IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Herbs and spices: Characterization and quantitation ofbiologically-active markers for routine quality control by multipleheadspace solid-phase microextraction combined with separative or non-separative analysis

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/152493 since 2016-11-21T21:49:16Z

Published version:

DOI:10.1016/j.chroma.2014.12.007

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

06 December 2024

UNIVERSITÀ DEGLI STUDI DI TORINO

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in *[Journal of Chromatography A Volume 2015 Jan 9;1376:9-17. doi: 10.1016/j.chroma.2014.12.007.*].

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

(1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.

(2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.

(3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en), [*http://dx.doi.org/10.1016/j.chroma.2014.12.007*]

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

Barbara Sgorbini, Carlo Bicchi*, Cecilia Cagliero, Chiara Cordero, Erica Liberto, Patrizia Rubiolo

Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, via P. Giuria 9, I-10125 Torino, Italy

*Corresponding author:

Prof. Dr. Carlo Bicchi

e-mail: carlo.bicchi@unito.it

Tel. +39 011 6707662

Fax +39 011 6707857

Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

2. Experimental

2.1. Materials and Reagents

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

2.2. SPME fibers

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

2.3. Sample preparation

2.3.1. Sampling conditions

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

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

Fingerprints were normalized by in-fiber external standardization: 1 μL of a 1000 μg mL^{-1} solution of nonane in dibutylphtalate was sampled for 20 minutes at 60°C [**17**].

2.3.2. Analysis conditions

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

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

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

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

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

2.4. Quantitation

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

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

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

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

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

2.6 Data processing

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

3. Results and discussion

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

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

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

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

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

3.2 Sample characterization by marker quantitation

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

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

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

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

$$\ln A_i = -q\,(i-1) + \ln A_1 \qquad \text{(Eq. 2)}$$

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

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

3.2.1. Determination of Q values

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

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

Moreover, as was pointed out by Kolb et al. [**7**], MHE can further be speeded-up, because the investigated markers can be quantified via a single-point calibration; this avoids the need to create a calibration curve, which of course can only be applied within the range of linearity across which the analyte has to be quantified. The linearity of the recoveries was here demonstrated by submitting standard mixtures of each marker to MHS-SPME, within the operative range of concentrations across which they are almost always present in the plant material. The linear regression equations and their correlation coefficients are in **Table 3SM**. The *r* values are all very high (all above 0.9987 for α-pinene in American peppertree), thus making the single-point calibration method applicable.

The accuracy of the reported results was confirmed by analyzing the same analytes quantitatively, on two samples for each lot investigated, by the standard addition method. These results are in line with those obtained with roasted coffee suspended in water [**14**] and with roasted hazelnuts as such [**17**].

3.4. Quantitative analysis by non-separative methods

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

chemical composition, such as spices and aromatic plants, non-separative methods are more complex than separative methods. Pepper and rosemary, for instance, contain several monoterpene hydrocarbon isomers, all characterized by very similar fragmentation patterns (e.g. m/z = 93); this impedes quantitation of one isomer, unless the contribution of each isomer to the total target ion intensity is known, and a correction factor can be determined [**14**]. In the present study, three of the spices/herbs investigated could be analyzed by non-separative methods, since they presented sufficiently specific diagnostic ions to quantify their markers or pairs of them, i.e. eugenol (m/z 164) in clove samples, the sum of thymol and carvacrol in thyme (m/z 135) and the sum of α- and β-thujones (m/z 110) in sage. **Table 3** reports the average concentrations (mg g^{-1}) of eugenol, thujones, and thymol and carvacrol, in clove, sage and thyme, respectively, quantified by a non-separative MHS-SPME-MS approach without applying any correction factor; the results are compared to those obtained with separative MHS-SPME-GC-MS, and the relative standard deviation (RSD%) between the two methods is given. The results are in general satisfactory since RSD% of more than 60% of the samples is below 10%. In all cases, those above 10%, comprise the sum of two analytes, and never exceed18%. These examples are briefly discussed below, to comment on the possibilities and limits of this approach.

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

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

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

3.3. Method repeatability, intermediate precision, LOD and LOQ

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

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

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

LOD values ranged from 20 ppb (ng/g) for limonene to 800 ppb for carvacrol; LOQ values were slightly higher, ranging from 60 ppb for phellandrene to 3 ppm (μg/g) for carvacrol.

4. Conclusions

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

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

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

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

Acknowledgments

This study was carried out within the project "*Studio di metaboliti secondari biologicamente attivi da matrici di origine vegetale*" financially supported by Ricerca Locale (Ex60%2013) of Turin University, Turin (Italy).

The authors are indebted to Cannamela-Bonomelli SpA (Zola Predosa (BO), Italy)) for supplying controlled lots of herbs and spices.

References

[1] J. Bruneton, Pharmacognosie, Phytochimie, plantes medicinales 4thed. 2009 Lavoisier ed.

[2] H.J.D. Dorman, S.G. Deans, Antimicrobial agents from plants: antibacterial activity of plant volatile oils, J. Appl. Microb. 88 (2000) 308-316.

