Journal of Chromatography A, 1218 (2011) 4517–4524

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Metabolite profiling on apple volatile content based on solid phase microextraction and gas-chromatography time of flight mass spectrometry

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ARTICLE INFO

Article history:
Received 17 November 2010
Received in revised form 2 March 2011
Accepted 9 May 2011
Available online 14 May 2011

Keywords:
SPME
GC–TOF-MS
Apple (*Malus domestica*)
Metabolite profiling
Metabolomics

ABSTRACT

A headspace SPME GC–TOF-MS method was developed for the acquisition of metabolite profiles of apple volatiles. As a first step, an experimental design was applied to find out the most appropriate conditions for the extraction of apple volatile compounds by SPME. The selected SPME method was applied in profiling of four different apple varieties by GC–EI-TOF-MS. Full scan GC–MS data were processed by MarkerLynx software for peak picking, normalisation, alignment and feature extraction. Advanced chemometric/statistical techniques (PCA and PLS-DA) were used to explore data and extract useful information. Characteristic markers of each variety were successively identified using the NIST library thus providing useful information for variety classification. The developed HS-SPME sampling method is fully automated and proved useful in obtaining the fingerprint of the volatile content of the fruit. The described analytical protocol can aid in further studies of the apple metabolome.

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1. Introduction

Apples are among the most widely cultivated tree fruits in temperate regions of the world. The commercial distribution and marketing of apples shows among others two important characteristics: (1) the long-term controlled-atmosphere storage which facilitates year-round availability of high quality fruit and (2) the numerous different apple varieties that are commercially available to encounter different consumer preferences [1].

Targeted analysis of apple volatiles has been used as a tool to characterise and classify varieties [2–4] or processing effects [5–7]. To the best of our knowledge the only untargeted approach published utilised proton transfer mass spectrometry (PTR-MS) without the prior application of separation method [8]. In that paper certain masses were identified as discriminators but definitive chemical identities were not reached.

Profiling of fruit metabolites can provide an effective tool to characterise the product and to extract useful information (e.g. on its quality or origin). A primary aim is the association between genes and metabolite content [8]. Our group's research is focused on the development of analytical methods to be used in population studies for the classification of fruits as a follow-up of the apple genomic sequence recently released by Velasco et al. [9]. To facilitate efficient profiling a strategy of two complementary analytical techniques was adapted: determination of volatiles conducted by GC–MS, along with LC–MS profiling of other organic metabolites including semi volatiles (methodologies will be described in future communications).

GC–MS based metabolomics studies typically utilise a dual derivatisation scheme that aims to decrease the polarity of small molecule metabolites so that the latter are analysed in GC with higher efficiency. This strategy covers important metabolite classes such as organic acids and amino acids, however derivatisation, being a chemical reaction, is dependent on various parameters and recent literature [10] shows that this process may introduce biases in metabolomics investigations. This may compromise the analytical results (introducing biases if samples are all simultaneously derivatised and are left in the autosampler awaiting analysis) or practically disable automation capabilities (in which case the analytical process is carried out sample by sample). Another important issue is the fact that derivatisation of the abundant primary metabolites may result to overloading the column or the detector. The concentration of primary metabolites can be up to eight orders of magnitude higher than that of certain secondary metabolites (such as a number of volatile compounds). This limits the possibility to concentrate the sample to enhance the detection of low abundance molecules, due to the need to protect, at the same time, the integrity of the analytical system.

In the present communication we report on using headspace solid phase microextraction coupled to gas chromatography time of flight mass spectrometry (HS-SPME GC–TOF-MS) as a methodology for metabolite profiling of the volatile content of an apple. An untargeted approach was employed using full scan MS data acquisition. SPME was selected for capturing the volatiles from the

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doi:10.1016/j.chroma.2011.05.019

samples and to provide simple and efficient sample preparation along with ready automation in the GC auto-sampler. SPME-GC–MS has been used in profiling volatiles from cacao beans [11], tomato fruit [12], raspberry [13], petunia flowers [14], bacterial samples [15] and human colon cell lines [16].

In our study SPME methodology was first optimised with the aid of software for experimental design. Subsequently, apple samples of four different varieties were analysed and the data were examined as chromatographic/mass traces but also after data reduction and multivariate statistical analysis. The apple varieties were chosen among the most representative cultigens. Granny Smith, Golden Delicious and Stark Delicious are historically “old” varieties, selected before 1900, originated respectively in Australia, West Virginia (USA) and Iowa (USA), while Pinova is a crossing between Golden Delicious and Clivia, originated in Dresden-Pillnitz (Germany) in 1965. The former three are among the major cultivars cultivated for the fresh market both in the Northern and Southern Hemispheres.

Short and longer term variations in the data were evaluated. The utility of freeze-drying for the removal of water from the samples and the facilitation of long-term storage prior to GC–MS analysis was also tested. Results show that HS-SPME GC–TOF-MS can provide a useful tool for the extraction of volatiles and the characterisation of apple varieties.

