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

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## UNIVERSITÀ DEGLI STUDI DI TORINO

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

3

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5

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8

9 **Astract:** This study describes a rapid total analysis system (TAS) to detect the authenticity of fruit-flavoured  
10 foods and beverages by on-line combining headspace solid phase microextraction (HS-SPME) with  
11 enantioselective GC-MS (Es-GC-MS) and statistical multivariate methods (PCA, HCA). Peach, coconut,  
12 apricot, raspberry, as fruits mainly characterized by  $\gamma$ - and  $\delta$ -lactones as chiral markers, strawberry ( $\alpha$ -ionone,  
13 linalool, nerolidol, ethyl 2-methylbutyrate, 2-methylbutyric acid and  $\gamma$ -lactones) and melon (ethyl 2-  
14 methylbutyrate and 2-methylbutanol) were investigated. The system was developed by a) optimizing non-  
15 equilibrium HS-SPME sample preparation, b) speeding-up ES-GC using cyclodextrin derivatives as chiral  
16 selectors with conventional and narrow-bore columns and c) elaborating data by multivariate methods. The  
17 resulting TAS affords a reduction of the time needed for the whole analytical process from about 150 min to  
18 20-50 minutes (67%-87% of the current routine method) depending on matrix, sampling and analysis  
19 conditions and Es-GC columns.

20

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

23

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

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## 26 1. Introduction

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

34 Enantiomer recognition in flavours and fragrances, and more in general in the food field, is very important,  
35 mainly because biological interactions and biosynthetic processes are mostly stereospecific, meaning that  
36 chiral components in natural products are often characterized by specific enantiomeric compositions (Bicchi,  
37 D'Amato & Rubiolo, 1999; Bicchi, Manzin, D'Amato & Rubiolo, 1995; Konig & Hochmuth, 2004; Mosandl,  
38 1995). On the other hand, essential oils and fruit flavours have great commercial relevance for food industry,  
39 and are quite often replaced by cheaper synthetic racemic compounds, products from other cheaper natural  
40 sources, or of different origins; these sometimes contain chiral components with different enantiomer  
41 compositions (Konig et al., 2004; Mosandl, 1995).

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

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

63 Recently, Es-GC analysis has been speeded up (Bicchi, Blumberg, Cagliero, Cordero, Rubiolo & Liberto,  
64 2010a; Bicchi, Liberto, Cagliero, Cordero, Sgorbini & Rubiolo, 2008; Liberto et al., 2008) by a suitable  
65 combination of short conventional or narrow bore columns and mass spectrometry as a second dimension in  
66 detection and used to locate the enantiomers in the chromatograms; they are afterward identified through their  
67 linear GC retention indices ( $I_r$ s) (Liberto et al., 2008). Two distinct but highly complementary approaches have  
68 been used: the first suitably combines narrow bore columns even shorter than the conventional 10 m length  
69 (e.g. 5 or 2 m) (Bicchi et al., 2008), with mass spectrometry in extract ion mode, while the second consists of  
70 finding the best speed/separation trade-off with a conventional column and translating the resulting analysis  
71 conditions to narrow bore columns by the method translation approach (Bicchi et al., 2010a).

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

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

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

## 84 2. Experimental

### 85 2.1 Samples

86 Pure standard mixtures of racemic  $\gamma$ - and  $\delta$ -lactones (both from C6 to C12),  $\alpha$ -ionone, linalool, nerolidol, ethyl  
87 2-methylbutyrate, 2-methylbutyric acid and 2-methylbutanol were from the collection of standards in the  
88 authors' laboratory. They were solubilised in cyclohexane at a concentration of 100 ppm each. Solvent was  
89 HPLC grade from Riedel-de Haen (Seelze, Germany).

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

95

### 96 2.2 Sample preparation and SPME extraction

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

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

106

### 107 2.3 GC-MS analysis

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

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

118 GC-MS conditions: temperatures: injector: 220°C, transfer line: 230°C; ion source: 200°C; carrier gas: He,  
119 flow control mode: constant linear velocity, initial flow rates and temperature programs are reported in the text.  
120 Injection: injection mode: split; split ratio 1:20 for the conventional columns, 1:300 for the 11m NB columns and  
121 1:400 for the 5m NB columns.

