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Chemical profiling of *Ocimum americanum* using external flavonoids

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Abstract

A HPLC survey was undertaken of the external flavonoids in 111 herbarium specimens of *Ocimum americanum* L. (*O. canum* Sims), which were largely collected from their natural habitats throughout Africa and Asia. The purpose of this study was to establish the flavonoid profiles of this species over the full range of its geographic distribution in order to use these for authentication purposes. Six different external flavonoid chemotypes were found. The major chemotype, present in circa 80% of the specimens of both var. *americanum* and var. *pilosum* collected throughout the distribution area of the species, was characterised by very high levels of nevadensin, slightly lower levels of salvigenin and much lower levels of up to 15 other external flavones. Of the remaining five chemotypes, two were found in var. *americanum* and three in var. *pilosum*. All specimens belonging to these chemotypes were collected in South or East Africa and represented by only a few specimens. These samples contained much smaller levels of flavones than present in the major chemotype of *O. americanum* and all lacked nevadensin. Xanthomicrol, a compound absent from the main chemotype, was the dominant flavone in two of the minor chemotypes. The external flavonoid profiles found in the six chemotypes of *O. americanum* were compared with those of *O.×citriodorum* (11 herbarium specimens studied) and seven other closely related species of *Ocimum*. The main nevadensin/salvigenin pattern present in *O. americanum* was also found in *O.×citriodorum*, *O. basilicum* and some specimens of *O. minimum*, but there were strong quantitative differences in external flavonoids among these taxa. The other chemotypes of *O. americanum* showed some similarities in their external flavone profiles to those found in the closely related East African species *O. fischeri*, *O. forskolei*, *O. kenyense* and *O. kilimandscharicum*, which occur in the same geographic areas. This suggests that the uncommon chemotypes of *O. americanum* may have originated by an exchange of genes with other *Ocimum* species, e.g. by introgressive hybridisation. Despite some similarities in profiles, chemical differences were also found among the species, so that it should be possible to authenticate a large proportion of leaf samples of *O. americanum* on the basis of external flavonoid profiles.

Keywords: *Ocimum americanum* var. *americanum*; *Ocimum americanum* var. *pilosum*; *O.×citriodorum*; Lamiaceae; Basil; External flavones; Chemotaxonomy; Infraspecific variability; Authentication

1. Introduction

For the commercial use of culinary and medicinal herbs, quality control is becoming an increasingly important issue. For example, medicinal species may wittingly or unwittingly be substituted by other species. These substitutes may be toxic (Kite et al., 2002), so that it is important for the users to be able to test the identity of the herb. Plant species can usually been identified by taxonomic experts when flowers and fruits are available, but the identification of leaf or root material is much more difficult, especially when it is ground. Plant anatomy and phytochemistry have been used for many years by pharmacognosists as tools to authenticate material of medicinal plants, and modern techniques such as HPLC coupled to photodiode array detection and mass spectrometry, which provide accurate compound profiles, have increased the reliability of these authentications. However, in order to use chemical profiles for the identification of herb species, their chemical infraspecific variability should be known.

Species belonging to the genus *Ocimum* L., basil (Lamiaceae), are examples of plants that are difficult to distinguish on the basis of just their leaf morphology, because of the wide range of leaf shapes within most species. Causes of this variation include hybridization among species and selection by humans for many centuries for various different uses often associated with the

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essential oils, e.g. as culinary herbs and as medicinal or insecticidal plants (Simon et al., 1999). Examples of *Ocimum* species used in this respect are *O. basilicum* L., *O. minimum* L., *O.×citriodorum* Vis., *O. americanum* L., *O. kilimandscharicum* Baker ex Gürke, *O. gratissimum* L. and *O. tenuiflorum* L. (=*O. sanctum* L.). However, profiles of essential oil cannot easily be used to distinguish these species, since they have been employed as a major character for selection and domestication of aromatic plants. For instance, volatile oil profiles rich in methyl (E) cinnamate are found in cultivars of several different *Ocimum* species (Vieira and Simon, 2000). Furthermore, at least six essential oil chemotypes are known to be present in *O. basilicum* (Grayer et al., 1996a) and three in *O. gratissimum* (Vieira et al., 2001). Flavonoid profiles have more potential as chemical fingerprints, as they are often characteristic for a certain species. Most species of *Ocimum* studied so far showed only little infraspecific variation of flavonoids. For example, there were only quantitative differences of surface flavonoids among many different accessions of *O. basilicum* studied (Grayer et al., 1996b). The same applied to internal flavonoids in accessions of *O. basilicum*, *O. minimum*, *O. gratissimum* and *O. tenuiflorum* (Grayer et al., 2002). However, in *O. gratissimum* three different external flavonoid chemotypes were found among eight accessions studied (Vieira et al., 2001), and the three different accessions investigated for *O. americanum* each showed totally different external flavonoid profiles (Grayer et al., 2001). For this reason it was decided to investigate the infraspecific variation of external flavonoids of *O. americanum* in more detail.

O. americanum L. (syn. *O. canum* Sims) is a widely distributed species in the tropics and subtropics of the Old and New World, but contrary to the name it is not native to America, but only to Africa and Asia (Paton et al., 1999). It is not often used as a culinary herb, unlike the related basil species *O. basilicum*, but more often as a medicinal plant. The essential oils found in this species have strong fungicidal activity against certain plant pathogens (Singh and Dwivedi, 1987; Shukla et al., 1990; Dubey, 1991). In Africa, leaves of *O. americanum* have been used as an insecticide for the protection against postharvest insect damage, especially that by bruchid beetles (Weaver et al., 1991). The active ingredients are also likely to be associated with the essential oils. However, some medicinal properties may be associated with the external flavonoids, as some specimens produce very high levels of these compounds, especially nevadensin (Grayer et al., 2001), which has antioxidant activity (Grayer et al., unpublished results). On the basis of morphological features two varieties of *O. americanum* are recognised, var. *americanum* and var. *pilosum* (Willd.) A.J. Paton. The former can generally be distinguished from the latter by having adpressed hairs on stems, smaller calyces, less densely pubescent calyx indumentum and more slender, shorter stems. However, there are a number of morphological intermediate specimens that are difficult to assign with certainty to either variety. Another complication is that *O.×citriodorum* Vis., putatively of hybrid origin from *O. americanum* var. *pilosum* in Africa and *O. basilicum* (Pushpangadan and Sobti, 1982, Paton et al., 1999), is morphologically similar to some specimens of *O. americanum* var. *americanum* in Africa. *O.×citriodorum* is naturalised in Asia and cultivated for its lemon-scented leaves which produce the essential oils citral and neral (Grayer et al., 1996a).

We have now surveyed a large number of herbarium specimens of *O. americanum* (both varieties) and *O.×citriodorum* and a few samples of closely related species for external flavonoids, to investigate whether these taxa can be distinguished on the basis of their flavonoid chemistry. A possible correlation between the geographic distribution of the plants and their flavonoid chemistry was also investigated.

