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TITLE:

Profiling Volatile Compounds in Blackcurrant Fruit Using Headspace Solid-phase Microextraction Coupled to Gas Chromatography-Mass Spectrometry

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SUMMARY:

A headspace solid-phase microextraction–gas-chromatography platform is described here for fast, reliable, and semi-automated volatile identification and quantification in ripe blackcurrant fruits. This technique can be used to increase knowledge about fruit aroma and to select cultivars with enhanced flavor for the purpose of breeding.

ABSTRACT:

There is an increasing interest in measuring volatile organic compounds (VOCs) emitted by ripe fruits for the purpose of breeding varieties or cultivars with enhanced organoleptic characteristics and thus, to increase consumer acceptance. High-throughput metabolomic platforms have been recently developed to quantify a wide range of metabolites in different plant tissues, including key compounds responsible for fruit taste and aroma quality (volatalomics). A method using headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) is described here for the identification and quantification of VOCs emitted by ripe blackcurrant fruits, a berry highly appreciated for its flavor and health benefits.

Ripe fruits of blackcurrant plants (*Ribes nigrum*) were harvested and directly frozen in liquid nitrogen. After tissue homogenization to produce a fine powder, samples were thawed and immediately mixed with sodium chloride solution. Following centrifugation, the supernatant was transferred into a headspace glass vial containing sodium chloride. VOCs were then extracted using a solid-phase microextraction (SPME) fiber and a gas chromatograph coupled to an ion trap mass spectrometer. Volatile quantification was performed on the resulting ion chromatograms by integrating peak area, using a specific *m/z* ion for each VOC. Correct VOC annotation was

confirmed by comparing retention times and mass spectra of pure commercial standards run under the same conditions as the samples. More than 60 VOCs were identified in ripe blackcurrant fruits grown in contrasting European locations. Among the identified VOCs, key aroma compounds, such as terpenoids and C6 volatiles, can be used as biomarkers for blackcurrant fruit quality. In addition, advantages and disadvantages of the method are discussed, including prospective improvements. Furthermore, the use of controls for batch correction and minimization of drift intensity have been emphasized.

INTRODUCTION:

Flavor is an essential quality trait for any fruit, impacting consumer acceptance and thus significantly affecting marketability. Flavor perception involves a combination of the taste and olfactory systems and depends chemically on the presence and concentration of a wide range of compounds that accumulate in edible plant parts, or in case of VOCs, are emitted by the ripe fruit[1,2]. While traditional breeding has focused on agronomic traits such as yield and pest resistance, fruit quality trait improvement, including flavor, has long been neglected due to the genetic complexity and the difficulty to properly phenotype these characteristics, leading to consumer discontent[3,4]. Recent advances in metabolomic platforms have been successful in identifying and quantifying key compounds responsible for fruit taste and aroma[5–8]. Furthermore, the combination of metabolite profiling with genomic or transcriptomic tools allows the elucidation of the genetics underlying fruit flavor, which in turn will help breeding programs develop new varieties with enhanced organoleptic characteristics[2,4,9–14].

Blackcurrant (*Ribes nigrum*) berries are highly appreciated for their flavor and nutritional properties, being widely cultivated across the temperate zones of Europe, Asia, and New Zealand[15]. Most of the production is processed for food products and beverages, which are very popular in the Nordic countries, mainly due to the berries' organoleptic properties. The intense color and flavor of the fruit are the result of a combination of anthocyanins, sugars, acids, and VOCs present in the ripe fruits[16–18]. The analysis of blackcurrant volatiles goes back to the 1960s[19–21]. More recently, several studies have focused on blackcurrant VOCs, identifying important compounds for fruit aroma perception and assessing the impact of genotype, environment, or storage and processing conditions on VOC content[5,17,18,22,23].

Because of its numerous advantages, the technique of choice for high-throughput volatile profiling is HS-SPME/GC-MS[24,25]. A silica fiber, coated with a polymeric phase, is mounted on a syringe device, allowing the adsorption of the volatiles in the fiber until an equilibrium phase is reached. Headspace extraction protects the fiber from the nonvolatile compounds present in the matrix[24]. SPME can successfully isolate a high number of VOCs present at highly variable concentrations (parts per billion to parts per million)[25]. In addition, it is a solvent-free technique that requires limited sample processing. Other advantages of HS-SPME are the ease of automation and its relatively low cost.

However, its success can be limited, depending on the chemical nature of the VOCs, the extraction protocol (including time, temperature, and salt concentration), sample stability, and the availability of sufficient fruit tissue[26,27]. This paper presents a protocol for blackcurrant VOCs

isolated by HS-SPME and analyzed by gas chromatography coupled with an ion trap mass spectrometer. A balance between the quantity of plant material, sample stability, and duration of extraction and chromatography was achieved to be able to process high numbers of blackcurrant samples, some of them presented in this study. In particular, VOC profiles and/or chromatograms of five cultivars ('Andega', 'Ben Tron', 'Ben Gairn', 'Ben Tirran', and 'Tihope') will be presented and discussed as example data. Furthermore, the same protocol has been successfully put into practice for VOC measurement in other fruit berry species such as strawberry (*Fragaria* x *ananassa*), raspberry (*Rubus idaeus*), and blueberry (*Vaccinium* spp.).

PROTOCOL:

1. Fruit harvesting

1.1. Grow between 4 to 6 plants per genotype and/or treatment to ensure sufficient fruit material and variability.

1.2. If possible, harvest the samples on the same date; if there is not enough fruit material, pool together samples harvested on different dates.

NOTE: It is recommended that the harvest time (morning, noon, afternoon) remains approximately identical as VOC profiles are affected by daytime/circadian rhythm[28–31].

1.3. Assess fruit ripening stage by visual observation[32]. Pool fruits from the same ripening stage, as ripening status strongly impacts VOC emission. Discard any damaged or pathogen-infected fruits.

NOTE: To better assess fruit ripeness, texture analysis can be performed[33]. In addition, counting days after flowering can be used to ensure that pooled fruits belong to a similar ripening stage.

