Analysis of Vervain Flavonoids by HPLC/Diode Array Detector Method. Its Application to Quality Control

Patrícia Valentão,[†] Paula B. Andrade,[†] Filipe Areias,[†] Federico Ferreres,[‡] and Rosa M. Seabra*[,†]

CEQUP/Laboratorio de Farmacognosia, Faculdade de Farmácia, Universidade do Porto, R. Aníbal Cunha, 4050 Porto, Portugal, and Laboratorio de Fitoquímica, Departamento de Ciencia y Tecnologia de Alimentos, CEBAS, CSIC, Apartado de Correos 4195, E-30080 Murcia, Spain

A reversed-phase HPLC procedure is proposed for the determination of seven flavonoids (luteolin, nepetin, hispidulin, jaceosidin, cirsimaritin, cirsilineol, and eupatorin) in vervain samples. A simple extractive technique was developed, involving only extraction with ethyl ether, which allowed the elimination of phenolic acids, yielding a sufficiently purified flavonoid fraction. All of the analyzed vervain samples showed a common flavonoid pattern, in which hispidulin and jaceosidin were the major compounds and nepetin was the minor compound.

Keywords: *Vervain (Lippia citriodora); HPLC/DAD detector; flavonoids; chemical fingerprint; quality control*

INTRODUCTION

Vervain or lemon verbena [*Lippia citriodora* (Ort.) HBK, *L. triphylla* O. Kze., *Aloysia citriodora* (Cav.) Ort., *Verbena citriodora* Cav., *V. triphylla* L'Her.] (Verbenaceae) is a shrub; the leaves, due to their agreeable scent, are largely used as an aromatic. In the United States they are listed as Generally Regarded as Safe (GRAS) for human consumption in alcoholic beverages. Vervain is also used in herbal teas because it is reputed to possess antispasmodic, antipyretic, sedative, and stomachic properties (Van Hellemont, 1986; Newall et al., 1996).

The Good Manufacturing Practices on Medicinal Plants demands higher and higher quality control, both of the raw materials and of the finished products. This induces us to improve methodologies that can be easily applied to routine quality control of plant material.

The chemical characterization of vervain is made, in some Pharmacopoeias (*Pharmacopée Française*, 1989), by the TLC detection of citral. However, in many European countries, plants are sold as mixtures, and the search for citral, given its widespread distribution, hardly can be used to guarantee the presence of vervain.

The complexity of the analysis of such mixtures requires that each species must be characterized not by one chemical marker but by the highest possible number of chemical compounds.

The fact that several flavonoids, previously described, exist in vervain (Skaltsa and Shammas, 1988) prompted us to search for a protocol, based on HPLC of this class of compounds, that could be easily fitted to routine analysis. HPLC coupled to diode array detection (DAD) has often proved to be suitable to this aim. Besides, this technique allows an easy quantification, which is also an important tool in the definition of a "chemical fingerprint".

To analyze the phenolic composition of vervain, several extracts were prepared and analyzed by HPLC, and our best results were obtained with ethyl ether extraction.

Table 1. Vervain Samples, Geographical Origins, Dates of Collection, and Total Flavonoids

year	sample	geographical origin[a]	month of collection	total flavonoids (mg/kg, dry basis)
1996	1	Vila da Feira	November	396.1
	2	Braga	December	301.6
1997	3	Custóias	July	1361.6
	4	Custóias	September	1405.6
	5	Penacova A[b]	July	606.7
	6	Penacova A[b]	September	692.1
	7	Vila da Feira	July	696.4
	8	Vila da Feira	September	1194.2
	9	Viana do Castelo	September	734.8
	10	Braga	July	654.0
1998	11	Custóias	July	907.5
	12	Custóias	September	1180.2
	13	Penacova A[b]	July	669.0
	14	Penacova A[b]	September	715.9
	15	Penacova B[b]	July	1867.4
	16	Penacova B[1b]	September	1619.3
	17	Penacova B[2b]	September	1693.6
	18	Braga	July	1281.6
	19	Braga	October	975.0
	20	Vila Nova de Gaia	July	758.0
	21	Vila Nova de Gaia	September	757.6
	22	Arouca	July	1279.2
	23	Arouca	October	1116.4
	24	Vila da Feira	July	1137.9
	25	Vila da Feira	September	2151.2
	26	Vila da Feira	October	1541.8
	27	Vila da Feira	November	947.1
	28	Vila da Feira	December	974.1

[a] Each geographical origin represents a different shrub. [b] Penacova A and B represent two shrubs from the same geographical origin; superscript 1 and 2 represent big and small leaves of the same shrub, respectively.

