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Analysis and retention behaviour in high-performance liquid chromatography of terpenic plant constituents (*Sideritis* spp.) with pharmacological interest

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Abstract

Terpenoids are natural products with an important pharmacological interest, which are present in a number of medicinal plants. The species of *Sideritis* genus are valuable due to their high content in those compounds and they have been used in the Mediterranean area in folk medicine as anti-inflammatory and anti-ulcer agents. The present study describes a gradient elution reversed-phase method that uses diode array detection to determine ten pharmacologically active diterpenoids occurring in 12 species of *Sideritis*. First, we studied the chromatographic behaviour of standard diterpenoids to analyse the variation on retention time and the chromatographic properties with the mobile phase. Standard calibration curves were generated by plotting the area of peaks against a concentration range of the compounds. Second, the validated method was applied to the analyses of hexanic and methanolic extracts from 12 species of *Sideritis*, which were collected from different areas of Spain. Finally, we established for this plant a relationship between their use in folk medicine and their diterpenoid content.

Keywords: Diterpenoids; Retention behaviour; *Sideritis* spp.; HPLC; Anti-inflammatory; Folk medicine

1. Introduction

Natural product research has lately undergone exponential growth owing to advances in isolation techniques and synthetic method design, as well as the finding of a wide range of biological properties exhibited by these compounds. Terpenoids are natural products with an important pharmacological interest, which are present in many medicinal plants. Diterpenoids are a large and ubiquitous family of isoprenoid products; most of them found in recent years have been isolated from the Compositae and Lamiaceae families. The species of genus *Sideritis* (Lamiaceae) are valuable for their high content in these compounds. This genus comprises about 140 species distributed in several countries of the Mediterranean region.

Several biological actions have been reported for diterpenes, including anti-bacterial, anti-fungal, anti-inflammatory, anti-leishmanial, cytotoxic and anti-tumour [1–8]. Plants from the *Sideritis* genus have been extensively used in Spanish traditional medicine for their anti-inflammatory and gastroprotective properties. Several natural products showing anti-inflammatory activity have been isolated from plants of this genus and they mainly include flavonoides [9–12] and terpenoids [1,13]. The frequent hybridisations between different species make their botanical determination in subspecies and varieties difficult, and thus, a reclassification according to the chemical composition of the plants is very necessary.

In the present study, diterpenoids components (serradiol, linearol, conchitriol, foliol, isofoliol, andalusol, lagascatriol, tobarrol, sidol and siderol) were determined by using a reversed-phase liquid chromatography. Detection was performed by using a photodiode array detector. First, we stud-

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ied the chromatographic behaviour of standard compounds to analyse the variation on retention times and the chromatographic properties with the mobile phase. The validated method was applied to the analyses of hexanic and methanolic extracts from different *Sideritis* spp., which were collected from different areas of Spain.

The method permitted the identification and quantification of the diterpenoids above mentioned in 12 species of the genus *Sideritis*, by direct injection, without any prior purification of the extracts. The separation by isocratic elution in HPLC was optimised with the aim of achieving well-resolved peaks and finding a relationship between their content of terpenoids and their use in folk medicine as anti-inflammatory and anti-ulcer agents.

Taking into account the considerations mentioned above, the aim of this work is the development of a HPLC method that allows not only the simultaneous determination of diterpenoids, contributing to their chemotaxonomic determination, but also, identification of the anti-inflammatory agents in order to establish a relationship between the plant use in folk medicine and their content of terpenoids.

2. Experimental

2.1. Reagents and standards

Methanol of HPLC quality was purchased from Scharlau (Barcelona, Spain). High-purity water was obtained by a Millipore system.

Solvents were filtered using 0.20 μm membrane filters (47 mm) (Millipore, Milford, MA, USA) and samples were filtered using 0.20 μm membrane filters (13 mm, Millipore, Milford, MA, USA). All solutions were degassed prior to use.

