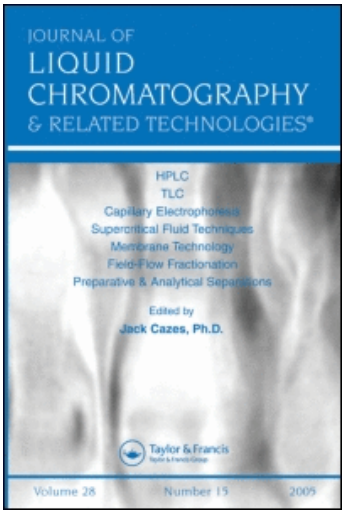

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CHROMATOGRAPHIC DETECTION OF SESQUITERPENE LACTONES IN PARTHENIUM PLANTS FROM NORTHWEST ARGENTINA

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ABSTRACT

Several chloroformic extracts containing some sesquiterpene lactones from autochtonous plants of *Parthenium hysterophorus*, collected in the province of Salta (Argentina), were separated and identified by high performance reversed-phase liquid chromatography with C_{18} column and propanol-water mobile phases, using gradient elution and concentrations of propanol larger than 10% (v/v). Lactones previously purified and characterized by H^1 RMN, C^{13} RMN, IR, and mass spectrometry, were used as standards.

The objective of the study was to check if the nature of the lactones was modified according to the altitude of the region where the plants were grown. Among the lactones, five were already described: coronopilin, hymenin, hysterin, parthenin, and tetraneurin A. *p*-Methoxybenzoic acid and two new ambrosanolides were also detected. Tetraneurin A and hymenin, on the one hand, and parthenin and coronopilin, on the other, were better resolved with a mobile phase of 6% propanol, run during 45 min.

INTRODUCTION

Parthenium hysterophorus L. is a species of the genus *Parthenium*, classified in the tribe Heliantheae from the Asteraceae family. Being native of America, it is found in central and northern Argentina and, nowadays, extended and naturalized in moderate and warm climates all over the world.[1] In 1950, Rollins[2] described a new species for the genus *Parthenium* found only in northern Argentina, *P. glomeratum* Rollins, which is characterized by the smaller size of the plant and by its tightly agglomerated capitula. Out of the diameter of the capitula, somewhat larger in the new Rollins species, all the other characteristics bring the existence of the two different species into question. According to Cabrera,[3] "*P. glomeratum* is similar to *P. hysterophorus*, being probably only an alpine species".

P. hysterophorus contents diverse allergenic sesquiterpene lactones, as shown by chemical, phytochemical and biological analysis.[4] Picman et al.[5] classified the plants collected in several continents in seven types, according to the lactones they found:

Type I	:	Parthenin, coronopilin and tetraneurin A
Type II	:	Parthenin, coronopilin
Type III	:	Coronopilin
Type IV	:	Hymenin, coronopilin and dihydrohymenin
Type V	:	Hymenin, coronopilin and hysterin
Type VI	:	Hymenin and hysterin
Type VII	:	Hymenin

Parthenin is considered to be the major allergen of the species. Some differences have been observed in the production of secondary metabolites between *P. hysterophorus* and *P. glomeratum*. Among other lactones, in the low regions for the former, hymenin has been detected, and in higher regions for the latter, parthenin is found. If both autochtonous and independent species really exist, it is difficult to establish following morphological and chemical criteria, the transition between them, that is, when *P. hysterophorus* disappears and from which point on the plant corresponds to *P. glomeratum*.

Chemical studies of plants collected in the same location, and in different maturing phases, have shown that only the relative percentage of lactones is modified, the nature of the isolated metabolic products being the same.

Sesquiterpene lactones are used as antiinflammatories, analgesics, antiexcematouses, and antifeverish drugs, and for the cure of herpes and rheumatism, among other diseases. However, their manipulation can produce in humans a contact dermatitis, especially in males, due to the presence of the compounds in the tricom of the vegetal, in a biological mechanism of defense.

