Fast headspace-enantioselective GC-mass spectrometric-multivariate statistical method for routine authentication of flavoured fruit foods

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Astract: This study describes a rapid total analysis system (TAS) to detect the authenticity of fruit-flavoured foods and beverages by on–line combining headspace solid phase microextraction (HS-SPME) with enantioselective GC-MS (Es-GC-MS) and statistical multivariate methods (PCA, HCA). Peach, coconut, apricot, raspberry, as fruits mainly characterized by γ- and δ-lactones as chiral markers, strawberry (α-ionone, linalool, nerolidol, ethyl 2-methylbutyrate, 2-methylbutyric acid and γ-lactones) and melon (ethyl 2-methylbutyrate and 2-methylbutanol) were investigated. The system was developed by a) optimizing non-equilibrium HS-SPME sample preparation, b) speeding-up ES-GC using cyclodextrin derivatives as chiral selectors with conventional and narrow-bore columns and c) elaborating data by multivariate methods. The resulting TAS affords a reduction of the time needed for the whole analytical process from about 150 min to 20-50 minutes (67%-87% of the current routine method) depending on matrix, sampling and analysis conditions and Es-GC columns.

Keywords: flavoured fruit products, chiral marker, non-equilibrium headspace-SPME, enantioselective GC-MS, multivariate statistical methods.

Running title: HS-SPME-Es-GC-MS-PCA/HCA TAS to authentify flavoured fruit products.

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1. Introduction

One of the most important tasks in the food field is how to meet the ever-increasing demand for control analyses to assure product authenticity and safety as well as to detect possible frauds or adulterations. One possible strategy is to develop fast and fully automatic analytical processes in which sample preparation, analysis and data elaboration are on-line integrated into a single step, resulting in the well-known “Total Analysis Systems” (TAS) (Manz, Graber & Widmer, 1990; Dittrich, Tachikawa & Manz, 2006). Their adoption has also contributed to the strong return to interest in headspace techniques for sample preparation and to the continual development of fast separation techniques.

Enantiomer recognition in flavours and fragrances, and more in general in the food field, is very important, mainly because biological interactions and biosynthetic processes are mostly stereospecific, meaning that chiral components in natural products are often characterized by specific enantiomeric compositions (Bicchi, D'Amato & Rubiolo, 1999; Bicchi, Manzin, D'Amato & Rubiolo, 1995; Konig & Hochmuth, 2004; Mosandl, 1995). On the other hand, essential oils and fruit flavours have great commercial relevance for food industry, and are quite often replaced by cheaper synthetic racemic compounds, products from other cheaper natural sources, or of different origins; these sometimes contain chiral components with different enantiomer compositions (Konig et al., 2004; Mosandl, 1995).

The discrimination between “natural” and “synthetic” flavoured food is also of great importance in view of the current European legislation (Reg. CE 1334/2008) that limits the use of the term “natural” on labelling only to flavoured preparation that contains exclusively “natural flavouring substances”, that are those obtainable enzymatically, microbiologically and by an appropriate physical process, the latter being: ”…a process which does not intentionally modify the chemical nature of the components of the flavouring….. “ (Article 3). In consequence, enantioselective analysis becomes a decisive tool with which a quality control laboratory can monitor conformity to both legislation and labelling regulations, and can check the authenticity and reveal any adulteration or fraud.

Enantioselective (Es)-GC-MS with cyclodextrin (CD) as chiral selectors, combined with automated headspace solid phase microextraction (HS-SPME) to differentiate natural flavour compounds from synthetic ingredients have recently successfully been applied to quality control of several fruit foods and beverages (Ebeler, Sun, Datta, Stremple & Vickers, 2001; Ravid, Elkabetz, Zamir, Cohen, Larkov & Aly, 2010). HS-SPME was here adopted because of its ability to reliably recover volatile analytes even under non-equilibrium conditions, and since it can operate automatically and may be on-line combined with GC-MS. Chiral recognition methods present some well-known limits including possible partial racemization during processing, and impossibility to detect an addition of enantiomerically pure but synthesized substances without an isotope ratio analysis. Moreover, these methods are in general time consuming, usually taking not less than 90 minutes because Es-GC separation is a bottle-neck due to the nature of the host-guest interaction mechanisms between each enantiomer and the CD chiral selector that lead the separation (Levkin & Schurig, 2008; Schurig, 2001). This limit is not in-line with the present trend in quality control that must be as fast as possible to satisfy the ever increasing demand for analyses.

Recently, Es-GC analysis has been speeded up (Bicchi, Blumberg, Cagliero, Cordero, Rubiolo & Liberto, 2010a; Bicchi, Liberto, Cagliero, Cordero, Sgorbini & Rubiolo, 2008; Liberto et al., 2008) by a suitable combination of short conventional or narrow bore columns and mass spectrometry as a second dimension in detection and used to locate the enantiomers in the chromatograms; they are afterward identified through their linear GC retention indices (I^Ts) (Liberto et al., 2008). Two distinct but highly complementary approaches have been used: the first suitably combines narrow bore columns even shorter than the conventional 10 m length (e.g. 5 or 2 m) (Bicchi et al., 2008), with mass spectrometry in extract ion mode, while the second consists of finding the best speed/separation trade-off with a conventional column and translating the resulting analysis conditions to narrow bore columns by the method translation approach (Bicchi et al., 2010a).

Last but not least, routine quality control usually involves the analyses of a large number of samples, and data processing must therefore also be considered in the total analysis time, and optimized if necessary. Chemometrics, through multivariate methods, can be a helpful and easy tool to separate useful from useless information (Beebe, 1998). Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA)

provide quick and automatic qualitative sample differentiation, in particular when quantitation or characterization of specific components of the matrix are not necessary.

This study describes a reliable total analysis system to determine the authenticity of commercial fruit-flavoured foods and beverages. Speeding-up of HS-SPME sampling and Es-GC analyses of different fruit matrices (peach, coconut, apricot, raspberry, strawberry and melon) were first investigated; the possibility to combine this technique on-line to multivariate methods to discriminate different commercial samples was then explored. The reliability of the method was finally verified by checking the stability of the enantiomeric composition of the analyte investigated under different conditions.

