Evaluation of volatile bioactive secondary metabolites transfer from medicinal and aromatic plants to herbal teas: Comparison of different methods for the determination of transfer rate and human intake

Evaluation of ~~Transfer of~~ volatile bioactive secondary metabolites transfer from medicinal and aromatic plants to herbal teas: comparison of different methods~~analytical strategies~~ for the determination of transfer rate and human intake.

Barbara Sgorbini[a]*, Cecilia Cagliero[a]*, Stefano Acquadro[a], Arianna Marengo[a], Chiara Cordero[a], Erica Liberto[a], Carlo Bicchi[a], Patrizia Rubiolo[a]

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

*Corresponding authors: barbara.sgorbini@unito.it, cecilia.cagliero@unito.it
Phone: +30 011 6707135, Fax: +39 011 6707162

Abstract

A correct botanical identification and analytical quality control of volatile key-markers responsible for aroma and biological activities ~~of plant material used for~~of ~~herbal teas~~ is necessary to monitor volatile compounds transferred ~~rate~~ from a plant to the related herbal tea and human intake to guarantee their safe use. This is mainly true for ~~plants containing components~~markers limited by regulations or by a recommended maximum amount of consumption per day.

GC-MS is the elective technique to analyze volatiles, provided~~ing~~ that for aqueous samples (herbal teas)~~, but the direct injection of aqueous samples (such as herbal teas) is poorly compatible with the most conventional GC stationary phases because of their possible degradation or interference with column stability. This limit can be overcome by~~ an appropriate sample preparation procedure, and/or ~~developing of~~ a water-compatible GC stationary phases are applied. Solid Phase Micro Extraction (SPME) on-line coupled to GC-MS in a fully automatic approach is here applied to sample and quantify key markers in plant material (headspace) and in the corresponding herbal tea (direct immersion). In parallel, a new generation of GC columns coated with ionic liquid based stationary phases compatible with aqueous samples (Watercol^{TM}) was applied to test direct injection of aqueous samples ~~in a GC-FID system~~ (DAI-GC-FID). The latter approach fully bypasses sample preparation thus speeding up quality control. This study deals with the quantitation of menthol, α- and β-thujone, estragole, and anethole contained in several plant species commonly used for herbal teas (i.e. peppermint, sage, wormwood, fennel, aniseed) and regulated by International Organizations. The two methods gave comparable results and are characterized by high repeatability, linearity and accuracy, although, as expected, their sensitivity was different because DAI-GC-FID implies injection of the sample as such without analyte concentration as for DI-SPME-GC-MS. For instance, LOD and LOQ of estragole were 0.03 and 0.1 mg L^{-1} with DI-SPME-GC-MS and 0.1 and 0.8 mg L^{-1} with DAI-GC-FID. The two methods are fully complementary and their adoption depends on the amount of marker(s) to be quantified.

KEYWORDS: herbal teas, aromatic plants, volatile secondary metabolites, Direct Immersion Solid Phase Microextraction, Direct Aqueous Injection, GC-MS/FID

1. Introduction

Herbal teas are very popular, being used both in traditional medicine and for food purposes: their pleasant aroma is characterized by several volatiles, many of which possess relevant biological activities [1]. From the safety standpoint, some volatile bioactive compounds have toxic effects if

consumed in large amounts; some concern thus surrounds the volatiles characteristic of herbal tea aromas. Moreover, the herb content is often not standardized, since herbal teas are not only prepared with commercially-available herbal teabags, but also using unspecified quantities of raw plant materials, as such or in a blend. Similar considerations may be made for their preparation procedure, and further, the number of cups consumed per day may also vary from person to person. The total amount of volatile bioactive compounds ingested per day, and thus their total intake, may differ significantly.

For these reasons, analytical and botanical quality control of herbal teas is mandatory to guarantee their effective and, above all, safe use. Quality control implies not only qualitative analysis of the volatile chemical composition, but also quantitation of the amount of bioactive volatiles ingested, in particular of those limited by international regulatory organizations (e.g. the European Medicinal Agency (EMA) or the World Health Organization (WHO)). For instance, EMA has established a maximum daily exposure of 6 and 3.5 mg [2,3], respectively, for α- and β-thujone, and 10 µg/Kg/day for estragole ~~10 µg/Kg/day~~ [4]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommended an accepted daily intake of below 2.0 mg/Kg/day and below 4.0 mg/Kg/day, respectively, for anethole and menthol [5,6].

A method to sample and analyze volatiles in quality control should be rapid, easy to use, automated and, when possible, avoid the use of solvents. The technique of choice to analyze volatiles is chromatography (GC), (conventional, fast or enantioselective) combined with FID or MS detector. Selection of the sample preparation approach, in particular in view of routine quantitative analysis, must take into account several points, principally the physical state of the sample (liquid or solid) and a suitable sensitivity for target analytes (main or trace components). The present study is in line with the above considerations, and aims to quantify markers in solid (plant material) and liquid aqueous (herbal teas) samples.

~~Plant materials are solid matrices for which this~~ This research group has recently shown that Multiple Headspace Extraction Solid-Phase Microextraction (MHS-SPME) ~~to be~~is a highly effective quantitative approach for sampling volatile analysis [7-9] from solid plant materials, since it ~~enabling~~ enables to overcome the matrix effect ~~to be overcome~~. ~~A number of applications to quantify volatiles from vegetable matrices by~~ MHS-SPME effectiveness in quantifying volatiles from solid vegetable matrices has been ~~shown~~ proved by a number of applications including ~~are reported in the literature; among others these concern~~ coffee [7], hazelnuts [8] and spices [9], rosemary extracts [10], tomato samples [11].

