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High performance Reversed-Phase Thin-Layer Chromatography-Desorption electrospray ionisation - time of flight high resolution mass spectrometric detection and imaging (HPTLC/DESI/ToFMS) of phytoecdysteroids

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ABSTRACT

Reversed-phase high performance thin-layer chromatography (RP-HPTLC) on C18 bonded silica gel was combined with desorption electrospray ionization (DESI) and high resolution time of flight mass spectrometry (HRToFMS) to detect, characterize and image (MSI) phytoecdysteroids (plant-derived insect moulting hormones) in ethanolic extracts of members of the *Silene* plant family. As seen previously for silica gel, DESI provided a simple and convenient method for recovering polar polyhydoxysteroids from RP-HPTLC plates for the purposes of both the MS and MSI of extracts obtained from three species of the *Silene* family *(Silene otites, S. nutans and S. viridiflora)*. Using RP-HPTLC/DESI/MSI/HRToFMS a number of ecdysteroids, including 20-hydroxyecdysone, polypodine-B, 2-deoxy-20-hydroxyecdysone and 2-deoxyecdysone were identified in these extracts. Differences were noted in the mass spectra obtained depending upon both the stationary phase on which they were separated, and the temperatures used in the heated transfer line used for introduction into the ion source. Ecdysteroids detected after chromatography on C18 bonded silica showed increased fragmentation due to water loss compared to those imaged from silica. In addition, the benefits of the additional resolution provided by 2-dimensional TLC for increasing spectral quality compared to a 1-dimensional separation are demonstrated.

1. Introduction.

The ecdysteroids, a group of over 500 polar, polyhydroxylated [1], steroids are involved in the development of insects and crustaceans where they are active as moulting hormones. They are also found in large numbers in plants where it is assumed that they act as a defence against insect predators. These plant-derived phytoecdysteroids are responsible for much of the structural diversity encountered in this class of natural product. In addition to their effects on arthropods these steroids also appear to have pharmacological effects in mammals, including anabolic effects in humans [2]. The parent of the series, ecdysone ((22R)-2β,3β,14α,22,25-pentahydroxy-5β-cholest-7-en-6-one) was first isolated from pupae of the silk moth (*Bombyx mori*) in 1954 [3], although its structure was only finally determined some 11 years later [4]. It is now widely accepted that ecdysone is a precursor for the active moulting hormone, originally named β-ecdysone, but which is now more commonly known as ecdysterone or 20-hydroxyecdysone (for a recent review of the field of ecdysteroid research see [5]). However, as has become clear in the intervening period, the presence of large numbers of structural variants of ecdysone, particularly plant-derived phytoecdysteroids, leads to a requirement for methods of their separation and identification in botanical materials. As is well accepted, TLC provides a robust and easily deployed method of separation, that is tolerant of often quite crude samples, making it popular for screening plant extracts. Simple visualization under UV light also enables a rapid evaluation of the TLC separation and provides a simple method for qualitative evaluation of the separation for screening purposes.

Nevertheless, despite these valuable attributes, TLC is at a disadvantage as far as resolution, specificity and identification are concerned when compared to techniques employing MS-based detection, such as e.

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g., HP and UHPLC/MS. Whilst there has long been interest in coupling TLC (directly and indirectly) with mass spectrometry (e.g. see [6]) the two techniques are not as easily hyphenated as LC/MS. However, developments such as MALDI (matrix assisted laser desorption ionization) and DESI (desorption electrospray ionization) have transformed this situation (reviewed in [7]), enabling the relatively facile acquisition of MS data from surfaces such as TLC plates. Thus, the availability of these TLC/MS techniques, either *in situ* employing "ambient ionization" techniques (MALDI, DESI), or via devices for the elution of analytes directly from the plate using special interfaces, has resulted in numerous applications to e.g., lipids and natural products [8–15]. Still, an advantage of MALDI and DESI (and other emerging techniques such as e.g., (LD-)LTP (low-temperature plasma probe)[16], EFISI (electrostatic field induced spray ionization) [17] and DINeC (cluster-induced desorption/ionization)[18]-based TLC/MS), that is not shared by the more targeted methods that use an interface to elute zone of interest into the MS for analysis, is that they enable plates to be accessed and imaged directly.

We have recently examined the imaging of ecdysteroids following normal phase (NP) HPTLC on silica gel plates using DESI, in combination with ion mobility (IM) spectrometry [14]. This showed that DESI/MS was well suited to this type of application. Indeed, the combination of HPTLC, DESI, and MS (with, or without, the inclusion of IM) confirmed the considerable advantages of this approach over the physical removal of regions of interest from the HPTLC plate for subsequent MS (e.g., see [19]) or the use of specialist interfaces to elute them from the silica and transport them into the ion source of the MS (e.g.[13]). DESI also has advantages over MALDI-based approaches as these require post-chromatographic preparation of the plate (such as e.g., [9]) which DESI does not. Encouraged by the results we obtained for DESI with silica HPTLC plates we have now investigated the use of DESI/MS and DESI/MS imaging (MSI), for the analysis of ecdysteroid-containing plant extracts, separated by one and two dimensional reversed-phase (RP) on C18 bonded HPTLC plates.

