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# Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity

C. Proestos [a], I.S. Boziaris [b], G.-J.E. Nychas [b], M. Komaitis [a,*]

[a] *Laboratory of Food Chemistry, Food Science and Technology Department, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece*
[b] *Laboratory of Microbiology and Biotechnology of Foods, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece*

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**Abstract**

The phenolic fraction of plant extracts has been linked to their antioxidant capacity and antimicrobial activity. In the present paper, RP-HPLC with UV detection was employed to distinguish phenolic compounds from the other constituents of the plants examined. The phenolic substances were identified and quantified after comparison with reference standards. A GC–MS method is also presented for characterization of different phenolics as trimethylsilyl derivatives. The antioxidant capacity was determined, in dried plants and in their methanol extracts, with the Rancimat test using sunflower oil as substrate. Both pulverized plants and extracts showed antioxidant capacity. Total phenolic content in the extracts was determined spectrometrically applying the Folin-Ciocalteu assay. It ranged from 2.9 to 28.2 mg gallic acid/100 g dry sample. Antimicrobial activity of the extracts against selected microorganisms was also investigated.


*Keywords:* Phenolic antioxidants; RP-HPLC; GC–MS; Trimethylsilyl derivatives; Antimicrobial activity

## 1. Introduction

There is intense interest in plant polyphenols as witnessed by the numerous papers devoted to various aspects of these compounds (Duthie & Crozier, 2000; Harborne & Williams, 2000; Tura & Robards, 2002). The use of plants, herbs as antioxidants in prossesed foods is becoming of increasing importance in the food industry as an alternative to synthetic antioxidants (Madsen & Bertelsen, 1995). They tend to be water soluble, because they frequently occur combined as glycosides and they are usually located in the cell vacuole (Harborne, 1998). Phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Pietta, 2000). They also have metal chelation properties (Kähkönen et al., 1999; Rice-Evans, Miller, & Paganga, 1997). Their significance for the human diet and antimicrobial activity has been recently established (Nychas, Tassou, & Skandamis, 2003; Rauha et al., 2000). The antioxidant properties of these compounds are often claimed for the protective effects of plant-based beverages against cardiovascular disease, certain forms of cancer and photosensitivity reactions (Haslam, 1998; Bravo, 1998). It was also found that they inhibit human immunodeficiency viral replication (HIV), human simplex virus (HSV), glucosyl transferases of *Streptococcus mutans* (dental carries), ascorbate auto-oxidation (green tea), cytotoxic effects, tumour promotion and xanthine, monoamine oxidases (Havsteen, 2002; Mattila, Astola, & Kumpulainen, 2000; Middleton, Kandaswami, & Theoharides, 2000). These studies provide the basis for the present rapidly increasing interest for the use of

* Corresponding author. Tel./fax: +30 210 5294681.
*E-mail address:* achem@aua.gr (M. Komaitis).

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natural antioxidants as functional food ingredients and/or as food supplements.

Recently, many separation techniques such as gas–liquid chromatography (GLC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) have been proposed to separate and identify phenolic compounds (Chu, Chang, Liao, & Chen, 2001; Deng et al., 1998; Zuo, Chen, & Deng, 2002). HPLC and CE, especially coupled a with photodiode array detector, do not require derivatization prior to qualitative and quantitative analysis. Hence, they have become the most commonly used techniques for the analysis of phenolic compounds in plants. However, often they do not provide satisfactory performance, and the UV–vis spectrum does not supply sufficient evidence for unambiguous identification (Chen, Zuo, & Deng, 2001; Suarez, Picinelli, & Mangas, 1996). For these reasons, capillary gas chromatography combined with mass spectrometry (CGC–MS) is an alternative useful technique, which can provide data sufficient for full structure analysis. More generally, it may be used to determine molecular masses and thus establish the respective substitution pattern on the phenolic ring(s). Trimethylsilyl (TMS) derivatives of phenolic compounds were prepared prior to CGC–MS analysis (Chu et al., 2001).

In the present study, the method of reversed-phase HPLC coupled with a UV–vis multiwavelength detector was used. This method enables the collection of on-line spectra and simultaneous quantification in several wavelengths. CGC–MS was also employed for the analysis of major phenolic substances present in some aromatic plants of Greece because of the combination of the separation capabilities of GC and the power of MS as an identification and confirmation method. HPLC–MS is also a useful method for structural identification. The combination of the chromatographic method and MS are very effective and elucidate better the structure of a particular compound. Finally, the antioxidant as well as the antimicrobial capacity/activity of these plant extracts is presented.

