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Elemental Content and Total Antioxidant Activity of *Salvia fruticosa*

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Abstract The determination of 18 elements (V, Cr, Cu, Co, Se, Sr, Sn, Sb, Ba, Bi, Pb, Cd, As, Ni, Mn, Fe, Mg and Zn) in leaves, flowers and the infusion from *Salvia fruticosa*, a sage grown in Greece, is described. For this purpose, flame atomic absorption spectrometry has been used for the determination of Fe, Mg, Zn and inductively coupled plasma-mass spectrometry has been used for the determination of V, Cr, Cu, Co, Se, Sr, Sn, Sb, Ba, Bi, Pb, Cd, As, Ni, Mn using ^{45}Sc ^{72}Ge, ^{115}In and ^{232}Th as internal standards. The elemental content was found to be in the range of 0.01 (Bi)-30.8 (Mn) mg/Kg (leaves), 0.30 (Bi)-39.1 (Mn) mg/Kg (flowers), 0.003 (Sb)-20.4 (Mn) mg/Kg (infusion) for V, Cr, Cu, Co, Se, Sr, Sn, Sb, Ba, Bi, Pb, Cd, As, Ni, Mn and in the range of 0.07 (Zn)-3.21 (Mg) g/kg (leaves) for Fe, Mg and Zn. The majority of the samples were collected from six sites in the island Crete and transplanted and grown in a model farm. Chemometric techniques were used to investigate the original site classification according to their elemental content, and it was proved that the initial cultivation sites were characterized by only five elements (Sb, V, Zn, Cd and Cr). The application of factor analysis revealed significant correlation between certain elements, denoting their common sources. In addition, the total antioxidant activity of the herbal preparation was determined by measuring the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity. Microwave-assisted extraction (MAE) was used to extract total antioxidants and the effect of temperature, time and solvent in the extraction efficiency was investigated. The determination of the antioxidant activity was based on the % inhibition of the absorbance signal of the radical DPPH at 515 nm, after the addition of herbal's extract. The IC_{50} values were found to be in the range of 10.6–40.1 mg/L.

Keywords Sage (*Salvia fruticosa*) · Herbs · Metal Contents · Elemental Correlations · ICP-MS · FAAS · Microwave-Assisted Extraction · DPPH Radical-Scavenging Method · Antioxidant Activity Index

Introduction

There is an increased interest in using plants for therapeutical purposes during the last decades (Caldas and Machado 2004). Trace elements play both a curative and a preventive role in combating diseases; therefore, determination of trace elements composition of foods and related products is essential for understanding their nutritive importance (Ajasa et al. 2004).

The determination of trace elements content of plants have been performed using various instrumental techniques including flame atomic absorption spectrometry (FAAS) (Ajasa et al. 2004; Narin et al. 2004) electrothermal atomic absorption spectrometry (Seenivasan et al. 2008) inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Özcan et al. 2008) and inductively coupled plasma-mass spectrometry (ICP-MS) (Nardi et al. 2009). Inductively coupled plasma-mass spectrometry is one of the most used techniques due to high sensitivity and selectivity, low analytical limits and multi-element capability so that it is an excellent tool for isotopic analysis and for detailed characterization of elemental composition. One of the most common calibration techniques used on ICP-MS is external

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calibration with internal standardization (Nardi et al. 2009; Sartoros and Salin 1999).

In addition, the use of plants and herbs as antioxidants in processed foods is of increasing importance in the food industry as an alternative to synthetic antioxidants (Proestos et al. 2006). It is accepted that the intake of compounds with antioxidant activity, like polyphenolics, reinforces the defense against reactive oxygen species (ROS) (Anesini et al. 2008; Issa et al. 2006). Consequently, the evaluation of antioxidant activity is of major interest in order to compare the potential antioxidant value of different foods and to evaluate antioxidant intake.

Several methods are used to measure the antioxidant activity. The most commonly used for their facility, speed and sensitivity are those involving chromogen compounds of a radical nature to simulate ROS (Karadag et al. 2009). The presence of an antioxidant leads to the disappearance of these radical chromogens, the two most widely used being $ABTS^+$ (Karadag et al. 2009; Labrinea and Georgiou 2004) and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Karadag et al. 2009; Arnao 2000; Atmani and Chaher 2009). The DPPH and $ABTS^+$ assays give comparable results (Thaipong et al. 2006). DPPH is a free radical that is acquired directly without preparation, while $ABTS^+$ must be generated by enzymatic or chemical reactions (Labrinea and Georgiou 2004). Thus, the use of DPPH is generally preferred.

The antioxidant properties of many species of genus *Salvia* have been discussed in the literature (Grzegorczyk et al. 2007). However, to the best of our knowledge, none is referred to *Salvia fruticosa*. *Salvia* species (sage) were reported to be used for memory-enhancing purposes in European folk medicine (Perry et al. 2003). This herb is grown in many places in Greece and in Mediterranean countries.

