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Ciclo XXX

# Titolo della tesi

"Tracciabilità dell'origine del latte alpino mediante lo studio del profilo alcaloidico naturale"

"Origin traceability of alpine milk using natural alkaloid profiles"

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**Abbreviations**

ACD: acridone
ACN: acetonitrile
AGC: automatic gain control
AIF: all ion fragmentation
ALK: alkaloid
BIQ: Benzylisoquinoline
BZP: benzophenantridine
CID: collision-induced dissociation
dd-MS/MS: data-dependent MS/MS
EIC: extracted ion chromatogram
ESI: electrospray ionization
FA: formic acid
FID: flame ionization detector
FSA: food standard agency
GC: gas chromatography
HCD: higher-energy collisional dissociation
HQOMS: hybrid quadrupole orbitrap mass spectrometer
HRMS: high resolution mass spectrometry
IND: indole
IQL: isoquinolone
IQN: isoquinoline
IRMS: isotope ratio mass spectrometry
IT: ion trap
LC: liquid chromatography
LOD: limit of detection
LOQ: limit of quantification
MeOH: methanol
MIRS: mid-infrared spectroscopies
MS/MS: mass/mass
NIRS: mid- and near-infrared spectroscopies
NMR: nuclear magnetic resonance
PDO: protected Designation of Origin
PGI: protected geographical indication
PPR: piperidine
PRA: protoalkaloid
PRR: pyrrole
PUR: purine
PYL: pyrrolidine
PYR: pyridine
PYZ: pyrrolizidine
QIT: quadrupole ion trap
QNL: quinoline
QNZ: quinolizidine
QqQ: triple quadrupole
RE: relative error

RSD: relative standard deviation
RT: retention time
S/N: signal-to-noise
SIM: single ion monitoring
SPE: solid-phase extraction
SRM: selected reaction monitoring
STR: steroidal
TIC: total ion current
TRN: terpene
TRP: tropane
UHPLC: ultra high performance liquid chromatography

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**ABSTRACT**

In 2015, 162.8 million tonnes of cow's milk were produced in the EU-28, headed by Germany and France with 33 and 26 million tonnes respectively, while Italy was in seventh place with 11 million tonnes (Eurostat statistics explained), and world milk production is forecast to grow. In a globalised market, demand is growing for secure information about product traceability, and the consumer trend is increasingly moving towards conscientious purchasing, which avoids adulteration and fraud. Traceability is a powerful tool for pursuing different objectives: reinforcing consumers' trust and loyalty to a product, peace of mind to promote product preference, and increasing confidence in the overall food chain quality system. As regards milk, in Italy the Decreto Interministeriale of 9 December 2016 made indication of the origin of raw milk materials on the packaging label mandatory, as stated by Regulation (EU) No. 1169/2011. In the same way, not only is the country of origin important, but also the differentiation between milk from highland and lowland areas, due to the evident implications for food quality. The different dietary regime of animals is the basis of the characteristics distinguishing alpine milk from intensive farming milk, and analytical controls usually exploit these properties.

This work aimed to propose new markers for alpine dairy product traceability by focusing on one of the most abundant and ubiquitous groups of secondary metabolites in plants, namely alkaloids.

In this work we developed a combined targeted and untargeted screening method for alkaloid profiling, using liquid chromatography coupled with high resolution mass spectrometry. Online SPE pre-treatment of herbal and milk sample extracts was proposed in order to reduce the impact of the matrix effect on instrumental response. Quantification of 41 analytes with reference to pure analytical standards, and putative identification of a further 116 alkaloids, confirmed on the basis of accurate mass, isotopic pattern, chromatographic retention time and fragmentation profile, were proposed.

Moreover, the alkaloid profiles of over 60 alpine herbs sampled in two natural pastures in the eastern Italian Alps were defined, providing evidence that alkaloid composition represents an interesting tool for individually characterising plant families, with the most encouraging results for the Poaceae species. The composition and variability of alkaloids ingested by dairy cows grazing on the two grasslands was also investigated, verifying the possibility of discriminating animal diets from different pastures.

Lastly, the variability of alkaloid profiles for milk samples produced by cows grazing on the two pastures was defined and the possibility of discriminating them with regard to pasture origin.

# 1. Introduction

## 1.1. Food authenticity and fraud

Food authenticity is misrepresentation by either mislabelling or adulteration, usually involving lower cost material in the case of food products. Food fraud is a global issue that damages the reputation of companies, hampers markets and reduces consumer confidence. The adulteration of prestigious foods has a high economic impact and deriving problems can be: incorrect description of the geographical or botanical origin of the species, failure to comply with established rules and legislative provisions, and implementation of unacceptable practices. Erroneous description of the name of the food and non-compliance with legal name requirements, adulteration of food or substitution with lower value ingredients, erroneous description of the geographical species and variety or the origin of production, failure to declare certain ingredients or processes in the preparation of foods, and incorrect ingredient quantity declarations are also covered and sanctioned by the European legislative framework (Carcea et al., 2009). The products most affected by this problem and reported in the international fraud databases are: spices and herbs, olive oil, seafood, dairy products, meat and other oils and fats (Weesepoel and van Ruth, 2015). Control measures, including analytical tests, are needed to prevent or discover fraud. Furthermore, the tests need to be improved and updated every time those perpetrating the fraud try to avoid and circumvent these methods. In the past, analytical methods focused mainly on single compound detection, while now they look for a more complete view through fingerprint evaluation. Due to the complexity of data, comparing these fingerprints requires advanced statistical modelling techniques. Although there has been a tendency to seek adulteration with individual components, there is now a trend to guarantee the overall authenticity of a product (van Ruth and Granato, 2017).

Globalisation means that an increasing range of foods are traded around the world and consumers come into contact with a great variety of foods, becoming more and more concerned about the origin of the products they eat. Traceability is a necessary tool for achieving a number of different objectives, helping to build trust, peace of mind, and increase confidence in the food system. Thus several definitions of traceability can be found, with classifications coming from organisations, legislation and research literature. ISO 8402 (1994) quality standards defined traceability as: “the ability to trace the history, application or location of an entity by means of recorded identification”. ISO 9000 (2005) standards extended the definition with ‘‘the ability to trace the history, application

or location of that which is under consideration". ISO guidelines further specify that traceability may refer to the origin of materials and parts, the processing history, and the distribution and location of the product after delivery (Aung, 2014). Three key features for traceability systems were identified: unit/batch identification of all ingredients and products, information on when and where they are moved and processed, and a system that links this data. It must be possible to trace the batch, commercial units and logistics units (Aung, 2014).

### 1.1.1. Milk characterisation and traceability

R.D. 9/5/29 no. 994 indicates that "Milk is the product obtained from regular, uninterrupted and complete milking of animals in good health and nutrition." Established by law, the generic term 'milk' refers to cow's milk, whereas for milk of other origin it is necessary to specify the animal (e.g. goat, sheep, donkey). Milk has a variable energy input, depending on skimming, ranging from 35 to 65 kcal/100 g and coming mainly from lipids (in whole milk) or carbohydrates (in skimmed milk). Fatty acids are generally saturated and carbohydrates predominantly simple glucides (lactose). Milk is particularly rich in riboflavin and vitamin A. With regard to mineral salts, milk contains considerable amounts of calcium and phosphorus, but also microelements such as zinc and selenium.

In 2015, 162.8 million tonnes of cow's milk were produced in the EU-28, headed by Germany and France with 33 and 26 million tonnes respectively, while Italy was in seventh place with 11 million tonnes (Eurostat statistics explained) and world milk production is forecast to grow (Griffin, 2016). In a globalised market, demand is growing for secure information about product traceability, and the consumer trend is increasingly moving towards conscientious purchasing, which avoids adulteration and fraud (Bitzios et al., 2017). As regards milk, in Italy the Interministerial Decree of 9 December 2016 made indication of the origin of raw milk material mandatory on the packaging label, as stated in Regulation (EU) No. 1169/2011. In the same way, not only is the country of origin important, but also differentiation of milk from highland or lowland areas, which is relevant in relation to quality. In particular, 182 Protected Designation of Origin (PDO) and 36 protected geographical indication (PGI) cheeses have been registered (Velcovska and Sadilek 2014).

In order to protect both consumers and honest producers from mislabelling fraud, objective and effective methods capable of identifying the origin of milk have been developed in the last few years. The use of High Resolution Magic Angle Spinning (NMR) spectroscopy was applied to evaluate the quality of cheese such as Parmigiano Reggiano, Emmental and ''Mozzarella di Bufala

Campana'' (Mazzei and Piccolo, 2012), milk terpene fraction analysis was used to discriminate French highland or lowland dairy products (Fernandez et al., 2003), flavonoid and other phenolic analysis was performed as a putative tool for the traceability of dairy products (Hocquette and Gigli, 2005), isotopic and elemental analysis methods were validated to protect against mislabelling PDO cheeses such as Parmigiano Reggiano and Grana Padano (Camin et al., 2015), Laser Induced Breakdown Spectroscopy was used for the detection of milk adulteration (Moncayo et al, 2017) and lanthanides were studied in the traceability of the milk production chain (Aceto et al., 2017).

### 1.1.2. Alpine milk

In the last few years, the link between the landscape and food production has ceased to exist in the mindset of modern industrialised societies. Although the European Union supports farmers by promoting dairy product quality policies, most foodstuffs have seen a drastic reduction in the amount of land necessary for their production, with crop farming being replaced regionally and locally with agribusiness characterised by a highly advanced division of labour and which involves the production, processing, transportation, storage and marketing of foodstuffs (Orland, 2004).

This is not only an environmental problem but also leads to a decline in milk quality. The quality of milk, which can be evaluated with sanitary, dietetic, nutritional and technological criteria, depends on multiple factors and their interaction. Most of these factors, such as fat, protein and lactose composition, their physical and chemical characteristics, as well as micro-compounds present regularly or occasionally, such as minerals, vitamins, minor fatty acids, conjugated linoleic acid, cholesterol and terpenes, depend on the farming system (Morand-Fehr, 2007). It is therefore important to control and protect against grazing milk fraud.

The different dietary regime of animals is the basis of the characteristics distinguishing alpine milk from intensive farming milk, and analytical controls usually exploit these properties. Enrichment or depletion of different stable isotope ratios of elements such as carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), hydrogen (D/H), and oxygen ($^{18}O/^{16}O$) in milk, can often be correlated with the geographical origin of a product (Camin et al. 2012; De la Fuente and Juarez 2005; Drivelos and Georgiou 2012; Kornexl et al. 1997; Manca et al. 2001, 2006; Scampicchio et al. 2012). In particular, the isotopic fractionation of $\delta\ ^{13}C$ is more negative with grass feeding than maize feeding (Balizs et al. 2005; De Smet et al. 2004), while $\delta\ ^{15}N$ can be lower than +4 parts per thousand for alpine products (Bontempo et al. 2012). Focusing on comparisons of feeding regimes, a few biomarkers including fatty acids have been proposed for the authentication of feeding practices (Bargo et al. 2006; Butler

et al. 2009; Collomb et al. 2008) and combining this profiling with multivariate analysis provides more in-depth study of food authenticity and geographical origin (Karoui and De Baerdemaeker 2007; Kim et al. 2014). Recently, by combining different techniques such as isotope ratio mass spectrometry (IRMS), mid- and near-infrared spectroscopy (MIRS and NIRS) and gas chromatography with a flame ionization detector (GC-FID), Scampicchio et al. (2016) classified alpine milk samples from the Tyrol region according to their geographical origin, heat treatment, and season of production.

### 1.1.3. Alkaloids in alpine herbs

The remarkable variability of geomorphological and ecological situations characterising the Alps justifies the presence of a markedly high number of different plant species. In terms of flora, 4,500 vascular plant species are present, representing 39% of European flora (Mörschel et al., 2004). Of plant secondary metabolites, alkaloids (alks), are the most regularly studied, as they play an important role in the interaction of plants with their environment (Fester, 2010). Alks are also responsible for the supposed beneficial effects of plant extracts in natural medicines. Among alpine plants, an initial example could be *Achillea millefolium*, a perennial and aromatic herb of the Asteraceae family, commonly used to treat wounds, digestive problems, respiratory infections, skin conditions, and more specifically, for liver diseases and as a mild sedative (Applequist and Moerman, 2011). Secondly, *Urtica dioica*, a perennial herb of the Lamiaceae family, well-known for its stinging hairs, has been proposed for the treatment of arthritis (Liao et al., 2016). Lastly, *Plantago major*, a herbaceous perennial plant belonging to the Plantaginaceae family, has traditionally been used for haemorrhoid treatment and respiratory diseases and has recently been proposed to aid recovery from skin wounds, burns and bleeding (Zubairab et al., 2015). Some alks have instead a highly toxic effect, in particular pyrrolizidines (Pyz), abundant in *Senecio vulgaris*, a flowering plant belonging to the Asteraceae family (Hartmann, 2007) as well as in *Arnica montana*, another flowering plant of the Asteraceae family, but to a lesser degree (Pabreiter, 1992).

Over ten thousand alks have been isolated from natural sources, the highest alk occurrence being in Ranunculaceae, Berberidaceae, Menispermaceae, Piperaceae, Cactaceae, Papaveraceae, Gentianaceae and Solanaceae (Yang et al., 2009). In many others, there is also high, though less pronounced occurrence, while it is almost zero in families such as Fagaceae, Betulacae, Casuarinaceae and Juglandaceae, suggesting alk occurrence can be treated as a general family characteristic (Li and Willaman, 1968). The supposed plant specificity of alks makes them worth

considering as potential chemical markers for studying the dietary composition of ruminants grazing on alpine pastures. Alks may play a role as tracers in assessing authenticity of origin, reassuring consumers about product identity and process compliance (Danezis et al., 2016; Camin et al., 2016).

### 1.1.4. Alks in milk

Recently, EFSA conducted a study requested by the European Commission, in which a large number of food samples, including milk, were analysed to quantify Pyz alk content, to deliver a scientific opinion on Pyzs in food and feed. The EFSA study reported only low Pyz levels in some milk products analysed, but the project investigated a limited number of samples (EFSA, 2011). Pyz-containing plants are generally unpalatable and normally avoided by grazing animals in the field, but in preserved and composed feed, this recognition is lost and toxic alks may be consumed by livestock (Hoogenboom et al. 2011). Various studies have been carried out regarding the analytical control of such compounds in milk. In 1982 Delnzer et al. (1982) already studied Pyz alk content in goat's milk, and subsequently Molyneux and James (1990) also controlled the presence of this type of alk in milk, while Smallwood et al. (1997) developed a method for the detection of Nicotine, Eserine, Strychnine, Cocaine and Cinchonidine in milk and other food commodities. In the last few years, the main interest has been directed at studying the effect on animals accidentally ingesting toxic alks, expecially Pyzs, and evaluating their transfer from herbs to milk. Patton (1976) showed that Colchicine and Vincristine led to a dramatic decrease in milk flow when infused into the udder of goats. Acute toxicity was observed when animals ingested *C. maculatum* vegetative and flowering plants and seeds (López et al., 1999). The piperidine (Ppr) alks γ-Coniceine and Coniine indeed lead to neuromuscular blockage, resulting in the death of the animal when the respiratory muscles are affected, while Prz alks induce genotoxicity, particularly carcinogenic effects (Fu et al., 2004).

Schumann et al. (2009) examined the effects of ergot contaminated concentrate in fistulated cows fed with a control contaminated diet. The study showed that approximately 67% of the alks fed were recovered in the duodenal ingesta, and 24% were excreted with the faeces. No alk residues could be detected in the blood or milk samples. Further details of the transfer of Pyzs to animal-derived products are reported in the literature, in particular for milk. The majority of alks ingested by cows were excreted through the urine, and the overall transfer to milk was relatively low, at 0.1% (Dickinson et al., 1976; Deinzer et al., 1982). In 2011 Hoogenboom et al. (2011) confirmed

that the transfer of Pyzs to milk reflected the data already reported by Dickinson et al. (1976), despite the fact that the ragwort dosage was 20–100 times lower, but highlighted that the value may be higher when considering only some alks, such as Jacoline (4–7%) and otonecine-type Pyzs.

## 1.2. Alks

With more than 12,000 different structures, alks are one of the most abundant groups of secondary metabolites, identified predominantly in plants (Angiosperms) and less frequently in fungi and animals (Schläger and Dräger, 2016). Alks are basic nitrogen-containing organic compounds deriving from amino acids (true alks, in which nitrogen is incorporated into a heterocyclic system, and proto-alks, where an acyclic unit appears) or arising from amination of another type of substrate, which may be acetate, phenylalanine, terpene or steroid (pseudo or crypto-alks). They are used by plants as storage reservoirs for nitrogen, protective agents for the plant against attack by predators, growth regulators, or as substitutes for minerals, such as potassium and calcium (Waller & Nowacki, 1978), playing an important role in the interaction of plants with their environment (Fester, 2010). Their pronounced biological activity, which is often associated with the presence of amine moieties, is manifested in humans and animals with a marked physiological action, converting the amine function into a quaternary system by protonation at physiological pH (El-Sakka, 2010). As regards this, some alks are considered to be responsible for the beneficial effects of traditional medicines (Bodirlau et al., 2009; Duarte et al., 2010; Chugh et al., 2012; Nilson et al., 2014), but some may have the harmful effects of poisons (Wiedenfeld and Edgar, 2010; Nebo et al., 2014).

Recognition of the biological activity of alks and the different content of these natural products in herbs and plants have made them an attractive field for chemical studies. The earliest articles on alks were published in 1975 and subsequently there have been about 30,000 articles published in many journals, relating to various aspects of alks, their biosynthesis, occurrence, chemistry, pharmacology and toxicology.

The study of alk distribution in plants has allowed taxonomic reclassification of some plant families or orders. The first examples are Papaveraceae and Fumariaceae, previously considered to be part of Rhoedales, because they were found to be rich in 1-benzyltetrahydroisoquinoline alks and poor sources of glucosinolates (Dey and Harborne, 1993), and Caryophyllales, which showed the exclusive presence of betalain (Mabry, 1966).

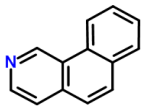
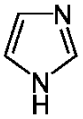
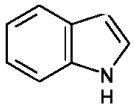
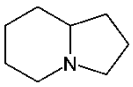
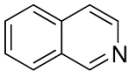
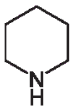
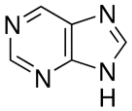
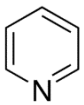
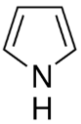
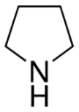
### 1.2.1. Biosynthesis of alkaloids

In 1910, in the book ‘‘Die Alkaloide’’ (1910) Winterstein and Trier had already formulated five principal mechanisms for the biosynthesis of these bases: methylation of imines, amines and amino

acids, methylation of phenols, condensation of phenols and alcohols, condensation of alkamines and condensation with ‘‘formic acid’’. They predicted that 3,4-dihydroxylated phenylethylamine could condense with 3,4-dihydroxylated phenylacetaldehyde, with a loss of water, to form the first alk, which today we call norlaudanosoline (tetrahydropapaveroline), the precursor of more than 2000 isoquinoline bases (Zenk and Juenger, 2007). More sophisticated schemes involving oxidative fission of the catechol ring, leading to intermediates that could explain the formation of the alks Strychnine, Serpentine, Ajmaline, Quinine, Emetine and other structural types of alks were proposed in 1948 (Woodward, 1948). A new hypothesis was formulated in 1957, according to which the oxidation of phenols by one-electron transfer afforded phenolic radicals, which through radical pairing were responsible for new C–C and/or C–O bonds, either intra- or intermolecularly (Barton and Cohen, 1957), explaining the formation of certain plant alks (e.g. salutaridine from Reticuline). Thirty years later, Zenk et al. (1989) found that the biocatalysts involved in this reaction were P450. The introduction of isotopes, in particular of $^{14}$C-specific labelled compound precursors fed to the organism to be metabolised into alk products, led to outstanding successes. These investigations led to the conclusion that most alks derived from a very small number of amino acids, namely tyrosine, phenylalanine, tryptamine, ornithine, lysine and histidine. In certain exceptions, alks arise from ammonia, terpenoid compounds, acetate or amino sugar (Dey and Harborne, 1993).

### 1.2.2. Classification

There are two worthwhile methods for classifying alks, one using chemical structure and one based on their biosynthesis route. Table 2 shows the biosynthetic origin of typical alks, and if present, the characteristic chemical structures.

| Alk type | Biosynthetic origin | Chemical group |
|---|---|---|
| Benzylisoquinoline | Phenilalanine, tyrosine |  |
| Betalain | Phenylalanine, tyrosine, proline | - |
| Carboline | Tryptophan | - |
| Cyanogen | Valine, phenylalanine | - |
| Diketopiperizine | Tryptophan | - |
| Glucosinolate | Homoserine, phenylalanine | - |
| Imidazole | Hstidine |  |
| Indole | Anthranilic acid, tryptamine, tryptophan |  |
| Indolizidine | Lysine, ornithine |  |
| Isoquinoline | Phenilalanine, tyrosine + C10 |  |
| Piperidine | Lysine, Acetate unit |  |
| Purine | Nucleotide catabolism |  |
| Pyridine | Nicotinic acid, putrescine |  |
| Pyrrole | Primary amine |  |
| Pyrrolidine | Ornithine |  |

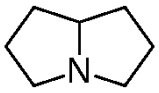
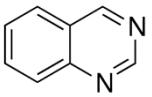
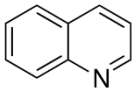
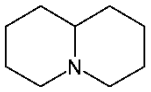
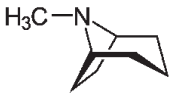
| | | |
|---|---|---|
| Pyrrolizidine | Putrescine |  |
| Quinazoline | Anthranilic acid |  |
| Quinoline | Anthranilic acid, tryptophan |  |
| Quinolizidine | Lysine |  |
| Steroidal | C30 | - |
| Terpene | C15, C20 | - |
| Tropane | Arginine, proline, ornithine |  |

**Table 2.** Alkaloid classification, biosynthetic origin and chemical structure

### 1.2.3. Indole alks

Indoles (Inds) are one of the largest classes of alks, containing more than 4100 different known compounds. Defined by the presence of a structural indole moiety, they are divided into 2 types: non-isoprenoid (simple Ind derivatives, β-carboline and pyroloindole) and isoprenoid (ergot, monoterpenoid Ind and bisindole) also called terpenoid-Ind alks. Inds are synthesized by several plant families, including Strychnaceae, Apocynaceae, Rutaceae and Rubiaceae, and are particularly prevalent in plant species such as *Rauvolfia verticillate, Catharanthus roseus, Camptotheca acuminata, Isatis indigotica* and *Lithospermum erythrorhizon*. Ind alks have been used as medicines for a long time, as in the case of Reserpine, from *Rauwolfia serpentine,* used as a hypotensor, or Vinblastine and Vincristine, produced by *C. roseus*, used directly or as derivatives for the treatment of several types of cancer (Huang et al., 2016). Inds can arise in plants either directly from anthranilic acid, or through the initial formation of tryptophan and tryptamine.

In biosynthesis of tryptamine by the shikimate pathway, the heterocyclic part comes from the shikimic acid pathway of plant metabolism (Mentzen et al. 2008; Gerhards et al. 2014; Xu et al. 2014) and the reaction is catalysed by shikimate kinase to generate shikimate-3-phosphate. After the

intermediate products, 5-enolpyruylshikimate-3-phosoate, chorismate acid, anthranilic acid and Indole-3-glycerol phosphate, the Ind compound is generated with a reaction catalysed by the tryptophan synthase α subunit. The tryptophan synthase β subunit catalyses the conversion of Ind into tryptophan, which generates tryptamine with a reaction catalysed by tryptophan decarboxylase (Radwanski et al. 1996). Tryptamine is the precursor of many terpenoid-Ind alks.

Isoprenoid alks, which have a common biosynthesis precursor with isopentenyl diphosphate and dimethylallyl diphosphate, come from the mevalonate (MVA) pathway, which takes place in cytosol and the methylerythritol-phosphate (MEP) pathway in plastids (Burlat et al. 2004, Figure 1.1.). Using different substrates, both produce the same precursors for the biosynthesis of isoprenoids. The central intermediate, strictosidine, is the precursor for several thousand MIAs with powerful biological activities. In the metabolic network, tryptamine, secologanin and strictosidine are the most important precursors (Huang et al., 2016).

Non-isoprenoid Ind alks, in particular simple Ind derivatives such as the biogenic amines tryptamine and 5-hydroxytryptamine (Huang et al., 2016) derived from tryptophan and the cytochrome P450, with the enzymes CYP79B2 and CYP79B3 involved in tryptophan conversion to Indole-3-acetaldoxime. Indole-3-acetaldoxime is converted by CYP71A13 to Indole-3-acetonitrile, and the last step, catalysed by another cytochrome P450 enzyme, multifunctional CYP71B15, acts upon either dihydrocamalexic acid or cysteine-indole-3-acetonitrile (Schuhegger et al. 2007; Moldrup et al. 2013a, 2013b; Figure 1.2.).

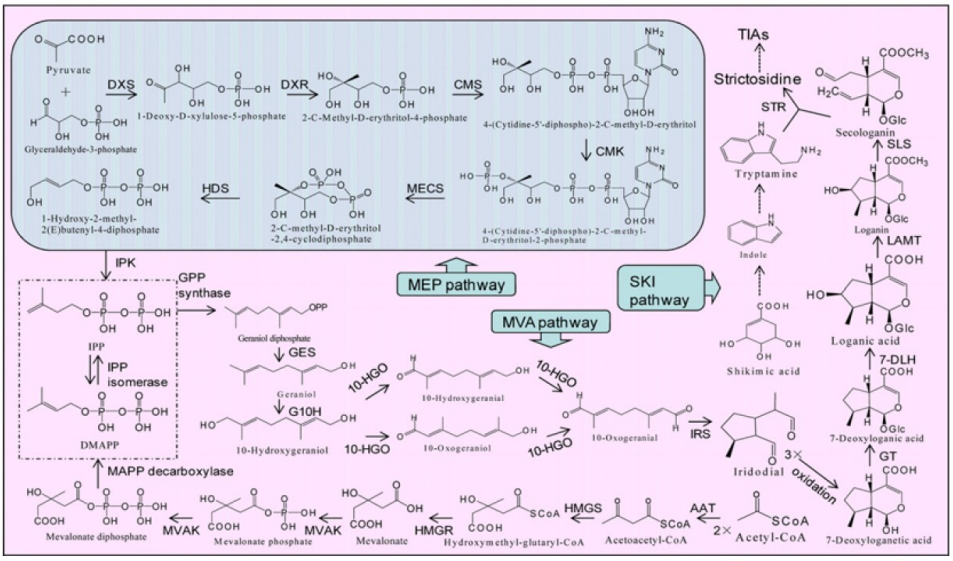



**Figure 1.1.** Biosynthesis of isoprenoid alkaloids (Huang et al., 2016)

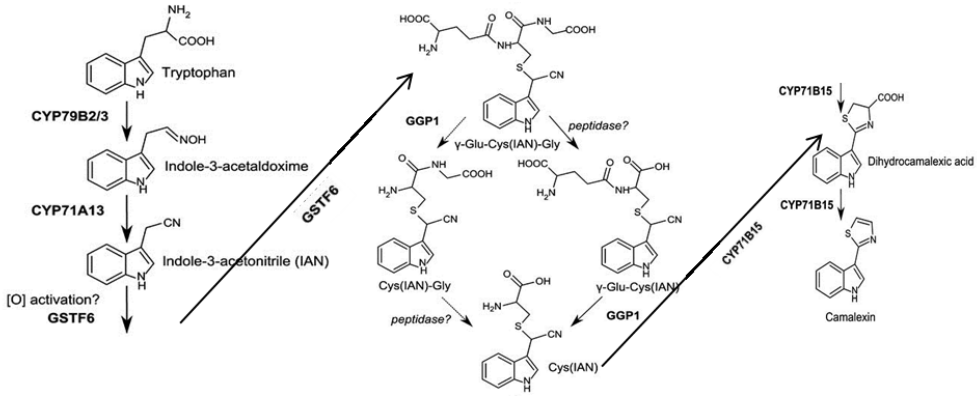



**Figure 1.2.** Biosynthesis of Camalexin a non-isoprenoid alkaloids (adapted from Moldrup et al. 2013a)

### 1.2.4. Pyrrole, pyrrolidine, pyridine and piperidine alks

Pyrrole (Prr) alks represent an important class of biologically active heterocyclic compounds that are also of pharmacological interest. They have a cyclic aromatic system, and differ from other alk types in terms of their non-basic character. Classic approaches to Prr biosynthesis include Hantzsch, Knorr, and Paal–Knorr synthesis, a reaction that generates pyrroles with the participation of a primary amine (Joule and Mills, 2010). There are few Prr alks, with one example being Brunfelsamidine (Pyrrole-3-carboxamide), a convulsant alkisolated from *Brunfelsia grandiflora* and the lethal principle of *Nierembergia hippomanica* (Dey & Harborne, 1993).

Pyrrolidine (Pyl), a tetrahydropyrrole without aromatic structure, again has basic characteristics. The Pyl family is larger and better known as pyrroles, with simple Pyls being Pyrrolidine and N-methylpyrrolidine, found in tobacco. Hygrine has been known for many years as an alk from *Erythroxylon coca*, notorious because it is also the source of Cocaine (Dey & Harborne, 1993). Recently, Hygrine has been studied as a precursor for Tropinone and terpenoid alks such as Hyoscyamine, Scopolamine, and Cocaine (Milen et al., 2014).

Pyridine (Pyr) alks are of historic and economic importance. Examples are Nicotine, Nornicotine, Anabasine and Anatabine, characteristic of the *Nicotiana* species. Nicotine biosynthesis starts with the N-methylation of putrescine to N-methylputrescine, catalysed by putrescine methyltransferase (PMT) (Hashimoto and Yamada, 1994). The gene encoding methylputrescine oxidase, a copper-containing specific diamine oxidase that catalyses the formation of N-methylaminobutanal, was only recently characterised in tobacco (Heim et al., 2007; Katoh et al., 2007). Nicotine is formed by

condensation of N-methylpyrrolinium and nicotinic acid, and can be further metabolised to other alks, such as Nornicotine, Nicotyrine and Myosmine (Figure 1.3.).

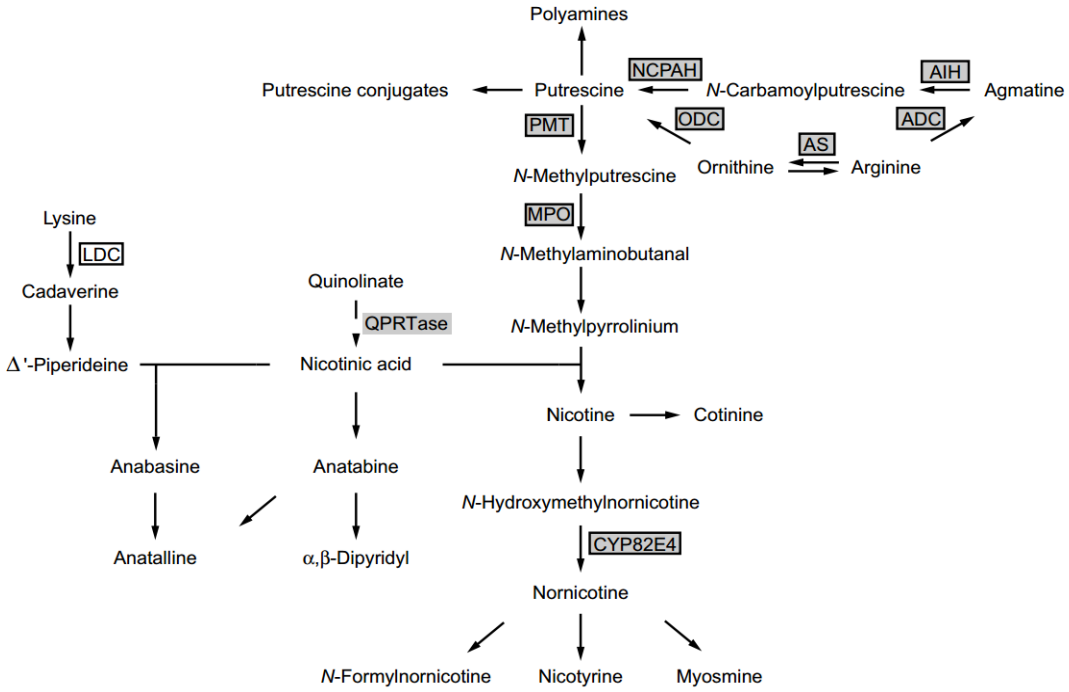



**Figure 1.3.** Pyridine alkaloids biosynthesis in *Nicotiana tabacum* (Häkkinen et al., 2007)

Pprs are various important alks that essentially have the piridine nucleus. The best known are Coniine, the toxic principle of poison hemlock, *Conium maculatum L.* (Umbelliferae), also found in the seeds of *Cicuta maculata L.* (Apiaceae), Lobeline obtained from the herb and seeds of *Lobelia inflata L.* (Lobeliaceae) and from the leaves of *Lobelia tupa L.* (Campanulaceae), Lobelanine, the most abundant alk of *Lobelia inflata L.* (Lobeliaceae) and Piperine, obtained from the dried unripe fruit of *Piper nigrum L.* (Black Pepper), *Piper longum L.*, *Piper retrofractum Vahl.* and *Piper clusii* and also present in the root bark of *Piper geniculatum* (Piperaceae).

An initial hypothesis borne out by many tracer experiments established that lysine or its biological equivalent were the precursor of the pyridine ring (Donovan and Keogh, 1968). Leete (1963) has demonstrated quite a different biosynthetic pathway for Coniine, formed from four acetate units and without the involvement of lysine. Gupta and Spenser (1967) have shown that Sedamine and Lobinaline, though structurally related to Coniine, are instead derived from lysine and phenylalanine (Donovan and Keogh, 1968).

### 1.2.5. Pyz alks

The Pyz alk group includes more than 400 different structures. They are produced by the plant and are believed to be part of its chemical defence against herbivores (Ober and Kaltenegger, 2009). Their presence is restricted to several unrelated angiosperm families, the subfamilies Senecioneae and Eupatorieae within the Asteraceae family, several genera of Boraginaceae, Apocynaceae, Fabaceae and some genera within the Orchid family. In addition, they occur in only a few or even a single species of the Ranunculaceae, Convolvulaceae, Celastraceae, Proteaceae, and Poaceae families (Hartmann and Ober, 2000; Hartmann and Witte, 1995; Koulman et al., 2008).

The structure of Pyzs is characterised by a nitrogen-containing bicyclic ring system, the necine base, which can be esterified with one or more aliphatic mono- or dicarboxylic acids (necic acids). Depending on the structure of the necine base, 4 possible types of Pyz can be found: the retronecine-type, heliotridine-type, otonecine-type and platynecine-type (Figure 1.4). According to the type of esterification, they can be differentiated into monoesters, diesters and macrocyclic diesters (Hartmann and Witte, 1995).

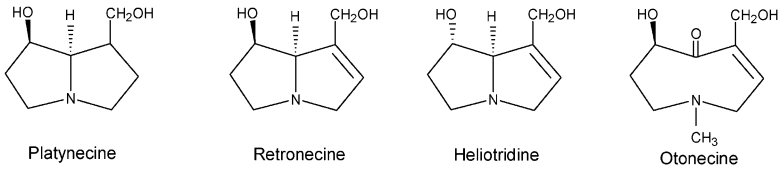



**Figure 1.4.** Common necine bases of pyrrolizidine alkaloids (Fu et al., 2002)

The biosynthesis of Pyz starts with transfer of the amino butyl moiety of spermidine to the diamine putrescine in a $NAD^+$-dependent reaction, catalysed by the enzyme homospermidine synthase. It results in synthesis of the symmetric triamine homospermidine, which shows no degradation but is exclusively incorporated into the necine base moiety of Pyzs (Figure 1.5.).

Ingestion of plants containing Pyzs by humans and animals has been recognised as a serious problem. They indeed cause acute toxicity, chronic toxicity and genotoxicity. Acute poisoning causes massive hepatotoxicity with hemorrhagic necrosis. Chronic poisoning takes place mainly in the liver, lungs and blood vessels, and in some instances in the kidneys, pancreas, gastrointestinal tract, bone marrow and brain (Fu et al., 2002). Serious economic losses are sustained annually due

to the death of livestock which has grazed on land where there are herbs with Pyzs (Dey & Harborne, 1993). Their toxicity is due to the structural features of 1,2-unsaturated Pyzs, which are substrates for cytochrome P450 enzymes located in the liver in vertebrates and insects. The resulting pyrrolic intermediates are cell-toxic, as they react with biological nucleophiles such as proteins and nucleic acids (Fu et al., 2002, 2004).

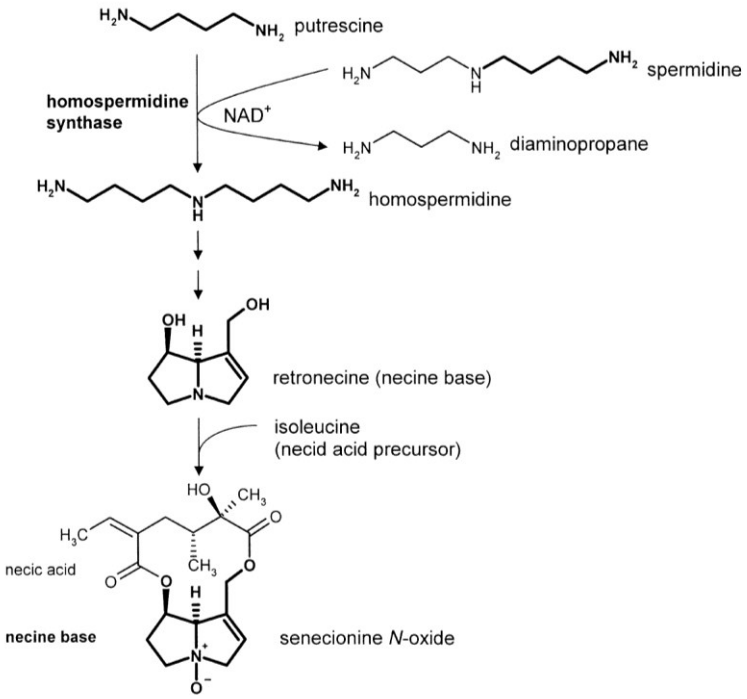



**Figure 1.5.** HSS catalyses the first specific step in the biosynthesis of the necine base moiety common to all Pyrrolizidine alkaloids. Exemplified with senecionine N-oxide (adapted from Reimann et al., 2004)

Despite the toxicity of Pyzs, some have been shown to be therapeutic. These useful alks contain a saturated Pyz nucleus, or are quaternary amines or N-oxides. Platyphylline obtained from *Senecio* spp. was used for treatment of hypertension and internal ulcers, while Indicine N-Oxide and Retronecine N-Oxide derivatives exhibit anti-tumoral activity (Dey & Harborne, 1993).

### 1.2.6. Quinoline, isoquinoline, isoquinolone and benzylisoquinoline alks

The best known quinoline (Qnl) alks are Quinine, Quinidine, Cinchonine and Cinchonidine deriving from *Chinchona*, used in medicine for a considerable time, Quinine as the prototype anti-malarial drug and Quinidine as an anti-arrhythmic drug. Unfortunately, they cause drug-induced

immunological thrombocytopaenia and great care must be exercised in their use (Dey & Harborne, 1993). The quinoline nucleus has also been demonstrated to have a critical role in the development of new anticancer drugs and some of its derivatives showed excellent results on various types of cancer cells, through different mechanisms of action (Afzal et al., 2015).

The majority of Qnls are typically present in Rutaceae and the Qnl nucleus derives from anthranilic acid, a metabolite formed from tryptophan through a sequence of enzymatic reactions, and one molecule of acetate/malonate to give 4-Hydroxy-1-methyl-2-quinolone (Diaz et al., 2015; Figure 1.6.).

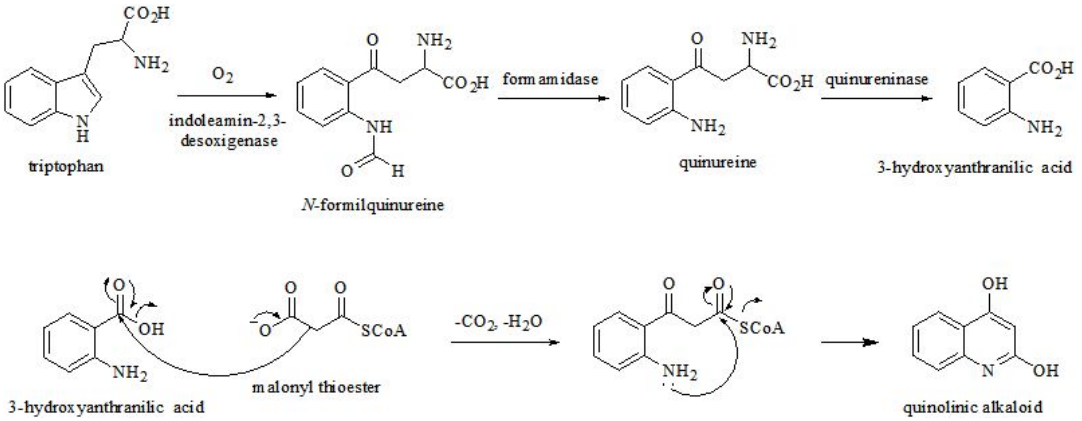



**Figure 1.6.** Biosynthesis of quinoline alkaloids (Diaz et al., 2015)

Isoquinoline (Iqn) and isoquinolone (Iql) alks form a group of about 130 compounds that only have the isoquinoline nucleus at different stages of oxidation. Iqls are probably the catabolic structure of Iqns. The benzylisoquinoline (Biq) group contains 100 different compounds, with the nitrogen ring partially or completely saturated.

The biosynthesis of Iqns proceeds from cyclization of the Schiff base formed by dopamine, obtained from the hydroxylation and decarboxylation of tyrosine, and an aliphatic aldehyde. A second intermediate, p-hydroxyphenyl acetaldehyde, can also be formed by transamination, decarboxylation and hydroxylation of tyrosine. The condensation of these intermediates, followed by a sequence of steps (cyclization, hydroxylation, and methylation) produces (S)-reticuline, a biosynthetic intermediate of all Iqn alks (Figure 1.7.; Diaz et al., 2015). In the case of Biqs the skeleton is formed from two tyrosine units (Dey & Harborne, 1993).

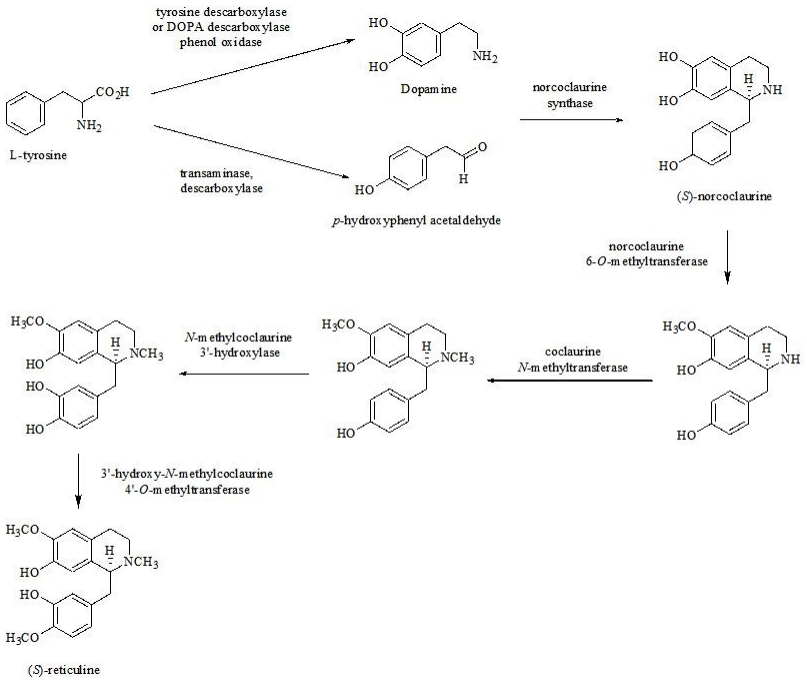



**Figure 1.7.** Biosynthesis of isoquinoline alkaloids (Diaz et al., 2015)

### 1.2.7. Quinolizidine alks

Quinolizidine (Qnz) alks occur mostly within the Leguminosae family, especially in the genera *Lupinus, Baptisia, Thermopsis, Genista, Cytisus* and *Sophora* (Ohmiya et al., 1995) and offer the plants protection against insect pests (Frick et.al, 2017). They are so-called because of their quinolizidine ring structure and can be divided into the following main structural classes: lupanine, angustifoline, lupinine, sparteine, multiflorine, aphylline, anagyrine and cytisine (Frick et al., 2017). Qnz alks are synthesized by the decarboxylation of lisine through the action of LDC, yielding cadaverine, which is further modified by various reactions. Lupinus accumulates two types of Qnz esters, the derivatives of Lupinine and Lupanine, assumed to be end products of biosynthesis and storage forms (Ohmiya et al., 1995). Two acyltransferases involved in ester biosynthesis have been identified: (–)-13α-hydroxymultiflorine/(+)-13α-hydroxylupanine O-tigloyltransferase (HMT/HLT), which catalyses the transfer of the tigloyl group from tigloyl-CoA to (–)-13α-hydroxymultiflorine or (+)-13α-hydroxylupanine, and p-coumaroyl-CoA/feruloyl-CoA: (+)-epilupinine/(–)-lupinine O-coumaroyl/feruloyltransferase (ECT/EFT-LCT/LFT), which catalyses the transfer of p-coumaroyl-CoA or feruloyl-CoA to the hydroxyl moiety of (+)-epilupinine or (–)-lupinine (Bunsupa et al., 2012; Figure 1.8).

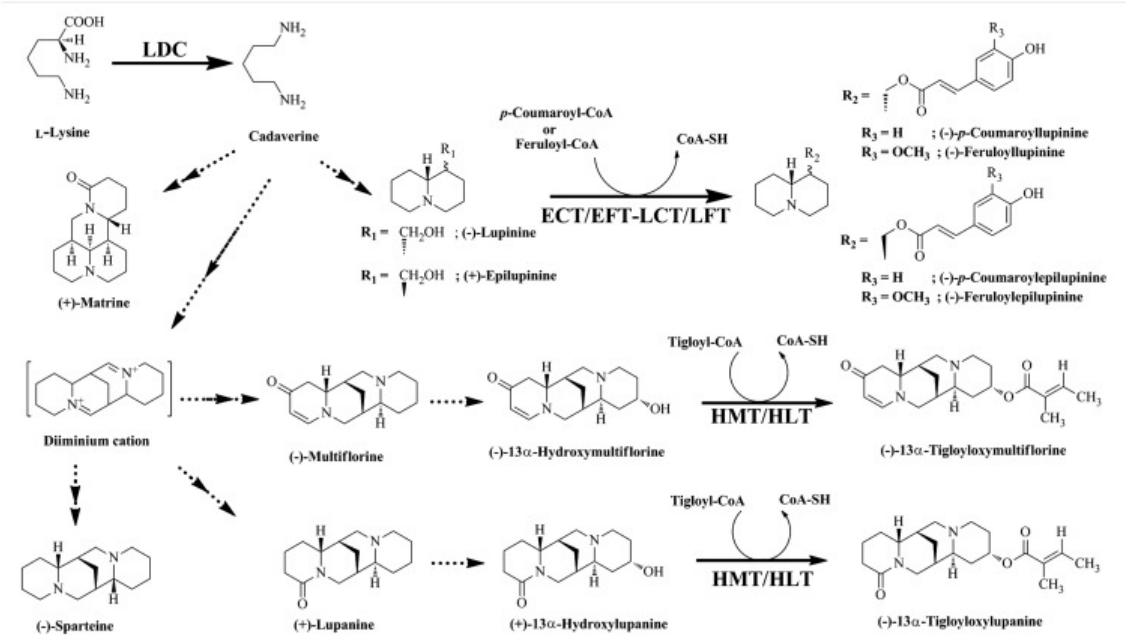



**Figure 1.8.** Biosynthetic pathway of quinolizidine alkaloids (Bunsupa et al., 2012)

High level consumption of plants containing Qnzs may result in acute anticholinergic toxicity, characterised by symptoms such as blurry vision, headache, weakness and nausea (Daverio et al., 2013). The lethal dose of Qnzs in children is estimated to be 11–25 mg total alks per kg of body weight, while no fatal poisonings have been reported in adults (Allen, 1998; Petterson et al., 1998). *L. angustifolius, L. albus, L. luteus* and *L. mutabilis* (Petterson et al., 1998) have recently been used for human food, despite undesirable traits, such as the remaining Qnzs accumulation. The grain, recognised as a health food due to the high protein and fibre content and its nutraceutical properties, has traditionally been used as animal feed (Petterson et al., 1997; Duranti et al., 2008; Sweetingham et al., 2008), although the presence of Qnzs has induced Australia and some European countries to define an legal threshold of 0.02% (Cowling et al., 1998; Boschin et al., 2008; Jansen et al., 2009). Qnzs do not only have toxic characteristics, but also a broad range of pharmacological properties, including cytotoxic, oxytocic, antipyretic, antibacterial, antiviral and hypoglycemic activities, as determined with in vivo pharmacological screening (Saito and Murakoshi, 1995). Some Qnz alk-containing plants, for example *Sophora flavescens*, have been used as sources of crude drugs in Chinese–Japanese medicine (KAMPO; Tang and Eisenbrand, 1992).

### 1.2.8. Steroidal and glycosteroidal alks

Steroidal (Str) alks have the basic steroidal (cyclopentanophenanthrene) skeleton with nitrogen incorporated as an internal part of the molecule, in the ring or in the side chain (Dey & Harborne, 1993). They may be divided into six groups based on their occurrence, two of which come from the animal world, while the other 4 have been obtained from plants and are Veratrum, Solanum and Buxus, along with Strs of Apocynaceae alk groups. Str alks are often bound to sugars such as solatriose (galactose-glucose-rhamnose) or chacotriose (glucose-rhamnose-rhamnose). These Str glycoalks are natural toxins commonly present in potato varieties (Gelder et al., 1988).

Veratrum alks represent the most important and medicinally significant class of Str alks. They can be divided into two classes: jerveratrum alks containing 1–3 oxygen atoms and having antiparasitic activity, and ceveratrum alks having a higher level of hydroxylation (7–9 oxygen atoms), which are responsible for the hypotensive activity of the *Veratrum* species (e.g. Germine or Protoverine; Kukula-Koch and Widelski, 2017). The powder of *Veratrum viride* root is used for toothache, the root sliced thin and boiled in vinegar is useful against Herpes milliaris and Protoveratrine is used in treatment of hypertension (Dey & Harborne, 1993).

Str alks can be differentiated into two types of structure: the oxo-aza spiro structure, as in the case of Tomatine from *Solanum lycopersicum* (tomato) and Solasonine from *Solanum melongena* (eggplant), and a cyclic amine structure, as in the case of Solanine and Chaconine from *Solanum tuberosum* (potato). Many alks isolated from *Solanum* spp. show pronounced antibacterial and antifungal activity (Dey & Harborne, 1993).

All these alks are biosynthesized from cholesterol (Figure 1.9.) via C-26 oxidation–amination, C-22 oxidation, C-16 oxidation and glycosylation of the C-3 hydroxy group (Friedman, 2002; Ginzberg et al., 2009; Petersen et al., 1993). The pro-R methyl group on C-25 of cholesterol is utilised during the biosynthesis of Tomatine and Solanine, whereas the pro-S methyl group is utilised during Solasonine biosynthesis (Rocchetti and Russo, 1974).

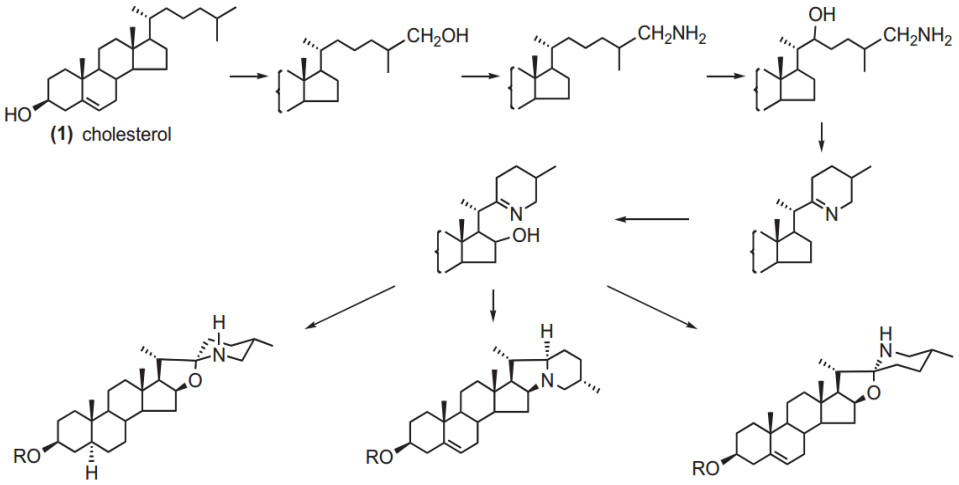



**Figure 1.9.** Biosynthesis of steroidal alkaloids from cholesterol (Ohyama et al., 2013)

### 1.2.9. Terpenoid alks

Terpenoid (Trp) alks are described as pseudo- or crypto-alks and 55,000 have been isolated in nature to date. Biosynthesis of these molecules begins first with prenyl units that are linked together to form phosphorylated hydrocarbon chains of varying lengths. These chains cyclise with Wagner–Meerwein rearrangements, forming a functionalised carbocyclic skeleton in the subsequent oxidase phase. In some cases, they undergo further rearrangement. Following introduction of nitrogen atom(s), the molecule can no longer be considered a terpene and is classified as a Trp-alk (Cherney and Baran, 2011; Figure 1.10.).

The richest source has been shown to be in the Raninculaceae genera *Aconitum, Consolida* and *Delphinium*. The monogeneric Garryaceae family is also a good source of diTrp. Dendrobine and Pumiliotoxins are smaller sesquiTrp-alks, which become increasingly complex as diTrp and triTrp-alks. Some have various types of biological activity, and they have been utilised in traditional medicine in China, Japan, Russia, Mongolia and India (Wang and Liiang, 2002), and as poisons for hunting, and later for homicides (Wang et al., 2010). Methyllycaconitine is considered to be the main agent responsible for the toxicity of larkspurs and causes the majority of cattle deaths in western North America (Pfister et al., 1999).

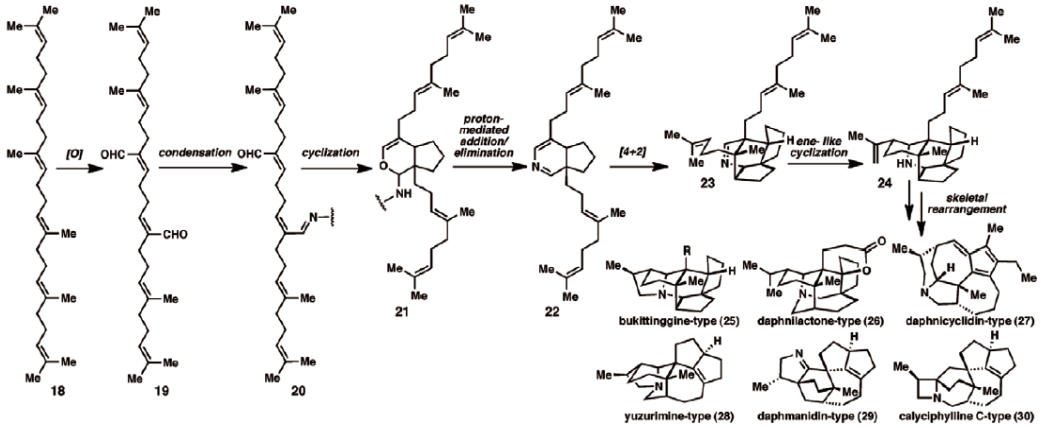



**Figure 1.10.** Proposed biosynthetic pathway for the production of Daphniphyllum alkaloids (Cherney and Baran, 2011)

### 1.2.10. Tropane alks

Tropane (Trp) alks are defined by their core structure, the 8-azabicyclo[3.2.1]octane nucleus. More than 200 structures are reported in the literature (Lounasmaa & Tamminen, 1993), of which Hyosciamine, its racemate Atropine, Hyoscine and Cocaine are the most medicinally important, used clinically on an everyday basis (Dey & Harborne, 1993). Trps are present in five major lineages of dicotyledons: peripheral Eudicots (Proteaceae), Malvid (Brassicaceae) and Fabid (Elaeocarpaceae, Erythroxylaceae, Moraceae, Phyllanthaceae, Rhizophoraceae) clusters of the Rosid lineage, peripheral Asterids (Olacaceae) and the Lamid cluster of the Asterid lineage (Solanaceae, Convolvulaceae) (Jirschitzka et al., 2013). Trps from solanaceous plants, in particular *Datura stramonium*, *Mandragora officinarum*, *Hyoscyamus niger* and *Atropa belladonna*, were important in medicine, witchcraft and divination, and were used as sedatives, sleep-inducing agents, aphrodisiacs and panaceas (Jirschitzka et al., 2013).