## 2. Materials and methods

### 2.1. Standards

Gallic acid, gentisic acid, *p*-coumaric acid, vanillic acid, ferulic acid, syringic acid, (+)-catechin, quercetin, apigenin, naringenin, eriodictyol were purchased from Sigma–Aldrich (Steinheim, Germany). Luteolin was from Roth (Karlsruhe, Germany). Caffeic acid was from Merck (Darmstadt, Germany). (−)-Epicatechin was from Fluka AG (Buchs, Switzerland). Rutin was from Alexis Biochemicals (Lausen, Switzerland). Hydroxytyrosol, *p*-hydroxybenzoic acid and butylated hydroxytoluene (BHT) were a kind donation from the National Agricultural Research Foundation (N.AG.RE.F, Greece). Quantification was done via a calibration with standards (external standard method). 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 between 0.5 and 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. The Folin-Ciocalteu reagent and silylation reagents, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were purchased from Merck (Darmstadt, Germany), respectively. Dimethyldichlorosilane (5%) (DMDCS) in toluene was obtained from Sigma–Aldrich (Steinheim, Germany), for deactivating glassware surfaces.

### 2.3. Plant material

Dried samples were obtained commercially or collected from different sites in Greece. Some samples were dried in the air (at 25 °C in the dark) and some were lyophilized. All samples were analyzed within 3 months of collection.

### 2.4. Sample preparation and derivatization

The extraction method used for dried samples had as follows: Forty milliliters of 62.5% aqueous methanol containing BHT (1 g $l^{-1}$) was 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 (Justesen, Knuthsen, & Leth, 1998), furthermore, 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 residual moisture. Then, 100 μl of the organic layer were derivatizized after evaporation of the solvent under nitrogen stream. For the silylation procedure, a mixture of TMCS (100 μl) and BSTFA (200 μl) were added and vortexed in screw cap glass tubes (priory deactivated with 5% DMDCS in toluene, and rinsed two times with toluene and three times with methanol), and consecutively placed in a water bath at 80 °C for 45 min. 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 adopted routinely (Harborne, 1998). For this reason, all steps were carried out in dark (flasks were covered with aluminum foil) and under nitrogen atmosphere (the headspace above the plant extract was under inert atmosphere created by a stream of nitrogen).

### 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 $4.6 \times 250$ 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, Heredia, & Troncoso, 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; post-time 10 min before next injection. The flow rate was 0.5 ml/min and the injection volume was 20 μl. The monitoring wavelength was 280 nm for the phenolic acids and 320 and 370 nm (flavones, flavonoles). 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 consisted 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 ($30 \text{ m} \times 0.32$ 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 stepped temperature program was as follows: from 70 to 135 °C with 2 °C/min, hold for 10 min, from 135 to 220 °C with 4 °C/min, hold for 10 min, from 220 to 270 °C with 3.5 °C/min 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. Identification of compounds was obtained 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.

### 2.7. Antioxidant capacity (Rancimat test)

Samples of sunflower oil (3.5 g) containing 0.02% w/w extract or 2% w/w ground material were subjected to oxidation at 110 °C (air flow 20 l/h). The standard compounds (0.02% addition) were also examined. Induction periods, IP (h), were recorded automatically. The protection factors (PF) were calculated according to the following formula: ($PF = IP_{extract}/IP_{control}$) (Exarchou et al., 2002).

### 2.8. Determination of total phenolic content of plant extracts

Total phenolic content was measured by the Folin-Ciocalteu assay (Kähkönen et al., 1999). Quantification was performed with the hydrolysed samples. Results were expressed as mg of gallic acid/g dry sample.

### 2.9. Antimicrobial assay

The methanolic extracts were tested against a panel of pathogenic microorganisms, including *Escherichia coli* 0157:H7 NCTC 12900, *Salmonella enteridis* PT4, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ScottA, *Pseudomonas putida* AMF178 and *Bacillus cereus* FSS134. Microorganisms were stored frozen in bead vials (Protect; Technical Service Consultants Ltd., Heywood, Lancashire, UK). Resuscitation of bacterial strains was carried out in 10 ml BH Broth (Merck Cat. No. 1.10493) incubated overnight at 37 °C for *E. coli* and *S. enteridis* PT4, 35 °C for *B. cereus* 30 °C for *L. monocytogenes* and *S. aureus* and 25 °C for *P. putida*.