2. Experimental

2.1. Materials and apparatus

Sodium chloride, ascorbic and citric acid, as well as 2-octanol and isoamyl formate used as internal standards (IS), were purchased by Sigma–Aldrich. MilliQ water was used for sample preparation. A Sunbeam Osterizer 4153 Blender apparatus designed for soft-tissue homogenisation was used for homogenising the samples and a laboratory “bench top” freeze dryer LIO-5P (Cinquepascal, Milan, Italy) for freeze-drying. (DVB/CAR/PDMS) Stableflex fibers were used (Supelco, Bellefonte, IL) for SPME.

2.2. Sample preparation

In the present work, apples (*Malus domestica*) from four different varieties were studied: Golden Delicious (GD), Granny Smith (GS), Pinova (PI) and Stark Delicious (SD). About 2 kg of apples per each variety were bought at a local market in Trento, Italy in April 2010. All the apple samples were produced in Italy in the year 2009.

Seventy grams from ten fruits were diced using a commercial cutter and were immediately inserted in a glass vessel where they were mixed with 75 mL of deionized water, 30 g of sodium chloride, 250 mg of ascorbic acid and 250 mg of citric acid. Water was added in the mixture to help the homogenising process and the dissolution of salt and acids. The acids were added to inhibit enzymatic reactions and consequent oxidation due to polyphenoloxidases [17] while sodium chloride is able to inhibit most enzymes thus helping in preserving the samples. Furthermore sodium chloride promotes extraction efficiency of volatile compounds due to the salting out effect [18]. From the homogenised samples a 10 g aliquot was inserted into a 20 mL screw cap vial, suitable for volatile analysis, and was spiked with 20 μL of 2-octanol (2.5 mg/L) and 20 μL of isoamyl formate (200 mg/L) both used as internal standards. The vials were placed in the thermostated autosampler tray at 10 °C before the HS-SPME GC–MS analysis.

In addition, in order to investigate the freeze drying storage condition effects five fruits of the same batch of one variety (GD) were divided in two portions. The first portion (5 half apples) was directly treated fresh as described above and subsequently SPME-GC–MS analysed. The other halves were diced and immediately immersed in liquid nitrogen. Next they were freeze-dried for 24 h. Then the amount of water that was lost during the freeze-drying procedure was added to the freeze-dried samples and the samples were analysed using the developed SPME-GC–MS method.

2.3. Headspace SPME and GC–TOF-MS

Headspace volatiles were extracted and concentrated on a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane fiber with the aid of a CTC Combi-PAL autosampler (Zwingen, Switzerland). The fiber was preconditioned before the analyses, according to manufacturer's instructions, performing two blank injections at a temperature of 270 °C. Prior to injections, the samples were stirred (250 rpm) at 40 °C for 10 min and then the fiber was exposed in the headspace. Volatiles, after 30 min of absorption, were thermally desorbed in splitless mode for 5 min in the GC injector port held at 250 °C. The GC–TOF-MS system consisted of an Agilent 6890N (Agilent Technologies, Palo Alto, CA) gas-chromatograph equipped with a split/splitless injector coupled to an GCT^{TM} Premier MS system (Waters Corporation, Milford, MA) operating under MassLynx software. Separation was achieved on a forte SolGel-wax fused-silica capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; SGE Analytical Science Pty Ltd., Victoria, Australia). The GC oven temperature program (53 min) applied was as follows: start at 40 °C for 3 min, then 40–220 °C at 4 °C min^{-1}, stable at 220 °C for 1 min, and then 220–250 at 10 °C min^{-1}, and finally 250 °C for 1 min. Helium was used as carrier gas with a constant column flow rate of 1.2 mL min^{-1}. The transfer line temperature was kept at 250 °C. The ion source was operating in electron ionization mode (EI, internal ionization source; 70 eV). Full scan TOF-MS data were acquired in a mass range of 40–400 amu. Heptacosa (perfluorotributylamine) from Sigma–Aldrich was used as lock mass for mass correction in order to produce m/z data within ±5 ppm accuracy.

2.4. Sample analysis

Forty apple extracts, ten from each of the four different varieties, were analysed in one sequence in a randomised order. The autosampler tray holder is equipped with a cooling system that allowed the samples to be kept at 4 °C during the waiting time before the analysis (PAL TrayCooler, ATAS GL International B.V., Veldhoven, Netherlands). SPME GC–TOF-MS profiles were obtained with the aim to detect potential discriminating variables among the varieties.

For estimating the freeze-drying storage effect analyses on both fresh and freeze-dried sample extracts were performed in triplicates.

In order to estimate the overall within-day variation of the obtained GC–TOF-MS profiles which comprise the variability of the SPME procedure plus the variability in the analytical performance of the instrument, one of the samples (Pinova) was aliquoted into ten vials and analysed in a sequence of 10 analytical runs. In addition inter-day repeatability was assessed by analysing three aliquots of one Pinova apple extract 4 times over a period of 10 days. The sample was kept at 4 °C during that period of time.

2.5. Data analysis

For the SPME parameter optimisation the STATGRAPHICS Plus 4.1 (StatPoint Technologies, Warrenton, Virginia, USA) software package was used. The raw spectrometric data were analysed by MarkerLynx applications manager version 4.1 (Waters, Manchester, UK) using the following parameters: The apex track peak parameters were set automatically, intensity threshold was set

at 10 counts, mass window at 0.05 Da, retention time window at 0.08 min and noise elimination level at 4. A list of 2307 features corresponding to pairs of retention time (RT) and *m/z* values was generated and exported to SIMCA-P+ software 12.0 (Umetrics, Umeå, Sweden) for multivariate analysis.

