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Analysis of strawberry volatiles using comprehensive two-dimensional gas chromatography with headspace solid-phase microextraction

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Abstract

The aims of the current study were to develop an enantioselective multi-dimensional gas chromatography (GC × GC) method for the examination of strawberry volatiles and to use this method to make comparisons between the volatile profiles of different cultivars and between fresh picked and post-harvest berries of the same cultivar. Strawberry volatiles were sampled using solid-phase microextraction (SPME), and the repeatability and reproducibility of this method was examined. Semi-quantitative analysis of the volatiles was conducted using the relatively new technique of comprehensive multi-dimensional gas chromatography, using enantioselective (chiral) columns for the differentiation of analyte enantiomers. Chiral GC × GC facilitated the detection of key enantiomers in strawberry flavour. The (−)-enantiomer of 2,5-dimethyl-4-hydroxy-(2*H*)-furan-3-one (DMHF) and the *S*-enantiomer of linalool were tentatively identified as the predominant forms in both the cultivars Selva and Adina. The compounds benzaldehyde and methyl hexanoate were shown to decrease in post-harvest berries, whilst DMHF and nerolidol increased upon storage.

Keywords: Solid-phase microextraction; Enantiomer separation; Gas chromatography, comprehensive two-dimensional

1. Introduction

The cultivated strawberry *Fragaria ananassa* represents an important fruit crop, popular worldwide as fresh fruit or flavour constituents in manufactured foods [1]. In Australia, the 1999 crop had an estimated value of A$ 111 million [2]. Although volatile compounds comprise only 0.01–0.001% of the fresh mass of strawberries, they have a great effect on the quality of the aroma [3] as the threshold for human perception of a volatile molecule can be as low as 0.007 mg/L in water [4]. Strawberries have a complex aroma with more than 360 identified constituents composed of alcohols, esters, aldehydes, ketones and furanones [5]. This complexity is compounded by the fact that aroma is known to vary with berry variety [6,7], season, and agronomic and climatic growing conditions [8]. Furthermore, Watson et al. [9], have shown that volatiles examined in the variety Elsanta, showed highly significant differences with harvest date (pick-to-pick variation; $P < 0.01$) and even between fruit on a single harvest date. Nevertheless, there has been considerable debate as to which volatiles are the most important in producing the characteristic strawberry aroma, although many authors suggest 2,5-dimethyl-4-hydroxy-(2*H*)-furan-3-one (DMHF, Furaneol®), 2,5-dimethyl-4-methoxy-(2*H*)-furan-3-one (mesifuran), ethyl hexanoate, hexanal, ethyl methyl butanoate and methyl butanoate as significant [5,6,10].

Many compounds present in strawberry fruits are chiral, and as such can exist as a racemates, or in specific enantiomeric ratios. In fact, many of the furanones are found as racemates [11] however excess of one enantiomer is often observed for natural chiral flavour compounds, because of the stereoselective nature of the enzymes which catalyze their biogenesis [12]. Enantiomers of a particular compound can exhibit vastly different sensory properties, for example the aroma of the *S*-enantiomer of 2-methyl butyric acid has been described as fruity and sweet [13] and pleasant, sweet, and elegant with a fruity note [14]. Conversely, the aroma of the *R*-enantiomer has been described as penetrating, cheesy and sweat-like [13,14]. The importance of enantiomeric characterisation is therefore critical for flavour evaluation, and enantiomeric ratios may also offer a means for varietal differentiation.

Methods for the isolation and enrichment of strawberry volatiles include liquid–liquid extraction, liquid-solid

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extraction, vacuum distillation and simultaneous steam distillation–extraction and headspace methods [8]. Liquid–liquid extraction is the most commonly used method for the isolation of strawberry volatiles, however this method is often tedious, requiring large volumes of high purity solvents [15], and may result in selective isolation. The fact that such isolations are labour intensive, time consuming, and susceptible to artefact formation has resulted in an increased popularity in headspace methods for volatile profiling.

Hakala et al. [6] used purge-and-trap headspace gas chromatography–mass spectrometry (GC–MS) for the analysis of the volatile composition of frozen strawberries whereby volatiles were collected using dynamic headspace sampling of thawed strawberry, and desorbed by backflushing into a cryofocusing module and a DB-1 phase coated capillary column cryogenic trap, coupled to a GC–MS fitted with an analytical fused silica capillary column of SPB-1701 phase. In total, 52 compounds were identified in the six strawberry varieties that were analysed. There is a consensus however that purge-and-trap and/or simultaneous steam distillation are expensive and time-consuming processes which can be prone to methodological difficulties [15] and artefacts [16].

Solid-phase microextraction (SPME) is a fast, solventless extraction method, developed by Arthur and Pawliszyn just over a decade ago [17]. This method has attracted widespread popularity for the analysis of volatile flavour components in various food, beverage and natural product matrices. SPME, because of its simplicity and speed was utilised in this work, with the aim of qualitatively and semi-quantitatively characterising the volatile composition of various strawberry varieties. Several authors have conducted SPME experiments for the analysis of strawberry volatiles, using various SPME fibres and conditions, although there is little consensus on the most suitable SPME method. Some have used whole fruits, with extraction times varying from 5 to 45 min, and equilibration times from 5 min to 2 h at temperatures up to 30 °C [10,15,18,19]. Others have used chopped or puréed fruits, with or without the addition of salts and internal standards, with or without stirring [18,20–23]. Little has been published on the reproducibility and repeatability of each method.

Several authors have investigated strawberry volatiles via SPME using PDMS fibres [10,20,21]. However, Song et al. [15] stated that the best fibre coating was PDMS/DVB based on its affinity for more polar molecules, although the important esters [5] and DMHF strawberry aroma components were extracted efficiently by all the fibres they examined, including PDMS. Thus, a PDMS coated fibre, was deemed suitable for the present study.

Different instrumental methods have been reported for analysis of strawberry volatile compounds, including direct atmospheric pressure chemical ionisation-gas phase analysis (APCI-GPA) of the headspace of macerated strawberry fruit, after calibration of the mass spectrometer with an ethyl butyrate standard [9]. Such direct sample introduction headspace mass spectrometric techniques obviate the need for sample extraction, and therefore eliminate the formation of artefacts. However, because compounds are resolved based on mass alone, the technique cannot differentiate between stereoisomers.