The leaves of this species are usually collected twice a year, in July and September. So, according to this, 28 samples, mainly collected in these two seasons at different places in northern and central Portugal (Table 1), were analyzed and the main flavonoids quantified. We also evaluated the evolution of flavonoid amounts

[†] Universidade do Porto.
[‡] CSIC.

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by collecting samples from the same origin until the end of the vegetative period.

MATERIALS AND METHODS

Vervain Samples and Standards. Healthy vervain samples (branches) were collected in different places in central and northern Portugal. All leaves were detached, dried at 30 °C, and ground in an hammer mill to pass 910 μm.

Eupatorin was obtained from Extrasynthése (Genay, France), and luteolin was obtained from Sigma (St. Louis, MO). Nepetin, hispidulin, cirsimaritin, and cirsilineol were previously obtained from *Salvia lavandulaefolia* (Tomás-Lorente et al., 1988), and jaceosidin was from *Salvia tomentosa* (Tomás-Barbéran, 1986).

HPLC grade methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Extraction of Flavonoids from Vervain Samples. For analytical purposes, vervain leaves (~ 2 g) were extracted, twice, with 50 mL of EtOH 40%, at room temperature, with agitation. The extract obtained was concentrated until dryness, under reduced pressure (30 °C). The residue was dissolved in 1 mL of methanol, and 20 μL was analyzed by HPLC. The entire protocol was repeated using EtOH 60%, EtOH, and ethyl ether as extractive solvents.

For quantification purposes 1 g of each powdered sample was extracted with 100 mL of ethyl ether for 20 min with agitation, followed by extraction with 50 mL of ethyl ether (twice) for 10 min. The extracts were gathered and filtered, and the ethyl ether was removed under reduced pressure (30 °C) until dryness. The residue was dissolved in 1 mL of methanol, and 20 μL was analyzed by HPLC.

HPLC Analysis of Flavonoids. Separation of flavonoids was achieved with an analytical HPLC unit (Gilson), using a reversed-phase Spherisorb ODS2 (5 μm particle size; 25.0 × 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; 60 min, 80% B; 65 min, 80% B; elution was performed at a solvent flow rate of 1 mL/min. For quantification purposes a solvent flow rate of 1 mL/min was used, starting with 50% methanol and installing a gradient to obtain 60% B at 5 min and 80% B at 30 min. Detection was accomplished with a 160 DAD, and chromatograms were recorded at 350 nm.

The compounds in each sample were identified by comparing their retention times and UV−vis spectra in the 200−400 nm range with those of the standards previously referred to. Peak purity was checked by means of the Gilson 160 Spectra Viewer software contrast facilities.

Flavonoid quantification was achieved by the absorbance recorded in the chromatograms relative to external standards of flavonoids with detection at 350 nm. Luteolin and eupatorin were quantified as themselves, and the other compounds were quantified as eupatorin.

RESULTS AND DISCUSSION

At the process of optimization of the extraction conditions, several extracts were prepared (EtOH 40%, EtOH 60%, EtOH, ethyl ether) and analyzed. When long gradients (65 min) were used, good resolutions were obtained, and the more polar extracts (EtOH 40% and EtOH 60%) were mainly characterized by the presence of verbascoside, identified by its UV spectra and retention time (Lamaison et al., 1993). The ethanolic extract showed, already, noticeable amounts of flavonoids but was still marked by the presence of verbascoside.

With the extract obtained with ethyl ether, no cinnamic acids were observed and significant amounts of flavonoids were detected. A shorter gradient was set up that was able to separate a set of eight flavonoids (Figure 1), seven of which were identified by comparison

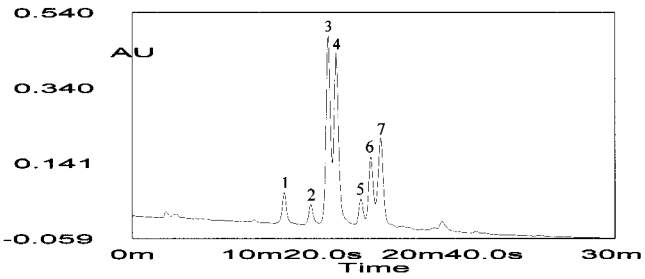

Figure 1. HPLC profile of a vervain sample, extracted with ethyl ether. Detection was at 350 nm. Peaks: (1) luteolin; (2) nepetin; (3) hispidulin; (4) jaceosidin; (5) cirsimaritin; (6) cirsilineol; (7) eupatorin. Gradient as follows: 0 min, 50% B; 5 min, 60% B; 30 min, 80% B.

