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Spectroscopic characterisation and identification of ecdysteroids using high-performance liquid chromatography combined with on-line UV–diode array, FT-infrared and ^{1}H-nuclear magnetic resonance spectroscopy and time of flight mass spectrometry

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Abstract

A prototype multiply hyphenated reversed-phase HPLC system has been applied to the analysis of a mixture of pure ecdysteroids and an ecdysteroid-containing plant extract. Characterisation was achieved via a combination of diode array UV, ^{1}H NMR, FT-IR spectroscopy and time of flight (TOF) mass spectrometry. This combination of spectrometers allowed the collection of UV, ^{1}H NMR, IR and mass spectra for a mixture of pure standards enabling almost complete structural characterisation to be performed. The technique was then applied to a partially purified plant extract in which 20-hydroxyecdysone and polypodine B were identified despite incomplete chromatographic resolution and the presence of co-chromatographing interferents. The experimental difficulties in the use of such a systems for these analytes are described.

Keywords: Multiple hyphenation; Mixture analysis; Detection, LC; Spectroscopic characterization; Plant extracts; Ecdysteroids

1. Introduction

The identification of analytes in complex mixtures via multiple hyphenation of chromatography with a range of spectroscopic detectors such as, e.g. HPLC–NMR–MS, etc. is becoming an established means for obtaining unambiguous characterisation (e.g., see [1–9], reviewed in Ref. [10]). Recently, we have begun to look at systems that enable ultraviolet (UV) and infrared (IR) spectra to be obtained in addition to NMR and MS [11–13]. The most recent version of these prototype systems combined a UV–diode array (DAD) detector, an FT-IR spectrometer, an NMR spectrometer and a time of flight (TOF) MS (providing the ability to determine molecular formulae via accurate mass determination) and was

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used initially for flow injection analysis [12]. Having successfully used the system for FIA an HPLC system was added to enable mixtures to be analysed, and this was successfully applied to a model mixture of non-steroidal anti-inflammatory drugs [13]. Here, we describe a further application of the system to ecdysteroids, either as pure compounds or present in a typical extract of the plant *Lychnis flos-coculi,* a member of the family Caryopyllaceae. This work represents an extension of our previous studies where the use of HPLC–NMR and HPLC–NMR–MS for ecdysteroid-containing plant extracts was examined [14,15], and represents an attempt by us to further explore the advantages and limitations of the use of complex multiply hyphenated systems which incorporate detectors of (potentially) widely differing sensitivities.

2. Experimental section

The ecdysteroids (ecdysone, makisterone A and 20-hydroxyecdysone) used as pure standards were gifts from a variety of sources. Samples of these pure standards were dissolved in deuterium oxide (D_2O) to give a solution containing 20 mg/ml of ecdysone and 20-hydroxyecdysone and 10 mg/ml of makisterone A.

The extract was obtained from finely ground air dried *Lycnis flos-coculi,* collected by one of the authors in the vicinity of ''Le Perray-en-Yvelines'', (near Rambouillet, France). An ethanol extract was prepared using 96% ethanol at 1 l/100 g dry weight of plant material with continuous stirring for 2–3 days. After extraction the sample was filtered, evaporated to dryness, re-dissolved in a small volume of methanol and centrifuged. Immediately prior to HPLC the sample was evaporated to dryness and taken up in a small volume of deuterium oxide.

The HPLC system consisted of a Bruker LC22 pump (Bruker, Coventry, UK) which delivered eluent at 1 ml min^{-1}. Typically 200 μl of sample were introduced into the flowing stream via a model 7125 Rheodyne injector (Rheodyne, USA) fitted with a 200 μl sample loop. HPLC was performed using either a 5 μm 10 cm×4.6 mm I.D. Hypersil HIRPB C_{18} or a 5 μm 25 cm×4.6 mm I.D. Hypersil H5BDS-C_{18} column (Hichrom, UK). The HPLC mobile phase consisted of acetonitrile (Pestanal Grade, Riedel-de Haen, Germany) and D_2O 99.8% isotopic purity (Fluorochem, UK) 20:80 v/v.

On emerging from the column the eluent flowed in to a Varian 9065 UV–diode array detector (Varian UK Ltd., Surrey, UK) via 30 cm of 0.005″ I.D. PEEK (polyethyl ether ketone) tubing. UV spectra were collected over the wavelength range 190–360 nm, using the Star Chromatography Workstation, Version 4.0 (Varian UK Ltd., Surrey, UK), and analysed for spectral information using Polyview Version 2.0 (Varian UK Ltd., Surrey, UK).