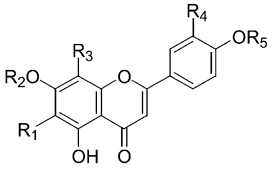
2. Results and discussion

2.1. Profiles of external flavones in *O. americanum*

Previous surveys of the surface flavonoids in species of *Ocimum* have been carried out by HPLC/DAD (Grayer et al., 1996b) and atmospheric pressure chemical ionisation (APCI) LC-MS (Grayer et al., 2001). LC-MS is of great benefit for the analysis and fingerprinting of extracts when the constituents are not known and when they do not have characteristic UV spectra. However, certain phenolics, especially methoxylated flavones, have such characteristic and specific UV spectra that the great majority of compounds can be identified on the basis of UV spectra alone, especially if retention times are also taken into consideration. As HPLC/DAD is a quicker and more sensitive procedure for the identification of surface flavonoids than LC-MS, and as the compounds in *O. americanum* had already been identified by means of APCI MS and NMR (Grayer et al., 2001), we used HPLC/DAD for the present survey. In the few cases where identification was ambiguous on the basis of UV spectra alone, the identifications were checked by APCI LC-MS.

Nineteen different external flavones were detected by HPLC in the diethyl ether extracts of 111 leaf specimens of *O. americanum* (74 of var. *americanum* and 37 of var. *pilosum*). Details of the compound identifications are given in the Experimental. The external flavones were (in sequence of their retention times): scutellarein (**1**), luteolin (**2**), cirsiliol (**3**), apigenin (**4**), pilosin (**5**), cirsimaritin (**6**), cirsilineol (**7**), ladanein (**8**), 5-desmethylsinensitin (**9**), xanthomicrol (**10**), 8-hydroxysalvigenin (**11**), nevadensin (**12**), acacetin (**13**), pectolinarigenin (**14**), gen-

kwanin (**15**), 5-desmethylnobiletin (**16**), salvigenin (**17**), gardenin B (**18**) and apigenin 7,4′-dimethyl ether (**19**) (see Fig. 1). Compound **11**, 8-hydroxysalvigenin (5,8-dihydroxy-6,7,4′-trimethoxyflavone), used to be called pedunculin and was first described by La Duke (1982) from *Tithonia pedunculata*. However, Horie et al. (1995) showed that the structure of the flavone from this plant is in fact 5,7-dihydroxy-6,8,4′-trimethoxyflavone (nevadensin), and that therefore the name pedunculin is a synonym of nevadensin and should no longer be used. On the other hand, the compound reported as pedunculin from species of *Ocimum* (Grayer et al., 2001) has the same UV spectrum as that determined by Horie et al. (1995) who synthesized the compound. Also, the APCI mass spectrum and CID spectrum are as expected for this flavone, so that the compound found in species of *Ocimum* really is 5,8-dihydroxy-6,7,4′-trimethoxyflavone. This compound has now also been detected in some species of *Nepeta* (Jamzad et al., in press), and the new trivial name 8-hydroxysalvigenin was proposed to show that it is likely to be biosynthesised by 8-hydroxylation of salvigenin. The distribution of the 19 flavones listed above in the different plant accessions of *O. americanum* is shown in Table 1. In this table the geographic area in which each herbarium specimen was collected is also given, to see whether there are any correlations between flavone profiles and geography. Additionally, the year of collection is presented, so that the profiles of old and more recent herbarium specimens can be compared. Three pluses (+ + +) were given to the compound present in the highest concentration in each plant accession, two pluses to the second most important compound and one plus to each of the remaining flavones found in the sample. When there were two major compounds in an extract, present in approximately equal amounts (when the HPLC peaks differed less than 10% in their absorbance), each received three pluses, etc. The retention times of cirsimaritin (**6**) and cirsilineol (**7**) were very similar in the solvent system used, so that it was very difficult to distinguish them. Usually they formed one peak on the HPLC chromatograms with a UV spectrum intermediate between that of the individual compounds. As only one peak area was obtained, the amounts for the combined rather than the separate compounds were used for Table 1. This does not have important chemotaxonomic implications, as the compounds are biogenetically closely related and occur together in most species of *Ocimum* (Grayer et al., 2001). In Table 1 a rough estimate of the approximate total amount of external flavones in mg per g dried leaf is also given for each plant accession. This was calculated by adding the total areas of the flavone HPLC peaks together after injecting a known amount of extract derived from 100 mg dried material and comparing this area with that of an injection of a known amount of a flavone standard (see Experimental).



	Compound	R_1	R_2	R_3	R_4	R_5
1	Scutellarein	OH	H	H	H	H
2	Luteolin	H	H	H	OH	H
3	Cirsiliol	OMe	Me	H	OH	H
4	Apigenin	H	H	H	H	H
5	Pilosin	OMe	H	OH	H	Me
6	Cirsimaritin	OMe	Me	H	H	H
7	Cirsilineol	OMe	Me	H	OMe	H
8	Ladanein	OH	Me	H	H	Me
9	5-Desmethylsinensetin	OMe	Me	H	OMe	Me
10	Xanthomicrol	OMe	Me	OMe	H	H
11	8-Hydroxysalvigenin	OMe	Me	OH	H	Me
12	Nevadensin	OMe	H	OMe	H	Me
13	Acacetin	H	H	H	H	Me
14	Pectolinarigenin	OMe	H	H	H	Me
15	Genkwanin	H	Me	H	H	H
16	5-Desmethylnobiletin	OMe	Me	OMe	OMe	Me
17	Salvigenin	OMe	Me	H	H	Me
18	Gardenin B	OMe	Me	OMe	H	Me
19	Apigenin 7,4'-dimethyl ether	H	Me	H	H	Me

Fig. 1. Chemical structures of flavones found in specimens of *Ocimum americanum*.

Principal component analysis of the results revealed that there are six different external flavone chemotypes among the specimens of *O. americanum* investigated (see Fig. 2), and therefore in Table 1 the accessions of *O. americanum* var. *americanum* and var. *pilosum* are arranged according to these chemotypes. Most chemotypes were characterised by the presence of two major flavonoids, so that it was decided to call the chemotypes after these two most important flavones (printed in bold):

I Nevadensin/salvigenin type (Fig. 3). This was the most important flavone chemotype, being found in 86% of the specimens of var. *americanum* and 78% of var. *pilosum*. This chemotype was also characterised by very high quantities of surface flavonoids, on average 7.3 mg/g of the dried leaf weight.

II Salvigenin/cirsimaritin type; found in 2 specimens (6%) of var. *pilosum*, again from East Tropical Africa. The flavonoid amounts were medium (on average 0.95 mg/g). This chemotype is in fact a very reduced version of chemotype 1; about half of the compounds present in chemotype 1 are missing, including nevadensin.

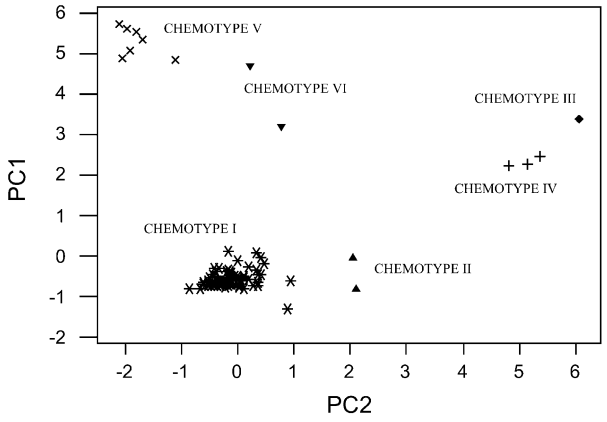


Fig. 2. Principal component analysis of external flavanoids from 107 herbarium samples of *Ocimum americanum*. Chemotype I (⋆); chemotype II (▲); chemotype III (◆); chemotype IV (+); chemotype V (x); chemotype VI (▼).

