UNIVERSITÀ DEGLI STUDI DI SALERNO

UNIVERSITÀ DEGLI STUDI DI SALERNO

Dipartimento di Farmacia

PhD Program in

Drug Discovery and Development

XXXV Cycle — Academic Year 2022/2023

PhD Thesis in

Analytical procedures for the safety assessment of dietary supplements and botanicals

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Data: 15/02/2023 16:09:37

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Abbreviations

AAS	Anabolic-androgenic steroids
$BMDL_{10}$	Benchmark Dose Lower Confidence Limit 10%
CD	Compound database
DHPA	Dehydropyrrolizidine alkaloids
DILI	Drug-induced liver damage
DS	Dietary supplements
EE	Extraction efficiency
EFSA	European Food Safety Authority
EIC	Extracted ion chromatogram
EU	European Union
FDA	Food and Drug Administration
FWHM	Full width at half maximum
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HQI	Hit Quality Index
HRMS	High-resolution mass spectrometry
IARC	International Agency for Research on Cancer
IL	Identification level
IR	Infrared
LC	Liquid Chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOI	Limit of identification
LOQ	Limit of quantification
ME	Matrix effect
ML	Maximum level
MOE	Margin of exposure
MS	Mass spectrometry
MSI	Metabolomics Standards Initiative
NIR	Near infrared
NMR	Nuclear magnetic resonance
PAs	Pyrrolizidine alkaloids
PANOs	Pyrrolizidine alkaloids N-Oxide
PDE5	Phosphodiesterase type 5
PDE5I	Phosphodiesterase type 5 inhibitors
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RASFF	Rapid Alert System for Food and Feed
RDS	Relative standard deviation
RL	Recommended level
RP	Reference Point
SALLE	Salting-out assisted liquid-liquid extraction

SERS	Surface-enhanced Raman scattering
SPE	Solid-phase extraction
TLC	Thin layer chromatography
UHPLC	Ultra-high performance liquid-chromatography
US	United States
VOD	Veno-occlusive disease
WADA	World Anti-Doping Agency

Introduction

The consumption of dietary supplements and botanicals is not always safe and free of potential health risks. In recent years, cases of contamination with hepatotoxic pyrrolizidine alkaloids, a group of plant secondary metabolites, have raised public health concern (EFSA CONTAM Panel, 2017). Besides their contamination, the growing use of these products has been accompanied by an increasing number of notifications of adverse reactions related to their adulteration with illicit and undeclared substances, such as pharmaceutical active ingredients or unauthorized plants (Ekar & Kreft, 2019). The lack of measures to combat these phenomena poses a serious health risk for consumers. Therefore, the collection of reliable and significative data on the occurrence, exposure, and toxicity of both recognized and newly identified contaminants and adulterants is necessary to assess the safety of these product categories. In this context, there is an ever-increasing demand for reliable analytical tools which can detect, identify, and quantify the wide range and variety of toxic and undesirable substances in these products. The presented doctoral thesis is aimed at providing innovative analytical procedures to assess the safety of these product categories and broaden the knowledge about the presence of noncompliant dietary supplements and botanicals on the health market.

In **CHAPTER 1**, the food safety issue of dietary supplements and botanicals regarding their contamination with hepatotoxic pyrrolizidine alkaloids and adulteration with illicit and undeclared substances is addressed from a legislative and scientific perspective.

In **CHAPTER 2**, the development of a sample preparation procedure, based on salting-out assisted liquid-liquid extraction (SALLE), for the fast and cheap extraction and clean-up of pyrrolizidine alkaloids and their N-oxides from different food matrices is discussed.

In **CHAPTER 3**, the design and development of an analytical platform for the detection and identification of pyrrolizidine alkaloids and their N-oxides is discussed; it includes a wide-scope suspect screening method, based on a diagnostic product ion filtering strategy for the characterization of unknown compounds, and a high-throughput target screening method for the detection and identification of 118 target compounds from a mass spectral library.

In **CHAPTER 4** the validation studies of the analytical platform, described in CHAPTER 3, are extended for quantitative purposes and the target screening method is applied to the analysis of a high number of real samples of the studied matrices, regarding the presence of 118 pyrrolizidine alkaloids. The large set of collected data are then discussed from a quali-quantitative point of view to provide an estimate of the contamination issue of the studied food matrices.

In **CHAPTER 5**, the development of a qualitative screening method, based on Surface-enhanced Raman scattering (SERS), for the rapid detection of illicit adulterants and botanical markers of unauthorized plants in dietary supplements using a portable analyzer is discussed.

CHAPTER 1

The food safety issue of dietary supplements and botanicals

1.1 Dietary supplements and botanicals on the EU market

1.1.1 Definitions, terminologies, and classification

Although the definition of "dietary supplement" within each individual legislation is quite precise, the main challenge regarding the regulation of these products is that there is no global consensus on what falls into this product category or how each category is called. For instance, the jurisdiction refers to "dietary supplements" in the United States (US), "food supplements" in the European Union (EU), "natural health products" in Canada, and "complementary medicines" in Australia. In addition, there are numerous substances which are classified as dietary supplements in some legislations and medicines in others. This situation becomes even more complicated when we consider that many of these products come from traditional health and care systems such as Traditional Chinese Medicine in China or the Ayurvedic Medicine in India. For this reason, we need to distinguish between how nations regulate the medical practice and how they regulate the commercial products used for the medical practice or as food (Dwyer et al., 2018). In the EU, dietary supplements are legally defined as foodstuffs intended to supplement the normal diet, as they are concentrated sources of vitamins, minerals, or other substances with a nutritional or physiological effect, marketed in pre-dosed forms. A wide range of other ingredients may be present in dietary supplements, such as amino acids, essential fatty acids, fibres, and various plants or herbal extracts. They are intended to supplement, and not replace, a balanced and varied diet by correcting nutritional deficiencies, maintaining an adequate intake of certain nutrients, and supporting specific physiological functions. They are not medicinal products and as such cannot prevent, treat, or cure any human disease (European Union, 2002).

Botanicals are preparations made from plants, algae, fungi, or lichens, and approved for the use in the formulation of dietary supplements. They have become widely available on the EU market and are generally labelled as natural foods, with a huge variety of claims about their possible health benefits. They can be purchased over the counter in pharmacies, supermarkets, herbalists, and online shops. Botanicals fall under different regulations in different countries and are mostly consumed without consulting healthcare professionals. In recent years, the use of herbal therapies has been extensively documented, giving rise to some concerns about their safety and quality. These include the risk of chemical or microbiological contamination and the need to ensure that concentrations of bioactive agents are within safe limits (Schilter et al., 2003).

1.1.2 EU regulatory framework

The term "food supplement" was introduced for the first time in the European legislation with the Directive 2002/46/EC (European Union, 2002). There is no centralized pre-marketing authorization for food supplements in the EU. Because food supplements are considered as foodstuffs, it is responsibility of the manufacturer, importer, supplier, or distributor to ensure that the product placed on the market is safe. The competent authority of the EU Member States can request to be informed when a certain food supplement is placed on the market in their territory and may monitor its use in that territory (Thakkar et al., 2020). The Regulation 2009/1170/EC establishes lists of vitamins and minerals which can be used in the manufacturing of food supplements (European Commission, 2009). The use of ingredients other than vitamins and minerals in the manufacturing of food supplements may be regulated by national or harmonized EU legislations. For instance, the use of novel foods – which are foods without a history of production or use before 1997, is regulated by the

Regulation (EC) 2015/2283 (European Union, 2015). The use of other substances with a nutritional and physiological effect om the human health, such as probiotics and prebiotics, botanicals and derived preparations, is not harmonized in the EU legislation but regulated on a national basis in each Member State.

Regarding botanicals, there are several reasons behind the lack of a harmonized legislation on their authorization and use. The main are the limited presence of evidence regarding the safety of these ingredients and the corresponding maximum daily dosages and the overlapping of botanical-based food supplements with traditional herbal medicines. Some botanicals are considered as traditional herbal medicinal plants and are used both in medicinal products and food supplements (Silano et al., 2011). To assist risk assessors on the evaluation of ingredients of concern in food supplements, the European Food Safety Authority (EFSA) published the EFSA Compendium of Botanicals in 2012, a database of botanicals whose content of ingredients can be potentially harmful to human health. The compendium is subjected to regular updates (the third version, which includes non-European botanical species, was released in 2016) and is intended to provide an additional tool for the safety evaluation of these substances and facilitate the recognition of any dangers (EFSA, 2012).

1.1.3 Consumption in Europe with one eye on the Italian market

The global market of dietary supplements and botanicals has steadily grown over the last twenty years. The increased use and demand of these products is due to different factors such as the growing inclination of consumers towards fitness and health, the newfound interest in preventing health disorders using supplements and functional foods, and the common belief that “natural” is healthy and plant-based products are safe. Moreover, the spread of chronic diseases such as diabetes, obesity,

and high blood pressure pushes people to lead a healthier life; in this context the use of dietary supplements has established as a good practice in maintaining an optimal state of well-being (Domínguez Díaz et al., 2020; Vargas-Murga et al., 2011).

The European market of dietary supplements was worth 13.2 billion euros in 2020 with Italy as market leader with a 29% share value among the other European countries (**Figure 1.1**). In detail, 87% of the Italian market value is divided into pharmacies and para-pharmacies, 8% in large-scale retailers, and the remaining 5% comes from e-commerce of pharmacies and para-pharmacies (Federsalus Research Centre, 2021). In Italy, 65% of the adult population uses dietary supplements (32 million people); therefore, the confidence in the benefits of supplements is quite high among Italian consumers. The average age of dietary supplements consumers is between 35–64 years old and they tend to be highly educated. Furthermore, according to recent surveys, it seems that the market is dominated by women, about 60% of whom use dietary supplements, while the rate is around 40% among men (Statista Research Department, 2021).

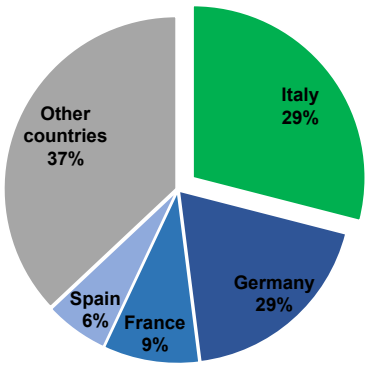

Figure 1.1. Contribution of different European countries to the dietary supplements market value of 2020 (Source: FederSalus elaboration on IQVIATM Consumer Health Global Insights and Multichannel View, MAT December 2020).

1.1.4 Major challenges of the dietary supplements and botanicals market

The growth of the dietary supplements and botanicals global market requires strengthening the regulations relating to their production, distribution, safety, and efficacy (Van Den Berg et al., 2011). The replacement of expensive ingredients with cheaper ones to increase profits exposes dietary supplements and botanicals to the risk of contamination with toxic substances; on the other hand, the fraudulent and intentional substitution or addition of a substance to rise market competitiveness increases the risk of adverse effects due to drug interactions between pharmaceuticals and botanical active substances (Rocha et al., 2016). Hence, the contamination with toxic substances and the fraudulent adulteration with illicit and undeclared substances are some of the major dietary supplements and botanicals market challenges regulatory agencies are facing in recent years (Costa et al., 2019; Czepielewska et al., 2018).

The presence of contaminated dietary supplements and botanicals on the market has become a major concern for public health; acute and chronic intoxications as well as other adverse reactions, attributable to the poor quality of these products, may be caused by prolonged or combined exposure to different classes of contaminants (Gil et al., 2021). Heavy metals, pesticides, dioxins, and polychlorinated biphenyls (PCBs), and various classes of natural toxins (cyanotoxins, mycotoxins, pyrrolizidine alkaloids) (**Figure 1.2**) are some of the most common contaminants of dietary supplements and botanicals (Costa et al., 2019).

The fraudulent adulteration of dietary supplements and botanicals with synthetic drugs (pharmaceuticals and their analogues) and/or unauthorized plants (**Figure 1.2**) is a serious food safety problem considering both the increasing use of these products and the consumers' lack of awareness of the risks associated with the presence of illicit substances, added to their formulations. Dietary supplements for weight loss and for

the enhancement of sports and sexual performances are the main categories prone to the adulteration risk (Rocha et al., 2016).

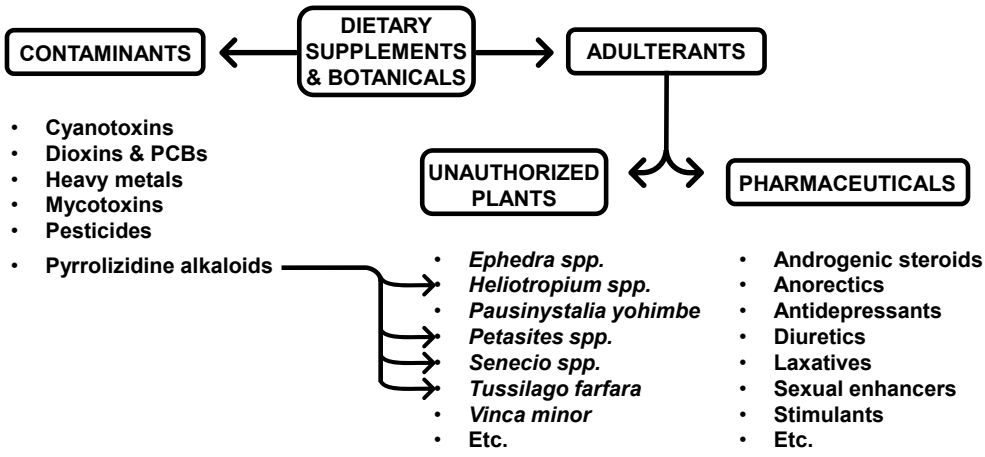

Figure 1.2. Main reasons behind the noncompliance of dietary supplements and botanicals.

1.2. Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PAs) are a large group of natural heterocyclic phytotoxins, which occur in approximately 3% of the world's flowering plants and whose role is to act as deterrents against herbivores and parasites. To date, more than 600 PAs have been identified in over 6000 plants. Most of these plants belong to the *Asteraceae*, *Boraginaceae*, *Orchidaceae*, and *Fabaceae* families, and half of them have been reported to be hepatotoxic (Schramm et al., 2019). PAs are considered among the most widespread natural toxins as they can affect wildlife, livestock, and humans through feed and food contamination. Acute PA-poisoning in humans is associated with liver damage, whereas a sub-acute or chronic poisoning may lead to liver cirrhosis and pulmonary arterial hypertension (Picron et al., 2018).

In 2011, the EFSA's Panel on Contaminants in the Food Chain (CONTAM) recognized the 1,2-unsaturated PAs as the most dangerous forms for human health and decided to focus on them for the risk assessment (EFSA CONTAM Panel, 2011). Based on the outcome of the EFSA 2011 and subsequent opinions and considering the available analytical standards, the European Commission selected 28 PAs as relevant in food samples (EFSA, 2016; EFSA CONTAM Panel, 2017). After that, the European Commission established maximum residue levels (MLs) for 35 PAs in certain food products (herbal infusions, teas, dried herbs, dietary supplements, and pollen), which is in force from July 2022 (European Commission, 2020). The International Agency for Research on Cancer (IARC) evaluated several PAs and PA-containing plants and classified lasiocarpine, monocrotaline and riddelliine in group 2B (possibly carcinogenic to humans), and jacobine, retrorsine, retrorsine N-oxide, seneciphylline and senkirkine in group 3 (not classifiable) (European Medicines Agency, 2014).

1.2.1 Chemical structure

The chemical structure of PAs consists of a bicyclic necine base to which aliphatic mono- or di-carboxylic acids, the necic acids, are generally linked by esterification (**Figure 1.3**). PAs can occur in plants as tertiary amines or N-oxides (PANOs) (**Figure 1.3**), which can easily be converted back to the tertiary amines by a reduction reaction (Moreira et al., 2018). Depending on the presence or not of a double bond between C1 and C2 of the necine base, PAs can be grouped in 1,2-unsaturated PAs and saturated PAs. 1,2-Unsaturated PAs are considered the most toxic because they can be oxidated in highly reactive pyrroles, which have been related to severe cases of hepatotoxicity, as consequence of acute toxicity, and carcinogenic and genotoxic effects, as consequence of chronic toxicity (**Figure 1.3**) (Schramm et al., 2019).

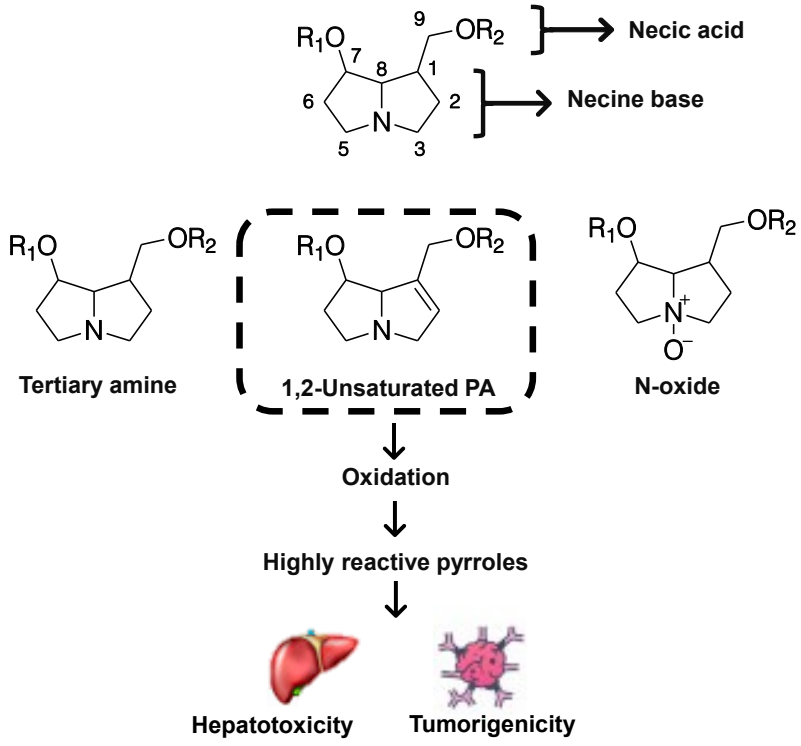

Figure 1.3. Basic chemical forms of pyrrolizidine alkaloids.

The necine bases commonly found in plants are: retronecine, heliotridine, otonecine, supinidine, trachelanthamidine, and platynecine (**Figure 1.4**). The first four types have an 1,2-unsaturation in the pyrrolizidine ring and are therefore considered of major toxicity whereas the platynecine and trachelanthamidine types have a saturated necine base and are generally regarded as non-toxic. Except for the otonecine type, in which N-oxides cannot be formed, N-oxides of the other types naturally occur and often coexist with basic PAs forms in plant materials. On the other hand, the type of esterification between the necic acids and the necine base allows to differentiate among three further types, which are monoesters, open-chained diesters, and cyclic diesters (**Figure 1.4**). The esterification often occurs at C-7 in monoesters and at C-7 and C-9 in open chained and cyclic diesters (EFSA CONTAM Panel, 2011; Moreira et al., 2018).

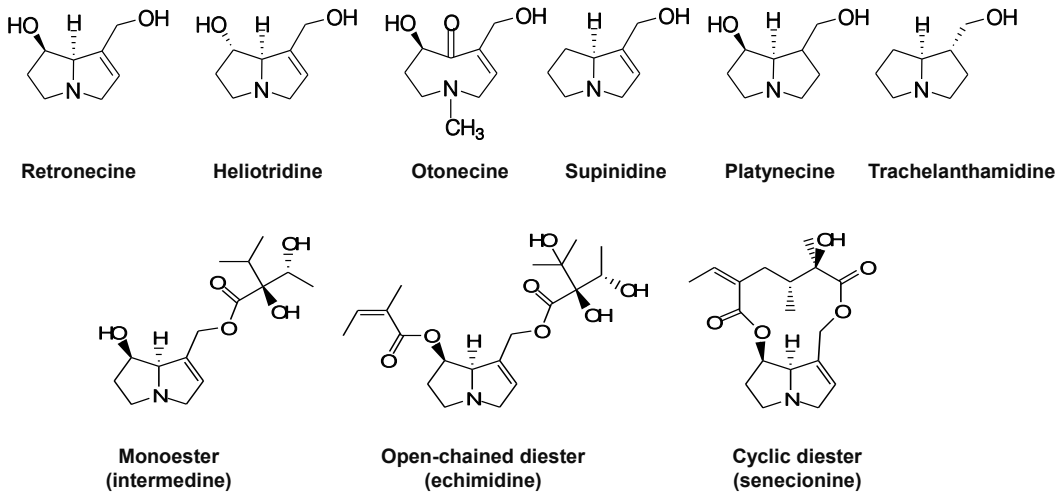

Figure 1.4. Necine base types and types of esterification depending on the linkage between the necic acids and the necine bases.

1.2.2 Toxicity and risks for human health

Due to their wide distribution in plants and their high prevalence of food contamination, PAs are listed among the most dangerous phytotoxins capable of

causing drug-induced liver damage (DILI). In general, the intake of a single potent dose of these toxins (acute toxicity) leads to severe cases of hepatotoxicity while the intake of small doses over a certain period of time (chronic toxicity) causes chronic liver diseases (Schrenk et al., 2020). The main structural features that a PA must possess to be considered capable of exerting toxicity are: (1) a double bond in the 1,2-position of the necine base, (2) an hydroxy-methylene substituent on the C-1 position and, preferably, a second hydroxyl group on the C-7 of the necine base, and (3) an esterification of the hydroxy-methylene at C-1 with a mono- or dicarboxylic acid containing a number of C atoms higher or equal to 5 (European Medicines Agency, 2014).

The metabolic pathway responsible of the PAs toxicity is the oxidation, which produces highly reactive pyrrole esters, called dehydropyrrolizidine alkaloids (DHPA). It is mainly carried out by a cytochrome P-450(CYP450)-dependent monooxygenase even if there are other isoforms involved in this reaction as well, such as CYP3A and CYP2B. After being formed, DHPAs can be conjugated by glutathione and excreted (detox route) or bind sulphur-, nitrogen-, and oxygen-containing groups of proteins and DNA to form toxic adducts (Schrenk et al., 2020).

Although the metabolic pathway is identical for all the types of PAs, the congeners differ widely in their relative toxicity. For instance, retronecine types of PAs are much more toxic than otonecine; in fact, it seems that pyrrole-protein adducts formed from otonecine in vitro are quantitatively less than those formed from retronecine bases with similar or identical necic acids. Cyclic diesters are more toxic than open-chained diesters and monoesters. The chirality at C-7 can also influence their toxicity; data have shown that cyclic open chained C-7-S diesters are more toxic than open chained C-7-R diesters, which are in turn more toxic than monoesters. The following general

scale can be concluded: C-7-S cyclic and open chained diesters > C-7-R cyclic and open chained diesters > C-7-S monoesters > C-7-R monoesters (Schrenk et al., 2020).

Currently, the risk assessment of PAs is based on the sum of selected PAs, assuming that they are all as toxic as the most dangerous ones, which are lasiocarpine and riddelliine. Furthermore, it seems that PANOs may also be responsible of liver damage since they can be reconverted into the relative PAs by the enzymes of the intestinal microbiota and the liver, after ingesting PANO-containing plants (Schrenk et al., 2020). Pyrrole-protein adducts can cross the space between the hepatocytes and the endothelial cells of the sinusoidal wall and attack the sinusoidal cells. The damage to hepatocytes and hepatic vein walls caused by toxic adducts leads to veno-occlusive disease (VOD) and liver cirrhosis (Moreira et al., 2018). Cases of neurotoxicity, teratogenicity, pulmonary arterial hypertension, and congestive heart failure have also been associated with intoxications by PAs (European Medicines Agency, 2014).

1.2.3 Occurrence in food

PAs are considered among the most widespread natural toxins as they can affect wildlife, livestock, and humans through feed and food contamination (Picron et al., 2018). As previously mentioned, PAs occur in a huge variety of plant species; however, the main sources of PAs are attributable to limited species of plants, which are all genera of the *Boraginaceae* family, the tribes *Senecioneae* and *Eupatorieae* of the *Asteraceae* family, and the *Crotalaria* genus of the *Fabaceae* family (EFSA CONTAM Panel, 2011). PA-producing plants are used for many different reasons, for instance some species belonging to the *Fabaceae* family are used as ground cover plants and soil improvers, others as ornamental plants and animal feeding. Many plants belonging to the *Boraginaceae* family are appreciated for the quality of the honey that comes from them. In general, the *Heliotropium* genus of *Boraginaceae* and the

Crotalaria genus of *Fabaceae* have been reported to be the main responsible of food poisoning in humans as they may be present as weeds of cereal and legume crops and their seeds be accidentally mixed with the main crop. As general rule, the closer the relationship between the food and the PA-producing plant is, the higher the level of PAs in the final product can be (EFSA CONTAM Panel, 2011).

Honey was the first food product to raise concerns about its safety regarding the contamination of PAs, both because it is a widely used product and is taken by vulnerable subjects, such as infants and children (Picron et al., 2018). To date, 25 PAs and 20 PANOs have been researched in commercially available honeys; among the most investigated PAs are echimidine, lycopsamine, retrorsine, senecionine, seneciphylline, heliotrine, senkirkine, intermedine, lasiocarpine, and monocrotaline, while among the PANOs, senecionine N-oxide, retrorsine N-oxide, monocrotaline N-oxide, and seneciphylline N-oxide are the most investigated ones. According to literature studies, the percentage of contamination of honey samples varies from 17% to 91% and the PAs responsible for the contamination are echimidine and lycopsamine-like compounds (Brugnerotto et al., 2021).

Other food categories at risk of contamination by PAs are botanical preparations, such as teas, herbal infusions, and plant-based dietary supplements. A recent study of Mulder and co-workers analyzed 168 samples of teas and herbal infusions regarding the presence of 28 PAs and found that 91% of them were contaminated by one or more PAs. Senecionine, retrorsine and their N-oxides were the most detected PAs in high concentrations, together with lycopsamine and heliotrine-like compounds (Mulder et al., 2018). Regarding plant-based dietary supplements, a study conducted by EFSA in 2016 revealed the presence of a number between 9 to 28 PAs in 278 samples of dietary supplements. The analyzed samples contained extracts of different PA-producing

plants (*Borago officinalis*, *Eupatorium cannabinum*, *Symphytum officinale*, *Tussilago farfara*); among them, lycopsamine, intermedine and their respective N-oxides represented the PAs detected in the highest concentrations while senkirkine was found in 80-90% of the samples containing extracts of *Tussilago farfara* (EFSA, 2016).

1.2.4 Legal requirements of contaminated food products

In 2017, the EFSA CONTAM Panel updated the risk assessment of human exposure to PAs and set a new Reference Point (RP) of 237 μg/kg body weight per day to assess the carcinogenic risks of PAs. The RP was calculated as benchmark dose lower confidence level for a 10% excess cancer risk ($BMDL_{10}$) of 70 μg/kg body weight per day, using the margin of exposure (MOE) approach (EFSA CONTAM Panel, 2017).

Subsequently, the European Commission published the Regulation (EU) 2020/2040 concerning the maximum residue levels of pyrrolizidine alkaloids in certain foodstuffs (European Commission, 2020). The document sets maximum levels (μg/kg) of 35 PAs (21 PAs and 14 coeluting isomers) for 11 foodstuff categories (**Table 1.1**).

Regarding the performance of analytical methods for contaminants in food chain, PAs are still not included in any European guidelines; however, some countries have adopted their internal regulation. Italy refers to the Decree of the Ministry of Health of 10 August 2018 (updated on 26 July 2019) which identifies the plants allowed for use in dietary supplements as part of the BelFrIt project, named after the three participating countries: Belgium, France, and Italy. Regarding PAs-producing plants, producers are required both to use parts of the plant specified in the document and to verify that PAs are absent in the final product with a limit of detection of 4 μg kg^{-1}.

Table 1.1. Annex of the Commission Regulation (EU) No 2020/2040.

Foodstuffs		Maximum level (*) (µg/kg)
8.4.	**Pyrrolizidine alkaloids**	
8.4.1.	Herbal infusions (dried product) with the exception of the herbal infusions referred to in 8.4.2. and 8.4.4.	200
8.4.2.	Herbal infusions of rooibos, anise (*Pimpinella anisum*), lemon balm, chamomile, thyme, peppermint, lemon verbena (dried product) and mixtures exclusively composed of these dried herbs with the exception of the herbal infusions referred to in 8.4.4.	400
8.4.3.	Tea (*Camellia sinensis*) and flavoured tea (*Camellia sinensis*) (dried product) with the exception of the tea and flavoured tea referred to in 8.4.4.	150
8.4.4.	Tea (Camellia sinensis), flavoured tea (Camellia sinensis) and herbal infusions for infants and young children (dried product)	75
8.4.5.	Tea (Camellia sinensis), flavoured tea (Camellia sinensis) and herbal infusions for infants and young children (liquid)	1,0
8.4.6.	Food supplements containing herbal ingredients including extracts with the exception of the food supplements referred to in 8.4.7.	400
8.4.7.	Pollen based food supplements Pollen and pollen products	500
8.4.8.	Borage leaves (fresh, frozen) placed on the market for the final consumer	750
8.4.9.	Dried herbs with the exception of the dried herbs referred to in 8.4.10.	400
8.4.10.	Borage, lovage, marjoram and oregano (dried) and mixtures exclusively composed of these dried herbs	1 000
8.4.11.	Cumin seeds (seed spice)	400

(*) The maximum level refers to the lowerbound sum of the following 21 PAs:
— intermedine/lycopsamine, intermedine-N-oxide/lycopsamine-N-oxide,
— senecionine/senecivernine, senecionine-N-oxide/senecivernine-N-oxide,
— seneciphylline, seneciphylline-N-oxide,
— retrorsine, retrorsine-N-oxide,
— echimidine, echimidine-N-oxide,
— lasiocarpine, lasiocarpine-N-oxide,
— senkirkine,
— europine, europine-N-oxide,
— heliotrine and heliotrine-N-oxide
and the following additional 14 PAs known to co-elute with one or more of the above identified 21 PAs, making use of certain currently used analytical methods:
— indicine, echinatine, rinderine (possible co-elution with lycopsamine/intermedine)
— indicine-N-oxide, echinatine-N-oxide, rinderine-N-oxide (possible co-elution with lycopsamine-N-oxide/intermedine-N-oxide)
— integerrimine (possible co-elution with senecivernine/senecionine)
— integerrimine-N-oxide (possible co-elution with senecivernine-N-oxide/senecionine-N-oxide)
— heliosupine (possible co-elution with echimidine)
— heliosupine-N-oxide (possible co-elution with echimidine-N-oxide)
— spartioidine (possible co-elution with seneciphylline)
— spartioidine-N-oxide (possible co-elution with seneciphylline-N-oxide)
— usaramine (possible co-elution with retrorsine)
— usaramine N-oxide (possible co-elution with retrorsine N-oxide)
PAs, which can be individually and separately identified with the used method of analysis, shall be quantified and included in the sum.

1.2.5 Analysis of pyrrolizidine alkaloids

The recent food safety issue regarding the presence of PAs-contaminated food products on the market has led to a significant increase in the number of analytical methodologies developed to detect and quantify these contaminants in different food matrices. Although the analysis of PAs has been performed with different analytical techniques over the years, such as thin layer chromatography (TLC), gas chromatography (GC), nuclear magnetic resonance (NMR), capillary electrophoresis, immunoaffinity or ultraviolet (UV) spectroscopy, liquid chromatography coupled to mass spectrometry (LC-MS) has recently been favoured for the analysis of food samples by the guidelines and recommendations of the official regulatory authorities (Casado et al., 2022a). For instance, EFSA suggested HPLC-MS as the most suitable analytical techniques for the determination of these toxins due to their high sensitivity and selectivity (EFSA CONTAM Panel, 2011). In fact, the newly developed analytical methods must allow for the accurate identification and quantification of these analytes at very low concentration levels in a wide range of food products (Casado et al., 2022a). Moreover, in the case of highly complex matrices, such as dietary supplements and botanicals, the sample preparation plays a critical role. A purification step based on solid-phase extraction (SPE) is often performed to obtain enriched PAs fractions. However, the affinity for PAs significantly varies depending on the type of SPE method used, which is a major limitation as it may lead to underestimation of certain PAs (Tsiokanos et al., 2023). In the last decade, QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), a type of dispersive SPE introduced in 2002 for the analysis of pesticide residues in plant matrices, showed to be a promising methodology for the selective extraction of PAs and PANOs from dietary supplements and botanical products, prior to the LC-MS analysis (Casado et al., 2022b).

1.3 Illicit substances as adulterants in dietary supplements and botanicals

Given the growing popularity of dietary supplements, the problem of their adulteration with illegal and undeclared substances has become a major concern. Dietary supplement manufacturers are not legally required to provide evidence that their product is safe or effective, contrary to what is required for medicines. This allows unscrupulous manufacturers to adulterate their products by adding synthetic drugs (or their analogues) or unauthorized botanicals to increase the efficacy of the final product. If the final product succeeds in producing the desired effects on consumers, the sales will increase and so will their profits (Czepielewska et al., 2018). The presence of adulterated dietary supplements on the market represents a serious health risk for consumers both because of side effects of the illicit drugs that may occur and interactions between the active pharmaceutical ingredient and other phytochemicals present in the formulation (Muschietti et al., 2020; Mathon et al., 2013).

1.3.1 Main classes of illicit substances

1.3.1.1 Active pharmaceutical and synthetic adulterants

1,3-Dimethylamylamine (DMAA) and analogues

DMAA is a phenethylamine alkaloid that naturally occurs in *Geranium* spp. It has been illicitly used in dietary supplements as fat burner, appetite inhibitor and stimulant. Its use was banned in dietary supplements both in EU and US after cases of hepatotoxicity attributed to the intake of a weight-loss supplement (OxyELITE Pro), known to contain it. Later, it was discovered that these cases were due to the presence of aegeline, a phenethylamine alkaloid of *Aegle marmelos* (Ronis et al., 2018). DMAA acts as indirect sympathomimetic drug on the central nervous system and can cause an increase in blood pressure, respiratory failure, tachycardia, and heart attack. 2-

dimethylaminoethanol (DMAE), 1,3-dimethylbutylamine (DMBA), and octodrine (DMHA) are analogues of DMAA detected as adulterants in dietary supplements (Pawar & Grundel, 2017; Cohen et al., 2018).

2,4-Dinitrophenol

2,4-Dinitrophenol is a slimming agent that promotes weight loss by inducing a hypermetabolic state of the body, which is followed by unwanted hyperthermia which is its main side effect. For this reason, after being marketed in numerous slimming products of the 90s, it was withdrawn from the market because it was considered a dangerous product (Koncz et al., 2021).

Anabolic-androgenic steroids (AAS)

AAS are a group of substances structurally related to testosterone and with similar effects to it (Abbate et al., 2015). Athletes use them to improve their physical and muscle strength. However, their prolonged use causes various side effects and creates unequal conditions for athletes in sports competitions; for this reason, their use in dietary supplements is strictly prohibited by the World Anti-Doping Agency (WADA). Androstenedione and methyltestosterone are just some of the AAS found as adulterants in dietary supplements (Odoardi et al., 2015).

Fluoxetine

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI). It is a psychotropic drug with anxiolytic and antidepressant action in human (Micheli et al., 2018). These properties have led to its illicit use in food supplements as a “mood enhancer”. Side effects due to misuse of fluoxetine are dry mouth, glaucoma, drowsiness, weakness, and uncontrollable tremors (Kim et al., 2014).

Phenolphthalein

Phenolphthalein is a laxative that works by increasing intestinal motility and facilitating the passage of stool. It was the main component of several 20th-century over-the-counter laxatives until, in 1997, the US Food and Drug Administration (FDA) proposed phenolphthalein to be classified as "not generally recognized as safe and effective" after a feeding study in rodents detected an increased incidence of the cancer of ovaries, adrenal glands, kidneys, and hematopoietic system in treated animals (Khazan et al., 2014).

Sibutramine

Sibutramine is a serotonin-norepinephrine reuptake inhibitor (SNRI). By preventing the reuptake of serotonin and noradrenaline, sibutramine increases the amount of these neurotransmitters in the brain. Clinical studies in humans have shown that sibutramine can reduce appetite exerting a slimming effect. For this reason, it was approved for the treatment of obesity in doses of 10 and 15 mg. However, sibutramine is banned due to severe side effects such as stroke, myocardial infarction, and cardiac arrest (Biesterbos et al., 2019).

Phosphodiesterase type 5 inhibitors (PDE5I)

PDE5I are a group of drugs used to treat erectile dysfunction. The inhibition of PDE5 enzyme causes the increase of cyclic guanosine monophosphate (cGMP) levels, which results in the relaxation of the smooth muscle of the penis followed by an increased blood flow and hence, the penile erection (Carson & Lue, 2005). In the EU, the drugs sildenafil, tadalafil, vardenafil, and avanafil are the only PDE5I approved by the competent authorities for the treatment of erectile dysfunction (Rocha et al., 2016). Being molecules with a vasodilating action, their intake could be considered a risk factor for subjects suffering from hypotension or cardiovascular disease (Venhuis &

De Kaste, 2012). Numerous cases of dietary supplement adulterations have confirmed not only the presence of approved PDE5I but also unapproved synthetic analogues of these drugs such as acetildenafil, amino tadalafil, homo sildenafil, thiosildenafil, xanthoanthrafil and many others (Venhuis & De Kaste, 2012). These analogues are often used by manufacturers as they are more difficult to detect in routine inspections using standard protocols. However, they pose an even greater risk to the consumers' health as their pharmacokinetics and safety profile are not known (Patel et al., 2014).

1.3.1.2 Botanical markers of unauthorized plants

Aristolochic acids

Aristolochic acid I and II are a group of modified aporphine produced from stephanine, a benzyl tetrahydroisoquinoline alkaloid. They occur in *Aristolochia*, a genus of plants commonly used in Chinese traditional medicines. Different studies have associated aristolochic acids with a form of nephropathy caused by a prolonged intake of a Chinese herb-based weight-loss preparation containing *Aristolochia fangchi* (Ioset et al., 2003). For this reason, most European Member States have restricted the use of *Aristolochia* species in botanical products. Despite this, plant-based dietary supplements and traditional medicines containing aristolochic acids are still available on the market (Abdullah et al., 2017).

Icariin

Icariin is one of the main flavonoids of *Herba epimedii*, a plant traditionally used in China, Japan, and Korea to treat osteoporosis, sexual dysfunction, and cardiovascular diseases. Extracts of *H. epimedii* are used in the formulation of dietary supplements because icariin inhibits PDE5 enzyme, albeit with a lower potency than sildenafil. Its toxicology has not been fully investigated and no maximum

recommended daily dose has been established for the safe intake of icariin in dietary supplements (Biesterbos et al., 2019).

Vinpocetine

Vinpocetine is a synthetic derivative of vincamine, an alkaloid of *Vinca minor*. While not regulated for the use in dietary supplements, this substance is often included as active ingredient in dosages of 5 to 40 mg for the prevention of cognitive impairment (Cohen, 2015). Its vasodilatory power leads manufacturers to claim cognitive enhancement due to the increased blood supply to the brain. In addition, it is also often added to muscle building supplements to increase the blood flow to the muscles and facilitate the supply of oxygen and nutrients to the muscle cells. Although extensive research has demonstrated the beneficial effects of vinpocetine, further studies are needed to define an effective dosage of both short- and long-term use of vinpocetine in dietary supplements (Zhang et al., 2018).

Yohimbine

Yohimbine is the primary alkaloid in Yohimbe bark (*Pausinystalia johimbe*), a banned product in dietary supplements (European Commission, 2019) but still available in products for sexual performance enhancement via internet retail stores. Yohimbine is an antagonist of α2-adrenergic receptor present in the central and peripheral nervous system. Blocking these receptors leads to an increase in the release of nitric oxide; the direct consequence is an increased blood flow to the penis which makes it easier for an erection to occur (Biesterbos et al., 2019). Yohimbine is a drug orally administered in the treatment of erectile dysfunction in a single dose range of 5-10 mg and a daily dose range of 10-30 mg. More than 40 mg/day of yohimbine can cause dangerous side effects, such as tachycardia, hypertension, insomnia, headache, anxiety, hallucinations, and panic attacks (EFSA ANS Panel, 2013).

1.3.1.3 Other potentially harmful substances

Synephrine

Synephrine is a phenethylamine alkaloid. High amounts of synephrine are found in bitter orange (*Citrus aurantium*) and is commonly used in weight-loss dietary supplements for athletes to increase energy expenditure, usually in combination with caffeine and/or multiple herbal ingredients (Stohs, 2017). Synephrine is a sympathetic adrenergic agonist that weakly activates the α-adrenergic receptors. These receptors typically respond to adrenaline causing an increase in heart rate or blood pressure. Not enough research has been done to establish a safe and effective dose of synephrine. However, several published case reports have associated products containing synephrine with various adverse effects, such as myocardial infarction, ischemic stroke, tachycardia, bradycardia, and acute arterial hypertension (Rossato et al., 2010; Biesterbos et al., 2019).