Resuscitated cultures were diluted tenfold in Ringer's solution (LabM) for the inoculation of 10 ml BH Broth (Merck Cat. No. 1.10493) to yield an initial suspension of approximately 10–100 cfu/ml. All broths were then incubated statically at the aforementioned temperatures for each microorganism, for 18–24 h to guarantee that all cells were in the stationary phase. Susceptibility of the test organism to the extract was determined by employing the standard disk diffusion technique. The bacterial suspensions were diluted tenfold in Ringer's solution (LabM), and 0.1 ml from the appropriate dilution was spread plated on BH Agar (Merck Cat. No. 1.13825) in order to give a population of approximately $10^6$ cfu/plate. Sterile paper discs with a diameter of 6.48 mm (Whatman No. 2) were placed onto the inoculated agar surface. Five microliters of each plant extract was added to the paper discs. Each experiment was carried out in triplicate. Petri dishes were incubated for 48 h, at 37 °C for *E. coli* 0157:H7 NCTC 12900 and *S. enteridis* PT4, 35 °C for *B. cereus* and 30 °C for *L. monocytogenes* ScottA and *S. aureus* ATCC 6538 and 25 °C for *P. putida*. After incubation, the inhibition zones were estimated by taking photos of Petrie's with a SONY

camera (x-wave HAD SSC-DC50AP) and processed using the Impuls Vision XL 2.5 software. Each inhibition zone diameter was measured three times (three different plates) and the average was taken.

## 3. Results and discussion

### 3.1. Antioxidant capacity

The antioxidant capacity (expressed as PF values) and the total phenolic content of all extracts are shown in Table 1. The amount of total phenolics varied slightly in plant materials and ranged from 2.9 to 28.2 mg of gallic acid/g dry sample. The highest amount was found in *Geranium purpureum*, and the lowest in *Humulus lupulus*. Similar amount in plant phenolics from herbs and medicinal plants collected in Finland have been reported recently (Kähkönen et al., 1999).

The outcome of the Rancimat test supports the hypothesis that aromatic plants are good sources of natural antioxidants such as phenolic compounds. When working accurately, this method offers an efficient, simple and automated assay. The antioxidant reactions involve multiple steps including the initiation, propagation, branching, and termination of free radicals. The antioxidants which inhibit or retard the formation of free radicals from their unstable precursors (initiation) are called the ''preventive'' antioxidants, and those which interrupt the radical chain reaction (propagation and branching) are the ''chain-breaking'' antioxidants (Ou, Hampsch-Woodill, & Prior, 2001). When ground material was added to sunflower oil, protection factors were slightly higher compared to the addition of methanol extracts. The PF values for the standard compounds (0.02% addition) examined ranged from 1.2 to 1.5 for all phenolic acids, except for gallic acid which had PF = 4.5. (+)-Catechin hydrated and (−)-epicatechin had PF values 1.8 and 2.5, respectively, whereas the flavonoids (rutin, quercetin, apigenin, luteolin, eriodictyol and naringenin) had PF values ranging from 1 to 1.2. The PF value for hydroxytyrosol was 1.4 and 1.8 for butylated hydroxytoluene. These values explain the antioxidant potential of the plant extracts examined and depend on the total phenol content, without being proportional. Similar PF values for ethanol and acetone extracts of plants of Greek origin have been reported (Exarchou et al., 2002). A wide range of color reactions has been used to determine total polyphenols, including the reaction with the Folin-Ciocalteau reagent. However, as each phenolic compound gives a different color and assays in general will be rather unspecific, the results of these determinations are of limited value.

### 3.2. HPLC analysis

Using the aforementioned procedure, the phenolic substances present in aromatic plants of Greek origin were separated and quantified. HPLC with UV–vis multiwavelength detector was used since all phenolic compounds show intense absorption in the UV region of the spectrum. The present method is simple, easy to use, and effective enough for the identification and quantification of major phenolic compounds in aromatic plants. A similar technique has been reported by other authors, for the analysis of major flavonoid aglycones (Justesen & Knuthsen, 2001; Mattila et al., 2000). Spherisorb® ODS2 stationary phase, which was used in this study to separate phenolic acids and flavonoids in the aforementioned wavelengths of aromatic plants, produced satisfactory results. After

