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Food Chemistry 87 (2004) 307–311
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC

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Received 2 October 2003; received in revised form 18 December 2003; accepted 18 December 2003

Abstract

An HPLC method for the determination of rosmarinic and caffeic acids in several aromatic herbs, namely, rosemary, sage, thyme, spearmint, balm, and lavender, has been developed and validated. The separation system consisted of a C18 reversed-phase column, a gradient elution system of methanol/water containing orthophosphoric acid, and a photodiode array detector. The content of rosmarinic and caffeic acids was found to be 2.0–27.4, and 0–0.4 mg/g, respectively, in the aromatic herbs analysed. The described method is simple, sensitive, reproducible and ideally suited for rapid routine analysis.

Keywords: Rosmarinic acid; Caffeic acid; Aromatic herb; HPLC

1. Introduction

Traditionally, aromatic herbs, such as rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.) and lavender (*Lavendula angustifolia* Mill.), which are native to the Mediterranean region and cultivated world-wide, and balm (*Melissa officinalis* L.), and spearmint (*Mentha spicata* L.) which are common plants in Britain and other European countries, have been used in folk remedies for exhaustion, weakness, depression, memory enhancement, circulation improvement and strengthening fragile blood vessels. Researchers have found that these plants are a source of compounds possessing high antioxidant (Zheng & Wang, 2001), anti-inflammatory (Al-Sereiti, Abu-Amer, & Sen, 1999), anti-allergy (Ito, Miyazaki, Ono, & Sakurai, 1998) and anti-depression (Takeda, Tsuji, Matsumiya, & Kubo, 2002) activity. This appears to be related to their content of phenolic compounds, amongst which, rosmarinic acid (Fig. 1), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, was found to be the most important (Wren, 1988). Extracts, in various forms, of these aromatic leaves are also widely used in various food, beverage and cosmetic applications either as astringents or as flavouring agents.

Despite many reports on the medicinal or functional properties of these aromatic leaves and their preparations, only a limited number of papers have been published on the determination of the phenolic constituents of these materials by either HPLC (Bandoniene & Murkovic, 2002) or GC (Kochan, Wysokinska, Chmiel, & Grabias, 1999).

This paper presents an HPLC method for the determination of rosmarinic and caffeic acids in the dried leaves of the aromatic herbs: rosemary, sage, thyme, balm and spearmint, and the flowers of lavender, and for compassion, in the leaves of the non-aromatic plant, self-heal (*Prunella vulgaris* L.).

2. Materials and methods

2.1. Materials

Samples of the dried leaves (or herb) of rosemary, thyme, spearmint, sage, balm and self-heal and flowers of lavender were obtained from the trade.

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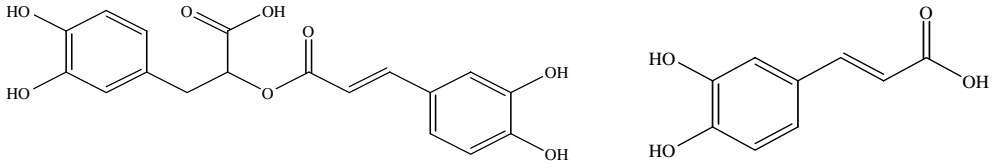
doi:10.1016/j.foodchem.2003.12.029



Rosmarinic acid Caffeic acid

Fig. 1. Chemical structures of caffeic acid and rosmarinic acid.

2.2. Reagents and chemicals

Methanol (HPLC grade), ethanol (analytical grade), acetone (analytical grade) and orthophosphoric acid (analytical grade) were purchased from Fisher Scientific (Essex, UK). The standard of rosmarinic acid was purchased from Extrasynthese (France). The standard of caffeic acid was purchased from Sigma (Dorset, UK). The water used in HPLC and for sample preparation was produced with a Super Purity Water System (Purite Ltd, England) with a resistivity over 17.5 MΩ cm.

2.3. Preparation of standard solution

Stock standard solutions were prepared by accurately weighing 10 mg of rosmarinic and caffeic acid reference standards into separate 10-ml volumetric flasks and dissolving in ethanol/water (30:70, v/v) with the aid of sonication. Working standard solutions, 5–100 μg/ml, were prepared by dilution from the stock standard solutions with ethanol/water (30:70, v/v).