Table 2. Vervain Flavonoids Separated by HPLC

no.	flavonoid	flavone structure	retention time
1	luteolin	5,7,3′,4′-tetrahydroxy-	9 min, 38 s
2	nepetin	5,7,3′,4′-tetrahydroxy-6-methoxy-	11 min, 25 s
3	hispidulin	5,7,4′-trihydroxy-6-methoxy-	12 min, 30 s
4	jaceosidin	5,7,4′-trihydroxy-6,3′-dimethoxy-	12 min, 58 s
5	cirsimaritin	5,4′-dihydroxy-6,7-dimethoxy-	14 min, 34 s
6	cirsilineol	5,4′- dihydroxy-6,7,3′-trimethoxy-	15 min, 14 s
7	eupatorin	5,3′- dihydroxy-6,7,4′-trimethoxy-	15 min, 48 s

with standards (Table 2). This gradient allowed the quantification of luteolin, nepetin, hispidulin, jaceosidin, cirsimaritin, cirsilineol, and eupatorin without problems of interference from other UV-absorbing substances.

These flavonoids were previously reported by other authors in vervain (Skaltsa and Shammas, 1988), with the exception of jaceosidin and cirsilineol, which, as far as we know, are now reported for the first time in this species.

The amounts of the different flavonoids present in the vervain samples analyzed are shown in Table 3. The most striking characteristic of the phenolic fingerprint of vervain is that all of the samples showed a common flavonoid pattern in which hispidulin and jaceosidin were the major compounds and nepetin was the minor one (Figures 2 and 3) with the exception of sample 27, which presented cirsilineol as the major compound, and samples 1, 8, and 21, in which cirsimaritin was present at the lowest amount.

A systematic study was initiated in 1997 with 8 samples collected in July and September and, in 1998, this study was extended to 15 more samples collected from seven different shrubs. Significant differences were observed in total flavonoids from both years. In 1998 leaves collected from Penacova B were separated into two samples of big leaves (sample 16) and small ones (sample 17) and analyzed separately; no noticeable differences were observed, which means that this factor was not responsible for the variation.

A geographical or climatic reason cannot be invoked as the main factor behind the variations observed, because, when samples 13 and 15, with the same origin and date of collection, are compared, a 3-fold increase is observed between the two samples. Samples from Penacova A present the lowest amounts of flavonoids in both years, and this may be attributed to the fact that this shrub is the only one that is not exposed to sunlight; besides, it is the shrub that showed less flavonoid variation between the two years and the two dates of collection. This is in accordance with the old hypothesis that flavonoids, acting as sun filters, have their contents related to sun exposure.