1.4. Include a minimum of 10–15 fruits per biological replicate (3 to 5) for VOC analysis.

NOTE: Here, three separate pools of 13–20 fruits (biological replicates) of 'Andega', 'Ben Tron', 'Ben Gairn', 'Ben Tirran', and 'Tihope' cultivars were harvested in two locations (Poland and Scotland) in summer 2018 and directly frozen in liquid nitrogen. Samples were then sent to the laboratory and processed as described below.

1.5. Once harvested, freeze all fruits immediately in liquid nitrogen, and subsequently store them at -80 °C until processing.

NOTE: If possible, fruits can be directly processed after harvest. In this case, fresh fruits can be homogenized in a mixer, weighed, and directly analyzed (step 3.1 onwards. However, to prevent fruits from further postharvest degradative processes, the fresh material should be stored in a cooler (4 °C) and processed as rapidly as possible. If not properly handled, liquid nitrogen can produce cold burns and can cause asphyxiation in poorly ventilated spaces.

2. Fruit sample and reagent preparation

2.1. Grind the fruits into a fine powder, taking care to always keep them frozen with the help of liquid nitrogen. Use a cryogenic mill, bead mill, or a mortar and pestle for homogenization. Precool stainless grinding jars or mortar and pestle with liquid nitrogen to avoid sample thawing.

NOTE: It is critical to homogenize samples to a fine powder to ensure proper VOC extraction.

2.2. Weigh 1 g of frozen material (from step 2.1.) in a 5 mL tube that is previously cooled in liquid nitrogen, and note the exact weight. Keep the material at -80 °C until processing step 3.1.

2.3. Include 'reference' or 'control' samples in the analysis to check technical variation, including VOC extraction and HS-SPME/GC-MS performance. For this purpose, pool together a mixture of randomly chosen fruit samples, and include at least one control sample per day for VOC analysis. In addition, use an internal standard, as described in step 2.5., to minimize the impact of intensity drift.

2.4. Prepare 20% (w/v) sodium chloride solution in high-performance liquid chromatography (HPLC) grade water (hereafter, referred to as NaCl solution). Dissolve NaCl with the help of a magnetic stirrer; ensure the availability of 1 mL of the solution per sample.

2.5. Prepare a 1 ppm solution in HPLC grade methanol of *N*-pentadecane (D32, 98%) from pure commercial standard (hereafter, referred to as the internal standard).

NOTE: *N*-pentadecane-d32 will be used as an internal standard, and 5 µL per sample will be needed. Methanol should be manipulated under a fume hood.

2.6. Prepare 1 ppm solutions in HPLC grade methanol of pure commercial standards for VOC identification (see **Table 1** for the list of commercial standards used in this study).

2.7. Prepare 10 mL screw-cap headspace vials by adding 0.5 g NaCl in each needed vial. Ensure that screw caps include a septum composed of a soft material, *i.e.*, silicone, with a thin polytetrafluoroethylene film on the inner side, to avoid contamination.

3. Sample preparation

3.1. Add 1 mL of NaCl solution to the 5 mL tube containing the weighed frozen sample. Shake the tube until the sample is completely thawed and homogenized.

3.2. Centrifuge at 5000 × *g* for 5 min at room temperature.

3.3. Transfer the supernatant with a 1000 µL pipette tip to the NaCl-containing headspace vial. Cut the end of the tip to facilitate this process.

3.4. Add 5 µL of internal standard to each sample-containing headspace vial.

4. HS-SPME/GC-MS data acquisition

4.1. Place the closed headspace vial in a GC-MS autosampler at room temperature, for an automated HS-SPME/GC-MS run, which is described in section 4. Do not place biological replicates in successive positions in the autosampler; instead, randomly distribute them to minimize the impact of intensity drift.

NOTE: Approximately 10–12 vials can be placed at once in the autosampler, without affecting sample stability.

4.2. Preincubate the headspace vials 10 min at 50 °C with agitation at 17 x *g*.

4.3. Insert an SPME device into the vial to expose the fiber to the headspace for VOC extraction for 30 min at 50 °C with agitation at 17 x *g*.

4.4. Introduce the fiber into the injection port for 1 min at 250 °C in splitless mode for volatile desorption.

4.5. Clean the fiber in an SPME cleaning station with nitrogen (1 bar N_2, ≥ 99.8% pure) for 5 min at 250 °C. Reuse the fiber approximately 100x.

4.6. Analyze VOCs with a gas chromatograph coupled to an ion trap mass spectrometer (see the **Table of Materials**), and perform chromatography under a constant flow of helium (He ≥ 99.9999% purity) of 1 mL/min, with a column that has dimensions of 60 m x 0.25 mm x 1 µm thickness. Use an oven temperature program that is isothermal at 40 °C for 3 min, followed by an 8 °C/min ramp to 250 °C and holding at 250 °C for 5 min. For mass spectrometry, set the transfer line and ion source temperatures to 260 °C and 230 °C, respectively. Set the ionization energy to 70 eV and the recorded mass range to *m/z* 35-220 at 6 scans per s.

4.7. Extract and analyze 1 ppm solutions of commercial standards as described above. In addition, run a mixture containing all the diluted commercial standards mixed with 300 µL NaCl solution and 900 µL HPLC grade water before sample data acquisition to check the correct calibration of the equipment. Furthermore, include a blank sample containing NaCl solution alone in every batch.

5. Analysis of GC-MS profile chromatograms: VOC identification and semi-quantification

5.1. Open raw GC-MS profile files with the software provided by the manufacturer. To identify compounds, compare their retention times and mass spectra and Kovats linear retention indices determined from the chromatograms of the samples with retention indices obtained from authentic standards. For each commercial standard, annotate retention time and the most

abundant *m/z* ions. Then, select a specific *m/z* ion for each VOC (**Table 1**).

5.2. Automatically integrate VOC peaks based on standard retention times and chosen *m/z* ions of the selected GC-MS raw files. For this, provide a list for each VOC with retention time and selected *m/z* ion. Although the software automatically integrates peak area corresponding to the same retention time and *m/z* ion as provided in the sequence setup, check the correct integration of each peak and correct it manually if necessary.

