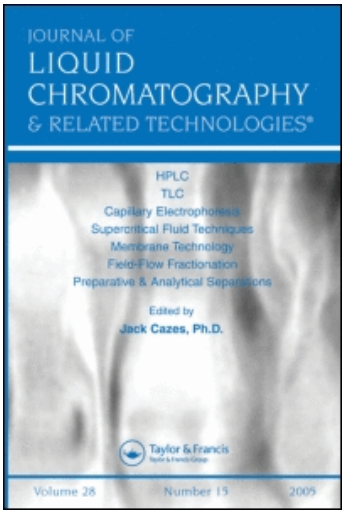

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SIMULTANEOUS DETERMINATION OF FLAVONOIDS, PHENOLIC ACIDS, AND COUMARINS IN SEVEN MEDICINAL SPECIES BY HPLC/DIODE-ARRAY DETECTOR

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ABSTRACT

A simple and accurate reversed phase HPLC procedure is proposed for the determination of 19 phenolic compounds (flavonoids, phenolic acids, and coumarins) in seven medicinal species. The sample preparation involved extraction, alkaline and acid hydrolysis, and purification through a Bond-Elut C18 column. The chromatographic separation was achieved using a reversed phase column Spherisorb ODS2 (5μm; 25.0 x 0.46 cm). Gradient elution was carried out using water-formic acid (19:1) (A) and methanol (B). A diode-array detector monitored the effluent and chromatograms were recorded at 280, 320, and 350 nm. The optimised methodology seems to be useful for the phytochemical analysis of plant extracts. A close correlation between the phenolic compound patterns and the botanical origin of plants was found.

Table 1

Phenolics Content of Plants

Compound	RT(min)	A*	B*	C*	D*	E*	F*	G*
2,4-di-OH-benzoic acid[a]	6m82s	-	-	+	-	-		-
Caffeic acid	7m29s	-	-	-	+	-	-	-
Syringic acid	9m35s	-	-	-	-	-	-	+
Vanillic acid	9m75s	+	-	-	-	-	-	-
p-courmaric acid	11m25s	+	-	-	-	-	-	+
Ferulic acid	14m01s	+	-	-	+	-	-	+
Sinapic acid	15m10s	+	-	-	-	-	-	+
o-coumaric acid	19m42s	-	-	-	-	-	+	-
Coumarin	24m69s	-	-	-	+	-	-	-
Eriodictyol	25m59s	-	-	-	-	-	+	-
3,4,5-triMeOcinnamic acid	29m35s	+	-	-	-	-	-	-
Herniarin	30m17s	-	-	-	+	-	-	-
4-MeOcinnamic acid[a]	36m22s	-	+	-	-	-	-	-
Quercetin	37m04s	+	-	+	-	-	-	-
Hesperetin	37m14s	-	-	-	-	-	+	-
Luteolin	39m56s	-	+	+	+	+	+	+
Kamepferol	44m62s	+	-	-	-	-	-	-
Apigenin	47m11s	-	+	-	+	+	+	+
Disometin	50m85s	-	-	-	-	+	+	-

*A-*Centaurea erythraea*; B-*Cynara cardunculus;* C-*Hypericum androsaemum;* D-*Lavandula officinalis;* E-*Lippia citriodora;* F-*Mentha piperita*; *G-Salvia officinalis.*

[a] 2,4-diOHbenzoic acid, 3,4,5-triMeOcinnamic acid, 4-MeOcinnamic acid are respectively, 2,4-dihydroxybenzoic acid, 3,4,5-trimethoxycinnamic acid and 4-methoxycinnamic acid.

INTRODUCTION

The volume of sales of species used in phytotherapy tends to increase in Portugal in the same way as they are increasing in the western European countries.

Centaurea erythraea, Cynara cardunculus, Hypericum androsaemum, Lavandula officinalis, Lippia citriodora, Mentha piperita and *Salvia officinalis* are some of the medicinal species of great consumption in Portugal.

