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High-performance liquid chromatography of flavonoids from *Sideritis* species[1]

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Abstract

The determination of flavonoids in the genus *Sideritis* (*Lamiaceae*) is described by a new reversed-phase high-performance liquid chromatography method. The ethyl acetate extract content of sixteen species of the genus *Sideritis* in isoscutelarein-7-O-[allosyl (1→2) glucoside], luteoline, hypolaetin-8-O-β-D-glucoside, chrysoeriol, apigenine, sideritoflavone, xantomicrol, gardenin D, 8-methoxy-cirsilineol and desmetilnobiletine is reported for the first time. Knowledge of the flavonoid content allows their relationship to the pharmacological potency to be established and contributes to the botanical determination of these species.

Keywords: *Sideritis*; Flavonoids

1. Introduction

The genus *Sideritis* (*Labiatae*) grows in different parts of the Mediterranean region and it is widely used in folk medicine because of its anti-inflammatory and anti-ulcer properties. The activity of these plants is mainly due to their flavonoid and terpenoid content, such as borjatriol [1–3], sideritoflavone [4], hypolaetin-8-O-β-D-glucoside [5–12] which have been isolated.

The frequent hybridizations between different species makes their botanical determination in subspecies and varieties difficult, and makes a reclassification, according to the chemical composition of the plants, necessary [13–19].

In this work we attempt to identify the flavonoids present in the ethyl acetate extracts from sixteen species of the genus *Sideritis* as a part of an investigation that includes the complete analysis of the flavonoids present in each taxon. Our aim is to contribute to their chemotaxonomic determination and to identify the anti-inflammatory agents.

2. Experimental

2.1. Reference substances

The HPLC-grade luteoline (**2**), chrysoeriol (**4**) and apigenine (**5**) were purchased by Extrasynthese (Genay, France). Isoscutelarein-7-O-[allosyl (1→2) glucoside] (**1**), hypolaetin-8-O-β-D-glucoside (**3**), sideritoflavone (**6**), xantomicrol (**7**), gardenin D (**8**), 8-methoxy-cirsilineol (**9**) and desmetilnobiletine (**10**)

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were isolated from different species of *Sideritis* and supplied by CSIC (Murcia, Spain).

2.2. Vegetal material

The sixteen species of the genus *Sideritis* were collected in Levante, East Andalusia and Guadalajara (Spain). A voucher specimen of each species is on deposit in the Botany Department Herbarium (M.A.F.) of the Faculty of Pharmacy, UCM, Madrid. The data for these species (name, voucher specimen Nos., date and collection place) are shown in Table 1.

All samples were dried at room temperature and powdered.

2.3. Flavonoids extraction

Each powdered sample underwent a maceration and percolation extraction process at room temperature with solvents of increasing polarity [*n*-hexane (4 × 100 ml, 48 h), EtOH–H_2O (70:30) (4 × 100 ml, 48 h)]. The hydroalcoholic extracts were concentrated to eliminate the EtOH, and the aqueous solutions were extracted successively with ethyl ether, EtOAc and *n*-butanol (10 × 25 ml). Each extract was concentrated to dryness below 50°C.

The ethyl acetate extract which showed the highest proportion of flavonoids, was subjected to further investigation.

2.4. Chromatographic system

The HPLC analyses were carried out on a Varian 2510 pump with Varian Polychrom 9065 photodiode-array detector operating at λ=254 nm, and a Varian DS 654 data processor. Chromatographic runs were performed on a Hypersil ODS 20 cm × 4.6 mm I.D., 5 μm, column (Shandon Science, Astmoon, UK) at a room temperature. Working solutions contained an exactly weighed quantity of 1–1.5 mg per 1 ml of the MeOH extract (HPLC grade Scharlau, Barcelona, Spain) and were filtered through a 0.45-μm filter. The eluents were water–acetic acid (98:2, v/v) from pump A (acetic acid was added to prevent tailing) and acetonitrile from pump B. All the solvents were of HPLC grade (Scharlau) and were filtered through a 0.45-μm filter and degassed before their use. Gradient elution was performed for 20 min and started with 70% A and 30% B, and followed as specified: $t_{10\ min}$=45% A, $t_{11\ min}$=20% A and $t_{20\ min}$=20% A. Flow-rate was 1 ml/min. Samples of 10 μl were injected.

The identification of the different compounds was achieved by comparison of both t_R and the absorption spectra obtained for each eluted peak with those obtained for the standards.

