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Unveiling elderflowers (*Sambucus nigra* L.) volatile terpenic and norisoprenoids profile: Effects of different postharvest conditions

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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.

1. Introduction

Sambucus 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 (CHMP, 2008). 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 (Jorgensen, Hansen, Christensen, Jensen, & Kaack, 2000; Kaack & Christensen, 2008). 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 (Jorgensen et al., 2000; Kaack, Christensen, Hughes, & Eder, 2006; Toulemonde & Richard, 1983). The monoterpenic metabolites, such as hotrienol, rose oxides, nerol oxide, linalool oxides, α-terpineol and linalool, were reported as the major contributors to the characteristic elderflower aroma (Jorgensen et al., 2000; Kaack et al., 2006). Despite the role of esters, alcohols and aldehydes, monoterpenes, such as limonene, terpinolene and terpinenes present a relevant contribution to the elderflowers' fruity aroma (Kaack et al., 2006). More exotic notes, such as woody and spicy, have been attributed to some mono- and sesquiterpenic compounds and norisoprenoids (Jorgensen et al., 2000; Kaack et al., 2006). 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 (Petronilho, Maraschin, Coimbra, & Rocha, 2012; Vinholes et al., 2014). According to the literature, 35 mono- and sesquiterpenic metabolites have been detected in elderflowers and related products, namely processed flowers or infusions (Eberhardt & Pfannhauser, 1985; Farré-Armengol, Filella, Llusià, & Peñuelas, 2015; Jorgensen et al., 2000; Kaack, 2008; Kaack & Christensen, 2008; Kaack et al., 2006; Toulemonde & Richard, 1983).

Flowering of *S. nigra* occurs from May to June, depending on the cultivars, geographic location and climatic conditions (Atkinson & Atkinson, 2002), 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 and texture. Elderflower formulations are normally prepared from fresh, frozen

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(Christensen, Kaack, & Fretté, 2008) or dried flowers (Kaack & Christensen, 2008), 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 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 (Kaack & Christensen, 2008); moreover, alterations in the levels of volatile terpenic components were reported, namely, linalool and linalool oxides when elderflowers were deep frozen or dried at 60 °C (Siegmund, Innerhofer, Pabi, Fedl, & Leitner, 2013).

Considering the interest in the volatile terpenic metabolites of elderflower aroma, and also taking into account their potential health benefits, the present study intended to establish the profile of 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). Norisoprenoids were also screened, as they may contribute to the elderflower's 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. Materials 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 norisoprenoid 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) (Sumner et al., 2007). These stages are described in detail in the following sub-sections.

2.1. 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); ( )-limonene oxide (97%, mixture of isomers) and methyl geranate were purchased from Aldrich (Milwaukee, WI); and linalool oxide (97% mixture of isomers) from TCI Europe (Zwijndrecht, Belgium). The retention index probe (a 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 (Bellefonte, PA). The SPME device included a 1-cm StableFlex™ fused silica fiber, coated with partially cross-linked 50/30 μm divinylbenzene/Carboxen™/poly(dimethylsiloxane) (DVB/CAR/PDMS). 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 °C for 60 min in the GC injector and daily for 10 min at 250 °C.

2.2. Elderflower sampling, handling and storage

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 (41.043233°N, 7.728820°W) of 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, *ca.* 1.5 kg per cultivar. Samples were immediately transported under refrigeration (2 4 °C) to the laboratory and then handled, stored and analyzed as described below (Fig. 1).

Fresh elderflower samples were first analyzed on the harvesting day. They were then submitted to different handling and storage conditions (Fig. 1): i) freezing and subsequent storage in polyethylene freezer bags at 20 °C (2 freezer bags were prepared for each cultivar); ii) air drying (flowers hung upside-down, at 19 21 °C 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 prior to 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 on 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. Volatile terpenic and norisoprenoid metabolites determination by HS-SPME/GC × GC-ToFMS

The HS-SPME and GC × GC-ToFMS experimental parameters were adapted from a previous study developed to characterize the volatile terpenic and norisoprenoid metabolites from elderberries (Salvador, Rudnitskaya, Silvestre, & Rocha, 2016). 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 sili cone/polytetrafluoroethylene septum and an aluminum cap (Chromacol Ltd, Welwyn Garden City, UK), and placed in a thermostated bath adjusted to 40.0 ± 0.1 °C. 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) consisting of an Agilent GC 7890A gas chromatograph (Agilent Technologies, Inc., Wilmington, DE), with a

