPLC-UV signal, the analytical standards used were: catechin and gallic acid at 280 nm; ellagic acid and benzoic acid at 250 nm; trans-ferulic acid, chlorogenic acid and *p*-coumaric acid at 320 nm; rutin, isorhamnetin, myricetin and quercetin at 350 nm. All the calibration curves had $R^2 \geq 0.999$ and were prepared using a concentration range between 0.5 and 100 mg L⁻¹ except for ellagic acid (2-20 mg L⁻¹) and *p*-coumaric acid (5-100 mg L⁻¹). The quantification of citric and isocitric acids was conducted in MS/MS mode using the 191 → 111 transition and a range of concentration from 1 to 25 mg L⁻¹ and $R^2=0.98$. The choice of using the UV or MS signals was related to the availability of the exact analytical standards, for example, quercetin was applied for the quantification of its derivatives. The lowest concentration of the reported ranges was our limits of quantification. Following are reported the limits of detection: 0.15 mg L⁻¹ for catechin, gallic acid, benzoic acid, trans-ferulic acid, chlorogenic acid, rutin, isorhamnetin, myricetin, citric acid and quercetin; 0.3 mg L⁻¹ for isocitric acid, 0.6 mg L⁻¹ for ellagic acid and 1.5 mg L⁻¹ for *p*-coumaric acid. For each extract, the percentage relative contribution of each characterized compound was evaluated considering the area under the peak determined in MS mode using the precursor ion, consequently, the % relative contribution of the single compound was calculated in relation to the sum of all the peak areas of the compounds found in the extract. The compounds with higher concentrations are indicated using an intense red.

4.2.6 *In vitro* antioxidant assays

For the DPPH assay, 4 mL of 0.004% MeOH solution of DPPH was prepared, and the solubilised extract was added to it. The mixture was incubated at r.t. for 30 minutes in the dark and then read at a wavelength of 517 nm²³.

For the ABTS assay, ABTS solution (7 mM) reacts with potassium persulfate (2.45 mM) forming ABTS⁺. ABTS solution was prior diluted with MeOH until the absorbance of 0.700 ± 0.02 at a wavelength of 734 nm was achieved. 2 mL of the ABTS solution was mixed with the sample solution. After 30 min of incubation (r.t.), the absorbance of the sample was read (734 nm)²⁴.

For the CUPRAC assay, a mixture of 1 mL of CuCl₂ (10 mM), 1 mL of neocuproine (7.5 mM) and 1 mL of NH₄Ac buffer (1 M and pH 7.0) was prepared and the sample solution (0.5 mL) added to it. The blank had a similar composition without the addition of CuCl₂.

After incubation (30 min, r.t.), the blank and the sample were read at a wavelength of 450 nm, and then the absorbance of the blank was deducted from that of the sample²⁵.

For the FRAP assay, 2 mL of FRAP reagent was mixed with acetate buffer at 0.3 M and pH 3.6, TPTZ (10 mM) in HCl (40 mM) and ferric chloride (20 mM) in the ratio of 10:1:1 (v/v/v), and then the sample solution was added to it. The incubation time was 30 minutes at r.t. and the wavelength used for the read was 593 nm²⁶.

For the PBD assay, the reagent solution (3 mL) composed of sulfuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM) was mixed with the sample solution, this mixture was incubated at 95°C for 90 minutes and read at 695 nm²⁷.

The MCA assay was performed by adding the 2 mL of the sample solution to a 0.05 mL of a solution of FeCl₂ (2 mM) and then 0.2 mL of ferrozine (5 mM). The blank was prepared similarly using 0.2 mL of water without ferrozine. After an incubation time of 10 minutes at r.t., the samples were read spectrophotometrically and the results were obtained by subtracting the absorbance of the blank from the absorbance of the sample at 562 nm²⁸.

The results of ABTS, DPPH, FRAP and CUPRAC assays are reported in mg Trolox equivalents/ g DE, those of PBD in mmol Trolox equivalents/ g DE and MCA in mg EDTAE/ g DE.

4.2.7 *In vitro* enzyme inhibition assays

For the Cholinesterase inhibition assay (Eldmann's method), the sample solution was added and mixed with 125 µL DTNB acid and 25 µL of AChE or BChE solution in the Tris-HCl buffer at pH = 8 in a 96 well-microplate. The mixture was incubated at 25°C for 15 minutes, then the reaction was started by adding 25 µL acetylthiocholine iodide or butyrylthiocholine chloride. The blank had the same composition, except for the absence of the enzyme solution. After an incubation time of 10 minutes at 25°C, the absorbances were read (405 nm) and the subtraction between the absorbance of the sample and that of the blank was done²⁹.

For the Tyrosinase inhibition assay (dopachrome method), the sample solution was put in a 96-well microplate along with 40 µL of tyrosinase solution and 100 µL of phosphate buffer (pH=6.8) and incubated (15 min, 25°C). 40 µL of L-DOPA was added to start the reaction. The absorbances of the sample and the blank, prepared without the enzyme, were registered at 492 nm after an incubation time of 10 minutes (25°C). The results were obtained by subtracting the blank absorbance from that of the sample³⁰.

For the α -amylase inhibition assay (iodine/potassium iodide method), 50 μ L of α -amylase solution in phosphate buffer (pH=6.9) with sodium chloride (6 mM) were mixed with the sample solution in the 96-well microplate and a pre-incubation was conducted for 10 minutes at 37°C. The reaction started after the addition of 50 μ L of the starch solution (0.05%) and subsequent incubation at 37°C for 10 minutes. The reaction was stopped by adding 25 μ L of HCl 1 M and then 100 μ L of the iodine-potassium iodine solution. The absorbances of the sample and the blank (same composition without the enzyme solution) were registered at 630 nm and that of the blank was subtracted from the absorbance of the sample³¹.

For the α -glucosidase inhibition assay (chromogenic PNPG method), the sample solution was added to 50 μ L of glutathione, 50 μ L of α -glucosidase solution in phosphate buffer (pH=6.8) and 50 μ L of PNPG in the 96-well microplate. After the incubation at 37°C for 15 minutes, the reaction was stopped adding 50 μ L of sodium carbonate 0.2 M. The sample and the blank (same composition without the enzyme) absorbances were registered at 400 nm. The absorbance of the blank was deducted from that of the sample³². The results of enzyme inhibition assays were expressed as mmol ACAE/ g DE for the α -glucosidase and α -amylase assays, mg GALAE/ g DE for AChE and BChE inhibition activity, mg KAE/ g DE for tyrosinase inhibition assay.

4.2.8 Statistical analysis

The experiments were performed in triplicate. Shapiro-Wilk test was used to evaluate the data normality. A p-value higher than 0.05 was considered. ANOVA test and Tukey's test were applied to determine the statistical differences among the extracts. The software used was Graph Pad Prism 9.2.

4.3 Results and Discussion

4.3.1 Extraction yields

The decoction has been the best extraction technique for Goji berries, raisins, rose hip and elderberry extracts. The extraction yields (measured as reported in paragraph 4.2.3) are reported in decreasing order:

- Goji berries extracts: 82 % DC, 81 % UAE and 47 % MAE.
- Raisins extracts: 80 % DC, 66 % MAE and 55 % UAE.
- Rose hip extracts: 55 % DC, 33 % MAE and 15 % UAE.
- Elderberries extracts: 47 % DC, 46 % MAE and 30 % UAE.

MAE gave the highest extraction yields for grape pomace and cranberry extracts:

- Grape pomace extracts: 36 % MAE, 32 % DC and 26 % UAE.
- Cranberries extracts: 63 % MAE, 55 % DC and 43 % UAE.

Polyphenols were soluble in the solvent mixture used and the temperature applied for decoction and MAE probably improved the mass transfer rate between the extraction solvent and the plant matrix. Thus, this can explain the better extraction yield in comparison with UAE. However, high temperatures ($\approx 70^{\circ}\text{C}$) along with the presence of O_2 , can reduce the anthocyanins content which goes through thermal degradation. This behaviour can explain the better yields of grape pomace and cranberry than DC and UAE.

4.3.2 TPC and TFC

The determination of the total phenolic and flavonoid content was conducted to provide an initial evaluation of the potential of the obtained extracts; the results are shown in Table 1.

	<i>Extraction method</i>	<i>TPC (mg GAE/g)</i>	<i>TFC (mg RE/g)</i>
<i>Grape pomace</i>	DC	67.42 ± 0.19 ^a	5.10 ± 0.09 ^c
	UAE	62.62 ± 1.25 ^b	6.85 ± 0.30 ^a
	MAE	62.83 ± 1.18 ^b	6.37 ± 0.22 ^b
<i>Cranberry</i>	DC	14.18 ± 0.85 ^j	1.82 ± 0.09 ^{ef}
	UAE	43.31 ± 0.33 ^c	1.93 ± 0.02 ^{ef}
	MAE	14.79 ± 0.33 ^{ij}	1.66 ± 0.03 ^{fg}
<i>Elderberry</i>	DC	17.20 ± 0.70 ^{gh}	3.80 ± 0.23 ^d
	UAE	34.73 ± 1.07 ^c	4.97 ± 0.16 ^c
	MAE	32.26 ± 0.37 ^f	4.78 ± 0.17 ^c
<i>Rose hip</i>	DC	38.50 ± 1.41 ^d	2.11 ± 0.12 ^e
	UAE	16.46 ± 0.22 ^{hi}	1.64 ± 0.05 ^{fg}
	MAE	19.21 ± 0.47 ^g	1.39 ± 0.02 ^g
<i>Goji berries</i>	DC	9.25 ± 0.20 ^k	0.55 ± 0.03 ^h
	UAE	9.60 ± 0.11 ^k	0.55 ± 0.02 ^h
	MAE	10.88 ± 0.16 ^k	0.53 ± 0.03 ^h
<i>Raisins</i>	DC	4.01 ± 0.18 ^l	0.22 ± 0.06 ^h
	UAE	4.11 ± 0.07 ^l	0.29 ± 0.06 ^h
	MAE	3.98 ± 0.05 ^l	0.30 ± 0.02 ^h

Table 1. TPC and TFC of the obtained extracts. Results are reported as the mean value ± SD of three parallel experiments. Different letters are put to indicate significant differences among the results ($p < 0.05$).

The highest total phenolic content determined by applying Folin-Ciocalteu assay was assigned to grape pomace extracts (62.62-67.42 mg GAE/g DE), cranberry UAE (43.31 mg GAE/ g DE) and rose hip DC (38.50 mg GAE/g DE). The TFC resulted higher in grape pomace extracts than the other types of extracts obtained, followed by elderberry extracts (3.80–4.97 mg RE/g DE). The extracts obtained from raisins had the lowest values of TPC and TFC. The extraction techniques seemed to have an impact on the TPC and TFC. Indeed, the extraction method should be selected depending on the plant matrix. For instance, the decoction is more convenient for hard plant material, indeed it was efficient for rose hip berries³³. Indeed, a previous study reported that the TPC of rosehip decoction was 96.7 mg GAE/250mL, while that of rose hip infusion was 68.2 mg GAE/250 mL³³. Cranberry and elderberries, being softer than rose hip berries, are more sensible to the penetration of ultrasonic waves³⁴. In a comparative study among Soxhlet extraction, UAE and shaking from American cranberry, UAE resulted in the best extraction technique in terms of phenolic compounds³⁵. Previous research articles also reported a positive impact on the extraction of phenolic compounds from grape pomace using the ultrasound-assisted extraction and microwave-assisted extraction^{36,37}.

The main drawback of the Folin-Ciocalteu assay is that this reagent can react and be reduced not only by phenolic compounds but also by some peptides, polysaccharides etc. obtaining not completely reliable results³⁸. Thus, an in-depth investigation of the phytochemical profile of each extract was conducted by HPLC-ESI-Q-TOF analyses.

4.3.3 Phytochemical characterization by HPLC-ESI-Q-TOF-MS

The investigation of the composition of each extract was performed by HPLC-ESI-Q-TOF-MS through the evaluation of the ion source fragmentation and the MS/MS fragmentation patterns, the accurate mass data, the accurate bibliographic investigation and the comparison with the available analytical standards. The identification of the phenolic acids and flavonoids in the extracts was conducted mainly in negative ion mode, while the presence of anthocyanins was evaluated in positive ion mode. The anthocyanins were detected only in grape pomace extracts. Their degradation could be related to multiple factors, *e.g.* storage temperature, pH, initial concentration, presence of enzymes, metallic ions or proteins^{39–41}.

A list of all the compounds characterized or tentatively characterized in the decoctions, ultrasound-assisted and microwave-assisted extracts are reported in Table 2.

t_R (min)	Observed [M-H] ⁻	Molecular formula	Fragment ions (m/z)	Assigned identification	Extract
1.699	377.0857	C ₁₂ H ₂₂ O ₁₁	341.1084, 221.0679, 179.0550 , 161.0440, 143.0354, 131.0367, 119.0371, 113.0240	Disaccharide (HCl adduct)	Cran, Goji
1.800	149.0098	C ₄ H ₆ O ₆	87.0088	Tartaric acid	Grape, Rai
1.916	191.0200	C ₆ H ₈ O ₇	173.0094, 129.0187, 111.0084 , 87.0090, 85.0294	Isocitric acid	Grape, Cran, Eld, Rose, Goji, Rai
1.978	133.0144	C ₄ H ₆ O ₅	115.0032 , 71.0141	Malic acid	Grape, Cran, Eld, Rose, Goji, Rai

t_R (min)	Observed [M-H] ⁻	Molecular formula	Fragment ions (m/z)	Assigned identification	Extract
2.532	191.0200	C ₆ H ₈ O ₇	173.0138, 129.0198, 111.0090 , 87.0090, 85.0299	Citric acid	All
2.558	117.0193	C ₄ H ₆ O ₄	99.0086, 73.0296	Succinic acid	Eld, Rose
2.956	205.0354	C ₇ H ₁₀ O ₇	111.0085 , 99.0472, 87.0087, 67.0189, 57.0365	Methylcitric acid	Cran, Rose
2.969	326.1245	C ₁₅ H ₂₁ NO ₇	164.0711 , 101.0242	Phenylalanine hexoside	Eld,
3.202	315.0723	C ₁₃ H ₁₆ O ₉	153.0194 , 109.0296	Protocatechuic acid- <i>O</i> - hexoside	Cran, Rai
3.210	169.0143	C ₇ H ₆ O ₅	125.0243	Gallic acid	Grape, Cran, Rose
3.278	164.0715	C ₉ H ₁₁ NO ₂	147.0446 , 103.0550, 91.0550, 72.0092	Phenylalanine	Eld,
3.703	331.0673	C ₁₃ H ₁₆ O ₁₀	169.0140 , 125.0247	Galloyl hexoside	Grape
3.888	315.1085	C ₁₄ H ₂₀ O ₈	153.0554 , 123.0446	Hydroxytyrosol hexoside	Grape, Eld,
4.017	329.0880	C ₁₄ H ₁₈ O ₉	167.0345 , 152.0110, 123.0454, 108.0202	Vanillic acid hexoside	Eld
4.311	219.0509	C ₈ H ₁₂ O ₇	173.0080, 129.0185, 111.0085 , 87.0079, 67.0193, 57.0349	Dimethylcitric acid	Grape, Cran, Rose, Rai
4.557	299.0773	C ₁₃ H ₁₆ O ₈	239.0587, 209.0440, 179.0339, 151.0383, 137.0238 , 119.0343, 113.0197	Monohydroxybenzoyl hexose	Cran
4.639	153.0556	C ₈ H ₁₀ O ₃	123.0445	Hydroxytyrosol	Grape
4.709	219.0514	C ₈ H ₁₂ O ₇	111.0091 , 87.0087, 67.0188, 57.0361	Dimethylcitric acid	Cran, Eld, Rose
4.991	359.0982	C ₁₅ H ₂₀ O ₁₀	197.0444 , 153.0570, 135.0431	Syringic acid hexoside	Grape, Rose
5.521	153.0194	C ₇ H ₆ O ₄	109.0293	Protocatechuic acid	Grape, Cran,

t_R (min)	Observed [M-H] ⁻	Molecular formula	Fragment ions (m/z)	Assigned identification	Extract
					Eld, Rose
5.434	353.0876	C ₁₆ H ₁₈ O ₉	191.0564 , 179.0349, 173.0428, 135.0447	Neochlorogenic acid	Eld
5.788	345.0826	C ₁₄ H ₁₈ O ₁₀	183.0294 , 168.0056, 124.0157	Methylgallate-hexoside	Rose
5.926	203.0826	C ₁₁ H ₁₂ N ₂ O ₂	186.0527, 159.0918, 142.0665, 116.0497 , 74.0243	L-Tryptophan	Eld
6.317	311.0408	C ₁₃ H ₁₂ O ₉	179.0344 , 149.0088, 135.0440	Caftaric acid	Grape, Rai
6.330	175.0613	C ₇ H ₁₂ O ₅	157.0501, 131.0711, 129.0574, 115.0397 , 113.0603, 85.0657	Isopropyl-malic acid	Grape
6.339	371.0980	C ₁₆ H ₂₀ O ₁₀	325.0879, 163.0392 , 119.0482	Coumaric acid- <i>O</i> -hexoside (formate adduct)	Cran, Eld, Goji
6.378	183.0299	C ₈ H ₈ O ₅	168.0061 , 124.0157	Methyl gallate	Grape
6.584	327.1094	C ₁₅ H ₂₀ O ₈	165.0525, 147.0458	Phenyllactic acid 2- <i>O</i> - hexoside	Rose
6.955	339.0726	C ₁₅ H ₁₆ O ₉	177.0194 , 149.0232, 133.0289	Esculetin hexoside	Cran
7.014	341.0875	C ₁₅ H ₁₈ O ₉	281.0664 , 251.0555, 221.0456, 179.0342, 135.0441	Caffeic acid- <i>C</i> -hexoside	Grape
7.132	487.1455	C ₂₁ H ₂₈ O ₁₃	323.0974, 163.0400 , 145.0291, 119.0457	Coumaric acid- <i>O</i> -dihexoside	Rose, Goji
7.137	577.1351	C ₃₀ H ₂₆ O ₁₂	451.1001, 425.0879 , 407.0771, 289.0704, 287.0557, 125.0237	(Epi)catechin-(epi)catechin (B-type)	Grape, Rose
7.429	515.1400	C ₂₂ H ₂₈ O ₁₄	353.0903, 341.0861, 323.0765 , 191.0549, 179.0355	Caffeoylglucosyl quinic acid	Goji
7.841	447.1501	C ₁₉ H ₂₈ O ₁₂	401.1451 , 269.1024, 161.0463, 131.0357	Benzyl alcohol hexose pentose (formate adduct)	Grape, Eld, Rai
7.865	337.0929	C ₁₆ H ₁₈ O ₈	163.0394 , 119.0503	3- <i>p</i> -Coumaroylquinic acid	Rose