The diterpenoid standards (serradiol, linearol, conchitriol, foliol, isofoliol, andalusol, lagascatriol, tobarrol, sidol and siderol) were isolated from different species of the genus *Sideritis* [14–17] and were kindly provided by Dr. B. Rodriguez (CSIC, Madrid. Spain).

Standard solutions of diterpenoids were prepared in methanol. Fresh working standard solutions were made daily by appropriate dilutions of standards in methanol.

2.2. Plant material

Sideritis spp.: *S. foetens*, *S. luteola*, *S. almeriensis*, *S. hirsuta*, *S. leucantha* var. *serratifolia*, *S. biflora*, *S. leucantha* spp. *incana* var. *meridionalis*, *S. bourgeana*, *S. pusilla* ssp. *almeriensis*, *S. ibanyezii*, *S. pusilla* ssp. *pusilla* var. *granatensis*, *S. leucantha* var. *incana*, *S. cillensis*, *S. pusilla*, *S. glauca*, *S. incana* ssp. *incana* were collected at the flowering stage in the eastern Andalucia, Guadalajara and Levante (Spain). A voucher specimen of each species was deposited in the Herbarium of the Botany Department (MAF), Faculty of Pharmacy, Universidad Complutense de Madrid, Spain. Plant material was left to dry at room temperature.

2.3. Extraction

Plant extracts were performed using a maceration and percolation extraction process at room temperature. Solvents of different polarities, *n*-hexane (3 ml × 150 ml, 72 h) and methanol (3 ml × 150 ml, 72 h) were used. The extracts obtained were concentrated to dryness below 35 °C.

2.4. Apparatus: equipment

The analyses were carried out in a Varian liquid chromatograph equipped with two pumps (Model 2510), an automated gradient controller (Varian model 2584), an injector (Rheodyne) with a 10 μl loop and a photodiode array detector (Varian Polychrom 9065), operating at λ = 220 nm. The analytical column was a Hypersil ODS (15 cm × 4.6 mm i.d.; 5 μm particle diameter) (Shandon Science; Astmoon, UK). The column was kept at room temperature.

2.4.1. Chromatographic conditions

The mobile phase was a mixture of water/methanol (30:70, v/v). The flow-rate was 1 ml/min. The injection volume was 10 μl and the detector was set at 220 nm.

2.4.2. Standard solutions and calibration graphs

Stock solutions of an accurately weighed amount of compounds were made in methanol and stored in darkness at 4 °C. Dilutions of different concentrations were made from those solutions.

Calibration graphs were obtained using six mixtures with all the standards at different concentrations. All samples were prepared and injected in triplicate.

2.4.3. Evaluation of peak purity and linearity

To check the peak purity, the eluates were monitored with a photodiode array detector (λ = 190–300 nm). The three spectra corresponding to the upslope, apex and downslope of each peak were computer normalised and superimposed. Peaks were considered pure when there was an exact coincidence among the three spectra (match factor ≥99.5).

The linearity of the detector responses for the prepared standards was assessed by means of a linear regression analysis regarding to the amounts of each standard (measures in μg) introduced in the loop of the chromatographic system and the area of the corresponding peak on the chromatogram.

3. Results

As the compounds have a different polarity, the mobile phase composition was first optimised to achieve an adequate separation of 10 diterpenoids in the same chromatogram and later the analysis of 12 *Sideritis* species was carried out.

The retention was influenced by the amount of methanol in the mobile phase, which produced changes in the column selectivity (Table 1), mainly in the case of the most polar

