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Short communication

GC–MS and LC–MS/MS workflows for the identification and quantitation of pyrrolizidine alkaloids in plant extracts, a case study: *Echium plantagineum*

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ABSTRACT

Workflows based on gas and liquid chromatography coupled to mass spectrometry for the identification of toxic pyrrolizidine alkaloids present in plants were developed and applied to *Echium plantagineum* L., Boraginaceae, extracts. GC–MS based determinations need reduction and derivatization steps prior to MS analysis, which is performed using a Full Scan and Single Ion Monitoring sequence for screening, identification and quantification purposes. The LC–(ESI)–MS/MS determination was performed directly from the extract without derivatization. Acetyl lycopsamine, echimidine, echimidine *N*-oxide, echiumine, echiumine *N*-oxide, lycopsamine, lycopsamine *N*-oxide, 7,9-ditigloylretronecine *N*-oxide and a not reported PA of *m/z* 466, were identified and quantified in *E. plantagineum* extracts, through three operating modes of LC-QTRAP: precursor ion scan, enhanced product ion scan and multiple reaction monitoring. Precursor ion scan detects all the ions that give rise to a daughter ion at *m/z* 120, the presence of the parent pyrrolizidine alkaloid is confirmed through its MS^2 spectrum (enhanced product ion) and quantified by multiple reaction monitoring. These workflows are general approaches to study chemical families using GC/LC-MS. For extracts suspicious of containing pyrrolizidine alkaloids, they are suitable tools for the quality and safety control of food, feed as well as phytotherapeutics.

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Introduction

Pyrrolizidine alkaloids occur as free necines or as mixtures of bases and their *N*-oxides (PANO). They can form single esters at C-9 or C-7, diesters at both C-7 and C-9, or macrocyclic lactone diesters linking C-7 with C-9.

The 1,2-unsaturated pyrrolizidine alkaloids show high acute and chronic toxicity as well as genotoxic properties (Mattocks, 1986). Macrocyclic pyrrolizidine alkaloids are more toxic than diesters, which are more toxic than monoesters (Codex Alimentarius Commision, 2011a).

There is increasing evidence that pyrrolizidine alkaloids can contaminate commonly consumed foods (grain, milk, meat, eggs, and honey). Therefore, the Joint FAO/WHO for food standards program of the Codex Alimentarius Commission (2011b) encouraged to develop analytical reference standards for pyrrolizidine alkaloids to enable the development of analytical methods, to gather data on pyrrolizidine alkaloids occurrence and perform their risk assessment.

Since analytical standards for the more than 300 pyrrolizidine alkaloids described until now are difficult to obtain, alternative routes are needed. High resolution chromatography (LC or GC) coupled to mass spectrometry could help to obtain standardized extracts from pyrrolizidine alkaloids containing plants. We present two workflows that enable the identification and quantification of toxic pyrrolizidine alkaloids when analytical standards are not available.

Materials and methods

All chemicals were HPLC or pa. grade. Acetyl lycopsamine standard was purchased from Phytolab GMBH (Germany).

GC-MS analysis were performed on a Hewlett Packard 6890 GC with a HP-5MS, 2,5 µm film, 30 m × 0.25 mm i.d, analytical col-

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umn, coupled to a single quadrupole mass spectrometer detector HP 5973 (Agilent Technologies,USA) with electron impact source (EI) and NIST 11 spectral library (NIST, 2018).

LC-MS/MS analysis were performed on a 1260 Infinity (Agilent Technologies, USA) HPLC fitted with a Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, 5 μm), coupled to a 4000 QTRAP® System (Sciex, Canada) with electrospray ionization (ESI) source.

The aerial parts of *Echium plantagineum* L., Boraginaceae, were collected. A voucher sample (MVFQ 4428) was identified and deposited at the Herbarium Jose Arechavaleta, Facultad de Química, Universidad de la República.

Two extracts of *E. plantagineum* were obtained

a) RT air dried plant material (870 g) were finely comminuted and extracted overnight with 10 l HCl 1% (v/v). After filtration, Zn granules were added and let to react for 1 h at 60 °C and left overnight at RT. The pH was adjusted to 9 with NH_4OH and the solution extracted with chloroform until colorless reaction to Draggendorff's reactive. Solvent evaporation yielded 1.44 g of the extract. Dry extract (100 mg) were dissolved in 10 ml of HCl 2% (v/v) in MeOH and stirred with 2 g of Dowex™-50WX2 (Dow Chemical,USA) resin during 15 h. The resin was eluted with basic KCl 3 M at pH 9-10. The eluate was extracted with chloroform as above.
b) Fresh aerial parts of *Echium plantagineum* (68 g) were comminuted and extracted with 500 ml of HCl 2% (v/v) under N_2 atmosphere. The extract was lyophilized and 4.44 g were reconstituted in 35 ml of MeOH.