With aqueous samples, like herbal teas, a logical solution for a routine method would appear to be directly injecting them in GC. However, with the commonest stationary phases this practice is

discouraged, because they are poorly compatible with water: the phase may undergo degradation, or the stability of column performance may be affected. Direct aqueous injection (DAI) is however possible with columns packed or wall-coated with molecular sieves, although they too possess several drawbacks, i.e. stationary phase degradation, poor sensitivity, poor efficiency, strong adsorption, low peak area repeatability [12,13]. To overcome these drawbacks, conventional but time-consuming, or poorly-sensitive conventional methods, of i) extraction of the target analytes, and/or ii) dilution of the samples with GC compatible solvents are adopted. In general, these approaches may produce analyte discrimination, reduction of sensitivity, and involve the use of organic solvents.

Alternatives overcoming the above drawbacks are, however, possible. The first involves green-chemistry approaches, based on solvent-free High Concentration Capacity Sampling techniques, such as Solid Phase Micro Extraction (SPME). SPME implies that the analyte(s) of interest are sorbed or adsorbed onto the polymeric coating of the fiber by direct immersion of the fiber directly into the sample (i.e. herbal tea) [14,15]. The sampled volatiles are then recovered on-line by thermal desorption into the injector of a GC or GC-MS system.

The second alternative exploits a very recent possibility for the direct injection of aqueous samples in GC, offered by the commercial introduction of ionic-liquid stationary phases (IL-SP) dedicated to water analysis. In 2012, Armstrong et al. introduced a new class of IL-SP based on phosphonium- and imidazolium-derived cations, combined with anions consisting of 2 or 3 units of trifluoromethanesulphonate; they compared the performance of these systems to that of other commercially-available columns currently in use [12] to quantify water as sample component. They showed that these columns had very good selectivity and stability in terms of performance over time for water analysis [16-18]. In 2014, Supelco commercially introduced this new generation of water-compatible columns under the trade name Watercol^{TM}, with stationary phases based on the ILs proposed by Armstrong. Cagliero et al. have very recently and successfully used these columns for the first time for the direct analysis of samples in the fragrance and essential oil fields, where water is the main solvent [13].

The aim of this study was to develop a method for routine quality control, with the final goal of measuring the release of biologically-active components, from the plant material to the resulting water infusion, and to monitor secondary metabolite total or daily intake to assure their safe use. In particular, it focused on raw plant materials and herbal teas, consisting of either single species or blends, containing volatile secondary metabolites limited or normed by regulatory authorities, such as thujones, estragole, anethole, menthol. The study proposes adopting two approaches for routine quantitation of the above volatiles in herbal teas: a) a multi-step method including a sample

preparation procedure, i.e. Direct Immersion Solid Phase Micro Extraction (DI-SPME) sampling followed by GC-MS analysis, and b) a single-step procedure entailing Direct Aqueous Injection (DAI) and GC-FID analysis with Watercol™ columns. Linearity, limit of detection, limit of quantitation, and precision were evaluated for both approaches applied to herbal teas prepared using blends of raw plant materials or commercially-available teabags. The complementarity of the two methods, and advantages and limitations with and without a sample preparation step are also critically discussed. This is the first time in which real samples containing water as the main solvent are directly injected in Watercol™ columns. The direct injection, avoiding sample preparation, results to be advantageous in terms of costs and total analysis time.

2. Experimental

2.1. Materials and Reagents

Dried samples of mint (*Mentha x piperita* L.) leaves and wormwood (*Artemisia absinthium* L.) aerial parts were kindly supplied by Chialvamenta (Pancalieri (TO), Italy). Dried samples of sage (*Salvia officinalis* L.) leaves, anise (*Pimpinella anisum* L.) fruits, fennel (*Foeniculum vulgare* Mill.) fruits, and commercial mixtures for herbal teas were purchased in different local supermarkets. **Table 1S** lists the raw plant materials and the commercially-available mixtures for herbal teas analyzed; the compositions of the commercial herbal teas investigated are those reported on the label. **Table 1** reports the list of bioactive secondary metabolites selected, together with their water solubility, partition coefficient values (log P), the target ions used for their quantitation both in the plant as such and in the blends and commercial herbal teas, and the maximum amount per day recommended by international regulatory authorities. Pure standard samples of anethole, camphor, 1,8-cineole, estragole, α– and β- thujone (mixture of isomers in a 95:5 ratio, respectively) were from Merck (Milan, Italy). Isoamyl acetate (3-methyl-1-butanol) used as Internal Standard (IS) was from Merck (Milan, Italy). Solvents (cyclohexane, ethyl acetate and ethanol) were all HPLC-grade from Sigma Aldrich (Milan, Italy).

2.2. SPME fibers

Carboxen/divinylbenzene/PDMS (CAR/DVB/PDMS) SPME fibres (2 cm long) were from Supelco Co. (Bellafonte, PA, USA). 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) [19,20]

2.3. Sample preparation conditions

Raw plant materials - 10 mg of wormwood, mint, sage, anise and fennel were introduced in a 20 mL headspace vial and submitted to MHS-SPME sampling for 30 minutes at 50°C using the CAR/DVB/PDMS SPME fibre. [9].

Herbal tea preparation - Herbal teas were prepared as per the European Pharmacopeia (IX edition) [21] with 2 grams of raw plant material or with a commercial teabag in 250 mL of boiling water for 5 minutes, and then filtered. Three preparations of each herbal tea were analyzed.

DI-SPME conditions: 5 mL of herbal teas were introduced into a 20 mL vial, diluted with 15 mL of water, spiked with a 0.0025 mg/ml standard solution of isoamyl acetate in water used as internal standard, and submitted to DI-SPME sampling for 30 minutes at room temperature using a 2 cm CAR/DVB/PDMS SPME fibre. After sampling, the fiber was automatically removed from the vapor phase or from the herbal tea, and transferred to the GC injection port for on-line thermal desorption of the sampled analytes into the GC column. Blank runs were carried out after DI-SPME GC-MS without detecting any carry over effects on the fiber.

