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Journal of Chromatography A, 1041 (2004) 11–18
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analytical procedure for the in-vial derivatization—extraction of phenolic acids and flavonoids in methanolic and aqueous plant extracts followed by gas chromatography with mass-selective detection

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Received 12 December 2003; received in revised form 23 April 2004; accepted 27 April 2004

Available online 25 May 2004

Abstract

An in-vial simple method for the combined derivatization and extraction of phenolic acids and flavonoids from plant extracts and their direct determination with GC–MS, is described. The method is taking advantage of the beneficial potentials of phase transfer catalysis (PTC). Catalysts in soluble and polymer-bound form were tested with the latter being the format of choice due to its high reaction yield and facile separation from the rest of the reaction system. Optimization of experimental conditions was established. Chromatographic separation of eight phenolic acids and four flavonoids methylated via the PTC derivatization step was achieved in 45 min. The detection limits for the described GC–MS(SIM) method of analysis ranged between 2 and 40 ng/ml whereas limits of quantitation fall in the range 5–118 ng/ml, with flavonoids accounting for the lowest sensitivity due to their multiple reaction behavior. Four methanolic extracts from *Tilia europea*, *Urtica dioica*, *Mentha spicata* and *Hypericum perforatum* grown wild in north-western Greece and four aquatic infusions from commercially available *Mentha spicata*, *Origanum dictamnus*, *Rosemarinus officinalis* and *Sideritis cretica* were analyzed. Good trueness of the method was demonstrated as no matrix effects were found for the analytes concerned.

Keywords: Derivatization, GC; Phase transfer catalysis; Phenolic acids; Flavonoids

1. Introduction

Phenolic acids and their derivatives are widely distributed in plants [1–3]. They occur as universal plant components bonded to lignins by ester bonds and play the role of inhibitors of cellulase secreted by pathogens across the membrane of cells and prevent the penetration of pathogens across the cell membrane. Phenolic compounds have been reported to accumulate in parts of plants infected by fungi [4] and have been shown to inhibit the in vitro oxidation of human low-density lipoprotein [5]. The role of the phenolics and flavonoids as natural antioxidants and free radical scavengers has attracted considerable interest [6,7] due to their pharmacological behavior [8,9].

There are many publications investigating the phenolic acid and flavonoid contents in plants. Most of the analytical protocols are based on high-performance liquid chromatography (HPLC) techniques, with UV spectrophotometry or electrochemical detection [10–14], or coupled with coulometric detection [15]. Mattila et al. determined flavonoids in plant material by HPLC with diode-array and electro-array detectors for improved peak identification [16]. Careri et al. devised a turbo-ionspray mass spectrometry detection method for the HPLC analysis of flavonoids [17]. Glowniak et al. have developed a procedure, which combines solid-phase extraction and reversed-phase HPLC for the isolation, purification as well as qualitative and quantitative determination of free phenolic acids in plants [18]. Fernandes et al. developed a capillary zone and micellar electrokinetic capillary chromatographic technique for the determination of a complex mixture of flavonoids, cinnamic acids and simple phenolic acids [19]. Gonzalez-SanJose et al. reported various applications of liquid chromatography–mass

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doi:10.1016/j.chroma.2004.04.041

spectrometry for the analysis of phenolic compounds [20]. Recently, hyphenated LC–UV–SPE–NMR using new cryoflowprobes has given a boost to the direct identification of compounds present in plant extracts [21].

Other methods than using liquid chromatography were also employed for the identification of some monomeric and dimeric phenolic acids [22]. Phenolic acids decompose when heated above their melting points (about 200 °C) [23]. Consequently, trimethylsilyl (TMS) derivatives of phenolic acids were prepared for gas chromatography analysis. Ng et al. developed a GC–MS method using anion-exchange disk extraction and TMS derivatives for the analysis of phenolic acids in distilled alcohol beverages [24]. Goldberg et al. applied a GC–MS method preceded by solid-phase extraction to measure the concentration of 15 phenolic components in wine [25] and Wu et al. used the method for the simultaneous determination of phenolic acids with allelopathic potential [26]. The application of microwave-accelerated digestion to promote the silylation reaction has also been reported [27]. The same silylation reaction was employed by other researchers to derivatize selected flavonoids [28–30], or flavonoids along with other acidic compounds [31]. Martinsen and Huhtikangas proposed a GC method for the determination of tropic, benzoic and cinnamic acid after derivatization by extractive alkylation with pentafluorobenzyl bromide [32].

