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Note

Analysis of ecdysteroids by supercritical-fluid chromatography

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Growth in insects is an intermittent process, in which their outer skin is shed, the body expands and the new skin then hardens. This process is initiated and controlled, in part, by short periods of production of moulting hormones, known as ecdysteroids, which are polyhydroxy steroidal ketones with a 5β-cholesterol skeleton. There are about 20 ecdysteroids (zooecdysteroids) known in insects and other invertebrates, and together with those found in plants (phytoecdysteroids), some 100 such compounds are now known[1]. Their importance in insect development is self evident, and their identification and quantitation is a matter of great interest to insect physiologists and those looking for new ways to control insect pests.

Ecdysteroids are found in insect tissues and eggs in μg/g to pg/g quantities. High sensitivity of detection is sought. They possess neither the thermal stability nor the volatility required for gas chromatography (GC) though they can be converted to suitable derivatives by trimethylsilylation of some or all of the hydroxyl groups[2]. As little as 10 ng can then be determined by flame ionization detection (FID) or about 50 pg by electron-capture detection[3]. However, because of the many hydroxyl groups, it is difficult to convert them into single derivatives. Generally, investigators avoid GC methods and prefer the much less sensitive (limit of 100–50 ng) UV detection with high-performance liquid chromatography (HPLC), due to the strong chromophore (λ 242 nm, ε 10 000–14 000) of the α,β-unsaturated ketone. Not only is detection less sensitive but analysis times are longer, and the range of conditions is limited to solvent-programmed reversed-phase, and isocratic normal-phase chromatography.

Any method that is simple to operate and capable of increasing the sensitivity of detection even by ten-fold would be valuable and welcome. Supercritical-fluid chromatography (SFC) promises to give more sensitive detection and shorter analysis time. We describe here our preliminary investigations of the use of SFC in the analysis of ecdysteroids.

MATERIALS AND METHODS

Ecdysteroids were purchased from Simes (Milan, Italy) or were gifts from the collection of Dr. I. D. Wilson (University of Keele). The compounds were dissolved

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in methanol and injected into the system with a Rheodyne injection valve (Phase Separations, Queensferry, U.K.) and loop. Chromatography was carried out on a column (100 × 0.46 mm I.D.) containing 5-μm Hypersil (Shandon Southern Products, Runcorn, U.K.) with carbon dioxide–methanol (4:1) at a flow-rate of 4 ml/min and a pressure of 300 bar, and temperature of 80°C, unless otherwise stated. A Hewlett-Packard 1046B high-performance liquid chromatograph (Hewlett-Packard, Bracknell, U.K.) modified[4] for SFC was used. Detection was at 235 nm.

For mass spectrometry (MS), a T-piece was inserted after the UV detector to deliver approximately half the flow to a modified Finnigan MAT thermospray ion source (Finnigan MAT, San José, CA, U.S.A.), modified as described by Games *et al.*[5] for SFC–MS in the filament-on mode. The hypodermic tubing leading from the T-piece was crimped at the end to maintain SFC conditions until the effluent left the vaporiser[5].

RESULTS AND DISCUSSION

There are many recent reports on the use of SFC for the analysis of non-polar mixtures, but very little on its use for polar compounds, although its utility for polar compounds was demonstrated at an early stage in its development[6]. Supercritical carbon dioxide is a non-polar fluid, with an elution power between hexane and dichloromethane, depending upon pressure. Because the ecdysteroids are highly polar, SFC on capillary columns is unsatisfactory. A high concentration of polar solvents (usually methanol) is required to elute them, and this produces a large standing current in the flame ionization detector. Moreover, sample size and injection volume are limited with capillary columns and elution times are long. Packed column SFC provides faster analysis than HPLC, better chromatographic efficiency, and lower detection limits[7].

The very sharp peaks and short elution times in SFC of the two most important ecdysteroids are shown in Fig. 1. The extremely narrow peak shape, reminiscent of capillary GC peaks, improves detection limits. Inokosterone (Fig. 2) occurs naturally in the form of two isomers (*R* and *S* at C-25). These were separated, almost to the baseline by SFC, much better than in HPLC[8]. The separation of polypodine B and 20-hydroxyecdysone is regarded as a difficult problem in HPLC[9], but is readily achieved by SFC with packed columns (Fig. 3). The effect of lowering the temperature to increase retention and improve separation are also illustrated here.