5.3. Calculate the peak area of each VOC relative to that of the internal standard to minimize instrumental variation and intensity drift.

NOTE: When analyzing fruit from different genotypes or growth and storage conditions, it is highly recommended to determine the VOC content relative to the fruit dry weight content to rule out dilution effects due to differences in water content.

5.4. For batch effect correction, normalize the VOC peak area of each sample to the corresponding peak area in the control sample analyzed in the same run.

NOTE: A relative VOC quantification is obtained; however, for the purpose of the experiment, VOC content can be then determined relative to any sample (*e.g.*, untreated fruits to compare the effect of storage upon VOC levels).

REPRESENTATIVE RESULTS:

High-throughput VOC profiling in a large set of fruit crops grown under different conditions or locations or belonging to distinct genotypes is necessary for accurate aroma phenotyping. Here, a fast and semi-automated HS-SPME/GC-MS platform for relative VOC quantification in blackcurrant cultivars is presented. VOC detection and identification were based on a library that was developed to profile berry fruit species (**Table 1**). A typical ripe blackcurrant fruit volatile profile (total ion chromatogram) obtained by HS-SPME/GC-MS in the aforementioned conditions is shown in **Figure 1A**. In total, 63 VOCs were identified, belonging to several chemical classes, the majority being esters (27), aldehydes (12), alcohols (8), ketones (7), terpenes (5), and furans (3) (**Table 1**).

Terpenoid compounds, esters, and C6 compounds have been described to dominate the blackcurrant volatilome and to be important for the aroma of the fresh fruit[5,17]. In agreement with these previous studies, some of the most abundant peaks observed in **Figure 1A** correspond to two monoterpenes (linalool and terpineol) and two C6 compounds ((*E*)-2-hexenal and (*Z*)-3-hexenal). Example mass spectra obtained from blackcurrant profiles and their comparison with spectra of pure commercial standards are shown for (*E*)-2-hexenal and terpineol in **Figure 1B** and **Figure 1C**, respectively.

[Place **Figure 1** here]

While terpenes have been depicted to be indicators of blackcurrant fruit freshness, C6

compounds are known as 'green leaf volatiles', imparting 'green' notes to fruit and vegetable aroma[34]. Thus, the semi-quantification of these VOCs emitted by ripe fruits of different blackcurrant varieties can be the first step in improving flavor-related traits. Furthermore, as the environment and plant growth conditions strongly impact fruit VOC content, which is one of the main drawbacks for aroma breeding, one of the objectives of this study was to validate the hypothesis that the semi-quantification of the identified VOCs in the same cultivars ('Ben Tron', 'Ben Gairn', 'Ben Tirran', and 'Tihope') was reproducible in diametrically opposed European locations such as Poland and Scotland. As expected, principal component analysis (PCA) of the VOC profiles of four different blackcurrant cultivars showed that the environment strongly impacts volatile content, as principal component (PC) 1 separates samples based on their location (**Figure 2**). However, the effect of genotype can be observed with PC2, as 'Ben Tirran' is clearly separated from the remaining cultivars (**Figure 2**).

Figure 3 shows the relative content of linalool and (*E*)-2-hexenal in the four assessed blackcurrant cultivars. For both locations, VOC content was normalized to the same control sample, for which the semi-quantification confirmed that linalool content was generally higher in Poland than in Scotland, whereas (*E*)-2-hexenal shows the opposite trend (**Figure 3**). This result demonstrates the environmental impact on VOC content in blackcurrant fruits, although the proportion of the two volatiles present in the four assessed cultivars was constant, with 'Ben Tirran' and 'Ben Tron' cultivars showing the highest amounts of linalool and (*E*)-2-hexenal, respectively (**Figure 3**). Taken together, these results indicate that the proposed method is valid to phenotype VOC content, and combined with genetic approaches, may be used for the purpose of fruit quality breeding.

[Place **Figure 2** here]

[Place **Figure 3** here]

[Place **Table 1** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Representative chromatograms from ripe blackcurrant fruit obtained by HS-SPME/GC-MS (from 'Andega' cultivar). (**A**) Total ion chromatogram. (*Z*)-3-hexenal (Retention time 14.33 min), (*E*)-2-hexenal (15.86 min), linalool (21.65 min), and terpineol (24.01 min) peaks are indicated with the numbers 1, 2, 3, and 4, respectively. (**B**) Mass spectrum corresponding to (*E*)-2-hexenal peak from a blackcurrant profile and comparison with a pure commercial standard. (**C**) Mass spectrum corresponding to terpineol peak from a blackcurrant profile and comparison with a pure commercial standard. Abbreviation: HS-SPME/GC-MS = headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry.

Figure 2: PCA to assess the variance among VOC profiles in the four blackcurrant cultivars grown in Poland and Scotland. PC1 (environment) explains 46.2% of the variability, while PC2 (genotype) contributes 24.8% of the variance in the dataset. Abbreviations: PCA = principal

component analysis; PC1 = first principal component; PC2 = second principal component; VOC = volatile organic compound.

Figure 3: Relative content of two representative VOCs in blackcurrant aroma profiles—linalool and (*E*)-2-hexenal, harvested in Scotland and Poland. Four different blackcurrant cultivars were assessed ('Ben Gairn', 'Ben Tirran', 'Ben Tron', and 'Tihope'). The bars represent the mean values of two biological replicates, and error bars represent the standard deviation. Statistical comparisons were performed by one-way ANOVA followed by Tukey's *post-hoc* test to determine significant differences in VOC content between cultivars and countries. For VOC contents with the same lowercase letters (a, ab, b), no significant differences were observed at $P < 0.05$. Abbreviations: VOCs = volatile organic compounds; ANOVA = analysis of variance.

Table 1: List of VOCs identified by HS-SPME/GC-MS in blackcurrant fruits. Retention time (min), selected *m/z* ion for VOC identification and semi-quantification, aroma description, chemical class and formula, and CAS number are indicated. Abbreviations: HS-SPME/GC-MS = headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry; VOCs = volatile organic compounds; KRI = Kovats retention index; CAS number = Chemical Abstracts Service registry number.