[3] B Sgorbini, M. Sganzerla, C. Cagliero, L. Boggia, C. Bicchi, P. Rubiolo, Exhaustive evaluation of volatiles in plants: two case studies menthol in peppermint (*Mentha x piperita* L.) and eugenol in clove (*Syzygium aromaticum* (L.) Merrill & Perry), submitted to Phytochemistry.

[4] C Bicchi, C Cordero, E Liberto, B Sgorbini, and P Rubiolo, Headspace Sampling in Flavor and Fragrance Field, in: J. Pawliszyn,L. Mondello,P. Dugo,(Eds), Comprehensive Sampling and Sample Preparation, Volume 4; Elsevier, Academic Press: Oxford, UK, 2012, pp 1–25.

[5] M. Suzuki, S. Tsuge, and T. Takeuki, Gas chromatographic estimation of occluded solvents in adhesive tape by periodic introduction method, Anal Chem 72 (1970) 1705.

450 [6] C. Mc Aucliffe, GC determination of solutes by multiple phase equilibration, Chem. Technol. 1
451 (1971) 46.
452 [7] B. Kolb, L.S. Ettre, “Static Headspace-Gas Chromatography, Theory and Practice”, Wiley –
453 VCH, New York, 1997.
454 [8] O. Ezquerro, B. Pons, M. T. Tena, Multiple headspace solid-phase microextraction for the
455 quantitative determination of volatile organic compounds in multilayer packagings, J. Chromatogr.
456 A 999 (2003) 155-164.
457 [9] J.D. Carrillo, M.T. Tena, Determination of volatile compounds in antioxidant rosemary extracts
458 by multiple headspace solid-phase microextraction and gas chromatography, Flav. Fragr. J. 21
459 (2006) 626-633.
460 [10] M. Flores, D. Hernandez, Optimization of multiple headspace solid-phase microextraction for
461 the quantification of volatile compounds in dry fermented sausages ,J. Agric. Food Chem. 55 (2007)
462 8688-8695.
463 [11] C. Pizarro, N. Perez-del-Notaro, J. M. Gonzàles-Sàiz, Multiple headspace solid-phase
464 microextraction for eliminating matrix effect in the simultaneous determination of haloanisoles and
465 volatile phenols in wines ,J. Chromatogr. A 1165 (2007) 1-8.
466 [12] E. Serrano, J. Beltràn, F. Hernandez, Application of multiple headspace-solid-phase
467 microextraction followed by gas chromatography-mass spectrometry to quantitative analysis of
468 tomato aroma components, J. Chromatogr. A 1216 (2009) 127-133.
469 [13] O. Ezquerro, G. Ortiz, B. Pons, M. T. Tena, Determination of benzene, toluene, ethylbenzene
470 and xylenes in soils by multiple headspace solid-phase microextraction, J. Chromatogr. A 1035
471 (2004) 17-22.
472 [14] C. Bicchi, M.R. Ruosi, C. Cagliero, C. Cordero, E. Liberto, P. Rubiolo, B. Sgorbini,
473 Quantitative analysis of volatiles from solid matrices of vegetable origin by high concentration
474 capacity headspace techniques: Determination of furan in roasted coffee, J. Chromatogr. A 1218
475 (2011) 753-762.

476 [15] R. Costa, L. Tedone, S. De Grazia, P. Dugo, L. Mondello, Multiple headspace-solid-phase
477 microextraction: An application to quantification of mushroom volatiles, Anal. Chim. Acta 770
478 (2013) 1–6.
479 [16] I. San Román, M.L. Alonso, L. Bartolomé, R.M. Alonso, R. Fañanás, Analytical strategies
480 based on multiple headspace extraction for the quantitative analysis of aroma components in
481 mushrooms, Talanta 123 (2014) 207–217.
482 [17] L. Nicolotti, C. Cordero, C. Bicchi, P. Rubiolo, B. Sgorbini, E. Liberto. Volatile profiling of
483 high quality hazelnuts (*Corylus avellana* L.): chemical indices of roasting, Food Chem. 138 (2013)
484 1723-1733.
485 [18] Y. Wang, J. O'Reilly, Y. Chen, J. Pawliszyn, Equilibrium in-fibre standardisation technique
486 for solid-phase microextraction, J. Chromatogr. A 1072 (2005) 13-17.
487 [19] C. Bicchi, C. Cordero, E. Liberto, B. Sgorbini, P. Rubiolo, Reliability of fibres in Solid Phase
488 Microextraction for routine analysis of the headspace of aromatic and medicinal plants, J.
489 Chromatogr. A, 1152 (2007) 138-149.
490 [20] P. Rubiolo, B. Sgorbini, E. Liberto, C. Cordero, C. Bicchi, Analysis of the plant volatile
491 fraction, in A. Herrmann (Ed), The Chemistry and Biology of Volatiles, 2010, Chapter 3, 50-93.
492 [21] B Sgorbini, C Bicchi, C. Cagliero, C Cordero, E Liberto, P. Rubiolo, Headspace sampling and
493 gaschromatography: A successful combination to study the composition of a plant volatile fraction,
494 in: K. Hostettmann, H. Stuppner, A. Marston, S. Chen (Eds), Handbook of Chemical and Biological
495 Plant Analytical Methods, , First Edition, 2014, Chapter 10.
496 [22] C. Cagliero, B Sgorbini, C Cordero, E Liberto, C Bicchi, P. Rubiolo, Analytical strategies for
497 multipurpose studies of a plant volatile fraction, in: K. Hostettmann, H. Stuppner, A. Marston, S.
498 Chen (Eds), Handbook of Chemical and Biological Plant Analytical Methods, First Edition, 2014,
499 Chapter 20.

