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Phytoecdysteroid analysis by high-performance liquid chromatography–thermospray mass spectrometry

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ABSTRACT

The potential of high-performance liquid chromatography–mass spectrometry with thermospray interface to identify and analyse ecdysteroids has been investigated. The response of eight different ecdysteroids and their acetonide derivatives is studied by positive- and negative-ion detection as well as with single-ion monitoring and scan mass detection modes. The usefulness of this technique for ecdysteroid identification and quantitation is discussed and, finally, the identification of a new phytoecdysteroid isolated from *Polypodium vulgare* is presented as an application of this technology. A fast and easy procedure for the extraction and purification of phytoecdysteroids is also described.

INTRODUCTION

Ecdysteroids are a family of compounds structurally related to the insect moulting hormones, ecdysone and 20-hydroxyecdysone. Briefly, they are highly hydroxylated 5β steroids with a 7-en-6-one group and are widely distributed in plants, insects and other Arthropod families as well as in other invertebrates [1]. In insects, they act as a signal to start the moulting process at the cellular level, but the whole regulation system, biosynthesis and transport are not well defined. More than 100 different ecdysteroids also have been isolated from plants (phytoecdysteroids) [2–4]. Although it is supposed that phytoecdysteroids could be a part of an integrated plant defence against insects, it is not clear if this is their only role [5]. On the other hand, it has been shown that ecdysteroids are able to participate in mammal biological processes [6] with a very low toxicity rate, which make these compounds interesting targets in the medical research field.

For several years, our group has been working with plant natural products, and recently we focused our attention on ecdysteroids. We are interested in compounds with anti-ecdysone activity in insects as well as in increasing ecdysteroid production in modified plants [7]. One of the main problems in ecdysteroid research is related to analysis owing to their presence in low amount in biological materials, their chromatographic behaviour (low number of theoretical plates per m of column) and their similar physical and chemical properties [8]. In order to resolve this problem we enhanced HPLC performance using 2-propanol–water as eluent at a temperature of 40–55°C [9], and later our objective was to improve the ecdysteroid identification techniques.

An ideal analytical method should be easy to implement, fast and sensitive. Mass spectrometry can approach that goal, especially when coupled with chromatographic methods. GC–MS is a well-known and easy technique, but previous reports in this field discouraged its use [10]. An

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easy and attractive idea is the combination TLC–fast atom bombardment MS [11], but TLC lacks the necessary resolution. On the other hand, we considered that HPLC–MS could combine the resolution and the specificity of these two techniques. For this purpose we have used a thermospray interface, which is one of the most widely known, because of its high sensitivity and good results in fragmentation of steroids [12] and other polar compounds. In this paper, we describe the application of HPLC–thermospray (TSP) MS to the identification and quantification of ecdysteroids.

MATERIALS AND METHODS

HPLC

A Waters (Milford, MA, USA) system with two 510 pumps, a 680 gradient controller, a Rheodyne 7125 injector (Cotati, CA, USA) and a lambda 530 UV detector was used. The HPLC column, Spheri-5-RP-18, 25 × 0.47 cm I.D., 5 μm particle size, was purchased from Brown-Lee Labs. (Sunnyvale, CA, USA).

MS system

A Model 5988A mass spectrometer with a thermospray probe and a Model 35741B data acquisition and treatment from Hewlett-Packard (Palo Alto, CA, USA) was used.

Chemicals and solvents

In all cases HPLC-grade solvents were used. Ammonium acetate and formate were obtained from Carlo Erba (Milan, Italy). All other reagents were obtained from usual vendors and used without further purification.

