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

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ABSTRACT

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

1. Introduction

Vaccinium padifolium, locally called Uveira, is a shrub from the family of Ericaceae endemic to Madeira Island (Portugal). It grows at relative high altitudes (800–1700 m) and its edible fruits (dark blue-black berries) have gained a remarkable worldwide interest due to appealing properties, such as color and size as well as reported benefits for human health associated with their consumption. These fruits, although not usually consumed directly, are used in a wide variety of foodstuffs, such as breakfast cereals, dairy products, juices, jams, liquors, jellies, yogurts, beverages and in dietary supplement forms (Seeram, 2008; Seeram et al., 2006). Furthermore, a growing body of evidence suggests that dietary intake of berries and berry phytochemicals has the potential to reduce the risk of various chronic diseases such as cancer, cardiovascular disease, respiratory diseases and cataracts as well as delay aging (Beattie, Crozier, & Duthie, 2005; Johnson, 2007). Other reports also point additional beneficial effects, such as anti-diabetic and anti-arthritis effects (Prior et al., 1998; Seeram, 2006; Seeram et al., 2006; Rivera & Obon, 1995; Vieira, 1992). These health-promoting properties are associated with the antioxidant and anti-inflammatory activities of a multitude of berry bioactive phytochemical components, including anthocyanins, phenolic acids, stilbenes, tannins and carotenoids. The identification of other secondary metabolites, namely volatile organic metabolites (VOMs), also needs to be explored.

Although Uveira berry aroma is widely recognized as an important attribute to quality and consumer acceptance, a limited number of studies deal with Uveira berries volatiles. This knowledge could be exploited by the food industry to improve the quality of endemic Uveira berries-based products. VOMs are biosynthesized through different metabolic pathways during fruit ripening (Aragüez & Valpuesta Fernández, 2013). Fatty acid metabolism leads to the formation of alcohols, aldehydes, ketones, acids, esters and lactones by lipoxygenase, α-oxidation and β-oxidation pathways (Aragüez & Valpuesta Fernández, 2013). In the same way, amino acid metabolism leads to the formation of benzenoids (C6-C1), phenylpropanoids (C6-C3), aldehydes, acids, alcohols, esters, C6-C2 compounds by biotransformation

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of L-phenylalanine and other amino acids through routes that compete with each other (Aragüez & Valpuesta Fernández, 2013). Several factors, such as the environmental conditions, genetic components and ripening stage, influence VOMs profiles and bioactive compound formation (El Hadi, Zhang, Wu, Zhou, & Tao, 2013).

To date, the effect of development and ripening on flavor compound accumulation has been investigated extensively in different fruits. In strawberries, C6 aldehydes are the major compounds in immature fruits, while furanones and esters are the main compounds in mature fruits (Ménager, Jost, & Aubert, 2004). In avocados, sesquiterpenes are the most abundant volatiles in early stages but these decrease during ripening (Pereira, Sargent, Tieman, Klee, & Huber, 2010). Volatile compounds are not only responsible for *V. padifolium* berry flavor, they also interact in the ecological network between plants and the environment, and respond to stress (e.g. herbivore attack or drought). Furthermore, terpenes are known to have bioactive properties (antimicrobial and anticarcinogen) (Paduch, Kandefer-Szerszeń, Trytek, & Fiedurek, 2007).

Despite studies conducted on *V. padifolium* berries, there is no information about the composition of volatile in Uveira berries cultivated on Madeira. Therefore, establishing the ripening volatomic pattern could provide important insights into biochemical transformations underlying the ripening physiological and biochemical processes, as key steps in crop quality providing useful information for producers in the appropriate selection of harvesting conditions and dates.

The main goal of this study was to investigate the evolution of volatile metabolites during ripening of Uveira berries, collected on Madeira (Portugal), helping to understand the significance of these compounds in the ripening process. For this purpose, three different ripening stages were considered (green, break and ripe). After optimization of the most relevant SPME experimental parameters, a detailed volatile profile was obtained using gas chromatography–quadrupole mass spectrometry (HS-SPME/GC-qMS) combined with multivariate statistical analysis. To best of our knowledge, there are no reported studies dealing with the effect of ripening stage on endemic Uveira berries volatile profile. The results obtained provide a powerful resource for uncovering components key to the regulation of metabolic networks and important information for future exploitation.

2. Material and methods

2.1. Chemical reagents

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

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

2.2. Sample preparation

Berries from endemic cultivar *of V. padifolium* (Uveira) were collected in a forest park (Montado do Pereiro, 32°42′40 N, 16°53′00 W) dominated by exotic and indigenous tree situated at the mountains (1200 meters above sea level) of Madeira Island, from an approximate area of 1 ha containing plants ≥ 20 years old. Uveira berries (100 g, green, breaker and ripe) were harvested morning (between 8 and 9 am) from six different plants to obtain representative samples for each ripening stage. Moreover, the samples were grown in the same agroclimatic conditions without any cultural intervention, minimizing the effects of different edaphoclimatic conditions on plant metabolism. Three lots of 100 g at different ripening stages were collected separately in sterile bags and immediately transported, under refrigeration (ca. 2–4 °C), to the laboratory, aliquoted (20 gr) and stored at −80 °C in amber vials until analysis.

2.3. HS-SPME optimization design

The development of HS-SPME procedures involves optimization of experimental factors that most influence the process in order to improve the extraction efficiency (Figueira, Câmara, Pereira, & Câmara, 2014). The effect of three independent factors (i) nature of the fibres, (ii) sample amount (2.5–5 g) and (iii) the exposure time (10–50 min) of the fibre to the headspace, on the SPME isolation of Uveira berry volatiles, were assayed and evaluated. For all the parameters, conditions were selected based on extraction efficiency expressed as numbers of isolated/identified volatile metabolites, total chromatographic area and reproducibility.