500 [23] E. Liberto, M.R. Ruosi, C. Cordero, P. Rubiolo, C. Bicchi, B. Sgorbini, Non-separative
501 headspace solid phase microextraction-mass spectrometry profile as a marker to monitor coffee
502 roasting degree, J. Agric. Food Chemistry 61 (2003) 1652-1660.
503 [24] P.S. Dittrich, K. Tachikawa, A. Manz, Micro total analysis systems. Latest advancements and
504 trends, Anal. Chem. 78 (2006) 3887-3907.
505 [25] A. Manz, N. Graber, H.M. Widmer, Miniaturized Total Chemical-Analysis Systems - a Novel
506 Concept for Chemical Sensing, Sensors Actuat. B-Chem. 1 (1990) 244-248.
507

Captions to figures

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

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

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

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

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

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

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

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

	Thyme (mg g^{-1})			
	Carv		Thy	
#	*Sp Q*	*Av Q*	*Sp Q*	*Av Q*
1	6.2	6.0	0.33	0.32
2	5.7	5.8	0.39	0.40
3	3.6	3.7	0.61	0.63
4	6.3	6.5	0.54	0.56
5	3.3	3.3	0.78	0.78
6	0.58	0.56	3.9	3.7
7	0.39	0.38	8.2	7.9
8	1.2	1.2	7.5	7.5
9	17	16	0.71	0.69

	American peppertree (mg g^{-1})							
	α-Pin		Δ-3-Car		α-Phel		Lim	
#	*Sp Q*	*Av Q*	*Sp Q*	*Av Q*	*Sp Q*	*Av Q*	*Sp Q*	*Av Q*
1	4.4	4.4	2.9	2.8	11.5	11.0	1.3	1.3
2	4.4	4.4	3.9	3.8	11.1	10.8	1.3	1.2
3	5.1	5.0	3.1	3.0	15.3	14.9	1.5	1.4
4	4.1	4.0	2.1	2.1	13.7	13.3	1.3	1.2
5	3.3	3.4	6.3	6.5	11.5	11.8	6.7	6.9
6	1.4	1.4	12.7	12.7	7.7	7.4	5.4	5.2
7	1.4	1.4	9.8	9.8	6.7	6.6	4.7	4.5
8	3.0	3.0	12.8	13.2	11.6	11.9	10.0	10.4
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})			
#	Lin	BorAc	α-Ter	Bor
1	0.035	0.034	1.5	0.30
2	0.069	0.045	1.7	0.37
3	0.005	0.007	0.33	0.044
4	0.031	0.025	1.4	0.24
5	0.022	0.020	1.1	0.24
6	0.020	0.037	1.1	0.22
7	0.077	0.051	1.9	0.35
8	0.052	0.032	1.5	0.29
9	0.036	0.046	1.7	0.33

	Sage (mg g^{-1})
#	Thuj
1	2.4
2	1.9
3	1.8
4	0.39
5	2.6
6	0.47
7	0.54
8	3.3

	White pepper (mg g^{-1})			
#	α-Phel	Car	Lim	α-Hum
1	0.023	4.6	0.11	0.40
2	0.019	5.3	0.10	0.49
3	0.034	4.3	0.11	0.44
4	0.12	4.8	0.16	0.42
5	0.063	5.8	0.26	0.49
6	0.36	2.7	0.77	0.23
7	0.16	0.94	0.26	0.08
8	0.059	10.4	0.22	0.81
9	0.025	6.0	0.13	0.48
10	0.011	3.5	0.09	0.28

	Cloves (mg g^{-1})
#	Eug
1	149
2	142
3	313
4	347
5	240
6	150
7	261
8	283
9	162
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

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

Cloves (eugenol)					
#	Sep Meth (mg g^{-1})	Non-sep Method (mg g^{-1})	RSD %	Corr. Non-sep Method (mg g^{-1})	RSD %
1	149	180	13.5	155	2.8
2	142	153	5.5	132	5.2
3	313	353	8.6	305	1.8
4	347	391	8.4	337	2.1
5	240	290	13.3	250	2.9
6	150	194	14.8	167	7.6
7	261	337	18.0	291	7.7
8	283	313	7.2	270	3.3
9	162	180	7.3	155	3.1
10	108	137	16.8	118	6.3