DAI conditions: herbal teas were spiked with a 0.1 mg/ml ethanolic solution of isoamyl acetate (IS) and directly injected into the GC-MS system.

2.4. Analysis conditions

HS-SPME and DI-SPME GC-MS 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 5975N MSD (Agilent, Little Falls, DE, USA).

GC-MS conditions: injector temperature: 230°C, injection mode: split, ratio: 20:1; carrier gas: helium, flow rate: 1 mL min^{-1}; fiber desorption and reconditioning time: 5 min; column: MEGA-5 (5%-phenyl-polymethylsiloxane d_f: 0.25 µm, d_c: 0.25 mm, length: 30 m) from Mega, Legnano (Milan), Italy. Temperature program: 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 to those available in commercial or home-made libraries or in the literature.

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

DAI-GC-FID conditions. Analyses were carried out on a Shimadzu GC–FID 2010 system provided with Shimadzu GC Solution 2.53SU software (Shimadzu, Milan, Italy). Instrumental conditions: injector temperature: 230°C, injection mode: split, ratio: 5:1; carrier gas: hydrogen, flow rate: 1 mL min^{-1}; column: Watercol™ 1460 (non-bonded tri (tripropylphosphoniumhexanamido)

trimethylamine trifluoromethanesulfonate d_f: 0.20 µm, d_c: 0.25 mm, length: 30 m) from Supelco, Bellefonte, USA. Temperature programs: from 40°C (5 min) to 250°C (2 min) at 3°C min^{-1}. Detector temperature: 250°C, FID sampling rate: 40 msec. Injection conditions: sample volume: 2 µL, injection was at high pressure (200 kPa for 1 min) to obtain a vapor volume compatible with the liner volume. Markers and components of the investigated matrices were identified by comparing their mass spectrum and retention times to those of authentic standards and/or commercially available library [22].

2.5. Quantitation

2.5.1. Raw plant materials

Stock standard mixtures of the selected markers for each raw plant material analyzed were prepared by adding 25 mg of pure standard to 1 mL of cyclohexane. Suitable dilutions of each stock standard mixture were then prepared. The resulting solutions (both stock and diluted) were stored at 0°C and renewed weekly. A calibration curve was built up by analyzing 1 µL of standard mixtures diluted at ten different concentration levels of the selected markers (range 0.01-25 mg/mL) in cyclohexane by full evaporation MHS-SPME (see Results and Discussion). Each level was analyzed three times.

MHS-SPME - MHS-SPME is an approach based on stepwise dynamic gas extraction of the target analytes from a single sample: their peak area decays exponentially with the number of extractions and the sum of the areas from each extraction is representative of the total amount present initially in a given sample [23, 7-9]. The total area of the target analyte(s) is determined through **Eq. 1**:

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

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

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

where A_i is the peak area obtained from the i^{th} extraction. Generally, a limited number of extractions (3-5) are sufficient for reliable area determination. The analytes can then be quantified by an external standard calibration approach, submitting standard mixtures of selected markers at different concentrations to MHS-SPME.

2.5.2. Herbal teas

Stock solutions, α- and β-thujone, and menthol were prepared at concentration of 0.1 mg/mL in water. Anethole and estragole stock solutions were prepared at a concentration of 100 mg/mL in ethyl acetate because of their low water solubility, and then diluted to 0.1 mg/mL in water. Suitable mixture dilutions in water were then prepared. The resulting solutions (both stock and diluted) were stored at 0°C and renewed weekly. DI-SPME calibration curves were built up within the expected range of concentration by analyzing 20 mL of standard mixtures diluted at ten different ~~concentrations~~ levels of the selected markers (range 10-0.01 mg/L) in water. The DAI calibration curve was built up by analyzing 1 µL of standard mixtures diluted at eight different concentrations of the selected markers (range 100-1 mg/L) (see Results and Discussion). Each level was analyzed three times.

2.6. Analytical performances

The analytical performances of the proposed methods were determined in terms of linearity, sensitivity (LOD and LOQ), repeatability, and intermediate precision. All samples (raw plant materials and herbal teas) were analyzed five times to evaluate repeatability. Intermediate precision was determined for each sample by analyzing it five times every four weeks over a period of three months. LOD and LOQ were experimentally determined on plant materials by analyzing decreasing amounts of the same sample diluted with an inert solid support (Celite® 545, Fluka) and for herbal teas on decreasing concentrations of marker standard mixture. The LOD of each analyte was calculated from the average area of the investigated marker divided by the average "peak to peak" noise values sampled in its region of elution in the chromatogram, with a coverage factor of 3. LOQ values were experimentally measured by analyzing decreasing concentration o~~i~~f standard solution of the investigated compounds. LOQ was the lowest concentration for which the error for in peak integration area determination (assignment) was ≤ 20%.

3. Results and discussion

~~Routine quality control of herbal teas, as well as of aromatic and medicinal plants used for their preparation, is mandatory for their safe use.~~ The qualitative and quantitative determination of volatile biologically-active secondary metabolites in herbal teas as well as in aromatic and medicinal plants requires methods in which, when possible, sample preparation and analysis are integrated on-line into a single step (total analysis system)~~; they must be highly reliable and at the same time rapid, cheap, easy to automate and solvent free~~.