2. Experimental

2.1. Chemicals and reagents

Acetone and ethanol were obtained from Sigma Aldrich (Dorset, UK). The ecdysteroids used as standards in this work were either isolated by us in previous studies or were obtained as gifts from a variety of sources and were used as supplied.

Samples: Four methanolic extracts, obtained from three species of the *Silene* family (*Silene otites* (SO*), S. viridiflora* (SV) and two extracts from *S. nutans* (SN1 and SN2)), prepared as described elsewhere [19] were used in this study.

2.2. Chromatography

RP-HPTLC was performed on glass backed C18 bonded silica gel glass backed silica gel HPTLC plates, 10 × 10 cm incorporating a fluorescent ($F_{254}S$) indicator (E. Merck, Darmstadt, Germany). Aliquots of 1 μL of each of methanolic solutions of the ecdysone and 20-hydroxyecdysone (ca. 1 mg/ml) standards were applied as spots to the plates manually using 1 μL glass capillaries (Drummond Scientific Company, Broomall, Pa, USA) to act as Rf markers. In the case of the plant extracts 2 μL of each of the samples were applied as narrow bands (ca. 5 mm) to the plates. One dimensional plate development was carried out in glass tanks (10 × 10 × 5 cm) using either ethanol–water 3:2 (v/v) or acetone–water 3:2 (v/v) as the developing solvent. After development the plates were air dried at ambient temperature and viewed under a UV lamp at 254 nm in order to assess their suitability for DESI/MS-based imaging.

The silica gel HPTLC plates reanalysed in the current investigation here to confirm the original MS results were obtained in our previous study on these samples [14].

For 2-Dimensional RP-HPTLC a 1 μL sample of the *Silene otites* extract was separated using both solvent systems, with acetone:water 3:2 (v/v) used for the first dimension and ethanol–water 3:2 (v/v) for the second.

2.3. DESI-MS imaging

Experiments were performed using a Xevo^{TM} G2-XS QTof mass spectrometer (Waters Corporation, Manchester, UK) operated in sensitivity mode (30,000 FWHM at *m/z* 500) and equipped with a two-dimensional DESI XS source, containing the High Performance DESI sprayer and Heated Transfer Line (HTL) (Waters Corporation, Manchester, UK). The DESI solvent composed of methanol/water (MeOH/H_2O) 95:5 (v/v) was delivered using an ACQUITY UPLC^{TM} microBSM system at a flow rate of 2 μL/min with an optimized nebulizing gas spray of nitrogen at a gas pressure of 10 Psi. An optimized voltage of 0.75 kV was applied to the spray. The heated transfer line (HTL) was set with no temperature applied (i.e., room temperature ca. 25 °C) or at 450 °C. DESI/MSI experiments were acquired in positive mode from *m/z* 100–1,200.

For imaging purposes, the DESI stage was set at a constant speed of 800 μm/s in the X direction with an MS scan time of 0.25 s, giving a pixel size of 200 μm in the X dimension. The distance between each line of DESI imaging, representing the pixel size in the Y dimension, during acquisition was also 200 μm. These instrumental setting thus resulted in a pixel size of 200 × 200 μm. These conditions represented a compromise between speed, resolution and signal intensity, enabling the differentiation of TLC spots with good spatial resolution and a relatively short acquisition time. Reducing the pixel size to 50 μm with the same stage speed, the MS scan time of would be 0.0625 s and the overall acquisition time would then be have been 43 h, but signal intensity would also be decreased significantly.

2.4. Data processing

Acquisition setup, processing and visualisation of imaging data were performed using High Definition Imaging (HDI) 1.6 (Waters Corporation, Manchester, UK). Data were acquired and mined using MassLynx^{TM} software version 4.2 (Waters Corporation, Manchester, UK).

Following the imaging of the 2D separation a number of regions of interest (ROI) were manually selected for more detailed examination of the mass spectrometric data that had been acquired on separated components of the extract to look for the presence of ecdysteroids.

3. Results

Separations for the plant extracts and ecdysteroid standards analysed in this study were, as described in the experimental section, undertaken using glass-backed C18 bonded silica gel HPTLC plates with either ethanol–water or acetone–water based solvent systems (both organic-water mixtures were 60:40 v/v). Ecdysone had Rf values of 0.56 and 0.46 in the ethanol and acetone-based solvent systems, respectively, whilst for 20-hydroxyecdysone the equivalent values were 0.63 and 0.64, respectively (Rf values for these RP-HPTLC systems, together with those obtained on silica gel HPTLC plates, for a range of ecdysteroids are provided in **Table S1** of the Supplementary Information).