Table 1
Capacity factors and selectivity for the different compounds analysed

Diterpene	Capacity factors and selectivity for each mobile phase percentage (water/methanol)											
	20:80		25:75		30:70		32:68		35:65		40:60	
	K'	α	K'	α	K'	A	K'	α	K'	α	K'	α
Serradiol	0.02	1	0.04	1	0.27	1	0.45	1	0.54	1	0.64	1
Linearol	0.42	10.50	0.71	17.75	4.11	15.22	4.56	10.13	6.26	11.59	9.02	14.09
Conchitriol	0.55	1.30	0.89	1.25	5.13	1.24	5.43	1.19	8.72	1.39	11.67	1.29
Foliol	0.62	1.12	1.02	1.14	5.37	1.04	6.00	1.10	8.87	1.02	12.36	1.06
Isofoliol	0.64	1.03	1.03	1.01	5.68	1.05	6.08	1.01	9.64	1.09	14.19	1.15
Andalusol	0.70	1.09	1.05	1.02	6.48	1.14	6.86	1.12	14.64	1.52	18.19	1.28
Lagascatriol	0.73	1.04	1.20	1.14	7.29	1.12	7.87	1.14	16.15	1.10	19.83	1.09
Tobarrol	1.20	1.64	2.02	1.68	15.74	2.15	18.13	2.30	34.01	2.10	43.79	2.21
Sidol	1.54	1.28	2.50	1.23	19.30	1.23	24.79	1.37	43.17	1.27	53.51	1.22
Siderol	2.58	1.67	4.21	1.68	23.67	1.23	34.06	1.37	83.93	1.94	91.61	1.71

Table 2
Variation in the retention time of terpenic compounds according to the percentage of methanol added to water in mobile phase

Diterpene	t_r (min)					
	20:80[a]	25:75[a]	30:70[a]	32:68[a]	35:65[a]	40:60[a]
Serradiol	1.24	1.35	1.65	1.88	2.00	2.13
Linearol	1.85	2.22	6.65	7.23	9.44	13.02
Conchitriol	2.01	2.46	7.97	8.36	12.63	16.46
Foliol	2.11	2.62	8.28	9.10	12.83	17.37
Isofoliol	2.14	2.64	8.69	9.21	13.83	19.74
Andalusol	2.21	2.66	9.72	10.22	16.81	24.94
Lagascatriol	2.25	2.86	10.7	11.54	22.29	27.08
Tobarrol	2.87	3.93	21.76	24.87	45.52	58.23
Sidol	3.30	4.55	26.38	33.53	57.42	70.86
Sideral	4.66	6.78	32.07	45.57	110.40	120.40

[a] Mobile phase percentage (water/methanol).

compounds. For this reason, it was considered that one of the mobile phase components should be water. To select its percentage, we tested solutions in which the proportion of water varied between 20 and 60%. It can be seen that as the water proportion increases, an increase in the retention, different for each compound, is produced.

As a consequence of this phenomenon, it could be deduced that water percentages over than 20% did not enhance the separation, whereas those proportions higher than 50% caused a high retention worsening the resolution, therefore, 30% of water was finally chosen.

The mobile phase composition that allowed the best separation was water/methanol (30:70) and, therefore it was finally selected. The retention times achieved on a mixture of standards is shown in Table 2 and the chromatogram is shown

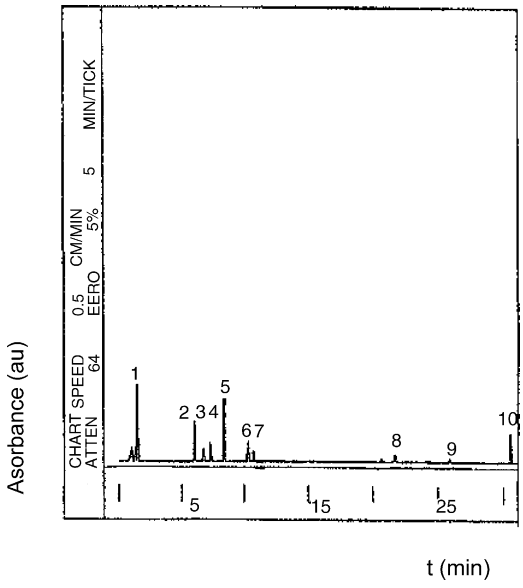

Fig. 1. Chromatogram of a standard solution. Peaks: (1) serradiol; (2) linearol; (3) conchitriol; (4) foliol; (5) isofoliol; (6) andalusol; (7) lagascatriol; (8) tobarrol; (9) sidol; (10) siderol.