2. Experimental

2.1 Samples

Pure standard mixtures of racemic γ- and δ-lactones (both from C6 to C12), α-ionone, linalool, nerolidol, ethyl 2-methylbutyrate, 2-methylbutyric acid and 2-methylbutanol were from the collection of standards in the authors' laboratory. They were solubilised in cyclohexane at a concentration of 100 ppm each. Solvent was HPLC grade from Riedel-de Haen (Seelze, Germany).

Six fruits were considered: peach (*Prunus persica* L.), coconut (*Cocos nucifera* L.), apricot (*Prunus armeniaca* L.), raspberry (*Rubus idaeus* L.), strawberry (*Fragraria x ananassa* Duch. Ex Rozier) and melon (*Cucumis melo* L.). Fruits and chiral markers investigated are reported in table 1. Fifty-eight commercial food and beverage products (juices, teas, yogurt, jam, desserts, milk, ice-cream and fruits) based on peach, coconut and strawberry, purchased at local supermarkets, and a synthetic peach aroma, were also analysed.

2.2 *Sample preparation and SPME extraction*

Whole fresh fruits were ground to produce a homogeneous pulp; foods and beverages were sampled as such. Six grams of each sample were placed in a 20 mL headspace vial together with a quantity of NaCl sufficient to oversaturate (2.2 g) liquid samples. Volatiles were sampled by automated headspace solid phase microextraction (auto-HS-SPME) using a SHIMADZU AOC 5000 autosampler on-line integrated with the GC-

MS system. A 2 cm Stableflex 50/30 µm DVB-Carboxen-PDMS fiber (Supelco, Bellefonte, USA) was used. After 5 min pre-equilibration of each sample at the sampling temperature, the SPME fibre was exposed to the headspace for the times and at the temperatures reported in table 2, conditions depending on the matrix, and under stirring at 250 rpm. Consistency of fibre performance was checked through in-fibre external standardization by analysing an undecane solution daily (5 µL of a 2 mg/mL solution).

2.3 *GC-MS analysis*

The analyses were carried out on a Shimadzu QP2010 GC-MS system provided with Shimadzu GC-MS Solution 2.51 software (Shimadzu, Milan, Italy).

Analyses were carried out on columns coated with $6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-$3^{I\text{-}VII}$-*O*-acetyl-β-CD (AcAc-CD) (Maas, Dietrich, Bartschat & Mosandl, 1995) and $6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-ethyl-$3^{I\text{-}VII}$-*O*-methyl-β-CD (EtMe-CD) (Bicchi et al., 2010b) as chiral stationary phases (CSP), both diluted at 30 % in PS086. For each chiral selector three different column dimensions were used: a conventional d_c column (25 m × 0.25 mm d_c × 0.25 µm d_f, for AcAc-CD and 25 m × 0.25 mm d_c × 0.15 µm d_f, for EtMe-CD) and two 0.10 mm d_c × 0.10 µm d_f narrow bore (NB) columns approximately 11 m (11.7 m for AcAc-CD and 11.3 m for EtMe-CD) and 5 m long, respectively. The exact length of the columns was determined by measuring the void time. All columns were from MEGA (Legnano, Italy).

GC-MS conditions: temperatures: injector: 220°C, transfer line: 230°C; ion source: 200°C; carrier gas: He, flow control mode: constant linear velocity, initial flow rates and temperature programs are reported in the text. Injection: injection mode: split; split ratio 1:20 for the conventional columns, 1:300 for the 11m NB columns and 1:400 for the 5m NB columns.

The MS operated in electron impact ionization mode (EI) at 70 eV, scan rate: 666 u/sec with conventional columns, 1666 u/sec for 11m NB columns and 2500 u/sec for 5m NB columns, mass range: 35–350 m/z (appropriate to cover the total fragmentation pattern of most fruit volatile components).

The chiral components were identified and their elution order assigned using a dedicated chiral library that interactively combines linear retention indices and mass spectra, developed in the authors' laboratory (Liberto et al., 2008).

3.4 *Statistical analysis*

The results obtained from the analyses of different peach and coconut flavoured foods and beverages were submitted to statistical elaboration using the Statistica 6.0 (StatSoft, Inc. 2001, Tulsa OK, USA) program. The percent areas of each enantiomer of the marker lactones (variables) calculated on the total area of each pair of enantiomers, determined by extracting the ions characterizing the two classes of compounds (i.e. 99 m/z for δ-lactones and 85 m/z for γ-lactones), were standardised and then submitted to statistical elaboration. Table 1 reports the marker lactones for peach and coconut flavoured foods.

Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were used as unsupervised pattern recognition techniques. For HCA, the single-link approach and the Euclidean distance were used to evaluate the analysis results of both peach and coconut flavoured foods.

3. Results and discussion

The main aim of this research was to prove the reliability of fast headspace sampling and enantioselective analysis of chiral markers in a set of fruits, for routine discrimination between natural and synthetic flavours added to fruit foods and beverages. Table 1 reports chiral components characterizing each fruit investigated, and their natural enantiomeric ratios (e.r.) reported in the available literature in the field.

This study involved: a) optimizing HS-SPME sample preparation, b) speeding-up chiral analyses with the aim of investigating a wide range of both vegetable matrices and target compounds, c) evaluating the stability of the enantiomeric composition under different conditions, and d) applying the methods to commercial fruit foods and beverages, and discriminating between them, using multivariate methods for data elaboration, to show the applicability of the method to routine quality control. In particular, step b) is here discussed in greater detail

because routine Es-GC is the most time consuming analysis step, and because the approaches adopted have only recently been introduced (Bicchi et al., 2010a; Bicchi et al., 2008).

