Flavonoids and Phenolic Acids of Sage: Influence of Some Agricultural Factors

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A reversed-phase high-performance liquid chromatography/diode-array detector procedure is proposed for the determination of six phenolic compounds (caffeic acid, luteolin 7-*O*-glucoside, rosmarinic acid, apigenin, hispidulin, and cirsimaritin) in sage. The chromatographic separation was achieved using a reversed-phase Spherisorb ODS2 (5-μm particle size, 25.0 × 0.46 cm) column. Of the several extractive solvents assayed only ethyl ether, ethyl acetate, and acetone were able to extract all the compounds mentioned. Best resolution was obtained using a gradient of water/phosphoric acid (999:1) and acetonitrile. Ten samples cultivated in two experimental fields (1997−1999) were analyzed and the individual compounds quantified. Four commercially available samples were also analyzed and the results are discussed.

Keywords: *Sage (Salvia officinalis); HPLC/DAD; phenolic profile; quality control*

INTRODUCTION

Sage (leaves of *Salvia officinalis* L. or *Salvia grandiflora* Tenore) is generally used as a culinary herb. The Council of Europe lists sage as a natural source of food flavoring (category N2). This category means that this flavoring can be used providing the final concentration of thuyones (central nervous system toxin causing convulsant activity) in foodstuff does not exceed fixed values (Newall et al., 1996; De Vincenzi et al., 1997). The drug is also used for its medicinal properties: antispasmodic, antiseptic, astringent, antihidrotic. As an herbal tea, sage is traditionally used to treat flatulent dyspepsia, dysmenorrhea, gastritis and sore throat (Newall et al., 1996; Evans, 1989; Van Hellemont, 1986).

Although sage is an herb considered mainly for its content of essential oil (it is an "aromatic herb"), it is largely used as an infusion where polar compounds play a central role. Among the more polar compounds, we can consider the presence of several phenolic acids and flavonoids (based in more than 15 aglycones) which are also described to occur (Wang et al., 2000; Bondy, 1997). Their presence usually is described on a phytochemical level, but no information is given about the relative amounts present. Phenolic diterpenes are also described to occur, mainly carnosic acid and carnosol, which, together with rosmarinic acid, are responsible for the potent antioxidant activities of sage extracts (Cuvelier et al., 1994, 1996; Schwarz and Ternes, 1992a−1995; Okamura et al., 1994).

The European Pharmacopoeia established the quality control of sage where the chemical identity is defined by the presence of cineole and thuyone. However, because flavonoids and phenolic acids have been applied successfully to quality control of several foodstuffs (Valentão et al., 1999; Tomás-Lorente et al., 1992), we tried to develop a methodology based on high-performance liquid chromatography (HPLC) of the phenolic compounds of sage which can be used for its routine quality control on a qualitative and quantitative level.

The developed methodology was applied to 10 samples cultivated in two experimental fields and to four commercially available samples.

MATERIALS AND METHODS

Plant Samples and Standards. Plant samples were commercially available (samples A, B, C, and D, bought during 1999) or collected from two experimental fields in the North of Portugal (Arcos and Arouca). These two fields are about 35 km from the sea and 100 km from each other; they are both at about 300 m altitude, so no big climatic differences are observed between them. Sage was sowed in February 1997 and transplanted in June to both fields; collections (leaves) were made in 1997 (September), 1998 (April and September), and 1999 (April and September).

Authentic standards were obtained from Sigma Chemical Co. or from Extrasynthése. Hispidulin and cirsimaritin were previously obtained from *Salvia lavandulaefolia* (Tomás-Lorente et al., 1988).

Extraction of Phenolic Compounds from Plants. For analytical purposes, 3 g of sage leaves were ground and extracted twice, with 50 mL of ethanol, 15 min each time, at room temperature, with agitation. The extract obtained was taken to dryness under reduced pressure at 30 °C. The residue was dissolved in 1 mL of methanol, and 20 μL were analyzed by HPLC. The entire protocol was repeated with other batches (3 g each) of the same sample; the batches were subjected to extraction with petroleum ether, chloroform, ethyl ether, ethyl acetate, acetone, methanol, ethanol 80% and 30%, and boiling water.

For quantification purposes, 3 g of each sample were ground to pass through a 910-μm sieve and extracted, at room temperature, with agitation, using acetone: 50 mL for 15 min, followed by 50 mL for 10 min, and, finally, 25 mL for 5 min. The three extracts were gathered, filtered, and taken to

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Table 1. Sage Phenolic Compounds Separated by HPLC

peak	compound	Rt gradient 1	Rt gradient 2
1	caffeic acid	6 min 48 s	8 min 43 s
2	luteolin 7-*O*-glucoside	22 min 38 s	20 min 05 s
3	rosmarinic acid	24 min 05 s	28 min 38 s
4	apigenin	47 min 31 s	46 min 48 s
5	hispidulin	48 min 10 s	47 min 17 s
6	cirsimaritin	52 min 53 s	51 min 41 s

dryness under reduced pressure at 30 °C. The residue was dissolved in 2 mL of methanol, and 20 μL were analyzed by HPLC.