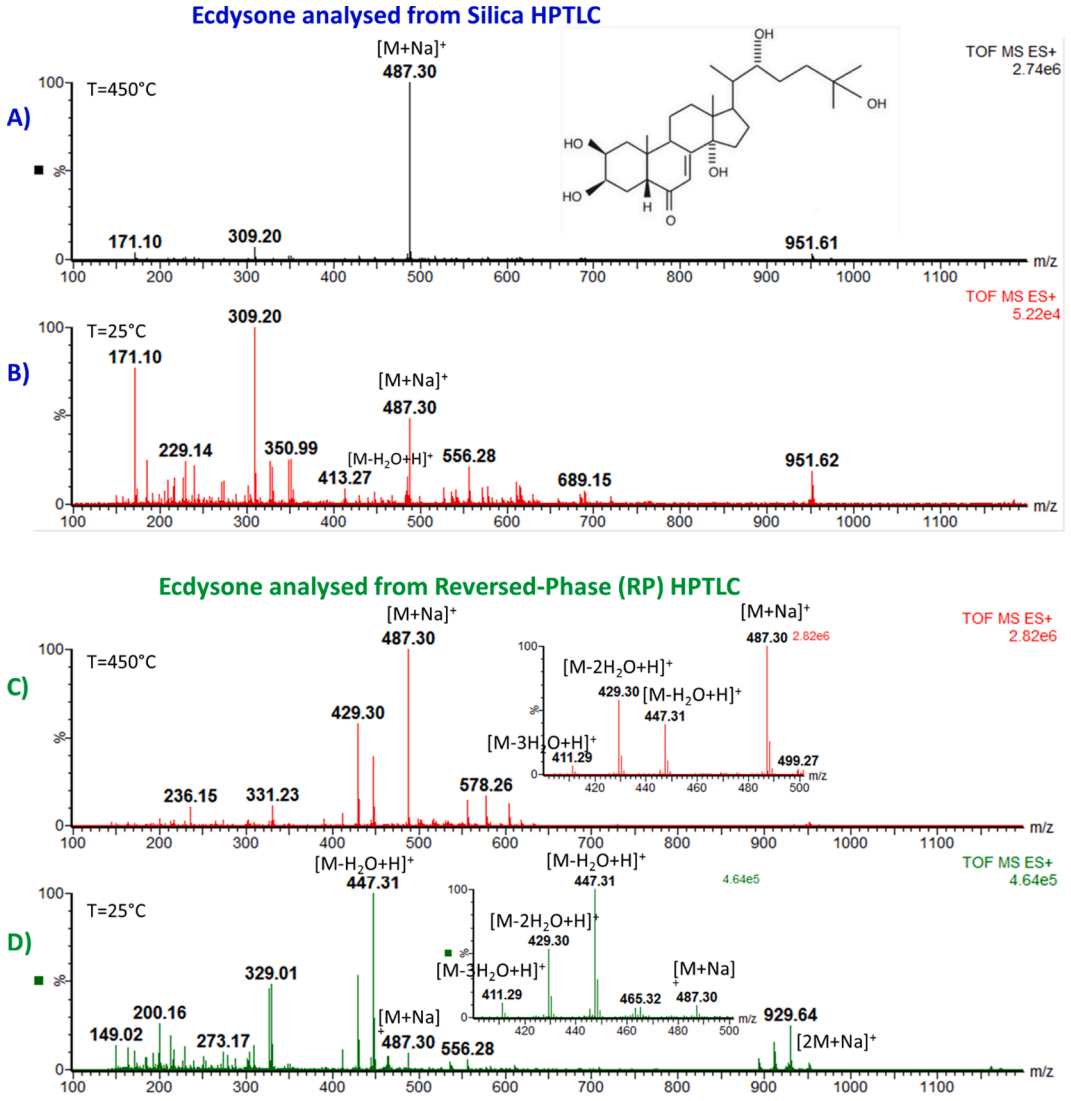
3.1. Effects of stationary phase and temperature on the DESI-MS of ecdysteroids

In our previous investigation of HPTLC/DESI/MS, carried out using silica based HPTLC, we found that the most intense ions obtained for ecdysteroids (such as ecdysone and 20-hydroxyecdysone) were the sodiated species, with little fragmentation observed [14]. As part of the present study, these DESI/MS experiments were repeated to enable a direct comparison with the RP-HPTLC data on the silica-HPTLC plates