t_R (min)	Observed [M-H]⁻	Molecular formula	Fragment ions (m/z)	Assigned identification	Extract
7.917	315.1086	C ₁₄ H ₂₀ O ₈	269.1029 , 161.0435, 153.0204	Benzyl glucopyranoside (formate adduct)	Cran
7.964	325.0930	C ₁₅ H ₁₈ O ₈	163.0399 , 119.0503	Coumaric acid- <i>O</i> -hexoside	Grape, Cran, Goji
8.114	577.1347	C ₃₀ H ₂₆ O ₁₂	451.1025, 425.0859 , 407.0748, 289.0700, 287.0558, 125.0246	(Epi)catechin-(epi)catechin (B-type)	Grape, Rose
8.272	341.0872	C ₁₅ H ₁₈ O ₉	281.0653 , 251.0558, 221.0449, 179.0333, 135.0447	Caffeic acid- <i>C</i> -hexoside	Grape
8.888	447.1496	C ₁₉ H ₂₈ O ₁₂	401.1453 , 293.0799, 269.0978, 233.0577, 161.0399	Benzyl alcohol hexose pentose (formate adduct)	Grape
8.950	289.0718	C ₁₅ H ₁₄ O ₆	245.0815 , 205.0506, 203.0715, 179.0338, 125.0237	Catechin	Grape, Rose
8.961	465.1033 (+)	C ₂₁ H ₂₀ O ₁₂	303.0497 , 257.0479, 229.0479	Delphinidin-3- <i>O</i> -hexoside	Grape
9.144	329.0880	C ₁₄ H ₁₈ O ₉	283.0822, 121.0292 , 77.0400	Benzoic acid- <i>O</i> -hexoside (formate adduct)	Cran
9.454	353.0879	C ₁₆ H ₁₈ O ₉	191.0555 , 179.0343, 161.0227, 135.0457 111.0085	Chlorogenic acid	Cran, Eld, Goji
9.699	461.1300	C ₁₉ H ₂₆ O ₁₃	415.1246 , 293.0882, 121.0293, 77.0407	Benzoic acid- <i>O</i> - pentosylhexoside (formate adduct)	Cran
9.750	431.1917	C ₂₀ H ₃₂ O ₁₀	385.1862 , 223.1308, 153.0927	Roseoside (formate adduct)	Grape, Cran, Rose
10.490	577.1345	C ₃₀ H ₂₆ O ₁₂	451.1000, 425.0866 , 407.0745, 289.0684, 287.0554, 125.0262	(Epi)catechin-(epi)catechin (B-type)	Grape
10.581	433.2077	C ₂₀ H ₃₄ O ₁₀	387.2010 , 225.1589, 179.0573, 161.0426, 113.0223	Dihydro-roseoside (formate adduct)	Rose
10.613	491.1194	C ₂₃ H ₂₄ O ₁₂	329.0653 , 315.0495, 300.0280, 299.0202, 271.0634	Tricin- <i>O</i> -hexoside	Grape

t_R (min)	Observed [M-H] ⁻	Molecular formula	Fragment ions (m/z)	Assigned identification	Extract
10.643	177.0187	C ₉ H ₆ O ₄	133.0293	Esculetin	Cran
10.848	449.1085 (+)	C ₂₁ H ₂₀ O ₁₁	287.0544	Cyanidin-3- <i>O</i> -hexoside	Grape
10.868	771.1982	C ₃₃ H ₄₀ O ₂₁	609.1467 , 462.0797, 301.0339, 178.9973, 151.0014	Quercetin- <i>O</i> -hexoside- <i>O</i> - rutinoside	Goji
11.016	577.1344	C ₃₀ H ₂₆ O ₁₂	451.1053, 425.0849 , 407.0772, 289.0721, 287.0540, 125.0242	(Epi)catechin-(epi)catechin (B-type)	Grape
11.461	179.0350	C ₉ H ₈ O ₄	135.0451	Caffeic acid	Cran
11.629	517.1561	C ₂₂ H ₃₀ O ₁₄	384.9326, 355.1009, 337.0938, 248.9599, 193.0502 , 175.0389, 149.0610, 134.0336	Ferulic acid- <i>O</i> -di-hexoside	Goji
11.783	479.1191 (+)	C ₂₂ H ₂₂ O ₁₂	317.0658	Petunidin- <i>O</i> -hexoside	Grape
12.338	289.0718	C ₁₅ H ₁₄ O ₆	245.0822 , 205.0502, 203.0713, 179.0343, 125.0243	(Epi)catechin	Grape
12.393	449.1083	C ₂₁ H ₂₂ O ₁₁	287.0563 , 269.0436, 259.0579	Dihydrokaempferol- <i>O</i> - hexoside	Rose
13.787	325.0933	C ₁₅ H ₁₈ O ₈	163.0410, 119.0506	Coumaric acid- <i>O</i> -hexoside	Rose
13.989	367.1027	C ₁₇ H ₂₀ O ₉	193.0541, 173.0437 , 111.0419	4-Feruloylquinic acid	Eld
14.060	463.1236 (+)	C ₂₂ H ₂₂ O ₁₁	301.0704 , 286.0453	Peonidin-3- <i>O</i> -hexoside	Grape
14.186	197.0455	C ₉ H ₁₀ O ₅	169.0152, 124.0160	Ethyl gallate	Grape
14.494	491.1189	C ₂₃ H ₂₄ O ₁₂	329.0657 , 314.0408, 313.0328, 299.0184	Tricin- <i>O</i> -hexoside	Grape
16.546	355.1032	C ₁₆ H ₂₀ O ₉	193.0504 , 134.0373	Ferulic acid- <i>O</i> -hexoside	Cran
16.736	163.0400	C ₉ H ₈ O ₃	119.0498	<i>p</i> -Coumaric acid	Cran, Eld, Rose, Goji

t_R (min)	Observed [M-H]⁻	Molecular formula	Fragment ions (m/z)	Assigned identification	Extract
16.869	625.1412	C ₂₇ H ₃₀ O ₁₇	463.0885 , 301.0368	Quercetin- <i>O</i> -dihexoside	Grape
16.917	435.0927	C ₂₀ H ₂₀ O ₁₁	303.0522, 285.0409, 177.0188, 151.0034 , 125.0232	Taxifolin- <i>O</i> -pentoside	Rose
17.025	755.2041	C ₃₃ H ₄₀ O ₂₀	591.1320, 489.1022, 301.0320, 300.0270 , 271.0254, 255.0285, 178.9984, 151.0062	Quercetin derivative	Eld
17.612	509.1299	C ₂₃ H ₂₆ O ₁₃	355.0663, 347.0759 , 329.0664, 193.0108, 149.0236	Unknown	Grape
17.911	191.0353	C ₁₀ H ₈ O ₄	176.0104 , 148.0142	Scopoletin	Goji
17.982	493.0623	C ₂₁ H ₁₈ O ₁₄	317.0308 , 178.9971, 151.0322	Myricetin- <i>O</i> -glucuronide	Grape
18.105	641.1357	C ₂₇ H ₃₀ O ₁₈	479.0828 , 317.0294	Myricetin- <i>O</i> -dihexoside	Grape
18.137	355.1032	C ₁₆ H ₂₀ O ₉	193.0503 , 134.0363	Ferulic acid- <i>O</i> -hexoside	Cran
18.167	479.0827	C ₂₁ H ₂₀ O ₁₃	317.0278, 316.0223 , 287.0183, 271.0249, 178.9981, 151.0009	Myricetin- <i>O</i> -hexoside	Grape
18.858	449.2033	C ₂₀ H ₃₄ O ₁₁	269.1387 , 225.1536	Apigenin derivative	Rose
19.221	449.1091	C ₂₁ H ₂₂ O ₁₁	287.0551 , 151.0041, 135.0465	Eriodictyol- <i>O</i> -hexoside	Rose
19.925	441.0822	C ₂₂ H ₁₈ O ₁₀	331.0419, 289.0702, 271.0577, 245.0943, 169.0137 , 125.0254	(Epi)catechin- <i>O</i> -gallate	Grape
20.046	303.0509	C ₁₅ H ₁₂ O ₇	285.0388 , 177.0180, 125.0246	Taxifolin	Cran
20.429	519.1132	C ₂₄ H ₂₄ O ₁₃	315.0481 , 300.0287, 299.0170	Isorhamnetin- <i>O</i> - acetylhexoside	Grape
20.726	609.1463	C ₂₇ H ₃₀ O ₁₆	301.0334, 300.0265 , 178.9964, 151.0037	Rutin	Grape, Eld, Goji, Rai
21.381	463.0878	C ₂₁ H ₂₀ O ₁₂	301.0339, 300.0274 , 271.0232, 255.0299, 178.9972, 151.0026	Quercetin- <i>O</i> -hexoside	All

t_R (min)	Observed [M-H]⁻	Molecular formula	Fragment ions (m/z)	Assigned identification	Extract
21.599	300.9992	C ₁₄ H ₆ O ₈	257.0091, 229.0137	Ellagic acid	Rose
21.689	477.0672	C ₂₁ H ₁₈ O ₁₃	301.0348 , 178.9984, 151.0028	Quercetin- <i>O</i> -glucuronide	Grape, Rose, Rai
21.812	463.0878	C ₂₁ H ₂₀ O ₁₂	301.0335, 300.0269 , 271.0247, 255.0277, 178.9968, 151.0033	Quercetin- <i>O</i> -hexoside	Grape, Cran, Rai
21.997	493.0987	C ₂₂ H ₂₂ O ₁₃	331.0445 , 330.0377, 316.0233, 315.0179	Mearnsetin- <i>O</i> -hexoside	Grape
22.120	435.0930	C ₂₀ H ₂₀ O ₁₁	303.0509 , 285.0398, 177.0183, 151.0037, 125.0250	Taxifolin- <i>O</i> -pentoside	Rose
22.564	579.2071	C ₂₈ H ₃₆ O ₁₃	417.1550 , 402.1324, 181.0477, 166.0344, 151.0153	Syringaresinol- <i>O</i> -hexoside	Eld
22.598	505.1346 (+)	C ₂₄ H ₂₄ O ₁₂	301.0702 , 286.0471	Peonidin-3- <i>O</i> -acetylhexoside	Grape
22.820	535.1455 (+)	C ₂₅ H ₂₆ O ₁₃	331.0813 , 315.0501, 299.0546, 287.0542, 270.0519, 242.0579, 179.0330	Malvidin-3- <i>O</i> -acetylhexoside	Grape
23.840	593.1512	C ₂₇ H ₃₀ O ₁₅	285.0396 , 255.0283, 229.0507, 151.0035	Kaempferol- <i>O</i> -rutinoside	Eld, Goji
23.907	447.0927	C ₂₁ H ₂₀ O ₁₁	285.0383, 284.0327 , 255.0292, 227.0333, 151.0046	Kaempferol- <i>O</i> -hexoside	Grape, Rai
24.055	641.1504 (+)	C ₃₁ H ₂₈ O ₁₅	317.0655 , 302.0412	Petunidin-(6- <i>O</i> - caffeoyl)hexoside	Grape
24.598	193.0506	C ₁₀ H ₁₀ O ₄	178.0247, 149.0604, 134.0366	Ferulic acid	Cran
24.653	623.1606	C ₂₈ H ₃₂ O ₁₆	315.0495 , 300.0266, 271.0224	Isorhamnetin- <i>O</i> -rutinoside	Eld, Goji
24.656	433.0763	C ₂₀ H ₁₈ O ₁₁	301.0349 , 178.9935, 151.0009	Quercetin- <i>O</i> -pentoside	Grape, Cran, Rose
24.993	611.1400 (+)	C ₃₀ H ₂₆ O ₁₄	303.0503 , 257.0428, 229.0570	Delphinidin-3-(6- <i>O</i> - coumaroyl)hexoside	Grape

t_R (min)	Observed [M-H]⁻	Molecular formula	Fragment ions (m/z)	Assigned identification	Extract
25.087	447.0930	C ₂₁ H ₂₀ O ₁₁	285.0386 , 284.0322, 255.0309, 227.0349, 151.0053	Kaempferol- <i>O</i> -hexoside	Grape
25.272	461.0725	C ₂₁ H ₁₈ O ₁₂	285.0406 , 175.0238, 113.0233	Scutellarin	Grape
25.334	447.0932	C ₂₁ H ₂₀ O ₁₁	301.0343 , 271.0241, 255.0270, 178.9975, 151.0028	Quercetin- <i>O</i> -deoxyhexoside	Grape, Cran, Rose
25.586	477.1030	C ₂₂ H ₂₂ O ₁₂	315.0539, 314.0416 , 150.9988	Isorhamnetin- <i>O</i> -hexoside	Grape, Eld
26.101	625.1559 (+)	C ₃₁ H ₂₈ O ₁₄	301.0709 , 286.0458, 258.0535	Peonidin-(6- <i>O</i> - caffeoyl)hexoside	Grape
26.243	655.1666 (+)	C ₃₂ H ₃₀ O ₁₅	331.0811 , 316.0574, 299.0539, 287.0545, 270.0515, 242.0571, 179.0314	Malvidin-(6- <i>O</i> - caffeoyl)hexoside	Grape
26.386	435.1291	C ₂₁ H ₂₄ O ₁₀	273.0753 , 167.0352	Phloretin- <i>O</i> -hexoside	Cran, Rose
27.009	595.1448 (+)	C ₃₀ H ₂₆ O ₁₃	287.0546	Cyanidin-(6- <i>O</i> - coumaroyl)hexoside	Grape
27.216	655.1666	C ₃₂ H ₃₂ O ₁₅	501.1029, 475.1221, 347.0760, 329.0659 , 303.0880, 193.0137, 149.0248	Unknown	Grape
27.278	317.0302	C ₁₅ H ₁₀ O ₈	178.9959 , 151.0022	Myricetin	Grape
27.530	625.1558 (+)	C ₃₁ H ₂₈ O ₁₄	317.0653 , 302.0423, 274.0446	Petunidin-(6- <i>O</i> - coumaroyl)hexoside	Grape
29.291	431.0974	C ₂₁ H ₂₀ O ₁₀	286.0470, 285.0385 , 284.0310, 255.0299, 229.0465, 227.0313	Kaempferol- <i>O</i> - deoxyhexoside	Grape, Cran
29.551	639.1715 (+)	C ₃₂ H ₃₀ O ₁₄	331.0813 , 315.0499, 299.0549, 287.0553, 270.0533, 242.0565, 179.0327	Malvidin-3-(6- <i>O</i> -coumaroyl) hexoside	Grape
29.653	609.1609 (+)	C ₃₁ H ₂₈ O ₁₃	301.0707 , 286.0465, 258.0515, 230.0617	Peonidin-3-(6- <i>O</i> - coumaroyl)hexoside	Grape

t_R (min)	Observed [M-H] ⁻	Molecular formula	Fragment ions (m/z)	Assigned identification	Extract
31.112	591.1352	C ₂₇ H ₂₈ O ₁₅	529.1330, 489.1027 , 447.0940, 301.0351, 300.0269, 178.9945	Quercetin-3- <i>O</i> -(4''- hydroxymethylglutaryl)- α - rhamnoside	Cran
31.179	312.1239	C ₁₈ H ₁₉ NO ₄	297.1011, 178.0499 , 148.0535, 135.0443	Feruloyltyramine	Goji
35.767	301.0355	C ₁₅ H ₁₀ O ₇	178.9990, 151.0035	Quercetin	Grape, Cran, Eld, Rose
36.716	567.1136	C ₂₈ H ₂₄ O ₁₃	301.0349, 300.0277 , 271.0269, 255.0383, 178.9984, 151.0021	Quercetin derivative	Cran
37.403	593.1300	C ₃₀ H ₂₆ O ₁₃	447.0908, 285.0395 , 284.0324, 257.0451	Kaempferol- <i>O</i> -(coumaroyl)- hexoside	Rose
38.605	327.2169	C ₁₈ H ₃₂ O ₅	229.1436, 211.1329, 183.1418, 171.1017	Oxo-dihydroxy-octadecenoic acid	Grape, Eld, Rai
38.871	271.0616	C ₁₅ H ₁₂ O ₅	177.0192, 151.0024 , 119.0525, 107.0134	Naringenin	Cran
40.117	345.0610	C ₁₇ H ₁₄ O ₈	330.0372 , 315.0123	Syringetin	Grape
40.172	329.2333	C ₁₈ H ₃₄ O ₅	229.1467 , 211.1354, 171.1049	Trihydroxy-octadecenoic acid	Grape, Eld, Goji, Rai
40.442	315.0506	C ₁₆ H ₁₂ O ₇	300.0264	Isorhamnetin	Grape

Table 2. Assigned identification and HPLC-MS parameters of all the compounds found in the extracts (DC, UAE and MAE) of grape pomace (Grape), cranberry (Cran), elderberry (Eld), rose hip berries (Rose), goji berries (Goji) and raisins (Rai). The base peak is marked in bold. (+): compounds detected in positive ion mode.

