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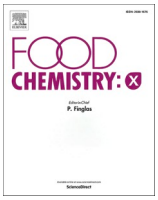

Fingerprinting alkaloids for traceability: Semi-untargeted UHPLC-MS/MS approach in raw lupins as a case study

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ABSTRACT

Lupin seeds are recognized for their nutritional value and potential health benefits, but they contain also a significant amount of alkaloids, an anti-nutritive class of compounds, which vary significantly in composition within and between species due to environmental factors. In this study, we developed a predictive multi-experiment approach using ultra-high performance liquid chromatography coupled with triple quadrupole with linear ionic trap tandem mass spectrometry (UHPLC-QqQ-LIT-MS/MS) for comprehensive alkaloid profiling and geographical classification of *Lupinus albus* L. samples originating from four different Italian regions. Six targeted quinolizidine alkaloids were detected and 21 other alkaloids were putatively identified. Hierarchical Cluster Analysis (HCA) and partial least squares discriminant analysis (PLS-DA) were applied to explore the data structure and successfully classify samples according to their geographical origin. The data demonstrate the efficacy of the developed approach in providing valuable insights in alkaloid profiles of lupin seeds and their potential as markers for geographical traceability.

1. Introduction

Lupin seeds are the edible portion of lupin plants, belonging to the genus *Lupinus*, within the Fabaceae family (Petterson, 2016). They are rich in proteins (>40 %) and fibers (>28 %) and traditionally used as animal feed. Due to their nutritional properties, they are increasingly considered as a cost-effective alternative to other legume such as soybeans. Moreover, they are gaining significant global interest for human gluten-free and plant-based diets, given their nutritional value and their low-fat content (<6 %) (Eugelio et al., 2024; Johnson, 2017; Khan et al., 2015). Lupin seeds also offer other nutrients like minerals and vitamins (Khan et al., 2015), along with bioactive phytochemicals such as polyphenols, phytosterols and squalene, which provide antioxidant and anti-inflammatory properties.

Numerous health benefits for the prevention and treatment of chronic diseases such as dyslipidemia, hyperglycemia and hypertension have been reported (Arnoldi et al., 2015; Van De Noort, 2017). Compared to other legumes, lupins can grow in areas with potential environmental stress and in a wide range of climatic conditions (Pereira et al., 2022). They are also recognized for their role in sustainable agriculture due to their nitrogen-fixing ability, which enhances soil fertility and reduces the need of synthetic fertilizers (Shrestha et al., 2021). Various lupin species are primarily considered in Europe, with *Lupinus albus* L. (*L. albus* L.) being particularly nutritionally valuable (Sujak et al., 2006). This species are suitable for a wide range of climatic conditions (Sujak et al., 2006), even it is mainly found around the Mediterranean area (Gresta et al., 2023).

Lupins contain also antinutritive compounds such as alkaloids, a group of neurotoxic secondary metabolites synthesized in the aerial parts of the plants and stored in their seeds (Boschin et al., 2008). These alkaloids act as defense mechanism against herbivores, pests, competing plants and microorganisms, owing to their typical bitter taste and toxic effects (El-Shazly et al., 2001). Lupin alkaloids (LAs) mainly include lysine-derived quinolizidine alkaloids (QAs) with a C_5NC_4 skeleton, in bicyclic, tricyclic or tetracyclic forms (Aniszewski, 2015). Additionally, some lupin species may produce bipiperidine alkaloids (BAs), like ammodendrine, and tryptophan-derived indole alkaloids (IAs), like gramine (Wink et al., 1995).

LAs levels can vary within species, upon the influence of different factors such as geographical origin, soil composition, climate and stress

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factors (Bundesinstitut Für Risikobewertung., 2017).

Most lupin species have a distinct alkaloid profile, known as "fingerprinting", comprising both major (relative abundance >1 % of total LAs) and minor alkaloids (relative abundance <1 % of total LAs) (Święcicki et al., 2019). The most abundant and distinctive LA in L. *albus* L. is lupanine, which contains two condensed quinolizidine units and may often contain a hydroxyl group as substituent (hydroxylupanine), existing in different isomeric forms. Other QAs include albine, multiflorine, and minor amounts of sparteine, angustifoline and some esters of lupanine such as angeloyl-, tigloyl-, cinnamoyl- or pyrroyl- moieties (Annicchiarico et al., 2014; Cely-Veloza et al., 2023).

The QAs biosynthetic pathway, which is species- and organ-specific, has not been fully elucidated considering that reported biosynthetic hypotheses during the last few decades are usually not always in accordance (Mancinotti et al., 2022; Ramírez-Betancourt et al., 2021). However, significant aspects have been identified, including the presence of key enzymes for QA biosynthesis (Wink, 1987; Wink & Hartmann, 1979). Some studies have extensively explored the LA profile of over 50 lupin species, characterizing about 100 alkaloids structures according to their mass fragmentation patterns using capillary gas-liquid chromatography-electron ionization-mass spectrometry (GLC-EI-MS) (Wink et al., 1995). While EI-MS has been a primary tool for in-depth LA characterization (El-Shazly et al., 2001), collision induced dissociation (CID), as analysis tool, has been less investigated.

To the best of our knowledge, few methods have been developed using high-performance liquid chromatography (HPLC) coupled to tandem MS (MS/MS) for comprehensive profiling of LAs or confirming geographic classification *via* multivariate regression models (Eugelio et al., 2023; Hwang et al., 2020). Few investigations provided a comprehensive understanding of mass fragmentation spectra of some lupanine, multiflorine and sparteine derivatives, often leveraging EI-MS and establishing the basis for distinguishing isomers and metamers (Wyrzykiewicz et al., 2000; Wyrzykiewicz & Wysocka, 1990). Despite the ongoing necessity of GC–MS to confirm the presence of QAs, when reference analytical standards are unavailable (Namdar et al., 2024), limited studies have employed CID spectra (Jasiewicz & Wyrzykiewicz, 2009, 2011). Furthermore, LC-MS/MS approaches are hindered by the scarcity of reference analytical standards, which limits the ability to achieve a comprehensive LA profile (Bundesinstitut Für Risikobewertung., 2017).

In this scenario, the proposed study introduces, for the first time, the development of a predictive multi-experiment approach combined with multivariate analysis to thoroughly survey the LA profile and determine the geographical origin of L. *albus* L. samples. The method employs ultra-high performance LC (UHPLC) coupled to a hybrid triple quadrupole-linear ion trap (QqQ-LIT) mass spectrometer, combining a survey scan in multiple reaction monitoring (MRM) with information-dependent acquisition (IDA) criteria, triggering experiments as enhanced product ion (EPI) (Palmieri et al., 2022). MRM transitions were generated by *in silico* experiments using a Competitive Fragmentation Modeling for Metabolite Identification (CFM-ID) (Wang et al., 2022) and included in an in-house MS/MS database. Unknown compounds were putatively identified by comparing MS/MS spectra acquired in EPI mode with those in the in-house database, simultaneously determining 6 targeted QAs and putatively identifying 21 semi-untargeted LAs, whose CID fragmentation spectra had not been previously reported.

