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Phenolic fingerprint of peppermint leaves

F.M. Areias [a,*], P. Valentão [a], P.B. Andrade [a], F. Ferreres [b], R.M. Seabra [a]

[a] *CEQUP/ Lab. Farmacognosia, Fac. Farmácia do Porto, R. Aníbal Cunha 164, 4050-047 Porto, Portugal*

[b] *Lab. Fitoquímica, Departamento de Ciencia y Tecnologia de Alimentos, CEBAS, CSIC, Apartado 4195-30080 Murcia, Spain*

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Abstract

A reversed-phase high-performance liquid chromatography procedure is proposed for the determination of 10 phenolic compounds (eriodictyol 7-*O*-rutinoside, eriodictyol 7-*O*-glucoside, luteolin 7-*O*-rutinoside, luteolin 7-*O*-glucoside, hesperetin 7-*O*-rutinoside, apigenin 7-*O*-rutinoside, rosmarinic acid, 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone, pebrellin and gardenin B) in peppermint. The chromatographic separation was achieved using a reversed-phase Spherisorb ODS 2 (5 μm particle size; 25.0×0.46 cm) column. Of the several extractive solvents tried, ethanol was the best for qualitative and quantitative analysis. Best resolution was obtained using a gradient of water-phosphoric acid (999:1) and acetonitrile. Fourteen samples were subjected to quantification and showed a common composition pattern.

Keywords: Peppermint; Phenolic fingerprint; HPLC

1. Introduction

Peppermint, *Mentha×piperita* L. (Lamiaceae) is a species cultivated as a source of essential oil, largely used as a condiment in several foodstuffs (non-alcoholic beverages, ice-creams, candies, chewing-gums, cakes, meats; De Vincenzi, Maialetti, Di Pasquale & Dessi, 1991). The leaves (*Menthae piperita folium* — European Pharmacopoeia, 1997) are used in traditional medicine as spasmolytics, antibacterial agents and promoters of gastric secretion (Tyler, 1993), but they are also used in several herbal mixtures just for their aromatizing activity.

The characteristic compounds are the terpenoids present in the essential oil, which have been the subject of numerous studies, and the European Pharmacopoeia, undertakes the chemical characterization of peppermint leaves by thin layer chromatography (TLC) detection of these compounds. However, several flavonoids have also been described, mainly flavones and flavanones, either in the glycosidic form (Guédon & Pasquier, 1994) or as free aglycones (Voirin & Bayet, 1992; Voirin, Saunois & Bayet, 1994). When peppermint is used as an infusion, only 21% of the original essential oil is found in the tisane, while 75% of the polyphenolic compounds are extracted (Duband, Carnat, Carnat, Petitjean-Freytet, Clair & Lamaison, 1992). These polyphenolics may well be responsible for the claimed physiological activity of this herbal tea, since many biological properties are now attributed to the phenolics (mainly rosmarinic acid and flavonoids) (Halliwell, Aeschbach, Löliger & Aruoma, 1995).

Since high-performance liquid chromatography (HPLC) of flavonoids and other phenolics is a widely used methodology and easily adapted to the quantification of individual compounds, it has, in contrast to the methodology employed by the Pharmacopoeia, the advantage of generating a chemical fingerprint, which can be useful in defining the identity and the quality of a given species.

The objective of the work herein was to develop an HPLC methodology applicable to the analysis of peppermint phenolics — rosmarinic acid and flavonoids, both in the glycosidic and aglyconic form — and apply it to the analysis of several samples, to check if there is some common compositional pattern among the individual phenolics. Several solvents were used for extraction, and ethanol was the one that extracted the greatest number of compounds that could be identified. The developed HPLC methodology was applied to the analysis of four commercially available samples and to 10 samples cultivated in two experimental fields during

* *Corresponding author.

E-mail address: rseabra@ff.up.pt (F. M. Areias).

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1997, 1998 and 1999. In all samples, 10 compounds were identified and quantified.

2. Materials and methods

2.1. Plant samples and standards

M.×piperita (black mint) was obtained from an experimental field (Viseu, Center of Portugal) in 1991 and was propagated and acclimatized in Lamaçães (North littoral) until 1997, when it was transplanted to two other experimental fields (Arcos and Arouca, North littoral). Plant samples were collected from these two fields during 1997, 1998 and 1999 (Table 1). The cultivation of the species was the responsibility of Direcção Regional de Agricultura de Entre Douro e Minho. Four commercially available samples (1999) were also analyzed.