In the literature, some reports have appeared on the separation of sesquiterpene lactones by gas chromatography and HPLC. Acetonitrile-water and methanol-water mixtures at different ratios[6-9] have been used, with isocratic and gradient elution, C_{18} reversed-phase columns, and UV-visible and mass spectrometry detection.[9]

In this work, the separation and identification by HPLC of some sesquiterpene lactones, obtained from autocthonous plants grown in diverse locations, in the region of Salta (Argentina), are shown. The sampling region comprised 72 km along road no. 33 (Salta Province), a region from Salta City (1220 m above sea level, a.s.l.) to Camino Mina Don Otto (3400 m a.s.l.), and Aguaray (400 m a.s.l.) and Tonco (2600 m a.s.l.). The purpose of the study was to confirm the presence of several sesquiterpene lactones, previously characterized by other instrumental techniques. The variation in the nature of the lactones, depending on the altitude of the region where the plants were collected, was studied.

Five previously described lactones were considered: coronopilin, hymenin, hysterin, parthenin and tetraneurin A (Figure 1). Two new ambrosanolides: 4α-O-acetyl-pseudoguaian-6β-olide (A1) and 1α-hydroxy-4β-O-acetyl-pseudoguaian-6β,12-olide (A2), corresponding to new natural products, found in Salta, were also studied, together with *p*-methoxybenzoic acid.

EXPERIMENTAL

Reagents

Standard solutions (100 μg/mL) of the compounds (coronopilin, hymenin, hysterin, parthenin, tetraneurin A, *p*-methoxybenzoic acid, 1α-hydroxy-4β-O-acetyl-pseudoguaian-6β,12-olide and 4α-O-acetyl-pseudoguaian-6β-olide) were prepared by dissolving 0.001 g in 1 mL of methanol and adding 10 mL of water, with the aid of an ultrasonic bath (TEST LAB, Model TB04, Bernal Oeste, Buenos Aires, Argentina).

