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Note

Packed column supercritical-fluid chromatography and linked supercritical-fluid chromatography–mass spectrometry for the analysis of phytoecdysteroids from *Silene nutans* and *Silene otites*

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The ecdysteroids are polar, polyhydroxylated steroids which are both involatile and thermally unstable, and thus unsuitable for gas–liquid chromatography unless derivatised, as for example, by trimethylsilylation of some, or all, of the hydroxyl groups. Currently, high-performance liquid chromatography (HPLC) methods are generally used for their analysis[1]. Two recent studies have shown them to be suitable candidates for supercritical-fluid chromatography (SFC) with packed columns [2,3]. It also proved to be possible to perform linked SFC–mass spectrometry (MS) for these compounds[3]. We wish to demonstrate the advantages of SFC methods in the examination of ecdysteroids and describe here its application to the screening of plants for phytoecdysteroids (*i.e*, plant-derived ecdysteroids) by using extracts of two plants of the genus *Silene, S. nutans* and *S. otites* (Caryophyllaceae). The presence of ecdysteroids in plants is of particular interest because, although the ecdysteroids are best known for their involvement in athropod development (particurlarly moulting in insects), plants frequently provide a much wider source, both in terms of quantity and variety of structural types. These phytoecdysteroids therefore are an invaluable source of material for the study of ecdysteroid metabolism in insects and other athropods. The greater chromatographic efficiency of SFC compared with HPLC[4], and the greater compatibility of SFC mobile phases with linked chromatography–mass spectrometry, make SFC an attractive technique for the rapid screening of plant extracts for ecdysteroids, as demonstrated in this paper.

MATERIALS AND METHODS

Ecdysteroid standards were gifts from a number of sources or had been isolated and fully characterised using NMR and MS (structures are shown in Fig.1). Air dried

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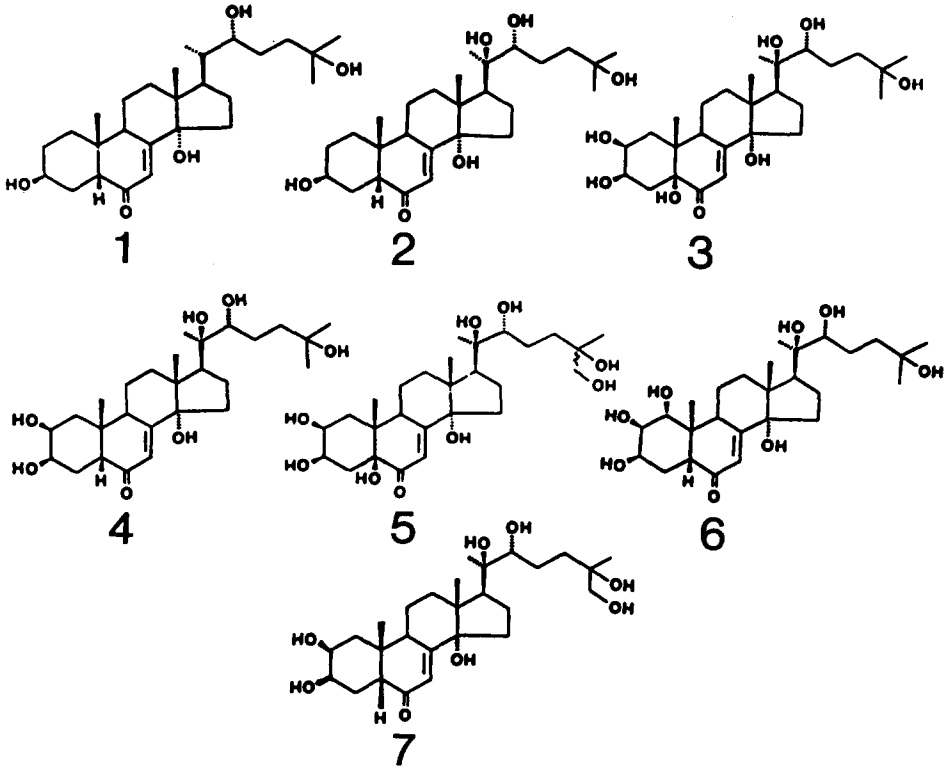