The compound with [M-H]⁻ value at m/z 377 was identified as a disaccharide (HCl adduct) observing its base peak at m/z 179 (hexoside), the neutral loss of 36 Da corresponding to the HCl and the fragmentation pattern typical of the hexosides (m/z 161, 143, 131, 119 and 113)⁴². Tartaric acid was characterized thanks to its molecular formula (C₄H₆O₆) and its typical fragmentation pattern (149→87)⁴³. The two compounds showing [M-H]⁻ at m/z 191, with the same molecular formula (C₆H₈O₇) and the following fragment

ions m/z 173, 129, 111, 87 and 85 were identified as citric and its isomer, isocitric acid, by comparison with the analytical standard citric acid. The compounds having $[M-H]^-$ at m/z 205 ($C_7H_{10}O_7$) and $[M-H]^-$ at m/z 219 ($C_8H_{12}O_7$) and a fragmentation pattern similar to citric acid, were tentatively characterized as methyleitric acid and dimethyleitric acid isomers based also on their molecular formulas. Malic acid was identified by observing its $[M-H]^-$ at m/z 133, the fragment ions at m/z 115 and 71 and its molecular formula. Succinic acid ($C_4H_6O_4$) was characterized thanks to its fragmentation pattern reported in literature by Tonidandel et al.⁴⁴. Phenylalanine was identified by comparison with data found in literature having $[M-H]^-$ at m/z 164 and fragment ions at m/z 147, 103, 91 and 72⁴⁵. The compound characterized as phenylalanine hexoside ($C_{15}H_{21}NO_7$) showed the fragmentation pattern $326 \rightarrow 164$, namely a neutral loss of 162 Da which corresponds to an hexoside moiety. Protocatechuic acid ($C_7H_6O_4$), fragmentation pattern ($153 \rightarrow 109$), was identified by comparison with the corresponding analytical standard. Protocatechuic acid-*O*-hexoside isomers have been detected in the extracts and identified observing $[M-H]^-$ at m/z 315, fragment ions at m/z 153 and 109 a neutral loss of 162 Da (hexoside moiety). Gallic acid ($C_7H_6O_5$), fragmentation pattern ($169 \rightarrow 125$), was characterized by comparison with the analytical standard, while the derivatives of gallic acid were tentatively characterized by observing the $[M-H]^-$, the neutral losses and molecular formulas. Specifically, to $[M-H]^-$ at m/z 331, neutral loss 162 Da and the typical fragment ions of gallic acid at m/z 169 and 125 correspond the galloyl hexoside, while methyl gallate and ethyl gallate were characterized by observing the molecular formula, fragmentation pattern and the neutral losses of 15 Da ($-CH_3$) and 28 Da ($-CH_2CH_2-$), respectively. Methyl gallate-hexoside, $[M-H]^-$ at m/z 345, was characterized thanks to its fragment ions (m/z 183, 168 and 124) and the neutral loss of the hexoside¹⁶. Hydroxytyrosol was identified thanks to its molecular formula ($C_8H_{10}O_3$) and fragmentation pattern $153 \rightarrow 123$, previously reported in literature⁴⁶. The similar fragmentation pattern, $[M-H]^-$ at m/z 315 and the neutral loss of a hexoside moiety yielding hydroxytyrosol at m/z 153, allowed the characterization of the hydroxytyrosol hexoside. Vanillic acid hexoside was identified based on $[M-H]^-$ at m/z 329, its fragment ions (m/z 167, 152, 123 and 108) typical of vanillic acid according to Santos et al.⁴⁷ According to bibliographic data, the compound showing deprotonated molecular ion at m/z 359 and fragment ions at m/z 197, 153 and 135, was identified as syringic acid hexoside (neutral loss: 162 Da)⁴⁸. Chlorogenic acid was identified by comparing the analytical standard which allow us to determine its retention time. Another compound, with the same $[M-H]^-$ at m/z 353, fragment ions and with molecular formula $C_{16}H_{18}O_9$ was detected and identified as

neochlorogenic acid. According to Feketeová et al., we identified L-tryptophan which has $[M-H]^-$ at m/z 203, m/z 186, 159, 142, 116 and 74 as fragment ions⁴⁹. $[M-H]^-$ at m/z 311, molecular formula $C_{13}H_{12}O_9$ and fragment ions at m/z 179, 149 and 135 identify caftaric acid, in agreement with bibliographic data⁵⁰. According to Ricciutelli et al., the isopropylmalic acid was characterized by evaluating the fragmentation pattern and the molecular formula⁵¹. *p*-coumaric acid and its derivatives were identified in our extract. *p*-coumaric acid (163→119) was compared to the analytical standard. Coumaric acid-*O*-hexoside (formate adduct) had $[M-H]^-$ at m/z 371, fragment ions at m/z 325, 163 and 119, the neutral loss of 46 Da corresponds to the formate adduct and 162 Da corresponds to the hexoside moiety. Coumaric acid-*O*-hexoside showed $[M-H]^-$ at m/z 325 and fragments at m/z 163 and 119. 3-*p*-coumaroylquinic acid was characterized following the scheme of Clifford et al., it had $[M-H]^-$ at m/z 337, fragment ions were at m/z 163 and 119⁵². Some coumaric acid-*O*-dihexoside isomers were tentatively identified based on the presence of the fragment ions at m/z 163 and 119 and the neutral loss of 324 Da (162 Da + 162 Da). According to Beelders et al., phenyllactic acid 2-*O*-hexoside isomers were characterized taking into account the $[M-H]^-$ at m/z 327 and its fragment ions (m/z 165, 147 and 103)⁵³. Esculetin was characterized according to Zhou et al. by observing the molecular formula and typical pattern 177→133⁵⁴. Given the $[M-H]^-$ at m/z 339, fragment ions at m/z 177, 149 and 133 and the neutral loss of 162 Da, esculetin hexoside was identified. Caffeic acid was identified comparing it to the analytical standard. Caffeic acid-*C*-hexoside isomers were identified according to Vallverdú-Queralt et al., they exhibited the same molecular formula and fragment ions at m/z 281, 251, 221, 179 and 135⁵⁵. The compounds showing $[M-H]^-$ at m/z 289 and the fragment ions at m/z 245, 205, 203, 179 and 125 were identified as catechin and (epi)catechin. The precise characterization was done using the analytical standard of catechin which allows us to determine its retention time and, consequently, that of (epi)catechin. The compounds presenting $[M-H]^-$ at m/z 577 and fragment ions at m/z 451, 425, 407, 289, 287 and 125, were characterized as (epi)catechin-(epi)catechin (B-type) isomers⁵⁶. (Epi)catechin-*O*-gallate displays $[M-H]^-$ at m/z 441 and fragment ions at m/z 331, 289, 271, 245, 169 and 125, showing a neutral loss corresponding to the galloyl moiety (441→289)⁵⁷. The caffeoylglucosylquinic acid (molecular formula: $C_{22}H_{28}O_{14}$; $[M-H]^-$ at m/z 515; fragment ions at m/z 353, 341, 323, 191 and 179) was determined following the previous literature⁵⁸. Benzyl alcohol hexose pentose (formate adduct), $[M-H]^-$ at m/z 447, was tentatively characterized using literature data⁵⁹. Benzyl glucopyranoside (formate adduct) was tentatively identified by observing the molecular formula, the neutral loss (46 Da), which is

related to the formate adduct. Based on the molecular formulas and the neutral loss corresponding to a pentoside moiety, benzyl glucopyranoside pentose (formate adduct) was characterized. Delphinidin-3-*O*-hexoside had $[M+H]^+$ at m/z 465, fragment ions at m/z 303, 257 and 229 and a neutral loss of 162 Da, the already reported fragmentation pattern allowed its identification⁶⁰. With the same fragment ions and $[M+H]^+$ at m/z 611 and the neutral loss of 308 Da, we identified the delphinidin-3-(6-*O*-coumaroyl)hexoside. Its molecular formula and the experimental data correspond to those reported in literature⁶⁰. Benzoic acid-*O*-pentosylhexoside (formate adduct) isomers were identified considering their molecular formula and that the fragment ions are at m/z 121 and 77, which are the typical fragments of benzoic acid⁶¹. Furthermore, they showed $[M-H]^-$ at m/z 461 and fragments at m/z 415, 293, 121 and 77. 46 Da and 294 Da (132 Da + 162 Da) are the neutral losses referred to the formate adduct and the pentosylhexoside moiety. With $[M-H]^-$ at m/z 329, the fragment ions at m/z 121 and 77 and the neutral loss of a hexoside moiety and the formate adduct, we also characterised the benzoic acid-*O*-hexoside (formate adduct). The compound having $[M-H]^-$ at m/z 315, its neutral loss of 162 Da and fragment ions at m/z 153, 152 and 108 was characterized as dihydrobenzoic acid hexoside according to Han et al.⁶². Roseoside (formate adduct) (C₂₀H₃₂O₁₀) showed $[M-H]^-$ at m/z 431 and fragmentation pattern (m/z 385, 223 and 153) in agreement with Spínola et al.⁶³ Dihydro-roseoside (formate adduct) (C₂₀H₃₄O₁₀), with $[M-H]^-$ at m/z 433, was tentatively characterized. The compounds presenting molecular formula C₂₃H₂₄O₁₂ and fragmentation pattern 491→329 were characterized as triclin-*O*-hexoside isomers⁶⁴. Cyanidin-3-*O*-hexoside and cyanidin-(6-*O*-coumaroyl)hexoside showed both the fragment ion at m/z 287 which correspond to cyanidin, due to the neutral losses of 162 Da and 308 Da, respectively, they were identified as hexoside and (6-*O*-coumaroyl)hexoside derivatives⁶⁰. Cyanidin-3-hexoside (C₂₁H₂₀O₁₁) was detected by observing the protonated molecular ion at m/z 449 and a fragment ion of cyanidin at m/z 287. Cyanidin-3-sambubioside (C₂₆H₂₈O₁₅) showed $[M+H]^+$ at m/z 581 and the fragment ion m/z 287 (cyanidin)⁶⁵. According to Sarnoski et al., procyanidins dimers B-type were characterized⁶⁶. Quercetin was identified using the analytical standard ($[M-H]^-$ at m/z 301, fragment ions at m/z 179 and 151). Numerous quercetin glycosides were detected in the extracts. Considering the neutral losses of 162 Da, 176 Da, 132 Da and 146 Da for, respectively, hexoside, glucuronide, pentoside and deoxyhexoside, were identified quercetin-*O*-dihexoside, quercetin-*O*-hexoside isomers, quercetin-*O*-glucuronide, quercetin-*O*-pentoside isomers and quercetin-*O*-deoxyhexoside. All showed the aglycone fragment at m/z 301 and the main fragment ions were m/z 179 and 151. According to Ek et

al., we characterized quercetin-3-*O*-(4''-hydroxymethylglutaryl)- α -rhamnoside which had $[M-H]^-$ at m/z 591 and fragment ions at m/z 529, 489, 447, 301, 300 and 179⁶⁷. Other quercetin derivatives were tentatively identified based on fragmentation patterns. Quercetin-*O*-hexoside-*O*-rutinoside, $[M-H]^-$ at m/z 771, was characterized based on the presence of quercetin typical fragment ions at m/z 301, 179 and 151 and the neutral losses 771 $\rightarrow$ 609 and 609 $\rightarrow$ 301. Ferulic acid exhibited $[M-H]^-$ at m/z 193, fragment ions at m/z 178, 149 and 134 and molecular formula $C_{10}H_{10}O_4$. Ferulic acid-*O*-hexoside isomers showed a neutral loss (162 Da) giving ferulic acid at m/z 193. Ferulic acid-*O*-di-hexoside had $[M-H]^-$ at m/z 517, fragment ions at m/z 193 (ferulic acid), 149 and 134 and the neutral losses (517 $\rightarrow$ 355 $\rightarrow$ 193) of two hexosides. Different petunidin glycosides have been identified considering the aglycone at m/z 317, in particular we found petunidin-*O*-hexoside(479 $\rightarrow$ 317), petunidin-(6-*O*-caffeoyl)hexoside (641 $\rightarrow$ 317) and petunidin-(6-*O*-coumaroyl)hexoside (625 $\rightarrow$ 317)⁶⁰. Kaempferol-*O*-hexoside isomers showed $[M-H]^-$ at m/z 447 with the fragment ions at m/z 285, 284, 255, 227 and 151. Kaempferol-*O*-deoxyhexoside, $[M-H]^-$ at m/z 431, showed the kaempferol aglycone at m/z 285 and the fragment ion at m/z 255. Kaempferol aglycone at m/z 285 is distinguished from luteolin thanks to the absence of the fragment ions at m/z 243 and 241. Kaempferol-*O*-rutinoside, $[M-H]^-$ at m/z 593, showed a neutral loss (308 Da) corresponding to the rutinoside moiety and fragments at m/z 285, 255, 229 and 151. Dihydrokaempferol-*O*-hexoside was identified given its molecular formula and the neutral loss of 162 Da giving the aglycone (m/z 287)⁶⁸. Kaempferol-*O*-(coumaroyl)-hexoside was identified following the data reported by Geng et al.⁶⁹ Based on the hierarchical scheme to identify chlorogenic acids of Clifford et al., 4-feruloylquinic acid ($C_{17}H_{20}O_9$) with a base peak at m/z 173 was identified⁵². Peonidin glycosides were also characterized by considering the fragment ion at m/z 301 (aglycone). Specifically, given the molecular formulas and their neutral losses, we characterized peonidin-3-*O*-hexoside, peonidin-3-*O*-acetylhexoside, peonidin-(6-*O*-caffeoyl)hexoside, and peonidin-3-(6-*O*-coumaroyl)hexoside⁶⁰. Observing the deprotonated molecular ion (m/z 303) and the typical fragment ions (m/z 285, 177 and 125), was identified taxifolin following the previously reported data by Hashim et al.⁷⁰ Taxifolin-*O*-pentoside isomers exhibited $[M-H]^-$ at m/z 435, neutral loss of 132 Da to give taxifolin at m/z 303 and fragment ions, as previously reported in literature, at m/z 285, 177 and 125⁷⁰. In agreement with Liu et al., we identified scopoletin with $[M-H]^-$ at m/z 191 and m/z 176 and 148 as fragment ions⁷¹. Myricetin, $[M-H]^-$ at m/z 317 and fragment ions at m/z 179 and 151, was identified by exploiting its analytical standard. Myricetin-*O*-hexoside, myricetin-*O*-glucoronide and myriceti-*O*-dihexoside were characterized by observing their

fragmentation pattern and the neutral losses. An apigenin derivative ($[M-H]^-$ at m/z 449) was tentatively characterized considering the apigenin base peak (m/z 269) and the main fragment ion at m/z 225. Eriodictyol-*O*-hexoside was identified following the literature data taking into consideration the neutral loss (162 Da) obtaining the aglycone at m/z 287 and also m/z 151 and 135 (fragment ions)¹⁶. Isorhamnetin (molecular formula: $C_{16}H_{12}O_7$, 315→300) was identified by comparison with the analytical standard. Given the neutral losses, molecular formula and fragment ions, were also characterized isorhamnetin-*O*-acetylhexoside (neutral loss: 204 Da), isorhamnetin-*O*-hexoside (162 Da) and isorhamnetin-*O*-rutinoside (308 Da). Rutin, $[M-H]^-$ at m/z 609 and fragment ions at m/z 301, 300, 179 and 151, was characterized by comparison with the analytical standard. Ellagic acid, $[M-H]^-$ m/z 301 and fragment ions at m/z 257 and 229, was identified by comparison with the analytical standard. Mearnsetin-*O*-hexoside was characterized according to bibliographic data showing $[M-H]^-$ at m/z 493 and neutral loss of 162 Da yielding mearnsetin (m/z 331)⁶². Based on the typical fragment ions of the syringaresinol (a lignin) and the neutral loss (162 Da), syringaresinol-*O*-hexoside was characterized⁷². Malvidin glycosides, showing m/z 331, were identified, specifically, malvidin-3-*O*-acetylhexoside, malvidin-(6-*O*-caffeoyl)hexoside and malvidin-3-(6-*O*-coumaroyl)hexoside⁶⁰. Scutellarin was characterized according to the data reported by Qu et al.⁷³ Phloretin-*O*-hexoside was characterized by observing its molecular formula, the neutral loss and the fragmentation pattern which was coherent with data literature⁷⁴. Considering the data reported by Martini et al., the molecular formula, the compound with $[M-H]^-$ at m/z 312 and fragment ions at m/z 297, 178, 148 and 135, feruloyltyramine was characterized⁷⁵. The oxylipins, oxo-dihydroxy-octadecenoic acid and trihydroxy-octadecenoic acid, exhibited an equal fragmentation pattern already reported by Van Hoyweghen et al.⁷⁶. Following the data mentioned in literature, we characterized naringenin given the molecular formula and its fragment ions (m/z 177, 151, 119 and 109)⁷⁷. Syringetin, $[M-H]^-$ at m/z 345 and fragment ions at m/z 330, 315 and 287, was characterized following Soltana et al.⁷⁸

All the detailed tables showing the characterization of the detected compounds in each typology of extract, referring to all the plant materials under examination, along with their chromatograms, heat maps and quantification of the main compounds are reported below.

Grape pomace

The major number of compounds was identified in grape pomace extracts (67) (Figure 1, Table 3). Heat Maps are reported (Figures 2 and 3) to show the % relative contribution of each identified or tentatively characterized compound on the total of them per each extract. Organic acids (tartaric acid and malic acid) represented $\approx 40\text{-}45\%$ of grape pomace extracts. Catechin, kaempferol and quercetin glycosides were the most abundant flavonoids, while among anthocyanins malvidin glycosides were prevalent, specifically malvidin-3-(6-O-coumaroyl) hexoside and malvidin-3- O-acetylhexoside accounted for the $\approx 50\%$ of all the anthocyanidins. The quantification of the prevalent compounds is shown in Table 4.

No.	t_R (min)	Observed [M-H] ⁻	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	DC	UAE	MAE
1	1.800	149.0098	C ₄ H ₆ O ₆	-4.05	87.0088	Tartaric acid	✓	✓	✓
					173.0094 129.0187				
2	1.916	191.0200	C ₆ H ₈ O ₇	-1.71	111.0084 87.0090 85.0294	Isocitric acid	✓	✓	✓
3	1.978	133.0144	C ₄ H ₆ O ₅	-0.82	115.0032 71.0141	Malic acid	✓	✓	✓
					173.0138 129.0198				
4	2.532	191.0200	C ₆ H ₈ O ₇	-1.26	111.0090 87.0090 85.0299	Citric acid	✓	✓	✓
5	3.210	169.0143	C ₇ H ₆ O ₅	-0.38	125.0243	Gallic acid	✓	✓	✓
6	3.703	331.0673	C ₁₃ H ₁₆ O ₁₀	-0.65	169.0140 125.0247	Galloyl hexoside	✓	✓	✓
7	3.888	315.1085	C ₁₄ H ₂₀ O ₈	0.07	153.0554 123.0446	Hydroxytyrosol hexoside	✓	✓	✓
8	4.639	153.0556	C ₈ H ₁₀ O ₃	-0.64	123.0445	Hydroxytyrosol		✓	
					111.0091 87.0087 67.0188 57.0361	Dimethylcitric acid	✓	✓	✓
10	4.991	359.0982	C ₁₅ H ₂₀ O ₁₀	0.17	197.0444 153.0570 135.0431	Syringic acid hexoside	✓	✓	✓
11	5.521	153.0194	C ₇ H ₆ O ₄	-0.97	109.0293	Protocatechuic acid	✓		
12	6.317	311.0408	C ₁₃ H ₁₂ O ₉	0.29	179.0344 149.0088 135.0440	Caftaric acid	✓	✓	✓
					157.0501 131.0711				
13	6.330	175.0613	C ₇ H ₁₂ O ₅	-0.34	129.0574 115.0397 113.0603	Isopropyl-malic acid	✓	✓	✓