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

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

128

### 129 3.4 *Statistical analysis*

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

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

139

## 140 3. Results and discussion

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

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

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

152

### 153 ***3.1 Sample preparation***

154 Headspace sampling by SPME has been chosen because of its ability to recover volatile analytes and its  
155 reliability, even in non-equilibrium HS conditions, and to eliminate any sample pre-treatments independently on  
156 the matrix investigated. The conditions to achieve a suitable trade-off between chiral marker representative  
157 recovery and sampling time were investigated for all fruits under study (peach, coconut, apricot, raspberry,  
158 strawberry and melon) and related matrices. The sampling conditions were optimized by applying an  
159 experimental Dohelert design to temperature and time (data reported in the supplement); these two variables  
160 were chosen after applying a fractional factorial design in which the significance of sample amount, salting out,  
161 temperature and time was investigated. The optimal temperature and time resulting from these experiments  
162 were 85°C and 60 minutes respectively for all matrices investigated. However, in the final method, both values  
163 were lowered because 1) the aim of this study is to develop a fast method therefore sampling time had to be  
164 logically compatible with analysis time, 2) the optimal conditions resulted in peak overloading (in particular with  
165 matrices containing artificial aromas) that affects e.r. calculation, and 3) the control of authenticity is based on  
166 the enantiomeric ratios of chiral markers so that recovery has not to be maximized. Table 2 reports sampling  
167 conditions selected for each fruit, food or beverage. Sampling temperatures ranged from 40°C to 60°C and  
168 sampling time from 10 min for strawberry and melon to 20 minutes for the other fruits investigated. Moreover,  
169 table 2 shows that, also under non-equilibrium but rigorously standardized conditions, marker recoveries are  
170 highly repeatable since RSD% calculated on the total area of the chiral markers of each fruit never exceeds  
171 6%.

172

### 173 ***3.2 Speeding-up of enantioselective analyses of different fruit matrices***

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

176 selector for one problem" approach (Bicchi, D'Amato & Manzin, 1997; Bicchi et al., 1995) had first to be found.  
177 6<sup>I-VII</sup>-*O*-TBDMS-2<sup>I-VII</sup>-3<sup>I-VII</sup>-*O*-acetyl- $\beta$ -CD (AcAc-CD) (Maas et al., 1995) was therefore used as chiral selector  
178 for fruits mainly containing  $\gamma$ - and  $\delta$ -lactones (peach, apricot, raspberry and coconut) and 6<sup>I-VII</sup>-*O*-TBDMS-2<sup>I-VII</sup>-  
179 ethyl-3<sup>I-VII</sup>-*O*-methyl- $\beta$ -CD (EtMe-CD) (Bicchi et al., 2010b) for fruits characterized by esters and alcohols other  
180 than  $\gamma$ -lactones (strawberry and melon).

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

### 185 3.2.1) *Optimization of Es-GC conditions of a peach sample with a conventional column*

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

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

201 ii) *determination of optimal multi-rate temperature program* – The above samples were then analysed by  
202 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/ $t_M$ ,  
203 15°C/ $t_M$ , using a flow rate of 1 mL/min and 50°C and 220°C as initial and final temperatures. The  
204 corresponding normalized heating rates were 1.9, 2.8, 4.7, 7.1, 9.5 and 14.3 °C/min, respectively. The  
205 enantiomers of both series of lactones are base-line separated until rate 7.5°C/ $t_M$ , while  $\gamma$ - and  $\delta$ -C12 lactones  
206 partially co-eluted from 10°C/ $t_M$ . On the other hand, the (S)- $\gamma$ -C6/(R)- $\gamma$ -C7 and (R)- $\delta$ -C6/(S)- $\delta$ -C8 enantiomer  
207 pairs overlapped at 5°C/ $t_M$  and 7.5°C/ $t_M$  heating-rates.

208 The analysis conditions were therefore modified, applying 90°C as initial temperature and 2°C/ $t_M$  and 3°C/ $t_M$   
209 as heating rates. The analysis at 2°C/ $t_M$  still showed a partial co-elution of (S)- $\gamma$ -C6/(R)- $\gamma$ -C7 and (S)- $\gamma$ -C7/(S)-  
210  $\delta$ -C6 pairs while that at 3°C/ $t_M$  gave a base-line separation of all chiral compounds. The best trade-off  
211 between separation and analysis time was therefore achieved with a multi-rate temperature program from  
212 90°C to 140°C at 3°C/ $t_M$  (i.e. after elution of  $\delta$ -C6), then to 220°C at 7.5°C/ $t_M$  and an Es-GC analysis time of  
213 29 min instead of 85 min with the original method.

