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1 **Herbs and spices: characterization and quantitation of biologically-active markers for routine**
2 **quality control by multiple headspace solid-phase microextraction combined with separative**
3 **or non-separative analysis.**

4

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15 **Abstract**

16 Herbs and spices are used worldwide as food flavoring, thus determination of their identity, origin,
17 and quality is mandatory for safe human consumption. An analysis strategy based on separative
18 (HS-SPME-GC-MS) and non-separative (HS-SPME-MS) approaches is proposed for the volatile
19 fraction of herbs and spices, for quality control and to quantify the aromatic markers with a single
20 analysis directly on the plant material as such. Eight-to-ten lots of each of the following
21 herbs/spices were considered: cloves (*Syzygium aromaticum* (L.) Merr. & Perry), American
22 peppertree (*Schinus molle* L.), black pepper and white pepper (*Piper nigrum* L.), rosemary
23 (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.).
24 Homogeneity, origin, and chemotypes of the investigated lots of each herb/spice were defined by
25 fingerprinting, through statistical elaboration with Principal Component Analysis (PCA).
26 Characterizing aromatic markers were directly quantified on the solid matrix through multiple
27 headspace extraction-HS-SPME (MHS-SPME). Reliable results were obtained with both separative
28 and non-separative methods (where the latter were applicable); the two were in full agreement,
29 RSD% ranging from 1.8 to 7.7% for eugenol in cloves, 2.2-18.4% for carvacrol+thymol in thyme,
30 and 3.1-16.8% for thujones in sage.

31

32 **KEYWORDS:** Herbs, Spices, Fingerprinting, Marker Quantitation, Separative method (Multiple
33 Head Space-Solid Phase MicroExtraction-Gas Chromatography-Mass Spectrometry), Non-
34 separative method (Multiple Head Space-Solid Phase MicroExtraction-Mass Spectrometry)

35

36

37 **1. Introduction**

38 Spices and herbs, as such or ground, alone or blended, are widely used for food flavoring. Many
39 volatiles characterizing spices possess relevant biological activities in addition to their flavor
40 (antibacterial, antiviral, antifungal, or toxic) [1,2] . Plant species for use as spices, as such or in

41 blends, must be submitted to quali- and quantitative controls to authenticate them and define their
42 quality and flavor profile; strict chemical and microbiological controls are also mandatory to
43 exclude contamination. Quali-quantitative analysis is an indispensable complement to botanical
44 identification, providing reliable definition of a plant's biological activity.

45 Conventional methods for volatile analysis very often entail isolation of the essential oil by
46 hydrodistillation, or solvent extraction followed by gas-chromatographic analysis; these are
47 effective but time-consuming for routine quality control. Moreover, although representative and
48 universally accepted, these two procedures frequently fail to recover the markers exhaustively from
49 the aromatic plant. Solvent extraction, when applied to a set of different-polarity analytes, may
50 discriminate between them thus altering recovery. Conversely, recovery of an analyte by isolating
51 the essential oil from the plant is closely conditioned by the analyte's water solubility: a recent
52 study in the authors' laboratory found that only 70-90% of the main components were recovered in
53 essential oil obtained by hydrodistillation, the remainder being solubilized in the residual water [3];
54 these results will be the object of a forthcoming publication.

55 For the above reasons, rapid, inexpensive, easily-automated and solventless analytical methods,
56 applicable directly to plant material, are needed for characterization, quality control and quantitation
57 of the biologically-active components of spices and herbs. For volatile markers, headspace sampling
58 (HS) meets these requirements in full, in particular when HS is carried out with high concentration
59 capacity techniques such as solid phase microextraction (SPME) [4]. Headspace sampling is also
60 ideal because it can be combined directly with MS in the so-called non-separative systems (perhaps
61 better known as MS-nose) that produce diagnostic MS profiles. However, quantitation with HS
62 techniques is quite complex, in particular when applied to solid matrices, as is the case of most
63 spices: the technique is conditioned by matrix effects, in other words matrix composition and
64 texture influence analyte release. HS quantitation of analytes in solid samples can be run either on
65 the matrix as such, or after suspending it in a liquid that, under the analysis conditions adopted, is
66 not volatilized (often water). The principal advantages of the latter approach are its greater

67 sensitivity for analytes that are poorly soluble in the suspension liquid, and the homogeneous
68 distribution of the internal standard. Conversely, direct quantitation on the solid sample is
69 indispensable if markers react with or are soluble in the suspension medium; however, it suffers
70 from two crucial drawbacks: the distribution of the internal standard within the matrix is non-
71 homogeneous and non-repeatable, and the internal standard interacts physico-chemically and
72 physically at the surface of the solid sample.

73 Multiple Headspace Extraction (MHE) is a possible approach to quantitation in solid samples; it
74 enables the matrix effect to be overcome. This quantitation approach was first proposed by Suzuki
75 et al. [5] and McAuliffe et al. [6] in the late 1960s, then developed by Kolb et al. [7] and recently
76 extended to include solid-phase microextraction (MHS-SPME) [8-13]. Ezquerro et al. [8] first
77 applied MHE to the quantitative determination of volatiles in multilayer packaging. MHS-SPME
78 was subsequently applied to quantify volatiles in antioxidant rosemary extracts [9] and in dry
79 fermented sausages [10], to determine haloanisoles and volatile phenols in wines [11], and aroma
80 components in tomato samples [12] and, more recently, in coffee [14], mushrooms [15,16] and
81 hazelnuts [17]. MHE is a stepwise quantitative approach based on dynamic gas extraction; it
82 enables the total peak area of an analyte in a matrix to be determined, excluding the matrix effect.
83 Despite this important advantage, this approach is not widely used because it is erroneously
84 considered to be complex and time-consuming.

85 This study aimed to meet the ever-increasing demand for routine control analyses to authenticate
86 and classify a group of spices through fingerprinting and profiling. In particular, seven aromatic
87 plants widely used as spices were investigated, i.e. cloves (*Syzygium aromaticum* (L.) Merr. &
88 Perry), American peppertree (*Schinus molle* L.), black pepper and white pepper (*Piper nigrum* L.),
89 rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.).
90 The main goal was to investigate the possibility of applying the above two approaches to routine
91 quality control, while significantly reducing total analysis time. Spice characterization was done in
92 a single analysis, by 1) fingerprinting it through its volatile fraction, by separative (HS-SPME-GC-

93 MS) and non-separative (HS-SPME-MS) methods in combination with Principal Component
94 Analysis (PCA), applied directly to solid matrices as such, and 2) quantitation through MHE of
95 selected key-markers known to be responsible for the flavor, and/or taxonomic classification, and/or
96 biological activity of the investigated spice, again by separative and, when possible, non-separative
97 methods.

98

99 **2. Experimental**

100 **2.1. Materials and Reagents**

101 Spice samples from lots of different geographical origins were kindly supplied by Cannamela (Zola
102 Predosa (BO), Italy), in particular ten samples of black pepper, white pepper (*Piper nigrum* L.),
103 and American peppertree (*Schinus molle* L.), and nine samples of thyme (*Thymus vulgaris* L.),
104 rosemary (*Rosmarinus officinalis* L.), and cloves (*Syzygium aromaticum* (L.) Merr. & Perry). Eight
105 samples of sage (*Salvia officinalis* L.) were purchased in different local supermarkets, being from
106 different origins according to the labels (1 from East Turkey, 3 from Central Turkey, and 4 from
107 Italy). **Table 1** lists the matrices analyzed and the target ions of the selected markers. Pure standard
108 samples of borneol, bornyl acetate, Δ -3-carene, carvacrol, β -caryophyllene, eugenol, α -humulene,
109 limonene, linalool, α -phellandrene, α -pinene, α -terpineol, thymol, α - and β - thujone were from
110 Sigma Aldrich (Milan, Italy). Solvents were all HPLC-grade from Sigma Aldrich (Milan, Italy).

111

112 **2.2. SPME fibers**

113 Polydimethylsiloxane (PDMS) and carboxen/divinylbenzene/PDMS (CAR/DVB/PDMS) SPME
114 fibers (1 cm long) were from Supelco Co. (Bellafonte, PA, USA). PDMS coating was used for
115 thyme, CAR/DVB/PDMS for all other matrices. Before use, all fibers were conditioned as
116 recommended by the manufacturer. Consistency of fiber performance was periodically checked
117 through in-fiber external standardization, by analyzing a standard aqueous solution containing some
118 of the selected markers (5 μ L of a 2 mg mL⁻¹ solution sampled for 30 minutes at 50°C) [**18, 19**]

119

120 **2.3. Sample preparation**

121 **2.3.1. Sampling conditions**

122 A series of experiments were run to determine the optimal HS-SPME sampling conditions: fiber
123 coating (PDMS, CAR-PDMS-DVB, PDMS-DVB), sampling time (15, 30, 45, 60 minutes) and
124 temperature (30, 50, 60°C), and vial volume (10 and 20mL).