III Cirsimaritin/cirsilineol type; found in just one specimen (3%) of var. *pilosum* of North East Tropical Africa. The flavone concentration was very low (0.1 mg/g dried leaf). In the principal component analysis this specimen seems close to chemotype IV (see Fig. 2), but the data in Table 1 show that there are several important chemical differences between these two chemotypes.

IV Apigenin7,4′-dimethyl ether/salvigenin type; found in 3 specimens (4%) of var. *americanum*, from East Tropical Africa. The average flavone concentration was very low (0.13 mg/g).

V Xanthomicrol/5-desmethylnobiletin type. This was present in seven specimens (9%) of var. *americanum* investigated, which had all been collected in South Africa, apart from one specimen originating from Botswana and from cultivated stock. The average flavone concentration in this group was 0.28 mg/g dried leaves.

VI Xanthomicrol/cirsimaritin type; present in 2 specimens (6%) of var. *pilosum* from East Tropical Africa. The average flavone concentration was very low (0.12 mg/g).

No surface flavones could be detected in another four specimens of var. *pilosum*, one of which was of cultivated stock. Perhaps the concentrations of the flavonoids were below the detection limits of the HPLC. It is also possible that the plant specimens lost their external flavonoids because they were not preserved properly or for some other artificial reason. However, we found that some genotypes of *O. americanum* used in cultivation are genuinely devoid of external flavonoids (Grayer et al., unpublished results), perhaps because they were selected for high production of essential oils, to the detriment of other surface compounds.

Plants belonging to the nevadensin/salvigenin chemotype contained up to fifteen additional flavones, but in much lower quantities than nevadensin and salvigenin. The most important of these minor compounds was pilosin (5,7,8-trihydroxy-6,4′-dimethoxyflavone), a compound recently isolated for the first time as a natural product from *O. americanum* var. *pilosum* (Grayer et al., 2001). Pilosin is thought to be the immediate biosynthetic precursor of nevadensin (5,7-dihydroxy-6,8,4′-trimethoxyflavone) and if this is so, it will be produced by all plants that synthesise nevadensin, but it is not necessarily accumulated in those plants in quantities detectable by HPLC. Ironically, this compound was detected more frequently in nevadensin-producing samples of var. *americanum* than of var. *pilosum* after which it was named. Another minor compound, 5-desmethylsinensetin, was more characteristic for var. *pilosum* (detected in 64% of the nevadensin-containing samples) than for *O. americanum* var. *americanum* (in only 6% of the specimens with the nevadensin/salvi-

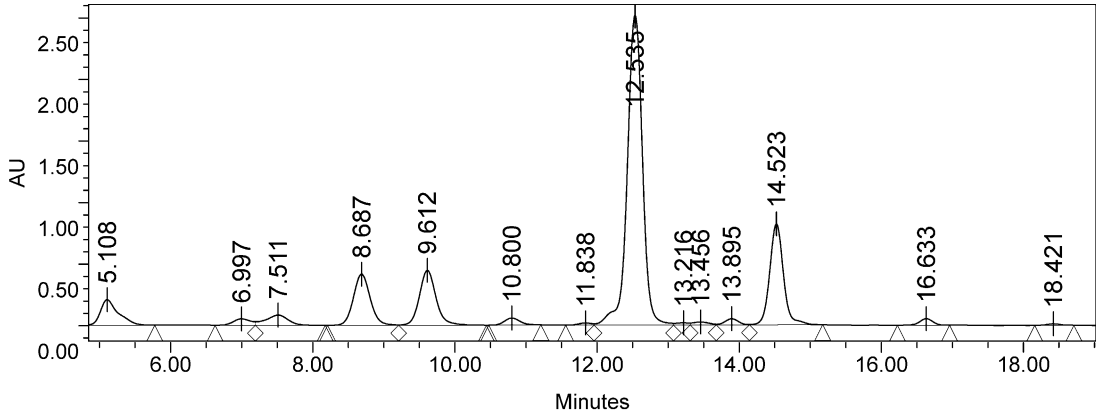


Fig. 3. HPLC chromatogram of the diethyl ether extract of herbarium sample No. 96, showing chemotype I of *Ocimum americanum*. The peaks are: scutellarein (R_t 5.108 min), cirsiliol (R_t 6.997 min), apigenin (R_t 7.511 min), pilosin (R_t 8.687 min), cirsimaritin (R_t 9.612 min), ladanein (R_t 10.800 min), 5-desmethylsinensetin (R_t 11.838 min), nevadensin (R_t 12.535 min), acacetin (R_t 13.216 min), pectolinarigenin (R_t 13.456 min), genkwanin (R_t 13.895 min), salvigenin (R_t 14.523 min), gardenin B (R_t 16.633) and apigenin 7,4′-dimethyl ether (R_t 18.421).

Table 1
Distribution of external flavones in *Ocimum americanum* and related species

