Universidade de Aveiro Departamento de Química
2017

Ângelo Miguel Correia Salvador

Caracterização química e avaliação biológica da baga e flor de *Sambucus nigra* L. com vista à sua valorização

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica da Doutora Sílvia Maria da Rocha Simões Carriço, Professora Auxiliar do Departamento de Química da Universidade de Aveiro, e do Doutor Armando Jorge Domingues Silvestre, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro.

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“Nothing under the sun is greater than education. By educating one person and sending him into the society of his generation, we make a contribution extending a hundred generations to come.”

Jigoro Kano

Aos meus pais por sempre acreditarem em mim.

Em homenagem aos meus avós.

o júri

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Professora auxiliar da Escola Superior de Biotecnologia da Universidade Católica Portuguesa

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palavras-chave

Sambucus nigra L., baga e flor de sabugueiro, cultivar, amadurecimento, condições pós-colheita, metabolitos secundários, compostos de aroma, compostos bioativos, diabetes.

resumo

Atualmente, há um crescente interesse na exploração de recursos naturais como fontes de compostos bioativos com potenciais efeitos benéficos para a saúde. A flor e a baga de sabugueiro (*Sambucus nigra* L.) têm sido amplamente usadas na alimentação e na medicina tradicional e mais recentemente no desenvolvimento de nutracêuticos, ilustrando o potencial e interesse em investigar esta espécie. Diversos compostos bioativos são reportados nesta planta, nomeadamente compostos terpénicos voláteis, triterpénicos, esteróis e compostos fenólicos. Nesta perspetiva, o conhecimento da composição química da baga e flor de *S. nigra* L., os fatores que a afetam (cultivar, grau de maturação, ano de colheita, condições pós-colheita, entre outros) e potenciais efeitos biológicos é extremamente importante para incrementar o seu valor e aplicabilidade. Assim, o objetivo principal desta tese foi a caracterização química da flor e baga de *S. nigra* L. e a avaliação de efeitos biológicos de extratos de baga, com vista à sua valorização.

Inicialmente foi estudado o perfil de compostos com potencial impacto no aroma da flor fresca, nomeadamente, os compostos terpénicos voláteis e os norisoprenóides. Sendo as flores uma matriz sazonal e perecível, foi ainda avaliado o impacto de diferentes condições pós-colheita, nomeadamente condições de manuseamento e armazenamento (congelamento, liofilização, secagem ao ar e embalamento a vácuo). As bagas foram estudadas ao nível de: *i)* perfil volátil (compostos terpénicos e norisoprenóides); *ii)* fração lipofílica (extratáveis em diclorometano); e *iii)* fração polar (extratáveis em metanol acidificado). Foi, também avaliado o impacto de alguns parâmetros de pré-colheita, nomeadamente, a cultivar, o estado de maturação e o ano de colheita. Na fase final, foi realizado um ensaio *in vivo*, com ratos Wistar diabéticos tipo-2, cuja dieta rica em lípidos foi suplementada, durante 4 semanas, com o extrato polar e lipofílico de bagas. A realização prévia de ensaios de toxicidade, permitiu definir as doses a usar para cada tipo de extrato: 190 e 350 mg/kg de peso corporal, para os extratos lipofílico e polar, respetivamente.

De um total 47 compostos monoterpénicos, 13 sesquiterpenos e 5 norisoprenóides detetados na flor, 38 compostos são reportados pela primeira vez nesta espécie. Para tempos de armazenamento intermédios (24-32 semanas), o embalamento a vácuo e o congelamento mostraram ser os mais adequados para preservar o teor total destes compostos. No entanto, para períodos mais longos (52 semanas), o congelamento revelou ser o tratamento mais adequado. Dado que os óxidos de linalol, ao longo do tempo de armazenamento, apresentaram uma tendência de variação similar ao teor total de metabolitos em estudo, estes podem ser futuramente usados como marcadores para estimar o impacto dos tratamentos de pós-colheita.

A caracterização química da baga de sabugueiro permitiu identificar 103 compostos pertencentes às frações volátil, lipofílica e polar, de entre os quais, 59 são reportados pela primeira vez na baga de *S. nigra* L. Os monoterpenos representaram entre 66% e 77% dos compostos identificados na fração volátil, sendo o limoneno e o *p*-cimeno os compostos maioritários desta fração. Os compostos triterpénicos foram a família química mais abundante da fração lipofílica, representando até 94% do total de componentes lipofílicos identificados. O ácido ursólico, seguido do ácido oleanólico e da β-amirina são os compostos maioritários desta fração. Por último, as antocianinas foram os principais compostos fenólicos presentes na fração polar, com a cianidina 3-glucósideo e cianidina 3-sambubiósideo a representarem até 73% dos compostos fenólicos identificados.

Ao longo da maturação, verificou-se um comportamento similar entre as famílias químicas presentes na fração lipofílica e para as três cultivares estudadas ‘Sabugueiro’, ‘Sabugueira’ e ‘Bastardeira’: observou-se um aumento inicial do conteúdo de cada família entre o primeiro e segundo grau de maturação, seguido de uma diminuição sistemática até ao estado maduro. As cultivares 'Sabugueira' e 'Bastardeira' maduras apresentaram teores mais elevados de compostos lipofílicos e particularmente de ácidos triterpénicos (p<0,05), para os dois anos de amostragem. Verificou-se ainda que os estados não maduros apresentaram um teor global superior da fração volátil, que diminuiu gradualmente ao longo do amadurecimento, sendo este comportamento consistente para as três cultivares em estudo. O estado de amadurecimento foi o parâmetro que teve maior impacto nos compostos voláteis terpénicos e norisoprenóides, seguido do parâmetro cultivar.

A suplementação da dieta de ratos Wistar diabéticos com o extrato polar corrigiu a hiperglicemia ao reduzir os níveis glicémicos em jejum, enquanto que a suplementação com o extrato lipofílico reduziu a secreção de insulina. Além disso, ambos os extratos reduziram a resistência à insulina, sem alterações significativas nos índices hematológicos, nos níveis séricos lipídicos e na homeostase sérica e tissular de microelementos.

Em conclusão, com este trabalho de doutoramento foram reportados pela primeira vez vários compostos na baga e flor de *S. nigra* L., cujo perfil foi modulado por parâmetros de pré- e pós-colheita. O efeito anti-diabético promovido pela suplementação com extratos de baga de sabugueiro, revelou o seu potencial impacto no controlo, através da dieta, de uma patologia que atualmente afeta milhões de pessoas em todo o mundo.

keywords

Sambucus nigra L., elderberry and elderflower, cultivar, ripening, postharvest conditions, secondary metabolites, aroma compounds, bioactive compounds, diabetes.

abstract

Nowadays, there is a growing interest on the exploitation of natural resources as sources of bioactive compounds with potential health benefits. Elderberry plant (*Sambucus nigra* L.) and particularly elderflowers and elderberries have been widely used on foods and on folk medicine, and more recently on nutraceuticals, which illustrates the potential and interest to study this species. Several bioactive compounds are reported on this plant, namely volatile terpenic, triterpenic, sterol and phenolic compounds. In this perspective, the knowledge of the chemical composition from *S. nigra* L. berry and flower, the factors that affect it (cultivar, berry ripening, harvesting season, postharvest conditions, etc.) and their potential health benefits are extremely important, contributing to improve their value and applicability. Therefore, the main objective of this PhD thesis was the chemical characterization of *S. nigra* L. berries and flowers, and the biological evaluation of berries extracts in view of their valorization.

The profile of the potential aroma compounds from fresh elderflowers, namely, volatile terpenic and norisoprenoids, was firstly established. Given that elderflowers are a seasonal and perishable matrix, the impact of different handling and storage conditions (freezing, freeze-drying, air drying and vacuum packing) was also assessed. Elderberries chemical profiling was studied on three groups: *i)* volatile profile (terpenic and norisoprenoids); *ii)* lipophilic fraction (dichloromethane extractives); and *iii)* polar fraction (acidified methanol extractives). The impact of pre-harvest parameters, namely cultivar, ripening stage and harvesting season, was also assessed. Finally, an *in vivo* assay was performed on type-2 diabetic Wistar rats, in which their high fat diet regimen was supplemented, during 4 weeks, with a polar and a lipophilic elderberries extract. Previous toxicity assays were employed, to define the administrated doses for each type of extract: 190 and 350 mg/kg body weight, of lipophilic and polar extract, respectively.

From 47 monoterpenic, 13 sesquiterpenes and 5 norisoprenoids detected elderflower components, 38 were reported for the first time on the flowers from *S. nigra* L. For intermediary storage times (24-32 weeks), vacuum packing and freezing seemed to be the most suitable methods to preserve the overall levels of the target metabolites. However, for a longer period (52 weeks), freezing revealed to be the most suitable method. Over the storage period, linalool oxides showed a tendency of variation similar to the overall metabolites content, thus, they were proposed as markers to estimate the impact of postharvest treatments.

The *S. nigra* L. berries chemical characterization revealed 103 components belonging to the volatile, lipophilic and polar fractions, being 59 reported for the first time in this matrix. Monoterpenes represented from 66% to 77% of the identified compounds from the volatile fraction, being limonene and *p*-cymene the major ones. Triterpenic compounds were the most abundant components of the lipophilic fraction, accounting for up to 94% of the total identified lipophilic components. Ursolic acid prevailed amongst triterpenic components, followed by oleanolic acid and β-amyrin. Anthocyanins were the major phenolic compounds present on the polar fraction, with cyanidin 3-glucoside and cyanidin 3-sambubioside representing up to 73% of the identified phenolic compounds.

Over ripening, a similar profile of the studied chemical families from the lipophilic fraction was found for the three studied cultivars 'Sabugueiro', 'Sabugueira', 'Bastardeira': an initial increasing of their content was observed (between the first and second ripening stage) followed by a decreasing until maturity. Regarding mature elderberries, 'Sabugueira' and 'Bastardeira' showed higher contents of lipophilics, and particularly of triterpenic acids ($p<0.05$), for the two harvesting periods. Similarly, for the three studied cultivars, the unripe elderberries stages had a higher overall content of volatile terpenic and norisoprenoids, which decreased over the ripening stages. Ripening stage was the parameter with higher impact on the volatile terpenic and norisoprenoids compounds, followed by the cultivar.

Diet supplementation of type-2 diabetic Wistar rats with the polar extract corrected hyperglycemia by reducing fasting blood glucose, while lipophilic extract supplementation lowered insulin secretion. Moreover, both extracts supplementation lowered insulin resistance, without remarkable alterations on hematological indices, sera lipids and on sera and tissular trace elements homeostasis.

In conclusion, with this PhD work several compounds were reported for the first time on *S. nigra* L. berries and flowers, whose profile was modulated by both, pre- and postharvest parameters. The anti-diabetic effect promoted by diet supplementation with elderberries extracts reveals their potential impact on management, through the diet, of a pathology that currently affects millions of people around the world.

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ABBREVIATIONS AND SYMBOLS

General abbreviations

^{1}D	First-dimension column
1D	One dimensional
$^{1}t_{R}$	Retention times for first dimension
^{2}D	Second-dimension column
2D	Two dimensional
$^{2}t_{R}$	Retention times for second dimension
ABTS	2,2´-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	One-way analysis of variance
AP1	Activator protein-1
ASCA	Analysis of Variance – Simultaneous Component Analysis
AST	Aspartate aminotransferase
bm	Body mass
bw	body weight
CFU	Colony-forming units
COX	Cyclooxygenase
CRP	C-reactive protein
CUPRAC	Cupric reducing antioxidant capacity
DAD	Diode array detector
DB	Diabetes
DBLE	Diabetic rats supplemented with lipophilic extract
DBPE	Diabetic rats supplemented with phenolic extract
DBNS	Diabetic rats, not supplemented
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DRV	Dietary reference values
DTIC	Deconvoluted total ion current

DVB/CAR/PDMS	50/30 μm Divinylbenzene/carboxen™/poly(dimethylsiloxane) StableFlex™
DW	Dry weight
EFSA	European Food Safety Authority
EI	Electron impact ionization
ESI	Electrospray ionization
FA	Fatty acids
FABP	Fatty-acid-binding proteins
FRAP	Ferric reducing antioxidant power
fw	Fresh weight
GC	Gas chromatography
GC×GC	Comprehensive two-dimensional gas chromatography
GC×GC-ToFMS	Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry analyzer
GC-MS	Gas chromatography coupled to mass spectrometry
GI	Gastrointestinal
HCA	Hierarchical Clusters Analysis
HF	High-fat diet
HGB	Blood hemoglobin
HOMA-IR	Insulin resistance index
HOMA-β	β Cells function index
HPLC	High-performance liquid chromatography
HS-SPME	Headspace solid-phase microextraction
I.D.	Internal diameter
IDDM	Insulin dependent diabetes mellitus
IKK	Inhibitory protein of κB kinase complex
IL	Interleukin
IRS	Insulin receptor substrate
JNK	c-Jun amino-terminal kinase
LCAA	Long-chain aliphatic alcohols
LC-MS	Liquid chromatography coupled to mass spectrometry
LDL	Low-density lipoprotein
LOQ	Limit of quantification
LXR	Liver X receptor

LYMPH	Lymphocytes
m/z	Mass-to-charge ratio
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MeOH	Methanol
MONO	Monocytes
MPV	Mean platelet volume
MS	Mass spectrometry
MSI	Metabolomics standards initiative
MS^n	Tandem MS
NDB	Non diabetic rats
NF-κB	Nuclear factor-κB
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorption capacity
PCA	Principal component analysis
PDW	Platelet distribution width
PERK	Pancreatic endoplasmic-reticulum kinase
p-LCR	Platelet large-cell ratio
PLT	Platelets
PPAR	Peroxisome-proliferator activated receptor
RBC	Red blood cell count
RDW-SD	Red cell distribution width based on standard deviation
RI	Retention index
Ricalc	Calculated retention index
RI_{lit}	Retention index from literature of the same GC column or equivalents
RLU	Relative light units
ROS	Reactive oxygen species
RSD	Relative standard deviation
ST	Sterols
STZ	Streptozotocin
T2D	Type 2 diabetes
TA	Titratable acidity

TAC	Total anthocyanin content
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TIC	Total ion chromatograms
TMS	Trimethylsilyl
TNF	Tumor necrosis factor
TPC	Total phenolic content
tr	traces
TSS	Total soluble solids content
TT	Triterpenoids
UHPLC	Ultra high-performance liquid chromatography
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
WBC	White blood cell count
WHO	World Health Organization
λmax	Wavelength for which a compound has a maximum ultraviolet absorbance

AIMS AND OUTLINE OF THIS THESIS

1.1. Context

In recent years there has been a growing interest on the exploitation of natural products as sources of bioactive compounds with potential health benefits, notably for humans, and particularly in the consumption of herbal products that are able to prevent or even ameliorate chronic diseases, and particularly of those with growing incidence in the advent of the 21st century, namely cardiovascular, cancer, diabetes and mental disorders [1].

Globally, and more recently in Portugal, there has been increasing interest in the elderberry plant (*Sambucus nigra* L.) due to its economic interest as raw material for the production of food and nutraceutical products derived from berries and flowers. In South Douro region, the production of elderberry reaches about 2500 tons/year, being mainly exported (refrigerated) for the production of juices and food colorants. The collection of flowers represent about 0.6 tons/year.

S. nigra flowers and berries have been widely used on folk medicine for numerous applications that include antimicrobial, anti-viral, antioxidant, anti-inflammatory, anti-cancer, antidiabetic, among others [2–4]. The detailed knowledge of the chemical composition of *S. nigra* is extremely important to understand its biological effects but also to improve its value and applicability. *S. nigra* flowers and berries received increased attention due to presence of phytochemicals with many reported health benefits, comprising monoterpenic, sesquiterpenic and triterpenic compounds, sterols and phenolic compounds, although more studies are needed to in-depth establish their profiles. Furthermore, different parameters, as cultivar, berry ripening stage, harvesting season, postharvest conditions, that might affect their chemical profile are still poorly explored and understood. Taking into account the increasing interest on the production of elderberries and flowers, a detailed chemical characterization of their bioactive components is still missing, along with the impact of different parameters like postharvest conditions, cultivar and ripening. This knowledge would open several possibilities for their valuation providing a significant scientific knowledge which will enable further development of value-added products from elderberry plant.

Despite several potential applications for *S. nigra*-based preparations, prevention and or amelioration of diabetes mellitus may represent a future relevant issue. In fact this disease is an increasing problem in modern society as clearly highlighted by World Health Organization [5], which demonstrates the need to develop preventive and therapeutic alternatives that, when supplemented on a diet may reduce the incidence of this public health concern.

1.2. Objectives and outline of the thesis

The main objective of this PhD thesis is the chemical characterization of *Sambucus nigra* L. berries and flowers, and the biological evaluation of elderberry extracts, in view of their valorization. In this context, different specific objectives were drawn:

- ✓ To establish the volatile terpenic and norisoprenoids metabolites profile from two elderflowers cultivars by employing comprehensive two dimensional gas chromatography-mass spectrometry and evaluate the impact of different postharvest conditions;
- ✓ To establish the lipophilic (dichloromethane extractives) profile from three elderberries cultivars by employing gas chromatography-mass spectrometry and evaluate the effect of ripening, cultivar and harvesting season parameters;
- ✓ To establish a metabolomic-based strategy for fingerprinting of elderberries volatile terpenic and norisoprenoids from three cultivars through ripening using comprehensive two dimensional gas chromatography-mass spectrometry;
- ✓ To evaluate the effect of the elderberry extracts supplementation on high fat fed diabetic rats.

The present PhD thesis is organized in six chapters, according to the organizational sequence illustrated in Figure 1.

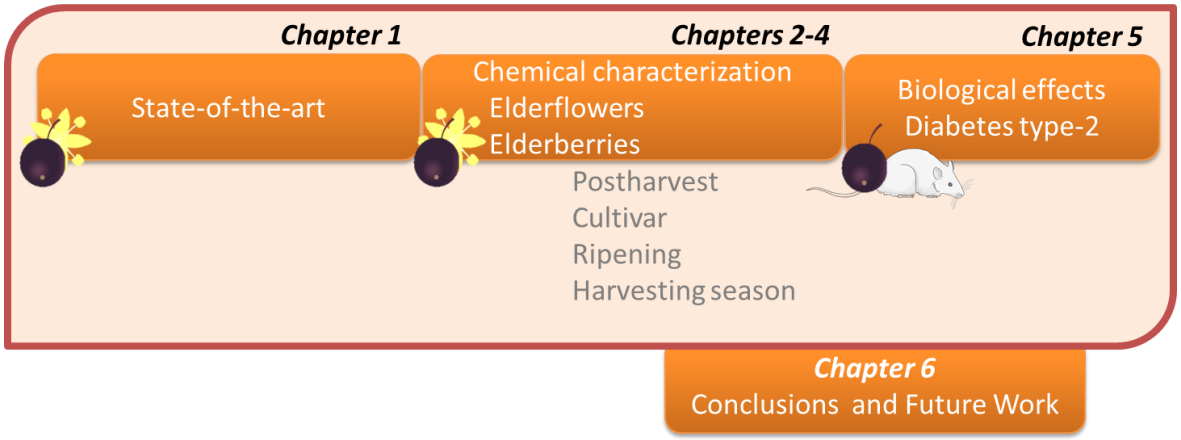


Figure 1. PhD thesis workflow: from the state-of-the-art to *S. nigra* L. flowers and berries chemical characterization and biological effects.

Chapter 1 reviews the most relevant literature data from *S. nigra* plant, namely its taxonomic classification, geographic distribution and applications, followed by a detailed revision of the chemical composition of the elderberries and elderflowers, focusing on terpenic, sterol and phenolic compounds, as well as the methodologies used for their characterization. The potential health benefits of elderflowers and elderberries and their relationship with chemical composition, with emphasis on diabetes type 2 are also discussed.

Chapters 2 to **5** describe the progresses beyond the state of the art in this PhD thesis. **Chapter 2** describes the research conducted on elderflower plant organ, while the remaining ones are related to elderberry research. The rationale underlying the chapters organization was to follow the plant phenology and also, as elderflowers are a very perishable matrix and therefore it was decided to study first their chemical profile and how different postharvest conditions affect it; the following chapters performed the elderberry chemical profiling and evaluated the effect of different parameters (**Chapters 3**-**4**), and on **Chapters 5**, the effect of elderberry extracts supplementation on the diet of diabetic rats was assessed.

Chapter 2 establishes the released volatile terpenic and norisoprenoid profile from two elderflowers cultivars using comprehensive two-dimensional gas chromatography. Due to the interest of this matrix as flavoring agent the impact of different postharvest conditions over the target chemical families, was assessed up to one storage year.

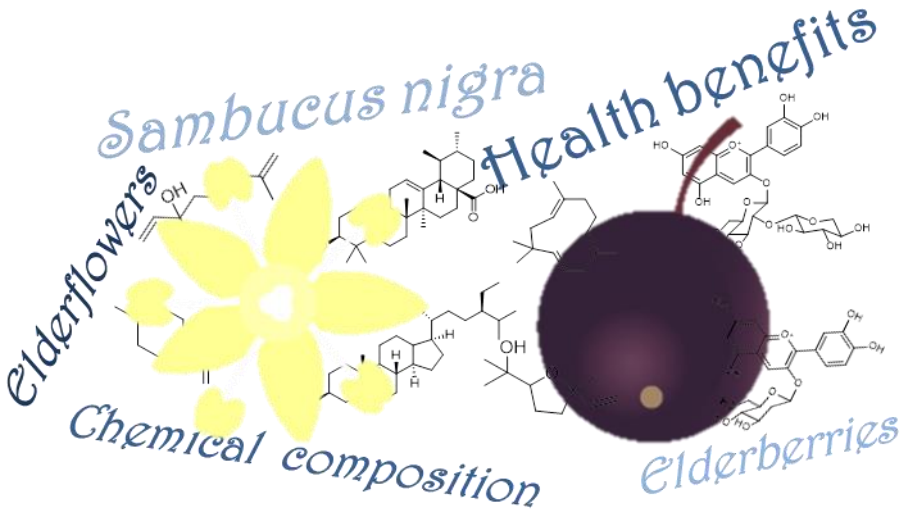
Chapter 3 discusses the elderberry lipophilic profile by gas chromatography-mass spectrometry. The lipophilic profile is established on three ripe elderberries cultivars, as a case study, and then, the influence of ripening, cultivar and harvesting season parameters over the target phytochemicals was assessed.

Chapter 4 sets up a metabolomic-based strategy for fingerprinting volatile terpenoids and norisoprenoids from *S. nigra* berries from three cultivars, through ripening by using comprehensive two-dimensional chromatography and multivariate statistical analysis.

Chapter 5 evaluates the effect of the supplementation of polar (acidified methanol extractives) and lipophilic (dichloromethane extractives) elderberry extracts on induced diabetic rats fed with a high fat diet. Firstly, the polar extract was characterized by ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-DAD-ESI-MS^n). *In vitro* toxicity assay was performed on both extracts. Then, the effects of dietary extracts supplementation were evaluated through the analysis of blood sera hematological and biochemical indices, and blood sera and tissular mineral status.

Chapter 6 highlights the main conclusions of this PhD thesis, as well some insights into future research in this domain.

CHAPTER 1. Bibliographic Revision



Adapted from:

ÂC Salvador, SM Rocha, AJD Silvestre, *Sambucus nigra* L.: A potential source of health-promoting components, Frontiers in Natural Product Chemistry, 2016, Vol. 2, Cap. 6, pp. 3-49.

1.1. General considerations about *Sambucus nigra* L.

Sambucus nigra L. is a plant whose berries and flowers are widely used on diverse formulations that range from food products to formulations that are applied on folk medicine, and more recently on supplements and nutraceuticals. The current market trends on agro-industries follow the consumers' desire for added value products, convenience, and foods which assist in disease prevention and healthy ageing, in response to the social and lifestyle changes [6,7]. These trends, allied with the fact that people can optimize the health-promoting capabilities of their diet though the consumption of foods that have been formulated or fortified to include health-promoting factors or by supplementation [6], reinforce the interest on studying plants that might contribute to the health status of an individual. Despite the interest of several macro and micronutrients, in the last decades, *S. nigra* flowers and berries secondary metabolites composition received increased attention due to presence of phytochemicals, with many health benefits that include monoterpenic, sesquiterpenic and triterpenic compounds, sterols and phenolic compounds. Considering that a wide range of biological properties have been reported for these secondary metabolites in other matrices or using standards, this chapter unveils the high interest in systematizing the information available related to *S. nigra* secondary metabolites composition, both in terms of their potential health benefits but also of their role in the plant metabolism and protection.

Among secondary metabolites the key families worth of consideration in the present context are mono and sesquiterpenes, sterols, triterpenes and phenolic compounds. Mono and sesquiterpenes play an important role in plant defense mechanisms being often reported as toxic to some microorganisms and are also involved in plant-insect interactions, such as pheromones, attractants and as feeding deterrents [8]. These compounds are also involved in the plant protection against oxidative stress, namely as thermo-tolerance mediators [9]. Beyond their protection and communication roles in plants, and their pleasant aroma properties, diverse potential health benefits are documented, namely their hepatoprotection [10], anti-inflammatory [11], analgesic [12] and antioxidant [13] properties. Sterols contribute to the regulation of the fluidity and permeability of cell membranes in plant cells. They are also substrates for the synthesis of numerous secondary plant metabolites and act as biogenic precursors of other compounds involved in plant growth [14]. Plant sterols are well known, since 1953, for their role in lowering the absorption of the low-density lipoprotein-cholesterol (LDL-cholesterol) in humans, which led to the development of several food supplements and nutraceuticals [15]. Triterpenic compounds have been described as plant defense agents against pathogens and herbivores [16,17], and exhibit anti-inflammatory, antimicrobial [18] and hepatoprotective potential [19], among others [20]. Finally, plant phenolic compounds have interested scientists for decades, as, on the one hand they are essential to plant

physiology, contributing e.g. to plant pigmentation, and they are also involved in growth, reproduction and provide plants resistance for pathogens and predators [21]. On the other hand, phenolic compounds exhibit a wide range of health benefits [22], such as anti-allergenic, anti-artherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic and cardioprotective effects. Within phenolic compounds, phenolic acids act as signaling molecules in plant-microbe symbioses, and are involved in the plant defense mechanisms when exposed to biotic and abiotic stress [23]. Regarding to their effects in human health, phenolic acids are often linked to potential antioxidant and anti-cancer activities [24]. On the other hand, flavonoids are involved on electron and proton transport, ion exchange and membrane potential, radicals formation; as regulators (inhibitors or activators in enzyme reactions) [25]; protection of the plant against the harmful effects of oxidative processes [24]; plant-microorganism communication; and attractants [24]. The potential health benefits of flavonoids are vast, although the interest is mainly associated with their antioxidant and anti-carcinogenic activities [24]. These compounds may also play important roles in the control of other human diseases, such as, among other, diabetes and cardiovascular diseases [24], being their activity deeply dependent of their structural features [26].

This chapter aims at giving a general perspective of *S. nigra* L. berries and flowers potential as sources of health-promoting compounds. This will cover aspects that range from its taxonomic classification and geographic distribution, current applications, a detailed overview of berries and flowers chemical composition, mainly focused on sterols, terpenic and phenolic components, and important factors that have impact on their biosynthesis and preservation. Finally, the main findings regarding their potential health benefits with special focus on diabetes mellitus and related complications.

1.2. *Sambucus nigra* taxonomic classification and geographic distribution

Elderberries belong to genus *Sambucus* being often included with the Caprifoliaceae family, but more recently, studies associate this plant to the Adoxaceae family [27,28]. Since no definitive taxonomic DNA-based studies have been conducted, and because species of this genus are difficult to delimit based solely on morphological characteristics, no clear consensus has been reached about the number of species it contains, ranging from 9 to 40 depending on the authors [28], being usual to find inconsistency in delimiting species, subspecies, and cultivars in regional floras. Several subspecies are described on Elder family, being the most common the American elder [*S. nigra* sbsp. *canadensis* (L.) R. Bolli] and black elder or European elder [*S. nigra* sbsp. *nigra* (L.) R. Bolli], being formerly used on literature as *S. canadensis* and *S. nigra*, respectively [29]. *S. nigra* L. is a deciduous multi-stemmed shrub where their suckering is made from the roots and branching

from the base of the main stems force the plant to form dense thickets. Elder shrub can reach up to 9 m in height, with average height values of 2-3 m (Figure 1.1A), and contains large leaves (5–15 cm long) with 5 to 11 leaflets bearing sharply serrated margins [28–31].

Usually, elderberry leaves emerge in February or March, and the shrubs blooms around May-June, depending on the geographical location and cultivar. The plant generates creamy-white flowers forming terminal clusters 10-25 cm across in flat, umbrella-like umbels (Figure 1.1B) [30]. Each flower has five petals, five stamens, and one inferior ovary [32]. Wind rather than insects is the main vector for elderflowers pollen distribution [29].

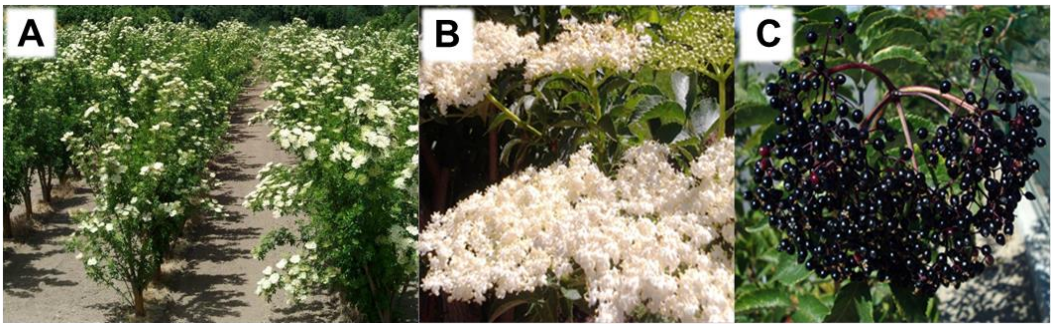


Figure 1.1. Elder shrub (A), elderflowers (B) and elderberries (C) from plantation in Dalvares, Tarouca, Portugal.

Berry ripening process happens in a 1 to 2 month period starting from a green color and when ripe they became deep purple [28]. At ripe stage, a single cluster contains hundreds of deep purple, almost black berries (Figure 1.1C). Individual berries range from 5.0 to 6.5 mm in diameter, and contain 3 to 5 oblong tan to yellowish one-seeded stones. Several cultivars from *S. nigra* are currently being explored for cultivation, with the most common ones being listed in Table 1.1.

Table 1.1. Common European elder (*S. nigra* L.) cultivars [33–41].

Cultivar	Country
Baalsta, Blute, DDR, Haschberg, Rubim, Rubini, Tattim	Austria
Albida, Allesö, Aurea, Bohatka, Dana, Haschberg, Korsör, Mammut, Pregarten, Riese aus Voßloch, Sambo, Sampo, Sambu, Sam dal, Samyl, Weihenstephan	Czech Republic
Allesoe, Blangstedgaard, Donau, Dresden, Esther, Finn Sam, Gentofte, Haschberg, Helene, Hellerup, Holstenshus, Hush, Jaegerspris, Jeberg, Karlskrona, Korsor, Mammut, Morsoe, Sambu, Samdal, Samidan, Samocco, Samyl, Sampo, Tvaerg Visby, Virum, Visby	Denmark
Road Due	England
Alleso, Kort, Dosw, Sampo, Samyl, Forma D Kortowo	Poland
Bastardeira, Sabugueira, Sabugueiro	Portugal
Baalsta	Sweden

S. nigra is classified as European temperate, being the ideal growth conditions for maximum yield and quality, in full sun or partially shaded exposures and in moist soils [42]. Plant growth occurs mainly throughout western Europe being the northern limit approximately 66° N [28], while the eastern limit of its distribution is approximately 55° E, and the limit of southern Europe differs, as illustrated in Figure 1.2.

Figure 1.2. Native distribution of *S. nigra* in Europe (dark shaded areas), adapted with permission [30].

The latitudinal and altitudinal distribution limits usually correspond to a mean October temperature below 7.2 °C, which may indicate that the seeds are unable to mature [30]. This was corroborated as *S. nigra* did not set seed on the southern limit in Europe and North Africa that corresponds approximately to a mean October temperature of 15 °C. *S. nigra* is not found at high altitudes (e.g. above 1500 m in the Alps). This species has been introduced into various parts of the world including Eastern Asia, North America, New Zealand and the southern part of Australia.

1.3. *S. nigra* applications – from food products to traditional medicine

Elder plant was used for centuries, as far back as the Ancient Rome for a broad range of applications, namely musical instruments, like flutes, whistles, or textile artifacts and dyes and even used on ritual ceremonies [29]. Roughly all parts of the plant (flowers, berries, leaves and sometimes also inner bark) have been known for their medicinal properties from the days of Hippocrates, being considered the medicinal “chest” of domestic medicine [43]. Leaves and inner bark have been used for their purgative, diuretic, laxative, expectorant and diaphoretic action [29]. Elderflowers and elderberries have long been used to alleviate or cure various illnesses in European

countries folk medicine. Elderflowers, for instance, were used to treat bronchial and pulmonary diseases, tumors and ulcers [30]. Their infusions have been reported as having soothing and laxative properties and were used to "sweat out fever" [44], for asthma treatment [45], and to treat toothache, colic, cold and rheumatic conditions [46]. Their decoctions are used for cold treatment [47], as well as, to treat bronchitis, whooping cough, asthma, hemorrhoids, as expectorant [45], insect bites and fever [46]. Elderflower traditional medicinal use for the relief of early symptoms of common cold has been found to fulfil the requirement of medicinal use for at least 30 years according to the Committee on Herbal Medicinal Products [3]. As reviewed by the European Medicines Agency [2], dried elderberries were traditionally used as a decoction to act as laxative, and elderberry juice or syrup were used as a diaphoretic. Elderberry decoction was also documented for fever reduction [44], while their infusions were consumed as an anti-rheumatic and to treat colic in children [28]. Their juice has also been used to treat sciatica, headache, dental pain, heart pain, nerve pain, while the syrup was recommend to treat coughs and colds [2].

Currently, food and beverage derived products followed by nutraceuticals are the main uses of elderberries and flowers [28,48,49]. Numerous products containing elderberry juice, pureed or dried elderberries, such as extracts, syrups, supplements, pies, ice creams, jellies, juices, beverages, beers, wines, liqueurs, fruit bars and coloring agents are currently available throughout the world [48–50]. The consumption of elderberry derived products is mainly associated with their high content in phenolic compounds, with remarkable antioxidant capacity compared to other red fruit juice concentrates [51]. The flowers are commonly used as ingredients for pastry products like pancakes, muffins, or waffles [28]. Flowers are also soaked in water and sugar with citrus juices to make a non-alcoholic cordial or, when fermented, a pale yellow wine can be produced. Infusions can also be prepared from the flowers [28,30]. It is worth mentioning that in England elderflowers are considered important wild plant resources, commercially exploited mainly for beverages production (e.g. flavored sparkling water and wine) [52].

1.4. Strategies for plant metabolites characterization

It is estimated that more than 200,000 metabolites exist in the plant kingdom [53], being present in a wide concentration and dynamic range, representing a huge chemical diversity, and complexity of natural plant-related samples [54]. Because of this complexity, there is no single methodology that can detect all metabolites present on a single organism. Historically, the metabolites identification/quantification was achieved either by spectrophotometric assays, capable of detecting single metabolites, or by simple chromatographic separation of mixtures of low complexity. More recently, several methods are able to perform the analysis of complex samples

with relevant accuracy and sensitivity, which include gas and liquid chromatography, capillary electrophoresis, often coupled with mass spectrometry, nuclear magnetic resonance, ultraviolet detection, photodiode array detection, fluorescence detection, refractive index, evaporative light scattering detection, etc. [55,56].

Prior to extraction and analysis of the target chemical components, sample harvesting and preparation has to be performed. The suitable sample preparation techniques for the extraction, clean-up, and pre-concentration of analytes from different matrices can effectively reduce matrix effects which has significant impact on the accuracy, precision and robustness of analytical methods. Common extraction methodologies include Soxhlet extraction, solid-phase extraction (SPE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), pressurized-liquid extraction (PLE), among others [56,57].

Aiming to identify and quantify a wide range of metabolites present on vegetable matrices, comprehensive approaches are employed, that include a set of different methodologies. The strategy used in the present PhD thesis for plant metabolites characterization is illustrated on Figure 1.3.

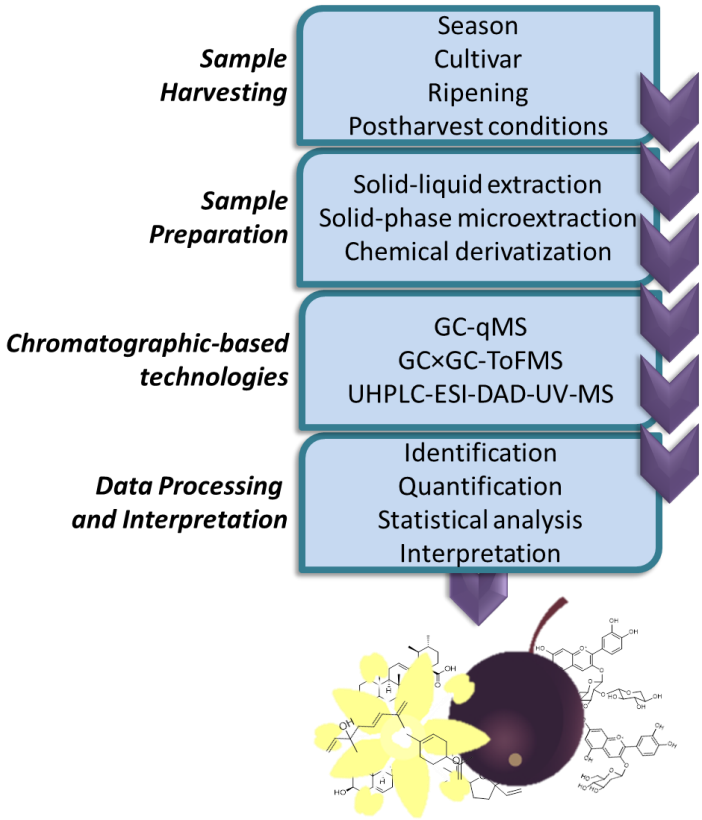


Figure 1.3. Strategy used in the present PhD thesis for plant metabolites characterization.

Four main steps are considered (often in iterative loops at one or more of these stages [58]):

- ✓ **Sample Harvesting** and **storage** (section 1.4.1);
- ✓ **Sample Preparation** comprises extraction of plant metabolites and chemical derivatization if used (section 1.4.2);
- ✓ **Chromatographic separation and detection** (section 1.4.3);
- ✓ **Data processing and interpretation** (section 1.4.4).

Plants chemical composition is likely to be altered during different physiological, edaphoclimatic and agricultural conditions, as well as with different genetic backgrounds. Comprehensive studies have been performed, aiming to characterize and follow the dynamic changes of the metabolites, which fall in the rational of the metabolomics studies. Metabolomics is the study of the low molecular metabolites (<1500 Da) in a biological sample, illustrating the physiological state of an organism or its organs, tissues, or cells [59]. It allows a comprehensive profiling of cellular metabolites at the systems level, thereby providing a direct readout of biochemical activity that can be correlated with the phenotype of an organism and its response to intrinsic or environmental changes [60].

Taking into consideration the goals of metabolomic studies, there are still challenges to solve in this field, for instance, the sample size; the multiple sources of variability of the samples, analytical methods, reagents, etc.; the chemical diversity and wide dynamic concentration range of small metabolites; the lack of analytical standards; the analysis time, among others [61].

Depending on the objectives of a given study, different strategies can be employed on metabolomics studies, namely metabolic fingerprinting, metabolic profiling, target analysis [62], among others. Accordingly to Hall [62], these concepts are defined as:

- ✓ **Metabolic fingerprinting**: a high throughput screening of the metabolic composition of the samples under study, aiming to compare and discriminate them. Usually, no attempt is initially made to identify the metabolites, as the bottom line of metabolic fingerprinting is to obtain enough information to unravel metabolic alterations;
- ✓ **Metabolic profiling**: identification and quantification of the metabolites present in an organism. This approach is often used for a limited number of components, due to practical limitations and are generally chosen on the basis of discriminant analysis or molecular relationships based upon molecular pathways/networks;
- ✓ **Target analysis**: is performed with higher detail on selected groups of metabolites by using optimized extraction and dedicated separation/detection techniques.

Metabolomics covers diverse application fields, namely on the medicine, plant and food sciences [63]. In the plant field, metabolomics face the challenge of understanding plants science biochemical complexity and to the effects of plant development, age, environment etc. [60]. In the

food sciences field, metabolomics aims, among others, to understand the molecular origins of the sensorial aspects of foods, namely aroma; supervise the production / processing of food products; evaluate processes of degradation and adulteration (quality control); to identify bioactive ingredients and their relation to health; to study the metabolites derived from the digestion of foods, their absorption in the gut, and biotransformation by the host tissues and the microbiota [64–66]. The integration of plant and food metabolomics can provide important insights into the biochemical mechanisms in response to developmental stages or different cultivars [67,68], as well as to study the impact of different processing/postharvest and storage conditions. This illustrates the interest to conduct these studies on *S. nigra* species. No metabolomics studies were conducted so far on *S. nigra* plant, as far as we know.

1.4.1. Sample harvesting and storage

Sample harvesting, the separation process to remove the required plant tissue from the original plant, is a key feature that deeply influences the chemical profile of a plant and related products. Different parameters are involved on the modulation of the plant chemical composition, namely environmental factors as harvesting time (day/night, season), light conditions, temperature, developmental stage of the plant or plant cells, the type of harvested tissue/plant organs, and genetic factors have to be considered [60]. The harvested samples must be representative of the population under study, being preferred biological replicates instead of technical replicates [69]. Storage time and conditions play a critical role to avoid the degradation and modification of metabolites in the sample. Plant tissues or organs are often stored at low temperatures or freeze-dried before analysis, to reduce the metabolic activity, namely enzymatic processes [60].

1.4.2. Sample preparation

The sample preparation step is crucial for the relevance of analytical results, and this step must be carefully planned in order to preserve the extracted compounds and to identify potential sources of experimental variability and errors [70]. Sampling and/or extraction methods are dependent on the type of sample and/or physicochemical properties of the analytes. Sample preparation for plant chemical characterization's studies include different stages as already reviewed [54]:

- ✓ **Extraction** (discussed in detail in the following subsections);
- ✓ **Pre-analytical sample preparation**, as chemical derivatization;

Extraction of plant metabolites has been used since ancient civilizations; in fact Egyptians, Phoenicians, Jews, Arabs, Indians, Chinese, Greeks and Romans, and even Mayas and Aztecs, used extraction processes (maceration, alembic distillation, etc.) to produce perfumes, medicines or foods [71]. Extraction step aims to transfer the analytes to a phase more compatible with the analytical technique, concentrate the compounds if these are present at trace levels in the original matrix and to eliminate undesirable interferences [72]. Nowadays, modern sampling approaches run towards automatization, miniaturization, easy manipulation, strong reduction or absence of organic toxic solvents, and low sample-volume requirements [72,73], although conventional methods are still the reference extraction procedures due to its simplicity, cost, and also the availability of literature data for comparative purposes. Different factors affect the extraction process, namely matrix properties, solvent and solvent/sample ratio, temperature, pressure and time [57]. Within the wide range of possibilities regarding the extraction techniques, only the methodologies employed in the present PhD thesis will be discussed, i.e., solid-liquid extraction (maceration and Soxhlet extraction) and solid-phase microextraction, which are resorted on methodologies often employed at our laboratory [74–76].

1.4.2.1. Solid-liquid extraction

Solid-liquid extraction is one of the oldest and commonly used extraction techniques. Its principle falls on the combination of the solid sample with a solvent in which the solute is soluble [77]. The criteria to select a specific solid-liquid extraction technique should take into consideration different variables, namely solvent nature, temperature, sample granulometry, partition coefficient and liquid-to-solid ratio [77,78]. In this PhD thesis, room temperature solid-liquid extraction and Soxhlet extraction were selected, for the isolation of phenolic and lipophilic fractions respectively, due to their simplicity, efficiency and due to the fact that these techniques are widely used on the extraction of metabolites from solid samples.

The employment of solid-liquid extraction, also called maceration, resorts on placing the plant material (often powdered) in contact with the solvent for a specific time that could range from few minutes to several days. Parameters like solid / liquid ratio, solvent type, among others, are usually explored in this method, in order to increase extraction efficiency and selectivity. This process can be performed at room temperature or at higher temperatures to enhance the extraction efficiency. This technique is one of the most commonly used on the extraction of phenolic compounds, including from *S. nigra* [35,79], in which solvents like acidified methanol (with HCl, acetic acid or formic acid) are used at room temperature [35,79,80].

Soxhlet extraction is solid-liquid extraction method suitable for non-thermolabile components. Soxhlet extractor was first proposed by German chemist Franz Ritter Von Soxhlet

(1879), being initially designed mainly for extraction of lipids but nowadays it is well-established technique, used for the extraction diverse plant components, as lipids, sterols and pentacyclic triterpenes [81,82], among others. The Soxhlet extractor system, has three main components, the top part is a solvent vapor reflux condenser; the middle part, where the sample is loaded, is a thimble holder with a siphon device and a side tube; and, at the bottom, a round-bottomed flask that is connected to the thimble holder [57,83]. The solvent vapor passes through the side tube and goes to the reflux condenser, where it condenses and drips back to the thimble chamber. When the condensed solvent fills the thimble holder and reaches the top of the siphon of the return tube, it is drained back into the flask, carrying the extractable compounds into the solvent reservoir [57,83]. This cycle repeats as many times as necessary to reach maximum extraction efficiency of analytes. Since the extracted analytes have higher boiling points than the extraction solvent, they accumulate in the flask while the solvent recirculates [83]. Sequential extraction using solvents with increasing polarities is often used to obtain different extracts with different chemical profiles.

Despite being simple and the inexpensive extraction apparatus, the long extraction time (6-48 h) and the possibility of degradation of thermolabile compounds are pointed out as a disadvantage of Soxhlet extraction methodology [83]. Comparing maceration with Soxhlet extraction, the latter usually requires a smaller quantity of solvent [84].

Finally, as toxic and large volumes of solvents are often used these extraction techniques do not fall on the principles of green analytical chemistry [72].

1.4.2.2. Solid-phase microextraction (SPME)

SPME is a solvent-free sample preparation technique based on sorption (absorption and/or adsorption), being used for simultaneous sampling, extraction, and concentration of the analytes [78]. This technique was first developed by Pawliszyn *et al.* [85], being initially applied for the analysis of water pollutants. SPME does not provide an exhaustive extraction of analytes, instead, only a small fraction of those is extracted, yet being representative of the overall composition if experimental conditions (fiber coating, temperature and extraction time, among others) are properly optimized and controlled.

SPME systems are available in different geometries, but the most common is a syringe (Figure 1.4) wherein the needle has a fused silica base coated with a thin layer of a stationary phase (among different ones commercially available). Those coatings have different polarities, film thicknesses and types of interaction with the analyte (absorption and adsorption), and their choice depends on the physicochemical properties of the analytes and the sample physicochemical properties [85].

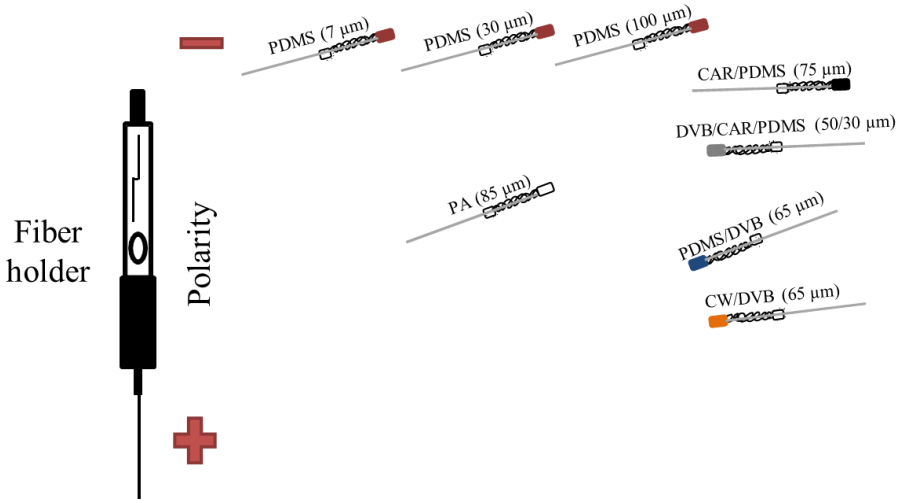


Figure 1.4. Common commercially available SPME fibers adapted from Kataoka et al. [85]**.**

The nonpolar polydimethylsiloxane (PDMS) fiber is suitable for nonpolar volatile compounds (Figure 1.4), and depending on its film thickness, the analytes can be more or less retained; polyacrylate (PA) fiber is preferred for the extraction of more-polar analytes (Figure 1.4). The mixed coating fibers, like divinilbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (the one used in this PhD thesis), has a high analyte retention capacity due to its combination of the three type of crosslinked coatings, being the resultant interaction promoted both by, adsorption and absorption [78,85].

The stationary phase can be immersed in the sample (for liquid samples) or introduced in the vapor phase above the sample (headspace-SPME, HS-SPME). On HS-SPME, the fiber has no direct contact with the sample, increasing the lifetime of the SPME fibers and the selectivity towards volatile and semi-volatile compounds [86]. Beyond its easy handling, high sensitivity and reproducibility and relatively short extracting time (of the order of minutes), this method does not require sample clean-up and can be easily used in combination with various types of analytical techniques, as for instance, gas (mainly) or liquid chromatography [87]. Additionally, this solvent-free microextraction technique takes into consideration the actual concerns of green analytical chemistry [72,78]. SPME technique has been employed in many types of matrices and for many applications, including, among others, biofluids, foods and medicinal plants [87], including *S. nigra* plant [34].

1.4.3. Chromatographic-based technologies

The wide spectrum of metabolites present on given a plant implies that characterization studies often combine multiple chromatographic-based platforms to enlarge metabolites coverage [54]. Furthermore, by coupling chromatographic systems with mass spectrometry (MS) brings together the high separation efficiency, selectivity and sensitivity of the former with a high in the identification power of the spectrometric data provided by the latter [59]. Most representative techniques of this type include (ultra-)high pressure liquid chromatography-mass spectrometry ((U)HPLC-MS) [88,89], gas chromatography-mass spectrometry (GC–MS) [55] and comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-ToFMS) [90]. Other techniques such as nuclear magnetic resonance (NMR) [70], are also very powerful tools in metabolites structural elucidation [54].

1.4.3.1. One and two dimensional gas chromatography

GC-MS technology has long been applied and optimized for analyses of metabolites in plant species [59]. The coupling of GC to electron impact ionization (EI) MS is possibly the oldest hyphenated technique, being often referred to as the "gold standard", as it is one of the most developed, robust, and highly sensitive technique for metabolite analysis [91,92].

The high reproducibility achieved with GC–MS analyses is partly the result of the electron impact ionization (EI) method generally employed in GC–MS, in which molecules interact with kinetically activated electrons with an accepted average standard energy of 70 eV [54]. Quadrupole (Q), ion trap, and time-of-flight (ToF) are commonly used mass analyzers for GC–MS plant characterization studies, in which the latter provides higher mass accuracy and faster acquisition times [54].

The operating principle of GC-MS system involves the volatilization of the sample in a heated inlet port (injector), components separation of the mixture in the chromatographic column, and detection of each component by the MS detector, being therefore restricted to volatile and thermally stable compounds [93].

This technique has already been used on the characterization of *S. nigra* plant, namely on elderflowers [39,94] and elderberries [95].

A basic schematic diagram of gas chromatographs coupled with mass spectrometers used along this PhD thesis is shown in Figure 1.5.

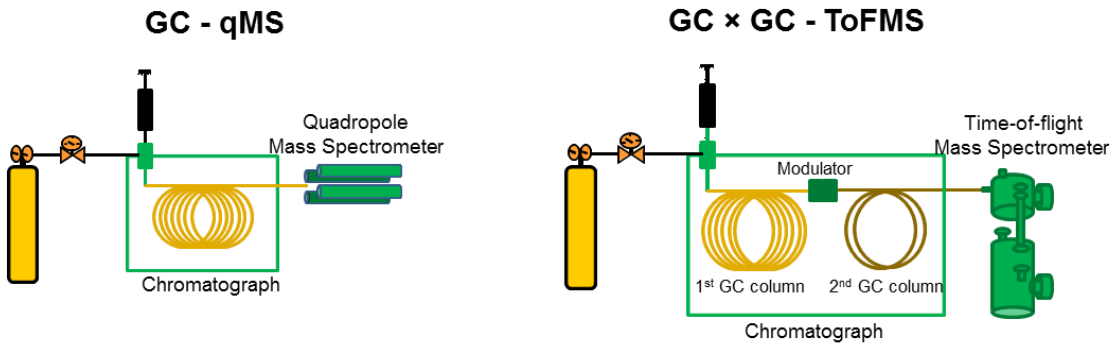


Figure 1.5. One- and two-dimensional gas chromatographic systems.

Depending on the target components, derivatization reactions are sometimes needed to transform analytes into volatile derivatives suitable to be eluted from the GC column without thermal decomposition [96]. Derivatization process can also improve detector response, peak resolution and peak symmetry [96].

Although one-dimensional chromatography (1D-GC) is widely used in the qualitative and quantitative analysis of a wide-range of samples, providing high quality analytical data, sometimes the complexity of the samples exceeds the separation capacity of a single chromatographic column [87,97]. In such cases, peaks co-elution might occur, which complicates the task of identification and quantification process. In order to increase chromatographic resolution, comprehensive two-dimensional gas chromatography (GC×GC) arises as a powerful solution, which includes two orthogonal mechanisms for separation of a mixture of components in a single analysis. This technique is based on the combination of two columns with different stationary phases such as, a nonpolar and a polar set of columns linked through a specific interface, designed the modulator (Figure 1.5). The modulator is a key component in a GC×GC system, transferring the analytes from the first to the second column, with high repeatability and without adding any extraneous chromatographic artifacts. Thermal modulation techniques are often employed by a rapid cooling (using a cryogenic jet, with liquid nitrogen for instance) followed by rapid heating (using a hot-gas jet, which immobilizes and then remobilizes the analytes). Very small fractions from the ^{1}D column are continuously focused in the cryogenic modulator and very rapidly re-injected into the second short and narrow GC column to enable very rapid separations [98]. To properly record very narrow peaks, with widths of typically 60-600 ms at the baseline, 10 data points are needed, implying a high data acquisition rate should be only possible with a time-of-flight-mass spectrometer (ToFMS) [98]. Thus, GC×GC-ToFMS provides information at four different dimension levels: i) the chromatographic separation on the ^{1}D; ii) the chromatographic separation on the ^{2}D; iii) the chromatographic area; and iv) the mass spectral information. The outcome of a GC×GC run is a large series of stacked side by side second-dimension chromatograms (Figure 1.6). GC×GC peak

integration is performed in such way that all the individual second-dimension peaks belonging to one analyte are integrated using conventional integration algorithms and, these are then summed [99–101]. Those are transformed into a 2D-matrix array and the visualization of the peaks is done by displaying them in a 2D plane through colors, shading or contour lines, which indicate the signal intensity; three-dimensional plots are also used (Figure 1.6) [99].

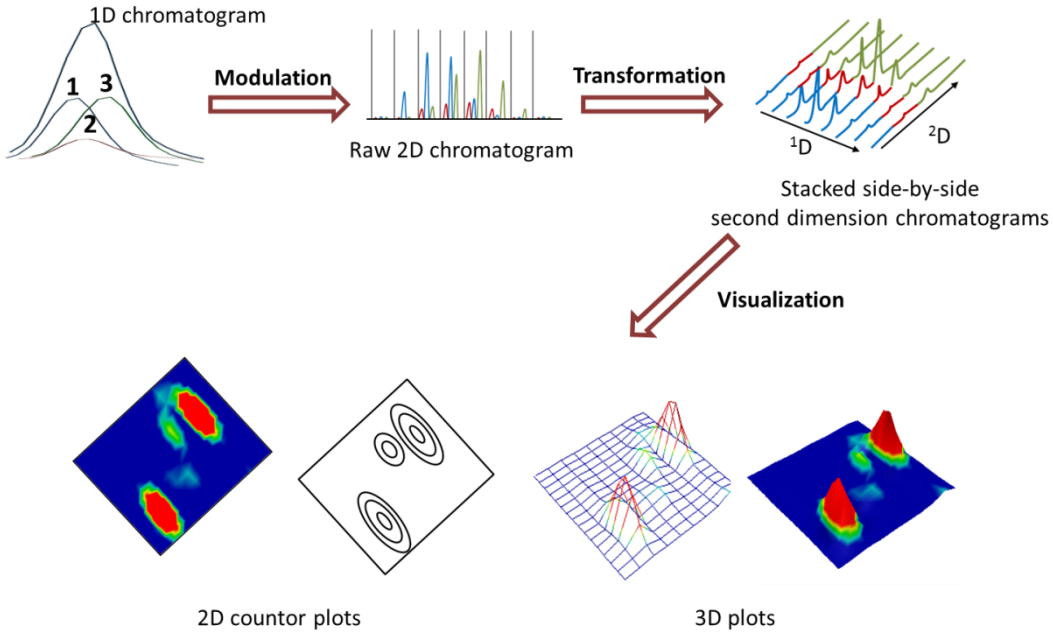


Figure 1.6. Generation and visualization of a GC×GC chromatogram (adapted from Dallüge et al. 2003 [99]**.**

The use of GC×GC coupled with time-of-flight mass spectrometry (ToFMS) has been used in the analysis of the volatile profile for different plants, namely basil, rice, etc. [90]. No studies were reported on *S. nigra* plant, as far as we know.

GC-based applications also have some limitations. One is that GC can only be used for low molecular weight (<1000 Da) compounds, which are either volatile at relatively low temperatures, or that can be chemically transformed into volatile derivatives [59]. Regarding comprehensive 2D-GC, despite it exceeds 1D-GC in separation power and detection sensitivity, its hurdles are the high complexity of the system and high costs of the equipment; operation, and maintenance; computationally intensive data handling; peak integration is a time-consuming task. However, and despite those limitations, GC×GC–TOFMS has the potential to be routinely implemented in plant characterization studies, yielding in concert with complementary techniques, such as LC–MS and NMR spectroscopy, a comprehensive picture of the system under investigation [97].

1.4.3.2. High-performance liquid chromatography

High-performance liquid chromatography-mass spectrometry shows as major advantage over GC–MS methods the potential to analyze thermolabile, polar metabolites (nonvolatile), and high-molecular weight compounds without previous derivatization [54]. The sample is first dissolved in a suitable solvent, and after being injected on HPLC system, the sample is then carried through a chromatographic column by a liquid mobile phase. The selection of the column stationary phase and the mobile phase, are crucial variables for the analytical outcome. Separation is determined by different interactions as liquid–solid adsorption, liquid–liquid partitioning, ion exchange and size exclusion, and by solute/mobile-phase interactions [102]. In reverse-phase chromatography, the more commonly encountered form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The separation of phenolic compounds by HPLC, the target chemical family of this PhD thesis, is generally performed in an octadecyl (C_{18}) bonded silica column. The mobile phase used for phenolic compounds analysis, through reversed-phase HPLC, is generally constituted by water and polar organic solvents (e.g. acetonitrile or methanol), and acetic, formic or phosphoric acids which are often added. Thus, less-polar compounds have a strong interaction with the stationary phase, and are more retained on the column than polar analytes [54].

Modern advances in column dimensions and particle size have been achieved, and it is now possible to analyze faster a wider range of different analytes and obtain very high separation efficiencies [54]. Within these new columns, core-shell particle stationary phases have been especially successful, with sub-2 μm particles, known as ultra-HPLC (UHPLC) columns, since these increase the optimum mobile phase velocity, supporting higher flow rates, maintaining high chromatographic efficiency, while decreasing analysis time, by working with high pressures (around 16,000 psi compared to ca. 6,000 psi for traditional HPLC systems) [103]. A basic schematic diagram of a possible configuration of an HPLC instrument is shown in Figure 1.7.

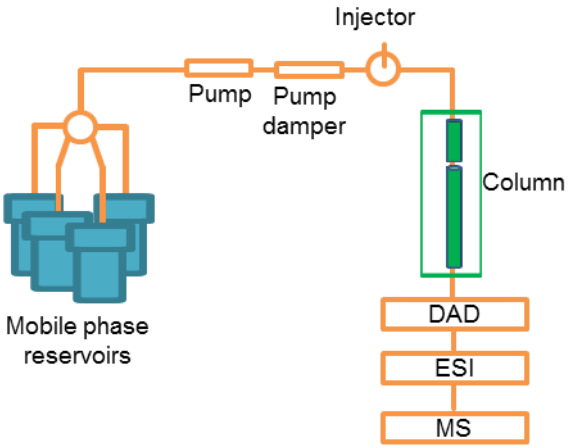


Figure 1.7. High-performance liquid chromatograph system.

One detector or more are placed at the end of the column, being single-wavelength ultraviolet-visible spectrophotometers (UV-Vis) and multi-wavelength diode array detectors (DAD) the most common, as well as mass spectrometers (Figure 1.7).

Electrospray ionization (ESI) is the most popular ionization method used on HPLC-MS systems, as it allows an efficient transfer and ionization of analytes to the gas phase [54]. However ESI is a soft-ionization technique; which, directly, provides little structural information. This information can be achieved by tandem mass spectrometry techniques (tandem-MS^n) in which analyte ions are further fragmented [54]. The most common tandem-in-time instruments are ion-trap mass spectrometers [54].

HPLC systems have been widely applied on the analysis of phenolic compounds from different plant extracts [104], including elderflowers [105] and elderberries [106].

1.4.4. Data processing and interpretation

Data processing and analysis is employed in order to extract relevant information needed to generate scientific hypotheses. The chemical complexity of natural matrices, the lack of reference mass spectra for all the compounds and the inherent variability in each sample due to the individuality of the each organism highlights the data analysis importance. Data analysis involves, different approaches from data pre-processing, pre-treatment, identification, quantification and processing to data post-processing, validation and interpretation (Table 1.2).

Table 1.2. Data pre-processing, pre-treatment, identification and quantification, processing, post-processing, validation and interpretation [58]**.**

Term	Objective	Examples
Pre-processing	From raw instrumental data to clean data for data processing	Deconvolution, alignment, base-line correction
Pre-treatment	Transforming the clean data to make them ready for data processing	Normalization, transformation, scaling
Identification and quantification	Conversion of raw data into biological context	Standards, MS fragmentation pattern; internal standard, instrument response
Processing	The actual data analysis	ANOVA, PCA, PLS, PLS-DA, ASCA
Post-processing	Transforming the results from the processing for interpretation and visualization	Visualization
Validation	Assure the quality of the conclusions drawn from the data analysis	Leave-one-out, cross validation
Interpretation	Hypothesis generated, pathways affected, or visualization of the data.	-

Data pre-processing and pre-treatment aims to identify and eliminate extraneous variability factors (human error, artifacts, instrument variation, etc.) from the intrinsic variations of the samples, playing a central role in data analysis [58]. These include, data deconvolution, alignment, base-line correction, normalization, transformation and scaling [58].

The identification of a chemical compound is a fundamental function that converts raw data into biological context. However, the exact basis for what constitutes a valid compound identification is still currently debated, and four levels identification can be defined based on Sumner et al. [107]:

1. Identified compounds (supported with the use of chemical reference standards);
2. Putatively identified compounds (without the use of chemical reference standards, based upon physicochemical and/or spectral properties);
3. Putatively characterized compound classes (based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class);
4. Unknown compounds (although unidentified or unclassified these metabolites can still be differentiated and quantified based upon spectral data).

The identification of a compound should be performed based on the combination of more than one information source to increase confidence. Most frequently, this approach is based on MS and retention data in combination with data bases and standards. However, the use of reference standards in co-injection is limited by the fact that they are often commercially unavailable or economically prohibitive. Additionally, sample processing flow (extraction, derivatization, injection mode, chromatographic column, etc.) can provide useful data to complement identification.

Two-dimensional GC×GC techniques bring a particular advantage, namely the structured chromatogram, which relies on the fact that similar chemical structures occupy the same 2D chromatographic space. For instance, if a nonpolar / polar set of GC columns is used in the first dimension they will be separated by volatility and in the second by polarity, providing additional information for the identification process [108].

Depending on the scope of the study, semi- and absolute-quantification methods may be applied [107]. Semi-quantification reports the instrument response from analytes abundances, which could be relative to an internal standard or other metabolites. The use of an internal standard relies on adding a known amount of a substance to the standards and to the samples to serve as a reference for the peak area, so that slight variations (ex. in injection technique and volume) are compensated by the fact that the internal standard peak and the analytes peaks are equally affected by these variations [109,110]. Regarding absolute quantification, the analytes absolute

concentrations are determined by the correlation between the response of the used instrument and known concentration series of the same analytes, using single or multiple calibration curves which can be obtained by an internal or external calibration [102,110]. Also, instrumental signal data has been used as an approach to estimate the relative content of different analytes, as it reports the response of the instrument to the analytes abundances, being often used on the evaluation of changes on analytes profile in response to a studied parameter [111].

Then, data processing includes different data analysis possibilities, namely multivariate analysis that is intended to distinguish classes in complex datasets. Multivariate analysis may be done in the form of unsupervised analysis, as principal component analysis (PCA) that is often the first step of the data analysis, and it aims to detect patterns in the measured data by the reduction of the data dimensionality, allowing their visualization retaining as much as possible the information present in the original data [112]. Clustering methods are also widely employed on statistical analysis, e.g. hierarchical cluster analysis, HCA, which is resorted on subdividing and ordering complex datasets into groups of data with a high degree of similarity [65].

Otherwise, supervised approaches, for instance partial-least squares discriminant analysis (PLS-DA), use the information about the class membership of the samples to a certain group (class or category) [58,112]. Analysis of variance simultaneous component analysis, ASCA is a generalized version of analysis of variance for univariate data to the multivariate case, being possible to isolate the variation in the data induced by a factor varied in the experimental design and by analyzing this isolated variation with simultaneous component analysis may reveal the relation between the samples and analytes [113]. In any case, the resulting plots are used to identify the variables that contribute to between-group variability based on separations observed between groups in the scores plot. Validation is a crucial step, especially used for supervised statistical methods (e.g. leave-one-out validation), in order to guarantee the reliability of the applied model. Data validation verifies the quality of the obtained discrimination models. Model validation process allows demonstrating that the models obtained by the supervised pattern recognition techniques are good enough to perform classification or discrimination of the samples [112]. The sample dimension is often a limitation on model validation.

Univariate methods are also used to individually study specific variables or chemical families that could be possibly altered between different groups. These tests include parametric methods for data that are normally distributed, as the analysis of variance (ANOVA) with *post hoc* tests [58].

1.5. Chemical composition of *S. nigra* berries and flowers

Nowadays, edible plant preparations are intended not only to provide necessary nutrients, but also to contribute directly to human health. Thus, in this perspective, the detailed knowledge of the chemical composition of *S. nigra* is extremely important to understand its biological effects but also to improve its value and applicability. This section, will first give an overview of the general composition of *S. nigra* berries and flowers, followed by a detailed overview of the secondary metabolites composition, namely the terpenic, sterol and phenolic families. Table 1.3, briefly shows the elderberry's general composition and its energetic value.

Table 1.3. *S. nigra* L. berry general composition, energetic value and dietary reference values (DRV).

Parameters	Values	DRV[a]	Reference
Weight (g)	0.08-0.21	-	[30,114,115]
Energy (kcal)	73	2000	[28]
Water (%)	76-85	-	[28,30,114,116]
pH	3.7-4.8	-	[35,95,114,116–120]
Lipids	0.5 g/100g fw	70 g	[28]
Volatiles	1.5-390 mg/100g fw	-	[34]
Carbohydrates	18.4 g/100g fw	260 g	[28]
Fiber	7.0 g/100g fw	25 g	[28]
Soluble Solids (º Brix)	10.1-17.5	-	[35,95,115,117,119]
Sugars	6.8-10.4 g/100g fw	90 g	[37]
Fructose (g/100g fw)	3.4-5.2	-	[37]
Glucose (g/100g fw)	3.3-5.0	-	[37]
Sucrose (g/100g fw)	0.047-0.17	-	[37]
Protein (N x 6.25, g/100g)	0.66	0.66 g/kg bw	[28]
Amino acids (mg/100g fw)	0.64	-	[28]
Titratable Acidity [b]	0.48-1.7	-	[35,115–117,119,120]
Citric acid (g/100g fw)	0.31-0.48	-	[37]
Malic acid (g/100g fw)	0.097-0.13	-	[37]
Shikimic acid (g/100g fw)	0.014-0.093	-	[37]
Fumaric acid (g/100g fw)	0.010-0.029	-	[37]
Mineral matter content (%)	0.90-0.99	-	[114]
Calcium	38.0-153 mg/100g fw	700 mg	[28,121]
Iron	1.4-8.5 mg/100g fw	10 mg	[28,121]
Potassium	288-549 mg/100g fw	3.1-3.5 g	[121]
Sodium	2.0-15 mg/100g fw	1.1-150 mg	[28,121]
Phosphorous	49.0-134 mg/100g fw	0.6-3.4 g	[28,121]
Zinc	0.2-1 mg/100g fw	7.3 mg	[121]
Manganese	0.4-1 mg/100g fw	2-5 mg [c]	[121]
Vitamin A (UI)	600	900 UI	[28]
Vitamin B6 (mg/100g fw)	0.23	2-3 mg	[28]
Vitamin C (mg/100g fw)	6-117	45 mg	[28,38,114,115,122]

fw – fresh weight; bw – body weight; [a] Dietary References Values (DRV) for adults per day according to European Food Safety Authority (EFSA) guidelines [123–126]; [b] g citric acid/100g fresh weight; [c] There is not a recommended diary intake but an estimated safe and adequate dietary intake was established.

