Journal of Mind and Medical Sciences

Volume 5 | Issue 2 Article 22

2018

Determination of pyrrolizidine alkaloids in dietary sources using a spectrophotometric method

Oana C. Șeremet
Carol Davila University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Pharmacology and Clinical Pharmacy, Bucharest, Romania

Octavian T. Olaru
Carol Davila University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Botany and Cellular Biology, Bucharest, Romania

Mihaela Ilie
Carol Davila University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Toxicology, Bucharest, Romania

Claudia M. Guțu
Carol Davila University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Toxicology, Bucharest, Romania

Mihai G. Nițulescu
Carol Davila University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Bucharest, Romania

See next page for additional authors

Determination of pyrrolizidine alkaloids in dietary sources using a spectrophotometric method

Authors
Oana C. Şeremet, Octavian T. Olaru, Mihaela Ilie, Claudia M. Guţu, Mihai G. Nițulescu, Camelia Diaconu, Catalina Motofei, Denisa Margină, Simona Negreș, Cristina E. Zbârcea, and Emil Ștefănescu

J Mind Med Sci. 2018; 5(2): 294-299
doi: 10.22543/7674.52.P294299

Research article

Determination of pyrrolizidine alkaloids in dietary sources using a spectrophotometric method

Oana C. Șeremet[1,2], Octavian T. Olaru[1,3], Mihaela Ilie[1,4], Claudia M. Guțu[1,4], Mihai G. Nițulescu[1,5], Camelia Diaconu[1,6], Catalina Motofei[7], Denisa Margină[1,8], Simona Negreș[1,2], Cristina E. Zbârcea[1,2], Emil Ștefănescu[1,2]

[1]Carol Davila University of Medicine and Pharmacy, Bucharest, Romania: [2]Faculty of Pharmacy, Department of Pharmacology and Clinical Pharmacy; [3]Faculty of Pharmacy, Department of Botany and Cellular Biology; [4]Faculty of Pharmacy, Department of Toxicology, [5]Faculty of Pharmacy, Department of Pharmaceutical Chemistry, [6]Internal Medicine Clinic of Emergency Hospital of Bucharest, Romania, [7]ASE Bucharest, Department of analysis and economic-financial evaluation, Statistics, Bucharest, Romania, [8]Faculty of Pharmacy, Department of Biochemistry, Bucharest, Romania

Abstract

Pyrrolizidine alkaloids (PAs) are a class of toxic compounds found in the composition of more than 6000 plants. People can be exposed to PAs by consuming phytotherapeutic products, food from crops contaminated with seeds of some species with high content of PAs, and/ or contaminated animal products like bee products. For this reason we developed and validated a method for quantitative determination of PAs, from the most frequently contaminated food sources, honey and flour. Colorimetric Ehrlich reagent method was used with standard addition (1mg/kg senecionine). The extraction solvent was methanol 50% acidified with citric acid to pH 2-3, as this solvent can be used for alkaloids and N-oxides. We found that, in extracting the alkaloid only once from the dietary sources, the percent of recovery is low (52.5% for honey, and 45.75% for flour). Using successive extractions, three times with the same solvent, the senecionine retrieval percentage increased to 86.0% for honey and 76.0% for flour. The method was validated using the following parameters: selectivity, linearity (0,25- 20 mg/ mL senecionine), accuracy (average recovery 93.5 - 107.93%) and precision (RSD 3,26-4.55%.). The calculated limit of quantification (0.174 mg/ mL) makes this method applicable for determining Pas occurring at toxic levels for consumers.

Keywords : pyrrolizidine alkaloids, spectrophotometry, contaminated food, Ehrlich reagent

Highlights

- ✓ It is presented a simple and inexpensive method for the quantitative determination of PAs in honey and flour, with a quantification limit (0.174 μg/mL).
- ✓ This method is applicable for the determination of PAs from food sources at toxic levels for consumers.