No.	Taxon	Collector and number	Geographic location	Date	Scutellarein	Luteolin	Cirsiliol	Apigenin	Pilosin	Cirsimaritin + Cirsilineol	Ladanein	5-Desmethylsinensetin	Xanthomicrol	8-Hydroxysalvigenin	Nevadensin	5-Desmethylnobiletin	Acacetin	Pectolinaringenin	Genkwanin	Luteolin 3',4'-dimethyl ether	Salvigenin	Gardenin B	Apigenin 7,4'-dimethyl ether	Total Flavones (mg/g)
Ocimum americanum var. *americanum*, chemotype I																								
6	var. *americanum*	Collenette 5586	Orient Arabia	1986	+			+	+	+	+				+ + +			+	+		+ +		+	4.0
7	var. *americanum*	Wood 1385	Orient Arabia	1976	+		+	+	+	+	+				+ + +		+		+		+ +	+	+	3.4
4	var. *americanum*	Comanor 783	India	1968	+			+	+	+	+				+ + +				+		+ +	+	+	7.4
9	var. *americanum*	Jayasuriya et al. 595	India	1972	+		+	+		+	+				+ +		+		+		+ + +	+	+	7.7
10	var. *americanum*	Worthington 3427	India	1948	+	+	+	+		+	+				+ + +		+	+	+		+ +	+	+	2.2
11	var. *americanum*	Ramesh Bedi 545	India	1971	+			+	+	+	+				+ + +				+		+ +	+	+	4.9
13	var. *americanum*	Mooney 2242	India	1943	+	+	+	+	+	+	+				+ + +			+	+		+ +	+	+	5.7
22	var. *americanum*	J.D. Hooker s.n.	Indo China	1950		+	+	+	+	+	+				+ + +				+		+ +		+	2.7
26	var. *americanum*	Everist 5109	Australia	1952	+	+	+	+	+	+	+				+ + +		+		+		+ +	+	+	8.9
27	var. *americanum*	Blake 21822	Australia	1962		+		+	+	+	+				+ + +				+		+ +	+	+	2.7
28	var. *americanum*	Meesulia s/n	Pacific Islands	1986	+		+		+	+	+				+ + +				+		+ +	+	+ +	10.0
29	var. *americanum*	Morton A3694	West Tropical Africa	1959			+	+	+	+	+ +				+ + +		+		+		+ +	+	+	2.0
30	var. *americanum*	Rose Innes 30738A	West Tropical Africa	1958		+	+	+	+	+	+				+ + +		+		+		+ +	+	+	3.5
31	var. *americanum*	Dalziel 951	West Tropical Africa	1915		+	+	+		+	+				+ + +		+		+		+ +		+	2.0
32	var. *americanum*	Sharland 674	West Tropical Africa	1979		+	+	+	+	+	+				+ + +		+		+		+ +	+	+	2.7
34	var. *americanum*	Madsen 5557	West Tropical Africa	1996		+	+	+	+	+ +	+				+ + +		+		+		+ +	+	+	4.1
35	var. *americanum*	Thomas 2652	West Tropical Africa	1914			+	+	+	+	+				+ + +		+	+	+		+ +		+	1.1
36	var. *americanum*	Morton A1676	West Tropical Africa	1955		+	+	+	+	+	+				+ + +		+		+		+ +	+	+	6.8
37	var. *americanum*	Baldwin 6794	West Tropical Africa	1947		+	+	+	+	+	+				+ + +		+		+		+ +	+	+	2.3
38	var. *americanum*	Morton 25350	West Tropical Africa	1954		+	+	+	+	+	+				+ + +				+		+ +	+	+	9.0
39	var. *americanum*	Noble A13	West Tropical Africa	1951	+		+	+	+	+	+	+			+ + +		+		+		+ +	+	+	24.5
41	var. *americanum*	Jean Louis 2382	W. Central Trop. Africa	1936		+		+	+	+	+				+ + +		+		+		+ +	+	+	1.4
42	var. *americanum*	Wilde 5325	W. Central Trop. Africa	1965		+	+			+	+				+ + +		+		+		+ +	+	+	2.5
43	var. *americanum*	Reekmans 3948	W. Central Trop. Africa	1974	+			+	+	+	+				+ + +		+		+		+ +	+	+	5.3
45	var. *americanum*	Wilde 4947	W. Central Trop. Africa	1964		+	+	+	+	+	+				+ + +		+		+		+ +	+	+	5.1
46	var. *americanum*	Hepper 1293	W. Central Trop. Africa	1957		+		+	+	+	+				+ + +		+		+		+ +		+	3.1
50	var. *americanum*	Friis et al. 6705	N. E. Tropical Africa	1995		+	+	+	+	+	+				+ + +		+		+		+ +		+	1.6
52	var. *americanum*	Lynes 344C	N. E. Tropical Africa	1921		+	+	+	+	+	+				+ + +			+	+		+ +	+	+	2.7
53	var. *americanum*	Friis et al. 2626	N. E. Tropical Africa	1982	+		+	+	+	+	+				+ + +		+		+		+ +	+	+	11.6
54	var. *americanum*	Burger 798	N. E. Tropical Africa	1961		+	+	+	+	+	+				+ + +			+	+		+ +	+	+	9.3
55	var. *americanum*	Gilbert 1185	N. E. Tropical Africa	1969		+	+	+	+	+	+				+ + +			+	+		+ +		+	3.5
56	var. *americanum*	Gilbert 1624	N. E. Tropical Africa	1969	+	+		+	+	+	+				+ + +			+	+		+ +		+	5.2
57	var. *americanum*	Deshmukh 396	N. E. Tropical Africa	1987		+	+	+	+	+	+				+ + +		+		+		+ +	+	+	1.9
58	var. *americanum*	Eagleton 98	N. E. Tropical Africa	1987	+	+	+	+	+ +	+	+				+ + +		+	+	+		+ +		+	7.7
61	var. *americanum*	Basinski 14A	N. E. Tropical Africa	1957	+		+	+	+	+	+				+ + +		+		+		+ +	+	+	11.3
62	var. *americanum*	Wickens 1148	N. E. Tropical Africa	1964		+				+	+				+ + +		+				+ +		+	0.4
63	var. *americanum*	Katende K105	East Tropical Africa	1970		+		+	+	+	+				+ + +		+		+		+ +	+	+	3.1
64	var. *americanum*	Langdale-Brown 2391	East Tropical Africa	1957	+		+	+	+	+	+				+ + +		+	+	+		+ +	+	+	14.9
67	var. *americanum*	Sampson 15	East Tropical Africa	1934	+			+	+	+	+				+ + +		+		+		+ +	+	+	6.0

(*continued on next page*)

Table 1 (*continued*)

No.	Taxon	Collector and number	Geographic location	Date	Scutellarein	Luteolin	Cirsiliol	Apigenin	Pilosin	Cirsimaritin + Cirsilineol	Ladanein	5-Desmethylsinensetin	Xanthomicrol	8-Hydroxysalvigenin	Nevadensin	5-Desmethylnobiletin	Acacetin	Pectolinaringenin	Genkwanin	Luteolin 3',4'-dimethyl ether	Salvigenin	Gardenin B	Apigenin 7,4'-dimethyl ether	Total Flavones (mg/g)
68	var. *americanum*	Newbold 3181	East Tropical Africa	1958		+		+	+ +	+	+				+ + +		+		+		+ +	+	+	23.7
69	var. *americanum*	Bally 4502	East Tropical Africa	1945		+	+	+	+	+	+				+ + +		+		+		+ +	+	+	28.9
70	var. *americanum*	Williams 147	East Tropical Africa	1952	+		+	+	+ +	+	+				+ + +		+	+	+		+ +	+	+	39.2
71	var. *americanum*	Faulkner 2490	East Tropical Africa	1960		+	+	+	+	+					+ + +		+	+	+		+ +	+	+	3.3
72	var. *americanum*	Jaasund 2100	East Tropical Africa	1967	+	+		+	+	+	+				+ + +		+	+	+		+ +		+	8.5
73	var. *americanum*	Batty 33	East Tropical Africa	1968	+	+	+	+	+	+	+				+ + +		+	+	+		+ +		+	9.5
75	var. *americanum*	Archbold 2970	East Tropical Africa	1982	+	+			+	+	+				+ + +		+	+	+		+ +		+	5.4
76	var. *americanum*	Archbold 2807	East Tropical Africa	1966	+	+		+	+	+	+				+ + +		+				+ +		+	7.6
77	var. *americanum*	Newman 103	East Tropical Africa	1966	+			+	+	+	+				+ + +		+	+	+		+ +	+	+	6.4
78	var. *americanum*	Sangai 905	East Tropical Africa	1973	+			+	+	+	+				+ + +		+	+	+		+ +	+	+	18.1
79	var. *americanum*	Newbold 3309	East Tropical Africa	1958		+		+	+	+	+				+ + +		+	+	+		+ +	+	+	15.6
80	var. *americanum*	Luke et al. 106	East Tropical Africa	1990	+	+		+	+	+	+				+ + +		+		+		+ +		+	4.2
90	var. *americanum*	Brummitt 11159	South Tropical Africa	1970		+	+	+		+	+				+ + +		+		+		+ +	+	+	2.2
96	var. *americanum*	Souza 103	South Tropical Africa	1970	+ +		+	+	+ +	+ +	+	+			+ + +		+	+	+		+ +	+	+	15.3
106	var. *americanum*	Killick & Leistner 3149	South Africa	1958		+	+			+	+				+ + +				+		+ +	+	+	3.0
116	var. *americanum*	Moll 4601	South Africa	1969					+	+		+			+ + +		+		+		+ +	+	+	4.8
118	var. *americanum*	Balkwill et al. 10882	South Africa	1999	+		+	+	+ +	+	+				+ + +		+		+		+ +	+	+	26.3
5	var. *americanum*	Bautista 465	East Tropical S. America	1981		+		+		+	+				+ + +				+		+ +		+	1.7
122	var. *americanum*	Pickersgill et al. 148	East Tropical S. America	1972			+	+	+	+					+ + +		+		+		+ +	+	+	2.8
123	var. *americanum*	MJPP/JKAM 478	East Tropical S. America	no date		+	+	+							+ + +		+		+		+ +	+	+	1.1
115	var. *americanum*	Nee 40518	West Tropical S. America	1991		+		+		+	+				+ + +		+		+		+ +		+	2.0
44	var. *americanum*	Reekmans 11080	W. Central Trop. Africa	1982	+		+	+	+	+	+				+ + +		+		+		+ +	+	+	7.8
125	var. *americanum*	Vieira & Simon 2182	Cultivated	1997		+	+			+	+	+			+ + +		+		+		+ +	+	+	1.2
126	var. *americanum*	Vieira & Simon 2151	Cultivated	1997				+	+	+					+ + +		+		+		+ +	+	+	1.5
127	var. *americanum*	Vieira & Simon 2144	Cultivated	1997	+		+	+	+ +	+	+				+ + +		+		+		+ +	+	+	13.6
Ocimum americanum var. *americanum*, chemotype IV																								
2	var. *americanum*	Newbould 6386	East Tropical Africa	1962						+ +		+ +									+ +	+	+ + +	0.2
65	var. *americanum*	Brenan et al. 14775	East Tropical Africa	1978						+											+ +	+ +	+ + +	0.1
66	var. *americanum*	Stannard & Gilbert 905-916	East Tropical Africa	1977						+ +									+		+ +	+	+ + +	0.1
Ocimum americanum var. *americanum*, chemotype V																								
95	var. *americanum*	R.C.Brown 8239	South Tropical Africa	1970						+ +			+ + +			+ + +								0.2
109	var. *americanum*	Dinter 407	South Africa	1910						+			+ + +			+ +			+			+ +	+	0.6
110	var. *americanum*	McGregor Museum s/n	South Africa	1934						+			+ + +			+ +						+		0.3
111	var. *americanum*	Leistner 1670	South Africa	1960						+			+ + +			+ +						+		0.1
112	var. *americanum*	Wadermann & Oberdieck 2332	South Africa	1959						+			+ +			+ + +						+ +	+	0.3
114	var. *americanum*	Gleiss 12768	South Africa	1973		+				+			+ + +			+ +						+		0.3
128	var. *americanum*	Vieira & Simon 2143	Cultivated	1997		+ +						+ +	+ + +			+ + +			+					0.2
Ocimum americanum var. *pilosum*, chemotype I																								
40	var. *pilosum*	Hooper & Townsend 545	W. Central Trop. Africa	1975		+	+			+		+			+ + +				+		+ +		+	0.7
47	var. *pilosum*	Lewalle 5294	W. Central Trop. Africa	1971		+	+			+	+	+			+ + +		+	+	+		+ +	+	+	1.7
48	var. *pilosum*	Jean Leburn 8922	W. Central Trop. Africa	1937		+	+			+	+	+			+ + +		+		+		+ +	+	+	2.1

