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Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS

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Abstract

It is well known that phenolic compounds are constituents of many plants and herbs, and they have attracted a great deal of public and scientific interest because of their health-promoting effects as antioxidants. Five plants, *Vitex agnus-castus* (Verbenaceae), *Origanum dictamnus* (Lamiaceae), *Teucrium polium* (Lamiaceae), *Lavandula vera* (Lamiaceae) and *Lippia triphylla* (Verbenaceae), were examined in order to determine their phenolic composition. Reversed phase high performance liquid chromatography was employed for the identification and quantification of phenolic compounds. Gas chromatography–mass spectometry(GC-MS) was also used for identification of phenolic compounds after silylation. Analysis of the non-volatile and thermolabile phenolic compounds by GC-MS presupposes their conversion into volatile and thermotolerant derivatives. The derivatization process was optimized against reagents, temperature and reaction time. A large excess of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing trimethylchlorosilane proved to be the best derivatization reagent to convert analytes into volatile trimethylsilyl derivatives. The most abundant phenolic compounds detected were caffeic acid (0.12–0.93 mg 100 g^{-1} dry sample), ferulic acid (0.34–1.52 mg 100 g^{-1} dry sample), and (+)-catechin (0.22–0.43 mg 100 g^{-1} dry sample).

Keywords: Phenolics; Silylation; Aromatic plants; HPLC; GC-MS

1. Introduction

Plant-derived polyphenols receive considerable interest because of their potential antioxidant and antimicrobial properties. Consumer concern over the possible adverse health effect of certain chemical preservatives, coupled with increasing demand for foods with long shelf-life, has resulted in increasing pressure on the food industry to withdraw chemically synthesized additives and to use ''natural'' alternatives (Roller, 1995). This consumer-led trend has fuelled a research for food preservatives derived from natural sources. Many plants are considered to be excellent sources of phenolic compounds that could be used, not only to preserve foods, but also to contribute to a healthy diet (Justesen & Knuthsen, 2001; Rice-Evans, Miller, & Paganga, 1997). The plant (poly)phenols are a diverse group of higher secondary metabolites, possessing an aromatic ring bearing one or more hydroxy substituents, derived from the shikimate pathway and phenylpropanoid metabolism (Ryan, Robards, Prenzler, & Antolovich, 1999). They include mainly simple phenols, phenolic acids, coumarins, tannins and flavonoids. These compounds usually occur in the form of glycosides or esters in plants. That is the reason for their tendency to be highly water-soluble (Harborne, 1998).

Phenolic compounds exhibit a considerable free-radical scavenging (antioxidant) activity, which is determined by their reactivity as hydrogen- or electron-donating agents, the stability of the resulting

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antioxidant-derived radical, their reactivity with other antioxidants and, finally, their metal chelation properties (Rice-Evans et al., 1997). Natural antioxidants, such as flavonoids, are associated with a reduced risk of cancer, chronic inflammation and cardiovascular disease (Middleton & Kandaswami, 1994). Although, these protective effects have been primarily attributed to food ingredients such as β-carotene and ascorbate, phenolic constituents may also play a significant role (Heim, Tagliaferro, & Bobilya, 2002; Hollman & Katan, 1999; Justesen & Knuthsen, 2001).

Plant polyphenols are considered to be antimicrobial agents, and they are proposed as potential food natural preservatives (Nychas, 1995; Tranter, Tassou, & Nychas, 1993). Although, the antimicrobial activity of phenolic compounds is recently established, the mechanism of their action on microorganisms has not yet been elucidated. Furthermore, contradictory data have been reported by different authors for the same antimicrobial compound (Nychas, Skandamis, & Tassou, 2003).

Undoubtedly, it is very important to determine phenols in aromatic plants, both qualitatively and quantitatively. A number of analytical methods have been proposed for the separation and determination of these compounds. Most of these protocols are based on a high performance liquid chromatography (HPLC) technique with UV spectrophotometry because derivatization is not required prior to analysis (Hertog, Hollman, & Venema, 1992; Justesen, Knuthsen, & Leth, 1998; Justesen & Knuthsen, 2001; Mattila, Astola, & Kumpulainen, 2000; Merken & Beecher, 2000; Mattila & Kumpulainen, 2002; Parrilla, Heredia, & Troncoso, 1999; Valentão, Andrade, Areias, Ferreres, & Seabra, 1999). However, compared to mass spectrometry (MS), the UV–vis spectrum does not supply sufficient identifying power (Chen, Zuo, & Deng, 2001; Suarez, Picinelli, & Mangas, 1996). Hence, capillary gas chromatography, coupled with mass spectometry (GC-MS) can provide more accurate results. Both methods have been used for the analysis of plant extracts. Analysis of the non-volatile and thermolabile phenolic compounds by GC-MS presupposes their conversion into volatile and thermotolerant ones by chemical derivatization (Angerosa, D'Alessandro, Konstantinou, & Di Giacinto, 1995; Chu, Chang, Liao, & Chen, 2001; Deng & Zito, 2003; Ryan et al., 1999; Van Beek, 2002; Zuo, Wang, & Zhan, 2002).