To cite this article: Șeremet OC, Olaru OT, Ilie M, Guțu CM, Nițulescu MG, Diaconu C, Motofei C, Margine D, Negreș S, Zbârcea CE, Ștefănescu E. Determination of pyrrolizidine alkaloids in dietary sources using a spectrophotometric method. *J Mind Med Sci*. 2018; 5(2): 294-299. DOI: 10.22543/7674.52.P294299

*Corresponding author: Emil Stefanescu, Carol Davila University of Medicine and Pharmacy, Department of Pharmacology and Clinical Pharmacy, Bucharest, Romania
Email: emil_e_stefanescu@yahoo.com

Introduction

Pyrolizidine alkaloids (PAs) are a class of natural compounds found in the composition of more than 6000 plants. More than 350 PAs are known to date, half of which are toxic (1). Toxicity of PAs in animal studies is characterised by hepatotoxicity, carcinogenicity, and genotoxicity (2, 3).

PAs are found in plants mainly as highly polar N-oxides, soluble in water and insoluble in most non-polar solvents or as tertiary bases, soluble in non-polar organic solvents (dichloromethane, chloroform), but also in polar solvents (eg. methanol). At low pH values, PAs are protonated and become water soluble (4, 5). N-oxides are easily converted to the corresponding toxic alkaloid base by enzymatic reactions in plants or in the digestive tract of animals or humans (6).

Besides the use of phytotherapeutic products with PAs, contaminated food products such as milk, eggs, bee products (honey, pollen), cereals and derived products (flour), and packaged salads, represent important sources of exposure to PAs (6, 7). In the literature, the products reported to be the most commonly contaminated are bee products and flour (5).

Various methods for the determination of PAs from food sources are known. Most are HPTLC (8), GC-MS (9, 10) or LC-MS methods (11). The chromatographic methods have the advantage of having very low detection limits, but they are expensive.

The literature cites numerous examples of spectrophotometric methods used for dosing alkaloids in food (12), pharmaceutical products (13), or plant products (14). For PAs, the best known are those described by Birecka et al. (1981) and Mattocks (1967) (15, 16). The first is based on the formation of a chloroform-soluble complex between PAs and methylorange, which in acid media releases the indicator that can be measured at 525 nm (15, 17). This method, however, is not characteristic for PAs, and can also be used for dosing other types of alkaloids. The second, based on reaction with the Ehrlich reagent, has the advantage of being able to quantify very small concentrations of alkaloids. This method is specific for alkaloids having an unsaturated pyrrole heterocycle at the 1-2 position, a double bond characteristic of PAs. It is also possible to determine the N-oxide content by this method because the PAs react directly with the Ehrlich reagent (18, 19).

Taking into account all these aspects, the purpose of this study was to develop and validate a simple and inexpensive spectrophotometric method able to determine PA presence in the most commonly contaminated food sources, honey and flour. We used the standard addition method (senecionine) at a concentration of 1mg/kg flour or honey.

Materials and Methods

Principle

PAs with a double bond in the 1,2-position of the heterocycle, in the presence of hydrogen peroxide, are converted to the corresponding N -oxides and acylated with acetic anhydride. By heating, acetic acid is removed and a pyrrole derivative is formed, which further reacts with 4-dimethylaminobenzaldehyde (Ehrlich's reagent) to form a violet-colored complex that can be spectrophotometrically measured. By omitting the first step (conversion of PAs to their N-oxides by hydrogen peroxide), only the N-oxides will be determined (Figure 1) (16, 20).

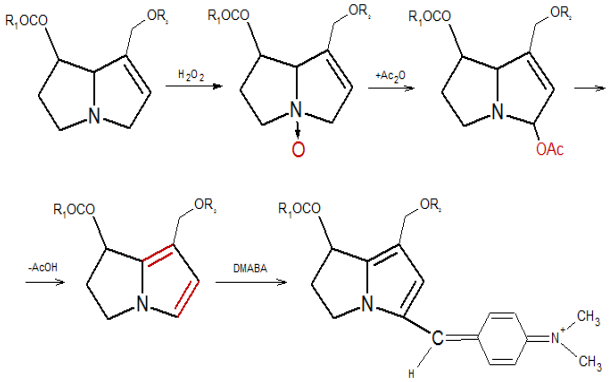

Figure 1. The coupling reaction of the double bond PAs with 4-dimethylaminobenzaldehyde (21)

Materials

– Standard solution

Standard solutions in methanol were prepared from a stock solution of senecionine (200 mg/mL, Carl Roth, Germany).