214 iii) *determination of optimal flow rate for the normalized optimal multi-rate temperature program* - The next step  
215 was flow rate optimization by determining the initial efficiency-optimized flow (EOF, i.e. initial flow that  
216 maximizes column efficiency and peak resolution) and, from it, by calculating the initial speed-optimized flow  
217 (SOF i.e. initial flow which minimizes analysis time at fixed efficiency, which is  $\text{SOF} = \sqrt{2} \text{ EOF}$ ) (Blumberg,  
218 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  
219 the GC method-translator to translate the temperature program for each value, so that the normalized  
220 temperature program was always the same. The initial EOF for the most critical pair (S)- $\gamma$ -C6/(S)- $\gamma$ -C7 was 1  
221 mL/min; as a consequence the calculated SOF was 1.4 mL/min (Blumberg, 1999) (figure 1b). The analysis  
222 time under SOF conditions was further reduced to 24 min.

### 223 3.2.2 Translation of the methods to narrow bore columns and analysis of different fruit matrices

224 The optimised EOF and SOF methods with conventional column were then translated to two 11 and 5 m long  
225 NB (0.1 mm  $\times$  0.1  $\mu\text{m}$ ) columns coated with the same stationary phase. The void time of the narrow bore  
226 columns was first measured to determine their exact length, before translating the methods; the calculated

227 lengths were 11.7 m and 5 m, respectively. The parameters of the translated SOF methods are reported in  
228 table 2 while figure 2 (a and b) reports the Es-GC patterns of  $\gamma$ -C6-C12-SS and  $\delta$ -C6-C12-SS analysed on the  
229 11 and 5 m NB columns, respectively. The analysis time with NB columns with the translated SOF methods is  
230 thus shortened to 12.1 minutes with the 11.7 m column, and to 3.4 minutes with the 5 m column, separation  
231 remaining exactly the same as for the conventional column. Table 2 also reports analysis time and %  
232 reduction, obtained in SOF mode, with the optimized methods applied to the columns investigated, compared  
233 to routine analysis: the analysis time reduction under optimal conditions with conventional columns was 72%,  
234 while with 11 m and 5 m narrow bore columns it was 86% and 96%, respectively.

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

245

### 246 ***3.3 Optimization of Es-GC conditions with EtMe-CD column for the headspace analysis of strawberry*** 247 ***and melon***

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

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

252 The same approach used for peach optimization was applied, first determining the void time (1.092 min) and  
253 the corresponding column length (25.6 m). After analysis under routine conditions, the same nominal  
254 temperature rates used for peach were applied to strawberry HS, with a flow rate of 1 mL/min, and 50°C and  
255 220°C as initial and final temperatures. The multi-rate temperature program was conditioned by  $\gamma$ -C12 lactone  
256 and by the two pairs of enantiomers of nerolidol diastereoisomers, that are only base-line separated until  
257 7.5°C/ $t_M$ . The following multi-rate temperature program was therefore chosen: from 50 to 185°C (after  $\gamma$ -C12  
258 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  
259 marker compounds were separated in an analysis time of about 19 min, instead of the 85 min of the routine  
260 method (table 2).

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

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

268 A procedure similar to that for strawberry was applied to melon, whose headspace is characterized by high  
269 volatility chiral compounds, in particular ethyl 2-methylbutyrate and 2-methylbutanol. 2-Methylbutanol  
270 enantiomers are base-line separated only at 3°C/ $t_M$ . The following multi-rate temperature program was  
271 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  
272 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  
273 columns (table 2). The analysis time ranged from 50 min in routine conditions to 3.9 min of the SOF analysis  
274 with the 5 m NB column.

275

276 ***3.4 Stability of enantiomeric composition in different conditions***

277 One of the main limitations on the use of enantiomeric recognition to determine the authenticity of a sample is  
278 that the enantiomeric ratio may vary under different conditions or during processing. The effect of sampling,  
279 storage and pH on the enantiomeric composition was therefore tested, by determining the consistency of the  
280 % area of the enantiomers of some target lactones in an unflavoured peach juice (taken as reference) and a  
281 flavoured tea. The results of these sets of experiments on the effect of sampling conditions on enantiomeric  
282 ratios of C8, C10, C11, C12  $\gamma$ -lactones in a peach-flavoured tea show that the values of % areas of the two  
283 enantiomers did not change when ionic strength, temperature or sampling time were varied. The effect of  
284 storage was tested by analysing the above samples stored in daylight at ambient temperature over a period of  
285 three months, without any variations in the lactone marker enantiomeric ratio being observed (data not  
286 reported).