Following DAD the flow went via 110 cm of 0.005″ I.D. PEEK tubing to a Bio-Rad FT-IR model 375C spectrometer (Cambridge, MA USA) fitted with a Spectra Tech (Stamford, CT USA) Macro Circle Cell ATR (attenuated total reflectance) stainless steel flow cell of 400 μl volume fitted with a zinc selenide ATR crystal. Spectra were acquired with the kinetics software collecting 20 scans per spectrum (5-s acquisition time) with a sensitive MCT (mercury cadmium telluride) liquid nitrogen cooled detector. The spectra were acquired at 8 cm^{-1} spectral resolution. The sample was ratioed against a background spectrum of the flowing solvent through the cell prior to injection of the sample solution thus automatically subtracting out the solvent spectrum from the sample spectra.

Following FT-IR the flow entered, via 150 cm of 0.005″ I.D. PEEK tubing, a Bischoff Lambda 1000 UV detector (Bruker, Coventry) set at 254 nm, after which the solvent stream was split 95:5 with 5% of the flow being directed to the mass spectrometer via 250 cm of 0.007″ I.D. PEEK tubing, and the remainder to the NMR via 280 cm of 0.01″ I.D. PEEK tubing. Further details concerning the layout of this system are provided in Ref. [13].

Mass Spectra were acquired on a Micromass LCT time of flight (TOF) mass spectrometer (Micromass, Altrincham, UK) using electrospray ionisation (ESI) with a Z Spray source. The nebuliser gas flow was set to 85 l h^{-1} and the desolvation gas to 973 l h^{-1}. Spectra were acquired in positive ion with a capillary voltage of 3.2 kV and a cone voltage of 25 V. The source temperature was set to 120°C and the desolvation temperature to 350°C. The pusher cycle time was 50 μs with 0.9 s acquisitions and an interacquisition delay of 0.1 s. The mass range was 100–900

Daltons. Caffeine, molecular mass 194.0804 Daltons, was used to provide a lock mass for the spectrometer and was introduced via a t-piece at 0.5 ml min^{-1} at a concentration of approximately 5 ng ml^{-1}.

All of the instrumentation described above was located outside the 5 Gauss line of the stray magnetic field generated by the 500-MHz NMR spectrometer.

NMR spectra were acquired using a Bruker DRX-500 NMR spectrometer in the stop–flow mode at 500.13 MHz using a flow-through probe of 4 mm I.D. with a cell volume of 120 μl. Spectra were acquired using the NOESYPRESAT pulse sequence (Bruker Spectrospin, UK) in order to suppress the ACN and residual water signal. Pulses of 90° were used with a relaxation delay of 2 s and a mixing time of 100 ms. FIDs were collected into 16K data points over a spectral width of 8278 Hz, resulting in an acquisition time of 0.99 s. Between 24 and 128 scans were acquired for each spectrum.

3. Results

Previous work with this prototype system indicated that it is possible to obtain ^{1}H NMR and IR spectra with as little as 50 μg of material [12,13]. However, as we were not limited in the amount of material available, we opted to use concentrated samples in order to minimise the amount of time required to obtain spectra, and thereby maximise throughput. The instrument layout used has been described in detail elsewhere [12,13] and was essentially as described in Ref. [13] and will not be discussed further.

3.1. Chromatography

3.1.1. Standards

The chromatographic system used a C_{18} bonded stationary phase in combination with a simple reversed-phase isocratic solvent system 80:20 mixture of D_2O and acetonitrile, which we have found to be satisfactory for this group of compounds in previous studies [14,15]. The separation obtained for this mixture of standards on a 10-cm column is shown in Fig. 1. Despite the relatively high sample loading used (ca. 2–4 mg/compound) good chromatographic peak shapes were obtained with baseline separation for all three analytes. The elution order was 20-hydroxyecdysone, makisterone A and ecdysone, with

