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Targeted and untargeted alkaloid characterisation of pasture herbs and milk from eastern Italian Alps using high resolution mass spectrometry

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Abstract. Alkaloids, widely present in herbs, have attracted the attention of phytochemists for over 150 years due to their potential value as medicinal agents or their toxic principles. In this work we developed a method for targeted and untargeted alkaloid investigation that automatically combines SPE-on line sample purification with UHPLC/ESI/High Resolution Mass Spectrometry (Q-Orbitap) detection. Thirty-five alkaloids were quantified in comparison with analytical standards, 48 were putatively identified and confirmed using the chromatographic retention time and the fragmentation profile obtained analysing the extracts of plants already well-documented in the literature, and further 200 using an in-house database built from literature information on exact mass and isotopic pattern. Besides, the alkaloid profile of 67 single herbage plants, characteristic of the local alpine flora, and that of 48 herbal mix samples representative of the exact daily intake of 8 cows grazing in three consecutive days on two distinct alpine pastures in north-eastern Italy, was described. Moreover, also the corresponding 48 milks produced by those cows were analysed. Among the quantified alkaloids, the most abundant were Lycopsamine and Gramine (42 and 31% of pasture samples, and 52 and 44% of milks, respectively; contents from 0.4 to 80 $\mu\text{g kg}^{-1}$ in herb, and 0.04-0.4 $\mu\text{g L}^{-1}$ in milk). The untargeted analysis found over 120 different alkaloids in herbal mixes and roughly 40 in milks.

Keywords. Alkaloids – Pasture plants – Orbitrap.

Caractérisation ciblée et non ciblée des alcaloïdes présents chez des plantes herbacées de pâturages et des échantillons de lait de vaches à l'aide de la spectrométrie de masse à haute résolution, dans les Alpes orientales italiennes

Résumé. Les alcaloïdes, très répandus dans les herbes, ont attiré l'attention des phytochimistes depuis plus de 150 ans, du fait de leur potentiel comme agents médicinaux ou de leurs propriétés toxiques. Ce travail a développé une méthode pour la détection ciblée et générale d'alcaloïdes, qui combine automatiquement la purification de l'échantillon par voie de SPE online avec UHPLC / ESI / High Resolution Mass Spectrometry (Q-Orbitap). Trente-cinq alcaloïdes ont été quantifiés par rapport aux normes analytiques pures. Quarante-huit alcaloïdes ont été confirmés en utilisant le temps de rétention chromatographique et le profil de fragmentation, en analysant les extraits d'herbes déjà bien documentées dans la littérature, et 200 autres alcaloïdes ont été provisoirement identifiés en utilisant une base de données construite à partir d'information de la littérature concernant les masses exactes et des motifs isotopiques. D'abord, le profil de composition de 67 plantes herbacées distinctes qui composent la flore alpine locale a été établi. Ensuite, 48 échantillons de mélange à base de plantes représentatives de l'ingestion quotidienne de la vache, recueillies à partir de deux alpages dans le nord-est de l'Italie, et 48 échantillons de lait produits par les vaches au pâturage sur ces alpages, ont été analysés. En ce qui concerne le contenu des 35 alcaloïdes quantifiés, lycopsamine et gramine étaient les plus répandues (42 et 31% des échantillons de pâturage, et 52 et 44% des laits, respectivement) avec une distribution de concentration dans les herbes de 0,4 à 80 $\mu\text{g kg}^{-1}$ et dans le lait de 0.04-0.4 $\mu\text{g L}^{-1}$. Plus de 120 alcaloïdes différents ont été détectés avec une analyse non ciblée dans les mélanges à base de plantes et à peu près 40 dans les laits.

Mots-clés. Alcaloïdes – Plantes de pâturages – Orbitrap.