As early as 1954, the amino acids ornithine and arginine were predicted to be the starting substrates in the biosynthesis of Trps (Leete et al., 1954), and in 1967 the incorporation of proline into the compounds Tropine and Scopolamine was reported (Liebisch & Schütte, 1967). The amino acids were interconvertible via the shared intermediate pyrroline-5-carboxylate. Labelling studies using [$^{2\text{-}14}$C]- Ornithine have produced conflicting results: symmetrical incorporation at positions C-1 and C-5 of the tropane ring has been reported for *Hyoscyamus albus* and *E. coca*, while asymmetrical labelling (at C-5 only) was reported in *D. stramonium* and *D. metel*. Selective methylation of ornithine could explain the asymmetrical pattern observed in *Datura* (Ahmad & Leete, 1970). The

production of putrescine can take place directly via the decarboxylation of ornithine, or indirectly from arginine. Following methylation, N-methylputrescine is oxidised to the intermediate 4-methylamino butanal, which spontaneously cyclises to yield the N-methyl-$\Delta^1$-pyrrolidinium cation. The first step in Trp alk biosynthesis and the enzymes involved are shown in Figure 1.11.

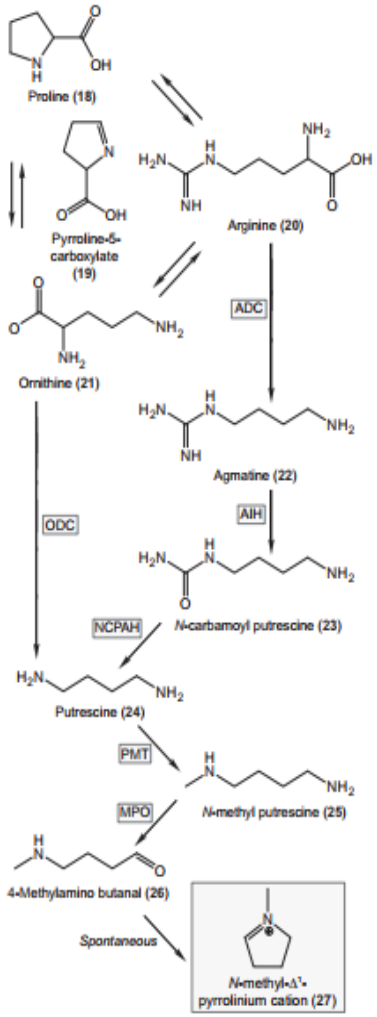



**Figure 1.11.** The first steps in tropane biosynthesis leading to formation of the N-methyl-$\Delta^1$-pyrrolidinium cation (Jirschitzka et al., 2013)

Specific details about how the second ring in the tropane skeleton is formed are not yet available and different substrates for condensation have been proposed. Acetyl-CoA can yield hygrine-1-carboxylate directly or indirectly via acetoacetate, or alternatively, 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoyl-CoA can be formed via decarboxylative condensation of two malonyl-CoA subunits. The keto function at the C-3 position is then reduced and the corresponding alcohol is esterified

using an acyl-CoA substrate. The epoxidation of Hyoscayamine is catalysed from one enzyme in a two-step process (Figure 1.12.; Jirschitzka et al., 2013).

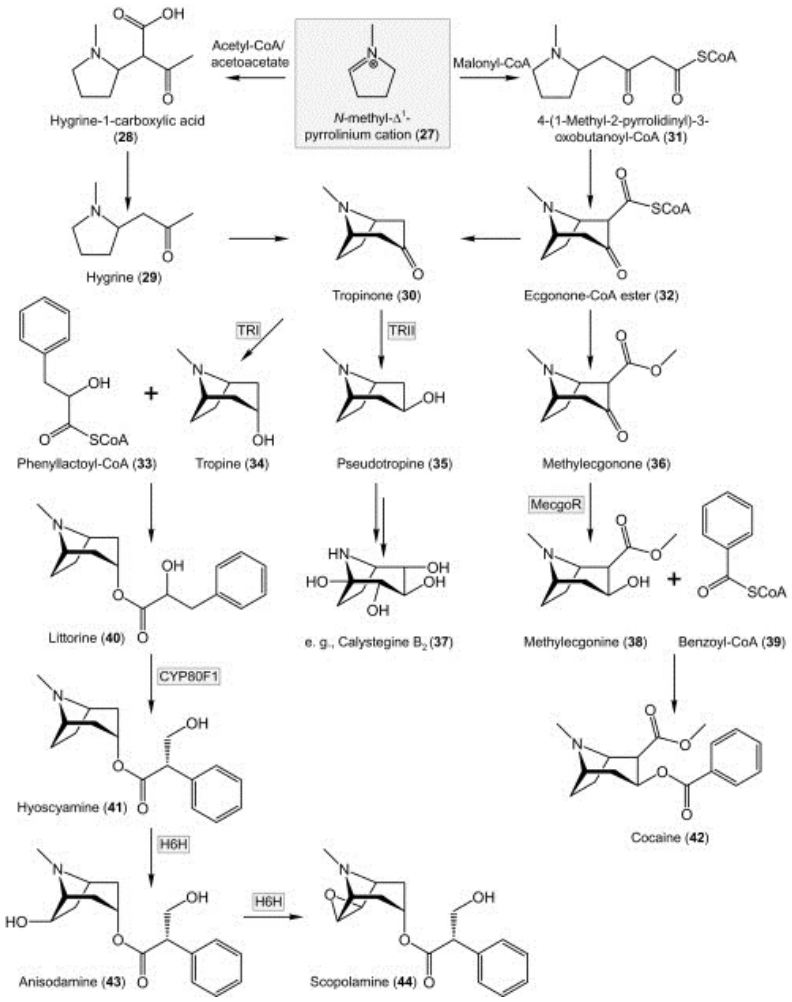



**Figure 1.12.** Mid and late biosynthetic reactions in tropane alkaloid production (Jirschitzka et al., 2013)

### 1.3. Liquid chromatography and mass spectrometry

High Performance Liquid Chromatography (HPLC) was developed in the 1960s, relying on the already developed liquid column chromatography, by improving column technology and instrumental components (pumps, injection valves and detectors) (Nielsen, 2017). During chromatographic separation, the different distribution of the sample components in two different phases, one fixed on a support (stationary phase) and the other passing through (mobile phase) allowed the separation of the analytes contained in a complex mixture. Depending on their nature, the analytes may be bound more or less strongly to the stationary phase and therefore elute with different retention times (RTs). In the case of a gas mobile phase, chromatography is defined as gas chromatography (GC), whereas in the case of liquid it is described as liquid chromatography (LC) (Moldoveanu and David, 2002).

By exploiting spectrochemical, electrochemical or other properties of solutes, analytes may be measured by a variety of instruments, translating sample concentration changes in the HPLC column effluent into electrical signals. The most widely used HPLC detectors are based on ultraviolet-visible (UV-Vis) and fluorescence spectrophotometry, refractive index determination, electrochemical analysis, light scattering and mass spectrometry (MS) (Nielsen, 2017).

HPLC coupled to MS technology has opened the analytical window to thermolabile polar compounds in the last 20 years (Krauss et al., 2010). By 1990, mass spectrometry had evolved as an important tool for solving problems in organic and inorganic chemistry (Nibbering, 2005) discriminating between ions of different *m/z* by subjecting them to constant, pulsed or periodically time-varying electric and/or magnetic fields (Gross, 2004).

In mass spectrometry, compounds are converted into molecular ions through ionization in the source to be analysed:

$$M + e^- \rightarrow M^{\bullet +} + 2e^-$$

Molecular ions obtained through ionization may undergo fragmentation, which in the case of a radical cation with an odd number of electrons can lead to either a radical and an ion with an even number of electrons, or to a molecule and a radical new cationic. The difference between these two types of ions is important and it is therefore also important to indicate them correctly:

$$M^{\bullet+} \rightarrow EE^{+} + R^{\bullet}$$
$$M^{\bullet+} \rightarrow OE^{\bullet+} + N$$

All these ions are distinguished by the mass spectrometer according to their mass-to-charge ratio (*m/z*) and are detected in proportion to their abundance, producing a mass spectrum, a diagram of the abundance of the ions compared to their *m/z*. The most intense peak is called the base peak and is arbitrarily assigned a relative abundance of 100%, while the other peaks are given as percentage proportionate to it. Furthermore, some molecules can also produce multiple charged ions and the precursor ion may fragment to produce fragment ions that provide information about the nature and structure of their molecular precursor.

*m/z* is the dimensional number, which was given a new unit, the Thompson (Th), whose fundamental definition is 1 Th = 1.036 426 x 10-8 kg/C (1 u/e, where e is the charge of an electron = 1.602 177×10−19 C and u is the atomic mass units =1.660 540×10−27 kg) (Hoffmann and Stroobant, 2007).

### 1.3.1. Average mass, nominal mass and monoisotopic mass

In mass spectrometry, average mass, nominal mass or monoisotopic mass can be used. The difference between the three masses can also arrive at several Da, depending on the number of atoms and their isotopic composition. The type of mass determined by the instrument depends largely on the resolution and accuracy of the analyser; monoisotopic mass is used when it is possible to distinguish isotopes, while average mass is used when the isotopes are not distinguishable. The average mass of a molecule is obtained by summing the average atomic masses of the constituent elements. The nominal mass is calculated by taking the weight of the most abundant isotope in nature for each element, and approximating to the nearest total figure corresponding to the mass number. In the case of isotopic mass (exact mass), the most abundant isotype is considered, but without approximation. Indeed, the exact masses of isotopes are not integers, since they differ slightly from the sum of the values for the masses of the constituent particles, which are protons, neutrons and electrons. These differences, called mass defects, are equivalent to the binding energy holding together these particles, and consequently each isotope has a unique and characteristic mass defect that has to be considered (Hoffmann and Stroobant, 2007).

### 1.3.2. Electrospray ionisation

Ionisation is the fundamental step allowing the transfer of the sample from HPLC to the mass spectrometer, by converting the elute into a gas-phase ion (Gross and Capriolli, 2007). The popularity of LC/MS techniques in MS spectrometry has made atmospheric pressure ionisation (API) sources the industry standard. API sources can be configured to operate in any of several API modes, including electrospray ionisation (ESI), heated-electrospray ionisation (H-ESI), atmospheric pressure chemical ionisation (APCI), and atmospheric pressure photo ionisation (APPI). ESI mode, invented by John Fenn at Yale University in the USA (Fenn et al., 1990), transforms ions in solution into ions in the gas phase. The range of molecular weights that can be analysed with ESI is greater than 100,000 u, due to multiple charging. Bulk solution is converted into a tiny, electrically charged droplets by applying a strong electric field to the liquid flowing through a capillary tube with a low flow (normally 1-10 µL/min) at atmospheric pressure (Mann, 1990; Figure 1.13).

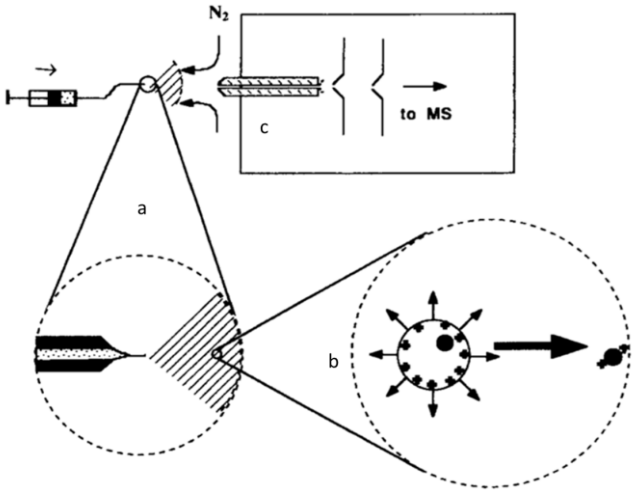



**Figure 1.13.** Representation of the three steps in electrospray ionisation (Mann, 1990)

In order to allow the formation of highly charged droplets, the electric field requires the application of a difference of potential of 3-6 kV between the capillary and the counter electrode. This configuration produces an electric field of the order of 106 V/m. This field induces charge accumulation at the liquid surface located at the end of the capillary, which will break to form highly charged droplets. A gas injected coaxially at a low flow rate allows the dispersion of the spray to be limited in space. These droplets then pass either through a curtain of inert (heated in HESI) gas, usually nitrogen, or through a heated capillary to remove the last solvent molecules. When a small amount of electrically conductive liquid is exposed to an electric field, the liquid

surface shape begins to deform due to loss of surface tension. By increasing the voltage, the effect of the electric field becomes greater and closer to the field and a force acts on the surface tension of the drop, forming a cone starting with a drop and convex tip. When a certain voltage threshold is reached, the slightly rounded tip inverts the voltage and emits a liquid jet referred to as the Taylor cone, the beginning of the electrospray process, in which ions can be transferred to the gaseous phase. From their observations Gomez and Tang concluded that breakage of the droplets can occur before reaching the limit estimated by Rayleigh's equation, because the droplets are mechanically deformed, with a consequential decrease in the repulsion required for their rupture (Gomez and Tang, 1994; Figure 1.13.a).

These small highly charged droplets continue to lose solvent and only when the electrical field on their surface becomes large enough does desorption of the ions occur (Kebarle and Tang, 1993). The excess charges accumulate on the surface of the droplet, while the electrolytes inside the solvent carry positive and negative charges in equal measure (Figure 1.13.b).

The molecular ions are then further desolvated in a counter-current flow of gas and collected inside the vacuum through a capillary. At the time of expansion at the exit of the capillary, the ions can be supplied within a certain energy range by changing the potential output of the capillary. The electron beam is then transmitted to the mass analyser (De Hoffmann and Stroobant, 2007; Figure 1.13.c).

### 1.3.3. Ion suppression and data quality

Ion suppression refers to the reducing of detector response due to ionisation of interfering compounds at the same time as the analyte of interest, which can be detected in traces or not detected at all (Knolhoff and Croley, 2016). In some cases, a correct number of sample dilutions reduces ion suppression and improves detection of the analytes of interest (Stahnke et al., 2012). To avoid this problem, sample extraction procedures are generally used, appropriate chromatography separation is developed, and where possible, high detection resolving power is exploited.

In screening and non-targeted analysis, the sample extraction method should not only be simple, fast and reproducible, but above all non-selective, to recover a wide range of chemically distinct compounds (Vuckovic, 2012). The sample will still be rich in analytes and possible interferences, so the suppression of ions can be prevented or at least reduced by efficient and selective chromatography (Knolhoff and Croley, 2016). Indeed, by minimising chemical noise and the co-

elution of isobaric compounds, ion cloud overlap and distortion of the measured frequency can be prevented (Croley et al., 2012).

### 1.3.4. Quadrupole analyser

A quadrupole mass analyser is one type of mass analyser that uses the stability of the trajectories in an oscillating electric field to separate the ions based on their *m/z* ratio. It is composed of four rods with a circular or ideally hyperbolic cross-section, which must be perfectly parallel to ensure the smooth passage of the ions (Ferguson et al., 1965). Ions travelling along the z axis are subject to the influence of a total electric field generated by the quadrupole, resulting from a constant potential applied to the bars.

$$\Phi_0 = +(U - V\cos\omega t) \text{ and } \Phi_0 = -(U - V\cos\omega t)$$

In this equation, $\Phi_0$ represents the potential applied to the rods, ω the angular frequency (in radians per second = 2πv, where v is the frequency of the RF field), U is the direct potential and V is the 'zero-to-peak' amplitude of the RF voltage. Typically, U will vary from 500 to 2000 V and V from 0 to 3000 V (from −3000 to +3000V peak to peak) (Hoffmann and Stroobant, 2007).

To increase the potential of the analyser, allowing mass/mass (MS/MS) function, three quadrupole analysers are normally used in series, making up the triple quadrupole (QqQ): a quadruple mass filter, an RF-only quadrupole that can be pressurised with a collision gas and a second quadrupole mass filter (Yost, 1979).

### 1.4. High Resolution Mass Spectrometry

Compared to the unit-mass-resolution approach, in the last few years high-resolution mass spectrometry (HRMS) has gained widespread diffusion due to improvement of detection specificity, allowing determination of the accurate mass of molecules (Kaufmann, 2012). Reflectron time-of-flight (TOF) and Fourier transform (FT) high-resolution mass analysers (orbitrap and ion cyclotron resonance) have a broadband resolving power of >10,000 (Marshall & Hendrickson, 2008).
Since 1980, roughly 2,400 studies have been carried out combining liquid chromatography with high-resolution mass spectrometry (LC-HRMS), dealing principally with clinical and forensic toxicology (Jiwan et al., 2011; Himmelsbach, 2012; Meyer and Maurer, 2012; Meyer et al., 2014; Maurer, & Meyer, 2016), omic sciences (proteomics, metabolomics and lipidomics; Gallien, & Domon, 2015; Lesur and Domon, 2015; Ghaste et al., 2016), food safety and control (Botitsi et al., 2011; Kaufmann, 2012; Hernández et al., 2014; Senyuva et al., 2015), and environmental pollution (Hernández et al., 2012; Gosetti et al., 2016).
The superior quality of HRMS means that instruments can provides more information on sample composition through the collection of full-scan spectra and consequently there is the possibility of performing retrospective data analysis (Righetti et al., 2016). Furthermore, HRMS can easily handle applications from targeted quantification to suspect screening and non-targeted experiments (Kaufmann, 2012).

Targeted analysis allows identification and quantification of the compounds of interest using reference standards. With this approach, triple quadrupole (QqQ) or quadrupole ion trap (QIT) have been widely used, thanks to the sensitivity and selectivity provided by selected reaction monitoring (SRM) of precursor-product ion transitions, with the limitation of requiring two transitions to exclude false positive identifications (Krauss et al., 2010). The two transitions limit SRM methods to around 100-150 target analytes dependent on chromatographic separation, due to insufficient temporal peak resolution or excessively short acquisition times respectively for single MS/MS, which results in a loss of precision or sensitivity. Furthermore, some transitions are not specific, such as the neutral loss of H2O or CO2, which are also common for matrix interference. Lastly, some analytes, especially those with low molecular weight, show only one transition, or in some cases, only one fragment shows a sufficiently intense signal, while others are below the limit of quantification (LOQ). The limits of the SRM approach can be overcome with HRMS, which allows

simultaneous detection of all the compounds present in a sample through the use of a full scan approach, without limiting the number of target compounds to be identified. In addition, through MS/MS data acquisition (dd-MS/MS), MS/MS analysis is enabled for each compound included in the target list or present in a minimum detectable quantity. (Krauss et al., 2010). However, HRMS selectivity requires good preceding LC separation in order to prevent co-elution of isobaric compounds, which could not be distinguished if filtered together for dd-MS/MS analysis.

In view of the ability of HRMS to discriminate between possible target compounds without the need for reference standards, the suspect screening approach is increasingly used (Senyuva, 2015). Since ESI predominantly forms $[M+H]^+$ and $[M-H]^-$ ions, identification of these molecules takes place by computing the exact mass starting from the molecular formula of the analytes, building up a database and extracting the ion-chromatogram from the high-resolution full-scan chromatogram. Identification of the compound can be confirmed by comparing the structural information derived from MS/MS with that available for the fragment ion spectra and verifying the correspondence of the isotopic pattern observed with the expected data (Krauss et al., 2010).

The non-targeted screening approach involves identifying unknown molecules without any prior knowledge of the compounds' occurrence. The use of several software packages based on different algorithms allows extraction of the accurate masses from the sample's total ionic current chromatogram (TIC), with deduction of the elementary formula and subsequently identification of the most plausible structure for the relative masses (Krauss et al., 2010). The accuracy measured must be less than 3 ppm and the relative isotopic ratio accuracy less than 5% (Kind and Fiehn, 2007).
A study of plausible structures for a given elementary formula generates an extensive list of compounds whose identification requires comparison of structural information derived from MS/MS with that reported in MS/MS spectral libraries. Due to the lack of reference standards and the difficult comparability of different ionisation sources, information on recorded spectra are however limited (Krauss et al., 2010). Comparison of experimental RT with the logical theoretical log $K_{ow}$, calculated on the basis of the predictive structure of database strikes, may also be useful in unknown structural explanation (Hogenboom et al., 2009).

However, unambiguous identification requires additional or standard reference techniques. For this reason, HRMS is usually combined with nuclear magnetic resonance spectroscopy (1H- and 13C), although these approaches require higher concentrations of the purified unknown compound.

### 1.4.1. Orbitrap

The Orbitrap is a high mass accuracy analyser developed and built by Alexander Makarov at the end of the 20$^{th}$ century (Makarov, 2000), following increasing demand for the analysis of extremely complex mixtures from genomics, proteomics and metabolomics in the field of mass spectrometry innovation (Hu et al., 2005). The Orbitrap mass analyser is similar to the Kingdon trap, as well as to two types of ion-trapping mass analysers, the Paul trap (QIT), and the Fourier transform ion cyclotron resonance instrument, but involves implementation of the orbital trapping method, which can itself be used for mass analysis (Hu et al., 2005).

The Orbitrap (Figure 1.14.) is a not a conventional IT. There is neither RF nor a magnet to hold ions inside, as the moving ions are trapped in an electrostatic field (Scigelova and Makarov, 2006). The mass analyser consists of a central spindle electrode and two surrounding barrel-like electrodes, co-axial with the inner one (Hu et al., 2005). The initial tangential velocity of the ions creates a centrifugal force that compensates the electrostatic attraction to the central electrode, in a very similar way to an orbiting satellite. The electrostatic field forces the ions to move in complex spiral patterns (Makarov, 2000; Hardman and Makarov, 2003). The axial component of these oscillations can be detected as the image current on the two halves of an electrode that encapsulates the orbit. Fourier transform is used to obtain the oscillation frequencies for different mass ions, resulting in accurate reading of their *m/z* (Scigelova and Makarov, 2006).

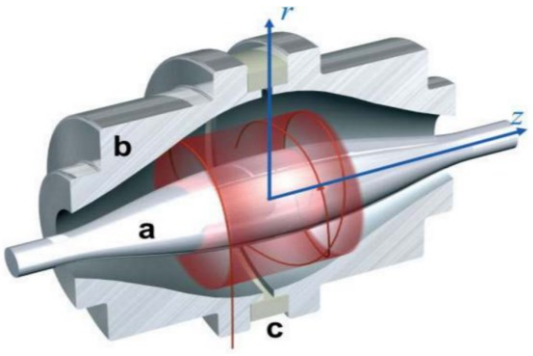



**Figure 1.14.** A cut-away model of the orbitrap mass analyser (a). An outer electrode (b) is split in half by an insulating ceramic ring (c). An image current induced by moving ions is detected via a

differential amplifier between the two halves of the outer orbitrap electrode. The *m/z* of different ions in the orbitrap can be determined from the respective frequencies of oscillation after Fourier transform (Scigelova and Makarov, 2006).

### 1.4.2. Hybrid instrument: Q-Orbitrap

In hybrid instruments (Figure 1.3.), the Orbitrap is combined with a low-resolution mass analyser set between the transfer multipole and the C-trap. This configuration allows isolation of precursor ions from the matrix background and defines the link precursor/product ions in the event of MS/MS experiments. When compared to the classic QqQ MS approach, hybrid Orbitrap ensures higher sensitivity in full-scan mode and accurate mass detection for both precursor and product ions (Gosetti et al., 2016).

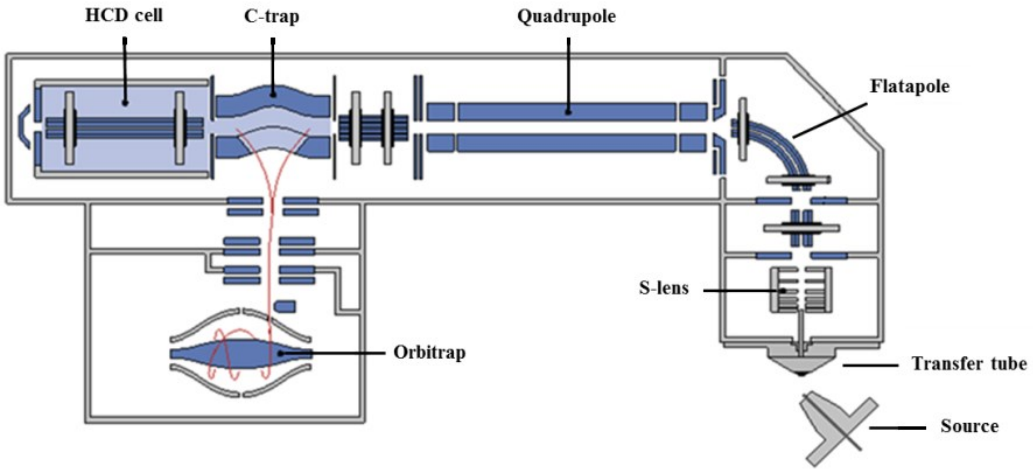



**Figure 1.15.** Hybrid Q-Orbitrap instrument (adapted from *www.planetorbitrap.com*)

Ions are formed at atmospheric pressure (ESI; atmospheric pressure chemical ionisation, APCI; atmospheric pressure photoionisation, APPI), moving through a transfer tube to a stacked-ring ion guide (S-lens) and then via an injection multipole into a bent flatapole (Michalski et al., 2011). Ion clusters and droplets from the S-lens fly unimpeded out of the flatapole, thanks to its 2-mm-distant rods and a short octapole that brings ions into a curved RF-only quadrupole, whose central axis follows a C-shaped arc (so called C-trap; Makarov et al., 2006).

Ions pass through the quadrupole and arrive at the C-trap, a device made up of hyperbolic rods enclosed by two flat lenses. Two lenses are placed at the sides of the C-trap, acting as electrode gates for C-trap ion entrance and ion exit. An innovative characteristic of the hybrid instrument is the automatic gain control (AGC) procedure, in which the low-resolution mass analyser carries out

a pre-scan of ions to determine the ion current within the mass range of interest, enabling storage of a defined number of ions (AGC target value) in the C-trap. Combining the AGC feature with determination of the ion injection time (IT) ensures stability and accuracy for high-resolution *m/z* measurements and allows accurate quantitative analysis (Scigelova and Makarov, 2013).

In the C-trap the ions lose energy in collision with nitrogen gas, without being fragmented thanks to the relatively low pressure of nitrogen (around 1 mTorr) and both the C-trap and the gates are compressed axially by applying 200 V. The ions form a thin thread along the curved axis and the RF voltage applied to the C-trap is then rapidly (over 100-200 ns) ramped down while DC pulses are applied to the electrodes as follows: 1200 V to the push-out electrode (i.e. the electrode furthest from the centre of C-trap curvature), 1000 V to the pull-out electrode (the electrode closest to the centre of curvature), and 1100 V to both the upper and lower electrodes. This voltage distribution forces ions orthogonally to the axis of the C-trap (centre of curvature of the C-trap), where they leave via a slot in the pull-out electrode (Makarov et al., 2006).

Before reaching the ultrahigh vacuum (circa $2 \times 10^{-10}$ mbar) compartment of the Orbitrap, the ions pass through three stages of differential pumping. In this way, by passing through curved ion optics, they are accelerated to high kinetic energy and converge into a tight cloud. In this form, they enter the Orbitrap tangentially through a small aperture on the outer curved electrode. The short transfer distance between the C-trap and the Orbitrap reduces time-of-flight separation, while the vertical displacement of ions through a dual electrostatic deflector avoids gas carryover to the mass analyser (Makarov et al., 2006).

As the ions enter the space between Orbitrap electrodes thanks to a rapid increase in the electric field, they gradually spread into rotating thin rings that oscillate axially along the central spindle electrode for a period proportional to $m/z^{1/2}$ (Makarov and Scigelova, 2010). During injection, narrow spatial (< few mm) and temporal distributions (<100-200 ns) of ions are required, in order to ensure their coherent motion during current signal detection (Perry et al., 2008). After the voltage of central electrode has been stabilised at around 3.5 kV, ion frequencies are measured through acquisition of the time-domain image current and then converted into a mass spectrum with fast Fourier transformation. Finally, mass spectral data can be stored in full- or reduced-profile format, in the latter case removing all data with the same intensity as the thermal noise of the pre-amplifier (Makarov et al., 2006).

Different experiments can be performed with a hybrid Orbitrap configuration, for example the ion pathway described above is characteristic of full MS acquisition mode. Another experiment is target

multiplexed single ion monitoring (targeted-SIM) mode, where selected ions, defined in an 'inclusion list', are filtered by a quadrupole capable of isolating ions with an isolation width ranging from 0.4 to 2.0 *m/z* (Michalski et al., 2011). In the case of targeted-$MS^2$ experiments, the quadrupole is also set to filter interesting precursors, but these ions are fragmented before entering the Orbitrap (Scigelova and Makarov, 2013). From the C-trap, ions are directed into the gas-filled HCD cell, where the required collision energy for ion fragmentation is provided by adjusting the inner axial field and the offset of the RF rods. The offset is negative compared to the C-trap and HCD exit lenses and all fragment ions produced are trapped inside the HCD cell until the end of fragmentation, when all fragments are transferred back into the C-trap and ejected into the Orbitrap (Michalski et al., 2011). Similarly, in full MS / dd-$MS^2$, a full MS scan is followed by selective fragmentation of ions that satisfy pre-defined criteria (data-dependent MS/MS). The all ion fragmentation (AIF) experiment can be performed if the quadrupole is not active and all the ionised molecules arrive in the C-trap and consequently at the HCD, which fragments all the molecules together and sends them into the C-trap and then to the Orbitrap. In full MS / AIF / NL dd-$MS^2$, a full MS scan is followed by an AIF-scan, in order to recognise user-defined *m/z* neutral losses (NL) between the two scan events and to automatically perform data-dependent MS/MS scans on selected precursor ions.

The quadrupole Orbitrap analyser combination enables multiplexed operation at MS and tandem MS level. This allows introduction of multiple precursor ions to the HCD and fragments them at their optimum collision energy, without compromising the storage of preceding injections. The overall ion population can then be transferred back into the C-trap, ejected into the Orbitrap analyser in a single detection cycle. In practice, the useful number of ion injections for single Orbitrap detection is limited by the sum of the individual injection times, being lower than the time for the Orbitrap scan (Michalski et al., 2011).

### 1.4.3. Resolving power and resolution

Hu et al. (2005) defined mass spectrometer resolving power, resolution, mass accuracy, mass range and ion dynamic range as the performance parameters that characterise the Orbitrap. The ability to distinguish between ions with differing mass-to-charge value is called resolving power and is characterised by the possibility to have a peak width (expressed in mass units, on the basis of mass) with at least two points on the peak, specifically at 5% and 50% of the maximum peak height

(IUPAC Gold Book, 2014). Resolving power depends closely on the acquisition time, while it is unaffected by the AGC target value. By fixing the acquisition time, the resolving power diminishes with the increase in ion mass, because the frequency of axial oscillation is inversely proportional to the square root of *m/z* (Makarov et al., 2006).

Resolution is calculated as $m/\Delta m$, where $m$ is the mass of the ion of interest and $\Delta m$ is the peak width or the spacing between two equal intensity peaks, with a valley between them of no more than 10% of their height (Murray et al., 2013). When the resolution is higher, mass analysis time increases, since the scan rate decreases. At a resolution of 17,500, the mass analysis time is 80 ms with a scan rate of 12 Hz, while on increasing the resolution to 140,000 the mass analysis time arrives at 700 ms, with a scan rate of 1.3 Hz.

Depending on the resolution power, it is possible to achieve very accurate ground accuracy with a typical error of <5 ppm for externally calibrated spectra and <2 ppm for internally calibrated spectra (Marshall and Hendrickson, 2008). External calibration is indeed influenced by the instability of the internal electrode potential, due to noise and thermal sensitivity. Thermal regulation of the Orbitrap and its high voltage supply makes it possible to keep mass error below 5 ppm for more than 20 h (Perry et al., 2008).

The mass range is the range of mass-charge ratios (*m/z*) that the instrument can analyse. When modified, the voltage on the central electrode quickly adjusts the amplitude of ionic motions during the axial oscillation period and prevents the loss of ions due to collisions with the external electrode (Marshall & Hendrickson, 2008). The ionic dynamic range is defined as the range within which the ionic signal is linear with the analyte concentration (Makarov et al., 2006).

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## 2. Objectives

The traceability and authentication of dairy products is important to ensure consumer safety and protect the market, while preserving the quality of products. The challenge for scientists is to find analytical methods capable of controlling and tracing the origin of milk. This thesis, which includes published (N=2), submitted (1) and *in litteris* (1) papers, presents a new analytical method for alkaloid analysis in herbal plants and milk, and proposes alkaloids as new interesting markers for dairy product traceability, focusing on:

- Investigation of targeted and untargeted high resolution screening experiments for analysis of alkaloids, and selectivity and sensitivity performance evaluation of this analytical approach in relation to herbs with a high alkaloid concentration;
- Investigation of targeted and untargeted screening of alkaloids in parts of plants used for herbalist purposes, to evaluate alkaloid composition;
- Definition of the targeted and untargeted alkaloid profiles of a wide selection of more than 60 alpine herbs characterising two natural pastures in the eastern Italian Alps;
- The ability of alkaloid profiling to distinguish herbs from different plant families;
- Estimation of the variability of alkaloid composition ingested by dairy cows grazing on the two types of alpine grasslands and discrimination of animal diet from different pastures;
- Definition of targeted and untargeted alkaloid profiles of milk collected from cows grazing in the two natural alpine pastures and investigation of the ability to discriminate milk origin.

## 3. Experimental section and results

### 3.1. Method development

#### 3.1.1. Targeted and untargeted profiling of alkaloids in herbal extracts using online solid-phase extraction and high-resolution mass spectrometry (Q-Orbitrap).

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### 3.1.1. Targeted and untargeted profiling of alkaloids in herbal extracts using online solid-phase extraction and high resolution mass spectrometry (Q-Orbitrap).

**(Herbal extract alkaloid analysis with high resolution mass spectrometry)**

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**Abstract**

The biological activity of alkaloids (ALKs) and the different content of these natural products in herbs and plants have made them an attractive field for chemical studies.

A screening method automatically combining online solid-phase purification and concentration of samples with analysis using ultra-high performance liquid chromatography coupled with a hybrid quadrupole orbitrap mass spectrometer was developed and is reported in this paper. The proposed quantification method was validated for 35 ALKs with reference to pure analytical standards. A further 48 ALKs were identified on the basis of their accurate mass and characterised for chromatographic retention time and fragmentation profile, following their confirmation in extracts of herbs already well documented in the literature. More than 250 other untargeted ALKs were also tentatively identified using literature information, such as exact mass and isotopic pattern. The mass spectrometer operated in positive ion mode and mass spectra were acquired, with full MS-data dependent MS/MS analysis (full MS–dd MS/MS) at a resolution of 140,000.

The method was linear up to an ALK concentration of 1000/3000 μg $L^{-1}$, with $R^2$ always > 0.99 and limits of detection ranging between 0.04-10 μg $L^{-1}$. Accuracy, expressed as the recovery relative error, had a median value of 7.4 %, and precision (RSD %) was generally lower than 10% throughout the quantitation range. The proposed method was then used to investigate the targeted

and untargeted ALK profile of a selection of 18 alpine herbal plants, establishing that pyrrolizidine, pyrrolidine and piperidine ALKs were the most well-represented.

**Key words:** alkaloids; herbal extracts; liquid chromatography; orbitrap; on-line solid-phase extraction.

## 1. Introduction

In the last few decades over ten thousand alkaloids (alkali-like; ALKs), an extremely varied group of natural, nitrogen-containing, basic organic compounds, have been isolated from natural sources, mainly in angiosperms (*Angiospermae* or *Magnoliophyta*)[1].

ALKs have been classified into three principal classes depending on precursors and final molecular structures: atypical, typical and pseudo-ALKs. Typical and atypical ALKs derive from amino acids such as ornithine, arginine, lysine, histidine, phenylalanine and tyrosine[2]. Atypical ALKs are non-heterocyclic compounds, sometimes called 'proto-ALKs' or biological amines, while typical ALKs are heterocyclic compounds that can themselves be classified into the following main groups: pyrrole, pyrrolidine, tropane, pyrrolizidine, piperidine, quinoline, isoquinoline, aporphine, quinolizidine, indole, indolizidine, pyridine, imidazole and purine compounds, according to their ring structure[3]. The third class of molecules, pseudo-ALKs, are basic compounds not deriving from amino acids, to wich diterpene and steroid groups belong[4].

Although ALK functions in plants are not yet fully understood, and even if it has been suggested that they could simply be the waste products of plant metabolic processes[5], their very differentiated chemical nature suggests that they fulfil various specific biological functions. In some plants, the concentration of ALKs increases just prior to seed formation and drops off later when the seed is ripe, suggesting that ALKs may play a triggering role in this process. Some evidence shows that ALKs actively protect plants against pathogen and herbivore attack[6,7], and that they can act as scavengers of reactive oxygen radicals, such as the singlet oxygen $^1O_2$, able to induce very damaging photodegradation processes in plant tissues[8].

Moreover, in addition to fulfilling these specific functions in plants, ALKs often manifest a marked physiological action on humans and animals, acting very quickly on specific areas of their nervous system. Some of them are regarded as responsible for the beneficial effects of traditional medicines[9,10,11,12], but some may instead have the harmful effects of poisons[13,14]. In particular,

pyrrolizidine ALKs have hepatotoxic, mutagenic and carcinogenic effects, and in accordance with the German Federal Institute for Risk Assessment (BfR), a daily intake limit of 0.007 μg $Kg^{-1}$ body weight (0.42 μg for a 60 Kg adult) was established for 1,2- unsaturated pyrrolizidine ALKs[15].

Several determination methodologies have been developed to detect and quantify ALKs in different commodities. Meaningful examples include high pressure liquid chromatography (HPLC) coupled with a diode array detector (DAD)[16] or a fluorimetric detector (FLD)[17], capillary electrophoresis[18] and also gas chromatography coupled with a mass spectrometer (GC-MS) [19,20]. Methods using HPLC–DAD/FLD are not generally sensitive and selective enough to analyse ALKs in traces, while the main limitation of GC-MS approaches is that ALKs cannot be directly analysed, but require time-consuming preventive steps for derivatization.

Last but not least, the complex matrix of plant or herbal extracts can definitely influence determination of ALKs, with suppression of the signal or false positive results. To overcome these problems, most analytical methods pretreat these samples using manual solid phase extraction (SPE), though this purification step is time-consuming and cost-intensive[21,22].

This work aimed to develop a method that would make it possible to investigate the broad profile of ALKs potentially present in herbal plants with a targeted and untargeted approach, by combining automatic online SPE clean-up to reduce matrix interference and the rapid and selective detection ability of hybrid quadrupole orbitrap mass spectrometry.

## 2. Materials and methods

### 2.1. Reagents and solutions

LC-MS grade acetonitrile (ACN), LC-MS grade methanol (MeOH), MS grade formic acid (FA, 98%), LC-MS grade ammonium acetate were purchased from Fluka (St. Louis, MO, USA) and ammonia solution 25% was purchased from Merk Millipore (Darmstadt, Germany). For mass calibration a standard mix of n-butylamine, caffeine, MRFA and Ultramark 1621 (Pierce® ESI Positive Ion Calibration Solution, Rockford, IL, USA) were used. Deionized water was produced with an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany).

Table 1S shows the technical characteristics of commercial ALKs used to implement the target method. Individual stock solutions of each ALK were prepared by dissolving the standard in a 50% aqueous methanol solution to reach a final concentration of about 100 mg $L^{-1}$. An aliquot of 2 mL of the mix solution produced from the single stock solutions, with a final concentration of 3 mg $L^{-1}$

of each single ALK, was transferred into an analytical vial and used for calibration in the range 0.02 – 3000 µg $L^{-1}$. The mix solution was prepared freshly before each analysis, while stock solutions were stored at -4°C.

2.2. Plant sampling and sample extract preparation

Eighteen herbal plants of typical Italian alpine flora were collected directly from mountain pastures in northern Italy. Eight of them were selected on the basis of well-documented ALK composition in the literature. The whole plant was sampled and kept frozen (-10 °C) until required for analytical preparation. Table 1 summarises the botanical characteristics of the plant samples.

Before ALK analysis, each solid plant sample was subjected to extraction using polyethylene 50 mL falcon tubes (Sartorius AG, Goettingen, Germany). A homogeneous aliquot of 2.5 g herb was added to 20 mL of extraction solution ($H_2O$/MeOH/FA; 44.5:44.5:1 v/v/v), sonicated for 10 minutes (LBS1 6Lt, FALC Instruments, Treviglio BG, Italy), and subjected to vertical shaking for 12 hours at 20 rpm (Rotoshake 24/16, Gerhardt GmbH & Co. KG, Königswinter, Germany). The mixtures were once again sonicated for 10 minutes, and the methanolic extract was separated after centrifugation (10 minutes at 4100 rpm; IEC CL31 Multispeed, Thermo Scientific, Sunnyvale, CA, USA). Finally, the extract was filtered with a 0.45 µm cellulose filter cartridge (Sartorius AG, Goettingen, Germany) and diluted 2 times with an ammonia solution (pH=10) before analysis.

2.3. Method development

2.3.1. SPE and chromatographic separation

Chromatographic separation was obtained using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA). A Rheodyne 6-port diverter valve allowed control of two independent fluid systems. The first system was dedicated to online SPE sample processing, while the second controlled chromatographic separation on the analytical column. Chromeleon™ 7.2 Chromatography Data System software (Thermo Scientific™ Dionex™) automatically piloted the switching valve and the chromatographic separation gradient. The autosampler was set at a temperature of 5 °C and the column at 35 °C.

In order to remove matrix interference, according to Barnaba at al.[23], different SPE cartridges were tested: HyperSep™ Retain PEP, 3.0 mm x 10 mm, 40-60 um; HyperSep™ Retain CX, 3.0 mm x 10 mm, 40-60 um; HyperSep™ Hypercarb, 3.0 mm x 10 mm, 40-60 um (Thermo Scientific,

Sunnyvale, CA, USA) and SolEx HRP, 2.1 mm × 20 mm, 12-14 µm, hydrophilic divinylbenzene (ThermoFisher, Sunnyvale, CA, USA).

In order to improve the chromatographic separation of many isomeric ALK compounds, 4 columns were tested: Raptor Biphenyl, 3 mm x 150 mm, 2.7 µm particle size (Restek, Bellefonte, PA, USA), Kinetex PFP, 3 mm x 150 mm, 2.6 µm particle size; Synergi Fusion-RP, 2 mm x 100 mm, 2.5 µm particle size (Phenomenex, Torrance, CA, USA) and Acclaim™ Trinity ™ P1, 2.1 mm x 100 mm, 3 µm particle size (Thermo Scientific, Sunnyvale, CA, USA).

Initially the online SPE UHPLC system operated an injection of sample (1 µL) into the sample loop, being the Rheodyne 6-port diverter valve in position 1-6, while pump 1 flushed the SPE online cartridge with 100% eluent A (4% MeOH with ammonia to pH=9) at 1 ml $min^{-1}$ in order to promote the retaining of ALKs and discharge as much as possible of the interfering matrix. After 1 minute, pump 1 switched to 100% eluent B (0.1% FA) and flushed the cartridge at 1 ml $min^{-1}$ for another minute to complete matrix interference removal. In the meantime, pump 2 conditioned the analytical column at 0.7 ml $min^{-1}$ with 70% of eluent C (0.1% FA with 5mM ammonium acetate) and 30% of eluent D (MeOH/ACN, 95:5 v/v, with 0.1% FA and 5mM ammonium acetate). Subsequently, the diverter valve was switched to position 1-2 and pump 2 eluted the retained analytes from the SPE cartridge to the analytical column in reverse-flow. Chromatographic separation was achieved with eluent D set at 30% from 2 to 4 minutes, then it was linearly increased to 80% from 4 to 25 minutes, and to 100% from 25 to 26 minutes. After 3 minutes at 100%, eluent D was linearly reduced to 30% in 0.5 minutes. Before each injection the analytical column was equilibrated for 2.5 minutes at 30% eluent D with the initial conditions, meanwhile pump 1 flow was set to 0.1 ml $min^{-1}$ and connected to the waste port. During the column rinse step, at 27 minutes, the valve was switched again to the initial 1-6 position and pump 1 flushed the SPE cartridge with 100% eluent E (MeOH with 1% FA) at 1 ml $min^{-1}$ in order to wash it, and then with 100% eluent A to re-equilibrate it before the next analysis.

### 2.3.2. Mass Spectrometry

A Q-Exactive™ hybrid quadrupole-orbitrap mass spectrometer (HQOMS, Thermo Scientific, Bremen, Germany) equipped with heated electrospray ionization (HESI-II) interface was used for ALK analysis. In the HESI II source, nitrogen was used as the drying and collision gas in positive ion mode.

HESI-II tune parameters were set according to the literature, aiming to find an acceptable compromise for optimization of all ALK molecules[24]. The heated capillary temperature was set at 330 °C, while the sheath gas flow rate was set at 30 arbitrary units, auxiliary gas flow rate at 10 arbitrary units, spray voltage at 3.5 kV, and auxiliary gas heater temperature at 300 °C.

Mass spectra were acquired in profile mode through full MS-data dependent MS/MS analysis (full MS–dd MS/MS). Full mass spectra were recorded at a resolution of 140,000 full width at half-maximum (FWHM, calculated for *m/z* 200, 1.5 Hz). The automatic gain control (AGC) target was set at $5 \cdot 10^6$ ions, the maximum inject time (IT) at 100 ms, while data-dependent mass spectra were recorded at a resolution of 17,500 FWHM (defined for *m/z* 200, 12 Hz; AGC target of $2 \cdot 10^5$ ions, IT of 50 ms). In order to obtain high-quality data dependent spectrograms, which could be used to compare the fragments generated with the reference ALK standards and in-house database confirmation fragments, an exclusion duration of 5 s was set in the dd-MS/MS experiment. This was the best compromise in order to avoid any loss of ionic fragment detection, the medium chromatographic peak width being generally 15-20 s. In order to obtain the most informative MS/MS spectra, containing both the precursor ion and fragments, normalized collision energy (NCE) for higher-energy collisional dissociation (HCD) was optimized by direct infusion of each target compound.

Accurate mass calibration was performed with the calibration solution, consisting of n-Butylamine (*m/z* 74.09643), Caffeine (*m/z* 195.08765), MRFA peptide (*m/z* 524.26496) and Ultramark 1621 (characteristic masses: *m/z* 922.01035, 1022.00397, 1121.99758, 1221.99119, 1269.97235, 1321.98481, 1421.97842, 1521.97203, 1621.96564, 1721.95926, 1821.95287, 1921.94648, 2021.94013). Chromeleon™ 7.2 Chromatography Data System software was used for acquisition control and target ALK data processing. In addition, Thermo Fisher Scientific TraceFinder™ software (Thermo Scientific, San Jose, CA, USA) was used for untargeted ALK data processing.

ALK molecules were identified as having a mass accuracy of below 5 ppm. Variability in isotopic pattern recognition was required not to exceed 20%, with at least one of the expected ion fragments being present.

## 2.4. Target method validation

The characteristics of the target ALK method were studied using 35 pure standards. The linearity range was evaluated considering the linear regression between the signal response (peak area) and the nominal concentration of eleven increasing levels from 0.02 to 3000 µg $L^{-1}$, each replicated with

seven different injections, for each ALK. The linearity range was defined as the maximum concentration allowing a correlation coefficient ($R^2$) higher than 0.99, starting from 0.02 μg $L^{-1}$. The limit of detection (LOD) was estimated as three standard deviations of ten replicated blank samples according to EURACHEM[25], and similarly, the limit of quantification (LOQ) was estimated as ten standard deviations of the same replicates.

Precision was estimated as the relative standard deviation (RSD%) of seven analytical replicates of a blank sample spiked at three increasing concentration levels covering the quantitation range of each ALK: 1xLOQ (Low), 2xLOQ (Medium), and 20xLOQ (high concentration) for Protoveratrine A; (1xLOQ), (10xLOQ), (100xLOQ) for Aconitine, alpha Solamargine, alpha Solanine, Harmaline, Senkirkin, Sipeimine, Solasodine, Strychnine and Tomatidine/Tomatine; (5xLOQ), (100xLOQ), (1000xLOQ) for Coniine, Echimidine, Erucifoline, Erucifoline-N-oxide, Jacobine, Jacobine-N-oxide, Jervine, Lycopsamine, Retrorsine N-oxide and Veratramine; (10xLOQ), (200xLOQ), (2000xLOQ) for alpha Solasonine, Heliotrine, Hyoscyamine/Atropine, Lasiocarpine, Monocrotaline, Retrorsine, Scopolamine, Senecionine N-oxide, Senecionine/Senecivernine, Seneciphylline and Veratridine; (20xLOQ), (500xLOQ), (5000xLOQ) for Gramine.

Method accuracy, expressed as relative error (RE%), was estimated as the percentage difference between the expected and the returned mean concentration of the same blank sample, spiked at low, medium and high concentration levels, each one analytically replicated seven times.

### 2.5. Untargeted study

In order to develop an untargeted method useful for ALK profiling of commercial herbal products, initial putative confirmation of the retention time and fragmentation of 48 non-commercially available ALKs was performed, using 8 plant samples with a well-documented ALK composition. Moreover, from systematic survey of the literature it was possible to implement the untargeted method with detailed mass information, producing a final database of 305 ALKs[24,26-37]. ALK name, molecular formula, parent *m/z* mass and, if present, *m/z* fragments are detailed in Tables 2S and 3S.

## 3. Results and discussion

### 3.1. SPE cartridge and analytical column optimization

Different SPE columns and loading/cleaning phases were tested. As regards ALK loading on SPE, a methanolic aqueous phase adjusted with ammonia to pH 9.0 was used[21]. HyperSep™ Retain CX, HyperSep™ Retain PEP, and HyperSep™ Hypercarb did not adequately retain ALKs with MeOH concentrations greater or equal to 4%, while SolEx HRP stopped all ALKs at 4%, although it did not retain Coniine, Monocrotaline and Lasyocarpine at concentrations greater or equal to 5%. Moreover, the elution of ALKs from HyperSep™ Retain CX, HyperSep™ Retain PEP, and HyperSep™ Hypercarb led to chromatographic separation with asymmetric and broad peaks, SolEx HRP being on the contrary characterised by the best chromatographic performance. As regards column cleaning and conditioning for preventing possible carryover between samples, effective SPE cartridge washing was obtained by fluxing for 2 minutes with a methanolic solution at 1% FA after ALK elution.

As regards chromatographic separation, Synergi Fusion-RP did not adequately retain ALKs, while with Acclaim ™ Trinity ™ P1 many chromatographic peaks were broad and irregular. Raptor Biphenyl and Kinetex PFP showed relatively similar performance and adequate chromatographic separation, but the former was more efficient and allowed better separation of isomeric compounds, although not all the target compounds could be individually isolated. The two structural isomers Senecionine and Senecivernine both eluted at 12.63 min, the two enantiomers Atropine and Hyoscyamine both eluted at 12.65 min, and Tomatidine and its glycosylated precursor Tomatine both eluted at 24.55 min. In particular, the parent ion of Tomatine was not detected, probably due to sugar loss in HESI or due to a hydrolysis reaction already occurring in the vial, forcing its identification as its aglyconic form.

### 3.2. Targeted method

The method allowed the quantification of 35 ALKs, using linear calibration curves that always had correlation coefficients ($R^2$) higher than 0.99. The range of quantitation went from the quantification limits to 500 µg $L^{-1}$ for Echimidine and alpha-Solanine; to 1000 µg $L^{-1}$ for Monocrotaline, Lycopsamine, Coniine, Erucifoline, Senecionine N-oxide, Erucifoline-N-oxide, Heliotrine, Senkirkin, Sipeimine, Veratramine, alpha-Solasonine and Solasodine; to 1500 µg $L^{-1}$ for Gramine, Jacobine-N-oxide, Retrorsine, Retrorsine N-oxide, Senecionine/ Senecivernine, Jacobine alpha-Solamargine, Protoveratrine A, Veratridine, Aconitine, Lasiocarpine and Strychnine; to 2000 µg $L^{-1}$ for Scopolamine, Seneciphylline, Hyoscyamine/Atropine, Jervine, Harmaline, and Tomatidine/ Tomatine.

Detectability was strongly dependant on the specific ionization efficiency of each compound and the LOD ranged from the lowest values for Heliotrine (0.04 µg $L^{-1}$), Monocrotaline, Senecionine N-oxide, and Gramine (0.05 µg $L^{-1}$), to the highest for Harmaline, Tomatidine/Tomatine, and Protoveratrine A (3.49, 5.99 and 10.71 µg $L^{-1}$ respectively). The method characteristics, namely, linearity, LOD and LOQ determined for each target ALK compound, are shown in Table 2.

Within-run precision (RSD %) was investigated for each ALK at low, medium and high concentration levels (Low, Medium and High), covering the entire quantitation range (Table 2). Considering the overall group of ALKs, the median precision values were 2.99, 1.60 and 1.02 % at the corresponding low, medium and high concentration respectively, always being lower than 10%, with the exception of Protoveratrine A and alpha-Solamargine (15.4 and 13.3% respectively at the lowest concentrations).

Accuracy, evaluated in terms of relative errors for all ALKs, had median values of 17, 3.3 and 7.5 respectively at the 3 increasing concentration levels (Low, Medium and High), with an overall figure of 7.4% over the entire range of quantitation (Table 2).

### 3.3. Untargeted ALK confirmation

To confirm the correct identification of tropane ALKs, analysis of *Datura stramonium* and *Hyoscyamus niger* was performed. *Datura stramonium* is a very toxic plant in the Nightshade family (Solanaceae). The literature documents the presence of tropane ALKs: 3.6-Diacetyltropine, 3-Acetyltropine, Apohyoscyamine, Hyoscyamine, and Tropinone[34]. In our experiments Hyoscyamine represented the highest signal and the retention time of 12.65 minutes and fragmentation ions (*m/z* 124.1122 and *m/z* 93.0702) found in the untargeted approach were confirmed by standard analysis. The other molecules mentioned above, with the exclusion of Apohyoscamine, were also detectable and chromatographically well separated, allowing the quadrupole to selectively isolate the masses of interest and ascertain the correct fragmentation pattern, while it was only possible to verify the retention time of 17.88 minutes for Apohyoscyamine. The retention times confirmed for 3-Acetyltropine, 3.6-Diacetyltropine, and Tropinone were 3.79, 3.86, and 14.08 minutes respectively.

*Hyoscyamus niger*, commonly known as henbane, black henbane or stinking nightshade, is another poisonous plant of the Solanaceae family, widely cultivated in Europe and Asia[38]. *Hyoscyamus niger* is also a known source of tropane ALKs, such as Atropine, Anisodamine, and Scopolamine[37]. We were able to confirm the presence of Atropine and Scopolamine using the

analytical standards (RT = 12.65 minutes and 10.25 respectively), while for Anisodamine the retention time (10.36 minutes) and fragmentation profile were defined.

Steroidal and glycosteroidal ALKs were investigated in extracts of *Solanum nigrum* (Morella), common name Nightshade. *Solanum nigrum*, a weed native to Eurasia, belongs to the Solanum genus, the largest and most important in the Solanaceae family[39], and was traditionally used for many disorders[33], although recent studies have highlighted the acute toxicity of Solanine, a neurotoxic glyco-ALK[40]. The ALKs *beta*-Solamargine, *alpha*-Solamargine, *alpha*-Solasonine and *alpha*-Solanine are generally present in this plant at a level of a few mg $g^{-1}$[33]. Our plant extract analysis, also in comparison with the standard compounds, confirmed the presence of *alpha*-Solamargine (20.00 minutes), *alpha*-Solanine (21.77 minutes), and *alpha*-Solasonine (RT = 19.83 minutes). The retention time of *beta*-Solamargine was identified as 21.75 minutes, although the low intensity of the signal did not make it possible to confirm the fragmentation.