					85.0657				
14	6.378	183.0299	C ₈ H ₈ O ₅	-0.39	168.0061 124.0157	Methyl gallate	✓	✓	
15	7.014	341.0875	C ₁₅ H ₁₈ O ₉	0.71	281.0664 251.0555 221.0456 179.0342 135.0441	Caffeic acid- <i>C</i> -hexoside	✓	✓	✓
16	7.137	577.1351	C ₃₀ H ₂₆ O ₁₂	0.51	451.1001 425.0879 407.0771 289.0704 287.0557 125.0237	(Epi)catechin-(epi)catechin (B-type)	✓	✓	✓
17	7.841	447.1501	C ₁₉ H ₂₈ O ₁₂	1.51	401.1451 269.1024 161.0463 131.0357	Benzyl alcohol hexose pentose (formate adduct)	✓	✓	✓
18	7.964	325.0930	C ₁₅ H ₁₈ O ₈	0.64	163.0399 119.0503	Coumaric acid- <i>O</i> -hexoside	✓	✓	
19	8.114	577.1347	C ₃₀ H ₂₆ O ₁₂	1.26	451.1025 425.0859 407.0748 289.0700 287.0558 125.0246	(Epi)catechin-(epi)catechin (B-type)	✓	✓	✓
20	8.272	341.0872	C ₁₅ H ₁₈ O ₉	1.63	281.0653 251.0558 221.0449 179.0333 135.0447	Caffeic acid- <i>C</i> -hexoside	✓	✓	✓
21	8.888	447.1496	C ₁₉ H ₂₈ O ₁₂	1.97	401.1453 293.0799 269.0978 233.0577 161.0399	Benzyl alcohol hexose pentose (formate adduct)	✓	✓	✓
22	8.950	289.0718	C ₁₅ H ₁₄ O ₆	-0.27	245.0815 205.0506 203.0715 179.0338 125.0237	Catechin	✓	✓	✓
23	8.961	465.1033 (+)	C ₂₁ H ₂₀ O ₁₂	-0.91	303.0497 257.0479 229.0479	Delphinidin-3- <i>O</i> -hexoside	✓	✓	✓
24	9.750	431.1917	C ₂₀ H ₃₂ O ₁₀	0.03	385.1862 223.1308 153.0927	Roseoside (formate adduct)	✓	✓	✓
25	10.490	577.1345	C ₃₀ H ₂₆ O ₁₂	1.41	451.1000 425.0866 407.0745 289.0684 287.0554 125.0262	(Epi)catechin-(epi)catechin (B-type)	✓	✓	✓
26	10.613	491.1194	C ₂₃ H ₂₄ O ₁₂	2.31	329.0653 315.0495 300.0280 299.0202 271.0634	Tricin- <i>O</i> -hexoside	✓	✓	✓
27	10.848	449.1085 (+)	C ₂₁ H ₂₀ O ₁₁	-1.24	287.0544	Cyanidin-3- <i>O</i> -hexoside	✓	✓	✓

28	11.016	577.1344	$C_{30}H_{26}O_{12}$	1.54	451.1053	(Epi)catechin- (epi)catechin (B-type)	✓	✓	✓
					425.0849				
					407.0772				
					289.0721				
					287.0540				
29	11.783	479.1191 (+)	$C_{22}H_{22}O_{12}$	-1.32	125.0242	Petunidin- <i>O</i> - hexoside	✓	✓	✓
					317.0658				
30	12.338	289.0718	$C_{15}H_{14}O_6$	0.1	245.0822	(Epi)catechin	✓	✓	✓
					205.0502				
					203.0713				
					179.0343				
					125.0243				
31	14.060	463.1236 (+)	$C_{22}H_{22}O_{11}$	-0.56	301.0704	Peonidin-3- <i>O</i> - hexoside	✓	✓	✓
					286.0453				
32	14.186	197.0455	$C_9H_{10}O_5$	0.34	169.0152	Ethyl gallate	✓	✓	
					124.0160				
33	14.494	491.1189	$C_{23}H_{24}O_{12}$	0.95	329.0657	Tricin- <i>O</i> - hexoside	✓	✓	✓
					314.0408				
					313.0328				
					299.0184				
34	16.869	625.1412	$C_{27}H_{30}O_{17}$	-0.51	463.0885	Quercetin- <i>O</i> - dihexoside		✓	
					301.0368				
35	17.612	509.1299	$C_{23}H_{26}O_{13}$	0.25	355.0663	Unknown	✓	✓	✓
					347.0759				
					329.0664				
					193.0108				
					149.0236				
36	17.982	493.0623	$C_{21}H_{18}O_{14}$	0.2	317.0308	Myricetin- <i>O</i> - glucuronide	✓	✓	✓
					178.9971				
					151.0322				
37	18.105	641.1357	$C_{27}H_{30}O_{18}$	0.89	479.0828	Myricetin- <i>O</i> - dihexoside	✓	✓	
					317.0294				
38	18.167	479.0827	$C_{21}H_{20}O_{13}$	0.83	317.0278	Myricetin- <i>O</i> - hexoside	✓	✓	✓
					316.0223				
					287.0183				
					271.0249				
					178.9981				
39	19.925	441.0822	$C_{22}H_{18}O_{10}$	1.03	151.0009	(Epi)catechin- <i>O</i> -gallate	✓	✓	✓
					331.0419				
					289.0702				
					271.0577				
					245.0943				
40	20.429	519.1132	$C_{24}H_{24}O_{13}$	1.82	169.0137	Isorhamnetin- <i>O</i> - acetylhexoside	✓	✓	✓
					125.0254				
41	20.726	609.1463	$C_{27}H_{30}O_{16}$	0.66	315.0481	Rutin	✓	✓	✓
					300.0287				
					299.0170				
					301.0334				
					300.0265				
42	21.381	463.0878	$C_{21}H_{20}O_{12}$	0.91	178.9964	Quercetin- <i>O</i> - hexoside	✓	✓	✓
					151.0037				
43	21.689	477.0672	$C_{21}H_{18}O_{13}$	0.73	301.0339	Quercetin- <i>O</i> - glucuronide	✓	✓	✓
					300.0274				
					271.0232				
					255.0299				
					178.9972				
43	21.689	477.0672	$C_{21}H_{18}O_{13}$	0.73	151.0026	Quercetin- <i>O</i> - glucuronide	✓	✓	✓
					301.0348				
					178.9984				
					151.0028				

44	21.812	463.0878	$C_{21}H_{20}O_{12}$	0.82	301.0335	Quercetin- <i>O</i> -hexoside	✓	✓	✓
					300.0269				
					271.0247				
					255.0277				
					178.9968				
45	21.997	493.0987	$C_{22}H_{22}O_{13}$	0.41	151.0033	Mearnsetin- <i>O</i> -hexoside	✓	✓	✓
					331.0445				
					330.0377				
					316.0233				
					315.0179				
46	22.598	505.1346 (+)	$C_{24}H_{24}O_{12}$	-0.78	301.0702	Peonidin-3- <i>O</i> -acetylhexoside	✓	✓	✓
					286.0471				
					331.0813				
					315.0501				
					299.0546				
47	22.820	535.1455 (+)	$C_{25}H_{26}O_{13}$	-1.66	287.0542	Malvidin-3- <i>O</i> -acetylhexoside	✓	✓	✓
					270.0519				
					242.0579				
					179.0330				
					285.0383				
48	23.907	447.0927	$C_{21}H_{20}O_{11}$	1.25	284.0327	Kaempferol- <i>O</i> -hexoside	✓	✓	✓
					255.0292				
					227.0333				
					151.0046				
					285.0383				
49	24.055	641.1504 (+)	$C_{31}H_{28}O_{15}$	-0.89	317.0655	Petunidin-(6- <i>O</i> -caffeoyl)hexoside		✓	✓
					302.0412				
					301.0349				
					178.9935				
					151.0009				
50	24.656	433.0763	$C_{20}H_{18}O_{11}$	3.97	178.9935	Quercetin- <i>O</i> -pentoside		✓	
					151.0009				
					303.0503				
					257.0428				
					229.0570				
51	24.993	611.1400 (+)	$C_{30}H_{26}O_{14}$	-0.52	257.0428	Delphinidin-3-(6- <i>O</i> -coumaroyl)hexoside	✓	✓	✓
					229.0570				
					285.0386				
					284.0322				
					255.0309				
52	25.087	447.0930	$C_{21}H_{20}O_{11}$	0.71	227.0349	Kaempferol- <i>O</i> -hexoside	✓	✓	✓
					151.0053				
					285.0406				
					175.0238				
					113.0233				
53	25.272	461.0725	$C_{21}H_{18}O_{12}$	-1.03	301.0343	Scutellarin		✓	
					271.0241				
					255.0270				
					178.9975				
					151.0028				
54	25.334	447.0932	$C_{21}H_{20}O_{11}$	-0.77	315.0539	Quercetin- <i>O</i> -deoxyhexoside	✓	✓	✓
					314.0416				
					150.9988				
					315.0539				
					314.0416				
55	25.586	477.1030	$C_{22}H_{22}O_{12}$	2.08	150.9988	Isorhamnetin- <i>O</i> -hexoside	✓	✓	✓
					301.0709				
					286.0458				
					258.0535				
					331.0811				
56	26.101	625.1559 (+)	$C_{31}H_{28}O_{14}$	-0.87	316.0574	Peonidin-(6- <i>O</i> -caffeoyl)hexoside	✓	✓	✓
					299.0539				
					287.0545				
					270.0515				
					242.0571				
57	26.243	655.1666 (+)	$C_{32}H_{30}O_{15}$	-1.6	179.0314	Malvidin-(6- <i>O</i> -caffeoyl)hexoside	✓	✓	✓
					331.0811				
					316.0574				
					299.0539				
					287.0545				
58	26.243	655.1666 (+)	$C_{32}H_{30}O_{15}$	-1.6	270.0515	Malvidin-(6- <i>O</i> -caffeoyl)hexoside	✓	✓	✓
					242.0571				
					179.0314				
					331.0811				
					316.0574				

58	27.009	595.1448 (+)	C ₃₀ H ₂₆ O ₁₃	-0.06	287.0546	Cyanidin-(6- <i>O</i> - coumaroyl)hex oside	✓	✓	✓
					501.1029 475.1221 347.0760				
59	27.216	655.1666	C ₃₂ H ₃₂ O ₁₅	0.55	329.0659	Unknown	✓	✓	✓
					303.0880 193.0137 149.0248				
60	27.278	317.0302	C ₁₅ H ₁₀ O ₈	-0.01	178.9959	Myricetin	✓	✓	✓
					151.0022				
61	27.530	625.1558 (+)	C ₃₁ H ₂₈ O ₁₄	-0.85	317.0653	Petunidin-(6- <i>O</i> - coumaroyl)hexo side	✓	✓	✓
					302.0423 274.0446				
62	29.291	431.0974	C ₂₁ H ₂₀ O ₁₀	2.27	285.0385	Kaempferol- <i>O</i> - deoxyhexoside	✓	✓	
					286.0470 284.0310 255.0299 229.0465 227.0313				
63	29.551	639.1715 (+)	C ₃₂ H ₃₀ O ₁₄	-1.27	331.0813	Malvidin-3-(6- <i>O</i> - coumaroyl) hexoside	✓	✓	✓
					315.0499 299.0549 287.0553 270.0533 242.0565 179.0327				
64	29.653	609.1609 (+)	C ₃₁ H ₂₈ O ₁₃	-0.82	301.0707	Peonidin-3-(6- <i>O</i> - coumaroyl)hexo side	✓	✓	✓
					286.0465 258.0515 230.0617				
65	35.767	301.0355	C ₁₅ H ₁₀ O ₇	-0.63	178.9990	Quercetin	✓	✓	✓
					151.0035				
66	38.605	327.2169	C ₁₈ H ₃₂ O ₅	2.22	171.1017	Oxo-dihydroxy- octadecenoic acid	✓	✓	✓
					229.1436 211.1329 183.1418				
67	40.117	345.0610	C ₁₇ H ₁₄ O ₈	1.22	330.0372	Syringetin	✓	✓	
					315.0123				
68	40.172	329.2333	C ₁₈ H ₃₄ O ₅	-0.29	229.1467	Trihydroxy- octadecenoic acid	✓	✓	✓
					211.1354 171.1049				
69	40.442	315.0506	C ₁₆ H ₁₂ O ₇	0.25	300.0264	Isorhamnetin	✓	✓	✓

Table 3. Compounds identified in grape pomace DC, UAE and MAE. (+) is used for the compounds detected in positive ion mode.

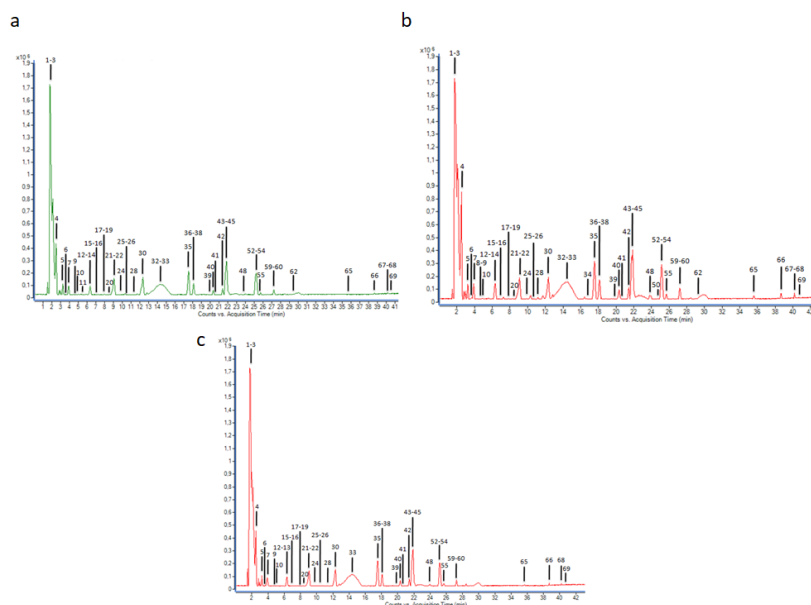


Figure 1. Base peak chromatograms (negative ion mode) of all the grape pomace extracts: a) DC, b) UAE, c) MAE.

Peak	Compound	Relative contribution (%)		
		DC	UAE	MAE
1	tartaric acid	20.96	16.4	17.2
2	isocitric acid	4.23	5.54	6.34
3	malic acid	22.18	22.22	27.03
4	citric acid	1.77	3	2.06
5	gallic acid	1.09	1.05	0.91
6	galloyl hexoside	0.37	0.38	0.36
7	hydroxytyrosol hexoside	1.48	1.54	1.33
8	Hydroxytyrosol	0	0.09	0
9	dimethylcitric acid	0.12	0.09	0.1
10	syringic acid- <i>O</i> -hexoside	0.16	0.19	0.17
11	protocatechuic acid	0.11	0	0
12	caftaric acid	0.23	0.23	0.22
13	isopropyl-malic acid	1.69	1.86	1.49
14	methyl gallate	0.2	0.14	0
15	caffeic acid- <i>C</i> -hexoside	0.33	0.37	0.26
16	(Epi)catechin-(epi)catechin (B-type)	0.48	0.43	0.5
17	benzyl alcohol hexose pentose (formate adduct)	0.33	0.34	0.32
18	coumaric acid- <i>O</i> -hexoside	0.21	0.23	0
19	(Epi)catechin-(epi)catechin (B-type)	0.39	0.45	0.57
20	caffeic acid- <i>C</i> -hexoside	0.39	0.45	0.25
21	benzyl alcohol hexose pentose (formate adduct)	0.24	0.24	0.23
22	Catechin	3.76	3.39	3.15
24	roseoside (formate adduct)	0.12	0.13	0.14

25	(Epi)catechin-(epi)catechin (B-type)	0.5	0.44	0.49
26	tricin- <i>O</i> -hexoside	0.35	0.39	0.37
28	(Epi)catechin-(epi)catechin (B-type)	0.5	0.48	0.49
30	(Epi)catechin	3.67	3.15	3.22
32	ethyl gallate	0.29	0.31	0
33	tricin- <i>O</i> -hexoside	7.1	7.08	7.11
34	quercetin- <i>O</i> -dihexoside	0	0.08	0
35	unknown	3.91	4.46	4
36	myricetin- <i>O</i> -glucuronide	0.24	0.25	0.22
37	myricetin- <i>O</i> -dihexoside	0.17	0.17	0
38	myricetin- <i>O</i> -hexoside	2.15	2.47	1.83
39	(Epi)catechin- <i>O</i> -gallate	0.39	0.38	0.34
40	isorhamnetin- <i>O</i> -acetylhexoside	0.87	1	0.87
41	rutin	0.18	0.16	0.15
42	quercetin- <i>O</i> -hexoside	1.14	1.26	1.11
43	quercetin- <i>O</i> -glucuronide	3.67	3.81	3.64
44	quercetin- <i>O</i> -hexoside	5.3	4.85	5.05
45	mearnsetin- <i>O</i> -hexoside	0.71	0.8	0.68
48	kaempferol- <i>O</i> -hexoside	0.53	0.57	0.56
50	quercetin- <i>O</i> -pentoside	0	0.07	0
52 + 54	kaempferol- <i>O</i> -hexoside + quercetin- <i>O</i> -deoxyhexoside	3.82	4.33	3.89
53	scutellarin	0	0.14	0
55	isorhamnetin- <i>O</i> -hexoside	0.48	0.51	0.55
59	unknown	1.11	1.34	1.02
60	Myricetin	0.19	0.25	0.17
62	kaempferol- <i>O</i> -deoxyhexoside	0.21	0.22	0
65	Quercetin	0.59	0.81	0.73
66	oxo-dihydroxy-octadecenoic acid	0.46	0.59	0.45
67	syringetin	0.17	0.28	0
68	trihydroxy-octadecenoic acid	0.39	0.49	0.36
69	Isorhamnetin	0.07	0.11	0.09

Figure 2. Heat Map obtained by HPLC-Q-TOF-MS of grape pomace DC, UAE and MAE (negative ion mode).

Peak	Compound	Relative contribution (%)		
		DC	UAE	MAE
23	delphinidin-3- <i>O</i> -hexoside	3.61	3.7	4.06
27	cyanidin-3- <i>O</i> -hexoside	1.87	1.57	1.88
29	petunidin- <i>O</i> -hexoside	11.79	12.18	11.83
31	peonidin-3- <i>O</i> -hexoside	10.61	10.41	10.38
46	peonidin-3- <i>O</i> -acetylhexoside	3.93	3.97	3.9
47	malvidin-3- <i>O</i> -acetylhexoside	31.72	31.18	29.92
49	petunidin-(6- <i>O</i> -caffeoyl)hexoside	0	0.81	0.81
51	delphinidin-3-(6- <i>O</i> -coumaroyl)hexoside	0.87	1.08	1.09
56	peonidin-(6- <i>O</i> -caffeoyl)hexoside	1.3	1.37	1.32
57	malvidin-(6- <i>O</i> -caffeoyl)hexoside	6.43	6.26	6.55
58	cyanidin-(6- <i>O</i> -coumaroyl)hexoside	0.78	0.65	0.77
61	petunidin-(6- <i>O</i> -coumaroyl)hexoside	2.91	3.13	3.28
63	malvidin-3-(6- <i>O</i> -coumaroyl) hexoside	20.83	20.51	20.8
64	peonidin-3-(6- <i>O</i> -coumaroyl)hexoside	3.35	3.15	3.4

Figure 3. Heat Map obtained by HPLC-Q-TOF-MS of grape pomace DC, UAE and MAE (positive ion mode).

