HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY (HPTLC) OF ECDYSTEROIDS PRESENT IN PLANT EXTRACTS COUPLED WITH *IN SITU* ANALYSIS AND IMAGING DESI/IMS/MS

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INTRODUCTION

The ecdysteroids form a group of polar, polyhydroxylated, steroids that are involved in arthropod development, particularly in regard to moulting. They have been also assessed for pharmacological properties such as e.g., anabolic effects in humans. When searching for phytoecdysteroids high performance thin-layer chromatography (HPTLC) provided a proven, robust and readily available method for screening extracts, of varying degrees of purity, but identification remains a problem. Moreover, ecdysteroids by nature contain numerous structural isomers when ion mobility separation adds another level of separation for more accurate characterization.

Here we describe the use of DESI/MS imaging (MSI) and DESI/IMS/MSI for the analysis of ecdysteroid-containing plant extracts, separated by HPTLC, as an example of its use for more complex mixtures.

METHODS

TLC chromatography preparation

Chromatography was performed by HPTLC on silica gel, on both ecdysteroid standards (aluminium backed HPTLC plates 5 x 7.5 cm) and plant extracts (glass backed silica gel HPTLC plates, 10 x 10 cm) incorporating a fluorescent indicator (E. Merck, Darmstadt, Germany). Plate development was performed using glass TLC tanks, in a fume hood, in with chloroform: ethanol 4:1 V/V as the developing solvent. Samples were applied to the plates manually using 1µl glass capillaries (Drummond Scientific Company, Broomall, Pa, USA). The quality of the separations achieved was assessed prior to further MS-based analysis by visualizing the plates under a UV lamp at 254 nm and, if suitable, were then taken for DESI.

Mass spectrometry

Experiments were performed using a SYNAPT XS High Definition MS (HDMS) system (Waters Corporation, Manchester, UK) and equipped with a 2D DESI stage from Prosolia (Indianapolis, IN, USA).

Ion Mobility MS:

Ionisation mode:	Positive
Mass range:	m/z 100-1,200
Trap DC bias:	50 V
IMS wave velocity:	Start 1,000 m/s, End 250 m/s
Transfer wave velocity:	175 m/s

DESI-MS:

Flow rate:	2 µl/min
Solvent composition:	MeOH/H_2O 98/2 (*v/v*)
Capillary voltage:	4 kV
Nebulising gas:	5 bar
Pixel sizes:	200 µm (lateral)

Data management

Acquisition setup, processing and visualisation of imaging data were performed using High Definition Imaging (HDI) 1.5 (Waters Corporation, Manchester, UK). Data were acquired and mined using MassLynx version 4.2 (Waters Corporation, Manchester, UK). Further data mining was carried out using DriftScope 2.9 (Waters Corporation, Manchester, UK) to visualise the IMS dimension of the data.

RESULTS

Five ecdysteroid standards were firstly analysed, spotted onto a Prosolia Teflon coated glass slide to ensure that the molecules could be detectedby DESI/IMS/MS. Secondly, the standards were then chromatographed individually on a glass-backed silica gel HTPLC plate.

In summary all standards were all detected as sodiated species with no or little presence od the protonated or potassiated species as seen in figure 1A. Isobaric Ecdysone and Ponasterone A were separated by TLC and ion mobility with retardation factor (Rf) and drift time (Dt) respectively being R_f 0.31/Dt 5.1 ms and Rf 0.55/Dt 5.37 ms (figure 1) . However isobaric Inokosterone and 20-hydroxyecdysone shared the similar R_f 0.25 and Dt 5.17 ms.

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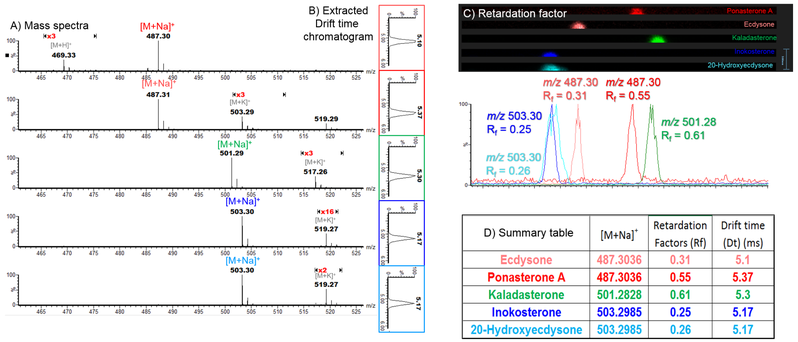

D) Summary table	[M+Na]+	Retardation Factors (Rf)	Drift time (Dt) (ms)
Ecdysone	487.3036	0.31	5.1
Ponasterone A	487.3036	0.55	5.37
Kaladasterone	501.2828	0.61	5.3
Inokosterone	503.2985	0.25	5.17
20-Hydroxyecdysone	503.2985	0.26	5.17

Figure 1: A and B) MS spectra of each ecdysteroid standards (ponasterone A, ecdysone, kaladasterone, inokosterone and 20-hydroxyecdysone) at the specific Drift time (Dt) of the sodiated molecular ion. C) Ion images of the five ecdysteroid standards chromatographed by TLC five and directly analysed by DESI MSI, with the overlay of extracted ion chromatograms of the ecdysteroid standards, D) summary table.