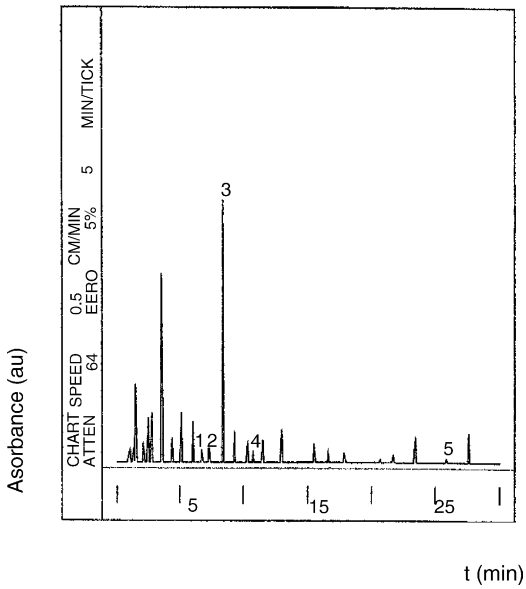

Fig. 2. Chromatogram of hexane extract of *Sideritis almeriensis* obtained using the following conditions: Column: Hypersil ODS 5 μm (15 cm × 0.46 cm); mobile phase: water/methanol (30:70); flow-rate: 1 ml/min; detection: UV, λ = 220 nm. Peaks: (1) linearol; (2) conchitriol; (3) isofoliol; (4) lagascatriol; (5) sidol.

Table 3
Diterpenoids content in the methanolic extracts, expressed as a percentage of dry plant mass

Sideritis	Methanolic extract									
	Serradiol	Linearol	Conchitriol	Foliol	Isofoliol	Andalusol	Lagascatriol	Tobarrol	Sidol	Siderol
S. almerienses		0.001	0.005		0.001		0.002		0.001	0.001
S. biflora		0.001	0.003	0.001						
S. bourgeana	0.058	0.002		0.001	0.001	0.009	Trazas			
S. cillensis	0.001	0.003				0.009				0.001
S. incana ssp. *incana*										
S. leucantha var. *incana*		0.001	0.001		0.001			0.002		
S. leucantha var. *serratifolia*					0.001					
S. leucantha ssp. *incana* var. *meridionales*		0.002		0.001	0.001			0.001		0.002
S. luteola	0.003					0.019	0.001			
S. pusilla (spike)								0.001		0.001
S. pusilla (stem)										
S. pusilla ssp. *almerienses*		0.002				0.012				
S. pusilla ssp. *pusilla* var. *granatensis*				0.001				0.001		

in Fig. 1. The retention times were highly reproducible among chromatograms, and the R.S.D.s obtained for a mixture of standards in six consecutive run, were lower than 1% for all the compounds.

3.1. Validation of the chromatographic method

Once the chromatographic conditions had been selected the method was validated paying attention to the linearity, accuracy, precision, selectivity, quantitation and stability of standards and samples.

3.2. Analyses of Sideritis spp.

We found that better separation conditions were reached when water/methanol (30:70) was used as the mobile phase under isocratic conditions. Fig. 2 shows an HPLC chromatogram obtained from a crude extract of *Sideritis almeriensis* monitored at 220 nm. Determination of ten diterpenoids was carried out in 35 min.

Once the chromatographic conditions for the separation had been set, the procedure was applied to the determination of terpenoid components in the hexanic and methanolic extracts from *Sideritis* spp. collected in different places of Spain. These studies were carried out using photodiode array detection. The identification of the different compounds was achieved by comparison of both retention time and the absorption spectra obtained for each eluted peak with those obtained for the standards.

The concentrations of the components were calculated from the chromatogram peak areas and the results are summarized in Tables 3 and 4.