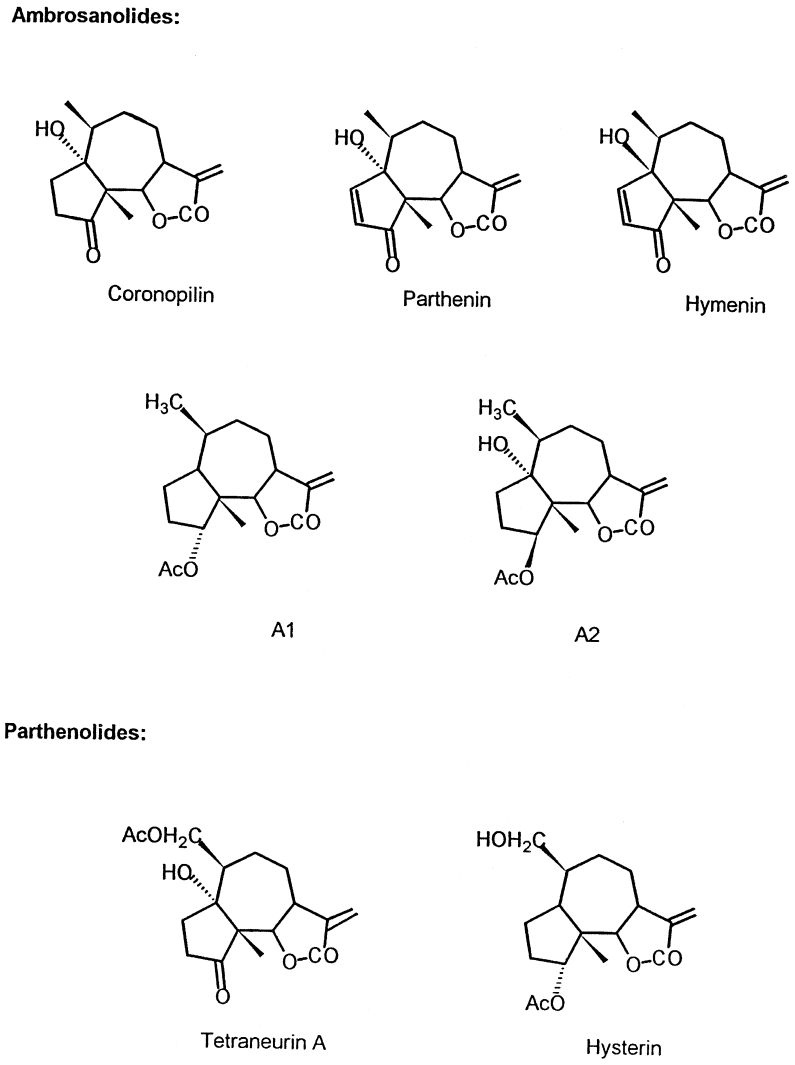

Figure 1. Structures of the sesquiterpene lactones.

The lactones used as standards were obtained from several plants, and characterized after purification of the chloroformic plant extracts through preparative columns (see below),[4] by ^{1}H NMR, ^{13}C NMR, IR, and mass spectrometry. The plant lots were collected along the years 1994 and 1995, and identified by Ing. Lázaro Novara. Voucher specimens are deposited at the Museo of the Facultad de Ciencias Naturales at the Universidad Nacional de Salta.

General Extraction Procedure

Samples of air dried above-ground parts were exhaustively extracted with $CHCl_3$ (Merck, Buenos Aires, Argentina). The residue obtained after evaporation of the solvent was dissolved in hot ethanol (Sorialxo, Buenos Aires, Argentina) and a solution of 4% lead acetate (Cicarelli, Santa Fe, Argentina) was added. After standing overnight the precipitate was filtered, the organic solvent evaporated, and the aqueous solution extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 (Cicarelli) and the solvent evaporated under reduced pressure to yield a gummy residue. All solvents were purified by fractionated distillation.

Isolation and Purification

The residues obtained as described above were fractionated by column chromatography, eluted with benzene (Cicarelli), and the polarity increased with ethyl acetate (Cicarelli) and acetone. Further purification of the fractions was achieved by dry column chromatography (coronopilin, hysterin, tetraneurin A, 1α-hydroxy-4β-O-acetyl-pseudoguaian-6β,12-olide and 4α-O-acetyl-pseudoguaian-6β-olide) and on Sephadex LH-20 (Sigma, St. Louis MO, USA) (hymenin and parthenin).

Chromatographic Analysis

For the chromatographic analysis of the lactones, 0.5 g of the plant extracts was weighed and dissolved in methanol (Merck), to a final volume of 10 mL. Methanol and 1-propanol (Dorwill, Buenos Aires, Argentina), used in the mobile phases, and the sesquiterpene lactone solutions were filtered through 0.2 μm and 0.45 μm Nylon membranes (Sartorius, Goettingen, Germany), respectively. 2-Propanol (Dorwill) was used to clean the chromatographic system. The location of the plants analyzed, their previously determined composition, and the composition found by HPLC in this work, are given in Table 1.

Apparatus

An Isco (Lincoln, Nebraska, USA) liquid chromatograph, provided with a ternary pump (model 2350), a Rheodyne valve (Cotati, CA, USA), including 20 and 50 μL loops, an elution gradient controller (model 2360), and a UV-visible detector (model 918, GBC Scientific Equipment, Dandenong, Victoria, Australia), with detection at 210 nm (wavelength near the maxima of the compounds), were used.

Table 1

Location of the Plant Extracts and Composition as Determined by ^{1}H NMR, ^{13}C NMR, IR and Mass Spectrometry (Previous Characterization) and by HPLC