Table 1
Species of the genus *sideritis*

Species	Subsection	M.A.F.	Collection place	Date
S. foetens	*Arborescens*	139030	Peñarrodá, Sierra Gádor (AL)	5/16/90
S. luteola	*Arborescens*	139042	Rambla de Aulago, Puente Navarro (AL)	5/15/90
S. almeriensis	*Flavovirens*		Las Negras (AL)	4/27/90
S. hirsuta	*Hirsuta*	139119	Bacares (AL)	7/5/90
S. leucantha var serratifolia		139035	Peñarrodá, Sierra Gádor (AL)	5/16/90
S. biflora	*Leucantha*	139041	Lucainena de las Torres (AL)	5/15/90
S. leucantha spp incana var meridionalis	*Leucantha*	139034	El Pinar de Védar (AL)	5/15/90
S. bourgeana	*Leucantha*		Montealegre (AB)	7/27/90
S. pusilla ssp almeriensis		139039	Rambla de Huéchar, Sierra Gádor (AL)	5/15/90
S. ibanyezii	*Flavovirens*	139040	Lorca (MU)	5/17/90
S. pusilla ssp pusilla var granatensis		139038	Sierra Alhamilla (AL)	5/15/90
S. leucantha var incana	*Leucantha*	139036	Vélez Rubio-Baza (AL)	7/5/90
S. cillensis			Cillas (GU)	8/12/88
S. pusilla	*Flavovirens*	139043	Almanzora (AL)	4/27/90
S. glauca	*Gymnocarpae*	139032	Sierra de Orihuela (A)	4/15/90
S. incana ssp incana	*Gymnocarpae*		Bicorp (V)	7/27/88

Table 2
Retention times, capacity factors and selectivity of flavonoids

Flavonoid	Abbreviation	t_R (min) (±S.D.)	k'	α
Isoscutelarein-7-O-[alosil (1→2)glucoside)	Isosc	1.897(±0.085)	0.66	–
Luteoline	Luteo	2.670(±0.093)	1.34	2.03
Hypolaetin-8-O-β-D-glucoside	Hypol	3.788(±0.037)	2.39	1.78
Chrysoeriol	Chrys	8.029(±0.618)	6.92	2.89
Apigenine	Apig	9.143(±0.335)	7.40	1.06
Sideritoflavone	Sider	11.349(±0.430)	9.69	1.30
Xantomicrol	Xant	12.648(±0.380)	10.06	1.03
Gardenin D	Gar-D	13.139(±0.276)	11.12	1.10
8-Methoxy-cirsilineol	8-Me-cirsil	13.391(±0.154)	11.16	1.003
Desmetilnobiletine	Desmet-nobilet	14.591(±0.045)	12.16	1.09

Hypersil ODS column: 20 cm × 4.6 mm, 5 μm . Solvent system: Water–acetic acid (98:2, v/v), acetonitrile, gradient (as described in Section 2.4). Total elution time, 20 min. Flow-rate, 1 ml/min.

To check the peak purity, the eluates were monitored with a photodiode-array detector (λ=200–400 nm). The three spectra corresponding to the upslope, apex and downslope of each peak were normalized and superimposed. Peaks were considered pure when there was an exact coincidence between the three spectra (match factor≥99.5).

3. Results and discussion

Table 2 shows the retention times ± S.D., capacity factors and selectivity of the standards (t_0= 1.10 min; flow-rate=1 ml/min). The final content in the ethyl acetate extract of each flavonoid, expressed in per cent of plant dry weight, is shown in Table 3.

Table 3
The content of flavonoids (%) in the EtOAc extract

Species[a]	Isosc	Luteo	Hypol	Chrys	Apig	Sider	Xant	Gar-D	8-Me-cirsil	Desmetnobilet
1	0.067	0.336						0.002		0.035
2	0.013	0.042								
3	0.781	0.125		0.400		0.050				
4	0.013	0.039		0.012		0.009	0.008	0.006		
5	0.125	0.530	0.014	0.076		0.004	0.001	0.001		
6				0.009		0.005	0.0003	0.0006	0.001	
7			0.207	0.033		0.018	0.001	0.005		0.083
8	0.215	0.761	0.038	0.203		0.067		0.013		
9	0.079	0.242		0.016						
10	0.427	0.902		0.198		0.193	0.017	0.052		0.435
11	0.093	0.171	0.075							
12	0.284	0.828		0.021						
13	0.148	0.604	0.008	0.119		0.098	0.006	0.004		
14	0.047	0.835	0.011	0.082				0.012		
15	0.005	0.056		0.002						
16	0.071	0.225		0.007		0.006				

For abbreviations used see Table 2.