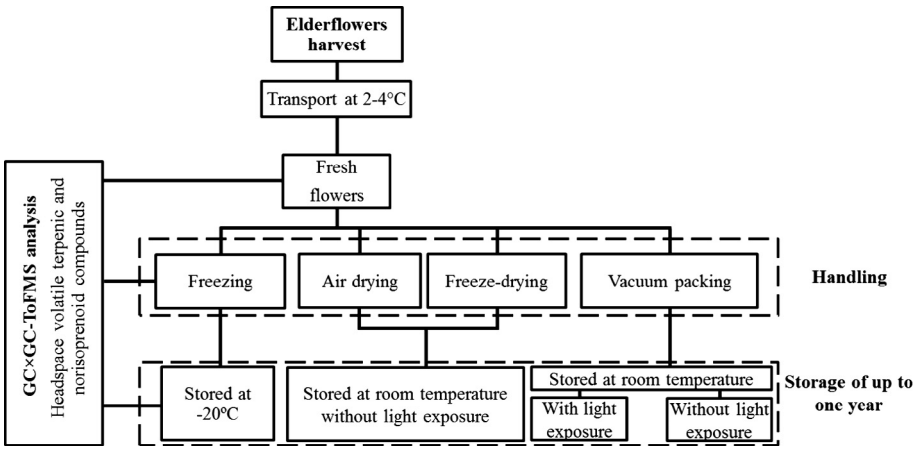

Fig. 1. Main stages of elderflower handling and storage.

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 °C 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 column (30 m × 0.32 mm I.D., 0.25 μm film thickness; Supelco, Bellefonte, PA) was used as the first-dimension column (^{1}D) and a DB-FFAP column (0.79 m × 0.25 mm I.D., 0.25 μm film thickness; J&W Scientific Inc., Folsom, CA) was used as the 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 °C (1 min) to 230 °C (3 min) at 10 °C/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 of 0.90 and 2.10 s, respectively. The mass spectrometer ran in 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 spectra 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, the GC peak area being proportional to the generated signal intensity (Dallüge, Beens, & Brinkman, 2003). Contour plots were used to evaluate the general separation quality and for manual peak identification. For identification purposes, the mass spectra 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 1 and Table S1 from Supplementary Information). Also, the mass spectrum of each detected metabolite was compared with 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 (van den Dool & Kratz, 1963). A C_8 C_{20} *n*-alkanes series was used for RI determination, comparing these values with reported in the literature for chromatographic columns similar to the above mentioned ^{1}D column (Adams, 1995; Dötterl, Wolfe, & Jürgens, 2005; Flamini, Cioni, & Morelli, 2002; Jalali et al., 2012, 2013; Palic, Stojanovic, Alagic, Nikolic, & Lepojevic, 2002; Petronilho, Maraschin, Delgadillo, Coimbra, & Rocha, 2011; Petronilho, Rocha, Ramírez-Chávez, Molina-Torres, & Rios-Chavez, 2013; Rocha, Coelho, Zrostlikova, Delgadillo, & Coimbra, 2007; Rocha et al., 2013; Salvador et al., 2013; Saraiva et al., 2014; Skaltsa, Demetzos, Lazari, & Sokovic, 2003). 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.

2.4. Statistical analysis

A full data matrix consisting of 65 variables (metabolites) and 156 observations was constructed (Table S1, from Supporting Information). 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 times during 1 year.

In order to evaluate the effect of the handling and storage conditions *versus* fresh elderflower 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 S1), 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 interface) (Xia, Sinelnikov, Han, & Wishart, 2015), was applied on these matrices (data from Table S1). 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 S1, from Supporting Information). Heat map visualization was applied on this sub-dataset, using absolute GC peak area and transforming each variable GC peak area by applying a logarithmic 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) (Xia et al., 2015), to further examine the