LC-MS has also been extensively used over the past years (Careri, Mangia, & Musci, 1998). The use of the hyphenated technique, liquid chromatography-mass spectrometry (LC-MS), in the analysis of plant extracts provides important advantages because of the combination of the separation capabilities of LC and the power of MS as an identification and confirmation method. There are recent reports in which steam-distilled material from Lamiaceae species was used to study the phenolics and antioxidant properties (Dorman, Bachmayer, Kosar, & Hiltunen, 2004; Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003).

The aim of this work was to determine plant phenolics by RP-HPLC, and to optimize a derivatization method prior to GC-MS analysis.

2. Materials and methods

2.1. Standards

Gallic acid, gentisic acid, *p*-coumaric acid, vanillic acid, ferulic acid, (+)-catechin, quercetin, apigenin, naringenin and eriodictyol were purchased from Sigma–Aldrich (Steinheim, Germany). Luteolin was from Roth (Karlsruhe, Germany). Caffeic acid was from Merck (Darmstadt, Germany). Rutin was from Alexis biochemicals (Lausen, Switzerland). Hydroxytyrosol, tyrosol, *p*-hydroxybenzoic acid and BHT (butylated hydroxytoluene) were a kind donation from the National Agricultural Research Foundation (N.AG.RE.F, Greece). All standards were prepared as stock solutions in methanol. Working standards were made by diluting stock solutions in 62.5% aqueous methanol containing BHT 1 g l^{-1}, and 6 M HCl to yield concentrations ranging from 0.5–25 mg l^{-1}. Stock working solutions of the standards were stored in darkness at −18 °C.

2.2. Solvents and reagents

All solvents and reagents from various suppliers were of the highest purity needed for each application. Silylation reagents, BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide), TMCS (trimethylchlorosilane), and HMDS (hexamethyldisilazane) were purchased from Merck (Darmstadt, Germany), respectively; 5% DMDCS (dimethyldichlorosilane) in toluene (used for deactivating glassware surfaces) was obtained from Sigma–Aldrich (Steinheim, Germany).

2.3. Samples

Samples of *Vitex agnus-castus* (Verbenaceae), *Origanum dictamnus* (Lamiaceae), *Teucrium polium* (Lamiaceae), *Lavandula vera* (Lamiaceae) and *Lippia triphylla* (Verbenaceae) were obtained from local stores. Leaves were dried at 25 °C in darkness and analyzed after grinding in a household blender. All samples were analyzed within 3 months of collection.

2.4. Sample preparation and derivatization

The extraction method used for dried samples was as follows: 40 ml of 62.5% aqueous methanol containing BHT (1 g l^{-1}) were added to 0.5 g of dried sample. Then

10 ml of 6 M HCl were added. The mixture was stirred carefully. In each sample, nitrogen was bubbled for ca. 40–60 s. The extraction mixture was then sonicated for 15 min and refluxed in a water bath at 90 °C for 2 h. The mixture was then: (a) filtered and made up to 100 ml with methanol (Hertog et al., 1992; Justesen et al., 1998), then filtered quickly through a 0.45 μm membrane filter (Millex-HV) and injected to HPLC or (b) extracted with 30 ml (3 × 10 ml) ethyl acetate. The organic layer was collected and reduced to 10 ml by rotary evaporation (37 °C) and centrifuged for 10 min. Anhydrous Na_2SO_4 was then added to remove moisture. Then, 100 μl of the organic layer were derivatized after evaporation of the solvent under a nitrogen stream. For the silylation procedure, a mixture of TMCS (100 μl) and BSTFA (200 μl) was added and vortexed in screw-cap glass tubes (previously deactivated with 5% DMDCS in toluene, and rinsed twice with toluene and thrice with methanol), and consecutively placed in a water bath, at 80 °C for 45 min. Apart from a water bath a microwave oven at high power (600 W) could have been used (Chu et al., 2001).

