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Packed column supercritical fluid chromatography of sesquiterpene lactones with different carbon skeletons

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Abstract

The analyses of eighteen different C-skeleton sesquiterpene lactone (SL) standards and of extracts of *Cardus benedictus* L. and *Artemisia umbelliformis* Lam. were carried out by SFC–UV. SLs of the groups of germacranolides, guaianolides, eudesmanolides, *seco*-eudesmanolides and helenanolides were investigated. Different stationary phases, different densities, several modifiers and modifier programmes were applied in order to optimize their separation. A CN stationary phase with MeOH–water (95:5) as modifier for supercritical CO₂ was the most successful combination in the separation of the SLs investigated. © 1997 Elsevier Science B.V.

Keywords: *Artemisia umbelliformis*; *Cardus benedictus*; Sesquiterpenes; Lactones

1. Introduction

Sesquiterpene lactones (SLs) are a class of secondary metabolites which are mainly found in several genera of Asteraceae [1], and in Umbelliferae [2], Magnoliaceae and Hepaticae. In general, SLs derive from the basic sesquiterpene carbon skeleton, in which one of the methyl groups of the isopropyl group is oxidized to the lactone group [2,3]. The SL group is known to contain over 500 compounds; they are of interest not only from chemical and chemotaxonomic standpoints, but also because many of them have different types of biological or therapeutic activity including antitumour, antileukaemic, cytotoxic, antimicrobial, antiphlogistic, spasmolytic, anthelmintic, sedative and anti-feeding [4–7].

These compounds, whose molecular masses gener-

ally range from 230 to 500, are similar in polarity; their volatility is low and many are thermolabile.

Reversed-phase high-performance liquid chromatography (RP–HPLC) is still the analytical technique of choice for SL analysis in crude plant extracts.

A previous article on HPLC coupled through a particle beam interface to mass spectrometry [in both the electron impact (EI) and chemical ionization (CI) modes] showed it to be successful in detecting and identifying twenty-two different C-skeleton SL standards analysed in four different groups and of two plant extracts, without preliminary isolation or chemical treatments [10].

Packed column supercritical fluid chromatography (SFC) has recently stimulated interest mainly thanks to the introduction of variable and programmable restrictors, on-line flow-rate controllers and high pressure cells for UV detectors, which have afforded reliable quali-quantitative results. Packed column SFC has a sample capacity similar to HPLC, oper-

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ates with the same columns, and in general requires shorter analysis times because of the higher theoretical plate number per unit time [8,9].

As part of a project aiming to evaluate packed column SFC–UV in the analysis of biologically active components in plant extracts [11,12], this article reports on the analyses of nineteen different C-skeleton SL standards and of two plant extracts by SFC–UV. The lactones investigated belong to the groups of the germacranolides, guaianolides, eudesmanolides, seco-eudesmanolides and helenanolides; Fig. 1 reports their structures, names, and molecular masses.

2. Experimental

2.1. Reference standards and plant material

Standard samples of SLs were supplied and/or isolated from several species belonging to different genera [10] by G. Appendino, Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin (Italy).

Artemisia umbelliformis Lam. was kindly supplied by L. Poggio, Giardino Botanico Alpino, Cogne (Aosta, Italy); voucher specimens are deposited in their herbarium collection. *Cardus benedictus* L. was supplied by Ulrich SpA (Turin, Italy).

2.2. Sample preparation

SL extracts from *Cardus benedictus* L. and from *Artemisia umbelliformis* Lam. for SFC–UV analyses were prepared with the methods previously reported for HPLC–MS analyses [10].

Cardus benedictus – 10 g of dried plant material was macerated overnight in MeOH (200 ml). The extract was evaporated to dryness and the residue treated with EtOH (20 ml) and 20 ml of a 3% (w/v) lead acetate solution and left for 1 h. After filtration and evaporation of the EtOH, the residue was diluted with water (20 ml) and extracted with CHCl₃ (3×25 ml). The CHCl₃ extract was then concentrated under vacuum and redissolved in an amount of solvent suitable to obtain a 2 mg ml⁻¹ solution, and analysed by SFC–UV.

Artemisia umbelliformis Lam. – Dried plant ma-

terial (10 g) was macerated overnight in MeOH (250 ml) and processed as reported above for *C. benedictus*. The extract, dissolved in CHCl₃, was analysed by SFC–UV.