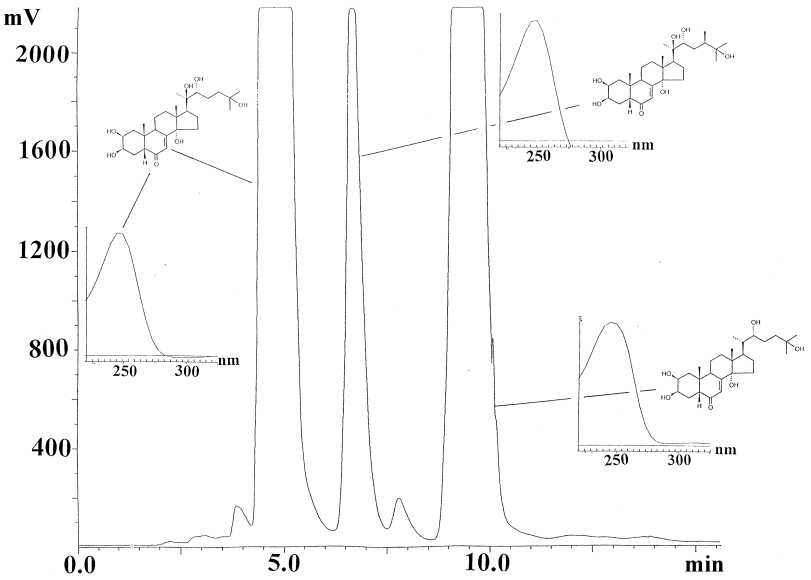

Fig. 1. The chromatogram obtained following the reversed-phase HPLC of the mixture of the 3 ecdysteroid standards. The elution order was 20-hydroxyecysone, makisterone A and ecdysone (structures inset to figure). The UV–DAD spectra obtained for each peak are also inset to the figure.

a total chromatographic run time of ca. 12.5 min. The structures of each of the standards are shown as insets to the figures.

3.1.2. *Lychnis flos-coculi*

Lychnis flos-coculi represents a rich source of phytoecdysteroids and has been shown to contain ca. 0.17% of 20-hydroxyecdysone relative to its dry weight. The main ecdysteroids present in extracts of this species have been shown to be 20-hydroxyecdysone and polypodine B (5β-hydroxy-20-hydroxyecdysone). Numerous minor ecdysteroids (including, e.g., 2-deoxy-20-hydroxyecdysone, dihydrorubrosterone, 20,26-dihydroxyecdysone, 20-hydroxyecdysone 2 and 3 acetates, 26-hydroxypolypodine B, integristerone A, makisterone A, poststerone, rubrosterone, taxisterone and viticosterone have also been detected [16]. In order to enhance the resolution of the ecdysteroids from the co-extracted contaminants, chromatography was performed on a 25-cm column. The HPLC trace for this extract is illustrated in Fig. 2. This shows a number of peaks eluting at or near the solvent front with a major UV-absorbing peak at ca. 34 min corresponding to the retention time of 20-hydroxyecdysone. In addition to this major UV-absorbing peak a number of minor UV-absorbing peaks were also observed in the sample eluting at ca. 36 and 39 min.

3.2. UV spectra

3.2.1. Standards

Typically the ecdysteroids are characterised by a strong UV absorption spectrum centred at ca. 240 nm. As we noted in our previous studies with this multiply hyphenated system the quantities of material used here to ensure that NMR and IR spectra could be easily obtained meant that the DAD was overloaded at anything other than the leading or tailing edge of the chromatographic peaks. The spectra obtained from the tailing edge of each of the peaks for these ecdysteroid standards are shown as insets to the chromatogram (Fig. 1). These are typical of those usually obtained for ecdysteroids and as the structural differences between the three compounds are minor, and distant from the chromophore, they were essentially indistinguishable from each other.

3.2.2. *Lychnis flos-coculi*

Despite the large quantities of the ecdysteroids

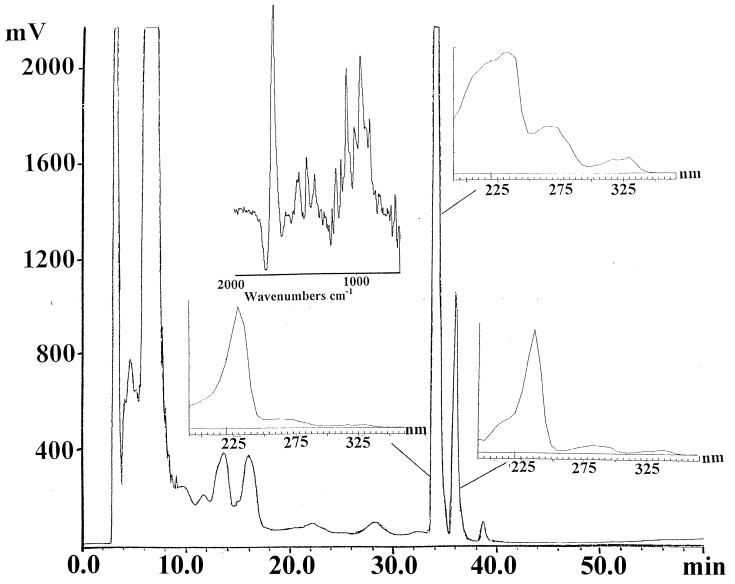

Fig. 2. The chromatogram obtained following the reversed-phase HPLC of the extract of *Lychnis flos-coculi*. The UV–DAD spectra obtained for the leading and trailing edges of the main peak, and for the close running UV-absorbing peak are given as insets to the chromatogram as is the FT-IR spectrum for the main peak.