Thyme (thymol + carvacrol)			
#	Sep Meth (mg g^{-1})	Non-sep Method (mg g^{-1})	RSD %
1	6.5	5,6	10.8
2	6.1	5.9	2.2
3	4.2	5.1	13.5
4	6.8	8.8	17.7
5	4.1	5.2	17.1
6	4.5	4.9	6.3
7	8.6	9.4	6.4
8	8.7	10.8	15.2
9	17.7	23	18.4

Sage (thujones)			
#	Sep Meth (mg g^{-1})	Non-sep Method (mg g^{-1})	RSD %
1	2,4	2.5	3.8
2	1.9	1.5	16.8
3	1.8	1.7	4.7
4	0.4	0.42	13.0
5	2.7	2.4	15.8
6	0.5	0.49	3.1
7	0.5	0.47	10.1
8	3.3	3	7.4

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
p-Mentha-1,5-dien-8-ol	1166	1170
p-Cymen-8-ol	1183	1183
Linalyl propionate	1192	/
δ-Elemene	1339	1338
Eugenol	1356	1359
α-Copaene	1376	1377
β-Elemene	1391	1391
t-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
t-β-Ocimene	1050	1050
γ-Terpinene	1062	1060
cis-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
t-β-Caryophyllene	1418	1419
α-Humulene	1454	1455
t-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
t-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
t-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
p-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
t-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-Bornyl formate	1233	1239
Carvacrol methyl ether	1244	1245
Bornyl acetate	1285	1289
Thymol	1290	1290
Carvacrol	1298	1299
Eugenol	1356	1359
t-β-Caryophyllene	1418	1419
Caryophyllene oxide	1581	1583
Cloves		
Eugenol	1356	1359
α-Copaene	1376	1377
t-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
p-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
t-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			
568			
569			

Table 2SM. *Q* value range, average, standard deviation and RSD% for the selected markers of each spice. Legend to the abbreviations: **α-Pin**: α-pinene; **Δ-3-Car**: Δ-3-Carene; **α-Phel**: α-Phellandrene; **Lim**: Limonene; **Lin**: Linalool; **BorAc**: Bornyl acetate; **α-Ter**: α-Terpinene; **Bor**: Borneol; **Car**: caryophyllene; **α-Hum:** α-Humulene; **Carv**:Carvacrol; **Thy:** Thymol; **α-Thuj:** α-Thujone; **β-Thuj:** β-Thujone; **Eug**: Eugenol

#	American peppertree - Q values			
	α-Pin	Δ-3-Car	α-Phel	Lin
1	0.85	0.79	0.82	0.85
2	0.80	0.72	0.75	0.78
3	0.80	0.72	0.76	0.78
4	0.81	0.75	0.78	0.74
5	0.86	0.80	0.82	0.79
6	0.83	0.80	0.80	0.77
7	0.82	0.78	0.78	0.76
8	0.83	0.80	0.80	0.79
9	0.86	0.81	0.84	0.81
10	0.87	0.81	0.83	0.80
Range	0.81-0.87	0.72-0.81	0.75-0.84	0.70-0.85
Average	0.83	0.78	0.80	0.77
Std dev	0.03	0.03	0.03	0.04
RSD%	3.1	4.5	3.6	5.8

#	Rosemary - Q values			
	Lin	BorAc	α-Ter	Bor
1	0.55	0.57	0.66	0.73
2	0.50	0.53	0.61	0.69
3	0.61	0.68	0.57	0.69
4	0.52	0.55	0.59	0.68
5	0.57	0.60	0.69	0.78
6	0.65	0.56	0.67	0.79
7	0.48	0.51	0.62	0.74
8	0.60	0.62	0.67	0.78
9	0.57	0.55	0.62	0.75
Range	0.50-0.65	0.51-0.68	0.57-0.69	0.68-0.79
Average	0.56	0.57	0.63	0.75
Std dev	0.05	0.05	0.05	0.04
RSD%	9.8	9.0	8.1	5.8

#	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	0.41
Range	0.81-0.93	0.41-0.52	0.62-0.82	0.36-0.48
Average	0.88	0.46	0.74	0.41
Std dev	0.03	0.04	0.07	0.04
RSD%	3.9	8.5	9.5	9.8

#	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	0.42
8	0.89	0.52	0.79	0.48
9	0.89	0.49	0.76	0.45
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

#	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

#	Q values - Cloves
	Eug
1	0.30
2	0.29
3	0.31
4	0.32
5	0.30
6	0.33
7	0.34
8	0.34
9	0.34
10	0.34
Range	0.29-0.34
Average	0.32
Std dev	0.02
RSD%	5.6

Table 3SM. Linear regression equations and correlation coefficients obtained by submitting standard mixtures of each marker to MHS-SPME-GC-MS.