According to Table 1.3, apart from water content, carbohydrates represent the major component of elderberries (up to 18% of the fresh weight) [28]. Within carbohydrates, fiber accounted for 7% of the berries weight [28], while sugars (mono and disaccharides) represent, between 6.8-10.4% [37]. Elderberries sugars are mainly composed of glucose and fructose, with sucrose being found in lower amounts.

Elderberries soluble solids content can vary from 10.1 to 17.5 Brix degree [35,95,115,117,119]. This parameter indicates the soluble sugars content, and thus delineates the elderberries ripe stage. Therefore, it is a key parameter for elderberry growers to define the technological maturity stage and consequently the harvest time and also their commercial value.

Titratable acidity of elderberries ranges from 0.48 to 1.7 g citric acid/100g fw [35,115–117,119], with citric and malic acids as the major contributors, followed by shikimic and fumaric acids. Titratable acidity is another important parameter as it is used as a decision making tool by growers to establish the berries technological maturity.

Volatile compounds are also reported in elderberries, belonging to diverse chemical families as terpenes, alcohols, aldehydes, ketones, esters, hydrocarbons, acids norisoprenoids, ranging from 1.5 to 390 mg/100g fw [34]. This diversity of volatiles compounds contributes to the elderberry aroma, namely the fruity notes of esters; the elderflower notes of terpenoids (oxides); the characteristic elderberry odor due to the norisoprenoids dihydroedulan and *β*-damascenone that result from carotenoids breakdown; and also compounds that result from fatty acids oxidation as aldehydes and alcohols (e.g. hexanal and hexanol) that contribute to grassy aroma [34,36,95,127].

The elderberries protein content, expressed as *N*-containing compounds, is 0.66 N×6.25 g/100g [28], which is within the common values for fruits (0.3-2 N×6.25, g/100g) [124].

It is worth to refer that as illustrated on Table 1.3, the parameters listed for elderberries can remarkable vary, as vitamin C that ranges from 6 to 117 mg/100g fw [28,38,114,115,122]. Those ranges reflect the use of different cultivars, agricultural management practices, the environment they were grown in and the processing and storage conditions [28].

The contribution of some parameters to the Dietary References Values (DRV), as the protein intake is low; however, for others their contribution is relevant to attain the reference dietary doses. For instance, a typical portion of elderberries (50g) permits to ingest up to 11% of the DRV of calcium and 7% of the DRV of zinc. It is important to note that the DRVs depends on several parameters, namely, individual (body size, diet, physical activity, etc.), health condition (e.g. digestive disorders); or environment ones (climate, sun exposure, etc.) [126].

Finally, regarding elderflowers, the available literature is scarce. In previous studies, 16 amino acids, 9 of which essential, were reported on flowers and, the total protein content was 2.4% on dry weight basis [128]. Elderflowers volatiles compounds represent on average 3,25 µg/mL of

extract (values unavailable on fresh or dry weight basis) [39], including several families, such as aliphatic alcohols, aldehydes, esters and ketones; terpenoids (mono and sesquiterpenic compounds); norisoprenoids, aromatic compounds, among others [39,129,130]. Their application as flavoring agents to produce soft drinks or infusions is linked to their intense, pleasant and characteristic aroma, currently named as elderflower aroma [129,130]. This aroma comprises a set of sensorial notes described as floral, fruity, grassy, woody, minty, spicy and herbaceous, being associated with the presence of several volatile compounds belonging to different chemical families, namely alcohols, aldehydes, ketones, esters, carboxylic acids, terpenic and norisoprenoids [39,94,130]. The major contributors for the characteristic elderflower aroma are the monoterpenic metabolites hotrienol, rose oxides, nerol oxide, linalool oxides, *α*-terpineol and linalool [39,130]. Despite the role of esters, alcohols and aldehydes, monoterpenes, as limonene, terpinolene and terpinene present a relevant contribution for the elderflowers fruitiness aroma [39]. Other monoterpenic compounds, along with sesquiterpenic and norisoprenoids compounds also contribute with more exotic notes, such as woody and spicy [39,130].

1.5.1. Terpenic compounds

Terpenic compounds form a large and structurally diverse family of natural products derived from C_5 isoprene units, with over 35 000 known structures [131]. The biochemical isoprene units may be derived by two pathways: through the intermediates mevalonic acid and 2-*C*-methyl-D-erythritol 4-phosphate [131]. Typical structures comprise carbon skeletons represented by $(C_5)_n$, and are classified as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), and tetraterpenes (C_{40}) [131].

Table 1.4 describes the terpenic compounds identified in *S. nigra*, corresponding to 32 compounds on elderberries and 41 on elderflowers. These are distributed over mono, sesqui and triterpenic compounds. As the information available on the analysis of berries and flowers terpenic composition is scarce, data about related minimum processed products, such as infusions, syrups and juices [36,39,95,130] was also included in Table 1.4. Most of the reported studies are focused on the identification of the analytes rather than on their quantitative analysis, however, when available, quantitative data is also provided.

Table 1.4. Terpenic compounds reported on *S. nigra* L. berries (B) and flowers (F) and related products, such as infusions, syrups, or juices.

Compound	Previously detected [a]		Reference
	Elderflowers	Elderberries	
Monoterpenic Compounds			
Monoterpenes			
3-Carene	✓	✓	[95,132]
β-Pinene	-	✓	[95]
D-Limonene [b]	✓	2.24-9.92	[34,94,95,130]
Myrcene	✓	✓	[94,95]
Terpinolene	✓	✓	[95,130]
α-Phellandrene	✓	✓	[34,39,130]
α-Terpinene	✓	✓	[34,39,130]
γ-Terpinene	✓	✓	[34,39,130]
Ocimene [b]	✓	1.55-9.32	[34,39,130]
p-Cymene	✓	-	[130]
o-Cymene	-	✓	[34]
Oxygen-containing type			
Linalool [b]	✓	1.18-128.89	[34,36,94]
Hydroxylinalool	✓	-	[39,130]
E-Linalool oxide (furanoid form)	✓	✓	[94,133]
Z-Linalool oxide (furanoid form)	✓	✓	[94,133]
E-Linalool oxide (pyranoid form)	✓	-	[39,130]
Z-Linalool oxide (pyranoid form)	✓	-	[39,130]
Hotrienol [b]	✓	2.56-8.08	[34,39,94,133]
E-Rose oxide	✓	✓	[36,39,94]
Z-Rose oxide [b]	✓	1.68-8.34	[34,36,39,94]
Nerol oxide [b]	✓	1.02-7.80	[34,36,39,94]
1,8-Cineole	✓	✓	[34,39,94]
α-Thujone	✓	-	[94]
β-Thujone	✓	-	[94]
α-Terpineol [b]	✓	70.85-2699.56	[34,36,94]
Citronellol	✓	✓	[39,94,133]
Camphor	✓	✓	[34,94]
Menthol	✓	✓	[34,94]
Carvone	✓	-	[94]
Terpinen-4-ol	✓	✓	[34,130]
Geraniol [a]	✓	1.05-7.21	[34,94]
Nerol	✓	✓	[34,94]
Nerolidol	✓	-	[94]
Thymol	✓	-	[94]
Carvacrol	✓	-	[94]
Borneol	-	✓	[34]
Hydroxycitronellol	-	✓	[34]
Citral	-	✓	[34]
Geranial	-	✓	[34]
Sesquiterpenes			
α-Humulene	-	✓	[95]
α-Copaene	✓	-	[94]
β-Caryophyllene	✓	-	[39,130]
Triterpenic Compounds			
α-Amyrin	✓	-	[134]
β-Amyrin	✓	-	[134]
Ursolic acid	✓	✓	[134,135]
Oleanolic acid	✓	✓	[135,136]
20*β*-hydroxyursolic acid	✓	-	[134]

Lupeol	✓	-	[137]

[a] when available, quantitative information was reported
[b] µg/kg of fresh berries
Marks "✓" correspond to non-quantified compounds or quantified but not expressed as berry or flower weight basis.

Mono and sesquiterpenic compounds, even present in trace amounts, are in part responsible for pleasant elderflower and elderberries odors, imparting fruity/sweet/flowery notes [33,95]. According to Table 1.4, the main volatile terpenic compounds present on elderberries are limonene (2.24-9.92 µg/kg of fresh berries), linalool (1.18-128.89 µg/kg of fresh berries) and *α*-terpineol (70.85-2699.56 µg/kg of fresh berries) (Figure 1.8) [34]. In elderberries juice, monoterpenic compounds ranged from 8.9-77.2 ng/mL, while sesquiterpenic compounds range from not detected up to 56.9 ng/mL, with limonene and linalool as the main monoterpenic components and *α*-humulene (Figure 1.8) as the unique sesquiterpene detected [95].

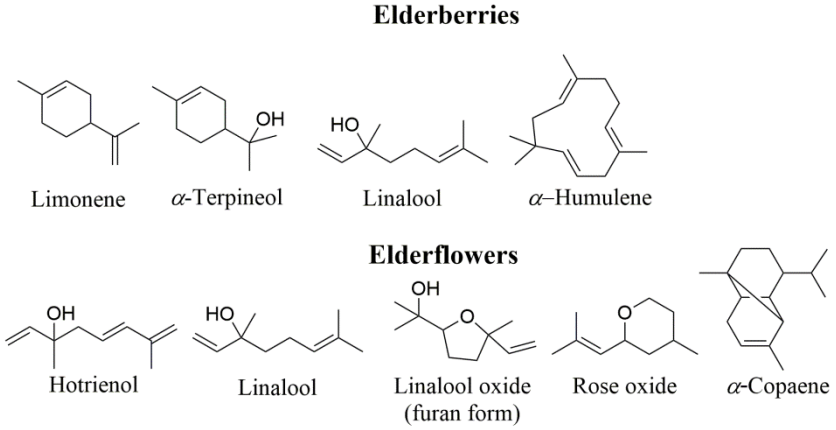


Figure 1.8. Main monoterpenic and sesquiterpenic compounds present on *S. nigra* L. berries and flowers.

On elderflowers, 34 volatile terpenic compounds have been reported, with 24 of those being oxygen-containing structures (Table 1.4). Hotrienol (13.7%, w/w), rose oxide (5.1%, w/w), linalool (3.7%, w/w), linalool oxide (furan forms, 3.1%, w/w), were the major monoterpenic compounds, while *α*-copaene was the only sesquiterpene detected (0.3%, w/w) (Figure 1.8) [94].

Pentacyclic triterpenic compounds are reported both in flowers and berries (Table 1.4, Figure 1.9), however, *α*- and *β*- amyrin, 20*β*-hydroxyursolic acid and lupeol were only reported on elderflowers.

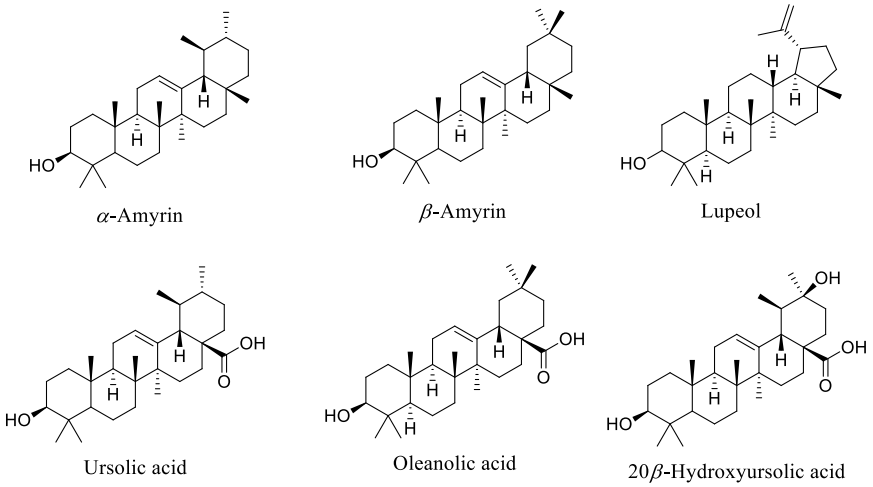


Figure 1.9. Pentacyclic triterpenic compounds present on *S. nigra* L. berries and flowers.

Ursolic and oleanolic acids, *α*- and *β*-amyrin, are the main triterpenic compounds found in *S. nigra* flowers, representing about 1% of the matrix [136].

1.5.2. Sterols

Plant sterols account for around 1% of the fresh weight of elderflowers [136], while for elderberries no studies are conducted so far regarding their content, as far as we know. It is noteworthy that the extraction methods, including extraction solvent, temperature, pressure, and time, may imply different sterols profile [138]. The sterols reported in *S. nigra* berries and flowers are listed on Table 1.5, and their structures shown in Figure 1.10.

Table 1.5. Sterols reported on *S. nigra* L. berries (B) and flowers (F).

Compound	Previously reported		Reference
	Elderflowers	Elderberries	
Methylenecycloartanol	✓	-	[134]
β-Sitosterol	✓	✓	[134,139]
Stigmasterol	✓	-	[136]
Campesterol	✓	-	[136]
Cholesterol	✓	-	[136]
β-Sitosterol-3-*O*-*β*-D-glucoside	✓	-	[134]

Marks "✓" correspond to non-quantified compounds or quantified but not expressed as berry or flower weight basis.

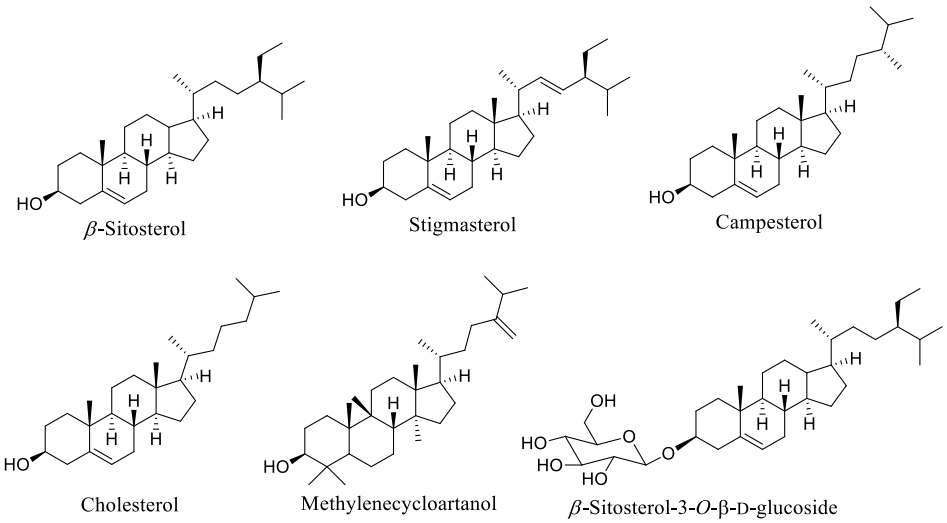


Figure 1.10. Sterols present on *S. nigra* L. flowers and berries.

From the reported sterols (Table 1.5), *β*-sitosterol is the major component present on flowers and the only one reported on elderberries. Although these compounds are often reported in their free form, the glycosylated form of *β*-sitosterol is also reported on elderflowers [134].

1.5.3. Phenolic compounds

Global phenolic content and antioxidant activity of elderberries and elderflowers are listed in the Table 1.6, revealing that phenolic acids, along with flavonols, exhibit higher contents on flowers than in berries, while anthocyanins are only reported in berries.

Table 1.6. Phenolic families' contents and antioxidant activity reported on elderberries and elderflowers.

Compound	Content [a]		Reference
	Elderflowers	Elderberries	
Phenolic Compounds			
Total Phenolic Content (TPC)	1.02	0.36-1.95	[35,105,120,140,141]
Total Anthocyanins Content (TAC)	-	0.17-1.00	[35,120,140]
Phenolic acids	0.51	0.03-0.04	[35,105,106]
Flavonoids	1-3	0.6-0.7	[136,142]
Flavonols	0.5-2.1	0.06-0.20	[35,105,142]
Anthocyanins	-	0.2-2.0	[35,141–143]
Proanthocyanidins (DP<10)	0.016	0.023	[105,141]
Antioxidant activity (% of inhibition)			
DPPH assay	91.9-94.2	50.2-67.7	[142]
β-Carotene	5.2-5.8	3.9-6.6	[142]

[a] g/100g fw

Table 1.7 lists the phenolic compounds identified in elderberries and elderflowers (31 and 24 respectively), being distributed over phenolic acids, flavonols, stilbenes, flavan-3-ols, flavanones,

anthocyanins and proanthocyanidins. As previous families, data from minimum processed products [105,106,143–145] was also included in Table 1.7.

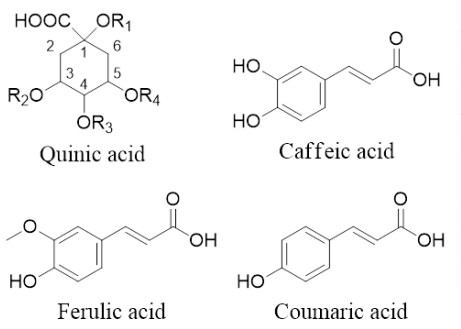
Table 1.7. Phenolic compounds reported on elderberries (B) and elderflowers (F).

Compounds	Previously reported [a,b]		Reference
	Elderflowers	Elderberries	
Phenolic acids			
Hydroxycinnamic acids			
Caffeic acid	✓	-	[136]
Ferulic acid	✓	-	[136]
3-Caffeoylquinic acid	51	1-4	[35,105,143,146]
4-Caffeoylquinic acid	3	1-2	[35,105,106]
5-Caffeoylquinic acid	278	26-36	[35,105,106,143]
3-*p*-Coumaroylquinic acid	4	-	[105,106]
5-*p*-Coumaroylquinic acid	1-8	-	[105,106]
1,5-Dicaffeoylquinic acid	✓	-	[106]
3,4-Dicaffeoylquinic acid	✓	-	[106]
3,5-Dicaffeoylquinic acid	✓	-	[106]
4,5-Dicaffeoylquinic acid	✓	-	[106]
Flavonols			
Quercetin	✓	3-5	[37,79,136]
Isoquercitrin (quercetin 3-glucoside)	20-90	2-26	[35,37,80,105,106,142]
Isorhamnetin 3-glucoside	6	0.3-2	[35,80,105,106]
Astragalin (kaempferol 3-glucoside)	0.4-20	-	[105,142]
Hyperoside (quercetin 3-galactoside)	1	0.1-0.2	[80,105,136]
Quercetin 3-(6″-acetyl)glucoside	✓	-	[106]
Quercetin 3-(6″-acetyl)galactoside	-	0.5-1.2	[80]
Rutin (quercetin 3-rutinoside)	330-2020	35-96	[35,37,43,80,105,142]
Nicotiflorin (kaempferol 3-rutinoside)	65	0.3-1	[35,80,105,146]
Isorhamnetin 3-rutinoside	89	0.8	[35,80,105,146]
Myricetin 3-rutinoside	-	0.3-0.4	[80]
Quercetin 3-vicianoside	-	0.2-3	[80]
Stilbenes			
trans-Resveratrol	-	✓	[144]
Flavan-3-ols			
Catechin	0.7	-	[105]
Epicatechin	25	✓	[105,144]
Gallocatechin	-	✓	[139]
Flavanones			
Naringenin	73	-	[105,146]
5,7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl)chroman-3-yl-3,4,5-trihydroxycyclohexanecarboxylate	-	✓	[147]
Anthocyanins			
Cyanidin (anthocyanidin)	-	✓	[145]
Cyanidin 3-glucoside	-	204-739	[35,37,141–143]
Pelargonidin 3-glucoside	-	*tr*-2	[35,141]
Cyanidin 3,5-diglucoside	-	8-23	[35,37,143]
Cyanidin 3-sambubioside	-	122-630	[35,37,141–143]
Cyanidin 3-rutinoside	-	*tr*-9	[35,37,141]
Delphinidin 3-rutinoside	-	*tr*	[35]
Pelargonidin 3-sambubioside	-	1	[141]
Cyanidin 3-sambubioside-5-glucoside	-	19-82	[35,37,141,143]

Cyanidin 3-*O*-(6-*O*-*p*-coumaroyl-2-*O*-*β*-D-xylopyranosyl)-*β*-D-glucoside-5-*O*-*β*-D-glucoside	-	✓	[148]
Proanthocyanidins			
Monomers	-	1	[141]
Dimers	-	11	[141]
Trimers	16	6	[105,141]
Tetramers to Hexamers	-	11	[141]

[a] when available, quantitative information was reported
[b] mg/100g fw
Marks "✓" correspond to non-quantified compounds or quantified but not expressed as berry or flower weight basis.

Phenolic acids present on elderberries and flowers are mainly hydroxycinnamic acids with a phenylpropane backbone, commonly esterified with quinic acid (Table 1.7, Figure 1.11). 5-Caffeoylquinic acid is the major phenolic acid present on both matrices, elderflowers [105] and elderberries [35] (Table 1.7).

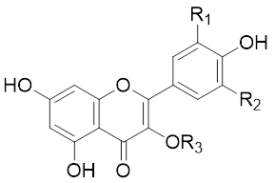


R_1	R_2	R_3	R_4	Compound
H	Caf	H	H	3-Caffeoylquinic acid
H	H	Caf	H	4-Caffeoylquinic acid
H	H	H	Caf	5-Caffeoylquinic acid
H	Coum	H	H	3-*p*-Coumaroylquinic acid
H	H	H	Coum	5-*p*-Coumaroylquinic acid
Caf	H	H	Caf	1,5-Dicaffeoylquinic acid
H	Caf	Caf	H	3,4-Dicaffeoylquinic acid
H	Caf	H	Caf	3,5-Dicaffeoylquinic acid
H	H	Caf	Caf	4,5-Dicaffeoylquinic acid

Figure 1.11. Phenolic acids present on *S. nigra* L. flowers and berries. Caf- caffeoyl; Coum- coumaroyl.

Flavonoids share the basic C_6-C_3-C_6 structural skeleton composed of two aromatic C_6 rings and a heterocyclic pyranic ring containing one oxygen atom, classified into several subgroups as mentioned above. The subgroups that prevail in elderflowers and elderberries are flavonols and anthocyanins, respectively (Table 1.6 and Table 1.7, Figure 1.12 and Figure 1.13).

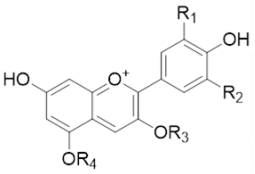
In elderflowers, flavonoids account for up to 3% of the elderflowers weight [136], and the major components belong to flavonols group, namely quercetin-3-rutinoside, quercetin-3-glucoside, kaempferol-3-glucoside, kaempferol-3-rutinoside and isorhamnetin-3-rutinoside (Figure 1.12) [105].



R_1	R_2	R_3	Compound
OH	H	H	Quercetin
OH	H	Glc	Isoquercitrin (quercetin 3-glucoside)
OCH_3	H	Glc	Isorhamnetin 3-glucoside
H	H	Glc	Astragalin (kaempferol 3-glucoside)
OH	H	Gal	Hyperoside (quercetin 3-galactoside)
OH	H	(6″-acetyl)Glc	Quercetin 3-(6″-acetyl)glucoside
OH	H	(6″-acetyl)Gal	Quercetin 3-(6″-acetyl)galactoside
OH	H	Rut	Rutin (quercetin 3-rutinoside)
H	H	Rut	Nicotiflorin (kaempferol 3-rutinoside)
OCH_3	H	Rut	Isorhamnetin 3-rutinoside
OH	OH	Rut	Myricetin 3-rutinoside
OH	H	Vic	Quercetin 3-vicianoside

Figure 1.12. Flavonols present on *S. nigra* L. flowers or berries. Glc- Glucoside; Gal- Galactoside; Rut- Rutinoside; Vic- Vicianoside.

In elderberries, anthocyanins are the main group of phenolic components with cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside, as the most abundant, followed by smaller amounts of cyanidin 3-rutinoside, pelargonidin 3-glucoside and pelargonidin 3-sambubioside (Table 1.6 and Table 1.7, Figure 1.13), accounting for ca. 1% of the fresh berries weight [141].



R_1	R_2	R_3	R_4	Compound
OH	H	H	H	Cyanidin (anthocyanidin)
OH	H	Glc	H	Cyanidin 3-glucoside
H	H	Glc	H	Pelargonidin 3-glucoside
OH	H	Glc	Glc	Cyanidin 3,5-diglucoside
OH	H	Sam	H	Cyanidin 3-sambubioside
OH	H	Rut	H	Cyanidin 3-rutinoside
OH	OH	Rut	H	Delphinidin 3-rutinoside
H	H	Sam	H	Pelargonidin 3-sambubioside
OH	H	Sam	Glc	Cyanidin 3-sambubioside-5-glucoside
OH	H	(6-*p*-Coum-2-Xyl*p*)-5-Glc	Glc	Cyanidin 3-*O*-(6-*O*-*p*-coumaroyl-2-*O*-*β*-D-xylopyranosyl)-*β*-D-glucoside-5-*O*-*β*-D-glucoside

Figure 1.13. Anthocyanins present on *S. nigra* L. berries. Glc- Glucoside; Sam- Sambubioside; Rut- Rutinoside; Coum- Coumaroyl.

Stilbenes, flavan-3-ols and flavanones are also reported for *S. nigra* berries and flowers (Table 1.6 and Table 1.7, Figure 1.14). *trans*-Resveratrol, was only reported on elderberries [144], while epicatechin and catechin were the only flavan-3-ols reported on elderflowers [105,144], with the former reported on berries [139], as well as gallocatechin [144]. The flavanones naringenin [105,146] and 5,7-dihydroxy-4-oxo-2-(3,4,5-trihydroxyphenyl)chroman-3-yl-3,4,5-trihydroxycyclohexanecarboxylate [147] were reported on flowers and berries, respectively. Interestingly, to the best of our knowledge, the last component, and particularly the 3,4,5-

trihydroxycyclohexanecarboxylate moiety is not reported elsewhere nor in *S. nigra* extracts nor in any other plant extract.

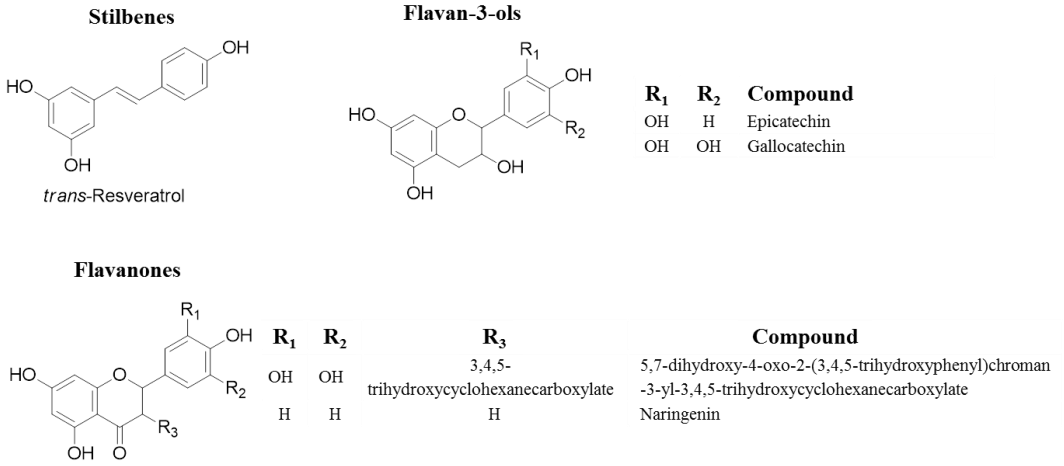


Figure 1.14. Stilbenes, flavan-3-ols, and flavanones present on *S. nigra* L. berries and flowers.

Proanthocyanidins (oligomeric and polymeric flavan-3-ols) were also reported on elderberries and elderflowers (Table 1.6 and Table 1.7, Figure 1.15). Up to now, their chemical structures were not fully elucidated on *S. nigra* berries and flowers, and only their degree of polymerization has been defined (trimers in elderflowers and up to decamers in elderberries) [105,141].

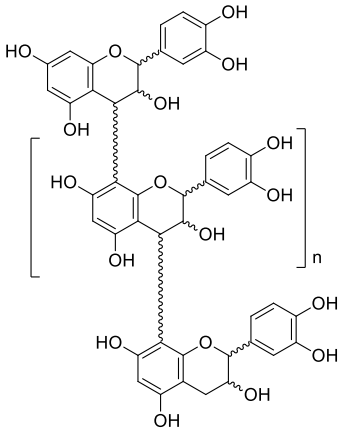


Figure 1.15. General skeleton of proanthocyanidins present on *S. nigra* L. berries and flowers.

1.5.4. Factors that affect *S. nigra* berries and flowers chemical composition

The exposure to biotic and abiotic stress may have an impact on the phenotypic trait of a plant, on plant metabolism, on its appearance, and on the phenological aspects, as dates of bud break, first and full flowering, and first ripening, vegetative growth, total yield, number and weight of cymes , and berries weight [31]. Different phenotypic traits are the result of complex metabolism alterations that ultimately might affect the chemical composition of berries and flowers. The biosynthesis and/or rearrangements of berries and flowers secondary metabolites Table 1.5-Table 1.7, can be modulated by the influence of different geoclimatic conditions, as well as, cultivar and ripening stage. Finally, postharvest handling and storage conditions may also imply chemical changes on berries and flowers composition.

1.5.4.1. Impact of ripening, cultivar and geoclimatic conditions

S. nigra berries and flowers composition depends on several factors, namely ripening, cultivar, harvesting location and year [80], however, the available information about these factors is scarce and disperse. In the following paragraphs an integrated perspective of the published data on this topic will be outlined.

During ripening, elderberries go through important biochemical changes. This process (Figure 1.16), happens from 1 to 2-month period, starting with a green, relatively compact, and oblong appearance and as they ripen over a period of 6 to 8 weeks from July to September (depending on the geographical location), they enlarge until they become spherical [28]. At ripe stage, the berries show a deep purple color. Diverse parameters that will affect the organoleptic characteristics of elderberries (used to define technological maturity), change through ripening, as the titratable acidity and total amino acid content (both decreasing, while total soluble solids increases) [28,30].

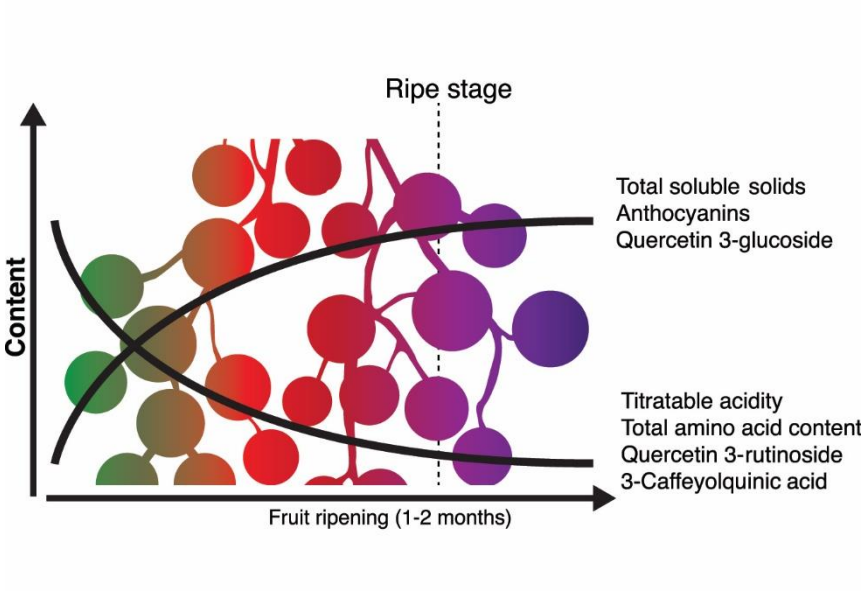


Figure 1.16. Main reported biochemical changes during elderberry's ripening [28,30,106]**.**

From the detailed composition point of view, the major differences along ripening are observed for phenolic components, in which their overall content tend to increase along ripening, being followed by higher antioxidant activity. This is mainly due to the fact that the concentration of the four main anthocyanins increased significantly along ripening [143] (Figure 1.16). For flavonols, while quercetin 3-glucoside tended to increase through ripening, it was observed that quercetin 3-rutinoside concentration decreased [143]. Finally, it was reported that the concentration of 5-caffeoylquinic acid was more or less unaffected by the ripening stage, while 3-caffeoylquinic acid decreased significantly through ripening (Figure 1.16) in most investigated genotypes [143].

The cultivar dependence of sensorial characteristics were reported in processed products obtained from different cultivars [39,127]. For instance, significant differences on the volatile compounds, namely on fatty acid derivatives (as aldehydes, ketones and alcohols) and mono and sesquiterpenic compounds amongst 89 elderflowers cultivars, have been reported [39]. Terpenic alcohols and oxides, the major group of volatile terpenic components from elderflowers, showed the largest variability between cultivars. Hotrienol ranged from 0.8 to 3870 ng/mL; *cis*-rose oxide from 1.2 to 2320 ng/mL; linalool from 2.3 to 1840 ng/mL; and *cis*-linalool oxide (furanic form) from 1.3 to 1100 ng/mL [39]. Significant differences among cultivars on linalool oxide (furanic form), that ranged from 6.3 to 210.8 ng/mL and in rose oxide which ranged from 84.8 to 439.9 ng/mL were also observed [130]. Differences in (*Z*)-and (*E*)-rose oxide levels (from undetectable up to 4.8 and 2.0 ng/mL, respectively) were also reported in elderberry juice from different cultivars [36], while limonene ranged from undetectable levels up to 12.5 ng/mL [95].

The phenolic composition of elderflowers was also cultivar dependent (44 studied cultivars); for example, 5-caffeyolquinic acid ranged from 1.0 to 63.8 mg/100g of extract [106]; considerable variations were also reported for flavonols, namely rutin, which ranged from 1.1 to 63.0 mg/100g of extract [106]. Beyond the cultivar, phenolic acids and flavonols content was also influenced by the harvesting season, both in elderflowers [149] and in elderberries [35].

Harvesting season and cultivar were also reported to affect anthocyanins profiles. For example in 'Haschberg' and 'Korsør' cultivars (from Austria and Denmark, respectively), cyanidin 3-glucoside accounted for 391.0 and 400.2 mg/100g of fresh berries respectively in one season, and for 656.5 and 806.1 mg/100g of fresh berries in the subsequent season [35]. In another study, 'Rubini' cultivar (from Austria) showed the highest amount of anthocyanins ever reported (1265 mg/100g of fresh berries), while the lowest anthocyanins content (603 mg/100g of fresh berries) was reported for 'Selection 14' cultivar (from Austria) [37]. Anthocyanins profile was also used for the discrimination of elderberries from different locations, mainly based on cyanidin 3-sambubioside-5-glucoside content [148].

Influence of altitude on the content of phenolic compounds of *S. nigra* wild populations revealed that elderflowers and elderberries collect at higher altitude (1000 m) exhibited higher levels of flavonol-3-glycosides, namely rutin, while the level of anthocyanins was lower for higher altitudes [149].

The influence of the discussed parameters over the triterpenic and sterols chemical families was not studied so far, as far as we know.

1.5.4.2. Impact of handling and storage

Elderflowers and elderberries go through different postharvest handling and storage conditions that precede the industrial processing, in order to prepare stable formulations for commercialization. Figure 1.17 shows some of the main steps from harvesting to storage of elderberries and elderflowers, and the main chemical changes that may occur throughout these processes [40,94,116,129,150,151]. Although these steps depend on the final applications, in this section a general description will be put forward.

Elderflowers and elderberries are collected, during the morning, in specific plastic crates that are able to avoid damage caused by their own weight and then transported to processing facilities. For berries, weight, acidity and Brix degree are determined (some batches the lactic acid content is also determined), and used as quality control parameters. Then, berries are washed, the stems removed and passed through a refrigerated tunnel, and stored in silos at subzero temperatures. Elderflowers can be processed fresh or may be dried or frozen. Elderberries can be submitted to the same processes of elderflowers or even being pulse light treated, and further crushed and mashed to

produce concentrate juices (Figure 1.17) [33,38,152,153]. These late steps promote the degradation of berries cell walls, contributing to increase the anthocyanin content of juices [116] (Figure 1.17). All these handling and storage processes may have impact on the chemical composition of these matrices, as will be discussed below.

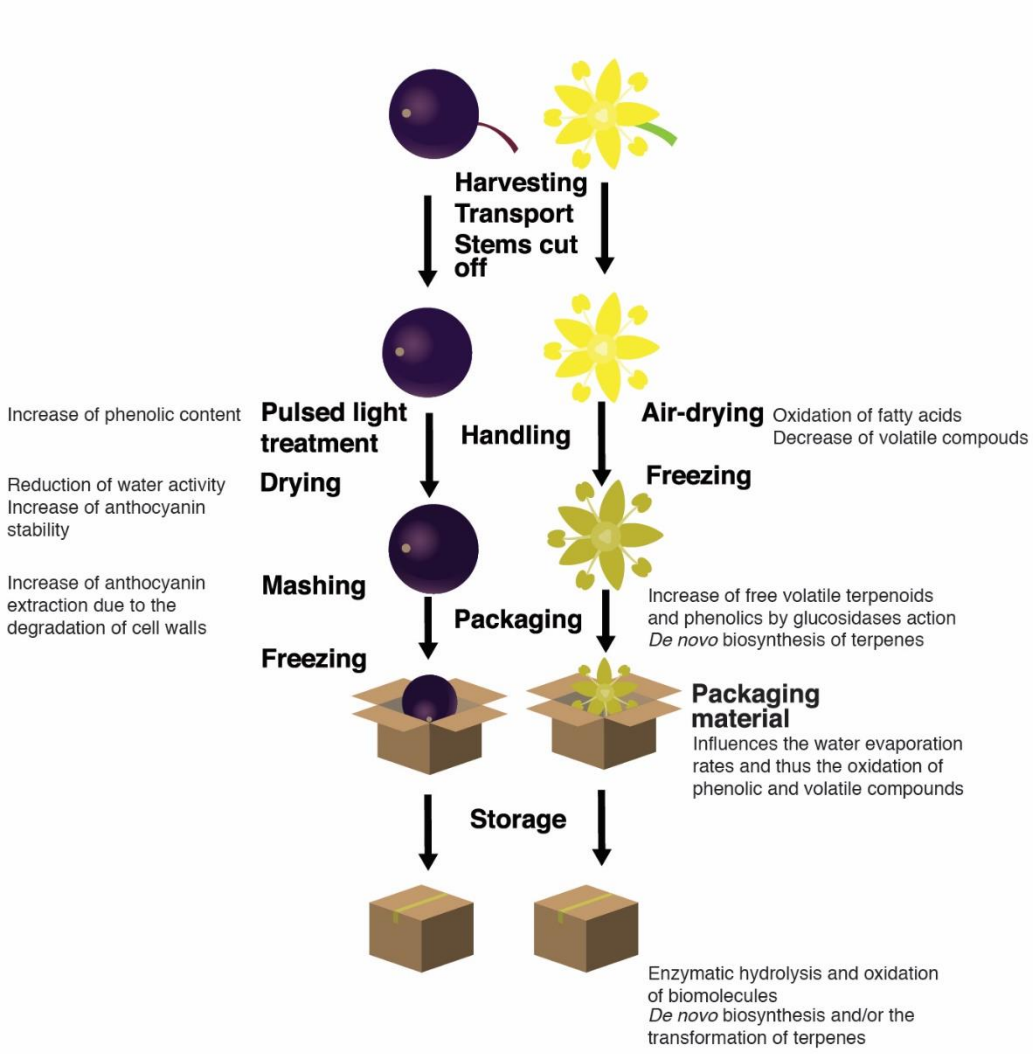


Figure 1.17. Several steps from harvesting to storage of elderberry and elderflower raw materials, including the main chemical changes that may occur through different steps [40,94,116,129,150,151]**.**

A decrease in the content of safranal, hydroxylinalool, limonene, *β*-caryophyllene was previously attributable to elderflowers air-drying process [129]. The production of fatty acids oxidation products, probably through the lipoxygenase action was also observed [94], explaining the presence of hexanal, heptanal, *trans*-2-hexenal, nonanal, and hexanol. The changes occurring during storage may be due enzyme-catalyzed hydrolysis of proteins and lipids resulting from disrupted plant tissue. Dried elderflowers packed in paper bags at normal pressure and in plastic and aluminum bags at 99% vacuum, were stored at 20 ºC and followed for up to 21 months [129].

Packaging material and storage time had a significant effect on the volatile composition, and 15 volatile compounds partially or completely vanished during storage. These included compounds with characteristic elderflower odor (*trans*- and *cis*-rose oxide and the terpene alcohols hotrienol, linalool, *α*-terpineol and hydroxylinalool), fruity notes (e.g., limonene), as well as volatile compounds with grassy (e.g., (*Z*)-3-hexen-1-ol) and sweet odors (e.g., 1-butanol). An increase of nerol oxide and linalool in plastic and aluminum bags and hydroxylinalool, hotrienol, *cis*-rose oxide, *trans*-rose oxide, *α*-phellandrene, *α*-terpinene in bags of the three packaging materials was also reported. The authors suggested that these terpenoids were glycosidically-linked, and were released through storage by enzymatic action (involving e.g. glucosidases), or chemical hydrolysis [129]. In addition, *de novo* biosynthesis and/or the transformation of terpenes may also explain their increase through storage [129].

Packaging materials and storage time also showed a significant impact on the content of phenolic acids and flavonoids [40]. A higher phenolic content of elderflowers stored in paper and plastic bags compared to aluminum foil bags was associated to different water evaporation rates (Figure 1.17). Regarding the storage time effect, 3-caffeyolquinic acid decreased after 21 months, while 3,5-dicaffeyolquinic acid increased. These changes were associated to enzymatic hydrolysis of glycoside precursors and oxidation and/or enzymatic degradation processes.

Drying of elderberries or their products promotes a water activity reduction, contributing to the preservation of the samples against microbial contamination and also decrease the degradation of anthocyanins [150], by increasing their stability, which might be due to reduced mobility of components and limited oxygen solubility [150]. Other strategies have been used to preserve the elderberries bioactive components or to enhance their nutritional value, as for instance their processing with pulsed ultraviolet light to enhance the phenolic content [151]. Although an increment on the elderberries phenolic content was observed, clear relationship with pulsed light treatment (or the associated thermal stress) was not unambiguously established.

1.6. *S. nigra* berries and flowers: potential health benefits

S. nigra flowers and berries have been widely used on folk medicine for numerous applications that include antimicrobial, anti-viral, antioxidant, anti-inflammatory, anti-cancer, antidiabetic, among others, which in some cases are nowadays sustained by detailed and published studies. An overview of the potential health benefits of berries and flowers preparations from this plant will be discussed in the coming sections, with emphasis on the use of elderberries and flowers preparations on the treatment of diabetes mellitus, mostly due to its high incidence on the modern society [5]. This disease often occurs along with other components of the metabolic syndrome such

as hypertension, dyslipidemia and obesity [154]. The relevance of diabetes and of its social impact is clearly highlighted by World Health Organization (WHO), which considers that further research is urgently needed to evaluate the effectiveness of interventions to prevent diabetes, notably by behavioral changes favoring a healthy diet and regular physical activity. This includes for example promotion of fruits and vegetables consumption thus, improving dietary patterns [155], notably with antidiabetic natural agents, such as *S. nigra*-based preparations.

1.6.1. Overview of the *S. nigra* potential health benefits

Antioxidant activity is one of the most studied biological activities of this plant, as a result of the growing interest in plant-derived antioxidants as a way to prevent the damaging effects of oxygen radicals and other reactive oxygen species (ROS) in human health [156,157]. Most studies have been performed *in vitro* and access the potential inhibition or scavenging capacity of aqueous or hydro-alcoholic *S. nigra* extracts, against ROS, using 2,2'-azobis(3-ethyl-benzothialzoline-6-sulfonic acid) (ABTS) [105,158], *β*-carotene/linoleic acid oxidation [142], 2,2-diphenyl-1-picrylhydrazyl (DPPH) [142,149,158–161], ferric reducing antioxidant power (FRAP) [105], oxygen radical absorption capacity (ORAC) [141], cupric reducing antioxidant capacity (CUPRAC) [162], among others. Studies performed so far include *in vitro* assays on elderflowers extracts [105,142,149,159,160,162,163], and on elderberries extracts *in vitro* [141,158,161,164–168], *in vivo* [169,170], and even in human trials [171,172].

Elderberry methanolic extract antioxidant activity is comparable to raspberry and blackberry extracts, showing one of the highest antioxidant activities (3.4-5.2 mmol Trolox eq./100g fw) within the analyzed dietary plants (cereals, roots, tubers, vegetables, fruits, berries, pulses, nuts, and dried fruits) [168]. This activity is mainly related to the overall phenolic content, although other components as vitamins, namely ascorbic acid are most likely involved [158,173–175].

The elderberry antioxidant activity can be exerted by the anthocyanins through an extracellular reduction of exposure to oxidative factors, which indirectly might protect the intracellular components of the cells, but also when they are absorbed, they confer a protective effect against oxidative species [166,176,177]. *In vivo* studies evaluated whether anthocyanins of *S. nigra* berries (elderberry concentrate, 2g/kg) had the potential to work synergistically with vitamin C and the antioxidant defense system in sparing vitamin E on Sprague-Dawley rats (during 4 weeks), although the tocopherols and lipidic patterns in plasma and liver were not significantly affected [170]. Nevertheless, elderberry ethanolic extracts supplementation (4%, during 4 weeks, Male Wistar rats with induced colitis) induced significantly higher levels of reduced glutathione (GSH) and glutathione-S-transferase (GST) in the colon and erythrocytes, respectively [169].

These enzymes participate in the detoxification of electrophilic decomposition of products resulting oxygen radicals on lipids and DNA [169].

Similarly, the data obtained on elderberry antioxidant studies conducted on humans are still insufficient: although an increase on the baseline plasma Trolox equivalent antioxidant capacity (TEAC), total radical-trapping antioxidant parameter (TRAP) after ingestion of 400 mL of elderberry juice was reported, 4h after dosing they returned to baseline, while another study revealed that a spray-dried elderberry juice (400 mg, equivalent to 5 mL of fresh juice) did not altered significantly the serum lipids, resistance to oxidation of LDL cholesterol and antioxidant vitamins pattern [171,172].

Elderberry and elderflower antioxidant activity is correlated with the overall amount of phenolic compounds [105], and it is also dependent on extraction conditions, namely temperature [142], time and solvent [105,160]. However, elderflower alcoholic extracts revealed higher antioxidant activity than elderberry (based on DPPH assays), which was linked to the higher flavonoids content, and particularly of flavonols of the former [142]. Aqueous elderflower extracts (1.5-6.2 mg/mL), also inhibited the neutrophil oxidative burst activated by periodontal pathogens (in *in vitro* assays) [163].

S. nigra berry extracts were also found to have a potential anti-inflammatory activity [178–180], which together with their antioxidant activity might play a key role in preventing the onset and development of non-communicable diseases [181], due to their regulatory effect over cytokines, which are mediators of the cellular immune system, whose balance controls inflammatory reactions [178]. In fact, it was demonstrated that an elderberry standardized extract (1/10 of dilution), regulated the imbalance of different tested cytokines, namely it increased the production of cytokines tumor necrosis factor α (TNF-α), and interleukins (IL-1β, IL-6 and IL-8) and one anti-inflammatory cytokine (IL-10), while an aqueous extract (0.1 mg of extract/mL) inhibited the expression of interleukin-1 (IL-1) [178–180]. On the other hand, an elderflower aqueous extract (1.6-6.2 mg/mL) inhibited the release of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) from macrophages, inhibited the transcriptional factor, nuclear factor-κB (NF-κB) activation and suppressed the activation of neutrophils [163], while ethanolic, methanolic and hexane elderflower extracts (1-30 μg/mL) inhibited the biosynthesis of interleukins (IL-1α and IL-1β), TNF-α [182] and carrageenan-induced footpad on rats [183]. Amongst the described studies, only one established a relationship between extracts anti-inflammatory activity and their composition, proposing that quercetin might explain the inhibition of macrophage release and activation of neutrophils [163].

Elderberry preparations also revealed antimicrobial activity against gram-negative bacteria, *Helicobacter pylori* (0.25-1% extract, solvent not described) [184], *Haemophilus influenza* and

Branhamella catarrhalis (5-20% of standardized elderberry extract, solvent not described) [185], and gram-positive bacteria, namely *Streptococcus* from Groups C and G and *Streptococcus pyogenes* [185]), however the antimicrobial action mechanisms were not discussed.

Anti-viral activity was also described for elderberry standardized extracts, in *in vitro* assays against influenza virus A or B, human immunodeficiency virus (HIV-1) and Herpes simplex [147,186,187]; as well as in *in vivo* assays, against influenza virus (in chimpanzees) [187,188]; and even in humans against influenza virus [186,189]. The anti-viral activity against influenza is related with the presence of flavonoids involving the stimulation of the immune system by enhancing production of cytokines, inhibition of the haemagglutination induced by influenza virus, and also through its anti-inflammatory activity [147,186,189,190]. Previous studies reported that phenolic compounds, including cyanidin derivatives [189], quercetin derivatives [147,190], and proanthocyanidins [190], might play a key role in the anti-viral activity of this type of extracts.

S. nigra berries (70% aqueous acetone and commercial extracts) also showed potential anti-carcinogenic activity inhibiting human colorectal adenocarcinoma HT29 cell lines growth, as well as, in the induction of quinone reductase (mouse hepatoma cell line) and inhibition of cyclooxygenase-2 (COX-2) (enzyme-linked immunosorbent assay), being indicative of cancer anti-initiation and anti-promotion properties, respectively [139,191]. It was proposed that the reported activities were related with the presence of anthocyanins [191], quercetin derivatives [139], proanthocyanidins [139], monoterpene glycosides [139], sesquiterpenes [139] and phytosterols [139].

Finally, the conducted studies using human models to evaluate the health benefits of *S. nigra*-based preparations are scarce and they only include a limited number of individuals [171,192,193]. From those, no significant alterations were reported on the lipidic profile, on cardiovascular disease risk biomarkers and on inflammatory biomarkers upon treatment with different elderberry derived formulations (elderberry juice enriched with elderflower extract, spray-dried juice and an extract with undetermined solvent) for a period of 2-12 weeks [171,192,193].

1.6.2. Effects on Type-2 Diabetes

WHO predicted that between 1997 and 2025, the number of diabetics will double from 143 to 300 million, with an estimated global prevalence of 9% in 2014 [155,194]. The term Diabetes mellitus describes a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism, resulting from defects in insulin secretion, insulin action, or both [154]. Type 2 diabetes (T2D), the non-insulin dependent form, comprises 90% of people with diabetes around the world [155], while type 1 diabetes is characterized by autoimmune destruction of the pancreatic islets [154]. The processes

that explain insulin resistance and islet β-cell dysfunction in T2D are oxidative stress, glucotoxicity, lipotoxicity, endoplasmic reticulum stress and amyloid deposition [195]. Insulin resistance is a state in which cells do not respond to normal levels of insulin, due to the excess release of fatty acids from visceral adipose tissue [196], as well as due to the release of certain hormones and cytokines from adipose tissue, e.g. TNF-α. TNF-α is crucial in the inflammatory process through stimulation the macrophages in the synthesis of other pro-inflammatory cytokines [197,198], and it is involved in the destruction of β-cells, in insulin-dependent Diabetes mellitus [199,200]. The key mechanisms involved in the pathogenesis of T2D and its relation to inflammatory reaction involve [195,201–203]:

- **Hypoxia** (macrophages accumulate at hypoxic sites, providing pathological link between adipose tissue expansion and inflammation);
- **Cell death** (adipocyte expansion seems to lead to adipocyte cell death);
- **IL-1 system as a sensor of metabolic stress** (high glucose concentrations induce IL-1β production by β-cells) (Figure 1.18A);
- **Chemokines** (play a crucial role in tissue infiltration by immune cells);
- **NF-κB and c-Jun amino-terminal kinase (JNK) pathways** (activation the inflammation- and stress-induced kinases) (Figure 1.18B);
- **IL-6 and insulin resistance**;
- **Adipokines** (hormones produced mainly or exclusively by adipocytes).

As a result of excessive levels of nutrients, including glucose and free fatty acids, stress will be induced in the pancreatic islets and insulin-sensitive tissues such as adipose tissue (Figure 1.18A), leading to an inflammatory response (production and release of cytokines and chemokines) [195]. Furthermore, a network of molecular pathways integrates the insulin action and inflammatory processes (Figure 1.18B) [204], where lipids and cytokines can trigger inflammatory kinases as c-Jun amino terminal kinase (JNK) and inhibitory protein of κB (IκB) kinase complex (IKK), and endoplasmic reticulum (ER) stress, which then regulate the production of various cytokines (as TNF-α). Insulin receptor substrate (IRS) plays an important role in insulin action by acting as signaling molecules, as well as pancreatic endoplasmic-reticulum kinase (PERK), that also activates IKK (being mediated by NF-κB) [205]. Metabolic inflammatory and immune processes are also regulated by lipids (where fatty-acid-binding proteins, FABP, regulate their biological activities), with PPAR and liver X receptor (LXR) transcription factors crucial for modulating these processes [204,206,207]. ROS that result from mitochondria and/or the ER, activate JNK and IKK and cause ER stress, blocking insulin action and producing more ROS, which leads to more inflammatory responses (Figure 1.18B) [208].

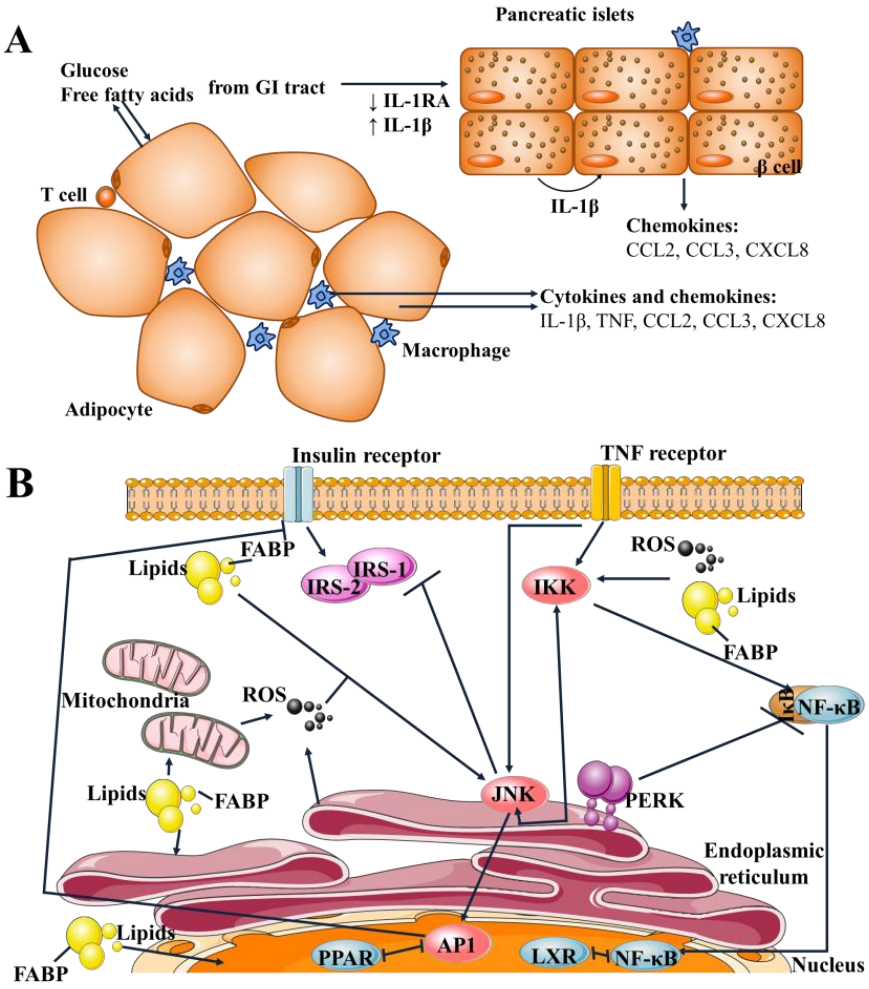


Figure 1.18. A: Inflammation in type-2 diabetes; B: Inflammatory responses with insulin action, adapted with permission from the following publications [195,204]**. IL- interleukin; GI – gastrointestinal; ROS-reactive oxygen species; FABP- fatty-acid-binding proteins; TNF- tumor necrosis factor; JNK- c-Jun amino terminal kinase; IKK- IκB kinase complex; NF-κB- nuclear factor κB; PERK- pancreatic endoplasmic-reticulum kinase; IRS- insulin receptor substrate; PPAR- peroxisome-proliferator activated receptor; AP1- activator protein-1; LXR- liver X receptor.**

The link between inflammatory, immunomodulatory and oxidative stress processes and diabetes clearly highlights the potentiality to apply different and complementary strategies that can help the treatment of type-2 diabetes through the administrating natural extracts that might act as adjuvants on diabetes treatment [5,194,209,210]. Several studies have demonstrated the potential of elderflowers [137,146,211–213] and elderberries [199,214–221], in this context.

Different elderflower extracts (hexane, dichloromethane, methanol, ethyl acetate and water) revealed, *in vitro*, an activation effect on the PPAR(α, δ, or γ), without stimulating adipocyte differentiation between 2.5 and 250 µg extract/mL being performed on mouse embryonic fibroblast and hepatoma cell lines [146,211]. The fatty acids α-linolenic and linoleic acid, as well the flavanone naringenin partially explained the documented bioactivities, while the main elderflower

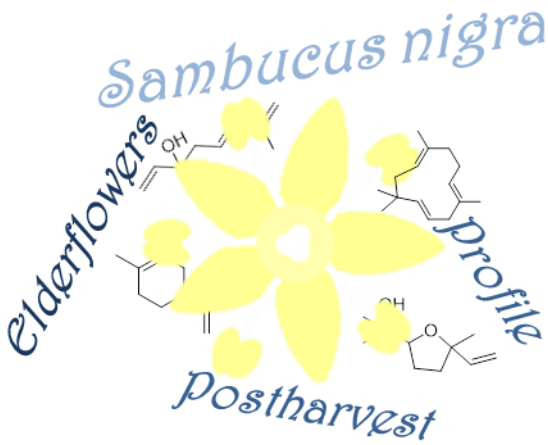
flavonols (quercetin, kaempferol and isorhamnetin derivatives) and 5-caffeoylquinic acid were shown to be inactive [146,211].

Supplementation with elderflower aqueous extracts (100-200 mg/kg body weight) and kaempferol (32-64 mg/kg body weight) revealed acute (6h) and sub-chronic (8 days) antidiabetic potential in alloxan-induced diabetic mice by lowering blood glucose, increasing mice weight and catalase serum levels [212]. The application of different elderflowers extracts (dichloromethane and methanol, from 6-1000 mg extract/L of solvent and aqueous extract 250-1000 mg elderflowers/L of water) has shown to increase the glucose uptake in the presence and absence of insulin (on mouse abdominal muscle), reduction of fat accumulation (*Caenorhabditis elegans* model) [213], and insulin secretion (clonal pancreatic β-cells) [137]. Naringenin and 5-*O*-caffeoylquinic acid partially explained the increase in glucose uptake in primary porcine myotube cultures, while naringenin and kaempferol were linked to the reduction of fat accumulation on *C. elegans* model. Rutin, lupeol and *β*-sitosterol, did not stimulate the insulin secretion [137].

Antidiabetic potential of elderberries were also evaluated through the analysis of serum lipidic, glycemic, inflammatory, oxidative, and immunological status of streptozotocin (STZ)-induced diabetic rats by supplementation of acidified (0.5% HCl) methanol extracts, with doses ranging from 28 to 70 mg of extract/kg of body weight during 12 to 16 weeks of treatment [199,214–221]. Reduction of the glycemic serum levels, and pro-inflammatory interleukins levels (as IL-6 and IL-1β) are documented with the supplementation with these extracts when compared to the diabetic/not treated group [214–218]. Hypolipidemic and hypocholesterolemic effects was also reported, through the significant reduction of total and LDL cholesterol and triglycerides, and increase of HDL cholesterol [215,218]. Serum oxidative status was altered, namely on the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH) enzymes, serum total antioxidant activity and reducing uric acid content when compared to diabetic group/not treated group [214–216,218]. The ability of phenolic compounds to decrease lipid peroxidation and LDL oxidation might be mediated through the uptake of lipid-peroxil radicals and lipooxygenases activity reduction [215,221], which is corroborated by the significant decrease of the serum malondialdehyde content (an index of lipid peroxidation) when it is compared the diabetic rats supplemented with these elderberry extracts and not treated diabetic rats [214,216]. Elderberry extracts also affected the immune system imbalances on diabetic rats, in which the authors beyond linked the potential antidiabetic activity to T2D, also connected with diabetes mellitus type 1, as the latter is an auto-immune illness mediated by the T cells [199,216–218,220]. An increase in the population of lymphocytes (T CD3+ and T helper naive CD4+CD45RA+) and in the levels of cytokines (TNF-α and interferon-γ, IFN-γ), and reduction of monocytes population and levels of

fibrinogen (a cardiac risk factor [219]) were reported, when compared to the group of diabetic and not treated rats [199,216–218,220].

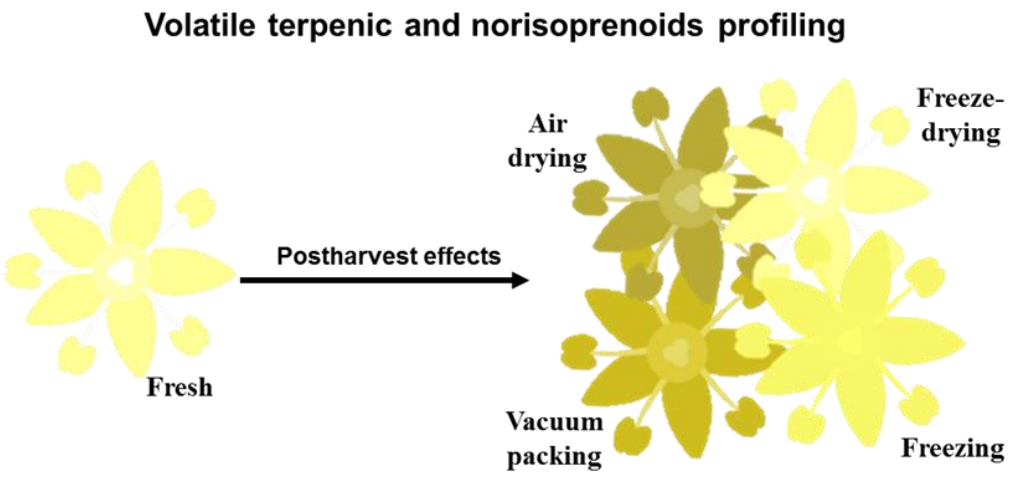
CHAPTER 2. Unveiling elderflowers volatile terpenic and norisoprenoids profile: effects of different postharvest conditions



Adapted from:

ÂC Salvador, SM Rocha, AJD Silvestre, *Unveiling elderflowers (Sambucus nigra L.) volatile terpenic and norisoprenoids profile: effects of different postharvest conditions;* Food Chem., 2017, 229, 276-285.

2.1. Abstract



The volatile terpenic and norisoprenoids profile from elderflowers (*Sambucus nigra* L.) was established for two cultivars by multidimensional gas chromatography. From 47 monoterpenic, 13 sesquiterpenes and 5 norisoprenoids components, 38 are reported for the first time on elderflowers. Elderflower seasonality implies proper handling and storage conditions, for further processing, thus the impact of freezing, freeze-drying, air drying and vacuum packing, was evaluated on these potential aroma metabolites. The most suitable preservation methods, regarding the total metabolites content, were vacuum packing and freezing for intermediary storage times (24-32 weeks) with a reported overall decrease of the volatile terpenic and norisoprenoids of up to 58.6%; and freezing, for longer period (52 weeks), with a decrease of up to 47.4% (compared to fresh elderflowers). This study presents the most detailed terpenic and norisoprenoids elderflower profiling and linalool oxides were proposed as markers for a more expedite assess to the impact of postharvest conditions.

2.2. Introduction

S. nigra L. is cultivated in various regions of the world, and several parts of the plant have been used in food, cosmetic and pharmaceutical areas; the flowers in particular are classified as a medicinal product according to European Medicines Agency [3]. They are mainly used as flavoring agents to produce soft drinks or infusions, and they are characterized by their intense, pleasant and characteristic aroma, currently named as elderflower aroma [129,130]. Olfactory studies revealed that the characteristic aroma of elderflowers comprised a set of sensorial notes described as floral, fruity, grassy, woody, minty, spicy and herbaceous. These notes have been associated with the presence of several volatile compounds belonging to different chemical groups, namely alcohols, aldehydes, ketones, esters, carboxylic acids, terpenic and norisoprenoids [39,94,130]. The monoterpenic metabolites such as hotrienol, rose oxides, nerol oxide, linalool oxides, α-terpineol and linalool were reported as the major contributors for the characteristic elderflower aroma [39,130]. Despite the role of esters, alcohols and aldehydes, monoterpenes, as limonene, terpinolene and terpinene present a relevant contribution for the elderflowers fruitiness aroma [39]. More exotic notes, such as woody and spicy have been attributed to some mono and sesquiterpenic compounds and norisoprenoids [39,130]. Beyond the role of the volatile terpenic metabolites as aroma contributors, these compounds have also been studied in several natural products due to their effect in the promotion of health benefits [10,222]. According to the literature, 35 mono and sesquiterpenic metabolites have been detected in elderflowers and related products, namely processed flowers or infusions [33,39,94,129,130,132,133].

Flowering of *S. nigra* occurs from May to June, depending on the cultivars, geographic location and climatic conditions [30], and the flowers should be collected and stored during this period to be used later. Thus, the seasonal harvesting of elderflowers represents a relevant challenge for farmers and industries as appropriate handling and storage conditions should be implemented to preserve their chemical composition and sensorial characteristics, such as aroma, color or texture. Elderflower formulations are normally prepared from fresh, frozen [106] or dried flowers [129], however the information about the impact of different handling and storage conditions on the volatile constituents, namely on the terpenic metabolites, is still scarce. The impact of air drying process and subsequent storage at room temperature (up to 21 months) has been evaluated, revealing that a network of effects, such as volatiles diffusion, enzymatic reactions and *de novo* biosynthesis may occur [129]; moreover, it was reported alterations on the levels of volatile terpenic components, namely, linalool and linalool oxides when elderflowers were deep frozen or dried at 60 ºC [223].

Considering the interest on the volatile terpenic metabolites for the elderflowers aroma properties, and also taking into account their potential health benefits, the present study is intended to establish of the profile the volatile terpenic metabolites from *S. nigra* fresh flowers, and to monitor their behavior under different handling and storage conditions currently used in the industry as preservation strategies (freezing, freeze-drying, air drying and vacuum packing with and without light exposure). Considering the commercial interest of fresh elderflowers as a starting point for the formulation of flavoring agents and the fact that this matrix is very perishable, particular attention was devoted to this stage. Norisoprenoids were also screened as they may contribute to distinguish and highlight elderflowers peculiar sensorial features. As these compounds are secondary metabolites whose biosynthesis is modulated by different factors including cultivars, 'Sabugueira' and 'Sabugueiro' cultivars grown in Varosa Valley, Portugal were used as case study samples.

2.3. Material and methods

Comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry detection (GC×GC-ToFMS), was employed to study in-depth the elderflowers released terpenic and norisoprenoids volatiles. The sampling, reporting of chemical analysis and metadata relative to data preprocessing, pretreatment, processing and interpretation were performed according to the Metabolomics Standards Initiative (MSI) [107]. These stages are described in detail in the following sub-sections.

2.3.1. Materials and reagents

For identification purposes, twenty-three standards, comprising monoterpenic (19) and sesquiterpenic compounds (3), and norisoprenoids (1) were used: (-)-*β*-caryophyllene (98.5%), citral (95% mixture of isomers), citronellal (95.0%), *α*-copaene (90.0%), *p*-cymene (99.5%), geraniol (98.0%), geranyl acetone (97%), humulene (96.0%), (R)-(+)-limonene (98%), (-)-linalool (95.0%), myrcene (90%), (+)-rose oxide (99%, mixtures of isomers), *γ*-terpinene (97%), (-)-terpinen-4-ol (95%) and (R)-(+)-*α*-terpineol (98%) were purchased from Fluka (Buchs, Switzerland); 1,8-cineole (98%) was purchased from Panreac (Barcelona, Spain); *α*-pinene (98%), (-)-*β*-pinene (99%), (-)-*α*-thujone (96%) and 1S-(-)-verbenone (94%) were purchased from Aldrich (St. Louis, MO, USA); (-)-limonene oxide (97%, mixture of isomers) and methyl geranate were purchased from Aldrich (Milwaukee, WI, USA); and linalool oxide (97% mixture of isomers) from TCI Europe (Zwijndrecht, Belgium). The retention index probe (an *n*-alkanes series of C_8 to C_{20} straight-chain alkanes, in *n*-hexane) was supplied from Fluka (Buchs, Switzerland). The solid-

phase microextraction (SPME) holder for manual sampling and the fiber coating used were purchased from Supelco (Aldrich, Bellefonte, PA, USA). The SPME device included a fused silica fiber coating, partially cross-linked with 50/30 μm divinylbenzene / carboxen™ / poly(dimethylsiloxane) StableFlex™ (DVB/CAR/PDMS) with 1 cm of length. The fiber presents a wide range capacity for adsorbing and absorbing compounds with different physicochemical properties with molecular weights ranging from 40 to 275. According to the producer's recommendations, the SPME fiber was initially conditioned at 270 ºC for 60 min in the GC injector and daily for 10 min at 250 ºC.