present in this plant the UV spectra obtained from the main chromatographic peak present in the crude Lychnis extract were not typical of those expected for ecdysteroids (and seen with the pure standards). Thus, UV maxima were observed at ca. 230 nm early in the peak with a gradual increase in the contribution of higher wavelength absorptions (260–280 nm) with time (see spectra inset to chromatogram, Fig. 2). Based on these spectra, it is unlikely that the peak would have been considered to be composed of ecdysteroid-like substances. Similarly, the UV spectra of the minor peaks eluting at ca. 36 and 39 min did not give ecdysteroid-like spectra (not shown).

3.3. IR spectra

3.3.1. Standards

On-flow spectra were obtained for all of the analytes in the mixture of standards. The spectra of all three ecdysteriods were dominated by the absorption at ca. 1645 cm^{-1} due to the presence of the carbonyl group in the en–one function. Fig. 3A–C. The fingerprint regions showed some differences between compounds enabling them to be distinguished from each other.

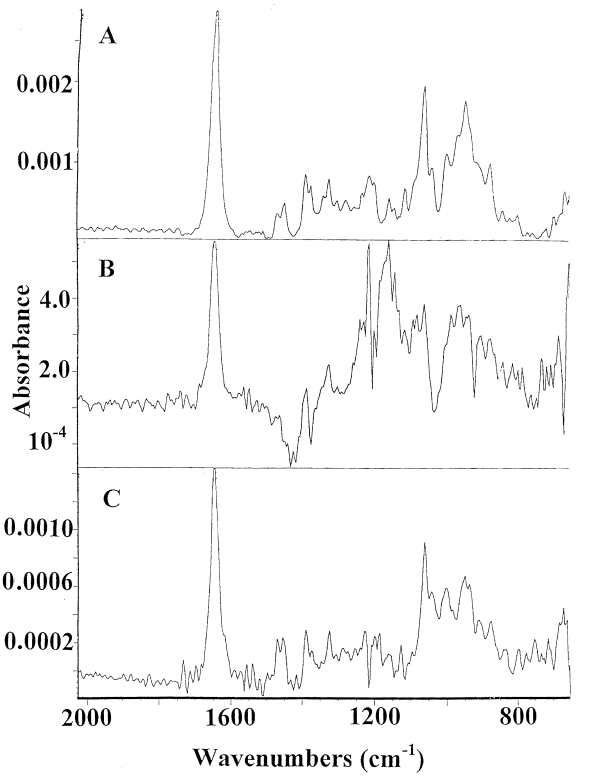

Fig. 3. The FT-IR spectra of: (A) 20-hydroxyecdysone; (B) makisterone A and (C) ecdysone obtained on the peaks of the mixture of ecdysteroid standards shown in Fig. 1.

3.3.2. Lychnis flos-coculi

The FT-IR spectrum of the main eluting peak for the plant extract, inset to Fig. 2 also shows a strong absorbtion at ca. 1645 cm^{-1}, with similarities in the fingerprint region to 20-hydroxyecdysone. The quality of the spectrum obtained enabled the identification of the material in the extract as 20-hydroxyecdysone when searched against a reference spectral library, giving a very good match of 92% to the reference spectrum for 20-hydroxyecdysone. In addition the ecdysteroid-containing peak was analysed by obtaining ''on-the-fly'' 5-s time slices throughout the peak. Typical spectra for the beginning, middle and trailing edge of the peak are shown in Fig. 4A–C. As this shows the position of the carbonyl peak maximum shifts with time from ca. 1645 cm^{-1} at the beginning and middle of the peak to ca. 1665 cm^{-1} in the trailing edge of the peak. Also, the carbonyl peak of the trailing edge spectrum clearly shows a shoulder at ca. 1645 cm^{-1} indicating the presence of at least two components. Thus, Fig. 4D–F shows the second derivative spectra of the beginning, middle and trailing edges of the ecdysteroid-containing peak. These clearly show the peak position, revealing that the beginning and middle spectra were obtained from one carbonyl entity (at ca. 1645 cm^{-1}), whereas the trailing edge of the peak clearly shows two carbonyl absorptions at 1667 and 1642 cm^{-1}, suggesting that at least two ecdysteroids were present in this peak. The similarity of the IR spectra in terms of the carbonyl absorption of the three ecdysteroid standards used in the model mixture suggests that, if the second carbonyl signal was due to an ecdysteroid, there must be some structural change close to the 7-en-6-one group. This is consistent with the known presence of polypodine B (structure inset to Fig. 8), which contains a 5-β hydroxyl, in the sample. Polypodine B also co-chromatographs with 20-hydroxyecdysone in many reversed-phase systems.