– Samples

Two samples of 30 g of polyfloral honey (SC Proapis SRL, Calarasi, Romania) and white wheat flour (Goldmaya, Satu Mare, Romania) were mixed with 150 ml of methanol 50% (Merck, Germany), acidified with citric acid to pH 2-3. Standard solution of senecionine (100 µg/mL, 0.3 ml) was added to each sample, mixed, and filtered. For one honey and one flour sample, the residue was again mixed with 150 mL of acidified 50% methanol and filtered. The operation was repeated twice and the filtrates combined.

All solutions were reduced to about 30 mL each and purified by liquid-liquid extraction 2 times with 30 mL

chloroform and 2 times with 30 mL ethyl ether. The samples were filtered, alkalinized with 25% ammonia solution to pH 9-10, and extracted 3 times with 30 mL chloroform. The chloroform solutions were reduced to about 5 mL using a rotary evaporator (Buchi, Switzerland) and brought to dryness under nitrogen with TechneDry-Block DB-3D (BibbyScientific Inc., Great Britain). The residue is dissolved in 3 mL of methanol and passed through 0.2 µm adaptive syringe filters (Pall Life Science, USA). In parallel, two control samples (honey and flour) were processed in the same way, but no standard solution was added.

The samples were noted as H1 (honey extracted once), H2 (honey extracted 3 times) and HC (honey control), respectively, and F1 (flour extracted once), F2 (flour extracted 3 times) and FC (flour control).

– Reagents

Oxidation reagent: 20 mL methanol (Merck, Germany) was mixed with 0.20 mL hydrogen peroxide 30% (Merck, Germany) containing sodium pyrophosphate (Merck, Germany) 5 mg/mL as a stabilizer, 0.20 mL ethylene glycol (Merck, Germany) and 20 mg butylated hydroxytoluene (Sigma-Aldrich, USA)

Diglyme: Diethylene glycol dimethyl ether (Merck, Germany) containing 5 mg/mL butylated hydroxytoluene (Sigma-Aldrich, USA). Acetic anhydride (Sigma Aldrich, USA) was redistilled and the fraction boiling between 136–139 °C was collected.

Modified Ehrlich's reagent: 4 mL boron trifluoride in methanol 14% (Acros Organics, Belgium) is diluted with 36 mL absolute ethanol (Scharlau Chemicals, Spain) and 0.8 g 4-dimethylaminobenzaldehyde (Sigma Aldrich, USA) are added.

Method

For the preparation of the calibration curve, volumes of the working standard solutions, corresponding to 2.5–20 µg senecionine, were evaporated under nitrogen jet; 0.5 mL oxidizing reagent was added and the test tubes were left in the boiling water bath WNB 10 (Memmert, Germany) for 20–30 min. 1 mL diglyme and 0.1 mL acetic anhydride were added, and the tubes were heated again in the water bath for 1 minute. After cooling the tubes at room temperature, 1 mL of modified Ehrlich reagent was added, and the tubes were heated in a water bath at 55–60 °C for 4–5 minutes. The samples were transferred in volumetric flasks and acetone (Chimopar, Romania) was added up to 10 mL.

For assessing the alkaloid content, 0.4 ml of each sample was evaporated under nitrogen stream and the same procedure was followed as described in the preparation of the calibration curve.

All measurements were performed on a Halo DB-20 (Dynamica, Great Britain) spectrophotometer, at 565 nm versus a blank (prepared in the same way but without standard addition). All assays were performed in triplicate and the results were expressed as mean±standard deviation (M±SD). Statistical analysis was performed using Microsoft Excel 2010 software (Microsoft Corp., USA) and GraphPad Prism v. 5.0. (GraphPad Software, USA).