Extraction into an appropriate solvent is the first step for the recovery of bioactive phytochemicals from plant materials. Microwave-assisted extraction (MAE) has many advantages over the classical methods, since it is fast and demands lower amounts of solvents (Guo et al. 2001; Gao et al. 2006). Methanol (Tepe et al. 2006) and ethanol (Pan et al. 2003) are the most used solvents for the extraction of antioxidants from herbs. Ethanol is often the final choice since it is non-toxic and can be mixed with water in different ratios, especially when MAE is used (Pan et al. 2003).

The aim of this work was the determination of the elemental content of leaves, flowers and their infusions and the investigation of the antioxidant activity of leaves and flowers of *S. fruticosa* (sage) of Crete Island for the first time. Plants of *S. fruticosa* from other places in Greece were also tested for comparison. A method was developed including microwave digestion and final determination by ICP-MS for the content of ^{51}V, ^{53}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{75}As, ^{82}Se, ^{88}Sr, ^{114}Cd, ^{118}Sn, ^{121}Sb, ^{137}Ba, ^{208}Pb and ^{209}Bi for leaves, flowers and infusions. Fe, Mg and Zn were determined in leaves by FAAS. The developed method was validated and the correlation among the elements was investigated. Supervised and unsupervised multivariate statistical techniques were used so that the plants' origin was tracked back and variables correlation was emphasized. The investigation of the antioxidant activity of the plant was based on the calculation of the % inhibition of the absorbance signal of the DPPH radical at 515 nm, after the addition of the herbal's extract. For this purpose, it was necessary to develop a rapid, reliable and reproducible microwave-assisted extraction method. The antioxidant activity of a Chinese white tea and of some reference antioxidants was also determined for comparison reasons.

Materials and Methods

Instrumentation

Two instruments were used for elemental determination: (a) an Agilent 7500s ICP-mass spectrometer equipped with a Fassel torch, a peristaltic pump and an autosampler; (b) a Perkin Elmer 2380 atomic absorption spectrometer with a deuterium background corrector. Helios Delta spectrometer was used for the DPPH absorption measurements.

ICP-MS operating conditions were as follows: rf power of 1,350 W; nebulizer argon flow, 1.15 L/min; number of replicates, ten. Between samples or standards, the sampling system was rinsed with 1% HNO_3 (*v*/*v*). ^{45}Sc, ^{72}Ge, ^{115}In and ^{232}Th (50 μg/L) were used as internal standards for elements in the mass range (m/z) 51–65, 75–82, 111–117 and 206–209, respectively.

The digestions and the extractions were performed on a microwave oven MARS X-Press (CEM Corporation).

Materials

The following reagents were used throughout this work. HNO_3 65% (*v*/*v*) *suprapur* grade was purchased from Merck, Germany. V, Cr, Mn, Co, Ni, Cu, As, Se, Sr, Cd, Sn, Sb, Ba, Pb and Bi working solutions prepared by diluting 10 mg/L stock solution of each analyte (High Purity Standards, Charleston, SC, USA) with ultra pure water (MilliQ water, Millipore, Bedford, MA, USA) and acidified to a final HNO_3 concentration of 1% *v*/*v*. Mg, Fe and Zn working solutions for FAAS prepared by diluting 1,000 mg/L stock solution of each analyte (Merck, Germany) with ultra pure water (MilliQ water) and acidified to a final HNO_3 concentration of 1% *v*/*v*. Stock solutions of In, Th, Sc and Ge (10 mg/L) were also used. Synthetic antioxidant 3,5-di-tert-butyl-4-hydroxytoluene, ≥99.9% (BHT; Supelco, USA); caffeic acid (Sigma, Germany) and rosmarinic acid,

97% (Aldrich, Germany); (+)-catechin hydrate, ≥98% (Sigma); quercetin hydrate, 95+% (Aldrich); 2-tert-butyl-4-methoxyphenol, ≥98% (Merck), were used as reference compounds. Absolute ethanol, 99.5% (Panreac, Spain); methanol, 99.9% (LAB-SCAN, UK) and ethyl acetate, 99.8% (LAB-SCAN) were used as solvents.

Preparation of Samples

Fifteen samples of leaves and flowers of *S. fruticosa* from six different local areas in Crete, namely I–VI, and four samples of sage leaves from other places in Greece, namely W (from "wild"), were dried at room temperature for 2 weeks and ground in a mortar.

Analytical Procedure for Elemental Determination

A 0.1250 g of the powdered samples (leaves or flowers) was weighed into the Teflon vessels and 5 mL of 65% HNO_3 was added. The samples were digested with MARS X-Press (CEM Corporation, NC, USA) microwave oven with a preselected program (first stage at 1,600 W and 165°C for 2 min; second stage at 1,600 W and 175°C for 8 min) and then diluted to a final volume of 20 mL with ultrapure water. The accuracy of the method was checked by spiking at four levels within the range of 0.8–6.4 mg/Kg for all the elements, except 0.08–0.64 mg/Kg for Cd and 1.6–12.8 mg/Kg for Ba, Sr. The recovery rates were calculated by external calibration with internal standardization. The precision was also checked by digesting six subsamples, following the whole procedure and calculating the (%) RSD.