Phytoecdysteroid isolation

Samples, usually 50 mg, of lyophilized vegetable material were homogenized in a Braun Potter S homogenizer (Melsungen, Germany) for 1 min at 1100 rpm with 2 × 5 ml of methanol and 2 × 5 ml of methanol–water (7:3). The combined extracts were washed with hexane, 2 × 10 ml, centrifuged and the methanol phase was evaporated under vacuum. The residue was redissolved in 3 ml of methanol–water (1:9) and poured into a preconditioned C_{18} Sep-Pak cartridge (Millipore, Milford, MA, USA). The pure ecdysteroid fraction was obtained after washing the cartridge with 10 ml of methanol–water (1:4) and eluting them with 10 ml of methanol–water (7:3).

Acetonide preparation

Standard ecdysteroids. Pure ecdysteroids (10 mg) were dissolved in 1 ml of 2,2-dimethoxypropane containing 0.5 mg of anhydrous *p*-toluensulphonic acid. After 10 min, 1 ml of 5% aqueous sodium hydrogencarbonate was added and the mixture extracted with chloroform (3 × 1 ml). The combined organic extracts were washed with water (2 × 1 ml), evaporated with nitrogen and dried under vacuum. The acetonides were stored at −20°C in a dry box filled with argon and dissolved immediately before their use. All acetonides were fully characterized by spectroscopic techniques.

Phytoecdysteroids. A 2-ml volume of the above methanol–water (7:3) eluate was evaporated to dryness (content in ecdysteroids between 100 and 5 μg depending on the plant source) and treated as above.

HPLC–TSP-MS conditions

The optimum temperature conditions were as follows: ion source 280°C, vapour 250°C, tip 190°C and steam 108°C. In all cases "filament on" ionization mode (ionization by an electron beam) was selected. The ecdysteroid acetonides were eluted with acetonitrile–buffer (7:3) at 1.4 ml/min with a mass scan range of 300–700 u and free ecdysteroids with 2-propanol–buffer (11:89) at 1 ml/min with 200–600 u scanning. The buffer was 0.2 *M* in ammonium acetate or formate.

RESULTS AND DISCUSSION

To test the usefulness of HPLC–TSP-MS in ecdysteroids analyses we used a set of eight common ecdysteroids (see Fig. 1 for structures) previously isolated and fully characterized (IR, NMR, MS) in our laboratory. HPLC conditions were those we reported previously [9], with the exception that the chromatography was carried

