TD

Exploring the Potential of Regional Fruits as Powerful Sources of Health-promoting Bioactive Compounds

DOCTORAL THESIS

José Aldónio Oliveira Figueira
DOCTORATE IN CHEMISTRY

UNIVERSIDADE da MADEIRA
A Nossa Universidade
www.uma.pt

June | 2021

Cofinanciado por:

MADEIRA 14-20
REGIÃO AUTÓNOMA DA MADEIRA
PORTUGAL 2020
UNIÃO EUROPEIA
Fundo Social Europeu
arditi

Exploring the Potential of Regional Fruits as Powerful Sources of Health-promoting Bioactive Compounds

DOCTORAL THESIS

José Aldónio Oliveira Figueira
DOCTORATE IN CHEMISTRY

ORIENTATION
José de Sousa Câmara

Acknowledgments

I would like to express my sincere gratitude to Professor José S. Câmara, my supervisor, for his guidance, enthusiastic encouragement and very useful constructive suggestions during the planning and development of this thesis. I would like to thank Centro de Química da Madeira (CQM) and Universidade da Madeira (UMa) for the facilites to perform this work. I acknowledge the technicians from UMa, Ana Paula Andrade and Paula Vieira for their support and assistance through the development of this thesis.

I also acknowledge the support by FCT-Fundação para a Ciência e a Tecnologia through the projects PEst-OE/QUI/UI0674/2019, the CQM Base Fund (UIDB/00674/2020, and Programmatic Fund—UIDP/00674/2020). Also ARDITI—Agência Regional para o Desenvolvimento da Investigação Tecnologia e Inovação, through the projects M1420-01-0145-FEDER-000005 – Centro de Química da Madeira—CQM+ (Madeira 14-20), PROEQUIPRAM - Reforço do Investimento em Equipamentos e Infraestruturas Científicas na RAM (M1420-01-0145-FEDER-000008), and Project M1420 – 09-5369-FSE-000001, for my Ph.D fellowship.

To all my colleagues and friends, that helped with my work. A special thanks to Priscilla Porto-Figueira, Jorge Pereira and João Gonçalves, for their friendship, happy moments and motivation to continue my work. For late, to the foundations of this PhD, but also in life, I would like to thank my family, to my father, now deceased, my mom and sisters, and my forever home, my wife Priscilla and son Cristiano.

Abstract

The work developed along the PhD, aimed the evaluation of the phytochemicals composition in fruits from regular consumption produced in Madeira Island, lemon (*Citrus limon* var. eureka), tangerine (*Citrus reticulata* var. setubalense), tomato (*Solanum lycopersicum* var. gordal) , pitanga (*Eugenia uniflora* var. red) and uva-da-serra (*Vaccinium padifolium*), and its potential bioactivity.

To achieve the proposed aims, diverse analytical approaches were developed and validated. Lycopene (727.1±13.8 mg/g), β-carotene (80.4±1.4 mg/g) and α-, γ-, δ-tocopherols were determined by LLUSAE/UHPLC-PDA/FLR, in tomatoes. For tocopherols this methodology presented LODs about 1000 times lower than those reported in literature, allowing the determination, for the first time, of δ-tocopherol in tomato.

QuEChERS combined with LC-ESI/MS/MS was applied to determine the free low-molecular weight phenolics in uva-da-serra. Twenty-six phenolic compounds were identified, being chlorogenic acid (17.4mg/g) the predominant.

The volatile composition of uva-da-serra and tangerine (129 volatiles), was established by HS-SPME/GC-qMS. Moreover, an emerging extraction technique, NTME, was used for the first time, to define the volatomic profile of foodstuff (lemon - 75 volatiles).

The volatile profile highlights terpenes as the dominant chemical family, and the relevant presence of phytochemicals with reported health-promoting benefits, such as limonene (lemon) and thymol (tangerine). The volatomic profile of uva-da-serra was analysed for the first time, being identified 72 volatiles. In addition, application of multivariate statistical analysis to the data results, allow the identification of variables that were able to differentiate among fruits according to species, variety,

sample type, and ripening stage, supporting the certification of their origin and authenticity, and improving crop quality.

The total phenolics, antioxidant, antidiabetic and antihypertensive activities of the target fruits confirmed the health-promoting potential of these fruits, highlighting the potential of added-value of the targeted fruit extracts, constituting a natural biosource of compounds to be used in different fields including food, cosmetics and pharmaceutical industries.

Keywords: fruits, volatile composition, bioactive compounds**,** antioxidant, antidiabetic, antihypertensive

Resumo

O trabalho desenvolvido ao longo do doutoramento teve como objetivo a avaliação da composição de fitoquímicos em frutos de consumo regular produzidos na Ilha da Madeira, limão (*Citrus limon* var. Eureka), tangerina (*Citrus reticulata* var. setubalense), tomate (*Solanum lycopersicum* var. gordal), pitanga (*Eugenia uniflora* var. red) e uva-da-serra (*Vaccinium padifolium*), e determinação do seu potencial bioativo.

Para atingir os objetivos propostos, foram desenvolvidas e validadas diferentes metodologias. Licopeno (727,1±13,8 mg/g), β-caroteno (80,4±1,4 mg/g) e α, γ, δ-tocoferóis foram determinados por LLUSAE/UHPLC-PDA/FLR, em tomate de diferentes variedades. Para os tocoferóis, esta metodologia apresentou LODs cerca de 1000 vezes inferiores aos reportados, permitindo pela primeira vez a determinação de δ-tocoferol em tomate.

O QuEChERS/LC-ESI/MS/MS foi aplicado para avaliar a composição de fenóis livres de baixo peso molecular em uva-da-serra. Foram identificados 26 compostos fenólicos, sendo o ácido clorogénico (17,4±1.7 mg/g) o predominante.

A composição volátil de uva-da-serra e tangerina, foi estabelecida por HS-SPME/GC-qMS. Além disso, uma técnica de extração emergente, NTME, foi usada pela primeira vez para definir o perfil volatómico de alimentos (limão).

Do perfil volátil dos frutos alvo destacam-se os terpenos como a família química dominante e a presença relevante de fitoquímicos reportados como benefícios para a saúde, como o limoneno (limão) e o timol (tangerina), sendo que o perfil volatómico da uva-da-serra foi analisado pela primeira vez (72 voláteis). A aplicação de análise estatística aos resultados, permitiu a identificação de variáveis capazes de diferenciar os frutos de acordo com a espécie, variedade, tipo de amostra e estágio

de maturação, ajudando na certificação de origem e autenticidade, e melhorando a qualidade do cultivo.

As atividades antioxidante, antidiabética e anti-hipertensiva e os fenóis totais, das frutas em estudo confirmaram o potencial benéfico para a saúde dessas frutas, destacando o potencial valor agregado de extratos de frutas direcionados, constituindo uma fonte biológica natural de compostos a serem usados em diferentes áreas, incluindo alimentos, cosméticos e indústrias farmacêuticas.

Palavras-chave: frutas, composição volátil, compostos bioativos, antioxidante, antidiabético, anti-hipertensivo

Abbreviations List

ACE	angiotensin-converting enzyme
ACN	acetonitrile
ANOVA	analysis of variance
CAR	carboxen
CV	coefficient of variation
DoE	experimental design
DVB	divinylbenzene
DW	dry weight
EtAc	ethyl acetate
EtOH	ethanol
FLMWP	free low-molecular weight phenolics
FLR	fluorescence detector
FW	fresh weight
GC–qMS	gas chromatography/quadrupole-mass spectrometry
HCA	hierarchical cluster analysis
HL	high level
HS-SPME	headspace-solid phase microextraction
LC-ESI/MS/MS	liquid chromatography electrospray ionization tandem mass spectroscopy
LDR	linear dynamic range
LL	low level
LLUSAE	liquid-liquid-based ultrasound-assisted extraction
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
ML	medium level
MVDA	multivariate data analysis
MWCNT	multi-walled carbon nanotubes
NIST	national institute of standards and technology
NTD	needle trap device
NTME	needle trap microextraction
ORAC	oxygen radical absorbance capacity
PA	polyacrylate
PC	principal component

PCA	principal component analysis
PDA	photodiode array detector
PDMS	polydimethylsiloxane
PEG	poliethyleneglicol
PLS-DA	partial least squares discriminant analysis
QuEChERS	quick, easy, cheap, effective, rugged, and safe
RI	retention index
RSD	relative standard deviations
RT	retention times
SD	standard deviation
TAC	total antioxidant capacity
TFC	total flavonoid content
TPC	total phenolic content
UdS	uveira-da-serra
UHPLC	ultra-high pressure liquid chromatography
VOCs	volatile organic compounds
VOMs	volatile organic metabolites

Table of Contents

List of Figures

Section 1.

Section 2.

Section 3.

Sub-Section 3.1.

Sub-Section 3.2.

Sub-Section 3.3.

Sub-Section 3.4.

Sub-Section 3.5.

Sub-Section 3.6.

Sub-Section 3.7.

Supplementary Material

List of Tables

Section 3.

Sub-Section 3.6.

Sub-Section 3.7.

Supplementary Material

Outline of the Thesis

This thesis was structured in four Sections, starting with the State of the Art that outlines the work developed (Section 1), followed by the Aims and Scope in Section 2. In Section 3, the results obtained as well as their discussion, will be presented by manuscripts, as they have already been published in scientific journals of reference. The main conclusions and future perspectives are described in Section 4.

Section 1. Introduction

In this section, we address the necessity to evaluate the phytochemical composition of foodstuff, identifying significant sources of phytochemicals with health benefits, exploring the potential of fruits as powerful sources of health-promoting bioactive compounds.

Section 2. Aims and Scope

The main objectives of this thesis, and the strategies to achieved them will be presented.

Section 3. Evaluation of fruits as sources of health-promoting bioactive compounds

In this section, analytical approaches were developed aiming the evaluation of the phytochemicals composition in fruits from regular consumption produced in Madeira Island, and its potential bioactivity. The results obtained as well as their discussion will be presented by manuscripts.

Section 4. Conclusions and Future Perspectives

Comprehensive and concise conclusions are presented, regarding the bioactive compounds (lycopene, β-carotene, α,γ,δ-tocopherols, phenolics and terpenes), identified on the selected fruit (tomato, tangerine, lemon, pitanga and uva-da-serra), and the correspondent health-promoting potential (total phenolics, antioxidant, antidiabetic and antihypertensive activities). Future works to consolidate the main conclusions and future perspectives will also be presented.

Section 1. Introduction

Exploring the potential of fruits as powerful sources of health-promoting bioactive compounds

Devasagayam *et al.* (2004)[2], defined that "*functional foods are those that provide more than simple nutrition, they supply additional physiological benefit to the consumer*". Moreover, according to Aune *et al.* (2017)[3], between 5.6 and 7.8 million premature deaths occurring worldwide in 2013, were attributable to a low fruit and vegetable intake, and Cheung *et al.* (2021)[4] reported that most of these mortality risks, mainly those related to cardiovascular disease, chronic diseases, and cancer, could be reduced by regular and varied consumption of fruit and vegetables [2, 5-9]. For these reasons, interest in the potential role of functional foods and nutraceuticals on preventing these diseases has increased. Drewnowski (2019)[10] review the food recommendations, fruits, vegetables, dairy, whole grains, and nuts and seeds, that resulted from food nutrients constitution. According to Mannucci (2021), fruits contain a wide range of secondary metabolites, such as alkaloids, polyphenols, and terpenoids which are called "phytochemicals", whose effects are protective for health. Different phytochemicals provide different levels of protection, so it is necessary to know the characteristics of the different phytochemicals.

1.1. Phytochemical compounds in fruits

Plants possess an immune system against environmental stresses, producing a mirage of molecules to enhance their physical and chemical immunity [11] . Fruits are generally consumed as sources of essential nutrients, due to the presence of numerous bioactive compounds, such as antioxidants, fibers, vitamins, minerals, and other nutrients, with associated potential beneficial health effects[12]. These secondary metabolites have the ability to help to mitigate oxidative stress, cardiovascular disease, obesity, Alzheimer's disease and cancer[13]. Phytochemicals can be distributed in three main groups: *i)* terpenes (e.g. monoterpenoids, iridoids, sesquiterpenoids, sesquiterpene lactones, diterpenoids, and carotenoids); *ii)* phenolics (e.g. anthocyanins, coumarins, lignans, phenols and phenolic acids, phenolic ketones, phenyl-propanoids, stilbenoids or tannins); and *iii)* alkaloids (e.g. betalain, diterpenoid, indole, isoquinoline, peptide, pyrroli-dine and piperidine,

pyrrolizidine, quinoline, or steroidal). From these groups of phytochemicals, were selected for study, carotenoids, tocopherols, polyphenols, phenolics and volatile composition (mainly terpenoids), due to their high bioactive effect.

1.1.1. Carotenoids

Carotenoids are isoprenoids (eight isoprene units), resulting in a C_{40} polyene backbone, biosynthesised from two C_{20} geranylgeranyl diphosphate molecules. There are known over 1100 carotenoids, but less than 10% are found in our daily foods [14, 15], and only around 10 % can be metabolised to retinol (vitamin A), having a β-ionone ring, along with one polyene chain. According with their structure, carotenoids can be classified in carotenes or carotenoids hydrocarbons, (composed by only carbon and hydrogen) and xanthophyls or oxygenated carotenoids (obtained by the addition of oxygen)[14, 16-18].

In plant cells, carotenoids biosynthesis occurs mainly in plastids [19]. The pathway starts with a small isoprenoid molecule, isopentyl diphosphate, derived from the methylerythritol phosphate and mevalonate pathways [20-22]. The sequential and linear addition of three isoprenoid molecules to one molecule of dimethylallyl diphosphate, first results in geranyl diphosphate, which may lead to monoterpenes, with second addition of isoprenoid molecules resulting in farnesyl diphosphate that may lead to sterols, sesquiterpenes or polyterpenes synthesis [20, 22-25]. A third isoprenoid addition results in geranylgeranyl pyrophosphate, which may lead to quinones, diterpenes, chlorophylls or tocopherols. Finally, the condensation of two geranylgeranyl pyrophosphate molecules, results in phytoene (a C_{40} molecule). Isomerisation and denaturation processes through enzymatic mediation, result in formation of lycopene, an C_{40} polyene backbone [22, 25-29]. Different paths can result from lycopene leading to the formation of provitamin A, retinol (vitamin A), xanthophylls or abscisic acid. In addition, β-carotene, a provitamin A, can result in one or two molecules of retinol [30-32], or lead to the formation of xanthophylls [22, 27-29,

[33]. The biosynthetic sequence of the carotenoids in plants can be seen in Fig. 1.1. and Fig. 1.2.

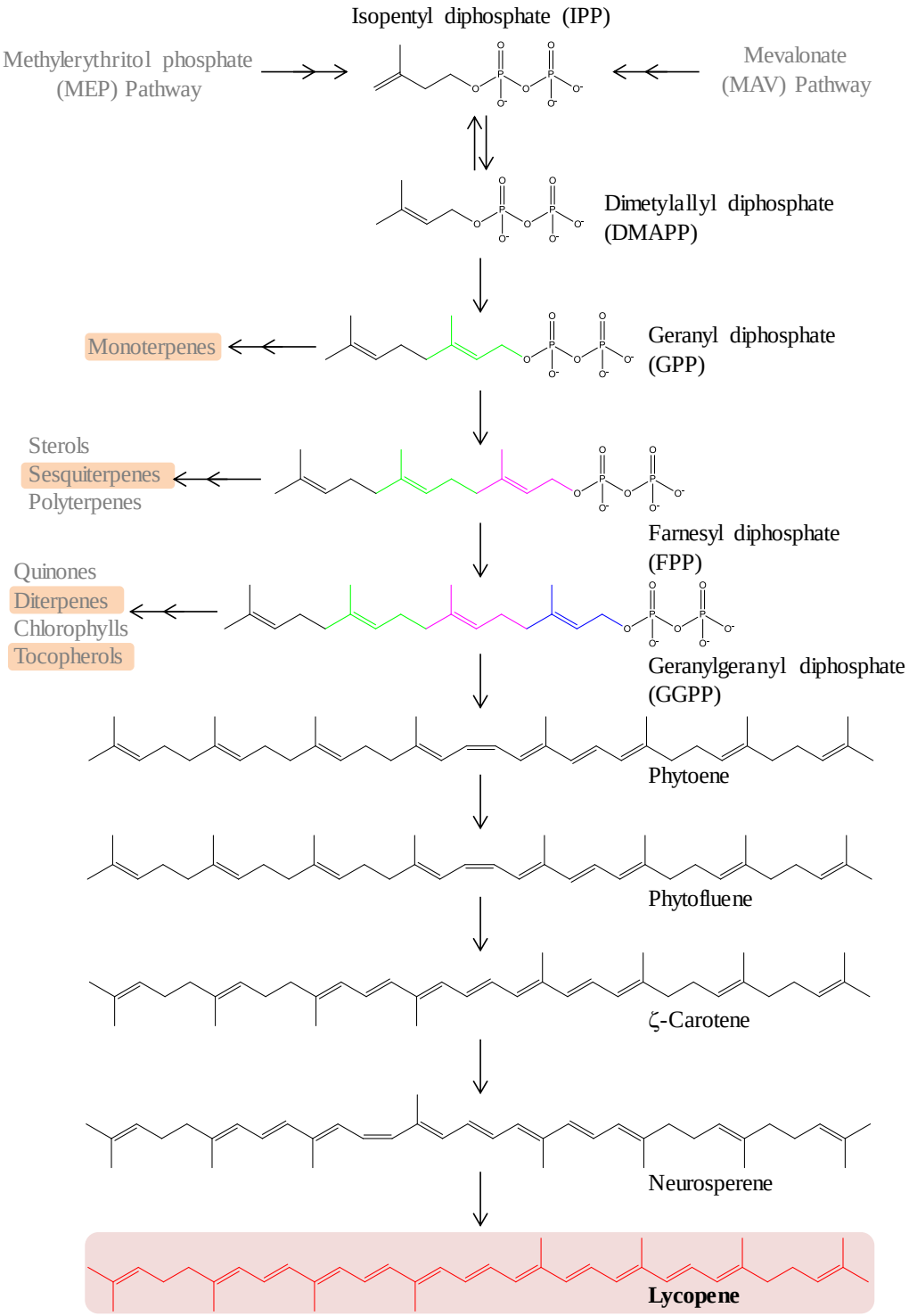


Fig. 1.1. Biosynthetic sequence of the carotenoids in plants – *Lycopene biosynthesis* [21-31, 34, 40-47].

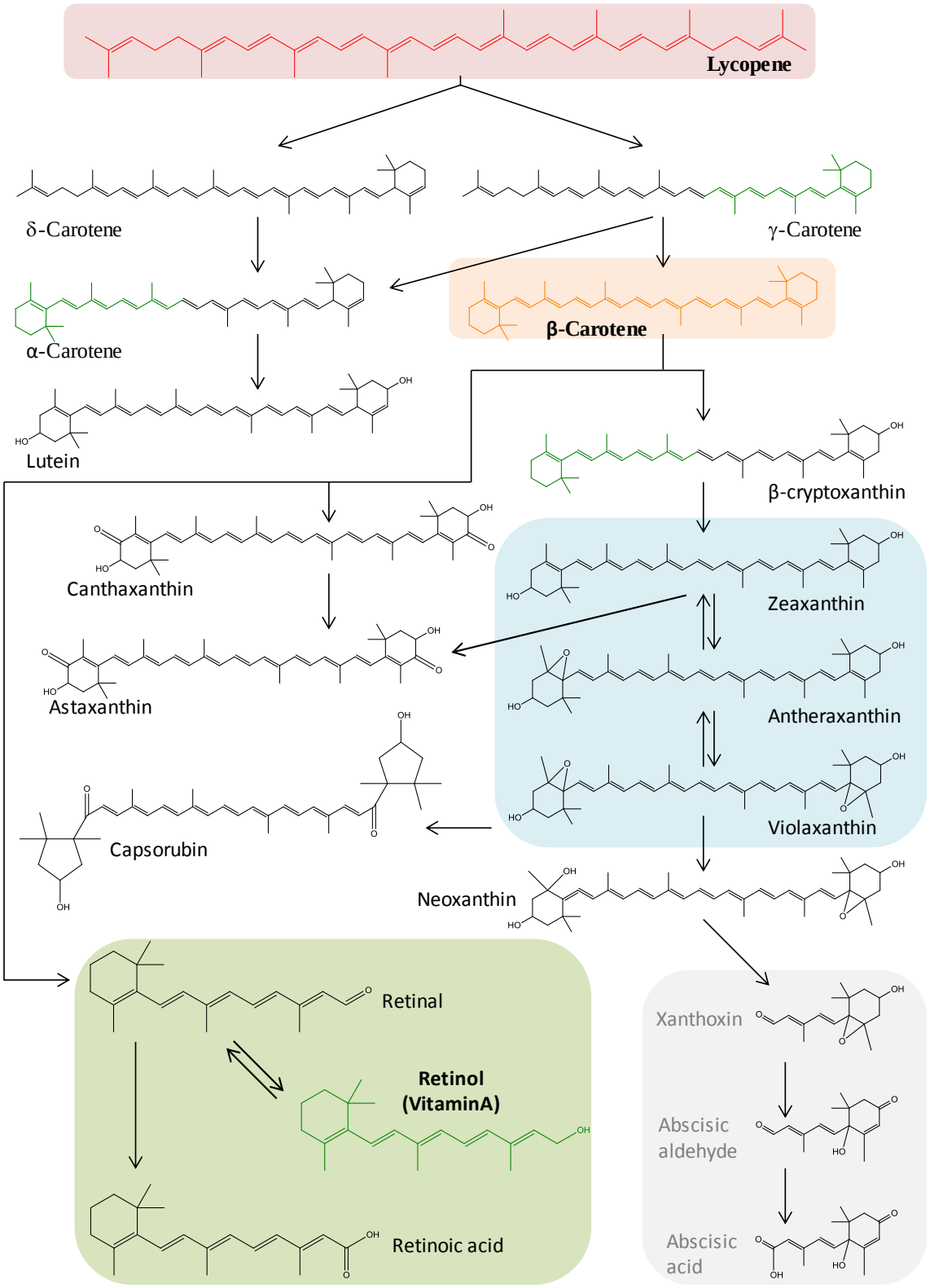


Fig. 1.2. Biosynthetic sequence of the carotenoids in plants – *Lycopene derivatives* [21-31, 34, 40-47].

Several studies demonstrate the health potential of several carotenoids. Astaxanthin, one of the most abundant aquatic carotenoids, stands out among carotenoids for its high antioxidant capacity, Donoso (2021) [34] review studies that associated

astaxanthin with several health benefits, including neuroprotective, cardioprotective and antitumoral properties, suggesting its therapeutic potential for the prevention or co-treatment of dementia, Alzheimer, Parkinson, cardiovascular diseases and cancer. Swapnil (2021)[15] review carotenoids vital role, summarizing potential health benefits of lycopene (prostate risk inhibition, antidiabetic activity, antitumor activity or cardiovascular disease protection) and β-carotene (inhibition of optical diseases, skin protection from UV-light, antitumor activity or arthrosclerosis disease protection), among others.

1.1.2. Tocopherols

Vitamin E, discovered in 1922 by Evans and Bishop [35], consists on a 6-chromanol ring, and with an alkyl C_{16} isoprenoid side chain [36, 37]. Vitamin E can be classified in tocopherols (α-, β-, γ- and δ-tocopherol) and tocotrienols (α-, β-, γ- and δ-tocotrienol), differing from tocopherols by having unsaturated side chains. In addition, tocopherols have much higher vitamin E activity. [35, 36, 38-40]. Concerning the antioxidant capacity of the different vitamin E isoforms, several studies have concluded that tocopherols and tocotrienols support a hydrogen-donating power in the order α > β > γ >δ. α-Tocopherol, one of the most abundant lipid-soluble antioxidant, is the most bioactive isoform, and the most abundant in the human body[36, 38, 39].

Biosynthesis of tocopherols in plants occurs in chloroplasts, where tocopherols results from the condensation of homogentisic acid, the final product of shikimate pathway, with phytyl diphosphate derived from chlorophyll *a* or GGPP, resulting in 2-methyl-6-phytylplastoquinol[22, 38, 41-44], a conjugation of a polar group from homogentisic acid with an alkyl side chain from phytyl diphosphate (Fig. 1.3).

Durazzo (2021) [40] review the occurrence of tocols in foods, and reported bioactivity, such as anticancer, antiobesity, antidiabetic, and cardioprotective effects. These positive potential health effects have inspired researchers to proceed with the evaluation of the phytochemical content of new fruits and vegetables. In addition,

recently in a review of phytochemicals and Mediterranean Diet related with COVID-19[45], underlined the relevance of tocopherols in the proper functioning of the immune system.

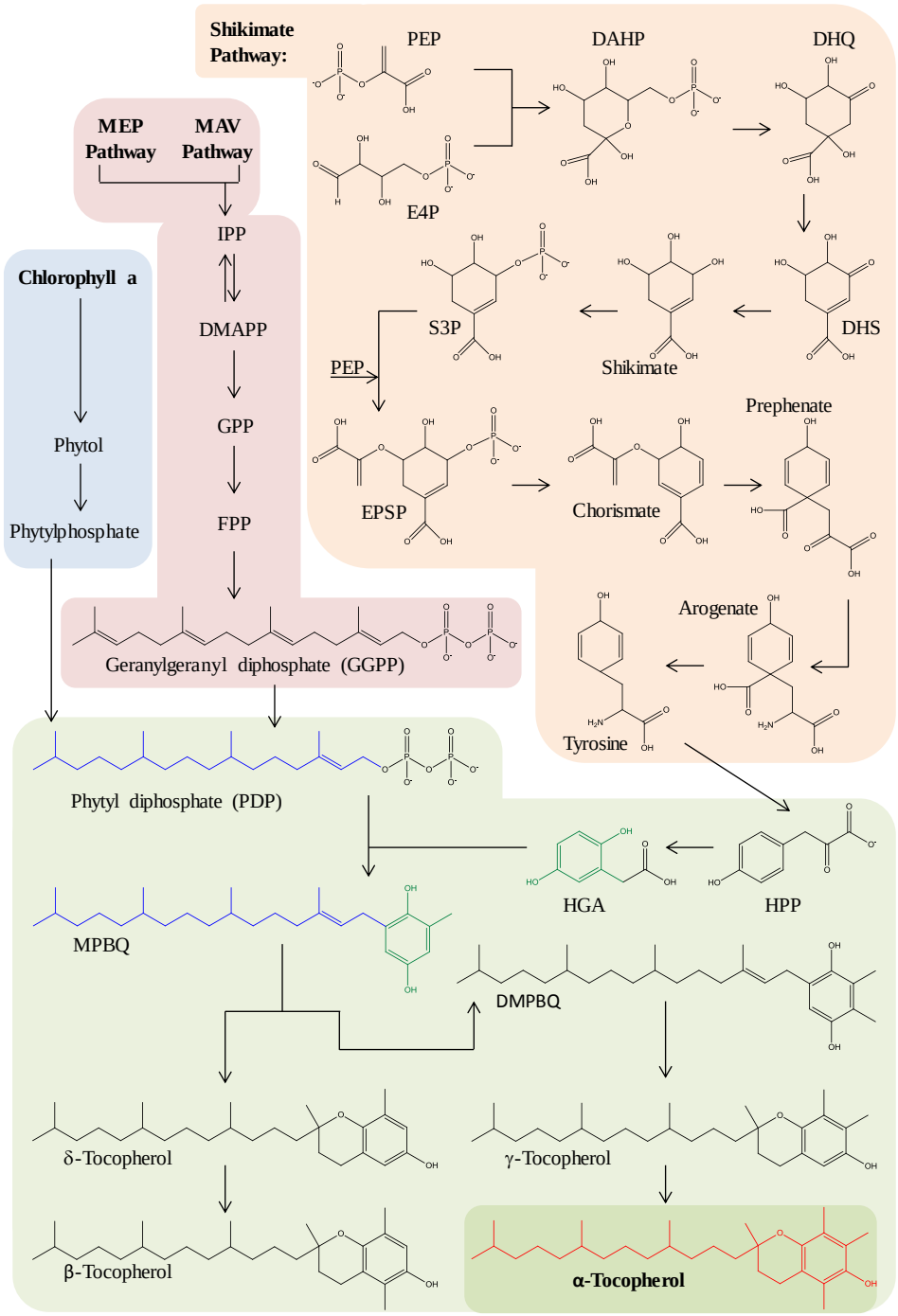


Fig. 1.3. Biosynthetic sequence of the tocopherols. IPP – isopentyl diphosphate, DMAPP – dimethylallyl diphosphate, GPP – geranyl diphosphate, FPP – farnesyl diphosphate, PeP – phosphoenol pyruvate, E4P – erythrose 4-phosphate, DAHP – 3-deoxy-D-arabino-heptulosonate 7-phosphate, DHQ – 3-dehydroquinate, DHS – dehydroshikimate, S3P – shikimate 3-phosphate, EPSP – 5-enolpyruvylshikimate 3-phosphate, HPP – 4-hydroxyphenylpyruvic acid, HGA – homogentisic acid, MPBQ – 2-methyl-6-phytylplastoquinol, DMPBQ – 2,3-dimethyl-5-phytyl-1, 4-benzoquinone [19, 23, 35, 38, 40-42]

1.1.3. Phenolics

Phenolics are secondary plant metabolites, characterized by having at least one aromatic ring with one (phenol) or more (polyphenols) hydroxyl groups attached [46]. Polyphenols can be classified in two classes: *i)* Flavonoids, consisted in a C15 backbone with in two aromatic rings (A and B) connected by a three carbon bridge (C6–C3–C6) that can form an oxygenated heterocyclic ring (C) (Fig. 1.4), and *ii)* Non-flavonoids, with more variated structures, constituted by three main subclasses phenolic acids, stilbenes and hydroxycinnamic acids (Fig. 1.4. and 1.5.) [46, 47]. In addition, Flavonoids can be subdivided in many other sub-classes, being the most relevant, the flavanones, flavones, flavonols, dihydroflavonols, flavan-3-ols, anthocyanidins, chalcones and isoflavones (Fig. 1.5.) [47]

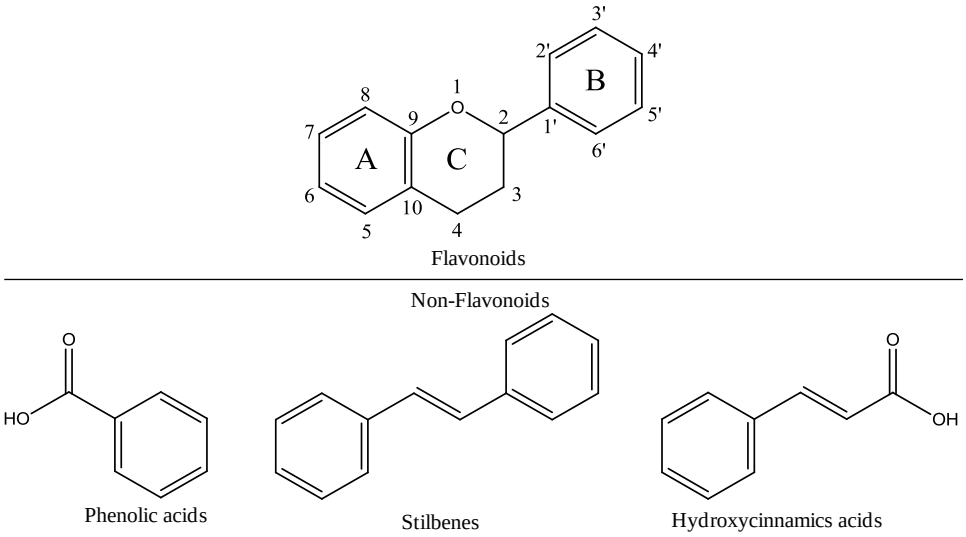


Fig. 1.4. General classification of polyphenols, according with their chemical structure. In non-flavonoids are presented the three principal subclasses, *i)* phenolic acids, *ii)* Stilbenes and *iii)* hydroxycinnamic acids. [46] To the simplified chemical structures of flavonoids and stilbenes, at least one -OH group must be attached to one of the aromatic rings.

In plants, most polyphenols have origin in the shikimate pathway, with the condensation of phosphoenolpyruvate with erythrose 4-phosphate to form 3-deoxy-D-heptulosonate 7-phosphate. As reported by Fraser *et al.* (2011) [48], subsequent ring closure, dehydration, and reduction lead to the production of shikimate, subsequent rearrangements will result in the more than 10000 known polyphenols (Fig. 1.5.) [46, 47]. Revi (2021) [49] and Pagano (2021) [50]reviewed the impact of dietary polyphenols,

where the phenolic compounds exhibit a wide range of physiological properties such as antioxidant, anticarcinogen, antimutagen, antiallergen and antiaging activity, and as so, have several fields of application, namely food industry, cosmetics and pharmaceuticals.

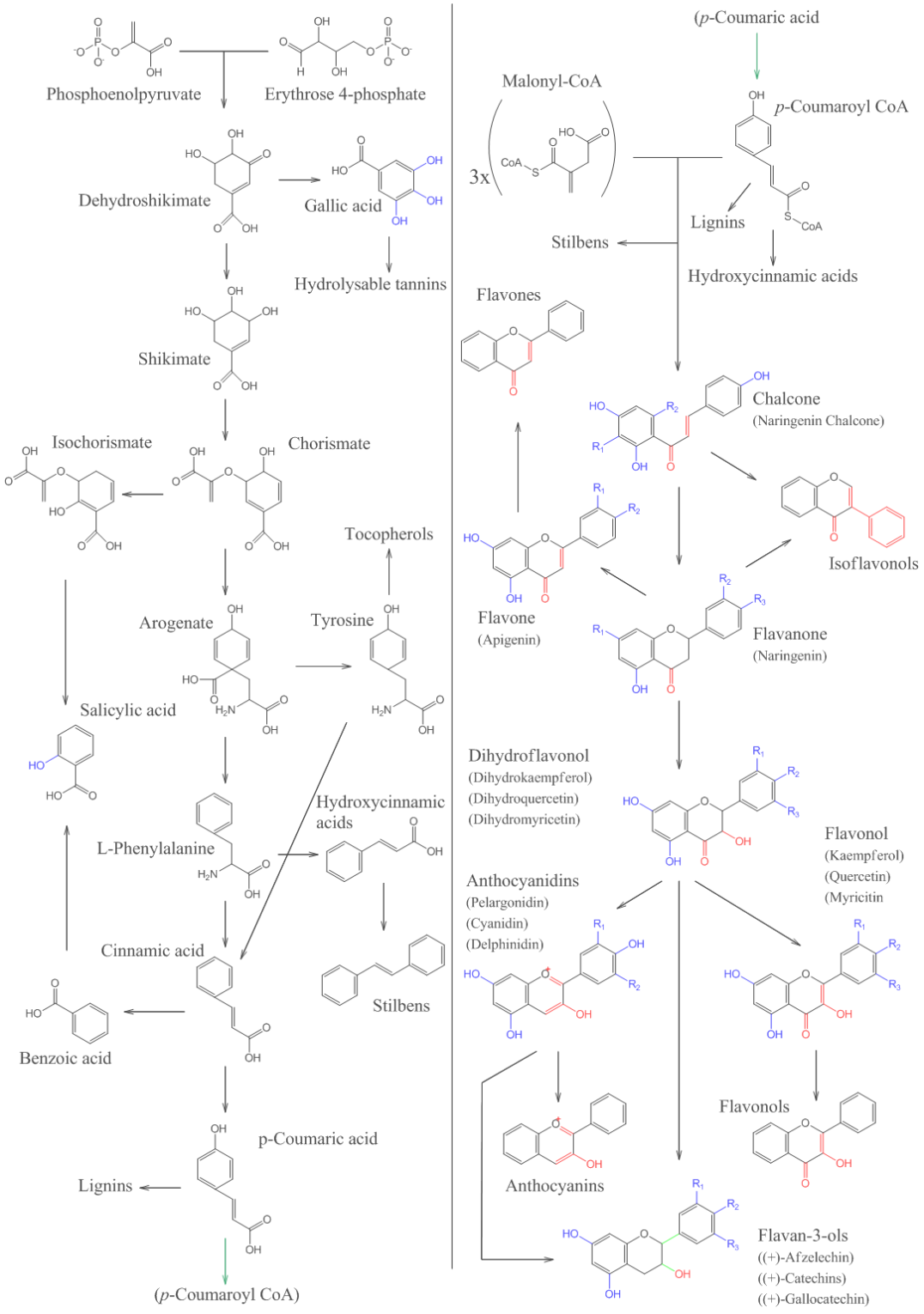


Fig. 1.5. Biosynthetic sequence of the phenolics. From Shikimate pathway (at left). [46]

1.1.4. Terpenes

Terpenes are the largest natural products, with more than 55000 known compounds [51]. Structurally, terpenes are derived from isoprene units, and according to the Isoprene Rule[52], the chemical compounds can be classified as hemi-, mono-, sesqui, -di-, sester-, tri-, sesquar-, tetra- (C5, C10, C15, C20, C25, C30, C35 and C40) [53]. Carotenoids can be classified as terpenes (tetraterpenes = four isoprenoid units), since terpenes are characterized by isoprene unit backbones, being the isopentenyl diphosphate unit and dimethylallyl diphosphate the biosynthesis precursors (Fig. 1.1.). Through processes of oxygenation, hydrogenation, or dehydrogenation, terpenes originate terpenoids. [51]. The lower the number of isoprene units on a terpene backbone, more volatile is the terpene, as so, big molecules as carotenoids are not classified as volatile, but smaller molecules, as the number generally accounts for their volatility, are known volatiles, such as hemiterpenes, monoterpenes and sesquiterpenes [51].

According with Ninkuu (2021) [51], terpenes are abundant in higher plants, citrus, conifers, eucalyptus, and are associated with antifungal, antimicrobial, antiviral, and antiparasitic proprieties [51]. Carvalho (2021) [54] recently reviewed brazilian native plants, and associate antioxidant, antimicrobial and anti-cancer activities to terpenoids. In addition, anti-inflammatory [55, 56], antidiabetic [57, 58], antileishmanial [59, 60], and antibacterial [58, 60-63] activities. Amparo (2021), in a review guided by in silico study, concluded that the top 14 compounds with potential to be used for a new anti-COVID-19 drug were terpenes (8 sesquiterpenes and 6 monoterpenes)[64].

There is a strong correlation between some terpenoids content and health benefits, but also between some carotenoids, tocopherols and phenolics, as well. It is necessary evaluate the phytochemical composition of foodstuff, identifying the potential nutraceutical foods, and significant sources of phytochemicals with high potential health benefits.

1.2. General Description of Studied Fruits

Fig. 1.6. Studied fruits. Uva-da-serra, pitanga, tomato, lemon and tangerine.

1.2.1. Tomato

Tomato (*Solanum lycopersicum* L.) is consumed fresh or processed in several foodstuffs. It is a known functional food, containing phytochemicals as carotenoids, tocopherols, polyphenols and terpenoids [65]. It is reported that these phytochemicals confers health protective activities, contributing to decrease the occurrence of chronic diseases, as cardiovascular diseases and certain types of cancer [66]. Recently, Sharma (2021) [67] review tomato implications in human health, reporting its antioxidant activity, along with antidiabetic and antiproliferative activity. Calniquer (2021) [68] reported that carotenoids and polyphenols cooperate in balancing UV-induced skin cell damage. Finally, in a review of 174 reported works, Li (2021) [69] conclude that the intake of tomato and lycopene had beneficial health effects.

1.2.2. Tangerine and Lemon

Citrus are one of the world's major fruit crops that are produced in many countries with tropical or subtropical and borderline temperatures [70,71]. *Citrus* is a nutritionally

important genus and a member of the subfamily Aurantioideae in the family Rutaceae.. It is in the *Atalantia* genera that we can find the most known and commercialized *Citrus* fruits, the *Citrus sinensis* (orange), *Citrus reticulata* (tangerine), *Citrus paradise* (grapefruit), *Citrus limon* (lemon), *Citrus aurantifolia* (lime) or *Citrus maxima* (pomelo) [71-73]. Tangerines combine the fresh and acidic notes of other citrus fruits like lemon or lime, with a honey-like sweetness, resulting in unique organoleptic properties that make them very appreciated by consumers [74-76]. Lemon along with orange are the most well-known citrus fruits.

These fruits are very rich in secondary metabolites with high nutraceutical value, such as vitamin C, folate, flavonoids, coumarins, limonoids, terpenoids and carotenoids [77]. Many of the volatiles identified in citrus fruits, as in many other fruits and food products, exhibit different bioactive properties (antioxidant, antidiabetic, antiproliferative, etc) and potential health benefits. Consequently, many of these fruits are considered functional foods with an added value. Adhikari-Devkota (2019) [78] attributed to tangerines an antidiabetic activity, whereas Hamdan (2020) [79] reported its anti-inflammatory activity. Barreca (2020) [80] review citrus bioactivity, related with its antioxidant, anti-inflammatory, antiviral, antimicrobial, and anticancer activities. Oboh (2017) [81] reported the inhibition of enzymes linked to type-2 diabetes and hypertension by essential oils from peels of lemon, while Tejpal (2020) [82] confirmed the angiotensin converting enzyme inhibition by lemon extracts.

1.2.3. Pitanga

The pitangueira (*Eugenia uniflora* L.) is a fruit-bearing tree native from Brazil widely distributed in South American tropical and subtropical regions, which produce small fruits (pitanga) that looks like a small pumpkin of about 3 cm in diameter [83-86]. The pitanga have an exotic and pleasant flavours, with a combination of sweet and sour makes, making them desirable for culinary purposes. In addition, Pitanga is used in popular medicine as a diuretic, anti-rheumatic, anti-febrile, and anti-inflammatory agent and as a therapeutic agent for stomach diseases [83, 85, 87]. These

medicine uses are associated with the bioactive compounds content, namely polyphenols, carotenoids, tannins and vitamin C [83-85, 88]. Lazzarotto-Figueiró (2021) [89] reported that pitanga seeds essential oils have a strong antioxidant activity, and suggest the ability of seeds to reduce cholesterol, prevent cardiovascular diseases and the inflammatory activity. In addition, the oils shown inhibition activity in relation with lactase, sucrase and maltase enzymes. Aranha (2019) [90] reported in vitro antiproliferative potential of essential oils of *Eugenia* spp, whereas Monteiro (2019) [91] showed the antibacterial and anti-inflammatory activity of pitanga, and Sobeh (2019) [92] demonstrated its anti-diabetic activity.

1.2.4. Uveira-da-serra

Vaccinium padifolium Sm. (Uveira-da-Serra), an endemic shrub of Madeira island, have as fruits blueberries, locally known as uva-da-serra. These fruits, although not usually consumed directly, are used in processed foodstuffs [93, 94]. It is reported that intake of berries has the potential to reduce the risk of various chronic disease conditions such as cancer, cardiovascular diseases and respiratory diseases [95, 96]. Ma (2018) [97] reviewed, in comprehensive manuscript databases with papers from 2008–2018, the health role of functional ingredients in blueberry, confirming its anticancer and anti-obesity activities, its prevention against degenerative diseases and its anti-inflammatory protective capacities. Despite the scarcity of scientific work on Uveira-da-serra, Carvalho (2017) [98] reported its antioxidant potential, while Spinola (2018) [99] confirmed that potential in addition to its antidiabetic activity.

1.3. Local agricultural production

Agriculture and local products result from the available resources and the consumer needs. They have a strong social impact, contributing not only to the population's traditions, but also their production and distribution practices are more environmentally friendly and enhance social equity for the community (farmers, producers and consumers) [422]. They are based on circular sustainability models,

where agricultural production is sustainably made, being regulated by the needs of nearby consumers and abiotic conditions [422, 423]. In addition, this kind of agricultural production, contributes for the preservation of biodiversity, where local varieties are not replaced by large-scale production varieties, which leads to an extraordinary loss of genetic diversity 424, 425. Local food products are generally distributed over short distances, increasing the accessibility to fresh products. Therefore, thus might provide health benefits due to their superior nutritional quality. In addition, this local procedure contributes to food and health security, reducing the time and intermediaries between the harvest and the consumer. Cases were also reported where consumers prefers traditional varieties due to their high organoleptic properties, tuned to consumer tastes over time [422, 425].

Due to population growth, it is estimated that agricultural production will have to increase at least by 60% until 2050 [423, 426]. One way to meet this need and to maintain local varieties and not replaced them by large-scale production varieties, is to maximize all the agricultural production obtained, namely, the food waste [424, 423]. These residues from agriculture, like inedible plant tissues such as seeds or peels, are rich in bioactive compounds, with reported antioxidant, anti-inflammatory, cardioprotective and anticancer capacities, among others [423]. These food wastes have potential applications, such as functional foods, nutraceuticals, cosmeceuticals, between others, and as a consequence, re-utilization of these agriculture residues drew attention, for the possibility of converting these unwanted by-products into high-value options, resulting in food waste valorization, along with a new foodstuff solution [423].

Section 2. Aims and Scope

Unhealthy diet is a key modifiable risk factor for noncommunicable diseases, that can lead to death, being estimated that these diseases can be responsible for about 35 million deaths each year. In addition, local cultures are an important source of biodiversity, having also appealing organoleptic qualities for the consumer. The work developed in this thesis project address this concern. In this context we aimed to assess the bioactive potential of selected fruits (including fruit waste), two strategic lines were adopted: *i)* determination of levels of carotenoids and tocopherols and phenolic compounds extracted by LLUSAE, followed by UHPLC; *ii)* determination of levels of tocopherols extracted by QuEChERS followed by LC-ESI/MS/MS; *iii)* establishment of the volatomic profile through HS-SPME and NTME extractions approaches followed by GC-qMS *iv)* After identification of bioactive compounds with health-promoting potential, an evaluation of the bioactive potential of the extracts of selected fruits obtained by LLUSAE was carried out.

The experimental design of developed work is summarized in the Fig. 2.1.:

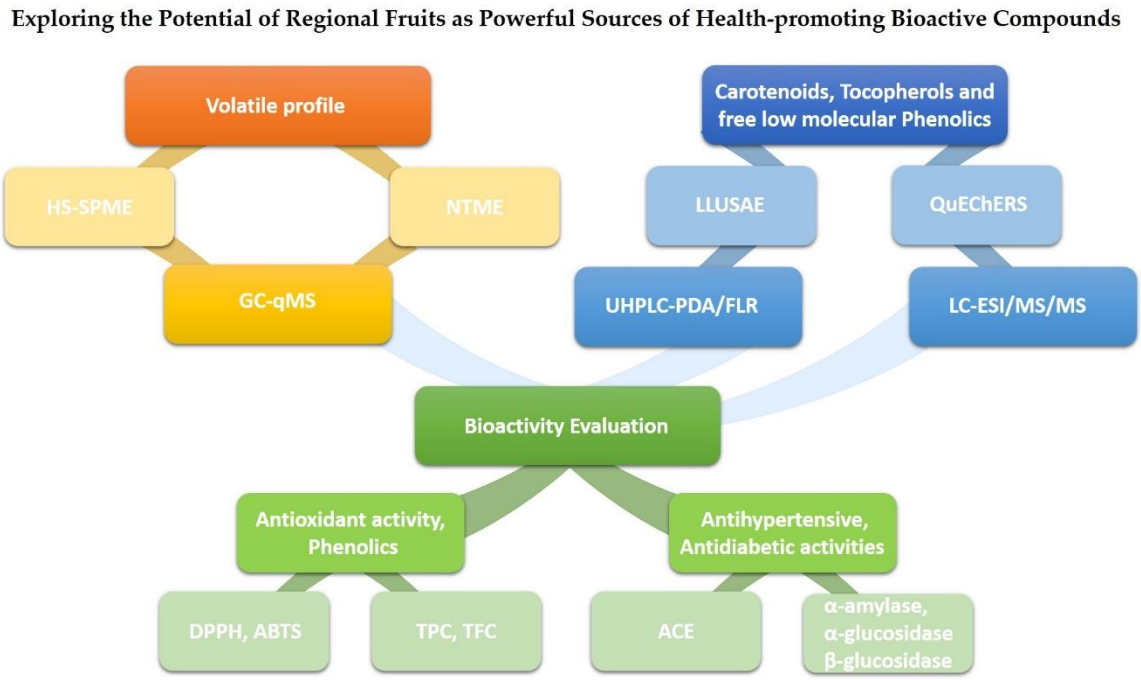


Fig. 2.1. General schematic of the study

The specific objectives of the thesis included:

1. Determination of carotenoids, tocopherols and phenolic compounds in tomato

A new extraction strategy (LLUSAE) was established and validated for the determination of the main carotenoids (lycopene and β-carotene) along with tocopherols (δ-, γ- and α-tocopherol), using UHPLC equipped with PDA system detector.

2. Determination of free low-molecular weight phenolics of *Vaccinium padifolium* Sm fruits

Determination of the phenolics from uva-da-serra, using QuEhERS for its extraction, followed by LC-ESI/MS/MS analysis.

3. Establishment volatomic profile of target fruits

The volatomic profile of *Vaccinium padifolium* Sm fruits and tangerine was achieved by HS-SPME followed by GC-qMS. It was used NTME, a new extraction approach, to define the volatile composition of citrus fruits was optimized and applied to lemon. This approach was used for the first time in food field.

4. Evaluation bioactivity of the selected fruits

Fruits extracts were evaluated in terms of its antioxidant activity, total phenolics, and antidiabetic and antihypertensive activities through different spectrometric assays.

Section 3. Evaluation of fruits as sources of health-promoting bioactive compounds

Sub-Section 3.1. Ultrasound-assisted liquid-liquid extraction followed by ultrahigh pressure liquid chromatography for the quantification of major carotenoids in tomato

Journal of Food Composition and Analysis 57 (2017) 87–93

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original research article

Ultrasound-assisted liquid-liquid extraction followed by ultrahigh pressure liquid chromatography for the quantification of major carotenoids in tomato

José A. Figueira[a], Jorge A.M. Pereira[a], Priscilla Porto-Figueira[a], José S. Câmara[a,b,*]

Published in:

Journal of Food Composition and Analysis (Impact Factor 4.556)

José A. Figueira, Jorge A.M. Pereira, Priscilla Porto-Figueira, José S. Câmara

http://dx.doi.org/10.1016/j.jfca.2016.12.022

Abstract

Lycopene and β-carotene, the main carotenoids present in different tomatoes varieties (gordal, cherry, roma and campari) of *Solanum lycopersicum* L. (*Solanaceae*), were investigated using ultrasound-assisted liquid-liquid extraction (LLUSAE) followed by ultra-high pressure liquid chromatography with PDA detection (UHPLC-PDA) analysis. Experimental parameters influencing the LLUSAE were optimized using a univariate design, resulting in a 30-min ACN/MeOH extraction by sonication of a lyophilized sample, followed by PSA/C18/MgSO4 clean-up and fast centrifugation before UHPLC analysis. Using this greener methodology, high recoveries (above 97%), good linearity (r2 > 0.98) and improved sensitivity, with limits of detection and quantification of 24.0 and 80.0 ng/mL for lycopene and 3.0 and 9.9 ng/mL for β-carotene, respectively, were obtained. This sensitivity is about five times better than previously reported in literature, making LLUSAE/UHPLC-PDA a promising strategy for lycopene and β-carotene quantification in tomato and eventually in other matrices. The carotenoids studied, lycopene and β-carotene, were found at highest concentrations in the gordal tomato variety, followed by cherry, roma and campari (727.1, 342.2, 267.2 and 218.2mg/g and 80.4, 44.0, 45.7 and 44.0mg/g for lycopene and β-carotene, respectively). Additionally, an exponential increase of both carotenoids occurs during ripening and mainly in the skin and locular cavity of the gordal variety. These results provide further evidences of the potential of tomatoes as an interesting source of lycopene and β-carotene.

Keywords: tomato; tomato maturation; *Solanum lycopersicum* L.; lycopene; β-carotene; LLUSAE; UHPLC-PDA; food analysis; food composition

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops worldwide, being included in different diets and consumed fresh or processed in several foodstuffs. A key reason for this popularity is the general awareness that tomato is a functional food, containing different compounds with antioxidant activity, namely carotenoids (as lycopene, β-carotene and lutein), tocopherols, flavonoids and ascorbic acid (reviewed in [65]). In fact, different epidemiologic studies correlate the consumption of tomato and tomato-based products with a reduced risk of chronic diseases, as cardiovascular diseases and certain types of cancer (reviewed in [66]). These protective effects have been extensively attributed to different antioxidants present in tomato, as tocopherols and carotenoids [100, 101]. In particular, there are growing evidence linking lycopene to the protection against prostate cancer (reviewed in [66]).

Carotenoids are the lipophilic terpenoids pigments synthesized during tomato ripening that confer the reddish colour to the fruit, being lycopene the most abundant (more than half of total carotenoids composition in ripe tomato) [102, 103]. These compounds cannot be synthesized by animals, but more than 20 carotenoids, mainly lycopene, β-carotene, α-carotene, lutein and β-cryptoxanthin, have been identified in human plasma and tissues [32]. This indicates that carotenoids are obtained from the diet [77], and tomato and tomato-based food products are important sources, particularly for lycopene [66]. Ultrasound (US), a high frequency pressure wave able to compress and expand the mediums it crosses, is particularly tailored for liquid, liquid-liquid (LL) or liquid-solid extractions of different matrices [104]. The use of US in sample extraction, ultrasound-assisted extraction (USAE), generates bubbles that growth and collapse (ultrasonic cavitation), facilitating, for instance, the destruction of surface materials or the disruption of the cell wall from the sample. In turn, this enhances the penetration of the solvent into the matrix and consequently a better extraction of the target analytes [105]. Accordingly, carotenoids extraction from different fruits and plants has being previously reported using USAE [106], as well as

similar approaches involving supercritical extraction [107] and microwave-assisted extraction [108].

In this work, we report an improved, sensitive and environmentally friendly methodology based on LLUSAE followed by UHPLC-PDA for lycopene and β-carotene quantification in tomatoes from different *S. lycopersicum* varieties. A univariate experimental design involving three independent parameters (extraction solvent, sonication time and clean-up sorbents), was performed to improve the extraction performance. Upon its validation, the methodology was used to assess the target carotenoids in tomatoes from different varieties (*gordal, cherry, roma* and *campari*), maturation stages and fruit sections.

2. Materials and methods

2.1. Reagents and standards

Lycopene (from tomato, 90%), β-carotene (HPLC grade, 95%), hydrochloric acid (HCl, ACS reagent, 37%), graphene oxide (2 mg/mL in H_2O) and carbon nanotubes (Multi-Walled Carbon Nanotubes, MWCNT) were acquired to Sigma-Aldrich (Buchs, Switzerland). Ethanol (EtOH, absolute PA, 99.5%) was acquired to Panreac, (Barcelona, Spain), acetonitrile (ACN) and methanol (MeOH) (both HPLC grade, 99.99%) to Thermo Fisher Scientific (Loughborough, UK) and PSA/C_{18}/MgSO4 (25mg/25mg/150mg, DisQuE) to Waters Corporation (Milford, MA, USA). Lycopene (12.43 μg/mL) and β-carotene (1000 μg/mL) stock solutions were prepared in pure ethanol. All solutions and samples extracts were filtered before use through a 0.20 μm PTFE syringe filter (Millipore Corporation, Bedford, MA, USA), except the UHPLC mobile phases, that were filtered using a 0.20 μm nylon filter (Millipore Corporation, Bedford, MA, USA).

2.2. Tomato samples

Gordal tomatoes (regional variety, 500 g) at different ripening stages (*immature green*, *full mature green*, *breaker* and *ripe*) were collected from different plants of the same

crop grown in green houses (to minimize abiotic interferences) at different time points for 90 days. The samples were frozen under liquid nitrogen, lyophilized (Christ Alpha 1-2 LD plus freeze dryer, Osterode am Harz, Germany), grounded to powder (IKA A11 basic analytical mill, Staufen, Germany) and immediately stored under nitrogen atmosphere at -80º C. The same procedure was applied to the ripe *gordal* tomatoes samples from which the different sections (skin, locular contents, inner and outer pericarps) were obtained, as well as to the *campari*, *cherry* and *roma* ripe whole tomatoes samples imported from mainland and acquired in a local market. Due to the oxygen and light sensitivity of carotenoids, all samples were processed fresh and analysed as fast as possible to minimize degradation. Furthermore, samples were fast frozen in liquid nitrogen before grinding and storage in dark flashes and vials, the procedures to isolate the different tomato sections were performed under dim light, the storage time was limited and whenever possible made in single-use aliquots in dark vials to avoid freeze/thaw cycles. Unless indicated, all procedures were repeated with at least three different samples analysed in triplicate (N=3, n=3)

2.3. Optimization of the experimental factors affecting LLUSAE performance

The starting conditions for the LLUSAE procedure were based in our previous experience with other vegetal matrices and involved 0.050 g of tomato sample diluted in 9 mL extraction solvent (MeOH) and a large excess of extraction solvent volume to tomato sample weight to favour the maximum extraction efficiency. The diluted sample was sonicated (BRANSON 2510E-DTH, 100 W, 40 KHz, Danbury, CT, USA) during 45 min at a 25-30 ºC, a temperature range suggested by [109], and submitted to a 5-min centrifugation step (5000 × g, Espresso Personal microcentrifuge, Thermo Scientific, Waltham, MA, USA). Finally, the supernatant was collected, homogenized in vortex prior to 5 min centrifugation at 5000 × g, cleaned up with 20 mg PSA/mL of tomato extract and filtrated (0.2 μm) before analysis. Then, four experimental parameters, (*i*) sample pre-treatment by freezing

in liquid nitrogen, lyophilisation and grinding to powder; (*ii*) extraction solvent: methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), ACN/MeOH (1:1; 1:4 and 4:1, v/v), ACN/EtOH (1:1) and MeOH/EtOH (1:1, v/v), (*iii*) sonication time (no sonication, 15, 30 and 45 min), and (*iv*) clean-up (20mg sorbent/mL tomato extract added to the sample, vortex homogenization and 5 min centrifugation at 5000 g): primary–secondary amine (PSA), PSA/C_{18}/$MgSO_4$, graphene oxide and multi-walled carbon nanotubes (MWCNT), were optimized following a univariate design to further improve the LL_{USAE}. Overall, the lyophilized diluted tomato samples (0.050 g in 9 mL ACN/MeOH (4:1, v/v), *n*=3) were sonicated for 30 min. Then, 1 mL of the supernatant obtained was subjected to a clean-up (20 mg of PSA/C_{18}/$MgSO_4$ (1:1:6; w/w/w)), filtrated and analysed by UHPLC-PDA. The selection of the best conditions was based on the highest total peak areas obtained for the target analytes. An overview of the whole experimental layout is detailed in Fig. 3.1.1.

2.4. UHPLC-PDA analysis and operating conditions

Lycopene and β-carotene analysis was carried out on the Waters Ultra Pressure Liquid Chromatographic Acquity system (UPLC Acquity H-Class, Waters Corporation, Milford, CT, USA), equipped with a quaternary solvent manager, a sample manager, a column heater and a Photodiode Array (PDA) detector. The whole configuration was driven by Empower software v2.0 from Waters Corporation. Optimum separation was achieved using 500 µL/min of ACN/MeOH (3:1; v/v) for 6 min and 2 min of re-equilibration between injections to avoid any carry-over effect. A maximum back pressure of 3.800 psi (far below the maximum capabilities of the UHPLC) was obtained. The extracts (2 µL) were loaded and separated in the Acquity UPLC BEH C_{18} analytical column (1.7 µm particle size, 2.1 mm × 50 mm, Waters), thermostated at 30º C, while the samples were kept at 20º C in the sample manager. The identification of lycopene and β-carotene in real samples chromatograms was based on the comparison of retention time and spectral characteristics with standards (PDA analysis was performed at 450 nm upon a

spectrum scanning in the 210-500 nm range) and confirmed using the standard addition method, which was also used to quantify the target carotenoids.

2.5. Method validation

LLUSAE/UHPLC-PDA methodology was validated in terms of selectivity, linearity, limits of detection (LOD) and quantification (LOQ), linear dynamic range (LDR), precision, accuracy and matrix effect (ME), according to our previous work [110]. Lycopene and β-carotene selectivity was assessed by the absence of interfering peaks in UV-vis spectra at 450 nm. In turn, linearity was evaluated by external standard addition method, through linear regression of the standards (n=3), using 6 different concentrations (from 0.25 up to 4.0 μg/mL) and applying the least-squares method to obtain the respective correlation coefficient (r^2). Sensitivity was assessed through LOD determination (the lowest analyte concentration that produces a response detectable above the noise level of the system) and LOQ (the lowest level of analyte that can be accurately and precisely measured), obtained from the linear regression. LOD was defined as "$a + 3S_{a/b}$" and LOQ as "$a + 10S_{a/b}$", where "a" represents origin ordinate, "S_a" the origin ordinate variance and "b" the slope. Precision is a function of concentration and it was calculated by dividing the standard deviation (SD) by the concentration means to obtain the coefficient of variation (CV). In turn, when CV is expressed on a percentage basis, gives the relative standard deviations (RSD). To assess the precision of the method developed, three concentrations, at low level (LL), medium level (ML) and high level (HL) (0.6, 1.0 and 2.0 μg/mL for lycopene, and 0.5, 1.0 and 3.0 μg/mL for β-carotene, respectively, see Table 3.1.1), were evaluated four times (n=4). Four trials were performed in the same day and in non-consecutive days to obtain the intra-day precision (method repeatability) and inter-day precision (method reproducibility), respectively. Accuracy was evaluated through a recovery study and expressed as recovery percentage (R%) according to the formula "$\% R = 100 \times [(S_F - S) / Std]$", where "$S_F$" represents concentration of target analytes in fortified sample, "S" represents the concentration of target analytes in sample, and

"Std" represents the concentration of target analytes added to sample. Three different standard concentrations levels corresponding to the LL, ML and HL were evaluated (n=3) in S_F and Std. The matrix effect (ME), which is the effect on an analytical method caused by all other components of the sample, was determined using the formula "%ME= 100 × (m_{Sol}/m_{FS})", where "m_{Sol}" represents the slope of standards linear regression and "m_{FS}" the slope of fortified sample linear regression.

3. Results and Discussion

To implement an efficient and sensitive methodology for the quantification of lycopene and β-carotene , the main carotenoids present in tomatoes, a LLUSAE/UHPLC-PDA analytical approach was developed to analyse tomato samples under several conditions, as detailed in sections 2.3 and 2.4 and synthetized in Fig. 3.1.1.

3.1. Optimization of the LL$_{USAE}$ procedure

3.1.1. Sample pre-treatment

According to our previous experience with the extraction of tomato samples [111], the sample pre-treatment was a crucial step to maximize carotenoids extraction. Furthermore, tomato peels and seeds are rich in the target analytes, but their extraction from these vegetable structures is hard to achieve using conventional approaches. We observed that just chopping the tomato samples in small fragments and then crushing and grinding them into a fine paste in a mortar was not a very effective extraction as a significant number of seeds and peel fragments remain intact. To avoid this samples were frozen in liquid nitrogen, lyophilized and reduced to powder using an analytical mill before extraction. This procedure is much more efficient as no more intact peels and seeds fragments were observed. In fact, as shown in Fig. 3.1.2A, the sample pre-treatment described allowed a huge increase in the extraction efficiency of the target analytes.