Song et al. [15] used SPME combined with GC–time-of-flight MS for the analysis of flavour volatiles in tomato and strawberry fruits. Rapid analysis was achieved by exploiting the fast mass spectral acquisition and deconvolution capabilities, which enabled compression of the chromatographic run time but was still able to identify overlapping eluting compounds. This method allowed the detection of important flavour compounds that were not detected by the slower, purge-and-trap/GC–MS sampling methods; 34 compounds were identified.

Due to the large number of components and the complexity of strawberry aroma, Urruty et al. [22] described the need for a technique that has a utility similar to that of a high-resolution photograph, as a tool to measure and compare strawberry aroma, and this description aptly applies to the capabilities of the technique of comprehensive multi-dimensional gas chromatography (GC × GC), which produces two-dimensional contour plots, or fingerprints very similar to photographs, of the sample volatile composition. GC × GC is now an established technique, offering superior separation capabilities afforded by high peak capacity offered by coupled GC columns, and sensitivity enhancement compared to routine single dimension GC. Only recently has GC × GC using enantioselective columns been implemented (e.g. [24,25]), and this work represents the first report of the application of enantioselective GC × GC to the characterisation of strawberry volatile composition.

2. Materials and methods

2.1. Standards

Twenty-one of the most relevant volatiles found in strawberries based upon literature data [5,6,10] were analysed individually and as part of a standard mixture by GC × GC to obtain retention time data for the first and second dimensions. DMHF, methyl butyrate, ethyl butyrate, methyl 2-methyl butyrate, butyl acetate, 2-furaldehyde, *trans*-2-hexen-1-al, hexyl acetate, *S*-(+)-2-methyl butyric acid, methyl acetate, ethyl acetate, benzaldehyde and tridecane were purchased from Fluka Chemie GmbH (Buchs, Switzerland), and hexanal, *n*-amyl acetate, methyl hexanoate, ethyl hexanoate, linalool and methyl cinnamate were purchased from Merck-Schuchardt (Hohenbrunn, Germany). Mesifuran and ethyl 2-methyl butyrate were supplied by Aldrich Chemical Co. (Milwaukee, USA) and analytical grade ethanol was supplied by Burdick & Jackson (Michigan, USA).

Tridecane was used as an internal standard, as it had been shown not to mask any of the peaks produced by strawberry

Table 1
Strawberry volatile standard mixture components, showing two-dimensional retention times and concentration

Peak number	Standard	^{1}D t_R (min)	^{2}D t_R (s)	Concentration in standard mix (μL/L)
1	Methyl acetate	3.7	0.75	SPME[a]
2	Ethyl acetate	5.2	0.60	SPME[a]
3	Methyl butyrate	8.1	0.93	411
4	Methyl-2-methyl butyrate[b]	9.7	1.11	274
		10.2	1.13	
5	Ethyl butyrate	10.5	1.05	247
6	Acetic acid	10.7	0.75	822
7	Ethyl-2-methyl butyrate[b]	11.6	1.30	274
		12.1	1.30	
8	Butyl acetate	12.4	1.12	205
9	Hexanal	12.7	1.13	205
10	2-Furaldehyde	16.8	1.40	219
11	*trans*-2-Hexen-1-al	17.3	1.40	178
12	*n*-Amyl acetate	17.4	1.37	137
13	Methyl hexanoate	17.5	1.63	137
14	Ethyl hexanoate	22.1	1.93	205
15	Benzaldehyde	25.1	2.10	178
16	Hexyl acetate	25.4	1.99	17
17	Mesifuran[b]	34.7	2.28	274
	2,5-Dimethyl-4-methoxy-(2*H*)-furan-3-one	Not separated		
18	*S*-(+)-2-Methyl butyric acid	35.5	1.43	137
19	Linalool[b]	37.8	2.05	274
	3,7-Dimethyl-1,6 octadien-3-ol	38.5	2.00	
20	Tridecane	43.7	2.21	96
21	Furaneol[b]	46.5	1.83	411
	2,5-Dimethyl-4-hydroxy-(2*H*)-furan-3-one	47.5	1.80	
22	Methyl cinnamate	53.4	2.60	437

Refer to Fig. 1 for the GC × GC result.
[a] Compounds with retention times which overlap the ethanol peak thus their ^{2}D retention times were determined via SPME of pure standards.
[b] Chiral compounds.

volatiles [26]. A standard mixture was prepared in ethanol, and volume concentrations (μL/L) of each component are listed in Table 1. This mixture was serially diluted to prepare calibration standards.

2.2. Strawberries

Strawberries (cv. Camarosa) used for SPME method optimisation and validation were purchased from a supermarket and stored at 4 °C prior to use. The variety used was dictated by supermarket availability at the time of investigation. All fruits were allowed to come to room temperature for equilibration between fruit and headspace before subsequent SPME extraction. Experimental samples (cultivars Adina and Selva) were picked on the morning of testing at a commercial strawberry farm in Coldsteam, Victoria.

2.3. Isolation of volatiles from strawberry using SPME

The SPME fibre holder and 100 μm polydimethylsiloxane (PDMS) fibre were purchased from Supelco Inc. (Bellefonte, PA, USA), and fibres were conditioned in a hot GC injection port at 250 °C for 30–60 min prior to sample extraction.

In order to optimise the isolation of volatiles by headspace SPME, several different methods of strawberry preparation were investigated: a whole single fruit, a chopped single fruit, a mixture of several chopped fruits, several whole fruits and several puréed fruits. Also examined were a variety of equilibration and extraction times. For whole berries both with and without the calyx, extraction times ranging between 15 and 45 min were trialed with equilibration times ranging from 15 min to 2 h. For the puréed matrix, four berries without sepals were puréed, and 5–10 mL subsamples with 15 μL of internal standard mix were equilibrated between 0–1 h and extracted over the range of 15–45 min. It was found that the largest number of volatiles was isolated using puréed or whole fruit (based upon detected GC × GC peaks), and the final optimised conditions used for both fruit matrices are as follows:

2.3.1. Whole fruit analysis

Four whole fruits (with calyx) were equilibrated in a 250 mL vial for 2 h prior to SPME extraction at room

temperature for 45 min based on experimental data, and optimised SPME equilibration conditions presented by Holt [16]. The SPME fibre was desorbed for 5 min in the GC injection port, and then further desorbed for 10 min in a separate injection port at 250 °C to prevent analyte carry over.