3.1 Sample preparation

Headspace sampling by SPME has been chosen because of its ability to recover volatile analytes and its reliability, even in non-equilibrium HS conditions, and to eliminate any sample pre-treatments independently on the matrix investigated. The conditions to achieve a suitable trade-off between chiral marker representative recovery and sampling time were investigated for all fruits under study (peach, coconut, apricot, raspberry, strawberry and melon) and related matrices. The sampling conditions were optimized by applying an experimental Dohelert design to temperature and time (data reported in the supplement); these two variables were chosen after applying a fractional factorial design in which the significance of sample amount, salting out, temperature and time was investigated. The optimal temperature and time resulting from these experiments were 85°C and 60 minutes respectively for all matrices investigated. However, in the final method, both values were lowered because 1) the aim of this study is to develop a fast method therefore sampling time had to be logically compatible with analysis time, 2) the optimal conditions resulted in peak overloading (in particular with matrices containing artificial aromas) that affects e.r. calculation, and 3) the control of authenticity is based on the enantiomeric ratios of chiral markers so that recovery has not to be maximized. Table 2 reports sampling conditions selected for each fruit, food or beverage. Sampling temperatures ranged from 40°C to 60°C and sampling time from 10 min for strawberry and melon to 20 minutes for the other fruits investigated. Moreover, table 2 shows that, also under non-equilibrium but rigorously standardized conditions, marker recoveries are highly repeatable since RSD% calculated on the total area of the chiral markers of each fruit never exceeds 6%.

3.2 Speeding-up of enantioselective analyses of different fruit matrices

Since a CD derivative with universal enantioselectivity is not available, a column coated with a CD selector able to simultaneously separate the chiral markers chosen for each fruit, in agreement with the “one chiral

selector for one problem" approach (Bicchi, D'Amato & Manzin, 1997; Bicchi et al., 1995) had first to be found. $6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-$3^{I\text{-}VII}$-*O*-acetyl-β-CD (AcAc-CD) (Maas et al., 1995) was therefore used as chiral selector for fruits mainly containing γ- and δ-lactones (peach, apricot, raspberry and coconut) and $6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-ethyl-$3^{I\text{-}VII}$-*O*-methyl-β-CD (EtMe-CD) (Bicchi et al., 2010b) for fruits characterized by esters and alcohols other than γ-lactones (strawberry and melon).

The speeding up of the enantioselective GC separation was achieved through the strategy reported in a previous article (Bicchi et al., 2010a) consisting of a) optimizing the chromatographic conditions affording the best speed/separation trade-off with a conventional d_c column, and b) translating the method to narrow-bore columns.

3.2.1) *Optimization of Es-GC conditions of a peach sample with a conventional column*

This part involved three main steps i) choice of initial conditions to optimize the process, ii) determination of optimal multi-rate temperature program for a predetermined fixed column flow rate, and iii) determination of optimal flow for the normalized optimal multi-rate temperature program. The samples resulting from the headspace sampling by HS-SPME of a peach fruit (for short peach HS), and γ-C6-C12 and δ-C6-C12 lactones standard solutions (γ-C6-C12-SS and δ-C6-C12-SS respectively) were used to optimize the ES-GC analysis.

i) *choice of initial conditions* - In agreement with Blumberg and Klee's strategy (Blumberg & Klee, 2000; Klee & Blumberg, 2002), the void time (t_M) of the AcAc-CD conventional column (25 m × 0.25 mm) was first measured, not only to confirm the exact length of the column (by comparing measured and predicted values), but also and mainly to determine the "normalized heating rate" r (defined as t_M x R_T, where R_T is the temperature rate), a parameter that must remain unvaried whatever translation the method is submitted to. The void time for this column was 1.052 min confirming a column length of 25.0 m. Peach HS and γ-C6-C12-SS and δ-C6-C12-SS were then analysed under routine conditions, i.e. helium flow rate 1 mL/min, heating rate from 50°C to 220°C at 2°C/min (figure 1a). Under these conditions the enantiomers of both γ- and δ-lactones were base-line separated but (S)-γ-C6/(S)-γ-C7 and (S)-γ-C7/(S)-δ-C8 pairs partially co-eluted; the resulting analysis time was 71 minutes.

ii) *determination of optimal multi-rate temperature program* – The above samples were then analysed by applying a set of different single-ramp heating rates, namely 2°C/t_M, 3°C/t_M, 5°C/t_M, 7.5°C/t_M, 10°C/t_M, 15°C/t_M, using a flow rate of 1 mL/min and 50°C and 220°C as initial and final temperatures. The corresponding normalized heating rates were 1.9, 2.8, 4.7, 7.1, 9.5 and 14.3 °C/min, respectively. The enantiomers of both series of lactones are base-line separated until rate 7.5°C/t_M, while γ-and δ-C12 lactones partially co-eluted from 10°C/t_M. On the other hand, the (*S*)-γ-C6/(*R*)-γ-C7 and (*R*)- δ-C6/(*S*)- δ-C8 enantiomer pairs overlapped at 5°C/t_M and 7.5°C/t_M heating-rates.

The analysis conditions were therefore modified, applying 90°C as initial temperature and 2°C/t_M and 3°C/t_M as heating rates. The analysis at 2°C/t_M still showed a partial co-elution of *(S)*-γ-C6/*(R)*-γ-C7 and *(S)*-γ-C7/*(S)*-δ-C6 pairs while that at 3°C/t_M gave a base-line separation of all chiral compounds. The best trade-off between separation and analysis time was therefore achieved with a multi-rate temperature program from 90°C to 140°C at 3°C/t_M (i.e. after elution of δ-C6), then to 220°C at 7.5°C/t_M and an Es-GC analysis time of 29 min instead of 85 min with the original method.

iii) *determination of optimal flow rate for the normalized optimal multi-rate temperature program* - The next step was flow rate optimization by determining the initial efficiency-optimized flow (EOF, i.e. initial flow that maximizes column efficiency and peak resolution) and, from it, by calculating the initial speed-optimized flow (SOF i.e. initial flow which minimizes analysis time at fixed efficiency, which is SOF = √2 EOF) (Blumberg, 1997). Seven different flow rates (i.e. 0.6, 0.7, 1.0, 1.4, 1.7, 2.0, 2.5 mL/min) were applied to the column using the GC method-translator to translate the temperature program for each value, so that the normalized temperature program was always the same. The initial EOF for the most critical pair *(S)*-γ-C6/*(S)*-γ-C7 was 1 mL/min; as a consequence the calculated SOF was 1.4 mL/min (Blumberg, 1999) (figure 1b). The analysis time under SOF conditions was further reduced to 24 min.