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, *t*-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

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

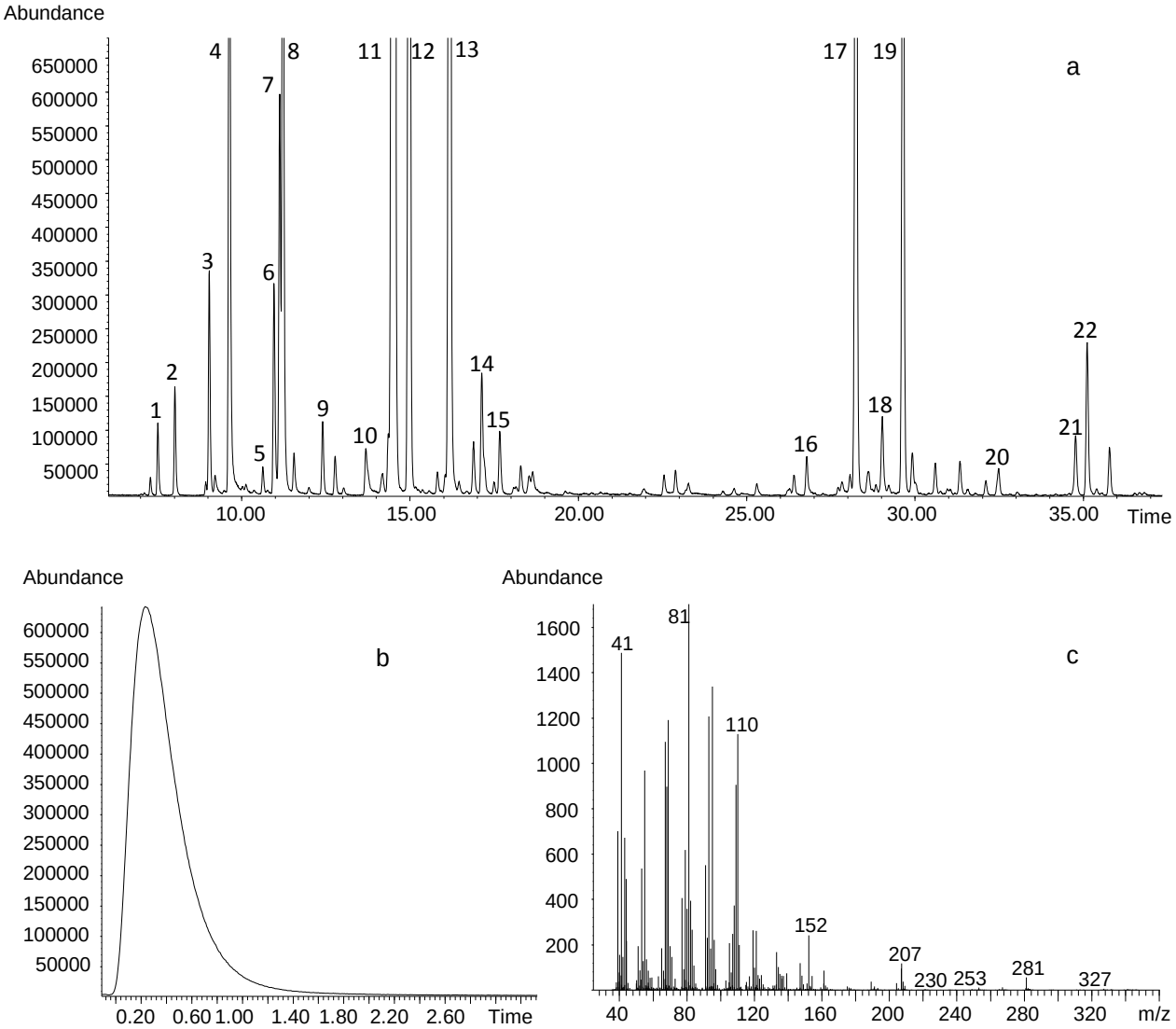
	Thyme - Repeatability	
#	Carv	Thy
1	11.6	9.7
2	5.8	6.4
3	9.4	10.6
4	10.5	11.0
5	10.8	10.9
6	5.8	5.8
7	12.8	7.6
8	3.9	9.5
9	0.6	11.0