HPLC Analysis of Phenolic Compounds. Separation of phenolic compounds was achieved with an analytical HPLC unit (Gilson), using a reversed-phase Spherisorb ODS2 (5-μm particle size, 25.0 × 0.46 cm) column. For analytical purposes the solvent system used (gradient 1) was a gradient of water/formic acid (19:1) (A) and methanol (B). The gradient was as follows: 0 min, 30% B; 15 min, 30% B; 20 min, 40% B; 30 min, 45% B; 50 min, 60% B; 51 min, 80% B; 52 min, 80% B. Elution was performed at a solvent flow rate of 1 mL/min. Detection was accomplished with a diode-array detector (DAD) and chromatograms were recorded at 280, 320, and 350 nm. The different phenolic compounds were identified by comparing their retention times and UV−vis spectra in the 200−400 nm range with authentic standards.

For quantitative purposes the solvent system (gradient 2) used was a gradient of water/phosphoric acid (999:1) (A) and acetonitrile (B). The gradient was as follows: 0 min, 17% B; 35 min, 23% B; 37 min, 36% B; 57 min, 56% B; 59 min, 100% B. Elution was performed at a solvent flow rate of 1 mL/min. Rt values of identified compounds in both gradients are in Table 1. Quantification of phenolic compounds was achieved by the absorbance recorded in the chromatograms relative to external standards at 330 nm. Hispidulin and cirsimaritin were quantified as apigenin and all other compounds as themselves.

Statistics. The results were analyzed by analysis of variance (ANOVA) methodology.

RESULTS AND DISCUSSION

Sage was initially subjected to extraction by several solvents and the extracts were analyzed by HPLC/DAD using the eluents more usually described for the analysis of phenolics (water with formic acid and methanol, gradient 1) (Andrade et al., 1998). Although numerous flavonoids and phenolic acids are described in sage, the only ones that were present in sufficient amounts to be identified and quantified were caffeic acid, luteolin 7-*O*-glucoside, rosmarinic acid, apigenin, hispidulin, and cirsimaritin. Some lipophilic compounds were found in the last part of the chromatograms, but their UV spectra were not identical to any of the flavonoids described. Rosmarinic acid, probably because it is the most abundant phenolic, was present in considerable amounts in all the extracts (Figures 1 and 2); the flavonoids apigenin, hispidulin, and cirsimaritin, visible in the final part of the chromatograms, were present in extracts obtained with several solvents, from petroleum ether to ethanol, although present in higher amounts in those obtained with ethyl ether, ethyl acetate, and acetone; in the infusion, only luteolin 7-*O*-glucoside and caffeic and rosmarinic acids were detected by HPLC (data not shown).

From the results above we considered that acetone was the best extract for routine quality control of sage because it is the one that exhibits the highest number of quantifiable compounds. On using acetone as extractive solvent and gradient 1 (Figure 2A), the pairs luteolin 7-*O*-heteroside/rosmarinic acid and apigenin/hispidulin were not well resolved although identifiable by the DAD detector. The signal due to the luteolin derivative could be attributed either to luteolin 7-*O*-glucoside or to luteolin 7-*O*-rutinoside, because both glycosyl derivatives have the same UV spectra and the same Rt in gradient 1. The resolution obtained with this gradient was far from good and, therefore, not quite suited to quantitative analysis. Besides, the signals of caffeic and rosmarinic acids were split by the *cis*/*trans* isomerism, which added an unnecessary complexity to the chromatogram. So, several other eluent systems

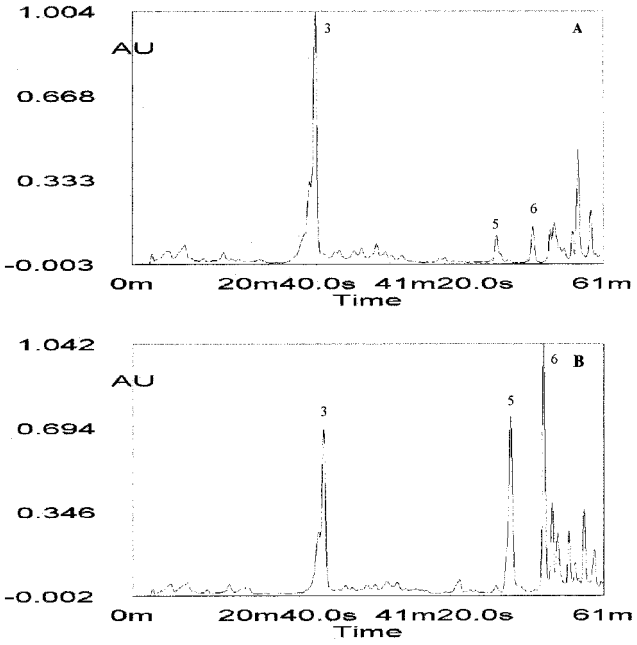

Figure 1. HPLC profile of a sage sample (gradient 1), extracted with petroleum ether (A) and chloroform (B). Detection at 330 nm. Peaks: (3) rosmarinic acid; (5) hispidulin; (6) cirsimaritin.