2.3.2. Puréed fruit analysis

Four whole fruits were puréed (calyx removed), at constant speed, using a hand-held blender. A sub sample (5 mL) of the purée was placed in a 20 mL flask and 15 μL of a tridecane/ethanol mix (2 μL tridecane in 5 mL ethanol) as an internal standard was added. The headspace of this sub sample was immediately extracted by SPME for 15 min with the provision of magnetic stirring. The fibre was desorbed as described for the whole fruit analysis.

2.3.3. Post-harvest analysis

Analysis of the cultivar Selva was performed on four fresh-picked whole berries and repeated on the same berries, after storage at 4 °C, 1 and 3 days post-harvest. The berries were allowed to come to room temperature before equilibration was commenced.

2.3.4. GC × GC

GC × GC analyses were conducted using a model 6890 GC (Agilent Technologies, Burwood, Australia) retrofitted with a longitudinally modulated cryogenic system (LMCS) from Chromatography Concepts (Doncaster, Australia). The LMCS was operated at a modulation period of 4 s, and Chemstation software (Agilent) was used to instruct the electronic modulator control to commence modulation at a predefined time. The first dimension of the column set was composed of two directly coupled enantioselective columns. The first was a EtTBS-β-CD coated column of dimensions 20 m × 0.25 mm × 0.25 μm (film thickness) (MeGA, Italy) and the second a CycloSil B coated column (26 m × 0.25 mm × 0.25 μm; J&W Scientific, USA). A short section of BPX5 (5% phenyl polysilphenylene-siloxane) column (0.14 m × 0.25 mm × 0.25 μm; SGE International, Australia) was used as a trapping column to improve focussing of more volatile compounds, between the first dimension and the more polar second dimension column (BPX50; 1 m × 0.1 mm × 0.1 μm; SGE International, Australia).

Flame ionisation detection was used to monitor the column effluent, and was operated at 250 °C with a data acquisition rate of 100 Hz. Injections were performed at 250 °C using either splitless or split (20:1) conditions, with a hydrogen carrier gas, flow rate of 2.5 mL/min, equivalent to an inlet pressure of approximately 28 psi, and an average linear velocity of 59 cm/s. The flow velocity in the second (narrow bore) column will be significantly larger than this, approximately 370 cm/s, which is higher than the optimum flow, but for chiral separations on the first column a higher flow rate elutes compounds at lower temperature which favours better enantio-resolution, and so the flow rate was chosen as above.

Temperature programming conditions were optimised for best separation of components of the standard mixture, initially based upon the method developed by Shao and Marriott [27] who used the present column set for the analysis of chiral compounds including various 2- and 3-methyl isomers of butyric acid relevant to wine volatile analysis. For Program 1, the GC oven was held for 5 min at 50 °C, ramped to 200 °C at 3 °C/min (held for 1 min), then increased to 240 °C at 20 °C/min (held for 10 min). For Program 2, an initial oven temperature of 60 °C was held for 5 min, ramped to 84 °C at 3 °C/min (held for 1 min), ramped to 86 °C at 0.5 °C/min (held for 2 min), ramped to 95 °C at a rate of 1 °C/min (held for 2 min), ramped to 200 °C at 3 °C/min (held for 1 min), and finally ramped to a final temperature of 240 °C at 20 °C/min (held for 10 min). The multi-step temperature program was employed to provide adequate chiral separation of some of the standard compounds.

GC × GC data were transformed using an in-house program and visualised as contour plots using Transform software (Fortner Research, Virginia, USA). For data analysis, including total peak height and area, Chemstation (Agilent) files were exported as "∗.csv" integration files prior to analysis using a Matlab program (L. Xie). The two-dimensional chromatograms produced represent a volatile profile for each cultivar. Principal component analysis (PCA), using the statistical package, Minitab (Version 13), was performed with the aim of analysing the differences in relative peak area for each cultivar, and to determine if there was a predictive set of compounds for the volatile profile of a particular cultivar. Data (not pre-treated) compiled from three, separate analyses, each from a set of four whole berries was used for the PCA. The Minitab program was instructed to extract five principal components and analysis was performed using a co-variance matrix with no transformation of the data or rotation of axes.

3. Results and discussion

3.1. GC × GC optimisation

The GC × GC process involves modulation of GC peaks eluting from the first column through trapping, focusing and effectively slicing them into a number of peak pulses for subsequent rapid analysis on the second column. Each analyte will be presented in the GC × GC chromatogram as a series of peak pulses with the envelope of the pulses forming the shape of the first dimension GC peak [28]. Importantly, the cryogenic modulation process used in this investigation is mass conservative, and so the area for each analyte is determined by adding the individual areas of each pulsed peak. Table 1 shows the concentration of each standard in the mixture, with concentrations purposefully selected to ensure similar peak responses for each analyte which indicate the different response factors inherent to each analyte, e.g. the detection of hexyl acetate is significantly more

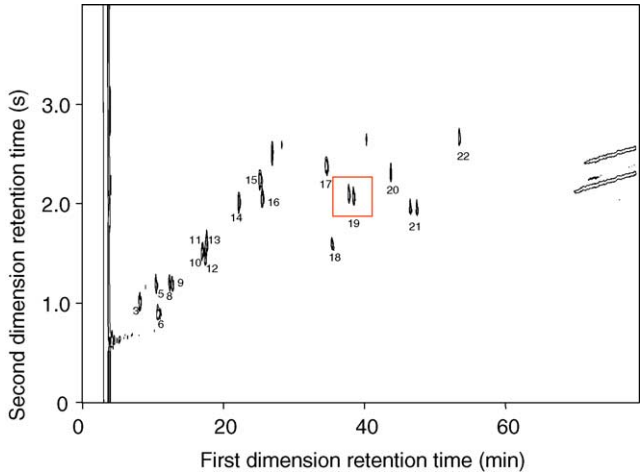

Fig. 1. Two-dimensional contour plot of direct solvent injection of the strawberry volatile standard mix, highlighting the separation of linalool enantiomers. (Refer to Table 1 for compound identification.) It should be noted that standards methyl-2-methyl butyrate and ethyl-2-methyl butyrate are not included as they cannot be adequately resolved at this contour setting.