Fig. 1. Structures of the ecdysteroids present in the plant extracts described: 1 = 2-deoxyecdysone; 2 = 2-deoxy-20-hydroxyecdysone; 3 = polypodine B; 4 = 20-hydroxyecdysone; 5 = 26-hydroxypolypodine B; 6 = integristerone A; 7 = 20,26-dihydroxyecdysone.

stems and leaves of *S. nutans* and *S. otites* were extracted with methanol, solid material was removed by filtration and the extract concentrated. The further precipitate which formed was removed by centrifugation, and the filtrate was partitioned between hexane and methanol–water (85:15) (to remove neutral lipids), followed by dilution of a methanol solution with acetone to precipitate phospholipids.

HPLC separation was achieved with a Zorbax-SIL column (25 cm × 4.6 mm I.D.) using dichloromethane–isopropanol–water (125:40:3) as moving phase at 1 ml min^{-1} with detection at 254 nm.

Initial studies were performed on 5 μm cyanopropyl Spherisorb (Phase Separations, Queensferry, U.K.) packed in stainless-steel columns (25 cm × 4.6 mm I.D.) using a mobile phase provided from a cylinder of carbon dioxide containing 10% methanol. The mobile phase was delivered using a modified Varian 8500 syringe pump (Varian, Walton-on-Thames, U.K.), operating at 290 atm, approximately 3 ml min^{-1} whilst the column itself was heated, at 60°C, using a Dupont Model 860 column oven. Detection of ecdysteroids was at 235 nm using a CE 2112 variable-wavelength detector (Cecil Instruments, Cambridge, U.K.) fitted with an 8-μl high pressure stainless-steel flow cell. Samples were dissolved in methanol for injection (5 μl) via a Rheodyne 7125 injector (Altech, Carnforth, U.K.). The pressure was main-

tained with a length of stainless-steel tubing (2 m × 0.006 in. I.D.) in a water bath at 40°C.

For SFC–MS, chromatography was performed on columns packed with 3 μm silica gel (2 cm × 4.6 mm I.D., Perkin Elmer, Beaconsfield, U.K.) using a mobile phase of carbon dioxide containing 22% of methanol. The mobile phase was delivered using a Hewlett-Packard 1046B HPLC system modified for SFC as described by Games *et. al.*[5] at a flow-rate of 4 ml min^{-1} (300 bar) and column temperature of 85°C, with UV detection at 235 nm.

A T-piece was inserted after the UV detector to deliver approximately half of the flow to a modified Finnigan MAT thermospray ion source, modified for SFC as described by Games and co-workers[5,6]. For these experiments the vapouriser and jet of the mass spectrometer was set at 95°C and 180°C respectively with the repeller voltage set at +10 V and a discharge voltage of 1700 V.

RESULTS AND DISCUSSION

In previous studies capillary and packed columns were examined[2] with supercritical carbon dioxide. This mobile phase proved unsuccessful with packed columns and only low polarity ecdysteroids could be eluted from a capillary column. In order to elute more polar ecdysteroids, carbon dioxide modified with methanol was used on either uncoated silica[3] or on cyanopropyl bonded silica[2] and gave excellent results with good peak shape and short analysis times. Initial studies, with normal-phase HPLC on the two plant extracts showed the *S. otites* extract to be rich in 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone and 20-hydroxyecdysone whilst the *S. nutans* extract contained large amounts of polypodine B, and 20-hydroxyecdysone together with smaller quantities of integristerone A, 20,26-dihydroxyecdysone and 26-hydroxypolypodine B (Fig. 2). HPLC analysis required analysis times of up to 45 min. SFC on cyanopropyl-bonded silica gel gave essentially the same pattern as