Melatonin and 5-hydroxytryptophan

Melatonin (N-acetyl-5-methoxytryptamine) and serotonin (5-hydroxytryptamine) are the primary hormones involved in the regulation of the circadian rhythm. Within the cells of the mammalian pineal gland, L-tryptophan is transformed in serotonin which is then converted into melatonin effects (Zhdanova & Tucci, 2003).

Melatonin is useful in treating insomnia. Furthermore, melatonin regulates the flow of energy to and from energy reserves, controlling the size and activity of brown adipose tissue as well as the browning process of white adipose tissue. Therefore, melatonin can exert weight-reducing effects (Zhdanova & Tucci, 2003).

5-Hydroxytryptophan is naturally produced by the body but can also be obtained by extraction from the seeds of the African plant *Griffonia simplicifolia*. It works by crossing the blood-brain barrier and increasing the synthesis of serotonin in the central

nervous system. Supplementing with 5-hydroxytryptophan leads to an increase in serotonin levels in the brain. It can also act as appetite suppressant by counteracting hunger-inducing hormones and, thus, helping to lose weight (Turner et al., 2006).

Due to their action, both melatonin and 5-hydroxytryptophan have often been found in the formulation of both relaxing and slimming products. The melatonin content in supplements ranges from 0.5 to 5 mg. However, based on the scientific opinion of EFSA, the authorized dose of melatonin as a sleep aid is 1 mg/day (EFSA NDA Panel, 2011). Higher doses could lead to side effects such as headaches, light-headedness, and vivid dreams; the recommended doses of 5-hydroxytryptophan are 100-300 mg/day for sleep and 250-300 mg/day for weight loss (Shin et al., 2022).

Piperin

Piperin is the main alkaloid of black pepper (*Piper nigrum*). It is used in dietary supplements, in doses between 5 and 30 mg, as a bioavailability enhancer of the active ingredients contained in the formulation (Ziegenhagen et al., 2021). Several studies have demonstrated the occurrence of interactions between piperin and different drugs, which have led to a greater bioavailability of those drugs when ingested together with piperin. Depending on the drug and the extent of the interaction, these interactions can lead to harmful effects. Although available human studies have rarely reported effects considered adverse, these are considered insufficient for a careful risk assessment (Lee et al., 2018).

1.3.2 EU authorities involved in the safety assessment of dietary supplements

The European market of dietary supplements is controlled by the Directorate-General for Health and Consumer Protection (DG-SANTE) and by the national health authorities of each member states. DG-SANTE collaborates with EFSA to discuss the specific requirements of the requests of the Member States, the Commission or of the

EU Parliament (Czepielewska et al., 2018). An important role in the assessment of the safety of dietary supplements is played by the Rapid Alert System for Food and Feed (RASFF), a network founded by the Council of the Member States in 2002 to enable the EU health authorities to take immediate action, warn consumers, and withdraw certain products from the market. The RASFF provides the supervisory authorities with a rapid and efficient mechanism for exchanging knowledge on the notifications issued by the different EU member states every time a food presents a serious risk to public health due to contamination, adulteration, or lack of framing in laws. The RASFF database can be searched by food type and product category; "Dietetic foods, food supplements, and fortified foods" is the category which refers to dietary supplements (Rocha et al., 2016). Since 2002, the number of RASFF notifications in the product category "dietetic products, food supplements and fortified foods" has steadily increased (**Figure 1.5**), positioning as second most notified category of the year 2020 and most notified category of both the years 2019 and 2018.

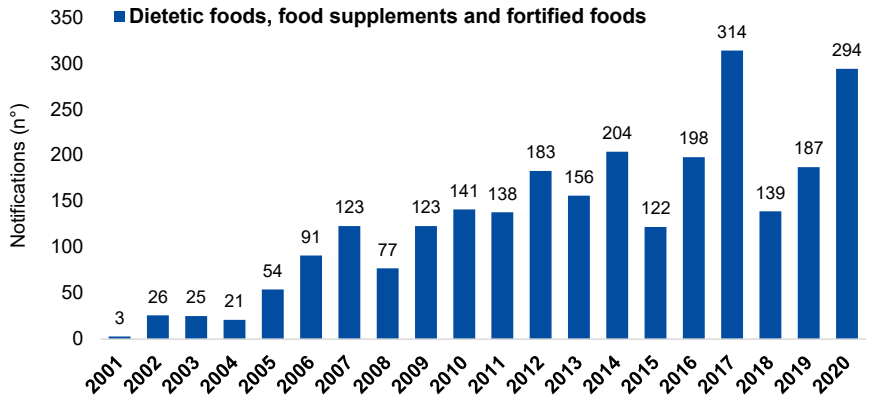

Figure 1.5. Total number of RASFF notifications in the product category "Dietetic foods, food supplements and fortified foods" from 2001 to 2020.

1.3.3 Analysis of illicit and undeclared substances

The growing production and use of dietary supplements and botanicals implies a greater need for analytical methods to detect an ever-wider range of adulterants. The new analytical methods must be quick and easy to use as well as flexible so that they can be adapted to the detection of ever "new" analytes (Vaclavik et al., 2014a). "Dilute and shoot" and QuEChERS procedures has been widely used as a simple and generic step of sample preparation prior to the instrumental analysis (Vaclavik et al., 2014b). Regarding the analytical technique, ultra-high performance liquid-chromatography (UHPLC) has been extensively documented in recent applications. UHPLC instrumentations in combination with UV, fluorometric, and MS detectors has been used for the analysis of different classes of adulterants in dietary supplements and botanicals (Vaclavik et al., 2014b). Mass spectrometric detectors are the most widely used for the detection and identification of adulterants in dietary supplements and botanicals as they meet the selectivity and sensitivity requirements needed for such analyses (Patel et al., 2014). However, the use of spectroscopic methods such as infrared (IR), near infrared (NIR), and Raman spectroscopy has grown in recent years. Spectroscopic methods are generally quick, easy to use, require no skilled technicians and little or no sample preparation, and are suitable for in-situ analyses. For these reasons, they are suitable for quick screening to be performed by inspectors at customs, since they usually deal with many products suspected of being adulterated, which must be confiscated and sent to a laboratory for inspection analyses (Deconinck et al., 2014).

CHAPTER 2

A fast and simple sample preparation procedure for the extraction and clean-up of pyrrolizidine alkaloids from different food matrices based on Salting-out Assisted Liquid-Liquid Extraction (SALLE)

Adapted from

Rizzo, S., Celano, R., Campone, L., Rastrelli, L., & Piccinelli, A. L. (2022). Salting-out Assisted Liquid-Liquid Extraction for the rapid and simple simultaneous analysis of pyrrolizidine alkaloids and related N-oxides in honey and pollen. *Journal of Food Composition and Analysis*, *108*, 104457

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Rizzo, S., Celano, R., Piccinelli, A. L., Serio, S., Russo, M., & Rastrelli, L. (2023). An analytical platform for the screening and identification of pyrrolizidine alkaloids in food matrices with high risk of contamination. *Food Chemistry*, *406*, 135058.

2.1 Introduction

The sample preparation is considered a bottleneck of the analysis of PAs and PANOs. Therefore, the choice of the analytical technique to use for their extraction and clean-up is crucial (Klein-Júnior et al., 2016). Solid-liquid extraction techniques, usually combined with a purification step based on SPE, represent the most used procedures. In the context of SPE, strong-cation exchange (SCX) sorbents have been widely used for the purification of PAs and PANOs from complex food samples. However, SPE-based methodologies are usually expensive, laborious, and time-consuming. For these reasons, the scientific community is now oriented towards the development of faster, simpler, and cheaper analytical procedures, which satisfy the principles of the green analytical chemistry (Casado et al., 2022a). Considering the co-occurrence of PAs and PANOs, a simultaneous extraction of both is required. Conventional liquid-liquid extraction (LLE) procedures are unable to perform their simultaneous extraction because of the semipolar-to-polar nature of these compounds; in fact, they are easily extracted with polar organic solvents or acidified aqueous solutions and poorly extracted with conventional water-immiscible organic solvents. To overcome the drawbacks of LLE procedures, the authors decided to explore the potential of salting-out assisted liquid-liquid extraction (SALLE) (Celano et al., 2019).

SALLE is commonly associated to QuEChERS, a type of dispersive SPE initially developed for the multiresidue analysis of pesticides in different food matrices (Sixto et al., 2019). Numerous studies have reported the use of SALLE procedures for partitioning polar analytes into an organic phase in a huge variety of applications, such as the analysis of drugs, toxicants, pesticides, and active pharmaceutical ingredients, and different matrices, including biological fluids, food products and water (Hammad et al., 2022). SALLE is based on the use of a water-miscible organic solvent as

extraction solvent and the addition of a pre-measured mixture of salts (salting-out agent) to the aqueous extracts containing the analytes. The addition of the salting-out agent facilitates the phase separation between the aqueous sample and the water-miscible organic solvent and favor the distribution of the polar analytes into the organic phase by increasing the ionic strength of the aqueous solution (Zhang et al., 2009; Avula et al., 2012; Wen et al., 2013). Compared to conventional LLE techniques, SALLE allows to expand the concept of LLE to a wider range of compounds, from high to low polarity; in addition, it has numerous advantages, including its simplicity, rapidity, low cost, and suitability for green chemistry applications, since it is possible to choose less toxic solvents (such as acetone, acetonitrile, ethanol, isopropanol, etc.) (Valente et al., 2013; Soylak & Yilmaz, 2020).

In the present study, a SALLE procedure was developed for the fast and simple simultaneous extraction and clean-up of PAs and PANOs from aqueous extracts of different food matrices at high risk of contamination, which are honey, pollen, herbal infusions, teas, and plant-based dietary supplements (various formulations: capsules, tablets, syrups, and infusions). Given the large number of PAs and PANOs currently known (> 600), the lack of reference standards, and the high cost of those currently available, a representative pool of analytes was selected to develop the procedure and evaluate its performances. The selection of the target analytes was made by considering their chemical structure, distribution in the food matrices of interest, and toxicological evidence; in detail, nine retronecine type PAs and PANOs were selected, of which three monoesters (the diastereoisomeric couple intermedine/lycopsamine and intermedine N-oxide), two open chained diesters (echimidine and its N-oxide), and four cyclic diesters (senecionine, retrorsine, and their N-oxides). The optimization of the experimental parameters affecting SALLE efficiency was carried out by

experimental design, with the aim of reducing the number of experiments and save time, costs, and efforts during the analyses. The optimization experiments were carried out on an artificial sugar solution, which was chosen as test matrix because of its high sugar content; this allowed to evaluate the performances of SALLE on the highest sugar content matrices (honey and dietary supplements in the form of syrups) first. The optimized procedure was then adapted to a wider number of analytes, since 21 further reference standards became available afterwards. The instrumental analyses were carried out by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) and the analytical performances evaluated in terms of extraction efficiency.

2.2 Materials and methods

2.2.1 Chemicals and standards

Analytical grade acetonitrile (MeCN), methanol (MeOH), isopropanol (iPrOH), magnesium sulfate heptahydrate ($MgSO_4$ $7H_2O$), sodium sulfate (Na_2SO_4), sodium chloride (NaCl), sodium acetate (CH_3COONa), ammonium sulfate ($(NH_4)_2SO_4$), sodium hydroxide (NaOH), sulfuric acid (H_2SO_4) and MS grade formic acid (HCOOH) were purchased from Merk Chemical (Milan, Italy). MS-grade MeCN and water (H_2O) were provided by Romil (Cambridge, UK). Ultrapure water (18 MΩ) was prepared using a Milli-Q purification system (Millipore, Bedford, USA). Reference standards (n = 9) (≥98% HPLC grade) of echimidine (Em), echimidine-N-oxide (EmNO), intermedine (Im), intermedine-N-oxide (ImNO), lycopsamine (Ly), retrorsine (Re), retrorsine-N-oxide (ReNO), senecionine (Se), senecionine-N-oxide (SeNO) were provided by Merk Chemical and used for the development and optimization of the procedure. Reference standards (n = 19) (85-98% HPLC grade) of erucifoline (Er), erucifoline N-oxide (ErNO), europine (Eu), europine N-oxide (EuNO), heliotrine (He), heliotrine N-oxide (HeNO), jacobine (Jb), jacobine N-oxide (JbNO), lasiocarpine (Lc), lasiocarpine N-oxide (LcNO), lycopsamine N-oxide (LyNO), monocrotaline (Mc), monocrotaline N-oxide (McNO), seneciphylline (Sp), seneciphylline N-oxide (SpNO), senkirkine (Sk), senecivernine (Sv), senecivernine N-oxide (SvNO), and trichodesmine (Td) were provided by Merck Chemical and used to extend the scope of the study. Standard stock solutions were prepared for each analyte at a concentration of 1 mg mL^{-1} in MeOH and stored at 4 °C. Diluted solutions and standard mixtures were prepared in H_2O/MeOH 7:3 v/v.

2.2.2 UHPLC-MS/MS analysis

UHPLC analyses were performed using a Shimadzu Nexera X2 UHPLC system (Shimadzu, Milan, Italy) coupled to a QTRAP 6500 mass spectrometer (AB Sciex, Milan, Italy) equipped with a TurboV ion source. Analyst software (version 1.6, Sciex) was used for instrument control, data acquisition, and data analysis. Chromatographic separation was performed using a Luna Omega Polar C18 (2.1 × 100 mm, 1.6 μm; Phenomenex, Bologna, Italy), thermostated at 40 °C, and a binary gradient of H_2O (A) and MeCN (B) both containing 0.1% HCOOH, at a flow rate of 400 μL min^{-1}. The eluting gradient was set as follows: 0−1 min, 2% B; 1−5.5 min, 2−8% B; 5.5−7.5 min, 8% B; 7.5−9.5 min, 8−12% B; 9.5−11 min, 12−18% B; 11−13 min, 18− 20% B. After each injection, cleaning (98% B, 6 min) and re-equilibration of the column (6 min) were performed. The injection volume was set at 5 μL. The mass spectrometer operated in positive ionization mode. Nitrogen was used as collision gas (CAD), and a medium value of pressure was set. The MRM parameters are reported in **Table 2.1**.

Table 2.1. UHPLC-MS/MS parameters of the 28 reference standards.

Analyte	Code	Retention time (min)	Q1 mass (*m/z*)	Q3 mass (*m/z*)	Collision energy (V)
Monocrotaline	Mc	3.40	326.2	120, 94, 121	31
Intermedine	Im	4.90	300.1	94, 120, 156	37
Monocrotaline N-oxide	McNO	5.05	342.2	137, 94, 119	45
Lycopsamine	Ly	5.10	300.0	94, 20, 156	37
Erucifoline	Er	5.20	350.2	120, 138, 84	40
Europine	Eu	5.40	330.2	138, 156, 254	32
Europine N-oxide	EuNO	5.80	346.2	172, 94, 256	36
Jacobine	Jb	6.00	352.1	120, 155, 122	36
Intermedine N-oxide	ImNO	6.10	316.0	172, 138, 94	43
Lycopsamine N-oxide	LyNO	6.30	316.1	172, 138, 94	32
Erucifoline N-oxide	ErNO	6.40	366.2	118, 136, 120	37
Jacobine N-oxide	JbNO	6.90	368.2	296, 120, 155	40
Trichodesmine	Td	7.70	354.2	222, 120, 308	41
Retrorsine	Re	7.90	352.1	94, 120, 138	65
Heliotrine	He	8.50	314.2	138, 156, 120	38
Retrorsine N-oxide	ReNO	8.60	368.0	119, 120, 136	56
Seneciphylline	Sp	9.10	334.2	120, 38, 94	38
Heliotrine N-oxide	HeNO	9.30	330.2	172, 111, 136	42
Seneciphylline N-oxide	SpNO	10.20	350.1	120, 119, 94	22
Senecivernine	Sv	11.10	336.1	120, 138, 94	38
Senecionine	Se	11.30	336.0	94, 120, 138	73
Senecivernine N-oxide	SvNO	11.50	352.1	120, 138, 118	36
Senecionine N-oxide	SeNO	11.80	352.0	118, 120, 136	33
Echimidine N-oxide	EmNO	12.40	414.0	254, 137, 120	39
Senkirkine	Sk	12.40	366.2	168, 150, 122	35
Echimidine	Em	12.40	398.0	120, 220	44
Lasiocarpine	Lc	14.30	412.2	120, 220, 336	37
Lasiocarpine N-oxide	LcNO	14.90	428.2	254, 136, 352	45

2.2.3 Experimental design

The experimental conditions affecting SALLE were optimized using a chemometric approach (response surface design), which was designed using the Statgraphics Centurion XVI software, version 18 (Statistical Graphics, Rockville, USA) The optimization experiments were carried out on a 25% w/v artificial sugar solution (supersaturated solution at 80% w/w), which was prepared by adding 45 g of fructose and 35 g of glucose to 100 g of water (Celano et al., 2019). A Box-Behnken design 2-factors interactions, with 21 degrees of freedom, 2 blocks of replicates, made of 16 randomized experimental runs and 4 center points for each block, was applied. The following independent variables were studied: (A) extraction solvent volume (MeCN, 1-4 mL); (B) concentration of $MgSO_4 \cdot 7H_2O$ (0-1 mol L^{-1}); and (C) concentration of Na_2SO_4 (0.5-1.5 mol L^{-1}). The aqueous phase volume was kept constant, at 2 mL. The minimum and maximum levels of the experimental factors A-C were selected based on preliminary experiments. The extraction efficiency (EE) of selected PAs and PANOs and the volumetric ratio ($r = MeCN_{preSALLE}/MeCN_{postSALLE}$) (hit target = 1) between the acetonitrile volume added before SALLE ($MeCN_{preSALLE}$) and the acetonitrile volume removed after the phase separation ($MeCN_{postSALLE}$), were considered as response variables. The experimental conditions and response factors are listed in the **Table 2.2**. The analysis of variance (ANOVA) allowed to determine the statistical significance of the experimental factors and their first order interactions. A response surface approach was used to estimate the statistical significance of the regression coefficients and calculate the coefficients of the proposed quadratic model.

Table 2.2. Experimental conditions of the response surface design (Box-Behnken design 2-factor interactions).

Experimental variables	Levels		
	Low	**Medium**	**High**
A: MeCN volume (mL)	**1**	**2.5**	**4**
B: $MgSO_4 \cdot 7H_2O$ (M)	**0**	**0.5**	**1**
C: Na_2SO_4 (M)	**0.5**	**1**	**1.5**

Experimental variables				Response factors (*r* and EE%)									
Block	**A**	**B**	**C**	***r***	**Im**	**Ly**	**Se**	**Re**	**Em**	**ImNO**	**SeNO**	**ReNO**	**EmNO**
1	2.5	0	1.5	0.84	19.63	19.08	85.85	63.24	83.38	26.38	70.53	41.40	68.35
1	4	0	1	1.08	94.43	93.33	89.46	100.00	98.03	54.10	100.66	76.88	100.37
1	2.5	0.5	1	1.23	102.39	101.67	92.50	98.56	98.03	97.14	115.89	108.06	112.09
1	2.5	1	0.5	1.20	88.59	86.67	87.94	95.69	91.27	71.62	99.01	89.78	91.58
1	2.5	0	0.5	1.14	114.59	116.67	105.41	122.18	116.62	108.57	134.77	129.57	126.01
1	4	1	1	1.18	75.86	76.08	84.52	89.12	85.63	67.24	90.73	80.65	84.25
1	2.5	0.5	1	1.19	83.29	82.58	98.20	106.37	111.55	106.67	133.11	127.96	123.08
1	1	0	1	1.15	96.55	95.83	111.87	117.66	113.24	88.00	119.54	109.14	112.09
1	2.5	0.5	1	1.14	110.34	108.33	96.87	110.06	107.04	106.67	121.52	115.05	115.75
1	1	0.5	0.5	1.18	80.11	78.58	79.01	81.52	80.56	64.76	83.77	73.66	79.85
1	4	0.5	1.5	1.18	75.33	74.92	85.28	88.50	84.51	67.43	92.05	81.72	86.45
1	4	0.5	0.5	1.17	49.71	48.00	85.85	84.39	99.15	65.05	107.62	84.95	100.37
1	1	0.5	1.5	1.11	102.39	102.50	92.69	103.29	101.41	92.48	109.27	103.76	105.49
1	2.5	0.5	1	0.98	68.97	48.33	103.13	82.96	98.03	39.90	78.81	56.45	76.19
1	1	1	1	1.18	98.14	98.33	100.47	110.68	106.48	85.05	118.87	106.99	109.16
1	2.5	1	1.5	1.08	98.14	98.33	95.54	106.78	103.10	58.19	104.64	80.65	104.03
2	2.5	0	1.5	0.85	25.15	23.92	92.88	70.64	84.51	31.33	70.20	43.28	73.26
2	4	0	1	1.07	103.45	102.50	92.12	98.97	90.70	67.52	110.93	91.40	113.55
2	2.5	0.5	1	1.22	87.53	85.83	87.56	82.55	77.18	80.67	92.05	88.71	93.77

Experimental variables	Levels		
	Low	Medium	High
A: MeCN volume (mL)	1	2.5	4
B: $MgSO_4 \cdot 7H_2O$ (M)	0	0.5	1
C: Na_2SO_4 (M)	0.5	1	1.5

Experimental variables				Response factors (*r* and EE%)									
Block	A	B	C	*r*	Im	Ly	Se	Re	Em	ImNO	SeNO	ReNO	EmNO
2	2.5	1	0.5	1.19	105.57	107.50	103.89	107.80	94.08	88.57	117.22	107.53	120.88
2	2.5	0	0.5	1.13	96.02	93.33	82.62	88.50	78.87	87.52	101.99	97.85	103.30
2	4	1	1	1.16	104.51	101.67	105.22	106.16	94.08	86.57	115.56	105.38	119.41
2	2.5	0.5	1	1.19	88.06	88.33	99.34	100.00	100.85	91.14	106.95	101.08	106.23
2	1	0	1	1.15	94.96	95.00	94.02	100.62	86.76	78.76	107.62	95.16	108.42
2	2.5	0.5	1	1.13	77.98	74.33	59.64	65.71	61.41	74.67	83.11	81.72	83.52
2	1	0.5	0.5	1.20	102.92	100.83	104.65	103.08	92.96	92.38	115.89	108.60	116.48
2	4	0.5	1.5	1.16	103.45	99.17	106.55	109.45	95.77	85.81	116.23	104.84	120.88
2	4	0.5	0.5	1.18	53.58	51.67	94.97	85.42	92.39	59.71	85.43	70.97	86.45
2	1	0.5	1.5	1.13	108.75	107.50	75.40	91.58	86.76	100.00	120.53	118.28	120.15
2	2.5	0.5	1	1.00	75.86	72.00	102.75	92.20	89.58	45.71	78.81	58.60	83.52
2	1	1	1	1.18	103.98	105.83	103.32	111.29	95.77	88.86	116.56	106.99	120.88
2	2.5	1	1.5	1.11	100.27	100.00	92.12	98.56	90.14	66.38	110.93	91.94	113.55

2.2.4 Sample pre-treatment

Honey and dietary supplements in form of syrups were diluted with distilled water to obtain a 25% w/v solution. Pollen and solid forms of plant-based dietary supplements (capsules and tablets) (1g) were extracted with an acidic solution (H_2SO_4, 0.05 M) (20 mL for pollen and 10 mL for dietary supplements) by sonication (15 min) after vortex-mixing (1 min) (Mulder et al., 2018). After centrifugation (5 min at 13,000 rpm), the supernatant was collected, and the solid residue was re-extracted under the same conditions. PA-producing plants were extracted like pollen. Infusions and teas (2g) were brewed with 150 mL of boiling water and left to infuse for 5 min (Mulder et al., 2018).

2.2.5 Salting-Out Assisted Liquid -Liquid Extraction (SALLE)

Under optimized conditions, a 10 mL aliquot of each aqueous sample was brought to a concentration of 1 M of $MgSO_4 \cdot 7H_2O$ and 1.5 M of Na_2SO_4. Samples were placed in a hot bath at 50 °C to facilitate the solubilization of the salts and the pH of the solution was adjusted at 9.6 by adding NaOH 5 N. Then, 2 mL of the solution were processed by SALLE by placing them into a 15 mL conical tube and adding 2 mL of acetonitrile. The solution was vortexed for 1 min to facilitate the dispersion of the extraction solvent into the aqueous solution and ensure the analytes extraction. Subsequently, the mixture was centrifuged at 13,000 rpm for 5 min to facilitate the phase separation. The extraction solvent (upper phase) was quantitatively transferred into a 2 mL Eppendorf tube and left to dry under nitrogen flow. The dried residue was redissolved with an appropriate volume of H_2O/MeOH 7:3 v/v before the chromatographic analysis commenced.

2.2.6 Analytical performance

The accuracy of SALLE was calculated as EE, according to the performance criteria established by the European analytical guidelines (Magnusson & Örnemark 2014). The experiments were conducted on 28 reference standards and performed on blank samples (previously identified) of six food matrices, including honey, pollen, back and green teas, herbal infusions, and plant-based dietary supplements. A representative sample of herbal infusion was prepared by mixing the same amount of chamomile, fennel, melissa, mint, and licorice, as these herbs were the most encountered during the collection of the samples. On the contrary, it was not possible to select or prepare a representative sample of a plant-based dietary supplement due to the high variability of their composition; for this reason, the experiments were carried out on 20 blank samples (10 samples × 2 replicates) of different composition and formulation. The EE was determined by pre- and post-spiking the target analytes at a concentration of 10 $\mu g\ L^{-1}$ of the SALLE extract (corresponding to 10 $\mu g\ kg^{-1}$ for honey, 100 $\mu g\ kg^{-1}$ for pollen, 75 $\mu g\ kg^{-1}$ for teas and infusions, and 50 $\mu g\ kg^{-1}$ for solid forms of plant-based dietary supplements) before and after the sample preparation procedure. Experiments were conducted in triplicate and EE was calculated as area ratio of pre- and post-spiked samples.

2.3 Results and discussion

The driving aim of the study was to develop a sample preparation procedure we could use for the extraction and clean-up of PAs and PANOs from aqueous extracts of different food matrices, which are honey, pollen, herbal infusions, black and green teas, and plant-based dietary supplements (various formulations: capsules, tablets, syrups, and infusions). However, the complex composition of these matrices and the low levels of PAs to be detected make it essential a pre-treatment and clean-up of the samples. Taking these steps allows to remove the matrix interferences and concentrate the analytes, prior to the instrumental analysis. The polar nature of PAs and PANOs, and consequently their poor solubility in non-polar solvents, make their simultaneous extraction with conventional LLE solvents difficult (Tighrine et al., 2019). SALLE was chosen as a promising technique for the extraction and clean-up of PAs and PANOs from different food matrices because it uses extraction solvents with high dielectric constants (such as acetone, acetonitrile, ethanol, and isopropanol), which favor the extraction of polar analytes (Hammad et al., 2022). However, being these solvents water-miscible, the addition of a salting-out agent is needed to increase the ionic strength of the aqueous solution and facilitate the separation of the two phases (Hammad et al., 2022).

To make the procedure suitable for the simultaneous extraction and clean-up of PAs and PANOs, the following parameters, affecting the performance of the procedure, were studied: pH of the aqueous solution, type of extraction solvent, and type and quantity of salting-out agents. Since SALLE is characterized by the addition of pre-measured amounts of a salting-out agent to the aqueous samples, we had to consider the chemical characteristics of all the food matrices to which SALLE was aimed and evaluate/predict the presence of any problems during the processing of the samples.

The main problem we had to face was the high sugar content of two of the considered matrices, which are honey and dietary supplements in the form of syrups. The addition of the salting-out agent to the aqueous samples of these matrices would subject them to a high risk of precipitation due to exceeding the saturation limit of the solution. For this reason, we decided to submit an artificial sugar solution, whose scope was to mimic the characteristics of the two abovementioned matrices, to the SALLE optimization experiments. This strategic choice allowed us to test the parameters affecting SALLE efficiency on these problematic matrices first to preserve their integrity. The selected parameters were then adapted to the analysis of the other food matrices.

2.3.1 Preliminary experiments

2.3.1.1 Effect of pH

It is well known that the pH of the solution has a strong influence on the extraction efficiency of ionizable compounds in LLE techniques (Campone et al., 2012). Since my research team already carried out experiments to assess the appropriate pH value to extract these contaminants from honey (Celano et al., 2019), the effect of pH on SALLE efficiency of this study was carried out by simply verifying and confirming the pH value of 9.6 previously selected (data not shown). An alkaline pH favors the shift of the equilibrium towards the unionized form of the analytes facilitating their distribution towards the organic solvent and, consequently, increasing the extraction efficiency of the procedure.

2.3.1.2 Selection of the extraction solvent

The selection of an appropriate extraction solvent is essential in LLE techniques as it plays an important role in enhancing the efficiency of the entire extraction process (Sarafraz-Yazdi & Amiri, 2010). An appropriate SALLE solvent should meet several

requirements, including a high affinity for the target analytes, water-miscibility, the ability to be readily separable from water after the addition of the salting-out agent, and good compatibility with LC systems (Magiera & Kwietniowska, 2016). Based on these requirements, MeCN and iPrOH were selected as possible SALLE solvents. The experiments were performed by mixing 2 mL of the extraction solvents with 2 mL of aqueous solution, salted-out with different types of salts ($MgSO_4 \cdot 7H_2O$, NaCl, CH_3COONa, and Na_2SO_4) at a concentration of 1 mol L^{-1}. Both solvents promote phase separation; however, iPrOH carried out with it a higher water content (r = 1.60-1.80) than MeCN (r = 1-1.40). A high water-content increases the risk of co-extracting matrix interferents and, therefore, decreases the accuracy and robustness of the entire procedure. Furthermore, the low solubility of some matrix components, such as lipids and proteins, in MeCN helps to enhance the selectivity of the procedure (Schenck et al., 2002). For these reasons, MeCN was selected as extraction solvent.

2.3.1.3 Selection of the salting-out agent

The choice of the salting agent is another key point of SALLE techniques as it influences the degree and efficiency of the phase separation between the aqueous sample and the organic solvent (Tighrine et al., 2019). An ideal salting-out agent should possess high solubility in water, poor/negligible solubility in the extraction solvent (Valente et al., 2013), and the ability to minimize the content of water in the organic phase to reduce/avoid matrix effects during LC-MS analyses. Four salts ($MgSO_4 \cdot 7H_2O$, NaCl, CH_3COONa, and Na_2SO_4) and their mixtures were tested as salting-out agents. The experiments were carried out using the following parameters as basal conditions: 2 mL of aqueous solution at a pH of 9.6 and a salt concentration of 1 mol L^{-1}, and 2 mL of MeCN. Results are shown in **Figure 2.1**. All the tested salts could promote phase separation; however, $MgSO_4 \cdot 7H_2O$ and Na_2SO_4 showed better

results, in terms of EE, for all the nine selected analytes (EE = 47-87% for $MgSO_4 \cdot 7H_2O$ and EE = 44-87% for Na_2SO_4). However, $MgSO_4 \cdot 7H_2O$ carried out with it a high water-content (r = 1.45), which prejudiced its use as single salting-out agent. The use of other salts in combination with $MgSO_4 \cdot 7H_2O$ is a commonly used strategy to reduce the water content from the organic phase ($r \approx 1$) and facilitate the phase separation (Valente et al., 2013). Therefore, the two best-performing salts ($MgSO_4 \cdot 7H_2O$ and Na_2SO_4) were tested in combination, at a concentration of 1 mol L^{-1} each. Their combination significantly improved the EE of the procedure, compared to their use as single salts (**Figure 2.1**); this improvement was particularly significant for the more polar analytes (Im, Ly and ImNO). The combination of the two salts was also able to reduce the water content from the organic phase ($r \approx 1$). Therefore, the combination of $MgSO_4 \cdot 7H_2O$ and Na_2SO_4 was selected as salting-out agent of the procedure.

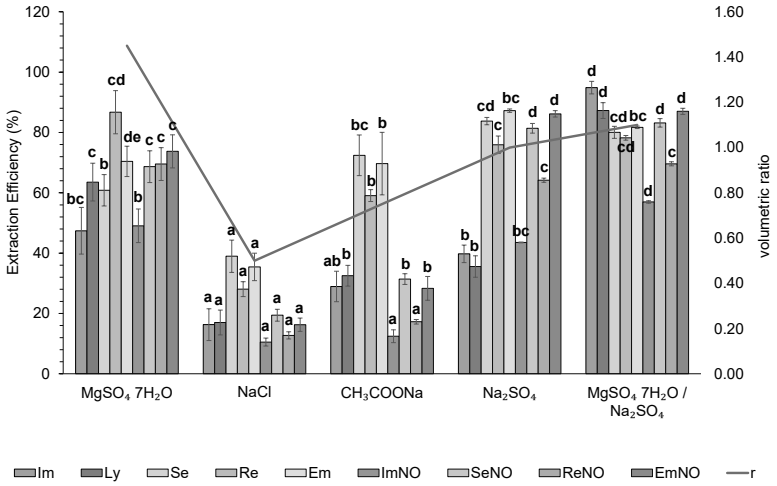


Figure 2.1. Effect of different salting-out agents on extraction efficiency and volumetric ratio (Different superscript letters within each compound indicate significant differences among experimental conditions; $p < 0.05$).

2.3.2 Optimization of SALLE

After conducting preliminary experiments to select the extraction solvent and salting-out agent to use, the extraction solvent volume and the quantity of salts were further investigated through chemometric approach to increase the EE of the nine target PAs and PANOs. The chemometric approach was used to study the influence of the individual experimental parameters and their first-order interactions on SALLE performance, with the aim of selecting the best extraction conditions. A Response surface model was evaluated by applying a Box-Behnken design 2-factors interactions (21 degrees of freedom, 2 blocks of replicates, made of 16 randomized experimental runs and 4 center points each) to simultaneously evaluate the influence of three independent variables: (A) extraction solvent volume (MeCN, 1-4 mL); (B) concentration of $MgSO_4 \cdot 7H_2O$ (0-1 mol L^{-1}); and (C) concentration of Na_2SO_4 (0.5-1.5 mol L^{-1}). The EE of each analyte (factors to be maximized) and the volumetric ratio (r = $MeCN_{post\text{-}SALLE}$ / $MeCN_{pre\text{-}SALLE}$, hit target = 1) were considered as response factors to be optimized. The experimental conditions and the values of each experimental response are reported in the **Table 2.2**. The statistical significance of the experimental response factors was determined by Analysis of variance (ANOVA) (**Table 2.3**) while the estimated standardized effects on the response factors are shown in the Pareto Charts in **Figure 2.2**.

Table 2.3. Analysis of variance (ANOVA) of the experimental variables (A-C) on the extraction efficiency (EE%) and volumetric ratio (r) of the nine target analytes.

Variable	p-value *r*	p-value Im	p-value Ly	p-value Se	p-value Re	p-value Em	p-value ImNO	p-value SeNO	p-value ReNO	p-value EmNO
A	0.0000	0.0027	0.0012	0.5042	0.2760	0.7603	0.0000	0.0107	0.0000	0.0333
B	0.0041	0.0000	0.0000	0.5638	0.1271	0.8355	0.0091	0.1107	0.0191	0.0773
C	0.0803	0.0059	0.0024	0.3302	0.2938	0.9026	0.0171	0.1074	0.0387	0.1150

A= MeCN volume (mL); B= $MgSO_4 \cdot 7H_2O$ (mol L^{-1}); C=Na_2SO_4 (mol L^{-1})

Se, Re, and Em did not show statistically significant effects ($p > 0.05$) on EE and *r* for all the studied variables (A-C). Therefore, they were not considered in the subsequent steps of the chemometric analysis. The increase in the volume of the extraction solvent (A) led to a significant increase of the EE of all the target analytes. This result is due to the aprotic polar nature of MeCN and, therefore, to its tendency to interact with the molecules of water of the sample. In other words, the greater the volume of MeCN used, the greater the possibility of dipole-dipole interactions between the two solvents. As direct consequence, the extraction of the analytes is favoured. Similarly, the increase in the concentration of the two salts (B-C) also led to an increase in the EE of all the target analytes except SeNO and EmNO. It is well known that an increase in the ionic strength of the aqueous solution decreases the water solubility of the analytes. Regarding SeNO and EmNO, no significant effects on EE were observed with varying B and C since they showed satisfactory EE values already during the preliminary experiments. Regarding the volumetric ratio (*r*), the variables A and B showed a statistically significant influence on this factor (**Figure 2.2**). In fact, the increase of both variables A and B led to a deviation of the volumetric ratio from the target value set ($r = 1$) as both variables favor the distribution of water into the organic solvent. Subsequently, the simultaneous effect of the three variables (A-C) on EE of the target analytes was investigated using a multiple response analysis.

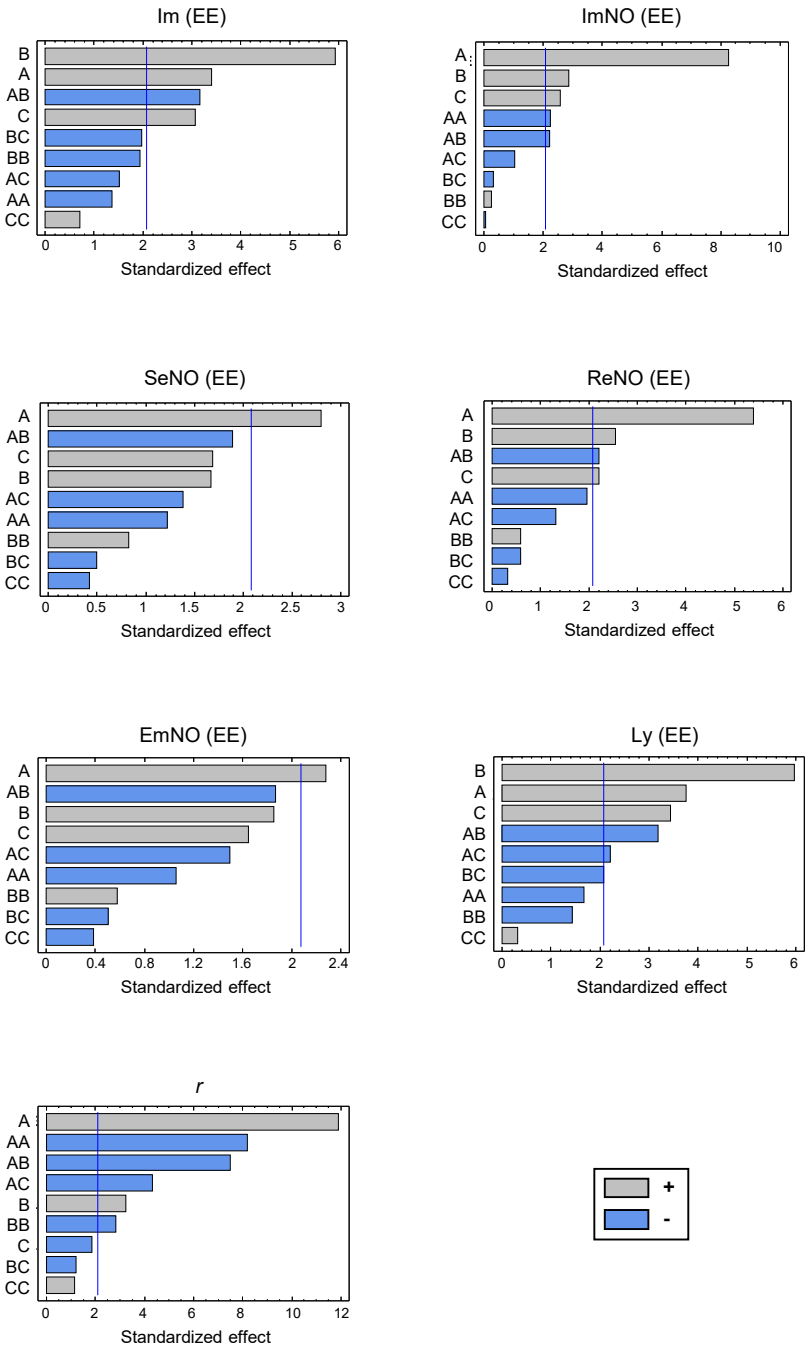


Figure 2.2. Standardized effects Pareto charts of volumetric ratio (*r*) and extraction efficiency (EE) of Im, Ly, ImNO, SeNO, ReNO and EmNO (differences in the bar shadings indicate positive and negative effects of the experimental factors on the independent variables A-C while the vertical line corresponds to statistical significance with a 95% confidence level).