For repeatability assessments, the data were processed by MarkerLynx separately applying the same parameters; the features exhibiting non-zero values in more than 70% of the samples (7 samples out of 10) were considered further for statistical analysis. Ion intensities were used to assess coefficient of variation (CV%) between profiles of the same sample. CV% calculations were also done based on the ratio of the ion intensities to the aggregate intensity of the internal standard(s).

3. Results and discussion

Sample preparation is often the most time and effort consuming step of an analytical process. Furthermore it is the most error prone analytical step [19]. Errors encountered during sample preparation due to human factors or due to the varying nature of the sample (e.g. different salinity or pH values) can be monitored or sometimes corrected with the use of appropriate internal standards. In holistic analytical approaches such as metabolite profiling, this may not be feasible because even the utilisation of several internal standards may not cover the whole metabolite complement [20]. As a result, the evaluation of method recovery or suitability is not straightforward. There is always the risk that biases introduced in sample preparation are perceived as variations of biological significance. There is therefore the need for careful study of the appropriateness of the applied protocols.

HS-SPME provides the advantage of easy automation thus minimizing human intervention while increasing analytical throughput (an important parameter in omics studies). In addition, longer GC column lifetime is achieved by introducing a minimal amount of unwanted molecules into the analytical system [21]. This can improve repeatability and retention time stability which is critical in long-term metabolomics studies [22]. Volatile compounds are analysed with lower interferences or biases from long derivatisation procedures (for a comprehensive study of biases introduced by derivatisation in GC–MS metabolomics can be found in Ref. [10]). Furthermore as HS-SPME is not an exhaustive extraction method the resulting background signal is much lower especially in comparison to liquid extraction and dual derivatisation. Hence we can conclude that HS-SPME offers distinct advantages and shows potential for application in metabolomics studies of volatile molecules.

3.1. SPME optimisation

In order to reach the widest coverage of volatile compounds, a mixed fiber material encompassing three phases (DVB/CAR/PDMS) was chosen [23]. To maximise the fiber's performance the optimisation of extraction parameters was performed by a three-level factorial design [24]. A factorial experiment is an experiment whose design consists of two or more factors, each with discrete possible values or "levels", and whose experimental units take on all possible combinations of these levels across all such factors. Such an experiment allows studying the effect of each factor on the response variable, as well as the effects of interactions between factors on the response variable. In the three-level factorial design all possible combinations of the three discrete values of the parameter are used. The parameters studied were the pre-heating time (time necessary to reach the equilibrium of the partitioning of volatile compounds between the matrix and the headspace at the chosen temperature), the extraction temperature and the extraction time. The major criterion for selecting the optimum extraction parameters was the maximisation of the aggregate peak area of the 41 most intense peaks observed in the TIC chromatogram. In Fig. 1a the Pareto chart based on 25 experimental values is showing each of the estimated effects in decreasing order of magnitude. The length of each bar is proportional to the standardized effect, which is the estimated effect divided by its standard error. This is equivalent to computing a *t*-statistic for each effect. Any parameters with standardized effect extending beyond the vertical line are considered as statistically significant at the 95.0% confidence level. Fig. 1a shows that temperature (*B*) and extraction time (*C*) are significant. In Fig. 1b the estimated fiber response as aggregate peak area is given in relation to the three considered factors based on experimental data. In each panel of the plot, the factor of interest is varied from its lowest level to its highest level, while all other factors are held constant at their central values. Based on this analysis the optimum conditions estimated were: pre-heating time of 16.5 min, extraction temperature of 50 °C and extraction time of 50 min. These theoretical optimum values were adapted properly in order to comply with the need of high throughput analysis without compromising sensitivity. Thus for practical reasons since the chromatographic run lasted 46 min, in order to synchronize the autosampler and to keep the total analysis time as short as possible the pre-heating time was set to 10 min and extraction time to 30 min. The extraction temperature was kept at 40 °C to minimize possible artifacts from thermally unstable compounds in the matrix. Using these parameter values the total chromatographic area measured was reduced by less than 20% compared to the total area measured using the optimal parameters. This would represent a reduction in sensitivity but with the advantage of higher throughput.

Under these conditions the system was able to detect a number of peaks capable of producing a rich profile of apple volatiles thus indicating that SPME GC–TOF-MS offers potential for the characterisation of the different varieties. Therefore the selected conditions were applied further to investigate differentiation of volatiles compounds in different apple varieties.