Eriodictyol 7-*O*-rutinoside, eriodictyol 7-*O*-glucoside, luteolin 7-*O*-glucoside, hesperetin 7-*O*-rutinoside, apigenin 7-*O*-rutinoside and rosmarinic acid were purchased from Extrasynthese.

2.2. Extraction of phenolic compounds from plants

For analytical purposes, 3 g of peppermint leaves were treated as before (Areias, Valentão, Andrade, Moreira, Amaral & Seabra, 2000). The entire extractive protocol was repeated with different batches of the same sample, using petroleum ether, chloroform, ethyl ether, ethyl acetate, acetone, methanol, ethanol, ethanol 80% and ethanol 30%. An extract with boiling water was also prepared according the protocol of the French Pharmacopoeia (5 g/l for 15 min). This infusion was not subjected to any concentration before analysis.

For quantification purposes, 3 g of each sample were ground to pass a 910 μm sieve and extracted, at room temperature, with agitation, using ethanol: 50 ml for 15 min, followed by 50 ml for 10 min and, finally, 25 ml for 5 min. The three extracts were combined, filtered and taken to dryness under reduced pressure at 30°C. The residue was dissolved in 5 ml of methanol and 20 μl were analyzed by HPLC.

2.3. 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 no. 1) was a mixture of water/formic acid (19:1; A) and methanol (B) with the following gradient: 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 (Andrade, Seabra, Valentão and Areias, 1998). Elution

Table 1
Phenolic contents of peppermint samples

Sample	Phenolic compounds[a] (mg/kg, dry basis)										
	Eriodictyol 7-rutinoside	Eriodictyol 7-glucoside	Luteolin 7-rutinoside	Luteolin 7-glucoside	Hesperetin 7-rutinoside	Apigenin 7-rutinoside	Rosmarinic acid	5,6-OH-7,8,3′,4′-OMe-flavone	Pebrellin	Gardenin B	Σ
18/07/97 Arcos	1085±35.26	112±10.00	365±9.63	51.8±8.10	790.1±63.47	84.9±12.11	2171±39.33	110±4.23	209.00±9.52	91.1±1.67	5071
24/07/97 Arouca	1810±17.79	169±13.04	526±13.16	82.3±17.42	1079±25.35	128±0.29	2817±173.90	152.40±1.22	248±1.42	109±7.71	7119
20/09/97 Arcos	2932±12.34	54.5±0.45	724±34.78	37.8±3.89	868±45.78	199±4.70	1623±97.65	345.51±23.50	599±50.23	259±0.89	7640
23/09/97 Arouca	3229±224.60	64.3±0.35	698±53.95	47.8±6.68	785±65.42	207.±5.53	1437±84.35	241.40±28.08	683±61.16	234±0.10	7627
10/07/98 Arcos	5153±82.11	248±17.05	1035±4.21	116±12.88	2153±30.39	269±11.61	4020±53.19	245.40±8.11	483±10.60	140±13.93	13863
07/07/98 Arouca	4844±386.40	311±26.03	1206±125.40	124±10.80	1976±170.60	220±19.57	3440±88.10	249.10±13.65	449±43.29	212±12.13	13032
18/09/98 Arcos	2706±7.96	197±14.79	747±14.94	153±13.40	1524±9.86	194.9±12.55	4429±17.69	464.70±12.75	482±11.06	156±16.68	11054
14/09/98 Arouca	4356±413.40	153±15.00	998±94.95	63±2.39	956±92.01	187±12.71	1559±129.80	279.80±21.79	723±59.70	336±42.27	9611
22/07/99 Arcos	2081±36.30	144±3.44	532±3.99	93.6±4.42	657±8.77	129±2.06	982±8.14	108.50±3.27	303±69.97	303±17.53	5332
13/07/99 Arouca	1843±13.16	114±10.54	636±6.37	119±3.07	615±33.82	120±2.30	1223±13.85	115.90±2.47	232±2.76	194±7.95	5211
Commercially available samples											
A	3001±155.80	149±2.47	804±53.04	138±10.27	897±25.50	211±8.66	1537±66.25	174.70±14.32	409±34.13	139±12.36	7459
B	3969±307.50	543±18.51	887±47.26	149±8.29	994±58.10	241±21.94	2045±172.30	163.50±13.01	457±41.96	144±11.60	9592
C	1695±53.43	174±7.52	1042±5.94	211±2.65	719±5.44	25±0.23	759±6.85	235.30±1.03	209±3.22	69.0±3.44	5138
D	1449±85.45	103±6.89	690±27.27	140±10.40	938±46.26	109±9.32	1368±10.93	296.80±12.90	538±23.92	235±22.92	5867

[a] Values are expressed as mean±standard deviation of two essays for each sample.

