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Free flavonoid aglycones as markers of parentage in *Mentha aquatica*, *M. citrata*, *M. spicata* and *M.* x *piperita*

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Abstract

External lipophilic methylated flavonoids have been extracted from dried leaves of *Mentha aquatica*, *M. spicata*, *M.* x *piperita* and *M. citrata*. After separation and purification, twenty flavonoids have been identified by means of spectrometric methods (UV, EIMS, ^{1}H NMR). The flavonoid patterns of these species and hybrid support the view that *M.* x *piperita* may be a hybrid of *M. aquatica* and *M. spicata* while a linalool-producing sample of *M. citrata* may be considered a variety of *M. aquatica*. Cytological data agree with the observed biochemical results. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Internal (vacuolar glycosides) and/or external (lipophilic methylated aglycones) flavonoids have successfully been used in several plant groups for the purpose of identifying the parental origin of hybrids of natural origin (Alston & Turner, 1963; Ferreres, Tomás-Lorente, Tomás-Barberán, Rivera, & Obon, 1989; Tomás-Lorente, Ferreres, Tomás-Barberán, Rivera, & Obon, 1989) or of cultivated origin (Crowden, Wright, & Harborne, 1977; Williams, Fronczyk, & Harborne, 1983). The present study of external leaf flavonoid aglycones was aimed at seeking objective markers of parentage among different species and a hybrid of the genus *Mentha*. This taxonomically complex genus, "has been subdivided into a number sections of which by far the largest and taxonomically most complex is section *Mentha*" (Harley & Brighton, 1977). Hybridization is a frequence occurrence in this section both in wild populations and in cultivation. Thus, in order to see if hybrids could be positively identified, a detailed investigation of external leaf flavonoids, identical to that previously realized on *M.* x *piperita* (Voirin & Bayet, 1992), was realized on its two parental species, *M. aquatica* L. and *M. spicata* L. and on one linalool–linalyl acetate producing sample, which, according to its terpene oil composition, could be considered either a notomorph of *M.* x *piperita*, *M.* x *piperita* nm *citrata* (Ehrh.) B. Boivin or a linalool-producing cultivar of *M. aquatica* known as 'erba-mentha' Harley & Brighton, 1977. This chemical investigation was carried out in conjunction with mostly classical taxonomic studies, namely chromosome number counts and determination of nuclear DNA content.

2. Results and discussion

2.1. Structural analysis of flavonoid aglycones

External lipophilic methylated flavonoids were extracted with diethyl ether from dried leaves according to a procedure previously described (Voirin & Bayet, 1992). Each crude total leaf extract was subjected to chromatographic procedures (TLC, HPLC). The aglycones, after isolation and purification, were identified using spectrophotometric techniques (Mabry, Markham, & Thomas, 1970; Voirin, 1983) and comparative TLC and HPLC with authentic markers and in some cases by EIMS and ^{1}H NMR data (see Table 1). Amongst the twenty identified compounds listed in Table 1 together

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Table 1
Distribution and HPLC analyses of free flavone aglycones from *Mentha* taxa studied