Table 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 in elderflowers[f]
Monoterpenic compounds											
Hydrocarbon-type											
1	306	0.400	α-Pinene	941	932	$C_{10}H_{16}$	Pine, turpentine	Jalali et al. (2012)	–	1	–
2	348	0.440	β-Pinene	987	975	$C_{10}H_{16}$	Turpentine, resinous	Jalali et al. (2012)	–	1	–
3	360	0.460	Myrcene	1008	988	$C_{10}H_{16}$	Balsamic, herbaceous	Jalali et al. (2012)	–	1	✓
4	378	0.450	3-Carene	1020	1009	$C_{10}H_{16}$	Turpentine	Jalali et al. (2012)	–	2	✓
5	396	0.490	Limonene	1031	1027	$C_{10}H_{16}$	Fruity, orange, lemon	Salvador et al. (2013)	–	1	✓
6	396	0.550	*p*-Cymene	1039	1027	$C_{10}H_{14}$	Fruity, citrus	Jalali et al. (2012)	–	1	✓
7	414	0.490	Ocimene	1043	1051	$C_{10}H_{16}$	Floral, sweet	Jalali et al. (2012)	–	2	✓
8	426	0.500	γ-Terpinene	1080	1063	$C_{10}H_{16}$	Fruity, lime	Rocha et al. (2007)	–	1	✓
9	444	0.590	2,6-Dimethyl-2,6-octadiene	1064	1082	$C_{10}H_{18}$	–	Rocha et al. (2007)	–	2	–
10	450	0.510	α-Terpinolene	1097	1093	$C_{10}H_{16}$	Fruity, citrus	Jalali et al. (2012)	–	2	✓
11	456	0.660	*p*-Cymenene	1090	1094	$C_{10}H_{12}$	Citrus	–	Flamini et al. (2002)	2	–
12	480	0.620	Cosmene isomer	1134	1122	$C_{10}H_{14}$	Floral	–	Flamini et al. (2002)	2	–
13	492	0.610	Cosmene isomer	1134	1134	$C_{10}H_{14}$	Floral	–	Flamini et al. (2002)	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	Rocha et al. (2007)	–	2	–
16	402	0.500	1,8-Cineole	1039	1034	$C_{10}H_{18}O$	Peppermint, menthol	Jalali et al. (2012)	–	1	✓
17	432	0.850	Myrcenol	1103	1069	$C_{10}H_{18}O$	Rose	Jalali et al. (2012)	–	2	–
18	438	0.680	Linalool oxide (furanoid) isomer[g]	1078	1076	$C_{10}H_{18}O_2$	Elderflower, sweet	Jalali et al. (2012)	–	1	✓
19	450	0.620	Fenchone	1093	1090	$C_{10}H_{16}O$	Camphoraceous	Jalali et al. (2012)	–	2	–
20	450	0.910	Hotrienol isomer	1122	1090	$C_{10}H_{16}O$	Elderflowers, floral	Jalali et al. (2012)	–	2	✓
21	456	0.640	Linalool oxide (furanoid) isomer[g]	1097	1094	$C_{10}H_{18}O_2$	Elderflower, sweet	Jalali et al. (2012)	–	1	✓
22	462	0.790	Linalool	1096	1101	$C_{10}H_{18}O$	Floral	Salvador et al. (2013)	–	1	✓
23	468	0.680	Thujone	1120	1108	$C_{10}H_{16}O$	Minty	Jalali et al. (2012)	–	1	✓
24	468	0.900	Hotrienol isomer	1122	1108	$C_{10}H_{16}O$	Elderflowers, floral	Jalali et al. (2012)	–	2	✓
25	474	0.640	Rose oxide[g]	1130	1115	$C_{10}H_{18}O$	Floral, elderflower	Jalali et al. (2012)	–	1	✓
26	492	0.490	Linalool, methyl ether	1137	1137	$C_{11}H_{20}O$	–	–	Adams (1995)	2	–
27	498	0.620	Limonene oxide[g]	1127	1144	$C_{10}H_{16}O$	Camphoraceous	Jalali et al. (2012)	–	1	–
28	504	0.650	Tagetone	1146	1150	$C_{10}H_{16}O$	Bitter fruity	–	Adams (1995)	2	–
29	510	0.620	Citronellal	1159	1154	$C_{10}H_{18}O$	Lemon, citronella	Jalali et al. (2012)	–	1	–
30	510	0.740	Lilac aldehyde isomer	1154	1155	$C_{10}H_{16}O_2$	Floral	–	Dötterl et al., 2005	2	–
31	516	0.600	Nerol oxide	1153	1162	$C_{10}H_{16}O$	Elderflower syrup	–	Adams (1995)	2	✓
32	516	0.675	Lilac aldehyde isomer	1163	1164	$C_{10}H_{16}O_2$	Floral	–	Dötterl et al., 2005	2	–
33	522	0.740	Lilac aldehyde isomer	1174	1172	$C_{10}H_{16}O_2$	Floral	Rocha et al. (2007)	–	2	–
34	528	1.090	Linalool oxide (pyranoid)	1174	1177	$C_{10}H_{18}O_2$	Floral	–	Flamini et al. (2002)	2	✓
35	534	0.700	4-Terpineol	1181	1181	$C_{10}H_{18}O$	Woody	Jalali et al. (2012)	–	1	✓
36	546	1.790	Myrtenol	1206	1194	$C_{10}H_{16}O$	Camphoraceous, minty	Jalali et al. (2012)	–	2	–
37	552	0.850	α-Terpineol	1206	1201	$C_{10}H_{18}O$	Sweet, floral	Jalali et al. (2012)	–	1	✓
38	564	0.880	Verbenone	1214	1216	$C_{10}H_{14}O$	Minty, spicy	Jalali et al. (2012)	–	1	–

(*continued on next page*)

Table 1 (*continued*)