(*continued on next page*)

Table 1 (*continued*)

No.	Taxon	Collector and number	Geographic location	Date	Scutellarein	Luteolin	Cirsiliol	Apigenin	Pilosin	Cirsimaritin + Cirsilineol	Ladanein	5-Desmethylsinensetin	Xanthomicrol	8-Hydroxysalvigenin	Nevadensin	5-Desmethylnobiletin	Acacetin	Pectolinaringenin	Genkwanin	Luteolin 3',4'-dimethyl ether	Salvigenin	Gardenin B	Apigenin 7,4'-dimethyl ether	Total Flavones (mg/g)
49	var. *pilosum*	Germain 1857	W. Central Trop. Africa	1944		+	+			+	+	+			+ + +				+		+ +	+	+	1.0
1	var. *pilosum*	Juniper & Jefford 68	East Tropical Africa	1958			+	+		+	+				+ + +			+	+		+ +	+	+	2.6
74	var. *pilosum*	Ruffo & Kisena 3293	East Tropical Africa	1991						+					+ + +						+ +			0.8
82	var. *pilosum*	Mathew & Hanid 6063	East Tropical Africa	1970			+	+		+	+	+			+ +		+		+		+ + +	+	+	3.2
85	var. *pilosum*	Advis. Bureaus D958	East Tropical Africa	1951				+	+	+	+				+ + +		+	+	+		+ +		+	0.6
88	var. *pilosum*	R. Welch s/n	East Tropical Africa	1954			+	+	+	+	+	+			+ + +			+	+		+ +	+	+	6.6
89	var. *pilosum*	Brummitt 8604	South Tropical Africa	1970		+	+	+	+	+	+	+			+ + +			+	+		+ +	+	+	2.8
91	var. *pilosum*	Jean Pawek 8169	South Tropical Africa	1974			+	+	+	+	+	+			+ + +			+	+		+ +	+	+	3.1
92	var. *pilosum*	Robinson 47	South Tropical Africa	1953			+			+	+	+			+ + +		+		+		+ +	+	+	2.6
93	var. *pilosum*	Philcox & Leppard 8807	South Tropical Africa	1981	+		+	+	+ +	+	+			+ +	+ + +		+		+		+ +	+	+	36.0
94	var. *pilosum*	P. A. Smith 2609	South Tropical Africa	1979			+		+	+	+	+			+ + +		+		+		+ +	+	+	0.9
97	var. *pilosum*	P.J.Mott 118	South Tropical Africa	1974	+		+	+	+ +	+	+				+ + +		+		+		+ +	+	+	25.4
98	var. *pilosum*	Manuel da Silva 95	South Tropical Africa	1970		+	+	+		+	+	+			+ + +			+	+		+ +	+	+	5.3
99	var. *pilosum*	Kaunda & Kwatha 612	South Tropical Africa	1987		+	+	+	+	+	+	+			+ + +		+		+		+ +	+	+	2.8
100	var. *pilosum*	Philcox & Leppard 8508	South Tropical Africa	1981			+	+	+	+	+	+			+ + +		+	+	+		+ +	+	+	7.7
101	var. *pilosum*	Robson 1298	South Tropical Africa	1959			+	+		+	+			+	+ + +			+	+		+ +	+	+	1.7
102	var. *pilosum*	Cecil Sandwilth 104	South Tropical Africa	1929			+	+		+	+	+			+ + +			+	+		+ +	+	+	2.2
103	var. *pilosum*	J.B.Philipps 2338	South Tropical Africa	1960		+	+	+		+	+	+			+ + +			+	+		+ +	+	+	3.3
104	var. *pilosum*	Clement et al. 2082	Madagascar	1992	+	+	+	+	+	+	+				+ + +		+		+		+ +	+	+	7.4
105	var. *pilosum*	M. R. Decary 15012	Madagascar	1939	+	+		+		+	+				+ + +		+	+	+		+ +	+	+	4.5
108	var. *pilosum*	H.&H.E Wantorp 593	South Africa	1968		+	+			+		+			+ + +						+ +			1.8
117	var. *pilosum*	Codd 6025	South Africa	1950	+		+	+	+ +	+	+				+ + +				+		+ +	+	+	29.5
119	var. *pilosum*	Long & Rae 714	South Africa	1987		+	+			+	+	+			+ + +				+		+ +	+	+	0.9
120	var. *pilosum*	Glocs 12491	South Africa	1973		+	+			+	+	+			+ + +				+		+ +	+	+	2.2
121	var. *pilosum*	Acocks 23348	South Africa	1963	+		+	+	+ +	+	+				+ + +		+	+	+		+ +	+	+	50.6
Ocimum americanum var. *pilosum*, chemotype II																								
81	var. *pilosum*	Bally 1797	East Tropical Africa	1941		+	+			+ +	+ +								+		+ + +		+	0.8
84	var. *pilosum*	Ardstein Lye 1656	East Tropical Africa	1969	+		+			+ +	+ +										+ + +		+	1.1
Ocimum americanum var. *pilosum*, chemotype III																								
59	var. *pilosum*	Mooney 4835	N. E. Tropical Africa	1953						+ + +									+		+		+	0.1
Ocimum americanum var. *pilosum*, chemotype VI																								
83	var. *pilosum*	Gillett 13987	East Tropical Africa	1952						+ +	+		+ + +							+				+	0.2
86	var. *pilosum*	Kayombo & Kunduketa 2905	East Tropical Africa	1999						+ +	+ +		+ + +							+ + +				+ +	0.0
Ocimum americanum var. *pilosum*, no external flavones																								
8	var. *pilosum*	Sabra s.n.	Orient Arabia	no date																				0.0
60	var. *pilosum*	Westphal & Stevels 370	N. E. Tropical Africa	1967																				0.0
87	var. *pilosum*	Ritchie et al. 3500-4000	East Tropical Africa	1929																				0.0
124	var. *pilosum*	Vieira & Simon 2146	Cultivated	1997																				0.0
Ocimum×*citriodorum*																								
3	*citriodorum*	Matthew 50886	India	1987						+					+ + +						+ +		+	0.2
15	*citriodorum*	Haines 59a	India	1903			+			+	+	+			+ + +				+		+ +		+	0.4