125 Appropriate amounts (1-20 mg depending on the matrix) of thyme, rosemary, black pepper, white
126 pepper, cloves, and sage in a 20 mL headspace vial were submitted to HS-SPME sampling for 30
127 minutes at 60°C. A known amount of cloves (1g) was diluted with an inert solid support (Celite®
128 545, Fluka) in a 1:20 ratio to obtain a mother sample, from which 2 mg samples containing 0.1 mg
129 of cloves were weighed out. Each sample was submitted to MHS-SPME three times, for a total of
130 nine extractions for each matrix. Blank runs were done, without detecting any carry-over effects.
131 After sampling, the fiber was automatically removed from the vapor phase, and inserted into the GC
132 injection port to desorb the sampled analytes thermally on-line into the GC column.

133 Fingerprints were normalized by in-fiber external standardization: 1 μL of a 1000 $\mu\text{g mL}^{-1}$ solution
134 of nonane in dibutylphthalate was sampled for 20 minutes at 60°C [17].

135

136 **2.3.2. Analysis conditions**

137 Analyses were carried out with a MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr,
138 Germany) installed on an Agilent 6890 GC unit coupled to an Agilent 5973N MSD (Agilent, Little
139 Falls, DE, USA). For the non-separative analyses, the GC injection port was connected directly to
140 the MS system through a length of deactivated fused silica tubing.

141 *Separative GC-MS method:* injector temperature: 230°C, injection mode: split, ratio: 1/20; liner:
142 Inlet Liner SPME Type (Sigma Aldrich); carrier gas: helium, flow rate: 1 mL min^{-1} ; fiber
143 desorption and reconditioning time: 5 min; column: MEGAWAX 20M (df 0.20 μm , dc 0.20 mm,
144 length 50 m) (Mega, Legnano (Milan), Italy). Temperature programs: for thyme and cloves, from

145 100°C (0 min) to 230°C (5 min) at 3°C min⁻¹; for white and black pepper, rosemary, American
146 peppertree, and sage, from 50°C (1 min) to 230°C (5 min) at 3°C min⁻¹. Markers were identified by
147 comparing their mass spectra and retention indices to those of authentic standards, or available in
148 commercial or home-made libraries, or reported in the literature.

149 *Non-separative MS method*: injector temperature: 250°C, injection mode: split, ratio: 1/20; carrier
150 gas: helium, flow rate: 0.4 mL min⁻¹; fiber desorption time and reconditioning: 5 min; transfer
151 column: deactivated fused silica tubing (dc 0.10 mm, length 6.70 m) (Mega, Legnano (Milan),
152 Italy); GC oven temperature: 250°C.

153 MSD conditions: MS operated in EI mode (70 eV), scan range: 35 to 350 amu; selected target ions
154 for quantitation are in Table 1; dwell time 40 ms, ion source temperature: 230°C; quadrupole
155 temperature: 150°C; transfer line temperature: 280°C.

156

157 **2.4. Quantitation**

158 Stock standard mixtures of the markers selected for each matrix were prepared by adding an aliquot
159 of pure standard to an appropriate volume of cyclohexane. Initial concentrations were 60 mg mL⁻¹,
160 with the exception of Δ -3-carene and α -humulene (70 mg mL⁻¹) and α -phellandrene (90 mg mL⁻¹).
161 Suitable dilutions (5-7) of each stock standard mixture in cyclohexane were then prepared in the
162 concentration range (0.002-90 mg mL⁻¹) reported in Table 3SM. The resulting solutions (stock and
163 diluted) were stored at 0°C and renewed weekly. Each calibration solution was analyzed in
164 triplicate by total vaporization MHS-SPME, under the conditions reported in paragraph 2.3.1.

165

166 **2.5. Method repeatability and intermediate precision, LOD and LOQ, method accuracy**

167 All matrices were analyzed three times on the same day by MHS-SPME to evaluate repeatability.
168 Intermediate precision was determined for each matrix, by analyzing it every four weeks over a
169 period of three months.

170 The LOD and LOQ values were determined experimentally by analyzing decreasing amounts of the
171 real-world samples diluted with an inert solid support (Celite® 545, Fluka). The LOD of each
172 analyte was calculated from the average area of the investigated marker divided by the average
173 “peak to peak” noise value, sampled in its region of elution in the chromatogram, with a coverage
174 factor of 3. LOQ was the lowest concentration for which the error in peak integration area
175 determination (assignment) was $\leq 20\%$.

176 The accuracy of the methods was evaluated by quantifying each marker in two samples, for each
177 spice and aromatic plant from different lots, in solid phase with the internal standard addition
178 approach, because of the lack of certified reference standard samples, and of methods exhaustively
179 recovering the markers investigated.

180

181 **2.6 Data processing**

182 Principal Component Analysis (PCA) was run with XLStat 2013 (Addinsoft, Paris, France). Data
183 for PCA and regression analysis were pre-treated by autoscaling.

184

185 **3. Results and discussion**

186 Quality control of aromatic plants used in the medicinal or food fields is a mandatory and crucial
187 step, which requires highly reliable, but at the same time simple and easily-automated, methods.
188 Recently, including in the plant field, non-separative methods have attracted considerable interest
189 alongside conventional separative methods, in particular when large numbers of samples are to be
190 analyzed.

191 In this connection, modern analysis strategies offer two complementary and related options:
192 fingerprinting and profiling. Fingerprinting generally involves untargeted methods: the sample
193 profile, a unique diagnostic parameter, is used to classify it within a set of samples, based on the
194 degree of similarity of their analytical patterns. Profiling involves targeted methods, in which a
195 sample is characterized and discriminated by the quantitative distribution of a number of known

196 target analytes, often descriptive of the sample's required characteristics. In this study, profiling
197 only involved quantitating the characterizing markers in terms of flavor [1,2].

198

199 **3.1 Sample discrimination by fingerprinting**

200 As said above, the fingerprinting approach entails defining a diagnostic profile, while analytes need
201 not be identified; samples are discriminated (evaluation of quality or origin) by processing the
202 analytical results with multivariate statistical analysis. The combination HS/GC-MS/multivariate
203 analysis is an established tool for aromatic plant classification [3, 20, 21, 22], whereas non-
204 separative methods (HS-SPME/MS/multivariate analysis) are little used, if at all [23]. In this study,
205 ten lots for cloves, American peppertree, black pepper, and white pepper, nine for rosemary and
206 thyme, and eight for sage were analyzed by both HS-SPME-GC-MS and HS-SPME-MS, under
207 rigorously standardized conditions: the resulting profiles were submitted to Principal Component
208 Analysis (PCA). PCA with conventional HS-SPME-GC-MS was run on the normalized area of all
209 peaks characterizing each spice/herb investigated (Table 1 SM). The list of volatile fraction
210 components of each spice/herb considered for PCA elaboration is reported in **Table 1SM**
211 (Supplementary Material). Figure 1 reports the HS-SPME GC-MS (1a) profile of a sage sample of
212 Italian origin (A4). **Figure 1SM** gives the HS-SPME-GC-MS patterns of the spices/herbs
213 investigated. Figure 2 reports the PCA scores of HS-SPME-GC-MS patterns of sage (2a) and thyme
214 (2c) samples.

215 The same plant samples from the same lots were then submitted to HS-SPME-MS analysis. **Figure**
216 **1** also reports TIC and MS pattern (1b and 1c) of the sage sample in Figure 1a, analyzed by HS-
217 SPME-MS. Again, the absolute intensity of all ions, diagnostic of the selected markers in the MS
218 profiles of each spice/herb, were considered for PCA (Table 1). **Figure 2** also gives the PCA plot of
219 HS-SPME-MS patterns of the same set of sage (2b) and thyme (2d) samples. The PCA results were
220 very similar with both separative and non-separative methods, and with both techniques
221 successfully classified the lots of each herb: the ten clove lots were divided into two groups (6 and 4

222 lots) corresponding to their geographical origins; American pepper, black pepper and white pepper
223 likewise produced a relatively uniform group, plus 2 or 3 outliers; rosemary lots were relatively
224 uniform, with only one outlier; sage lots were distributed across the statistical plane with one
225 outlier, as expected, because of their declared differing origins; lastly, thyme lots were in two main
226 groups, corresponding to the species' two well-known chemotypes (i.e. thymol and carvacrol).

227 *A series of non-equilibrium HS-SPME experiments at ever decreasing sampling times (20, 10, 5*
228 *minutes) was also run, to speed up discriminative control. The PCA results were fully comparable*
229 *to those described above (data not reported).*

230 Fingerprinting with non-separative methods, in combination with multivariate statistical analysis,
231 was found to give results that were fully comparable to those obtained with separative methods.
232 Both approaches can be equally useful to check homogeneity, and to classify lots and samples; the
233 presence of different chemotypes, as in the case of rosemary and thyme, can very quickly be
234 detected. The unquestioned advantage of non-separative methods is that analysis time is limited to
235 the time required for sample preparation, and is thus markedly reduced compared to that required
236 for separative methods.