2.3.2. Elderflower sampling, handling and storage conditions

Elderflowers from *S. nigra* L. cultivars 'Sabugueira' and 'Sabugueiro' were supplied by the Cooperativa do Vale do Varosa - RégieFrutas (Tarouca, Portugal). The samples were collected in an experimental field (41.043233ºN, 7.728820ºW) with 0.5 ha, from 12/13-year old plants. This field was selected in order to harvest the two cultivars within the same location and minimize the effect of different edaphoclimatic conditions on plant metabolism. The 'Sabugueira' and 'Sabugueiro' elderflowers were harvested on-site between 9 and 12 a.m. (May 25th, 2012). Approximately 3 kg of elderflowers were harvested, being collected ca. 1.5 kg per cultivar, which were mixed together to prepare a pool, from which several aliquots were arranged. Samples were immediately transported under refrigeration (ca. 2-4 ºC) to the laboratory and then handled, stored and analyzed as described below (Figure 2.1).

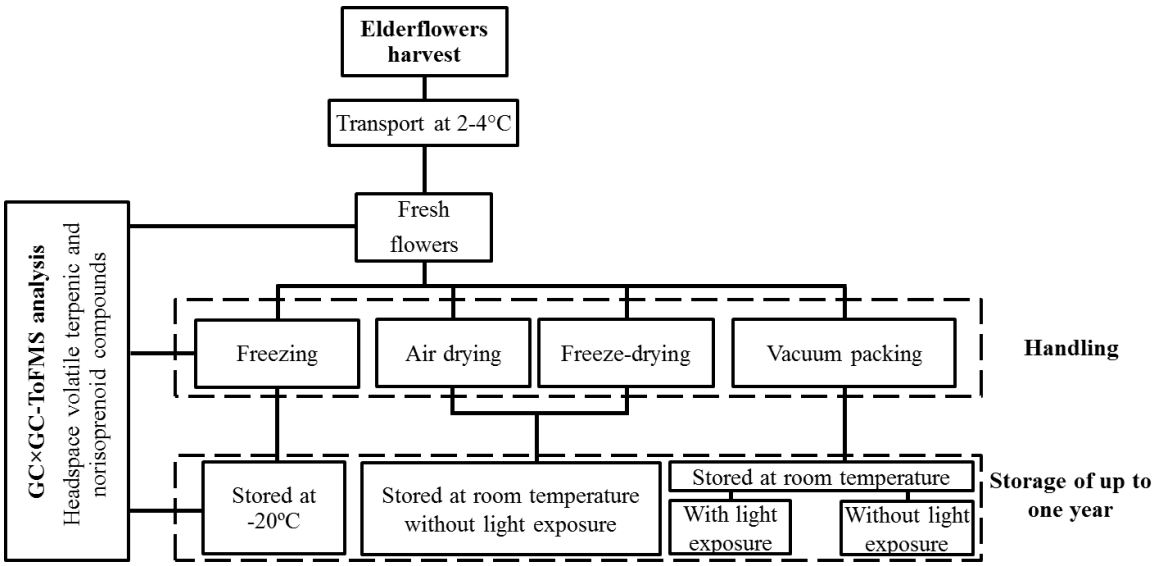


Figure 2.1. Main stages of elderflower's handling and storage.

Fresh elderflowers samples were first analyzed on the harvesting day. Those were then submitted to different handling and storage conditions (Figure 2.1): i) freezing and subsequent storage in polyethylene freezer bags at -20 ºC (2 freezer bags were prepared for each cultivar); ii) air drying (hanged flowers upside down, at 19-21 ºC with relative humidity of 53-55%) and subsequent storage at room temperature without light exposure in polypropylene sample pots (2 pots were prepared for each cultivar); iii) freeze-drying and subsequent storage at room temperature without light exposure in polypropylene sample pots (2 pots were prepared for each cultivar); and submitted to vacuum packing subsequent storage at room temperature, iv) with light exposure, and v) without light exposure. In the particular case of vacuum packing, to ensure that the samples were under vacuum conditions until the time of analysis, one bag was prepared per cultivar and time of analysis. Vacuum packing (Albipack Packaging Solutions, Aveiro, Portugal) was performed at 99% vacuum in heat-sealed polyamide-polyethylene bags (PA/PE-90, Albipack Packaging Solutions, Portugal).

In order to highlight the impact of the storage effect over the target metabolites, samples were analyzed at different storage phases: i) fresh elderflowers; ii) frozen samples: after 20, 32 and 52 weeks of storage; iii) air dried samples: after 1, 3, 16, 32 and 52 weeks of storage; iv) freeze-dried samples: after freeze-drying process (2 days), and after 1, 16, 32 and 52 weeks of storage; and for vacuum packing v) with and vi) without light exposure: after 1, 2, 4, 8, 24 and 52 weeks of storage.

2.3.3. Volatile terpenic and norisoprenoid metabolites determination by HS-SPME/GC×GC-ToFMS

About 0.4 g of elderflowers were weighed and placed into a 12 mL glass vial, corresponding to a ratio of the solid phase volume to the headspace volume ($1/\beta$) of 0.5. Then, the vial was capped with a silicone/polytetrafluoroethylene septum and an aluminum cap (Chromacol LTD, Herts, UK), and placed in a thermostated bath adjusted to 40.0 ± 0.1 ºC. The DVB/CAR/PDMS SPME fiber was inserted in the vial headspace for 20 min. In order to avoid any crossover contamination due to the fiber coating, blanks, corresponding to analysis of the SPME fiber not submitted to any extraction procedure, were run between sets of three analyses.

The volatiles adsorbed and absorbed on the SPME fiber coating were determined using a LECO Pegasus 4D GC×GC–ToFMS system (LECO, St. Joseph, MI, USA) consisting of an Agilent GC 7890A gas chromatograph (Agilent Technologies, Inc., Wilmington, DE), with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven, and a mass spectrometer equipped with a ToF analyzer. After the extraction/concentration step, the SPME fiber was manually introduced into the port at 250 ºC for analytes desorption. The injection port was lined

with a 0.75 mm I.D. glass liner. Splitless conditions (30 s) were used. An Equity-5 30 m × 0.32 mm I.D., 0.25 μm film thickness (Supelco, Bellefonte, PA, USA) was used as first-dimension column (^{1}D) and a DB-FFAP 0.79 m × 0.25 mm I.D., 0.25 μm film thickness (J&W Scientific Inc., Folsom, CA, USA) was used as a second-dimension column (^{2}D). The carrier gas was helium at a constant flow rate of 2.50 mL/min. The primary oven temperature was programmed from 40 ºC (1 min) to 230 ºC (3 min) at 10 ºC/min and the secondary oven program was 30 °C offset above the primary one. Both the MS transfer line and MS source temperatures were set at 250 °C. The modulation period was 6 s, keeping the modulator at 20 °C offset above primary oven, with hot and cold pulses by periods of 0.90 and 2.10 s, respectively. The mass spectrometer was running in the EI mode at 70 eV and detector voltage of -1456 V, using an *m/z* range of 35-300.

Total ion chromatograms were processed using the automated data processing software ChromaTOF® (LECO) at signal-to-noise threshold of 100. Spectral deconvolution was computationally processed, being intended to reconstruct clean mass spectrum for each component; while, the GC×GC peak area was obtained by transforming the series of side-by-side second-dimension chromatograms, into a two-dimensional chromatogram, being the GC peak area proportional to the generated signal intensity [99]. Contour plots were used to evaluate the separation general quality and for manual peak identification. For identification purposes, the mass spectrum and retention times (^{1}D and ^{2}D) of the analytes were compared with standards, when available (23 standards were used, as shown in Table 2.1 and Table A2.1 from annex). Also, the mass spectrum of each detected metabolite was compared with the mass spectral libraries, namely an in-house library of standards and two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0 –Mainlib and Replib). A mass spectral match factor, similarity >850/1000, was used to decide whether a peak was correctly identified. Moreover, a manual analysis of mass spectra was performed, combining additional information like retention index (RI) value, which was experimentally determined according to van den Dool and Kratz RI equation [224]. A C_8-C_{20} *n*-alkanes series was used for RI determination, comparing these values with reported ones in existing literature for chromatographic columns similar to ^{1}D column above mentioned [75,76,108,111,225–233]. The calculated retention index (RI_{calc}) only differed 0–5% when compared to literature data (RI_{lit}). The DTIC (deconvoluted total ion current) GC×GC peak area data were used as an approach to estimate the relative content of each metabolite. Three independent aliquots of each sample were analyzed.

2.3.4. Data statistical analysis

A full data matrix consisting of 65 variables (metabolites) and 156 observations was constructed (Table A2.1, from annex). The 156 observations correspond to 2 cultivars × 26 conditions (each one with 3 independent aliquots). The 26 conditions include fresh elderflowers and the samples submitted to 4 handling and 5 storage conditions, analyzed at several moments through 1 year.

In order to evaluate the effect of the handling and storage conditions versus fresh elderflowers composition, i.e., freezing, freeze-drying, air drying and vacuum packing (with and without light exposure), and storage of up to one year, on the global content or on the content of each chemical family under study, different matrices were constructed from the above full dataset (Table A2.1), by using only the observations respective to each handling and storage condition. One-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's HSD) using the MetaboAnalyst 3.0 (web interfaces) [234], was applied on these matrices (data from Table A2.1). It was considered statistically significant when $p<0.05$.

To rapidly and visually assess the metabolites profile of fresh elderflowers, a reduced dataset was prepared from the previous full dataset using only data corresponding to fresh elderflowers, i.e. comprising data from the 65 variables and 6 observations (2 fresh cultivars, each one with 3 independent aliquots) (Table A2.1, from annex). Heatmap visualization was applied on this sub-dataset, using absolute GC peak area and transforming each variable GC peak area by applying a logarithm function, using the Unscrambler® X (30-day trial version, CAMO Software AS, Oslo, Norway). Additionally, Hierarchical Clusters Analysis (HCA) was performed on this sub-dataset using MetaboAnalyst 3.0 (web interfaces) [234], to further examine the differences and similarities between the fresh elderflowers metabolite profiles. HCA was applied to the absolute and log transformed GC peak areas of the 65 variables, each one corresponding to three independent assays. Ward's minimum variance algorithm method and squared Euclidean distances were employed.

2.4. Fresh elderflowers volatile terpenic and norisoprenoids profile

Considering the commercial interest of fresh elderflowers as a starting point for the formulation of flavoring agents and the fact that this matrix is very perishable, particular attention was devoted to this stage. Thus, the volatile terpenic and norisoprenoid profile was first established on fresh elderflowers samples for the two cultivars.

The most reliable way to confirm the identification of each metabolite is based on authentic standard co-injection, which in several cases is economically prohibitive, and often unachievable in the time available for analysis, or because standards are not commercially available. Thus, in this study, a strategy was attempted to comprise the arrangement of several parameters. Therefore, criteria for putative identification was based on the *i*) calculation of RI index (RI_{calc}) and comparison with the literature data (RI_{lit}) for the 1D column or equivalents, *ii*) analysis of the retention times of the first and second dimensions, as similar chemical structures are grouped into the same 2D chromatographic space, which provides useful information based on both boiling point and polarity (if NP / P set of columns is used) – principle of the structured chromatogram, *iii*) analysis of the MS fragmentation pattern of each analyte and comparison with databases and standards and *iii*) MS spectral similarity value in relation with commercial and house-made libraries. Figure 2.2 exhibits the mass spectra and the main fragmentations of representative components from the three chemical families under study. The mono and sesquiterpenic compounds and norisoprenoids present a diversity of chemical structures, i.e. linear and cyclic structures with one or more rings, and with different functional groups. Their mass spectra reveal this chemical structural diversity, however it is possible to observe a fragmentation pattern common within each chemical family. The mass spectra of the target chemical families commonly show ions at *m/z* 41, 43, and 69 (e.g. Figure 2.2), but these ions are also reported on other chemical families frequently found in plant extracts (i.e. alkanes, alkenes, aliphatic ketones, among others) [78]. Thus, others fragments are currently used to check the identification of the mono and sesquiterpenic compounds and norisoprenoids. For instance, ions at *m/z* 93, 121 and 136 (molecular ion) are often used for the detection of monoterpenic compounds (Figure 2.2A) [78]. The ion at *m/z* 161 is commonly used for sesquiterpenes (Figure 2.2B), as well as, the ion at *m/z* 204 (molecular ion) [78]. Regarding norisoprenoids, those also share a great structural diversity with backbones from C_{10} to C_{18} (Table 2.1). For instance, the mass spectrum and fragmentation pattern of the dihydroedulan, detected in the elderflower (Table 2.1) and previously detected in the elderberries [36], is illustrated on Figure 2.2C. Along with the common ions at *m/z* 43 and 69, the ions at *m/z* 194 (molecular ion), 179 (loss of methyl group) and 107 (subsequent loss of the oxane structure)

were obtained. Furthermore, the product ion at *m/z* 93 was obtained which is often detected on norisoprenoids.

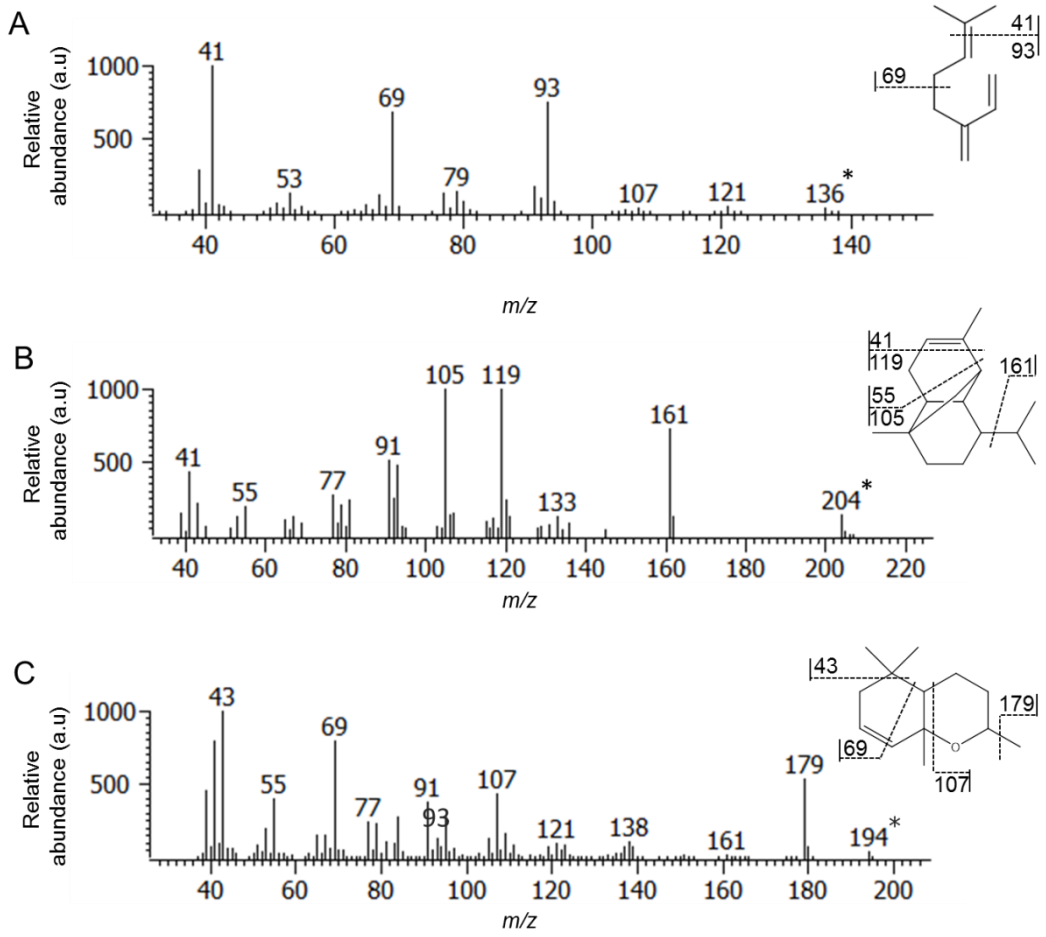


Figure 2.2. Illustrative examples of the mass spectra and main fragmentations of representative components of the three target families under study: (A) myrcene, (B) α-copaene and (C) dihydroedulan. * molecular ion (*m/z*)

A representative extracted ion GC×GC–ToFMS chromatogram contour plot from the fresh 'Sabugueiro' elderflower cultivar is illustrated in Figure 2.3.

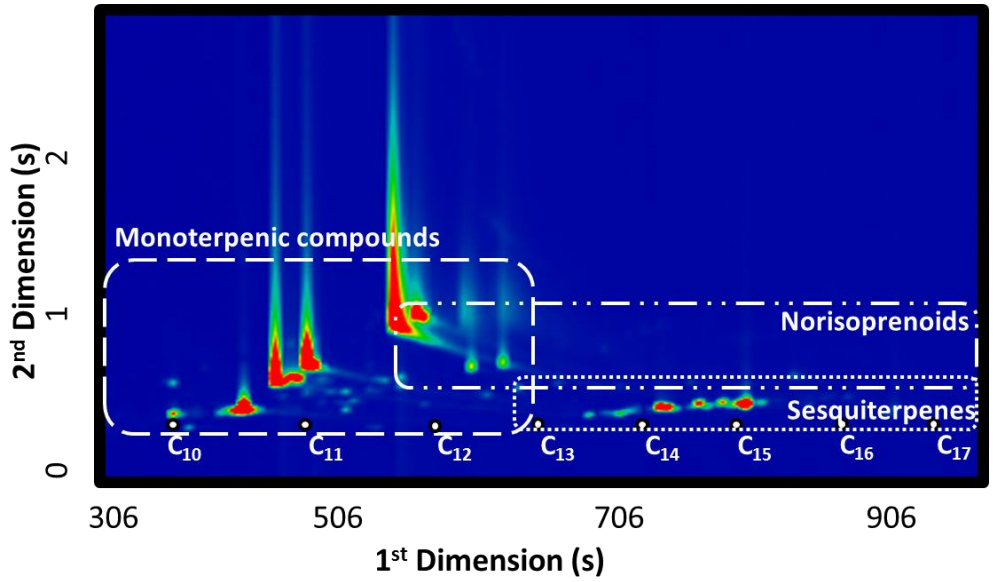


Figure 2.3. Blow up of GC×GC–ToFMS extracted ion chromatogram contour plot of *m/z* 93, 161, and 204 of the released volatile compounds from 'Sabugueiro' fresh elderflowers. The white spots indicate the position of the series of alkanes (C_{10}-C_{17}). The chromatographic spaces corresponding to monoterpenic, norisoprenoid and sesquiterpenes compounds are highlighted.

The extracted ion contour plot, using specific *m/z* diagnostic ions selected to identify the terpenic (monoterpenic and sesquiterpenic) and norisoprenoid metabolites, was applied in order to increase the sensitivity and specificity, thus minimizing the contribution of co-eluted compounds. The contour plot illustrates that the analytes were separated according to their physicochemical characteristics, through volatility (^{1}D) and polarity (^{2}D). Thus, structurally related compounds occupy similar 2D spaces, being highlighted chromatographic spaces that include along ^{1}D the monoterpenic (C_{10}, and its derivatives, C_{11}), norisoprenoids (C_{10}-C_{18}) and sesquiterpenes metabolites (C_{15}), and along ^{2}D, from hydrocarbon to oxygen-containing structures (from lower to higher ^{2}D retention times).

The high chromatographic resolution, low detection limits, and sensitivity of the GC×GC-ToFMS allowed the identification of 65 metabolites on 'Sabugueira' and 'Sabugueiro' *S. nigra* flowers (Table 2.1), being distributed over 47 monoterpenic (monoterpenes and oxygen-containing monoterpenes), 13 sesquiterpenes and 5 norisoprenoid metabolites. Among these, 38 are reported for the first time in elderflowers (highlighted in Table 2.1), resulting on a significant expansion of the *S. nigra* flowers chemical profiling only possible through the use of 2D orthogonal GC analysis.

Table 2.1. Volatile terpenic and norisoprenoid metabolites detected in elderflowers (*S. nigra* L.) using HS-SPME/GC×GC-ToFMS, including relevant chromatographic data used to assess metabolite identification and the corresponding aroma descriptors.

Peak Nº	^1t_R [a] (s)	^2t_R [a] (s)	Compound	RI_{lit} [b]	RI_{calc} [c]	Chemical Formula	Aroma descriptor [d]	ref RI_{lit} for 2D-GC	ref RI_{lit} for 1D-GC	MSI level [e]	Compound Previously Reported on Elderflowers [f]
			Monoterpenic compounds								
			Hydrocarbon-type								
1	306	0.400	*α*-Pinene	941	932	$C_{10}H_{16}$	Pine, turpentine	[225]	-	1	-
2	348	0.440	*β*-Pinene	987	975	$C_{10}H_{16}$	Turpentine, resinous	[225]	-	1	-
3	360	0.460	Myrcene	1008	988	$C_{10}H_{16}$	Balsamic, herbaceous	[225]	-	1	✓
4	378	0.450	3-Carene	1020	1009	$C_{10}H_{16}$	Turpentine	[225]	-	2	✓
5	396	0.490	Limonene	1031	1027	$C_{10}H_{16}$	Fruity, orange, lemon	[108]	-	1	✓
6	396	0.550	*p*-Cymene	1039	1027	$C_{10}H_{14}$	Fruity, citrus	[225]	-	1	✓
7	414	0.490	Ocimene	1043	1051	$C_{10}H_{16}$	Floral, sweet	[225]	-	2	✓
8	426	0.500	*γ*-Terpinene	1080	1063	$C_{10}H_{16}$	Fruity, lime	[226]	-	1	✓
9	444	0.590	2,6-Dimethyl-2,6-octadiene	1064	1082	$C_{10}H_{18}$	-	[226]	-	2	-
10	450	0.510	*α*-Terpinolene	1097	1093	$C_{10}H_{16}$	Fruity, citrus	[225]	-	2	✓
11	456	0.660	*p*-Cymenene	1090	1094	$C_{10}H_{12}$	Citrus	-	[229]	2	-
12	480	0.620	Cosmene isomer	1134	1122	$C_{10}H_{14}$	Floral	-	[229]	2	-
13	492	0.610	Cosmene isomer	1134	1134	$C_{10}H_{14}$	Floral	-	[229]	2	-
14	624	0.630	*m/z* 43,94,55,68	-	1288	-	-	-	-	3	-
			Oxygen containing								
15	378	0.520	Dehydroxylinalool oxide	1006	1008	$C_{10}H_{16}O$	Green, minty	[226]	-	2	-
16	402	0.500	1,8-Cineole	1039	1034	$C_{10}H_{18}O$	Peppermint, menthol	[225]	-	1	✓
17	432	0.850	Myrcenol	1103	1069	$C_{10}H_{18}O$	Rose	[225]	-	2	-
18	438	0.680	Linalool oxide (furanoid) isomer [g]	1078	1076	$C_{10}H_{18}O_2$	Elderflower, sweet	[225]	-	1	✓
19	450	0.620	Fenchone	1093	1090	$C_{10}H_{16}O$	Camphoraceous	[225]	-	2	-
20	450	0.910	Hotrienol isomer	1122	1090	$C_{10}H_{16}O$	Elderflowers, floral	[225]	-	2	✓
21	456	0.640	Linalool oxide (furanoid) isomer[g]	1097	1094	$C_{10}H_{18}O_2$	Elderflower,	[225]	-	1	✓

22	462	0.790	Linalool	1096	1101	$C_{10}H_{18}O$	sweet Floral	[108]	-	1	✓
23	468	0.680	Thujone	1120	1108	$C_{10}H_{16}O$	Minty	[225]	-	1	✓
24	468	0.900	Hotrienol isomer	1122	1108	$C_{10}H_{16}O$	Elderflowers, floral	[225]	-	2	✓
25	474	0.640	Rose oxide[g]	1130	1115	$C_{10}H_{18}O$	Floral, elderflower	[225]	-	1	✓
26	492	0.490	Linalool, methyl ether	1137	1137	$C_{11}H_{20}O$	-	-	[228]	2	-
27	498	0.620	Limonene oxide[g]	1127	1144	$C_{10}H_{16}O$	Camphoraceous	[225]	-	1	-
28	504	0.650	Tagetone	1146	1150	$C_{10}H_{16}O$	Bitter fruity	-	[228]	2	-
29	510	0.620	Citronellal	1159	1154	$C_{10}H_{18}O$	Lemon, citronella	[225]	-	1	-
30	510	0.740	Lilac aldehyde isomer	1154	1155	$C_{10}H_{16}O_2$	Floral	-	[231]	2	-
31	516	0.600	Nerol oxide	1153	1162	$C_{10}H_{16}O$	Elderflower syrup	-	[228]	2	✓
32	516	0.675	Lilac aldehyde isomer	1163	1164	$C_{10}H_{16}O_2$	Floral	-	[231]	2	-
33	522	0.740	Lilac aldehyde isomer	1174	1172	$C_{10}H_{16}O_2$	Floral	[226]	-	2	-
34	528	1.090	Linalool oxide (pyranoid)	1174	1177	$C_{10}H_{18}O_2$	Floral	-	[229]	2	✓
35	534	0.700	4-Terpineol	1181	1181	$C_{10}H_{18}O$	Woody	[225]	-	1	✓
36	546	1.790	Myrtenol	1206	1194	$C_{10}H_{16}O$	Camphoraceous, minty	[225]	-	2	-
37	552	0.850	α-Terpineol	1206	1201	$C_{10}H_{18}O$	Sweet, floral	[225]	-	1	✓
38	564	0.880	Verbenone	1214	1216	$C_{10}H_{14}O$	Minty, spicy	[225]	-	1	-
39	564	0.925	Lilac alcohol	1237	1217	$C_{10}H_{18}O_2$	Green, grassy	[226]	-	2	-
40	582	0.980	Nerol	1242	1236	$C_{10}H_{18}O$	Floral, citrus	[225]	-	2	✓
41	588	0.770	Citral[g]	1241	1244	$C_{10}H_{16}O$	Lemon, lime peel	[226]	-	1	-
42	594	1.040	Geraniol	1235	1254	$C_{10}H_{18}O$	Woody, leaf	[225]	-	1	✓
43	600	0.580	Methyl citronellate	1261	1261	$C_{11}H_{20}O_2$	Fruity, apple	-	[228]	2	-
44	612	0.620	Citronellyl formate	1275	1274	$C_{11}H_{20}O_2$	Fruity	-	[228]	2	-
45	612	0.800	Geranial	1287	1274	$C_{10}H_{16}O$	Lemon	[226]	-	2	-
46	654	0.500	Methyl geranate	1323	1325	$C_{11}H_{18}O_2$	Waxy	-	[228]	1	-
47	654	1.080	*m/z* 81,67,43,153	-	1327	-	-	-	-	3	-
			Sesquiterpenes								
48	678	0.460	α-Cubebene	1354	1354	$C_{15}H_{24}$	Herbaceous	[75]	-	2	-
49	702	0.470	α-Copaene	1375	1385	$C_{15}H_{24}$	Woody	[76]	-	1	✓
50	708	0.480	β-Bourbonene	1379	1393	$C_{15}H_{24}$	Herbaceous,	[76]	-	2	-

							woody				
51	714	0.490	*β*-Elemene	1380	1401	$C_{15}H_{24}$		[76]	-	2	-
52	726	0.567	*α*-Bergamotene	1439	1416	$C_{15}H_{24}$	Woody	[75]	-	2	-
53	732	0.500	*β*-Caryophyllene	1417	1432	$C_{15}H_{24}$	Woody, spicy	[76]	-	1	✓
54	756	0.515	Aromadendrene	1447	1455	$C_{15}H_{24}$	Cucumber	[75]	-	2	-
55	762	0.540	*α*-Humulene	1450	1462	$C_{15}H_{24}$	Woody	[76]	-	1	-
56	780	0.540	Germacrene D	1473	1486	$C_{15}H_{24}$	Woody	[76]	-	2	-
57	798	0.520	*α*-Farnesene	1507	1510	$C_{15}H_{24}$	Herbal	[227]	-	2	-
58	810	0.540	Δ-Cadinene	1525	1528	$C_{15}H_{26}$	Woody	[76]	-	2	-
59	810	0.600	Calamenene	1525	1528	$C_{15}H_{22}$	Spicy, floral	[230]	-	2	-
60	1014	0.680	*m/z* 69, 41, 55	-	1841	-	-		-	3	-
			Norisoprenoids								
61	558	0.705	Safranal	1241	1201	$C_{10}H_{14}O$	Herbaceous, saffron	[226]	-	2	✓
62	572	0.705	*β*-Cyclocitral	1261	1225	$C_{10}H_{16}O$	Woody	[226]	-	2	-
63	632	0.515	Dihydroedulan	1289	1300	$C_{13}H_{22}O$	Elderberry	-	[232]	2	-
64	756	0.660	Geranyl acetone	1454	1455	$C_{13}H_{22}O$	Floral, rose	[111]	-	1	-
65	1020	0.570	Phytone	1856	1854	$C_{18}H_{36}O$	-	-	[233]	2	-

[a] Retention times for first (^1t_R) and second (^2t_R) dimensions in seconds.
[b] RI, Retention Index reported in the literature for Equity-5 GC column or equivalents.
[c] RI: Retention Index obtained through the modulated chromatogram.
[d] Aroma descriptor based from the literature [36,129,235–237].
[e] Level of metabolite identification according to Metabolomics Standards Initiative: (l) Identified compounds using standards coinjection; (2) putatively annotated compounds; (3) putatively characterized compound classes; and (4) unknown compounds [107].
[f] Compound previously reported on elderflowers [39,94,129,130,132,133].
g The used standards are a mixture of isomers.

The aroma descriptors associated to the elderflowers volatile terpenic compounds and norisoprenoids (Table 2.1) may contribute to explaining the sensory properties attributed to elderflowers, which are in line with the sensorial characteristics described on previous olfactory studies [39,94,130]. Amongst the metabolites described for the first time in *S. nigra* flowers, several are described according to the literature as having aroma notes of pine, turpentine, resinous, citric and floral (hydrocarbon-type monoterpenes); green, grassy, minty, camphoraceous, waxy, floral, rose, citronella, fruity, lemon, lime, apple and spicy (oxygen containing monoterpenic compounds); herbaceous, woody, cucumber, spicy and floral (sesquiterpenes); and finally, woody, elderberry, floral and rose (norisoprenoids) [36,129,235–237] (Table 2.1). These compounds may contribute to explain the floral, fruity, grassy, woody, minty, spicy and herbaceous notes [130] that currently characterize the aroma of elderflowers. It is worth mentioning that the major contributors for the characteristic elderflower aroma, i.e., hotrienol, rose oxide, nerol oxide, linalool oxides, *α*-terpineol and linalool [39,130] were identified on fresh elderflowers (Table 2.1).

The heatmaps from Figure 2.4 reveal information about the volatile terpenic compounds and norisoprenoids profiles from fresh elderflowers, based on the absolute (A) and logarithmized GC peak areas of the 65 metabolites under study (B). Each metabolite content was illustrated using different colors, from blue (minimum), to red (maximum).

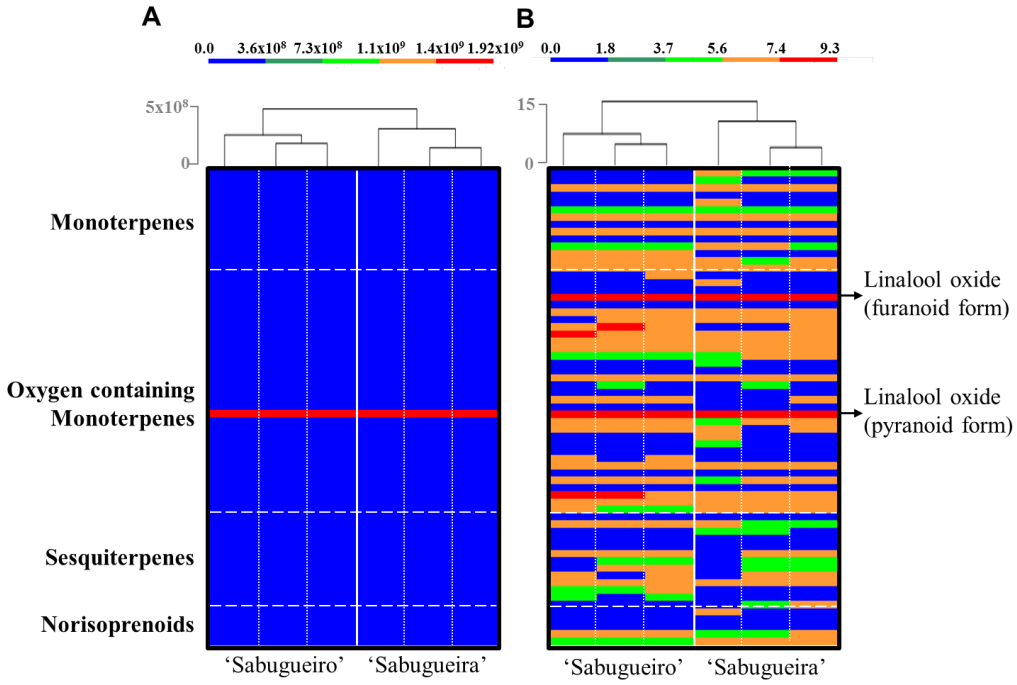


Figure 2.4. Heatmap representation of the 65 metabolites (putatively) identified from 'Sabugueira' and 'Sabugueiro' fresh elderflowers: (A) GC peak areas, expressed as arbitrary units. (B) GC peak areas normalized by logarithm function. Each compound content was illustrated through different colors (from blue, minimum, to red, maximum). Dendrogram for the HCA results using Ward's cluster algorithm to the data set was also included. The Euclidean distances are presented on the HCA Y-axis.

As observed by the red color, linalool oxide (in pyranoid form) represents the major component for both cultivars (Figure 2.4A and Table A2.1 from annex), accounting from 82.5% to 86.9% of GC peak area for 'Sabugueiro' and 'Sabugueira', respectively. The corresponding furanoid form represents 9.0% to 9.3% of the total GC peak area, for 'Sabugueira' and 'Sabugueiro' cultivars, respectively. A Log transformation was performed in order to in-depth analysis of all the detected metabolites (Figure 2.4B). Oxygen containing monoterpenes prevailed (colors orange and red) (Figure 2.4B), and those represented 97.9% in 'Sabugueiro' and 98.7% in 'Sabugueira' of the identified metabolites, while, sesquiterpenic and norisoprenoids represented, respectively, 0.6% and 0.1% for both cultivars (Table A2.1 from annex). Hierarchical clusters analysis (included on Figure 2.4A and B) revealed that the samples were clustered based on cultivars. The metabolites that contributed more to the cultivars clustering were the monoterpenes, ocimene (7), 2,6-dimethyl-2,6-octadiene (9), cosmene isomers (12, 13), the oxygen containing monoterpenes hotrienol isomers (20, 24), linalool (22), 4-terpineol (35), the sesquiterpene *α*-copaene (49) and the norisoprenoid geranyl acetone (64) on 'Sabugueiro' cultivar, while for 'Sabugueira' cultivar it was the monoterpenic compounds, *α*-pinene (1), *p*-cymenene (11), citronellal (29), and the sesquiterpene *β*-bourbonene (50). According to the literature (Table 2.1), the aroma descriptors of the metabolites that clustered 'Sabugueiro' cultivar are floral, rose, elderflower, sweet and woody notes; while the metabolites with citronella, citrus, lemon, herb, woody, pine and turpentine aroma notes were linked to 'Sabugueira' clustering. The specific cultivar metabolite profile with analogous aroma descriptors may imply differences at the sensorial level on elderflowers-based products, as already documented for other elderflowers cultivars [39,130]. Although more studies, including sensorial ones, should be performed to relate the elderflowers volatile composition from different cultivars and its potential impact on the elderflower peculiarities.

2.5. Insights of handling and storage impact on the volatile terpenic and norisoprenoids profile

To evaluate the impact of different handling and storage conditions currently used in the industry as preservation strategies (freezing, freeze-drying, air drying and vacuum packing), volatile terpenic compounds and norisoprenoids were monitored for up for 1 year. In the first step, the overall content of the released volatile terpenic and norisoprenoids (expressed as GC peak area), and the linalool oxides (major metabolites), were monitored (Figure 2.5) and in the second step, a more detailed discussion, highlighting the influence of the different handling and storage strategies on the different studied chemical families, was put forward (Figure 2.6).

After an intermediary storage time (24-32 storage weeks), a decrease of the total content of the target metabolites was observed (p<0.05), with overall losses that ranged from 27.5-47.4% (frozen elderflowers, Figure 2.5C) to 36.2-85.2% (freeze-dried elderflowers, Figure 2.5B), depending on the storage time and cultivar. In fact, the freeze-drying process by itself, led to overall losses ranging between 36.2% and 45.7% (p<0.05), depending on the cultivar. Regarding the remaining handling and storage conditions, it was observed an overall decrease of metabolites content of 27.0-58.6% and 30.7-52.2%, for vacuum packing with light and without light exposure (Figure 2.5D and E), respectively, and 31.5-80.3% for air drying (Figure 2.5A). Considering longer storage times, i.e., after one year of storage, the reported decrease of the total content of the target metabolites was up to 47.4% for frozen elderflowers (Figure 2.5C); up to 67.1% and 70.7%, when the elderflowers were vacuum packed and kept under light exposure (Figure 2.5D) and without light exposure (Figure 2.5E), respectively; up to 81.9% for air drying (Figure 2.5A); and up to 85.2% for freeze-dried elderflowers (Figure 2.5B). Light parameter was evaluated for vacuum packing, in order to simulate real storage conditions and also to infer if this parameter affected the studied chemical families, as light may affect the pattern of secondary metabolites [238]. Under vacuum packing, the light parameter impact was not clear, since a similar trend is noticeable between the two conditions (Figure 2.5D and Figure 2.5E).

It is worth to mention that a general perspective of postharvest impact is presented, although, a complex and dynamic process was observed, being discussed in detail with support of Figure 2.6.

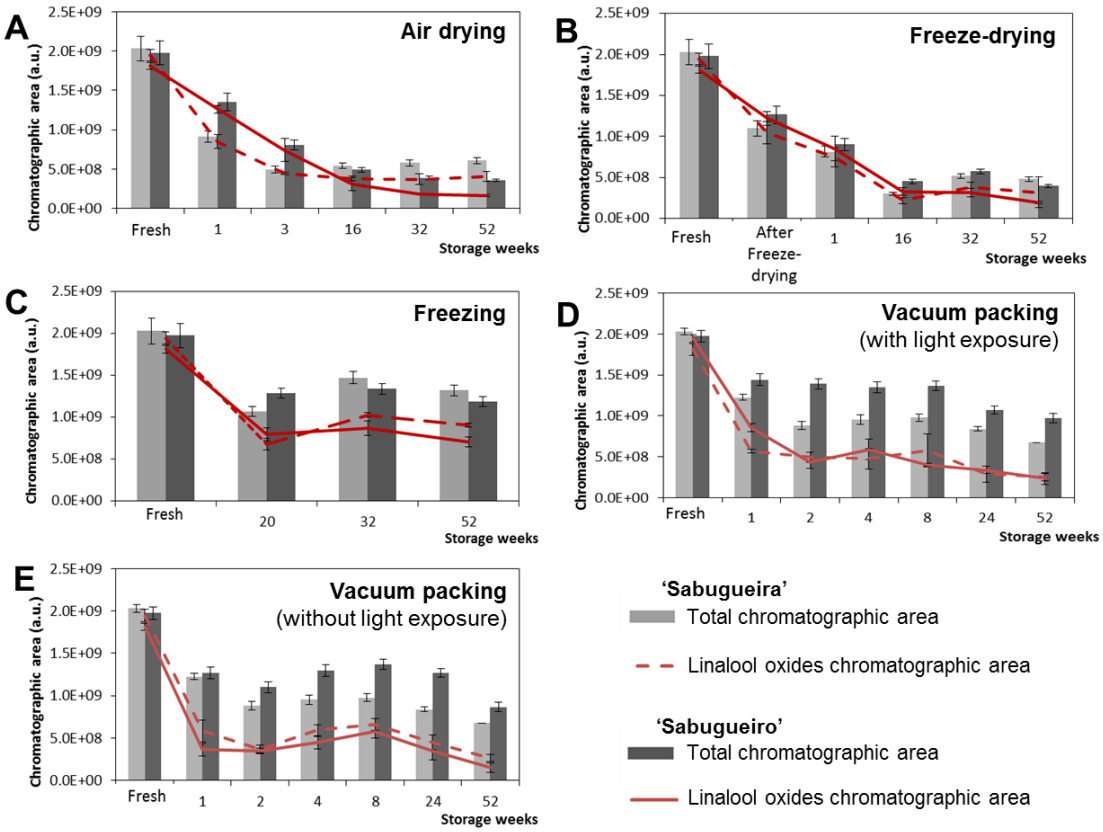


Figure 2.5. Released content of terpenic and norisoprenoid metabolites from 'Sabugueira' and 'Sabugueiro' elderflowers handled for different processes and storage conditions for up to 1 year: Air drying (A) and freeze-drying (B) and subsequent storage at room temperature; freezing (C) and subsequent storage at –20 ºC; and vacuum packing stored at room temperature with (D) and without (E) light exposure. Bars: expressed as total chromatographic areas; Lines: expressed as linalool oxides chromatographic areas. a.u.: arbitrary units.

The reported results for dried elderflowers (Figure 2.5A and 4B), are in line with previous studies, as most of drying methods often fail to fully preserve volatile aroma compounds [239], namely, on air and oven dried vegetable matrices, as elderflowers [129,223], thyme leafs (*Thymus vulgaris* L.) and sage leafs (*Salvia officinalis* L.) [240,241]; as well as, on freeze-dried bay leaves (*Laurus nobilis* L.) [242] and parsley leaves (*Petroselinum crispum* L.) [243]. Likewise, for the other postharvest processes, the reported results are in agreement with previously reported data, namely for frozen elderflowers [223], and others plants, such as *L. nobilis* leaves [242]; and also tomatoes [244] and several apples cultivars [245] stored under sub-atmospheric pressure storage.

Figure 2.5A to E show that, in general, the overall trends observed for the targeted chemical families were defined by the major metabolites, linalool oxides, which, hence, may be suggested as markers that illustrate the impact of the studied postharvest conditions over the overall terpenic and norisoprenoids metabolites.

In order to better understand the underlying complexity of the impact of different postharvest handling and storage conditions over the volatile terpenic and norisoprenoid metabolites, it is important to follow-up the behavior of these different chemical families. Therefore, the variation trends of oxygen containing monoterpenes, hydrocarbon monoterpenes, sesquiterpenes and norisoprenoids (Figure 2.6A to D), towards the different handling and storage conditions were analyzed. In addition, the main physicochemical processes that might explain the observed variations are included based on literature data for elderflowers and other vegetable matrices [129,240–242,246].

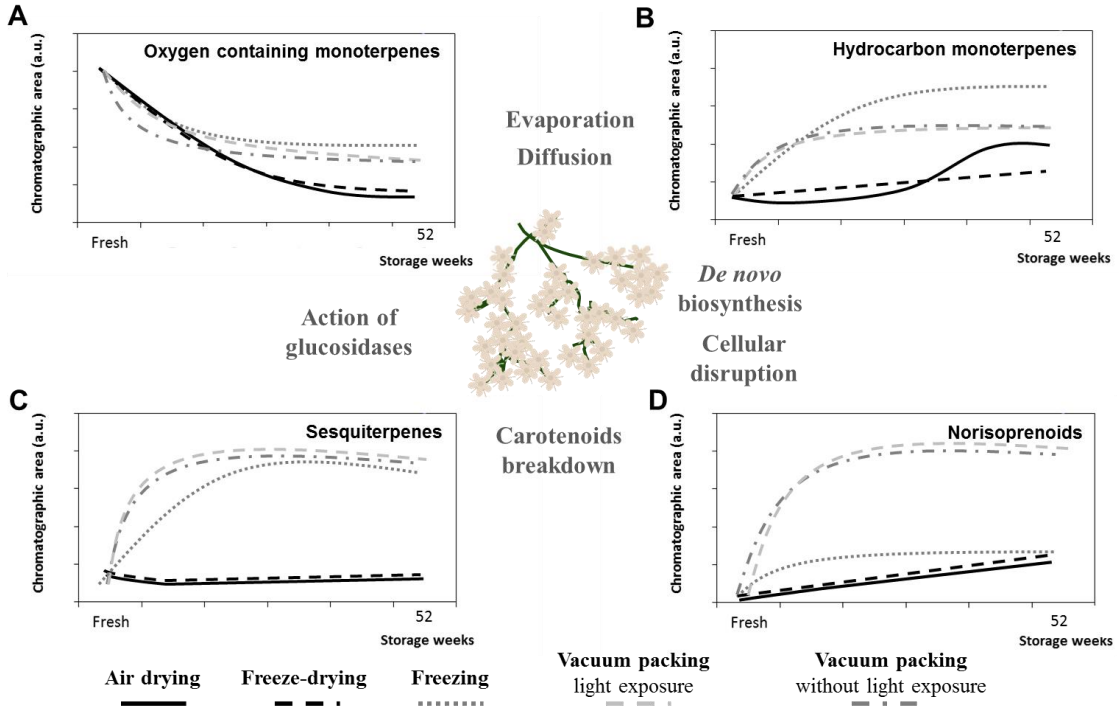


Figure 2.6. Variation trends of the GC peak areas of oxygen containing monoterpenes (A**), hydrocarbon monoterpenes (**B**), sesquiterpenes (**C**) and norisoprenoids (**D**) towards the different handling and storage conditions for up to 1 year. Main physicochemical processes that explain the observed variations are also listed in the central part of the figure** [129,240–242,246]**. a.u.: arbitrary units.**

The oxygen containing monoterpenes tended to decrease along storage (Figure 2.6A), which may be explained mainly due to diffusion and evaporation processes [129,240,241]; cellular disruption caused by freezing may also enhance the release of volatile compounds that were retained on vegetable cells [246]; and upon vacuum, the removal of aroma compounds occurs [244,245]. Despite the overall decrease of oxygen containing monoterpenes, it is important to note that the content of some particular volatile monoterpenoids increased. For instance, hotrienol isomer, with an elderflower/floral aroma note, increased on air dried elderflowers, which has already been reported for this matrix and process, being associated with the action of enzymes such

as glucosidases that unbound the volatile components from glycosides present on the matrix [129]. The increasing content of this metabolite along storage is also observed for the other postharvest conditions studied here (freeze-drying, freezing and vacuum packing). The levels of nerol oxide and *α*-terpineol, other important contributors for the characteristic elderflower aroma, were also higher on stored elderflowers than in fresh ones, for all storage conditions and for both cultivars.

Concerning hydrocarbon monoterpenes (Figure 2.6B) and norisoprenoids (Figure 2.6D), it is possible to observe that after one year of storage their levels are higher than in fresh flowers. In fact, vacuum packing (both with and without light exposure) was a suitable postharvest condition to preserve these two chemical families, particularly for norisoprenoids ones. Likewise, the sesquiterpenes levels tended to increase through storage (Figure 2.6) under freezing and vacuum packing. The trends of hydrocarbon monoterpenes and norisoprenoids were defined by limonene and geranyl acetone, for air drying and freeze-drying processes and on both cultivars, which are metabolites described to have fruity, orange, lemon, floral and rose aroma notes. For freezing storage, the metabolites that defined these changes were ocimene (floral and sweet aroma notes), *β*-caryophyllene (woody and spicy), *α*-farnesene (herbal) on 'Sabugueira'; and ocimene (floral and sweet), *α*-pinene (pine and turpentine), *β*-caryophyllene (woody and spicy), and geranyl acetone (floral and rose) on 'Sabugueiro'. Regarding vacuum packing, limonene (fruity, orange and lemon), *β*-caryophyllene (woody and spicy) and dihydroedulan (elderberry) contributed to the higher levels of mono-, sesquiterpenes and norisoprenoids on both cultivars. The *de novo* biosynthesis of terpenes and carotenoids breakdown during the drying process may explain the higher levels of these families [129]. Increasing of the levels of sesquiterpenes and norisoprenoids were also reported on other matrices, namely on frozen raspberries (*Rubus idaeus* L.) [246] and *L. nobilis* leaves [242]. During freezing storage, the rate of the biological processes that take place in vegetative tissue are reduced, although the different levels of these families might indicate that secondary plant metabolism still remains active [247], contributing to explaining, at least in part, the observed variations. The losses of the major metabolite, linalool oxide, exhibiting a floral aroma descriptor, and changes observed on the levels of other important metabolites for the characteristic elderflower aroma, allows to infer that these handling and storage conditions might have impact on the characteristic aroma of fresh elderflowers, which in fact was sensorially observed during this work (data not shown as planned sensorial assays were not performed).

2.6. Conclusions

The volatile terpenic and norisoprenoid profile from *S. nigra* flowers from two cultivars was established in-depth using GC×GC-ToFMS, revealing 65 metabolites, of which 38 are reported for the first time on this species. The data regarding the metabolites reported for the first time, complemented with data collected on the literature about their respective aroma descriptors, highlighted the relation of these metabolites with the elderflowers aroma profile, namely camphor, mint, flower, rose, citronella, fruit, citric, apple, spice, pine, resin, wax, wood, turpentine, green, herb and grass notes. Thus, this study contributes for *S. nigra* flowers terpenic and norisoprenoid profile construction, providing a step forward on their valorization and understanding of their role as flavoring agents.

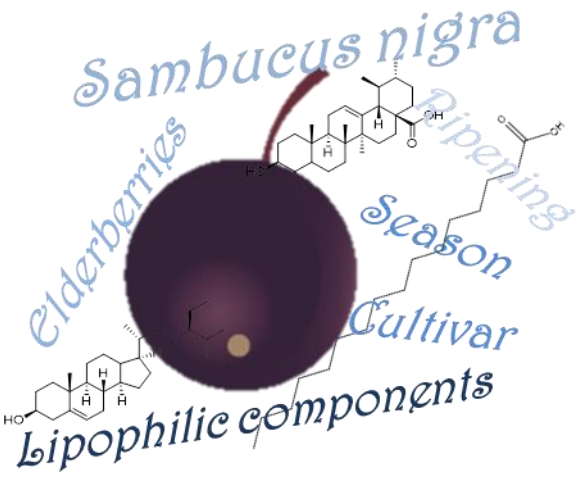
Postharvest impact was assessed during up to one year of storage, being observed a decrease on the overall volatile terpenic and norisoprenoids content for all tested conditions, when compared to fresh elderflowers ($p<0.05$). However, levels of monoterpenic hydrocarbons and norisoprenoids were higher on stored samples than in fresh ones, especially the norisoprenoids increased significantly upon vacuum packing. For intermediary storage times (24-32 weeks), both freezing and vacuum packing (with and without light exposure), seemed to be the most appropriate methods to preserve the total metabolites content, while for longer storage times, freezing seemed the most suitable method. Otherwise, freeze-drying promoted the higher decreasing on the terpenic and norisoprenoids overall levels. Linalool oxides defined, in general, the observed overall trends, being, hence, suggested as expedite markers to estimate the impact of the studied postharvest conditions over the overall terpenic and norisoprenoid metabolites.

The observed modifications along handling and storage conditions may be attributable to a dynamic and complex network of enzymatic and physical-chemical phenomena. All this comprehensive data should be helpful understand the underlying mechanisms of their postharvest variation, which may be later used to manage and control the production of elderflowers flavoring agents.

CHAPTER 3. Lipophilic phytochemicals from elderberries

3.1. Berry lipophilic constituents from three *S. nigra* L. cultivars grown in Portugal

3.2. Lipophilic phytochemicals from elderberries: Influence of ripening, cultivar and season

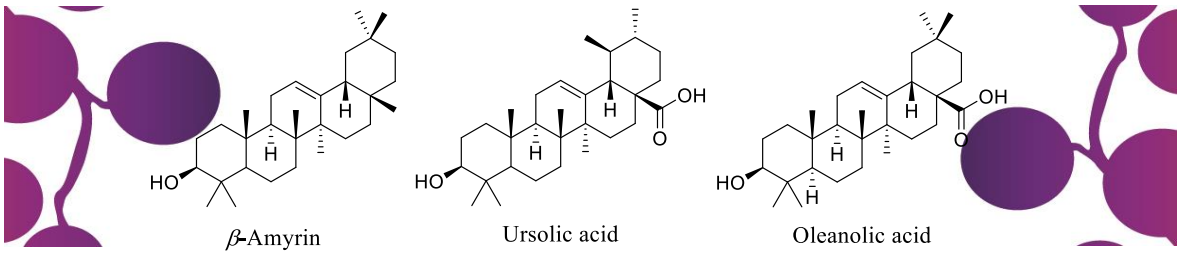


3.1. Berry lipophilic constituents from three important *S. nigra* L. cultivars grown in Portugal

Adapted from:

ÂC Salvador, D Charlebois, AJD Silvestre, SM Rocha, *Berry lipophilic constituents from three important Sambucus nigra L. cultivars grown in Portugal – Preliminary results*, Horticola Acta, 2015, 1061, 53-60;

3.1.1. Abstract



Lipophilic constituents (triterpenoids, fatty acids, fatty alcohols, and sterols) from the berries of the three major *S. nigra* L. cultivars grown in Portugal (‘Sabugueira’, ‘Sabugueiro’, and ‘Bastardeira’) were characterized by gas chromatography-mass spectrometry. Triterpenoids were the most abundant constituents, accounting for up to 92% of the lipophilic extract in all studied cultivars. Our results indicated that ursolic and oleanolic acid were the major triterpenoids identified, accounting for 1403 to 2080 mg/kg dry weight for the cultivars ‘Sabugueiro’ and ‘Sabugueira’, respectively. All cultivars under study presented a similar lipophilic profile, however significant differences (p<0.05) on the overall constituents content were noticed. This information is likely to be useful for the growers and processing industries interested in the formulation of foods and/or nutraceuticals, and can help increase the value and efficacy of elderberry-based natural products.

3.1.2. Introduction

European elder (*S. nigra* L.) berries are used worldwide as a food source, food additive, in nutraceuticals, and in folk medicine. Relevant potential human health benefits of these berries include antiviral [189], anti-inflammatory [179], and antioxidant activities [164], among others. Although several bioactive compounds may contribute to the mentioned health benefits, most studies dealing with elderberries have focused on the phenolic compounds from hydrophilic extracts [147,166]. Other families of secondary metabolites, namely those present in the lipophilic-like fraction (e.g., compounds present in dichloromethane extracts), have been poorly studied. Furthermore, it is known that a specific biological activity of a plant extract might be due to synergistic, cumulative, or antagonist effects of several components [248]. Such complex interactions make it difficult to fully understand reported beneficial effects of nutraceuticals or plant extracts used in folk medicine. It is therefore important to better characterize plant extracts in order to adequately support health claims and provide additional information needed for new applications. A better knowledge of the biochemical composition of these extracts is likely to have a positive impact on the production and use of this plant material.

To the best of our knowledge, only a single and recent study reported the presence of ursolic and oleanolic acids on elderberries [135]. Furthermore, it is important to characterize berries from bushes grown in specific geographical regions, since any given genotype grown in a variety of environments may show significant differences in the chemical composition of its berries. Such differences can have an impact on the nutraceutical properties of elderberry-based products [106,143].

In northern Portugal (Varosa Valley), elderberry plants benefit from excellent edaphoclimatic conditions for their development [249] which have stimulated its cultivation, especially during the last decade [148]. However, research on the chemical characterization of the berries, and more specifically the lipophilic fraction, is lacking. In this perspective, the present work aims at providing an insight into the chemical composition of the lipophilic fraction of berries from the three main Portuguese cultivars produced in the Varosa Valley. Particular attention was given to the oleanane and ursane pentacyclic triterpenoids families as they are the most abundant components of the lipophilic extracts.

3.1.3. Material and methods

The sampling, reporting of chemical analysis and metadata relative to data preprocessing, pretreatment, processing and interpretation were performed according to the Metabolomics Standards Initiative (MSI) [107]. These stages are described in detail in the following sub-sections.

3.1.3.1. Material and reagents

Ursolic (98%) and oleanolic acid (98%) were purchased from Aktin Chemicals (Chengdu, China); *β*-sitosterol (99%) was purchased from Fluka Chemie (Madrid, Spain). Palmitic acid (99%), dichloromethane (99%), pyridine (99%), *N,O*-bis(trimethylsilyl)trifluoroacetamide (99%), trimethylchlorosilane (99%), and tetracosane (99%) were supplied by Sigma Chemical Co (Madrid, Spain).

3.1.3.2. Samples

In 2012, berries from *S. nigra* cultivars 'Sabugueiro', 'Sabugueira', and 'Bastardeira' were supplied by the Adega Cooperativa do Vale Varosa, RégieFrutas (41.043233°N, 7.728820°W) from an experimental field on 12-year-old plants. This field was selected in order to harvest the three cultivars within the same location and minimize the effect of different edaphoclimatic parameters on extracts composition. Field management and interventions in this experimental field included soil pH adjustment to *ca.* 6.0 using 1500 kg/ha Tudidol (dolomite: 10% MgO and 43% CaO, Lusical, Alcanede, Portugal), addition of 1000 kg/ha of organic fertilizer Biorgano (Bricoagro21, Monção, Portugal), winter fertilization with 300 kg/ha of single superphosphate, $Ca(H_2PO_4)_2$ (18% Ca), and foliar application of aqueous sodium tetraborate decahydrate at 100 mL/hL, using 2 L/ha. A Confidor Classic insecticide from group 4A (Bayer CropScience, Carnaxide, Portugal) at concentration of 50 mL/hK of water, using 0.5 L/ha, was also applied. Samples were harvested on August, 23rd between 9 and 12 a.m. For each cultivar, five average size bunches were collected from the same shrub and mixed together. In order to have an indication of fruit ripening state, total soluble solids content (TSS) expressed as °Brix was measured. Samples were immediately transported under refrigeration (ca. 2-4°C) to the laboratory and then stored at -20°C until analysis. Prior to analysis, elderberries were freeze-dried using VirTis BenchTop K (SP Industries, NY, USA).

3.1.3.3. Extraction of the lipophilic compounds

The lipophilic fraction of the freeze-dried elderberries (approximately 2.5 g) was Soxhlet extracted with ca. 80 ml of dichloromethane for 8 h [74]. The solvent was evaporated to dryness, the extracts weighed and the results expressed in percent of dry weight (% dw). Dichloromethane was used since it is a fairly specific solvent for lipophilic extraction for analytical purposes [74]. An exploratory alkaline hydrolysis was also performed to improve the in-depth analysis of this fraction, but no significant additional data was observed (data not shown); direct analysis of the dichloromethane extract was therefore carried out.

3.1.3.4. Gas chromatography-mass spectrometry analysis

Before GC-MS analysis, about 20 mg of each extract was converted into trimethylsilyl (TMS) derivatives according to a previously optimized methodology [74]. GC-MS analyses were performed using a Trace Gas Chromatograph 2000 Series equipped with a Thermo Scientific DSQ II mass spectrometer using helium as carrier gas (35 cm/s) equipped with a DB-1 J&W capillary column (30 m×0.32 mm i.d., 0.25 μm film thickness). The chromatographic conditions were as follows: initial temperature of 80°C for 5 min; temperature rate of 4°C/min up to 260°C, and 2°C/min until the final temperature of 285°C; then maintained at 285°C for 10 min; injector temperature of 250°C; transfer-line temperature of 290°C; split ratio: 1:50. The MS was operated in the electron impact mode with electron impact energy of 70 eV and data collected at a rate of 1 scan/s over a range of *m/z* 33-700. The ion source was maintained at 250°C. Compounds were identified as TMS derivatives by comparing their mass spectra with the GC-MS spectral library (Wiley-NIST Mass Spectral Library, 1999) and also by injection of standards (*β*-sitosterol, ursolic, oleanolic and palmitic acid). For quantitative analysis, GC-MS was calibrated with the same standards relative to tetracosane, used as internal standard. The respective response factors needed to obtain correct quantification of the peak areas were calculated based on three standards concentrations as an average of three GC-MS runs of each concentration. Two independent aliquots of each extract were derivatized and submitted to GC-MS analysis. Each aliquot was injected in duplicate. The presented results are the average of the concordant values obtained for each sample (n=4).

3.1.3.5. Water content and total soluble solids (ºBrix)

The water content of the elderberries was determined based on the Association of Analytical Communities protocol [250] with slight modifications. Briefly, samples were placed in a drying oven at 105 ± 1°C until constant weight (approximately 8 h). For determination of TSS the elderberries were crushed and the juice suspension was centrifuged at 3000 rpm for 5 min at room temperature to obtain a clarified juice. The juice was then filtered through 0.45 μm pore size membrane filters. Total soluble solids content was determined based on the corresponding °Brix using an A. KRÜSS Optronic (Hamburg, Germany) refractometer.

3.1.3.6. Statistical analysis

The effect of cultivar on the content of the different chemical families identified was analyzed by one-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's HSD), using the GraphPad Prism version 6 for Windows (trial version, GraphPad Software, San Diego California, USA). It was considered statistically significant when p<0.05.

3.1.4. Elderberry lipophilic constituents

TSS-based ripening trend of the three cultivars is shown in Figure 3.1. For the studied harvest season, maturity was achieved simultaneously for the three cultivars around August 23rd. It was therefore assumed that all analyzed berries were at the same maturation state (p>0.05).

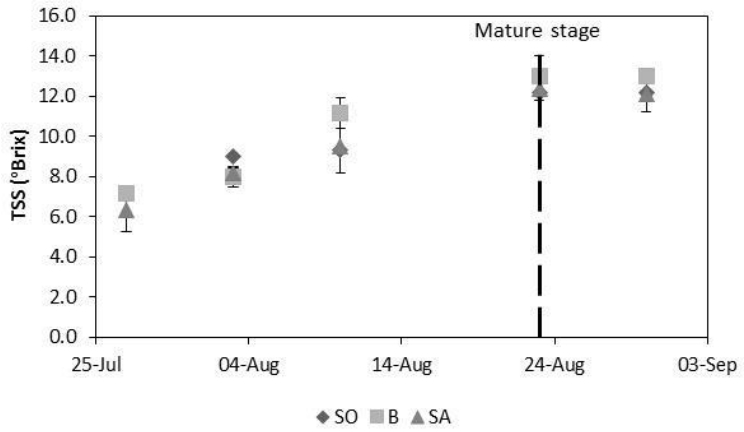


Figure 3.1. Total soluble solids content (TSS) through ripening of elderberry fruits at Varosa Valley, Portugal, 2012.

At maturity, the TSS ranged from 12.2 to 13.0 °Brix for cultivar 'Sabugueiro' and 'Bastardeira', respectively. These values are within the range commonly reported for ripe elderberries [35]. Since the berries were harvested in the same field, grown using the same phytochemical treatments and agricultural practices, it is assumed that the observed variance of the lipophilic composition is mainly cultivar-dependent.

The dichloromethane extraction yields ranged from 0.74% to 0.95% (w/dw) for 'Sabugueiro' and 'Bastardeira' cultivars, respectively.

The elderberry lipophilic extracts composition (fatty acids, fatty alcohols, phytosterols, and triterpenic compounds) is shown on Table 3.1, which were identified as TMS derivatives. Different parameters were used to perform the compounds identification, which included the co-injection of reference standards, being used ursolic and oleanolic acids, *β*-sitosterol and hexadecanoic acid. Furthermore, other criteria was applied for putative identification, namely, comparison of retention times with the literature data (for identical GC apparatus, columns and methodologies), analysis of the MS fragmentation pattern of the TMS derivatives of each analyte and comparison with GC-MS spectral libraries. The *m/z* 73 $[(CH_3)_3Si]^+$ and *m/z* 75 $[(CH_3)_2SiOH]^+$ not provide any useful information to confirm the analyte identification, however they are ubiquitous in the mass spectra of TMS derivatives confirming the silylation reaction (Figure 3.2 to Figure 3.4).

On Figure 3.2 to Figure 3.4, it is illustrated the mass spectra and respective fragmentation pattern of the major components of each chemical family under study: hexadecanoic acid, *β*-sitosterol, ursolic and oleanolic acids. For instance, the mass spectrum (Figure 3.2A) of the hexadecanoic acid TMS exhibits several ions (*m/z*), corresponding to the molecular fragmentation illustrated on Figure 3.2B. The *m/z* of 328 corresponds to the molecular ion. The loss of a methyl radical from the TMS group originates the fragment *m/z* 313, which is useful to assign the molecular weight of the TMS fatty acid ester, since the molecular ion peak can be sometimes absent in the EI mass spectra. Other fragments regarding the EI fragmentation of fatty acid TMS esters are commonly observed, i.e. *m/z* 145 (1,3-hydrogen transfer), 132 (McLafferty rearrangement), 129 (loss of methane) and 117 (loss of methyl radical).

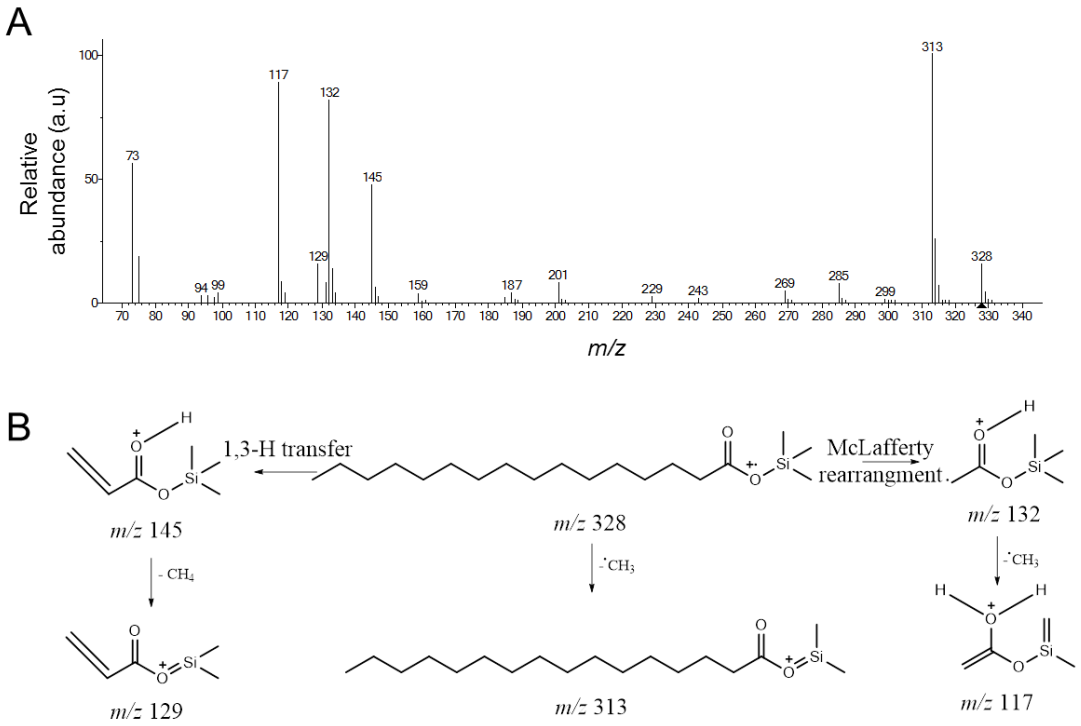


Figure 3.2. Mass spectrum (A) and respective fragmentation pattern (B) of the hexadecanoic acid trimethylsilyl ester.

Other component reported on *S. nigra* berries is the *β*-sitosterol (Table 3.1), and the mass spectrum of its TMS ether derivatives and respective fragmentation pattern are illustrated on Figure 3.3. In general, in the first step, the mass spectra (Figure 3.3A) of TMS ethers of sterols are analyzed to search for the molecular ion fragment or $[M\text{-}CH_3]^+$ ion, which are very useful to extract information about the analyte molecular weight. The *m/z* at 486 represent the molecular ion of TMS derivative of *β*-sitosterol and the loss of a methyl radical afforded the product ion at *m/z* 471 (Figure 3.3B).