No.	Assigned identification	DC (mg/gDE)	UAE (mg/gDE)	MAE (mg/gDE)
<i>Flavonoids</i>				
22	Catechin	0.91 ± 0.06 ^a	0.97 ± 0.07 ^a	0.89 ± 0.06 ^a
30	(epi)catechin	1.00 ± 0.07 ^a	1.00 ± 0.07 ^a	1.01 ± 0.07 ^a
37+38	myricetin- <i>O</i> -dihexoside + myricetin- <i>O</i> -hexoside	0.33 ± 0.02 ^a	0.38 ± 0.03 ^{ab}	0.42 ± 0.03 ^b
40	isorhamnetin- <i>O</i> -acetylhexoside	0.25 ± 0.02 ^a	0.24 ± 0.02 ^a	0.30 ± 0.02 ^b
42	quercetin- <i>O</i> -hexoside	0.24 ± 0.02 ^a	0.29 ± 0.02 ^b	0.31 ± 0.02 ^b
43+44 +45	quercetin- <i>O</i> -glucuronide + quercetin- <i>O</i> -hexoside + mearnsetin- <i>O</i> -hexoside	1.5 ± 0.1 ^a	1.8 ± 0.1 ^b	1.8 ± 0.1 ^b
48	kaempferol- <i>O</i> -hexoside	0.20 ± 0.01 ^a	0.23 ± 0.02 ^{ab}	0.26 ± 0.02 ^b
52+54	kaempferol- <i>O</i> -hexoside + quercetin- <i>O</i> -deoxyhexoside	0.74 ± 0.04 ^a	0.89 ± 0.04 ^b	0.94 ± 0.04 ^b
65	Quercetin	0.15 ± 0.01 ^a	0.16 ± 0.01 ^a	0.20 ± 0.01 ^b
Total		5.3 ± 0.2^a	6.0 ± 0.2^b	6.1 ± 0.2^b
<i>Phenolic acids</i>				
5	gallic acid	0.22 ± 0.02 ^a	0.21 ± 0.01 ^a	0.22 ± 0.02 ^a
<i>Others</i>				
2	isocitric acid	1.5 ± 0.1 ^a	1.5 ± 0.1 ^a	1.8 ± 0.1 ^b
4	citric acid	0.76 ± 0.05 ^a	0.84 ± 0.06 ^{ab}	0.95 ± 0.07 ^b
Total		2.3 ± 0.1^a	2.3 ± 0.1^a	2.8 ± 0.1^b
TIPC		7.8 ± 0.2^a	8.6 ± 0.2^b	9.2 ± 0.2^c

Table 4. Quantification of the main compounds found in grape pomace extracts. Results are the mean ± SD of three measurements. TIPC= total individual phenolic content (represent the sum of all the phytochemicals identified).

Cranberry

In cranberry DC, UAE and MAE 44 compounds were identified (Figure 4, Table 5). The extracts showed a high content of citric and isocitric acids ($\approx 30\%$) and benzoic derivatives ($\approx 30\%$) (Figure 5). Among flavonoids, numerous quercetin derivatives were identified. The high presence of benzoic acid derivatives and flavonol glycosides was reported also in previous studies^{79,80}. The main characterized compounds were quantified (Table 6), there wasn't a significant difference among the minor compound's concentrations. Considering the reported quantification of the phytochemicals, MAE gave the worst yields and UAE the best ones.

No.	t _R (min)	Observed [M-H] ⁻	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	DC	UAE	MAE
1	1.699	377.0857	C ₁₂ H ₂₂ O ₁₁	-0.97	341.1084	Disaccharide (HCl adduct)			✓
					221.0679				
					179.0550				
					161.0440				
					143.0354				
					131.0367				
2	1.908	191.0204	C ₆ H ₈ O ₇	-3.25	119.0371	Isocitric acid	✓	✓	✓
					113.0240				
					173.0086				
					129.0189				
					111.0086				
3	2.093	133.0141	C ₄ H ₆ O ₅	0.86	87.0090	Malic acid	✓	✓	✓
					85.0297				
					115.0035				
					71.0138				
4	2.648	191.0198	C ₆ H ₈ O ₇	-0.28	173.0062	Citric acid	✓	✓	✓
					129.0176				
					111.0085				
					87.0090				
5	2.956	205.0354	C ₇ H ₁₀ O ₇	-0.2	85.0289	Methylcitric acid	✓	✓	✓
					111.0085				
					99.0472				
					87.0087				
					67.0189				
6	3.202	315.0723	C ₁₃ H ₁₆ O ₉	-0.78	57.0365	Protocatechuic acid- <i>O</i> - hexoside	✓	✓	✓
					153.0194				
7	3.270	169.0142	C ₇ H ₆ O ₅	0.16	109.0296	Gallic acid		✓	✓
8	3.695	315.0721	C ₁₃ H ₁₆ O ₉	0.01	153.0179	Protocatechuic acid- <i>O</i> - hexoside	✓	✓	✓
					109.0243				
					108.0200				
9	4.311	219.0509	C ₈ H ₁₂ O ₇	0.64	173.0080	Dimethylcitric acid	✓	✓	✓
					129.0185				
					111.0085				
					87.0079				
					67.0193				
					57.0349				

10	4.557	299.0773	C ₁₃ H ₁₆ O ₈	-0.16	239.0587	Monohydroxy benzoyl hexose	✓	✓	✓
					209.0440				
					179.0339				
					151.0383				
					137.0238				
11	4.681	219.0510	C ₈ H ₁₂ O ₇	-0.11	119.0343	Dimethylcitric acid	✓	✓	✓
					113.0197				
					173.0088				
					157.0501				
					111.0085				
12	5.622	153.0193	C ₇ H ₆ O ₄	-0.05	87.0089	Protocatechuic acid	✓	✓	✓
					67.0192				
					57.0348				
					109.0293				
13	6.339	371.0980	C ₁₆ H ₂₀ O ₁₀	1.07	325.0879	Coumaric acid- <i>O</i> - hexoside (formate adduct)	✓	✓	✓
					163.0392				
					119.0482				
14	6.955	339.0726	C ₁₅ H ₁₆ O ₉	-3.31	177.0194	Esculetin hexoside	✓	✓	✓
					149.0232				
					133.0289				
15	7.875	325.0932	C ₁₅ H ₁₈ O ₈	-1.33	163.0395	Coumaric acid- <i>O</i> - hexoside		✓	✓
					119.0493				
16	7.917	315.1086	C ₁₄ H ₂₀ O ₈	-0.18	269.1029	Benzyl glucopyranosi de (formate adduct)	✓	✓	✓
					161.0435				
					153.0204				
17	8.040	447.1502	C ₁₉ H ₂₈ O ₁₂	1.31	401.1467	Benzyl glucopyranosi de pentose (formate adduct)	✓	✓	✓
					269.1044				
					161.0460				
					131.0344				
18	8.467	447.1501	C ₁₉ H ₂₈ O ₁₂	1	401.1448	Benzyl glucopyranosi de pentose (formate adduct)	✓	✓	✓
					269.1013				
					161.0470				
					131.0341				
19	9.021	461.1293	C ₁₉ H ₂₆ O ₁₃	1.6	415.1212	Benzoic acid- <i>O</i> - pentosylhexosi de (formate adduct)	✓	✓	✓
					293.0839				
					121.0289				
					77.0405				
20	9.144	329.0880	C ₁₄ H ₁₈ O ₉	-0.54	283.0822	Benzoic acid- <i>O</i> -hexoside (formate adduct)	✓	✓	✓
					121.0292				
					77.0400				
21	9.454	353.0879	C ₁₆ H ₁₈ O ₉	-0.21	191.0555	Chlorogenic acid	✓	✓	✓
					179.0343				
					161.0227				
					135.0457				
					111.0085				
22	9.699	461.1300	C ₁₉ H ₂₆ O ₁₃	0.45	415.1246	Benzoic acid- <i>O</i> - pentosylhexosi	✓	✓	✓
					293.0882				
					121.0293				
					77.0407				

						de (formate adduct)			
23	10.192	431.1918	C ₂₀ H ₃₂ O ₁₀	1.11	385.1860 223.1325 153.0907	Roseoside (formate adduct)	✓	✓	✓
24	10.643	177.0187	C ₉ H ₆ O ₄	2	133.0293	Esculetin	✓	✓	✓
25	11.461	179.0350	C ₉ H ₈ O ₄	0.35	135.0451	Caffeic acid	✓	✓	✓
26	16.546	355.1032	C ₁₆ H ₂₀ O ₉	0.65	193.0504 134.0373	Ferulic acid- <i>O</i> -hexoside	✓	✓	✓
27	16.736	163.0400	C ₉ H ₈ O ₃	0.47	119.0498	<i>p</i> -Coumaric acid	✓	✓	✓
28	18.137	355.1032	C ₁₆ H ₂₀ O ₉	0.91	193.0503 134.0363	Ferulic acid- <i>O</i> -hexoside	✓	✓	✓
29	20.046	303.0509	C ₁₅ H ₁₂ O ₇	-0.41	285.0388 177.0180 125.0246	Taxifolin	✓	✓	✓
30	21.342	463.0879	C ₂₁ H ₂₀ O ₁₂	0.82	301.0342 300.0269 271.0269 255.0304 178.9987 151.0037	Quercetin- <i>O</i> -hexoside	✓	✓	✓
31	21.853	463.0873	C ₂₁ H ₂₀ O ₁₂	2.13	301.0322 300.0278 271.0246 255.0317 178.9973 151.0036	Quercetin- <i>O</i> -hexoside	✓	✓	✓
32	23.257	433.0770	C ₂₀ H ₁₈ O ₁₁	1.18	301.0345 300.0268 271.0256 255.0281 178.9983 151.0016	Quercetin- <i>O</i> -pentoside	✓	✓	✓
33	23.988	433.0785	C ₂₀ H ₁₈ O ₁₁	-2.2	301.0328 300.0280 271.0607 255.0284 178.9944 151.0023	Quercetin- <i>O</i> -pentoside	✓	✓	✓
34	24.517	433.0777	C ₂₀ H ₁₈ O ₁₁	-0.02	301.0342 300.0263 271.0232 255.0227 178.9970 151.0015	Quercetin- <i>O</i> -pentoside	✓	✓	✓
35	24.598	193.0506	C ₁₀ H ₁₀ O ₄	0.4	178.0247 149.0604 134.0366	Ferulic acid	✓	✓	✓
36	25.128	447.0930	C ₂₁ H ₂₀ O ₁₁	0.47	301.0345 300.0266 271.0242 255.0292 178.9981 151.0030	Quercetin- <i>O</i> -deoxyhexoside	✓	✓	✓
37	26.386	435.1291	C ₂₁ H ₂₄ O ₁₀	0.68	273.0753 167.0352	Phloretin- <i>O</i> -hexoside	✓	✓	✓
38	29.210	431.0981	C ₂₁ H ₂₀ O ₁₀	-0.2	286.0437 285.0389 284.0326	Kaempferol- <i>O</i> -deoxyhexoside	✓	✓	✓

					255.0272				
					529.1330				
					489.1027	Quercetin-3-			
					447.0940	O-(4"-			
					301.0351	hydroxylmeth	✓	✓	✓
					300.0269	ylglutaryl)-α-			
					178.9945	rhamnoside			
					178.9980				
					151.0039	Quercetin	✓	✓	✓
					301.0349				
					300.0277	Quercetin			
					271.0269	derivative	✓	✓	✓
					255.0383				
					178.9984				
					151.0021				
					301.0326				
					300.0271	Quercetin			
					271.0248	derivative	✓	✓	✓
					255.0292				
					243.0303				
					178.9981				
					151.0031				
					177.0192				
					151.0024	Naringenin	✓	✓	✓
					119.0525				
					107.0134				

Table 5. Compounds identified in cranberry DC, UAE and MAE.

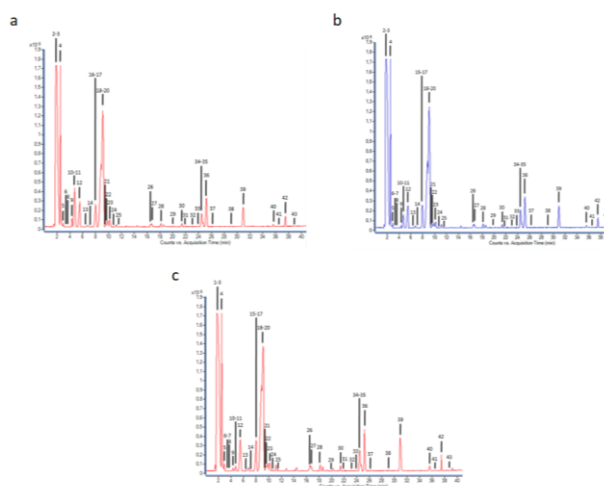


Figure 4. Base peak chromatograms (negative ion mode) of all the cranberry extracts: a) DC, b) UAE, c) MAE.

Peak	Compound	Relative contribution (%)		
		DC	UAE	MAE
1	disaccharide (HCl adduct)	0	0	1.35
2	isocitric acid	29.37	29.29	23.13
3	malic acid	1.05	1.4	1.06
4	citric acid	8.12	8.44	8.24
5	methylcitric acid	0.36	0.45	0.57
6	protocatechuic acid- <i>O</i> -hexoside	0.1	0.09	0.14
7	gallic acid	0	0.11	0.15
8	protocatechuic acid- <i>O</i> -hexoside	0.08	0.11	0.11
9	dimethylcitric acid	0.53	0.21	0.19
10	monohydroxybenzoyl hexose	0.2	0.25	0.28
11	dimethylcitric acid	4.7	1.6	0.75
12	protocatechuic acid	3.46	3.21	3.9
13	coumaric acid- <i>O</i> -hexoside (formate adduct)	0.2	0.21	0.24
14	esculetin hexoside	0.09	0.11	0.13
15	coumaric acid- <i>O</i> -hexoside	0	0.18	0.2
16	benzyl glucopyranoside (formate adduct)	3.18	3.16	3.7
17	benzyl glucopyranoside pentose (formate adduct)	1.05	1.02	1.16
18	benzyl glucopyranoside pentose (formate adduct)	0.5	0.46	0.54
19	benzoic acid- <i>O</i> -pentosylhexoside (formate adduct)	10.95	11.63	13.48
20	benzoic acid- <i>O</i> -hexoside (formate adduct)	18.05	18.99	18.58
21	chlorogenic acid	0.36	0.5	0.55
22	benzoic acid- <i>O</i> -pentosylhexoside (formate adduct)	1.16	1.08	1.13
23	roseoside (formate adduct)	0.86	0.93	1.1
24	Esculetin	0.12	0.14	0.16
25	caffeic acid	0.24	0.21	0.29
26	ferulic acid- <i>O</i> -hexoside	0.67	0.78	0.9
27	<i>p</i> -coumaric acid	0.74	0.79	0.86
28	ferulic acid- <i>O</i> -hexoside	0.68	0.81	0.91
29	Taxifolin	0.09	0.1	0.12
30	quercetin- <i>O</i> -hexoside	0.66	0.66	0.76
31	quercetin- <i>O</i> -hexoside	0.13	0.13	0.15
32	quercetin- <i>O</i> -pentoside	0.17	0.18	0.21
33	quercetin- <i>O</i> -pentoside	0.19	0.2	0.22
34	quercetin- <i>O</i> -pentoside	0.73	0.7	0.78
35	ferulic acid	2.22	2.77	2.79
36	quercetin- <i>O</i> -deoxyhexoside	3.84	3.86	4.31
37	phloretin- <i>O</i> -hexoside	0.06	0.06	0.07
38	kaempferol- <i>O</i> -deoxyhexoside	0.17	0.17	0.19
39	quercetin-3- <i>O</i> -(4''-hydroxymethylglutaryl)- α -rhamnoside	2.66	2.82	3.82
40	Quercetin	0.99	0.98	1.39
41	quercetin derivative	0.17	0.15	0.19
42	quercetin derivative	1.04	0.94	1.12
43	Naringenin	0.08	0.1	0.1

Figure 5. Heat Map obtained by HPLC-Q-TOF-MS of cranberry DC, UAE and MAE (negative ion mode).

No.	Assigned identification	DC (mg/gDE)	UAE (mg/gDE)	MAE (mg/gDE)
<i>Flavonoids</i>				
30	quercetin- <i>O</i> -hexoside	0.18 ± 0.01 ^b	0.19 ± 0.01 ^b	0.14 ± 0.01 ^a
36	quercetin- <i>O</i> -deoxyhexoside	0.48 ± 0.03 ^{ab}	0.51 ± 0.04 ^b	0.42 ± 0.03 ^a
39	quercetin-3- <i>O</i> -(4''-hydroxylmethylglutaryl)- α - rhamnoside	0.29 ± 0.02 ^a	0.30 ± 0.02 ^a	0.25 ± 0.02 ^a
40	Quercetin	0.16 ± 0.01 ^{ab}	0.17 ± 0.01 ^b	0.14 ± 0.01 ^a
42	quercetin derivative	0.17 ± 0.01 ^b	0.17 ± 0.01 ^b	0.12 ± 0.01 ^a
Total		1.28 ± 0.04^b	1.34 ± 0.05^b	1.07 ± 0.04^a
<i>Phenolic acids</i>				
12	protocatechuic acid	0.44 ± 0.03 ^a	0.44 ± 0.03 ^a	0.40 ± 0.03 ^a
19 + 20	benzoic acid- <i>O</i> -pentosylhexoside (formate adduct) + benzoic acid- <i>O</i> -hexoside (formate adduct)	10.1 ± 0.7 ^a	11.1 ± 0.8 ^a	9.7 ± 0.7 ^a
22	benzoic acid- <i>O</i> -pentosylhexoside (formate adduct)	0.43 ± 0.03 ^{ab}	0.47 ± 0.03 ^b	0.38 ± 0.03 ^a
Total		11.0 ± 0.7^a	12.0 ± 0.8^a	10.5 ± 0.7^a
<i>Others</i>				
2	isocitric acid	10.3 ± 0.7 ^b	11.4 ± 0.8 ^b	7.7 ± 0.5 ^a
4	citric acid	4.2 ± 0.3 ^b	4.5 ± 0.3 ^b	3.2 ± 0.2 ^a
Total		14.5 ± 0.8^b	15.9 ± 0.9^b	10.9 ± 0.5^a
TIPC		26.8 ± 1.0^b	29.2 ± 1.2^c	22.5 ± 0.9^a

Table 6. Quantification of the main compounds found in cranberry extracts. Results are the mean ± SD of three measurements. TIPC= total individual phenolic content (represent the sum of all the phytochemicals identified).