Table 3. Phenolic Contents of Vervain Samples

	phenolic compounds[a] (mg/kg, dry basis)						
sample	luteolin	nepetin	hispidulin	jaceosidin	cirsimaritin	cirsilineol	eupatorin
1	17.3 ± 0.38	26.5 ± 0.06	117.1 ± 3.09	125.7 ± 1.34	12.5 ± 0.07	56.1 ± 0.15	40.9 ± 0.02
2	6.5 ± 0.32	nq[b]	111.3 ± 6.45	98.3 ± 0.50	11.5 ± 0.07	39.0 ± 0.78	35.0 ± 0.52
3	39.6 ± 0.67	25.5 ± 0.24	343.5 ± 7.01	464.0 ± 17.62	90.4 ± 5.46	210.9 ± 12.54	187.7 ± 27.97
4	49.2 ± 1.23	31.2 ± 0.60	381.7 ± 4.91	437.8 ± 4.71	94.6 ± 2.90	219.2 ± 7.81	192.0 ± 13.78
5	12.7 ± 0.55	8.2 ± 0.19	179.1 ± 2.36	201.1 ± 0.86	23.4 ± 0.05	103.0 ± 0.19	79.3 ± 0.32
6	19.2 ± 0.70	10.2 ± 0.33	192.4 ± 2.38	246.4 ± 1.12	22.2 ± 0.06	113.4 ± 0.77	88.4 ± 0.50
7	32.0 ± 0.79	17.0 ± 0.08	210.0 ± 7.13	232.7 ± 0.98	23.4 ± 0.48	103.3 ± 1.55	78.0 ± 1.73
8	69.2 ± 1.10	41.1 ± 0.06	332.0 ± 2.13	392.1 ± 0.20	36.1 ± 0.86	197.8 ± 2.71	125.9 ± 5.72
9	30.5 ± 0.79	17.2 ± 0.08	217.2 ± 0.24	253.6 ± 2.00	22.5 ± 0.44	112.9 ± 1.19	80.8 ± 0.26
10	15.1 ± 0.38	7.5 ± 0.09	230.9 ± 2.19	220.6 ± 3.28	26.2 ± 0.60	82.9 ± 0.83	70.9 ± 0.90
11	91.6 ± 0.18	31.6 ± 0.19	305.8 ± 0.16	235.1 ± 0.35	42.4 ± 1.37	98.2 ± 2.58	102.8 ± 4.18
12	57.5 ± 0.61	33.4 ± 0.12	332.1 ± 3.17	415.5 ± 5.08	40.8 ± 1.26	179.1 ± 0.58	121.7 ± 0.44
13	13.8 ± 0.08	6.4 ± 0.58	216.4 ± 2.38	218.5 ± 0.91	19.1 ± 0.55	123.5 ± 0.51	71.3 ± 0.45
14	18.6 ± 0.21	11.2 ± 0.37	218.3 ± 5.17	241.0 ± 4.69	26.0 ± 4.35	121.1 ± 0.95	79.5 ± 0.19
15	52.1 ± 0.53	21.5 ± 0.17	493.7 ± 23.44	617.6 ± 9.55	97.5 ± 1.83	390.0 ± 11.56	195.0 ± 2.19
16	61.3 ± 0.76	34.9 ± 0.14	394.1 ± 3.97	562.0 ± 4.72	88.1 ± 1.87	305.7 ± 2.62	173.2 ± 1.50
17	76.3 ± 0.65	43.1 ± 1.77	402.8 ± 8.07	591.0 ± 4.21	90.2 ± 3.09	304.4 ± 1.77	185.8 ± 1.86
18	51.1 ± 0.75	24.9 ± 0.02	385.6 ± 3.07	404.4 ± 4.74	41.1 ± 0.93	246.2 ± 6.43	128.3 ± 11.69
19	43.6 ± 0.12	22.5 ± 0.17	262.4 ± 1.69	299.7 ± 0.31	30.6 ± 0.90	219.2 ± 1.12	97.0 ± 0.48
20	nq[b]	nq[b]	274.3 ± 2.21	276.5 ± 1.81	18.4 ± 0.12	112.4 ± 0.04	76.4 ± 0.40
21	50.4 ± 0.61	27.9 ± 0.31	209.1 ± 0.39	282.6 ± 2.72	15.9 ± 0.48	102.9 ± 0.40	68.9 ± 0.62
22	43.0 ± 0.12	23.5 ± 0.50	325.2 ± 2.87	419.8 ± 2.45	76.5 ± 0.75	221.2 ± 0.02	170.0 ± 0.04
23	43.3 ± 0.45	20.7 ± 0.22	288.4 ± 2.49	339.6 ± 2.85	34.8 ± 0.22	278.5 ± 0.52	111.0 ± 0.89
24	53.0 ± 1.61	31.1 ± 0.72	340.6 ± 18.24	352.5 ± 7.97	42.0 ± 1.68	214.3 ± 6.97	104.3 ± 2.92
25	124.1 ± 5.59	60.9 ± 2.46	523.2 ± 32.38	702.4 ± 29.62	107.4 ± 9.10	383.4 ± 24.84	249.9 ± 26.41
26	90.0 ± 2.23	45.6 ± 0.91	379.1 ± 7.76	499.2 ± 20.88	64.2 ± 21.57	297.6 ± 12.39	166.0 ± 15.21
27	49.4 ± 0.63	29.0 ± 0.31	228.8 ± 1.16	256.1 ± 1.28	28.3 ± 0.78	271.8 ± 0.92	83.7 ± 0.29
28	40.0 ± 0.63	23.4 ± 0.09	263.4 ± 2.48	269.0 ± 0.86	39.7 ± 0.08	240.2 ± 3.76	98.4 ± 0.26

[a] Values are expressed as mean ± standard deviation of two assays for each sample. [b] nq, not quantified.