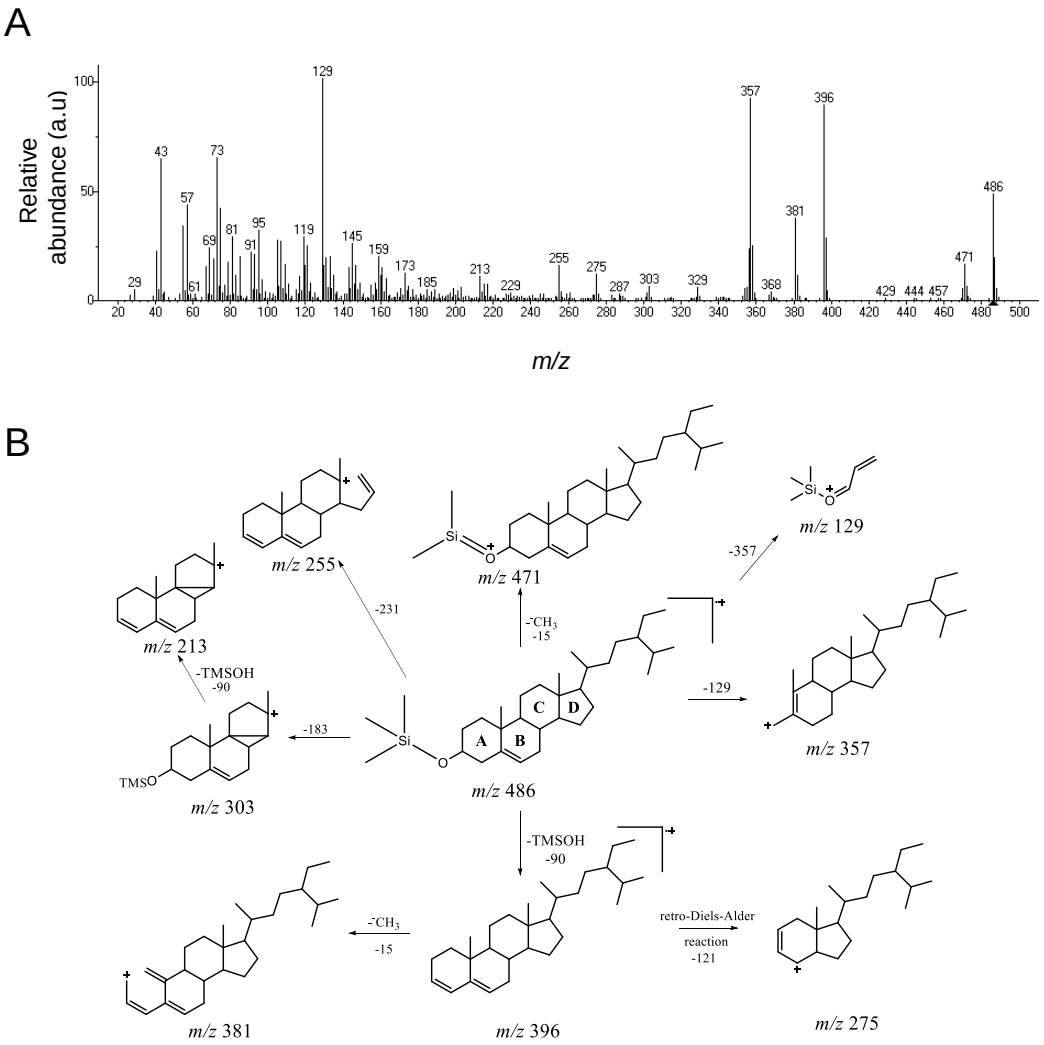


Figure 3.3. Mass spectrum (A) and respective fragmentation pattern (B) of the *β*-sitosterol trimethylsilyl eter.

The ions at *m/z* 129 and 357 $[M-129]^+$ which correspond to the loss of the TMS group with a three carbon fragment are characteristic of this compound [93,251]. The *m/z* at 396 corresponding to the 1,2-elimination of the trimethylsilanol group $[M-90]^+$ is another important feature for the identification [93,251]. The retro-Diels-Alder reaction on the fragment ion at *m/z* 396 originates the fragment ion at *m/z* 275 that results from the loss of the A and B-rings. Also, the fragment ion at *m/z* 396 suffers the loss of a methyl radical yields the fragment ion at *m/z* 381 [251]. The *β*-sitosterol trimethylsilyl eter may present loss of the side chain and the D-ring (-183 Da) and loss of the trimethylsinalol (-90 Da) originates the product ion at *m/z* 213. The fragment ion at *m/z* 255 is originated due to the loss of side chain, partial loss of D-ring and the loss of trimethylsilanol [251,252].

The triterpenic compounds, oleanolic and ursolic acids are also reported on this plant (Table 3.1), and their TMS derivatives are identified based on the co-injection of reference standards. These analytes are structurally similar, as well as their respective mass spectra (Figure 3.4A-B). Thus, their identification based on the fragmentation patterns (Figure 3.4A-B) and elution order, represent a huge challenge [20]. The ursolic acid presented a longer retention time than oleanolic acid (Table 3.1) which may be related to the positions of the two methyl groups of ring E (Figure 3.4C). These groups are in equatorial position in ursolic acid causing an increase in the planarity of the molecule, while oleanolic acid present the methyl groups in axial position [20].

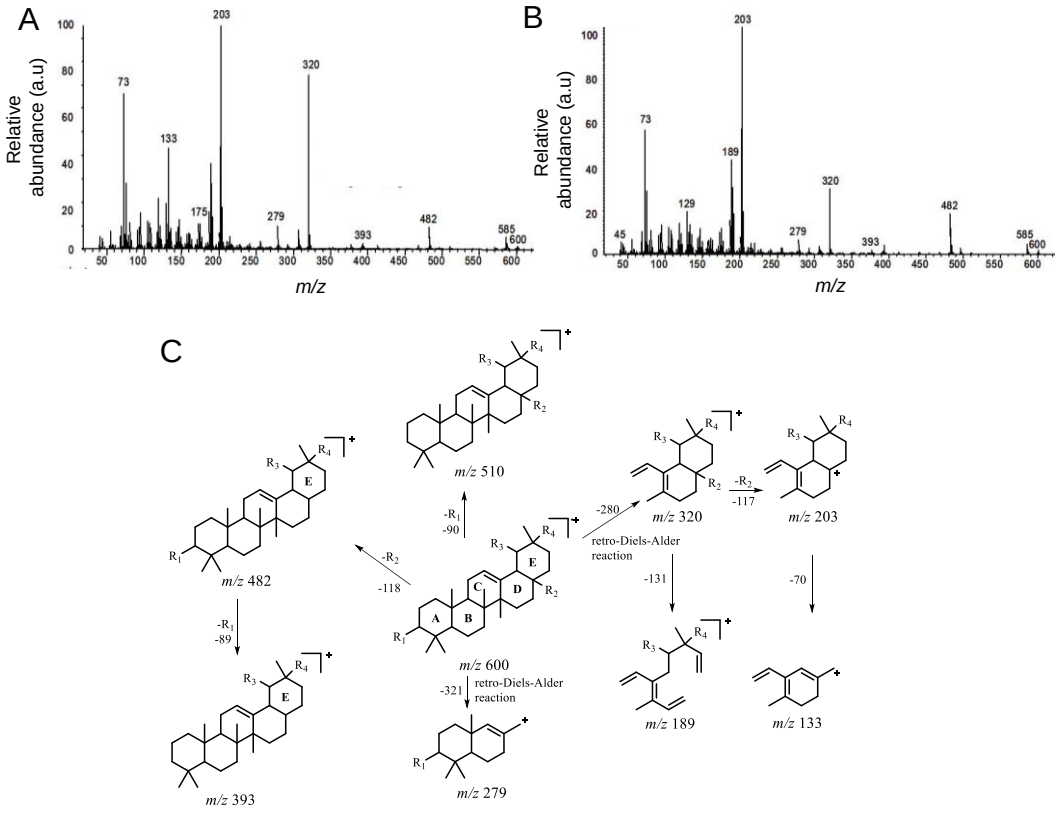


Figure 3.4. Mass spectra of ursolic (A) and oleanolic (B) acids and the (C) fragmentation patterns of triterpenoids TMS derivatives with ursane (R_3=CH_3; R_4=H) and oleanane (R_3=H; R_4=CH_3) type structures with a double bond at C12(13). R_1=OTMS; R_2=COOTMS.

As partially illustrated on the Figure 3.4C, under EI, the most important signals are found at *m/z* 600 assigned to the molecular $[M]^{+\cdot}$; the 585 due to the loss of a methyl group; 510 corresponding to the loss of a silylated hydroxyl group $[M\text{-}TMSOH]^+$; 495 $[M\text{-}TMSOH\text{-}CH_3]^+$; 482 by the loss of a silylated carboxyl group $[MTMSOOCH]^+$; 393 due to the loss of silylated hydroxyl and carboxyl groups $[M\text{-}TMSOH\text{-}TMSOOC]^+$; and 392 $[M\text{-}TMSOH\text{-}TMSOOCH]^+$ [20]. Although, as previously reviewed [20], the most characteristic MS fragmentation of TMS derivatives of all compounds with a 12-unsaturated oleanene or ursene skeleton arise from the

presence of a double bond at C12(13), resulting on a retro-Diels-Alder reaction and in the formation of fragments containing rings A, B and part of C (*m/z* 279) and containing rings D, E and part of C (*m/z* 320).

As presented on Table 3.1, dichloromethane extracts are mainly composed of triterpenic compounds such as ursolic and oleanolic acids, accounting for 1138.6 to 1751.7 mg/kg and 264.8 to 363.4 mg/kg, respectively. *β*-amyrin (7.1 to 9.0 mg/kg) and small amounts of unidentified triperpenoids (15.1 to 41.4 mg/kg) were also present. All these compounds totaled 1452.5, 2102.1, and 2044.1 mg/kg for the cultivars ‘Sabugueiro’, ‘Sabugueira’ and ‘Bastardeira’, respectively.

Table 3.1. Composition (mg/kg dry berry weight) of dichloromethane extracts from three elderberry cultivars harvested from Varosa Valley, Portugal, 2012

t_R (min)	Compound	MSI level[*1]	Cultivar		
			'Sabugueiro'	**'Sabugueira'**	**'Bastardeira'**
Fatty acids					
33.63	Hexadecanoic acid	1	104.6 ± 3.1	158.1 ± 1.9	145.6 ± 1.2
37.93	Octadecanoic acid	2	13.1 ± 1.1	4.1 ± 0.1	11.1 ± 1.0
41.90	Eicosanoic acid	2	5.5 ± 0.3	*tr*	3.3 ± 0.3
	Sub-total (mg/kg)		**123.2[a]**	**162.2[b]**	**160.0[b]**
	Sub-total (%)[*2]		**7.7**	**7.0**	**7.2**
Long chain aliphatic alcohols					
31.95	Hexadecanol	2	5.4 ± 0.2	21.8 ± 0.3	*tr*
36.30	Octadecanol	2	*tr*	1.9 ± 0.0	*tr*
	Sub-total (mg/kg)		**5.4[a]**	**23.7[b]**	*tr*
	Sub-total (%)[*2]		**0.3**	**1.0**	*tr*
Sterols					
56.76	Stigmasterol	2	4.2 ± 0.6	3.6 ± 0.3	5.1 ± 2.5
57.96	*β*-Sitosterol	1	9.4 ± 0.9	12.4 ± 0.8	12.9 ± 2.0
	Sub-total (mg/kg)		**13.6[a]**	**16.0[a]**	**18.0[a]**
	Sub-total (%)[*2]		**0.9**	**0.7**	**0.8**
Triterpenoids					
58.48	*β*-Amyrin	2	7.7 ± 0.7	7.1 ± 0.2	9.0 ± 1.0
63.00	Oleanolic acid	1	264.8 ± 11.3	328.3 ± 15.3	363.4 ± 7.0
64.00	Ursolic acid	1	1138.6 ± 41.5	1751.7 ± 18.0	1632.2 ± 31.4
	Unidentified triterpenoids	3	41.4	15.1	39.5
	Sub-total (mg/kg)		**1452.5[a]**	**2102.1[c]**	**2044.1[b]**
	Sub-total (%)[*2]		**91.1**	**91.3**	**92.0**
	Total content		1594.7[a]	2304.0[c]	2222.1[b]

[*1] Level of compound identification according to Metabolomics Standards Initiative: (l) Identified compounds using standards coinjection; (2) putatively annotated compounds; (3) putatively characterized compound classes; and (4) unknown compounds [107].
[*2] The relative amount (%) for the different identified chemical families represents their relative content compared to the total content.
tr = trace, < LOQ (limit of quantification) of each analyte.
[a, b, c] Different letters indicate statistical significant differences, $p < 0.05$

Differences in the triterpenic acids contents were significant (p<0.05) between the three cultivars sampled. Differences in total amount of sugars, organic acids, and flavonoids have already been reported for elderberry cultivars [37]. Such variations in chemical composition may have an impact on elderberry-based products, as already reported on juices from elderberries of different genotypes, affecting physicochemical properties such as turbidity, soluble solids, titratable acidity, and content of phenolic acids and flavonoids [143].

Overall, triterpenoids accounted for 91.1 to 92.0% of the mass of the lyophilized extract. Within this chemical group, ursolic acid represents between 71.4 and 76.0% of the total identified lipophilic compounds for 'Sabugueiro' and 'Sabugueira' respectively, followed by oleanolic acid which accounts from 14.2 to 16.6% of the total lipophilic compounds for 'Sabugueiro' and 'Sabugueira', respectively. Their content expressed as fresh weight (fw) comprises values between 233.4 and 359.1 mg/kg fw for ursolic acid and 54.3 to 74.5 mg/kg fw for oleanolic acid. The triterpenoids contents of the studied cultivars are within the ranges reported in the literature for other berries, such as cranberry fruit (*Vaccinium macrocarpon*), northern cranberry (*V. oxycoccus*), lowbush blueberry (*V. angustifolium*) and partridge berry, also known as lingonberry (*V. vitis-idaea*) where ursolic acid and the corresponding hydroxycinnamoyl esters content ranged from 120 to 1090 mg/kg of fruit fw [253]. Ursolic and oleanolic acids have also been reported in elderberries [135], and other plant organs of *S. nigra* such as the bark [254] and leaves [255].

Triterpenoids, and triterpenic acids in particular, are well known as bioactive compounds with a panoply of described biological activities [256–258]. Within this range of biological activities, and in particular those associated with ursolic acid (the major triterpenoid present in the studied elderberries), it is important to point out that several biological activities that are commonly associated with elderberries are also reported for ursolic acid, either in other extracts or as a standard. Such activities include: antiviral [259], anti-tumorigenic and chemopreventive [260], anti-oxidative and anti-inflammatory activities [261]. Additionally, it is also important to emphasize that the reported biological activities are expressed at relatively moderate concentrations. For instance, EC_{50} of 6.6 mg/L was reported for ursolic acid antiviral activity on herpes viruses HSV-1 [259], which corresponds to a consumption of ca. 22 g of fresh elderberries (a typical portion is about 50 g).

Free fatty acids were the second most important family, accounting for 7.0 to 7.7% of the elderberry extracts, with palmitic acid (hexadecanoic acid) the most abundant component (104.6 to 158.1 mg/kg dw). Fatty alcohols and phytosterols represent minor components of the extracts under study. Fatty alcohols and fatty acids are in the free form, the even-members being predominant. As observed for triterpenoids, significant differences (p<0.05) were found among cultivars on the fatty acids content.

Phytosterols accounted from 0.7% for 'Sabugueira' to 0.9% for 'Sabugueiro' cultivars, of the lipophilic fraction, ranging from 13.6 to 18.0 mg/kg dw depending on the cultivar (corresponds to 2.8 to 3.7 mg/kg fw). Two sterols were identified, stigmasterol and β-sitosterol, where the latter represented from 68.7% ('Sabugueiro') to 77.4% ('Sabugueira') of the total phytosterols content. The content of this chemical class for the samples under study is well below the range previously

reported [262], for a set of fruits and berries: between 60 mg/kg fw for red currant (*Ribes rubrum* L.) and 279 mg/kg fw for lingonberry.

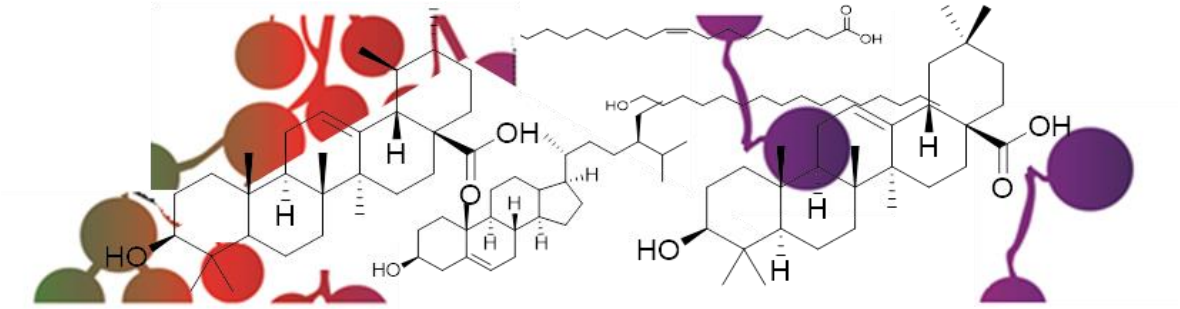
3.1.5. Conclusions

The lipophilic fraction (dichloromethane extractives) of the 'Sabugueiro', 'Sabugueira', and 'Bastardeira', produced in the Varosa Valley (Portugal) was characterized. All cultivars under study presented a similar lipophilic profile, mainly composed of triterpenic acids, followed by fatty acids, phytosterols, and fatty alcohols. However, significant differences ($p<0.05$) were observed in the total amount of the different chemical families. Ursolic acid and oleanolic acid represented the major triterpenoids. This study allowed the identification of several relevant bioactive compounds in the elderberries lipophilic extracts indicating that they are a valuable source of triterpenic bioactive compounds. More detailed studies tackling variables that can modulate plant metabolism, such as harvesting season and ripening stage are needed.

3.2. Lipophilic phytochemicals from elderberries: Influence of ripening, cultivar and season

Adapted from:
ÂC Salvador, SM Rocha, AJD Silvestre, *Lipophilic phytochemicals from elderberries (Sambucus nigra L.): Influence of ripening, cultivar and season*, Industrial Crops and Products, 2015, 71, 15-23.

3.2.1. Abstract



The chemical composition of the lipophilic extracts of three Portuguese elderberries cultivars belonging to the *S. nigra* L. species (‘Bastardeira’, ‘Sabugueira’ and ‘Sabugueiro’), was studied by gas chromatography–mass spectrometry. The influence of the harvesting season (2012 and 2013), cultivar and the ripening stage was evaluated. Regarding the amount of lipophilic extractives, they ranged from 0.56% to 1.84% of the dry weight. The major chemical families present in these fractions were triterpenoids and fatty acids accounting with 84.9–93.8% and 4.3–11.4% of the total amount of lipophilic components, respectively. The most abundant compounds, identified as elderberries components were ursolic and oleanolic acids, followed by smaller amounts of long chain aliphatic alcohols and sterols. During ripening, a similar profile of the studied chemical families was found for the two sampling seasons and the three cultivars, with an initial growth of their content followed by a systematic decrease until maturity, yet, a higher lipophilic content (p<0.05) is reported for the 2013 harvest. Regarding mature elderberries, ‘Sabugueira’ and ‘Bastardeira’ showed higher contents of lipophilics, and particularly of triterpenic acids (p<0.05), for the two sampling years. In-depth study of elderberries, lipophilic extractives can contribute to the valuation of this natural product, being in this study highlighted the profile of these bioactive compounds, as well, the parameters that affect their content. Additionally, since considerable amounts of unripe and overripe elderberries are produced on harvesting, and that our results showed considerable amounts of lipophilics for both ripening stages, this highlights that these wastes can be seen as a promising sources for the preparation of lipophilic extracts enriched in bioactive compounds.

3.2.1. Introduction

Black elderberry or European elderberry (*S. nigra* L.) is a deciduous shrub that grows to a height of 4-6 m, and produces dark violet-black drupes which grow in clusters [185]. Since ancient times, elder trees are referred as the "medicine chest", being used on the formulation of diverse medicinal preparations to prevent and treat several diseases [30]. *In vitro* and *in vivo* studies supported some of the traditional health claims, indicating many biological effects such as anti-inflammatory [178,179], anti-hepatotoxic [263], as well anti-oxidant [164,166,171] and anti-viral effect against influenza [189]. Although few studies establish a relationship between berries composition and the reported biological activities, and when such relationship is established, it is almost often linked to the phenolic fraction [179]. However, the several other families of extractives are also frequently related with the biological activity of plant-related materials, including berries. That is for example the case of the lipophilic fraction, typically rich in unsaturated fatty acids, phytosterols and triterpenic compounds which are frequently associated with a panoply of health benefits [20,264,265]. While, the phenolic composition of elderberries has already been well explored in detail [148,266,267]; only a single and recent study [135] report the composition of berries, berries, showing they were rich in ursolic and oleanolic acids. Furthermore, the presence of these compounds in the bark [254] and leaves [255] of this species has been reported several decades ago.

In the last decade, the production of elderberries increased considerably in Varosa Valley, northern of Portugal, where *S. nigra* benefits from excellent edaphoclimatic conditions for their development [41,249]. These conditions combined with the implementation of several National and European programmes during the last decade led to the growth of *S. nigra* cultivated area [148]. Presently, between 1500 and 2500 tons of elderberries are produced annually, being almost totally exported to other European countries, as refrigerated berries, for further industrial processing. In this region three main cultivars are being explored, namely 'Bastardeira', 'Sabugueira' and 'Sabugueiro' [41].

Elderberry physiological development and ripening process generally starts in July, becoming fully ripe and ready to be harvested by the end of August. Considering the growing interest on elderberries, and in order to improve the knowledge about their chemical composition and potential human health benefits, the aim of this research work was to carry out a detailed chemical characterization of the lipophilic components of this crop. Furthermore, as it is known that the secondary metabolites composition might be affected by edaphoclimatic and genetic factors [117], as well as by maturation state [268], the elderberry lipophilic profile was also

followed along ripening period during two years (2012 and 2013) for the three main cultivars, to access the impact of these variables over the chemical composition of this fraction.

3.2.2. Material and methods

The sampling, reporting of chemical analysis and metadata relative to data preprocessing, pretreatment, processing and interpretation were performed according to the Metabolomics Standards Initiative (MSI) [107]. These stages are described in detail in the following sub-sections.

3.2.2.1. Materials and reagents

Ursolic (98%), and oleanolic acid (98%) were purchased from Aktin Chemicals (Chengdu, China), cholesterol (99%) was purchased from Fluka Chemie (Madrid, Spain). Palmitic acid (99%), dichloromethane (p.a.≥99.9%), pyridine (99%), *N,O*-bis(trimethylsilyl)trifluoroacetamide (99%), trimethylchlorosilane (99%), and tetracosane (99%) were supplied by Sigma Chemical Co (Madrid, Spain).

3.2.2.2. Samples

Elderberries from *S. nigra* L. cultivars 'Sabugueiro', 'Sabugueira', and 'Bastardeira' were supplied by the Adega Cooperativa do Vale Varosa, RégieFrutas from 12-year-old plants of an experimental field (41.043233ºN, 7.728820ºW). This field was selected in order to harvest the three cultivars within the same location and to minimize the effect of different edaphoclimatic and soil characteristics on the elderberries composition. Management and interventions in this experimental field included soil pH adjustment to ca. 6.0 using 1500 kg/ha Tudidol (dolomite: 10% MgO and 43% CaO, Lusical, Alcanede, Portugal), addition of 1000 kg/ha of organic fertilizer Biorgano (Bricoagro21, Monção, Portugal), winter fertilization with 300 kg/ha of single superphosphate, $Ca(H_2PO_4)_2$ (18% Ca), and foliar application of aqueous sodium tetraborate decahydrated at 100 mL/hL, using 2 L/ha. A Confidor Classic insecticide from group 4A (Bayer CropScience, Carnaxide, Portugal) at concentration of 50 mL/hL of water, using 0.5 L/ha, was also applied.

Samples from the three cultivars were harvested on-site between 9 and 12 a.m., for each ripening degree. For each cultivar and sampling moment a pool containing, on average, five bunches from the same shrub were prepared. The different stages of ripening corresponded to the harvesting dates of 27th July, 3rd, 10th, 23rd and 30th August of 2012, and 2nd, 6th, 21st, 28th August, and 5th September of 2013. The mature stage was established by the local company based on the total soluble solids (Brix degree should be at least 12) and the homogeneous pigmentation of the elderberries clusters (to be harvest, 75% of the berries from the cluster must be dark-violet), and corresponding to August 23rd and 28th from 2012 and 2013 seasons, respectively.

After harvesting, the samples were immediately transported under refrigeration (ca. 2-4ºC) to the laboratory and then stored at -20ºC until analysis. Prior to analysis, elderberries were freeze-dried using a VirTis BenchTop K (SP Industries, NY, USA), and the water content determined (105 ºC – 8h).

3.2.2.3. Extraction of the lipophilic compounds

The lipophilic fraction of the freeze-dried elderberries (approximately 2.5 g) was Soxhlet extracted with ca. 80 mL of dichloromethane for 8 h [74] The solvent was evaporated to dryness, the extracts weighed and the results expressed in percent of dry weight (% dw). Dichloromethane was used since it is a fairly specific solvent for lipophilic components extraction for analytical purposes [74].

3.2.2.4. Gas chromatography-mass spectrometry analysis

Before GC–MS analysis, about 20 mg of each extract was converted into trimethylsilyl (TMS) derivatives according to a previously optimized methodology [74].

GC–MS analyses were performed using a Trace Gas Chromatograph 2000 Series equipped with a Thermo Scientific DSQ II mass spectrometer using helium as carrier gas (35 cm/s) equipped with a DB-1 J&W capillary column (30 m×0.32 mm i.d., 0.25 μm film thickness). The chromatographic conditions were as follows: initial temperature 80ºC for 5 min; temperature rate of 4ºC/min up to 260ºC, and 2ºC/min until the final temperature 285ºC; then maintained at 285ºC for 10 min; injector temperature of 250ºC; transfer-line temperature 290ºC; split ratio: 1:50. The MS was operated in the electron impact mode with electron impact energy of 70 eV and data collected at a rate of 1 scan/s over a range of m/z 33–700. The ion source was maintained at 250ºC.

Compounds were identified as TMS derivatives by comparing their mass spectra with the GC–MS spectral library (Wiley-NIST Mass Spectral Library 1999), with literature MS fragmentation [20,269–272] and also by injection of standards, namely ursolic and oleanolic acids for the corresponding compounds and hexadecanoic acid. Manual MS identification of TMS derivatives was also performed as briefly described on **Chapter 3.1**. For quantitative analysis, GC–MS was calibrated with cholesterol for phytosterols, ursolic, oleanolic acids for the corresponding compounds (and their average for unidentified triterpenoids) and hexadecanoic acid for fatty acids and alcohols relative to tetracosane, used as internal standard as described in detail in previous studies e.g., [270,271]. The respective response factors needed to obtain correct quantification of the peak areas were calculated based on three standards concentrations as an average of three GC–MS runs of each concentration. Two independent aliquots of each extract were derivatized and

submitted to GC-MS analysis. Each aliquot was injected in duplicate. The presented results are the average of the concordant values obtained for each sample (n=4).

3.2.2.5. Statistical analysis

Statistical analyses were performed, in order to evaluate the effect of the cultivar (3 cultivars), harvesting season (2 seasons) and ripening stage (5 stages) on the total content of each identified chemical family, being considered statistically significant when $p<0.05$. Thus, one-way analysis of variance (ANOVA) was performed followed by a multiple comparison test (Tukey's HSD), using the GraphPad Prism version 6 for Windows (trial version, GraphPad Software, San Diego California, USA).

3.2.3. Chemical composition of the elderberry lipophilic extracts

The elderberry water content, obtained upon freeze-drying ranged from 74.8 to 76.8%. The lipophilic extractives yield of the different elderberries cultivars harvested from unripe to ripe fruits, through two consecutive harvesting seasons, ranged from 0.56% to 1.84% of the elderberries dry weight (Figure 3.5).

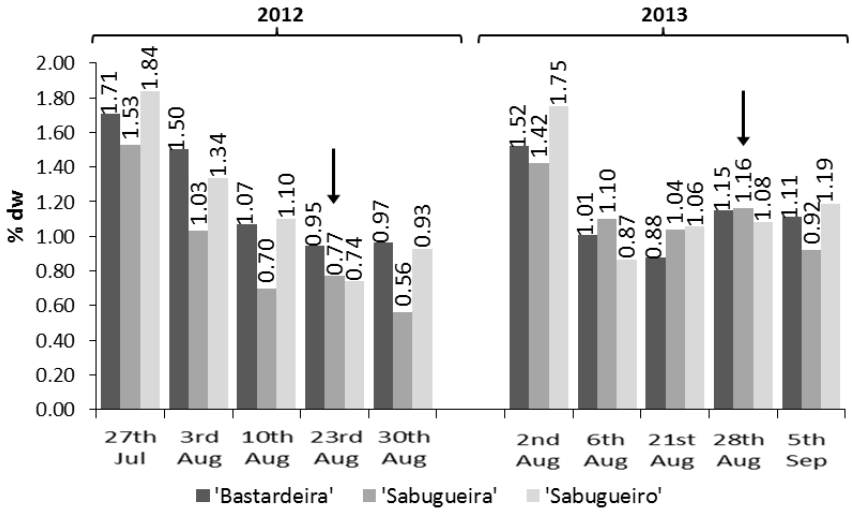


Figure 3.5. Lipophilic extractive yields expressed in dry weight (% dw) for each harvesting date and elderberry cultivars under study. The arrows point out the mature stage.

For both seasons, it was observed that this parameter tends to decrease through ripening. At maturity stage, which is highlighted with an arrow on Figure 3.5, in 2012 the extraction yield ranged from 0.74% to 0.95% of the dry weight for the cultivars 'Sabugueiro' and 'Bastardeira', respectively, while in 2013, the extraction yield ranged from 1.08% to 1.16% of the dry weight for

the cultivars ‘Sabugueiro’ and ‘Sabugueira’, respectively. The low lipidic content of elderberries is in agreement with previously published results obtained for other berries, as for different mulberries species (*Morus* spp.) that ranged between 0.14% and 1.10% [273,274].

The lipophilic extracts chemical composition from three elderberry cultivars, at different ripening stages, and two harvesting seasons, was studied in detail by GC–MS. The identification and quantification data (expressed as mg of compound per kg of dry elderberries berry weight) about the lipophilic compounds is summarized in annexes (Tables A3.2.1-A3.2.3) for the ‘Bastardeira’, ‘Sabugueira’ and ‘Sabugueiro’ cultivars, respectively. An alkaline hydrolysis of the extracts was also performed in order to detect compounds eventually present in esterified forms [275], but no significant information was obtained after this procedure (data not shown), therefore, only the direct analysis of the dichloromethane extract is reported.

The GC–MS analysis revealed the presence of up to 17 compounds distributed over four chemical families, namely triterpenoids, that accounted for 84.9–93.8% of all identified compounds, followed by free fatty acids (C_{10}–C_{26}), which accounted with 4.3–11.4% of the identified compounds, sterols (0.0–5.2%) and long chain aliphatic alcohols (C_{18} and C_{26}, from 0.0 to 0.3%).

Ursolic and oleanolic acids correspond to the main triterpenoids identified in the lipophilic extracts of the different elderberry cultivars, which accounted from 1138.6 to 3126.6 mg/kg and 264.8 to 1205.0 mg/kg, respectively (Tables A3.2.1-A3.2.3). *β*-Amyrin ranged from 0.6 to 63.5 mg/kg and other minor unidentified triterpenoids were also quantified. Therefore, the triterpenoids content represented between 1452.5 and 4494.9 mg/kg of the extracts, corresponding to 218–674 mg/kg of elderberries fresh weight. These concentration range is within the range presented in other berries, namely in cranberry fruit (*Vaccinium macrocarpon*), northern cranberry (*Vaccinium oxycoccus*), lowbush blueberry (*Vaccinium angustifolium*) and partridge berry, also known as lingonberry (*Vaccinium vitis-idaea*), where ursolic acid and the corresponding hydroxycinnamoyl esters content ranged from 120 to 1090 mg/kg of fruit fresh weight [253].

Free fatty acids, the second most abundant family, accounted for 111.7–375.8 mg/kg of the lipophilic extract of elderberry cultivars (Tables A3.2.1-A3.2.3), where hexadecanoic acid was the most abundant component, representing up to 97.5% of the identified fatty acids. The identified fatty acids ranged from decanoic to hexacosanoic acids, including octadec-9-enoic acid ($C_{18:1}$). A wide range of fatty acids, including unsaturated ones were already reported on *S. nigra* L. seeds, representing 3.06×10^5 mg/kg of dry seed oil [276]. This last value is much higher than the one determined on berries, which is expected considering the typically high lipidic content of seeds.

Long chain aliphatic alcohols and sterols represented the minor lipophilic components of the elderberry extracts. According to the Tables A3.2.1-A3.2.3, three sterols were identified,

namely campesterol, stigmasterol and *β*-sitosterol, ranging from traces to 200.5 mg/kg. Two long chain aliphatic alcohols were identified (C_{18} and C_{26}), ranged from 0.6 to 13.5 mg/kg. The content of sterols found in elderberries is lower than the values reported for other berry crops, which ranged from 370 to 2930 mg/kg of dry weight [262,277].

The composition of elderberries demonstrates that these fruits might be an interesting source of valuable bioactive compounds, and particularly of triterpenic compounds. Indeed ursolic acid and oleanolic acid are common triterpenoids in plant kingdom [19,20] and both were deeply studied on diverse biological activities [74] and have been often used in cosmetics and nutraceutical products [278]. Additionally, phytosterols, although in much lower concentration are reported as LDL cholesterol lowering agents, and are also shown to promote an anti-inflammatory and immune modulatory activity [279]. Furthermore, based on several human studies, the European Food Safety Authority (EFSA) considers that there are already scientific substantiation of health claims related to plant sterols and lower/reduced blood cholesterol [280].

3.2.4. Influence of ripening on the chemical composition of the elderberry lipophilic extracts throughout two harvesting seasons

The lipophilic composition of the elderberry extracts was followed throughout five ripening stages, during two consecutive harvesting seasons. In Figure 3.6, it is illustrated the overall content of the two major chemical families (triterpenoids, Figure 3.6A, and fatty acids, Figure 3.6B).

Specifically, for the triterpenoids (Figure 3.6A), it can be seen that for all cultivars the content slightly increased from the first to the second harvesting moment, and then, it gradually decreased until the last harvested ripening stage. Regarding the fatty acids content of the three studied cultivars with different ripening stages (Figure 3.6B), it can be observed that at the mature stage (marked with an arrow in the figure), the content is one of the lowest, when compared with the remaining ripening stages. A similar behavior is observed for the two harvesting seasons, both for triterpenic compounds (Figure 3.6A) and fatty acids (Figure 3.6B). A similar trend was also observed for the other chemical families in study (graphics not shown for sterols and aliphatic alcohols, c.f. Tables A3.2.1-A3.2.3), but, despite this trend concerning the sterols chemical family, a much higher content is reported in 2013 when compared with 2012 (at least 3-fold).

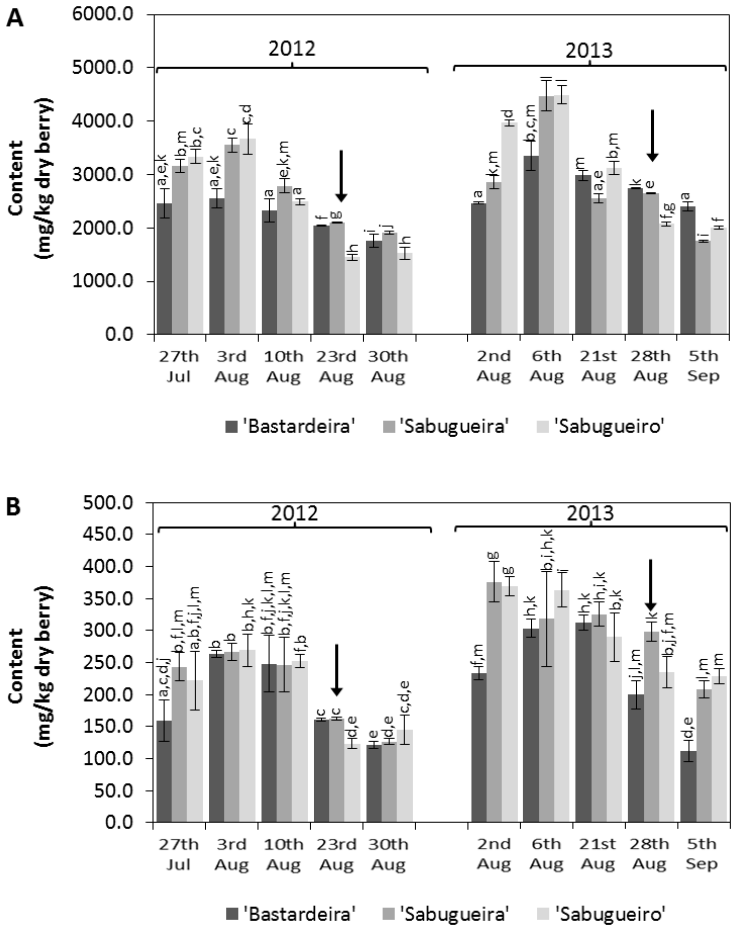


Figure 3.6. Follow up of the triterpenoids (A) and fatty acids (B) content through ripening, for the three elderberry cultivars under study, during two consecutive harvesting seasons. The arrows point out the mature stage. Different letters indicate statistical significant differences, $p<0.05$.

The presented behavior is in consonance to what was previously reported on other crops namely: triterpenic compounds on *Vitis vinifera* berries [281]; fatty acids on *Arbutus unedo* L. berries [282]; and sterols on *V. vinifera* berry skins [283]. Despite the similar observed trend over the studied chemical families on both harvesting seasons, and that has also been demonstrated for other fruits, this is not a general rule in fruit ontogeny [281], where for instance triterpenoids content can increase during crops development [284]. The decrease of fatty acids over ripening, is reported as a biochemical mediated process where membrane lipids and free fatty acids are highly susceptible to oxidation [285]. The action of the degradative lipolytic enzymes [286] that are able to degrade endogenous lipids, thus changing the lipid bilayer and accelerating permeability in senescing membranes [287]. Due to the contribution of these enzymes, a reduction of the fruit firmness occurs during ripening [288,289]. Again, as for triterpenic compounds, this is not a general rule for fatty acids in fruit development, being already documented the accumulation of these compounds through fruit ripening [290].

3.2.5. Influence of cultivar and harvesting season on the chemical composition of the lipophilic extracts from mature elderberries

Regarding the mature elderberries, which are highlighted as gray cells in Tables A3.2.1-A3.2.3 and summarized in Figure 3.7, it can be observed that the content of the different chemical families differs substantially between cultivars and harvesting season. Significant differences in the content of triterpenic compounds were observed among the three sampled cultivars, on both harvesting seasons ($p<0.05$). Furthermore, it is important to note that the amount of triterpenoids in 'Sabugueiro' was significantly lower than in 'Sabugueira' and 'Bastardeira'. In 2012, the amount of triterpenoids in 'Sabugueiro' accounted for 1452.5 mg/kg dw, while for the other cultivars, it accounted for 2045.1 ('Bastardeira') and 2102.1 ('Sabugueira') mg/kg dw, and in 2013, it accounted for 2074.1 mg/kg dw ('Sabugueiro'), while for 'Bastardeira' it accounted for 2741.6 mg/kg dw and for 'Sabugueira', 2650.1 mg/kg dw. Significant differences in the lipophilic composition of different cultivars have already been reported in other berries [281,291]. The elderberry cultivar differences are reflected, for instance, on the leaves characteristics (color, number and format), on the color of the clusters (white/green or purple) or on the berries size. The results obtained also allow inferring that differences in secondary metabolism may be reported, which may explain the differences observed among the three cultivars for both harvesting seasons.

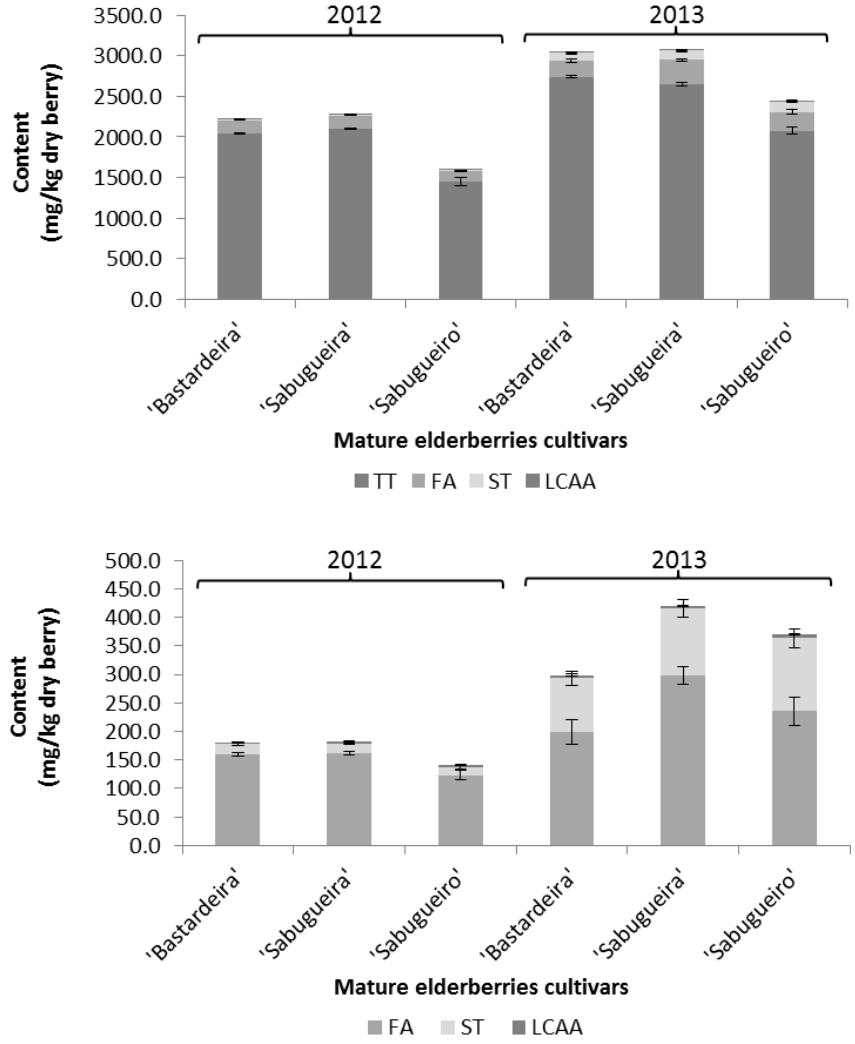


Figure 3.7. Content expressed as mg/kg of dry berry for the major chemical families of lipophilic components identified in the dichloromethane extracts of the mature elderberry cultivars from Varosa Valley, harvested during two seasons, Portugal (TOP) and an expansion of the graphic for the less abundant compounds (bottom). TT: triterpenoids. FA: fatty acids. ST: sterols. LCAA: long-chain aliphatic alcohols.

Additionally, comparison between the harvest seasons of 2012 and 2013 allowed to observe that the later one exhibited a higher content of the lipophilic compounds ($p<0.05$). In the case of triterpenoids, an increment of 1.3–1.4-fold is reported for 2013 when compared to the content of 2012.

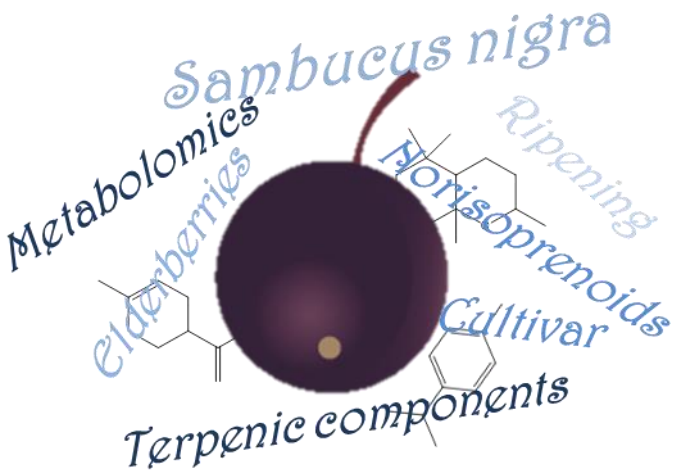
The plant metabolism results from a network of effects which are regulated by intrinsic factors, as the genetic load, as well as, extrinsic factors namely the climatic conditions, that imply different levels of radiation, temperature, water, being those, season dependent [292]. Consequently, the observed differences in the elderberries lipophilic profiles should be explained not based on one factor, but as the result of the complex network of external and internal ones.

3.2.6. Conclusions

In the present study an evaluation of the lipid profile of the ‘Sabugueiro’, ‘Sabugueira’, and ‘Bastardeira’ *S. nigra* L. cultivars is reported. The cultivar, ripening stage and harvesting season influence were studied. The results showed that up to 17 lipophilic components were identified and quantified, belonging to triterpenoids, fatty acids, sterols and long chain aliphatic alcohols. Globally, ursolic and oleanolic acid represent the major components of the extracts under study, which have been previously reported in *S. nigra* bark [254] and leaves [255], and more recently in berries [135].

During ripening a similar profile of variation of triterpenic acids (as well as for the other studied families) was found in the two sampling years for the three cultivars, with an initial increasing followed by a systematic decrease until maturity. For mature fruits it was observed that in the two sampling years ‘Sabugueira’ and ‘Bastardeira’ showed higher contents of lipophilics and of triterpenic acids in particular. To deeply understand the ripening and cultivar effects over the elderberry composition, this study was broadened to other chemical families, namely volatiles (e.g., mono and sesquiterpenes) (**Chapter 4**).

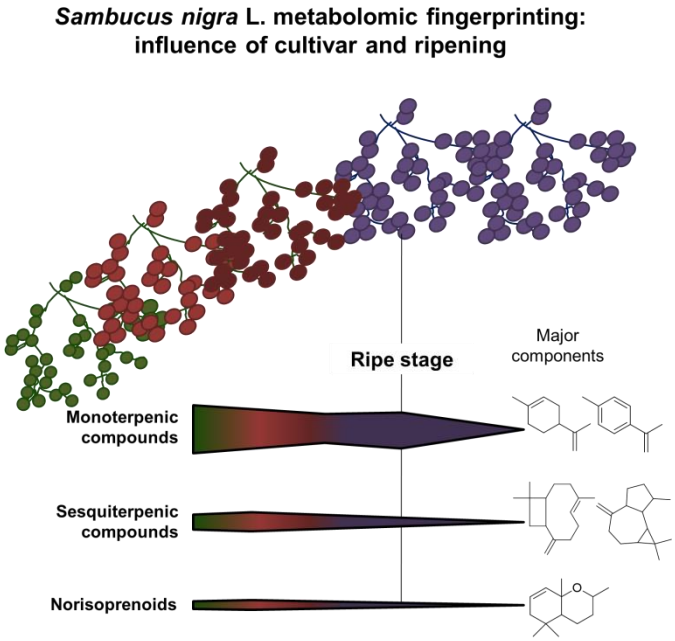
CHAPTER 4. Metabolomic-based strategy for fingerprinting of *S. nigra* L. berries volatile terpenoids and norisoprenoids: influence of ripening and cultivar



Adapted from:

ÂC Salvador, A Rudnitskaya, AJD Silvestre, SM Rocha, *Metabolomic-based strategy for fingerprinting of Sambucus nigra L. berries volatile terpenoids and norisoprenoids: influence of ripening and cultivar*, Journal of Agricultural and Food Chemistry, 2016, *64, 5428-5438*.

4.1. Abstract



The integration of plant metabolomics to support preharvest fruit development studies can provide important insights into the biochemical mechanisms involved and lately support producers on harvesting management. A metabolomic-based strategy for fingerprinting of volatile terpenoids and norisoprenoids from *S. nigra* L. berries from three cultivars, through ripening, was established. From 42 monoterpenic, 20 sesquiterpenic, and 14 norisoprenoid compounds, 48 compounds are reported for the first time as *S. nigra* berries components. Chemometric tools revealed that ripening was the factor that influenced more the volatile fraction profile and physicochemical parameters (pH, TA, and TSS), followed by cultivar. For the unripe stages, a higher overall content of the studied metabolites was observed, which gradually decreased over the ripening stages, being consistent for the three cultivars. These trends were mainly ruled by limonene, *p*-cymene, aromadendrene, *β*-caryophyllene, and dihydroedulan, which might therefore be used by producers as an additional simple decision making tool in conjunction with physicochemical parameters.

4.2. Introduction

Plant metabolomics comprises the study of all low molecular weight metabolites in plants, those being the end products of cellular function, which may be used to evaluate the response of biological systems to edaphoclimatic conditions and genetic manipulation, among others [68]. This approach has been applied to support preharvest fruit physiology studies, namely, to explore the effects of the growing conditions, developmental stages of different cultivars, and thus, provide important insights into the biochemical mechanisms involved [67,68], as well as key information to improve crop quality (e.g., sensorial characteristics, nutritional value, or other biological activities) by the adequate selection of harvesting conditions and date. Secondary metabolites are well positioned for metabolomics studies as these compounds are responsive to environmental and genetic factors [117]. Because of the commercial interest of plant secondary metabolites, that can be further exploited by the pharmaceutical, cosmetic or food industries, their metabolomic fingerprinting may be of utmost importance for crop valorization. Volatile terpenic compounds (mono and sesquiterpenic ones) and norisoprenoids have been studied in several plant matrices as they are responsible for a wide range of biological properties, such as potential health benefits, and also explain the peculiar aroma properties of plant related products [293]. For instance, sesquiterpenic compounds revealed hepatoprotective, antiradical, and antiproliferative [10,13], as well as anti-inflammatory [222] activities; while monoterpenic compounds showed, among others, immunomodulatory and anti-inflammatory potential [294], and influenced positively the lipid and glucose metabolism [295]. The bioprospection of these metabolites seems to be relevant for berry valorization, as these small fruits have been reported as potential sources of this kind of compounds [296].

Berries from *S. nigra* have been used for generations in folk medicine [30]. More recently, *in vivo* driven studies were performed in order to establish a biochemical rationale to understand the claimed health benefits of elderberries or derived products [179,188,189,297]. Although, those effects are mainly linked to phenolic compounds, the bioprospection of other secondary metabolites, namely, volatile and semivolatile terpenic compounds and norisoprenoids may also be explored, with the latter ones being particularly interesting to explain elderberry's peculiar aroma [36]. This knowledge could be explored by food industries to understand and improve the quality of elderberry based products [127]. To the best of our knowledge, 34 volatile terpenic and norisoprenoid compounds have been reported so far in *S. nigra* berries, namely, 31 monoterpenic compounds (among which 20 are oxygenated species), 2 norisoprenoids, and 1 sesquiterpenic compounds [34,36,95,127].

Fruit ripening affects several physiological and biochemical processes, resulting in changes in fruit characteristics [298]. Thus, a deeper understanding of the biochemical transformations underlying this process and their effect on fruit composition might be a key step to improve crop management and quality [299]. Furthermore, comparison of cultivar composition may also help producers in agricultural management, as cultivars may reveal different composition and environmental adaptation. To the best of our knowledge, there are no reported studies dealing with the effect of ripening stage on elderberry's volatile terpenic and norisoprenoid profile. However, differences have been reported on the terpenic and norisoprenoids compounds from elderberry juice, for different genotypes [36,95,127], which allows one to infer that more studies regarding the elderberry's chemical composition are needed, especially for unstudied geographic locations, as they could reveal peculiar metabolite profiles. In northern Portugal (Varosa Valley), elderberry plants benefit from favorable edaphoclimatic conditions for their growth [249], which has stimulated its cultivation, especially during the past decade.

In order to improve the elderberry valorization as a crop with wide a range of biomolecules, as well as to create support-decisions for elderberries producers, the aim of this study was to look in-depth at fingerprinting the volatile terpenoids and norisoprenoids released from *S. nigra* L. berries. As a case study, the three main cultivars grown in Varosa Valley, Portugal were selected, namely 'Sabugueiro', 'Sabugueira', and 'Bastardeira', and studied through ripening, allowing one to understand the plant response regarding ripening stage and cultivar. A detailed profiling was done using an advanced gas chromatographic methodology (GC×GC–ToFMS), a highly suited technique for the analysis of complex plant related matrices [225,226]. Classical parameters used to establish the berries technological ripening, corresponding to the harvesting day, were also screened (total soluble solids, titratable acidity, and pH), and special attention was devoted to ripen berries, the stage currently used for industrial applications.

4.3. Material and methods

The main stages for elderberry metabolomics profiling, including elderberry collection, sample preparation, and metabolites extraction, GC×GC-ToFMS analysis and data processing (Figure 4.1), are described in detail in the following subsections. The sampling, reporting of chemical analysis, and metadata relative to data preprocessing, pretreatment, processing, validation and interpretation were performed according to the Metabolomics Standards Initiative (MSI) [107].

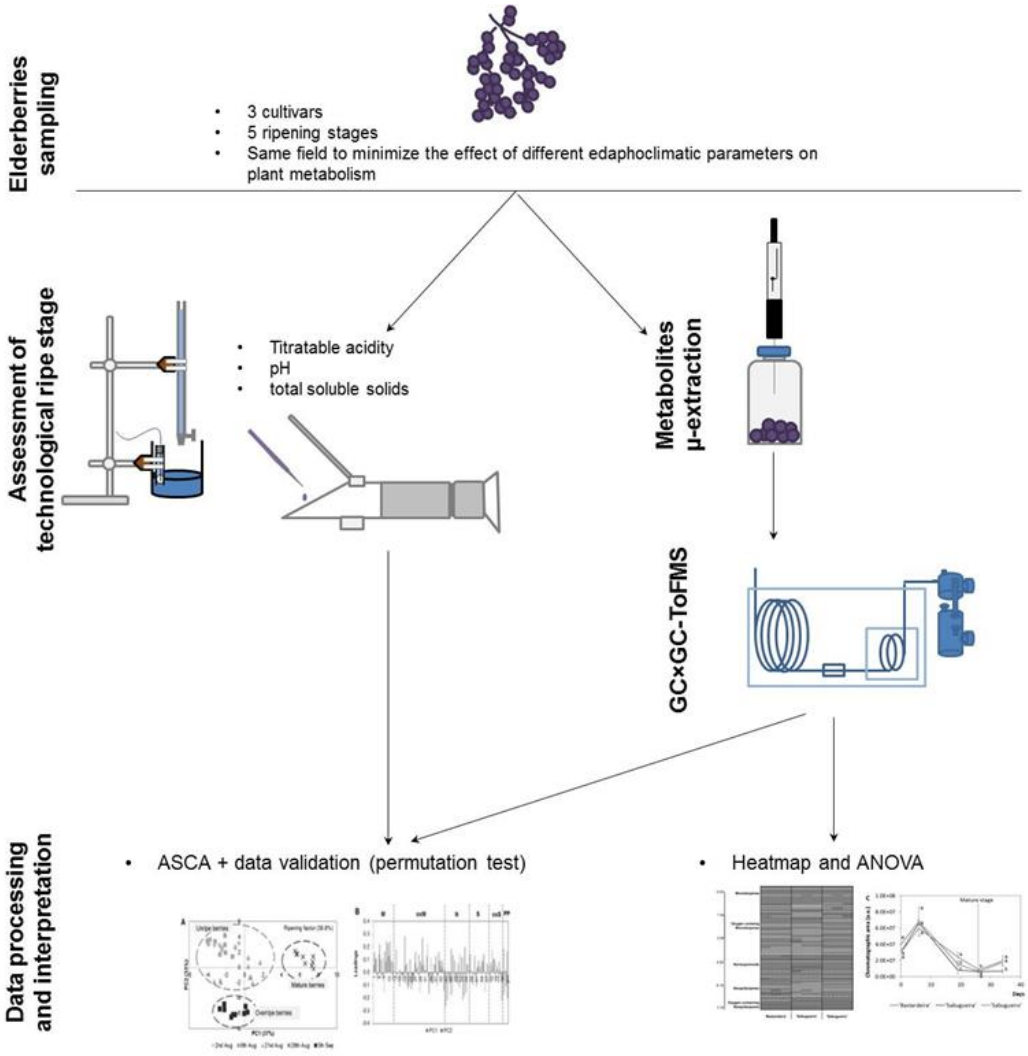


Figure 4.1. Main stages for *S. nigra* L. berry metabolomics fingerprinting: berry sampling, metabolites extraction, GC×GC-ToFMS analysis, and data processing. Also, parameters used for technological ripe stage assessment were included.

4.3.1. Materials and reagents

Eighteen standards, comprising monoterpenic (13) and sesquiterpenic compounds (3) and norisoprenoids (2) were used in order to confirm the identification of the corresponding metabolites: (R)-(+)-limonene (98%), *p*-cymene (99.5%), (-)-linalool (95.0%), (R)-(+)-citronellal (95.0%), menthol (99%), borneol (97.0%), *α*-terpineol (98%), *β*-citronellol (95.0%), *trans*-geraniol (98.0%), citral (95% mixture of isomers), carvone (98.5%), nerol (97.0%), geranyl acetone (97%), *β*-ionone (97%),*α*-copaene (90.0%), (-)-*β*-caryophyllene (98.5%) and humulene (96.0%), were purchased by Fluka (Buchs, Switzerland) and linalool oxide (97% mixture of isomers) from TCI Europe (Zwijndrecht, Belgium). Sodium hydroxide was purchased from Panreac (Barcelona, Spain). The retention index probe (an *n*-alkanes series of C_8 to C_{20} straight-chain alkanes, in *n*-

hexane) was supplied from Fluka (Buchs, Switzerland). The solid-phase microextraction (SPME) holder for manual sampling and the fiber coating used were purchased from Supelco (Aldrich, Bellefonte, PA, USA). The SPME device included a fused silica fiber coating partially cross-linked with divinylbenzene/carboxen™/polydimethylsiloxane StableFlex™ (DVB/CAR/PDMS) with 50/30 μm thickness and 1 cm of length. The fiber presents a wide range capacity of sorbing compounds with different physicochemical properties with molecular weights ranging from 40 to 275. According to the producer's recommendations, the SPME fiber was initially conditioned at 270 ºC for 60 min in the GC injector and daily for 10 min at 250 ºC.

4.3.2. Elderberry samples

Elderberries from *S. nigra* L. cultivars 'Sabugueira', 'Sabugueiro', and 'Bastardeira' were supplied in 2013 by the Cooperativa do Vale do Varosa - RégieFrutas (Tarouca, Portugal). The samples were collected in an experimental field (41.043233ºN, 7.728820ºW) with 0.5 ha, from 12/13-years old plants, where each plant produces approximately 15 kg of elderberries per year, with similar production yields between cultivars. This field was selected in order to harvest the three cultivars within the same location and minimize the effect of different edaphoclimatic conditions on plant metabolism. Agricultural management of this experimental area included soil pH adjustment to ca. 6 using 1500 kg/ha Tudidol (dolomite: 10% MgO and 43% CaO, Lusical, Alcanede, Portugal), addition of 1000 kg/ha of organic fertilizer Biorgano (Bricoagro21, Monção, Portugal), winter fertilization with 300 kg/ha of single superphosphate, $Ca(H_2PO_4)_2$ (18% Ca), and foliar feeding with aqueous sodium tetraborate decahydrate at 100 mL/hL, using 2L/ha. A Confidor Classic insecticide from group 4A (Bayer CropScience, Carnaxide, Portugal) at concentration of 50 mL/hL of water, using 0.5 L/ha, was also applied. The 'Sabugeira', 'Sabugueiro' and 'Bastardeira' elderberries were harvested over five ripening stages, being picked on August 2nd, 8th, 21st and 28th, and September 5th in 2013 (Table 4.1). For each ripening stage, elderberries from the three cultivars were collected between 9 and 12 a.m. For each cultivar and sampling moment, five average size bunches were collected from three shrubs and mixed together to prepare a pool, from which several aliquots were prepared and further analyzed. Samples were immediately transported under refrigeration (ca. 2-4ºC) to the laboratory and then stored at -20ºC and analyzed within 1 week after harvesting.

4.3.3. Assessment to elderberry's technological ripe stage

To assess the technological elderberry's ripe stage, three parameters were determined: titratable acidity, pH and total soluble solids. First, the elderberries were crushed, and the juice suspension was centrifuged at 3000 rpm for 5 min at room temperature to obtain a clarified juice.

The juice was then filtered through 0.45 μm pore size membrane filters. Total soluble solids content (TSS) was determined based on the corresponding ºBrix using an A.KRÜSS Optronic refractometer (Hamburg, Germany). Titratable acidity (TA) was determined by titrating each sample with a standardized NaOH solution (0.1 N) to pH 8.1 using a Crison micro pH 2000 (Barcelona, Spain) pH meter. TA was expressed in citric acid equivalents (g citric acid / 100g fresh berries). The pH was determined using the same equipment. For all determinations reported above, three independent aliquots of each elderberry cultivar were used.

4.3.4. Volatile terpenic and norisoprenoids metabolites determination by HS-SPME/GC×GC-ToFMS

About 5.5 g of thawed berries were weighed, crushed, and placed into a 20 mL glass vial (corresponding to a ratio of the volume of the liquid phase to the headspace volume, 1/β, of 0.5). Then, the vial was capped with a silicone/polytetrafluoroethylene septum and an aluminum cap (Chromacol LTD, Herts, UK), and placed in a thermostated bath adjusted to 60.0 ± 0.1 ºC. The SPME fiber was inserted in the vial headspace for 30 min. In order to avoid any crossover contamination due to the fiber coating, blanks, corresponding to analysis of the SPME fiber not submitted to any extraction procedure, were run between sets of three analyses. The sorbed volatiles on the SPME fiber coating were determined using a LECO Pegasus 4D GC×GC–ToFMS system (LECO, St. Joseph, MI, USA) consisting of an Agilent GC 7890A gas chromatograph (Agilent Technologies, Inc., Wilmington, DE), with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven, and mass spectrometer equipped with a ToF analyzer. After the extraction/concentration step, the SPME fiber was manually introduced into the injection port at 250ºC for analyte desorption. The injection port was lined with a 0.75 mm i.d. splitless glass liner. Splitless conditions (30s) were used. The detector was a high-speed ToF mass spectrometer. An Equity-5 30 m×0.32 mm i.d., 0.25 μm film thickness (Supelco, Bellefonte, PA, USA) was used as the first-dimension column (^{1}D) and a DB-FFAP 0.79 m×0.25 mm I.D., 0.25 μm film thickness (J&W Scientific Inc., Folsom, CA, USA) was used as a second-dimension column (^{2}D). The carrier gas was helium at a constant flow rate of 2.50 mL/min. The primary oven temperature was programmed from 40 ºC (1 min) to 230 ºC (2 min) at 10 ºC/min. The secondary oven temperature program was 30°C offset above the primary oven. Both the MS transfer line and MS source temperatures were 250°C. The modulation period was 6 s, keeping the modulator at 20°C offset above primary oven, with hot and cold pulses by periods of 0.90 and 2.10 s, respectively. The mass spectrometer was running in the EI mode at 70 eV and detector voltage of -1456 V, using an *m/z* range of 35-300. Total ion chromatograms were processed using the automated data processing software ChromaTOF® (LECO) at a signal-to-noise threshold of 100. Contour plots were used to

evaluate the separation general quality and for manual peak identification. For (putative) identification purposes, the mass spectrum and retention times (^{1}D and ^{2}D) of the analytes were compared with standards, when available (18 standards were used, as shown in Table 4.2 and annexes on Table A4. Total soluble solids (TSS), titratable acidity (TA) and pH from 'Sabugueira', 'Sabugueiro'). Also, the mass spectrum of each metabolite detected was compared to the mass spectral libraries, namely, an in-house library of standards and two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0 –Mainlib and Replib). A mass spectral match factor, similarity >850 out of 1000, was used to decide whether a peak was correctly identified. Moreover, the MS analysis was done (as briefly described on **Chapter 2**), and by combining additional information like retention index (RI) value, which was experimentally determined according to the van den Dool and Kratz RI equation [224]. A C_8-C_{20} *n*-alkanes series was used for RI determination (the solvent *n*-hexane was used as the C_6 standard), comparing these values with reported ones in existing literature for chromatographic columns similar to the ^{1}D column mentioned above [75,76,111,225,228–230,232,300–305]. The calculated retention index (RI_{calc}) differed 0–5% when compared to the literature data (RI_{lit}) for the ^{1}D column or equivalents. The DTIC (deconvoluted total ion current) GC×GC peak area data were used as an approach to estimate the relative content of each metabolite. Three independent aliquots of each sample were analyzed, and the reproducibility expressed as relative standard deviation (RSD) was calculated and presented in the annexes (Table A4.1-A4.3).

4.3.5. Statistical analysis

A full data matrix from elderberries that consists of 45 observations (3 cultivars × 5 ripening stages, each one by 3 independent assays) and two types of variables, 76 metabolites (Table A4.1-A4.3, from annexes) and 3 physicochemical parameters (TA, pH and TSS, Table 4.1), was constructed. In order to evaluate influence of ripening and cultivar on the elderberry metabolite composition and on physicochemical parameters an Analysis of Variance – Simultaneous Component Analysis (ASCA) was applied to the above data set. Significance of each effect was assessed using a permutation test. Data were permuted 2000 times and the percentage of the variance explained by each submodel in the total model was used as quality-of-fit criterion for the permutation test. Log transformation was used for data pretreatment to make the profiles comparable in size. ASCA is a combination of analysis of variance (ANOVA) and principal component analysis (PCA) and was proposed for the analysis of multivariate data sets from designed experiments [306,307]. The original data matrix is partitioned into a set of matrices corresponding to design factors and their interactions and running PCA on each of these matrices. ASCA and permutation tests were conducted by MATLAB, v. 7.12. In order to evaluate the effect

of cultivar and ripening stage on the global content of each chemical family under study, another matrix was constructed from the above full data set, using only as variables the data from volatile metabolites. One-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's HSD) using GraphPad Prism® version 6, for Windows (30-day trial version, GraphPad Software, San Diego California, USA), was applied. It was considered statistically significant when $p<0.05$. Finally, to rapidly and visually assess the metabolomics of ripe elderberry profiling, a reduced data set was prepared from the previous subdata set using only data corresponding to ripe elderberries (9 observations at ripe state = 3 cultivars, each one by 3 independent assays and 76 variables) (Table A4.1-A4.3, from annexes, harvesting date in light gray represents the ripe stage). Heatmap visualization was applied on this sub-dataset, using the absolute GC peak area and normalizing each variable area by applying a logarithm function, using the Unscrambler® X (30-day trial version – CAMO Software AS, Oslo, Norway).

4.4. Assessment of elderberry's technological ripe stage

In order to identify the technological ripe stage and establish a harvest schedule, pH, total soluble solids (sugar), and titratable acidity are currently used [28,293]. Total soluble solids (TSS), titratable acidity (TA) and pH from 'Sabugueira', 'Sabugueiro' and 'Bastardeira' elderberries, at five ripening stages, were determined (Table 4.1).

Table 4.1. Total soluble solids (TSS), titratable acidity (TA) and pH from 'Sabugueira', 'Sabugueiro' and 'Bastardeira' elderberries, at five ripening stages from the harvesting season of 2013. The harvesting date in light gray represents the ripe berries.

Cultivar	Harvesting date	TSS (ºBrix)	TA[a]	pH
'Sabugueira'	Aug. 2nd	7.3 ± 0.1	1.63 ± 0.03	3.47 ± 0.03
	Aug. 8th	9.8 ± 0.4	1.23 ± 0.09	4.04 ± 0.02
	Aug. 21st	11.4 ± 0.6	1.09 ± 0.04	4.30 ± 0.08
	Aug. 28th	12.7 ± 1.2	1.01 ± 0.21	4.64 ± 0.03
	Sep. 5th	12.8 ± 1.2	1.16 ± 0.13	4.47 ± 0.06
'Sabugueiro'	Aug. 2nd	7.3 ± 0.2	1.43 ± 0.11	3.57 ± 0.09
	Aug. 8th	8.2 ± 0.3	1.22 ± 0.20	3.63 ± 0.06
	Aug. 21st	10.7 ± 0.6	1.04 ± 0.06	3.88 ± 0.15
	Aug. 28th	12.4 ± 1.2	1.00 ± 0.11	4.40 ± 0.20
	Sep. 5th	10.5 ± 0.6	1.07 ± 0.09	4.60 ± 0.08
'Bastardeira'	Aug. 2nd	7.3 ± 0.2	1.35 ± 0.03	3.76 ± 0.23
	Aug. 8th	9.0 ± 0.4	1.09 ± 0.25	3.95 ± 0.08
	Aug. 21st	10.7 ± 1.2	0.94 ± 0.30	4.46 ± 0.07
	Aug. 28th	12.3 ± 0.6	0.81 ± 0.09	4.62 ± 0.01
	Sep. 5th	11.2 ± 0.3	0.92 ± 0.09	4.70 ± 0.15

[a] g of citric acid/100g of fresh fruits.

For all the cultivars, the pH and TSS values tended to increase through ripening, ranging from 3.47 to 4.70 and 7.3 to 12.8 ºBrix, respectively, reaching a maximum for TSS on August 28th, with a slight decrease after that (September 5th), while pH remained nearly constant. The TA values decreased along the different ripening stages until August 28th, and then kept constant, ranging from 0.81 to 1.63 g of citric acid/100g fresh weight (FW) berries. The data obtained suggest that August 28th is the nearest point to the ripe stage. Regarding the three parameters under study, no significant differences were observed between the three ripe cultivars (p>0.05). Indeed, at ripe stage (Table 4.1), the pH ranged from 4.40 to 4.64, TA from 0.81 to 1.01 g of citric acid/100g FW berries and TSS from 12.3 to 12.7 ºBrix. These data reveals that the pH, TA and TSS levels of the berries from Varosa Valley are within values reported in the literature for ripe elderberries collected from other regions (pH ranging from 3.8 to 4.8, TA from 0.48 to 1.43 g citric acid/100g FW berries and TSS from 10.1 to 17.5 ºBrix) [35,95,117].