For piperidine-type ALKs we analysed an extract of *Lobelia inflata,* a plant belonging to the Campanulaceae family. The most important ALK of this plant is Lobeline, but it also contains 8,10-Diethyllobelidiol, 8-Ethyl-10-phenylnorlobelidione, 8-Ethylnorlobelol, 8-Methyl-10-phenyllobelidiol, Allosedamine, Isolobinanidine, Isolobinine, Lelobanidines I and II, Lobelanine, Lobelanidine, Lobinaline, Lobinanidine, Lobinine, Norallosedamine, Norlelobanidine, Norlobelanidine, and Norlobelanine[29]. However, in our study on *Lobelia inflata* extracts, we could not detect 8-Ethyl-10-henylnorlobelidione, Allosedamine, and Norlobelanidine. The extracted ion chrogmatogram (EIC) referred to the two isomers, 8-Methyl-10-phenyllobelidiol and Norlelobanidine in the literature, corresponded to three peaks, suggesting the existence of a possible unidentified isomer. In consideration of the strong similarity of the molecular ion fragmentation spectra with the most abundant fragment accurate mass *m/z* 156.1378, the three peaks were tentatively labelled as 8-Methyl-10-phenyllobelidiol/Norlelobanidine (RT = 15.53, 16.08 and 16.6 minutes). Similarly, retention times were putatively assigned to the two pairs of isomers Isolobinine/Lobinine and Lelobanidine I/Lelobanidine II (16.84 and 19.99; 16.91 and 17.41 minutes). The EIC of the two isomers Isolobinanidine and Lobinanidine corresponded to three peaks having similar data-dependent spectra and retention times - 16.23, 17.40 and 17.64 minutes respectively - but the existence of a third isomer, *beta*-Lobinanidine, has also been confirmed by the literature[41]. The EICs of Lobelanidine, Lobelanine and Lobeline showed two peaks for each of these ALKs (RT = 20.17, 20.86 minutes; 20.66, 21.15 and 20.23, 20.54 respectively). The presence of two *cis/trans* forms for Lobelanidine is reported by Felpin & Lebreton[29], making it reasonable

to also surmise the existence of similar *cis/trans* isomers for Lobelanine and Lobeline. The EIC of 8,10-Diethyllobelidiol showed two peaks at 12.05 and 13.07 minutes, suggesting the existence of other possible isomers, while the EICs of 8-Ethylnorlobelol, Lobinaline, Norallosedamine and Norlobelanine had only one peak at 5.60, 28.49, 19.76 and 20.89 minutes respectively.

To confirm the correct identification of pyrrolizidine and pyrrolidine ALKs, *Senecio vulgaris* and *Arnica montana extracts* were analysed. *Senecio vulgaris,* a tenacious annual herb present in worldwide habitats, belongs to the Asteraceae family. It contains toxic pyrrolizidine ALKs such as Integerrimine, Integerrimine-N-oxide, Retrorsine, Riddelline, Riddelline-N-oxide, Senecionine, Seneciphylline, Seneciphylline N-oxide, Spartioidine, Spartioidine-N-oxide, Usamarine, and Usamarine-N-oxide[21,42]. Retrorsine (RT= 10.73 minutes), Seneciphylline (11.40 minutes), and Senecionine (12.63 minutes), were confirmed in comparison with the analytical standards, while Riddelline-N-oxide and Usamarine-N-oxide were not found. Due to the presence of several isobaric compounds, assignment of the correct retention times to other ALKs was sometimes very complicated. The EIC corresponding to Senecionine, Integerrimine and Senecivernine, showed only one chromatographic peak at 12.63 minutes in this extract, possibly of three coeluted ALKs. The EIC of the 6 isomers Senecionine-N-oxide (RT = 8.97 minutes), Retrorsine (10.73 minutes), Jacobine (14.05 minutes), Integerrimine-N-oxide, Senecivernine-N-oxide, and Usamarine, showed 4 peaks, the one at 11.22 minutes being different to those of the target compounds. Riddelline, Seneciphylline N-oxide and Spartioidine-N-oxide, with the same mass as Erucifoline (RT = 8.44 minutes), showed a single chromatographic peak with a retention time of 9.52 minutes. Spartioidine showed one peak at a retention time of 11.39 minutes.

The medicinal herb *Arnica montana,* belongs to the Asteraceae family and is endemic in Europe.

A study of the flower heads of different Arnica species showed the presence of 2 pyrrolidines, 2-Pyrrolidineacetic acid and 2-Pyrrolidineacetic methyl ester, and of 8 pyrrolizidines, Tussilagine, Isotussilagine, 1-epimers of Tussilagine and 1-epimers of Isotussilagine, Tussilaginic acid, Isotussilaginic acid, 1-epimers of Tussilaginic acid and 1epimers of Isotussilaginic acid[35], but unfortunately, only 2-Pyrrolidineacetic acid was detected in our sample of *Arnica Montana* at 3.51 minutes.

In order to confirm the correct identification of indole ALKs, we investigated extract of *Gelsemium sempervirens*, a climbing plant indigenous to the southern USA belonging to the Loganiaceae family. It contains extremely toxic ALKs, with Gelsemine being the most abundant, but Gelsemicine the most toxic[3]. Other ALKs are 14,15-Dihydroxygelsenicine, 16-epi-Voacarpine,

19Z-16-epi-Voacarpine, Gelsemoxonine, Gelsempervine-A, Gelsempervine-B, Gelsempervine-C, Gelsempervine-D, Sempervilam, and Sempervirine[30,31]. Our extract analysis did not find Sempervilam and Sempervirine. The EICs of the pair of isomers Gelsempervine-A and Gelsempervine-C, Gelsempervine-B and Gelsempervine-D, 19Z-16-epi-Voacarpine and 16-epi-Voacarpine each showed two peaks (RT= 15.32 and 15.62 minutes; 18.54 and 18.91; 16.30 and 17.40 respectively), whilst 14,15-Dihydroxygelsenicine and Gelsemoxonine showed a single peak at 28.37 minutes. Unfortunately, due to insufficient quadrupole selectivity, the fragmentation spectra of the latter 2 ALKs could not be defined. The EICs of Gelsemine and Gelsemicine showed a single peak at 11.85 and 19.59 minutes respectively.

Finally, to confirm quinoline-type ALKs we studied an extract of *Ranunculus montanus,* a plant in the Ranunculaceae family common in alpine meadows at high altitude. Magnoflorine, a positively charged molecule, previously quantified at 24 µg $g^{-1}$ in dry rhizomes of *Ranuncolus*[36], was also confirmed to be present in our experiment, with a single peak at 15.04 minutes in the selected EIC. Figures 1a and 1b show the MS fragmentation profiles of ALKs that were not previuosly documented in literature[43-47].

Table 3 shows accurate masses of the selected precursor ion and fragments, and the NCE of the detected targeted (N=35) and untargeted (49) ALKs.

### 3.4. ALK characterisation of a selection of 18 alpine herbs

#### 3.4.1 Targeted profile

Table 4 shows the content of the 35 ALKs quantified in the herbal extracts. The concentration of ALKs is relatively diversified in the plant species. Pyrrolizidines were the most commonly present ALKs (44% of samples), with concentrations generally ranging from 0.01 to 0.5 mg $Kg^{-1}$, with the exception of Retrorsine, Senecionine/Senecivernine and Seneciphylline, which showed higher content in *Senecio vulgaris* (87, 179, and 246 mg $Kg^{-1}$ respectively), confirming the documented presence of pyrrolizidine ALKs in many *Senecio* spp. [21,42,48]. The most well-represented was Echimidine (present in 39% of samples) with an average concentration of 0.02 mg $Kg^{-1}$, whereas Erucifoline, Erucifoline-N-oxide, Jacobine-N-Oxide, Lasiocarpine, Monocrotaline, Seneciphylline and Senkirkin were never present.

Important concentrations of tropane ALKs were detected in Solanaceae family plants, in particular Hyoscyamine/Atropine and Scopolamine (204 and 136 mg $Kg^{-1}$, respectively) in *Hyoscyamus niger* and Hyoscyamine/Atropine (34 mg $Kg^{-1}$) in *Datura stramonium*[34,37,49].

Glycosteroidal and steroidal ALKs were detected in 30% of samples, with concentrations ranging from 0.1 to 1.3 mg $Kg^{-1}$, while Jervine, Tomatine/Tomadine, Sipeimine, Veratrine, Veratramine and Protoveratrine A were never detected. The highest concentrations were found for Solasodine (24 mg $Kg^{-1}$) in *Solanum nigrum*[50].

For indole ALKs, Gramine was found in quantifiable amounts only in *Hyoscyamus niger* (0.05 mg $Kg^{-1}$), whilst Strychnine and Harmaline were never present.

As regards diterpene ALKs, Aconitine was quantified only in *Phytolacca decandra*, while the piperidine ALK Coniine was never present in our samples.

3.4.2 Untargeted profiling

In order to carry out characterisation of the herbal samples, tentative identification of other ALKs was performed through comparison with the previously mentioned database. The study of untargeted ALKs was limited to the compounds providing a sufficient detectable response (area > 100000 area units). For compounds whose recognition was based only on parent ion accurate mass and isotopic pattern, many chromatographic peaks at different retention times were sometimes found.

As many as 101 different ALKs were detected in untargeted analysis of the 18 herbs based on our 305-ALK database. The results are shown in Table 5, summarising the ALKs, sorted by chemical/botanical group. ALKs (at least one) belonging to the pyrrolizidine, pyrrolidine and piperidine groups were present in all the samples. Pyridines, quinolines, tropanes, protoALKs, indoles and quinazolines were widely present (from 83% to 50 % of analysed samples), whilst isoquinoline, steroidal, pyrrole, imidazoline, azepine and aconitine-related ALKs were detected in less than 22% of plant samples.

In Solanaceae family plants (*Datura stramonium*, *Hyoscyamus niger* and *Solanum nigrum*) 39 different ALKs were detected. Of these, piperidines (N=10 ALKs), pyrrolizidines (7) and tropanes (4) were the most well-represented groups. For the single ALK, 2-Pyrrolidineacetic acid, 3-Acetyltropine, Lobinaline, one isomer of Lobinanidine/Isolobinanidine/betha-Lobinanidine, Magnoflorine, Pycnarrhine, and Tropinone were always present in the three plants.

*Lobelia inflata* (Campanulaceae family) was the richest of these nitrogen active compounds, having as many as 37 ALKs. The most well-represented groups were piperidine (N=22 ALKs; 8-Ethylnorlobelol, 3 isomers of 8-Methyl-10-phenyllobelidiol/Norlelobanidine, 2 isomers of 8,10-Diethyllobelidiol, 2 isomers of LelobanidineI/LelobanidineII, 3 isomers of

Lobinanidine/Isolobinanidine/betha-Lobinanidine, 2 isomers of cis-Lobelanidine/trans-Lobelanidine, 2 isomers of cis-Lobelanine/trans-Lobelanine, 2 isomers of cis-Lobeline/trans-Lobeline, Lobinaline, 2 isomers of Lobinine/Isolobinine, Norallosedamine, and Norlobelanine), as already reported in the literature[29] and tropane ALKs (6; 3,6-diacetyltropine, 3-Acetyltropine, Anisodamine, Apohyoscyamine, Bellendin, and Tropinone).

In *Senecio vulgaris* (Senecionaeae family) 36 ALKs were found. According to the literature[21,42,48] the most well-represented groups were pyrrolizidine (N=16 ALKs; Acetylerucifoline-N-oxide, Dehydrojaconine, Jacoline, Jacoline-N-oxide, Jacozine-N-oxide, Junceine, Monocrotaline N-oxide, Riddelline-N-oxide, Spartioidine, Trichodesmine, one isomer of Riddelline/Seneciphylline N-oxide/Spartioidine N-oxide, 4 isomers of Usamarine/Integerrimine-N-Oxide/Jacobine/Retrorsine/Senecionine/Senecivernine-N-Oxide, and one isomer of Integerrimine/Senecionine/Senecivernine) and tropane (8; 3,6-diacetyltropine, 3-Acetyltropine, Arecoline, Ecgonine, Ferruginine, Scopine, Tropinone, and Valerine).

29 ALKs were detected in *Convallaria majalis* (Asparagaceae family), these being pyperidine (N=5 ALKs; one isomer of Lelobanidine I/Lelobanidine II, one isomer of Lobinaline, one isomer of Lobinanidine/Isolobinanidine/betha-Lobinanidine, one isomer of Lobinine/Isolobinine, and one isomer of Lobinine/Isolobinine), tropane ALKs (N=4; 3-Acetyltropine, Anisodamine, Ferruginine, and Tropinone) and pyrrolizidine (N=3; Jacoline-N-oxide, Monocrotaline N-oxide, and Spartioidine) being the most well-represented.

Ranunculaceae (*Ranunculus montanus* and *Trollius europaeus*) and Asteraceae (*Arnica montana* and *Lactuca Virosa*) had the same number of ALKs (N=27), albeit with different profiles. The most well-represented groups in Ranunculaceae herbs were piperidine (N=5 ALKs), quinoline (5), and pyrrolizidine (4) compounds, Reticuline, 2-Pyrrolidineacetic acid, Chavicine, Indigotin, Indirubin, one isomer of Integerrimine/Senecionine/Senecivernine, Lobinaline, Nicotine, Piperine, Pycnarrhine, and Spartioidine being present in both plants, whilst in Asteraceae herbs the most well-represented were pyrrolizidine (9) and tropane (4) groups, 2-Pyrrolidineacetic acid and Lobinaline being common to the two plants.

22 ALKs were detected in *Gelsemium sempervirens* (Gelsemiaceae family), indole ALKs (N=6 ALKs; Gelsemicine, Gelsemine, one isomer of Gelsempervine-A/Gelsempervine-C, one isomer of Gelsempervine-B/Gelsempervine, one isomer of 14,15-Dihydroxygelsenicine/Gelsemoxonine, and one isomer of 19Z-16-epi-Voacarpine/16-epi-Voacarpine), as previously reported in the literature[30,31], and piperidine ALKs (5; 8-Ethylnorlobelol, Lobinaline, one isomer of Lelobanidine

I/Lelobanidine II, and 2 isomers of Lobinanidine/Isolobinanidine/betha-Lobinanidine) being the most well-represented.

21 ALKs were identified in *Phytolacca decandra* (Phytolaccaceae), 9 of them belonging to the piperidine group (8-Ethylnorlobelol, Lobinaline, 2 isomers of 8,10-Diethyllobelidiol, one isomer of cis-Lobelanidine/trans-Lobelanidine, one isomer of Lelobanidine I/Lelobanidine II, 2 isomers of Lobinanidine/Isolobinanidine/betha-Lobinanidine, and one isomer of 8-Methyl-10-phenyllobelidiol/Norlelobanidine).

*Scrophularia nodosa* (Scrophulariaceae) and *Dryopteris filix-mas* (Dryopteridaceae) had 18 different ALKs. The most well-represented in the Scrophulariaceae herb belonged to pyrrolizidine ALKs (N=3 ALKs; Monocrotaline N-oxide, one isomer of Integerrimine/Senecionine/Senecivernine, and one isomer of Usamarine /Integerrimine-N-oxide/Senecivernine-N-oxide/Jacobine), indole ALKs (3; Gelsemicine, Gelsemine, and Yohimbine), and tropane ALKs (3; 3-Acetyltropine, Anisodamine, and Tropinone), whilst in the Dryopteridaceae herb the most well-represented was the indole group (5; Corynoxin B, Gelsemicine, Gelsemine, Isorhynchophyllin, and Rhynchophylline).

*Rhododendron ferrugineum* (Ericaceae) and *Hypericum perforatum* (Hypericaceae) had 13 different ALKs. The most well-represented in the Ericaceae herb were piperidine ALKs (N=3 ALKs; Lobinaline, Tuberostemonine, and one isomer of 8-Methyl-10-phenyllobelidiol/ Norlelobanidine) and quinoline ALKs (3; Magnoflorine, Quinidine and Quinine), whilst in the Hypericaceae herb the most well-represented was the pyrrolizidine group (4; Europine N-oxide, Florosenine, Neo-Senkirkine and Usaramine-N-oxide).

The herbs showing the lowest number of ALKs turned out to be *Gentiana lutea* (Gentianaceae) and *Cyclamen libanoticum* (Primulaceae), with 7 ALKs (2-Pyrrolidineacetic acid, 5-Methoxyvascicinol, Lobinaline, Jacozine, Mefenamic acid, Scrophularianine C, and Tropinone) and 6 ALKs (2-Pyrrolidineacetic acid, Gelsemine, isomer of Lelobanidine I/Lelobanidine II, Lobinaline, Magnoflorin, and Nicotine) respectively.

## 4. Conclusions

The proposed method, using liquid chromatographic separation coupled with an high resolution mass with targeted and untargeted approaches, made it possible to define the alkaloid profile in more detail. The quantification of 35 alkaloids and untargeted screening of a further 305 alkaloids

in herbal extracts was possible with reduced analysis times and automation, through SPE online pretreatment of herbal extracts in order to minimise the matrix effects on instrumental response.
This broad and rapid ALK characterisation can represent a useful tool for assessing the healthiness of human food and animal feed in toxicology screening.

**Captions to tables**

**Table 1**

Botanical characteristics of herbal samples

**Table 2**

Performance characteristics of the targeted alkaloids method.

**Table 3**

Retention time, precursor ion and fragments accurate masses of targeted and untargeted ALKs.

**Table 4**

ALK contents (mg $Kg^{-1}$) of 18 alpine plant extracts.

**Table 5**

Summary of the untargeted ALK profile of 18 alpine plant extracts, sorted by chemical/botanical groups.

**Captions to figures**

**Figure 1a**

MS fragmentation profiles of ALKs detected in 8 alpine herb extracts.

**Figure 1b**

MS fragmentation profiles of ALKs detected in 8 alpine herb extracts.

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**Table 1**

| Species | Common name | Family |
|---|---|---|
| *Cyclamen libanoticum* | Cyclamen | Primulaceae |
| *Convallaria majalis* | Lily of the valley | Asparagaceae |
| *Dryopteris filix-mas* | Male fern | Dryopteridaceae |
| *Phy0tolacca decandra* | Pokeweed | Phytolaccaceae |
| *Gelsemium sempervirens* | Yellow jessamine | Gelsemiaceae |
| *Hyoscyamus niger* | Henbane | Solanaceae |
| *Lactuca Virosa* | Wild lettuce | Asteraceae |
| *Lobelia inflata* | Indian tobacco | Campanulaceae |
| *Solanum nigrum* | Black nightshade | Solanaceae |
| *Scrophularia nodosa* | Figwort | Scrophulariaceae |
| *Senecio vulgaris* | Common groundsel | Senecionaeae |
| *Datura stramonium* | Jimson weed | Solanaceae |
| *Arnica montana* | Wolf's bane | Asteraceae |
| *Trollius europaeus* | Globeflower | Ranunculaceae |
| *Ranunculus montanus* | Mountain buttercup | Ranunculaceae |
| *Rhododendron ferrugineum* | Rhododendron | Ericaceae |
| *Gentiana lutea* | Great yellow gentian | Getianaceae |
| *Hypericum perforatum* | Perforate St John's-wort | Hypericaceae |

**Table 2**

| Compound | Linearity range (µg $L^{-1}$) | $R^2$ | LOD* (µg $L^{-1}$) | LOQ** (µg $L^{-1}$) | Precision (RSD%[a]) | | | Accuracy (RE%[b]) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | low conc. | medium conc. | high conc. | low conc. | medium conc. | high conc. |
| Monocrotaline | 0.17-1000 | 0.998 | 0.05 | 0.17 | 3.0 | 0.6 | 0.7 | 14.2 | 2.7 | 5.7 |
| Lycopsamine | 0.40-1000 | 0.998 | 0.12 | 0.4 | 1.0 | 0.6 | 1.0 | 15.8 | 3.4 | 5.1 |
| Coniine | 0.71-1000 | 0.998 | 0.21 | 0.71 | 3.2 | 1.4 | 1.2 | 27.7 | 3.3 | 5.3 |
| Erucifoline | 0.25-1000 | 0.998 | 0.07 | 0.25 | 2.0 | 0.8 | 0.6 | 7.4 | 2.1 | 6.8 |
| Senecionine N-oxide | 0.17-1000 | 0.997 | 0.05 | 0.17 | 2.3 | 1.9 | 1.2 | 11.9 | 5.0 | 8.2 |
| Gramine | 0.16-1500 | 0.998 | 0.05 | 0.16 | 3.0 | 1.8 | 1.3 | 21.4 | 1.6 | 2.0 |
| Scopolamine | 0.65-2000 | 0.996 | 0.20 | 0.65 | 1.6 | 1.0 | 1.1 | 16.4 | 3.3 | 9.1 |
| Jacobine-N-oxide | 0.94-1500 | 0.996 | 0.28 | 0.94 | 1.9 | 0.9 | 0.8 | 11.8 | 1.1 | 9.5 |
| Erucifoline-N-oxide | 1.18-1000 | 0.996 | 0.35 | 1.18 | 2.5 | 0.5 | 0.7 | 7.2 | 0.6 | 7.6 |
| Heliotrine | 0.14-1000 | 0.998 | 0.04 | 0.14 | 2.6 | 1.0 | 0.6 | 16.0 | 3.4 | 7.4 |
| Retrorsine | 0.83-1500 | 0.996 | 0.25 | 0.83 | 2.4 | 0.8 | 0.8 | 21.1 | 6.9 | 18.2 |
| Seneciphylline | 0.58-2000 | 0.996 | 0.14 | 0.56 | 1.7 | 1.1 | 1.0 | 10.4 | 3.1 | 7.8 |
| Retrorsine N-oxide | 1.43-1500 | 0.996 | 0.43 | 1.43 | 3.1 | 3.1 | 0.8 | 25.3 | 7.1 | 5.3 |
| Senecionine/ Senecivernine | 0.46-1500 | 0.993 | 0.14 | 0.46 | 1.5 | 0.7 | 0.8 | 23.1 | 4.0 | 13.1 |
| Hyoscyamine/ Atropine | 0.51-2000 | 0.994 | 0.15 | 0.51 | 1.4 | 1.1 | 0.6 | 20.6 | 1.9 | 8.6 |
| Echimidine | 0.25-500 | 0.999 | 0.08 | 0.25 | 4.9 | 1.3 | 0.7 | 18.0 | 1.7 | 5.8 |
| Senkirkin | 2.22-1000 | 0.998 | 0.66 | 2.22 | 1.5 | 0.9 | 0.8 | 13.6 | 1.8 | 6.5 |
| Jacobine | 0.95-1500 | 0.997 | 0.28 | 0.95 | 2.5 | 0.9 | 0.6 | 12.6 | 1.2 | 8.2 |
| Lasiocarpine | 0.41-1500 | 0.998 | 0.12 | 0.41 | 2.4 | 0.9 | 1.1 | 18.3 | 2.7 | 6.3 |
| Strychnine | 1.86-1500 | 0.993 | 0.59 | 1.86 | 6.3 | 1.9 | 1.3 | 39.2 | 6.9 | 13.2 |
| Harmaline | 11.63-3000 | 0.996 | 3.49 | 11.6 | 8.3 | 6.0 | 2.0 | 34.7 | 5.9 | 8.7 |
| Sipeimine | 3.92-1000 | 0.998 | 1.18 | 3.92 | 4.2 | 2.9 | 0.8 | 9.9 | 4.7 | 8.1 |
| Veratramine | 0.59-1000 | 0.995 | 0.18 | 0.59 | 6.7 | 6.3 | 2.3 | 17.9 | 1.9 | 10.4 |
| alpha-Solasonine | 0.25-1000 | 1,000 | 0.1 | 0.35 | 3.0 | 1.8 | 0.9 | 17.6 | 3.1 | 2.3 |
| Jervine | 0.86-2000 | 0.995 | 0.26 | 0.86 | 4.5 | 2.4 | 1.3 | 32.4 | 4.7 | 14.2 |
| alpha-Solamargine | 0.11-1500 | 0.999 | 2.73 | 9.1 | 13.3 | 2.9 | 1.1 | 28.6 | 2.4 | 1.0 |
| Protoveratrine A | 35.71-1500 | 0.996 | 10.7 | 35.7 | 15.4 | 6.4 | 3.7 | 45.0 | 13.7 | 2.9 |
| Veratridine | 0.45-1500 | 0.998 | 0.14 | 0.45 | 9.9 | 6.3 | 1.9 | 7.7 | 5.2 | 4.4 |
| alpha-Solanine | 4.26-500 | 0.998 | 1.28 | 4.26 | 5.4 | 2.5 | 4.1 | 53.0 | 8.6 | 7.2 |
| Solasodine | 2.16-1000 | 0.993 | 0.65 | 2.16 | 6.0 | 8.6 | 3.2 | 14.6 | 4.4 | 10.4 |
| Aconitine | 4.12-1500 | 0.999 | 1.24 | 4.12 | 2.3 | 1.8 | 0.7 | 14.6 | 1.6 | 3.3 |
| Tomatidine/ Tomatine | 19.97-3000 | 0.991 | 5.99 | 20.0 | 5.0 | 7.9 | 2.5 | 2.9 | 5.2 | 12.5 |

* LOD = limit of detection; ** LOQ = limit of quantitation; [a] RSD% = relative standard deviation; [b] RE% = relative error.

## Table 3

| Compound name | RT* (min) | $[M+H]^+$ (*m/z*) | Δ *m/z*** (ppm) | NCE**** | MS/MS fragments (*m/z*) |
|---|---|---|---|---|---|
| *Target compounds* | | | | | |
| Monocrotaline | 5.25 | 326.1594 | 1.23 | 60 | 120.0801; 121.0889; 94.0653 |
| Lycopsamine | 6.96 | 300.1796 | 3.00 | 30 | 138.0903; 94.0652 |
| Coniine | 7.59 | 128.1433 | 0.78 | 45 | 69.0700 |
| Erucifoline | 8.44 | 350.1587 | 3.06 | 60 | 20.0802; 94.0652; 67.0549 |
| Senecionine N-oxide | 8.97 | 352.1750 | 1.42 | 50 | 120.0802; 155.1054; 122.0960 |
| Gramine | 9.28 | 175.1228 | 1.14 | 30 | 130.0655; 113.0590 |
| Scopolamine | 10.25 | 304.1537 | 1.97 | 30 | 138.0903; 156.1018; 121.065 |
| Jacobine-N-oxide | 10.52 | 368.1694 | 2.72 | 50 | 120.0804; 296.1477; 119.0732 |
| Erucifoline-N-oxide | 10.66 | 366.1540 | 1.91 | 60 | 94.0652; 118.0654; 119.0730 |
| Heliotrine | 10.71 | 314.1956 | 1.91 | 30 | 138.091; 156.1017 |
| Retrorsine | 10.73 | 352.1750 | 1.42 | 50 | 120.0803; 94.0652; 165.0136 |
| Seneciphylline | 11.40 | 334.1641 | 2.39 | 50 | 120.0804; 94.0653; 306.1704 |
| Retrorsine N-oxide | 11.89 | 368.1694 | 2.72 | 60 | 94.0652; 120.0803 |
| Senecionine/Senecivernine | 12.63 | 336.1795 | 3.24 | 50 | 120.0803;138.0902; 94.0652 |
| Hyoscyamine/Atropine | 12.65 | 290.1746 | 1.72 | 45 | 124.1122; 93.0702 |
| Echimidine | 13.64 | 398.2169 | 1.00 | 30 | 120.0803; 84.0491; 220.1317 |
| Senkirkin | 13.94 | 366.1906 | 1.37 | 30 | 168.1023; 122.0595; 150.0937 |
| Jacobine | 14.05 | 352.1749 | 1.70 | 50 | 118.0652; 120.0803; 136.0759 |
| Lasiocarpine | 15.80 | 412.2325 | 1.21 | 30 | 120.0803; 220.1314; 336.1808 |
| Strychnine | 16.33 | 335.1746 | 2.39 | 60 | 184.0703; 222.0962 |
| Harmaline | 16.42 | 215.1175 | 1.86 | 50 | 200.0931; 174.0902 |
| Sipeimine | 17.55 | 430.3309 | 1.63 | 50 | 138.1274; 214.1384 |
| Veratramine | 19.68 | 410.3050 | 0.97 | 30 | 295.2022; 84.0824 |
| alpha-Solasonine | 19.83 | 884.4976 | 2.94 | 50 | 85.0288; 71.0498; 157.1012 |
| Jervine | 19.85 | 426.3000 | 0.70 | 30 | 126.1372; 313.2073 |
| alpha-Solamargine | 20.00 | 868.5041 | 1.38 | 45 | 85.0288; 71.0498 |
| Protoveratrine A | 20.62 | 740.4309 | 1.51 | 50 | 658.3609; 436.6457 |
| Veratridine | 21.65 | 674.3515 | 2.97 | 60 | 456.2718; 165.0534; 438.2591 |
| alpha-Solanine | 21.77 | 868.5041 | 1.38 | 50 | 98.0966; 398.3385 |
| Solasodine | 23.60 | 414.3355 | 2.90 | 60 | 157.1017; 70.0658; 159.1158 |
| Aconitine | 23.68 | 646.3195 | 4.02 | 50 | 105.0330; 368.1839 |
| Tomatidine/Tomatine | 24.54 | 416.3520***** | 1.44 | 60 | 161.1319; 70.0658; 147.1161 |
| *Untargeted compounds* | | | | | |
| 2-Pyrrolidineacetic acid | 3.51 | 130.0861 | 1.54 | 30 | 70.0652; 84.0448 |

| | | | | | |
|---|---|---|---|---|---|
| 3-Acetyltropine | 3.79 | 184.1329 | 1.63 | 30 | 107.9599; 78.8800 |
| 3,6-Diacetyltropine | 3.86 | 226.1433 | 2.21 | 30 | 110.0598; 71.0494 |
| 8-Ethylnorlobelol | 5.60 | 158.1536 | 1.90 | 30 | 84.9598; 140.1424; 98.9750 |
| Usamarine /Integerrimine-N-oxide/Senecivernine-N-oxide/Senecionine-N-oxide | 8.97 | 352.1748 | 1.99 | 50 | 120.0805; 324.1392; 138.0918 |
| Riddelline/Seneciphylline N-oxide/Spartioidine N-oxide | 9.52 | 350.1591 | 2.00 | 30 | 120.0809; 138.0901; 322.1627 |
| Anisodamine | 10.36 | 306.1690 | 3.27 | 50 | 140.1060; 122.0965 |
| Usamarine /Integerrimine-N-oxide/Senecivernine-N-oxide/Retrorsine | 10.73 | 352.1747 | 2.27 | 50 | 94.0660; 138.0918; 120.0805 |
| Usaramine/Integerrimine-N-oxide/Senecivernine-N-oxide | 11.22 | 352.1747 | 2.27 | 50 | 120.0815; 352.1751; 138.0918 |
| Spartioidine | 11.39 | 334.1642 | 2.09 | 30 | 120.0803; 138.0919 |
| Gelsemine | 11.85 | 323.1743 | 3.47 | 30 | 70.0654; 236.1065 |
| 8,10-Diethyllobelidiol | 12.05 | 244.2262 | 3.69 | 60 | 98.0962; 226.2176; 58.0654 |
| Integerrimine/Senecionine/Senecivernine | 12.63 | 336.1798 | 2.38 | 50 | 120.0815; 138.0919; 94.0659 |
| 8,10-Diethyllobelidiol | 13.07 | 244.2264 | 2.87 | 60 | 81.0704; 226.2148; 152.1423 |
| Usamarine /Integerrimine-N-oxide/Senecivernine-N-oxide/Jacobine | 14.05 | 352.1750 | 1.42 | 50 | 94.0652; 120.0805; 138.0919 |
| Tropinone | 14.08 | 140.1067 | 2.14 | 30 | 108.0442; 126.0551 |
| Magnoflorine | 15.04 | 342.1692***** | 2.34 | 30 | 297.1140; 265.0847; 58.0657 |
| Gelsempervine-A/Gelsempervine-C | 15.32 | 383.1958 | 1.83 | 30 | 180.1011; 321.1599; 166.0864 |
| 8-Methyl-10-phenyllobelidiol/Norlelobanidine | 15.53 | 278.2108 | 2.52 | 30 | 156.1376; 138.1270 |
| Gelsempervine-A/Gelsempervine-C | 15.62 | 383.1958 | 1.83 | 30 | 180.1015; 321.1600; 166.0864 |
| 8-Methyl-10-phenyllobelidiol/Norlelobanidine | 16.08 | 278.2110 | 1.80 | 30 | 156.1378; 171.1374; 202.1578 |
| Lobinanidine, Isolobinanidine, beta-Lobinanidine | 16.23 | 290.2108 | 2.41 | 30 | 168.1375; 96.0807; 272.1996 |
| 19Z-16-epi-Voacarpine/16-epi-Voacarpine | 16.30 | 369.1802 | 1.90 | 30 | 321.1591; 206.9745; 112.7368 |
| 8-Methyl-10-phenyllobelidiol/Norlelobanidine | 16.60 | 278.2109 | 2.16 | 30 | 156.1378; 171.1370; 138.1269 |
| Lobinine/Isolobinine | 16.84 | 288.1952 | 2.08 | 30 | 162.0893; 111.0802; 99.0440 |
| Lelobanidine I/Lelobanidine II | 16.91 | 292.2263 | 2.40 | 40 | 170.1540; 202.1580; 98.0961 |
| Lobinanidine, Isolobinanidine, beta-Lobinanidine | 17.40 | 290.2109 | 2.07 | 30 | 50.0655; 164.1063; 200.1426 |
| 19Z-16-epi-Voacarpine/16-epi-Voacarpine | 17.40 | 369.1814 | 1.35 | 30 | 321.1591; 206.9745; 112.7370 |
| Lelobanidine I/Lelobanidine II | 17.41 | 292.2271 | 2.74 | 40 | 170.1538; 202.1580; 98.0959 |
| Lobinanidine, Isolobinanidine, beta-Lobinanidine | 17.64 | 290.2115 | 2.07 | 30 | 50.0655; 168.1375; 200.1425 |
| Apohyoscyamine | 17.88 | 272.1645 | 2.57 | 50 | / |
| Gelsempervine-B/Gelsempervine-D | 18.54 | 425.2071 | 1.88 | 40 | 172.0752; 180.1009; 158.0608 |
| Gelsempervine-B/Gelsempervine-D | 18.91 | 425.2071 | 1.65 | 40 | 172.0752; 180.1009; 158.0608 |
| Gelsemicine | 19.59 | 359.1965 | 2.23 | 30 | / |

| | | | | | | |
|---|---|---|---|---|---|---|
| Norallosedamine | 19.76 | 206.1539 | 1.46 | 30 | 84.0384; 122.0964; 105.0699 |
| Lobinine/Isolobinine | 19.99 | 288.1958 | 2.08 | 30 | 162.0893; 111.0802; 99.0440 |
| *cis*-Lobelanidine/*trans*-Lobelanidine | 20.17 | 340.2271 | 2.06 | 30 | 202.1584; 218.15441; 98.0962 |
| *cis*-Lobeline/*trans*-Lobeline | 20.23 | 338.2115 | 2.37 | 30 | 216.1361; 96.0808 |
| *cis*-Lobeline/*trans*-Lobeline | 20.54 | 338.2115 | 2.37 | 30 | 216.1511; 216.1386; 96.0808 |
| *cis*-Lobelanine/*trans*-Lobelanine | 20.66 | 336.1958 | 1.78 | 30 | 96.0814; 216.1378; 290.1744 |
| *cis*-Lobelanidine/*trans*-Lobelanidine | 20.86 | 340.2271 | 2.35 | 30 | 218.1544; 202.1583; 98.0961 |
| Norlobelanine | 20.89 | 322.1802 | 2.48 | 30 | 202.1223; 82.0657; 171.1392 |
| *cis*-Lobelanine/*trans*-Lobelanine | 21.15 | 336.1958 | 0.59 | 30 | 96.0813; 216.1380 |
| beta-Solamargine | 21.75 | 868.5057 | 0.23 | 45 | / |
| Gelsemoxonine/14,15-Dihydroxygelsenicine | 28.37 | 359.1622 | 2.78 | 30 | / |
| Lobinaline | 28.49 | 387.2795 | 2.07 | 30 | / |

*RT= retention time; **Δ *m/z* (ppm)= error of accurate mass respect to exact mass; ***NCE= normalized collision energy; ****Tomatine=$[M+H-C_{23}H_{38}O_{19}]^+$; *****$[M]^+$;

## Table 4

| 3 | Acontitine | alfa-Solanine | alpha-Solasonine | alpha-Solamargine | Echimidine | Erucifoline | Gramine | Heliotrine | Hyoscyamine/Atropine | Jacobine | Lycopsamine | Retrorsine | Retrorsine N-oxide | Scopolamine | Senecionine N-oxide | Senecionine/ Seffnecivernine | Solasodine |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Cyclamen libanoticum* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | <0.002 | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Convallaria majalis* | <0.07 | **0.64** | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | <0.002 | <0.008 | <0.015 | **0.528** | <0.013 | **0.024** | <0.01 | <0.003 | <0.007 | <0.04 |
| *Dryopteris filixn.d.mas* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | <0.002 | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Phytolacca decandra* | **0.09** | <0.07 | <0.006 | <0.15 | **0.016** | <0.004 | <0.003 | <0.002 | **0.103** | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | **0.006** | <0.007 | <0.04 |
| *Gelsemium sempervirens* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | <0.002 | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Hyoscyamus niger* | <0.07 | <0.07 | **0.221** | **0.37** | **0.017** | <0.004 | **0.049** | **0.088** | **204** | <0.015 | <0.006 | <0.013 | **0.024** | **136** | <0.003 | <0.007 | <0.04 |
| *Lactuca Virosa* | <0.07 | <0.07 | <0.006 | <0.15 | **0.017** | <0.004 | <0.003 | <0.002 | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Lobelia inflata* | <0.07 | <0.07 | <0.006 | <0.15 | **0.019** | <0.004 | <0.003 | <0.002 | **1.31** | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Solanum nigrum* | <0.07 | <0.07 | **0.103** | <0.15 | **0.017** | <0.004 | <0.003 | **0.021** | <0.008 | <0.015 | **0.252** | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | **24.0** |
| *Scrophularia nodosa* | <0.07 | **0.13** | <0.006 | <0.15 | **0.017** | <0.004 | <0.003 | **0.019** | **1.50** | <0.015 | <0.006 | <0.013 | **0.027** | **0.18** | <0.003 | **0.012** | **0.13** |
| *Senecio vulgaris* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | <0.002 | <0.008 | **0.116** | <0.006 | **87.3** | **0.064** | <0.01 | <0.003 | **179** | <0.04 |
| *Datura stramonium* | <0.07 | <0.07 | **0.376** | **1.31** | **0.029** | <0.004 | <0.003 | <0.002 | **34.2** | <0.015 | <0.006 | <0.013 | <0.023 | **2.21** | <0.003 | <0.007 | <0.04 |
| *Arnica montana* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | <0.002 | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Trollius europaeus* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | <0.002 | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Ranunculus montanus* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | **0.018** | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Rhododendron ferrugineum* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | <0.002 | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Gentiana lutea* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | <0.004 | <0.003 | <0.002 | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |
| *Hypericum perforatum* | <0.07 | <0.07 | <0.006 | <0.15 | <0.004 | **0.009** | <0.003 | **0.011** | <0.008 | <0.015 | <0.006 | <0.013 | <0.023 | <0.01 | <0.003 | <0.007 | <0.04 |

## Table 5

| Herb sample | Family | Aconitine-related | Azepine | Imidazoline | Indole | Isoquinoline | Piperidine | Protoalkaloid | Pyridine | Pyrrole | Pyrrolidine | Pyrrolizidine | Quinazoline | Quinoline | Steroidal | Tropane |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Convallaria majalis* | Asparagaceae | x | x | x | ✓ | x | ✓ | ✓ | ✓ | x | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| *Arnica montana* | Asteraceae | x | x | x | x | x | ✓ | x | ✓ | x | ✓ | ✓ | x | x | x | x |
| *Lactuca Virosa* | Asteraceae | x | x | x | ✓ | x | ✓ | ✓ | x | x | ✓ | ✓ | ✓ | ✓ | x | ✓ |
| *Lobelia inflata* | Campanulaceae | x | x | ✓ | ✓ | x | ✓ | x | ✓ | x | ✓ | ✓ | ✓ | ✓ | x | ✓ |
| *Dryopteris filix-mas* | Dryopteridaceae | x | x | x | ✓ | x | ✓ | ✓ | ✓ | x | ✓ | ✓ | ✓ | x | x | ✓ |
| *Rhododendron ferrugineum* | Ericaceae | x | x | x | x | x | ✓ | ✓ | ✓ | x | ✓ | ✓ | x | ✓ | x | ✓ |
| *Gelsemium sempervirens* | Gelsemiaceae | x | x | x | ✓ | ✓ | ✓ | ✓ | ✓ | x | ✓ | ✓ | x | ✓ | x | ✓ |
| *Gentiana lutea* | Gentianaceae | x | x | x | x | x | ✓ | ✓ | ✓ | x | ✓ | ✓ | ✓ | x | x | ✓ |
| *Hypericum perforatum* | Hypericaceae | x | x | x | x | x | ✓ | x | ✓ | x | ✓ | ✓ | x | ✓ | x | ✓ |
| *Phytolacca decandra* | Phytolaccaceae | x | x | x | x | ✓ | ✓ | ✓ | ✓ | x | ✓ | ✓ | ✓ | ✓ | x | ✓ |
| *Cyclamen libanoticum* | Primulaceae | x | x | x | ✓ | x | ✓ | x | ✓ | x | ✓ | ✓ | x | ✓ | x | x |
| *Ranunculus montanus* | Ranunculaceae | x | x | x | ✓ | ✓ | ✓ | ✓ | ✓ | x | ✓ | ✓ | x | ✓ | x | ✓ |
| *Trollius europaeus* | Ranunculaceae | x | ✓ | x | ✓ | ✓ | ✓ | x | ✓ | x | ✓ | ✓ | x | ✓ | x | x |
| *Scrophularia nodosa* | Scrophulariaceae | x | x | x | ✓ | x | ✓ | x | ✓ | x | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| *Senecio vulgaris* | Senecionaeae | x | x | x | x | x | ✓ | ✓ | ✓ | x | ✓ | ✓ | ✓ | ✓ | x | ✓ |
| *Datura stramonium* | Solanaceae | ✓ | x | x | x | x | ✓ | ✓ | x | x | ✓ | ✓ | ✓ | ✓ | x | ✓ |
| *Hyoscyamus niger* | Solanaceae | x | x | x | ✓ | x | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | x | ✓ | ✓ | ✓ |
| *Solanum nigrum* | Solanaceae | x | x | x | ✓ | x | ✓ | ✓ | ✓ | x | ✓ | ✓ | x | ✓ | x | ✓ |

✓= detected; ×= not detected

**Figure 1a**

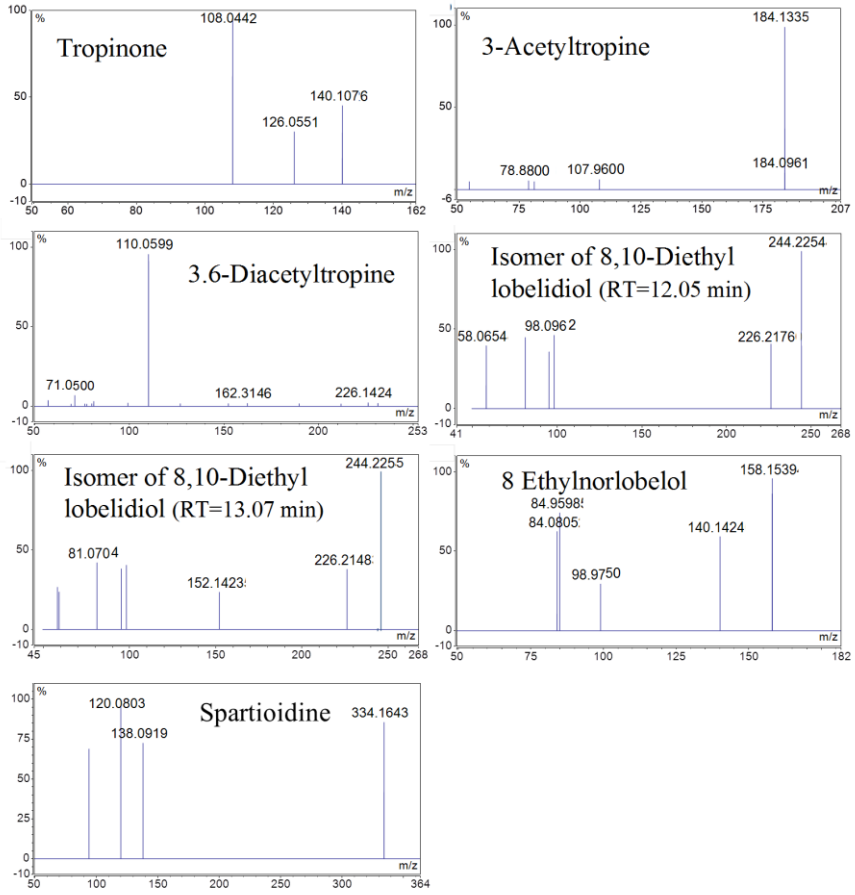
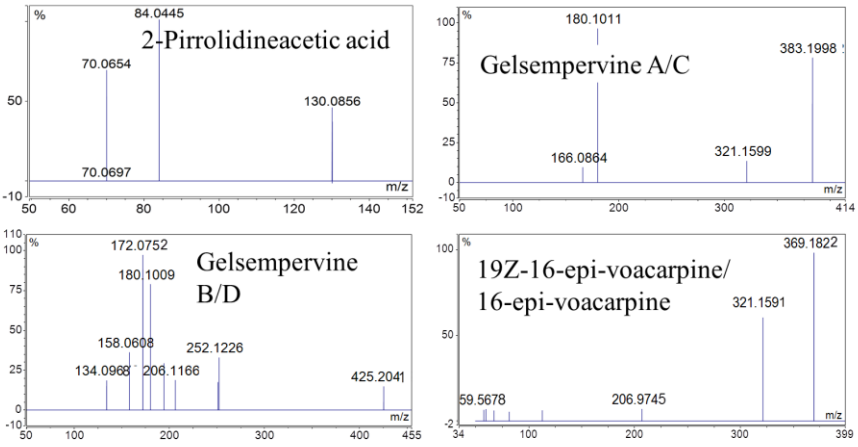



**Figure 1b**



## Supplementary material

## Captions to supplementary tables

### Table 1S

Technical characteristics of the ALK analytical standards.

### Table 2S

Untargeted in-house database of ALKs sorted by chemical/botanical group.

### Table 3S

Untargeted in-house database of ALKs sorted by chemical/botanical group.

### Table 1S

| Group | Common name | Chemical formula | CAS number | Purity (%) | Supplier |
|---|---|---|---|---|---|
| Diterpene | Aconitine | $C_{34}H_{47}NO_{11}$ | 302-27-2 | 96.2 | a |
| Glycosteroidal | alpha-Solamargine | $C_{45}H_{73}NO_{15}$ | 20311-51-7 | 96.7 | a |
| Glycosteroidal | alpha-Solanine | $C_{45}H_{73}NO_{15}$ | 20562-02-1 | 99.1 | a |
| Glycosteroidal | alpha-Solasonine | $C_{45}H_{73}NO_{16}$ | 19121-58-5 | 96.1 | a |
| Glycosteroidal | Tomatine | $C_{50}H_{83}NO_{21}$ | 17406-45-0 | 98.0 | a |
| Indole | Gramine | $C_{11}H_{14}N_2$ | 87-52-5 | 99.6 | a |
| Indole | Harmaline | $C_{13}H_{14}N_2O$ | 6027-98-1 | >95% ($HCl{\cdot}2H_2O$) | b |
| Indole | Strychnine | $C_{21}H_{22}N_2O_2$ | 57-24-9 | not declared | b |
| Piperidine | Coniine | $C_8H_{17}N$ | 15991-59-0 | 99.8 | a |
| Pyrrolizidine | Echimidine | $C_{20}H_{31}NO_7$ | 520-68-3 | 96.6 | a |
| Pyrrolizidine | Erucifoline | $C_{18}H_{23}NO_6$ | 40158-95-0 | 99.8 | a |
| Pyrrolizidine | Erucifoline-N-oxine | $C_{18}H_{23}NO_7$ | 123864-94-8 | 98.4 | a |
| Pyrrolizidine | Heliotrine | $C_{16}H_{27}NO_5$ | 303-33-3 | 91.0 | a |
| Pyrrolizidine | Jacobine | $C_{18}H_{25}NO_6$ | 6870-67-3 | 100 | a |
| Pyrrolizidine | Jacobine-N-oxide | $C_{18}H_{25}NO_7$ | 38710-25-7 | 98.9 | a |
| Pyrrolizidine | Lasiocarpine | $C_{21}H_{33}NO_7$ | 303-34-4 | 99.1 | a |
| Pyrrolizidine | Lycopsamine | $C_{15}H_{25}NO_5$ | 10285-07-1 | 100 | a |
| Pyrrolizidine | Monocrotaline | $C_{16}H_{23}NO_6$ | 315-22-0 | 98.9 | a |
| Pyrrolizidine | Retrorsine | $C_{18}H_{25}NO_6$ | 480-54-6 | 100 | a |
| Pyrrolizidine | Retrorsine-N-oxide | $C_{18}H_{25}NO_7$ | 15503-86-3 | 99.6 | a |

| | | | | | |
|---|---|---|---|---|---|
| Pyrrolizidine | Senecionine | $C_{18}H_{25}NO_5$ | 130-01-8 | 99.3 | a |
| Pyrrolizidine | Senecionine-N-oxide | $C_{18}H_{25}NO_6$ | 13268-67-2 | 99.6 | a |
| Pyrrolizidine | Seneciphylline | $C_{18}H_{23}NO_5$ | 480-81-9 | 99.8 | a |
| Pyrrolizidine | Senecivernine | $C_{18}H_{25}NO_5$ | 72755-25-0 | 97.9 | a |
| Pyrrolizidine | Senkirkine | $C_{19}H_{27}NO_6$ | 2318-18-5 | 98.5 | a |
| Steroidal | Jervine | $C_{27}H_{39}NO_3$ | 469-59-0 | 99.2 | a |
| Steroidal | Protoveratrine A | $C_{41}H_{63}NO_{14}$ | 143-57-7 | not declared | a |
| Steroidal | Sipeimine | $C_{27}H_{43}NO_3$ | 61825-98-7 | 99.9 | a |
| Steroidal | Solasodine | $C_{27}H_{43}NO_2$ | 126-17-0 | 100 | a |
| Steroidal | Tomatidine | $C_{27}H_{45}NO_2$ | 6192-62-7 | 98.21 (HCl) | a |
| Steroidal | Veratramine | $C_{27}H_{39}NO_2$ | 60-70-8 | 99.8 | a |
| Steroidal | Veratridine | $C_{36}H_{51}NO_{11}$ | 71-62-5 | 98.7 | a |
| Tropane | Atropine | $C_{17}H_{23}NO_3$ | 55-48-1 | 99.44 ($SO_4^{=}$) | a |
| Tropane | Hyoscyamine | $C_{17}H_{23}NO_3$ | 620-61-1 | 100 ($SO_4^{=}$) | a |
| Tropane | Scopolamine | $C_{17}H_{21}NO_4$ | 114-49-8 | 100 (HBr) | a |

a= PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany

b= Sigma, St. Louis, MO, USA

## Table 2S

| ***Group*** **Name** | **Chemical formula** | **$[M+H]^+$ (*m/z*)** | **Name** | **Chemical formula** | **$[M+H]^+$ (*m/z*)** | **Name** | **Chemical formula** | **$[M+H]^+$ (*m/z*)** |
|---|---|---|---|---|---|---|---|---|
| *aconitum* | | | | | | | | |
| Hypaconitine | $C_{33}H_{45}NO_{10}$ | 616.3116 | Lappaconitine | $C_{32}H_{44}N_2O_8$ | 585.3170 | Mesaconitine | $C_{33}H_{45}NO_{11}$ | 632.3065 |
| *azepine* | | | | | | | | |
| Azepine | $C_6H_7N$ | 94.0651 | Chalciporone | $C_{16}H_{21}NO$ | 244.1696 | Isochalciporone | $C_{16}H_{21}NO$ | 244.1696 |
| Tolazamide | $C_{14}H_{21}N_3O_3S$ | 312.1376 | | | | | | |
| *benzazepine* | | | | | | | | |
| Benazepril | $C_{24}H_{28}N_2O_5$ | 425.2071 | | | | | | |
| *benzodiazepine* | | | | | | | | |
| Diazepam | $C_{16}H_{13}ClN_2O$ | 285.0789 | | | | | | |
| *benzothiadiazine* | | | | | | | | |
| Bendroflumethiazide | $C_{15}H_{14}F_3N_3O_4S_2$ | 422.0451 | Hydrochlorothiazide | $C_7H_8ClN_3O_4S_2$ | 297.9717 | Methyclothiazide | $C_9H_{11}Cl_2N_3O_4S_2$ | 359.9641 |
| *Imidazole* | | | | | | | | |
| Clonidine | $C_9H_9Cl_2N_3$ | 230.0246 | Phentolamine | $C_{17}H_{19}N_3O$ | 282.1601 | | | |
| *imidazolidine* | | | | | | | | |
| Phenytoin | $C_{15}H_{12}N_2O_2$ | 253.0971 | | | | | | |
| *indole* | | | | | | | | |
| 14,15-dihydroxygelsenicine | $C_{19}H_{22}N_2O_5$ | 359.1601 | 19Z-16-epi-Voacarpine/16-epi-Voacarpine | $C_{21}H_{24}N_2O_4$ | 369.1809 | Ajmalicine | $C_{21}H_{24}N_2O_3$ | 353.1860 |