Elderberry

28 compounds were characterized or tentatively characterized in elderberry berries DC, UAE and MAE (Figure 6) and are reported in Table 7. In agreement with previously published studies, elderberry extracts resulted in being rich in citric, isocitric and malic acids, rutin and protocatechuic acid^{65,81}. Quercetin glycosides were the major flavonoids detected in our extracts. In a previous work, quercetin glycosides were reported as highly abundant in elderberry extracts along with kaempferol and isorhamnetin derivatives, but the last ones weren't particularly abundant in our extracts compared to the berries extracts⁸². Furthermore, phenylalanine hexoside was very abundant in our extract, as shown in the Heat Map (Figure 7); to the best of our knowledge this data was not previously mentioned. Table 8 displays the quantifications conducted on elderberry extracts; the ultrasound-assisted extract exhibited the highest concentration of phenolic acids and flavonoids, 1.56 mg g⁻¹ DE and 5.4 mg g⁻¹ DE respectively. Moreover, rutin was particularly abundant (3.7 mg g⁻¹ DE for DC, 4.6 mg g⁻¹ DE for UAE and 4.0 mg g⁻¹ DE for MAE).

No.	t_R (min)	Observed [M-H] ⁻	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	DC	UAE	MAE
1	2.045	191.0200	C ₆ H ₈ O ₇	-1.36	173.0086 129.0190 111.0085 87.0089 85.0295	Isocitric acid	✓	✓	✓
2	2.107	133.0143	C ₄ H ₆ O ₅	-0.12	115.0032 71.0141	Malic acid	✓	✓	✓
3	2.558	117.0193	C ₄ H ₆ O ₄	0.28	99.0086 73.0296	Succinic acid	✓	✓	✓
4	2.600	191.0197	C ₆ H ₈ O ₇	-0.18	173.0072 129.0192 111.0085 87.0088 85.0297	Citric acid	✓	✓	✓
5	2.969	326.1245	C ₁₅ H ₂₁ NO ₇	0.06	164.0711 101.0242	Phenylalanine hexoside	✓	✓	✓
6	3.278	164.0715	C ₉ H ₁₁ NO ₂	1.23	147.0446 103.0550 91.0550 72.0092	Phenylalanine	✓	✓	✓
7	3.832	315.1081	C ₁₄ H ₂₀ O ₈	1.41	153.0549 123.0449	Hydroxytyrosol- O-hexoside	✓	✓	✓
8	4.017	329.0880	C ₁₄ H ₁₈ O ₉	-0.15	167.0345 152.0110 123.0454 108.0202	Vanillic acid hexoside	✓	✓	✓
9	4.793	219.0513	C ₈ H ₁₂ O ₇	-2.45	111.0082 87.0098 72.9921 67.0204 57.0360	Dimethylcitric acid		✓	
10	5.434	353.0876	C ₁₆ H ₁₈ O ₉	0.88	191.0564 179.0349 173.0428 135.0447	Neochlorogenic acid		✓	✓
11	5.557	153.0193	C ₇ H ₆ O ₄	0.03	109.0292	Protocatechuic acid	✓	✓	✓
12	5.926	203.0826	C ₁₁ H ₁₂ N ₂ O ₂	-0.08	186.0527 159.0918 142.0665 116.0497 74.0243	L-Tryptophan	✓	✓	✓
13	7.831	325.0926	C ₁₅ H ₁₈ O ₈	0.97	163.0398 119.0498	Coumaric acid- O-hexoside	✓	✓	✓
14	8.508	447.1503	C ₁₉ H ₂₈ O ₁₂	0.73	401.1443 293.0763 269.1023 233.0662 161.0446	Benzyl alcohol hexose pentose (formate adduct)	✓	✓	✓
15	8.816	137.0242	C ₇ H ₆ O ₃	1.49	93.0344	Hydroxybenzoic acid	✓	✓	✓
16	9.490	353.0876	C ₁₆ H ₁₈ O ₉	0.54	191.0559 179.0350 173.0443 135.0457	Chlorogenic acid	✓	✓	✓
17	13.496	165.0557	C ₉ H ₁₀ O ₃	0.44	147.0447 119.0494 103.0548 91.0545	Unknown	✓	✓	✓

					72.9931				
18	13.989	367.1027	C ₁₇ H ₂₀ O ₉	1.13	193.0541 173.0437 111.0419	4-Feruloylquinic acid	✓	✓	✓
19	14.174	525.1971	C ₂₅ H ₃₄ O ₁₂	1.41	479.1915 327.1223 312.0993 297.0784 179.0576	Unknown	✓	✓	✓
20	16.779	163.0398	C ₉ H ₈ O ₃	1.27	119.0499	<i>p</i> -Coumaric acid	✓	✓	✓
21	17.025	755.2041	C ₃₃ H ₄₀ O ₂₀	0.01	591.1320 489.1022 301.0320 300.0270 271.0254 255.0285 178.9984 151.0062	Quercetin derivative	✓	✓	✓
22	20.751	609.1458	C ₂₇ H ₃₀ O ₁₆	0.56	301.0350 300.0266 178.9985	Rutin	✓	✓	✓
23	21.706	463.0879	C ₂₁ H ₂₀ O ₁₂	0.32	301.0343 300.0279 271.0230 255.0311 178.9990 151.0026	Quercetin- <i>O</i> -hexoside	✓	✓	✓
24	22.564	579.2071	C ₂₈ H ₃₆ O ₁₃	2.56	417.1550 402.1324 181.0477 166.0344 151.0153	Syringaresinol- <i>O</i> -hexoside			✓
25	23.840	593.1512	C ₂₇ H ₃₀ O ₁₅	0.26	285.0396 255.0283 229.0507 151.0035	Kaempferol- <i>O</i> -rutinoside	✓	✓	✓
26	24.653	623.1606	C ₂₈ H ₃₂ O ₁₆	1.93	315.0495 300.0266 271.0224	Isorhamnetin- <i>O</i> -rutinoside	✓	✓	✓
27	25.697	477.1031	C ₂₂ H ₂₂ O ₁₂	0.22	315.0497 314.0414 300.0117 271.0268	Isorhamnetin- <i>O</i> -hexoside	✓	✓	✓
28	27.336	238.1083	C ₁₂ H ₁₇ NO ₄	0.94	164.1071 94.0295	Unknown	✓	✓	✓
29	35.549	301.0355	C ₁₅ H ₁₀ O ₇	-0.29	178.9983 151.0024	Quercetin	✓	✓	✓
30	38.700	327.2176	C ₁₈ H ₃₂ O ₅	0.12	229.1444 211.1329 183.1385 171.1019	Oxo-dihydroxy-octadecenoic acid	✓	✓	✓
31	40.199	329.2335	C ₁₈ H ₃₄ O ₅	-0.67	229.1434 211.1337 171.1023	Trihydroxy-octadecenoic acid	✓	✓	✓

Table 7. Compounds identified in elderberry DC, UAE and MAE.

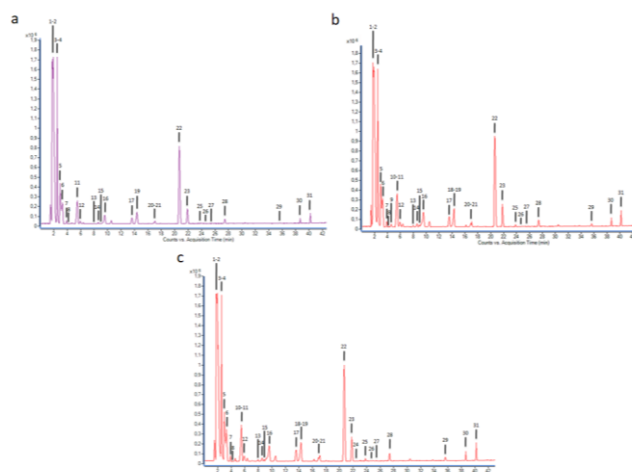


Figure 6. Base peak chromatograms (negative ion mode) of all the elderberry extracts: a) DC, b) UAE, c) MAE.

Peak	Compound	Relative contribution (%)		
		DC	UAE	MAE
1	isocitric acid	30.35	28.19	21.79
2	malic acid	2.94	6.84	5.19
3	succinic acid	1.41	1.29	1.37
4	citric acid	12.06	9.19	8.02
5	phenylalanine hexoside	9.82	9.66	10.56
6	Phenylalanine	2.42	2.05	2.67
7	hydroxytyrosol- <i>O</i> -hexoside	1.05	1.03	1.12
8	vanillic acid hexoside	0.12	0.15	0.15
9	dimethylcitric acid	0	0.31	0
10	neochlorogenic acid	0	0.33	0.42
11	protocatechuic acid	5.03	5.37	6.55
12	L-tryptophan	0.57	0.55	0.62
13	coumaric acid- <i>O</i> -hexoside	0.64	0.7	0.83
14	benzyl alcohol hexose pentose (formate adduct)	1.04	0.88	1.26
15	hydroxybenzoic acid	0.57	0.64	0.72
16	chlorogenic acid	2.24	2.46	2.72
17	Unknown	1.86	2.01	2.49
18	4-feruloylquinic acid	0	0.26	0.31
19	Unknown	3.41	3.71	4.28
20	<i>p</i> -coumaric acid	0.27	0.28	0.34
21	quercetin derivative	0.95	1.04	1.24
22	Rutin	14.8	14.04	16.43
23	quercetin- <i>O</i> -hexoside	3.04	3.29	3.84
24	syringaresinol- <i>O</i> -hexoside	0	0	0.1
25	kaempferol- <i>O</i> -rutinoside	0.51	0.57	0.7
26	isorhamnetin- <i>O</i> -rutinoside	0.21	0.23	0.26
27	isorhamnetin- <i>O</i> -hexoside	0.16	0.17	0.19

28	Unknown	1.39	1.36	1.62
29	Quercetin	0.94	1	1.28
30	oxo-dihydroxy-octadecenoic acid	0.75	0.87	1.01
31	trihydroxy-octadecenoic acid	1.46	1.53	1.92

Figure 7. Heat Map obtained by HPLC-Q-TOF-MS of elderberry DC, UAE and MAE (negative ion mode).

No.	Assigned identification	DC (mg/gDE)	UAE (mg/gDE)	MAE (mg/gDE)
<i>Flavonoids</i>				
22	Rutin	3.7 ± 0.3 ^a	4.6 ± 0.3 ^b	4.0 ± 0.3 ^{ab}
23	quercetin- <i>O</i> -hexoside	0.49 ± 0.03 ^a	0.55 ± 0.04 ^a	0.47 ± 0.03 ^a
29	Quercetin	0.19 ± 0.01 ^{ab}	0.21 ± 0.01 ^b	0.17 ± 0.01 ^a
Total		4.4 ± 0.3^a	5.4 ± 0.3^b	4.6 ± 0.3^a
<i>Phenolic acids</i>				
11	protocatechuic acid	0.68 ± 0.05 ^a	0.79 ± 0.06 ^a	0.68 ± 0.05 ^a
16	chlorogenic acid	0.64 ± 0.04 ^a	0.77 ± 0.05 ^b	0.70 ± 0.05 ^{ab}
Total		1.32 ± 0.06^a	1.56 ± 0.08^b	1.38 ± 0.07^a
<i>Others</i>				
1	isocitric acid	9.5 ± 0.7 ^a	9.7 ± 0.7 ^a	8.6 ± 0.6 ^a
4	citric acid	4.6 ± 0.3 ^b	4.6 ± 0.3 ^b	3.7 ± 0.3 ^a
Total		14.1 ± 0.8^b	14.3 ± 0.8^b	12.3 ± 0.7^a
TIPC		19.8 ± 0.9^{ab}	21.3 ± 0.9^b	18.3 ± 0.8^a

Table 8. Quantification of the main compounds found in elderberry extracts. Results are the mean ± SD of three measurements. TIPC= total individual phenolic content (represent the sum of all the phytochemicals identified).

Rose Hip berries

In rose hip berries extracts (DC, UAE and MAE), 38 compounds were identified (Table 9, Figure 8). The main compounds found in these extracts were organic acids (isocitric/citric acid and malic acid), kaempferol-*O*-(coumaroyl)-hexoside and methylgallate hexoside, followed by catechin, protocatechuic acid and quercetin-*O*-deoxyhexoside (Figure 9). Malic acid and citric acid, as well as the phenolic compounds, were already detected in *R. Canina*⁸³. Isocitric acid (9.8-12.5 mg g⁻¹ DE) and citric acid (4.4-5.7 mg g⁻¹ DE) were the most abundant compounds in our rose hip berries extracts (Table 10). Catechin was also particularly abundant, more than previously reported in literature⁸⁴.

No.	t_R (min)	Observed [M-H] ⁻	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	DC	UAE	MAE
1	1.905	191.0202	C ₆ H ₈ O ₇	-2.46	173.0085 129.0190 111.0086 87.0089 85.0296	Isocitric acid	✓	✓	✓
2	2.057	133.0142	C ₄ H ₆ O ₅	-0.21	115.0033 71.0140	Malic acid	✓	✓	✓
3	2.550	117.0193	C ₄ H ₆ O ₄	-0.2	99.0090 73.0297	Succinic acid	✓	✓	✓
4	2.611	191.0197	C ₆ H ₈ O ₇	-0.05	173.0099 129.0187 111.0085 87.0087 85.0294	Citric acid	✓	✓	✓
5	2.981	205.0358	C ₇ H ₁₀ O ₇	-1.68	111.0084 87.0093 67.0175 57.0342	Methylcitric acid	✓	✓	✓
6	3.227	169.0143	C ₇ H ₆ O ₅	-0.63	125.0242	Gallic acid	✓	✓	✓
7	3.753	315.0720	C ₁₃ H ₁₆ O ₉	-0.34	153.0545 152.0098 108.0225	Dihydrobenzoic acid hexoside	✓		
8	4.307	219.0510	C ₈ H ₁₂ O ₇	-0.12	111.0085 87.0090 67.0193 57.0351	Dimethylcitric acid	✓	✓	✓
9	4.677	219.0511	C ₈ H ₁₂ O ₇	-0.28	111.0083 87.0087 67.0190 57.0346	Dimethylcitric acid	✓	✓	✓
10	4.970	359.0970	C ₁₅ H ₂₀ O ₁₀	3.1	197.0443 153.0561 135.0419	Syringic acid hexoside			✓
11	5.603	153.0194	C ₇ H ₆ O ₄	-0.89	109.0287	Protocatechuic acid	✓	✓	✓
12	5.788	345.0826	C ₁₄ H ₁₈ O ₁₀	0.39	183.0294 168.0056 124.0157	Methylgallate-hexoside	✓	✓	✓
13	6.584	327.1094	C ₁₅ H ₂₀ O ₈	-1.57	165.0525 147.0458	Phenyllactic acid 2- <i>O</i> -hexoside			✓
14	7.234	577.1350	C ₃₀ H ₂₆ O ₁₂	1.06	451.1029 425.0868 407.0764 289.0712 287.0694	Procyanidin dimer B-type	✓	✓	✓
15	7.865	337.0929	C ₁₆ H ₁₈ O ₈	-0.05	163.0394 119.0503	3- <i>p</i> -Coumaroylquinic acid	✓	✓	✓
16	8.050	325.0934	C ₁₅ H ₁₈ O ₈	-0.79	163.0395 119.0500	Coumaric acid- <i>O</i> -hexoside	✓	✓	✓
17	8.159	577.1349	C ₃₀ H ₂₆ O ₁₂	1.25	451.1001 425.0869 407.0757 289.0706 287.0535	Procyanidin dimer B-type		✓	✓
18	9.090	289.0717	C ₁₅ H ₁₄ O ₆	0.27	245.0817 205.0495 203.0713	Catechin	✓	✓	✓

					179.0347 151.0397 125.0236				
19	9.337	327.1084	C ₁₅ H ₂₀ O ₈	0.82	165.0571 147.0449 103.0550	Phenyllactic acid 2- <i>O</i> - hexoside	✓	✓	✓
20	9.724	431.1918	C ₂₀ H ₃₂ O ₁₀	-1.54	385.1856 223.1363 153.0945	Roseoside (formate adduct)	✓	✓	✓
21	10.581	433.2077	C ₂₀ H ₃₄ O ₁₀	0.18	387.2010 225.1589 179.0573 161.0426 113.0223	Dihydro- roseoside (formate adduct)	✓	✓	✓
22	10.835	248.0928	C ₁₃ H ₁₅ NO ₄	0.1	204.1022 154.9737 126.0558 112.9850 105.0349 84.0461	Unknown	✓	✓	✓
23	12.393	449.1083	C ₂₁ H ₂₂ O ₁₁	2.04	287.0563 269.0436 259.0579	Dihydrokaempferol- <i>O</i> -hexoside	✓	✓	✓
24	13.787	325.0933	C ₁₅ H ₁₈ O ₈	-1.94	163.0410 119.0506	Coumaric acid- <i>O</i> -hexoside	✓	✓	✓
25	16.776	163.0397	C ₉ H ₈ O ₃	1.33	119.0500	<i>p</i> -Coumaric acid		✓	✓
26	16.917	435.0927	C ₂₀ H ₂₀ O ₁₁	1.36	303.0522 285.0409 177.0188 151.0034 125.0232	Taxifolin- <i>O</i> - pentoside	✓	✓	✓
27	18.858	449.2033	C ₂₀ H ₃₄ O ₁₁	-0.39	269.1387 225.1536	Apigenin derivative	✓	✓	✓
28	19.221	449.1091	C ₂₁ H ₂₂ O ₁₁	-0.49	287.0551 151.0041 135.0465	Eriodictyol- <i>O</i> - hexoside	✓	✓	✓
29	19.412	551.2701	C ₂₅ H ₄₄ O ₁₃	1.29	505.2639 359.1455 179.0544 161.0431 149.0454 143.0346 131.0359 119.0358 113.0221	Unknown	✓	✓	✓
30	21.319	463.0883	C ₂₁ H ₂₀ O ₁₂	-0.12	301.0351 300.0276 178.9978 151.0031	Quercetin- <i>O</i> - hexoside	✓	✓	✓
31	21.599	300.9992	C ₁₄ H ₆ O ₈	-1.05	257.0091 229.0137	Ellagic acid	✓	✓	✓
32	21.751	477.0671	C ₂₁ H ₁₈ O ₁₃	0.43	301.0350 178.9982 151.0021	Quercetin- <i>O</i> - glucuronide	✓	✓	✓
33	21.932	463.0875	C ₂₁ H ₂₀ O ₁₂	0.57	301.0346 300.0262 271.0229 183.0286 178.9967 151.0070	Quercetin- <i>O</i> - hexoside	✓	✓	✓

34	22.120	435.0930	$C_{20}H_{20}O_{11}$	0.6	303.0509	Taxifolin- <i>O</i> -pentoside	✓	✓	✓
					285.0398				
					177.0183				
					151.0037				
					125.0250				
35	23.327	433.0766	$C_{20}H_{18}O_{11}$	1.88	301.0344	Quercetin- <i>O</i> -pentoside	✓	✓	✓
					300.0263				
					271.0216				
					255.0269				
					229.0488				
36	24.696	433.0778	$C_{20}H_{18}O_{11}$	-0.45	178.9962	Quercetin- <i>O</i> -pentoside	✓	✓	✓
					151.0007				
					301.0331				
					300.0273				
					271.0591				
37	25.164	447.0927	$C_{21}H_{20}O_{11}$	1.3	178.9974	Quercetin- <i>O</i> -deoxyhexoside	✓	✓	✓
					150.9982				
					301.0346				
					255.0283				
					227.0339				
38	26.395	435.1294	$C_{21}H_{24}O_{10}$	0.52	179.0001	Phloretin- <i>O</i> -hexoside	✓	✓	✓
					151.0044				
39	35.643	301.0349	$C_{15}H_{10}O_7$	1.06	273.0762	Quercetin	✓	✓	✓
					167.0333				
40	37.403	593.1300	$C_{30}H_{26}O_{13}$	0.08	179.0015	Kaempferol- <i>O</i> -(coumaroyl)-hexoside	✓	✓	✓
					151.0019				
					447.0908				
					285.0395				
					284.0324				
					257.0451				

Table 9. Compounds identified in rose hip berries DC, UAE and MAE.