The optimal experimental conditions predicted by the response surface design were (A) 1.5 mL of MeCN, (B) 1 mol L^{-1} of $MgSO_4$ $7H_2O$ and (C) 1.5 mol L^{-1} of Na_2SO_4 (degree of desirability = 77.4%). The optimized conditions were then tested on further analytes (previously unavailable) to provide an exhaustive extraction of all the available reference standards. Since the extraction solvent volume resulted the variable that most influenced the EE of the analytes, we decided to test a further volume of MeCN (2.0 mL) to improve the EE of the less effectively extracted analytes (**Figure 2.3**). An increased EE of the 28 target analytes was observed: from 51-96% (when using 1.5 mL of MeCN) to 68-106% (when using 2.0 mL of MeCN), especially for the less effectively extracted polar analytes, such as EuNO (from 51 to 71%), ImNO (from 53 to 74%), LyNO (from 51 to 68%) and McNO (from 54 to 75%). Although the use of a larger volume of MeCN caused a slight increase in the volumetric ratio (from 1 to 1.2), it did not adversely affect the accuracy and sensitivity of the method. Based on these results, a volume of 2.0 mL of MeCN was selected.

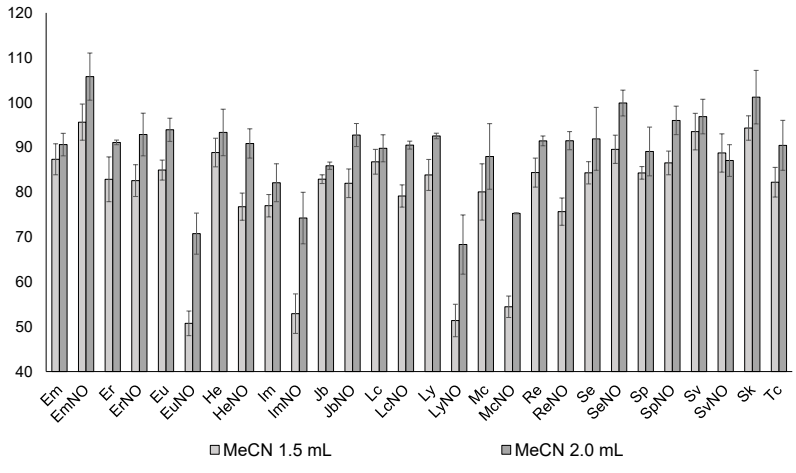


Figure 2.3. Extraction efficiency of the target analytes in optimal SALLE conditions using two different extraction solvent volumes.

2.3.3 SALLE application to different food matrices

An aqueous pre-treatment of the investigated matrices was needed to bring the analytes into water and allow the simultaneous extraction of PAs and PANOs from the food matrices of interest before subjecting the samples to SALLE, which was used as clean-up step. Honey and dietary supplements in form of syrups were simply diluted with water to obtain a 25% w/v solution. Pollen and solid forms of dietary supplements (capsules and tablets) were extracted with acidified water, an extensively used solvent for the extraction of these alkaloids from different food matrices due to the ability to provide exhaustive extraction and cleaner extracts (Casado et al., 2022a; Kaltner et al., 2019; Mulder et al., 2018). Teas and infusions were extracted by infusion with boiling water to mimic the real exposure scenario to these contaminants (Casado et al., 2022a; Mulder et al., 2018; Picron et al., 2018).

The EE of the entire sample preparation procedure was evaluated for 28 target analytes in six food matrices (honey, pollen, herbal infusions, black and green teas, and plant-based dietary supplements); the procedure resulted highly efficient in extracting the analytes from the tested matrices (EE = 68-106 %) (**Table 2.4**). The SALLE procedure was also applied to aqueous extracts of PA-producing plants to evaluate its efficiency in extracting PAs and PANOs others than the target ones. No differences were observed between the profiles of the aqueous and SALLE extracts, indicating the efficiency of the procedure in extracting the target PAs and PANOs from PA-producing plants.

Table 2.4. Extraction efficiency (EE, %) of the target analytes in different food matrices.

Analyte	Honey	Pollen	Black tea	Green tea	Herbal infusion	Dietary supplement
	EE (SD)	EE (SD)	EE (SD)	EE (SD)	EE (SD)	EE (SD)
Em	80.1 (2.8)	94.8 (0.4)	89.6 (3.2)	94.9 (8.9)	83.5 (8.2)	95.5 (1.6)
EmNO	98.7 (2.4)	100.3 (2.8)	109.1 (5.0)	94.0 (7.2)	106.0 (6.5)	105.5 (0.6)
Er	94.3 (7.8)	95.6 (8.3)	88.8 (5.3)	89.6 (7.6)	93.5 (6.2)	101.7 (5.5)
ErNO	92.9 (4.4)	87.2 (7.2)	89.8 (7.1)	91.3 (2.4)	94.3 (5.9)	85.1 (2.3)
Eu	96.4 (4.9)	97.7 (5.7)	96.5 (5.6)	92.1 (2.7)	90.4 (3.2)	91.1 (4.4)
EuNO	71.4 (4.5)	81.4 (9.1)	72.6 (5.6)	75.9 (5.8)	73.1 (6.9)	70.8 (3.2)
He	87.1 (6.3)	98.5 (4.0)	91.5 (6.3)	90.9 (5.2)	96.1 (7.1)	93.8 (2.6)
HeNO	90.9 (3.0)	84.2 (1.0)	88.6 (4.9)	88.0 (6.1)	87.2 (5.7)	86.4 (0.6)
Im	91.2 (1.6)	90.6 (1.5)	93.3 (0.9)	95.4 (3.7)	88.9 (0.9)	89.8 (6.9)
ImNO	75.3 (0.8)	78.3 (3.7)	73.1 (3.7)	80.4 (4.3)	76.5 (1.4)	70.8 (2.6)
Jb	91.8 (8.0)	94.3 (11.1)	95.2 (3.9)	85.9 (9.1)	89.7 (7.1)	104.5 (6.0)
JbNO	86.1 (5.6)	95.4 (6.2)	87.6 (5.9)	89.7 (3.9)	92.8 (2.4)	88.2 (1.6)
Lc	89.8 (2.7)	99.2 (5.1)	100.2 (5.1)	99.1 (7.1)	95.7 (5.7)	96.9 (5.9)
LcNO	90.5 (0.8)	89.8 (4.6)	95.0 (4.3)	93.8 (2.3)	92.0 (6.2)	97.4 (4.0)
Ly	91.1 (0.4)	92 (2.1)	93.5 (1.6)	91.6 (2.4)	87.3 (0.5)	91.9 (4.0)
LyNO	69.7 (5.7)	73.6 (0.7)	68.8 (4.5)	69.3 (5.8)	72.4 (5.3)	70.2 (6.1)
Mc	94.2 (4.9)	89.9 (8.4)	91.2 (3.0)	93.3 (6.1)	90.8 (7.1)	98.7 (5.5)
McNO	74.9 (5.7)	76.7 (0.9)	75.3 (6.9)	77.4 (5.3)	77.0 (5.4)	79.6 (6.9)
Re	100.7 (2.5)	80.0 (3.3)	98.8 (3.3)	104.2 (13.2)	74.1 (7.9)	99.2 (1.2)
ReNO	83.2 (3.1)	108.3 (5.2)	106.2 (3.2)	90.2 (8.6)	112.9 (4.0)	95.9 (1.6)
Se	77.8 (2.6)	90.7 (9.3)	88.8 (3.3)	96.4 (5.7)	102.3 (5.4)	99.5 (2.6)
SeNO	101.8 (3.4)	99.5 (6.8)	101.1 (2.8)	87.1 (5.8)	102.4 (4.2)	93.6 (1.0)
Sp	84.9 (3.6)	89.5 (7.1)	93.3 (0.8)	89.1 (4.8)	88.9 (0.9)	102.4 (3.9)
SpNO	91.9 (6.4)	101.4 (4.0)	88.8 (4.1)	89.6 (4.6)	93.5 (6.2)	91.5 (0.5)
Sv	81.3 (0.0)	86.0 (9.1)	91.6 (5.0)	88.3 (5.3)	93.4 (4.3)	100.9 (6.4)
SvNO	94.3 (5.6)	90.9 (8.4)	96.9 (3.1)	96.0 (3.7)	91.5 (6.4)	92.8 (0.9)
Sk	99.2 (8.7)	102.6 (0.6)	96.8 (8.7)	97.2 (7.9)	94.3 (3.9)	98.1 (5.2)
Tc	95.2 (8.2)	97.3 (0.2)	90.5 (5.0)	92.3 (7.1)	88.2 (3.2)	96.2 (3.1)

2.4 Conclusions

A simple, fast, and cheap sample preparation procedure for the extraction and clean-up of PAs and PANOs from different food matrices was developed in this study. The parameters that mainly influence the performances of SALLE were carefully studied using a chemometric approach, which allowed to obtain excellent values of EE for all target analytes, which resulted comparable to the previously used techniques for the extraction of PAs and PANOs from food samples (Ma et al., 2018; Casado et al., 2022a). The authors conclude that SALLE is a valid alternative to the laborious and expensive sample preparation procedures commonly used for the analysis of PAs and PANOs of food samples.

CHAPTER 3

An analytical platform for the screening and identification of pyrrolizidine alkaloids in food matrices with high risk of contamination

Adapted from

Rizzo, S., Celano, R., Piccinelli, A. L., Serio, S., Russo, M., & Rastrelli, L. (2022). An analytical platform for the screening and identification of pyrrolizidine alkaloids in food matrices with high risk of contamination. *Food Chemistry*, 406, 135058.

3.1 Introduction

The analysis of PAs and PANOs is a challenging task as the high variety of both necine bases and necic acids results in a huge number of different structures and numerous stereoisomers; to date, well over 600 PAs are known (Moreira et al., 2018; Schramm et al., 2019). In general, based on the necine bases, PAs can be classified into six types: retronecine (R) and its 7-stereoisomer heliotridine (H), otonecine (O), supinidine (S), platynecine (P), and trachelanthamidine (T) (**Figure 3.1**). R, H, O, and S types have 1,2-unsaturation in the pyrrolizidine ring and are, therefore, considered of major toxicity since they may cause liver damage and cancer in humans, whereas P and T types have a saturated necine base and generally regarded as non-toxic. Except for O type, in which N-oxides cannot be formed, N-oxides of the other types naturally occur and often coexist with basic PAs forms in plant materials. Furthermore, based on the type of esterification, PAs can be classified into three sub-types: monoesters (m), open chained diesters (d), and cyclic diesters (c) (**Figure 3.1**) (Moreira et al., 2018; Schramm et al., 2019).

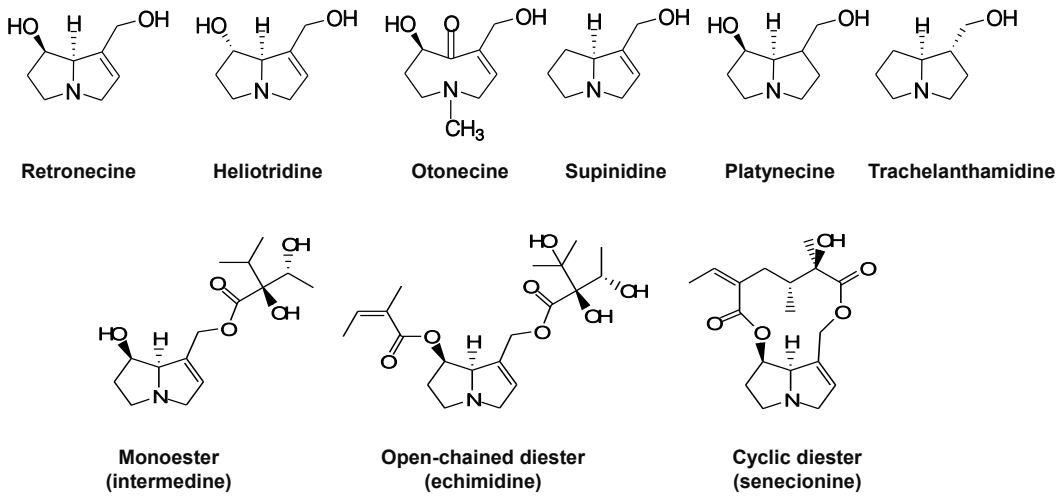


Figure 3.1. Necine base types and types of esterification depending on the linkage between the necic acids and the necine bases.

The implementation of measures to mitigate the contamination of food products by PAs and the development of new sensitive analytical procedures to collect occurrence data are important aspects to evaluate and reduce their chronic exposure (EFSA, 2016; EFSA CONTAM Panel 2017). Currently available methods for the sensitive determination of PAs in various matrices are based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with unit resolution spectrometric analyzers (triple quadrupole and ion trap). Selected reaction monitoring (SRM), also called multiple reaction monitoring (MRM), is a well-established MS/MS acquisition mode for the targeted analysis of PAs, due to its high selectivity, sensitivity, and robustness (Casado et al., 2022a; Ma et al., 2018; Mulder et al., 2018; Picron et al., 2018). Although it ensures excellent analytical performance, which easily meet the quality criteria required in food safety control, this approach presents limitations on the number of compounds to be analyzed in one run, requires the availability of reference standards, and it does not provide suitable MS/MS spectra for the screening and structural elucidation of unknown or suspected compounds (Hird et al., 2014; Righetti et al., 2016). In recent years, high-resolution mass spectrometry (HRMS) has been increasingly used as complementary method for the analysis of trace-level contaminants in food matrices since it allows the simultaneous screening of target, suspect, and untarget compounds. Moreover, the acquisition of accurate MS and MS/MS spectra (resolution < 5 ppm) offers the possibility to detect a theoretically unlimited number of molecules without the need of a compound-specific tune, carry out the retrospective data analysis, and perform structural characterization of unknown or suspected compounds (Hird et al., 2014; Menger et al., 2020; Rajski et al., 2021; Righetti et al., 2016).

The regulated list of PAs and PANOs to monitor is limited (21 compounds and their 14 co-eluting isomers) and the development of advanced analytical approaches to detect further PAs, which can potentially contaminate plant-based matrices, is of huge importance to better understand the presence of these contaminants in food matrices and guarantee their safety (Casado et al., 2022a). This study aimed to develop an analytical platform for the rapid and automated screening and identification of PAs and PANOs at trace levels in various food matrices to broaden the knowledge about the distribution of these contaminants in foods. To achieve this goal, an analytical procedure combining SALLE of aqueous extracts with UHPLC-HRMS/MS was developed. A systematic workflow, based on a huge database (778 molecules) and a diagnostic product ion filtering strategy, was designed to first characterize PAs and PANOs from PA-producing plants and then create an internal HRMS/MS spectral library. Furthermore, two software-assisted processing methods were implemented to automate and facilitate the detection and characterization of PAs and PANOs (wide-scope suspect screening method) and perform the rapid and reliable screening of 118 target PAs and PANOs in commercial samples (high-throughput target screening and identification method). The proposed platform was validated for six food matrices according to the European guidelines for qualitative screening methods (Magnusson & Örnemark, 2014; Pihlström et al., 2017). After that, 282 commercial samples were screened to test the applicability of the method and investigate the contamination profile of six food matrices at high risk of contamination, which are honey, pollen, black and green teas, herbal infusions, and plant-based dietary supplements.

3.2 Materials and methods

3.2.1 Chemicals and standards

See CHAPTER 2, paragraph 2.2.1

3.2.2 PA-producing plants and samples

Ten PA-producing plants, four of which belonging to the *Asteraceae* family (*Eupatorium cannabinum*, *Petasites hybridus*, *Senecio vulgaris*, *Tussilago farfara*) and the other six to the *Boraginaceae* family (*Anchusa officinalis*; *Borago officinalis*, *Echium italicum*, *Heliotropium europaeum*, *Lithospermum officinale*, *Symphytum officinale*) were provided by Giardino della Minerva (Orto botanico della Scuola Medica Salernitana, Salerno, Italy).

A total number of 282 commercial samples were analyzed. Honey (n = 72) and pollen (n = 6) samples from different botanical and geographical origins were obtained from Italian supermarkets, online shops, and local beekeepers. Herbal infusions (n = 101, including 21 labelled as dietary supplements), black teas (n = 31), green teas (n = 20), and plant-based dietary supplements (n = 44 in solid form and n = 8 as syrups) were purchased from herbalist's and chemist's shops. Honey samples were stored at 4°C until the analysis. Regarding herbal infusions, teas, and solid forms of plant-based dietary supplements, 50% of units of each package were combined and milled to form a representative aggregate sample. Each aggregate sample was appropriately coded and kept in plastic containers at room temperature and protected from light until the analysis.

3.2.3 Sample preparation

Samples of honey, pollen, herbal infusions, black and green teas, and plant-based dietary supplements (various formulations: capsules, tablets, syrups, and infusions) were pre-treated and extracted with the sample preparation procedure previously developed (see CHAPTER 2, paragraphs 2.2.4 and 2.2.5). The dried residues of SALLE extracts were redissolved with an appropriate volume of H_2O/MeOH 7:3 v/v: 125 µL for honey, 250 µL for solid forms of plant-based dietary supplements, and 200 µL for herbal infusions and teas.

3.2.4 UHPLC-HRMS analysis

The analyses were conducted on an UltiMate 3000 UHPLC system (ThermoFisher Scientific, Milano, Italy) interfaced via a heated electrospray ionization source (HESI-II) to a Q-Exactive mass spectrometer (ThermoFisher Scientific, Milano, Italy). The UHPLC system was equipped with a Luna Omega Polar C18 (2.1 × 100 mm, 1.6 µm; Phenomenex, Bologna, Italy) column, operated at 40°C with a flow rate of 400 µL min^{-1}. The chromatographic separation was achieved using a binary gradient of H_2O (A) and MeCN (B), both containing 0.1% of formic acid; the elution gradient was as follows: 0–1 min, 2% B; 1–5.5 min, 2–8% B; 5.5–7.5 min, 8% B; 7.5–9.5 min, 8–12% B; 9.5–11 min, 12–18% B; 11–13 min, 18–20% B; 13–15 min, 20-40% B; 15-17 min, 40-60% B; 17-19 min, 60-80% B. After each injection, washing (98% B, 4 min) and re-equilibration of the column (2% B, 5 min) were performed. The injection volume was set at 5 µL.

The mass spectrometer operated in positive ionization mode with the following instrument parameters: spray voltage, 3.5 kV; sheath gas flow rate, 50; auxiliary gas flow rate, 13; capillary and auxiliary gas heater temperatures, 300 °C; S-lens level, 55. Nitrogen was used as collision gas of the higher-energy collisional dissociation (HCD)

cell. Data were acquired in Full MS/dd-MS^2 mode. The resolution of the Full MS scans (scan range 250-500 *m/z*) was set at 70k (FWHM), the Automatic Gain Control (AGC) target at 3e6, and the maximum IT (Injection time) at 250 ms. Each time the detector detected a peak corresponding to the accurate mass (± 5ppm) of a certain precursor ion of the inclusion list associated to the method, these ions were isolated in the quadrupole, accumulated in the C-trap, and finally accelerated in the HCD cell to be fragmented. The inclusion list associated to the acquisition method was filled with 112 masses of precursor ions ($[M+H]^+$) (**Table 3.1**). The fragmentation was performed using the NCE (Normalized Collision Energy) technology, which applies a stepped collisional energy scheme by combining low, medium, and high collision energies capable of increasing the diversity of fragment ions generated; a range of collision energies between 40 and 60 was applied in this study. The recording parameters of the dd-MS^2 scans were set as follows: mass resolution, 17.5 k (FWHM); AGC target, 2e4; maximum IT, 80 ms; isolation window, *m/z* 1.5; intensity threshold, 1.3e4; and dynamic exclusion: 2.0 s. The TopN parameter, which refers to the number of ions to be triggered after a Full MS scan, was disabled to prevent precursor ions other than those contained in the inclusion list from being isolated. Xcalibur software version 4.4 (ThermoFisher Scientific, Milano, Italy) was used for instrument control and data acquisition.

Table 3.1. Inclusion list of the Full MS/dd-MS² acquisition method.

Molecular formula	$[M+H]^+$ (calculated *m/z*)	Molecular formula	$[M+H]^+$ (calculated *m/z*)	Molecular formula	$[M+H]^+$ (calculated *m/z*)	Molecular formula	$[M+H]^+$ (calculated *m/z*)
$C_{13}H_{19}NO_4$	254.1387	$C_{18}H_{23}NO_5$	334.1649	$C_{20}H_{31}NO_6$	382.2224	$C_{21}H_{29}NO_8$	424.1966
$C_{14}H_{25}NO_3$	256.1907	$C_{18}H_{25}NO_5$	336.1805	$C_{18}H_{25}NO_8$	384.1653	$C_{22}H_{33}NO_7$	424.2330
$C_{14}H_{25}NO_4$	272.1856	$C_{18}H_{27}NO_5$	338.1962	$C_{19}H_{29}NO_7$	384.2017	$C_{20}H_{27}NO_9$	426.1759
$C_{14}H_{19}NO_5$	282.1336	$C_{17}H_{25}NO_6$	340.1755	$C_{20}H_{33}NO_6$	384.2381	$C_{21}H_{31}NO_8$	426.2122
$C_{15}H_{23}NO_4$	282.1700	$C_{18}H_{29}NO5$	340.2118	$C_{18}H_{27}NO_8$	386.1809	$C_{21}H_{33}NO_8$	428.2279
$C_{16}H_{27}NO_3$	282.2064	$C_{16}H_{23}NO_7$	342.1547	$C_{18}H_{29}NO_8$	388.1966	$C_{22}H_{37}NO_7$	428.2643
$C_{15}H_{25}NO_4$	284.1856	$C_{17}H_{27}NO_6$	342.1911	$C_{18}H_{26}ClNO_6$	388.1521	$C_{20}H_{31}NO_9$	430.2072
$C_{15}H_{27}NO_4$	286.2013	$C_{16}H_{25}NO_7$	344.1704	$C_{20}H_{25}NO_7$	392.1704	$C_{20}H_{33}NO_9$	432.2228
$C_{17}H_{21}NO_3$	288.1594	$C_{16}H_{27}NO_7$	346.1860	$C_{20}H_{27}NO_7$	394.1860	$C_{23}H_{31}NO_7$	434.2173
$C_{14}H_{19}NO_6$	298.1285	$C_{18}H_{23}NO_6$	350.1598	$C_{20}H_{29}NO_7$	396.2017	$C_{22}H_{33}NO_8$	440.2279
$C_{15}H_{23}NO_5$	298.1649	$C_{18}H_{25}NO_6$	352.1755	$C_{21}H_{33}NO_6$	396.2381	$C_{21}H_{31}NO_9$	442.2072
$C_{16}H_{27}NO_4$	298.2013	$C_{20}H_{33}NO_4$	352.2482	$C_{20}H_{31}NO_7$	398.2173	$C_{22}H_{37}NO_8$	444.2592
$C_{15}H_{25}NO_5$	300.1805	$C_{18}H_{27}NO_6$	354.1911	$C_{18}H_{25}NO_9$	400.1602	$C_{23}H_{31}NO_8$	450.2122
$C_{16}H_{29}NO_4$	300.2169	$C_{17}H_{25}NO_7$	356.1704	$C_{19}H_{29}NO_8$	400.1966	$C_{23}H_{35}NO_8$	454.2435
$C_{15}H_{27}NO_5$	302.1962	$C_{18}H_{29}NO_6$	356.2068	$C_{20}H_{33}NO_7$	400.2330	$C_{22}H_{33}NO_9$	456.2228
$C_{17}H_{21}NO_4$	304.1543	$C_{17}H_{27}NO_7$	358.1860	$C_{18}H_{26}ClNO_7$	404.1471	$C_{21}H_{31}NO_{10}$	458.2021
$C_{16}H_{23}NO_5$	310.1649	$C_{19}H_{25}NO_6$	364.1755	$C_{18}H_{29}NO_9$	404.1915	$C_{22}H_{35}NO_9$	458.2385
$C_{16}H_{25}NO_5$	312.1805	$C_{18}H_{23}NO_7$	366.1547	$C_{21}H_{27}NO_7$	406.1860	$C_{21}H_{30}ClNO_8$	460.1733
$C_{15}H_{23}NO_6$	314.1598	$C_{19}H_{27}NO_6$	366.1911	$C_{20}H_{25}NO_8$	408.1653	$C_{22}H_{37}NO_9$	460.2541
$C_{16}H_{27}NO_5$	314.1962	$C_{18}H_{25}NO_7$	368.1704	$C_{21}H_{29}NO_7$	408.2017	$C_{23}H_{31}NO_9$	466.2072
$C_{15}H_{25}NO_6$	316.1755	$C_{20}H_{33}NO_5$	368.2431	$C_{20}H_{27}NO_8$	410.1809	$C_{23}H_{35}NO_9$	470.2385
$C_{16}H_{29}NO_5$	316.2118	$C_{18}H_{27}NO_7$	370.1860	$C_{21}H_{31}NO_7$	410.2173	$C_{22}H_{33}NO_{10}$	472.2177
$C_{15}H_{27}NO_6$	318.1911	$C_{18}H_{29}NO_7$	372.2017	$C_{20}H_{29}NO_8$	412.1966	$C_{22}H_{35}NO_{10}$	474.2334
$C_{17}H_{25}NO_5$	324.1805	$C_{17}H_{27}NO_8$	374.1809	$C_{21}H_{33}NO_7$	412.2330	$C_{25}H_{37}NO_8$	480.2592
$C_{16}H_{23}NO_6$	326.1598	$C_{20}H_{25}NO_6$	376.1755	$C_{20}H_{31}NO_8$	414.2122	$C_{23}H_{35}NO_{10}$	486.2334
$C_{16}H_{25}NO_6$	328.1755	$C_{19}H_{25}NO_7$	380.1704	$C_{20}H_{33}NO_8$	416.2279	$C_{25}H_{37}NO_9$	496.2541
$C_{16}H_{27}NO_6$	330.1911	$C_{20}H_{29}NO_6$	380.2068	$C_{19}H_{28}ClNO_7$	418.1627	$C_{23}H_{35}NO_{11}$	502.2283
$C_{15}H_{25}NO_7$	332.1704	$C_{19}H_{27}NO_7$	382.1860	$C_{18}H_{26}ClNO_8$	420.1420	$C_{25}H_{37}NO_{10}$	512.2490

3.2.5 Data processing

The data processing was performed using TraceFinder software version 5.1 (ThermoFisher Scientific, Milano, Italy). In detail, two processing methods were built to automate and facilitate the data treatment, according to the specific objectives of the study. The first one, named as wide-scope suspect screening method, was developed to detect and characterize suspect PAs and PANOs from PAs-producing plants and commercial samples; the second one, named as high-throughput screening and identification method, to rapidly perform the screening of a huge number of commercial samples regarding the presence of the 118 target PAs and PANOs of the

spectral library. Both methods were created using the "Target screening method" workflow of the software.

3.2.5.1 Wide-scope suspect screening method

The Compound Database (CD) was built by importing a csv file, containing the list of 112 precursor ions of the inclusion list, associated with the instrumental acquisition method (**Table 3.1**), into the software. Then, 30 key product ions for the characterization of PAs and PANOs (*m/z* 120.0808, 138.0913, 150.0913, 168.1019, 124.1121, 142.1226, 122.0964, 140.107, 156.1019, 94.0651, 96.0808, 110.0964, 122.0964, 180.1019, 198.1125, 83.0491, 220.1332, 238.1438, 158.1176, 136.0757, 137.0835, 158.1176, 139.0992, 111.0679, 172.0968, 118.0651, 119.0729, 113.0835, 174.1125, 121.0886, 214.1074, 254.1387 were associated with each precursor ion. A master method was then created with the following processing parameters: a range-integrated detection type over the entire chromatographic run; a response threshold (peak area) of 10e5; a mass tolerance of ± 5 ppm; and at least three product ions. The suspect compounds were flagged as "detected" (green flag) when all the criteria were fulfilled. This allowed the method to detect the presence of PAs/PANOs analogues whenever a peak matched the molecular formula of the relative precursor ion (± 5 ppm) and at least three diagnostic product ions (± 5 ppm) over the entire chromatographic run.

3.2.5.2 High-throughput screening and identification method

The high-throughput screening and identification method is linked to the construction of an in-house HRMS/MS spectral library. Therefore, the initial step involved the construction of the library, which was created using mzVault software version 2.3 (ThermoFisher Scientific, Milano, Italy) by uploading UHPLC-HRMS/MS information of the 118 target PAs and PANOs (**Table 3.3**). Then, the CD was built by

importing into the master method a csv containing the acquired mass spectra information (retention time, molecular formula, precursor ions, five most abundant product ions and their ratios) of the 118 compounds of the library. The spectral library was associated to the processing method as additional identification tool. The following identification criteria were set: a retention time variation of ± 0.2 min, a response threshold of 10e4, a mass tolerance of 5 ppm for both precursor and product ions, a minimum of three product ions required for the identification, and a library match score higher than 70%. The target compounds were flagged as "identified" (green flag) when all the criteria were fulfilled, "found" when only the precursor ion was encountered at the expected retention time (red flag), and "not found" (yellow flag) when none of the criteria were met.

3.2.6 Validation of the screening and identification method

The high-throughput screening and identification method was validated in terms of specificity, limit of identification (LOI), and precision (expressed as false negative rates), according to the performance criteria of qualitative screening methods established by the European analytical guidelines (Magnusson & Örnemark, 2014; Pihlström et al., 2017). The validation studies were conducted on 28 out of 30 reference standards (indicine and indicine N-oxide were excluded for co-elution reasons) in six food matrices: honey, pollen, back and green teas, herbal infusions, and plant-based dietary supplements. The experiments were performed on blank samples, previously identified through analysis. A representative sample of herbal infusion was prepared by mixing the same amount of chamomile, fennel, melissa, mint, and licorice, as these herbs were the most encountered during the collection of the samples. On the contrary, it was not possible to select or prepare a representative sample of a plant-based dietary supplement due to the high variability of their composition. The

specificity was evaluated by processing spiked (10 µg L^{-1}) and non-spiked SALLE extracts of blank samples of each studied matrix. LOIs of herbal infusions, honey, pollen, black and green teas were evaluated by fortifying blank samples at eight concentration levels, ranging from 0.4 to 2 µg L^{-1} of the SALLE extracts. LOIs were assigned for each target analyte at the concentration level that met all the identification criteria of the high-throughput target screening and identification method. Regarding plant-based dietary supplements, since it was not possible to find a representative sample, LOIs were estimated as the lowest concentration at which a compound was identified in at least 95% of the blank samples. For this purpose, 20 blank samples (10 samples × 2 replicates) of different composition were spiked before the extraction at 10 and 20 µg kg^{-1}. The precision of the method, estimated as false negative rates, was determined by fortifying 72 blank samples (36 samples × 2 replicates), including honey samples (n = 3), pollen samples (n = 3), herbal infusions (n = 10), black (n = 5) and green (n = 5) teas, and dietary supplements (n = 10) at concentration of 2 µg L^{-1} (corresponding to 2 µg kg^{-1} for honey, 20 µg kg^{-1} for pollen, 15 µg kg^{-1} for teas and herbal infusions, and 10 µg kg^{-1} for solid forms of dietary supplements) and 4 µg L^{-1} (corresponding to 4 µg kg^{-1} for honey, 40 µg kg^{-1} for pollen, 30 µg kg^{-1} for teas and herbal infusions, and 20 µg kg^{-1} for solid forms of dietary supplements) of the SALLE extracts, which correspond to the tenth and the fifth part of the lower limit of the Regulation (EU) 2020/2040 (Tea, Camellia sinensis, ML of 150 µg kg^{-1}).

3.3 Results and discussion

3.3.1 UHPLC-HRMS/MS analysis

The setting of the UHPLC conditions aimed at solving/minimizing one of the main problems encountered during the chromatographic analysis of PAs and PANOs, which is the co-elution of structural isomers impossible to be distinguished by their MS/MS spectra. Examples are some of the isomeric groups listed in the Regulation (EU) 2020/2040, such as indicine/intermedine/lycopsamine, echinatine/rinderine, integerrimine/senecionine/senecivernine, echimidine/heliosupine, seneciphylline/spartioidine, and their N-oxides (Casado et al., 2022a; Kaltner et al., 2019). The chromatographic conditions were carefully optimized on both the 28 reference standards and the extracts of the PA-producing plants to obtain the separation of regulated structural isomers and further isomers for which no reference standards are available. As already reported by Kaltner and co-workers, the best chromatographic conditions of the isomers are obtained using acidified solvents (Kaltner et al., 2019). The optimized conditions allowed to achieve a good separation for most of the abovementioned PAs and PANOs isomers (**Figure 3.2**), within a run time of 17 min. In addition, many of the indistinguishable isomers, characterized from the extracts of PA-producing plants, (7-acetylintermedine/7-acetyllycopsamine, amabiline/supinine, asperumine/heliosupine, lasiocarpine/7-tigloyleuropine, their N-oxides, and neosenkirkine/senkirkine) resulted in well separated peaks. Indicine/lycopsamine and their N-oxides and integerrimine/senecionine or senecivernine were the only isomers that could not be resolved. Even the isomers putatively identified as echinatine and rinderine, their 7-acetyl analogues, and their N-oxides were not sufficiently resolved under the chromatographic conditions used.

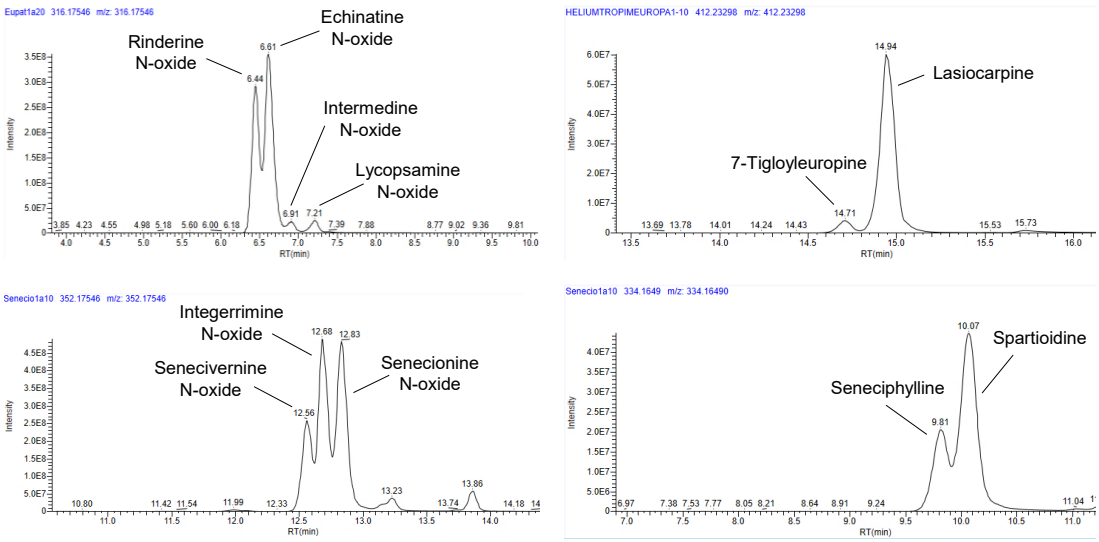


Figure 3.2. (+)-Extracted ion chromatogram (EIC) profiles of different isomeric groups, chromatographically resolved under the optimized conditions (5 ppm).

Different HRMS/MS acquisition modes were considered to evaluate the suitability of the detection method to the structural characterization and identification of PAs. Eventually, a data-dependent acquisition mode (Full MS/dd-MS^2), with an inclusion list of prioritized masses, was selected as it proved to be efficient in terms of selectivity and ability to detect the target analytes at trace levels and provide high quality HRMS/MS spectra. The quality of the MS/MS spectra is crucial to obtain reliable identifications of molecules in complex matrices, and the data-dependent acquisition mode provides MS/MS spectra from specific precursor ions by dismissing the other precursor ions, which reduces the risk of background noise and signal interferences (Rajski et al., 2021). The Full MS/dd-MS^2 was adapted to the detection and characterization of a wide range of PAs at low concentration levels (ppb) in complex food matrices. For this purpose, the mass range of the Full MS scan (*m/z* 250-550) was defined to cover the entire range of molecules of the internal database. The dd-MS^2 scan was triggered on an inclusion list of accurate masses of $[M+H]^+$ ions obtained from the internal database. This allowed to fragment the suspected PAs over matrix-

interfering ions, even when they were present as minor compounds. The TopN function, which selects the most abundant ions of every single Full MS scan, was disabled as it is not suitable for trace analyses in complex matrices; in fact, in such conditions, the selected ions would correspond to the matrix interferences. Furthermore, to detect and confirm the target analytes at low contamination levels, the minimum AGC target of the dd-MS^2 scan was set to a much lower value (10e3) than that commonly used in Full MS/dd-MS^2 analyses (10e5-10e6). The optimal collision energies were determined by analyzing the reference standards to obtain fragmentation spectra with significant product ions. The developed acquisition method allowed to detect (Full MS) and identify (dd-MS^2) target PAs and PANOs up to a concentration close to 1 μg L^{-1}, with enough data points across the chromatographic peaks (Full MS extracted ion chromatogram) and reliable fragmentation profiles.

3.3.2 Identification strategy

3.3.2.1 Database of PAs and PANOs

A wide database of PAs and PANOs was created from a systematic survey of EFSA reports, literature studies, and online databases, to support the identification strategy. The list of known compounds was also implemented with "expected unknowns", intended as unreported compounds that can be predicted based on the chemical features of this class of alkaloids (e. g. N-oxide derivatives). The database (778 molecules) was filled with structural information (CAS number, elemental composition, molecular weight, accurate mass of precursor ions), and the groups of PAs with identical molecular formula were further classified into different subgroups according to the N-oxidation, the necine base, and the type of esterification (**Tab. 3.2**). The full database is available as supplementary material of Rizzo et al., 2023.

Tab. 3.2. Representation of a section of the internal database of PAs and PANOs.

Name	Expected Unknowns [a]	CAS Number	Molecular formula	Molecular weight	$[M+H]^+$ (calculated *m/z*)	Necine base type	Esterification type	N-oxide	Isomeric group	Isomeric subgroup
Vulgarine		846552-52-1	**C20H31NO7**	397.2095	398.2173	retronecine	monoester		68	A
Echihumiline		174285-73-5	C20H31NO7	397.2095	398.2173	retronecine	open chained diester		68	B
Echimidine		520-68-3	C20H31NO7	397.2095	398.2173	retronecine	open chained diester		68	B
2'-epi-Heliosupine	X	-	C20H31NO7	397.2095	398.2173	heliotridine	open chained diester		68	C
Asperumine		32779-96-7	C20H31NO7	397.2095	398.2173	heliotridine	open chained diester		68	C
Heliosupine		32728-78-2	C20H31NO7	397.2095	398.2173	heliotridine	open chained diester		68	C
3'-Angeloylindicine N-oxide		2361519-38-0	C20H31NO7	397.2095	398.2173	retronecine	monoester	X	68	D
3'-Angeloylintermedine N-oxide		NF	C20H31NO7	397.2095	398.2173	retronecine	monoester	X	68	D
3'-Angeloyllycopsamine N-oxide		NF	C20H31NO7	397.2095	398.2173	retronecine	monoester	X	68	D
Anadoline		28513-29-3	C20H31NO7	397.2095	398.2173	retronecine	monoester	X	68	D
Scorpioidine N-oxide		2699638-71-4	C20H31NO7	397.2095	398.2173	retronecine	monoester	X	68	D
7-Angelyindicine N-oxide		NF	C20H31NO7	397.2095	398.2173	retronecine	open chained diester	X	68	E
Echiumine N-oxide		685554-68-1	C20H31NO7	397.2095	398.2173	retronecine	open chained diester	X	68	E
Echiupinine N-oxide		138590-58-6	C20H31NO7	397.2095	398.2173	retronecine	open chained diester	X	68	E
Echiuplatine N-oxide		850143-95-2	C20H31NO7	397.2095	398.2173	retronecine	open chained diester	X	68	E
Myoscorpine N-oxide		138663-95-3	C20H31NO7	397.2095	398.2173	retronecine	open chained diester	X	68	E
Symlandine N-oxide		182967-33-5	C20H31NO7	397.2095	398.2173	retronecine	open chained diester	X	68	E
Symphytine N-oxide		72698-57-8	C20H31NO7	397.2095	398.2173	retronecine	open chained diester	X	68	E
Symviridine N-oxide		NF	C20H31NO7	397.2095	398.2173	retronecine	open chained diester	X	68	E
7-Tigloyindicine N-oxide	X	-	C20H31NO7	397.2095	398.2173	retronecine	open chained diester	X	68	E
3'-Angeloylechinatine N-oxide		NF	C20H31NO7	397.2095	398.2173	heliotridine	monoester	X	68	F
3'-Angeloylrinderine N-oxide		NF	C20H31NO7	397.2095	398.2173	heliotridine	monoester	X	68	F

[a] NF = Not Found

3.3.2.2 Diagnostic product ions filtering strategy

The structural diversity of PAs appears in the MS/MS fragmentation pattern. Depending on the necine base, necic acids, esterification type and N-oxidation of the pyrrolizidine ring, PAs and PANOs show characteristic and predictable product ions with specific ion ratios. This behavior was used to develop a HRMS/MS approach for their detection and characterization without the need for reference standards. Thus, a diagnostic product ion filtering strategy was designed for the characterization of PAs and PANOs through their HRMS/MS spectra. A systematic flowchart (**Figure 3.3**) was designed to delineate the fragmentation patterns of PAs and PANOs by studying the HRMS/MS spectra of the reference standards, online spectral libraries, and previous studies (Mädge et al., 2020; Ruan et al., 2012; These et al., 2013). Important clarifications also arose during the collection of HRMS/MS spectra of the spectral library, in particular regarding the ion ratios.