2.3. SFC–UV analysis

Packed column SFC analyses were carried out on a Gilson SF3 SFC–UV system (Middleton, WI, USA) provided with a Gilson 160 diode array UV detection (DAD) system or a UV 119 multiwavelength detector, equipped with a high pressure cell. Separations were carried out with the following columns: Hibar pre-packed column LiChrospher 100 RP-18 (C₁₈) (5 μm, 250×4 mm) from Merck (Darmstadt, Germany); Hibar pre-packed column Lichrosorb RP-8 (C₈) (10 μm, 250×4 mm) from Merck; Hibar pre-packed column LiChrosorb Diol (Diol) (5 μm, 250×4 mm) from Merck; S3W Silica Spherisorb (Sil) (3 μm, 150×4.6 mm) from Phase Separations (Deeside, UK); S3-Nitrile Spherisorb (CN) (3 μm, 150×4.6 mm) from Phase Separations.

2.3.1. Analysis conditions

Analysis was done on a CN column using supercritical CO₂ modified with MeOH–water (95:5) as mobile phase; flow-rate was 2.5 ml min⁻¹; UV detection wavelength: 254 nm.

SLs were located and identified in both standard and plant SFC–UV patterns by comparison of their retention times and UV spectra, obtained by DAD, and quantitated at 254 nm, with authentic samples. Standard mixtures of SLs, varying from 0.5 to 5 μg μl⁻¹ in respect of their UV molar absorptivity were prepared.

3. Results and discussion

One of the main aims of this study was to find suitable stationary phases, analysis conditions and modifiers to analyse as many lactones as possible. Therefore columns packed with RP-18, RP-8, Diol, CN and Sil stationary phases were tested under different CO₂ pressures (isobaric or programmed), modifiers [MeOH, MeOH–water, EtOH, iso-PrOH, CHCl₃, acetonitrile (ACN), isocratic or gradient],