Table 1
Total phenolics in plant extracts and their antioxidant capacity (expressed as PF values)

| Family | Species | Part examined | Drying method[a] | Total phenolics[b] (mg gallic acid/g ds) | PF[c] (ground material) | PF (methanol extracts) |
|---|---|---|---|---|---|---|
| Capparaceae | *Capparis spinosa* | Leaves | F/v | 15.9 ± 0.2 | 1.6 | 1.5 |
| Cupuliferae | *Castanea vulgaris* | Leaves | F/v | 11.9 ± 0.2 | 1.8 | 1.6 |
| Geraniaceae | *Geranium purpureum* | Leaves | F/v | 28.2 ± 0.1 | 3.1 | 2.9 |
| Labiateae | *Nepeta cataria* | Herb | Air | 19.2 ± 0.3 | 2.5 | 2.1 |
| | *Origanum dictamnus* | Leaves | Air | 13.6 ± 0.4 | 1.4 | 1.2 |
| Leguminosae | *Spartium junceum* | Flower | Air | 4.8 ± 0.3 | 1.3 | 1.2 |
| Oleaceae | *Jasminum officinalis* | Flower | F/v | 6.6 ± 0.2 | 1 | 0.8 |
| Phytolaccaceae | *Phytolacca americana* | Leaves | F/v | 9.2 ± 0.2 | 1.5 | 1.3 |
| Rutaceae | *Ruta graveolens* | Leaves | F/v | 4.3 ± 0.4 | 1.7 | 1.6 |
| Styracaceae | *Styrax officinalis* | Leaves | F/v | 18.4 ± 0.3 | 1.8 | 1.7 |
| Umbelliferae | *Cuminum cyminum* | Seeds | Air | 5.2 ± 0.2 | 1.2 | 1.1 |
| | *Foeniculum vulgare* | Herb | Air | 10.5 ± 0.3 | 1.5 | 1.4 |
| Urticaceae | *Humulus lupulus* | Leaves | F/v | 2.9 ± 0.1 | 1.1 | 0.9 |
| | *Urtica dioica* | Leaves | F/v | 6.9 ± 0.1 | 1.2 | 1 |

[a] Air, air drying; F/v, freeze vacuum, i.e., lyophilization.
[b] Mean of duplicate assays; ds, dry sample.
[c] PF, protection factor.

Table 2
Content of phenolic acids in the examined plant extracts

| Plant | Content (mg/100 g dry sample)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Gallic acid | Gentisic acid | Caffeic acid | *p*-Coumaric acid | Vanillic acid | Syringic acid | Ferulic acid | *p*-Hydroxybenzoic acid |
| *Capparis spinosa* | ND | ND | 6.5 ± 0.02 | 4.3 ± 0.02 | 4.8 ± 0.02 | ND | 13.2 ± 0.04 | ND |
| *Castanea vulgaris* | 3.4 ± 0.01 | ND | ND | ND | ND | ND | 6.7 ± 0.02 | ND |
| *Cuminum cyminum* | 1.2 ± 0.02 | ND | 4.9 ± 0.01 | ND | ND | ND | 5.8 ± 0.02 | ND |
| *Foeniculum vulgare* | 3.7 ± 0.01 | ND | 5.6 ± 0.02 | ND | ND | ND | 4.1 ± 0.01 | ND |
| *Geranium purpureum* | 14 ± 0.02 | 3.2 ± 0.03 | 20 ± 0.03 | 4.1 ± 0.02 | 2 ± 0.02 | 1.1 ± 0.02 | ND | 5.4 ± 0.01 |
| *Humulus lupulus* | 3.2 ± 0.02 | ND | ND | 3.8 ± 0.01 | ND | ND | ND | 1.5 ± 0.02 |
| *Jasminum officinalis* | 2.2 ± 0.01 | ND | ND | 8.1 ± 0.03 | ND | ND | ND | ND |
| *Nepeta cataria* | 1.9 ± 0.02 | ND | 38.4 ± 0.06 | ND | ND | ND | 30.8 ± 0.07 | ND |
| *Origanum dictamnus* | 0.7 ± 0.02 | ND | 23 ± 0.04 | 4.4 ± 0.01 | ND | ND | ND | 1.3 ± 0.02 |
| *Phytolacca americana* | ND | ND | 11.2 ± 0.02 | 4.5 ± 0.02 | 3.9 ± 0.02 | ND | 41.6 ± 0.03 | 2 ± 0.02 |
| *Ruta graveolens* | ND | 2.4 ± 0.03 | 2.1 ± 0.02 | ND | ND | ND | 4.9 ± 0.02 | 1.2 ± 0.01 |
| *Spartium junceum* | ND | 1.5 ± 0.02 | 37.5 ± 0.05 | 22.5 ± 0.03 | 0.8 ± 0.02 | ND | ND | ND |
| *Styrax officinalis* | 1.3 ± 0.01 | 2.5 ± 0.02 | 17.3 ± 0.03 | 6.5 ± 0.02 | 1.1 ± 0.01 | ND | 42 ± 0.04 | 2.1 ± 0.01 |
| *Urtica dioica* | 2.9 ± 0.01 | ND | ND | ND | ND | 1.5 ± 0.01 | 12.4 ± 0.02 | ND |