Considering the variability in alkaloid profiles, some studies have suggested the use of LAs as traceability markers, showing a significant genetic variation in alkaloid content based on species and geographical origin (Boschin et al., 2008; Święcicki et al., 2019). Therefore, the developed approach was applied on raw L. *albus* L. samples from four different regions of central and southern Italy. An unsupervised chemometric approach based on Hierarchical Cluster Analysis (HCA) was applied, followed by a supervised Partial Least Squares Discriminant Analysis (PLS-DA), since the advantages of multivariate analysis and the employment of chemometric models were already demonstrated in other studies to explain phytochemical diversity (Palmieri et al., 2019; Turi et al., 2015). The PLS-DA model accurately classified these samples according to their geographical origin, highlighting the significant differences in alkaloid profiles and the substantial contribution of the putatively identified LAs.

2. Materials and methods

2.1. Raw lupin samples

Different raw L. *albus* L. seeds were harvested and collected in 2022 year, at commercial maturity, from different central and southern Italy regions, such as Abruzzo (Gida Srl, Castel di Sangro, AQ), Lazio (Azienda Imperoli Emma Ss agricola, Latina, LT), Campania (Il lupino DOC Srl, Caserta, CE) and Puglia (Albano Srl, Lucera, FG) and supplied by Madama Oliva srl Company (Carsoli, AQ, Italy). All lupin samples were characterized by the same size (between 13 and 15 mm) and morphology. Before extraction, in order to guarantee their representativeness, each sample was ground and homogenized using a blender for 3 cycles of 2 min each, obtaining a flour-like fine powder and then stored in the dark, at room temperature, until further analysis.

2.2. Chemicals

The alkaloid standards (+)-lupanine (perchlorate) and 13α-hydroxylupanine were purchased from Vinci-Biochem Srl (Firenze, Italy); (−)-angustifoline, multiflorine, and albine (hydrochloride) were purchased from Phytoplan (Heidelberg, Germany); (+)-sparteine and heptafluorobutyric acid (HFBA) were purchased from Merck Life Science Srl (Darmstadt, Germany). Water (H_2O), methanol (MeOH) and acetonitrile (ACN), all UPLC-MS grade, were purchased from VWR (Radnor, PA, USA).

2.3. In silico spectra prediction

A semi-untargeted method was developed to putatively identify lupin alkaloids commonly present in L. *albus* L. species, whose analytical standards were not available. At first, a thorough literature search on all known alkaloids already found in different lupins species (including conjugated forms) was performed to create a preliminary list. Subsequently, for each identified chemical structure, canonical SMILES was annotated using PubChem, an open chemistry database (https://pubchem.ncbi.nlm.nih.gov/), or ACD/ChemSketch, from Advanced Chemistry Development (Toronto, Canada). The SMILES format was used to enter each molecule on the web tool CFM-ID, that employs a predictive machine learning algorithm based on an MS/MS fragmentation database (Wang et al., 2022). For each chemical structure, the *in silico* predicted ESI-MS/MS spectrum was generated at multiple collision energies (10 eV, 20 eV and 40 eV), allowing the observation of each peak with probable fragment ion abundances. For each *in silico* experiment, the precursor ion, *m/z* fragments and relative intensity values were extrapolated and annotated. From this, the most five intense fragments for each molecule were carefully selected and used to create a comprehensive in-house MS/MS database, that was then incorporated into the MRM survey scan method, allowing for the search of these compounds in lupin samples by instrumental analysis.

2.4. Extraction and clean-up procedure

The extraction and clean-up procedures were performed according to Eugelio et al. (2023). Briefly, 200 mg of each milled lupin sample were extracted with 1 mL of a MeOH:H_2O 60:40 (v:v) solution by a Precellys Evolution homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France) at 7000 rpm for 3 cycles of 10 s each, with a 45 s stop between each cycle, followed by centrifugation for 10 min at 11424*g* at 4 °C. After that, 50 μL of the supernatant were collected and

diluted in 1 mL, with a final ratio of a H_2O:MeOH 90:10 (v:v) solution, with the aim of being subjected to a clean-up step performed by Solid Phase Extraction (SPE). According to this, Strata-XL 100 μm Polymeric Reversed Phase cartridges (330 mg mL^{-1}) from Phenomenex (Torrance, CA, USA) were conditioned with 1 mL of MeOH and subsequently equilibrated, sample-loaded and washed with 1 mL of H_2O:MeOH 90:10 (v:v) for each step. Finally, the analytes were eluted with 1 mL of MeOH and further subjected to UHPLC-MS/MS analysis.

2.5. Instrumental analysis

2.5.1. UHPLC-MS/MS

The chromatographic separation of alkaloids was performed according to Eugelio et al. (2023), with slight modifications. An ACQUITY UPLC H-Class System from Waters Corporation (Milford, Connecticut, United States) was used with an Excel 2 C18-PFP (10 cm × 2.1 mm) column from ACE (Aberdeen, UK), packed with 2 μm particles and equipped with a security guard column. The mobile phases consisted of H_2O with 0.1 % of HFBA (A) and ACN:MeOH 50:50 (v:v) with 0.1 % of HFBA (B). The elution of the analytes was carried out with the following gradient: start with 10 % of phase B for 0.1 min; linear increase at 50 % of B in 2.15 min; isocratic step at 50 % of B for 3 min; linear increase at 99 % of B in 1.5 min; isocratic step at 99 % of B for 0.38 min; back to the initial conditions (10 % of B) in 0.37 min. The injection volume and the flow rate were set at 3 μL and 0.400 mL min^{-1}, respectively. The separation of the analytes took place in 7.5 min, and the total duration of the analysis was 9 min, including 2 min to equilibrate the column to the initial conditions. The UHPLC system was coupled with a 4500 QTRAP hybrid QqQ-LIT mass spectrometer from Sciex (Sciex, Toronto, ON, Canada), equipped with a Turbo V Electrospray Ionization (ESI) source operating in positive ionization mode (ESI+), with a capillary voltage of 5500 V, nebulizer gas (air) and turbo gas (nitrogen) at 40 psi, and source temperature at 500 °C. The analyses were performed in MRM-IDA-EPI in a single run: the MRM acquisition was used as survey scan for the IDA criteria, which was employed to trigger the EPI experiments.

For the six targeted analytes, instrumental parameters, such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP), were tuned by infusion of the standard methanolic solutions (10 ng/mL) at a flow rate of 10 μL/min. Data collection and processing were achieved with Analyst 1.7.2 software; the peak integration was executed by Multiquant 3.0.3 software (Sciex).