Table 2
Peppermint phenolic compounds separated by high performance liquid chromatography

No.	Compound	RT (min s) gradient No. 1	RT (min s) gradient No. 2
a	Eriodictyol 7-*O*-heteroside	09.22	–
1	Eriodictyol 7-*O*-rutinoside	–	12.05
2	Eriodictyol 7-*O*-glucoside	–	14.19
b	Luteolin 7-*O*-heteroside	22.43	–
3	Luteolin 7-*O*-rutinoside	–	18.02
4	Luteolin 7-*O*-glucoside	–	20.08
5	Hesperetin 7-*O*-rutinoside	–	23.46
c	Apigenin 7-*O*-heteroside	26.48	–
6	Apigenin 7-*O*-rutinoside	–	25.22
7	Rosmarinic acid	24.53	28.10
8	5,6-OH-7,8,3′,4′-OMe-flavone	54.38	52.29
9	Pebrellin (5,6-dihydroxy-7,8,4′trimethoxyflavone)	56.14	55.55
10	Gardenin B (5-hydroxy-6,7,8,4′-tetramethoxyflavone)	60.29	62.19

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 when available.

For quantitative purposes the solvent system (gradient No.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; 63 min, 100% B. Elution was performed at a solvent flow rate of 1 ml/min. Quantification of phenolics was achieved by the absorbance recorded (peak area) in the chromatograms relative to external standards at 320 nm. 5,6-Dihydroxy-7,8,3′,4′-tetramethoxyflavone, pebrellin and gardenin B were quantified as eupatorin and luteolin 7-*O*-rutinoside was quantified as luteolin 7-*O*-glucoside. RT values obtained with gradients no.1 and no.2 are listed in Table 2.

2.4. Statistics

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

3. Results and discussion

Polar extracts of peppermint are characterized by the presence of eriocitrin, luteolin 7-*O*-rutinoside, hesperidin and rosmarinic acid as the main constituents (Duband et al., 1992; Guédon & Pasquier, 1994) while, in non-polar extracts, 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone, pebrellin and gardenin B are the prevalent compounds confirming previously obtained results (Voirin & Bayet, 1992; Voirin, Saunois & Bayet, 1994) (Tables 1 and 2).

Bearing in mind that peppermint has no characteristic chemical marker, we must consider that it will be best characterized, by the highest number of compounds that can be identified in it. Furthermore, a quantitative profile can be useful as a chemical fingerprint as is revealed on analyzing vervain and lavender (Areias et al., 2000; Valentão, Andrade, Areias, Ferreres & Seabra, 1999). So, a systematic analysis of the available samples of peppermint leaves was carried out, subjecting samples to extraction by solvents of increasing polarity, in order to choose the one able to extract the highest number of identifiable and quantifiable compounds. All the extracts were analyzed using gradient No. 1. In extracts obtained with chloroform, ethyl ether (Fig. 1a) and ethyl acetate, compounds 8, 9 and 10 were predominant; according to the literature, the UV spectra (Fig. 2) and the chromatographic behaviour, they were identified as 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone, pebrellin and gardenin B, respectively. The identities of these compounds were also confirmed by comparison of their spectra with those obtained from the same compounds identified in *Thymus piperella* (Barberan, Hernández, Ferreres & Tomás, 1985) and *Siderites* species (Tomás-Lorente, Ferreres, Tomás-Barbéran, Rivera & Obón, 1988).

On using acetone or ethanol, extracts became richer in glycosides and rosmarinic acid (Fig. 1b and Fig. 3), but the main flavones are still detectable and quantifiable. The infusion showed the presence of only eriodictyol 7-*O*-rutinoside, luteolin 7-*O*-rutinoside and rosmarinic acid. Therefore, the ethanolic extract is a useful chemical fingerprint for peppermint.

On analyzing ethanolic extract with gradient No. 1 (Fig. 1b) it was possible to identify the following phenolic compounds: an eriodictyol 7-*O*-glycoside (a), a luteolin 7-*O*-glycoside (b), rosmarinic acid (7), an apigenin 7-*O*-glycoside (c), 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone (8), pebrellin (9) and gardenin B (10). The signals due to the eriodictyol, luteolin and apigenin

derivatives can be attributed either to 7-*O*-glucoside or to 7-*O*-rutinoside since both derivatives have the same UV spectra and the same RT when using gradient No. 1 (Table 2). The resolution obtained with this gradient is far from good and, therefore, not suited to qualitative or quantitative analysis. Besides, the signal of rosmarinic acid is split by the *cis*/*trans* isomerism, which adds an unnecessary complexity to the chromatogram. Several other eluent systems were tried and best results were obtained by employing water/phosphoric acid and acetonitrile with gradient No. 2. (Fig. 3).