Ecdysteroids are found frequently as esters of inorganic and organic acids. We have also examined 20-hydroxyecdysone 2-cinnamate as a representative of the organic group and observed that it has similar, slightly shorter retention under the same conditions.

A plot of peak area against amount injected was found to be linear for ecdysone over the range 10–500 ng. At present the detection limit is 10 ng, but as conditions have not yet been optimised, we expect that it can be lowered further.

Coupling a mass spectrometer to an SFC system is simpler than in HPLC, because of the lower volumes of mobile phase to be removed. The subject of SFC–MS has been reviewed[10], and some of the practical problems of operating with packed columns have recently been described[5]. Using a T-piece to deliver 50% of the effluent to the mass spectometer as described in Materials and Methods, spectra close

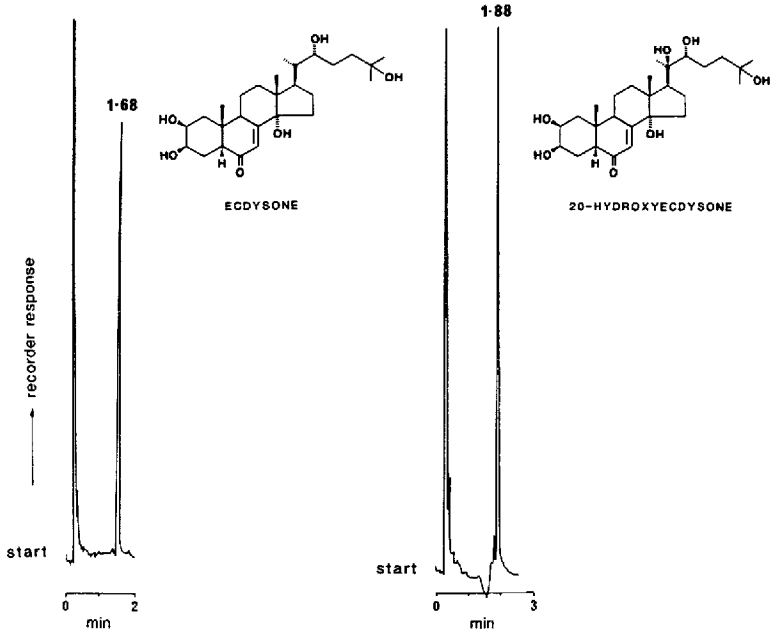

Fig. 1. Packed-column SFC chromatography of ecdysone and 20-hydroxyecdysone, on Hypersil with carbon dioxide–methanol as the mobile phase. For details see Materials and Methods.

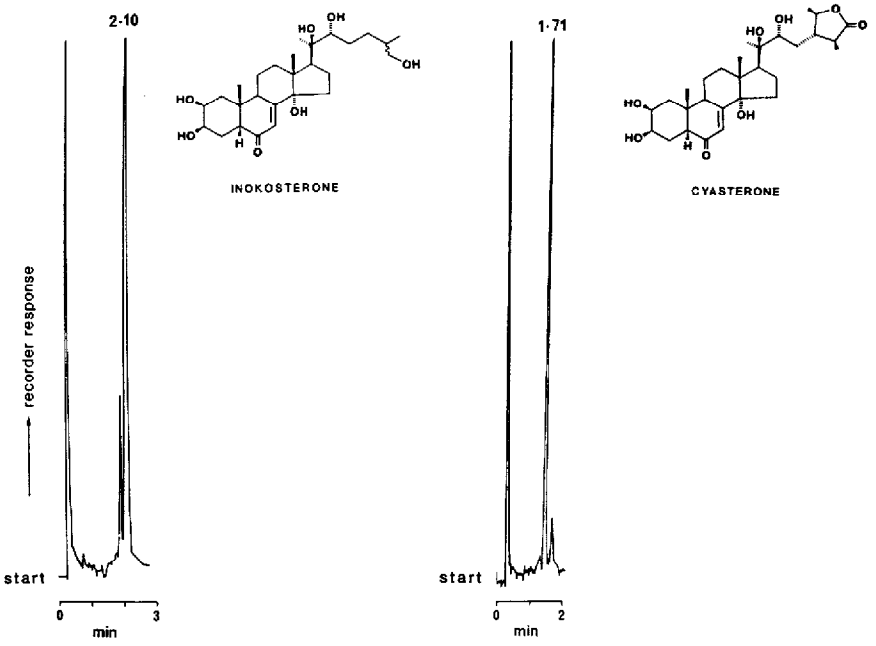

Fig. 2. Examples of SFC of two phytoecdysones: inokosterone, which consist of a mixture of 25-*R* and 25-*S* isomers, and cyasterone, which contains a side-chain lactone. Conditions as in Fig. 1.