For the elemental determination in the infusion, the following protocol was used: 0.2500 g of the powdered sample (leaves) was put into 50 mL of boiled water for about 5 min and, after filtering the sample, the final volume was adjusted to 50 mL with 1% (*v*/*v*) HNO_3. The samples should be measured within 2 days. The accuracy was again checked by spiking subsamples at three different concentration levels of each analyte and following the whole procedure. The precision was also checked by calculating the (%) RSD of six independent infusion preparations.

The (%) migration of each element from leaves to infusions was calculated as the ratio of the elemental content in the infusion to the respective content in the leaves multiplied by 100.

Chemometric Evaluation of the Origin of the Samples

Two multivariate statistical techniques were used in order to classify the samples according to their origin. Discriminant analysis (DA; a supervised technique) and cluster analysis (CA; an unsupervised one) were applied to the data of leaves' analysis. The latter data set were chosen as their elemental values were elevated and comparison with corresponding results from plants grown in different Greek areas besides Crete was feasible. Discriminant analysis was applied to the initial data, providing the classification matrix for every individual site (area) having exploited only nine of the available elements: V, Sb, Co, As, Mn, Cu, Cd, Se and Zn. Hierarchical CA was performed on the standardized (means of zero and SD of one) data by means of the Ward's method, for revealing similarities between the different areas.

Elemental Correlation

Factor analysis (FA) using varimax rotation was also used in this work for revealing variables' correlation, in order to investigate the possible similar sources of the element in the plant. Specifically, varimax normalization is a rotation method that makes possible the characterization of each variable by a single factor, which is much simpler (the rule is: few variables with high loadings and many variables with low loadings in a particular factor) (Adams 2004). Moreover, Spearman's non-parametric correlation was performed for confirmation of the FA results.

Microwave-Assisted Extraction for Antioxidant Activity Investigation

The extraction was performed on a microwave oven MARS X-Press (CEM Corporation) at 400 W using magnetic stirring at 50% of nominal power, using two vessels in a batch. Three different solvents (methanol, ethanol and ethyl acetate) along with ethanol–water mixtures, extraction temperatures and extraction time were evaluated. The extraction protocol was as follows: 0.5 g of ground leaves were extracted with 10 mL of ethanol. The extracts were filtered and the filtrate transferred into a rotary evaporator, where the extracting solvent was removed at 50°C (Grzegorczyk et al. 2007). The residue was weighted and dissolved in an appropriate volume of ethanol (100 mL) to obtain a standard solution. The approximate concentration for most of the extracts was 0.6 mg/mL. The % inhibition of the absorbance signal of the radical DPPH at 515 nm after the addition of the herbal's extract was measured.

DPPH Radical Scavenging

A similar procedure as that of Grzegorczyk et al. (2007) was followed for the determination of DPPH radical scavenging activity. The sage extracts at seven different concentrations (ranging from 3.0 mg/L to 21.0 mg/L) were mixed with 2 mL of 0.126 mM ethanolic solution of DPPH, concentration which was considered from Scherer and Godoy (2009) as into the linearity range of the radical.

The disappearance of DPPH was estimated spectrometrically (Helios Delta spectrometer) at 515 nm. The steady state was achieved within 20 min after the incubation at room temperature. Free radical scavenging activity was calculated by Eq. 1:

$$(\%)\,\text{Inhibition} = (A_0 - A_{20}) \times 100/A_0 \quad (1)$$

were:

A_0 the absorbance of DPPH at t=0
A_{20} the absorbance at t=20 min

From the results obtained, the IC_{50} (defined as the concentration of the sample at which the 50% of maximum scavenging activity was recorded) was calculated from the calibration graph, plotting (%) Inhibition=f (concentration of sage extract) for each sample. Correlation coefficients higher than 0.995 were always achieved.

The results were also expressed as the antioxidant activity index (AAI) (Scherer and Godoy 2009). The AAI calculated as follows:

$$\text{AAI} = \text{final concentration of DPPH}\,(\text{mg/L})/\text{IC}_{50}(\text{mg/L}) \quad (2)$$

In this work, it is considered that the plant extracts showed poor antioxidant activity when AAI<0.5, moderate antioxidant activity when AAI was between 0.5 and 1.0, strong antioxidant activity when AAI was between 1.0 and 2.0 and very strong antioxidant activity when AAI>2.0 (Scherer and Godoy 2009).

All the calculations and plots were made using Excel 2003 by MicroSoft and Statistica for Windows, Version 7.0 by StatSoft Inc., 2004.