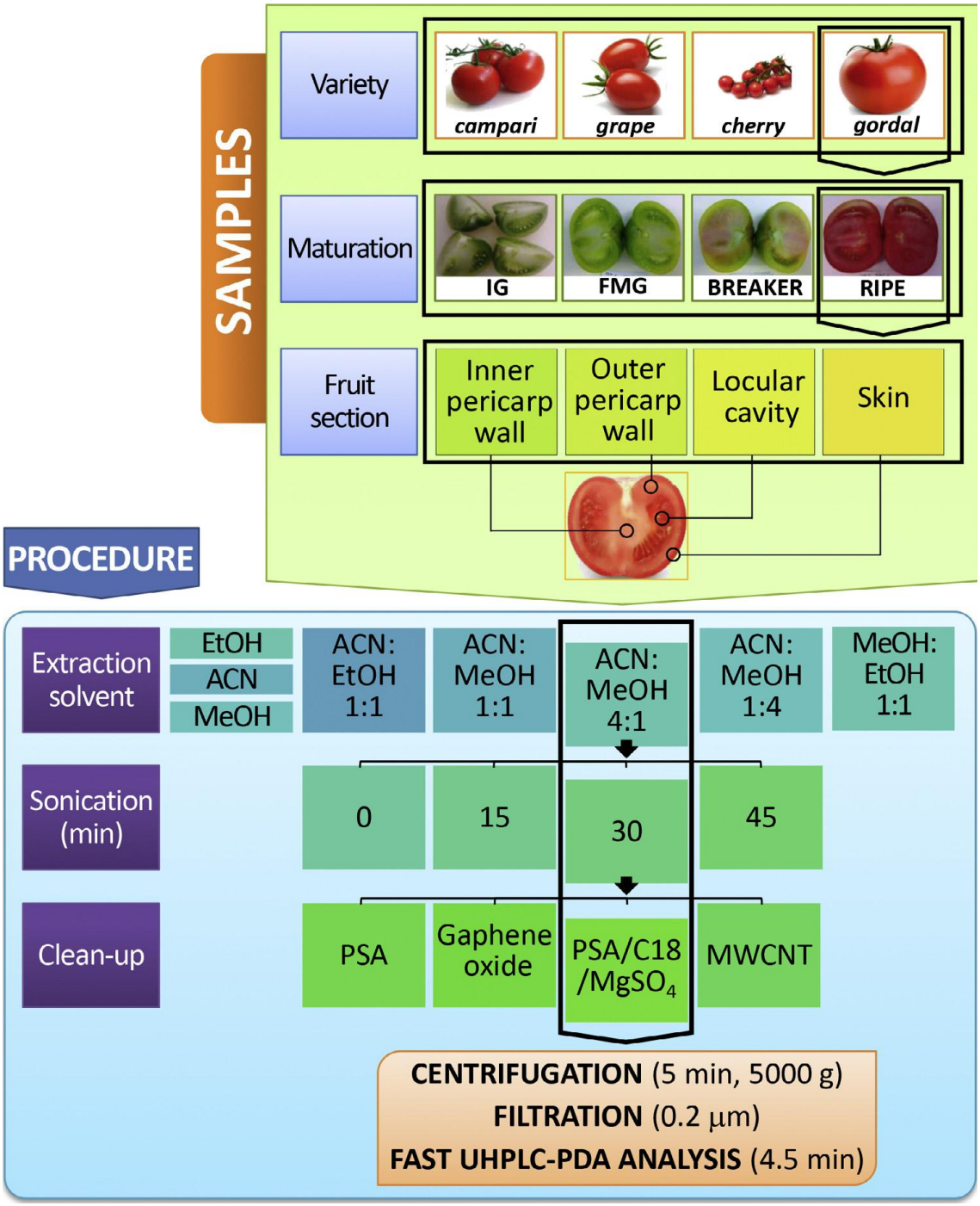


Fig. 3.1.1. Overview of the experimental layout followed in this work.

3.1.2. Extraction solvent

To select the best extraction solvent, the organic solvents ACN, MeOH, EtOH and different combinations of those (described in the section 2.3) were assayed. As shown in Fig. 3.1.2B, the best results were obtained with ACN/EtOH (1:1; v/v) and ACN/MeOH (4:1, v/v) (columns 7 and 8 in Fig. 3.1.2B, respectively). This second

condition was selected to minimize solvent variations in the following chromatographic separation using ACN and MeOH as mobile phases.

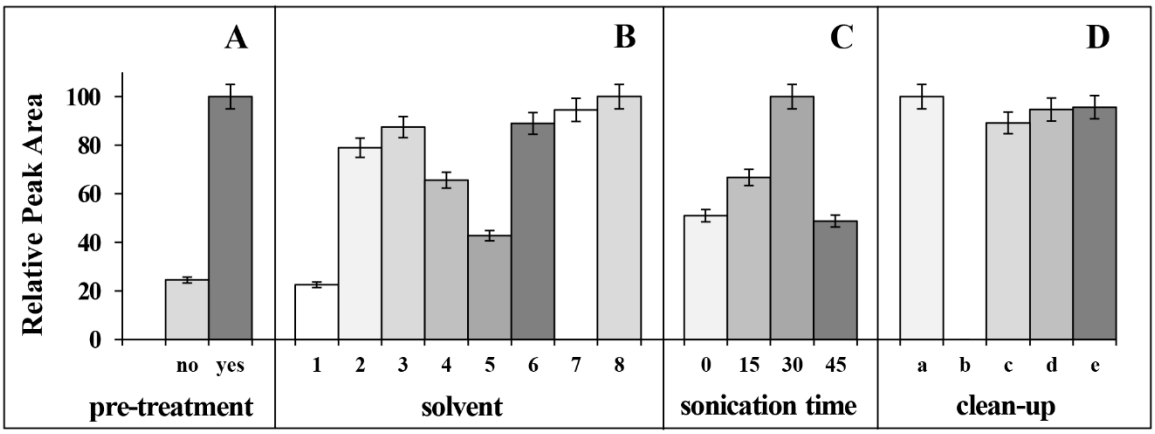


Fig. 3.1.2. Optimization of LL_{USAE} extraction: pre-treatment (A), solvent (B), sonication time (min) (C) and clean-up sorbents (D). Legend: 1–methanol (MeOH), 2–acetonitrile (ACN), 3–ethanol (EtOH), 4 – MeOH:EtOH (1:1, v/v), 5–ACN:MeOH (1:4, v/v), 6–ACN:MeOH (1:1, v/v), 7–ACN:MeOH (4:1, v/v), 8–ACN:EtOH (1:1, v/v), a – Sample, b – Sample + MWCNT, c – Sample + PSA, d – Sample + Graphene oxide, e – Sample + PSA/C_{18}/$MgSO_4$. For each parameter, the best condition (higher area) was set to 100% and remain conditions expressed as relative areas of the former.

3.1.3. Sonication time

The initial condition for the sonication time was 45 min. Longer times of operation would constitute a bottleneck in the experimental layout as sample deterioration could occur and the cumulative extraction time by sample would be very high. Therefore, we have assayed 0, 15, 30 and 45 min of sonication. The results obtained show that the optimum condition is 30 min (Fig. 3.1.2C). Moreover, the amount of the target analytes detected with 45 min of sonication is equivalent to the control without sonication, suggesting that carotenoid deterioration is occurring upon 30 min of sonication.

3.1.4. Sample clean-up

Following the extraction, different sample clean-up procedures were assayed to discard interfering compounds and simplify the tomato matrix. Additionally, this procedure allows a significant noise reduction in the baseline of the chromatographic separation (data not shown) and consequently better analytical performance can be attained. The clean-up options assayed were PSA, graphene oxide, PSA/C_{18}/$MgSO_4$ and MWCNT. As shown in Fig. 3.1.2D, except for MWCNT (column b) that completely deplete the target carotenoids from the tomato extract (in agreement with previous reports [112]), sample clean-up using PSA, graphene oxide or PSA/C_{18}/$MgSO_4$ (Fig. 3.1.2D, columns c, d and e, respectively) involve minimum lycopene and β-carotene losses. PSA/C_{18}/$MgSO_4$ (column e) was the selected option because it allows a consistent improvement of the baseline chromatographic separation (data not shown). Additionally, it is commercialised in a SPE-like pre-loaded format that minimize the user intervention, thus allowing faster and more reliable experimental procedures.

3.2. Method validation

Following the LLUSAE/UHPLC-PDA optimization, the methodology was validated for the determination of lycopene and β-carotene in tomato. The selectivity of the method was demonstrated by the absence of any signal at the expected retention times for the specific wavelength of lycopene and β-carotene (Fig. 3.1.3). Linearity was evaluated through external standard addition method, applying the least-squares method to obtain the respective correlation coefficient ($r^2 > 0.985$), with a LDR from 0.25 to 2.0 μg/mL for lycopene, and 0.25 to 4.0 μg/mL for β-carotene. The LODs and LOQs were calculated from ordinary least squares regression data. As can be verified in Table 3.1.1, very low LODs and LOQs (24.0 and 80.0 ng/mL for lycopene and 3.0 and 9.9 ng/mL for β-carotene, respectively) were obtained. These values are about five times lower than previously reported for tomato samples [113, 114],

or other matrices, as serum [115]. This makes LLUSAE/UHPLC-PDA a powerful strategy for lycopene and β-carotene quantification.

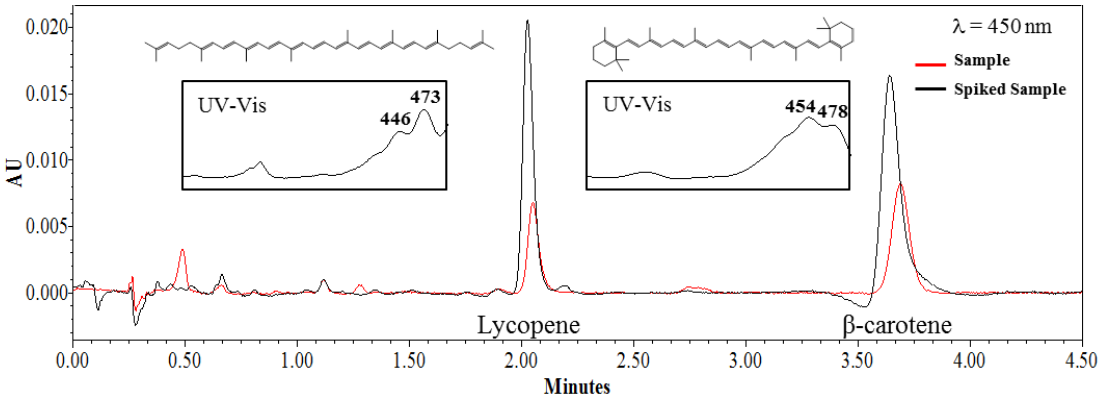


Fig. 3.1.3. Evaluation of the method selectivity for lycopene and β-carotene upon fortification of a tomato sample with lycopene and β-carotene standards. The specific retention time and PDA spectrum for both carotenoids were also matched with pure standards solutions.

For the precision assessment, three concentrations (LL, ML and HL, see Table 3.1.1) were evaluated ($n = 4$) and the RSD, intra-day precision (repeatability) and inter-day precision (reproducibility) determined. The precision results obtained are presented in Table 3.1.1 and range from 0.1 % (β-carotene) to 1.9 % (lycopene). As expected, repeatability is lower than reproducibility for both carotenoids. The accuracy of the method was assessed through a recovery study. This involved spiking tomato samples at three concentration levels (LL, ML and HL), with known amounts of each carotenoid (see Table 3.1.1). The recovery percentages, ranging between 97.1±3.8 % (lycopene) and 108.3±9.9 % (β-carotene), were determined using the equation presented in Section 2.6. These values are within the tolerance range (80 to 120%) [116], and in agreement with matrix effect results obtained (98.5 % and 98.6 % for lycopene and β-carotene, respectively).

Table 3.1.1. Figures of merit of the proposed LL_{USAE}/UHPLC-PDA methodology.

Carotenoids	Retention Time	Linearity		Sensitivity		Spiking Levels	Precision (%)		Recovery	ME
(λ_{max}=450 nm)	(min)	LDR (µg/mL)	r^2	LOD (ng/mL)	LOQ (ng/mL)	(µg/mL)	Intra-day	Inter-day	(%)	(%)
Lycopene	20	0.25 – 2.0	0.9911	24.0	80.0	0.6 (LL)	0.6	1.9	92.8	985
						1.0 (ML)	0.5	1.0	99.4	
						2.0 (HL)	0.2	0.5	99.2	
						Average	**0.4**	**1.2**	**97.1**	
β-Carotene	3.6	0.25 – 4.0	0.9854	3.0	9.9	0.5 (LL)	0.6	1.8	118.5	986
						1.0 (ML)	0.9	0.5	107.7	
						3.0 (HL)	0.1	0.4	98.7	
						Average	**0.5**	**0.9**	**108.3**	

LDR - linear dynamic range

r^2 - correlation coefficient

LOD - limits of detection

LOQ - limits of quantification

ME - matrix effect

LL - low level

ML - medium level

HL - high level

The methodology here validated for the analysis of lycopene and β-carotene presents several advantages when compared to other methods reported so far. Firstly, the sample pre-treatment by freezing with liquid nitrogen, lyophilisation and grinding to powder allows a more efficient extraction of the target analytes. Particularly, the levels of lycopene obtained are very high and only observed in enzyme-added extractions [117]. Then, the use of ACN/MeOH (4:1, v/v), which are less expensive and less toxic extraction solvents than the often-used hexane, makes the methodology cheaper and more environmentally friendly. Furthermore, the experimental layout is very simple and straightforward, and although a 30 min US step is involved in the sample extraction, it is followed by a fast-chromatographic analysis (4 min) using a very low sample injection volume (2 µL). Overall, excellent LODs and LOQs were obtained, highlighting a detection capacity about 30 times

lower than other methods using UV detection and similar to the ones involving expensive mass detection [118]. A detailed comparison between different chromatographic methodologies reported so far for lycopene and β-carotene quantification can be further appreciated in the Supplementary Table 3.1.1.

3.3. Lycopene and β-carotene determination in tomato samples

To verify the efficiency of the methodology developed, the selected carotenoids were quantified in tomato samples from *gordal* variety in four ripening stages, *immature green*, *full growth*, *breaker* and *ripe* (Fig. 3.1.4A).

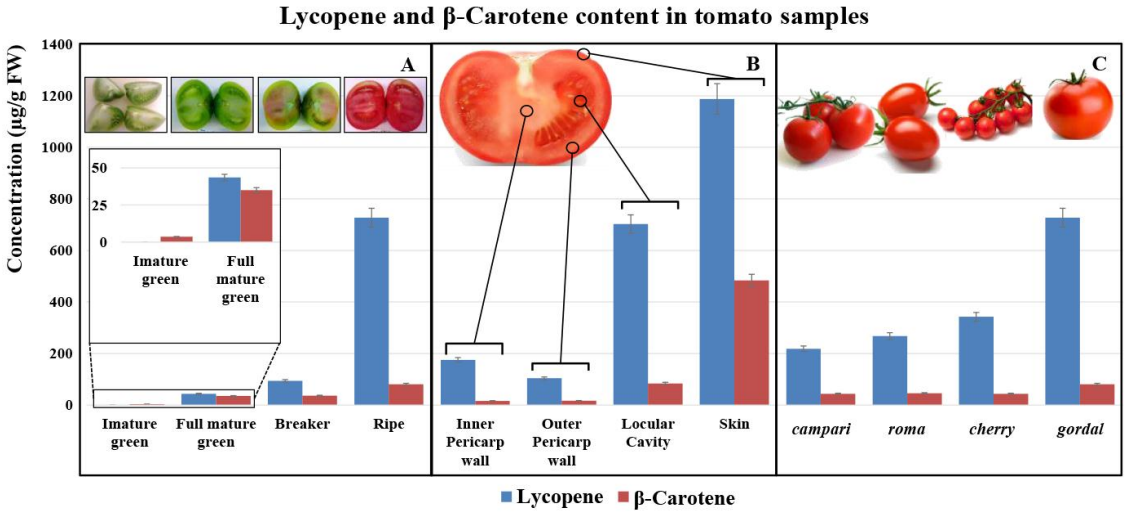


Fig. 3.1.4. Variation of lycopene and β-carotene levels in tomato according to the fruit ripening (A), section (B) and variety (C). Details about the preparation of the samples can be found in the Sampling subsection of Material and methods.

As can be observed, lycopene and β-carotene concentrations increase during maturation, reaching their maximum at the ripe stage. Nevertheless, lycopene variation is much more pronounced than β-carotene, being absent in the immature green stage and increasing about seven times from the breaker to ripe stages up to a final concentration around 700 µg/g of tomato. In contrast, maximum β-carotene levels fall below 100 µg/g of tomato. These carotenoid levels, particularly those referring to lycopene, are very high and only comparable to enzyme-added

extraction procedures [117]. Remarkably, the lycopene levels we have found in ripe *gordal* tomatoes are four times higher than those reported for three high-lycopene (HLY 13, HLY 18 and Lyco 2) tomato cultivars [119]. Nevertheless, the absolute levels of lycopene and β-carotene here reported are hardly compared with other methodologies because the experimental layouts used in those works could be not very efficient in carotenoids extraction, therefore affecting the final carotenoids quantification. Moreover, lycopene and β-carotene concentrations in tomato are also highly dependent of the variety and abiotic conditions where the fruits grow [119-124]. There are evidences that annual weather fluctuations are an important factor affecting anthocyanin and carotenoid composition and content. Furthermore, lower concentrations of lycopene were reported for table varieties grown in green houses than tomato varieties grown on open field [125]. A more detailed comparison of the abundances in lycopene and β-carotene reported in different matrices, particularly different tomato cultivars grown worldwide, can be found in the Supplementary Table 3.1.1. Overall, and despite these differences in concentration, the variations in lycopene and β-carotene levels during tomato maturation are in agreement with other reports, particularly the exponential increase in the lycopene levels that occurs in the transition from the breaker to the fully ripe state [114, 119, 126-128], and correlate with the carotenoids biosynthesis kinetics. In this sense, during ripening, the chlorophylls green colour of the immature (green) tomatoes is gradually substituted by the reddish of carotenoids as the firsts are reduced and degraded (changing chloroplasts colour from green to white), while carotenoid synthesis is triggered by lycopene [129]. In the immature green stage, lycopene production is very low and this precursor is not detected because it is readily convert to α- and β-carotene (Fig. 3.1.4A). However, as ripening progress, lycopene production bursts, leading to its accumulation to much higher levels than β-carotene [114, 130]. Lycopene and β-carotene distribution in the fruit, however, is not uniform. Using ripe *gordal* tomatoes, we could observe that the selected carotenoids are quite more abundant in the skin, followed by the locular cavity, being much less represented in the pericarp (inner

and outer walls, Fig. 3.1.4B). These results support the statement that peeling tomatoes, a commonly practice both at home and processing industry, strongly affect lycopene and β-carotene levels (above 70% and 50% decrease, respectively, according to [131]). As previously referred, the tomato variety also affects very significantly the levels of carotenoids the fruit can accumulate, and this was also observed in this work. In fact, lycopene and β-carotene levels present considerable differences between the four varieties analysed, *gordal* (local crop), and *campari*, *roma* and *cherry* (imported from mainland). As can be observed in Fig. 3.1.4C, *gordal* contains much higher concentrations (almost doubling) of the selected carotenoids. In fact, as far we may know, not only this is the first study using *gordal* tomatoes, as the carotenoids abundance they exhibit, particularly of lycopene, is very high (further details can be found in the Supplementary Table 3.1.1). For the future, it will be very relevant to study with more detail this *gordal* variety, particularly in what concerns to the levels of carotenoids we detected.

4. Conclusions

A sensitive and efficient methodology involving a LLUSAE followed by a fast UHPLC-PDA analysis has been developed to assess lycopene and β-carotene in tomatoes. Although the methodology developed does not discriminate the different isomers of the referred carotenoids, it is suitable for naturally occurring isomers (about 90% predominance), and it is much more environmentally friendly as the extraction step is carried out using ACN and MeOH rather than toxic solvents, such as hexane. Furthermore, this was achieved without compromising the analytical performance of the methodology, which is comparable to the one obtained with mass spectrometry detection. The method developed was successfully applied to tomato samples in different ripening stages, fruit sections and varieties, unveiling the higher abundance of lycopene and β-carotene in the *gordal* variety which is above the other tomato varieties analysed or any other reported so far.

Sub-Section 3.2. Quantification of δ-, γ- and α-tocopherol in tomatoes using an improved liquid-dispersive solid-phase extraction combined with ultrahigh pressure liquid chromatography

Food Anal. Methods (2017) 10:2507–2517
DOI 10.1007/s12161-017-0799-0

Quantification of δ-, γ- and α-Tocopherol in Tomatoes Using an Improved Liquid-Dispersive Solid-Phase Extraction Combined with Ultrahigh Pressure Liquid Chromatography

José A. Figueira [1] · Jorge A. M. Pereira [1] · José S. Câmara [1,2]

Published in:

Food Analytical Methods (Impact Factor 2.667)

José A. Figueira, Jorge A.M. Pereira, José S. Câmara

http://dx.doi.org/ 10.1007/s12161-017-0799-0

Abstract

Tomato (*Solanum lycopersicum* L.) consumption has been correlated with a lower incidence of cardiovascular diseases and cancer. This protective effect has been ascribed to different bioactive compounds present in this fruit. Therefore, to gain insights on the potential of *S. lycopersicum* L. as bioactive food, a fast and sensitive methodology, based on liquid–liquid extraction (LLE), dispersive solid phase extraction (dSPE) followed by ultrahigh pressure liquid chromatography (UHPLC-FLR) analysis, was developed and validated to quantify δ-, γ- and α-tocopherol in tomatoes. Upon the optimization of different parameters, a fast extraction and separation, and simultaneously, increased resolution and sensitivity was attained. The methodology was validated, retrieving better analytical performance than most methods reported so far. This included good linearity, (r2 > 0.99) and precision (<6.4%), high recoveries (>79.5%) and improved limits of detection and quantification (LODs of 2.15, 5.52 and 1.67 ng/mL and LOQs of 7.18, 18.40 and 5.58 ng/mL, for δ- γ- and α-tocopherol, respectively). These limits are about 1000 times lower than those reported in literature. Furthermore, as far we are aware, this is the first time δ-tocopherol presence in tomato is fully characterized and quantified. The methodology was applied to different tomato varieties, ripening stages and fruit sections, revealing high levels of δ-tocopherol that increase along fruit ripening, while the α-tocopherol follows the inverse trend. Moreover, δ-tocopherol is almost fully concentrated in the seeds and skin of ripe tomato. Finally, ORAC and DPPH assays revealed that the selected tocopherols contribute to approximately half of tomato total antioxidant capacity.

Keywords: LLE-dSPE. UHPLC-FLR. δ- γ- and α–tocopherols. Method validation. *Solanum lycopersicum* L

1. Introduction

Originally from the Andean region, tomatoes (*Solanum lycopersicum* L.) arrived in Europe around the 15th century, being nowadays one of the most popular and extensively consumed vegetable crops worldwide [132, 133]. This fruit presents a high water content and up to 10% of dry matter and organic acids (mainly citric acid and malic acid) [111, 134]. Nevertheless, the most interesting constituents of tomato are the bioactive compounds, as tocopherols, carotenes, lycopene, ascorbic acid, chlorogenic and gallic acids (phenolic acids), and the flavonoids quercetin, kaempferol, rutin, myricetin and naringenin [135, 136]. All these compounds have been widely associated with additional protection against different diseases, namely cancer and cardiovascular diseases [137-139]. Tomato is therefore regarded as a functional food, being an important constituent of different diets across the planet, notably the Mediterranean diet. Tocopherols (α, β, γ and δ isoforms, differing in the number and position of alkyl groups) and tocotrienols (also α, β, γ and δ isoforms, differing from tocopherols in the unsaturated side chains) (Fig. 3.2.1.) are important naturally occurring plant antioxidants [140]. These compounds constitute the forms of vitamin E characterized in 1922 by Evans and Bishop [35, 141] and are considered the most important lipid soluble antioxidants in our organism [142].

The α-tocopherol is the most bioactive of the tocopherols isoforms, being widely distributed in plant tissues, while δ-tocopherol is much less abundant and simultaneously the less bioactive isoform [143, 144]. Nonetheless, γ- and δ-tocopherol have been suggested to have stronger anti-inflammatory activity than α-tocopherol [145, 146], and have shown greater ability to reduce inflammation, cell proliferation, and tumour burden [145, 147]. Considering specifically the fruit, vitamin E activity is usually assessed by the levels of α-tocopherol, which is reported to be mainly found in the seeds [148] and is comparable to β-carotene, another important dietary antioxidant (up to 1.8 mg / 100 g FW) [120, 133, 149]. The vitamin E activity of tocopherols, however, is not limited to their antioxidant capacity which lies in ability to donate phenolic

hydrogens [143, 146, 150]. Instead, also include the regulation of the activity of important

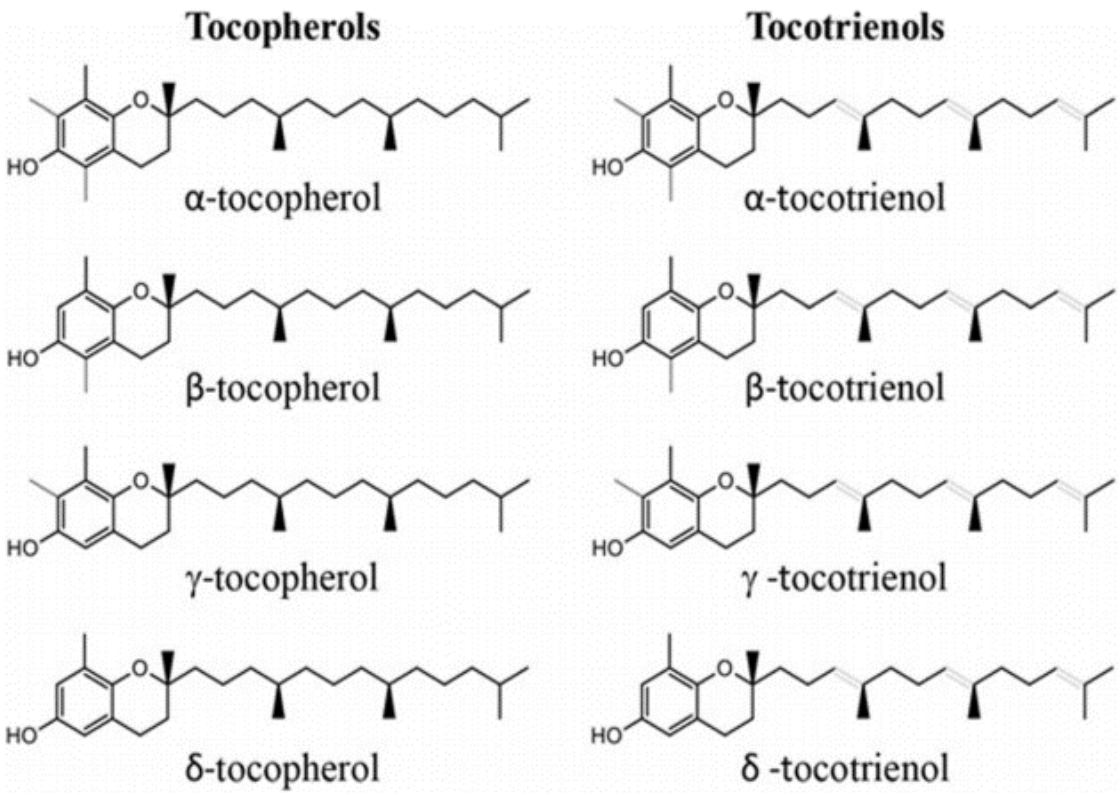


Fig. 3.2.1. Tocopherols and tocotrienols structures

enzymes, as the inhibition of cyclooxygenase-2 and 5-lipoxygenase (involved in the synthesis of inflammatory mediators such as prostaglandin E2 and leukotriene B4) and SR-A and CD36 (inhibits the uptake of oxidised LDL into monocyte-derived macrophages) [143]. Moreover, tocopherols have been associated to the inhibition of monocyte-endothelial cell adhesion and platelet adhesion and aggregation, as well as to the modulation of gene expression and cellular signalling [143, 151-153]. The evaluation of the total antioxidant capacity (TAC) of a certain bioactive compound can be obtained through different assays, being the oxygen radical absorbance capacity (ORAC) [154] and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays [155-157] often used.

In this work, we report a noteworthy improved, fast and reliable methodology based on LLE-dSPE followed by UHPLC-FLR analysis for quantification of δ- γ- and α-

tocopherol in tomato fruits from *Solanum lycopersicum* L. species. A univariate experimental design involving independent variables, extraction solvent and clean-up sorbents, was performed and used to investigate the effects of experimental variables on the extraction performance. The analytical performance of the proposed LLS-dSPE/UHPLC-FLR was evaluated in terms of selectivity, linear dynamic range, LOD, LOQ, precision, accuracy and uncertainty. The antioxidant profiles of four *S. lycopersicum L.* varieties were evaluated by using DPPH and TBARS assays.

2. Materials and Methods

2.1. Reagents, standards and materials

The tocopherols (α- and γ-tocopherol, HPLC grade 96%, and δ- tocopherol, 90%) were purchased to Sigma-Aldrich (St. Louis, MO, USA). Ethanol (absolute PA, 99.5%) was acquired to Panreac (Valencia, Spain), and acetonitrile (ACN) and methanol (MeOH) (both HPLC grade, 99.99%) Thermo Fisher Scientific (Leicestershire, UK). The clean-up salts multi-walled carbon nanotubes (MWCNT), Primary Secondary Amine (PSA), graphene oxide and PSA/C18/$MgSO_4$ (25mg /25mg/150mg, DisQuE) were purchased to Waters (Milford, MA, USA).

2.2. Tomato samples

Gordal tomatoes variety (regional variety, 1500 g) at different ripening stages (full mature green -FMG, breaker and ripe) were collected from different plants of the same crop at different time points (during 90 days), while *campari, cherry* and *roma* samples (200 g) were imported from mainland and acquired in the local market. The samples were lyophilized (Christ Alpha 1-2 LD plus freeze dryer, Osterode am Harz, Germany), grounded to powder (IKA A11 basic analytical mill, Staufen, Germany) and immediately stored under nitrogen at -80º C, in several aliquots, which were used only once to prevent sample degradation.

2.3. Optimization of experimental factors affecting LLE-dSPE performance

Different parameters affecting the efficiency of the extraction procedure were tested and optimized. This included the (*i*) extraction solvent (MeOH, ethanol (EtOH), ACN, ACN/MeOH 4:1 and MeOH/EtOH 4:1), and (*ii*) clean-up salts (PSA, graphene oxide, MWCNT and PSA/C18/MgSO4). The selection of the best conditions was based in the highest total peak areas for the target analytes and and resolution

2.4. LLE-dSPE procedure

Upon the tomato samples processing described above, sample aliquots of 0.50 g were diluted (1:10) with 5 mL of ACN: MeOH (4:1, v/v) and vortexed during 1 min to homogenise. Then, 1 mL of the extract was collected to eppendorfs (*n*=3), mixed with 20 mg of PSA/C18/$MgSO_4$ (1:1:6; w, w, w) and submitted to centrifugation (5000 g, Espresso Personal microcentrifuge, Thermo Fisher Scientific (Leicestershire, UK) for 5 min. The supernatant was collected and evaporated (Heidolph Collegiate, Schwabach, Germany) to dryness and the residue reconstituted in 500 µL of initial mobile phase. After filtration over a PTFE syringe filter (0.20 µm; 13 mm, Millipore Corporation, Bedford, USA), the extract was collected in a 200-µL insert and placed into a LC amber glass vials for further UHPLC-FLR analysis.

2.5. UHPLC-FLR analysis and operating conditions

Analysis of tocopherols was carried out on a Waters Ultra Pressure Liquid Chromatographic Acquity system (UPLC, Acquity H-Class) combined with a Waters Acquity quaternary solvent manager (QSM), an Acquity sample manager (SM), a column heater, and a FLR detector. The whole configuration was driven by Empower software v2.0 from Waters (Milford, MA, USA). Optimum separation was achieved with a binary mobile phase composed by (A) ACN and (B) MeOH, with a constant flow rate of 500 µL min^{-1} and the gradient conditions: 75% A until 1 min, increasing to 78% A (3 min), continuing up to 4 min, returning to 75% A (5 min),

remaining until the end of the run. A re-equilibration time of 2 min regenerate the column to the initial conditions after each analysis was used. Overall, during the 8 min run, a maximum back pressure of 3.800 psi was reached, which is within the capabilities of the UHPLC. The samples were kept at 20º C in the SM and 2 µL injected in the thermostated (30º C) Acquity UPLC BEH C18 analytical column (1.7 µm particle size, 2.1 mm × 50 mm, Waters, Milford, MA, USA USA). For quantification purposes the FLR detection was conducted by using a channel with λ_{Exc} = 296 nm and λ_{Em} = 330 nm. The identification of tocopherols in real samples chromatograms was based on the comparison of retention time and spectral characteristics with standards and confirmed using the standard addition method. Quantification was also based on the standard addition method.

2.6. Method validation

After the sample extraction optimization, the performance of the proposed LLE/UHPLC-FLR approach was assessed by studying the selectivity, linearity, limits of detection (LOD) and quantification (LOQ), linear dynamic range (LDR), precision, accuracy and matrix effect. The selectivity of the method for tocopherols was assessed by the absence of interfering peaks in fluorescence spectra with λ_{Exc} = 296 nm and λ_{Em} = 330 nm. Linearity was evaluated using the external standard addition method, through analytes standards linear regression (n=3). This involved 8 different concentrations and the least-squares method to obtain the respective correlation coefficient (r^2). Sensitivity of the method was assessed through determination of the LOD (the lowest analyte concentration that produces a response detectable above the noise level of the system) and LOQ (the lowest level of analyte that can be accurately and precisely measured), obtained from the linear regression, being LOD defined as ($a + 3S_{a/b}$) and LOQ as ($a + 10S_{a/b}$), where "a" represents origin ordinate, "S_a" the origin ordinate variance and "b" the slope. Precision is a function of concentration and it was calculated by dividing the standard deviation (SD) by the means of concentration to obtain the coefficient of

variation, which when expressed on a percentage basis gives the relative standard deviations (RSD). For method precision assessment, 3 concentrations, low level (LL), medium level (ML) and high level (HL), were evaluated four times (n=4). Four trials were executed in the same day, resulting in intra-day precision which retrieved the repeatability. Other four trials were executed in non-consecutive days, resulting in inter-day precision, retrieving the reproducibility. Accuracy was evaluated through a recovery study and expressed as recovery percentage (R%) according to the following formula: $\% R = 100 \times [(S_F - S) / Std]$, where "$S_F$" represents concentration of target analytes in fortified sample, "S" represents the concentration of target analytes in sample, and "Std" represents the concentration of target analytes added to sample. Three different standard concentrations levels corresponding to the LL, ML and HL were evaluated (n=3) in S_F and Std. Matrix effect (ME) is the effect on an analytical method caused by all other components of the sample, and was determinated according to the formula: $\% ME = 100 \times (m_{Sol}/m_{FS})$, where "$m_{Sol}$" represents the slope of standards linear regression and "m_{FS}" the slope of fortified sample linear regression.

2.7. Total antioxidant capacity (TAC)

Tomato TAC determination was performed using the ORAC and DPPH assays. The ORAC assay measures the oxidative degradation of a florescent probe, fluorescein, by a peroxyl radicals ($ROO^{\bullet}$) generator, as the azo-initiator 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH). This degradation is obviously affected by the quenching ability of the sample extract being measured, allowing its TAC determination. The methodology here used was adapted from [158]. Briefly, 25 µL of sample (diluted 1000 times) were added to 150 µL of fluorescein solution (40.0 nM), incubated at 37º C during 30 min and added 25 µL AAPH (153.0 mM). The values of fluorescence ($\lambda_{Exc.}$ 485 nm, $\lambda_{Em.}$ 520 nm) were subsequently determined every 90 s, for about one hour through Victor3 Multilabel Plate Counter 1420 fluorescence reader (Perkin Elmer, Waltham, USA). Instead of the 25 µL sample, it

was used 25 μL of 10 mM phosphate buffer at pH 7.4 for the reaction control or different trolox solutions (ranging from 1 to 60 μM) to obtain the standards linear regression. The blank was prepared using only 200 μL of phosphate buffer. The results were expressed in mM Trolox / 100g FW.

The DPPH methodology relies in the scavenging ability of the antioxidants present in the matrix being assayed against the free radical DPPH. This compound has deep violet colour (maximum absortion around 515 nm in alcoholic solution) that is loss upon its reduction [155-157, 159]. The DPPH assays here used were adapted from Xie [155] with minor differences. Briefly, 10 mg of DPPH were dissolved in 250 mL of MeOH and rest overnight (DPPH stock solution). Then 500 μL of sample extracts (diluted ten times) were mixed in 1000 μl DPPH stock solution and rest 10 min in the dark. Finally, the absorbance was taken at 515 nm using a UV-Vis spectrophotometer (UV-Vis LAMBDA 25, Perkin Elmer, Waltham, USA). The blank assays were prepared using MeOH instead of sample extract. The DPPH % inhibition was obtained using the formula $((A_{Ctr}-A_S)/A_{Ctr}) \times 100$, where A_{Ctr} is the absorbance of the control reaction and A_S is the absorbance of the samples extracts or standards used, as described by Okoh [159].

3. Results and discussion

To implement a fast and sensitive method for the quantification of tocopherols, a LLE approach was developed, optimized and combined with a fast UHPLC-FLR analysis.

3.1. Optimization of the LLE procedure

LLE optimization involved the selection of the best extraction solvent time and sample extracts clean-up.

3.1.1. Extraction solvent

To select the best extraction solvent, ACN, MeOH and different ratios between these two solvents (4:1; 1:1 and 1:4, v/v) were tested and compared. As shown in Fig.

3.2.2A, although the best results are obtained with MeOH, there isn't a significant difference for the other conditions assayed and so ACN/MeOH (4:1; v/v) was selected to match the conditions used in the following chromatographic separation. In addition, MeOH extraction is very broad, extracting many interferents [160], while the selected ACN:MeOH mixture promotes protein precipitation [161], allowing the obtention of cleaner extracts (data not shown).

3.1.2. Sample clean-up

To simplify even more the extracts composition before the chromatographic separation, discarding part of the interferents that could affect tocopherols analysis and quantification, different sorbents, namely MWCNT, PSA, graphene oxide and PSA/C18/$MgSO_4$ were used. As shown in Fig. 3.2.2B, this procedure did not affect tocopherols extraction, with exception of MWCNT, which shows a very significant retention of the target analytes. Therefore, the selection of the best clean-up sorbent was made between PSA, graphene oxide and the PSA/C18/$MgSO_4$ mixture. It was selected the last option due to the cleaner extracts it produces (observed by the lower noise signals in the chromatographic separations, data not shown).

3.2. Method validation

The optimized LLE-dSPE/UHPLC-FLR was validated for the determination of δ-, γ- and α-tocopherol using ripe tomato from *gordal* variety. First, the method was applied to a mixture of tocopherol standards, yielding three distinct peaks with retention time of 1.25 min (δ-tocopherol), 1.45 min (γ-tocopherol) and 1.60 min (α-tocopherol). The selectivity of the method was therefore confirmed by the absence of any interferent in the chromatographic separation of the selected tocopherols using their specific excitation and emission wavelengths (Fig. 3.2.3.).

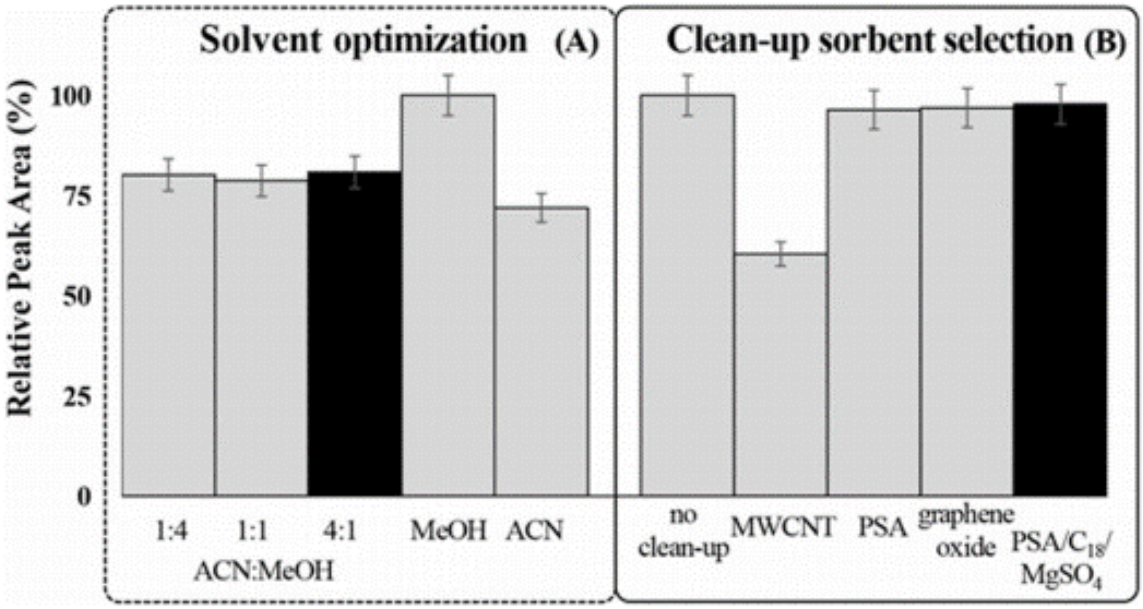


Fig. 3.2.2. Experimental optimization of the LLUSAE procedure: a solvent optimization using methanol (MeOH), acetonitrile (ACN) and three ACN/MeOH gradients (1:4, 4:1, and 1:1); b clean-up sorbent selection among multiwalled carbon nanotubes (MWCNT), PSA, graphene oxide and a PSA/C18/MgSO4 mixture. Selection of the best conditions was based in the relative peak area and chromatographic conditions involved (as detailed in the text).

Linearity was evaluated through external standard addition method, by applying the least-squares method elsewhere. A good correlation coefficient ($r^2 > 0.997$) was obtained in the LDR 0.01-4.0 μg/mL (Table 3.2.1). Regarding LODs and LOQs, determined from ordinary least squares regression data, the limits obtained (LODs of 2.15/5.52/1.67 ng/mL and LOQs of 7.18/18.40/5.58 ng/mL for δ-/γ-/α-tocopherol, respectively, Table 3.2.1) are substantially lower than the reported in literature for tomato extracts (1000 times lower) [133, 149] and serum (10 times lower) [162-164], making LLE-dSPE/UHPLC-FLR a powerful strategy for tocopherols quantification. A further comparison of the analytical performance of selected methodologies to quantify tocopherols can be appreciated in Table 3.2.2.

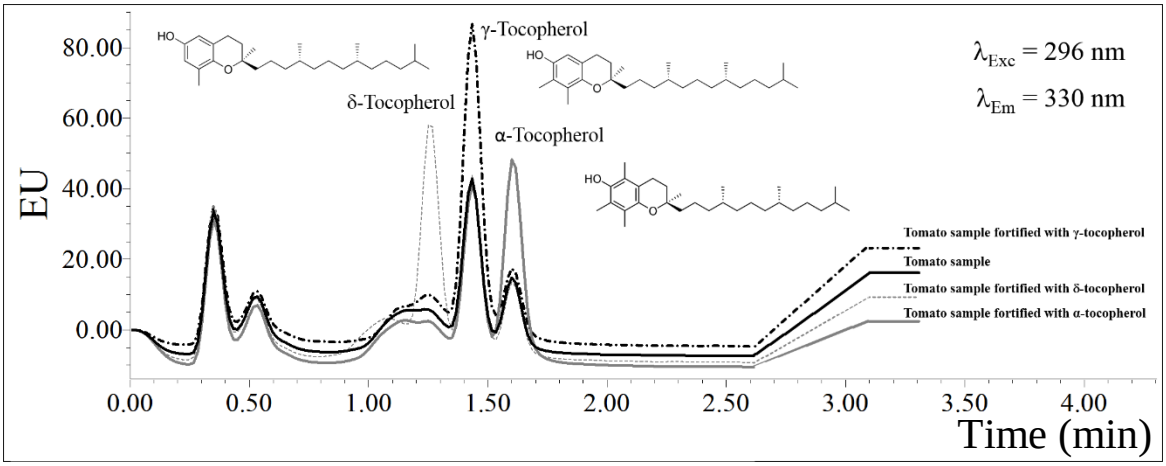
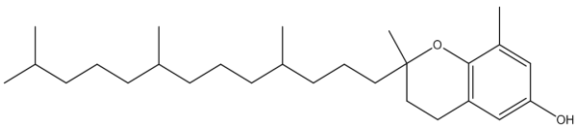
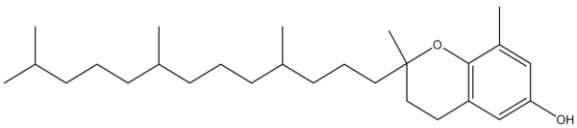
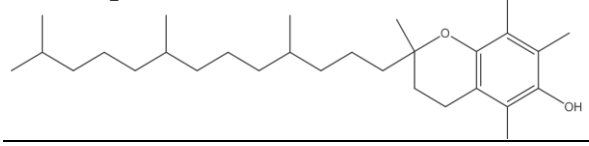


Fig. 3.2.3. Representative UHPLC-FLR chromatograms obtained at λExc = 296 nm and λEm = 330 nm for gordal tomato sample (Tom) spiked with δ-, γ- and α-tocopherol standards (δ-, γ- and α-Toc, respectively)

Table 3.2.1. Validation parameters of LLE-dSPE/UHPLC-FLR for tocopherols determination.

Tocopherols λ_{exc} = 296nm λ_{em} = 330 nm	**RT (min)**	**Linearity**		**Sensitivity**		**Spiking Levels**	**Precision (%)**		**Recovery (%)**	**Matrix Effect (%)**
		LDR	r^2	**LOD**	**LOQ**		**Intra-day** (n=4)	**Inter-day** (n=9)		
δ-Tocopherol 	1.2	0.01 - 4.0	0.9987	2.15	7.18	0.1 (LL)	3.0	6.4	105.3	96.8
						1.0 (ML)	2.1	5.8	105.3	
						4.0 (HL)	2.1	4.9	96.9	
						Average	**2.4**	**5.7**	**102.5**	
γ-Tocopherol 	1.4	0.01 - 4.0	0.9974	5.52	18.40	0.1 (LL)	4.0	6.3	81.6	98.8
						1.0 (ML)	3.6	3.4	96.1	
						4.0 (HL)	1.9	2.6	94.1	
						Average	**3.2**	**5.7**	**90.6**	
α-Tocopherol 	1.6	0.01 - 4.0	0.9998	1.67	5.58	0.1 (LL)	3.0	6.3	80.1	84.9
						1.0 (ML)	2.2	6.1	79.5	
						4.0 (HL)	1.9	5.8	85.2	
						Average	**2.4**	**6.1**	**81.6**	

RT - Retention Time; LDR - Linear Dynamic Range (µg/mL); LOD - limit of detection (ng/mL); LOQ - limit of quantification (ng/mL); Spiking Levels (µg/mL); LL - low level; ML - medium level; HL - high level.

For precision assessment, three concentrations were evaluated (LL, ML and HL, *n*=4) and the RSD calculated. Intra-day precision (repeatability) and inter-day precision (reproducibility) were also calculated using the same concentration levels (LL, ML and HL, *n*=9). The results obtained (Table 3.2.1) range between 2.4 to 6.1 %. As expected, repeatability is lower than reproducibility and both are far below the reference limit of 20% [116, 165]. In addition to the evaluation of the method accuracy, a recovery study was carried out by spiking a tomato sample at three concentration levels, with a known amount of each tocopherol (see Table 3.2.1). The average recoveries obtained, ranging from 81.6 % to 102.5 % with RSDs lower than 6.1 % (Table 3.2.1) are within the tolerance range (80 to 120%) [116] and in agreement with the matrix effect results. These ranged between 84.9 % and 98.8, being therefore also within the tolerance range (80 to 120 %) [116, 166].

3.3 Determination of δ-, γ- and α-tocopherol in tomato by LLE-dSPE/UHPLC-FLR

Tocopherols composition in plant and fruits is affected by several abiotic and biotic factors, as temperature of the cultivation area, intercepted solar radiation to the plants, ripening stage and genotypic variety (reviewed in [167]). In sea buckthorn berries, for instance, the abundance of δ-tocopherol is greatly affected by the ripening stage of the fruit, but also the cultivars and season harvesting [168, 169]. Therefore, we use the methodology developed, LLE-dSPE/UHPLC-FLR, to assess δ-, γ- and α-tocopherol content in tomato samples from different ripening stages, varieties and fruit sections. The results obtained reveals that α-tocopherol is the most abundant of the selected tocopherols, followed by γ-tocopherol and finally δ-tocopherol with much lower levels than the other tocopherols analysed. Furthermore, while α- and γ-tocopherols levels are affected by the ripening stage of the fruit, δ-tocopherols remains almost constant during this stage (Fig. 3.2.4A). Accordingly, the levels of α-tocopherol decrease almost one third to 23.95 μg/ g FW as the fruit ripening progress from the full mature green (FMG) to breaker and finally ripe stage, the γ- isoform displays the opposite trend, rising its initial

concentration from 7.1 up to 13.0 μg/ g FW and δ-tocopherols reveals a very narrow variation from 0.9 to 1.3 μg/ g FW during tomato maturation (Fig. 3.2.4A, left dashed box). Following this, tocopherols levels were assessed in different tomato varieties, namely the regional *gordal* variety and the *campari*, *cherry* and *grape* varieties imported from mainland, in the ripe stage. As the results show, tocopherols levels present some variations among the four varieties analysed, but the three isoforms are significantly more abundant in the *gordal* variety (Fig. 3.2.4A, righ box). This results agrees with previous reports showing evidences of the great influence of the tomato genetic diversity in its antioxidant potential and consequently in the relative composition of the antioxidant compounds [170, 171]. Regardless of the ripening stage considered, the δ-tocopherol levels we found are particularly interesting because δ-tocopherol is rarely quantified in tomato and the amounts reported range from not detected [172, 173], to trace levels [148] and some mg/ kg of industrial tomatoes dry wheight [174]. Even for most vegetables and other fruits, δ-tocopherol has been scarcely reported [175, 176] and we were able to find this tocopherol described only in some legumes [177], banana [175, 176], and a few other tropical fruits with a very limited production and consumption [149, 167, 168, 178, 179]. Previously, it has been reported that the relative abundance of tocopherols can vary significantly in different tomato sections, being α-tocopherol mainly found in the outside (59%) and inside layers (39%) [180]. Here, we have performed a more detailed analysis of δ-, γ- and α-tocopherol isoforms distribution in tomato, considering five different fruit sections, inner and outer pericarp walls, locular cavity, skin and seeds of the ripe *Gordal* variety.

Table 2. Comparison of the proposed extraction procedure with other published methods for the extraction of tocopherols in different samples.

Sample	Extraction (method / solvents)	Analytical Conditions (hardware / mobile phase / volume injected)	Detection FLD (λ_{Exc} / λ_{Em})[1] DAD (λ)[2]	TA[3] (min)	LODs (δ-T/γ-T/α-T)[4] (ng/mL)	Recovery (δ-T/γ-T/α-T)[4] (%)	Ref.
Tomatoes	LLE-dSPE	UHPLC: Acquity BEH C18 column (50 × 2.1 mm, 1.7 µm) / ACN:MeOH (75:25, v/v) / 2 µL	296 / 330	2	2.2/5.5/1.7	96.8/98.8/84.9	**Method proposed**
Mushrooms	LLE / MeOH and Hex	HPLC: Polyamide II column (250 × 4.6 mm, 5 µm) / Hex:ethyl acetate (70:30, v/v) / 20 µL	290 /330	27	-/20/8	99/110/114	[181]
Corn, walnut, grape seed, rice, virgin olive, sesame, peanut, sunflower oils	LLE / MeOH:Hex: tetrahydrofuran (80:10:10, v/v/v)	HPLC: Alltima RP C-18 column (250 × 4.6 mm, 5 µm) / ACN:MeOH (50: 50, v/v) / 20 µL	290 / 325	10	8/8/9	101/99/98	[182]
Human plasma	LLE / H_2O, EtOH and Hex	UHPLC: Acquity BEH C18 column (150 × 2.1 mm, 1.7 µm) / ACN and MeOH / 10 µL	295 / 330	5	-/10/50	-/97/99	[183]
Butter	LLE with H_2O and 2-propanol	HPLC: Phenomenex Luna PFP column (150 × 4.6 mm, 3 µm) / MeOH:H_2O, (93:7 v/v) / 10 µL	295 / 330	13	0.4/0.2/0.5	-	[184]
Carrot, broccoli, red pepper, green pepper, spinach, green beans, kohlrabi, tomato and celery	LLE / acetone (0.025% BHT)	UHPLC: Kinetex PFP column (150 × 3 mm, 2.6 µm) / MeOH:H_2O (85:15, v/v) and MTBE:MeOH:H_2O (80:18:2, v/v/v) / 10 µL	295 / 330	24	100/100/500	88-100/85-98/99-108	[185]
Grass	LL-USAE / BHT:EtOH (10:1, w/v) and calcium carbonate:acetone (1:2, w/v), acetone, H_2O, diethyl ether with BHT	HPLC: Zorbax RX-SIL column (25 cm × 4.6 mm, 5 µm) / Hex:2-propanol (99.3/0.7, v/v) / 20 µL	290 / 330	20	38/50/72	104-135	[186]
Lipid emulsions	LLE / MeOH, and Hex (0.05% BHT)	HPLC: Pinnacle DB silica column (100 × 2.1mm, 1.9 µm) / 1,4-dioxane:Hex (2:98, v/v) / 20 µL	292 / 330	11	6/12/98	98/107/101	[187]
Powdered milk	LLE / Hex and EtOH	HPLC: Tracer Spherisorb ODS2 C18 column (250 × 4.6 mm, 5 µm) / MeOH / 20 µL	292	7	21/33/33	101/98/98	[188]

Oils of date fruits	Soxhlet extractor / petroleum ether as a solvent, plus saponification	UHPLC: Acquity BEH C18 column (150 × 2.1 mm, 1.7 µm) / ACN:F.A. (99.9/0.1, v/v) / 10 µL	291	10	-/-/83	-/-/97	189
Human plasma	LLE / H_2O, EtOH and Hex	HPLC: Discovery HS C18 column (150 × 4 mm, 5 µm) / MeOH and EtOH / 10 µL	292	5	-/-/13	-/-/96	190
Guava, watermelon,tamarillo, carrot, pumpkin, papaya, physalis,corn, sweetpotato, nectarine, broccoli and spinach	LLE / H_2O and Hex:acetone (1:1 v/v)	HPLC: C18 Poroshell 120 column (5 cm × 4.6 mm, 2,7 µm) / ACN, MeOH and ethyl acetate / 10-20 µL	285	5	67/7/25	-/-/96	191
Almonds, cashew nuts, hazelnuts, peanuts, tiger nuts, sun flower seeds and pistachios	QuEChERS / MeOH (PSA was selected as the clean-up)	HPLC: Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 5 µm) / MeOH:H_2O (98:2, v/v) / 20 µL	205	9	210/40/80	-/80/78	160
Moringa oleifera Lam.	Saponification followed by LLE / ether	UPC[2]: BEH 2-EP column (3.0 × 100 mm, 1.7 lm) / CO_2 and MeOH:isopropanol (1:1, v/v) / 1 µL	294	3	47/23/49	94/91/98	192
Human serum	LLE / EtOH and DCM:Hex (1:5, v/v)	HPLC: Spheri-5-ODS column (220 × 4.6 mm, 5 µm) / ACN:MeOH (85:15) and ACN:DCM:MeOH (70:20:10) / 10 µL	294	10	-/-/2154	-/-/95	193
		UHPLC: HSS T3 column (100 × 2.1 mm, 1.8 µm) / ACN:MeOH (85:15) and ACN:DCM:MeOH (70:20:10) / 1 µL		3			
Human serum	LLE / H_2O:MeOH (1:2, vv), followed by Hex	HPLC: Pursuit PFP (150 × 2 mm) / H_2O:MeOH:F.A. (97.9/2/0.1, v/v/v/), MeOH:F.A. (99.9/0.1, v/v) / 8 µL	MS/MS (+): Prec Ion 431 Prod Ion 165	28	-/-/430.7	-/-/101	194

[1] λ_{Exc} – excitation wavelength and λ_{Em} – emission wavelength in nm; [2] wavelengths in nm; [3] TA – Time of analysis; [4] δ-, γ- and α-tocopherol.

Abbreviations: ACN – acetonitrile, BHT – butylhydroxytoluene, DAD – diode array detector, DCM – dichloromethane, dSPE – dispersive solid phase extraction, EtOH – ethanol, F.A. – formic acid, FLD – fluorescence detector, Hex – hexane, LLE – liquid-liquid extraction, LODs – limits of detection, MeOH – methanol, MTBE - methyl tert-butyl ether, PSA – primary–secondary amine, UPC[2] – Ultra-performance convergence chromatography.

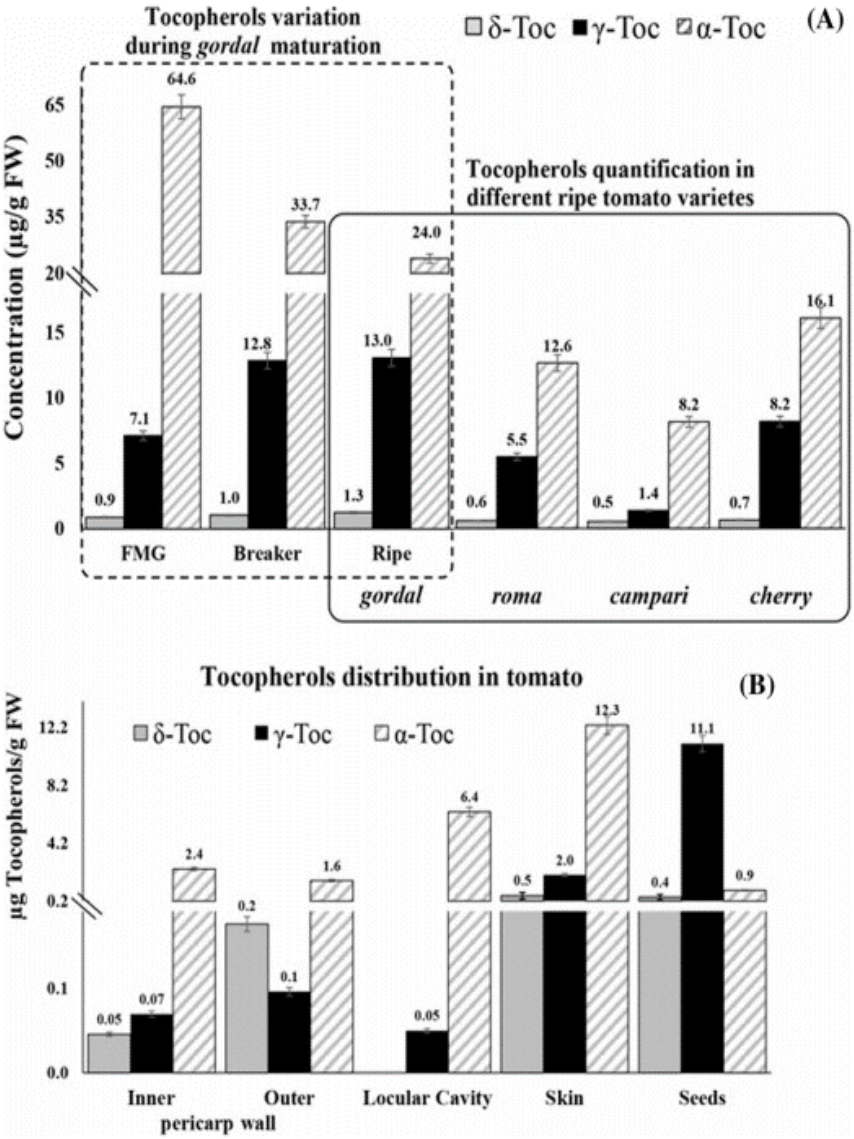


Fig. 3.2.4. Selected tocopherols concentration in different a tomato ripening stage (full mature green—FMG, breaker and ripe) and variety (gordal, campari, cherry and grape) and b fruit sections (inner and outer pericarps walls, locular cavity, skin and seeds).

The results shown in Fig. 3.2.5B confirm the heterogeneous distribution of δ-, γ- and α-tocopherol in the fruit, being the α-tocopherol more abundant in the skin, followed by locular cavity and minor amounts in the pericarp walls and seeds. In turn, δ-tocopherol is almost exclusively found in the skin and seeds, minor amounts in the outer pericarp wall, being vestigial in the inner pericarp walls and not detected

in the locular cavity. Finally, γ-tocopherol is almost totally concentrated in the seeds, minor levels in the skin and vestigial in the remain sections analysed.

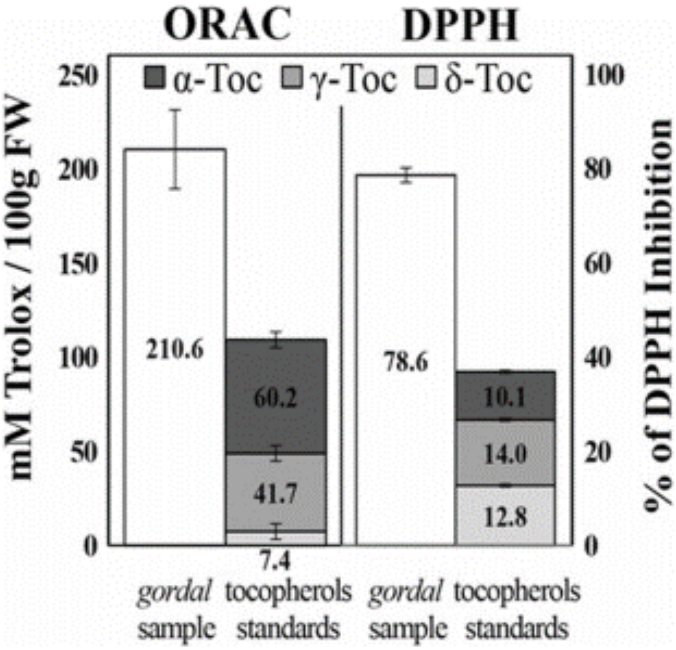


Fig. 3.2.5. Evaluation of tomato TAC and contribution of the selected tocopherols for this activity. TAC activity was assessed through the ORAC and DPPH assays as described in the experimental section (Total antioxidant capacity (TAC)) and using ripe gordal tomato samples. The relative contribution of δ-, γ- and α-tocopherol for TAC (δ-, γ- and α-Toc bars) was estimated using pure standards of the selected tocopherols in

3.4 Contribution of δ-, γ- and α-tocopherols for the Total antioxidant capacity (TAC)

The evaluation of tomato TAC and the respective contribution α- and δ-tocopherols for this activity was performed through the ORAC and DPPH assays, using ripe *gordal* samples and the pure standards. As shown in Fig. 3.2.5, δ-, γ- and α-tocopherol antioxidant potential is quite significant, representing half of tomato TAC. This contribution could be even more relevant if synergetic effects with other tomato antioxidants, namely between α-tocopherol and β-carotene [195, 196], could be assayed. Furthermore, if we take in account that these tocopherols are much more abundant in the tomato skin and seeds, as discussed in the previous section (Fig. 3.2.4B), then our results about the contribution of δ-, γ- and α-tocopherol for the

tomato TAC agrees and support the observation that peeling and seeding tomatos for cooking considerably affects their nutrional value [131].

4. Conclusions

This paper reports the successful development, validation and application of a fast, simple and reliable LLE-dSPE/UHPLC-FLR methodology for the characterization of δ-, γ- and α-tocopherol. Moreover, the methodology developed is precise, accurate and sensitive, retrieving LODs and LOQs about 1000 times lower than previously reported in literature and 10 times lower than the tocopherols levels found in serum. This anticipates the use of the devevlopped LLE/UHPLC-FLR methodology as a powerful strategy for tocopherols quantification in other matrices beyond tomato extracts. It was also shown that δ-, γ- and α-tocopherol localize preferentially in tomato skin and seeds and have a very important contribution for tomato TAC. Therefore, at the one hand this rises important nutritional concerns regarding the tomato peeling and seeding habits, particularly before its processing and cooking. At the other hand, tomato by-products contains high levels of tocopherols [174], having therefore great potential as ingredients in the food chain as shown very recently for the tomato seed oil [197].