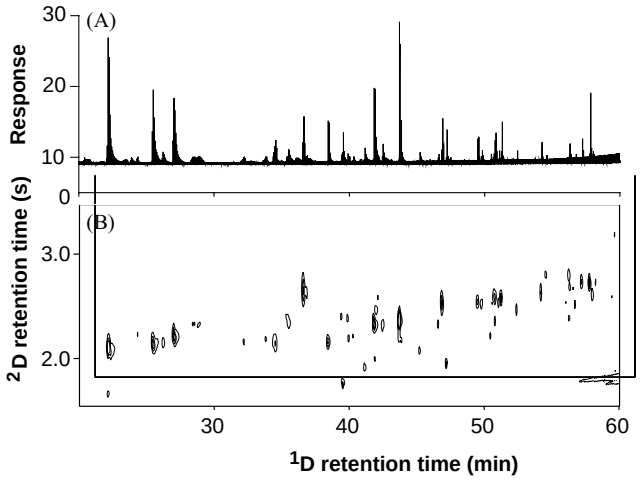

Fig. 2. Modulated chromatogram (A) and corresponding two-dimensional contour plot (B) for puréed fruit analysis. Contour plot levels were set at 8, 10, 12 and 15 pA.

sensitive compared to that of acetic acid. The linearity of the GC × GC method was validated through the construction of four-point standard calibration curves, and R^2 values of at least 0.996 were obtained over a concentration range of 150–1000 μL/L.

Determination of the best temperature program was achieved using the standard mixture of strawberry flavourants. The initial temperature program (Program 1) was that used by Shao and Marriott [27] for the separation of enantiomers of ethyl lactate, 2-methyl butyric acid and 2-methyl butanol in wine, and for their separation from the 3-methyl isomers. This program was then optimised to give the best resolution of the standards contained in the mixture (Fig. 1). Fig. 1 demonstrates the chiral separation achieved for analyte enantiomers through the use of shape selective capillary columns, and shows that for enantiomeric compounds, such as linalool (19) and DMHF (21), an elution pattern typical of enantiomeric pairs. Resolved enantiomers exhibit slight differences in retention time, so one enantiomer will elute into the second column at a slightly higher temperature; its higher elution temperature leads to less retention. Enantiomers therefore appear as two peaks in the two dimensional plot, separated slightly in the *y*-dimension [23] (Fig. 1).

Resolution of enantiomers of DMHF (21) was successful, despite literature reports to the contrary [12], thereby enabling the enantiomeric ratio of DMHF in strawberry fruit to be established, and the potential to quantify individual enantiomer concentrations. Raab et al. [12] believe racemisation occurs in the injection port, and that GC analysis is unsuitable for the determination of DMHF due to uncertainty of absolute enantiomeric ratio. Resolution of the enantiomers of the structurally similar mesifuran (17) however could not be achieved, despite temperature programming adjustments. The structure of mesifuran is conducive to keto-enol tautomerism (with the keto form enantiomeric). If this structural interconversion occurs rapidly on the GC column, only a single, averaged peak will be seen (peak coalescence of the third kind [29]).

3.2. Strawberry analysis

SPME analysis with GC × GC of both whole berries and puréed berries was undertaken to determine the most suitable matrix for the representative headspace extraction of fruit volatiles. Fig. 2 depicts both the expanded modulated chromatogram (maximum response ~55 pA) and its corresponding two-dimensional contour plot obtained for puréed fruit analysis, and highlights the benefit of GC × GC for the improved resolution of a significantly greater number of strawberry volatiles. Vertically aligned contour peaks co-elute on the first column, and are separated on the second. The sensitivity enhancement due to the zone compression effect increases peak responses and smaller peaks can be better delineated compared with the single column experiment. Contour levels for Fig. 2B were set at 8 (just below baseline), 10 (just above baseline), 12 and 15 pA, and from Fig. 2A it is clear which peaks can be sampled at each contour setting. Any peak less than 10 pA will not be drawn on the contour diagram. Tailing peaks seen in Fig. 2A can be recognised in Fig. 2B as contours distorted to longer retention time. In absence of zone focussing (which gives about a 10–20-fold increased in response magnitude), most peaks will have responses less than about 3 pA above baseline, which would make many peaks indistinguishable. Despite the successful separation of analytes in the two-dimensional space, both strawberry matrices were found to contain very few of the 21 standard compounds. Since all analytes of the standard mixture could be detected using headspace SPME/GC × GC analysis of their solution (with the exception of methyl

and ethyl acetate which co-eluted with the ethanol solvent), the PDMS fibre is able to sorb these analytes. This suggests that either these volatile components were not present in the strawberry samples, or were present at concentrations below detection limits. Despite an attractive appearance, the strawberries were judged by the experimenters as decidedly lacking in flavour, especially compared to those sampled at the peak of the previous season. The fact that the present season was initially very cold, followed by several days of high temperature, may have hindered the development of the volatile components, thus explaining the resultant bland, firm and watery fruits. Watson et al. [9] demonstrated that even relatively short periods of lowered light levels can affect production of volatiles, and that plants subjected to 47% shading showed significantly reduced headspace concentrations of hexenal, hexanal, ethyl methyl butyrate and methyl butyrate compared to control fruits. They hypothesised that a reduction in photosynthesis, caused by shading, could reduce the amount of primary metabolites produced by the plant, consequently reducing the raw materials available for synthesis of volatiles [9]. Thus, it is plausible that the low levels of volatiles detected in this investigation may be a direct result of poor and overcast weather experienced throughout the fruit development.