A different method of silylation was also tried, in duplicate, upon two plants, *V. agnus-castus* and *T. polium*: HMDS + TMCS + pyridine in proportions of 3:1:9 (v/v/v), respectively, were used as derivatizing reagents This method has been applied to the silylation of standard phenolic compounds by Creaser, Koupai-Abyazani, and Stephenson (1989). From the silylated mixture, 1 μl was directly analyzed by CGC-MS.

To prevent enzymic oxidation, extraction of the polyphenols from plants with boiling alcohol is essential and should be routinely adopted (Harborne, 1998). For the same reason, all this work was carried out in the dark and under a nitrogen atmosphere.

2.5. HPLC analysis

The analytical HPLC system employed consisted of a JASCO high performance liquid chromatograph coupled with a UV–vis multiwavelength detector (MD-910 JASCO). The analytical data were evaluated using a JASCO data processing system (DP-L910/V). The separation was achieved on a Waters Spherisorb® 5 μm ODS2 4.6 × 250 mm column at ambient temperature. The mobile phase consisted of water with 1% glacial acetic acid (solvent A), water with 6% glacial acetic acid (solvent B), and water–acetonitrile (65:30 v/v) with 5% glacial acetic acid (solvent C). The gradient used was similar to that used for the determination of phenolics in wine (Parrilla et al., 1999) with some modifications: 100% A 0–10 min, 100% B 10–30 min, 90% B/ 10% C 30–50 min, 80% B/ 20% C 50–60 min, 70% B/ 30% C 60–70 min, 100% C 70–105 min, 100% A 105–110 min; post-time 10 min before next injection. The flow rate was 0.5 ml min^{-1} and the injection volume was 20 μl. The monitoring wavelength was 280 nm. The identification of each compound was based on a combination of retention time and spectral matching.

2.6. CGC-MS

The silylated samples were injected into a CGC-MS system consisting of a Fisons GC 8000 Series, model 8060 gas chromatograph coupled with a Fisons MD 800 mass spectrometer in the EI (Electron Impact) mode with the electron energy set at 70 eV and the mass range at *m/z* 25–700. A capillary column Low-bleed CP-Sil 8 CB-MS (30 m × 0.32 mm, i.d.), of 0.25 μm film thickness of coated material was used. The injector was set at 280 °C and the detector at 290 °C. GC was performed in the splitless mode with 1 min splitless-time. The temperature programme was as follows: from 70 to 135 °C with 2 °C min^{-1}, hold for 10 min, from 135 to 220 °C with 4 °C min^{-1}, hold for 10 min, from 220 to 270 °C with 3.5 °C $min^{-1}$and then hold for 20 min. A post-run of 10 min at 70 °C was sufficient for the next injection. The flow rate of carrier gas (helium) was maintained at 1.9 ml min^{-1}. Identification of compounds was achieved by comparing the retention times with those of authentic compounds and the spectral data obtained from the Wiley and NIST libraries. Each determination was carried out in duplicate.

3. Results and discussion

3.1. HPLC analysis

Before HPLC analysis, hydrolysis of glycosides or esters was necessary, so as to determine phenolic content, since a considerable fraction is in bound form (Lee & Widmer, 1996).

Extraction was performed with a mixture of 62.5% aqueous methanol. Methanol has a protective role. It can prevent phenolic compounds from being oxidized by enzymes, such as phenoloxidases (Harborne, 1998).

Columns employed to separate phenolics are almost exclusively reversed-phase. This system is a high resolution chromatographic technique widely used for simultaneous separation and quantification of phenolic substances.

The identification of each compound was based on a combination of retention time and spectral matching, since polyphenols absorb in the ultraviolet (UV) region. According to the literature, most benzoic acid derivatives show an absorption maximum at 246–262 nm with a shoulder at 290–315 nm, except gallic acid that shows a maximum at 271 nm (Lee & Widmer, 1996). Two absorption bands are characteristic of flavonoids. Band II with a maximum in the 240–285 nm range, is believed to arise from the A-ring. Band I, with a maximum in the