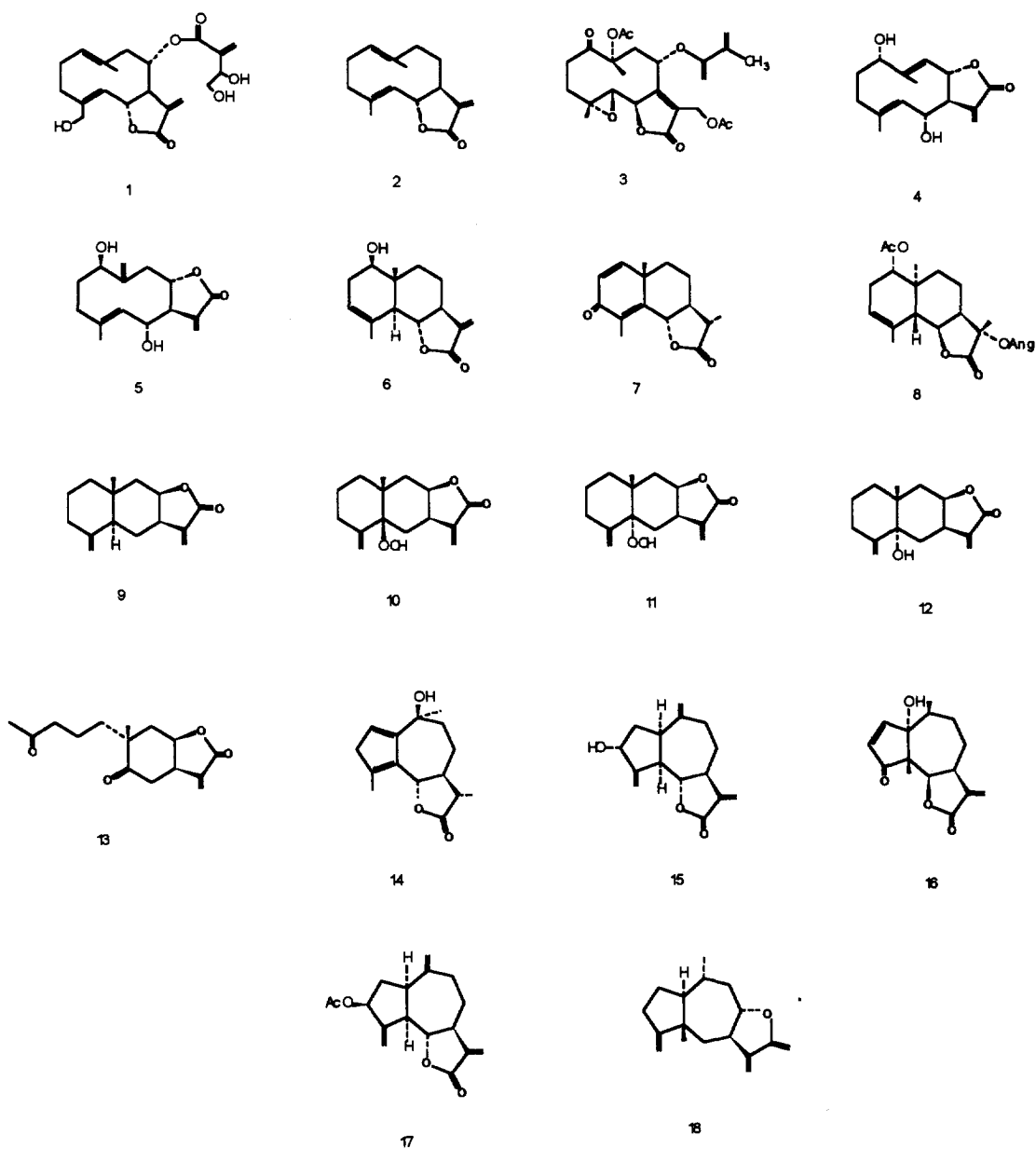


Fig. 1. Structures, names and molecular masses of the SLs under investigation: (1) cnicin (378), (2) costunolide (232), (3) glaucolide A (464), (4) tatrindine A (264), (5) tatrindine B (264), (6) santamarine (248), (7) α -santonine (246), (8) isosilerolide (418), (9) isoallantolactone (232), (10) 5-deoxy-5-hydroperoxy-5-epitelekinone (AU1) (264), (11) 5-deoxy-5-hydroperoxy-5-telekinone (AU2) (264), (12) telekinone (248), (13) umbellifolide (264), (14) artabsine (248), (15) epizaluzanine C (246), (16) parthenine (262), (17) zaluzanine D (288), (18) graveolide (248).

flow-rates (1 to 3 ml min⁻¹) and temperatures (40°C to 60°C).

Slightly polar stationary phases (C₁₈, C₈) were

initially tested; however the SLs that eluted were not separated at all. It was then decided to try some more polar phases (Diol, CN, Sil): the most interesting

results were obtained with a chromatographic system consisting of a CN column using CO₂ modified with MeOH–water (95:5) as eluent. With this stationary phase in combination with a suitable mobile phase composition, good separations of eighteen SLs, analysed in two sub-groups according to vegetable origin, skeleton and polarity, were obtained. The two sub-groups were analysed with CO₂ at two different pressures; the less polar SLs were separated at low pressure, while the more polar ones required high pressure. Moreover, some water had to be added to the mobile phase to reduce the activity of the column and to improve SL separation. CHCl₃ was chosen as sample solvent for SL SFC analysis, because it is well soluble in CO₂ and this avoids the analytes under investigation suffering band broadening or, worse, peak splitting [13]. The first standard mixture (ST1) contained costunolide (2), isosilerolide (8), zaluzanine D (17), graveolide (18), artabsine (14), santamarine (6), and glaucolide A (3) and was analysed with CO₂ at 14 MPa and 50°C using MeOH–water (95:5) as modifier; a modifier gradient from 0 to 10% at 0.25% per min was applied. Fig. 2 reports the SFC–UV pattern of ST1. The second standard mixture (ST2) contained isoallantolactone (9), telekine (12), umbellifolide (13), α-santonine (7), epizaluzanine C (15), tatrindine A (4), tatrindine B (5), parthenine (16), and cnicin (1) and was analysed with CO₂ at 20 MPa and 50°C using MeOH–water (95:5) as modifier; a modifier gradient from 0 to 10% at 0.5% per min was applied. Fig. 3 reports the SFC–UV pattern of ST2. AU1 (10) and AU2 (11) were not included in these standard mixtures, being only available in very small amounts.