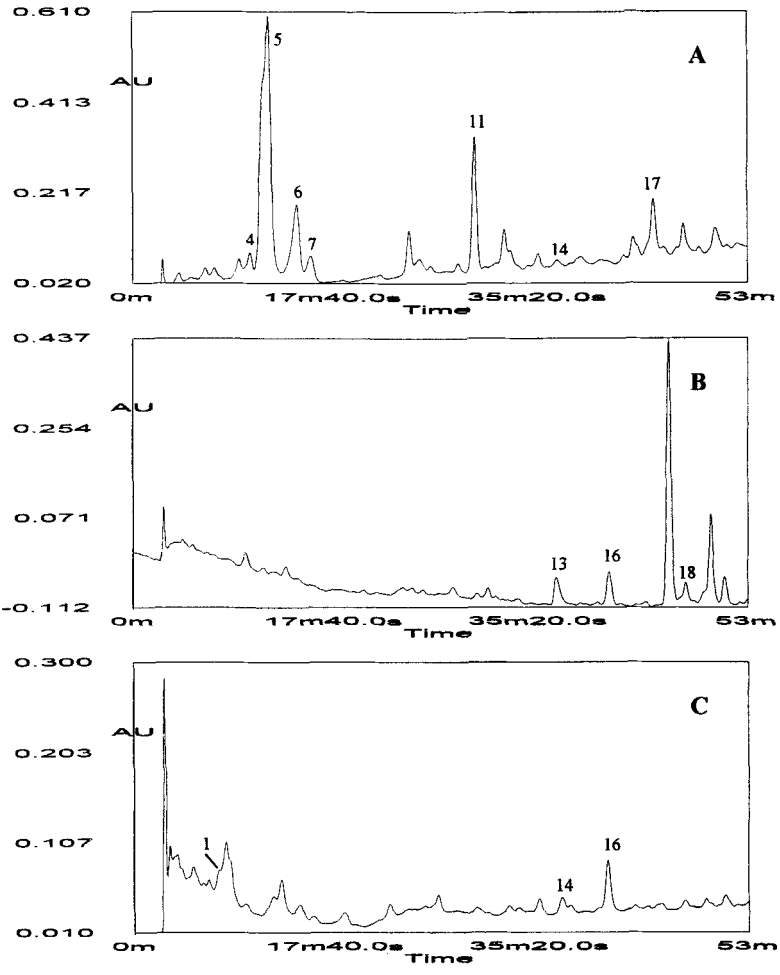
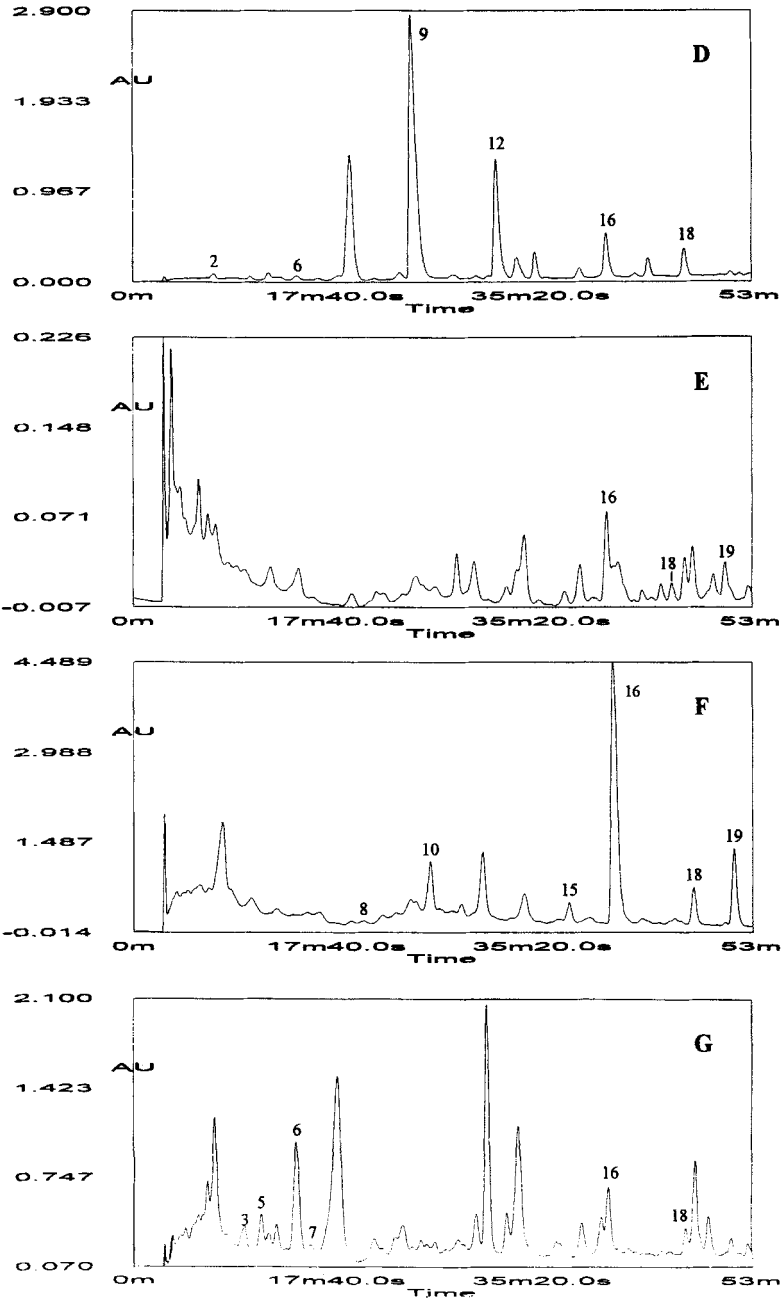