Results and Discussion

Validation of the ICP-MS Method

As referred in "Preparation of Samples", for the validation of ICP-MS method, recovery tests were performed. The calculated recoveries (% recovery±SD, n=6) were found in the range of (69.1±2.1%; ^{121}Sb) to (103±7%; ^{114}Cd) for the leaves, (67.3±3.1%; ^{121}Sb) to (115±12%; ^{65}Cu) for the flowers and (58±10%; ^{209}Bi) to (106±9%; ^{59}Co) for the infusions. The obtained recovery values show that the method produce accurate results in general, but there were problems in the determination of ^{121}Sb in leaves and flowers (69.1% and 67.3%, respectively) and ^{209}Bi in leaves, flowers and infusions (80.4%, 74.5% and 57.5%, respectively). The precision was also checked and the relative standard deviation (n=6) was ranged from 1.3% (62.0 mg/Kg ^{55}Mn) to 10% (0.2 mg/Kg ^{82}Se) for the leaves, 2.4% (1.3 mg/Kg ^{60}Ni) to 14% (0.06 mg/Kg ^{121}Sb) for the flowers and 3.7% (2.5 mg/Kg ^{88}Sr) to 9.8% (0.02 mg/Kg ^{75}As) for the infusions. The method LODs were determined to be in the range of 1.6 μg/Kg (Cd) to 68.8 μg/Kg (Bi) for the leaves and the flowers and in the range of 2.0 μg/Kg (Cd) to 86.0 μg/Kg (Bi) for the infusion. The above results are satisfactory taking into account the low concentration levels of some metals in the samples.

Metal Content

The fifteen samples of *S. fruticosa* from Crete were collected as young plants from six different locations in the island (coded as I to VI), transferred in the same place and left to grow all together. Four more samples were collected from other Greek areas (coded as W). A total of 15 elements (^{51}V, ^{53}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{75}As, ^{82}Se, ^{88}Sr, ^{114}Cd, ^{118}Sn, ^{121}Sb, ^{137}Ba, ^{208}Pb and ^{209}Bi) were determined in leaves, flowers and infusions of *S. fruticosa* by ICP-MS and other three (Mg, Fe and Zn) only in the leaves by FAAS. The results are presented in Table 1.

From this study, it was revealed that:

a. Some elements, like Se, Sr, Ba and Zn showed higher concentration in Crete's sage than in herbs from the other locations. Especially Se, which has a strong antioxidant activity (Kumar and Krishnaswarny 1997; Reilly 1996), had six times higher content in Crete's samples than in the other locations. Se was also migrated into the infusion.
b. Herbs from Crete and Thermopyles had higher levels of Pb and Cd than the other samples, because the examined populations were cultivated nearby a highway. However, the (%) migration of both elements was low, as Table 1 reveals (6.5% for Pb and 0.0% for Cd).
c. The leaves and the flowers presented similar elemental content, generally higher than the infusions, as expected. The only exceptions were Sn and Bi, which showed higher levels in flowers than in leaves.
d. Some elements, like V, Cr, Cd, Sb and Sn, present in the leaves of sage, did not migrate into the infusion and others like Co, Se, Pb migrated at a very low percentage (5.8%, 6.0% and 6.5%, respectively) and only in the samples from Crete, as Table 1 shows. Ni, Cu, As, Sr and Ba showed high (%) migration levels, but with high variation (high standard deviation) between the samples. Similar (%) migrations for Cr, Cd, Pb, Co and Ba were reported from Başgel and Erdemoğlu (2006), who investigated the elemental content in infusions from Turkey's sage (*Salvia officinalis*) using FAAS and ICP-AES, whereas there was a significant difference in the (%) migration of Cu, Sr, Mn and Ni. In general, it is observed that most elements in

Table 1 Elemental content (mg/Kg) of *Salvia fruticosa* of Greece and the (%) migration from the leaves to infusions

Element	Area									Migration (%)[c]
	Crete[a]			Amorgos		Thermopyles		Lesvos[b]		
	Leaves	Flowers	Infusions	Leaves	Infusions	Leaves	Infusions	Leaves	Infusions	
	Elemental content (mg/Kg)									
V	1.11±0.53	1.24±0.32	<0.03	0.66	<0.03	1.23	<0.03	0.59±0.16	<0.03	0.0
Cr	5.6±3.6	2.60±0.74	<0.05	2.1	<0.05	3.4	<0.05	2.2±0.85	<0.05	0.0
Mn	28.4±7.1	39.2±9.2	7.9±3.4	36.6	20.4	28.7	13.9	36.0±2.9	14.0±3.1	26±14
Co	0.31±0.16	0.36±0.12	0.02±0.01	0.22	<0.01	0.27	<0.01	0.20±0.00	<0.01	5.8±5.1
Ni	3.6±1.5	1.37±0.23	0.40±0.06	2.22	0.14	4.16	0.14	2.58±0.31	0.28±0.10	12.0±7.8
Cu	10.8±3.1	12.2±1.9	4.0±1.3	7.3	1.7	6.6	4.8	5.9±1.0	2.3±1.0	42±24
As	0.34±0.20	0.22±0.08	0.05±0.02	0.18	<0.006	0.20	0.01	0.43±0.3	0.050±0.004	14.8±7.0
Se	0.66±0.39	0.44±0.16	0.04±0.03	0.098	<0.01	0.096	<0.01	0.096±0.006	<0.01	6.0±4.0
Sr	24.0±8.2	27.3±6.3	8.1±3.1	7.5	4.6	14.8	8.2	8.7±0.39	5.1±0.15	43±19
Cd	0.05±0.02	0.10±0.09	<0.002	0.03	<0.002	0.08	<0.002	0.020±0.013	<0.002	0.0
Sn	0.16±0.03	1.18±0.13	<0.008	0.06	<0.008	0.08	<0.008	0.09±0.008	<0.008	0.0
Sb	0.07±0.03	0.06±0.01	<0.014	0.08	<0.014	<0.011	<0.014	<0.011	<0.014	0.0
Ba	18.±13	16.7±5.6	2.8±1.0	7.6	3.2	12.8	4.3	10.0±0.15	1.90±0.17	21±10
Pb	3.30±0.51	1.10±0.45	0.2±0.1	1.8	<0.04	2.3	<0.04	2.1±0.08	<0.04	6.5±5.7
Bi	<0.07	0.28±0.06	<0.09	<0.07	<0.09	<0.07	<0.09	<0.07	<0.09	0.0
Zn	86±37	–[d]	–	32.9	–	25.0	–	40.4±5.5	–	–
Fe	526±100	–	–	365	–	529	–	357±67	–	–
Mg	3,139±685	–	–	3,837	–	3,804	–	3,505±334	–	–