3.2. Effect of freeze-drying process on metabolite profiles

Freeze-drying is often used for long-term storing of different types of samples. It is the method of choice when large number of samples are distributed among different labs, being usually a convenient choice for collaborative research projects. The elimination of water before storing is desirable for quenching the enzymatic activity especially in fruits which contain almost 80% of water. However there is a significant risk of loss of volatiles during this process, a fact that may alter the samples' GC–MS profiles [25,26]. Despite this rather obvious possibility we decided to compare GC–TOF-MS profiles of 5 samples of one variety (Golden Delicious) before and after freeze-drying to investigate the extent of this reduction. Freeze-drying procedure, as expected, altered the volatiles' profile in the apple fruit with the reduction or the elimination of compounds that could provide useful characteristic markers. Examination of the acquired profiles from the same sample freshly prepared and after freeze-drying showed that many chromatographic peaks exhibited decreased signals or were practically disappearing after freeze-drying. In Fig. 2a the overlaid chromatographic traces of fresh and freeze-dried apple are given for a certain time frame showing the reduction of the signal of certain peaks in freeze-dried samples. *t*-Test was performed in the total number of features detected by MarkerLynx in samples before and after freeze-drying. It was found that 17% of the features gave *p* values of <0.05, thus indicating significant differentiation due to freeze-drying sample processing. From the same dataset it was found that the sum of peak areas was decreased by ca. 33% compared to freshly prepared samples. The peaks found with *p*

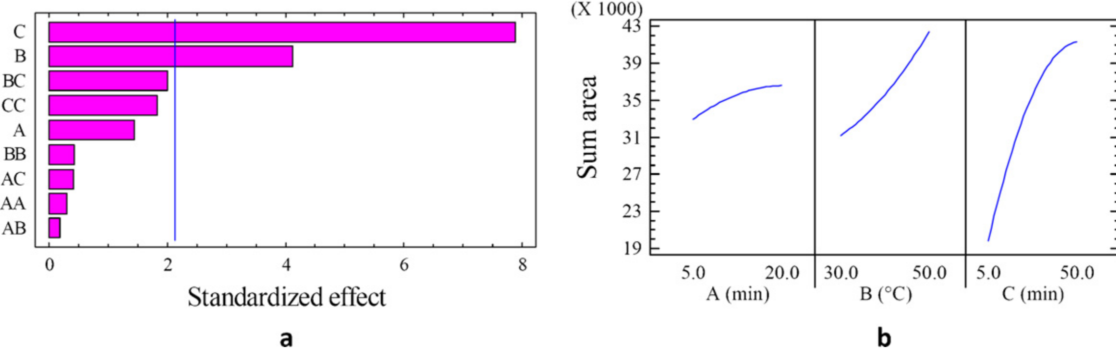

Fig. 1. (a) Standardized Pareto chart. A: pre-heating time; B: extraction temperature; C: extraction time; BC, CC, AC, AA, AB denote combinations of two of the parameters. (b) Main effect plot. Estimated total areas as functions of each experimental factor. A: pre-heating time; B: extraction temperature; C: extraction time.

values lower than 0.01 were then examined in the mass chromatographic traces. Some of these were of high significance being characteristic of the variety. For example the peak at retention time 13.87 min which was found strongly reduced in the freeze-dried extract (mean decrease of peak areas in the 5 fruits was found to be 95%) corresponds to 3-methyl-1-pentanol, a candidate marker potentially useful for Golden Delicious variety characterization (as shown in the analysis presented in the next part of the manuscript). Significant decrease of 58% and 61% was also observed for hexylacetate (RT 11.33 min) and for a sesquiterpene (29.61 min), respectively. Thus it can be concluded that useful information can be lost during freeze-drying.