4.5. Ripe elderberry volatile terpenic and norisoprenoids profile

Because of the commercial interest of ripe berries, particular attention was devoted to this stage. Volatile terpenic and norisoprenoids metabolites released from ripe elderberries are listed in Table 4.2.

Table 4.2. Volatile terpenic and norisoprenoids metabolites detected in elderberries (*S. nigra* L.) using HS-SPME/GC×GC-ToFMS, including relevant chromatographic data used to assess metabolites identification.

Peak Number	^1t_R [a] (s)	^2t_R [a] (s)	Compound	RI_{lit} [b]	RI_{calc} [c]	Chemical Formula	Ref. RI_{lit} for 2D-GC	Ref. RI_{lit} for 1D-GC	MSI level [d]	Compound Previously Reported on elderberries [e]
			Monoterpenic compounds							
			Hydrocarbon-type							
1	312	0.430	*α*-Pinene	941	938	$C_{10}H_{16}$	[225]		2	-
2	330	0.490	Verbenene	967	957	$C_{10}H_{14}$	[225]		2	-
3	342	0.401	Camphene	960	961	$C_{10}H_{16}$	[225]		2	-
4	348	0.470	*β*-Pinene	987	975	$C_{10}H_{16}$	[225]		2	✓
5	360	0.500	Myrcene	1008	988	$C_{10}H_{16}$	[225]		2	✓
6	378	0.447	*β*-Phellandrene	1035	1008	$C_{10}H_{16}$	[225]		2	✓
7	396	0.500	Limonene	1035	1027	$C_{10}H_{16}$	[225]		1	✓
8	396	0.570	*p*-Cymene	1039	1027	$C_{10}H_{14}$	[225]		1	-
9	426	0.590	1,3,8-*p*-Menthatriene	1121	1060	$C_{10}H_{14}$	[75]		2	-
10	456	0.530	Terpinolene	1097	1094	$C_{10}H_{16}$	[225]		2	✓
11	456	0.620	*p*-Cymenene	1090	1094	$C_{10}H_{12}$		[229]	2	-
12	582	0.620	*m/z* 93, 43, 77	-	1237	-			3	-
			Oxygen containing							
13	438	0.640	Linalool oxide isomer (furanoid) [f]	1078	1074	$C_{10}H_{18}O_2$	[225]		1	✓
14	438	0.730	Dihydromyrcenol	1076	1074	$C_{10}H_{20}O$	[226]		2	-
15	456	0.720	Linalool oxide isomer (furanoid) [f]	1097	1094	$C_{10}H_{18}O_2$	[225]		1	✓
16	462	0.820	Linalool	1108	1101	$C_{10}H_{18}O$	[225]		1	✓
17	468	0.950	Hotrienol isomer	1122	1108	$C_{10}H_{16}O$	[225]		2	✓
18	474	0.510	Rose oxide isomer	1117	1114	$C_{10}H_{18}O$	[225]		2	✓
19	480	0.870	Fenchol	1118	1121	$C_{10}H_{18}O$	[225]		2	-
20	492	0.510	Rose oxide isomer	1130	1131	$C_{10}H_{18}O$	[225]		2	✓
21	498	0.600	Artemisia alcohol	1083	1141	$C_{10}H_{18}O$		[228]	2	-
22	504	0.740	Camphor	1147	1148	$C_{10}H_{16}O$	[225]		2	✓
23	510	0.650	Citronellal	1159	1154	$C_{10}H_{18}O$	[225]		1	-
24	516	0.990	Hotrienol isomer	1122	1162	$C_{10}H_{16}O$	[225]		2	✓
25	522	0.690	Pinocarvone	1164	1167	$C_{10}H_{14}O$	[225]		2	-
26	528	0.850	Menthol	1173	1174	$C_{10}H_{20}O$		[228]	1	✓
27	528	0.990	Borneol	1172	1174	$C_{10}H_{18}O$	[225]		1	✓
28	528	1.070	Epoxylinalool	1173	1175	$C_{10}H_{18}O_2$		[301]	2	-
29	534	0.787	Terpinen-4-ol	1181	1181	$C_{10}H_{18}O$	[225]		2	✓
30	540	1.380	*p*-Cymen-8-ol	1203	1188	$C_{10}H_{14}O$	[225]		2	-
31	546	0.500	3-Caren-2-ol	1211	1194	$C_{10}H_{16}O$	[225]		2	-
32	546	0.920	*α*-Terpineol	1206	1194	$C_{10}H_{18}O$	[225]		1	✓
33	576	0.950	*β*-Citronellol	1237	1230	$C_{10}H_{20}O$	[225]		1	✓
34	576	1.050	*trans*-Geraniol	1235	1230	$C_{10}H_{18}O$	[225]		1	✓
35	588	0.750	*m/z* 59, 68, 43	-	1244	-			3	-

36	588	0.800	Citral isomer[f]	1241	1244	$C_{10}H_{16}O$	[226]		1	✓
37	588	0.910	Carvone	1245	1244	$C_{10}H_{14}O$	[225]		1	-
38	600	1.070	Nerol	1242	1258	$C_{10}H_{18}O$	[225]		1	-
39	612	0.820	Citral isomer[f]	1287	1272	$C_{10}H_{16}O$	[226]		1	✓
40	684	0.600	*β*-Terpinyl acetate	-	1357	$C_{12}H_{20}O_2$			2	-
41	702	0.607	Geranyl acetate	1383	1385	$C_{12}H_{20}O_2$		[228]	2	-
42	714	0.680	10-(Acetylmethyl)-3-carene	1380	1401	$C_{13}H_{20}O$		[302]	2	-
			Nosiroprenoids							
43	402	0.620	2,2,6-Trimethyl-cyclohexanone	1051	1034	$C_9H_{16}O$	[303]		2	-
44	504	1.080	Ketoisophorone	1171	1148	$C_9H_{12}O_2$	[303]		2	-
45	552	0.830	Safranal	1241	1201	$C_{10}H_{14}O$	[226]		2	-
46	570	0.670	*β*-Cyclocitral	1261	1222	$C_{10}H_{16}O$	[226]		2	-
47	636	0.570	Dihydroedulan	1289	1300	$C_{13}H_{22}O$		[232]	2	✓
48	654	0.500	Theaspirane	1308	1322	$C_{13}H_{22}O$		[302]	2	-
49	708	0.760	Damascenone	1379	1393	$C_{13}H_{18}O$		[302]	2	✓
50	738	0.740	*α*-Ionone	1422	1432	$C_{13}H_{20}O$		[302]	2	-
51	744	0.950	Megastigma-3,5-dien-9-ol	1430	1440	$C_{13}H_{22}O$		[302]	2	-
52	750	0.850	*β*-Ionon-5,6-epoxide	1428	1447	$C_{13}H_{20}O_2$		[302]	2	-
53	756	0.700	Geranyl acetone	1454	1455	$C_{13}H_{22}O$	[111]		1	-
54	780	0.680	*α*-methyl ionone	1486	1486	$C_{14}H_{22}O$	[305]		2	-
55	780	0.780	*β*-Ionone	1481	1487	$C_{13}H_{20}O$		[302]	1	-
56	900	0.970	Methyl dihydrojasmonate	1650	1660	$C_{13}H_{22}O_3$		[228]	2	-
			Sesquiterpenic compounds							
			Hydrocarbon-type							
57	678	0.490	*β*-Cubebene	1387	1354	$C_{15}H_{24}$	[76]		2	-
58	702	0.500	*α*-Copaene	1375	1385	$C_{15}H_{24}$	[76]		1	-
59	708	0.510	*β*-Bourbonene	1379	1393	$C_{15}H_{24}$	[76]		2	-
60	714	0.540	*β*-Elemene	1380	1401	$C_{15}H_{24}$	[76]		2	-
61	738	0.515	Longifolene	1395	1431	$C_{15}H_{24}$	[76]		2	-
62	738	0.540	*β*-Caryophyllene	1417	1432	$C_{15}H_{24}$	[76]		1	-
63	750	0.480	Aromadendrene	1447	1447	$C_{15}H_{24}$	[75]		2	-
64	762	0.560	*α*-Humulene	1450	1462	$C_{15}H_{24}$	[76]		1	✓
65	792	0.506	*α*-Muurolene	1490	1499	$C_{15}H_{24}$	[76]		2	-
66	810	0.570	*Δ*-Cadinene	1525	1528	$C_{15}H_{26}$	[76]		2	-
67	810	0.630	Calamenene	1525	1528	$C_{15}H_{22}$	[230]		2	-
68	828	0.680	*α*-Calacorene	1554	1556	$C_{15}H_{20}$	[76]		2	-
			Oxygen containing							
69	822	0.810	*β*-bourbonen-13-ol	-	1547	$C_{15}H_{24}O$			2	-
70	840	0.670	Epiglobulol	1588	1572	$C_{15}H_{26}O$		[304]	2	-
71	858	0.710	Caryophyllene oxide	1610	1601	$C_{15}H_{24}O$	[76]		2	-
72	870	0.730	Globulol	1592	1618	$C_{15}H_{26}O$	[75]		2	-
73	888	0.730	Cubenol	1643	1643	$C_{15}H_{26}O$	[76]		2	-
74	894	0.830	*t*-Cadinol	1650	1651	$C_{15}H_{26}O$	[76]		2	-

75	918	0.900	*m/z* 91, 119, 105	-	1685	-	3	-
76	1014	0.710	*m/z* 69, 41, 55	-	1841	-	3	-
Number of Detected Compounds			76					

[a] Retention times for first (^1t_R) and second (^2t_R) dimensions in seconds.
[b] RI, Retention Index reported in the literature for Equity-5 GC column or equivalents.
[c] RI: Retention Index obtained through the modulated chromatogram.
[d] Level of metabolite identification according to Metabolomics Standards Initiative. (1) Identified compounds using standards co-injection; (2) Putatively annotated compounds; (3) Putatively characterized compound classes; (4) Unknown compounds [107]
[e] Compound previously reported on elderberries.[34,95,127]
[f] The used standards are a mixture of isomers

A representative total ion GC×GC-ToFMS chromatogram contour plot from the ripe cultivar 'Sabugueiro' (harvested on August 28th, 2013) is illustrated (Figure 4.2), revealing the complexity of the matrix and illustrating the potentials of the comprehensive GC×GC to generate a structured chromatogram, where the analytes are separated according to their physicochemical characteristics, through volatility (^{1}D) and polarity (^{2}D).

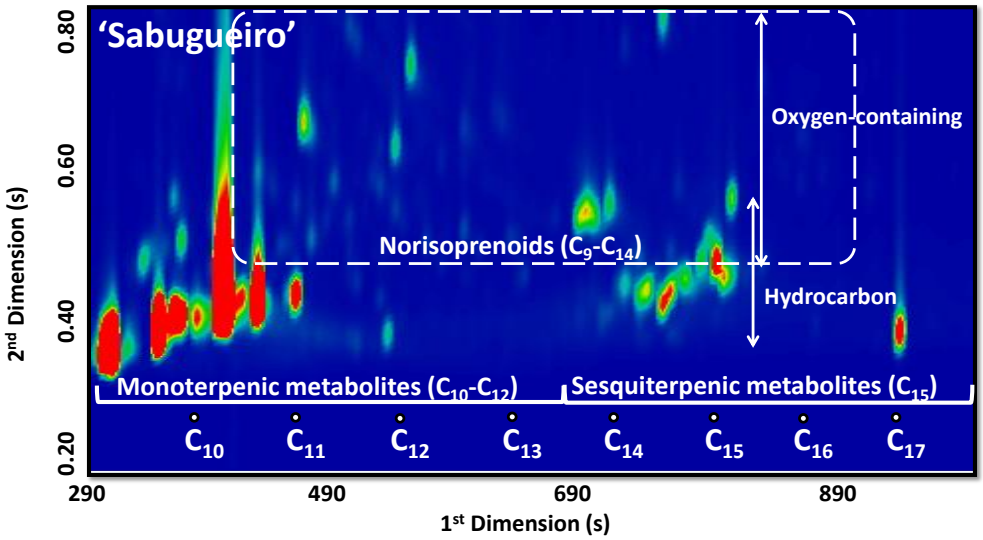


Figure 4.2. Blow up of total ion GC×GC–ToFMS chromatogram contour plot from 'Sabugueiro' ripe elderberries selected to illustrate structured chromatogram principle: clusters formed by structurally related compounds are highlighted. The white spots indicate the position of the series of alkanes (C_{10}-C_{17}).

Thus, structurally related metabolites occupy similar 2D spaces, and specific regions are established for the families under study: along ^{1}D, the metabolites are distributed in the following order: monoterpenic (C_{10}-C_{12}), norisoprenoid (C_9-C_{14}), and sesquiterpenic metabolites (C_{15}), and along ^{2}D, from hydrocarbon to oxygen-containing structures. The structured chromatogram principle is particularly interesting for the putative identification of components of complex matrices, when standards are not commercially available. The high chromatographic resolution, low detection limits, and sensitivity of the GC×GC-ToFMS allowed the detection of 76 compounds in the studied samples (Table 4.2), being distributed over the monoterpenic (monoterpenes and oxygen-containing monoterpenes), norisoprenoids, and sesquiterpenic (sesquiterpenes and oxygen-containing sesquiterpenes) families. From these, 48 are reported for the first time [34,95,127], resulting in a significant expansion of the metabolomic coverage of elderberries only possible through the use of 2D orthogonal GC analysis. Furthermore, it is important to highlight that HS-SPME was used to simultaneously extract and concentrate volatile metabolites directly from berries, through a short extraction time and moderate temperature (30 min / 60 ºC), avoiding any

solvent or chemical addition. This procedure minimizes the formation of artifacts, suggesting that the monoterpenoid alcohols identified on elderberries (e.g., linalool and α-terpineol) and oxides (e.g., rose oxides) are naturally present on elderberries, rather than being produced during hydrolysis promoted by extraction, as previously suggested [36].

A heatmap representation was constructed (Figure 4.3) using data from 'Sabugueiro', 'Sabugueira' and 'Bastardeira'. Each compound content was illustrated through different colors (from blue, minimum to red, maximum), allowing a rapid and visual assessment of its relative abundance. This provides information of the different intensities of each of the 76 metabolites based on the absolute (A) and normalized (logarithmized) GC peak areas (B).

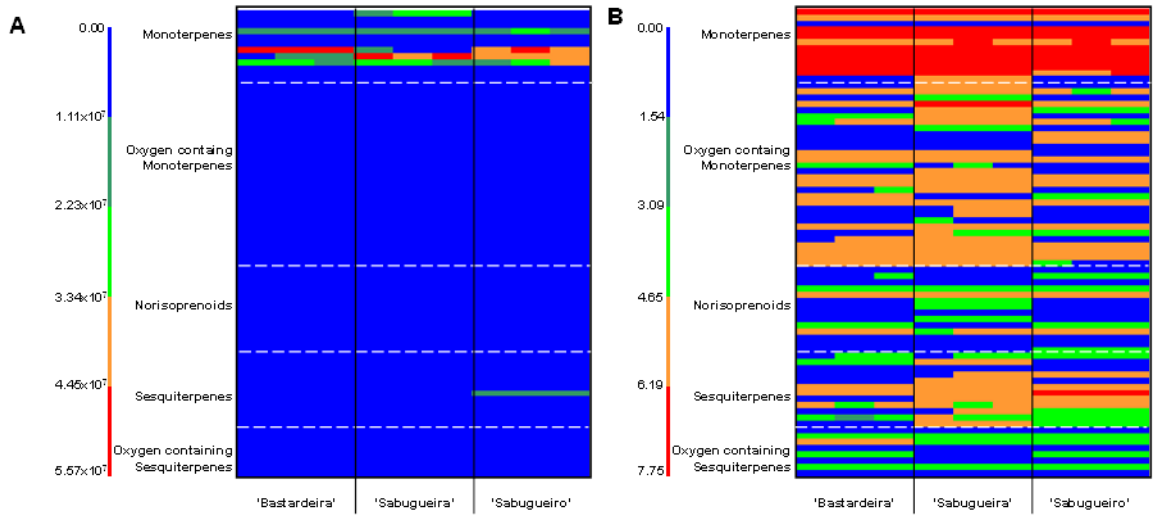


Figure 4.3. Heatmap representation of the 76 metabolites (putatively) identified from 'Bastardeira', 'Sabugueira' and 'Sabugueiro' ripe elderberries: A. GC peak areas, expressed as arbitrary units B. GC peak areas normalized by logarithmization. Each compound content was illustrated through different colors (from blue- minimum to red – maximum).

As can be seen by the color (red), the headspace monoterpenes largely prevailed for the three studied cultivars. Those represented from 66.5% for 'Sabugueira' to 77.2% for 'Bastardeira' of the overall terpenic and norisoprenoid content (the relative content of each chemical family is provided in annex, Table A4.1-A4.3).

This may be also noticed from the chromatogram contour plot (Figure 4.2) observation, where the peak intensities of the compounds show C_{10}-C_{12} prevalence. In order to perform in-depth analysis of all metabolite patterns, a log transformation was performed to make this highly skewed profile less skewed (Figure 4.3B), making patterns in the data more interpretable.

Within the monoterpenes class, limonene was the most abundant compound for the ripe cultivars 'Bastardeira' and 'Sabugueiro', while for the cultivar 'Sabugueira', *p*-cymene prevailed (Table A4.1-S4.3). Through the analysis of the heatmap and with the annexed data support,

significant differences (p<0.05) among the studied cultivars on the overall content of the studied chemical families (with the exception of hydrocarbon monoterpenes) are observed. For instance, the headspace oxygen containing monoterpenes prevailed for the cultivar ‘Sabugueira’ (p<0.05) compared to the other studied cultivars, which can be seen on the heatmap (Figure 4.3B). Regarding the number of detected terpenic and norisoprenoid compounds on the three ripe cultivars (Table A4.1-S4.3), 43, 46, and 57 compounds were detected on the cultivars ‘Bastardeira’, ‘Sabugueiro’ and ‘Sabugueira’, respectively.

These differences in the volatile profile are expected to have an impact on the sensorial characteristics of the studied elderberry samples, as previously demonstrated for different ripe elderberry cultivars or elderberry-based products [34,95,127].

4.6. In-depth fingerprinting of three elderberry cultivars at different ripening stages

The integration of metabolomics data with complementary information from conventional parameters (TA, pH, and TSS), used to evaluate technological ripe stage, was performed as a system biological strategy that would help to better describe the complexity of volatile metabolite variation during ripening and between cultivars. Actually, during ripening several phenomena take place, such as the accumulation of sugars and decrease in acidity, and these attributes have been routinely used by producers as a guide to decision-making. However, other phenomena occur, namely, the biosynthesis and degradation of a wide range of components that may have direct relevance to elderberry quality, but this information is not currently available, nor can it easily be included as part of the decision-making package of elderberry producers. The volatile terpenic and norisoprenoids metabolites’ profile is a combination of two pathways, the mevalonate and the methylerythritol phosphate (2-*C*-methyl-D-erythritol 4-phosphate) pathways [131]. During fruit ripening, these pathways can be modulated by enzymes, namely, terpene synthases and degradation processes, such as carotenoid cleavage (Figure 4.4) [289,308], resulting in variations in the elderberry metabolite profiles along ripening and between cultivars.

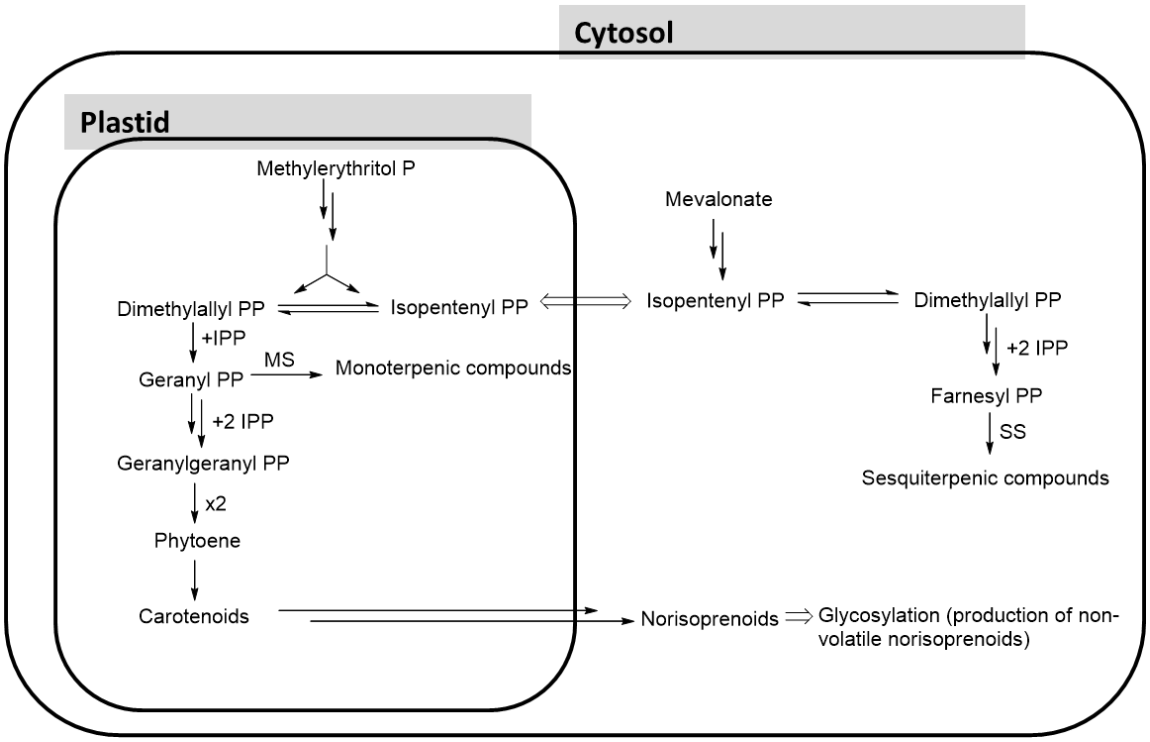


Figure 4.4. Summary of target metabolite modulation that occurs on elderberry ripening mapped along a simplified biochemical pathway [289,308]**. MS – monoterpene synthases; SS – sesquiterpene synthases; PP – pyrophosphate.**

First, the total content of each chemical family through ripening was followed, and in a second step, a detailed fingerprinting was performed by using the metabolites data through ripening stages and for three cultivars, using ASCA chemometrics tool, which highlights the influence of ripening (independently of the analyzed cultivar); cultivar (independently of the ripening stage); and their interaction (cultivar×ripening).

Despite, significant differences (p<0.05), which may be observed for specific harvesting moments, total metabolite content for the three families under study exhibited a similar trend of variation through ripening for the three cultivars (Figure 4.5). The exception was perceived for sesquiterpenic compounds from ‘Bastardeira’, for which the highest content reached before the ripe stage, followed by a decrease through ripening.

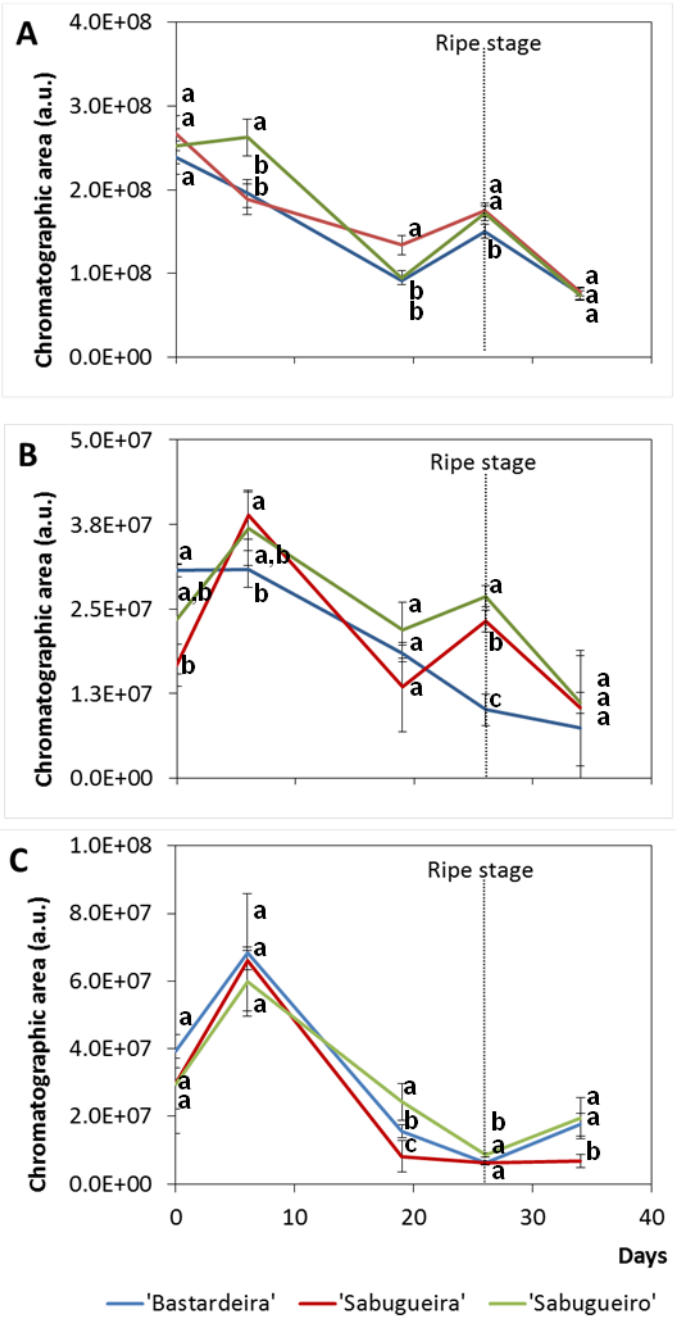


Figure 4.5. Follow up of monoterpenic (A), sesquiterpenic (B) and norisoprenoid (C) metabolites (expressed as total chromatographic areas) through ripening from 'Bastardeira', 'Sabugueira' and 'Sabugueiro' elderberries. a. u.: arbitrary units. Different letters indicate statistically significant differences between cultivars at each harvesting date, $p<0.05$.

These overall trends were ruled by the major components of each chemical family: limonene (7) and *p*-cymene (8), for monoterpenic compounds, *β*-caryophyllene (62) and aromadendrene (63), for sesquiterpenic compounds, and worth highlighting, dihydroedulan (47) for norisoprenoids, given its relevant impact on elderberry odor [95]. These results are in agreement with data reported

for other berries, such as strawberry tree berries (*A. unedo* L.) [309] and raspberries (*Rubus idaeus*) [310], where a decrease in volatiles component content through ripening was also noticed. Furthermore, the increase of free norisoprenoids content at the beginning of ripening is expected, as it is linked to the carotenoid breakdown, while the subsequent decrease could be explained by their glycosylation [308], as the berries' sugar content (TSS) increased during this period (Table 4.1).

Scores and loadings plots of the ASCA submodels ripening, cultivar, and cultivar×ripening are illustrated in the Figure 4.6A-E. The *p*-values were significant for all submodels under study ($p<0.0005$ for ripening and cultivar×ripening, and $p<0.001$ for cultivar), explaining 52.8% of the total data set variance.

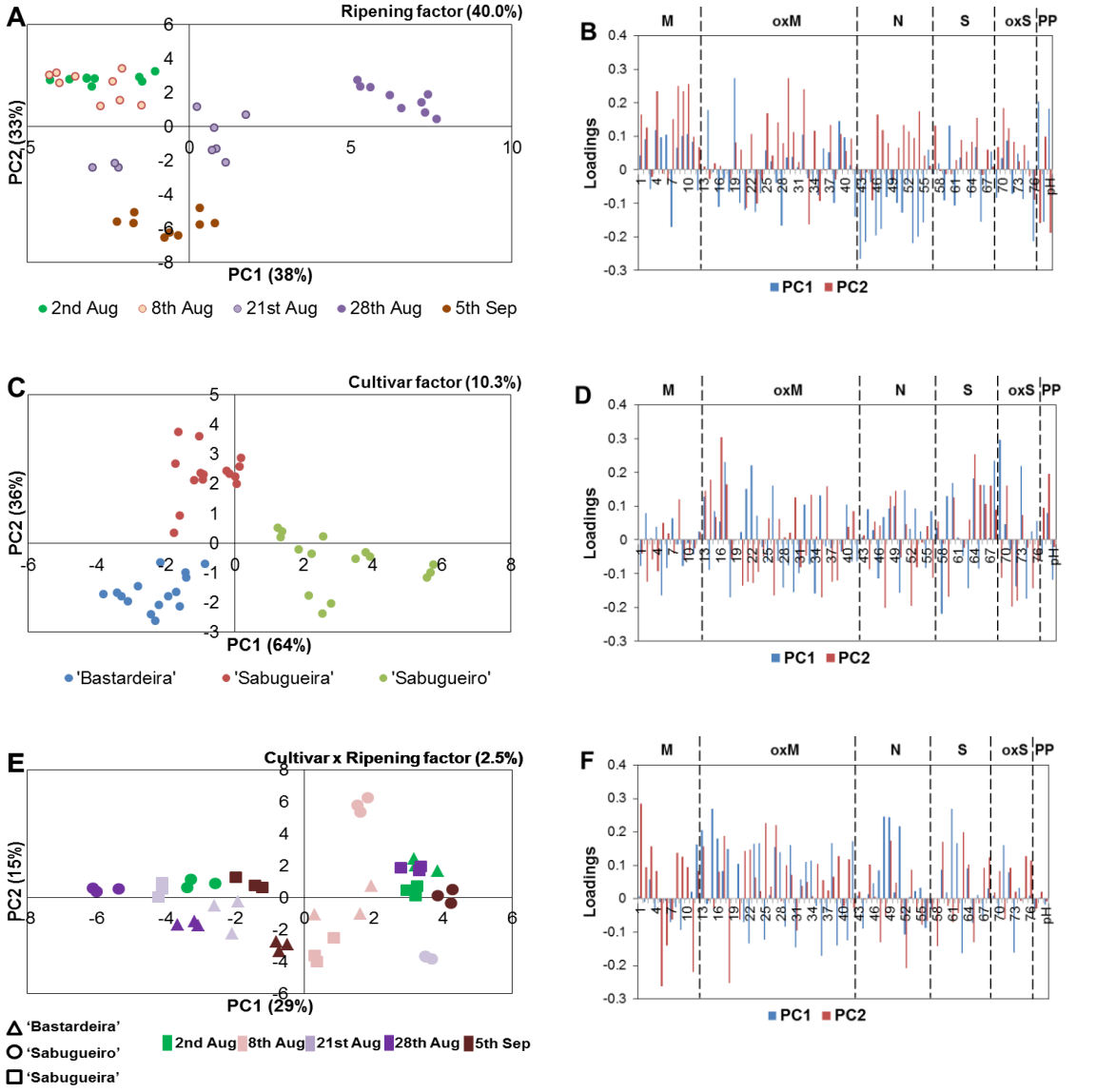


Figure 4.6. ASCA scores plot for ripening (A), cultivar (C) and cultivar × ripening (E) factors, and the corresponding variable loadings plot (B, D and F respectively) of elderberries from three cultivars at five ripening stages each. The set of 76 metabolites identified by GC×GC–ToFMS (peak number attribution shown in Table 4.2) and the physicochemical parameters (pH, TA and Brix degree shown in Table 4.1) used to assess the technological ripe stage were used. M- Monoterpenes; oxM- oxygen containing Monoterpenes; N-Norisoprenoids; S- Sesquiterpenes; oxS- oxygen containing Sesquiterpenes; PP- Physicochemical parameters.

Ripening stage was the largest source of variance for all cultivars accounting for 40.0% (Figure 4.6A-B), followed by the cultivar accounting for 10.3% (Figure 4.6C-D) and the interaction cultivar×ripening submodel accounting for the lowest percentage of variance, 2.5% (Figure 4.6E-F). The number of PCs used in the ASCA submodels of the analyzed data was 2 components for the cultivar and ripening, and 4 for the cultivar×ripening. A clear distinction between the ripe

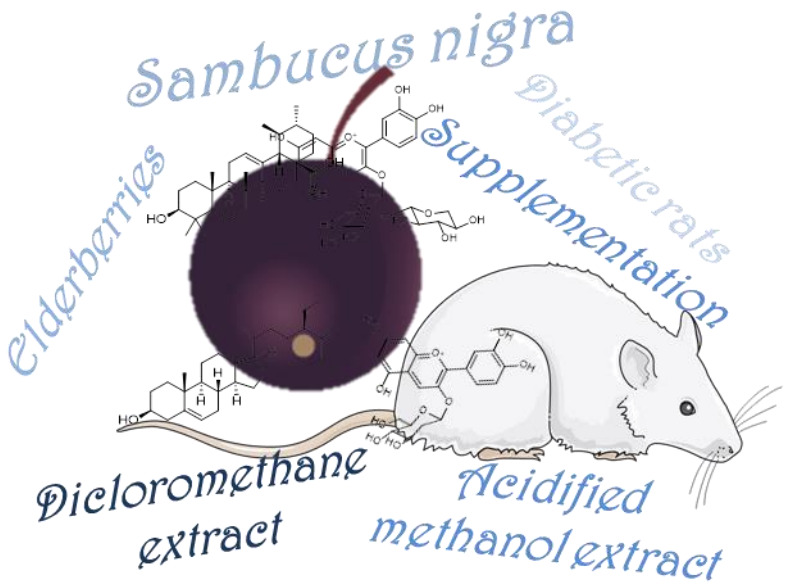
berries (August 28th), and the remaining ripening stages was observed for all cultivars (Figure 4.6A), with ripe berries being located on the PC1 positive (first quadrant), while the berries from the first stages are located on the opposite position (PC1 negative). According to loading weight (Figure 4.6B), PC1 positive is characterized mainly by the variability on monoterpenic compounds, namely by *β*-pinene (4), 1,3,8-*p*-menthatriene (9), terpinolene (10), dihydromyrcenol (14), fenchol (19), *α*-terpineol (32) and citral isomer (39), and sesquiterpene *β*-elemene (60) as well. The first two ripening stages (August 2nd and 8th) were located on the second quadrant of the PCA being distinguished mainly by the norisoprenoids, *β*-cyclocitral (46), dihydroedulan (47), megastigma-3,5-dien-9-ol (51), geranyl acetone (53), *α*-methyl ionone (54) and *β*-ionone (55), as well as limonene (7), epoxylinalool (28), and TA. The prevalence of norisoprenoids on the first ripening stages is in accordance with their higher overall content (Table A4.1-A4.3), and their relative decrease along ripening should be linked to the glycosylation of these compounds into nonvolatile forms [308], as illustrated in Figure 4.4. For instance, the norisoprenoid *β*-cyclocitral (46) was up to 32-fold higher on unripe elderberries when compared with that of ripe elderberries. The overripe berries (September 5th) were located on the PC2 negative, and the variables that contributed more to their position were artemisia alcohol (21), citronellal (23), *β*-citronellol (33), safranal (45), and the physicochemical parameters TSS and pH.

Regarding the evaluation of the cultivar factor, a distinction was observed between the three cultivars according to the PC1×PC2 space. The ‘sabugueiro’ cultivar was located on the positive PC1 scores plot (Figure 4.6C), being characterized mainly by hotrienol isomer (17), camphor (22), *β*-elemene (60), *α*-calacorene (68), *β*-bourbonen-13-ol (69) and cubenol (73) (Figure 4.6D). ‘Sabugueira’ and ‘Bastardeira’ cultivars were located mainly on PC1 negative, being distinguished by PC2. Dihydromyrcenol (14), linalool (16), *α*-humulene (64), and epiglobulol (70), as well, as the TA parameter contributed to the location of the ‘Sabugueira’ cultivar on PC2 positive, while the menthol (26), dihydroedulan (47), *β*-ionon-5,6-epoxide (52), caryophyllene oxide (71), and globulol (72) contributed to the position of the cultivar ‘Bastardeira’ on PC2 negative. The submodel cultivar×ripening interaction explained low variance (2.5%), and no clear tendency may be extracted by the interpretation of the respective PCA scores and loadings (Figure 4.6E-F).

4.7. Conclusions

In summary, the integration of metabolomics data with complementary information from conventional parameters (TA, pH, and TSS), currently used to evaluate technological ripe stage, was performed as a strategy that would help to better describe the complexity of volatile metabolite variation during ripening and between cultivars. During ripening was observed an accumulation of sugars, a decrease in acidity, and also modifications in the profiles of terpenes and carotenoid cleavage products (i.e., norisoprenoids), revealing that several biochemical phenomena took place during elderberry ripening. Regarding the TSS, TA, and pH values, no significant differences were observed between the three ripe cultivars (p>0.05) under the previous study from Varosa Valley (Portugal), and they exhibited values which are within those reported in the literature for other regions [35,95,117]. In addition, the total metabolite content for the three families under study exhibited a similar trend of variation through ripening for the three cultivars, and these trends were ruled by the major components of each chemical family. Thus, limonene (7), *p*-cymene (8), dihydroedulan (47), *β*-caryophyllene (62), and aromadendrene (63) may be suggested as quality markers to follow-up the content of these the corresponding families. Additionally, the detailed analysis of the mono and sesquiterpenic profiles and norisoprenoids, based on GC×GC-ToFMS tandem with the ASCA chemometric tool, allowed to detect 76 components, of which 48 were reported for the first time as *S. nigra* L. berries and revealed differences between ripening stage and cultivar. Despite, the higher impact of the ripening stage, cultivar also seems to have impact on the modulation of the secondary metabolites profiles. Yet, terpene metabolism remains poorly understood, and their evolution during berry ripening could be species and even variety-dependent [311].

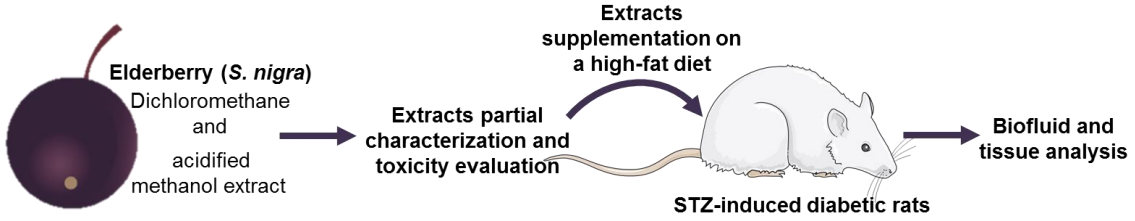
CHAPTER 5. Effect of elderberry extracts supplementation in STZ-induced diabetic rats fed with a high-fat diet



Adapted from:

ÂC Salvador, E Król, V C. Lemos, SAO Santos, FPMS Bento, CP Costa, A Almeida, D Szczepankiewicz, B Kulczyński, Z Krejpcio, AJD Silvestre, SM Rocha, *Effect of elderberry (Sambucus nigra L.) extracts supplementation in STZ-induced diabetic rats fed with a high-fat diet*, International Journal of Molecular Sciences, 2017, 18, 13.

5.1. Abstract



Elderberry (*S. nigra* L.) lipophilic and polar extracts dietary supplementation effects were evaluated on diabetes management indices, using an *in vivo* model. A research pipeline was constructed, that ranged from extracts preparation, partial chemical characterization and toxicity evaluation, to examining elderberry extracts dietary supplementation effects on biofluid and tissues. Extracts toxicity was screened using *Aliivibrio fischeri* bioluminescence model. A concentration of up to 60 mg/L was selected, and rat doses for oral supplementation were computed applying the interspecies correlation between *A. fischeri* and rats. Wistar type-2 diabetic rats, induced by streptozotocin (STZ), were fed with high-fat diet and supplemented for 4 weeks at doses of 190 and 350 mg/kg body weight/day, of lipophilic and polar extract, respectively. As far as we know, lipophilic elderberry extract supplementation was assessed for the first time, while polar extract was administrated at higher doses and for a shorter period compared to previous studies, aiming to evaluate subacute supplementation effects. The polar extract supplementation modulated glucose metabolism by correcting hyperglycemia, while the lipophilic extract supplementation lowered insulin secretion. Both extracts lowered insulin resistance, without remarkable alterations on hematological indices, sera lipids and sera and tissular trace elements homeostasis. In conclusion, elderberries are a potential source of bioactive compounds for formulations to be used as co-adjuvants on diabetes management.

5.2. Introduction

Diabetes mellitus is a multiple etiology metabolic disorder with abnormalities on the metabolism of carbohydrate, fat and protein, being characterized by chronic hyperglycemia and defects in insulin secretion, insulin action, or both [154]. It is projected that diabetes will affect over 300 million people by the year 2030 [155,194,312]. The relevance of this disorder, namely its social impact is clearly highlighted by World Health Organization (WHO) which proposes that further research is urgently needed to evaluate the effectiveness of interventions to prevent it, including behavioral changes, favoring a diet with increased fruits and vegetables consumption and thus, improving dietary patterns [155]. Diets or food supplements that contribute to control and/or prevent hyperglycemia might be crucial to the reduce diabetes incidence [215], namely through the development of alternative sources of antidiabetic agents.

European elderberry (*S. nigra* L.) is a deciduous shrub that produces violet-black drupes which grow in clusters, holding hundreds of berries [30]. This plant is considered from the days of Hippocrates the "medicine chest" [43], and has been used on the formulation of diverse medicinal preparations to prevent and/or control different diseases [4]. Several bioactive compounds are reported on elderberries, namely phenolic compounds as anthocyanin derivatives, including cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-sambubioside-5-glucoside and cyanidin 3,5-diglucoside (Table 1.7 & [35,37,141]); as well as triterpenic compounds such as ursolic and oleanolic acids, and sterols, as *β*-sitosterol were reported as elderberries bioactive components (**Chapter 3** & [135]).

The huge importance of searching for alternative sources of antidiabetic agents and the limited number of studies dealing with elderberry extracts supplementation to reduce diabetes complications, highlights the need to conduct more detailed studies on this topic. Previous studies revealed the potential of elderberry extracts in diabetes status management [199,214–218,220]. The effects of acidified methanol elderberry extracts dietary supplementation (28 to 70 mg of extract/kg body weight (b.w.) streptozotocin (STZ)-induced diabetic Wistar rats, during 12 to 16 weeks) were evaluated showing a reduction in serum glycemic and lipidic levels (cholesterol and triacylglycerol); reduction in the levels of oxidative markers (as superoxide dismutase and glutathione peroxidase activities) and inflammatory markers (as interleukin-6); and an increase on immunological parameters from T lymphocytes populations [199,214–218,220]. The metabolic effects of elderberry extract supplementation in an obese mouse model C57BL/6J were also evaluated (20 to 200 mg of extract/kg b.w., fed either with a low-fat diet and high-fat diet, for 16 weeks), and decreased serum triacylglycerol, inflammatory markers (as TNF-α) and insulin resistance were reported [313]. Despite the claim that these effects were attributable to phenolic

components [199,214–218,220], structure-activity relationship studies were not conducted. Furthermore, only a few *in vivo* studies were performed, in which diets were supplemented with bioactive components present in elderberries, such as cyanidin 3-glucoside, quercetin 3-rutinoside and ursolic acid. Cyanidin 3-glucoside dietary supplementation (0.2% of the diet during 5 weeks) promoted a reduction in the blood glucose levels and an enhancement of insulin sensitivity in type 2 diabetic KK-A^y mice [314]; quercetin 3-rutinoside supplementation (25 to 100 mg/kg during 45 days) revealed antihyperglycemic and antioxidant activity on STZ-injected Wistar rats [315]; while ursolic acid supplementation lowered the urine excretion and renal oxidative stress level on STZ-injected Wistar rats (0.2% of the diet during 16 weeks) [316]. The fact that elderberry extracts and their major components, are linked with different health benefits including on diabetes complications, it illustrates the interest to consider the use of elderberry enriched extracts to access their potential antidiabetic effect.

It is worth noting that any compound that interferes with a biological system, might raise toxicity concerns, thus a screening of plant extracts toxicity before *in vivo* assays is of major importance. Different models are currently used for this purpose, namely based on the use of microorganisms. *Aliivibrio fischeri* bioluminescence method is widely used to evaluate the toxicity response as it correlates bioluminescence signal and viable counts, where light output reflects the cells metabolic rate, being therefore a rapid, sensitive and cost-effective option [317,318]. Disturbances on the bacterial metabolism implies alterations on light production, as the *A. fischeri* cellular respiration and light emission metabolic pathway are intrinsically linked [319].

From this perspective, this study aims to evaluate the *S. nigra* L. lipophilic (dichloromethane) and polar (acidified methanol) extracts dietary supplementation effects on an animal model of diabetes, in order to obtain insights on their effects over diabetes and related complications. Wistar streptozotocin (STZ)-induced diabetic rats fed with a high-fat diet were enrolled in this study, and non-diabetic and diabetic rats without supplementation were also used as controls. Hematological and biochemical blood indices, as well as blood and tissular trace elements were assessed. A specific set of biochemical parameters were analyzed, namely fasting blood glucose and insulin, as there have been increasing efforts in search of bioactive compounds or extracts that can improve insulin action and lower blood glucose levels [320]. Furthermore, type 2 diabetes allied with a high fat diet regimen, might induce changes in lipidic patterns, as well as hepatic dysfunctions, highlighting the need to understand whether elderberry extract supplementation might improve these conditions. In order to obtain fractions enriched in lipophilic and polar bioactive components (e.g. in triterpenic acids and in phenolic compounds), dietary elderberry supplementations were performed using extracts instead of whole elderberries. Thus, firstly the polar extract was characterized by ultra-high-pressure liquid chromatography-tandem

mass spectrometry (UHPLC-MS^n) analysis, while lipophilic fraction characterization was already performed by GC-MS (**Chapter 3**). Secondly, elderberry extracts toxicity was evaluated using the bioluminescence *A. fischeri* assay. Diets were then prepared and administrated to rats, using doses selected based on the preliminary toxicity assays.

5.3. Material and methods

The experimental setup of this study (Figure 5.1) includes extracts preparation and partial chemical characterization (phenolic composition by UHPLC-MS^n analysis), evaluation of extracts toxicity; animals, diet and supplementation; and finally, the biofluid and tissue analysis, as described in detail in the following sections.

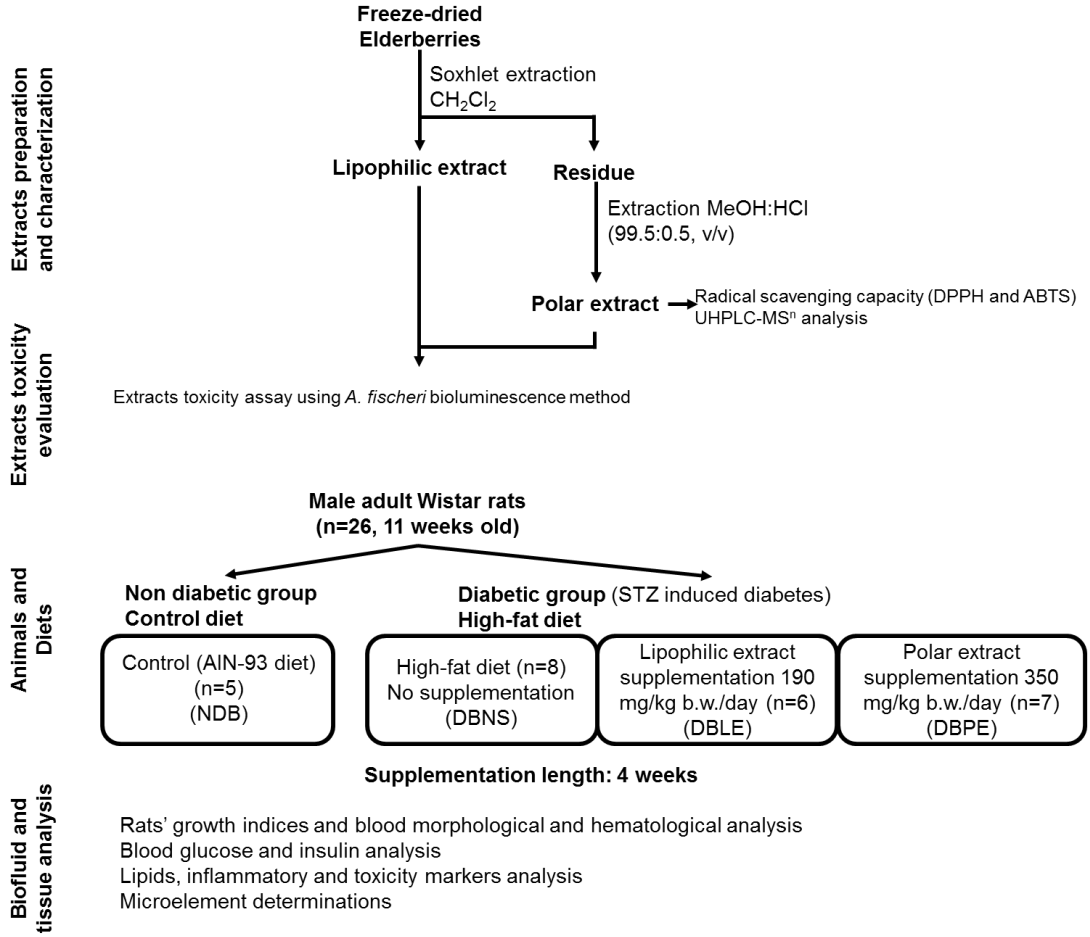


Figure 5.1. Main stages of elderberry extracts preparation, partial characterization, toxicity evaluation; followed by Wistar rats elderberry extracts supplementation and biofluid and tissue analysis. NDB: non-diabetic group; DBNS: diabetic group/not supplemented; DBLE: diabetic group/supplemented with lipophilic extract; and DBPE: diabetic group/supplemented with polar extract.

5.3.1. Reagents

Methanol (≥99.9%) was purchased from Panreac (Barcelona, Spain). Dichloromethane (≥99.9%) was supplied by Sigma Chemical Co. (Madrid, Spain). Hydrochloric acid (37%, w/w) was purchased from Riedel-De Haën, Sigma (Seelze, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH·), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ≥97%), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, >98%), cyanidin 3-glucoside chloride (≥95%) and quercetin-3-glucoside (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Maltodextrin was purchased from Nowamyl (Łobez, Poland). Nitric acid (65%, w/w) was purchased from Merck (Darmstadt, Germany). Formic acid (p.a. ≥98%) was purchased from Fluka Chemie (Madrid, Spain). HPLC-grade methanol and water were supplied from Fisher Scientific Chemicals (Loures, Portugal) and further filtered using a Solvent Filtration Apparatus 58,061 from Supelco (Bellefonte, PA, USA). Reference Bovine Liver, NIST-1577C was purchased from LGC standards (Dziekanów Leśny, Poland).

5.3.2. Elderberry samples

Elderberries (*S. nigra* L.) were supplied by the Adega Cooperativa do Vale Varosa - RégieFrutas (Tarouca, Portugal). The samples were collected on an experimental field (41.043233°N, 7.728820°W) with 0.5 ha, from 13/14-years old plants, where each plant produces approximately 15 kg of elderberries per year.

Samples were harvested on the same day between 9 and 12 a.m., in which several bunches from diverse shrubs were randomly harvested and mixed together. Samples were immediately transported under refrigeration (ca. 2-4 °C) to the laboratory and then stored at -20 °C. Prior to extraction, elderberries were freeze-dried using a VirTis BenchTop K (SP Industries, Stone Ridge, NY, USA).

5.3.3. Extracts preparation

The lipophilic extract (LE) was obtained as previously described (**Chapter 3**). Briefly, freeze-dried elderberries (approximately 850 g) were Soxhlet extracted using dichloromethane for 8 h. The solvent was evaporated to dryness in a rotary evaporator and the extracts weighed. This extract was previously chemically characterized by gas chromatography-mass spectrometry analysis (**Chapter 3**). The resulting lipophilic-free solid residue was then extracted (m/v 1:5) with acidified methanol (0.5% HCl), for one hour under constant stirring, based on previous publications [214,321]. The suspension was then filtered and the extraction process repeated 5 times. The extracts were combined was then evaporated to dryness by low-pressure evaporation. The extract

was freeze-dried to ensure the absence of water. As this extract is highly hygroscopic, it was mixed with maltodextrin at a ratio of 1:0.7 (m/m, extract/maltodextrin).

5.3.4. Phenolic compounds analysis

5.3.4.1. UHPLC-MS^n analysis

UHPLC-MS^n analysis were established based on previous methodologies developed in our lab [322], in which the UHPLC system consisted of a variable loop Accela autosampler (200 vial capacity set at 16 °C), an Accela 600 LC pump and an Accela 80 Hz PDA detector (Thermo Fisher Scientific, San Jose, CA, USA). Before UHPLC injection, each extract was dissolved in methanol (HPLC grade), with a concentration of 15 mg/mL, being subsequently filtered with a 0.2 µm PTFE syringe filter. A gradient elution program was carried out for the separation of the analytes, using a Kinetex C_{18} (100 mm × 2.1 mm × 1.7 µm) column supplied by Phenomenex (Torrance, CA, USA), at 45°C and a flow rate of 0.39 µL/min. The injection volume was 20 µL and the mobile phase consisted of methanol (A) and water:formic acid (95:5, v/v) (B). It was applied a linear gradient that consisted of: 0–3 min: 1%A, 3–8 min: 1–10%A, 8–21 min: 10–28%A, 21–28 min: 28–65%A, 28–31 min: 65%A, 31–35 min: 65-1%A, followed by 4 min of column re-equilibration before the next run. Detection was carried out in the diode array detector (DAD) at 280, 340 and 520 nm, and UV spectra in a range of 210–600 nm were also recorded. A LCQ Fleet ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA), equipped with an electrospray ionization source and operating in negative and positive modes was used to perform tandem mass spectrometry analysis. The nitrogen sheath and auxiliary gas were 40 and 10 (arbitrary units), respectively. The capillary temperature was 330 °C and the spray voltage was 5 kV. The capillary and tune lens voltages were set at 41 V and 110 V for positive mode and for negative mode at −36 V and −120 V. CID-MS^n experiments were performed on mass-selected precursor ions in the range of *m/z* 100–1500. The isolation width of precursor ions was 1.0 mass units. Collision energy was optimized between 20 and 35 (arbitrary units), using helium as collision gas and scan time was equal to 100 ms. Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA) was used for data acquisition.

Cyanidin 3-glucoside and quercetin 3-glucoside standard solutions (in methanol, with five different concentrations each, between 0.1 and 20 µg/mL), were used for quantification using UHPLC-DAD system. Limits of detection (LOD) and quantification (LOQ) were also estimated using the S/N approach (n=5). Individual compounds quantification was accomplished with calibration data for the most similar standard in terms of maximum wavelength absorption, when no pure reference compounds were available. The concentration of each compound was expressed as mean value (n= 3).

5.3.4.2. Radical scavenging capacity

DPPH and ABTS radical scavenging capacities were determined using Lambda 35 spectrophotometer (Perkin-Elmer, Waltham, MA, USA) following previously described procedures [323,324]. The samples were appropriately diluted in methanol. Calibration curves were performed using Trolox as standard, with concentrations between 0.10 and 0.40 mg/mL (r^2= 0.9946) for ABTS assay and 0.02 and 0.20 mg/mL (r^2= 0.9955) for DPPH assay. The results are expressed in mmol Trolox equivalents. All determinations were performed in triplicate.

5.3.5. Sugar content of the polar extract

Phenol-sulfuric acid colorimetric method was used to determine the sugar content of the polar extract [325]. It was added to the extract 1 mL of H_2SO_4 72% (w/w) and 160 µL of phenol 5% (w/w). The tubes were heated at 100 °C during 5min, cooled to room temperature and stirred. Absorbance was measured at 490 nm (Lambda 35, Perkin-Elmer, USA) and a calibration curve was prepared using glucose as standard (0–1 mg/mL). The determinations were performed in triplicate.

5.3.6. *A. fischeri* bioluminescence assay

Toxicity was evaluated through the bioluminescence assay using the bacteria *A. fischeri*, based on previous established methodology [318]. A bioluminescent marine bacterium *A. fischeri* ATCC 49387 (USA) bacterial strain was used (stored at -80 °C in 10% glycerol). The bioluminescent *A. fischeri* fresh plate cultures were maintained at 4 °C in solid BOSS medium (1% peptone, 0.3% beef extract, 0.1% glycerol, 3% NaCl, 1.5% agar, pH 7.3). One isolated colony was aseptically inoculated in liquid BOSS medium (30 mL), and kept at 26 °C under constant stirring (170 rpm) during 18 h. Then, an aliquot (200 µL) was sub-cultured in BOSS medium (30 mL), and grew at 26 °C under stirring (170 rpm) overnight. The colony-forming units (CFU) and the bioluminescent signal (in relative light units, RLU) correlation of *A. fischeri* was also assessed [318].

For bioassays purpose, an overnight culture of *A. fischeri* was used after a ten-fold dilution in phosphate buffered saline (PBS: 30 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per litre; pH 7.4). For each, 15 mL of bacterial suspension were aseptically distributed in 100 mL acid-washed and sterilized glass beakers containing appropriate amounts of *S. nigra* extract to achieve a final concentration between 0 (control) and 1995 mg of extract/L, respectively. For the lipophilic (dichloromethane) extract, 2% (v/v) of dimethyl sulfoxide (DMSO) was added in order to dissolve this extract. Previously to the development of toxicity tests, solutions of 2% DMSO were analyzed to check the absence of toxic effects for *A. fischeri*, revealing no toxic effects for up to 110 min. All beakers were wrapped with aluminum foil to protect from light exposure and incubated under 120

rpm stirring at 25 °C. Aliquots, 500 µL, of treated and control samples were collected at time 10, 15, 25, 40, 70, 90 and 110 min and the bioluminescence signal was measured in a luminometer (peak wavelength detected at 420 nm, standard range: 300–650 nm) (Promega Glomax 20/20 luminometer, Turner Designs, Inc., Madison, WI, USA). The tested extract concentrations were selected to have a wide range of concentrations (from 0 to 1995 mg of extract/L), in order to establish the non-toxic doses to be administrated to rats. These doses were calculated based on previous established correlations between the *A. fischeri* bioluminescent model and rat toxicity assays [326,327]. Three independent experiments for each tested condition were done.

5.3.7. Animals, diets and elderberry extracts supplementation

All animal procedures and the protocol were conducted according to EU Directive 2010/63/EU for animal experiments and approved by the Local Animal Bioethics Committee in Poznan, Poland (No 3/2015 - 16 January 2015). All necessary efforts were made to minimize the number of animals used and their suffering.

Male adult Wistar rats (n = 26, 11 weeks old) were purchased from the Licensed Laboratory of the Animal Breeding Center (Poznan, Poland). After arrival at the animal care facility, rats were kept under controlled temperature (21 ± 2 °C) and humidity (55-60%) with a 12h/12 h day/night cycle throughout the experiment. After a 5 day adaptation period, animals were divided into 4 groups (initial mean body weight = 330 g), and kept in metal-free individual cages: NDB (non-diabetic group, n= 5), DBNS (diabetic group/not supplemented, n= 8), DBLE (diabetic group/supplemented with lipophilic extract, n= 6) and DBPE (diabetic group/supplemented with polar extract, n= 7). Animals were fed *ad libitum* for 2 weeks: i) non-diabetic group was fed with semi-synthetic standard composed by casein (14%), sunflower oil (10%), wheat starch (56.5%), sucrose (10%), potato starch (5%), vitamin mix AIN-93M (1%) and mineral mix AIN-93M (3.5%), while ii) the three diabetic groups (DBNS, DBLE and DBPE) received high fat (HF) diet (40% calories from fat), which were obtained from the basal AIN-93M diet [328], by replacement of wheat starch with fat, being thereby composed by casein (14%), sunflower oil (10%), wheat starch (46.5%), lard (10%), sucrose (10%), potato starch (5%), vitamin mix AIN-93M (1%) and mineral mix AIN-93M (3.5%). Polar extract was incorporated on wheat starch, while lipophilic extract was mixed with sunflower oil. An excessive amount of fat in the diet is one of factors contributing to insulin resistance on animal models, and thus, the group of rats fed with a high-fat diet was formed to elucidate changes associated with this syndrome [320].

After 2 weeks of controlled diet, the three diabetic groups (DBNS, DBLE and DBPE) were subjected to multiple intraperitoneal injection of STZ freshly dissolved in 0.1 M-citrate buffer (pH 4.4), given in 3 subsequent doses: 20, 10 and 25 mg/kg body weight, in weekly intervals, while

NDB group were injected in the same manner, but with the carrier alone (citrate buffer). The approach with multiple doses of STZ combined with a high-fat diet has been shown to be more efficient and stable animal model of diabetes type-2 [329]. The presence of diabetes in rats was confirmed by measuring fasting blood glucose concentration in blood samples (>11 mmol/L) withdrawn from the tail tip after 48 h using a glucometer iXell®, Genexo (Warsaw, Poland). After that, dietary supplementation was performed using 500 mg of polar extract/100 g HF diet for DBPE group and 190 mg of lipophilic extract /100 g HF diet for DBLE group.

All diets were prepared weekly and stored in sealed containers at 4 ± 1 °C. Food intake was measured daily and body mass every 7 days.

After 4 weeks of feeding and overnight fast, the animals were anesthetized with CO_2 inhalation and dissected to collect blood and the internal organs. Blood samples were drawn from the heart aorta into Vacutest tubes with plasma coagulant Medlab-Products (Raszyn, Poland), coagulated at room temperature for 20 min, and centrifuged at 4000 rpm. Inner organs (liver, kidneys, heart, spleen, pancreas and testes) and femoral bones were also removed, being washed in a saline solution (0.9% NaCl), weighed and stored at -70 °C. Serum samples were separated and kept in aliquots at -70 °C for biochemical assays.

5.3.8. Biochemical analyses

Blood morphology and biochemical analyses were conducted in a certified laboratory (Laboratorium Medyczne Synevo, Poznan).

5.3.8.1. Blood morphology

The Drabkin cyanmethemoglobin method was employed to determine blood hemoglobin (HGB) concentration [330]. The remaining parameters were obtained using the CELLDYN-1700 analytical hematology system [331], being analyzed the following parameters: red blood cell count (RBC), hematocrit, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width based on standard deviation (RDW-SD), white blood cell count (WBC), monocytes (MONO), lymphocytes (LYMPH), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW) and platelet large-cell ratio (p-LCR). For each analyzed parameter, three replicates were performed for each animal.

5.3.8.2. Blood biochemical indices

The serum glucose concentration was determined by the hexokinase method [332], while the total cholesterol, LDL-c and HDL-c levels and triacylglycerol levels were all determined using Olympus AU 560 equipment by the colorimetric methods [333–335]. The colorimetric method using Biuret method [336], was used to measure total protein concentration, while the Jaffe kinetic method with picric acid was employed to analyze the creatinine levels [337]. The kinetic method using urease and glutamine dehydrogenase was used to determine urea concentration [337]. Enzyme activities of alanine aminotransaminase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) were measured by kinetic methods [338]. Plasma insulin concentration was measured by the RIA method using kits specific for rats, Linco Research (St. Charles, MO, USA) [339]. The efficacy of glucose utilization, insulin resistance and β-cell function were characterized by the homeostasis model assessment (HOMA) indices [340]. For each analyzed parameter, three replicates were performed for each animal.

5.3.8.3. Trace element (Fe, Cu and Zn) status in blood sera and organs

Trace element analysis were based on previous established methodologies [341], in which the rat tissues were digested in spectra pure HNO_3 (65%, w/w) in the Microwave Digestion System (MARS 5, CEM). Flame atomic-absorption spectrometry F-AAS method (AAS-3 spectrometer, Zeiss, with BC, Jena, Germany), was used to measure Fe, Zn and Cu concentrations in the mineral solutions. Simultaneous analyses of certified reference material (Bovine Liver, NIST-1577C for tissues, (Gaithersburg, MD, USA), HUMASY CONTROL 2 for serum (Randox, London, U.K.)) were performed to assure the accuracy of quantitative determinations of Fe, Zn and Cu. Water content of the tested organs was determined for the expression of the results on dry basis. Ca. 1g of each sample was weighed and kept overnight at 105 °C. Zn and Cu concentrations in sera samples were determined by F-AAS after diluting these samples with 0.01% Triton-X100 solution (Merck, Germany). The serum Fe concentration was determined by the Guanidine/Iron-Zine method [342,343]. Zn, Cu and Fe were selected as their metabolism might be disturbed in insulin resistance and in diabetic states. Particularly, Fe overload may affect glucose homeostasis, while alterations in Zn and Cu metabolism may increase oxidative damage of cells and exacerbate complications in diabetes [320]. For each analyzed parameter, three replicates were performed for each animal.

5.3.9. Statistical analysis

All the results presented in the tables are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by a Fisher's Least Significant Difference (LSD) test using the GraphPad Prism version 7 for Windows (trial version, GraphPad Software, San Diego California, USA), was applied for the obtained results. It was considered statistically significant when $p<0.05$.

5.4. Elderberry extracts preparation, radical scavenging activity and UHPLC-MS^n analysis

The strategy followed in this work is illustrated on Figure 5.1, in which the two extracts were firstly obtained from freeze-dried berries and partially characterized. The dichloromethane Soxhlet extraction yield was 1.69 ± 0.25% (m/m, d.w.), being within the values previously reported for this matrix (**Chapter 3**). The detailed composition of this extract was already characterized by GC-MS (**Chapter 3**), showing that triterpenic acids, namely ursolic and oleanolic acids, were detected as the major components (ca. 85-90% of the identified compounds), followed by fatty acids, fatty alcohols and sterols. The overall content of the identified compounds on elderberry dichloromethane extracts ranged from 19.6 to 26.4 g/100 g extract, with overall values of triterpenic compounds of 16.3 to 22.3 g/100 g extract.

The yield of the polar extract, obtained after removal of the lipophilic components (Figure 5.1) and the antioxidant activity, obtained by the DPPH and ABTS assays, are shown in Table 5.1. The extraction yield (58.2% m/m, d.w.), was similar to the values reported before (60.2%) for *S. nigra* berries using the same solvent [321]. Elderberry polar extract showed ABTS and DPPH radical scavenging activities of 2.37 and 0.63 mmol trolox equivalents (TE)/g extract, respectively. These values are in agreement with those previously published for aqueous elderberry extracts (1.74 - 2.20 mmol TE/g extract and 0.63 - 0.89 mmol TE/g extract, for ABTS and DPPH radical scavenging, respectively) [297].

Table 5.1. Extraction yield and antioxidant activities of *S. nigra* L. polar berry extract.

Parameter		Values
Extraction yield (%, m/m, d.w.)		58.2 ± 6.3
Antioxidant activity	ABTS[a]	2.37 ± 0.28
	DPPH[a]	0.63 ± 0.03

Values expressed as mean ± SD, n=3; d.w. – dry weight; [a] expressed in mmol TE/g extract.

The phenolic composition of the elderberry polar extract was studied by ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS^n). Phenolic compounds were quantified by UHPLC-UV using calibration curves of reference compounds representative of each chemical family (Table 5.2).

Table 5.2. Calibration data used for the UHPLC-UV quantification of phenolic compounds in *S. nigra* L. polar berry extract.

Compound	λ (nm)	Conc. Range (μg/mL)	Calibration Curve[a]	r^2	LOD[b] (μg/mL)	LOQ[b] (μg/mL)
Quercetin 3-glucoside	340	1.00×10^{-1} – 20.0	$y = 137729\,x + 112942$	0.994	1.92	6.39
Cyanidin 3-glucoside	520	1.00×10^{-1} – 20.0	$y = 293965\,x + 55885$	0.999	7.40×10^{-1}	2.49

[a] *y*: peak area; *x*: concentration in μg/mL
[b] LOD: limit of detection; LOQ: limit of quantification

A strategy that comprises the analysis of different parameters was attempted in order to accomplish the phenolic compounds identification, which included the co-injection of standards (cyanidin 3-glucoside and quercetin 3-glucoside), retention times, evaluation of the maximum UV wavelengths absorption, and the analysis of corresponding $[M-H]^-$ or $[M+H]^+$ ions and the key MS^n fragmentation product ions relevant for their identification (summarized in Table 5.3).

Two illustrative examples of the mass spectra and respective interpretation relative to the two major chemical families under study are shown on Figure 5.2: cyanidin 3-glucoside (Figure 5.2A) representative of anthocyanins and quercetin 3-rutinoside (Figure 5.2B), representative of flavonols. Regarding the first one, i.e., cyanidin 3-glucoside, it is observed the loss of 162, which is a typical loss from cyanidin glycosides (Figure 5.2A), and corresponds to the loss of a hexose moiety (as glucose or galactose). Regarding elderberries only glucose moieties are reported so far (Table 1.7) [35,37,141–143]. From other cyanidin glycosides containing sugar moieties different than glucose, such as cyanidin 3-sambubioside, a neutral loss of 294 is observed that corresponds to sambubioside moiety. This disaccharide can be also elucidated by neutral losses of 132 and 162, corresponding to their pentose (xylose) and hexose (glucose) moieties. The substituents from B ring defines the anthocyanidin types and allow their identification based on specific fragmentation pattern [344]. Considering the cyanidin derivatives, the most common anthocyanin present on *S. nigra* berries, a typical product ion at *m/z* 287 is detected (Figure 5.2A).