237

238 **3.2 Sample characterization by marker quantitation**

239 The approach described gave useful indications concerning the homogeneity and classification of
240 the lots investigated, in agreement with the available information. In cases where the results can be
241 compared to reference results, i.e. if a reference data collection for each spice/herb is available, the
242 results might also provide information about the quality and economic value of the spices/herbs
243 investigated. To characterize a spice/herb fully, however, the volatile markers of sensory quality,
244 and/or taxonomy, and/or biological activity must be quantified directly on the plant material. The
245 volatile markers characterizing the investigated spices/herbs are known from the literature [1]; in
246 particular thymol and carvacrol for thyme; α -pinene, Δ -3-carene, α -phellandrene and limonene for
247 American peppertree; eugenol for clove; linalool, bornyl acetate, α -terpineol and borneol for

248 rosemary; α -phellandrene, limonene, α -humulene and β -caryophyllene for white pepper; Δ -3-
249 carene, limonene, α -humulene and β -caryophyllene for black pepper; and α - and β - thujone for
250 sage . Headspace sampling was used not only because it is quick and easily automated, but also
251 because it has been proved to provide quantitative results closer to the true content of plant markers
252 than any other technique (hydrodistillation, solvent extraction, etc.); this is because the reduced
253 number of sample treatments reduces losses or artifact formation. MHS-SPME was selected
254 because it is considered to be the most appropriate approach for volatile component quantitation in
255 solid matrices. Its theoretical foundations derive from the model developed by Kolb et al. for MHE-
256 static HS [7]. Both MHS-SPME and MHE are based on stepwise dynamic gas extraction of the
257 investigated analyte from a single sample: the analyte peak area decays exponentially with the
258 number of extractions, and the sum of the areas from each extraction corresponds to the amount
259 present initially in a given matrix. The total area of the analyte(s) under investigation for
260 quantitation is determined through equation 1:

261
262
263 (Eq. 1)
264

$$A_T = \sum_{i=1}^{\infty} A_i = \frac{A_1}{(1 - e^{-q})} = A_1 / (1 - Q)$$

265
266 where A_1 is the analyte area after the first extraction, A_T is the total analyte area; $Q: e^{-q}$, $-q$ is a
267 constant that can be calculated from the following linear regression analysis equation:

268
269 $\ln A_i = -q (i-1) + \ln A_1$ (Eq. 2)
270

271 where A_i is the peak area obtained from the i^{th} extraction. In everyday practice, extractions need not
272 be continued until all the analyte has been removed from the sample: a small number of extractions
273 (generally 3-5) are sufficient to obtain a reliable exponential equation describing analyte decay,
274 from which the total area of the analyte in the sample can be extrapolated. The extrapolated analyte
275 area can then be quantified by an external standard approach, by submitting mixtures of selected
276 markers at different concentrations to MHS-SPME.

277 MHS-SPME can also be carried out under non-equilibrium conditions [13], provided that sampling
278 parameters are rigorously standardized. The main advantage of this method is that several analytes
279 can be quantified simultaneously, without requiring the addition of internal standards and without
280 requiring recovery determination; this provides the analyte absolute total area in the investigated
281 sample, and is not affected by the matrix effect. The limitations of MHS-SPME under non-
282 equilibrium conditions are that i) correctly determined Q value(s) must be used and, ideally, ii) a Q
283 value for each sample should be measured. The second drawback can be overcome with sets of
284 homogeneous samples of the same matrix [14, 17] (see 3.2.1). Figure 3 shows the GC-MS extracted
285 ion chromatograms for eugenol ($m/z=164$) in a clove sample, corresponding to three consecutive
286 extractions (A), and its linear decay diagram (B).

287

288

289 **3.2.1. Determination of Q values**

290 In previous work [14, 17] the authors showed that, with samples possessing similar matrix effects
291 (e.g. ground roasted coffee, and roasted hazelnuts) the Q value for a given analyte tends to be
292 constant, thus making it possible to adopt an average Q to quantify an analyte in a single analysis.
293 In this study, the first step aimed to verify whether the average Q value can also be applied to
294 matrices that are less “standardized” than roasted coffee or roasted hazelnuts, and that are
295 characterized by relatively low homogeneity, be it due to their different origins, different growing
296 or storage conditions, or to the soft technological process to which they are submitted. In this study,

297 Q values for each spice/herb in terms of RSD% were very satisfactory, ranging from 2.6% for Δ -3-
298 carene in black pepper to 10.0% for β -thujone in sage. **Table 1** reports the average Q and its RSD%
299 for each selected marker, together with the decay correlation coefficients (r) (eq. 2), for all samples
300 of all spices/herbs investigated. The results show that the Q values for the markers of each of the six
301 spices/herbs investigated fell within a very narrow range; this means that an average Q value can be
302 adopted for routine marker quantitation also for herbs and spices (**Table 2 SM**); in particular,
303 RSD% values for markers belonging to different classes of secondary metabolites from different
304 plants were very satisfactory; in no case did they exceed 5% for the markers of thyme and
305 American peppertree, and 10% for those of rosemary, cloves, black and white pepper, and sage.
306 These results are especially significant because each of the samples came from a different
307 commercial lot.

308 The reliability of the Q value was also confirmed by the correlation coefficients for all markers: all
309 were above 0.9977 (i.e. limonene in black pepper), and several above 0.999 (Table 1). These results
310 confirm that the total area of the investigated markers can be determined from a single
311 sampling/extraction, provided that marker concentration is in the range across which the average Q
312 value has been calculated. As a general consideration, the possibility, in routine analyses, to
313 quantify several markers in the same run, while adopting the average Q value for each of them
314 within the same matrix, markedly reduces the total number of analyses and, as a consequence, the
315 analysis time. This is particularly true for solid matrices, and makes MHS-SPME highly
316 competitive with other approaches usually adopted (i.e. standard addition and Stable Isotope
317 Dilution Assay, SIDA).

318

319 **3.3. Quantitative analysis by separative method**

320 The selected markers were initially quantified by applying both sample-specific and average Q
321 values, in order to determine the manner in which they may be applied correctly to all samples of a
322 given plant species. **Table 2** reports the average concentrations (expressed as mg g⁻¹) of selected

323 thyme and American peppertree markers, calculated with both specific and average Q values. The
324 results show that the amount of a marker in a matrix, calculated by MHS-SPME with average Q, is
325 either identical or very close to the amount calculated applying the specific Q value. Similar results
326 were obtained for the other spices and herbs investigated. The possibility to quantify a marker with
327 a single peak area makes MHS-SPME a very rapid approach, suitable for application in routine
328 quality control [7]. **Table 2** also reports the average concentrations (expressed as mg g⁻¹) of the
329 selected markers of cloves, white pepper and black pepper, rosemary and sage, calculated with the
330 average Q values.

331 Moreover, as was pointed out by Kolb et al. [7], MHE can further be speeded-up, because the
332 investigated markers can be quantified via a single-point calibration; this avoids the need to create a
333 calibration curve, which of course can only be applied within the range of linearity across which the
334 analyte has to be quantified. The linearity of the recoveries was here demonstrated by submitting
335 standard mixtures of each marker to MHS-SPME, within the operative range of concentrations
336 across which they are almost always present in the plant material. The linear regression equations
337 and their correlation coefficients are in **Table 3SM**. The *r* values are all very high (all above 0.9987
338 for α -pinene in American peppertree), thus making the single-point calibration method applicable.
339 The accuracy of the reported results was confirmed by analyzing the same analytes quantitatively,
340 on two samples for each lot investigated, by the standard addition method. These results are in line
341 with those obtained with roasted coffee suspended in water [14] and with roasted hazelnuts as such
342 [17].

343

344 **3.4. Quantitative analysis by non-separative methods**

345 Whether or not non-separative methods may be applied depends on both the chemical composition
346 of the matrix under investigation, and the nature of its markers. Simple matrices containing markers
347 characterized by specific diagnostic m/z fragments are suitable for quantitative non-separative
348 analysis. Conversely, to quantify markers in matrices with volatile fractions having a complex

349 chemical composition, such as spices and aromatic plants, non-separative methods are more
350 complex than separative methods. Pepper and rosemary, for instance, contain several monoterpene
351 hydrocarbon isomers, all characterized by very similar fragmentation patterns (e.g. $m/z = 93$); this
352 impedes quantitation of one isomer, unless the contribution of each isomer to the total target ion
353 intensity is known, and a correction factor can be determined [14]. In the present study, three of the
354 spices/herbs investigated could be analyzed by non-separative methods, since they presented
355 sufficiently specific diagnostic ions to quantify their markers or pairs of them, i.e. eugenol (m/z
356 164) in clove samples, the sum of thymol and carvacrol in thyme (m/z 135) and the sum of α - and
357 β -thujones (m/z 110) in sage. **Table 3** reports the average concentrations (mg g^{-1}) of eugenol,
358 thujones, and thymol and carvacrol, in clove, sage and thyme, respectively, quantified by a non-
359 separative MHS-SPME-MS approach without applying any correction factor; the results are
360 compared to those obtained with separative MHS-SPME-GC-MS, and the relative standard
361 deviation (RSD%) between the two methods is given. The results are in general satisfactory since
362 RSD% of more than 60% of the samples is below 10%. In all cases, those above 10%, comprise the
363 sum of two analytes, and never exceed 18%. These examples are briefly discussed below, to
364 comment on the possibilities and limits of this approach.