DISCUSSION:

Breeding for fruit aroma has long been hindered by the complex genetics and biochemistry underlying the synthesis of volatile compounds and the lack of technologies for proper phenotyping. However, recent advances in metabolomic platforms, combined with genomic tools, are finally allowing the identification of the metabolites responsible for consumer preferences and to breed crops with improved flavor[3]. While most progress has been achieved in the model fruit, tomato[9,10], similar results could be achieved in other economically relevant crop species such as strawberry, apple, or blueberry[2,12,35,36].

This paper presents a fast and reproducible HS-SPME/GC-MS-based platform that has been successfully used for measuring VOC content in different berry species, including blackcurrant, a fruit highly appreciated for its delicate flavor and remarkable nutritional value. Compared to previously published methods, the main improvement was achieved by decreasing the total chromatographic run time. Indeed, it was possible to increase the temperature ramp from 5 °C/min to 8 °C/min with adequate resolution, reducing the chromatographic time from 50 min to 35 min (**Figure 1A**)[27]. Furthermore, the high amount of NaCl added to the samples (1 mL of 20% NaCl solution + 0.5 g of solid NaCl) seems to positively impact sample stability over time. Indeed, volatile profiles were stable over time, and combined with faster chromatography, allowed the measurement of up to 20–22 samples per day.

The use of an internal standard, such as *N*-pentadecane-d32, together with a proper distribution of the biological replicates along the run, is necessary to prevent intensity drift[37]. In addition, control or reference samples must be run at least once per day of analysis for batch correction. Variations between batches are mainly caused by changes in detector sensitivity or by fiber aging[27]. While this protocol enabled the detection of more than 60 VOCs present in the

headspace of ripe blackcurrant fruits, readers must take into account that this number can be easily increased by adding pure commercial standards in the proposed library (**Table 1**). For example, published studies detected a high number of terpenoid compounds that were not included in this analysis[5,17] . In this sense, a more blackcurrant-aroma-specific VOC library may be readily put together, if necessary. However, the goal of this study was to adapt a previously established library[27] for VOC measurement in different berries, including raspberry, strawberry, and blackcurrant fruits.

It is noteworthy that the protocol presented here has several advantages and disadvantages, like other HS-SPME/GC-MS platforms, which have already been discussed elsewhere[25,26,38]. While it offers ease of automation, making it the technique of choice when large number of samples are required to be analyzed, its main drawback is its susceptibility to matrix effects[38]. In addition, special caution should be taken during SPME fiber-coating selection and with sampling conditions depending on the chemical nature of the targeted VOCs[25,27]. To conclude, a rapid and semi-automated protocol for VOC profiling in berry fruit headspace is presented here and could be easily adapted for use with an increased library size, if required. It is expected that this platform can be adapted to other fruit species and when combined with genomic studies and/or sensory analysis panel will help crop aroma profiling and improvement.

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DISCLOSURES:

The authors declare no conflict of interest.

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38. Burzynski-Chang, E. A. et al. HS-SPME-GC-MS analyses of volatiles in plant populations-quantitating compound × individual matrix effects. *Molecules.* **23** (10), 2436 (2018).