[a] Each value is the mean (mg/100 g dry sample) of two replications ± standard deviation; ND, not detected.

extraction and acid hydrolysis the content of phenolic substances was determined. Quantification was done via a calibration with standards (external standard method). The amount of phenolic acids detected in the analysed samples is shown in Table 2. Additionally, the content of flavonoids identified in the same plant extracts is shown in Table 3. Results are expressed in mg/100 g dry sample.

Another phenolic compound which was detected as well in some samples was hydroxytyrosol. Hydroxytyrosol (also known as dihydroxyphenylethanol), is a main bioactive constituent in olives (O'Dowd et al., 2004). *Capparis spinosa* and *G. purpureum* contained large amounts of this compound (11.2 and 9.8 mg/100 g dry sample, respectively), whereas *Styrax officinalis* and *Spartium junceum* contained a moderate amount (5.3 and 4.9 mg/100 g dry sample, respectively). In most of the other plant extracts hydroxytyrosol was detected in traces. The most abundant phenolic acids were ferulic (4.1–42 mg/100 g dry sample) and caffeic acid (2.1–38.4 mg/100 g dry sample). *S. officinalis* contained the highest amount of ferulic acid (42 mg/100 g dry sample). Syringic acid was detected only in *G. purpureum* and *Urtica dioica*. (+)-Catechin, rutin (quercetin 3-*o*-rhamnose glycoside) and quercetin were the most abundant flavonoids. Apigenin was detected only in *Castanea vulgaris*. Naringenin was detected only in *S. officinalis* and *C. vulgaris*. Another flavanone, eriodictyol, was also detected in other plant extracts (Table 3). The levels of phenolic compounds were as expected according to previous investigations of these compounds in similar herbs, plants (Zheng & Wang, 2001). The flavonol quercetin and the two major flavones luteolin and apigenin have been identified and simultaneously quantified in 62 types of edible tropical plants in Malaysia (Miean & Suhaila, 2001).

Table 3
Flavonoid content in the examined plant extracts

| Plant | Content (mg/100 g dry sample)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Quercetin | Apigenin | Luteolin | Naringenin | Eriodictyol | Rutin | (+)-Catechin hydrated | (−)-Epicatechin |
| *Capparis spinosa* | 1.5 ± 0.01 | ND | ND | ND | ND | 1.3 ± 0.01 | ND | ND |
| *Castanea vulgaris* | 3.6 ± 0.01 | 4 ± 0.01 | ND | 1.7 ± 0.02 | ND | 2.6 ± 0.01 | ND | ND |
| *Cuminum cyminum* | ND | ND | 3.1 ± 0.02 | ND | 0.3 ± 0.02 | ND | ND | ND |
| *Foeniculum vulgare* | 3.6 ± 0.02 | ND | ND | ND | ND | 1.9 ± 0.01 | ND | 4.5 ± 0.04 |
| *Geranium purpureum* | 11.2 ± 0.02 | ND | ND | ND | ND | 4.5 ± 0.01 | 1.5 ± 0.01 | 5.6 ± 0.02 |
| *Humulus lupulus* | 1.2 ± 0.02 | ND | ND | ND | 0.1 ± 0.01 | ND | ND | ND |
| *Jasminum officinalis* | 3 ± 0.02 | ND | ND | ND | ND | ND | ND | ND |
| *Nepeta cataria* | ND | ND | 3.6 ± 0.02 | ND | 1.4 ± 0.01 | ND | ND | 1.2 ± 0.01 |
| *Origanum dictamnus* | 1.3 ± 0.03 | ND | ND | ND | ND | ND | 2.3 ± 0.02 | ND |
| *Phytolacca americana* | ND | ND | ND | ND | ND | 4.3 ± 0.02 | ND | ND |
| *Ruta graveolens* | 3.1 ± 0.01 | ND | ND | ND | ND | 3.1 ± 0.01 | 10.5 ± 0.02 | ND |
| *Spartium junceum* | 3.5 ± 0.02 | ND | 2.5 ± 0.01 | ND | ND | ND | ND | ND |
| *Styrax officinalis* | 4.1 ± 0.01 | ND | ND | 1.2 ± 0.01 | ND | ND | 8.3 ± 0.03 | 0.4 ± 0.01 |
| *Urtica dioica* | ND | ND | ND | ND | ND | ND | 1.1 ± 0.02 | 2 ± 0.02 |