The flowchart was divided in two subsets since the HRMS/MS spectra immediately allowed to differentiate PAs (**Figure 3.3A**) from PANOs (**Figure 3.3B**). The different necine base types of PAs are easily recognized by the presence of characteristic product ions: *m/z* 120.0808 and 138.0910 for both R and H types, *m/z* 150.0913 and 168.1019 for O type, *m/z* 122.0964 and 140.1070 for both P and S types, and *m/z* 124.1121 and 142.1226 for T type (**Figure 3.3A**). The product ions of higher intensity were placed on the top of each subset by adding in succession characteristic product ions for each subgroup as far as it was possible. Regarding R and H types, PA monoesters are easily distinguished from diesters as they show the distinctive product ion at *m/z* 156.1019. Depending on the base peak, monoesters can be differentiated into R (bp at *m/z* 94.0654) and H types (bp at *m/z* 138.0910). Cyclic and open-chained diesters of R/H type can be differentiated based on the relative intensity of the product

ions at *m/z* 94.0654, 120.0808, and 138.0910: they show comparable intensities (> 20%) in cyclic forms, and a base peak at *m/z* 120.0808 and low intensities (< 10%) at *m/z* 94.0651 and 138.0913 in open-chained diester forms. The presence of product ions at *m/z* 180.1019 and 198.1125 identify an acetyl group at C-7 while product ions at *m/z* 83.0491, 220.1332, and 238.1438 identify an angeloyl/tigloyl group at the same position. Regarding S types, the product ions at *m/z* 94.0654 and 110.0964 are crucial for their identification (**Figure 3.3A**).

PANOs show more complex HRMS/MS spectra than PAs and characteristic product ion clusters (**Figure 3.3B**). The cluster 136 to 138 (*m/z* 136.0757, 137.0835, and 138.0913) identifies R and H PANOs. Whitin R and H types, the base peak at *m/z* 172.0968 and the product ion at *m/z* 111.0679 identify the monoester subgroups and allow to distinguish them from the diester subgroups, which show the cluster 118 to 120 (*m/z* 118.0651, 119.0729, and 120.0808). Depending on the relative intensities of the cluster 136 to 138, monoesters can be differentiated into R (higher intensities) and H (lower intensities) types. The same applies to open-chained diesters, albeit in inverted ratios. In both groups, the product ions at *m/z* 214.1074 or 254.1387 indicate the presence of an acetyl or angeloyl/tigloyl group respectively at the C-7 position of the open-chained diesters. S and P types of PANOs are instead characterized by the cluster 138 to 140 (*m/z* 138.0913, 139.0992, and 140.1070); the base peak at *m/z* 156.1019 and the product ion at *m/z* 139.0992 allow to differentiate S from P types (**Figure 3.3B**).

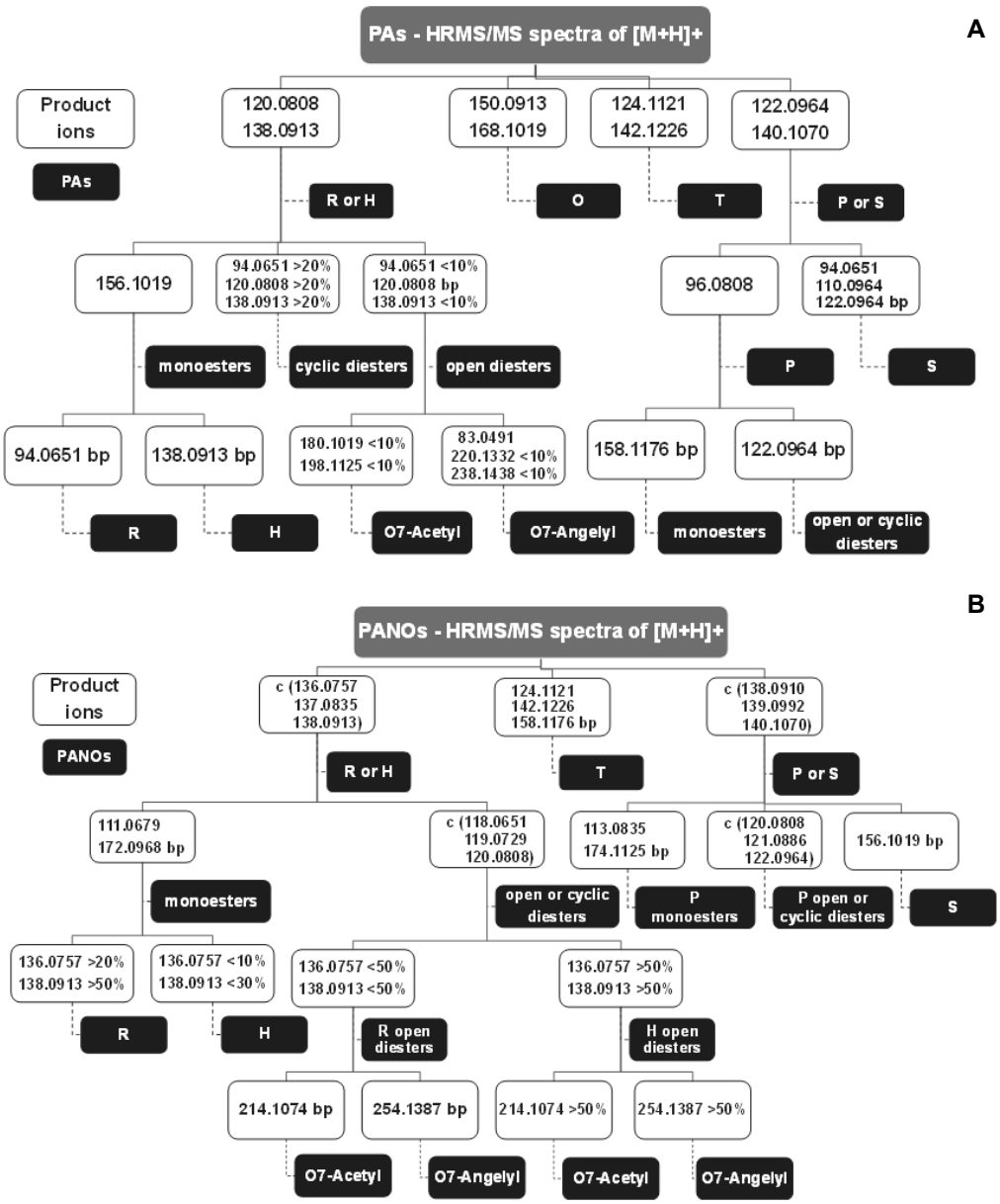


Figure 3.3. Flowchart of key diagnostic product ions for the identification of PAs (A) and PANOs (B).

Figure 3.4 and **Figure 3.5** show the chemical structures, molecular formulas, and exact masses of the key product ions required for the subdivision of PAs and PANOs into the different groups and subgroups of the **Figure 3.3**.

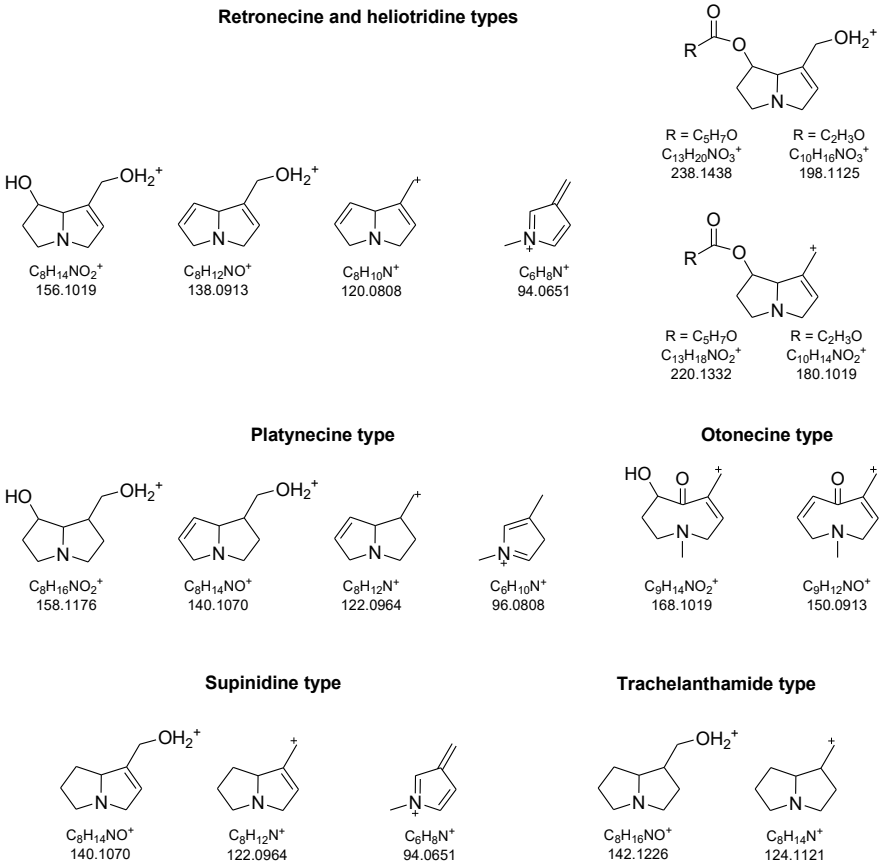


Figure 3.4. Characteristic product ions of different groups of PAs.

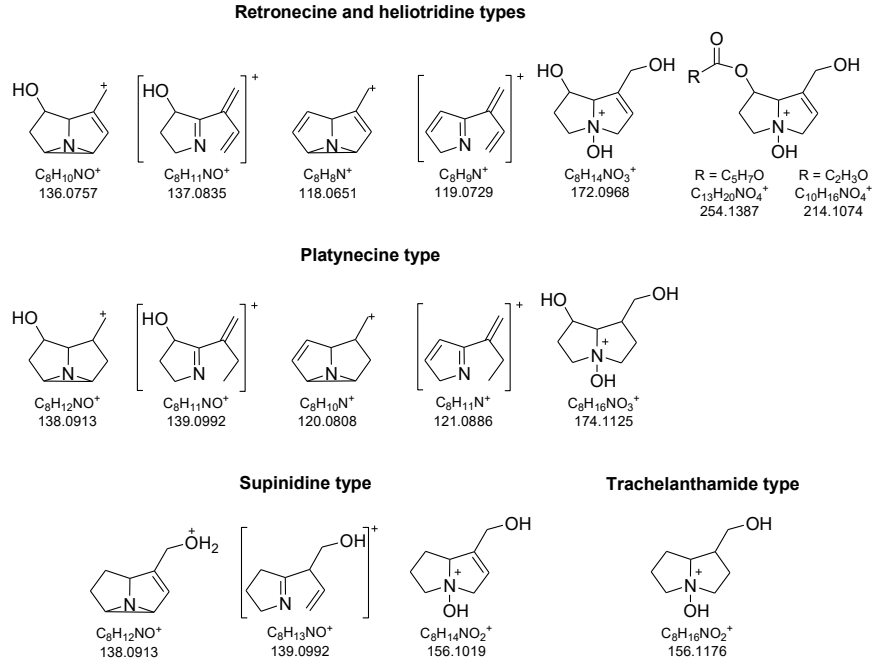


Figure 3.5. Characteristic product ions of different groups of PANOs.

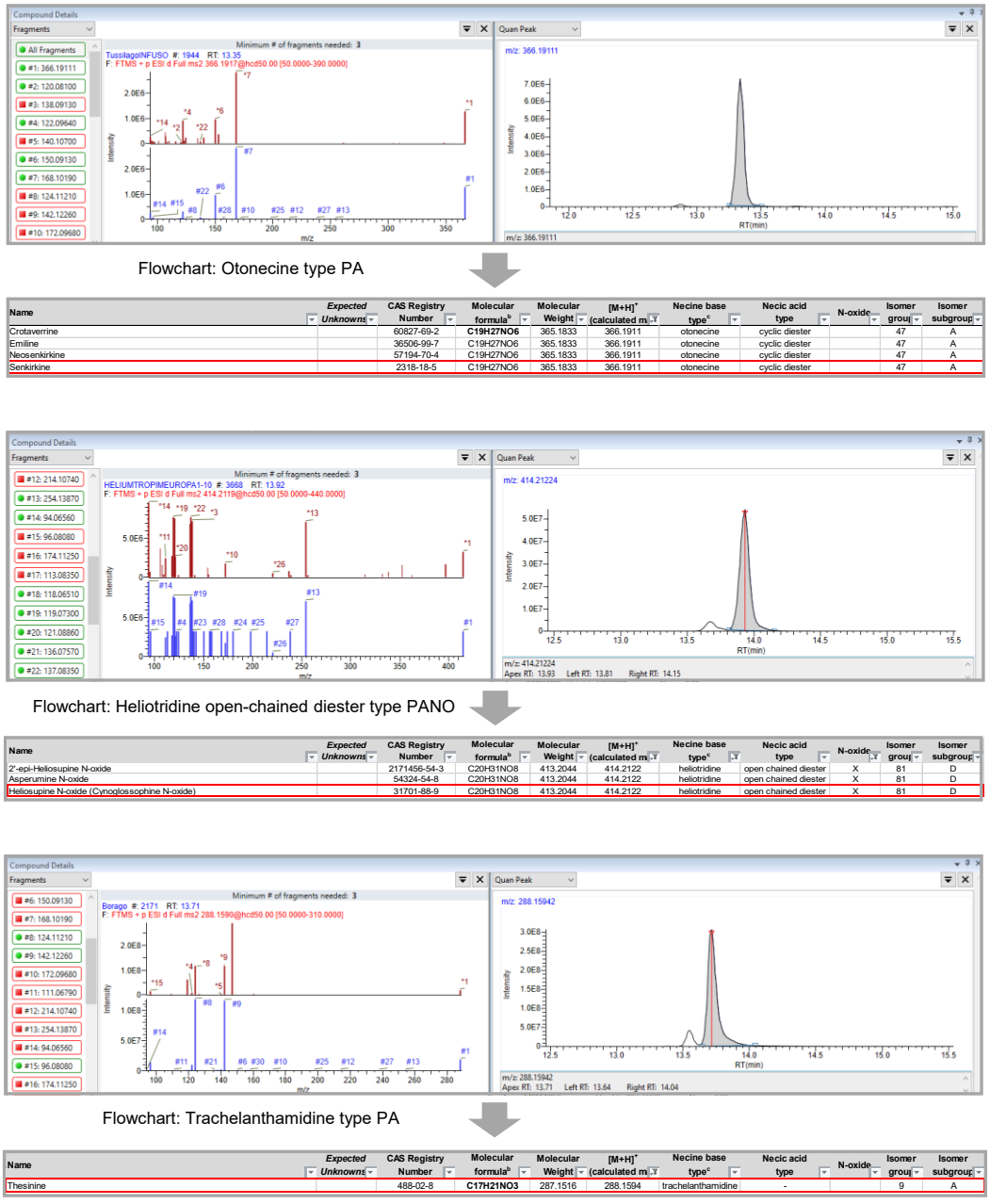
3.3.2.3 Wide-scope suspect screening method

A reliable informatic solution was elaborated to handle with the large amount of HRMS/MS data and to automate and facilitate the detection and characterization of PAs and PANOs. The wide-scope suspect screening method was developed by associating each precursor ion of the inclusion list to a set of diagnostic product ions. This allowed the software to process the raw data, flagging as putative PAs/PANOs the only peaks with a molecular formula corresponding to that of the compounds of the database (± 5 ppm) and at least three diagnostic product ions (± 5 ppm). The product ions (*m/z* values and ion ratios) of suspected peaks were first matched with the information reported in the flowchart (**Figure 3.3**) to establish the group and subgroup of the detected compound, and then the presumed structures were searched into the database to verify the match with a collected analogue. The presumed identity of the detected PA/PANO was confirmed by comparison with the reference standards (MSI, L1 – Metabolomics Standards Initiative, Level 1), or putatively assigned based on literature studies and online databases (MSI, L2). When no spectrum or literature information was available, the detected PA/PANO was tentatively assigned to the compound suggested by the proposed identification strategy, when present (MSI, L3). **Figure 3.6** shows three examples of application of the diagnostic product ions filtering strategy during the identification of suspect PAs.

3.3.3 HRMS/MS spectral library

The identification strategy was applied to 10 PAs-producing plants to detect and identify as much compounds as possible and collect their spectra into an HRMS/MS spectral library. The plant profiles were defined by comparing the information of the diagnostic product ions filtering strategy with literature information, MS/MS spectra available on online databases and libraries, and chemotaxonomic data. The latter

resulted essential in discriminating structural isomers with superimposable MS/MS spectra (echiumine in *Echium italicum*, echinatine/rinderine in *Eupatorium cannabinum*, heliosupine in *Heliotropium europaeum*, and symphytine in *Symphytum officinale*).



Name	Expected Unknowns	CAS Registry Number	Molecular formula[b]	Molecular Weight	[M+H]+ (calculated m	Necine base type[c]	Necic acid type	N-oxide	Isomer group	Isomer subgroup
Crotaverrine		60827-69-2	**C19H27NO6**	365.1833	366.1911	otonecine	cyclic diester		47	A
Emiline		36506-99-7	C19H27NO6	365.1833	366.1911	otonecine	cyclic diester		47	A
Neosenkirkine		57194-70-4	C19H27NO6	365.1833	366.1911	otonecine	cyclic diester		47	A
Senkirkine		2318-18-5	C19H27NO6	365.1833	366.1911	otonecine	cyclic diester		47	A

Name	Expected Unknowns	CAS Registry Number	Molecular formula[b]	Molecular Weight	[M+H]+ (calculated m	Necine base type[c]	Necic acid type	N-oxide	Isomer group	Isomer subgroup
2'-epi-Heliosupine N-oxide		2171456-54-3	C20H31NO8	413.2044	414.2122	heliotridine	open chained diester	X	81	D
Asperumine N-oxide		54324-54-8	C20H31NO8	413.2044	414.2122	heliotridine	open chained diester	X	81	D
Heliosupine N-oxide (Cynoglossophine N-oxide)		31701-88-9	C20H31NO8	413.2044	414.2122	heliotridine	open chained diester	X	81	D

Name	Expected Unknowns	CAS Registry Number	Molecular formula[b]	Molecular Weight	[M+H]+ (calculated m	Necine base type[c]	Necic acid type	N-oxide	Isomer group	Isomer subgroup
Thesinine		488-02-8	**C17H21NO3**	287.1516	288.1594	trachelanthamidine	-		9	A

Figure 3.6. Mode of operation of the diagnostic product ions filtering strategy for the identification of (A) senkirkine (MSI, L1), (B) heliosupine N-oxide (MSI, L2), and (C) thesinine (MSI, L3).

84 PAs and PANOs other than the reference standards were detected, including two "expected unknowns": canescine/canescenine N-oxide (*m/z* 416.2275, $C20H34NO8^+$) and lithosenine N-oxide (*m/z* 432.2223, $C20H34NO9^+$) in *L. officinale*. Their structures, hypothesized on the basis of the key product ions of R/H open-chained diester type of PANOs (**Figure 3.7**), were further supported by chemotaxonomic data (El-Shazly & Wink, 2014; Kopp et al., 2020). Moreover, the product ion at *m/z* 272.1492 ($C13H22NO5^+$) corresponds to a hydroxyisovaleroyl residue (typical necic acid of canescine and lithosenine) at the C-7 position. Besides, the clusters at *m/z* 136 to 138 in the spectra of lithosenine N-oxide (low intensity; < 50%) and canescine N-oxide (high intensity; > 50%) supported their assignments as R and H types (**Figure 3.3B**).

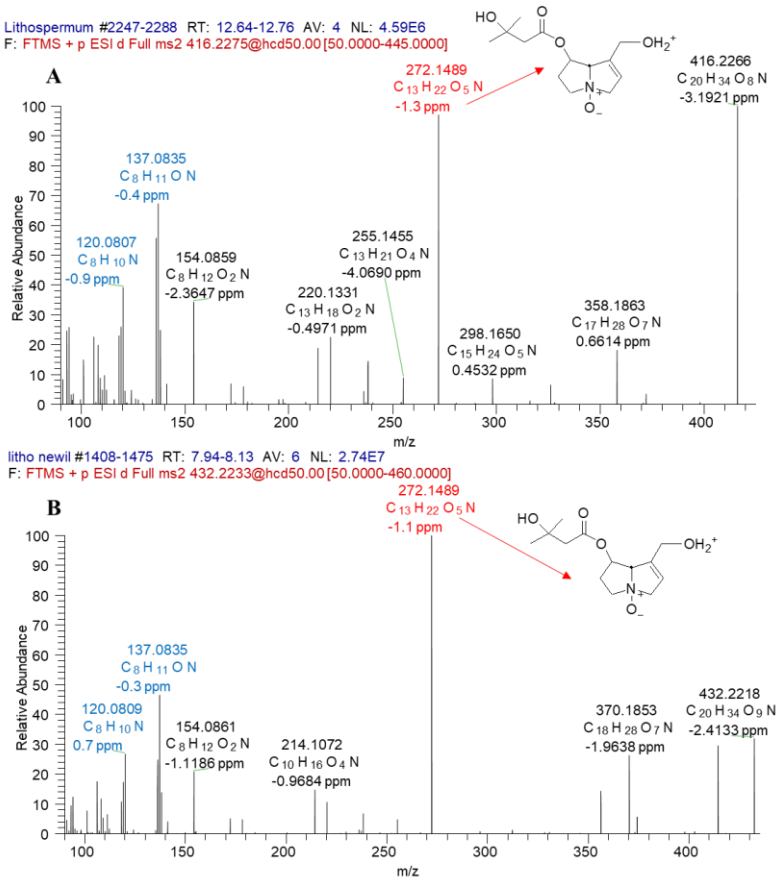


Figure 3.7. HRMS/MS spectra of (A) canescine/canescenine N-oxide and (B) lithosenine N-oxide.

A spectral library of 114 total compounds was built (**Table 3.3**). Among these, 30 were reference standards (MSI, L1), 52 were putatively assigned based on their MS similarity with literature information and online databases (MSI, L2), and 32 were assigned based on the diagnostic product ions filtering strategy and chemotaxonomic data (MSI, L3). During the analysis of the commercial samples, four further compounds were characterized, bringing the number of spectra of the library to 118. The library includes all the PAs of the EFSA's list (28) and the Regulation 2020/2040/EU (21). Most of the library compounds are 1,2 unsaturated PA (103 compounds of which 58% R type and 34% H type) and, therefore, they are considered the most toxic due to their ability to form adducts with DNA and proteins.

Table 3.3. HRMS/MS spectral library of the 118 target PAs and PANOs.

Compound name	Necine base[a]	Esterification type[b]	Molecular formula	$[M+H]^+$ (*m/z*)[c]	Rt (min)	Diagnostic product ions, *m/z* (relative abundance)	IL[d]
7-Tigloylretronecine N-oxide	R	m	$C_{13}H_{19}NO_4$	254.1387	7.2	106.0653 (100); 83.0496 (34); 111.0679 (28); 136.0757 (21); 94.0654 (18)	2
7-Angeloylretronecine N-oxide	R	m	$C_{13}H_{19}NO_4$	254.1387	7.4	106.0654 (100); 83.0496 (46); 136.0758 (32); 94.0655 (31); 111.0680 (30)	2
9-Tigloylretronecine N-oxide	R	m	$C_{13}H_{19}NO_4$	254.1387	10.4	93.0577 (88); 136.0757 (77); 138.0913 (35); 137.0833 (30); 94.0654 (30); 108.0809 (20)	2
9-Angeloylretronecine N-oxide	R	m	$C_{13}H_{19}NO_4$	254.1387	10.7	93.0577 (93); 136.0757 (66); 154.0861 (56); 83.0496 (46); 94.0654 (36); 137.0834 (34); 138.0913 (33)	2
Supinine	S	-	$C_{15}H_{25}NO_4$	284.1856	8.1	122.0965 (100); 140.1069 (66); 70.0657 (23); 110.0967 (12); 94.0655 (11)	2
Amabiline	S	-	$C_{15}H_{25}NO_4$	284.1856	8.4	122.0964 (100); 140.1069 (90); 70.0657 (28); 110.0967 (16); 94.0655 (13)	2
Spilanthine	T	-	$C_{15}H_{25}NO_4$	284.1856	9.2	142.1227 (100); 124.1122 (56); 96.0811 (8); 70.0657 (6)	2
Viridiflorine	T	-	$C_{15}H_{27}NO_4$	286.2013	7.9	142.1227 (100); 125.1198 (7); 70.0657 (6); 124.1121 (5); 96.0814 (1)	2
Cynaustraline	T	-	$C_{15}H_{27}NO_4$	286.2013	8.2	142.1225 (100); 124.1120 (52); 70.0657 (12); 86.0968 (9); 96.0810 (5)	2
Thesinine	T	-	$C_{17}H_{21}NO_3$	288.1594	13.7	147.0441 (100); 142.1227 (40); 124.1122 (39); 119.0493 (18); 96.0812 (5)	3
Heleurine	S	-	$C_{16}H_{27}NO_4$	298.2013	12.0	122.0965 (100); 140.1069 (57); 94.0655 (13); 110.0966 (12); 70.0657 (11)	2
Intermedine	R	m	$C_{15}H_{25}NO_5$	300.1805	5.3	94.0654 (100); 156.1018 (47); 138.0912 (39); 120.0807 (16); 82.0656 (7)	1
Indicine	R	m	$C_{15}H_{25}NO_5$	300.1805	5.5	94.0654 (100); 156.1016 (46); 138.0912 (35); 120.0808 (18); 82.0655 (7)	1
Lycopsamine	R	m	$C_{15}H_{25}NO_5$	300.1805	5.6	94.0654 (100); 156.1018 (55); 138.0912 (35); 120.0807 (18); 82.0656 (7)	1
Rinderine	H	m	$C_{15}H_{25}NO_5$	300.1805	5.9	138.0913 (100); 156.1019 (50); 120.0808 (31); 94.0655 (24); 82.0656 (12)	2
Echinatine	H	m	$C_{15}H_{25}NO_5$	300.1805	6.0	138.0912 (100); 156.1018 (55); 120.0808 (33); 94.0654 (26); 82.0656 (13)	2
Supinine N-oxide	S	-	$C_{15}H_{25}NO_5$	300.1805	8.7	156.1020 (100); 139.0992 (34); 120.0809 (24); 122.0965 (22); 121.0887 (9); 138.0913 (7); 960812 (7)	2
Amabiline N-oxide	S	-	$C_{15}H_{25}NO_5$	300.1805	9.1	156.1019 (100); 139.0991 (32); 120.0808 (26); 122.0965 (26); 121.0887 (10); 138.0913 (8); 960812 (7)	3
Curassavine	T	-	$C_{16}H_{29}NO_4$	300.2169	11.9	142.1227 (100); 124.1122 (75); 156.1022 (12); 70.0658 (12); 96.0812 (10)	2
Dihydroechinatine (rinderine)	P	m	$C_{15}H_{27}NO_5$	302.1962	6.5	158.1176 (100); 140.1069 (56); 122.0963 (41); 96.0812 (18)	3
Dihydrointermedine	P	m	$C_{15}H_{27}NO_5$	302.1962	6.6	158.1175 (100); 140.1068 (55); 122.0964 (36); 96.0811 (14)	3
Dihydrolycopsamine	P	m	$C_{15}H_{27}NO_5$	302.1962	6.7	158.1175 (100); 140.1069 (55); 122.0965 (34); 96.0810 (12)	3
Viridiflorine N-oxide	T	-	$C_{15}H_{27}NO_5$	302.1962	8.5	158.1176 (100); 124.1121 (16); 141.1148 (9), 140.1071 (7); 122.0966 (2)	3
Helioamplexine	R	m	$C_{16}H_{27}NO_5$	314.1962	7.4	94.0655 (100); 156.1019 (36); 138.0914 (30); 120.0809 (17); 82.0657 (9)	2
Heliotrine	H	m	$C_{16}H_{27}NO_5$	314.1962	9.2	138.0912 (100); 156.1017 (43); 94.0654 (29); 120.0808 (27); 82.0656 (14); 108.0809 (9)	1
Heleurine N-oxide	S	-	$C_{16}H_{27}NO_5$	314.1962	12.4	156.1019 (100); 120.0808 (20); 139.0991 (18); 122.0965 (16); 138.0914 (8); 121.0889 (5); 96.0811 (3)	2
5'-Hydroxyindicine	R	m	$C_{15}H_{25}NO_6$	316.1755	1.9	94.0654 (100); 138.0912 (43); 156.1017 (25); 120.0808 (18); 82.0656 (5); 108.0809 (2)	2

Compound name	Necine base[a]	Esterification type[b]	Molecular formula	$[M+H]^+$ (*m/z*)[c]	Rt (min)	Diagnostic product ions, *m/z* (relative abundance)	IL[d]
5'-Hydroxyintermedine (lycopsamine)	R	m	$C_{15}H_{25}NO_6$	316.1755	3.0	94.0654 (100); 138.0912 (40); 156.1018 (26); 120.0808 (16); 82.0655 (5); 108.0809 (2)	3
5'-Hydroxyechinatine (rinderine)	H	m	$C_{15}H_{25}NO_6$	316.1755	3.5	138.0913 (100); 94.0653 (28); 72.0813 (23); 156.1017 (21); 120.0809 (8)	2
Rinderine N-oxide	H	m	$C_{15}H_{25}NO_6$	316.1755	6.4	172.0965 (100); 138.0912 (19); 111.0680 (18); 94.0654 (18); 136.0757 (7); 137.0835 (3); 155.0939 (18)	2
Echinatine N-oxide	H	m	$C_{15}H_{25}NO_6$	316.1755	6.6	172.0965 (100); 138.0912 (20); 111.0680 (20); 94.0654 (19); 136.0757 (7); 137.0833 (3); 155.0939 (16)	2
Intermedine N-oxide	R	m	$C_{15}H_{25}NO_6$	316.1755	6.9	172.0965 (100); 138.0912 (53); 94.0654 (37); 111.068 (25); 155.0938 (18); 136.0756 (15); 137.0836 (6)	1
Indicine N-oxide	R	m	$C_{15}H_{25}NO_6$	316.1755	7.2	172.0964 (100); 138.0912 (53); 94.0654 (37); 111.0679 (23); 136.0754 (18); 137.0833 (7); 155.0938 (1)	1
Lycopsamine NO	R	m	$C_{15}H_{25}NO_6$	316.1755	7.2	172.0966 (100); 138.0912 (64); 94.0654 (41); 111.0681 (24); 136.0758 (19); 155.0939 (18); 137.0834 (6)	1
Dihydrointermedine N-oxide	P	m	$C_{15}H_{27}NO_6$	318.1911	7.6	174.1122 (100); 113.0837 (20); 96.0810 (3); 140.1069 (2); 138.0913 (1); 139.0990 (1)	3
Dihydrolycopsamine N-oxide	P	m	$C_{15}H_{27}NO_6$	318.1911	7.7	174.1122 (100); 113.0837 (20); 96.0811 (3); 140.1070 (2); 138.0912 (1); 139.0990 (1)	3
Dihydrorinderine N-oxide	P	m	$C_{15}H_{27}NO_6$	318.1911	8.0	174.1123 (100); 96.0811 (26); 113.0837 (23); 140.1068 (22); 138.0913 (10); 139.0994 (2)	3
Dihydroechinatine N-oxide	P	m	$C_{15}H_{27}NO_6$	318.1911	8.1	174.1123 (100); 96.0811 (41); 140.1068 (25); 113.0837 (25); 138.0912 (13); 139.0994 (2)	3
Monocrotaline	R	c	$C_{16}H_{23}NO_6$	326.1598	2.7	120.0808 (82); 121.0885 (80); 94.0654 (31); 237.1358 (21); 194.1175 (20); 280.1540 (16); 138.0911 (15); 298.1642 (6)	1
Europine	H	m	$C_{16}H_{27}NO_6$	330.1911	5.9	138.0913 (100); 156.1019 (41); 94.0655 (22); 120.0809 (18); 254.1383 (11); 82.0657 (11); 108.0810 (5)	1
Helioamplexine N-oxide	R	m	$C_{16}H_{27}NO_6$	330.1911	9.2	172.0966 (100); 138.0913 (83); 94.0654 (65); 111.0681 (32); 155.0939 (29); 136.0757 (24); 137.0837 (8)	3
Heliotrine N-oxide	H	m	$C_{16}H_{27}NO_6$	330.1911	10.2	172.0964 (100); 111.0679 (17); 138.0912 (13); 94.0654 (12); 155.0938 (6); 136.0754 (6); 137.0834 (3);	1
5'-Hydroxyechinatine (rinderine) N-oxide	H	m	$C_{15}H_{25}NO_7$	332.1704	1.9	172.0965 (100); 111.0681 (19); 155.0937 (7); 136.0757 (2); 137.0837 (3); 138.0912 (1); 94.0654 (1)	2
5'-Hydroxyintermedine (lycopsamine) N-oxide	R	m	$C_{15}H_{25}NO_7$	332.1704	3.0	172.0965 (100); 138.0912 (30); 94.0654 (24); 111.0680 (19); 155.0938 (16); 136.0757 (11); 137.0835 (5)	2
Spartioidine	R	c	$C_{18}H_{23}NO_5$	334.1649	9.8	120.0807 (67); 94.0654 (41); 138.0912 (35); 306.1696 (21)	2
Seneciphylline	R	c	$C_{18}H_{23}NO_5$	334.1649	10.1	120.0807 (58); 94.0654 (50); 138.0912 (38); 306.1697 (22)	1
Senecivernine	R	c	$C_{18}H_{25}NO_5$	336.1805	12.1	120.0808 (41); 138.0913 (27); 308.1855 (23); 94.0654 (18)	1
Senecionine	R	c	$C_{18}H_{25}NO_5$	336.1805	12.3	120.0808 (51); 94.0654 (45); 138.0912 (33); 308.1851 (19)	1
Monocrotaline N-oxide	R	c	$C_{16}H_{23}NO_7$	342.1547	5.2	137.0833 (76); 119.0729 (39); 120.0808 (33); 136.0755 (24); 118.0651 (23); 236.1278 (19); 94.0654 (19); 296.1491 (8); 138.0913 (7); 314.1587 (4)	1
3'-Acetylintermedine	R	m	$C_{17}H_{27}NO_6$	342.1911	8.8	94.0655 (100); 138.0914 (22); 120.0809 (20); 156.1017 (15); 282.1697 (6)	2
3'-Acetylrinderine	H	m	$C_{17}H_{27}NO_6$	342.1911	8.9	138.0913 (100); 120.0809 (36); 94.0655 (27); 156.1017 (19); 282.1707 (6)	2

Compound name	Necine base[a]	Esterification type[b]	Molecular formula	$[M+H]^+$ (*m/z*)[c]	Rt (min)	Diagnostic product ions, *m/z* (relative abundance)	IL[d]
7-Acetylrinderine	H	d	$C_{17}H_{27}NO_6$	342.1911	9.6	120.0808 (100); 180.1016 (6); 94.0657 (4); 138.0913 (3);	3
7-Acetylechinatine	H	d	$C_{17}H_{27}NO_6$	342.1911	9.7	120.0808 (100); 138.0913 (6); 198.1123 (5); 94.0653 (3); 282.0629 (2); 180.1021 (2)	3
3'-Acetyllycopsamine	R	m	$C_{17}H_{27}NO_6$	342.1911	9.8	94.0656 (100); 138.0913 (27); 120.0810 (20); 156.1020 (14); 282.1695 (4)	2
3'-Acetylechinatine	H	m	$C_{17}H_{27}NO_6$	342.1911	10.2	138.0912 (100); 120.0809 (28); 94.0654 (26); 156.1018 (14); 282.1696 (3)	2
7-Acetylintermedine	R	d	$C_{17}H_{27}NO_6$	342.1911	10.5	120.0807 (100); 198.1122 (6); 94.0654 (5); 180.1016 (5); 138.0912 (3)	2
7-Acetyllycopsamine	R	d	$C_{17}H_{27}NO_6$	342.1911	10.7	120.0807 (100); 94.0654 (7); 198.1123 (7); 180.1014 (4); 138.0913 (3)	2
Europine N-oxide	H	m	$C_{16}H_{27}NO_7$	346.1860	6.5	172.0965 (100); 111.0680 (16); 155.0939 (12); 256.1175 (12); 138.0912 (10); 94.0655 (10); 136.0754 (5); 137.0834 (2)	1
Erucifoline	R	c	$C_{18}H_{23}NO_6$	350.1598	5.5	120.0808 (67); 138.0911 (39); 94.0653 (33); 322.1643 (5)	1
Riddelliine	R	c	$C_{18}H_{23}NO_6$	350.1598	7.1	120.0807 (62); 94.0654 (48); 138.0912 (42); 322.1644 (23)	2
Spartioidine N-oxide	R	c	$C_{18}H_{23}NO_6$	350.1598	11.0	120.0807 (91); 118.0652 (84); 119.0729 (77); 94.0654 (72); 136.0756 (39); 138.0912 (25); 322.1647 (10); 137.0833 (4)	2
Seneciphylline N-oxide	R	c	$C_{18}H_{23}NO_6$	350.1598	11.2	120.0808 (85); 94.0654 (79); 118.0652 (68); 119.0729 (54); 136.0756 (38); 138.0913 (25); 322.1646 (8); 137.0832 (5)	1
Retrorsine	R	c	$C_{18}H_{25}NO_6$	352.1755	8.8	120.0808 (51); 94.0654 (36); 138.0912 (34); 324.1798 (18)	1
Jacobine	R	c	$C_{18}H_{25}NO_6$	352.1755	10.4	120.0808 (100); 155.1065 (62); 122.0964 (57); 123.1043 (37); 94.0655 (34); 280.1547 (28); 140.1068 (11); 138.0913 (9)	1
Senecivernine N-oxide	R	c	$C_{18}H_{25}NO_6$	352.1755	12.6	120.0807 (57); 118.0652 (54); 94.0654 (53); 119.0731 (35); 136.0757 (21); 138.0912 (15); 324.1804 (9); 137.0838 (3)	1
Integerrimine N-oxide	R	c	$C_{18}H_{25}NO_6$	352.1755	12.7	118.0652 (68); 120.0807 (56); 94.0654 (52); 119.0729 (50); 136.0756 (41); 138.0912 (16); 324.1799 (8); 137.0833 (4)	2
Senecionine N-oxide	R	c	$C_{18}H_{25}NO_6$	352.1755	12.8	118.0651 (55); 120.0808 (50); 94.0654 (50); 136.0756 (43); 119.0730 (37); 138.0913 (17); 324.1799 (6); 137.0836 (5)	1
Trichodesmine	R	c	$C_{18}H_{27}NO_6$	354.1911	8.1	222.1487 (100); 120.0808 (83); 94.0654 (30); 164.1069 (19); 308.1850 (17); 138.0912 (16)	1
Uplandicine	R	d	$C_{17}H_{27}NO_7$	358.1860	6.2	120.0808 (100); 94.0655 (6); 180.1018 (4); 198.1127 (3); 138.0915 (2)	2
3'-Acetylrinderine N-oxide	H	m	$C_{17}H_{27}NO_7$	358.1860	9.9	172.0966 (100); 298.1646 (37); 138.0912 (28); 94.0655 (26); 111.0681 (26); 155.0939 (18); 136.0756 (11); 137.0834 (4)	2
7-Acetylintermedine N-oxide	R	d	$C_{17}H_{27}NO_7$	358.1860	10.7	214.1070 (100); 137.0834 (50); 180.1016 (43); 136.0756 (24); 120.0808 (22); 119.0731 (19); 118.0651 (14)	2
3'-Acetylintermedine N-oxide	R	m	$C_{17}H_{27}NO_7$	358.1860	10.8	172.0967 (100); 138.0913 (73); 94.0655 (61); 298.1649 (54); 111.0682 (33); 136.0757 (23); 155.0939 (21); 137.0837 (8)	3
3'-Acetylechinatine N-oxide	H	m	$C_{17}H_{27}NO_7$	358.1860	10.9	172.0965 (100); 138.0912 (37); 298.1647 (35); 94.0654 (33); 111.0680 (23); 155.0938 (17); 136.0756 (16); 137.0833 (4)	3
7-Acetyllycopsamine N-oxide	R	d	$C_{17}H_{27}NO_7$	358.1860	11.0	214.1071 (100); 180.1015 (53); 137.0835 (51); 136.0757 (25); 120.0807 (23); 119.0732 (16); 118.0652 (14)	2
7-Acetylrinderine N-oxide	H	d	$C_{17}H_{27}NO_7$	358.1860	11.3	214.1070 (100); 137.0835 (81); 119.0730 (75); 120.0807 (62); 136.0756 (28); 118.0650 (25); 180.1019 (10); 298.1652 (5); 138.0911 (5);	3
7-Acetylechinatine N-oxide	H	d	$C_{17}H_{27}NO_7$	358.1860	11.4	214.1072 (100); 137.0835 (76); 120.0808 (62); 119.0731 (60); 106.0654 (50); 136.0757 (25); 118.0653 (22); 298.1662 (3); 138.0915 (6)	3