On using these conditions, compounds 1 and 2 showed the same UV spectra and were identified, by comparison with standards commercially available, as eriodictyol 7-*O*-rutinoside (1), and eriodictyol 7-*O*-glucoside (2); the second set of compounds presented the UV spectra characteristic of luteolin 7-*O*-glycosides and they were identified as luteolin 7-*O*-rutinoside (3) and luteolin 7-*O*-glucoside (4), according to the literature (Duband et al., 1992; Guédon & Pasquier, 1994) and chromatographic behaviour, although no standard of luteolin 7-*O*-rutinoside is available on the market.

Considering these results, we consider that this last-mentioned method is quite useful for the definition of a chemical fingerprint for peppermint, since all the main constituents — polar and non polar — can be identified and quantified.

For quantification purposes, and in order to guarantee a full extraction of the phenolic compounds and the

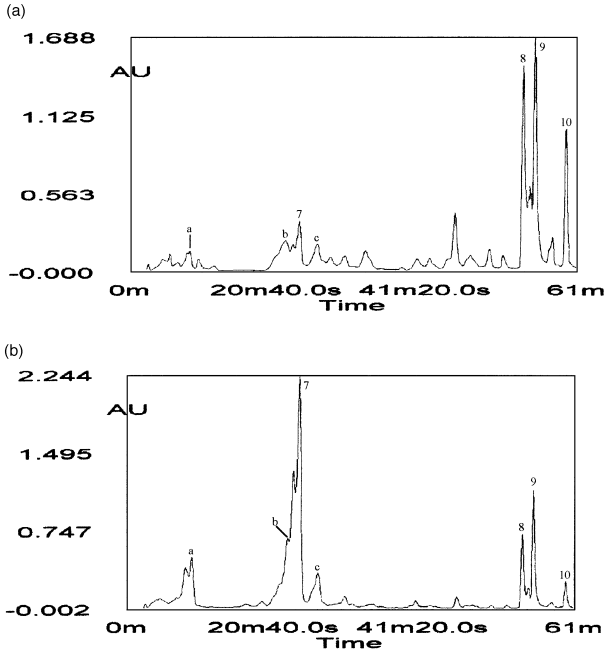

Fig. 1. High-performance liquid chromatography profile of a peppermint sample (gradient No. 1), extracted with ethyl ether (A) and ethanol (B). Detection at 320 nm. Peaks: (a) eriodictyol 7-*O*-glycoside, (b) luteolin 7-*O*-glycoside, (7) rosmarinic acid, (c) apigenin 7-*O*-glycoside, (8) 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone, (9) pebrellin and (10) gardenin B.

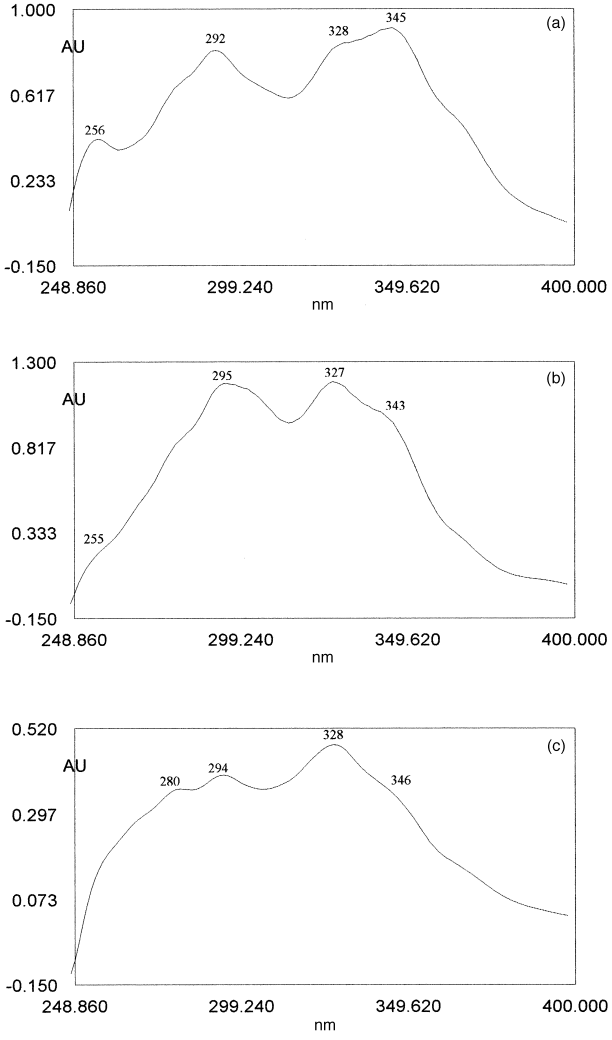

Fig. 2. UV spectra of 5,6-OH-7,8,3′,4′-OMe-flavone (A), pebrellin (B) and gardenin B (C) of *Mentha×piperita* recorded on-line by the diode-array detector and using eluent No. 2.