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

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

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

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

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

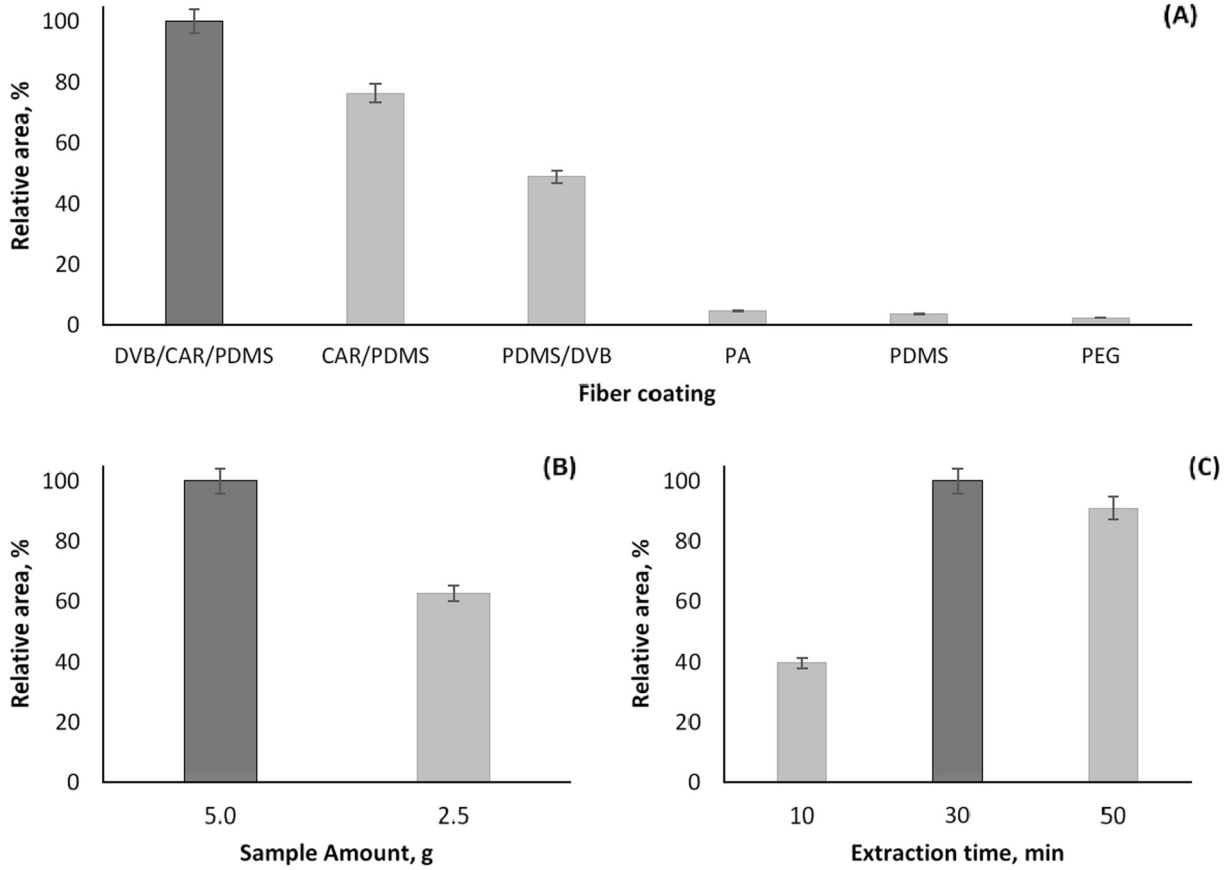

Fig. 1. Optimization of the HS-SPME-influencing extraction parameters on the extraction efficiency of volatile compounds from endemic Uveira (*V. padifolium*) berries (A) Influence of fibre coating (best fibre: DVB/CAR/PDMS); (B) Influence of sample amount (best sample amount: 5 g using 10% w/w NaCl, DVB/CAR/PDMS fibre coating and 30 min of extraction) and (C) Influence of extraction time (best extraction time: 30 min, using 10% w/w NaCl, DVB/CAR/PDMS fibre coating and 5g of sample). Thermal desorption of metabolites was carried out at 250 °C for 6 min. The results are the means of triplicates of the total areas.

initial temperature of 60 °C for 4 min, raised to 120 °C at 1 °C/min, held there for 4 min. Then, raised to 220 °C at 4 °C/min and held there for 5 min. The injection and ion source temperatures were 250 and 220 °C, respectively. The mass spectrometer was operated in electron-impact (EI) mode at 70 eV. The electron multiplier was set to the auto tune procedure. Data acquisition was performed in scanning mode (mass range *m/z* 35–300; six scans per second). Acquisition was made using MSD ChemStation software (Agilent Technologies, Palo Alto, CA, USA). Chromatograms and spectra were recorded and processed using the Enhanced ChemStation software for GC-$_q$MS (Agilent). The identification of volatile metabolites was based on comparisons between GC retention times (RT) for sample chromatogram peaks with those, when available, of authentic standards (ST) run under the same conditions. MS fragmentation patterns were compared with those of pure compounds, and a mass spectrum database search was performed, using the National Institute of Standards and Technology (NIST) MS 05 spectral database. Finally, confirmation was also achieved through determination of RI values for each metabolite, using a C_8-C_{20} *n*-alkanes series. Experimental RI values were compared with values reported in the literature for similar chromatographic columns. Chromatographic peak areas, expressed in arbitrary units (a.u.) of area, were used as an indirect approach to estimate the relative content of each volatile metabolite. For semi-quantification purposes, each sample was injected in triplicate and peak areas were determined by a reconstructed full-scan chromatogram using, for each volatile metabolite, some specific quantification ions: ionic fragment corresponding to peak base (100% intensity), molecular ion (M^+) and another characteristic ions for each molecule.

2.5. Data processing and statistical analysis

HS-SPME/GC-$_q$MS data matrix was submitted to statistical treatment to identify significant differences among Uveira ripening stages, using SPSS version 22.0 (SPSS Inc., 2006). Normality was tested using the Shapiro-Wilk test. Outliers were determined either numerically (normalized values higher than 3 and lower than −3) or graphically (boxplot chart) for all factor groups. The data were evaluated by one-way analysis of variance followed by Bonferroni's Post Hoc test, when homogeneity of variances was assumed, and Games-Howell's Post Hoc test when this assumption was not met. For all the analyses, a significance level of 5% (*p-value* < .05) was assumed to be significant.

Principal component analysis (PCA) was processed to reduce the dimensionality of dataset and, in addition, should reveal those variables or combination of variables that determine some inherent structure in the data. The number of principal components was based on the eigenvalue criterion. PCA reduces the dimensionality of the original data matrix while retaining the maximum amount of variability (Câmara, Alves, & Marques, 2006; Worley, Halouska, & Powers, 2013). This reduction allows visualization of the different ripening stages of Uveira berries in a two- or three-dimensional space, allowing identification of the directions in which most of the information is retained. With this unsupervised technique, it is possible to explain differences between ripening stages and visualize those variables that most contribute most to these differences (Câmara et al., 2006). The stability of the model was evaluated by "cross validation" to test the predictive capacity of the discriminant model (Ballabio & Consonni, 2013; Worley et al., 2013).