	Compound	Trivial name	*M. citrata*	*M. aquatica*	*M.* x *piperita* (Jullien, Voirin, Bernillon, & Favre-Bonvin, 1984; Voirin & Bayet, 1992)	*M. spicata*	HPLC	
							peak no.	Rt[a]
1	5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone		+	++	+++	+++ [b c]	9	38.78
2	5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone	5-*O*-desmethylnobiletin	tr	tr	+	+ [b]	15	50.17
3	5,7,4′-trihydroxyflavone	apigenin	tr	tr (Burzanska-Hermann et al., 1977)	tr	tr (Sankara-Subramanian & Nair, 1972)	5	31.84
4	5,7-dihydroxy-4′-methoxyflavone	acacetin	tr	tr (Burzanska-Hermann et al., 1977)	tr	tr	14	51.81
5	5,4′-dihydroxy-6,7,8-trimethoxyflavone	xanthomicrol	tr	tr	tr	−	11	43.91
6	5,6-dihydroxy-7,8,4′-trimethoxyflavone	pebrellin	++	+	+++	−	13	47.89
7	5-hydroxy-6,7,4′-trimethoxyflavone	salvigenin	+ (Passannanti et al., 1990)	+	+	−	16	52.81
8	5-hydroxy-6,7,8,4′-tetramethoxyflavone	gardenin B	+++ (Passannanti et al., 1990)	+++	++	−	17	55.97
9	5,6,4′-trihydroxy-7-methoxyflavone	sorbifolin	−	−	tr	tr [b]	1	25.56
10	5,7,3′,4′-tetrahydroxyflavone	luteolin	−	− (Burzanska-Hermann et al., 1977)	tr	tr (Sankara-Subramanian & Nair, 1972)	1	25.56
11	5,6,4′-trihydroxy-7,8-dimethoxyflavone	thymusin	−	−	+	+ [b]	3	29.49
12	5,6,4′-trihydroxy-7,8,3′-trimethoxyflavone	thymonin	−	−	+	+++ [b] (Tomás-Barberán et al., 1983)	4	30.78
13	5,6-dihydroxy-7,3′,4′-trimethoxyflavone	−	−	+	++ [b]		6	33.76
14	5,3′,4′-trihydroxy-6,7,8-trimethoxyflavone	sideritoflavone	−	−	tr	+++ [b]	7	34.52
15	5,6,4′-trihydroxy-7,3′-dimethoxyflavone	−	−	−	++ [b] (Tomás-Barberán et al., 1983)	2	26.35	
16	5,4′-dihydroxy-6,7,3′-trimethoxyflavone	cirsilineol	−		−	+ [b]	8	37.73
17	5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone	−	−	+	[b]	12	45.46	
18	5-hydroxy-6,7,3′,4′-tetramethoxyflavone	−	−		+	[b]	13	47.89
19	5,6-dihydroxy-7,4′-dimethoxyflavone	ladanein	−	−	+	−	10	42.49
20	5,3′-dihydroxy-6,7,8,4′-tetramethoxyflavone	gardenin D	−	−	+	−	12	45.46

[a] Rt: retention time (min).
[b] EIMS recorded.
[c] ^{1}H NMR recorded (see references).Excretion levels: −: flavonoid not detected; tr: 0–1%; +: 1–5%; ++: 5–20%; +++: >20%.

with the restricted bibliographic data (Sankara-Subramanian & Nair, 1972; Burzanska-Hermann, Rzadkowska-Bodalska, & Olechnowiez-Stepien, 1977; Tomás-Barberán, Husain, Gil, 1983; Passannanti, Paternostro, Piozzi, 1990), sixteen have been described in our previous work (Jullien, Voirin, Bernillon, & Favre-Bonvin, 1984; Voirin & Bayet, 1992); the structures of the four remaining aglycones, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone (**15**), 5,4′-dihydroxy-6,7,3′-trimethoxyflavone (cirsilineol, **16**), 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone (**17**) and 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**18**), isolated from *M. spicata*, have been established by UV and MS data. It is interesting to note that this latter compound presents in HPLC the same retention time (peak 13, Table 1) as 5,6-dihydroxy-7,8,4′-trimethoxyflavone (pebrellin, **6**) isolated from *M.* x *piperita* leaves. However, the UV spectrum recorded on this peak by the array detector distinguishes easily each compound since the first presents two main UV maxima at 276 (band II) and 339 nm (band I) while, for the second, band II is located at 292 nm and band I at 331 nm. Moreover the R_f values in TLC are different for these two compounds (see Section 3). Finally, the UV spectra in MeOH + NaOMe distinguishes between 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone (**17**) and 5,3′-dihydroxy-6,7,8,4′-tetramethoxyflavone (**20**, gardenin D).