Results

A six points linear calibration curve of senecionine in the 0.25 – 2 µg/mL range, with good linearity (R = 0.9996) was obtained (Figure 2)

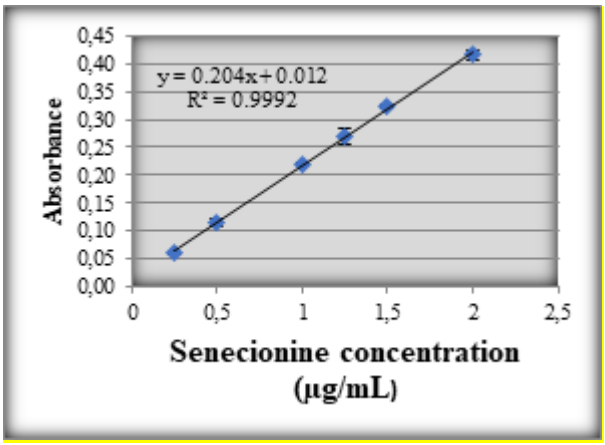

Figure 2. The standard calibration curve and equation for senecionine

Validation of the newly established method was performed according to analytical guidelines (22), with results summarized in Table I.

Table I. Characteristics of regression line, detection and quantification limit

Validation criterion	Parameter	Results
Linearity	Slope	0.2045 ± 0.002839
	Y-intercept when X=0.0	0.01278 ± 0.003502
	Correlation coefficient	0.9996
Precision*		RDS=3.84%
Range		0.25 – 2 µg/mL.
Detection limit (LD)**		0. 058 µg/mL
Quantification limit (LQ)**		0.174 µg/mL

* from 3 replicate for 3 different concentrations corresponding to 0.6 µg/mL; 0.8 µg/mL and 1 µg/mL senecionine; **based on the standard deviation of the response and slope

The results of the quantitative determinations are presented in Table II. By twice washing the residue obtained after filtration with acidified methanol 50%, the recovery percentage of senecionine increased from 52.5% to 86.00% in honey and from 45.75% to 76.00% in flour.

Table II. Recovery of senecionine from food sources.

Sample	Added senecionine (mg/kg)	Recovery (mg/kg)	Recovery (%)
H_1	1.00	0.525	52.50 %
H_2		0.860	86.00 %
F_1		0.468	45.75 %
F_2		0.760	76.00 %

Discussions

Contamination of food with PAs is a serious health issue. Kakar et al. (2010) reported 67 cases of veno-ocular disease in Afghanistan due to the consumption of bakery products made from flour obtained from wheat crops contaminated with Heliotropium species. Flour samples were taken from households where these cases were recorded and an average concentration of 5.6 mg PAs/ kg flour was found (11). Azadbakh and Talavaki (2002) determined the PA concentration from 40 samples of flour obtained from wheat contaminated with Senecio vulgaris in the province of Mazandaran, Iran. The total PA concentration was between 40-100 mg/kg flour (22, 23).

Also, numerous cases of honey contaminated with PAs have been reported. Deinzer et al. (1977) detected a concentration of up to 3900 µg PAs/kg in honey from Senecio jacobaea (24, 25). Other reports found concentrations of PAs up to 1480 µg/kg or 2850 µg/kg in honey (26-28). If honey contains about 2500 µg PAs/kg, an adult who consumes a daily average portion of 40 g of honey would be exposed to 100 µg PAs per day, a quantity higher than the acceptable European doses (6).

Taking into account these premises, we developed a method for the quantitative determination of PAs from food sources (honey and flour). Acidified 50% methanol was used as the extraction solvent because both the alkaloid bases (which at this pH is converted to soluble salts) and N-oxides are soluble in this solvent. We have established that by using only one solvent extraction of alkaloids from food sources, the recovery percentage of senecionine is low (52.5% for honey and 45.75% for flour). By successive extractions, three times, with the same solvent, the sencecione retrieval percentage increases to 63.81% in honey and 66.12% in flour.

The method was validated by the following parameters: selectivity, linearity (on the 0.25-20 µg/ mL range), accuracy (average recovery yield between 93.5 and 107.93%) and precision (RSD between 3.26 - 4.55%).

Conclusions

Because of the health hazards raised by the consumption of food contaminated with PAs, we developed a simple and inexpensive method for the quantitative determination of PAs in honey and flour, with a quantification limit (0.174 µg/mL) that makes the method applicable for the determination of PAs from food sources at toxic levels for consumers.