In the paper herein, we investigated the potential of employing a new GC–MS method for the determination of phenolic acids and selected flavonoids. Methylated derivatives in methanolic plant extracts and aqueous infusions by means of two- and three-phase transfer catalysis (PTC) systems were prepared and chromatographed. Plant aqueous extracts were subjected directly to a combined process of derivatization–extraction, whereas methanolic extracts were processed in a like manner after condensation and redilution of a specific amount of the extract.

2. Experimental

2.1. Reagents and chemicals

Riedel-de Haën supplied GC grade solvents *n*-hexane, toluene, *tert*-butyl methyl ether, ethyl acetate and dichloromethane. Methyl iodide was of 99% purity (GC-grade), *n*-pentadecane, phase transfer catalysts [tetrabutylammonium bromide (TBAB), tetrahexylammonium bromide (THAB), cetyltrimethylammonium bromide (CTAB) and polymer-bound tri-*n*-butylmethylphosphonium chloride (1.4 mmol Cl^-/g resin)], phenolic acids and flavonoids, ACS grade sodium chloride, sodium hydroxide, sodium dihydrogen phosphate monohydrate, anhydrous sodium sulfate, and hydrochloric acid were all obtained from Sigma–Aldrich.

Methyliodide is a cancer suspect agent, so all work was carried out in a hood.

2.2. Solutions

Stock solutions of the individual phenolic acids and flavonoids in the mg/ml range, were prepared in a mixture of double distilled water (DDW)/methanol 50% under ultrasonication to facilitate dissolution, when necessary. A series of working standard solutions were prepared by serial dilution to fulfill the requirements for the construction of the calibration curves. Phosphate buffer solution (1.0 M) was prepared by dissolving 13.8 g of sodium dihydrogen phosphate monohydrate in 100 ml DDW; the pH was adjusted to 8.0 or 10.0 with NaOH.

TBAB, THAB and CTAB stock solutions were prepared in ethyl acetate and dichloromethane at concentrations 0.1 M. Stock solution of *n*-pentadecane, used as internal standard to compensate for differences in injection volumes, was prepared at a concentration of 2.12 mg/ml in both dichloromethane and ethyl acetate.

2.3. Instrumentation

The GC analyses were performed using a Hewlett-Packard 6890 Series GC System interfaced to an HP 5973 mass-selective detector. The mass spectra for the methylated analytes were obtained via electron impact ionization (EI) at 70 eV. The MS detector transfer line was maintained at 280 °C and tuning was performed on a daily basis with perfluorotributylamine (PFTBA) with the masses *m*/*z* 69, 219, 502. Detection was performed in the selective ion monitoring (SIM) mode and peaks were identified and quantitated using target ions as given in Tables 1 and 2.

The GC was equipped with split/splitless HP6890 Series injectors with auto sampler controller and a Supelco SPBM-5 capillary column (Bellefonte, PA, USA) (30 m × 0.32 mm i.d., 0.25-μm film thickness) for the separation of the derivatives, with helium of 99.999% purity as carrier gas.

2.4. Sample preparation

2.4.1. Methanolic extracts

An amount of 18 g of dried aerial parts of the plants: *Mentha spicata*, *Tilia europea*, *Urtica dioica* and *Hypericum perforatum*, which were collected in the period form spring to summer of 2003, from the region of Epirus, northwestern Greece were ground to pass a 0.4 mm sieve and extracted with methanol in a Soxhlet apparatus for 12 h. The excess methanol was evaporated in vacuo in a flash evaporator and the completely condensed extracts were kept at −40 °C. For the analyses, specific quantities of the extracts were redissolved in phosphate buffer/methanol solution, 80:20 (v/v).

2.4.2. Aqueous infusions

Aqueous infusions were prepared by adding 2 g of the commercially available dried plants of *Mentha spicata*,

Table 1
Characteristic ions and relative abundances for the major chromatographic peaks of the derivatized phenolic acids

Analyte	MW of the derivative formed	M^+	$M^+ - 15$	$M^+ - 29$ (−28)	$M^+ - 31$ (−30)	Other ions
p-Hydroxy benzoic acid	138 + 2Me = 166	166 (30)[a]			135 (100)[a]	107 (10), 92 (10), 77 (15)
trans-Cinnamic acid	148 + Me = 162	162 (60)[a]			131 (100)[a]	103 (80), 77 (50), 51 (40)
Homovanillic acid	182 + 2Me = 210	210 (10)[a]		181 (100)[a]		169 (10), 161 (10)
Vanillic acid	168 + 2Me = 196	196 (90)[a]	181 (5)		165 (100)[a]	137 (40), 125 (30)
2-Hydroxy cinnamic acid	164 + 2Me = 192	191 (60)[a]			161 (100)[a]	179 (25), 137 (30), 131 (15), 118 (20)
4-Hydroxy cinnamic acid	164 + 2Me = 192	192 (70)[a]			161 (100)[a]	178 (5), 133 (30), 118 (10)
Syringic acid	198 + 2Me = 226	226 (100)[a]	211 (60)[a]		195 (50)	154 (30), 125 (20), 77 (40)
Ferulic acid	194 + 2Me = 222	222 (100)[a]	207 (15)		191 (60)[a]	178 (15), 164 (25), 147 (20), 133 (30)