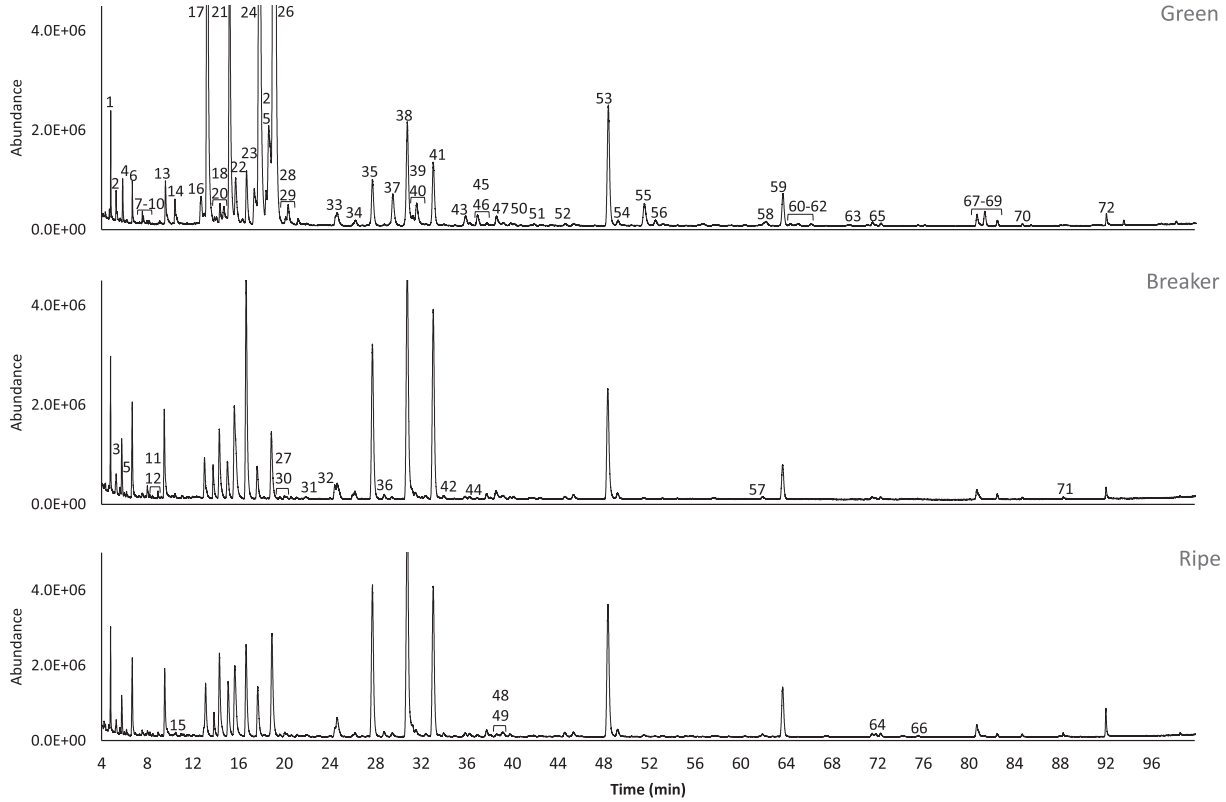

Fig. 2. Chromatographic profile of endemic Uveira fruits at different maturation stages, by HS-SPME using DVB/CAR/PDMS fibre (For identification numbers see Table 1).

3. Results and discussion

3.1. Optimization of HS-SPME extraction

The optimization of HS-SPME experimental parameters was crucial to improve extraction efficiency. Therefore, some key experimental factors, namely time required for the target analytes to reach equilibrium, nature of fibre coating and sample amount, were investigated. This procedure was conducted using univariate experimental design, considering one parameter at a time, while keeping all other variables the same.

3.1.1. SPME fibre selection

Six SPME fibres (PDMS, PEG, PA, CAR/PDMS, PDMS/DVB and DVB/CAR/PDMS) were chosen for assessment. The effectiveness of SPME fibres was assessed by semi-quantitative and qualitative analysis of chromatograms. Fig. 1A shows a typical comparison of the extraction efficiency of different fibre coatings for Uveira berries volatile metabolites. Non-polar (PDMS) and polar (PEG and PA) fibre coatings revealed a fair ability for the extraction of Uveira berries volatiles compared to semi-polar fibre coatings (i.e., DVB/CAR/PDMS, CAR/PDMS and PDMS/DVB) (Fig. 1A). The poor afinity of PEG and PA coatings for the terpenes and sesquiternes explained the trend observed (Fig. 1A). Considering the number of volatiles identified, the best performance was achieved by DVB/CAR/PDMS (58 metabolites) followed by CAR/PDMS (33 metabolites) > PDMS/DVB (18 metabolites) > PA (18 metabolites) > PEG (17 metabolites) and PDMS (13 metabolites), respectively. DVB/CAR/PDMS coating performed best for isolation of Uveira berry volatiles, achieving the highest number of extracted volatiles, highest signal intensity and best reproducibility (RSD < 10%). The bipolar DVB/CAR/PDMS coating (molecular weight ranging from 40 to 275) combined the absorption properties of the liquid polymer with the adsorption properties of porous particles, which contains macro (> 500 Å), *meso* (20–500 Å) and microporous (2–20 Å) {what? missing word – ed.}. The mutually synergetic effect of adsorption and absorption of the stationary phase explains its high retention capacity compared to PDMS coatings (Ceva-Antunes, Bizzo, Silva, Carvalho, & Antunes, 2006; Ong et al., 2008; Rega, Fournier, & Guichard, 2003).

3.1.2. Sample amount

Theoretically, optimum sample amounts can be selected based on the estimated sample/headspace/coating distribution constant. Generally, the amount of extracted analyte increases with the sample content up to a point, after which the sensitivity does not increase with further increasing sample amounts (Pawliszyn, 1997; Pawliszyn, 1999). In this assay, two different amounts (2.5 and 5.0 g) of ripe Uveira berries were used. The effect of sample amount on the extraction efficiency of VOMs is shown in Fig. 1B. The best performance was achieved using 5.0 g of sample, corresponding to a ratio of the volume of the liquid phase to the headspace volume (1/β) of about 0.5. For this reason, the amount used in subsequent experiments was 5.0 g.