(*continued on next page*)

Table 1 (*continued*)

No.	Taxon	Collector and number	Geographic location	Date	Scutellarein	Luteolin	Cirsiliol	Apigenin	Pilosin	Cirsimaritin + Cirsilineol	Ladanein	5-Desmethylsinensetin	Xanthomicrol	8-Hydroxysalvigenin	Nevadensin	5-Desmethylnobiletin	Acacetin	Pectolinaringenin	Genkwanin	Luteolin 3',4'-dimethyl ether	Salvigenin	Gardenin B	Apigenin 7,4'-dimethyl ether	Total Flavones (mg/g)
16	*citriodorum*	Venugopal 15792	India	1978		+	+			+	+				+ + +				+		+ +		+	1.1
17	*citriodorum*	s.coll s/n	India	1825		+	+	+	+	+	+				+ + +		+		+		+ +		+	5.7
18	*citriodorum*	Jacquemont 333	India	no date	+		+	+		+ +	+	+			+ + +				+		+ + +	+	+ +	1.1
19	*citriodorum*	Sibil 199	Malay Islands	1993			+	+		+	+	+			+ + +						+ +	+	+	1.0
20	*citriodorum*	Kadi A2582	Malay Islands	1949			+	+		+ +	+	+			+ + +				+		+ + +	+	+ +	2.0
21	*citriodorum*	Kerr 3396	Indo China	1914			+	+		+ +	+	+			+ + +				+		+ + +	+	+ +	3.9
23	*citriodorum*	Talmy 76	Indo China	1868	+		+	+		+ +	+	+			+ + +				+		+ + +	+	+	2.3
24	*citriodorum*	Schiefenhoevel 162	New Guinea	1971			+			+	+	+			+ + +		+		+		+ +	+	+ + +	2.1
129	*citriodorum*	Vieira & Simon 2163	Cultivated	1997			+			+ +	+	+			+ + +				+		+ + +	+	+ +	1.7
Other species of *Ocimum*																								
137	*circinatum*	Glover & Gillidand 403	N. E. Tropical Africa	1944	+ +		+			+			+ + +						+					0.1
131	*fischeri*	Abdallah et al. 96/183	East Tropical Africa	1996		+	+			+ +	+								+		+ + +			1.8
132	*fischeri*	Verdcourt 2075	East Tropical Africa	1957						+ +	+								+		+ + +			1.8
133	*fischeri*	Gilbert 6098	East Tropical Africa	1981			+			+ +	+								+		+ + +			1.7
134	*forskolei*	Adamson 690	East Tropical Africa	1957																				0.0
135	*forskolei*	Gilbert & Thulin 1390	East Tropical Africa	1978						+						+ +	+		+				+ + +	0.2
136	*forskolei*	R.B. & A.J. Faden 74/779	East Tropical Africa	1974																				0.0
138	*kenyense*	Greenway 6948	East Tropical Africa	1944						+ + +														0.1
139	*kenyense*	Bogdan AB4428	East Tropical Africa	1957						+ + +														0.2

+ + +—Flavone showing the highest UV absorbance on the HPLC chromatogram, when extracted at 335 nm, for that particular plant accession. + +—Flavone showing the second highest UV absorbance on the HPLC chromatogram extracted at 335 nm. +—Any other flavone detectable on the HPLC chromatogram extracted at 335 nm.

genin profile). The only other minor compound that showed differences in distribution among the two varieties was scutellarein, which occurred quite frequently in specimens of *O. americanum* var. *americanum* (although it was almost totally absent from West African plants of this variety), but was absent from most samples of var. *pilosum*. However, it was present in the few specimens of this variety that lacked 5-desmethylsinensetin.

The four remaining flavonoid chemotypes found in specimens of *O. americanum* were all characterised by much smaller total amounts of flavonoids, the absence of nevadensin and sometimes also the absence of salvigenin. Besides, they were restricted in distribution to South Africa or Tropical East Africa. It is interesting that the two major flavones found in chemotype V, xanthomicrol and 5-desmethylnobiletin, were not present in any of the plants with nevadensin and salvigenin as main constituents (chemotype I). However, both xanthomicrol and 5-desmethylnobiletin have the same 5-hydroxy-6,7,8-trimethoxy A-ring substitution pattern as gardenin B, which compound was generally present as a minor constituent in chemotype I, so that the biochemistry of chemotypes I and V may not be as different from each other as they seem.

2.2. Comparison of the external flavonoid profiles of *O. americanum* with those of *O.×citriodorum* and other related species

To investigate whether the surface flavonoid profiles found in *O. americanum* are distinctive and cannot be confused with those of closely related species, they were compared with those of *O.×citriodorum* and seven other closely related species, all belonging to the same section (*Ocimum* section *Ocimum*). Eleven herbarium specimens were studied for *O.×citriodorum*, three herbarium specimens of both *O. fischeri* Gürke and *O. forskolei* Benth., two specimens of *O. kenyense* Ayobangira ex A.J. Paton and one of *O. circinatum* A.J. Paton. The results of their flavonoid analysis are presented in Table 1. The profiles of *O. basilicum*, *O. minimum* and *O. kilimandscharicum* were studied previously in the same way, so that the results are comparable (Grayer et al., 1996b, 2001).

Table 2 gives a comparison between the external flavonoid profiles found in each of the *O. americanum* chemotypes with those found in related species. In this table three pluses are given for the most abundant flavone (on average) in each taxon, two pluses for the second most abundant flavone, and one plus for diagnostic minor compounds. The average flavone concentration is also given for each taxon for quantitative comparisons and also the average nevadensin/salvigenin ratios in taxa that accumulate these two compounds.