For both standard mixtures, the injected amounts varied from 0.5 to 5 μg μl⁻¹. At the selected UV wavelength, detection limits varied from 10 to 150 ng μl⁻¹ in respect of the molar absorptivity of each SL. A threshold of 10 was taken for the signal-to-noise ratio. For obvious reasons, only SLs with absorbing chromophoric groups other than the lactone carbonyl in their structure were analysed. For those SLs which are not UV absorbing, other detection systems, e.g. evaporative light scattering detection (ELSD) or mass spectrometry (MS), must, of course, be used. ELSD seems to be very effective for SFC, because although not as sensitive as UV, its response is not affected by the analyte structure (it is a universal detector) and its cost is reasonable

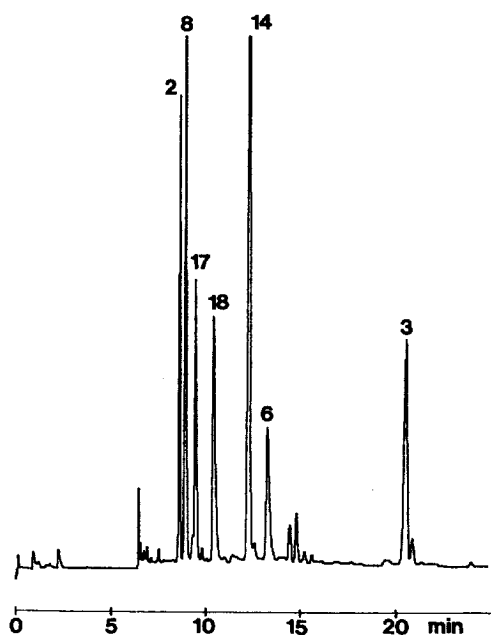


Fig. 2. SFC–UV pattern of ST1 SL standard mixture. Analysis conditions: pressure: 14 MPa, temperature: 50°C; modifier: MeOH–water (95:5), programme: from 0 to 10% at 0.25% per min.

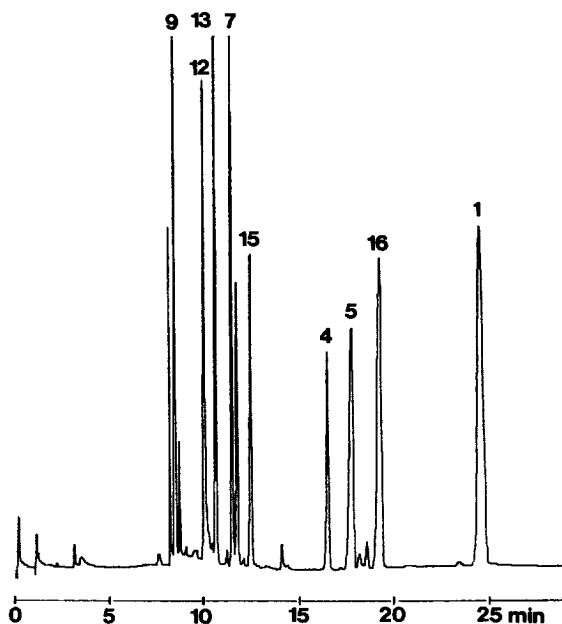


Fig. 3. SFC–UV pattern of ST2 SL standard mixture. Analysis conditions: pressure: 20 MPa, temperature: 50°C; modifier: MeOH–water (95:5), programme: from 0 to 10% at 0.5% per min.

[14,15]. ELSD has recently been used with success as an SFC detector to analyse vegetable extracts [16], and in particular for quantitative determination of artemisinin, an SL from *Artemisia annua* that is highly active against several chloroquine-resistant malaria plasmodia [17].

The SL extracts from *Cardus benedictus* L. and *Artemisia umbelliformis* Lam. were also analysed. *C. benedictus* mainly contains cnicin and is widely used in folk-medicine as cholagogue and anti-inflammatory agent [18]; *A. umbelliformis* contains AU1 (10), AU2 (11), and umbellifolide (13), among others, and is an important ingredient in several typical alpine liqueurs because of its aromatic and SL-related bitter properties [19,20].

Cnicin (1) was the only SL detected in *C. benedictus* extract, which was analysed under different SFC–UV conditions to shorten analysis time, as were the pure SL standards. Fig. 4 reports the SFC–UV pattern of a *C. benedictus* extract carried out at 30 MPa with the MeOH–water (95:5) modifier programmed from 3% to 10% at a rate of 1% per min.