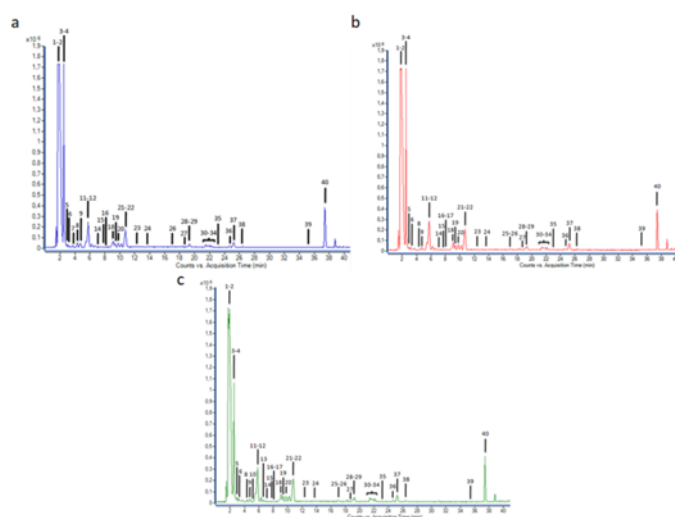


Figure 8. Base peak chromatograms (negative ion mode) of all the rose hip berries extracts: a) DC, b) UAE, c) MAE.

Peak	Compound	Relative contribution (%)		
		DC	UAE	MAE
1	isocitric acid	42.16	38.75	39.61
2	malic acid	6.91	7.88	8.12
3	succinic acid	0.93	0.93	0.92
4	citric acid	16.39	14.67	9.6
5	methylcitric acid	0.42	0.4	0.6
6	gallic acid	0.42	0.44	0.54
7	dihydrobenzoic acid hexoside	0.26	0	0
8	dimethylcitric acid	0.47	0.13	0.25
9	dimethylcitric acid	1.08	0.65	0.8
10	syringic acidhexoside	0	0	0.11
11	protocatechuic acid	1.54	1.8	2.23
12	methyl gallate-hexoside	6.17	7.21	8.14
13	phenyllactic acid 2- <i>O</i> -hexoside	0	0	0.17
14	procyanidin dimer B-type	0.19	0.2	0.17
15	3- <i>p</i> -coumaroylquinic acid	0.23	0.24	0.23
16	coumaric acid- <i>O</i> -hexoside	0.19	0.22	0.26
17	procyanidin dimer B-type	0	0.24	0.26
18	Catechin	2.01	2.04	2.26
19	phenyllactic acid 2- <i>O</i> -hexoside	0.44	0.48	0.62
20	roseoside (formate adduct)	1.21	1.38	1.4
21	dihydro-roseoside (formate adduct)	0.25	0.32	0.31
22	Unknown	4.77	5.55	6.24
23	dihydrokaempferol- <i>O</i> -hexoside	0.23	0.31	0.28
24	coumaric acid- <i>O</i> -hexoside	0.31	0.33	0.4
25	<i>p</i> -coumaric acid	0	0.14	0.13
26	taxifolin- <i>O</i> -pentoside	0.39	0.53	0.61
27	apigenin derivative	0.09	0.16	0.11
28	eriodictyol- <i>O</i> -hexoside	1.18	1.31	1.51
29	unknown	0.38	0.77	0.48
30	quercetin- <i>O</i> -hexoside	0.66	0.8	0.89
31	ellagic acid	0.64	0.91	0.83
32	quercetin- <i>O</i> -glucuronide	0.6	0.71	0.8
33	quercetin- <i>O</i> -hexoside	0.42	0.56	0.58
34	taxifolin- <i>O</i> -pentoside	0.63	0.8	0.9
35	quercetin- <i>O</i> -pentoside	0.12	0.15	0.17
36	quercetin- <i>O</i> -pentoside	0.4	0.49	0.52
37	quercetin- <i>O</i> -deoxyhexoside	1.47	1.77	1.81
38	phloretin- <i>O</i> -hexoside	0.36	0.43	0.47
39	Quercetin	0.24	0.26	0.35
40	kaempferol- <i>O</i> -(coumaroyl)-hexoside	5.82	6.04	6.33

Figure 9. Heat Map obtained by HPLC-Q-TOF-MS of rose hip berries DC, UAE and MAE (negative ion mode).

No.	Assigned identification	DC (mg/gDE)	UAE (mg/gDE)	MAE (mg/gDE)
<i>Flavonoids</i>				
18	Catechin	0.60 ± 0.04 ^{ab}	0.67 ± 0.05 ^b	0.55 ± 0.04 ^a
37	quercetin- <i>O</i> -deoxyhexoside	0.24 ± 0.02 ^b	0.27 ± 0.02 ^b	0.19 ± 0.01 ^a
40	kaempferol- <i>O</i> -(coumaroyl)-hexoside	0.50 ± 0.04 ^b	0.49 ± 0.03 ^b	0.35 ± 0.02 ^a
Total		1.34 ± 0.06^b	1.43 ± 0.06^b	1.09 ± 0.05^a
<i>Phenolic acids</i>				
11 + 12	protocatechuic acid + methyl gallate-hexoside	0.87 ± 0.06 ^{ab}	0.93 ± 0.06 ^b	0.78 ± 0.05 ^a
<i>Others</i>				
1	isocitric acid	12.5 ± 0.9 ^b	11.3 ± 0.8 ^{ab}	9.8 ± 0.7 ^a
4	citric acid	5.7 ± 0.4 ^b	5.3 ± 0.4 ^b	4.4 ± 0.3 ^a
31	ellagic acid	0.30 ± 0.02 ^a	0.38 ± 0.03 ^b	0.27 ± 0.02 ^a
Total		19 ± 1^b	17.0 ± 0.9^b	14.5 ± 0.8^a
TIPC		21.0 ± 1.0^b	19.7 ± 0.9^b	16.6 ± 0.8^a

Table 10. Quantification of the main compounds found in rose hip berries extracts. Results are the mean ± SD of three measurements. TIPC= total individual phenolic content (represent the sum of all the phytochemicals identified).

Goji berries

In goji berries extracts (DC, UAE and MAE) were characterized 20 compounds (Table 11). A disaccharide at retention time 1.719 resulted in being the most abundant along with organic acids, while the main phenolics were *p*-coumaric acid and rutin (Figure 10). The *p*-coumaric content (0.37–0.46 mg g⁻¹ DE) was reported in Table 12 along with its derivatives content, rutin and organic acids. Rutin (0.32 mg g⁻¹ DE) content was higher in MAE than in the other extraction techniques.

No.	t _R (min)	Observed [M-H] ⁻	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	DC	UAE	MAE
1	1.719	377.0859	C ₁₂ H ₂₂ O ₁₁	-0.62	341.1082	Disaccharide (HCl adduct)	✓		✓
					215.0313				
					179.0551				
					161.0448				
					143.0356				
					119.0360				
2	2.027	191.0194	C ₆ H ₈ O ₇	1.29	113.0241	Isocitric acid	✓	✓	✓
					173.0117				
					129.0188				
					111.0083				
					87.0086				
3	2.089	133.0141	C ₄ H ₆ O ₅	0.97	85.0295	Malic acid	✓	✓	✓
					115.0030				
4	2.581	191.0199	C ₆ H ₈ O ₇	-0.91	71.0140	Citric acid	✓	✓	✓
					173.0070				
					129.0197				

					111.0087 87.0094 85.0290				
5	6.342	371.0981	C ₁₆ H ₂₀ O ₁₀	0.58	325.0916 163.0394 119.0493	Coumaric acid- <i>O</i> -hexoside (formate adduct)	✓	✓	✓
6	7.132	487.1455	C ₂₁ H ₂₈ O ₁₃	0.06	323.0974 163.0400 145.0291 119.0457	Coumaric acid- <i>O</i> -dihexoside	✓	✓	✓
7	7.429	515.1400	C ₂₂ H ₂₈ O ₁₄	0.71	353.0903 341.0861 323.0765 191.0549 179.0355	Caffeoylglucosy l quinic acid	✓	✓	✓
8	7.855	325.0929	C ₁₅ H ₁₈ O ₈	-0.23	163.0394 119.0487	Coumaric acid- <i>O</i> -hexoside	✓	✓	✓
9	9.309	487.1452	C ₂₁ H ₂₈ O ₁₃	1.41	427.1220 323.0966 163.0391 145.0288 119.0504	Coumaric acid- <i>O</i> -dihexoside	✓	✓	✓
10	9.500	353.0877	C ₁₆ H ₁₈ O ₉	-0.11	191.0561 179.0348 173.0645 135.0479	Chlorogenic acid	✓	✓	✓
11	10.479	487.1451	C ₂₁ H ₂₈ O ₁₃	1.12	323.0897 307.0776 229.0511 163.0390 145.0294 119.0494	Coumaric acid- <i>O</i> -dihexoside	✓	✓	✓
12	10.868	771.1982	C ₃₃ H ₄₀ O ₂₁	0.72	609.1467 462.0797 301.0339 178.9973 151.0014	Quercetin- <i>O</i> - hexoside- <i>O</i> - rutinoside	✓	✓	✓
13	11.629	517.1561	C ₂₂ H ₃₀ O ₁₄	1.1	384.9326 355.1009 337.0938 248.9599 193.0502 175.0389 149.0610 134.0336	Ferulic acid- <i>O</i> - di-hexoside	✓	✓	✓
14	16.664	163.0400	C ₉ H ₈ O ₃	0.04	119.0501	<i>p</i> -Coumaric acid	✓	✓	✓
15	17.911	191.0353	C ₁₀ H ₈ O ₄	-1.99	176.0104 148.0142	Scopoletin	✓	✓	
16	18.273	245.0932	C ₁₃ H ₁₄ N ₂ O ₃	-0.01	203.0817 154.9732	Unknown	✓	✓	✓
17	20.691	609.1461	C ₂₇ H ₃₀ O ₁₆	-0.03	301.0337 300.0271 271.0246 255.0295 178.9976 151.0031	Rutin	✓	✓	✓
18	23.918	593.1503	C ₂₇ H ₃₀ O ₁₅	0.26	285.0388 284.0319 255.0292 229.0583 150.9995	Kaempferol- <i>O</i> - rutinoside	✓	✓	✓

19	24.591	623.1608	C ₂₈ H ₃₂ O ₁₆	1.2	315.0512	Isorhamnetin- <i>O</i> -rutinoside	✓	✓	✓
					300.0264				
					299.0196				
					271.0247				
20	31.179	312.1239	C ₁₈ H ₁₉ NO ₄	1.79	297.1011	Feruloyltyramine	✓		✓
					178.0499				
					148.0535				
					135.0443				
21	40.245	329.2332	C ₁₈ H ₃₄ O ₅	-0.94	229.1429	Trihydroxy-octadecenoic acid		✓	✓
					211.1300				
					171.1007				

Table 11. Compounds identified in goji berries DC, UAE and MAE.

Peak	Compound	Relative contribution (%)		
		DC	UAE	MAE
1	disaccharide (HCl adduct)	7.88	0	7.19
2	isocitric acid	42.53	52.29	42.93
3	malic acid	8.6	8.96	13.85
4	citric acid	21.53	16.62	17.72
5	coumaric acid- <i>O</i> -hexoside (formate adduct)	1.32	1.6	1.36
6	coumaric acid- <i>O</i> -dihexoside	1.45	1.78	1.29
7	caffeoylglucosyl quinic acid	0.9	0.93	0.73
8	coumaric acid- <i>O</i> -hexoside	1.62	1.86	1.4
9	coumaric acid- <i>O</i> -dihexoside	1.48	1.58	1.41
10	chlorogenic acid	0.85	1.01	0.94
11	coumaric acid- <i>O</i> -dihexoside	1.58	1.88	1.47
12	quercetin- <i>O</i> -hexoside- <i>O</i> -rutinoside	1.19	1.27	1.14
13	ferulic acid- <i>O</i> -di-hexoside	0.62	0.61	0.51
14	<i>p</i> -coumaric acid	2.4	2.7	2.18
15	Scopoletin	0.19	0.19	0
16	Unknown	1.68	1.99	1.68
17	Rutin	3.55	4.12	3.38
18	kaempferol- <i>O</i> -rutinoside	0.18	0.21	0.17
19	isorhamnetin- <i>O</i> -rutinoside	0.23	0.27	0.23
20	Feruloyltyramine	0.21	0	0.3
21	trihydroxy-octadecenoic acid	0	0.11	0.13

Figure 10. Heat Map obtained by HPLC-Q-TOF-MS of goji berries DC, UAE and MAE (negative ion mode).

No.	Assigned identification	DC (mg/gDE)	UAE (mg/gDE)	MAE (mg/gDE)
<i>Flavonoids</i>				
17	Rutin	0.26 ± 0.02 ^a	0.30 ± 0.02 ^{ab}	0.32 ± 0.02 ^b
<i>Phenolic acids</i>				
9	coumaric acid- <i>O</i> -dihexoside	0.37 ± 0.03 ^a	0.36 ± 0.03 ^a	0.44 ± 0.03 ^b
11	coumaric acid- <i>O</i> -dihexoside	0.36 ± 0.03 ^a	0.36 ± 0.02 ^a	0.44 ± 0.03 ^b
14	<i>p</i> -coumaric acid	0.37 ± 0.03 ^a	0.37 ± 0.03 ^a	0.46 ± 0.03 ^b
Total		1.10 ± 0.05^a	1.09 ± 0.05^a	1.34 ± 0.05^b
<i>Others</i>				
2	isocitric acid	4.5 ± 0.3 ^a	4.9 ± 0.4 ^a	5.8 ± 0.4 ^b
4	citric acid	2.1 ± 0.2 ^a	2.2 ± 0.2 ^a	2.6 ± 0.2 ^a
Total		6.6 ± 0.4^a	7.1 ± 0.4^a	8.4 ± 0.4^b
TIPC		8 ± 0.4^a	8.5 ± 0.4^a	10.0 ± 0.4^b

Table 12. Quantification of the main compounds found in goji berries extracts. Results are the mean ± SD of three measurements. TIPC= total individual phenolic content (represent the sum of all the phytochemicals identified).

Raisins

Raisin extracts (DC, UAE and MAE) were very poor in phenolic compounds. Indeed, only 16 compounds were identified/tentatively characterized (Table 13). As shown in Figure 11, tartaric acid, citric/isocitric and malic acids were the most abundant. The principal polyphenols detected were caftaric acid and the derivatives of quercetin, but their concentrations were very low. For the quantification data of these extracts, see Table 14.

No.	t _R (min)	Observed [M-H] ⁻	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	DC	UAE	MAE
1	1.799	149.0103	C ₄ H ₆ O ₆	-7.34	87.0089	Tartaric acid	✓	✓	✓
					173.0094				
					129.0190				
2	1.947	191.0203	C ₆ H ₈ O ₇	-3.05	111.0086	Isocitric acid	✓	✓	✓
					87.0089				
					85.0296				
3	2.008	133.0143	C ₄ H ₆ O ₅	-0.5	115.0030	Malic acid	✓	✓	✓
					71.0140				
					131.0302				
					119.0342				
4	2.223	179.0561	C ₆ H ₁₂ O ₆	-1.34	113.0222	Hexose	✓	✓	✓
					89.0242				
					59.0142				
					173.0087				
					129.0187				
5	2.501	191.0199	C ₆ H ₈ O ₇	-0.83	111.0084	Citric acid	✓	✓	✓
					87.0090				

					85.0296				
					153.0597				
6	3.708	315.0718	C ₁₃ H ₁₆ O ₉	1.02	152.0107 109.0355 108.0191	Protocatechuic acid- <i>O</i> -hexoside	✓		
					111.0083 87.0092 67.0211 57.0347	Dimethylcitric acid	✓		✓
8	6.324	311.0410	C ₁₃ H ₁₂ O ₉	-0.58	179.0349 149.0096 135.0451	Caftaric acid	✓	✓	✓
9	8.908	447.1505	C ₁₉ H ₂₈ O ₁₂	-1.24	401.1465 293.0926 269.1010 233.0636 161.0433	Benzyl alcohol hexose pentose (formate adduct)			✓
					301.0335				
10	20.732	609.1455	C ₂₇ H ₃₀ O ₁₆	1.2	300.0272 271.0239 178.9960 151.0033	Rutin			✓
					301.0329				
11	21.434	463.0879	C ₂₁ H ₂₀ O ₁₂	0.37	300.0263 271.0189 178.9968 151.0011	Quercetin- <i>O</i> - hexoside			✓
					301.0349 255.0340 178.9980 151.0049	Quercetin- <i>O</i> - glucuronide	✓	✓	✓
13	21.855	463.0877	C ₂₁ H ₂₀ O ₁₂	1.03	301.0341 300.0273 271.0278 255.0296 178.9986 151.0057	Quercetin- <i>O</i> - hexoside	✓	✓	✓
					285.0404				
14	25.177	447.0927	C ₂₁ H ₂₀ O ₁₁	1.41	284.0318 255.0283 227.0297 151.0082	Kaempferol- <i>O</i> - hexoside	✓	✓	✓
					229.1428				
15	38.732	327.2169	C ₁₈ H ₃₂ O ₅	2.59	211.1333 183.1494 171.1004	Oxo-dihydroxy- octadecenoic acid	✓	✓	✓
					229.1431				
16	40.109	329.2332	C ₁₈ H ₃₄ O ₅	0.04	211.1338 171.1022	Trihydroxy- octadecenoic acid	✓	✓	✓

Table 13. Compounds identified in raisin DC, UAE and MAE.