Here, twenty-four samples of plant materials and commercial teabags were analyzed with GC-MS combined with Head Space Solid Phase Micro Extraction (MHS-SPME), and the corresponding herbal teas with GC-MS combined with Direct Immersion Solid Phase Micro Extraction (DI-SPME) and GC-FID with Direct Aqueous Injection in dedicated GC columns (DAI combined with Watercol^{TM} columns).

3.1 Quantitation of volatile bioactive secondary metabolites in raw plant materials

The first step comprised quantifying target analytes in mono-component raw plant materials, to evaluate the rate of extraction during the infusion process.

~~MHS-SPME is an approach based on stepwise dynamic gas extraction of the target analytes from a single sample: their peak area decays exponentially with the number of extractions and the sum of the areas from each extraction is representative of the total amount present initially in a given sample[23, 7-9]. The total area of the target analyte(s) is determined through **Eq. 1:**~~

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

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

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

~~where A_i is the peak area obtained from the i^{th} extraction. Generally, a limited number of extractions (3-5) are sufficient for reliable area determination. The analytes can then be quantified by an external standard calibration approach, submitting standard mixtures of selected markers at different concentrations to MHS-SPME.~~

The volatile bioactive secondary metabolites quantitated were menthol for peppermint (*Mentha x piperita* L.), α- and β- thujone for sage (*Salvia officinalis* L.) and wormwood (*Artemisia absinthium* L.), respectively, estragole for fennel (*Foeniculum vulgare* Mill.) and anethole for anise (*Pimpinella anisum* L.).

Table 2 reports the linearity range and the equations of calibration curves of the investigated volatiles (obtained by MHS-SPME-GC-MS of suitable concentration levels of standards in

cyclohexane), together with their regression coefficients (R^2), LODs and LOQs. These results indicate a very good linearity, being the regression coefficient always higher than 0.998. ~~and~~ LOD and ~~/~~LOQ values for markers are by far lower than the concentrations usually present in the samples under analysis (2 µg Kg^{-1} to 2 mg Kg^{-1}).

Table 3 gives the amount (expressed as mg) of the investigated markers in peppermint, wormwood, fennel, and anise, chosen as model plant materials and referred to the quantity of plant used to prepare the herbal teas. The content of the investigated compounds in the analyzed herbal teas was always below the maximum amount per day recommended by EMA or FAO/WHO, with the exception for estragole. The percent of extracted compounds in herbal teas (i.e. transfer rate) was in line with the literature data [24].

3.2 Analytical performance of the DI-SPME-GC-MS and of the DAI-GC-FID methods

The second step of this study involved the quantitation of some selected volatile secondary metabolites in herbal teas, prepared as the indications of the European Pharmacopoeia, using both monocomponent raw plant materials and multicomponent commercial teabags, by. a) a DI-SPME sampling followed by GC-MS analysis, and b) a DAI GC-FID analysis using the Watercol™ 1460 column.

The analytical performances of the two approaches were first evaluated in terms of linearity, sensitivity, repeatability and intermediate precision. **Table 4** reports concentration range, calibration curves, regression coefficient, LOD and LOQ, repeatability and intermediate precision (both calculated on five replicates) for each analyte investigated, analyzed with both DI-SPME-GC-MS and DAI-GC-FID. The R^2 values show that the linearity of both methods in the concentration range investigated is very good, being always higher than 0.998 for DI-SPME-GC-MS and 0.994 for DAI-GC-FID. ~~as are~~ Also repeatability and intermediate precision were ~~very good~~highly satisfactory (see section 4). ~~and s~~Sensitivity was in line with the method purposes with DI-SPME-GC-MS LOD and LOQ values were lower than those obtained with DAI-GC-FID. The DI-SPME-GC-MS approach was more sensitive (and thus had lower LOD/LOQ) and more informative for identification than DAI-GC-FID, thanks to the ability of the SPME coating to accumulate the target analytes, and also to the use of MS as detector. On the other hand, sampling by DI-SPME can produce discrimination in the recoveries of analyte(s) with different chemical structure(s) because of their different interaction with the fiber coating material. Conversely, the DAI-GC-FID method did not discriminate among the analytes~~,~~ because of their different affinities with the SPME coating material. However, the DAI-GC-FID method was sensitive enough (with LOD generally below 1 ppm) to quantify the amount of target analytes present in herbal teas. **Figure 1** compares the MHS-

SPME-GC-MS, DI-SPME-GC-MS and DAI-GC-FID patterns of peppermint plant material of and related herbal tea prepared with commercially-available teabags, as an example.

Table 3 also shows the percent rate of the selected markers transferred from plant material to herbal teas (for quantitation of analytes in herbal teas, see paragraph 3.3). The data show that extracted percent rate of investigated markers was below 30% in all cases, in agreement with their relatively low solubility in water and high log P values.

3.3 Quantitation of volatile bioactive secondary metabolites in herbal teas

Figure 2 shows the content of selected markers, in all herbal teas analyzed, as mg/cup (250 mL) obtained by both DI-SPME-GC-MS and DAI-GC-FID. Quantitative data obtained with the two methods on menthol, anethole, and estragole were found to be quite consistent in all herbal teas, being the differences in the amounts quantified with the two methods in no case exceeding 15%. The DAI method combined satisfactory sensitivity for the quantitative determination of all selected analytes, the amount of compound in the herbal teas being in all cases well above the LOQ, with a remarkably faster speed than DI-SPME, the total analysis time being reduced to less than half.

In terms of food safety, the results show that consumption of the herbal teas, based on peppermint, wormwood, anise, fennel, and sage, leads to an intake of the target active principle within the limits recommended by the regulatory organizations. Special care should to be taken for children (because of their lower body weight) and for multiple cup consumption in the case of some herbal teas that contain specific secondary metabolites of concern, which are rather soluble: for example, those containing estragole. In this study, for instance, two 250 mL cups of herbal tea prepared with the investigated fennel overcomes the maximum amount per day established by regulatory authorities for estragole, since two of its cups account for 840 μg of it, i.e. an amount higher than that established by EMA (600 μg/day for an adult of 60 Kg).