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 in elderflowers[f]
39	564	0.925	Lilac alcohol	1237	1217	$C_{10}H_{18}O_2$	Green, grassy	Rocha et al. (2007)	–	2	–
40	582	0.980	Nerol	1242	1236	$C_{10}H_{18}O$	Floral, citrus	Jalali et al. (2012)	–	2	✓
41	588	0.770	Citral[g]	1241	1244	$C_{10}H_{16}O$	Lemon, lime peel	Rocha et al. (2007)	–	1	–
42	594	1.040	Geraniol	1235	1254	$C_{10}H_{18}O$	Woody, leaf	Jalali et al. (2012)	–	1	✓
43	600	0.580	Methyl Citronellate	1261	1261	$C_{11}H_{20}O_2$	Fruity, Apple	–	Adams (1995)	2	–
44	612	0.620	Citronellyl formate	1275	1274	$C_{11}H_{20}O_2$	Fruity	–	Adams (1995)	2	–
45	612	0.800	Geranial	1287	1274	$C_{10}H_{16}O$	Lemon	Rocha et al. (2007)	–	2	–
46	654	0.500	Methyl geranate	1323	1325	$C_{11}H_{18}O_2$	Waxy	–	Adams (1995)	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	Petronilho, Rocha, Ramírez-Chávez, Molina-Torres, & Rios-Chavez (2013)	–	2	–
49	702	0.470	α-Copaene	1375	1385	$C_{15}H_{24}$	Woody	Jalali et al. (2013)	–	1	✓
50	708	0.480	β-Bourbonene	1379	1393	$C_{15}H_{24}$	Herbaceous, woody	Jalali et al. (2013)	–	2	–
51	714	0.490	β-Elemene	1380	1401	$C_{15}H_{24}$		Jalali et al. (2013)	–	2	–
52	726	0.567	α-Bergamotene	1439	1416	$C_{15}H_{24}$	Woody	Petronilho et al. (2013)	–	2	–
53	732	0.500	β-Caryophyllene	1417	1432	$C_{15}H_{24}$	Woody, spicy	Jalali et al. (2013)	–	1	✓
54	756	0.515	Aromadendrene	1447	1455	$C_{15}H_{24}$	Cucumber	Petronilho et al. (2013)	–	2	–
55	762	0.540	α-Humulene	1450	1462	$C_{15}H_{24}$	Woody	Jalali et al. (2013)	–	1	–
56	780	0.540	Germacrene D	1473	1486	$C_{15}H_{24}$	Woody	Jalali et al. (2013)	–	2	–
57	798	0.520	α-Farnesene	1507	1510	$C_{15}H_{24}$	Herbal	Saraiva et al. (2014)	–	2	–
58	810	0.540	δ-Cadinene	1525	1528	$C_{15}H_{26}$	Woody	Jalali et al. (2013)	–	2	–
59	810	0.600	Calamenene	1525	1528	$C_{15}H_{22}$	Spicy, floral	Petronilho et al. (2011)	–	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	Rocha et al. (2007)	–	2	✓
62	572	0.705	β-Cyclocitral	1261	1225	$C_{10}H_{16}O$	Woody	Rocha et al. (2007)	–	2	–
63	632	0.515	Dihydroedulan	1289	1300	$C_{13}H_{22}O$	Elderberry	–	Skaltsa et al. (2003)	2	–
64	756	0.660	Geranyl acetone	1454	1455	$C_{13}H_{22}O$	Floral, rose	Rocha et al. (2013)	–	1	–
65	1020	0.570	Phytone	1856	1854	$C_{18}H_{36}O$	–	–	Palic et al. (2002)	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 (Breitmaier, 2006; Burdock, 2009; Jensen et al., 2001; Kaack & Christensen, 2008; Surburg & Panten, 2006).
[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 (Sumner et al., 2007).
[f] Compound previously reported on elderflowers (Eberhardt & Pfannhauser, 1985; Farré-Armengol et al., 2015; Jorgensen et al., 2000; Kaack & Christensen, 2008; Kaack et al., 2006; Toulemonde & Richard, 1983).
[g] The used standards are a mixture of isomers.

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.

3. Results and discussion

3.1. Fresh elderflowers volatile terpenic and norisoprenoid profile

A representative extracted ion GC × GC–ToFMS chromatogram contour plot from the fresh 'Sabugueiro' elderflower cultivar is illustrated in Fig. 2. 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 sesquiterpene 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 in 'Sabugueira' and 'Sabugueiro' *S. nigra* flowers (Table 1), being distributed over 47 monoterpenic (monoterpenes and oxygen-containing monoterpenes), 13 sesquiterpenes and 5 norisoprenoid metabolites. Among