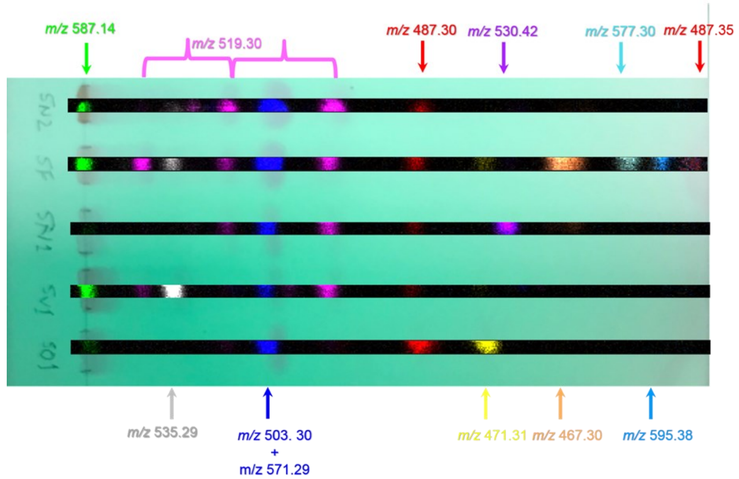

Figure 2: Overlay of the UV absorbing trace of the TLC plate before DESI acquisition with the ion images of the different colour coded spots of different m/z for S, otites (SO1), S. viridiflora (SV1), two S. nutans (SN1 and SN2) and the S. fimbriata plant extracts.

Five methanolic plant extracts (*S.fimbriata*) were subject to HTPLC separation and examined under UV light (λ=254 nm) and imaged by DESI IMS MS. From the UV trace, a number of compounds with strong UV absorbion (figure 2) were detected with the most intense spot being at a R_f of 0.28 with the highest MS peak at *m/z* 503.29. Based on mass accuracy and R_f, it could therefore be putatively identified as either inokosterone or 20-hydroxyecdysone.

Interestingly three TLC spots were detected for *m/z* 519.29 at R_f's of 0.09, 0.23 and 0.39 (figure 2) which one could corresponds to polypodine B (PPB) ($C_{27}H_{44}O_8$, Na^+) which is a very common phytoecdysteroid in plants.

When PPB standard was analysed by DESI/IMS, one IMS peak was detected for *m/z* 519.29 with a Dt of 5.43 ms (figure 3,A1). In Figure 3,A2, the extracted mobilogram for *m/z* 519.29 for *S. fimbriata* extract shows three peaks with Dt's of 4.7, 5.23 and 5.43 ms respectively. The peak with the greatest intensity was found to have a Dt of 5.43 ms and can be putatively identified as polypodine B on the basis of mass spectral and drift time data.

According to the Ecdysteroids database (http://ecdybase.org), there are at least 24 different isomers reported in the literature. The two *S. nutans* extracts (SN1 and SN2) had the same IMS profile and DESI traces (figure 3,B2 and B3) showing 8 different DESI TLC spots sharing the three different drift

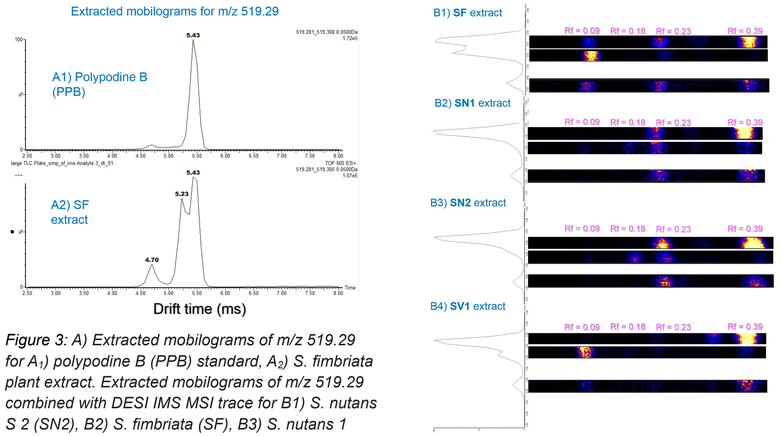

Figure 3: A) Extracted mobilograms of m/z 519.29 for A_1) polypodine B (PPB) standard, A_2) S. fimbriata plant extract. Extracted mobilograms of m/z 519.29 combined with DESI IMS MSI trace for B1) S. nutans S 2 (SN2), B2) S. fimbriata (SF), B3) S. nutans 1 (SN1) and B4) S. viridiflora (SV1) plant extracts.

CONCLUSION

- **DESI/MS coupled with imaging of HPTLC plate was an effective method for preliminary screening of plant extracts for target compounds such as ecdysteroids.**
- **More analytes were detected by DESI MSI compared to the equivalent TLC UV absorption traces, allowing a richer fingerprints of the plant extracts.**
- **Deeper differentiation between extracts was achieved with the incorporation of ion mobility to separate ecdysteroid isomers.**