Sub-Section 3.3. Free low-molecular weight phenolics composition and bioactivity of *Vaccinium padifolium* Sm fruits

Journal Pre-proofs

Free low-molecular weight phenolics composition and bioactivity of *Vaccinium padifolium* Sm fruits

José A. Figueira, Priscilla Porto-Figueira, Jorge A. M. Pereira, José S. Câmara

PII: S0963-9969(21)00479-8
DOI: https://doi.org/10.1016/j.foodres.2021.110580
Reference: FRIN 110580

To appear in: *Food Research International*

Received Date: 11 November 2020
Revised Date: 28 April 2021
Accepted Date: 25 June 2021

Published in:

Food Research International (Impact Factor 6.475)

José A. Figueira, Priscilla Porto-Figueira, Jorge A.M. Pereira, José S. Câmara

https://doi.org/10.1016/j.foodres.2021.110580

Abstract

Uveira-da-serra (*Vaccinium padifolium* Sm) is a native blueberry from Madeira Island (Portugal). In this study, the free low-molecular weight phenolic composition of *Vaccinium padifolium* berries (uva-da-serra - UdS), was established using a modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) strategy combined with liquid chromatography electrospray ionization tandem mass spectroscopy (LC-ESI/MS/MS). In addition the total phenolic content (TPC), and total flavonoid content (TFC), were evaluated, and in vitro bioactivity assessed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical-scavenging activities, and oxygen radical absorbance capacity (ORAC). Twenty-six phenolic compounds were identified in the UdS, being chlorogenic acid (17.4 mg/g DW), epigallocatechin (2.33 mg/g DW), caffeic acid (0.66 mg/g DW), quercetin-3-glucoside (0.38 mg/g DW) and myricetin (0.33 mg/g DW) the predominant compounds. As far we are aware, this is the first time that the free low molecular weight phenolic composition of Vaccinium padifolium Sm is characterized, also unveiling (-)epigallocatechin gallate, o-coumaric acid and m-coumaric acids presence in a Vaccinium specie. TPC (3021.8 mg GAE/100g DW), TFC (2645.2 mg QE/100g DW), DPPH (20509.0 μmol TE/g DW), ORAC (18510.0 μmol TE/g DW) and ABTS (19338.0 μmol TE/g DW) suggest a high antioxidant potential which is to health benefits including on cardiovascular and neurodegenerative disease prevention, , making UdS a useful biosource with potential applications in food, pharmaceutical and cosmetic industries.

Keywords: *Vaccinium padifolium*; phenolic compounds; chlorogenic acid; Total Phenolic and Flavonoid Content; QuEChERS; LC-ESI/MS/MS; Bioactivity evaluation

1. Introduction

Polyphenols, are secondary metabolites from plants and certain fungal species, characterized by high structural diversity, and their common occurrence in plants renders them intrinsic to dietary components. The interest in their consumption has increased considerably due to their wide bioactive potential, namely antioxidant [198, 199], anti-inflammatory [199-201], antihypertensive [202, 203], anticancer [204-208], as well antiviral properties [199, 209]. Notably, a recent anti-Covid-19 potential application was also explored by Chojnacka et al [209, 210].

The Madeira archipelago is a group of volcanic islands located in the Atlantic Ocean that has been isolated from the European continent for millions of years. For this reason, the flora of Madeira island is very rich and old, being the Laurisilva forest, classified as World Heritage by UNESCO, one of its best examples. Moreover, the rich environmental heterogeneity, isolation and relatively stable climate of Madeira Island promoted a high level of endemism. In this context, *Vaccinium padifolium* Sm., a shrub from the family of Ericaceae producing edible ovoid-shape berries of blue-black colour, when riped, locally known as uva-da-serra (UdS), is a typical example of an endemic specie [211-213] with high nutritional interest for human health.

It is widely reported in the literature that significant amounts of polyphenols are found in highly pigmented blueberries, raspberries, and blackberries as well as in a diverse range of wild berries [214-217]. Similarly, the genus *Vaccininum* contains vitamin C, E and carotenoids, high content of anthocyanins and phenolic compounds [218-220], among others, with potential human health. Moreover, UdS berries are widely used in Madeira Island for fresh consumption (natural berries) and for liqueurs, jams, and others food derivatives (after processing the fruit). In traditional and local ethnopharmacology, this fruit is frequently used namely against bronchitis, cough, dysentery [213]. For this reason, there is a great interest in identifying *Vaccinium padifolium* Sm. bioactive constituents beyond the anthocyanins and conjugated phenolics ([221]; Spinola, Pinto, & Castilho,2018). The free low-molecular weight phenolics (FLMWP), in particular the most easily extracted phenolics from

vegetables and fruits [222] presenting high bioavailability and bioassimilation rates [219, 223, 224], have never been studied in detail in UdS. To achieve this goal, an improved and highly efficient QuEChERS-*d*SPE approach (quick, easy, cheap, rugged, and safe extraction with dispersive solid-phase clean-up) followed by LC/ESI-MS/MS analysis was employed to characterize the FLMWP of UdS berries. Also, considering the protective health effects associated with dietary polyphenols consumption, the potential of UdS as a bioactive food was assessed through the determination of total phenolic content (TPC), total flavonoid content (TFC), and the total antioxidant capacity (TAC).

2. Materials and Methods

2.1. Chemical Reagents

Analytical standard of phenolic compounds gallic acid, *p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid, gentisic acid, cinnamic acid, vanillic acid and ferulic acid were purchased from Fluka Biochemica AG (Buchs, Switzerland). (-)-Epicatechin, catechin, caffeic acid, syringic acid, trans-resveratrol, protocatechuic, chlorogenic acid syringaldehyde, rutin, quercetin-3-glucoside, luteolin, kaempferol, apigenin, *p*-hydroxybenzoic acid, (-)-epigallocatechin, and (-)-epigallocatechin gallate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Quercetin, myricetin, ABTS, fluorescein sodium salt, AAPH, potassium persulfate ($K_2S_2O_8$), aluminium chloride, ($AlCl_3$) and sodium nitrite ($NaNO_2$) were obtained from Sigma-Aldrich (Steinheim, Germany) and Acros Organics (NJ, USA), respectively. All standards were of analytical grade, presenting purity greater than 95%. Ultrapure water (18 MΩ cm at 23 °C) was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). Acetonitrile (ACN), methanol (MeOH) and ethyl acetate (EtAc) HPLC grade from Fischer Scientific were obtained from JM Santos. Ethanol absolute (EtOH, 99.5%) was obtained from Panreac (Valencia, Spain). The magnesium sulphate anhydrous, DPPH, Folin-Ciocalteu phenol reagent and trolox were obtained from Fluka (Madrid, Spain), sodium chloride (NaCl) and sodium

hydroxide (NaOH) from Fischer Scientific (Loughborough, UK), sodium citrate tribasic dehydrate from Sigma-Aldrich (St. Louis, MO, USA) and sodium citrate dibasic sesquihydrate from Fluka (Madrid, Spain) were obtained from LaborSpirit (Portugal). Multiwalled carbon nanotubes clean-up salt (MWCNTs), primary secondary amine (PSA), graphene oxide and PSA/C18/$MgSO_4$ (25/25/150 mg, DisQuE) were purchased from Waters (Milford, MA, USA). All chromatographic solvents were filtered using a 0.20 μm membrane nylon filter and all solutions and samples extracts were filtered using a 0.20 μm PTFE syringe filter (ϕ4 mm, SPECANALITICA, Portugal).

2.2. Sample preparation

The UdS samples were collected in Montado do Pereiro (Madeira Island, 32° 42'33''N 16° 53'14''W, 1350m altitude), on an approximate area of 20 m^2. The berries were randomly collected in the largest possible number of different plants to obtain representative samples (Fig. 3.3.1). The collection was carried out in August of 2016. The berries were lyophilized (76.4 % ± 1.3 % of water loss), homogenized, triturated using an analytical mill (IKA) and stored under $N_{2\,(g)}$ atmosphere at -80 °C in dark glasses until analysis.

Fig. 3.3.1. *Vaccinium padifolium* Sm. Plant (A), flower (B), and fruit (berries) under different ripening stages, from unripe (green), medium ripe (light blue) up to fully ripe (dark blue) (C).

2.3. QuEChERS extraction and clean-up

The QuEChERS procedure used was based on the methodology proposed by [225] with the addition of citrate buffer and additional improvements in terms of sample mass and agitation mode as reported by [226]. Briefly, 50 mg of the lyophilized sample was added to a 15 mL PTFE centrifuge tube containing buffered salts, trisodium citrate dihydrate (1 g), disodium hydrogencitrate sesquihydrate (0.5 g), NaCl (1 g) and anhydrous $MgSO_4$ (4 g) and 5 mL of organic solvent (ACN, MeOH, EtAc, ACN:MeOH 1:1 w/w, ACN:EtAC 1:1 w/w). Each tube was shaken by ultrasound (US, BRANSON 2510, 100 W) for 1, 5 and 10 min and centrifuged (Rotofix32A) at 5000 rpm for 5 min at room temperature. An aliquot (1 mL) from the upper part of the extract (organic phase) was transferred into a 2-mL PTFE *d*SPE clean-up tubes containing 25 mg of PSA, 25 mg of C_{18} sorbent and 150 mg $MgSO_4$ and subjected to a *d*SPE clean-up. The mixture was shaken in a vortex (30 s) and centrifuged for 5 min at 3000 rpm. Then, the extracts purified were filtered through a 0.20 μm. For sample extraction, QuEChERS was performed using 50 mg of sample, using the best solvent and US agitation, and following the same procedure described above.

2.4. LC-ESI/MS/MS

Liquid chromatography was performed on a Waters Alliance 2695 system consisting of a quaternary, low-pressure mixing pump, on-line vacuum degasser, autosampler and column compartment. Separation of phenolic compounds was achieved on a column Zorbax XDB C18, (150 mm × 2.1 mm, 3.5 μm), kept at 40 °C. A binary mobile phase with a gradient program was used, combining solvent A (acetic acid solution in water, ρ= 0.1 mL glacial acetic acid /L) and solvent B (acetic acid solution in acetonitrile, ρ= 0.1 mL glacial acetic acid /L) as follows: 100% A (0 min), 0% A (20-22 min), 80% A (22.10-30 min) and 100% A (35 min). The flow rate of the mobile phase was set to 0.3 mL/min and the injection volume of both standard solutions and sample extracts was 20 μL. The system was re-equilibrated with the initial composition for 5 min, before next injection. Mass spectrometry was performed on a Micromass Quattro Micro triple-quadrupole equipped with an electrospray

ionization (ESI) source, operating in the positive ion mode. Data acquisition, data processing and instrument control were performed through Microsoft Windows-NT (v4.1)-based MassLynx software. The mass spectra were acquired over the mass range 50 to 1000 m/z. The ionization source working conditions were as follows: source temperature, 140 °C; capillary voltage, 2.9 kV; cone gas flow rate, 80 L/h; desolvation gas flow rate 650 L/h; desolvation temperature, 350 °C. Nitrogen (99% purity) and argon (99% purity) were used as nebulizing and collision (product ion scan, MS/MS) gasses, respectively. Flow injection of the phenolic compounds (standard solutions of (-)epicatechin, vanillic acid, *trans*-resveratrol, syringaldehyde, syringic acid, rutin, quercetin, quercetin-3-glucoside, protocatechuic, *p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid, myricetin, luteolin, kaempferol, gentisic acid, gallic acid, ferulic acid, cinnamic acid, chlorogenic acid, catechin, caffeic acid, apigenin, *p*-hydroxybenzoic acid, (-)-epigallocatechin and (-)-epigallocatechin gallate prepared in the range 0.001-1 mg mL^{-1}) was used to optimize the multiple reaction monitoring (MRM) conditions. For each phenolic compound in this study, the cone energy and the ions to get the most abundant MRM transition were optimized for confirmation of the presence of each compound.

2.5. Total Phenolic and Flavonoid Content and Bioactivity Evaluation

2.5.1. Total Phenolic Content (TPC)

TPC, adapted from the Folin-Ciocalteu procedure, involves electron transfer from phenolic compounds which is measured through the absorbance of the blue coloured complexes formed at 765 nm [227]. To determine TPC, water was added to the lyophilized phenolic extract (50 mg) up to 1 mL, followed by the addition of 100 µL of Folin-Ciocalteu solution, 400 µL of Na_2CO_3 (20%), and another 500 µL of water. After 1h, the reaction was analysed at 765 nm using a UV–Vis spectrophotometer (UV–Vis LAMBDA 25, Perkin Elmer, Waltham, USA).

2.5.2 Total Flavonoid Content (TFC)

TFC was evaluated using the aluminium chloride colourimetric method, through which $AlCl_3$ forms acid-stable complexes with the C-4 keto groups and the C-3 or the C-5 hydroxyl group of flavones and flavonols [228]. To prepare the assay, methanol (70%) was added to the lyophilized phenolic extract (50 mg) up to 1 mL, followed by 60 µL of $NaNO_2$ (5%). After 5 min in the dark, 60 µL $AlCl_3$ (10%) were added, then wait another 5 min and added 400 µL of NaOH (1M), after 2 min reaction, 480 µL of methanol (70%) were added and the solution submitted to UV-Vis analysis at 510 nm.

2.5.2. Total Antioxidant Capacity (TAC): DPPH, ORAC and ABTS assays

TAC was assessed through the DPPH, ORAC and ABTS assays. For all assays (DPPH, ORAC and ABTS), a trolox standard calibration curve was established, and the results expressed as µmol TE / g DW. DPPH methodology relies on the scavenging ability of the antioxidants present in the sample extract, being evaluated against the free-radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). This compound has a characteristic deep violet colour, being the DPPH reduction (and colour loss) measured during 10 min. DPPH was performed according to [229]. Briefly, methanol was added to the lyophilized phenolic extract (50 mg) up to 500 µL, followed by the addition of 1 mL of DPPH, being stored for 10 min in the dark, before UV-Vis analysis at 515 nm.

ORAC (Oxygen radical absorbance capacity) measures the oxidation of fluorescein by peroxyl radicals, generated by an azo-initiator like 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH). This degradation is affected by the quenching ability of the sample extract being measured, allowing its antioxidant activity determination. Also, the monitoring of the fluorescein decay can be achieved by making use of the area under the curve (AUC), as follows:

$$AUC = (0.5 + f1/f0 + f2/f0 + \cdots + fi/f0) \times CT$$

Where *f0* is the initial fluorescence reading, *f1*, *f2* and *fi* are the fluorescence readings at cycles *1*, *2* and *i*; *CT* is the total cycle time, in min. ORAC assay was adapted from

[158]. Briefly, to a Greiner 96 well Cellstar plate, black with a flat bottom, it were added 25 μL of the trolox solution (standards linear regression), 25 μL of the sample (diluted 1000 times), and 25 μL of 10.00 mM phosphate buffer at pH 7.40 (for control assays), or 200 μL of 10.00 mM phosphate buffer (blank assays). Finally, 150 μL of fluorescein solution (10.00 nM) was added to all wells, with exception of the blank assay's wells. After 30 min of incubation at 37º C, 25 μL AAPH (153.0 mM) was added to all wells, again with exception of the wells corresponding to the blank assays. Exterior wells and spaces between wells were filled with water and serve as a thermal reservoir. The values of fluorescence (λ_{Exc} = 485 nm, λ_{Em} = 520 nm) were subsequently determined every 90 s, for about one hour, through Victor3 Multilabel Plate Counter 1420 fluorescence reader (Perkin Elmer, Waltham, USA).

ABTS assay results from the generation of the ABTS [2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation, through the reaction between ABTS and potassium persulfate. The antioxidants present in the sample under evaluation, will reduce the ABTS and decolorate the characteristic blue/green colour of ABTS solution [230]. The ABTS methodology used was adapted from [231]. Briefly, an ABTS stock solution was prepared by mixing ABTS (7.3 mM) with potassium persulfate (2.59 mM) in ethanol and allowing the mixture to stand in the dark at room temperature for 16 h before use. The working ABTS solution was obtained by dilution of the stock solution 100 times in ethanol, adjusting the dilution to obtain an absorbance of 0.70 at 734 nm. Ethanol was added to the lyophilized phenolic extract (50 mg) up to 500 μL, followed by the addition of 1.9 mL of ABTS diluted solution, being stored for 2 h in the dark, before UV-Vis analysis at 734 nm. For TAC evaluation, sample extraction was obtained according to [232]. Briefly, 50 mg of the lyophilized sample was diluted with 9 mL of ACN:MeOH (4:1, v/v), sonicated for 30 min and centrifuged. Then, the supernatant obtained was subjected to a clean-up with PSA/C18/$MgSO_4$ and filtrated before storage in aliquots on dark glasses until analysis.

3. Results and discussion

The results obtained can be essentially considered into three steps: (*i*) optimization of the QuEChERS-*d*SPE extraction procedure (US extraction time and solvent); (*ii*) characterization of the UdS FLMWP composition by LC-ESI/MS/MS and (*iii*) evaluation of TPC, TFC and the antioxidant activity (DPPH, FRAP and ABTS assays)

.

3.1. QuEChERS-dSPE optimization

In its most simple definition, QuEChERS is a single step buffered acetonitrile extraction promoted by salting-out effect with anhydrous magnesium sulphate. Therefore, its efficiency can be affected by several parameters, including sample agitation, time of extraction and nature of the sample and extraction solvent. According to our previous work, US usage can accelerate mass transfer between the matrix sample and the extraction solvent. Consequently, extraction equilibrium can be met faster, while the time the organic solvent is dissolved in the aqueous phase also increases [226, 232].

For this reason, the use of US during the QuEChERS extraction and the type of solvent were thoroughly assayed for all the 26 phenolics analysed in this work and using lyophilized UdS samples. The experimental conditions tested included 1, 5 and 10 min of US and the solvents ACN, MEOH, EtAc and equimolar mixtures of ACN:MeOH and ACN:EtAc (Fig. 3.3.2 and Supplementary Fig. 3.3.1.). The main reason for this exhaustive assay for all phenolics is justified by the disparity found for the best extraction conditions (best solvent and US time). These results are summarized in Fig. 3.3.2 and detailed in Supplementary Fig. 3.3.1.. Accordingly, MeOH and ACN:MeOH (50:50) are the solvents that allow the best extraction for a higher number of phenolics. Such a result is in agreement with the literature as methanol has been generally found to be more efficient in the extraction of lower molecular weight polyphenols [227, 233, 234]. Nevertheless, this only applies to less than half of the 26 phenolics (11 in MeOH and 8 in MeOH:ACN). Moreover, several phenolics can be hardly extracted under these conditions. This is the case of (-)-

epigallocatechin gallate, which requires EtAc and *p*-hydroxybenzoic acid, *p*-coumaric acid, vanillic acid and syringaldehyde that prefer ACN:EtAc (50:50, v/v) (Supplementary Fig. 3.3.1). In contrast, the gain in extending the use of US is not so obvious and overall, there is not a major difference in terms of extraction efficiency between the best condition (5 min US for 11 phenolics). Moreover, the use of US for more than one min does not favour the extraction of luteolin, apigenin or kaempferol (Supplementary Fig. 3.3.2).

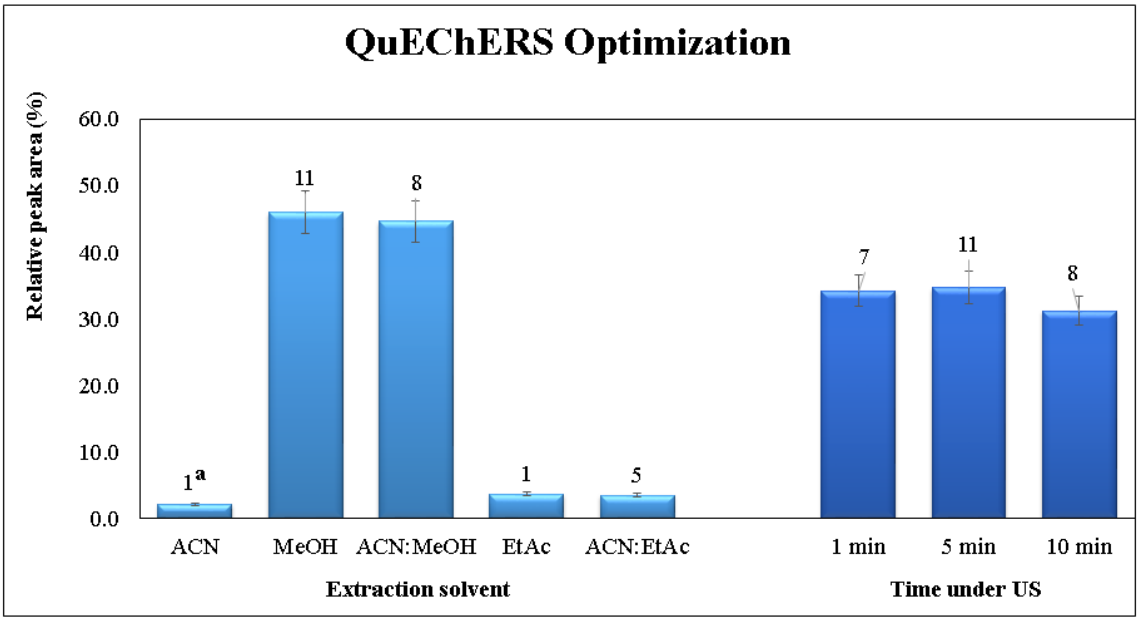


Fig. 3.3.2. Distribution of polyphenols by relative quantity (% of the total), according to the individual results presented in Supplementary Fig. 3.3.1. a – number of polyphenols in each condition.

3.2. Free low molecular weight phenolics composition of UdS.

The LC-ESI/MS/MS conditions were optimized to determine unequivocally the identity of each phenolic compound, even if present in low concentrations, as described in Materials and Methods. Parent, quantifier, and qualifier ions for each target analyte were chosen as a function of their intensities to get maximum sensitivity. The precursor ions showing the highest abundance were, in most cases,

protonated $[M^+H]^+$ species. All tuning data acquired automatically were manually examined to ensure proper selection of product ions and collision energy. The MS conditions were first optimized in quadrupole 1 (Q1), which transmits only an ion of m/z. After collision-induced dissociation (CID) studies, the conditions were adjusted for the third quadrupole (Q3) to provide optimal signals from the daughter ions. The monitored transitions, dwell times, cone voltages, collision energies and delays are listed in Table 3.3.1.

Table 3.3.1. Transition Reactions Monitored by LC-ESI-MS/MS for identification of each phenolic compound and extraction solvent

Phenolic Compound	RT	Precursor ions (*m/z*)	Molecular ion	Product ions (*m/z*)			Dwell (s)	Cone (V)	Coll (eV)
				T. 1	T. 2	T. 3			
Gallic acid	11.97	169	$[M - H]^-$	169 > 125			0.1	25.0	15.0
(-)Epigallocatechin	13.17	305	$[M - H]^-$	305 > 125	305 > 179		0.1	25.0	25.0, 20.0
Gentisic acid	13.38	153	$[M - H]^-$	153 > 108	153 > 109		0.1	20.0	25.0, 15.0
Protocatechuic acid	13.58	153	$[M - H]^-$	154 > 109			0.1	20.0	15.0
Chlorogenic acid	13.74	353	$[M - H]^-$	353 > 191			0.1	20.0	20.0
(-)Epicatechin	14.37	289	$[M - H]^-$	289 > 179	289 > 205	289 > 245	0.1	30.0	15.0
Catechin	14.45	289	$[M - H]^-$	289 > 179	289 > 205	289 > 245	0.1	25.0	15.0, 20.0
(-)Epigallocatechin gallate	14.59	457	$[M - H]^-$	457 > 169	457 > 301	457 > 331	0.1	25.0	20.0, 15.0
Rutin	14.75	609	$[M - H]^-$	609 > 151	609 > 179	609 > 301	0.1	40.0	45.0, 40.0, 25.0
p-Hydroxibezoic acid	15.05	137	$[M - H]^-$	137 > 93			0.1	20.0	15.0
Syringic acid	15.09	197	$[M - H]^-$	197 > 153	197 > 182		0.1	25.0	15.0
Caffeic acid	15.16	179	$[M - H]^-$	179 > 135	179 > 135		0.1	25.0	15.0
Vanilic acid	15.20	167	$[M - H]^-$	167 > 123	167 > 152		0.1	20.0	15.0
Quercetin-3-glucoside	15.34	463	$[M - H]^-$	463 > 300	463 > 301		0.1	35.0	30.0, 25.0
o-Coumaric acid	16.09	163	$[M - H]^-$	163 > 119			0.1	20.0	15.0
m-Coumaric acid	16.30	163	$[M - H]^-$	163 > 119			0.1	25.0	15.0
p-Coumaric acid	16.41	163	$[M - H]^-$	163 > 119			0.1	20.0	15.0
Ferulic acid	16.56	193	$[M - H]^-$	193 > 134	193 > 149	193 > 178	0.1	25.0	15.0, 10.0
Syringaldehyde	16.97	183	$[M + H]^+$	183 > 155			0.1	20.0	10.0
Myricetin	17.12	317	$[M - H]^-$	317 > 151	317 > 179		0.1	30.0	25.0, 20.0
trans-Resveratrol	18.03	227	$[M - H]^-$	227 > 143	227 > 159	227 > 185	0.1	30.0	30.0, 20.0
Kaempferol	18.20	285	$[M - H]^-$	285 > 133	285 > 151	285 > 169	0.1	35.0	30.0, 20.0, 25.0
Luteolin	18.31	285	$[M - H]^-$	285 > 133	285 > 151	285 > 175	0.1	30.0	30.0, 25.0
Quercetin	18.52	301	$[M - H]^-$	301 > 151	301 > 179		0.1	30.0	25.0, 20.0
Apigenin	19.52	269	$[M - H]^-$	269 > 151	269 > 227		0.1	35.0	25.0
Cinnamic acid	19.63	147	$[M - H]^-$	147 > 103			0.1	20.0	10.0

The identification procedure was carried out using the retention time of two transitions being the most intense transition used for quantification (through the calibration curve made with standards for each phenolic compound). From the experimental results and literature, we proposed a fragmentation pattern for the principal phenolic compounds (chlorogenic acid, (-)-epigallocatechin, caffeic acid and myricetin) identified in UdS samples (Fig. 3.3.3).

After the optimization of the LC-ESI/MS/MS conditions, a total of 26 different phenolic compounds of different classes were identified in UdS samples (Fig. 3.3.3). Although the majority of polyphenols in plants exist as glycosides with different sugar units and acylated sugars at different positions of the polyphenol skeleton [216], there is also a wide diversity of free low molecular phenolics that we target in this study. The 26 phenolics compound identified in UdS composition can be divided into flavonoids – flavanones, flavones, flavonols, dihydroflavonols and flavon-3-ols and non-flavonoids – phenolic acids, hydroxycinnamic acids and stilbenes (Fig. 3.3.4).

The diversity of the phenolic composition of UdS can be easily observed by comparison with the phenolic compounds found in different *Vaccinium* species reported in the literature (Table 3.3.2). Regarding this, [235] identified several phenolic compounds, including flavan-3-ols, proanthocyanidins, flavonols and their glycosides, and various phenolic acid conjugates in *Vaccinium vitis-idaea*, *Vaccinium myrtillus* L. and *Vaccinium* x *intermedium Ruthe* L. More recently [218] reported a wide diversity of phenolics in the European cranberry (*Vaccinium oxycoccos*), while [236] quantify phenolics in blueberries (*Vaccinium Angustifolium*) and pink-fruited blueberry clones (*Vaccinium* × hybrids). Concerning the UdS (*Vaccinium padifolium* Sm.), previously, [221] identified twenty anthocyanins, and more recently [237], also reported an abundant phenolic composition, mostly glycosylated with different sugar units and acylated sugars at different positions of the polyphenol skeleton. Therefore, as far we are aware, this is the first study focusing on the extraction and characterization of the FLMWP of Vaccinium padifolium Sm. fruits. Overall, taking

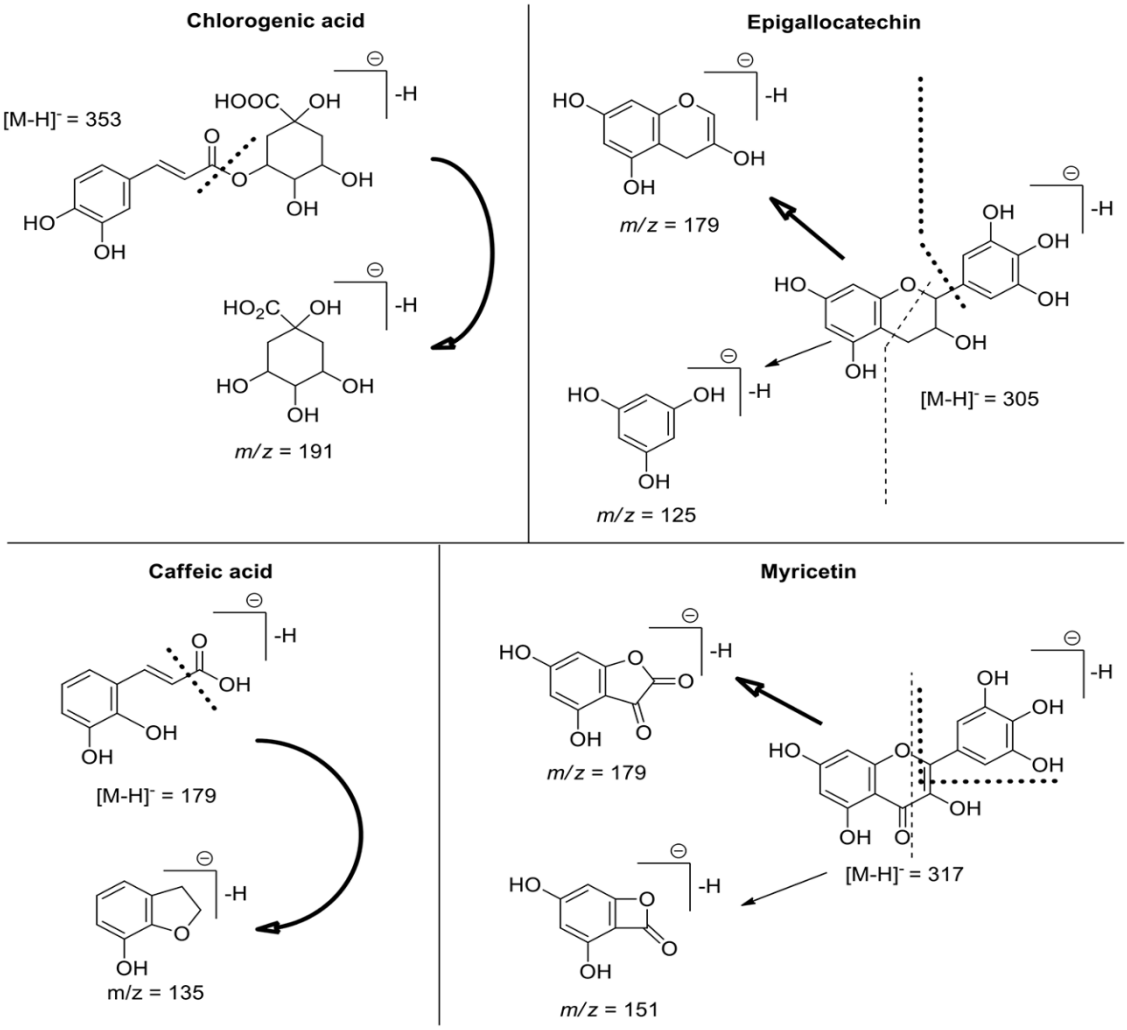


Fig. 3.3.3. Proposed fragmentation pattern for the main phenolic compound, the formation of precursor ions and respective daughter ions from [M-H]- for chlorogenic acid, epigallocatechin, caffeic acid and myricetin.

into consideration the 26 phenolics identified in UdS, the strategy applied seems to be efficient as remaining studies reported much less phenolics in the fruits of the respective *Vaccinium* species (less than 20 FLMWP associated to *V. myrtillus* and *V. corymbosum* fruits, and about half of that number in the remaining *Vaccinium* fruits, Table 2). Additionally, this composition is also very diverse, being quercetin the only FLMWP identified in the 10 *Vaccinium* specifies considered. Caffeic acid, (-)-epicatechin, ferulic acid were also often found, as well as the chlorogenic acid, which was the most abundant FLMWP found in UdS, and was reported in all species with exception of *V. bracteatum* and *V. uliginosum* fruits. In contrast, (-)-epigallocatechin

Table 3.3.2. Low molecular weight phenolic composition of UdS fruits, concentration (mg/100g FW) obtained using an extraction method based on QuEChERS-*d*SPE (extraction with MeOH and 5 min under US) followed by LC/ESI-MS/MS. Survey of the phenolic compounds reported in the literature in different *Vaccinium* species fruits.

	Phenolic Compound																								
***Vaccinium* species**	Gallic acid	(-) Epigallocatechin	Gentisic acid	Protocatechuic	Chlorogenic acid	(-) Epicatechin	Catechin	(-) Epigallocatechin gallate	Rutin	*p*-Hydroxibezoic acid	Syringic acid	Caffeic acid	Vanilic acid	Quercetin-3-glucoside	*o*-Coumaric acid	*m*-Coumaric acid	*p*-Coumaric acid	Ferulic acid	Syringaldeyde	Myricetin	trans-Resveratrol	Kaempferol	Luteolin	Quercetin	Apigenin	Cinnamic acid	**Ref.**
V. padifolium	**48**	**2331.9**	**1.3**	**2.3**	**17438.6**	**156.9**	**143**	**0.50[a]**	**129.5**	**2**	**1.6**	**662.7**	**4.8**	**384.5**	**0.1**	**1.90[b]**	**19.8**	**19.4**	**2.7**	**328.6**	**0.3**	**68**	**4.1**	**49.7**	**1.40[c]**	**3.1**	**§**
	1.13	55.01	0.03	0.05	411.38	3.7	3.37	0.01	3.05	0.05	0.04	15.63	0.11	9.07	0	0.04	0.47	0.46	0.06	7.75	0.01	1.6	0.1	1.17	0.03	0.07	[d]
V. myrtillus	0-0.8			0-4.75						0-0.7	0-0.06	0-2.08	0-0.37				0.11-2.95	0.01-0.28									[238]§
				0.081								0.038					0.032										[239]
										0.7		9.2					29	27.5		6.2		2.6		21.4			[240]#
												0.4					2.4			1.2				1.7			[241]
		0.07				1.75	0.2					0.16		0.99			0.02							0.03			[235]#
	6.2				23.1	2	0.2		0.2			0	0.3				0.3	0.4		0.4	0.2			0.8			[242]
																	3.2-10.0			0.3-4.7				0.2-15.9			[243]
V. ashei	2.77-16.16				0.04-2.46	0.06-6.65	0.13-1.15		0.04-6.74								0.001-0.76	0.03-12.90						0.05-3.68			[244]
					10.5-68.6									6.7-32.5							0.06-0.7						[245]
V. vitis-idaea				D		D	D						D									D					[246]
					D	D	D							D										D			[247]
										2.1		2.6						19.9	7		2.6		1.6	63			[240]#
							0.02					0.61		3.87										0.17			[235]#
					D	D	D															D		D			[248]
V. angu					15.74-30.13							0.40-1.96					0.55	0.2									[249]

					D	D	D						D								D				247
	5.4				3311	74.6	117.5					35.8	189.8						0.4		15.4				236§
					115.9-293.7							4.65-18.2			1.82-5.17	0-4.41									250§
V. arctostaphylos					45.6-17.66							0.28-0.84			0.005-0.61						0.7				249
	0.0003		0.0002	0.0056					0.0023	0.0055	0.0311				0.0163	0.0014									251
				0.005	0.019						0.034				0.029										239
V. floribundum					7.9-12.6								D												252
	3.1				17	8	10		2.1		D	5			1.7	D	2.6				35				253
V. bracteatum						D	D				D														254
																		122	392	970	248				255
V. corymbosum		2.10-3.99			33.08-120.75		0.67-2.32		0.54-1.00	0.34-1.27		0.11-0.27			0.08-0.56	0.18-1.70			0-0.73		2.88-53.69		0.03-0.07		256#
									D		1.5-6.4				0.5	66.5-68.3	3.7-3.8		0.6-1.0		18.3-26.0				240#
					15.6-34.9											57.3-131.7									257
	1.8				70	0.5	1.8	3.1			0.2				D	2.2		0.4			0.1				242
V. oxycoccos									0.4		3.3				2.1	18.4	13.5		1.4		59.2				240#
					96.3	6.3					1.4				77						15.4				218
V. Uliginosum	0.014			0.061						0.022	0.351					0.008	0.009								258
						D	D						D								D				247

[a] – extracted with ethyl acetate, [b] – extracted with ACN:MeOH 1:1 w/w, [c] - extracted with CAN, [d] Calculated considering 76.41 % of water content removed in sample lyophilization. D – detected, § – results expressed as µg/g DW, # – results expressed as %.

gallate, o-coumaric acid and m-coumaric acids are reported for the first time in *Vaccinium* fruits, while gentisic acid, apigenin and cinnamic acid were only reported in another *Vaccinium* fruit beyond the ones of *Vaccinium padifolium* Sm. (Table 2). In

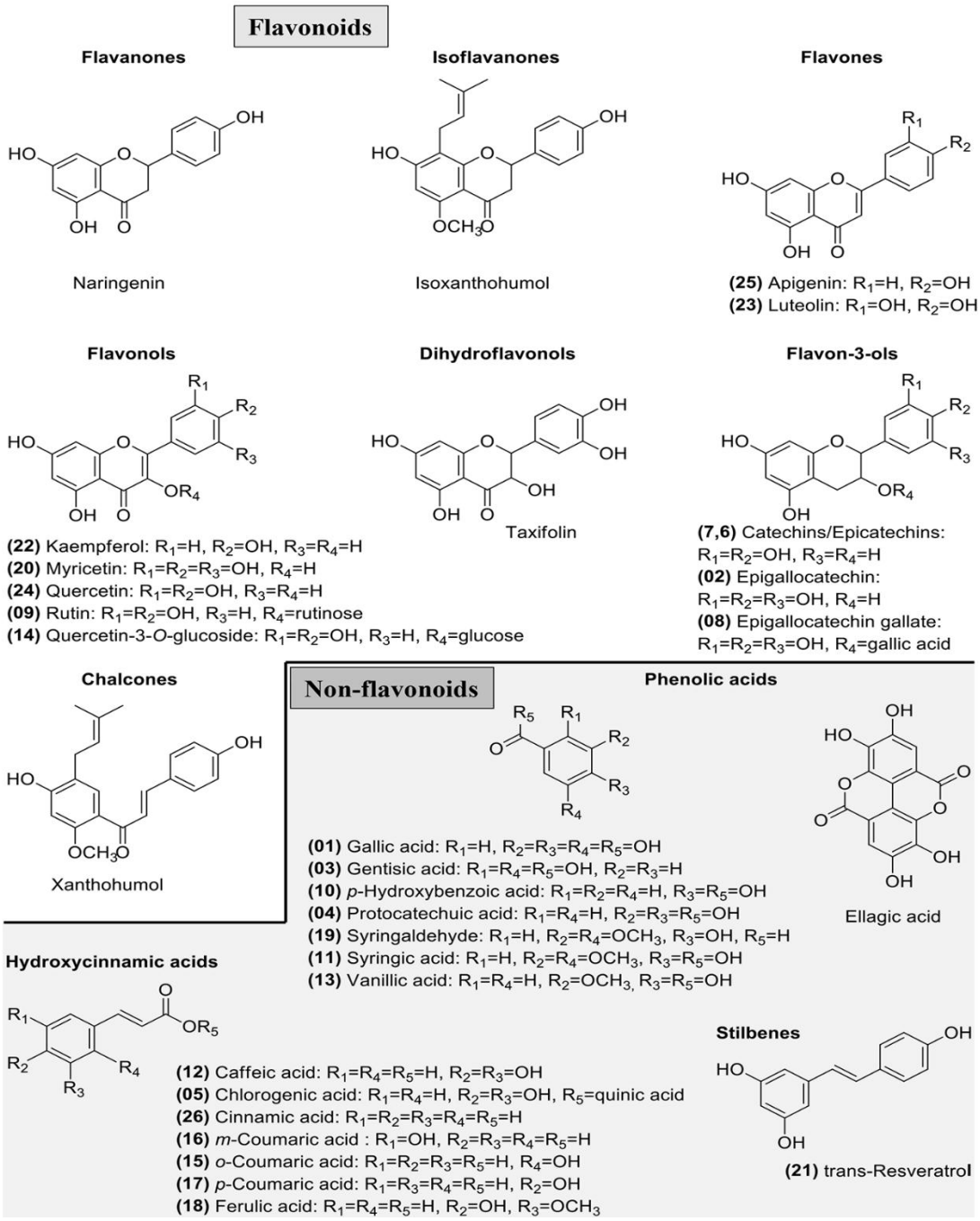


Fig. 3.3.4. Chemical structures of main phenolic compounds classified by family. The phenolic compounds identified in Vaccinium padifolium berries are numbered according to retention time indicated in Table 3.3.2.

In turn, (-)-epigallocatechin, which was the second most abundant polyphenol found in UdS, was only reported in *V. myrtillus* and *V. corymbosum* fruits [210, 235].

But, beyond the diversity in FLMWP, it is important to highlight that comparatively with the remaining *Vaccinium* fruits, UdS presents a high abundance for chlorogenic acid (17438.6µg/g DW), epigallocatechin (2331.9 µg/g DW), caffeic acid (662.7 µg/g DW), quercetin-3-glucoside (384.5µg/g DW) and myricetin (328.6 µg/g DW). In fact, chlorogenic acid has been widely reported as one of the main polyphenols in blueberries (see Table 2). However, the concentration found in UdS largely exceeds the expected values. [259] and [242], for instance, reported values under 1 mg/g for blueberry cultivars, while in a previous work, using acidic methanol extraction, [237] reported 8.88 mg of chlorogenic acid / g DW of UdS. This result strongly suggests that the QuEChERS approach we used is more efficient for the chlorogenic acid extraction from UdS samples. But eventually, a more meaningful comparison can be established with coffee berries, where chlorogenic acid is widely known as a reference. According to [260], chlorogenic acid ranges from 5 mg/g on dark roast samples to 41 mg/g in green coffee. This means that the chlorogenic acid levels found in UdS are in the same range to those reported in coffee berries. Finally, it should be also referred that the high abundance of phenolic compounds in UdS is also favoured by the orography and edaphic conditions in which the *Vaccinium padifolium* Sm. plants grow. These include the high altitude and consequent lower temperatures, leading to a shift in the secondary metabolism in plants with accumulation of hydroxycinnamic acids. Furthermore, an enhanced UV-B radiation that occurs in Madeira island throughout the whole year, causes an increased synthesis of phenolic acids in the plants and fruits [261, 262].

3.3. Total Phenolic and Flavonoid content and Bioactivity Evaluation of UdS

The wide diversity of the phenolic composition of UdS is indicative of a high bioactive potential for these fruits and, not surprisingly medicinal effects have been reported for *Vaccinium padifolium* Sm. plants and fruits [213]. This certainly involves

many, if not all, of the FLMWP identified in UdS composition (Table 3.3.2). Most of these FLMWP have been widely shown to elicit health protective effects. To gain insights into the health potential of UdS berries extracts, the total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC) assays were performed (Table 3.3.3). TPC results show a high total phenolic content (3021.8 mg GAE/100g DW) which can be attributed to the rich profile of phenolic compounds identified in the berries under study, with levels slightly higher than the ones reported by [236] for blueberries (*Vaccinium angustifolium*, Table 3.3.3). TPC is unquestionably correlated with the levels of the phenolics analysed and thus higher levels of individual compounds correspond to a higher TPC of the respective sample. In agreement with the TPC results, TFC shows a high level of flavonols (2645.2 mg QE / 100g DW). As previously stated, the orographic and edaphic characteristics where UdS growth may have an important contribution to flavonol content, particularly the high UV radiation that stimulates a higher content of flavonoids [218, 261]. TAC was evaluated through three different methodologies but using the same standard (trolox) to establish the antioxidant activity. The correlation coefficient for the trolox standard curve was r^2 = 0.9950, 0.9774 and 0.9991 for DPPH, ORAC and ABTS, respectively. The results obtain for DPPH (20509.0 μmol TE/g DW), ORAC (18510.0 μmol TE/g DW) and ABTS (19338.0 μmol TE/g DW) agree with each other. The TAC obtained for *Vaccinium padifolium* Sm. fruits is simultaneously much higher than the ones reported for other *Vaccinium* species (Table 3.3.3). In fact, UdS berries performed better than all related *Vaccinium* fruits reported in the literature regardless of the bioactive assays performed (Table 3.3.3). Regarding this, is noteworthy to refer that the extraction procedure can bias such comparison, as reviewed by [227]. Nevertheless, UdS presents higher TPC and TFC by comparison with extracts obtained using similar extractions methodologies (Table 3.3.3).

Table 3.3.3 Antioxidant evaluation of the UdS fruits (*Vaccinium padifolium*) through Total Phenolics, Flavonoids and Antioxidant Capacity assays. Comparison with values obtained for other *Vaccinium* species.[238, 239, 244, 257, 258, 263, 264]

	TPC (mg GAE / 100g sample		TFC (mg QE / 100g sample		TAC						
					DPPH (µmol TE / g sample)		ORAC (µmol TE / g sample)		ABTS (µmol TE / g sample)		
Sample	**DW**	**FW**	**DW**	**FW**	**DW**	**FW**	**DW**	**FW**	**DW**	**FW**	**Ref.**
Vaccinium padifolium	3021.8	755.4	2645.21	624.0	20509	4838.1	18510	4366.5	19338	4561.8	this work
Vaccinium myrtillus		2636.24				87.8					258
	3715.21				122.69						238
		215.12		91.69							249
		576							103		
	10200				1867						227
		13400				1658				199	
	7218										
Vaccinium corymbosum		626									259
		400									257
		296.9						287.5			264
		2740									
Vaccinium arctostaphylos		2494.26									258
		193.19		77.8							249
Vaccinium oxycoccos		819.5						98.8			264
		374.2				68.8				16.4	227
Vaccinium uliginosum		679		458		175		84			258
Vaccinium angustifolium	2450								127		236
Vaccinium ashei	2500										244
Vaccinium vitisidaea	1720										263
Vaccinium meridionale		758.6							45.5		227

Legend: ABTS **-** 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), DPPH - 2,2-diphenyl-1-picrylhydrazyl, DW – dry weight, FW **–** fresh weight**,** GAE – gallic acid equivalents, ORAC - Oxygen radical absorbance capacity, TAC - Total Antioxidant Capacity, TFC - Total Flavonoid Content, TPC - Total Phenolic Content.

4. Conclusions

In this work an improved US-assisted QuEChERS procedure was coupled to LC-ESI-MS/MS to extract and analyse the free and low molecular weight phenolic composition of UdS berries (*Vaccinium padifolium* Sm. fruits), unveiling the presence of (-) epigallocatechin gallate, o-coumaric acid and m-coumaric acids, which are reported for the first time in a *Vaccinium* specie. Overall, this efficient methodology allowed the unequivocal identification and quantification of 26 FLMWP belonging to different classes of phenolic compounds, being chlorogenic acid, epigallocatechin, caffeic acid, quercetin-3-glucoside and myricetin the most abundant. Notably, the high concentration of chlorogenic acid in UdS, in the same range of the levels found in coffee beans, makes this fruit an exceptional source of this important bioactive compound. The high potential of UdS as a functional food is further supported by the bioactivity evaluation that is also very appreciable, as shown by the high TPC,

TFC and TAC values obtained. Future *in vivo* assays will certainly confirm the high bioactive potential of UdS as functional fruits.

Sub-Section 3.4. Exploring a volatomic-based strategy for a fingerprinting approach of *Vaccinium padifolium* Sm berries at different ripening stages

Food Chemistry 245 (2018) 141–149

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Exploring a volatomic-based strategy for a fingerprinting approach of *Vaccinium padifolium* L. berries at different ripening stages

Priscilla Porto-Figueira[a], José A. Figueira[a], Pedro Berenguer[a], José S. Câmara[a,b,*]

Published in:

Food Chemistry (Impact Factor 6.306)

Priscilla Porto-Figueira, **José A. Figueira**, Pedro Berenguer, José S. Câmara

http://dx.doi.org/ 10.1016/j.foodchem.2017.10.049

Abstract

The effect of ripening on the evolution of the volatomic pattern from endemic *Vaccinium padifolium* Sm (Uveira) berries was investigated using headspace-solid phase microextraction (HS-SPME) followed by gas chromatography/quadrupole-mass spectrometry (GC–qMS) and multivariate statistical analysis (MVA). The most significant HS-SPME parameters, namely fibre polymer, ionic strength and extraction time, were optimized in order to improve extraction efficiency. Under optimal experimental conditions (DVB/CAR/PDMS fibre coating, 40 °C, 30 min extraction time and 5 g of sample amount), a total of 72 volatiles of different functionalities were isolated and identified. Terpenes followed by higher alcohols and esters were the predominant classes in the ripening stages – green, break and ripe. Although significant differences in the volatomic profiles at the three stages were obtained, cis-β-ocimene (2.0–40.0%), trans-2-hexenol (2.4–19.4%), cis-3-hexenol (2.5.16.4%), β-myrcene (1.9–13.8%), 1-hexanol (1.7–13.6%), 2-hexenal (0.7–8.0%), 2-heptanone (0.7–7.7%), and linalool (1.9–6.1%) were the main volatile compounds identified. Higher alcohols, carboxylic acids and ketones gradually increased during ripening, whereas monoterpenes significantly decreased. These trends were dominated by the higher alcohols (1-hexanol, cis-3-hexenol, trans-2-hexenol) and monoterpenes (β-myrcene, cis-β-ocimene and trans-β-ocimene). Partial least squares regression (PLSR) revealed that ethyl caprylate (1.000), trans-geraniol (0.995), ethyl isovalerate (−0.994) and benzyl carbinol (0.993) are the key variables that most contributed to the successful differentiation of Uveira berries according to ripening stage. To the best of our knowledge, no study has carried out on the volatomic composition of berries from endemic Uveira.

Keywords: *V. padifolium* L., Ripening, Volatile composition, HS-SPME/GC-qMS, PCA

1. Introduction

Vaccinium padifolium, locally called Uveira, is a shrub from the family of Ericaceae endemic to Madeira Island (Portugal). It grows at relative high altitudes (800 – 1700 m) and in local ethnopharmacology is used against colds bronchitis, dysentery, cough, among others [213, 265]. In fact, *Vaccinium* species and their its edible fruits (dark blueblack berries), have gained a remarkable worldwide interest due to their excellent and appealing properties, namely the such as color and size, as well as to the reported benefits on human health associated to with their consumption. These fruits, although not usually consumed directly, are used in a wide variety of foodstuffs such as breakfast cereals, dairy products, juices, jams, liquors, jellies, yogurts, beverages and in dietary supplement forms [93, 94].

Furthermore, a considerably growing body of evidence suggests that dietary intake of berries and berry phytochemicals has the potential to reduce the risk of various chronic disease conditions such as cancer, cardiovascular diseases, respiratory diseases, stroke, and cataracts, as well as delay aging [95, 96]. Other reports also point additional beneficial effects such as anti-diabetic and anti-arthritis effects [93, 266, 267]. These health-promoting properties are greatly associated to the antioxidant and anti-inflammatory activities of a multitude of berry bioactive phytochemical components, including anthocyanins, phenolic acids, stilbenes, tannins and carotenoids. The identification of other secondary metabolites, namely volatile organic metabolites (VOMs), may also be explored.

Although Uveira berry, aroma is widely recognized as an important attribute to fruit quality and consumer acceptance, a limited number of studies deal with the analysis of Uveira berries volatiles. This knowledge could be explored by food industries to improve the quality of endemic Uveira berries-based products. VOMs are biosynthesized through different metabolic pathways during fruit ripening [268]. Fatty acid metabolism lead to the formation of alcohols, aldehydes, ketones, acids, esters and lactones by lipoxygenase, α-oxidation and β-oxidation pathways [268]. In the same

way, amino acid metabolism lead to the formation of benzenoids (C6-C1), phenylpropanoids (C6-C3), aldehydes, acids, alcohols, esters, C6-C2 compounds by biotransformation of L-phenylalanine and other amino acids through routes that compete with each other [268]. Several factors such as the environmental conditions, the genetic component and the ripening stage influence the VOMs profile and bioactive compounds formation [269]. To date, the effect of development and ripening on flavor compound accumulation has been extensively investigated in different fruits. In strawberries, C6 aldehydes were the major compounds in immature fruits, while furanones and esters are the main compounds in mature fruits [270]. In avocados, sesquiterpenes are the most abundant volatiles in early stages decreasing during ripening [271]. Volatile compounds are not only responsible for the *V. padifolium* berry flavor, they also interact in the ecological network between plants and the environment and respond to stress conditions (e.g. herbivore attack or drought). Furthermore, terpenes are known to have bioactive properties (antimicrobial and anticarcinogen) [272].

Despite the studies conducted on the *V. padifolium* berries, there is no information available about the volatile composition of endemic Uveira berries cultivated at Madeira Island. Therefore, the establishment of the ripening volatomic pattern can provide important insights on the biochemical transformations underlying the ripening physiological and biochemical processes as a key step to improve crop quality and useful information to producers by the appropriate selection of harvesting conditions and date.

The main goal of this study is to investigate the evolution of volatile metabolites through ripening of Uveira berries, collected at Madeira Island, Portugal, helping to understand the significance of those compounds in the ripening process of this fruit. For this purpose, three different ripening stages were considered (green, break and ripe). After optimization of the most relevant SPME experimental parameters, a detailed volatile profile was done using gas chromatography–quadrupole mass spectrometry (HS-SPME/GC-qMS) data combined with multivariate statistical

analysis. To best of our knowledge there are no reported studies dealing with the effect of ripening stage on endemic Uveira berries volatile profile. The obtained results will provide a powerful resource for uncovering key components in the regulation of metabolic networks and give the important information for consumer-oriented management.

2. Material and methods

2.1. Chemical Reagents

All chemicals used were of analytical grade and obtained from several suppliers. Standards used for confirmation (purity level higher than 98.5 %) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and sodium chloride (99.5%) from Merck (Darmstadt, Germany). Deionised water was supplied from a Milli-Q water purification system (Millipore, Bedford, PA, USA). The retention index probe, an *n*-alkanes mixture containing C_8–C_{20} straight-chain alkanes, in hexane, was supplied from Fluka (Buchs, Switzerland). Helium, ultra-pure grade (Air Liquide, Portugal) was used as carrier gas in the GC system.

The SPME holder for manual sampling, the fibres coating used and the clear glass screw cap vials for SPME with PTFE/silica (film thickness 1.3 mm) septa were purchased from Supelco (Bellefonte, PA, USA). The SPME fibres were thermally conditioned in the GC injector according to the producer's recommendations, and daily for 10 min before first extraction.

2.2 Sample preparation

*B*erries from endemic cultivar *of V. padifolium* (Uveira) were collected in a forest park (Montado do Pereiro, 32º42'40 N, 16º53'00 W) dominated by exotic and indigenous tree situated at the mountains (1200 meters above sea level) of Madeira Island, with an approximate area of 1 ha, from plants with ≥20 years old. Uveira berries were harvested morning (between 8-9 a.m.) over three ripening stages (green, breaker and ripe) from six different plants to obtain a representative sample for each ripening

stage. Moreover, the samples grown in the same agroclimatic conditions and without any cultural intervention, minimizing the effect of different edaphoclimatic conditions on plant metabolism. Three lots of Uveira berries with 100gr at each different ripening stage were collected separately in sterile bags and immediately transported, under refrigeration (ca. 2-4ºC), to the laboratory, aliquoted (20 gr) and stored at -80 °C in amber vials until analysis.

2.3. HS-SPME optimization design

The development of HS-SPME procedure involves the optimization of experimental factors that most influence the process in order to improve the extraction efficiency [273]. The effect of three independent factors (*i*) nature of the fibres, (*ii*) sample amount (2.5-5 g) and (*iii*) the exposure time (10–50 min) of the fibre to the headspace, on the SPME isolation of Uveira berry volatiles, were assayed and evaluated. For all parameters, the best conditions were selected based on extraction efficiency performance expressed as number of isolated/identified volatile metabolites, total chromatographic area and reproducibility.

The SPME fibre optimization step was carried out by testing six commercially available silica SPME fibres with different polarities, thickness of stationary phase, retention abilities and coatings. The polymers: polydimethylsiloxane (PDMS, 100 µm), polyacrylate (PA, 85 µm) and poliethyleneglicol (PEG, 60 µm), were selected for absorption of volatiles (solid coatings). On the other hand, mixed-phase fibres such as divinylbenzene/carboxen on polydimethylsiloxane (DBV/CAR/PDMS 50/30 µm), carboxen/polydimethylsiloxane (CAR/PDMS, 75 µm) and polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 µm), were selected to represent the adsorption mechanism. All fibres were 1 cm long and were conditioned, prior to their first use, according to the manufacturer's instructions by heating into the injection port of the GC. Before each sampling, blank runs were carried out to ensure no carry-over of analytes from the previous extraction.

The influence of sample amount was evaluated using, 2.5 and 5.0 g of Uveira berries at ripe stage. SPME as a measure of free concentration of analytes, is an equilibrium extraction technique, therefore the selection of the optimum extraction time is a critical step. In order to improve the extraction efficiency of volatiles from Uveira berries, 10, 30 and 50 min of extraction times were evaluated and compared. All assays were carried out in triplicate.

2.4. Volatomic pattern establishment by HS-SPME/GC-qMS

For each ripening stage, 5.0 g of homogeneized Uveira berries, previously thawed, were placed into a 10 mL headspace glass vial (1/β≈0.5) containing a magnetic microstirring bar and covered with PTFE-lined silicon septa. 10 % (w/w) of NaCl was added to the sample matrix to decrease the solubility of volatile metabolites in the water phase (‘*salting-out*’ effect). HS-SPME extractions were carried out by exposing manually the SPME fibres to the headspace of the glass vial, for 30 min at 40 ºC. All the experiments were performed in triplicate under constant stirring (800 rpm) in order to improve the extraction, since the static layer resistant to mass transfer is destroyed (facilitating mass transfer from the bulk of the aqueous sample to the headspace). After extraction, the needle was pulled back into the needle, the whole fibre was removed from the vial, and subsequently put into the GC injection system for termal desorption of volatile metabolites.

The sorbed volatiles on the DVB/CAR/PDMS fibre were determined in an Agilent 6890N gas chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a BP-20 fused silica capillary column (60 m × 0.25 mm I.D. × 0.25 μm film thickness) and Agilent 5975 quadrupole inert mass selective detector. Splitless injections were used with helium as the carrier gas (Helium N60, Air Liquide, Portugal) at a constant flow rate of 1.0 $mL.min^{-1}$. The GC oven temperature program was set at an initial temperature of 60 ºC for 4 min, raised to 120 ºC at 1 ºC/min, held there for 4 min. Then raised to 220 ºC at 4 ºC/min and held there for 5 min. The injection and ion source temperatures were 250 and 220 ºC, respectively. The mass

spectrometer was operated in electron-impact (EI) mode at 70 eV. The electron multiplier was set to the auto tune procedure. Data acquisition was performed in scanning mode (mass range *m*/*z* 35–300; six scans per second). Acquisition was made using MSD ChemStation software (Agilent Technologies, Palo Alto, CA, USA). Chromatograms and spectra were recorded and processed using the Enhanced ChemStation software for GC-$_q$MS (Agilent). The identification of volatile metabolites was based on the comparison between the GC retention times (RT) of the chromatographic peaks with those, when available, of authentic standards (ST) run under the same conditions. MS fragmentation patterns were compared with those of pure compounds, and mass spectrum database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral database. Finally, confirmation was also conducted by determining the RI of each metabolite, using a C_8-C_{20} *n*-alkanes series. Experimental RI values were compared with values reported in the literature for similar chromatographic columns. Chromatographic peak areas, expressed in arbitrary units (a.u.) of area, were used as an indirect approach to estimate the relative content of each volatile metabolite. For semi-quantification purposes, each sample was injected in triplicate, and the chromatographic peak areas were determined by a reconstructed full-scan chromatogram using for each volatile metabolite some specific quantification ions: ionic fragment corresponding to peak base (100% intensity), molecular ion (M^+) and another characteristic ion for each molecule.

2.6. Data processing and statistical analysis

HS-SPME/GC-$_q$MS data matrix was evaluated and significant differences among the Uveira ripening stages, were investigated using SPSS program, version 22.0 (SPSS Inc., 2006). A test of Normality was carried out through a Shapiro-Wilk test. Outliers were determined either numerically (normalized values higher than 3 and lower than -3) and graphically (boxplot chart) for all factor's groups. The data was tested by one-way analysis of variance procedure followed by Bonferroni's Post Hoc test

when homogeneity of variances was assumed or Games-Howell's Post Hoc test when this assumption was not met. For all the analysis, a significance level of 5% ($p < 0.05$) was assumed to be statistically significant.

Principal component analysis (PCA) was processed to reduce the dimensionality of dataset, and in addition can reveal those variables, or combination of variables that determine some inherent structure in the data. The number of principal components was based on the eigenvalue criterion and the total variance explained. PCA reduces the dimensionality of the original data matrix retaining the maximum amount of variability [274, 275]. This reduction allows visualization of the different ripening stages of Uveira berries in a two-dimensional space and identifying the directions in which most of the information is retained. With this unsupervised technique it is possible to explain the differences between ripening stages and visualize the variables that most contribute to those differences [274]. The stability of the model was evaluated by "cross validation" in order to test the prediction capacity of the discriminant model [275, 276]. PLS-DA does not allow for other response variables than the one for defining the groups of individuals.

3. Results and discussion

3.1. Optimization of HS-SPME extraction

The optimization step of HS-SPME methodology is crucial for good extraction efficiency. Therefore, some key experimental factors, namely time required for the target analytes to reach equilibrium, nature of fibre coating and sample amount, were optimized in order to investigate the evolution of the Uveira berries volatiles through ripening. This procedure was conducted using a univariate experimental design, considering one parameter at a time, while keeping all other variables.

3.1.1. SPME Fibre Selection

Six SPME fibres: PDMS, PEG, PA, CAR/PDMS, PDMS/DVB and DVB/CAR/PDMS, were chosen for assessing their performance on VOMs extraction emitted by Uveira

berries. The effectiveness of SPME fibres was assessed by semi-quantitative and qualitative analysis of chromatograms. Fig. 3.4.1A shows the comparison of the extraction efficiency of different fibre coatings towards volatiles Uveira berries composition. Non-polar (PDMS) and polar (PEG and PA) fibre coatings revealed fair ability for the extraction of Uveira berries volatiles compared to semi-polar fibre coatings (i.e., DVB/CAR/PDMS, CAR/PDMS and PDMS/DVB) (Fig. 3.4.1A). The low ability of PEG and PA (Fig. 3.4.1A) coatings for the terpenes and sesquiternes, explain the observed trend. Considering the number of identified volatiles, the best performance was achieved by DVB/CAR/PDMS (58 metabolites) followed by CAR/PDMS (33 metabolites) > PDMS/DVB (18 metabolites) > PA (18 metabolites) > PEG (17 metabolites) and PDMS (13 metabolites), respectively. According to the results, DVB/CAR/PDMS coating showed the best performance for isolation of Uveira berry volatiles achieving the highest number of extracted volatiles, highest signal intensity and best reproducibility (RSD lower than 10%). DVB/CAR/PDMS coating (molecular weight ranging from 40 to 275) combines the absorption properties of the liquid polymer with the adsorption properties of porous particles, which contains macro (>500 Å), meso (20–500 Å) and microporous (2–20 Å) and has bi-polar properties. The mutually synergetic effect of adsorption and absorption of the stationary phase explains its high retention capacity compared to a coating that consists only of PDMS [277-279].