	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

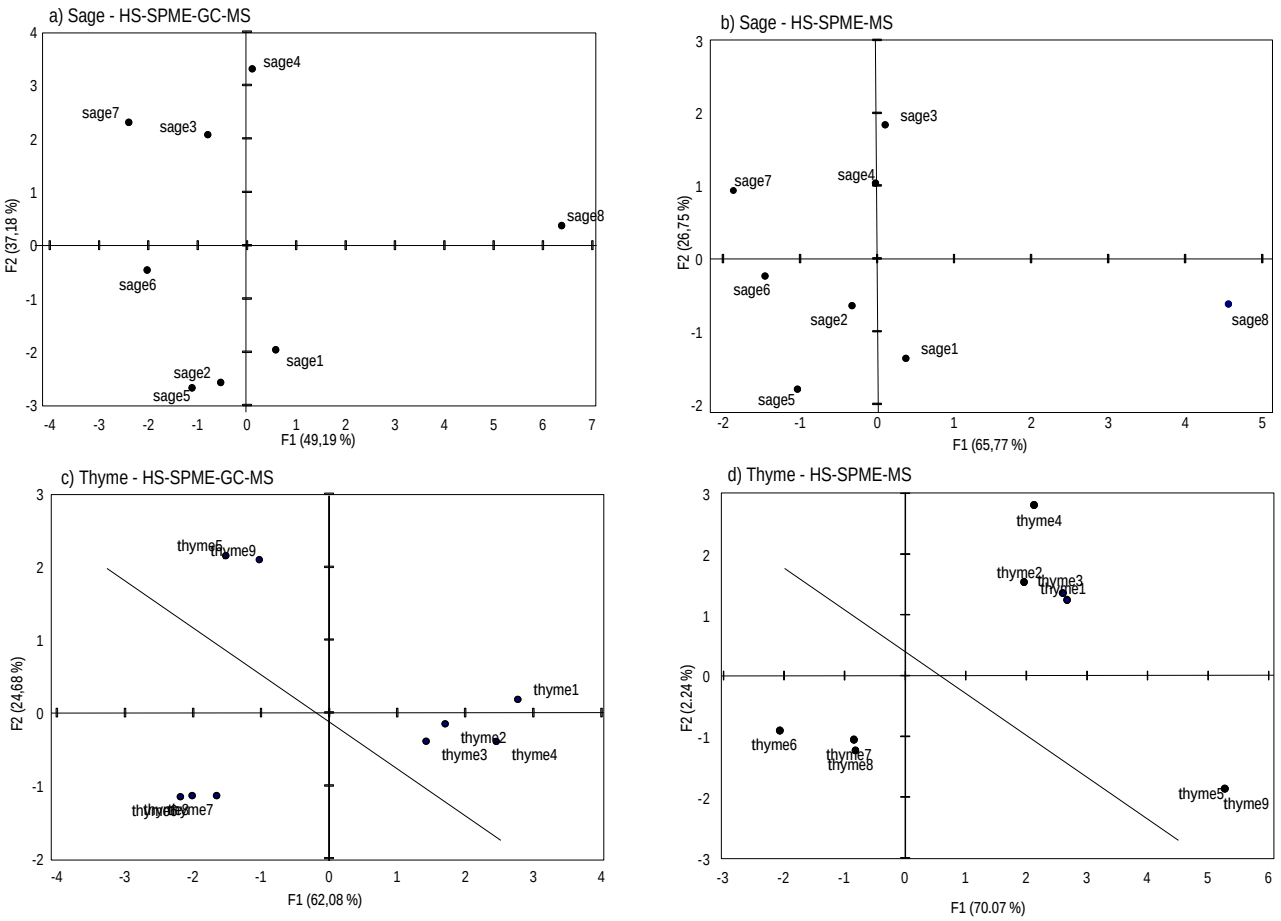
	American peppertree – Interm. precision			
#	α-Pin	Δ-3-Car	α-Phel	Lim
1	7.9	5.7	5.9	5.4
2	12.8	9.9	8.7	8.9
3	6.2	6.1	6.8	6.8
4	8.4	8.9	8.5	7.9
5	14.3	10.0	9.2	7.5
6	12.5	10.7	10.3	9.4
7	9.8	6.8	6.9	10.4
8	10.6	11.5	11.2	12.2
9	11.5	13.9	12.4	10.4
10	6.8	12.6	8.1	12.4

734


Abundance
650000
600000
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
a
10.00
15.00
20.00
25.00
30.00
35.00
Time
Abundance
600000
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
b
0.20
0.60
1.00
1.40
1.80
2.20
2.60
Time
Abundance
1600
1400
1200
1000
800
600
400
200
41
81
110
152
207
230
253
281
327
c
40
80
120
160
200
240
280
320
m/z