The aim of this investigation was to gain qualitative and semi-quantitative volatile profiles for direct comparison of strawberry fruit varieties. Therefore, it was necessary to evaluate the reliability of the SPME data obtained from both whole fruit and puréed fruit analyses with respect to extraction repeatability and reproducibility, by monitoring area response values for selected GC × GC peaks. Thus, peaks (unidentified) were randomly selected from across the entire temperature program range, ensuring that analytes with significantly different boiling points and polarities were assessed, rather than a closely eluting range of analytes that may have similar SPME extraction performance.

For the purposes of the qualitative SPME study, repeatability was undertaken using the same fruit sample (whole fruits or purée) and thus the same sample headspace, whilst reproducibility investigations used new fruit samples of the same fruit variety and batch. Repeatability studies evaluate the performance and reliability of the SPME extraction method, whilst reproducibility investigations determine the extent of the variation between fruit samples and thus the feasibility of the SPME approach for the quantitative comparison of fruit samples of the same variety, harvested at different intervals. Replicate repeatability experiments using the same fruit sample were not considered feasible; due to the long equilibration time used (2 h), fruit began to degrade faster in the enclosed containers at room temperature, indicating that artefacts relating to respiration and enzymatic breakdown may be forming. This also limits the number and comparability of repeat analyses that can be conducted for reproducibility purposes. The results obtained for these investigations are discussed below.

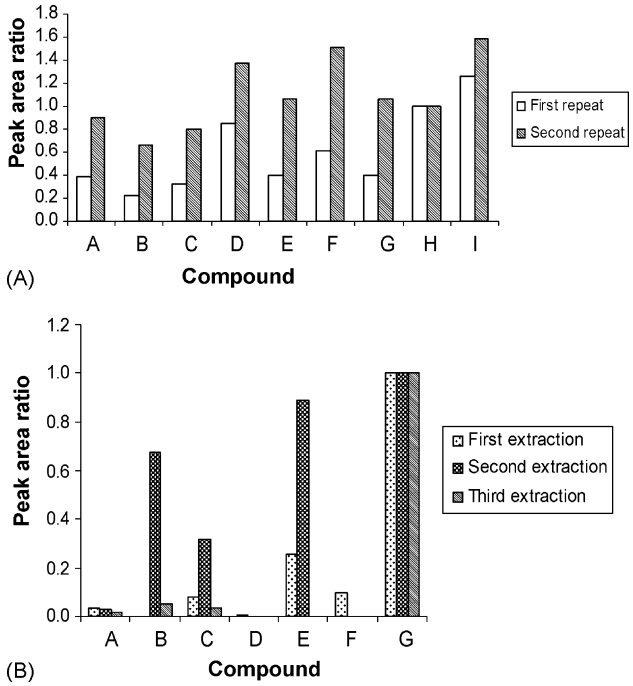

Fig. 3. (A) Repeatability of SPME headspace extraction from puréed fruit. (B) Reproducibility of SPME headspace extraction from sets of puréed fruit.

3.3. *Puréed fruit analysis*

Puréed fruit allows internal standard to be spiked into the mixture, which should improve measurement of the efficiency of the SPME extraction process and compensating for any variability in GC injection. However, the repeatability of the method was less than that desired, with percentage standard deviation for the same compound, from replicate analysis of the same sample averaging around 70%, caused by increasing analyte concentrations as a result of extended equilibration time (Fig. 3A). Whether this was caused by oxidation or enzymatic breakdown of other compounds was not established, although formation of new compounds was detected by their appearance and increasing concentration over successive analyses. Although a corresponding decrease in other compounds was not detected, other authors have observed the generation of significant quantities of *trans*-2-hexen-1-ol, 1-hexanal and *trans*-2-hexenal by enzymatic oxidative cleavage of linoleic and linolenic acids in the presence of oxygen during homogenisation of the fruits [5]. These C_6 aldehydes and alcohols may account for up to 55% of the volatile profiles of homogenized fruit [30], but account for less than 0.1% of the volatiles collected from whole fruit [31].

Variations in reproducibility, between three different samples, each from a purée of four different fruits from the same harvest (Fig. 3B) show much larger percentage standard deviations, as high as 173% for compounds D and F, with an average percentage standard deviation of 127%. This is most probably due to the natural variation within the strawberry

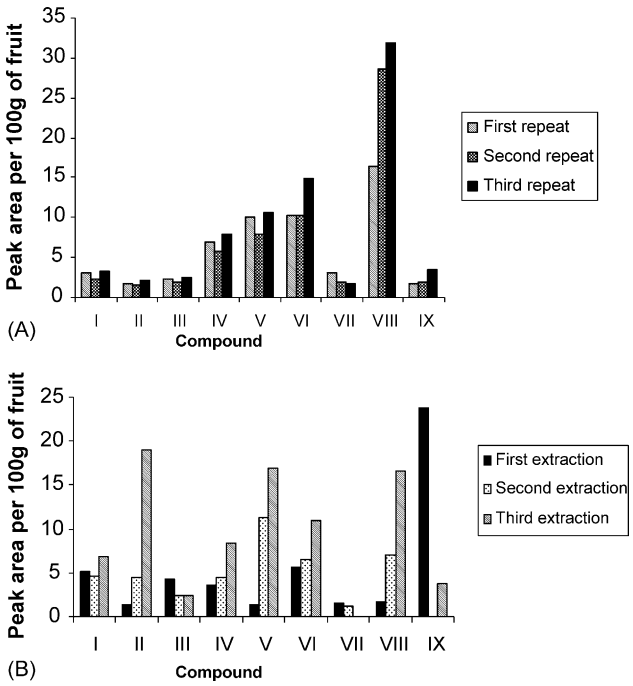

Fig. 4. (A) Repeatability of SPME headspace extraction from whole fruits. (B) Reproducibility of SPME headspace extraction from sets of whole fruits.

population but coupled with the variations in repeatability make this a rather inaccurate sampling method for strawberry volatiles. It must be noted that the reproducibility and repeatability studies were completed using different temperature programs and thus the identity of the compounds cannot be correlated between the two analyses.