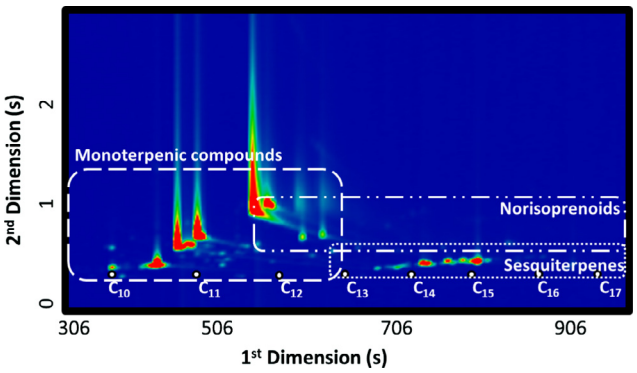

Fig. 2. Blow up of GC × GC–ToFMS extracted ion chromatogram contour plot of *m*/*z* 93, 161, and 204 of the released volatile compounds from 'Sabugueiro' fresh elderflowers. The white spots indicate the position of the series of alkanes (C_{10} C_{17}). The chromatographic areas corresponding to monoterpenic, norisoprenoid and sesquiterpene compounds are highlighted.

these, 38 are reported for the first time in elderflowers (highlighted in Table 1), resulting in a significant expansion of the *S. nigra* flower chemical profile, only possible through the use of 2D orthogonal GC analysis.

The aroma descriptors associated with the elderflowers volatile terpenic compounds and norisoprenoids (Table 1) may contribute to explaining the sensory properties attributed to elderflowers, which are in line with the sensorial characteristics described in previous olfactory studies (Jorgensen et al., 2000; Kaack et al., 2006; Toulemonde & Richard, 1983). 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) (Breitmaier, 2006; Burdock, 2009; Jensen, Christensen, Hansen, Jørgensen, & Kaack, 2001; Kaack & Christensen, 2008; Surburg & Panten, 2006) (Table 1). These compounds may contribute to explain the floral, fruity, grassy, woody, minty, spicy and herbaceous notes (Jorgensen et al., 2000) that currently characterize the aroma of elderflowers. It is worth mentioning that the major contributors to the characteristic elderflower aroma, i.e., hotrienol, rose oxide, nerol oxide, linalool oxides, α-terpineol and linalool (Jorgensen et al., 2000; Kaack et al., 2006) were identified in fresh elderflowers (Table 1).

The heat maps from Fig. 3 reveal information about the volatile terpenic compounds and norisoprenoid 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). As observed by the red color, linalool oxide (in pyranoid form) represents the major component for both cultivars (Fig. 3A and *Table S1 from* Supplementary Information), 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 allow in-depth analysis of all the detected metabolites (Fig. 3B). Oxygen-containing monoterpenes prevailed (colors orange and red) (Fig. 3B), 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 S1 from Supplementary Information). Hierarchical cluster analysis (included in Fig. 3A 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 1),

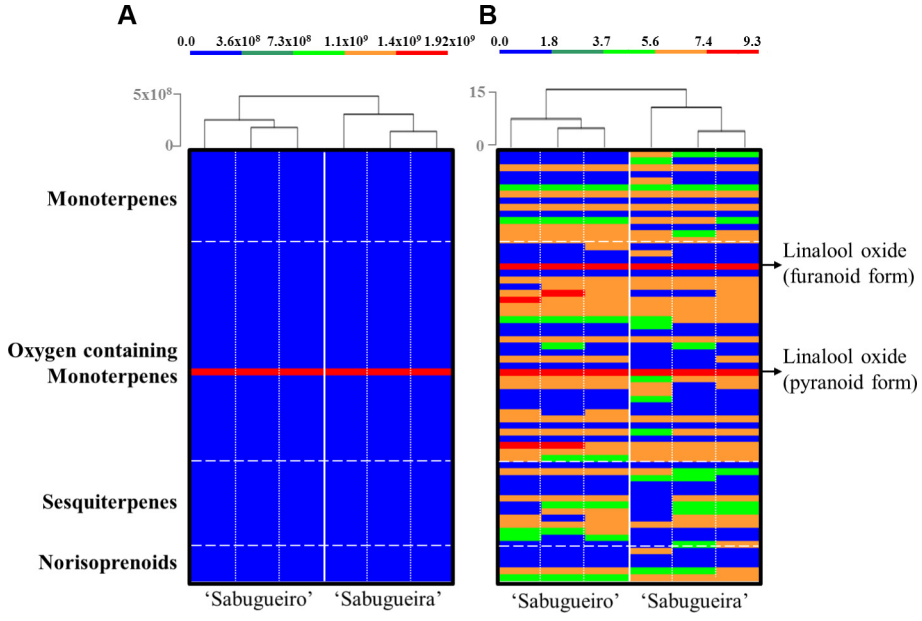

Fig. 3. Heat map representation of the 65 metabolites (putatively) identified from 'Sabugueira' and 'Sabugueiro' fresh elderflowers: (A) GC peak areas, expressed as arbitrary units. (B) GC peak areas normalized by logarithm function. Each compound content was illustrated through different colors (from 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.