For GC analysis, the organic extract was reconstituted in 1 ml of dry chloroform, N_2 was bubbled and 50 μl of bis-trimethylsilylacetamide was added and stirred for 30 min at 40 °C.

GC-MS was operated under full scan acquisition that enabled spectral library comparison and SIM mode (selected ion monitoring). The injection volume was 1 μl at split (1:20) mode, with He as carrier gas and the mass spectra were recorded at 70 eV. Kempf et al (2008) procedure was followed.

Both extracts were analyzed by liquid chromatography coupled to mass spectrometry.

The mobile phase was composed of (A) 0.1% formic acid in water and (B) acetonitrile at a flow rate of 0.5 ml/min. The HPLC conditions were those described by Avula et al. (2012) with modifications. The elution gradient was: 0 min, 87% A/13% B isocratic for 1 min, next 7 min to 50% A/50% B and 7 min to 100% B. Afterwards, 3 min equilibration with 87% A/13% B. Injection volume 10 μl.

ESI source was operated in positive ion mode with a capillary voltage of 3500 V under standard conditions, optimized for acetyl lycopsamine and the extracts obtained. The LC–MS/MS was run in multiple ions detection in the first quadrupole (Q1 MI) with declustering potential of 125 V, enhanced product ion scan (EPI) with collision energy of 10 KeV, precursor ion scan (PIS) and multiple reaction monitoring (MRM).

Results and discussion

GC–MS has been the most popular method to determine pyrrolizidine alkaloids as commercial libraries at 70 ev EI mass spectra can be used for identification purposes (El-Shalzy et al., 1996; Pedersen and Larsen, 1970). The organic solvent extract showed four compounds with a base peak at *m/z* 220 and also the ion series, *m/z* 136, 120, 93 characteristics of 1,2-unsaturated diester pyrrolizidine alkaloids. The base peak at *m/z* 220 is the result of the cleavage of the allylic ester bond (Pedersen and Larsen, 1970). Results are shown in Table 1.

Echiumine and echimidine were identified through spectral data comparison with bibliography (El-Shazly and Wink, 2014; NIST, 2018). Echimidine was also identified as the TMS derivative. It showed ions at *m/z* 93,120, 136 and 220, characteristic of open chain diesters type pyrrolizidine alkaloids, in addition to the ion M + at *m/z* 395, the tri-TMS acid at C-9.

The compound at t_R = 10.4 min was not identified through GC-MS. However, the LC-MS/MS analysis permitted to assign it to an angeloyl (or tigloyl) derivative of echiumine as discussed below.

The proposed working protocol to characterize pyrrolizidine alkaloids is to identify them through a post run analysis scheme, using the Reconstructed Ion Chromatogram (RIC) sequence looking for the coincidences at *m/z* 136, 120 and 93 ions in full scan chromatogram, the main fragmentation pathway of the unsaturated necine bases (El-Shalzy et al., 1996). Further analysis through SIM acquisition mode allows the precise quantitation of pyrrolizidine alkaloids present in these extracts.

SIM monitoring of highly specific pattern such as the one exhibited by *m/z* 136, 120 and 93 can be used also as a general screening technique of unsaturated pyrrolizidine alkaloids.

LC-MS/MS has been applied to pyrrolizidine alkaloids analysis recently (Hwan Yoon et al., 2015; Mroczek et al., 2006), taking the advantage that extracts can be analyzed as such (Fragoso-Serrano et al., 2012). Tandem mass spectrometry, either QqQ or Qtrap configurations opened a palette of operational procedures useful to find structurally related compounds.

EPI experiment allows to select ions of specific *m/z,* to fragment and to drive them to the detector, giving a MS^2 spectrum whose spectral pattern can be used for comparison as traditional EI-MS (70 ev) spectrum. The characteristic ions of *m/z* 220 and 120 are due to the necine nucleus after the loss of the acid in C9 and C7, respectively, as well as the ions of *m/z* 138 and 94 which are typical of an unsaturated necine with a hydroxyl in C7 suggesting a lycopsamine structure. These ions allowed the detection of the presence of lycopsamine, lycopsamine *N*-oxide, echimidine, echimidine *N*-oxide and echiumine, by comparing their spectral data with literature (Siciliano et al., 2005; Mroczek et al., 2006; Liu et al., 2009; Carvalho et al., 2013; Hwan Yoon et al., 2015). Acetyl lycopsamine and 7,9-ditiglioyl retronecine *N*-oxide were confirmed after optimization of DP and CE conditions (Table 2).