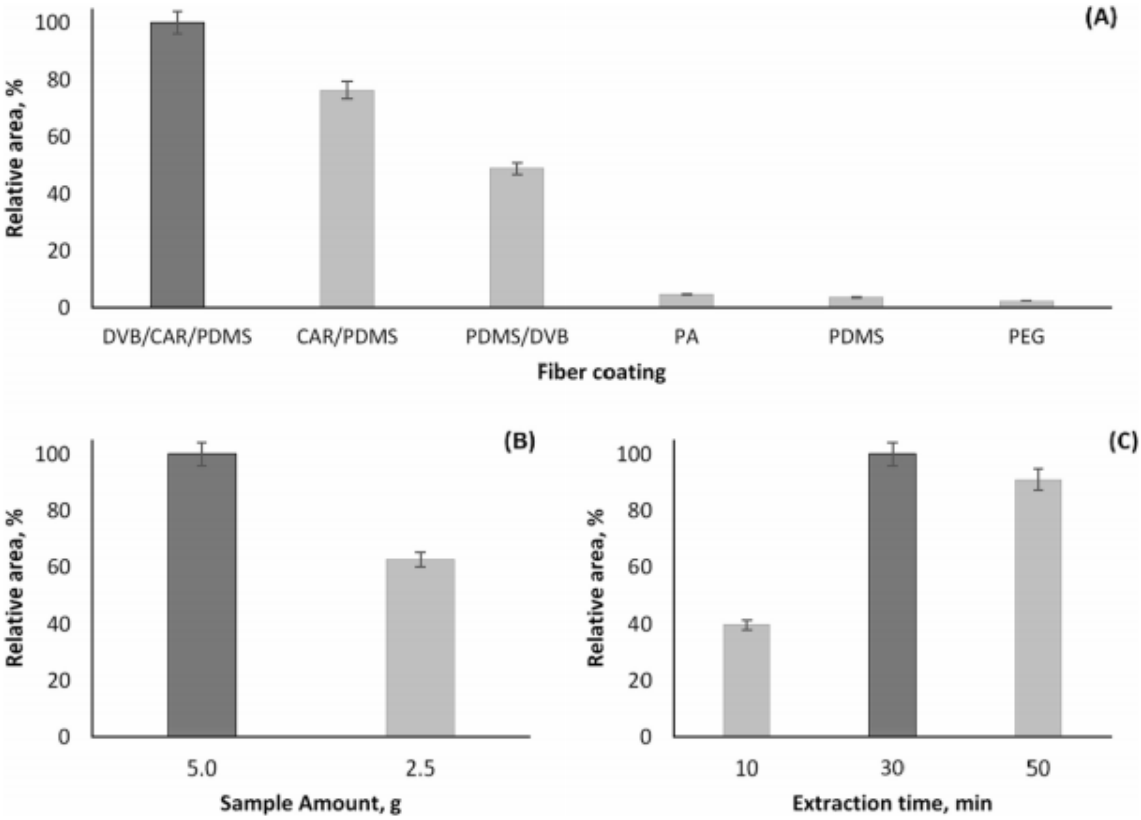


Fig. 3.4.1. Optimization of the HS-SPME-influencing extraction parameters on the extraction efficiency of volatile compounds from endemic Uveira (*V. padifolium*) berries (A) Influence of fibre coating (best fibre: DVB/CAR/PDMS); (B) Influence of sample amount (best sample amount: 5 g using 10% w/w NaCl, DVB/CAR/PDMS fibre coating and 30 min of extraction) and (C) Influence of extraction time (best extraction time: 30 min, using 10% w/w NaCl, DVB/CAR/PDMS fibre coating and 5g of sample). Thermal desorption of metabolites was carried out at 250 °C for 6 min. The results are the means of triplicates of the total areas.

3.1.2 Sample amount

Theoretically, optimum sample amount can be selected based on the estimated sample/headspace/coating distribution constant. Generally, the amount of extracted analyte increases with the sample content up to a point, after which the sensitivity does not increase with further increasing in sample amount [280, 281]. For this experiment, two different amounts (2.5 and 5.0 g) of Uveira berries, at ripe stage, were used. The effect of the sample amount on the extraction efficiency of VOMs is

shown in Fig. 3.4.1B. The best performance was achieved using 5.0 g of sample, corresponding to a ratio of the volume of the liquid phase to the headspace volume (1/β) of about 0.5. For this reason, the sample amount used in the following experiments was 5.0 g.

3.1.3. Extraction time

SPME is an equilibrium extraction technique [282, 283] therefore the time required for volatiles sampling is a parameter that also needs to be optimized. The influence of the extraction time was evaluated by exposing DVB/CAR/PDMS fibre into the headspace of ripe Uveira berries for differentiated times ranging between 10 to 50 min. As shown in Fig. 3.4.1C, the best performance was obtained using 30 min of extraction. Since the extraction efficiency can be affected by the mass transfer kinetics of the volatiles in the HS-SPME procedure, the best time should correspond to a period of equilibrium between analyte and fibre coating. In general, a longer extraction period leads to an increase in the signal intensity of volatile compounds (until the equilibrium is achieved). However, increasing the extraction time 50 min, no significant changes were observed (Fig. 3.4.1C). In addition, the number of VOMs isolated/identified using 30 and 50 min of extraction time is almost the same. Consequently, and in order to implement an expeditious procedure, 30 min was the time selected for extraction of VOMs from Uveira fruits.

3.2 *Fingerprint of volatile profile of Uveira berries at different ripening stages*

The fingerprint of the variation of volatile metabolites of Uveira berries through ripening stage, using HS-SPME/GC-qMS under optimal analytical conditions (i.e.: DVB/CAR/PDMS fibre; 30 min extraction time; 40 ± 1 ºC extraction temperature; salt addition: NaCl (10 % (w/w)), 5.0 g of sample, GC-qMS see section 2.4), is illustrated in Fig. 3.4.2.

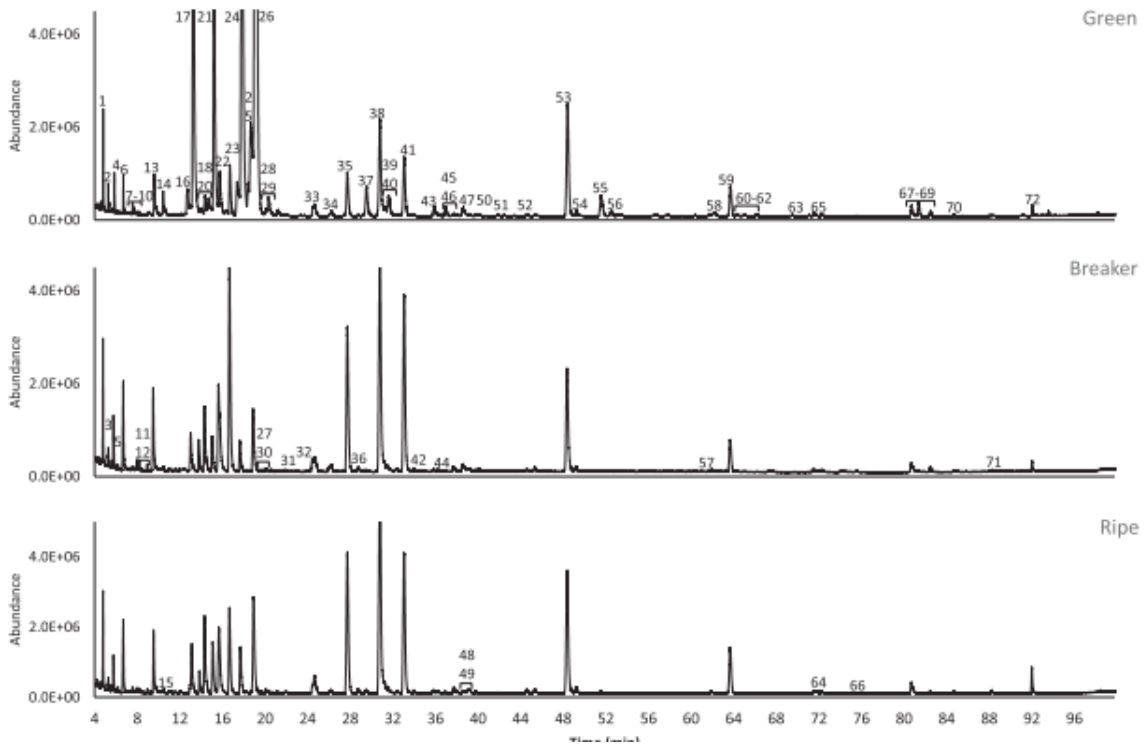


Fig. 3.4.2. Chromatographic profile of endemic Uveira fruits at different maturation stages, by HS-SPME using DVB/CAR/PDMS fibre (For identification numbers see Table 3.4.1).

HS-SPME was used to simultaneously extract and concentrate VOMS from berries through a short tima (30 min) and low temperature (40 ºC), without any solvent or chemical addition minimizing the formation of artifacts.

GC-MS chromatographic profiles revealed the complexity of the matrix indenpendently of the ripening stage. The list of volatile metabolites identified at different ripening stages of Uveira berries is presented in Table 3.4.1. Altogether, 72 VOMs were identified in the studied samples being distributed over monoterpenes and sesquiterpenes, higher alcohols, aldehydes, ketones, and fatty acids (Fig. 3.4.2, Table 3.4.1). Monoterpenes (24 identified volatiles, ranging between 31-37 % of total volatile fraction) were the most abundant chemical group found in the investigated endemic Uveira berries, followed by higher alcohols (16 volatiles, ranging between 19-28 %), esters (13 volatiles, ranging between 15-23 %), sesquiterpenes (7 volatiles, around 11 % of the volatile fraction from green stage), aldehydes (5 volatiles, ranging

between 7-8 %), ketones (3 volatiles, ranging around 5 %), fatty acids (2 volatiles, ranging 3-4 %) and others (2 volatiles, ranging between 3-4 %) (Fig. 3.4.3A).

Table 3.4.1. Volatile metabolites identified at different ripening stages of Uveira berries using HS-SPMEDVB/CAR/PDMS/GC–MS methodology (extraction time: 30 min: extraction temperature: 40 °C; desorption of metabolites: 250 °C for 7 min).

#	[a] RT	Common Name	[b] KI Exp	[c] KI Teo	Ions	Maturation Stage (× 10^6)		
						Green	Breaker	Ripe
	Higher Alcohols							
4	6.05	Ethanol *	927	926	**31**; 45; 46	16.9 ^{e}	27.3 ^{f}	44.2 ^{g}
5	6.19	Pentyl Alcohol [(c)]	936	941	**55**; 70; 42; 41	n.d. ^{e}	0.6 ^{f}	0.8 ^{g}
15	11.37	sec-Isoamyl alcohol [(c)]	1121	1124	**45**; 55; 27; 73	n.d.	n.d.	2.1 ^{e}
33	24.84	2-Heptanol	1319	1319	**45**; 55; 27	10.3 ^{e}	13.2 ^{f}	22.1 ^{g}
35	27.93	1-Hexanol	1353	1356	**56**; 43; 55; 31	32.3 ^{e}	101.9 ^{f}	128.7 ^{g}
36	28.96	trans-3-Hexenol	1364	1366	**41**; 67; 55; 82	0.7 ^{e}	2.2 ^{f}	3.2 ^{g}
38	31.01	cis-3-Hexenol	1384	1386	**67**; 41; 82; 55	52.2 ^{e}	121.4 ^{f}	161.3 ^{g}
41	33.28	trans-2-Hexenol	1405	1406	**57**; 41; 82;67	49.0 ^{e}	145.3 ^{f}	155.1 ^{g}
42	34.19	cis-2-Hexenol	1415	1418	**57**; 41; 67; 82	n.d.	1.8 ^{f}	2.7 ^{f}
46	37.97	3-Octenol	1452	1451	**57**; 72	2.6 ^{e}	4.9 ^{f}	8.0 ^{g}
48	39.36	6-Methylhept-5-en-2-ol	1465	1466	**91**; 41; 69; 55	0.7 ^{e}	0.8 ^{f}	1.4 ^{g}
51	42.11	2-Ethylhexanol	1489	1489	**57**; 41; 43; 56	1.3 ^{e}	1.6 ^{f}	1.6 ^{g}
54	49.48	1-Octanol	1558	1561	**55**; 56; 41; 69	1.3 ^{e}	1.4 ^{f}	2.6 ^{g}
64	72.03	1-Decanol	1763	1763	**55**; 41; 56; 69	n.d.	n.d.	0.9 ^{e}
69	82.71	Phenylcarbinol	1875	1876	**108**; 79; 107; 77	2.2 ^{e}	1.9 ^{f}	1.3 ^{g}
70	84.88	Benzyl Carbinol	1899	1899	**91**; 92; 122	1.6 ^{e}	1.4 ^{f}	2.9 ^{g}
		∑ Areas by variety				**171.1** ^{e}	**425.7** ^{f}	**538.8** ^{g}
		Nº of Compounds				**12**	**14**	**16**
	Aldehydes							
3	5.80	Butyraldehyde	911	912	**57**; 41; 58; 86	0.4 ^{e}	1.8 ^{f}	1.8 ^{f}
13	9.78	Hexanal	1082	1083	**44**; 56; 41; 43	9.4 ^{e}	18.2 ^{f}	18.5 ^{f}
23	16.91	2-Hexenal	1219	1220	**41**; 55; 69; 83	13.8 ^{e}	59.6 ^{f}	32.3 ^{g}
31	22.07	Octanal [(c)]	1287	1287	**43**; 44; 56; 84	0.6 ^{e}	n.d.	n.d.
52	44.84	Benzaldehyde	1515	1515	**105**; 77; 106; 51	1.2 ^{e}	1.5 ^{f}	3.9 ^{g}
		∑ Areas by variety				**25.4** ^{e}	**81.1** ^{f}	**56.5** ^{g}
		Nº of Compounds				**5**	**4**	**4**
	Fatty acids							
71	88.49	trans-2-Hexenoic acid	1972	1967	**73**; 42; 55; 99	0.4 ^{e}	0.6 ^{f}	1.6 ^{g}
72	92.25	Octanoic Acid	2045	2050	**60**; 73; 43; 55	3.7 ^{e}	3.7 ^{e}	10.7 ^{f}
		∑ Areas by variety				**4.1** ^{e}	**4.3** ^{e}	**12.3** ^{f}
		Nº of Compounds				**2**	**2**	**2**
	Esters							
2	5.47	Ethyl Acetate	882	885	**43**; 61; 70	9.7 ^{e}	5.4 ^{f}	3.8 ^{g}

9	8.19	Ethyl butanoate	1032	1036	**43**; 71; 29; 88	0.5 ^{e}	2.0 ^{f}	1.1 ^{g}
11	8.65	Ethyl α-methylbutyrate	1048	1046	**57**; 102; 85; 41	n.d.	0.6 ^{e}	0.8 ^{f}
12	9.13	Ethyl isovalerate	1063	1062	**88**; 57; 85; 41	0.1 ^{e}	1.2 ^{f}	0.9 ^{g}
27	19.80	Z-Methyl 3-hexenoate	1259	1253	**41**; 68; 74; 59	n.d.	0.5 ^{e}	0.9 ^{f}
30	20.80	Hexyl acetate	1272	1274	**43**; 56; 84; 69	n.d.	1.5 ^{e}	1.4 ^{f}
32	24.63	3-Hexen-1-ol, acetate	1317	1314	**67**; 43; 82	5.5 ^{e}	8.0 ^{f}	8.5 ^{g}
39	31.47	Methyl caprylate	1388	1387	**74**; 87; 43; 55	5.6 ^{e}	5.7 ^{e}	8.6 ^{e}
43	36.11	Ethyl caprylate	1435	1435	**88**; 101; 127	3.5 ^{e}	0.8 ^{f}	1.5 ^{g}
47	38.81	cis-3-Hexenyl butyrate $^{(c)}$	1460	1459	**67**; 82; 71; 43	6.0 ^{e}	4.2 ^{f}	0.9 ^{g}
50	40.37	trans-2-Hexenyl Butyrate	1474	1475	**71**; 43; 55; 67	1.2 ^{e}	2.0 ^{f}	0.2 ^{g}
57	62.11	α-Methylcaproic acid	1673	1625	**74**; 57;87;43	2.5 ^{e}	2.7 ^{f}	3.1 ^{g}
63	71.77	Methyl salicylate	1761	1758	**120**; 92; 152; 121	3.2 ^{e}	2.1 ^{f}	3.4 ^{e}
		∑ Areas by variety				**37.8 ^{e}**	**36.7 ^{f}**	**35.1 ^{g}**
		Nº of Compounds				**10**	**13**	**13**
	Ketones							
1	4.88	Acetone	811	813	**43**; 58	2.6 ^{e}	3.1 ^{f}	4.2 ^{g}
19	14.56	2-Heptanone	1182	1184	**43**; 58; 71	14.1 ^{e}	49.2 ^{f}	75.9 ^{g}
34	26.47	Methyl heptenone	1338	1339	**43**; 69; 108; 55	2.8 ^{e}	3.5 ^{f}	2.7 ^{e}
		∑ Areas by variety				**19.6 ^{e}**	**55.9 ^{f}**	**82.9 ^{g}**
		Nº of Compounds				**3**	**3**	**3**
	Monoterpenes							
7	7.77	α-Pinene $^{(c)}$	1017	1019	**93**; 92; 91; 77	2.0 ^{e}	0.4 ^{f}	0.4 ^{g}
8	7.86	α-Thujene	1021	1023	**93**; 91; 77; 92	1.3 ^{e}	0.5 ^{f}	0.4 ^{g}
14	10.63	β-Pinene $^{(c)}$	1105	1108	**93**; 41; 69; 77	9.8 ^{e}	n.d.	n.d.
16	12.91	β-Phellandrene	1153	1163	**93**; 77; 91; 136	10.6 ^{e}	n.d.	n.d.
17	13.48	β-Myrcene	1163	1165	**93**; 41; 69; 79	284.5 ^{e}	14.6 ^{f}	29.5 ^{g}
18	14.28	2-Carene $^{(c)}$	1177		**93**; 121; 136; 91	1.3 ^{e}	n.d.	n.d.
20	14.90	Limonene $^{(c)}$	1188	1189	**68**; 93; 67; 79	3.2 ^{e}	n.d.	n.d.
21	15.41	D-Limonene	1196	1200	**68**; 93; 67; 136;121	93.9 ^{e}	10.8 ^{f}	19.6 ^{g}
22	15.95	Eucalyptol	1205	1209	**93**; 43; 55; 108	9.3 ^{e}	7.6 ^{f}	7.0 ^{g}
24	18.03	trans-β-Ocimene	1236	1232	**93**; 91; 92; 79	406.8 ^{e}	15.2 ^{f}	31.0 ^{f}
25	18.62	γ-Terpinene	1244	1243	**93**; 91; 136; 121	7.6 ^{e}	n.d.	n.d.
26	19.29	cis-β-Ocimene	1253	1256	**93**; 91; 79; 77	826.4 ^{e}	28.4 ^{f}	60.5 ^{g}
28	20.32	m-Cymene	1266	1270	**119**; 134; 91	16.6 ^{e}	1.7 ^{f}	2.5 ^{g}
29	20.54	o-Cymene $^{(c)}$	1269	1268	**119**; 134; 91	16.9 ^{e}	n.d.	n.d.
37	29.71	Neo-allo-ocimene	1371	1378	**121**; 105; 136; 79	22.0 ^{e}	1.5 ^{f}	3.3 ^{g}
40	31.81	trans-allo-ocimene	1391	1400	**121**; 136; 105; 79; 91	10.4 ^{e}	1.1 ^{f}	2.6 ^{g}
44	36.42	Linalool oxide	1438	1443	**59**; 94; 43; 111	0.9 ^{e}	1.0 ^{e}	1.8 ^{f}
45	37.15	Cosmene	1445	1460	**119**; 91; 134; 77	5.0 ^{e}	0.6 ^{f}	0.5 ^{f}
49	39.99	Dihydromyrcenol	1471	1471	**59**; 43; 55; 67	1.3 ^{e}	1.4 ^{e}	2.8 ^{f}
53	48.64	Linalool	1551	1552	**71**; 93; 41; 55	40.0 ^{e}	39.8 ^{e}	60.1 ^{f}
59	63.92	α-Terpineol	1689	1689	**59**; 93; 121; 136	12.0 ^{e}	11.6 ^{e}	23.8 ^{f}
65	72.51	Citronellol	1767	1764	**69**; 41; 81; 67	0.8 ^{e}	0.8 ^{e}	1.2 ^{f}
66	75.75	cis-Geraniol	1795	1794	**69**; 41; 93; 68	0.4 ^{e}	0.4 ^{e}	0.9 ^{f}
67	80.91	trans-Geraniol	1855	1854	**69**; 41; 93	5.7 ^{e}	4.7 ^{f}	8.1 ^{g}

		∑ Areas by variety				**1788.8** [e]	**142.1** [f]	**256.0** [g]
		Nº of Compounds				**24**	**18**	**18**
	Sesquiterpenes							
55	51.75	Caryophyllene	1578	1580	**93**; 133; 91; 69	5.0 [e]	n.d.	n.d.
56	52.76	Aromandendrene [(c)]	1587	1600	**161**; 41; 93; 107	1.1 [e]	n.d.	n.d.
58	62.40	Viridiflorene [(c)]	1676	1671	**107**; 93; 161; 119	0.8 [e]	n.d.	n.d.
60	64.56	α-Guaiene	1694	1652	**105**; 93; 79; 147	0.3 [e]	n.d.	n.d.
61	65.22	α-Selinene [(c)]	1700	1705	**189**; 204; 93; 107	0.5 [e]	n.d.	n.d.
62	66.40	Bicyclogermacrene [(c)]	1711	1706	**121**; 93; 41; 107	0.9 [e]	n.d.	n.d.
68	81.59	Curzerene [(c)]	1862	1874	**108**; 148	8.5 [e]	n.d.	n.d.
		∑ Areas by variety [(c)]				**17.1** [e]	**n.d.**	**n.d.**
		Nº of Compounds				**7**	**0**	**0**
	Others							
6		2-Ethylfurane	949	945	**81**; 96; 53	0.4 [e]	1.8 [f]	2.7 [g]
10		Toluene	1039	1037	**91**; 92	0.9 [e]	1.5 [f]	1.6 [g]
		∑ Areas by variety				**1.4** [e]	**3.3** [f]	**4.4** [g]
		Nº of Compounds				**2**	**2**	**2**
		Total Compounds identified by maturation stage				**65**	**56**	**58**
		Total ∑ Area				**2065.4**	**749.1**	**986.0**
		% RSD (n = 3)						

[a]RT – Retention time (minutes); KI – Kovats Index ([b]Exp – experimental, [c]Lit – theoretical KI value reported in literature.)* Not higher alcohol n.d. – not detected. Means followed by different letters (e,f,g) for a given parameter are significantly different at P < 0.05 (Bonferroni and Games-Howell [(d)] tests).

The qualitative composition at each ripening stage and the number of identified volatiles is slightly different according to the ripening stage of Uveira berries (Fig. 3.4.3A and 3.4.3B). Its formation is result of different metabolic pathways (as direct or indirect products) [268].

Some volatile compounds were found at all ripening stages (Table 3.4.1), while others were found at a specific ripening stage (Table 3.4.1). In fact, 51 from the 72 volatiles compounds identified in targeted Uveira berries ripening stages are common to all stages (see Table 3.4.1). On the other hand, 14 of identified volatiles, mainly mono- and sesquiterpenes, were found only at green ripening stage, whereas some higher alcohols, such as sec-Isoamyl alcohol and 1-decanol, were found only at ripe stage. Significant differences ($p<0.05$) were observed for all chemical groups according to the ripening stage (Table 3.4.1). Earlier studies using *V. ashei* and *V.*

stamineum [284, 285] showed that the volatile composition change according the ripening stage.

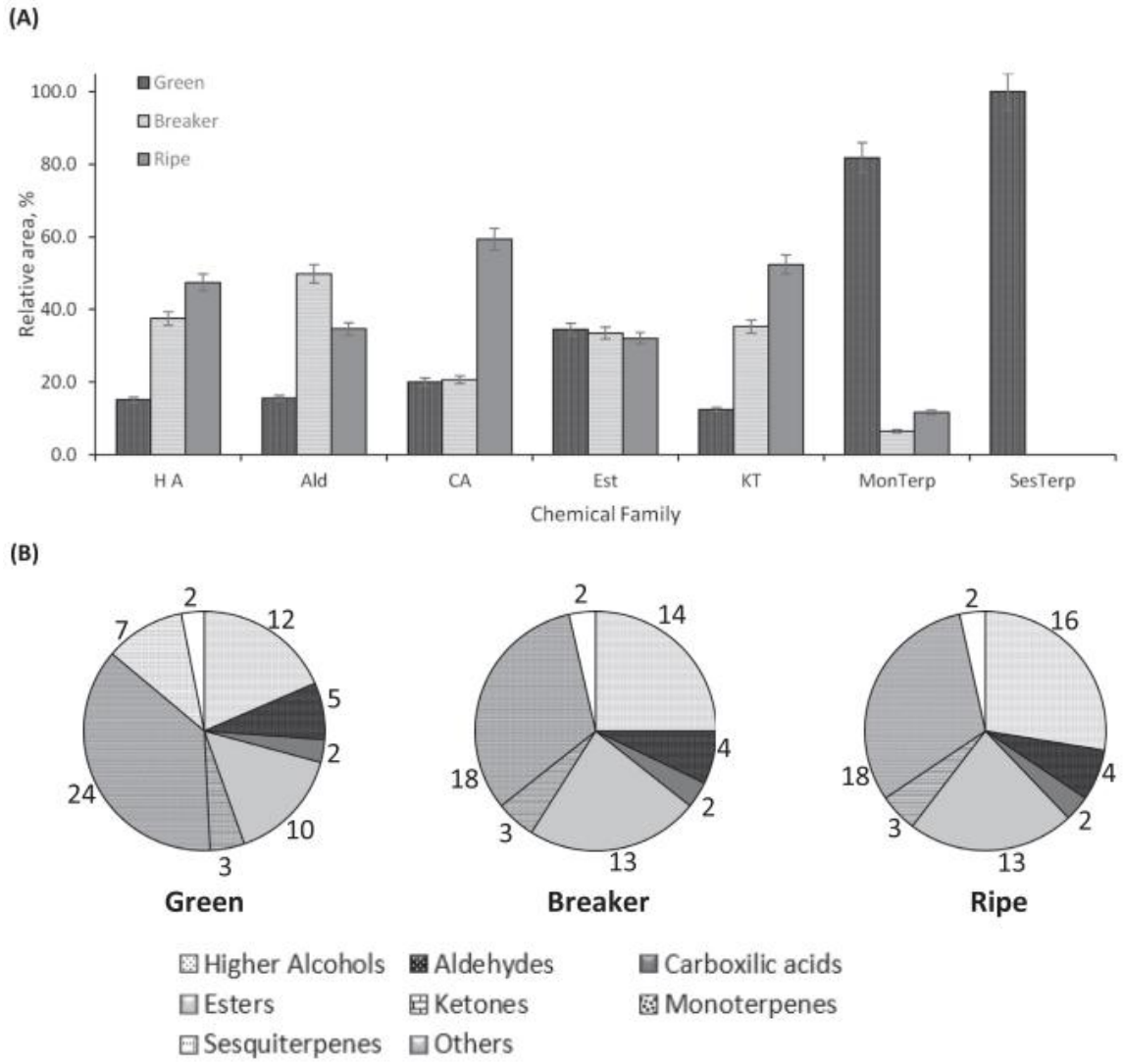


Fig. 3.4.3. (A) Volatile profile of endemic Uveira berries distributed by chemical groups; (B) Number of volatile compounds identified for each ripening stage (green, break and ripe) after extraction by HS-SPME according the chemical group.

Regarding the volatile profile of Uveira according to ripening stage (Fig. 3.4.3, Table 3.4.1) the number of identified volatiles was similar between stages (65 identified compounds for green, 56 for breaker and 58 for ripe stage). However, the volatile expression of different chemical groups changes during ripening. At green stage, it is clear that the monoterpenes have a high expression in terms of volatile profile

(with 24 from 65 volatile compounds identified), followed by higher alcohols (12 identified compounds), esters (10 compounds), sesquiterpenes (7 compounds), aldehydes (5 compounds), ketones (3 compounds) and carboxylic acids (2 compounds). The most abundant volatile identified at green stage was *cis*-β-ocimene. This terpene accounts for more than 40 % of the volatile profile of endemic Uveira berries, which might contribute to a *citrus* and *herbal* aroma of green Uveira berries [286]. [287] related this metabolite to a large use in flavors, food supplement fragrances and as building block for pharmaceuticals. [288] also related this metabolite with anti-tumour activity. Furthermore, *trans*-β-ocimene (*herba*l notes) and β-myrcene (*herbaceous, resinous, green, balsamic, fresh hops* notes), are also present in high percentage, ranging from 14 to 20% from the volatile fraction. Regarding the presence of β-myrcene, [289] showed its beneficial effects against Ischemia/Reperfusion-Mediated Oxidative (I/R) and Neuronal Damage in the brain. They clearly indicated that β-myrcene treatment ameliorates the neurodegenerative effects caused by global cerebral I/R in C57BL/J6 mice and concluded that β-myrcene attenuates the neuronal damage caused by global cerebral I/R in the brain. This effect is associated with its antioxidant properties and is correlated with a decrease in oxidative stress [289]. In addition, D-limonene contributes to approximately 5 % of the volatile composition of Uveira green berries. This compound imparts a citrus aroma and is strongly related to health benefits. In fact, [290] demonstrated for the first time that D-limonene can inhibit proliferation of colorectal cancer cells, whereas [291] provides evidence that D-limonene protects against the development of dyslipidemia and hyperglycemia in HFD-fed mice. Moreover, D-limonene improves insulin resistance and regulates lipid profiles, which appear to be mediated through the activation of PPARα and the inhibition of LXRβ signalling [291]. Furthermore [292] showed that administration by inhalation of D-limonene exerted anxiolytic-like effects in the elevated plus maze test, but not related to benzodiazepine receptors.

At breaker stage of *Uveira* berries, monoterpenes are also the major chemical group (18 identified monoterpenes of 56 compounds). Higher alcohols were the second

most abundant chemical group (14 identified compounds), followed by esters (13 identified compounds) and aldehydes (4 identified compounds). The remaining chemical groups maintained the number of identified compounds at green ripening stage. Sesquiterpenes were not identified at this ripening stage (Fig. 3.4.4).

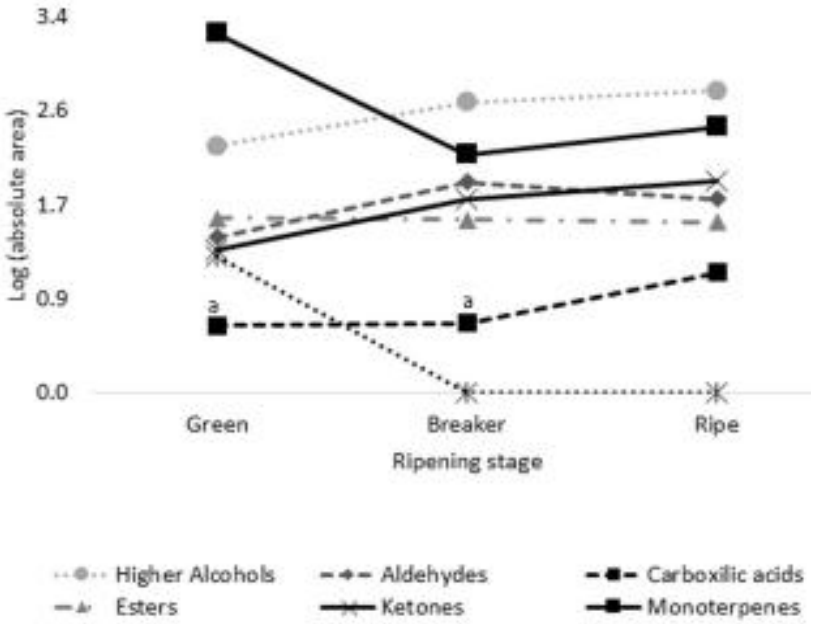


Fig. 3.4.4. Evaluation of the ripening effect on the principal chemical groups identified in endemic Uveira berries

Trans-2-hexenol is the most abundant volatile, accounting for around 19% of the volatile fraction of breaker berries, followed by *cis*-3-hexenol (16% of the total volatile composition). These alcohols impart a *green* and *grassy* aroma notes. According [293] they are derived from linolenic acid via lipoxygenase activity and are, therefore, indicators of the presence of free fatty acids classified as essential to the human diet. Moreover [294] showed that the exposure of the mouse to *cis*-3-hexenol induced anxiolytic behavior in the elevated plus maze test, which demonstrate the pharmacological potential of this compound. 1-Hexanol (~ 14 % of volatile profile) formed by the breakdown of linoleic acid [293] contribute with characteristics notes like *fruity*, *alcoholic* and *sweet*. (E)-2-Hexenal, responsible for the *apple*, *fruity*, *green*, *herbal*

and *leafy* aroma characteristics [286]. was also identified at significant level (8 % of the volatile composition of breaker berries). This metabolite was reported by [295] as having a significant inhibitory effect against pathogen microorganisms isolated from raw materials (*E. coli, S. enteritidis,* and *L. monocytogenes*) when inoculated in both model and real systems at low concentration (20 ppm).

The prominent contribution from the esters, was given by 2-heptanone (~7 %). This compound imparts a *banana-like, fruity* odor. Belonging to the class of terpenes, linalool (~ 5 % of volatile composition) contributes with *floral notes, wood, spicy, and lavender* notes. Linalool is one of the volatile compounds most investigated by scientists [296] due to their biological properties like sedative, anxiolytic, anticonvulsant, analgesic, antidepressant and anti-inflammatory activity [296, 297].

The ripe ripening stage is characterized by high levels of monoterpenes (18 identifed volatile compounds) and higher alcohols (16 of 58 identified compounds) which may related to the enzymatic activity characteristic of the ripe fruits [298]. Esters are the third most abundant chemical group with 13 identified volatile compounds followed by aldehydes (4 identified volatile compounds), and other chemical groups which includes ketones, carboxylic acids and furans (7 identified volatiles). Similarly, to the observed at breaker stage, in ripe berries the most abundant volatiles are *cis*-3-hexenol and *trans*-2-hexenol (16 % of volatile composition) followed by 1-hexanol, 2-heptanone and linalool (with 13 %, 8 % and 6 % of volatile composition). At this ripening stage, *cis*-β-ocimene, was also identified in significant abundance (around 6 % of volatile profile).

With this result, although the differences in terms of expression of the chemical families during the maturation, is possible observe the bioactive potential from berries of *V. padifolium* in all maturation stages.

3.3 Multivariate Analysis

To better understand the usefulness of the volatile composition to differentiate between the three ripening stages of Uveira berries and detect the potential

relationships/variables responsible for differentiation, a principal component analysis (PCA) was performed, using the normalized data, considering the principal components (PCs) with eigenvalues > 1. The data, presented as a bidimensional plot of sample scores in the space defined by the two first PCs, enables the formation of three differentiated clusters according to ripening stage (Fig. 3.4.5). Figure 3.4.5A demonstrates a clear separation between ripening stages of Uveira berries.

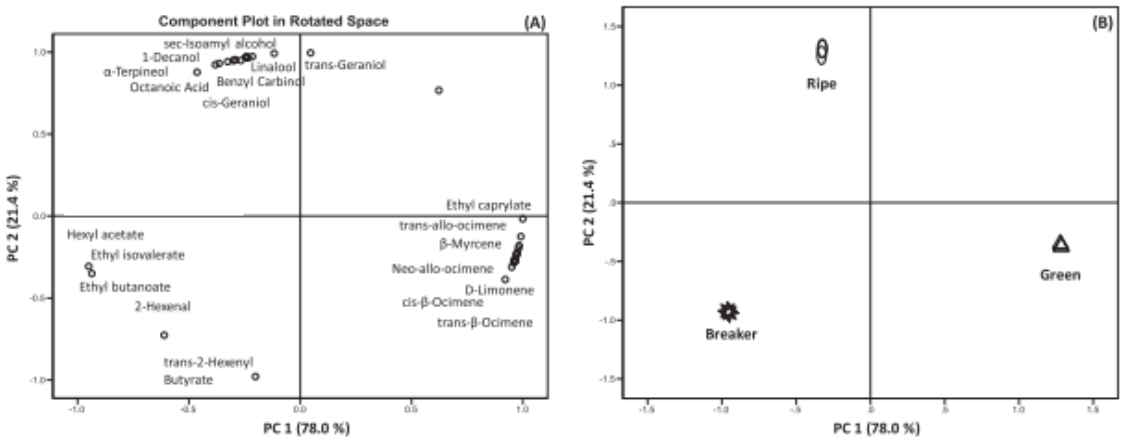


Fig. 3.4.5. PC1 and PC2 scatter plot of the main sources of variability between the different reipening stages of berries from endemic Uveira (A) Relation between the compounds (loadings) and (B) Distinction between the samples (scores).

The first three PCs explain about 99.4 % of the total variance (Supplementary Table 3.4.1.). The first principal component (PC1) explains 78.0 % of the variance and separates the ripe and breaker stages from the green ripening stage. The most relevant volatiles contributing to the separation are ethyl caprylate (1.000), ethyl isovalerate (-0.994), *trans*-allo-ocimene (0.991), D-limonene (0.984), neo-allo-ocimene (0.981), hexyl acetate (-0.975), *m*-cymene (0.974), β-myrcene (0.974), *cis*-β-ocimene (0.971), *trans*-β-ocimene (0.970), ethyl butanoate (-0.950) and 2-hexenal (-0.950). The second principal component (PC2), 21.4 % of the total variance, was strongly characterized by *trans*-geraniol (0.995), benzyl carbinol (0.993), *trans*-2-hexenyl

butyrate (-0.979), α-terpineol (0.973), sec-isoamyl alcohol (0.973), 1-decanol (0.969), octanoic acid (0.968), *cis*-geraniol (0.968) and linalool (0.966).

Partial Least Squares Discriminant Analysis (PLS-DA) was performed in order to obtain the separation between groups of observations, to understand which variables carry the class separating information [275, 276]. Using PLS-DA the relevant sources of data variability are modelled by the so-called Latent Variables (LVs), which are linear combinations of the original variables and, consequently, it allows graphical visualization. With this methodology is possible to obtain the probability of each sample belonging to a specific ripening stage. Two statistical significant functions were obtained with eigenvalues 52073 and 8027. The first function (F1) account for 86.6 % of the variability and the second function (F2) for 13.4 %. A clearly separation of the samples according the ripening stage can be observed in Supplementary Fig. 3.4.1. (Suplementary Material). The prediction capacity of the discriminant model was evaluated by the ''leave-one-out'' cross-validation (Supplementary Table 3.4.2. Supplementary Fig. 3.4.1. (Suplementary Material)). The discriminant analysis model based on volatile profile of Uveira berries at different ripening stage was correctly classified with 100 % of the observations based on cross-validation.

4. Conclusions

In this study, the evolution of volatile compounds of endemic Uveira berries was investigated. HS-SPME/GC-qMS revealed a powerful methodology for the establishment of the volatomic profile of Uveira berries providing an appropriate and selective way to better understand the volatile composition changes during Uveira berries ripening. A detailed anlysis of the obtained chromatograms allowed the unequivocal identification (using the KI, NIST database and in some cases pure standards) of 72 volatile metabolites distributed by targeted ripening stages, revealing significant differences among them. The main volatile compounds identified in Uveira berries belonging to monoterpenes, followed by higher alcohols

and esters. From 72 identified metabolites, 51 are common to all ripening stages, with differences in terms of abundance. Some variations on volatile profile during ripening were observed. The accumulation of the main compounds derived from carotenoids-cleavage deacrease dramatically during ripening in opposite to the increase of alcohols, fatty acids and ketones, revealing that several biochemical transformations take place during ripening. As far we know, this is the first study reporting the volatomic pattern of Uveira berries through ripening stages providing important insights on the biochemical transformations underlying the ripening physiological and biochemical processes as a key step to improve crop quality and useful information to producers.

Sub-Section 3.5. Tangerines cultivated on Madeira island – a high throughput natural source of bioactive compounds

foods

MDPI

Article

Tangerines Cultivated on Madeira Island—A High Throughput Natural Source of Bioactive Compounds

José A. Figueira [1], Priscilla Porto-Figueira [1], Jorge A. M. Pereira [1] and José S. Câmara [2,*]

Published in:

Foods (Impact Factor 4.350)

José A. Figueira, Priscilla Porto-Figueira, Jorge A.M. Pereira, José S. Câmara

http://dx.doi.org/ 10.3390/foods9101470

Abstract

Tangerines (Citrus reticulata) are popular fruits worldwide, being rich in many bioactive metabolites. The setubalense variety cultivated on Madeira Island has an intense aroma easily distinguishable from other tangerines, being traditionally used to enrich several foods and beverages. Nonetheless, setubalense volatile composition has never been characterized and we aimed to unveil the bioactive potential of peels and juices of setubalense tangerines and compare them with the murcott variety grown in Portugal mainland. Using headspace solid-phase microextraction coupled to gas chromatography mass spectrometry (HS-SPME/GC-MS), we identified a total of 129 volatile organic metabolites (VOMs) in the juice and peels, with D-limonene, γ-terpinene, β-myrcene, α- and β-pinene, o-cymene and terpinolene the most dominant in both cultivars. In contrast, setubalense juices are richer in terpenes, many of them associated with health protection. Discriminant analysis revealed a pool of VOMs, including β-caryophyllene and E-ocimene, with bioactive properties able to differentiate among tangerines according to variety and sample type (peel vs juice). This is the first report on the volatile composition of setubalense tangerines grown on Madeira Island revealing that its pungent aroma is constituted by secondary metabolites with specific aroma notes and health properties. This is strong evidence of the higher nutraceutical value of such fruit for the human diet.

Keywords: tangerine; D-limonene; thymol; HS-SPME; GC-MS

1. Introduction

Citrus fruits are one of the world's major fruit crops, being produced in many countries of the tropical, subtropical and temperate borderlines [70]. According to the recent data released by United States Department of Agriculture (USDA), citrus production in 2019/20 is estimated around 92 million metric tons, representing oranges and tangerines 46.1 and 31.6 million tons, respectively [299]. Citrus fruits are very rich in secondary metabolites with high nutraceutical value, such as vitamin C, folate, flavonoids, coumarins, limonoids, terpenoids and carotenoids [77]. Some of these metabolites, namely terpenes, are volatile organic compounds (VOMs), giving the fruit a rich, pungent and distinctive aroma [75, 300]. For this reason, citrus fruits and by-products have very important applications, spanning the food industry, cosmetics and medicine [300]. Moreover, citrus VOMs also constitute a valuable tool for the identification and differentiation of cultivars, hybrids and genotypes [70, 75, 300]. Citrus fruits are a very heterogeneous group, with dozens of citrus species and varieties, being *Citrus reticulata* one of the most popular. Their fruits are commonly known as mandarins and tangerines are one of its varieties [75]. Tangerines combine the fresh and acidic notes of other citrus fruits like lemon or lime, with a honey-like sweetness, resulting in unique organoleptic properties that make them very appreciated by consumers [74-76]. The core aroma of mandarin juice is essentially composed by nine volatiles, namely limonene (citrus-like), linalool (floral, citrus), α-terpineol (floral), terpinen-4-ol (woody, earthy), nonanal (piney, floral, citrus), decanal (fatty, musty), α-pinene (pine-like), β-myrcene (musty, wet soil) and carvone (spearmint, car-away) [74, 76]. Additionally, thymol and dimethyl anthranilate have been also mentioned as relevant in the aroma of mandarins [75, 76, 301]. Many of these volatiles identified in citrus fruits, as in many other fruits and food products, exhibit different bioactive properties (antioxidant, antidiabetic, antiproliferative, etc) and potential health benefits. Consequently, many of these fruits are considered functional foods with an added value. In this context, current consumers have a growing interest in the provenience and authenticity of their food products as a

guarantee of their quality. Therefore, the identification of suitable markers to discriminate these products from others that can be very similar, is a crucial regulatory requirement for consumers' confidence. In this context, the development of fast and reliable methods for volatile analysis is received much attention. To achieve this, headspace solid phase microextraction (HS-SPME) combined with gas chromatography–mass spectrometry (GC–MS) is a well-established methodology for VOMs analysis, retrieving high sensitivity, reproducibility, and robustness [111]. In recent years, HS-SPME/GC-MS strategy has been successfully applied in the characterization of the volatile profile of new fruits [302]. Furthermore, the use of multivariate statistical to process the volatomic data, allowed the discriminated between different cultivars, varieties and species, and even between different maturation stages of the different fruits [111, 302-304].

The main purpose of this study was to establish the volatile profile of the juice and peels of *setubalense* tangerines grown on Madeira Island, assess their authenticity by comparison with the closely related *murcott* variety cultivated in the mainland, Portugal, and explore its potential as a very rich natural source of several important bioactive compounds with differentiated health properties, namely thymol, in the variety cultivated in Madeira island.

2. Materials and Methods

2.1. Chemicals and Materials

All standards used in this work for VOMs confirmation (purity higher than 98.5%) and the C_8 – C_{20} n-alkanes mixture, were obtained from Sigma-Aldrich (St. Louis, MO, USA). The carrier gas in the GC system was helium (ultra-pure grade, Air Liquide, Portugal). The SPME holder for manual sampling, the fibres (divinylbenzene/carboxen on polydimethylsiloxane - DBV/CAR/PDMS, 50/30 µm thickness and 1 cm length) and the clear glass screw cap vials for SPME with PTFE/silica septa (film thickness 1.3 mm) were purchased from Supelco (Bellefonte, PA, USA).

2.2. Tangerine samples

Tangerine samples of the varieties *setubalense* (grown in Madeira island) and *murcott* (grown in Portugal mainland) were selected randomly (10 fruits of each variety) from a local market (Madeira island), as purchased for consumption. The collection was performed at the beginning of winter (December), which is the optimal time for harvesting. After careful visual inspection of the absence of any sign of fruit degradation, peels and juices of individual fruits were collected and stored under N_2 (g) atmosphere at −80 °C until analysis, in single-use aliquots (250 mg and 5 ml of peels and juice, respectively) to prevent sample degradation. Aliquots from three different fruits of each tangerine variety were selected and analysed in triplicate.

2.3. HS-SPME procedure

The extraction procedure was adopted from previous studies in our laboratory [302, 303] with minor modifications. Briefly, 5 mL of tangerine juice were placed into 20 mL headspace glass vial ($1/\beta \approx 0.5$) containing a magnetic microstirring bar. NaCl 10% (w/v) was added to the sample matrix to promote the 'salting-out' effect to decrease the solubility of volatile metabolites in the water-based phase). Before sealing the vial, 100 μL of 3-octanol (16.4 μg/L) were also added as internal standard (IS). For the peels, 250 mg of sample was placed into 20 mL of extraction tubes, added the same amount of IS (100 μL of 3-octanol 16.4 μg/L) and the extraction tubes sealed. HS-SPME extractions were carried out by exposing the SPME fibre to the headspace of the glass vial (placed in the middle of the vial headspace, about 2.5 cm above the sample to avoid splashing the fibre with juice sample during agitation) for 40 min at 40 °C. Finally, the fibre was injected into the GC-MS system at 250 °C for 10 min to attain the thermal desorption of the extracted VOCs. Before each run, blank samples were carried out to ensure any carry-over from the previous analysis. All the experiments were performed in triplicate (n = 3) under constant stirring (800 rpm) to improve the extraction. The SPME fibres were thermally conditioned in the GC

injector according to the producer's recommendations, and daily for 10 min before the first extraction.

2.4. Gas chromatography–mass spectrometry analysis (GC–MS)

The analysis was carried out on an Agilent 6890N gas chromatograph system (Agilent Technologies, Palo Alto, CA, USA), equipped with a BP-20 polar column, and coupled to an Agilent 5975 quadrupole inert mass selective detector. The full settings and procedure were described previously by [303]. Briefly, trapped VOMs were loaded in the GC-MS using a 10-min splitless injection using He (1.0 mL/min) and separated in BP20 fused silica using a temperature gradient in the oven starting at 45 °C (held for 2 min), followed by a temperature gradient (2 °C /min) up to 90 °C, held 3 min, then another gradient (3 °C /min) up to 160 °C, held for 6 min, and finally from 160 °C to 220 °C (6 °C /min) and held for 15 min. The resulting chromatograms were processed using the Enhanced ChemStation software for GC-qMS (Agilent Technologies, Palo Alto, CA, USA). The identification of the VOMs involved the comparison between the GC retention times (RT) of the chromatographic peaks with those of authentic standards processed under the same conditions, as well as the determination of the RI of each peak of the C_8-C_{20} n-alkanes series. Each sample was injected in triplicate.

2.5. Multivariate statistical analysis

The volatomic data generated in this work was processed with the MetaboAnalyst 4.0 web-based tool [305] and according to [303]. Briefly, the raw data was normalized (IS ratio correction, sample median, data transformation by cubic root and data scaling by autoscaling). Then, variance (ANOVA, $p < 0.05$) and partial least square discriminant analysis (PLS-DA), were used for variable reduction. PLS-DA reduces the size of the data matrix by eliminating redundant variables (VOMs), thus defining the set of volatiles which define the best separation among the different groups analysed. Ward's linkage algorithm and Euclidean distance analysis were used to

calculate the ratio of the VOMs and the resulting metabolic alterations (top VOMs with VIP (variable importance in projection) > 1) were subjected to used to HCA analysis to depict distinct clustering patterns among the studied groups.

3. Results and Discussion

3.1. Volatomic profile of tangerines

The volatile profiles from the two tangerines varieties analysed in this work are quite different, being the *setubalense* juice clearly richer than *murcott* in terms of number and intensity of VOMs characterized (Fig. 3.5.1). Regarding the peels, we observe that their volatile composition is much richer (number and intensity of VOMs) than the juices, while minor differences have been observed between varieties (Fig. 3.5.1). Overall, 128 different VOMs (plus IS) have been identified, from which 109 in the *murcott* and 117 in the *setubalense* variety. The detailed list of all VOMs identified and respective experimental data, including the retention time and relative peak area is available in the Supplementary Table 3.5.1. The contribution of each VOM for the total volatile fraction expressed as relative peak area was calculated according to equation (1):

$$Peak\ area\ of\ analyte / Peak\ area\ of\ internal\ standard \quad (1)$$

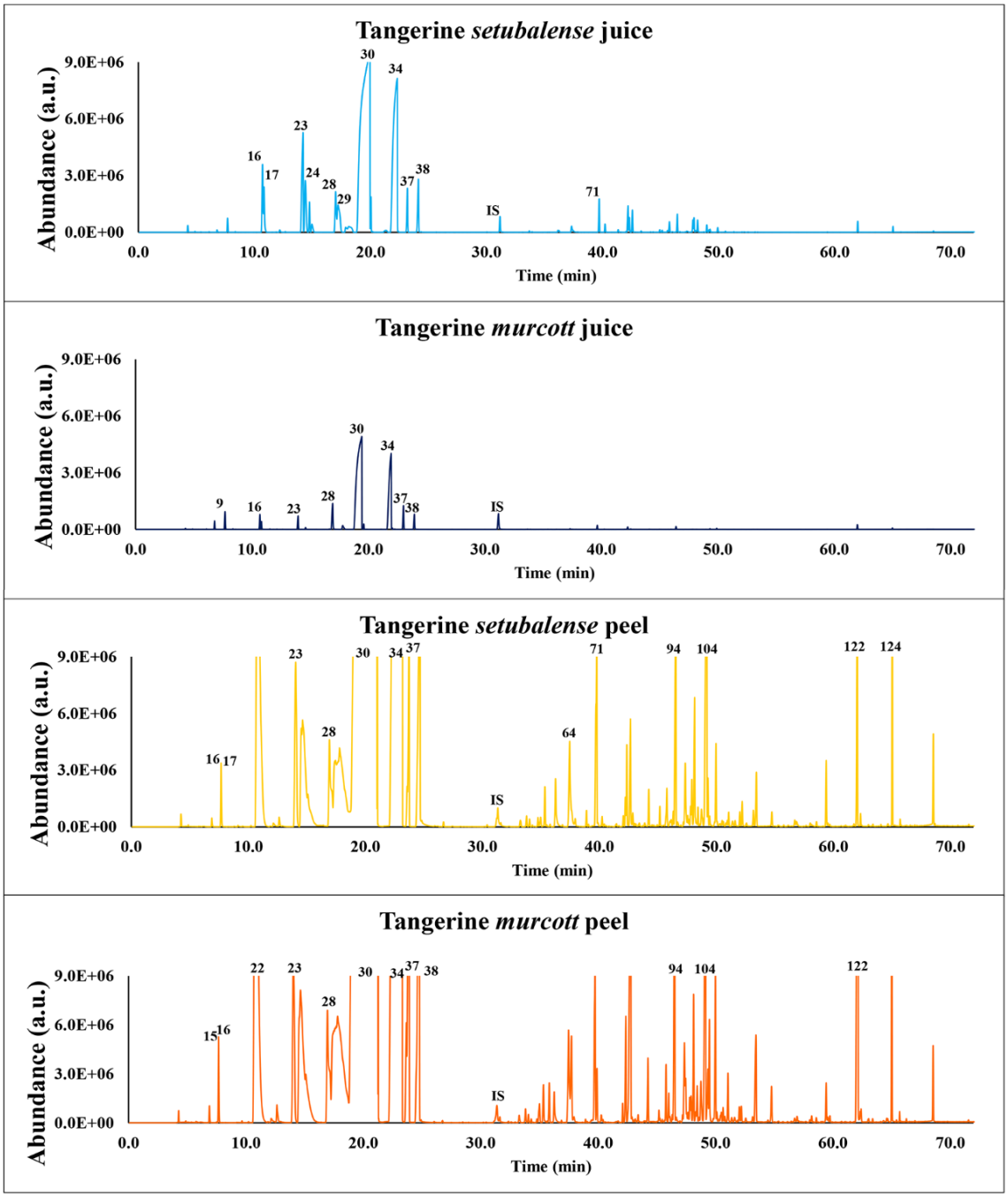


Fig. 3.5.1. Representative chromatograms of the samples analysed in this work. Numbers above the peaks refer to the VOMs identified in the Supplementary Table 3.5.1. Peak number identification: 9 - ethanol, 15 - 1-penten-3-one, 16 - α-pinene, 17 - α-thujene, 22 - hexanal, 23 - β-pinene (isomer 1), 24 - β-pinene (isomer 2), 28 - β-myrcene, 29 - α-terpinene, 30 - D-limonene, 34 - γ-terpinene, 37 - o-cymene, 38 - terpinolene, IS – internal standard (3-octanol), 64 - decanal, 71 - linalool, 94 - α-terpineol, 104 - α-farnesene, 122 - dimethyl anthranilate, 124 - thymol.

During sample preparation, we observed a stronger and distinctive smell for the *setubalense* tangerines. This agrees with the fact that the sum of the relative peak areas of the 75 VOMs identified in *setubalense* juices is 5 times higher than the 56 VOMs identified in *murcott* juices. In contrast, the sum of the relative peak areas obtained for the 89 VOMs identified in *setubalense* peels is only 20% higher than 91 VOMs identified in *murcott* peels samples (Supplementary Table 3.5.1.). Monoterpenes hydrocarbons are by far the functional class more abundant in all samples analysed, representing over 93% and 84% of the volatile fractions of tangerine juices and peels, respectively (Fig. 3.5.2). Such abundance is mainly due to D-limonene, followed by γ-terpinene, β-myrcene, β-pinene, o-cymene, α-pinene and terpinolene, which are the most abundant VOMs identified in all tangerine samples (Supplementary Table 3.5.1). This suggests that the VOMs with lower abundance should have an important contribution for the distinct profile of *setubalense* juices and peels in comparison with *murcott*. Further olfactometry analysis would be very interesting to clarify this question.

3.1.1. Volatomic profile of tangerine juices

As can be observed in Fig. 3.5.2, sesquiterpene hydrocarbons and oxygenated terpenes are clearly more abundant in *setubalense* juices, both in terms of the number of VOMs identified, as in relative peak areas for each family of compounds. In contrast, the relative peak areas for alcohols and esters are much higher in *murcott*. Ethyl acetate, for instance, is three times more abundant in *murcott* comparatively to *setubalense* juices. Beyond the relative peak areas, 28 VOMs were exclusively identified in the *setubalense* variety juices (7 monoterpenes, 8 sesquiterpenes and 10 terpenoids, Supplementary Table 3.5.1.). Since terpenes result from the Methylerythritol 4-phosphate (MEP) and the Mevalonate (MAV) pathways [1, 306] (Fig. 3.5.3), this result suggests a lower activity of these pathways in the *murcott* relative to the *setubalense* variety, particularly in the cytosol where the final steps of sesquiterpenes biosynthesis occur. In opposition, ethyl isobutyrate, propyl acetate

and ethyl 2-butenoate were exclusively identified in *murcott* juices. The close related mandarim juice contains nine volatiles that are considered as core aromatic volatiles, namely linalool, α-terpineol, terpinen-4-ol, nonanal, decanal, carvone, limonene, α-pinene and β-myrcene [74, 76], and all these VOMs were identified in the tangerine samples analysed in this work. Beyond this, more VOMs, namely terpenes, were identified in this study in comparison to a recent report [307].

3.1.2. Volatomic profile of tangerine peels

When compared to the juice samples, the volatomic profile of tangerine peels is more complex, being the sum of relative peak areas 5 times higher in the *murcott* variety and 1.25 times higher in the *setubalense* variety than the respective juice samples. Regarding the number of VOMs, there was also an increment in the number of VOMs identified in the peels. Overall, 89 VOMs were identified in *murcott* peels (56 in the juices), while in *setubalense* variety, the variation was lower (87 vs 75 VOMs in peels and juices, respectively, Supplementary Table 3.5.1.). As shown in Fig. 3.5.2, these differences are mainly due to aldehydes and oxygenated terpenes, whose number of VOMs triplicates from the juices to the peels. The variation in the number of oxygenated terpenes found in the peel samples, when compared to juices samples, can be easily explained by the interaction with the atmosphere air that favours the oxidation of many metabolites, including VOMs. Similarly, 3 ketones and 3 furans, grouped as "others" in Fig. 3.5.2, exhibit a robust predominance in the volatile composition of the peels. Regarding this, 1-penten-3-one accounts for half of the relative peak area of the "others" family of the tangerine peel samples from the *murcott* variety. In contrast, esters almost disappear, being only ethyl acetate identified in the peels of both varieties. Overall, 33 more VOMs were identified in the *murcott* variety and more 12 VOMs in the *setubalense* variety.

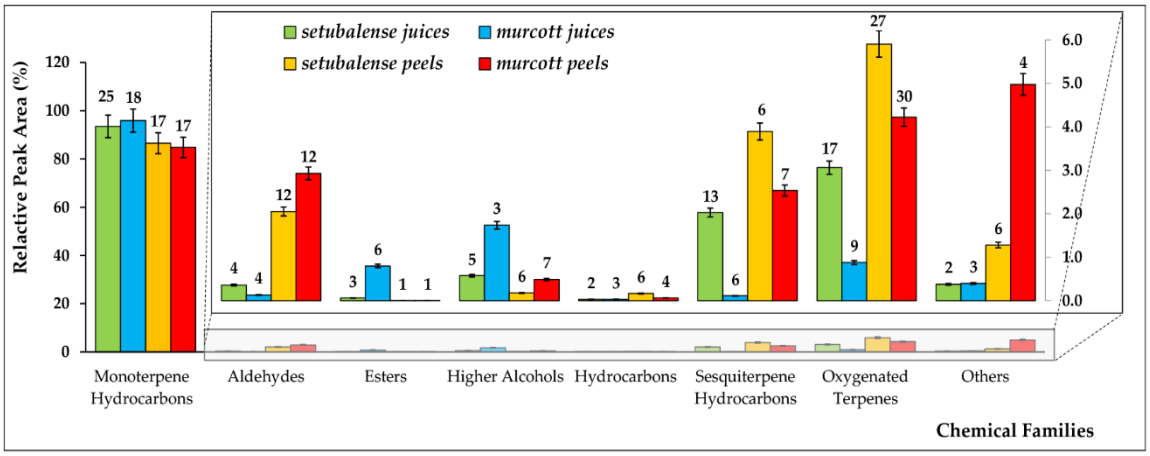


Fig. 3.5.2. VOMs identified in juices and peels of setubalense and murcott tangerines, by chemical family. The number of VOMs identified for each chemical family is indicated above the respective bars.

3.2. Tangerines bioactive potential

Many of the compounds identified in *setubalense* and *murcott* tangerines have been previously reported with a myriad of bioactivities. This includes antioxidant [56, 308-310], anti-inflammatory [56], antidiabetic [57, 309], antileishmanial [59, 311], antimicrobial [309, 311-313], cytotoxic [59, 311], antitumor [314] and antiproliferative activities [315, 316]. Furthermore, there are evidence pointing to other protective effects against Alzheimer [317] and tuberculosis [318]. Most of these bioactive VOMs are terpenes that, as already referred, can be obtained from the MEP and MAV pathways through the isopentyl diphosphate (IPP) precursor [1, 306]. Fig. 3.5.3 shows a simplified diagram of the reaction cascade producing the most abundant terpenes identified in the *setubalense* and *murcott* tangerines analysed in this work.

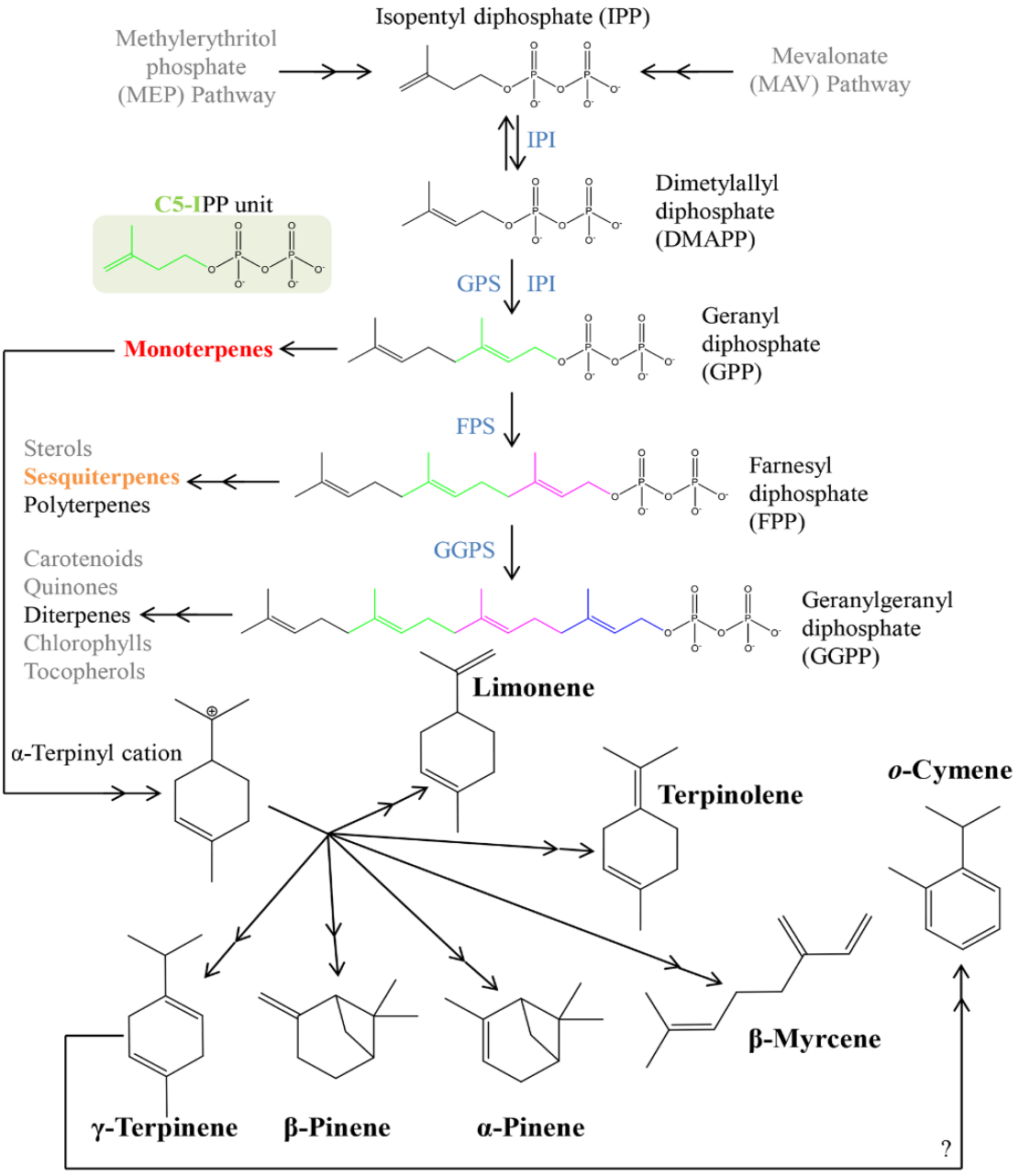


Figure 3.5.3. Main reactions cascade involved in the formation of the most abundant terpenoids identified in this work (adapted from [1]).