2.5.2. MRM-IDA-EPI acquisition method

An MRM-IDA-EPI acquisition method was developed to putatively identify the numerous predicted MRM transitions obtained using the CFM-ID tool. Over 100 MRM transitions were used to create a comprehensive MS/MS spectral library, which was integrated into the MRM survey scan. The IDA criteria were set to use this survey scan and to trigger EPI-dependent scans when ion intensities exceeded a specified threshold (500 cps), with the dynamic background subtraction (DBS) algorithm enabled. This approach generated enhanced fragmentation spectra for the analytes detected in the survey scan.

The EPI acquisition was performed with DP and EP values set to 75 eV and 10 eV, respectively. The scan rate was set at 10000 Da/s, covering a molecular weight range of 50 to 500 Da, while the CE for fragmentation was set at 45 ± 10 eV, according to literature and *in silico* experiments. Once the characteristic fragmentation spectra for each analyte were obtained through EPI experiments, they were subsequently matched, with a mass tolerance of 0.6 Da, with the previous MS/MS library and with known fragmentation patterns of the structurally similar analytical standards to confirm the molecules. In Table S1, all the putative identified compounds from the MRM-IDA-EPI acquisition method are reported.

2.6. Multivariate statistical analysis

Python open-source programming language (Rossum & Drake, 2009) was used for all data processing steps. The dataset consisted in 400 observations (100 observations for each Italian region) and 27 variables (the 6 targeted and 21 semi-untargeted alkaloids). No missing data were present in the dataset.

The dataset of the areas obtained from the UHPLC-MS/MS analysis was reported in supplementary material (Table S2a). A percentage conversion was applied to the dataset to ensure that each variable contributed equally to the analysis, preventing variables with larger ranges to dominate the results (Table S2b). Before any statistical analysis, the features were standardized by removing the mean and scaling the variance to unity, making the data comparable in terms of variance. Different python libraries, such as pandas (McKinney, 2010), numpy (Harris et al., 2020), sklearn (Pedregosa et al., 2011), matplotlib (Hunter, 2007), seaborn (Waskom, 2021) and scipy (Virtanen et al., 2020), were used to perform unsupervised hierarchical clustering and supervised PLS-DA, as reported in the python script (script_lupin.py), in the supplementary material. To avoid the problem of overfitting, PLS-DA was carried out using a 10-fold cross-validation approach, randomly splitting the dataset with a train-test ratio of 90:10. The four Italian regions (Abruzzo, Campania, Lazio and Puglia) were used as the PLS-DA classes. The procedure was automated taking advantage of the script facilities provided by "Spyder", a python open-source Integrated Development Environment (IDE).

3. Results and discussion

3.1. Putative identification of lupin alkaloids by MRM-IDA-EPI acquisition method

The proposed method was applied on raw L. *albus* L. samples to explore the entire LA profile with a single analysis. A similar approach was successfully applied also in other studies for bioactive compounds in food matrices (Oliva et al., 2022; Viteritti et al., 2022).

The determination of QAs (the most abundant alkaloids among LAs) was performed and experimental MS/MS spectra were acquired. To tentatively identify these compounds, information about their biosynthetic pathways was used and a comparison with theoretical MS/MS spectra was conducted as, to our best knowledge, their CID fragmentation was not deeply investigated in previous works. Examples of structures for some considered alkaloids are reported in Fig. S1.

The MRM-IDA-EPI method was developed merging targeted and semi-untargeted approaches in a single acquisition. The quantitative determination of lupanine, hydroxylupanine, multiflorine, albine, angustifoline and sparteine in L. *albus* L. samples was conducted in targeted mode using reference analytical standards, as reported in previous work (Eugelio et al., 2023).

The putative identification of the other 21 LAs was achieved with a semi-untargeted approach, leveraging the capabilities of a hybrid QqQ-LIT analyzer that allowed to acquire MS/MS spectra for each chromatographic peak above the selected threshold. Given the basic nature of alkaloids, the instrumental analysis was performed in positive ionization mode (ESI+). Each MS/MS spectrum was then compared with the in-house library built by using both CFM-ID and known fragmentation patterns of structurally similar analytical standards, which spectra are reported in Fig. S2, S3 and S4. This approach provided a powerful tool to obtain a putative identification of LA profile, even for minor LAs lacking commercially available reference standards, enabling comprehensive alkaloid fingerprinting. Table S1 reports all the putatively identified compounds from the semi-untargeted approach.

Various LAs structures were detected during the analysis, such as lupanine-, multiflorine- and sparteine-derivative QAs, including naturally occurring structural isomers and conjugated forms, such as esters with short chain fatty acids or phenolic acids. For example, several esters

of hydroxylupanine, already known to occur in L. *albus* L., such as angeloyloxylupanine (or tigloyloxylupanine), propionyloxylupanine, valeroyloxylupanine (Boschin & Resta, 2013) and two isomeric forms of benzoyloxylupanine (Mühlbauer et al., 1988) were detected at different retention times (RT). The MS/MS spectra of the hydroxylupanine derivatives exhibited several common fragments, such as 247 *m/z* ([[lupanine-2H] + H]$^+$) and, additionally, 193, 148, and 112 m/z fragments, in common with lupanine and hydroxylupanine MS/MS spectra, confirming the presence of a common moiety.

Here we report some examples of application of the developed methodology for putative identification of lupanine- and multiflorine-derivatives, hydroxysparteine and ammodendrine.

In Fig. 1, a peak detected at RT 3.50 min corresponds to a precursor ion of 347.0 *m/z*, putatively identified as angeloyloxylupanine (or *cis* isomer tigloyloxylupanine). Being a lupanine-type QA, it is characterized by two condensed quinolizidine units forming a bridged bisquinolizidine, with a carbonyl group and containing an ester bond with angelic acid (or *cis* isomer tiglic acid) (Cely-Veloza et al., 2023). The EPI experiments produced a specific MS/MS spectrum for this analyte, in which the main fragments were reported and matched with the in-house database and with the lupanine and hydroxylupanine MS/MS spectra. The main common fragments for this compound were 265.0 *m/z*, corresponding to [hydroxylupanine+H]$^+$ structure, and 246.8 m/z corresponding to the same m/z of [[lupanine-2H] + H]$^+$ structure; the latter was obtained by the neutral loss of the angelic ester moiety (−98 m/z) and consequent unsaturation of the ring (−2 *m/z*). Additionally, several fragments, such as 193.0, 148.0, 133.9, 112.0, 97.8 and 84.1 m/z were also successfully matched with the lupanine MS/MS spectrum (Fig. S2). The fragment at 193.0 *m/z* fits with a methyl bonded tricyclic QA skeleton, formed by the cleavage of the lactam moiety; 148.0 m/z corresponds to a tri-unsaturated bicyclic structure bonded to a methyl group; the fragment corresponding to the methyl group loss was also present (133.9 *m/z*). Eventually, the fragments 112.0 and 98.0 *m/z* represent an ethyl- and methyl- piperideine form, respectively, with 84.1 m/z indicating a piperidine structure.