Figure 1

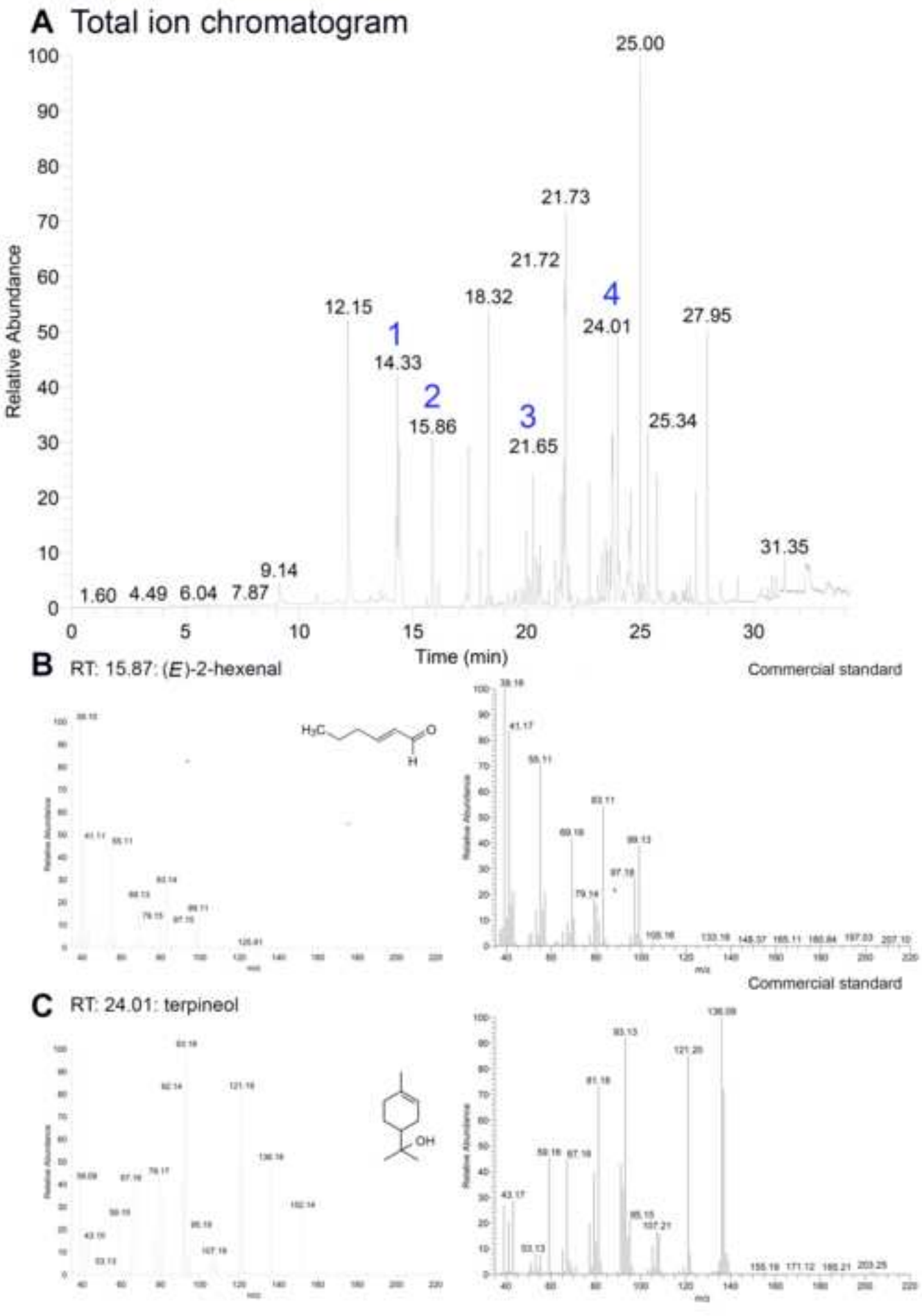

Figure 2

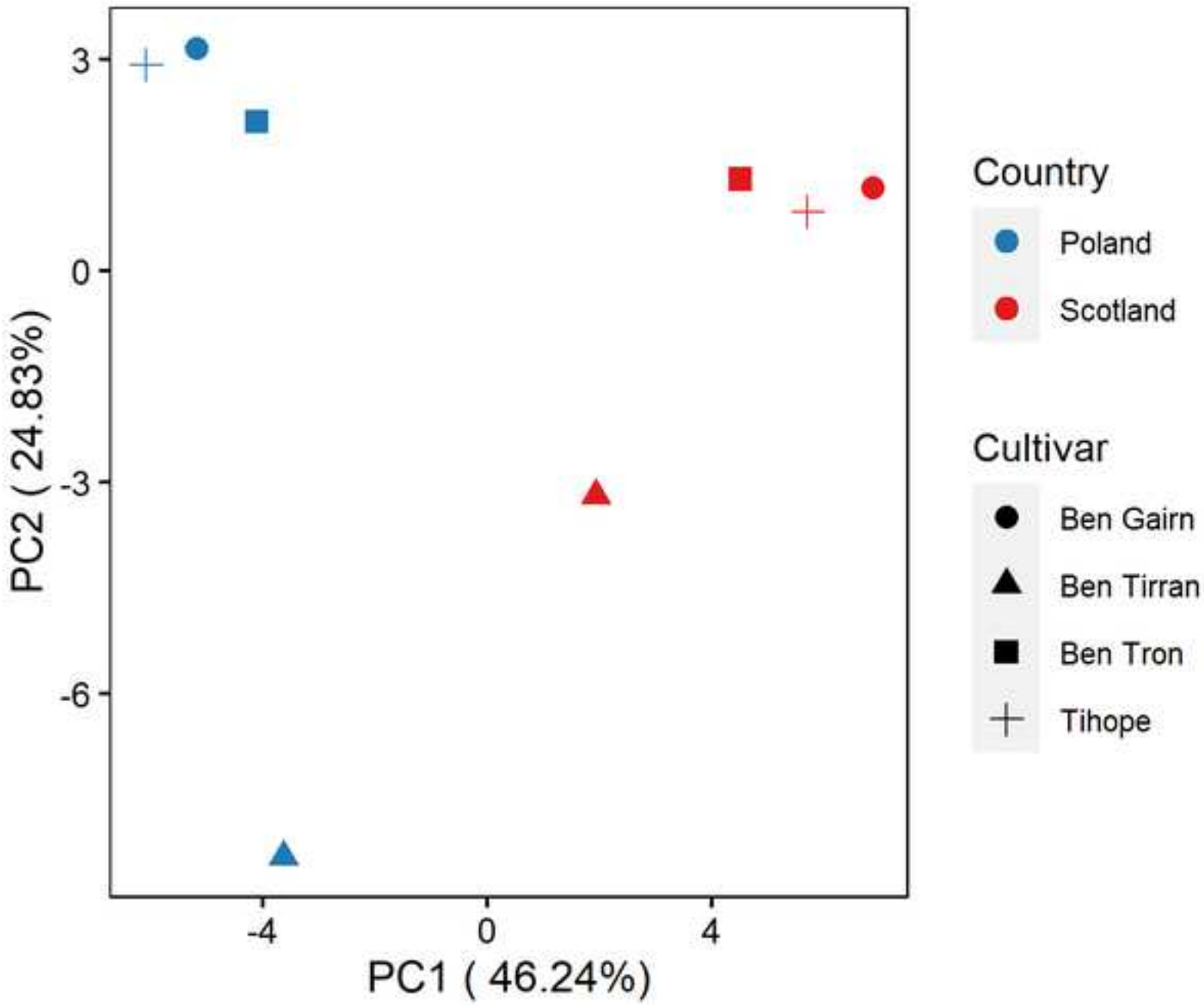

Figure 3

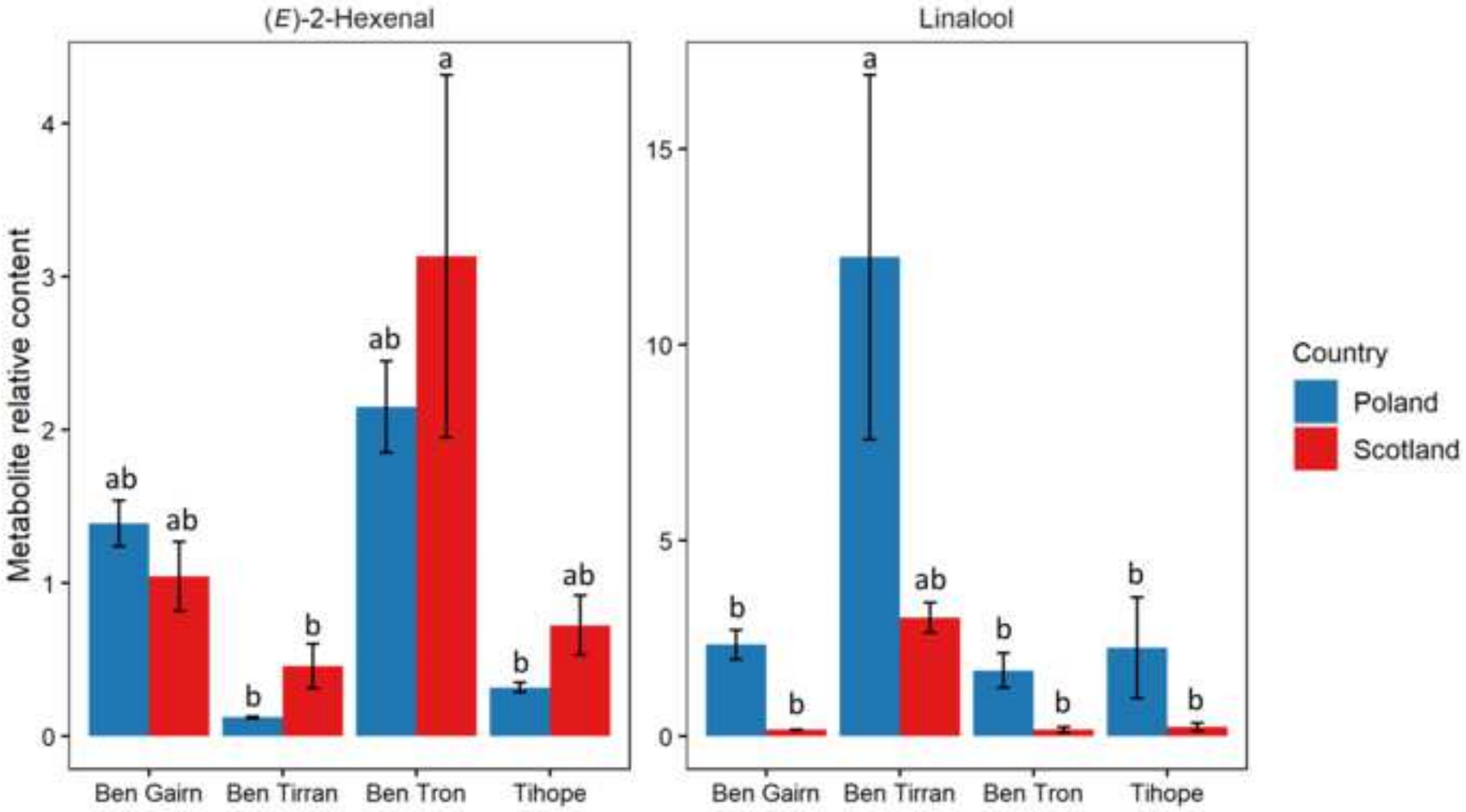

Table 1

Standard	Retention time	*m/z* ion	KRI	Chemical class	Aroma description	Chemical formula	CAS number
1-Penten-3-ol	11.09	57	1661/1678	alcohol	green	C5H10O	616-25-1
(*E*)-2-Hexen-1-ol	16.02	67	1743/1756	alcohol	green	C6H12O	928-95-0
1-Hexanol	16.04	56	1718/1726	alcohol	green	C6H14O	111-27-3
2-Heptanol	16.92	45	1800/18002	alcohol	citrus, fruity	$C_7H_{16}O$	543-49-7
1-Octanol	20.88	69	1920/1912	alcohol	waxy	C8H18O	111-87-5
Myrtenol	24.14	79	200672017	alcohol	herbal, minty	C10H14O	515-00-4
1-Decanol	24.98	69	1890/1895	alcohol	fatty	C10H22O	112-30-1
Eugenol	26.90	91	2039/2020	alcohol	spicy	C10H12O2	97-53-0
Pentanal	11.56	58	1683/1688	aldehyde	fermented, winey	C5H10O	110-62-3
(*E*)-2-Pentenal	13.19	55	1795/1799	aldehyde	green	C5H8O	1576-87-0
(*Z*)-3-Hexenal	14.33	69	1677/1692	aldehyde	green	C6H10O	6789-80-6