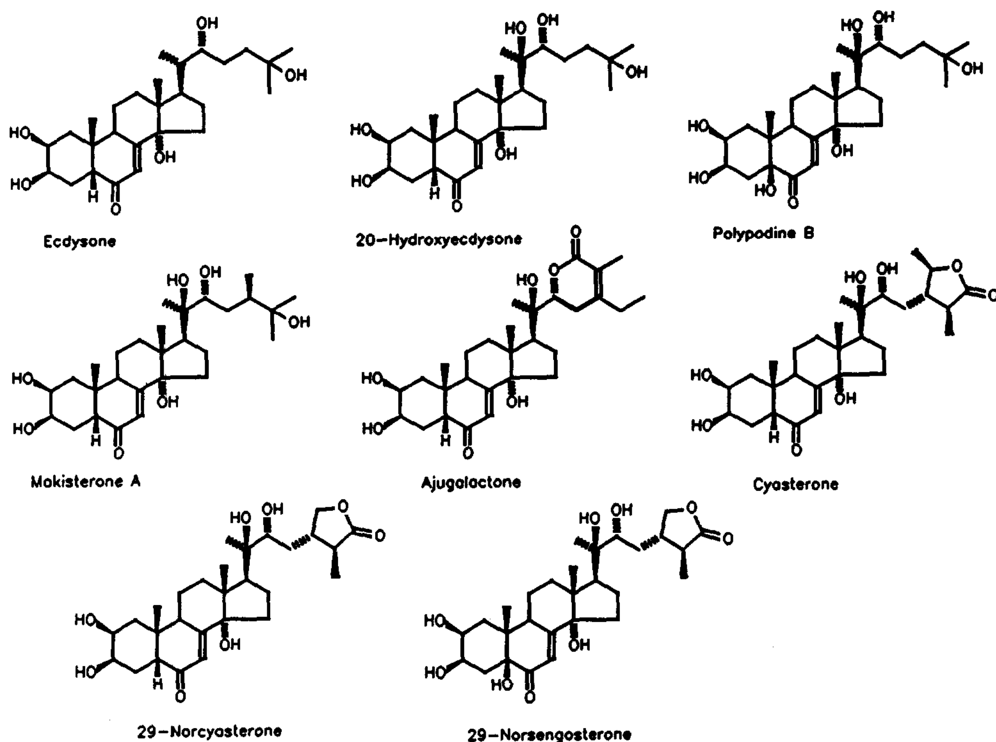

Fig. 1. Structures of the eight standard ecdysteroids used in this work: ecdysone (E), 20-hydroxyecdysone (20E), polypodine B (PB), makisterone (MK), ajugalactone (AJ), cyasterone (C), norcyasterone (NC) and norsengosterone (NS).

out at room temperature, and with buffer added to the eluent. The electrolyte presence causes only a slight decrease in the column efficiency (about 5%) when compared with our previous conditions.

The main fragmentation of ecdysteroids in HPLC–TSP-MS conforms to a common pattern (giving the most important fragments) with a secondary one derived from each peculiar structure (see Fig. 2 and Table I). MS spectra obtained resemble chemical ionization (CI) spectra, giving high intensity of high-mass fragments, especially those derived from electrolyte adduct $[M+H]^+$ and its dehydration ions (only in

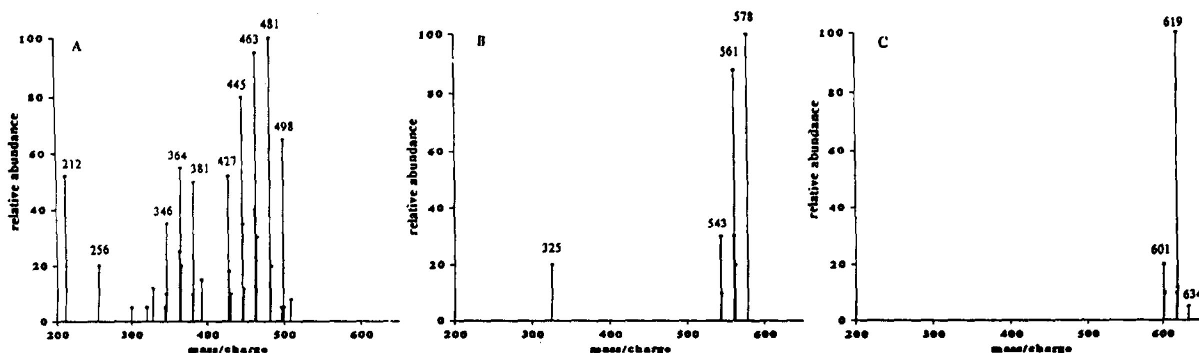

Fig. 2. Fragmentation patterns, in direct flow injection for (A) 25 μg of 20E, positive-ion detection mode, eluent 2-propanol–buffer (11:89), 1 ml/min; (B) 50 ng of 20E diacetonide, positive-ion detection, eluent acetonitrile–buffer (70:30), 1.3 ml/min and (C) 50 ng of 20E diacetonide, negative-ion detection, the same eluent as B. Buffer ammonium acetate 0.2 *M*.

TABLE I

MAIN COMMON FRAGMENTATION PATTERNS OF ECDYSTEROIDS AS A FUNCTION OF THE DETECTION MODE AND THE ECDYSTEROID FORM

Ecdysteroid	Fragment list
Free ecdysteroid, positive ions	$[M+NH_4]^+$, $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-3H_2O]^+$, $[M+H-[C20-C22]]^+$ (side chain, SC, cleavage between C-20 and C-22), $[M+2H-SC-H_2O]^+$, $[M+H-SC-2H_2O]^+$
Acetonides, positive ions	$[M+NH_4]^+$, $[M+H]^+$, $M+H-H_2O]^+$
Acetonides, negative ions	$[M+HCOO]^-$ or CH_3COO^- (electrolyte anion)

positive ion detection). In this work we also establish the sensitivity level of detection of ecdysteroids with this methodology and test it with pure ecdysteroid mixtures and with vegetable samples.