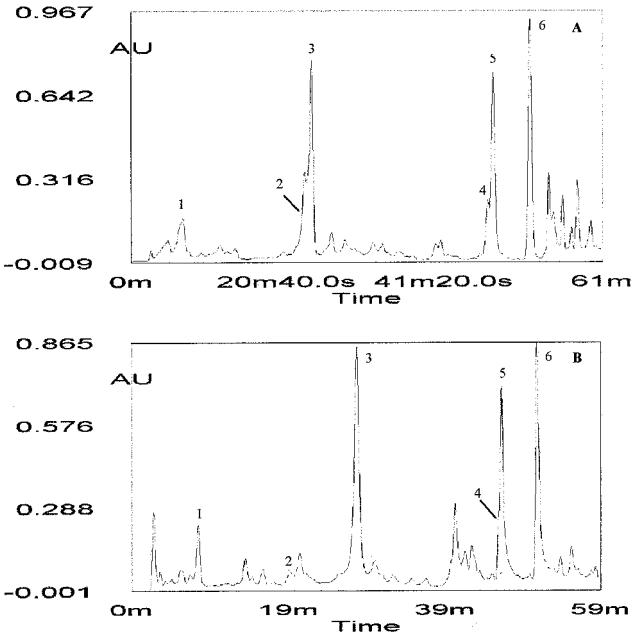

Figure 2. HPLC profile of a sage sample extracted with acetone with gradient 1 (A) and gradient 2 (B). Detection at 330 nm. Peaks: (1) caffeic acid; (2) luteolin 7-*O*-glucoside; (3) rosmarinic acid; (4) apigenin; (5) hispidulin; (6) cirsimaritin.

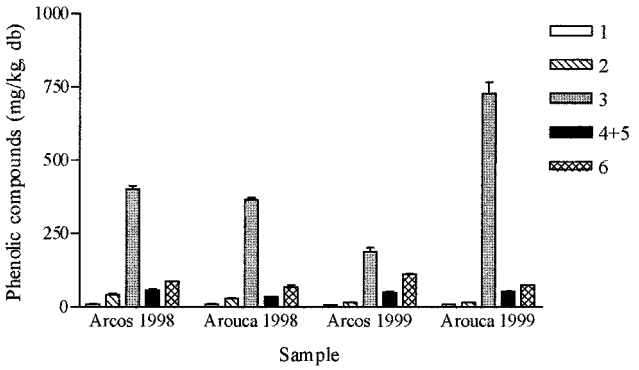

Figure 3. Phenolic acid amounts from the samples collected in April: (1) caffeic acid; (2) luteolin 7-*O*-glucoside; (3) rosmarinic acid; (4 + 5) apigenin + hispidulin; (6) cirsimaritin. Error bars represent the standard deviation of two assays for each sample.

were tried, and best results were obtained using acetonitrile and gradient 2. (Figure 2B). On using this gradient, the two possible luteolin derivatives had different Rt values, and on comparison with standards, it was possible to ensure that the compound present in sage is luteolin 7-*O*-glucoside. Rosmarinic acid was well separated from luteolin 7-*O*-glucoside, and the cinnamic acids were not split. Hispidulin, however, was, collapsed with apigenin and both compounds were quantified as apigenin.

For quantification purposes, and to guarantee a full extraction of the phenolic compounds and the repeatability of the method, one sample was subjected to a set of six extraction conditions, using different times and volumes of solvent (data not shown). Best results were obtained using a 3-fold extraction (see Materials and Methods). Quantification was performed at 330 nm, a wavelength at which all the compounds are detected and can be quantified by the software available. The use of only one wavelength makes routine analysis more feasible, allowing the quantification of all the compounds in one run, even when a DAD is not available.

The tested analytical conditions were applied to several samples collected during 1997, 1998, and 1999 on the two mentioned experimental fields and to four commercially available samples (Materials and Methods). Sage was sown in February 1997 and transplanted in July; first collections were made in September. During 1998 and 1999, two collections a year were made (April and September).