that had originally been analysed in our previous investigation. Two temperatures, ambient (ca. 25 °C) and 450 °C, were used for the HTL responsible for transferring the analytes from the plate to the ion source of the MS. These investigations confirmed the original observations made for silica, and the resulting mass spectra for ecdysone are shown in Fig. 1. As shown in Fig. 1**A and 1B** the signal of the $[M + Na]^+$ ion for ecdysone increased in intensity significantly at 450 °C vs. ambient temperature. The $[M + H]^+$ molecular ion for ecdysone itself was absent. However, in the present study involving separation by RP-HPTLC, on a C18-bonded phase as opposed to silica, a somewhat different outcome was seen. Thus, when ambient temperature was used with the C18 bonded material, the most intense peaks detected for ecdysone corresponded to the single and multiple losses of H_2O (*m/z* 447.31, 429.30 and 411.29) (see Fig. 1**D**) with a low intensity signal for the protonated ($M + H^+$) molecular ion of ecdysone (*m/z* 465.32 present, as well as a low intensity ion corresponding to the Na^+ adduct. In contrast to the results obtained for silica, protonated and sodiated ions for ecdysone (and other ecdysteroids) were only detected at low intensity when C18 bonded silica formed the substrate (see Fig. 1 **D).** When the temperature used for the HTL was raised to 450 °C, the peaks corresponding to the characteristic losses of H_2O from ecdysone, were still detected, although not ecdysone itself, and the sodiated ion molecular ion once again became the most abundant peak in the MS spectrum, with a significant increase in intensity of the $[M + Na]^+$ molecular ion as shown in Fig. 1**C.** For both stationary phases, dimers resulting from ecdysone (*m/z* 929.64 corresponding to and $[2\ M-H_2O]^+$ (Fig. 1**C and D**) and *m/z* 951.62 to $[2\ M + Na]^+$ (Fig. 1 **A** and **B**) at 450 °C respectively) were also observed. Similar results were obtained for 20-hydroxyecdysone and are shown in supplementary **Figure S1.** However, for the initial screening/imaging of the ecdysteroids on RP-TLC plates the use of the HTL at 450 °C offered the advantage of greater sensitivity compared to the ambient mode. Thus, the HTL operated at the higher temperature, provided ca. 100 fold higher intensity signals than seen using ambient conditions, making it more suitable for rapid screening/imaging, and therefore this mode has been used here for MS imaging experiments.

3.2. 1D-RP-HPTLC/DESI/MS analysis of Silene (SO)extracts

The four methanolic extracts, one each from *S. otites* and *S. viridiflora* and 2 from *S. nutans*, together with ecdysone and 20-hydroxyecdysone standards (used as Rf markers), were separated by 1D RP-HPTLC using acetone- or ethanol–water based solvents as described in the



https://commons.wikimedia.org/w/index.php?search=20-Hydroxyecdysone&title=Special:MediaSearch&go=Go&type=image

Fig. 1. DESI-MS analysis of the ecdysone standard following HPTLC on silica and RP-HPTLC plates: A) Analysis from silica with the HTL at 450 °C, B) at 25 °C, C) analysis from C18 with the HTL at 450 °C, D) at ca. 25 °C.

experimental section. After preliminary evaluation by visualization under UV light (λ = 254 nm) MS-based DESI imaging was undertaken with no further sample preparation. Using this system, it is possible to image either particular regions, or the whole plate. However, clearly the larger the area to image, the longer it takes to complete the analysis. In these experiments, the DESI stage speed was 800 μm/s and 4 pixels/sec were acquired. For rapid analysis, at this speed, a single track would take only 2 min of DESI MS acquisition time. Here however, imagining the whole "active" area of the plate on which the extracts and standards were dispersed required ca. 10 hr of acquisition time.

As observed in our previous NP-HPTLC study of these extracts [14], ions corresponding to the sodiated $[M + Na]^+$ ions of 20-hydroxyecdysone (*m/z* 503.30), 2-deoxy-20-hydroxyecdysone/ecdysone/ponasterone A(*m/z* 487.30), 2-deoxyecdysone (*m/z* 471.31) and polypodine B (*m/z* 519.30), or other isobaric ecdysteroids were detected in some or all of these plant extracts. In addition to the sodiated molecular ions, it was also possible to detect ions corresponding to sequential losses of H_2O from the ecdysteroids present in the extracts as was noted for the standards. As is clear from the images in Fig. 2**,** which are composites of a number of different ions as indicated in the caption, the acetone and ethanol-based solvent systems show slightly different selectivities from each other, providing different "fingerprints" for the various extracts. Further images, generated from single ions, are provided in the supplementary data (using the *m/z* data of single ions).

The resulting spectra from a number of the bands present in the *S. otites* extract are shown in Fig. 3. These were for the sodiated ions corresponding to 20-hydroxyecdysone (*m/z* 503.30), 2-deoxy-20-hydroxyecdysone (*m/z* 487.30) and 2-deoxyecdysone (*m/z* 471.31). Such a result is consistent with previous publications (using both HPTLC [19] and HPLC/MS [20] and HPLC/NMR [21]-based analyses). However, interestingly, in addition to the bands containing ions consistent with the sodium adducts of 20-hydroxyecdysone (*m/z* 503.30) and 2-deoxyecdysone (*m/z* 471.31), two further bands were visible, of similar Rf, which corresponded in mass to sodiated 2-deoxy-20-hydroxyecdysone (or an isomer such as 5-α-2-deoxy-20-hydroxyecdysone) (*m/z* 487.30). The higher Rf component was shown to co-chromatograph with an authentic standard of 2-deoxy-20-hydroxyecdysone in both solvent systems. Examination of the mass spectra containing for these 2 components whilst indicative was somewhat inconclusive given the large number of other ions detected at the same Rf (see Fig. 3). Images showing the individual ions obtained using DESI/MS are provided in the Supplementary data (**Figure S2**).