[a] Each value is the mean (mg/100 g dry sample) of two replications ± standard deviation; ND, not detected.

Phenolic compounds are found usually in nature as esters and rarely as glycosides or in free form (Rice-Evans, Miller, & Paganga, 1996). Thus, hydrolysis was needed for their identification and quantitative determination. Flavonoids are also present in plants in the form of glycosides. Any flavonoid may occur in a plant in several glycosidic combinations. For this reason, hydrolysis was used to release the aglycones which they can be further investigated by HPLC. The data presented in Tables 1–3 are considered as indicative of phenolic content of these aromatic plants. Papers about most of the examined plant extracts are very scarce in the literature. Among others, time of harvest, storing conditions, is considered responsible for the observed variations in the phenolic contents.

### 3.3. CGC–MS analysis

The isolation and quantification of phenolic compounds in plant material is difficult because of their chemical complexity. The sensitivity and resolving power of capillary GC–MS make this technique particularly suitable for unambiguous detection of phenolic compounds in the hydrolysed samples.

Silylation is the most widely used derivatization procedure for sample analysis by GC. The derivatives are generally less polar, more volatile and more thermally stable. The silylation reaction is a nucleophilic substitution reaction. The strong electron withdrawing group ($CF_3$) of BSTFA stabilizes the negative charge of the leaving group, and thereby facilitates the occurrence of a substitution reaction (Chu et al., 2001). TMCS is a silylation catalyst, rarely used alone but typically mixed with other silylation reagents to increase their reactivity in derivatization. Several variables were examined to determine their roles in the derivatization procedure: (1) temperature; (2) reaction time; and (3) the amount of the silylating reagent required to complete the derivatization. In our study, the temperature and reaction time used was 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 most cases 1% TMCS in BSTFA is sufficient to achieve the desired derivatization. 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, Wang, & Zhan, 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 the present study, 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 TMS derivatives towards moisture. Therefore, anhydrous $Na_2SO_4$ was added.

The GC oven temperature program, as well as the injector and detector temperatures, were 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. Data obtained showed excellent resolution between all compounds of interest. Molecular weights (MWs) and important ions present in the mass spectra of silylated phenolic compounds in the examined plant extracts are presented in Table 4. Three more phenolic compounds, cinnamic acid, protocatechuic acid and *p*-hydroxyphenylacetic acid were identified by the present

Table 4
Molecular weights (MWs) and important ions present in the mass spectra of silylated phenolic compounds[a] in the examined plant extracts by CGC–MS

| Compound | MW (silylated compounds) | Identified ions (*m*/*z*) |
|---|---|---|
| *p*-Hydroxybenzoic acid | 282 | 267 (100%), 193, 223, 282 |
| Vanillic acid | 312 | 149 (100%), 312, 223, 165 |
| Gentisic acid | 370 | 355 (100%), 281, 147, 223, 267, 370 |
| Gallic acid | 458 | 281 (100%), 458, 179, 147 |
| *p*-Coumaric acid | 308 | 219 (100%), 293, 308, 249 |
| Ferulic acid | 338 | 338 (100%), 308, 323, 249, 293, 219, 279 |
| Caffeic acid | 396 | 219 (100%), 396, 381, 191 |
| Quercetin | 647 | 575 (100%), 647, 487 |
| (+)-Catechin | 650 | 368 (100%), 355, 650, 267, 383, 179, 297 |
| Hydroxytyrosol | 370 | 267 (100%), 193, 179, 370 |
| Syringic acid | 342 | 327 (100%), 312, 297, 342 |
| (−)-Epicatechin | 650 | 368 (100%), 355, 267, 147, 649 |
| *p*-Hydroxyphenylacetic acid | 296 | 179 (100%), 164, 149, 296 |
| Protocatechuic acid | 370 | 193 (100%), 223, 370, 267 |
| Cinnamic acid | 220 | 131 (100%), 205, 103 161, 220 |

[a] Identified as trimethylsilyl (TMS) derivatives.