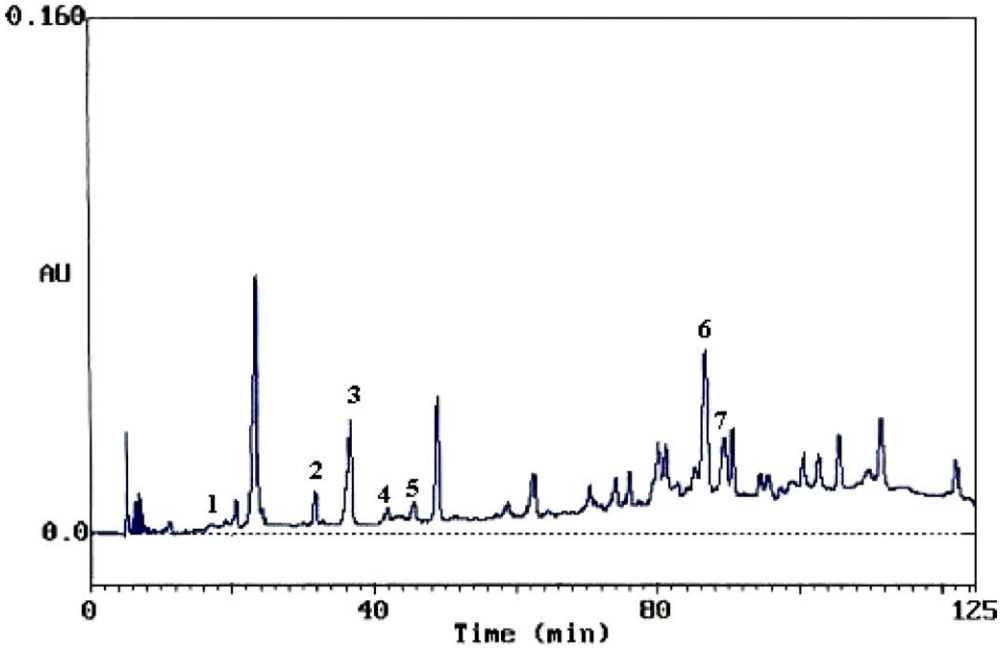

Fig. 1. Typical HPLC chromatogram of *Lavandula vera*: (1) gallic acid, (2) *p* -hydroxybenzoic acid, (3) (+)-catechin, (4) vanillic acid, (5) caffeic acid, (6) ferulic acid and (7) naringenin.

Table 1
Contents of phenolic compounds in five aromatic plant extracts

	Vitex agnus-castus		*Origanum dictamus*		*Teucrium polium*		*Lavandula vera*		*Lippia triphylla*	
	mg 100 g^{-1} dry sample[a]	RSD[b] (%)	mg 100 g^{-1} dry sample	RSD (%)	mg 100 g^{-1} dry sample	RSD (%)	mg 100 g^{-1} dry sample	RSD (%)	mg 100 g^{-1} dry sample	RSD (%)
Phenolic compounds										
Gallic acid	tr[c]	–	tr	–	ND[d]	–	tr	–	ND	–
p-Hydroxybenzoic acid	0.15 ± 0.01	6.7	ND	–	ND	–	0.18 ± 0.01	5.6	ND	–
Gentisic acid	ND	–	ND	–	ND	–	ND	–	ND	–
Hydroxytyrosol	ND	–	ND	–	ND	–	ND	–	0.40 ± 0.02	5.0
Tyrosol	0.13 ± 0.01	7.7	ND	–	0.42 ± 0.01	2.4	ND	–	ND	–
(+)-Catechin	ND	–	0.22 ± 0.01	4.5	ND	–	0.43 ± 0.01	2.3	ND	–
Vanillic acid	ND	–	ND	–	ND	–	0.11 ± 0.01	9.1	ND	–
Caffeic acid	0.93 ± 0.02	2.2	ND	–	0.65 ± 0.01	1.5	0.12 ± 0.01	8.3	0.84 ± 0.02	2.4
p-Coumaric acid	0.75 ± 0.02	2.7	ND	–	ND	–	ND	–	ND	–
Rutin	1.58 ± 0.01	0.6	ND	–	ND	–	ND	–	ND	–
Ferulic acid	1.52 ± 0.02	1.3	0.34 ± 0.01	2.9	0.95 ± 0.02	2.1	0.53 ± 0.01	1.8	0.82 0.02	2.4
Naringenin	ND	–	ND	–	ND	–	0.26 ± 0.01	3.8	ND	–
Eriodictyol	ND	–			ND	–	ND	–	ND	–
Apigenin	ND	–	ND	–	ND	–	ND	–	0.24 ± 0.01	4.2
Luteolin	ND	–	ND	–	0.48 ± 0.01	2.1	ND	–	ND	–
Quercetin	ND	–	ND	–	ND	–	ND	–	ND	–

[a] Each value is the mean (mg 100 g^{-1} dry sample) of three replications ± standard deviation.
[b] RSD = SD/mean * 100.
[c] tr: concentration < 1.16 mg l^{-1}.
[d] ND: not detected.