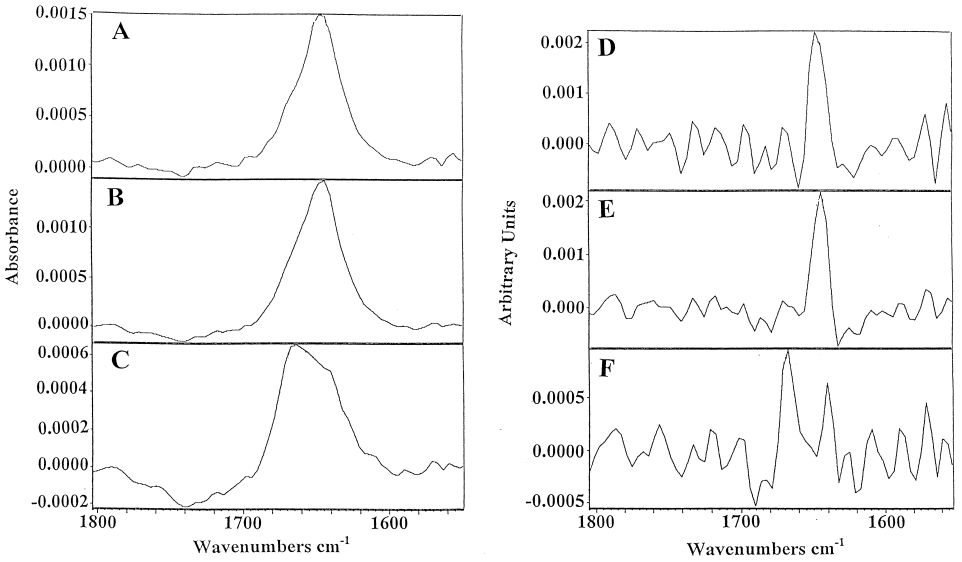

Fig. 4. The FT-IR spectra of the ecdysteroid-containing peak of the *Lychnis flos-coculi* extract shown in Fig. 2, showing the carbonyl absorption at: (A) the beginning; (B) the middle and (C) the trailing edge of the chromatographic peak and the corresponding second derivative FT-IR spectra of the carbonyl absorptions showing: (D) the beginning; (E) the middle and (F) the trailing edge of the chromatographic peak.

3.4. NMR spectra

3.4.1. Standards

As in our previous HPLC study with this system [13], NMR spectra were obtained in the stop–flow mode. Thus, ^{1}H NMR spectra shown in Fig. 5A–C acquired for each of the ecdysteroids in the mixture were acquired with between 24 and 128 scans per

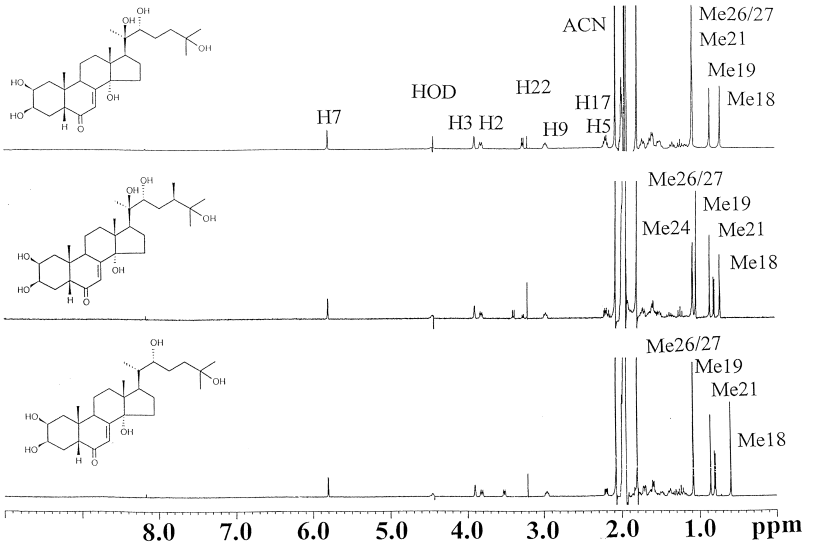

Fig. 5. The ^{1}H HPLC–NMR spectra of: (A) 20-hydroxyecdysone; (B) makisterone A and (C) ecdysone obtained in stop–flow mode on the separation illustrated in Fig. 1.