2.2. *Flavonoid patterns of* M. aquatica, M. spicata *and* M. *x* piperita

The four aglycones 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone (**1**), 5-*O*-desmethylnobiletin (**2**), apigenin (**3**) and acacetin (**4**), shared by the two clones investigated for *M. aquatica* and *M. spicata*, were also identified in the putative hybrid. In addition, each of the presumed parents produced specific compounds. So, the *M. aquatica* clone investigated is characterised by four *O*-methylated apigenin derivatives namely xanthomicrol (**5**), pebrellin (**6**), salvigenin (**7**) and gardenin B (**8**) while the *M. spicata* clone, which presents a more diversified pattern, contained mainly *O*-methylated luteolin derivatives, thymonin (**12**), 5,6-dihydroxy-7,3′,4′-trimethoxyflavone (**13**), sideritoflavone (**14**), 5,6,4′-trihydroxy-7,3′-dimethoxyflavone (**15**), cirsinileol (**16**), 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone (**17**) and 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**18**). From a biochemical viewpoint, these results show that each parent contains specific biosynthetic pathways. The *M. aquatica* clone produces specifically salvigenin (**7**) and gardenin B (**8**), i.e. 6- and 4′-*O*-methylated derivatives of sorbifolin (**9**) and thymusine (**11**), while the *M. spicata* clone, although producing these latter two compounds, is unable to carry out their methylation either at C-4′ or at C-6′. On the other hand, only the *M. spicata* clone which produces 5,6,4′-trihydroxy-7,3′-dimethoxyflavone (**15**), 5,6-dihydroxy-7,3′,4′-trimethoxyflavone (**13**), 5,4′-dihydroxy-6,7,3′-trimethoxyflavone (**16**) and 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**18**), is able to produce the sequential *O*-methylation either at C-4′ or at C-6 of 5,6,7-trisubstituted A-ring chrysoeriol derivatives.

The flavonoid pattern of the *M.* x *piperita* clone seems to reflect the profile of each putative parent since both *O*-methylated apigenin (**6**–**8**) and *O*-methylated luteolin derivatives (**12**–**14**), present at a high percentage in both parents, were inherited by this hybrid. However, the four *O*-methylated chrysoeriol derivatives (**15**–**18**) present in *M. spicata* could not be detected in the hybrid. "The fact that occasionally parental compounds do not appear in the hybrid examined is not really unexpected" according to Williams et al. (1983), "undoubtedly, the quantitative balance of flavonoid synthesis could well be upset by hybridization so that the production of a particular component in one or other species is lowered to an amount below the level of ready detection". The comparisons of percentage for pebrellin (**6**) and gardenin B (**8**) observed in *M. aquatica* and *M.* x *piperita* on the one hand and for thymonin (**12**) and sideritoflavone (**14**) in *M. spicata* and *M.* x *piperita* on the other hand shows that the hybrid may possess its own balance of flavonoid biosynthesis. In addition, *M.* x *piperita* produced extra *O*-methylated flavones, ladanein (**19**) and gardenin D (**20**). The production of new compounds in the hybrid "could be due to gene interactions changing the specificities in flavonoid synthesis" (Williams et al., 1983).

Thus, with the above exceptions, the hybrid *M.* x *piperita* showed a flavonoid pattern which contains the compounds which are typical of both parents. However, all these conclusions have to be drawn with some caution, because only one clone was investigated for each species or hybrids except for *M.* x *piperita* (Voirin, Saunois, & Bayet, 1994) and no information was obtained about possible infraspecific variation of the flavonoid profiles within these taxa.

2.3. *Flavonoid patterns of linalool-producing sample of* M. citrata

The results reported in Table 1 show that the linalool-producing sample was unable to synthesize the *O*-methylated luteolin derivatives characteristic of *M. spicata* clone investigated, even those present at a high percentage (**12**, **14**). In contrast, all the flavonoids identified in *M. aquatica* were also identified in this sample. Thus according to its flavonoid pattern, this latter may be a *M. aquatica* form and not a hybrid *M.* x *piperita* variety *citrata*.

The cytological results (chromosome numbers (Maia, Gilly, Poupet, Chambon, & Ammeux, 1984) and determination of nuclear DNA content) reported in Table 2 entirely confirm the above biochemical results. Thus *M. citrata* ($2n = 96$) has to be an *M. aquatica* in agreement with the data of Harley and Brighton, 1977. Unfortunately it has not been possible to obtain and analyse

Table 2
Nuclear DNA content and chromosome number count of *Mentha* taxa studied

Mint genotype	2C DNA value (pg)	95% confidence interval	Chromosome number (2*n*) (Maia et al., 1984)
M. citrata	3.19	3.04–3.34	96
M. aquatica	3.13	3.02–3.24	96
M. x *piperita*	2.28	2.23–2.33	72
M. spicata	1.74	1.72–1.76	48

See reference.

the notomorph *M.* x *piperita citrata*, described by the two latter authors, for which the chromosome number is 108 (2*n*) in order to demonstrate its potential hybrid origin.