300–550 nm range is attributed to the B-ring (Merken & Beecher, 2000).

After extraction and acid hydrolysis, the content of phenolic substances was determined by quantitative HPLC analysis. A typical HPLC chromatogram of *L. vera* is presented in Fig. 1. The amounts of phenolic compounds detected in the samples are presented in Table 1. Results are expressed in mg 100 g^{-1} of dry sample. The most abundant phenolic compounds detected were caffeic acid (0.12–0.93 mg 100 g^{-1} dry sample), ferulic acid (0.34–1.52 mg 100 g^{-1} dry sample), and (+)-catechin (0.22–0.43 mg 100 g^{-1} dry sample).

Table 2
Linear calibration curves for the HPLC analysis of the most abundant phenolic compounds

Compound	$y = \alpha\chi + b$		R^2	LOD[a] (mg/l)
	Slope ($\alpha \pm$ SD[b])	Intercept ($b \pm$ SD)		
(+)-Catechin	0.004 ± 3.02 E − 05	+0.002 ± 0.002	0.9997	1.65
Caffeic acid	2.127 ± 0.019	−1.766 ± 0.355	0.9998	0.5
Ferulic acid	2.067 ± 0.003	−0.350 ± 0.033	0.9999	0.05

[a] Limit of detection.
[b] Standard deviation.

V. agnus-castus leaves contained the highest amount of caffeic and ferulic acids (0.93 mg caffeic acid 100 g^{-1} dry sample and 1.52 mg ferulic acid 100 g^{-1} dry sample). (+)-Catechin was detected only in *O. dictamnus* (0.22 mg (+)-catechin 100 g^{-1} dry sample) and *L. vera* (0.43 mg (+)-catechin 100 g^{-1} dry sample).

Linear regression analysis, using the least squares method, was used to evaluate the calibration curve of each analyte as a function of its concentration. The limit of detection (LOD) was estimated as 3.3 S_b/slope of the calibration curve, where S_b was the standard error of the intercept (b) (95% confidence limit). Regression analysis of the peak area ratio (y) vs. concentration (x) for the most abundant phenolic compounds is shown in Table 2. Concentrations of phenolics (mg l^{-1}) were above the corresponding limit of detection.

3.2. GC-MS analysis

Silylation is an ideal procedure for the GC analysis of non-volatile and thermolabile compounds. Compared to their parent compounds, TMS derivatives are more volatile, less polar and more thermotolerant. In silylation, an active hydrogen in –OH, –COOH, =NH, $–NH_2$ or –SH is replaced by a trimethylsilyl group. Silylation is a nucleophilic substitution reaction. It is viewed as a nucleophilic attack upon the silicon atom of the silyl donor, producing a bimolecular transition state. The silyl compound leaving group must be of low basicity and able to stabilize a negative charge in the transition state (Chu et al., 2001).

Several variables were examined to determine their roles in the derivatization process: the reactivity of variable derivatizing agents, reaction time, temperature, and the amount of the silylating agent required to complete the derivatization.

For the preparation of the TMS derivatives, mixtures such as HMDS, TMCS and pyridine 3:1:9 (v/v/v) and BSTFA, TMCS 2:1 (v/v), have been employed. Silylation reagents are influenced by both the solvent system and the addition of a catalyst. A catalyst (e.g., TMCS or pyridine) increases the reactivity of the reagent.

Attempts to silylate phenolic compounds with mixtures of HMDS, TMCS and pyridine proved to be unsuccessful for most of the compounds, because HMDS was a rather weak trimethylsilyl donor. In contrast to the unsuccessful silylation with HMDS, all the phenolic compounds were successfully converted into TMS derivatives with BSTFA and TMCS. In a recent report, a large excess of the derivatization reagent BSTFA and TMCS was used for the determination of phenolic antioxidants in American cranberry juice (Zuo et al., 2002). BSTFA was also used for the derivatization of phenolic constituents in wines (Soleas, Diamandis, Karumanchiri, & Goldberg, 1997), and in white juices and wines from Spain (Betés-Saura, Andrés-Lacueva, & Lamuela-Raventós, 1996).

In our study, the temperature and reaction time used were sufficient for the silylation of phenolic compounds. Apigenin, luteolin, naringenin and eriodictyol displayed very poor sensitivity to the derivatization process, even at concentrations as high as 30 mg l^{-1}. In our case, BSTFA and TMCS were used in excess to ensure that the silylation was complete. Care was taken to ensure anhydrous conditions during the derivatization process because of the high sensitivity of trimethylsilyl (TMS) derivatives toward moisture. For this purpose, anhydrous Na_2SO_4 was added.