Extract	Altitude[a]	Previous Characterization	HPLC Identification
Aguaray[b]	400	Hymenin and coronopilin	Hymenin, coronopilin, A2 and other compounds
Salta City[c]	1220	Hymenin, coronopilin and hysterin	Hymenin, coronopilin, hysterinin and A1 or p-methoxybenzoic acid
El Infiernillo[d]	1620	Terraneurin A and coronopilin	Tetraneurin A, coronopilin A1, A1 or p-methoxybenzoic acid and another compound
El Infiernillo[e]			Tetraneurin A, coronopilin, A2 and another compound
San Martin[e]	2500	Tetraneurin A, coronopilin, parthenin and p-methoxybenzoic acid	Tetraneurin A, coronopilin, parthenin, hysterin, A2 and other compounds
Tonco[e]	2600	No previous study	Tetraneurin A, coronopilin, parthenin, hysterin, A2, A1 or p-methoxybenzoic acid and other compounds
Valle Encantado[d]	3000	Coronopilin and p-methoxybenzoic acid	Tetraneurin A, coronopilin, parthenin, A2 and other compounds
Valle Encantado[c]			Tetraneurin A, coronopilin parthenin, A2, A1 or p-methoxybenzoic acid and other compounds
Mina Don Otto[c]	3400	Coronopilin, hysterin, A2, A1 and p-methoxybenzoic acid	Coronopilin, hysterin, A2 and other compounds

[a] m. a.s.1 - above sea level, [b] July 1994, [c] March, 1995, [d] February 1994, [e] December 1995.

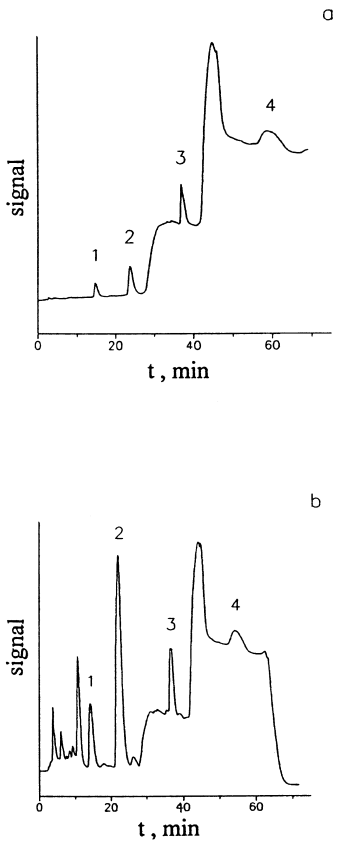

Figure 2. Chromatograms of: a) a mixture of standards of 100 µg/mL coronopilin (1), 100 µg/mL hysterin (2), 300 µg/mL A2 (3), and 140 µg/mL A1 (4), and b) a raw extract from Mina Don Otto, both dissolved in 1:9 methanol/water. Gradient elution was made.

A Nucleosil C_{18} column (5 µm particle size, 250 mm x 4 mm I.D.) (Macherey-Nagel, Oensingen, Switzerland), and a 3-cm precolumn of similar characteristics, were employed. The flow-rate was 1 mL/min. Data acquisition was made through the GBC 918 software.

The dead time (measured in each chromatogram from the first significant deviation of the base-line), the capacity factors, efficiencies and asymmetry factors, were obtained with an MS-DOS program called MICHROM, which was developed by Dr. José Ramón Torres.[10]

RESULTS AND DISCUSSION

Optimization of Mobile Phase Composition

The sesquiterpene lactones were first eluted with mobile phases of methanol-water of diverse composition: 30:70, 40:60, and 50:50 (v/v). An increase in the amount of methanol produced a reduction of retention, together with a deterioration of the efficiencies and asymmetry factors. The best resolution was achieved with 30:70 methanol-water, but the retention times were excessive (coronopilin, 23.2 min; hymenin, 12.9 min; hysterin, 32.1 min; parthenin, 24.4; tetraneurin A, 14 min; A1 and A2, > 60 min).

Very asymmetrical peaks were obtained with methanol-water mobile phases of larger elution strength. It was, therefore, decided to change to a stronger solvent, such as propanol.

The peaks of tetraneurin A, coronopilin, and hysterin were sufficiently resolved with 10:90 propanol-water in less than 25 min, but A1 and A2 did not appear before 60 min. For these compounds, a larger amount of propanol was required. Finally, a stepwise gradient was designed: 10:90 propanol-water (0-20 min), 22:78 propanol-water (20-34 min), and 37:63 propanol-water (34-57 min). This permitted the separation of the mixture of compounds, with some exceptions, as commented below. The reequilibration of the column was made with 10:90 propanol-water during 15 min, before the next analyses.