Figure 1. HPLC of phenolic compounds in medicinal plants. A-*Centaurea erythraea*; B-*Cynara cardunculus*; C-*Hypericum androsaemum*. (1) 2,4-dihydroxybenzoic acid; (4) vanillic acid; (5) p-coumaric acid; (6) ferulic acid; (7) sinapic acid; (11) 3,4,5-trimethoxycinnamic acid; (13) 4-methoxycinnamic acid; (14) quercetin; (16) luteolin; (17) kaempferol; (18) apigenin. Detection at 320 nm.

The quality control of medicinal species is established in the Pharmacopoeias and is based on several parameters including a chemical characterisation. This chemical characterisation is made by TLC of the constituents that ideally would be chemical markers of each species, although we are still far from that ideal.



HPLC is a more promising technique because it allows high resolution and a rapid and reproducible determination, even of trace amounts of compounds. That is why Pharmacopoeias are changing the chemical control of plants from TLC to HPLC as, for example, with garlic.[1]

On the other hand a simple, rapid and involving method is needed to be applied to routine analysis of plants in general.

Once phenolic compounds are widespread in nature and have been successfully applied to quality control of plant foodstuffs,[2-8] namely to fruit derivatives, we tried to put to work a methodology, based on HPLC of phenolic compounds.

However, phenolic compounds exist in plants in combinations, namely with sugars, and it is common to find, in a single plant, a great number of derivatives of the same phenolic. This renders the chromatograms very complex and implies the need for a set of conditions to resolve the complex profile of each plant.

In order to develop a methodology that can simplify the chromatograms and which can be applied to routine analysis, the methanolic extracts of the plants were previously subjected to hydrolysis. An alkaline hydrolysis was carried out to break ester complexes, the most common form of phenolic acids, followed by an acidic hydrolysis to break the glycoside linkages.

Besides the simplification of the chromatograms, this also allows the characterisation of the species to be made on the basis of compounds accessible on the market, which is a point to keep in mind when developing a methodology for a simple and cheap analysis.