As reported in Table S1, the analysis revealed the presence of two peaks at RT 2.45 and 2.62 min, characterized by the same precursor ion 279.2 *m/z*, corresponding to two isomers of hydroxyoxolupanine, already found in L. *albus* L. (Mohamed et al., 1994). The MS/MS spectrum shared major product ions 246.8, 150.0, 134.0, 112.1, 84.0 and 70.1 *m/z* in common with the lupanine fragmentation pattern, and the most intense peak at 166.0 *m/z*, found in the hydroxylupanine MS/MS spectrum; all these fragments were also successfully matched with the database.

Two esters of hydroxymultiflorine, such as angeloyloxymultiflorine (or the *cis* isomer tigloyloxymultiflorine) commonly occurring in L. *albus*

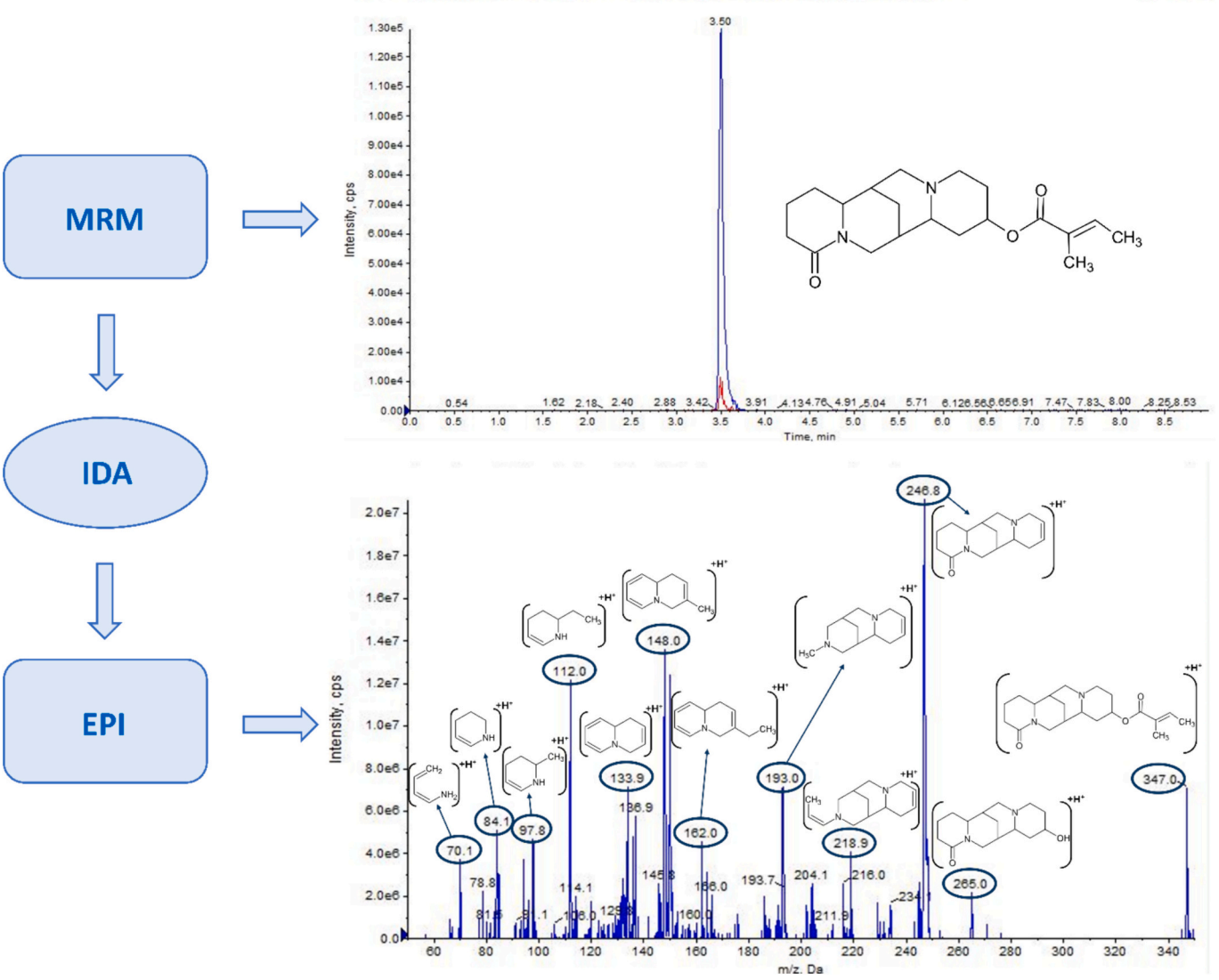

Fig. 1. Angeloyloxylupanine MS/MS spectrum putatively identified in MRM-IDA EPI-mode.

L. (Boschin & Resta, 2013) and cinnamoyloxymultiflorine, already found in traces amounts in L. *gibertianus* L. (Planchuelo-Ravelo et al., 1993; Wink et al., 1995), were also putatively identified. In particular, Fig. 2 shows a peak detected at RT 3.46 min, corresponding to a precursor ion of 345.2 *m/z*, identified as angeloyloxymultiflorine (or the *cis* isomer). Being a multiflorine-type QA, its structure primarily differs from lupanine-type by the presence of a double bond (−2 m/z) and may contain an ester bond with angelic acid (or the *cis* isomer tiglic acid) (Cely-Veloza et al., 2023). The EPI experiments conducted in this work matched in the built-in-house database and with the multiflorine MS/MS spectrum, showing a common fragment at 245.0 *m/z*, which is most intense peak and corresponding to the m/z of $[[\text{multiflorine-2H}] + \text{H}]^+$ structure. This behavior is similar to the already described fragmentation of lupanine ester, detected by the neutral loss of the angelic ester moiety (−98 *m/z*) plus the unsaturation of the ring (−2 m/z). As further evidence, the fragments of the putative multiflorine ester at m/z below 245 (146.0, 132.0 and 96.0 m/z; Fig. 2), correspond to the lupanine ester series of fragments with a difference of $[\text{M-2H}]^+$, (148.0, 134.0 and 98.0 *m/z*; Fig. 1), due to the unsaturation. Similar to lupanine esters, the 84.0 m/z (Figs. 1 and 2) matches to piperideine, which in this MS/MS spectrum was also found in ethyl-substituted (112.0 m/z; Fig. 2) and di-unsaturated methyl-substituted (96 m/z) forms.

As reported in Table S1, other multiflorine-derivative QAs were putatively identified, for the three chromatographic peaks detected at RT 2.12, 2.48, 2.90 min. All these peaks have the same precursor ion at 263.2 m/z and the fragmentation pattern can be associated to three isomers of hydroxymultiflorine, which have been previously reported in L. *albus* L. (Boschin & Resta, 2013). The main fragments (244.9, 132.0, 112.0, 84.0, 96.0 *m/z*) successfully matched with angeloyloxymultiflorine MS/MS spectrum in database. Two peaks at 2.13 and 2.43 min were also detected, with a precursor ion of 277.2 *m/z*, and tentatively identified as two isomers of methoxymultiflorine, previously found in *L. albescens* L., L. *gibertianus* L. and *L. linearis* L. (Wink et al., 1995). In this case, the fragments at 149.0, 131.9, and 81.8 m/z matched the angeloyloxymultiflorine MS/MS spectrum, and those at 164.8 and 112.0 m/z were matched the multiflorine MS/MS spectrum (Fig. S3) and the database.