Overall, bioactivity is recognized not only to the major VOMs identified in the tangerines, but also to other present in lower abundance, as thymol and dimethyl anthranilate, whose benefits to human health are widely known [319, 320] (Table 3.5.1.). Thymol and dimethyl anthranilate have also been reported as having an important contribution to the mandarin aroma, being used in synthetic tangerine flavour [76, 301]. According to Ladanyia and Ladanyia [321], the distinctive notes in the aroma of the

Mediterranean mandarin oil were due this two VOMs, accounting dimethyl anthranilate to 0.85 % and thymol to 0.08 % of the volatomic profile. In this work, dimethyl anthranilate and thymol represent 1.18 % and 2.35 %, and thymol 0.87 % and 0.58 %, of the peels of *setubalense* and *murcott* varieties, respectively. Such abundance in thymol and dimethyl anthranilate supports the use of *setubalense* tangerine as a potential and interesting alternative to the thyme oils, which are often associated to some allergic reactions, even when it is used diluted [320]. Another interesting VOM identified in this work is cosmene. This compound represents almost 20% of the oil obtained from the roots of Eup*atorium adenophorum* Spreng (Asteraceae) [322]. This native plant from Central America, commonly known as Mexical devil became invasive worldwide, being used in the folk medicine for its bioactive properties (antimicrobial, antiseptic, etc. [322]). In fact, [322] reported antimicrobial, antioxidant and phytotoxic properties for the root's oils of this plant and such effect will be certainly elicit by cosmene given its high abundance in the roots extracts.

Table 3.5.1. VOMs identified in this work with potential bioactive effects.

		juice		peels		VOMs potential bioactive effects[3]										
PN[1]	**VOMs**	***set/mu r* ratio[2]**	**T test *(p<0.05)***	***set/mur* ratio[2]**	**T test *(p<0.05)***	Antibacterial	Antidepress	Antidiabetic	Anti-	Antileishma	Antifungal	Antioxidant	Antiprolifer	Antitumor	Cytotoxic	**References**
16	α-pinene	8.50	0.0218	0.40	0.0367			ǂ		ǂ	ǂ	ǂ	ǂ	ǂ	ǂ	57, 59, 308, 311, 312, 314-316
17	α-thujene	10.0	0.0135	**39.8**	0.0002	ǂ					ǂ	ǂ		ǂ		323, 324
21	camphene	15.8	0.0079	0.10	0.0025	ǂ						ǂ	ǂ			308, 312, 316
23	β-pinene	17.5	0.0182	1.00	0.3719						ǂ	ǂ	ǂ	ǂ		308, 312, 314, 316
25	sabinene	23.3	0.0115	---	---	ǂ				ǂ				ǂ	ǂ	311, 312, 314
28	β-myrcene	5.60	0.0054	0.70	0.3413			ǂ	ǂ	ǂ	ǂ	ǂ	ǂ	ǂ	ǂ	56, 309, 311, 312, 314, 316
29	α-terpinene	7.50	0.0076	0.90	0.0854	ǂ				ǂ	ǂ			ǂ	ǂ	311, 312, 314
30	D-limonene	4.40	0.0086	1.20	0.0979	ǂ		ǂ	ǂ		ǂ	ǂ	ǂ	ǂ	ǂ	57, 59, 308-316
31	β-phellandrene	7.90	0.0201	0.70	0.0827	ǂ		ǂ		ǂ		ǂ			ǂ	309, 311, 314
33	(E)-ocimene	31.3	0.0052	---	---	ǂ		ǂ	ǂ			ǂ				56, 309
34	γ-terpinene	4.90	0.0001	2.10	0.0283						ǂ	ǂ		ǂ		308, 312, 314
37	o-cymene	3.00	0.0033	0.20	0.0015	ǂ			ǂ		ǂ	ǂ			ǂ	323, 325, 326
38	terpinolene	6.00	0.0001	1.60	0.0013	ǂ								ǂ		312, 314

53	p-cymenene	7.00	0.0004	1.90	0.2046	ǂ					ǂ	ǂ				ǂ	325, 326
55	cosmene[4]	---	---	**3.40**	0.0000	ǂ					ǂ	ǂ					322
71	linalool	17.3	0.0000	1.90	0.0000	ǂ		ǂ	ǂ			ǂ	ǂ				56, 310, 312, 313, 316
79	thymol methyl ether	8.34	0.0004	**3.00**	0.0000	ǂ			ǂ		ǂ	ǂ	ǂ				319, 320
82	β-caryophyllene	62.5	0.0009	1.20	0.0000	ǂ		ǂ		ǂ		ǂ	ǂ			ǂ	59, 310-312, 315, 316
94	*α*-terpineol	13.7	0.0010	1.60	0.0030	ǂ				ǂ	ǂ	ǂ		ǂ		ǂ	59, 308, 312-314
107	perilla aldehyde	9.40	0.0017	1.40	0.0164	ǂ	ǂ		ǂ			ǂ					327
122	dimethyl anthranilate	4.94	0.0105	0.6	0.0012	ǂ					ǂ						328
124	thymol	11.5	0.0073	1.9	0.0004	ǂ			ǂ		ǂ	ǂ					319, 320

Legend: [1] VOMs peak number in the chromatograms (Figure 3.5.1); [2] ratio between the relative peak areas obtained for *setubalense* and *murcott* tangerines; [3] Potential bioactive effect indicates the type of bioactive effects reported for the each of the VOMs referred in the table, *mur* – *murcott* cultivar, *set* – *setubalense* cultivar; [4] strong evidence for the indicated bioactivity.

3.3. Classification of tangerine samples

To evaluate the potential of the volatile fingerprints obtained in this work to discriminate tangerine samples according to the variety and sample type (juice vs. peel), a statistical analysis of the volatomic data matrix was performed using MetaboAnalyst 4.0 web-based tool [305]. The complexity of the data was reduced through normalization, as described in the experimental section. Then univariate statistical analysis (ANOVA, $p \leq 0.05$) was carried out to verify the significant statistical differences among the concentrations of VOMs in each sample group ($p < 0.05$, Supplementary Table 3.5.2.). Multivariate statistical analysis (MVSA) using PLS-DA was then performed to assess if there were significant VOMs signatures between each group. The results obtained show four different clusters discriminating each of the groups under study (Fig. 3.5.4).

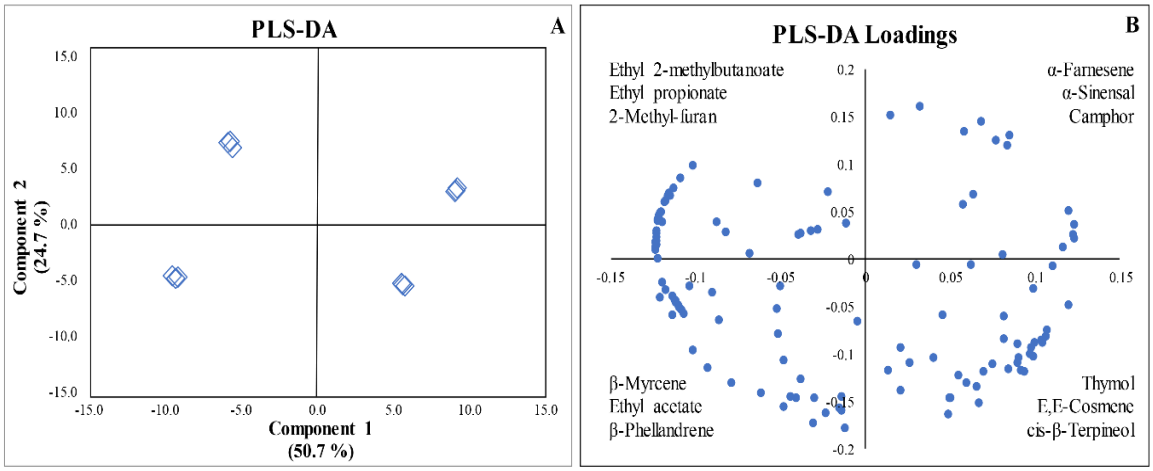


Fig. 3.5.4. Multivariate statistical analysis (MVSA) using partial least square discriminant analysis (PLS-DA) of the volatomic data obtained in this work (A) and identification of the variables (VOMs) responsible for the differentiation of the samples (B).

As Fig. 3.5.4A shows, the first component explains 50.7 % of the variance and separate the tangerine "juice" from "peel". The second component contributes for 24.7 % of the total variance of the model and separate the tangerine variety *murcott* from *setubalense*. Fig. 3.5.4B presents the variables that most contributed for the differentiation of the

tangerines by variety and type of sample. Accordingly, ethyl 2-methylbutanoate, ethyl propionate and 2-methyl-furan are more associated to *setubalense* juice, ethyl acetate, β-myrcene and β-phellandrene to *murcott* juice, α-farnesene, α-sinensal, camphor more associated to *setubalense* peel, and cis-β-terpineol, cosmene and thymol to *murcott* peel.

Hierarchical cluster analysis (HCA) was also performed using the 64 most significant VOMs identified in tangerine samples, as described in section 2.5. This strategy allows a better identification of the inherent clustering patterns between each variety, in complementarity with the statistical analysis carried out previously. The result of this treatment can be visualized in the heatmap plot (Fig. 3.5.5) and as a dendrogram (Supplementary Figure 3.5.1).

Overall, the set of variables that contributed to the differentiation of the tangerines by variety, have the potential to be used for product identification and authenticity. Furthermore, tangerines of the *setubalense* variety contain higher amounts of bioactive analytes than *murcott* tangerines (Table 3.5.1). Therefore, this *bioactive signature* not only supports higher health benefits for the *setubalense* tangerines grown in Madeira Island, as can be used to the discrimination of the two varieties analysed in this work.

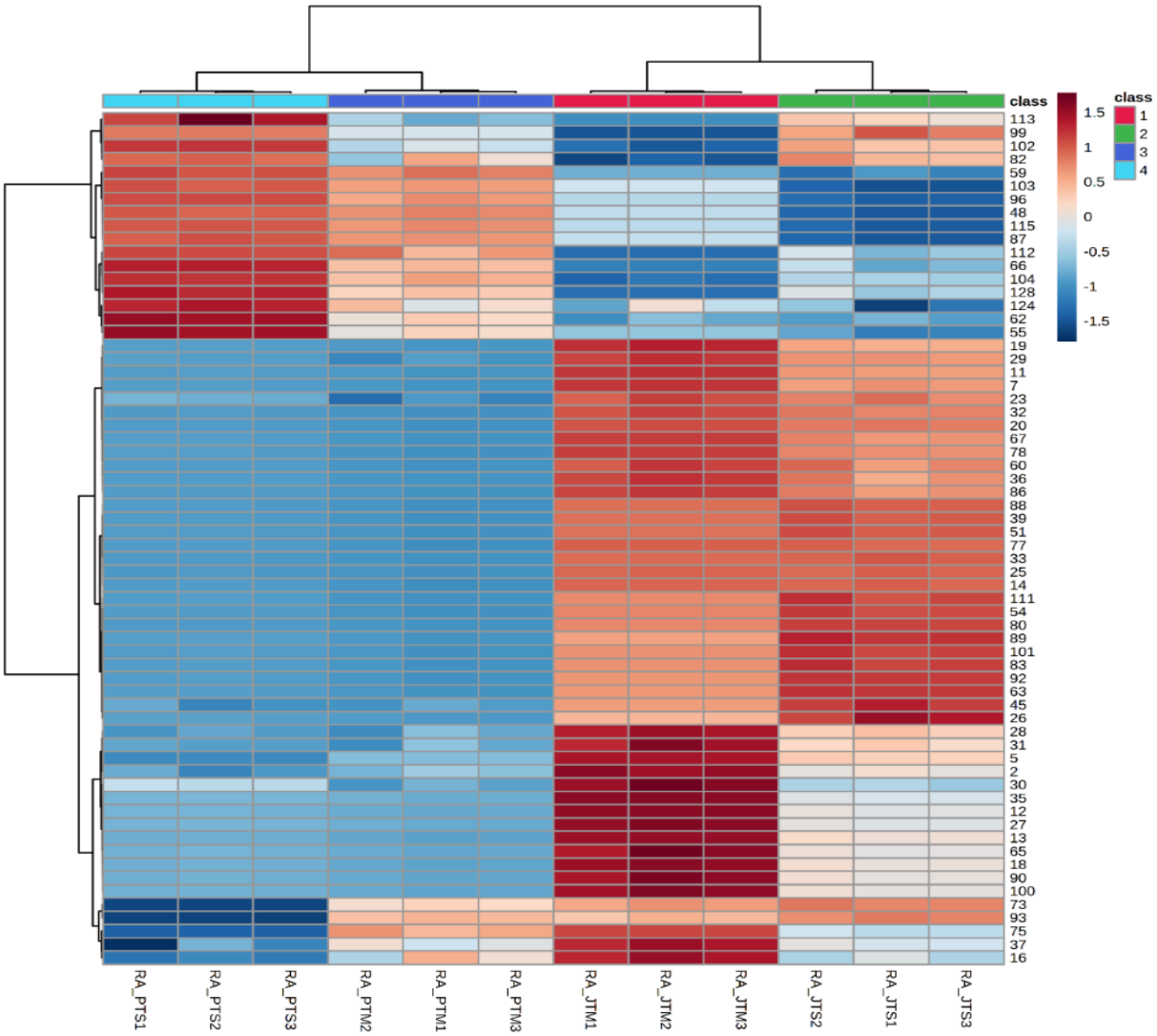


Fig. 3.5.5. Hierarchical cluster analysis (HCA) analysis of the volatomic data obtained in this work, as described in Materials and Methods section.

4. Conclusions

In this work HS-SPME-GC/MS was used to characterize for the first time the volatile composition of a tangerine variety cultivated in Madeira island (*setubalense*) and compare it with the *murcott* variety grown in mainland. This comparison involved the juice and peels of both varieties and allowed the identification of 129 VOMs, 107 VOMs in *murcott* and 115 VOMs in *setubalense* varity, which is considerably higher than most of the previous reports using the same methodology to analyse different tangerine varieties. To the best of our knowledge, only the tangerine hybrid *9-4* ×

Blood4x analysed by [75] presented a similar number of VOMs (118 VOMs) than the *setubalense* variety. The volatile composition of the peels is more complex than the juices and the *setubalense* distinctive aroma not only correspond to a higher abundance of common VOMs, as most of them have been previously reported with important bioactive properties. D-limonene, γ-terpinene, β-myrcene, β-pinene, o-cymene, α-pinene and terpinolene are among the most abundant VOMs, but *setubalense* tangerines are also very rich in thymol, making this fruit a very interesting alternative to thyme oils.

The uniqueness of *setubalense* tangerines was shown through a statistical analysis that differentiate this variety from the common *murcott* grown in Portugal mainland. Accordingly, ethyl 2-butenoate, (E)-2-decenal, cycloheptane and carvone associated to the *murcott* variety, and α-himachalene, β-santalene, α-selinene and α-sinensal associated to the *setubalense* variety, were the VOMs that most contributed to the differentiation of two tangerine varieties. Moreover, the set of variables that contributed for the differentiation of the tangerines by variety, have the potential to be used for product identification and authenticity.

Sub-Section 3.6. A comprehensive platform based on NTME/GC-MS data and chemometrics for discrimination of lemons according to geographical origin

Microchemical Journal 157 (2020) 104933

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

A comprehensive methodology based on NTME/GC-MS data and chemometric tools for lemons discrimination according to geographical origin

José A. Figueira[a], Priscilla Porto-Figueira[a], Jorge A.M. Pereira[a], José S. Câmara[a,b,*]

Published in:

Microchemical Journal (Impact Factor 4.821)

José A. Figueira, Priscilla Porto-Figueira, Jorge A.M. Pereira, José S. Câmara

http://dx.doi.org/ 10.1016/j.microc.2020.104933

Abstract

In the present work we report a comprehensive knowledge on the volatile composition of lemon (Eureka variety) peel (exocarp) from different geographical regions Portugal (mainland), Madeira Island (Portugal), Argentine and South Africa, using a new and high throughput isolation technique, the Needle Trap Microextraction (NTME), combined with GC-MS analysis and chemometric tools, as an innovative approach to identify a set of geographic molecular markers very useful for lemons discrimination according to its geographical origin. The most important NTME-influencing extraction parameters, namely extraction volume, sample temperature, headspace volume and equilibration time, were optimized using an experimental design (DoE) procedure. Overall, 75 volatile organic compounds (VOCs), belonging to different chemical groups, namely monoterpenes, sesquiterpenes, alcohols and carbonyl compounds, were identified in the peel of targeted citrus. The most dominant volatiles are D-limonene, α-pinene, β-pinene, sabinene, β-myrcene and γ-terpinene, accounting to more than 50% of the volatile compositions from studied lemons.

The VOCs data matrix was submitted to principal component analysis and hierarchical clustering statistics allowing to discriminate lemons considering geographical origin, based on the volatomic fingerprint of its peels. The variables which contributed largely to the geographical origin classification includes butanal, α-pinene, α-thujene, 1-butanol, 2-heptanone, D-limonene, 2-methyl-2-heptenal, nonanal, decanal, 1-octanol, limonene oxide, β-caryophyllene and 2,6-dimethyl-2,6-octadiene, and therefore, can be used as potential useful geographical markers. This study suggested that approach based on NTMS/GC-MS combined with multivariate statistical analysis, is a powerful and rapid strategy to differentiate lemons from Eureka variety from different geographical regions and support its origin and authenticity.

Keywords: Lemon, Eureka variety, NTME-GC-MS; VOCs, geographical origin, multivariate statistical analysis

1. Introduction

Agro-food products, including *citrus* fruits, have in its composition some geographic-based characteristics, arising from terroir and edafo-climatic conditions. These both factors might influence the metabolomics biosignatures which can change the quality and the value of the product [1,2] and therefore determine its acceptance by consumers. The use of geographical indication allows producers to obtain premium price and market recognition.

Lemon (*Citrus limon*) is a well-known citrus fruit widely used throughout the world [70, 71]. It is an important source of secondary metabolites useful for human nutrition and industrial applications. In addition to vitamin C, lemon contains several phytochemicals, including polyphenols (flavonoids and non-flavonoids), limonoids and terpenoids, which play a key role as nutraceuticals [300, 329]. Some of these metabolites have been shown to possess anticancer, antimicrobial, antioxidant and antidiabetic properties. Furthermore, essential oils from lemons and other citrus fruits are considered excellent alternatives to chemical additives in the food industry [70, 330, 331], encompassing both the need for safety and the consumers demand for natural food components.

The volatile composition of lemons and other food matrices is one of the most important factors influencing the food flavor and therefore the consumer acceptance [300]. It is widely reported that the VOCs metabolomic pathways and its composition in lemons are influenced by several factors related with maturity stage of the fruit, cultivar, hybrids, genotypes and geographical origin [70, 300]. To understand the volatile composition of peel from lemons of *Eureka* variety and its differentiation according geographical origin, some factors should be considered. Firstly, is crucial to establish the volatomic fingerprint in the lemon peel and reveal the differences of volatile compounds among lemons from different geographic regions. Moreover, it is important to identify the specific VOCs responsible for the unique characteristics of lemons according to its origin. Nowadays, different methods have been developed for the establishment of volatile composition in lemons and other food matrices and food-

related samples. Gas chromatography-mass spectrometry (GC–MS) has been used as a golden standard instrumental technique for VOCs analysis in food and food-related samples [332-334]. However, before the instrumental analysis, sample preparation should be taken for concentrating the VOCs and remove interferences from the complicated matrices [334, 335]. The most used extraction techniques including solvent extraction, distillation and headspace techniques, were mainly based on the solubility or volatility of the VOCs and provides a fingerprint of volatile composition and therefore a comprehensive information on its flavor/aroma. Solid phase microextraction (SPME) is a well-established technique in the field of VOCs analysis [336-338], however the extraction capacity is hindered by the small amount of sorbent normally used (60-100 ◎m). In addition, as non-exhaustive technique, it depends of the mass transfer equilibrium between a small portion of analytes toward the extracting media, and larger amounts of analytes remain in the sample solution/matrix [336, 339, 340]. In recent years needle trap microextraction (NTME) has been introduced as a simple and fast isolation/extraction technique for VOCs. NTME can be considered to be mechanically more robust than SPME, since its steel needle protects sorbent particles inside of the needle trap device (NTD) (Fig. 3.6.1.) [335], and it is an exhaustive technique, meaning that the analytes may be completely extracted by the sorbent bed before a breakthrough occurs. In addition the sensitivity can be improved by increasing the sample volume and its capacity can be expanded by increasing the volume of the packed sorbent in the NTD [336, 341, 342]. Since NTME requires small sample volumes to extract large amounts of analytes, normally a sampling volume smaller than the breakthrough volume was used [336, 343]. The analyte concentration (C_0) can be calculated using the following equation: $n = C_0V$, where n is the extracted mass by the NTD, C_0 is the concentration of analyte, and V is the sample volume [343, 344].

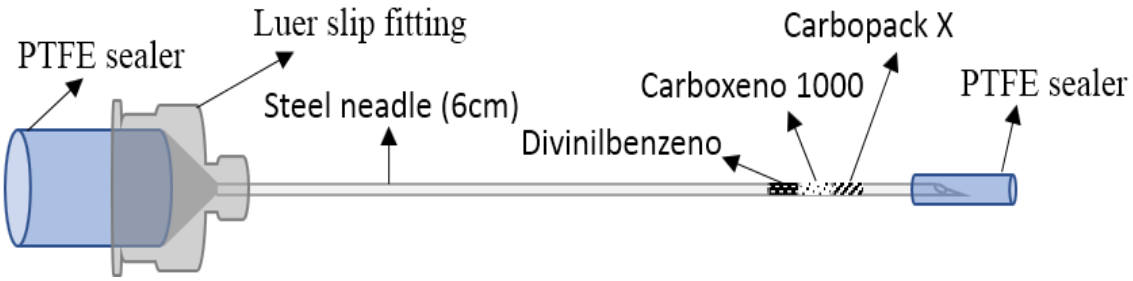


Fig. 3.6.1. Design of the NTD used in this study (NeedleEx): sharp end with triple bed sorbent (DVB/Car1000/CarX) configuration.

In NTME, the VOCs passes over the sorbent packed in the needle, with the analytes being trapped into sorbent [340, 345, 346]. The choice of appropriate sorbent material is one of the critical factors to obtain good recovery and high enrichment factor [336]. There are several commercially available sorbents, such as divinylbenzene (DVB), polydimethylsiloxane (PDMS), DVB and carboxen particles Carbopack X, Tenax TA, Porapak Q, as well different sorbents combinations [334, 335, 340, 345]. In order to improve selectivity as well specify towards target compounds, new sorbent materials, including synthetic polymers and nanostructured based materials, are being developed, such as SiO2@cis- 9-octadecenoic acid, molecularly imprinted sol–gel xerogel [334] carbon nanotubes (CNTs), CNT/silica composite, multi-walled carbon nanotubes/silica composite (MWCNTs/Si), graphene (G), graphene nano-platelets silica composite (G/Si), polythiophene-silver nanocomposite (PT-Ag), graphene oxide (GO) and nanoporous silica aerogel (NPSA) [334, 340, 342, 346]. Other experimental factors such as extraction time, sample amount and headspace volume, are key parameters on the NTME efficiency.

Desorption of the analyte requires a single-stage thermal desorption, with the process taking place into the injection port in a few seconds, transferring the analytes effectively to the GC-MS system, with minimum or no carryover [340, 345-347]. The thermal desorption from the NTD is faster than the SPME fibre under the same GC injection conditions, overcorrecting the downside of the SPME technique with a highest

sensibility and speed [332, 346]. As summarized by Barkhordari et. al. (2017), NTME has been used mainly for isolation of VOCs from exhaled breath samples [346].

Since the geographical origin will influence the Lemon composition, the potential health benefits and nutritional qualities of this fruit will be dependent on the growing conditions associated. In this context, the main purpose of this work was to explore the potential of an integrated strategy based on the development of a novel analytical approach, NTME/GC-MS, for identification of geographical markers of lemons of a single botanical origin (Eureka) cultivated at different countries - Portugal (mainland and Madeira Island), Argentina and South Africa. The key NTME experimental parameters that can influence the extraction efficiency, namely extraction temperature, equilibration time, headspace volume and sample amount, were optimized. The acquired data set of non-targeted fingerprints was then processed using multi-dimensional chemometric strategies in order to find marker compounds that positively distinguish lemons from different geographical origins. This is the first work reporting the high throughput potential of NTME in food field with enormous potential to be applied to a wide range of food samples in addition to different research fields.

2. Materials and methods

2.1. Chemicals and Materials

All standards used for VOCs confirmation (purity higher than 98.5%) and the *n*-alkanes mixture containing C_8 – C_{20} straight-chain alkanes in hexane, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Helium, ultra-pure grade (Air Liquide, Portugal) was used as carrier gas in the GC system. Clear glass screw cap vials for extraction with PTFE/silica septa were purchased from Supelco (Bellefonte, PA, USA). The NTDs used in this work, "NeedleEx", were custom manufactured by Shinwa Ltd., Japan (60 mm × 0:41 mm id, 0.72 mm od, triple bed configuration Divinylbenzene/Carboxen 1000/Carbopack X - DVB/Car1000/CarX) and purchased to PAS Technology (Magdala, Germany). Prior to their use, NTDs were conditioned in a

special custom-made heating device (PAS Technology, Magdala, Germany) at 250 ºC, under permanent helium flow for at least 20 h to eliminate any contaminations from the manufacturing process or shipping. Afterwards, both ends of the needles were sealed with Teflon caps and stored. Before being used, the NTDs were conditioned again for 30 min in the heating device.

2.2. Lemon samples

Lemon samples from same variety (*Eureka*) from different geographical origins (Portugal - mainland and Madeira island), Argentina and South Africa) were selected randomly from a local market, as purchased for consumption. After selection, the peel (exocarp) of each lemon was individually collected, and immediately stored under nitrogen at −80 °C, in 250 mg aliquots, which were used only once to prevent sample degradation.

2.3. Optimization of needle trap microextraction (NTME)

To increase the NTME efficiency, key experimental parameters were optimized [348, 349], including *(i)* the extraction temperature (30 °C, 40 °C and 50 °C), *(ii)* the equilibration time (10, 30, 50 min), and *(iii)* the headspace volume (20, 30 and 40 ml), using a 'Design of Experiments' (DoE) optimisation approach. All extractions were performed in triplicate. The DoE is relatively straightforward and can greatly facilitate the optimisation assays, generated a model with 16 combinations, and the resulting data matrix was submitted to statistical treatment.

2.3.1. NTME Procedure

Following the optimization step, 250 mg of sample was placed into 20 ml of extraction tubes and added 100 µL of 2-heptanol (30 ppm) as internal standard. The extraction tubes were sealed, and the system equilibrated for 10 min at 50 ± 1 °C. Then, the NTDs pre-attached to a disposable 1 ml syringe were inserted into the headspace of the extraction tube, and 30 mL of the gas phase were manually loaded through the sorbent

(30 withdraw-loading cycles, average speed 10 ± 2 ml min^{-1}). After the extraction, the syringe was discarded and the NTD was sealed in both ends with PTFE caps. Finally, the NTD was injected into GC-MS system at 250 °C for 60 seconds to thermal desorption of the extracted VOCs. Before the next extraction, the sorbent was reactivated by placing the NTDs in a conditioner at 250 °C under constant flow of helium (purity 5.0, Air Liquid, Portugal) at a constant pressure of 1 bar for 30 min. Unless indicated, all procedures were repeated with at least three different samples (N = 3) analysed in triplicate (n = 3).

2.4. Gas chromatography–quadrupole mass spectrometry analysis (GC–qMS)

The analysis was carried out with an Agilent 6890N gas chromatograph system (Agilent Technologies, Palo Alto, CA, USA) coupled with an Agilent 5975 quadrupole inert mass selective detector. The separation of the extracted compounds was performed on a BP-20 fused silica capillary column (60 m × 0.25 mm I.D. × 0.25 μm film thickness). Splitless injection was employed using helium as carrier gas at a constant flow rate of 1.0 $mLmin^{-1}$. Oven temperature conditions were: 45 ºC (held for 2 min), followed by a gradient temperature ramp from 45 ºC held for 1 min, then up to 90 ºC, held for 3 min at a rate of 2 ºC min^{-1}, followed by a flow rate of 3 ºC min^{-1} until 160 ºC (held for 6 min), and finally from 160 ºC to 220 ºC held for 15 min at a rate of 6 ºC min^{-1}. The injection and ion source temperatures were 250 ºC and 230 ºC, respectively. The mass spectra of the compounds were acquired in electron-impact (EI) mode at 70 eV. The electron multiplier was set to the auto tune procedure. Data acquisition was performed in scanning mode (mass range m/z = 35 – 300 amu; six scans per second). Chromatograms and spectra were recorded and processed using the Enhanced ChemStation software for GC-MS (Agilent Technologies, Palo Alto, CA, USA). VOCs identification was based on the comparison between the GC retention times (RT) of the chromatographic peaks with those, when available, of authentic standards run under the same conditions. MS fragmentation patterns were compared with those of pure compounds, and mass spectrum database search was performed

using the National Institute of Standards and Technology (NIST) MS 05 spectral database. Finally, confirmation also involved the determination of the RI of each peak of C_8-C_{20} *n*-alkanes series. Once again, the values were compared, when available, with values reported in the literature for similar chromatographic columns. Chromatographic peak areas, expressed in arbitrary units (a.u.) of area, were determined using the Full Scan chromatogram, and were used as an approach to estimate the relative content of each volatile metabolite. For semi-quantification purposes, each sample was injected in triplicate, and the chromatographic peak areas (as kcounts amounts) were determined by a reconstructed full-scan chromatogram using for each compound some specific quantification ions: these corresponded to base ion (m/z 100 % intensity), molecular ion (M^+), and another characteristic ion for each molecule.

2.5. Multivariate statistical analysis

The multivariate data analysis (MVDA) was performed using the MetaboAnalyst 4.0 web-based tool [305]. The raw GC-qMS data was firstly pre-processed by normalization (to sample median, data transformation by cubic root and data scaling by autoscaling). The analysis of variance (ANOVA, $p < 0.05$), including PCA, was used for variable reductions and to convert a set of highly correlated variables to a set of independent variables by using linear transformations.

Hierarchical cluster analysis (HCA) was carried out using the 40 most significant VOCs identified in lemon samples obtained by ANOVA (generated using Ward algorithm and Pearson distance analysis).

Principal component analysis (PCA) was used as an unsupervised pattern for statistical procedure that converts a set of observations of possible correlated variables into a set of values of linearly uncorrelated variables (principal components) using orthogonal transformation.

3. Results and discussion

3.1. Optimization of NTME Procedure

A properly optimized method ensures good accuracy and precision in addition with high sensitivity. The most influencing NTME parameters - sample amount, extraction temperature, equilibration time and headspace volume, were optimized using DoE optimization approach (Supplementary Table 3.6.1). From the obtained results (Fig. 3.6.2.), DoE will predict

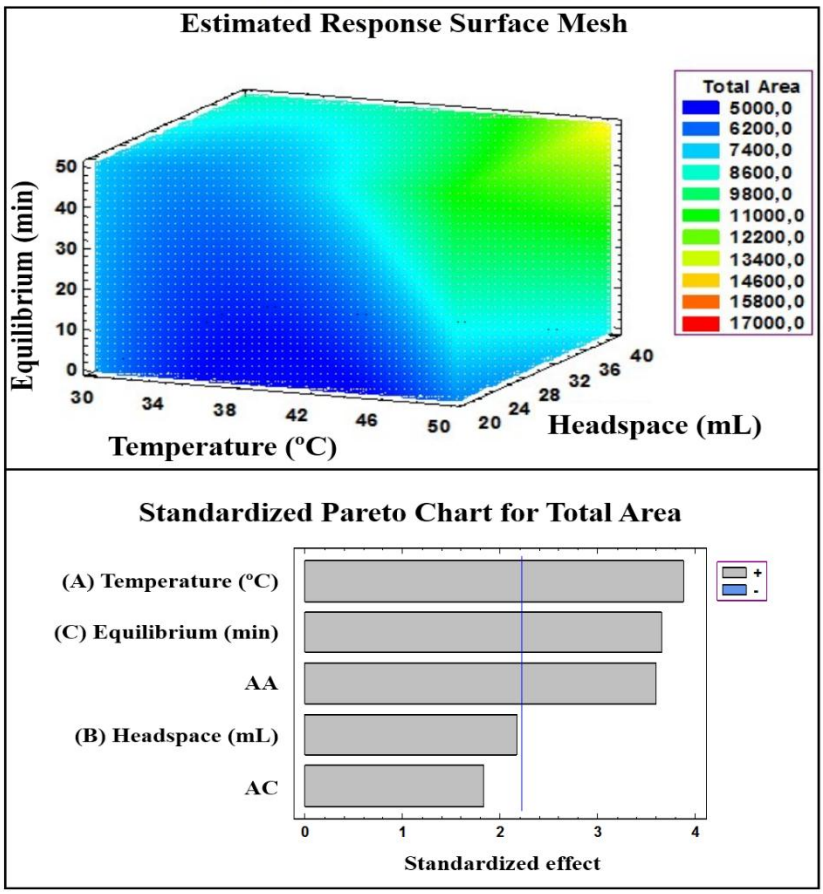


Fig. 3.6.2. DoE results as Estimated Response Surface Mesh and Standardized Pareto Chart for Total Area, from different key parameters that influence NTME: Temperature (º C), Equilibrium Time (min) and Headspace Volume (mL).

the influence between the parameters, and the outcome of different combination along the range between the maximum and minimum values of the studied parameters.

3.1.1. Sample amount

The sample amount should be selected based on the established distribution constant (K_D) of the volatile composition. Depending on the sorbent and nature of the volatiles, the K_D may vary substantially, leading to different extraction yields. The dependence of the extraction efficiency on the sample amount gives useful information on NTME method development. Firstly, 1 g of lemon peel was tested. This amount revealed to be too high for a good chromatogram resolution and therefore the sample amount was downsized until 250 mg of lemon peel, where respective chromatograms show a good peak resolution and sensitivity. In addition, the effect of the particle size of the lemon peel amount was evaluated, confronting 1 piece of 250 mg *vs* 250 mg of sample sliced into ±1 mm^2 (through disperser ULTRA-TURRAX T25). As expected, due to highest surface area, 250 mg of sample sliced into 1 mm^2 presented the best results and therefore, this condition was used in all further assays.

3.1.2. Extraction temperature

Extraction temperature is one of the most important parameters for the evaluation of extraction efficiency in NTME. Kinetically, high extraction temperature improves the kinetic of mass transfer from the bulk sample to the headspace increasing the extraction efficiency of the method [335, 342, 345]. However, too high temperatures decreases the trapping capability due to the exothermic effect of sorption process [342, 350], in addition to thermal degradation of the most labile VOCs and isomerisations. Therefore, the use of too high temperatures is not recommended.

The best results were obtained using 50 ºC as extraction temperature (Fig. 3.6.2). It is observed a direct correlation between extraction temperature and total instrument signal. The statistic results (Fig. 3.6.2) shows that the extraction temperature is the main factor in the extraction process.

3.1.3. Equilibration time

The selection of a proper equilibration time has direct influence on the sensitivity and precision of the NTME method [335]. NTME is an equilibrium-based process, where the amount of analyte extracted by the sorbent is proportional to equilibration time, until the equilibrium state between the headspace and sorbent occur, after that the recovery remains constant [335, 342, 345]. The results (Fig. 3.6.2) shows that the equilibration time was the second most important parameter in the optimization model. A linear correlation among equilibration time and instrument signal was obtained meaning that how much higher the extraction time, higher the instrument signal (within the time range studied).

3.1.4. Headspace volume

Since NTD is an exhaustive technique, in theory, the response will be proportional to the sample headspace volume that is loaded through the sorbent ($n = C_0V$), until the fibre breakpoint (where the saturation is reached) [343, 344]. Therefore, when we increase the headspace volume of the sample, we will increase the extraction efficiency. According Trefz et al the extractive response of the sorbent was greater when larger sample headspace volumes were used, at least for a set of model metabolites like isoprene, pentane, toluene and pentanal [351]. In conventional NTDs the breakthrough is about 0.5 mg for a packing length of 1 cm [352], moreover, according to Trefz et al, for DVB/CAR/CAR fibre the breakthrough volume was not reached up to 60 mL[351] of headspace volume. Based on the obtained results, and in order to minimize the extraction time, avoid saturation of signal on the GC-MS and increase the reusability of the sorbents, 30 mL was selected as the appropriate headspace sample volume. This is in agreement with the results obtained in DoE (Fig. 3.6.2).

3.2. Fingerprint of VOCs profile of lemon peels from different geographical regions

As can be seen in Fig. 3.6.3 and Table 3.6.1, the volatile profile from peel of the *Eureka* lemon samples from different geographical regions shows a similar volatomic pattern.

The total VOCs identified range from 69 (South Africa) to 72 (Portugal). A total of 75 different VOCs were identified in all samples based on comparison of mass spectra with the reference database (MS) and calculated retention indices (RI_{calc}) with values reported in the literature (RI_{lit}) for BP-20 fused silica (or equivalent) capillary column (Table 3.6.1). The retention indices of the experimental data were in good agreement with those reported on the literature, and with the correspondent linear retention having an r^2 = 0.995 (Supplementary Figure 3.6.1.). The contribution of each VOC for the total volatile fraction es expressed as relative peak area calculated as follow:

$$Peak\ area\ of\ analyte / Peak\ area\ of\ internal\ standard$$

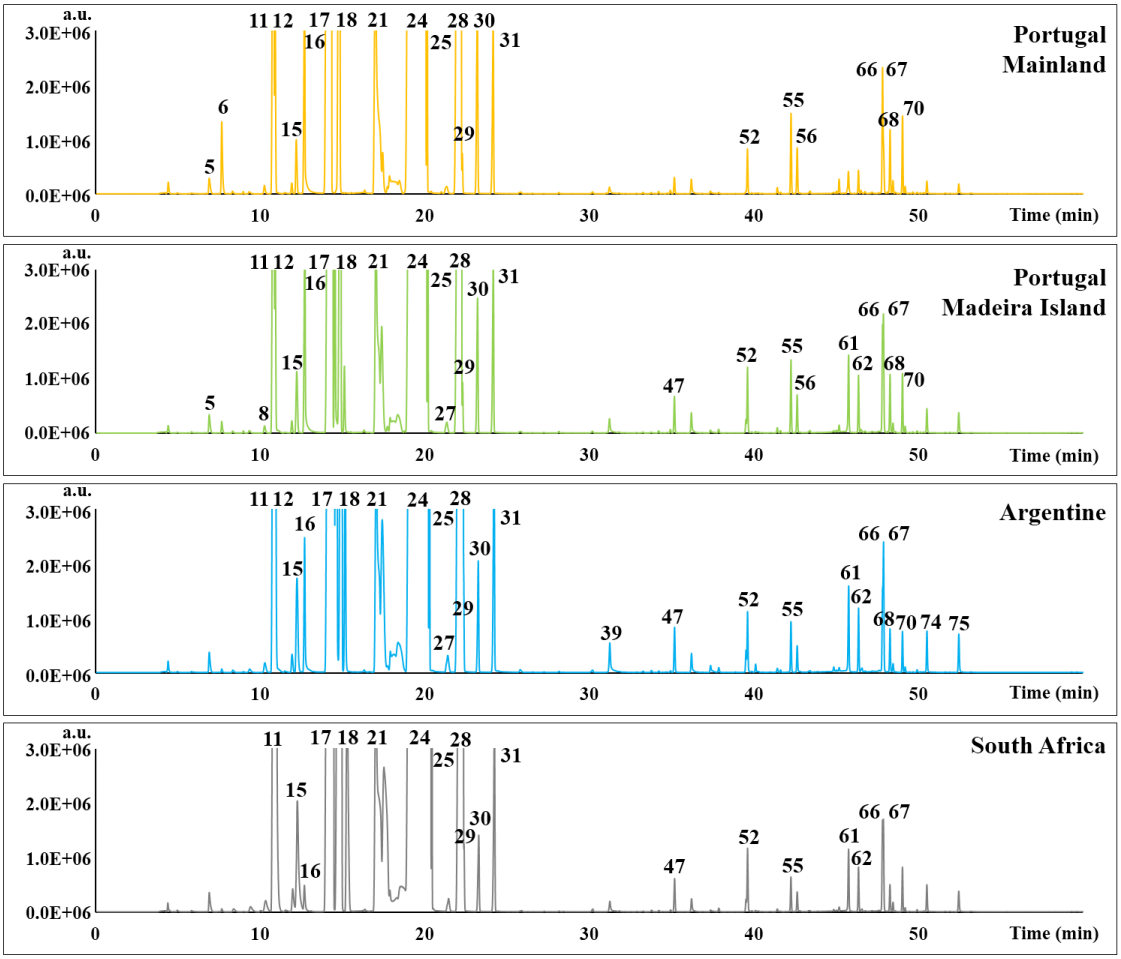


Fig. 3.6.3. NTME/GC-MS typical profile of peel from lemons of *Eureka* variety from geographical regions investigated in the study: Portugal (mainland and Madeira Island), Argentine and South Africa. VOCs that contributed with more than 0.05% to total peak area are: 5- methanol, 6- ethanol, 11- α-pinene, 12- α -thujene, 15- camphene, 16- hexanal, 17- β-pinene, 18- sabinene, 21- β-myrcene, 24- d-limonene, 25- β-phellandrene, 27- (E)-ocimene, 28- γ-terpinene, 29-(Z)-ocimene, 30- *o*-cimene, 31- terpinolene, 39- nonanal, 47- trans-β-terpineol, 52- linalool, 55- α-bergamotene, 56- β-caryophyllene, 61- neral, 62- α-terpineol, 66- neryl acetate, 67- valencene, 68- β-bisabolene, 70- geranyl acetate, 74- nerol, 75- geraniol.

Table 3.6.1. Volatile organic compounds (VOCs) identified in the peel of lemons from *Eureka* variety, from different geographical origins.

VOCs (organized by chemical family)		RT[a] (min)	RI[b]calc	RI[c]lit	Relative Peak Area (×10^{-2})[d] (RSD<10%)			
					Portugal (mainland)	Portugal (Madeira Island)	Argentine	South Africa
Aldehydes								
1[e]	Acetaldehyde	5.0	725	723	0.64	0.58	0.26	1.35
4	Butanal	6.7	843	860	0.12	0.12	0.02	0.07

8	Pentanal	9.0	946	968	0.77	0.89	0.14	0.36
16	Hexanal	12.7	1045	1083	140.34	139.49	20.07	30.71
37	2-Methyl-2-heptenal	28.4	1298	1342	nd[f]	nd	nd	0.41
39	Nonanal	31.2	1343	1390	3.51	12.39	8.31	11.06
49	Decanal	37.4	1446	1481	1.15	1.83	1.84	1.48
				Total	146.53	155.26	30.65	45.44
Esters								
3	Methyl acetate	6.0	798	813	0.23	0.13	0.05	0.11
26	Ethyl hexanoate	21.0	1190	1213	0.82	nd	nd	nd
44	Ethyl octanoate	34.0	1385	1428	0.18	0.07	nd	nd
				Total	1.24	0.20	0.05	0.11
Alcohols								
5	Methanol	7.0	860	866	12.04	20.08	5.12	21.08
6	Ethanol	7.7	899	917	44.36	6.97	0.89	2.24
19	1-Butanol	15.5	1099	1148	0.30	0.38	0.09	nd
20	1-Penten-3-ol	16.4	1116	1161	1.45	1.76	0.58	1.11
32	(Z)-2-Penten-1-ol	25.8	1263	1304	0.85	1.15	nd	0.42
33	2-Heptanol (*IS*) [g]	25.9	1265	1319	1.00	1.00	1.00	1.00
36	1-Hexanol	28.1	1294	1339	0.63	1.07	0.30	0.63
38	3-Hexen-1-ol	30.2	1327	1384	1.18	1.71	1.08	2.80
40	3-tert-Butylphenol	31.8	1352	-	0.37	0.49	0.13	0.56
53	1-Octanol	40.1	1494	1530	0.30	0.62	1.72	0.69
58	1-Nonanol	44.9	1597	1661	nd	nd	0.53	1.07
				Total	62.48	35.23	11.45	31.60
Ketones								
2	Acetone	5.8	785	775	0.44	0.79	0.18	0.76
22	2-Heptanone	17.8	1140	1180	4.07	3.46	1.00	nd
35	6-Methyl-5-hepten-2-one	27.2	1282	1322	0.16	0.42	0.12	0.54
				Total	4.67	4.67	1.30	1.29
Monoterpene Hydrocarbons								
9	Fenchene	9.4	957	-	2.21	2.41	0.74	6.93
10	Tricyclene	10.2	985	1003	6.29	8.34	3.18	15.19
11	α-Pinene	10.8	1001	1007	351.84	608.49	236.22	1315.55
12	α-Thujene	10.9	1003	1017	95.58	150.98	59.17	nd
14	α-Fenchene	11.9	1027	1052	6.51	8.43	2.98	16.52
15	Camphene	12.2	1034	1063	33.44	56.10	22.61	120.12
17	β-Pinene	14.3	1078	1094	1533.12	2408.58	1010.20	3005.88
18	Sabinene	14.8	1086	1109	405.39	589.44	285.60	635.61
21	β-Myrcene	17.0	1127	1169	308.92	507.92	207.17	740.51
23	α-Terpinene	17.8	1141	1181	8.67	2.61	1.16	4.11
24	D-Limonene	20.0	1176	1188	6179.13	8098.05	3138.40	12291.42
25	β-Phellandrene	20.1	1177	1197	70.31	105.95	43.58	136.96
27	(E)-Ocimene	21.3	1196	1240	6.64	15.45	7.58	18.56
28	γ-Terpinene	22.2	1210	1243	1277.39	1742.15	753.91	2247.46
29	Z-Ocimene	22.3	1210	1245	13.18	26.60	9.04	27.72
30	o-Cymene	23.2	1225	1260	103.82	88.27	24.78	61.49
31	Terpinolene	24.1	1239	1274	85.05	122.41	55.45	161.47
43	p-Cymenene	33.8	1383	1421	0.55	0.53	0.31	1.03
				Total (Monoterpene)	10488.03	14542.70	5862.07	20806.52
Sesquiterpene Hydrocarbons								
54	α-Santalene	41.6	1526	1555	0.58	1.25	0.39	0.25
55	α-Bergamotene	42.3	1542	1584	31.29	34.09	8.79	25.20
56	β-Caryophyllene	42.6	1548	1615	18.58	18.49	4.84	14.82
57	α-Himachalene	43.4	1566	1649	1.05	1.08	0.66	0.72
59	beta-Santalene	45.0	1599	1649	0.78	0.84	0.21	0.56
60	2,6-Dimethyl-2,6-octadiene	45.2	1604	-	4.89	2.82	0.61	2.69
63	(Z)-β-Farnesene	46.5	1636	1670	1.32	1.88	0.31	0.72
67	Valencene	47.9	1669	1718	15.32	52.22	21.05	49.75
68	β-Bisabolene	48.3	1678	1723	24.15	27.76	6.80	20.06
69	Bicyclogermacrene	48.5	1683	1735	4.66	4.93	1.37	7.04

72	α-Panansinene	49.6	1709	-	0.80	0.54	0.09	0.17
				Total	103.42	145.91	45.12	121.97
Oxygenated Terpenes								
41	Perillene	32.9	1369	1415	0.23	0.31	0.09	0.35
45	Limonene oxide	34.2	1388	1442	1.07	1.12	0.29	0.99
46	trans-Limonene oxide	34.9	1398	1472	1.64	2.10	0.33	1.49
47	*trans*-β-Terpineol	35.2	1404	1563	6.25	17.98	8.73	25.04
48	(3R)-(+)-Citronellal	36.2	1423	1493	6.87	14.44	4.81	12.23
50	Camphor	37.9	1455	1512	0.65	2.17	0.98	2.67
51	*cis*-β-Terpineol	39.5	1484	1616	0.84	6.19	3.55	7.88
52	Linalool	39.6	1486	1522	15.42	29.85	11.22	43.73
61	Neral	45.8	1619	1689	9.44	41.12	15.22	45.36
62	α-Terpineol	46.4	1633	1692	7.57	25.15	10.44	30.26
64	Borneol	46.6	1638	1698	nd	nd	0.50	1.14
65	Piperitone	47.6	1662	1705	0.24	0.42	0.17	0.45
66	Neryl acetate	47.8	1666	1708	46.53	44.28	11.82	64.61
70	Geranyl acetate	49.0	1694	1752	27.21	23.90	6.72	31.47
71	(R)-Citronellol	49.2	1699	-	2.31	2.73	1.00	2.45
73	Perilla aldehyde	49.9	1716	1776	0.57	1.12	0.51	1.52
74	Nerol	50.5	1731	1794	4.39	11.36	8.25	18.95
75	Geraniol	52.5	1778	1840	3.75	10.40	8.24	16.41
				Total	134.99	234.63	92.89	307.02
Others								
7	2-Ethyl-Furan	8.3	922	945	1.71	1.49	0.45	3.16
13	Toluene	11.1	1008	1019	0.25	0.21	0.10	nd
34	Tridecane	26.5	1273	1300	0.22	0.18	0.08	0.15
42	Tetradecane	33.3	1375	1400	0.58	0.52	0.22	0.78
				Total	2.77	2.40	0.85	4.09
				Total relative peak area (vs Internal standard)	10944.13	15121.04	6044.38	21318.06
				TOTAL VOCs	72	71	71	69

[a] RT: retention time expressed in min.
[b] RI_{calc}: experimental Kovat's index.
[c] RI_{lit}: Kovat's index reported in the literature.
[d] Relative Peak Area ($\times 10^{-2}$): (VOC peak area/Internal Standard peak area).
[e] Peak number ordered by VOC retention time.
[f] nd: not detected.
[g] IS: Internal Standard (2-heptanol).

3.2.1. *Eureka lemon from Portugal*

Lemons from Portugal presented the highest number of VOCs (72 identified volatiles) (Table 3.6.1) in peel volatile fraction, with a high relative peak area (when compared with Argentine and South Africa lemon peels). In terms of chemical families, the samples from Portugal presents just 3 esters, and for these, ethyl hexanoate is only present in lemons from mainland. The lemon peel from Portugal also presented a very high presence of ethanol, 6 to 45 times higher than found in other investigated samples. The total area of ketones was similar to that found in Madeira island lemons peel, but 4 times higher than lemon peels from Argentine and South Africa. We also

identified 19 monoterpenes contributing to a total volatile fraction 2 times higher than obtained for lemon peels from Argentina but about 50% lower than obtained in lemon peels from Madeira Island and South Africa. Regarding sesquiterpenes and terpenoids the lemon peels from Portugal presented a total peak area higher than obtained for Argentine lemon peel, but lower than obtained for Madeira Island and South Africa lemon peels.

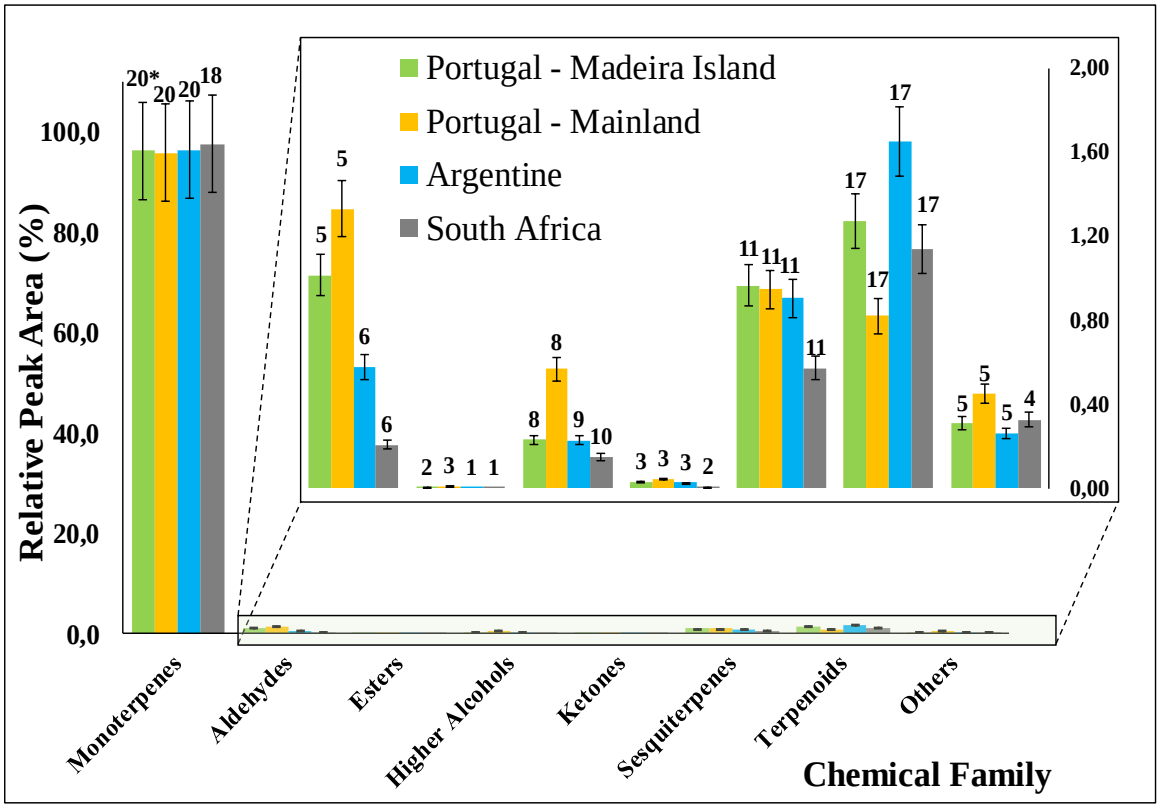


Fig. 3.6.4. Volatile metabolites identified in each lemon sample (*Eureka* variety from the 4 geographic regions indicated), after extraction by NTME, by chemical family (*number of VOCs identified is indicated above each column).

3.2.2. *Eureka lemons from Madeira Island*

A total of 71 VOCs were identified in lemon peels from *Eureka* variety cultivated at Madeira Island. The number of volatiles present in each chemical family was very similar to obtained in samples from lemons from Portugal mainland (Fig. 3.6.4). However, in terms of contribution of each chemical family, we can observe an increase

of total area for aldehydes, monoterpenes, sesquiterpenes and oxygenated terpenes. The volatiles present in theses chemical families are frequently associated in literature to bioactivity and beneficial properties (see section 3.3.).

3.2.3. *Eureka lemons from Argentine*

With a total of 71 VOCs, lemon peels from Argentine presented the lower total peak area, and the same trend for identified VOCs 2-Heptanone presenting a peak area 5 times that determined in Eureka lemons from Portugal mainland and Madeira Island. This volatile was not detected in peels of Eureka lemons from South Africa. the relative peak areas, the distribution along the chemical families (Fig. 3.6.4), when comparing with the other lemon samples, can be observed a higher area for the terpenoids and monoterpenes. On the other hand, we can see a lower area for the alcohols, esters and aldehydes (Fig. 3.6.4).

3.2.4. *Eureka lemons from South Africa*

Although having the lowest identified number of VOCs (69), the lemon peels of Eureka variety from South Africa presented the highest total area. This trend is explained by its high monoterpenes content with values 3.5, 2.0 and 1.5 times higher than determined for Eureka lemon peels from Argentine, Portugal and Madeira Island, respectively. The major contribution is determined by the major VOCs, D-limonene, followed by α- and β-pinene, β-myrcene and γ-terpinene. 2-Methyl-2-heptenal was identified only in Eureka lemon peels from South Africa, being considered a potential geographical marker. In opposite, 1-butanol, 2-heptanone, α-thujene and toluene, were not identified in any of lemon peel samples from South Africa.

The differences on the distribution of the VOCs along the respective chemical family, namely higher relative areas of aldehydes, esters, alcohols and ketones, suggest that the two samples from Portugal have higher metabolic expression of the Fatty acid biosynthesis than the lemon samples from Argentina and South Africa.

Volatile composition of lemon peels from Eureka variety has been subject of a number of papers in the scientific literature, employing a range of analytical techniques. In comparison with similar published works on volatile fingerprint of peel from Eureka lemons Lota et al. [353] identified 22 VOCs, Zhong et al. [354] identified 34 VOCs and Zhang et al. [355] identified 54 VOCs. In addition Zhang et al. [355] reported 67 VOCs for *Limonia* lemon peel. The 75 VOCs reported in this work revealed the high throughput analytical potential of the proposed methodology in comparison to the used in described works. In agreement with our results, these works indicate a rich-monoterpene volatile fingerprint and a similar volatile composition for the major VOCs namely D-limonene, β-pinene, γ-terpinene, β-myrcene and α-pinene.

Monoterpenes were the most abundant chemical family identified in lemons from Eureka variety, accounting for about 95% of the volatomic fingerprint (Fig. 3.6.4). The most dominant VOCs, D-limonene (about 54.9±2.6%), β-pinene (about 15.2±1.4 %) and γ-terpinene (about 11.6±0.8 %) accounting for more than 81.6± 0.7 % of the total volatile composition expressed in VOCs peak area. The chemical families with most important contributions to the volatomic fingerprint of Eureka lemon peels, includes terpenoids (18 VOCs), sesquiterpenes (11 VOCs) and alcohols (10 VOCs).

3.3. *Potential of Eureka variety lemons*

The main monoterpenes present in the volatile composition of lemons peel, are reported as potential bioactive compounds (see Table 3.6.2), being also identified on studies of *Thymus vulgaris* L.[62], *Rosmarinus officinalis* L.[62], *Myrtus communis* L.[62], *Pistacia lentiscus* var. *chia* [316], *Araucaria heterophylla* [315], *Araucaria bidwillii* [315], *Citrus hystrix* [308], *Citrus aurantifolia* [55], and other plants. In addition, some studies focus some bioactive compounds like isoprenoids [356] or D-limonene [61].

Table 3.6.2. Reported bioactivity of some monoterpenes, that are between the main VOCs present in the establish volatile profile of the lemons under study.

#	Terpene	Bioactivity	Ref.
11	α-Pinene	Antioxidant, Antidiabetic, Antitumor, Antimicrobial, Antiproliferative, Antileishmanial and Cytotoxic activity	57-60, 62, 308, 314-316, 357
15	Camphene	Antioxidant, Antibacterial and Antiproliferative activity	62, 308, 316
17	β-Pinene	Antioxidant, Antitumor, Antimicrobial and Antiproliferative activity	62, 308, 314, 316, 358
18	Sabinene	Antitumor, Antibacterial, Antileishmanial and Cytotoxic activity	60, 62, 314
21	β-Myrcene	Antioxidant, Antidiabetic, Antitumor, Antimicrobial, Antiproliferative, Anti-inflammatory, Antileishmanial and Cytotoxic activity	56-60, 62, 308, 314, 316, 358
23	α-Terpinene	Antitumor, Antibacterial, Antileishmanial and Cytotoxic activity	60, 62, 314
24	D-Limonene	Antioxidant, Antidiabetic, Antitumor, Antimicrobial, Antiproliferative, Antileishmanial and Cytotoxic activity	57-62, 308, 313-316, 356-359
25	β-Phellandrene	Antioxidant, Antidiabetic, Antitumor, Antibacterial, Antileishmanial and Cytotoxic activity	58, 60, 314
27	(E)-Ocimene	Antioxidant, Antidiabetic, Antibacterial and Anti-inflammatory activity	56, 58
28	γ-Terpinene	Antioxidant, Antitumor and Antimicrobial activity	62, 308, 314, 357
31	Terpinolene	Antitumor and Antibacterial activity	62, 314
56	β-Caryophyllene	Antioxidant, Antidiabetic, Antibacterial, Antiproliferative, Antileishmanial and Cytotoxic activity	59, 60, 62, 315, 316, 337, 359, 360
52	Linalool	Antioxidant, Antidiabetic, Antibacterial, Antiproliferative and Anti-inflammatory activity	56, 57, 62, 63, 308, 313, 316, 359
62	α-Terpineol	Antioxidant, Antitumor, Antimicrobial, Antileishmanial and Cytotoxic activity	59, 62, 308, 313, 314
73	Perilla aldehyde	Antioxidant, Antidepressant-like effect and Anti-inflammatory activity	55, 361
75	Geraniol	Antioxidant, Antibacterial and Antiproliferative activity	63, 308, 356, 360

As can be seen in Table 3.6.2, a brief research in recent papers (mostly 2018 and 2019) allowed the identification of sixteen of lemon peel VOCs, being associated to antioxidant [55, 56, 58, 308, 359], anti-inflammatory [55, 56], antidiabetic [57, 58], antileishmanial [59, 60], antibacterial [58, 60-63], antimicrobial [313, 357, 358], cytotoxic [59, 60], antitumor [314] and antiproliferative activities [315, 316, 356, 360]. It is reported others benefits related to health, such as on Alzheimer [317] and tuberculosis [318] symptoms. This suggest a strong correlation between the main VOCs identified on the volatile profile of lemon peels

and the health benefits, adding nutraceutical value to the lemons and lemon-based foodstuffs.

The sent of lemon is recognized worldwide, D-limonene is fundamental for the resulting aroma, dominating the volatile profile, contributing to more than half of the total volatile profile, giving that strong and characteristic citrus notes. β-Phellandrene helps give fresh notes with a distinctive mint smell, such as hexanal with the typical fresh grass smell. α- and β-pinene have a pine, woody smell, just as terpinolene. Sabinene and β-myrcene add some spicy notes. The acidic, fresh and sweet notes mixt with a lithe touch of wood, pine result in a distinctive and appealing aroma. in such a way that lemon aroma is widely used by the industry linked to cosmetics, perfumes and cleaning products. Connecting the pleasant aroma with health benefits, lemon and its aroma are also being used in beauty treatments, and aromatherapy.

3.4. *Multivariate analysis – Geographical discrimination*

To evaluate the ability of the identified VOCs to discriminate Eureka lemons according to geographical origin, the volatomic data were processed using different statistical tools. The data matrix was subjected to a statistical analysis using MetaboAnalyst 4.0 web-based tool [305]. To reduce the data complexity, a normalization method, described in the experimental section, was applied to the raw data previously to the univariate statistical analysis (ANOVA test, $p \leq 0.05$). The ANOVA test was carried out to evaluate the significant statistical differences among the concentrations of VOCs in each sample group. A total of 65 VOCs were found as statistically significant for ($p < 0.05$, Supplementary Table 3.6.2.). Following this, to assess if there were significant VOCs signatures between each group, multivariate statistical analysis (MVSA) was performed using no supervised (PCA) analysis. The obtained results show four different clusters segregating each group under study (Fig. 3.6.5).