3.4. Whole fruit analysis

Volatiles were extracted from the headspace of whole fruits (calyx attached), and analysed as described previously. This situation differs from that of the puréed fruit, whereby the calyx was removed from the fruits prior to maceration. Whole, intact berries were sampled to avoid the formation of secondary products that may occur when the cells are disrupted as in a purée, or when the calyx is removed, which may stimulate the production of new volatiles as part of the fruit's wounding response.

Fig. 4A depicts the repeatability for three successive extractions from the same set of four whole fruit. Amounts extracted for each analyte were variable, however the headspace concentrations of some analytes (identity unknown) increased with each extraction (compounds VI, VIII, IX). The observed variation is most likely due to changes in headspace composition over time. Percentage standard deviations for analytes were much lower than for puréed fruit, the highest being 42% for compound IX. The average relative standard deviation for repeatability of this method was around 30% which was much lower than for puréed fruits although still well above the generally accepted 7% for trace organic analysis [32]. One factor that may have contributed to this is that due to the long chromatographic run time (79 min), replicate samples had a much longer equilibration time compared to the 2 h equilibration time used in the first extraction. Nevertheless, a 2 h equilibration time was shown to give the best comparative recovery of compounds within a reasonable time, based upon equilibration time optimisation. Reproducibility studies showed even greater percentage standard deviations (Fig. 4B), as high as 114% (II), on par with those obtained from puréed fruit. Part of this variation must be attributed to natural variance between the fruits [9] since different fruits were used for reproducibility studies, but also limitations to the SPME technique. Its ease and speed are counterbalanced by problems relating to its reproducibility and quantification [32].

SPME analysis of whole berries enables a better understanding of the headspace composition of the complete, intact fruit, which is what the consumer first bases their choice on, and minimises the creation of secondary products; however, analysis of headspace compounds is dependent upon their individual vapour pressure. Thus, the more-volatile analytes might be more easily extracted, and appear at comparatively higher concentrations. This reflects the compound's contribution to the fruit aroma, but does not give its true concentration in the tissue [7]. Ultimately this method of extraction could not be used to accurately quantify the level of compounds in the sample, a problem other authors have encountered with this sampling approach [32,15]. Thus, compounds present in the cultivars analysed are presented as relative concentrations per 100 g of fruit.

3.5. Differentiation of strawberry varieties

Volatile analytes from whole berries were identified by comparison of their two-dimensional retention time co-ordinates with that of the standards, and included methyl butyrate, methyl cinnamate, DMHF, linalool, benzaldehyde, butyl acetate and methyl hexanoate. A single GC–MS analysis of an Adina sample also revealed the presence of 2-methyl-octyl butanoate, (2)-3-hexenyl-1-acetate, methyl hexanoate, hexyl acetate, octyl butanoate and 3,7,11-trimethyl-1,6,10-dodecatrien-3-ol (nerolidol). Assignment of these compounds to the GC × GC volatile profile however could not be achieved in this preliminary investigation, since GC–MS analysis was performed using different GC conditions and column (e.g. with an achiral column).

DMHF was detected at relatively low levels in both varieties, however concentrations were higher for Adina fruit. This is not unexpected as Adina is a much tastier variety and DMHF is considered to be an essential component of strawberry flavour. In fact, DMHF has a very low odour threshold, thus a small difference in concentration may account for a great difference in taste. The concentration of this compound is known to vary widely between cultivars [33]. Similarly, methyl butyrate, described as a character-impact

compound in strawberries, [9] was found to have a slightly higher average peak area in cv. Adina (4.8 versus 2.3 per 100 g of fruit). Only one enantiomer (the first eluting enantiomer) of DMHF was detected in fresh strawberries, and has been tentatively identified as the (−) enantiomer as this was found to elute first in both capillary electrophoresis and chiral-phase HPLC [12], although with different enantioselectors used in these experiments, the assignment is far from certain. None of the (+) enantiomer was detected although it has been reported that DMHF isolated from strawberries is racemic [12]. Mesifuran, created via the methylation of DMHF [34], which is considered to play a considerable role in strawberry flavour was not detected in either variety at any stage. It has been suggested that the concentration of mesifuran depends more on the availability of its precursor, DMHF, rather than on the methyl transferase that creates it [34], thus its absence may be due to low levels of DMHF.

Only one enantiomer of the terpene alcohol linalool ((±)-3,7-dimethyl-1,6-octadien-3-ol) was detected in the cultivars examined. Each enantiomer of this alcohol evokes different neural responses in humans [35], the enantiomer isolated from both varieties is tentatively identified as the *S* form, the same as is present in tomato fruits, on the basis of peak elution order determined by analysis of a sample of lavender essential oil on the enantioselective column set. Relatively high levels of the linalool enantiomer were detected in both cultivars, although on average they were much higher in Selva. A high relative concentration of benzaldehyde was also detected in Selva fruit, in agreement with the findings of Pelayo et al. [36]. Both of these compounds are noted to be important aroma contributors [8]. The majority of unidentified analytes were present in greater concentrations in Selva compared to the much tastier Adina variety. It could therefore be postulated that the unknown analytes could be composed of more volatile alcohols and acids which play little role in overall flavour due to higher odour thresholds [5].

Because only eight compounds could be unequivocally identified in the GC × GC contour plots on the basis of available standards, an additional 12 unidentified compounds that showed significant peak areas were selected and included in PCA for the differentiation of strawberry varieties. Examination of the variation between the cultivars via principal component analysis, using data compiled from triplicate whole berry analyses (expressed as average analyte peak area per 100 g of fresh fruit) shows that the differences in concentrations of nerolidol, linalool and benzaldehyde appear to be most significant. A score plot defined by the first two principal components was considered sufficient to visualise the differences between the varieties as it explains most (>84%)