3.2.2 Translation of the methods to narrow bore columns and analysis of different fruit matrices

The optimised EOF and SOF methods with conventional column were then translated to two 11 and 5 m long NB (0.1 mm × 0.1 μm) columns coated with the same stationary phase. The void time of the narrow bore columns was first measured to determine their exact length, before translating the methods; the calculated

lengths were 11.7 m and 5 m, respectively. The parameters of the translated SOF methods are reported in table 2 while figure 2 (a and b) reports the Es-GC patterns of γ-C6-C12-SS and δ-C6-C12-SS analysed on the 11 and 5 m NB columns, respectively. The analysis time with NB columns with the translated SOF methods is thus shortened to 12.1 minutes with the 11.7 m column, and to 3.4 minutes with the 5 m column, separation remaining exactly the same as for the conventional column. Table 2 also reports analysis time and % reduction, obtained in SOF mode, with the optimized methods applied to the columns investigated, compared to routine analysis: the analysis time reduction under optimal conditions with conventional columns was 72%, while with 11 m and 5 m narrow bore columns it was 86% and 96%, respectively.

The translated methods were then applied, besides peach, to the analysis of apricot, coconut and raspberry, whose aromas are also mainly characterized by lactones. The SOF analysis of all fruits with 11m and 5m NB columns demonstrated that it was possible to determine reliably the enantiomeric composition, and hence the authenticity of food based on "lactone-characterizable" fruit, in less than 4 minutes. The full method was also applied to a synthetic peach aroma (1μL of aroma in 20 mL vial sampled for 2 min at room temperature without pre-equilibrium); its HS-SPME-Es-GC profile with the conventional column is reported in figure 1b. The comparison between the profiles of the fruit and the synthetic aroma shows that they can easily be discriminated, the synthetic flavour being characterized by racemic lactones including γ-undecalactone (i.e. a non-natural lactone), and the natural fruit by a large excess of the (R)-enantiomer (in agreement with findings of other studies, see table 1).

3.3 Optimization of Es-GC conditions with EtMe-CD column for the headspace analysis of strawberry and melon

Other fruit matrices were then considered, to extend the range of chiral markers that characterize fruit flavours, and as a consequence the possibility of determining the authenticity of other fruit-based products.

Strawberry is characterized by α-ionone, linalool, nerolidol, ethyl 2-methylbutyrate, 2-methylbutyric acid, in addition to γ-lactones, and requires EtMe-CD as chiral selector to separate all chiral markers in a single run.

The same approach used for peach optimization was applied, first determining the void time (1.092 min) and the corresponding column length (25.6 m). After analysis under routine conditions, the same nominal temperature rates used for peach were applied to strawberry HS, with a flow rate of 1 mL/min, and 50°C and 220°C as initial and final temperatures. The multi-rate temperature program was conditioned by γ-C12 lactone and by the two pairs of enantiomers of nerolidol diastereoisomers, that are only base-line separated until 7.5°C/t_M. The following multi-rate temperature program was therefore chosen: from 50 to 185°C (after γ-C12 elution) at 7.5°C/t_M, then to 220°C at 15°C/t_M. Under these conditions and with a SOF of 1.4 mL/min, all marker compounds were separated in an analysis time of about 19 min, instead of the 85 min of the routine method (table 2).

The method was then translated to two 11.3 m and 5 m, 0.1 mm × 0.1 μm NB columns. The result of the SOF translated methods was an analysis time of 14.4 min and 4.4 min, for the 11.3 and 5 m NB columns, respectively, with a reduction compared to routine conditions of a factor 6 and 19.

The methods were also applied to two strawberry yogurts, one of them flavoured with a synthetic flavour, to evaluate the ability of this method to discriminate between them: the results showed that the enantiomeric composition of unflavoured yogurt complies with that of the fruit, while the flavoured yogurt contains racemic linalool, ethyl 2-methylbutyrate, γ-C6 and γ-C10.

A procedure similar to that for strawberry was applied to melon, whose headspace is characterized by high volatility chiral compounds, in particular ethyl 2-methylbutyrate and 2-methylbutanol. 2-Methylbutanol enantiomers are base-line separated only at 3°C/t_M. The following multi-rate temperature program was therefore applied: from 50°C to 70°C at 3°C/t_M (after elution of 2-methylbutanol) then to 150°C at 15°C/t_M. In this case, too, the method was translated to a SOF of 1.4 mL/min and then to the 11.3 and the 5 m NB columns (table 2). The analysis time ranged from 50 min in routine conditions to 3.9 min of the SOF analysis with the 5 m NB column.

3.4 Stability of enantiomeric composition in different conditions

One of the main limitations on the use of enantiomeric recognition to determine the authenticity of a sample is that the enantiomeric ratio may vary under different conditions or during processing. The effect of sampling, storage and pH on the enantiomeric composition was therefore tested, by determining the consistency of the % area of the enantiomers of some target lactones in an unflavoured peach juice (taken as reference) and a flavoured tea. The results of these sets of experiments on the effect of sampling conditions on enantiomeric ratios of C8, C10, C11, C12 γ-lactones in a peach-flavoured tea show that the values of % areas of the two enantiomers did not change when ionic strength, temperature or sampling time were varied. The effect of storage was tested by analysing the above samples stored in daylight at ambient temperature over a period of three months, without any variations in the lactone marker enantiomeric ratio being observed (data not reported).