365 The determination of eugenol in cloves was affected by the contribution made by eugenyl acetate to
366 its target ion intensity (see figure 3); a correction factor was therefore determined in the attempt to
367 improve between quantitative results of non-separative and separative methods. The percentage of
368 interference by eugenyl acetate in the intensity of the eugenol target ion was determined as follows:
369 the 10 samples of cloves were analyzed by the separative method, with MS in Selected Ion
370 Monitoring; the average contribution of eugenyl acetate to the total intensity of the eugenol target
371 ion at 164 m/z was 15.9%. Adoption of this correction factor, markedly improved agreement
372 between quantitative data, since the RSD% *versus* the separative method dropped to 7.7% , as
373 shown in **Table 3**.

374 The situation was different for thyme: the relative abundance of thymol and carvacrol varies in what
375 appears to be a random manner, depending on the analyzed chemotype and, within a single
376 chemotype, depending on origin (par. 3.2). Thymol and carvacrol are isomers with very similar
377 mass spectra. It is thus not possible to calculate the average contribution of one of them to the target
378 ion intensity, but only to quantify the sum of the two markers. In sage samples too, the contribution
379 of α - and β - thujone to the target ion cannot be distinguished, although no interference from other
380 compounds was observed. In this case, however, no correction factor was necessary; quantitative
381 discrimination between α - and β - thujone is not required under EU law, restrictions due to the
382 compounds' toxicity concerning the total amount and not each isomer.

383 These results also show that correct quantitation of the markers of a complex matrix with a non-
384 separative HS-SPME-MS method can successfully be guided by preliminary fingerprinting
385 analysis, which helps to define plant chemotype, quality, and origin as an indication of the quali-
386 quantitative chemical composition.

387

388 **3.3. Method repeatability, intermediate precision, LOD and LOQ**

389 The repeatability of the method was evaluated by analyzing all samples of the spices/herbs
390 investigated, three times on the same day, by MHS-SPME-GC-MS. Intermediate precision was
391 determined by submitting all samples to MHS-SPME-GC-MS every four weeks for a period of
392 three months. **Table 4SM** reports the relative standard deviations (RSD%) of the markers of the
393 volatile fraction of thyme and American pepper. Repeatability and intermediate precision were
394 highly satisfactory, RSD% never exceeding 11% and 15%, respectively, for the two species. The
395 results were similar for all other matrices.

396 Repeatability and intermediate precision with non-separative MHS-SPME-MS was determined on
397 the total area of the TIC profile, in the same way as for the separative method. In this case, too, the
398 results were highly satisfactory, RSD% never exceeding 13% and 18%, respectively.

399 In consideration of the very small amount of plant material processed (1-5 mg) both repeatability
400 and intermediate precision should be considered very satisfactory, in particular for the non
401 separative HS-SPME-MS method, in which data are obtained via the TIC profile.

402 LOD values ranged from 20 ppb (ng/g) for limonene to 800 ppb for carvacrol; LOQ values were
403 slightly higher, ranging from 60 ppb for phellandrene to 3 ppm ($\mu\text{g/g}$) for carvacrol.

404

405 **4. Conclusions**

406 The results of this study show that MHS-SPME, combined with either separative (GC-MS) or non-
407 separative (MS) techniques, is an effective Total Analysis System [24, 25] for the reliable quali-
408 quantitative characterization of spices and aromatic plants. Both separative and non-separative
409 methods, in a single step, enable the analyst a) to discriminate between qualities, origins, and
410 chemotypes, since they provide diagnostic sample fingerprinting for correct sample classification in
411 combination with PCA, and b) to quantify the aromatic markers characteristic of the plant's flavor
412 directly on the solid matrix, by MHS-SPME.

413 The results also enhance the reliability of MHE when used to quantify volatile markers directly in
414 solid matrices, by showing that an average Q value may be used to quantify one or more analytes
415 with one automatic extraction (experiment) for each sample. This is particularly significant when a
416 large number of samples of the same homogeneous matrix are to be analyzed. In addition, MHE is
417 also confirmed as a time-competitive approach for routine analysis compared to other HS
418 quantitation methods, again when the number of analyses is large, and the time necessary to
419 determine a significant average Q value is compensated by the higher analysis throughput. MHS-
420 SPME can also be successfully combined with non-separative methods (MHS-SPME-MS) to speed
421 up control analysis when one or more markers from solid matrices must be quantified, provided that
422 they present specific diagnostic ion(s) in the total MS fingerprint. Separative and non-separative
423 approaches are closely complementary; they can be carried out with the same instrumentation and

424 adopted impartially, since they produce fully comparable qualitative results and, where MHS-
425 SPME-MS is applicable, highly compatible quantitative results.

426 More in general, the consistency between separative and non-separative methods, combined with
427 the complementarity of the results on fingerprinting and marker quantitation, show that the
428 proposed MHS-SPME-GC-MS or MHS-SPME-MS method can be adopted as a routine strategy of
429 choice to characterize aromatic plants and spices, directly and as such, in a single analytical step.

430

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437

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507

508 **Captions to figures**

509 Figure 1 Sage sample of Italian origin (A4): (a) HS-SPME GC-MS profile, (b) HS-SPME-TIC-MS
510 pattern, and (c) MS pattern.

511 Peak identification: 1) α -pinene, 2) camphene, 3) β -pinene, 4) myrcene, 5) α -terpinene, 6) p-
512 cymene, 7) limonene, 8) 1,8-cineole, 9) γ -terpinene, 10) α -terpinolene, 11) α -thujone, 12) β -
513 thujone, 13) camphor, 14) borneol, 15) 4-terpineol, 16) β -bourbonene, 17) β -caryophyllene, 18)
514 aromadendrene, 19) α -humulene, 20) δ -cadinene, 21) caryophyllene oxide, 22) viridiflorol.

515

516 Figure 2 PCA scores of the HS-SPME-GC-MS patterns of the set of sage (2a) and thyme (2c)
517 samples and of HS-SPME-MS patterns of the same set of sage (2b) and thyme (2d) samples.

518

519 Figure 3 GC-MS extracted ion chromatograms of eugenol ($m/z=164$) in a clove sample from three
520 consecutive extractions (a) together with its linear decay diagram (b).

521

522 Figure 1SM HSSPME-GC-MS patterns of black pepper and white pepper (a and b), American
523 peppertree (c), rosemary (d), thyme (e) and cloves (f).

524

525 Table 1. List of the investigated matrices together with target ion (in bold) and qualifier ions of the
 526 selected markers. For each marker the average Q values with their RSD% and r coefficients are
 527 reported. Legend of acronyms. Thyme: *Thymus vulgaris* L.; Amer. Pep.: American peppertree,
 528 *Schinus molle* L.; Cloves: *Syzygium aromaticum* (L.) Merr. & Perry; Rosem.: rosemary,
 529 *Rosmarinus officinalis* L.; White pep. and Black pep.: pepper, *Piper nigrum* L.; Sage: *Salvia*
 530 *officinalis* L.

	<i>m/z</i> fragments		Thyme	Amer. pep.	Cloves	Rosem	White pep.	Black pep.	Sage
Thymol	135,150,91	Aver Q RSD% <i>r</i>	0.81 3.2 0.9997						
Carvacrol	135,150,91	Aver Q RSD% <i>r</i>	0.82 3.0 0.9997						
α-Pinene	93,79,136	Aver Q RSD% <i>r</i>		0.83 3.1 0.9992					
Δ-3-carene	93,91,136	Aver Q RSD% <i>r</i>		0.78 4.5 0.9998				0.89 2.6 0.9988	
α-Phellandrene	93,91,136	Aver Q RSD% <i>r</i>		0.80 3.6 0.9999			0.88 3.9 0.9983		
Limonene	68,93,136	Aver Q RSD% <i>r</i>		0.77 5.8 0.9999			0.74 9.5 0.9985	0.74 7.5 0.9977	
α-Humulene	93,121,204	Aver Q RSD% <i>r</i>					0.25 9.6 0.9990	0.41 9.9 0.9993	
Eugenol	164,149,77	Aver Q RSD% <i>r</i>			0.32 5.6 0.9989				
Linalool	71,121,136	Aver Q RSD% <i>r</i>				0.56 9.8 0.9993			
Bornyl acetate	95,136,154	Aver Q RSD% <i>r</i>				0.57 9.0 0.9980			
α-Terpineol	59,121,136	Aver Q RSD% <i>r</i>				0.63 8.1 0.9993			
Borneol	95,67,139	Aver Q RSD% <i>r</i>				0.75 5.8 0.9980			
β-Caryophyllene	93,133,204	Aver Q RSD% <i>r</i>					0.46 8.5 0.9980	0.45 8.4 0.9985	
α-Thujone	81,110,152	Aver Q RSD% <i>r</i>							0.71 9.7 0.9999
β-Thujone	81,110,152	Aver Q RSD% <i>r</i>							0.71 10.0 0.9999