Three peaks at RT 2.45, 3.00 and 3.19 min were putatively identified as isomers of dehydromultiflorine, already found in L. *albus* L. (Bundesinstitut Für Risikobewertung., 2017). The precursor ion was at 245.2 *m/z* and the fragmentation pattern matched with the multiflorine MS/MS spectrum and the database.

As shown in Fig. 3, the MRM-IDA-EPI analysis also revealed the presence of three isomers of hydroxysparteine. Three peaks were detected at different RT (2.66, 3.01 and 3.10 min), all presenting a similar fragmentation pattern, with a common precursor ion of 251.0 *m/*

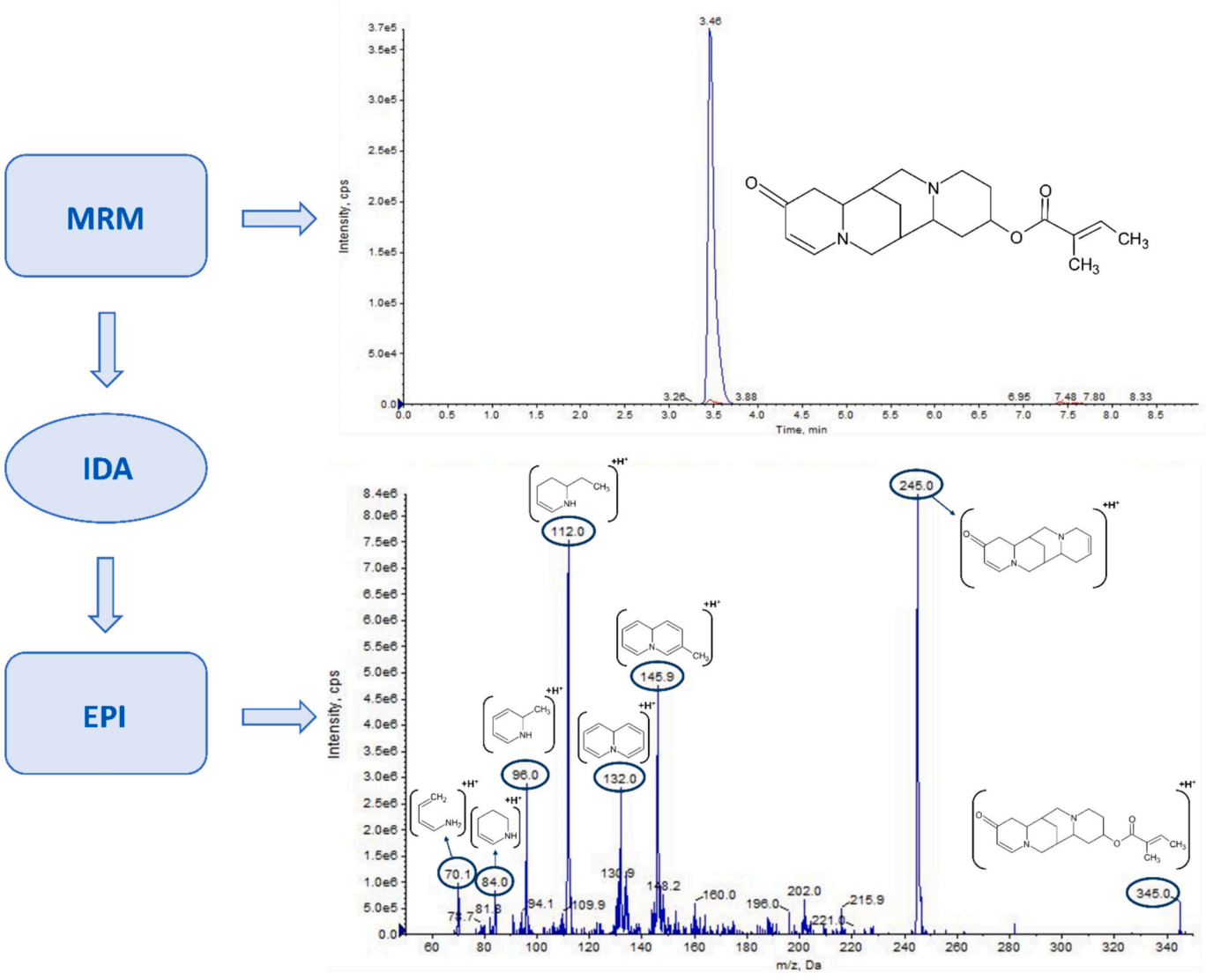

Fig. 2. Angeloyloxymultiflorine MS/MS spectrum putatively identified in MRM-IDA EPI-mode.