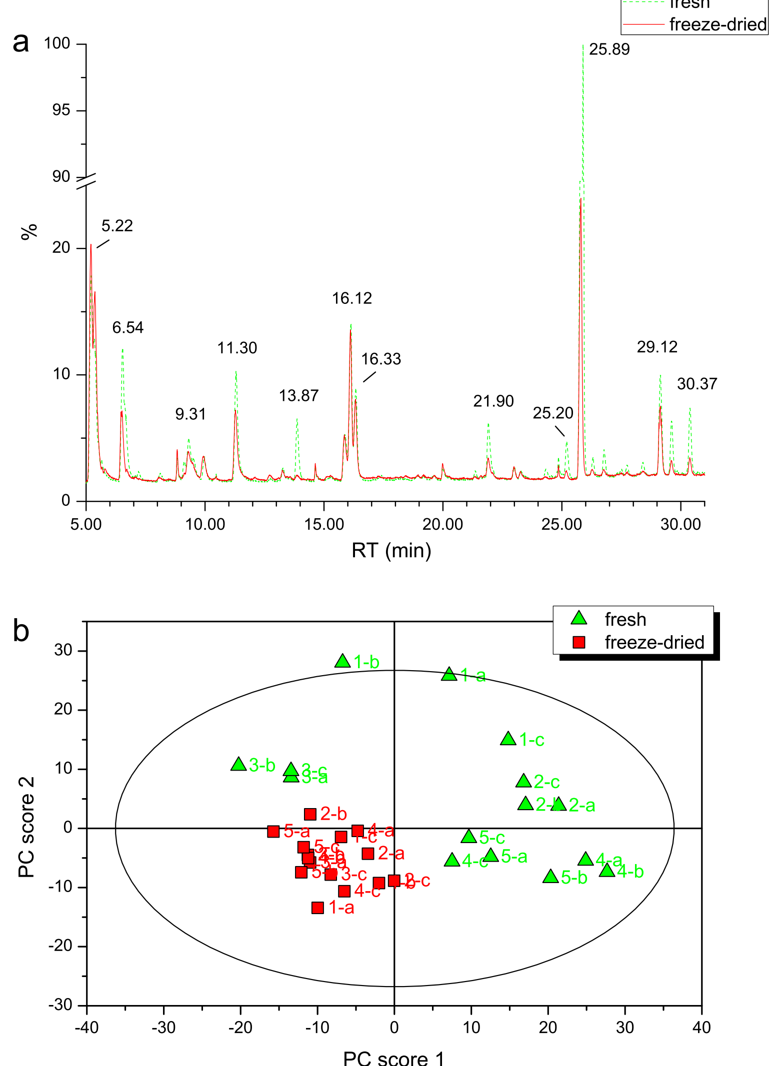

Fig. 2. (a) GC–TOF-MS profile of one of the samples analysed fresh (green dashed trace) and after freeze-drying (red continuous trace) and (b) PCA scores plot of data originating from triplicate HS-SPME-GC-TOF-MS analysis of five Golden Delicious apples extracted fresh (green triangles) and after freeze-drying (red squares). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

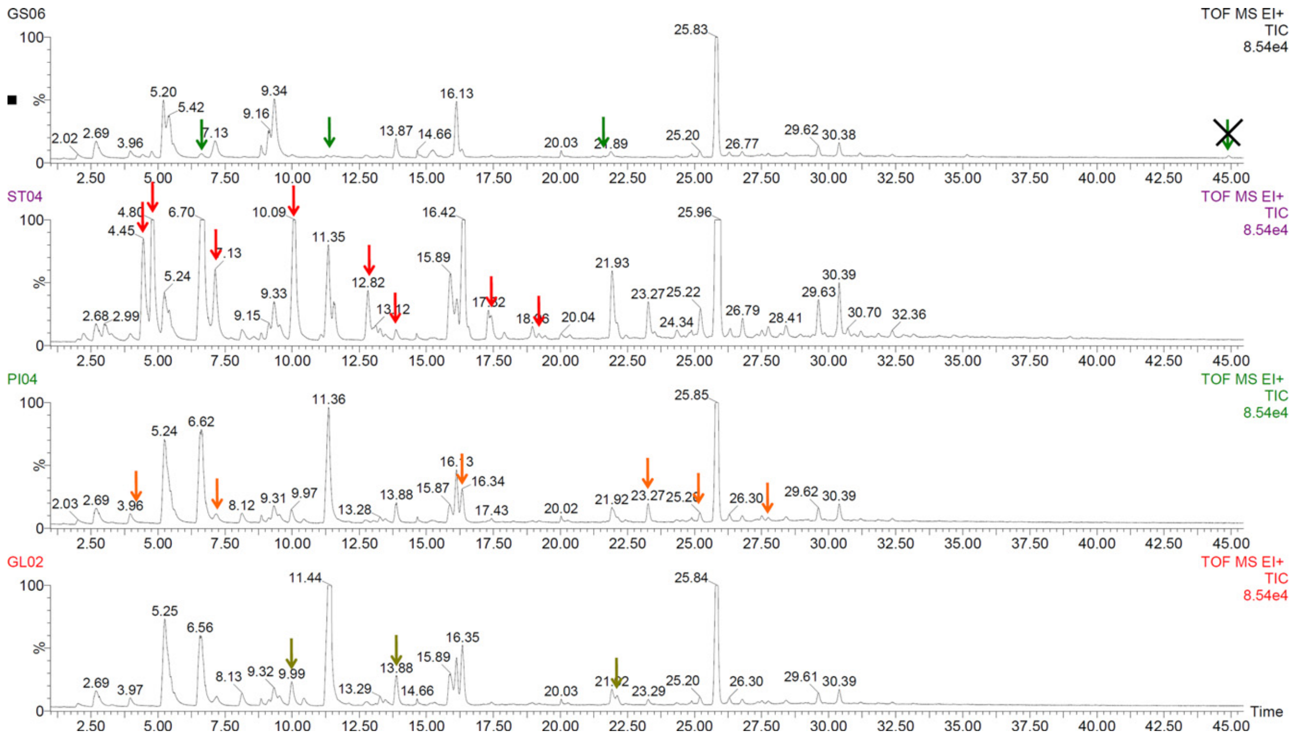

Fig. 3. GC–TOF-MS chromatograms generated from the analysis of four different apple varieties studied. The arrows are indicating characteristic peaks for each variety (for the meaning of the deleted arrow, the reader is referred to the text). GL02: Golden Delicious; GS06: Granny Smith; PI04: Pinova; ST04: Stark Delicious.

In the second step to further highlight the differentiation of freeze-dried samples, multivariate statistical analysis was applied to the GC profiles of fresh samples vs freeze-dried. Principal Component Analysis (PCA), an unbiased statistical analysis method was applied to reveal patterns and trends in the data. The PCA scores plot in Fig. 2b showes that the profiles of freeze-dried samples were differentiated from the corresponding profiles of freshly prepared extracts. In addition PCA revealed that the biological variability within group was strongly reduced after freeze-drying; in Fig. 2b the freeze-dried samples (red square symbols) are tightly clustered compared to the fresh samples (green triangle symbols). Furthermore the differentiation between individual samples is lost as the samples are shown "mingled" in the plot. This trend may result from masking of the natural variation associated with the individual samples. The latter needs to be taken under consideration when one performs variety characterisation studies with the aim to achieve robust classification models. Thus for similar studies on the volatile fraction, freeze drying cannot be recommended. All further SPME analyses in the present study were conducted using fresh samples. The issue of optimising the storing strategies remains open, as it is necessary for long term studies.