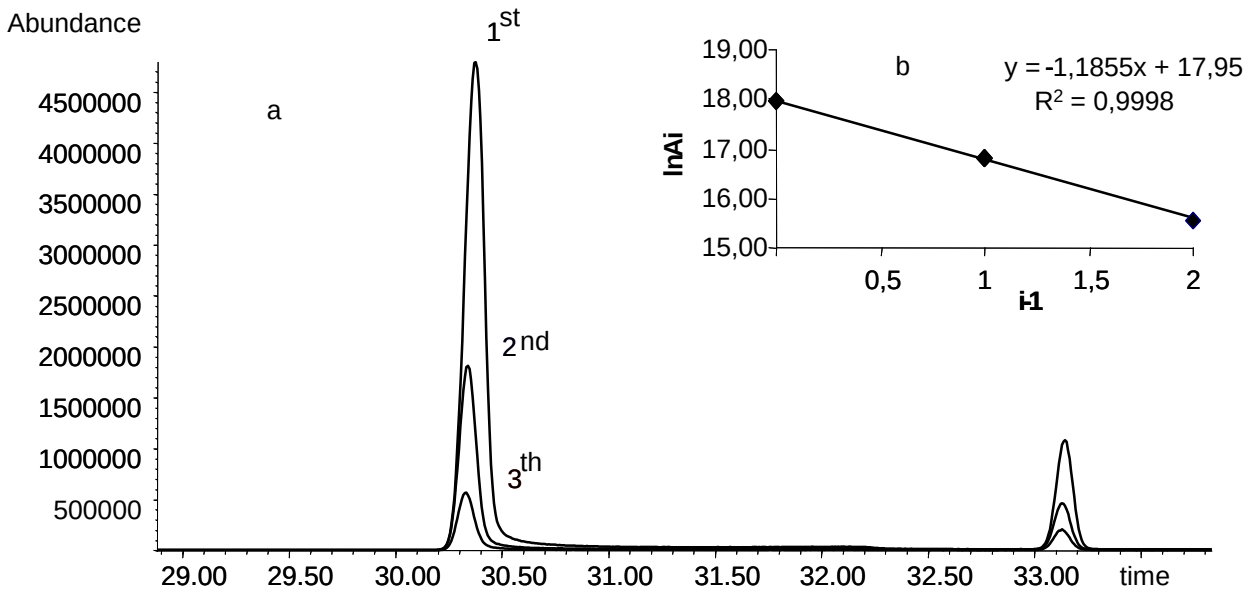
735

736


a) Sage - HS-SPME-GC-MS
F2 (37,18 %)
F1 (49,19 %)
sage4
sage7
sage3
sage8
sage6
sage1
sage2
sage5
b) Sage - HS-SPME-MS
F2 (26,75 %)
F1 (65,77 %)
sage3
sage7
sage4
sage6
sage2
sage8
sage1
sage5
c) Thyme - HS-SPME-GC-MS
F2 (24,68 %)
F1 (62,08 %)
thyme5
thyme9
thyme1
thyme2
thyme3
thyme4
thyme7
d) Thyme - HS-SPME-MS
F2 (2,24 %)
F1 (70.07 %)
thyme4
thyme2
thyme3
thyme1
thyme6
thyme7
thyme8
thyme5
thyme9