3.3. 2D-RP-HPTLC/DESI/MSI analysis of a S. Otites extract

The use of 2-Dimensional separations in TLC, which enables the analyst to access the benefits of greater chromatographic resolution of components, remains a valuable and readily implemented tool for the characterization of complex mixtures. In order to demonstrate the utility of 2D-HPTLC in combination with DESI/MSI for improving spectral quality in this type of application, the *S. otites* extract was analysed by developing plates first with the acetone–water based solvent system followed by redevelopment in the 2nd dimension ethanol–water. The resulting UV_{254}-based image obtained following the 2D separation illustrated in Fig. 4 (upper) is shown together with the composite ion DESI/MS image Fig. 4 (lower). The DESI/MSI image of the HPTLC plate acquired, like the images for the 1D plates, over 10 hr, clearly reveals many more components than the UV-based image with respect to the complexity of the extract as non– or/poorly-UV_{254} absorbing components were also detected. Mass spectra were obtained for various circled regions of interest (ROI) from the DESI/MSI image (identified by "spot" numbers). Representative mass spectra obtained from the 2D separation shown in Fig. 4 are provided in Fig. 5 for four of these ROI (4,8,9 and 10). Mass spectra for the remaining ROI (1–3,6,7,11–18) are provided in the supplementary data. In addition to 20-hydroxyecdyone (ROI 10), 2-deoxy-20-hydroxyecdysone (ROI 8) (and the isobaric compound of slightly higher Rf (ROI 9, potentially the 5-α-isomer) and 2-deoxyecdysone (ROI 4) (mass spectra in Fig. 5**).** For the two MS spectra containing

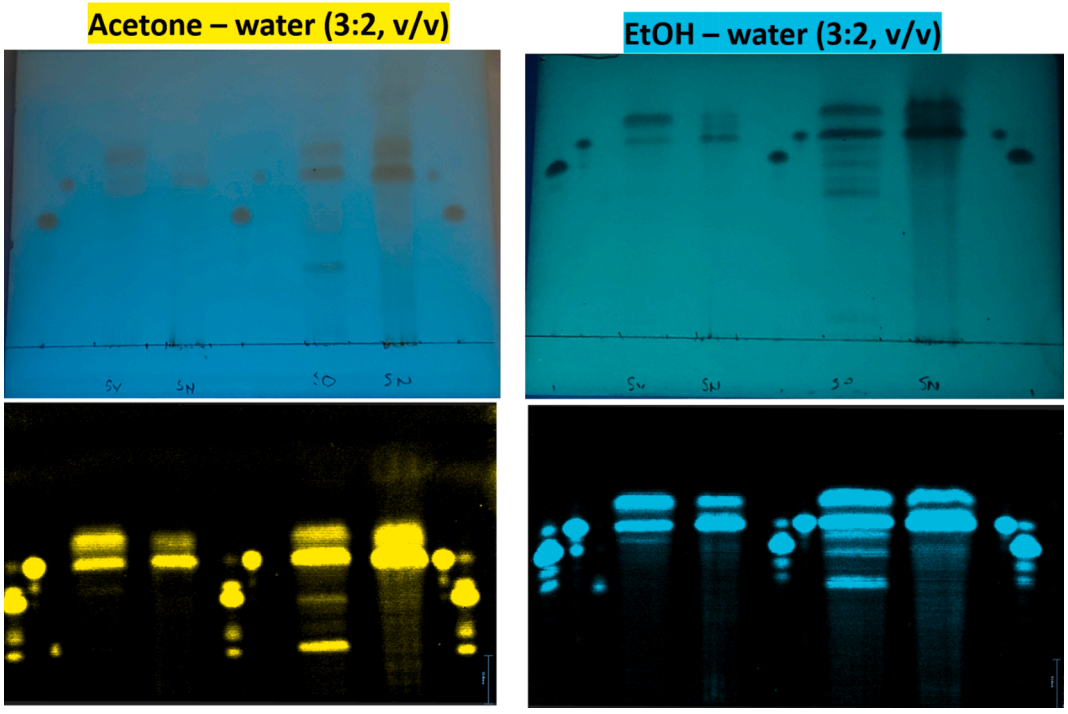


Fig. 2. Images for the UV_{254} (upper) and composite DESI overlay (lower) of the ions *m/z* 413.3, 427.3, 487.30, 497.3, 503.3, 517.3, 519.3 and 547.2 (HTL set at 450 °C). The plates were developed using acetone:water 60:40 (left) or ethanol:water 60:40 (right) using the same plate layout for samples for both. Starting from the left, ecdysone and 20-hydroxyecdysone as spots; partially purified extracts of *S. viridiflora* and *S. nutans,* as bands; ecdysone and 20-hydroxyecdysone as spots; partially purified extract of *S. otites* and crude extract of *S. nutans* as bands; 20-hydroxyecysone and ecdysone as spots.