[a] The results are the mean of 15 different samples

[b] The results are the mean of two different samples from the same location

[c] The value is the mean (%) migration±SD (n=19) of all the infusions experiment

[d] The elements were not determined in flowers and the infusions

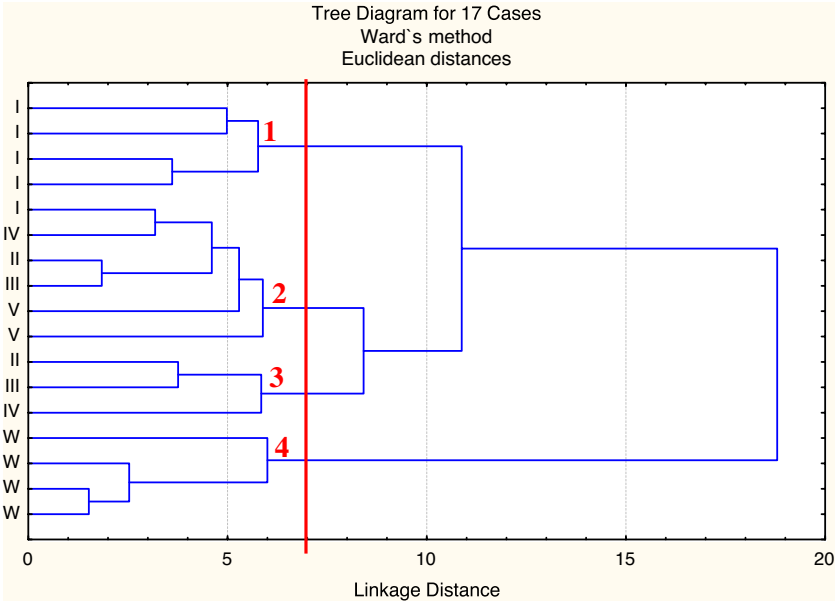

Fig. 1 Dendrogram of the cluster analysis of the samples using Ward's method/Euclidean distances

herbal tea powders were also released into the infusions at different percentages depending on types of herbs (Nookabkaew et al. 2006).

Chemometric Data Evaluation

First, a site classification was attempted, tracking back the origin of the initial sage samples. Samples classification was succeeded with the help of two multivariate techniques: cluster analysis (CA) and discriminant analysis (DA). Figure 1 shows the dendrogram obtained from hierarchical CA. Plants from areas I and W were easily differentiated, while areas II to V seem to be confused due to the limited data represented them. Thus, four clusters were identified. The first one (1) corresponded to area I, while the next two (clusters 2 and 3) contained samples from all areas. All the rest of the sampling sites (outside Crete) comprised the fourth cluster (code W) due to its unique elemental characteristics. These results show the potential of chemometric techniques to track the origin of plants according to their elemental content, even though these plants were transferred from their original cultivation site and left to grown all together in another field.

For DA, three models (the standard, forward and backward stepwise) were tested, in order to determine the most significant parameters (elements) for the site classification. Discriminant analysis constructed the models and evaluated them through the classification matrix (CM), succeeding high percentages in the classification evaluation of all the cases. Thus, all models gave CMs with 100% correct predictions (Table 2) using nine, eight or six variables, respectively, for the standard, forward and backward stepwise approaches. All of them showed that Sb, V, Zn, Cd and Cr are the more important parameters (five out of eleven), while Se, Co, Mn and As are insignificant.