spectrum (see Experimental). The spectra contained, in addition to the resonances for the ecdysteroids, signals for residual HOD and acetonitrile. However, these additional signals did not result in significant problems for the spectroscopy of the ecdysteroids used in this mixture. Thus, in the case of the first eluting component 20-hydroxyecdysone, all of the methyl resonances are clearly visible between 0.6 and 1.2 ppm, together with the diagnostic 7-en proton (ca. 5.8 ppm) (see Fig. 5A). Similarly, all of the diagnostic methyl resonances for makisterone A and ecdysone are visible in the stop–flow ^{1}H NMR spectra obtained for them in this experiment (see Fig. 5B and C). The assignment of the HPLC–NMR spectra of the ecdysteroids is considered in more detail in Ref. [14].

3.4.2. Lychnis flos-coculi

It was immediately apparent from the ^{1}H NMR spectra of the early portion of the main UV-absorbing peak in the plant extract that, whilst there were indeed signals that were consistent with the presence of ecdysteroids, centred around the methyl region between 0.6 and 1.2 ppm, and at ca. 5.8 ppm corresponding to the 7-ene proton, the peak also contained significant quantities of other materials. In particular, there appeared to be a number of anomeric protons consistent with the presence of sugars. We therefore proceeded to analyse the ecdysteroid-containing peak by the use of the ''time-slicing'' technique whereby stopped-flow spectra were obtained every 20 s through the peak. Typical spectra for the beginning, middle and tailing edge of the peak are shown in Fig. 6A–C. As can be seen, the impurity profile within the peak changed with time. In addition, as the peak eluted from the column, it was observed that the region of the ^{1}H NMR spectrum containing the resonance for the 7-ene proton gradually acquired a second signal. The NMR spectrum of the first eluting component of this ecdysteroid mixture corresponded to 20-hydroxyecdysone, whilst the chemical shift of the second eluting C-7 proton observed in subsequent spectra was consistent with the presence of polypodine B (C-5 hydroxyl-20-hydroxyecdysone), supporting the suggestion provided by the second derivative FT-IR spectrum that the structural difference between the two ecdysteroids was close to the en–one group. As indicated above, this result is in accordance with the known chromatographic properties of these two compounds in RP-HPLC systems. The ^{1}H NMR spectra obtained for this peak therefore clearly

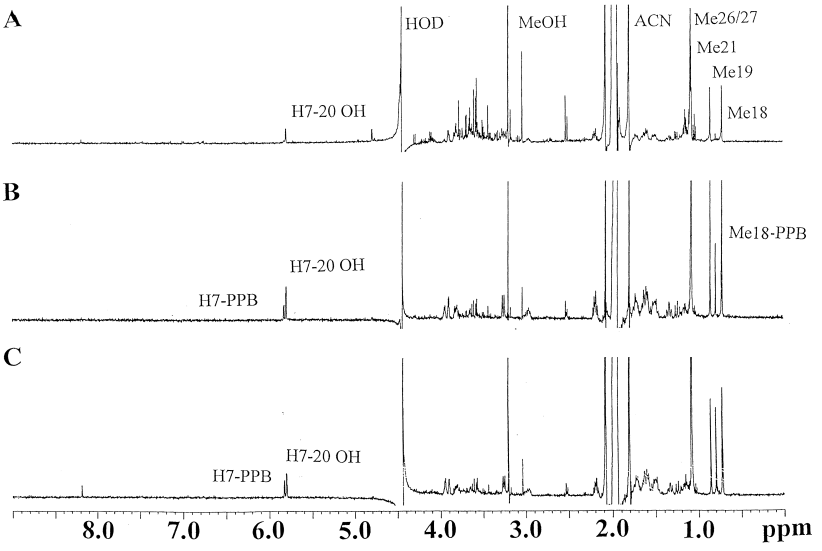

Fig. 6. The ^{1}H HPLC–NMR spectra obtained for: (A) the beginning; (B) the middle and (C) the trailing edge of the ecdysteroid-containing peak of the *Lychnis flos-coculi* extract shown in Fig. 2.

indicated the presence, and proportions, of the two major ecdysteroids present in the extract together with the co-eluting impurities.