Compound name	Necine base[a]	Esterification type[b]	Molecular formula	$[M+H]^+$ (*m/z*)[c]	Rt (min)	Diagnostic product ions, *m/z* (relative abundance)	IL[d]
3'-Acetyllycopsamine N-oxide	R	m	$C_{17}H_{27}NO_7$	358.1860	11.7	172.0968 (100); 138.0914 (87); 94.0656 (77); 298.1648 (53); 111.0682 (33); 136.0758 (29); 155.0939 (17); 137.0837 (9)	2
Erucifoline N-oxide	R	c	$C_{18}H_{23}NO_7$	366.1547	6.2	118.0651 (93); 119.0730 (86); 94.0654 (80); 120.0808 (78); 136.0755 (68); 137.0835 (9); 138.0913 (10)	1
Riddelliine N-oxide	R	c	$C_{18}H_{23}NO_7$	366.1547	7.7	120.0808 (100); 94.0654 (99); 118.0652 (78); 119.0730 (70); 136.0757 (51); 138.0913 (26); 338.1598 (7); 137.0832 (6)	2
Neosenkirkine	O	c	$C_{19}H_{27}NO_6$	366.1911	12.9	168.1021 (100); 150.0915 (52); 122.0603 (25)	2
Senkirkine	O	c	$C_{19}H_{27}NO_6$	366.1911	13.3	168.1018 (100); 150.0911 (34); 122.0600 (34); 348.1821 (2)	1
Jacobine N-oxide	R	c	$C_{18}H_{25}NO_7$	368.1704	7.3	120.0808 (100); 296.1488 (62); 94.0654 (28); 118.0651 (25); 119.0729 (22); 139.0992 (10); 138.0914 (8)	1
Retrorsine N-oxide	R	c	$C_{18}H_{25}NO_7$	368.1704	9.4	120.0808 (68); 118.0652 (67); 94.0654 (67); 136.0757 (53); 119.0731 (43); 138.0912 (24); 340.1743 (7); 137.0835 (7)	1
Uplandicine N-oxide	R	d	$C_{17}H_{27}NO_8$	374.1809	6.2	214.1070 (100); 137.0835 (35); 180.1015 (19); 136.0756 (16); 120.0807 (15); 119.0731 (9); 118.0651 (6)	2
Acetylseneciphylline N-oxide	R	c	$C_{20}H_{25}NO_6$	376.1755	16.0	118.0653 (100); 120.0809 (94); 94.0655 (76); 119.0731 (57); 136.0758 (47); 332.1490 (21); 138.0915 (16); 137.0594 (2)	2
Symphytine isomer 1	R	d	$C_{20}H_{31}NO_6$	382.2224	15.4	120.0808 (100); 83.0496 (22); 238.1436 (6); 138.0914 (4); 94.0654 (2)	2
Symphytine isomer 2	R	d	$C_{20}H_{31}NO_6$	382.2224	15.6	120.0808 (100); 83.0496 (61); 138.0914 (8); 238.1432 (8); 94.0655 (4); 220.1331 (1)	2
Echiumine	R	d	$C_{20}H_{31}NO_6$	382.2224	15.7	120.0807 (100); 138.0912 (41); 94.0654 (15); 83.0496 (5); 220.1332 (5); 238.1439 (2)	2
5'-Acetyleuropine N-oxide	H	m	$C_{18}H_{29}NO_8$	388.1966	11.3	172.0965 (100); 137.0834 (58); 328.1749 (38); 111.0679 (11); 138.0911 (11); 136.0753 (5)	2
7-Angeloylheliotrine	H	d	$C_{21}H_{33}NO_6$	396.2381	16.4	120.0809 (100); 138.0913 (6); 94.0654 (3)	3
Asperumine	H	d	$C_{20}H_{31}NO_7$	398.2173	13.1	120.0809 (100); 138.0913 (6); 94.0653 (3); 238.1425 (2); 83.0495 (1)	2
Echimidine isomer 1	R	d	$C_{20}H_{31}NO_7$	398.2173	13.2	120.0808 (100); 83.0497 (15); 238.1438 (2); 138.0916 (2); 94.0656 (2); 220.1332 (1)	3
Heliosupine	H	d	$C_{20}H_{31}NO_7$	398.2173	13.3	120.0809 (100); 138.0915 (4); 238.1434 (2); 220.1333 (2); 94.0654 (2); 83.0496 (2)	2
Echimidine isomer 2	R	d	$C_{20}H_{31}NO_7$	398.2173	13.4	120.0808 (100); 83.0495 (63); 138.0913 (5); 238.1424 (3); 94.0654 (3);	3
Echimidine	R	d	$C_{20}H_{31}NO_7$	398.2173	13.4	120.0808 (100); 83.0496 (20); 238.1431 (2); 138.0913 (2); 94.0656 (2);	1
Symphytine N-oxide	R	d	$C_{20}H_{31}NO_7$	398.2173	15.5	254.1383 (100); 83.0496 (86); 137.0834 (53); 220.1330 (49); 120.0807 (39); 136.0758 (36); 119.0729 (27); 118.0652 (23)	2
Echiumine N-oxide	R	d	$C_{20}H_{31}NO_7$	398.2173	15.7	83.0496 (100); 254.1385 (75); 137.0834 (40); 220.1329 (38); 136.0757 (37); 120.0809 (29); 119.0730 (17); 118.0651 (15); 138.0913 (6)	2
Canescine (canescenine)	H	d	$C_{20}H_{33}NO_7$	400.2330	12.4	120.0809 (100); 94.0655 (9); 138.0914 (5); 256.1535 (3); 83.0496 (1)	3
7-Tigloyleuropine	H	d	$C_{21}H_{33}NO_7$	412.2330	14.9	120.0809 (100); 138.0915 (4); 94.0655 (3); 238.1434 (2); 220.1334 (1); 83.0495 (1)	2
Lasiocarpine	H	d	$C_{21}H_{33}NO_7$	412.2330	15.1	120.0807 (100); 138.0911 (5); 94.0654 (4); 238.1437 (3); 220.1321 (2); 83.0495 (1)	1
7-Angeloylheliotrine N-oxide	H	d	$C_{21}H_{33}NO_7$	412.2330	16.7	120.0809 (100); 94.0655 (87); 138.0912 (52); 254.138 (51); 119.0731 (48); 136.0757 (47); 137.0836 (30); 118.0653 (25)	2
Echihumiline N-oxide	R	d	$C_{20}H_{31}NO_8$	414.2122	13.2	254.1387 (100); 137.0836 (44); 83.0496 (44); 120.0808 (26); 136.0757 (23); 220.1331 (16); 119.0732 (13); 118.0655 (11)	3

Compound name	Necine base[a]	Esterification type[b]	Molecular formula	$[M+H]^+$ (*m/z*)[c]	Rt (min)	Diagnostic product ions, *m/z* (relative abundance)	IL[d]
Echimidine N-oxide	R	d	$C_{20}H_{31}NO_8$	414.2122	13.4	254.1384 (100); 83.0496 (40); 137.0834 (34); 120.0807 (32); 136.0756 (21); 220.1331 (20); 119.0730 (17); 118.0653 (8); 138.0913 (3)	1
Vulgarine N-oxide	R	m	$C_{20}H_{31}NO_8$	414.2122	13.5	172.0967 (100); 256.1178 (49); 94.0656 (43); 138.0914 (42); 136.0757 (27); 111.0682 (17); 155.0938 (11)	3
Asperumine N-oxide	H	d	$C_{20}H_{31}NO_8$	414.2122	13.7	119.0731 (100); 120.0809 (76); 137.0836 (74); 94.0655 (64); 254.1384 (59); 136.0757 (56); 138.0913 (35); 121.0889 (34); 118.0652 (34)	3
Heliosupine N-oxide	H	d	$C_{20}H_{31}NO_8$	414.2122	14.1	94.0655 (100); 119.0731 (81); 137.0836 (81); 120.0809 (80); 254.1384 (75); 138.0915 (76); 136.0758 (74); 118.0652 (30)	2
Lithosenine	R	d	$C_{20}H_{33}NO_8$	416.2279	8.3	120.0807 (100); 94.0654 (8); 138.0913 (4); 256.1540 (1)	3
Canescine (canescenine) N-oxide	H	d	$C_{20}H_{33}NO_8$	416.2279	12.7	272.1491 (90); 137.0835 (58); 136.0757 (41); 120.0809 (33); 119.0730 (23); 118.0651 (23); 138.0913 (21)	3
7-Tigloyleuropine N-oxide	H	d	$C_{21}H_{33}NO_8$	428.2279	15.6	119.0730 (100); 120.0808 (95); 254.1381 (83); 137.0834 (81); 136.0756 (65); 118.0651 (32); 138.0913 (33)	2
Lasiocarpine N-oxide	H	d	$C_{21}H_{33}NO_8$	428.2279	15.8	94.0654 (100); 254.1384 (91); 120.0808 (90); 119.0731 (83); 136.0757 (77); 137.0835 (76); 138.0913 (72); 118.0652 (60)	1
Lithosenine N-oxide	R	d	$C_{20}H_{33}NO_9$	432.2228	8.7	272.1490 (100); 137.0835 (46); 120.0809 (26); 136.0757 (25); 119.0731 (18); 138.0916 (14); 118.0652 (11)	3
Thesinine-4'-ramnoside	T	-	$C_{23}H_{31}NO_7$	434.2173	13.6	147.0440 (100); 142.1227 (26); 124.1121 (25); 119.0493 (17); 288.1594 (15)	3
3'-Acetylheliosupine	H	d	$C_{22}H_{33}NO_8$	440.2279	15.0	120.0809 (100); 138.0913 (4); 238.1438 (2); 83.0495 (2); 220.1335 (1)	2
3'-Acetylechiumine N-oxide	R	d	$C_{22}H_{33}NO_8$	440.2279	16.7	83.0496 (100); 254.1387 (40); 380.2062 (34); 220.1331 (34); 136.0755 (32); 137.0835 (30); 120.0809 (30); 118.0652 (18); 119.0730 (15)	3
Thesinine-4'-glucoside	T	-	$C_{23}H_{31}NO_8$	450.2122	11.1	147.0440 (100); 142.1222 (20); 124.1122 (20); 119.0493 (17); 288.1591 (6)	3
5'-Acetyllasiocarpine	H	d	$C_{23}H_{35}NO_8$	454.2435	16.4	120.0808 (100); 138.0912 (7); 238.1439 (6); 94.0654 (3); 220.1333 (2); 83.0495 (2)	2
3'-Acetylheliosupine N-oxide	H	d	$C_{22}H_{33}NO_9$	456.2228	15.6	94.0655 (100); 119.0731 (95); 138.0913 (83); 120.0808 (61); 136.0759 (55); 254.1386 (52); 137.0836 (38); 118.0652 (35); 396.2015 (3)	2
5'-Acetyllasiocarpine N-oxide	H	d	$C_{23}H_{35}NO_9$	470.2385	16.7	94.0655 (100); 120.0809 (87); 254.1382 (75); 138.0914 (73); 136.0757 (65); 119.0731 (63); 137.0834 (58); 118.0650 (30); 410.2153 (25)	2

[a] Type of necine base: Retronecine (R) heliotridine (H), otonecine (O) trachelanthamidine (T), platynecine (P), and supinidine (S)

[b] Type of esterification: monoester (m), open-chained diester (d), and cyclic diester (c)

[c] Exact mass

[d] Identification level (IL) according to the Metabolomics Standards Initiative

3.3.4 High-throughput target screening and identification method

The in-house spectral library was associated to the high-throughput target screening and identification method. The identification criteria were set as follows: the presence of the precursor ion, a mass tolerance < ± 5 ppm, the expected retention time (± 0.2 min), at least three product ions (± 5 ppm), and a library match score higher than 70%. The post-acquisition data evaluation was combined with the sample preparation procedure and the PA-tailored UHPLC-HRMS/MS method for the high-throughput target screening and identification of numerous PAs in food matrices with high risk of contamination.

Figure 3.8 shows an example of the method ability to identify europine N-oxide and distinguish it from a close interfering peak in a dietary supplement sample. Europine N-oxide (**Figure 3.8A**) met all the identification criteria (mass tolerance of the precursor ion, 0.7 ppm; 4 product ions with mass tolerance < 5 ppm; library match score, 86%), while the interfering peak (**Figure 3.8B**) only met two of them (mass tolerance of precursor ion, 0.5 ppm; retention time within the range). These results show that the HRMS/MS criteria were the strictest and demonstrate the ability of the method to detect the target analytes with high reliability.

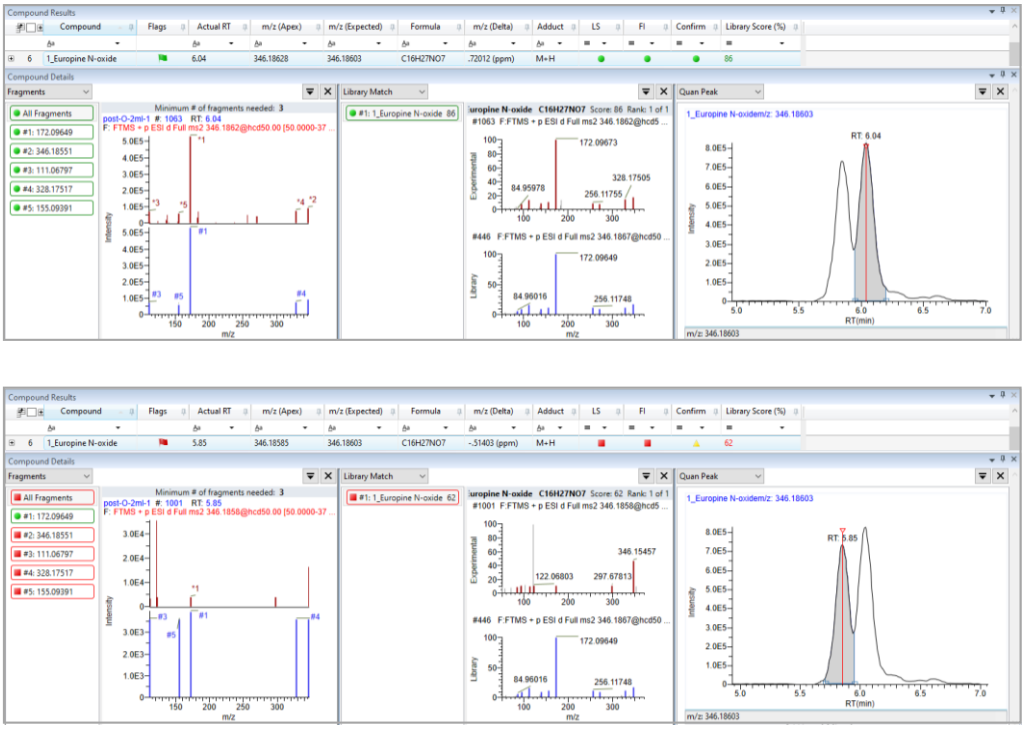


Figure 3.8. Specificity of the high-throughput target screening and identification method in identifying europine N-oxide (A) and distinguishing it from a close interfering peak in a dietary supplement (B).

3.3.5 Qualitative analytical performance

A qualitative validation was performed since the aim of the proposed study was to develop an analytical platform for the detection and identification of PAs in complex matrices at relevant contamination levels. The method specificity, LOIs, and precision (false negative rate) were evaluated on 28 reference standards for all the investigated matrices, according to the performance criteria of screening methods (Magnusson & Örnemark, 2014; Pihlstrom et al., 2017). Regarding the remaining PAs and PANOs, for which no reference standards were available, the detection and identification can be achieved although it is not possible to specify qualitative performance parameters (Pihlstrom et al., 2017). The method specificity, defined as the ability of the method to distinguish the analyte from any other matrix interferences, was evaluated by comparison between different blank and spiked samples of the studied matrices. No

interfering peaks were observed at the expected retention time for all the 28 reference standards in honey and pollen samples. On the contrary, some plant-based samples showed the presence of interfering peaks close to some of the target analytes. The proposed method provided satisfactory specificity and the matrix interferents were either chromatographically or spectrally discriminated; moreover, the number of false positives dropped to zero when all the identification criteria were considered. To achieve an accurate identification of the target analytes and minimize the risk of false positives, diagnostic information, that meets the defined criteria, is required (Lehotay et al., 2015). The LOIs, defined as the lowest concentration that fulfill all the identification criteria of the method, were established to estimate the threshold concentrations at which the identification become reliable. LOIs of the 28 target analytes in the six tested matrices ranged from 0.6 to 30 μg kg^{-1} (**Table 3.4**). The method was able to detect and identify all the target analytes in the SALLE extract at a concentration of 2 μg L^{-1}, except for Em, EmNO, Er, Jb, Mc, Re, ReNO, SpNO, Sv, and SvNO, which were detected from 4 μg L^{-1} in dietary supplements. The LOIs demonstrated to be fit-for-purpose for PA-monitoring applications; LOIs were much lower than the MLs (17-119 times in pollen, 21-67 times in herbal infusions, 10-50 times in teas, and 20-40 times in dietary supplements). The precision of the method was calculated as false negative rate. Considering the calculated LOIs and the regulatory MLs, two cut-off levels, 2 and 4 μg L^{-1} of the SALLE extract, were defined to achieve the best suited false negative rate; the guidelines require identification methods to accomplish a false negative rate ≤ 5% (Lehotay et al., 2015). The overall false negative rate evaluated on 36 blank samples spiked at the two abovementioned levels and processed in duplicates, was lower than 5 % (0-1.3%) at 4 μg L^{-1} of the SALLE extract for all the 28 analytes. However, the method achieved reliable

identification results (< 5% of false negatives) at the lowest level tested (2 μg L^{-1}) as well for most of the analytes, excluding those with a LOI of 20 μg kg^{-1} in dietary supplements (**Table 3.4**) and ErNO, Se, Sp and Sv in teas and infusions.

Table 3.4. Limits of identification (LOIs) of the 28 target PAs in different food matrices.

Analyte	Honey	Pollen	Black tea	Green tea	Herbal infusion	Dietary supplement
	LOI (μg kg^{-1})	LOI (μg kg^{-1})	LOI (μg kg^{-1})	LOI (μg kg^{-1})	LOI (μg kg^{-1})	LOI (μg kg^{-1})
Em	0.6	10.4	3.0	3.2	3.0	20.0
EmNO	0.6	10.4	3.0	9.4	3.0	20.0
Er	0.6	15.6	7.5	11.7	3.0	20.0
ErNO	0.6	10.4	4.5	9.4	3.0	10.0
Eu	1.3	15.6	9.4	9.4	9.4	10.0
EuNO	0.6	15.6	6.0	9.4	7.5	10.0
He	0.6	4.2	3.0	4.7	3.0	10.0
HeNO	0.6	12.5	7.5	7.8	3.0	10.0
Im	0.6	8.3	4.5	9.4	3.0	10.0
ImNO	0.6	8.3	6.0	9.4	3.0	10.0
Jb	0.8	15.6	9.4	11.7	3.0	20.0
JbNO	0.6	12.5	4.5	7.8	3.0	10.0
Lc	0.6	10.4	3.0	4.7	3.0	10.0
LcNO	0.6	12.5	4.5	15.0	3.0	10.0
Ly	0.6	4.2	3.0	3.2	3.0	10.0
LyNO	0.6	4.2	3.0	3.2	3.0	10.0
Mc	1.3	15.6	6.0	11.7	4.5	20.0
McNO	1.3	15.6	7.5	9.4	4.5	10.0
Re	0.6	15.6	4.5	6.2	4.5	20.0
ReNO	0.6	30.0	15.0	11.7	9.4	20.0
Se	0.6	15.6	6.0	11.7	3.0	10.0
SeNO	0.6	20.0	3.0	11.7	3.0	10.0
Sp	0.6	15.6	3.0	9.4	3.0	10.0
SpNO	0.6	10.4	4.5	9.4	3.0	20.0
Sv	0.6	20.0	9.4	7.8	3.0	20.0
SvNO	0.6	4.2	3.0	7.8	3.0	20.0
Sk	0.6	4.2	3.0	3.2	3.0	10.0
Tc	1.3	15.6	4.5	7.8	3.0	10.0

3.3.6 Analysis of commercial samples

A huge number of commercial samples (n = 282) was screened against the 118 target PAs and PANOs to demonstrate the applicability of the analytical platform and investigate the profile of different food matrices. The collected samples represent food matrices susceptible to the contamination of PAs and relevant to consumer intake; they include honey, pollen, black and green teas, herbal infusions, and plant-based dietary supplements. Qualitative data only are discussed in this CHAPTER since the suitability of the procedure for the quantitative determination of the analytes in the studied matrices will be discussed in CHAPTER 4. The wide-scope suspect screening method was applied to the commercial samples to interrogate them regarding the presence of PAs and PANOs other than those already characterized from PA-producing plants. This allowed to detect four additional PAs: helioamplexine and two isomers of echimidine in honey samples, and acetylseneciphylline N-oxide in a dietary supplement, which were added to the HRMS/MS spectral library and to the high-throughput screening and identification method. The qualitative analysis of the samples revealed the presence of 60 PAs/PANOs in 59% of the analyzed samples (**Table 3.5**); among these, 21 PAs/PANOs were listed in the Regulation (EU) 2020/2040 (echimidine, europine, heliotrine, intermedine, lasiocarpine, lycopsamine, retrorsine, senecionine, seneciphylline, senecivernine, their N-oxides, and senkirkine), 8 belonged to the list of 14 coeluting isomers to be monitored (echinatine, heliosupine, indicine, integerrimine, rinderine, spartioidine, usaramine, and their N-oxides) and 28 were PAs included in the HRMS/MS spectral library but not mentioned in the regulation or in the EFSA's list of relevant contaminants of plant matrices. Among the studied matrices, honey was found to be the most contaminated one as 89% of the samples tested positive to the presence of PAs. In decreasing order of contamination,

follow dietary supplements (58%), pollen (50%), herbal infusions (46%), and teas (39%).

Table 3.5. Type of PAs/PANOs and number of times they were detected in the analyzed samples.

Analyte	Honey	Pollen	Infusions	Teas	DS[a]	Total
N° of samples	**72**	**6**	**80**	**51**	**73**	**282**
N° of contaminated samples	**64**	**3**	**37**	**20**	**42**	**166**
Echimidine	53	3	0		4	60
Echinatine/rinderine	25		3	9	20	57
Echimidine isomer 2	38	3	0		1	42
Intermedine	29	3	2		8	42
Lycopsamine	24	2	6	1	8	41
Echimidine isomer 1	35	2	0		1	38
Echinatine N-oxide	3		5	12	8	28
5'-Hydroxyindicine	26	1	0			27
Senecionine N-oxide	1		14	4	8	27
Heliotrine	3		6		15	24
Europine	2		4		16	22
Echimidine N-oxide	15	3	1		1	20
Heliotrine N-oxide	1		9		9	19
Integerrimine N-oxide			6	8	5	19
Intermedine N-oxide	8	2	4	1	4	19
Lycopsamine N-oxide		2	3	8	5	18
Senecionine	3		4	1	9	17
Symphytine (sum of isomers 1 and 2)	14	2	1			17
Lasiocarpine			5		11	16
Lasiocarpine N-oxide			10		6	16
Europine N-oxide			8		6	14
Uplandicine	11		0			11
Heleurine N-oxide			6		4	10
7-Acetylintermedine	8	1	0			9
Amabiline	8		0		1	9
Retrorsine	3		2		4	9
Rinderine N-oxide			4	2	3	9
Seneciphylline N-oxide			6	1	2	9
Senkirkine	2		3		4	9
7-Acetyllycopsamine	6	1	0		1	8
Helioamplexine	7		0		1	8
Retrorsine N-oxide			2	6		8
Senecivernine	2		0	1	5	8
Seneciphylline	2		0		5	7
Senecivernine N-oxide			3	1	3	7
Thesinine rhamnoside			5		2	7
Supinine	4		0		1	5
Spartioidine	2		0		2	4
Thesinine-glucoside				1	3	4
7-Tigloyleuropine N-oxide			2		1	3
Echihumiline N-oxide	1	2	0			3
Heliosupine			1		2	3

Analyte	Honey	Pollen	Infusions	Teas	DS[a]	Total
N° of samples	**72**	**6**	**80**	**51**	**73**	**282**
N° of contaminated samples	**64**	**3**	**37**	**20**	**42**	**166**
Heliosupine N-oxide			3			3
Monocrotaline N-oxide		2			1	3
Thesinine				1	2	3
Tigloyleuropine			1		2	3
Amabiline N-oxide					2	2
Cynaustraline			1		1	2
Heleurine					2	2
Helioamplexine N-oxide					2	2
Monocrotaline			1		1	2
Riddelliine			1		1	2
Spartioidine N-oxide			1	1		2
Supinine N-oxide			1		1	2
Viridiflorine					2	2
5'-Hydroxyechinatine	1		0			1
5'-Hydroxyintermedine/ 5'-hydroxylycopsamine		1				1
7-Acetylrinderine					1	1
Viridiflorine N-oxide					1	1
Symphytine N-oxide						0

[a] Dietary supplements

Regarding the contamination profile, **Figure 3.9** shows the PAs and PANOs detected in honey, herbal infusions, and plant-based dietary supplements, which turned out to be the matrices with the widest contamination profile (32, 34 and 49 detected analytes, respectively). In detail, the most frequently detected and identified compounds (> 20% of the contaminated samples of each matrix) were echimidine and its two isomers, echimidine N-oxide, echinatine/rinderine, 5-hydroxyindicine, intermedine, lycopsamine, and symphytines for honey; europine N-oxide, heliotrine N-oxide, lasiocarpine N-oxide, and senecionine N-oxide for herbal infusions; and echinatine/rinderine, europine, heliotrine, heliotrine N-oxide, lasiocarpine, and senecionine for dietary supplements. The qualitative data on the distribution of PAs indicated that the PAs and PANOs of the Regulation (EU) 2020/2040 contribute to almost the total content for herbal infusions (86%) and dietary supplements (83%). On the other hand, 47% of the PAs detected in honey were not included in the lists of relevant PAs to be monitored; echimidine isomer 1 (55%) and 2 (59%), 5-

hydroxyindicine (41%) and the sum of symphytines 1 and 2 (22%) were the most prevalent.

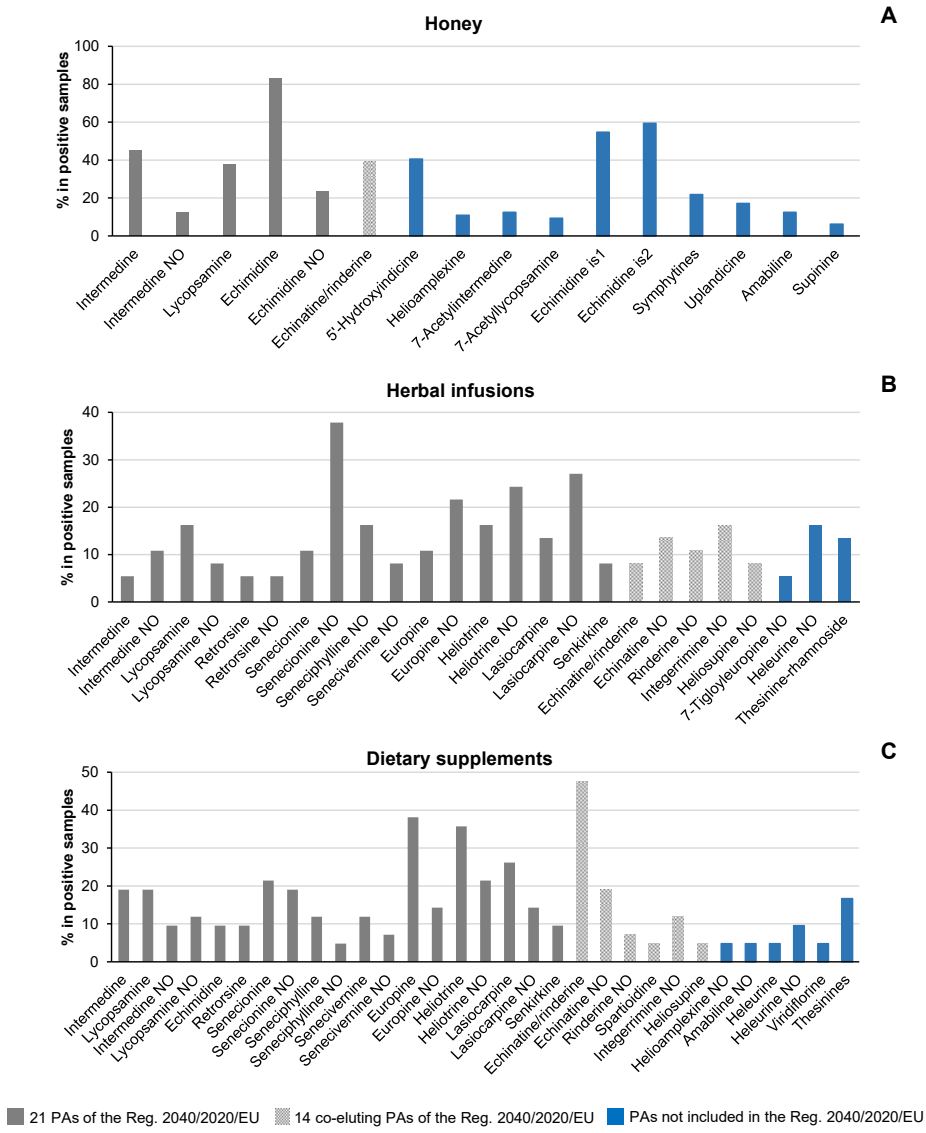


Figure 3.9. Contamination profiles of honey (A), herbal infusions (B), and dietary supplements (C) (the percentage on each bar represents the prevalence of each PA/PANO in positive samples; only PAs present in more than 5 % of the positive samples are shown).

3.4 Conclusions

The present study proposes an analytical platform for the rapid and automated detection of pyrrolizidine alkaloids in food matrices with high risk of contamination. It consists of an easy and cheap sample preparation followed by a PA-tailored UHPLC-HRMS/MS analysis, which combined with the identification strategy and a post-acquisition data evaluation allow to detect, identify, and characterize a wide range of compounds at the required levels. This analytical platform offers the possibility to interrogate the samples on the presence of 118 target PAs and PANOs and identify additional unreported analogues. The complementary mode of operation of the wide-scope suspect screening method and the high-throughput target screening and identification method makes the procedure versatile and state-of-the-art. The HRMS/MS spectral library can be continuously implemented with newly identified compounds according to the proposed strategy. Furthermore, the possibility of adding further molecular masses to the inclusion list of the Full MS/dd-MS^2 acquisition method, each time a PA is identified, allows to considerably broaden the identification range since each molecular mass can identify multiple structural isomers. Finally, the non-dependence of the platform on the purchase of reference standards not only lowers the cost of the procedure but also solves the problem of the lack of reference standards of these toxins.

CHAPTER 4

Target screening method for the quantitative determination of 118 pyrrolizidine alkaloids in dietary supplements, herbal infusions, honey and teas by liquid chromatography coupled to quadrupole Orbitrap mass spectrometry

4.1 Introduction

The occurrence of PAs and PANOs in food has recently become an emerging food safety issue. Numerous scientific reports have revealed a high incidence of PAs contamination in foods and the number of alerts on the RASFF portal has notably increased in recent years (Casado et al., 2022a). PAs can be introduced into the food chain from various contamination routes. Apart from the direct consumption of PA-producing plants, the major dietary sources of contamination seem to be plant-based products containing PA-producing plants, which grow in the field as weeds and accidentally contaminate the crops (Casado et al., 2022a; EFSA, 2016; Schrenk et al., 2020). Furthermore, the collection of nectar and pollen from PA-containing plants by bees can contaminate beehive products such as honey (Brugnerotto et al., 2021).

PAs are considered among the most widespread and dangerous phytotoxins capable of causing liver damage. In fact, 1,2-Unsaturated PAs/PANOs exhibit a strong hepatotoxic, genotoxic, tumorigenic, and neurotoxic activity. Their intake can lead to severe cases of hepatotoxicity (acute toxicity) or to slowly progress to chronic diseases (Dusemund et al., 2018; EFSA CONTAM Panel, 2011; Schrenk et al., 2020). For this reason, EFSA recommended a $BMDL_{10}$ of 237 $\mu g\ kg^{-1}$ body weight per day to estimate the exposure dose for humans and assess the carcinogenic risks of 1,2-unsaturated PAs (EFSA CONTAM Panel, 2011; EFSA 2016; EFSA CONTAM Panel, 2017). EFSA scientific reports concluded that there is a possible human health concern related to chronic cumulative exposure to PA-contaminated food products. The main foods contributing to the human exposure to PAs are teas and herbal infusions, but also pollen and herbal dietary supplements can significantly contribute, although the lack of sufficient occurrence data (EFSA, 2016; EFSA CONTAM Panel 2017). Based on EFSA outcomes, the European Commission has recently set maximum levels (MLs)

of PAs and PANOs as sum in certain foodstuffs (European Commission, 2020). MLs were set for 21 compounds, belonging to three widespread groups of PAs, with the highest toxic potential (Casado et al., 2022a): heliotrine-type, lycopsamine-type, and senecionine-type. The list of PAs of the Regulation (EU) 2020/2040 also includes 14 co-eluting isomers of the 21 compounds, which should be monitored if the chromatographic method allows it. Moreover, the regulation recommends including in the sum other PAs which can be identified with the method of analysis used.

According to the legislation, highly sensitive analytical methods should be used to monitor these contaminants in food. Currently employed methods are based on target LC-MS/MS analyses and require the availability of reference standards (Casado et al., 2022a; Ma et al., 2018; Mulder et al., 2018; Picron et al., 2018). An additional issue is that PAs show a striking structural variety (more than 600 compounds are known) (Moreira et al., 2018; Schramm et al., 2019) but only a few are available as reference standards. Therefore, monitoring programs should be extended to PAs other than those included in the legislation, which can potentially contaminate foods and have the same toxic potential (Casado et al., 2022a; Louisse et al., 2022). Thus, novel analytical approaches are needed to broaden the knowledge about the distribution of these contaminants in foods and identify additional compounds, which are not yet included in the list of relevant PAs to be monitored.

In CHAPTER 3, the development of a HRMS-based analytical platform for the screening and identification of 118 PAs and PANOs in different food matrices was discussed. In this CHAPTER, an extension of the validation studies for quantitative purposes and the application of the validated procedure to the analysis of a large number of samples are discussed. The aim was to provide occurrence data regarding the contamination of high-risk samples of different food matrices.

The analytical procedure was validated according to the European guidelines for 28 reference standards, which include 21 regulated PAs, in five food matrices, which are honey, herbal infusions, dietary supplements, black and green teas. After conducting the validation studies, it was applied to the analysis of 281 commercial samples of the abovementioned matrices, covering four food categories included in the Regulation (EU) 2020/2040 (8.4.1, 8.4.2, 8.4.3, and 8.4.6). The accurate quantitative determination of the 28 reference standards was achieved through a matrix-matched calibration or standard addition approach while the levels of the remaining target analytes were estimated by linking them to a structurally related reference standard.

4.2 Materials and methods

4.2.1 Chemicals and standards

See CHAPTER 2, paragraph 2.2.1

4.2.2 Samples

A total number of 281 samples were collected between 2019 and 2021 from different supermarkets, herbalists, pharmacies, and online stores between the Italian and Belgian market. Herbal infusions of mixed plants (n = 60, Foodstuffs at 8.4.1), herbal infusions of rooibos, anise, lemon balm, chamomile, thyme, peppermint, lemon verbena and mixtures (n = 25, Foodstuffs at 8.4.2), teas of *Camellia sinensis* and flavoured teas (n = 51, Foodstuffs at 8.4.3), and plant-based dietary supplements (n = 73, Foodstuffs at 8.4.6), including 44 formulated as solid forms, 21 as infusions, and 8 as syrups or liquid forms, were collected. Among the 8.4.1 samples, three were infusions of PA-producing plants (*Borago officinalis*, *Symphytum officinale*, and *Tussilago farfara*). In addition to the regulated food matrices, 72 collected samples of honey were also analyzed.

4.2.3 Sample preparation

Samples of honey, herbal infusions, black and green teas, and plant-based dietary supplements (various formulations: capsules, tablets, syrups, and infusions) were pre-treated and extracted with the sample preparation procedure previously developed (see CHAPTER 2, paragraphs 2.2.4 and 2.2.5). The dried residues of SALLE extracts were redissolved with an appropriate volume of H_2O/MeOH 7:3 v/v: 125 µL for honey, 250 µL for solid forms of plant-based dietary supplements, and 200 µL for herbal infusions and teas.

4.2.4 UHPLC-HRMS/MS analysis

The UHPLC-HRMS/MS analysis was performed using the instrumental method previously developed (see CHAPTER 3, paragraph 3.2.4).

4.2.5 Data processing and quantitative determination

Data processing was performed using TraceFinder software (Version 5.1, ThermoFisher Scientific). A high-throughput processing method was developed using the "Target screening method" workflow of the software. A compound database of 118 target PAs (**Table 4.1**) was created in the master method by uploading into the software a csv file generated from an in-house HRMS spectral library of the target PAs, which was built using mzVault software (ThermoFisher Scientific, version 2.3). The csv file contained all the information to detect and identify the target PAs (retention time, molecular formula, precursor ion, five most abundant product ions and relative ion ratios). The detection parameters were set as follows: a single-detected detection type within a time range of 60 sec, a response threshold (peak area) of 10e4, a mass tolerance of ± 5 ppm, at least three product ions required for the identification, and a library match score higher than 70%. The latter was possible by uploading the HRMS spectral library into the "Library selection" section of the software configuration. The extracted ion chromatograms of the target compounds (precursor ion, $[M+H]^+$), with a mass selection window of 5 ppm, were used for the quantitation and semi-quantitation of the target compounds.

The parameters for the quantitative determination of the 28 reference standards were set as follows: external standard mode, linear calibration curve and no weighting factor applied. The semi-quantitative determination of the remaining target PAs was achieved by indicating one of the 28 reference standards as linked compound; the latter

was chosen based on structural similarity, giving priority to the belonging of the same type of necine base first and the same type of esterification then (**Table 4.1**).

After processing of raw data, the software flagged a target compound as "found" (green flag) every time a precursor ion was detected with a mass tolerance of ± 5 ppm at the set retention time (± 0.2 min), together with the typical set of product ions and a library match score > 70%. Afterwards, the detected compounds were quantified by the software quantification algorithm, which directly interpolated the area of each peak in the matrix-matched calibration curve of the relative matrix, injected within the same batch of the same day. The concentration levels of PAs and PANOs of dietary supplements were estimated using solvent-based calibration curves or the standard addition method. Different conversion factors (0.25 for honey, 7.5 for herbal infusions and teas, and 2.5 for solid forms of dietary supplements) were used to convert the quantitative data from μg L^{-1} of SALLE extracts to μg kg^{-1} of each matrix. When calculating the total content of the samples, the analyte concentrations below the LOD (Limit of detection) were considered as 0.0 μg kg^{-1} while the concentrations between LOD and LOQ (Limit of quantification) were summed as 0.5 times the LOQ value.

Table 4.1. UHPLC-HRMS/MS information of the 118 target PAs and PANOs.