The effects of acidification and basification on the enantiomeric composition of γ-C10 were then evaluated on both the above samples. A 1ppm solution of (R)-γ-C10 was added to both samples (juice: pH 3.4, tea: pH 4.2) acidified with H_3PO_4 (up to pH 1.5 for juice and pH 3.0 for tea) and HCl (up to pH <0.5 for both samples), or basified with NaOH (up to pH 11 for both samples), and then analysed over a period of three weeks (table 3). The results of acidification compared to those of the original samples show that: i) γ-C10 in the flavoured tea is racemic at both native and acid pH, ii) the enantiomeric ratio due to added (*R*)-γ-C10 to the flavoured tea does not change after acidification and storage, iii) the natural (R)-γ-C10 enantiomeric ratio of the juice does not decrease after acidification. The results after basic treatment indicate a strong decrease of absolute area of lactones, probably due to hydrolysis of the lactone group, but the enantiomeric ratio is kept probably because the reaction is not stereospecific

Analysis of commercially available samples and statistical discrimination

Table 4 reports ranges of concentration and enantiomeric ratios of γ-decalactone and δ-decalactone taken as representative of all markers together with the number of analyzed samples and LOD in the different peach and coconut based food matrices investigated. The average LODs for each matrix investigated are much lower of both the range of concentrations and odour thresholds of the two markers considered in table 4,

showing the reliability of the proposed method. The reduction of analysis times for all matrices has been the same as that reported in table 2 for the fruits thanks to the choice of HS-SPME as sampling technique that affords a selective recovery of the volatile fraction, thus excluding lower volatility matrix components in general co-extracted with conventional methods that can affect the time required for GC-MS.

The results reported in the previous paragraphs confirmed that analysis of the enantiomeric composition of the volatile fraction of a food is an effective tool to discriminate between natural and synthetic flavoured products. However, routine quality control analyses often entail analysing large numbers of samples, and data processing must therefore also be included in the total analysis time. Chemometrics, and in particular Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), can be effective tools for the quick discrimination between different samples. The 38 variously-labelled commercially-available peach-flavoured samples and the 18 coconut-flavoured foods were analysed twice, and the average percent areas of the enantiomers, calculated over each pair *per* marker compound (table 1), were submitted to PCA. The authenticity of the sample can quickly be determined by extracting the percentage of each enantiomer within the pair from the analysis report file, and automatically transferring them to the PCA matrix. The PCA score plot of the peach-flavoured foods (figure 3a) showed a very good separation between the natural and synthetic flavoured samples. The “natural” samples were more homogeneous than the synthetic samples, probably because of different origins of the flavours and different sample formulations. Similar results were obtained from the cluster analysis. Figure 3b reports the plot of the loadings: the natural markers, i.e. the *(R)*-enantiomers of even lactones, are mutually correlated and are all located to the right of the plot, while the synthetic compounds are on the left and include *(S)*-enantiomers of all lactones and both enantiomers of γ-undecalactone. Similar results were obtained for the coconut flavoured products: both PCA and HCA elaborations clearly discriminated between natural and synthetic flavoured samples, as shown in the HCA dendrogram in figure 3c. With both sets of flavoured foods and beverages, experimental results in all cases agreed with the commercial information reported on the labels.

4. Conclusions

The above results show that a fully automatic total analysis system can be developed to check the authenticity of fruit-based foods and beverages, thanks to the full compatibility between HS-SPME, Es-GC-MS chiral recognition and statistical elaboration. The system reduced the time needed for the entire analytical procedure, from about 150 min to 20 or 50 minutes (i.e. with a time reduction ranging from about 67% to 87%) depending on the investigated matrix, sampling and analysis conditions and Es-GC column dimensions. These reductions in total analysis time were made possible by a non-equilibrium but highly repeatable HS-SPME sampling procedure, fast Es-GC enantiomer separation using suitable CD chiral selectors, and chromatographic conditions optimized in agreement with the method translation approach, and on-line statistical elaboration.

The main limit of this approach is that while the conventional routine method is generally applied as such to any matrix, only selecting the most effective chiral column, the proposed approach requires the development of dedicated analysis conditions for each group of chiral markers characterizing a given fruits matrix. In any case, the time for method development is fully compensated for by the time saved in routine analysis.

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Captions to figures

Figure 1. Es-GC-MS profiles of peach HS fruit (—), synthetic aroma (- - -) and γ-C6-C12 and δ-C6-C12 (—) standard solutions on a 30% AcAc-CD/PS086 conventional inner diameter column a) routine conditions, b) optimized temperature and flow rate conditions. For analysis conditions see text and table 2

Peak identification: 1: γ-hexalactone, 2: γ-heptalactone, 3: γ-octalactone, 4: γ-nonalactone, 5: γ-decalactone, 6: γ-undecalactone, 7: γ-dodecalactone, 8: δ-hexalactone; 9: δ-octalactone, 10: δ-nonalactone, 11: δ-decalactone, 12: δ-undecalactone, 13: δ-dodecalactone; a: *(R)*-enantiomer, b: *(S)*-enantiomer.

Figure 2. Es-GC-MS profiles γ-C6-C12 and δ-C6-C12 standard solutions on two 30% AcAc-CD/PS086 narrow bore columns a) length: 11.3 m , b) length: 5 m. For analysis conditions see text and table 2. For peak identification see caption to figure 1

Figure 3. PCA score (a) and loading plots (b) of the peach flavoured foods and beverages investigated. HCA dendrogram (c) of the coconut flavoured foods and beverages investigated. Fr: fruits, Ar: synthetic aroma, j: juice, t: tea, y: yogurt, m: jam, d: dessert, i: ice cream, k: milk.

Table 1: Chiral markers and related enantiomer natural abundances in the investigated fruit matrices reported in the literature. Absent: not naturally occurring in the matrix according to the literature, not reported: naturally occurring in the fruit, but enantiomeric composition not reported in literature