As a general rule, there are no significant differences between samples from the two experimental fields (Figures 3 and 4) ($P > 0.05$); such differences were not expected because the climatic characteristics of both are similar. Only one difference was observed in April 1999, when the sample from Arcos exhibited a considerably lower amount of phenolics (mainly rosmarinic acid) than that from Arouca; this can be attributed to the fact that some of the plants were infested by *Phomopsis* sp and *Peronospera* sp.

An increase of total phenolics from 1997 to 1998 was also observed, which is not surprising, because sage was sowed in 1997 and, because it is a perennial shrub, an increase may be expected in the production of such metabolites.

Following the tradition sage leaves are collected before flowering, which, in Portugal, happens from May through July. This is probably because the content of essential oil is higher during that season. However, this is not the case with phenolics because a significant increase ($P < 0.05$) was observed in total phenolics from April to September. This rise is due to an increase of rosmarinic acid, but mainly to a 5−6-fold increase of apigenin/hispidulin and cirsimaritin.

Some observations can be made concerning the quantitative pattern of individual compounds. In all the samples, commercially available or cultivated in the North of Portugal, caffeic acid and luteolin 7-*O*-glucoside were always the minor compounds; together they account for 2.5−8% of the total phenolics. Rosmarinic acid was always the major compound in the cultivated samples, ranging from 50 to 83% in samples collected in April, and from 35 to 62% in samples collected in September; however, in commercially available samples (Figure 5), cirsimaritin equals or overpasses rosmarinic acid. Major differences between cultivated and commercially available samples concern the quantitative distribution of rosmarinic acid, apigenin/hispidulin, and cirsimaritin, with these three phenolics having a more equilibrated distribution in commercially available samples.

From the study presented herein we can conclude that in a 3-year cultivation and without any fertilizing, it is possible, in the North of Portugal, to attain total phenolics equivalent to those registered in commercially available samples, the amount of rosmarinic acid, probably the most interesting compound when sage is used as an infusion, being even greater. However, more studies are necessary to determine the factors responsible for the quantitative patterns of individual com-

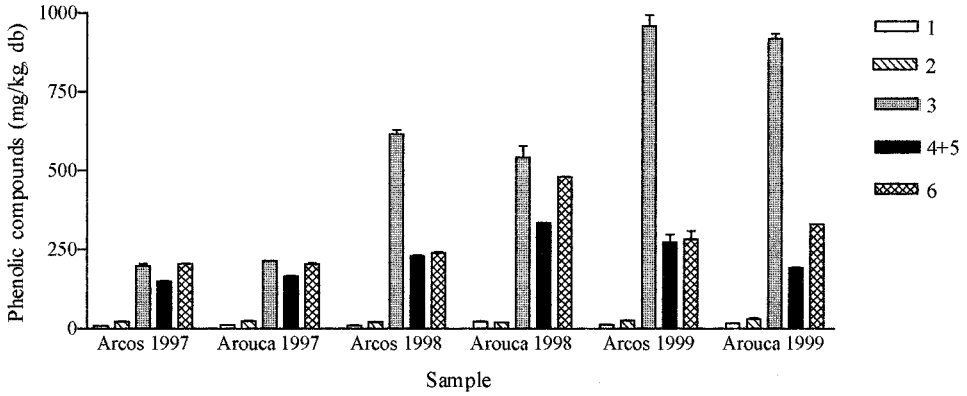

Figure 4. Phenolic acid amounts from the samples collected in September: (1) caffeic acid; (2) luteolin 7-*O*-glucoside; (3) rosmarinic acid; (4 + 5) apigenin + hispidulin; (6) cirsimaritin. Error bars represent the standard deviation of two assays for each sample.

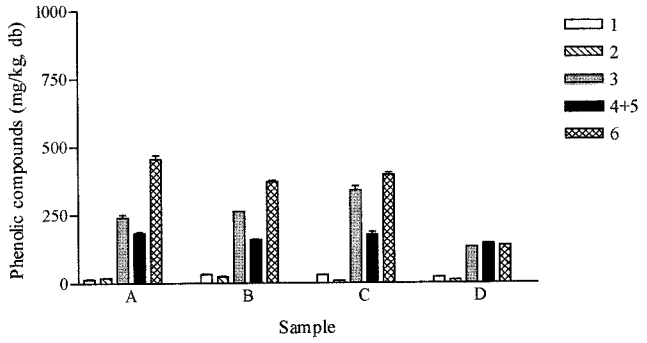

Figure 5. Phenolic acid amounts from the commercially available samples: (1) caffeic acid; (2) luteolin 7-*O*-glucoside; (3) rosmarinic acid; (4 + 5) apigenin + hispidulin; (6) cirsimaritin. Error bars represent the standard deviation of two assays for each sample.

pounds, namely for the increase of the methoxylated flavonoids in commercially available samples.

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