Then, the correlation between the elements was investigated by factor analysis in order to investigate possible common sources of the elements. Five factors succeeded to interpret the original structure explaining 84.2% of the initial variance. Factor analysis applied to the initial data and indentified the factors determining the plants origin. Specifically, according to Kaiser's criterion (eigenvalues >1), five factors had to be selected. Cattell's criterion demanded six to eight factors due to the scree-plot. Furthermore, for explaining 80–90% of the original variance (Massart et al. 1988), five to seven factors had to be retained. Finally, five factors were selected and their loadings are presented in Table 3.

Table 2 Details for DA models classification of *Salvia fruticosa* leaves according to their origin

	Standard	Forward stepwise	Backward stepwise
Variables in the model	Sb>V>Zn>Cd>Cr>Se>Co>Mn>As	Cr>Cd>Sb>V>Se>Zn>Co>Mn	V>Sb>Cr>Cd>Zn>Se
Variables not in the model	–	As	Co>Mn>As
Correct percentages	100% for all I to V and W sites	100% for all I to V and W sites	100% for all I to V and W sites

Table 3 Factor loadings (Varimax normalized, marked loadings are higher than 0.70)

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
V	**0.967**	−0.088	0.043	0.024	0.152
Cr	0.020	**0.782**	0.147	0.007	0.491
Mn	−0.179	−0.545	**−0.727**	−0.210	−0.044
Co	0.464	−0.457	−0.373	0.234	0.342
Ni	0.072	**0.812**	0.009	−0.131	0.291
^{63}Cu	0.386	−0.129	−0.296	0.550	0.591
As	0.350	−0.585	−0.061	0.027	0.178
Se	−0.051	0.351	0.145	−0.163	**0.879**
Sr	0.449	0.535	−0.084	0.420	0.503
^{114}Cd	0.403	0.536	0.135	−0.489	0.150
Sn	0.419	0.049	0.203	0.113	**0.792**
^{123}Sb	**0.849**	0.114	0.009	0.060	0.287
Ba	−0.265	0.603	−0.330	0.414	0.192
^{208}Pb	0.661	0.073	0.349	0.326	0.515
Mo	0.300	0.180	0.030	0.164	**0.820**
Zn	0.182	−0.018	0.298	**0.867**	0.068
Fe	**0.949**	−0.137	0.136	0.056	0.047
Mg	−0.074	0.093	**−0.908**	−0.052	−0.207
Eigenvalue	6.74	3.66	2.00	1.61	1.13
Variance explained (%)	37.4	20.4	11.1	8.9	6.3

The loadings for the first Varimax Factor (VF1) indicated that this component was determined by V, Sb and Fe, explaining 37.4% of the original variance. VF2, explaining 20.4% of the total data variance, represented the metals Cr and Ni, a significant correlation found also in a previous study (Nookabkaew et al. 2006). The elements Mn and Mg contributed to the third factor VF3 (explaining 11.1% of the total data variance). The fourth factor (VF4) reflected Zn, while the last factor (VF5) represented mainly Se, Sn and Mo. All the above correlations were confirmed by Spearman's coefficients (data not shown).

Effect of Temperature of Microwave-Assisted Extraction for the Investigation of Antioxidant Activity

Generally, higher extracting temperature favours the extraction, but also the risk to destroy the antioxidants is increased (Liazid et al. 2007). Several studies suggest 60°C to 120°C as the optimum temperature range for microwave-assisted extraction of herbs, especially when using ethanol as extraction solvent (Pan et al. 2003; Pan et al. 2002; Hemwimon et al. 2007). In this study, the optimum extraction temperature for all potential solvents (ethanol, methanol and ethyl acetate) was investigated. Figure 2 shows the optimum temperature for each solvent (stronger % inhibition of the radical DPPH), which was

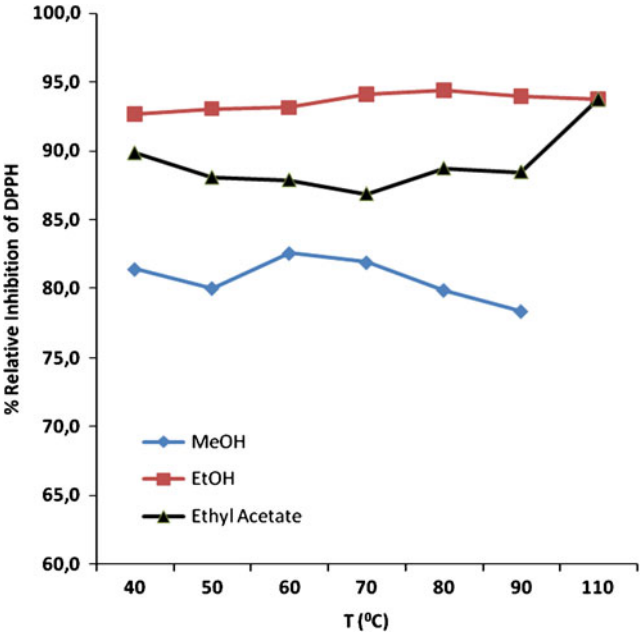

Fig. 2 Effect of extraction temperature on the radical scavenging activity of *Salvia fruticosa's* extract

90°C for ethanol, 60°C for methanol and 100°C for ethyl acetate. These temperatures are within the optimum temperature range of other similar studies (Guo et al. 2001; Pan et al. 2002, 2003; Hemwimon et al. 2007) and below 125°C, where a significant degradation of phenolic compounds was observed (Liazid et al. 2007).