Analyte	Necine base[a]	Esterification type[b]	Molecular formula	$[M+H]^+$ (*m/z*)[c]	Rt (min)	IL[d]	Linked compound
7-Tigloylretronecine N-oxide	R	m	$C_{13}H_{19}NO_4$	254.1387	7.2	2	Intermedine N-oxide
7-Angeloylretronecine N-oxide	R	m	$C_{13}H_{19}NO_4$	254.1387	7.4	2	Intermedine N-oxide
9-Tigloylretronecine N-oxide	R	m	$C_{13}H_{19}NO_4$	254.1387	10.4	2	Intermedine N-oxide
9-Angeloylretronecine N-oxide	R	m	$C_{13}H_{19}NO_4$	254.1387	10.7	2	Intermedine N-oxide
Supinine	S	-	$C_{15}H_{25}NO_4$	284.1856	8.1	2	Intermedine
Amabiline	S	-	$C_{15}H_{25}NO_4$	284.1856	8.4	2	Intermedine
Spilanthine	T	-	$C_{15}H_{25}NO_4$	284.1856	9.2	2	Intermedine
Viridiflorine	T	-	$C_{15}H_{27}NO_4$	286.2013	7.9	2	Intermedine
Cynaustraline	T	-	$C_{15}H_{27}NO_4$	286.2013	8.2	2	Intermedine
Thesinine	T	-	$C_{17}H_{21}NO_3$	288.1594	13.7	3	Intermedine
Heleurine	S	-	$C_{16}H_{27}NO_4$	298.2013	12.0	2	Heliotrine
Intermedine	R	m	$C_{15}H_{25}NO_5$	300.1805	5.3	1	-
Indicine	R	m	$C_{15}H_{25}NO_5$	300.1805	5.5	1	-
Lycopsamine	R	m	$C_{15}H_{25}NO_5$	300.1805	5.6	1	-
Rinderine	H	m	$C_{15}H_{25}NO_5$	300.1805	5.9	2	Intermedine
Echinatine	H	m	$C_{15}H_{25}NO_5$	300.1805	6.0	2	Intermedine
Supinine N-oxide	S	-	$C_{15}H_{25}NO_5$	300.1805	8.7	2	Intermedine N-oxide
Amabiline N-oxide	S	-	$C_{15}H_{25}NO_5$	300.1805	9.1	3	Intermedine N-oxide
Curassavine	T	-	$C_{16}H_{29}NO_4$	300.2169	11.9	2	Intermedine
Dihydroechinatine/Dihydrorinderine	P	m	$C_{15}H_{27}NO_5$	302.1962	6.5	3	Intermedine
Dihydrointermedine	P	m	$C_{15}H_{27}NO_5$	302.1962	6.6	3	Intermedine
Dihydrolycopsamine	P	m	$C_{15}H_{27}NO_5$	302.1962	6.7	3	Intermedine
Viridiflorine N-oxide	T	-	$C_{15}H_{27}NO_5$	302.1962	8.5	3	Intermedine N-oxide
Helioamplexine	R	m	$C_{16}H_{27}NO_5$	314.1962	7.4	2	Intermedine
Heliotrine	H	m	$C_{16}H_{27}NO_5$	314.1962	9.2	1	-
Heleurine N-oxide	S	-	$C_{16}H_{27}NO_5$	314.1962	12.4	2	Heliotrine N-oxide
5'-Hydroxyindicine	R	m	$C_{15}H_{25}NO_6$	316.1755	1.9	2	Intermedine
5'-Hydroxyintermedine/5'-Hydroxylycopsamine	R	m	$C_{15}H_{25}NO_6$	316.1755	3.0	3	Intermedine
5'-Hydroxyechinatine/5'-Hydroxyrinderine	H	m	$C_{15}H_{25}NO_6$	316.1755	3.5	2	Intermedine
Rinderine N-oxide	H	m	$C_{15}H_{25}NO_6$	316.1755	6.4	2	Intermedine N-oxide
Echinatine N-oxide	H	m	$C_{15}H_{25}NO_6$	316.1755	6.6	2	Intermedine N-oxide
Intermedine N-oxide	R	m	$C_{15}H_{25}NO_6$	316.1755	6.9	1	-
Indicine N-oxide	R	m	$C_{15}H_{25}NO_6$	316.1755	7.2	1	-
Lycopsamine N-oxide	R	m	$C_{15}H_{25}NO_6$	316.1755	7.2	1	-
Dihydrointermedine N-oxide	P	m	$C_{15}H_{27}NO_6$	318.1911	7.6	3	Intermedine N-oxide
Dihydrolycopsamine N-oxide	P	m	$C_{15}H_{27}NO_6$	318.1911	7.7	3	Intermedine N-oxide
Dihydrorinderine N-oxide	P	m	$C_{15}H_{27}NO_6$	318.1911	8.0	3	Intermedine N-oxide
Dihydroechinatine N-oxide	P	m	$C_{15}H_{27}NO_6$	318.1911	8.1	3	Intermedine N-oxide
Monocrotaline	R	c	$C_{16}H_{23}NO_6$	326.1598	2.7	1	-

Analyte	Necine base[a]	Esterification type[b]	Molecular formula	$[M+H]^+$ (*m/z*)[c]	Rt (min)	IL[d]	Linked compound
Europine	H	m	$C_{16}H_{27}NO_6$	330.1911	5.9	1	-
Helioamplexine N-oxide	R	m	$C_{16}H_{27}NO_6$	330.1911	9.2	3	Intermedine N-oxide
Heliotrine N-oxide	H	m	$C_{16}H_{27}NO_6$	330.1911	10.2	1	-
5'-Hydroxyechinatine N-oxide/5'-Hydroxyrinderine N-oxide	H	m	$C_{15}H_{25}NO_7$	332.1704	1.9	2	Intermedine N-oxide
5'-Hydroxyintermedine N-oxide/5'-Hydroxylycopsamine N-oxide	R	m	$C_{15}H_{25}NO_7$	332.1704	3.0	2	Intermedine N-oxide
Spartioidine	R	c	$C_{18}H_{23}NO_5$	334.1649	9.8	2	Seneciphylline
Seneciphylline	R	c	$C_{18}H_{23}NO_5$	334.1649	10.1	1	-
Senecivernine	R	c	$C_{18}H_{25}NO_5$	336.1805	12.1	1	-
Senecionine	R	c	$C_{18}H_{25}NO_5$	336.1805	12.3	1	-
Monocrotaline N-oxide	R	c	$C_{16}H_{23}NO_7$	342.1547	5.2	1	-
3'-Acetylintermedine	R	m	$C_{17}H_{27}NO_6$	342.1911	8.8	2	Intermedine
3'-Acetylrinderine	H	m	$C_{17}H_{27}NO_6$	342.1911	8.9	2	Intermedine
7-Acetylrinderine	H	d	$C_{17}H_{27}NO_6$	342.1911	9.6	3	Lasiocarpine
7-Acetylechinatine	H	d	$C_{17}H_{27}NO_6$	342.1911	9.7	3	Lasiocarpine
3'-Acetyllycopsamine	R	m	$C_{17}H_{27}NO_6$	342.1911	9.8	2	Intermedine
3'-Acetylechinatine	H	m	$C_{17}H_{27}NO_6$	342.1911	10.2	2	Intermedine
7-Acetylintermedine	R	d	$C_{17}H_{27}NO_6$	342.1911	10.5	2	Echimidine
7-Acetyllycopsamine	R	d	$C_{17}H_{27}NO_6$	342.1911	10.7	2	Echimidine
Europine N-oxide	H	m	$C_{16}H_{27}NO_7$	346.1860	6.5	1	-
Erucifoline	R	c	$C_{18}H_{23}NO_6$	350.1598	5.5	1	-
Riddelliine	R	c	$C_{18}H_{23}NO_6$	350.1598	7.1	2	Erucifoline
Spartioidine N-oxide	R	c	$C_{18}H_{23}NO_6$	350.1598	11.0	2	Seneciphylline N-oxide
Seneciphylline N-oxide	R	c	$C_{18}H_{23}NO_6$	350.1598	11.2	1	-
Retrorsine	R	c	$C_{18}H_{25}NO_6$	352.1755	8.8	1	-
Jacobine	R	c	$C_{18}H_{25}NO_6$	352.1755	10.4	1	-
Senecivernine N-oxide	R	c	$C_{18}H_{25}NO_6$	352.1755	12.6	1	-
Integerrimine N-oxide	R	c	$C_{18}H_{25}NO_6$	352.1755	12.7	2	Senecionine N-oxide
Senecionine N-oxide	R	c	$C_{18}H_{25}NO_6$	352.1755	12.8	1	-
Trichodesmine	R	c	$C_{18}H_{27}NO_6$	354.1911	8.1	1	-
Uplandicine	R	d	$C_{17}H_{27}NO_7$	358.1860	6.2	2	Echimidine
3'-Acetylrinderine N-oxide	H	m	$C_{17}H_{27}NO_7$	358.1860	9.9	2	Intermedine N-oxide
7-Acetylintermedine N-oxide	R	d	$C_{17}H_{27}NO_7$	358.1860	10.7	2	Echimidine N-oxide
3'-Acetylintermedine N-oxide	R	m	$C_{17}H_{27}NO_7$	358.1860	10.8	3	Intermedine N-oxide
3'-Acetylechinatine N-oxide	H	m	$C_{17}H_{27}NO_7$	358.1860	10.9	3	Intermedine N-oxide
7-Acetyllycopsamine N-oxide	R	d	$C_{17}H_{27}NO_7$	358.1860	11.0	2	Echimidine N-oxide
7-Acetylrinderine N-oxide	H	d	$C_{17}H_{27}NO_7$	358.1860	11.3	3	Lasiocarpine N-oxide
7-Acetylechinatine N-oxide	H	d	$C_{17}H_{27}NO_7$	358.1860	11.4	3	Lasiocarpine N-oxide
3'-Acetyllycopsamine N-oxide	R	m	$C_{17}H_{27}NO_7$	358.1860	11.7	2	Intermedine N-oxide
Erucifoline N-oxide	R	c	$C_{18}H_{23}NO_7$	366.1547	6.2	1	-
Riddelliine N-oxide	R	c	$C_{18}H_{23}NO_7$	366.1547	7.7	2	Erucifoline N-oxide
Neosenkirkine	O	c	$C_{19}H_{27}NO_6$	366.1911	12.9	2	Senkirkine
Senkirkine	O	c	$C_{19}H_{27}NO_6$	366.1911	13.3	1	-

Analyte	Necine base[a]	Esterification type[b]	Molecular formula	$[M+H]^+$ (*m/z*)[c]	Rt (min)	IL[d]	Linked compound
Jacobine N-oxide	R	c	$C_{18}H_{25}NO_7$	368.1704	7.3	1	-
Retrorsine N-oxide	R	c	$C_{18}H_{25}NO_7$	368.1704	9.4	1	-
Uplandicine N-oxide	R	d	$C_{17}H_{27}NO_8$	374.1809	6.2	2	Echimidine N-oxide
Acetylseneciphylline N-oxide	R	c	$C_{20}H_{25}NO_6$	376.1755	16.0	2	Seneciphylline N-oxide
Symphytine isomer 1	R	d	$C_{20}H_{31}NO_6$	382.2224	15.4	2	Echimidine
Symphytine isomer 2	R	d	$C_{20}H_{31}NO_6$	382.2224	15.6	2	Echimidine
Echiumine	R	d	$C_{20}H_{31}NO_6$	382.2224	15.7	2	Echimidine
5'-Acetyleuropine N-oxide	H	m	$C_{18}H_{29}NO_8$	388.1966	11.3	2	Europine N-oxide
7-Angeloylheliotrine	H	d	$C_{21}H_{33}NO_6$	396.2381	16.4	3	Lasiocarpine
Asperumine	H	d	$C_{20}H_{31}NO_7$	398.2173	13.1	2	Lasiocarpine
Echimidine isomer 1	R	d	$C_{20}H_{31}NO_7$	398.2173	13.2	3	Echimidine
Heliosupine	H	d	$C_{20}H_{31}NO_7$	398.2173	13.3	2	Lasiocarpine
Echimidine isomer 2	R	d	$C_{20}H_{31}NO_7$	398.2173	13.4	3	Echimidine
Echimidine	R	d	$C_{20}H_{31}NO_7$	398.2173	13.4	1	-
Symphytine N-oxide	R	d	$C_{20}H_{31}NO_7$	398.2173	15.5	2	Echimidine N-oxide
Echiumine N-oxide	R	d	$C_{20}H_{31}NO_7$	398.2173	15.7	2	Echimidine N-oxide
Canescine/canescenine	H	d	$C_{20}H_{33}NO_7$	400.2330	12.4	3	Lasiocarpine
7-Tigloyleuropine	H	d	$C_{21}H_{33}NO_7$	412.2330	14.9	2	Lasiocarpine
Lasiocarpine	H	d	$C_{21}H_{33}NO_7$	412.2330	15.1	1	-
7-Angeloylheliotrine N-oxide	H	d	$C_{21}H_{33}NO_7$	412.2330	16.7	2	Lasiocarpine N-oxide
Echihumiline N-oxide	R	d	$C_{20}H_{31}NO_8$	414.2122	13.2	3	Echimidine N-oxide
Echimidine N-oxide	R	d	$C_{20}H_{31}NO_8$	414.2122	13.4	1	-
Vulgarine N-oxide	R	m	$C_{20}H_{31}NO_8$	414.2122	13.5	3	Intermedine N-oxide
Asperumine N-oxide	H	d	$C_{20}H_{31}NO_8$	414.2122	13.7	3	Lasiocarpine N-oxide
Heliosupine N-oxide	H	d	$C_{20}H_{31}NO_8$	414.2122	14.1	2	Lasiocarpine N-oxide
Lithosenine	R	d	$C_{20}H_{33}NO_8$	416.2279	8.3	3	Echimidine
Canescine N-oxide/canescenine N-oxide	H	d	$C_{20}H_{33}NO_8$	416.2279	12.7	3	Lasiocarpine N-oxide
7-Tigloyleuropine N-oxide	H	d	$C_{21}H_{33}NO_8$	428.2279	15.6	2	Lasiocarpine N-oxide
Lasiocarpine N-oxide	H	d	$C_{21}H_{33}NO_8$	428.2279	15.8	1	-
Lithosenine N-oxide	R	d	$C_{20}H_{33}NO_9$	432.2228	8.7	3	Echimidine N-oxide
Thesinine-4'-ramnoside	T	-	$C_{23}H_{31}NO_7$	434.2173	13.6	3	Intermedine
3'-Acetylheliosupine	H	d	$C_{22}H_{33}NO_8$	440.2279	15.0	2	Lasiocarpine
3'-Acetylechiumine N-oxide	R	d	$C_{22}H_{33}NO_8$	440.2279	16.7	3	Echimidine N-oxide
Thesinine-4'-glucoside	T	-	$C_{23}H_{31}NO_8$	450.2122	11.1	3	Intermedine
5'-Acetyllasiocarpine	H	d	$C_{23}H_{35}NO_8$	454.2435	16.4	2	Lasiocarpine
3'-Acetylheliosupine N-oxide	H	d	$C_{22}H_{33}NO_9$	456.2228	15.6	2	Lasiocarpine N-oxide
5'-Acetyllasiocarpine N-oxide	H	d	$C_{23}H_{35}NO_9$	470.2385	16.7	2	Lasiocarpine N-oxide

[a] Type of necine base: Retronecine (R) heliotridine (H), otonecine (O) trachelanthamidine (T), platynecine (P), and supinidine (S)

[b] Type of esterification: monoester (m), open-chained diester (d), and cyclic diester (c)

[c] Exact mass

[d] Identification level (IL) according to the Metabolomics Standards Initiative

4.2.6 Quality control

The stability of the reference standards in the SALLE extracts and under the sample preparation conditions was checked by UHPLC-HRMS analysis. PAs and PANOs resulted quite stable, which allowed to process the samples up to three days prior to the injection. Sensitivity tests were performed before each batch of samples by analyzing in triplicate a solution of analytes at the concentration level of 2 μg L^{-1}, prepared in solvent. To ensure total absence of carryover, a sample of solvent (H_2O/MeOH 7:3 v/v) was injected after each calibration curve and after every ten runs. Additionally, the 10 μg L^{-1} level of the solvent calibration curve was injected every ten samples of a batch to ensure the stability of the detector response. A tolerated deviation of ± 15% from the theoretical concentration of the calculated values was required for the batch of samples to be considered qualified for the analysis.

4.2.7 Targeted method validation

The performances of the proposed method were assessed according to the quantitative criteria established by the European analytical guidelines (Magnusson & Örnemark, 2014; Pihlstrom et al., 2018). The method validation was carried out for 28 reference standards in five food matrices by studying limits of detection (LODs) and quantification (LOQs), matrix effect (ME), linearity, extraction efficiency (EE) and intra-day repeatability (expressed as relative standard deviation, RSD). The validation experiments were performed on blank samples, previously identified through analysis, for honey and black and green teas. A representative sample of herbal infusion was prepared for the validation studies by mixing the most representative herbs of the collected samples: chamomile (28%), fennel (56%), melissa (32%), mint (47%) and licorice (44%). Blank samples of these herbs were selected after processing them with the target screening method. It was not possible to select or prepare a representative

sample of herbal food supplement to be used for the validation experiments due to the high variability of their composition. Thus, ten blank samples of herbal dietary supplements of different composition were used to study the following validation parameters: LODs, LOQs, EEs, and intra-day repeatability (Relative standard deviation, RSD).

LODs and LOQs of the 28 reference standards in the investigated matrices were calculated using the calibration-based approach (Wenzl et al., 2016). In detail, blank samples of each matrix were fortified at concentration levels close to the expected LOD (range 0-1.5 μg L^{-1} of the SALLE extracts) and processed in duplicate (independent replicates). For some analyte/matrix combinations the experiments were repeated at higher concentrations.

The matrix-matched calibration curves were prepared by spiking blank SALLE extracts of honey, herbal infusions, black and green teas at eight concentration levels covering the range 1-100 μg L^{-1} (corresponding to 0.25-25 μg kg^{-1} for honey, and 7.5-750 μg kg^{-1} for teas and infusions). Each concentration level was injected in triplicate to evaluate the linearity of each curve with the analysis of variance (ANOVA). A linear model was found appropriate over the tested concentration range ($R^2 \geq 0.999$) for all the analytes in the studied matrices. The ME was evaluated by comparing the slope of the matrix-matched calibration curves (post-spiked samples) with that of the solvent calibration curves (H_2O/MeOH 7:3 v/v) of the reference standards in the concentration range 1-100 μg L^{-1} of the SALLE extracts. MEs were defined as ratio between the slopes of matrix-matched and solvent-based calibration curves.

The accuracy of the method, expressed as EE, was calculated by spiking the reference standards before (pre-spiked samples) and after (post-spiked samples) the sample preparation procedure at two concentration levels: a low level close to the LOQ

of 2 μg L^{-1} of the SALLE extracts (corresponding to 0.5, 5 and 15 μg kg^{-1} for honey, dietary supplements and teas/infusions, respectively), and a high level of 100 μg L^{-1} of the SALLE extracts (corresponding to 25, 250 and 750 μg kg^{-1} for honey, dietary supplements and teas/infusions, respectively). The EE at low level of ReNO in green teas was evaluated with the spike level of 37.5 μg kg^{-1} (5 μg L^{-1} of the SALLE extract). Experiments were conducted in triplicate. Intra-day repeatability was obtained by the same set of accuracy experiments, evaluating the responses of the pre-spiked samples at two concentration levels. The precision was expressed as RSD of the PA/PANO contents (three replicates).

4.3 Results and discussion

The aim of this study was to validate a previously developed analytical procedure for the screening and identification of PAs and PANOs in different food matrices to make the method capable of performing the quantitative determination of a high number of analytes and collect occurrence data from a large number of samples.

The quantitative determination of PAs and PANOs in commercial samples was performed applying the sample procedure and UHPLC-HRMS/MS method described in CHAPTER 2. In particular, the quantification and semi-quantification of the analytes was performed by extracting the accurate masses of precursor ions in full MS traces (< 5 ppm) in the range *m/z* 250-500. Regarding the sample preparation, the reconstitution volume of the SALLE extracts was chosen according to the required sensitivity and regulated MLs, by considering the matrix effects. For this purpose, the SALLE extracts of the five investigated matrices were dissolved in different volumes and spiked at 10 μg L^{-1} of each PA and PANO.

4.3.1 Quantitative validation studies

The analytical performances were assessed for 28 commercially available reference standards (including 21 regulated compounds) in five food matrices: herbal dietary supplements, herbal infusions, honey, black and green teas. The analytical procedure was validated in terms of sensitivity (LODs and LOQs), matrix effect (ME), linearity, extraction efficiency (EE) and intra-day repeatability (RSD).

4.3.1.1 Sensitivity (LOD and LOQ)

LOD (the lowest analyte concentration that can be detected at a specified confidence level) and LOQ (the lowest analytes concentration that can be quantified with a reasonable level of accuracy) were determined using the calibration-based approach (Wenzl et al., 2016). Calculated values of LOQ for selected PAs in each

studied matrix are shown in the **Table 4.2**; the achieved LOQs were very low, ranging from 0.1 to 2.1 μg kg^{-1} in solid matrices (honey and dietary supplements) and from 1 to 12 μg kg^{-1} in infusions and teas. Retrorsine N-oxide only showed a higher LOQ value in green tea. The overall LOQs were within the recommended quantification limits set by EFSA (10 μg kg^{-1} for PA sum, and 0.1-5 μg kg^{-1} for individual content of PAs and PANOs) (EFSA CONTAN Panel, 2011), and showed to be lower than those established by the regulation, which demonstrate the suitability of the method for the determination of PAs and PANOs at trace levels in all the studied matrices.

Table 4.2. Limits of quantification (LOQs), extraction efficiencies (EEs) and precision (RDS) of the target PAs in the studied food matrices.

Analyte	Honey			Black tea			Green tea			Herbal infusion			Dietary supplements		
	EE Low[a] (RSD)	EE High[b] (RSD)	LOQ (µg kg^{-1})	EE Low[a] (RSD)	EE High[b] (RSD)	LOQ (µg kg^{-1})	EE Low[a] (RSD)	EE High[b] (RSD)	LOQ (µg kg^{-1})	EE Low[a] (RSD)	EE High[b] (RSD)	LOQ (µg kg^{-1})	EE Low[a] (RSD)	EE High[b] (RSD)	LOQ (µg kg^{-1})
Em	72.6 (5.4)	85.6 (2.7)	0.2	87.3 (8.1)	95.1 (2.3)	5.4	82.1 (11.6)	98.7 (7.1)	3.3	75.4 (16.8)	84.8 (8.4)	2.4	82.9 (4.0)	103.0 (1.9)	1.1
EmNO	89.2 (3.8)	95.7 (2.3)	0.1	102.6 (4.9)	101.2 (3.0)	3.6	90.3 (6.0)	100.0 (7.9)	11.7	104.1 (7.0)	113.5 (5.8)	4.1	97.4 (5.2)	111.2 (0.1)	0.9
Er	88.7 (13.0)	99.6 (7.3)	0.2	80.1 (13.7)	99.2 (2.1)	5.6	83.7 (7.4)	97.1 (10.1)	2.3	80.9 (9.8)	92.1 (7.3)	3.1	99.8 (6.3)	102.1 (5.5)	2.1
ErNO	92.7 (6.7)	94.3 (1.3)	0.2	89.0 (10.2)	95.3 (11.5)	2.8	88.1 (1.4)	101.7 (5.5)	2.3	92.2 (9.7)	95.9 (7.1)	3.3	75.6 (4.4)	92.6 (3.5)	1.2
Eu	83.8 (7.9)	103.9 (4.8)	0.2	94.2 (9.6)	97.0 (1.0)	6.0	84.0 (5.0)	97.6 (1.2)	4.6	90.1 (3.1)	95.9 (7.4)	1.9	89.0 (8.4)	96.7 (8.6)	1.3
EuNO	63.3 (14.1)	70.0 (7.1)	0.1	72.2 (6.9)	77.9 (0.3)	2.7	74.0 (6.8)	76.4 (13.6)	3.2	85.0 (12.7)	70.1 (11.3)	5.5	70.5 (4.0)	71.3 (6.0)	0.8
He	85.2 (8.3)	93.2 (4.3)	0.2	91.3 (8.9)	99.0 (4.1)	9.2	78.3 (4.5)	92.5 (4.6)	2.4	95.9 (9.3)	101.4 (1.7)	3.2	90.4 (2.9)	97.8 (0.1)	0.7
HeNO	80.5 (0.6)	98.4 (1.0)	0.2	87.2 (7.2)	90.3 (3.8)	2.0	78.5 (6.5)	81.6 (11.4)	5.7	85.8 (8.3)	88.4 (4.2)	5.1	81.9 (2.4)	91.9 (1.1)	1.3
Im	89.0 (5.1)	83.4 (0.6)	0.2	90.1 (2.2)	94.8 (2.2)	7.1	89.8 (0.1)	100.7 (9.1)	2.1	88.5 (0.4)	88.2 (1.4)	3.7	88.4 (10.0)	95.5 (1.6)	1.3
ImNO	75.0 (0.6)	78.6 (2.7)	0.2	65.0 (12.6)	77.1 (2.9)	2.8	78.3 (1.7)	81.7 (6.7)	3.4	75.7 (4.5)	82.6 (0.9)	3.3	70.4 (6.3)	72.5 (2.3)	0.7
Jb	103.7 (11.5)	93.4 (9.5)	0.2	93.3 (4.8)	96.8 (4.8)	6.4	78.2 (9.3)	90.4 (10.7)	3.3	79.3 (4.5)	97.2 (5.0)	11.1	96.7 (12.4)	102.4 (2.7)	1.1
JbNO	81.6 (4.6)	91.7 (10.4)	0.2	75.0 (10.1)	81.2 (3.3)	2.6	83.3 (0.4)	90.9 (6.5)	2.1	89.5 (4.0)	103.2 (0.8)	3.3	81.8 (6.3)	92.2 (0.6)	0.7
Lc	88.4 (4.4)	90.3 (4.1)	0.1	90.7 (6.7)	100.2 (1.2)	9.0	111.0 (2.9)	106.6 (6.6)	2.7	86.2 (7.8)	101.3 (1.4)	3.9	92.2 (5.3)	101.5 (3.8)	0.8
LcNO	90.1 (0.2)	91.7 (5.0)	0.2	89.4 (8.9)	96.3 (3.3)	1.0	83.4 (7.0)	90.8 (1.4)	3.5	86.4 (11.5)	92.5 (1.7)	6.0	95.2 (2.3)	101.9 (3.4)	1.5
Ly	83.4 (2.6)	95.1 (0.2)	0.2	88.4 (4.0)	98.0 (1.0)	5.8	89.4 (2.5)	90.2 (2.1)	5.3	80.9 (5.1)	88.5 (0.2)	2.9	88.6 (5.8)	93.3 (2.6)	1.4
LyNO	63.3 (14.7)	71.4 (5.9)	0.2	63.9 (10.0)	70.0 (3.6)	1.7	65.2 (10.4)	71.0 (10.3)	3.0	63.0 (8.1)	66.0 (3.2)	4.3	65.1 (8.4)	70.2 (14.4)	0.5
Mc	89.5 (5.3)	95.7 (6.4)	0.4	90.9 (2.9)	98.7 (3.1)	5.9	88.6 (3.9)	94.8 (5.1)	2.7	86.3 (6.1)	94.8 (2.7)	3.8	91.1 (8.0)	101.4 (2.3)	1.9
McNO	72.7 (7.8)	74.9 (13.0)	0.4	71.2 (8.3)	80.9 (3.2)	7.4	68.0 (8.0)	81.4 (8.4)	1.6	69.4 (10.4)	77.2 (3.8)	3.8	70.9 (9.4)	80.8 (6.1)	0.7
Re	98.4 (6.0)	100.0 (0.7)	0.1	94.1 (6.3)	102.1 (1.0)	4.4	99.7 (15.1)	104.2 (16.4)	3.1	66.4 (14.6)	78.6 (9.3)	4.3	90.9 (1.4)	101.9 (1.5)	1.2
ReNO	78.1 (6.4)	84.3 (1.3)	0.3	96.8 (3.1)	103.2 (4.1)	9.6	82.6 (8.2)[c]	91.4 (10.0)	23.4	101.0 (5.2)	116.9 (2.6)	4.7	93.5 (4.5)	96.1 (7.0)	1.9
Se	71.4 (9.2)	77.1 (3.8)	0.2	84.3 (1.8)	87.4 (4.3)	9.4	94.1 (1.9)	100.4 (6.7)	3.0	100.1 (5.5)	105.6 (3.0)	3.7	98.7 (4.7)	104.8 (1.4)	1.6
SeNO	93.1 (9.8)	102.0 (4.6)	0.2	93.5 (4.9)	100.4 (3.1)	6.0	80.8 (7.1)	91.1 (11.3)	2.2	98.3 (3.3)	94.6 (2.3)	5.1	87.3 (5.6)	98.1 (3.6)	1.2
Sp	80.8 (3.2)	89.4 (3.4)	0.2	91.2 (4.2)	99.4 (0.1)	9.6	80.4 (11.7)	89.3 (14.7)	3.6	82.6 (1.0)	95.2 (2.6)	2.1	97.7 (8.0)	107.9 (4.8)	1.3
SpNO	89.6 (10.1)	95.9 (5.6)	0.2	81.1 (7.3)	90.0 (4.0)	2.3	88.8 (2.9)	95.9 (8.3)	2.6	84.8 (14.0)	95.2 (4.9)	4.1	81.1 (3.7)	92.7 (0.4)	1.2
Sv	78.1 (1.8)	85.8 (4.7)	0.2	103.5 (8.6)	95.6 (0.5)	9.4	83.2 (4.0)	80.5 (9.0)	3.3	91.1 (9.0)	97.4 (2.8)	3.2	98.6 (9.9)	104.7 (4.3)	0.9
SvNO	93.5 (8.1)	100.6 (2.2)	0.2	90.5 (7.4)	100.9 (2.1)	2.6	93.7 (0.3)	101.5 (4.6)	1.9	86.8 (10.4)	93.0 (8.2)	3.9	80.8 (2.6)	98.8 (2.2)	1.0
Sk	89.8 (9.8)	104.7 (7.4)	0.2	86.4 (6.0)	97.0 (0.4)	3.6	85.3 (7.9)	102.8 (2.0)	1.5	89.2 (6.6)	94.3 (0.4)	4.3	92.5 (9.6)	101.4 (5.6)	0.9
Td	87.6 (11.4)	99.0 (6.4)	0.4	88.3 (5.8)	96.8 (1.7)	6.1	92.0 (8.2)	95.6 (7.1)	3.6	85.9 (7.7)	93.7 (2.4)	3.8	96.0 (5.1)	97.8 (0.8)	1.6

[a] Spike level of 2 µg L^{-1} of SALLE extract; [b] spike level of 100 µg L^{-1} of SALLE extract; [c] spike level of 5 µg L^{-1} of SALLE extract.

4.3.1.2 Accuracy and precision

The accuracy (expressed as EE) and precision (expressed as intra-day repeatability, RSD), were assessed at two concentration levels: low level (2 μg L^{-1}) and high level (100 μg L^{-1}). Results are shown in **Table 4.2**. EEs in the range 63-117% were obtained for the 28 reference PAs in all the studied matrices, indicating the efficiency of the proposed procedure in providing an exhaustive extraction. The lowest values of EE were observed for LyNO. RSD values below 17% were achieved, indicating the high precision of the procedure.

4.3.1.3 Matrix effect and linearity

The ME phenomenon (suppression or enhancement of the instrumental response due to the co-elution of matrix interferences) can compromise the sensitivity of the analytical method and the accuracy of the data; therefore, the validation studies must include an evaluation of the ME to establish the most suitable quantification method. The ME of the 28 reference PAs in herbal infusions, honey, black and green teas showed to be variable (61-149%) depending on the matrix type and the type of analyte; however, it resulted negligible (80-120%) for 93% of the target analytes in herbal infusions, 96% and 93% of them in black and green teas respectively, and 89% of them in honey (**Figure 4.1**). These results confirmed the efficiency of the sample preparation procedure in removing or reducing the matrix interferences from all the tested matrices, despite their complex nature. Unlike QuEChERS, the adopted sample preparation procedure consisted in an acid aqueous extraction of PAs from the studied matrices rather than an acetonitrile/water mixture. Thus, SALLE was applied to cleaner extracts, which resulted in a reduced co-extraction of matrix interferents. Moreover, this procedure does not include the addition of clean-up sorbents as part of the sample purification step, which makes it faster and less expensive.

Despite the good ME values achieved, the PAs/PANOs content of contaminated samples of herbal infusions, honey and teas were quantified on matrix-matched calibration curves to obtain more accurate data. On the other hand, the PAs/PANOs content of contaminated dietary supplements was estimated using solvent-based calibration curves due to their high variable composition.

The linearity of the solvent-based and matrix-matched calibration curves was calculated in the range 1−100 μg L^{-1} of SALLE extracts and resulted in excellent correlation coefficients ($R^2 > 0.998$ for all the 28 reference compounds).

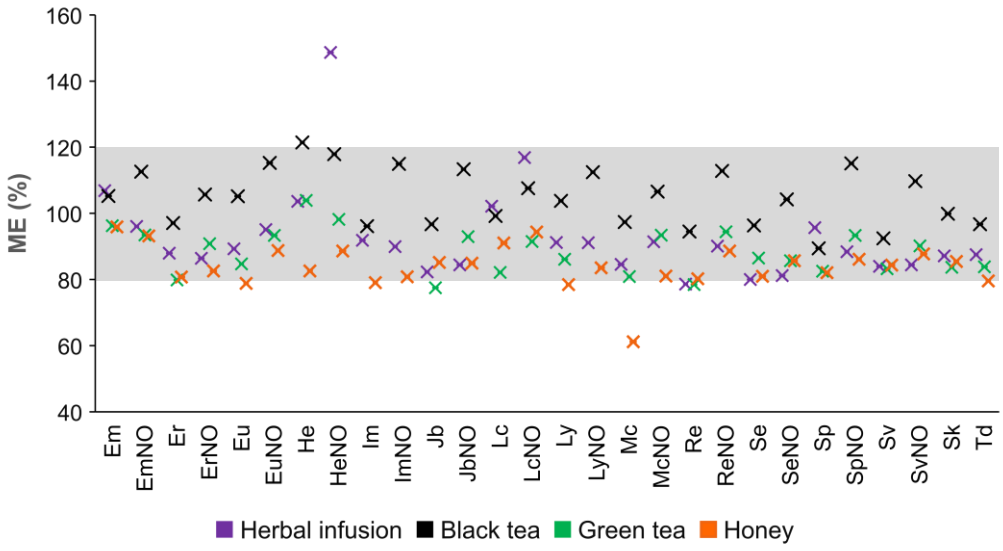


Figure 4.1. Matrix effects (MEs) of the 28 target PAs/PANOs in herbal infusions, black and green teas, and honey.

4.3.2 Analysis of commercial samples

The food matrices of this study were selected based on the data available on their contamination incidence and consumers intake, according to EFSA reports, literature studies, and current legislation. Hence, herbal dietary supplements, infusions, honey, black and green teas were selected, and a high number (n = 281) of commercial samples were collected and categorized according to the foodstuff categories of the

Regulation (EU) 2020/2040. The sampling of herbal dietary supplements and infusions was guided by the composition of the products, which means that those containing the plants most susceptible to the contamination were selected. The collected samples were subjected to the target screening of 118 PAs and PANOs, which allowed to provide an accurate quantitative determination of the 28 reference standards, including the 21 regulated analytes, and a semi-quantitative determination of the remaining target analytes. Quantitative data were expressed as concentration of each PA and PANO and total PA content, to evaluate the PA contamination profiles of the investigated matrices and to verify the compliance with the MLs of the regulation.

The contamination rate of the studied matrices as function of the total PAs content is summarized in **Figure 4.2**, where the total PAs content of the contaminated samples is divided in four sections (from LOQ to 0.5 times the ML; from 0.5 times the ML to ML; from ML to 2 times the ML; and more than 2 times the ML) based on the regulated MLs of herbal infusions (8.4.1 and 8.4.2), teas (8.4.3), and herbal dietary supplements (8.4.6) and the recommended level (RL) for the maximum daily intake of honey (Brugnerotto et al., 2021; EFSA CONTAM Panel, 2017).

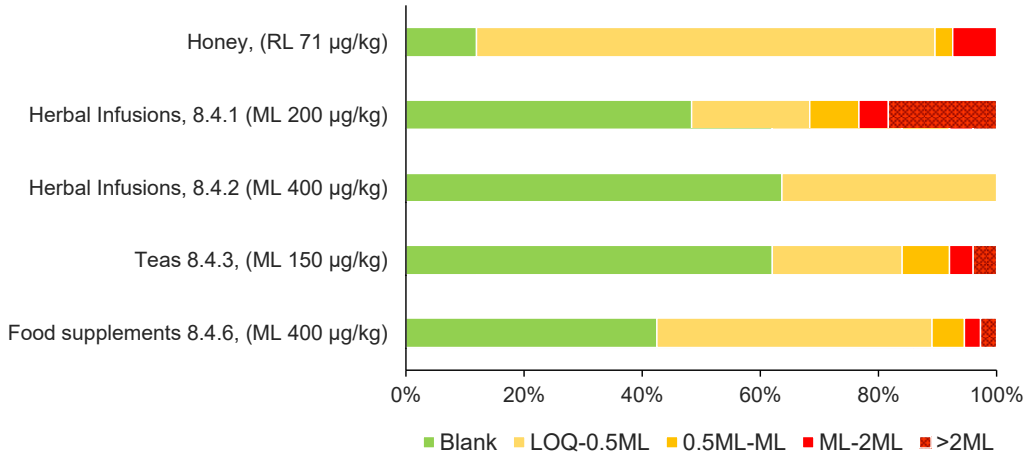


Figure 4.2. Contamination rate of the studied categories as function of the total PAs content.

In general, 56% of the analyzed samples resulted positive to the presence of at least one of the 118 target PAs/PANOs (a measurable amount was detected), with contamination levels ranging from 0.1 to 218381 μg kg^{-1}. Moreover, 9.6% of the positive samples exceeded the MLs (or RL) (**Figure 4.2**).

The contamination profile of the studied matrices was assessed by grouping the target analytes into three main classes: (i) the 21 regulated PAs/PANOs of the Reg. (EU) 2020/2040, (ii) their 14 co-eluting isomers, and (iii) additional PAs (not included in the regulation), to evaluate the contribution of the three classes to the overall contamination level of the samples (**Figure 4.3**). The 21 regulated PAs/PANOs covered almost all the total PAs content in herbal infusions (89%) and dietary supplements (87%) while the 14 co-eluting isomers significantly contributed to the total PAs content of honey (36%) and teas (48%). The contribution of additional PAs and PANOs was only relevant for honey (16%) and dietary supplements (8%) (**Figure 4.3**). A detailed discussion of the contamination data of the studied matrices will be addressed in the next paragraphs.

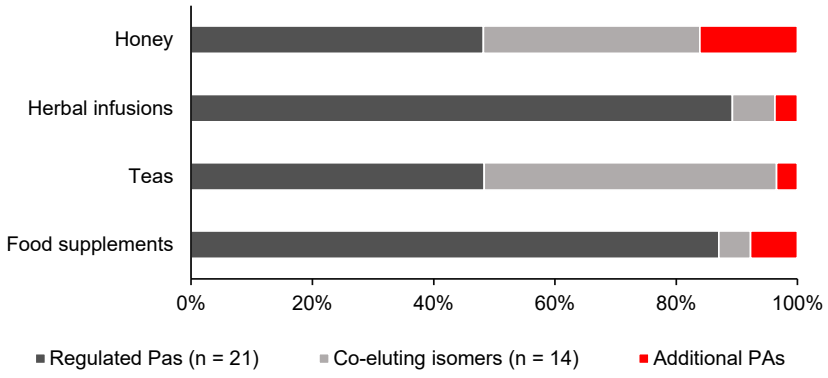


Figure 4.3. Contribution of the 21 regulated PAs and PANOs, their 14 co-eluting isomers, and additional PAs to the overall contamination of the studied samples.

4.3.2.1 Honey

The European regulation does not set a ML for honey, for which EFSA recommended a $BMDL_{10}$ of 237 μg kg^{-1} body weight per day. The BMDL allowed us to estimate the exposure dose for humans and calculate a recommended level (RL) for adults and children (Brugnerotto et al., 2021; EFSA CONTAM Panel, 2017). The exposure dose for humans can be calculated by the following equation (**Equation 4.1**):

$$\text{Exposure dose} = \frac{\text{BMDL}}{\text{MOE}} \qquad \textbf{Equation 4.1}$$

where MOE is the margin of exposure. MOE is ≥ 10,000 for genotoxic and carcinogenic substances such as PAs; hence, the estimated exposure dose is 0.0237, which means that the PAs intake limit is 1.42 μg per day for a 60 kg man and 0.47 μg per day for a 20 kg child. These values must be compared to the average human daily intake of honey, which is 20 g per day, and we were able to obtain the RLs of PAs per kg of honey to refer in this study, which are 71.1 μg kg^{-1} for adults and 23.7 μg kg^{-1} for children.