Therefore, an HPLC methodology was developed to separate 19 phenolic compounds usually described in these species and involving two flavanones (eriodictyol and hesperetin), three flavones (luteolin, apigenin and diosmetin), two flavonols (quercetin and kaempferol), two coumarins (coumarin and herniarin), and 10 phenolic acids (caffeic, ferulic, syringic, sinapic, vanillic, 2,4-dihydroxybenzoic, 4-methoxycinnamic, 3,4,5-trimethoxycinnamic, *o*-coumaric and *p*-coumaric).

Figure 2 (left). HPLC of phenolic compounds in medicinal plants. D-*Lavandula officinalis*; E-*Lippia citriodora*; F-*Mentha piperita*; G-*Salvia officinalis*. (2) caffeic acid; (3) syringic acid; (5) p-coumaric acid; (6) ferulic acid; (7) sinapic acid; (8) o-coumaric acid; (9) coumarin; (10) eriodictyol; (12) herniarin; (15) hesperetin; (16) luteolin; (18) apigenin; (19) diosmetin. Detection at 320 nm.

EXPERIMENTAL

Plant Samples and Standards

Plant samples were commercially available and authentic standards were obtained from Sigma Chemical Co. or from Extrasynthése.

Extraction of Phenolic Compounds from Plants

In each case 1 g of part used was subjected to methanolic extraction until negative reaction with aqueous NaOH; the extract was filtered, taken to dryness, and 10 mL of 2N NaOH were added. The solution was kept in darkness for 4 h, acidified by HCl, passed through a C18 Bond Elut, and the phenolic compounds were solubilised by MeOH; this solution was taken to dryness and 10 mL of 2N HCl were added; this acidic solution was heated for 45 min, passed through a C18 Bond Elut, and the phenolic compounds were solubilised by MeOH. This solution was taken to dryness, dissolved in 1 mL of MeOH, and 20 μL were analysed 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 (5μm, particle size; 25.0 x 0.46 cm) column. The solvent system used 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.- 100% B, 53 min.- 100% B. Elution was performed at a solvent flow rate of 1 mL/min. Detection was accomplished with a diode-array detector, 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.

RESULTS AND DISCUSSION

To optimise the HPLC conditions for the analysis of phenolic compounds in plants, an artificial mixture was prepared containing 19 phenolic compounds, usually described in the species herein analysed. Under the conditions described in the Materials and Methods section, the retention times obtained were those indicated in Table 1. The repeatability of the method was high, with respect to retention times.

The optimised HPLC methodology was then applied to the analysis of phenolic compounds in *Centaurea erythraea, Cynara cardunculus, Hypericum androsaemum* (Fig.1, A-C and Table1), *Lavandula officinalis, Lippia citriodora, Mentha piperita* and *Salvia officinalis* (Fig. 2, D-G and Table 1).

With these experimental conditions it was possible to identify each specie by a set of phenolic compounds as can be seen in Figures 1 and 2.

Some compounds were detected in only one medicinal specie. Thus, caffeic acid, coumarin, and herniarin were only detected in *Lavandula officinalis;* 4-methoxycinnamic acid was only detected in *Cynara cardunculus;* 2,4-dihydroxybenzoic acid was only detected in *Hypericum androsaemum,* while 3,4,5-trimethoxycinnamic acid, kaempferol, and vanillic acid were only detected in *Centaurea erythraea.* This last specie was also characterised by several unidentified polymethoxylated xanthones. On the other hand luteolin and apigenin were detected in the majority of the analysed samples.

It is evident from Figures 1 and 2, that several phenolic compounds remained unidentified. Isolation and identification of these compounds are in progress, once they can be useful as chemical markers.

In conclusion, this study suggests that the technique herein is quite useful for the analysis of phenolic compounds in medicinal species, allowing their quality control.

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