[a] Target ions used for the identification and quantitation.

Table 2
Characteristic ions and relative abundances for the major chromatographic peaks of the derivatized flavonoids

Analyte	MW of the derivative formed	M^+	$M^+ - 18$	$M^+ - 29$ (−28)	$M^+ - 31$ (−30)	Other ions
Naringenin	272 + 2Me = 300	300 (90)[a]	283 (5)		272 (5)	193 (20), 166 (25), 134 (100)[a], 121 (80)
Galangin	270 + 3Me = 312	311 (100)[a]	293 (40)[a]		281 (30)	221 (30), 181 (20), 164 (10), 142 (40)
Kaempferol	286 + 3Me = 328	327 (100)[a]	309 (20)		297 (30)	285 (50)[a], 167 (15), 135 (35), 107 (10)
Luteolin	286 + 3Me = 328	328 (100)[a]		299 (20)[a]		206 (20), 166 (40)[a], 150 (40)

[a] Target ions used for the identification and quantitation.

Origanum dictamnus, *Rosemarinus officinalis* and *Sideritis cretica* to 250 ml of boiling DDW and stirred for 30 min. The plant residue was then filtered and the filtrate was analyzed for phenolic acids and flavonoid content.

2.5. Analytical procedure

In a typical procedure, a portion of 10 ml of a standard solution or of a plant extract, containing 0.5 ml of phosphate buffer of pH 8.0 for the analysis of phenolic acids or of pH 10.0 for analysis of both phenolic acids and flavonoids, was transferred to a tube with PTFE-lined screw-caps. To this solution were added the appropriate amount of phase-transfer catalyst in the soluble or polymer-bound form, 1 ml of extraction solvent, 10 μl of the internal standard and 180 mg methyl iodide. The reaction tube was sealed and vigorously stirred with a magnetic stirrer for 30 min, for analyzing phenolic acids and 90 min for total analysis, at 70 °C, so that the vortex formed was spread throughout the liquid volume and the two phases were suitably in close contact. The mixture was allowed to cool down and was saturated with sodium chloride for the complete extraction of the derivatives into the organic phase. After phase separation, the organic layer was dried with anhydrous sodium sulfate and was subjected to GC analysis.

An analytical set for the construction of calibration curve consisted of triplicate six analytical standards of various concentrations. Recalibration was taking place for checking the stability of the system and chromatographic column performance. The derivatives were quantified by the area ratios relative to the IS.

2.6. Chromatographic analysis

An aliquot of 1 μl was injected splitless, with a 50:1 split being activated 1 min after the injection. The injector and detector temperatures were set at 260 and 280 °C, respectively. The oven temperature for GC analysis started at 50 °C with 5 min hold. Then temperature was programmed at 5 °C/min to 150 °C and from 150 to 210 °C at 10 °C/min with 11 min hold; the total run time was 45 min. The mass spectrometer started its run 3 min after the injection and stopped at the end of the GC run, whereby the mass range from 50 to 500 was recorded. The control of the GC–MS system as well as the acquisition and processing of the chromatograms received, were carried out by means of the Enhanced ChemStation G1701AA, Version A.03.00 (copyright © HP 1989–1996).

3. Results and discussion

3.1. Confirmation of the derivatives

The selection of the phenolic acids and flavonoids to be studied was based on their abundance in the plants extracted and their structural relevance (e.g. isomers like 2-hydroxy cinnamic and 4-hydroxy cinnamic acid, kaempferol and luteolin). The chemical structures of the studied compounds are illustrated in Fig. 1.