The GC oven temperature programme, as well as the injector and detector temperatures, was based on previous experience with the analysis of marker compounds in *Ginkgo biloba* L. extract (Deng & Zito, 2003).

Prior to employing GC-MS for the determination of phenolic compounds in plant extracts, a standard mixture of all substances was tested, after derivatization. A TIC chromatogram of phenolics from *L. vera* is presented in Fig. 2. Data obtained showed excellent resolution between all compounds of interest. Retention times of silylated phenolic compounds in the examined plant extracts are presented in Table 3. Their molecular weights (MW) and characteristic fragments are presented in Table 4. For example, hydroxytyrosol shows a molecular ion (M^+) at m/z 370 and a main peak at m/z 267 ($M^+ - 103$). The fragmentation mechanism of simple phenols, such as hydroxytyrosol, has already been studied by other researchers (Angerosa et al., 1995). Peaks related to complex phenols with high molecular masses were also identified by the electronic libraries. However, their mass spectral fragmentations have been fully interpreted in the literature (Owen et al., 2003; Zuo et al., 2002). MS spectrograms of three

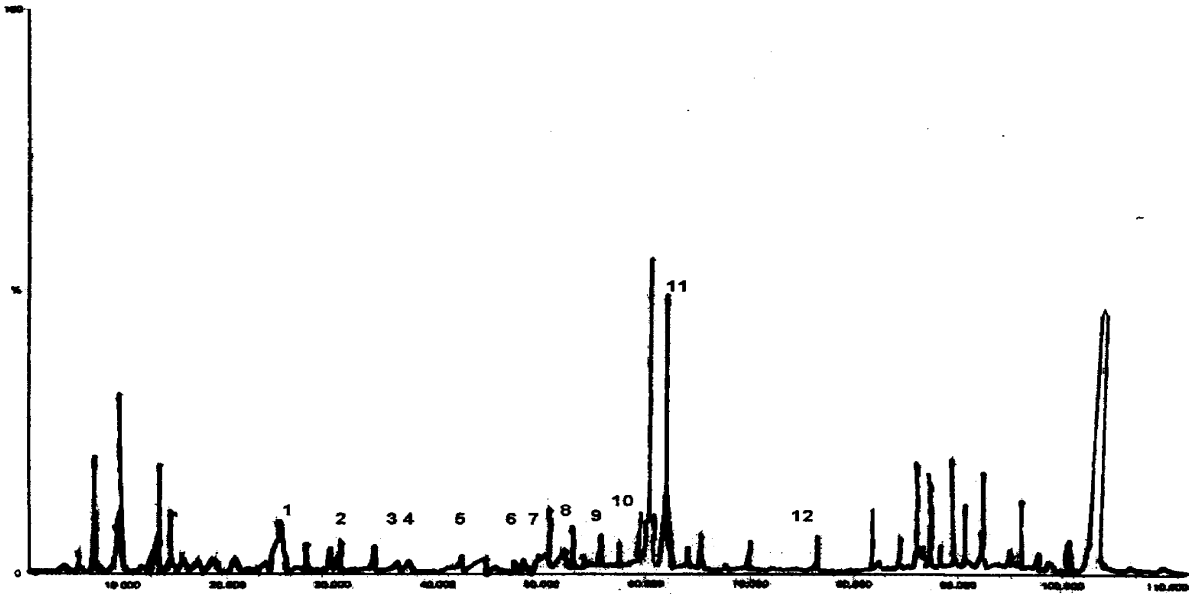

Fig. 2. TIC chromatogram of phenolics from *Lavandula vera*: (1) coumarin, (2) tyrosol, (3) hydroxytyrosol, (4) *p*-hydroxybenzoic acid, (5) *p*-hydroxyphenylpropionic acid, (6) vanillic acid, (7) *p*-coumaric acid, (8) *o*-coumaric acid, (9) hydroxycaffeic acid, (10) gallic acid, (11) caffeic acid and (12) 3-nitro-phthalic acid. Retention times of the examined plant extract are those presented in Table 3.