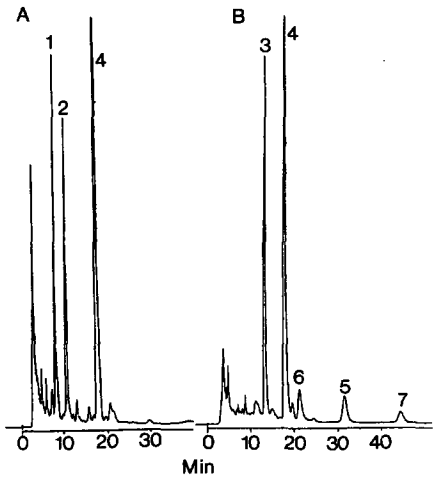

Fig. 2. Normal phase HPLC of ecdysteroid-containing plant extracts from (A) *Silene otites* and (B) *Silene nutans*. See Fig. 1 for peak identification.

normal phase HPLC. A minor exception was that 26-hydroxypolypodine B eluted before integristerone A on the cyano column in SFC and after it on silica gel in HPLC separation. A noteworthy feature was the much reduced analysis times, less than 2 min (Fig. 3A) attained with SFC with near baseline resolution for the ecdysteroids present in the *S. nutans* extract. Similarly, the results obtained for the *S. otites* extract (Fig. 3B) showed a large reduction in analysis time from over 40 min to under 3 min, albeit with the loss of resolution between the three minor components. Under the conditions described here the seven ecdysteroids studied had the following elution order and retention times: 2-deoxyecdysone, 1.4 min; 2-deoxy-20-hydroxyecdysone, 1.6 min; polypodine B, 1.7 min; 20-hydroxyecdysone, 1.9 min; 26-hydroxypolypodine B, 2.3 min; integristerone A, 2.5 min; 20,26-dihydroxyecdysone, 2.7 min. As we have observed before, there is no simple correlation between the number of a hydroxyl groups in ecdysteroids and elution order. In this SFC system, which has similar chromatographic elution order to normal phase HPLC, addition of a hydroxyl group in the 5β-position seems to reduce retention time compared to similar compounds with 5β-H. Sensitivity of detection was not examined here. Each injection contained approximately 25 μg of each major ecdysteroid. In earlier work[3] the limit of detection was found to be below 50 ng.

SFC on silica gel required both higher temperatures, and more methanol in the mobile phase. Nevertheless with such modification very short analysis times were possible. 20-Hydroxyecdysone eluted in 2.06 min using two linked 2-cm silica gel columns and in 1.15 min with a short 2-cm column. Mass spectra were obtained on the substances separated on the silica gel columns, as described below.

Supercritical-fluid chromatography linked to mass spectrometry

With the linked SFC–MS system described by Games *et al.*[5] spectra close to electron impact (EI) type are obtained with carbon dioxide, but in the presence of increasing amounts of methanol, spectra increasingly closer to chemical ionization

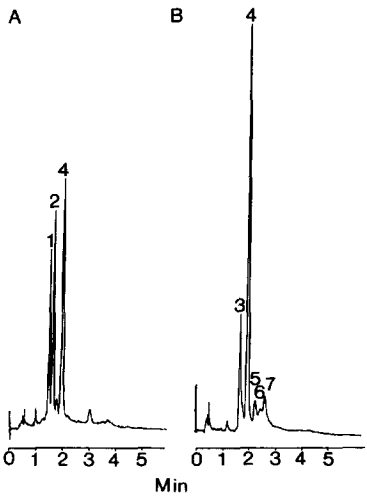

Fig. 3. SFC of ecdysteroids on 5 μm cyanopropyl bonded silica gel with carbon dioxide–methanol (9:1) as mobile phase at 3 ml min^{-1}, 60°C and 290 bar. (A) *S. nutans*, (B) *S. otites*. See Fig. 1 for peak identification.