2.4. Sample preparation

Approximately 50 mg of ground sample were accurately weighed into a 30-ml tube, and extracted with 25 ml ethanol/water (30:70, v/v) with the aid of sonication for 10 min. The resulting mixture was centrifuged at 4500 rpm for 5 min, and the supernatant transferred to a 50-ml volumetric flask. The residual solid was further extracted with 20 ml of the same ethanol/water mixture with sonication for 5 min, and centrifuged as above. The supernatants were combined, and made to 50 ml with water. All samples were centrifuged at 13,000 rpm for 10 min prior to injection for HPLC analysis.

2.5. Instrumentation

An HP 1100 series liquid chromatograph system comprising vacuum degasser, quaternary pump, autosampler, thermostatted column compartment, and diode array detector was used. The column, a Kingsorb 5μ C18, (150 × 4.6 mm) was maintained at 30 °C. Solvents used for separation were 0.1% orthophosphoric acid in water (v/v) (eluent A) and 0.1% orthophosphoric acid in methanol (v/v) (eluent B). The gradient used was: 0–10 min, linear gradient from 40% to 50% B; 10–15 min, linear gradient from 50% to 60% B, maintain at 60% B until 25 min. The flow rate was 1.0 ml min^{-1}. Detection wavelength was 330 nm. The sample injection volume was 10 μl. The chromatographic peaks of rosmarinic acid and caffeic acid were confirmed by comparing their retention times and UV spectra with that of their reference standards. Working standard solutions were injected into the HPLC and peak area responses obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph.

3. Results and discussion

3.1. Separation of rosmarinic and caffeic acids

Several mobile phases, including methanol–water and acetonitrile–water in combination with acetic acid or phosphoric acid, were tested. Eventually, it was found that a water–methanol system containing phosphoric acid, as described in Section 2.5, gave the best separation of rosmarinic and caffeic acids. Fig. 2 demonstrates the separation obtained for a typical sample of rosemary leaves, and Figs. 3 and 4 show the spectra of rosmarinic and caffeic acids, respectively. It can be seen that a good separation can be achieved within 20 min using the conditions described. The remainder of the gradient conditions ensures efficient column washing.

3.2. Comparison of different solvents for the extraction of rosmarinic and caffeic acids

Ethanol, methanol, acetone, and acetonitrile, all at 30% in water (v/v), were used to investigate the effect of solvents on the extraction of rosmarinic and caffeic acids. The results were compared to those obtained with water as the extraction solvent. It was found that there was little difference using ethanol, methanol, acetonitrile or acetone. However, with water alone as an extraction solvent, the content of rosmarinic acid was about 20% lower than for the other four solvents. Based on the

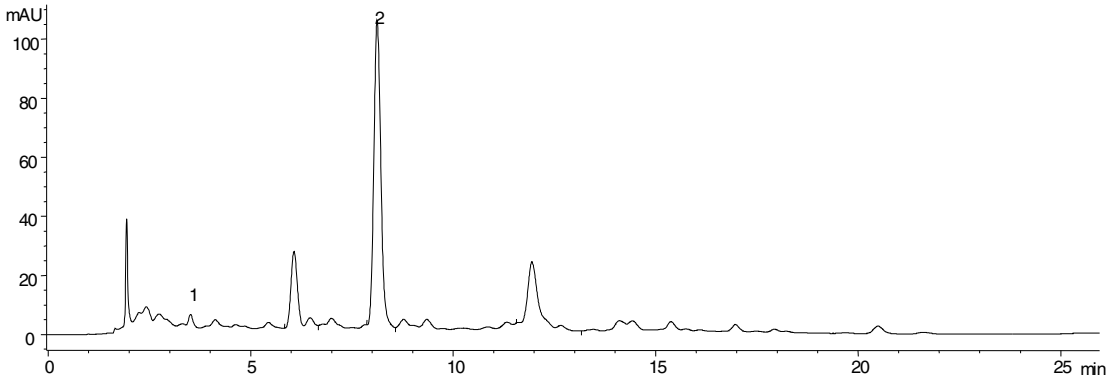


Fig. 2. Chromatogram of an extract from rosemary leaves. Peak identification: 1 – caffeic acid; 2 – rosmarinic acid.