The chromatograms of 1:9 methanol-water solutions of a mixture of coronopilin, hysterin, A1 and A2 standards, and of a raw extract from Mina Don Otto, are compared in Figure 2. Figure 3 shows the chromatograms of a methanolic solution of a plant extract from Tonco. Tetraneurin A (average retention time: 8.3±0.4 min), hymenin (8.2±0.4 min), coronopilin (13.9±0.7 min), parthenin (15.3±0.9 min), and hysterin (21.3±0.6 min), were eluted from different extracts in the first gradient step.

As observed, tetraneurin A and hymenin showed the same retention times and the peaks of coronopilin and parthenin partially overlapped with 10:90 propanol-water. The ambrosanolide A2 (36.6±0.7 min) appeared in the second gradient step, and an unresolved mixture of A1 (54±1 min) and *p*-methoxybenzoic acid was eluted in the third step.

Resolution of Tetraneurin A and Hymenin

Confirmation of the identity of peak 1 in Figure 3 (*i.e.*, tetraneurin A or hymenin) was not possible using a 10:90 propanol-water eluent. Although both

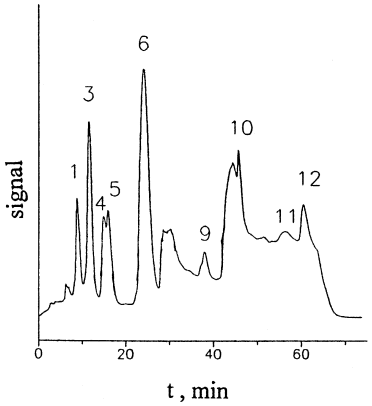

Figure 3. Chromatogram of an extract from Tonco dissolved in methanol and obtained with gradient elution. Peaks: tetraneurin A (1), coronopilin (4), parthenin (5), hysterin (6), A2 (9), A1 or p-methoxybenzoic acid (11), unknown compounds (3, 10 and 12).

lactones have not been found simultaneously in the same plant extract, their characterization requires an adequate separation. Therefore, a mobile phase of lower elution strength, containing 6% (v/v) propanol, was further used (Figure 4a). This permitted the identification of tetraneurin A and hymenin, in plant extracts from Tonco (Figure 4b) and Salta (Figure 4c), respectively. The identification of tetraneurin A could also be performed in the extracts from Valle Encantado and El Infiernillo.

Resolution of Coronopilin and Parthenin

The resolution of the peaks of coronopilin and parthenin, which are frequently present simultaneously in the same plant extracts, with 10:90 propanol-water, is sufficient to allow their identification, but the determination of the relative amount of these compounds would be difficult. Knowledge of this parameter is important, owing to the probable relationship with the altitude of the location of the plant. In order to analyze raw extracts with a high coronopilin/parthenin ratio, where the peak of parthenin can be completely overlapped, a study was performed where the amount of 1-propanol was decreased from 10 to 6% (v/v). Figure 5a-c shows the modification of the chromatogram of a mixture of coronopilin and parthenin, upon variation of the amount of alcohol. A mobile phase containing 6% propanol was again useful to increase peak resolution. Complete separation was not achieved, but it was enough for quantitation purposes.