4. Discussion

The plant kingdom is a potential source of terpenoids that act as anti-inflammatory drugs, a vast number of terpenoids have been evaluated as potential anti-inflammatory molecules in vivo animal models and ex vivo cultures of cells compromised in the inflammatory response [18–21].

Terpenoids generally occur as complex mixtures, which can be extracted from the natural source by a great variety of methods. Care must be taken during the extraction, and

Table 4
Diterpenoids content in the hexane extracts, expressed as a percentage of dry plant mass

Sideritis	Hexane extract									
	Serradiol	Linearol	Conchitriol	Foliol	Isofoliol	Andalusol	Lagascatriol	Tobarrol	Sidol	Siderol
S. almerienses		0.001	0.001		0.002				0.002	0.001
S. biflora	0.003	0.001	0.002	0.030	0.001	0.030	0.001		0.003	
S. bourgeana	0.004			0.004	0.003	0.026				
S. cillensis	0.002			0.008			0.001		0.002	0.001
S. incana ssp. *incana*	0.004						0.001			
S. leucantha var. *incana*		0.002	0.004		0.002		0.002			
S. leucantha var. *serratifolia*	0.007				0.001				0.001	0.005
S. leucantha ssp. *incana* var. *meridionales*		0.002	0.004		0.001	0.033			0.002	
S. luteola	0.005				0.002					
S. pusilla (spike)								0.001		
S. pusilla (stem)					0.005	0.032				
S. pusilla ssp. *almerienses*	0.007	0.002		0.004		0.030			0.002	0.001
S. pusilla ssp. *pusilla* var. *granatensis*		0.001	0.004	0.004						

also during the isolation process to avoid the occurrence of artefacts. HPLC chromatography is often found to be the most adequate method for isolation of the pure individual constituents in adequate amounts [22–29].

It is of great importance to develop of new methods for the chemical analysis of crude extract and discovering new molecules present in them. With this aim we developed a method for the analysis of terpenic constituents from *Sideritis* spp. by HPLC using suitable (water/methanol with isocratic elution) and economic mobile phase and short analysis times, and appearing optimal to the study of the genus *Sideritis*.

The proposed method allowed the quantitation of foliol, serradiol, linearol, conchitriol, isofoliol, andalusol, lagascatriol, tobarrol, sidol and siderol in 12 *Sideritis* spp. Applied it to several species of the genus *Sideritis* we could determine important differences among them in their composition. There was a direct relation for the species studied between the diterpenoid content and their use in folk medicine, for instance, andalusol, diterpenoid which possess an important anti-inflammatory activity [19] is the main compound in the species which have been used for their anti-inflammatory properties, *S. biflora*, *S. bourgeana*, *S. cillensis*, *S. leucantha* ssp. *incana* var. *meridionales*, *S. luteola* and *S. pusilla* spp. *almeriensis*.

Besides its important as active compound diterpenoid content, maybe is useful as a chemotaxonomic marker, which is very valuable when applying it to the genus *Sideritis*. Previous studies have shown that two different species of *Sideritis* (*S. almerienses* Pau and *S. pusilla* (Lage) Pau spp. *almeriensis* (Pau) H.T. Malagarriga) very near botanically, possessed different flavonoids content [9]. In the present work we corroborated that finding by determining different diterpenoids content (Tables 2 and 3).