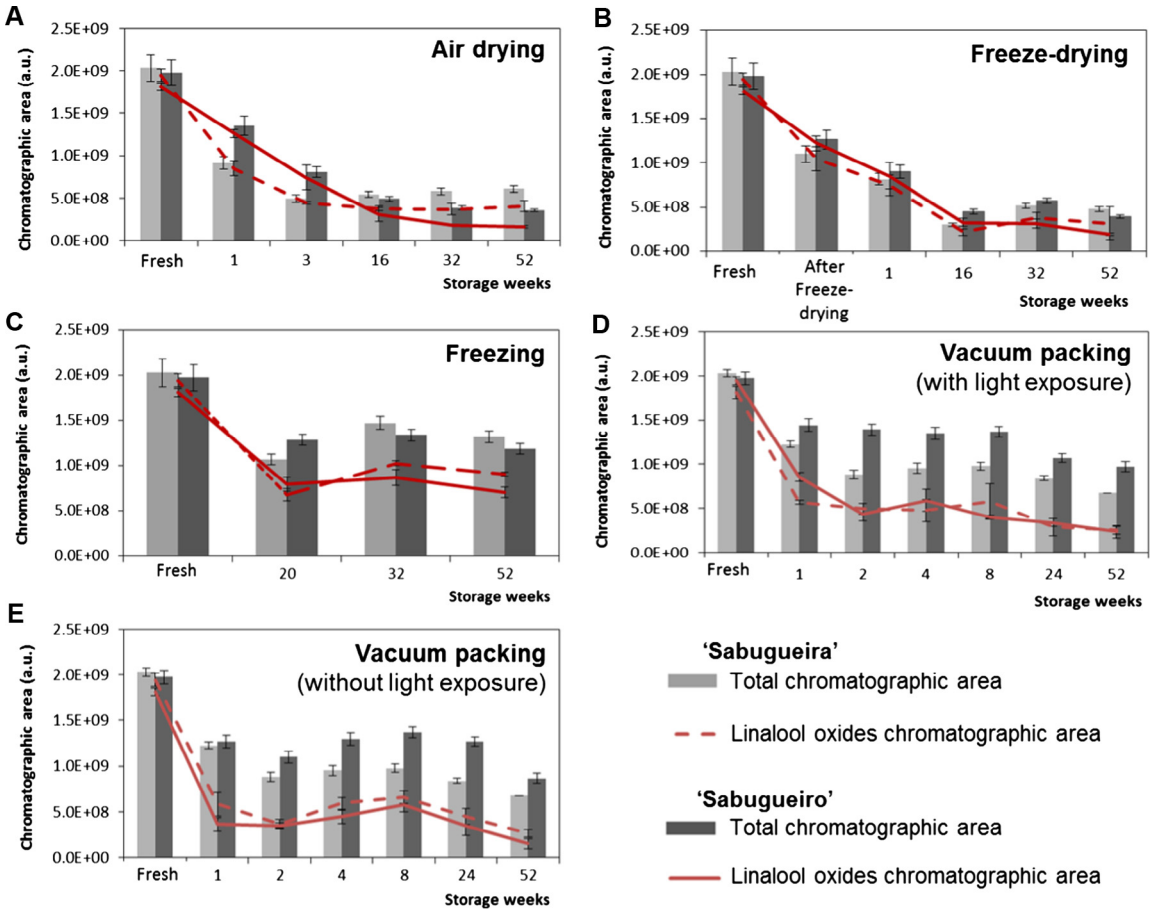

Fig. 4. Released content of terpenic and norisoprenoid metabolites from 'Sabugueira' and 'Sabugueiro' elderflowers handled by different processes and storage conditions for up to 1 year: Air drying (A) and freeze-drying (B) and subsequent storage at room temperature; freezing (C) and subsequent storage at −20 °C; 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 aroma descriptors of the metabolites associated with 'Sabugueiro' cultivar are floral, rose, elderflower, sweet and woody; while the metabolites with citronella, citrus, lemon, herb, woody, pine and turpentine aroma notes were linked to 'Sabugueira'. The specific cultivar metabolite profile with analogous aroma descriptors may imply differences at the sensorial level in elderflower-based products, as already documented for other elderflower cultivars (Jorgensen et al., 2000; Kaack et al., 2006). More studies, including sensory ones, should be performed to relate the elderflower volatile composition of different cultivars to their overall character.

3.2. Insights of handling and storage impact on the volatile terpenic and norisoprenoid 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 to 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 (Fig. 4). 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 (Fig. 5).