Table 3
Results after GC-MS analysis of the derivatized plant extracts with BSTFA and TMCS

	Vitex agnus-castus		*Origanum dictamus*		*Teucrium polium*		*Lavandula vera*		*Lippia triphylla*	
	t_R[a](min)	RSD[b] (%)	t_R (min)	RSD (%)	t_R (min)	RSD (%)	t_R (min)	RSD (%)	t_R (min)	RSD (%)
Phenolic compounds										
Coumarin	ND[c]	–	ND	–	ND	–	25.1 ± 0.1	0.4	ND	–
Tyrosol	ND	–	ND	–	ND	–	33.2 ± 0.2	0.6	ND	–
o-Hydroxybenzoic acid	ND	–	ND	–	36.2 ± 0.1	0.3	ND	–	36.3 ± 0.2	0.5
Hydroxytyrosol	ND	–	36.8 ± 0.1	0.3	36.9 ± 0.1	0.3	36.8 ± 0.2	0.5	36.9 ± 0.2	0.5
p-Hydroxybenzoic acid	37.5 ± 0.2	0.5	ND	–	37.3 ± 0.2	0.5	37.4 ± 0.2	0.5	ND	–
p-Hydroxyphenylpropionic acid	ND	–	41.3 ± 0.3	0.7	ND	–	41.9 ± 0.3	0.7	ND	–
Vanillic acid	48.3 ± 0.3	0.6	ND	–	48.2 ± 0.3	0.4	48.2 ± 0.3	0.6	ND	–
Gentisic acid	ND	–	ND	–	49.2 ± 0.2	0.4	ND	–	ND	–
p-Coumaric acid	49.8 ± 0.2	0.4	ND	–	ND	–	49.7 ± 0.2	0.4	ND	–
o-Coumaric acid	ND	–	ND	–	ND	–	50.6 ± 0.2	0.4	ND	–
3,4-Dihydroxybenzoic acid	52.0 ± 0.2	0.3	ND	–	ND	–	ND	–	ND	–
Hydroxycaffeic acid	55.1 ± 0.2	0.4	55.2 ± 0.2	0.4	ND	–	55.1 ± 0.3	0.5	55.2 ± 0.3	0.5
Gallic acid	ND	–	ND	–	ND	–	57.0 ± 0.2	0.3	ND	–
Ferulic acid	60.2 ± 0.2	0.3	ND	–	60.5 ± 0.2	0.3	ND	–	ND	–
Caffeic acid	61.4 ± 0.2	0.3	61.7 ± 0.2	0.3	61.7 ± 0.1	0.2	61.5 ± 0.2	0.3	ND	–
3-Nitro-phthalic acid	74.6 ± 0.1	0.1	ND	–	74.5 ± 0.3	0.4	74.3 ± 0.4	0.5	74.4 ± 0.3	0.4
(+)-Catechin	ND	–	88.9 ± 0.1	0.1	ND	–	ND	–	ND	–
Naringenin	ND	–	ND	–	ND	–	ND	–	ND	–
Eriodictyol	ND	–	ND	–	ND	–	ND	–	ND	–
Apigenin	ND	–	ND	–	ND	–	ND	–	ND	–
Luteolin	ND	–	ND	–	ND	–	ND	–	ND	–
Quercetin	ND	–	ND	–	94.1 ± 0.2	0.2	ND	–	ND	–
Rutin	ND	–	ND	–	ND	–	ND	–	ND	–

[a] Each value is the mean retention time after two replications ± standard deviation.
[b] RSD = SD/mean * 100.
[c] ND: not detected.

Table 4
Molecular weight and characteristic fragments of TMS derivatives

Phenolic compounds	Molecular weight of TMS derivatives	Characteristic fragments
p-Hydroxybenzoic acid	282	267, 193, 223, 282
Vanillic acid	312	297, 267, 312, 223, 253, 282, 126, 193
Gentisic acid	370	355, 281, 147, 223, 267, 370
Gallic acid	458	281, 458, 443, 355, 399, 179, 147
p-Coumaric acid	308	219, 293, 308, 249
Ferulic acid	338	338, 308, 323, 249, 293, 219, 279
Caffeic acid	396	219, 396, 381, 191
Hydroxycaffeic acid	469	179, 398, 267, 469
Quercetin	647	575, 647, 487
(+)-Catechin	650	368, 355, 650, 267, 383, 179, 297
Hydroxytyrosol	370	267, 193, 179, 370