Upon the simultaneous PTC methylation–extraction, hydroxyl groups of flavonoids and hydroxyl and carboxyl groups of phenols are converted into their corresponding methyl ethers and esters and subsequently extracted in

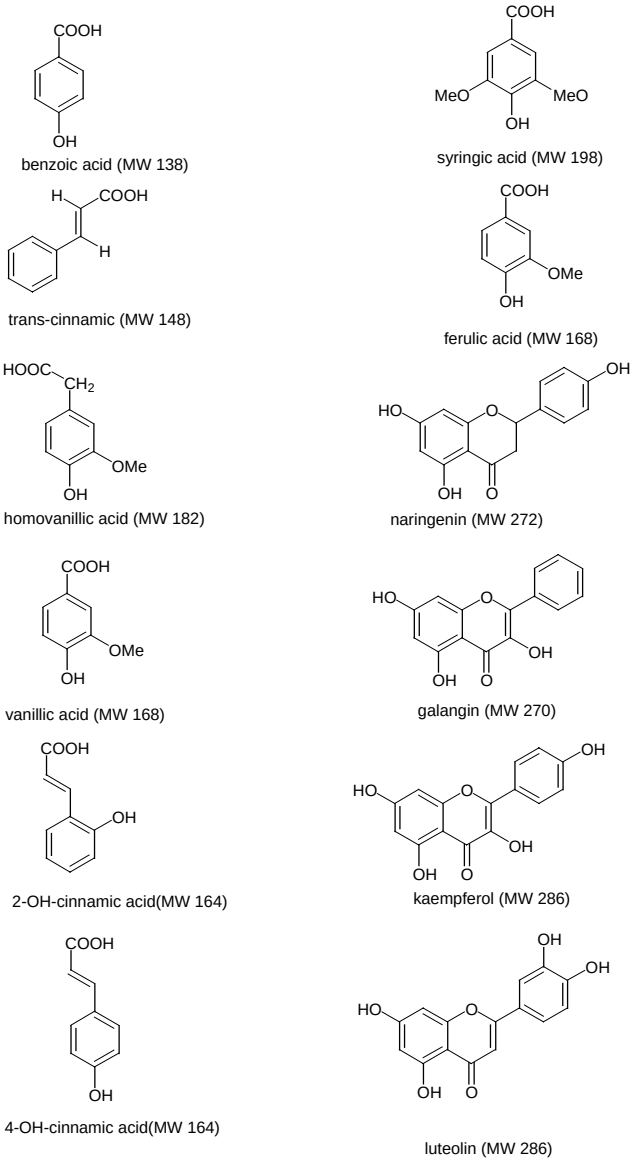

Fig. 1. Chemical structures of the selected analytes.

the minor organic phase. Based on the spectra information gathered in Tables 1 and 2, it is evident that in the majority of the derivatives the base peaks coincide with the molecular ions or with the $[M-31]^+$. Prominent ion fragments originate, mostly, from the cleavage of bonds between phenolic oxygen and carbon of methyl-group (i.e. $[M-15]^+$) and the detachment of the methoxy group from the formed ester (i.e. $[M-31]^+$). It is to be noted that for flavonoids, methylation renders more than one derivative and the most intense chromatographic peak was chosen for the analysis. Flavonoids follow distinct fragmentation pattern with detachment of the B ring (phenyl) and/or the B with parts of the C ring (pyranone). The methylation of naringenin generated the dimethoxy derivative, 5-hydroxy-4′,7-dimethoxyflavanone (RT 30.92 min), as main product, with fragmentation pattern matching the respective spectrum of the NIST/EPA Mass Spectra Library. Four trimethoxy isomeric derivatives of naringenin observed to a much lesser degree, account for methylation in the carbonyl oxygen, as well. Galangin gave one major trimethoxy derivative, the 2,5,7-trimethoxyflavone (RT 32.37 min) with MS pattern matching the respective of the MS library. The dimethoxy derivative was scarcely observed. The derivatization of kaempferol led to a trimethoxy (RT 34.88 min) and a tetramethoxy derivative with the latter being of no use to quantitation purposes. Finally, luteolin, upon methylation, gave only one trimethoxy derivative (RT 38.44 min) intensive enough for quantitation.

3.2. Optimization of derivatization conditions

The concept of the simultaneous PTC derivatization and extraction is rationalized as follows: The hydroxyl and carboxyl groups of the compounds present in the aqueous phase are deprotonated and the anionic nucleophiles are transferred into the organic phase as ion-pairs. The “naked” analyte anions in the organic phase or at the interface, react with methyl iodide towards the formation of methylated derivatives, which remain in the organic phase [33].

In order to optimize the conditions for PTC-based analysis of the phenolic acids and flavonoids, several sets of experiments should be conducted including: kind and concentration of catalyst, pH, organic solvent, time and temperature needed, stirring and concentration of methyl iodide.