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|---|---|---|---|---|---|---|---|---|
| Betanidin | $C_{18}H_{16}N_2O_8$ | 389.0979 | Betanin | $C_{24}H_{26}N_2O_{13}$ | 550.1429* | Brucine | $C_{23}H_{26}N_2O_4$ | 395.1965 |
| Catharanthine | $C_{21}H_{24}N_2O_2$ | 337.1910 | Chlorthalidone | $C_{14}H_{11}ClN_2O_4S$ | 339.0201 | Corynoxin B | $C_{22}H_{28}N_2O_4$ | 385.2122 |
| Evodiamine | $C_{19}H_{17}N_3O$ | 304.1444 | Gelsemicine | $C_{20}H_{26}N_2O_4$ | 359.1965 | Gelsemine | $C_{20}H_{22}N_2O_2$ | 323.1754 |
| Gelsemoxonine | $C_{19}H_{22}N_2O_5$ | 359.1601 | Gelsempervine-A | $C_{22}H_{26}N_2O_4$ | 383.1965 | Gelsempervine-B | $C_{24}H_{28}N_2O_5$ | 425.2071 |
| Gelsempervine-C | $C_{22}H_{26}N_2O_4$ | 383.1965 | Gelsempervine-D | $C_{24}H_{28}N_2O_5$ | 425.2071 | Indapamide | $C_{16}H_{16}ClN_3O_3S$ | 366.0674 |
| Indigotin | $C_{16}H_{10}N_2O_2$ | 263.0815 | Indirubin | $C_{16}H_{10}N_2O_2$ | 263.0815 | Indomethacin | $C_{19}H_{16}ClNO_4$ | 358.0841 |
| Isomitraphyllin | $C_{21}H_{24}N_2O_4$ | 369.1809 | Isorhynchophyllin | $C_{22}H_{28}N_2O_4$ | 385.2122 | Koenigicin | $C_{20}H_{21}NO_3$ | 324.1594 |
| Koenimbin | $C_{19}H_{19}NO_2$ | 294.1489 | Mahanimbin | $C_{23}H_{25}NO$ | 332.2009 | Mitraphyllin | $C_{21}H_{24}N_2O_4$ | 369.1809 |
| Reserpin | $C_{33}H_{40}N_2O_9$ | 609.2807 | Rhynchophylline | $C_{22}H_{28}N_2O_4$ | 385.2122 | Rutaecarpine | $C_{18}H_{13}N_3O$ | 288.1131 |
| Sempervilam | $C_{19}H_{16}N_2O$ | 289.1335 | Sempervirine | $C_{19}H_{17}N_2$ | 274.1464 | Tabersonine | $C_{21}H_{24}N_2O_2$ | 337.1910 |
| Tadalafil | $C_{22}H_{19}N_3O_4$ | 390.1448 | Uncarin C | $C_{21}H_{24}N_2O_4$ | 369.1809 | Uncarin D | $C_{21}H_{24}N_2O_4$ | 369.1809 |
| Uncarin E | $C_{21}H_{24}N_2O_4$ | 369.1809 | Yohimbine | $C_{21}H_{26}N_2O_3$ | 355.2016 | | | |
| *isoquinoline* | | | | | | | | |
| (-)-Coclaurine | $C_{17}H_{19}NO_3$ | 286.1438 | (-)-Limacine | $C_{37}H_{40}N_2O_6$ | 609.2959 | (-)-Mecambrine | $C_{18}H_{17}NO_3$ | 296.1281 |
| (-)-Nuciferine | $C_{19}H_{21}NO_2$ | 296.1645 | (+)-Linearisine | $C_{18}H_{21}NO_3$ | 300.1594 | (+)-Reticuline | $C_{19}H_{23}NO_4$ | 330.1700 |
| Berberine | $C_{20}H_{18}NO_4$ | 336.1230* | Columbamine | $C_{20}H_{20}NO_4$ | 338.1387* | Gliquidone | $C_{27}H_{33}N_3O_6S$ | 528.2163 |
| Hydroflumethiazide | $C_8H_8F_3N_3O_4S_2$ | 331.9981 | Hydrohydrastinine | $C_{11}H_{13}NO2$ | 192.1019 | Mollinedine | $C_{18}H_{13}NO_4$ | 308.0917 |
| Palmatine | $C_{21}H_{22}NO_4$ | 352.1543* | Phellodendrine | $C_{20}H_{24}NO_4$ | 342.1700* | Pycnarrhine | $C_{11}H_{14}NO_2$ | 192.1019* |
| Trolline | $C_{12}H_{13}NO_3$ | 219.0890* | | | | | | |
| *piperazine* | | | | | | | | |
| Vardenafil | $C_{23}H_{32}N_6O_4S$ | 489.2278 | | | | | | |
| *piperidine* | | | | | | | | |
| 1-Deoxynojirimycin | $C_6H_{13}NO_4$ | 164.0917 | 2-ß-D glycopyranosyl-2-undecil-3,5-dihydroxy-6-carboxy piperidine | $C_{25}H_{47}NO_{10}$ | 522.3273 | 8-Methyl-10-phenyllobelidiol | $C_{17}H_{27}NO_2$ | 278.2114 |
| 8,10-Diethyllobelidiol | $C_{14}H_{29}NO_2$ | 244.2271 | 8-Ethyl-10-phenylnorlobelidione | $C_{17}H_{24}NO_2$ | 275.1880 | 8-Ethylnorlobelol | $C_9H_{19}ON$ | 158.1539 |
| 8-Methyl-10-ethyllobelidiol | $C_{13}H_{26}NO_2$ | 229.2036 | Acronycine | $C_{20}H_{19}NO_3$ | 322.1438 | Allosedamine | $C_{14}H_{21}NO$ | 220.1696 |
| Ammodendrine | $C_{12}H_{20}N_2O$ | 209.1648 | beta-Lobinanidine | $C_{18}H_{27}NO_2$ | 290.2115 | Chavicine | $C_{17}H_{19}NO_3$ | 286.1438 |
| cis-Lobelanidine | $C_{22}H_{29}NO_2$ | 340.2271 | cis-Lobelanine | $C_{22}H_{25}NO_2$ | 336.1958 | cis-Lobeline | $C_{22}H_{27}NO_2$ | 338.2115 |
| Isolobinanidine | $C_{18}H_{27}NO_2$ | 290.2115 | Isolobinine | $C_{18}H_{25}NO_2$ | 288.1958 | Isopelletierine | $C_8H_9NO$ | 369.1809 |
| Lelobanidine I | $C_{18}H_{29}NO_2$ | 292.2271 | Lelobanidine II | $C_{18}H_{29}NO_2$ | 292.2271 | Ligustrazine | $C_8H_{12}N_2$ | 137.1073 |
| Lobinaline | $C_{27}H_{34}N_2$ | 387.2795 | Lobinanidine | $C_{18}H_{27}NO_2$ | 290.2115 | Lobinine | $C_{18}H_{25}NO_2$ | 288.1958 |
| Minoxidil | $C_9H_{15}N_5O$ | 210.1349 | Multiflorine | $C_{15}H_{22}N_2O$ | 247.1805 | Norallosedamine | $C_{13}H_{19}NO$ | 206.1539 |
| Norlelobanidine | $C_{17}H_{27}NO_2$ | 278.2115 | Norlobelanibine | $C_{21}H_{24}NO$ | 307.1931 | Norlobelanine | $C_{21}H_{23}NO_2$ | 322.1802 |
| Piperettine | $C_{19}H_{21}NO_3$ | 312.1594 | Piperic acid | $C_{12}H_{10}O_4$ | 219.0652 | Piperidine | $C_5H_{11}N$ | 86.0964 |
| Piperine | $C_{17}H_{19}NO_3$ | 286.1438 | Piptanthine | $C_{20}H_{35}N_3$ | 318.2904 | Repaglinide | $C_{27}H_{36}N_2O_4$ | 453.2748 |
| Rimonabant | $C_{22}H_{21}Cl_3N_4O$ | 463.0854 | Sedamine | $C_{14}H_{21}NO$ | 220.1696 | *trans*-Lobelanidine | $C_{22}H_{29}NO_2$ | 340.2271 |
| *trans*-Lobelanine | $C_{22}H_{25}NO_2$ | 336.1958 | *trans*-Lobeline | $C_{22}H_{27}NO_2$ | 338.2115 | Tuberostemonine | $C_{22}H_{33}NO_4$ | 376.2482 |
| *protoalkaloid* | | | | | | | | |
| 1,3-Dimethylamylamine | $C_7H_{17}N$ | 116.1434 | Acetohexamide | $C_{15}H_{20}N_2O_4S$ | 325.1216 | Aristolochic Acid | $C_{17}H_{11}NO_7$ | 342.0608 |
| Aristolochic Acid II | $C_{16}H_9NO_6$ | 312.0503 | beta-Methylphenethylamine | $C_9H_{13}N$ | 136.1121 | Bumetanide | $C_{17}H_{20}N_2O_5S$ | 365.1166 |

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|---|---|---|---|---|---|---|---|---|
| Cathine | $C_9H_{13}NO$ | 152.1070 | Chloropropamide | $C_{10}H_{13}ClN_2O_3S$ | 277.0408 | Colchicine | $C_{22}H_{25}NO_6$ | 400.1755 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | Fenfluramine | $C_{12}H_{16}F_3N$ | 232.1308 | Fluoxetin | $C_{17}H_{18}F_3NO$ | 310.1413 |
| Glibenclamide | $C_{23}H_{28}ClN_3O_5S$ | 494.1511 | Glipizide | $C_{21}H_{27}N_5O_4S$ | 446.1856 | Mefenamic Acid | $C_{15}H_{15}NO_2$ | 242.1176 |
| Metformin | $C_4H_{11}N_5$ | 130.1087 | Methamphetamine | $C_{10}H_{15}N$ | 150.1277 | Metoprolol tartrate | $C_{15}H_{25}NO_3$ | 268.1907 |
| Nateglinide | $C_{19}H_{27}NO_3$ | 318.2064 | Norephedrine | $C_9H_{13}NO$ | 152.1070 | Orlistat | $C_{29}H_{53}NO_5$ | 496.3996 |
| Phenformin | $C_{10}H_{15}N_5$ | 206.1400 | Phentermine | $C_{10}H_{15}N$ | 150.1277 | Ricinine | $C_8H_8N_2O_2$ | 165.0658 |
| Sertraline | $C_{17}H_{17}Cl_2N$ | 306.0811 | Sibutramine | $C_{17}H_{26}ClN$ | 280.1826 | Tolbutamide | $C_{12}H_{18}N_2O_3S$ | 271.1111 |
| Topiramate | $C_{12}H_{21}NO_8S$ | 340.1061 | Amygdalin | $C_{20}H_{27}NO_{11}$ | 458.1657 | Capsaicin | $C_{18}H_{27}NO_3$ | 306.2064 |
| N-methylatanine | $C_4H_9NO_2$ | 104.0706 | Prunasin | $C_{14}H_{17}NO_6$ | 296.1129 | Sambunigrin | $C_{14}H_{17}NO_6$ | 296.1129 |
| Vicianin | $C_{19}H_{25}O_{10}$ | 414.1520 | | | | | | |

*$[M]^+$

## Table 3S

| ***Group*** **Name** | **Chemical formula** | **$[M+H]^+$ (*m/z*)** | **Name** | **Chemical formula** | **$[M+H]^+$ (*m/z*)** | **Name** | **Chemical formula** | **$[M+H]^+$ (*m/z*)** |
|---|---|---|---|---|---|---|---|---|
| *pyrazole* | | | | | | | | |
| Sildenafil | $C_{22}H_{30}N_6O_4S$ | 475.2122 | | | | | | |
| *pyrazolidine* | | | | | | | | |
| Oxyphenbutazone | $C_{19}H_{20}N_2O_3$ | 325.1547 | Phenylbutazone | $C_{19}H_{20}N_2O_2$ | 309.15975 | | | |
| *pyridine* | | | | | | | | |
| Amlodipine | $C_{20}H_{25}ClN_2O_5$ | 409.1525 | Amphetamine | $C_9H_{13}N$ | 136.1121 | Aphylline | $C_{15}H_{24}N_2O$ | 249.1961 |
| Betalamic acid | $C_9H_9NO_5$ | 212.0553 | Felodipine | $C_{18}H_{19}Cl_2NO_4$ | 384.0764 | Gentiatibetine | $C_9H_{11}NO_2$ | 166.0863 |
| Ginkgotoxine | $C_9H_{13}NO_3$ | 152.1070 | Indicaxanthin | $C_{14}H_{16}N_2O_6$ | 309.1081 | Lupanine | $C_{15}H_{24}N_2O$ | 249.1962 |
| Muscopyridine | $C_{16}H_{25}N$ | 232.2060 | Myosmine | $C_9H_{10}N_2$ | 147.0917 | Navenone A | $C_{15}H_{15}NO$ | 226.1226 |
| Nicotine | $C_{10}H_{14}N_2$ | 163.1230 | Nicotinic acid | $C_6H_5NO_2$ | 124.0393 | Pyridine | $C_5H_5N$ | 80.0495 |
| Rosiglitazone | $C_{18}H_{19}N_3O_3S$ | 358.1220 | Scrophularianine A | $C_9H_7NO_2$ | 162.0550 | Scrophularianine B | $C_9H_9NO_2$ | 164.0706 |
| Scrophularianine C | $C_{10}H_{11}NO_3$ | 194.0812 | Trigonelline | $C_7H_7NO_2$ | 138.0550 | Venoterpine | $C_9H_{11}NO$ | 150.0913 |
| Wilforin | $C_{43}H_{49}NO_{18}$ | 868.3022 | | | | | | |
| *pyrimidine* | | | | | | | | |
| Pioglitazone | $C_{19}H_{20}N_2O_3S$ | 357.1267 | | | | | | |
| *Pyrrole* | | | | | | | | |
| Gliclazide | $C_{15}H_{21}N_3O_3S$ | 324.1376 | Linarinic acid | $C_{12}H_{12}N_2O$ | 201.1022 | Lycorine | $C_{16}H_{17}NO_4$ | 288.1230 |
| Pyrrole | $C_4H_5N$ | 68.0495 | Ternatusine A | $C_{16}H_{21}NO_8$ | 356.1340 | Ternatusine B | $C_{24}H_{23}N_3O_7$ | 466.1609 |
| Ternatusine C | $C_{30}H_{33}O_{11}N_3$ | 612.2188 | | | | | | |
| *pyrrolidine* | | | | | | | | |
| (-)-N-methylproline | $C_6H_{11}NO_2$ | 130.0863 | 2-Pyrrolidineacetic acid | $C_6H_{11}NO_2$ | 130.0863 | 2-Pyrrolidineacetic methyl ester | $C_7H_{13}NO_2$ | 144.1019 |
| Cuscohygrine | $C_{13}H_{24}N_2O$ | 225.1961 | Hygrine | $C_8H_{15}NO$ | 142.1226 | Pyrrolidine | $C_4H_9N$ | 72.0808 |
| Stachydrine | $C_7H_{14}NO_2Cl$ | 179.0708* | Tricholeine | $C_6H_{11}NO_2$ | 130.0862 | Trichostachine | $C_{16}H_{17}NO_3$ | 272.1281 |
| *pyrrolizidine* | | | | | | | | |
| 1-epimers of Isotussilagine | $C_{10}H_{18}NO_3$ | 201.1359 | 1-epimers of Isotussilaginic acid | $C_9H_{16}O_3N$ | 187.1203 | 1-epimers of Tussilagine | $C_{10}H_{18}NO_3$ | 201.1359 |
| 1-epimers of Tussilaginic acid | $C_9H_{16}O_3N$ | 187.1203 | Acetylerucifoline | $C_{20}H_{25}NO_7$ | 392.1705 | Acetylerucifoline-N-oxide | $C_{20}H_{25}NO_8$ | 408.1653 |
| Acetylseneciphylline | $C_{20}H_{25}NO_6$ | 376.1755 | Acetylseneciphylline-N-oxide | $C_{20}H_{25}NO_7$ | 392.1705 | Dehydrojaconine | $C_{18}H_{24}ClNO_6$ | 386.1365 |
| Desacetyldoronine | $C_{19}H_{28}ClNO_7$ | 418.1627 | Doronine | $C_{21}H_{30}ClNO_8$ | 460.1733 | Europine | $C_{16}H_{27}NO_6$ | 330.1911 |
| Europine N-oxide | $C_{16}H_{27}NO_7$ | 346.1860 | Floridanine | $C_{21}H_{31}NO_9$ | 442.2072 | Florosenine | $C_{21}H_{29}NO_8$ | 424.1966 |
| Heliotrine N-oxide | $C_{16}H_{27}NO_6$ | 330.1911 | Indicine | $C_{15}H_{25}NO_5$ | 300.1805 | Indicine N-oxide | $C_{15}H_{25}NO_6$ | 316.1755 |
| Integerrimine | $C_{18}H_{25}NO_5$ | 336.1805 | Integerrimine-N-oxide | $C_{18}H_{25}NO_6$ | 352.1756 | Intermedine | $C_{15}H_{25}NO_5$ | 300.1805 |
| Intermedine-N-oxide | $C_{15}H_{25}NO_6$ | 316.1755 | Isotussilagine | $C_{10}H_{18}NO_3$ | 201.1359 | Isotussilaginic acid | $C_9H_{16}O_3N$ | 187.1203 |
| Jacoline | $C_{18}H_{27}NO_7$ | 370.1860 | Jacoline-N-oxide | $C_{18}H_{27}NO_8$ | 386.1809 | Jaconine | $C_{18}H_{26}ClNO_6$ | 388.1521 |
| Jaconine-N-oxide | $C_{18}H_{26}ClNO_7$ | 404.1471 | Jacozine | $C_{18}H_{23}NO_6$ | 350.1598 | Jacozine-N-oxide | $C_{18}H_{23}NO_7$ | 366.1547 |
| Junceine | $C_{18}H_{27}NO_7$ | 370.1860 | Lasiocarpine N-oxide | $C_{21}H_{33}NO_8$ | 428.2279 | Lycopsamine-N-oxide | $C_{15}H_{25}NO_6$ | 316.1754 |
| Monocrotaline N- | $C_{16}H_{23}NO_7$ | 342.1547 | Neo-Senkirkine | $C_{19}H_{27}NO_6$ | 366.1911 | Onetine | $C_{19}H_{29}NO_8$ | 400.1966 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| oxide | | | | | | | | |
| Otosenine | $C_{19}H_{27}NO_7$ | 382.1860 | Riddelline | $C_{18}H_{23}NO_6$ | 350.1598 | Riddelline-N-oxide | $C_{18}H_{23}NO_7$ | 366.1547 |
| Seneciphylline-N-oxide | $C_{18}H_{23}NO_6$ | 350.1598 | Senecivernine-N-oxide | $C_{18}H_{25}NO_6$ | 352.1755 | Spartioidine | $C_{18}H_{23}NO_5$ | 334.1649 |
| Spartioidine N-oxide | $C_{18}H_{23}NO_6$ | 350.1598 | Trichodesmine | $C_{18}H_{27}NO_6$ | 354.1911 | Tussilagine | $C_{10}H_{18}NO_3$ | 201.1359 |
| Tussilaginic acid | $C_9H_{16}NO_3$ | 187.1203 | Usamarine | $C_{18}H_{25}NO_6$ | 352.1755 | Usaramine-N-oxide | $C_{18}H_{25}NO_7$ | 368.1704 |
| *quinazoline* | | | | | | | | |
| 5-Hydroxy vasicine | $C_{11}H_{12}N_2O_2$ | 205.0971 | 5-Methoxyvasicine | $C_{12}H_{14}N_2O_2$ | 235.1077 | 5-Methoxyvasicinol | $C_{12}H_{14}N_2O3$ | 219.113 |
| Adhatodine | $C_{20}H_{21}N_3O_2$ | 336.1706 | Adhavasicinone | $C_{12}H_{12}N_2O_3$ | 233.0921 | Aniflorine | $C_{20}H_{21}N_3O_3$ | 352.1656 |
| Anisotine | $C_{20}H_{19}N_3O_3$ | 350.1499 | Deoxyaniflorine | $C_{20}H_{21}N_3O_2$ | 336.1706 | Deoxy-methoxy vasnetine | $C_{20}H_{21}N_3O_3$ | 352.1656 |
| Deoxyvasicine | $C_{11}H_{12}N_2$ | 173.1073 | Methoxy-adhatodine | $C_{21}H_{23}N_3O_3$ | 366.1812 | Methoxy-anisotine | $C_{21}H_{21}N_3O_4$ | 380.1605 |
| Methoxy-vasicoline | $C_{20}H_{23}N_3O$ | 322.1914 | Methoxy-vasnetine | $C_{20}H_{19}N_3O_4$ | 366.1448 | Metolazone | $C_{16}H_{16}ClN_3O_3S$ | 366.0674 |
| N-demethyl-adhatodine | $C_{19}H_{19}N_3O_2$ | 322.1550 | Prazosin | $C_{19}H_{21}N_5O_4$ | 384.1666 | Vasicine | $C_{11}H_{13}N_2O$ | 190.1100 |
| Vasicine glycoside | $C_{17}H_{22}N_2O_6$ | 351.1551 | Vasicinol | $C_{11}H_{12}N_2O_2$ | 205.0971 | Vasicinolone | $C_{11}H_{10}N_2O_3$ | 219.0764 |
| Vasicinone | $C_{11}H_{11}N_2O2$ | 204.0893 | Vasicoline | $C_{19}H_{21}N_3$ | 292.1808 | Vasicolinone | $C_{19}H_{19}N_3O$ | 306.1601 |
| Vasnetine | $C_{19}H_{17}N_3O_3$ | 336.1343 | | | | | | |
| *quinoline* | | | | | | | | |
| Aurachin A | $C_{25}H_{33}NO_3$ | 396.2533 | Evolitrine | $C_{13}H_{11}NO_3$ | 230.0812 | Graveoline | $C_{17}H_{13}NO_3$ | 280.0968 |
| Haplophyllidine | $C_{18}H_{23}NO_4$ | 318.1700 | Kokusagine | $C_{13}H_9NO_4$ | 244.0604 | Leiokinine A | $C_{14}H_{17}NO_2$ | 232.1332 |
| Magnoflorine | $C_{20}H_{24}NO_4$ | 342.1700* | Montanine | $C_{17}H_{19}NO_4$ | 302.1387 | Perforine | $C_{18}H_{25}NO_5$ | 336.1805 |
| Quinidine | $C_{20}H_{24}N_2O_2$ | 325.1910 | Quinine | $C_{20}H_{24}N_2O_2$ | 325.1910 | Ravenine | $C_4H_9NO_2$ | 104.0706 |
| Ribalinine | $C_{15}H_{17}NO_3$ | 260.1281 | Veprisine | $C_{17}H_{19}NO_4$ | 302.1387 | | | |
| *quinolizidine* | | | | | | | | |
| Angustifoline | $C_{14}H_{22}N_2O$ | 235.1805 | Lupinine | $C_{10}H_{19}NO$ | 170.1539 | Matrine | $C_{15}H_{24}N_2O$ | 249.1961 |
| *ß-carbonil* | | | | | | | | |
| Harmalol | $C_{12}H_{12}N_2O$ | 201.1022 | Harmine | $C_{13}H_{12}N_2O$ | 213.1022 | | | |
| *steroidal* | | | | | | | | |
| alpha-Chaconin | $C_{45}H_{73}NO_{14}$ | 852.5104 | beta-Solamargine | $C_{45}H_{73}NO_{15}$ | 868.5053 | Cevadin | $C_{32}H_{49}NO_9$ | 592.3480 |
| Cyclopamine | $C_{27}H_{41}NO_2$ | 412.3210 | Peimin | $C_{27}H_{45}NO_3$ | 432.3472 | Peiminin | $C_{27}H_{43}NO_3$ | 430.3316 |
| *tropane* | | | | | | | | |
| 3,6-Diacetyltropine | $C_{12}H_{19}NO_3$ | 226.1438 | 3-Acetyltropine | $C_{10}H_{17}NO_2$ | 184.1332 | Anisodamine | $C_{17}H_{23}NO_3$ | 306.1700 |
| Anisodine | $C_{17}H_{21}NO_5$ | 320.1492 | Apohyoscyamine | $C_{17}H_{21}NO_2$ | 272.1645 | Arecoline | $C_8H_{13}NO_2$ | 156.1019 |
| Bellendine | $C_{12}H_{15}NO_2$ | 206.1176 | Brugine | $C_{12}H_{19}NO_2S_2$ | 274.0930 | Catuabin A | $C_{25}H_{22}O_{10}$ | 483.1286 |
| Chalcostrobamine | $C_{17}H_{19}NO_2$ | 270.1489 | Cochlearine | $C_{15}H_{19}NO_3$ | 262.1438 | Ecgonine | $C_9H_{15}NO_3$ | 186.1125 |
| Ferrugine | $C_{15}H_{19}NO$ | 230.1539 | Ferruginine | $C_{10}H_{15}NO$ | 166.1226 | Homatropine | $C_{16}H_{21}NO_3$ | 276.1594 |
| Scopine | $C_8H_{13}NO_2$ | 156.1019 | Tropinone | $C_8H_{13}NO$ | 140.1070 | Valerine | $C_8H_{15}NO_2$ | 158.1176 |
| *other* | | | | | | | | |
| Valsartan | $C_{24}H_{29}N_5O_3$ | 436.2343 | | | | | | |

*$[M]^+$

## 3.2. Alkaloid profiles of herbal and alpine plants

### 3.2.1. Alkaloid profiling of herbal drugs using high resolution mass spectrometry

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### 3.2.2. Targeted and untargeted profiling of alkaloids in Italian alpine herbs using high resolution mass spectrometry and their use for distinguishingcriminating the herbage grazed by dairy cows

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### 3.2.1. Alkaloid profiling of herbal drugs using high resolution mass spectrometry

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**Abstract**

Herbal infusions are consumed worldwide thanks to their "natural" beneficial effects, also due to the presence of alkaloids, although these compounds can have poisonous effects. A method combining online solid-phase purification with high resolution mass spectrometry was used to define the alkaloid profiles of 117 herbs and 7 commercial blends. 41 alkaloids were quantified in reference to analytical standards, while the presence of a further 116 was confirmed on the basis of accurate mass, retention time and fragmentation profile. The targeted study showed that 52% of herbs and 42% of commercial blends contained at least one alkaloid, and pyrrolizidines were the most commonly present (26% of samples), with concentrations generally ranging from the quantification limit to roughly 100 μg $kg^{-1}$. Moreover, a homemade infusion was studied, finding on average 45% and 6% lower extraction for pyrrolizidine and steroidal alkaloids, respectively. Nevertheless, the migration of pyrrolizidines was confirmed. The study confirmed the frequent presence, natural or accidental, of alkaloids in commercial infusion herbs, highlighting the urgent need for routine and accurate controls.

**Keywords:** alkaloids; herbal infusion; liquid chromatography; Orbitrap™; on-line solid-phase extraction

**Introduction**

Alkaloids (alks), basic nitrogen-containing organic components, are a heterogeneous class of compounds produced by plants as secondary metabolites[1]. They are classified into three principal classes, depending on precursors and final molecular structures: atypical, typical and pseudo alks. Typical and atypical compounds are both derived from amino acids, although typical alks have heterocyclically bound nitrogen. Pseudo alks do not derive from amino acids but arise from amination of another type of substrate, which may be acetate derived, phenylalanine derived, terpene or steroid. Their pronounced biological activity, which is often associated with the presence of amine moieties, plays an important role in the interaction of plants with their environment[2]. In humans, alks manifest a marked physiological action, converting the amine function into a quaternary system by protonation at physiological pH[3]. As regards this, some alks are considered to be responsible for the beneficial effects of traditional medicines[4-7] and their intake is often associated with widespread consumption of herbal infusions, used worldwide as 'natural' home medical treatments. In contrast, some alks may have the harmful effects of poisons[8,9]. In particular, pyrrolizidine (Pyz) alks have hepatotoxic, mutagenic and carcinogenic effects. Pyz alks and their N-oxide forms, mainly found in the families of the Boraginaceae (all genera), Asteraceae (Senecioneae and Eupatorieae tribes) and Fabaceae (Crotalaria genus), are composed of a core necine base condensed with one or two necic acids, but only Pyz alks with double bonding in the 1,2-position of the Pyz ring (dehydro Pyz alks), such as retronecine-, heliotridine-, otonecine- and supinidine-types demonstrated these effects[10]. Some Pyz alks are absorbed from the intestine and partly hydrolysed by esterases in non-toxic compounds excreted by the kidneys, but most alks are transported to the liver, where monooxygenases metabolise them into pyrrole derivatives through hydrolysis, N-oxidation and dehydrogenation of the Pyz ring, inducing intoxication reactions[11]. In addition to the liver, blood vessels, lungs, kidneys, the gastro-intestinal tract, the pancreas and bone marrow may be damaged[12]. The British "Committee on Toxicity of Chemicals in Food, Consumer Products and Environment"[13], concluded that doses of Pyz alks below 0.007 μg/kg body weight/day would not be problematic for health. In accordance with the German Federal Institute for Risk Assessment (BfR), a daily intake limit of 0.007 μg $kg^{-1}$ body weight (0.42 μg for a 60 kg adult) was established for dehydro Pyzs[14]. Recently, a scientific opinion was requested from EFSA in relation to the possible human and animal health risk due to tropane (Trp) alks present in food and feed. Although, more than 200 different Trp alks have been identified in several plant families, including Brassicaceae, Solanaceae and Erythroxylaceae, risk assessment has however only been performed on (-) hyoscyamine and (-) scopolamine, the two compounds for which presence and

toxicity data were available. The CONTAM Panel established an Acute Reference Dose (ARfD) of 0.016 µg/kg body weight, expressed as the sum of (-) hyoscyamine and (-) scopolamine, based on decreased heart rate results in a human volunteer study[15].

Following these claims, various studies have focused their attention on the alkaloid content of various plant species used for infusions. Even restricting their research to Pyz alks and Trp alks, they observed that commercial preparations may contain high amounts of alks[10,16,17].

The toxicity of other groups such as quinolizidine (Qnz) alks and glycosteroid (Gst) alks has been demonstrated and evaluated in other food commodities such as the lupin bean, potato, tomato and eggplant[18,19], but there have been no studies about infusion herbs. Qnz alk intoxication is characterised by trembling, shaking, excitation and convulsions, and in more severe cases there can be acute oral toxicity due to neurological effects, leading to loss of motor co-ordination and muscular control. The health authorities of some countries (Great Britain, France, Australia and New Zealand) have established a maximum limit of 200 mg $kg^{-1}$ of Qnz alks for lupin flour and food[18]. As regards Gst alks, toxicity may be due to the effect induced by hepatic ornithine decarboxylase on the central nervous system and disruption of the cell membrane affecting the digestive system. The acute toxic dose is estimated to be 1.75 mg $kg^{-1}$ of body weight, and the lethal dose 3-6 mg $kg^{-1}$[19].

Several methodologies have been developed to detect and quantify alks, including high performance liquid chromatography (HPLC) coupled with a diode array detector (DAD)[20] or fluorimetric detector (FLD)[21], capillary electrophoresis[22], and gas chromatography (GC) coupled with a mass spectrometer (MS)[23,24]. The use of HPLC–DAD/FLD often does not allow adequate sensitivity and selectivity to be obtained for alks present at trace levels, and GC-MS approaches require time-consuming preventive derivatisation steps. Even with regard to HPLC-MS methods, to remove matrix interference, samples were manually pretreated using solid phase extraction (SPE)[17,25], a time-consuming and cost-intensive step.

This work aimed to carry out broad alk profiling of a wide selection of commercial herbal products, including both quantification of 41 alks with reference to pure analytical standards, and putative identification of a further 116 alks, confirmed on the basis of accurate mass, isotopic pattern, chromatographic retention time (RT) and fragmentation profile, obtained by analysing extracts of herbs already well documented in the literature.

Alk characterisation was carried out on 117 commercial single-herb products and 7 herbal blends by combining automatic online SPE clean-up and the rapid and selective detection ability of hybrid quadrupole orbitrap mass spectrometry.

Moreover, intake of alks due to domestic consumption of hot water infusions of these herbal products was investigated.

## Materials and Methods

*Reagents and solutions*

LC-MS grade acetonitrile (ACN), LC-MS grade methanol (MeOH), MS grade formic acid (FA, 98%) and LC-MS grade ammonium acetate were purchased from Fluka (St. Louis, MO, USA) and ammonia solution 25% was purchased from Merk Millipore (Darmstadt, Germany). For mass calibration, a standard mix of n-butylamine, caffeine, MRFA and Ultramark 1621 (Pierce® ESI Positive Ion Calibration Solution, Rockford, IL, USA) were used. Deionized water was produced with an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany).

Table 1 shows the technical characteristics and validation parameters of commercial alks used for the targeted method. Individual stock solutions of each alk were prepared by dissolving the standard in a 50% aqueous methanol solution to reach a final concentration of about 100 mg $L^{-1}$. An aliquot of 2 mL of the mix solution produced from the single stock solutions, with a final concentration of 1 mg $L^{-1}$ of each single alk, was transferred into an analytical vial and used for calibration in the range 0.02 – 1000 µg $L^{-1}$, injecting 1ul for each level. The mix solution was prepared freshly before each analysis, while stock solutions were stored at -4°C.

*Plant sampling and sample extract preparation*

117 single-plant samples and 7 commercial herbal blends for infusions were purchased from different herbalist shops in the north of Italy. Table 2 summarises the botanical characteristics of the herb samples.

For alk profile evaluation, each herbal sample was subjected to extraction using polyethylene 50 mL falcon tubes (Sartorius AG, Goettingen, Germany). A homogeneous aliquot of 2.5 g of the herb was added to 20 mL of extraction solution ($H_2O$/MeOH/FA; 49.5:49.5:1 v/v/v), sonicated for 10 minutes (LBS1 6Lt, FALC Instruments, Treviglio BG, Italy), and subjected to vertical shaking for 12 hours at 20 rpm (Rotoshake 24/16, Gerhardt GmbH & Co. KG, Königswinter, Germany). The

mixtures were once again sonicated for 10 minutes, and the methanolic extract was separated after centrifugation (10 minutes at 4100 rpm; IEC CL31 Multispeed, Thermo Scientific, Sunnyvale, CA, USA). Finally, the extract was filtered with a 0.45 µm cellulose filter cartridge (Sartorius AG, Goettingen, Germany) and diluted 2 times with a $H_2O$/MeOH solution (50:50 v/v) and 10 µL was injected.

For evaluation of the real alk content of a herbal infusion extract, the weight of one spoon of 8 herbal samples (*Acorus calamus, Cassia angustifolia, Cuminum cyminum, Escholtzia californiea, Malva sylvestris, Origanum vulgare, Parietaria officinalis,* and *Pimpinella anisum*) previously shown to have a diversified alk content and high concentration were extracted with 4 mL of hot water for 15 minutes. The mixtures were then separated after centrifugation and the extract was filtered with a 0.45 µm cellulose filter cartridge, diluted twice with a H2O/MeOH solution (50:50 v/v) and injected 10 µL.

*Chromatographic separation*

Chromatographic separation was obtained using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA), furnished with a Rheodyne 6-port automated switching valve and a pump module that allowed control of two independent fluid systems. The method used the same approach proposed by Nardin and colleagues[26].

Online clean-up was performed using a SolEx HRP SPE cartridge (2.1 mm × 20 mm, 12-14 µm, ThermoFisher, Sunnyvale, CA, USA), loaded with 10 µL of sample and flushed with 4% MeOH adjusted to pH=9 with ammonia (eluent A; flow rate of 1 mL $min^{-1}$). After 1 minute, 0.1% FA flushed the cartridge at 1 mL $min^{-1}$ for another minute to complete matrix interference removal. A Rheodyne valve switched the position and analytical mobile phase. 70% of 0.1% FA with 5mM ammonium acetate (eluent B) and 30% of MeOH/ACN 95:5 v/v with 0.1% FA and 5 mM ammonium acetate (eluent C) at a flow rate of 0.700 mL $min^{-1}$ flowed through the SPE cartridge, progressively removing the retained analytes and transferring them to the analytical column (Raptor Biphenyl, 3 mm x 150 mm, 2.7 µm particle size, Restek, Bellefonte, PA, USA). Chromatographic separation was achieved with eluent C set at 30% from 2 to 4 minutes, then it was linearly increased to 80% from 4 to 25 minutes, and to 100% from 25 to 26 minutes. After 3 minutes at 100%, eluent C was linearly reduced to 30% in 0.5 minutes. Before each injection, the analytical column was equilibrated for 2.5 minutes with 30% eluent C in the initial conditions, meanwhile the SPE

cartridge was flushed with MeOH with 1% FA at 1 mL $min^{-1}$ in order to wash it, and then with eluent A to re-equilibrate it before the next analysis.

Chromeleon™ 7.2 Chromatography Data System software (Thermo Scientific™ Dionex™) automatically piloted the switching valve and the chromatographic separation gradient. The autosampler was set at a temperature of 5 °C and the column at 35 °C.

*Mass Spectrometry*

A Q-Exactive™ hybrid quadrupole-orbitrap mass spectrometer (HQOMS, Thermo Scientific, Bremen, Germany) equipped with heated electrospray ionisation (HESI-II) interface was used for alk analysis. In the HESI II source, nitrogen was used as the drying and collision gas in positive ion mode. Mass spectra were acquired in profile mode through a full MS-data dependent MS/MS (dd MS/MS; a fixed number of precursor ions are subjected to a second MS/MS analysis) experiment, as in the method proposed by Nardin and colleagues[26]..

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software was used for instrument control and for data processing and evaluation.

*Targeted method validation*

The characteristics of the targeted alks method were studied using full mass spectral data of 41 pure standards. Matching *m/z* values (mass tolerance < 5 ppm[27]), RT and isotope pattern were used to identify targeted alks in sample analysis data, and in order to confirm them dd-MS/MS spectra samples were compared with those collected from available standards. The precursor ion detected in the extracted ion chromatograms (EICs) and corresponding to the protonated molecules $[M-H]^+$ was always used for quantification.

External solvent calibration curves were used for analyte quantification. The limit of detection (LOD) was estimated as three standard deviations of ten replicated blank samples according to EURACHEM[28], and similarly, the limit of quantification (LOQ) was estimated as ten standard deviations of the same replicates. Standard levels allowing a regression coefficient ($R^2$) of at least 0.990 were included in the linearity range. Precision was estimated as the relative standard deviation (RSD%) of seven analytical replicates of a blank sample, spiked at three increasing concentration levels covering the quantitation range of each alk. Method accuracy, expressed as relative error (RE%), was estimated as the percentage difference between the expected and the returned mean concentration of the same blank sample, spiked at low, medium and high

concentration levels, each one analytically replicated seven times. Table 1 summarises the exact mass, the normalized collision energy (NCE) used for MS/MS experiments, fragment ions, linearity range, precision and accuracy of targeted compounds.

*Untargeted study*

In order to develop an untargeted method useful for alk profiling of commercial herbal infusion products, initial putative confirmation of the RT and fragmentation of 116 non-commercially available alks was performed, using 25 plant samples with a well-documented alk composition. The plants were extracted and injected for initial evaluation. EICs, matching *m/z* values with a mass tolerance < 5 ppm[27]) compared to the exact mass of the alks reported in the literature, were investigated to confirm the presence of such compounds in our extracts. If a peak signal was present, the RT of the alk was confirmed and a second injection was performed to collect fragmentation profile information.

*Statistical study*

Bivariate analysis was performed by combining targeted and untargeted alk data, using Statistica 13.1 software (StatSoft; Tulsa, OK, USA). As regards targeted alks, statistical processing was carried out based on concentration values, while for untargeted alks the peak area was used. The correlation matrices were obtained considering alks present in at least 10% of samples ($p<0.05$).

**Results**

*Targeted profile*

The targeted alk standards, linearity, LOD and LOQ, precision, and accuracy determined for each compound are shown in Table 1. Detectability was strongly dependent on the specific ionisation efficiency of each compound and the LOD ranged from the lowest values for Heliotrine (0.04 µg $L^{-1}$), to the highest for Nicotine (15 µg $L^{-1}$). The range of quantitation went from the quantification limits to 500/1500 µg $L^{-1}$, depending on the compounds. Table 3 shows herbal (62) and commercial blends (3) in which at least one of the 41 tested targeted alks was present above the LOD. As regards individual herbal teas, 52% of samples contained at least one targeted alk. *Cinchona succirubra* showed a total concentration of 23 mg $kg^{-1}$, the highest of all the herbal samples, while the alk content in other samples ranged from 1.5 to 1100 µg $kg^{-1}$. In the case of commercial blends,

42% of samples contained at least one targeted alk. Specifically, alfa-Solasonine and alpha-Solamargine were quantified, with concentrations ranging from 6 to 90 µg $kg^{-1}$, along with Lycopsamne, at a concentration of 0.9 µg $kg^{-1}$.

In general, Pyzs were the most commonly present alks (26% of samples), with concentrations generally ranging from 1 to 115 µg $kg^{-1}$, with the exception of Heliotrine and Lasiocarpine which had higher content in *Cuminum cyminum* (285 and 283 µg $kg^{-1}$, respectively), and Monocrotaline in *Cassia angustifolia* (176 µg $kg^{-1}$). The most common was Lycopsamine (present in 13% of samples) with an average concentration of 10 µg $kg^{-1}$, whereas Erucifoline and Senecionine-N-oxide were never present.

A significant percentage of purine (Pun) alks (10 %) were also found in the samples analysed. Caffeine was the most common (9.6%), with an average concentration of 47 µg $kg^{-1}$.

Gst alks and Qnz alks were detected in 8% of samples. Gst alk concentrations ranged from 6 to 400 µg $kg^{-1}$ and quinoline (Qnl) from 10 µg $kg^{-1}$ to 22 mg $kg^{-1}$. The highest concentrations of Quinidine and Quinine were in *Cinchona succirubra* (6 and 22 mg $kg^{-1}$, respectively) as reported in the literature[29].

Piperidine (Ppr) and Str alks were detected in 4% of samples, with concentrations generally ranging from 1 to 35 µg $kg^{-1}$, whilst Protoveratrine A, Tomatidine/Tomatine, and Veratramine were never present.

Indole (Ind) alks were detected in 3% of samples, and diterpene and pyridine (Pyr) alks in 2%, while Trp alks were never detected.

*Confirmation of untargeted alks*

In the previous work proposed by Nardin and colleagues[26], the RT and fragmentation of 48 alks was confirmed by analysing extracts of *Datura stramonium* and *Hyoscyamus niger* for 4 Trp alks, *Solanum nigrum* for 1 Gst alk, *Lobelia inflata* for 23 Ppr-type alks, *Senecio vulgaris* for 10 Pyz alks, *Arnica montana* for 1 pyrrolidine (Pyl) alk, *Gelsemium sempervirens* for 8 Ind alks, and *Ranunculus montanus* for 1 Qnl-type alk.

In this work, we implemented the previous database with a further 68 alks confirmed by analysing the extracts of 17 plants of well-documented composition. Table 4 shows the RTs and accurate masses of the selected precursor ion and fragments.

Acridone-type (Acd) alks were studied in *Ruta graveolens* extract. This spontaneous plant of the Rutaceae family was popularly known as an abortive remedy, and preclinical studies suggested that

its aqueous extract could induce apoptosis in different malignant cell lines of glioblastoma. Evoxanthine, Rutacridone and Arborinine, the most important alks for this plant, were detected in our extract at 13.4, 24.3 and 25.5 minutes, respectively.

Correct identification of Ind alks was performed in *Passiflora incarnate* extracts. Extracts of this fast-growing perennial plant have sedative effects similar to chamomile, due to its Ind alk content. The literature documents the presence of Harmalol, Harmaline, Norharmane, Harmol, Harmine and Harmane, and we confirmed the presence of the first 5 compounds at 11.3, 11.8, 14.2, 14.4 and 15.0 minutes, and of two possible isomers of Harmane at 15.5 and 15.9 minutes, respectively.

Isoquinoline (Iqn) and benzophenantridine (Bzp) alks were investigated in extracts of *Escholtzia californiea* and *Fumaria officinalis. Escholtzia californiea*, a herbaceous perennial plant native to Arizona, California and Oregon, was used in the past by Native North Americans for medicinal purposes. Modern medicine uses it to treat insomnia and anxiety, due to its antispasmodic properties as a result of action on the central nervous system, and also for childhood neuropathies. The literature reports the presence of Caryachine, Escholtzina, N-Methyllaurotetanine, *o*-Methylcaryachine, Protopin and Sanguinarine. The EICs of Iqn alks Escholtzina and N-Methyllaurotetanine showed one peak respectively at 18.6 and 18.0 minutes, while the other Iqn alks showed more than one peak, probably due to the presence of different isomers. Caryachine showed 4 peaks at 14.5, 15.5, 17.8, and 19.3 minutes, whilst *o*-Methylcaryachine had 2 peaks at 16.0 and 18.3 minutes. The EICs of the Bzp alks Protopin and Sanguinarine showed peaks at 19.5 and 25.3 minutes, respectively. *Fumaria officinalis*, a spontaneous herbaceous annual plant belonging to the Fumariaceous family, has been shown to have depurative, hepatoprotective and anti-spastic properties, especially within the bile duct but also in the gastrointestinal tract. Recent studies emphasised the interesting inhibitory activity of *Fumaria officinalis* alks against acetylcholinesterase, suggesting its use may be a valid aid in the treatment of Alzheimer's disease. In our plant extract, the RTs of Cheilanthifoline, Coridamine, Fumaricine, Fumariline, Fumaritine, Fumaropycine, methyl-Fumariphycine, Sinactine and Stylopine were 17.2, 22.4, 18.3, 18.3, 18.3, 16.6, 19.3, 19.1, and 19.2 respectively, whilst Parfumidine had 2 isomers, with peaks at 18.9 and 19.7.

Ppr-type alks were studied in black pepper grains, the fruits of *Piper nigrum,* a plant belonging to the Piperacee family, traditionally used by Ayurveda, Siddha and Unani medicine in India as an anti inflammatory and anti malarial drug, and currently used in anti-leukaemia treatments. In our extract,

Dihydropiperlonguminine, Piperanine, Piperine and Piperlonguminine showed peaks at 22.6, 25.1, 25.5 and 23.0 minutes, respectively.

Protoalkaloids (PrAs) were investigated in *Galega officinalis*, a plant of the Fabaceae family known for its galactagogue properties and that is also used in diabetes and liver treatments. The presence of the 2 isomers Galegin and Peganin was found at 15.6 and 15.9 minutes.

Of the Pyr alks, the toxic neurotoxin Ginkgotoxin was studied in *Ginko biloba*, the last surviving species of the Ginkgoaceae family, native to China. Its exocarp extract was shown to have immune promotion and cancer inhibition effects. The EIC showed a peak at 5.5 minutes for Ginkgotoxin.

*Achillea millefolium*, a perennial and aromatic herb of the Asteraceae family, was analysed to confirm the Pyl alks Betonicine and Stachydrine. This plant, one of the most widely used medicinal plants in the world, is commonly used primarily to treat wounds, digestive problems, respiratory infections and skin conditions, and more specifically, for liver diseases and as a mild sedative. Betonicine had one peak at 3.0 minutes, while Stachydrine showed two peaks at 3.5 and 4.1 minutes.

Pyz alks were identified in *Mentha piperita* and *Urtica dioica. Mentha piperita,* a highly aromatic herbaceous perennial plant that belongs to the Lamiaceae family, is native to Europe and is widely cultivated throughout the world. Its essence is mostly used to prepare drinks and confectionery products and it is used in cases of indigestion, nausea, diarrhoea, colds, flu, acne, toothache and migraine. *Urtica dioica,* a perennial herb native to Europe, Asia, northern Africa and North America, well-known for its stinging hairs, was proposed for the treatment of arthritis. Due to the frequent presence of isobaric masses among Pyz alks, it was only possible to confirm the compounds with fragments reported in the literature. In *Mentha piperita* extract, considering the *m/z* 254.1374 fragment, Lasiocarpine N-oxide was attributed to the peak at 19.0 minutes, whilst in *Urtica dioica*, considering the *m/z* 118.0357 fragment, Seneciphylline N-Oxide was attributed to the peak at 22.0 minutes.

Qnl alks were confirmed by analysing *Cinchona succirubra* and *Plantago major* extracts. *Cinchona succirubra* is a genus of woody plants from the Andes belonging to the Rubiaceae family, with antimalarial, analgesic and antipyretic properties. The presence of different Cinchonanines, A, B, C, D, E, and F, is documented in *Cinchona succirubra* extracts, but in our extract, we could not detect Cinchonanine A and D. Cinchonanine B, C and F showed 2 peaks for each alk respectively at 19.5 and 23.4 minutes, 14.8 and 16.3 minutes, and 12.0 and 19.0 minutes, while Cinchonanine E had a single peak at 15.7 minutes. *Plantago major*, a herbaceous perennial plant belonging to the

Plantaginaceae family, traditionally used for haemorrhoid treatment and respiratory diseases, was recently proposed for the cure of skin wounds, burns and bleeding. The literature documents the presence of Indicain and Plantagonin and we were able to tentatively identify them at 5.8 and 4.4 minutes respectively, although the low intensity of signals did not make it possible to confirm the fragmentation.

Qnz alks were studied in *Salvia officinalis*, *Vaccinium myrtillus*, and *Veratrum album*. *Salvia officinalis*, commonly used to flavour dishes despite the proven toxicity of thujone present in traces, is also used to treat depression, memory disorders and age-related memory decline. Heimidine is reported as a *Salvia officinalis* Qnz alk, and our EIC showed 2 peaks at 21.2 and 22.3 minutes. *Vaccinium myrtillus,* a shrub belonging to the Ericaceae family, is regarded by traditional medicine as a useful plant with antidiabetic properties. The literature reports the presence of Epimyrtine and Myrtin, two isomeric compounds which were detected at a single peak at 6.9 minutes. *Veratrum album*, a toxic rhizomatous plant belonging to the Liliaceae family, contains Cevadine, Jervine and Pseudojervine alks. The presence of Jervine was also confirmed in comparison with the analytical standard at 19.9 minutes, while two peaks at 17.2 and 18.1 minutes were attributed to Cevadine, and one at 18.5 minutes to Pseudojervine.

Finally, terpenoid (Trn) alks were studied in *Glycyrrhiza glabra* and *Valeriana officinalis* extracts. *Glycyrrhiza glabra*, a perennial herbaceous plant belonging to the Fabaceae family, is traditionally used in Asian medicine and preventive effects against atherosclerosis have been reported. The presence of Mesaconine and Aconine in this plant is documented and 2 peaks for each alk were found respectively at 16.3 and 16.6 minutes, and 17.2 and 17.5 minutes. *Valeriana officinalis* is a flowering plant belonging to the Valerianaceae family, known for its sedative and calming properties and recently studied as a cancer treatment. The literature documents the presence of Actinidine, Cathinine, Valerianine, and Valerine and we confirmed Actinidine at 13.0, Cathinine at 5.3, two possible isomers of Valerianine at 9.0 and 10.1, and Valerine at 5.2 minutes.

References to the botanical, pharmacological and chemical information presented above are given in Table 5.

*Untargeted profiling*

Broad characterisation with tentative identification of commercial herbal samples was performed through comparison with the previously mentioned database. The study of untargeted alks was

limited to compounds providing a sufficient detectable response (area > 15000 area units for herbs, and area > 1000 for commercial blends).

An overview of the alk profiles in herbs and commercial blends, sorted by chemical/botanical groups, is presented in Table 6. As many as 92 different alks were detected with untargeted analysis of the 117 herb samples, and 10 alks in the 7 commercial blends. As regards single herbs, the alks sorted by groups were: Iqns (present in 40% of herb samples), Trns (39%), Qnls (36%), Inds (35%), Pyl (33%), Pprs (30%), Bzps (26%), Qnzs (21%), PrAs and Pyrs (16%), Pyzs (7%), Trps (6%) and Acds (3%). *Escholtzia californiea* and *Citelidonium majus,* both belonging to the Papaveraceae family, and *Fumaria officinalis,* in the Fumariaceae family, showed the highest number of alks (28, 24 and 26, respectively).

In the families with the largest number of analysed samples, Asteraceae (14 herbs) were found to be rich in Iqns (Caryachine, Escholtzina, Fumaropycine, methyl-Fumarophycine and N-methyllaurotetanine, o-Methylcaryachine and Parfumidine), Qnls (Cinchonanine B, Cinchonanine C and Cinchonanine E/ Cinchonine, Cinchonanine F, Cinchonanine G and Magnoflorine), Pprs (Dihydropiperlonguminine, Isolobinanidine, Isolobinine/Lobinine and Norlelobanidine/8-Methyl-10-phenyllobelidiol), and Inds (Harmalol, Harmane, Harmine, Harmol and Norharmane). Valerianine (Trn) was the most frequently found (6 herbs). Lamiaceae (12 herbs) were found to be rich in Iqns (Caryachine, Escholtzina, Fumaricine and N-methyllaurotetanine, o-Methylcaryachine and Sinactine), Qnls (Cheilanthifoline, Cinchonanine C, Cinchonanine E/ Cinchonine, Cinchonanine F and Magnoflorine). Isolobinine/Lobinine (Ppr) was the most frequently found (5 herbs). Apiaceae (10 herbs) were found to be rich in Inds (Harmalol; Harmane, Harmine, Harmol and Norharmane) and Iqns (Corydamine, Fumariline, methyl-Fumarophycine and N-methyllaurotetanine). The alks most frequently found (3 different herbs) were Harmane, Harmol and Norharmane (Inds), Tropinone (Pyr), and Stachydrine (Pyl). Rosaceae (6 herbs) were found to be rich in Inds (Harmane, Harmine, Harmol and Norharmane) and Iqns (Escholtzina, Fumariline, Fumaropycine, ethyl-Fumarophycine and N-methyllaurotetanine). Harmane (Ind) and Myrtine/Epimyrtine (Qnz) were the most frequently found (5 and 4 herbs, respectively). Fabaceae (4 herbs) were found to be rich in Trns (Aconine and Mesaconine) and Pyls (2 Pyrrolidineacetic acids and Stachydrine). N-methyllaurotetanine (Iqn), Stachydrine (Pyl), and Magnoflorine (Qnl) were the most frequently found (2 herbs each). Polygonaceae (4 herbs) were found to be rich in Iqns (Fumariline, Fumaropycine, methyl-Fumarophycine and N-methyllaurotetanine), Magnoflorine

(Qnl) being the most frequently found (2 herbs). Finally, Zingiberaceae (4 herbs) showed only the presence of Stachydrine (Pyl).

As regards commercial blends, Mix#4 had the highest number of alks (N=4; Harmane, Norlelobanidine/8 Methyl-10-phenyllobelidiol, Plantagonin and Myrtine/Epimyrtine). Mix#5 had 3 alks (Plantagonin, Lasiocarpine and Valerianine), while Mix#2 and Mix#6 had 2 alks (Dihydropiperlonguminine and Magnoflorine, and Integerrimine/Perforine and Mesaconitine, respectively). Mix#1 and Mix#7 had only 1 alk (N-methyllaurotetanine and Valerianine, respectively).

*Statistical study*

Studies in the literature report that only some alkaloids can be recognised as markers of taxonomic plant grouping, such as glucosinolates for Capparaceae and Cruciferae, and 1-benzyltetrahydroisoquinoline alks for Papaveraceae and Fumariaceae[67,68]. In contrast, others such as Pyzs, predominantly present in families such as Boraginaceae, Asteraceae and Fabaceae[69], are certainly rarer in Sapotaceae, Ranunculaceae and Monocotyledonae, suggesting that they cannot be proposed as possible markers for this family.

Statistical analysis did not make it possible to highlight any alkaloids as possible taxonomic markers for the plant families considered in our study. In specific groups of plants, significant correlations ($p<0.05$) were found between pairs of alks belonging to Ind, Qnl and Trn groups. As regards Inds in particular, Harmane and Norharmane showed a correlation in *Cuminum cyminum* and *Coriandrum sativum* (Apiaceae), *Calendula officinalis* and *Hieracium pilosella* (Asteraceae), *Bursa pastoris* (Brassicaceae), *Alchemilla vulgaris* and *Rubus idaeus* (Rosaceae), and *Asperula odorata* (Rubiaceae), with a correlation index of 0.15. 2 isomers of Harmane (RT 15.5 and 15.9 min) in *Cuminum cyminum* and *Coriandrum sativum* (Apiaceae), *Arctium lappa* and *Calendula officinalis* (Asteraceae) and *Bursa pastoris* (Brassicaceae) showed a correlation index of 0.96. In *Cuminum cyminum* (Apiaceae), *Calendula officinalis, Hieracium pilosella* and *Solidago virga-aurea* (Asteraceae), *Bursa pastoris* (Brassicaceae), *Ocymum basilicum* (Lamiaceae), and *Alchemilla vulgaris* (Rosaceae) Norharmane and Harmine had a correlation index of 0.31. In *Cuminum cyminum* (Apiaceae), *Taraxacum officinale, Arctium lappa* and *Calendula officinalis* (Asteraceae), *Bursa pastoris* (Brassicaceae) and *Alchemilla vulgaris* (Rosaceae) Harmine and Harmane had a correlation index of 0.10. The families showing this phenomenon were often recurring, as in the case of Apiaceae, Asteraceae, Brassicaceae and Rosaceae, and it is particularly interesting to note

that *Cuminum cyminum, Calendula officinalis* and *Bursa pastoris* always showed a correlation between Inds.

As regards Qnls, N-methyllaurotetanine and Magnoflorine showed a correlation in *Angelica archangelica* (Apiaceae), *Brassica alba* (Brassicaceae), *Melissa officinalis* (Lamiaceae), *Galega officinalis* (Fabaceae), *Rumex crispus* (Pollygonaceae) and *Rubus idaeus* (Rosaceae), with a correlation index of 0.79. Cinchonanine E/ Cinchonine and Quinine were correlated in *Petroselinum crispum* (Apiaceae), *Cichorium intybu, Arctium lappa, Heterotheca inuloides, Calendula officinalis* and *Cynara scolymus* (Asteraceae), *Lavandula hybrid* (Lamiaceae) and *Cinchona succirubra* (Rubiaceae), with a correlation index of 0.99. Cinchonanine E/Cinchonine and Cinchonanine F were correlated in *Cichorium intybus, Arctium lappa, Heterotheca inuloides, Calendula officinalis* and *Cynara scolymus* (Asteraceae), *Lavandula hybrid* (Lamiaceae) and *Cinchona succirubra* (Rubiaceae), with a correlation index of 0.99. These plants, most of which belonging to the Asteraceae family, showed a significant correlation between Cinchonanine E/Cinchonine and Cinchonanine F, also showing a correlation between Cinchonanine E/ Cinchonine and Quinine.

As regards Trns, Valerianine and Valerine showed a correlation in *Heterotheca inuloides, Cynara scolymus* and *Solidago virga-aurea* (Asteraceae), *Sisymbrium officinale* (Brassicaceae), *Filipendula ulmaria max* and *Rubus idaeus* (Rosaceae), with a correlation index of 0.52.