3.1.3. Extraction time

SPME is an equilibrium extraction technique (Pawliszyn & Pedersen-Bjergaard, 2006; Pontes, Marques, & Câmara, 2009) and, therefore, the time required for volatile sampling also needs to be optimized. The influence of extraction time was evaluated by exposing DVB/CAR/PDMS fibre to the headspace of ripe Uveira berries for times ranging between 10 and 50 min. As shown in Fig. 1C, the best performance was obtained at 30 min. Since extraction efficiency can be affected by mass transfer kinetics of the volatiles in the HS-SPME procedure, the best time should correspond to a period of equilibrium between analyte and fibre coating. In general, a longer extraction period leads to an increase in the signal intensity of volatile compounds (until equilibrium is achieved). However, no significant changes were observed on increasing the extraction time up to 50 min (Fig. 1C). In addition, the numbers of VOMs isolated/identified using 30 and 50 min were almost

Table 1
Volatile metabolites identified at different ripening stages of Uveira berries using HS-SPME$_{DVB/CAR/PDMS}$/GC–MS methodology (extraction time: 30 min: extraction temperature: 40 °C; desorption of metabolites: 250 °C for 7 min).

#	[a]RT	Common Name	[b]KI $_{Exp}$	[c]KI $_{Teo}$	Ions	Maturation Stage (× 10^6)		
						Green	Breaker	Ripe
Higher alcohols								
4	6.05	Ethanol*	927	926	**31**; 45; 46	16.9^{e}	27.3^{f}	44.2^{g}
5	6.19	Pentyl Alcohol(c)	936	941	**55**; 70; 42; 41	n.d.^{e}	0.6^{f}	0.8 ^{g}
15	11.37	sec-Isoamyl alcohol(c)	1121	1124	**45**; 55; 27; 73	n.d.	n.d.	2.1^{e}
33	24.84	2-Heptanol	1319	1319	**45**; 55; 27	10.3^{e}	13.2^{f}	22.1^{g}
35	27.93	1-Hexanol	1353	1356	**56**; 43; 55; 31	32.3^{e}	101.9^{f}	128.7^{g}
36	28.96	trans-3-Hexenol	1364	1366	**41**; 67; 55; 82	0.7^{e}	2.2^{f}	3.2^{g}
38	31.01	cis-3-Hexenol	1384	1386	**67**; 41; 82; 55	52.2^{e}	121.4^{f}	161.3^{g}
41	33.28	trans-2-Hexenol	1405	1406	**57**; 41; 82;67	49.0^{e}	145.3^{f}	155.1^{g}
42	34.19	cis-2-Hexenol	1415	1418	**57**; 41; 67; 82	n.d.	1.8^{f}	2.7^{f}
46	37.97	3-Octenol	1452	1451	**57**; 72	2.6^{e}	4.9^{f}	8.0^{g}
48	39.36	6-Methylhept-5-en-2-ol	1465	1466	**91**; 41; 69; 55	0.7^{e}	0.8^{f}	1.4^{g}
51	42.11	2-Ethylhexanol	1489	1489	**57**; 41; 43; 56	1.3^{e}	1.6^{f}	1.6^{g}
54	49.48	1-Octanol	1558	1561	**55**; 56; 41; 69	1.3^{e}	1.4^{f}	2.6^{g}
64	72.03	1-Decanol	1763	1763	**55**; 41; 56; 69	n.d.	n.d.	0.9^{e}
69	82.71	Phenylcarbinol	1875	1876	**108**; 79; 107; 77	2.2^{e}	1.9^{f}	1.3^{g}
70	84.88	Benzyl Carbinol	1899	1899	**91**; 92; 122	1.6^{e}	1.4^{f}	2.9^{g}
		Σ Areas by variety				171.1^{e}	425.7^{f}	538.8^{g}
		N° of Compounds				12	14	16
Aldehydes								
3	5.80	Butyraldehyde	911	912	**57**; 41; 58; 86	0.4^{e}	1.8^{f}	1.8^{f}
13	9.78	Hexanal	1082	1083	**44**; 56; 41; 43	9.4^{e}	18.2^{f}	18.5^{f}
23	16.91	2-Hexenal	1219	1220	**41**; 55; 69; 83	13.8^{e}	59.6^{f}	32.3^{g}
31	22.07	Octanal (c)	1287	1287	**43**; 44; 56; 84	0.6^{e}	n.d.	n.d.
52	44.84	Benzaldehyde	1515	1515	**105**; 77; 106; 51	1.2^{e}	1.5^{f}	3.9^{g}
		Σ Areas by variety				25.4^{e}	81.1^{f}	56.5^{g}
		N° of Compounds				5	4	4
Fatty acids								
71	88.49	trans-2-Hexenoic acid	1972	1967	**73**; 42; 55; 99	0.4^{e}	0.6^{f}	1.6^{g}
72	92.25	Octanoic Acid	2045	2050	**60**; 73; 43; 55	3.7^{e}	3.7^{e}	10.7^{f}
		Σ Areas by variety				4.1^{e}	4.3^{e}	12.3^{f}
		N° of Compounds				2	2	2
Esters								
2	5.47	Ethyl Acetate	882	885	**43**; 61; 70	9.7^{e}	5.4^{f}	3.8^{g}
9	8.19	Ethyl butanoate	1032	1036	**43**; 71; 29; 88	0.5^{e}	2.0^{f}	1.1^{g}
11	8.65	Ethyl α-methylbutyrate	1048	1046	**57**; 102; 85; 41	n.d.	0.6^{e}	0.8^{f}
12	9.13	Ethyl isovalerate	1063	1062	**88**; 57; 85; 41	0.1^{e}	1.2^{f}	0.9^{g}
27	19.80	Z-Methyl 3-hexenoate	1259	1253	**41**; 68; 74; 59	n.d.	0.5^{e}	0.9^{f}
30	20.80	Hexyl acetate	1272	1274	**43**; 56; 84; 69	n.d.	1.5^{e}	1.4^{f}
32	24.63	3-Hexen-1-ol, acetate	1317	1314	**67**; 43; 82	5.5^{e}	8.0^{f}	8.5^{g}
39	31.47	Methyl caprylate	1388	1387	**74**; 87; 43; 55	5.6^{e}	5.7^{e}	8.6^{e}
43	36.11	Ethyl caprylate	1435	1435	**88**; 101; 127	3.5^{e}	0.8^{f}	1.5^{g}
47	38.81	cis-3-Hexenyl butyrate (c)	1460	1459	**67**; 82; 71; 43	6.0^{e}	4.2^{f}	0.9^{g}
50	40.37	trans-2-Hexenyl Butyrate	1474	1475	**71**; 43; 55; 67	1.2^{e}	2.0^{f}	0.2^{g}
57	62.11	α-Methylcaproic acid	1673	1625	**74**; 57;87;43	2.5^{e}	2.7^{f}	3.1^{g}
63	71.77	Methyl salicylate	1761	1758	**120**; 92; 152; 121	3.2^{e}	2.1^{f}	3.4^{e}
		Σ Areas by variety				37.8^{e}	36.7^{f}	35.1^{g}
		N° of Compounds				10	13	13
Ketones								
1	4.88	Acetone	811	813	**43**; 58	2.6^{e}	3.1^{f}	4.2^{g}
19	14.56	2-Heptanone	1182	1184	**43**; 58; 71	14.1^{e}	49.2^{f}	75.9^{g}
34	26.47	Methyl heptenone	1338	1339	**43**; 69; 108; 55	2.8^{e}	3.5^{f}	2.7^{e}
		Σ Areas by variety				19.6^{e}	55.9^{f}	82.9^{g}
		N° of Compounds				3	3	3
Monoterpenes								
7	7.77	α-Pinene(c)	1017	1019	**93**; 92; 91; 77	2.0^{e}	0.4^{f}	0.4^{g}
8	7.86	α-Thujene	1021	1023	**93**; 91; 77; 92	1.3^{e}	0.5^{f}	0.4^{g}
14	10.63	β-Pinene(c)	1105	1108	**93**; 41; 69; 77	9.8^{e}	n.d.	n.d.
16	12.91	β-Phellandrene	1153	1163	**93**; 77; 91; 136	10.6^{e}	n.d.	n.d.
17	13.48	β-Myrcene	1163	1165	**93**; 41; 69; 79	284.5^{e}	14.6^{f}	29.5^{g}
18	14.28	2-Carene(c)	1177		**93**; 121; 136; 91	1.3^{e}	n.d.	n.d.
20	14.90	Limonene(c)	1188	1189	**68**; 93; 67; 79	3.2^{e}	n.d.	n.d.
21	15.41	D-Limonene	1196	1200	**68**; 93; 67; 136;121	93.9^{e}	10.8^{f}	19.6^{g}
22	15.95	Eucalyptol	1205	1209	**93**; 43; 55; 108	9.3^{e}	7.6^{f}	7.0^{g}
24	18.03	trans-β-Ocimene	1236	1232	**93**; 91; 92; 79	406.8^{e}	15.2^{f}	31.0^{f}
25	18.62	γ-Terpinene	1244	1243	**93**; 91; 136; 121	7.6^{e}	n.d.	n.d.
26	19.29	cis-β-Ocimene	1253	1256	**93**; 91; 79; 77	826.4^{e}	28.4^{f}	60.5^{g}
28	20.32	m-Cymene	1266	1270	**119**; 134; 91	16.6^{e}	1.7^{f}	2.5^{g}