The concentration of TBAB, THAB and of the cationic surfactant CTAB tested as soluble catalysts was varied from 10^{-4} to 10^{-1} M. It was found that the extent of methylation is increased with the increasing amount of PTC. The THAB at a concentration of 10^{-3} M was proved to be better suited than the reported TBAB and CTAB in terms of reaction product yields and time required, despite its noteworthy higher hydrophilicity.

Apart from the quaternary salts in the soluble form, for the methylation and extraction of the studied analytes the polymer-bound tri-*n*-butylmethylphosphonium chloride was employed as a PTC, which behaves as an ion-exchange resin and can bound to anionic analytes [34]. The catalyst recovery and regeneration from the reaction mixture is a unique advantage of the polymer-bound tri-*n*-butylmethylphosphonium chloride as a PTC [35]. An amount of more than 15 mg of the aforementioned catalyst was enough to attain better results than those of the catalysts in soluble form, as we inferred from the reaction yields. The amount of 17 mg was finally selected for subsequent experiments to compensate for higher concentrations of phenolic acids, flavonoids and possible interferences, which may interact with the resin.

Bearing in mind that the ionized analytes are the reactive species to be transferred into the organic phase as ion-pairs, the optimum pH value was tested in the pH range 4–11 by successive PTC methylation reactions. It is, therefore, advisable to raise the pH at least two units above the pK_a value

of the weakest acid to ensure all analytes are ionic. The phenolic acids concerned do not vary in acidity having pK_{a1} and pK_{a2} values in water around 4.5 and 9.5, respectively [36,37]. In contrast, the pK_a values of most flavonoids are unknown. Some of them have been reported to vary from 7.3 to 12.5 [38]. The ionization degree of hydroxyl groups is related to the number and position of the free hydroxyl groups on the flavonoid skeleton. At pH 8.0 adequate methylation for the phenolic acids occurs but even better yields were acquired at pH 10.0 where analytes appear completely as monoanions and partially as dianions. As for flavonoids, at pH < 9 they are scarcely detected as methylated derivatives in the organic phase while their derivatization yield increases at higher pHs. Despite the high yields, the catalysts involved in soluble and polymer-bound format are unstable under highly basic pH conditions [39]. Taking account of the above-mentioned, a pH $= 10$ was selected as the optimum. However, it is advisable that the derivatization can be performed at pH 8.0 if only the analysis of phenolic acids is aimed.

The distribution of PTC reagents between water and an organic medium depends, to a large extent, on the nature of the latter. Several organic solvents immiscible with water such as hexane, toluene, dichloromethane, *tert*-butyl methyl ether and ethyl acetate were tested. Dichloromethane and ethyl acetate showed, by far, the most favorable behavior with respect to extraction and derivatization yield. But dichloromethane was preferred to ethyl acetate due to its capability to exclude extraneous interfering peaks from the chromatograms, the better volatility and its greater immiscibility with water. Dichloromethane as the organic solvent in combination with saturated NaCl for salting out effects after the derivatization, enhanced the performance of the reaction system.

The extractive methylation reactions in aqueous solutions were further examined using dichloromethane as the organic phase containing methyl iodide as derivatising agent at basic pH ambience with the tri-phase PTC system, under stirring. The methylation of the phenolic acids at pH 10.0, progresses rapidly in the first 10 min and reaches completeness after almost 15 min. In pH 8.0 the reaction needs 20 min to finish. The derivatization reaction of the flavonoids was a pronouncedly slower procedure progressing to completeness in 90 min at pH 10.0. The absence of the acidic moiety and the multiple electronic effects in the aromatic rings of the flavonoid molecules are mainly responsible for the striking difference in the derivatization time and the multiple methylation products formed.

Temperature is expected to be a rate-determining parameter on the reaction kinetics and product yield. Increasing temperature accelerates the product formation, which is the case for both types of analytes. However, high temperatures can be the cause of degradation of the tri-phase PTC system due to its low thermal stability at elevated temperatures. For the above reasons, the temperature of 70 °C was selected as the optimum.