In the PIS experiment, all ions that give rise to a daughter ion of a specific *m/z* are selected and registered. Toxic alkaloids contain an unsaturated pyrrolizidine which yields a characteristic ion at *m/z* 120. Scanning for the precursor ions of the fragment *m/z* 120 showed $[M+H]^+$ ions at *m/z* 382.5, 398.4, 420.6 and 466.2. They correspond to the main alkaloids present in the extract: echiumine, echimidine, and sodium echimidine respectively for the former three ions, which is in agreement with previous findings for *Echium* genus (El-Shazly and Wink, 2014). Additionally, an unknown compound of $[M+H]^+$ 466.2 was detected. None of the 232 possible pyrrolizidine alkaloids listed in Pubchem showed such a molecular weight. According to the possible structures present, we looked for acylatedechiumine derivatives. A $[M+H]^+$ 466 could correspond either to an alkaloid $C_{24}H_{35}O_8N$ or $C_{25}H_{39}O_7N$. The former formula corresponds to a diacetylatedechiumine and it was ruled out, as the loss of water could not be explained, leaving as the only possibility a monoacylatedechiumine with angelic acid which was tentatively confirmed after careful inspection ofthe MS^2 spectrum. The proposed structure (where R1 and R2 where angelic acid and/or H) and the EPI experiment are shown in Fig. 1. We suggest that the acyl angelate lies at 2-C of the *treo* structure of the necic acid. The structure is proposed based in the common fragmentation pathways for ESI in MS/MS according to the comprehensive description of the degradation mechanisms for pyrrolizidine alkaloids described in the literature (Hwan Yoon et al., 2015; Demarque et al., 2016). The positive charge is located at the heterocyclic nitrogen and

Table 1
GC–MS data from the pyrrolizidine alkaloids found.

Compound	Retention time (min.)	Characteristic ions *m/z* (relative abundance)
Echiumine	7.3	220 (100), 136 (69.9), 120 (39.8), 93 (49.6)
Echimidine	9.8	220 (100), 136 (42.2), 120 (54.4), 93 (34.5)
TMS echimidine	10.1	220 (100), 136 (29.7), 120 (64.0), 93 (20.1)
Unknown	10.4	220 (100), 136 (35.8), 120 (47.1), 93 (28.2)

Table 2
LC/MS-MS results. Multiple reaction monitoring conditions, characteristic fragmentation ions and quantification results.

Compound	Transition	DP (V)	CE (KeV)	Characteristic ion *m/z*	Organic solvent extract (mg/l)[a]	Aqueous extract (mg/l)[a]
Acetyllycopsamine	342.3 – 198.4 342.3 – 138.3 342.3 – 120.2 342.3 – 94.2	53	38 36 36 60	198.4, 138.3, 120.2, 94.2	6.31	3.34
Echimidine	398.3 – 220.3 398.3 – 120.3	75	22 35	380.6, 336,7, 220.4, 119.8	109.2	3.82
Echimidine *N*-oxide	414.4 – 396.4 414.4 – 254.0	80	35 41	396.2, 352.3, 254.2, 220.0	1.23	13.11
Echiumine	382.5 – 220.3 382.5 – 120.3	51	25 38	220.1, 119.8	107.1	3.13
Echiumine *N*-oxide	398.3 – 220.4 398.3- 120.2	80	22 35	220.4, 120,2	0.09	1.72
Lycopsamine	300.2 – 138.2 300.2 – 120.3	60	30 32	137.8,119.8, 93.8	0.19	1.81
Lycopsamine *N*-oxide	316.3 – 138.2 316.3 – 94.0	118	29 44	138.3, 120.2, 94.2	0.21	2.15
7,9-ditigloylretronecine *N*-oxide	336.0 – 138.2 336.0 – 120.2	60	42	254.2,156.0,138.1,120.	0.08	ND

ND, not detectable.
[a] Expressed in mg/l of acetyllycopsamine.