*Comparison between lab solvent extraction and domestic hot water infusions*

In order to evaluate the real health risk of alk consumption through domestic herbal infusions, the 8 herbal samples richest in alks (*Acorus calamus, Cassia angustifolia, Cuminum cyminum, Escholtzia californiea, Malva sylvestris, Origanum vulgare, Parietaria officinalis* and *Pimpinella anisum*) were also extracted using a common home recipe for water infusion (100°C, 15 minutes). The average alk content, grouped by chemical/botanical group, found both in hot water extracts and methanol/water/formic acid (49.5:49.5:1) extracts of the 8 herbs were compared. To allow comparison of the data obtained with the 2 different extractive procedures, the values were converted from μg $L^{-1}$ (extract content) to μg $kg^{-1}$ (herbal content). The average Pyr content determined in methanolic solvent extracts was 155 μg $kg^{-1}$, while in water extracts it was 69 μg $kg^{-1}$. For Gst+Str alks the content in solvent extract was also higher than in water extracts, 21 μg $kg^{-1}$ *vs* 1 μg $kg^{-1}$, respectively. As regards Ind, Pun, and Qnl alks, the content in solvent extract was 13, 3, and 28 μg $kg^{-1}$, respectively, while in water extracts these alks were not found.

**Discussion**

In recent years, several studies have demonstrated that a real danger is posed by Pyz alks extracted from herbs in teas and infusions[10,17,70]. In line with previous studies[17,71], our study confirmed the migration of Pyz alks from herbs to water infusions, albeit at a lower rate compared to the potential content. Moreover, this work investigated the possible migration of other alk groups such as Gst and Str in hot water. We could not find any evidence in the literature regarding the behaviour of these alks, but we believe that these results pave the way for broader assessment of the migration of alks from herbs to infusions, not focusing only on Pyzs.

Assessing our targeted study, despite the rare presence of Pyz alks at high concentrations, it is theoretically possible to exceed the recommended daily intake limit suggested by German Federal Institute for Risk Assessment (0.42 µg for a 60 kg adult; BfR). Indeed, considering *Cuminum cyminum,* with a total Pyz alk content of 518 µg $kg^{-1}$ (methanolic extract), a teacup of infusion prepared with 2 teaspoons of the herb (about 2 g) would provide up to 1 µg of Pyz alks, greater than the limit established, even considering the reduced extraction capability of hot water. As regards Gst alks, the abovementioned acute toxic dose (105 mg for a 60 kg adult), could not be reached or exceeded even with abundant consumption. For example, a cup with 2 teaspoons of *Mentha Piperita*, the plant with the highest content at 658 µg $kg^{-1}$, provides only 1.3 µg of the measured Gst alks, far less than the acute toxic dose. As regards the other classes for which a toxicological limit has been reported, Trp and Qnz alks, our untargeted study showed their presence in 6% and 25% of samples respectively, but without allowing evaluation useful for establishing respect for toxicology limits.

As further evidence of possible accidental contamination of commercial herb samples with fragments of other plants occurring during harvest and preparation processes[72] we found significant amounts of Atropine in a single commercial sample of *Parietaria officinalis* (2.8 µg $kg^{-1}$), and alpha-Solasonine and alpha-Solamargine in a sample of *Escholtzia californiea* (2.3 and 3 mg $kg^{-1}$, respectively). In effect, this anomalous presence, never previously documented in botanical literature, was not confirmed on repeating the analysis with other commercial samples of the same herbs. Moreover, we observed the almost total absence of alks in commercial blends, perhaps due primarily to the evident dilution of alks possibly present in a single herb in a more complex mixture, but also possibly highlighting the effectiveness of industrial quality control along the production chain.

Finally, it should be emphasised that in general, people consume no more than one/two cups of herb tea/infusions per day, and that several other foods may also contain alks. As regards Pyzs, the most widely studied alks, commodities such as honey, cereals, milk and green salads could also definitely affect our daily intake[10,19,73].

**Conclusions**

The proposed liquid chromatography - high resolution mass method confirmed the interesting ability of this new approach to define broad targeted and untargeted alkaloid profiles for herbal infusion products. This extensive survey of individual commercial untreated herbs showed the considerable variability of plant alkaloid content, and also the correlation between alkaloids belonging to indoles, quinolines and tropanes for some plant groups. The detected alkaloid profiles were generally characteristic for herbs, and were rarely dangerous, but the accidental presence of some undesired alkaloids was noted, in particular pyrrolizidines, suggesting that greater attention should be paid during harvest and processing processes. On the other hand, the industrial blends sampled did not show any alk contamination.

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## Tables

### Table 1

Performance characteristics of the targeted Alks method.

| Compound | RT (min) | $[M+H]^+$ (m/z) | Δ*m/z* (ppm) | NCE | MS/MS fragments (m/z) | LOD (µg $L^{-1}$) | LOQ (µg $L^{-1}$) | Linearity range (µg $L^{-1}$) | $R^2$ | Precision (RSD%) | | | Accuracy (RE%) | | | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | L | M | H | L | M | H | |
| Nicotine | 4.73 | 163.1227 | 1.84 | 35 | 132.0806<br>130.0645<br>106.0652 | 15 | 50 | 50-4000 | 0.990 | 9.6 | 9.0 | 0.8 | 7.6 | 7.0 | 7.1 | 1 |
| Monocrotaline | 5.25 | 326.1587 | 1.23 | 60 | 120.0801<br>121.0889<br>94.0653 | 0.05 | 0.17 | 0.17-1000 | 0.998 | 3.0 | 0.6 | 0.7 | 14.2 | 2.7 | 5.7 | 1 |
| Lycopsamine | 6.96 | 300.1805 | 3.00 | 30 | 138.0903<br>94.0652 | 0.12 | 0.4 | 0.40-1000 | 0.998 | 1.0 | 0.6 | 1.0 | 15.8 | 3.4 | 5.1 | 1 |
| Coniine | 7.59 | 128.1433 | 0.78 | 45 | 69.0700 | 0.21 | 0.71 | 0.71-1000 | 0.998 | 3.2 | 1.4 | 1.2 | 27.7 | 3.3 | 5.3 | 1 |
| Erucifoline | 8.44 | 350.1598 | 3.06 | 60 | 20.0802<br>94.0652<br>67.0549 | 0.07 | 0.25 | 0.25-1000 | 0.998 | 2.0 | 0.8 | 0.6 | 7.4 | 2.1 | 6.8 | 1 |
| Senecionine N-oxide | 8.97 | 352.1755 | 1.42 | 50 | 120.0802<br>155.1054<br>122.0960 | 0.05 | 0.17 | 0.17-1000 | 0.997 | 2.3 | 1.9 | 1.2 | 11.9 | 5.0 | 8.2 | 1 |
| Gramine | 9.28 | 175.1229 | 1.14 | 30 | 130.0655<br>113.0590 | 0.05 | 0.16 | 0.16-1500 | 0.998 | 3.0 | 1.8 | 1.3 | 21.4 | 1.6 | 2.0 | 1 |
| Theobromine/ Theophylline | 9.57 | 181.072 | 1.66 | 35 | 124.0504<br>116.9859<br>167.0554 | 2.80 | 9.3 | 9.3-500 | 0.999 | 16.9 | 11.8 | 5.7 | 37.9 | 5.7 | 1.0 | 1 |
| Scopolamine | 10.25 | 304.1543 | 1.97 | 30 | 138.0903<br>156.1018<br>121.065 | 0.20 | 0.65 | 0.65-2000 | 0.996 | 1.6 | 1.0 | 1.1 | 16.4 | 3.3 | 9.1 | 1 |
| Jacobine-N-oxide | 10.52 | 368.1703 | 2.72 | 50 | 120.0804<br>296.1477<br>119.0732 | 0.28 | 0.94 | 0.94-1500 | 0.996 | 1.9 | 0.9 | 0.8 | 11.8 | 1.1 | 9.5 | 1 |
| Erucifoline-N-oxide | 10.66 | 366.1547 | 1.91 | 60 | 94.0652<br>118.0654<br>119.0730 | 0.35 | 1.18 | 1.18-1000 | 0.996 | 2.5 | 0.5 | 0.7 | 7.2 | 0.6 | 7.6 | 1 |
| Heliotrine | 10.71 | 314.1961 | 1.91 | 30 | 138.0910<br>156.1017 | 0.04 | 0.14 | 0.14-1000 | 0.998 | 2.6 | 1.0 | 0.6 | 16.0 | 3.4 | 7.4 | 1 |
| Retrorsine | 10.73 | 352.1754 | 1.42 | 50 | 120.0803<br>94.0652<br>165.0136 | 0.25 | 0.83 | 0.83-1500 | 0.996 | 2.4 | 0.8 | 0.8 | 21.1 | 6.9 | 18.2 | 1 |
| Seneciphylline | 11.40 | 334.1649 | 2.39 | 50 | 120.0804<br>94.0653<br>306.1704 | 0.14 | 0.56 | 0.58-2000 | 0.996 | 1.7 | 1.1 | 1.0 | 10.4 | 3.1 | 7.8 | 1 |
| Retrorsine N-oxide | 11.89 | 368.1703 | 2.72 | 60 | 94.0652<br>120.0803 | 0.43 | 1.43 | 1.43-1500 | 0.996 | 3.1 | 3.1 | 0.8 | 25.3 | 7.1 | 5.3 | 1 |
| Senecionine/ Senecivernine | 12.63 | 336.1806 | 3.24 | 50 | 120.0803<br>138.0902<br>94.0652 | 0.14 | 0.46 | 0.46-1500 | 0.993 | 1.5 | 0.7 | 0.8 | 23.1 | 4.0 | 13.1 | 1 |
| Hyoscyamine/ Atropine | 12.65 | 290.1751 | 1.72 | 45 | 124.1122<br>93.0702 | 0.15 | 0.51 | 0.51-2000 | 0.994 | 1.4 | 1.1 | 0.6 | 20.6 | 1.9 | 8.6 | 1 |
| Echimidine | 13.64 | 398.2173 | 1.00 | 30 | 120.0803<br>84.0491<br>220.1317 | 0.08 | 0.25 | 0.25-500 | 0.999 | 4.9 | 1.3 | 0.7 | 18.0 | 1.7 | 5.8 | 1 |
| Senkirkin | 13.94 | 366.1911 | 1.37 | 30 | 168.1023<br>122.0595 | 0.66 | 2.22 | 2.22-1000 | 0.998 | 1.5 | 0.9 | 0.8 | 13.6 | 1.8 | 6.5 | 1 |
| Jacobine | 14.05 | 352.1754 | 1.70 | 50 | 150.0937<br>118.0652 | 0.28 | 0.95 | 0.95-1500 | 0.997 | 2.5 | 0.9 | 0.6 | 12.6 | 1.2 | 8.2 | 1 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Caffeine | 15.09 | 195.088 | 1.03 | 50 | 120.0803<br>136.0759<br>138.0660<br>110.0714 | 0.92 | 3.06 | 3.06-500 | 0.998 | 9.7 | 16.3 | 8.3 | 33.7 | 26.2 | 0.3 | 1 |
| Lasiocarpine | 15.80 | 412.2329 | 1.21 | 30 | 120.0803<br>220.1314<br>336.1808 | 0.12 | 0.41 | 0.41-1500 | 0.998 | 2.4 | 0.9 | 1.1 | 18.3 | 2.7 | 6.3 | 1 |
| Strychnine | 16.33 | 335.1754 | 2.39 | 60 | 184.0703<br>222.0962 | 0.59 | 1.86 | 1.86-1500 | 0.993 | 6.3 | 1.9 | 1.3 | 39.2 | 6.9 | 13.2 | 2 |
| Harmaline | 16.42 | 215.1179 | 1.86 | 50 | 200.0931<br>174.0902 | 3.49 | 11.6 | 11.63-3000 | 0.996 | 8.3 | 6.0 | 2.0 | 34.7 | 5.9 | 8.7 | 2 |
| Sipeimine | 17.55 | 430.3315 | 1.63 | 50 | 138.1274<br>214.1384 | 1.18 | 3.92 | 3.92-1000 | 0.998 | 4.2 | 2.9 | 0.8 | 9.9 | 4.7 | 8.1 | 1 |
| Quinine | 18.30 | 325.1904 | 1.99 | 60 | 81.0702<br>172.0753<br>160.07533 | 1.95 | 6.5 | 6.5-200 | 0.996 | 12.1 | 7.2 | 4.8 | 35.9 | 0.7 | 2.5 | 1 |
| Quinidine | 18.82 | 325.1905 | 1.69 | 60 | 81.0702<br>172.0754<br>160.0754 | 2.90 | 9.7 | 9.7-500 | 0.995 | 15.0 | 3.6 | 5.7 | 42.9 | 12.7 | 1.9 | 1 |
| Veratramine | 19.68 | 410.3053 | 0.97 | 30 | 295.2022<br>84.0824 | 0.18 | 0.59 | 0.59-1000 | 0.995 | 6.7 | 6.3 | 2.3 | 17.9 | 1.9 | 10.4 | 1 |
| alpha-Solasonine | 19.83 | 884.5002 | 2.94 | 50 | 85.0288<br>71.0498<br>157.1012 | 0.10 | 0.35 | 0.25-1000 | 1,000 | 3.0 | 1.8 | 0.9 | 17.6 | 3.1 | 2.3 | 1 |
| Jervine | 19.85 | 426.3002 | 0.70 | 30 | 126.1372<br>313.2073 | 0.26 | 0.86 | 0.86-2000 | 0.995 | 4.5 | 2.4 | 1.3 | 32.4 | 4.7 | 14.2 | 1 |
| alpha-Solamargine | 20.00 | 868.5052 | 1.38 | 45 | 85.0288<br>71.0498 | 2.73 | 9.1 | 0.11-1500 | 0.999 | 13.3 | 2.9 | 1.1 | 28.6 | 2.4 | 1.0 | 1 |
| Protoveratrine A | 20.62 | 794.4321 | 1.51 | 50 | 658.3609<br>436.6457 | 10.7 | 35.7 | 35.71-1500 | 0.996 | 15.4 | 6.4 | 3.7 | 45.0 | 13.7 | 2.9 | 1 |
| Veratridine | 21.65 | 674.3534 | 2.97 | 60 | 456.2718<br>165.0534 | 0.14 | 0.45 | 0.45-1500 | 0.998 | 9.9 | 6.3 | 1.9 | 7.7 | 5.2 | 4.4 | 1 |
| alpha-Solanine | 21.77 | 868.5050 | 1.38 | 50 | 438.2591<br>98.0966<br>398.3385 | 1.28 | 4.26 | 4.26-500 | 0.998 | 5.4 | 2.5 | 4.1 | 53.0 | 8.6 | 7.2 | 1 |
| Solasodine | 23.60 | 414.3367 | 2.90 | 60 | 157.1017<br>70.0658<br>159.1158 | 0.65 | 2.16 | 2.16-1000 | 0.993 | 6.0 | 8.6 | 3.2 | 14.6 | 4.4 | 10.4 | 1 |
| Aconine | 23.68 | 646.3221 | 4.02 | 50 | 105.0330<br>368.1839 | 1.24 | 4.12 | 4.12-1500 | 0.999 | 2.3 | 1.8 | 0.7 | 14.6 | 1.6 | 3.3 | 1 |
| Tomatidine/ Tomatine | 24.54 | 416.3523* | 1.44 | 60 | 161.1319<br>70.0658<br>147.1161 | 5.99 | 20.0 | 19.97-3000 | 0.991 | 5.0 | 7.9 | 2.5 | 2.9 | 5.2 | 12.5 | 1 |

Note: RT= retention time; Δ *m/z* (ppm)= accurate mass error compared to exact mass; NCE= normalized collision energy; LOD= limit of detection; LOQ= limit of quantitation; RSD%= relative standard deviation; RE% = relative error; L, M, H= spiked blank sample solutions at low, medium and high concentration respectively; S= Supplier (1= PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany; 2= Sigma, St. Louis, MO, USA); *=Tomatine $[M+H-C_{23}H_{38}O_{19}]^+$;

**Table 2**

Botanical characteristics of herbal samples.

| Family | Common name | Species | Type |
|---|---|---|---|
| Adoxaceae | Elder | *Sambucus nigra* | herb |
| Anacardiaceae | Pink pepper | *Scitinus molle* | herb |
| Apiaceae | Anise | *Pimpinella anisum* | seeds |
| Apiaceae | Caraway | *Carum carvi* | herb |
| Apiaceae | Centella asiatica | *Hydrocotyle asiatica* | herb |

| | | | |
|---|---|---|---|
| Apiaceae | Chervil | *Anthriscus cerefolium* | herb |
| Apiaceae | Coriander | *Coriandrum sativum* | herb |
| Apiaceae | Cumin | *Cuminum cyminum* | herb |
| Apiaceae | Dill | *Anethum graveolens* | herb |
| Apiaceae | Garden angelica | *Angelica archangelica* | herb |
| Apiaceae | Parsley | *Petroselinum crispum* | herb |
| Apiaceae | Wild carrot | *Daucus carota* | herb |
| Araceae | Calamus | *Acorus calamus* | herb |
| Araliaceae | Eleuthero | *Eleutherococcus senticosussenticosus* | herb |
| Araliaceae | Ginseng | *Panax ginseng* | herb |
| Asteraceae | Absinthium | *Absinthium absinthium* | herb |
| Asteraceae | Artichoke | *Cynara scolymus* | herb |
| Asteraceae | Cardus marianus | *Silybum marianum* | herb |
| Asteraceae | Chamomile | *Anthemis nobilis* | herb |
| Asteraceae | Chicory | *Cichorium intybus* | herb |
| Asteraceae | Dandelion | *Taraxacum officinale* | herb |
| Asteraceae | European goldenrod | *Solidago virga-aurea* | herb |
| Asteraceae | Great Valley gumplant | *Grindelia robusta* | herb |
| Asteraceae | Greater burdock | *Arctium lappa* | herb |
| Asteraceae | Mexican arnica | *Heterotheca inuloides* | herb |
| Asteraceae | Mouse-ear hawkweed | *Hieracium pilosella* | herb |
| Asteraceae | Pot marigold | *Calendula officinalis* | flowers |
| Asteraceae | Southernwood | *Artemisia abrotanum* | herb |
| Asteraceae | Yarrow | *Achillea millefolium* | herb |
| Berberidaceae | European barberry | *Berberis vulgaris* | herb |
| Betulaceae | Silver birch | *Betula pendula* | herb |
| Bignoniaceae | Pink ipê | *Tabebuia avellandese* | herb |
| Boraginaceae | Common comfrey | *Symptitum officinale* | herb |
| Brassicaceae | Hedge mustard | *Sisymbrium officinale* | herb |
| Brassicaceae | Shepherd's-purse | *Bursa pastoris* | herb |
| Brassicaceae | White mustard | *Brassica alba* | herb |
| Burseraceae | Salai | *Gomma resina* | herb |
| Cesalpinaceae | Egyptian senna | *Cassia angustifolia* | herb |
| Crassulaceae | Golden root | *Rhodiola rosea* | herb |
| Equisetaceae | Horsetail | *Equisetum arvense* | herb |
| Ericaceae | Bilberry | *Vaccinium myrtillus* | herb |
| Ericaceae | Pointleaf manzanita | *Arctostaphylos pungens* | herb |
| Fabaceae | Fenugreek | *Trigonella foenum* | herb |

| | | | |
|---|---|---|---|
| | | *graecum* | |
| Fabaceae | Galega | *Galega officinalis* | herb |
| Fabaceae | Liquorice | *Glycyrrhiza glabra* | roots |
| Fabaceae | Spiny restharrow | *Ononis spinose* | herb |
| Fagaceae | Sessile oak | *Quercus petraea* | herb |
| Fucaceae | Bladder wrack | *Fucus vesiculosus* | herb |
| Fumariaceae | Fumitory | *Fumaria officinalis* | herb |
| Getianaceae | Great yellow gentian | *Gentiana lutea* | herb |
| Ginkoaceae | Ginkgo | *Ginko biloba* | herb |
| Hypericaceae | Perforate St John's-wort | *Hypericum perforatum* | herb |
| Juglandaceae | Walnut | *Juglans regia L.* | seeds |
| Lamiaceae | Balm | *Melissa officinalis* | leaves |
| Lamiaceae | Basil | *Ocymum basilicum* | herb |
| Lamiaceae | Breckland thyme | *Thymus serphyllum* | herb |
| Lamiaceae | Grapple plant | *Harpagophytum procumbens* | herb |
| Lamiaceae | Hyssop | *Hyssopus officinalis* | herb |
| Lamiaceae | Java’s tea | *Orthosiphon stamineus* | herb |
| Lamiaceae | Java’s tea | *Orthosiphonstamineus* | herb |
| Lamiaceae | Lavender | *Lavandula hybrids* | flowers |
| Lamiaceae | Marjoram | *Origanum majorana* | herb |
| Lamiaceae | Oregano | *Origanum vulgare* | herb |
| Lamiaceae | Peppermint | *Mentha piperita* | herb |
| Lamiaceae | Sage | *Salvia officinalis* | herb |
| Lannabinaceae | Common hop | *Humulus lupulus* | herb |
| Lauraceae | Cinnamon | *Cinammomun zeylanicum* | bark |
| Liliaceae | Butcher's-broom | *Ruscus aculeatus* | herb |
| Liliaceae | Sarsaparilla | *Similax medica* | herb |
| Loranthaceae | European mistletoe | *Viscum album* | herb |
| Malvaceae | Mallow | *Malva sylvestris* | herb |
| Malvaceae | Marsh-mallow | *Althaea officinalis* | herb |
| Monimiaceae | Boldo | *Pneumus boldus* | herb |
| Myristicaceae | Nutmeg | *Myristica fragrans* | herb |
| Myrtaceae | Allspice | *Pimenta officinalis* | herb |
| Myrtaceae | Cloves | *Syzygium aromaticum Merrill Eugenia* | flowers |
| Oleaceae | Olive | *Olea europaea* | herb |
| Onaeraceae | Smallflower hairy willowherb | *Epilobium parviflorum* | herb |
| Papaveraceae | California poppy | *Escholtzia californiea* | herb |
| Papaveraceae | Celandine | *Citelidonium majus* | herb |

| | | | |
|---|---|---|---|
| Passifloraceae | Purple passionflower | *Passiflora incarnate* | herb |
| Piperaceae | Black pepper | *Piper nigrum* | herb |
| Plantaginaceae | English plantain | *Plantago lanceolata* | herb |
| Plantaginaceae | Psyllium | *Plantago psyllium* | seeds |
| Poaceae | Couch grass | *Agropyron repens* | herb |
| Poaceae | Mais | *Zea mays* | seeds |
| Polygonaceae | Ceterach | *Ceterach officinarium* | herb |
| Polygonaceae | Curly dock | *Rumex crispus* | herb |
| Polygonaceae | False rhubarb | *Rheum rhaponticum* | herb |
| Polygonaceae | Knotgrass | *Polygonum aviculare* | herb |
| Ramnaceae | Alder buckthorn | *Rhamnus frangula* | bark |
| Rosaceae | Agrimony | *Agrimonia eupatoria* | herb |
| Rosaceae | Hawthorn | *Crataegus oxyacantha* | flowers |
| Rosaceae | Lady's mantle | *Alchemilla vulgaris* | herb |
| Rosaceae | Prunus cerasus | *Prunus cerasus* | herb |
| Rosaceae | Raspberry | *Rubus ipaeus* | herb |
| Rosaceae | Spirea ulmaria | *Filipendula ulmaria max* | herb |
| Rubiaceae | Asperula | *Asperula odorata* | herb |
| Rubiaceae | Cleavers | *Galium aparine* | herb |
| Rubiaceae | Red cinchona | *Cinchona succirubra* | herb |
| Rutaceae | Rue | *Ruta graveolens* | herb |
| Rutaceae | Bitter orange | *Citrus aurantium var bigaradia bigaradia* | herb |
| Salicaceae | Willow | *Salix alba* | herb |
| Sapindaceae | Guarana | *Paullina cupana* | herb |
| Schisandraceae | Five-flavor berry | *Schisandra chinensis* | herb |
| Scrophulariaceae | Figwort | *Schrophularia nodosa* | herb |
| Scrophulariaceae | Heath speedwell | *Veronica officinalis* | herb |
| Theaceae | Thea | *Thea sinensis* | herb |
| Ulmaceae | Elm | *Ulmus campestris* | herb |
| Urticaceae | Nettle | *Urtica dioica* | herb |
| Urticaceae | Pellitory-of-the-wall | *Parietaria officinalis* | herb |
| Valerianaceae | Valerian | *Valeriana officinalis* | herb |
| Verbenaceae | Lemon verbena | *Lippia citriodora* | herb |
| Violaceae | Heartsease | *Viola tricolor* | herb |
| Zingiberaceae | Ginger | *Zingiber officinale* | roots |
| Zingiberaceae | Grains of paradise | *Aframomum melegueta* | herb |
| Zingiberaceae | True cardamom | *Elettaria cardamomum* | herb |
| Zingiberaceae | Turmeric | *Curcuma domestica* | herb |

| | | | |
|---|---|---|---|
| Mix 1 *"Buon respiro"* | | | commercial blend |
| Fabaceae | Liquorice | *Glycyrrhiza glabra* | roots |
| Illiciaceae | Star anise | *Illicium verum* | fruits |
| Malvaceae | Mallow | *Malva sylvestris* | leaves |
| Malvaceae | Marshmallow | *Althaea officinalis* | leaves and roots |
| Lamiaceae | Breckland thyme | *Thymus serphyllum* | herb |
| Papaveraceae | Papaver | *Papaver rhoeas L.* | flowers |
| Scrophulariaceae | Black mullein | *Verbascum nigrum* | flowers |
| Mix 2 *"Depur mix"* | | | commercial blend |
| Apiaceae | Garden Angelica | *Angelica archangelica* | roots |
| Apiaceae | Fennel | *Foeniculum vulgare* | seeds |
| Asteraceae | Greater burdock | *Arctium iappa* | roots |
| Betulaceae | Silver birch | *Betula pendula* | leaves |
| Brassicaceae | Common horsetail | *Equisetum arvense* | herb |
| Equisetaceae | Shepherd's purse | *Capsella bursa-pastoris* | herb |
| Ericaceae | Bearberry | *Arctostaphylos uva-ursi* | leaves |
| Fabaceae | Liquorice | *Glycyrrhiza glabra* | roots |
| Fabaceae | Senna Tinnevelly | *Cassia angustifolia* | leaves |
| Fabaceae | Spiny restharrow | *Ononis spinosa* | roots |
| Hypericaceae | Perforate St John's-wort | *Hypericum perforatum* | herb |
| Poaceae | Weed | *Cynodon dactylon* | roots |
| Rhamnaceae | Buckthorn | *Rhamnus frangula* | bark |
| Smilacaceae | Catbriers | *Smilax officinalis* | roots |
| Solanaceae | Bittersweet | *Solanum dulcamara* | stems |
| Urticaceae | Nettle | *Urtica dioica* | herb |
| Mix 3 *"Intestino pigro"* | | | commercial blend |
| Apiaceae | Anise | *Pimpinella anisum* | seeds |
| Apiaceae | Caraway | *Carum carvi* | leaves |
| Apiaceae | Coriander | *Coriandrum sativum* | seeds |
| Apiaceae | Fennel | *Foeniculum vulgare* | seeds |
| Equisetaceae | Horsetail | *Equisetum arvense* | herb |
| Ericaceae | Bearberry | *Arctostaphylos uva-ursi* | leaves |
| Fabaceae | Senna Tinnevelly | *Cassia angustifolia* | leaves |
| Lamiaceae | Melissa | *Melissa officinalis* | leaves |
| Lamiaceae | Peppermint | *Mentha piperita* | leaves |
| Lamiaceae | Rosemary | *Rosmarinus officinalis* | leaves |

| | | | |
|---|---|---|---|
| Poaceae | Weed | *Cynodon dactylon* | roots |
| Rhamnaceae | Buckthorn | *Rhamnus frangula* | bark |
| Mix 4 *"Linea snella"* | | | commercial blend |
| Apiaceae | Fennel | *Foeniculum vulgare* | seeds |
| Apiaceae | Anise | *Pimpinella anisum* | seeds |
| Bromeliaceae | Pineapple | *Ananas comosus* | stems |
| Lamiaceae | Melissa | *Melissa officinalis* | leaves |
| Mix 5 *"Notte serena"* | | | commercial blend |
| Apiaceae | Anise | *Pimpinella anisum* | herb |
| Asteraceae | Chamomile | *Matricaria chamomilla L.* | flowers |
| Lamiaceae | Melissa | *Melissa officinalis* | leaves |
| Lamiaceae | Lavender | *Lavandula angustifolia* | herb |
| Lamiaceae | Peppermint | *Mentha piperita* | flowers |
| Lamiaceae | Rosemary | *Rosmarinus officinalis* | herb |
| Papaveraceae | Papaver | *Papaver rhoeas L.* | flowers |
| Passifloraceaeae | Maypop | *Passiflora incarnata* | herb |
| Rutaceae | Orange | *Citrus sinensis* | leaves |
| Rosaceae | Common hawthorn | *Crataegus monogyna* | herb |
| Valerianaceae | Valerian | *Valeriana officinalis* | herb |
| Mix 6 *"Tisana alle erbe"* | | | commercial blend |
| Apiaceae | Fennel | *Foeniculum vulgare* | seeds |
| Asteraceae | Chamomile | *Matricaria chamomilla L.* | flowers |
| Fabaceae | Rooibos | *Aspalathus linearis* | herb |
| Lamiaceae | Melissa | *Mentha × piperita* | herb |
| Lamiaceae | Mint | *Melissa officinalis* | leaves |
| Poaceae | Lemongrass | *Cymbopogon citratus* | herb |
| Rosaceae | Blackberry | *Rubus ulmifolius* | leaves |
| Tiliaceae | Lime trees | *Tilia L.* | flowers |
| Verbenaceae | Verbena | *Verbena officinalis L.* | herb |
| Mix 7 *"Tonico"* | | | commercial blend |
| Caricaceae | Papaya | *Carica papaya* | fruits |
| Rosaceae | Elmleaf blackberry | *Rubus ulmifolius* | leaves |
| Verbenaceae | Lemon verbena | *Aloysia citriodora* | herb |
| Zingiberaceae | Ginger | *Zingiber officinale* | roots |

## Table 3

Alk content (μg $kg^{-1}$) of 61 herbal infusion samples and 3 commercial blends.

| Herb sample | Aconine | alfa-Solanine | alfa-Solasonine | alpha-Solamargine | Caffeine | Coniine | Echimidine | Erucifoline | Gramine | Harmaline | Heliotrine | Jacobine | Jacobine-N-oxide | Lasiocarpine | Lycopsamine | Monocrotaline | Nicotine | Quinidine | Quinine | Retrorsine | Retrorsine N-oxide | Senecionine/Seneciverine | Seneciphylline | Senkirkin | Sipeimine | Solasodine | Theobromine/Theophylline | Veratridine |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Apiaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Anethum graveolens* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | 3.4 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Coriandrum sativum* | <2 | <2 | 93 | 93 | <2 | 4.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Cuminum cyminum* | <2 | <2 | <0.2 | 13 | 8 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | 286 | <0.4 | <0.4 | 233 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Hydrocotyle asiatica* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 29 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Petroselinum crispum* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | 10 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Pimpinella anisum* | <2 | <2 | 118 | 25 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | 54 | <0.4 | <0.4 | 31 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Araceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Acorus calamus* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | 115 | <1 | <2 | 20 | <4.5 | <0.2 |
| Araliaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Eleutherococcus senticosus* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | 0.8 |
| Asteraceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Absinthium absinthium* | 27 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Achillea millefolium* | 32 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Arctium lappa* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | 17 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Calendula officinalis* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | 49 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Cichorium intybus* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | 34 | 1068 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Cynara scolymus* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | 192 | <5 | 56 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Grindelia robusta* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | 118 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Heterotheca inuloides* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | 42 | 679 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Hieracium pilosella* | <2 | <2 | <0.2 | <4 | 120 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | 21 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Silybum marianum* | <2 | <2 | <0.2 | <4 | <2 | 35 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Solidago virga-aurea* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 4 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Berberidaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Berberis vulgaris* | <2 | <2 | <0.2 | <4 | 11 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Betulaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Betula pendula* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | 3 | <2 | <1 | <4.5 | <0.2 |
| Bignoniaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Tabebuia avellanadae* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | 8 | <2 | <1 | <4.5 | <0.2 |
| Brassicaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Bursa pastoris* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 5 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Cesalpinaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Cassia angustifolia* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | 19 | <0.4 | <0.2 | <0.2 | 176 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Equisetaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Equisetum arvense* | <2 | 89 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Ericaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Vaccinium myrtillus* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 1 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Fabaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Galega officinalis* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | 2 |
| *Ononis spinosa* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | 191 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Trigonella foenum graecum* | <2 | <2 | <0.2 | <4 | 5 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | 11 | <1 | <4.5 | 1 |
| Fucaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Fucus vesiculosus* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | 21 | <0.2 |
| Fumariaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Fumaria officinalis* | <2 | 12 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | 67 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | 4 | 9 | <2 | <1 | <4.5 | <0.2 |
| Ginkoaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Ginko biloba* | <2 | <2 | <0.2 | <4 | 253 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Lamiaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Harpagophytum procumbens* | <2 | <2 | <0.2 | <4 | 102 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Lavandula hybrid* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | 60 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Melissa officinalis* | <2 | <2 | 61 | 83 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Mentha piperita* | <2 | <2 | 402 | 256 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | 93 | 54 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | 36 | <4.5 | <0.2 |
| *Origanum majorana* | <2 | <2 | <0.2 | <4 | 21 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Origanum vulgare* | <2 | <2 | <0.2 | <4 | 7 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | 200 | 5 | <0.2 | <0.2 | <0.1 | <24 | 54 | 30 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Orthosiphon stamineus* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 9 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | 3 | <2 | <1 | <4.5 | <0.2 |
| *Salvia officinalis* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 79 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Liliaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Similax medica* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 21 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Loranthaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Viscum album* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | 34 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Malvaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Althaea officinalis* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | 6 | <2 | <1 | <4.5 | <0.2 |
| *Malva sylvestris* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | 101 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Myrtaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Syzygium aromaticum Merrill Eugenia* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 4 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Papaveraceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Citelidonium majus* | <2 | <2 | <0.2 | <4 | <2 | 25 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Escholtzia californiea* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | 12 | <0.1 | <0.1 | <6 | <0.1 | 7 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | 3 | <0.7 | 16 | <0.2 | <1 | <2 | 9 | <4.5 | <0.2 |
| Passifloraceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Passiflora incarnata* | <2 | <2 | 70 | 147 | <2 | <0.3 | <0.1 | 78 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Poaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Agropyron repens* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 4 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Polygonaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Ceterach officinarium* | <2 | <2 | <0.2 | <4 | 8 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Rheum rhaponticum* | 32 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Rosaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Alchemilla vulgaris* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 6.9 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Rubiaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Cinchona succirubra* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | 48 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | 6001 | 21860 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Galium aparine* | <2 | <2 | <0.2 | <4 | <2 | 9 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Rutaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Citrus aurantium var bigaradia* | <2 | <2 | 31 | 21 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Sapindaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Paullina cupana* | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | 11 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Schrophulariaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Schrophularia nodosa* | <2 | <2 | <0.2 | <4 | 4 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 6 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Veronica officinalis* | <2 | <2 | <0.2 | <4 | 7 | <0.3 | 15 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 3 | 6 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Urticacee | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Parietaria officinalis* | <2 | <2 | <0.2 | <4 | 6 | <0.3 | 62 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| *Urtica dioica* | <2 | <2 | <0.2 | <4 | <2 | 40 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <1 | <4.5 | <0.2 |
| Violaceae | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Viola tricolor* | <2 | <2 | <0.2 | <4 | 19 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <2 | <4.5 | <0.2 |
| Commercial blend | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Mix 2 | <2 | <2 | 19 | 90 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <2 | <4.5 | <0.2 |
| Mix 4 | <2 | <2 | <0.2 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | 1 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <2 | <2 | <4.5 | <0.2 |
| Mix 6 | <2 | <2 | 6 | <4 | <2 | <0.3 | <0.1 | <0.1 | <0.1 | <6 | <0.1 | <0.4 | <0.4 | <0.2 | <0.2 | <0.1 | <24 | <5 | <3 | <0.4 | <0.7 | <0.2 | <0.2 | <1 | <4 | <2 | <4.5 | <0.2 |

**Table 4**

Retention time and accurate masses of precursor ion and fragments of untargeted Alks.

| Compound name | RT (min) | $[M+H]^+$ (*m/z*) | $\Delta m/z$ (ppm) | NCE | MS/MS fragments (*m/z*) |
|---|---|---|---|---|---|
| Betonicine | 3.00 | 160.0962 | 2.4 | 60 | 72.0812; 132.1214 |
| Stachydrine | 3.50 | 144.1017 | 1.4 | 60 | 127.0864; 110.0601 |
| 2-Pyrrolidineacetic acid | 3.51 | 130.0861 | 1.5 | 30 | 70.0652; 84.0448 |
| 3-Acetyltropine | 3.79 | 184.1329 | 1.6 | 30 | 107.9599; 78.8800 |
| 3,6-Diacetyltropine | 3.86 | 226.1433 | 2.2 | 30 | 110.0598; 71.0494 |
| Stachydrine | 4.06 | 144.1017 | 1.2 | 60 | 127.0865; 110.0602 |
| Plantagonin | 4.36 | 178.0857 | 3.2 | 35 | / |
| Valerine | 5.20 | 158.1173 | 1.9 | 65 | 96.081; 122.0965 |
| Cathinine | 5.26 | 150.0911 | 1.5 | 45 | 132.1018; 135.0677 |
| Ginkotoxin | 5.46 | 184.0965 | 1.7 | 35 | 152.0719; 134.05962 |
| 8-Ethylnorlobelol | 5.60 | 158.1536 | 1.9 | 30 | 84.9598; 140.1424; 98.9750 |
| Indicain | 5.80 | 162.0912 | 0.7 | 35 | / |
| Myrtine /Epimyrtine | 6.90 | 168.1382 | 0.4 | 35 | 89.056; 151.0961 |
| Usamarine /Integerrimine-N-oxide/Senecivernine-N-oxide/Senecionine-N-oxide | 8.97 | 352.1748 | 2.0 | 50 | 120.0805; 324.1392; 138.0918 |
| Valerianine | 9.03 | 178.1225 | 0.8 | 65 | 134.0963; 119.0730 |
| Riddelline/Seneciphylline N-oxide/Spartioidine N-oxide | 9.52 | 350.1591 | 2.0 | 30 | 120.0809; 138.0901; 322.1627 |
| Valerianine | 10.05 | 178.1223 | 1.6 | 65 | 118.0731; 146.0959 |
| Anisodamine | 10.36 | 306.1690 | 3.3 | 50 | 140.1060; 122.0965 |
| Usamarine /Integerrimine-N-oxide/Senecivernine-N-oxide/Retrorsine | 10.73 | 352.1747 | 2.3 | 50 | 94.0660; 138.0918; 120.0805 |
| Usaramine/Integerrimine-N-oxide/Senecivernine-N-oxide | 11.22 | 352.1747 | 2.3 | 50 | 120.0815; 352.1751; 138.0918 |
| Harmalol | 11.30 | 201.1019 | 1.3 | 35 | 144.9972; 187.0076 |
| Spartioidine | 11.39 | 334.1642 | 2.1 | 30 | 120.0803; 138.0919 |
| Harmaline | 11.80 | 215.1176 | 1.5 | 50 | 197.1071; 70.0655 |
| Gelsemine | 11.85 | 323.1743 | 3.5 | 30 | 70.0654; 236.1065 |
| Cinchonanine F | 12.00 | 327.2060 | 2.2 | 35 | 253.15267; 261.16428 |
| 8,10-Diethyllobelidiol | 12.05 | 244.2262 | 3.7 | 60 | 98.0962; 226.2176; 58.0654 |
| Integerrimine/Senecionine/Senecivernine | 12.63 | 336.1798 | 2.4 | 50 | 120.0815; 138.0919; 94.0659 |
| Actinidine | 13.00 | 148.1118 | 2.1 | 60 | 130.0861; 120.0196 |
| 8,10-Diethyllobelidiol | 13.07 | 244.2264 | 2.9 | 60 | 81.0704; 226.2148; 152.1423 |
| Evoxanthine | 13.34 | 284.0913 | 1.3 | 35 | 108.0446; 158.0598 |
| Usamarine /Integerrimine-N-oxide/Senecivernine-N-oxide/Jacobine | 14.05 | 352.1750 | 1.4 | 50 | 94.0652; 120.0805; 138.0919 |
| Tropinone | 14.08 | 140.1067 | 2.1 | 30 | 108.0442; 126.0551 |

| | | | | | |
|---|---|---|---|---|---|
| Norharmane | 14.20 | 169.0758 | 1.2 | 55 | 81.0722; 109.0647 |
| Harmol | 14.40 | 199.0862 | 1.9 | 35 | 181.1111; 199.0862 |
| Caryachine | 14.50 | 326.1382 | 1.5 | 35 | 281.0805; 251.0703 |
| Cinchonanine C | 14.80 | 311.1747 | 2.4 | 35 | 293.1642; 136.1119 |
| Harmine | 15.00 | 213.1020 | 0.9 | 35 | 213.1017; 195.0907 |
| Magnoflorine | 15.04 | 342.1692* | 2.3 | 30 | 297.1140; 265.0847; 58.0657 |
| Gelsempervine-A/Gelsempervine-C | 15.32 | 383.1958 | 1.8 | 30 | 180.1011; 321.1599; 166.0864 |
| Harmane | 15.50 | 183.0914 | 1.8 | 55 | 95.0857; 123.0803 |
| 8-Methyl-10-phenyllobelidiol/Norlelobanidine | 15.53 | 278.2108 | 2.5 | 30 | 156.1376; 138.1270 |
| Galegin /Peganin | 15.60 | 328.1538 | 1.8 | 35 | 231.62611; 259.1478 |
| Gelsempervine-A/Gelsempervine-C | 15.62 | 383.1958 | 1.8 | 30 | 180.1015; 321.1600; 166.0864 |
| Cinchonanine E | 15.70 | 295.1801 | 1.5 | 35 | 277.1693; |
| Harmane | 15.90 | 183.0914 | 1.8 | 55 | 95.0857; 123.0805 |
| Galegin /Peganin | 15.90 | 328.1537 | 2.0 | 35 | 231.6262; |
| o-Methylcaryachine | 15.99 | 340.1537 | 1.9 | 35 | 295.0957; 263.07 |
| Cinchonanine G | 16.00 | 329.1854 | 1.9 | 35 | 309.1955; 160.0755 |
| 8-Methyl-10-phenyllobelidiol/Norlelobanidine | 16.08 | 278.2110 | 1.8 | 30 | 156.1378; 171.1374; 202.1578 |
| Lobinanidine, Isolobinanidine, beta-Lobinanidine | 16.23 | 290.2108 | 2.4 | 30 | 168.1375; 96.0807; 272.1996 |
| 19Z-16-epi-Voacarpine/16-epi-Voacarpine | 16.30 | 369.1802 | 1.9 | 30 | 321.1591; 206.9745; 112.7368 |
| Mesaconine | 16.30 | 486.2689 | 1.9 | 20 | 70.06558; 130.08618 |
| Cinchonanine C | 16.30 | 311.1748 | 1.9 | 35 | 293.1641; 130.0649 |
| 8-Methyl-10-phenyllobelidiol/Norlelobanidine | 16.60 | 278.2109 | 2.2 | 30 | 156.1378; 171.1370; 138.1269 |
| Mesaconine | 16.60 | 486.2696 | 0.5 | 20 | 70.06584; 130.08654 |
| Fumaropycine | 16.60 | 398.1591 | 1.8 | 35 | 338.1377; 277.0851 |
| Lobinine/Isolobinine | 16.84 | 288.1952 | 2.1 | 30 | 162.0893; 111.0802; 99.0440 |
| Lelobanidine I/Lelobanidine II | 16.91 | 292.2263 | 2.4 | 40 | 170.1540; 202.1580; 98.0961 |
| Cevadine | 17.17 | 592.3474 | 1.0 | 35 | 574.3358; 556.3259 |
| Aconine | 17.20 | 500.2849 | 1.0 | 20 | 84.06105; 141.10161 |
| Cheilanthifoline | 17.20 | 326.1384 | 0.8 | 35 | 178.0833; 151.0724 |
| Lobinanidine, Isolobinanidine, beta-Lobinanidine | 17.40 | 290.2109 | 2.1 | 30 | 50.0655; 164.1063; 200.1426 |
| 19Z-16-epi-Voacarpine/16-epi-Voacarpine | 17.40 | 369.1814 | 1.4 | 30 | 321.1591; 206.9745; 112.7370 |
| Lelobanidine I/Lelobanidine II | 17.41 | 292.2271 | 2.7 | 40 | 170.1538; 202.1580; 98.0959 |
| Protopin | 17.45 | 354.1332 | 1.1 | 35 | 149.0595; 189.0782 |
| N-Methyllaurotetanine | 17.50 | 342.1695 | 1.3 | 35 | 280.1089; 296.1037 |
| Aconine | 17.50 | 500.2836 | 3.5 | 20 | 84.06099; 141.10164 |

| | | | | | |
|---|---|---|---|---|---|
| Lobinanidine, Isolobinanidine, beta-Lobinanidine | 17.64 | 290.2115 | 2.1 | 30 | 50.0655; 168.1375; 200.1425 |
| Caryachine | 17.80 | 326.1382 | 1.5 | 35 | 311.1134; 244.15536 |
| Apohyoscyamine | 17.88 | 272.1645 | 2.6 | 50 | / |
| Cevadine | 18.07 | 592.3471 | 1.5 | 35 | 574.3346; 474.2847 |
| o-Methylcaryachine | 18.26 | 340.1538 | 1.5 | 35 | 309.1112; 188.0702 |
| Fumaricine | 18.30 | 370.1641 | 2.2 | 35 | 309.0755; 352.1535 |
| Fumaritine | 18.30 | 355.1411 | 0.9 | 35 | 336.1228; 323.0901 |
| Fumariline | 18.30 | 352.1170 | 2.5 | 35 | 337.1299; 322.1435 |
| Escholtzina | 18.54 | 324.1227 | 0.9 | 35 | 293.0803; 188.07034 |
| Pseudojervine | 18.54 | 588.3525 | 1.0 | 35 | 526.849; 188.4952 |
| Gelsempervine-B/Gelsempervine-D | 18.54 | 425.2071 | 1.9 | 40 | 172.0752; 180.1009; 158.0608 |
| Parfumidine | 18.90 | 368.1484 | 2.3 | 35 | 58.0657; 323.0909 |
| Gelsempervine-B/Gelsempervine-D | 18.91 | 425.2071 | 1.9 | 40 | 172.0752; 180.1009; 158.0608 |
| Lasiocarpine N-oxide | 19.00 | 428.2269 | 2.3 | 35 | 254.1374; 410.2173 |
| Cinchonanine F | 19.00 | 327.2059 | 2.6 | 35 | 309.1953; 184.0754 |
| Sinactine | 19.10 | 340.1536 | 2.1 | 35 | 324.1221; 309.0942 |
| Stylopine | 19.20 | 324.1227 | 1.0 | 35 | 336.8412; 232.9228 |
| methyl-Fumariphycine | 19.30 | 412.1748 | 1.8 | 20 | 277.0855; 249.0906 |
| Caryachine | 19.34 | 326.1382 | 1.5 | 35 | 295.0885; 190.0876 |
| Cinchonanine B | 19.50 | 309.1592 | 1.7 | 35 | 136.11162; 121.0885 |
| Gelsemicine | 19.59 | 359.1965 | 2.2 | 30 | / |
| Parfumidine | 19.70 | 368.1486 | 1.7 | 35 | 58.0657; 323.0909 |
| Norallosedamine | 19.76 | 206.1539 | 1.5 | 30 | 84.0384; 122.0964; 105.0699 |
| Lobinine/Isolobinine | 19.99 | 288.1958 | 2.1 | 30 | 162.0893; 111.0802; 99.0440 |
| cis-Lobelanidine/*trans*-Lobelanidine | 20.17 | 340.2271 | 2.1 | 30 | 202.1584; 218.15441; 98.0962 |
| cis-Lobeline/*trans*-Lobeline | 20.23 | 338.2115 | 2.4 | 30 | 216.1361; 96.0808 |
| cis-Lobeline/*trans*-Lobeline | 20.54 | 338.2115 | 2.4 | 30 | 216.1511; 216.1386; 96.0808 |
| cis-Lobelanine/*trans*-Lobelanine | 20.66 | 336.1958 | 1.8 | 30 | 96.0814; 216.1378; 290.1744 |
| cis-Lobelanidine/*trans*-Lobelanidine | 20.86 | 340.2271 | 2.4 | 30 | 218.1544; 202.1583; 98.0961 |
| Norlobelanine | 20.89 | 322.1802 | 2.5 | 30 | 202.1223; 82.0657; 171.1392 |
| cis-Lobelanine/*trans*-Lobelanine | 21.15 | 336.1958 | 0.6 | 30 | 96.0813; 216.1380 |
| Heimidine | 21.20 | 454.2216 | 1.8 | 35 | 408.21603; |
| beta-Solamargine | 21.75 | 868.5057 | 0.2 | 45 | / |
| Seneciphylline N-oxide | 22.00 | 350.1591 | 1.9 | 35 | 263.7915; 118.0357 |
| Heimidine | 22.30 | 454.2214 | 2.1 | 35 | 408.21579; |
| Coridamine | 22.40 | 351.1323 | 4.6 | 35 | 293.1339; 336.1199 |
| Dihydropiperlonguminine | 22.60 | 276.1590 | 1.4 | 35 | 155.0436; 161.0594 |

| | | | | | |
|---|---|---|---|---|---|
| piperlonguminine | 23.00 | 274.1433 | 1.8 | 35 | 201.0541; 135.0438 |
| Cinchonanine B | 23.40 | 309.1591 | 2.0 | 35 | 121.0084; 184.0733 |
| Piperanine | 25.10 | 288.1587 | 2.5 | 35 | 203.1059; 135.0438 |
| Sanguinarine | 25.30 | 332.0911* | 1.9 | 35 | 304.0961; 317.0673 |
| Rutacridone | 25.30 | 308.1276 | 1.6 | 35 | 278.0805; 290.1169 |
| Arborinine | 25.50 | 286.1069 | 1.7 | 35 | 271.0834; 253.0729 |
| Piperine | 25.50 | 286.1431 | 2.4 | 35 | 201.0542; 135.0438 |
| Gelsemoxonine/14,15-Dihydroxygelsenicine | 28.37 | 359.1622 | 2.8 | 30 | / |
| Lobinaline | 28.49 | 387.2795 | 2.1 | 30 | / |

Note: RT= retention time; $\Delta$ *m/z* (ppm)= accurate mass error compared to exact mass; NCE= normalized collision energy; *= $[M]^+$;

## Table 5

Botanical, pharmacological and chemical references in the untargeted plant study.

| **Herb** | **Botanical and pharmacological references** | **Alkaloid** | **Alkaloid References** |
|---|---|---|---|
| *Ruta graveolens* | [30-32] | Arborinine, Evoxanthine, Rutacridone | [33] |
| *Passiflora incarnate* | [34] | Harmaline, Harmalol, Harmane, Harmine, Harmol, Norharmane | [35] |
| *Escholtzia californiea* | [36] | Caryachine, Escholtzina, N-Methyllaurotetanine, *o*-Methylcaryachine, Protopin, | [37] |
| | | Sanguinarine | [38] |
| *Fumaria officinalis* | [39,40] | Cheilanthifoline, Coridamine, Fumaricine, Fumariline, Fumaritine, Fumaropycine, methyl-Fumariphycine, Sinactin, Stylopine | [40] |
| *Piper nigrum* | [41] | Dihydropiperlonguminine, Piperanine, Piperine, Piperlonguminine | [42] |
| *Galega officinalis* | [43,44] | Galegin, Peganin | [45] |
| *Ginko biloba* | [46] | Ginkgotoxin | [47] |
| *Achillea millefolium* | [48] | Betonicine, Stachydrine | [49] |
| *Mentha piperita* | [50] | Lasiocarpine N-oxide | [17,51] |
| *Urtica dioica* | [52] | Seneciphylline N-Oxide | [17,51] |
| *Cinchona succirubra* | [53] | Cinchonanine A, B, C, D, E, F | [54] |
| *Plantago major* | [55] | Indicain, Plantagonin | [56] |
| *Salvia officinalis* | [57,58] | Heimidine | [59] |
| *Vaccinium myrtillus* | [60] | Epimyrtine, Myrtin | [61] |
| *Veratrum album* | | Cevadine, Jervine, Pseudojervine | [62] |
| *Glycyrrhiza glabra* | [63] | Aconine, Mesaconine | [64] |
| *Valeriana officinalis* | [65] | Actinidine, Cathinine, Valerianine, Valerine | [66] |

## Table 6

Summary of the untargeted Alk profile of 52 herbal infusion samples and 6 commercial blends, sorted by chemical/botanical groups.