531

532 Table 2. Average concentration (mg g⁻¹) of selected markers in spices and aromatic plants
 533 investigated. If not specified quantity is calculated with Av Q. (Sp: specific; Av: average)

Thyme (mg g ⁻¹)					American peppertree (mg g ⁻¹)								
#	Carv		Thy		#	α-Pin		Δ-3-Car		α-Phel		Lim	
	Sp Q	Av Q	Sp Q	Av Q		Sp Q	Av Q	Sp Q	Av Q	Sp Q	Av Q	Sp Q	Av Q
1	6.2	6.0	0.33	0.32	1	4.4	4.4	2.9	2.8	11.5	11.0	1.3	1.3
2	5.7	5.8	0.39	0.40	2	4.4	4.4	3.9	3.8	11.1	10.8	1.3	1.2
3	3.6	3.7	0.61	0.63	3	5.1	5.0	3.1	3.0	15.3	14.9	1.5	1.4
4	6.3	6.5	0.54	0.56	4	4.1	4.0	2.1	2.1	13.7	13.3	1.3	1.2
5	3.3	3.3	0.78	0.78	5	3.3	3.4	6.3	6.5	11.5	11.8	6.7	6.9
6	0.58	0.56	3.9	3.7	6	1.4	1.4	12.7	12.7	7.7	7.4	5.4	5.2
7	0.39	0.38	8.2	7.9	7	1.4	1.4	9.8	9.8	6.7	6.6	4.7	4.5
8	1.2	1.2	7.5	7.5	8	3.0	3.0	12.8	13.2	11.6	11.9	10.0	10.4
9	17	16	0.71	0.69	9	2.4	2.5	5.1	5.3	16.9	17.3	10.8	11.2
					10	3.8	3.8	4.2	4.3	13.8	14.1	5.3	5.5

Rosemary (mg g ⁻¹)					Sage (mg g ⁻¹)	
#	Lin	BorAc	α-Ter	Bor	#	Thuj
1	0.035	0.034	1.5	0.30	1	2.4
2	0.069	0.045	1.7	0.37	2	1.9
3	0.005	0.007	0.33	0.044	3	1.8
4	0.031	0.025	1.4	0.24	4	0.39
5	0.022	0.020	1.1	0.24	5	2.6
6	0.020	0.037	1.1	0.22	6	0.47
7	0.077	0.051	1.9	0.35	7	0.54
8	0.052	0.032	1.5	0.29	8	3.3
9	0.036	0.046	1.7	0.33		

White pepper (mg g ⁻¹)					Cloves (mg g ⁻¹)	
#	α-Phel	Car	Lim	α-Hum	#	Eug
1	0.023	4.6	0.11	0.40	1	149
2	0.019	5.3	0.10	0.49	2	142
3	0.034	4.3	0.11	0.44	3	313
4	0.12	4.8	0.16	0.42	4	347
5	0.063	5.8	0.26	0.49	5	240
6	0.36	2.7	0.77	0.23	6	150
7	0.16	0.94	0.26	0.08	7	261
8	0.059	10.4	0.22	0.81	8	283
9	0.025	6.0	0.13	0.48	9	162
10	0.011	3.5	0.09	0.28	10	108

Black pepper				
#	Δ-3-Car	Car	Lim	α-Hum
1	2.3	3.5	3.0	0.27
2	2.1	3.9	2.6	0.32
3	2.0	5.2	0.93	0.37
4	1.7	6.2	0.59	0.43
5	1.2	5.1	0.60	0.36
6	1.1	4.1	0.38	0.32
7	2.0	6.4	1.3	0.41
8	0.84	7.3	1.5	0.49
9	0.75	4.9	0.90	0.38
10	2.3	5.8	1.2	0.39

553

554 Table 3. Average concentration (mg g^{-1}) of eugenol, thujones and thymol and carvacrol in clove,
 555 sage and thyme respectively quantified with separative (MHS-SPME-GC-MS) and non-separative
 556 (MHS-SPME- MS) approaches, together with RSD% between the two methods. For cloves, RSD%
 557 is calculated for both non-separative and corrected non-separative methods *versus* separative
 558 method.

Cloves (eugenol)						Thyme (thymol + carvacrol)				Sage (thujones)			
#	Sep Meth (mg g^{-1})	Non-sep Method (mg g^{-1})	RSD %	Corr. Non-sep Method (mg g^{-1})	RSD %	#	Sep Meth (mg g^{-1})	Non-sep Method (mg g^{-1})	RSD %	#	Sep Meth (mg g^{-1})	Non-sep Method (mg g^{-1})	RSD %
1	149	180	13.5	155	2.8	1	6.5	5,6	10.8	1	2,4	2.5	3.8
2	142	153	5.5	132	5.2	2	6.1	5.9	2.2	2	1.9	1.5	16.8
3	313	353	8.6	305	1.8	3	4.2	5.1	13.5	3	1.8	1.7	4.7
4	347	391	8.4	337	2.1	4	6.8	8.8	17.7	4	0.4	0.42	13.0
5	240	290	13.3	250	2.9	5	4.1	5.2	17.1	5	2.7	2.4	15.8
6	150	194	14.8	167	7.6	6	4.5	4.9	6.3	6	0.5	0.49	3.1
7	261	337	18.0	291	7.7	7	8.6	9.4	6.4	7	0.5	0.47	10.1
8	283	313	7.2	270	3.3	8	8.7	10.8	15.2	8	3.3	3	7.4
9	162	180	7.3	155	3.1	9	17.7	23	18.4				
10	108	137	16.8	118	6.3								

559

560

561

562 Table 1SM. List of the identified components for each investigated spice together with experimental
 563 and tabulated linear retention indices (I^T) on a 5% phenyl polymethylsiloxane column.
 564

White pepper		
Compound	Exp. I^T	Tab. I^T
α -Pinene	939	939
β -Pinene	980	979
Myrcene	991	991
α -Phellandrene	1005	1003
Δ -3-Carene	1011	1012
Limonene	1031	1029
γ -Terpinene	1062	1060
α -Terpinolene	1088	1089
Linalool	1098	1097
<i>p</i> -Mentha-1,5-dien-8-ol	1166	1170
<i>p</i> -Cymen-8-ol	1183	1183
Linalyl propionate	1192	/
δ -Elemene	1339	1338
Eugenol	1356	1359
α -Copaene	1376	1377
β -Elemene	1391	1391
<i>t</i> - β -Caryophyllene	1418	1419
α -Humulene	1454	1455
δ -Cadinene	1524	1523
Caryophyllene oxide	1581	1583
Black pepper		
α -Thujene	931	930
α -Pinene	939	939
Sabinene	976	975
Myrcene	991	991
α -Phellandrene	1005	1003
Δ -3-Carene	1011	1012
Limonene	1031	1029
<i>t</i> - β -Ocimene	1050	1050
γ -Terpinene	1062	1060
<i>cis</i> -Sabinene hydrate	1068	1070
α -Terpinolene	1088	1089
Linalool	1098	1097
4-Terpineol	1162	1176
Linalyl propionate	1192	/
δ -Elemene	1339	1338
Eugenol	1356	1359
α -Copaene	1376	1377
β -Elemene	1391	1391
<i>t</i> - β -Caryophyllene	1418	1419
α -Humulene	1454	1455
<i>t</i> - β -Farnesene	1458	1457
β -Selinene	1485	1490

α -Selinene	1494	1498
β -Bisabolene	1509	1506
δ -Cadinene	1524	1523
Elemol	1549	1550
Nerolidol	1564	1563

American peppertree

α -Thujene	931	930
α -Pinene	939	939
Camphene	953	954
Sabinene	976	979
β -Pinene	980	981
Myrcene	991	991
α -Phellandrene	1005	1003
Δ -3-Carene	1011	1012
β -Phellandrene + limonene	1031	1030
<i>t</i> - β -Ocimene	1050	1050
γ -Terpinene	1062	1060
α -Terpinolene	1088	1089
Sabinol	1140	1143
δ -Elemene	1339	1338
Citronellyl acetate	1354	1353
Eugenol	1356	1359
α -Copaene	1376	1377
β -Elemene	1391	1391
<i>t</i> - β -Caryophyllene	1418	1419
Germacrene D	1480	1485
Bicyclogermacrene	1494	1500
α -Farnesene	1508	1506
δ -Cadinene	1524	1523
Elemol	1549	1550