Using this technique, free ecdysteroids exhibit complex fragmentation patterns, which are advantageous for identification purposes, however this implies a reduction in the sensitivity with concomitant problems for quantification. To overcome this problem we decided to protect the glycol groups in the form of acetonides because the silyl ethers or acetate esters used as classical derivatives in GC–MS analysis are not stable enough under HPLC–TSP-MS conditions. We slightly modified one well-known acetonization procedure using anhydrous media and 2,2-dimethoxypropane as solvent. These changes led us to a very easy, fast and quantitative derivatization procedure that was highly efficient in all assayed cases. Likewise, to apply this procedure to real samples we optimized an easy and reproducible method for ecdysteroid extraction and purification that requires very little vegetable material. This method had been successfully used for quantitative ecdysteroid analysis of different tissue culture samples of *Polypodium vulgare*. The recovery is better than 94% for ecdysone (calculated from three replicates of the whole procedure with samples containing radiolabelled ecdysone). Subsequent to the isolation procedure, a Sep-Pak prepurification [13] was performed and, when it was needed, the acetonide derivatization was carried out as before.

For acetonide derivatives we could not find any appropriate chromatographic conditions to resolve completely the eight-ecdysteroid mixture, as we had accomplished for the free ecdysteroids [9]. With solvent mixtures containing methanol or isopropanol, the acetonides coeluted in groups, according to the number of glycol groups in the molecule and the presence or absence of a hydroxyl group at carbon 5 (see Fig. 3). However, this characteristic and constant behaviour was also useful for us because, as we will explain below, it gave some structural information when we tried to identify a new ecdysteroid. The best performance was obtained with acetonitrile as organic modifier and, in this case, the electrolyte addition only decreased the capacity factor by only a small amount. It is important to note that chromatographic efficiency for separation of acetonides is much better than that of free ecdysteroids, thus considerably improving the sensitivity. This effect could be attributed to the change in the column efficiency N (theoretical plates per m), which, in the case of 20-hydroxyecdysone and its diacetonide, had the value of 15 200 and 27 600, respectively, very close to the average value of N in the set ecdysteroids or ecdysteroid acetonides studied (13 900 and 27 100).

The acetonide fragmentation in HPLC–TSP-MS was very low but gave enough structural information (working with positive ions, see Table I) to allow positive identification of known ecdysteroids with a sensitivity similar to classical UV, which could be increased working in single-ion monitoring (SIM) mode with detection of