Honey resulted to be the food matrix with the highest prevalence of contamination; at least one PA/PANO above the LOQ was found in 78% of the samples with concentrations ranging from 0.2 to 129.2 μg kg^{-1}. The mean (15.5 μg kg^{-1}) and median (2.9 μg kg^{-1}) values of the positive samples were well below the RL for adults. Even though 90% of the samples contained negligible levels of PAs (< 0.5 RL), 7% of them (five samples of multifloral honey from extra-European countries) exceeded the RL for adults, and 3% of them (two samples) exceeded the RL for children (**Figure 4.2**). These data confirm that the daily consumption of honey represents a health risk for consumers, especially for children. Regarding the contamination profile, the most abundant PAs of the contaminated honey samples were lycopsamine-type compounds (59% of the total content of the samples), mainly present as tertiary amines. Among

the regulated PAs, Em (73%, range 0.2-16.2 μg kg^{-1}), Im (43%, 0.2-57.0 μg kg^{-1}), Ly (25%, 0.2-3.6 μg kg^{-1}), EmNO (23 %, 0.1-1.3 μg kg^{-1}), and ImNO (14%, 0.3-0.7 μg kg^{-1}) were the most frequently detected in the positive samples (**Figure 4.4**). These data suggest that the botanical species responsible for the PA contamination of honey belong to *Boraginaceae* and *Asteraceae* families (Brugnerotto et al., 2021). Among the 14 co-eluting isomers, echinatine (32%, 0.2-69.1 μg kg^{-1}) was the most frequently detected in the positive samples, with a contribution of 36% to the total PAs content of all the samples (**Figure 4.4**). In fact, some samples exceeded the RL because of the contribution of echinatine (45.8-69.1 μg kg^{-1}) to the total content of PAs. This proves the importance to chromatographically separate co-eluting isomers of PAs from the regulated PAs to provide more accurate data on the botanical origin of the contaminated honey samples. Regarding the PAs not included in the regulation, 11 additional compounds were detected, 7 of which in more than 10% of the positive samples: two isomers of echimidine (isomer 1, 52% and isomer 2, 34%), 5-hydroxyindicine (43%), uplandicine (20%), symphytines (sum of two isomers, 18%), amabiline (14%) and 7-acetylintermedine (11%) (**Figure 4.4**). Among them, the isomers of echimidine, symphytines, as well as uplandicine, 7-acetylintermedine, 7-acetyllycopsamine and amabiline are typical PAs of the main plants responsible for the contamination of honey (*Echium* spp., *Symphytum* spp., *Senecio* spp., *Eupatorium* spp. and *Borago* spp.) (Casado et al., 2022a; Mädge et al., 2020). On the contrary, 5-hydroxyindicine and helioamplexine have been recently reported in Australian honey as result of a contamination with *Heliotropium amplexicaule* (Carpinelli De Jesus et al., 2019). These data suggest that the contamination profile of honey can be extremely variable as it depends on the botanical origin of the geographical area. Thus, it is

necessary to expand the pool of PAs to be monitored in honey and related products to provide a more accurate contamination profile and cover the mean PAs content.

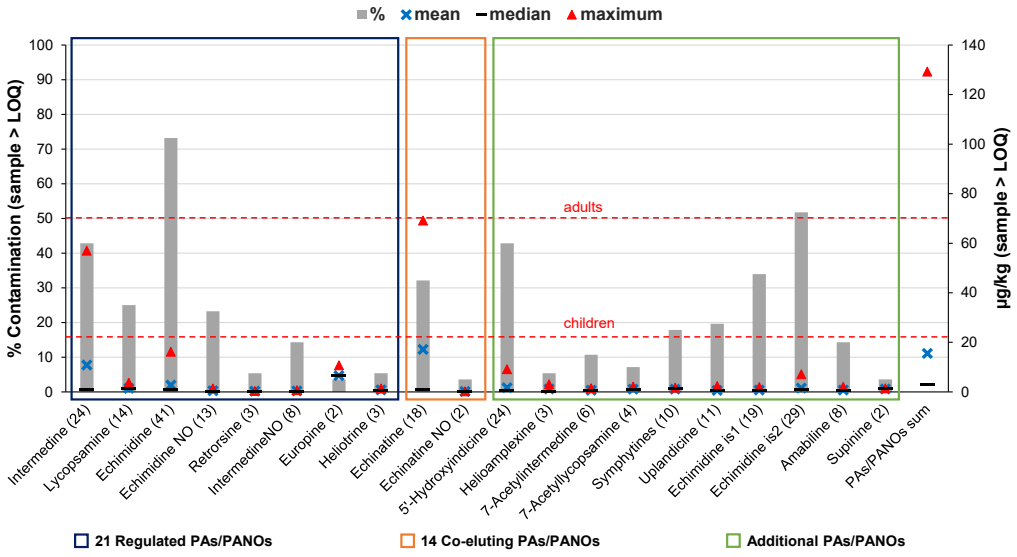


Figure 4.4. Contamination profile of honey: prevalence and levels of each PA/PANO in positive samples (only PAs present in more than 2% of positive samples are shown).

4.3.2.2 Herbal infusions and teas

The Regulation (EU) 2020/2040 divides herbal infusions and teas into three categories: herbal infusions of mixed plants (8.4.1, ML = 200 µg kg^{-1}), herbal infusions of rooibos, anise, lemon balm, chamomile, thyme, peppermint, verbena, and their mixtures (8.4.2, ML = 400 µg kg^{-1}), and *Camellia sinensis* teas (8.4.3, ML = 150 µg kg^{-1}). According to EFSA reports and literature studies, 8.4.2 infusions and teas are the food matrices with the highest contamination rate (EFSA CONTAM Panel, 2017; Mulder et al., 2018; Picron et al., 2018). However, in this study they resulted to be the least contaminated matrices (**Figure 4.2**); this is likely due to the application of good agricultural and harvesting practices after the reporting of worrying levels of contamination in these food matrices.

The 8.4.2 category of infusions showed the lowest contamination rate (32% of the samples were above the LOQ) with much lower levels than the ML (6.5-97.7 μg kg^{-1}; mean and median of 44 μg kg^{-1}). A similar contamination rate was observed in tea samples (39% of the samples were above the LOQ; range 6.9-415.7 μg kg^{-1}). However, four samples of black teas (8%) exceeded the ML (**Figure 4.2**). In accordance with the literature data, senecionine-type N-oxides were prevalent in tea samples (64% of the total content of all samples). Retrorsine N-oxide (30%, 33.2-258.9 μg kg^{-1}), lycopsamine N-oxide (40%, 6.9-20.9 μg kg^{-1}) and senecionine N-oxide (10%, 2.6-76.4 μg kg^{-1}) were the regulated PANOs most frequently detected. Among the 14 co-eluting isomers, echinatine N-oxide (60%, 8.5-76.3 μg kg^{-1}), echinatine (45%, 8.3-33.8 μg kg^{-1}) and integerrimine N-oxide (40%, 8.0-245.5 μg kg^{-1}) were frequently found in the contaminated samples, with a contribution to the overall contamination levels of teas of 8, 14 and 22% respectively, which is comparable to the percentages of the regulated PAs (**Figure 4.5**).

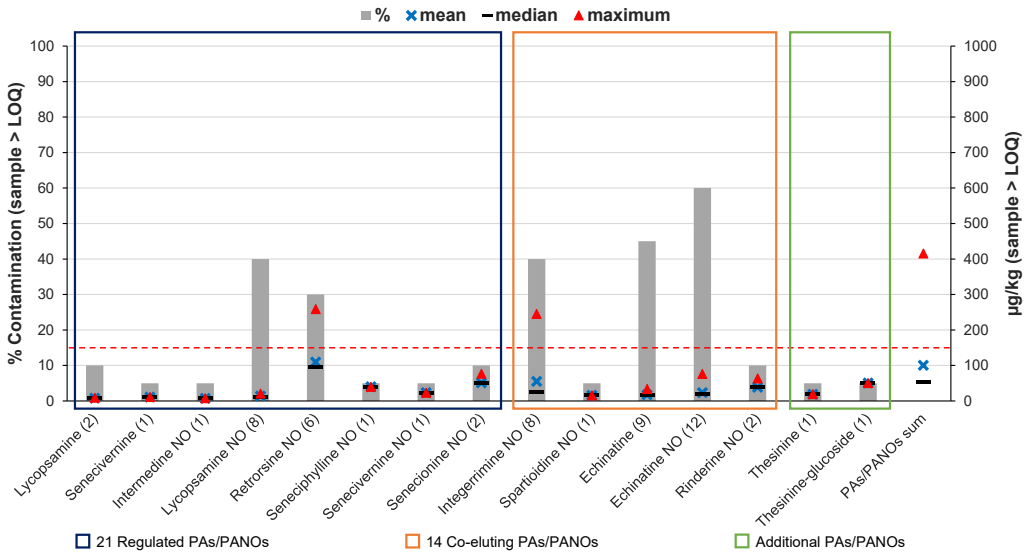


Figure 4.5. Contamination profile of *Camelia sinensis* teas: prevalence and levels of each PA/PANO in positive samples (only PAs present in more than 2% of positive samples are shown).

Differently, 8.4.1 infusions resulted much more contaminated (52% of the samples were above the LOQ), with higher total levels up to 218381 μg kg^{-1} (mean of 14025 μg kg^{-1} and median of 127 μg kg^{-1}). In fact, 23% of these samples exceeded the ML of their category (**Figure 4.2**). Among the 60 analyzed samples, three samples containing PA-producing plants resulted contaminated with very high levels (865-218381 μg kg^{-1}), well above the ML. Except for the infusion of *Tussilago farfara*, where senkirkine was the main compound, the contamination of the infusions of *Borago officinalis* and *Symphytum officinale* was mainly due to lycopsamine-type monoesters (76%). The contamination data of the PA-producing plants infusions were not included in the discussion of the results of this category (8.4.1) to avoid an overestimation of the data. However, even excluding these data from the total content, the contamination levels of this category resulted conspicuous (maximum level of 953.6 μg kg^{-1}, mean of 225 μg kg^{-1} and median of 110 μg kg^{-1}) with a mean value exceeding the ML (**Figure 4.6**). These data indicate a possible risk for human health associated to the consumption of infusions of mixed plants, even if they do not contain PA-producing plants. The contamination profile of the 8.4.1 infusions showed a prevalence of heliotrine-type and senecionine-type PAs, which were responsible for 59% and 24% of the overall contamination, respectively (**Figure 4.6**). These results clearly demonstrate that this matrix is mainly subjected to the contamination of *Heliotropium* and *Senecio* spp. Among the 21 regulated PAs and PANOs, the most detected ones were SeNO (32%) LcNO (32%), EuNO (29%), HeNO (29%), SpNO (21%) and He (21%). The main contributors to the total contents, with mean concentrations higher than 100 μg kg^{-1}, were ReNO (136-421 μg kg^{-1}), Eu (30-398 μg kg^{-1}), EuNO (39-337 μg kg^{-1}), and LcNO (31-217 μg kg^{-1}) (**Figure 4.6**). Co-eluting isomers and additional PAs contributed the least amount to the contamination

level of this matrix (7% and 3%, respectively). Echinatine N-oxide (18%), rinderine N-oxide (14%) and integerrimine N-oxide (14%) were the main detected co-eluting isomers. Among the 1,2-unsatured additional PAs, heleurine N-oxide was found in 18% of the contaminated samples (**Figure 4.6**); it is a supinidine-type of PAs, characteristic of *Heliotropium* spp. (Carpinelli De Jesus et al., 2019; Louisse et al., 2022) and mainly detected in highly contaminated samples by heliotrine-type PAs of these species.

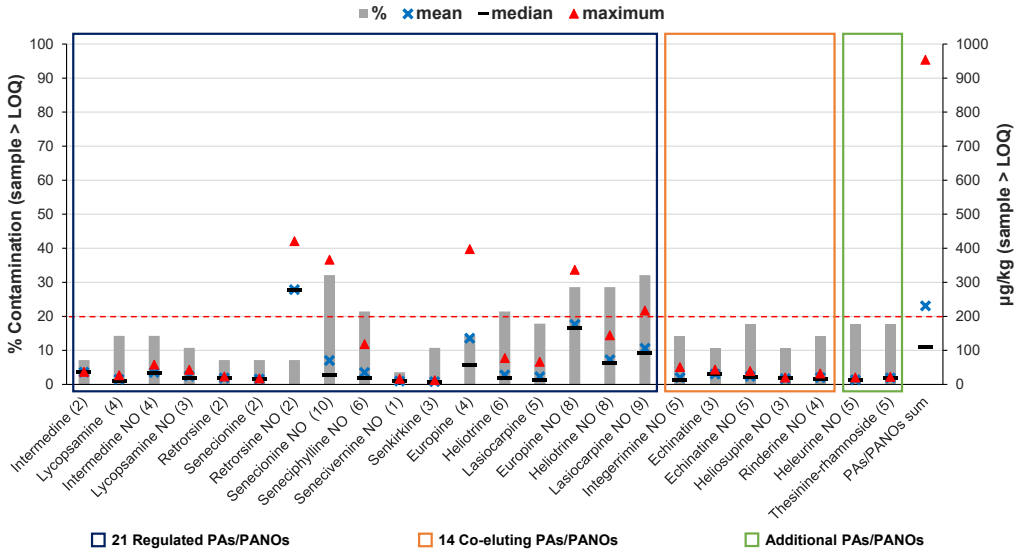


Figure 4.6. Contamination profile of 8.4.1 infusions: prevalence and levels of each PA/PANO in positive samples (only PAs present in more than 5% of positive samples are shown).

4.3.2.3 Herbal dietary supplements

Collected samples of herbal dietary supplements showed a huge diversity in their composition, with added plant extracts, various herbs, spices, flowers, and roots. Therefore, the contamination levels of PAs and PANOs of these samples were calculated using solvent-based calibration curves as it was not possible to select or prepare a representative matrix to mimic the composition of a representative number

of real samples. Contaminated samples with PAs levels close to or above the ML (400 μg kg^{-1}) were accurately quantified using the standard addition method.

In total, 58% of the analyzed samples contained at least one PA/PANO above the LOQ while only 6% of the positive samples exceeded the ML; however, two of the exceeding samples contained more than 1000 μg kg^{-1} of total PAs. The ingredients of these two samples were mainly raw plant materials of well-known herbal products to be contaminated with significant levels of PAs (leaves of rosemary and peppermint, fruits of anise, cumin, and fennel). Heliotrine-type PAs covered 75% of the overall contamination of dietary supplements. According to these results, *Heliotropium* spp seemed to be the most common weed of crops used for the manufacturing of herbal dietary supplements. The most frequently occurring regulated PAs of positive samples were Eu (38%), He (33%), Lc (26%) and HeNO (21%). On the contrary, the quantitatively predominant analytes (mean above 100 μg kg^{-1}) were SpNO (5 and 231 μg kg^{-1}), EuNO (4-924 μg kg^{-1}), HeNO (3-1374 μg kg^{-1}) and LcNO (3-652 μg kg^{-1}) (**Figure 4.7**). The highest levels of these analytes were detected in samples formulated as infusions, and therefore, containing dried plant materials. The content of co-eluting isomers was not significant for dietary supplements (5%), and the most detected compounds of this group were echinatine (45%), echinatine N-oxide (19%) and integerrimine N-oxide (12%). On the other hand, numerous additional PAs (23 out of 49) were detected but only heleurine N-oxide (10%), thesinine and its glycosides (17%) frequently occurred in positive samples (**Figure 4.7**). It is noteworthy the diverse distribution of PAs observed in dietary supplements of different composition; in fact, N-oxide forms were prevalent in infusion samples (86% of the overall contamination) while tertiary amines significantly contributed to the contamination of plant extracts-based dietary supplements (59% of the overall contamination). This is

likely due to higher water solubility of PANOs compared to PAs, which leads to the loss of PANOs during the extraction processes employed to produce plant extracts used in the formulations of dietary supplements.

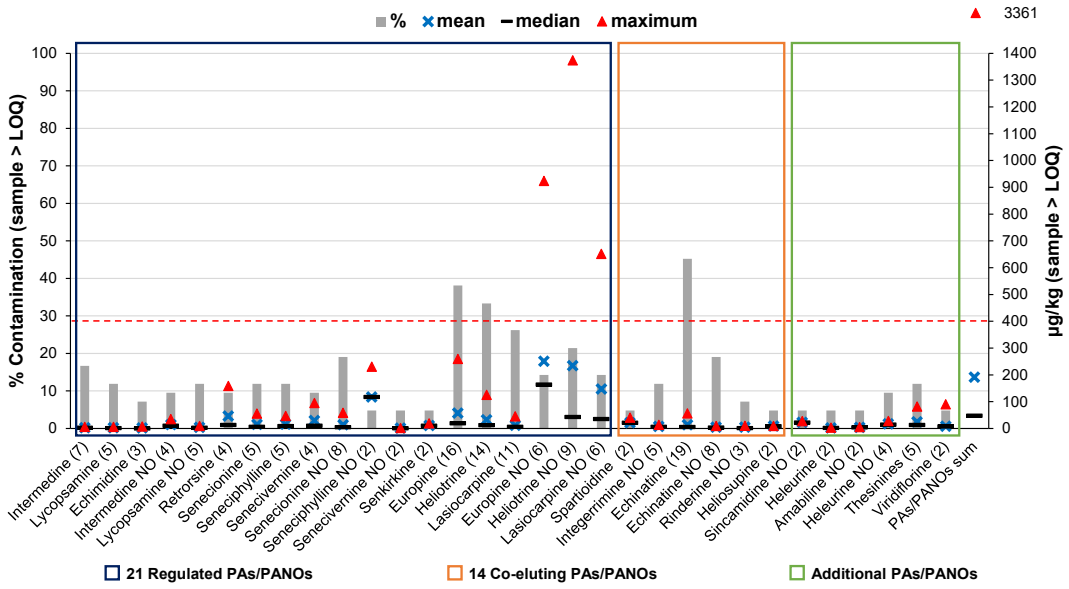


Figure 4.7. Contamination profile of dietary supplements: prevalence and levels of each PA/PANO in positive samples (only PAs present in more than 5% of the positive samples are shown).

4.4 Conclusions

In this CHAPTER, the previously developed analytical platform (see CHAPTER 3) was validated for quantitative purposes and applied to the analysis of a high number of real samples (n = 281) of different food matrices, which were screened against the presence of 118 target PAs and PANOs. The proposed procedure showed good analytical performance in detecting 28 reference standards of PAs and met the requirements of analytical methods for trace level contaminants analysis in food samples. Therefore, we conclude that it can be successfully used for the determination of PAs and PANOs in honey, herbal infusions, dietary supplements, black and green teas. Even if the present study could provide accurate quantitative data for 28 analytes only (no reference standards are available for the other compounds), it is certainly worth using to estimate the total PA content and direct high-PA-content samples to further quality control investigations.

A high rate of contamination (above 50% of the analyzed samples) was determined in honey, 8.4.1 infusions, and dietary supplements. Samples exceeding the MLs were found for all the analyzed food categories (except 8.4.2 infusions), with the highest percentage found for 8.4.1 infusions (23%). This means that there is a possible health risk for consumers associated with the consumption of these products, especially those with habits of drinking herbal infusions.

Regarding the contamination profiles, the co-eluting isomers significantly contributed to the overall contamination levels of honey (36%) and teas (48%) and a high number of additional PAs (not included in the regulation) was detected in honey and herbal dietary supplements, even if their contribution to the overall contamination level was only significant for honey. These results indicate the need to expand the pool of PAs to be monitored in honey to provide a more accurate contamination profile of

honey and honey-based products. It is also important to continue collecting occurrence data in different food matrices, especially in herbal infusions and dietary supplements, which cover a large slice of the health market and may represent a source of health risk for consumers. Occurrence data collection is also fundamental to provide the regulatory agencies with a broader picture regarding the distribution of these toxins in foods and enable them to strengthen the current regulatory framework.

CHAPTER 5

A multi-analyte screening method for the rapid detection of illicit adulterants in dietary supplements using a portable SERS analyzer

5.1 Introduction

The popularity and number of dietary supplements on the health market have experienced an unprecedented boost in recent years. Simultaneously, their increased use has been accompanied by an increase in acute intoxication cases linked to the adulteration of these products with illicit and undeclared substances (Wheatley & Spink, J, 2013; Manning et al., 2022; Biesterbos et al., 2019). The presence of such adulterated products in the marketplace is a worldwide problem and their consumption poses major health risks to consumers (Ekar & Kreft, 2019; Başaran et al., 2022). Traditional analytical methods for the determination of adulterants in dietary supplements are based on liquid chromatography combined with mass spectrometry; however, these methods are time-consuming, costly and depend on laboratory-based analyses (Muschietti et al., 2020). Hence, for a better overview of the food fraud landscape on dietary supplements, a rapid screening methodology that could be applied on site would be very beneficial.

Surface-enhanced Raman scattering (SERS) has recently attracted a lot of attention for the detection of food adulterations as it meets these requirements (Petersen et al., 2021). It is based on the reciprocal interaction between an analyte and a metallic substrate to detect its intrinsic fingerprint and amplify the Raman signal making this technique sufficiently sensitive to detect single molecules on a surface. The advantages of this methodology are its simplicity, high sensitivity, rapidity, low cost, and in-situ sampling and monitoring (Yang et al., 2021). The technique is also relatively insensitive towards the pharmaceutical excipients used during the manufacturing of dietary supplements, which is a great advantage for the detection of single, specific adulterants (De Veij et al., 2009).

The aim of the present study was to explore the use of SERS for the qualitative screening and detection of illicit adulterants in dietary supplements using portable devices, with the goal of developing a rapid screening method suitable for this purpose. This includes an appropriate evaluation of any interferences due to the presence of plant-based extracts and other ingredients present in the formulation of dietary supplements as well as the setting of detection ranges for the studied adulterants.

23 pharmaceutically active adulterants were selected, and their Raman activity examined using a benchtop Raman spectrometer. Subsequently a SERS-based methodology was developed and assessed for the pure adulterants, the adulterants mixed with a representative mixture of excipients, and the adulterants mixed with 18 commercial dietary supplements. A portable analyzer and silver printed-SERS substrates were used to enhance the signal, requiring less than 20 minutes of sample preparation prior to the analysis. A spectral library was then built and applied to the screening of the artificially adulterated dietary supplements prepared. The method was successful in the qualitative identification of 11 out of 23 illicit adulterants in the dietary supplements, demonstrating the potential of SERS-based methodologies in the analysis of forensic applications in this field.

5.2 Materials and methods

5.2.1 Chemicals and standards

Analytical grade ethanol (EtOH), acetone (Me_2CO), and acetonitrile (MeCN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (H_2O, 18 MΩ) was prepared by a Milli-Q purification system (Millipore, Bedford, USA).

The following reference standards of 23 target adulterants were purchased from Biosynth International (Naperville, IL): acetildenafil (ACD), amino tadalafil (ATAD), homo sildenafil (HSIL), DL-5-hydroxytryptophan (HTP), piperin (PIP), tadalafil (TAD), thiosildenafil (TSIL), vardenafil hydrochloride (VAR), xanthoanthrafil (XAN), and Sigma-Aldrich (St. Louis, MO, USA): 4-androstene-3,17-dione (AND), 1,3-dimethylamylamine (DMAA), 2-dimethylaminoethanol (DMAE, ≥ 99.5%), 1,3-dimethylbutylamine (DMBA, 98%), 2,4-dinitrophenol (DNP), fluoxetine hydrochloride (FLU), melatonin (MEL, ≥ 98%), phenethylamine (PEA, 99%), phenolphthalein (PNP), sibutramine (SIB), sildenafil citrate (SIL), synephrine (SYN), vinpocetine (VIN), yohimbine hydrochloride (YOH, ≥ 98%). They were selected based on alerts from the RASFF portal.

The following excipients were provided by Sigma-Aldrich (St. Louis, MO, USA): arabic gum from the acacia tree, calcium hydrogen phosphate, carboxymethylcellulose sodium salt, cellulose microcrystalline, gelatin, hydroxypropylmethylcellulose, lactose, magnesium stearate, methyl cellulose, polyvinylpyrrolidone, silicon dioxide, starch, stearic acid, and titanium oxide.

5.2.2. Commercial samples

A pool of commercial dietary supplements samples (n = 18) were sourced and included nine weight-loss and energy boosting supplements (W1-9) and nine sexual and sport performance enhancement supplements (S1-9). The samples were purchased

through different online e-commerce. One of the weight-loss and energy boosting supplements was formulated as powder, four as capsules, and four as tablets. Three of the sexual and sport performance enhancement supplements were formulated as tablets and six as capsules. All the samples were manufactured in different countries of the EU and the United Kingdom. The excipients listed on the label of the samples were mainly microcrystalline cellulose, magnesium stearate, hydroxypropyl methylcellulose, and silicon dioxide for both capsules and tablets. Among the weight-loss and the energy boosting supplements, the principal plant-based ingredients claimed on the label were Garcinia cambogia fruit extract (*Garcinia gummi-gutta*), green tea (*Camellia sinensis*) leaf extract, and green coffee (*Coffea robusta*) dried extract. Among the sexual and sport performance enhancement supplements, the principal plant-based ingredients claimed on the label were maca root (*Lepidium meyenii*), gokshur (*Tribulus terrestris*), ginkgo (*Ginkgo biloba*), ashwagandha (*Withania somnifera*), rhodiola (*Rhodiola rosea*), stone pine (*Pinus pinea*), fennel seed (*Foeniculum vulgare*), fenugreek (*Trigonella foenum-gra cum*), psyllium husk powder (*Plantago ovata*), aloe leaf extract (*Aloe vera*), black chia seed powder (*Salvia hispanica*), and flaxseed powder (*Linum usitatissimum*), broccoli dried extract (*Brassica oleracea*), ginseng dried extract (*Panax ginseng*). Ten of the supplements also included different vitamins (B5, B6, B9, B12, C, D3, E, K) and minerals (calcium, chromium, magnesium, selenium, zinc) in their formulation. The average weight of the supplements was 1000 mg with supplements ranging from a minimum weight of 200 mg up to a maximum of 1800 mg. The recommended dosage for all the supplements ranged from 2 to 4 tablets/capsules per day.

5.2.3 Sample preparation

5.2.3.1 Artificially adulterated excipients mixture

The detectability range of each SERS active adulterant was assessed by measuring eight levels of adulteration, in the range 0.1-50.0% w/w, within a basic formulation of dietary supplements (excipients only; no other additional ingredients were added). In detail, a mixture of excipients was prepared to be used as filling material and it was prepared as follows: microcrystalline cellulose (95.0% w/w), magnesium stearate (3.0% w/w), silicon dioxide (1.0% w/w), hydroxypropyl methylcellulose (0.5% w/w), and titanium oxide (0.5% w/w). The excipients were selected to reflect a composition of excipients as representative as possible of the real formulations by observing the composition of the excipients on the labels of various dietary supplements present on the market. The final percentage of each component of the mixture was chosen based on the experience of the researchers who have worked on the project. No plant-based extracts were used during this step since it was not possible to choose a representative set of plant-based ingredients due to the enormous variability in their composition within the formulation of dietary supplements.

5.2.3.2 Artificially adulterated dietary supplements

The commercial dietary supplements (n = 18) were used as filling materials for the preparation of five levels of adulteration, in the range 0.1%-5.0% w/w, to test the applicability of the SERS method to the routine screening of suspected fraudulent dietary supplements and estimate the limit of identification (LOI) of the target adulterants, which is the lowest concentration for which the identification criteria are met. To do so, the target adulterants were divided into two groups based on the frequency of their detection as adulterants in the considered categories of dietary supplements; thus, FLU, HTP, MEL, PEA, SYN, and VIN were used to adulterate nine

weight-loss and energy boosting supplements (W1-9) while ACD, HSIL, PIP, SIL, TSIL, VAR and YOH were used to adulterate nine sexual and sport performance enhancement supplements (S1-9).

The mixing of all the excipients (necessary for the preparation of the mixture) as well as the preparation of the adulteration levels of all the target analytes were carried out using the geometric dilution method, commonly used in the pharmaceutical industry to produce formulations with a low content of active ingredients (Alyami et al., 2017). It consists of the gradual addition of equal portions of an excipient to the pharmacologically active ingredient, taking care to double the present quantity each time, to achieve an equitable distribution of the active ingredient particles within the mixture. During the analysis of each batch, both 100% w/w (pure standard) and 0% w/w (mixture of excipients, blank sample) were also tested.

5.2.4 Raman and SERS spectroscopy

5.2.4.1 Benchtop FT-Raman spectrometer

The Raman activity of the pure target adulterants was tested on the solid or liquid materials using a benchtop RAM II FT-IR Raman module (Bruker Nederland B.V., Leiderdorp, The Netherlands) coupled to a FT-IR VERTEX 70 spectrometer. The spectrometer was equipped with a 1064 nm laser and the spectra were recorded with a resolution of 4 cm^{-1} in the range of 50 to 3600 cm^{-1}, resulting in an average of 32 consecutive scans. The laser output power was set at 300 mW. Three spectra were recorded for each sample and averaged in the following step of data analysis. Spectral data were acquired using OPUS software (version 7.2, Bruker).

5.2.4.2 Portable SERS analyzer

The SERS analyses were performed using a Metrohm Instant SERS analyzer (MISA) (Metrohm, Laramie, WY, USA) equipped with a 785 nm ± 0.5 nm laser and

an Orbital Raster Scan (ORS™) technology where a focused laser spot (30 μm in diameter) is continuously rasterized over a region of ≈ 2 mm in diameter on the substrate, resulting in a higher sample coverage and better averaging. Spectra were recorded with a resolution of 8 cm^{-1} (FWHM) in the range of 400 to 2300 cm^{-1} obtaining an average of three successive scans. The laser output power was 100 mW. Three spectra were recorded for each sample and averaged in the following step of data analysis. Silver printed-SERS (p-SERS) substrates and a specific p-SERS Attachment (Metrohm, Laramie, WY, USA) were used to carry out the SERS measurements. Gold p-SERS substrates were also tested for adulterants which were silver p-SERS unactive. Daily calibrations of the instrument were performed using the appropriate calibration standard (ASTM 1840 reference sample). Spectral data were acquired using the instrument-associated MISA Cal software (Metrohm) with the following standard operating procedure: Smart Acquire mode on, laser power level set to 5, auto integration on, averages of three successive scans, and raster on.

5.2.5 SERS procedure

Each artificially adulterated dietary supplement was processed as follows: 10 mg of each sample were first weighed into an Eppendorf tube (1.5 mL) and then diluted with 100 μL of EtOH or H_2O to prepare a super-concentrated solution of the analyte. EtOH was used as diluting solvent for all the studied adulterants except for HTP and YOH. Instead, H_2O was used for the latter to achieve an appropriate SERS signal during the instrumental analysis. Each sample was then vortexed for 20 seconds and centrifugated at 806,400 RCF for 2 minutes. Afterwards, 10 μL of the supernatant were pipetted onto the silver p-SERS strip and left to dry for 15 minutes after which the instrumental analysis commenced. Measurements were conducted in triplicates.

5.2.6 Spectral library creation and identification strategy

The MISA identification of unknowns is carried out by correlating a sample spectrum with the spectra of the library. Therefore, it was first necessary to build a spectral library of adulterants through the libraries section of the MISA Cal software. The library was then used for the screening of artificially adulterated samples of dietary supplements. All the spectra of the library were collected by diluting the pure adulterants in EtOH, except for HTP and YOH which were diluted with H_2O for signal strength reasons, at a concentration of 1 mg mL^{-1}. The software is equipped with a correlation algorithm for spectral comparison to identify an unknown spectrum against a spectral library. The library is selected for matching when the operating procedure is created. The software searches the library and returns an HQI (Hit Quality Index) value that indicates the level of correlation defined by a user-defined threshold. When analyzing a sample, the spectrum of the sample will then be compared with all the spectra present in the selected library. The measured sample will be identified as one of the library samples and displayed as the 'Identification Result'. If the sample does not match a library, 'No Results' will be displayed. Moreover, the Confusion Matrix tab of the libraries section allows for evaluation of the accuracy of the classification. The software warns the user about the presence of any matches between the spectra of the library through three Warning categories, highlighted by the three colours: red (match score > 0.95), orange (match score > 0.85), and yellow (match score > 0.75). Similarity scores below 0.75 are not highlighted as they are not considered warning scores.

5.2.7 Data analysis

Data analysis was performed using the instrument-associated MISA Cal software. The HQI matching was used to identify an unknown spectrum. The correlation

coefficient HQI for the unknown scan compared to the library spectrum is calculated using the least square dot product of the mean centered unknown spectrum and the library spectrum, represented by the **Equation 5.1**:

$$\mathrm{HQI} = \frac{(\mathrm{Library} \times \mathrm{Unknown})^{2}}{(\mathrm{Library} \times \mathrm{Library})(\mathrm{Unknown} \times \mathrm{Unknown})} \qquad \textbf{Equation 5.1}$$

The HQI values range from 0 to 1, with 0 representing no match and 1 representing a perfect match.

A second check of the data analysis was also carried out using an in-house script developed using R software version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) and RStudio version 5501.9.1.0. The R packages prospectr_0.2.0, signal_0.7-6, Rtools_4.0.3.0, and pracma were used. The statistical correspondence scores between Raman spectra of the thirteen target adulterants and the artificially adulterated dietary supplements at different concentration levels were calculated as Pearson's correlation between the maximum/minimum intensity value of the reference peak (first derivative) and the values at the corresponding wavelength points of each unknown spectrum, using a second order filter. Subsequently, for each reference spectrum of the first derivative, the spectral features were identified using a generic peak-finder algorithm (pracma). Match scores range from 0 (no match) to 1 (perfect match). However, score values below 0 were also observed and interpreted as "absence of any similarity".

5.3 Results and discussion

5.3.1 Approach

Twenty-three pharmaceutically active adulterants were used in the study, and their Raman activity examined using a benchtop FT-Raman spectrometer and a portable SERS analyzer. Only 13 out of 23 adulterants were found to be SERS active and used for the subsequent steps of method development. Then, a spectral library was created using the software associated to the portable instrument to verify the existence of any similarities between the SERS spectra of the target adulterants by applying basic statistical matching. Afterwards, a simple dilution and pre-concentration step of the analytes was developed and optimized before the instrumental analysis. The developed procedure was applied to the SERS analysis of different concentrations of each adulterant mixed with a representative mixture of excipients (range 0.1–50.0% w/w) in order to estimate the detectability range of each adulterant; the same was done by testing low concentrations of the adulterants mixed with 18 commercially sourced dietary supplements with claims to reduce weight, enhance sexual performances, and build muscles (range 0.1–5.0% w/w) to test the applicability of the developed procedure in real conditions of adulteration and set a LOI for each adulterant. After the latter experiments, two adulterants were removed from the list of 13 as they resulted undetectable in all the commercial dietary supplements tested, reducing the number of adulterants included in the method to 11. These include the following target compounds: ACD, HSIL, SIL, TSIL, VAR, PEA, SYN, MEL, VIN, FLU, and PIP.

5.3.2 Evaluation of the Raman activity of the target adulterants

5.3.2.1 Raman spectroscopy

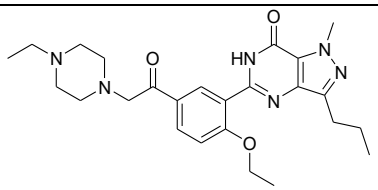
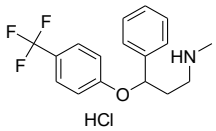
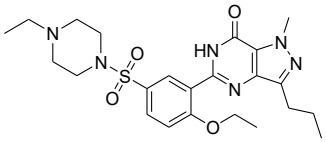
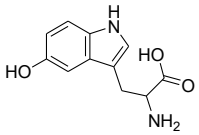
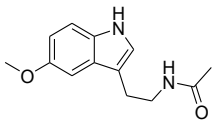
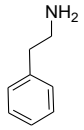
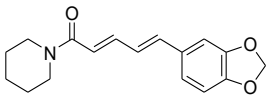
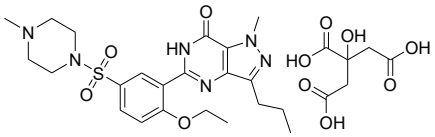
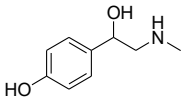
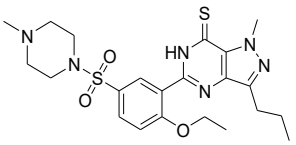
The Raman activity of pure powdered (n = 19) and liquid (n = 4) adulterants was first tested using a benchtop FT-Raman instrument; the aim was to establish if the

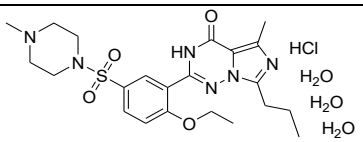
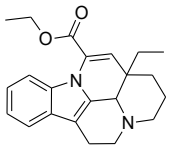
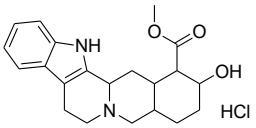
selected adulterants were Raman active before proceeding to study the improvement of the signal induced by their interaction with the p-SERS substrate. Aside from seven compounds (AND, DMAA, DMAE, DMBA, TSIL, VAR, and XAN), all the other target analytes exhibited a characteristic Raman shift when tested with the benchtop FT-Raman instrument, albeit at very low intensities.

5.3.2.2 Surface-enhanced Raman scattering (SERS)

As the aim of the study was to develop a rapid screening method using a portable analyzer, the SERS activity of the target adulterants was further investigated using the MISA device. The use of silver p-SERS substrates provided enhancement factors ranging from 10^3 to 10^4 for thirteen target adulterants and allowed to detect TSIL and VAR, which resulted Raman unactive using the benchtop instrument; this is likely due to a good interaction between the analytes and the SERS substrates. **Table 5.1** shows the chemical structures, therapeutic daily doses, and European Commission (EC) status of the thirteen SERS active adulterants. Regarding the remaining SERS inactive adulterants, the SERS inactivity observed on the benchtop instrument was confirmed for AND, DMAA, DMAE, DMBA, and XAN while for ATAD, DNP, PNP, SIB and TAD the SERS inactivity is probably due to a lack of interaction of the analytes with the silver p-SERS substrate. Additional tests were also carried out on the latter five adulterants using gold p-SERS substrates; these tests further confirmed the inactivity of the tested analytes.

Table 5.1. Chemical structures, therapeutic daily doses, and European Commission (EC) status regarding the use of SERS active adulterants in dietary supplement formulations.

Adulterant	EC status	Chemical structure	Daily doses (mg)
Acetildenafil (ACD)	Forbidden		–
Fluoxetine (FLU)	Forbidden		10–60
Homo sildenafil (HSIL)	Forbidden		–
5-Hydroxytryptophan (HTP)	Allowed		100-300
Melatonin (MEL)	Allowed		0.5–5
Phenethylamine (PEA)	Forbidden		100–500
Piperin (PIP)	Allowed		5–30
Sildenafil (SIL)	Forbidden		25–100
Synephrine (SYN)	Allowed		30
Thiosildenafil (TSIL)	Forbidden		–

Adulterant	EC status	Chemical structure	Daily doses (mg)
Vardenafil (VAR)	Forbidden		5–20
Vinpocetine (VIN)	Allowed		5–40
Yohimbine (YOH)	Forbidden		10–30

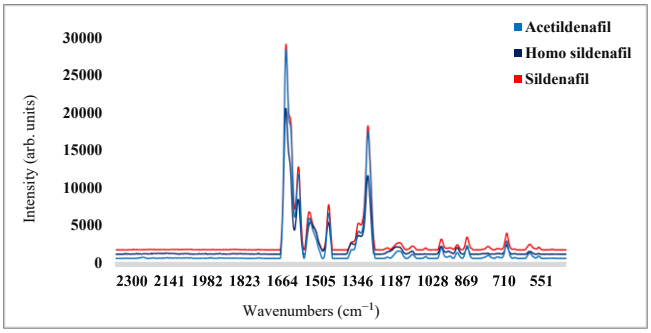
5.3.3 Evaluation of the Raman activity of the excipients

The Raman activity of the excipients was tested to determine the existence of any interferences between them and the target adulterants. This evaluation was necessary since the excipients are the basic ingredients of the main pharmaceutical forms used to formulate dietary supplements (capsules and tablets). Aside from titanium oxide, none of the tested excipients exhibited any pronounced Raman activity using both the benchtop FT-Raman instrument and the portable SERS analyzer. In the case of titanium oxide, its Raman activity was not considered a major issue as it only exhibited a strong Raman activity on the benchtop FT-Raman instrument. Moreover, this ingredient has recently been banned for its use in foodstuffs (including food supplements) according to the Regulation 2022/63/EC (European Commission, 2022).

5.3.4 Spectral library and identification results

A SERS spectral library of the 13 active adulterants was first built and then used to screen all the artificially adulterated dietary supplements; before doing so, it was necessary to establish the existence of any similarities between the spectra of the target adulterants to prevent the software from providing false assignments when screening the samples. The software showed three red match scores between the target

adulterants due to match scores of 0.99 between ACD and SIL, 0.98 between ACD and HSIL, and 0.97 between HSIL and SIL. This led to the conclusion that the spectra of these three adulterants are indistinguishable. **Figure 5.1** shows SERS spectra of ACD, HSIL, and SIL as confusion matrices of the library. Although the unfeasibility of distinguishing these three adulterants might seem like a drawback of the procedure, it is not since they belong to the same chemical-pharmacological class which is used for the same fraudulent purpose in sexual performance enhancement dietary supplements. Therefore, it is not a huge issue that the algorithm is not able to discriminate them. Rather, this could benefit the procedure since there is a chance that these adulterants could be added together within the same formulation. This would result in an increase of the signal intensity by superimposing the intensities of the single analytes. No orange and yellow matches were present among the spectra of the other adulterants of the library, indicating the absence of any other warning similarities and therefore, the risk of false positives.