Fruit	*Chiral marker*	*Natural abudance*	*Ref.*	*Fruit*	*Chiral marker*	*Natural abudance*	*Ref.*
Peach	γ-C6	R>>S 90/10	Bernreuther, et al., 1989	Coconut	δ-C6	R>>S 85/15-87/13	Lehemann, et al., 1995
	γ-C8	R>>S 87/13	Bernreuther, et al., 1989		δ-C8	R>>S 90/10-96/4	Lehemann et al., 1995; Nago et al., 1993
	γ-C10	R>>S 86/14-89/6	Bernreuther et al., 1989; Bernreuther et al., 1990; Ebeler et al., 2001; Lehemann, et al., 1993		δ-C10	R>>S 75/25-86/14	Lehemann et al., 1995; Nago et al., 1993
	γ-C11	absent	Ebeler et al., 2001		δ-C12	R>>S 60/40-81/19	Lehemann et al., 1995; Nago et al., 1993
	γ-C12	R>>S 96/4	Bernreuther et al., 1989	Strawberry	Linalool	S>>R 97/3-99/1	Bernreuther et al., 1991b
	δ-C10	R>>S			Ethyl 2-methylbutanoate	S>>R 98/2-99/1	Kreck, et al., 2001
Apricot	γ-C6	R>>S 90/10	Mosandl et al., 1992; Bernreuther et al., 1989		2-Methylbutanoic acid	S>>R 99/1	Kreck, et al., 2001
	γ-C7	R>>S 82/18	Bernreuther et al., 1989		α-ionone	R>>S 99/1	Kreck, et al., 2001
	γ-C8	R>>S 89/11	Mosandl et al., 1992; Bernreuther et al., 1989		γ-C8	R>>S 96/4-99/1	Kreck, et al., 2001
	γ-C9	absent	Ebeler et al., 2001		γ-C10	R>>S 97/3-99/1	Ebeler et al., 2001; Kreck et al., 2001
	γ-C10	R>> S 94/6	Mosandl et al., 1992; Bernreuther et al., 1991a; Ebeler et al., 2001		γ-C12	R>>S 98/2-99/1	Kreck, et al., 2001
	γ-C11	absent	Ebeler et al., 2001	Melon	Ethyl 2-methylbutanoate	not reported	Senesi, et al., 2002
	γ-C12	R>>S 99/1	Mosandl et al., 1992; Bernreuther et al., 1989; Ebeler et al., 2001		2-Methylbutanol	not reported	Senesi, et al., 2002
Raspberry	α-ionone	not reported	Aprea, et al., 2009				
	δ-C8	S>>R 96/4	Bernreuther et al., 1991a; Mosandl et al., 1992				
	δ-C10	S>>R 99/1	Bernreuther et al., 1991a; Mosandl et al., 1992				

Table 2: Sampling and analysis conditions, sampling, analysis and total times and related percent reduction for each fruit *and related food products investigated*.

	Sample preparation: HS-SPME					**Analysis: Es-GC**						**TAS**	
	Temp. (°C)	Eq. time (min)	Sampl. time (min)	Area RSD %	**Tot. time (min)**	Col. dim. L (m) d_c (mm)	Initial flow (mL/min) *SOF*	Void time (min)	Temperature program (°C/min)	**Time (min)**	*% red.*	**Time (min)**	*% red.*
Peach, apricot, raspberry, coconut and related food products													
Column									*AcAc-CD*				
Routine	85	5	60		**65**		1.00		50°C/*2.0*/220°C	**85.0**		**150.0**	
Optimised methods	60	5	20	6.0	**25**	25.0 0.25	1.40	0.87	90°C/*3.4*/140°C/*8.6*/220°C	**24.0**	*71.8*	**49.0**	*67.3*
						11.7 0.10	0.56	0.44	90°C/*6.8*/140°C/*16.9*/220°C	**12.1**	*85.8*	**37.1**	*75.3*
						5.0 0.10	0.56	0.13	90°C/*24.0*/140°C/*60.0*/220°C	**3.4**	*96.0*	**28.4**	*81.1*
Strawberry and related food products													
Column									*EtMe-CD*				
Routine	85	5	60		**65**		1.00		50°C/*2.0*/220°C	**85.0**		**150.0**	
Optimised methods	40	5	10	3.5	**15**	25.6 0.25	1.40	0.92	50°C/*8.2*/185°C/*16.2*/220°C	**18.7**	*78.0*	**33.7**	*77.5*
						11.3 0.10	0.56	0.43	50°C/*10.6*/185°C/*21.0*/220°C	**14.4**	*83.1*	**29.4**	*80.4*
						5.0 0.10	0.56	0.13	50°C/*34.8*/185°C/*68.7*/220°C	**4.4**	*94.8*	**19.4**	*87.1*
Melon and related food products													
Column									*EtMe-CD*				
Routine	85	5	60		**65**		1.00		50°C/*2.0*/150°C	**50.0**		**115.0**	
Optimised methods	40	5	10	3.8	**15**	25.6 0.25	1.40	0.92	50°C/*3.2*/70°C/*16.2*/150°C	**11.2**	*77.6*	**26.2**	*77.2*
						11.3 0.10	0.56	0.43	50°C/*4.1*/70°C/*21.0*/150°C	**8.7**	*82.6*	**23.7**	*79.4*
						5.0 0.10	0.56	0.13	50°C/*11.7*/70°C/*68.7*/150°C	**2.9**	*92.4*	**17.9**	*84.4*

Table 3: Effect of acidification (H_3PO_4 85% and HCl 37%) and basification (NaOH 2M) on the % areas ratio of the two enantiomers of C10 γ-lactones on a natural peach fruit and a peach flavoured tea as such and spiked with a 1ppm solution of (*R*)-γ-C10 lactone

	Week 1						Week 2				Week 3			
Juice jF														
	Ref.	+ *HCl*	+ *H_3PO_4*	+ *NaOH*	+ *STD (1ppm)*	+ *HCl +STD (1ppm)*	+ *HCl*	+ *NaOH*	+ *STD (1ppm)*	+ *HCl +STD (1ppm)*	+ *HCl*	+ *NaOH*	+ *STD (1ppm)*	+ *HCl +STD (1ppm)*
(*R*)-γ-C_{10} %	81.6	84.3	82.2	82.5	99.1	99.2	84.0	82.1	97.4	97.8	79.6	84.8	97.2	97.6
(*S*)-γ-C_{10} %	18.4	15.7	17.8	17.5	0.9	0.8	16.0	17.9	2.6	2.2	20.4	15.2	2.8	2.4
Tea tC														
	Ref.	+ *HCl*	+ *H_3PO_4*	+ *NaOH +STD (2ppm)*	+ *STD (1ppm)*	+ *HCl +STD (1ppm)*		+ *NaOH +STD (2ppm)*	+ *STD (1ppm)*	+ *HCl +STD (1ppm)*		+ *NaOH +STD (2ppm)*	+ *STD (1ppm)*	+ *HCl +STD (1ppm)*
(*R*)-γ-C_{10} %	51.5	51.7	50.9	94.2	70.7	70.3		92.5	70.2	70.0		91.9	70.7	69.7
(*S*)-γ-C_{10} %	48.5	48.3	49.1	5.8	29.3	29.8		7.5	29.8	30.0		8.1	29.3	30.4

STD = (*R*)-γ C10 in MeOH

Table 4: number of analyzed samples, average LOD, ranges of concentration and enantiomeric ratios of γ-decalactone and δ-decalactone in the different peach and coconut based food matrices investigated.