(*continued on next page*)

Table 1 (*continued*)

#	[a]RT	Common Name	[b]KI $_{Exp}$	[c]KI $_{Teo}$	Ions	Maturation Stage ($\times 10^6$)		
						Green	Breaker	Ripe
29	20.54	o-Cymene[(c)]	1269	1268	**119**; 134; 91	16.9[e]	n.d.	n.d.
37	29.71	Neo-allo-ocimene	1371	1378	**121**; 105; 136; 79	22.0[e]	1.5[f]	3.3[g]
40	31.81	trans-allo-ocimene	1391	1400	**121**; 136; 105; 79; 91	10.4[e]	1.1[f]	2.6[g]
44	36.42	Linalool oxide	1438	1443	**59**; 94; 43; 111	0.9[e]	1.0[e]	1.8[f]
45	37.15	Cosmene	1445	1460	**119**; 91; 134; 77	5.0[e]	0.6[f]	0.5[f]
49	39.99	Dihydromyrcenol	1471	1471	**59**; 43; 55; 67	1.3[e]	1.4[e]	2.8[f]
53	48.64	Linalool	1551	1552	**71**; 93; 41; 55	40.0[e]	39.8[e]	60.1[f]
59	63.92	α-Terpineol	1689	1689	**59**; 93; 121; 136	12.0[e]	11.6[e]	23.8[f]
65	72.51	Citronellol	1767	1764	**69**; 41; 81; 67	0.8[e]	0.8[e]	1.2[f]
66	75.75	cis-Geraniol	1795	1794	**69**; 41; 93; 68	0.4[e]	0.4[e]	0.9[f]
67	80.91	trans-Geraniol	1855	1854	**69**; 41; 93	5.7[e]	4.7[f]	8.1[g]
		Σ Areas by variety				1788.8[e]	142.1[f]	256.0[g]
		N° of Compounds				24	18	18
Sesquiterpenes								
55	51.75	Caryophyllene	1578	1580	**93**; 133; 91; 69	5.0[e]	n.d.	n.d.
56	52.76	Aromandendrene[(c)]	1587	1600	**161**; 41; 93; 107	1.1[e]	n.d.	n.d.
58	62.40	Viridiflorene[(c)]	1676	1671	**107**; 93; 161; 119	0.8[e]	n.d.	n.d.
60	64.56	α-Guaiene	1694	1652	**105**; 93; 79; 147	0.3[e]	n.d.	n.d.
61	65.22	α-Selinene[(c)]	1700	1705	**189**; 204; 93; 107	0.5[e]	n.d.	n.d.
62	66.40	Bicyclogermacrene[(c)]	1711	1706	**121**; 93; 41; 107	0.9[e]	n.d.	n.d.
68	81.59	Curzerene[(c)]	1862	1874	**108**; 148	8.5[e]	n.d.	n.d.
		Σ Areas by variety[(c)]				17.1[e]	n.d.	n.d.
		N° of Compounds				7	0	0
Others								
6		2-Ethylfurane	949	945	**81**; 96; 53	0.4[e]	1.8[f]	2.7[g]
10		Toluene	1039	1037	**91**; 92	0.9[e]	1.5[f]	1.6[g]
		Σ Areas by variety				1.4[e]	3.3[f]	4.4[g]
		N° of Compounds				2	2	2
Total Compounds identified by maturation stage						65	56	58
Total Σ Area						2065.4	749.1	986.0
% RSD (n = 3)								

[a]RT – Retention time (minutes); KI – Kovats Index ([b]Exp – experimental, [c]Lit – theoretical KI value reported in literature.) *Not higher alcohol n.d. – not detected. Means followed by different letters (e,f,g) for a given parameter are significantly different at $P < .05$ (Bonferroni and Games-Howell [(d)] tests).

the same. Consequently, in order to implement an expeditious procedure, 30 min was selected for the extraction of VOMs from Uveira fruits.