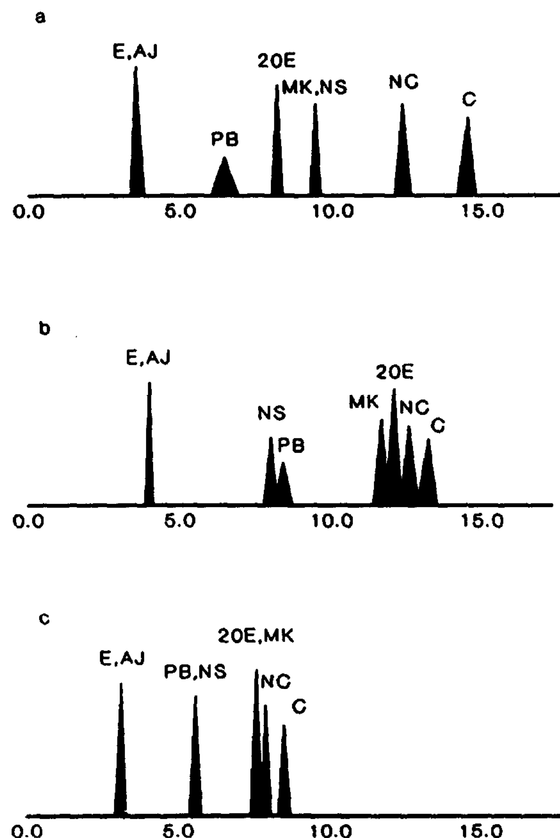

Fig. 3. HPLC elution profiles of ecdysteroid acetonides under several eluting systems. (a) acetonitrile–buffer (70:30), 1.3 ml/min; (b) methanol–buffer (75:25), 1.3 ml/min; and (c) 2-propanol–buffer (11:89), 1.0 ml/min. Buffer ammonium acetate 0.2 *M*; column RP-18, 25 × 0.47 cm I.D.

negative ions (see Table II). Previous reports using supercritical fluid chromatography–MS showed a sensitivity similar to that obtained by us with free ecdysteroids [14].

The second step in our work was to test the system performance when injecting a test mixture containing different amounts of the eight standard ecdysteroids working at different conditions (see Fig. 4). We obtained similar relative responses as when single pure ecdysteroid acetonides were injected. This was the first evidence that HPLC–TSP-MS could be a good ecdysteroid analytical method for quantification and identification.

Finally, we used HPLC–TSP-MS to analyse the ecdysteroid content (see Fig. 5) of the fern *Polypodium vulgare*. Since we began working with this fern, the existence of other ecdysteroids in this plant, in addition to those previously described (ecdysone, 20-hydroxyecdysone and polypodine B), was supported by the appearance of other small peaks during quantitative analysis by HPLC–UV of the isolated ecdysteroid fraction. Finally, with the optimization of this analytical technique we found that, for one of these minor compounds, the fragmentation pattern in positive- and negative-ion mode was similar to the behaviour of the standard ecdysteroid acetonides. The main fragments for this compound (617 and 634 in positive and 661 in negative), as well as its chromatographic

TABLE II

EXPERIMENTAL HPLC–TSP-MS SENSITIVITY OF THE EIGHT STANDARD ECDYSTEROIDS EXPRESSED AS AMOUNT OF COMPOUND NEEDED FOR A SIGNAL-TO-NOISE RATIO OF 5:1 AS A FUNCTION OF ECDYSTEROID FORM AND ION MONITORING MODE

PI = Positive-ion mode; NI = negative-ion mode

Ecdysteroid	Free scan, PI (μg)	Acetonide scan, PI (ng)	Acetonide scan, NI (ng)	Acetonide SIM, NI (ng)
E	7	50	25	3
20E	5	20	8	1
PB	18	100	50	6
MK	6	50	30	3
AJ	7	50	20	2
C	12	80	30	4
NC	8	70	30	3