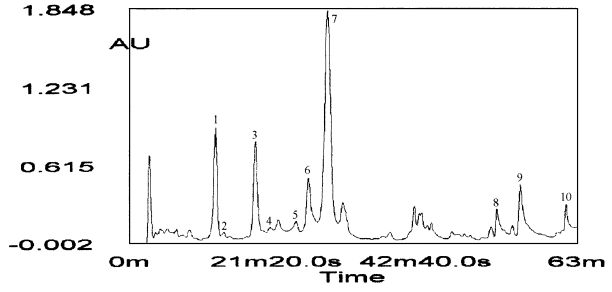

Fig. 3. High-performance liquid chromatography profile of a peppermint sample extracted with ethanol with gradient No. 2. Detection at 320 nm. Peaks: (1) eriodictyol 7-*O*-rutinoside, (2) eriodictyol-7-O-glucoside, (3) luteolin 7-*O*-rutinoside, (4) luteolin 7-*O*-glucoside, (5) hesperetin 7-*O*-rutinoside, (6) apigenin 7-*O*-rutinoside, (7) rosmarinic acid, (8) 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone, (9) pebrellin and (10) gardenin B.

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 three-fold extraction (Section 2). Quantification was performed at 320 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 diode-array is not available.

The tested analytical conditions were applied to four commercially available samples and to samples collected during 1997, 1998 and 1999 in the two mentioned experimental fields (Table 1). Since peppermint is traditionally collected from July to October, samples were collected in July and September.

There are no significant differences in total phenolics between the samples from the two experimental fields ($P > 0.05$). Also, between July and September, there were no big differences ($P > 0.05$). An increase in total phenolics was observed from 1997 to 1998 ($P < 0.01$); this may be explained by a better adaptation in 1998, since peppermint was transferred to the fields only in February 1997.

The main individual compounds are eriocitrin and rosmarinic acid: together, they account for 59 to 67% of total phenolics. Luteolin 7-*O*-rutinoside and hesperidin also play an important role and they account for 7–12% and 10–16% of total phenolics, respectively. On quantifying the individual compounds in all cultivated samples and analyzing the results in terms of percentage in each sample, we can obtain a chemical fingerprint for the 10 samples cultivated in the North of Portugal (Fig. 4).

Treating the results obtained with the commercially available samples (unknown origin and date of collection) in the same way, an identical graph is obtained (data not shown).

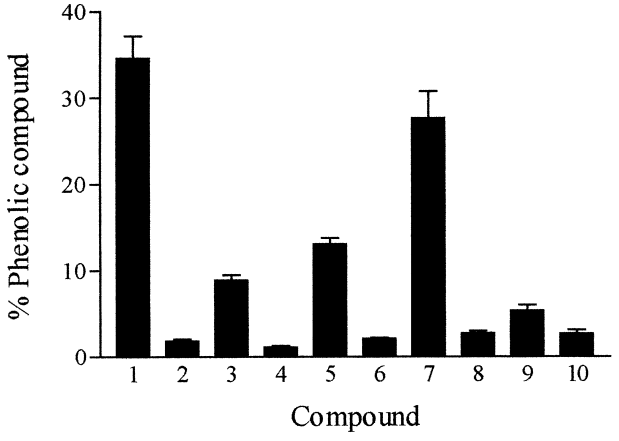

Fig. 4. Phenolic fingerprint of the samples cultivated in the North of Portugal. Results are mean of all the analyzed samples and standard error bars are on the top of each column. (1) eriodictyol 7-*O*-rutinoside, (2) eriodictyol-7-O-glucoside, (3) luteolin 7-*O*-rutinoside, (4) luteolin 7-*O*-glucoside, (5) hesperetin 7-*O*-rutinoside, (6) apigenin 7-*O*-rutinoside, (7) rosmarinic acid, (8) 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone, (9) pebrellin and (10) gardenin B.

Although more samples should be analyzed (other geographic origins and years of collection), the work herein seems to indicate that the analysis of the phenolic compounds allows a phenolic fingerprint of peppermint leaves that can be used for checking their authenticity and quality.

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