3.5. Mass spectra

3.5.1. Standards

In the case of 20-hydroxyecdysone and makisterone A, the masses of the deuterated molecules were readily observed at 488 and 502 amu, with base peak ions noted at 469 and 483 amu respectively. These spectra are illustrated in Fig. 7A and B. In the case of ecdysone a weak molecular ion was detected at 471 amu, with the base peak ion at 452 amu (Fig. 7C). In each case the spectrum was dominated by an ion 19 amu down from the deuterated molecule. This we presume is the loss of HOD from the parent. In our previous HPLC–NMR–MS study of these compounds, where we employed an ion trap mass spectrometer the pseudo molecular ions tended to be the base peak (e.g. Ref. [15]. It is well documented that the loss of water from the ecdysteroids is facile and we assume that the predominance of ions showing a loss of 19 amu in our spectra is due to the cone voltage being too high, although not excessive. We have not been able to establish this loss as being definitely due to the elimination of HOD but this seems likely and will be investigated further in subsequent studies. Other ions present were related to sodium and acetonitrile adducts (see below also). We were unable to obtain any satisfactory accurate mass data due to the fact that such large amounts of sample were loaded to enable relatively easy NMR and IR detection. The amount of sample involved overloaded both the ion source and detector system of the LCT.

3.5.2. Lychnis flos-coculi

In the case of the plant extract it was possible to obtain spectra consistent with the presence of 20-hydroxyecdysone and polypodine B co-eluting in the major UV-absorbing peak. Representative spectra are shown in Fig. 8A and B. For 20-hydroxyecdysone, a pseudo molecular ion was observed at m/z 488, together with ions at m/z 469 (loss of HOD from the parent) and 529 (acetonitrile adduct). These results are similar to those obtained for the standards as described above. The case of polypodine B proved to be somewhat more complex with, instead of a single ion corresponding to the pseudo molecular ion, a cluster of ions were observed around the expected pseudo molecular ion at m/z 505 (see Fig. 8A). Such

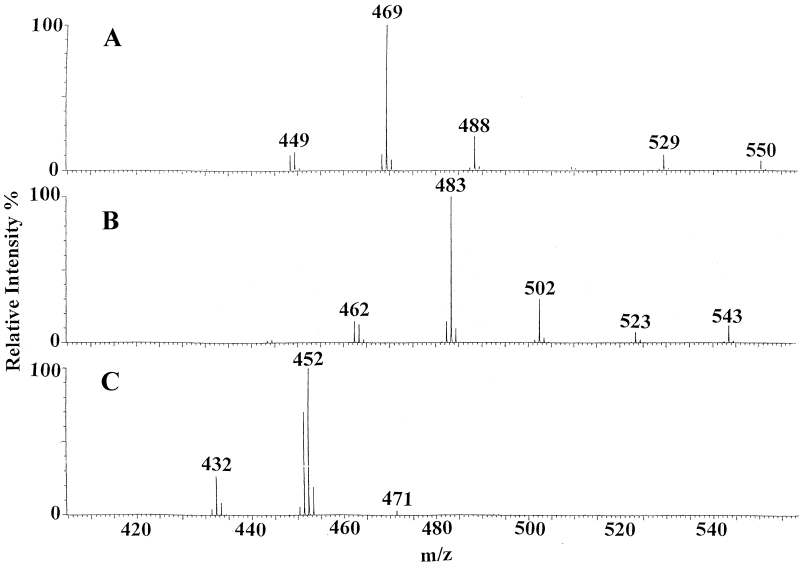

Fig. 7. The HPLC–TOF mass spectra of: (A) 20-hydroxyecdysone; (B) makisterone A and (C) ecdysone obtained on the peaks of the ecdysteroid mixture shown in Fig. 1.

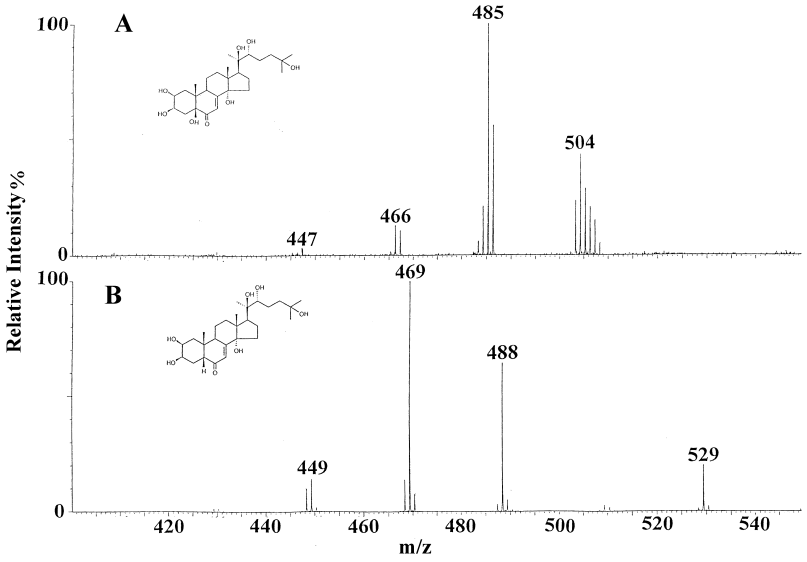

Fig. 8. The HPLC–TOF mass spectra of: (A) polypodine B and (B) 20-hydroxyecdysone obtained for the ecdysteroid containing chromatographic peak of the *Lychnis flos-coculi* extract shown in Fig. 2.