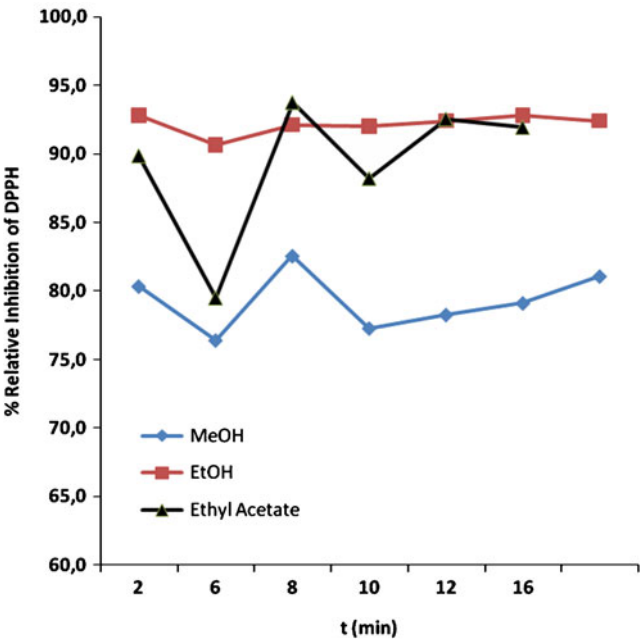

Fig. 3 Effect of extraction time on the radical scavenging activity of *Salvia fruticosa's* extract

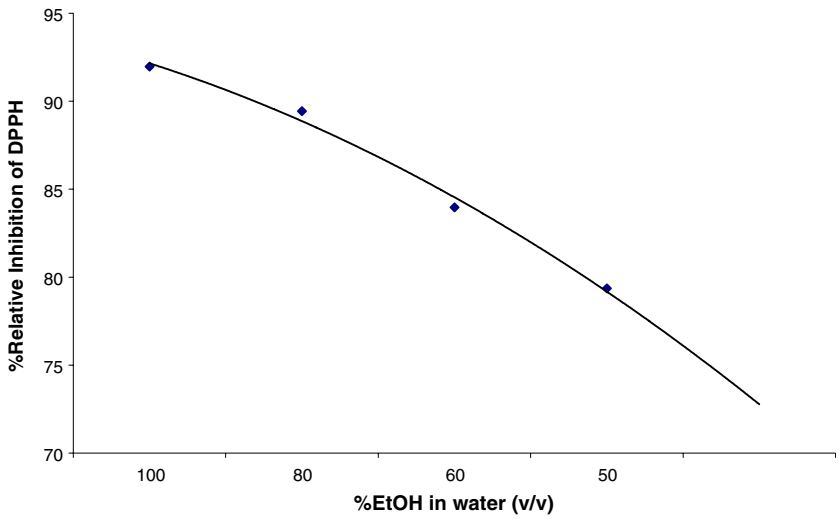

Fig. 4 Effect of the % (*v*/*v*) of ethanol in water on the radical scavenging activity of *Salvia fruticosa's* extract

Effect of Time of Microwave-Assisted Extraction

The duration of the microwave radiation is also an important factor of the extraction. Extraction time differs from method to method and between different compounds. In this study, 0.5 g of leaves was extracted with all three solvents at the optimum temperature. Figure 3 shows the optimum time for each solvent (stronger % inhibition of the radical DPPH). Extraction time higher than 10 min resulted in consistent and reproducible results for all the solvents and a time of 12-min extraction was chosen as the optimum. These results are similar to those obtained from Hemwimon et al. (2007) who used the same instrumentation and the same DPPH method.

Selection of the Solvent of Microwave-Assisted Extraction

Three moderate polar solvents were tested (pure methanol, pure ethanol and pure ethyl acetate). A mixture of ethanol–water was also tested at different ratios. The results obtained showed that ethanol and ethyl acetate produced similar yields (similar % inhibition), higher than methanolic extracts (Figs. 2 and 3). Ethanol is a safe solvent, compatible with food industry, providing good yield, so it was chosen as the appropriate solvent.