Hexanal	14.38	56	1655/1640	aldehyde	green	C6H12O	66-25-1
(*E*)-2-Hexenal	15.86	83	1896/1888	aldehyde	green	C6H10O	6728-26-3
Heptanal	17.05	81	1917/1913	aldehyde	green	C7H14O	111-71-7
(*E*)-2-Heptenal	18.45	83	1764/1771	aldehyde	green	C7H12O	18829-55-5
Benzaldehyde	18.94	105	180871819	aldehyde	fruity	C7H6O	100-52-7
Octanal	19.49	69	1831/1807	aldehyde	waxy, citrus	C8H16O	124-13-0
(*E,Z*)-2,4-Heptadienal	19.48	81	1815/1820	aldehyde		C9H14O	4313-02-4
E-2-Octenal	20.78	83	1956/1950	aldehyde	fatty	C8H14O	2548-87-0
3,4-dimethylbenzaldehyde	24.60	133	1926/1917	aldehyde		C9H10O	5973-71-7
Decane	19.35	57	1885/1897	alkane	-	C8H14O2	124-18-5
Ethyl acetate	9.13	61	1618/1624	ester	ethereal, fruity, ove	C4H8O2	141-78-6
Isopropyl acetate	10.47	61	1675/1652	ester	ethereal, fruity, swe	C5H10O2	108-21-4
Methyl butyrate	12.15	74	1689/1704	ester	fruity	C5H10O2	623-42-7
Ethyl butanoate	14.28	71	1918/1915	ester	fruity	C6H12O2	105-54-4
Butyl acetate	14.60	61	1785/1794	ester	ethereal, fruity	C6H12O2	123-86-4