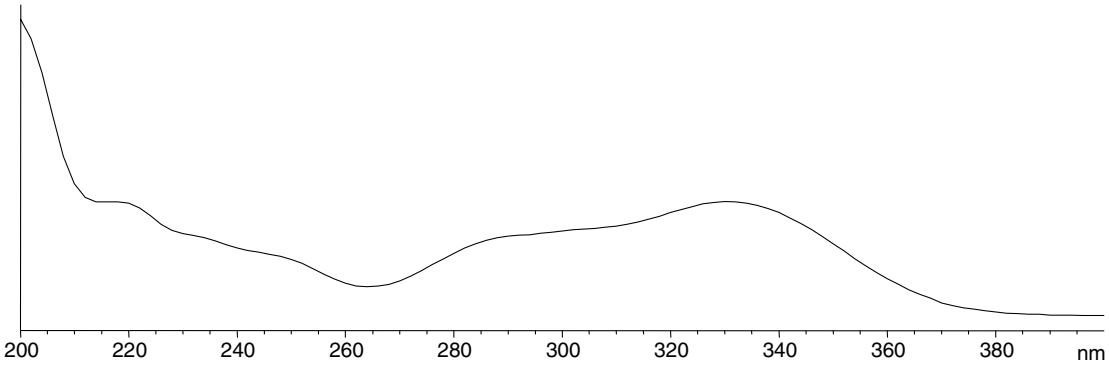


Fig. 3. Spectrum of rosmarinic acid.

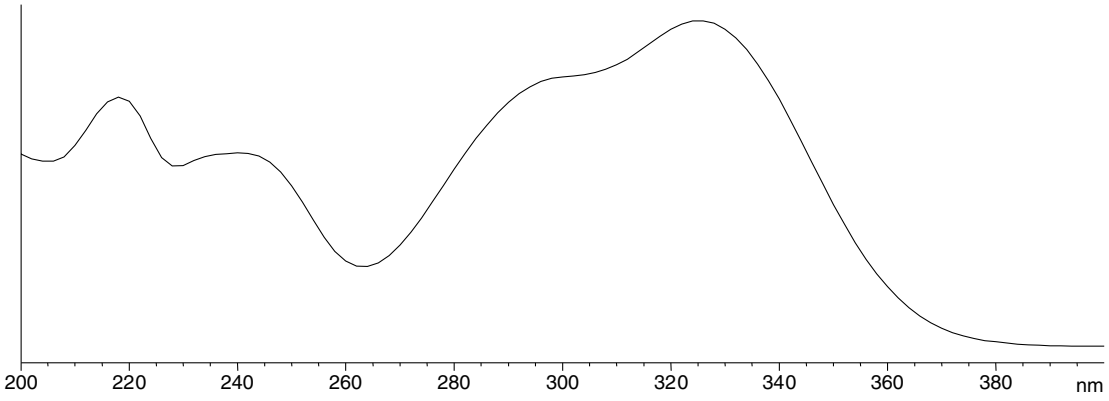


Fig. 4. Spectrum of caffeic acid.

above observations and the fact that various concentrations of ethanol are usually used to prepare commercial extracts from the raw materials, a mixture of ethanol and water was selected to be the solvent for sample preparation. Various concentrations of ethanol in water from 15% to 96% (v/v) were investigated for the extraction of rosmarinic and caffeic acids. It was found that ethanol concentration between 30% and 60% (v/v) gave the highest extraction yield for both acids. However, acceptable separation could not be achieved when the ethanol concentration was 96% (v/v) due to injection solvent effects: the injection solvent being stronger than the mobile phase. The same phenomenon was noticed in analyses of catechins (Wang, You, & Helliwell, 2000) and hamamelitannin (Wang, Porvan, & Helliwell, 2003).

The effect of extraction time on the content of rosmarinic acid was investigated using 30% ethanol/water

(v/v) as the solvent. It was found that 10 min of sonication was sufficient to extract the analytes.