O. americanum chemotype I (the nevadensin/salvigenin profile) was also found in *O.×citriodorum* (see Table 1) and in *O. basilicum* and some plants of *O. minimum* (Grayer et al., 1996b, 2001). As nevadensin is a very unusual compound in the Lamiaceae, these four taxa should be very closely related indeed. However, the profile found in *O. americanum* chemotype I can be distinguished from those in *O. basilicum* and its very close relative *O. minimum* on both a qualitative and a quantitative basis. Some of the minor compounds in *O. americanum* chemotype I, such as pilosin, scutellarein and pectolinarigenin were absent from *O. basilicum*. They were also absent or rare in *O.×citriodorum*. Quantitative differences among the four species include total amounts of external flavonoids and the ratio between the amounts of nevadensin and salvigenin. The average nevadensin/salvigenin ratio was much higher in *O. americanum* (3.1) than in *O. basilicum* (0.7) and *O. minimum* (0.6), although occasional specimens of these three species were found that showed uncharacteristic ratios. The average value for *O.×citriodorum* (1.4), was intermediate and partly overlapped with those found in specimens of *O. americanum* and *O. basilicum*. Similarly, the total amounts of external flavones determined were on average highest in *O. americanum* chemotype I (7.3 mg/g dried leaves), lowest in *O. basilicum* (0.19 mg/g) and *O. minimum* (0.16 mg/g) and intermediate in *O.×citriodorum* (1.96 mg/g). Pushpangadan and Sobti (1982) postulated that *O.×citriodorum* is a hybrid between *O. basilicum* and *O. americanum*, because of a number of intermediate characters. The intermediate surface flavonoid profile of *O.×citriodorum* supports this view, although chemical characters of hybrid species are not necessarily intermediate between those of the parents.

Specimens of *O. americanum* and *O.×citriodorum* of the nevadensin/salvigenin chemotype collected in the 19th and early 20th century contained the same range of compounds and in similar concentrations as specimens recently collected. Therefore, no deterioration or alteration of flavonoids seems to have occurred during the long storage of the plant material. However, once extracted, the two flavones containing an unsubstituted 8-hydroxyl group, pilosin and 8-hydroxysalvigenin, had disappeared from the extracts when they were rerun on the HPLC a week later, so they must have degraded in the aqueous methanol of the solutions. We have found before that flavones with a free 8-hydroxyl group are unstable in solution, e.g. 8-hydroxycirsimaritin (=isothymusin), which occurs in *Ocimum grandiflorum* (=*Becium grandiflorum*) (Grayer and Veitch, 1998). This compound is converted (probably oxidised) into a non-flavonoid substance with a different UV spectrum and a much shorter retention time (it is not the 8-methoxyderivative, xanthomicrol). All other surface flavones in *O. americanum*, which have free hydroxyl groups in different positions of the flavone molecule, were stable in aqueous methanol, as they were still present in the

Table 2
Comparison of the external flavonoid profiles of *O. americanum* with those of related species

Taxon	Natural distribution	Flavonoid content (mg/g)	Nevadensin	Salvigenin	Ratio Nevad./ Salvig.	Pilosin	5-Desmethyl sinensetin	Cirsimaritin/ Cirsilineol	Genkwanin	Apigenin 7,4′-dimeth. ether.	Xantho microl	5-Desmethyl nobiletin	Gardenin B	Scutellarein	Pectolin arigenin
O. amer. var. *amer.* Chemotype I	Africa, Asia	7.29	+++	++	3.3	+	(+)	+	+	+	–	–	+	+	(+)
O. amer. var. *pilosum* Chemotype I	Africa, Asia	7.49	+++	++	2.8	(+)	+	+	+	+	–	–	+	(+)	(+)
O.×citriodorum	Asia	1.96	+++	+++	1.4	(+)	+	+	+	+	–	–	+	(+)	–
O. basilicum *	Asia	0.19	++	+++	0.7		(+)	+	+	+	–	–	+	–	–
O. minimum ˆ	Asia	0.16	(++)	+++	0.6	–	–	++	+	+	–	–	+	–	–
O. amer. var. *pilosum* Chemotype II	East Africa	0.95	–	+++	0	–	–	++	+	+	–	–	–	(+)	–
O. fischeri	East Africa	1.8	–	+++	0	–	–	++	+	–	–	–	–	–	–
O. kilimandscharicum ˆ	East Africa	0.5	–	+++	0	–	–	++	+	–	–	–	+	–	–
O. amer. var. *pilosum* Chemotype III	N.-E. Africa	0.1		+	0	–	–	+++	+	+	–	–	–	–	–
O. kenyense	East Africa	0.12	–	–	0	–	–	+++	–	–	–	–	–	–	–
O. amer. var. *amer.* Chemotype IV	East Africa	0.13	–	++	0	–	(+)	++	(+)	+++	–	–	+	–	–
O. forskolei	East Africa	0.18	–	–	0	–	–	+	+	+++	–	++	–	–	–
O. amer. var. *amer.* Chemotype V	South Africa	0.28	–	–	0	–	(+)	+	(+)	(+)	+++	++	+	–	–
O. amer. var. *pilosum* Chemotype VI	East Africa	0.12	–	–	0	–	–	++	+	+	+++	–	–	–	–
O. circinatum	East Africa	0.11	–	–	0	–	–	+	+	–	+++	–	–	++	–

+++—major flavone. ++—second major flavone. +—minor, but diagnostic flavone. (+)—peak present in less than half of the specimens of the taxon. – —compound not detected. * Results published in Grayer et al. (1996b). ˆ Results published in Grayer et al. (2001).

extracts in the same concentrations after a month or more. Because of the instability in solution of pilosin and 8-hydroxysalvigenin, no subdivisions within chemotype I were made based on the presence or absence of these compounds.

The **salvigenin/cirsimaritin** profile present in *O. americanum* var. *pilosum* chemotype II was also found in *O. fischeri* and *O. kilimandscharicum*. As these three taxa all occur in the same geographic area (East Africa), it is possible that the specimens of *O. americanum* var. *pilosum* showing this chemotype acquired their external flavonoid profile by introgressive hybridisation with related species showing this profile. *O. americanum* chemotype II, *O. fischeri* and *O. kilimandscharicum* can be distinguished chemically on the basis of the presence or absence of apigenin 7,4′-dimethyl ether (present in *O. americanum* chemotype II only), and of gardenin B, which was present in *O. kilimandscharicum* only.

The **cirsimaritin/cirsilineol** profile found in *O. americanum* var. *pilosum* chemotype III, was also found in *O. kenyense*. However, cirsimaritin and cirsilineol were the only flavones in *O. kenyense*, whereas the specimen showing chemotype III also produced small amounts of genkwanin, apigenin 7,4′-dimethyl ether and salvigenin.

The **apigenin 7,4′-dimethyl ether/salvigenin** profile found in chemotype IV of *O. americanum* var. *americanum* resembled that of one specimen out of three studied of *O. forskolei* (the other two specimens of *O. forskolei* did not contain external flavones). The majority of specimens containing high concentrations of apigenin 7,4′-dimethyl ether were collected in East Africa, so that again there may have been an exchange of genes. However, the flavonoid profiles of these two taxa were distinguished by the production of 5-desmethylnobiletin in *O. forskolei* only, and this was the second most abundant surface flavonoid in this species instead of salvigenin.