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, Fig. 4C) to 36.2 85.2% (freeze-dried elderflowers, Fig. 4B), 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, an overall decrease of metabolites content of 27.0 58.6% and 30.7 52.2% was observed for vacuum packing with light and without light exposure (Fig. 4D and E), respectively, and 31.5 80.3% for air drying (Fig. 4A). 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 (Fig. 4C); up to 67.1% and 70.7%, when the elderflowers were vacuum packed and kept under light exposure (Fig. 4D) and without light exposure (Fig. 4E), respectively; up to 81.9% for air drying (Fig. 4A); and up to 85.2% for freeze-dried elderflowers (Fig. 4B). 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 (Rahimmalek & Goli, 2013). Under vacuum packing, the light parameter impact was not clear, since a similar trend is noticeable between the two conditions (Fig. 4D and E).

It is worth mentioning that a general perspective of postharvest impact is presented, although, a complex and dynamic process was observed, being discussed in detail with the support of Fig. 5. The reported results for dried elderflowers (Fig. 4A and B), are in line

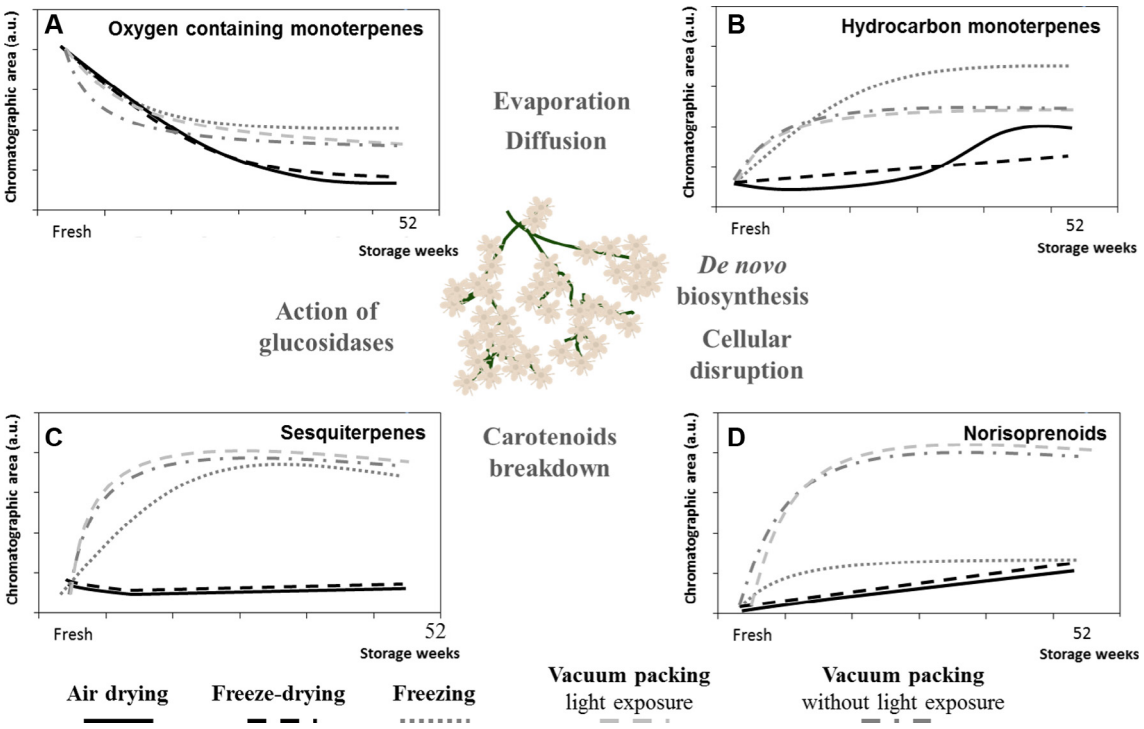

Fig. 5. Variation trends of the GC peak areas of oxygen-containing monoterpenes (A), hydrocarbon monoterpenes (B), sesquiterpenes (C) and norisoprenoids (D) towards the different handling and storage conditions for up to 1 year. Main physicochemical processes that explain the observed variations are also listed in the central part of the figure (de Ancos et al., 2000; Díaz-Maroto et al., 2002a; Kaack & Christensen, 2008; Venskutonis, 1997; Venskutonis et al., 1996). a.u.: arbitrary units.

with previous studies, as most drying methods often fail to fully preserve volatile aroma compounds (Abascal, Ganora, & Yarnell, 2005) in air dried vegetable matrices, such as elderflowers (Kaack & Christensen, 2008), thyme leaves (*Thymus vulgaris* L.) and sage leaves (*Salvia officinalis* L.) (Venskutonis, 1997; Venskutonis, Poll, & Larsen, 1996); as well as freeze-dried bay leaves (*Laurus nobilis* L.) (Díaz-Maroto, Pérez-Coello, & Cabezudo, 2002a) and parsley leaves (*Petroselinum crispum* L.) (Díaz-Maroto, Pérez-Coello, & Cabezudo, 2002b). Likewise, for the other postharvest processes, the reported results are in agreement with previously reported data, namely for frozen elderflowers (Siegmund et al., 2013), and others plants, such as *L. nobilis* leaves (Díaz-Maroto et al., 2002a), tomatoes (Wu, Jadhav, & Salunke, 1972) and several apple cultivars (Wang & Dilley, 2000) stored under sub-atmospheric pressure.