287 The effects of acidification and basification on the enantiomeric composition of  $\gamma$ -C10 were then evaluated on  
288 both the above samples. A 1ppm solution of (R)- $\gamma$ -C10 was added to both samples (juice: pH 3.4, tea: pH 4.2)  
289 acidified with H<sub>3</sub>PO<sub>4</sub> (up to pH 1.5 for juice and pH 3.0 for tea) and HCl (up to pH <0.5 for both samples), or  
290 basified with NaOH (up to pH 11 for both samples), and then analysed over a period of three weeks (table 3).  
291 The results of acidification compared to those of the original samples show that: i)  $\gamma$ -C10 in the flavoured tea is  
292 racemic at both native and acid pH, ii) the enantiomeric ratio due to added (R)- $\gamma$ -C10 to the flavoured tea does  
293 not change after acidification and storage, iii) the natural (R)- $\gamma$ -C10 enantiomeric ratio of the juice does not  
294 decrease after acidification. The results after basic treatment indicate a strong decrease of absolute area of  
295 lactones, probably due to hydrolysis of the lactone group, but the enantiomeric ratio is kept probably because  
296 the reaction is not stereospecific

### 297 298 ***Analysis of commercially available samples and statistical discrimination***

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

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

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

#### 327 4. Conclusions

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

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

340

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345

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431

432 Captions to figures

433

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

437 Peak identification: 1:  $\gamma$ -hexalactone, 2:  $\gamma$ -heptalactone, 3:  $\gamma$ -octalactone, 4:  $\gamma$ -nonalactone, 5:  $\gamma$ -decalactone,  
438 6:  $\gamma$ -undecalactone, 7:  $\gamma$ -dodecalactone, 8:  $\delta$ -hexalactone; 9:  $\delta$ -octalactone, 10:  $\delta$ -nonalactone, 11:  $\delta$ -  
439 decalactone, 12:  $\delta$ -undecalactone, 13:  $\delta$ -dodecalactone; a: (*R*)-enantiomer, b: (*S*)-enantiomer.

440

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

444

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

448

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

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

452

453 Table 2: Sampling and analysis conditions, sampling, analysis and total times and related percent reduction for  
 454 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 <sub>c</sub> (mm)	Initial flow (mL/min) SOF	Void time (min)	Temperature program (°C/min)	Time (min)	% red.	Time (min)	% red.	
<i>Peach, apricot, raspberry, coconut and related food products</i>													
<i>Column</i>						<i>AcAc-CD</i>							
Routine	85	5	60	<b>65</b>		1.00		50°C/2.0/220°C	<b>85.0</b>		<b>150.0</b>		
Optimised methods	60	5	20	6.0	<b>25</b>	25.0	1.40	0.87	90°C/3.4/140°C/8.6/220°C	<b>24.0</b>	71.8	<b>49.0</b>	67.3
						0.25							
						11.7	0.56	0.44	90°C/6.8/140°C/16.9/220°C	<b>12.1</b>	85.8	<b>37.1</b>	75.3
						0.10							
						5.0	0.56	0.13	90°C/24.0/140°C/60.0/220°C	<b>3.4</b>	96.0	<b>28.4</b>	81.1
						0.10							
<i>Strawberry and related food products</i>													
<i>Column</i>						<i>EtMe-CD</i>							
Routine	85	5	60	<b>65</b>		1.00		50°C/2.0/220°C	<b>85.0</b>		<b>150.0</b>		
Optimised methods	40	5	10	3.5	<b>15</b>	25.6	1.40	0.92	50°C/8.2/185°C/16.2/220°C	<b>18.7</b>	78.0	<b>33.7</b>	77.5
						0.25							
						11.3	0.56	0.43	50°C/10.6/185°C/21.0/220°C	<b>14.4</b>	83.1	<b>29.4</b>	80.4
						0.10							
						5.0	0.56	0.13	50°C/34.8/185°C/68.7/220°C	<b>4.4</b>	94.8	<b>19.4</b>	87.1
						0.10							
<i>Melon and related food products</i>													
<i>Column</i>						<i>EtMe-CD</i>							
Routine	85	5	60	<b>65</b>		1.00		50°C/2.0/150°C	<b>50.0</b>		<b>115.0</b>		
Optimised methods	40	5	10	3.8	<b>15</b>	25.6	1.40	0.92	50°C/3.2/70°C/16.2/150°C	<b>11.2</b>	77.6	<b>26.2</b>	77.2
						0.25							
						11.3	0.56	0.43	50°C/4.1/70°C/21.0/150°C	<b>8.7</b>	82.6	<b>23.7</b>	79.4
						0.10							
						5.0	0.56	0.13	50°C/11.7/70°C/68.7/150°C	<b>2.9</b>	92.4	<b>17.9</b>	84.4
						0.10							