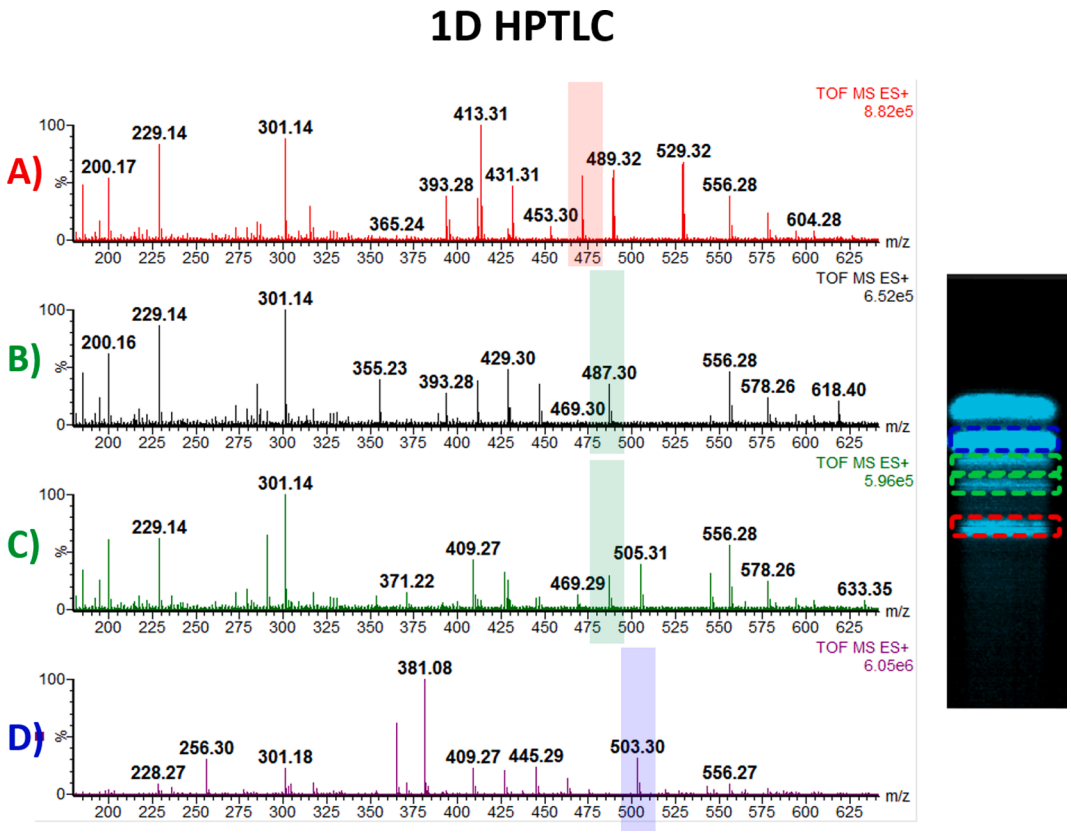


Fig. 3. Mass spectra (HTL at 450 °C) acquired from C18-bonded HPTLC plates, developed using acetone:water 60:40 (v/v) for the *S. otites* extract. The spectra show the sodiated ions corresponding to 2-deoxyecdysone (m/z 471.31) (A), then the two below to 2-deoxy-20-hydroxyecdysone (m/z 487.30) (B and C) and at the bottom 20-hydroxyecdysone (m/z 503.30) (D). The MSI of the HPTLC track for this extract is inset to the right of the spectra.

the ion *m/z* 471.31 (ROI 8 and 9) the improvements spectral quality provided by the 2D separation allowed them to be readily distinguished from each other. As indicated above, a 1D-HPTLC experiment using an authentic standard showed that the ecdysteroid with the lower Rf comigrated with 2-deoxy-20-hydroxyecdysone, increasing confidence in its positive identification. However, the identity of the remaining compound, and indeed confirmation that it indeed represents an ecdysteroid remains a matter of conjecture requiring further investigations beyond the scope of the present preliminary study.

The DESI/MS spectra for the other ROI, provided in the supplementary data, may well contain features indicative of the presence of other, minor, ecdysteroids of which many have been identified in this species (see ref [20]). However, to obtain further information would require additional MS and MS/MS-based studies (including applying the HTL at ambient temperatures). Such investigations would also perhaps also benefit from the use of ion mobility spectrometry (IMS) as described elsewhere [14] which is also beyond the scope of this preliminary work.

4. Discussion

The results provided here demonstrate that DESI combined with high resolution ToF/MS provides a rapid method for the profiling of plant extracts separated in either one or two dimensions on RP-TLC plates. Using the technique allowed the relatively rapid MS-based screening of such extracts for phytoecdysteroids. This enabled the more confident identification of known/expected compounds for the purposes of dereplication and highlighted potential regions of interest that might indicate the presence of novel phytoecdysteroids for further investigation. Intriguingly, significant differences were seen in the mass spectral information seen when using C18 bonded silica-based HPTLC plates compared to those seen for silica. Whatever the reason for these differences, the increased fragmentation observed when using a C18 bonded silica gel HPTLC plate, rather than one based on silica gel, may prove to be useful for identification and characterization purposes. Such differences also suggest that, depending on the purpose of the analysis, careful selection/screening of the type stationary phase used for the TLC separation might be appropriate.