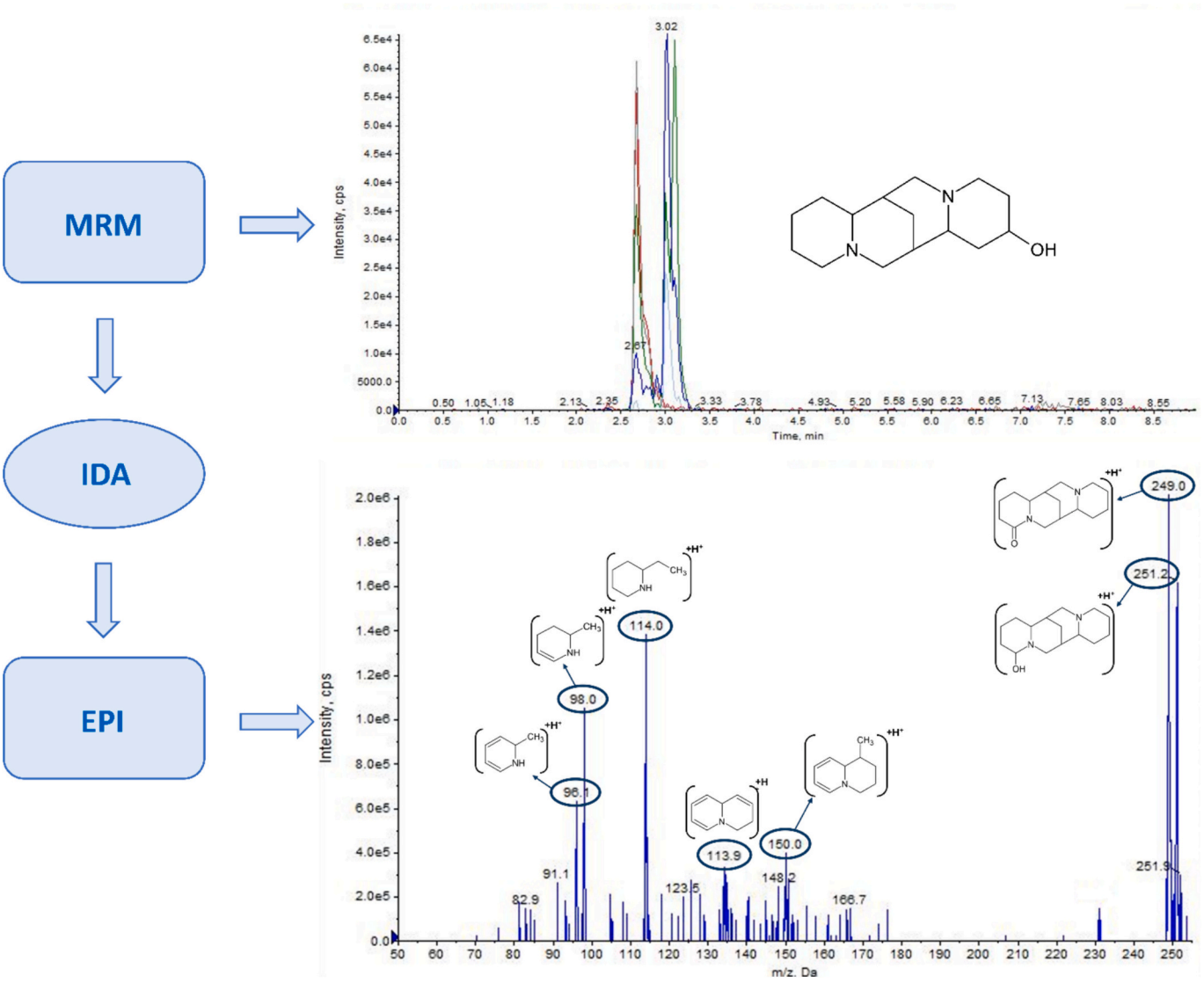

Fig. 3. Three isomers of hydroxysparteine MS/MS spectrum putatively identified in MRM-IDA EPI-mode.

z. This alkaloid was previously found in L. *polyphyllus* L. and *L. sericeus* L. (Wink et al., 1995) and the presence can be attributed to the catalytic hydrogenation of multiflorine (Michael, 2003). The hydroxysparteine structure is characterized by two condensed quinolizidine units forming a bridged bisquinolizidine, differing from lupanine by the absence of the carbonyl group (Cely-Veloza et al., 2023).

Each of the EPI experiments allowed the acquisition of a common fragmentation pattern for the three peaks. The main fragments were compared with the sparteine MS/MS spectrum (Fig. S4) and the built-in-house database. The most intense fragment ion was 249.0 m/z, probably obtained by the loss of 2H (−2 m/z), leading to formation of the carbonyl group corresponding to same m/z as $[\text{lupanine}+\text{H}]^+$.

Several fragments, such as 150.0, 133.9, 114.0, 98.0 and 96.0 m/z were also successfully matched with database and the lupanine MS/MS spectrum; among them, 150.0 m/z probably corresponds to a di-unsaturated bicyclic structure bonded to a methyl group, while 133.9, 98.0 and 96.1 matched with the sparteine fragmentation pattern.

Eventually, the BA ammodendrine, which corresponds to another biosynthetic product from the L-lysine metabolic pathway (Mancinotti et al., 2022) and found in L. *albus* L. (Bundesinstitut Für Risikobewertung., 2017), was putatively identified. As depicted in Fig. 4, it was represented by a peak detected at RT 2.85 min. The precursor ion was at 209.2 m/z and the main generated fragment ions were 165.0, 150.1, 122.2, 105.0, 91.0 and 79.1 m/z, among which 150.1, 122.2 and 79.1 m/z were confirmed by the database. The 165.0 m/z product ion (−44 m/z) likely corresponds to the ammodendrine skeleton with the loss of the aldehyde group and relative unsaturation, while the other fragments are formed by the cleavage of the bicyclic skeleton.

3.2. Unsupervised analysis: hierarchical clustering with heatmap

The chemical profile of alkaloids in lupins was analyzed to characterize raw lupin samples coming from four different Italian regions (Abruzzo, Lazio, Campania, Puglia; 100 samples for each region). The dataset of the areas obtained by the UHPLC-MS/MS analysis (Table S2a) was normalized reporting the peaks area in percentage (Table S2b). The mean and coefficient of variation (CV%) of each region group data were calculated and reported as well in supplementary material (Table S2c).

All regions were characterized by a distinct alkaloids profile suggesting a different alkaloid response to geographical and environmental conditions; remarkably, the presence of semi-untargeted analytes varied significantly among regions. Isomers of hydroxymultiflorine and methoxymultiflorine had high coefficients of variation highlighting a relevant variability within each region. On the other hand, the targeted alkaloids exhibited a relatively uniform presence across regions, except for sparteine and angustifoline, showing a more heterogeneous