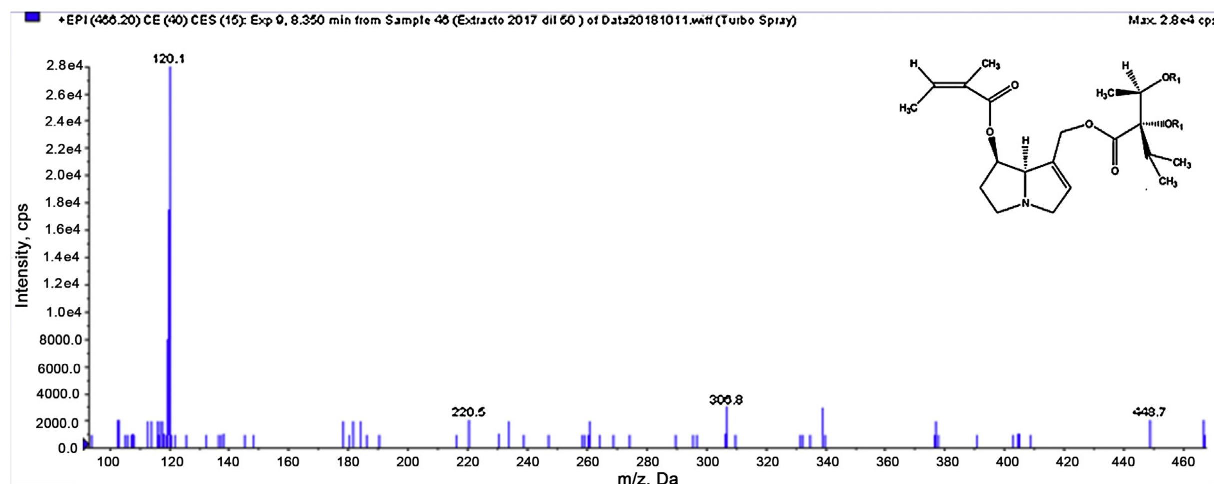

Fig. 1. Proposed structure and EPI experiment of *m/z* 466 compound (R1 is indistinctly either H or $C_5H_7O_2$ groups).

neutral losses are seen (Hwan Yoon et al., 2015). The characteristic pyrrolizidine alkaloids ions of *m/z* 220 and 120 of open chain diesters are present. Loss of water [M + −18] followed by CO_2 extrusion (Hwan Yoon et al., 2015), yields the signal at 405 amus that after losing angelic acid yields the signal at 306. The ion at 405 amus, can also suffer the loss of an ethylene group yielding the ion at 377 amus. These mechanistic pathways could confirm that the hydroxyl group at C-3 of the acid is free and the angelic acid esterify the hydroxyl at the quaternary carbon, α- to the carboxylic acid. More experiments are needed to definitively prove this assumption.

Pyrrolizidine alkaloids and pyrrolizidine alkaloids *N*-oxide in both extracts were quantified through MRM using two ions, a qualifier (for identification) that corresponds to the daughter ion giving the less intense signal and the most intense one, the quantifier (for quantification purposes) (Sante, 2017).The optimized conditions for MRM monitoring the pyrrolizidine alkaloid and pyrrolizidine alkaloid *N*-oxide content of the two extracts are presented in Table 2.

Pyrrolizidine alkaloids quantitation was performed using a six points linear calibration function (area = 11,467,656; conc. + 86,536 (R^2 = 0,998)) of acetyl lycopsamine in acetonitrile from 0.042 to 0.55 mg/l. The PAs concentration were expressed as acetyl lycopsamine.

As the aqueous extract was obtained under N_2 atmosphere and lyophilized, the relationship between *N*-oxides and tertiary bases should not be modified by air oxidation and it represents the occurrence of these compounds in the plant. This study is only possible with the LC–MS/MS workflow. Additionally, it was observed that

not all the pyrrolizidine alkaloids *N*-oxide are recovered quantitatively during Zn reduction, as in the case of lycopsamine.

Pyrrolizidine alkaloids *N*-oxide are the predominant species in the aqueous extracts, in agreement with previous findings (Hartmann et al., 1989; Hartmann and Dierich, 1998; Boppre, 2011). Nevertheless, free echiumine predominated over its *N*-oxide in this extract.

Different strategies were presented to produce a standardized extract of pyrrolizidine alkaloids from plants. GC-MS based workflow requires time consuming derivatization steps and acid reduction that did not yield quantitative results. LC-MS/MS was the most suitable one, as the extract can be analyzed directly, providing structural information, which is relevant in toxicity assessment.

The proposed LC-MS/MS workflow is: first to look at the PIS, then, to perform the EPI experiments to get the MS^2. The MS^2 of each compound allows the selection of the main ions to perform the MRM for pyrrolizidine alkaloids quantification. This sequence allowed the characterization of several pyrrolizidine alkaloids and pyrrolizidine alkaloids *N*-oxide in the plant extracts and even a non-described pyrrolizidine alkaloid for *Echium* spp. was identified. The method is applicable in general for the characterization of families of compounds that bear common structural features.

These protocols permit to identify pyrrolizidine alkaloids in extracts of plants and to standardize their content. The standardized extracts can then be used as secondary standards seeking the trace determination of pyrrolizidine alkaloids for the safe consume of food, feed and phytomedicines.

Conflicts of interest

The authors declare no conflicts of interest.

Author's contributions

AS (PhD student) contributed in collecting plant samples, the chromatographic, data analysis, and drafted the paper. AP contributed to the GC-MS analysis, data discussion and revision of the manuscript whereas SN helped in the LC-MS experimental design and determinations as well as on the analysis and discussion of the data. HH participated in the experiments planning, supervised the laboratory work, the structure elucidation of the unknown compound and contributed to critical reading and improvement of the manuscript. All the authors have read the final manuscript and approved its submission.

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