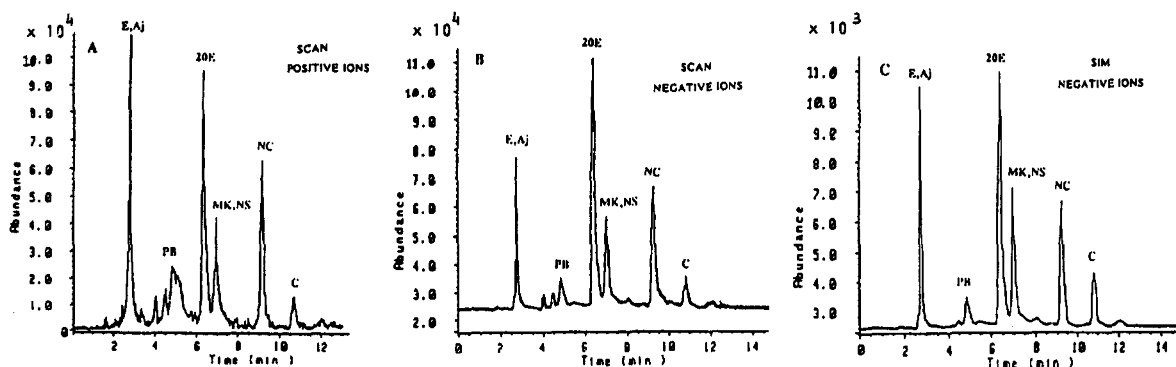

Fig. 4. HPLC–TSP-MS chromatogram of a mixture of standard ecdysteroid acetonides in acetonitrile–ammonium formate 0.2 M buffer (70:30), 1.8 ml/min (AJ 0.2 μg, E 0.2 μg, PB 0.1 μg, 20E 0.3 μg, MK 0.1 μg, NS 0.1 μg, NC 0.3 μg and C 0.1 μg; in SIM mode ten-fold less) in scan mode monitoring: (A) positive and (B) negative ions; and (C) SIM mode monitoring simultaneously the following negative ions (m/z 549, 601, 605, 619, 621, 631, 645, 647 u).

behaviour (long retention time), indicates a structure such as the triacetonide of the 20,26-dihydroxyecdysone (see Fig. 5). Prior to derivatization this compound exhibited higher polarity than the other ecdysteroids in the mixture. Later, by isolating larger amounts of this compound, its structure was completely confirmed by spectroscopic techniques (IR, ^{1}H-NMR and ^{13}C-NMR).

CONCLUSIONS

The results of this work show that HPLC–TSP-MS is a useful analytical tool in the ecdysteroid field. Working with free ecdysteroids it is possible to obtain enough structural information, but the sensitivity is very low compared with UV detection. When acetonide derivatives were used the sensitivity increased, with the additional advantage of giving complementary structural information, especially when positive- and negative-ion detection are used sequentially. For quantitation, the method requires internal standard calibration (preferably another ecdysteroid) because of the large response variations of the HPLC–TSP-MS system and negative-ion detection. This technique could be the first choice in the research of new ecdysteroids of plant or insect origin.

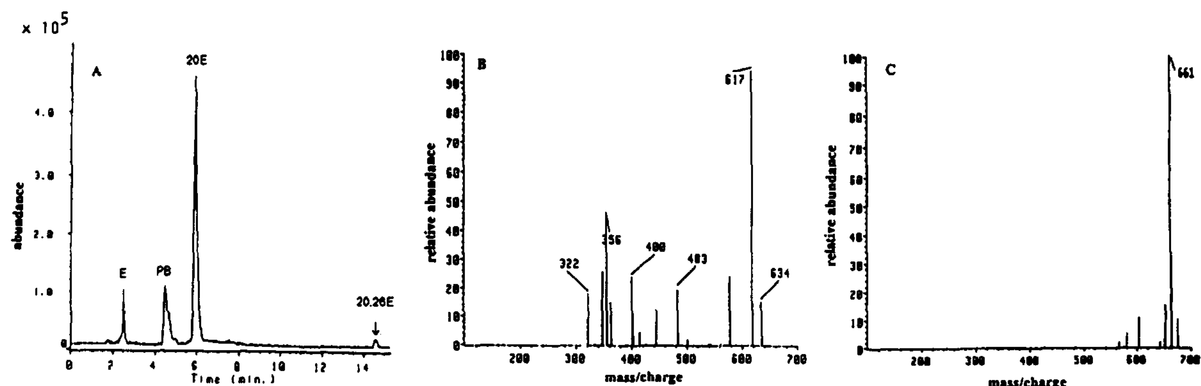

Fig. 5. HPLC–TSP-MS of a sample of *Polypodium vulgare*, one-fifth fraction of purified extract from 50 mg of starting plant, derivatized and diluted to 100 μl. (A) Total ion current of 25 μl of the above sample eluted with acetonitrile–ammonium formate 0.2 M buffer (70:30), 1.8 ml/min in positive scan mode. (B and C) Fragmentograms obtained in positive- and negative-ion detection modes for the peak at 16.2 min, assigned as 20,26-dihydroxyecdysone.

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