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

The fingerprint of volatile metabolites from Uveira berries through ripening stages, using HS-SPME/GC-qMS under optimal analytical conditions (i.e.: DVB/CAR/PDMS fibre; 30 min extraction time; 40 ± 1 °C extraction temperature; salt addition: NaCl (10% (w/w)), 5.0 g of sample, GC-qMS see section 2.4), is illustrated in Fig. 2.

HS-SPME was used to extract and concentrate simultaneously VOMS from berries over a short time (30 min) at low temperatures (40 °C) without any solvent or chemical addition, minimizing the formation of artifacts.

GC–MS chromatographic profiles revealed the complexity of the matrix indenpendent of the ripening stage. The list of volatile metabolites identified at different ripening stages is listed in Table 1. Altogether, 72 VOMs were identified in the samples studied and included monoterpenes and sesquiterpenes, higher alcohols, aldehydes, ketones, and fatty acids (Fig. 2, Table 1). Monoterpenes (24 identified volatiles, ranging between 31 and 37% of total volatile fraction) were the most abundant chemical group found in the endemic Uveira berries investigated followed by higher alcohols (16 volatiles, ranging between 19 and 28%), esters (13 volatiles, ranging between 15 and 23%), sesquiterpenes (7 volatiles, around 11% of the volatile fraction from green stage), aldehydes (5 volatiles, ranging between 7 and 8%), ketones (3 volatiles, ranging around 5%), fatty acids (2 volatiles, ranging 3–4%) and others (2 volatiles, ranging between 3 and 4%) (Fig. 3A).

The qualitative composition at each ripening stage, and the number of volatiles identified, were slightly different according to ripening stage (Fig. 3A and B), revealing different priority metabolic pathways during ripening (as direct or indirect products) (Aragüez & Valpuesta Fernández, 2013).

Some volatile compounds were found at all ripening stages (Table 1), while others were found at a specific ripening stage (Table 1). In fact, 51 from the 72 volatiles compounds identified in Uveira berries were common to all stages (see Table 1). On the other hand, 14, mainly mono- and sesquiterpenes, were found only during the green stage, whereas some higher alcohols, such as sec-isoamyl alcohol and 1-decanol, were found only once ripe. Significant differences ($p < .05$) were observed for all chemical groups according to the ripening stage (Table 1). Earlier studies using *V. ashei* and *V. stamineum* (Horvat, Schlotzhauer, Chortyk, Nottingham, & Payne, 1996; Horvat & Senter, 1985) reported that the volatile composition changes according the ripening stage.

Regarding the volatile profile of Uveira, according to ripening stage (Fig. 3, Table 1), the number of volatiles identified was similar (65 identified compounds for green, 56 for breaker and 58 for ripe stage). However, the expression of different chemical groups changed during ripening. At the green stage, monoterpenes were most common (24 monoterpenes of 65 volatile compounds identified) followed by higher alcohols (12 identified compounds), esters (10 compounds), sesquiterpenes (7 compounds), aldehydes (5 compounds), ketones (3 compounds) and carboxylic acids (2 compounds). *Cis*-β-ocimene was the most abundant. This terpene accountsed for more than 40% of the volatile profile of endemic green Uveira berries, which might contribute to their *citrus* and *herbal* aroma (El-Sayed, 2015). He, Xie, Tang, and Qi (2012) associated this metabolite with use in flavors, food supplement fragrances, and as building block for pharmaceuticals. Bowen and Ali (2007) linked this metabolite with anti-tumor activity. Furthermore,