Table 5
Antimicrobial activity of plant extracts (sample amount 5 μl; $n = 3$)

| Plant extracts | *Escherichia coli* 0157:H7 NCTC12900 | *Salmonella enteridis* PT4 | *Staphylococcus aureus* ATCC 6538 | *Listeria monocytogenes* ScottA | *Bacillus cereus* FSS134 | *Pseudomonas putida* AMF178 |
|---|---|---|---|---|---|---|
| *Capparis spinosa* | –[a] | – | ∼[b] | ++[d] | ∼ | +[c] |
| *Astanea vulgaris* | ∼ | – | – | ++ | ∼ | ∼ |
| *Cuminum cyminum* | – | – | – | ∼ | – | – |
| *Foeniculum vulgare* | – | – | – | ∼ | – | ∼ |
| *Geranium purpureum* | ++ | – | + | ++ | + | ++ |
| *Humulus lupulus* | – | – | – | ++ | – | + |
| *Jasminum officinalis* | – | – | – | + | – | – |
| *Nepeta cataria* | – | – | – | – | – | ∼ |
| *Origanum dictamnus* | ∼ | – | + | ++ | ∼ | ∼ |
| *Phytolacca americana* | – | – | ∼ | ++ | – | ∼ |
| *Ruta graveolens* | – | – | – | ∼ | – | – |
| *Spartium junceum* | – | – | – | ∼ | – | ∼ |
| *Styrax officinalis* | ∼ | – | – | – | ∼ | ∼ |
| *Urtica dioica* | ∼ | – | ++ | ++ | ++ | ∼ |

[a] No antimicrobial capacity, i.z. of sample < i.z. of solvent (62.5% aqueous methanol).
[b] Slight antimicrobial capacity, i.z. of sample 1–3 mm > i.z. of solvent.
[c] Moderate antimicrobial capacity, i.z. of sample 3–4 mm > i.z. of solvent.
[d] Clear antimicrobial capacity, i.z. of sample 4–10 mm > i.z. of solvent.

method as TMS derivatives, based upon the Wiley and NIST libraries.

### 3.4. Antimicrobial activity

Antimicrobial assays described in the literature include measurement of: (i) the radius or diameter of the zone of inhibition of bacterial growth around paper disks impregnated with (or wells containing) an antimicrobial compound on agar media; (ii) the inhibition of bacterial growth on an agar medium with the antimicrobial compound diffused in the agar (iii) the minimum inhibitory concentration (MIC) of the antimicrobial compound in liquid media; (iv) the changes in optical density or impedance in a liquid growth medium containing the antimicrobial compound. To screen the antimicrobial activity of 'unknown' compounds the second methodology mentioned above is considered to be the simplest where the results are obtained rapidly. Vardar-Ünlü et al. (2003) have used the disk diffusion method to determine the antimicrobial activity of essential oil and methanol extracts using several microbial strains. Antimicrobial activity of *Decalepsis hamiltonii* roots against foodborne pathogens using the same method has been reported (Thangadurai, Anitha, Pullaiah, Reddy, & Ramachandraiah, 2002).

The antimicrobial activity of plant extracts are shown in Table 5. The plant extracts found to be more effective on inhibiting the microorganisms tested were: *G. purpureum*, *U. dioica* and *Origanum dictamnus*. *L. monocytogenes* ScottA was the most sensitive microorganism to the plant extracts examined in this study. On the contrary, all the extracts failed to inhibit *S. enteridis* PT4. The results in Table 5 revealed that Gram (+) bacteria are more sensitive to the plant extracts than Gram (−) bacteria especially the Enterobacteriaceae (*E. coli* 0157:H7 NCTC12900 and *S. enteridis* PT4). It should be taken into account that the comparatively weak inhibition found in this study could be influenced by the fact that the inhibition area depends on the ability of the antimicrobial compound to diffuse uniformly through the agar. Thus, a greater inhibition might be revealed if alternative(s) methodologies are applied.

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