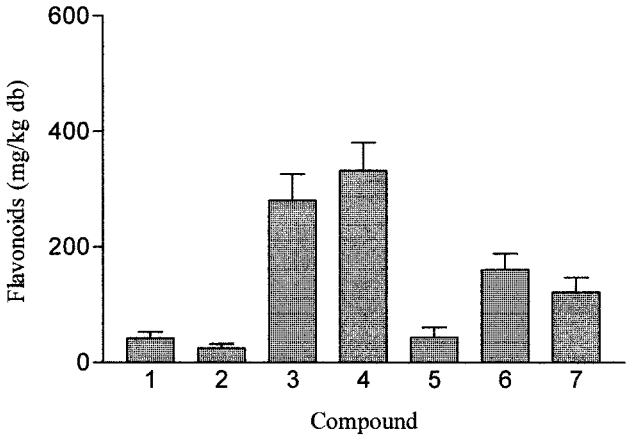

Figure 2. Flavonoid amounts from the samples collected in September 1997: (1) luteolin; (2) nepetin; (3) hispidulin; (4) jaceosidin; (5) cirsimaritin; (6) cirsilineol; (7) eupatorin. Results are mean of four replicates, and standard error bars are on the top of each column.

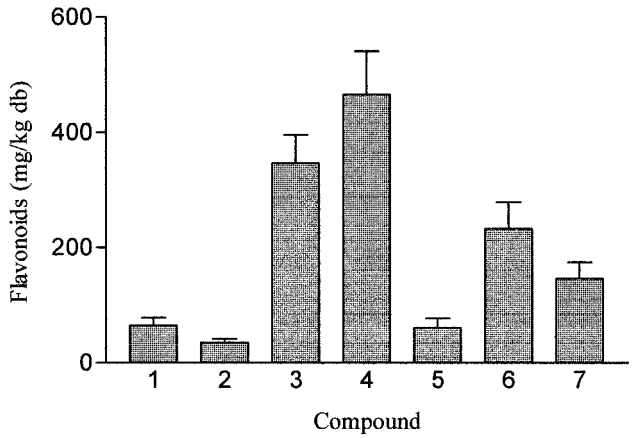

Figure 3. Flavonoid amounts from the samples collected in September 1998: (1) luteolin; (2) nepetin; (3) hispidulin; (4) jaceosidin; (5) cirsimaritin; (6) cirsilineol; (7) eupatorin. Results are mean of six replicates, and standard error bars are on the top of each column.

Samples from Vila Nova de Gaia (samples 20 and 21) also presented flavonoid levels <1000 mg/kg; although exposed to the sunlight, this shrub and those from Penacova A and Custóias are the only ones that are not subjected to pruning, and this can be a major factor responsible for the low levels observed. Other variations are probably due to climatic reasons, or example, watering and depth of pruning. For instance, we are aware that the shrub from Vila da Feira is subjected to watering and to a deep pruning every year. This may be the reason samples from this shrub had the biggest increase from 1997 to 1998 (~74%) and, within each year, from July to September (from 70 to 90%).

With the shrub from Vila da Feira we also carried on a study of the variation from July to December (samples 24−28). A noticeable decrease was observed near the end of the vegetative period (Figure 4), which is in

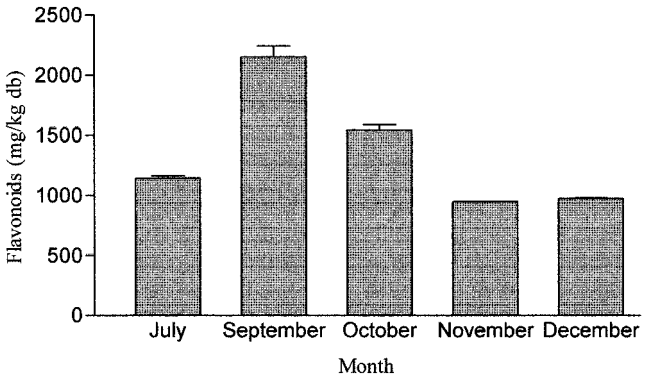

Figure 4. Evolution of the total flavonoid contents between July and December on the samples from Vila da Feira in 1998. Results are mean of two replicates, and standard error bars are on the top of each column.

accordance with the small amounts found in samples 1 and 2.

In conclusion, this study suggests that the technique herein described is quite useful for the analysis of the vervain flavonoids because it puts in evidence the striking characteristic pattern of its main seven flavonoids from the ethyl ether extract. Besides, the flavonoids are resolved enough to allow its quantification, which can be used for quality control.

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