4. Data precision

Tables 2 and 4 report the repeatability and intermediate precision of the data obtained with the two approaches applied, expressed as RSD%. Relative standard deviations (RSD%) values of the selected markers for both repeatability and intermediate precision were highly satisfactory, RSD% never exceeding 5.5% for menthol in peppermint with DI-SPME-GC-MS and 10.5% for estragole in fennel for DAI-GC-FID, respectively. The results were similar for all samples. Watercol™ column stability was also monitored through periodical injection of Grob test: the results showed that column performance was unvaried after 400 injections of aqueous samples.

5. Conclusions

Quality control is a fundamental step to guarantee the safe use of herbal teas, in particular for those containing regulated or limited biologically-active volatiles. The results reported here show that analytes' transfer from plant material to infusion can be determined, and thus that consumers' intake of biologically-active or toxic compounds from the related herbal tea can be monitored. In this study, routine methods for quality control were developed i) for plant material (MHS-SPME-GC-MS), and ii) for related herbal teas. In the latter case, two approaches were adopted: i) the first, and more "conventional", method combines Direct Immersion SPME of volatile secondary metabolites with GC-MS analysis, with a column coated with a conventional stationary phase, ii) the second adopts -direct injection of the aqueous samples (DAI) into a GC-FID system, using water-compatible (Watercol^{TM}) ionic liquid coated columns. The DAI-GC-FID method complies with all specifications characteristic of routine quality control analyses, providing the required sensitivity together with reliable, accurate and repeatable results, while reducing total analysis time by a factor of two (from 100 min to 50 min), and eliminating intermediate steps, the use of consumables (e.g. SPME fibers) and additional instrumentation (e.g. thermostatic system). A further advantage of the DAI method is that it does not suffer from discrimination between analytes. Moreover, Watercol^{TM} columns are here applied for the first time for routine analysis of real-world samples where water is the main solvent. Conversely, its main limitations are i) ~~its~~ a lower sensitivity than that of DI-SPME-GC-MS, because of the lack of a concentration step of the target analytes, and ii) at present, the lower identification power due to the use of FID, a detector unable to provide analyte structure information. However, the results show that the two methods are fully complementary and their choice is conditioned by the number and amount of markers to be quantified. Further investigation is under way to improve GC performance with Watercol^{TM} columns having different characteristics (i.e. narrow-bore columns) and to explore the possibility of combining DAI-GC directly with a diagnostic spectroscopic detector (MS).

Acknowledgment

The authors are indebted to Robertet SA (Grasse, France) for financial support to the laboratory. This study was supported financially by the Ricerca Locale (Ex60%2016-2017) of University of Turin, Turin (Italy).

References

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

[2] European Medicines Agency - Committee on Herbal Medicinal Products (HMPC). European Union herbal monograph on *Salvia officinalis* L. folium, EMA/HMPC/277152/2015. www.ema.europa.eu/documents/herbal-monograph/draft-european-union-herbal-monograph-salvia-officinalis-l-folium_en.pdf

[3] European Medicines Agency - Committee on Herbal Medicinal Products (HMPC). Public statement on the use of herbal medicinal products containing thujone, EMA/HMPC/732886/2010. www.ema.europa.eu/documents/public-statement/draft-public-statement-use-herbal-medicinal-products-containing-thujone_en.pdf.

[4] European Medicines Agency - Committee on Herbal Medicinal Products (HMPC). Public statement on the use of herbal medicinal products containing estragole, EMA/HMPC/137212/2005 Rev 1. www.ema.europa.eu/documents/public-statement/public-statement-use-herbal-medicinal-products-containing-estragole_en.pdf.

[5] Joint FAO/WHO Expert Committee on Food Additives, WHO food additives series: 42, 1998.

[6] Joint FAO/WHO Expert Committee on Food Additives, JECFA/86/SC, 2018.

[7] C. Bicchi, M.R. Ruosi, C. Cagliero, C. Cordero, E. Liberto, P. Rubiolo, B. Sgorbini, Quantitative analysis of volatiles from solid matrices of vegetable origin by high concentration capacity headspace techniques: Determination of furan in roasted coffee, J. Chromatogr. A 1218 (2011) 753-762.

[8] L. Nicolotti, C. Cordero, C. Bicchi, P. Rubiolo, B. Sgorbini, E. Liberto. Volatile profiling of high quality hazelnuts (*Corylus avellana* L.): chemical indices of roasting, Food Chem. 138 (2013) 1723-1733.

[9] B. Sgorbini, C. Bicchi, C. Cagliero, C. Cordero, E. Liberto, P. Rubiolo. 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, J. Chromatogr. A 1376 (2015) 9-17.

[10] J.D. Carrillo, M.T. Tena, Determination of volatile compounds in antioxidant rosemary extracts by multiple headspace solid-phase microextraction and gas chromatography, Flav. Fragr. J. 21 (2006) 626-633.

[11] E. Serrano, J. Beltràn, F. Hernandez, Application of multiple headspace-solid-phase microextraction followed by gas chromatography-mass spectrometry to quantitative analysis of tomato aroma components, J. Chromatogr. A 1216 (2009) 127-133.