The importance of magnetic stirring in comparison with static conditions as well as with sonication was brought out. A close dependence of derivative formation and stirring speed indicates that the anticipated interfacial phenomena are consequential. The reaction kinetics are significantly faster when the surface of the reaction interface is increased by increasing stirring rate and pronouncedly lower under no-stirring conditions. In addition to above, it was demonstrated that sonication along with stirring is quite advantageous to the PTC-methylation of phenolics, unequivocally better than mere sonication and slightly better than stirring alone. In our experiments, vigorous stirring under heating

Table 3
Analytical figures of merit of the method

Analyte	Calibration curve		r^{2a}	DLR[b] (μg/ml)	LOD[c] (μg/ml)	LOQ[d] (μg/ml)
	Slope ± s[e]	Intercept ± s[e]				
p-Hydroxy benzoic acid	0.0263 ± 0.0004	−0.0195 ± 0.0012	0.9900	0.024–2.579	0.008	0.024
trans-Cinnamic acid	0.0133 ± 0.0004	−0.0051 ± 0.0004	0.9978	0.024–2.183	0.008	0.024
Homovanillic acid	0.0876 ± 0.0008	0.0059 ± 0.0004	0.9980	0.009–1.984	0.003	0.009
Vanillic acid	0.0595 ± 0.0003	0.0004 ± 0.0004	0.9992	0.009–2.976	0.003	0.009
2-Hydroxy cinnamic acid	0.0426 ± 0.0009	−0.0169 ± 0.0011	0.9978	0.012–2.579	0.004	0.012
4-Hydroxy cinnamic acid	0.0635 ± 0.0004	−0.0261 ± 0.0014	0.9980	0.012–2.381	0.004	0.012
Syringic acid	0.0348 ± 0.0003	0.0070 ± 0.0004	0.9984	0.012–2.579	0.004	0.012
Ferulic acid	0.0360 ± 0.0004	−0.0415 ± 0.0017	0.9964	0.012–5.357	0.004	0.012
Naringenin	0.0858 ± 0.0005	−0.0036 ± 0.0004	0.9984	0.012–1.235	0.004	0.012
Galangin	0.0040 ± 0.0004	−0.0033 ± 0.0004	0.9984	0.115–2.103	0.038	0.115
Kaempferol	0.0105 ± 0.0001	−0.0114 ± 0.0022	0.9996	0.118–2.222	0.040	0.118
Luteolin	0.0042 ± 0.0004	−0.0021 ± 0.0005	0.9992	0.062–1.706	0.021	0.062

[a] Correlation coefficient. Calculated from three replicates for six concentration levels.
[b] DLR: dynamic linear range.
[c] Limit of detection.
[d] Limit of quantitation.
[e] ±Significance level 95%.

Table 4
Statistical treatment for the recovery function $X_{\text{std.addition}} = \alpha + b \times X_{\text{std.calibration}}$ ($n = 6$)

Analyte	$b \pm tS_b$ [a]	$\alpha \pm tS_\alpha$ [a]	t-Calculated b	t-Calculated α [b]
p-Hydroxy benzoic acid	0.96 ± 0.05	3.85 ± 11.18	2.0	1.0
trans-Cinnamic acid	0.98 ± 0.06	7.16 ± 10.44	1.0	1.9
Homovanillic acid	1.03 ± 0.08	4.50 ± 10.55	1.0	1.2
Vanillic acid	1.07 ± 0.09	3.66 ± 9.36	2.2	1.1
2-Hydroxy cinnamic acid	1.02 ± 0.03	5.45 ± 14.05	2.0	1.1
4-Hydroxy cinnamic acid	1.01 ± 0.03	5.28 ± 12.17	1.0	1.2
Syringic acid	0.97 ± 0.04	3.54 ± 10.88	2.1	0.9
Ferulic acid	1.08 ± 0.08	4.22 ± 14.29	2.8	0.8
Naringenin	1.03 ± 0.04	5.11 ± 12.62	2.1	1.1
Galangin	1.07 ± 0.08	2.95 ± 11.22	2.4	0.7
Kaempferol	1.09 ± 0.09	4.78 ± 10.16	2.8	1.3
Luteolin	1.02 ± 0.06	4.87 ± 11.08	1.0	1.2

Significance level 95%, t-tabulated ($\nu = 4$) = 2.8.
[a] S_b, S_α: standard deviation of slope and intercept, respectively.
[b] t-Calculated $b = (0 - b)/S_b$, t-calculated $\alpha = (0 - \alpha)/S_\alpha$.