References

[1] A. Navarro, B. de Las Heras, A. Villar, Planta Med. 66 (2000) 289.
[2] B. de las Heras, B. Rodríguez, L. Boscá, A.M. Villar, Curr. Top. Med. Chem. 3 (2003) 171.
[3] R.M. Diaz, A.A. Garcia-Granados, E. Moreno, A. Parra, J. Quevedo-Sarmiento, A. Saenz de Buruaga, J.M. Saenz de Buruaga, Planta Med. 54 (1988) 301.
[4] E. Scio, A. Ribeiro, T.M.A. Alves, A.J. Romanha, J.D. de Souza Filho, G.A. Cordell, C.L. Zani, Phytochemistry 64 (2003) 1125.
[5] G.E. Feresin, A. Tapia, A. Gimenez, A. Ravelo, S. Zacchino, M. Sortino, G. Schmeda-Hirschmann, J. Ethnopharmacol. 89 (2003) 73.
[6] S. Habtemariam, BMC Pharmacol. 3 (2003) 6 (Electronic resource).
[7] N. Tan, M. Kaloga, O.A. Radtke, A.F. Kiderlen, S. Oksüz, A. Ulubelen, H. Kolodziej, Phytochemistry 61 (2002) 881.
[8] C. Pessoa, E.R. Silveira, T.L. Lemos, L.A. Wetmore, M.O. Moraes, A. Leyva, Phytother. Res. 14 (2000) 187.
[9] O.M. Palomino, P. Gomez-Serranillos, E. Carretero, A. Villar, J. Chromatogr. A 731 (1996) 103.
[10] M. Hernández-Pérez, R.M. Rabanal, J. Ethnopharmacol. 81 (2002) 43.
[11] J.L. Rios, M.C. Recio, S. Mañez, R.M. Giner, Natural triterpenoids as antinflammatory agents, in: A. ur-Rahman (Ed.), Natural Proucts Chemistry. Part C. Bioactive Natural Products, vol. 22, Elsevier, Amsterdam, 2000, p. 93.
[12] F.A. Tomas-Barberan, M. Rejdali, J.B. Harborne, V.H. Heywood, Phytochemistry 27 (1988) 165.
[13] E.A. Aboutabl, M.I. Nassar, F.M. Elsakhawy, Y.A. Maklad, A.F. Osman, E.A.M. El-Khrisy, J. Ethnopharmacol. 82 (2002) 177.
[14] T.G. de Quesada, B. Rodríguez, S. Valverde, Tetrahedron Lett. 22 (1972) 2187.
[15] T.G. de Quesada, B. Rodríguez, S. Valverde, Phytochemistry 14 (1975) 517.
[16] M.A. López, C. Von Carstenn-Lichterfelde, B. Rodríguez, J. Org. Chem. 42 (14) (1977) 2517.
[17] F. Martín Panizo, B. Rodríguez, S. Valverde, An. Quim. 70 (1974) 164.
[18] B. de Las Heras, J.M. Vivas, A. Villar, Planta Med. 56 (1990) 58.
[19] B. de Las Heras, A. Navarro, M.J. Diaz-Guerra, P. Bermejo, A. Castrillo, L. Bosca, A. Villar, Br. J. Pharmacol. 128 (2000) 605.
[20] A. Godoy, B. De Las Heras, J.M. Vivas, A. Villar, Biol. Pharm. Bull. 23 (2000) 1193.
[21] C. Villena, J.M. Vivas, A.M. Villar, J. Ethnopharmacol. 71 (2000) 301.
[22] K. Hostettman, A. Marston, M. Hostettman, Preparative chromatographic techniques, in: Applications in Natural Products Isolation, second ed., Springer–Verlag, Berlin, Heidelberg, 1998.
[23] B. Avula, R.B. Manyam, E. Bedir, I.A. Khan, Die Pharmazie. 58 (2003) 494.
[24] B.H. Oliveira, A.E.G. Sant'Ana, D.Z.L. Bastos, Phytochem. Anal. 13 (2002) 368.
[25] N. Kolb, J.L. Herrera, D.J. Ferreyra, R.F. Uliana, J. Agric. Food Chem. 49 (2001) 4538.
[26] G. Vogg, S. Achatz, A. Kettrup, H. Sandermann Jr., J. Chromatogr. A 855 (1999) 563.
[27] T. Cserháti, E. Forgács, J. Chromatogr. A 936 (2001) 119.
[28] F. Yang, Y. Ito, J. Chromatogr. A 923 (2001) 281.
[29] L. Suntornsuk, J. Pharm. Biom. Anal. 27 (5) (2002) 679.