[12] DA Jayawardhana ~~DA~~, RM Woods ~~RM~~, Y Zhang ~~Y~~, CL Wang ~~CL~~, DW Armstrong ~~DW~~. Rapid, Efficient Quantification of Water in Solvents and Solvents in Water Using an Ionic Liquid-Based GC Column. LC GC N Am. 30(2) (2012)142

[13] C. Cagliero, C. Bicchi, C. Cordero, E. Liberto, P. Rubiolo, B. Sgorbini, Ionic liquids as water-compatible GC stationary phases for the analysis of fragrances and essential oils, Analytical and Bioanalytical Chemistry 410 (2018) 4657-4668

[14] A. Spietelun, Ł. Marcinkowski, M. la Guardia, J. Namieśnik, Recent developments and future trends in solid phase microextraction techniques towards green analytical chemistry, J. Chromatogr. A 1321 (2013) 1-13.

[15] H. Yu, T.D. Ho, J.L. Anderson, Ionic liquid and polymeric ionic liquid coatings in solid-phase microextraction, TrAC Trends in Analytical Chemistry 45 (2013) 219-232

[16] LA Frink, CA Weatherly, DW Armstrong. Water determination in active pharmaceutical ingredients using ionic liquid headspace gas chromatography and two different detection protocols. J Pharmaceut. Biomed. 94 (2014) 111-117.

[17] LA Frink, DW Armstrong. Water Determination in Solid Pharmaceutical Products Utilizing Ionic Liquids and Headspace Gas Chromatography. J Pharm Sci-Us. 105 (2016) 2288-2292.

[18] LA Frink, DW Armstrong. The utilisation of two detectors for the determination of water in honey using headspace gas chromatography. Food Chem. 205 (2016) 23-27.

[19] Y. Wang, J. O'Reilly, Y. Chen, J. Pawliszyn, Equilibrium in-fibre standardisation technique for solid-phase microextraction, J. Chromatogr. A 1072 (2005) 13-17.

[20] C. Bicchi, C. Cordero, E. Liberto, B. Sgorbini, P. Rubiolo, Reliability of fibres in Solid Phase Microestraction for routine analysis of the headspace of aromatic and medicinal plants, J. Chromatogr. A, 1152 (2007) 138-149.

[21] European Pharmacopeia, IX edition.

[22] R Adams. Identification of EO component by gas chromatography/mass spectrometry. 4th ed. Allured, Carol Stream, 2007

[23] B. Kolb, L.S. Ettre, "Static Headspace-Gas Chromatography, Theory and Practice", Wiley – VCH, New York, 1997.

[24] C Tschiggerl, F Bucar, The volatile fraction of herbal teas, Phytochem. Rev. 11 (2012)245–254

Captions to figures

Figure 1 - MHS-SPME-GC-MS DI-SPME-GC-MS and DAI-GC-FID patterns of peppermint plant material and related herbal tea prepared with commercially available teabags. Peak identification: 1) limonene, 2) 1,8-cineole, 3) γ-terpinene, 4) menthone, 5) isomenthone, 6) menthol, 7) isomenthol, 8) pulegone, 9) piperitone, 10) menthyl acetate, 11) β-bourbonene, 12) *trans*-β-caryophyllene, 13) germacrene D.

Figure 2 - Content of selected markers in the analyzed herbal teas.

Captions to tables

Table 1. List of bioactive secondary metabolites selected together with their water solubility, target ions used for quantification analysis, maximum amount per day recommended by international regulatory authorities.

Table 2. Linearity range and equations of calibration curves obtained by MHS-SPME-GC-MS together with the regression coefficient (R^2) for each quantitated compounds, root mean square error, LOD and LOQ, repeatability and intermediate precision (n=5).

Table 3. Amount of selected markers in peppermint, wormwood, fennel and anise (referred to the quantity used to prepare herbal teas) and percent extraction rate of the selected markers transferred to herbal teas.

Table 4. Concentration range adopted, equation of calibration curve together with the regression value, root mean square error, LOD and LOQ values, repeatability (n = 5) and intermediate (int.) precision for each analyte investigated for DI-SPME-GC-MS and DAI-GC-FID approaches.

Table 1S. List of raw plant materials and commercially available blends used to prepare the herbal teas analyzed; for commercial herbal teas, the labelled composition is reported.