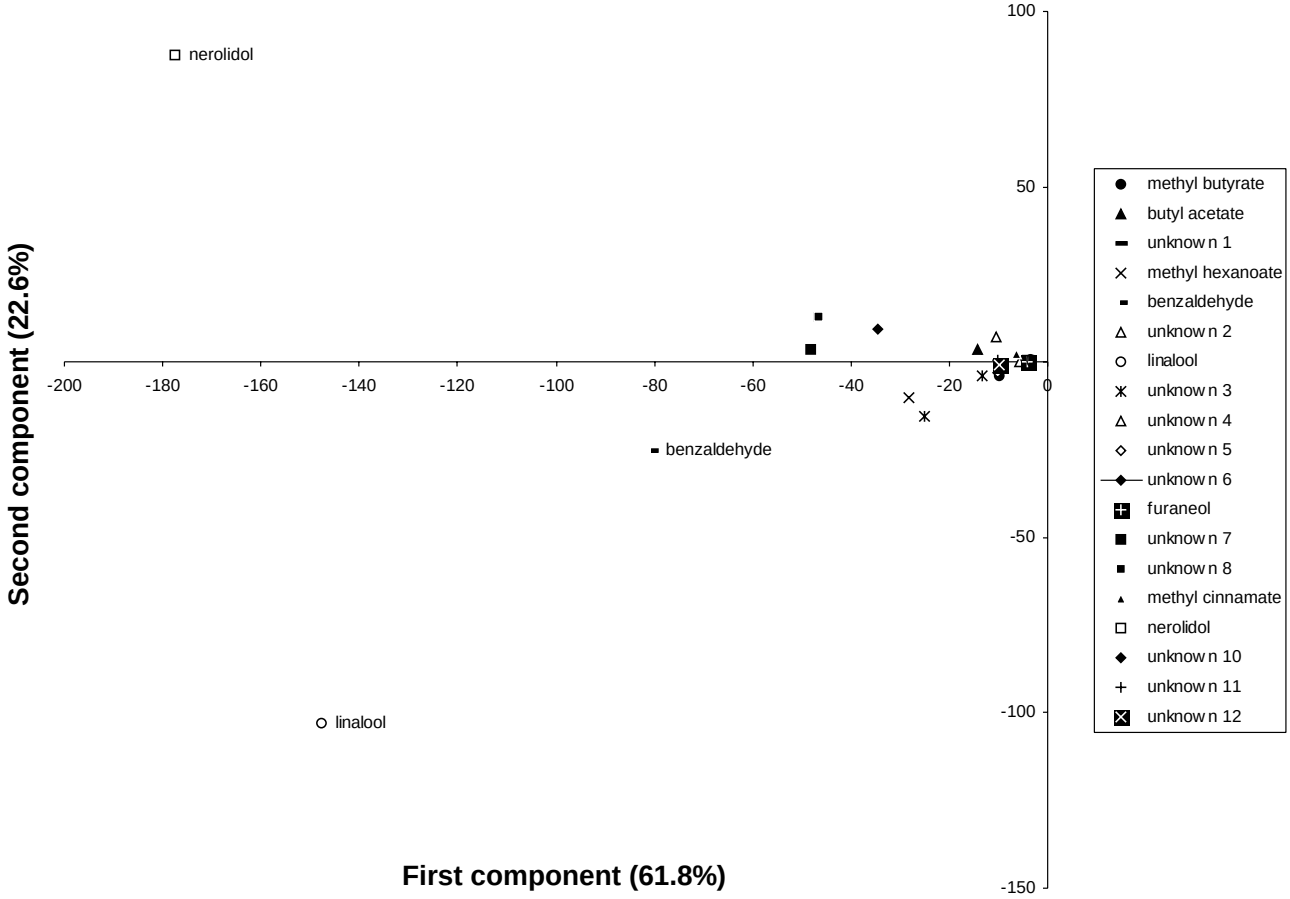

Fig. 5. Principal component analysis of cultivars Adina and Selva.

of the variation (Fig. 5). The first component explains only 61.8% of the differences in peak area observed between the varieties whilst the second component accounts for 22.6%, thus separations of compounds in both dimensions is relatively important. Separation of the scores of each compound along the *x*-axis (first PCA component) appears to correlate with increased peak area in Selva, whilst separation along the *y*-axis (second PCA component) correlates with a larger peak area in Adina for compounds with greater differences in peak area.

It cannot be said that the relative peak areas for each variety correlates with compounds that produce attractive flavours in strawberries, as some of the more important compounds are present in low concentrations and thus do not contribute significantly to the principal components. However, it does reveal that there are similarities between the concentrations of many compounds in both varieties, many of which are most likely esters as these are the most predominant qualitative and quantitative components of strawberry volatiles [5]. Although other authors have successfully used PCA to differentiate between the volatile profiles of several cultivars [6,33] this could not be done on the basis of these experiments. This may be due to the fact that whilst the cultivars selected had quite different tastes, at the time the studies were conducted the fruit was not at its most flavoursome, or simply that not enough repetitions were performed to overcome the variation within a cultivar. Hakala et al. [6] found that the effect of environmental conditions in the year of harvest could have a greater effect on volatile concentration than the difference between varieties. Thus, it may be that there are significant differences between the volatile profiles of these varieties that were not detectable in this study.

3.6. Comparison between fresh picked and post-harvest fruit

Analysis of the cultivar Selva was performed on fresh picked whole berries and repeated on the same berries after storage at 4 °C, 1 and 3 days post-harvest. The compounds were identified by comparison of retention times in first and second dimensions with those of the standards, and the area response values for selected GC × GC peaks were used for PCA.