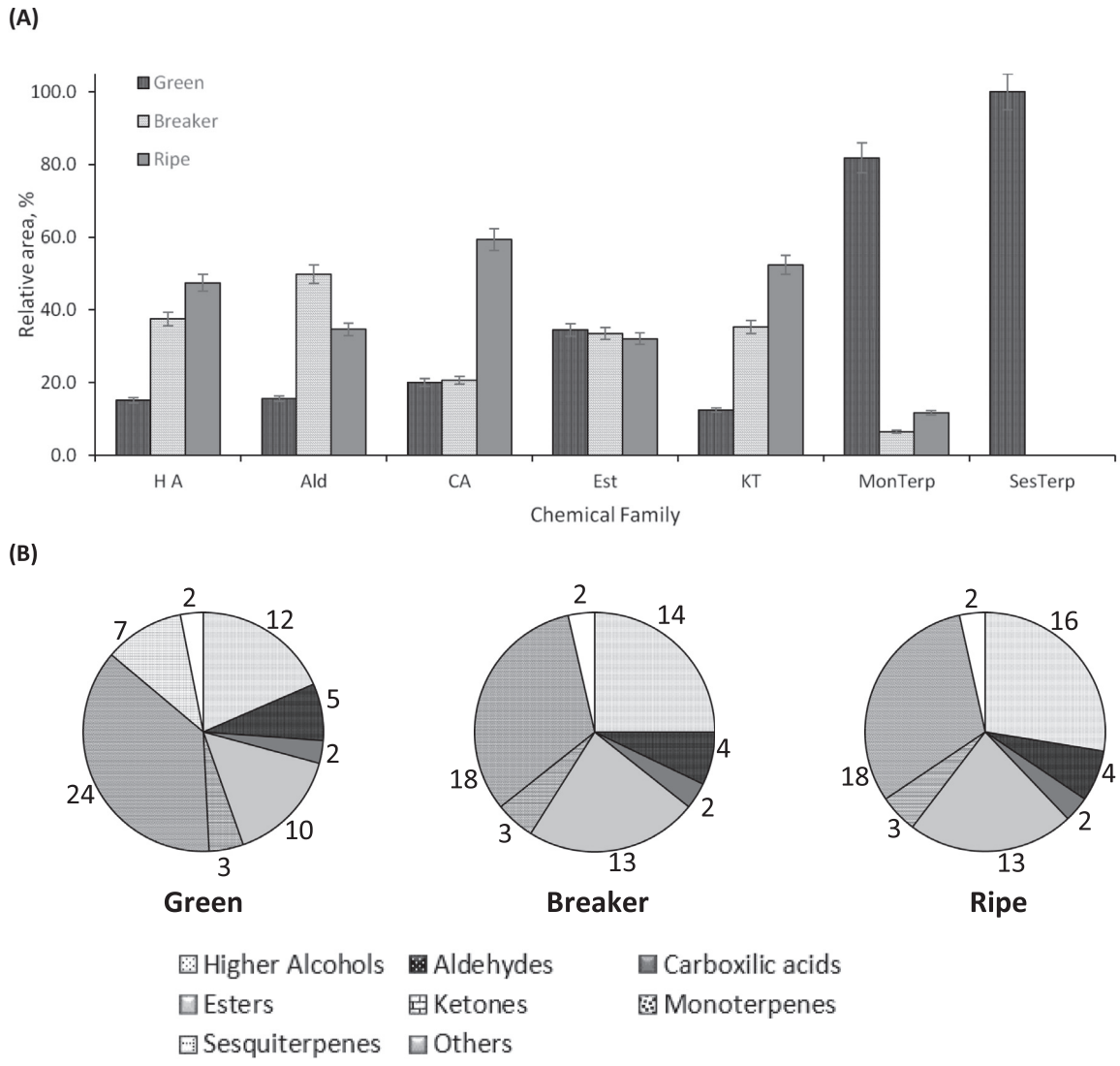

Fig. 3. (A) Volatile profile of endemic Uveira berries distributed by chemical groups; (B) Number of volatile compounds identified for each ripening stage (green, break and ripe) after extraction by HS-SPME according the chemical group.

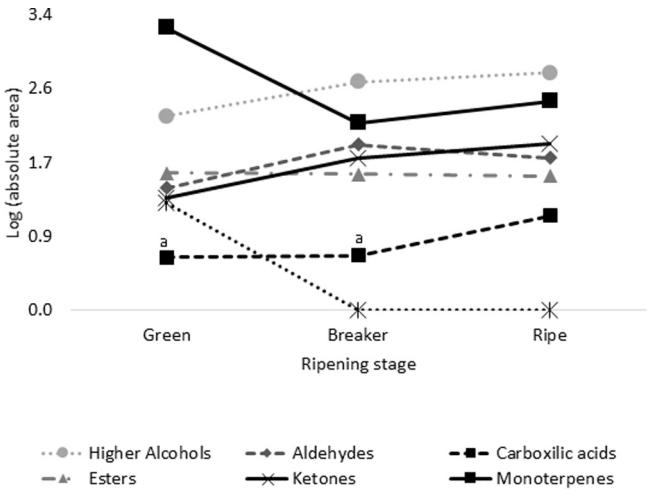

Fig. 4. Evaluation of the ripening effect on the principal chemical groups identified in endemic Uveira berries.

trans-β-ocimene (*herbal* notes) and β-myrcene (*herbaceous, resinous, green, balsamic, fresh hops* notes) were present at high percentages, ranging from 14 to 20% of the total volatile fraction. Ciftci, Oztanir, and Cetin (2014) reported the beneficial effect of β-myrcene against ischemia/reperfusion-mediated oxidative (I/R) and neuronal damage in the brain. They suggested β-myrcene ameliorated neurodegenerative effects caused by global cerebral I/R in C57BL/J6 mice and concluded that β-myrcene attenuated neuronal damage caused by global cerebral I/R in the brain. These effects are associated with antioxidant properties and correlated with decreased oxidative stress (Ciftci et al., 2014). D-limonene contributed approximately 5% of the volatile composition of green Uveira berries. This compound imparts a citrus aroma and is strongly associated with health benefits. In fact, Murthy, Jayaprakasha, and Patil (2012) demonstrated that D-limonene can inhibit proliferation of colorectal cancer cells, while Jing et al. (2013) provided evidence that D-limonene protected against development of dyslipidemia and hyperglycemia in HFD-fed mice. Moreover, D-limonene improved insulin resistance and regulated lipid profiles, which appears to be mediated through activation of PPARα and inhibition of LXRβ signalling (Jing et al., 2013). Furthermore Lima et al. (2013) showed that inhalation of D-limonene exerted anxiolytic-like effects in the elevated plus maze test, but was unrelated to benzodiazepine receptors.

At the breaker stage, monoterpenes were also the major chemical

group (18 identified monoterpenes of 56 compounds). Higher alcohols were the second most abundant (14 identified compounds) followed by esters (13 identified compounds) and aldehydes (4 identified compounds). Sesquiterpenes were not identified at this stage (Fig. 4).

Trans-2-hexenol was the most abundant volatile, accounting for around 19% of the volatile fraction of breaker berries, followed by *cis*-3-hexenol (16% of the total volatile composition). These alcohols impart *green* and *grassy* aroma notes. According Goff and Klee (2006), they derive from linolenic acid via lipoxygenase activity and are, therefore, indicatative of the presence of free fatty acids, which are essential to the human diet. Tokumo, Tamura, Hirai, and Nishio (2006) showed that exposure of mice to *cis*-3-hexenol induced anxiolytic behavior in the elevated plus maze test, which demonstrated the pharmacological potential of this compound. 1-Hexanol (~14% of volatile profile) formed during the breakdown of linoleic acid (Goff & Klee, 2006) contributed *fruity, alcoholic* and *sweet* notes while (E)-2-hexenal (8% of the volatile composition of breaker berries) is responsible for the *apple*, *fruity*, *green*, *herbal* and *leafy* notes (El-Sayed, 2015). This metabolite was reported by Lanciotti et al. (2003) to have a significant inhibitory effect against pathogen microorganisms isolated from raw materials (*E. coli*, *S. enteritidis*, and *L. monocytogenes*) when inoculated in both model and real-world systems at low concentration (20 ppm).

The prominent contribution of ketones was provided by 2-heptanone (~7%). This compound imparts a *banana-like, fruity* odor. Belonging to the class of terpenes, linalool (~5% of volatile composition) contributes *floral notes as well as wood, spicy, and lavender* notes. Linalool is one of the most investigated volatile compounds (Aprotosoaie, Hăncianu, Costache, & Miron, 2014) due to its biological properties, like sedative, anxiolytic, anticonvulsant, analgesic, antidepressant and anti-inflammatory activity (Aprotosoaie et al., 2014; Guzmán-Gutiérrez, Bonilla-Jaime, Gómez-Cansino, & Reyes-Chilpa, 2015).