737

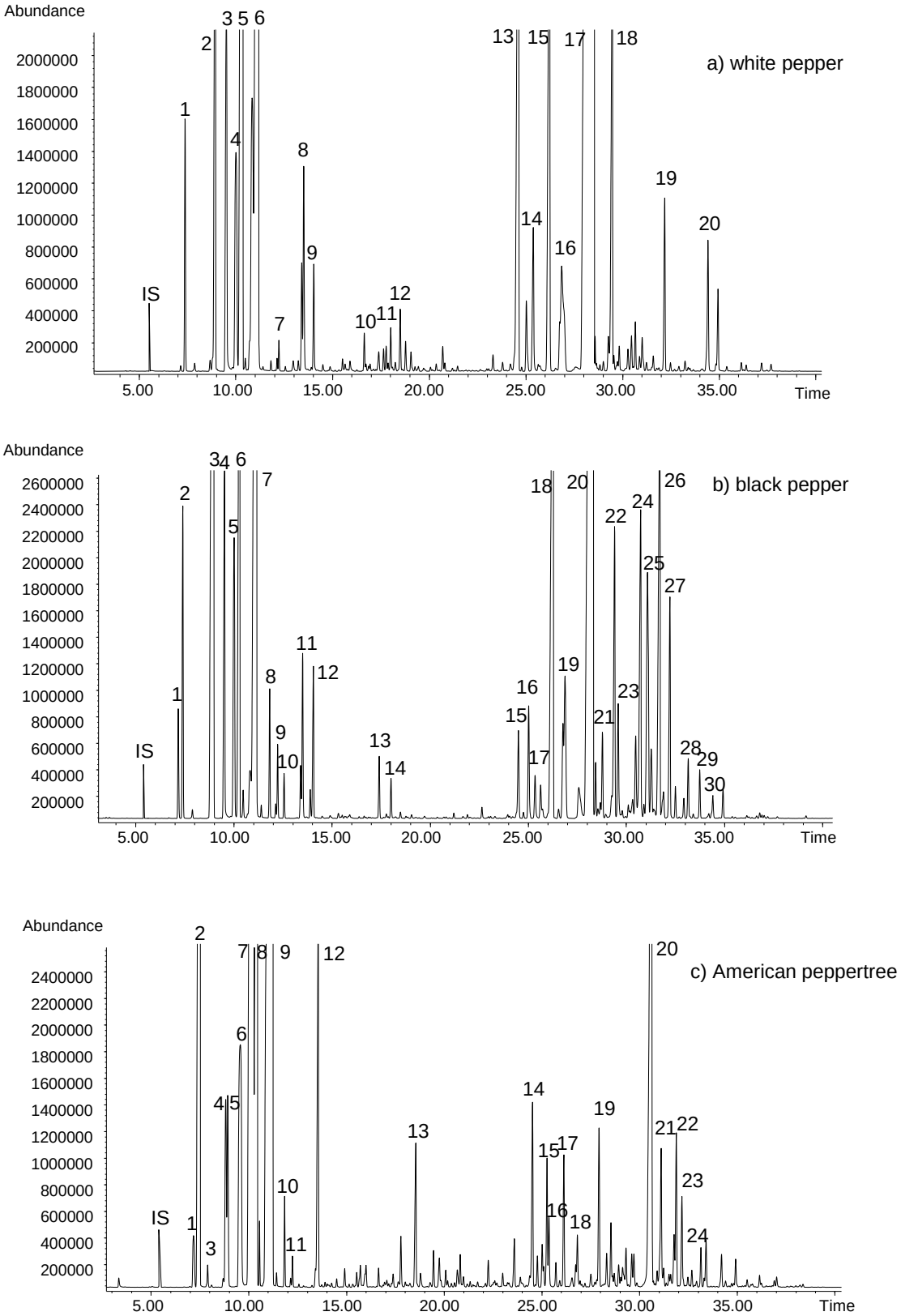
738


Abundance
1st
a
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
2nd
3th
29.00
29.50
30.00
30.50
31.00
31.50
32.00
32.50
33.00
time
b
y = -1,1855x + 17,95
R2 = 0,9998
lnAi
19,00
18,00
17,00
16,00
15,00
0,5
1
1,5
2
i-1

739

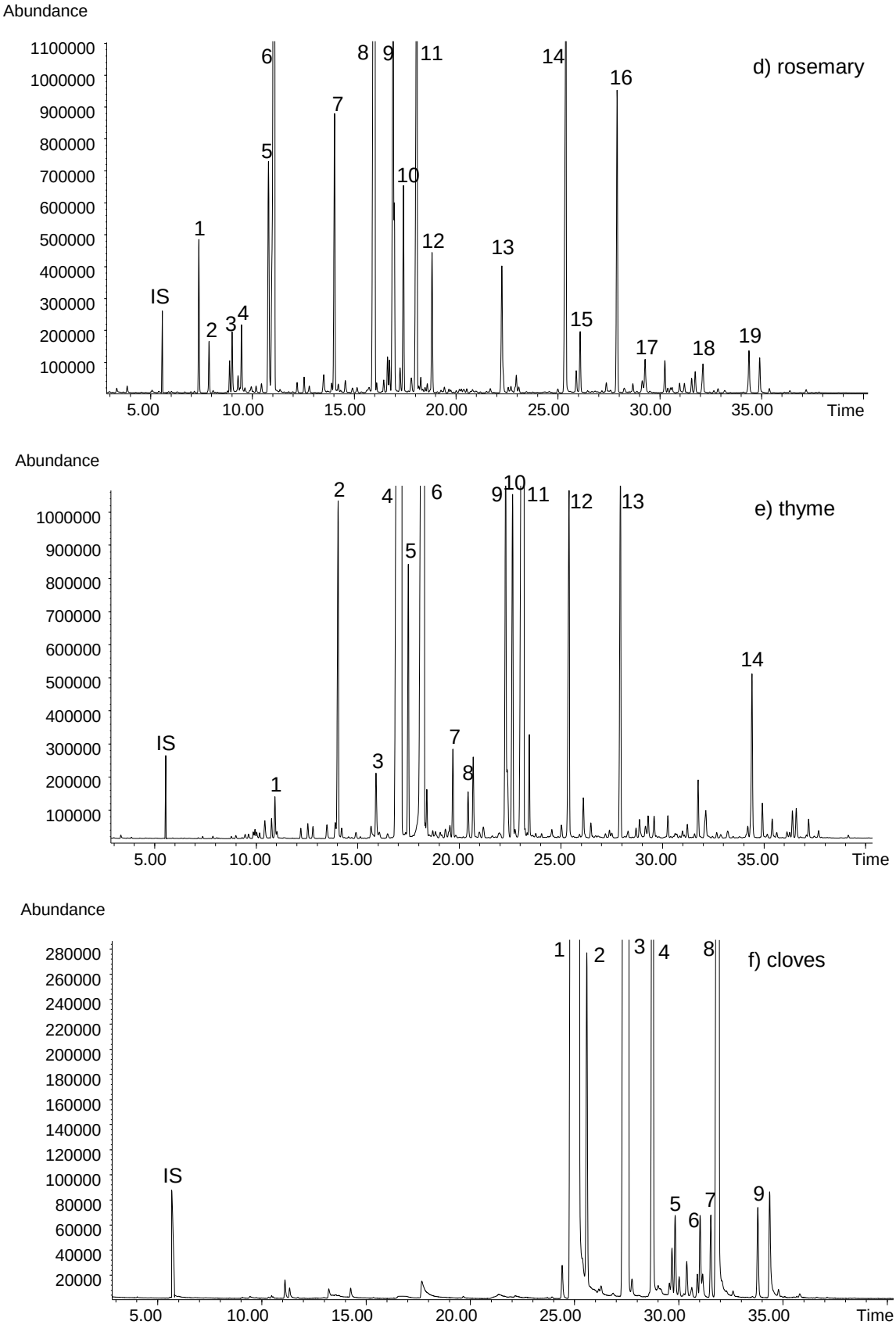
740

Figure 1SM



741

742


Abundance
d) rosemary
IS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
Time
Abundance
e) thyme
IS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time
Abundance
f) cloves
IS
1
2
3
4
5
6
7
8
9
Time

743