In particular the $[M + Na]^+$ ions that dominated the silica-derived spectra were much less prominent than seen for the RP-HPTLC-derived results which instead showed an $[M + H]^+$ ion and significant fragmentation through loss of water. The reason(s) for these differences in mass spectrometric behaviour seen when imaging from either silica or C18 bonded silica HPTLC phases are not obvious and are deserving of further investigation. However, irrespective of the reason, the increased fragmentation observed when using a C18 bonded silica gel HPTLC plate, rather than one based on silica gel, may prove to be useful for identification and characterization purposes. Such differences also suggest that, depending on the purpose of the analysis, careful selection/screening of the type stationary phase used for the TLC separation might be appropriate.

Overall, whilst the use of HTL at different temperatures may be beneficial for structural identification, the significant increase in signal intensities, for both reference standards and sample components, regardless of the stationary phase used, means that in practice for initial screening, the use of the HTL at high temperatures is to be recommend. The high temperature mode provides the highest signal intensities, which facilitates analyte detection (including less abundant species) as well as increasing the speed of MSI analysis.

As indicated in the results, in addition to the detection of the expected 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone and 2-dexyecdysone in the *S. otites* extracts, it was also possible to profile a number of additional spots which could be further investigated for the

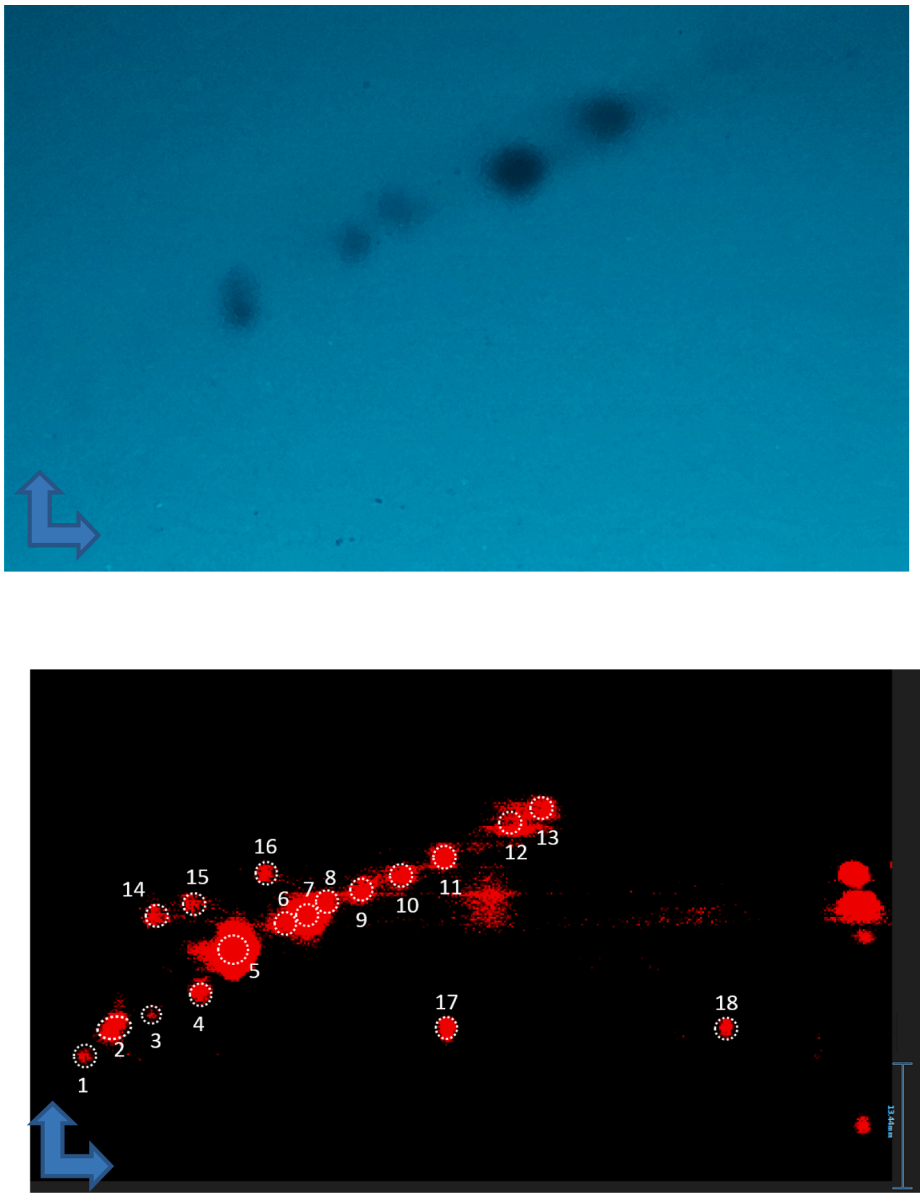


Fig. 4. Images of the 2D HPTLC separation of the *S. otites* (crude) extract following separation with the aqueous acetone and ethanol-based solvents used for the 1st (vertical) and 2nd (horizontal) dimensions respectively (acquired with the HTL set at 450 °C). The upper image shows the UV_{254} illuminated image whilst the composite overlay DESI/MS ion result, with regions of interest circled and numbered, is shown in the lower image. (Spectra for all of the ROI are provided in Supplementary information).

presence of phytoecdysteroid-like structures. However, by enabling the rapid identification of likely candidates for further *in situ* MS (and possibly IMS) experiments such information highlights targets for further investigation and characterization. The study also reinforces the benefits of 2D separations as an easily implemented means of improving the quality of the subsequent spectral data.