Once more, the present results show that the *O*-methylated flavonoid aglycone analyses are potentially useful tools in the identification of hybrids. Moreover our results are in agreement with the limited previous results focused on the distribution of specific flavonoids in *Mentha spicata* (Sankara-Subramanian & Nair, 1972; Tomás-Barberán et al., 1983; Passannanti et al., 1990), *M. citrata* (Passannanti et al., 1990) and *M. aquatica* (Burzanska-Hermann et al., 1977) although rigorous comparisons between the results of these authors and ours are difficult owing to the different extraction procedures.

3. Experimental

Peppermint plants (clone 19) were the Black mint variety of *M.* x *piperita*; *M. aquatica* (clone 77), *M. spicata* (clone 94) and *M. citrata* (clone 86) were obtained from I.N.R.A. Antibes (06-France). Structural studies have been carried out from dried and powered leaves of *M.* x *piperita* (750 g), *M. spicata* (200 g), *M. aquatica* (200 g) and *M. citrata* (200 g). The analyses, performed on field grown plants, were repeated twice from each clone.

The extraction and separation (TLC) of free flavonoid aglycones have been previously described (Voirin & Bayet, 1992). Flavones (**15**), (**16**), (**17**) and (**18**) isolated from *M. spicata* leaves and pebrellin (**6**) isolated from *M.* x *piperita*, studied by TLC on Polyamide DC6 (Merck, solvent: toluene–petrol (b.p. 100–140°C)–MeCOEt–MeOH, 5:5:2:1) and cellulose (Merck, solvent AcOH–H_2O, 4:6), present the following R_f values: 5,6,4′-trihydroxy-7,3′-dimethoxyflavone (**15**), 0.03 and 0.2; pebrellin (**6**), 0.32 and 0.5; cirsinileol (**16**), 0.33 and 0.5; 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone (**17**), 0.5 and 0.52 and 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**18**), 0.69 and 0.6, respectively. The identification of flavonoids was based on chromatographic comparisons (TLC and HPLC) with authentic samples and confirmed by comparisons with published spectral data for all compounds (Voirin, 1983) and in some cases by EIMS and ^{1}H NMR (see Table 1). The HPLC analysis conditions were also previously described (Voirin & Bayet, 1992).

The chromosome number counts were established by Maia et al. (1984).

3.1. Determination of nuclear DNA content

This content was assessed by flow cytometry on nuclei prepared from newly expanded leaves. For calculation of the absolute DNA value (expressed as pg per 2C nucleus) propidium iodide was used as DNA specific fluorochrome and leaf nuclei of *Petunia hybrida* 'PxPc6' (2C = 2.85 pg) and/or *Medicago sativa* 'du Puits' (2C = 3.44 pg) were included as internal standards (Brown, Bergougnioux, Tallet, & Marie, 1991). To release nuclei, leaf patches (about 25 mm^2 for mint samples and half size for the standard) were chopped (Galbraith et al., 1983) with a razor blade in Petri dishes containing 400 µl of the following chopping buffer: 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate pH 7, 10 mM sodium bisulfite, 1% (W/V) Triton X-100 (Marie & Brown, 1993). The suspension of nuclei were then passed through a 30 µm nylon mesh and stained for 30 min with 50 µg ml^{-1} propidium iodide + 50 µg ml^{-1} RNase. Analyses were conducted with an Epics V flow cytometer (Coulter, Hialeah, FL) using a 100 µm nozzle and an argon laser (Spectra-Physic 2025-05) set at 400 mW for an excitation wavelength of 514 nm. The red fluorescence emission of propidium iodide was measured with 540 LP (absorptive) and 610 LP (interferential) filters. Each fluorescence histogram was obtained from 5000 nuclei. The DNA contents were defined by the ratio of modal positions of the 2C peaks of the standard and of mint nuclei. Each sample was analysed in 5 replicates and each measure was repeated twice.

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