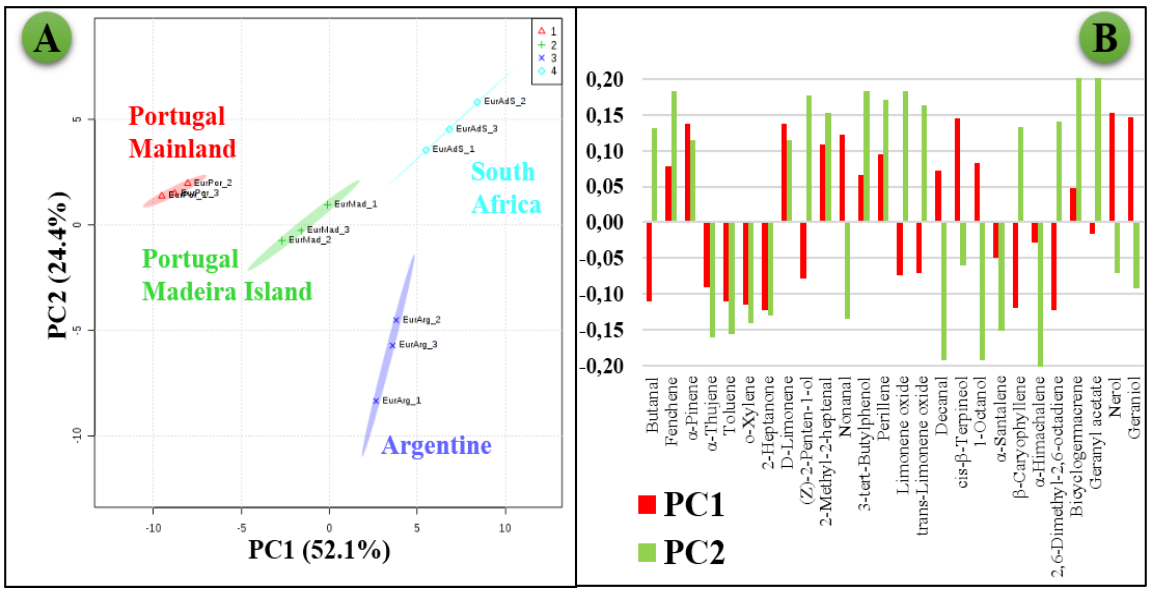


Fig. 3.6.5. (A) Score scatter plot based on two principal components (PC1 and PC2) using the VOCs obtained by NTME/GC-MS, and (B) the related variables that most contributed for the PCA differentiation.

In terms of PCA, the first principal component (PC1) explains 52.1 % of the variance and separate varieties produced in Portugal - Mainland and Madeira island from varieties produced in South Africa and Argentine (ethanol, ethyl octanoate, trans-β-terpinol, *α*-panansinene, perilla aldehyde and nerol). The second principal component (PC2) contributes for 24.4 % of the total variance of the model and separate the varieties produced in South Africa from those produced in Argentine (*α*-pinene, *α*-thujene, toluene, 1-butanol, *D*-limonene and 2-methyl-2-heptenal).

Following the PCA analysis, HCA was also performed using the 40 most significant VOCs identified in lemon samples, obtained by ANOVA (was generated using Ward algorithm and Pearson distance analysis), allowing a better identification of the inherent clustering patterns between each geographic origin, in complementarity with the statistical analysis already carried out previously. The difference of potential VOCs that allow the differentiation between groups were visualized in the heatmap plot (Fig. 3.6.5) and in a dendrogram (Figure S1). The ratio of VOCs was first calculated by average algorithm and Pearson distance analysis, and then the metabolic

alterations were demonstrated as log10 (ratio) depicting distinct clustering patterns among the studied groups (Fig. 3.6.6).

Table 3.6.3. Most significant potential geographical marker compounds, responsible for the distinction between *Eureka* lemons according to production region.

Portugal (mainland)	**Portugal (Madeira Island)**	**Argentina**	**South Africa**
Butanal	α-Thujene	Nonanal	Fenchene
Limonene oxide	Toluene	Decanal	Tricyclene
(Z)-2-Penten-1-ol	1-Butanol	Cis-β-Terpineol	α-Pinene
β-Caryophyllene	2-Heptanone	1-Octanol	Camphene
2,6-Dimethyl-2,6-octadiene	α-Santalene	Nerol	D-Limonene
Ethyl hexanoate[a]	α-Himachalene	Geraniol	2-Methyl-2-heptenal[a]

[a] VOC identified only in this geographical region

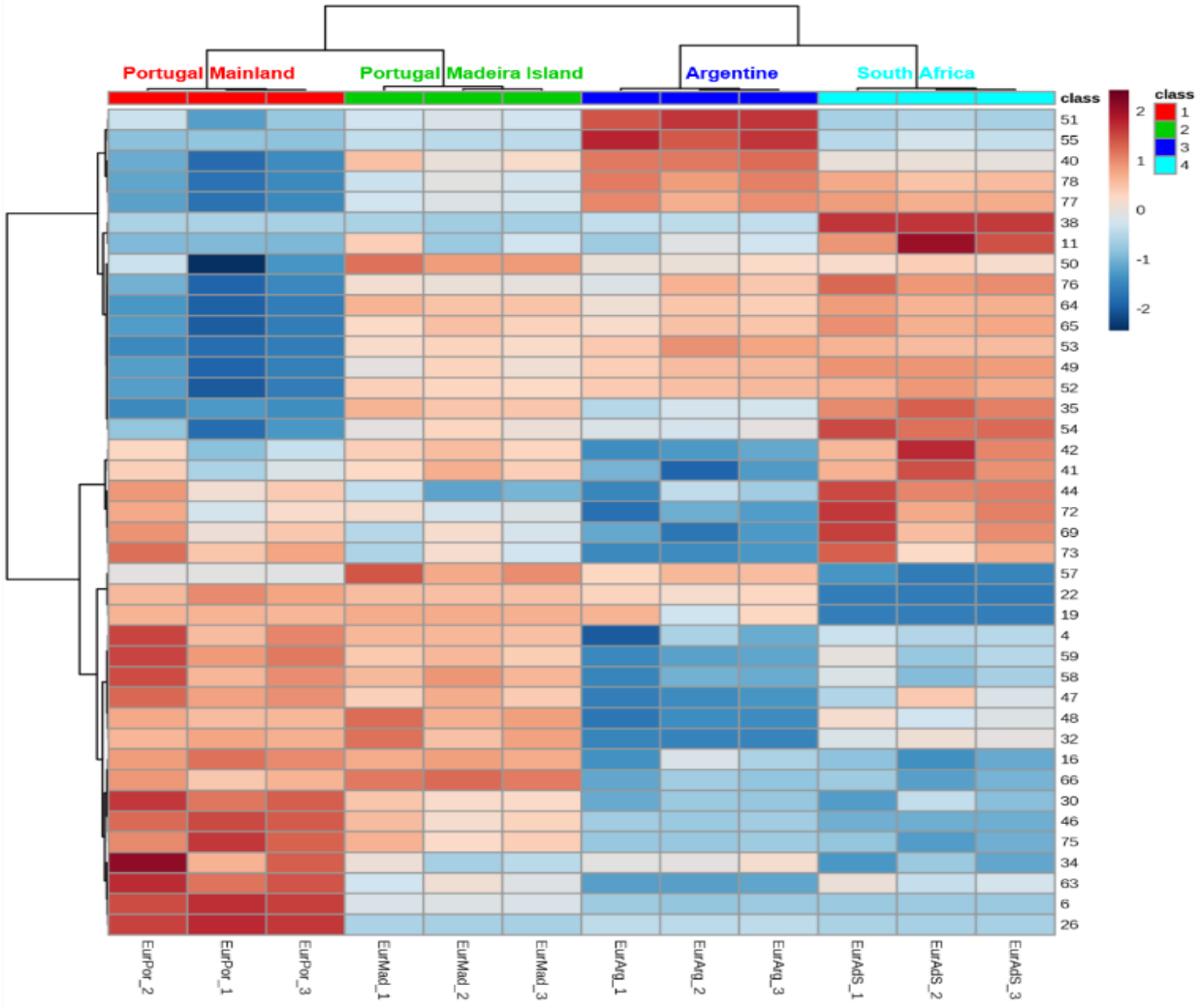


Fig. 3.6.6. Hierarchical cluster analysis (HCA). The heat map with the 40 most significant volatiles identified in lemon samples obtained by ANOVA, was generated using Ward algorithm and Pearson distance analysis.

4. *Conclusions*

The identification of lemons according to geographical origin based on a simple analytical sequence and a relatively low-cost experimental set-up was presented. The optimized high throughput analytical approach, NTME/GC-MS, allowed a deep and comprehensive insight on the volatile composition of lemon peels (exocarp) from *Eureka* variety cultivated at different geographical origins – Portugal Madeira Island (Portugal), Argentine and South Africa. A total of 75 VOCs were identified in lemon peels from Eureka variety, a number slightly higher than those reported in previous published works for the same variety. The monoterpenes family are the most

dominant VOCs contributing for about 95% of the volatomic composition of lemon peels from Eureka variety. D-limonene, β-pinene and γ-terpinene are the major volatiles identified in lemon peels from the targeted geographical origins.

The identified VOCs were able to differentiate lemons according to its geographical origin, being butanal, *α*-pinene, *α*-thujene, 2-heptanone, D-limonene, 2-methyl-2-heptenal, nonanal, decanal, 1-octanol, limonene oxide, β-caryophyllene and 2,6-dimethyl-2,6-octadiene, the VOCs that most contributed for the discrimination.

In addition, this analytical approach provides a feasible strategy for authentication of citrus fruits based on volatile fingerprint of its exocarp. NTME/GC-MS reveals a great application potential to other fruits and food matrices, regarding its analytical characterization and authentication based on its volatomic composition, enabling effective strategies to support food integrity. The results also suggested a wide range of potential applications for lemon peels from Eureka variety based on identified VOCs, namely health benefits, potential food additives, as flavour and fragance agents and cosmetic industry.

Sub-Section 3.7. Evaluation of the health-promoting properties of selected fruits

molecules

MDPI

Article

Evaluation of the Health-Promoting Properties of Selected Fruits

José A. Figueira [1], Priscilla Porto-Figueira [1], Cristina Berenguer [1], Jorge A. M. Pereira [1] and José S. Câmara [1,2,*]

Published in:

Molecules (Impact Factor 4.411)

José A. Figueira, Priscilla Porto-Figueira, Cristina Berenguer, Jorge A.M. Pereira, José S. Câmara

https://doi.org/10.3390/molecules26144202

Abstract

In this study, the health-promoting benefits of different fruits grown in Madeira Island, namely lemon (*Citrus limon* var. eureka), tangerine (*Citrus reticulata* var. setubalense), pitanga (*Eugenia uniflora* var. red), tomato (*Solanum lycopersicum* var. gordal) and uva-da-serra, an endemic blueberry (*Vaccinium* padifolium Sm.), were investigated. The phenolic composition (total phenolics and total flavonoids content) and antioxidant capacity (assessed through ABTS and DPPH assays) were measured revealing a high phenolic potential for all fruits, except tomato, while uva-da-serra is particularly rich in flavonoids. Regarding the antioxidant capacity, the highest values were obtained for pitanga and uva-da-serra extracts. The bioactive potential was also assessed through the ability of the extracts to inhibit digestive enzymes linked to diabetes (α-amylase, α- and β-glucosidases) and hypertension (angiotensin-converting enzyme, ACE). The results obtained point to a very high bioactive potential with the selected samples exhibiting very important ACE anti-enzymatic capacities. A statistical analysis of the data obtained reveals a very strong correlation between ABTS and TPC and a strong contribution of the fruit polyphenols for enzyme inhibition, and therefore presenting high antihypertensive and antidiabetic capacities. Overall, the results obtained clearly show a high bioactive potential of the selected fruits that should be further studied in terms of specific phenolic composition. Moreover, these results strongly support the valorisation of pitanga seeds usually discarded as a waste, and uva-da-serra, an endemic and wild bush, as potential bioresources of bioactive compounds with impact in human diet.

Keywords: antioxidant, antidiabetic, antihypertensive *Vaccinium padifolium* Sm., nutraceutical fruits, pitanga seeds

1. Introduction

It is widely known that the consumption of fruits and vegetable elicit health protection against different diseases. According to [3], between 5.6 and 7.8 million premature deaths occurring worldwide in 2013, were attributable to a low fruit and vegetable intake (lower than 500 and 800 g/day), respectively. In this context [4] pointed that most of these mortality risks, mainly those related to cardiovascular disease, chronic diseases, and cancer, could be reduced by regular and varied consumption of fruit and vegetables,. These protective effects are largely attributed to secondary metabolites including polyphenols, glucosinolates, carotenoids, terpenoids, alkaloids, saponins, vitamins, among others, present in fruits and vegetables[9] exhibiting antioxidant, antiatherogenic, anti-inflammatory, antimicrobial, and cardioprotective effects [9] [8]. These bioactive compounds are mostly produced by plants to cope with different challenges particularly those related to adverse environmental conditions (hydric stress, high temperatures and humidity levels). In this sense, Madeira Island has very challenging climate conditions, with very hot and humid conditions all over the year, high thermic variations and pronounced slopes which correlate with the high bioactive potential and complex volatile compositions exhibited by fruits growing in Madeira Island in comparison with same varieties from other geographical regions. Previously, we have shown that tomato (*Solanum lycopersicum* L.) from gordal variety grown in Madeira island presented higher tocopherols content when compared with other varieties commonly consumed like campari, roma and cherry [229]. In addition, recently [45] underlined the relevance of tocopherols, as vitamin E, on the proper functioning of the immune system, acting as an antioxidant in the context of the importance of phytochemicals of the Mediterranean diet against COVID-19 effects. Similarly, polyphenols have been suggested as a therapeutic adjuvant in the treatment of COVID-19 patients [362]. These classes of secondary metabolites with high nutraceutical value are widely found in nature including in citrus fruits. Among them, flavonoids have been previously associated with a positive effect in the treatment of different diseases, including arthritis, diabetes mellitus, cancer and

neurodegenerative disorders, as well as liver, kidney and heart diseases [363]. Overall, this protection elicited by metabolites present in citrus fruits contributes to strengthening their general awareness of functional foods [364]. Lemon, for instance, is very rich in a large variety of secondary metabolites, mainly monoterpenes, which are used in nutraceutical and food industries [365]. Tangerines are another widely consumed citrus fruit that contains a similar bioactive content profile to lemon, namely in what concerns monoterpenes [364]. According to Figueira *et al.*[364, 365] both lemons and tangerines grown in Madeira Island were shown to have a very complex volatile composition with some of identified VOCs being responsible for health benefits. Tutunchi *et al.* [366] and Alberca *et al.*[367] reported the potential effects of naringenin, a flavanone with antiviral and anti-inflammatory activities, as a promising treatment strategy against COVID-19.

Vaccinium padifolium Sm. is an endemic blueberry tree from Madeira Island, locally known as uveira-da-serra, whose interest and consumption of its berries increased in recent years due to its very high nutritional value [98], related to the high content of phenolic compounds [99, 368].

Eugenia uniflora L. fruits, popularly known as "Suriname cherry" or “pitanga” is an exotic fruit native from Brazil [84], but widely available in Madeira Island. Pitanga is appreciated by consumers for its softness, aromatic and bittersweet flavour, and presents a low lipid and caloric content and high amounts of phenolic compounds [369, 370], carotenoids and anthocyanins [371]. Several ethnomedical uses of *E. uniflora* have been documented, especially those related to leaves and oils extracts [92], which present in vitro antiproliferative potential [90]. The leaves and fruits extracts have also shown stimulant, febrifuge, aromatic and antidiarrheal characteristics [372], and pitanga juices, anti-inflammatory properties [373]. Pitanga seeds, however, are usually discarded although [374] reported a promising antioxidant potential requiring further studies to explore its different fields of application.

In this context, this study aimed to evaluate the health-promoting proprieties of fruits of regular consumption grown in Madeira Island, including the total phenolic and

flavonoid contents (TPC and TFC, respectively), antioxidant capacity (ABTS and DPPH assays) and its ability to inhibit digestive enzymes linked to diabetes (α-amylase, α- and β-glucosidase) and hypertension (angiotensin-converting enzyme),. The fruits were selected according to its high bioactive potential, previously reported, namely tomato (gordal variety) [229, 232], lemon (eureka variety) [303], tangerine (setubalense variety) [364], uva-da-serra [368], and pitanga. Liquid-liquid-based ultrasound-assisted extraction (LLUSAE), an efficient extraction procedure for vegetable matrices, previously optimized in our lab [232], was used to obtain the extracts.

2. Materials and Methods

2.1. Chemicals and Materials

Angiotensin-converting enzyme (ACE, from human, 95%), hydrochloric acid (HCl, ACS reagent, 37%), trisodium citrate dihydrate (C6H5Na3O7·2H2O, 99%), fluorescein, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), sodium carbonate (Na2CO3), 3,5-Dinitrosalicylic acid (DNS) colour reagent, aluminium chloride (AlCl3), so-dium nitrite (NaNO2 ACS reagent, 97.0%), α- and β-glucosidase, α- and β-pNPG, and α-amylase were acquired to Sigma-Aldrich (Buchs, Switzerland). Potassium phosphate dibasic trihydrate (K2HPO4.3H2O) was acquired from Merck (Buchs, Switzerland), ethanol (EtOH, absolute PA, 99.5%), potassium dihydrogen phosphate (KH2PO4, 99.5%), sodium chloride (NaCl, 99.8%), N-[3-(2-furyl)acryloyl]-Phe-Gly-Gly (FAPGG) and sodium hy-droxide (NaOH) were acquired to Panreac, (Barcelona, Spain). Folin-Ciocalteu solution, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, 98%), and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were acquired to Fluka (Munich, Germany). Acetonitrile (ACN) and methanol (MeOH) (both HPLC grade, 99.99%) to Thermo Fisher Scientific (Loughborough, UK), and PSA/C18/Mg2SO4 (25/25/150 mg, DisQuE) was purchased from Waters (Milford, MA, USA). The ultrapure water used on the assays was obtain using an Ultrapure water purification system (Milli-Q® Direct 8 at 18 MW cm, 23 °C, Milli-pore Corporation, USA).

2.2. Samples

Lemon samples (eureka variety), tangerine (setubalense variety) and tomato (gordal variety) were selected randomly from a local market (Madeira Island), as purchased for consumption (Fig. 3.7.1). Red pitanga and uva-da-serra samples were randomly harvested directly from trees in local productions in Madeira Island. After selection, five hundred grams of the peels and juice from lemon and tangerines, the seeds and pulp from pitanga and the whole tomato and uva-da-serra fruits were collected and immediately stored under N2 (g) atmosphere at −80 °C. Then, with exception of the citrus juices, all samples were lyophilized (Christ Alpha 1–2 LD plus freeze dryer, Osterode am Harz, Germany), grounded to powder (IKA A11 basic analytical mill, Staufen, Germany) and immediately stored under nitrogen at −80 °C, in several aliquots. All aliquots were used only once to prevent sample degradation.

Fig. 3.7.1. Fruits under study: Pitanga (*Eugenia uniflora* variety red) seeds and pulp, uva-da-serra (*Vaccinium padifolium* Sm.), tomato (*Solanum lycopersicum* variety gordal), lemon (*Citrus limon* variety *eureka*) peels and juice and tangerine (*Citrus reticulata* variety setubalense) peels and juice.

2.3. Extraction

Sample extraction was performed according to the procedure previously optimized for vegetable matrices by [232]. Briefly, 50 mg of the lyophilized samples for each fruit were diluted with 9 mL of ACN: MeOH (4:1, v/v), sonicated (BRANSON 2510E-DTH, 100 W, 40 kHz, Danbury, CT, USA) for 30 min at 25 °C and centrifuged during 5 min (5000 × g, Espresso Personal microcentrifuge, Thermo Scientific, Waltham, MA, USA). Finally, the supernatant was collected, and 180 mg PSA/C18/Mg2 SO4 (1:1:6; w, w, w) / mL of sample extract was added for cleaned up, being homogenized in the vortex, centrifuged again (5 min, 5000 × g), and filtrated (0.2 μm) before analysis.

2.3. Bioactivity Evaluation

2.3.1. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The TPC was determined using a modified Folin-Ciocalteu procedure. Briefly, fruit extracts were diluted in water up to 1 mL final volume, added 100 μL of Folin-Ciocalteu solution, 400 μL of Na2CO3 (20%), and 500 μL of water. After 1h, the electron transfer from phenolic compounds is measured by UV-Vis at λ = 765 nm. TFC assay was performed using the aluminium chloride colorimetric method. Briefly, fruits extracts were diluted in methanol (70%) up to 1 mL final volume, added 60 μL of NaNO2 (5%) and rest 5 min in the dark. Then, added 60 μL AlCl3 (10%), rest another 5 min before the addition of 400 μL of NaOH (1M), a 2-min rest and finally 480 μL of methanol (70%). The acid-stable com-plexes formed by the AlCl3 with flavones and flavonols were measured at λ = 510 nm.

2.3.2. Total Antioxidant Capacity (TAC)

DPPH assay was performed according to [232]. Briefly, 500 μL methanol was added to the fruit extracts, followed by 1 mL of free-radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and stored 10 min in the dark, before UV-Vis analysis at λ = 515 nm (Lambda 25, Perkin Elmer, Belgium) to measure the free-radical reduction. ABTS assay was adapted from the procedure reported by [231]. Briefly, a stock solution of 2,29-azinobis-

(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS, 7.3 mM and potassium persulfate 2.59 mM) was prepared in ethanol and rest in the dark for 16h, at room temperature, before use. Ethanol was added to the fruits extracts up to a 100 µL final volume and added 1.9 mL of 100x diluted ABTS solution (in ethanol). After 2 h storage in the dark, the reduction of the radical cation was measured at 734 nm.

2.3.3. Antihypertensive Capacity

The antihypertensive capacity was assessed using the ACE-inhibition activity assay reported by [375] with minor changes. Briefly, 50 µL of FAPGG (2 mM) were diluted in 450 µL of Tris-HCl buffer (50 mM, with 300 mM of NaCl and HCl 0.1 M at pH 8.3). After homogenization by vortex (1 min), 400 µL of water were added, then 50 µL of the sample, followed by homogenization before adding 50 µL of ACE (125 mU diluted from a stock solution of 25 U in the phosphate-potassium buffer - KH_2PO_4 9.3 mM and $K_2HPO_4.3H_2O$ 0.7 M; with 300 mM NaCl at pH 8.3), and incubate 3 min at 37 °C. Finally, the absorbance was measured every 2 min for 20 min at λ = 328 nm.

2.3.4. Antidiabetic Capacity

The study of the antidiabetic capacity was estimated through the inhibition of the digestive enzymes α- and β-glucosidases and α-amylase. For α- and β-glucosidases assay, 50 µL of the respective enzyme (1 U/mL) were added to 25 µL of the sample extract and incubated 10 min at 37 °C. 100 µL of the substrate α-pNPG (5mM) or β-pNPG (5mM), respectively, were added and incubated 30 min at 37 °C.

The reaction was terminated by adding 180 µL of Na_2CO_3 (0.1M) and the absorbance measured at λ = 405 nm. α-Amylase inhibition was evaluated by adding 400 µL of the substrate starch (1%) to 200 µL of the sample extract followed by 3 min incubation at 37 °C, and addition of 200 µL of the enzyme (13 U/mL) followed by a new 3 min of incubation at 37 °C. It was adicionad200 µL of DNS colour reagent (DNS 96 mM, potassium sodium tartrate 5.31 M in NaOH 2M), finally, the mixture was incubated at 95 °C for 10 min in a dry bath (Block heating system Grant QBD1, Frilabo, Portugal),

the reaction stopped with the addition of 900 μL of cold water, and absorbance measured at λ = 540 nm.

2.4. Statistical Analysis

The statistical analysis was performed using the MetaboAnalyst 4.0 web-based tool [305]. The raw data obtained in the bioactive assays (TPC, TFC, ABTS, DPPH, α-amylase, α-glucose, β-glucose, and ACE-inhibition) was pre-processed by normalization (to sample median, data transformation by Log_{10} normalization and data scaling by autoscaling). Additionally, one-way Analysis of Variance (ANOVA, $p < 0.05$) was carried out, followed by posthoc analyses Tukey's Honestly Significant Difference (Tukey's HSD, $p \leq 0.05$), used for mean comparisons among dates. The correlations between variables were examined by Pearson's correlation ($p < 0.05$).

3. Results

In previous works, a high concentration in different bioactive compounds, particularly phenolic compounds, has been observed in the fruits analysed in this work [229, 232, 303, 364, 368]. This observation led us to investigate the bioactive potential of the juice and peels of lemon and tangerine, pulp and seeds of pitanga, and the whole fruit of tomato and uva-da-serra. The bioactivity was assessed by measuring the phenolic content (total phenolic content, TPC, and total flavonoid content, TFC), the antioxidant capacity (total antioxidant assays, TAC, DPPH and ABTS), as well the inhibition of key enzymes associated with antihypertensive and antidiabetic effects. To allow a systematic comparison between the fruit extracts, seven extraction conditions were assayed to find the most suitable to all samples. Accordingly, TPC and TFC were determined for 35 different conditions (five sample extracts *vs* seven extraction conditions). The data obtained (Supplementary Table 3.7.1.) were normalized as log_{10} of the antioxidant assays values determined to allow the identification of the best extraction conditions. As shown in Fig. 3.7.1, the solvent mixtures ACN:MeOH (4:1, v/v), MeOH:FA (19:1, v/v) and ACN retrieve the best results. ACN:MeOH (4:1, v/v)

was the selected condition as it was previously used with success in the extraction of hydrophilic and lipophilic bioactive compounds (carotenoids or tocopherols) [229, 232].

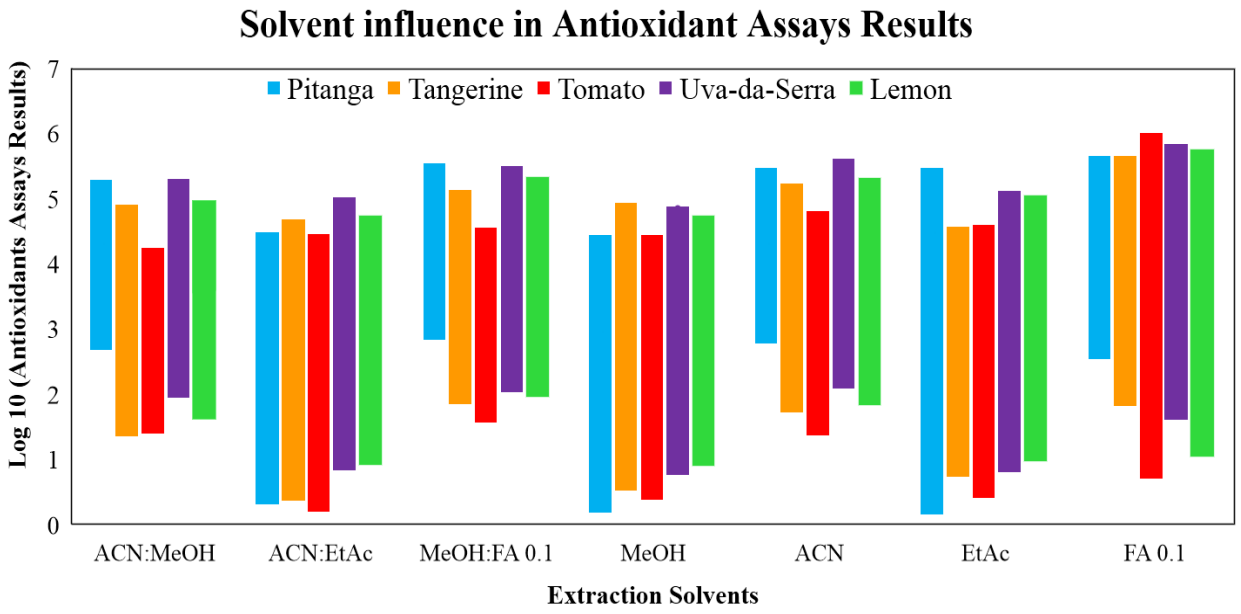


Fig. 3.7.2. The influence of the extraction conditions on the antioxidants assays performance was assessed by measuring the TAC, TPC and TFC for each fruit extract (pitanga, tangerine, tomato, uva-da-serra and lemon) obtained using each of the seven solvent extraction conditions: methanol - MeOH, acetonitrile – ACN, ethyl acetate - EtAc, formic acid 0.1 % - FA, ACN:MeOH (4:1, v/v), ACN:EtAc (1:1, v/v) and MeOH:FA (19:1, v/v). Log_{10} was applied to results obtained normalize the data obtained and allow a better comparison of the best extraction condition.

3.1. Phenolic composition and antioxidant capacity of the selected fruits extracts

The TPC and TFC were determined to get a snapshot of the phenolic amount of the selected fruits. As shown in Fig. 3.7.2A, pitanga seeds and uva-da-serra berries exhibit the highest phenolic content, while peels from lemon and tangerines and the pulp from pitanga also contain very interesting TPCs, being much lower for tomato and vestigial in tangerine and lemon juices. Although most of these individual results are in agreement with the previous literature results for uva-da-serra [99], tangerine [74] and lemon [376] and tomato [377], however, pitanga seeds present a TPC that is being, to the best of our knowledge, reported for the first time.

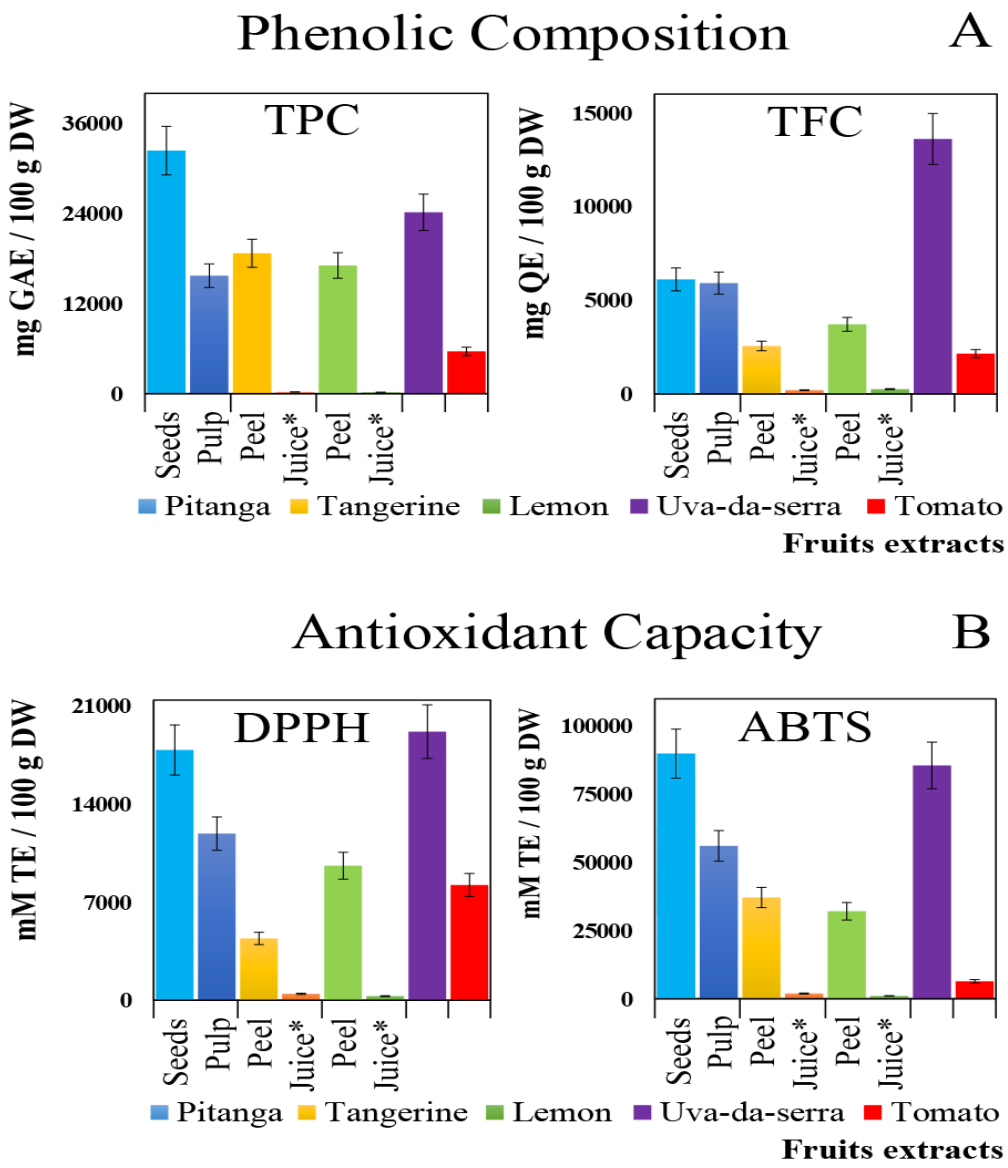


Fig. 3.7.3. Evaluation of the phenolic composition (A) and antioxidant capacity (B) of the selected fruit extracts, pitanga (seeds and pulp), tangerine (peel and juice), lemon (peel and juice), uva-da-serra (whole fruits) and tomato (whole fruits). The phenolic composition was evaluated through the assessment of the total phenolic composition (TPC) and total flavonoid content (TFC), while the total antiox-idant capacity (TAC) was evaluated using DPPH and ABTS assays; * by 100 mL of fresh juice instead of dry weight (DW). Legend: ABTS - 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay, ACE – angiotensin-converting enzyme, DPPH - 2,2-diphenyl-1-picrylhydrazyl free radical assay, GAE – gallic acid equivalents, QE - quercetin equivalents, TFC - total flavonoid content, TE – Trolox equivalents, TPC - total phenolic content.

Very relevant is the high flavonoid composition of uva-da-serra which is almost three times higher than the levels found in pitanga seeds, the second extract with the highest TFC value (Fig. 3.7.2A, right panel). Again, this result is corroborated by our previous results[329] which revealed that 8 of the top 10 free low molecular polyphenols identified in uva-da-serra are flavonoids. To understand the impact of the TPC and TFC values found in the selected fruits, a literature survey was performed to compare the results obtained with the ones previously reported in other studies involving the same or similar fruits (Supplementary Table 3.7.2.). A systematic comparison among all data collected is not totally feasible due to the variations in the experimental conditions used in the different reports, namely the fruits extracts preparation and extraction technique. Despite that fact, the potential of the fruits studied in this work is very relevant and promising as they present the richest phenolics content and antioxidant capacities. To unveil putative correlations between the high phenolic content and antioxidant capacity, the antioxidant capacity of the 8 extracts analysed was assessed through DPPH and ABTS assays. As Fig. 3.7.2B shows, the antioxidant capacity of pitanga (seeds and pulp) and uva-da-serra replicate the trends observed for the phenolic content, being these extracts that present the highest values for the DPPH and ABTS assays. The data obtained is supported by previous reports for the different fruits studied, namely tomato [377], uva-da-serra [99], pitanga [372] and lemon [378, 379]. Detailed data is listed in Supplementary Table 3.7.2.

Overall, the TAC results point very clearly to the promising antioxidant proprieties of the endemic blueberry uva-da-serra and pitanga. Moreover, the obtained results show that the seeds of pitanga, which are not edible and generally discarded as waste, can be explored as a bioresource of natural antioxidants and nutraceuticals unveiling important applications in the food industry as a bioresource of natural antioxidant compounds.

3.2. Enzymatic Inhibition Capacity

To obtain further evidence of the bioactive potential of the fruits analysed beyond their high antioxidant properties, enzyme inhibition assays were performed using selected enzymes to verify putative antidiabetic and antihypertensive effects. The antidiabetic potential was estimated through the inhibition of the digestive enzymes α- and β-glucosidases and α-amylase. The α-glucosidase and α-amylase are the main enzymes that mediate the metabolism of dietary carbohydrates [380]. Also, α- and β-glucosidase are responsible for the conversion of glycosidic bond into oligosaccharide and finally into monosaccharide [381]. Accordingly, the inhibition of these enzymes will delay glucose absorption, preventing post-meal peaks of glucose in blood that eventually trigger diabetes development[381]. The results obtained (Fig. 3.7.3) reveal a high antidiabetic potential, mostly above 50% inhibition of α- and β-glucosidases and α-amylase. Pitanga seeds extracts, however, are particularly effective against α-glucosidase, reaching a total inhibitory effect.

The antihypertensive capacity of the selected extracts was assessed through the ACE-inhibition activity assay. As can be observed in Fig. 3.7.3, except for uva-da-serra, which presents an inhibitory effect of around 90%, the remaining fruit extracts achieved almost full ACE-inhibition. Overall, these results agree with previous observations taking into account that fruits rich in flavonoids, as the ones studied, exert important inhibitory effects on ACE [382]..

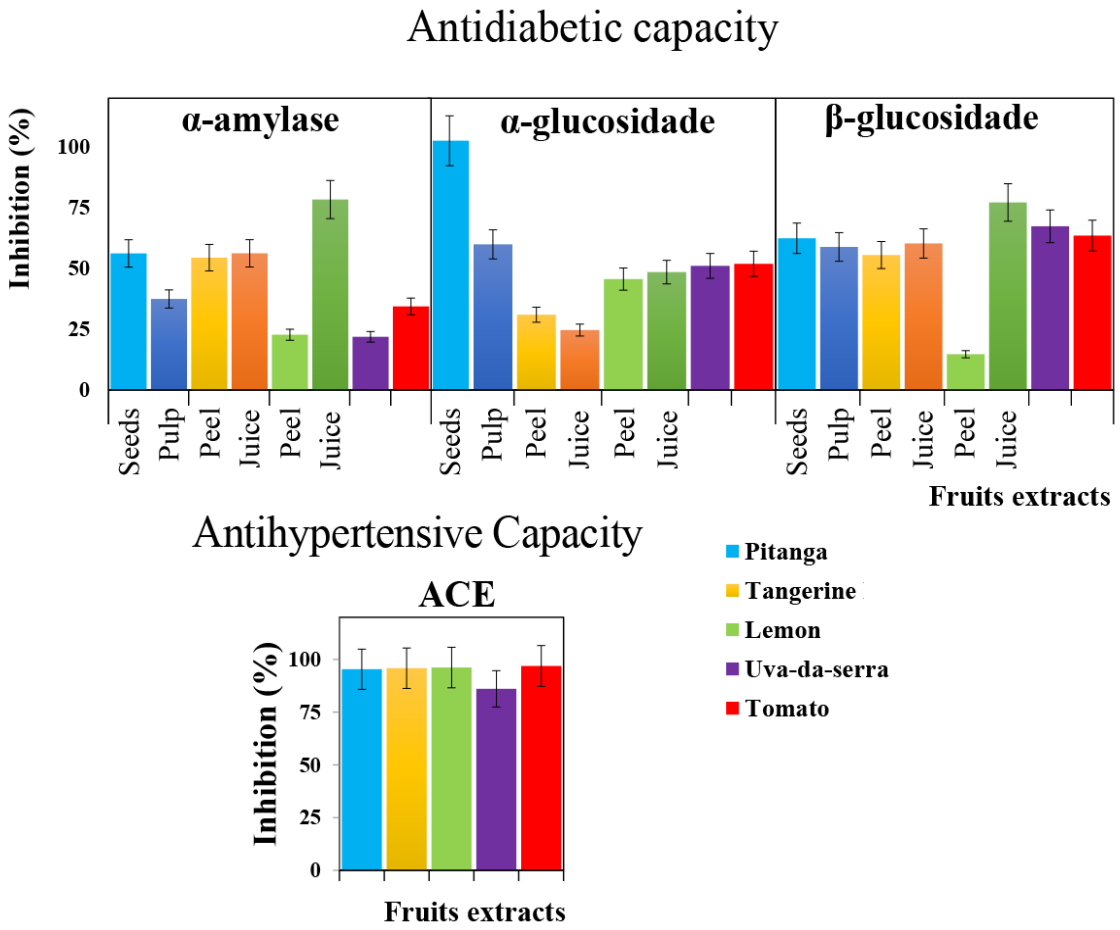


Fig. 3.7.4. In vitro inhibitory activities (of the selected fruits samples towards antidiabetic- (α-amylase – 83 µL extract / 1 U, α- and β-glucosidase – 500 µL extract / 1 U) and antihypertensive- (ACE – 8 µL extract / 1 U)) model enzymes. For legend simplification, fruits sections were organized by colours: pitanga seeds and pulp in light and dark blue, respectively, tangerine peel and juice in yellow and light orange respectively, lemon peel and juice in light and dark green, respectively. Legend: ACE - Angiotensin-converting enzyme.

4. Statistical Analysis

To unveil a possible correlation between the phenolic composition, antioxidant capacity and antihypertensive and antidiabetic potential for the selected fruits extracts, correlation coefficients (r) between the TPC and TFC assays, TAC, and enzyme inhibition of ACE, α -amylase and α- and β-glucosidases were performed (Table 3.7.1).

According to the correlation matrix obtained, DPPH presents a strong inverse correlation to ABTS and TFC, and a moderate inverse correlation to TPC. In fact, DPPH assay is very sensitive to the nature of the antioxidants present in the extracts analysed and may manifest positive or negative correlations with those [383].

Table 3.7.1. Pearson correlation coefficients between phenolic composition, antioxidant capacity and selected enzymes inhibition for the fruit extracts analysed in this work.

DPPH	TFC	TPC	ABTS	β-glu	α-glu	α-amy	ACE
1.00	-0.80	-0.65	-0.83	0.22	0.13	0.02	-0.11
	1.00	0.27	0.57	-0.21	-0.27	-0.33	-0.12
		1.00	0.92	0.48	0.59	0.74	0.77
			1.00	0.35	0.29	0.47	0.51
				1.00	0.75	0.81	0.72
					1.00	0.92	0.96
						1.00	0.94
							1.00

Legend: a_amy - α-amylase, a_glu - α-glucosidase, b_gluc - β-glucosidase, ABTS - 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay, ACE – angiotensin-converting enzyme, DPPH - 2,2-diphenyl-1-picrylhydrazyl free radical assay, TAC – total antioxidant capacity, TFC - total flavonoid content, TPC - total phenolic content.

In turn, there is a strong correlation between ABTS and TPC. Regarding the enzymatic inhibition assays, there is a strong correlation between themselves, which suggests that the nutraceuticals presented in the samples may have common anti-enzymatic effects. To further understand the translation of these correlations in the selected fruit extracts, correlations heatmaps were produced for each sample (Supplementary Fig. 3.7.1.). The results obtained are evidence important of variations in the correlation between phenolic composition, antioxidant capacity and enzyme inhibition, that were summarized in an overall correlation heatmap (Fig. 3.7.4). Accordingly, it is easily

observed that the contribution of the phenolics of each fruit extract to the antioxidant capacity is very different among fruits (uva-da-serra vs tomato, for instance), as well as among fruit sections (pitanga seeds vs pulp or tangerine and lemon peels vs the respective juices). Concerning the enzyme inhibition assays, Fig. 3.7.5 reveals a strong contribution of the phenolics present in pitanga seeds to the inhibition of the selected enzymes. Such behaviour is different from what can be observed for tangerine, lemon or uva-da-serra, in which the respective phenolics seems to be more effective against one enzyme than another. As an example of such correlation, despite the higher phenolic composition of the uva-da-serra extracts, which correlate with a high antioxidant capacity, such phenolic composition is not very effective in the inhibition of α-amylase or ACE. In contrast, an overall positive correlation is particularly evident for pitanga seed extracts that present the highest phenolic content (Fig. 3.7.2A), highest antioxidant capacity (Fig. 3.7.2B) and highest enzyme inhibition (Fig. 3.7.4). Similar correlation patterns were previously reported in citrus fruits by [384].

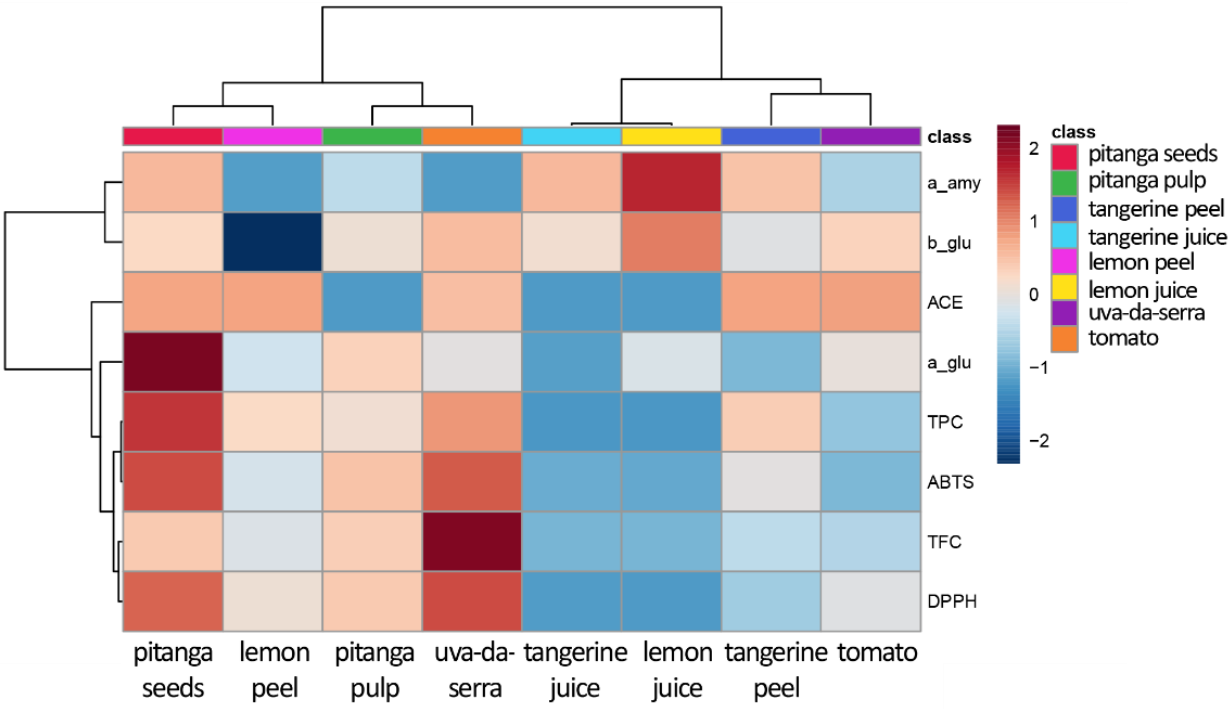


Fig. 3.7.5. Correlation heatmap to evaluate the putative contribution of the phenolics and flavonoids present in the samples extracts to the TAC and key enzymes inhibition. The different assays were performed as described in Materials and Methods. Legend: a_amy - α-amylase, a_glu - α-glucosidase, b_gluc - β-glucosidase, ABTS - 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay, ACE – angiotensin-converting enzyme, DPPH - 2,2-diphenyl-1-picrylhydrazyl free radical assay, TAC – total antioxidant capacity, TFC - total flavonoid content, TPC - total phenolic content.

5. Conclusions

In this work, the health-promoting benefits of different fruits grown in Madeira Island - lemon (*Citrus limon* var. eureka), tangerine (*Citrus reticulata* var. setubalense), pitanga (*Eugenia uniflora* var. red), tomato (*Solanum lycopersicum* var. gordal) and uva-da-serra, (*Vaccinium padifolium* Sm.), were investigated. to evaluate its bioactive potential based on TPC, TFC, antioxidant capacities, antihypertensive and antidiabetic properties. Overall, the analysis of seeds and pulp of pitanga, and peels and juices tangerine and lemon, uva-da-serra and tomato, reveal a high bioactive potential that justifies further and deeper studies uncovering the specific nutraceutical composition, namely the phenolic content of the fruits studied. This is particularly relevant in the context of the

production of new functional foods with antihypertensive and antidiabetic capacities. In this context, pitanga seeds, which are inedible, at least in their raw presentation, and thus discarded as waste, have a great potential to explore. In turn, uva-da-serra, the berry of the wild bush *Vaccinium padifolium* is fairly unknown and so there is also a great potential in its use in the human diet, taking into consideration the high bioactive potential determined in this study.

Section 4. Conclusions and Future Perspectives

Unhealthy diet is a key modifiable risk factor for Noncommunicable diseases, that can lead to death, being estimated that Noncommunicable diseases can be responsible for about 35 million deaths each year. The work described in this thesis address this concern, identifying bioactive compounds present in fruit samples, and the fruits extracts with relevant health-promoting benefits, highlighting the ones with higher potential. In addition, when comparing the fruits produced in Madeira Island against same fruits imported, it was possible to discriminate the different fruits according to their geographical origin, being able to function as a support for geographic authenticity.

The main conclusions of this thesis:

- ✓ The developed analytic procedure LLUSAE/UHPLC-PDA/FLR revealed to be a good approach for determination of carotenoids and tocopherols in fruit samples.
- ✓ LLUSAE/UHPLC-PDA/FLR was able to quantify lycopene (727.1±13.8 mg/g), β-carotene (80.4±1.4 mg/g) and α-, γ-, δ-tocopherols (24.0, 13.0 and 0.6 μg/g, respectively) in ripe tomato from gordal variety.
- ✓ Tomato from gordal variety has twice the carotenoid content (218.2, 342.2 and 267.2 mg/g for lycopene vs. 727.1 mg/g, and 44.0, 44.4 and 45.7 mg/g for β--carotene vs. 80.4 mg/g), and tocopherol content (12.6, 8.2 and 16.1 μg/g for α-tocopherol, 5.5, 1.4 and 8.2 μg/g for γ-tocopherol, and 0.6, 0.5 and 0.7 μg/g δ-tocopherol), that the ones presented by other common tomato varieties (roma, campari and cherry respectively).
- ✓ LLUSAE/UHPLC-PDA/FLR presented a good linearity, precision, high recoveries, and low LODs and LOQs. In Addition, LOD an LOQ for tocopherols are about 1000 times lower than those reported in literature. Furthermore, as far as we know, this is the first time that δ-tocopherol was quantified in tomato

- ✓ QuEChERS combined LC-ESI/MS/MS, shown to be an excellent approach for determination of free low-molecular weight polyphenols, being used for the first time in uva-da-serra.

- ✓ Twenty-six phenolic compounds were identified in the uva-da-serra, being chlorogenic acid (17.4 mg/g DW), epigallocatechin (2.33 mg/g DW), caffeic acid (0.66 mg/g DW), quercetin-3-glucoside (0.38 mg/g DW) and myricetin (0.33 mg/g DW) the predominant . In addition, in uva-da-serra the chlorogenic acid present similar concentrations to those determined in coffee.

- ✓ The volatomic profile of the endemic *Vaccinium padifolium* Sm fruits were carried out for the first time, being established the volatomic profile of uva-da-serra at different stages of ripeness.
- ✓ A total of 72 volatiles of different functionalities were extracted and identified. Terpenes followed by higher alcohols and esters are the predominant chemical families in this matrix.
- ✓ The concentration of volatile compounds varies according to the stage of maturation. *cis*-β-Ocimene (2.0–40.0%), *tra*ns-2-hexenol (2.4–19.4%), *cis*-3-hexenol (2.5.16.4%), β-myrcene (1.9–13.8%), 1-hexanol (1.7–13.6%), 2-hexenal (0.7–8.0%), 2-heptanone (0.7–7.7%), and linalool (1.9–6.1%) were the main volatile compounds identified in uva-da-serra.
- ✓ Partial least squares regression revealed that ethyl caprylate (1.000), trans-geraniol (0.995), ethyl isovalerate (−0.994) and benzyl carbinol (0.993) are the key variables that most contributed to the successful differentiation of uva-da-serra according to ripening stage.

- ✓ HS-SPME/GC-MS was able to be identify a total of 129 volatiles in juice and peels from two varieties of tangerine (setubalense and murcott), with D-

limonene, γ-terpinene, β-myrcene, α- and β-pinene, o-cymene and terpinolene being the most dominant in both cultivars.

- ✓ Setubalense tangerine juices are richer in terpenes, many of them associated with health protection effects. In addition, setubalense tangerines has proved to be a rich source of thymol, making this fruit a very interesting alternative to thyme oils.
- ✓ Discriminant analysis revealed a pool of VOMs, including β-caryophyllene and E-ocimene, with bioactive properties able to differentiate among tangerines according to variety and sample type (peel *vs* juice).

- ✓ NTME was used for the first time in foodstuff, being optimized and applied for establishment the volatile profile of lemon peel, being identified 75 volatiles.
- ✓ The most dominant volatiles are D-limonene, α-pinene, β-pinene, sabinene, β-myrcene and γ-terpinene, accounting for more than 95% of the volatile compositions from studied lemons. In addition, the main VOCs identified on the volatile profile of lemon peels are reported as having health protective effects.
- ✓ NTME combined with GC-MS analysis and chemometric tools shown to be an excellent strategy for lemons discrimination according to its geographical origin. The variables which contributed largely to the geographical origin classification includes butanal, α-pinene, α-thujene, 1-butanol, 2-heptanone, D-limonene, 2-methyl-2-heptenal, nonanal, decanal, 1-octanol, limonene oxide, β-caryophyllene and 2,6-dimethyl-2,6-octadiene, and therefore, can be used as potential useful geographical markers.

- ✓ Pitanga seeds and uva-da-serra shown high antioxidant capacity, however uva-da-serra present high levels of flavonoids.
- ✓ The analysis of seeds and pulp of pitanga, peels and juices of tangerine and lemon, uva-da-serra and tomato, reveal high bioactive potential, expressed by

high antidiabetic (mostly above 50% inhibition of α- and β-glucosidases and α-amylase) and antihypertensive potential (above 90% of ACE-inhibition activity), and also relevant terpenes, carotenoids, tocopherols and phenolic contents with reported antioxidant activity, and associated to health benefits.

- ✓ All fruit samples exhibit very important ACE anti-enzymatic capacities.
- ✓ Statistical analysis reveals a strong contribution of the fruit polyphenols for enzyme inhibition, and therefore presenting high antihypertensive and antidiabetic capacities.

Future Perspectives

- ✓ Consolidation of the results obtained, performing all bioactive compounds evaluation assays (determination of carotenoids, tocopherols, polyphenols and volatile profile) to all fruits.
- ✓ Valorisation of the fruit waste (lemon and tangerine peel, and pitanga seeds), as powerful bioactive sources.
- ✓ Utilization of fruit extracts with bioactive compounds for use in the preparation of food with functional proprieties.
- ✓ Characterization of locally produced fruit cultures, distinguishing them from others on the market, which may lead to a Controlled Denomination of Origin (DOC).

References

1. J. Degenhardt, T. G. Kollner and J. Gershenzon, *Phytochemistry*, 2009, **70**, 1621-1637.
2. T. Devasagayam, J. Tilak, K. Boloor, K. Sane, S. Ghaskadbi and R. Lele, *Japi*, 2004, **52**, 794-804.
3. D. Aune, E. Giovannucci, P. Boffetta, L. T. Fadnes, N. Keum, T. Norat, D. C. Greenwood, E. Riboli, L. J. Vatten and S. Tonstad, *Int. J. Epidemiol.*, 2017, **46**, 1029-1056.
4. J. T. H. Cheung, J. Lok, S. Gietel-Basten and K. Koh, *Nutrients*, 2021, **13**, 148.
5. C. H. Foyer and G. Noctor, *Antioxid. Redox Signal.*, 2009, **11**, 861-905.
6. V. Fuchs-Tarlovsky, *Nutrition*, 2012, **29**, 15-21.
7. R. Sharma and M. Vinayak, *Mol. Biol. Rep.*, 2013, **40**, 839–849.
8. J. S. Câmara, B. R. Albuquerque, J. Aguiar, R. C. Corrêa, J. L. Gonçalves, D. Granato, J. A. Pereira, L. Barros and I. C. Ferreira, *Foods*, 2021, **10**, 37.
9. C. Mannucci, M. Casciaro, E. E. Sorbara, F. Calapai, E. Di Salvo, G. Pioggia, M. Navarra, G. Calapai and S. Gangemi, *Antioxidants*, 2021, **10**, 261.
10. A. Drewnowski, J. Dwyer, J. C. King and C. M. Weaver, *Nutrition Rev.*, 2019, **77**, 404-416.
11. G. Z. Han, *New Phytol.*, 2019, **222**, 70-83.
12. S. K. Chang, C. Alasalvar and F. Shahidi, *Crit. Rev. Food Sci. Nutr.*, 2019, **59**, 1580-1604.
13. R. Guan, Q. Van Le, H. Yang, D. Zhang, H. Gu, Y. Yang, C. Sonne, S. S. Lam, J. Zhong and Z. Jianguang, *Chemosphere*, 2021, 129499.
14. J. Pereira, J. Gonçalves, C. L. Silva, B. Mendes, P. Silva, V. Alves, J. Figueira and J. S. Câmara, in *Liquid Chromatography: Principles, Technology and Applications*, ed. F. Ramos, Nova Science Publishers, New York, EUA, 2013, pp. 269-290.
15. P. Swapnil, M. Meena, S. K. Singh, U. P. Dhuldhaj, Harish and A. Marwal, *Current Plant Biology*, 2021, **26**.
16. M. Al-Duais, J. Hohbein, S. Werner, V. Bo̊hm and G. Jetschke, *J. Agric. Food Chem.*, 2009, **57**, 5420-5427.
17. S. Rivera and R. Canela-Garayoa, *J. Chromatogr. A*, 2011, **1224**, 1-10.
18. S. A. R. Paiva and R. M. Russell, *J. Am. Coll. Nutr.*, 1999, **18**, 426-433.
19. P. Swapnil, M. Meena, S. K. Singh, U. P. Dhuldhaj, Harish and A. Marwal, *Current Plant Biology*, 2021, **26**, 100203.
20. F.-L. Hsieh, T.-H. Chang, T.-P. Ko and A. H.-J. Wang, *Plant Physiol.*, 2011, **155**, 1079-1090.
21. L. Zhao, W.-c. Chang, Y. Xiao, H.-w. Liu and P. Liu, *Annu. Rev. Biochem.*, 2013, **82**, 497-530.
22. D. DellaPenna and B. J. Pogson, *Annu. Rev. Plant Biol.*, 2006, **57**, 711-738.
23. F. Cunningham Jr and E. Gantt, *Annu. Rev. Plant Biol.*, 1998, **49**, 557-583.
24. S. Kajiwara, P. Fraser, K. Kondo and N. Misawa, *Biochem. J.*, 1997, **324**, 421-426.
25. P. Botella-Pavía and M. Rodríguez-Concepción, *Physiol. Plant.*, 2006, **126**, 369-381.
26. Z.-W. Ye, J.-G. Jiang and G.-H. Wu, *Biotechnol. Adv.*, 2008, **26**, 352-360.
27. S. Lu and L. Li, *J. Integr. Plant Biol.*, 2008, **50**, 778-785.
28. G. Giuliano, R. Tavazza, G. Diretto, P. Beyer and M. A. Taylor, *Trends Biotechnol.*, 2008, **26**, 139-145.
29. Z. Li, *Regulation of carotenoid and vitamin E content and their functions in photosynthetic organisms*, ProQuest LLC, Berkeley, 2008.
30. J. G. Lashbrooke, P. R. Young, S. J. Dockrall, K. Vasanth and M. A. Vivier, *BMC Plant Biol.*, 2013, **13**, 156.
31. A. Eroglu and E. H. Harrison, *J. Lipid Res.*, 2013, **54**, 1719-1730.

32. E. Fernández-García, I. Carvajal-Lérida, M. Jarén-Galán, J. Garrido-Fernández, A. Pérez-Gálvez and D. Hornero-Méndez, *Food Res. Int.*, 2012, **46**, 438-450.
33. A. H. Wright, J. M. DeLong, A. H. Gunawardena and R. K. Prange, *Photosynth. Res.*, 2011, **107**, 223-235.
34. A. Donoso, J. González, A. A. Muñoz, P. A. González and C. Agurto-Muñoz, *Pharmacol. Res.*, 2021, 105479.
35. J.-M. Zingg, *Mol. Aspects Med.*, 2007, **28**, 400-422.
36. C. M. Seppanen, Q. Song and A. S. Csallany, *J. Am. Oil Chem. Soc.*, 2010, **87**, 469-481.
37. A. Crozier, I. B. Jaganath and M. N. Clifford, *Nat. Prod. Rep.*, 2009, **26**, 1001-1043.
38. J. Almeida, L. Quadrana, R. Asís, N. Setta, F. de Godoy, L. Bermúdez, S. N. Otaiza, J. V. Corrêa da Silva, A. R. Fernie, F. Carrari and M. Rossi, *J. Exp. Bot.*, 2011, **62**, 3781-3798.
39. M. G. Annunziata, A. Attico, P. Woodrow, M. A. Oliva, A. Fuggi and P. Carillo, *J. Food Compost. Anal.*, 2012, **27**, 145-150.
40. A. Durazzo, A. Nazhand, M. Lucarini, A. M. Delgado, M. De Wit, K. L. Nyam, A. Santini and M. Fawzy Ramadan, *J. Food Qual.*, 2021, **2021**.
41. I. Ajjawi and D. Shintani, *Trends Biotechnol.*, 2004, **22**, 104-107.
42. P. Dörmann, *Planta*, 2007, **225**, 269-276.
43. L. Quadrana, J. Almeida, S. N. Otaiza, T. Duffy, J. V. C. da Silva, F. de Godoy, R. Asís, L. Bermúdez, A. R. Fernie and F. Carrari, *Plant Mol. Biol.*, 2013, **81**, 309-325.
44. H. E. Valentin, K. Lincoln, F. Moshiri, P. K. Jensen, Q. Qi, T. V. Venkatesh, B. Karunanandaa, S. R. Baszis, S. R. Norris and B. Savidge, *Plant Cell*, 2006, **18**, 212-224.
45. P. Detopoulou, C. A. Demopoulos and S. Antonopoulou, *Nutrients*, 2021, **13**, 462.
46. F. Truzzi, C. Tibaldi, Y. Zhang and G. Dinelli, *Int. J. Mol. Sci.*, 2021, **22**, 5514.
47. J. S. Câmara, J. Figueira, R. Perestrelo, C. Silva and J. Pereira, in *Polyphenols: Food Sources, Bioactive Properties and Antioxidant Effects*, ed. D. T. Cobb, Nova Science Publishers, New York, EUA, 2014, ch. 7, pp. 131-156.
48. C. M. Fraser and C. Chapple, *Arabidopsis Book*, 2011, **9**.
49. N. Revi and A. K. Rengan, *Neurol. Sci.*, 2021, 1-19.
50. I. Pagano, L. Campone, R. Celano, A. L. Piccinelli and L. Rastrelli, *J. Chromatogr. A*, 2021, 462295.
51. V. Ninkuu, L. Zhang, J. Yan, Z. Fu, T. Yang and H. Zeng, *Int. J. Mol. Sci.*, 2021, **22**, 5710.
52. L. Ruzicka, *Experientia*, 1953, **9**, 357-367.
53. M. Zielińska-Błajet, P. Pietrusiak and J. Feder-Kubis, *Int. J. Mol. Sci.*, 2021, **22**, 4763.
54. A. P. A. de Carvalho and C. A. Conte-Junior, *Trends Food Sci. Technol.*, 2021.
55. N. Halla, S. A. Heleno, P. Costa, I. P. Fernandes, R. C. Calhelha, K. Boucherit, A. E. Rodrigues, I. C. F. R. Ferreira and M. F. Barreiro, *Ind. Crops Prod.*, 2018, **119**, 249-254.
56. H. Li, Y. Ge, Z. Luo, Y. Zhou, X. Zhang, J. Zhang and Q. Fu, *J. Food Sci. Techno.*, 2017, **54**, 1882-1890.
57. F. Alam, Z. Shafique, S. T. Amjad and M. H. H. Bin Asad, *Phytother. Res.*, 2019, **33**, 41-54.
58. M. F. Mahomoodally, A. Mollica, A. Stefanucci, M. Zakariyyah Aumeeruddy, R. Poorneeka and G. Zengin, *Ind. Crops Prod.*, 2018, **126**, 13-21.
59. V. P. da Silva, C. C. F. Alves, M. L. D. Miranda, L. C. Bretanha, M. P. Balleste, G. A. Micke, E. V. Silveira, C. H. G. Martins, M. A. L. V. Ambrosio, T. de Souza Silva, D. C. Tavares, L. G. Magalhães, F. G. Silva and M. B. Egea, *Ind. Crops Prod.*, 2018, **123**, 638-645.