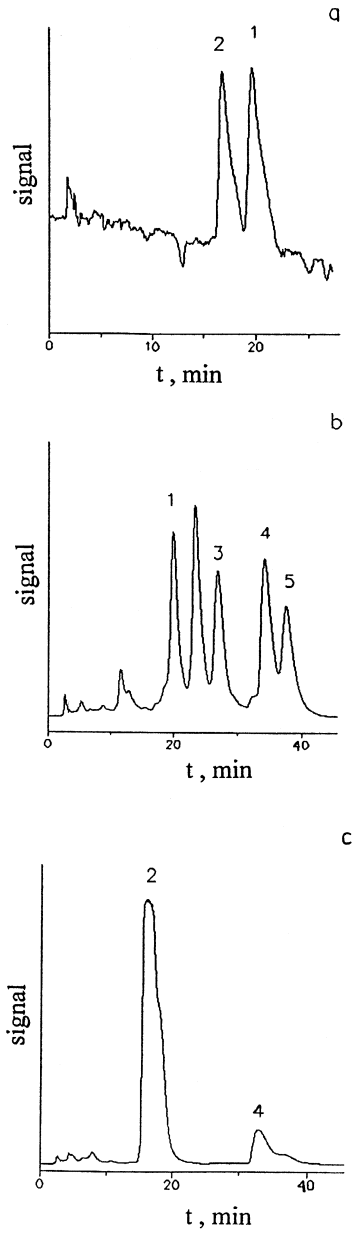

Figure 4. Chromatograms: a) a mixture of 50 μg/mL standard solutions of tetraneurin A and hymenin, b) extract from Tonco, c) extract from Salta, obtained with 6% (v/v) PrOH. tetraneurin A (1), hymenin (2), coronopilin (4), parthenin (5), unknown compound (3).

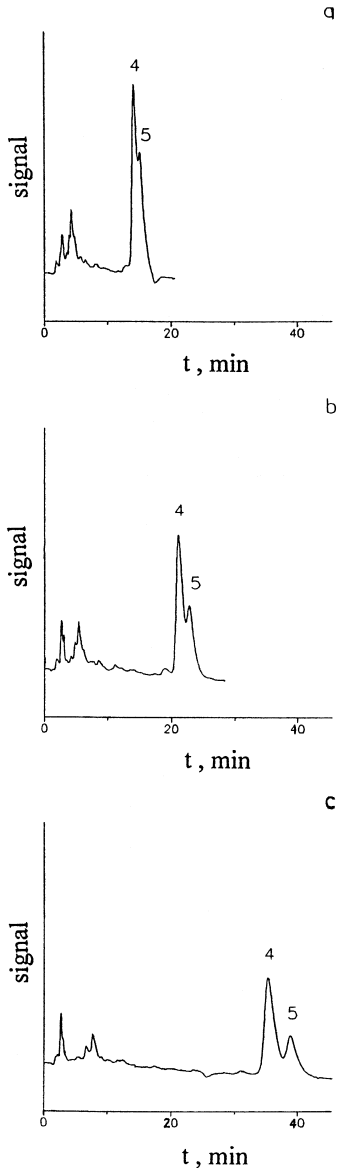

Figure 5. Chromatograms of a mixture of 50 µg/mL of both coronopilin (4) and parthenin (5) standards, dissolved in methanol and eluted with: a) 10%, b) 8%, and c) 6% propanol.

The use of a mobile phase of lower elution strength was not convenient, because of the long retention times of the compounds. Parthenin was confirmed in the extracts from Valle Encantado collected in december 95, but not in the samples collected in february 94. A clear identification of parthenin was also made in the extracts from Tonco (Figure 4b), whereas its absence was confirmed in the extracts from El Infiernillo.

CONCLUSIONS

The analysis of 24 extracts of different lots of *Parthenium* plants, collected in different months and altitudes, showed how the altitude is an important factor that affects the nature of sesquiterpene lactones. It was confirmed that the identity of the lactones can be made by using HPLC with mobile phases of propanol-water, which alternatively should be performed with ^{1}H NMR, ^{13}C NMR, IR and mass spectrometry. In some plants, the HPLC technique showed the presence of compounds undetected by these techniques, as occurred with tetraneurin A in the extract from Valle Encantado or hysterin in the extract from San Martín. Likewise, in diverse raw extracts, the presence of an unknown component, with a retention time similar to that of A1 and *p*-methoxybenzoic acid, was observed. As the compounds investigated covered a wide range of polarity, sesquiterpene lactones from other genera probably will be also separated following the proposed procedure.

As observed in Table 1, the composition of the plant extracts varied with the altitude. Hymenin was found between 400 and 1220 m, tetraneurin A between 1620 and 3000 m, parthenin at 2500-3000 m, and hysterin was usually found at the highest altitudes. Coronopilin appeared in all the extracts analyzed. The same occurred with A2, which however was not detected in the extracts from Salta.

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