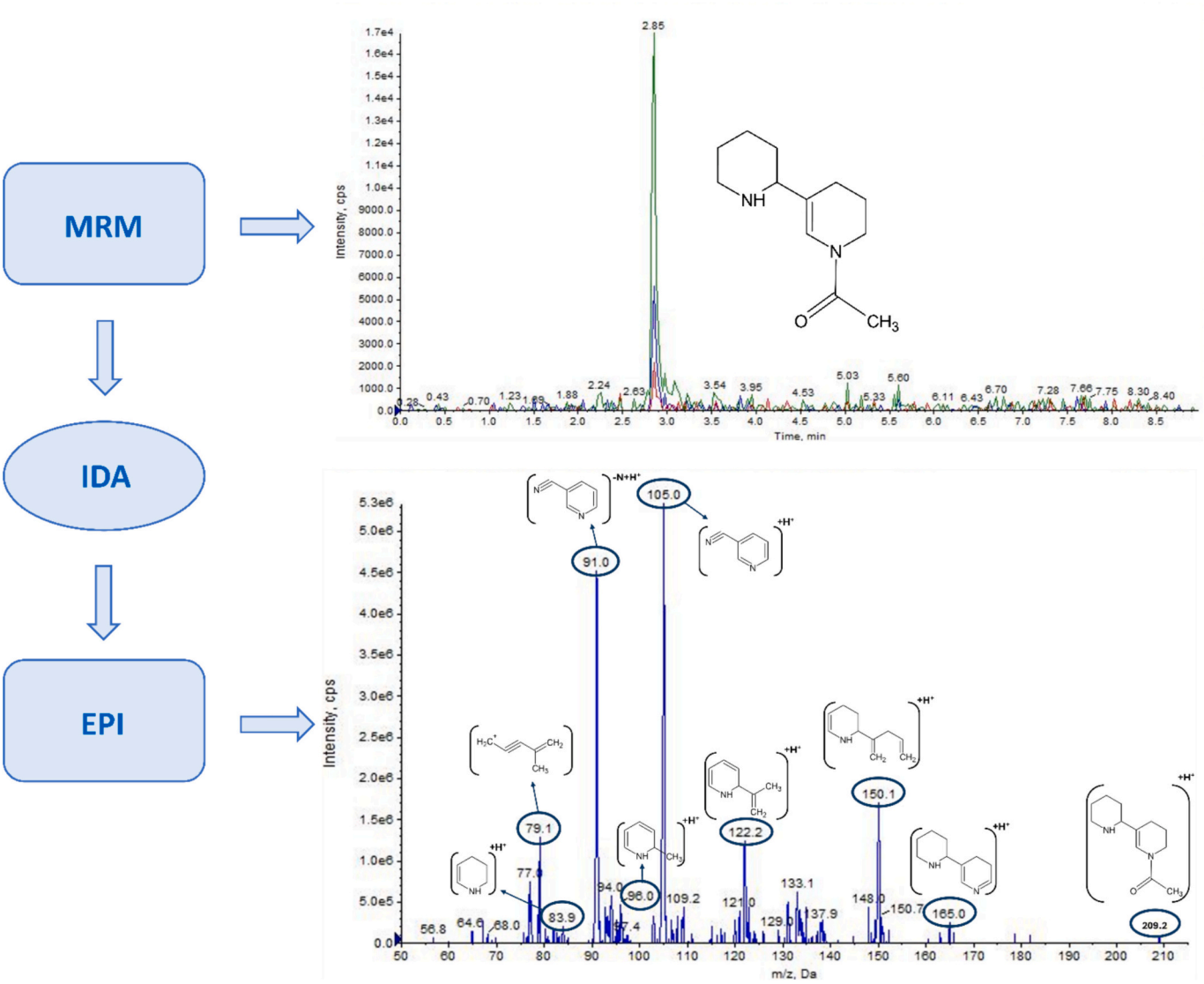

Fig. 4. Ammodendrine MS/MS spectrum putatively identified in MRM-IDA EPI-mode.

distribution.

To uncover the complex relationships in the dataset, an exploratory unsupervised HCA combined with a heatmap was carried out. As shown in Fig. 5 both clustering of the alkaloid features (on the x-axis) and the lupin samples (on the y-axis) were performed.

Abruzzo and Campania lupin samples showed a perfect regional grouping, indicating homogeneity regarding the measured alkaloid profiles. Lazio and Puglia exhibited mixing in clusters, suggesting that alkaloid profiles were not strictly specific for these regions.

Alkaloids were grouped, based on their cooperative patterns across all samples, in three main clusters. The first cluster (sparteine, methoxymultiflorine (I), hydroxyoxolupanine (I), benzoyloxylupanine (II), hydroxymultiflorine (III), angeloyloxylupanine, benzoyloxylupanine (I), propionyloxylupanine and valeroyloxylupanine) was relevant to differentiate between Abruzzo and Campania. The second cluster (ammodendrine, hydroxymultiflorine (I), dehydromultiflorine (II), dehydromultiflorine (III), angeloyloxymultiflorine, hydroxysparteine (II), hydroxysparteine (III)) was important in separating Abruzzo and Campania from the Lazio-Puglia mixed cluster. The alkaloids in the third cluster (hydroxylupanine, lupanine, angustifoline, hydroxysparteine (I), multiflorine, albine, dehydromultiflorine (I), hydroxymultiflorine (II), methoxymultiflorine (II), hydroxyoxolupanine (I) and cinnamoyloxymultiflorine) were spread across the four regions, indicating variability and overlap.

3.3. *Supervised analysis: PLS-DA performances using a targeted and semi-untargeted approach*

HCA was used for exploring the data structure of the dataset without pre-specifying the number of clusters, providing an unbiased lupin samples grouping based on alkaloid features; however, PLS-DA supervised method was carried out to build a predictive model to correctly classify the lupin samples into the four regions and to identify the most discriminant alkaloids. Such a robust method is widely applied in food science, offering a direct way to manage the outcomes.

PLS-DA was run using the dataset obtained from the UHPLC-MS/MS analysis, the same input as HCA, to capture the direct relationship between features and class labels, preserving the alkaloids information. Utilizing alkaloids features facilitated a clearer interpretation of each feature's contribution to the classification, which was the main purpose of our findings. This approach also avoided potential information loss from data summarization into clusters, ensuring all data variability and nuances were considered.

To enhance the accuracy of the PLS-DA and mitigate the risk of overfitting, a nested 10-fold cross-validation approach was adopted to validate the results. To evaluate the relevance of the developed semi-