3.3. Validation of the method

Calibration graphs for rosmarinic and caffeic acids were constructed using seven levels of concentration which covered the concentration ranges expected in the various samples. The linearity range for rosmarinic and caffeic acids was determined to be 2–100 and 0.4–100 μg/ml with 0.99995 and 0.99999 of the square of correlation coefficient (R^2), respectively, and on-line linearity (LOL) was 99.72% and 99.92%, respectively, according to the following equation (Garćia, Cuadros, Alés, Román, & Sierra, 1997; Natera, Castro, Garća-Moreno, Rowe, & Barroso, 2002):

$$\mathrm{LOL}\ (\%) = 100 - \mathrm{RSD}(b),$$

where RSD(b) is the relative standard deviation of the slope (expressed as a percentage).

According to an ALAMIN program (Garćia et al., 1997), analytical sensitivity (AS) is determined by the ratio of S_s/b, in which S_s is the residual standard deviation and b is the slope of the calibration curve. The limit of detection ($\mathrm{LOD}_{\mathrm{approx}}$) is determined by the following equation:

$$\mathrm{LOD}_{\mathrm{approx}} = 3(S_s/b) \times \left[\frac{(n-2)}{(n-1)}\right]^{1/2},$$

where n is the number of total measurements for the calibration set. The limit of quantitation ($\mathrm{LOQ}_{\mathrm{approx}}$) is calculated by replacing 3 with 10 in the above equation.

Table 1
Performance characteristics

	AS (μg/ml)	$\mathrm{LOD}_{\mathrm{approx}}$ (μg/ml)	$\mathrm{LOQ}_{\mathrm{approx}}$ (μg/ml)
Rosmarinic acid	0.259	0.708	2.361
Caffeic acid	0.056	0.150	1.345

Table 2
The content of rosmarinic acid (mg/g)

Samples	RA	CA
Rosemary 1	10.0	0.1
Rosemary 2	10.0	0.1
Rosemary 3	11.0	0.2
Sage 1	8.7	0.3
Sage 2	14.1	0.3
Sage 3	8.5	0.4
Thyme 1	8.7	0.3
Thyme 2	4.5	0.1
Spearmint 1	14.3	0.3
Spearmint 2	7.1	0.2
Balm	27.4	0.3
Self-heal	21.7	1.8
Lavender	2.0	nd

RA – rosmarinic acid; CA – caffeic acid; nd – not detectable.

The results for the AS, LOD and LOQ were listed in Table 1. It can be seen from these results that the limits are low enough to determine rosmarinic acid and caffeic acid in the aromatic herbs.

Recovery was determined by spiking a sample with three different additions of rosmarinic acid and caffeic acid standard solutions. The average recovery was found to be 97.3% and 100.1% for the rosmarinic acid and caffeic acid, respectively.

To evaluate the precision of the system, a sample solution kept at ambient temperature was analysed three times in one day and three times over seven days. As a result, the intra-day precision was found to be 1.18% and 1.31%, and inter-day precision, 4.43% and 2.72% for rosmarinic acid and caffeic acid, respectively. Moreover, if the sample was kept in a freezer at −10 °C, the inter-day precision over seven days was less than 0.56% for both compounds.

3.4. Quantitative measurement of different samples

Table 2 shows the content of rosmarinic and caffeic acids in three samples of rosemary, three of sage, two of thyme, two of spearmint, one of balm and one of lavender. It can be seen from the table that the content of rosmarinic acid ranged from 2.0 to 27.4 mg/g, and caffeic acid from 0 to 0.4 mg/g. Rosmarinic and caffeic acids are also found in some non-aromatic herbs, for example, self-heal, the leaves of a sample of which contained about 21.7 and 1.8 mg/g of rosmarinic and caffeic acids, respectively.

4. Conclusions

This method is simple and sensitive, and the limits of detection and quantitation are low enough to analyse rosmarinic and caffeic acids in aromatic herbs, such as rosemary, sage, thyme, lavender, balm and spearmint. The method is thought to be ideally suited for rapid routine analysis.

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