Table 5
GC–MS analysis of herbal methanolic extracts expressed in ng/mg of dried plant extract

Analyte	*Tilia europea*	*Urtica dioica*	*Mentha spicata*	*Hypericum perforatum*
p-Hydroxy benzoic acid	37.3 ± 1.4	nd	280.6 ± 5.3	62.5 ± 1.7
trans-Cinnamic acid	139.4 ± 4.2	nd	nq	nd
Homovanillic acid	58.0 ± 3.0	3.0 ± 0.2	296.7 ± 4.2	124.1 ± 1.2
Vanillic acid	nq	2.5 ± 0.2	123.4 ± 3.7	nd
2-Hydroxy cinnamic acid	nd	nd	nd	nd
4-Hydroxy cinnamic acid	71.3 ± 3.5	43.65 ± 5.1	379.5 ± 6.6	24.1 ± 0.9
Syringic acid	2.89 ± 0.18	nq	nq	0.25 ± 0.08
Ferulic acid	41.59 ± 2.85	574.8 ± 5.6	5729 ± 6	71.8 ± 2.9
Naringenin	1.38 ± 0.12	nq	465.7 ± 7.0	23.6 ± 4.1
Galangin	nd	nd	nd	nd
Kaempferol	nd	nd	nd	236.6 ± 5.7
Luteolin	nd	nd	20245 ± 37	170.6 ± 6.8

Mean values of three replicates ± S.D.; nd, not detected; nq, not quantified.

was judged to be sufficient to drive the reaction yield to its highest within 20 min for the methylation of phenolic acids alone or 90 min for the analysis of both phenolic acids and flavonoids.

Methyl iodide is a very efficient methylating agent leading to high derivatization yields in rather short reaction time. It was used in the overwhelming excess of 180 mg/reaction vial, to ensure complete derivatization reaction.

Table 6
GC–MS analysis of herbal aqueous infusions expressed in μg/ml of water extract

Analyte	*Mentha spicata*	*Origanum dictamnus*	*Rosemarinus officinalis*	*Sideritis cretica*
p-Hydroxy benzoic acid	0.906 ± 0.003	nd	nd	2.791 ± 0.005
trans-Cinnamic acid	0.408 ± 0.002	0.399 ± 0.002	nd	0.615 ± 0.007
Homovanillic acid	0.377 ± 0.004	nd	1.142 ± 0.008	0.847 ± 0.004
Vanillic acid	nd	nd	nd	0.040 ± 0.005
2-Hydroxy cinnamic acid	nd	0.682 ± 0.003	nd	nd
4-Hydroxy cinnamic acid	0.790 ± 0.005	0.327 ± 0.003	0.765 ± 0.006	1.004 ± 0.004
Syringic acid	nd	6.705 ± 0.004	nd	1.186 ± 0.006
Ferulic acid	6.717 ± 0.012	0.057 ± 0.002	12.305 ± 0.007	4.878 ± 0.021
Naringenin	0.055 ± 0.005	nd	nd	nd
Galangin	nd	nd	nd	nd
Kaempferol	nd	1.217 ± 0.006	nd	nd
Luteolin	3.891 ± 0.008	nd	nd	nd

Mean values of three replicates ± S.D.; nd, not detected.

3.3. Method validation

3.3.1. Calibration curves

A series of standard composite mixture solutions were prepared in triplicate over the range of 0.005–5.357 μg/ml. The amounts injected and their respective response ratios (analyte-to-IS) were used for the construction of the calibration plots and quantification. A summary of the analytical data obtained is presented in Table 3. Satisfactory linearity was obtained for the employed GC–MS(SIM) method as demonstrated by correlation coefficients higher than 0.9900 throughout the method validation.

3.3.2. Limits of detection (LOD) and quantitation (LOQ)

The limit of detection (LOD) was estimated as $3S_b$/slope of the calibration curve where S_b is the standard deviation of the blank measurements ($n = 10$). The actual LOD was then determined by the analysis of samples of known concentrations and visually found to be between 3 and 40 ng/ml, for a sample volume of 10 ml. Similarly, the limit of quantitation (LOQ) was estimated as $10S_b$/slope of the calibration curve where S_b is the standard deviation of the blank measurements ($n = 10$). The method enabled quantitation of the compounds in the range 9–118 ng/ml, with flavonoids to possess the lowest sensitivity due to their multiple reaction behavior.

3.3.3. Method precision

To verify the precision of the proposed method, within-day and between-day precision of processed standards in the range 0.15–0.45 μg/ml were obtained. The overall relative standard deviations of the within-day repeatability ($n = 5$) and between-day reproducibility (five consecutive days, three replicates each day) were <2.3, and <4.8%, respectively. This demonstrates perfect repeatability of this method down to trace levels even for the flavonoids, which, in most instances, produce multiple products.