Figure 1 revised

Figure 1

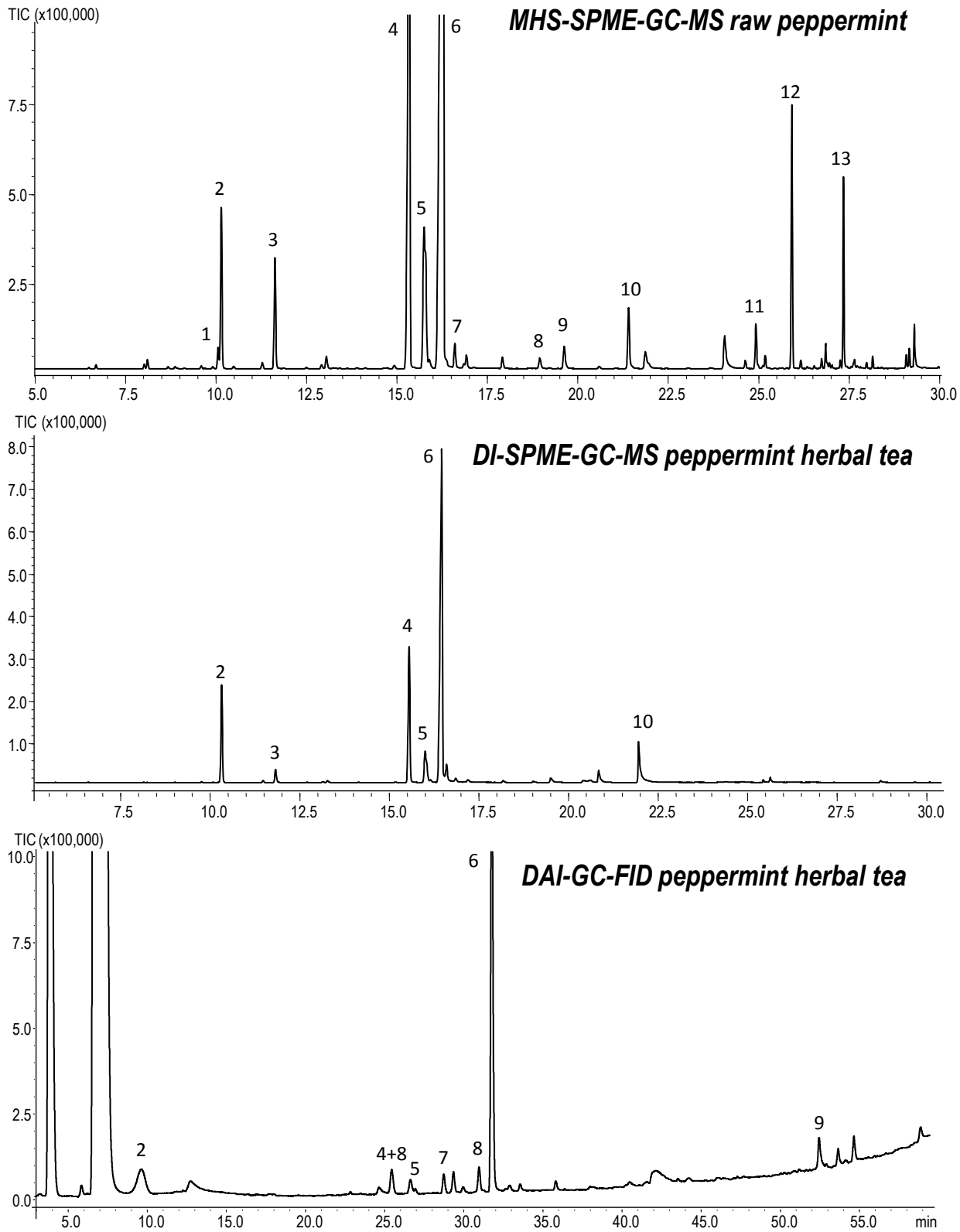

Figure 2 revised

Figure 2

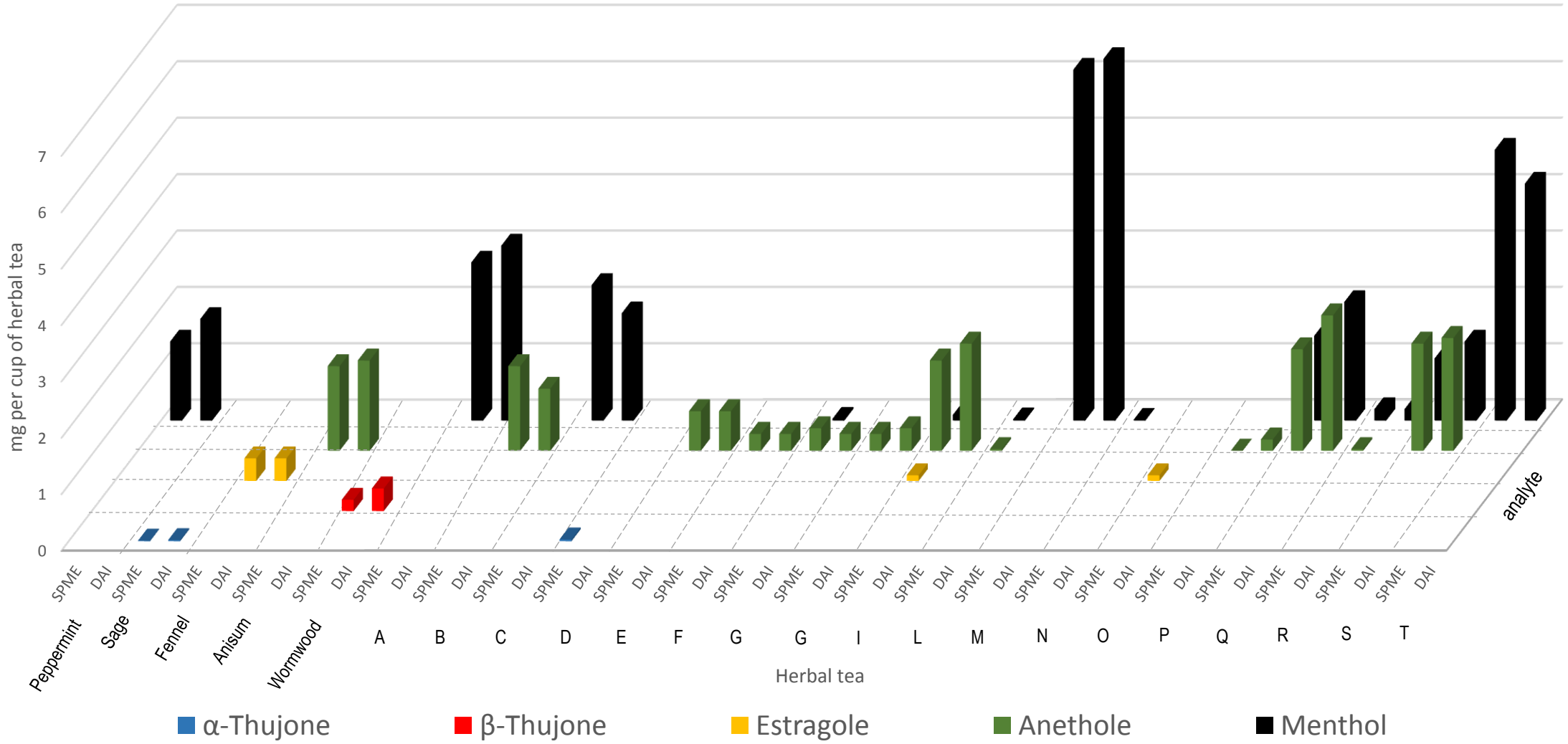

Table 1 revised

Table 1. List of bioactive secondary metabolites selected together with their water solubility, target ions used for quantification analysis, maximum amount per day recommended by international regulatory authorities.