[a] Species: 1 = *Sideritis foetens*; 2 = *S. luteola*; 3 = *S. almeriensis*; 4 = *S. hirsuta*; 5 = *S. leucantha var. serratifolia*; 6 = *S. biflora*; 7 = *S. leucantha ss. incana var. meridionalis*; 8 = *S. bourgeana*; 9 = *S. pusilla ss. almeriensis*; 10 = *S. ibanyeszii*; 11 = *S. pusilla ss. pusilla var. granatensis*; 12 = *S. leucantha var. incana*; 13 = *S. cillensis*; 14 = *S. pusilla*; 15 = *S. glauca*; 16 = *S. incana ss. incana*.

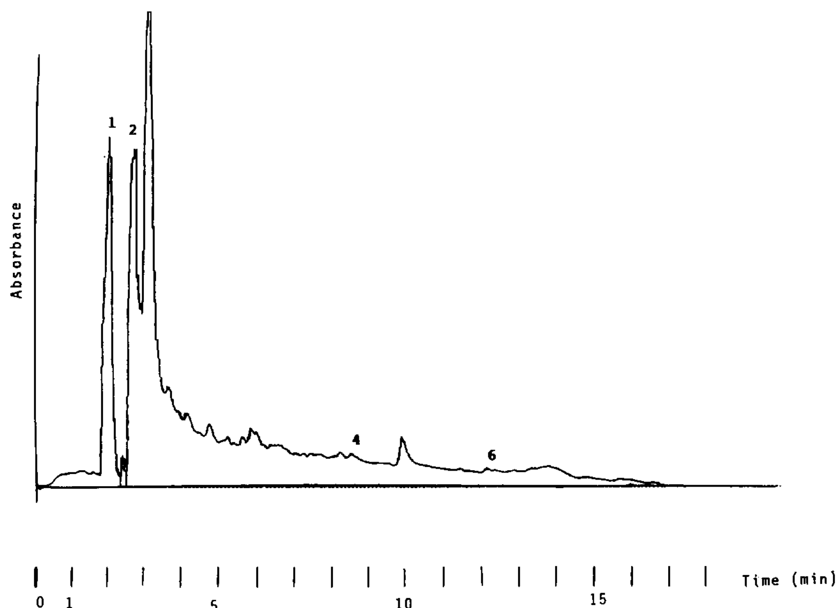

Fig. 1. Chromatogram of the EtOAc extract from *Sideritis almeriensis* under the analytical conditions described in Section 2.

Fig. 1, Fig. 2 and Fig. 3 show the chromatograms of the ethyl acetate extract of *Sideritis almeriensis*, *Sideritis pusilla ssp almeriensis* and *Sideritis luteola*, respectively, under the analytical conditions previously described.

Study of published HPLC methods for flavonoids induced us to start with an isocratic elution with H_2O–formic acid (98:2, v/v) from pump A and acetonitrile from pump B, but good resolution was not obtained. Next, a gradient elution starting with 70% A and decreasing to 30% A in 20 min was tested; this improved the resolution of the peaks that showed the largest overlap.

The presence of formic acid interfered with the photodiode-array detector and forced us to change it for acetic acid, which does not absorb at the critical wavelength and is as efficient as formic acid in suppressing tailing.

Successive assays, varying the mobile-phase gradient starting with 10% A and increasing up to 100% A, led to a mobile-phase composition that provided best resolution: H_2O–formic acid (98:2, v/v) from pump A and acetonitrile from pump B in a gradient elution for 11 min followed by an isocratic elution up to 20 min as described in Section 2.4.