Based on the data acquired in this preliminary study, the use of this instrumental setup appears to be well suited to the relatively rapid imaging of both 1- and 2D-TLC plates. If only simple "lane" scanning of single tracks, rather than full images, were required then an analysis time of 2 min/track could form the basis of a relatively rapid screening program for natural product studies. Clearly on the basis of the qualitative studies performed here the ready detection of the ecdysteroids and low background from the RP-HPTLC plates observed here suggest that the use of this approach for quantification should also be possible. Investigation of this option will be the subject of future studies. As such RP (or NP) HPTLC/DESI/MS could represent an important addition to the armamentum of those performing dereplication analyses, seeking novel compounds, attempting to confirm authenticity or ensuring that plant-based supplements, such as those provided by various phytoecdysteroid-derived products, actually contain the correct ingredients in the amounts specified etc.

5. Conclusions

This study, using RP-HPTLC in combination with DESI/MSI provides another demonstration of the utility of the technique for both the MSI imaging and MS of ecdysteroids. HPTLC, used in this way, clearly provides an excellent method for the assessment of crude plant extracts for target compounds such as phytoecdysteroids without requiring the prior recovery of the analytes from the plate or the application of matrices for MS or imaging via MALDI. The differences seen in the mass spectra obtained after NP and RP-HPTLC are intriguing, and worthy of further investigation, including on alternative phases. DESI/MSI also provides detailed images for 2D-HPTLC which may prove especially useful where the differences in selectivity provided by the various solvent systems employed offer greater resolution of a complex mixture. Whilst applied here to plant extracts for ecdysteroids the technique has obvious applications in many other areas of phytochemistry and biochemistry.

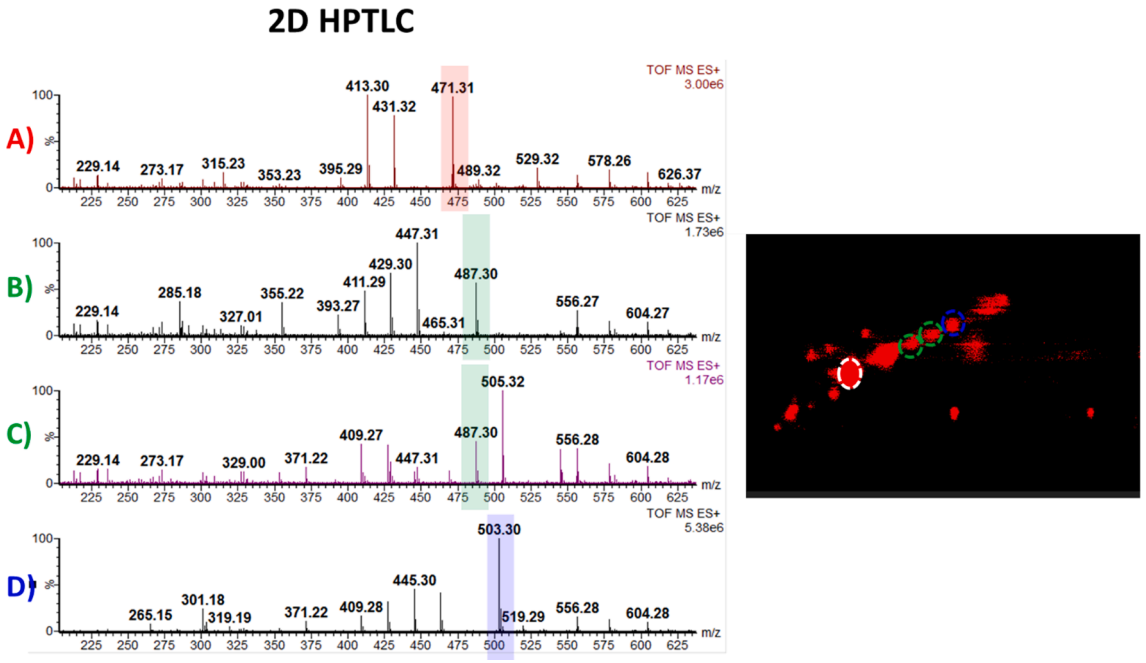


Fig. 5. Mass spectra (HTL at 450 °C) acquired from C18-bonded HPTLC plates, developed using 2D development, with acetone–water 60:40 (v/v) for the 1st dimension and ethanol:water 60:40 for the 2nd, for *the S. otites* extract. The spectra show the sodiated ions in Fig. 4 corresponding to 20-hydroxyecdysone (*m/z* 503.30, D; ROI 10), then the two below to 2-deoxy-20-hydroxyecdysone (*m/z* 487.31, B and C; ROI's 8 and 9 respectively) and 2-deoxyecdysone (*m/z* 471.31, A; ROI 4), together with ions corresponding to the dehydration of these molecules.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123265.

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