Analyte	Water solubility (mg L^{-1} 25°C)	Log P	Target ion (m/z)	Daily exposure (EMA)	Acceptable Daily Intake (FAO/WHO)
α-Thujone*	407	2.65	110	< 6.0 mg	
β-Thujone**	407	2.65	110	< 3.5 mg	
Estragole***	84.6	3.15	148	10 µg/Kg/day	
Anethole$	98.7	3.17	148		< 2.0 mg/Kg/day
Menthol$$	456	3.20	71		< 4.0 mg/Kg/day

* EMA/HMPC/277152/2015; ** EMA/HMPC/732886/2010; *** EMA/HMPC/137212/2005; $ Joint FAO/WHO Expert Committee on Food Additives JECFA 1998; $$ JECFA 2018

Table 2 revised

Table 2. Linearity range and equations of calibration curves obtained by MHS-SPME-GC-MS together with the regression coefficient (R^2) for each quantitated compounds, LOD and LOQ, repeatability and intermediate precision (n=5)

Analyte	Linearity range (µg g^{-1})	Equation	Regression RMSE*	Linearity (R^2)	LOD (µg g^{-1})	LOQ (µg g^{-1})	Repeatability (1 µg g^{-1}) %RSD	Int. precision (1 µg g^{-1}) %RSD
α-Thujone	0.25-10	y = 196670238x - 565943445	0.029	1.000	0.3	1	0.80	5.6
β-Thujone	0.25-50	y = 244188329x - 52857221	3.5	0.9990	0.5	2	3.2	10.71
Estragole	0.25-10	y = 282318851x - 1528473710	0.59	1.000	0.03	0.1	1.3	8.1
Anethole	0.25-10	y = 170617591x - 240758991	0.25	0.9980	0.002	0.01	2.2	5.9
Menthol	0.25-10	y = 14453638x - 17434.345	1.1	0.9984	0.003	0.02	5.3	6.6

*root mean square error

Table 3 revised

Table 3. Amount of selected markers in peppermint, wormwood, fennel and anise (referred to the quantity used to prepare herbal teas) and percent extraction rate of the selected markers transferred to herbal teas

Plant material	Compound	Total amount in plant material (mg)	DI-SPME-GC-MS		DAI-GC-FID	
			Herbal tea (mg)	% Extraction in herbal tea	Herbal tea (mg)	% Extraction in herbal tea
Salvia officinalis L. (sage)	α-Thujone	0.040 ± 0.001	0.014 ± 0.002	35%	0.017 ± 0.002	42%
Artemisia absinthium L. (wormwood)	β-Thujone	1.2 ± 0.03	0.27 ± 0.02	23%	0.33 ± 0.03	27%
Mentha x piperita L. (peppermint)	Menthol	9.8 ± 0.1	1.4 ± 0.02	14%	1.8 ± 0.1	18%
Foeniculum vulgare Mill. (fennel)	Estragole	2.3 ± 0.04	0.42 ± 0.03	18%	0.40 ± 0.04	17%
Pimpinella anisum L. (anise)	Anethole	4.9 ± 0.2	1.5 ± 0.04	31%	1.6 ± 0.2	33%

Table 4 revised

Table ~~3~~4. Concentration range adopted, equation of calibration curve together with the regression value, LOD and LOQ values, repeatability (n = 5) and intermediate (int.) precision (n=5) for each analyte investigated for DI-SPME-GC-MS and DAI-GC-FID approaches

DI-SPME-GC-MS								
Compound	**Linearity range (mg L^{-1})**	**Equation**	**Regression RMSE***	**Linearity (R^2)**	**LOD (mg L^{-1})**	**LOQ (mg L^{-1})**	**Repeatability (1 mg L^{-1}) %RSD**	**Int. precision (1 mg L^{-1}) %RSD**
α-Thujone	0.25-10	y = 2911206x - 14877801	0.34	1.000	0.01	0.05	0.8	3.8
β-Thujone	0.25-50	y = 6103196x - 2128019	0.91	0.9990	0.01	0.05	3.2	6.4
Estragole	0.25-10	y = 360270x - 986331	1.7	0.99630	0.03	0.1	1.3	1.8
Anethole	0.25-10	y = 761575x - 4780742	0.70	0.9994	0.002	0.01	2.2	9.4
Menthol	0.25-10	y = 3873205x - 47618621	1.2	0.9985	0.02	0.1	5.3	7.7
DAI-GC-FID								
Compound	**Linearity range (mg L^{-1})**	**Equation**	**Regression RMSE***	**Linearity (R^2)**	**LOD (mg L^{-1})**	**LOQ (mg L^{-1})**	**Repeatability (1 mg L^{-1}) %RSD**	**Int. precision (1 mg L^{-1}) %RSD**
α-Thujone	0.5-95	y=1282x+2429	2.2	0.9959	0.5	1.9	3.5	6.9
β-Thujone							4.6	7.9
Estragole	1-100	y=1418x+527	1.9	0.9974	0.1	0.8	4.9	10.51
Anethole	1-100	y=1841x-2066	2.8	0.9943	0.2	0.8	1.7	6.4
Menthol	1-100	y=2244x-652	1.3	0.9999988	0.1	0.7	1.5	8.6

*root mean square error