Methyl pentanoate	15.01	74	1794/1799	ester	fruity	C6H12O2	624-24-8
Isopentyl acetate	16.27	70	1853/1863	ester	fruity	C7H14O	123-92-2
Propyl butanoate	16.79	71	1854/1858	ester	fruity	C7H14O2	105-66-8
Methyl thiobutanoate	16.86	71	1799/1804	ester	cheesy, musty	C5H10OS	2432-51-1
Pentyl acetate	17.15	61	1893/1904	ester	fruity	C7H14O2	628-63-7
Prenyl acetate	17.35	68	1855/1867	ester	fruity	C7H14O2	1191-16-8
Methyl hexanoate	17.46	74	1857/1862	ester	fruity	C7H14O2	106-70-7
Methyl hex-2-enoate	18.55	97	1767/1774	ester	fatty	C7H12O2	2396-77-2
Butyl butanoate	19.15	89	1874/1884	ester	fruity	C8H16O2	109-21-7
Ethyl hexanoate	19.20	99	1711/1719	ester	fruity	C8H16O2	123-66-0
(*Z*)-3-Hexen-1-ol acetate	19.37	67	1865/1873	ester	green	C8H16O	3681-71-8
Hexyl acetate	19.49	61	1728/1730	ester	fruity	C8H16O2	142-92-7
Methyl benzoate	21.86	105	1925/1920	ester	phenolic	C8H8O2	93-58-3
Methyl octanoate	21.99	74	1936/1942	ester	waxy, green	C9H18O2	111-11-5
E -2-Nonenal	22.96	81	1926/1922	ester	green	C9H16O	18829-56-6
Benzyl acetate	23.10	108	1986/1977	ester	floral, fruity	C9H10O2	140-11-4
Ethyl octanoate	23.43	88	1933/1937	ester	waxy	C10H20O2	106-32-1
Octyl acetate	23.70	83	1936/1942	ester	floral, waxy	C10H20O2	112-14-1
Methyl decanoate	25.90	74	2025/2005	ester	fermented, fatty	C11H22O2	110-42-9
Myrtenyl acetate	26.42	91	2018/2027	ester	herbal	C12H18O2	1079-01-2
Octyl butanoate	27.20	89	2016/2003	ester	fruity, waxy	C12H24O2	110-39-4
Decyl acetate	27.39	83	2001/1994	ester	waxy	C12H24O2	112-17-4
2-Pentylfuran	19.25	138	1895/1907	furan	fruity, green	C10H22	3777-69-3
Gamma-decalactone	28.83	85	2030/2008	furan	fruity	C10H18O2	706-14-9
Gamma-dodecalactone	32.62	85	1996/2013	furan	fruity	C12H22O2	2305-05-7
Acetone	6.42	43	1484/1478	ketone	solvent	C3H6O	67-64-1
1-Penten-3-one	11.56	86	1605/1589	ketone	spicy, pungent	C5H8O	1629-58-9
2-Heptanone	16.69	58	1897/1903	ketone	cheesy	C7H14O	110-43-0
6-Methyl-5-hepten-2-one	19.00	108	1889/1916	ketone	citrus	C8H14O	110-93-0
Acetophenone	21.30	105	1793/1797	ketone	floral	C8H8O	98-86-2
2-Nonanone	21.40	58	1804/1813	ketone	fruity, cheesy	C9H18O	821-55-6
Cis-geranylacetone	28.25	107	2017/2001	ketone	floral	C13H22O	211-711-2
Limonene	20.42	93	1753/1746	terpene	citrus	C10H16	5989-27-5
Linalool	21.66	93	1732/1738	terpene	floral	C10H18O	78-70-6
Terpineol	24.01	93	197871983	terpene	terpenic, citrus	C10H18O	98-55-5
Nerol	24.28	93	1970/1977	terpene	floral, citrus	C10H18O	106-25-2
Nerolidol	30.20	93	2027/2010	terpene	floral, green	C15H26O	7212-44-4

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10 mL screw top headspace vials	Thermo Scientific	10-HSV	
18 mm screw cap Silicone/PTFE	Thermo Scientific	18-MSC	
5 mL Tube with HDPE screw cap	VWR	216-0153	
Centrifuge	Thermo Scientific	75002415	
Methanol for HPLC	Merck	34860-1L-R	
N-pentadecane (D32, 98%)	Cambridge Isotope Laborato	DLM-1283-1	
Sodium chloride	Merck	S9888	
SPME fiber PDMS/DVB	Merck	57345-U	
Stainless grinding jars for TissueLyser	Qiagen	69985	
TissueLyser II	Qiagen	85300	Can be subsituted by mortar and pestle or cryogenic mill
Trace GC gas chromatograph-ITQ900 ion trap mass spectrometer	Thermo Scientific		
Triplus RSH autosampler with automated SPME device	Thermo Scientific	1R77010-0450	
Water for HPLC	Merck	270733-1L	
Xcalibur 4.2 SP1	Thermo Scientific		software

To:
Editor of *JoVE*

Dear Editor,

Enclosed, please, find the revision of the manuscript entitle "**HS-SPME/GC-MS method for the identification and quantification of volatile organic compounds in blackcurrant fruit**" by Delphine M. Pott, José G. Vallarino and Sonia Osorio.

We have followed the detailed comments of the reviewers which has resulted in a streamlined and more concise manuscript addressing all of the raised points.

A detailed point-by-point response to your and the reviewers' comments follows below. For clarity, our responses are presented in a blue font right after each particular point of the reviews.

Best regards,
Sonia

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have double check the text

2. Please consider revising the title to "HS-SPME/GC-MS method for the identification and quantification of volatile 3 organic compounds in blackcurrant fruit". Terms like "Improved" may not be relevant in the future.

We have delete "improved" from the title

3. Please revise the following lines to avoid previously published work: 80-83.

It has been revised and rewritten

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns have been removed

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., TissueLyser, Xcalibur software, Qual browser, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

We have taken care in removing any commercial language from the MS.

6. Line 96-111: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

As suggested, imperative tense has been used in this section. In addition, safety procedures for liquid nitrogen and methanol have been added.

7. Please use standard abbreviations for SI units when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2 (Line: 135,140,151,154,184, etc.).