3.3. Inter- and intra-day variations

The raw data set of 10 consecutive analyses of different aliquots of the same sample (Pinova variety) was extracted with the MarkerLynx software (using the parameters given in Section 2) to provide a peak list file containing 859 variables. From these features, only those that were present in at least seven out of the ten samples were taken under consideration for further statistical analysis. Thus peak-list was reduced to 636 features, a number that corresponds to ca. 75% of the total number of features detected. Signal variability was evaluated on the basis of coefficient of variation (CV%) of the intensity values in the 10 replicate analyses and was found to be less than 20% for the 45% of the features (287 features out of the 636) and less than 30% for 63.5% of the features (404 features out of the 636). Of the remaining 232 features that were found with CV > 30%, the 213 (92%) exhibited very low intensity values (areas) in the range of 0–3; a finding that is expected since ions which are close to the intensity threshold are not picked-up by the software constantly in all the samples resulting to a number of zero values in the peak-table and consequently high CV% values.

The mean values of CV% calculated for all the detected features deriving from the two IS ranged from 13 to 15%. Integration of IS peaks at 16.2 min (2-octanol) and 5.27 min (isoamyl formate) and CV% assessment on peak areas gave similar CV% values. In general the overall repeatability coincides with previous reported findings in the analysis of the volatiles in raspberry fruit [27].

For the long-term variability, raw data deriving from the 3 aliquots analysed in 4 different days over a course of 10 days were processed together providing 881 features. From these, only the features that were present in all three replicates from the different days of analysis were examined (339 features). The intermediate precision of intensities expressed as CV% was found to be at the same levels as for the within day repeatability; thus 42% of the features were found with CV < 20% and 60% of the features were found with CV < 30%.

A general finding for both short and long term stability studies is that calculations of CV% based on correction by the IS intensities did not show to improve the overall repeatability of the data.

3.4. Variety classification

Typical SPME GC–TOF-MS chromatograms for the four different apple varieties obtained under the optimised conditions are shown in Fig. 3. From the first look, the chromatographic profiles in the form of GC–EI-MS total ion currents are showing differences in the four varieties. Certain peaks can be observed being higher in one apple variety extract compared to the others. To effectively examine trends in the data and correlations among the samples, multivariate analysis was necessary. Hence PCA was performed

first in order to reveal patterns in the data and then to looking for characteristic characteristic peaks.

Processing of the data by MarkerLynx provided 2320 features which were first normalized to unit variance and Log transformed since such a data pre-treatment before PCA gives better results when variable's values are spread over different orders of magnitude [28]. PCA revealed grouping of the apples according to variety. The samples are plotted in the first three principal component scores of PCA (describing 38% of the variation) as shown in Fig. 4. It is observed that Granny Smith (GS) and Stark Delicious (SD) groups are better separated from the other two varieties; Pinova (PI) and Golden Delicious (GD) are partially overlapping.

To obtain a higher level of group separation and enhance recognition of variables responsible for classification, PLS-DA, a supervised technique, was applied [29]. Briefly, PLS-DA is used to sharpen the separation between groups of observations, by rotating PCA components in a way that a maximum separation among classes is obtained, and to understand which variables carry the class separating information. The PLS-DA scores plot obtained is given in Fig. 5 showing clear separation of the four apple varieties. This model was subsequently validated to demonstrate its robustness and reliability in the classification of unknown samples. The stability of the model, and thus the reliability of discriminant variables, was tested by cross validation [30]. In cross-validation, the

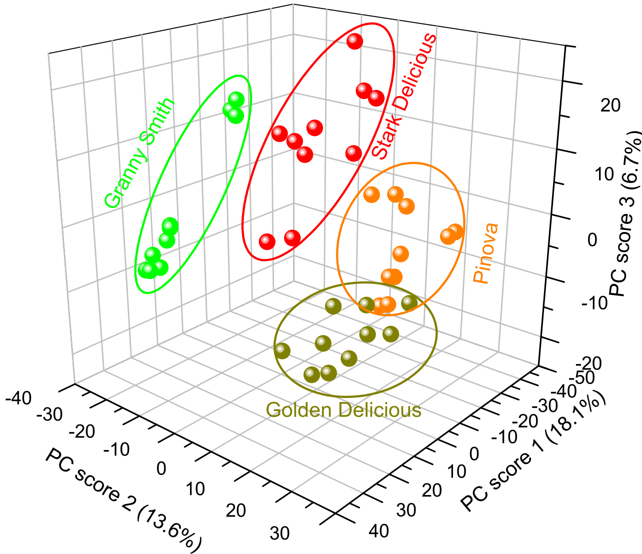

Fig. 4. PCA scores plot of the first three principal components showing grouping of the samples according to variety. The explained variance for each component is reported in parentheses.

Table 1
Differentiating metabolites found by SPME-GC–TOF-MS and identified with NIST-MS database.