Rosemary

α -Pinene	939	939
Camphene	953	954
β -Pinene	980	981
Myrcene	991	991
<i>p</i> -Cymene	1026	1025
1,8-Cineole	1033	1031
Linalool	1098	1097
Camphor	1143	1146
Borneol	1165	1169
4-Terpineol	1177	1176
Linalyl propionate	1192	/
Verbenone	1204	1205
Bornyl acetate	1285	1289
Eugenol	1356	1359
α -Copaene	1376	1377
<i>t</i> - β -Caryophyllene	1418	1419

α -Humulene	1454	1455
δ -Cadinene	1524	1523
Thyme		
Limonene	1031	1029
Linalool	1098	1097
Camphor	1143	1146
Borneol	1165	1169
4-Terpineol	1177	1176
Linalyl propionate	1192	/
<i>i</i> -Bornyl formate	1233	1239
Carvacrol methyl ether	1244	1245
Bornyl acetate	1285	1289
Thymol	1290	1290
Carvacrol	1298	1299
Eugenol	1356	1359
<i>t</i> - β -Caryophyllene	1418	1419
Caryophyllene oxide	1581	1583
Cloves		
Eugenol	1356	1359
α -Copaene	1376	1377
<i>t</i> - β -Caryophyllene	1418	1419
α -Humulene	1454	1455
Germacrene D	1480	1485
α -Farnesene	1508	1506
δ -Cadinene	1524	1523
Eugenyl acetate	1525	/
Caryophyllene oxide	1581	1583
Sage		
α -Pinene	939	939
Camphene	953	954
β -Pinene	980	979
Myrcene	991	991
α -Terpinene	1018	1017
<i>p</i> -Cymene	1026	1025
Limonene	1031	1029
1,8-Cineole	1033	1031
γ -Terpinene	1062	1060
α -Terpinolene	1088	1089
α -Thujone	1101	1102
β -Thujone	1113	1114
Camphor	1143	1146
Borneol	1165	1169
4-Terpineol	1177	1176
β -Bourbonene	1384	1388
<i>t</i> - β -Caryophyllene	1418	1419
Aromadendrene	1439	1441
α -Humulene	1454	1455
δ -Cadinene	1524	1523

565	Caryophyllene oxide	1581	1583
566	Viridiflorol	1590	1593
567			
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569			

570 Table 2SM. Q value range, average, standard deviation and RSD% for the selected markers of each
 571 spice. Legend to the abbreviations: **α-Pin**: α-pinene; **Δ-3-Car**: Δ-3-Carene; **α-Phel**: α-Phellandrene;
 572 **Lim**: Limonene; **Lin**: Linalool; **BorAc**: Bornyl acetate; **α-Ter**: α-Terpinene; **Bor**: Borneol; **Car**:
 573 caryophyllene; **α-Hum**: α-Humulene; **Carv**:Carvacrol; **Thy**: Thymol; **α-Thuj**: α-Thujone; **β-Thuj**:
 574 β-Thujone; **Eug**: Eugenol
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#	American peppertree - Q values			
	α-Pin	Δ-3-Car	α-Phel	Lim
1	0.85	0.79	0.82	0.859
2	0.80	0.72	0.75	0.800
3	0.80	0.72	0.76	0.811
4	0.81	0.75	0.78	0.822
5	0.86	0.80	0.82	0.833
6	0.83	0.80	0.80	0.844
7	0.82	0.78	0.78	0.855
8	0.83	0.80	0.80	0.866
9	0.86	0.81	0.84	0.877
10	0.87	0.81	0.83	0.888
Range	0.81-0.87	0.72-0.81	0.75-0.84	0.70-0.85
Average	0.83	0.78	0.80	0.827
Std dev	0.03	0.03	0.03	0.040
RSD%	3.1	4.5	3.6	5.8

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#	Rosemary - Q values			
	Lin	BorAc	α-Ter	Bor
1	0.55	0.57	0.66	0.596
2	0.50	0.53	0.61	0.597
3	0.61	0.68	0.57	0.598
4	0.52	0.55	0.59	0.599
5	0.57	0.60	0.69	0.600
6	0.65	0.56	0.67	0.601
7	0.48	0.51	0.62	0.602
8	0.60	0.62	0.67	0.603
9	0.57	0.55	0.62	0.604
Range	0.50-0.65	0.51-0.68	0.57-0.69	0.68-0.79
Average	0.56	0.57	0.63	0.626
Std dev	0.05	0.05	0.05	0.040
RSD%	9.8	9.0	8.1	5.8

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#	White pepper - Q values			
	α -Phel	Car	Lim	α -Hum
1	0.81	0.41	0.77	0.36
2	0.87	0.43	0.82	0.39
3	0.89	0.48	0.75	0.39
4	0.93	0.48	0.75	0.38
5	0.89	0.49	0.64	0.47
6	0.91	0.42	0.62	0.38
7	0.88	0.46	0.72	0.42
8	0.87	0.52	0.82	0.48
9	0.84	0.41	0.81	0.44
10	0.87	0.49	0.71	0.44
Range	0.81-0.93	0.41-0.52	0.62-0.82	0.36-0.48
Average	0.88	0.46	0.74	0.42
Std dev	0.03	0.04	0.07	0.04
RSD%	3.9	8.5	9.5	9.8

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#	Black pepper - Q values			
	Δ -3-Car	Car	Lim	α -Hum
1	0.85	0.43	0.78	0.38
2	0.87	0.45	0.80	0.39
3	0.92	0.42	0.73	0.37
4	0.92	0.42	0.71	0.37
5	0.89	0.49	0.69	0.46
6	0.92	0.40	0.62	0.38
7	0.90	0.45	0.75	0.43
8	0.89	0.52	0.79	0.44
9	0.89	0.49	0.76	0.44
10	0.89	0.47	0.73	0.42
Range	0.85-0.92	0.40-0.52	0.62-0.80	0.38-0.48
Average	0.89	0.45	0.74	0.41
Std dev	0.02	0.04	0.05	0.04
RSD%	2.6	8.4	7.5	9.8

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#	Thyme - Q values	
	Carv	Thy
1	0.80	0.78
2	0.85	0.83
3	0.81	0.80
4	0.81	0.80
5	0.82	0.81
6	0.83	0.84
7	0.84	0.81
8	0.82	0.81
9	0.77	0.75
Range	0.77-0.85	0.75-0.84
Average	0.82	0.81
Std dev	0.02	0.03
RSD%	3.0	3.2

#	Sage - Q values	
	α -Thuj	β -Thuj
1	0.65	0.64
2	0.76	0.77
3	0.70	0.70
4	0.60	0.59
5	0.78	0.76
6	0.67	0.69
7	0.74	0.75
8	0.80	0.80
Range	0.65-0.80	0.59-0.80
Average	0.71	0.71
Std dev	0.07	0.07
RSD%	9.7	10.0

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#	Q values - Cloves	
	Eug	670
1	0.30	671
2	0.29	672
3	0.31	673
4	0.32	674
5	0.30	675
6	0.33	676
7	0.34	677
8	0.34	678
9	0.34	679
10	0.34	680
Range	0.29-0.34	681
Average	0.32	682
Std dev	0.02	683
RSD%	5.6	684

685

686 Table 3SM. Linear regression equations and correlation coefficients obtained by submitting
 687 standard mixtures of each marker to MHS-SPME-GC-MS.
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Sample	Markers	Concentration range (mg mL ⁻¹)	Equation	r
Sage	α -thujone + β -thujone	0.1 – 5	y=11909x+7887994 y=14629x+383582	0.9998 0.9999
Thyme	thymol, carvacrol	0.25 – 10 0.25 – 20	y=8936x+5061613 y=6335x+4608817	0.9994 0.9991
Rosemary	linalool, borneol, bornyl acetate, α -terpineol	0.002 – 2 0.1 – 2 0.1 – 2 0.1 – 6	y=6944x+71523 y=17877x+1317504 y=8405x-164025 y=2591x-4377	0.9995 0.9996 0.9998 0.9988
American peppertree	α -pinene, Δ -3-carene, limonene, α -phellandrene	2 – 40 7 – 70 2 – 60 10 – 90	y=9368x+9875445 y=6676x+18933679 y=5540x+7798181 y=8092x+7369394	0.9987 0.9996 0.9996 0.9990
Cloves	eugenol	2 – 60	y=6947x+13637306	0.9994
White pepper and black pepper	Δ -3-carene, limonene, <i>t</i> - β -caryophyllene, α -humulene	0.5 – 10 0.5 – 10 1 – 20 0.25 - 70	y=8777x+584746 y=7061x+15247 y=4615x-240498 y=15813x-273280	0.9987 0.9988 0.9988 0.9993

712

713 Table 4SM. Repeatability and intermediate precision expressed as relative standard deviation
 714 (RSD%) of the selected markers for thyme and American peppertree analyzed by MHS-SPME-GC-
 715 MS. Legend to the abbreviations: **α -Pin**: α -pinene; **Δ -3-Car**: Δ -3-Carene; **α -Phel**: α -Phellandrene;
 716 **Lim**: Limonene; **Carv**: Carvacrol; **Thy**: Thymol;