455

456 Table 3: Effect of acidification (H<sub>3</sub>PO<sub>4</sub> 85% and HCl 37%) and basification (NaOH 2M) on the % areas ratio of  
 457 the two enantiomers of C<sub>10</sub>  $\gamma$ -lactones on a natural peach fruit and a peach flavoured tea as such and spiked  
 458 with a 1ppm solution of (*R*)- $\gamma$ -C<sub>10</sub> lactone

	Week 1					Week 2					Week 3			
	<i>Juice jF</i>													
	Ref.	+ HCl	+ H <sub>3</sub> PO <sub>4</sub>	+ NaOH	+ STD (1ppm)	+ HCl +STD (1ppm)	+ HCl	+ NaOH	+ STD (1ppm)	+ HCl +STD (1ppm)	+ HCl	+ NaOH	+ STD (1ppm)	+ HCl +STD (1ppm)
( <i>R</i> )- $\gamma$ -C <sub>10</sub> %	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
( <i>S</i> )- $\gamma$ -C <sub>10</sub> %	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
	<i>Tea tC</i>													
	Ref.	+ HCl	+ H <sub>3</sub> PO <sub>4</sub>	+ NaOH +STD (2ppm)	+ STD (1ppm)	+ HCl +STD (1ppm)	+ NaOH +STD (2ppm)	+ STD (1ppm)	+ HCl +STD (1ppm)	+ NaOH +STD (2ppm)	+ STD (1ppm)	+ HCl +STD (1ppm)		
( <i>R</i> )- $\gamma$ -C <sub>10</sub> %	51.5	51.7	50.9	94.2	70.7	70.3	92.5	70.2	70.0	91.9	70.7	69.7		
( <i>S</i> )- $\gamma$ -C <sub>10</sub> %	48.5	48.3	49.1	5.8	29.3	29.8	7.5	29.8	30.0	8.1	29.3	30.4		

459  
 460 STD = (*R*)- $\gamma$  C<sub>10</sub> in MeOH  
 461

462 Table 4: number of analyzed samples, average LOD, ranges of concentration and enantiomeric ratios of  $\gamma$ -  
 463 decalactone and  $\delta$ -decalactone in the different peach and coconut based food matrices investigated.

<b>Peach</b>				
<i><math>\gamma</math>-decalactone (odor threshold = 11 ppb)</i>				
	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
<b>Coconut</b>				
<i><math>\delta</math>-decalactone (odor threshold = 100 ppb)</i>				
	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

464  
 465  
 466 Effect of acidification ( $H_3PO_4$  85% and HCl 37%) and basification (NaOH 2M) on the % areas ratio of the two  
 467 enantiomers of C10  $\gamma$ -lactones on a natural peach fruit and a peach flavoured tea as such and spiked with a  
 468 1ppm solution of (R)- $\gamma$ -C10 lactone

	<b>Week 1</b>					<b>Week 2</b>					<b>Week 3</b>			
	<b>Juice jF</b>													
	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)- $\gamma$ -C <sub>10</sub> %	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)- $\gamma$ -C <sub>10</sub> %	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
	<b>Tea tC</b>													
	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)- $\gamma$ -C <sub>10</sub> %	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)- $\gamma$ -C <sub>10</sub> %	48.5	48.3	49.1	5.8	29.3	29.8	7.5	29.8	30.0	8.1	29.3	30.4		

469  
 470 STD = (R)- $\gamma$  C10 in MeOH  
 471

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