RT	Assigned compound	Identity[a]	Golden Delicious	Pinova	Stark Delicious	Granny Smith	
4.43	Ethyl butanoate	F			+		
7.20	Propyl 2-methylbutanoate	T	+				
9.97	Isobutyl 2-methylbutanoate	T	+				
13.02	5-Hexenyl acetate	T			+		
13.87	3-Methyl-1-pentanol	T	+				HO
15.87	Butyl hexanoate	F			+		
16.33	2-Ethyl phenol	T		+			OH
16.41	Hexyl 2-methylbutanoate	F		+			
22.11	n-Butyl tiglate	T				–	
27.73	Sesquiterpene (m/z: 107.083; 135.116; 204.187; 93.068; 91.053; 105.069)	T				–	?

[a] F indicates fully identified compound by means matching of RT and MS profile with injected pure compound. T indicate compound tentatively identified by means NIST library and RT. The symbol "–" indicates compounds characterizing varieties for their lowest amount

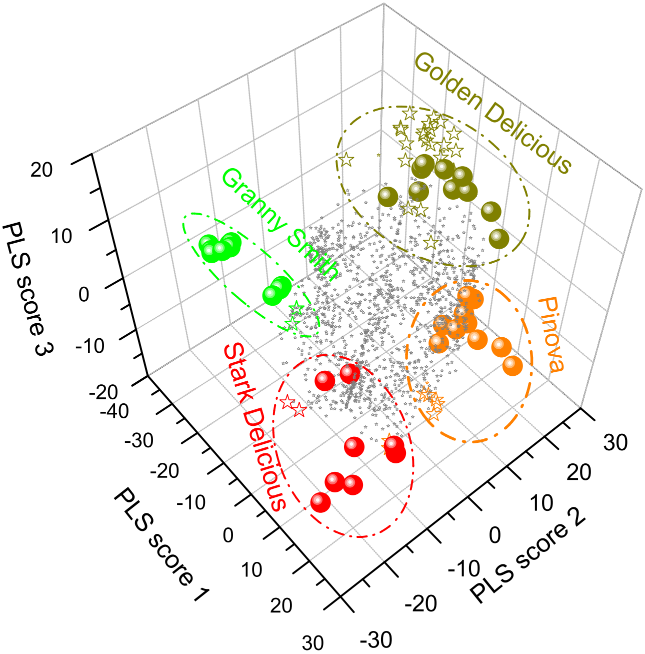

Fig. 5. PLS-DA plot showing grouping of samples according to variety. Loadings with variable importance values (VIP) > 1 are also shown (in grey). Significant characteristic loadings (VIP > 1.7) for each variety are shown as stars.

prediction ability of the model is determined by developing a model with part of the data set (training or learning set) and using another part of data (test set) for testing the model. Both, training and test sets, contain samples representative of each class. This procedure, consisting of model development and model testing, is repeated several times so that the same samples have the probability to be used as training and as test objects. In leave one out cross validation only one sample at a time is removed from the training set and considered as a test set. Two cross validation procedures were considered, one by dividing samples data set in training (70% of the samples) and test set (30% of the samples) and the second by adopting a leave one out cross validation. Both cross validation procedures gave a 100% of correct classification.

To further validate the model, a permutation test was performed [31]. The R^2 and Q^2 intercept values were 0.37 and −0.27 for GD, 0.37 and −0.31 for GS, 0.41 and −0.25 for Pi, 0.41 and −0.29 for SD after 400 permutations. The negative values of Q^2 intercept indicate the robustness of the models and thus a low risk of overfitting. Cross-validation assesses the predictive power of a model and permutation test assesses the statistical significance of the estimated predictive power, in our case both verifications proved the reliability of the adopted classification model.

Next, from the developed PLS-DA model the variable importance values (VIP) were calculated [32]. VIP values mainly reflect the correlation of the terms to all the responses and those having VIP values larger than 1 are the most relevant for explaining Y. In total 1019 variables exhibited VIP values higher than 1. To aid visualization and data exploration, loadings having a VIP higher than 1 are plotted as grey dots in Fig. 5 and those with VIP higher than 1.7 (30 variables) are indicated with stars and are colored according the closest group. Variables sharing the same retention time window were scrutinised as if originating from the same metabolite since MS data were acquired in EI mode that causes strong ion fragmentation. This could practically mean that the 30 variables indicated with stars in Fig. 5 correspond to much fewer compounds. These ions were further examined by the inspection of the GC mass chromatograms. The corresponding peaks were finally annotated by retention time indices and spectral matching using NIST library. Identification of the compounds of interest were additionally done by standards' injection in the cases that standard was available (e.g. butyl hexanoate and hexyl 2-methylbutanoate). In Table 1 the compounds that could be identified as the most characteristic markers of the different varieties are reported. It was observed that the variation of characteristic compounds that could be identified among the different apples of the same variety was relatively high e.g. 3-methylpentanol, a characteristic peak for the Golden Delicious variety, showed a value of 36% as CV of the peaks areas in the 10 apples; while the IS (2-octanol) belonging to the same class of compounds showed 11% as a CV value in the same samples. In Fig. 6 peaks areas of some volatile metabolites that were found to vary among the four varieties are given as box