| Herb sample | Acridinone (N=3) | Benzophenantridine (N=2) | Indole (N=15) | Isoquinoline (N=17) | Piperidine (N=28) | Protoalkaloid (N=2) | Pyridine (N=3) | Pyrrolidine (N=5) | Pyrrolizidine (N=5) | Quinoline (N=11) | Quinolizidine (N=3) | Terpenoid (N=10) | Tropane (N=4) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Adoxaceae | | | | | | | | | | | | | |
| *Sabucus nigra* | X | X | X | X | 1 | X | X | 1 | X | X | X | X | X |
| Apiaceae | | | | | | | | | | | | | |
| *Angelica archangelica* | X | X | X | 2 | X | X | X | X | X | 1 | X | 1 | X |
| *Anthriscus cerefolium* | X | X | X | X | X | X | X | X | X | X | 1 | X | X |
| *Coriandrum sativum* | X | 1 | 5 | X | X | X | X | X | X | X | X | X | X |
| *Cuminum cyminum* | X | X | 5 | 1 | 1 | 1 | X | X | 1 | X | X | X | X |
| *Hyrocotyle asiatica* | X | X | 2 | X | X | X | X | 1 | X | X | 1 | X | X |
| *Petroselinum crispum* | X | 1 | 1 | 1 | X | X | X | 2 | X | 1 | X | 1 | X |
| *Pimpinella anisum* | X | X | X | X | X | X | X | 1 | X | X | X | X | X |
| Araceae | | | | | | | | | | | | | |
| *Acorus calamus* | X | X | 1 | 2 | X | 1 | X | X | 2 | 1 | X | X | X |
| Araliaceae | | | | | | | | | | | | | |
| *Eleutherococcus senticosus* | X | X | 1 | 3 | X | X | X | X | X | 1 | X | 2 | X |
| Asteraceae | | | | | | | | | | | | | |
| *Absinthium absinthium* | X | X | X | X | X | X | X | 3 | X | X | X | 2 | 1 |
| *Achillea millefolium* | X | X | 1 | X | X | X | X | 2 | X | X | 1 | 1 | X |
| *Arctium lappa* | X | X | 5 | X | X | X | X | 2 | X | 2 | X | X | X |
| *Calendula officinalis* | X | X | 6 | 1 | 1 | 1 | X | X | X | X | 1 | 1 | 1 |
| *Cichorium intybus* | X | X | X | X | 1 | X | X | X | X | 6 | X | X | X |
| *Cynara scolymus* | X | 1 | X | 6 | X | 1 | X | 1 | 1 | X | X | 2 | X |
| *Grindelia robusta* | X | 1 | X | X | X | X | X | X | X | 1 | X | X | X |
| *Heterotheca inuloides* | X | X | X | X | 2 | X | X | X | 1 | 7 | X | 3 | X |

| Herb sample | Acridinone (N=3) | Benzophenantridine (N=2) | Indole (N=15) | Isoquinoline (N=17) | Piperidine (N=28) | Protoalkaloid (N=2) | Pyridine (N=3) | Pyrrolidine (N=5) | Pyrrolizidine (N=5) | Quinoline (N=11) | Quinolizidine (N=3) | Terpenoid (N=10) | Tropane (N=4) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Malvaceae | | | | | | | | | | | | | |
| *Althaea officinalis* | X | 1 | 3 | 7 | X | X | X | 1 | X | 1 | 1 | X | X |
| *Malva sylvestris* | X | 1 | X | 6 | X | 2 | 1 | 1 | X | 1 | X | 1 | X |
| Monimiaceae | | | | | | | | | | | | | |
| *Pneumus boldus* | X | X | X | 5 | X | 2 | X | X | X | 1 | X | X | X |
| Myrtaceae | | | | | | | | | | | | | |
| *Pimenta officinalis* | X | 2 | 1 | X | 1 | X | X | X | X | 1 | X | X | X |
| *Syzygium aromaticum Merrill Eugenia* | X | X | X | X | X | X | 1 | X | X | X | X | X | X |
| Oleaceae | | | | | | | | | | | | | |
| *Olea europaea* | X | X | X | 1 | X | X | X | X | 1 | X | X | 1 | X |
| Onaeraceae | | | | | | | | | | | | | |
| *Epilobium parviflorum* | X | 2 | 1 | 12 | X | 2 | X | X | X | 2 | X | X | X |
| Papaveraceae | | | | | | | | | | | | | |
| *Citelidonium majus* | X | 2 | 4 | 10 | 2 | 2 | X | 1 | X | 2 | 1 | X | X |
| *Escholtzia californiea* | X | 2 | X | 15 | 1 | 2 | 1 | 1 | 1 | 2 | 1 | 2 | X |
| Passifloraceae | | | | | | | | | | | | | |
| *Passiflora incarnata* | 1 | X | 7 | X | 2 | X | X | 1 | X | X | X | 3 | 2 |
| Piperaceae | | | | | | | | | | | | | |
| *Piper nigrum* | X | X | X | X | 5 | X | X | X | X | 1 | X | X | X |
| Plantaginaceae | | | | | | | | | | | | | |
| *Plantago lanceolata* | X | X | X | X | X | 1 | X | X | X | X | 1 | 2 | X |
| Poaceae | | | | | | | | | | | | | |
| *Agropyron repens* | X | X | 5 | X | X | X | X | X | X | X | X | X | X |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Hieracium pilosella* | X | 1 | 3 | 2 | X | X | X | 1 | X | X | 1 | 1 | X |
| *Silybum marianum* | X | X | X | X | 3 | X | X | X | X | X | X | 1 | X |
| *Solidago virga-aurea* | X | X | 3 | X | 1 | 1 | X | X | X | X | X | 3 | X |
| *Taraxacum officinale* | X | 2 | 2 | 8 | 1 | 1 | X | 1 | X | X | X | X | X |
| Berberidaceae | | | | | | | | | | | | | |
| *Berberis vulgaris* | X | 1 | X | 13 | 1 | 2 | X | X | X | 2 | X | X | 1 |
| Betulaceae | | | | | | | | | | | | | |
| *Betula pendula* | X | X | 1 | X | X | X | X | X | X | X | X | 4 | X |
| Bignoniaceae | | | | | | | | | | | | | |
| *Tabebuia avellanadae* | X | X | X | 3 | X | X | X | 1 | X | 1 | X | 1 | X |
| Brassicaceae | | | | | | | | | | | | | |
| *Brassica alba* | X | X | X | 4 | 1 | 1 | 1 | X | X | 2 | X | X | X |
| *Bursa pastoris* | X | X | 4 | 1 | 2 | X | 1 | 3 | X | X | 1 | X | X |
| *Sisymbrium officinale* | X | X | X | X | X | X | X | 1 | X | 1 | 1 | 3 | X |
| Cesalpinaceae | | | | | | | | | | | | | |
| *Cassia angustifolia* | X | 1 | X | X | X | X | X | 1 | X | X | X | X | X |
| Equisetaceae | | | | | | | | | | | | | |
| *Equisetum arvense* | X | 1 | X | 1 | X | X | 2 | X | X | X | X | 2 | X |
| Ericaceae | | | | | | | | | | | | | |
| *Arctostaphylos pungens* | X | X | X | 2 | X | X | X | X | X | X | X | X | X |
| *Vaccinium myrtillus* | X | 1 | 1 | 8 | X | 2 | X | X | X | 2 | 1 | 1 | X |
| *Fabaceae* | | | | | | | | | | | | | |
| *Galega officinalis* | X | 2 | X | 2 | X | 2 | X | 1 | X | 1 | X | X | X |
| *Glycyrrhiza glabra* | X | X | 1 | 1 | X | X | X | 3 | X | X | X | 4 | X |
| *Trigonella foenum graecum* | X | X | X | X | X | X | X | X | X | 1 | X | X | X |
| Fumariaceae | | | | | | | | | | | | | |
| *Fumaria officinalis* | X | 2 | X | 16 | 2 | 2 | X | X | X | 2 | 1 | X | 1 |
| Getianaceae | | | | | | | | | | | | | |
| *Gentiana lutea* | X | X | X | X | X | X | X | 1 | X | X | X | X | X |
| Ginkoaceae | | | | | | | | | | | | | |
| *Ginko biloba* | X | X | X | X | X | X | 2 | X | X | X | X | 1 | X |
| Juglandaceae | | | | | | | | | | | | | |
| *Juglans regia L.* | X | X | X | X | X | X | 1 | X | X | X | X | 1 | X |
| Lamiaceae | | | | | | | | | | | | | |
| *Harpagophytum procumbens* | X | X | X | 1 | X | X | X | X | X | X | X | 1 | X |
| *Lavandula hybrid* | 1 | 1 | X | 1 | 1 | X | X | X | X | 2 | X | 1 | X |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Zea mais* | X | 1 | X | 5 | X | 1 | X | 1 | X | X | X | X | X |
| Polygonaceae | | | | | | | | | | | | | |
| *Ceterach officinarium* | X | 1 | X | 3 | X | X | X | X | X | X | X | X | X |
| *Rumex crispus* | X | X | X | 1 | X | X | X | X | X | 1 | X | X | X |
| *Rheum rhaponticum* | X | X | 2 | X | 1 | X | X | X | X | X | X | X | X |
| *Polygonum aviculare* | X | X | X | X | X | X | X | 1 | X | 1 | 1 | X | X |
| Ramnaceae | | | | | | | | | | | | | |
| *Rhamnus frangula* | X | 1 | X | X | X | X | X | X | X | 1 | X | X | X |
| Rosaceae | | | | | | | | | | | | | |
| *Agrimonia eupatoria* | X | X | 1 | 1 | X | X | X | 1 | X | X | 1 | X | X |
| *Alchemilla vulgaris* | X | 1 | 5 | 2 | 2 | X | X | 1 | X | X | 1 | 1 | X |
| *Crataegus oxyacantha* | X | X | 1 | X | X | X | X | X | X | 1 | X | 1 | X |
| *Filipendula ulmaria max* | X | X | 2 | X | X | X | X | X | X | 1 | 1 | 3 | X |
| *Prunus cerasus* | X | X | 1 | X | 1 | X | X | X | X | X | X | 1 | X |
| *Rubus idaeus* | X | X | 3 | 4 | X | X | X | 1 | X | 1 | 1 | 3 | 1 |
| Rubiaceae | | | | | | | | | | | | | |
| *Asperula odorata* | X | X | 2 | X | X | X | X | 1 | X | 1 | 1 | 1 | X |
| *Cinchona succirubra* | X | X | 1 | X | X | X | X | X | X | 8 | X | X | X |
| *Galium aparine* | X | X | X | X | 1 | X | 1 | X | X | X | X | X | X |
| Rutaceae | | | | | | | | | | | | | |
| *Citrus aurantium var bigaradia* | X | X | 1 | X | 1 | X | 1 | 2 | X | X | X | 2 | X |
| *Ruta graveolens* | 3 | 2 | 5 | 1 | 2 | 1 | X | 4 | X | X | X | 2 | X |
| Salicaceae | | | | | | | | | | | | | |
| *Salix alba* | X | 1 | X | 1 | X | X | X | X | X | 1 | X | 1 | X |
| Sapindaceae | | | | | | | | | | | | | |
| *Paullina cupana* | X | X | X | 1 | 1 | X | X | 1 | X | 1 | X | X | X |
| Schrophulariaceae | | | | | | | | | | | | | |
| *Schrophularia nodosa* | X | X | X | 3 | X | X | X | X | X | X | X | 1 | X |
| *Veronica officinalis* | X | X | 2 | X | 2 | X | X | X | X | X | 1 | 1 | X |
| Theaceae | | | | | | | | | | | | | |
| *Thea sinensis* | X | X | 2 | X | X | 1 | X | X | X | X | X | X | X |
| Urticaceae | | | | | | | | | | | | | |
| *Parietaria officinalis* | X | X | 1 | X | X | X | X | 1 | X | X | X | 3 | X |
| *Urtica dioica* | X | 1 | 1 | 2 | 1 | X | 1 | X | 1 | X | X | 3 | X |
| Valerianaceae | | | | | | | | | | | | | |
| *Valeriana officinalis* | X | X | X | 1 | X | 1 | 2 | X | X | 1 | X | 4 | X |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Melissa officinalis* | X | 1 | X | 9 | 1 | 2 | X | 1 | X | 3 | X | X | X |
| *Mentha piperita* | X | 1 | X | 1 | 1 | X | 1 | 2 | 2 | X | 1 | 1 | X |
| *Ocymum basilicum* | X | X | 2 | X | 1 | X | X | X | X | X | X | 1 | X |
| *Origanum majorana* | X | X | X | X | 1 | X | X | X | X | X | X | X | X |
| *Origanum vulgare* | X | 1 | X | X | 1 | 1 | X | 2 | X | X | X | 1 | X |
| *Orthosiphon stamineus* | X | X | X | X | X | X | X | X | X | X | 1 | X | X |
| *Ortosiphon stamineus* | X | X | X | X | X | X | X | X | X | X | 1 | 2 | X |
| *Salvia officinalis* | X | X | X | X | 1 | X | X | 2 | X | X | 3 | 1 | X |
| *Lannabinaceae* | | | | | | | | | | | | | |
| *Humulus lupulus* | X | X | X | X | X | X | X | 1 | X | 1 | X | X | X |
| *Lauraceae* | | | | | | | | | | | | | |
| *Cinammomun zeylanicum* | X | X | X | 2 | 1 | 2 | X | X | X | 1 | X | X | X |
| Loranthaceae | | | | | | | | | | | | | |
| *Viscum album* | X | X | 3 | X | X | X | X | X | X | X | X | X | X |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Verbenaceae | | | | | | | | | | | | | |
| *Lippia citriodora* | X | X | X | X | 3 | X | X | 1 | X | X | X | 3 | 1 |
| Violaceae | | | | | | | | | | | | | |
| *Viola tricolor* | X | 1 | 1 | 6 | X | 1 | X | X | X | 1 | 1 | X | X |
| Zingiberaceae | | | | | | | | | | | | | |
| *Zingiber officinale* | X | X | X | X | X | X | X | 1 | X | X | X | X | X |
| | | | | | | | | | | | | | |
| Mix | | | | | | | | | | | | | |
| Mix 1 | X | X | X | 1 | X | X | X | X | X | X | X | X | X |
| Mix 2 | X | X | X | X | 1 | X | X | X | X | 1 | X | X | X |
| Mix 4 | X | X | 1 | X | 1 | X | 1 | X | X | X | 1 | X | X |
| Mix 5 | X | X | X | X | X | X | 1 | X | 1 | X | X | 1 | X |
| Mix 6 | X | X | X | X | X | X | X | X | 1 | X | X | 1 | X |
| Mix 7 | X | X | X | X | X | X | X | X | X | X | X | 1 | X |

### 3.2.2. Targeted and untargeted profiling of alkaloids in Italian alpine herbs using high resolution mass spectrometry and their use for distinguishing the herbage grazed by dairy cows

**ALKALOIDS FOR DISCRIMINATING COW GRAZED HERBS**

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**Abstract**

Understanding herbivores' feeding preferences on complex natural polyphyte grassland such as alpine pastures is pivotal for evaluating the nutrient intake of animals and understanding the effect of grazing on vegetation. In this study, 62 different herbal plants, sampled at the same phenological stage in two types of alpine pasture in north-eastern Italy (*Poin alpinae* and *Seslerion caeruleae*), and 48 herbage samples assembled mimicking the grazing preferences of a group of 16 Italian Simmental cows in triplicate, using the hand plucking technique, were characterized to determine the alkaloid profile. Forty-one alkaloids (N=16 pyrrolizidine alkaloids; 11 steroidal, 3 indole, 3 purine, 3 tropane, 2 quinoline, 1 piperidine, 1 pyridine, and 1 terpenoid) were quantified and another 116 were putatively identified with high resolution mass spectrometry.

As regards the herbal plants, a Partial Least Squares – Discriminant Analysis model based on the alkaloid profiles of the 3 most well-represented plant families, Poaceae (9 species), Asteraceae (7) and Lamiaceae (7), correctly reclassified the plants with an average accuracy of 91%.

The composition of the herbages made it possible to determine firstly the potential alkaloid intake of cows grazing in the 2 pastures in relation to the different botanical composition. Generally, the

cows showed a preference for plants belonging to families such as Fabaceae, Rosaceae, Plantaginaceae, Cariophyllaceae and Apiaceae. Secondly, a Partial Least Squares – Discriminant Analysis model applied to the alkaloid profiles of herbages allowed us to discriminate between the diets of cows grazing on the different pastures with an overall accuracy of 85%.

**Keywords**: alkaloid; orbitrapTM; alpine herb; grazing; cow selection.

## Introduction

The remarkable variability of geomorphological and ecological situations characterizing the Alps justifies the presence of a markedly high number of different plant species. In terms of flora, 4,500 vascular plant species are present, representing 39% of European flora (Mörschel et al., 2004). The composition of these plants is not frequently addressed in the literature and there are few studies on the contribution of alpine plants to the nutrient intake of herbivores (Lefebvre et al., 2016; Peiretti et al., 2017). In particular, to the best of our knowledge, no systematic and exhaustive studies are available for some of the most important secondary metabolites, alkaloids (alks), in alpine herbage. Alks, basic nitrogen-containing organic compounds, are also used by plants as protective agents against predator attack (Dobler, 2001), playing an important role in the interaction of plants with their environment (Fester, 2010). Some alks are responsible for the beneficial effects of plant extracts in humans in traditional medicine (e.g. Achillea millefolium, used to treat wounds, digestive problems and respiratory infections; Plantago major, traditionally used for haemorrhoid treatment and respiratory diseases, and recently proposed to alleviate skin wounds, burns and bleeding; Urtica dioica, proposed for the treatment of arthritis (Applequist and Moerman, 2011; Zubairab et al., 2015; Liao et al., 2016). Nevertheless, other alks show highly toxic effects. In particular, pyrrolizidine (Pyz) alks are hepatotoxic, mutagenic and carcinogenic (e.g. Senecio vulgaris the most common Alpine plant rich in Pyz alks and Arnica Montana an Alpine plant with reduced content of Pyzs) (Pabreiter, 1992; Hartmann, 2007). Over ten thousand alks have been isolated from natural sources, the highest alk occurrence being in Ranunculaceae, Berberidaceae, Menispermaceae, Piperaceae, Cactaceae, Papaveraceae, Gentianaceae and Solanaceae (Yang et al., 2009), while the occurrence is almost zero in families such as Fagaceae, Betulacae, Casuarinaceae and Juglandaceae, suggesting alk occurrence can be treated as a general family characteristic (Li and Willaman, 1968).

Estimation of herbivore selection on complex natural grassland such as alpine pasture is important for evaluating the nutrient intake of animals and understanding the effect on vegetation. Of the techniques available to estimate dietary composition and the intake of grazing herbivores, marker-based ones are considered advantageous for various reasons, extensively discussed elsewhere (Langlands, 1987; Piasentier et al., 1995; Malossini et al., 1996). Since the 1980s, the most widely used plant markers have been alkanes (Mayes et al., 1986; Malossini et al., 1994). However, evaluation of dietary composition in rich multispecies association requires an increase in the number of suitable markers useful to identify even individual plants (Malossini et al.,1990; Mayes and Dove, 2000). The supposed plant specificity of alks makes them worthy of consideration as potential chemical markers for studying the dietary composition of ruminants grazing on alpine pastures. Alks may play a role as tracers in assessing the authenticity of origin, reassuring consumers about product identity and process compliance (Camin et al., 2016; Danezis et al., 2016). This study aimed first of all to define the alk profiles of a large selection of herbs (N=62), characterizing two natural pastures in the eastern Italian Alps; secondly, it aimed to estimate the variability of alk compounds ingested by dairy cows grazing on the two types of alpine grasslands and verify the discriminability of animal diet from different pastures. Characterization of alk profiles was obtained using liquid chromatography, combined with online SPE clean-up of plant extracts, and the rapid and selective detection ability of high resolution mass spectrometry.

## 1. Materials and methods

### 1.1. Grazed pastures and the composition of flora

The study was carried out on a traditional Alpine farm in north-eastern Italy (Malga Montasio; 46°24′45″N, 13°25′53″E), during the summer grazing period. Two different types of pasture were considered, *Poion alpinae* (PO), a nutrient-rich pasture located at 1,500 m above sea level, and Seslerion caeruleae (SE), a nutrient-poor one at 1,700 m a.s.l. (Bovolenta et al., 2014).

Characterization of the flora was performed by an experienced botanist in 100m2 sample areas at the beginning of the grazing period, according to the different altitude of pastures, with five and nine replicates on PO and SE respectively. In each replicate, the individual plant species were identified (Poldini et al., 2002) and sampled, taking note of their relative abundance by visually evaluating the percentage of surface coverage, as described by Pasut (2016). In particular, the 53 species most abundant in both pastures and some sporadic species (9), selected on the basis of

observation of cow grazing behaviour, were identified and individually sampled. Their replication frequency and average coverage in the two pastures are shown in Table 1. Data were not tabulated for sporadic species, i.e. those recorded in only one replicate, with coverage of < 1%.

### 2.2. Grazing animals and herbage selection

A herd of 110 Italian Simmental dairy cows grazed the two pastures at the same phenological stage (Poaceae flowering period), according to the altitudinal gradient of the vegetation. The cows had access to pasture during the day and night, and received on average 2.5 kg/head per day of feed supplement (mixed concentrate based on maize, barley, beet pulp, soy and wheat). In both pastures, a group of eight lactating cows was selected from the herd, taking care to balance feeding requirements between the groups. The average production data recorded during the two-week preliminary periods were as follows: milk yield (17.4 ± 2.1 kg/d; average ± SD), stage of lactation (148 ± 59.8 Days In Milk), fat (3.84 ± 0.46%), protein (3.14 ± 0.15%), lactose (4.68 ± 0.14%) and Somatic Cell Count (124,800 ± 117,000 cells/mL). All the cows involved in the trial had already grazed at this alpine farm for at least one season. The herbage selected by each cow was manually sampled, using a hand plucking technique that mimics animal intake (Langlands, 1974; Berry et al., 2002). The sampling was repeated for three consecutive days for both pastures, gathering 48 herbage mixtures.

At the same time, immediately before the cows entered the pasture, samples of available herbage were collected. For each grazing day the grass was cut from two representative sample areas (1 m2) using electric grass shears.

Bulked samples of available herbage and selected herbage from each pasture type were described according to the main botanical families, which contribute was determined on a weight basis and expressed as a dry matter percentage (Table 2).

### 2.3. Plant sample preparation

After harvesting, the herbal samples were stored at -18 °C while awaiting chemical analysis.

Alk profile evaluation was carried out on both individual plant samples and hand-plucked herbage samples. An aliquot of 2.5 g of homogenised herbal sample (particle diameter of roughly < 2 mm) was directly weighed in 50 mL polyethylene falcon tubes (Sartorius AG, Goettingen, Germany) and extracted, adding 20 mL of extraction solution (H2O/MeOH/FA; 49.5:49.5:1 v/v/v). The mixture was sonicated for 10 minutes (LBS1 6Lt, FALC Instruments, Treviglio BG, Italy), subjected to

vertical shaking for 12 hours at 20 rpm (Rotoshake 24/16, Gerhardt GmbH & Co. KG, Königswinter, Germany), and once again sonicated for 10 minutes. The methanolic extract was separated with centrifugation (10 minutes at 4100 rpm; IEC CL31 Multispeed, Thermo Scientific, Sunnyvale, CA, USA), filtered with a 0.45 µm cellulose filter cartridge (Sartorius AG, Goettingen, Germany), diluted twice with a H2O/MeOH solution (50:50 v/v) and injected (10 µL).

### 2.4. Reagents and solutions

LC-MS grade acetonitrile (ACN), LC-MS grade methanol (MeOH), MS grade formic acid (FA, 98%) and LC-MS grade ammonium acetate were purchased from Fluka (St. Louis, MO, USA) and ammonia solution 25% was purchased from Merk Millipore (Darmstadt, Germany). For mass calibration, a standard mix of n-butylamine, caffeine, MRFA and Ultramark 1621 (Pierce® ESI Positive Ion Calibration Solution, Rockford, IL, USA) were used. Deionized water (H2O) was produced with an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany). Alk standards (Table 3) were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany), except for Striknine and Harmaline, which were purchased from Sigma (St. Louis, MO, USA).

Individual stock solutions of each alk (100 mg/L) were prepared by dissolving the standard powder in an aqueous methanol solution (50:50, v/v). A mix solution (1 mg/L of each individual alk) was prepared from the single stock solutions and used for calibration in the range 0.02 – 1000 µg/L, injecting 1ul for each level. The mix solution was prepared freshly before each analysis, while stock solutions were stored at -4°C.

### 2.5. Chromatographic separation

Chromatographic separation was obtained using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA), furnished with a Rheodyne 6-port automated switching valve and a pump module that allowed control of two independent fluid systems. The method used the same approach proposed by Nardin et al. (2016).

A SolEx HRP SPE cartridge (2.1 mm × 20 mm, 12-14 µm, ThermoFisher, Sunnyvale, CA, USA) was used to perform online clean-up, loading 10 µl of the sample and flushing with 4% MeOH adjusted to pH=9 with ammonia (eluent A, flow rate of 1 mL/min) and with 0.1% FA for another minute to complete matrix interference removal. The position of the Rheodyne valve was switched and the analytical mobile phase, 70% of 0.1% FA with 5mM ammonium acetate (eluent B) and

30% of MeOH/ACN 95:5 v/v with 0.1% FA and 5 mM ammonium acetate (eluent C), flowed through the SPE cartridge at a flow rate of 0.7 mL/min, progressively removing the retained analytes and transferring them to the analytical column (Raptor Biphenyl, 3 mm x 150 mm, 2.7 µm particle size, Restek, Bellefonte, PA, USA). Isocratic elution with 30% of eluent C was run from 2 to 4 minutes, then gradient elution was performed from 30% to 80% (eluent C) from 4 to 25 minutes, and from 80% to 100% (eluent C) from 25 to 26 minutes, and held until 28 minutes. Eluent C was then linearly reduced to 30% in 0.5 minutes and the analytical column was equilibrated for 2.5 minutes with the initial conditions. Meanwhile, the SPE cartridge was flushed with 1% FA at 1 mL/min with MeOH in order to wash it, and then with eluent A to re-equilibrate it before the next analysis. The autosampler was set at a temperature of 5 °C and the column at 35 °C. Chromeleon™ 7.2 Chromatography Data System software (Thermo Scientific™ Dionex™) automatically piloted the switching valve and the chromatographic separation gradient.

### 2.6. Mass spectrometry analysis

A Q-ExactiveTM hybrid quadrupole-orbitrap mass spectrometer (HQOMS, Thermo Scientific, Bremen, Germany) equipped with heated electrospray ionization (HESI-II) interface was used for alk analysis. In the HESI II source, nitrogen was used as the drying and collision gas in positive ion mode. The heated capillary temperature was set at 330 °C, while the sheath gas flow rate was set at 30 arbitrary units, auxiliary gas flow rate at 10 arbitrary units, spray voltage at 3.5 kV, and auxiliary gas heater temperature at 300 °C. Mass spectra were acquired in profile mode through a full MS-data dependent MS/MS experiment (full MS–dd MS/MS), as in the method proposed by Nardin et al. (2016).

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System software was used for instrument control and for data processing and evaluation.

### 2.7. Targeted method validation

The characteristics of the targeted alks method were studied using the full mass spectral data of 41 pure standards. The precursor ion detected in the extracted ion chromatograms (EICs) and corresponding to the protonated molecules $[M-H]^+$ (mass tolerance < 5 ppm; European Commission Guidelines) was always used for quantification. Retention time (RT) and isotope pattern were used to identify targeted alks in sample analysis data, and dd-MS/MS spectra compared with those collected from available standards were used in order to confirm them.

Quantification was performed with a 5-point calibration curve, allowing a regression coefficient (R2) of at least 0.990, included in the linearity range. The limit of detection (LOD) was estimated as three standard deviations of ten replicated blank samples according to EURACHEM (2014), and similarly, the limit of quantification (LOQ) was estimated as ten standard deviations of the same replicates. Table 3 shows the name, linearity range, LOD and LOQ of alk standards.

### 2.8. Untargeted study

In order to extend alpine herb alk characterization, a suspect screening approach was adopted according to previous studies performed by the same authors (Nardin et al., 2016; Nardin et al., 2017). RT and mass fragmentation of an initial group of 48 alks found in 8 plants (*Datura stramonium, Hyoscyamus niger, Solanum nigrum, Lobelia inflate, Senecio vulgaris, Arnica montana, Gelsemium sempervirens and Ranunculus montanus*) and a further group of 68 alks present in 17 other plants (*Ruta graveolens, Passiflora incarnate, Escholtzia californiea, Fumaria officinalis, Piper nigrum, Galega officinalis, Ginko biloba, Achillea millefolium, Mentha piperita, Urtica dioica, Cinchona succirubra, Plantago major, Salvia officinalis, Vaccinium myrtillus, Veratrum album, Glycyrrhiza glabra and Valeriana officinalis*) respectively were defined. Peak signals were confirmed with the EIC matching *m/z* values, with mass tolerance of < 5 ppm (European Commission Guidelines) compared to the exact mass of alks reported in the literature. Untargeted detection was limited to those alks providing a sufficient detectable response (area > 100 area units). Possible isomers were differentiated, indicating the name of the alk putatively identified and the experimental RT (alk name@RT in minutes; e.g. 8,10-Diethyllobelidiol@12.1)

### 2.9. Statistical analysis

Univariate and multivariate analysis were performed using Statistica 13.1 (StatSoft; Tulsa, OK, USA) software and Unscrambler X 10.4 (CAMO software AS, Oslo, Norway). Statistical processing was carried out on concentration values for targeted alks, while the ionization intensity expressed as peak area was used for untargeted alks. Data not normally distributed (Kolmogorov-Smirnov test, $p<0.01$) were normalized by applying Box-Cox transformation.

Tukey's Honestly Significant Difference (HSD) test for an unequal number of samples ($p<0.05$) was performed to identify significant differences in the alk content of different plant families or selected diets from different pastures. The correlation between alks was evaluated with Pearson's test.

Partial Least Squares – Discriminant Analysis (PLS-DA) (Chevallier et al., 2006) was applied to check the efficacy of the alk profile of individual plant species or hand-plucked samples in discriminating the botanical family or selected diet from the two grazed pastures. With this aim, a PLS-DA model was built using the alk matrixes (X) and the family or grazed pasture matrix (Y), which was created by defining a dummy variable for each family and pasture type considered.

The optimum number of PLS components was estimated using full cross-validation. The significance of alk predictors was evaluated with Marten's uncertainty test. Classification performance was assessed in the validation phase in terms of sensitivity, specificity and accuracy, adopting a cut-off value of 0.5. Thus, samples with a predicted Y-value greater than 0.5 were identified as belonging to one class, whilst those with predicted Y-values lower than 0.5 were predicted as belonging to the other class (Camin et al., 2017).

## 3. Results and discussion

### *3.1. Alk profiling of alpine herbs*

#### *3.1.1. Targeted alk profile*

The targeted alk standards, linearity, LOD and LOQ, precision and accuracy determined for each compound are shown in Table 3. The LOD ranged from the lowest values for Heliotrine (0.04 µg/L), to the highest for Nicotine (15 µg/L), and the quantification range went from the quantification limit to 500/1500 µg/L, depending on the compounds. Table 4 shows plants (six) in which we found at least one of the 41 tested targeted alks to be above the LOD. Only 10% of sampled species contained at least one targeted alk, with Pyzs in particular being the most commonly present alks (7% of sampled species): we found Lycopsamine in *Capsella bursa pastoris*, with a concentration of 6 µg/kg, and in *Veronica chamaedrys*; we found Seneciphylline in *Potentilla crantzii*, with a concentration of 112 µg/kg; both Erucifoline and Heliotrine occurred in *Hypericum maculatum,* with a concentration of 9 µg/kg and 11 µg/kg, respectively. We detected one indole (Ind) alk, Gramine, in *Betonica alopecurus* at 118 µg/kg and found one steroidal (Str) alk, Veratramine, in *Phleum rhaeticum* at 10 µg/kg.

#### *3.1.2. Untargeted profiles*

We detected up to 85 different alks with the suspect screening approach in the 62 alpine plant samples. In particular, we detected Inds, pyrrolidines (Pyls), piperidines (Pprs) and terpenoids

(Trns) in 100% of samples, quinolines (Qnls) in 87%, pyridine (Pyr) in 83%, quinolizidines (Qnzs) in 76%, Pyzs in 74%, Strs in 45%, isoquinolines (Iqns) in 30%, acridones (Acds) in 27%, tropanes (Trps) in 24%, benzophenantridines (Bzps) in 13%, and protoalkaloids (PrAs) in 8% of samples. Considering the individual plants, *Carlina acaulis, Capsella bursa-pastoris* and *Plantago media* showed the highest number of alks (33, 34 and 41, respectively).

Following the approach suggested by Li and Willaman (1968), we analyzed the occurrence of alks in Alpine herbs using the family criterion. Table 5 presents an overview of the chemical groups of alks identified in Alpine herbs, sorted by botanical family. Below we describe the alk profiles of the most well-represented families.

Asteraceae: In at least one of the seven Asteraceae species we found Pprs (8-Ethylnorlobelol, 8,10-Diethyllobelidiol@12.1, 8,10-Diethyllobelidiol@13.1, Dihydropiperlonguminine, Isolobinine /Lobinine@20.0 Lelobanidine I/Lelobanidine II@16.9, Lelobanidine I/Lelobanidine II@17.4, Norallosedamine, Norlelobanidine/8-Methyl-10-phenyllobelidiol@15.5, Norlelobanidine/8-Methyl-10-phenyllobelidiol@16.1, Norlelobanidine/8-Methyl-10-phenyllobelidiol@16.6, Piperanine, Piperine and Piperlonguminine), Inds (14,15-Dihydroxygelsenicine/Gelsemoxonine, Gelsemine, Harmaline, Harmane@15.5, Harmane@15.9, Harmine, Harmol and Norharmane), Trns (Aconine@17.2, Aconine@17.5, Actinidine, Cathinine, Mesaconine@16.3, Mesaconine@16.6, Valerianine@8.9, Valerianine@13.0 and Valerine), Qnls (Cinchonanine B@19.4, Cinchonanine F@12.0, Cinchonanine F@19.1, Indicain and Magnoflorine), Iqns (Fumaricine, Fumaritine, *o*-Methylcaryachine@18.3 and Parfumidine@19.7), Pyzs (Integerrimine/Perforine, Lasiocarpine N-oxide and Seneciphylline N oxide), Pyrs (Ginkgotoxin, Plantagonin and Tropinone), Pyls (Stachydrine@3.5 and Stachydrine@4.1), Strs (Cevadine@17.2 and Cevadine@18.1), Trps (Anisodamine and Apohyoscyamine), Acds (Arborinine), Bzps (Protopin), and Qnzs (Myrtine/Epimyrtine). The alks Myrtine/Epimyrtine, Norharmane, Stachydrine@4.1, and Valerianine@8.9 were present in all the 7 investigated Asteraceae plants. Furthermore, we only detected Apohyoscyamine, Cinchonanine B@19.4, Fumaritine and 8,10-Diethyllobelidiol@13.1 in this family.

We found Caryophyllaceae: Caryophyllaceae (four species) to be rich in Pprs (8-Ethylnorlobelol, Dihydropiperlonguminine, Isolobinine/Lobinine@20.0, Norallosedamine, Norlelobanidine/8-Methyl-10-phenyllobelidiol@15.5, Norlelobanidine/8-Methyl-10-phenyllobelidiol@16.6 and Piperine), Inds (14,15-Dihydroxygelsenicine/Gelsemoxonine, Gelsemicine, Gelsemine, Harmaline, Harmalol, Harmane@15.5, Harmane@15.9, Harmine, Harmol and Norharmane), Trns

(Aconine@17.2, Aconine@17.5, Actinidine, Mesaconine@16.3, Mesaconine@16.6, Valerianine@8.9, Valerianine@13.0 and Valerine), Qnls (Cinchonanine F@12.0, Cinchonanine F@19.1, Indicain and Magnoflorine), Pyzs (Integerrimine/Perforine, Lasiocarpine N-oxide and Seneciphylline N-oxide), Trps (3-Acetyltropine, 3,6-Diacetyltropine and Anisodamine), Pyrs (Ginkgotoxin and Tropinone), Strs (Cevadine@17.2, Cevadine@18.1 and Pseudojervine), Pyls (Stachydrine@3.5 and Stachydrine@4.1) Acds (Arborinine), and Qnzs (Myrtine/Epimyrtine). Gelsemine, Harmane@15.5, Harmine, Harmol, Norharmane, Stachydrine@3.5, Stachydrine@4.1, Valerianine@8.9, Valerianine@13.0, Indicain, Tropinone and Lasiocarpine N-oxide were present in all the 4 sampled plants.

We found Fabaceae: Fabaceae (five species) to be rich in Inds (14,15-Dihydroxygelsenicine/Gelsemoxonine, Gelsemicine, Gelsemine, Gelsempervine C /Gelsempervine A, Harmaline, Harmalol, Harmane@15.5, Harmane@15.9, Harmine, Harmol and Norharmane), Pprs (8-Ethylnorlobelol, 8,10-Diethyllobelidiol, Dihydropiperlonguminine, Lelobanidine I/Lelobanidine II@17.4, Norallosedamine, Norlelobanidine/8-Methyl-10-phenyllobelidiol@15.3, Norlelobanidine/8-Methyl-10-phenyllobelidiol@16.1, Norlelobanidine/8-Methyl-10-phenyllobelidiol@16.6 and Piperine), Trns (Aconine@17.2, Aconine@17.5, Actinidine, Mesaconine@16.3, Valerianine@8.9, Valerianine@13..0 and Valerine), Qnls (Cinchonanine F@12.0, Cinchonanine F@19.1, Cinchonanine G, Indicain and Magnoflorine), Iqns (Caryachine@14.5, Cheilanthifoline and N-methyllaurotetanine), Pyzs (Integerrimine/Perforine, Lasiocarpine N-oxide, Seneciphylline N oxide and Spartioidine), Pyrs (Ginkgotoxin, Plantagonin and Tropinone), Trps (3-Acetyltropine, 3,6-Diacetyltropine and Anisodamine), Pyls (Betonicine, Stachydrine@3.5 and Stachydrine@4.1), Strs (Cevadine@17.2 and Cevadine@18.1), Bzps (Protopin), PrAs (Galegin/Peganin@15.6), and Qnzs (Myrtine/Epimyrtine). We detected 8-Ethylnorlobelol, Gelsemine, Harmane@15.5, Harmane@15.59, Harmol, Indicain, Myrtine/Epimyrtine, Norharmane, Stachydrine@3.5, Stachydrine@4.1 and Valerianine@8.9 in all 5 Fabacee plants.

We found Lamiaceae: Lamiaceae (seven species) to be rich in Pprs (8- Ethylnorlobelol, Dihydropiperlonguminine, Isolobinanidine/Lobinanidine@16.2, Isolobinine/Lobinine@20.0, Lelobanidine I/Lelobanidine II@16.9, Lelobanidine I/Lelobanidine II@17.4, Norallosedamine, Norlelobanidine/8 Methyl-10- phenyllobelidiol@15.5, Norlelobanidine/8-Methyl-10 phenyllobelidiol@16.1, Norlelobanidine/8-Methyl-10- phenyllobelidiol@16.6, Piperanine and Piperine), Inds (14,15-Dihydroxygelsenicine/Gelsemoxonine, 19Z-16-epi-Voacarpine/16-epi-

Voacarpine@16.3, 19Z-16-epi Voacarpine/16-epi-Voacarpine@17.4, Gelsemicine, Gelsemine, Harmaline, Harmane@15.5, Harmane@15.9, Harmine, Harmol and Norharmane), Trns (Aconine@17.2, Actinidine, Mesaconine@17.2, Mesaconine@17.6, Valerianine@8.9, Valerianine@13.0 and Valerine), Iqns (Cheilanthifoline, Fumaricine, Fumaropycine and N-methylllaurotetanine), Qnls (Cinchonanine F@19.1, Indicain and Magnoflorine), Pyrs (Ginkgotoxin and Tropinone), Pyzs (Lasiocarpine N-oxide and Seneciphylline N-oxide), Trps (3-Acetyltropine, 3,6-Diacetyltropine and Anisodamine), Pyls (Betonicine, Stachydrine@3.5 and Stachydrine@4.1), Acds (Arborinine), Bzps (Protopin), Qnzs (Myrtine/Epimyrtine), and Strs (Cevadine@17.2). 8-Ethylnorlobelol, Norharmane, Stachydrine@3.5, Stachydrine@4.1, Valerianine@8.9, and Valerianine@13.0 were present in all 7 Lamiaceae plants.

We found Poaceae: Poaceae (nine species) to be rich in Inds (14,15-Dihydroxygelsenicine/Gelsemoxonine, Gelsemicine, Gelsemine, Gelsempervine D /Gelsempervine B@18.5, Harmaline, Harmalol, Harmane@15.5, Harmane@15.9, Harmine, Harmol and Norharmane), Pprs (8-Ethylnorlobelol, Dihydropiperlonguminine, Isolobinanidine/Lobinanidine@17.4, Isolobinine/Lobinine@20.0, Norallosedamine, Norlelobanidine/8-Methyl-10-phenyllobelidiol@15.5, Norlelobanidine/8-Methyl-10-phenyllobelidiol@16.1, Norlelobanidine/8-Methyl-10-phenyllobelidiol@16.6 and Piperlonguminine), Trns (Aconine@17.2, Aconine@17.5, Actinidine, Mesaconine@16.3, Mesaconine@16.6, Valerianine@13.0, Valerianine@8.9 and Valerine), Qnls (Cinchonanine C@16.3, Cinchonanine F@12.0, Cinchonanine F@19.1, Cinchonanine G and Indicain), Pyrs (Plantagonin and Tropinone), Pyzs (Integerrimine/Perforine, Lasiocarpine N-oxide and Spartioidine), Trps (3 Acetyltropine, 3,6-Diacetyltropine and Anisodamine), Pyls (Betonicine, Stachydrine@3.5 and Stachydrine@4.1), Strs (Cevadine@17.2 and Pseudojervine), Acds (Arborinine), Iqns (Fumaropycine), and Qnzs (Myrtine/Epimyrtine). We detected Lobinaline, 2-Pyrrolidineacetic acid, 14,15-Dihydroxygelsenicine/Gelsemoxonine, Gelsemine, Harmol, Norharmane, Stachydrine@3.5, Stachydrine@4.1, Valerianine@8.9 and Valerianine@13 in all the 9 investigated Poaceae plants, and Cinchonanine C and Isolobinanidine/Lobinanidine@17,4 were present exclusively in this family.

Other families: we then considered alks in botanical families comprising three species each. We found 3,6-Diacetyltropine, 8-Ethylnorlobelol, 14,15-Dihydroxygelsenicine/Gelsemoxonine, Arborinine, Harmane@15.5, Harmol, Indicain, Isolobinine/Lobinine@20.0, Lasiocarpine N-oxide,

Myrtine/Epimyrtine, Norharmane, Stachydrine, Tropinone, Valerianine@8.9, Valerianine@13.0 and Valerine in all plants belonging to the Apiaceae family.

We found 3-Acetyltropine, 8-Ethylnorlobelol, 14,15-Dihydroxygelsenicine/Gelsemoxonine, Caryachine, Harmane, Mesaconine@16.6, Myrtine/Epimyrtine, Norharmane, Plantagonin, Stachydrine and Valerianine in all plants belonging to the *Plantaginaceae* family.

We found 8-Ethylnorlobelol, 14,15-Dihydroxygelsenicine/Gelsemoxonine, Dihydropiperlonguminine, Harmane, Harmane@15.5, Harmane@15.9, Harmol, Myrtine/Epimyrtine, Norharmane, Stachydrine@4.1, Tropinone, Valerianine@8.9, Valerianine@13.0 and Valerine in all plants belonging to the Poligonaceae family. 8-Ethylnorlobelol, Harmane@15.5, 14,15-Dihydroxygelsenicine/Gelsemoxonine, Harmane@15.9, Harmol, Indicain, Norharmane, Stachydrine@3.5, Stachydrine@4.1, Tropinone, Valerianine@8.9 and Valerianine@13.0 were present in all Rosaceae plants. We found 8-Ethylnorlobelol, Gelsemine, Harmane@15.5, Indicain, Integerrimine/Perforine, Norharmane, Stachydrine@3.5, Stachydrine@4.1, Tropinone, Valerianine@8.9 and Valerianine@13.0, in all plants belonging to the Rubiaceae family.

### *3.2. Discrimination of botanical family from alk variability*

After a detailed description of alks in the chemical groups detected in the main botanical families, we carried out statistical analysis to evaluate the effectiveness of the examined compounds in discriminating plant taxonomy.

#### *3.2.1. Individual alk variability*

Significant differences (Tukey's HSD test) characterized the individual alk content in the main herb families, highlighting a particular relationship between alks (data not shown). Considering families represented by at least 3 sampled plants, the following alk variability is worthy of consideration. Caryophyllaceae were significantly richer in Harmol than Plantaginaceae. Caryachine@14.5, present in all the analyzed Plantaginaceae, was significantly higher in this family than in the others. Furthermore, Plantaginaceae had a higher content of Methyl-fumarophycine, *o*-methylcaryachine@16.0, Parfumidine@18.9 and Galegin/Peganin@15.9 than the other families. Isolobinanidine/Lobinanidine@17.6 was higher in Apiaceae than in the other families. Stachidrine@3.1 was significantly lower in Poligonaceae than in Asteraceae, Caryophyllaceae,

Fabaceae and Plantaginaceae. Finally, Valerianine@8.9 was significantly higher in Asteraceae and Lamiaceae than in Poaceae.

Taking into account only the three most well-represented families (i.e. Asteraceae, Lamiaceae and Poaceae) Pearson's test ($p<0.001$) highlighted significant correlations between Valerianine@13.0 and Valerianine@8.9 ($r \geq 0.98$ in the three families).

In Asteraceae plants, Valerianine@13.0 and Valerianine@8.9 were also both correlated with Stachydrine@3.5 (r=0.93 and r=0.87, respectively), 8-ethylnorlobelol (r=0.89 and r=0.91, respectively), and Norharmane (r=0.81 and r=0.85, respectively). Harmol was correlated with Indicain (r=0.68) and this last was correlated with Stachydrine@3.5 (r=0.67). Norharmane was correlated with Stachydrine@3.5 (r= 0.74) and Dihydropiperlonguminine (r=0.69).

In Lamiaceae, Norlelobanidine/8-methyl-10-phenyllobelidiol@16.6 was correlated with Mesaconina@16.6 (r=0.99), Valerine with 8-ethylnorlobelol (r=0.91), and Norharmane with Magnoflorine (r=0.77) and Harmol (r=0.88). This last was also correlated with Gelsemine (r=0.81). Harmine was correlated with Mesaconine@16.3 (r=0.99), whereas Lelobanindine I/LelobanidineII@17.4 was correlated with Magnoflorine, Valerianine@13.0 and Valerianine@8.9 (r=0.99, r=0.94 and 0.91, respectively).

Finally, in Poaceae plants Gelsemine was correlated with Harmol, Norharmane and Stachydrine@4.1 (r=0.80, 0.90 and -0.80, respectively), while Harmane@15.5 was correlated with Tropinone and Valerianine@8.9 (r= 0.90 and 0.71, respectively). Harmol was also correlated with Stachydrine@4.1 (r=-0.75). Moreover, we found significant correlations between Norharmane and Harmol (r=0.79), and Norharmane and 8-ethylnorlobelol (r=0.69).

### *3.3. Discrimination of botanical family based on the alk profile*

To assess whether plants belonging to the same taxonomic group were characterized by a peculiar alk distribution and consequently whether the alk profiles could allow prediction of the family of origin, we proposed a PLS-DA model. To assure suitable representability for the botanical family, we based the model on the alk profile of the three most abundant families - Poaceae, Asteraceae and Lamiaceae - respectively comprising nine, seven and seven individual herb species (X-matrix). We then defined three dummy variables (Y-matrix), one for each family. After having retained the 11 alks with *P* values for Beta coefficients $\leq 0.10$, we obtained a three-factor PLS-DA discriminant model. The relationship between the two sets of variables is represented in Figure 1. The families are located within the outer ring of the plot, well separated in three different quadrants, indicating

that the alk profile of herbs may be a viable tool for grouping them according to botanical family. The coefficients of prediction ($R^2_C$) were 0.87, 0.48 and 0.53 respectively for the Poaceae, Asteraceae and Lamiaceae classes, with total explained variance of 62.8% per family.

We give the full cross-validation results of the PLS-DA model in Table 6, together with evaluation of prediction performance. Measurement of the quality of each binary classification model comes from the confusion matrix, which records correctly and incorrectly recognized plants for each botanical family. We calculated the quality of the overall model as a multiclass overall classification (Sokolova and Lapalme, 2009). The average accuracy of the discriminant multiclass model was 91%. Asteraceae and Lamiaceae discriminant models achieved the same accuracy percentage (87%), misclassifying two out of seven species of the selected family (sensitivity = 0.71). The false negative herbs were *Carlina acaulis* and *Crepis aurea* for Asteraceae and *Acinos alpinus* and *Betonica alopecurs* for Lamiaceae, which did not attain the predictive Y-value of 0.5 required to be validated as belonging to the true family. Moreover, both models were affected by one false positive family identification of individual plants (specificity = 0.94). Indeed, *Betonica alopecurs* was erroneously validated as an Asteraceae species while *Crepis aurea* was recognized as a Lamiaceae. By contrast, the model for binary classification of the Poaceae species provided the maximum expected performance, with 100% accuracy. Lobinaline and two Inds, Harmalol and Harmane@15.9, were the most powerful alks for discriminating Poaceae from the components of the other two families (Figure 1). The inclusion of less numerous families worsened the overall accuracy of the multiclass model. However, Poaceae still remained the most reliable family, testifying to the consistency of the alk profile for grasses.

### *3.4. Alk profiling of pastures*

#### *3.4.1. Targeted profiles*

We report the targeted alks detected in selected herbage samples in Table 7, showing the minimum, median and maximum of the four alks above the LOD in at least one sample. Lycopsamine (Pyz) was the most frequently found alk, quantified in 46% of samples collected in PO pasture, with a concentration between the LOD and 8.5 µg/kg, and in 38% of samples of SE pasture, with a concentration between the LOD and 3.4 µg/kg. We quantified Gramine (Ind) in 29% of PO samples, with a concentration between the LOD and 86.1 µg/kg, and in 33% of SE samples, with a concentration between the LOD and 64.7 µg/kg. Veratramine (Str) was only found in herbs collected in PO pasture, with a concentration between the LOD and 357 µg/kg. As shown in Table

4, of the species considered, this alk was found only in *Phleum r.*, a Poaceae detected in all botanical assessments of PO pasture, where its coverage surface averaged 14.6% (Table 1). We found Veratridine (Str) in one of the samples collected in SE at 15.6 µg/kg, probably carried by a sporadic unidentified herb. The lack of Erucifoline, Heliotrine and Seniciphylline detection in the hand-plucked samples is probably a consequence of the selective behavior of cows, avoiding plants (i.e. *Hypericum m.* and *Potentilla c.*, Table 4) containing unpalatable substances.

*3.4.2. Untargeted profiles*

As regards the occurrence of untargeted alks in the selected samples (Table 8), we detected Ind, Ppr and Pyl in all the samples from both pastures, while Bzp alks were identified only in one sample (4%) per pasture. As far as the other alk groups are concerned, PO turned out to be numerically richer than SE in Pyrs (88% and 83% respectively), Trns (83% and 79%), Trps (83% and 62%), Strs (58% and 38%), and Acds (8% and 4%). Conversely, SE was found to be richer than PO in Qnzs (79% and 50% respectively), Pyzs (58% and 33%), Iqns (50% and 21%), Qnls (37% and 17%), and PrAs (17% and none found).

### *3.5. Discrimination of grazed pastures from alk variability*

*3.5.1. Individual alks variability between pasture*

Evaluating the alk content of the two different pastures, Tukey's HSD test highlighted the following significant differences (data not shown): PO was significantly richer ($P<0.05$) in Harmane@15.5 and Lobinaline, while conversely 3-Acetyltropine and Aconine@17.2 were more abundant in SE.

Considering only alks detected in at least 50% of the 48 herbage mixtures, Pearson's test ($p<0.001$) highlighted significant correlations between Stachidrine@3.5 and Stachidrine@4.1 ($r=0.6$) in terms of PO alk content, while in SE it showed significant correlations of Norharmane with Myrtine/Epimyrtine ($r=0.5$), Harmane@15.5 ($r=0.7$) and Lobinaline ($r=0.6$), and Stachidrine@3.1 with Myrtine/Epimyrtine ($r=0.7$) and Stachidrine@4.1 ($r=0.6$).

*3.5.2. Discrimination of pastures from alk profile*

We considered a PLS-DA model to verify the viability of the alk profile, to discriminate between the diet selected by dairy cows grazing in two different pastures. The details of the model are summarized in Figure 2, while the cross-validation results of the model are shown in Figure 3.

After having considered all the available alks, we built the proposed bi-factorial model considering only those with a P-value of Beta coefficient of <0.2. Those with the highest discriminant power, significant according to the Merten's uncertainty test, were Lobinaline, Harmane@15.5, Pseudojervine, Isolobinine/Lobinine@20.0 and Stachydrine@4.1, closer to Poin alpinae pasture, and Parfumidine@18.9, Aconine@17.2 and 3-Acetyltropine, closer to Seslerion caeruleae pasture. The performance of the model was satisfying, with total accuracy of 85%. Indeed, four out of 24 samples hand-plucked in PO pasture and three out of 24 samples selected in SE pasture were incorrectly assigned to the other pasture. The hand-plucked sample distribution was not related to the cow or day of collection, as proved by the score scattering highlighted in Figure 3.

## 4. Discussion

### 4.1. *Discrimination of pastures*

We recognized two categories of powerful discriminant alks. The first comprises alks detected in a few hand-plucked samples mainly collected in only one pasture. These compounds, which mostly occurred in a limited number of species or were strongly selected only in one pasture, may be regarded as qualitative markers of pasture type. Isolobinine/Lobinine@20.0 and Pseudojervine for PO, and Parfumidine@18.9 for SE belong to this category. Isolobinine/Lobinine@20.0 was particularly rich in Achillea m. (Asteraceae), a plant with 100% frequency of occurrence (F) in PO pasture (compared to 22% in SE; Table 1). As discussed above, we detected Pseudojervine with a high peak signal in Veratrum a. (Liliaceae) and Silene a. (Cariophyllaceae), and a low signal in a few Poaceae species. Phleum r., one of these grasses, made a major contribution to PO pasture cover (14.6%). Veratrum a. is a species with a high plant mass and very frequent in PO (F = 80%), but due to its poor palatability it is almost completely avoided by dairy cows (Table 2). Clearly, its low intake (0.48% DM) was enough to cause the presence of Pseudojervine in 6 hand-plucked samples out of 24 collected in PO pasture. On the other hand, Silene a. was present exclusively in SE (F = 56%) and had a reduced plant mass. Its contribution to the cows' diet did not appear in the analysis of selected herbage. We only found Parfumidine@18.9 in three species (P. media, Rhinanthus g. and P. atrata). This alk characterized SE pasture. Indeed, Plantaginaceae contributed to 8.95% DM of the cows' diet in SE and Rhinanthus g. is regarded as an inedible species.

The second discriminant category comprises alks that may be seen as quantitative markers, i.e. alks widely detected in hand-plucked samples from both pastures, but with a signal of different intensity.

Lobinaline, Harmane@15.5, Stachydrine@4.1, Aconine@17.2 and 3-Acetyltropine belong to this category. They occurred in many species, and with the exception of Harmane@15.9 and Aconine@17.2, also in many selected herbage samples (between 20 and 24 for each pasture; Table 8). Most of them have previously been mentioned as being characterized by a significant difference in peak signal in the two pastures. Lobinaline, found in 47 out of 62 studied herbs, was abundant in Poaceae, Poligonaceae, Ranunculaceae, Rosaceae, Rubiaceae and Scrofulariaceae. This result may be related to the widespread presence of species such as Phleum r., Poa a., Ranunculus a. and Alchemilla ssp in PO. We found Stachidrine@4.1 in all the species analyzed, mainly in Poaceae, Cyperaceae, Fabaceae, Rosaceae and Asteraceae. 3-Acetyltropine was particularly present in Cyperaceae, Scrofulariaceae and Plantaginaceae, more frequently in SE.

### *4.2. Herbage selectivity of dairy cows in alpine pastures*

Temperate cattle are bulk and roughage feeders, included in the fresh grass-eater class. They feed selectively, depending on the range of available nutritive classes (Van Soest, 1994). Therefore, a different herbage intake composition was expected in the two experimental pastures, due to the different botanical composition.

Table 2 shows the results of botanical analysis of bulked samples, of both available and selected herbage, from the two experimental pasture types. As expected, plant species referable to the *Poales* order, comprising grass families, were by far the most abundant in the available and selected herbage masses (% DM; dry matter). Data on “other families” confirmed the complexity of natural Alpine pastures and the effect on cattle feeding behavior. Particularly in the PO pasture, families not belonging to the top ten represented 2.36% of available herbage but reached almost 20% of the selected herbage mass, with a selectivity index (ratio between family percentages in selected and available mass) equal to 8.4. Apart from this group, the most selected families were Fabaceae (selectivity index = 4.2), Rosaceae (3.6) and Cariophyllaceae (3.4) in PO pasture, and Apiaceae (3.0), Plantaginaceae (2.8) and again Fabaceae (1.6) in SE pasture. Other families were instead avoided or under-grazed by dairy cows, particularly Liliaceae (selectivity index = 0.1 in PO pasture), Asteraceae (0.2 and 0.4 in Se and PO, respectively) and Hypericaceae (0.3 and 0.8 in Se and PO, respectively).

The Liliaceae data is almost exclusively related to *Veratrum album*. Although it is a poisonous plant, during the experiment we noted that several cows ate the apical parts of young plants. Some authors report that ingestion of *Veratrum* spp. can cause deformation in fetuses, and in the most

severe cases, the death of cows (Binns et al., 1972; Mulligan and Munro, 1987). Veratrum alks occur as glycosides, aglycones or in the form of esters with various acids. The most important are alks of the Jervanine and Veratranine type with a Steroidian skeleton and alks with Cevanine skeleton (Grancai and Grancaiova, 1994; Gaillard and Gilbert, 2001). Of these untargeted alks, several have been found with a noticeable signal in Alpine herbs. Cevadine@17.2 and Cevadine@18.1 occurred in many species belonging to different botanical families (Table 5), but were highly signaled only in *Veratrum a.* (peak signal 60 times higher than in the second species). Their toxicity is probably linked to a fairly high threshold, perhaps exceeded only by *Veratrum a.* among the examined species. Similar considerations may be extended to Pseudojervine, another Str alk reported among the toxic alks (Schep et al., 2006). We identified it in *Silene alpestris,* with a peak signal comparable to that of *Veratrum a.*, approximately 40 times higher than three Poaceae species. However, the lack of a standard did not make it possible to establish the real concentration of these untargeted alks. Nevertheless, their tentative identification does not exclude possible isomers, perhaps endowed with different biological activity. Integerrimine is a Pyzs also present in other non-edible species such as *Cruciata laevipes*, *Galium* spp., and *Rhinanthus glacialis*. In a study on mice, Gimmler-Luz et al. (1990) showed that Integerrimine causes chromosomal aberrations in bone marrow cells.

Asteraceae was a well-represented family, covering 3.5-4% of the surface area of the two pastures, mainly with *Crepis aurea* and *Achillea clavanae* respectively in PO and SE, which also shared a relatively wide occurrence of *Carlina acaulis* (Table 1). This family group includes various species, from edible plants (e.g. *Lactuca Sativa* and *Taraxacum officinale*) to plants toxic due to the Pyz content (*Senecio spp*.). As regards Asteraceae herbs present in PO and SE, these are edible and/or medicinal plants and no information is available in the literature about toxic alkaloids contained in them. Despite this, we found the signal of the Pyz alk Seneciphylline N-Oxide in *Hieracium pilosum* and determined the presence of Lasiocarpine N-xide in *Achillea millefolium, Carlina acaulis* and *Leontodon hispidus*. Their analysed alk profile, as already discussed, was quite complex, with significant differences between species. Indicain, Lelobanidine I/Lelobanidine II@17.4 and Valerianine @8.9 and @12.9 allowed the discrimination between Poaceae and Lamiaceae (Fgure 1).