Library Sample	Match	Match Score
Acetildenafil	Sildenafil	0.99
Acetildenafil	Homo sildenafil	0.98
Homo sildenafil	Sildenafil	0.97

Figure 5.1. SERS spectra of ACD, HSIL, SIL, and library match scores among the three adulterants obtained by the MISA Cal software.

5.3.5 Optimization of the SERS procedure

Before proceeding to the screening of the artificially adulterated dietary supplements, the SERS procedure was carefully optimized to select the optimum solvent to dilute the adulterants of interest and its appropriate amount. Solubility tests

were carried out to select the best diluting agent for the screening of the selected adulterants; five different solvents: H_2O, EtOH, Me_2CO, MeCN, and the mixture EtOH/H_2O 50:50 v/v were tested. Among the tested solvents, EtOH was established as the most suitable solvent to dilute all the adulterants except for HTP and YOH, which showed good signals only if diluted with H_2O. The mixture EtOH/H_2O 50:50 v/v was discarded because the results obtained from the two solvents (individually used) showed to be better, in terms of signal enhancement, than those obtained from the mixture. Moreover, all the selected excipients were SERS inactive when diluted with both EtOH and H_2O; most of the tested excipients were not dissolved at all within the two solvents. For these reasons, H_2O was selected as diluting solvent for HTP and YOH while EtOH was used for all the remaining adulterants.

Once the diluting agents were chosen, subsequent tests were carried out to determine the amount of solvent to be used when performing the screening of artificially adulterated dietary supplements. To do so, three different amounts, 0.1, 0.5 and 1 mL of each solvent were selected. It is important to mention that for this step of choosing the correct amount of solvent, the solubility limits of the selected compounds were not considered as the purpose of the present study was not to develop a quantitative screening method, which would have required not exceeding the limits of solubility of the selected compounds. The aim was instead to develop a rapid screening method providing a rapid pass/fail answer regarding the presence of the selected adulterants in dietary supplements using a portable device. Any positive sample must be subjected to further investigations to confirm the presence of the suspected adulterants and establish its amount; LC-MS methods are currently used for this purpose.

5.3.6 Screening of artificially adulterated dietary supplements

5.3.6.1 Analysis of artificially adulterated excipients mixture

To estimate a detectability range, eight levels of adulteration in the range of 0.1–50.0% w/w were prepared for each analyte using the mixture of excipients as filling material. The concentration range was chosen to cover the therapeutic range of the adulterants of interest. In detail, a calculation of the hypothetical concentration levels of adulteration was made, according to the active therapeutic doses of the target adulterants when added to formulations ranging from 100 to 2000 mg per unit to select the concentration range to test. Then, the entire concentration range of all the target adulterants was analyzed using the portable SERS analyzer and it showed to be able to detect the target adulterants covering the entire concentration range (0.1-50.0% w/w) with correlation scores ranging from 0.93 to 0.99 for ACD, HSIL, SIL, TSIL, VAR, and VIN, the concentration range of 0.5–50.0% w/w with correlation scores ranging from 0.65 to 0.99 for FLU, HTP, MEL, PEA, and YOH, and the concentration range of 2.5–50.0% w/w with correlation scores ranging from 0.73 to 0.99 for PIP, and SYN. The detectable concentration ranges as well as the Pearson's correlation scores of each adulterant obtained using the MISA Cal and R software are shown in the **Table 5.2**.

Table 5.2. Detected ranges of artificially adulterated samples and comparison of Person's correlation scores between MISA Cal and R software.

Analyte	MISA Cal software		R software	
	Detected range (% w/w)	Library match score	Detected range (% w/w)	Library match score
ACD[a]	0.1–50.0	0.99–0.99	0.1–50.0	1.00–1.00
FLU	0.5–50.0	0.76–0.96	0.5–50.0	0.91–0.99
HSIL[a]	0.1–50.0	0.99–0.99	0.1–50.0	1.00–1.00
HTP	0.5–50.0	0.65–0.82	0.1–50.0	0.74–0.92
MEL	0.5–50.0	0.79–0.98	0.1–50.0	0.75–0.99
PHE	0.5–50.0	0.78–0.99	0.5–50.0	0.95–1.00
PIP	2.5–50.0	0.89–0.98	0.5–50.0	0.81–0.95
SIL[a]	0.1–50.0	0.99–0.99	0.1–50.0	1.00–1.00
SYN	2.5–50.0	0.73–0.99	1.0–50.0	0.72–1.00
TSIL	0.1–50.0	0.98–0.99	0.1–50.0	1.00–1.00
VAR	0.1–50.0	0.93–0.99	0.1–50.0	0.98–1.00
VIN	0.1–50.0	0.95–0.99	0.1–50.0	0.92–0.99
YOH	0.5–50.0	0.71–0.99	0.1–50.0	0.62–1.00

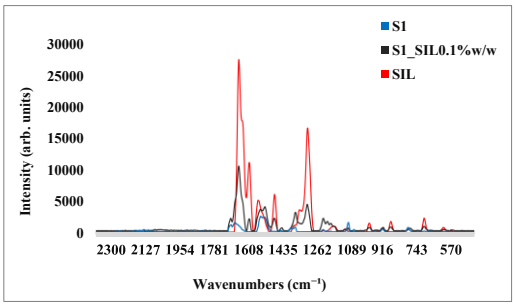
[a] SERS spectra of ACD, HSIL, and SIL are indistinguishable.

5.3.6.2 Analysis of artificially adulterated dietary supplements

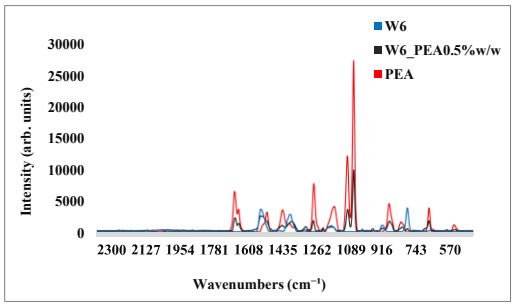
The last phase of this study involved the analysis of 18 artificially adulterated commercial dietary supplements to evaluate the applicability of the developed SERS screening method to real conditions of adulteration and estimate the Limit of Identification (LOI) for the target adulterants. The LOI is calculated to estimate the lowest concentration of the analytes of interest for which correct identifications can be made from a defined database (Massarini et al., 2015); a library match score higher than 0.70 was established as identification criterion to estimate the LOI for each adulterant.

The blank commercial dietary supplements were first analyzed to confirm if they tested negative to the presence of the adulterants of interest and, therefore, to verify that the method does not provide false positive results. Then, the samples were artificially adulterated using the adulterants of interest, at low concentration levels

(range 0.1–5.0% w/w), and the commercial dietary supplements as filling materials. **Figure 5.2** shows the SERS spectra of two blank dietary supplements (S1 and W6), their artificially adulterated forms with SIL and PEA at the LOI levels (0.1 and 0.5% w/w respectively), and the relative match scores with the library reference spectra of the two adulterants, which are also shown in the figure.



Hazard Level	Name	Sample type	Source library	Match Score
Alarm	Sildenafil	Illicit Drug	Adulterants	0.82
Alarm	Acetildenafil	Illicit Drug	Adulterants	0.82
Alarm	Homo sildenafil	Illicit Drug	Adulterants	0.79



Hazard Level	Name	Sample type	Source library	Match Score
Alarm	Phenethylamine	Illicit Drug	Adulterants	0.81

Figure 5.2. SERS spectra of two blank dietary supplements (S1 and W6), their artificially adulterated forms at LOI levels (SIL 0.1% w/w and PEA 0.5% /w), and library match scores with the spectra of the two reference adulterants (SIL and PEA).

Under real conditions of adulteration, the developed screening method showed to be highly effective in detecting all the PDE5I tested (ACD, HSIL, SIL, TSIL, and VAR), with high match scores (0.79–0.99) even at the lowest concentration level tested (LOI of 0.1% w/w). This means that the developed screening method allows for the detection of PDE5I, added as adulterants in dietary supplements, below the concentration levels of the active therapeutic doses of the related pharmaceutical products on the market (Viagra® for sildenafil and Levitra® for vardenafil). Regarding the remaining analytes, the screening method showed to be capable of detecting VIN with a LOI of 0.5% w/w, PEA and PIP with a LOI of 2.5% w/w, and MEL, FLU, and SYN with a LOI of 5.0% w/w. The results are shown in the **Table 5.3**. Eventually, HTP and YOH were excluded from the detection method as, in real conditions of

adulteration, H_2O did not prove to be the suitable diluting solvent as it failed in detecting both analytes in all the commercial dietary supplements used as filling material; this is likely due to the presence of SERS active matrix interferents which cover the analytes signal when the samples are diluted with water.

Table 5.3. Limits of identification (LOIs) of artificially adulterated samples (S1-9 and W1-9), number of commercial dietary supplements where adulterants could be detected, and Pearson's correlation scores.

Analyte	DS[a] type	DS[a] n.	LOI (% w/w)	Library match score
ACD	S1–9	9	0.1	0.79–0.99
FLU	W1–9	4	5.0	0.71–0.83
HSIL	S1–9	9	0.1	0.79–0.99
HTP	W1–9	–	ND[b]	–
MEL	W1–9	5	5.0	0.79–0.93
PEA	W1–9	8	0.5	0.81–0.94
PIP	S1–9	7	2.5	0.70–0.92
SIL	S1–9	9	0.1	0.79–0.99
SYN	W1–9	5	5.0	0.70–0.81
TSIL	S1–9	9	0.1	0.60–0.99
VAR	S1–9	9	0.1	0.81–0.95
VIN	W1–9	9	0.5	0.71–0.98
YOH	S1–9	–	ND[b]	–

[a] Dietary supplements
[b] Non detected

5.4 Conclusions

This study developed a multi-analyte screening method for the rapid detection of illicit adulterants in dietary supplements. The method proved to be highly effective in detecting adulterations of the target PDE5I in sexual and sport performance enhancement supplements at very low concentration levels (0.1% w/w). Regarding the other adulterants covered by this study, it was not possible to detect them in all the tested samples at low concentration levels (the lowest detected concentrations range from 0.5 to 5% w/w). However, the present study demonstrated the great potential of using SERS-based techniques to detect adulterants of different chemical classes in complex food matrices such as dietary supplements. Moreover, the portability of the instrumentation used makes sampling and monitoring in-situ possible. The high selectivity of the silver p-SERS substrates for the tested adulterants and the simplicity of the sample pre-treatment step makes the whole procedure fast, effective, and reachable for all the operators in the field. Furthermore, the possibility of adding new SERS spectra of adulterants to the internal library of the software allows to re-interrogate a previously acquired sample to test its positivity to a new identified adulterant. The developed SERS screening method offers the possibility to quickly identify the presence of the adulterants in a capsule or tablet of dietary supplements, which is a valuable tool for law enforcement and the pharmaceutical industry to combat the occurrence of food fraud in this field.

References

Abbate, V., Kicman, A. T., Evans-Brown, M., McVeigh, J., Cowan, D. A., Wilson, C., Coles, S. J., & Walker, C. J. (2015). Anabolic steroids detected in bodybuilding dietary supplements–a significant risk to public health. *Drug testing and analysis*, *7*(7), 609-618.

Abdullah, R., Diaz, L. N., Wesseling, S., & Rietjens, I. M. (2017). Risk assessment of plant food supplements and other herbal products containing aristolochic acids using the margin of exposure (MOE) approach. *Food Additives & Contaminants: Part A*, *34*(2), 135-144.

Alyami, H., Dahmash, E., Bowen, J., & Mohammed, A. R. (2017). An investigation into the effects of excipient particle size, blending techniques and processing parameters on the homogeneity and content uniformity of a blend containing low-dose model drug. *PloS one*, *12*(6), e0178772.

Avula, B., Wang, Y. H., Wang, M., Smillie, T. J., & Khan, I. A. (2012). Simultaneous determination of sesquiterpenes and pyrrolizidine alkaloids from the rhizomes of Petasites hybridus (L.) GM et Sch. and dietary supplements using UPLC-UV and HPLC-TOF-MS methods. *Journal of pharmaceutical and biomedical analysis*, *70*, 53-63.

Başaran, N., Paslı, D., & Başaran, A. A. (2022). Unpredictable adverse effects of herbal products. *Food and Chemical Toxicology*, *159*, 112762.

Biesterbos, J. W., Sijm, D. T., van Dam, R., & Mol, H. G. (2019). A health risk for consumers: the presence of adulterated food supplements in the Netherlands. *Food Additives & Contaminants: Part A*, *36*(9), 1273-1288.

Brugnerotto, P., Seraglio, S. K. T., Schulz, M., Gonzaga, L. V., Fett, R., & Costa, A. C. O. (2021). Pyrrolizidine alkaloids and beehive products: A review. *Food Chemistry*, *342*, 128384.

Campone, L., Piccinelli, A. L., Celano, R., & Rastrelli, L. (2012). pH-controlled dispersive liquid–liquid microextraction for the analysis of ionisable compounds in complex matrices: Case study of ochratoxin A in cereals. *Analytica Chimica Acta*, *754*, 61-66.

Carpinelli de Jesus, M., Hungerford, N. L., Carter, S. J., Anuj, S. R., Blanchfield, J. T., De Voss, J. J., & Fletcher, M. T. (2019). Pyrrolizidine alkaloids of Blue Heliotrope (Heliotropium amplexicaule) and their presence in Australian honey. *Journal of agricultural and food chemistry*, 67(*28*), 7995-8006.

Carson, C. C., & Lue, T. F. (2005). Phosphodiesterase type 5 inhibitors for erectile dysfunction. *BJU international*, *96*(3), 257-280.

Casado, N., Morante-Zarcero, S., & Sierra, I. (2022a). The concerning food safety issue of pyrrolizidine alkaloids: An overview. *Trends in Food Science & Technology*.

Casado, N., Morante-Zarcero, S., & Sierra, I. (2022b). Application of the QuEChERS Strategy as a Useful Sample Preparation Tool for the Multiresidue Determination of Pyrrolizidine Alkaloids in Food and Feed Samples: A Critical Overview. *Applied Sciences*, *12*(9), 4325.

Celano, R., Piccinelli, A. L., Campone, L., Russo, M., & Rastrelli, L. (2019). Determination of selected pyrrolizidine alkaloids in honey by dispersive liquid–liquid microextraction and ultrahigh-performance liquid chromatography–tandem mass spectrometry. *Journal of agricultural and food chemistry*, *67*(31), 8689-8699.

Cohen, P. A. (2015, October). Vinpocetine: an unapproved drug sold as a dietary supplement. In *Mayo Clinic Proceedings* (Vol. *90*, No. 10, p. 1455). Elsevier.

Cohen, P. A., Travis, J. C., Keizers, P. H., Deuster, P., & Venhuis, B. J. (2018). Four experimental stimulants found in sports and weight loss supplements: 2-amino-6-methylheptane (octodrine), 1, 4-dimethylamylamine (1, 4-DMAA), 1, 3-dimethylamylamine (1, 3-DMAA) and 1, 3-dimethylbutylamine (1, 3-DMBA). *Clinical toxicology*, *56*(6), 421-426.

Costa, J. G., Vidovic, B., Saraiva, N., Do Céu Costa, M., Del Favero, G., Marko, D., Oliveira, N. G., & Fernandes, A. S. (2019). Contaminants: a dark side of food supplements?. *Free radical research*, *53*(sup1), 1113-1135.

Czepielewska, E., Makarewicz-Wujec, M., Różewski, F., Wojtasik, E., & Kozłowska-Wojciechowska, M. (2018). Drug adulteration of food supplements: A threat to public health in the European Union?. *Regulatory Toxicology and Pharmacology*, *97*, 98-102.

De Veij, M., Vandenabeele, P., De Beer, T., Remon, J. P., & Moens, L. (2009). Reference database of Raman spectra of pharmaceutical excipients. *Journal of Raman Spectroscopy: An International Journal for Original Work in all Aspects of Raman Spectroscopy, Including Higher Order Processes, and also Brillouin and Rayleigh Scattering*, *40*(3), 297-307.

Deconinck, E., Cauwenbergh, T., Bothy, J. L., Custers, D., Courselle, P., & De Beer, J. O. (2014). Detection of sibutramine in adulterated dietary supplements using attenuated total reflectance-infrared spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, *100*, 279-283.

Domínguez Díaz, L., Fernández-Ruiz, V., & Cámara, M. (2020). The frontier between nutrition and pharma: The international regulatory framework of functional foods, food supplements and nutraceuticals. *Critical Reviews in Food Science and Nutrition*, *60*(10), 1738-1746.

Dusemund, B., Nowak, N., Sommerfeld, C., Lindtner, O., Schäfer, B., & Lampen, A. (2018). Risk assessment of pyrrolizidine alkaloids in food of plant and animal origin. *Food and Chemical Toxicology*, *115*, 63-72.

Dwyer, J. T., Coates, P. M., & Smith, M. J. (2018). Dietary supplements: regulatory challenges and research resources. *Nutrients*, *10*(1), 41.

EFSA Panel on Contaminants in the Food Chain (CONTAM) (2011). Scientific opinion on pyrrolizidine alkaloids in food and feed. *EFSA Journal*, *9*(11), 2406.

EFSA Panel on Contaminants in the Food Chain (CONTAM) (2017). Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. *EFSA Journal*, *15*(7), e04908.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) (2011). Scientific Opinion on the substantiation of a health claim related to melatonin and reduction of sleep onset latency (ID 1698, 1780, 4080) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006. *EFSA Journal*, *9*(6), 2241.

EFSA Panel on Food Additives and Nutrient Sources Added to Food (ANS). (2013). Scientific Opinion on the evaluation of the safety in use of Yohimbe (*Pausinystalia yohimbe* (K. Schum.) Pierre ex Beille). *EFSA Journal*, *11*(7), 3302.

Ekar, T., & Kreft, S. (2019). Common risks of adulterated and mislabeled herbal preparations. *Food and chemical toxicology*, *123*, 288-297.

El-Shazly, A., & Wink, M. (2014). Diversity of pyrrolizidine alkaloids in the Boraginaceae structures, distribution, and biological properties. *Diversity*, *6*(2), 188-282.

European Commission (2009). Commission Regulation (EC) No 1170/2009 of 30 November 2009 amending Directive 2002/46/EC of the European Parliament and of Council and Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards the lists of vitamin and minerals and their forms that can be added to foods, including food supplements. *Official Journal of the European Union*, *50*, 36.

European Commission (2019). Commission Regulation (EU) 2019/650 of 24 April 2019 amending Annex III to Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards Yohimbe (*Pausinystalia yohimbe* (K. Schum) Pierre ex Beille). *Official Journal of the European Union*, L110, 21–22.

European Commission (2020). Commission Regulation (EU) 2020/2040 of 11 December 2020 amending Regulation (EC) No 1881/2006 as regards maximum levels of pyrrolizidine alkaloids in certain foodstuffs. *Official Journal of the European Union*, L 420/1-5.

European Commission (2022). Commission Regulation (EU) 2022/63 of 14 January 2022 amending Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council as regards the food additive titanium dioxide (E 171). *Official Journal of the European Communities*, *65* (2022) 1–5.

European Food Safety Authority (EFSA) (2012). Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements. *EFSA Journal*, *10*(5), 2663.

European Food Safety Authority (EFSA) (2016). Dietary exposure assessment to pyrrolizidine alkaloids in the European population. *EFSA Journal*, *14*(8), e04572.

European Union (2002). Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. *Official Journal of the European Communities: Legislation*, *45*, 51-57.

European Union (2015). Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. *Official Journal of the European Union*, *327*, 1-27.

Federsalus Research Centre, 2021. VI Indagine di settore: La filiera italiana degli integratori alimentari. Available at https://managefedersalus.it/72630-2/.

Gil, F., Hernández, A. F., & Martín-Domingo, M. C. (2021). Toxic contamination of nutraceuticals and food ingredients. In *Nutraceuticals* (pp. 1145-1158). Academic Press.

Hammad, S. F., Abdallah, I. A., Bedair, A., & Mansour, F. R. (2022). Homogeneous liquid–liquid extraction as an alternative sample preparation technique for biomedical analysis. *Journal of Separation Science*, *45*(1), 185-209.

Hird, S. J., Lau, B. P. Y., Schuhmacher, R., & Krska, R. (2014). Liquid chromatography-mass spectrometry for the determination of chemical contaminants in food. *TrAC Trends in Analytical Chemistry*, *59*, 59-72.

Ioset, J. R., Raoelison, G. E., & Hostettmann, K. (2003). Detection of aristolochic acid in Chinese phytomedicines and dietary supplements used as slimming regimens. *Food and Chemical Toxicology*, *41*(1), 29-36.

Kaltner, F., Stiglbauer, B., Rychlik, M., Gareis, M., & Gottschalk, C. (2019). Development of a sensitive analytical method for determining 44 pyrrolizidine alkaloids in teas and herbal teas via LC-ESI-MS/MS. *Analytical and bioanalytical chemistry*, *411*(27), 7233-7249.

Khazan, M., Hedayati, M., Kobarfard, F., Askari, S., & Azizi, F. (2014). Identification and determination of synthetic pharmaceuticals as adulterants in eight common herbal weight loss supplements. *Iranian Red Crescent Medical Journal*, *16*(3).

Kim, H. J., Lee, J. H., Park, H. J., Cho, S. H., Cho, S., & Kim, W. S. (2014). Monitoring of 29 weight loss compounds in foods and dietary supplements by LC-MS/MS. *Food Additives & Contaminants: Part A*, *31*(5), 777-783.

Klein-Júnior, L. C., Vander Heyden, Y., & Henriques, A. T. (2016). Enlarging the bottleneck in the analysis of alkaloids: A review on sample preparation in herbal matrices. *TrAC Trends in Analytical Chemistry*, *80*, 66-82.

Koncz, D., Tóth, B., Roza, O., & Csupor, D. (2021). A systematic review of the European Rapid Alert System for Food and Feed: tendencies in illegal food supplements for weight loss. *Frontiers in pharmacology*, *11*, 611361.

Kopp, T., Abdel-Tawab, M., & Mizaikoff, B. (2020). Extracting and analyzing pyrrolizidine alkaloids in medicinal plants: A review. *Toxins*, *12*(5), 320.

Lee, S. H., Kim, H. Y., Back, S. Y., & Han, H. K. (2018). Piperine-mediated drug interactions and formulation strategy for piperine: Recent advances and future perspectives. *Expert opinion on drug metabolism & toxicology*, *14*(1), 43-57.

Lehotay, S. J., Sapozhnikova, Y., & Mol, H. G. (2015). Current issues involving screening and identification of chemical contaminants in foods by mass spectrometry. *TrAC Trends in Analytical Chemistry*, *69*, 62-75.

Louisse, J., Mulder, P. P., Gerssen, A., Stoopen, G., Rijkers, D., van de Schans, M. G., & Peijnenburg, A. A. (2022). Bioassay-directed analysis-based identification of relevant pyrrolizidine alkaloids. *Archives of Toxicology*, 1-19.

Ma, C., Liu, Y., Zhu, L., Ji, H., Song, X., Guo, H., & Yi, T. (2018). Determination and regulation of hepatotoxic pyrrolizidine alkaloids in food: A critical review of recent research. *Food and Chemical Toxicology*, *119*, 50-60.

Mädge, I., Gehling, M., Schöne, C., Winterhalter, P., & These, A. (2020). Pyrrolizidine alkaloid profiling of four Boraginaceae species from Northern Germany and implications for the analytical scope proposed for monitoring of maximum levels. *Food additives & contaminants: part a*, *37*(8), 1339-1358.

Magiera, S., & Kwietniowska, E. (2016). Fast, simple and efficient salting-out assisted liquid-liquid extraction of naringenin from fruit juice samples prior to their enantioselective determination by liquid chromatography. *Food chemistry*, *211*, 227-234.

Magnusson, B., & Örnemark, U. (2014). Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics (2nd ed. 2014). *Eurachem*. Available from www.eurachem.org.

Manning, L., Bieniek, M., Kowalska, A., & Ward, R. (2022). Dietary supplements, harm associated with synthetic adulterants and potential governance solutions. *Crime, Law and Social Change*, *78*(5), 507-533.

Massarini, E., Wästerby, P., Landström, L., Lejon, C., Beck, O., & Andersson, P. O. (2015). Methodologies for assessment of limit of detection and limit of identification using surface-enhanced Raman spectroscopy. *Sensors and Actuators B: Chemical*, *207*, 437-446.

Mathon, C., Duret, M., Kohler, M., Edder, P., Bieri, S., & Christen, P. (2013). Multi-targeted screening of botanicals in food supplements by liquid chromatography with tandem mass spectrometry. *Food chemistry*, *138*(1), 709-717.

Menger, F., Gago-Ferrero, P., Wiberg, K., & Ahrens, L. (2020). Wide-scope screening of polar contaminants of concern in water: A critical review of liquid chromatography-high resolution mass spectrometry-based strategies. *Trends in Environmental Analytical Chemistr*y, *28*, e00102.

Micheli, L., Ceccarelli, M., D'Andrea, G., & Tirone, F. (2018). Depression and adult neurogenesis: positive effects of the antidepressant fluoxetine and of physical exercise. *Brain research bulletin*, *143*, 181-193.

Moreira, R., Pereira, D. M., Valentão, P., & Andrade, P. B. (2018). Pyrrolizidine alkaloids: chemistry, pharmacology, toxicology and food safety. *International journal of molecular sciences*, *19*(6), 1668.

Mulder, P. P., López, P., Castelari, M., Bodi, D., Ronczka, S., Preiss-Weigert, A., & These, A. (2018). Occurrence of pyrrolizidine alkaloids in animal-and plant-derived food: results of a survey across Europe. *Food Additives & Contaminants: Part A*, *35*(1), 118-133.

Muschietti, L., Redko, F., & Ulloa, J. (2020). Adulterants in selected dietary supplements and their detection methods. *Drug testing and analysis*, *12*(7), 861-886.

Odoardi, S., Castrignanò, E., Martello, S., Chiarotti, M., & Strano-Rossi, S. (2015). Determination of anabolic agents in dietary supplements by liquid chromatography-high-resolution mass spectrometry. *Food Additives & Contaminants: Part A*, *32*(5), 635-647.

Patel, D. N., Li, L., Kee, C. L., Ge, X., Low, M. Y., & Koh, H. L. (2014). Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: analytical techniques and challenges. *Journal of pharmaceutical and biomedical analysis*, *87*, 176-190.

Pawar, R. S., & Grundel, E. (2017). Overview of regulation of dietary supplements in the USA and issues of adulteration with phenethylamines (PEAs). *Drug testing and analysis*, *9*(3), 500-517.

Petersen, M., Yu, Z., & Lu, X. (2021). Application of Raman spectroscopic methods in food safety: a review. *Biosensors*, *11*(6), 187.

Picron, J. F., Herman, M., Van Hoeck, E., & Goscinny, S. (2018). Analytical strategies for the determination of pyrrolizidine alkaloids in plant based food and examination of the transfer rate during the infusion process. *Food chemistry*, *266*, 514-523.

Pihlström, T., Fernández-Alba, A. R., Gamón, M., Amate, C. F., Poulsen, M. E., Lippold, R., & Anastassiades, M. (2017). Analytical quality control and method validation procedures for pesticide residues analysis in food and feed. *Sante*, *11813*, 21-22.

Rajski, Ł., Petromelidou, S., Díaz-Galiano, F. J., Ferrer, C., & Fernández-Alba, A. R. (2021). Improving the simultaneous target and non-target analysis LC-amenable pesticide residues using high speed Orbitrap mass spectrometry with combined multiple acquisition modes. *Talanta*, *228*, 122241.

Righetti, L., Paglia, G., Galaverna, G., & Dall'Asta, C. (2016). Recent advances and future challenges in modified mycotoxin analysis: why HRMS has become a key instrument in food contaminant research. *Toxins*, *8*(12), 361.

Rocha, T., Amaral, J. S., & Oliveira, M. B. P. (2016). Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review. *Comprehensive reviews in food science and food safety*, *15*(1), 43-62.

Ronis, M. J., Pedersen, K. B., & Watt, J. (2018). Adverse effects of nutraceuticals and dietary supplements. *Annual review of pharmacology and toxicology*, *58*, 583.

Rossato, L. G., de Pinho, P. G., Silva, R., Carmo, H., Carvalho, F., de Lourdes Bastos, M., Costa, M., & Remião, F. (2010). Development and validation of a GC/IT-MS method for simultaneous quantitation of para and meta-synephrine in biological samples. *Journal of pharmaceutical and biomedical analysis*, *52*(5), 721-726.

Ruan, J., Li, N., Xia, Q., Fu, P. P., Peng, S., Ye, Y., & Lin, G. (2012). Characteristic ion clusters as determinants for the identification of pyrrolizidine alkaloid N-oxides in pyrrolizidine alkaloid–containing natural products using HPLC–MS analysis. *Journal of mass spectrometry*, *47*(3), 331-337.

Sarafraz-Yazdi, A., & Amiri, A. (2010). Liquid-phase microextraction. *TrAC Trends in Analytical Chemistry*, *29*(1), 1-14.

Schenck, F. J., Callery, P., Gannett, P. M., Daft, J. R., & Lehotay, S. J. (2002). Comparison of magnesium sulfate and sodium sulfate for removal of water from pesticide extracts of foods. *Journal of AOAC International*, *85*(5), 1177-1180.

Schilter, B., Andersson, C., Anton, R., Constable, A., Kleiner, J., O'Brien, J., Renwick, A. G., Korver, O., Smit, F., & Walker, R. (2003). Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements. *Food and Chemical Toxicology*, *41*(12), 1625-1649.

Schramm, S., Köhler, N., & Rozhon, W. (2019). Pyrrolizidine alkaloids: biosynthesis, biological activities and occurrence in crop plants. *Molecules*, *24*(3), 498.

Shin, D., Kwon, J., Kang, H. S., Suh, J., & Lee, E. (2022). The presence of unauthorized ingredients in dietary supplements: An analysis of the risk warning data in Korea. *Journal of Food Composition and Analysis*, *108*, 104462.

Silano, V., Coppens, P., Larrañaga-Guetaria, A., Minghetti, P., & Roth-Ehrang, R. (2011). Regulations applicable to plant food supplements and related products in the European Union. *Food & function*, *2*(12), 710-719.

Sixto, A., Niell, S., & Heinzen, H. (2019). Straightforward determination of pyrrolizidine alkaloids in honey through simplified methanol extraction (QuPPE) and LC-MS/MS modes. *Acs Omega*, *4*(27), 22632-22637.

Soylak, M., & Yilmaz, E. (Eds.). (2020). New Generation Green Solvents for Separation and Preconcentration of Organic and Inorganic Species. *Elsevier*.

Statista Research Department, 2021. Health and food supplements in Italy - Statistics & facts. Available at https://www.statista.com/topics/5149/health-supplements-market-in-italy/.

Stohs, S. J. (2017). Safety, efficacy, and mechanistic studies regarding Citrus aurantium (bitter orange) extract and p-synephrine. *Phytotherapy Research*, *31*(10), 1463-1474.

Thakkar, S., Anklam, E., Xu, A., Ulberth, F., Li, J., Li, B., Hugas, M., Sarma, N., Crerar, S., Swift, S., Hakamatsuka, T., Curtui, V., Yan, W., Geng, X., Slikker, W., & Tong, W. (2020). Regulatory landscape of dietary supplements and herbal medicines from a global perspective. Regulatory Toxicology and Pharmacology, 114, 104647.

These, A., Bodi, D., Ronczka, S., Lahrssen-Wiederholt, M., & Preiss-Weigert, A. (2013). Structural screening by multiple reaction monitoring as a new approach for tandem mass spectrometry: presented for the determination of pyrrolizidine alkaloids in plants. *Analytical and bioanalytical chemistry*, *405*(29), 9375-9383.

Tighrine, A., Amir, Y., Alfaro, P., Mamou, M., & Nerín, C. (2019). Simultaneous extraction and analysis of preservatives and artificial sweeteners in juices by salting out liquid-liquid extraction method prior to ultra-high performance liquid chromatography. *Food chemistry*, *277*, 586-594.

Tsiokanos, E., Tsafantakis, N., Obé, H., Beuerle, T., Leti, M., Fokialakis, N., & Grondin, A. (2023). Profiling of pyrrolizidine alkaloids using a retronecine-based untargeted metabolomics approach coupled to the quantitation of the retronecine-core in medicinal plants using UHPLC-QTOF. *Journal of Pharmaceutical and Biomedical Analysis*, *224*, 115171.

Turner, E. H., Loftis, J. M., & Blackwell, A. D. (2006). Serotonin a la carte: supplementation with the serotonin precursor 5-hydroxytryptophan. *Pharmacology & therapeutics*, *109*(3), 325-338.

Vaclavik, L., Krynitsky, A. J., & Rader, J. I. (2014a). Mass spectrometric analysis of pharmaceutical adulterants in products labeled as botanical dietary supplements or herbal remedies: a review. *Analytical and bioanalytical chemistry*, *406*(27), 6767-6790.

Vaclavik, L., Krynitsky, A. J., & Rader, J. I. (2014b). Targeted analysis of multiple pharmaceuticals, plant toxins and other secondary metabolites in herbal dietary supplements by ultra-high performance liquid chromatography–quadrupole-orbital ion trap mass spectrometry. *Analytica chimica acta*, *810*, 45-60.

Valente, I. M., Gonçalves, L. M., & Rodrigues, J. A. (2013). Another glimpse over the salting-out assisted liquid–liquid extraction in acetonitrile/water mixtures. *Journal of Chromatography A*, *1308*, 58-62.

Van Den Berg, S. J., Serra-Majem, L., Coppens, P., & Rietjens, I. M. (2011). Safety assessment of plant food supplements (PFS). *Food & function*, *2*(12), 760-768.

Vargas-Murga, L., Garcia-Alvarez, A., Roman-Viñas, B., Ngo, J., Ribas-Barba, L., Van Den Berg, S. J., Williamson, G., & Serra-Majem, L. (2011). Plant food supplement (PFS) market structure in EC Member States, methods and techniques for the assessment of individual PFS intake. *Food & Function*, *2*(12), 731-739.

Venhuis, B. J., & De Kaste, D. (2012). Towards a decade of detecting new analogues of sildenafil, tadalafil and vardenafil in food supplements: a history, analytical aspects and health risks. *Journal of pharmaceutical and biomedical analysis*, *69*, 196-208.

Wen, Y., Li, J., Yang, F., Zhang, W., Li, W., Liao, C., & Chen, L. (2013). Salting-out assisted liquid–liquid extraction with the aid of experimental design for determination of benzimidazole fungicides in high salinity samples by high-performance liquid chromatography. *Talanta*, *106*, 119-126.

Wenzl, T., Haedrich, J., Schaechtele, A., Robouch, P., & Stroka, J. (2016). Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food. In *European Union Reference Laboratory*.

Wheatley, V. M., & Spink, J. (2013). Defining the public health threat of dietary supplement fraud. *Comprehensive Reviews in Food Science and Food Safety*, *12*(6), 599-613.

Yang, Y., Creedon, N., O'Riordan, A., & Lovera, P. (2021, December). Surface Enhanced Raman spectroscopy: Applications in agriculture and food safety. In *Photonics* (Vol. *8*, No. 12, p. 568). MDPI.

Zhang, J., Wu, H., Kim, E., & El-Shourbagy, T. A. (2009). Salting-out assisted liquid/liquid extraction with acetonitrile: a new high throughput sample preparation technique for good laboratory practice bioanalysis using liquid chromatography–mass spectrometry. *Biomedical chromatography*, *23*(4), 419-425.

Zhang, Y. S., Li, J. D., & Yan, C. (2018). An update on vinpocetine: new discoveries and clinical implications. *European journal of pharmacology*, *819*, 30-34.

Zhdanova, I. V., & Tucci, V. (2003). Melatonin, circadian rhythms, and sleep. *Current treatment options in neurology*, *5*(3), 225-229.

Ziegenhagen, R., Heimberg, K., Lampen, A., & Hirsch-Ernst, K. I. (2021). Safety Aspects of the Use of Isolated Piperine Ingested as a Bolus. *Foods*, *10*(9), 2121.

Concluding remarks

The presented work in this thesis is a collection of scientific articles published or submitted in international peer reviewed journals.

Full papers

Rizzo, S., Celano, R., Campone, L., Rastrelli, L., & Piccinelli, A. L. (2022). Salting-out Assisted Liquid-Liquid Extraction for the rapid and simple simultaneous analysis of pyrrolizidine alkaloids and related N-oxides in honey and pollen. *Journal of Food Composition and Analysis*, *108*, 104457.

Rizzo, S., Celano, R., Piccinelli, A. L., Serio, S., Russo, M., & Rastrelli, L. (2023). An analytical platform for the screening and identification of pyrrolizidine alkaloids in food matrices with high risk of contamination. *Food Chemistry*, *406*, 135058.

Rizzo, S., Celano, R., Piccinelli, A. L., Serio, S., Russo, M., & Rastrelli, L. Target screening method for the quantitative determination of 118 pyrrolizidine alkaloids in food supplements, herbal infusions, honey and teas by liquid chromatography coupled to quadrupole Orbitrap mass spectrometry. Submitted to *Food Chemistry*.

Rizzo, S., Weesepoel, Y., Erasmus, S., Sinkeldam, J., Piccinelli, A. L., & van Ruth, S. A multi-analyte screening method for the rapid detection of illicit adulterants in dietary supplements using a portable SERS analyzer. Submitted to *Food Control*.

Poster Presentations

Rizzo, S., Celano, R., Piccinelli, A. L., & Rastrelli, L. A general analytical platform and strategy for the screening and determination of pyrrolizidine alkaloids in food matrices with high risk of contamination using high-resolution mass spectrometry (Q-Orbitrap). 14-23 September 2021, SCI2021 (online event).

Rizzo, S., Celano, R., Piccinelli, A. L., & Rastrelli, L. A general analytical platform and strategy for the screening and determination of pyrrolizidine alkaloids in food matrices with high risk of contamination using high-resolution mass spectrometry (Q-Orbitrap). 20-22 June 2022, MASSA2022, Carlentini, Italy.

Oral Communication

Rizzo, S., Celano, R., Piccinelli, A. L., & Rastrelli, L. An analytical platform for the screening an identification of pyrrolizidine alkaloids in food matrices with high risk of contamination. 5-7 October 2022, 7thMSFoodDay2022, Florence, Italy.

Schools and Courses

1st HR-MS SCHOOL, 2-3 December 2019. The Course was organized by the Department of Chemistry and Biology of the University of Salerno, Fisciano, Italy.

Costruzione di un metodo LCMS: dalla progettazione allo sviluppo del metodo analitico, 30 April, 2020. The Course was organized by DASP S.r.l., Milan, Italy (online event).

24° CORSO DI SPETTROMETRIA DI MASSA, 5-9 October 2020. The Course was organized by the Italian Chemical Society, Division of Mass Spectrometry, Certosa di Pontignano, Siena, Italy.

8th International Course of Advanced Food Analysis, 24-28 January 2022. The Course was organized by the Graduate School VLAG, in cooperation with the laboratories of Food Chemistry and Organic Chemistry of Wageningen University & Research, Wageningen, the Netherlands (online event).

The co-authors' contributions to the articles presented in CHAPTERS 2-5 are specified as follows:

The studies presented were all carried out under the supervision of Prof. Dr. **Anna Lisa Piccinelli** at the Department of Pharmacy of the University of Salerno, Italy. Prof. Piccinelli contributed to the conception of this thesis and always gave valuable and essential advice during the planning of all the laboratory experiments, the interpretation of the results, and the proof-reading of all the manuscripts.

Prof. Dr. **Luca Rastrelli** co-supervised the studies presented in the CHAPTERS 2, 3, and 4, providing counsel and proof-reading of the manuscripts, and was responsible of all the formal aspects.

Prof. Dr. Ir. **Saskia van Ruth** supervised the study presented in the CHAPTER 5; she contributed to the conception of the study, provided counsel and proof-reading of the manuscript, and was responsible of all the formal aspects.

Dr. **Rita Celano** contributed to the design and proof-reading of the study outlined in the CHAPTER 2, and contributed to the evaluation, interpretation, and discussion of the results of the studies presented in the CHAPTERS 2, 3, and 4.

Dr. Ir. **Yannick Weesepoel** co-supervised the study presented in the CHAPTER 5; he contributed to the planning and implementation of all the laboratory experiments and provided proof-reading of the manuscript.

Dr. **Sara Erasmus** provided counsel on creating the manuscript and contributed to the proof-reading of the study outlined in the CHAPTER 5.

Ms. **Simona Serio** assisted in conducting the laboratory experiments of the study outlined in the CHAPTER 3.

Ms. **Carmen Guacci** assisted in conducting the laboratory experiments of the study outlined in the CHAPTER 4.

Mr. **Joost Sinkeldam** assisted in using the scientific equipment and conducting part of the laboratory experiments of the study outlined in the CHAPTER 5.