60. V. D. da Silva, F. Almeida-Souza, A. M. Teles, P. A. Neto, R. Mondego-Oliveira, N. E. M. Filho, N. N. Taniwaki, A. L. Abreu-Silva, K. d. S. Calabrese and V. E. M. Filho, *Ind. Crops Prod.*, 2018, **119**, 201-208.
61. M. D. S. Costa, J. E. Rocha, F. F. Campina, A. R. P. Silva, R. P. Da Cruz, R. L. S. Pereira, L. J. Quintans-Júnior, I. R. A. De Menezes, A. A. De S. Araújo, T. S. De Freitas, A. M. R. Teixeira and H. D. M. Coutinho, *Eur. J. Pharm. Sci.*, 2019, **128**, 158-161.
62. M. Fadil, K. Fikri-Benbrahim, S. Rachiq, B. Ihssane, S. Lebrazi, M. Chraibi, T. Haloui and A. Farah, *Eur. J. Pharm. Biopharm.*, 2018, **126**, 211-220.
63. S. Nardoni, V. V. Ebani, C. D'Ascenzi, L. Pistelli and F. Mancianti, *Front. Pharmacol.*, 2018, **9**.
64. T. R. Amparo, J. B. Seibert, B. M. Silveira, F. S. F. Costa, T. C. Almeida, S. F. P. Braga, G. N. da Silva, O. D. H. Dos Santos and G. H. B. de Souza, *Phytochem. Rev.*, 2021, 1-20.
65. R. K. Saini, S. H. Nile and S. W. Park, *Food Res. Int.*, 2015, **76, Part 3**, 735-750.
66. R. Perveen, H. A. Suleria, F. M. Anjum, M. S. Butt, I. Pasha and S. Ahmad, *Crit. Rev. Food Sci. Nutr.*, 2015, **55**, 919-929.
67. S. Sharma, V. Katoch, S. Kumar and S. Chatterjee, *J. Nutr. Biochem.*, 2021, 108615.
68. G. Calniquer, M. Khanin, H. Ovadia, K. Linnewiel-Hermoni, D. Stepensky, A. Trachtenberg, T. Sedlov, O. Braverman, J. Levy and Y. Sharoni, *Molecules*, 2021, **26**, 1931.
69. N. Li, X. Wu, W. Zhuang, L. Xia, Y. Chen, C. Wu, Z. Rao, L. Du, R. Zhao and M. Yi, *Food Chem.*, 2020, 128396.
70. A. Geraci, V. Di Stefano, E. Di Martino, D. Schillaci and R. Schicchi, *Nat. Prod. Res.*, 2017, **31**, 653-659.
71. G. Kamal, F. Anwar, A. Hussain, N. Sarri and M. Ashraf, *Int. Food Res. J.*, 2011, **18**.
72. Y. Nagano, T. Mimura, N. Kotoda, R. Matsumoto, A. J. Nagano, M. N. Honjo, H. Kudoh and M. Yamamoto, *Tree Genet. Genom.*, 2018, **14**, 6.
73. X. Wang, Y. Xu, S. Zhang, L. Cao, Y. Huang, J. Cheng, G. Wu, S. Tian, C. Chen and Y. Liu, *Nat. Genet.*, 2017, **49**, 765.
74. L. Goldenberg, Y. Yaniv, R. Porat and N. Carmi, *J. Sci. Food Agric.*, 2018, **98**, 18-26.
75. T. Miyazaki, A. Plotto, K. Goodner and F. G. Gmitter, Jr., *J. Sci. Food Agric.*, 2011, **91**, 449-460.
76. Z. Tietel, A. Plotto, E. Fallik, E. Lewinsohn and R. Porat, *J. Sci. Food Agric.*, 2011, **91**, 14-23.
77. J. Kaur and G. Kaur, *Journal of Functional Foods*, 2015, **13**, 239-261.
78. A. Adhikari-Devkota, S. M. I. Elbashir, T. Watanabe and H. P. Devkota, *Nat. Prod. Res.*, 2019, **33**, 1670-1673.
79. D. I. Hamdan, R. A. El-Shiekh, M. A. El-Sayed, H. M. Khalil, M. R. Mousa, A. A. Al-Gendy and A. M. El-Shazly, *Food Funct.*, 2020, **11**, 8214-8236.
80. D. Barreca, G. Mandalari, A. Calderaro, A. Smeriglio, D. Trombetta, M. R. Felice and G. Gattuso, *Plants (Basel, Switzerland)*, 2020, **9**.
81. G. Oboh, T. A. Olasehinde and A. O. Ademosun, *Int. J. Food Sci. Prop.*, 2017, **20**, S586-S594.
82. S. Tejpal, A. M. Wemyss, C. C. Bastie and J. Klein-Seetharaman, *Nutrients*, 2020, **12**, 2348.
83. A. L. Oliveira, R. B. Lopes, F. A. Cabral and M. N. Eberlin, *Food Chem.*, 2006, **99**, 1-5.
84. J. L. Bicas, G. Molina, A. P. Dionísio, F. F. C. Barros, R. Wagner, M. R. Maróstica Jr and G. M. Pastore, *Food Res. Int.*, 2011, **44**, 1843-1855.

85. A. G. V. Costa, D. F. Garcia-Diaz, P. Jimenez and P. I. Silva, *J. Funct. Foods*, 2013, **5**, 539-549.
86. R. Ferreira-Ramos, K. A. G. Accoroni, A. Rossi, M. C. Guidugli, M. A. Mestriner, C. A. Martinez and A. L. Alzate-Marin, *Genet. Resour. Crop Evol.*, 2014, **61**, 267-272.
87. F. N. Victoria, E. J. Lenardão, L. Savegnago, G. Perin, R. G. Jacob, D. Alves, W. P. d. Silva, A. d. S. d. Motta and P. d. S. Nascente, *Food Chem. Toxicol.*, 2012, **50**, 2668-2674.
88. F. S. Malaman, L. A. B. Moraes, C. West, N. J. Ferreira and A. L. Oliveira, *Food Chem.*, 2011, **124**, 85-92.
89. J. Lazzarotto-Figueiró, A. Capelezzo, M. Schindler, J. Fossá, D. Albeny-Simões, L. Zanatta, J. Oliveira and J. Dal Magro, *Braz. J. Biol.*, 2020.
90. E. S. P. Aranha, S. G. de Azevedo, G. G. dos Reis, E. S. Lima, M. B. Machado and M. C. de Vasconcellos, *Ind. Crops Prod.*, 2019, **141**, 111736.
91. J. R. B. Monteiro, J. S. Ardisson, B. R. Athaydes, R. d. C. R. Gonçalves, R. P. Rodrigues, R. M. Kuster and R. R. Kitagawa, *Braz. Arch. Biol. Technol.*, 2019, **62**.
92. M. Sobeh, M. El-Raey, S. Rezq, M. A. Abdelfattah, G. Petruk, S. Osman, A. M. El-Shazly, H. A. El-Beshbishy, M. F. Mahmoud and M. Wink, *J. Ethnopharmacol.*, 2019, **240**, 111939.
93. N. P. Seeram, L. S. Adams, Y. Zhang, R. Lee, D. Sand, H. S. Scheuller and D. Heber, *J. Agric. Food Chem.*, 2006, **54**, 9329-9339.
94. N. P. Seeram, *J. Agric. Food Chem.*, 2008, **56**, 630-635.
95. I. T. Johnson, *Proc. Nutr. Soc.*, 2007, **66**, 207-215.
96. J. Beattie, A. Crozier and G. G. Duthie, *Current Nutrition & Food Science*, 2005, **1**, 71-86.
97. L. Ma, Z. Sun, Y. Zeng, M. Luo and J. Yang, *Int. J. Mol. Sci.*, 2018, **19**, 2785.
98. M. J. Carvalho, C. S. Gouveia, A. C. Vieira, A. C. Pereira, M. Â. Carvalho and J. C. Marques, *J. Food Sci.*, 2017, **82**, 2554-2561.
99. V. Spínola, J. Pinto and P. C. Castilho, *J. Funct. Foods*, 2018, **40**, 595-605.
100. P. Palozza, R. E. Simone, A. Catalano and M. C. Mele, *Cancers (Basel)*, 2011, **3**, 2333-2357.
101. J. Karppi, J. A. Laukkanen, T. H. Makikallio, K. Ronkainen and S. Kurl, *Atherosclerosis*, 2013, **226**, 172-177.
102. J. Burns, P. D. Fraser and P. M. Bramley, *Phytochemistry*, 2003, **62**, 939-947.
103. S. Srivastava and A. K. Srivastava, *Journal of Food Science and Technology*, 2013, **52**, 41-53.
104. J. Regueiro, M. Llompart, C. Garcia-Jares, J. C. Garcia-Monteagudo and R. Cela, *J. Chromatogr. A*, 2008, **1190**, 27-38.
105. J. M. Prado, P. C. Veggi and M. A. A. Meireles, *Current Analytical Chemistry*, 2014, **10**, 29-66.
106. E. Luengo, S. Condon-Abanto, S. Condon, I. Alvarez and J. Raso, *Sep. Purif. Technol.*, 2014, **136**, 130-136.
107. A. F. Silva, M. M. R. de Melo and C. M. Silva, *J. Supercrit. Fluids*, 2014, **95**, 618-627.
108. B. Hiranvarachat and S. Devahastin, *J. Food Eng.*, 2014, **126**, 17-26.
109. Y. Sun, G. Ma, X. Ye, Y. Kakuda and R. Meng, *Ultrason. Sonochem.*, 2010, **17**, 654-661.
110. V. Spinola, B. Mendes, J. S. Camara and P. C. Castilho, *Anal. Bioanal. Chem.*, 2012, **403**, 1049-1058.
111. J. Figueira, H. Camara, J. Pereira and J. S. Camara, *Food Chem.*, 2014, **145**, 653-663.
112. L. Stobinski, J. Mazurkiewicz, H. M. Lin and P. Tomasik, *J Nanosci Nanotechnol*, 2005, **5**, 2121-2127.

113. L. Frusciante, P. Carli, M. R. Ercolano, R. Pernice, A. Di Matteo, V. Fogliano and N. Pellegrini, *Mol. Nutr. Food Res.*, 2007, **51**, 609-617.
114. V. Garcia-Valverde, I. Navarro-Gonzalez, J. Garcia-Alonso and M. J. Periago, *Food and Bioprocess Technology*, 2013, **6**, 391-402.
115. B. Chauveau-Duriot, M. Doreau, P. Noziere and B. Graulet, *Anal. Bioanal. Chem.*, 2010, **397**, 777-790.
116. V. P. Shah, K. K. Midha, J. W. Findlay, H. M. Hill, J. D. Hulse, I. J. McGilveray, G. McKay, K. J. Miller, R. N. Patnaik and M. L. Powell, *Pharm. Res.*, 2000, **17**, 1551-1557.
117. S. M. Choudhari and L. Ananthanarayan, *Food Chem.*, 2007, **102**, 77-81.
118. H. G. Daood, G. Bencze, G. Palotás, Z. Pék, A. Sidikov and L. Helyes, *J. Chromatogr. Sci.*, 2013, **52**, 985-991.
119. R. Ilahy, C. Hdider, M. S. Lenucci, I. Tlili and G. Dalessandro, *J. Food Compost. Anal.*, 2011, **24**, 588-595.
120. M. Gomez-Romero, D. Arraez-Roman, A. Segura-Carretero and A. Fernandez-Gutierrez, *J. Sep. Sci.*, 2007, **30**, 452-461.
121. A. M. Adalid, S. Roselló and F. Nuez, *Journal of Food Composition and Analysis*, 2010, **23**, 613-618.
122. A. P. Breksa Iii, L. D. Robertson, J. A. Labate, B. A. King and D. E. King, *Journal of Food Composition and Analysis*, 2015, **42**, 16-25.
123. J. L. Guil-Guerrero and M. M. Rebolloso-Fuentes, *Journal of Food Composition and Analysis*, 2009, **22**, 123-129.
124. C. Moraru, L. Logendra, T.-C. Lee and H. Janes, *Journal of Food Composition and Analysis*, 2004, **17**, 141-154.
125. A. Sass-Kiss, J. Kiss, P. Milotay, M. M. Kerek and M. Toth-Markus, *Food Res. Int.*, 2005, **38**, 1023-1029.
126. A. Carrillo-Lopez and E. M. Yahia, *J Food Sci Technol*, 2014, **51**, 2720-2726.
127. J. W. Qin, K. L. Chao and M. S. Kim, *J. Food Eng.*, 2011, **107**, 277-288.
128. Y. C. Cheng, T. T. Wang, J. H. Chen and T. T. Lin, *Postharvest Biol. Technol.*, 2011, **62**, 17-25.
129. T. Tohge, S. Alseekh and A. R. Fernie, *J. Exp. Bot.*, 2014, **65**, 4599-4611.
130. D. DellaPenna and B. J. Pogson, *Annu. Rev. Plant Biol.*, 2006, **57**, 711-738.
131. A. F. Vinha, R. C. Alves, S. V. P. Barreira, A. Castro, A. S. G. Costa and M. B. P. P. Oliveira, *LWT-Food Sci. Technol.*, 2014, **55**, 197-202.
132. E. Capanoglu, J. Beekwilder, D. Boyacioglu, R. Hall and R. De Vos, *J. Agric. Food. Chem.*, 2008, **56**, 964-973.
133. L. Frusciante, P. Carli, M. R. Ercolano, R. Pernice, A. Di Matteo, V. Fogliano and N. Pellegrini, *Mol. Nutr. Food Res.*, 2007, **51**, 609-617.
134. J. Shi and M. Le Maguer, *Crit. Rev. Food Sci. Nutr.*, 2000, **40**, 1-42.
135. E. Hallmann, *J. Sci. Food Agric.*, 2012, **92**, 2840–2848.
136. S. Georgé, F. Tourniaire, H. Gautier, P. Goupy, E. Rock and C. Caris-Veyrat, *Food Chem.*, 2011, **124**, 1603-1611.
137. Y. Sharoni, K. Linnewiel-Hermoni, M. Khanin, H. Salman, A. Veprik, M. Danilenko and J. Levy, *Mol. Nutr. Food Res.*, 2012, **56**, 259-269.
138. A. V. Rao and L. G. Rao, *Pharmacol. Res.*, 2007, **55**, 207-216.
139. M. Giovannetti, L. Avio, R. Barale, N. Ceccarelli, R. Cristofani, A. Iezzi, F. Mignolli, P. Picciarelli, B. Pinto, D. Reali, C. Sbrana and R. Scarpato, *Br. J. Nutr.*, 2012, **107**, 242-251.
140. K. Chong-Han, *Crit. Rev. Food Sci. Nutr.*, 2010, **50**, 931-937.

141. A. Azzi, *Free Radic. Biol. Med.*, 2007, **43**, 16-21.
142. H. Sircelj and F. Batic, *J Appl Bot Food Qual*, 2007, **81**, 121-125.
143. C. Schneider, *Mol. Nutr. Food Res.*, 2005, **49**, 7-30.
144. R. Stocker and J. F. Keaney, *Physiol. Rev.*, 2004, **84**, 1381-1478.
145. S. Wada, *Curr. Pharm. Biotechnol.*, 2012, **13**, 156-164.
146. C. S. Yang, G. Li, Z. Yang, F. Guan, A. Chen and J. Ju, *Cancer Lett.*, 2013, **334**, 79-85.
147. A. K. Smolarek and N. Suh, *Nutrients*, 2011, **3**, 962-986.
148. N. K. Marsiv, H. Sircelj and D. Kastelec, *J. Agric. Food Chem.*, 2010, **58**, 390-397.
149. J. Chun, J. Lee, L. Ye, J. Exler and R. R. Eitenmiller, *Journal of Food Composition and Analysis*, 2006, **19**, 196-204.
150. A. Kamal-Eldin and L.-Å. Appelqvist, *Lipids*, 1996, **31**, 671-701.
151. P. Borel, D. Preveraud and C. Desmarchelier, *Nutr. Rev.*, 2013, **71**, 319-331.
152. R. Brigelius-Flohé and M. G. Traber, *The FASEB Journal*, 1999, **13**, 1145-1155.
153. S. Salinthone, A. R. Kerns, V. Tsang and D. W. Carr, *Mol. Immunol.*, 2013, **53**, 173-178.
154. G. Cao, H. M. Alessio and R. G. Cutler, *Free Radic. Biol. Med.*, 1993, **14**, 303-311.
155. J. Xie and K. M. Schaich, *J. Agric. Food. Chem.*, 2014, **62**, 4251-4260.
156. J. Tabart, C. Kevers, J. Pincemail, J.-O. Defraigne and J. Dommes, *Food Chem.*, 2009, **113**, 1226-1233.
157. S. B. Kedare and R. Singh, *Journal of Food Science and Technology*, 2011, **48**, 412-422.
158. N. Bernaert, D. De Paepe, C. Bouten, H. De Clercq, D. Stewart, E. Van Bockstaele, M. De Loose and B. Van Droogenbroeck, *Food Chem.*, 2012, **134**, 669-677.
159. S. O. Okoh, O. T. Asekun, O. B. Familoni and A. J. Afolayan, *Antioxidants (Basel)*, 2014, **3**, 278-287.
160. M. M. Delgado-Zamarreño, C. Fernández-Prieto, M. Bustamante-Rangel and L. Pérez-Martín, *Food Chem.*, 2016, **192**, 825-830.
161. C. Polson, P. Sarkar, B. Incledon, V. Raguvaran and R. Grant, *J. Chromatogr. B*, 2003, **785**, 263-275.
162. M. G. Traber, *Annu. Rev. Nutr.*, 2007, **27**, 347-362.
163. M. F. Charão, A. M. Moro, N. Brucker, R. P. Bulcão, M. Baierle, F. Freitas, J. Durgante, S. Nascimento, G. B. Bubols and P. H. Saldiva, *Journal of the Brazilian Chemical Society*, 2012, **23**, 1441-1449.
164. B. Chauveau-Duriot, M. Doreau, P. Nozière and B. Graulet, *Anal. Bioanal. Chem.*, 2010, **397**, 777-790.
165. I. Naidis and S. Turpeinen, *Guidance for the validation of analytical methodology and calibration of equipment used for testing of illicit drugs in seized materials and biological specimens*, United Nations Publications, Vienna, Austria, 2009.
166. S. A. Rodrigues, S. S. Caldas, M. H. S. Kurz, L. da Costa Cabrera, F. A. Duarte, R. Zanella and E. G. Primel, *Analytical Methods*, 2012, **4**, 1820-1824.
167. R. Monge-Rojas and H. Campos, *J. Food Compost. Anal.*, 2011, **24**, 202-216.
168. S. C. Andersson, K. Rumpunen, E. Johansson and M. E. Olsson, *J. Agric. Food Chem.*, 2008, **56**, 6701-6706.
169. L. M. Bal, V. Meda, S. N. Naik and S. Satya, *Food Res. Int.*, 2011, **44**, 1718-1727.
170. H. T. Aldrich, K. Salandanan, P. Kendall, M. Bunning, F. Stonaker, O. Külen and C. Stushnoff, *J. Sci. Food Agric.*, 2010, **90**, 2548-2555.
171. P. M. Hanson, R.-y. Yang, J. Wu, J.-t. Chen, D. Ledesma, S. C. S. Tsou and T.-C. Lee, *J. Am. Soc. Hort. Sci.*, 2004, **129**, 704-711.

172. E. Vági, B. Simándi, K. P. Vásárhelyiné, H. Daood, Á. Kéry, F. Doleschall and B. Nagy, *The Journal of Supercritical Fluids*, 2007, **40**, 218-226.
173. C. Botinestean, M. Schreiner and I. Jianu, *Influence of Solvent Used for Extraction on Tocopherols Content of Tomato Seed Oil*, 2013.
174. N. Kalogeropoulos, A. Chiou, V. Pyriochou, A. Peristeraki and V. T. Karathanos, *LWT - Food Science and Technology*, 2012, **49**, 213-216.
175. S. Caretto, R. Nisi, A. Paradiso and L. De Gara, *Mol. Nutr. Food Res.*, 2010, **54**, 726-730.
176. V. Piironen, E. L. Syvaoja, P. Varo, K. Salminen and P. Koivistoinen, *J. Agric. Food Chem.*, 1986, **34**, 742-746.
177. N. Kalogeropoulos, A. Chiou, M. Ioannou, V. T. Karathanos, M. Hassapidou and N. K. Andrikopoulos, *Food Chem.*, 2010, **121**, 682-690.
178. I. Konczak and P. Roulle, *Food Res. Int.*, 2011, **44**, 2339-2344.
179. P. A. d. Costa, C. A. Ballus, J. Teixeira-Filho and H. T. Godoy, *Food Res. Int.*, 2010, **43**, 1603-1606.
180. C. Seybold, K. Frohlich, R. Bitsch, K. Otto and V. Bohm, *J. Agric. Food Chem.*, 2004, **52**, 7005-7010.
181. L. Barros, D. M. Correia, I. C. Ferreira, P. Baptista and C. Santos-Buelga, *Food Chem.*, 2008, **110**, 1046-1050.
182. C. Bele, C. T. Matea, C. Raducu, V. Miresan and O. Negrea, *Not. Bot. Horti Agrobot. Cluj Napoca*, 2013, **41**, 93-96.
183. E. C. Bell, M. John, R. J. Hughes and T. Pham, *J. Chromatogr. Sci.*, 2014, **52**, 1065-1070.
184. P. Górnaś, A. Siger, J. Czubinski, K. Dwiecki, D. Segliņa and M. Nogala-Kalucka, *Eur. J. Lipid. Sci. Technol.*, 2014, **116**, 895-903.
185. K. Knecht, K. Sandfuchs, S. E. Kulling and D. Bunzel, *Food Chem.*, 2015, **169**, 20-27.
186. I. Valdivielso, M. Á. Bustamante, J. C. R. de Gordoa, A. I. Nájera, M. de Renobales and L. J. R. Barron, *Food Chem.*, 2015, **173**, 709-717.
187. Z. Xu, K. A. Harvey, T. M. Pavlina, G. P. Zaloga and R. A. Siddiqui, *Eur. J. Lipid. Sci. Technol.*, 2015, **117**, 15-22.
188. B. R. Mendoza, S. M. Pons, A. C. Bargalló and M. Lopez-Sabater, *J. Chromatogr. A*, 2003, **1018**, 197-202.
189. H. M. Habib, H. Kamal, W. H. Ibrahim and A. S. Al Dhaheri, *Ind. Crops Prod.*, 2013, **42**, 567-572.
190. R. Kanďár, P. Novotná and P. Drábková, *J. Chem.*, 2013.
191. C. M. Stinco, A. M. Benítez-González, D. Hernanz, I. M. Vicario and A. J. Meléndez-Martínez, *J. Chromatogr. A*, 2014, **1370**, 162-170.
192. N. Qi, X. Gong, C. Feng, X. Wang, Y. Xu and L. Lin, *Food Chem.*, 2016, **207**, 157-161.
193. F. Granado-Lorencio, C. Herrero-Barbudo, I. Blanco-Navarro and B. Pérez-Sacristán, *Anal. Bioanal. Chem.*, 2010, **397**, 1389-1393.
194. A. A. Albahrani, V. Rotarou, P. J. Roche and R. F. Greaves, *The Journal of steroid biochemistry and molecular biology*, 2016, **159**, 41-53.
195. Z. Kotíková, J. Lachman, A. Hejtmánková and K. Hejtmánková, *LWT - Food Science and Technology*, 2011, **44**, 1703-1710.
196. A. Zanfini, G. Corbini, C. La Rosa and E. Dreassi, *LWT - Food Science and Technology*, 2010, **43**, 67-72.
197. D. Shao, C. Venkitasamy, X. Li, Z. Pan, J. Shi, B. Wang, H. E. Teh and T. H. McHugh, *LWT - Food Science and Technology*, 2015, **63**, 191-197.

198. V. S. Arya, S. K. Kanthlal and G. Linda, *J. Food Biochem.*, 2020, DOI: 10.1111/jfbc.13369, e13369.
199. Q. Huang, B. H. Braffett, S. J. Simmens, H. A. Young and C. L. Ogden, *Journal of the Academy of Nutrition and Dietetics*, 2020, DOI: https://doi.org/10.1016/j.jand.2020.06.016.
200. D. Margină, A. Ungurianu, C. Purdel, G. M. Nitulescu, D. Tsoukalas, E. Sarandi, M. Thanasoula, T. I. Burykina, F. Tekos and A. Buha, *Food Chem. Toxicol.*, 2020, 111558.
201. F. Paciello, A. Di Pino, R. Rolesi, D. Troiani, G. Paludetti, C. Grassi and A. R. Fetoni, *Food Chem. Toxicol.*, 2020, **143**, 111555.
202. M. Fidelis, M. A. Vieira do Carmo, L. Azevedo, T. M. Cruz, M. B. Marques, T. Myoda, A. S. Sant'Ana, M. M. Furtado, M. Wen, L. Zhang, N. D. Rosso, M. I. Genovese, W. Y. Oh, F. Shahidi, N. Pap and D. Granato, *Food Chem. Toxicol.*, 2020, **142**, 111439.
203. P. Marcolongo, A. Gamberucci, G. Tamasi, A. Pardini, C. Bonechi, C. Rossi, R. Giunti, V. Barone, A. Borghini and P. Fiorenzani, *Molecules*, 2020, **25**, 3758.
204. S.-T. Wang, W.-Q. Cui, D. Pan, M. Jiang, B. Chang and L.-X. Sang, *World J. Gastroenterol.*, 2020, **26**, 562.
205. P. Selvakumar, A. Badgeley, P. Murphy, H. Anwar, U. Sharma, K. Lawrence and A. Lakshmikuttyamma, *Nutrients*, 2020, **12**, 761.
206. T. Mitra and R. Bhattacharya, *J. Cell. Physiol.*, 2020, **235**, 7696-7708.
207. S. Ding, S. Xu, J. Fang and H. Jiang, *Front. Immunol.*, 2020, **11**.
208. X. Montane, O. Kowalczyk, B. Reig-Vano, A. Bajek, K. Roszkowski, R. Tomczyk, W. Pawliszak, M. Giamberini, A. Mocek-Plociniak and B. Tylkowski, *Molecules*, 2020, **25**.
209. J. S. Mani, J. B. Johnson, J. C. Steel, D. A. Broszczak, P. M. Neilsen, K. B. Walsh and M. Naiker, *Virus Res.*, 2020, **284**, 197989.
210. K. Chojnacka, A. Witek-Krowiak, D. Skrzypczak, K. Mikula and P. Młynarz, *Journal of Functional Foods*, 2020, **73**, 104146.
211. C. Hobohm, *Endemism in vascular plants*, Springer, 2014.
212. J. R. Press and M. J. Short, *Flora of Madeira*, HMSO Publications Centre, 1994.
213. D. Rivera and C. Obon, *J. Ethnopharmacol.*, 1995, **46**, 73-93.
214. C. Manach, A. Scalbert, C. Morand, C. Remesy and L. Jimenez, *Am. J. Clin. Nutr.*, 2004, **79**, 727 - 747.
215. J. Oszmiański, A. Wojdyło, J. Gorzelany and I. Kapusta, *J. Agric. Food Chem.*, 2011, **59**, 12830-12835.
216. R. Tsao, *Nutrients*, 2010, **2**, 1231-1246.
217. I. Khalifa, W. Zhu, K.-k. Li and C.-m. Li, *J. Funct. Foods*, 2018, **40**, 28-43.
218. T. Jurikova, S. Skrovankova, J. Mlcek, S. Balla and L. Snopek, *Molecules*, 2019, **24**, 24.
219. L. Lavefve, L. R. Howard and F. Carbonero, *Food Funct.*, 2020, **11**, 45-65.
220. A. Aires, R. Carvalho, M. Matos, V. Carnide, A. P. Silva and B. Gonçalves, *J. Food Biochem.*, 2017, **41**, e12414.
221. L. Cabrita and O. M. Andersen, *Phytochemistry*, 1999, **52**, 1693-1696.
222. C. S. Dzah, Y. Duan, H. Zhang, N. A. Serwah Boateng and H. Ma, *Trends Food Sci. Technol.*, 2020, **99**, 375-388.
223. A. K. Rai, A. Pandey and D. Sahoo, *Trends Food Sci. Technol.*, 2019, **83**, 129-137.
224. D. Carregosa, R. Carecho, I. Figueira and C. u. N Santos, *J. Agric. Food Chem.*, 2019, **68**, 1790-1807.
225. M. Anastassiades, S. J. Lehotay, D. Stajnbaher and F. J. Schenck, *J. AOAC Int.*, 2003, **86**, 412-431.
226. P. Porto-Figueira, I. Camacho and J. S. Camara, *J. Chromatogr. A*, 2015, **1408**, 187-196.

227. G. I. Hidalgo and M. P. Almajano, *Antioxidants (Basel)*, 2017, **6**, 7.
228. G. Bag, P. G. Devi and T. Bhaigyabati, *International Journal of Pharmaceutical Sciences Review and Research*, 2015, **30**, 154-159.
229. J. A. Figueira, J. A. M. Pereira and J. S. Câmara, *Food Anal. Methods*, 2017, **10**, 2507-2517.
230. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, *Free Radic. Biol. Med.*, 1999, **26**, 1231-1237.
231. K. Thaipong, U. Boonprakob, K. Crosby, L. Cisneros-Zevallos and D. H. Byrne, *Journal of food composition and analysis*, 2006, **19**, 669-675.
232. J. A. Figueira, J. A. M. Pereira, P. Porto-Figueira and J. S. Câmara, *J. Food Compost. Anal.*, 2017, **57**, 87-93.
233. J. Dai and R. J. Mumper, *Molecules*, 2010, **15**, 7313-7352.
234. P. Porto-Figueira, J. A. Figueira, J. A. Pereira and J. S. Camara, *J. Chromatogr. A*, 2015, **1424**, 1-9.
235. J. Hokkanen, S. Mattila, L. Jaakola, A. M. Pirttilä and A. Tolonen, *J. Agric. Food Chem.*, 2009, **57**, 9437-9447.
236. M. H. Grace, J. Xiong, D. Esposito, M. Ehlenfeldt and M. A. Lila, *Food Chem.*, 2019, **277**, 336-346.
237. V. Spinola, J. Pinto and P. C. Castilho, *Journal of Functional Foods*, 2018, **40**, 595-605.
238. N. Colak, A. K. Primetta, K. R. Riihinen, L. Jaakola, J. Grúz, M. Strnad, H. Torun and F. A. Ayaz, *Food Bioscience*, 2017, **20**, 67-78.
239. N. Colak, H. Torun, J. Gruz, M. Strnad, M. Subrtova, H. Inceer and F. A. Ayaz, *Polish journal of food and nutrition sciences*, 2016, **66**, 85-92.
240. S. Häkkinen, M. Heinonen, S. Kärenlampi, H. Mykkänen, J. Ruuskanen and R. Törrönen, *Food Res. Int.*, 1999, **32**, 345-353.
241. S. H. Häkkinen and A. R. Törrönen, *Food Res. Int.*, 2000, **33**, 517-524.
242. S. p. Može, T. Polak, L. Gasperlin, D. Koron, A. Vanzo, N. a. Poklar Ulrih and V. Abram, *J. Agric. Food Chem.*, 2011, **59**, 6998-7004.
243. K. Riihinen, L. Jaakola, S. Kärenlampi and A. Hohtola, *Food Chem.*, 2008, **110**, 156-160.
244. X. Guofang, X. Xiaoyan, Z. Xiaoli, L. Yongling and Z. Zhibing, *Int. J. Food Sci. Prop.*, 2019, **22**, 320-329.
245. S. Y. Wang, H. Chen, M. J. Camp and M. K. Ehlenfeldt, *Food Chem.*, 2012, **132**, 855-864.
246. S. Ek, H. Kartimo, S. Mattila and A. Tolonen, *J. Agric. Food Chem.*, 2006, **54**, 9834-9842.
247. M. H. Grace, D. Esposito, K. L. Dunlap and M. A. Lila, *J. Agric. Food Chem.*, 2013, **62**, 4007-4017.
248. M. Zorzi, F. Gai, C. Medana, R. Aigotti and P. G. Peiretti, *Plant Foods for Human Nutrition (Dordrecht, Netherlands)*, 2020.
249. O. T. Okan, I. Deniz, N. Yayli, İ. G. ŞAT, Ö. Mehmet and G. H. SERDAR, *Not. Bot. Horti Agrobot. Cluj Napoca*, 2018, **46**, 639-652.
250. W. Kalt and J. E. McDonald, *J. Am. Soc. Hortic. Sci.*, 1996, **121**, 142-146.
251. F. A. Ayaz, S. Hayirlioglu-Ayaz, J. Gruz, O. Novak and M. Strnad, *J. Agric. Food Chem.*, 2005, **53**, 8116-8122.
252. F. P. Prencipe, R. Bruni, A. Guerrini, D. Rossi, S. Benvenuti and F. Pellati, *J. Pharm. Biomed. Anal.*, 2014, **89**, 257-267.
253. C. Vasco, K. Riihinen, J. Ruales and A. Kamal-Eldin, *J. Agric. Food Chem.*, 2009, **57**, 8274-8281.
254. S. Lee, E. S. Jung, S.-G. Do, G.-y. Jung, G. Song, J.-m. Song and C. H. Lee, *J. Agric. Food Chem.*, 2014, **62**, 2126-2133.

255. L. Wang, H.-N. Xu, H. Yao and H. Zhang, *Int. J. Food Sci. Prop.*, 2011, **14**, 721-725.
256. M. F. Akšić, D. D. Zagorac, M. Sredojević, J. Milivojević, U. Gašić, M. Meland and M. Natić, *Molecules*, 2019, **24**, 4310.
257. D. V. Costa, D. P. Almeida and M. Pintado, *J. Sci. Food Agric.*, 2018, **98**, 5052-5061.
258. N. Colak, H. Torun, J. Gruz, M. Strnad, I. Hermosín-Gutiérrez, S. Hayirlioglu-Ayaz and F. A. Ayaz, *Food Chem.*, 2016, **201**, 339-349.
259. M. Fotirić Akšić, D. Dabić Zagorac, M. Sredojević, J. Milivojević, U. Gašić, M. Meland and M. Natić, *Molecules*, 2019, **24**, 4310.
260. I. A. Ludwig, M. N. Clifford, M. E. Lean, H. Ashihara and A. Crozier, *Food Funct.*, 2014, **5**, 1695-1717.
261. M. Mikulic-Petkovsek, V. Schmitzer, A. Slatnar, F. Stampar and R. Veberic, *J. Sci. Food Agric.*, 2015, **95**, 776-785.
262. A. Sharma, B. Shahzad, A. Rehman, R. Bhardwaj, M. Landi and B. Zheng, *Molecules*, 2019, **24**, 2452.
263. O.-C. Bujor, C. Ginies, V. I. Popa and C. Dufour, *Food Chem.*, 2018, **252**, 356-365.
264. P. Denev, A. Lojek, M. Ciz and M. Kratchanova, *Bulg J Agric Sci*, 2013, **19**, 22-27.
265. R. Vieira, *Coleccao natureza e paisagem*, 1992.
266. N. P. Seeram, in *Nutritional oncology*, eds. D. Heber, G. Blackburn, V. Go and J. Milner, Academic Press, 2006, pp. 615 - 625.
267. R. L. Prior, G. Cao, A. Martin, E. Sofic, J. McEwen, C. O'Brien, N. Lischner, M. Ehlenfeldt, W. Kalt, G. Krewer and C. M. Mainland, *J. Agric. Food Chem.*, 1998, **46**, 2686-2693.
268. I. Aragüez and V. Valpuesta Fernández, *Biotechnology journal*, 2013, **8**, 1144-1158.
269. M. A. El Hadi, F. J. Zhang, F. F. Wu, C. H. Zhou and J. Tao, *Molecules*, 2013, **18**, 8200-8229.
270. I. Ménager, M. Jost and C. Aubert, *J. Agric. Food Chem.*, 2004, **52**, 1248-1254.
271. M. E. C. Pereira, S. A. Sargent, D. M. Tieman, H. J. Klee and D. J. Huber, 2010.
272. R. Paduch, M. Kandefer-Szerszeń, M. Trytek and J. Fiedurek, *Arch. Immunol. Ther. Exp.*, 2007, **55**, 315-327.
273. J. Figueira, H. Câmara, J. Pereira and J. S. Câmara, *Food Chem.*, 2014, **145**, 653-663.
274. J. S. Câmara, M. A. Alves and J. C. Marques, *Talanta*, 2006, **68**, 1512-1521.
275. B. Worley, S. Halouska and R. Powers, *Anal. Biochem.*, 2013, **433**, 102-104.
276. D. Ballabio and V. Consonni, *Anal Methods*, 2013, **5**, 3790.
277. P. M. N. Ceva-Antunes, H. R. Bizzo, A. S. Silva, C. P. S. Carvalho and O. A. C. Antunes, *LWT-FOOD SCI. TECHNOL.*, 2006, **39**, 437-443.
278. B. T. Ong, S. A. H. Nazimah, C. P. Tan, H. Mirhosseini, A. Osman, D. Mat Hashim and G. Rusul, *J. Food Compost. Anal.*, 2008, **21**, 416-422.
279. B. Rega, N. Fournier and E. Guichard, *J. Agric. Food Chem.*, 2003, **51**, 7092-7099.
280. J. Pawliszyn, *Solid phase microextraction: theory and practice*, John Wiley & Sons, 1997.
281. J. Pawliszyn, *Applications of solid phase microextraction*, Royal Society of Chemistry, 1999.
282. M. Pontes, J. Marques and J. Câmara, *Microchem. J.*, 2009, **93**, 1-11.
283. J. Pawliszyn and S. Pedersen-Bjergaard, *J. Chromatogr. Sci.*, 2006, **44**, 291-307.
284. R. J. Horvat, W. S. Schlotzhauer, O. T. Chortyk, S. F. Nottingham and J. A. Payne, *J. Essent. Oil Res.*, 1996, **8**, 645-648.
285. R. J. Horvat and S. D. Senter, *J. Food Sci.*, 1985, **50**, 429-431.

286. A. El-Sayed, The pherobase: database of insect pheromones and semiochemicals, http://www.pherobase.com (accessed 29-02-2016).
287. J. He, M. Xie, X. Tang and X. Qi, *J. Phys. Org. Chem.*, 2012, **25**, 373-378.
288. *United States Pat.*, 2007/0259056 A1, 2007.
289. O. Ciftci, M. N. Oztanir and A. Cetin, *Neurochem. Res.*, 2014, **39**, 1717-1723.
290. K. N. C. Murthy, G. K. Jayaprakasha and B. S. Patil, *Life Sci.*, 2012, **91**, 429-439.
291. L. Jing, Y. Zhang, S. Fan, M. Gu, Y. Guan, X. Lu, C. Huang and Z. Zhou, *Eur. J. Pharmacol.*, 2013, **715**, 46-55.
292. N. G. Lima, D. P. De Sousa, F. C. F. Pimenta, M. F. Alves, F. S. De Souza, R. O. Macedo, R. B. Cardoso, L. C. S. de Morais, F. Margareth de Fátima and R. N. de Almeida, *Pharmacol. Biochem. Behav.*, 2013, **103**, 450-454.
293. S. A. Goff and H. J. Klee, *Science*, 2006, **311**, 815-819.
294. K. Tokumo, N. Tamura, T. Hirai and H. Nishio, *Behav. Brain Res.*, 2006, **166**, 247-252.
295. R. Lanciotti, N. Belletti, F. Patrignani, A. Gianotti, F. Gardini and M. E. Guerzoni, *J. Agric. Food Chem.*, 2003, **51**, 2958-2963.
296. A. C. Aprotosoaie, M. Hăncianu, I. I. Costache and A. Miron, *Flavour Fragrance J.*, 2014, **29**, 193-219.
297. S. L. Guzmán-Gutiérrez, H. Bonilla-Jaime, R. Gómez-Cansino and R. Reyes-Chilpa, *Life Sci.*, 2015, **128**, 24-29.
298. C. Brady, *Annual review of plant physiology*, 1987, **38**, 155-178.
299. USDA, Citrus: World Markets and Trades, https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf, (accessed 25.09.2020).
300. A. Rodríguez, J. E. Peris, A. Redondo, T. Shimada, E. Costell, I. Carbonell, C. Rojas and L. Peña, *Food Chem.*, 2017, **217**, 139-150.
301. H. Maarse, *Volatile Compounds in Foods and Beverages*, CRC Press, New York, 1991.
302. P. Porto-Figueira, J. A. Figueira, P. Berenguer and J. S. Câmara, *Food Chem.*, 2018, **245**, 141-149.
303. J. A. Figueira, P. Porto-Figueira, J. A. M. Pereira and J. S. Câmara, *Microchem. J.*, 2020, **157**, 104933.
304. P. Porto-Figueira, A. Freitas, C. J. Cruz, J. Figueira and J. S. Camara, *Food Res. Int.*, 2015, **77**, 408-418.
305. J. Chong, O. Soufan, C. Li, I. Caraus, S. Li, G. Bourque, D. S. Wishart and J. Xia, *Nucleic Acids Res.*, 2018, **46**, W486–W494.
306. M. El Hadi, F.-J. Zhang, F.-F. Wu, C.-H. Zhou and J. Tao, *Molecules*, 2013, **18**, 8200-8229.
307. Y. Yu, J. Bai, C. Chen, A. Plotto, E. A. Baldwin and F. G. Gmitter, *J. Sci. Food Agric.*, 2018, **98**, 1124-1131.
308. M. Lubinska-Szczygieł, A. Różańska, J. Namieśnik, T. Dymerski, R. B. Shafreen, M. Weisz, A. Ezra and S. Gorinstein, *Food Control*, 2018, **89**, 270-279.
309. M. Fawzi Mahomoodally, A. Mollica, A. Stefanucci, M. Zakariyyah Aumeeruddy, R. Poorneeka and G. Zengin, *Ind. Crops Prod.*, 2018, **126**, 13-21.
310. H. M. Ahmed and S. Tavaszi-Sarosi, *Food Chem.*, 2019, **275**, 730-738.
311. V. D. da Silva, F. Almeida-Souza, A. M. Teles, P. A. Neto, R. Mondego-Oliveira, N. E. Mendes Filho, N. N. Taniwaki, A. L. Abreu-Silva, K. d. S. Calabrese and V. E. Mouchrek Filho, *Ind. Crops Prod.*, 2018, **119**, 201-208.
312. M. Fadil, K. Fikri-Benbrahim, S. Rachiq, B. Ihssane, S. Lebrazi, M. Chraibi, T. Haloui and A. Farah, *Eur. J. Pharm. Biopharm.*, 2018, **126**, 211-220.

313. E. Dănilă, Z. Moldovan, M. Popa, M. C. Chifiriuc, A. D. Kaya and M. A. Kaya, *Ind. Crops Prod.*, 2018, **122**, 483-492.
314. C. E. Assmann, F. C. Cadoná, B. d. S. R. Bonadiman, E. B. Dornelles, G. Trevisan and I. B. M. da Cruz, *Biomed. Pharmacother.*, 2018, **103**, 1253-1261.
315. W. M. Elkady and I. M. Ayoub, *Ind. Crops Prod.*, 2018, **118**, 188-195.
316. K. Spyridopoulou, A. Tiptiri-Kourpeti, E. Lampri, E. Fitsiou, S. Vasileiadis, M. Vamvakias, H. Bardouki, A. Goussia, V. Malamou-Mitsi, M. I. Panayiotidis, A. Galanis, A. Pappa and K. Chlichlia, *Scientific Reports*, 2017, **7**, 3782-3782.
317. T. Satou, Y. Hanashima, I. Mizutani and K. Koike, *Flavour Fragrance J.*, 2018, **33**, 230-234.
318. E. Sieniawska, R. Sawicki, M. Swatko-Ossor, A. Napiorkowska, A. Przekora, G. Ginalska, M. Swatko-Ossor and E. Augustynowicz-Kopec, *Molecules*, 2018, **23**, 176.
319. J. M. Lorenzo, A. Mousavi Khaneghah, M. Gavahian, K. Marszałek, I. Eş, P. E. Munekata, I. C. Ferreira and F. J. Barba, *Crit. Rev. Food Sci. Nutr.*, 2019, **59**, 2879-2895.
320. B. Salehi, A. P. Mishra, I. Shukla, M. Sharifi-Rad, M. d. M. Contreras, A. Segura-Carretero, H. Fathi, N. N. Nasrabadi, F. Kobarfard and J. Sharifi-Rad, *Phytother. Res.*, 2018, **32**, 1688-1706.
321. M. Ladanyia and M. Ladaniya, *Citrus fruit: biology, technology and evaluation*, Academic press, 2010.
322. V. Ahluwalia, R. Sisodia, S. Walia, O. P. Sati, J. Kumar and A. Kundu, *J. Pest Sci.*, 2013, **87**, 341-349.
323. C. E. Galvez, C. M. Jimenez, A. d. l. A. Gomez, E. F. Lizarraga and D. A. Sampietro, *Nat. Prod. Res.*, 2020, **34**, 1442-1445.
324. W. Elghwaji, A. M. El-Sayed, K. S. El-Deeb and A. M. ElSayed, *Pharmacogn. Mag.*, 2017, **13**, S446.
325. J. Y. Nie, R. Li, Z. T. Jiang, Y. Wang, J. Tan, S. H. Tang and Y. Zhang, *J. Sci. Food Agric.*, 2020, **100**, 3481-3487.
326. A. A. Safar, A. O. Ghafoor and D. Dastan, *Polish Journal of Environmental Studies*, 2020, **29**.
327. Y. Song, R. Sun, Z. Ji, X. Li, Q. Fu and S. Ma, *Life Sci.*, 2018, **206**, 117-124.
328. M. K. Okla, S. A. Alamri, M. Z. Salem, H. M. Ali, S. I. Behiry, R. A. Nasser, I. A. Alaraidh, S. M. Al-Ghtani and W. Soufan, *Processes*, 2019, **7**, 363.
329. J. A. Figueira, P. Porto-Figueira, J. A. M. Pereira and J. S. Câmara, *J. Funct. Foods*, (In Press).
330. A. Gök, Ş. İsmail Kirbaşlar and F. Gülay Kirbaşlar, *J. Essent. Oil Res.*, 2015, **27**, 17-22.
331. S. M. Abd-Elwahab, N. D. El-Tanbouly, M. Y. Moussa, A. R. Abdel-Monem and N. M. Fayek, *J. Essent. Oil Bear. Plants*, 2016, **19**, 1932-1942.
332. U. J. Go and I.-Y. Eom, *J. Chromatogr. A*, 2014, **1361**, 88-94.
333. S. Król, B. Zabiegała and J. Namieśnik, *TrAC, Trends Anal. Chem.*, 2010, **29**, 1101-1112.
334. S. Banihashemi and H. Bagheri, *J. Sep. Sci.*, 2017, **40**, 1985-1992.
335. Y. Li, J. Li and H. Xu, *RSC Advances*, 2017, **7**, 11959-11968.
336. K. Kędziora and W. Wasiak, *J. Chromatogr. A*, 2017, **1505**, 1-17.
337. A. Poormohammadi, A. Bahrami, M. Farhadian, F. G. Shahna and A. Ghiasvand, *J. Chromatogr. A*, 2017, **1527**, 33-42.
338. P. Trefz, S. Kischkel, D. Hein, E. S. James, J. K. Schubert and W. Miekisch, *J. Chromatogr. A*, 2012, **1219**, 29-38.
339. Z. Ayazi, *The Analyst*, 2017, **142**, 721-739.

340. M. R. Azari, A. Barkhordari, R. Zendehdel and M. Heidari, *Microchem. J.*, 2017, **134**, 270-276.
341. L. M. Feijó Barreira, Y. Xue, G. Duporté, J. Parshintsev, K. Hartonen, M. Jussila, M. Kulmala and M.-L. Riekkola, *Atmos. Meas. Tech.*, 2016, 3661-3671.
342. N. Heidari, A. Ghiasvand and S. Abdolhosseini, *Anal. Chim. Acta*, 2017, **975**, 11-19.
343. M. Heidari, A. Bahrami, A. R. Ghiasvand, F. G. Shahna and A. R. Soltanian, *Anal. Chim. Acta*, 2013, **785**, 67-74.
344. I.-Y. Eom, A.-M. Tugulea and J. Pawliszyn, *J. Chromatogr. A*, 2008, **1196**, 3-9.
345. H. Bagheri, Z. Ayazi and A. Aghakhani, *Anal. Chim. Acta*, 2011, **683**, 212-220.
346. A. Barkhordari, M. R. Azari, R. Zendehdel and M. Heidari, *Environ. Monit. Assess.*, 2017, **189**, 171.
347. I. Y. Eom and J. Pawliszyn, *J. Sep. Sci.*, 2008, **31**, 2283-2287.
348. M. Mieth, S. Kischkel, J. K. Schubert, D. Hein and W. Miekisch, *Anal. Chem.*, 2009, **81**, 5851-5857.
349. J. Gonçalves, J. Figueira, F. Rodrigues and J. S. Câmara, *J. Sep. Sci.*, 2012, 2282-2296.
350. X. Zang, W. Liang, Q. Chang, T. Wu, C. Wang and Z. Wang, *J. Chromatogr. A*, 2017, **1513**, 27-34.
351. P. Trefz, L. Rosner, D. Hein, J. K. Schubert and W. Miekisch, *Analytical and bioanalytical chemistry*, 2013, **405**, 3105-3115.
352. J. M. Sanchez, *J. Chromatogr. A*, 2014, **1369**, 18-25.
353. M.-L. Lota, D. de Rocca Serra, F. Tomi, C. Jacquemond and J. Casanova, *J. Agric. Food Chem.*, 2002, **50**, 796-805.
354. S. Zhong, J. Ren, D. Chen, S. Pan, K. Wang, S. Yang and G. Fan, *Food Sci. Technol. Res.*, 2014, **20**, 167-174.
355. H. Zhang, Y. Xie, C. Liu, S. Chen, S. Hu, Z. Xie, X. Deng and J. Xu, *Food Chem.*, 2017, **230**, 316-326.
356. H. Mo, R. Jeter, A. Bachmann, S. T. Yount, C.-L. Shen and H. Yeganehjoo, *Front. Pharmacol.*, 2019, **9**.
357. H. Zhang, Z. Lou, X. Chen, Y. Cui, H. Wang, X. Kou and C. Ma, *J. Food Eng.*, 2019, **244**, 126-135.
358. G. d. S. Dannenberg, G. D. Funck, W. P. d. Silva and Â. M. Fiorentini, *Food Control*, 2019, **95**, 115-120.
359. H. M. Ahmed and S. Tavaszi-Sarosi, *Food Chem.*, 2019, **275**, 730-738.
360. L. T. García, A. F. Leal, É. M. Moreno, E. E. Stashenko and H. J. Arteaga, *Ind. Crops Prod.*, 2017, **96**, 140-148.
361. Y. Song, R. Sun, Z. Ji, X. Li, Q. Fu and S. Ma, *Life Sci.*, 2018, **206**, 117-124.
362. M. A. El-Missiry, A. Fekri, L. A. Kesar and A. I. Othman, *Phytother. Res.*, 2020, DOI: 10.1002/ptr.6992.
363. O. M. Ahmed, S. F. AbouZid, N. A. Ahmed, M. Y. Zaky and H. Liu, *Curr. Pharm. Des.*, 2021, **27**, 513-530.
364. J. A. Figueira, P. Porto-Figueira, J. A. Pereira and J. S. Câmara, *Foods*, 2020, **9**, 1470.
365. J. A. Figueira, P. Porto-Figueira, J. A. M. Pereira and J. S. Câmara, *Microchem. J.*, 2020, DOI: https://doi.org/10.1016/j.microc.2020.104933, 104933.
366. H. Tutunchi, F. Naeini, A. Ostadrahimi and M. J. Hosseinzadeh-Attar, *Phytother. Res.*, 2020, **34**, 3137-3147.
367. R. W. Alberca, F. M. E. Teixeira, D. R. Beserra, E. A. de Oliveira, M. M. de Souza Andrade, A. J. Pietrobon and M. N. Sato, *Front. Immunol.*, 2020, **11**.

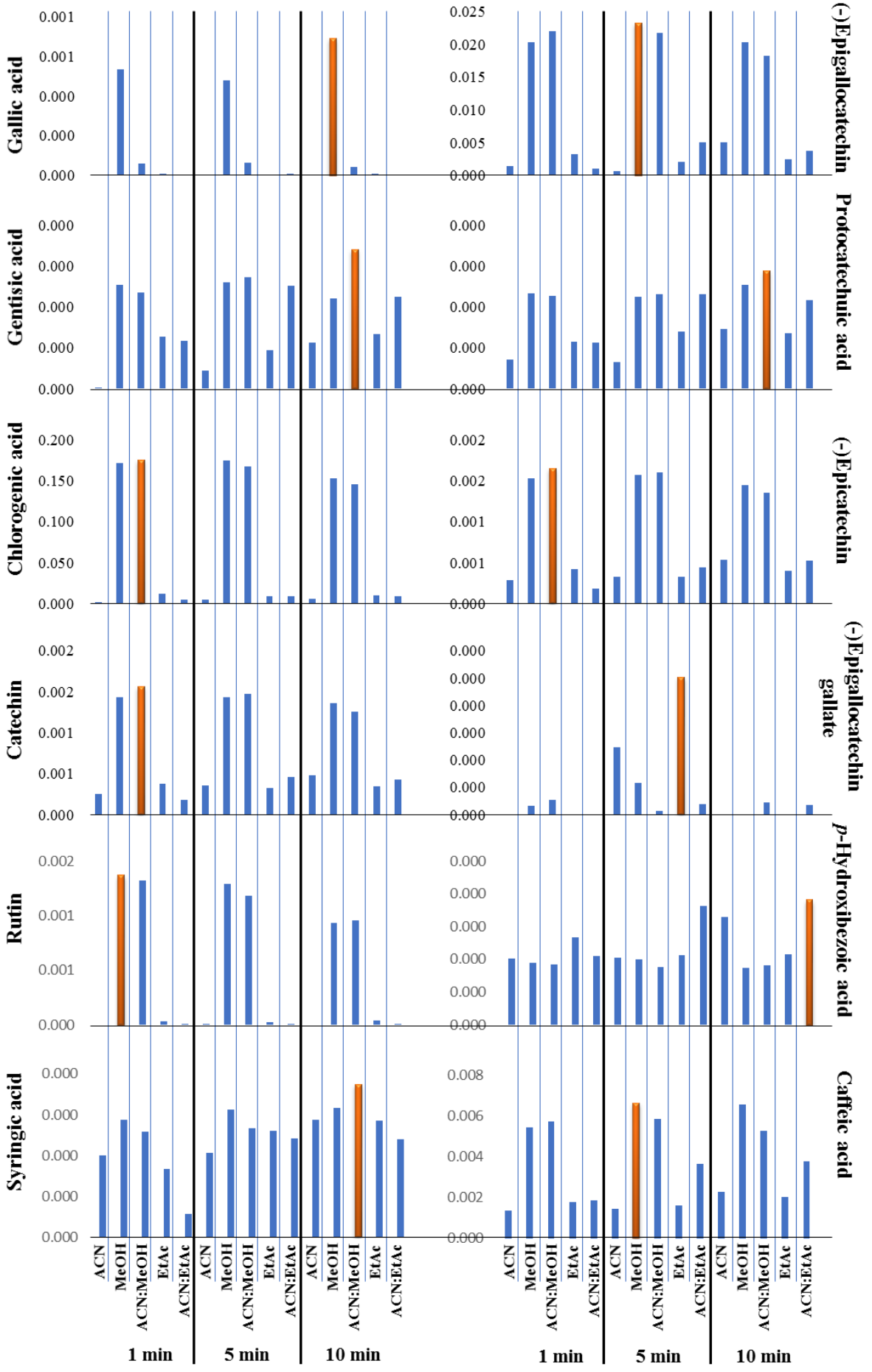
368. J. A. Figueira, P. Porto-Figueira, J. A. M. Pereira and J. S. Câmara, *Food Res. Int.*, (In Press).
369. A. C. Rodrigues, F. G. Zola, B. D. Ávila Oliveira, N. T. B. Sacramento, E. R. da Silva, M. C. Bertoldi, J. G. Taylor and U. M. Pinto, *J. Food Sci.*, 2016, **81**, M2538-M2544.
370. D. A. Siebert, F. de Mello, M. D. Alberton, L. Vitali and G. A. Micke, *Nat. Prod. Res.*, 2020, **34**, 2683-2688.
371. K. R. Biazotto, L. M. de Souza Mesquita, B. V. r. Neves, A. R. C. Braga, M. M. P. Tangerina, W. Vilegas, A. Z. Mercadante and V. V. De Rosso, *J. Agric. Food Chem.*, 2019, **67**, 1860-1876.
372. D. N. Santos, L. L. de Souza, C. A. F. de Oliveira, E. R. Silva and A. L. de Oliveira, *Food Bioscience*, 2015, **12**, 93-99.
373. D. J. Soares, J. Walker, M. Pignitter, J. M. Walker, J. M. Imboeck, M. M. Ehrnhoefer-Ressler, I. M. Brasil and V. Somoza, *Food Funct.*, 2014, **5**, 2981-2988.
374. A. L. Oliveira, E. Destandau, L. Fougère and M. Lafosse, *Food Chem.*, 2014, **145**, 522-529.
375. B. Holmquist, P. Bünning and J. F. Riordan, *Anal. Biochem.*, 1979, **95**, 540-548.
376. S.-Y. Lu, Y.-L. Chu, K. Sridhar and P.-J. Tsai, *LWT*, 2021, **138**, 110511.
377. J. Aguiar, J. L. Gonçalves, V. L. Alves and J. S. Câmara, *Antioxidants (Basel)*, 2020, **9**.
378. M. A. Raspo, M. B. Vignola, A. E. Andreatta and H. R. Juliani, *Food Bioscience*, 2020, **36**, 100651.
379. A. Gironés-Vilaplana, D. A. Moreno and C. García-Viguera, *Food Funct*, 2014, **5**, 764-772.
380. N. Leyva-López, E. P. Gutiérrez-Grijalva, G. Vazquez-Olivo and J. B. Heredia, *Molecules*, 2017, **22**, 989.
381. Z. Ilyas, H. S. Shah, R. Al-Oweini, U. Kortz and J. Iqbal, *Metallomics*, 2014, **6**, 1521-1526.
382. W. Błaszczak, M. Jeż and A. Szwengiel, *Food Chem.*, 2020, **314**, 126181.
383. F. Rey, L. Zacarías and M. J. Rodrigo, *Antioxidants*, 2020, **9**, 1296.
384. M. H. Alu'datt, T. Rababah, M. N. Alhamad, M. A. Al-Mahasneh, K. Ereifej, G. Al-Karaki, M. Al-Duais, J. E. Andrade, C. C. Tranchant, S. Kubow and K. A. Ghozlan, *Food Funct.*, 2017, **8**, 3187-3197.
385. L. Csambalik, A. Diveky-Ertsey, Z. Pap, C. Orban, M. Stegerne Mate, A. Gere, E. Stefanovits-Banyai and L. Sipos, *J. Food Sci.*, 2014, **79**, C2192-2202.
386. R. K. Toor, C. E. Lister and G. P. Savage, *Int. J. Food Sci. Nutr.*, 2005, **56**, 597-605.
387. K. K. H. Y. Ho, M. G. Ferruzzi, A. M. Liceaga and M. F. S. Martin-Gonzalez, *Lwt-Food Science and Technology*, 2015, **62**, 160-168.
388. M. Knoblich, B. Anderson and D. Latshaw, *J. Sci. Food Agric.*, 2005, **85**, 1166-1170.
389. S. Kumcuoglu, T. Yilmaz and S. Tavman, *Journal of Food Science and Technology*, 2013, **51**, 4102-4107.
390. S. Stajčić, G. Ćetković, J. Čanadanović-Brunet, S. Djilas, A. Mandić and D. Četojević-Simin, *Food Chem.*, 2015, **172**, 225-232.
391. V. García-Valverde, I. Navarro-González, J. García-Alonso and M. J. Periago, *Food Bioprocess Technol.*, 2013, **6**, 391-402.
392. P. Gupta, Y. Sreelakshmi and R. Sharma, *Plant Methods*, 2015, **11**, 5.
393. M. M. Maurer, J. R. Mein, S. K. Chaudhuri and H. L. Constant, *Food Chem.*, 2014, **165**, 475-482.
394. Y. Tang, X. Li, P. X. Chen, B. Zhang, M. Hernandez, H. Zhang, M. F. Marcone, R. Liu and R. Tsao, *Food Chem.*, 2015, **174**, 502-508.