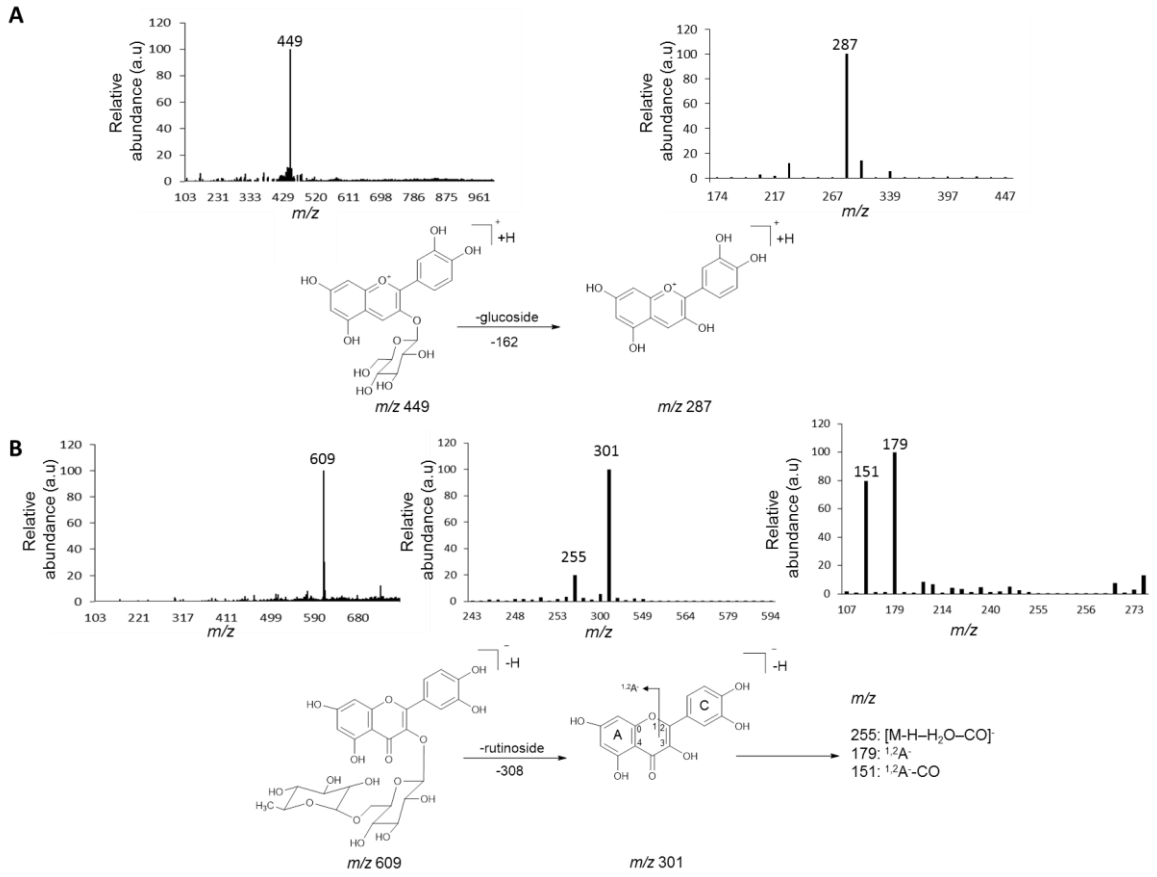


Figure 5.2. Mass spectra and main fragmentation product ions of the major compounds from each chemical family present on acidified methanol elderberry extract: (A) cyanidin 3-glucoside and (B) quercetin 3-rutinoside. $^{1,2}A^{-}$: the superscript numbers indicate the C-ring bonds that have been broken and A denotes product ions containing an intact A ring (nomenclature adapted from Ma et al. [345]**).**

The quercetin derivatives identified on elderberry acidified methanol extract were also identified by their typical losses, being exemplified the main product ions of quercetin 3-rutinoside on Figure 5.2B. The loss of 308 corresponds to the neutral loss of the disaccharide rutinoside giving the aglycone at *m/z* 301. Flavonol aglycone fragmentation pathways are recognized by the typical retro-Diels-Alder fissions [35], in which the main fragmentation product ions are illustrated on Figure 5.2B with nomenclature adapted from Ma et al. [345]: the values of *m/z* lower than the flavonol aglycone, like *m/z* 179 ($^{1,2}A^{-}$) and 151 ($^{1,2}A^{-}$-CO), are typical of retro Dies-Alder, while the product ion at *m/z* 255 corresponds to $[M\text{-}H\text{–}H_2O\text{–}CO]^{-\cdot}$ [346,347].

Hence, eight phenolic compounds were identified, representing a total amount of 12.64 g/100 g extract (Table 5.3), namely: caffeyolquinic acid, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside-5-glucoside, cyanidin 3-glucoside, cyanidin 3-sambubioside, quercetin 3-glucoside, quercetin 3-rutinoside and quercetin.

Table 5.3. Phenolic compounds identified in *S. nigra* L. berry polar extract and corresponding MS^n fragmentation profiles.

t_R (min)	Compound	λ (nm)	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)	Content (g/100 g extract)	Identification
2.1	Caffeyolquinic acid	280	-	-	-	353	191, 179	-	*tr*	[35]
9.4	Cyanidin 3,5-diglucoside[a]	514, 278	611	**449**[c], 287	287	-	-	-	0.11 ± 0.02	[35]
10.9	Cyanidin 3-sambubioside-5-glucoside[a]	514, 278	743	581, **449**[c], 287	287	-	-	-	0.80 ± 0.22	[35]
12.9	Cyanidin 3-glucoside[a]	514, 278	449	287	-	-	-	-	4.46 ± 0.74	coinjection
13.3	Cyanidin 3-sambubioside[a]	514, 278	581	449, 287	-	-	-	-	4.80 ± 0.91	[161]
17.9	Quercetin 3-glucoside[b]	349, 265	-	-	-	463	**301**[c], 179	179, 151	0.87 ± 0.20	coinjection
18.5	Quercetin 3-rutinoside[b]	340, 258	-	-	-	609	**301**[c], 255	179, 151	1.43 ± 0.02	[35]
23.3	Quercetin[b]	259, 367	-	-	-	301	179, 151	-	0.18 ± 0.06	[322]
								Total	12.64 ± 2.21	

Content values expressed as mean ± SD, n=3; t_R - retention time; *tr* – trace; Calibration curves used: [a] cyanidin 3-glucoside, [b] quercetin 3-glucoside; [c] product ions at bold were subjected to further MS^3 fragmentation.

All the phenolic compounds reported here have been previously reported in elderberries [35,37,141–143]. Cyanidin 3-glucoside and cyanidin 3-sambubioside were the major phenolic compounds found in *S. nigra* polar extract, accounting for 4.46 and 4.80 g/100 g extract (368 and 396 mg/100 g fresh berries weight (f.w.)), respectively. These two anthocyanins were previously reported as the major phenolic compounds present in ripe elderberries, accounting for 204-739 mg/100 g f.w., and 122-630 mg/100 g f.w. of cyanidin 3-glucoside and cyanidin 3-sambubioside, respectively [35,37,141–143]. Quercetin 3-rutinoside was the major flavonol detected in this extract, accounting for 1.43 g/100 g extract (118 mg/100 g f.w.), being in agreement with published data (up to 96 mg/100 g f.w.) [35].

The overall sugar content of the polar extract was also determined, representing 67 ± 8 g sugars/ 100 g of extract. As elderberries sugar fraction is composed mainly by fructose and glucose [37], it may be inferred that this fraction also contains these sugars. Thus, it is expected that these contribute to the overall energy intake of the rats' diet.

5.5. Elderberry extracts toxicity evaluation using *A. fischeri* bioluminescence model

In order to evaluate elderberry extracts toxicity using *A. fischeri* bioluminescence assay, firstly the correlation between the colony-forming units (CFU) and the bioluminescence signal (relative light units, RLU) of *A. fischeri* was carried out [318]. A linear correlation between CFU and the bioluminescence signal was obtained with r^2 of 0.965 and $\log_{RLU} = 0.84 \log_{CFU} + 0.72$, confirming that the bioluminescence reflects the viable bacterial activity. Then, the *A. fischeri* bioluminescence was assessed when exposed to polar and lipophilic elderberry extracts during up to 110 min and at concentrations ranging from 9 to 1995 mg/L (Figure 5.3). For each extract, a control (0 mg/L) condition was also considered.

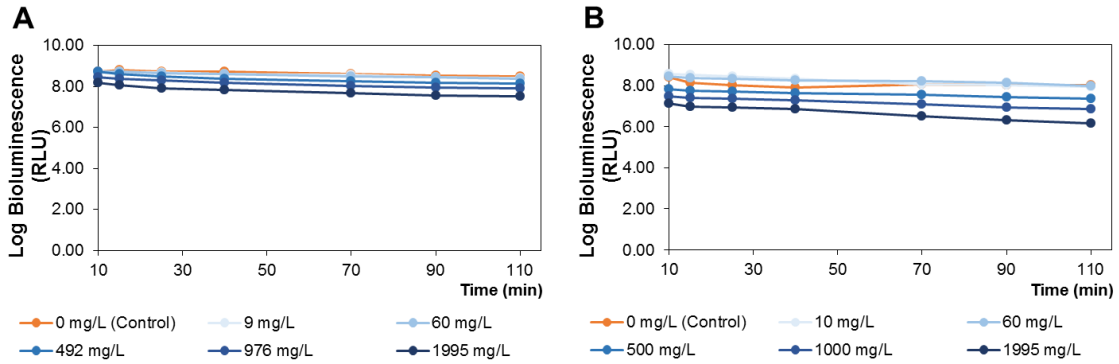


Figure 5.3. Bioluminescence monitoring of *A. fischeri* treated with elderberry polar (A) and lipophilic (B) extracts.

For both extracts, at lower concentrations (9 to 60 mg/L), the bioluminescence signal (expressed as RLU) decreased from 0 to 1.5% in comparison with control (Figure 5.3), which allowed to infer that these concentration levels were innocuous for *A. fischeri*. Considering the polar extract, for the highest concentration (1995 mg/L), the RLU decreased from 6 to 12% (Figure 5.3A), unveiling a slight impact in the viable bacterial activity, while for the lipophilic extract, the bioluminescence signal decreased until 23% at the same concentration (Figure 5.3B). Thus, 60 mg/L was selected, and the corresponding dose to be administrated to rats was calculated by applying the equation of interspecies correlation between *A. fischeri* and rats for oral administration ($\log_{extract\ concentration\ A.\ fischeri} = 0.55 \log_{extract\ dose\ mouse} - 0.13$; also a mouse to rat dosage conversion factor was used) [327], resulting on a dosage of up to 5194 mg/kg of body weight. Therefore, the diets were prepared ensuring that both extracts doses' did not exceed this value, and also taking into consideration the relative proportion of the target chemical families in each extract, which corresponded to 190 and 500 mg/100 g of diet of the lipophilic and polar extracts, respectively.

5.6. Effect of the elderberry extracts supplementation on dietary intake, body weight and on blood morphology and hematology indices

The dietary intake, overall growth and organ indices were determined (Table 5.4). Results indicate that there were no significant differences in food intake on the four experimental groups ($p>0.05$). The daily extract intake represented 350 and 190 mg/kg b.w. for the polar and lipophilic extracts' supplementation, respectively. These doses correspond to, 35, 9 and 31-42 mg/kg b.w. in terms of anthocyanin-derivatives, quercetin-derivatives, and triterpenic-derivatives, respectively. For an average person of 70 kg, this would correspond to 397, 102 and 352-477 mg/day of anthocyanin-derivatives, quercetin-derivatives, and triterpenic-derivatives, respectively.

Table 5.4. Effects of dietary supplementation of elderberry polar and lipophilic extracts on the rats' overall growth indices. Diabetic rats without dietary supplementation and non-diabetic rats were also followed-up.

Index	Non-Diabetic rats *(n=5)*	Diabetic rats with high-fat diets		
		Not supplemented (n=8)	*Polar extract (n=7)*	*Lipophilic extract (n=6)*
Diet intake (g/24h)	20.1 ± 1.1[a]	20.9 ± 2.1[a]	22.7 ± 2.9[a]	20.8 ± 2.2[a]
Daily extract intake (mg/kg b.w.)	-	-	350 ± 3	190 ± 3
Average body weight at the beginning / end of experiment (g)	424.56 / 443.88	383.31 / 383.38	370.73 / 368.41	410.77 / 415.67
Body weight variation (g/24h)	0.69 ± 0.08[a]	0.00 ± 0.34[b]	-0.08 ± 0.38[b]	0.18 ± 0.65[a,b]
Body mass / body length ratio (g/cm)	16.7 ± 1.3[a]	14.7 ± 1.3[a]	14.3 ± 1.1[a]	16.1 ± 1.5[a]
Liver (% b.m.)	3.04 ± 0.24[a]	3.38 ± 0.32[a]	3.39 ± 0.22[a]	3.26 ± 0.21[a]
Kidneys (% b.m.)	0.60 ± 0.04[a]	0.85 ± 0.11[b]	0.88 ± 0.11[b]	0.78 ± 0.12[b]
Heart (% b.m.)	0.28 ± 0.02[a]	0.29 ± 0.03[a]	0.29 ± 0.02[a]	0.30 ± 0.07[a]
Testes (% b.m.)	0.93 ± 0.04[a]	0.97 ± 0.07[a]	0.98 ± 0.15[a]	0.61 ± 0.35[a]
Pancreas (% b.m.)	0.24 ± 0.04[a]	0.26 ± 0.02[a]	0.26 ± 0.04[a]	0.22 ± 0.04[a]
Spleen (% b.m.)	0.15 ± 0.02[a]	0.15 ± 0.02[a]	0.13 ± 0.01[a]	0.14 ± 0.02[a]
Left femur (% b.m.)	0.21 ± 0.02[a]	0.24 ± 0.02[a]	0.25 ± 0.02[a]	0.23 ± 0.02[a]
Right femur (% b.m.)	0.22 ± 0.01[a]	0.25 ± 0.02[a]	0.25 ± 0.02[a]	0.23 ± 0.03[a]

Values expressed as mean ± SD; b.w. – body weight; b.m. – body mass; Mean values with unlike letters in rows show statistically significant differences (p<0.05, LSD's Fisher test).

A significant difference (p<0.05) is observed in the body weight variation, between non-diabetic (NDB) and the two diabetic groups, DBNS and DBPE, while within the STZ-induced diabetic rats (DB groups), no statistical differences were observed (p>0.05). Additionally, no significant changes were observed regarding the body mass/body length ratio (p>0.05). The HF diet and diabetic status had a significant effect (p<0.05) on the rats' relative kidney mass (% of body weight), of up to 1.4-fold higher than NDB group, which is in agreement with previously reported data for HF/STZ-induced diabetic rats [348]. Other tissues such as liver, spleen, heart, testes, pancreas and femur bones were not remarkably affected (p>0.05). The lower body weight variation in STZ-injected rats is probably connected to poor glycemic control, accompanied with a protein catabolism to provide amino acids for gluconeogenesis, that results in muscle wasting and weight loss in diabetic rats [349], while the increase in the weight of kidney (hypertrophy) in proportion to the body weight in STZ-injected rats was suggested to be linked to local alterations in the production of one or more growth factors and/or their receptors in insulin dependent diabetes mellitus [350].

Blood morphological and hematological indices were also determined in order to follow the general health status of the rats along the experiment (Table 5.5). In fact, the assessment of hematological parameters can be used to reveal the deleterious effects of foreign compounds.

Moreover, it may evidence abnormalities on enzymes, metabolic products, hematology, and/or normal functioning of the organs [351].

Table 5.5. Effects of dietary supplementation of elderberry polar and lipophilic extracts on the rats' blood morphology and hematology indices. Diabetic rats without dietary supplementation and non-diabetic rats were also followed-up.

Index	Non-Diabetic rats *(n=5)*	Diabetic rats with high-fat diets		
		Not supplemented (n=8)	*Polar extract (n=7)*	*Lipophilic extract (n=6)*
RBC (10^{12}/L)	9.42 ± 0.17^{a}	9.77 ± 0.46^{a}	9.88 ± 0.54^{a}	9.50 ± 0.37^{a}
HGB (mmol/L)	15.24 ± 0.38^{a}	15.95 ± 0.56^{a}	16.13 ± 0.82^{a}	15.53 ± 0.39^{a}
Hematocrit (%)	44.64 ± 1.58^{a}	46.14 ± 2.28^{a}	44.57 ± 3.01^{a}	43.28 ± 1.70^{a}
MCV (10^{-15} L)	47.39 ± 1.32^{a}	47.30 ± 2.77^{a}	45.17 ± 2.78^{a}	45.63 ± 2.82^{a}
MCH (10^{-15} kg)	16.18 ± 0.23^{a}	16.40 ± 0.87^{a}	16.34 ± 0.62^{a}	16.37 ± 0.42^{a}
MCHC (10^{-2} kg/L)	34.14 ± 0.55^{a}	34.71 ± 1.04^{a,b}	36.23 ± 1.01^{b}	35.92 ± 1.33^{a,b}
WBC (10^{9}/L)	4.51 ± 1.04^{a}	2.80 ± 0.98^{b}	3.03 ± 0.49^{b}	2.97 ± 0.68^{b}
MONO (10^{3}/µL)	0.44 ± 0.15^{a}	0.17 ± 0.08^{b}	0.24 ± 0.06^{b}	0.21 ± 0.09^{b}
LYMPH (10^{3}/µL)	3.72 ± 1.01^{a}	1.92 ± 0.53^{b}	2.35 ± 0.51^{a,b}	2.38 ± 0.63^{a,b}
PLT (10^{12}/L)	1129 ± 99^{a}	866 ± 129^{b,c}	960 ± 114^{a,c}	855 ± 144^{b,c}
PDW (10^{-15}/L)	9.10 ± 0.44^{a}	9.54 ± 0.24^{a}	9.73 ± 0.56^{a}	10.22 ± 1.52^{a}
MPV (10^{-15}/L)	7.92 ± 0.20^{a}	8.11 ± 0.29^{a}	8.04 ± 0.51^{a}	8.25 ± 0.41^{a}
p-LCR (%)	10.70 ± 1.60^{a}	12.13 ± 1.91^{a}	11.86 ± 3.71^{a}	13.20 ± 3.25^{a}
RDW-SD (10^{-15} L)	30.68 ± 1.72^{a}	35.45 ± 1.96^{b}	33.01 ± 2.01^{a,b}	33.58 ± 2.07^{a,b}

Values expressed as mean ± SD; Mean values with unlike letters in rows show statistically significant differences (p<0.05, LSD's Fisher test); RBC - red blood cell count; HGB - blood hemoglobin concentration; MCV - mean corpuscular volume; MCH - mean corpuscular hemoglobin; MCHC - mean corpuscular hemoglobin concentration; WBC - white blood cell count; MONO - monocyte count; LYMPH - lymphocyte count; PLT - platelet count; PDW - platelet distribution width; MPV - mean platelet volume; p-LCR - platelet large-cell ratio; RDW-SD - red cell distribution width based on standard deviation.

Most of these indices were not markedly different amongst the four tested groups (p>0.05), however MCHC, WBC, MONO, LYMPH, PLT and RDW-SD showed significant differences (p<0.05), between at least two groups. In particular MCHC and RDW-SD values were slightly altered (p<0.05) between NDB and DBPE, and NDB and DBNS, respectively, although the remaining parameters were not altered owing the red blood cells' and hemoglobin' status. Taking into consideration that alterations on red blood cells' parameters are indicators of anemic status [351], these results suggests the absence of anemic status. The reduction of PLT levels in diabetic rats induced with streptozotocin was confirmed in this study in relation to the non-diabetic rats (NDB), being known that the STZ administration implies abnormalities on the platelets' function [352]. STZ-diabetes induction (DB groups) led to a decrease on WBC, MONO and LYMPH levels (p<0.05) when compared to NDB rats, as already reported [351]. Streptozotocin suppresses the immune system by damaging WBC and its differentials, such as monocytes and lymphocytes [351].

5.7. Effect of the elderberry extracts supplementation on fasting blood glucose and insulin

The levels of fasting blood glucose and insulin levels, as well as insulin resistance and β cells function indices are presented in Figure 5.4. After 4 weeks, the STZ injection (DBNS) caused an almost 1.6-fold increase in blood glucose levels (Figure 5.4A), attaining a median glucose level of 14 mmol/L. Polydipsia and polyuria was observed on the animals in this group, as already reported [353]. After elderberry polar extract supplementation (DBPE), a significant decrease (p<0.05) of the fasting blood glucose was observed when compared to DBNS (Figure 5.4A), with values similar to the NDB group (p>0.05). Significant reduction on glycaemia levels on STZ-induced diabetes rats after supplementation of the same extract but at lower doses and longer times was already reported (50 mg/kg b.w. during 16 weeks) [214,215]. No significant differences (p>0.05) between the DBNS and the STZ-induced diabetic rats supplemented with lipophilic extract (DBLE) are reported.

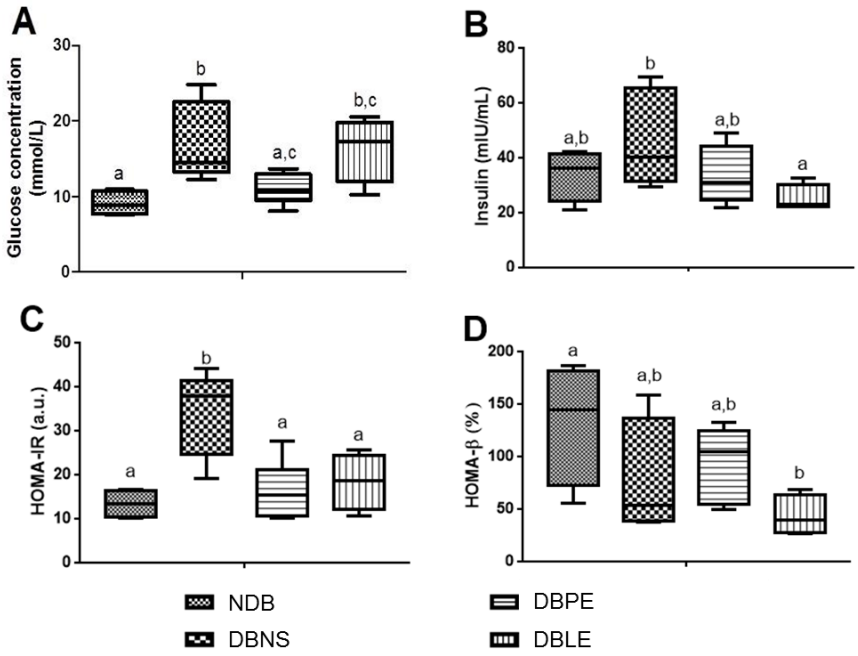


Figure 5.4. Elderberry extracts dietary supplementation effects on (A) fasting blood glucose concentration, (B) fasting plasma insulin, (C) insulin resistance index (HOMA-IR) and (D) β cells function index (HOMA-β) on high-fat diet fed STZ-induced diabetic rats. Diabetic rats without dietary supplementation and non-diabetic rats were also followed-up. NDB: non-diabetic group; DBNS: diabetic group/not supplemented; DBPE: diabetic group/supplemented with polar extract; and DBLE: diabetic group/supplemented with lipophilic extract. Values with unlike letters show statistically significant differences (p<0.05, LSD's Fisher test).

Regarding, the plasma insulin levels (Figure 5.4B), it was found that the DBLE group had a lower insulin level ($p<0.05$) compared to DBNS, being the values similar to the NDB group ($p>0.05$). For DBPE, the insulin levels did not differed from NDB and DBNS groups ($p>0.05$). Insulin resistance is a significant feature of diabetes, resulting in the deregulation of carbohydrate metabolism and decreased activity of glycolysis enzymes, which ultimately causes impaired peripheral glucose utilization and augmented hepatic glucose production [353]. Our results suggested an ameliorating effect of elderberry preparations on insulin resistance (Figure 5.4C), being most likely caused by decreasing fasting blood glucose and by insulin secretion modulation. No significant differences are reported between the two diabetic supplemented groups ($p>0.05$) (Figure 5.4C). Regarding β cells' function index (HOMA-β), our results showed no differences amongst the three diabetics groups (Figure 5.4D).

5.8. Effect of the elderberry extracts supplementation on fasting lipids, inflammatory and toxicity markers

Sera lipidic markers were assessed on the different groups (Table 5.6). No significant differences ($p>0.05$) are reported regarding the total cholesterol, HDL-c and LDL-c, as well as in triacylglycerols, among the four experimental groups. Previous results showed lower levels of total cholesterol, HDL-c, LDL-c, and triacylglycerol on STZ-induced diabetic rats after supplementation with polar extract (50 mg/kg b.w. during 16 weeks) when compared with non-supplemented diabetic rats [215]. The different diet, administrated dosages and supplementation length may explain these differences.

Table 5.6. Effects of dietary supplementation of elderberry polar and lipophilic extracts on the rats' sera lipids, inflammatory and toxicity indices. Diabetic rats without dietary supplementation and non-diabetic rats were also followed-up.

Index	Non-Diabetic rats *(n=5)*	Diabetic rats with high-fat diets		
		Not supplemented (n=8)	*Polar extract (n=7)*	*Lipophilic extract (n=6)*
Total cholesterol concentration (mg/dL)	99.34 ± 15.01[a]	83.34 ± 17.04[a]	90.79 ± 11.16[a]	90.92 ± 14.25[a]
HDL cholesterol concentration (mg/dL)	72.80 ± 3.19[a]	64.69 ± 13.32[a]	71.37 ± 10.09[a]	71.28 ± 11.25[a]
LDL cholesterol concentration (mg/dL)	12.15 ± 8.86[a]	4.36 ± 1.89[a]	7.13 ± 5.03[a]	6.90 ± 5.45[a]
Triacylglycerol (mg/dL)	88.44 ± 45.21[a]	110.79 ± 77.90[a]	65.52 ± 14.91[a]	82.50 ± 28.87[a]
Total protein (10^{-2} kg/L)	6.86 ± 0.40[a]	6.36 ± 0.52[a,b]	6.09 ± 0.52[a,b]	6.45 ± 0.38[a,b]
Creatinine (µmol/L)	34.12 ± 5.25[a]	48.51 ± 7.79[b]	38.65 ± 8.38[a,b]	34.92 ± 6.19[a]
Urea (mmol/L)	4.88 ± 0.44[a]	10.98 ± 2.61[b]	15.03 ± 4.76[b]	7.78 ± 3.02[a,b]
ALT (U/L)	26.00 ± 10.32[a]	53.00 ± 26.38[a,b]	69.43 ± 30.54[b]	54.67 ± 21.02[a,b]
AST (U/L)	78.00 ± 32.60[a]	97.43 ± 18.21[a]	128.14 ± 69.37[a]	104.20 ± 33.21[a]
ALP (U/L)	60.20 ± 8.73[a]	165.00 ± 53.71[b]	152.29 ± 34.75[b]	108.67 ± 39.05[a,b]

Values expressed as mean ± SD; Mean values with unlike letters in rows show statistically significant differences ($p<0.05$, LSD's Fisher test). HDL - high-density lipoprotein; LDL - low-density lipoprotein; ALT - alanine aminotransaminase; AST - aspartate aminotransferase; ALP - alkaline phosphatase.

Renal and hepatic function markers were also assessed (Table 5.6). Although no significant differences ($p>0.05$) on the total protein levels amongst the three diabetic groups were observed, higher creatinine and urea levels were observed for DBNS group when compared to NDB group ($p<0.05$). This suggests alterations on renal function on diabetic rats after the STZ-induction [349], which is corroborated by the kidney hypertrophy reported on Table 5.4. Elderberry extracts dietary supplementation (DBPE and DBLE) did not show statistical differences of the urea levels compared to DBNS ($p>0.05$). Regarding the creatinine parameter, DBLE led to a significant decrease being lower than DBNS ($p<0.05$).

The levels of intracellular enzymes alanine aminotransferase (ALT) (particularly found in liver), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) (both enzymes found in different tissues) were also assessed in blood sera. Higher levels of ALP ($p<0.05$) after diabetes induction with STZ injection (DBNS) were observed, but no statistical differences were found for AST and ALT ($p>0.05$) when compared DBNS and NDB. Likewise, no significant differences were reported amongst the three diabetic groups ($p>0.05$), for the three analyzed enzymes. Enzyme leakage into circulation is an indication of damaged cells due to inflammation or necrosis, being this toxicity possibly induced by STZ administration [354]. Elderberry extracts dietary supplementation have not shown an additional toxicity as the values did not differ statistically amongst the three diabetic groups ($p>0.05$).

5.9. Effect of the elderberry extracts supplementation on sera and tissular Zn, Fe and Cu levels

The status of some trace elements might be disturbed in chronic hyperglycemia [341], and minerals such as Zn and Cu can modulate glucose and lipid homeostasis [355]. Furthermore, the components present on elderberry extracts might interact with these trace elements, which possibly affect *in vivo* mineral absorption and metabolism [320]. In these perspectives, the tissular and sera mineral status of the rats (Fe, Zn and Cu) were analyzed after 4 weeks of *S. nigra* berry extracts dietary supplementation. The results are included on Table 5.7.

Table 5.7. Effects of dietary supplementation of elderberry polar and lipophilic extracts on the rats' sera and tissular Zn, Fe and Cu levels. Diabetic rats without dietary supplementation and non-diabetic rats were also followed-up.

Index	Non-Diabetic rats *(n=5)*	Diabetic rats with high-fat diets		
		Not supplemented (n=8)	*Polar extract (n=7)*	*Lipophilic extract (n=6)*
Zn (µg/g dry mass)				
Liver	144.69 ± 16.78^{a}	141.48 ± 9.47^{a}	138.56 ± 17.36^{a}	144.50 ± 41.78^{a}
Kidney	94.63 ± 4.06^{a}	114.04 ± 12.61^{b}	120.77 ± 17.81^{b}	114.04 ± 17.89^{a,b}
Sera (µg/dL)	133.81 ± 23.56^{a}	121.27 ± 19.26^{a}	128.10 ± 21.06^{a}	121.15 ± 12.55^{a}
Fe (µg/g dry mass)				
Liver	427.63 ± 51.20^{a}	487.06 ± 108.45^{a}	459.61 ± 127.83^{a}	512.50 ± 189.15^{a}
Kidney	301.93 ± 33.20^{a}	353.06 ± 58.20^{a}	299.16 ± 56.05^{a}	360.12 ± 48.02^{a}
Sera (µg/dL)	116.76 ± 14.75^{a}	141.11 ± 21.25^{a}	139.77 ± 36.31^{a}	149.23 ± 22.38^{a}
Cu (µg/g dry mass)				
Liver	20.08 ± 1.11^{a}	18.30 ± 2.53^{a}	17.60 ± 1.57^{a}	18.63 ± 6.50^{a}
Kidney	26.87 ± 5.60^{a}	54.29 ± 18.13^{b}	59.87 ± 16.94^{b}	46.55 ± 18.81^{a,b}
Sera (µg/dL)	120.12 ± 8.14^{a}	94.50 ± 19.30^{b}	101.45 ± 16.15^{a,b}	92.20 ± 9.29^{b}

Values expressed as mean ± SD; Mean values with unlike letters in rows show statistically significant differences ($p<0.05$, LSD's Fisher test).

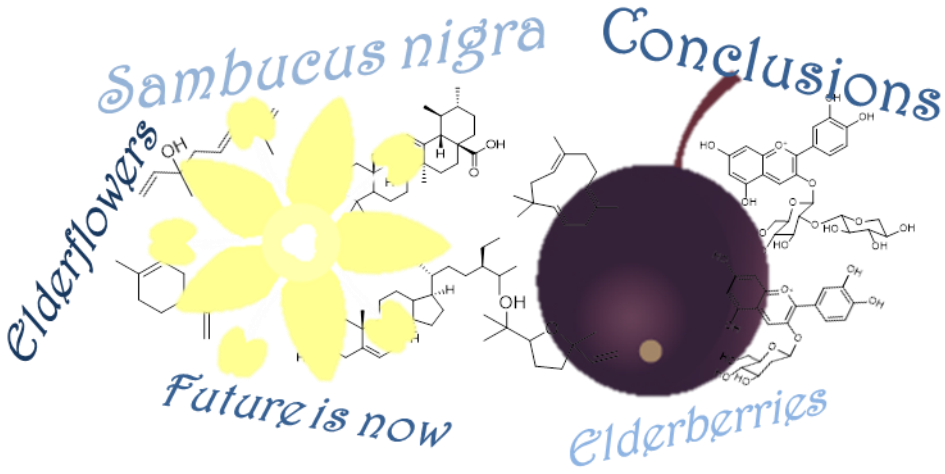
These results indicate that diabetes caused by STZ injection altered the mineral homeostasis of Zn on kidneys, as well as Cu on sera and kidneys ($p<0.05$). Liver and sera Zn and liver Cu were not altered amongst the four tested groups ($p>0.05$), neither both, tissular or sera Fe ($p>0.05$). For the cases were mineral homeostasis were changed after STZ-injection, no significant changes were observed after supplementation with two extracts ($p>0.05$), which allowed to infer that STZ administration had a significant role ($p<0.05$) on the trace elements homeostasis, particularly Zn (kidney) and Cu (kidney and sera), which might be associated with metabolic disturbances occurring in diabetes mellitus [341]. The elderberry dietary extracts supplementation did not show a significant additional effect, as the trace element levels did not differ amongst the three diabetic groups on all tested tissues and blood sera ($p>0.05$).

5.10. Conclusions

The lipophilic and polar elderberry extracts dietary supplementation effects on STZ-induced diabetic Wistar rats fed with a high-fat regimen were evaluated. Extracts toxicity was first assessed using *A. fischeri* model, revealing that both extracts not altered significantly the viable bacterial metabolic activity at concentrations of up to 60 mg/L. Therefore, by applying the equation of interspecies correlation between *A. fischeri* and rats (oral administration), the resultant daily dietary intake of the lipophilic and polar extracts, was 190 and 350 mg/kg b.w., respectively. Elderberry polar extract led to a reduction on fasting blood glucose, while lipophilic extract decreased insulin levels. Furthermore, both extracts lowered insulin resistance, without remarkable alterations in the hematological indices, sera lipidic pattern and on the homeostasis of trace elements (Zn, Fe, Cu) from sera and tissues (kidney and liver). The highlighted results are the result of a elderberry polar extract dietary supplementation during a shorter period and at higher doses compared to literature (four-fold lower length and five-fold higher doses) [199,214–218,220]. Thus, considering this dietary supplementation length, these findings illustrate that a subacute elderberry dietary regimen ameliorated diabetic complications. Regarding elderberry lipophilic extract supplementation, it was tested for the first time, as far as we know. Additionally, the dietary *S. nigra* extracts supplementation effects on STZ-diabetic Wistar rats fed with HF diet on trace elements, as well as on the insulin status have not been studied previously, as far as we know.

The observed improvement of the studied diabetic indices caused by elderberry extract supplementation, demonstrates their potential as suitable substrates for the development of new dietary adjuncts that could help alleviate metabolic disorder in diabetes type 2. To go further with valorization of these extracts, it is crucial to establish in detail their chemical profile, aiming at extracts standardization and by relating elderberry biological effects with their molecular structures.

CHAPTER 6. Conclusions and Future Work



6.1. Concluding remarks

The exploitation of *S. nigra* L. plant has gained increasing attention in the last decade. Literature highlights that this plant has been used both for the formulation of food products and on folk medicine. More recently, herbal supplements are available as nutraceuticals. Dozens of bioactive compounds, are reported on this plant, namely volatile terpenic, triterpenic, sterol, phenolic compounds, among others. Given the diversity of biological activities reported for these families, it illustrates an additional valorization pathway of *S. nigra* plant.

This thesis accomplished the main objective, i.e., the chemical characterization of *S. nigra* L. berries and flowers and biological evaluation of berry extracts, in view of their valorization. Specifically, the interest of elderflowers' volatile terpenic metabolites, namely for aroma properties, led to establish their profile in two cultivars by multidimensional gas chromatography: 38 components were reported for the first time on elderflowers, amongst the total of 65 identified monoterpenic, sesquiterpenic and norisoprenoids components. The generated data, revealed the identification of several metabolites that potentially contribute to the fresh elderflower aroma profile. The impact of common postharvest strategies was evaluated, among which for intermediary storage times (24-32 weeks), vacuum packing and freezing seemed to be the most appropriate preservation methods, while for longer storage times (52 weeks), freezing seemed to the most suitable preservation postharvest condition (in terms of the overall levels of these potential aroma metabolites). The observed overall trends were defined, in general, by linalool oxides, being these suggested as markers to estimate the impact of the studied postharvest conditions over the overall terpenic and norisoprenoid metabolites. This knowledge contributed to understand the underlying postharvest impact on elderflower's metabolites variation, which may be used by growers as a support for decision-making tool and explored to implement strategies regarding the valuation of elderflowers namely on the production of flavoring agents.

Regarding elderberry metabolites profiling, this PhD thesis focused on three main groups: 1) volatile profile (terpenic and norisoprenoids compounds); 2) lipophilic fraction (dichloromethane extractives), and 3) polar fraction (acidified methanol extractives). The chemical analysis of *S. nigra* berries revealed the presence of 103 components from the three studied main groups, being 59 reported for the first time on this matrix. Monoterpenes represented from 66% to 77% of the fraction, being limonene and *p*-cymene the major metabolites. Triterpenoids (*β*-amyrin, ursolic and oleanolic acids) were the most abundant constituents of the lipophilic fraction accounting for up to 94% of the extract. Finally, anthocyanins were the major chemical family amongst the identified phenolic compounds, with cyanidin 3-glucoside and cyanidin 3-sambubioside representing up to 73% of the fraction.

S. nigra berries exposure to different biotic and abiotic stresses may result on complex metabolism alterations which lately affect the profile of its bioactive components. The assessment of the influence of these parameters over the quality of the resulting extracts might however play a critical role in a valorization perspective, namely in the selection of the most appropriate conditions to prepare extracts that preserve most of their health benefits potential [142]. In this perspective, the influence of diverse parameters like ripening stage, cultivar and season was assessed over the elderberry metabolites profile, by using different analytical tools. Regarding lipophilic fraction, during ripening, a similar profile of the studied chemical families was found for the two sampling seasons and the three cultivars, with an initial growth of their content followed by a systematic decrease until maturity. This behavior was consistently observed on two studied harvesting seasons. Similarly, for volatile terpenic and norisoprenoids metabolites, the unripe stages had a higher overall content of the studied metabolites, which gradually decreased over the ripening stages, being consistent for the studied three cultivars. Despite the higher impact of the ripening stage, cultivar also had a significant impact the secondary metabolites profile. All this comprehensive data should be also helpful for producers as a way to manage elderberry harvesting schedule.

Beyond the diverse potential health benefits of elderberry and elderflower extracts, special attention has been given here to Diabetes mellitus, a disease considered as a major threat to human health in the 21st century [5]. Current data about *S. nigra* berry extracts effects on the diabetic complications is still insufficient, illustrating the need to conduct more studies that are able to understand their effects over the disease. Thus, after a detailed chemical characterization of elderberry fractions, an *in vivo* study was conducted that aimed to evaluate extracts dietary supplementation on diabetes management indices. Two extracts were selected to be administrated to STZ-induced diabetic (type-2) Wistar rats, a polar (acidified methanol) and a lipophilic (dichloromethane) one. Extracts toxicity were first screened using *A. fischeri* bioluminescence model, to select a non-toxic dose to be administrated as oral supplementation, being the resultant daily dietary intake of the lipophilic and polar extracts 190 and 350 mg/kg b.w., respectively. Rats were fed with a high-fat diet and supplemented for 4 weeks. Polar extract modulated the glucose metabolism by correcting hyperglycemia, while lipophilic extract lowered insulin secretion. Additionally, both extracts lowered insulin resistance, without remarkable alterations on hematological indices, sera lipids and on sera and tissular trace elements homeostasis. Such results, demonstrated elderberry extracts potential as suitable substrates for the development of new dietary adjuncts that could help alleviate metabolic disorder in diabetes type 2.

The acquired scientific knowledge further demonstrates that elderberry and elderflower matrices can be potentially exploited as a source of bioactive compounds, which illustrates the

interest to be exploited on production of herbal supplements or new food products. In particular, elderberries are a potential source of bioactive compounds for new formulations to be used as co-adjuvants on diabetes management.

6.2. Future work

Following the main objective of this PhD thesis and considering the main results and conclusions there are some challenges that could be considered to further valorize the gathered knowledge:

- ✓ to understand the impact of processing conditions on the elderflower sensorial properties;
- ✓ to study the valorization of *S. nigra* berries flowers as well as by-products (e.g. unripe berries) through the isolation of valuable fractions using benign extraction systems and accessing their biological activity and application in nutraceutical/cosmetic applications;
- ✓ to combine metabolomics with transcriptomics or genomics to go further with regard to terpene metabolism and its relationship with species or cultivar characteristics and ripening stage;

Altogether and beyond the scientific knowledge obtained during this PhD thesis, our goal is to perform different formulations that can be, in a near future, industrially explored on food or nutraceutical markets, which is under development.

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ANNEXES

Annex from Chapter 2

Table A2.1 Full data set including the volatile terpenic and norisoprenoid compounds identified by HS-SPME/GC×GC-ToFMS in two *S. nigra* cultivars under study: 'Sabugueiro' (SO) and 'Sabugueira' (SA) at different handling and storage conditions. Chromatographic area expressed as arbitrary units. F: fresh, AD: air drying, FD: freeze-drying; Fr: freezing; VP_L: vacuum packing with light exposure; VP_NL: vacuum packing without light exposure.

Peak Nº	^1t_R[a] (s)	^2t_R[a] (s)	Compound	Formula	RI $_{Lit}$[b]	RI $_{Calc}$[c]	MSI level	Fresh elderflowers					
								SO_F 1	SO_F 2	SO_F 3	SA_F 1	SA_F 2	SA_F 3
								Peak area (x10^6) (expressed as arbitrary units)					
			Monoterpenic Compounds										
			Hydrocarbon-type										
1	306	0.400	*α*-Pinene	$C_{10}H_{16}$	941	932	1	0.00	0.00	0.00	0.12	0.87	0.11
2	348	0.440	*β*-Pinene	$C_{10}H_{16}$	987	975	1	0.00	0.00	0.00	0.00	0.21	0.00
3	360	0.460	Myrcene	$C_{10}H_{16}$	1008	988	1	1.45	1.70	1.32	1.70	1.55	1.53
4	378	0.450	3-Carene	$C_{10}H_{16}$	1020	1009	2	0.00	0.00	0.00	0.00	0.00	0.00
5	396	0.490	Limonene	$C_{10}H_{16}$	1031	1027	1	0.00	0.00	0.00	0.00	1.21	0.00
6	396	0.550	*p*-Cymene	$C_{10}H_{14}$	1039	1027	1	0.09	0.24	0.24	0.20	0.36	0.26
7	414	0.490	Ocimene	$C_{10}H_{16}$	1043	1051	2	19.28	19.44	18.67	9.82	3.68	9.31
8	426	0.500	*γ*-Terpinene	$C_{10}H_{16}$	1080	1063	1	0.00	0.00	0.00	0.00	0.00	0.00
9	444	0.590	2,6-Dimethyl-2,6-octadiene	$C_{10}H_{18}$	1064	1082	2	1.11	1.27	0.85	0.69	0.42	0.57
10	450	0.510	*α*-Terpinolene	$C_{10}H_{16}$	1097	1093	2	0.00	0.00	0.00	0.00	0.00	0.00
11	456	0.660	*p*-Cymenene	$C_{10}H_{12}$	1090	1094	2	0.23	0.25	0.33	0.49	0.46	0.36
12	480	0.620	Cosmene isomer	$C_{10}H_{14}$	1134	1122	2	3.38	4.72	2.95	0.00	0.00	0.00
13	492	0.610	Cosmene isomer	$C_{10}H_{14}$	1134	1134	2	2.51	2.93	2.05	0.15	0.44	0.85
14	624	0.630	*m/z* 43,94,55,68	-	-	1288	3	0.56	0.77	0.64	2.60	1.46	2.00
			Subtotal (GC Peak Area)						**29.0**			**13.8**	
			Oxygen containing										
15	378	0.520	Dehydroxylinalool oxide	$C_{10}H_{16}O$	1006	1008	2	0.00	0.57	0.00	0.00	0.00	0.00
16	402	0.500	1,8-Cineole	$C_{10}H_{18}O$	1039	1034	1	0.00	0.00	0.00	0.00	0.90	0.00
17	432	0.850	Myrcenol	$C_{10}H_{18}O$	1103	1069	2	0.00	0.00	0.00	0.00	0.00	0.00
18	438	0.680	Linalool oxide (furanoid) isomer[e]	$C_{10}H_{18}O_2$	1078	1076	1	155.31	152.93	221.03	174.58	150.68	174.73
19	450	0.620	Fenchone	$C_{10}H_{16}O$	1093	1090	2	0.00	0.00	0.00	0.00	0.00	0.00
20	450	0.910	Hotrienol isomer	$C_{10}H_{16}O$	1122	1090	2	2.53	2.96	2.74	1.22	0.63	1.44
21	456	0.640	Linalool oxide (furanoid) isomer[e]	$C_{10}H_{18}O_2$	1097	1094	1	11.47	7.56	0.00	14.98	19.25	11.49

22	462	0.790	Linalool	$C_{10}H_{18}O$	1096	1101	1	28.37	15.88	15.76	0.00	0.00	0.49
23	468	0.680	Thujone	$C_{10}H_{16}O$	1120	1108	1	6.17	6.18	33.44	11.59	7.71	9.09
24	468	0.900	Hotrienol isomer	$C_{10}H_{16}O$	1122	1108	2	10.06	10.29	8.89	4.77	4.19	5.80
25	474	0.640	Rose oxide[e]	$C_{10}H_{18}O$	1130	1115	1	1.36	1.58	19.89	2.23	0.83	2.05
26	492	0.490	Linalool, methyl ether	$C_{11}H_{20}O$	1137	1137	2	0.30	0.35	0.35	0.44	0.13	0.48
27	498	0.620	Limonene oxide[e]	$C_{10}H_{16}O$	1127	1144	1	0.00	0.00	0.00	0.00	0.36	0.00
28	504	0.650	Tagetone	$C_{10}H_{16}O$	1146	1150	2	0.00	0.00	0.00	0.00	0.00	0.00
29	510	0.620	Citronellal	$C_{10}H_{18}O$	1159	1154	1	1.74	3.28	2.80	5.28	3.24	3.95
30	510	0.740	Lilac aldehyde isomer	$C_{10}H_{16}O_2$	1154	1155	2	0.11	0.00	0.00	0.14	0.00	0.00
31	516	0.600	Nerol oxide	$C_{10}H_{16}O$	1153	1162	2	0.04	0.10	0.00	0.20	0.00	0.06
32	516	0.675	Lilac aldehyde isomer	$C_{10}H_{16}O_2$	1163	1164	2	1.39	1.00	1.06	0.00	0.00	1.21
33	522	0.740	Lilac aldehyde isomer	$C_{10}H_{16}O_2$	1174	1172	2	0.00	0.00	0.00	0.00	0.00	0.00
34	528	1.090	Linalool oxide (pyranoid)	$C_{10}H_{18}O_2$	1174	1177	2	1824.10	1520.93	1540.27	1824.10	1749.31	1716.27
35	534	0.700	4-Terpineol	$C_{10}H_{18}O$	1181	1181	1	0.66	0.78	1.06	0.39	0.22	0.38
36	546	1.790	Myrtenol	$C_{10}H_{16}O$	1206	1194	2	3.15	2.36	3.36	0.00	1.03	1.00
37	552	0.850	*α*-Terpineol	$C_{10}H_{18}O$	1206	1201	1	0.00	0.10	0.12	0.30	0.46	0.00
38	564	0.880	Verbenone	$C_{10}H_{14}O$	1214	1216	1	0.00	0.00	0.00	0.00	0.20	0.00
39	564	0.925	Lilac alcohol	$C_{10}H_{18}O_2$	1237	1217	2	0.00	0.00	0.00	0.00	0.00	0.00
40	582	0.980	Nerol	$C_{10}H_{18}O$	1242	1236	2	0.00	3.11	10.32	0.00	0.00	0.00
41	588	0.770	Citral[e]	$C_{10}H_{16}O$	1241	1244	1	5.16	5.48	8.28	5.43	2.53	5.57
42	594	1.040	Geraniol	$C_{10}H_{18}O$	1235	1254	1	0.00	0.00	0.00	0.00	0.00	0.00
43	600	0.580	Methyl citronellate	$C_{11}H_{20}O_2$	1261	1261	2	0.95	1.12	1.15	0.54	0.35	0.68
44	612	0.620	Citronellyl formate	$C_{11}H_{20}O_2$	1275	1274	2	0.00	0.00	0.00	0.00	0.00	0.00
45	612	0.800	Geranial	$C_{10}H_{16}O$	1287	1274	2	29.01	19.62	65.00	11.53	11.02	11.14
46	654	0.500	Methyl geranate	$C_{11}H_{18}O_2$	1323	1325	1	8.75	9.95	7.00	12.02	18.08	19.52
47	654	1.080	*m/z* 81,67,43,153	-	-	1327	3	0.24	0.28	0.40	0.72	0.42	0.93
			Subtotal (GC Peak Area)						**1933.3**			**2002.6**	
			Sesquiterpenic Compounds										
48	678	0.460	*α*-Cubebene	$C_{15}H_{24}$	1354	1354	2	0.00	0.00	0.00	0.00	0.00	0.00
49	702	0.470	*α*-Copaene	$C_{15}H_{24}$	1375	1385	1	0.61	1.55	1.30	0.15	0.38	0.35
50	708	0.480	*β*-Bourbonene	$C_{15}H_{24}$	1379	1393	2	0.00	0.00	0.00	0.32	0.20	0.00
51	714	0.490	*β*-Elemene	$C_{15}H_{24}$	1380	1401	2	0.00	0.00	0.00	0.00	0.00	0.00
52	726	0.567	*α*-Bergamotene	$C_{15}H_{24}$	1439	1416	2	0.00	0.00	0.00	0.00	0.00	0.00
53	732	0.500	*β*-Caryophyllene	$C_{15}H_{24}$	1417	1432	1	6.90	7.86	8.86	4.59	2.95	2.73

54	756	0.515	Aromadendrene	$C_{15}H_{24}$	1447	1455	2	0.24	0.23	0.00	0.24	0.00	0.15
55	762	0.540	α-Humulene	$C_{15}H_{24}$	1450	1462	1	0.67	1.10	0.00	0.26	0.00	0.27
56	780	0.540	Germacrene D	$C_{15}H_{24}$	1473	1486	2	0.00	1.22	0.56	0.70	0.00	0.51
57	798	0.520	α-Farnesene	$C_{15}H_{24}$	1507	1510	2	8.98	7.64	22.47	6.52	8.16	6.75
58	810	0.540	Δ-Cadinene	$C_{15}H_{26}$	1525	1528	2	0.10	0.46	0.17	0.00	0.00	0.00
59	810	0.600	Calamenene	$C_{15}H_{22}$	1525	1528	2	0.00	0.03	0.03	0.00	0.00	0.00
60	1014	0.680	*m/z* 69, 41, 55	-	-	1841	3	0.00	0.00	0.00	0.28	0.00	0.50
			Subtotal (GC Peak Area)						**12.0**			**12.0**	
			Norisoprenoids										
61	558	0.705	Safranal	$C_{10}H_{14}O$	1241	1201	2	0.00	0.00	0.00	0.00	0.59	0.00
62	572	0.705	β-Cyclocitral	$C_{10}H_{16}O$	1261	1225	2	0.00	0.00	0.00	0.00	0.00	0.00
63	632	0.515	Dihydroedulan	$C_{13}H_{22}O$	1289	1300	2	0.00	0.00	0.00	0.00	0.00	0.00
64	756	0.660	Geranyl acetone	$C_{13}H_{22}O$	1454	1455	1	0.56	0.52	0.56	0.26	0.18	0.43
65	1020	0.570	Phytone	$C_{18}H_{36}O$	1856	1854	2	0.27	0.24	0.28	0.97	0.61	0.60
			Subtotal (GC Peak Area)						**0.8**			**1.2**	
			Total						**1975.1**			**2029.6**	

[a] Retention times for first (^1t_R) and second (^2t_R) dimensions in seconds.

[b] RI, Retention Index reported in the literature for Equity-5 GC column or equivalents.

[c] RI: Retention Index obtained through the modulated chromatogram.

[d] Level of metabolite identification according to Metabolomics Standards Initiative: (l) Identified compounds using standards coinjection; (2) putatively annotated compounds; (3) putatively characterized compound classes; and (4) unknown compounds [107].

[e] The used standards are a mixture of isomers.

Table A2.1 (continuation) Full data set including the volatile terpenic and norisoprenoid compounds identified by HS-SPME/GC×GC-ToFMS in two *S. nigra* cultivars under study: 'Sabugueiro' (SO) and 'Sabugueira' (SA) at different handling and storage conditions. Chromatographic area expressed as arbitrary units. F: fresh, AD: air drying, FD: freeze-drying; Fr: freezing; VP_L: vacuum packing with light exposure; VP_NL: vacuum packing without light exposure.

Air drying
Peak area (x10^6) (expressed as arbitrary units)

Peak Nº	Compound	1 week						3 week						4 months						8 months						1 year					
		SO_{AD} 1	SO_{AD} 2	SO_{AD} 3	SA_{AD} 1	SA_{AD} 2	SA_{AD} 3	SO_{AD} 1	SO_{AD} 2	SO_{AD} 3	SA_{AD} 1	SA_{AD} 2	SA_{AD} 3	SO_{AD} 1	SO_{AD} 2	SO_{AD} 3	SA_{AD} 1	SA_{AD} 2	SA_{AD} 3	SO_{AD} 1	SO_{AD} 2	SO_{AD} 3	SA_{AD} 1	SA_{AD} 2	SA_{AD} 3	SO_{AD} 1	SO_{AD} 2	SO_{AD} 3	SA_{AD} 1	SA_{AD} 2	SA_{AD} 3
	Monoterpenic Compounds																														
	Hydrocarbon-type																														
1	*α*-Pinene	0.00	0.00	0.00	0.09	0.00	2.85	1.33	0.85	0.60	0.69	1.83	0.48	1.26	0.79	1.31	1.18	0.86	0.95	9.02	8.41	11.00	8.11	6.05	7.90	12.53	15.50	28.17	3.94	7.77	2.34
2	*β*-Pinene	0.00	0.00	0.00	0.00	0.00	0.56	6.47	4.64	3.49	3.21	1.42	2.37	4.13	4.25	2.72	3.16	3.34	2.78	6.80	6.20	7.23	2.65	4.54	4.86	2.60	2.72	1.83	1.18	0.91	6.95
3	Myrcene	0.36	0.00	0.00	0.11	0.13	0.16	0.43	0.26	0.13	0.63	0.27	0.30	0.72	1.05	0.80	1.47	1.22	0.94	1.23	1.47	1.25	2.49	2.60	2.45	1.83	1.16	1.81	2.70	0.89	1.92
4	3-Carene	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	1.43	1.26	0.87	1.63	1.52	1.13	2.88	3.50	1.66	1.61	1.74	1.18	2.63	1.11	5.32	1.45	0.87	0.64
5	Limonene	0.63	0.91	0.65	0.62	0.12	1.37	11.20	10.11	9.12	9.31	6.20	8.29	28.08	26.63	24.69	24.96	26.29	25.98	62.80	48.25	56.26	37.02	29.23	57.67	37.93	30.95	32.31	39.59	31.05	39.53
6	*p*-Cymene	0.12	0.13	0.13	0.16	0.27	0.33	7.38	6.27	4.86	1.38	0.61	1.16	6.07	5.31	5.18	4.55	4.21	4.17	12.28	10.88	12.23	8.57	9.34	11.00	11.92	12.63	13.22	1.81	1.25	1.46
7	Ocimene	0.24	0.51	0.31	0.10	0.00	0.39	3.07	2.59	2.05	0.18	1.34	0.17	0.29	0.27	0.39	0.66	3.23	0.53	0.00	0.39	0.37	0.56	0.65	0.00	3.22	5.38	7.55	0.61	0.00	0.00
8	*γ*-Terpinene	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.00	3.16	0.00	2.34	3.14	2.61	2.56	3.10	0.00	3.11	1.93	1.90	2.24	2.11	2.34	2.28	3.69	1.13	2.85	6.29	0.49	5.17
9	2,6-Dimethyl-2,6-octadiene	0.82	1.68	0.89	0.33	1.31	0.38	0.22	0.20	0.16	0.25	0.18	0.18	1.01	0.98	1.21	0.93	0.69	0.87	0.85	0.76	1.31	0.83	0.68	1.04	1.46	4.93	2.57	0.12	0.24	0.19
10	*α*-Terpinolene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.10	0.36	0.40	0.30	0.58	0.49	0.36	0.38	0.77	0.68	0.50	0.70	0.69	0.00	0.20	0.25	7.73	7.23	5.22
11	*p*-Cymenene	0.54	0.71	0.38	0.41	0.42	0.46	0.48	0.54	0.43	0.34	0.28	0.26	0.52	0.62	0.00	0.08	0.00	0.00	0.65	1.29	1.63	0.00	0.00	0.95	2.76	5.46	2.74	0.92	0.00	1.02
12	Cosmene isomer	2.72	3.15	2.66	0.00	0.00	0.00	0.76	0.66	0.60	0.00	0.00	0.00	2.72	2.74	2.70	0.00	0.00	0.00	0.19	0.68	0.38	0.00	0.00	0.00	1.43	1.91	1.93	0.00	0.00	0.00
13	Cosmene isomer	1.94	2.29	1.89	0.65	2.53	0.65	0.54	0.47	0.43	0.62	0.35	0.00	2.72	2.74	2.70	1.47	1.39	1.52	0.19	0.00	0.14	0.64	0.00	0.18	0.99	1.25	0.00	0.17	0.00	2.69
14	*m/z* 43,94,55,68	2.17	2.31	1.99	1.67	0.94	1.06	0.30	0.26	0.24	0.37	0.37	0.32	0.71	0.85	1.25	1.56	1.30	1.51	0.63	0.50	0.00	2.37	1.99	2.26	0.00	0.99	0.72	1.03	1.19	1.02
	Subtotal (GC Peak Area)		**10.0**			**6.1**			**27.0**			**16.4**			**50.1**			**44.6**			**93.7**			**73.3**			**89.9**			**62.5**	
	Oxygen containing																														
15	Dehydroxylinalool oxide	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.53	1.81	0.00	1.51
16	1,8-Cineole	0.00	0.00	0.00	0.00	0.00	1.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.06	8.80	5.45	5.27	2.34	5.08	0.00	0.00	0.00	0.00	0.00	0.00
17	Myrcenol	5.38	6.08	4.93	4.31	7.51	2.92	0.00	0.00	0.00	0.55	0.52	0.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.15	19.03	12.01
18	Linalool oxide (furanoid) isomer [e]	12.98	14.63	14.90	13.62	13.35	12.70	6.22	5.51	4.60	12.24	12.91	10.88	56.56	58.13	59.22	26.16	28.62	10.79	58.08	52.36	57.23	14.77	13.73	12.07	35.63	36.36	42.77	70.83	83.47	65.81

19	Fenchone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.66	0.14	0.15	0.13
20	Hotrienol isomer	0.36	0.48	0.40	0.09	0.36	0.12	0.39	0.39	0.33	0.11	0.00	0.00	0.52	0.55	0.66	0.45	0.37	0.48	0.35	0.36	0.39	0.57	0.41	0.61	0.00	0.24	0.31	0.10	0.17	0.12
21	Linalool oxide (furanoid) isomer[e]	4.36	5.60	4.37	1.86	2.19	1.97	1.25	1.07	0.86	1.86	1.04	1.59	13.16	11.69	17.20	14.36	13.27	15.54	9.99	9.90	11.21	15.79	15.78	17.05	3.04	5.63	7.49	5.49	7.53	5.12
22	Linalool	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	45.46	41.55	39.78
23	Thujone	13.92	0.00	21.59	8.10	9.69	6.15	6.95	7.03	5.08	6.96	4.87	6.60	44.88	51.30	55.91	49.03	69.29	51.74	52.49	38.33	36.82	67.69	49.03	50.49	29.18	17.89	22.14	17.32	32.26	13.75
24	Hotrienol isomer	16.74	19.15	16.76	7.77	8.21	7.42	15.77	15.62	14.73	16.24	9.14	9.19	30.63	30.09	29.98	15.47	18.28	15.15	18.57	19.25	20.09	21.64	22.80	25.33	29.12	18.86	30.16	20.61	25.71	24.55
25	Rose oxide[e]	0.06	0.17	0.14	0.08	0.08	0.10	0.04	0.07	0.00	0.14	0.07	0.09	1.46	1.66	1.74	2.79	2.47	2.80	1.38	1.76	1.91	3.98	3.32	3.66	1.05	2.03	1.15	1.92	2.27	1.53
26	Linalool, methyl ether	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.79	0.20	0.44	0.78	0.43
27	Limonene oxide[e]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.92	4.10	2.05	0.49	0.00	0.00
28	Tagetone	8.53	10.94	9.21	0.80	2.39	0.96	1.41	1.28	0.99	0.90	0.67	0.71	14.28	16.74	18.99	1.32	1.20	1.47	10.63	10.55	11.22	1.26	1.20	1.32	0.10	0.11	0.17	0.08	0.09	0.07
29	Citronellal	0.92	0.66	1.57	1.40	2.04	1.54	0.31	0.21	0.11	0.76	0.35	0.45	5.63	5.83	6.39	3.02	7.51	9.36	0.74	1.21	4.37	1.04	1.02	1.55	2.50	3.50	1.54	0.57	0.35	1.00
30	Lilac aldehyde isomer	0.00	0.00	0.71	1.44	0.17	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.67	0.00	0.00	4.91	0.00	5.18	0.00	0.00	0.00	3.48	3.95	5.94
31	Nerol oxide	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.88	0.49	1.43	1.77	1.33
32	Lilac aldehyde isomer	0.00	0.00	0.35	0.47	0.70	0.00	0.15	0.14	0.28	0.30	0.00	0.24	1.87	1.46	0.00	0.00	2.47	3.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.38	0.50	0.42
33	Lilac aldehyde isomer	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.00	2.05	0.00	0.00	0.00	0.67	0.62	0.95	2.25	2.12	2.44	0.95	1.53	0.82	0.00	0.77	0.00
34	Linalool oxide (pyranoid)	1247.93	1284.12	1191.46	757.12	812.74	927.46	602.90	705.55	897.07	444.61	420.00	428.20	228.55	164.29	320.44	355.06	320.94	350.44	111.81	116.52	118.24	390.01	268.45	366.49	115.49	115.26	129.84	271.73	373.56	335.53
35	4-Terpineol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	2.13	0.00	1.65
36	Myrtenol	24.77	33.18	29.99	12.48	44.61	11.26	4.72	7.06	5.01	4.28	4.24	2.70	0.00	0.00	0.00	0.00	0.00	0.00	6.73	6.65	6.61	7.82	1.10	1.50	9.49	10.30	9.71	2.85	0.00	2.55
37	α-Terpineol	0.00	0.00	0.00	0.00	0.00	0.61	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.39	0.22	0.00	0.39	0.38	0.67	0.00	0.14	0.21	0.32	5.35	4.90
38	Verbenone	0.00	0.52	0.50	0.00	0.00	0.20	0.00	0.00	0.00	0.16	0.00	0.00	2.29	2.47	3.63	3.74	3.42	3.50	0.68	0.34	0.49	1.81	1.80	1.95	0.00	0.00	0.00	0.00	0.00	0.00
39	Lilac alcohol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.08	0.60	1.05	0.00	0.00	0.00	0.00	0.00	0.00
40	Nerol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.12	3.39	6.18	0.00	0.00	9.95	1.94	3.19	4.88	8.31	5.96	8.00	9.59	9.01	9.11	1.94	2.41	3.38
41	Citral[e]	0.00	0.00	0.00	0.00	0.00	0.00	0.83	1.34	1.03	1.76	0.00	1.09	2.79	0.00	3.59	2.77	2.89	3.87	2.49	1.63	1.71	0.00	2.58	2.82	1.95	1.66	1.28	3.96	0.56	3.54
42	Geraniol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.92	1.45	2.30	0.00	0.00	0.00
43	Methyl citronellate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
44	Citronellyl formate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.00	0.00	0.01	0.13	3.31	0.84	3.56
45	Geranial	0.50	1.39	0.94	0.41	0.56	0.68	1.00	1.00	0.98	1.41	0.61	1.09	0.00	8.45	0.00	4.47	4.86	5.84	2.17	1.40	2.63	3.08	6.88	4.38	0.57	4.57	6.21	0.00	0.00	0.00
46	Methyl geranate	0.00	0.23	0.00	1.12	0.35	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.12	0.24	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
47	*m/z* 81,67,43,153	0.43	0.54	0.42	0.34	0.46	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.29	0.37	0.58	0.79	0.00	0.12	0.13	0.35	0.62	0.54	0.60	0.00	0.12	0.18	0.15	0.22	0.24
	Subtotal (GC Peak Area)		**1337.6**			**897.7**			**773.1**			**470.2**			**429.9**			**480.1**			**280.7**			**488.3**			**251.1**			**533.4**	
	Sesquiterpenic Compounds																														
48	α-Cubebene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.08	0.08	0.06

49	α-Copaene	0.34	0.00	0.25	0.53	0.34	0.94	0.00	0.00	0.00	0.00	0.24	0.00	1.10	0.22	0.19	0.49	0.13	0.30	1.76	0.00	0.21	0.00	0.00	0.00	1.97	2.52	2.42	0.26	0.00	0.50
50	β-Bourbonene	0.00	0.00	0.00	0.66	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.33	0.00	0.00	0.00	0.00	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.10	0.09
51	β-Elemene	0.29	0.00	0.48	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.00	0.29	0.26	0.00	0.00	0.00	0.00	0.00	0.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00
52	α-Bergamotene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
53	β-Caryophyllene	0.00	0.00	0.42	3.26	3.21	3.50	1.92	1.55	2.15	2.67	3.17	2.61	1.46	1.92	2.62	2.12	1.90	1.68	1.35	1.67	1.97	1.05	8.84	1.31	2.56	1.95	2.15	5.62	4.34	3.29
54	Aromadendrene	0.00	0.00	0.00	0.00	0.24	0.25	0.00	0.00	0.00	0.00	0.24	0.00	0.00	0.00	0.00	0.57	0.00	0.49	2.21	6.06	1.08	0.00	0.00	0.00	2.49	2.45	2.65	0.00	0.00	1.82
55	α-Humulene	0.00	0.00	0.00	0.85	0.43	0.21	0.55	0.40	0.00	0.30	0.14	0.20	0.00	0.00	0.00	1.20	1.36	1.12	0.46	0.66	0.66	1.28	1.99	1.31	0.00	0.00	0.00	0.00	0.00	0.00
56	Germacrene D	0.00	0.00	0.00	0.98	0.43	0.38	0.15	0.41	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.56	0.83	1.00	0.35	0.00	0.00	0.00	0.00	0.87	0.00	0.00	0.00	0.00	0.00	0.00
57	α-Farnesene	1.12	1.34	1.50	0.63	0.46	0.34	0.54	0.51	0.46	0.86	0.74	0.73	0.00	0.00	0.00	1.12	1.01	1.46	1.62	0.00	0.00	0.00	0.91	0.00	0.36	0.30	0.30	0.00	0.00	0.40
58	Δ-Cadinene	0.11	0.00	0.12	0.39	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.55	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.17	0.28	0.00	0.00	0.00	0.00	0.00	0.00
59	Calamenene	0.05	0.07	0.06	0.03	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.05	0.00	0.00	0.00	0.10	0.07	0.00	0.07	0.09	0.11	0.00	0.08	0.09	0.00	0.00	0.00
60	*m/z* 69, 41, 55	0.16	0.34	0.00	0.63	0.24	0.17	0.17	0.29	0.15	0.22	0.18	0.40	0.61	0.54	0.48	0.19	0.16	0.19	0.11	0.00	0.00	0.13	0.21	0.17	0.00	0.00	0.00	0.11	0.57	0.19
	Subtotal (GC Peak Area)		**2.2**			**6.5**			**3.1**			**4.4**			**3.4**			**6.2**			**6.9**			**6.5**			**7.5**			**5.9**	
	Norisoprenoids																														
61	Safranal	0.00	0.00	0.00	0.00	0.00	0.49	0.00	0.00	0.00	0.00	0.39	0.00	0.49	0.43	0.55	0.59	1.24	0.81	0.37	0.00	0.00	0.64	0.63	2.08	1.34	1.79	1.21	2.50	2.00	1.94
62	β-Cyclocitral	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.90	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.00	0.00	0.00	0.00	0.91	0.00	0.00	0.00	0.00
63	Dihydroedulan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
64	Geranyl acetone	1.85	3.19	2.62	4.21	3.35	1.84	1.67	1.86	1.52	2.32	1.26	2.10	5.56	5.52	7.44	10.78	8.86	9.41	3.98	3.37	8.08	6.58	5.85	7.15	4.96	5.19	6.74	3.90	5.25	3.18
65	Phytone	0.62	0.75	1.05	0.23	0.46	0.36	0.26	0.32	0.43	0.43	0.32	0.42	0.82	0.81	0.94	0.56	0.41	0.79	0.73	0.83	3.39	1.44	0.50	1.11	1.39	1.29	1.27	0.12	0.40	0.54
	Subtotal (GC Peak Area)		**3.4**			**3.6**			**2.0**			**2.4**			**8.2**			**11.1**			**7.1**			**8.7**			**8.7**			**6.6**	
	Total		**1353.2**			**913.9**			**805.2**			**493.5**			**491.5**			**542.1**			**388.3**			**576.8**			**357.2**			**608.4**	

Table A2.1 (continuation) Full data set including the volatile terpenic and norisoprenoid compounds identified by HS-SPME/GC×GC-ToFMS in two *S. nigra* cultivars under study: 'Sabugueiro' (SO) and 'Sabugueira' (SA) at different handling and storage conditions. Chromatographic area expressed as arbitrary units. F: fresh, AD: air drying, FD: freeze-drying; Fr: freezing; VP_L: vacuum packing with light exposure; VP_NL: vacuum packing without light exposure.

