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

Ângelo C. Salvador [a,b], Armando J.D. Silvestre [b], Sílvia M. Rocha [a,*]

[a] Department of Chemistry, QOPNA, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[b] Department of Chemistry, CICECO, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

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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

* Corresponding author.
E-mail address: smrocha@ua.pt (S.M. Rocha).

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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