Hypericaceae were represented almost exclusively by one species: *Hypericum maculatum*. This plant contained significant quantities of Seneciphylline N oxide and Erucifoline, two compounds

also found in toxic plants such as *Senecio jacobea* (Hartmann and Toppel, 1987; Macel et al., 2004).

Ranunculaceae (*Ranunculus acris* and *Ranunculus repens*), usually considered to be toxic and avoided (selectivity index= 0.6) in both pasture types, did not show significant amounts of any toxic alkaloid among those identified.

## 5. Conclusions

Alpine herbs synthesize a wide and complex range of alkaloids, most of which have been identified without evaluating the actual concentration, because of the lack of standards. Within these analytical limits, the study provides evidence that alkaloid profiles represent a potential tool for distinguishing Alpine plants belonging to some family groups. We obtained the most encouraging results for the Poaceae species. Moreover, the alkaloid profiles allowed us to discriminate the herbage selected by dairy cows in two northern Italian natural Alpine pastures, acting as both qualitative and quantitative markers. Understanding the metabolic fate of these compounds and assess them and their metabolites in milk and derivatives, this evidence permits us to suggest the possible application of alk profiles to dairy product traceability, as potential compositional markers of origin.

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**Table 1**
Individual plant species identified in the experimental Alpine pastures (Poion alpinae, PO; Seslerion caeruleae, SE), their frequency of occurrence (F, %) in flora assessment replicates and their relative average coverage (AC, %) in pasture.

| Family | Species | PO | | SE | | Family | Species | PO | | SE | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | F | AC* | F | AC | | | F | AC | F | AC |
| Apiaceae | *Carum carvi* | 80 | 0.6 | | | Lamiaceae | *Lamium album* | | | | |
| Apiaceae | *Heracleum sphondylium* | 40 | 0.8 | | | Lamiaceae | *Prunella grandiflora* | | | 78 | 8.5 |
| Apiaceae | *Laserpitium peucedanoides* | | | 56 | 0.8 | Lamiaceae | *Prunella vulgaris* | | | | |
| Asteraceae | *Achillea clavennae* | 20 | 0.5 | 100 | 1.1 | Lamiaceae | *Thymus polytrichus* | 100 | 2.2 | 100 | 1.0 |
| Asteraceae | *Achillea millefolium* | 100 | 0.8 | 22 | 0.4 | Lamiaceae | *Urtica dioica* | 40 | 0.3 | | |
| Asteraceae | *Carlina acaulis* | 20 | 1.0 | 78 | 0.8 | Liliaceae | *Veratrum album* | 80 | 2.9 | 56 | 0.5 |
| Asteraceae | *Centaurea jacea* | | | 56 | 0.7 | Plantaginaceae | *Plantago atrata* | 100 | 3.8 | 78 | 0.6 |
| Asteraceae | *Crepis aurea* | 60 | 1.2 | 40 | 0.4 | Plantaginaceae | *Plantago major* | | | | |
| Asteraceae | *Hieracium pilosum* | | | 100 | 0.5 | Plantaginaceae | *Plantago media* | 20 | 0.5 | | |
| Asteraceae | *Leontodon hispidus* | 40 | 2.3 | | | Poaceae | *Agrostis capillaris* | | | 33 | 1.9 |
| Brassicaceae | *Biscutella laevigata* | 80 | 0.4 | 89 | 0.5 | Poaceae | *Briza media* | | | 44 | 3.5 |
| Brassicaceae | *Capsella bursa pastoris* | | | | | Poaceae | *Dactylis glomerata* | 20 | 5.0 | | |
| Campanulaceae | *Campanula scheuchzeri* | | | 44 | 0.4 | Poaceae | *Deschampsia caespitosa* | 20 | 0.5 | 11 | 0.4 |
| Caprifoliaceae | *Valeriana collina* | | | | | Poaceae | *Festuca calva* | 20 | 3.0 | 11 | 0.4 |
| Cariophyllaceae | *Cerastium arvense* | 100 | 1 | 67 | 0.4 | Poaceae | *Festuca rubra* | 100 | 9.2 | 89 | 9.1 |
| Cariophyllaceae | *Dianthus sylvestris* | 20 | 0.5 | 89 | 0.5 | Poaceae | *Phleum rhaeticum* | 100 | 14.6 | 33 | 1.5 |
| Cariophyllaceae | *Silene alpestris* | | | 56 | 0.4 | Poaceae | *Poa alpina* | 100 | 15.4 | 89 | 6.5 |
| Cariophyllaceae | *Silene nutans* | 60 | 0.5 | 11 | 0.4 | Poaceae | *Sesleria caerulea* | 20 | 0.5 | 100 | 25.9 |
| Cyperaceae | *Carex atrata* | | | 11 | 0.4 | Poligonaceae | *Polygonum viviparum* | 40 | 0.5 | 100 | 1.8 |
| Cyperaceae | *Carex sempervirens* | 20 | 0.5 | 89 | 8.7 | Poligonaceae | *Rumex acetosa* | 40 | 2.5 | 33 | 0.4 |
| Dipsaceae | *Knautia longifolia* | 20 | 1.0 | 33 | 0.4 | Poligonaceae | *Rumex alpinus* | | | | |
| Dipsaceae | *Scabiosa lucida* | 40 | 0.8 | 67 | 0.5 | Ranunculaceae | *Ranunculus acris* | 100 | 5.8 | 44 | 0.4 |
| Fabaceae | *Anthyllis vulneraria* | 20 | 0.5 | 100 | 1.1 | Ranunculaceae | *Ranunculus repens* | | | | |
| Fabaceae | *Lotus corniculatus* | 40 | 0.5 | | | Rosaceae | *Alchemilla gr. vulgaris* | 100 | 4.8 | 56 | 0.5 |
| Fabaceae | *Medicago lupulina* | | | | | Rosaceae | *Potentilla crantzii* | 20 | 0.5 | 67 | 2.5 |
| Fabaceae | *Trifolium pratense* | 100 | 3.2 | 33 | 2.9 | Rosaceae | *Potentilla erecta* | | | 22 | 1.1 |
| Fabaceae | *Trifolium repens* | 100 | 3.8 | 67 | 1.1 | Rubiaceae | *Cruciata laevipes* | | | | |
| Hypericaceae | *Hypericum maculatum* | 90 | 2.1 | 78 | 0.4 | Rubiaceae | *Galium album* | 20 | 0.5 | | |
| Juncaceae | *Luzula multiflora* | 80 | 0.5 | 78 | 0.4 | Rubiaceae | *Galium anisophyllum* | 100 | 0.5 | 89 | 0.4 |
| Lamiaceae | *Acinos alpinus* | 40 | 0.8 | 78 | 1.6 | Scrophulariaceae | *Rhinanthus glacialis* | 20 | 0.5 | 100 | 5.4 |
| Lamiaceae | *Betonica alopecurus* | 40 | 1.8 | 89 | 1.7 | Scrophulariaceae | *Veronica chamaedrys* | 100 | 1.6 | 22 | 0.4 |

*Species without numerical values were sporadic.

**Table 2**

Contribute (expressed as a dry matter percentage) of the main botanical families to the available and the selected herbage masses of 2 experimental alpine pastures (Poion alpinae, PO; Seslerion caeruleae, SE).

| PO | | | SE | | |
|---|---|---|---|---|---|
| **Botanical families** | **Available** | **Selected** | **Botanical families** | **Available** | **Selected** |
| *Poaceae** | 66.5 | 41.5 | *Poaceae** | 61.8 | 57.5 |
| *Ranuncolaceae* | 7.37 | 4.29 | *Asteraceae* | 8.47 | 2.10 |
| *Liliaceae* | 5.11 | 0.48 | *Fabaceae* | 5.36 | 8.63 |
| *Asteraceae* | 3.53 | 1.36 | *Rosaceae* | 3.33 | 3.66 |
| *Poligonaceae* | 3.35 | 1.27 | *Plantaginaceae* | 3.17 | 8.95 |
| *Fabaceae* | 2.82 | 11.8 | *Lamiaceae* | 2.93 | 2.45 |
| *Hypericaceae* | 2.47 | 2.00 | *Hypericaceae* | 2.85 | 0.92 |
| *Rosaceae* | 2.38 | 8.45 | *Scrophulariaceae* | 2.22 | 2.08 |
| *Scrophulariaceae* | 2.17 | 2.37 | *Poligonaceae* | 1.66 | 1.54 |
| *Cariophyllaceae* | 1.96 | 6.59 | *Ranuncolaceae* | 1.64 | 0.92 |
| Other families | 2.36 | 19.9 | *Apiaceae* | 1.59 | 4.83 |
| | | | Other families | 4.98 | 6.42 |

* *Poaceae* includes other families belonging to the *Poales* order, such as *Cyperaceae* and *Juncaeae*

**Table 3**

Validation parameters for the 41 alk analytical standards.

| Compound | RT (min) | LOD (µg/kg) | LOQ (µg/kg) | $R^2$ | Compound | RT (min) | LOD (µg/kg) | LOQ (µg/kg) | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|
| Nicotine | 4.7 | 24 | 80 | 0.990 | Senkirkin | 14.5 | 1.1 | 3.6 | 0.998 |
| Monocrotaline | 5.3 | 0.1 | 0.3 | 0.998 | Caffeine | 15.1 | 1.5 | 4.9 | 0.998 |
| Lycopsamine | 7.0 | 0.2 | 0.6 | 0.998 | Lasiocarpine | 15.8 | 0.2 | 0.7 | 0.998 |
| Coniine | 7.6 | 0.3 | 1.1 | 0.998 | Striknine | 16.3 | 0.9 | 3.0 | 0.993 |
| Erucifoline | 8.4 | 0.1 | 0.4 | 0.998 | Harmaline | 16.4 | 5.6 | 19 | 0.996 |
| Senecionine N-oxide | 9.0 | 0.1 | 0.3 | 0.997 | Sipeimine | 17.6 | 1.9 | 6.3 | 0.998 |
| Gramine | 9.3 | 0.1 | 0.3 | 0.998 | Quinine | 18.3 | 3.1 | 10 | 0.996 |
| Theobromine/ Theophylline | 9.6 | 4.5 | 15 | 0.999 | Quinidine | 18.8 | 4.6 | 16 | 0.995 |
| Scopolamine | 10.3 | 0.3 | 1.0 | 0.996 | Veratramine | 19.7 | 0.3 | 0.9 | 0.995 |
| Jacobine N-oxide | 10.5 | 0.5 | 1.5 | 0.996 | alpha-Solasonine | 19.8 | 0.2 | 0.6 | 1.000 |
| Erucifoline N-oxide | 10.7 | 0.6 | 1.9 | 0.996 | Jervine | 19.9 | 0.4 | 1.4 | 0.995 |
| Heliotrine | 10.7 | 0.1 | 0.2 | 0.998 | alpha-Solamargine | 20.0 | 4.4 | 15 | 0.999 |
| Retrorsine | 10.7 | 0.4 | 1.3 | 0.996 | Protoveratrine A | 20.6 | 17 | 57 | 0.996 |
| Seneciphylline | 11.4 | 0.2 | 0.9 | 0.996 | Veratridine | 21.7 | 0.2 | 0.7 | 0.998 |
| Retrorsine N-oxide | 11.9 | 0.7 | 2.3 | 0.996 | alpha-Solanine | 21.8 | 2.0 | 6.8 | 0.998 |
| Senecionine/ Senecivernine | 12.6 | 0.2 | 0.7 | 0.993 | Solasodine | 23.6 | 1.0 | 3.5 | 0.993 |
| Hyoscyamine/ Atropine | 12.7 | 0.2 | 0.8 | 0.994 | Aconitine | 23.7 | 2.0 | 6.6 | 0.999 |
| Echimidine | 13.6 | 0.1 | 0.4 | 0.999 | Tomatidine/ Tomatine | 24.5 | 10 | 32 | 0.991 |
| Jacobine | 14.1 | 0.4 | 1.5 | 0.997 | | | | | |

RT= retention time; LOD= limit of detection; LOQ= limit of quantification.

**Table 4**

Targeted alk content (µg/kg) in Alpine herbs.

| Alkaloid | *Betonica alopecurus* | *Capsella-bursa pastoris* | *Hypericum maculatum* | *Phleum rhaeticum* | *Potentilla crantzii* | *Veronica chamaedrys* |
|---|---|---|---|---|---|---|
| Erucifoline | <0.1 | <0.1 | 9 | <0.1 | <0.1 | <0.1 |
| Gramine | 108 | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Heliotrine | <0.1 | <0.1 | 11 | <0.1 | <0.1 | <0.1 |
| Lycopsamine | <0.2 | 6 | <0.2 | <0.2 | <0.2 | 6 |
| Seneciphylline | <0.2 | <0.2 | <0.2 | <0.2 | 112 | <0.2 |
| Veratramine | <0.3 | <0.3 | <0.3 | 10 | <0.3 | <0.3 |

**Table 5**

Summary of the chemical groups of untargeted alks identified in Alpine plants, sorted by botanical family.

| | Acridone (N=3)[1] | Benzophenantridine (3) | Indole (15) | Isoquinoline (19) | Piperidine (27) | Protoalkaloid (2) | Pyridine (3) | Pyrrolidine (4) | Pyrrolizidine (7) | Quinoline (12) | Quinolizidine (3) | Steroid (3) | Terpenoid (10) | Tropane (4) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Apiaceae | | | | | | | | | | | | | | |
| *Carum carvi* | 1 | - | 4 | 1 | 5 | - | 1 | 3 | 2 | 1 | 1 | - | 4 | 1 |
| *Heracleum sphondylium* | 1 | - | 7 | - | 2 | - | 1 | 2 | 3 | 1 | 1 | - | 3 | - |
| *Laserpitium peucedanoides* | 1 | - | 5 | - | 9 | - | 2 | 2 | 1 | 1 | 1 | 2 | 3 | 1 |
| Asteraceae | | | | | | | | | | | | | | |
| *Achillea clavanae* | - | - | 7 | - | 4 | - | 1 | 2 | - | 2 | 1 | - | 6 | 1 |
| *Achillea millefolium* | - | - | 8 | - | 7 | - | 2 | 2 | 2 | 2 | 1 | 1 | 4 | 2 |
| *Carlina acaulis* | - | - | 7 | 3 | 8 | - | 1 | 2 | 2 | 2 | 1 | 1 | 7 | - |
| *Centaurea jacea* | - | - | 4 | - | 2 | - | 1 | 2 | - | 1 | 1 | 1 | 5 | - |
| *Crepis aurea* | - | - | 6 | - | 8 | - | - | 2 | - | 1 | 1 | - | 5 | 1 |
| *Hieracium pilosum* | 1 | - | 7 | 1 | 5 | - | 1 | 2 | 2 | 1 | 1 | - | 5 | 1 |
| *Leontodon hispidus* | - | 1 | 7 | - | 7 | - | 1 | 2 | 1 | 3 | 1 | - | 5 | 1 |
| Brassicaceae | | | | | | | | | | | | | | |
| *Biscutella laevigata* | - | - | 7 | - | 4 | - | 1 | 2 | 2 | 1 | 1 | 1 | 4 | 1 |
| *Capsella bursa-pastoris* | 1 | - | 9 | 1 | 4 | - | - | . | 3 | 2 | 1 | 2 | 8 | 1 |
| Campanulaceae | | | | | | | | | | | | | | |
| *Campanula scheuchzeri* | - | - | 7 | 1 | 2 | - | 1 | 2 | 3 | - | 1 | 2 | 7 | - |
| Caprifolliaceae | | | | | | | | | | | | | | |
| *Valeriana collina* | - | - | 5 | - | 2 | - | 2 | 2 | - | - | - | - | 6 | - |
| Cariophyllaceae | | | | | | | | | | | | | | |
| *Cerastium arvense* | 1 | - | 9 | - | 4 | - | 1 | 2 | 1 | 2 | - | - | 7 | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Dianthus sylvestris* | - | - | 8 | - | 3 | - | 1 | 2 | 2 | 2 | - | 2 | 5 | - |
| *Silene alpestris* | - | - | 8 | - | 3 | - | 2 | 2 | 1 | 2 | 1 | 2 | 5 | 1 |
| *Silene nutans* | - | - | 9 | - | 3 | - | 1 | 2 | 2 | 2 | - | 2 | 4 | 3 |
| Cyperaceae | | | | | | | | | | | | | | |
| *Carex atrata* | - | - | 9 | 1 | 7 | - | 1 | 2 | 1 | 2 | - | - | 7 | - |
| *Carex sempervirens* | - | - | 8 | - | 7 | - | 1 | 2 | 3 | 2 | - | 1 | 5 | 1 |
| Dipsaceae | | | | | | | | | | | | | | |
| *Knautia longifolia* | 1 | 1 | 6 | 3 | 4 | - | 2 | 2 | 1 | 2 | 1 | - | 4 | 1 |
| *Scabiosa lucida* | 1 | 1 | 3 | 2 | 2 | - | 1 | 2 | 1 | 2 | - | - | 3 | 1 |
| Fabaceae | | | | | | | | | | | | | | |
| *Anthyllis vulneraria* | - | - | 5 | 2 | 3 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 2 | 1 |
| *Lotus corniculatus* | - | - | 8 | 1 | 5 | - | 2 | 2 | 3 | 3 | 1 | - | 7 | - |
| *Medicago lupulina* | - | - | 8 | - | 2 | - | - | 2 | 3 | 1 | 1 | 2 | 3 | 1 |
| *Trifolium pratense* | - | 1 | 8 | - | 3 | - | 1 | 3 | - | 3 | 1 | - | 5 | 2 |
| *Trifolium repens* | - | - | 7 | - | 4 | - | 2 | 3 | 2 | 2 | 1 | - | 2 | 1 |
| Hypericaceae | | | | | | | | | | | | | | |
| *Hypericum maculatum* | - | - | 4 | - | 7 | - | - | 2 | 3 | - | 1 | - | 6 | - |
| Juncaceae | | | | | | | | | | | | | | |
| *Luzula multiflora* | - | - | 5 | - | 4 | - | - | 2 | 1 | - | 1 | 2 | 5 | - |
| Lamiaceae | | | | | | | | | | | | | | |
| *Acinos alpinus* | - | - | 6 | - | 4 | - | - | 2 | 1 | - | 1 | - | 4 | - |
| *Betonica alopecurus* | 1 | 1 | 6 | 3 | 6 | - | - | 3 | 1 | 1 | - | - | 5 | 1 |
| *Lamium album* | 1 | - | 8 | - | 2 | - | 1 | 3 | - | 1 | 1 | 1 | 6 | 2 |
| *Prunella grandiflora* | - | - | 7 | 1 | 9 | - | 2 | 2 | 1 | 3 | - | 1 | 4 | - |
| *Prunella vulgaris* | - | - | 8 | - | 6 | - | - | 2 | 2 | 2 | 1 | - | 7 | 1 |
| *Thymus polytrichus* | 1 | - | 2 | - | 3 | - | - | 2 | - | 1 | - | - | 5 | - |
| *Urtica dioica* | - | - | 7 | - | 5 | - | 1 | 2 | 2 | 2 | - | - | 5 | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Liliaceae | | | | | | | | | | | | | | |
| *Veratrum album* | - | - | 6 | - | 3 | - | 2 | 2 | 2 | 1 | 1 | 3 | 5 | - |
| Plantaginaceae | | | | | | | | | | | | | | |
| *Plantago atrata* | - | 1 | 6 | 6 | 3 | - | - | 2 | - | - | 1 | - | 4 | 3 |
| *Plantago major* | - | - | 6 | 2 | 5 | 2 | 3 | 2 | 1 | 2 | 1 | 1 | 5 | 1 |
| *Plantago media* | - | 1 | 7 | 10 | 5 | 2 | 2 | 2 | 2 | 3 | 1 | - | 5 | - |
| Poaceae | | | | | | | | | | | | | | |
| *Agrostis capillaris* | 1 | - | 8 | - | 2 | - | 1 | 2 | - | 1 | 1 | - | 6 | 1 |
| *Briza media* | - | - | 9 | - | 4 | - | 1 | 2 | 2 | 2 | 1 | 1 | 4 | 1 |
| *Dactylis glomerata* | - | - | 7 | - | 2 | - | 1 | 2 | - | 1 | 1 | - | 6 | 1 |
| *Deschampsia caespitosa* | 1 | - | 9 | - | 1 | - | 1 | 3 | - | 2 | 1 | - | 6 | - |
| *Festuca calva* | 1 | - | 7 | - | 7 | - | 1 | 3 | 1 | 2 | - | - | 5 | 1 |
| *Festuca rubra* | - | - | 9 | - | 4 | - | 1 | 2 | - | 5 | 1 | 1 | 6 | 1 |
| *Phleum rhaeticum* | - | - | 6 | - | 4 | - | 1 | 2 | - | 1 | 1 | 1 | 4 | 1 |
| *Poa alpina* | - | - | 7 | 1 | 7 | - | 2 | 2 | 2 | 2 | 1 | 1 | 7 | 1 |
| *Sesleria caerulia* | - | - | 5 | - | 3 | - | 1 | 2 | 2 | 2 | 1 | 1 | 6 | - |
| Poligonaceae | | | | | | | | | | | | | | |
| *Polygonum viviparum* | - | - | 6 | - | 4 | - | 2 | 2 | - | 2 | 1 | 2 | 4 | 1 |
| *Rumex acetosa* | - | - | 6 | - | 3 | - | 1 | 1 | - | - | 1 | 1 | 4 | - |
| *Rumex alpinus* | 1 | - | 6 | - | 2 | - | 1 | 1 | 1 | 1 | 1 | - | 4 | 1 |
| Ranunculaceae | | | | | | | | | | | | | | |
| *Ranunculus acris* | - | - | 5 | - | 4 | - | - | 2 | 1 | - | 1 | - | 3 | - |
| *Ranunculus repens* | - | - | 5 | - | 3 | - | 1 | 2 | 4 | 1 | 1 | - | 5 | 1 |
| Rosaceae | | | | | | | | | | | | | | |
| *Alchemilla gr. vulgaris* | - | - | 5 | - | 3 | - | 1 | 2 | 1 | - | 1 | - | 5 | - |
| *Potentilla crantzii* | - | - | 7 | - | 2 | - | 1 | 2 | 3 | 2 | - | - | 2 | - |
| *Potentilla erecta* | - | - | 7 | - | 6 | - | 1 | 3 | 2 | - | 1 | - | 6 | - |
| Rubiaceae | | | | | | | | | | | | | | |
| *Cruciata laevipes* | - | - | 6 | - | 4 | - | 1 | 2 | 3 | 2 | - | 2 | 8 | 1 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Galium album* | 1 | 1 | 6 | 2 | 2 | - | 1 | 2 | 4 | 1 | 1 | - | 3 | 1 |
| *Galium anisophyllum* | 1 | - | 10 | - | 4 | - | 2 | 2 | 1 | 2 | 1 | - | 4 | 2 |
| Scrofulariaceae | | | | | | | | | | | | | | |
| *Rhinanthus glacialis* | - | - | 4 | 8 | 4 | 2 | - | 2 | 3 | 2 | 1 | - | 5 | - |
| *Veronica chamaedrys* | - | - | 6 | 3 | 2 | 2 | 2 | 2 | 1 | 1 | - | - | 4 | 2 |

[1] number of alks in the chemical group.

**Table 6**

Botanical family classification of herb species according to their alkaloid profile using a Partial Least Squares – Discriminant Analysis multiclass model.

| | Binary discrimination of family | | | Multiclass |
|---|---|---|---|---|
| | Asteraceae | Lamiaceae | Poaceae | overall classification[1] |
| Confusion matrix (no. of validation samples) | | | | |
| true positive ($tp_i$) | 5 | 5 | 9 | |
| false negative ($fn_i$) | 2 | 2 | 0 | |
| true negative ($tn_i$) | 15 | 15 | 14 | |
| false positive ($fp_i$) | 1 | 1 | 0 | |
| Performance evaluation[1] | | | | |
| sensitivity | 0.71 | 0.71 | 1.00 | 0.83 |
| specificity | 0.94 | 0.94 | 1.00 | 0.96 |
| (average) accuracy | 0.87 | 0.87 | 1.00 | 0.91 |

[1] $Sensitivity_i = tp_i/(tp_i+fn_i)$; $Sensitivity_\mu = \Sigma\, tp_i/\Sigma\, (tp_i+fn_i)$;
$Specificity_i = tn_i/(fp_i+tn_i)$; $Specificity_\mu = \Sigma\, tn_i/\Sigma\, (fp_i+tn_i)$;
$Accuracy_i = (tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)$; Average accuracy $= \Sigma\, [(tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)]\ /3xc$.

**Table 7**

Minimum, median and maximum values for alk content (µg/kg) in the herbage selected in experimental pastures (*Poion alpinae*, PO; *Seslerion caeruleae*, SE).

| Alkaloid | PO | | | | SE | | | |
|---|---|---|---|---|---|---|---|---|
| | n. | Min | Med | Max | n. | Min | Med | Max |
| Gramine | 7 | <0.1 | <0.1 | 86 | 8 | <0.1 | <0.1 | 64 |
| Lycopsamine | 11 | <0.2 | <0.2 | 8.5 | 9 | <0.2 | <0.2 | 3.4 |
| Veratramine | 4 | <0.3 | <0.3 | 354 | 0 | <0.3 | <0.3 | <0.3 |
| Veratridine | 0 | <0.2 | <0.2 | <0.2 | 1 | <0.2 | <0.2 | 15 |

Min = minimum; Med = median; Max = maximum.

**Table 8**

Untargeted alk profile of the herbage selected in the experimental pasture (number of hand plucked samples containing each individual alk; *Poion alpinae*, PO; *Seslerion caeruleae*, SE).

| | PO (N=24) | SE (24) | Compound | PO | SE | Compound | PO | SE |
|---|---|---|---|---|---|---|---|---|
| Acridinone | | | 8,10-Diethyllobelidiol@13.1 | 0 | 2 | Lasiocarpine N-oxide | 5 | 7 |
| Arborinine | 2 | 1 | cis-Lobelanidine/trans Lobelanidine@20.2 | 0 | 1 | Seneciphylline N-oxide | 0 | 1 |
| Benzophenantridine | | | cis-Lobelanidine/trans Lobelanidine@20.9 | 1 | 0 | Spartioidine | 4 | 7 |
| Protopin | 1 | 1 | Dihydropiperlonguminine | 5 | 2 | Quinoline | | |
| Indole | | | Isolobinine/Lobinine@20.0 | 6 | 0 | Cinchonanine F@12.0 | 3 | 7 |
| 14,15-Dihydroxygelsenicine/ Gelsemoxonine | 22 | 24 | Lelobanidine I/ Lelobanidine II@16.91 | 1 | 2 | Cinchonanine F@19.1 | 1 | 3 |
| Gelsemicine | 3 | 1 | Lelobanidine I/ Lelobanidine II@17.4 | 1 | 4 | Indicain | 0 | 1 |
| Gelsemine | 6 | 7 | Lobinaline | 24 | 22 | Quinolizidine | | |
| Harmaline | 3 | 0 | Norallosedamine | 11 | 3 | Myrtine/Epimyrtine | 12 | 19 |
| Harmalol | 0 | 1 | Norlelobanidine/8 Methyl 10 phenyllobelidiol@15.5 | 5 | 4 | Steroid | | |
| Harmane@15.5 | 19 | 15 | Norlelobanidine/8 Methyl 10 phenyllobelidiol@16.1 | 3 | 2 | Cevadine@17.2 | 1 | 4 |
| Harmane@15.9 | 3 | 3 | Norlelobanidine/8 Methyl 10 phenyllobelidiol@16.6 | 14 | 10 | Cevadine@18.1 | 13 | 8 |
| Harmine | 2 | 1 | Piperlonguminine | 2 | 2 | Pseudojervine | 6 | 0 |
| Harmol | 13 | 10 | Protoalkaloid | | | Terpenoid | | |
| Norharmane | 16 | 16 | Galegin/Peganin@15.6 | 0 | 4 | Aconine@17.2 | 5 | 8 |
| Isoquinoline | | | Pyridine | | | Aconine@17.5 | 3 | 6 |
| Caryachine@14.2 | 0 | 1 | Ginkgotoxin | 0 | 1 | Actinidine | 1 | 0 |
| Caryachine@15.5 | 4 | 4 | Tropinone | 21 | 20 | Cathinine | 13 | 10 |
| Fumaricine | 1 | 0 | Pyrrolidine | | | Mesaconine@16.3 | 5 | 2 |
| *o*-Methylcaryachine@16.0 | 0 | 1 | 2-Pyrrolidineacetic acid | 24 | 24 | Mesaconine@16.6 | 5 | 7 |
| o-Methylcaryachine@18.3 | 0 | 2 | Betonicine | 2 | 4 | Valerianine@13.0 | 0 | 3 |
| Parfumidine@18.9 | 1 | 9 | Stachydrine@3.5 | 18 | 20 | Valerianine@8.9 | 12 | 9 |
| Parfumidine@19.7 | 0 | 1 | Stachydrine@4.1 | 24 | 24 | Valerine | 0 | 2 |
| Piperidine | | | Pyrrolizidine | | | Tropane | | |
| 8-Ethylnorlobelol | 0 | 3 | beta-Solamargine | 0 | 2 | 3-Acetyltropine | 20 | 21 |
| 8,10-Diethyllobelidiol@12.1 | 3 | 8 | Integerrimine/Perforine | 1 | 2 | Anisodamine | 0 | 1 |

**Figure legends**

**Figure 1**

Correlation loadings for factors 1 and 2 in a three-factor Partial Least Squares – Discriminant Analysis model for the classification of herb species according to the botanical family: *Asteraceae, Lamiaceae* and *Poaceae* (red dots, Y-matrix), based on their alkaloid profile (blue dots, X-matrix). Significant alkaloids (Marten's uncertainty test) for family prediction are ringed.

**Figure 2**

Correlation loadings in a bi-factor Partial Least Squares – Discriminant Analysis model ($R^2_C$ = 0.54) for the classification of cow diet samples (hand plucked) according to the type of grazed pasture (red dots, Y-matrix, PO= *Poion alpinae*; SE= *Seslerion caeruleae*), based on their alkaloid profile (blue dots, X-matrix). Significant alkaloids (Marten's uncertainty test) for pasture type prediction are ringed.

**Figure3**

Score-plot of cow diet samples (hand-plucked) from the Partial Least Squares – Discriminant Analysis model of grazed pasture type (PO= *Poion alpinae*; SE= *Seslerion caeruleae*) classification based on their alkaloid profile. Incorrectly classified samples, both false positive and negative, are ringed. Samples from the same cow are linked by dotted lines (four examples out of eight animals per each pasture).

**Figure 1**

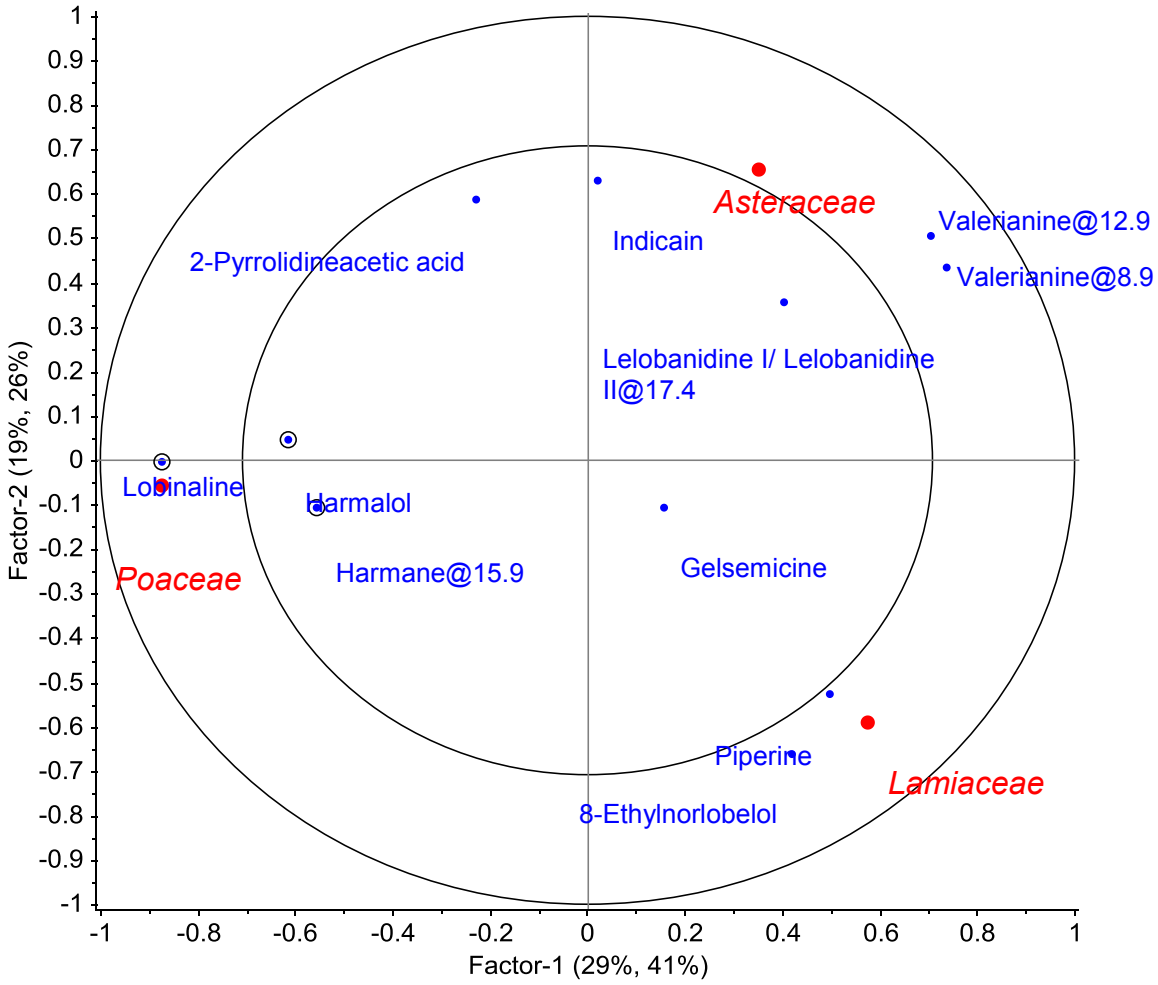

Asteraceae
Valerianine@12.9
Valerianine@8.9
Indicain
2-Pyrrolidineacetic acid
Lelobanidine I/ Lelobanidine II@17.4
Lobinaline
Harmalol
Poaceae
Harmane@15.9
Gelsemicine
Piperine
Lamiaceae
8-Ethylnorlobelol
Factor-2 (19%, 26%)
Factor-1 (29%, 41%)

**Figure 2**

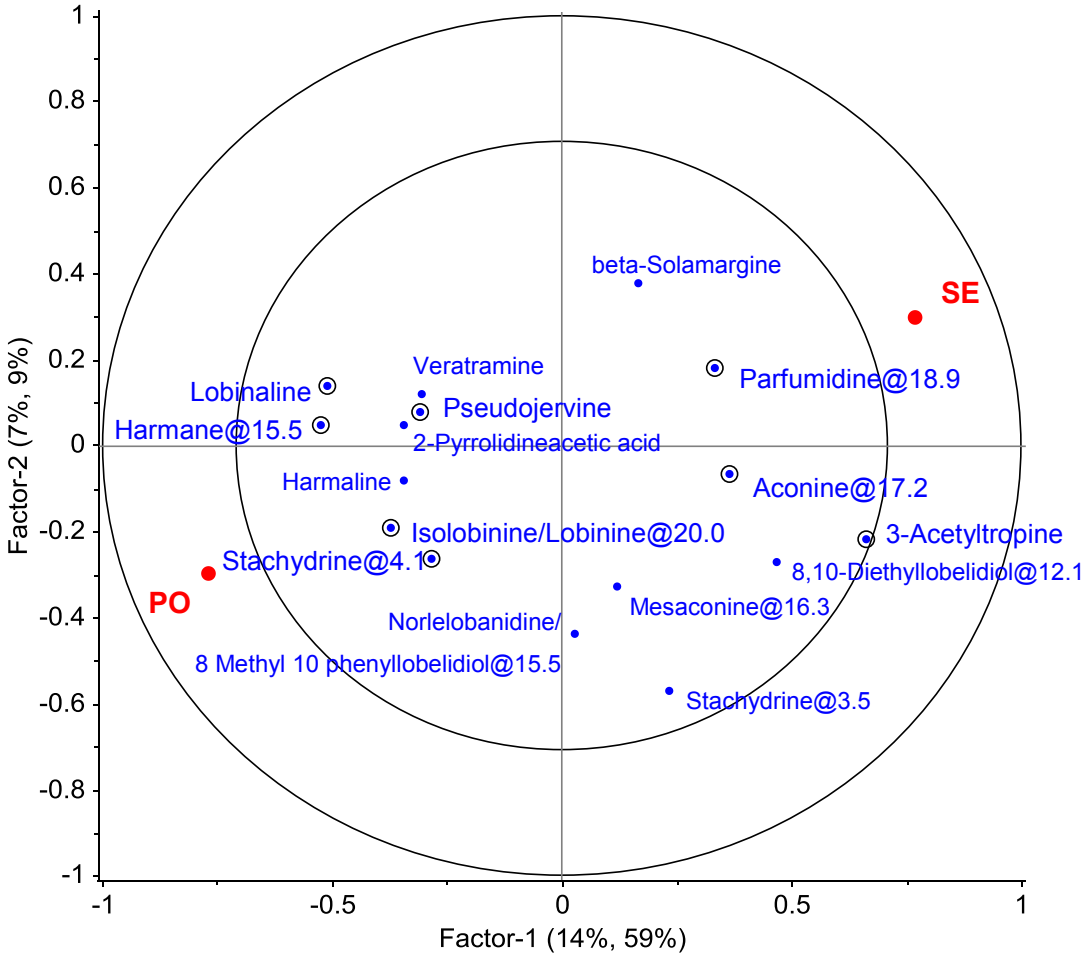
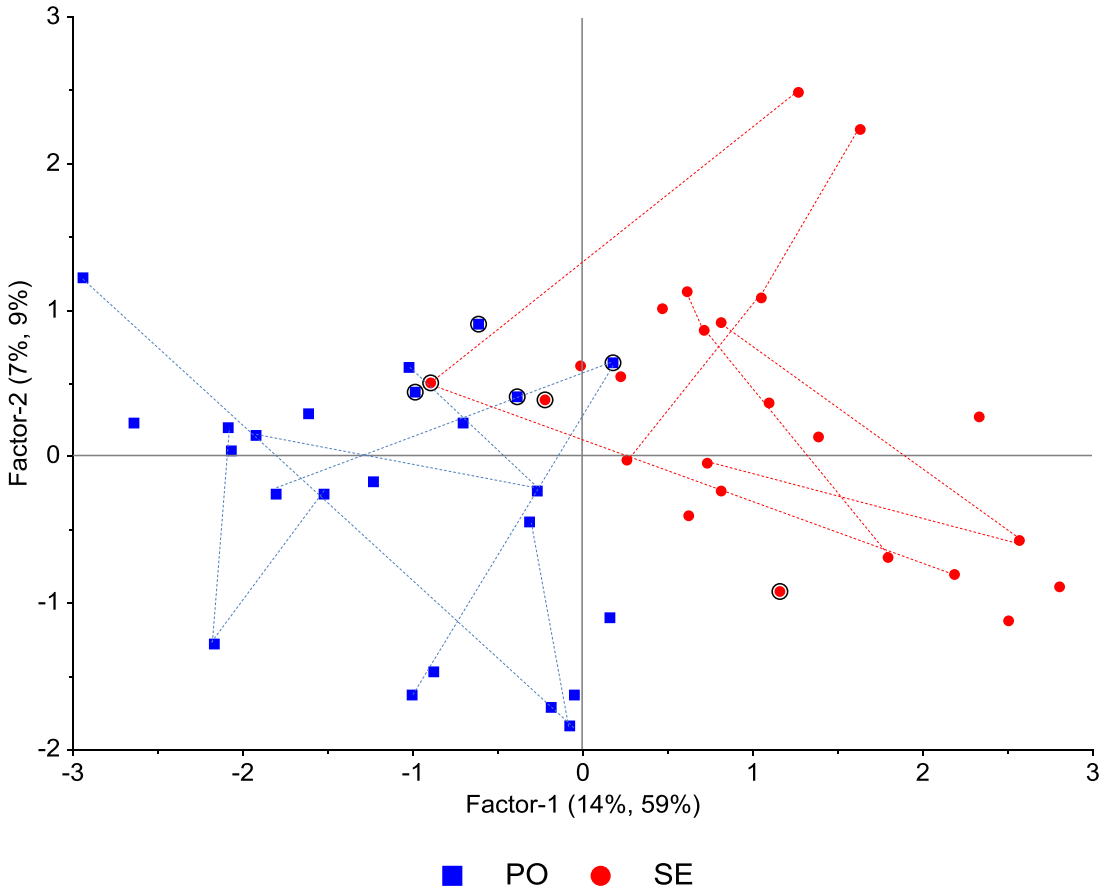

beta-Solamargine
SE
Veratramine
Parfumidine@18.9
Lobinaline
Pseudojervine
Harmane@15.5
2-Pyrrolidineacetic acid
Harmaline
Aconine@17.2
Isolobinine/Lobinine@20.0
3-Acetyltropine
Stachydrine@4.1
8,10-Diethyllobelidiol@12.1
PO
Mesaconine@16.3
Norlelobanidine/
8 Methyl 10 phenyllobelidiol@15.5
Stachydrine@3.5
Factor-2 (7%, 9%)
Factor-1 (14%, 59%)
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-0.5
0.5

**Figure 3**



### 3.3. Alkaloid profiles of alpine milk samples

#### 3.3.1. Alkaloid profiles for dairy product traceability using high resolution mass spectrometry

The work of this chapter is still *in litteris*: Nardin T., Larcher R., Romanzin A., Piasentier E. Alkaloid profiles for dairy product traceability using high resolution mass spectrometry, 2018.

### 3.3.1 Alkaloid profiles for dairy product traceability using high resolution mass spectrometry

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**Abstract**

Supplying consumers with guarantees concerning the origin of dairy products requires reliable methods for milk chain production characterisation. This paper focuses on alkaloids, plant secondary metabolites, and their use as possible markers for this purpose. The wide and complex variety of alkaloids synthesised by alpine plants leads to a predictable high alkaloid intake for grazing cows. The supposed alkaloid transfer from herbs to milk was studied by considering 48 herbage mixes gathered using the hand-plucking technique, applied to mimic the natural pasturing behaviour of 16 lactating Italian Simmental cows grazing for 3 consecutive days on two distinct alpine pastures (*Poin alpinae* and *Seslerion caeruleae* in north-eastern Italy), and the corresponding 48 milk samples, collected daily from the same 16 cows. Moreover, a further 12 mass milk samples obtained from morning and evening milking of the entire herd of 110 cows grazing on the two pastures over the same three days were considered. Both herbage and milk samples were analysed using liquid chromatography coupled with high resolution mass spectrometry to determine the alkaloid profile, quantifying 41 alkaloids (N=16 pyrrolizidine

alkaloids; 11 steroidal, 3 indole, 3 purine, 3 tropane, 2 quinoline, 1 piperidine, 1 pyridine, and 1 terpenoid), and putatively identifying another 116.

The average transfer from herbage to milk was assessed as 0.7% of the overall daily dose of Lycopsamine and 0.6% of Gramine, while for untargeted alkaloids potential transfer was observed, especially for pyrrolidine alkaloids (2-Pyrrolidineacetic acid, Betonicine, Stachydrine@3.5 and Stachydrine@4.1).

Finally, in order to verify if alkaloids could be suggested as possible markers for milk traceability, a Partial Least Squares – Discriminant Analysis model based on the 48 milk sample profiles was developed. The model allowed the correct reclassification of milk samples with an average accuracy of 77.8%. Stachidrine@3.5 and Stachidrine@4.1 were the most predictive alkaloids for discriminating milk collected from Poin alpinae, while 3-Acetyltropine and Valerianine@8.9 alkaloids were the best for discriminating milk collected from Seslerion caeruleae.

Principal Component Analysis of mass milk profiles suggested 2 isomers of Stachydrine and Lycopsamine for *Poin alpinae* pasture and 8-Ethylnorlobelol and 2 isomers of Valerianine for *Seslerion caeruleae* pasture as possible markers.

**Key words:** alkaloid; milk; traceability; Orbitrap™;

## 1. Introduction

In a globalised market, an increasing range of foods are traded around the world and consumers come into contact with a wide variety of products, becoming more and more concerned about the

origin of what they eat. In 2015, 162.8 million tonnes of cow's milk were produced in the EU-28, headed by Germany and France with 33 and 26 million tonnes respectively, while Italy was in seventh place with 11 million tonnes (Eurostat statistics explained), but world milk production is growing steadily (Griffin, 2016). The trend is toward conscientious purchasing, which avoids adulteration and fraud (Bitzios et al., 2017), and traceability is a necessary tool for achieving a number of different objectives, helping to build trust, peace of mind, and increase confidence in the food system.

As regards milk, in Italy the Interministerial Decree of 9 December 2016 made indication of the origin of raw milk material mandatory on the packaging label, as declared by Regulation (EU) No. 1169/2011. In the same way, not only is the country of origin important, but the differentiation of milk from highland or lowland areas is also relevant to quality.

Milk is one of the most important foods, and many scientific studies on milk traceability have been reported. For example, the use of high resolution magic angle spinning (NMR) spectroscopy was applied to characterise cheese such as Parmigiano Reggiano, Emmental and 'Mozzarella di Bufala Campana' (Mazzei and Piccolo, 2012). Milk terpene fraction was used to discriminate French highland or lowland dairy products (Fernandez et al., 2003), while flavonoids and other phenols were suggested for the traceability of dairy products (Hocquette and Gigli, 2005). The combined use of isotopes and elemental profile made it possible to protect PDO cheeses such as Parmigiano Reggiano and Grana Padano from mislabelling (Camin et al., 2015). Laser induced breakdown spectroscopy has been suggested for milk adulteration detection (Moncayo et al, 2017) and lanthanides for milk production chain traceability (Aceto et al., 2017). Enrichment or depletion of different stable isotope ratios of elements such as carbon ($^{13}C/^{12}C$),

nitrogen ($^{15}N/^{14}N$), hydrogen (D/H) and oxygen ($^{18}O/^{16}O$) in milk, dependent on animal feed, were correlated with geographical origin (Camin et al. 2012; De la Fuente and Juarez 2005; Drivelos and Georgiou 2012; Kornexl et al. 1997; Manca et al. 2001, 2006; Scampicchio et al. 2012). Focusing on the comparison of feeding regimes, biomarkers including fatty acids have been proposed for the authentication of feeding practices (Bargo et al. 2006; Butler et al. 2009; Collomb et al. 2008). Recently, by combining different techniques such as isotope ratio mass spectrometry (IRMS), mid- and near-infrared spectroscopy (MIRS and NIRS) and gas chromatography with a flame ionization detector (GC-FID), Scampicchio et al. (2016) classified alpine milk samples from the Tyrol region according to their geographical origin, heat treatment, and season of production.

Animal-derived products, such as milk, meat and eggs, are often investigated for alkaloid (alk) contamination, in particular pyrrolizidine (Pyz) alks, due to the natural consumption of pasture herbs or contaminated animal feed products by livestock. These secondary plant metabolites derive from amino acids (typical and atypical alks) or arise from amination of another type of substrate, which may be acetate, phenylalanine, terpene or steroid (pseudo alks). Herbs may contain different chemical classes of alks, often characteristic for different plant families. There is a high incidence of alk occurrence in Ranunculaceae, Berberidaceae, Menispermaceae, Piperaceae, Cactaceae, Papaveraceae and Gentianaceae, while in Fagaceae, Betulacae, Casuarinaceae and Juglandaceae the occurrence is almost zero. (Li and Willaman, 1968). Alks are not always toxic, indeed some even have beneficial effects, and herbs are commonly used in traditional medicine (Bodirlau et al., 2009; Duarte et al., 2010; Nilson et al., 2014)

A recent EFSA study investigated Pyz alks, in particular 1,2-unsaturated Pyzs, for their genotoxic and carcinogenic effect on humans, in a large number of food samples. The study indicated low Pyz levels in some of the few analysed milk products (EFSA, 2011). The transfer of Pyz alks to animal-derived products has also been reported in the literature (Dickinson et al., 1976; Edgar and Smith, 2000; Mulder et al., 2016). For milk, in particular, the majority of the ingested alks are excreted through urine, and the overall transfer into milk is relatively low (0.1%), but the specific value is decidedly higher if only alks such as jacoline (4–7%) and otonecine-type Pyzs are considered (Hoogenboom et al., 2011).

This work aimed to study alk transfer from herbage to milk and milk alk profiles in depth, in order to verify their possible use as effective markers for origin traceability. The nutritional behaviour of 16 Italian Simmental lactating cows grazing in two different alpine pastures was studied for 3 consecutive days. For each cow, one herbal mix sample mimicking natural plant intake and one milk sample were sampled daily, collecting a total of 48 milk samples and 48 herbage mixes. Furthermore, 12 milk samples were collected from the entire herd of 110 cows grazing on the same pastures, milked twice a day for the 3 consecutive days. Broad targeted and untargeted alk profiles of herbage and milk samples, defined using liquid chromatography coupled with high resolution mass spectrometry, were investigated to establish the possible plant-cow-milk transfer of alks.

## 2. Materials and methods

### *2.1. Herbage and milk sample selection*

A group of 16 lactating cows was selected from a herd of 110 Italian Simmental dairy cows grazing on two different pastures: *Poion alpinae* type (PO), a nutrient-rich pasture located at 1,500 m above sea level, and *Seslerion caeruleae* type (SE) a nutrient-poor one at 1,700 m a.s.l. at a traditional Alpine farm in north-eastern Italy (Malga Montasio; 46°24′45″N, 13°25′53″E). The cows had access to pasture during the day and the night, and received on average 2.5 kg/head per day of feed supplement (mixed concentrate based on maize, barley, beet pulp, soy and wheat). All the cows involved in the trial had already grazed at this alpine farm for at least one season. The herbage selected by each cow was manually sampled, using a hand plucking technique that mimicked animal intake (Langlands, 1974; Berry et al., 2002). The sampling was repeated for three consecutive days for both pastures, gathering 48 herbage mixtures.

The average production data, recorded during two-week preliminary periods, were as follows: milk yield (17.4 ± 2.1 kg/d; average ± SD), stage of lactation (148 ± 59.8 Days In Milk), fat (3.84 ± 0.46%), protein (3.14 ± 0.15%), lactose (4.68 ± 0.14%) and Somatic Cell Count (124,800 ± 117,000 cells/mL). 48 milk samples were collected from the 16 cows, milked once a day for 3 consecutive days. Furthermore, the entire herd was milked twice a day, in the morning and in the evening, for the same three days, collecting 12 milk mass samples.

2.2 Reagents and solutions

LC-MS grade acetonitrile (ACN), LC-MS grade methanol (MeOH), MS grade formic acid (FA, 98%), HPLC grade hexane 97% and LC-MS grade ammonium acetate were purchased from Fluka (St. Louis, MO, USA) and ammonia solution 25% was purchased from Merk Millipore (Darmstadt, Germany). For mass calibration, a standard mix of n-butylamine, caffeine, MRFA

and Ultramark 1621 (Pierce® ESI Positive Ion Calibration Solution, Rockford, IL, USA) were used. Deionized water ($H_2O$) was produced with an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany). Alk standards (Table 1) were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany), except for Strychnine and Harmaline, which were purchased from Sigma (St. Louis, MO, USA).

A mix solution (1 mg $L^{-1}$ of each single alk) was created, starting from individual stock solutions of each alk (100 mg $L^{-1}$), prepared by dissolving the standard powder in an aqueous methanol solution (50:50, *v/v*) and used for calibration in the range 0.02 – 1000 µg $L^{-1}$, injecting 1ul for each level. The mix solution was prepared freshly before each analysis, while stock solutions were stored at -4°C.

2.3 Sample preparation

Alk profile evaluation was carried out on the herbage samples (N=48), the milk samples (48 individual and 12 mass samples) and the mixed concentrate used for feed supplement.

The herbage samples were stored at -18 °C, then an aliquot of 2.5 g of homogenised sample (particle diameter of roughly < 2 mm) was weighed into 50 mL polyethylene falcon tubes (Sartorius AG, Goettingen, Germany) and extracted, adding 20 mL of extraction solution ($H_2O$/MeOH/FA; 49.5:49.5:1 v/v/v). The mixture was sonicated for 10 minutes (LBS1 6Lt, FALC Instruments, Treviglio BG, Italy), subjected to vertical shaking for 12 hours at 20 rpm (Rotoshake 24/16, Gerhardt GmbH & Co. KG, Königswinter, Germany), and once again sonicated for 10 minutes. The methanolic extract was separated with centrifugation (10 minutes at 4100 rpm; IEC CL31 Multispeed, Thermo Scientific, Sunnyvale, CA, USA), filtered with a

0.45 µm cellulose filter cartridge (Sartorius AG, Goettingen, Germany), diluted twice with a $H_2O$/MeOH solution (50:50 v/v) and injected (10 µL).

For milk samples, a homogeneous aliquot of 5 g was added to 2 mL of extraction solution ($H_2O$/MeOH/FA; 40:40:20 v/v/v) in polyethylene 50 mL falcon tubes and sonicated for 15 minutes. Then 1 mL hexane was added, the samples were shaken for 10 minutes and then the two phases were separated after centrifugation (10 minutes at 4100 rpm). The hexane phase was removed and the water layer was filtered with a 0.45 µm PVDF filter cartridge, diluted twice with $H_2O$ and injected (30 µL).

For the mixed concentrate, an aliquot of 2.5 g of homogenised sample (particle diameter of roughly < 2 mm) was directly weighed into polyethylene 50 mL falcon tubes and extracted by adding 20 mL of extraction solution ($H_2O$/MeOH/FA; 49.5:49.5:1 v/v/v). The mixture was sonicated for 10 minutes (LBS1 6Lt, FALC Instruments, Treviglio BG, Italy), subjected to vertical shaking for 12 hours at 20 rpm (Rotoshake 24/16, Gerhardt GmbH & Co. KG, Königswinter, Germany), and once again sonicated for 10 minutes. The methanolic extract was separated with centrifugation (10 minutes at 4100 rpm), filtered with a 0.45 µm cellulose filter cartridge, diluted twice with a $H_2O$/MeOH solution (50:50 v/v) and injected (15 µL).

## 2.4. Method development

Chromeleon™ 7.2 Chromatography Data System software (Thermo Scientific™ Dionex™) software was used for instrument control and for data processing and evaluation.

### 2.4.1 Chromatographic separation

Chromatographic separation was obtained using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA), equipped with a Rheodyne 6-port automated switching valve and a pump module that allowed control of the two independent fluid systems. The method used the same approach proposed by Nardin et al. (2016).

Online clean-up was performed by loading 10 µl of the sample on a SolEx HRP SPE cartridge (2.1 mm × 20 mm, 12-14 µm, ThermoFisher, Sunnyvale, CA, USA) flushed with 4% MeOH adjusted to pH=9 with ammonia (eluent A, flow rate of 1 mL $min^{-1}$) for 2 minutes and with 0.1% FA for another minute to complete matrix interference removal. The Rheodyne valve switched position and the analytical mobile phase, 70% of 0.1% FA with 5mM ammonium acetate (eluent B) and 30% of MeOH/ACN 95:5 v/v with 0.1% FA and 5 mM ammonium acetate (eluent C), flowed through the SPE cartridge at a flow rate of 0.700 mL $min^{-1}$, progressively removing the retained analytes and transferring them to the analytical column (Raptor Biphenyl, 3 mm x 150 mm, 2.7 µm particle size, Restek, Bellefonte, PA, USA). 30% of eluent C and 70% of eluent B flowed isocratically from 2 to 4 minutes, then gradient elution from 30% to 80% (eluent C) was performed between 4 and 25 minutes, from 80% to 100 % (eluent C) from 25 to 26 minutes and held until 28 minutes. Finally, eluent C was linearly decreased to 30% in 0.5 minutes and the analytical column was equilibrated for 2.5 minutes with the initial conditions. Meanwhile, in order to wash the SPE cartridge, MeOH with 1% FA was flushed through at 1 ml $min^{-1}$ for 1 minute before re-equilibration with eluent A. The autosampler was set at a temperature of 5 °C and the column at 35 °C.