results are only explicable in terms of varying degrees of deuteration of the exchangeable protons of polypodine B, which logically must be related to the presence of the 5-β-hydroxyl, given the rapid and complete deuteration of the 20-hydroxyecdysone in the same sample. The two most significant ions in the spectrum of polypodine B were observed at m/z 485 and 486, corresponding to the loss of 19 (HOD) from the fully deuterated (seven hydroxyl protons replaced) and the hexadeuterated steroid respectively. The relatively greater proportions of the ions at m/z 504 and 485 compared to those at m/z 486 and 505 suggest that the hydroxyl in the 6 position is less easily deuterated than the other hydroxyl groups and this is supported by previous literature reports [17]. In order to confirm the identification of polypodine B, mass spectrometry was repeated using protonated solvents which gave the expected molecular ions for both 20-hydroxyecdysone (m/z 481) and polypodine B (m/z 497) (together with ions corresponding to the loss of water). This slow exchange may prove to be useful diagnostically when trying to determine the sites of hydroxylation of novel ecdysteroids.

As was the case with both IR and NMR measurements, which both indicated the presence of more than one component in the major chromatographic peak, careful examination of the ion traces for the individual ecdysteroids also revealed their co-elution (with the leading edge of the peak composed predominantly of 20-hydroxyecdysone and the tailing edge containing both compounds).

4. Discussion

As indicated, this system was assembled to begin to explore the feasibility and usefulness of an integrated approach to the spectroscopic characterisation of column eluates using commercially available instrumentation. The alternative approaches of either off line characterisation of isolated components, or the use of several chromatographic separations, with each optimised for a specific spectroscopic detector are clearly viable and have clear advantages in terms of simplicity vs. the complexity of the system used here. These are powerful arguments for their use and off-line techniques may be essential for minor components in a mixture where long analysis times are required for FT-based techniques (NMR, IR), or where careful optimisation of the response may be required (MS). Against this the fully integrated approach offers the benefit that one chromatographic run only is required, thus ensuring that all of the spectrometers see the same separation. A fully

integrated system may thus provide a rapid and efficient means of obtaining data on the major components of a mixture.

It was clearly the case with the standards that obtaining good quality UV, IR, NMR and MS data did not prove to be particularly problematic. The major difficulty encountered here was the difference in the sensitivities of the detectors for these analytes. Thus, whilst the NMR and IR detectors were relatively well matched, the UV–DAD and TOF-MS were clearly overloaded and this did pose some problems insofar as obtaining UV spectra and accurate mass data were concerned. However, it should be noted that this overloading did not prevent the acquisition of diagnostic mass and UV spectra. Furthermore, the UV spectra that were obtained for the plant extract were less useful anyway because of strongly UV-absorbing, but minor, impurities that co-chromatographed with the major components in the peak of interest.

In future studies we aim to solve the problem of UV detector overloading by placing the UV–DAD in line with the MS, after the flow is split (95:5), with a make-up flow to dilute the sample and bring it into the linear range of the UV. This will also have the advantage of diluting the sample prior to TOF-MS, thereby enabling accurate masses to be obtained (if required) more easily (although further dilution may be necessary). Clearly, the need for extra dilution of the column eluates will not simplify the construction of an already complex system. However, the current system was constructed using readily available equipment that, with the exception of the TOF-MS, did not represent the current state of the art. The rapid technical advances that are being made in terms of sensitivity in NMR and IR will, in the (near) future, inevitably result in a dramatic reduction in the sample requirements for such a system and therefore, the need for the overcomplex multiple splitting of eluates.

Despite the difficulties highlighted above, when applied to the plant extract this combination of detectors was able to provide a wealth of information on the main ecdysteroid-containing peak in the chromatogram, yielding information on the identity of the components present and their proportions, and the type and quantity of impurities present.

Whilst we would not claim for a moment that the demonstration system described here provides the basis for a robust and reliable installation, the results give an indication of the potential of a multiple (hyper) hyphenated system for extracting information from chromatographic peaks. Experience gained with this and similar assemblies should, by highlighting the difficulties and advantages inherent in such devices, enable an informed debate to be held on the place of such instruments for mixture analysis.

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