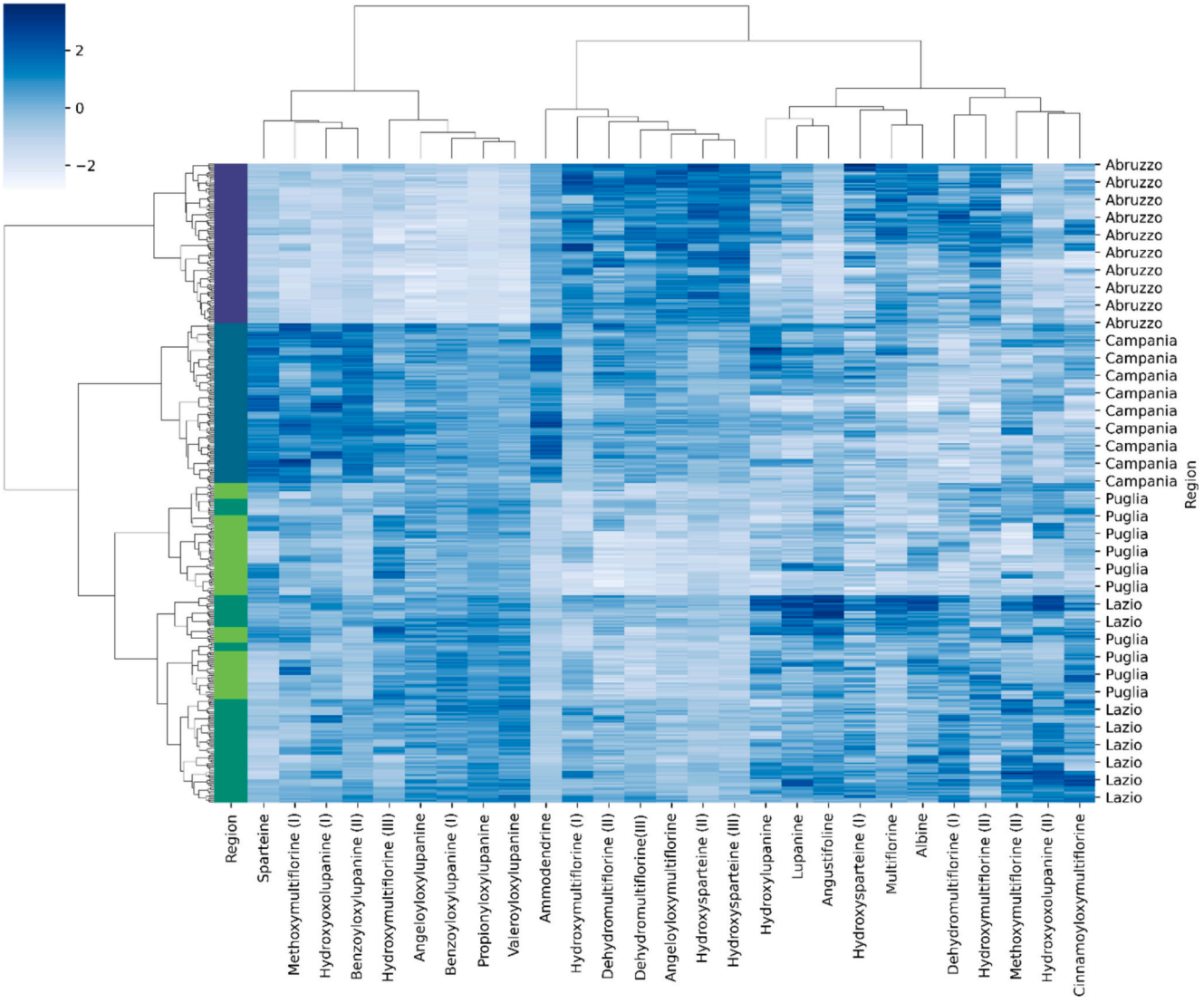

Fig. 5. Heatmap of the standardized data set. Both Italian regions and LAs were organized according to HCA.

untargeted approach, the lupin dataset was divided into two subsets: the first, constituted by the 6 QAs detected with targeted approach (analytical standards available), the second included all 27 LAs identified through the EPI-based method of this work.

The classification procedure was run for both datasets, and the prediction results obtained from the test step are reported in Table 1. Detailed train and test statistical metrics are provided in Table S3.

The targeted method did not precisely predict the lupin samples originating from Abruzzo, Lazio, and Puglia, demonstrating notably low accuracy. Particularly, the method was ineffective in correctly identifying true positive samples from all regions, leading to a significantly low sensitivity in all models. The exception was for samples from Abruzzo, where the classification accuracy was relatively higher, with 95 % accuracy.

Table 1
Results of PLS-DA classification using nested 10-fold cross-validation for both targeted and semi-untargeted approaches.

Test metrics	Abruzzo			Campania			Lazio			Puglia		
Targeted approach												
Accuracy	0.950	±	0.088	0.628	±	0.082	0.400	±	0.075	0.498	±	0.167
F1 Score	0.709	±	0.137	0.657	±	0.072	0.476	±	0.106	0.389	±	0.167
Precision	0.950	±	0.088	0.628	±	0.082	0.400	±	0.075	0.498	±	0.167
Recall (sensitivity)	0.580	±	0.162	0.700	±	0.105	0.600	±	0.176	0.340	±	0.184
Specificity	0.898	±	0.038	0.893	±	0.032	0.845	±	0.052	0.798	±	0.044
Semi-untargeted approach												
Accuracy	1.000	±	0.000	0.982	±	0.038	0.989	±	0.035	0.956	±	0.074
F1 Score	1.000	±	0.000	0.990	±	0.020	0.957	±	0.061	0.972	±	0.051
Precision	1.000	±	0.000	0.982	±	0.038	0.989	±	0.035	0.956	±	0.074
Recall (sensitivity)	1.000	±	0.000	1.000	±	0.000	0.930	±	0.095	0.990	±	0.032
Specificity	1.000	±	0.000	1.000	±	0.000	0.978	±	0.030	0.997	±	0.011

On the other hand, the semi-untargeted that used all the 27 LAs features resulted in significantly improvement of PLS-DA performances, achieved with the same 6 latent variables (LVs) used in the targeted approach. To highlight the role of alkaloids in determining origin, Table S4 detailed the importance of the features in the discrimination process. Notably, the enhanced classification of lupin samples from the Abruzzo, Campania, Lazio and Puglia regions was predominantly due to the relative contributions of dehydromultiflorine (III), hydroxysparteine (III), ammodendrine, angeloyloxymultiflorine and benzoyloxylupanine (II). It is important to notice that the correct classification resulted from the combined effect of the alkaloids identified through the semi-untargeted method. No distinct contribution of any targeted feature was observed for the correct classification of the lupin samples; among them, only multiflorine slightly contributed to the classification rate of lupin samples from Puglia.

The results of this work demonstrated that the developed semi-untargeted method allowed a strong classification of the regional origins of lupin samples, emphasizing the synergic cooperation of the identified alkaloids. The application of supervised multivariate analysis reinforced the significance of these alkaloids, which are often present in lower amounts, demonstrating the chemical variability of the geographical origin. The results clearly suggested that the targeted approach alone is insufficient for a comprehensive geographical characterization of the lupin samples profiles, opening the way for a more thorough approach to classify origins by broadening the range of alkaloids descriptors.

4. Conclusions

In this study, a semi-untargeted approach was developed to detect the alkaloid profile in L. *albus* L. samples using an MRM-IDA-EPI acquisition *via* UHPLC-MS/MS, leveraging the capabilities of hybrid QqQ-LIT mass spectrometry. This method enabled the acquisition of fragmentation spectra of various LAs structure, which are often inadequately described in CID fragmentation studies. This approach facilitated the detection of LAs without relying on GC–MS, as commonly described in the literature, or on analytical reference standards, which are unavailable for all compounds, thereby providing a complementary tool for LA profile identification.

Moreover, to underscore the significance of this achievement, the method was applied to analyze lupins from four Italian regions. Supervised classification, based solely on standard alkaloids, clearly demonstrated that targeted approach alone is insufficient for comprehensive analysis of LA profile variation across different geographical origins. Integrating the semi-untargeted method with multivariate chemometric techniques successfully distinguished lupin samples from all the four regions, highlighting the synergistic contribution of the new alkaloids identified form semi-untargeted approach.

Ultimately, we believe this proposed method could serve as a valuable tool for extensive exploration of intra-species LAs content and as possible markers of geographical origin. This could enhance product traceability and have broader implications for food safety, providing consumers with valuable information for quality assessment and traceability of the food samples.

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CRediT authorship contribution statement

Fabiola Eugelio: Writing – original draft, Validation, Methodology, Investigation. **Sara Palmieri:** Validation, Investigation, Data curation. **Marcello Mascini:** Writing – original draft, Data curation. **Francesco Della Valle:** Investigation, Formal analysis. **Federico Fanti:** Writing – review & editing, Resources, Methodology, Conceptualization. **Eleonora Oliva:** Formal analysis. **Michele Del Carlo:** Supervision, Project administration, Conceptualization. **Dario Compagnone:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Manuel Sergi:** Writing – review & editing, Supervision, Methodology, Conceptualization.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101769.

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