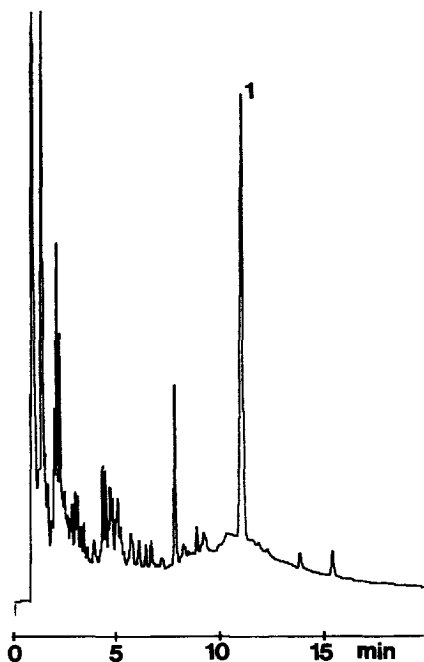


Fig. 4. SFC–UV pattern of a *C. benedictus* SL extract. 1: Cnicin; analysis conditions: pressure: 30 MPa, temperature: 50°C; modifier: MeOH–water (95:5), programme: from 3 to 10% at 1% per min.

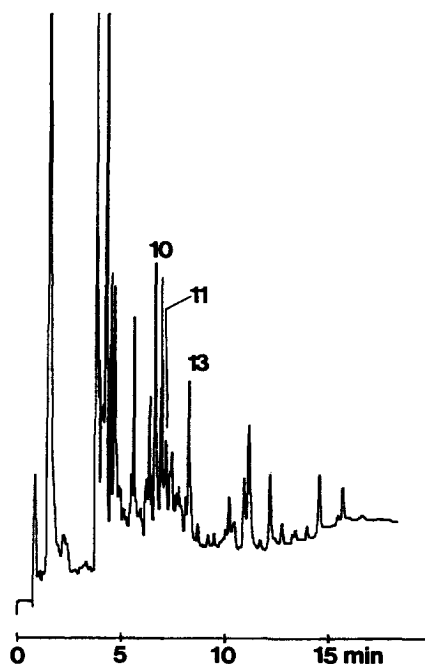


Fig. 5. SFC–UV pattern of the *Artemisia umbelliformis* SL extract. 10: AU1, 11: AU2, 13: Umbellifolide; analysis conditions: pressure: 20 MPa, temperature: 50°C; modifier: MeOH–water (95:5), programme: from 0 to 10% at 0.5% per min.

AU1 (10), AU2 (11), and umbellifolide (13) were detected in the *A. umbelliformis* extract; Fig. 5 reports the SFC–UV pattern of the *Artemisia umbelliformis* extract carried out at 20 MPa; the MeOH–water (95:5) modifier was programmed from 0 to 10% at 0.5% per min. It is interesting to note that umbellifolide and AU1 reverse their elution order when analysed at different pressures, without varying modifier composition or mobile phase flow-rate: at 20 MPa, AU1 elutes before umbellifolide, while at 30 MPa, the contrary occurs.

The SFC patterns of the SL extracts had fewer peaks in the first part of the chromatogram than the RP–HPLC patterns, because with SFC, the polar stationary phase retained the polar components, which are eluted in the first part of RP–HPLC chromatogram. Nevertheless, and very importantly, in comparison to HPLC, SFC allowed more consistent column conditions during repeated analyses. It generally happens that, in routine HPLC of SL extracts, column pressure increases after about twenty injections; the column must then be washed with a

series of solvents of decreasing polarity and the frits must be cleaned, procedures that SFC does not require.

In conclusion, SFC–UV, using a polar stationary phase (CN) and CO₂ modified with MeOH–water as eluent, was successful as a technique for the analysis of different skeleton SLs; the method described can be considered of general use since most of the common SL skeletons are included in the eighteen analysed SL standards. Separation was achieved by dividing the SL standards into two sub-groups according to their skeleton and polarity, and separating them with supercritical CO₂ at two different pressures. Pressure and modifier programmes were chosen to minimize matrix interferences in the analysis of plant extracts.

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