This method, which shows good reproducibility,

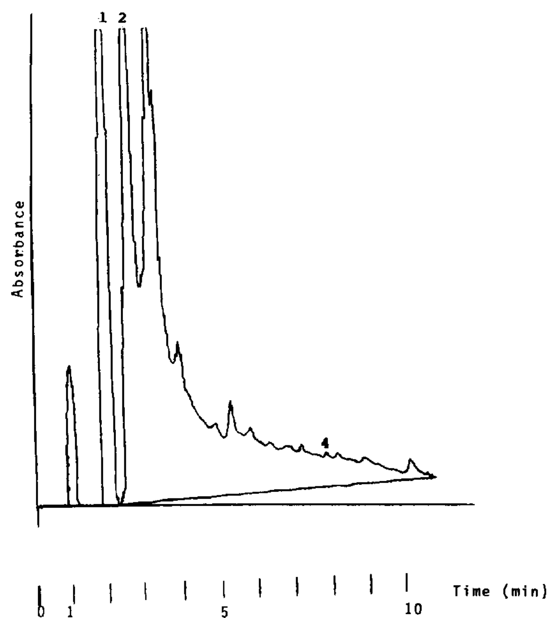

Fig. 2. Chromatogram of the EtOAc extract from *Sideritis pusilla ssp almeriensis* under the analytical conditions described in Section 2.

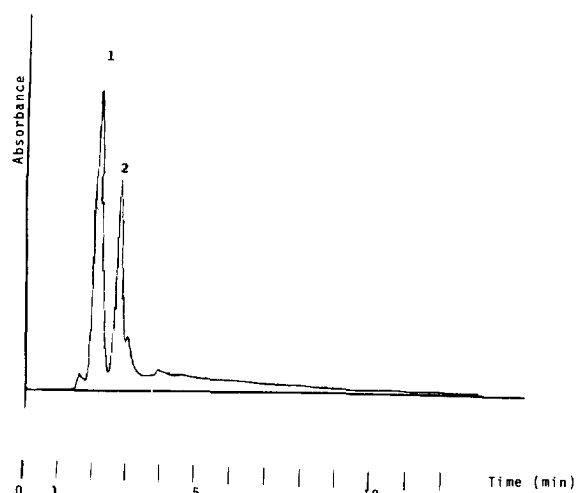

Fig. 3. Chromatogram of the EtOAc extract from *Sideritis luteola* under the analytical conditions described in Section 2.

allowed us to obtain a good resolution of the flavonoids using an easy and economic mobile phase and short analysis times (20 min), and appeared optimal to study the genus *Sideritis* and other vegetal extracts.

Our studies show that all species contained isoscutelarein-7-glucoside (excepting *Sideritis biflora*) and luteoline, the latter being the most abundant in every extract.

The flavonoid distribution in the ethyl acetate extract compared to the flavonoid percentages in the dry plant that were first reported for these species, allows us to divide them into two different groups. The first group includes species with a higher content of polar flavonoids: *S. foetens*, *S. luteola*, *S. leucantha var incana*, *S. cillensis*, *S. pusilla*, *S. glauca* and *S. incana ssp incana*; the second group, formed by *S. almeriensis*, *S. hirsuta*, *S. leucantha var serratifolia*, *S. biflora*, *S. leucantha ssp incana var meridionalis*, *S. bourgeana*, *S. pusilla ssp almeriensis*, *S. ibanyezii* and *S. pusilla ssp pusilla var granatensis*, shows, in addition to the most abundant polar flavonoids, an appreciable quantity of the apolar flavonoids.

The flavonoid distribution we detected in *S. almeriensis* Pau. and *S. pusilla ssp almeriensis* (Pau.) Malagarriga showed that their composition differs both qualitatively and quantitatively: *S. almeriensis* is poorer than *S. pusilla ssp almeriensis* in isoscutelarein-7-glucoside, but contains sideritoflavone, xantomicrol and gardenin D, which are not present in the second species. *S. almeriensis* lacks 8-methoxy-cirsilineol, a compound that is quite abundant in *S. pusilla ssp almeriensis*. Finally, the percentage of desmetilnobiletine is higher in *S. pusilla ssp almeriensis* than in *S. almeriensis*. All these facts demonstrate that both denominations are not synonymous, as reported in the literature [15,20], and reclassification is necessary.

Our last objective is to justify the popular use of these species because of their richness in anti-inflammatory flavonoids. The decoction of the plant allows the extraction of the most polar flavonoids such as isoscutelarin-7-glucoside and hypolaetin-8-glucoside, which are well documented for their anti inflammatory and anti-ulcer activity, and luteoline, which shows vasodilatatory activity [21]. Species containing high proportions of these flavonoids will show the strongest activity, and justify the popular use of *S. foetens*, *S. hirsuta*, *S. leucantha var serratifolia*, *S. bourgeana*, *S. ibanyezii* and *S. pusilla*; moreover, we can propose *S. biflora*, *S. leucantha var meridionalis*, *S. pusilla ssp pusilla var granatensis* and *S. cillensis* for the same use.

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