		Freeze-drying Peak area (x10^6) (expressed as arbitrary units)																													
		After freeze-drying						1 week						4 months						8 months						1 year					
Peak Nº	Compound	SO_{FD} 1	SO_{FD} 2	SO_{FD} 3	SA_{FD} 1	SA_{FD} 2	SA_{FD} 3	SO_{FD} 1	SO_{FD} 2	SO_{FD} 3	SA_{FD} 1	SA_{FD} 2	SA_{FD} 3	SO_{FD} 1	SO_{FD} 2	SO_{FD} 3	SA_{FD} 1	SA_{FD} 2	SA_{FD} 3	SO_{FD} 1	SO_{FD} 2	SO_{FD} 3	SA_{FD} 1	SA_{FD} 2	SA_{FD} 3	SO_{FD} 1	SO_{FD} 2	SO_{FD} 3	SA_{FD} 1	SA_{FD} 2	SA_{FD} 3
	Monoterpenic Compounds																														
	Hydrocarbon-type																														
1	*α*-Pinene	2.82	2.07	1.54	4.40	2.26	3.07	1.55	1.73	0.87	2.11	0.94	0.65	1.17	1.54	1.41	0.62	0.51	0.00	1.21	1.35	1.30	1.39	1.77	1.86	1.07	0.92	0.82	1.67	2.75	2.71
2	*β*-Pinene	6.04	7.03	5.76	9.74	7.71	9.01	6.09	6.75	4.49	9.02	6.17	4.81	1.73	1.62	1.58	4.30	4.22	3.18	0.59	1.03	0.66	0.62	0.74	0.72	2.33	1.71	2.34	0.18	0.17	0.19
3	Myrcene	0.83	0.96	0.86	1.65	1.63	1.58	1.24	1.10	0.82	1.94	1.29	1.01	1.59	1.93	1.61	1.58	2.51	1.59	2.18	2.37	2.23	1.02	0.78	1.08	9.52	7.73	5.63	1.72	1.51	1.85
4	3-Carene	0.57	0.72	0.59	0.94	0.59	0.47	0.83	0.86	0.63	1.17	0.66	0.50	2.40	2.53	2.13	0.97	1.17	0.95	2.91	2.31	2.24	2.06	1.47	2.15	4.09	3.27	3.23	2.79	0.00	4.74
5	Limonene	12.86	13.72	10.30	10.57	14.08	13.32	13.50	13.54	11.37	13.71	16.40	14.85	19.53	20.37	18.83	13.83	14.70	13.19	12.67	15.64	13.80	19.58	17.05	20.71	13.68	12.31	14.94	10.32	14.40	10.58
6	*p*-Cymene	2.67	3.02	2.48	2.63	1.96	1.12	3.24	3.03	2.28	7.33	5.20	4.46	4.82	5.30	5.86	0.00	6.47	0.72	4.68	5.24	5.15	3.98	0.00	0.00	0.98	0.27	0.99	3.85	2.77	3.92
7	Ocimene	0.68	1.67	1.58	0.94	0.69	0.55	2.34	1.82	2.43	0.95	0.85	0.78	2.06	2.66	2.62	0.47	0.41	0.40	1.70	1.65	1.71	0.62	0.62	0.90	1.14	1.19	1.66	1.96	1.49	1.06
8	*γ*-Terpinene	4.38	4.30	4.16	5.94	5.71	5.79	4.33	4.34	3.56	7.03	5.84	5.29	1.52	1.47	1.44	1.14	1.20	1.13	1.71	1.79	1.62	1.02	0.74	0.97	9.74	1.17	1.92	1.31	2.76	1.51
9	2,6-Dimethyl-2,6-octadiene	0.12	0.18	0.13	0.12	0.29	0.13	0.21	0.14	0.15	0.20	0.28	0.17	4.87	1.52	1.74	0.89	1.13	0.88	3.96	4.24	4.43	1.21	0.93	1.52	4.07	2.67	4.66	1.81	2.33	6.52
10	*α*-Terpinolene	0.29	0.33	0.30	0.64	0.58	0.69	0.33	0.29	0.23	0.72	0.48	0.41	0.18	0.27	0.54	0.18	0.20	0.18	0.00	0.30	0.24	0.27	0.14	0.16	0.26	0.26	0.10	0.21	0.21	0.27
11	*p*-Cymenene	0.25	0.15	0.18	0.20	0.20	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.43	0.57	0.47	0.60	0.00	0.48	0.00	0.61	0.28	0.22	1.55	0.17	6.68	3.18	3.14	3.65	4.33	0.00
12	Cosmene isomer	0.26	0.44	0.32	0.00	0.00	0.00	0.43	0.20	0.22	0.00	0.00	0.00	3.42	4.51	4.66	0.00	0.00	0.00	8.38	10.56	11.39	0.00	0.00	0.00	11.62	11.43	18.46	0.00	0.00	0.00
13	Cosmene isomer	0.17	0.31	0.23	0.00	0.09	0.00	0.19	0.20	0.15	0.00	0.19	0.00	3.42	4.51	3.77	2.16	2.25	1.48	5.53	10.56	11.39	1.06	2.08	1.67	0.74	0.60	8.70	0.41	0.51	0.82
14	*m/z* 43,94,55,68	0.00	0.00	0.00	0.26	0.35	0.34	0.18	0.13	0.12	0.70	0.67	0.64	0.32	0.57	0.47	1.90	1.44	1.33	1.16	1.25	1.16	3.27	1.86	2.38	2.35	2.17	3.09	1.35	1.73	1.95
	Subtotal (GC Peak Area)		31.8			36.8			32.0			39.1			48.0			30.1			54.4			33.4			62.3			34.1	
	Oxygen containing																														
15	Dehydroxylinalool oxide	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.03	3.57	3.59	2.65	3.05	2.93
16	1,8-Cineole	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
17	Myrcenol	0.11	0.44	0.42	0.95	3.23	1.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.43	0.00	0.00	0.00	0.00	0.00	1.43	1.42	1.29	4.04	4.66	3.85
18	Linalool oxide (furanoid) isomer [e]	8.68	12.71	13.50	15.58	17.06	18.97	49.38	23.64	0.00	24.55	34.98	25.01	59.59	59.38	64.42	57.55	58.83	57.90	17.36	17.32	16.80	96.17	100.66	105.78	11.35	10.53	18.00	69.90	84.21	67.03
19	Fenchone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.91	4.66	0.00	0.43	1.30	0.00	3.21	2.73	3.09	1.75	2.17	2.37
20	Hotrienol isomer	0.00	0.10	0.10	0.07	0.10	0.12	0.05	0.00	0.00	0.00	0.00	0.00	0.51	0.75	0.68	0.20	0.25	0.20	1.65	1.47	1.59	0.36	0.39	0.71	0.44	0.21	0.00	0.14	0.11	0.11

21	Linalool oxide (furanoid) isomer[e]	1.06	1.96	2.13	2.29	4.18	3.16	0.35	0.66	0.00	0.00	0.54	0.95	11.47	18.04	17.89	7.95	7.71	7.20	27.00	21.70	23.69	19.66	16.64	22.50	19.34	15.72	30.24	9.27	11.50	12.60
22	Linalool	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.32	0.15	0.16	0.12	0.12	0.71
23	Thujone	0.42	0.57	0.48	1.94	2.51	2.16	0.00	0.00	0.00	5.60	5.85	0.00	0.43	0.59	0.21	4.66	0.66	4.80	0.00	0.00	0.00	8.31	8.69	9.07	0.00	0.11	0.08	5.00	6.97	6.53
24	Hotrienol isomer	0.85	2.47	1.60	1.39	3.84	1.20	1.60	1.82	1.54	12.55	11.38	11.27	30.29	36.63	32.57	19.61	22.56	17.49	90.03	90.03	90.85	29.17	23.37	26.80	56.86	36.59	56.71	24.54	28.25	29.87
25	Rose oxide[e]	0.00	0.08	0.07	0.11	0.16	0.13	0.18	0.17	0.21	0.38	0.26	0.27	0.76	0.78	0.75	0.43	0.46	0.41	2.27	1.09	2.84	0.87	0.87	1.06	0.00	1.43	2.24	1.00	1.73	1.29
26	Linalool, methyl ether	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.04	2.21	2.49	1.90	1.68	1.86
27	Limonene oxide[e]	0.30	0.25	0.25	0.57	1.02	0.86	0.75	0.57	2.19	3.22	1.63	1.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.55	1.91	1.99	3.09	2.72	5.92
28	Tagetone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.31	0.34	0.09	0.00	0.00	1.03	0.95	3.53	0.59	0.72	0.77
29	Citronellal	0.22	0.23	0.29	0.24	0.28	0.58	0.22	0.14	0.24	0.18	0.26	0.19	0.00	0.00	0.74	2.51	2.84	3.19	0.00	0.00	0.00	3.39	3.60	3.63	2.08	1.73	2.72	1.02	0.00	0.00
30	Lilac aldehyde isomer	0.00	0.00	0.00	0.00	0.65	0.00	0.82	0.48	0.54	1.21	0.09	1.69	0.62	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.03	0.00	0.79	7.16	0.00	7.79	2.79	4.18	4.71
31	Nerol oxide	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.37	0.33	0.00	0.00	0.00	0.49	0.32	0.31	0.33	0.24	0.59
32	Lilac aldehyde isomer	0.00	0.00	0.00	0.09	0.27	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.43	0.00	0.69	0.70	0.75	0.00	0.00	0.00	0.00	1.79	0.00	5.29	6.77	4.56	3.90	4.76	6.06
33	Lilac aldehyde isomer	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.26	0.88	1.06	0.00	0.81	0.38	0.45	2.00	0.00
34	Linalool oxide (pyranoid)	1170.71	1148.49	1296.52	970.20	933.68	1179.78	964.09	797.93	722.48	818.09	739.97	601.55	212.71	245.18	294.16	121.70	190.75	142.47	212.21	307.50	297.51	202.52	303.21	281.02	156.49	154.94	155.95	312.87	354.81	282.9
35	4-Terpineol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.36	6.25	9.69	3.82	4.15	8.24
36	Myrtenol	0.00	0.78	0.00	0.00	1.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	70.43	59.88	61.89	6.39	4.15	3.66	7.48	5.76	7.88	3.99	4.86	2.35
37	α-Terpineol	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.13	0.14	0.00	0.00	0.00	0.15	0.15	0.22	7.95	9.35	8.34
38	Verbenone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
39	Lilac alcohol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
40	Nerol	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.69	0.00	0.00	0.48	18.95	11.84	25.48	26.70	8.01	8.25	16.88	15.35	16.47	38.15	30.24	41.87	11.69	12.69	10.00	13.28	17.06	11.07
41	Citral[e]	0.00	0.11	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.45	0.78	0.83	0.00	0.00	0.00	0.00	0.00	0.00	0.62	0.00	0.00	6.38	8.28	1.04	4.07	3.76	3.84
42	Geraniol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.39	1.65	3.67	9.08	11.06	10.29
43	Methyl citronellate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
44	Citronellyl formate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.84	0.11	0.01	5.53	4.85	4.65
45	Geranial	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.81	1.59	1.43	0.00	0.00	0.00	0.00	0.54	0.00	0.88	0.00	0.00	1.37	2.07	8.00	0.00	1.07	2.89
46	Methyl geranate	0.25	0.61	0.58	0.58	1.01	0.84	0.87	0.48	0.85	1.94	1.91	1.69	0.90	1.51	1.55	1.52	1.55	1.46	1.05	0.78	0.72	1.65	1.38	2.07	0.00	0.00	1.87	0.00	0.00	0.00
47	*m/z* 81,67,43,153	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.40	0.33	0.59	0.54	0.55	0.75	0.75	0.73	0.85	0.66	0.97	0.77	0.76	1.21	0.39	0.52	0.59
	Subtotal (GC Peak Area)		**1222.6**			**1057.5**			**857.9**			**769.7**			**386.6**			**261.2**			**499.5**			**470.2**			**310.4**			**430.6**	
	Sesquiterpenic Compounds																														
48	α-Cubebene	0.00	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
49	α-Copaene	0.44	0.30	0.53	0.00	0.00	0.11	0.59	0.38	0.09	0.00	0.00	0.00	0.08	0.00	0.60	0.00	0.18	0.20	0.00	0.29	0.67	0.31	0.58	0.57	0.00	0.00	0.00	0.00	0.78	0.00
50	β-Bourbonene	0.00	0.00	0.00	0.00	0.04	0.00	0.13	0.10	0.00	0.08	0.14	0.07	0.00	0.00	0.00	0.49	0.00	0.14	0.00	0.00	0.00	0.16	0.17	0.00	0.21	0.00	0.00	0.00	0.00	0.30

51	*β*-Elemene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
52	*α*-Bergamotene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
53	*β*-Caryophyllene	4.25	4.88	3.85	1.73	1.92	1.90	3.17	1.48	2.69	1.44	1.10	1.17	4.12	5.83	5.41	2.86	2.39	2.19	6.20	6.60	5.86	3.30	3.09	2.94	5.62	5.73	3.43	3.76	5.46	0.68
54	Aromadendrene	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.43	0.04	0.41	0.00	0.60	0.44	0.47	2.99	5.06	0.85
55	*α*-Humulene	0.11	0.45	0.73	0.00	0.05	0.05	0.98	0.57	0.81	0.10	0.06	0.07	0.33	0.50	0.44	0.20	0.15	0.14	1.83	1.20	0.41	0.57	0.44	0.58	0.00	0.00	0.00	0.00	0.00	0.00
56	Germacrene D	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.25	0.00	0.00	0.00	0.00	0.00	1.76	0.00	1.34	0.00	0.00	0.00
57	*α*-Farnesene	0.54	0.23	0.63	0.38	0.54	0.48	0.87	0.50	0.66	0.55	0.53	0.48	0.00	0.30	0.00	0.29	0.23	0.22	0.00	0.00	0.00	0.46	0.00	0.00	0.00	1.24	2.03	0.46	0.69	0.78
58	Δ-Cadinene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.58	0.00	0.00	0.00
59	Calamenene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.12	0.08	0.06	0.10	0.06	0.04	0.05	0.13	0.00	0.00	0.05
60	*m/z* 69, 41, 55	0.00	0.11	0.22	0.11	0.14	0.18	0.98	1.00	0.76	0.28	0.22	0.23	0.24	0.31	0.22	0.40	0.40	0.89	0.35	0.21	0.26	0.64	0.85	0.55	0.12	0.23	0.52	0.13	0.35	0.64
	Subtotal (GC Peak Area)		**5.9**			**2.5**			**5.2**			**2.2**			**6.1**			**3.8**			**8.6**			**5.3**			**8.6**			**7.7**	
	Norisoprenoids																														
61	Safranal	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.21	0.32	0.18	0.19	0.20	0.73	0.69	0.69	0.38	0.33	0.41	2.07	1.58	3.47	1.06	1.00	1.83
62	*β*-Cyclocitral	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.39	0.38	0.51	0.49	0.44	0.00	0.58	0.55	1.15	1.06	1.34	1.14	1.13	5.99	0.65	0.74	1.11
63	Dihydroedulan	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.35	0.00	0.65	0.51	0.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00
64	Geranyl acetone	0.40	0.47	1.06	0.85	0.49	0.47	3.35	4.45	1.42	0.90	1.31	1.06	3.09	6.15	4.03	3.67	4.29	5.65	7.32	5.79	5.92	5.07	5.06	6.32	4.40	4.63	7.04	2.48	3.68	4.90
65	Phytone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.16	0.00	0.16	0.18	0.17	0.25	0.28	0.32	0.80	0.26	0.38	0.51	0.50	0.47	0.51	0.48	0.78	0.26	0.43	0.42
	Subtotal (GC Peak Area)		**0.7**			**0.6**			**3.1**			**1.2**			**5.2**			**5.5**			**8.9**			**7.9**			**11.1**			**6.2**	
	Total		**1260.9**			**1097.5**			**898.1**			**812.2**			**445.9**			**300.6**			**571.4**			**516.8**			**392.3**			**478.5**	

Table A2.1 (continuation) Full data set including the volatile terpenic and norisoprenoid compounds identified by HS-SPME/GC×GC-ToFMS in two *S. nigra* cultivars under study: 'Sabugueiro' (SO) and 'Sabugueira' (SA) at different handling and storage conditions. Chromatographic area expressed as arbitrary units. F: fresh, AD: air drying, FD: freeze-drying; Fr: freezing; VP_L: vacuum packing with light exposure; VP_NL: vacuum packing without light exposure.

Peak Nº	Compound	Freezing Peak area (x10^6) (expressed as arbitrary units)																	
		5 months						8 months						1 year					
		SO_{Fr} 1	SO_{Fr} 2	SO_{Fr} 3	SA_{Fr} 1	SA_{Fr} 2	SA_{Fr} 3	SO_{Fr} 1	SO_{Fr} 2	SO_{Fr} 3	SA_{Fr} 1	SA_{Fr} 2	SA_{Fr} 3	SO_{Fr} 1	SO_{Fr} 2	SO_{Fr} 3	SA_{Fr} 1	SA_{Fr} 2	SA_{Fr} 3
	Monoterpenic Compounds																		
	Hydrocarbon-type																		
1	*α*-Pinene	0.45	0.80	0.42	0.12	0.00	0.36	33.95	46.89	45.56	0.13	0.14	0.16	47.33	46.34	42.18	0.64	0.78	0.57
2	*β*-Pinene	0.44	0.91	0.38	0.12	0.25	0.21	5.14	6.20	7.40	1.72	1.79	1.54	8.37	6.91	7.36	6.72	7.76	6.95
3	Myrcene	7.19	9.65	9.61	8.17	8.44	9.52	18.11	16.04	16.55	4.64	4.68	4.60	6.48	6.16	6.37	4.50	5.55	4.81
4	3-Carene	1.06	1.68	0.00	0.00	0.00	0.12	3.67	3.70	3.28	5.81	5.60	4.11	0.00	0.00	0.00	5.53	5.02	5.02
5	Limonene	40.60	55.27	41.81	1.40	1.09	1.43	30.22	28.45	49.76	2.73	3.28	5.78	17.92	16.49	16.58	3.46	4.59	3.33
6	*p*-Cymene	3.21	5.09	2.64	1.28	1.25	1.50	5.55	4.54	5.30	2.93	3.54	7.44	0.00	0.41	0.53	0.00	0.00	0.00
7	Ocimene	11.37	20.68	13.11	59.21	59.40	55.92	14.50	14.44	12.47	67.32	77.26	68.18	76.44	62.16	61.10	58.78	71.43	46.04
8	*γ*-Terpinene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.62	0.50	0.66
9	2,6-Dimethyl-2,6-octadiene	8.56	9.94	12.55	3.80	4.02	4.28	9.82	7.62	8.20	5.24	5.72	8.89	6.76	8.31	8.02	14.02	12.75	12.35
10	*α*-Terpinolene	0.82	2.41	0.97	0.20	0.00	0.32	3.07	4.88	3.66	3.54	3.11	3.82	8.40	8.34	7.34	2.53	2.94	2.46
11	*p*-Cymenene	1.30	0.00	2.09	0.00	1.57	0.00	1.37	1.71	1.67	1.33	1.17	1.72	3.13	4.99	4.88	3.22	2.68	3.00
12	Cosmene isomer	14.99	16.57	21.59	0.00	0.00	0.00	20.99	13.61	17.04	0.00	0.00	0.00	10.16	15.55	12.94	0.00	0.00	0.00
13	Cosmene isomer	14.99	16.57	21.59	5.44	3.98	9.47	17.74	13.61	11.89	8.88	7.02	7.44	0.77	0.30	0.49	19.29	13.53	11.74
14	*m/z* 43,94,55,68	6.76	5.85	4.17	6.46	9.57	8.34	1.76	1.09	1.78	7.26	9.49	10.68	2.02	2.50	2.76	6.39	7.40	6.02
	Subtotal (GC Peak Area)		**129.4**			**89.1**			**171.1**			**119.6**			**178.9**			**121.2**	
	Oxygen containing																		
15	Dehydroxylinalool oxide	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.93	3.79	0.00	0.00	0.00
16	1,8-Cineole	0.00	2.40	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
17	Myrcenol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.54	20.12	19.93	0.04	6.30	6.69
18	Linalool oxide (furanoid) isomer [e]	229.98	267.18	295.89	230.52	202.40	245.57	265.49	279.58	214.63	245.59	270.09	278.83	169.85	152.73	123.64	200.10	202.56	204.76
19	Fenchone	5.89	5.89	5.78	5.89	2.86	1.02	0.00	0.00	0.00	5.84	5.82	5.09	0.34	0.00	0.20	0.25	0.30	0.38
20	Hotrienol isomer	19.25	17.36	20.50	7.94	6.47	7.31	11.63	9.92	11.58	8.63	8.15	8.96	12.55	14.27	13.97	5.06	6.88	7.63
21	Linalool oxide (furanoid) isomer [e]	83.52	83.07	82.23	21.01	21.17	85.50	76.06	54.57	80.28	188.13	187.79	189.32	71.46	71.95	78.15	174.61	177.28	193.19
22	Linalool	48.95	40.40	42.06	41.07	26.26	39.95	10.13	11.10	19.62	26.97	26.63	26.26	10.54	14.68	10.11	28.74	21.54	21.36
23	Thujone	76.12	76.12	77.26	43.85	40.03	46.47	35.60	30.94	36.70	56.34	45.68	57.45	38.23	34.47	39.24	39.61	44.33	57.80
24	Hotrienol isomer	47.96	49.98	80.01	19.41	24.02	23.94	70.78	37.13	35.64	30.64	34.82	30.42	38.18	36.82	45.48	30.51	29.07	36.36

25	Rose oxide[e]	14.38	15.66	25.66	8.54	9.08	7.31	13.13	19.77	15.16	0.89	1.01	1.06	8.35	8.43	8.70	0.57	0.74	0.78
26	Linalool, methyl ether	2.73	3.05	2.31	2.90	2.80	3.02	2.11	1.59	2.09	3.45	3.33	3.42	0.00	0.00	0.00	0.00	0.00	0.00
27	Limonene oxide[e]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	2.84	3.55	0.95	2.40	1.91
28	Tagetone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00
29	Citronellal	13.74	17.88	10.85	6.63	8.16	8.43	22.09	17.55	25.40	1.28	1.24	1.39	22.95	10.94	10.23	1.51	1.84	1.80
30	Lilac aldehyde isomer	0.00	0.00	0.00	1.20	1.66	1.37	0.00	0.00	0.00	1.59	1.13	1.68	3.50	2.07	1.27	1.04	1.37	1.02
31	Nerol oxide	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.59	0.44	0.65	0.52	0.61	1.10
32	Lilac aldehyde isomer	12.75	14.47	10.32	0.00	0.00	0.00	9.30	13.35	12.55	5.39	5.82	9.75	23.56	20.02	17.98	2.51	1.51	2.75
33	Lilac aldehyde isomer	0.00	0.00	0.00	1.50	2.50	0.00	0.00	0.51	0.42	4.28	4.59	2.68	3.89	4.24	4.12	5.37	5.19	4.44
34	Linalool oxide (pyranoid)	480.89	463.26	400.83	401.70	404.48	417.00	513.83	542.67	582.31	561.41	579.52	576.99	492.65	447.33	504.19	510.86	531.29	517.42
35	4-Terpineol	8.14	6.42	8.60	1.70	2.01	1.63	15.00	10.41	10.74	2.64	2.30	3.02	5.37	4.12	6.25	3.92	3.29	2.84
36	Myrtenol	0.00	0.00	0.00	0.00	0.00	0.00	9.65	5.95	7.58	5.09	5.34	7.59	8.87	5.49	9.44	4.84	2.94	3.54
37	*α*-Terpineol	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.09	0.11	0.12	0.10	0.85	0.11
38	Verbenone	0.18	0.00	0.43	0.21	0.00	0.00	0.53	0.30	0.35	0.00	0.30	0.15	0.00	0.00	0.00	0.00	0.00	0.00
39	Lilac alcohol	0.40	0.41	0.24	0.00	0.00	0.00	0.25	0.00	0.30	2.53	3.48	0.00	0.00	0.00	0.00	0.00	0.00	0.00
40	Nerol	18.02	20.71	27.41	1.03	1.81	5.44	15.11	18.33	19.61	19.65	18.33	12.18	14.06	19.81	19.81	12.84	12.49	12.94
41	Citral[e]	8.61	4.34	4.28	2.57	3.71	3.44	10.87	18.75	17.72	3.04	3.32	3.45	19.08	13.00	19.85	3.03	2.83	2.09
42	Geraniol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.73	3.20	4.29	5.36	4.22	6.45
43	Methyl citronellate	1.01	1.18	1.09	0.99	4.08	4.57	8.82	7.92	8.83	3.34	4.17	5.26	0.00	0.00	0.00	0.00	0.00	0.00
44	Citronellyl formate	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.20	0.12	0.00	0.00	0.00
45	Geranial	13.84	22.97	13.10	16.61	14.73	15.27	15.96	14.13	13.75	29.56	16.97	12.14	15.01	11.62	15.71	15.65	10.19	14.82
46	Methyl geranate	9.04	10.23	11.29	76.63	78.43	87.38	8.39	7.62	9.47	68.61	66.43	75.83	12.24	1.82	11.00	85.31	84.52	83.16
47	*m/z* 81,67,43,153	0.21	0.21	0.21	4.24	9.79	4.97	0.21	0.13	0.26	3.63	6.34	5.05	2.26	2.83	3.59	3.33	4.99	4.91
	Subtotal (GC Peak Area)		**1113.2**			**924.1**			**1114.1**			**1299.7**			**958.7**			**1162.2**	
	Sesquiterpenic Compounds																		
48	*α*-Cubebene	0.99	0.00	1.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.99	0.56	0.54	0.22	0.32	0.22
49	*α*-Copaene	5.70	6.76	8.66	0.59	0.94	0.97	8.39	6.02	6.94	3.84	1.01	0.65	1.98	1.70	0.00	1.24	0.96	0.63
50	*β*-Bourbonene	0.00	0.00	0.00	0.73	1.23	0.94	0.00	0.00	0.00	0.00	0.67	3.43	1.45	2.81	0.00	0.92	0.95	6.29
51	*β*-Elemene	1.28	0.00	1.47	0.91	2.40	0.76	1.32	0.00	1.07	0.99	0.00	0.69	0.00	0.00	0.00	0.00	0.00	0.00
52	*α*-Bergamotene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
53	*β*-Caryophyllene	11.27	10.43	15.31	18.95	13.55	10.94	13.59	19.33	17.97	11.89	10.57	18.86	14.08	15.57	18.25	8.78	9.39	8.80
54	Aromadendrene	0.00	0.00	9.07	0.40	1.89	0.52	0.00	0.00	1.58	0.00	0.00	0.00	8.65	4.97	8.43	0.00	0.45	2.28
55	*α*-Humulene	9.43	8.61	1.71	1.05	0.17	2.57	9.75	5.57	8.85	0.99	0.58	0.00	0.00	0.00	0.00	0.00	0.00	0.00
56	Germacrene D	1.24	1.34	1.25	5.36	4.72	5.10	9.24	5.61	0.00	2.00	2.09	0.00	8.49	0.00	9.73	1.56	0.00	3.46

57	*α*-Farnesene	0.73	0.73	0.57	21.38	20.29	26.93	7.19	3.67	8.21	20.67	23.39	26.55	2.99	4.23	3.42	10.89	18.48	19.26
58	Δ-Cadinene	2.54	1.26	2.83	0.37	0.66	1.25	3.70	1.27	1.66	1.30	0.93	2.17	0.00	0.00	0.99	0.24	0.52	0.24
59	Calamenene	0.22	0.20	0.27	0.10	0.10	0.13	0.23	0.13	0.21	0.09	0.09	0.19	0.18	0.13	0.17	0.11	0.12	0.11
60	*m/z* 69, 41, 55	0.75	0.67	0.81	0.00	1.62	1.25	1.07	0.44	0.00	0.26	0.33	0.37	0.99	0.93	0.89	1.11	1.07	0.61
	Subtotal (GC Peak Area)		**35.8**			**49.6**			**47.7**			**44.9**			**37.7**			**33.1**	
	Norisoprenoids																		
61	Safranal	0.49	0.69	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.07	1.09	1.36	1.35	0.00	0.49
62	*β*-Cyclocitral	0.41	0.93	0.54	0.15	0.24	0.16	0.53	0.27	0.44	0.00	0.34	0.33	0.30	0.26	0.44	0.33	0.34	0.36
63	Dihydroedulan	4.82	3.17	4.73	1.12	1.55	1.45	3.19	2.15	1.75	0.37	0.96	1.91	0.00	0.00	0.00	0.00	0.00	0.00
64	Geranyl acetone	4.33	4.50	4.53	1.73	2.18	3.40	4.92	3.00	2.13	1.36	1.58	2.49	6.27	5.57	5.42	2.02	2.23	2.08
65	Phytone	2.41	2.38	1.25	2.25	1.46	1.59	1.82	1.70	1.47	3.69	3.51	3.45	1.63	2.49	3.75	2.39	2.23	2.59
	Subtotal (GC Peak Area)		**11.7**			**5.8**			**7.8**			**6.7**			**9.9**			**5.5**	
	Total		**1290.1**			**1068.5**			**1340.6**			**1470.8**			**1185.2**			**1321.9**	

Table A2.1 (continuation) Full data set including the volatile terpenic and norisoprenoid compounds identified by HS-SPME/GC×GC-ToFMS in two *S. nigra* cultivars under study: 'Sabugueiro' (SO) and 'Sabugueira' (SA) at different handling and storage conditions. Chromatographic area expressed as arbitrary units. F: fresh, AD: air drying, FD: freeze-drying; Fr: freezing; VP_L: vacuum packing with light exposure; VP_NL: vacuum packing without light exposure.

Vacuum packing - Light exposure

Peak area (x10^6) (expressed as arbitrary units)

Peak Nº	Compound	1 week						2 weeks						1 month						2 months						6 months						1 year					
		SO_VP_L1	SO_VP_L2	SO_VP_L3	SA_VP_L1	SA_VP_L2	SA_VP_L3	SO_VP_L1	SO_VP_L2	SO_VP_L3	SA_VP_L1	SA_VP_L2	SA_VP_L3	SO_VP_L1	SO_VP_L2	SO_VP_L3	SA_VP_L1	SA_VP_L2	SA_VP_L3	SO_VP_L1	SO_VP_L2	SO_VP_L3	SA_VP_L1	SA_VP_L2	SA_VP_L3	SO_VP_L1	SO_VP_L2	SO_VP_L3	SA_VP_L1	SA_VP_L2	SA_VP_L3	SO_VP_L1	SO_VP_L2	SO_VP_L3	SA_VP_L1	SA_VP_L2	SA_VP_L3
Monoterpenic Compounds																																					
Hydrocarbon-type																																					
1	*α*-Pinene	0.58	0.83	0.91	11.81	12.34	21.74	1.55	1.70	1.24	11.81	16.43	14.90	2.21	1.90	1.24	7.40	5.41	4.79	2.14	3.42	4.35	2.43	3.56	3.95	1.13	3.67	1.56	0.00	1.66	16.70	6.35	1.24	1.81	0.00	0.00	0.00
2	*β*-Pinene	1.13	0.00	0.94	0.35	0.80	1.33	2.50	2.42	2.37	0.22	0.26	24.35	0.58	0.60	0.55	1.48	1.01	0.59	0.72	1.04	1.21	1.63	1.17	1.33	4.36	3.44	6.30	2.89	2.72	2.03	0.00	0.00	0.00	2.91	2.20	0.44
3	Myrcene	8.11	7.56	5.64	2.01	1.75	2.76	39.95	23.97	30.91	3.77	4.14	3.60	6.88	8.88	6.48	0.96	2.76	1.30	6.72	11.61	10.52	4.69	3.92	11.72	4.16	6.53	4.70	0.00	1.88	3.41	14.64	12.85	15.72	0.00	0.00	0.00
4	3-Carene	1.09	0.00	0.25	30.36	29.83	37.45	3.31	3.16	3.37	28.61	31.06	26.94	1.37	1.30	1.21	1.73	1.25	0.99	0.89	1.02	2.83	0.00	0.90	1.03	0.00	0.00	0.00	0.00	0.00	0.00	0.39	0.00	0.00	0.00	0.00	0.28
5	Limonene	18.08	18.26	18.93	14.21	10.68	17.12	11.89	13.80	13.78	39.49	38.51	31.79	48.70	50.27	53.24	56.09	37.90	40.57	34.17	44.32	37.66	21.06	28.41	34.97	96.40	65.29	97.98	20.83	29.34	35.99	41.12	43.81	46.72	14.95	9.34	9.14
6	*p*-Cymene	0.00	1.55	0.00	0.40	0.00	0.54	5.47	5.15	4.54	1.87	4.85	1.05	4.24	3.03	4.95	14.38	16.54	10.06	1.94	1.19	3.88	19.94	11.57	13.08	24.14	19.91	14.41	15.19	19.80	28.31	13.03	11.76	17.87	39.60	32.80	20.72
7	Ocimene	9.76	12.03	8.63	8.43	5.99	9.69	34.14	10.74	78.67	8.94	8.65	6.90	6.41	6.96	0.42	0.78	1.02	0.53	3.51	2.19	5.76	0.00	2.62	6.22	0.00	1.20	0.00	0.00	0.00	0.00	1.53	0.75	0.41	4.55	2.09	0.35
8	*γ*-Terpinene	0.00	0.00	0.00	0.00	0.00	1.01	0.00	0.00	0.00	0.00	0.34	0.00	0.00	0.70	0.00	0.00	0.68	0.00	0.70	0.48	0.93	0.53	0.75	1.02	2.01	0.96	1.94	6.88	15.25	4.55	10.60	13.60	14.72	0.00	0.00	0.00
9	2,6-Dimethyl-2,6-octadiene	10.51	3.39	4.40	2.76	0.00	0.00	4.74	0.00	3.66	3.17	3.70	3.07	3.49	3.22	3.36	1.23	3.28	3.22	3.94	0.05	2.07	1.59	1.04	1.89	2.20	1.47	1.15	60.71	25.29	6.68	3.56	3.36	4.47	40.52	78.23	30.01
10	*α*-Terpinolene	0.91	0.68	0.77	0.00	0.19	0.00	12.65	14.39	14.57	12.32	15.03	13.03	0.97	1.09	0.97	0.47	1.08	0.64	0.94	1.52	1.26	0.71	0.99	1.43	1.40	1.48	1.77	0.00	1.16	0.69	0.74	0.00	0.00	0.00	0.00	0.00
11	*p*-Cymenene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.56	1.08	1.44	1.99	0.00	0.00	0.00	0.00	0.49	0.29	0.80	0.79	0.30	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.12	0.12	0.00	0.00	0.00
12	Cosmene isomer	0.00	2.85	68.28	0.00	0.00	0.00	0.55	0.41	0.56	0.00	0.00	0.00	2.54	0.00	6.54	0.00	0.00	0.00	0.00	37.71	4.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	40.24	22.50	17.90	0.00	0.00	0.00
13	Cosmene isomer	0.00	2.85	68.28	0.00	0.00	0.00	0.55	1.41	0.56	0.00	0.00	0.00	2.54	0.00	4.47	0.00	1.53	0.00	0.00	37.71	1.29	0.00	0.00	11.99	1.51	0.96	1.23	0.00	0.80	0.00	0.00	0.00	0.29	3.85	3.19	3.79
14	*m/z* 43,94,55,68	3.79	4.83	5.07	0.00	0.00	1.19	3.76	4.31	3.72	0.00	0.00	2.16	17.82	17.34	10.23	1.09	1.60	1.52	3.00	3.37	3.51	7.08	6.30	10.14	0.00	0.00	0.00	2.75	3.44	3.18	0.00	0.00	0.00	0.00	0.00	0.00
	Subtotal (GC Peak Area)		**97.0**			**74.9**			**122.7**			**121.8**			**95.6**			**74.9**			**95.3**			**73.3**			**124.4**			**104.0**			**120.8**			**99.7**	
Oxygen containing																																					
15	Dehydroxylinalool oxide	0.00	0.00	0.00	2.05	2.32	3.26	0.00	0.00	0.00	0.00	0.00	0.00	1.27	1.11	0.00	0.00	0.00	3.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.26	10.33	0.00	4.34	0.86	1.45	1.60	1.49	1.40	1.99	1.49
16	1,8-Cineole	3.59	3.03	8.82	0.00	0.00	0.00	28.28	96.67	79.05	2.77	2.32	2.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.07	4.59	0.00	7.25	69.89	54.90	0.00	0.00	0.00	2.02	2.55	2.40	0.47	0.99	0.83
17	Myrcenol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.36	0.00	0.00	1.23	0.00	0.00	1.22	0.00	0.00	3.47	0.00	4.86	0.24	0.00	0.00	2.53
18	Linalool oxide (furanoid) isomer[e]	257.51	316.10	364.82	484.71	425.20	468.06	295.67	292.60	264.27	242.50	217.42	175.03	199.66	340.81	186.59	310.64	245.06	114.97	259.84	246.71	263.60	51.50	77.03	139.79	12.84	17.76	13.87	117.56	193.70	130.78	32.68	25.79	29.85	139.58	186.52	111.69
19	Fenchone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.18	0.00	0.00	2.99	2.95	9.43	12.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.69	5.90	11.60	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.52	0.31	0.30	0.21	0.33
20	Hotrienol isomer	2.50	4.49	4.37	0.62	0.30	0.71	3.92	3.57	4.24	0.00	0.00	0.00	0.90	1.19	0.91	0.41	0.34	0.00	4.52	4.80	5.57	4.77	3.97	6.64	5.54	8.47	6.53	4.37	5.12	6.87	3.83	8.56	7.43	0.00	0.00	0.00
21	Linalool oxide (furanoid) isomer[e]	55.16	57.79	76.28	58.89	59.64	53.07	40.94	51.29	53.43	42.36	18.82	0.00	63.54	54.88	77.78	37.38	80.03	91.74	79.39	87.41	78.27	60.36	82.29	105.19	73.69	92.63	100.65	84.82	89.14	89.80	1.26	13.08	16.88	28.87	11.32	17.46

22	Linalool	32.05	0.00	0.00	3.54	3.05	1.15	0.00	0.00	0.00	0.00	0.00	0.00	14.88	11.79	19.52	7.09	0.00	0.00	81.49	94.82	38.75	12.59	12.82	12.82	2.80	20.60	14.09	10.06	15.49	18.07	17.02	14.22	16.87	0.00	0.00	0.00
23	Thujone	14.66	15.12	17.44	43.28	47.24	42.85	10.31	9.73	10.26	22.04	20.34	20.84	17.12	12.54	18.39	16.14	24.09	21.78	14.17	13.82	15.07	18.06	16.38	23.65	0.00	0.00	0.00	1.16	3.56	0.00	4.35	4.23	4.08	1.39	1.99	2.11
24	Hotrienol isomer	30.71	41.51	49.30	15.95	26.65	33.11	25.25	29.90	27.66	10.40	17.51	14.72	49.75	65.19	54.42	16.78	22.87	19.32	38.19	43.85	46.35	43.81	53.43	86.33	17.77	15.92	16.43	43.44	48.97	51.33	0.00	0.00	0.00	19.51	19.84	22.75
25	Rose oxide[e]	95.98	90.23	81.45	66.14	43.98	66.61	71.45	65.52	71.06	33.78	32.73	47.96	119.35	119.10	117.40	86.10	63.79	74.70	110.56	113.06	129.33	17.14	21.24	23.55	6.51	8.81	30.63	10.42	13.43	16.31	47.46	18.52	23.95	14.96	15.09	18.39
26	Linalool, methyl ether	1.06	0.00	1.49	2.71	1.40	2.76	0.95	0.00	0.00	1.40	0.00	0.00	1.45	0.00	1.40	1.05	0.00	1.91	1.03	1.12	1.48	0.97	1.14	1.50	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.30	2.53	0.22	0.17	0.14
27	Limonene oxide[f]	0.00	0.77	0.00	0.00	0.00	0.00	0.00	0.42	0.70	0.00	0.00	0.00	0.00	1.68	93.10	0.48	0.68	0.41	0.42	0.71	0.00	0.90	0.00	1.94	0.00	5.26	51.07	0.32	0.00	0.00	2.29	0.00	0.00	2.25	2.75	3.02
28	Tagetone	0.00	0.74	1.29	0.00	0.00	0.00	0.69	0.62	0.74	0.00	0.00	0.00	1.28	1.15	0.84	0.15	0.00	0.00	0.68	1.27	0.95	0.82	0.74	1.34	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.23	0.29	0.59	0.87	0.56
29	Citronellal	0.00	26.94	0.00	1.54	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.11	0.00	0.00	0.00	0.00	72.49	4.53	5.69	0.00	0.00	0.00	10.46	0.72	21.64	20.22	21.49	7.40	4.12	1.80	7.07	7.42	7.28	3.46	4.19	5.26
30	Lilac aldehyde isomer	0.44	0.00	0.00	1.06	1.57	1.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	41.84	31.76	52.47	5.37	3.51	15.34	85.02	106.00	90.05	6.78	7.75	6.80
31	Nerol oxide	13.82	5.38	5.24	0.00	0.00	0.00	11.06	8.05	10.46	11.64	13.25	11.12	21.35	21.34	27.84	8.23	0.00	17.88	10.58	14.31	17.82	45.00	41.72	62.32	10.22	10.75	10.49	15.53	24.00	27.11	13.41	12.46	13.89	19.24	15.75	19.90
32	Lilac aldehyde isomer	0.00	0.00	0.00	9.41	0.00	4.51	0.00	11.26	9.27	1.75	1.79	3.21	77.06	0.23	0.00	0.00	9.52	0.00	6.61	21.01	25.35	0.00	0.00	1.25	0.00	10.93	6.60	0.00	6.14	1.25	0.00	0.00	0.00	0.00	0.00	0.00
33	Lilac aldehyde isomer	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.46	0.00	10.63	14.96	16.15	1.64	1.73	2.76	22.31	24.21	17.13	2.91	2.15	1.65
34	Linalool oxide (pyranoid)	165.17	252.53	172.55	314.63	360.46	352.53	108.92	194.20	184.05	279.05	182.14	142.36	149.13	162.60	210.44	269.09	375.14	223.54	239.42	250.43	234.83	262.46	229.78	202.55	181.29	216.88	156.75	108.11	105.23	105.44	158.35	272.31	211.46	84.60	57.07	69.66
35	4-Terpineol	2.82	1.70	3.18	0.95	0.00	0.00	0.00	0.00	9.65	1.30	1.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.49	0.00	0.32	0.97	26.28	0.00	0.00	0.67	63.63	62.39	66.60	0.58	0.13	0.33
36	Myrtenol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	45.21	38.80	40.00	22.22	4.21	20.57	78.87	73.15	79.32	52.81	55.91	20.53	1.67	1.83	2.30	0.90	0.83	0.54
37	α-Terpineol	0.74	0.59	0.71	0.00	0.22	0.35	63.34	64.63	77.71	74.63	16.72	63.84	1.26	1.74	1.37	0.78	0.59	0.50	1.24	1.47	0.00	2.70	2.92	4.11	12.50	14.15	79.04	1.48	0.00	9.11	37.84	39.53	40.65	3.96	7.70	3.25
38	Verbenone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.51	0.41	0.94	3.88	3.52	2.71	0.81	0.69	0.20	1.74	1.71	1.73	0.19	0.12	0.11
39	Lilac alcohol	2.32	3.26	3.67	1.57	1.82	2.23	0.00	0.00	0.00	0.00	0.00	2.41	1.14	6.10	3.04	0.00	1.94	1.74	4.01	2.92	3.24	2.85	1.94	2.75	3.47	0.00	3.96	5.80	2.32	12.46	0.00	6.89	0.00	0.00	0.00	0.00
40	Nerol	47.28	31.97	25.29	37.57	0.00	1.31	26.63	26.21	27.50	5.60	5.09	5.63	115.06	131.47	146.55	11.60	22.54	0.00	185.41	181.25	168.35	7.08	57.87	8.18	26.33	29.98	24.93	0.91	7.68	22.88	3.77	2.42	4.61	3.95	3.20	2.27
41	Citral[f]	55.78	38.75	43.87	18.35	12.27	29.82	74.70	20.50	37.63	22.25	33.42	14.01	13.72	19.60	16.99	2.80	5.67	21.81	5.14	12.39	7.60	3.69	7.72	6.33	22.63	23.46	21.30	3.89	3.13	2.24	7.80	7.48	6.01	10.35	14.95	13.86
42	Geraniol	2.25	2.55	2.85	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.09	3.10	3.32	0.00	0.00	0.00	0.51	0.48	0.41	0.18	0.00	0.00	6.04	6.73	6.06	1.19	1.26	1.09	3.04	2.44	2.72	10.46	10.69	8.88
43	Methyl citronellate	1.16	0.00	0.00	0.74	0.00	2.10	1.40	0.00	10.27	0.00	0.00	0.00	14.95	5.42	1.04	2.71	1.33	0.32	6.22	7.65	7.22	0.00	0.00	4.20	5.16	4.61	3.81	2.67	0.00	6.20	1.35	0.92	1.29	0.00	0.00	0.00
44	Citronellyl formate	1.13	1.44	0.85	0.58	0.00	0.00	1.53	0.00	1.25	0.00	0.00	0.00	0.00	0.00	0.00	0.55	0.00	0.88	1.86	2.02	1.43	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.39	0.36	0.46	0.95	0.91	0.89
45	Geranial	97.45	47.23	13.29	24.02	33.02	65.30	36.27	28.91	53.54	20.39	36.21	23.07	28.00	30.10	28.55	0.00	22.56	12.35	8.93	21.20	35.03	4.20	0.00	9.11	39.53	16.63	19.61	5.97	3.38	0.00	11.18	19.60	11.10	0.00	0.00	0.00
46	Methyl geranate	35.74	42.38	43.38	27.28	16.01	34.40	26.69	19.49	34.28	29.43	18.36	14.71	38.70	43.32	43.87	24.62	30.32	37.80	35.86	41.75	41.89	21.79	20.94	32.34	11.86	11.34	11.79	1.12	2.37	2.62	28.16	28.63	29.68	2.95	2.66	2.11
47	*m/z* 81, 67, 43, 153	1.25	1.71	1.75	2.48	1.43	13.66	0.98	1.14	1.03	2.99	2.73	2.75	0.87	1.68	1.25	2.24	0.00	3.11	1.44	1.22	1.32	2.46	2.04	3.78	1.78	3.47	1.20	2.26	3.01	2.55	2.81	2.78	5.10	5.96	4.98	5.68
	Subtotal (GC Peak Area)		**1254.6**			**1111.3**			**1150.8**			**706.8**			**1149.0**			**838.0**			**1172.8**			**841.5**			**837.1**			**677.0**			**769.4**			**527.5**	
Sesquiterpenic Compounds																																					
48	α-Cubebene	0.47	0.91	0.44	0.00	0.00	0.20	1.06	0.63	0.91	0.45	0.76	0.77	1.57	0.96	0.00	0.00	0.00	0.00	0.28	0.44	0.51	0.16	0.19	0.62	0.00	3.22	0.87	0.00	0.00	0.00	0.00	0.68	0.00	0.00	0.00	0.00
49	α-Copaene	3.08	2.56	3.76	1.24	1.12	1.48	0.00	9.41	0.00	2.95	1.17	1.57	4.59	9.10	3.90	0.29	0.00	0.00	2.58	3.43	2.82	0.32	0.63	0.83	2.11	1.52	1.10	0.88	0.00	1.14	2.93	0.00	3.14	0.00	0.00	0.00
50	β-Bourbonene	1.22	1.06	0.00	0.58	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.14	1.31	1.66	1.16	0.00	0.96	0.00	0.00	0.00	0.00	0.00	0.00	2.07	2.45	2.02	0.00	0.64	0.00
51	β-Elemene	0.55	0.72	1.05	0.00	0.00	0.00	0.00	0.72	0.00	1.10	0.00	0.00	0.40	0.36	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.01	6.91	6.06	0.00	0.00	0.00	1.09	1.18	1.09	3.94	4.56	3.22
52	α-Bergamotene	0.34	0.71	1.05	0.00	0.00	0.00	1.11	1.46	1.85	0.00	0.00	0.00	1.02	1.93	1.52	0.00	0.00	0.00	0.68	0.00	0.00	1.94	2.46	4.66	4.69	3.29	1.55	1.30	2.13	1.25	3.46	2.46	3.14	5.97	1.48	6.16
53	β-Caryophyllene	20.86	20.39	16.25	16.56	12.31	19.30	34.57	32.25	38.24	20.65	26.01	24.92	33.71	28.83	24.93	12.75	13.48	14.31	24.17	29.28	25.86	15.91	17.11	17.73	39.78	37.93	23.66	14.38	16.68	17.55	35.10	35.66	31.82	13.95	14.02	17.52

54	Aromadendrene	0.00	0.82	0.00	0.35	0.00	0.00	0.00	0.66	0.00	0.00	0.00	0.00	0.00	0.00	7.88	0.00	0.46	0.00	0.00	0.00	0.00	1.46	0.00	0.00	3.08	6.68	8.18	0.00	0.73	3.72	0.00	0.00	0.00	0.00	0.00	0.00
55	α-Humulene	9.14	5.82	7.52	9.97	9.41	6.24	46.03	18.74	12.09	4.43	4.96	4.50	5.50	0.00	0.00	1.49	1.80	0.00	7.30	9.07	0.00	0.51	3.33	0.00	0.00	3.11	2.63	0.00	0.00	1.48	0.00	0.00	0.00	0.00	0.00	0.00
56	Germacrene D	0.00	0.00	0.00	0.00	0.00	1.42	0.00	4.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.55	0.00	0.00	0.22	0.00	0.28	0.00	0.13	0.00	0.00	0.00	0.13	0.29	0.23	0.38	1.94	1.29	2.11
57	α-Farnesene	13.51	14.37	12.76	5.89	0.00	8.75	0.31	0.98	0.00	0.00	0.00	0.00	28.06	0.00	8.54	1.43	2.62	3.42	6.81	11.90	11.90	3.83	4.93	7.07	3.04	2.98	2.07	0.94	1.74	1.04	4.53	2.26	4.44	0.00	0.00	0.00
58	Δ-Cadinene	0.81	0.31	0.00	0.00	0.00	0.00	0.00	0.00	1.86	3.16	2.15	3.21	0.00	0.00	0.37	0.00	0.18	0.00	0.00	0.47	0.45	0.28	0.28	0.58	0.18	0.16	2.80	0.00	0.00	0.00	0.00	0.00	0.21	0.00	0.00	0.00
59	Calamenene	0.24	0.18	0.14	0.15	0.07	0.12	0.00	0.00	0.00	0.39	0.39	0.76	0.17	0.18	0.14	0.13	0.12	0.11	0.08	0.11	0.13	0.05	0.08	0.13	0.06	0.09	0.00	0.00	0.00	0.09	0.17	0.19	0.18	0.10	0.08	0.14
60	*m/z* 69,41,55	0.62	0.53	3.11	0.58	0.46	1.50	0.51	0.50	0.66	0.26	0.26	0.32	0.40	0.00	0.00	0.00	0.21	1.11	0.00	0.49	0.45	0.37	0.00	0.39	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Subtotal (GC Peak Area)		**48.4**			**32.6**			**69.5**			**35.0**			**54.8**			**18.0**			**48.0**			**29.5**			**58.6**			**21.7**			**47.1**			**25.7**	
	Norisoprenoids																																				
61	Safranal	0.00	0.00	0.00	0.00	0.00	0.00	4.91	0.00	0.00	5.33	8.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.76	0.48	0.91	0.78	1.45	3.04	3.25	3.06	2.00	0.00	0.00	5.01	7.48	7.89	0.00	1.51	2.13
62	β-Cyclocitral	0.00	2.01	0.64	0.00	0.45	0.80	1.89	2.08	2.92	2.13	3.04	1.99	0.97	4.14	1.80	5.38	5.81	7.23	0.93	1.14	0.97	1.60	1.69	2.24	0.00	1.34	2.58	0.72	1.26	1.27	1.33	1.43	1.14	2.20	1.94	2.10
63	Dihydroedulan	32.09	51.74	29.58	3.19	3.31	5.38	28.99	29.30	42.68	2.19	10.21	6.10	40.10	49.52	46.72	14.40	10.49	11.77	54.10	48.29	33.33	13.69	18.64	51.03	42.40	42.83	41.89	25.94	29.82	36.91	26.33	24.89	25.35	19.46	13.40	17.88
64	Geranylacetone	3.99	2.70	0.00	4.10	1.83	3.74	8.71	5.07	11.37	5.79	4.43	3.77	5.38	0.00	6.46	2.21	2.79	3.38	2.23	4.37	4.07	1.51	1.99	1.87	4.22	3.83	5.37	1.66	2.86	5.13	4.22	3.94	4.65	3.59	4.22	3.99
65	Phytone	0.90	1.10	1.31	0.44	0.24	0.55	0.52	0.59	0.54	0.75	0.62	0.97	0.69	0.90	0.85	0.94	0.98	0.90	0.89	0.62	0.73	0.49	0.66	0.61	1.47	0.97	0.96	0.87	1.49	1.08	0.00	0.00	0.00	0.00	0.00	0.00
	Subtotal (GC Peak Area)		**42.0**			**8.0**			**46.5**			**18.7**			**52.5**			**22.1**			**51.1**			**33.1**			**52.4**			**37.0**			**37.9**			**24.1**	
	Total		**1442.0**			**1226.8**			**1389.5**			**882.4**			**1351.9**			**952.9**			**1367.1**			**977.4**			**1072.5**			**839.8**			**975.1**			**677.0**	

Table A2.1 (continuation) Full data set including the volatile terpenic and norisoprenoid compounds identified by HS-SPME/GC×GC-ToFMS in two *S. nigra* cultivars under study: 'Sabugueiro' (SO) and 'Sabugueira' (SA) at different handling and storage conditions. Chromatographic area expressed as arbitrary units. F: fresh, AD: air drying, FD: freeze-drying; Fr: freezing; VP_L: vacuum packing with light exposure; VP_NL: vacuum packing without light exposure.

Peak Nº	Compound	Vacuum packing - Without light exposure, Peak area (x10^6) (expressed as arbitrary units): 1 week						2 weeks						1 month						2 months						6 months						1 year					
		SOVP_NL 1	SOVP_NL 2	SOVP_NL 3	SAVP_NL 1	SAVP_NL 2	SAVP_NL 3	SOVP_NL 1	SOVP_NL 2	SOVP_NL 3	SAVP_NL 1	SAVP_NL 2	SAVP_NL 3	SOVP_NL 1	SOVP_NL 2	SOVP_NL 3	SAVP_NL 1	SAVP_NL 2	SAVP_NL 3	SOVP_NL 1	SOVP_NL 2	SOVP_NL 3	SAVP_NL 1	SAVP_NL 2	SAVP_NL 3	SOVP_NL 1	SOVP_NL 2	SOVP_NL 3	SAVP_NL 1	SAVP_NL 2	SAVP_NL 3	SOVP_NL 1	SOVP_NL 2	SOVP_NL 3	SAVP_NL 1	SAVP_NL 2	SAVP_NL 3
Monoterpenic Compounds																																					
Hydrocarbon-type																																					
1	*α*-Pinene																																				
2	*β*-Pinene	0.23	0.26	0.62	5.70	8.39	6.44	5.30	4.66	5.95	15.16	13.08	10.37	1.83	2.35	2.94	3.97	3.10	3.56	3.27	2.75	5.10	1.91	3.56	3.15	0.00	0.68	1.70	0.86	2.55	1.23	2.99	3.09	7.59	17.46	25.33	12.74
3	Myrcene	0.63	0.91	0.91	1.77	2.07	1.48	0.28	0.21	0.21	2.56	2.02	2.43	0.65	0.36	0.78	0.89	0.72	0.73	1.18	1.13	1.42	0.96	1.23	1.39	0.00	0.00	8.19	0.00	0.00	0.00	2.82	4.34	3.24	0.11	0.86	0.24
4	3-Carene	7.30	6.30	8.22	2.22	1.69	1.79	3.29	3.67	2.95	3.70	2.80	2.95	5.70	4.44	6.75	3.30	2.97	2.73	6.15	6.62	6.17	4.12	4.82	4.43	6.34	6.19	7.13	1.23	2.11	1.88	7.44	6.06	10.11	6.69	2.23	5.50
5	Limonene	0.22	1.17	0.57	0.64	0.28	1.20	3.17	2.99	2.09	3.16	2.08	2.85	0.98	1.22	1.24	0.00	0.00	0.00	0.91	0.79	1.42	1.19	0.52	0.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.98	0.00	1.75	1.46	1.65
6	*p*-Cymene	46.73	51.36	39.70	39.03	25.10	22.53	35.27	41.69	49.29	57.27	53.87	52.21	35.54	35.71	49.66	34.98	33.91	15.42	57.34	48.46	50.01	25.40	31.79	30.89	63.93	31.47	37.11	77.78	23.99	84.58	50.34	43.43	74.88	23.86	28.10	24.75
7	Ocimene	0.00	0.27	0.00	0.00	0.00	0.00	0.72	0.74	0.78	0.00	0.00	0.00	0.00	0.83	0.00	0.00	0.00	0.00	2.90	5.07	0.00	0.00	0.00	0.00	11.60	16.44	49.01	2.77	1.05	8.49	21.80	17.95	11.23	7.08	6.41	7.23
8	*γ*-Terpinene	33.70	22.75	21.49	0.00	3.17	7.83	47.05	38.48	35.80	2.99	2.38	2.79	24.47	17.97	17.84	4.60	4.88	6.39	37.00	18.02	26.37	2.80	3.43	0.00	0.00	0.49	0.00	0.82	0.12	0.00	2.20	1.05	0.73	1.28	1.08	1.09
9	2,6-Dimethyl-2,6-octadiene	0.00	0.00	0.00	0.00	1.73	0.68	32.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.86	0.92	0.92	0.00	1.05	0.00	0.42	0.80	0.00	26.06	0.00	13.05	0.00	0.27	0.98	0.28	0.00	0.12	0.18	0.16	0.39
10	*α*-Terpinolene	5.13	4.96	4.65	4.14	4.00	2.60	3.49	4.46	4.71	5.52	3.09	4.17	5.71	3.94	3.92	0.75	1.82	1.12	2.92	1.38	2.81	2.51	2.89	2.62	4.33	4.47	2.11	1.21	1.08	1.06	5.15	3.91	5.56	2.33	2.82	2.60
11	*p*-Cymenene	0.66	0.77	1.33	0.00	0.00	0.30	1.47	1.23	1.07	0.13	0.11	0.14	0.92	1.18	0.85	0.00	0.47	0.50	1.64	1.22	0.00	0.73	0.21	0.00	1.54	1.45	1.57	0.00	1.20	1.06	3.75	2.17	2.72	1.42	1.48	8.17
12	Cosmene isomer	0.51	0.00	1.08	0.00	0.00	0.00	0.00	0.74	3.34	0.11	0.11	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.09	0.06	9.93	7.29	8.84
13	Cosmene isomer	10.92	11.07	4.10	0.00	0.00	0.00	4.84	6.21	2.27	0.00	0.00	0.00	0.00	0.00	1.52	0.00	0.00	0.00	0.00	0.00	0.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
14	*m/z* 43,94,55,68	4.19	4.07	4.10	1.59	0.00	0.00	4.84	4.07	4.66	6.53	4.28	2.90	0.00	0.00	1.52	0.00	0.00	0.00	0.00	0.00	0.55	0.00	0.87	1.29	1.68	0.00	0.00	0.00	0.00	0.00	0.28	0.32	0.00	0.35	0.47	0.87
Subtotal (GC Peak Area)		3.78	2.95	5.16	1.36	1.29	1.48	1.19	1.43	1.39	2.80	3.10	2.01	21.68	21.65	9.86	8.31	8.14	10.81	0.00	27.59	25.96	5.23	8.02	6.44	46.97	33.13	35.03	6.43	7.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oxygen containing			**104.3**			**50.2**			**122.7**			**89.9**			**94.7**			**52.3**			**115.9**			**51.5**			**137.2**			**76.7**			**98.9**			**74.7**	
15	Dehydroxylinalool oxide																																				
16	1,8-Cineole	3.16	6.94	2.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.18	1.03	0.00	0.00	0.00	2.34	0.00	0.00	1.12	0.00	0.00	0.00	0.40	0.00	6.75	3.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00
17	Myrcenol	0.00	82.14	0.00	22.90	0.00	0.00	3.57	3.86	1.32	70.83	190.58	66.00	12.07	10.67	0.00	0.00	0.00	34.76	0.00	0.00	0.00	4.57	5.79	0.00	0.00	0.00	4.08	0.00	0.00	0.00	1.39	1.51	1.59	1.38	1.75	0.00
18	Linalool oxide (furanoid) isomer[e]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	14.04	0.00	0.00	0.00	0.00	0.00	0.00	2.49	0.00	0.00	0.00	0.00	0.00	2.25	6.21	5.62	0.00	0.00	0.00
19	Fenchone	255.99	161.60	187.89	161.24	303.95	259.48	127.84	130.75	115.54	340.22	115.83	120.55	213.57	282.75	210.74	161.24	194.53	174.02	294.96	296.21	262.54	174.23	234.24	171.33	29.86	82.62	37.76	115.47	128.00	188.72	11.92	35.83	32.14	20.28	51.64	45.51
20	Hotrienol isomer	0.00	0.00	0.00	0.00	0.00	0.00	2.82	0.00	1.96	0.00	3.78	3.42	14.92	12.52	0.00	0.00	0.00	0.00	47.52	2.03	90.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.21	1.60	1.28	0.88	1.44	1.37

21	Linalool oxide (furanoid) isomer[c]	2.53	1.41	3.13	3.93	2.40	3.38	6.31	7.92	7.57	3.88	5.56	4.34	12.20	9.95	0.99	5.55	7.52	6.74	10.53	10.58	10.19	10.08	11.80	9.27	9.14	4.21	7.32	4.34	3.09	4.76	7.76	9.64	8.11	4.54	7.11	5.17
22	Linalool	32.48	55.22	59.59	24.876	59.65	52.30	73.42	74.26	85.63	55.50	54.28	47.73	95.77	64.32	64.80	89.81	95.39	122.02	71.25	71.25	74.29	124.76	144.02	124.43	89.86	7.31	78.57	73.21	46.27	95.95	12.01	17.04	11.45	149.76	153.99	153.96
23	Thujone	97.97	88.24	78.01	29.24	30.70	54.40	5.97	5.43	7.20	0.00	56.24	0.00	0.00	66.86	50.31	82.22	81.00	86.72	0.00	0.00	1.76	0.00	0.00	0.00	219.65	122.65	190.14	97.16	70.09	83.61	184.62	175.59	42.50	13.69	15.24	14.87
24	Hotrienol isomer	81.87	87.84	82.90	62.74	54.43	56.03	92.02	109.96	99.4	56.20	60.09	47.69	109.8	17.50	17.12	42.92	44.53	44.06	16.15	16.56	15.40	72.02	77.13	64.50	37.91	37.32	58.01	17.75	11.38	13.95	1.18	1.33	3.27	0.96	2.07	1.41
25	Rose oxide[e]	65.35	46.19	41.89	33.64	32.13	31.76	34.18	47.83	43.84	17.61	21.95	22.54	75.86	75.48	64.17	29.23	29.96	37.73	76.03	86.06	71.84	40.81	47.84	40.05	166.29	192.90	136.65	55.91	48.03	65.83	129.83	141.63	102.57	15.41	17.69	18.39
26	Linalool, methylether	86.23	39.43	80.77	53.28	44.72	58.32	13.97	10.53	15.36	43.33	42.56	38.82	40.24	10.22	12.21	101.49	100.36	94.18	17.72	16.12	16.90	88.19	96.58	85.74	29.52	32.91	20.07	106.69	101.45	117.72	18.43	21.57	11.82	18.42	15.34	20.60
27	Limonene oxide[e]	1.26	2.07	0.00	2.20	1.40	0.00	0.00	0.00	0.00	0.00	2.78	1.56	0.00	5.82	0.95	0.00	0.00	0.00	0.00	1.68	0.75	1.58	0.93	1.21	0.00	0.00	0.00	0.00	0.00	0.00	1.28	1.58	2.28	0.17	0.17	0.22
28	Tagetone	0.00	0.00	0.94	0.00	1.53	0.00	59.10	66.61	11.85	0.00	0.00	2.56	28.63	27.13	1.97	0.00	0.00	0.00	25.83	20.12	21.34	1.06	1.64	0.00	0.00	0.00	0.00	0.00	2.53	0.00	2.29	0.00	2.38	1.76	3.29	4.12
29	Citronellal	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	3.14	4.01	2.87	0.32	0.48	0.34	4.98	0.00	0.00	3.26	0.00	4.65	0.03	0.03	0.03	1.10	1.83	1.59
30	Lilacaldehyde isomer	0.92	10.29	0.00	3.56	4.87	37.99	0.00	0.00	0.00	0.00	0.00	0.00	11.38	0.00	0.00	7.36	14.28	7.44	1.68	1.91	2.22	2.98	0.00	2.89	22.25	0.00	0.00	1.13	0.00	1.09	0.83	0.10	0.09	5.89	7.66	8.21
31	Nerol oxide	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.26	0.00	0.00	0.00	2.59	5.20	9.85	0.00	2.07	0.00	24.13	39.03	50.15	2.54	2.79	2.81	8.25	8.57	8.11	5.03	5.80	5.01
32	Lilacaldehyde isomer	0.00	4.28	7.78	0.00	0.00	0.00	3.87	4.18	4.89	0.00	0.00	0.00	5.58	59.38	26.84	17.60	15.95	12.53	85.26	81.17	81.81	11.74	4.92	11.00	12.99	5.69	65.79	30.42	69.93	46.97	111.52	85.52	88.39	59.77	75.05	72.31
33	Lilacaldehyde isomer	0.00	0.00	1.43	5.92	0.00	8.50	0.00	33.62	1.45	3.14	11.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.27	0.00	0.00	0.00	28.43	0.00	11.36	5.69	9.96	0.00	0.00	0.00	0.80	0.77	0.44	0.11	0.62	0.84
34	Linalool oxide (pyranoid)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.97	0.00	5.60	0.00	0.84	0.00	6.54	6.97	7.29	0.23	0.25	3.33	1.22	1.62	1.95
35	4-Terpineol	115.24	118.05	103.06	21.047	138.95	31.791	149.55	163.93	123.52	137.13	111.27	125.87	140.02	119.89	153.24	283.83	310.10	360.32	230.05	278.65	165.24	359.69	316.45	348.41	273.62	243.33	208.46	237.44	215.44	262.27	78.74	102.50	156.63	99.69	66.69	52.40
36	Myrtenol	0.00	7.98	0.00	0.41	1.48	0.97	0.00	0.00	0.00	0.00	0.00	0.77	0.00	0.00	0.00	12.88	0.00	0.00	1.11	0.95	2.38	0.00	0.00	0.00	0.00	0.00	0.69	0.00	0.00	0.00	46.78	43.70	42.06	3.47	2.16	0.67
37	α-Terpineol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	22.38	28.95	45.23	0.00	37.31	25.11	94.05	81.13	71.70	9.76	8.97	7.65	0.45	0.76	0.59	0.00	0.69	1.40
38	Verbenone	0.35	0.48	0.57	0.31	0.43	0.34	82.36	75.95	49.65	73.82	56.07	48.16	2.82	3.24	1.16	0.84	0.46	1.32	4.12	4.62	3.82	0.68	0.82	1.06	11.95	16.95	1.79	1.81	2.58	0.00	12.90	11.14	15.99	5.44	7.29	6.55
39	Lilacalcohol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.33	0.00	0.00	0.00	0.00	0.00	0.00	1.90	1.36	1.26	1.90	3.33	4.53	3.31	1.11	2.97	0.00
40	Nerol	3.19	2.51	2.82	2.49	4.98	0.00	6.47	0.00	3.04	6.97	5.13	2.78	1.71	1.74	1.99	2.55	3.44	0.41	1.79	2.47	0.00	5.40	9.11	14.24	9.96	81.06	9.41	6.54	0.00	7.63	3.76	4.42	7.49	6.44	5.05	9.61
41	Citral[e]	343.77	190.63	229.78	82.71	35.45	58.48	118.68	132.68	104.00	55.96	61.47	51.87	265.17	199.63	389.44	144.8	51.34	36.71	124.78	165.55	144.11	0.00	28.60	57.91	35.43	10.62	18.32	16.30	35.59	28.11	34.39	24.63	30.13	26.91	28.65	37.19
42	Geraniol	15.17	18.14	32.84	25.12	29.72	45.63	46.80	42.48	41.71	31.09	18.08	33.96	24.77	66.39	0.00	14.12	18.36	22.11	17.38	19.30	16.27	5.05	4.87	6.87	20.36	1.42	20.36	3.93	3.08	3.50	6.71	1.97	0.00	0.00	0.00	0.00
43	Methyl citronellate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.86	1.23	1.00	1.45	3.24	3.04	3.17	0.26	0.36	0.37	0.00	0.00	4.06	0.57	0.00	0.67	0.93	1.43	0.00	0.00	0.00	0.00
44	Citronellyl formate	0.00	0.00	1.62	0.00	0.00	0.98	5.21	43.18	7.31	0.66	0.98	0.00	4.55	3.98	0.00	1.42	3.13	1.21	4.73	0.87	2.93	0.00	0.00	0.00	3.22	2.38	1.87	5.36	8.65	6.70	2.03	0.28	0.42	0.00	0.00	0.00
45	Geranial	0.71	0.97	1.35	0.54	0.00	1.05	0.84	1.51	1.27	1.34	1.70	1.70	0.00	0.00	0.00	0.00	2.09	0.00	0.00	0.91	1.12	3.27	3.70	3.28	0.00	0.00	0.00	0.00	0.00	0.00	2.96	3.93	3.64	0.77	0.92	0.93
46	Methyl geranate	22.85	5.97	52.37	25.00	30.17	51.32	69.60	72.01	51.90	9.18	22.07	36.21	92.82	52.62	31.42	26.86	39.85	57.26	24.98	19.96	20.94	7.91	10.33	8.43	17.21	10.50	13.57	7.69	4.33	1.81	12.55	12.39	15.53	0.00	3.28	4.54
47	*m/z* 81,67,43,153	45.91	39.87	47.93	40.51	31.18	41.53	44.73	55.99	47.85	25.47	25.67	28.31	44.52	48.36	41.50	26.55	26.97	41.85	35.12	36.60	34.23	27.50	23.56	27.45	10.80	1.56	9.34	0.73	3.94	1.17	11.83	15.78	10.65	7.39	9.01	9.34
Subtotal (GC Peak Area)		1.30	1.09	1.50	11.80	2.90	3.29	1.23	2.13	1.56	7.34	2.91	3.13	1.56	1.66	1.95	1.75	3.79	4.26	1.45	1.60	1.39	3.71	5.07	4.97	1.84	0.00	1.57	0.00	4.76	4.24	3.02	3.94	3.34	2.68	4.11	3.90
Sesquiterpenic Compounds			**1056.0**			**973.8**			**890.9**			**832.6**			**1108.1**			**1039.7**			**1140.8**			**1020.1**			**1047.0**			**851.3**			**690.5**			**476.5**	
48	α-Cubebene																																				