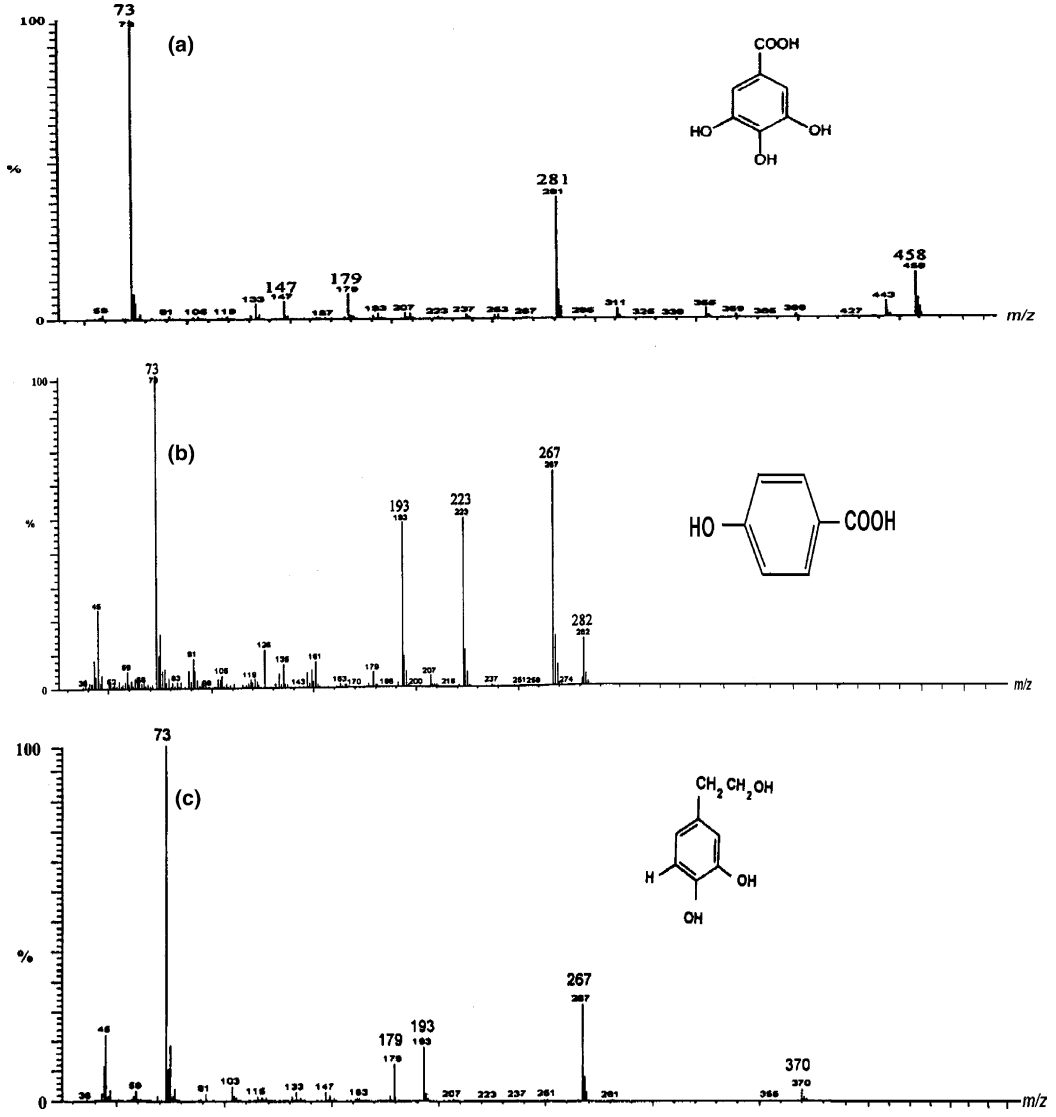

Fig. 3. MS spectrograms of TIC peaks. Peaks: A, gallic acid; B, *p*-hydroxybenzoic acid; C, hydroxytyrosol.

total ion chromatogram (TIC) peaks are presented in Fig. 3.

Other phenolic compounds, such as *o*-hydroxybenzoic acid, *p*-hydroxyphenylpropionic acid, *o*-coumaric acid, 3,4-dihydroxybenzoic acid, hydroxycaffeic acid and 3-nitro-phthalic acid, were identified by the present method as TMS derivatives, based upon the Wiley and NIST libraries.

4. Conclusions

Reversed phase high performance liquid chromatography, coupled with a UV–vis multiwavelength detector allows the collection of on-line spectra and simultaneous quantification at several wavelengths. This experiment proved that silyl derivatization offers a very good alternative for the identification of phenolic compounds. However, it should be stressed that more research is needed on the identification of silyl derivatives. This procedure may solve many problems regarding, not only the determination of phenolics, but also their fate in foodstuffs.

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