Peak	Compound	Relative contribution (%)		
		DC	UAE	MAE
1	tartaric acid	70.29	73.77	48.21
2	isocitric acid	12.43	10.83	18.31
3	malic acid	9.96	8	19.13
4	Hexose	0.84	0.85	1.19
5	citric acid	3.06	3.96	8.83
6	protocatechuic acid- <i>O</i> -hexoside	0.24	0	0
7	dimethylcitric acid	0.25	0	0.22
8	caftaric acid	0.94	0.64	0.81
9	benzyl alcohol hexose pentose	0	0	0.18
10	Rutin	0	0	0.14
11	quercetin- <i>O</i> -hexoside	0	0	0.16
12	quercetin- <i>O</i> -glucuronide	0.53	0.43	0.61
13	quercetin- <i>O</i> -hexoside	0.62	0.75	1.19
14	kaempferol- <i>O</i> -hexoside	0.27	0.29	0.44
15	oxo-dihydroxy-octadecenoic acid	0.21	0.17	0.24
16	trihydroxy-octadecenoic acid	0.37	0.3	0.35

Figure 11. Heat Map obtained by HPLC-Q-TOF-MS of raisin DC, UAE and MAE (negative ion mode).

No.	Assigned identification	DC (mg/gDE)	UAE (mg/gDE)	MAE (mg/gDE)
<i>Flavonoids</i>				
12 + 13	quercetin- <i>O</i> -glucuronide + quercetin- <i>O</i> -hexoside	0.13 ± 0.01 ^a	0.15 ± 0.01 ^a	0.15 ± 0.01 ^a
<i>Phenolic acids</i>				
8	caftaric acid	0.071 ± 0.005 ^a	0.067 ± 0.005 ^a	0.071 ± 0.005 ^a
<i>Others</i>				
2	isocitric acid	1.2 ± 0.1 ^{ab}	1.0 ± 0.1 ^a	1.3 ± 0.1 ^b
5	citric acid	0.34 ± 0.02 ^b	0.27 ± 0.02 ^a	0.36 ± 0.02 ^b
Total		1.5 ± 0.1 ^{ab}	1.3 ± 0.1 ^a	1.7 ± 0.1 ^b
TIPC		1.7 ± 0.1 ^{ab}	1.5 ± 0.1 ^a	1.9 ± 0.1 ^b

Table 14. Quantification of the main compounds found in raisin extracts. Results are the mean ± SD of three measurements. TIPC= total individual phenolic content (represent the sum of all the phytochemicals identified).

4.3.4 Antioxidant activity

DPPH and ABTS assays were used to define the radical scavenging properties of each extract (Table 15). Grape pomace extracts gave the best antioxidant results in both assays, followed by Cranberry UAE and Rose Hip DC. Raisin extracts gave the worst results, in agreement with the poor total phenolic and flavonoid contents detected. CUPRAC and FRAP assays (Table 15) highlighted the electron donation ability of the extracts, indeed they are based on the process of electron transfer from an antioxidant sample to metal ions. Grape pomace extracts, in agreement with the TPC and TFC, had the highest results. The different extracts obtained from grape pomace (DC, UAE and MAE) gave very similar results in CUPRAC and FRAP assays, while the extraction technique seemed to influence the antioxidant results of the other considered plant matrix extracts. This could be due to the multiplicity of biologically active compounds found in grape pomace, such as polyunsaturated fatty acids as well as polyphenols, which contain hydroxyl groups. Thus, the low variability in these antioxidant results can be related to the poor specificity of the extraction procedure. Furthermore, the best results in DPPH, ABTS, CUPRAC and FRAP could be related to the significant content of quercetin derivatives and catechin^{85,86}. A Pearson correlation analysis was performed revealing the strong correlation between TPC and the antioxidant abilities of the extracts (Figure 12), thus, polyphenols had a key role in conferring these properties. MCA assay (Table 15) was performed because of the role of metal chelation in inducing the reduction of hydroxyl radicals produced by the Fenton reaction. In this assay, goji berries DC and elderberry MAE gave the highest results. The lack of correlation between the TPC and this antioxidant assay can be related to the presence of non-phenolic compounds, *e.g.* sulphides and peptides, which act as chelators^{87,88}. The PBD assay (Table 15) is based on the reduction of molybdenum by antioxidant samples in acidic conditions. The best results were given from grape pomace extracts followed by elderberry extracts and rose hip extracts.

In general, the extraction method applied influenced the antioxidant properties of the berry extract except for grape pomace, *e.g.* the results of DPPH were 15.62 mg TE/g for cranberry DC and ≈ 3 times higher for cranberry UAE (44.13 mg TE/g), while decoction gave the best results for rose hip. This is related to the characteristics of the plant matrix, as explained in paragraph 4.3.2.

		<i>DPPH</i> (mg TE/g)	<i>ABTS</i> (mg TE/g)	<i>CUPRAC</i> (mg TE/g)	<i>FRAP</i> (mg TE/g)	<i>MCA</i> (mg EDTAE/g)	<i>PBD</i> (mmol TE/g)
<i>Grape pomace</i>	DC	45.05±0.22 ^a	61.70±0.01 ^a	172.26±7.25 ^a	121.04±0.12 ^a	9.24±0.29 ^{cf}	1.89±0.09 ^a
	UAE	45.23±0.13 ^a	61.54±0.04 ^a	178.47±0.98 ^a	115.58±1.60 ^b	8.30±0.36 ^{efg}	1.97±0.06 ^a
	MAE	45.07±0.16 ^a	61.53±0.02 ^a	177.68±3.78 ^a	116.49±4.02 ^b	8.32±0.30 ^{efg}	1.86±0.05 ^a
<i>Cran</i>	DC	15.62±0.72 ^f	36.19±0.46 ^e	41.78±1.69 ^h	24.62±0.96 ⁱ	7.42±0.53 ^{fg}	0.82±0.01 ^h
	UAE	44.13±0.51 ^a	61.57±0.06 ^a	136.25±3.61 ^b	87.04±0.77 ^c	12.81±0.66 ^{bc}	1.32±0.01 ^d
	MAE	17.07±0.98 ^f	37.88±1.05 ^e	44.11±0.47 ^h	26.38±0.30 ⁱ	8.36±0.18 ^{efg}	0.90±0.05 ^{gh}
<i>Eld</i>	DC	16.78±0.86 ^f	36.21±0.33 ^e	42.09±0.43 ^h	24.26±1.63 ⁱ	14.42±0.44 ^b	0.82±0.03 ^h
	UAE	39.29±0.50 ^b	58.96±0.22 ^b	86.31±1.27 ^d	55.39±1.42 ^e	14.00±0.15 ^b	1.29±0.07 ^d
	MAE	36.89±0.63 ^c	57.26±0.83 ^b	71.02±4.13 ^c	45.24±0.63 ^f	17.01±1.33 ^a	1.71±0.02 ^b
<i>Rose hip</i>	DC	43.95±0.30 ^a	61.70±0.03 ^a	121.20±2.41 ^c	75.54±0.60 ^d	13.15±0.45 ^{bc}	1.46±0.05 ^c
	UAE	22.02±0.42 ^c	43.35±0.93 ^d	52.68±1.19 ^g	30.84±0.88 ^h	10.09±0.85 ^{de}	0.88±0.04 ^{gh}
	MAE	28.80±0.74 ^d	49.70±0.20 ^c	60.29±0.09 ^f	40.24±0.86 ^g	11.58±1.44 ^{cd}	0.90±0.02 ^{gh}
<i>Goji</i>	DC	4.70±0.47 ^h	26.09±0.74 ^g	13.48±0.35 ⁱ	9.18±0.16 ^j	18.40±0.36 ^a	1.20±0.04 ^{de}
	UAE	6.16±0.16 ^{gh}	26.29±1.48 ^g	13.39±0.03 ⁱ	9.05±0.13 ^j	13.23±0.48 ^{bc}	1.26±0.05 ^d
	MAE	6.60±0.47 ^g	28.72±0.14 ^f	15.10±0.29 ⁱ	10.83±0.32 ^j	16.56±0.57 ^a	1.31±0.04 ^d
<i>Rais</i>	DC	0.91±0.06 ^j	15.20±0.58 ^h	9.43±0.13 ⁱ	7.37±0.52 ^j	7.64±0.52 ^{fg}	0.93±0.05 ^{ef}
	UAE	1.50±0.23 ^{ij}	14.93±0.27 ^h	9.54±0.05 ⁱ	10.52±1.56 ^j	6.65±0.29 ^g	1.07±0.04 ^{gh}
	MAE	2.50±0.12 ⁱ	15.59±0.51 ^h	9.04±0.16 ⁱ	7.73±0.66 ^j	7.43±0.76 ^{fg}	0.98±0.03 ^{fg}

Table 15. Results of the antioxidant assays. Values are expressed as mean ± SD of 3 parallel experiments. Different letters are used to indicate significant differences among the tested samples (p <0.05). Cran: cranberry; Eld: elderberry; Rais: raisin.

4.3.5 Enzyme inhibition abilities

The determination of the inhibition ability of the extracts is important to understand their potential pharmaceutical use being enzymes involved in numerous diseases. The performed assays aimed to determine the capability to inhibit amylase and glucosidase, essentials in the control of the sugar blood levels of diabetic patients, cholinesterase, for the improvement of the cognitive functions in patients affected by Alzheimer's, and tyrosinase^{58,89}. The study of plant extracts for the inhibition of these enzymes could be an interesting alternative to pharmaceutical enzyme inhibitors with different unpleasant long-term side effects. The results of our work are reported in Table 16.

		<i>AChE</i> (mg GALAE/g)	<i>BChE</i> (mg GALAE/g)	<i>Tyrosinase</i> (mg KAE/g)	<i>Amylase</i> (mmol ACAE/g)	<i>Glucosidase</i> (mmol ACAE/g)
Grape pomace	DC	1.88 ±0.03 ^{cd}	2.83 ±0.11 ^c	54.40 ±0.31 ^a	0.77 ±0.01 ^a	1.69 ±0.01 ^a
	UAE	1.79 ±0.01 ^{de}	2.33 ±0.04 ^{de}	54.35 ±0.63 ^a	0.78 ±0.04 ^a	1.68 ±0.01 ^a
	MAE	1.71 ±0.04 ^e	2.66 ±0.14 ^{cd}	54.50 ±0.45 ^a	0.78 ±0.02 ^a	1.64 ±0.01 ^a
Cranberry	DC	2.05 ±0.03 ^a	4.33 ±0.07 ^{ab}	20.52 ±0.90 ^{cd}	0.48 ±0.04 ^{bcd}	1.19 ±0.07 ^{bc}
	UAE	1.88 ±0.01 ^{bcd}	4.07 ±0.25 ^b	20.87 ±1.05 ^{cd}	0.53 ±0.01 ^{bc}	1.28 ±0.03 ^b
	MAE	2.10 ±0.04 ^a	4.62 ±0.05 ^a	23.55 ±0.58 ^{bc}	0.55 ±0.07 ^b	1.05 ±0.03 ^{cd}
Elderberry	DC	1.84 ±0.02 ^{de}	4.65 ±0.07 ^a	21.62 ±1.65 ^{cd}	0.52 ±0.01 ^{bc}	0.90 ±0.03 ^d
	UAE	1.30 ±0.02 ^f	2.09 ±0.16 ^{efg}	na	0.55 ±0.02 ^b	0.61 ±0.07 ^{ef}
	MAE	1.77 ±0.03 ^d	4.42 ±0.05 ^{ab}	13.18 ±0.96 ^e	0.56 ±0.01 ^b	0.93 ±0.06 ^d
Rose Hip	DC	2.04 ±0.09 ^{ab}	4.44 ±0.16 ^{ab}	25.35 ±2.61 ^b	0.50 ±0.04 ^{bcd}	1.17 ±0.04 ^{bc}
	UAE	2.04 ±0.05 ^{ab}	4.31 ±0.02 ^{ab}	20.34 ±2.19 ^d	0.50 ±0.04 ^{bcd}	1.20 ±0.08 ^{bc}
	MAE	2.03 ±0.11 ^{abc}	4.45 ±0.07 ^{ab}	16.01 ±1.14 ^c	0.49 ±0.02 ^{bcd}	1.18 ±0.11 ^{bc}
Goji berries	DC	0.94 ±0.09 ^{gh}	2.14 ±0.04 ^{efg}	na	0.44 ±0.02 ^{cde}	0.64 ±0.05 ^{ef}
	UAE	0.86 ±0.04 ^h	1.88 ±0.01 ^{fg}	na	0.37 ±0.02 ^{ef}	0.66 ±0.04 ^{ef}
	MAE	0.93 ±0.01 ^h	1.81 ±0.05 ^g	na	0.40 ±0.03 ^{def}	0.74 ±0.04 ^e
Raisins	DC	1.09 ±0.06 ^g	2.31 ±0.36 ^{efg}	na	0.32 ±0.04 ^f	0.66 ±0.03 ^f
	UAE	1.10 ±0.08 ^g	2.11 ±0.23 ^{def}	na	0.42 ±0.05 ^{def}	0.57 ±0.04 ^{ef}
	MAE	0.01 ±0.01 ⁱ	1.89 ±0.07 ^{fg}	na	0.35 ±0.01 ^{ef}	0.60 ±0.04 ^{ef}

Table 16. Results of the enzyme inhibition assays reported as mean ± SD of 3 measurements. Different letters are used to indicate significant differences among the tested samples ($p < 0.05$).

Rose hip berries and cranberry extracts showed a higher inhibition effect against AChE and BChE. Grape pomace extracts had the highest tyrosinase effects, while the extracts of goji berries and raisins weren't effective. Some compounds found in the extracts could influence the inhibition abilities of the samples, for instance, quercetin and catechin (found in grape pomace extracts) are tyrosinase inhibitors^{90,91}. The good tyrosinase inhibition of cranberry MAE could be related to its high concentration of benzoic acid derivatives⁹². Regarding amylase inhibition, the best results were given by grape pomace extracts, followed by elderberry extracts and cranberry extracts, while the highest inhibition effect against glucosidase was obtained by grape pomace extracts followed by rose hip extracts and cranberry extracts. These results seemed in agreement with TPC, indeed previous research works reported an effective inhibition of glucosidase *in vitro* and *in silico* performed by phenolic compounds^{93–95}.

Overall, the results are influenced by the applied extraction techniques, since the composition in terms of bioactive compounds changes based on them. However, the extracts obtained with a hydroalcoholic mixture have a complex composition in terms of phytochemicals. Indeed, non-phenolic compounds as well as polyphenols can be extracted and interact in different ways with the active/allosteric site of the considered enzymes contributing to conferring the inhibition capability.

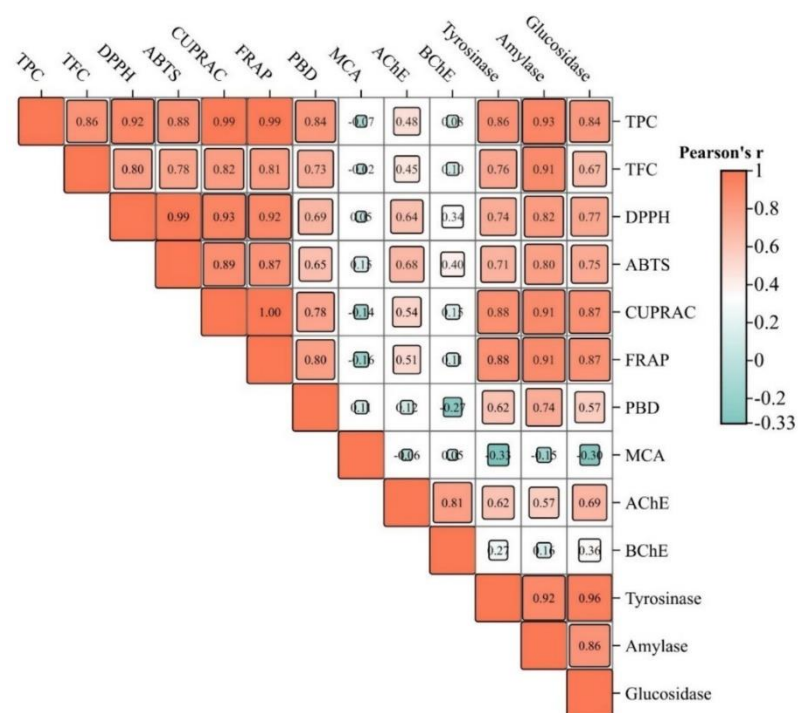


Figure 12. Pearson correlation determined between the total biologically active components and the biological activities.

4.4 Conclusion

This work was focused on the comparison of the phytochemical profiles and biological *in vitro* activities of grape pomace and berries/fruit extracts. Three types of extraction techniques were considered to prepare the extracts, which had consequently different phytochemical profiles (in terms of polyphenols detected) and antioxidant and enzyme inhibition properties.

The extracts prepared from grape pomace had the highest TPC and TFC detected spectrophotometrically with small differences among them, while the other matrices follow peculiar trends. MAE gave the best grape pomace extraction yield. From the HPLC-MS analyses, grape pomace extracts resulted to be rich in biologically active compounds which confer antioxidant properties and enzyme inhibition abilities to them. Comparing the obtained data with those of the other plant matrices extracts, grape pomace was the one with the highest number of biologically active compounds determined, TPC and TFC, radical scavenging and reducing power abilities and tyrosinase, amylase and glucosidase inhibition activities.

Currently, grape pomace is mainly used to produce spirits. In the European Union, wine pomace was delivered to distilleries to produce ethanol until 2010. With the introduction of EC Regulation 5396/2008, which implemented EC Regulations 479/2008 and 555/2008, there was an extension in the applications of wine pomace further on distillations, *e.g.* as fertilizers, a component of animal feeds or for applications in cosmetic and pharmaceutical sectors⁹⁶.

The results of our research work highlight the potentiality of this by-product of the wine-making process against commercially available products, prompting its valorization and future applications as a nutraceutical or food ingredient.

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Chapter 5:
Conclusions

The common thread running through this thesis was the sustainability of the processes, the use of green procedures and the valorization of food waste deriving from agri-food chains. The aims pursued were the extraction of valuable compounds with a beneficial effect on human health to produce novel nutraceuticals and enriched foods. A brief recap of the presented research works is reported below.

Chapters 2 and 3 were focused on the extraction of lycopene from tomato skins, a waste generated during tomato industrial processing. The employment of the natural deep eutectic solvent menthol-thymol opens a new perspective for further studies on its application in green extractions of hydrophobic compounds. The achieved removal of the solvent represents an advance in this sector. Tomato skins were also used for the extraction and simultaneous enrichment of an extra virgin olive oil, producing an enriched food with improved antioxidant and enzyme inhibition effects, which was safe on the PNT1A normal prostate cell line.

Chapter 4 was focused on the valorization of grape pomace extracts through a comparative study with commercial berries extracts. Grape pomace extracts showed the best antioxidant and enzyme inhibition effects; furthermore, the phytochemical profile was determined by HPLC-ESI-Q-TOF-MS highlighting the array of beneficial compounds contained in them. The future perspective of this work is the employment of this food waste as a novel ingredient for the enrichment of foods or for the production of nutraceuticals and food supplements.

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