It has been corrected

8. Line 163: Please convert the agitation in rpm to centrifugal force (x g).

It has been corrected

9. Line 171: Please specify the rate of Helium flow. Is there a specific concentration used?

It has now included the purity of He and the flow rate (1 ml/min).

10. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Editor´s comment has been considered

11. Figure 2: Please define the symbols "a" and "b" used to describe the statistics in the Figure Legends.

It has been changed

12. Please sort the Table of Materials in alphabetical order.

It has been modified

Reviewers' comments:

Reviewer #1:

Line 90: author should add also blueberry

Following reviewer´s suggestion, it has been added.

Line 102: please add some references about it

We have now included references as suggested

Lines 104-106: is it so easy and precise for an operator to discriminate between ripe and overripe blackcurrant fruit based only on color? As far as i know, blackcurrant color is not changing a lot in these last ripening stages. What about using some non destructive texture analysis?

We have included a "note" including other method to asses ripening stage in fruit

Line 123: should be the tube also cold (liquid nitrogen)? should you work in cold condition? after weighting the powdered sample, at which temperature do you have to store it?

The reviewer is right. You have always to work in cold conditions. We have added more precise information about this issue

Lines 144-145: are all these steps really necessary? In my opinion, you might lose lot of VOCS in the moment you are transferring the samples from the 5 ml tube(after centrifuge) to the 10 ml vial. Did you test the methodic without these steps? Is it really necessary to keep only the supernatant? What is the advantage to do that? Why did not author consider to add any antioxidant solution (i.e.ascorbic and/or citric acid) to the sample?

We need to centrifuge the sample and we only keep the supernatant as previously published in Olbricht et al. (2011). By using this procedure, we obtained better reproducibility than using whole mixture. We did not add any antioxidant solutions, as other published protocols did not (Olbricht et al., 2011; Rambla et al., 2015) and also because we wanted to avoid any interference with samples. We would like to highlight that the samples are immediately analyzed (< 6h after processing). We need to use plastic tubes to process the samples, as headspace vials are made of glass, and it is not suitable for liquid nitrogen.

-What is the temperature of the autosampler cooler? And how many samples can you run without having any time (in the autosampler) effect?

The closed vial are placed into GC-MS autosampler at room temperature. We tested that between 10-12 samples can be prepared at once and placed into the autosampler without affecting sample stability but not higher number. We have included these details in the MS.

Line 222: around 20-25 samples a day and 35 min chromatographic run: Can this technique be considered "fast" and "high throughput"? have authors considered to adopt a Fast GC-MS?

We shorten chromatographic run time in comparison to published protocol, and VOC measurements are (at least partially) semi-automatized. We have texted to analyze higher number of samples but we did not obtain good reproducibility between samples.

Lines 239-254: author may ass a PCA analysis to compare the whole VOC profile of samples from Poland and Scotland. In addition, in figure 2, samples from Poland and Scotland should be compared in the same figure.
Following reviewer´s suggestion, we have included a PCA analysis and Figure 2 (now Figure 3) has been modified.

Line 296-297: can author show some results of the effect of different NaCl content?

We did some tests, using strawberry fruit samples, with and without using solid NaCl but we did not text different % of NaCl solution (we based on previous protocols stablished for strawberry and tomato fruits). We observed much better reproducibility when used solid NaCl plus NaCl solution at 20%.

Table 1: Authors should add information about the chemical class of each compounds, CAS No, chemical formula and Kovats retention index

Following reviewer´s suggestion, we have included more information about each compounds in Table 1.

Reviewer #2:
Major Concerns:
Abstract doesn't consist any information about obtained results and conclusion, it contains only a general information and methodology description. The abstract cannot be accepted in this form. Keywords: instead of metabolomics, I recommend to write "volatolomics". What about analysis of fresh pulp? I recommend to add a notice that if it is possible fresh material should be used to analyze VOC. All the processes can avoid to loss of some volatiles. Just the fresh material need to be properly protected from spoilage.

Following the advises of the reviewer, we have prepared an updated version of the abstract. We have changed “metabolomics” to “volatolomics” in the keywords.

For technical reasons, most of the times it is not possible to harvest fruits (in the field/greenhouse) and analyze them directly in the GC-MS. However, a note, as suggested by the reviewer, has been added.

2.3. Also an internal standard can be added to each sample to check the correctness of analysis.

That is correct. We describe the use of d32-pentadecane as internal standard (Step 2.5). More clear information has been added about this step.

What is the producer of autosampler, please provide name of the equipment also.

These details are included in the Table of Materials, as no commercial language/brand can be included in the manuscript.

The quality of chromatograms is low, need to improve it.

It has been improved

In the beggining there is no information about cultivars, it strangely apperead at the last pages with diagram description. Put the information in materials part.

Following reviewer´s suggestion, information about cultivars are now presented in the introduction and method part.

instead of retention time, most of highly-valued manuscripts give an information about linear retension indices (LRIs), the experimental and from libraries, which are prepared according to run n-alkenes. I recommend to work more on the look of the table 1 and 2. In the table 1 i recommend to add also a aroma description to each compound, which can be easily found in the databases. In table 2 I recommend to shorten the table, by group chemical compounds with ions e.g. esters: 73, 88, 89, etc. In this form i cannot recommend this publication to be published.

As suggested for the reviewer, we have included more information about each compounds in Table 1. In particular, Kovats retention index, chemical formula, CAS number and also aroma description when possible. In the MS, we did not present Table 2, in table 1, m/z ion is also indicated for each compound.

Reviewer #3:

Manuscript Summary:

The manuscript "Improved HS-SPME/GC-MS method for the identification and quantification of volatile organic compounds in blackcurrant fruit" is an interesting work. The manuscript is well written and organized. The article is completely clear and understandable, and presents originality. The description of the methodology and the

discussion of the results are clear, argued and adequate.

We thank the reviewer for this comment

Minor Concerns:

It is recommended to present the graphs and chromatograms in a better definition and resolution, since they appear pixelated in the document.

It has been improved