Fig. 4A to E show that, in general, the overall trends observed for the targeted chemical families were defined by the major metabolites, linalool oxides, which may be suggested as markers that illustrate the impact of the studied postharvest conditions on the overall terpenic and norisoprenoid metabolites. In order to better understand the underlying complexity of the impact of different postharvest handling and storage conditions on 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 (Fig. 5A 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 (de Ancos, Ibañez, Reglero, & Cano, 2000; Díaz-Maroto et al., 2002a; Kaack & Christensen, 2008; Venskutonis, 1997; Venskutonis et al., 1996).

The oxygen-containing monoterpenes tended to decrease with storage (Fig. 5A), which may be explained mainly due to diffusion and evaporation processes (Kaack & Christensen, 2008; Venskutonis, 1997; Venskutonis et al., 1996); cellular disruption caused by freezing may also enhance the release of volatile compounds that were retained in vegetable cells (de Ancos et al., 2000); and upon vacuum, the removal of aroma compounds occurs (Wang & Dilley, 2000; Wu et al., 1972). 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 in air-dried elderflowers, which has already been reported for this matrix and process, being associated with the action of enzymes such as glucosidases that unbind the volatile components from glycosides present in the matrix (Kaack & Christensen, 2008). The increasing content of this metabolite during storage is also observed for the other studied postharvest conditions (freeze-drying, freezing and vacuum packing). The levels of nerol oxide and α-terpineol, other important contributors to the characteristic elderflower aroma, were also higher in stored elderflowers than in fresh ones, for all storage conditions and for both cultivars.

Concerning hydrocarbon monoterpenes (Fig. 5B) and norisoprenoids (Fig. 5D), 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. Likewise, the sesquiterpene levels tended to increase through storage (Fig. 5C) 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 in both cultivars; they are metabolites described as having fruity, orange, lemon, floral and rose aroma notes. For frozen storage, other metabolites that defined these changes were ocimene (floral and sweet aroma notes), β-caryophyllene (woody and spicy), α-farnesene (herbal) in 'Sabugueira'; and ocimene (floral and sweet), α-pinene (pine and turpentine), β-caryophyllene (woody and spicy), and geranyl acetone (floral and rose) in 'Sabugueiro'. Regarding vacuum packing, limo-

nene (fruity, orange and lemon), β-caryophyllene (woody and spicy) and dihydroedulan (elderberry) contributed to the higher levels of mono-, sesquiterpenes and norisoprenoids in both cultivars. The *de novo* biosynthesis of terpenes and carotenoids breakdown during the drying process may explain the higher levels of these families (Kaack & Christensen, 2008). Increasing levels of sesquiterpenes and norisoprenoids were also reported in other matrices, namely in frozen raspberries (*Rubus idaeus* L.) (de Ancos et al., 2000) and *L. nobilis* leaves (Díaz-Maroto et al., 2002a). During frozen 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 (Kjeldsen, Christensen, & Edelenbos, 2003), 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 in the levels of other important metabolites characteristic of the elderflower aroma, suggests that these handling and storage conditions might have impact on the characteristic aroma of fresh elderflowers.

4. Conclusions

The volatile terpenic and norisoprenoid profiles from *S. nigra* flowers of two cultivars were established in-depth using GC × GC-ToFMS, revealing 65 metabolites, of which 38 are reported for the first time in this species. The data regarding the metabolites reported for the first time, alongside data collected from the literature about their respective aroma descriptors, highlighted the relation of these metabolites with the elderflower 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 to *S. nigra* flowers terpenic and norisoprenoid profile construction, providing a step forward in their valorization and understanding of their role as flavoring agents.

Postharvest impact was assessed up to one year of storage, a decrease in the overall volatile terpenic and norisoprenoids content for all tested conditions being observed, when compared to fresh elderflowers ($p < 0.05$). However, levels of monoterpenic hydrocarbons and norisoprenoids were higher in 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 decrease in the terpenic and norisoprenoid overall levels. Linalool oxides defined, in general, the observed overall trends, being, suggested as markers to quickly estimate the impact of the studied postharvest conditions on 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 physicochemical phenomena. All this comprehensive data should be helpful in understanding the underlying mechanisms of postharvest variation, which may be later used to manage and control the production of elderflower flavoring agents.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.037.

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