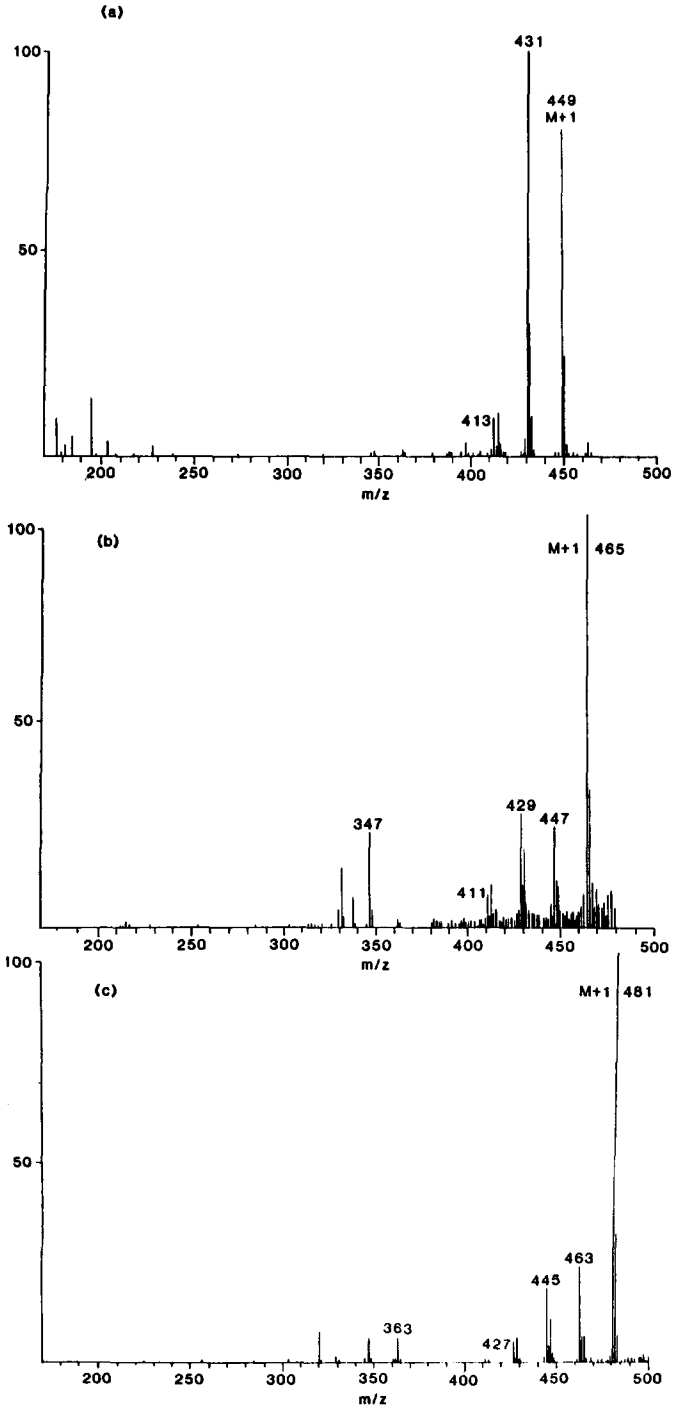

Fig. 4. Mass spectra of (a) 2-deoxyecdysone, (b) 2-deoxy-20-hydroxyecdysone and (c) 20-hydroxyecdysone from *S. otites* following SFC as described in Materials and Methods.

(CI) type are obtained. This has advantages and disadvantages. The advantage of greater sensitivity is obtained with the CI type spectra and they are useful for obtaining molecular masses, particularly with the ecdysteroids which tend to lose one, two, three or four molecules of water in the spectrometer. On the other hand, the CI spectra give little or no structural information to help identification.