3.3.4. Matrix effects

The accuracy of the developed method was assessed by applying the standard addition method. In this way, the influence of matrix on analytes' reliable detection was ascertained. The method of standard addition was applied by analyzing unspiked methanolic extract of *Mentha spicata* and extract spiked to obtain concentrations up to four times higher than that of the unspiked sample. By plotting the "found concentrations" versus the original calibration concentrations for the twelve analytes the recovery curves were calculated. From Table 4 it is seen that the *t*-calculated are lower than *t*-tabulated at the 95% significance level. It can therefore be inferred that the slope and intercept of the recovery curves do not differ significantly from the ideal values of 1 and 0, respectively. Thus there is no evidence that the calibration curve obtained by spiking the extract differ significantly from that using standard solutions. The GC–MS(SIM) analysis of a fortified extract, as portrayed in Fig. 2, showed no interfering peaks in the proximity of the peaks of interest, allowing for baseline separation and absence of major chromatographic interferences. All four ether derivatives of the flavonoids are chromatographed shortly after the phenolic acid derivatives as a result of their lower volatility. These results, manifest absence of matrix effects and reliable measurement of phenolic acids and flavonoids by the developed PTC method.

3.3.5. Sample analysis

The method practicability and applicability under normal laboratory conditions was confirmed by the analysis of plant extracts for the determination of the 12 analytes. Initially, known quantities of condensed methanolic extracts of *Tilia europea*, *Urtica dioica*, *Mentha spicata* and *Hypericum perforatum* were analyzed in triplicate. The results of the analysis are tabulated in Table 5 as ng/mg of dried extract with the respective relative standard deviations varying up to 9.0%. Of relevance are the results obtained from the analysis of

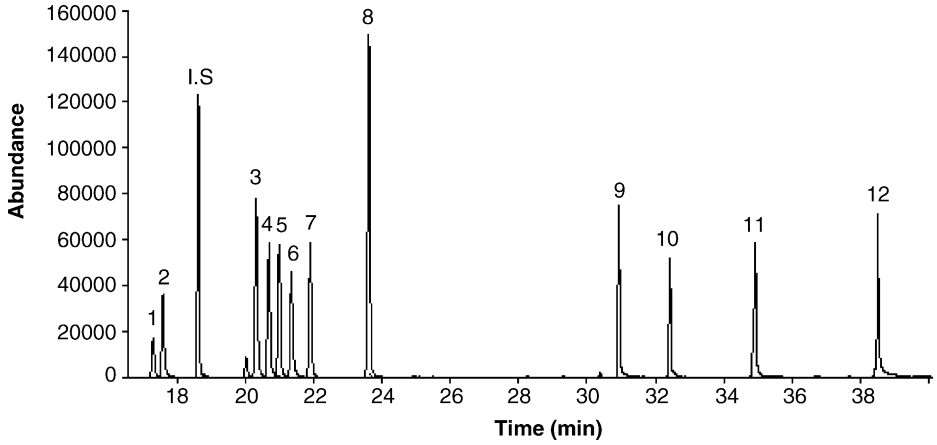

Fig. 2. The GC–MS(SIM) chromatogram of a *Mentha spicata* fortified extract. Peak assignment: (1) *p*-hydroxy benzoic acid; (2) *trans*-cinnamic acid; (3) homovanillic acid; (4) vanillic acid; (5) 2-hydroxy cinnamic acid; (6) 4-hydroxy cinnamic acid; (7) syringic acid; (8) ferulic acid; (9) naringenin; (10) galangin; (11) kaempferol; (12) luteolin; IS, internal standard.

the commercially available *Mentha spicata*, *Origanum dictamnus*, *Rosemarinus officinalis* and *Sideritis cretica* herbal infusions (Table 6). Although different from the quantitative point of view, the phenolic acid and flavonoid content of *Mentha spicata* in both samples present a remarkably similar qualitative pattern.

The total analysis time for an extract ranges from 40 to 50 min for phenolic acids and from 130 to 140 min for the sum of the twelve analytes.

4. Conclusions

The use of SIM acquisition during GC–MS in relation to the PTC process provided adequate detection limits for selected phenolic acids and flavonoids in methanolic extracts and aqueous infusions. The method combines the transfer of analytes into the organic phase with the in-situ derivatization and extraction, before the injection into the GC. As far as this is guaranteed from the derivatization conditions, this PTC method could be extended for the analysis of a large number of derivatized phenolic acids and flavonoids depending solely on the chromatographic conditions.

Acknowledgements

Funding for Dr. Fiamegos was generously provided by EU Large Scale Facilities program, contract HPRI-CT-2001-00178 ‘LC-NMR analysis of traditional herbal pharmaceuticals’ and ‘On line LC-SPE-NMR system in analyzing the bioactive constituents of *Artemisia* extract’ of the Wageningen NMR centre as well as HPRI-CT-1999-500187 Capillary NMR.

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