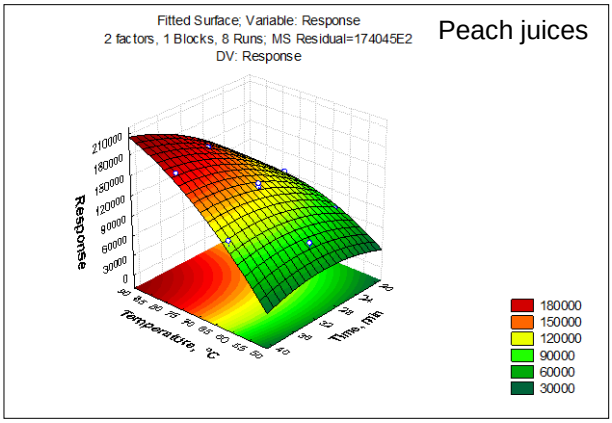
Peach				
	γ-decalactone (odor threshold = 11 ppb)			
	Number of samples	Average LOD (ppb)	Concentration range (ppb)	e.r. range *(R)/(S)*
Juice (natural)	16	2	100-480	85/15 -97/3
Juice (synthetic flavoured)	4	2	500-7000	52/48 - 63/37
Tea (synthetic flavoured)	10	1	1000-40000	49/51 - 68/32
Yogurt (synthetic flavoured)	6	3	1500-5500	49/51 - 65/35
Jam (natural)	2	2	130-250	78/22 - 80/20
Coconut				
	δ-decalactone (odor threshold = 100 ppb)			
	Number of samples	Average LOD (ppb)	Concentration range (ppb)	e.r. range *(R)/(S))*
Desserts (natural)	9	2	900-4400	87/13 - 89/11
Yogurt (synthetic flavoured)	7	1	400-5800	70/30 - 55/45
Milk and icecream (natural)	2	1	1000-4000	86/14 - 89/11

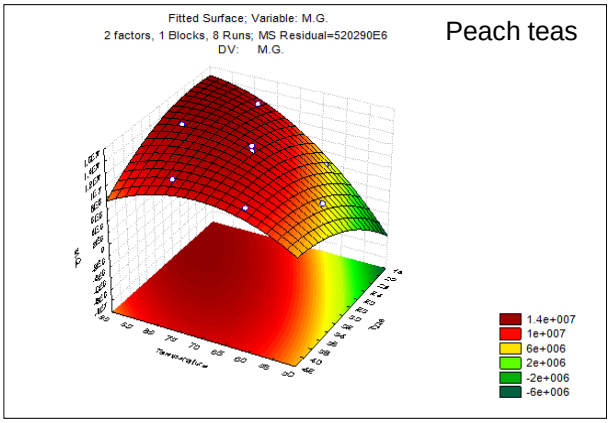
Effect of acidification (H_3PO_4 85% and HCl 37%) and basification (NaOH 2M) on the % areas ratio of the two enantiomers of C10 γ-lactones on a natural peach fruit and a peach flavoured tea as such and spiked with a 1ppm solution of (*R*)-γ-C10 lactone

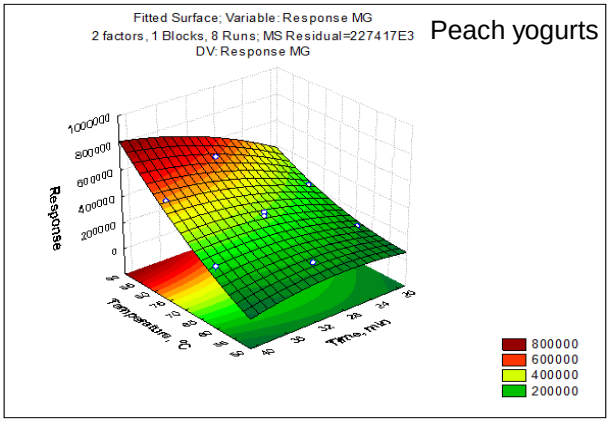
	Week 1						**Week 2**				**Week 3**			
Juice jF														
	Ref.	*+ HCl*	*+ H_3PO_4*	*+ NaOH*	*+ STD (1ppm)*	*+ HCl +STD (1ppm)*	*+ HCl*	*+ NaOH*	*+ STD (1ppm)*	*+ HCl +STD (1ppm)*	*+ HCl*	*+ NaOH*	*+ STD (1ppm)*	*+ HCl +STD (1ppm)*
(*R*)-γ-C_{10} %	81.6	84.3	82.2	82.5	99.1	99.2	84.0	82.1	97.4	97.8	79.6	84.8	97.2	97.6
(*S*)-γ-C_{10} %	18.4	15.7	17.8	17.5	0.9	0.8	16.0	17.9	2.6	2.2	20.4	15.2	2.8	2.4
Tea tC														
	Ref.	*+ HCl*	*+ H_3PO_4*	*+ NaOH +STD (2ppm)*	*+ STD (1ppm)*	*+ HCl +STD (1ppm)*		*+ NaOH +STD (2ppm)*	*+ STD (1ppm)*	*+ HCl +STD (1ppm)*		*+ NaOH +STD (2ppm)*	*+ STD (1ppm)*	*+ HCl +STD (1ppm)*
(*R*)-γ-C_{10} %	51.5	51.7	50.9	94.2	70.7	70.3		92.5	70.2	70.0		91.9	70.7	69.7
(*S*)-γ-C_{10} %	48.5	48.3	49.1	5.8	29.3	29.8		7.5	29.8	30.0		8.1	29.3	30.4