From SFC–MS, spectra of the CI type (Fig. 4) were obtained for 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone and 20-hydroxyecdysone from the crude extract of *S. otites*. These spectra show prominent M + 1 ions, and losses of up to three molecules of water, the (M + 1) − 18 ion at *m/z* 431 being the base peak for 2-deoxyecdysone. Similar spectra were obtained for 20-hydroxyecdysone and polypodine B from *S. nutans*, with weak ions in the latter showing losses of four molecules of water.

In order to obtain more diagnostic fragments, the temperature of the block was raised to 260°C, which altered the spectra. That of 2-deoxyecdysone did not give many fragment ions, but in the case of 20-hydroxyecdysone (Fig. 5) and polypodine B characteristic fragment ions were obtained. All ecdysteroids with hydroxyl groups at C-20 and C-22 show a characteristic cleavage between these atoms to give prominent ions, with daughter ions at 18 and 36 a.m.u. less. Thus for 20-hydroxyecdysone the prominent ions are at *m/z* 363 ($C_{21}H_{34}O_5$), 345 and 327 from the nucleus and *m/z* 99 and 81 from the side chain. At the higher temperature *m/z* 363 became the base peak (Fig. 5) with 345 and 327 prominent. These spectra are clearly due to greater fragmentation and not pyrolysis. The result of pyrolysis of ecdysteroids is dehydration, with successive loss of the molecular ion, M − 18, M − 26, etc. The molecular ion is still clearly seen in Fig. 5 and is the strongest of that cluster of ions. The ion at *m/z* 363 arises only by fragmentation and not by dehydration. Similarly, 2-deoxy-20-hydroxyecdysone gave *m/z* 347 ($C_{21}H_{34}O_4$ base peak), 329 and 311 and polypodine B gave *m/z* 379 ($C_{21}H_{34}O_6$), 361 and 343. These fragment ions, due to cleavage between C-20 and C-22, together with the molecular ions are useful because they tell how many hydroxyl groups are in the nucleus and how many in the side chain, an impor-

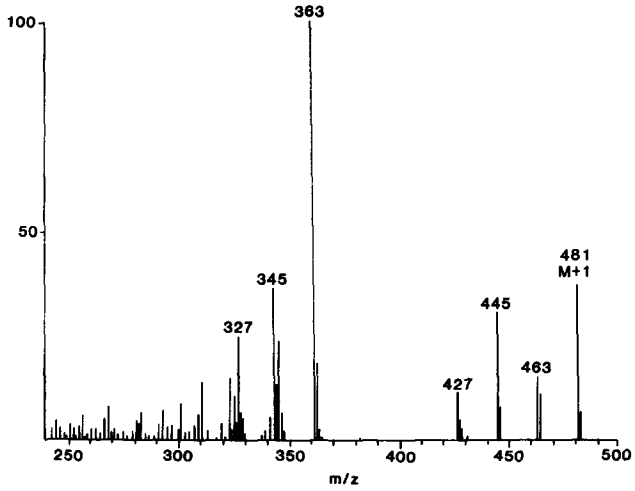

Fig. 5. SFC–MS of 20-hydroxyecdysone showing the increased fragmentation obtained with the jet block temperature set at 260°C.

tant early piece of information in identification of ecdysteroids. Spectra were not scanned to low masses because of the presence of cluster ions of carbon dioxide and methanol. We did not explore the limits of sensitivity. The spectra shown were obtained with microgram quantities.

Good quality spectra were not obtained from the minor components present in *S. nutans* extract, however this indicates the limits of the present equipment rather than the ultimate capabilities of the SFC–MS technique.

CONCLUSIONS

We have shown that supercritical-fluid chromatography can be used for very rapid separation of the polar sterols in ecdysteroid-containing plant extracts. Combined with mass spectrometry the technique can give both the molecular masses of these compounds and, by varying temperature, the characteristic fragmentation, so useful in electron impact mass spectrometry for compound identification.

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