#	Thyme - Repeatability	
	Carv	Thy
1	11.6	9.7
2	5.8	6.4
3	9.4	10.6
4	10.5	11.0
5	10.8	10.9
6	5.8	5.8
7	12.8	7.6
8	3.9	9.5
9	0.6	11.0

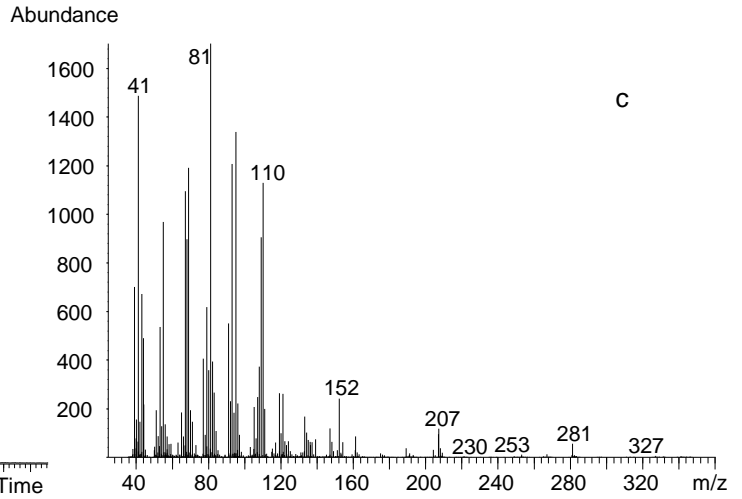
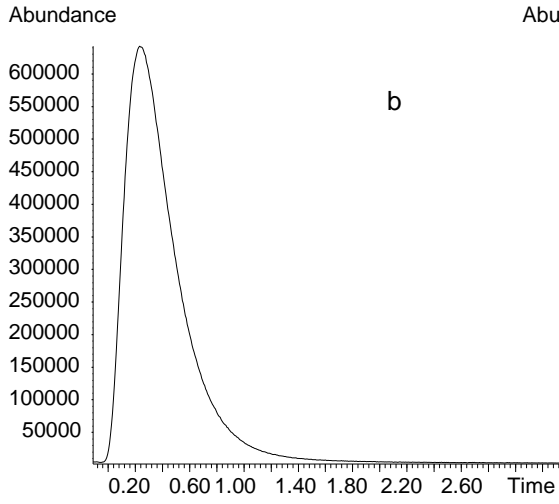
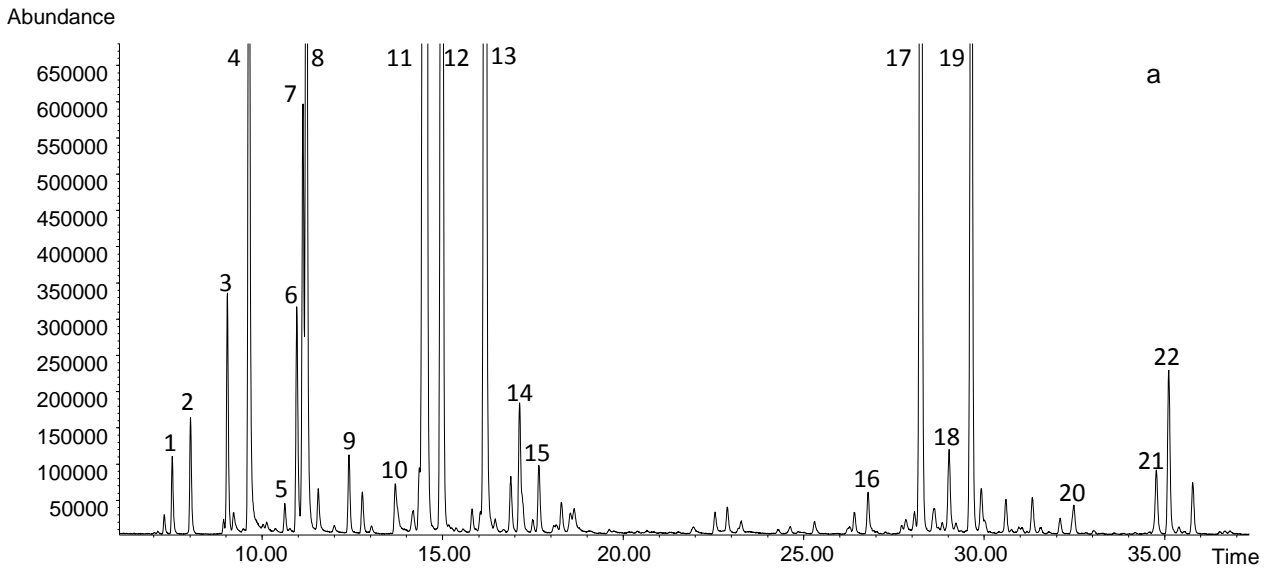
#	American peppertree - Repeatability			
	α -Pin	Δ -3-Car	α -Phel	Lim
1	6.4	0.7	0.9	0.4
2	11.0	8.6	7.1	6.7
3	0.6	2.1	3.8	3.5
4	6.9	1.9	2.4	3.4
5	12.1	9.0	7.0	5.2
6	10.0	9.4	8.3	7.4
7	7.7	2.0	3.1	7.3
8	9.4	10.5	10.2	10.2
9	9.5	11.9	9.4	8.4
10	0.7	10.2	0.5	9.1

#	Thyme - Interm. precision	
	Carv	Thy
1	12.6	11.7
2	8.8	6.9
3	9.8	11.8
4	11.5	12.1
5	12.3	14.5
6	6.9	7.2
7	13.5	9.3
8	6.7	11.2
9	5.3	13.2

#	American peppertree - Interm. precision			
	α -Pin	Δ -3-Car	α -Phel	Lim
1	7.9	5.7	5.9	5.4
2	12.8	9.9	8.7	8.9
3	6.2	6.1	6.8	6.8
4	8.4	8.9	8.5	7.9
5	14.3	10.0	9.2	7.5
6	12.5	10.7	10.3	9.4
7	9.8	6.8	6.9	10.4
8	10.6	11.5	11.2	12.2
9	11.5	13.9	12.4	10.4
10	6.8	12.6	8.1	12.4