I – Introduction

Alkaloids are an extremely varied group of natural, nitrogen-containing and basic organic compounds. Currently over ten thousand alkaloids have been isolated from various natural sources, especially in plants. Despite this, their physiological function in herbs is not yet fully understood. Usually considered simply waste products of plant metabolic processes (Hartmann, 2007), the highly differentiated chemical structure suggests that they play various specific biological roles, from plant protection against pathogen and herbivore attacks (Rasmann and Agrawal, 2008) to scavenger activity (Larson and Marley, 1894). Alkaloids have been classified into three principal classes depending on precursors and final molecular structures: atypical (non-heterocyclic compounds, sometimes called 'proto-alkaloids' or biological amines), typical (heterocyclic compounds further classified into: pyrrole, pyrrolidine, tropane, pyrrolizidine, piperidine, quinoline, isoquinoline, aporphine, quinolizidine, indole, indolizidine, pyridine, imidazole, and purine groups), and pseudo-alkaloids (basic compounds, not deriving from amino acids). Acting very quickly on specific areas of the nervous system, alkaloids can often manifest a marked physiological action on humans and animals. Some of them are responsible for the beneficial effects of traditional medicines, such as Corynoline, an isoquinoline alkaloid belonging to *Papaveraceae* family and used in traditional Chinese Pharmacopoeia (Liu *et al.*, 2016), or pterogynine and pterogynidine, isolated from *Pterogyne nitens* and tested for their effect on a human infiltrating ductal carcinoma cell line (ZR-7531) (Duarte *et al.*, 2010). However, some alkaloids may instead have the harmful effects of poisons. Pyrrolizidine (PA), found primarily in the plant families of Boraginaceae, Compositae and Leguminosae, is the most studied alkaloid group due to increased awareness of its potential risk and the European Food Safety Authority (EFSA) has published a scientific opinion on PAs in food and feed (Mudge *et al.*, 2015). In particular, 1,2-dehydro pyrrolizidine ester alkaloids are toxic for humans and livestock and globally many episodes of PA intoxication have been reported involving humans as well as ruminants (Wiedenfeld and Edgar, 2011). Furthermore, some studies have investigated and confirmed the potential transfer of PAs present in herbs to milk, although in much lower concentrations and supposedly not dangerous for health (Hoogenbooma *et al.*, 2011; Rouge *et al.*, 2013). The objective of the study was the characterisation of the alkaloid profile of herbs and milks produced by cows grazing on alpine pastures in order to evaluate the extent of the transfer of these nitrogen compounds from the grass to the milk as possible markers of the floristic composition ingested.

II – Materials and methods

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).

Analytical standards were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany), and Harmaline, Strychnine from Sigma (St. Louis, MO, USA). The standards were dissolved in a 50% aqueous methanol solution to reach a final concentration of about 3 mg L⁻¹ of each individual alkaloid, and used for calibration in the range 0.05 – 3000 µg L⁻¹.

2. Plant and milk sampling and sample extract preparation

Two pastures with different vegetation types (Bovolenta *et al.*, 2014) were grazed, at the same phenological stage, by a herd of Simmental dairy cows. During 3 days for each pasture, samples of

herbage consumed by 8 previously selected cows were collected using the hand-plucking technique. For herb samples, an homogeneous aliquot of 2.5 g herb sample was added to 20 mL of extraction solution (H₂O/MeOH/FA; 44.5:44.5:1 v/v/v) in polyethylene 50 mL falcon tubes (Sartorius AG, Goettingen, Germany), sonicated for 10 minutes (LBS1 6Lt, FALC Instruments, Treviglio BG, Italy), and left under 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.

During the milking (twice a day for 3 days) individual milk samples were collected from selected cows. For milk samples, a homogeneous aliquot of 5 g was added to 2 mL of extraction solution (H₂O/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 and the samples were shaken for 10 minutes. 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 (Sartorius AG, Goettingen, Germany), and diluted 2 times with water.

3. Method

On-line purification was performed with a SolEx HRP spe cartridge, loading the sample with ammonia: methanol (96:4 v/v; pH = 9) at 1 ml min⁻¹, during chromatographic separation with a Raptor Biphenyl analytical column, managing gradient elution in 32 minutes from 30% to 100% of organic solvent (eluent A: water/0,1% formic acid/5mM ammonium acetate; B: methanol:acetonitrile (95:5 v/v)/0,1% formic acid/5mM ammonium acetate). The mass spectrometer was operated with a heat source in positive ion mode using the following parameters: sheath gas flow rate set at 30 arbitrary units; aux gas flow rate at 10 arbitrary units; spray voltage at 3.5 kV; capillary temperature at 330 °C; aux gas heater temperature at 300 °C; Mass spectra were acquired in full MS-data dependent MS/MS analysis (full MS—dd MS/MS) at a mass resolving power of 140,000.

III – Results and discussion

A group of 35 alkaloids were quantified with reference to pure analytical standards. The method was linear up to concentrations of 1000/3000 µg L⁻¹ with R² always > 0.99, and the limits of quantification ranged between 0.15 and 37 µg kg⁻¹ for herbs and 0.01-3 µg L⁻¹ for milk. Accuracy, expressed as the average of relative errors, was less than 10% for 71% of compounds, and precision (as RSD%) was generally < 10% throughout the quantitation range. For other 48 alkaloids, whose analytical standards were not commercially available, the presence was confirmed using the chromatographic retention time and the fragmentation profile as defined analysing the extract of plants of already well-documented composition (*Arnica montana*, *Lobelia inflata*, *Gelsemium sempervirens*, *Ranunculus montanus*, *Senecio vulgaris*, *Datura stramonium*, *Hyoscyamus niger*, and *Solanum nigrum*). Besides, more than 200 other alkaloids were tentatively identified using an extensive in-house database with exact masses and molecular formulas (isotopic patterns) found in literature.

Overall, the 67 plant extracts contained 146 different alkaloids (or their isomers): 4 were identified by comparison with the analytical standards, 8 comparing the retention time and fragmentation profile, and 154 tentatively identified using the in-house database. The most frequently present alkaloids were Allosedamide, Piperidine and 8-ethylnorlobelol (belonging to the piperidine group); 5-methoxyvascine (quinazoline); one isomer of Nicotinic acid (pyridine); Europine/Heliotrine N-oxide (pyrrolizidine); Fluoxetine (protoalkaloid), and Quinidine (quinoline). The plants with the largest number of detected alkaloids were *Carex sempervirens*, *Biscutella laevigata* and *Leontodon hispidus*, having over 50 different alkaloids, or their isomers.