The ripe stage was characterized by large amounts of monoterpenes (18 identifed volatile compounds) and higher alcohols (16 of 58 identified compounds), which may be related to enzymatic activity characteristic of ripe fruits (Brady, 1987). Esters were the third most abundant chemical group with 13 identified followed by aldehydes (4 identified volatile compounds) and other chemical groups, which included ketones, carboxylic acids and furans (7 identified volatiles). As with the breaker stage, most abundant volatiles in ripe berries were *cis*-3-hexenol and *trans*-2-hexenol (16% of volatile composition) followed by 1-hexanol, 2-heptanone and linalool (with 13%, 8% and 6% of volatile composition). At this stage, *cis*-β-ocimene was also identified in significant amounts (around 6% of volatile profile).

3.3. Multivariate analysis

To better understand the usefulness of volatile composition to differentiate between ripening stages and determine the potential relationships/variables responsible for this, a principal component analysis (PCA) was performed, using normalized data, considering principal components (PCs) with eigenvalues > 1. The data, presented as a bidimensional plot of sample scores in the space defined by the two first PCs, enabled formation of three differentiated clusters according to ripening stage (Fig. 5). Fig. 5A demonstrates a clear separation between ripening stages of Uveira berries.

The first three PCs explained about 99.4% of the total variance (Table 1 SM). The first principal component (PC1) explains 78.0% of the variance and separated the ripe and breaker stages from the green stage. The most relevant volatiles contributing to the separation were ethyl caprylate (1.000), ethyl isovalerate (−0.994), *trans*-allo-ocimene (0.991), D-limonene (0.984), neoallo-ocimene (0.981), hexyl acetate (−0.975), *m*-cymene (0.974), β-myrcene (0.974), *cis*-β-ocimene (0.971), *trans*-β-ocimene (0.970), ethyl butanoate (−0.950) and 2-hexenal (−0.950). The second principal component (PC2), 21.4% of the total variance, was characterized strongly by *trans*-geraniol (0.995), benzyl carbinol (0.993), *trans*-2-hexenyl butyrate (−0.979), α-terpineol (0.973), *sec*-isoamyl alcohol (0.973), 1-decanol (0.969), octanoic acid (0.968), *cis*-geraniol (0.968) and linalool (0.966).

Partial least squares discriminant analysis (PLS-DA) was performed to obtain separation between groups and understand which variables carry the class separating information (Ballabio & Consonni, 2013; Worley et al., 2013). Using PLS-DA, the relevant sources of data variability were modelled by the so-called latent variables (LVs), which are linear combinations of the original variables and, consequently, allow graphical visualization. With this methodology, it is possible to obtain probabilities for each sample belonging to a specific ripening stage. Two statistical significant functions were obtained with eigenvalues 52073 and 8027. The first function (F1) accounted for 86.6% of the variability and the second function (F2) for 13.4%. A clear separation of samples according ripening stage can be observed in Fig. 1SM (Suplementary Material). The predictive capacity of the discriminant model was evaluated using "leave-one-out" cross-validation (Table 2 SM and Fig. 1SM (Suplementary Material)). The discriminant analysis model, based on volatile profile of Uveira berries at different ripening stage, was classified correctly with 100% of observations based on cross-validation.

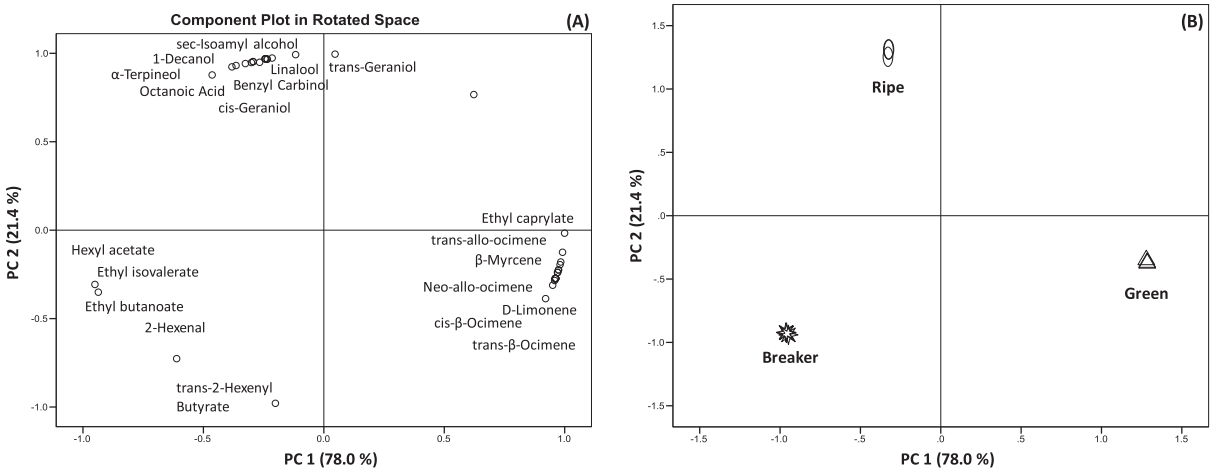

Fig. 5. PC1 and PC2 scatter plot of the main sources of variability between the different reipening stages of berries from endemic Uveira (A) Relation between the compounds (loadings) and (B) Distinction between the samples (scores).

4. Conclusions

In this study, the evolution of endemic Uveira berries volatile compounds was investigated. HS-SPME/GC-qMS was revealed as a powerful methodology for establishing the volatomic profile of Uveira berries, providing an appropriate and selective way to better understand the volatile composition of Uveira berries during ripening. A detailed anlysis of the chromatograms obtained allowed the unequivocal identification (using the KI, NIST database and in some cases pure standards) of 72 volatile metabolites and revealed significant differences among ripening stages. The main volatile compounds identified in Uveira berries were monoterpenes followed by higher alcohols and esters. From 72 metabolites identified, 51 were common to all stages but there were differences in abundance. Some variations on volatile profile during ripening were observed. Accumulation of the main compounds derived from carotenoids-cleavage deacrease dramatically during ripening in contrast to the higher alcohols, fatty acids and ketones, revealing that several biochemical transformations take place during ripening. As far we know, this is the first study reporting the volatomic pattern of Uveira berries during ripening and provides important insights into the biochemical transformations underlying ripening, as a key step to improve crop quality and provides useful information to producers.

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Conflit of interest

All co-authors declare NO conflits of interest.

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.2017.10.049.

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