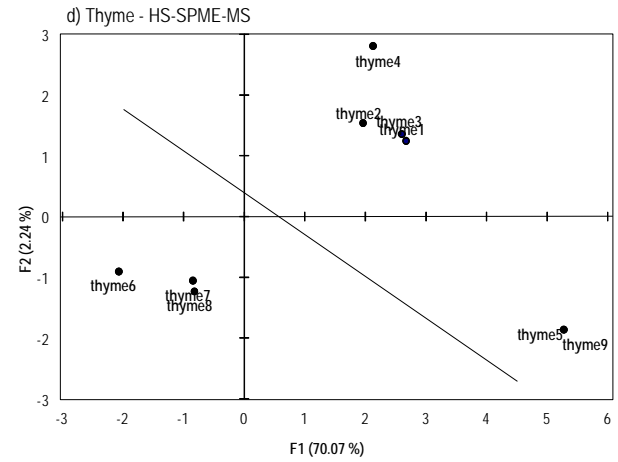
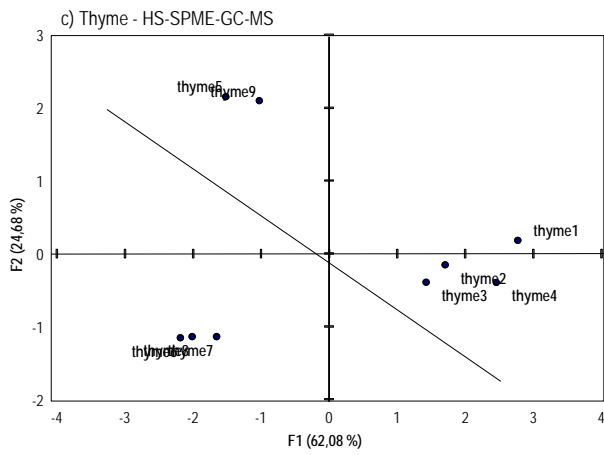
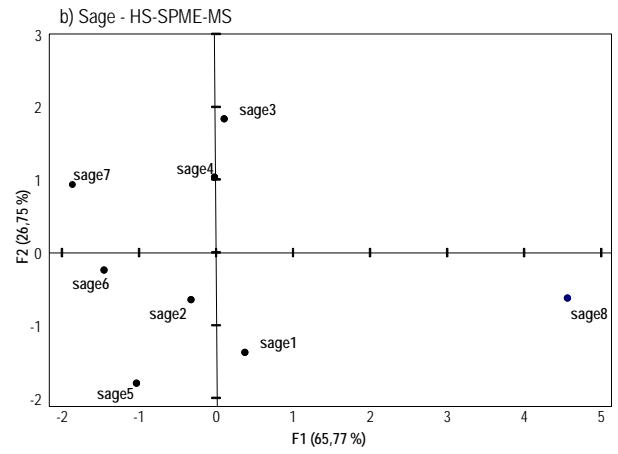
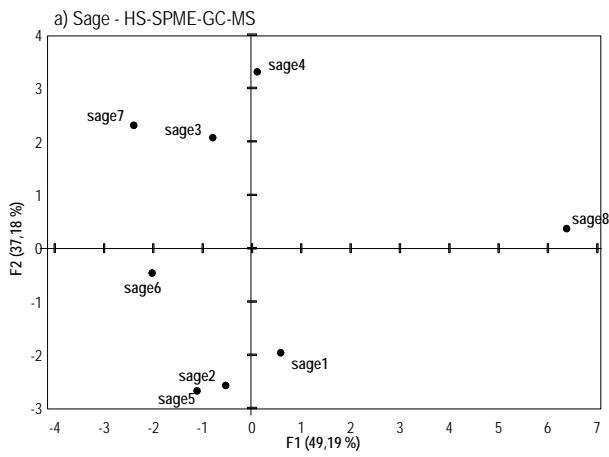
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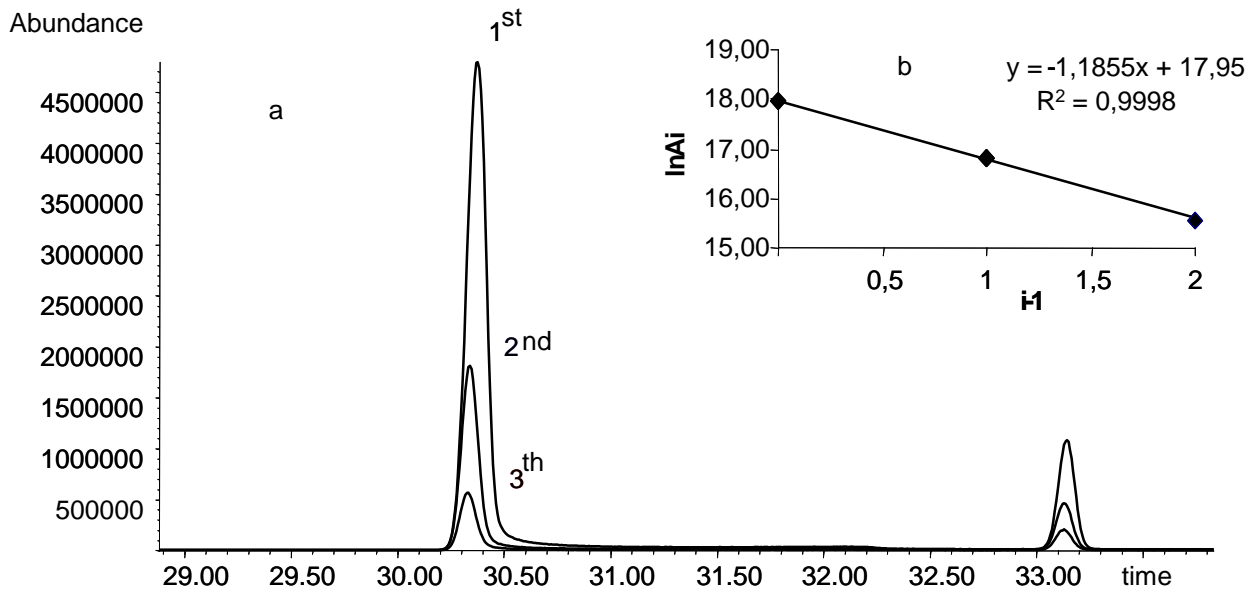
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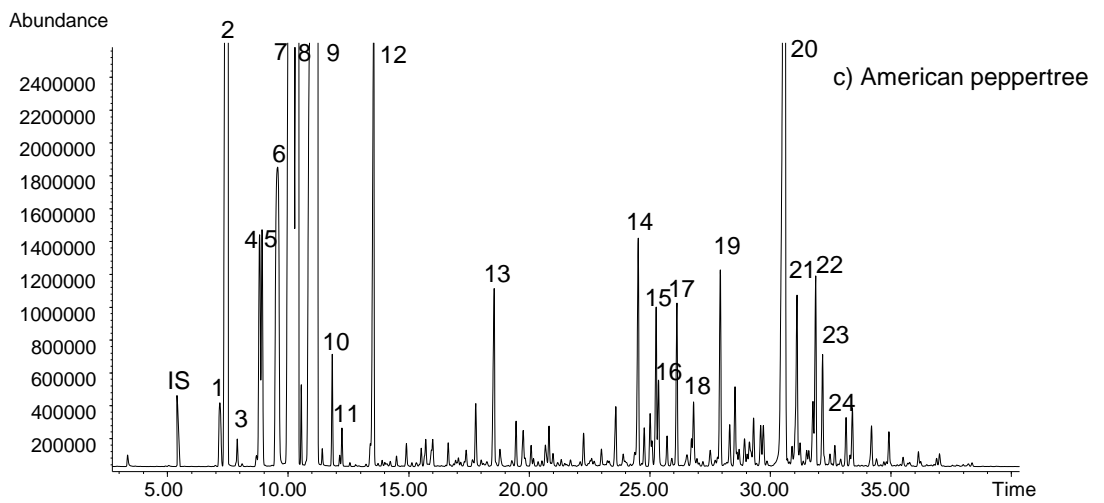
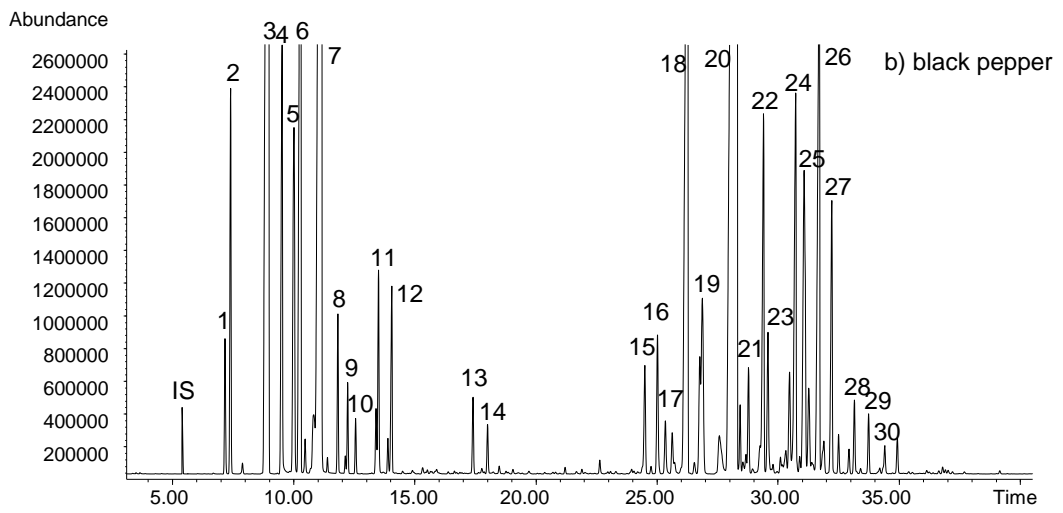
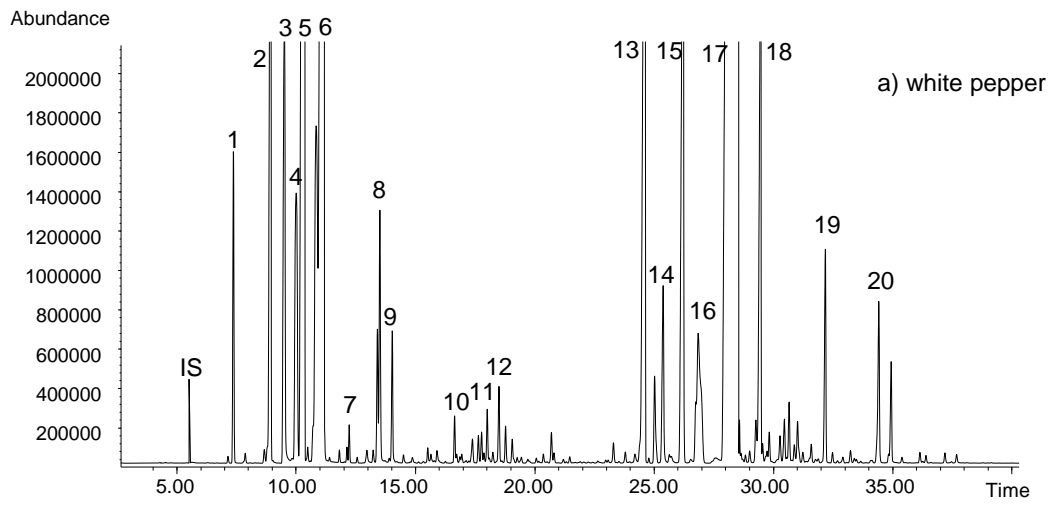
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Figure 1SM



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