For the herbal mixes, target analysis allowed to detect 3 alkaloids (Lycopsamine in 42% of samples; Gramine, 31%; and Veratramine, 8%) with concentrations ranging from 0.4 to 8.5, 3.7-65, and 40-350 $\mu\text{g kg}^{-1}$, respectively. The untargeted analysis showed the presence of 120 other different alkaloids. The most frequent were Nicotinic acid, Venoterpine, Fluoxetine, 8-ethylnorlobelol, Ephedrine/Feruginine, Bellendine, 2-Pyrrolidineacetic methyl ester, 5-methoxyvascicolol, Gentiatibetine, Onetine, Piperidine, 5-methoxyvascine, Gelsemine, and Yohimbine, or the corresponding isomers.

As regards milk samples, the target analysis allowed to quantify 3 alkaloids (Lycopsamine in 52% of samples; Gramine, 44%; and Senkirkin, 8%) with concentrations ranging from 0.04 to 0.1, 3.7-65, and 0.4-17 $\mu\text{g kg}^{-1}$, respectively, while the untargeted analysis detected 40 other alkaloids, being 8-ethylnorlobelol, 2-Pyrrolidineacetic methyl ester, (-)-Coclaurine/Chavicine/Piperine, 5-methoxyvascicolol, Valerine, 3-acetyltropine, and one isomer of 8,10-diethyllobelidiol the most frequently present.

The results showed for herbal mixes and milks very differentiated and complex alkaloid profiling. Reasonably, the assessment of transfer mechanisms of alkaloids from herbs to milk must then take into account also factors such as their bacterial or enzymatic degradation, the metabolic waste disposal, and the reduced assimilation during digestive processes.

IV – Conclusions

The proposed analytical method allowed to define a wide and detailed database regarding alkaloid composition, and represented a useful tool for the compositional description of a large selection of herbs sampled in mountain pastures. Moreover, the first findings on the presence of the same alkaloids in both the herbal mixes consumed daily by cows, and the corresponding produced milks, suggest interesting perspectives of feed traceability.

References

- Bovolenta S., Romanzin A., Corazzin M., Spanghero M., Aprea E., Gasperi F. and Piasentier E., 2014.** Volatile compounds and sensory properties of Montasio cheese made from the milk of Simmental cows grazing on alpine pastures. *Journal of Dairy Science*, 97, 1-13.
- Duarte R.A., Mello E.R., Araki C., Bolzani V.S., Silva D.H.S., Regasini L.O., Silva T.G.A., Morais M.C.C., Ximenes V.F. and Soares C.P., 2010.** Alkaloids extracted from *Pterogyne nitens* induce apoptosis in malignant breast cell line. *Tumor Biology*, 31, 513-522.
- Hartmann T., 2007.** From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Science Direct*, 68, 2831-2846.
- Hoogenbooma L.A.P., Muldera P.P.J., Zeilmakerb M.J., Van den Topab H.J., Rimmelink G.J., Brandonb E.F.A., Klijnstra M., Meijerc G.A.L., Schothorstb R. and Van Egmond H.P., 2011.** Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows. *Food Additives and Contaminants*, 28, 359-372.
- Larson R.A. and Marley K.A., 1894.** Quenching of singlet oxygen by alkaloids and related nitrogen heterocycles. *Pytochemistry*, 23, 2351-2354.
- Liu R., Gu P., Wang L., Cheng M., Wu Y., Zheng L., Liu Y. and Ding L.m 2016.** Study on the pharmacokinetic profiles of corynoline and its potentia interaction in traditional Chinese medicine formula Shuanghua Baihe tablets in rats by LC-MS/MS. *Journal of Pharmaceutical and Biomediacal Analysis*, 117, 247-254.
- Mudge E.M., Maxwell A., Jones P. and Brown P.N., 2015.** Quantification of pyrrolizidine alkaloids in North American plants and honey by LC-MS: single laboratory validation. *Food Additives & Contaminants: Part A*, 32, 1-7.
- Rasmann S. and Agrawal A.A., 2008.** In defence of roots: A research agenda for studying plant resistance to belowground herbivory. *Plant Physiology*, 146, 875-880.
- Rouge P., Cornu A., Biesse-Martin A.-S., Lyan B., Rochut N. and Graulet B., 2013.** Identification of quinoline, corboline and glycinamide compounds in cow milk using HRMS and NRM. *Food Chemistry*, 141, 1888-1894.
- Wiedenfeld H. and Edgar J., 2011.** Toxicity of pyrrolizidine alkaloids to humans and ruminants. *Phytochem Reviews*, 10, 137-151. DOI 10.1007/s11101-010-9174-0.