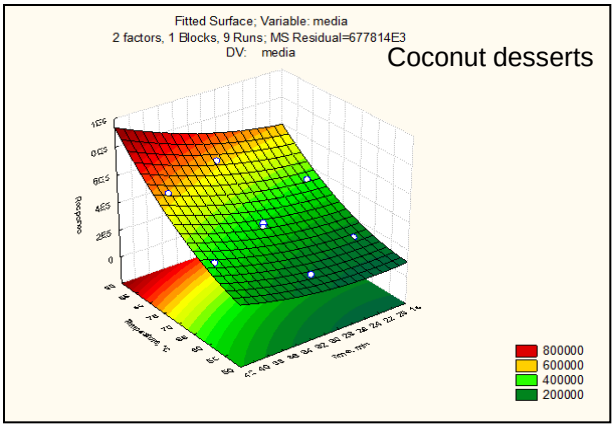
STD = (*R*)-γ C10 in MeOH

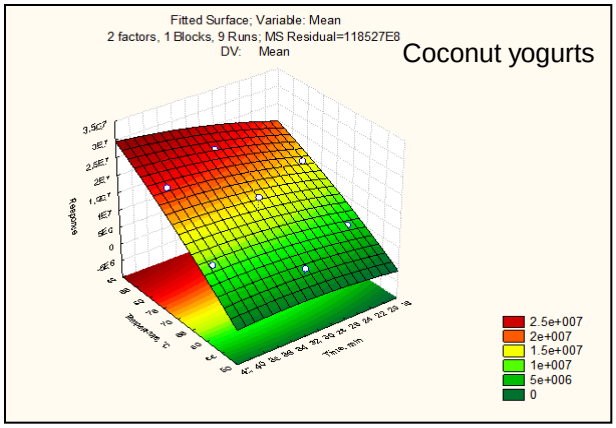
Dohelert response surface to optimize HS-SPME sampling conditions of peach and coconut matrices investigated

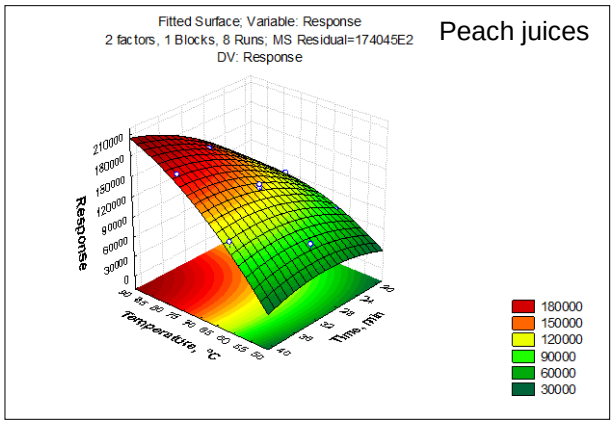








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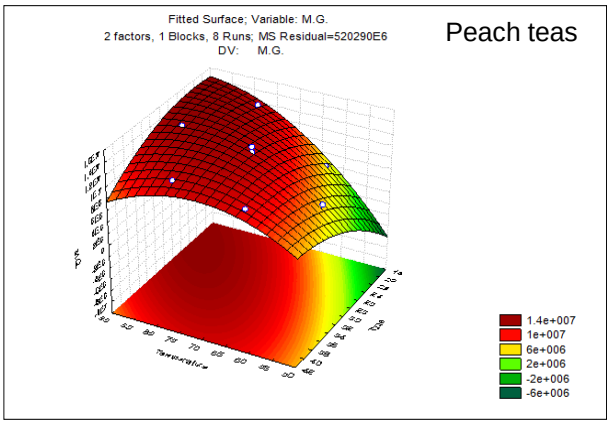
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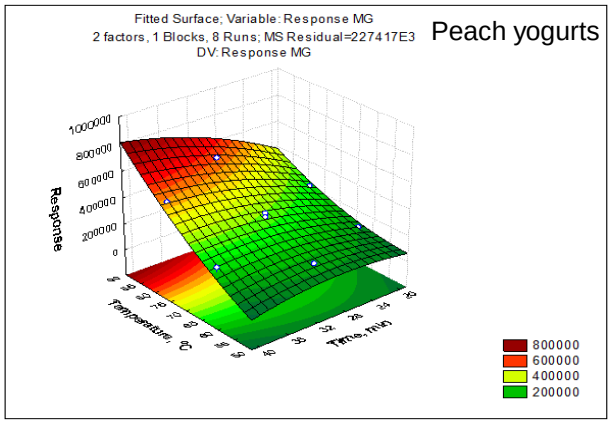
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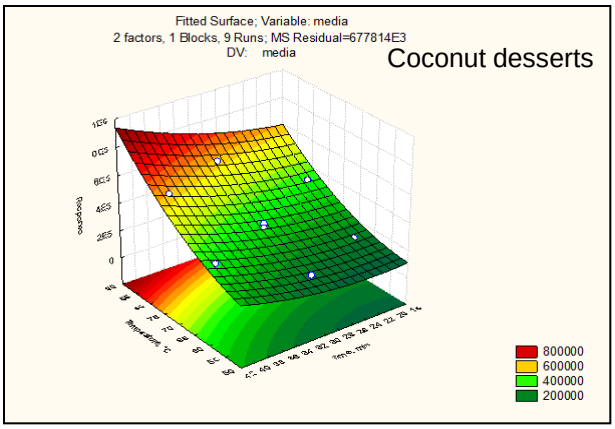
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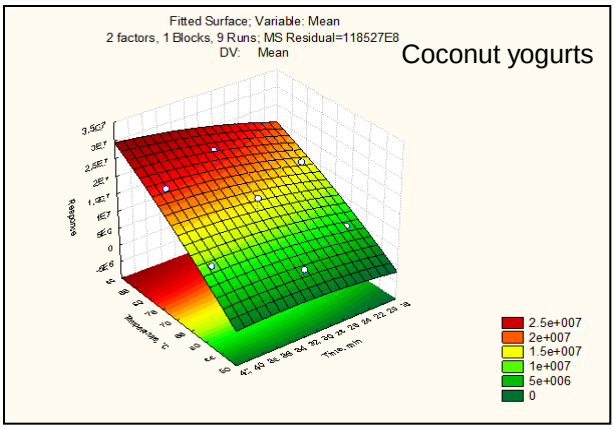
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