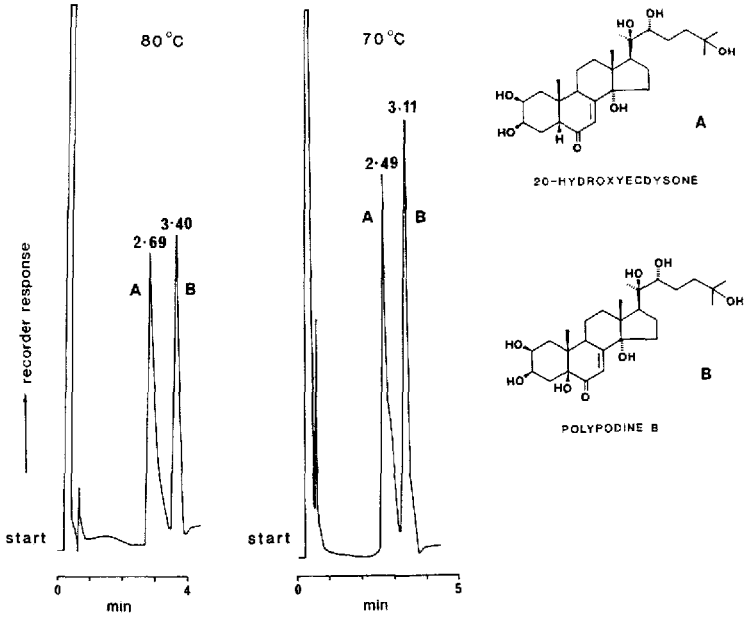

Fig. 3. Separation of polypodine B and 20-hydroxyecdysone on packed-column SFC. The two compounds differ in that the former has an extra 5β-OH group. The second tracing shows the effect of lowering the temperature to 70°C.

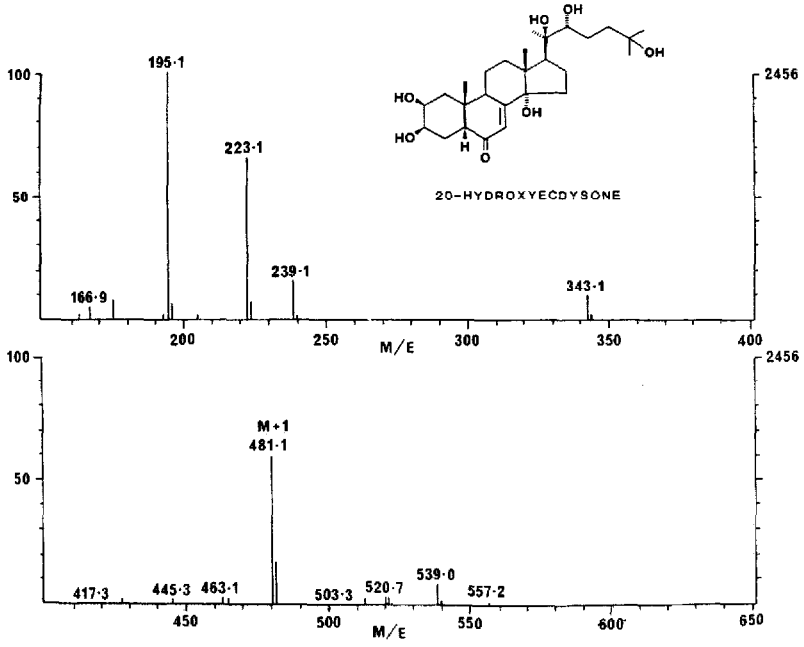

Fig. 4. Mass spectrum of 20-hydroxyecdysone, obtained under thermospray conditions by leading half of the SFC column effluent straight into the source of the spectrometer.

to the electron impact type are obtained with carbon dioxide, but in the presence of methanol spectra closer to the chemical ionization type are obtained, and sensitivity is improved.

In the spectrum of 20-hydroxyecdysone (Fig. 4) there is a prominent $[M + 1]^+$ ion, with minor fragment ions at m/z 463, 445, and 427, indicating successive loss of three molecules of water. Complete interpretation of this type of mass spectrum will require the analysis of a number of spectra of this group.

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