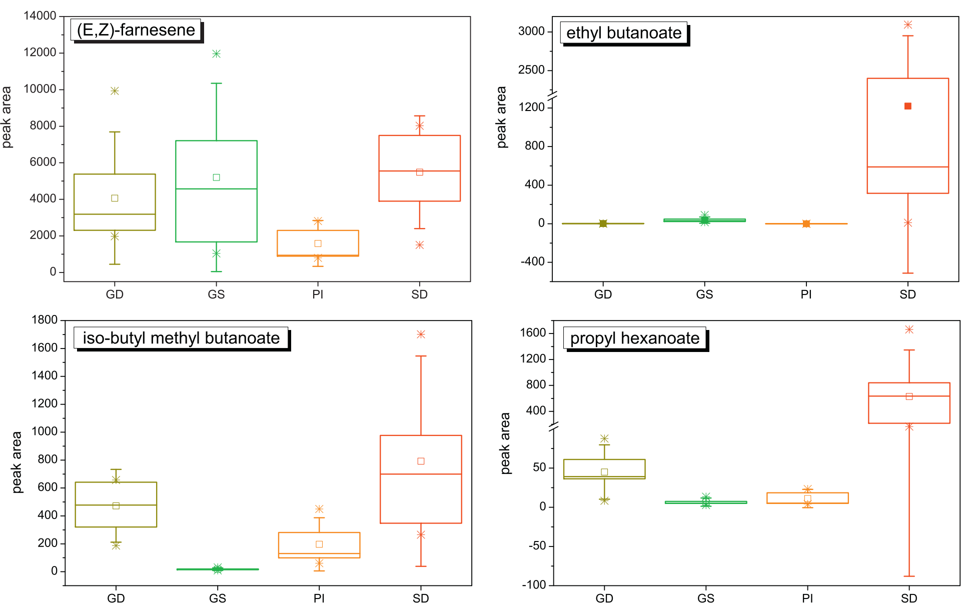

Fig. 6. Box plots of peak areas for four of the identified compounds showing variation in the four apple varieties. Boxes represent the 25–75% percentile; whiskers indicate the 1.5 × SD; asterisks the min and max values and small inner boxes the mean values. GD: Golden Delicious; GS: Granny Smith; PI: Pinova; SD: Stark Delicious.

plots (box is presenting the 25–75% percentile and whiskers the $1.5 \times$ SD values of peak areas). It can be observed that the peak areas for characteristic compounds like those given in Fig. 6 differ significantly between varieties. Thus these metabolites can be considered as potential variety classifiers. It must be considered that since the production of volatiles depends also on the storage conditions and is strictly controlled by ethylene [33], these markers should be further investigated on larger sample set of apples under strictly controlled conditions. However, the findings of this preliminary survey demonstrates the classification power of this technique applied to the product on the market.

According to data reported in Table 1, GD apples are characterised by higher amount of propyl 2-methylbutanoate, isobutyl 2-methylbutanoate and 3-methyl-1-pentanol; PI apples show higher emission of 2-ethyl phenol and hexyl 2-methylbutanoate; SD apples show higher emission of ethyl butanoate, 5-hexenyl acetate and butyl hexanoate. Finally GS apples are characterised by lowest emission for butyl tiglate and a not full identified sesquiterpene (possibly α-patchoulene). N-phenylaniline (peak at RT = 44.90 min) was identified as a possible marker for GS apples indicated by the PLS-DA model. This peak was found only in GS apples and was completely absent in the other three varieties. N-phenylaniline is used as a pre- or postharvest scald inhibitor for apples [34]. Its anti-scald activity is the result of its antioxidant properties, which protect the apple skin from the oxidation products of alpha-farnesene during storage. Granny Smith apples are particularly prone to scald [35] and thus the presence of this peak can be due to the residuals of N-phenylaniline after storage. This indicates that data analysis gives discriminating features which need to be carefully investigated for their biological significance to avoid misinterpretation.

It was also observed that one of the main differences between GD and SD varieties are related to the kind of esters, branched in the case of GD and linear in the case of SD (Table 1). This may suggest a difference in the enzymatic activities and/or a different amino acid metabolism of the different apple varieties [36]. GS apples showed lower overall levels of volatile production in comparison to the other apple varieties in agreement with literature data [37,38] and, in fact, the two markers found are characterised to be the lowest among the four varieties.

4. Conclusions

In the present communication further evidence is provided that headspace SPME without the application of derivatisation can provide a useful tool for the extraction of volatiles from real samples, in this case homogenised apple flesh. Following a three parameter experimental design, SPME conditions were selected and were successfully applied in sampling volatiles for the characterisation and safe classification of four different apple varieties by GC–TOF-MS. Untargeted TOF-MS analysis revealed markers specific for each apple variety and was proven a useful tool for further studies on the apple metabolome. It was seen that volatile content of the fruit may provide useful insight to characteristics of quality. It was also concluded that despite its advantages for long term sample storage freeze-drying is not a method of choice for sample preparation prior to GC–MS profiling of volatile molecules.

Acknowledgements

This research was supported by the Autonomous Province of Trento, Italy, “ADP2010” Project. Urska Vrhovsek is supported by the MetaQuality project, funded by the autonomous Province of Trento (Italy).

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