Acronyms and abbreviations

- GC-MS - gas chromatography coupled with mass spectrometry
- HPTLC - high performance thin layer chromatography
- LC-MS - liquid chromatography coupled with mass spectrometry
- PAs - pyrrolizidine alkaloids
- RDS – relative standard deviation

† In Memoriam: The authors would like to respectfully dedicate this article to Ms. Mihaela Ilie, who passed away on 1 January 2018.

Conflict of interest disclosure

The authors declare that there are no conflicts of interest to be disclosed for this article.

References

1. Fu PP, Xia Q, Lin G, Chou MW. Pyrrolizidine alkaloids – genotoxicity, metabolism enzymes, metabolic activation and mechanisms. *Drug Metab Rev*. 2004; 36(1): 1-55. DOI: 10.1081/DMR-120028426
2. WHO-IPCS (World Health Organisation-International Programme on Chemical Safety). Pyrrolizidine alkaloids. *Environmental Health Criteria*. 80. 1988.

http://www.inchem.org/documents/ehc/ehc/ehc080.htm.

3. Şeremet OC, Bărbuceanu F, Ionică FE, Margină DM, Guţu CM, Olaru OT, Ilie M, Gonciar V, Negreş S, Chiriţă C. Oral toxicity study of certain plant extracts containing pyrrolizidine alkaloids. *Rom J Morphol Embryol*. 2016; 57(3): 1017–23.
4. Mattocks AR. Chemistry and toxicology of pyrrolizidine alkaloids. London, New York, Academic Press, 1986.
5. EFSA (European Food Safety Authority), Scientific Opinion on Pyrrolizidine alkaloids in food and feed. *The EFSA Journal*. 2011; 9(11): 2406-40. DOI: DOI: 10.2903/j.efsa.2011.2406
6. Committee on Herbal Medicinal Products. Public statement on the use of herbal medicinal products containing toxic, unsaturated pyrrolizidine alkaloids (EMA/HMPC/893108/2011), 2014. Available on-line: http://www.ema.europa.eu/docs/en_GB/document_library/Public_statement/2014/12/WC500179559.pdf
7. Molyneux RJ, Gardner DL, Colegate SM, Edgar JA. Pyrrolizidine alkaloid toxicity in livestock: a paradigm for human poisoning? *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2011; 28(3): 293-307. DOI: 10.1080/19440049.2010.547519
8. Seremet OC, Olaru OT, Ilie M, Negres S, Balalau D. HPTLC Evaluation Of The Pyrollizidine Alkaloid Senecionine In Certain Phytochemical Products. *Farmacia*. 2013; 61(4): 756 – 63.
9. Kempf M, Wittig M, Reinhard A, von der Ohe K, Blacquière T, Raezke KP, Michel R, Schreier P, Beuerle T. Pyrrolizidine alkaloids in honey: comparison of analytical methods. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2011; 28(3): 332-47. DOI: 10.1080/19440049.2010.521772
10. Seremet OC, Olaru OT, Gutu CM, Nitulescu GM, Ilie M, Negres S, Zbarcea CE, Purdel CN, Spandidos DA, Tsatsakis AM, Coleman MD, Margina DM. Toxicity of plant extracts containing pyrrolizidine alkaloids using alternative invertebrate models. *Mol Med Rep*. 2018; 17(6): 7757-63. DOI: 10.3892/mmr.2018.8795.
11. Kakar F, Akbarian Z, Leslie T, Mustafa ML, Watson J, van Egmond HP, Omar MH, Mofleh J. An outbreak of hepatic veno-occlusive disease in Western Afghanistan associated with exposure to wheat flour contaminated with pyrrolizidine alkaloids. *J Toxicol*. 2010; 2010: 313280. DOI: 10.1155/2010/313280
12. Shar Z, Chippa H, Hussain N, Arain M, Khan A, et al. Spectrophotometric Determination of Caffeine in Selected Pakistani Beverages. *J Food Processing & Beverages*. 2017; 5(1): 1-4.
13. Begum J, Rao KS, Rambabu C. Assay of yohimbine chloride in bulk samples and pharmaceutical formulations by extractive spectrophotometry. *Asian Journal of Chemistry*. 2006; 18(2): 1417-22.
14. Vachnadze V, Dzhakeli EZ, Dadeshidze IA, Kintsurashvili LG. Quantitative spectrophotometric determination of alkaloids in roots of Vinca herbacea. *Pharmaceutical Chemistry Journal*. 2010; 44(4): 199-201.
15. Birecka H, Catalfamo JL, Eisen N. A sensitive method for detection and quantitative determination of pyrrolizidine alkaloids. *Phytochemistry*. 1981; 20(2): 343-4.
16. Mattocks AR. Spectrophotometric determination of unsaturated pyrrolizidine alkaloids. *Anal Chem*. 1967; 39(4): 443-7.
17. Şeremet OC, Olaru OT, Ilie M, Negreş S, Bălălău D. Phytotoxicity Assessment of Certain Phytochemical Products Containing Pyrrolizidine Alkaloids. *Acta Medica Marisiensis*. 2013; 59(5): 250-253. DOI: 10.2478/amma-2013-0057
18. Roeder E, Liu K, Mütterlein R. Quantitative photometrische Bestimmung der Pyrrolizidinalkaloide in Symphyti Radix. *Fresenius J Anal Chem*. 1992; 343(8): 621-624. DOI: 10.1007/BF00324124
19. Motofei IG, Rowland DL, Georgescu SR, Tampa M, Baconi D, Stefanescu E, Baleanu BC, Balalau C, Constantin V, Paunica S. Finasteride adverse effects in subjects with androgenic alopecia: A possible therapeutic approach according to the lateralization process of the brain. *J Dermatolog Treat*. 2016; 27(6): 495-497. DOI: 10.3109/09546634.2016.1161155
20. Şeremet OC, Olaru OT, Bălălău D, Negreş S. The effect of certain plant extracts containing pyrrolizidine alkaloids on Lactuca sativa radicle growth. *Rom J Biopys*. 2014; 24(1): 1-9.
21. Mattocks AR. Spectrophotometric determination of pyrrolizidine alkaloids – some improvements. *Anal Chem*. 1968; 40(11): 1749-50.
22. Barko Bartkowski JP, Wiedenfeld H,Roeder E. Quantitative Photometric Determination of Senkirkine in Farfarae Folium. *Phytochemical Analysis*. 1997; 8(1): 1–4. DOI:

10.1002/(SICI)1099-1565(199701)8:1<1::AID-PCA328>3.0.CO;2-H

23. ICH-Q2 (R1) Validation of Analytical Procedures: Text and Methodology, 1995 (CPMP/ICH/381/95). Available on-line: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002662.pdf
24. Azadbakht M, Talavaki M. Qualitative and quantitative determination of pyrrolizidine alkaloids of wheat and flour contaminated with Senecio in Mazandaran province farms. *Iranian Journal of Pharmaceutical Research*. 2003; 2(3): 179-183.
25. Diaconu CC, Dragoi CM, Bratu OG, Neagu TP, Pantea Stoian A, Cobelschi PC, Nicolae AC, Iancu MA, Hainarosie R, Stanescu AMA, Socea B. New approaches and perspectives for the pharmacological treatment of arterial hypertension. Farmacia 2018, 66(3):408-415.
26. Deinzer ML, Thomson PA, Burgett DM, Isaacson DL. Pyrrolizidine alkaloids: their occurrence in honey from tansy ragwort (Senecio jacobaea L.). *Science*. 1977; 195(4277): 497-9.
27. Crews C, Startin JR, Clarke PA. Determination of pyrrolizidine alkaloids in honey from selected sites by solid phase extraction and HPLC-MS. *Food Additives and Contaminants*. 1997; 14(5): 419–428. DOI: 10.1080/02652039709374547
28. Betteridge K, Cao Y, Colegate SM. Improved method for extraction and LCMS analysis of pyrrolizidine alkaloids and their N-oxides in honey: Application to Echium vulgare honeys. *J Agric Food Chem*. 2005, 53(6): 1894–902. DOI: 10.1021/jf0480952