2.4.2 Mass Spectrometry

All the chromatograms were recorded in profile mode through a full MS-data dependent MS/MS experiment (full MS–dd MS/MS), employing a Q-Exactive™ hybrid quadrupole-orbitrap mass spectrometer (HQOMS, Thermo Scientific, Bremen, Germany) equipped with heated electrospray ionization (HESI-II) interface. Tune parameters were set, aiming to find an acceptable compromise for optimisation of all alks. The heated capillary temperature was set at 330 °C, while the sheath gas flow rate was set at 30 arbitrary units, auxiliary gas flow rate at 10 arbitrary units, spray voltage at 3.5 kV, and auxiliary gas heater temperature at 300 °C, as in the method proposed by Nardin et al. (2016). In the HESI II source, nitrogen was used as the drying and collision gas in positive ion mode. Accurate mass calibration was performed with the calibration solution, consisting of n-butylamine (*m/z* 74.09643), caffeine (*m/z* 195.08765), MRFA peptide (*m/z* 524.26496) and Ultramark 1621 (characteristic masses: *m/z* 922.01035, 1022.00397, 1121.99758, 1221.99119, 1269.97235, 1321.98481, 1421.97842, 1521.97203, 1621.96564, 1721.95926, 1821.95287, 1921.94648, 2021.94013).

2.5 Targeted method validation

The exported chromatograms (EICs) corresponding to the protonated molecules $[M-H]^+$ were used for the identification of compounds. A mass error of less than 5 ppm (European Commission Guidance), comparing the measured mass with the exact calculated mass, was required for positive identification. The characteristics of the targeted alks method were studied using full mass spectral data of 41 pure standards. RT, isotope pattern and dd-MS/MS spectra compared with those collected from available standards were used to confirm targeted alks in sample analysis data.

Quantification was performed from a 5-point calibration curve allowing a regression coefficient ($R^2$) of at least 0.990 included in the linearity range. The limit of detection (LOD) was estimated as three standard deviations of ten replicated blank samples according to EURACHEM (2014), and similarly, the limit of quantification (LOQ) was estimated as ten standard deviations of the same replicates. Table 1 reports accurate mass, linearity range, LOD and LOQ of alk standards.

2.6 Untargeted study

In order to extend milk and pasture alk characterisation, a suspect screening approach was exploited. A previous work by Nardin et al. (2016) confirmed the RT and fragmentation of 48 alks by analysing extracts of 8 well-documented alkaloid composition plants (*Datura stramonium*, *Hyoscyamus niger, Solanum nigrum, Lobelia inflate, Senecio vulgaris, Arnica montana*, *Gelsemium sempervirens* and *Ranunculus montanus).* A second work (Nardin et al., 2017) implemented the screening database with a further 68 alks, confirmed by analysing the extracts of a further 17 plants (*Ruta graveolens, Passiflora incarnate, Escholtzia californiea, Fumaria officinalis, Piper nigrum, Galega officinalis, Ginko biloba, Achillea millefolium, Mentha piperita*, *Urtica dioica, Cinchona succirubra*, *Plantago major, Salvia officinalis*, *Vaccinium myrtillus*, *Veratrum album, Glycyrrhiza glabra*, and *Valeriana officinalis*). After preparation, the plant extracts were injected and evaluated for the presence of the alks reported in the literature as typical of that plant. Peak signals were studied in the EICs matching m/z values, with a mass tolerance of < 5 ppm (European Commission Guidance) compared to the exact mass of alks reported in the literature. When present, the RT of alks were confirmed and a second

injection was performed to collect fragmentation profile information. The study of untargeted alks was limited to compounds providing a sufficient detectable response (area > 100 area units).

2.7 Statistical analysis

Univariate and multivariate analysis were performed using Statistica 13.1 (StatSoft; Tulsa, OK, USA) software and Unscrambler X 10.4 (CAMO software AS, Oslo, Norway). Statistical processing was carried out on concentration values for targeted alks, while the ionisation intensity, expressed as peak area, was used for untargeted alks. Data not normally distributed (Jarque-Bera test, $p<0.05$) were normalised by applying Box-Cox transformation.

Tukey's Honestly Significant Difference (HSD) test for an unequal number of samples ($p<0.05$) was performed to identify significant differences in the alk content of different milk samples collected from cows grazing on 2 distinct pastures.

Partial Least Squares – Discriminant Analysis (PLS-DA) (Chevallier et al., 2006) was used to test the efficacy of alk profiles in discriminating the different pasture origin of milk samples. A PLS-DA model was built using the alk matrixes (X) and the milk sample matrix (Y), which was created by defining a dummy variable for each milk origin pasture type considered. The optimum number of PLS components was estimated using full cross-validation. The significance of alk predictors was evaluated with Marten's uncertainty test. Classification performance was assessed in the validation phase in terms of sensitivity, specificity and accuracy, adopting a cut-off value of 0.5. Thus, samples with a predicted Y-value of over 0.5 were identified as belonging to one class, whilst those with predicted Y-values lower than 0.5 were predicted to belong to the other class (Camin et al., 2017).

## 3. Results

### 3.1 Targeted profile

Tables 2a and 2b include the minimum, maximum and median concentrations for the alks detected at least once in herbage, milk samples and mixed concentrate.

As regards herbage samples, Lycopsamine (Pyz) was the most frequently found alk, quantified in 46% of samples collected in PO pasture, with an average concentration of 1.2 µg $kg^{-1}$, and in 38% of samples of SE pasture, with an average concentration of 1.0 µg $kg^{-1}$. We found Gramine (indole alk; Ind) in 29% of PO samples and 33% of SE samples, with an average concentration of 24 µg $kg^{-1}$ and 13 µg $kg^{-1}$, respectively. Veratramine (steroidal alk; Str) was found only in one herb collected in PO pasture, with 357 µg $kg^{-1}$, while Veratridine (Str) was detected in one single sample collected in SE, with 15.6 µg/kg.

As regards the mixed concentrate used for feeding cows, no alkaloid was detected above the LOD.

Considering the 48 individual milk samples collected from cows grazing on the two different pastures, Lycopsamine and Senkirkin (Pyr) were detected in milk deriving from both pastures (58% and 4% respectively in milk from PO, and 58% and 13% respectively in milk from SE). In PO milk samples Lycopsamine was quantified above the LOQ in 13 samples, with an average concentration of 0.02 µg $kg^{-1}$, while Senkirkin was quantified in only one sample, with 4.8 µg $kg^{-1}$. In SE milk samples Lycopsamine was quantified above the LOQ in 11 samples, with 0.012 µg $kg^{-1}$, while Senkirkin was detected above the LOQ in 3 samples, with an average

concentration of 1.2 μg $kg^{-1}$. Gramine (Ind) was quantified in 54% of PO milk samples and 33% of SE samples, with an average concentration of 0.1 μg $kg^{-1}$ and 0.09 μg $kg^{-1}$ respectively. Veratridine (Str) was quantified in only one milk sample from SE pasture, with 1.6 μg $kg^{-1}$.

Considering the 12 mass milk samples collected from the entire herd, only Lycopsamine and Senkirkin were detected. Lycopsamine had an average concentration of 0.03 μg $kg^{-1}$ for PO milk samples while was founf above LOQ only in one SE milk sample at 0.02 μg $kg^{-1}$. Senkirkin was quantified above LOQ in one PO milk sample, with 0.27 μg $kg^{-1}$.

3.2 Untargeted profiles

An overview of the alk profiles in the selected herbage in alpine milk samples and the mixed concentrate, sorted by chemical/botanical groups, is presented in Table 3.

As regards the occurrence of untargeted alks in the selected herbage samples, 68 different alks were detected. Ind, piperidine (Ppr), and pyrrolidine (Pyl) were present in all the samples from both pastures, while benzophenantridine (Bzp) alks were identified in only one sample (4%) per pasture. As far as the other alk groups are concerned, PO turned out to be numerically richer than SE in pyridines (Pyrs; 88% and 83% respectively), terpene (Trns; 83% and 79%), tropane (Trps; 83% and 62%), Strs (58% and 38%) and acridones (Acds; 8% and 4%). Conversely, SE was found to be richer than PO in quinolizidines (Qnzs; 79% and 50% respectively), Pyzs (58% and 33%), isoquinolines (Iqns; 50% and 21%), quinolines (Qnls; 37% and 17%), and protoalkaloids (PrAs; 17% and none found).

Taking into account the milk samples, a significantly lower number of alkaloids (32) were detected. Inds, Pprs, Pyls, and Trns were present in all the milk samples from both the PO and

SE pastures, while Pyrs were detectable in 100% of PO samples and 91% of SE samples. Considering the other groups, milk samples from PO turned out to be numerically richer than SE in Iqns (75% and 67 % respectively) and Qnls (29% and 17%), while conversely, SE milk samples were found to be richer than PO in Qnzs (46% and 38%, respectively) and in Trps (21% and 17%). Pyzs and PrAs were detected only in SE milk samples (17% and 4%, respectively).

As regards the mixed concentrate, 5 Ind alks (14,15-Dihydroxygelsenicine/Gelsemoxonine, Harmaline, Harmane@15.91, Harmine and Harmol), 4 Iqns (Caryachine@17.5, Fumaricine, Fumariline and Parfumidine@18.9), 2 Pprs (Isolobinine/Lobinine@20.0 and Lobinaline), 2 Qnls (Cinchonanine and Indicain ), one Pyl (2-Pyrrolidineacetic acid) and one Trp (Apohyoscyamine) were detected.

3.3 Discrimination of pastures

The PLS-DA model correctly reclassified the most frequently present plant families in PO and SE pastures - Poaceae (9 species), Asteraceae (7) and Lamiaceae (7) - with an average accuracy of 91%, confirming the differences in plant taxonomy with the alkaloid profiles. Furthermore, the herbage diets of the cows grazing on the 2 different pastures were also differentiated, with an overall accuracy of 85%, with Lobinaline, Harmane@15.9, Pseudojervine, Isolobinine/Lobinine@20.0 and Stachydrine@4.1 being the most predictive for PO pasture, according to Marten's uncertainty test, and Parfumidine@18.9, Aconine@17.2 and 3-Acetyltropine for SE pasture.

3.4 Carry-over of alkaloids from herbs to milk

Figure 1 compares the presence of alks in the herbage and milk samples from the 2 pastures (as a percentage). Alks also present in the mixed concentrate are shown underlined.

Of the targeted compounds, the most abundant alks in herbs and milk samples were Lycopsamine and Gramine. On the basis of estimated milk production of about 20 L $day^{-1}$ $cow^{-1}$ and a daily herbage intake of 60 Kg, the average transfer from herb to milk was assessed at 0.7% of the overall daily dose of Lycopsamine and 0.6% of Gramine, confirming the limited alk transfer of these Pyz and Ind alks. Similarly, Hoogenboom et al. (2011) found an estimated overall carry-over of around only 0.1% for other Pyz alks, but reaching 4% for Jacoline.

As regards untargeted alks, Figure 1 highlights the transfer of Pyl alks (2-Pyrrolidineacetic acid, Betonicine, Stachydrine@3.5 and Stachydrine@4.1) in particular, as also reported in Table 3. Other alks showing remarkable carry-over in both pastures were Lobinaline, 14,15-Dihydroxygelsenicine/Gelsemoxonine, Tropinone, Valeriani@8.9, Norlelobanidine/8-Methyl-10-phenyllobelidiol@15.5 and Lelobanidine I/Lelobanidine II@17.4.

Veratridine, Seneciphylline N-Oxide and 8-Ethylnorlobelol, although rare, were particularly interesting, being found only in milk samples produced from SE pasture.

3.5 Discrimination of alpine milk samples

Alks also present in the mixed concentrate were not taken into account in statistical analysis. As regards single alks present in the individual milk samples collected in the 2 different alpine pastures, HSD Tukey's test ($p<0.05$) was performed on normally distributed compounds and on those normalised by applying Box-Cox transformation (Heimidine@22.3 and beta-Solamargine; Jarque-Bera test, $p<0.05$). The test highlighted that PO milks were significantly richer in

Stachidrine@3.5, Stachidrine@4.1 and Plantagonin, while conversely Valerianine@8.9 was more abundant in SE milk samples. To investigate whether alk profiles could allow prediction of milk origin, a PLS-DA model was proposed. The X-matrix was composed of the individual milk samples, while for the Y-matrix it were defined 2 dummy variables, one for each pasture considered. The 3 alks with P values for Beta coefficients $\leq 0.10$ were used to obtained a PLS-DA discriminant model. The relationship between the two sets of variables (Figure 2) total explained variance of 77% for milk pasture origin. The full cross-validation results are shown in Figure 3 and Table 4 reports the evaluation of prediction performance. Measurement of the quality of each binary classification model came from the confusion matrix, which correctly recorded and incorrectly recognised plants for each milk pasture origin. The average accuracy of the discriminant multiclass model was 77%. The model for milk from PO pasture misclassified 5 out of 24 milk samples, while the model for the classification of the SE milk samples misclassified 6 milk samples out of 24. Lycopsamine was the most powerful alks for discriminating milk collected from PO, while Cathinine and Valerianine@8.9 alks were the best for discriminating milk collected from SE (Figure 2).

For mass milk samples, to assess single alk variability in mass samples from the 2 different pastures, HSD Tukey's test ($p<0.05$) was performed on normally distributed compounds and on those normalised by applying Box-Cox transformation (Senkirkin, 8-Ethylnorlobelol and 3-Acetyltropine; Jarque-Bera test, $p<0.05$). PO milks were significantly richer in Lycopsamine and Stachidrine@4.1, while conversely Valerianine@8.9 was more abundant in SE mass milk samples.

Principal Component Analysis (PCA) was performed to evaluate our alk variables, to find out which guaranteed the most variability. The bi-plot of loadings and scores for the milk alk study (Figure 4a and 4b) shows that Lycopsamine, Stachydrine@3.5, Stachydrine@4.1, 8-Ethylnorlobelol, Valerianine@8.9 and Valerianine@13.0 explained 69% of variance in the 2 alpine milks. In particular, Lycopsamine, Stachydrine@3.5 and Stachydrine@4.1 were close to milk from PO pasture, and 8-Ethylnorlobelol, Valerianine@8.9 and Valerianine@13.0 to milk from SE pasture.

### 3.6 Conclusions

A wide and complex range of alkaloids are synthesized by Alpine plants and become part of cows' intake. This study allowed assessment of the carry-over of alks from grazed herbs to the milk produced by cows at pasture in two different northern Italian Alpine grasslands, albeit with the limit of not being able to extensively calculate the alk transfer ratio because of the limited availability of pure commercial standards. Within these analytical limits, the study provided evidence that alkaloid profiles represent a potential tool for distinguishing Alpine milk from two different pastures, both by evaluating the milk collected from individual cows and the overall milk produced by a herd grazing on the 2 pastures.

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## Table 1

Validation parameters for the 41 alk analytical standards.

| Compound | RT | $[M+H]^+$ ($\Delta m/z$) | Herb (µg $kg^{-1}$) | | Milk (µg $kg^{-1}$) | | Animal feed (µg $kg^{-1}$) | | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | (min) | ($m/z$; ppm) | LOD | LOQ | LOD | LOQ | LOD | LOQ | |
| Nicotine | 4.8 | 163.1232 (1.3) | 24.0 | 80.0 | 0.41 | 1.35 | 15.9 | 53.0 | 0.990 |
| Monocrotaline | 5.3 | 326.1589 (0.5) | 0.10 | 0.30 | 0.001 | 0.005 | 0.05 | 0.18 | 0.998 |
| Lycopsamine | 7.1 | 300.1809 (1.3) | 0.20 | 0.60 | 0.003 | 0.01 | 0.13 | 0.42 | 0.998 |
| Coniine | 8.6 | 128.1429 (3.1) | 0.30 | 1.10 | 0.01 | 0.02 | 0.22 | 0.75 | 0.998 |
| Erucifoline | 8.8 | 350.1599 (0.9) | 0.10 | 0.40 | 0.002 | 0.01 | 0.07 | 0.27 | 0.998 |
| Senecionine N-oxide | 9.2 | 352.1751 (1) | 0.10 | 0.30 | 0.001 | 0.005 | 0.05 | 0.18 | 0.997 |
| Gramine | 9.6 | 175.1225 (2.3) | 0.10 | 0.30 | 0.001 | 0.004 | 0.05 | 0.17 | 0.998 |
| Theobromine/ Theophylline | 9.6 | 181.0725 (2.8) | 4.50 | 15.0 | 0.08 | 0.25 | 2.97 | 9.86 | 0.999 |
| Scopolamine | 10.6 | 304.1539 (1.3) | 0.30 | 1.00 | 0.01 | 0.02 | 0.21 | 0.69 | 0.996 |
| Jacobine-N-oxide | 10.9 | 368.1715 (3.3) | 0.50 | 1.50 | 0.01 | 0.03 | 0.30 | 1.00 | 0.996 |
| Erucifoline-N-oxide | 11.1 | 366.1548 (0.3) | 0.60 | 1.90 | 0.01 | 0.03 | 0.37 | 1.25 | 0.996 |
| Heliotrine | 11.1 | 314.196 (0.3) | 0.10 | 0.20 | 0.001 | 0.004 | 0.04 | 0.15 | 0.998 |
| Retrorsine | 11.2 | 352.1755 (0.3) | 0.40 | 1.30 | 0.01 | 0.02 | 0.27 | 0.88 | 0.996 |
| Seneciphylline | 12.0 | 334.1655 (1.8) | 0.20 | 0.90 | 0.00 | 0.02 | 0.15 | 0.59 | 0.996 |
| Retrorsine N-oxide | 12.5 | 368.1708 (1.4) | 0.70 | 2.30 | 0.01 | 0.04 | 0.46 | 1.52 | 0.996 |
| Senecionine/ Senecivernine | 13.2 | 336.1801 (1.3) | 0.20 | 0.70 | 0.004 | 0.01 | 0.15 | 0.49 | 0.993 |
| Hyoscyamine/ Atropine | 13.3 | 290.1755 (1.5) | 0.20 | 0.80 | 0.004 | 0.01 | 0.16 | 0.54 | 0.994 |
| Echimidine | 14.3 | 398.2172 (0.3) | 0.10 | 0.40 | 0.002 | 0.01 | 0.08 | 0.27 | 0.999 |
| Senkirkin | 14.5 | 366.1918 (1.9) | 1.10 | 3.60 | 0.02 | 0.06 | 0.70 | 2.35 | 0.998 |
| Jacobine | 14.9 | 352.1762 (2.3) | 0.50 | 1.50 | 0.01 | 0.03 | 0.30 | 1.01 | 0.997 |
| Caffeine | 15.1 | 195.0881 (2.1) | 1.50 | 4.90 | 0.02 | 0.08 | 0.98 | 3.24 | 0.998 |
| Lasiocarpine | 16.5 | 412.2325 (1) | 0.20 | 0.70 | 0.003 | 0.01 | 0.13 | 0.43 | 0.998 |
| Striknine | 17.2 | 335.1751 (0.9) | 0.90 | 3.00 | 0.02 | 0.05 | 0.63 | 1.97 | 0.993 |
| Harmaline | 17.3 | 215.1185 (2.9) | 5.60 | 19.0 | 0.09 | 0.31 | 3.70 | 12.30 | 0.996 |
| Sipeimine | 18.0 | 430.3311 (0.9) | 1.90 | 6.30 | 0.03 | 0.11 | 1.25 | 4.16 | 0.996 |
| Quinine | 18.2 | 325.1909 (0.5) | 3.10 | 10.0 | 0.05 | 0.18 | 2.07 | 6.89 | 0.995 |
| Quinidine | 18.2 | 325.1901 (2.9) | 4.60 | 16.0 | 0.08 | 0.26 | 3.07 | 10.28 | 0.998 |
| Veratramine | 20.7 | 410.3069 (3.9) | 0.30 | 0.90 | 0.005 | 0.02 | 0.19 | 0.63 | 0.995 |
| alpha-Solasonine | 20.7 | 884.5008 (0.7) | 0.20 | 0.60 | 0.003 | 0.01 | 0.11 | 0.37 | 1,000 |
| Jervine | 20.8 | 426.3005 (0.7) | 0.40 | 1.40 | 0.01 | 0.02 | 0.28 | 0.91 | 0.995 |
| alpha-Solamargine | 21.1 | 868.5056 (0.5) | 4.40 | 15.0 | 0.07 | 0.25 | 2.89 | 9.65 | 0.999 |
| Protoveratrine A | 21.2 | 794.4322 (0.1) | 17.0 | 57.0 | 0.29 | 0.96 | 11.34 | 37.84 | 0.996 |
| alpha-Solanine | 22.1 | 868.5059 (1) | 0.20 | 0.70 | 0.004 | 0.01 | 0.15 | 0.48 | 0.998 |
| Veratridine | 22.2 | 674.3521 (1.9) | 2.00 | 6.80 | 0.03 | 0.12 | 1.36 | 4.52 | 0.998 |
| Solasodine | 24.5 | 414.3375 (2) | 1.00 | 3.50 | 0.02 | 0.06 | 0.69 | 2.29 | 0.993 |
| Aconitine | 24.5 | 646.3229 (1.2) | 2.00 | 6.60 | 0.03 | 0.11 | 1.31 | 4.37 | 0.999 |
| Tomatidine/ Tomatine | 25.4 | 416.3535* (2.9) | 10.0 | 32.0 | 0.16 | 0.54 | 6.35 | 21.20 | 0.991 |

Note: RT= retention time; Δ m/z (ppm)= accurate mass error compared to exact mass; LOD= limit of detection; LOQ= limit of quantitation;

*=Tomatine $[M+H-C_{23}H_{38}O_{19}]^+$;

**Table 2a**

Minimum, median and maximum values for alk content (µg/kg) in the herbage selected in experimental pastures (*Poion alpinae*, PO; *Seslerion caeruleae*, SE).

| Alkaloid | Herbage from PO | | | | Herbage from SE | | | |
|---|---|---|---|---|---|---|---|---|
| | Samples (N>LOD) | Min | Med | Max | Samples (N>LOD) | Min | Med | Max |
| Lycopsamine | 11 | <0.2 | <0.2 | 8.5 | 9 | <0.1 | <0.1 | 3.4 |
| Gramine | 7 | <0.1 | <0.1 | 86 | 8 | <0.1 | <0.1 | 64 |
| Veratramine | 4 | <0.3 | <0.3 | 354 | 0 | <0.3 | <0.3 | <0.3 |
| Veratridine | 0 | <0.2 | <0.2 | <0.2 | 1 | <0.2 | <0.2 | 15 |

Min = minimum; Med = median; Max = maximum;

**Table 2b**

Minimum, median and maximum values for alk content (µg/kg) in individual and mass milk samples from the two experimental pastures (*Poion alpinae*, PO; *Seslerion caeruleae*, SE).

| Alkaloid | Individual milk from PO (N=24) (µg $kg^{-1}$) | | | | Individual milk from SE (N=24) (µg $kg^{-1}$) | | | | Mass milk from PO (N=6) (µg $kg^{-1}$) | | | | Mass milk from SE (N=6) (µg $kg^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Samples (N>LOD) | Min | Med | Max | Samples (N>LOD) | Min | Med | Max | Samples (N>LOD) | Min | Med | Max | Samples (N>LOD) | Min | Med | Max |
| Gramine | 13 | <LOD | 0.09 | 0.1 | 8 | <LOD | <LOD | 0.12 | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD |
| Lycopsamine | 14 | <LOD | 0.01 | 0.03 | 11 | <LOD | <LOD | 0.01 | 6 | 0.01 | 0.02 | 0.06 | 1 | <LOD | <LOD | 0.02 |
| Senkirkin | 1 | <LOD | <LOD | 4.85 | 3 | <LOD | <LOD | 2.6 | 2 | <LOD | <LOD | 0.27 | 3 | <LOD | <LOD | <LOQ |
| Veratridine | 0 | <LOD | <LOD | <LOD | 1 | <LOD | <LOD | 1.6 | 0 | <LOD | <LOD | <LOD | 0 | <LOD | <LOD | <LOD |

Min = minimum; Med = median; Max = maximum;

**Table 3**

Untargeted alk profile of the selected herbage, individual and mass milk samples from the two experimental pastures (number of samples containing each individual alk; *Poion alpinae*, PO; *Seslerion caeruleae*, SE).

| Compound | Herbage samples | | Individual milk samples | | Mass milk samples | | Mixed concentrate (N=1) |
|---|---|---|---|---|---|---|---|
| | PO (N=24) | SE (24) | PO (N=24) | SE (24) | PO (N=6) | SE (6) | |
| Acridinone | | | | | | | |
| Arborinine | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| Benzophenantridine | | | | | | | |
| Protopin | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Indole | | | | | | | |
| 14,15-Dihydroxygelsenicine/ Gelsemoxonine | 22 | 24 | 24 | 24 | 6 | 6 | 1 |
| Gelsemicine | 3 | 1 | 0 | 0 | 0 | 0 | 0 |
| Gelsemine | 6 | 7 | 0 | 0 | 0 | 0 | 0 |
| Harmaline | 3 | 0 | 0 | 0 | 0 | 0 | 1 |
| Harmalol | 0 | 1 | 2 | 1 | 0 | 0 | 0 |
| Harmane@15.5 | 19 | 15 | 2 | 2 | 0 | 0 | 0 |
| Harmane@15.9 | 3 | 3 | 0 | 0 | 0 | 0 | 1 |
| Harmine | 2 | 1 | 0 | 0 | 0 | 0 | 1 |
| Harmol | 13 | 10 | 0 | 0 | 0 | 0 | 1 |
| Heimidine@22.3 | 0 | 0 | 7 | 4 | 0 | 0 | 1 |
| Norharmane | 16 | 16 | 3 | 1 | 5 | 4 | 0 |
| Isoquinoline | | | | | | | |
| Caryachine@14.2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Caryachine@15.5 | 4 | 4 | 0 | 0 | 0 | 0 | 0 |
| Caryachine@17.5 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Fumaricine | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Fumariline | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *o*-Methylcaryachine@16.0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| o-Methylcaryachine@18.3 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Parfumidine@18.9 | 1 | 9 | 0 | 0 | 0 | 0 | 1 |
| Parfumidine@19.7 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Piperidine | | | 0 | 0 | 0 | 0 | 0 |
| 8-Ethylnorlobelol | 0 | 3 | 0 | 2 | 0 | 3 | 0 |
| 8,10-Diethyllobelidiol@12.1 | 3 | 8 | 0 | 2 | 0 | 0 | 0 |
| 8,10-Diethyllobelidiol@13.1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| cis-Lobelanidine/trans Lobelanidine@20.2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| cis-Lobelanidine/trans Lobelanidine@20.9 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| cis Lobelanine/ trans Lobelanine@21.2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Dihydropiperlonguminine | 5 | 2 | | | | | |
| Isolobinine/Lobinine@20.0 | 6 | 0 | 0 | 0 | 0 | 0 | 1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Lelobanidine I/ Lelobanidine II@16.91 | 1 | 2 | 0 | 0 | 0 | 0 | 0 |
| Lelobanidine I/ Lelobanidine II@17.4 | 1 | 4 | 5 | 1 | 0 | 0 | 1 |
| Lobinaline | 24 | 22 | 24 | 22 | 6 | 6 | 1 |
| Norallosedamine | 11 | 3 | 0 | 0 | 0 | 0 | 0 |
| Norlelobanidine/8 Methyl 10 phenyllobelidiol@15.5 | 5 | 4 | 3 | 3 | 0 | 0 | 0 |
| Norlelobanidine/8 Methyl 10 phenyllobelidiol@16.1 | 3 | 2 | 1 | 2 | 0 | 0 | 0 |
| Norlelobanidine/8 Methyl 10 phenyllobelidiol@16.6 | 14 | 10 | 0 | 1 | 0 | 0 | 0 |
| Piperlonguminine | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| Protoalkaloid | | | | | | | |
| Galegin/Peganin@15.6 | 0 | 4 | 0 | 1 | 0 | 0 | 0 |
| Pyridine | | | | | | | |
| Ginkgotoxin | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Plantagonin | 0 | 0 | 22 | 18 | 6 | 6 | 0 |
| Tropinone | 21 | 20 | 19 | 16 | 0 | 0 | 0 |
| Pyrrolidine | | | | | | | |
| 2-Pyrrolidineacetic acid | 24 | 24 | 24 | 24 | 6 | 6 | 1 |
| Betonicine | 2 | 4 | 18 | 16 | 4 | 4 | 0 |
| Stachydrine@3.5 | 18 | 20 | 24 | 23 | 6 | 6 | 0 |
| Stachydrine@4.1 | 24 | 24 | 19 | 15 | 6 | 6 | 0 |
| Pyrrolizidine | | | | | | | |
| beta-Solamargine | 0 | 2 | 8 | 7 | 1 | 4 | 0 |
| Integerrimine/Perforine | 1 | 2 | 0 | 0 | 0 | 0 | 0 |
| Lasiocarpine N-oxide | 5 | 7 | 0 | 0 | 0 | 0 | 0 |
| Seneciphylline N-oxide | 0 | 1 | 0 | 2 | 0 | 0 | 0 |
| Spartioidine | 4 | 7 | 0 | 2 | 0 | 0 | 0 |
| Quinoline | | | | | | | |
| Cinchonanine E/ Cinchonine | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| Cinchonanine F@12.0 | 3 | 7 | 0 | 0 | 0 | 0 | 0 |
| Cinchonanine F@19.1 | 1 | 3 | 1 | 0 | 0 | 0 | 0 |
| Cinchonanine G | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Indicain | 0 | 1 | 5 | 3 | 0 | 0 | 1 |
| Quinolizidine | | | | | | | |
| Myrtine/Epimyrtine | 12 | 19 | 3 | 1 | 0 | 0 | 0 |
| Steroid | | | | | | | |
| Cevadine@17.2 | 1 | 4 | 0 | 0 | 0 | 0 | 0 |
| Cevadine@18.1 | 13 | 8 | 0 | 0 | 0 | 0 | 0 |
| Pseudojervine | 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| Terpenoid | | | | | | | |
| Aconine@17.2 | 5 | 8 | 0 | 0 | 0 | 0 | 0 |
| Aconine@17.5 | 3 | 6 | 2 | 0 | 0 | 0 | 0 |
| Actinidine | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| Cathinine | 13 | 10 | 2 | 8 | 1 | 5 | 0 |
| Mesaconine@16.3 | 5 | 2 | 0 | 0 | 0 | 0 | 0 |
| Mesaconine@16.6 | 5 | 7 | 0 | 0 | 0 | 0 | 0 |
| Valerianine@13.0 | 0 | 3 | 0 | 0 | 2 | 4 | 0 |
| Valerianine@8.9 | 12 | 9 | 24 | 23 | 6 | 6 | 0 |
| Valerine | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Tropane | | | | | | | |
| 3-Acetyltropine | 20 | 21 | 4 | 5 | 0 | 3 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Anisodamine | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Apohyoscyamine | 0 | 0 | 0 | 0 | 0 | 0 | 1 |

**Table 4**

Pasture origin classification of milk samples according to their alkaloid profile using a Partial Least Squares – Discriminant Analysis multiclass model.

| | Binary discrimination | |
|---|---|---|
| | Milk from PO | Milk from SE |
| Confusion matrix (no. of validation samples) | | |
| true positive ($tp_i$) | 19 | 19 |
| false negative ($fn_i$) | 5 | 5 |
| true negative ($tn_i$) | 18 | 18 |
| false positive ($fp_i$) | 6 | 6 |
| Performance evaluation[1] | | |
| sensitivity | 0.79 | 0.79 |
| specificity | 0.75 | 0.75 |
| (average) accuracy | 0.77 | 0.77 |

[1] $Sensitivity_i = tp_i/(tp_i+fn_i)$; $Sensitivity_\mu = \Sigma\, tp_i/\Sigma\, (tp_i+fn_i)$;

$Specificity_i = tn_i/(fp_i+tn_i)$; $Specificity_\mu = \Sigma\, tn_i/\Sigma\, (fp_i+tn_i)$;

$Accuracy_i = (tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)$; Average accuracy= $\Sigma\, [(tp_i+tn_i)/(tp_i+fn_i+fp_i+tn_i)]$ /3xc.

**Captions**

**Figure1**

Alkaloid presence in the 48 selected herbage mixes (hand-plucked) and in the milk samples collected from the cows investigated.

**Figure 2**

Correlation loadings for factors 1 and 2 in a Partial Least Squares – Discriminant Analysis model for the classification of individual milk samples according to the two alpine pastures (PO= *Poion alpinae*; SE= *Seslerion caeruleae*).

**Figure 3**

Score-plot of cow individual milk samples from the Partial Least Squares – Discriminant Analysis model of grazed pasture type (PO= *Poion alpinae*; SE= *Seslerion caeruleae*) classification based on their alkaloid profile. Incorrectly classified samples, both false positive and negative, are ringed.

**Figure 4a/b**

Principal Component Analysis of mass milk samples according to the two alpine pastures (PO= *Poion alpinae*; SE= *Seslerion caeruleae*).

**Figure 1**

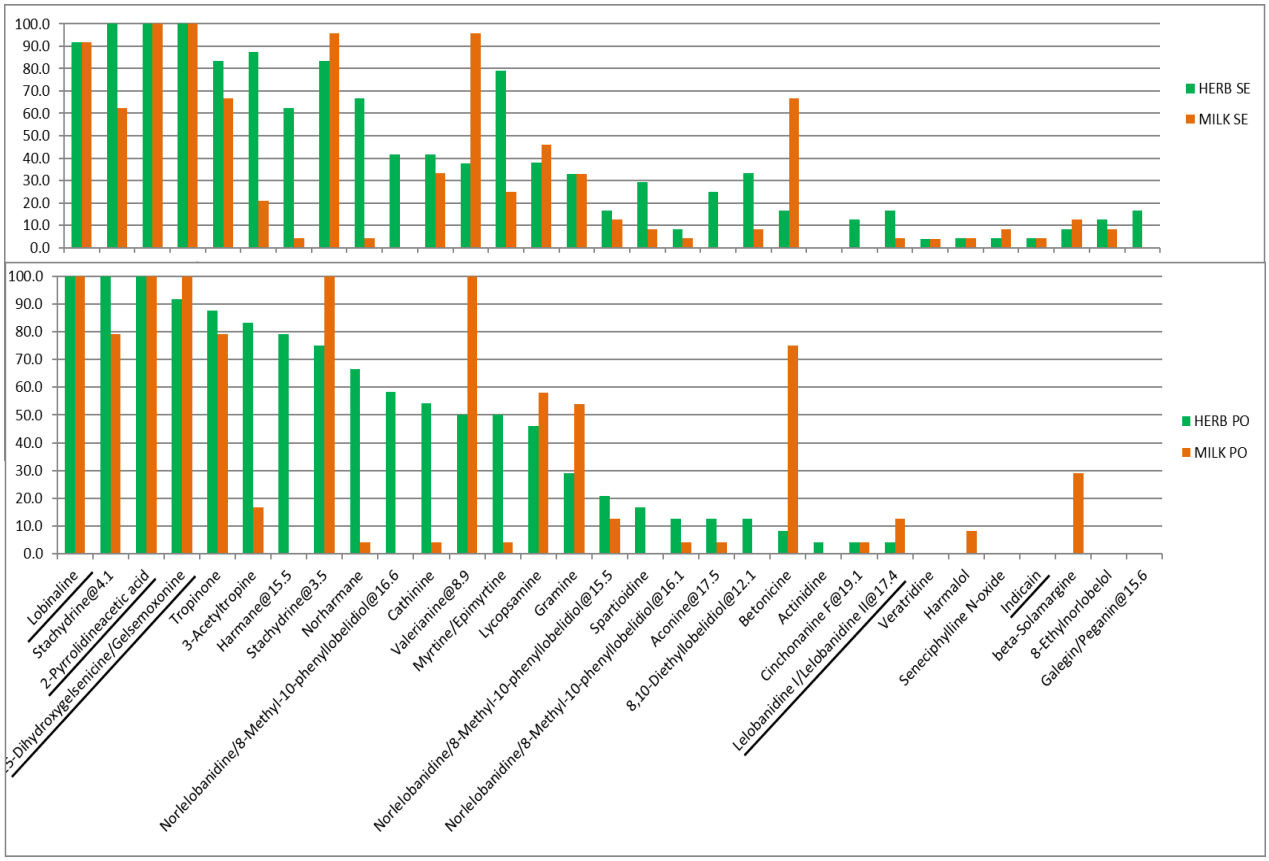

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
HERB SE
MILK SE
HERB PO
MILK PO
Lobinaline
Stachydrine@4.1
2-Pyrrolidineacetic acid
5-Dihydroxygelsenicine/Gelsemoxonine
Tropinone
3-Acetyltropine
Harmane@15.5
Stachydrine@3.5
Norharmane
Norlelobanidine/8-Methyl-10-phenyllobelidiol@16.6
Cathinine
Valerianine@8.9
Myrtine/Epimyrtine
Lycopsamine
Gramine
Norlelobanidine/8-Methyl-10-phenyllobelidiol@15.5
Spartioidine
Norlelobanidine/8-Methyl-10-phenyllobelidiol@16.1
Aconine@17.5
8,10-Diethyllobelidiol@12.1
Betonicine
Actinidine
Cinchonanine F@19.1
Lelobanidine I/Lelobanidine II@17.4
Veratridine
Harmalol
Seneciphylline N-oxide
Indicain
beta-Solamargine
8-Ethylnorlobelol
Galegin/Peganin@15.6

**Figure 2**

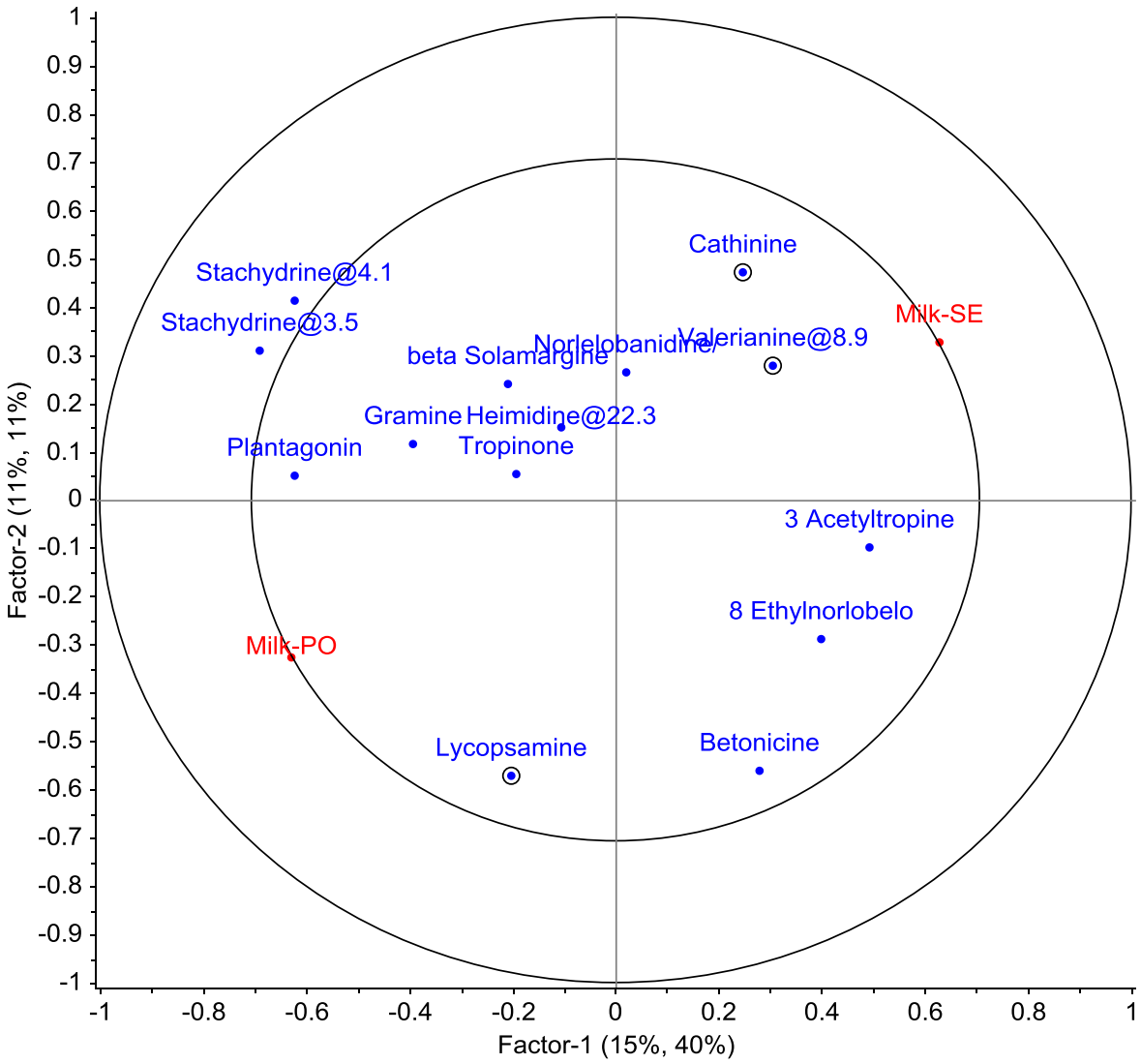

Cathinine
Stachydrine@4.1
Milk-SE
Stachydrine@3.5
Norlelobanidine/
Valerianine@8.9
beta Solamargine
Gramine
Heimidine@22.3
Plantagonin
Tropinone
3 Acetyltropine
8 Ethylnorlobelo
Milk-PO
Lycopsamine
Betonicine
Factor-2 (11%, 11%)
Factor-1 (15%, 40%)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

**Figure 3**

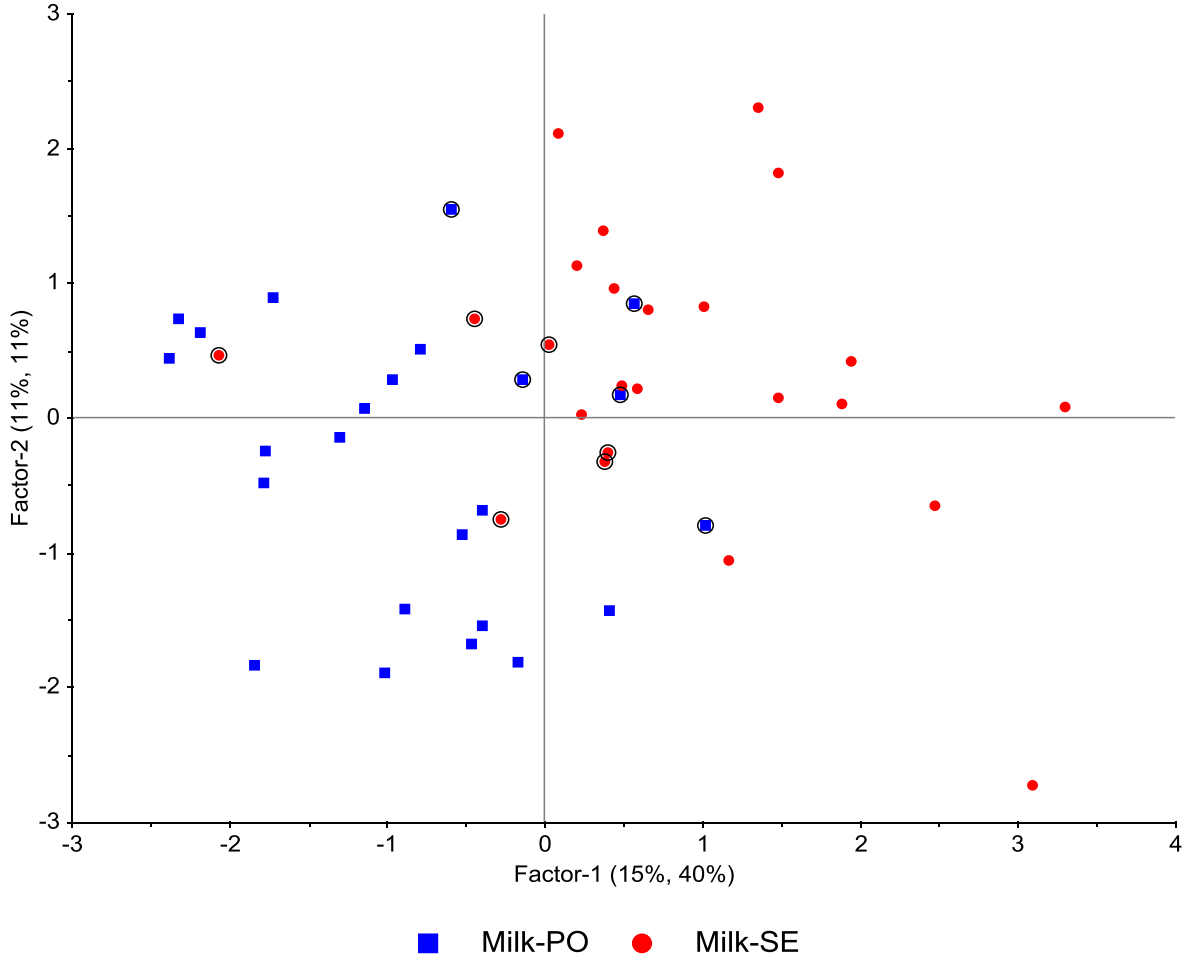


**Figure 4a**

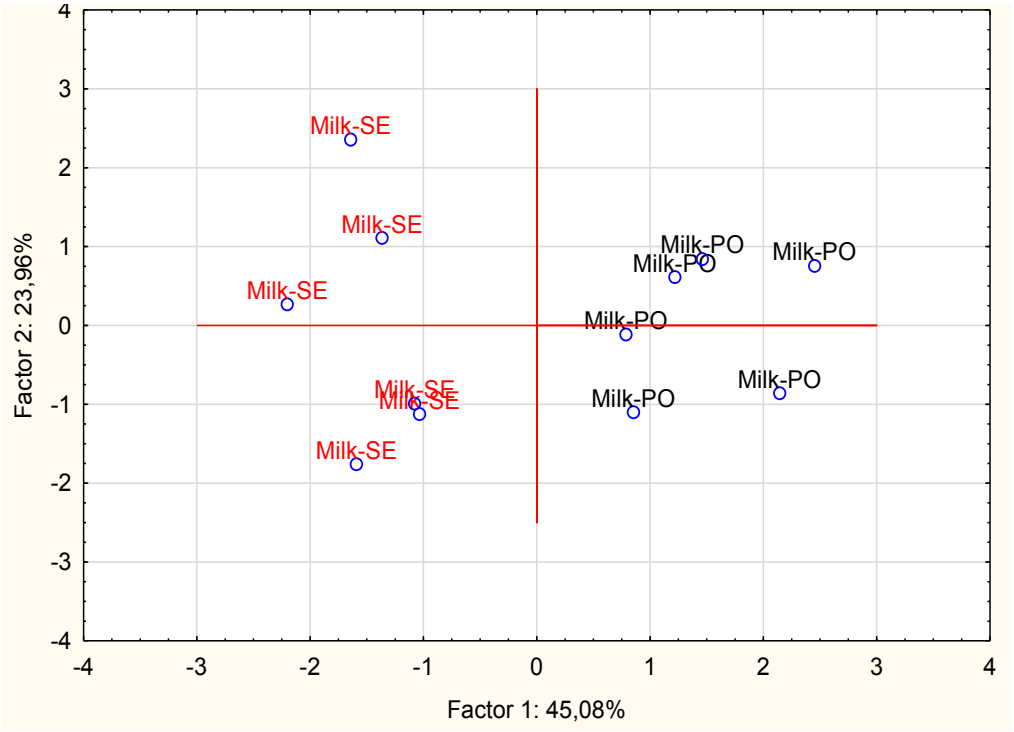
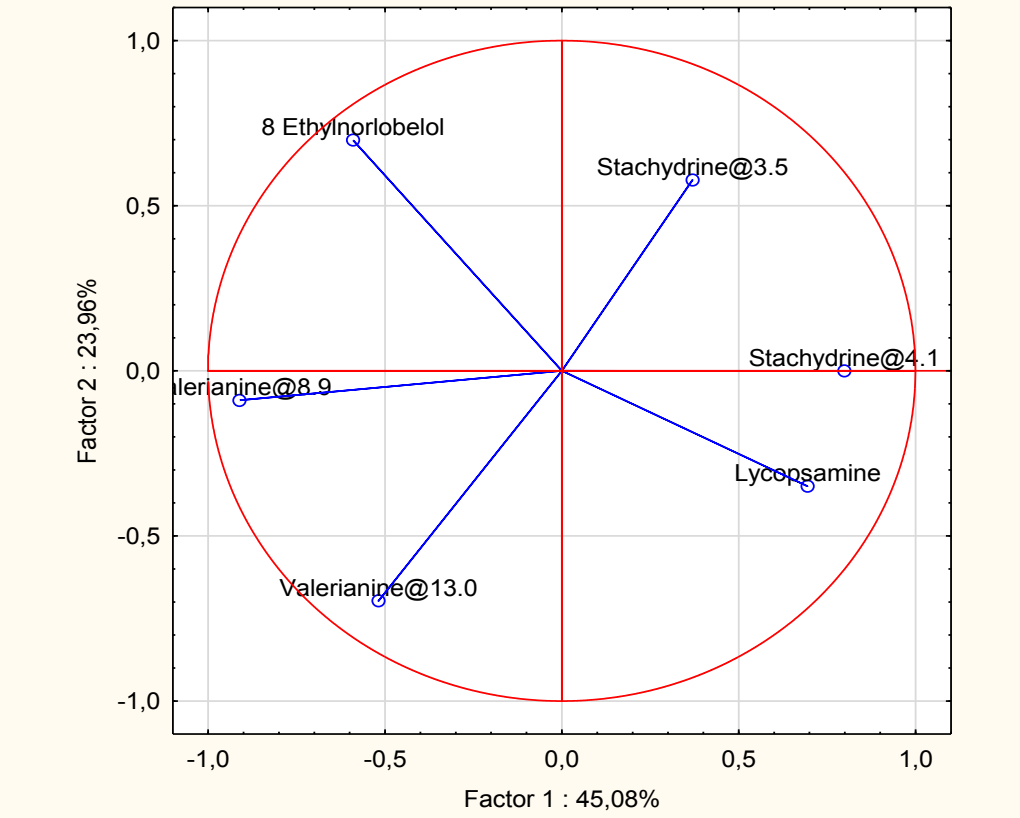



**Figure 4b**



## 4. Conclusions to the thesis

In this work we proposed a very selective and sensitive method able to quantify up to 41 alkaloids and putatively identify more than 100 untargeted alkaloids in herbal and milk extracts using liquid chromatographic separation coupled with high resolution mass spectrometry. Using SPE online pre-treatment it was possible to reduce analysis time and minimise the matrix effect impact on instrumental response.

The proposed method permitted us to define broad, targeted and untargeted alkaloid profiles for herb and milk products. An extensive survey of alkaloid profiles for a wide selection of plant products commercially used for herbalist remedies proved the effectiveness of the proposed method in describing the considerable variability of alkaloid content, and also the correlations existing between alkaloids belonging to indoles, quinolines and tropanes of some plant groups.

Moreover, the wide and complex alkaloid content in Alpine herbs was evaluated, analysing more than 60 different plants collected in two northern Italian natural Alpine pastures. Despite the limited availability of pure commercial standards, the combination of targeted and untargeted approaches provided good evidence of the ability of alkaloid profiles to represent a potential tool for classifying alpine plants on the basis of the corresponding family groups, especially for the Poaceae species, the most representative in the pastures. Furthermore, the alkaloid profiles allowed discrimination of the herbage selected by dairy cows grazing on the two different pastures, acting as both qualitative and quantitative diet markers.

The alkaloid profiles of milk samples collected from the cows grazing on the two pastures also showed evidence of the possible application of alkaloid composition to dairy product traceability, as a novel potential marker of origin.

## 5. Other scientific contributions

### Conference

Poster

- Nardin T., Barnaba C., Nicolini G., Malacarne M., & Larcher R. Simultaneous determination of amino acids and biogenic amines after derivatization using hybrid quadrupole-orbitrap mass spectrometer. X In Vino Analytica Scientia Symposium (IVAS), Salamanca, 17-20 July 2017.
- Nardin T., Barnaba C., Larcher R. Herbal infusion alkaloid profiles by high resolution mass spectrometry. The 1st Food Chemistry Conference, October 30 – November 01 2016.
- Nardin T., Piasentier E., Barnaba C., & Larcher R. Rapid target and untarget analytical method for alkaloids analysis in herbal extracts, 7th International Symposium on recent advances in food analysis, Prague, November 3-6 2015.
- Nardin T., Barnaba C., Nicolini G., Malacarne M., & Larcher R. A new comprehensive method for the characterisation of simple phenols in alcoholic beverages by high resolution mass spectrometry. IX In Vino Analytica Scientia Symposium (IVAS), Trento, 14-17 Luglio 2015.
- Nardin T., Malacarne M., Bertoldi D., & Larcher R. A case of food traceability: tannins. 5° Advanced Food Analysis, Wageningen, The Netherlands, 26 - 30 January 2015.

Oral presentation (Underlined the speaker)

- T. Nardin, R. Larcher, New Methods for Quat Pesticides Analysis Using Cationic Chromatography Coupled with High Resolution Mass Spectrometry, 5th MS-Food Day, Bologna, October 11-13, 2017.
- T. Nardin, Distribution of atypical ageing defect precursors in berry fractions (skin, pulp and seeds) by high resolution mass spectrometry, 2nd MS-Wine Day, MS for grapes, wine, spirits. Conegliano, 9-10 May, 2017.
- Nardin, T., Barnaba, C., Larcher, R. Food profiling: new horizons of high-resolution mass spectrometry application, International conference MASSA 2016, Rome, September 6-8, 2016.

- Nardin T., Piasentier E., Barnaba C., Romanzin A., Larcher R., Targeted and untargeted alkaloids characterization of pasture herbs in eastern Italian alps using high resolution mass spectrometry, 19th Meeting of the FAO-CIHEAM Mountain Pastures sub-network, Zaragoza, June 14-16 2016.
- Nardin T., Piasentier E., Barnaba C., Larcher R., Targeted and untargeted alkaloids profiling of alpine flora using high resolution mass spectrometry (Q-Orbitrap), Incontro di Spettroscopia Analitica, Matera, May 29 –June 01 2016.
- Nardin T., Barnaba C., Larcher R., Target and untargeted analytical method for alkaloids analysis in herbal extracts by high resolution mass spectrometry, 4th MS Food Day, Foggia, October 7-9 2015.

**Workshop**

Oral presentation (Underlined the speaker)

- Nardin T., Larcher R., Study of alkaloid profiles of alpine herbs and milk by high resolution mass spectrometry, European Food and Environmental Seminars, Bologna, 14 November 2017.
- Nardin T., IC e LC accoppiate alla massa ad alta risoluzione (Q-Orbitrap) per la determinazione di erbicidi cationici in matrici agroalimentari, Soluzioni innovative nel campo dell'analisi elementare in tracce, Rome, 23 May 2017.
- Nardin T., IC e LC accoppiate alla massa ad alta risoluzione (Q-Orbitrap) per la determinazione di erbicidi cationici in matrici agroalimentari, Soluzioni innovative nel campo dell'analisi elementare in tracce, Milan, 25 May 2017.
- Nardin T., CM: Profiling Processing in LC/IC – HRMS, System (CDS) Software User Meeting, Milan, 22 March.
- Nardin T., Larcher R., Barnaba C., "Food profiling": nuovi orizzonti applicativi per la spettrometria di massa ad alta risuoluzione, Sicurezza alimentare ed autenticità dei cibi, Bologna, 23 giugno 2016.
- Nardin T., Barnaba C., Larcher R., Caratterizzazione degli alcaloidi da estratti vegetali tramite LC Orbitrap e gestione dati con software Chromeleon, Seminario Cromatografia Liquida, Thermo Scientific, Pomezia, 9 dicembre 2015.

- Nardin T., Barnaba C., Larcher R., Malacarne M., SPE On-line-UHPLC: possibili applicazioni in enologia, Seminario Tecniche analitiche innovative nell'analisi del vino, Thermo Scientific, Asti, Italia, 26 Marzo 2015.