Principal component analysis was carried out for the same 20 compounds analysed for the cultivar study. The results displayed in Fig. 6, show that the first principal component accounts for 90.9% of variation and that the second component for 8.8% of variation. Compounds with larger negative values in the *x*-axis have higher concentrations in mature fruit and compounds with larger negative values in the *y*-axis are those with higher peak areas in post-harvest fruit. As the first component explains most of the variability, the

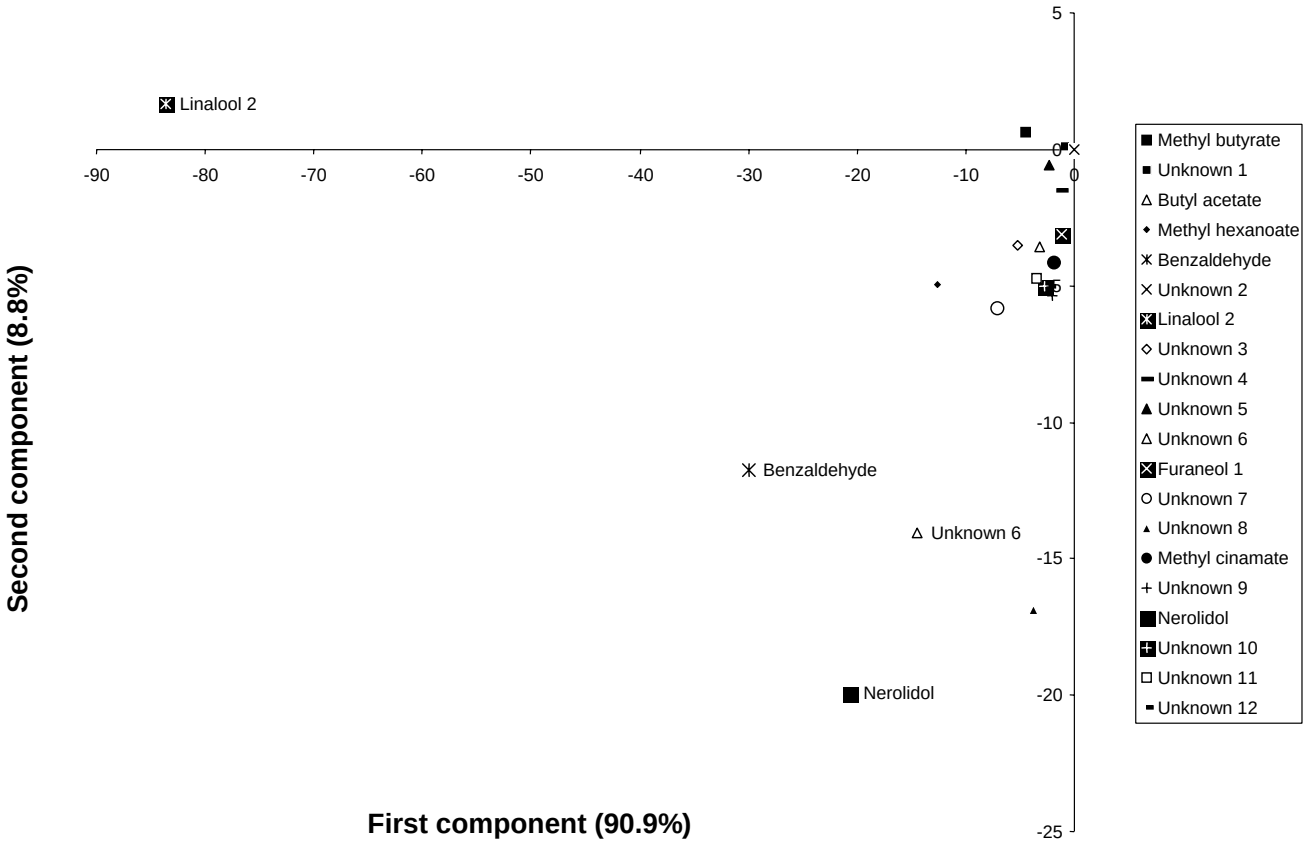

Fig. 6. Principal component analysis comparing fresh and post-harvest Selva fruit.

separation along the *x*-axis of the components gives stronger correlation with differences in the volatile profile of the samples. Linalool appears separated from the rest of compounds indicating that linalool is prevalent in fresh picked fruit. Benzaldehyde, nerolidol, unknown 6 and methyl hexanoate are also separated from most compounds in the *x*-axis meaning that they are significantly different in the profiles of mature and post-harvest fruit.

Benzaldehyde was found to decrease in post-harvest fruit, a similar result to that of Pelayo et al. [36] who found that Selva had a high concentration of benzaldehyde that ranged from 70 to 228 nL/L, that decreased to 78–126 nL/L in post-harvest fruit stored at 5 °C for 7–11 days. Methyl butyrate and methyl hexanoate were found to decrease after 3 days post-harvest whilst ethyl esters were absent in mature and post-harvest fruit. These results agree with those of Pelayo et al. [36] who reported that methyl esters were more prevalent in Selva cultivars, and also found that Selva fruits stored under cooling conditions had higher amounts of methyl esters than ethyl esters after 9 days of storage. Pérez et al. [37] have demonstrated that ratio of methyl/ethyl esters is variety dependent. (−)-DMHF was the only isomer detected in Selva fruits, and showed an increase after 3 days of storage at 4 °C. Higher levels of furanones have also been observed by other authors, when strawberry samples are stored under cooling conditions. Pérez et al. [38] reported that DMHF levels increased from 8.7 ± 1.4 μg/g to 12.8 ± 1.3 μg/g when stored at 1 °C in polypropylene filmed baskets for up to 9 days. Pelayo et al. [36] found that the levels of furanones in Selva increased two-fold at the end of post-harvest life (11 days) stored in air at 5 °C.

4. Conclusion

The SPME method used for the extraction of strawberry volatiles ensured qualitative differentiation of the strawberry cultivars, Adina and Selva. However, SPME appears to be only suited to semi-quantitative analysis, and whilst it is a fast and effective qualitative tool its limitations must be recognised. Some of these limitations may be attributed to the fact that headspace SPME was performed manually; automated SPME in combination with solvent extraction may ensure more repeatable and reproducible methods for quantitative strawberry volatile analysis. Enantioselective GC × GC served as a valuable tool for the sensitive enantiomeric characterisation of strawberry volatiles. Specifically, enantiomers of each of the compounds linalool and DMHF, which are significant contributors to strawberry flavour, could be resolved by the described method, but only one enantiomer for each compound was identified in the fruit. Investigations into the effects of post-harvest storage showed definite changes in the volatile composition of the fresh and post-harvested fruits, with the latter showing increased levels of DMHF and nerolidol, and reduced levels of benzaldehyde and methyl hexanoate. A number of unknown analytes in the volatile profiles were separated using GC × GC, and thus (chiral) GC × GC–MS analysis is recommended for future investigations for the improved volatile characterisation of strawberry cultivars.

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