49	α-Copaene	0.61	0.83	0.86	0.12	0.00	0.00	0.83	0.89	0.75	0.48	0.33	0.66	0.00	0.35	0.32	0.00	0.00	0.00	0.42	0.31	0.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.97	0.00	0.54	1.08	0.49	1.17	1.08	4.25
50	β-Bourbonene	2.60	2.26	2.57	0.83	0.00	0.00	2.14	2.41	2.23	3.05	3.54	1.01	0.71	1.21	2.17	0.00	0.00	0.00	1.86	1.96	3.06	0.70	0.70	0.98	2.06	2.94	1.08	0.00	0.18	0.00	2.57	1.70	1.40	0.96	1.14	5.57
51	β-Elemene	0.00	1.03	1.12	0.00	0.00	0.97	0.00	0.00	0.00	2.39	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.77	0.72	0.69	0.00	0.00	0.27
52	α-Bergamotene	2.89	1.14	1.05	0.00	0.00	0.00	0.00	0.97	0.00	0.00	0.63	2.15	0.47	0.00	0.41	0.47	0.47	0.56	0.00	0.00	0.00	0.00	0.00	0.00	3.83	2.56	3.18	0.95	0.49	0.57	0.34	0.45	0.34	0.95	1.03	1.31
53	β-Caryophyllene	0.00	0.00	0.00	0.00	0.00	0.00	1.71	1.61	1.48	0.00	0.00	0.00	3.24	14.60	2.01	0.68	0.32	0.00	2.58	2.61	1.90	1.08	1.87	0.40	2.63	2.50	1.65	4.21	5.29	5.17	2.17	2.64	2.85	1.71	3.38	2.44
54	Aromadendrene	38.80	38.55	33.32	13.01	13.06	14.31	37.95	35.01	34.72	10.24	15.90	13.88	37.44	37.25	33.02	9.47	16.78	9.34	22.50	35.33	36.36	11.01	13.95	19.40	32.44	31.05	38.85	6.71	4.43	4.70	36.79	31.08	22.94	6.98	7.12	8.86
55	α-Humulene	0.00	0.00	0.00	4.40	4.91	4.60	0.00	0.00	0.50	4.86	8.88	7.76	5.74	0.00	7.65	0.72	0.73	0.00	0.00	0.00	0.00	2.90	0.00	0.00	2.79	0.19	2.03	0.00	0.07	0.00	4.05	2.52	2.47	0.00	0.00	0.00
56	Germacrene D	6.87	13.13	11.57	5.37	6.91	8.28	0.00	0.04	0.00	0.86	0.00	0.78	0.00	0.00	0.00	0.00	0.91	0.98	3.14	3.43	7.38	1.10	2.56	2.41	0.00	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
57	α-Farnesene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.57	0.00	0.00	0.00	0.00	0.33	0.00	0.21	0.23	0.00	0.32	0.49	0.48
58	Δ-Cadinene	25.37	21.18	19.70	15.48	14.94	16.42	10.07	17.80	10.07	0.00	0.00	0.00	13.98	0.00	7.65	9.71	0.00	14.70	17.70	20.49	11.42	22.85	13.79	8.89	6.24	0.26	5.56	9.50	4.05	4.92	2.35	2.92	2.34	1.36	2.09	1.99
59	Calamenene	0.22	0.93	0.00	0.00	0.00	0.00	2.85	1.62	2.00	5.03	2.44	4.32	0.00	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.20	0.00	0.00	0.19	0.34	0.00	0.00	0.00
60	*m/z* 69, 41, 55	0.19	0.18	0.29	0.14	0.11	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.13	0.42	0.14	0.10	0.11	0.15	0.16	0.12	0.09	0.14	0.13	0.08	0.00	0.00	0.00	0.16	0.13	0.19	0.22	0.14	0.13	0.23	0.57
Subtotal (GC Peak Area)		0.31	0.00	3.77	0.36	0.17	0.00	0.40	0.00	0.61	0.52	0.47	0.56	0.41	0.39	0.00	0.00	0.00	0.29	0.28	1.00	0.63	0.57	0.25	0.34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Norisoprenoids			**77.1**			**41.5**			**56.2**			**30.2**			**56.6**			**22.2**			**59.0**			**35.6**			**47.4**			**18.0**			**42.6**			**18.6**	
61	Safranal	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.39	2.72	0.00	0.00	0.83	1.32	1.16	1.01	2.42	2.91	1.53	1.69	1.82	1.48	2.85	0.00	0.00	6.60	4.96	5.04	0.00	0.00	0.00	2.97	2.31	5.29
62	β-Cyclocitral	0.65	0.82	1.10	0.33	0.24	0.00	2.04	2.21	0.00	1.97	1.39	2.00	1.37	1.64	2.21	0.74	0.51	0.00	2.05	1.36	2.42	0.65	0.61	0.84	0.00	0.00	2.16	0.00	0.00	0.00	6.41	9.01	6.19	0.90	0.97	0.71
63	Dihydroedulan	25.46	15.21	27.19	7.03	4.73	6.32	11.77	24.92	33.06	4.06	10.61	10.32	26.82	30.31	32.57	14.46	15.65	31.19	43.57	48.25	45.14	32.93	32.59	11.37	35.45	28.57	33.92	21.08	13.62	35.95	20.29	28.77	22.26	12.70	19.77	10.08
64	Geranyl acetone	4.60	0.00	13.29	3.61	2.74	0.00	5.00	4.32	3.16	5.07	3.38	6.09	4.39	3.75	6.33	4.23	4.26	4.60	2.40	2.30	3.83	3.05	2.98	2.93	2.45	0.36	2.78	2.03	3.41	2.07	5.04	6.27	6.33	4.48	8.85	9.19
65	Phytone	0.68	0.72	0.80	0.54	0.67	0.68	0.73	1.12	0.76	0.63	0.63	0.82	0.62	0.78	0.90	0.63	1.18	0.85	0.46	0.52	0.61	0.88	0.79	1.03	1.49	0.16	1.00	0.94	1.36	1.29	0.00	0.00	0.00	0.00	0.00	0.00
Subtotal (GC Peak Area)			**30.2**			**9.0**			**29.7**			**17.0**			**37.5**			**27.3**			**53.3**			**31.9**			**37.1**			**32.8**			**36.9**			**26.1**	
	Total		**1267.5**			**1074.5**			**1099.6**			**960.9**			**1296.9**			**1141.4**			**1369.0**			**1139.0**			**1268.7**			**978.9**			**868.9**			**595.9**	

Annex from Chapter 3.2

Table A3.2.1. Composition (mg/kg dry berry weight) of dichloromethane extracts from 'Bastardeira' elderberry cultivar, harvested at five ripening stages during two seasons from Varosa Valley, Portugal.

t_R (min)	Compound	MSI level[a]	Content (mg/kg dry berry)																			
			Harvesting date																			
			2012 Season										2013 Season									
			27th Jul	RSD	3rd Aug	RSD	10th Aug	RSD	23rd Aug	RSD	30th Aug	RSD	2nd Aug	RSD	6th Aug	RSD	21st Aug	RSD	28th Aug	RSD	5th Sep	RSD
Fatty acids																						
18.07	Decanoic acid	2	-		-		-		-		-		2.5	15.7	3.4	2.2	2.5	15.7	4.0	2.5	3.5	14.0
23.84	Dodecanoic acid	2	-		-		-		-		-		3.2	23.3	7.5	2.6	11.0	9.8	14.3	22.9	0.0	0.0
28.88	Tetradecanoic acid	2	-		-		-		-		-		12.2	5.9	24.6	2.1	12.0	6.1	18.7	6.5	9.5	11.7
33.63	Hexadecanoic acid	1	144.1	21.8	229.8	1.3	228.1	18.3	145.6	0.8	109.7	3.6	189.4	2.5	188.7	1.5	245.4	3.2	104.8	8.1	45.6	21.1
37.31	Octadec-9-enoic acid	2	tr		5.3	20.3	tr		tr		tr		-		-		-		-		-	
37.93	Octadecanoic acid	2	12.2	6.7	21.5	6.5	17.0	11.7	11.1	9.0	7.7	9.7	15.0	17.6	39.8	20.0	25.9	1.9	26.8	13.3	14.3	2.6
41.91	Eicosanoic acid	2	3.5	4.8	6.4	5.7	3.1	4.0	3.3	9.2	3.6	14.8	8.5	1.4	17.0	1.0	8.4	6.5	11.0	10.5	11.8	9.4
49.03	Tetracosanoic acid	2	-		-		-		-		-		0.0	0.0	9.6	23.3	3.5	15.1	15.5	21.4	17.4	15.2
51.08	Hexacosanoic acid	2	-		-		-		-		-		2.6	12.3	12.8	0.1	3.8	3.7	4.3	19.4	9.7	5.6
	Sub-total (mg/kg)		159.8	20.3	262.9	2.2	248.1	17.7	160.0	1.5	121.0	4.3	233.3	4.1	303.4	4.6	312.4	3.8	199.4	11.0	111.7	14.2
Long chain aliphatic alcohols																						
36.30	Octadecanol	2	0.6	5.1	3.4	10.1	3.2	20.0	0.6	7.6	-		-		-		-		-		-	
51.08	Hexacosanol	2	tr		2.3	3.6	tr		2.1	12.4	3.6	17.6	6.7	3.0	11.6	22.2	4.5	5.9	5.7	23.1	6.2	13.1
	Sub-total (mg/kg)		0.6	5.1	5.8	7.4	3.2	20.0	2.7	11.4	3.6	17.6	6.7	3.0	11.6	22.2	4.5	5.9	5.7	23.1	6.2	13.1
Sterols																						
56.13	Campesterol	2	tr		9.3	24.3	tr		tr		tr		14.2	5.2	-		7.5	20.9	9.6	11.8	10.1	2.6
56.76	Stigmasterol	2	tr		5.0	3.5	9.2	13.2	5.1	15.6	6.1	9.1	16.3	1.1	11.5	0.2	8.1	16.1	13.1	8.5	10.8	11.3

57.96	β-Sitosterol	1	3.1	4.3	26.8	12.7	13.9	15.2	12.9	15.5	9.6	9.7	20.5	6.9	71.2	19.7	75.3	15.7	30.7	9.9	14.6	23.2
	Unidentified sterols	3	-		-		-		-		-		44.8	3.3	51.3	1.2	23.1	9.3	40.4	18.6	45.9	6.7
	Sub-total (mg/kg)		3.1	4.3	41.0	14.2	23.0	14.4	18.0	15.6	15.7	9.5	95.8	4.0	134.0	11.0	114.0	14.8	93.8	13.6	81.5	9.8
Triterpenoids																						
58.48	*β*-Amyrin	2	6.1	6.0	14.7	6.8	7.5	19.4	9.0	9.9	7.7	2.7	21.5	3.6	32.8	2.1	30.8	5.7	26.6	4.3	34.3	8.2
63.00	Oleanolic acid	1	484.2	14.5	478.9	7.3	331.8	8.3	364.4	0.9	320.4	12.1	596.5	0.6	955.2	1.2	702.6	5.5	652.7	1.5	563.8	7.6
64.00	Ursolic acid	1	1960.8	10.3	2026.7	6.8	1970.2	8.4	1632.2	0.4	1345.1	4.9	1771.3	0.8	2239.4	10.7	2132.8	2.1	1949.3	0.3	1689.1	1.8
	Unidentified triterpenoids	3	13.9	10.6	41.5	9.9	20.5	7.5	39.5	3.7	86.1	7.0	79.6	0.8	124.9	13.9	117.4	3.5	113.1	3.9	118.2	6.6
	Sub-total (mg/kg)		2464.9	11.1	2561.9	7.0	2330.0	9.3	2045.1	0.6	1759.3	6.6	2468.8	0.8	3352.3	8.1	2983.6	3.0	2741.6	0.5	2405.3	3.7
	Total content (mg/kg)		2628.4		2871.6		2604.4		2225.7		1899.7		2804.7		3801.3		3414.6		3040.5		2604.7	

[a] Level of compound identification according to Metabolomics Standards Initiative: (l) Identified compounds using standards coinjection; (2) putatively annotated compounds; (3) putatively characterized compound classes; and (4) unknown compounds [107].
The harvesting date in light gray represents the mature stage of the elderberries. tr = trace, < LOQ (limit of quantification) of each analyte.

Table A3.2.2. Composition (mg/kg dry berry weight) of dichloromethane extracts from 'Sabugueira' elderberry cultivar, harvested at five ripening stages during two seasons from Varosa Valley, Portugal.

t_R (min)	Compound	MSI level[a]	Content (mg/kg dry berry)																			
			Harvesting date																			
			2013 Season																			
			27th Jul	RSD	3rd Aug	RSD	10th Aug	RSD	23rd Aug	RSD	30th Aug	RSD	2nd Aug	RSD	6th Aug	RSD	21st Aug	RSD	28th Aug	RSD	5th Sep	RSD
Fatty acids																						
18.07	Decanoic acid	2	-		-		-		-		-		3.7	13.6	2.2	20.5	4.1	11.4	6.0	5.8	11.7	22.5
23.84	Dodecanoic acid	2	-		-		-		-		-		12.3	23.0	5.0	14.5	19.3	5.8	13.6	4.1	9.4	3.0
28.88	Tetradecanoic acid	2	-		-		-		-		-		19.8	11.5	23.3	24.2	30.1	10.1	48.8	2.9	23.5	4.7
33.63	Hexadecanoic acid	1	228.9	9.0	253.1	4.7	239.8	17.3	158.1	1.2	122.5	3.7	250.4	6.7	216.6	24.0	197.9	4.7	182.6	4.7	129.4	4.6
37.31	Octadec-9-enoic acid	2	tr		tr		0.2	21.1	tr		tr		-		-		-		-		-	
37.93	Octadecanoic acid	2	9.0	10.9	9.9	3.1	3.6	15.7	4.1	16.7	4.0	12.9	24.2	13.8	28.2	24.9	7.6	3.3	3.4	5.5	2.5	1.8
41.91	Eicosanoic acid	2	5.6	9.8	3.7	19.2	3.1	23.8	tr		tr		13.2	8.5	15.2	11.4	21.9	5.0	13.4	8.0	7.9	4.4
49.03	Tetracosanoic acid	2	-		-		-		-		-		20.1	23.7	10.1	22.6	22.6	2.7	10.7	21.7	8.2	10.5
51.08	Hexacosanoic acid	2	-		-		-		-		-		31.9	0.4	17.7	24.8	22.1	16.1	19.2	2.1	15.5	18.3
	Sub-total (mg/kg)		243.5	9.1	266.7	4.8	246.7	17.3	162.2	1.6	126.4	4.0	375.8	8.5	318.3	23.3	325.6	5.9	297.6	5.0	208.1	6.8
Long chain aliphatic alcohols																						
36.30	Octadecanol	2	1.0	12.4	1.7	14.5	2.2	4.0	1.9	22.4	1.6	13.9	-		-		-		-		-	
51.08	Hexacosanol	2	-		6.6	15.1	1.8	21.2	1.8	22.1	3.0	14.1	5.1	3.6	13.5	22.2	6.4	2.2	4.3	14.0	4.1	1.6
	Sub-total (mg/kg)		1.0	12.4	8.2	15.0	4.0	11.8	3.7	22.2	4.6	14.0	5.1	3.6	13.5	22.2	6.4	2.2	4.3	14.0	4.1	1.6
Sterols																						
56.13	Campesterol	2	-		-		-		-		-		10.4	17.8	-	0.0	11.6	6.1	10.6	11.3	-	0.0
56.76	Stigmasterol	2	5.0	16.9	6.7	3.9	3.0	9.2	3.6	8.6	0.7	21.7	7.9	6.1	46.2	13.5	34.7	3.1	-	0.0	6.5	16.5

57.96	β-Sitosterol	1	4.6	8.5	22.6	9.6	9.7	14.9	12.4	6.8	7.8	20.7	24.2	11.0	64.6	24.7	17.4	21.9	31.8	3.5	21.7	8.2
	Unidentified sterols	3	-		-		-		-		-		19.6	10.3	57.2	24.2	54.5	2.7	75.1	17.0	50.9	4.2
	Sub-total (mg/kg)		9.5	12.9	29.3	8.3	12.7	13.6	16.0	7.3	8.4	20.8	62.1	11.3	168.0	21.4	118.2	6.0	117.5	12.9	79.0	6.3
Triterpenoids																						
58.48	*β*-Amyrin	2	10.0	20.6	10.7	0.7	4.6	6.2	7.1	14.5	0.6	6.6	24.5	14.2	63.5	22.6	30.9	4.6	26.0	1.2	22.8	2.9
63.00	Oleanolic acid	1	624.3	4.2	682.0	6.7	338.1	6.9	328.3	3.5	324.8	1.4	633.8	3.4	1205.0	12.8	590.7	1.4	584.3	2.5	423.8	1.3
64.00	Ursolic acid	1	2497.7	3.8	2782.4	2.5	1225.8	3.5	1751.7	0.3	1567.8	1.3	2015.6	3.8	3100.1	5.1	1727.9	2.2	1813.8	0.4	1202.0	0.3
	Unidentified triterpenoids	3	32.7	10.0	79.4	6.4	31.5	23.2	15.1	10.6	25.7	14.3	188.5	1.0	106.5	5.7	212.3	4.6	226.0	1.8	104.8	1.2
	Sub-total (mg/kg)		3164.7	4.0	3554.5	3.6	1600.0	4.8	2102.1	0.5	1918.9	1.5	2862.4	4.4	4475.1	6.3	2561.9	3.3	2650.1	0.6	1753.4	1.0
	Total content (mg/kg)		3418.8		3858.8		1863.4		2284.0		2058.3		3305.4		4974.9		3012.0		3069.5		2044.5	

[a] Level of compound identification according to Metabolomics Standards Initiative: (l) Identified compounds using standards coinjection; (2) putatively annotated compounds; (3) putatively characterized compound classes; and (4) unknown compounds [107].
The harvesting date in light gray represents the mature stage of the elderberries. tr = trace, < LOQ (limit of quantification) of each analyte.

Table A3.2.3. Composition (mg/kg dry berry weight) of dichloromethane extracts from 'Sabugueiro' elderberry cultivar, harvested at five ripening stages during two seasons from Varosa Valley, Portugal.

t_R (min)	Compound	MSI level[a]	Content (mg/kg dry berry)																		
			Harvesting date																		
			2013 Season																		
			27th Jul	RSD	3rd Aug	RSD	10th Aug	RSD	23rd Aug	RSD	30th Aug	RSD	2nd Aug	RSD	6th Aug	RSD	21st Aug	RSD	28th Aug	RSD	5th Sep	RSD
Fatty acids																					
18.07	Decanoic acid	2	-		-		-		-		-		4.0	20.7	5.1	15.9	3.6	6.7	4.0	8.3	2.6	5.4
23.84	Dodecanoic acid	2	-		-		-		-		-		3.7	17.6	3.7	22.5	10.4	16.5	12.5	11.5	16.5	13.2
28.88	Tetradecanoic acid	2	-		-		-		-		-		47.1	9.7	71.7	4.0	37.6	12.0	29.4	6.9	26.4	2.2
33.63	Hexadecanoic acid	1	199.9	20.4	239.0	8.2	116.1	9.2	104.6	5.4	137.7	16.2	227.9	2.4	206.5	6.8	174.9	13.0	121.7	12.7	112.2	2.9
37.31	Octadec-9-enoic acid	2	3.7	18.9	tr		tr		tr		tr		-		-		-		-		-	
37.93	Octadecanoic acid	2	16.3	22.1	22.6	0.0	11.8	2.5	13.1	7.1	3.4	13.3	30.9	3.4	22.4	18.0	30.0	14.4	32.8	10.2	25.5	7.9
41.91	Eicosanoic acid	2	1.6	8.9	7.3	17.8	124.3	0.1	5.5	2.3	3.6	13.3	7.6	2.7	15.7	6.8	13.0	19.4	13.6	2.3	10.9	2.7
49.03	Tetracosanoic acid	2	-		-		-		-		-		19.3	4.5	17.1	11.5	9.9	10.1	10.4	7.4	18.8	19.5
51.08	Hexacosanoic acid	2	-		-		-		-		-		28.1	5.4	20.8	6.0	10.5	8.8	11.1	7.1	15.4	2.2
	Sub-total (mg/kg)		221.5	20.4	269.0	9.3	252.2	4.4	123.2	5.4	144.7	16.1	368.7	4.1	363.0	7.4	289.8	13.1	235.3	10.4	228.5	5.4
Long chain aliphatic alcohols																					
36.30	Octadecanol	2	tr		2.9	5.7	1.1	19.9	tr		2.2	19.1	-		-		-		-		-	
51.08	Hexacosanol	2	4.0	22.6	6.9	21.0	3.1	4.4	4.3	20.1	1.4	11.6	5.9	7.6	13.2	11.5	6.9	10.4	6.3	8.0	6.0	4.7
	Sub-total (mg/kg)		4.0	22.6	9.8	16.4	4.2	8.4	4.3	20.1	3.6	16.1	5.9	7.6	13.2	11.5	6.9	10.4	6.3	8.0	6.0	4.7
Sterols																					
56.13	Campesterol	2	tr		12.9	13.1	14.4	12.6	tr		tr		24.2	2.4	-	0.0	14.1	13.2	11.7	8.5	10.9	0.8
56.76	Stigmasterol	2	tr		5.6	12.1	5.8	0.7	4.2	24.9	6.5	7.1	28.4	5.2	21.5	14.3	-	0.0	-	0.0	-	0.0

57.96	β-Sitosterol	1	tr		8.0	9.4	0.7	7.5	9.4	20.2	11.4	17.4	103.4	2.8	48.2	11.5	28.5	12.0	32.2	5.5	27.1	7.5
	Unidentified sterols	3	tr		-		-		-		-		44.6	24.0	58.0	14.5	82.7	6.6	84.3	16.0	51.6	10.7
	Sub-total (mg/kg)		tr		26.6	11.8	20.9	9.1	13.6	21.6	18.0	13.6	200.5	7.8	127.8	13.4	125.3	8.6	128.3	12.7	89.5	8.5
Triterpenoids																						
58.48	*β*-Amyrin	2	9.2	22.0	22.6	11.4	17.0	5.5	7.7	9.1	6.3	17.8	48.3	1.6	51.3	1.6	34.1	8.7	24.4	5.9	21.3	3.4
63.00	Oleanolic acid	1	625.7	3.1	697.6	7.9	453.5	1.5	264.8	8.9	286.5	3.1	893.6	2.1	1203.5	9.8	682.7	7.4	526.6	3.0	468.8	1.3
64.00	Ursolic acid	1	2641.4	3.9	2862.0	7.5	1944.4	2.5	1138.6	2.7	1205.3	8.7	2720.6	0.9	3126.6	2.9	2253.9	3.2	1400.9	1.7	1399.4	1.0
	Unidentified triterpenoids	3	62.2	19.6	84.5	0.0	75.5	10.7	41.4	3.9	26.9	16.4	305.9	1.7	113.5	12.2	158.0	4.5	122.2	1.8	116.5	0.9
	Sub-total (mg/kg)		3338.6	4.1	3666.7	7.6	2490.5	2.6	1452.5	3.4	1525.0	7.8	3968.4	1.4	4494.9	3.8	3128.7	3.7	2074.1	2.2	2005.9	1.5
	Total content (mg/kg)		3564.1		3972.1		2767.8		1593.5		1691.2		4543.5		4998.8		3550.6		2444.0		2329.9	

[a] Level of compound identification according to Metabolomics Standards Initiative: (l) Identified compounds using standards coinjection; (2) putatively annotated compounds; (3) putatively characterized compound classes; and (4) unknown compounds [107].
The harvesting date in light gray represents the mature stage of the elderberries.tr = trace, < LOQ (limit of quantification) of each analyte.

Annex from Chapter 4

Table A4.1 Monoterpenic, sesquiterpenic and norisoprenoid metabolites detected in 'Sabugueira' elderberries (*S. nigra* L.) during ripening, from the harvesting season of 2013. The harvesting date in light gray corresponds to the ripe stage.

Peak number	^1t_R [a] (s)	^2t_R [a] (s)	Compound	RI_{lit} [b]	RI_{calc} [c]	GC Peak Area ($x10^6$)[d] and RSD (%)				
						Harvesting date				
						2nd Aug	8th Aug	21st Aug	28th Aug	5th Sep
			Monoterpenic compounds							
			Hydrocarbon-type							
1	312	0.430	*α*-Pinene	941	938	5.99 (7.3)	1.08 (2.1)	14.15 (13.6)	24.02 (13.4)	2.36 (2.1)
2	330	0.490	Verbenene	967	957	-	-	-	-	0.08[f]
3	342	0.401	Camphene	960	961	-	0.20 (10.7)	0.07[f]	0.64 (11.2)	-
4	348	0.470	*β*-Pinene	987	975	13.54 (22.3)	2.83 (9.7)	3.45 (2.8)	14.93 (25.1)	1.37 (19.6)
5	360	0.500	Myrcene	1008	988	2.17 (15.7)	2.62 (12.3)	1.60 (2.5)	6.82 (3.3)	0.43 (9.0)
6	378	0.447	*β*-Phellandrene	1035	1008	0.58 (10.1)	0.85 (16.4)	0.32 (27.9)	1.72 (12.1)	0.13 (10.8)
7	396	0.500	Limonene [h]	1035	1027	126.90 (17.9)	118.78 (28.9)	81.98 (10.1)	11.65 (4.1)	26.20 (2.0)
8	396	0.570	*p*-Cymene [h]	1039	1027	26.23 (12.3)	16.48 (27.5)	14.56 (28.2)	46.62 (11.2)	6.47 (22.7)
9	426	0.590	1,3,8-*p*-Menthatriene	1121	1060	12.02 (9.2)	10.11 (17.3)	7.09 (16.5)	24.86 (9.6)	4.83 (16.3)
10	456	0.530	Terpinolene	1097	1094	1.48 (20.1)	0.90 (20.2)	0.46 (29.3)	2.34 (1.8)	0.20 (8.6)
11	456	0.620	*p*-Cymenene	1090	1094	1.37 (22.8)	1.18 (5.2)	0.46 (24.0)	2.58 (19.5)	-
12	582	0.620	*m/z* 93, 43, 77	-	1237	-	0.42 (22.9)	-	0.10 (3.5)	-
				Subtotal (GC Peak Area)		**190.27**	**155.15**	**124.09**	**136.27**	**42.03**
					Subtotal (%)[g]	**60.6**	**52.8**	**79.8**	**66.5**	**44.2**
				Subtotal (Number of Compounds)		**9**	**11**	**10**	**11**	**9**
			Oxygen containing							
13	438	0.640	Linalool oxide isomer (furanoid) [h, i]	1078	1074	1.94 (25.4)	-	-	0.53 (4.4)	-
14	438	0.730	Dihydromyrcenol	1076	1074	1.05 (4.4)	-	-	1.47 (28.7)	1.39 (26.2)
15	456	0.720	Linalool oxide isomer (furanoid) [h, i]	1097	1094	0.17 (11.9)	-	-	0.14 (9.3)	-
16	462	0.820	Linalool [h]	1108	1101	16.03 (26.1)	8.62 (6.6)	2.72 (5.8)	8.10 (16.0)	15.13 (12.2)
17	468	0.950	Hotrienol	1122	1108	3.06 (28.5)	-	0.45 (5.2)	1.21 (4.5)	1.12 (6.1)
18	474	0.510	Rose oxide	1117	1114	1.09 (16.9)	2.55 (9.1)	-	0.51 (13.5)	0.15 (18.3)
19	480	0.870	Fenchol	1118	1121	-	-	-	0.34 (22.9)	-
20	492	0.510	Rose oxide isomer	1130	1131	-	1.57 (22.1)	-	0.12 (11.4)	-
21	498	0.600	Artemisia alcohol	1083	1141	0.50 (23.4)	-	0.74 (9.1)	-	0.70 (10.9)
22	504	0.740	Camphor	1147	1148	-	-	-	-	0.04[f]
23	510	0.650	Citronellal [h]	1159	1154	0.17 (24.6)	-	-	-	0.60 (20.6)

24	516	0.990	Hotrienol isomer	1122	1162	20.27 (4.3)	0.52 (5.0)	-	3.13 (6.1)	8.46 (14.6)
25	522	0.690	Pinocarvone	1164	1167	0.09 (20.5)	0.10 (7.3)	0.51 (20.0)	1.42 (6.8)	0.45 (28.0)
26	528	0.850	Menthol [h]	1173	1174	-	-	0.10 (1.2)	0.10 (9.7)	-
27	528	0.990	Borneol [h]	1172	1174	1.71 (18.9)	-	-	0.96 (13.2)	-
28	528	1.070	Epoxylinalool	1173	1175	19.50 (12.3)	4.49 (23.8)	1.38 (5.1)	1.67 (28.7)	1.38 (27.5)
29	534	0.787	Terpinen-4-ol	1181	1181	0.46 (6.9)	0.88 (1.9)	0.26 (19.9)	0.98 (21.6)	-
30	540	1.380	*p*-Cymen-8-ol	1203	1188	0.40 (23.4)	-	-	0.57 (15.8)	-
31	546	0.500	3(10)-Caren-2-ol	1211	1194	0.26 (29.3)	0.43 (1.1)	0.25 (11.1)	-	0.27 (20.4)
32	546	0.920	*α*-Terpineol [h]	1206	1194	1.01 (1.4)	1.00 (28.8)	0.48 (17.8)	2.21 (13.5)	0.29 (16.7)
33	576	0.950	*β*-Citronellol [h]	1237	1230	0.40 (21.3)	-	-	0.33 (14.0)	1.77 (22.2)
34	576	1.050	*trans*-Geraniol [h]	1235	1230	0.08[f]	-	-	0.62 (2.8)	-
35	588	0.750	*m/z* 59, 68, 43	-	1244	-	-	-	0.05[f]	0.04[f]
36	588	0.800	Citral isomer [h, i]	1241	1244	0.72 (13.5)	1.11 (26.7)	0.64 (17.6)	1.65 (2.5)	0.23 (22.1)
37	588	0.910	Carvone [h]	1245	1244	-	0.21[e] (0.4)	-	0.19 (11.6)	-
38	600	1.070	Nerol [h]	1242	1258	2.57 (23.3)	0.29 (4.3)	-	0.77 (13.3)	1.23 (14.7)
39	612	0.820	Citral isomer [h, i]	1287	1272	1.95 (8.8)	1.12 (25.6)	1.48 (24.9)	2.03 (17.7)	1.97 (22.7)
40	684	0.600	*β*-Terpinyl acetate	-	1357	0.84 (28.6)	1.08 (2.4)	0.20 (21.2)	4.84 (18.4)	0.55 (21.9)
41	702	0.607	Geranyl acetate	1383	1385	1.32 (19.9)	1.31 (29.5)	0.48 (9.7)	1.62 (17.2)	0.18 (31.1)
42	714	0.680	10-(Acetylmethyl)-3-carene	1380	1401	1.61 (26.1)	8.74 (6.8)	0.13 (4.0)	4.10 (12.0)	0.11 (24.0)
				Subtotal (GC Peak Area)		**77.12**	**33.67**	**9.75**	**39.26**	**35.81**
				Subtotal (%)		**24.6**	**11.5**	**6.3**	**19.2**	**37.7**
				Subtotal (Number of Compounds)		**24**	**16**	**14**	**26**	**20**
			Nosiroprenoids							
43	402	0.620	2,2,6-Trimethyl-cyclohexanone	1051	1034	1.13 (23.7)	2.60 (13.2)	0.48 (10.3)	-	1.44 (1.6)
44	504	1.080	Ketoisophorone	1171	1148	0.32 (12.4)	0.18 (27.5)	0.15 (16.5)	-	0.06 (11.1)
45	552	0.830	Safranal	1241	1201	-	-	2.07 (22.8)	-	-
46	570	0.670	*β*-Cyclocitral	1261	1222	1.24 (27.3)	2.90 (27.1)	0.54 (21.7)	0.47 (17.2)	0.45 (1.2)
47	636	0.570	Dihydroedulan	1289	1300	22.09 (20.8)	56.02 (10.0)	1.37 (19.6)	3.66 (7.6)	2.67 (14.9)
48	654	0.500	Theaspirane	1308	1322	0.27 (6.5)	0.25 (17.9)	-	0.15 (3.9)	-
49	708	0.760	Damascenone	1379	1393	0.38 (17.0)	-	-	0.16 (7.3)	0.32 (20.8)
50	738	0.740	*α*-Ionone	1422	1432	0.17 (21.3)	-	0.07 (2.3)	-	-
51	744	0.950	Megastigma-3,5-dien-9-ol	1430	1440	0.10 (20.3)	0.32 (17.9)	-	0.23 (4.9)	-
52	750	0.850	*β*-Ionon-5,6-epoxide	1428	1447	0.34 (28.8)	0.11 (12.1)	0.04[f]	-	-
53	756	0.700	Geranyl acetone [h]	1454	1455	3.20 (16.6)	2.91 (29.0)	3.20 (15.5)	1.52 (6.7)	1.95 (22.6)
54	780	0.680	*α*-Methyl ionone	1486	1486	0.19 (23.8)	0.18 (11.5)	0.07 (7.1)	-	-
55	780	0.780	*β*-Ionone [h]	1481	1487	0.26 (13.7)	0.64 (3.7)	0.26 (12.1)	-	-

56	900	0.970	Methyl dihydrojasmonate	1650	1660	0.09[f]	-	-	-	-
					Subtotal (GC Peak Area)	**29.74**	**66.10**	**8.15**	**6.20**	**6.88**
					Subtotal (%)	**9.5**	**22.5**	**5.2**	**3.0**	**7.2**
					Subtotal (Number of Compounds)	**13**	**10**	**10**	**6**	**6**
			Sesquiterpenic compounds							
			Hydrocarbon-type							
57	678	0.490	*β*-Cubebene	1387	1354	0.26 (16.4)	0.22 (29.1)	0.21 (16.0)	0.10 (0.7)	0.10 (2.3)
58	702	0.500	*α*-Copaene [h]	1375	1385	-	1.12 (6.3)	0.80 (14.6)	0.78 (22.8)	0.23 (6.3)
59	708	0.510	*β*-Bourbonene	1379	1393	-	-	-	-	-
60	714	0.540	*β*-Elemene	1380	1401	-	-	0.92 (17.7)	0.69 (25.8)	-
61	726	0.540	Longifolene	1395	1431	1.09 (18.3)	3.80 (4.3)	-	1.82 (17.4)	-
62	738	0.540	*β*-Caryophyllene [h]	1417	1432	4.34 (6.3)	11.84 (6.4)	3.27 (28.7)	4.84 (17.9)	-
63	750	0.480	Aromadendrene	1447	1447	2.65 (20.7)	13.10 (12.0)	4.28 (8.5)	6.54 (19.8)	7.23 (7.9)
64	762	0.560	*α*-Humulene [h]	1450	1462	2.05 (23.9)	2.68 (17.3)	1.03 (14.9)	4.50 (17.7)	0.60 (17.5)
65	792	0.506	*α*-Muurolene	1490	1499	1.78 (18.0)	3.38 (10.8)	0.16 (13.9)	0.49 (23.9)	0.11 (7.0)
66	810	0.570	*Δ*-Cadinene	1525	1528	0.54 (18.7)	0.86 (15.0)	0.38 (2.2)	0.74 (11.3)	0.16 (16.0)
67	810	0.630	Calamenene	1525	1528	0.14 (6.3)	0.38 (26.5)	0.59 (24.2)	0.33 (12.5)	0.14 (20.2)
68	828	0.680	*α*-Calacorene	1554	1556	-	-	0.08[f]	0.56 (3.0)	0.15 (19.8)
					Subtotal (GC Peak Area)	**12.85**	**37.37**	**11.66**	**20.89**	**8.57**
					Subtotal (%)	**4.1**	**12.7**	**7.5**	**10.2**	**9.0**
					Subtotal (Number of Compounds)	**8**	**9**	**10**	**11**	**8**
			Oxygen containing							
69	822	0.810	*β*-Bourbonen-13-ol	-	1547	-	-	-	-	-
70	840	0.670	Epiglobulol	1588	1572	0.56 (9.0)	0.42 (28.0)	0.52 (14.0)	0.66 (13.2)	0.45 (12.0)
71	858	0.710	Caryophyllene oxide	1610	1601	1.53 (4.2)	0.77 (16.6)	0.11 (8.3)	1.45 (18.0)	0.42 (9.2)
72	870	0.730	Globulol	1592	1618	-	-	-	-	-
73	888	0.730	Cubenol	1643	1643	0.04[f]	0.03 (1.5)	0.61 (17.2)	-	0.42 (28.1)
74	894	0.830	*t*-Cadinol	1650	1651	0.85 (10.6)	0.03 (2.3)	-	-	-
75	918	0.900	*m/z* 91, 119, 105	-	1685	0.25 (16.6)	0.46 (17.9)	0.27 (29.5)	0.22 (16.9)	0.23 (8.6)
76	1014	0.710	*m/z* 69, 41, 55	-	1841	0.61 (12.1)	0.06[f]	0.28 (26.7)	-	0.25 (13.7)
					Subtotal (GC Peak Area)	**3.82**	**1.53**	**1.79**	**2.32**	**1.77**
					Subtotal (%)	**1.2**	**0.5**	**1.2**	**1.1**	**1.9**
					Subtotal (Number of Compounds)	**6**	**6**	**5**	**3**	**5**
					Total	**313.81**	**293.82**	**155.44**	**204.94**	**95.06**
					Number of Identified Compounds	**60**	**52**	**49**	**57**	**48**

a Retention times for first (1tR) and second (2tR) dimensions in seconds.

b RI, Retention Index reported in the literature for Equity-5 GC column or equivalents.

c RI: Retention Index obtained through the modulated chromatogram.

d Mean of three independent assays (*n*=3). GC peak areas were expressed as arbitrary units.

e The compound was detected in two assays.

f The compound was detected in one assay.

g The relative amount (%) for the different identified chemical families represents their relative content compared to the total content.

h Identification confirmed by chemical standards

i The used standards are a mixture of isomers

Table A4.2 Monoterpenic, sesquiterpenic and norisoprenoid metabolites detected in 'Sabugueiro' elderberries (*S. nigra* L.) during ripening, from the harvesting season of 2013. The harvesting date in light gray corresponds to the ripe stage.

Peak number	^1t_R [a] (s)	^2t_R [a] (s)	Compound	RI_{lit} [b]	RI_{calc} [c]	GC Peak Area ($x10^6$)[d] and RSD (%)				
						Harvesting date				
						2nd Aug	8th Aug	21st Aug	28th Aug	5th Sep
			Monoterpenic compounds							
			Hydrocarbon-type							
1	312	0.430	*α*-Pinene	941	938	15.82 (28.5)	12.21 (8.0)	2.49 (2.5)	2.95 (27.7)	1.66 (24.1)
2	330	0.490	Verbenene	967	957	-	0.08[f]	-	-	0.05[f]
3	342	0.401	Camphene	960	961	0.12 (9.0)	0.24 (19.4)	-	0.74 (21.3)	0.15 (27.5)
4	348	0.470	*β*-Pinene	987	975	16.57 (25.1)	9.08 (10.7)	2.42 (29.3)	20.99 (8.2)	2.41 (15.8)
5	360	0.500	Myrcene	1008	988	1.87 (24.3)	-	0.88 (27.7)	5.92 (15.9)	1.10 (28.2)
6	378	0.447	*β*-Phellandrene	1035	1008	0.97 (20.5)	-	0.24 (23.8)	1.72 (22.7)	0.29 (21.0)
7	396	0.500	Limonene [h]	1035	1027	144.71 (13.8)	142.23 (18.3)	52.89 (21.0)	43.75 (8.2)	36.60 (26.7)
8	396	0.570	*p*-Cymene [h]	1039	1027	16.29 (18.0)	30.85 (15.4)	7.11 (9.7)	38.22 (8.7)	2.58 (1.0)
9	426	0.590	1,3,8-*p*-Menthatriene	1121	1060	18.75 (13.6)	20.25 (15.5)	3.18 (27.9)	28.60 (23.7)	1.86 (1.2)
10	456	0.530	Terpinolene	1097	1094	1.15 (15.4)	1.51 (15.8)	0.28 (23.1)	2.42 (25.8)	0.34 (22.0)
11	456	0.620	*p*-Cymenene	1090	1094	1.36 (13.4)	-	0.10 (2.3)	1.85 (16.3)	0.59 (22.3)
12	582	0.620	*m/z* 93, 43, 77	-	1237	-	0.38 (4.6)	-	-	0.28 (16.1)
				Subtotal (GC Peak Area)		**217.62**	**216.78**	**69.59**	**147.15**	**47.88**
				Subtotal (%)[g]		**71.3**	**60.4**	**49.4**	**71.0**	**45.8**
				Subtotal (Number of Compounds)		**10**	**9**	**9**	**10**	**12**
			Oxygen containing							
13	438	0.640	Linalool oxide isomer (furanoid) [h, i]	1078	1074	-	0.44 (20.2)	0.04[f]	-	0.54 (25.3)
14	438	0.730	Dihydromyrcenol	1076	1074	-	-	-	0.34 (21.2)	-
15	456	0.720	Linalool oxide isomer (furanoid) [h, i]	1097	1094	0.08[f]	-	0.20 (6.4)	-	0.12 (22.0)
16	462	0.820	Linalool [h]	1108	1101	2.51 (5.1)	11.55 (25.9)	7.46 (10.5)	2.31 (10.3)	7.48 (13.6)
17	468	0.950	Hotrienol	1122	1108	0.87 (21.1)	2.59 (6.3)	1.98 (1.6)	0.19 (10.5)	0.94 (8.9)
18	474	0.510	Rose oxide	1117	1114	0.67 (19.2)	-	1.26 (22.9)	-	0.34 (9.5)
19	480	0.870	Fenchol	1118	1121	-	-	-	0.30 (26.5)	-
20	492	0.510	Rose oxide isomer	1130	1131	0.24 (7.8)	0.39 (26.6)	0.25 (25.9)	-	0.10 (10.6)
21	498	0.600	Artemisia alcohol	1083	1141	1.18 (28.1)	0.35 (9.9)	0.29 (19.6)	0.45 (3.6)	1.87 (21.8)
22	504	0.740	Camphor	1147	1148	0.74 (17.1)	0.14 (29.3)	-	0.65 (24.8)	-

23	510	0.650	Citronellal [h]	1159	1154	-	0.56 (6.3)	0.35 (16.2)	-	0.42 (7.7)
24	516	0.990	Hotrienol isomer	1122	1162	2.86 (30.4)	2.04 (0.5)	2.00 (5.6)	-	1.67 (15.8)
25	522	0.690	Pinocarvone	1164	1167	1.11 (6.7)	1.58 (11.9)	-	1.34 (18.9)	-
26	528	0.850	Menthol [h]	1173	1174	0.14 (17.9)	0.14 (2.4)	0.21 (2.1)	-	0.14[e] (25.1)
27	528	0.990	Borneol [h]	1172	1174	-	1.54 (22.7)	-	-	-
28	528	1.070	Epoxylinalool	1173	1175	2.43 (14.1)	2.86 (29.0)	1.83 (14.2)	0.47 (10.7)	3.06 (29.5)
29	534	0.787	Terpinen-4-ol	1181	1181	1.07 (17.8)	1.00 (13.7)	-	1.93 (28.9)	-
30	540	1.380	*p*-Cymen-8-ol	1203	1188	-	-	-	-	-
31	546	0.500	3(10)-Caren-2-ol	1211	1194	0.47 (11.3)	-	0.25 (6.6)	0.19 (9.4)	-
32	546	0.920	*α*-Terpineol [h]	1206	1194	1.67 (17.9)	1.53 (22.1)	0.77 (9.6)	1.57 (27.7)	0.73 (6.8)
33	576	0.950	*β*-Citronellol [h]	1237	1230	-	-	-	-	0.89 (14.3)
34	576	1.050	*trans*-Geraniol [h]	1235	1230	-	-	-	-	-
35	588	0.750	*m/z* 59, 68, 43	-	1244	0.38 (16.2)	0.60 (22.6)	-	-	0.12 (24.6)
36	588	0.800	Citral isomer [h, i]	1241	1244	1.36 (1.7)	0.90 (25.1)	-	1.76 (13.6)	0.28 (29.9)
37	588	0.910	Carvone [h]	1245	1244	0.17 (21.3)	-	-	0.22 (6.4)	-
38	600	1.070	Nerol [h]	1242	1258	2.03 (13.7)	2.51 (14.2)	0.99 (22.1)	-	1.40 (8.2)
39	612	0.820	Citral isomer [h, i]	1287	1272	2.11 (27.2)	1.96 (16.7)	0.90 (4.1)	3.00 (13.9)	1.07 (3.3)
40	684	0.600	*β*-Terpinyl acetate	-	1357	3.85 (16.6)	2.32 (8.9)	0.65 (15.2)	6.20 (9.6)	0.43 (16.0)
41	702	0.607	Geranyl acetate	1383	1385	6.53 (19.4)	0.93 (22.9)	-	3.63 (24.1)	0.42 (22.8)
42	714	0.680	10-(Acetylmethyl)-3-carene	1380	1401	1.95 (28.2)	9.71 (18.6)	5.73 (23.7)	0.04[f]	4.18 (2.9)
					Subtotal (GC Peak Area)	**34.34**	**45.64**	**25.12**	**24.57**	**26.03**
					Subtotal (%)	**11.3**	**12.7**	**17.8**	**11.9**	**24.9**
					Subtotal (Number of Compounds)	**22**	**21**	**17**	**17**	**20**
			Nosiroprenoids							
43	402	0.620	2,2,6-Trimethyl-cyclohexanone	1051	1034	1.59 (29.9)	1.62 (7.4)	0.78 (5.7)	-	0.76 (27.4)
44	504	1.080	Ketoisophorone	1171	1148	0.19 (11.3)	0.74 (14.9)	0.11 (21.1)	0.06 (9.7)	0.07 (24.8)
45	552	0.830	Safranal	1241	1201	-	-	-	-	0.56 (5.3)
46	570	0.670	*β*-Cyclocitral	1261	1222	1.18 (21.4)	0.89 (7.3)	0.46 (117.)	0.20 (6.1)	0.24 (20.7)
47	636	0.570	Dihydroedulan	1289	1300	20.67 (30.7)	49.97 (8.9)	18.57 (23.0)	6.77 (0.6)	14.50 (2.6)
48	654	0.500	Theaspirane	1308	1322	-	0.27 (17.0)	0.11 (19.4)	-	0.17 (28.4)
49	708	0.760	Damascenone	1379	1393	-	0.23 (28.4)	0.18 (28.6)	-	0.16 (15.9)
50	738	0.740	*α*-Ionone	1422	1432	0.10 (7.2)	-	-	-	-
51	744	0.950	Megastigma-3,5-dien-9-ol	1430	1440	0.14 (6.2)	0.33 (7.9)	0.28 (18.6)	-	0.30 (15.8)
52	750	0.850	*β*-Ionon-5,6-epoxide	1428	1447	0.69 (24.8)	0.09 (18.3)	0.17 (29.1)	0.06 (19.9)	-
53	756	0.700	Geranyl acetone [h]	1454	1455	4.91 (19.2)	5.47 (27.1)	3.27 (22.4)	1.48 (24.8)	2.70 (2.1)
54	780	0.680	*α*-Methyl ionone	1486	1486	0.19 (18.5)	0.11 (26.4)	0.10 (6.0)	-	-

55	780	0.780	*β*-Ionone [h]	1481	1487	-	0.13 (11.9)	0.23 (4.0)	-	-
56	900	0.970	Methyl dihydrojasmonate	1650	1660	0.09[e] (9.7)	-	0.07 (12.1)	0.08 (27.8)	-
					Subtotal (GC Peak Area)	**29.62**	**59.84**	**24.29**	**8.65**	**19.47**
					Subtotal (%)	**9.7**	**16.7**	**17.2**	**4.2**	**18.6**
					Subtotal (Number of Compounds)	**10**	**11**	**12**	**6**	**9**
			Sesquiterpenic compounds							
			Hydrocarbon-type							
57	678	0.490	*β*-Cubebene	1387	1354	0.03[f]	0.73 (8.4)	2.33 (12.4)	0.19 (14.6)	-
58	702	0.500	*α*-Copaene [h]	1375	1385	0.89 (27.9)	-	1.41 (26.0)	-	-
59	708	0.510	*β*-Bourbonene	1379	1393	-	2.70 (8.4)	-	-	1.17 (19.5)
60	714	0.540	*β*-Elemene	1380	1401	-	-	0.72 (24.4)	1.06 (23.5)	0.05[f]
61	726	0.540	Longifolene	1395	1431	-	2.24 (16.0)	2.71 (14.0)	-	2.89 (8.1)
62	738	0.540	*β*-Caryophyllene [h]	1417	1432	-	6.14 (14.7)	2.81 (20.8)	1.16 (7.9)	1.55 (10.6)
63	750	0.480	Aromadendrene	1447	1447	10.82 (10.1)	13.96 (11.3)	-	16.81 (13.1)	1.04 (17.0)
64	762	0.560	*α*-Humulene [h]	1450	1462	0.13 (20.5)	5.19 (20.5)	2.16 (23.6)	1.27 (26.5)	2.65 (20.9)
65	792	0.506	*α*-Muurolene	1490	1499	3.89 (13.7)	-	0.22 (25.7)	3.09 (23.3)	-
66	810	0.570	*Δ*-Cadinene	1525	1528	0.94 (11.7)	1.29 (17.1)	4.30 (9.2)	0.21 (26.2)	0.16 (15.4)
67	810	0.630	Calamenene	1525	1528	0.30 (15.6)	0.23 (13.4)	0.22 (16.4)	0.21 (24.1)	0.07 (16.8)
68	828	0.680	*α*-Calacorene	1554	1556	0.03 (21.6)	0.06 (8.4)	0.25 (28.3)	0.03 (8.6)	0.05[f]
					Subtotal (GC Peak Area)	**16.79**	**32.55**	**17.13**	**24.04**	**9.56**
					Subtotal (%)	**5.5**	**9.1**	**12.2**	**11.6**	**9.2**
					Subtotal (Number of Compounds)	**8**	**9**	**10**	**9**	**9**
			Oxygen containing							
69	822	0.810	*β*-Bourbonen-13-ol	-	1547	0.48 (25.6)	0.51 (21.2)	0.38 (21.7)	-	-
70	840	0.670	Epiglobulol	1588	1572	2.60 (14.3)	1.18 (28.1)	0.50 (9.0)	0.69 (28.2)	0.04[f]
71	858	0.710	Caryophyllene oxide	1610	1601	0.52 (26.6)	0.67 (1.8)	1.54 (29.5)	1.45 (20.5)	0.79 (27.3)
72	870	0.730	Globulol	1592	1618	-	-	-	-	-
73	888	0.730	Cubenol	1643	1643	1.15 (16.8)	0.48 (18.4)	1.59 (10.1)	0.41 (8.6)	-
74	894	0.830	*t*-Cadinol	1650	1651	-	-	-	-	-
75	918	0.900	*m/z* 91, 119, 105	-	1685	0.48 (6.3)	0.27 (7.1)	0.31 (14.9)	0.27 (2.2)	0.28 (26.5)
76	1014	0.710	*m/z* 69, 41, 55	-	1841	1.44 (22.0)	0.84 (19.5)	0.40 (17.3)	-	0.49 (25.7)
					Subtotal (GC Peak Area)	**6.68**	**4.33**	**4.72**	**2.82**	**1.57**
					Subtotal (%)	**2.2**	**1.2**	**3.4**	**1.4**	**1.5**
					Subtotal (Number of Compounds)	**6**	**6**	**6**	**4**	**4**
					Total	**305.04**	**359.14**	**140.86**	**207.23**	**104.52**

	Number of identified compounds	**56**	**56**	**54**	**46**	**54**

a Retention times for first (1tR) and second (2tR) dimensions in seconds.

b RI, Retention Index reported in the literature for Equity-5 GC column or equivalents.

c RI: Retention Index obtained through the modulated chromatogram.

d Mean of three independent assays (*n*=3). GC peak areas were expressed as arbitrary units.

e The compound was detected in two assays.

f The compound was detected in one assay.

g The relative amount (%) for the different identified chemical families represents their relative content compared to the total content.

h Identification confirmed by chemical standards

i The used standards are a mixture of isomers

Table A4.3 Monoterpenic, sesquiterpenic and norisoprenoid metabolites detected in 'Bastardeira' elderberries (*S. nigra* L.) during ripening, from the harvesting season of 2013. The harvesting date in light gray corresponds the ripe stage.

Peak number	$^{1}t_R$ [a] (s)	$^{2}t_R$ [a] (s)	Compound	RI_{lit} [b]	RI_{calc} [c]	GC Peak Area ($x10^6$)[d] and RSD (%)				
						Harvesting date				
						2nd Aug	8th Aug	21st Aug	28th Aug	5th Sep
			Monoterpenic compounds							
			Hydrocarbon-type							
1	312	0.430	*α*-Pinene	941	938	14.61 (18.5)	7.94 (11.9)	7.31 (0.3)	10.45 (2.8)	2.21 (8.0)
2	330	0.490	Verbenene	967	957	0.09 (12.0)	-	-	-	-
3	342	0.401	Camphene	960	961	0.17 (19.0)	0.12 (1.2)	0.16 (10.4)	0.25 (14.5)	-
4	348	0.470	*β*-Pinene	987	975	6.34 (6.8)	7.45 (14.5)	7.15 (10.2)	17.51 (14.3)	1.75 (15.8)
5	360	0.500	Myrcene	1008	988	2.83 (27.5)	2.47 (29.7)	3.39 (20.6)	5.41 (8.7)	0.55 (23.4)
6	378	0.447	*β*-Phellandrene	1035	1008	1.47 (32.4)	0.36 (9.0)	0.59 (25.3)	0.76 (9.9)	0.27 (24.8)
7	396	0.500	Limonene [h]	1035	1027	130.17 (32.3)	112.77 (21.7)	13.26 (14.3)	53.03 (4.6)	47.80 (19.9)
8	396	0.570	*p*-Cymene [h]	1039	1027	17.38 (28.2)	18.96 (11.6)	16.63 (14.8)	11.57 (11.7)	4.82 (17.7)
9	426	0.590	1,3,8-*p*-Menthatriene	1121	1060	14.43 (19.3)	13.57 (24.6)	21.17 (20.0)	25.31 (12.0)	4.45 (24.0)
10	456	0.530	Terpinolene	1097	1094	1.41 (23.9)	0.87 (21.6)	1.17 (22.1)	2.18 (12.3)	0.19 (8.3)
11	456	0.620	*p*-Cymenene	1090	1094	1.69 (5.3)	1.35 (20.2)	1.53 (21.3)	2.25 (5.0)	0.13 (25.9)
12	582	0.620	*m/z* 93, 43, 77	-	1237	0.18 (8.7)	0.29 (7.8)	-	-	-
					Subtotal (GC Peak Area)	**190.15**	**166.07**	**72.36**	**128.74**	**62.14**
					Subtotal (%)[g]	**61.6**	**56.3**	**57.8**	**77.2**	**61.7**
					Subtotal (Number of Compounds)	**12**	**11**	**10**	**10**	**9**
			Oxygen containing							
13	438	0.640	Linalool oxide isomer (furanoid) [h, i]	1078	1074	-	-	0.05[f]	-	-
14	438	0.730	Dihydromyrcenol	1076	1074	-	-	-	0.88 (10.2)	1.21 (16.1)
15	456	0.720	Linalool oxide isomer (furanoid) [h, i]	1097	1094	0.25 (25.5)	-	-	-	-
16	462	0.820	Linalool [h]	1108	1101	6.37 (8.3)	5.56 (7.1)	3.06 (24.9)	2.03 (9.9)	1.87 (14.4)
17	468	0.950	Hotrienol	1122	1108	4.52 (24.0)	0.50 (8.2)	-	-	0.20[e] (6.7)
18	474	0.510	Rose oxide	1117	1114	0.87 (17.9)	2.03 (27.3)	0.55 (19.7)	0.15 (25.4)	0.79 (22.7)
19	480	0.870	Fenchol	1118	1121	-	-	-	0.37 (26.1)	-
20	492	0.510	Rose oxide isomer	1130	1131	0.25 (2.7)	0.44 (27.0)	0.17 (2.5)	-	0.37 (8.4)
21	498	0.600	Artemisia alcohol	1083	1141	0.33 (27.6)	0.14 (25.9)	1.18 (9.9)	-	0.45 (4.6)
22	504	0.740	Camphor	1147	1148	0.36 (14.1)	-	-	-	-

23	510	0.650	Citronellal [h]	1159	1154	0.49 (17.3)	0.09 (5.4)	-	-	0.26 (10.8)
24	516	0.990	Hotrienol isomer	1122	1162	1.37 (21.5)	0.62 (9.8)	0.77 (11.9)	0.50 (14.0)	0.40 (23.7)
25	522	0.690	Pinocarvone	1164	1167	1.71 (26.9)	0.50 (32.5)	0.98 (12.2)	1.41 (15.5)	-
26	528	0.850	Menthol [h]	1173	1174	-	-	0.15 (24.5)	0.13 (28.8)	-
27	528	0.990	Borneol [h]	1172	1174	0.72 (4.9)	1.14 (20.8)	0.20[e] (20.1)	-	-
28	528	1.070	Epoxylinalool	1173	1175	21.32 (23.6)	2.22 (22.1)	2.77 (31.9)	1.84 (16.1)	2.87 (7.7)
29	534	0.787	Terpinen-4-ol	1181	1181	0.63 (11.6)	0.70 (31.1)	0.57 (28.7)	1.14 (11.3)	-
30	540	1.380	*p*-Cymen-8-ol	1203	1188	0.23 (12.3)	0.06[f]	0.06[e] (5.4)	0.01[f]	-
31	546	0.500	3(10)-Caren-2-ol	1211	1194	0.24 (24.1)	0.39 (12.8)	0.30 (23.6)	1.11 (7.2)	0.14 (7.7)
32	546	0.920	*α*-Terpineol [h]	1206	1194	1.19 (29.0)	0.90 (21.4)	0.80 (26.7)	2.35 (18.2)	0.18 (10.2)
33	576	0.950	*β*-Citronellol [h]	1237	1230	0.72 (0.4)	-	-	-	0.46 (10.3)
34	576	1.050	*trans*-Geraniol [h]	1235	1230	0.70 (4.9)	1.13 (31.6)	-	-	-
35	588	0.750	*m/z* 59, 68, 43	-	1244	-	0.09[f]	0.03[f]	-	0.83 (27.5)
36	588	0.800	Citral isomer [h, i]	1241	1244	-	0.48 (29.8)	0.85 (4.8)	1.09 (1.4)	0.36 (21.8)
37	588	0.910	Carvone [h]	1245	1244	0.16 (19.1)	0.16 (30.2)	0.22 (29.2)	-	-
38	600	1.070	Nerol [h]	1242	1258	2.09 (25.2)	2.11 (17.9)	1.71 (3.8)	0.40 (15.4)	0.76 (8.5)
39	612	0.820	Citral isomer [h, i]	1287	1272	1.44 (22.4)	1.81 (13.2)	1.54 (22.8)	3.27 (18.6)	1.40 (15.1)
40	684	0.600	*β*-Terpinyl acetate	-	1357	1.64 (6.6)	0.46 (13.7)	2.31 (15.9)	3.63 (5.2)	0.18 (20.7)
41	702	0.607	Geranyl acetate	1383	1385	0.70 (11.6)	2.83 (16.5)	0.44 (13.2)	0.89 (20.3)	0.17 (25.2)
42	714	0.680	10-(Acetylmethyl)-3-carene	1380	1401	0.71 (15.0)	6.53 (16.9)	1.34 (24.0)	0.55 (26.2)	1.31 (1.7)
					Subtotal (GC Peak Area)	**48.28**	**29.8**	**18.85**	**21.6**	**13.62**
					Subtotal (%)	**15.7**	**10.1**	**15.0**	**13.0**	**13.5**
					Subtotal (Number of Compounds)	**24**	**23**	**22**	**18**	**19**
			Nosiroprenoids							
43	402	0.620	2,2,6-Trimethyl-cyclohexanone	1051	1034	5.96 (20.7)	4.43 (16.1)	1.18 (17.1)	-	0.75 (3.4)
44	504	1.080	Ketoisophorone	1171	1148	0.79 (7.5)	0.23 (15.5)	0.08 (28.8)	0.06[f]	0.05 (18.9)
45	552	0.830	Safranal	1241	1201	0.53 (3.4)	0.03[f]	-	-	-
46	570	0.670	*β*-Cyclocitral	1261	1222	4.77 (29.8)	3.70 (29.4)	0.65 (15.0)	0.15 (9.1)	0.27 (15.2)
47	636	0.570	Dihydroedulan	1289	1300	21.98 (24.5)	53.47 (5.8)	9.93 (22.4)	4.63 (16.2)	14.74 (15.9)
48	654	0.500	Theaspirane	1308	1322	0.08[f]	0.37 (8.8)	-	-	-
49	708	0.760	Damascenone	1379	1393	1.59 (3.9)	-	-	-	-
50	738	0.740	*α*-Ionone	1422	1432	0.10 (18.7)	0.37[e] (9.0)	0.14 (20.2)	-	0.08 (2.4)
51	744	0.950	Megastigma-3,5-dien-9-ol	1430	1440	0.16 (18.5)	0.21 (16.8)	-	-	-
52	750	0.850	*β*-Ionon-5,6-epoxide	1428	1447	-	0.41 (13.7)	0.25 (4.5)	0.10 (14.8)	0.11 (9.6)
53	756	0.700	Geranyl acetone [h]	1454	1455	3.52 (16.0)	4.42 (14.7)	3.22 (26.6)	1.49 (29.1)	1.77 (9.8)
54	780	0.680	*α*-Methyl ionone	1486	1486	0.14 (13.8)	0.20 (18.7)	0.07 (7.0)	-	-

55	780	0.780	*β*-Ionone [h]	1481	1487	0.30[e] (10.1)	0.85 (30.4)	0.22 (15.9)	-	0.07 (26.4)
56	900	0.970	Methyl dihydrojasmonate	1650	1660	-	-	0.10 (5.7)	-	0.02[f]
					Subtotal (GC Peak Area)	**39.31**	**68.4**	**15.67**	**6.4**	**17.54**
					Subtotal (%)	**12.7**	**23.2**	**12.5**	**3.8**	**17.4**
					Subtotal (Number of Compounds)	**12**	**12**	**10**	**5**	**9**
			Sesquiterpenic compounds							
			Hydrocarbon-type							
57	678	0.490	*β*-Cubebene	1387	1354	0.08 (2.4)	0.18 (26.0)	1.26 (4.7)	0.06 (26.1)	0.08[f]
58	702	0.500	*α*-Copaene [h]	1375	1385	0.52 (30.3)	0.82 (19.1)	0.60 (6.8)	0.58 (20.7)	0.27 (4.6)
59	708	0.510	*β*-Bourbonene	1379	1393	-	0.83 (15.4)	-	-	-
60	714	0.540	*β*-Elemene	1380	1401	-	-	-	-	-
61	726	0.540	Longifolene	1395	1431	1.40 (14.8)	3.16 (10.3)	-	-	0.72 (24.5)
62	738	0.540	*β*-Caryophyllene [h]	1417	1432	1.42 (10.7)	17.16 (14.8)	0.40 (22.5)	1.88 (6.3)	0.31 (24.2)
63	750	0.480	Aromadendrene	1447	1447	17.05 (5.5)	3.58 (10.1)	6.35 (25.0)	2.50 (11.8)	5.03 (23.8)
64	762	0.560	*α*-Humulene [h]	1450	1462	1.58 (7.3)	2.41 (21.0)	0.66 (5.0)	-	-
65	792	0.506	*α*-Muurolene	1490	1499	1.30 (21.5)	0.26 (18.3)	2.34 (19.7)	1.12 (10.3)	-
66	810	0.570	*Δ*-Cadinene	1525	1528	0.32 (29.2)	0.15 (23.3)	1.04 (25.8)	-	0.28 (26.1)
67	810	0.630	Calamenene	1525	1528	0.21 (28.1)	0.11 (25.8)	1.17 (23.9)	0.04 (29.3)	0.10 (19.6)
68	828	0.680	*α*-Calacorene	1554	1556	0.08[f]	0.11e (3.4)	-	-	-
					Subtotal (GC Peak Area)	**23.69**	**28.7**	**13.11**	**6.2**	**6.74**
					Subtotal (%)	**7.7**	**9.7**	**10.5**	**3.7**	**6.7**
					Subtotal (Number of Compounds)	**10**	**11**	**8**	**6**	**7**
			Oxygen containing							
69	822	0.810	*β*-Bourbonen-13-ol	-	1547	-	-	-	-	-
70	840	0.670	Epiglobulol	1588	1572	0.68 (28.2)	0.49 (21.8)	0.68 (16.4)	1.04 (27.1)	-
71	858	0.710	Caryophyllene oxide	1610	1601	2.34 (25.1)	0.72 (13.7)	2.14 (1.0)	2.25 (29.7)	0.33 (16.0)
72	870	0.730	Globulol	1592	1618	2.79 (4.3)	0.13 (3.1)	-	-	-
73	888	0.730	Cubenol	1643	1643	-	-	-	0.30 (16.7)	-
74	894	0.830	*t*-Cadinol	1650	1651	0.21 (2.0)	0.19 (6.0)	1.45 (22.1)	-	-
75	918	0.900	*m/z* 91, 119, 105	-	1685	0.34 (13.3)	0.30 (19.1)	0.45 (6.4)	0.33 (5.6)	0.21 (2.4)
76	1014	0.710	*m/z* 69, 41, 55	-	1841	0.60 (26.7)	0.64 (23.8)	0.57 (15.4)	-	0.11 (13.4)
					Subtotal (GC Peak Area)	**7.05**	**2.2**	**5.27**	**3.9**	**0.66**
					Subtotal (%)	**2.3**	**0.7**	**4.2**	**2.4**	**0.7**
					Subtotal (Number of Compounds)	**6**	**6**	**5**	**4**	**3**
					Total	**308.47**	**295.2**	**125.27**	**166.8**	**100.70**
					Number of identified compounds	**64**	**63**	**55**	**43**	**47**

a Retention times for first (1tR) and second (2tR) dimensions in seconds.

b RI, Retention Index reported in the literature for Equity-5 GC column or equivalents.

c RI: Retention Index obtained through the modulated chromatogram.

d Mean of three independent assays (n=3). GC peak areas were expressed as arbitrary units.

e The compound was detected in two assays.

f The compound was detected in one assay.

g The relative amount (%) for the different identified chemical families represents their relative content compared to the total content.

h Identification confirmed by chemical standards

i The used standards are a mixture of isomers