Different mixtures of ethanol–water were applied, from 50% to 100% of ethanol, and it was found that the yield of extraction (% inhibition of radical) increased with the increase of ethanol concentration (Fig. 4). This is also

Table 4 IC_{50} and antioxidant activity index (AAI) values for ethanolic extracts of the leaves and some flowers of *Salvia fruticosa* and the respective values of some reference compounds

Plant				Reference compounds		
Sample	Part	IC_{50} (mg/L)	AAI		IC_{50} (mg/L)	AAI
I (Crete)	Leaf	18.9±5.0[a]	2.65±0.77[a]	Caffeic acid	3.3	14.7
	Flower	39±14[b]	1.29±0.49[b]	Rosmarinic acid	4.7	10.7
II (Crete)	Leaf	19.2±3.7[b]	2.61±0.52[b]	BHT	40.9	1.2
III (Crete)	Leaf	14.1±5.0[b]	3.5±1.4[b]	(+) Catechin	4.8	10.4
IV (Crete)	Leaf	17.8±2.2[b]	2.81±0.35[b]	Quercetin	3.2	15.6
V (Crete)	Leaf	19.2±8.3[b]	2.6±1.2[b]	2-tert-butyl-4-methoxyphenol	4.5	11.1
VI (Crete)	Leaf	27.4±1.6[b]	1.82±0.11[b]			
Thermopyles	Leaf	27.9	1.79			
Amorgos	Leaf	40.1	1.25			
Gera I (Lesvos)	Leaf	14.3	3.50			
Gera II (Lesvos)	Leaf	32.2	1.55			
White tea	Leaf	9.4	5.32			

[a] The results are the mean of five different samples from the same location

[b] The results are the mean of two different samples from the same location

supported from the findings of Guo et al. (2001), where the higher ethanol concentration, the higher is the yield. Maisuthisakul et al. (2008) proved that the scavenging effects of plant extracts on the DPPH radical are strongly correlated with total phenolic and flavonoid contents. *S. fruticosa* contains high amount of polyphenols like caffeic acid, rosmarinic acid, chlorogenic acid, coumaric acid and 4-hydroxyacetophenone which are usually attracted with less polar solvents like ethanol (Lu and Foo 2000; Suhaj 2006). Our results denote that the extraction of radical scavenging compounds is favoured with 100% (*v*/*v*) ethanol.

Antioxidant Activity of Ethanolic Extracts of *S. fruticosa*

The radical scavenging activity was expressed as the percentage of the initial DPPH absorbance by the extracts studied. The IC_{50} values and the AAI values, for these extracts were calculated and compared with those obtained using some reference compounds and one synthetic antioxidant (BHT). The IC_{50} and AAI values obtained for the reference compounds in this work were compared with those obtained from Scherer and Godoy (2009), who used the same DPPH scavenging method. The calculated IC_{50} and AAI values of the reference compounds from Scherer and Godoy (2009) were similar with these reported in this work.

Fifteen samples of leaves and two samples of flowers from Crete (from six different local areas) were examined for their antioxidant activity. The IC_{50} and the AAI values for these extracts were calculated and compared with those from other places in Greece (Thermopyles, Amorgos and Lesvos). The antioxidant activity of a Chinese white tea leaves was also examined. The results are presented in Table 4.

The results obtained showed that the ethanolic extracts of the leaves of *S. fruticosa* presented high antioxidant activity, higher than that of the flowers. The plant extracts presented higher (%) inhibition than that of the synthetic antioxidant BHT. The Crete's sage presented the best IC_{50} values, ranging between 14.1 and 27.4 mg/L, whereas the IC_{50} values from the other samples ranged between 14.3 and 40.1 mg/L. From the AAI values, as Scherer and Godoy (2009) suggested, almost all the samples from Crete and Gera I from Lesvos had very strong antioxidant activity (AAI>2.0), whereas the other samples had just strong antioxidant activity (1.0<AAI<2.0). However the higher antioxidant activity was obtained from the extract of Chinese white tea, as expected.

In conclusion, an ICP-MS method for the elemental determination of leaves, flowers and infusions of *S. fruticosa* was developed and validated. The results obtained indicated that *S. fruticosa* contains large amounts of nutrients and is rich in Fe and Mg. *S. fruticosa* also contained Cr, Cu, Zn and Mn which play an important role in the metabolism of cholesterol as well as to heart diseases. Flowers and leaves of *S. fruticosa* contained also some toxic heavy metals (Pb and Cd) at very low concentrations which did not migrate in the infusion.

A significant correlation between Cr and Ni in leaves was proved by using factor analysis and Spearman correlation. Other significant correlations between V, Sb, Fe, or Mn, Mg or Se, Sn and Mo were revealed with the use of FA, denoting common sources. Discriminant and cluster analysis were also used to confirm correlations between the origin of the samples and the concentration of various elements. The classification matrix of three DA models gave high classification accuracy (100%) with the use of the five most significant variables (Sb, V, Zn, Cd and Cr), while CA visualized the samples' classification according to their initial origin.

In addition, a fast (12 min) and quantitative method for the microwave-assisted extraction of antioxidants from sage was also developed. During the development of the method, several parameters were evaluated such as the extraction solvent, temperature and time. The antioxidant activity was determined using the DPPH scavenging method.

According to this work, strong antioxidant effects obtained from the herb *S. fruticosa* grown in all over Greece. Nevertheless, Crete's sage exhibited the stronger antioxidant activity among the tested samples. Furthermore, it was also found that extracts of sage flowers exhibited strong antioxidant activity, similar to that of synthetic antioxidant BHT.

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