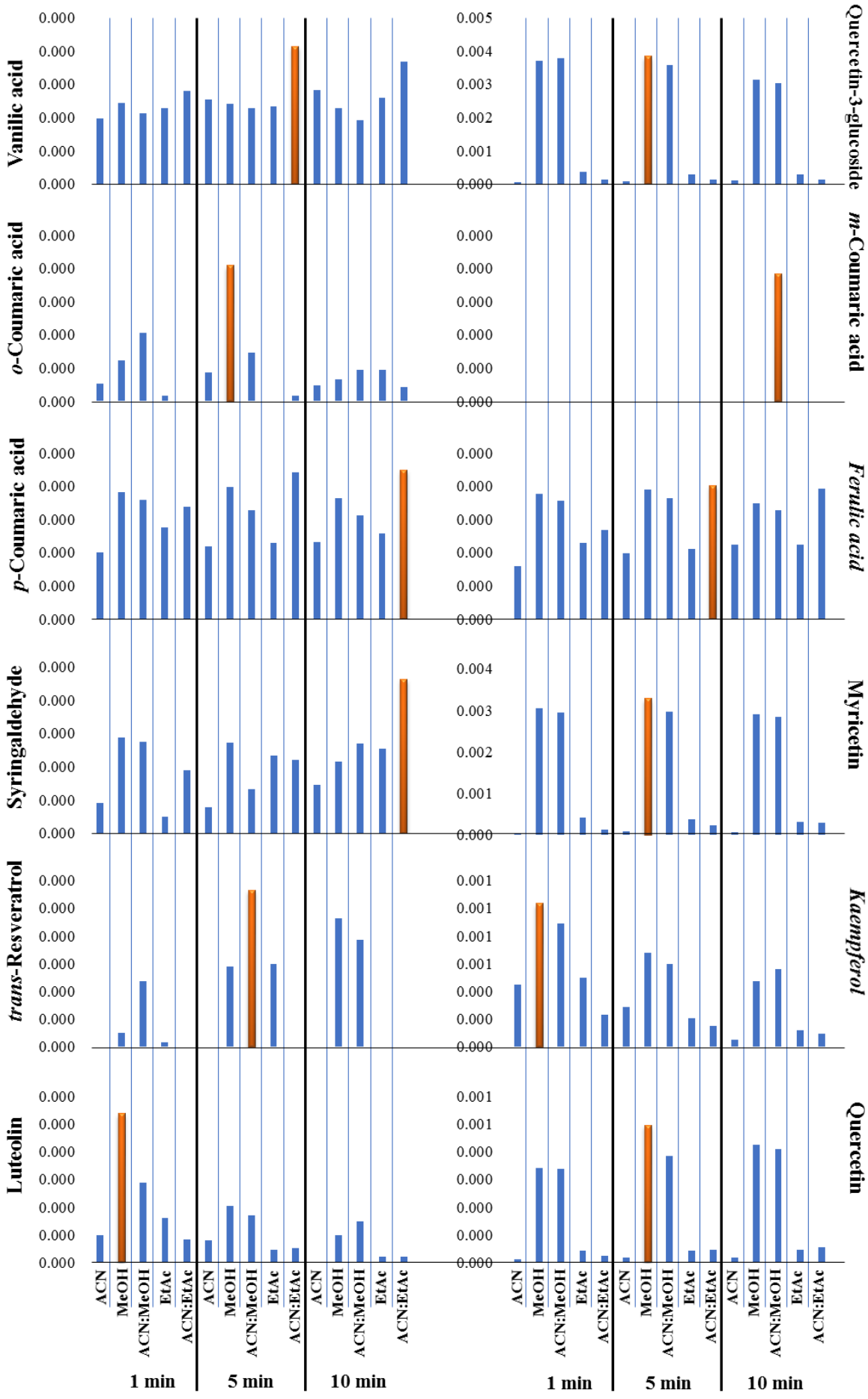
395. C. Assumpção, I. Nunes, T. Mendonça, A. Jablonski, S. Flôres and A. d. O. Rios, *J. Am. Oil Chem. Soc.*, 2014, **91**, 2035-2042.
396. A. Karioti, M.-C. Bergonzi, A. R. Bilia and F. F. Vincieri, *J. Agric. Food Chem.*, 2014, **62**, 12529−12535.
397. R. E. Kopec, R. M. Schweiggert, K. M. Riedl, R. Carle and S. J. Schwartz, *Rapid Commun. Mass Spectrom.*, 2013, **27**, 1393-1402.
398. C. Nimalaratne, C. Sun, J. Wu, J. M. Curtis and A. Schieber, *Food Res. Int.*, 2014, **66**, 69-77.
399. J. S. da Costa, E. d. N. S. da Cruz, W. N. Setzer, J. K. d. R. da Silva, J. G. S. Maia and P. L. B. Figueiredo, *Biomolecules*, 2020, **10**, 1155.
400. J. Vinholes and M. Vizzotto, *Pharmacognosy Res.*, 2017, **9**, 101.
401. J. Vinholes, G. Lemos, R. Lia Barbieri, R. Franzon and M. Vizzotto, *Journal*, 2017.
402. J. Vinholes, S. F. Reis, G. Lemos, R. L. Barbieri, V. de Freitas, R. C. Franzon and M. Vizzotto, *Food Funct.*, 2018, **9**, 6380-6390.
403. R. T. Correia, K. C. Borges, M. F. Medeiros and M. I. Genovese, *Food Sci. Technol. Int.*, 2012, **18**, 539-547.
404. K. Senthil Kumar, M. Gokila Vani, C.-S. Wang, C.-C. Chen, Y.-C. Chen, L.-P. Lu, C.-H. Huang, C.-S. Lai and S.-Y. Wang, *Plants*, 2020, **9**, 770.
405. V. Sicari, T. M. Pellicanò, A. M. Giuffrè, C. Zappia and M. Capocasale, *J. Food Meas. Charact.*, 2016, **10**, 773-780.
406. A. Gironés-Vilaplana, D. Moreno and C. García-Viguera, *Journal*, 2014.
407. S. Hussein, A. Kaluf, Y. Ahmed, B. Ahmed and A. Iyad, *The Iraqi Journal of Agricultural Science*, 2020, **51**, 411-421.
408. S. Fabroni, G. Ballistreri, M. Amenta, F. V. Romeo and P. Rapisarda, *J. Sci. Food Agric.*, 2016, **96**, 4713-4723.
409. V. Spínola, J. Pinto and P. C. Castilho, *Food Chem.*, 2015, **173**, 14-30.
410. I. R. Flores, M. S. Vásquez-Murrieta, M. O. Franco-Hernández, C. E. Márquez-Herrera, A. Ponce-Mendoza and M. del Socorro López-Cortéz, *Food Chem.*, 2021, **344**, 128608.
411. S. Tan, Z. Ke, D. Chai, Y. Miao, K. Luo and W. Li, *Food Chem.*, 2021, **338**, 128062.
412. J. Aguiar, J. L. Gonçalves, V. L. Alves and J. S. Câmara, *Antioxidants*, 2020, **9**, 305.
413. F. Bot, M. Anese, G. Hungerford and M. Lemos, *Ital. J. Food Sci.*, 2017, **29**, 424-433.
414. M. M. Belović, A. Gironés-Vilaplana, D. A. Moreno, I. L. Milovanović, A. R. Novaković, M. A. Karaman and N. M. Ilić, *J. Food Process. Preserv.*, 2016, **40**, 1229-1237.
415. P. Sricharoen, S. Techawongstein and S. Chanthai, *Asia Pac. J. Sci. Technol.*, 2015, **20**, 66-74.
416. D. Biswas, M. M. Uddin, L. L. Dizdarevic, A. Jørgensen and A. K. Duttaroy, *Eur. J. Nutr.*, 2014, **53**, 1699-1706.
417. H. Zhong, M. Zhao, J. Tang, L. Deng and F. Feng, *J. Sci. Food Agric.*
418. M. Yousefi, M. Shadnoush, N. Khorshidian and A. M. Mortazavian, *Phytother. Res.*, 2021, **35**, 846-863.
419. S. Tian, X. Li, S. Zang, Y. Jin, Z. Zhang and Y. Yu, *J. Food Process. Preserv.*, 2021, **45**, e15087.
420. B. Barut, E. N. Barut, S. Engin, A. Özel and F. S. Sezen, *Turk. J. Pharm. Sci.*, 2019, **16**, 175.
421. D. Sarkar, W. Agustinah, F. Woods, E. Coneva, E. Vinson and K. Shetty, *J. Berry Res.*, 2017, **7**, 163-177.

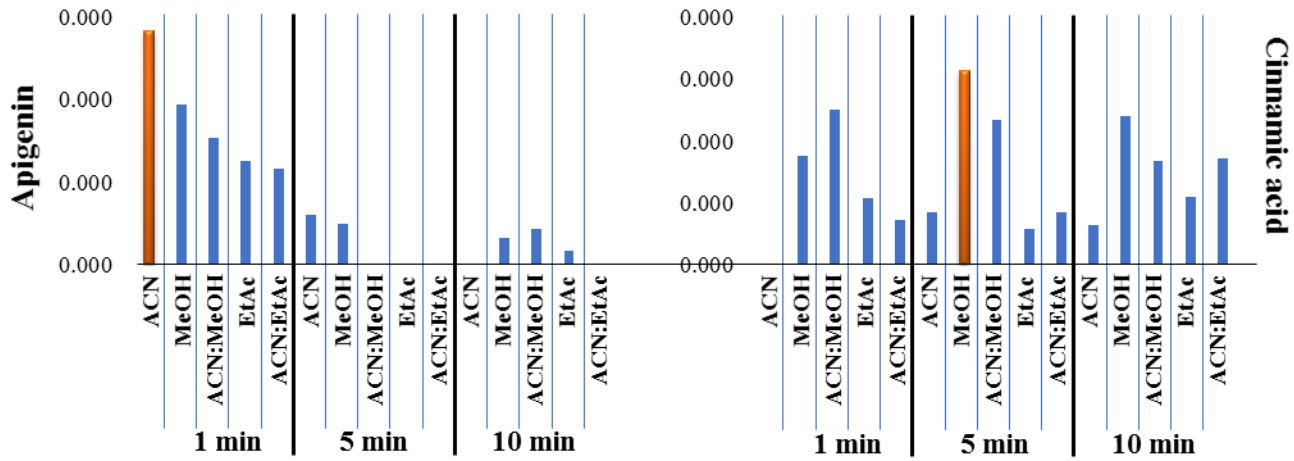
422. Art1 V. Verner, P. Novy, J. Tauchen, L. Huml, J. W. Soon, T. Kudera, F. Laupua and L. Kokoska, Sustainability, 2020, 12, 10014.
423. C. Jimenez-Lopez, M. Fraga-Corral, M. Carpena, P. García-Oliveira, J. Echave, A. Pereira, C. Lourenço-Lopes, M. Prieto and J. Simal-Gandara, Food Funct., 2020, 11, 4853-4877.
424. C. Pérez-Caselles, M. Brugarolas and L. Martínez-Carrasco, Sustainability, 2020, 12, 4517.
425. E. Strobl, Am. J. Agric. Econ., 2021.
426. A. M. Loboguerrero, B. M. Campbell, P. J. Cooper, J. W. Hansen, T. Rosenstock and E. Wollenberg, Sustainability, 2019, 11, 1372.

Supplementary Material

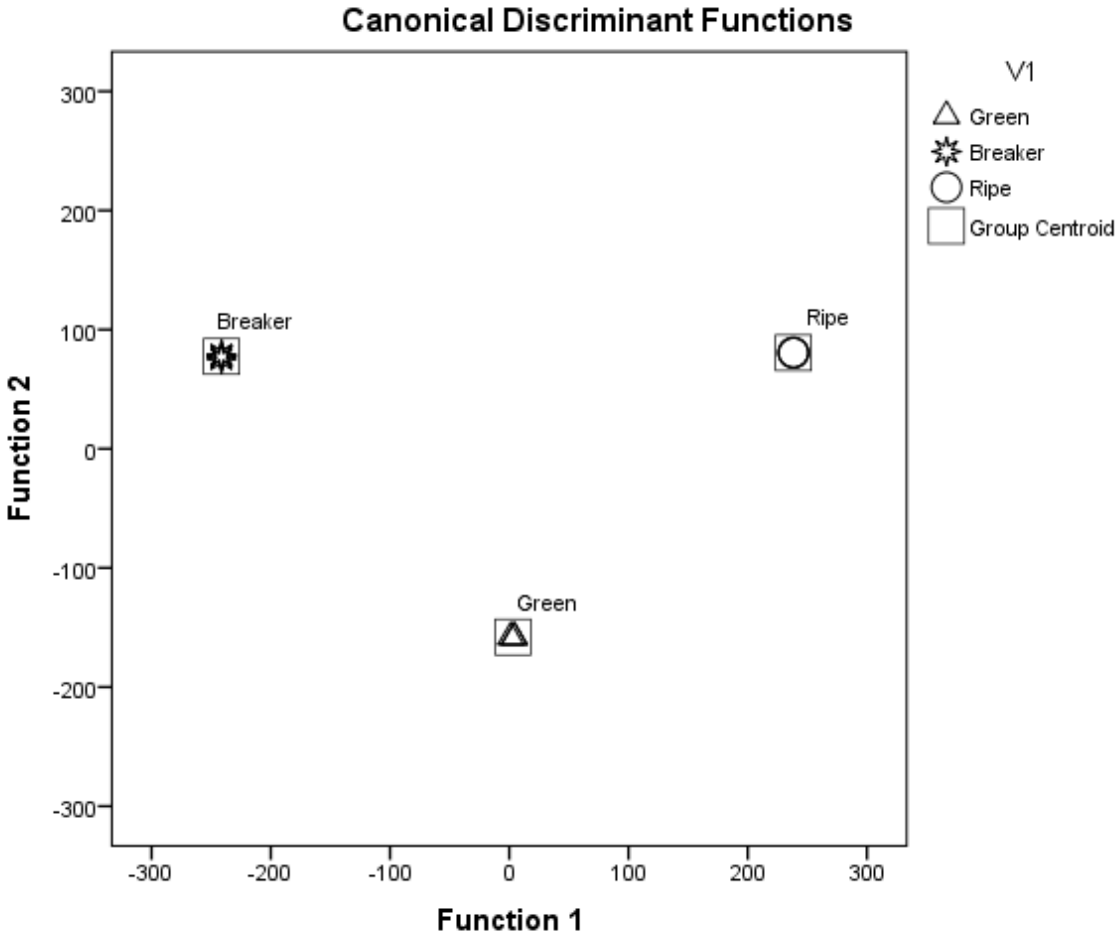
Supplementary Figures


Gallic acid
(-)Epigallocatechin
Gentisic acid
Protocatechuic acid
Chlorogenic acid
(-)Epicatechin
Catechin
(-)Epigallocatechin gallate
Rutin
p-Hydroxibezoic acid
Syringic acid
Caffeic acid
ACN
MeOH
ACN:MeOH
EtAc
ACN:EtAc
1 min
5 min
10 min

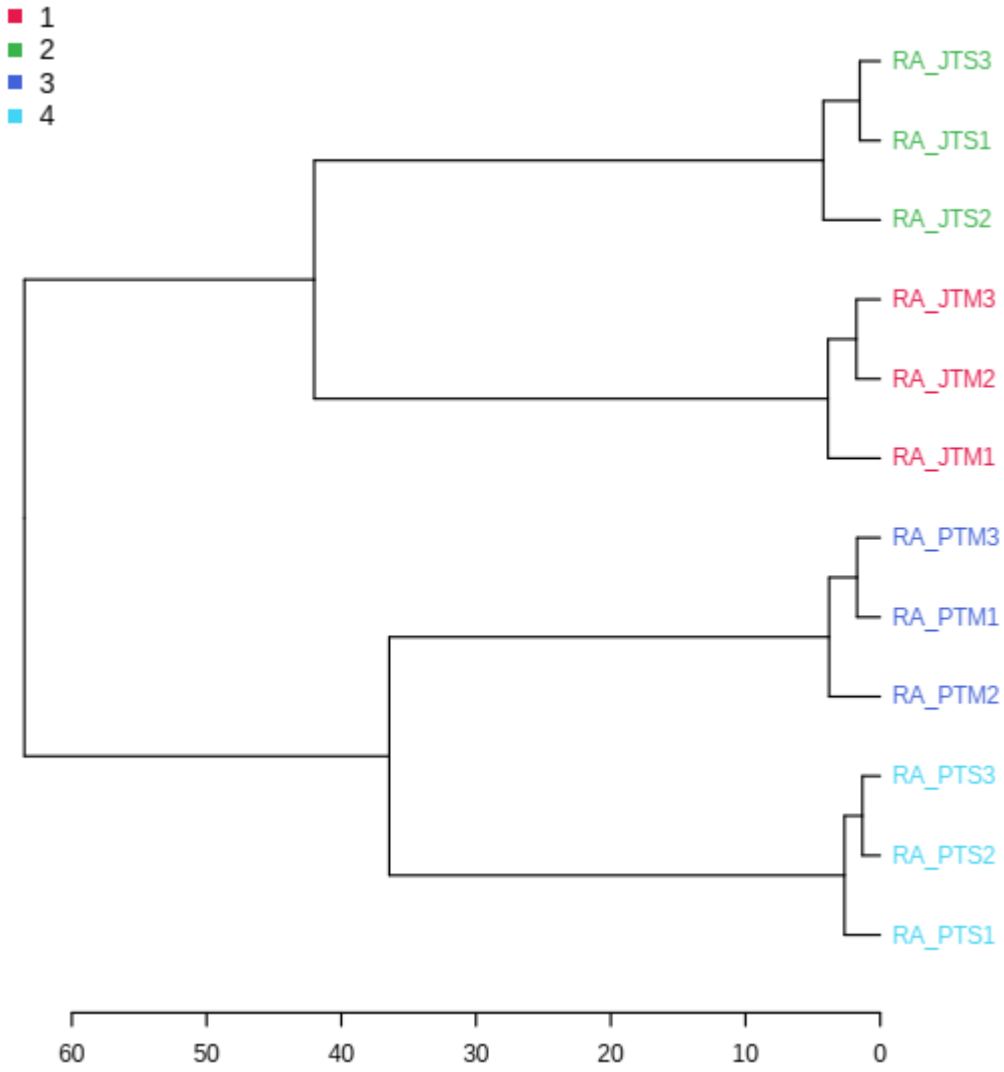

Vanilic acid
o-Coumaric acid
p-Coumaric acid
Syringaldehyde
trans-Resveratrol
Luteolin
Quercetin-3-glucoside
m-Coumaric acid
Ferulic acid
Myricetin
Kaempferol
Quercetin
ACN
MeOH
ACN:MeOH
EtAc
ACN:EtAc
1 min
5 min
10 min



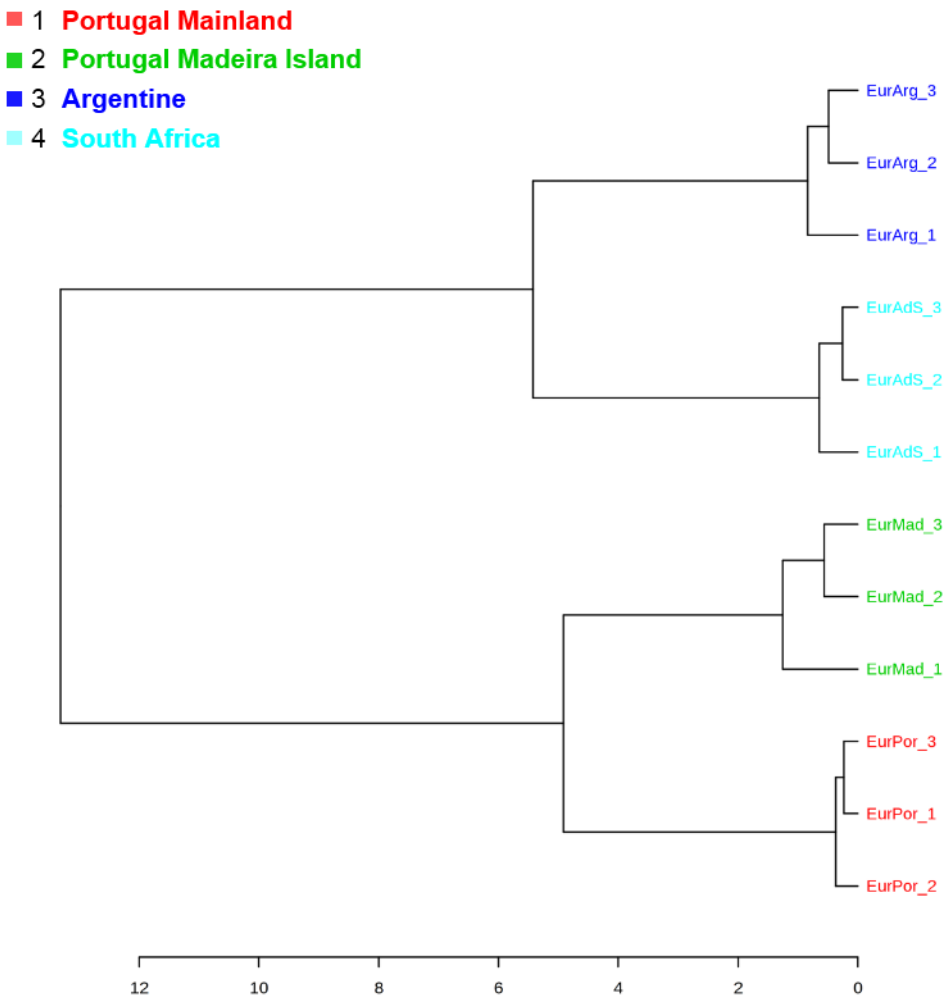
Supplementary Fig. 3.3.1. Distribution of individual polyphenols extraction by the different extraction solvents



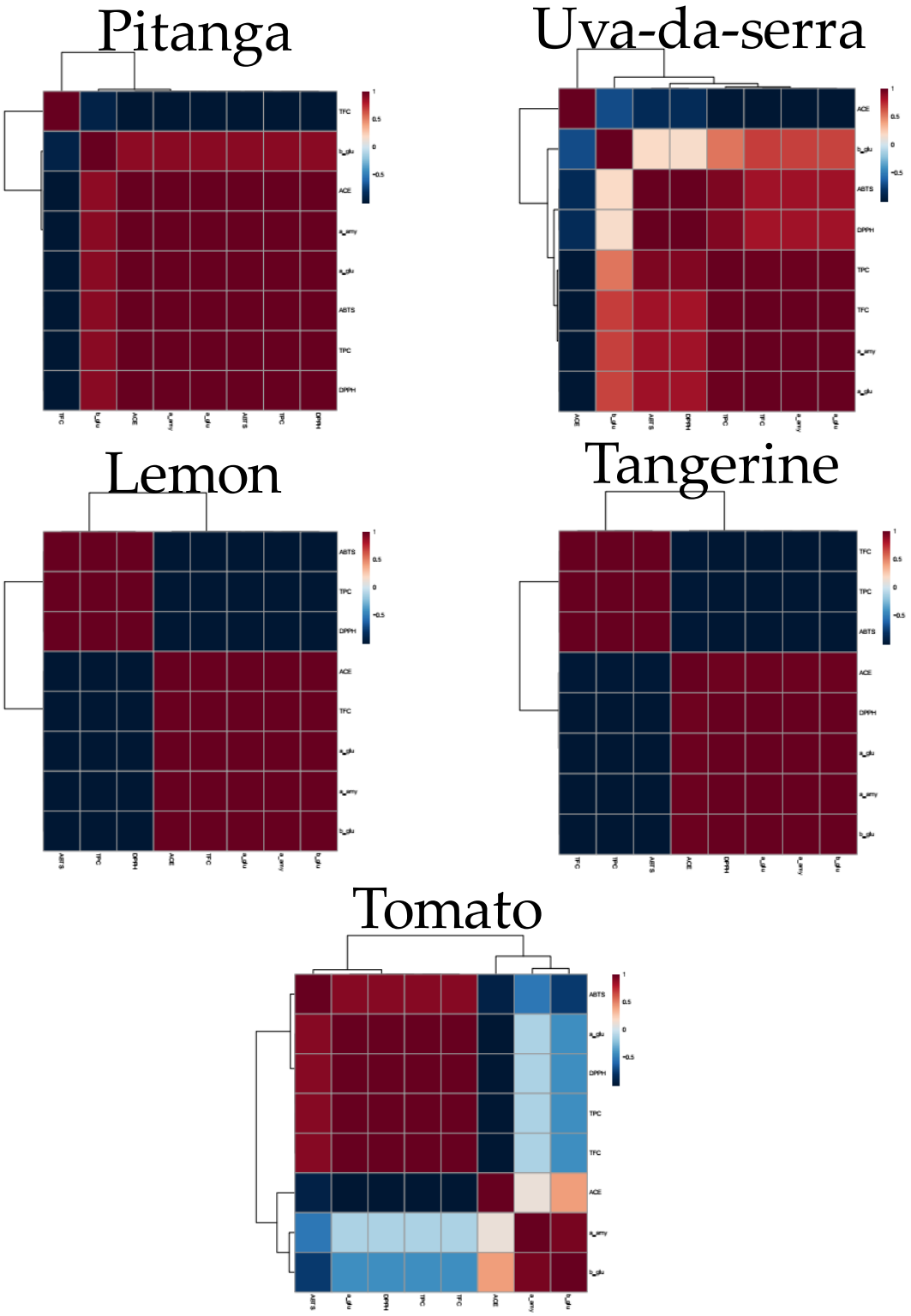
Supplementary Fig. 3.4.1. Canonical discriminant functions scatter plot.



Supplementary Fig. 3.5.1. Clustering result shown as dendrogram (distance measure using euclidean, and clustering algorithm using ward.D).



Supplementary Fig. 3.6.1. Clustering result shown as dendrogram (distance measure using euclidean, and clustering algorithm using ward.D).



Supplementary Fig. 1.7.1 Correlation heatmaps between the bioactive assays for each fruit extract

Supplementary Tables

Supplementary Table 3.1.1. Comparison of the proposed extraction procedure with other published methods for the extraction of lycopene and β-carotene from food matrices.

Sample	Extraction method/solvents	Analysis (main features: methodology / mobile phase / sample injection volume / λ)	Time[1]	LODs (Ly/β-C)[2] (ng/mL)	Recovery (Ly/β-C)[2] (ng/mL)	Abundance (Ly/β-C)[2] (μg/g FW)	Ref.
Tomatoes (*gordal*, *cherry*, *campari*, *grape*)	LL_{USAE}/ACN:MeOH	UHPLC/ACN, MeOH / 2 μL	5/4	24.0/3.0	97.1/108.3	727.1/80.4	**Method proposed in this manuscript**
Tomatoes (*cherry*, *campari*, *grape*)	LL/Hex, acetone	UV-Vis (453, 505, 645 and 663 nm)	-	-	-	88.0-158.0/7.0-16.0	131
Tomatoes (several varieties)	LLE/1.2-dichloroethane, H_2O	HPLC (MS)/H_2O, acetone	21/25	15.6/8.3	89-97	37.7-84.6/0.9-1.68	118
Tomatoes (*cherry*)	-	Colorimetric measurements	-	-	-	62.0-149.0/5.2-10.3	385
Tomatoes (*campari*)	LL/Hex, acetone	UV-Vis (472 nm)	-	-	-	452.0/- (DM^3)	386
Tomato peels	LLmae/H_2O, Hex:ethyl acetate	HPLC/MeOH:ammonium acetate, ethyl acetate / 10 μL	11	166	73.3/-	135.9/---	387
Tomatoes byproducts	Enzyme- aided extraction	UV-Vis (445, 472 and 502 nm)	-	-	-	108.0-1104.0/---	117
Tomato byproducts	LLE/THF:MeOH.	HPLC/ACN:MeOH:DCM, 0.1% BHT and trimethylamine / 50 μL	-	-	-	130.0-734.0/-14.4-29.3	388
Tomato byproducts	UAE/COSE/BHT (0.05%) in hexane:acetone:ethanol (2:1:1)	Spectrophotometric method	-	-	-	39.5-93.9/---	389
Tomato byproducts	high performance homogenizer/Hex	-	-	-	-	134.0-815.4/86.4-501.4 (DM^3)	390
Tomato (*ronaldo*, *zoco*, *pera* and *cherry pera*)	LL/MeOH:THF, 0.1% BHT	HPLC/MeOH, MTBE	-	-	-	0.6-116.7	391
Tomatoes, Arabidopsis leaf and green capsicum	LLE/chloroform:DCM in MeOH/MTBE	HPLC/MeOH:H_2O, MTBE / 20 μL	12	75	93.6/80.0	66.2/5.1	392
Broccoli, lettuce, carrot, and tomato	LL_{USAE}/acetone:MeOH, Hex	UHPLC/ACN:H_2O:Hex (0.1% acetic acid v/v) and ACN:butanol:Hex (0.1% acetic acid v/v) / 2 μL	10	60	> 97	17.0/1.7	393
Vegetables and fruits	LLE/H_2O, Hex:acetone	HPLC/ACN, MeOH, ethyl acetate / 10-20 μL	8	70/19	92.1 to 107.6	-	191

Quinoa	Soxhlet/MTBE:THF; LLE: Hex	HPLC/Hex:MTBE	-	280	98.6	-	[394]
Grass	LL_{USAE}/BHT:EtOH, calcium carbonate:acetone, acetone, H_2O, diethyl ether with BHT	HPLC/MeOH, MTBE, H_2O / 15 µL	72	-/629	-/70.4 to 116.0	-	[186]
Virgin and refined organic grape seed oil	LLE (saponification/chilled acetone)	HPLC/MeOH, MTBE, H_2O / 5 µL	46	-/44.6	-	-	[395]
Goji Berry	LL_{USAE}/H_2O, Hex:acetone	HPLC/acetone:MeOH / 5 µL	-	-/ 42.5	-/ 94.9 to 107.4	-	[396]
Human plasma	LLE/EtOH	HPLC/MeOH:H_2O, MTBE/MeOH/H_2O (0.04% ammonium acetate) / 20 µL	16	24.1/31.8	100.2/99.5	-	[397]
Infant formula and dietary supplements	LL_{USAE}/Hex:ethyl acetate	UHPLC(MS)/MeOH:H_2O, MTBE:MeOH / 3 µL	14	-/10	-/103.5	-	[398]

[1] Time (min) required for the carotenoids extraction/chromatographic separation and identification. The time for sample preparation and carotenoids extraction was estimated according to the methodology described in the respective report, [2] Ly/β-C – lycopene/β-C, [3] DM – abundance is reported in µg of carotenoid/ g of powered (Dry Mater) tomato; ACN – acetonitrile, BHT - butylhydroxytoluene, DCM - dichloromethane, EtOH – ethanol, FW - fresh weight, Hex - hexane, HL - high level, LL - low level, LLE - Liquid-liquid extraction, LL_{USAE}/UHPLC-PDA – ultrasound-assisted liquid-liquid extraction ultra-high performance liquid analysis, LDR - linear dynamic range, LLmae - Liquid-Liquid microwave assisted extraction, LOD - limit of detection, LOQ - limit of quantification, λ – wavelength (nm), ME – matrix effect, ML - medium level, MeOH – methanol, MTBE - methyl tert-butyl ether, PDA - photodiode array detector, PSA - primary–secondary amine, THF - tetrahydrofuran, US – Ultrasound

Supplementary Table 3.4.1. Percentage of variance and percentage of cumulative variance explained by the three extracted principal components.

Total Variance Explained

Component		Initial Eigenvalues[a]			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
		Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
Raw	1	55.4	78.0	78.0	55.4	78.0	78.0	43.5	61.3	61.3
	2	15.2	21.4	99.4	15.2	21.4	99.4	27.0	38.1	99.4
	3	0.3	0.4	99.8	0.3	0.4	99.8	0.3	0.4	99.8
Rescaled	1	55.4	78.0	78.0	55.4	78.0	78.0	43.5	61.3	61.3
	2	15.2	21.4	99.4	15.2	21.4	99.4	27.0	38.1	99.4
	3	0.3	0.4	99.8	0.3	0.4	99.8	0.3	0.4	99.8

Extraction Method: Principal Component Analysis.

a. When analyzing a covariance matrix, the initial eigenvalues are the same across the raw and rescaled solution.

Supplementary Table 3.4.2. Classification and cross-validation results

Classification Results[a,c]

V1			Predicted Group Membership			Total
			Green	Breaker	Ripe	
Original	Count	Green	3	0	0	3
		Breaker	0	3	0	3
		Ripe	0	0	3	3
	%	Green	100.0	0.0	0.0	100.0
		Breaker	0.0	100.0	0.0	100.0
		Ripe	0.0	0.0	100.0	100.0
Cross-validated[b]	Count	Green	3	0	0	3
		Breaker	0	3	0	3
		Ripe	0	0	3	3
	%	Green	100.0	0.0	0.0	100.0
		Breaker	0.0	100.0	0.0	100.0
		Ripe	0.0	0.0	100.0	100.0

a. 100.0% of original grouped cases correctly classified.

b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

c. 100.0% of cross-validated grouped cases correctly classified.

Supplementary Table 3.5.1. Volatile organic metabolites (VOMs) identified in tangerines juices and peels from the *setubalense* and *marcott* varieties.

	VOMs (organized by chemical family)	Cas number	RT (min) [a]	RI_{calc} [b]	RI_{lit} [c]	Relative Peak Area[d], RSD<10%			
						Juice		Peel	
						marcott	*setubalense*	*marcott*	*setubalense*
Aldehydes									
2[e]	**Acetaldehyde**	75-07-0	4.90	716	725	1.18	2.33	12.46	5.82
22	**Hexanal**	66-25-1	12.70	1045	1045	0.42	2.67	3626.29	122.31
40	Octanal	124-13-0	25.09	1252	1268	nd[f]	nd	95.49	14.04
48	**Nonanal**	124-19-6	31.41	1345	1343	nd	nd	290.35	423.45
64	**Decanal**	112-31-2	37.40	1447	1446	3.26	66.26	804.32	2402.25
67	**Benzaldehyde**	100-52-7	38.20	1462	1471	0.13	0.66	nd	nd
69	(E)-2-Nonenal	18829-56-6	39.00	1476	1500	0.14	nd	7.68	25.20
70	cis-4-Decenal	21662-09-9	39.42	1484	1523	nd	nd	5.86	14.64
87	(E)-2-Decenal	3913-81-3	44.22	1584	1597	nd	nd	200.17	384.60
96	**Dodecanal**	112-54-9	47.36	1656	1685	nd	nd	317.24	914.88
115	2-Dodecenal	4826-62-4	53.41	1800	1830	nd	nd	324.62	635.96
116	(E,Z)-2,6-nonadienal	557-48-2	54.75	1829	1601	nd	nd	96.76	167.40
117	Tetradecanal	124-25-4	56.69	1869	1911	nd	nd	22.76	39.36
	Σ=[g]	13		**Total Area[h]:**		5.13	71.91	5803.99	5149.89
Esters									
5	**Ethyl acetate**	141-78-6	6.80	849	856	26.17	8.90	4.72	0.87
11	**Ethyl propionate**	105-37-3	8.40	925	931	1.41	2.57	nd	nd
12	**Ethyl isobutyrate**	97-62-1	8.60	932	954	0.78	nd	nd	nd
13	**Propyl acetate**	109-60-4	8.80	939	962	0.14	nd	nd	nd
19	Ethyl 2-methylbutanoate	7452-79-1	11.50	1018	1015	1.21	1.25	nd	nd
27	Ethyl 2-butenoate	10544-63-5	16.70	1121	1122	1.18	nd	nd	nd
	Σ=	6		**Total Area:**		30.89	12.72	4.72	0.87
Alcohols									
6	**Methanol**	67-56-1	6.89	854	860	nd	nd	147.18	76.14
8	**Isopropyl Alcohol**	67-63-0	7.50	888	884	nd	nd	725.84	nd
9	**Ethanol**	64-17-5	7.60	893	899	65.68	65.71	2.72	355.93
41	(Z)-4-Hexen-1-ol	543-49-7	26.35	1269	1280	nd	nd	nd	6.75
44	**1-Hexanol**	111-27-3	28.46	1296	1294	nd	nd	7.74	1.09
46	3-Hexen-1-ol	544-12-7	30.40	1328	1327	nd	0.78	18.44	9.03
47	**3-Octanol (IS)[i]**	**589-98-0**	**31.10**	**1339**	**1368**	**100.00**	**100.00**	**100.00**	**100.00**
73	**1-Octanol**	111-87-5	40.20	1498	1494	1.12	44.42	65.38	nd
90	**1-Nonanol**	143-08-8	45.00	1600	1639	0.29	nd	nd	nd
111	(E)-2-Nonen-1-ol	31502-14-4	51.30	1750	1722	nd	4.01	nd	nd
	Σ=	10		**Total Area:**		67.09	114.93	967.30	448.94
Hydrocarbon									
1	**1,4-pentadiene**	591-93-5	4.65	695	646	nd	nd	0.88	2.20
36	1-Dodecene	112-41-4	22.30	1211	1232	1.05	3.39	nd	nd
42	**Tridecane**	629-50-5	26.40	1270	1273	nd	0.73	7.83	79.67
43	(E)-6-Tridecene	6434-76-0	28.23	1293		nd	nd	nd	6.32
49	(3E,5Z)-1,3,5-Undecatriene	51447-08-6	31.65	1348	1382	nd	nd	68.74	71.39
52	**Tetradecane**	629-59-4	33.30	1374	1375	nd	nd	36.36	104.59
60	**1-Tetradecene**	1120-36-1	35.40	1408	1429	0.30	1.94	nd	nd
68	**Pentadecane**	629-62-9	38.94	1475		nd	nd	13.35	135.34
84	**Hexadecane**	544-76-3	43.73	1574		nd	nd	3.88	6.97
119	3-propyl-Cyclohexene	3983-06-0	60.69	1968		nd	nd	nd	11.31
	Σ=	10		**Total Area:**		1.35	6.06	131.04	417.78
Monoterpene Hydrocarbons									
14	Tricyclene	508-32-7	10.20	984	995	nd	1.08	nd	nd
16	**α-Pinene**	7785-70-8	10.70	998	1001	50.49	429.86	8108.73	3108.77
17	α-Thujene	2867-05-2	10.80	1001	1003	24.03	240.76	78.23	3114.79
20	**α-Fenchene**	471-84-1	11.90	1027	1027	0.18	1.69	nd	nd
21	**Camphene**	79-92-5	12.19	1034	1034	0.88	13.91	620.88	31.74
23	β-Pinene (isomer 1)	127-91-3	14.10	1073	1078	52.67	921.27	2706.30	2832.13

24	β-Pinene (isomer 2)	18172-67-3	14.40	1079		nd	368.04	nd	nd
25	**Sabinene**	3387-41-5	14.79	1086	1086	6.29	146.39	nd	nd
26	β-Thujene	28634-89-1	14.90	1088	1107	nd	51.44	nd	nd
28	**β-Myrcene**	123-35-3	17.00	1127	1127	142.08	794.50	2964.61	2064.57
29	**α-Terpinene**	99-86-5	17.80	1141	1141	28.58	213.43	109.88	102.70
30	**D-Limonene**	5989-27-5	19.50	1169	1176	2346.61	10337.65	113983.25	134417.14
31	**β-Phellandrene**	555-10-2	19.70	1172	1177	13.85	109.10	704.76	523.59
32	(Bis(1-methylethylidene)-cyclobutane	3642-14-6	19.90	1175	1460	0.33	2.46	nd	nd
33	**(E)-Ocimene**	3779-61-1	21.10	1192	1196	0.91	28.44	nd	nd
34	**γ-Terpinene**	99-85-4	21.80	1203	1210	877.32	4327.34	29060.28	61526.81
35	**Z-Ocimene**	3338-55-4	22.10	1208	1210	3.26	nd	nd	nd
37	**o-Cymene**	527-84-4	23.00	1221	1225	93.05	276.77	4501.03	929.45
38	**Terpinolene**	586-62-9	24.00	1236	1239	61.99	373.69	4944.98	7887.33
39	2-Carene	554-61-0	24.30	1241	1273	nd	1.69	nd	nd
45	(4E,6Z)-allo-Ocimene	7216-56-0	30.00	1321	1327	nd	1.91	3.90	2.87
53	p-Cymenene	1195-32-0	33.80	1382	1383	1.02	7.19	77.78	151.49
54	1,3,8-p-Menthatriene	21195-59-5	33.90	1383	1387	nd	3.32	nd	nd
55	E,E-Cosmene	460-01-5	34.20	1388	1460	nd	0.66	36.15	123.76
91	2,6-Dimethyl-2,6-octadiene	2792-39-4	45.20	1604	1604	0.17	9.92	26.10	179.54
	Σ=	25		**Total Area:**		3703.72	18662.53	167926.86	216996.67
Sesquiterpene Hydrocarbons									
61	α-Cubebene	17699-14-8	35.70	1414	1435	0.16	0.33	109.55	27.20
63	δ-Elemene	20307-84-0	36.25	1425	1465	nd	7.39	nd	nd
65	Copaene	3856-25-5	37.50	1449	1454	0.54	nd	nd	nd
72	β-Cubenene	13744-15-5	39.85	1492	1519	nd	nd	137.26	
77	α-Santalene	512-61-8	41.50	1526	1526	nd	0.96	nd	nd
78	Allo-Aromadendrene	25246-27-9	41.60	1528	1618	nd	0.34	nd	nd
80	α-Bergamotene	17699-05-7	42.20	1541	1542	0.97	147.31	nd	nd
82	**β-Caryophyllene**	87-44-5	42.60	1550	1548	1.94	121.19	1460.97	1766.45
83	**α-Himachalene**	3853-83-6	43.40	1567	1566	nd	5.84	nd	nd
88	Elixene	3242-08-8	44.40	1587	1514	nd	1.49	nd	nd
89	β-Santalene	511-59-1	44.90	1598	1599	nd	12.36	nd	nd
92	(Z)-β-Farnesene	28973-97-9	45.60	1614	1613	nd	6.57	nd	nd
101	α-Selinene	473-13-2	48.00	1671	1719	nd	5.40	nd	nd
102	β-Bisabolene	495-61-4	48.20	1676	1723	0.37	60.69	426.24	1672.20
103	γ-Elemene	30824-67-0	48.47	1682	1651	nd	nd	119.02	239.37
104	α-Farnesene	502-61-4	49.00	1694	1744	0.41	35.23	2383.36	5960.78
106	-δ-Cadinene	483-76-1	49.45	1705	1716	nd	nd	385.27	95.52
	Σ=	17		**Total Area:**		4.40	405.09	5021.67	9761.52
Oxygenated Terpenes									
50	Perillene	539-52-6	32.97	1369	1369	nd	nd	2.21	2.14
56	**cis-Limonene oxide**	4680-24-4	34.33	1390	1388	nd	nd	11.50	16.47
57	**trans-Limonene oxide**	6909-30-4	35.04	1401	1398	nd	nd	199.31	168.22
59	cis-β-Terpineol	7299-41-4	35.30	1406	1404	nd	0.58	322.93	422.05
62	Citronellal	106-23-0	36.20	1424	1457	0.40	8.57	360.03	1034.77
66	**Camphor**	76-22-2	37.90	1456	1455	nd	1.77	66.47	119.87
71	**Linalool**	78-70-6	39.60	1487	1486	10.52	182.49	1559.91	2959.94
74	(E)-p-2-Menthen-1-ol	29803-81-4	40.45	1503	1552	nd	nd	1.83	2.05
76	Isopulegol	89-79-2	40.82	1511	1565	nd	nd	5.01	9.67
81	**Terpinen-4-ol**	562-74-3	42.30	1543	1585	6.77	82.96	523.22	883.81
85	Umbellulone	24545-81-1	43.93	1578	1610	nd	nd	14.04	nd
93	**α-Caryophyllene**	106-26-3	45.80	1619	1641	0.55	45.42	181.72	nd
94	**α-Terpineol**	98-55-5	46.40	1633	1633	7.71	105.29	1951.38	3213.62
95	**Borneol**	507-70-0	46.70	1641	1638	nd	nd	18.52	75.25
97	**Piperitone**	89-81-6	47.66	1663	1662	nd	nd	56.80	79.86
98	Neryl propionate	105-91-9	47.80	1667	1758	nd	45.99	76.06	160.10
99	Geranial	141-27-5	47.88	1668	1714	nd	49.01	141.05	553.47
100	**Carvone**	2244-16-8	47.89	1668	1715	0.34	nd	nd	nd
105	**(R)-Citronellol**	1117-61-9	49.30	1701	1699	2.28	16.33	305.96	455.00
107	**Perilla aldehyde**	2111-75-3	49.90	1716	1716	2.89	27.13	636.71	921.07
108	y Isogeraniol	13066-51-8	50.12	1721	1800	nd	nd	55.34	nd

109	**Nerol**	106-25-2	50.60	1733	1731	nd	2.97	87.03	33.62
110	(E,E)-2,4-Decadienal	25152-84-5	51.04	1744	1771	nd	nd	116.69	144.06
112	**cis-Carveol**	1197-06-4	52.00	1767	1832	nd	2.58	127.82	154.01
113	**Cherry propanol**	1197-01-9	52.60	1781	1840	nd	2.06	20.20	45.33
114	**Carveol**	99-48-9	53.20	1795	1845	nd	4.17	14.77	176.11
118	**Perillyl alcohol**	536-59-4	59.69	1940	1959	nd	nd	25.02	35.15
120	**Nerolidol**	7212-44-4	61.06	1978	1990	nd	nd	nd	9.33
123	**Eugenol**	97-53-0	64.58	2069	2117	nd	nd	48.28	nd
124	**Thymol**	89-83-8	65.00	2080	2166	2.49	28.63	1143.30	2190.10
125	4-Isopropyl-3-methylphenol	3228-02-2	65.68	2097		nd	nd	72.95	50.82
128	α-Sinensal	17909-77-2	68.50	2166	2271	nd	4.95	219.08	878.83
	Σ=	32		**Total Area:**		33.94	610.90	8365.13	14794.71
Others									
3	Carbon disulfide	75-15-0	5.08	731	745	nd	nd	3.55	1.11
4	**Acetone**	67-64-1	5.76	782	775	nd	nd	5.12	1.96
15	**1-penten-3-one**	1629-58-9	10.24	985	991	nd	nd	4771.24	4.54
7	**2-Methyl-furan**	534-22-5	6.90	854	858	0.42	1.25	nd	nd
10	2-Ethyl-furan	3208-16-0	8.26	920	922	nd	nd	12.64	1.48
18	**Toluene**	108-88-3	11.10	1008	1008	0.24	nd	nd	nd
51	2,3-Dihydro-2-methylbenzofuran	1746-11-8	33.10	1371		nd	1.71	nd	nd
58	**Acetic acid**	64-19-7	35.10	1402	1403	nd	0.45	335.74	12.00
75	6,6-Dimethyl-2-methylidene-norpinan-3-one	16812-40-1	40.57	1505		nd	nd	0.45	0.75
79	**Thymol methyl ether**	1076-56-8	42.00	1537	1563	0.43	3.59	55.95	169.84
86	**p-Tolualdehyde**	104-87-0	44.10	1581	1605	0.86	3.12	nd	nd
121	**Octanoic acid**	124-07-02	61.75	1996	2021	nd	nd	nd	30.41
122	**Dimethyl anthranilate**	85-91-6	62.00	2003	2042	13.19	65.20	4651.75	2970.61
126	**Methyl anthranilate**	134-20-3	66.20	2110	2198	0.18	nd	10.62	4.78
127	**n-Decanoic acid**	334-48-5	67.16	2134	2229	nd	nd	6.10	6.57
129	**Indole**	120-72-9	71.58	2238	2398	nd	nd	3.11	10.80
	Σ=	16		**Total Area:**		15.32	75.31	9856.28	3214.85
				TOTAL AREA (relative peak area):		3861.83	19959.46	198076.99	250785.22
				TOTAL VOMs (number):		56	75	89	87

[a] RT: retention time expressed in min.

[b] RI_{calc}: experimental Kovat's index.

[c] RI_{lit}: Kovat's index reported in the literature.

[d] Relative Peak Area: (VOM peak area/Internal Standard peak area).

[e] Peak identification number ordered by the VOC retention time.

[f] nd - Not decteted

[g] Sum of VOMs in the chemical familie

[h] Total relative area in the chemical familie

[i] IS - Internal Standard (3-octanol).

VOCs indicated in **bold** were confirmed against commercial standards

Supplementary Table 3.5.2: VOM identified by One-way ANOVA and post-hoc analysis, found as statistically significant for ($p < 0.05$).

VOM	chi.squared	p.value	=-LOG10(p)	FDR	Post-Hoc
3	10.532	0.014546	1.8373	0.022136	NA
4	10.532	0.014546	1.8373	0.022136	NA
6	10.532	0.014546	1.8373	0.022136	NA
8	10.532	0.014546	1.8373	0.022136	NA
10	10.532	0.014546	1.8373	0.022136	NA
15	10.532	0.014546	1.8373	0.022136	NA
24	10.532	0.014546	1.8373	0.022136	NA
26	10.532	0.014546	1.8373	0.022136	NA
39	10.532	0.014546	1.8373	0.022136	NA
41	10.532	0.014546	1.8373	0.022136	NA
43	10.532	0.014546	1.8373	0.022136	NA
44	10.532	0.014546	1.8373	0.022136	NA
48	10.532	0.014546	1.8373	0.022136	NA
50	10.532	0.014546	1.8373	0.022136	NA
51	10.532	0.014546	1.8373	0.022136	NA
52	10.532	0.014546	1.8373	0.022136	NA
54	10.532	0.014546	1.8373	0.022136	NA
55	10.532	0.014546	1.8373	0.022136	NA
56	10.532	0.014546	1.8373	0.022136	NA
59	10.532	0.014546	1.8373	0.022136	NA
63	10.532	0.014546	1.8373	0.022136	NA
66	10.532	0.014546	1.8373	0.022136	NA
68	10.532	0.014546	1.8373	0.022136	NA
70	10.532	0.014546	1.8373	0.022136	NA
72	10.532	0.014546	1.8373	0.022136	NA
74	10.532	0.014546	1.8373	0.022136	NA
75	10.532	0.014546	1.8373	0.022136	NA
76	10.532	0.014546	1.8373	0.022136	NA
78	10.532	0.014546	1.8373	0.022136	NA
83	10.532	0.014546	1.8373	0.022136	NA
84	10.532	0.014546	1.8373	0.022136	NA
85	10.532	0.014546	1.8373	0.022136	NA
87	10.532	0.014546	1.8373	0.022136	NA
88	10.532	0.014546	1.8373	0.022136	NA
89	10.532	0.014546	1.8373	0.022136	NA
92	10.532	0.014546	1.8373	0.022136	NA
95	10.532	0.014546	1.8373	0.022136	NA
96	10.532	0.014546	1.8373	0.022136	NA
97	10.532	0.014546	1.8373	0.022136	NA
98	10.532	0.014546	1.8373	0.022136	NA
101	10.532	0.014546	1.8373	0.022136	NA
103	10.532	0.014546	1.8373	0.022136	NA
106	10.532	0.014546	1.8373	0.022136	NA
108	10.532	0.014546	1.8373	0.022136	NA
110	10.532	0.014546	1.8373	0.022136	NA
111	10.532	0.014546	1.8373	0.022136	NA

112	10.532	0.014546	1.8373	0.022136	NA
113	10.532	0.014546	1.8373	0.022136	NA
114	10.532	0.014546	1.8373	0.022136	NA
115	10.532	0.014546	1.8373	0.022136	NA
116	10.532	0.014546	1.8373	0.022136	NA
119	10.532	0.014546	1.8373	0.022136	NA
120	10.532	0.014546	1.8373	0.022136	NA
121	10.532	0.014546	1.8373	0.022136	NA
123	10.532	0.014546	1.8373	0.022136	NA
127	10.532	0.014546	1.8373	0.022136	NA
128	10.532	0.014546	1.8373	0.022136	NA
2	10.421	0.015306	1.8151	0.022136	NA
13	10.421	0.015306	1.8151	0.022136	NA
67	10.421	0.015306	1.8151	0.022136	NA
5	10.385	0.015564	1.8079	0.022136	NA
7	10.385	0.015564	1.8079	0.022136	NA
9	10.385	0.015564	1.8079	0.022136	NA
11	10.385	0.015564	1.8079	0.022136	NA
12	10.385	0.015564	1.8079	0.022136	NA
18	10.385	0.015564	1.8079	0.022136	NA
19	10.385	0.015564	1.8079	0.022136	NA
20	10.385	0.015564	1.8079	0.022136	NA
21	10.385	0.015564	1.8079	0.022136	NA
23	10.385	0.015564	1.8079	0.022136	NA
27	10.385	0.015564	1.8079	0.022136	NA
30	10.385	0.015564	1.8079	0.022136	NA
32	10.385	0.015564	1.8079	0.022136	NA
33	10.385	0.015564	1.8079	0.022136	NA
34	10.385	0.015564	1.8079	0.022136	NA
35	10.385	0.015564	1.8079	0.022136	NA
36	10.385	0.015564	1.8079	0.022136	NA
53	10.385	0.015564	1.8079	0.022136	NA
60	10.385	0.015564	1.8079	0.022136	NA
61	10.385	0.015564	1.8079	0.022136	NA
65	10.385	0.015564	1.8079	0.022136	NA
73	10.385	0.015564	1.8079	0.022136	NA
80	10.385	0.015564	1.8079	0.022136	NA
86	10.385	0.015564	1.8079	0.022136	NA
90	10.385	0.015564	1.8079	0.022136	NA
91	10.385	0.015564	1.8079	0.022136	NA
100	10.385	0.015564	1.8079	0.022136	NA
102	10.385	0.015564	1.8079	0.022136	NA
104	10.385	0.015564	1.8079	0.022136	NA
126	10.385	0.015564	1.8079	0.022136	NA
117	10.116	0.017607	1.7543	0.024765	NA
16	9.9744	0.018785	1.7262	0.025855	NA
69	9.9744	0.018785	1.7262	0.025855	NA
1	9.8038	0.02031	1.6923	0.0262	NA
42	9.8038	0.02031	1.6923	0.0262	NA

49	9.8038	0.02031	1.6923	0.0262	NA
29	9.6667	0.021623	1.6651	0.0262	NA
38	9.6667	0.021623	1.6651	0.0262	NA
82	9.6667	0.021623	1.6651	0.0262	NA
14	9.5957	0.022334	1.651	0.0262	NA
40	9.5957	0.022334	1.651	0.0262	NA
45	9.5957	0.022334	1.651	0.0262	NA
57	9.5957	0.022334	1.651	0.0262	NA
58	9.5957	0.022334	1.651	0.0262	NA
77	9.5957	0.022334	1.651	0.0262	NA
99	9.5957	0.022334	1.651	0.0262	NA
125	9.5957	0.022334	1.651	0.0262	NA
129	9.5957	0.022334	1.651	0.0262	NA
124	9.5128	0.023195	1.6346	0.0262	NA
46	9.4917	0.02342	1.6304	0.0262	NA
109	9.4917	0.02342	1.6304	0.0262	NA
17	9.4615	0.023744	1.6244	0.0262	NA
22	9.4615	0.023744	1.6244	0.0262	NA
28	9.4615	0.023744	1.6244	0.0262	NA
31	9.4615	0.023744	1.6244	0.0262	NA
79	9.4615	0.023744	1.6244	0.0262	NA
25	9.359	0.02488	1.6042	0.026539	NA
37	9.359	0.02488	1.6042	0.026539	NA
62	9.359	0.02488	1.6042	0.026539	NA
93	9.359	0.02488	1.6042	0.026539	NA
81	8.8974	0.030686	1.5131	0.032461	NA
107	8.6923	0.033674	1.4727	0.03533	NA
105	8.5385	0.0361	1.4425	0.037568	NA
71	8.0769	0.044448	1.3521	0.045515	NA
122	8.0769	0.044448	1.3521	0.045515	NA

Supplementary Table 3.6.1. Analysis of variance for total area, of the three parameters studied in the optimization, Equilibration Time (min), Headspace Volume (mL) and Temperature (º C).

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value
A: Temperature	2.09323E7	1	2.09323E7	15.02	0.0031
B: Headspace	6.5399E6	1	6.5399E6	4.69	0.0555
C: Equilibrium	1.8545E7	1	1.8545E7	13.31	0.0045
AA	1.79909E7	1	1.79909E7	12.91	0.0049
AC	4.69865E6	1	4.69865E6	3.37	0.0962
Total error	1.39342E6	10	1.39342E6		
Total (corr.)	8.26409E7	15			

Supplementary Table 3.6.2. ANOVA test ($p \leq 0.05$)

Compound	*f*-value	*p*-value	-LOG10(p)	FDR
38	8271.2	2.66E-14	13.576	1.96E-12
26	1594.4	1.91E-11	10.719	7.07E-10
6	945.8	1.54E-10	9.814	3.79E-09
22	285.1	1.81E-08	7.743	3.34E-07
12	253.7	2.87E-08	7.542	4.25E-07
46	235.0	3.88E-08	7.411	4.26E-07
55	232.8	4.03E-08	7.395	4.26E-07
35	205.4	6.60E-08	7.180	6.11E-07
13	143.3	2.72E-07	6.565	2.22E-06
53	139.7	3.01E-07	6.522	2.22E-06
67	126.5	4.44E-07	6.353	2.94E-06
77	124.2	4.77E-07	6.322	2.94E-06
78	104.3	9.42E-07	6.026	5.29E-06
32	102.7	1.00E-06	5.999	5.29E-06
63	94.0	1.41E-06	5.851	6.95E-06
48	91.9	1.54E-06	5.814	7.11E-06
57	88.3	1.80E-06	5.745	7.29E-06
49	88.2	1.80E-06	5.745	7.29E-06
64	87.4	1.87E-06	5.728	7.29E-06
52	85.1	2.07E-06	5.684	7.67E-06
66	80.6	2.55E-06	5.593	8.99E-06
61	73.3	3.69E-06	5.433	1.22E-05
75	72.7	3.80E-06	5.420	1.22E-05
65	70.0	4.39E-06	5.358	1.35E-05
40	68.0	4.92E-06	5.308	1.46E-05
70	66.2	5.45E-06	5.264	1.55E-05
19	65.1	5.81E-06	5.236	1.59E-05
51	61.7	7.12E-06	5.148	1.88E-05
8	59.6	8.16E-06	5.088	2.08E-05
59	46.1	2.15E-05	4.668	5.23E-05
30	45.9	2.19E-05	4.660	5.23E-05
54	42.9	2.83E-05	4.548	6.54E-05
47	37.8	4.54E-05	4.343	0.000102
62	33.0	7.43E-05	4.129	0.000162
58	32.1	8.27E-05	4.083	0.000175
76	31.7	8.65E-05	4.063	0.000178
16	27.7	0.000142	3.849	0.000283
23	24.9	0.000207	3.684	0.000403
24	23.3	0.000261	3.583	0.000495
73	22.6	0.000292	3.535	0.00054
72	21.2	0.000367	3.436	0.000662
41	20.0	0.000447	3.350	0.000787
69	19.6	0.000484	3.315	0.000834

71	19.4	0.000497	3.304	0.000836
42	18.4	6.00E-04	3.222	0.000983
44	18.3	0.000611	3.214	0.000983
11	17.5	0.000706	3.151	0.001112
37	17.4	0.000723	3.141	0.001115
4	17.0	0.000786	3.105	0.001186
34	16.4	0.000889	3.051	0.001316
1	16.3	0.000907	3.042	0.001316
68	15.5	0.00108	2.967	0.001537
27	14.3	0.001409	2.851	0.001967
25	12.0	0.002461	2.609	0.003372
39	10.6	0.003725	2.429	0.005012
50	9.6	0.004941	2.306	0.00653
31	9.3	0.005577	2.254	0.007241
29	9.0	0.006157	2.211	0.007856
28	8.1	0.008331	2.079	0.010449
3	6.2	0.017679	1.753	0.021804
2	5.4	0.02544	1.595	0.030862
15	5.1	0.029636	1.528	0.035372
9	4.9	0.032677	1.486	0.038383
17	4.7	0.035436	1.451	0.040972
5	4.6	0.038153	1.419	0.043435

Legend: *The correspondence between each VOC number and its identification is presented in the Table A.1; FDR – False discovery rate.

Supplementary Table 3.7.1. Optimization of extraction solvents for TAC, TPC and TFC assays. Assays results from the five fruits samples with seven extraction solvents were normalize by Log10 application.

	Solvents[1]	Pitanga Seeds	Tangerine Peel	Tomato	Uva-da-serra	Lemon Peel
DPPH	ACN:MeOH	2.12	0.51	0.78	1.15	0.85
	ACN:EtAc	0.29	0.35	0.19	0.82	0.90
	MeOH:FA 0.1	2.32	1.05	0.82	1.25	1.17
	MeOH	0.17	0.50	0.37	0.75	0.88
	ACN	2.25	0.96	0.82	1.32	1.05
	EtAc	0.13	0.72	0.40	0.79	0.96
	FA 0.1%	1.96	1.14	0.69	1.59	1.03
ABTS	ACN:MeOH	4.31	3.92	3.16	4.29	3.86
	ACN:EtAc	-	-	-	-	-
	MeOH:FA 0.1	4.30	4.17	3.72	4.31	4.28
	MeOH	-	-	-	-	-
	ACN	4.31	3.94	2.94	4.31	4.16
	EtAc	-	-	-	-	-
	FA 0.1%	4.20	3.81	-	-	-
TFC	ACN:MeOH	4.69	3.83	3.28	5.14	4.31
	ACN:EtAc	3.95	3.97	4.20	5.02	4.16
	MeOH:FA 0.1	4.98	4.45	4.11	5.26	4.71
	MeOH	2.95	3.47	2.81	4.85	3.80
	ACN	5.01	4.90	4.84	5.40	4.88
	EtAc	5.48	4.51	4.31	5.11	5.05
	FA 0.1%	5.42	5.81	6.01	5.83	5.53
TPC	ACN:MeOH	5.49	5.23	4.57	5.35	5.18
	ACN:EtAc	4.48	4.68	4.46	4.74	4.74
	MeOH:FA 0.1	5.74	5.36	4.71	5.58	5.53
	MeOH	4.44	4.93	4.44	4.88	4.73
	ACN	5.62	5.35	4.71	5.68	5.47
	EtAc	4.48	4.56	4.60	4.68	4.70
	FA 0.1%	5.73	5.16	4.91	5.48	5.76

1 – MeOH – methanol, ACN – acetonitrile, EtAc – ethyl acetate - EtAc, FA 0.1 – formic acid 0.1 %.

2 – The mixture of extracts with ABTS solvents resulted in a suspension solution.

Supplementary Table 3.7.2. Total phenolic content (TPC), total flavonoid content (TFC), total antioxidant capacity assessed through the DPPH and ABTS assays, antidiabetic capacity through the ability to inhibit digestive enzymes (α-amylase, α- and β-glucosidase), and antihypertensive capacity through ACE-inhibition activity, bioactivity associated to pitanga, tangerine, lemon, tomato and uva-da-serra extract samples.

	Extracts conditions	TPC	TFC	DPPH	ABTS	α-amylase	α-glucosidase	β-glucosidase	ACE	Ref
Pitanga	oils from leaves			15350 - 40030 mgTE/100g						399
	supercritical extract of seeds	0.003-7.3 mgGAE/100g		(IC_{50}) 460.1-5024.6 mg/100g	532-3337 µMTE/100g					372
	pressurised fluid extraction of seeds (dried- 12.7% H_2O)	420-1680 mgGAE/100g DW								374
	pulp homogenized in etanolic soluction	663.8 mgCAE/100g		IC_{50} 15.45 µg/mL			IC_{50} Acarbose 413.6 µg/mL extract 0.26 µL/mL			400
		517-908 mgCAE/100g FW		IC_{50} 212-317 µg/mL			IC_{50} Acarbose 413.6 µg/mL extract 66-212 µg/mL			401
		226.88 mgCAE/100mL		85.9 % DPPH inhibition			74.2 %			402
	juice	36.7 mgGAE/100mL					5 mg/mL inhibits 69.47 %			370
	pulp homogenized in metanolic soluction	4253 mgCAE/100g		20668 mM TE/100g		IC50 5.7 mg/mL reaction solution	IC50 1.15 mg/mL reaction solution			403
	leaves	19306 mgGAE/100g	2864 mgGAE/100g							91
Tange	oil								59.20 %	404

	oil			750-2000 mgTE/100mL	1550-3000 mgTE/100mL				378
	juices			24-68 % DPPH inhibition					74
	juices	92 mgGAE/100mL	11 mgGAE/100mL	64.53 % DPPH inhibition	0.98 % ABTS inhibition		IC_{50} 12 mg/1mL		405
	freeze dried			2.50 mMTE/100g DW	6.47 mMTE/100g DW				406
Lemon	-	4750 mg/100g		94.4% DPPH inhibition		87.3 %			407
	oil							24.79 %	404
	lyophilized juice							18 %	82
	oil			2000-2750 mgTE/100mL	600-1500 mgTE/100mL				378
	oil	1951-3197 mgGAE/100g	229-414 mgQE/100g	49.29-52.32 % DPPH inhibition		26 %	36 %		376
	hidrolysis of peels	488 mgGAE/100g DW				-11.6 %	100 %	100 %	384
	oil					IC_{50} Acarbose 7.45 µg/mL extract 8.16 µg/mL	IC_{50} Acarbose 8.44 µg/mL extract 7.56 µg/mL	IC_{50} 26.17 µg/mL	81
	juices			24-5-42.0 % DPPH inhibition					74
	young lemon shoots	8930 mgGAE/100g DW							408
	juices	103 mgGAE/100mL	14 mgGAE/100mL	42.28 % DPPH inhibition	0.72 % ABTS inhibition				405
	juices	222-236 mgGAE/100g	170-189 mgQE/100g	1558-1761 mMTE/100g					409

Tomato	pulp homogenized in metanolic soluction	48 mgGAE/100g							410
	lyophilized samples	2080-3360 mgGAE/100g							411
	locular gel and the serum	50-110 mgGAE/100g		210-380 mMTE/100g	350-700 mMTE/100g				203
	QuEChERS	52 mgGAE/100g	24 mgQE/100g	98 mMTE/100g	114 mMTE/100g				412
	lyophilized samples							IC_{50} 18.5-57.4 mg/mL	382
	hidrolysis of peels	365 mgGAE/100g				-37.7 %	42.5 %	100 %	384
	juice	192 mgGAE/100g					72 %		413
	lyophilized samples	784-1315 mgGAE/100g		IC_{50} 146-175 µg/mL					414
	freeze-dried frozen puree			664-1472 mMTE/100g	674-1650 mMTE/100g				415
	freeze-dried frozen puree							30-40 %	416
Uva-da-serra	myrtle oil							93.6 %	404
	blueberry juice	128 mgGAE/100mL			63 mMTE/100mL	IC_{50} 2.67 mg/mL	IC_{50} 40.68 mg/mL		417
		7.5-24.5 mgGAE/100mL	0.2-12.6						418
	blueberry pulp homogenized in metanolic soluction	185.6-929 mgGAE/100mL	39.5-64.6 mgQE/100mL						419

blueberry air dried, in ethanol, methanol and water extractions	1642-4442 mgGAE/100g DW	140-922 mgQE/100g DW	IC_{50} 141-263 µg/mL			IC_{50} Acarbose 31 µg/mL extract 301-591 µg/mL		420
lyophilized	885 mgGAE/100g DW	1467 mgRuE/100g DW		180 mMTE/100g	IC_{50} Acarbose 20 µg/mL extract 2630 µg/mL	IC_{50} Acarbose 2060 µg/mL extrat 1030 µg/mL	IC_{50} 9360 µg/mL	99
dehydrated at 105 °C	345-426 mgGAE/100g FW		11-12 mMTE/100g FW					98
blueberry water-based extraction	80-140 mgGAE/100g FW		70-80 % DPPH inhibition		50-90%	55-80%		421


UNIVERSIDADE da MADEIRA
CQM
CENTRO DE QUÍMICA
DA MADEIRA
OPERAÇÃO CQM+
FCT
Fundação para a Ciência e a Tecnologia
arditi
MADEIRA
14-20
PORTUGAL
2020
COMPETE
2020
UNIÃO EUROPEIA
Fundo Europeu
de Desenvolvimento Regional