Xanthomicrol was the major external flavone in chemotypes V and VI of *O. americanum* and in *O. circinatum*. The profiles of these three taxa could be easily distinguished, however, because the second most abundant flavone was different. This was 5-desmethylnobiletin in *O. americanum* chemotype V, cirsimaritin in chemotype VI and scutellarein in *O. circinatum*.

Despite the fact that there are several external flavone chemotypes in *O. americanum*, that profiles similar to these chemotypes have been found in related species, and that some specimens were devoid of external flavonoids, it should be possible to identify and authenticate a large proportion of leaf samples of *O. americanum* using external flavonoid profiles. However, the distinction of chemotype I with *O.×citriodorum* is not very sharp and neither is that of chemotype II with *O. fischeri*.

This research has shown that in order to identify and authenticate a species with certainty using chemical profiling, it is necessary to study the profiles in as many as specimens as possible, collected from different areas of the geographical range of the species. Furthermore, these chemical profiles should be compared with those of all related species.

3. Experimental

3.1. Plant material

The plant material for the flavonoid survey was obtained from the collections of the Herbarium, Royal Botanic Gardens, Kew. The collector and number of each specimen, the year and the geographic area where each plant accession was collected are given in Table 1. The identification of each specimen was thoroughly checked by the authors (R.F.V. and A.J.P).

3.2. Extraction of the plant material

From each herbarium specimen 100 mg of dried leaves were taken and these were extracted for 24 h in 10 ml of diethyl ether. The extract was then filtered and evaporated to dryness at ambient temperature in a fume cupboard. Just prior to HPLC, the dried extract was redissolved in 1.0 ml of 80% aqueous MeOH and filtered through a Gelman Nylon Acrodisc 13 filter (pore size 0.45 μl) into a HPLC autosampler vial.

3.3. HPLC with diode array detection (HPLC-DAD) of the plant extracts

Each extract was analysed for external flavonoids by means of a HPLC system consisting of a Waters LC 600 pump, Waters 996 photodiode array detector and Waters 717 plus autosampler. A LiChrosper 100RP-18 (5 μm) column was used (4.0 mm i.d.×250 mm, Merck) and for elution of the compounds a gradient of two solvents denoted A and B was employed. A was 2% aqueous HOAc, whereas B was MeOH–HOAc–H_2O, 18:1:1. Initial conditions were 40% A and 60% B, with a linear gradient reaching B = 100% at $t = 15$ min. This was followed by an isocratic elution (B = 100%) until $t = 20$ min, after which the programme returned to the initial solvent composition. At $t = 30$ min the injection of the next sample took place. The flow rate used was 1.0 ml min^{-1}, and the temperature of the column was 30 °C throughout the HPLC run. For each extract a 40 μl injection was made using an autosampler. If the sample appeared to be overloaded, i.e. if the UV absorbance of one or more peaks was more than 3.0, smaller injections were also made (5 or 10 μl). The retention times and UV spectra of the flavonoids in the extracts were compared with those of marker compounds.

Table 3
HPLC retention times and UV absorption maxima of external flavones found in species of *Ocimum*

Trivial name of flavone	Semi-systematic name of flavone	R_t (min)	UV λ_{max} (nm)
Scutellarein	5,6,7,4′-Tetrahydroxyflavone	5.1	285, 338
Luteolin	5,7,3′,4′-Tetrahydroxyflavone	5.4	255, 267*sh*, 350
Cirsiliol	5,3′,4′-Trihydroxy-6,7-dimethoxyflavone	7.0	252, 274, 348
Isothymusin	5,8,4′-Trihydroxy-6,7-dimethoxyflavone	7.4	282*sh*, 304, 335*sh*
Apigenin	5,7,4′-Trihydroxyflavone	7.5	268, 338
Pilosin	5,7,8-Trihydroxy-6,4′-dimethoxyflavone	8.7	287*sh*, 299, 332
Cirsilineol	5,4′-Dihydroxy-6,7,3′-trihydroxyflavone	9.6	252, 274, 345
Cirsimaritin	5,4′Dihydroxy-6,7-dimethoxyflavone	9.6	276, 337
Ladanein	5,6-Dihydroxy-7,4′-dimethoxyflavone	10.8	285, 334
5-Desmethylsinensetin	5-Hydroxy-6,7,3′,4′-tetramethoxyflavone	11.8	252, 273, 342
Xanthomicrol	5,4′-Dihydroxy-6,7,8-trimethoxyflavone	12.1	283, 294*sh*, 336
8-Hydroxysalvigenin	5,8-Dihydroxy-6,7,4′-trimethoxyflavone	12.2	282*sh*, 304, 330*sh*
Nevadensin	5,7-Dihydroxy-6,8,4′-trimethoxyflavone	12.5	281, 294*sh*, 332
Acacetin	5,7-Dihydroxy-4′-methoxyflavone	13.2	268, 334
Pectolinarigenin	5,7-Dihydroxy-6,4′-dimethoxyflavone	13.5	273, 334
Genkwanin	5,4′-Dihydroxy-7-methoxyflavone	13.9	268, 337
5-Desmethylnobeletin	5-Hydroxy-6,7,8,3′,4′-pentamethoxyflavone	14.3	283, 342
Salvigenin	5-Hydroxy-6,7,4′-trimethoxyflavone	14.5	275, 333
Gardenin B	5-Hydroxy-6,7,8,4′-tetramethoxyflavone	16.6	283, 294*sh*, 330
Apigenin 7,4′-dimethyl ether	5-Hydroxy-7,4′-dimethoxyflavone	18.4	269, 331

sh, Denotes shoulder.

3.4. Identification of the flavonoids in the extracts

The external flavonoids in the extracts of herbarium material of *O. americanum* and *O.×citriodorum* were identified by comparison of their HPLC retention times and UV spectra with the compounds found in freshly prepared extracts of previously analysed plants of *O. americanum* (accessions BI 6445 and BI 6419) and *O. grandiflorum* Lam. (BI 6418). The compounds from these plant accessions had been isolated during a previous survey of *Ocimum* species and identified by NMR spectroscopy (nevadensin and pilosin) or by comparison of their atmospheric pressure chemical ionisation (APCI) mass spectra and UV spectra with those of standards (Grayer et al., 2001). During APCI mass spectrometry of these flavones it was found that the product ions formed by collision induced dissociation (CID) of the protonated molecule provided structural information about the substitution pattern (hydroxylation and methoxylation) of the A-ring of 6- and 8-oxygenated flavones (Grayer et al., 2001). This method makes it possible to identify many external flavones even when no standards are available for comparison. The flavonoids currently detected, their UV spectra, retention times and identification are presented in Table 3.

3.5. Quantification of the flavonoids

The maximum UV absorbance of each HPLC flavonoid peak was recorded at 335 nm for each extract, which is the approximate λ_{max} of the most abundant flavones found in *O. americanum*. The UV absorbance of solutions containing known concentrations of the flavone cirsiliol (purchased from Apin) were plotted against the concentration to make a standard curve, and this was used to calculate the approximate amounts of flavones present in each of the extracts. As the original weight of the dried leaves used for extraction was known, and also the amount injected into the HPLC column, the total amount of flavonoids per gram dry weight could be calculated from the UV absorbance of the peaks and adding these together. Although these figures are not very accurate, they represent a reasonable estimate of the amounts present and the values among the specimens are comparable.

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