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DEVELOPMENT AND VALIDATION OF A QuEChERS METHOD COUPLED TO HRMS DETECTION TO DETERMINE PYRROLIZIDINE AND TROPANE ALKALOIDS IN HONEY

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There is a growing interest in natural toxins in recent years, due to their potential hazard for human and animal health related to their presence as contaminants in food and feed and the occurrence of botanical toxins especially at sub-chronic levels, is possibly underestimated.

Awareness about the presence of potentially dangerous natural toxins in food was raised by the European Food Safety Authority through scientific opinions on pyrrolizidine alkaloids (PAs) and tropane alkaloids (TAs) in food, stressing the lack of data and gaps of knowledge required to improve the current risk assessment strategy



2007 Opinion of the scientific panel on contaminants in the food chain on a request from the European Commission related to **pyrrolizidine alkaloids** as undesirable substances in animal feed. *EFSA Journal*, 447, 1-51.

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

2013 Scientific opinion on **tropane alkaloids** in food and feed. EFSA Panel of Contaminants in the Food Chain. *EFSA Journal*, 11(10), 3386.

Pyrrolizidine alkaloids (PAs)

PAs are probably the most widely distributed natural toxins, over 350 PAs have been identified so far, diffused in more than 6,000 species. The main sources are the families of the *Boraginaceae* (all genera), *Asteraceae* (tribes *Senecioneae* and *Eupatorieae*) and *Fabaceae* (genus *Crotalaria*).



Borago officinalis



Senecio jacobea

Poisoning : associated with acute hepatic veno-occlusive disease (HVOD), with hepatomegaly, ascites and massive pleural effusion. The acute disease can cause high mortality, whereas a sub-acute or chronic onset may lead to liver cirrhosis.

In different animal models PAs induce DNA mutations, teratogenicity and carcinogenicity. Based on the actual knowledge, EFSA concluded that PAs may act as carcinogens in humans.

One of the most common sources of PAs exposure in humans is honey consumption.

Pyrrolizidine alkaloids (PAs)



Although the “matter of PAs” is now well known, a common European legislation about PAs to establish consumption or concentrations limits in foods is not yet available.

Tropane alkaloids (TAs)

TAs are secondary metabolites which naturally occur in plants of several families including *Solanaceae* (the most common sources being *Atropa belladonna* and genus *Datura*), *Erythroxylaceae*, *Proteaceae*, *Euphorbiaceae*, *Rhizophoraceae*, *Convolvulaceae* and *Cruciferae*.



Atropa belladonna

The group of TAs comprises more than 200 compounds but the only data available concerning the occurrence in food and feed regard atropine and scopolamine.



Datura stramonium

Toxicological effects: related to the inhibition of muscarinic acetylcholine receptors in the central and autonomic nervous system (dryness of the mucosa of the upper digestive and respiratory tract, constipation, pupil dilatation and disturbance of vision, photophobia, hypotension, bradycardia or tachycardia, arrhythmias, nervousness, and, in case of overdose, hypertension, restlessness, irritability, disorientation, ataxia, seizures and respiratory distress).

Tropane alkaloids (TAs)



In June 2015 the European Commission issued the **Recommendation (EU) 2015/976** on the monitoring of the presence TAs in food.

In February 2016 entered into force the **Commission Regulation (EU) 2016/239**, amending Regulation (EC) n. 1881/2006, which set maximum levels for some contaminants in foodstuffs, adding TAs in the list of the “regulated” contaminants.

20.2.2016

EN

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COMMISSION REGULATION (EU) 2016/239

of 19 February 2016

amending Regulation (EC) No 1881/2006 as regards maximum levels of tropane alkaloids in certain cereal-based foods for infants and young children

(Text with EEA relevance)

Regulation (EU) 2016/239 states that the maximum levels of atropine and of scopolamine in certain cereal-based foods for infants and young children should be 1.0 µg/kg.

The present study aimed at the elaboration and **validation** of a rapid method for the simultaneous determination of PAs and TAs in a complex food matrix such as honey.

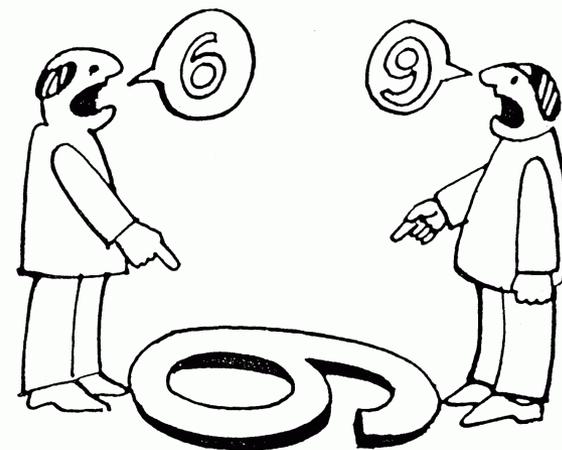
VALIDATION



“Confirmation by **examination** and the provision of **objective evidence** that the particular requirements of a **specific intended use** are fulfilled”

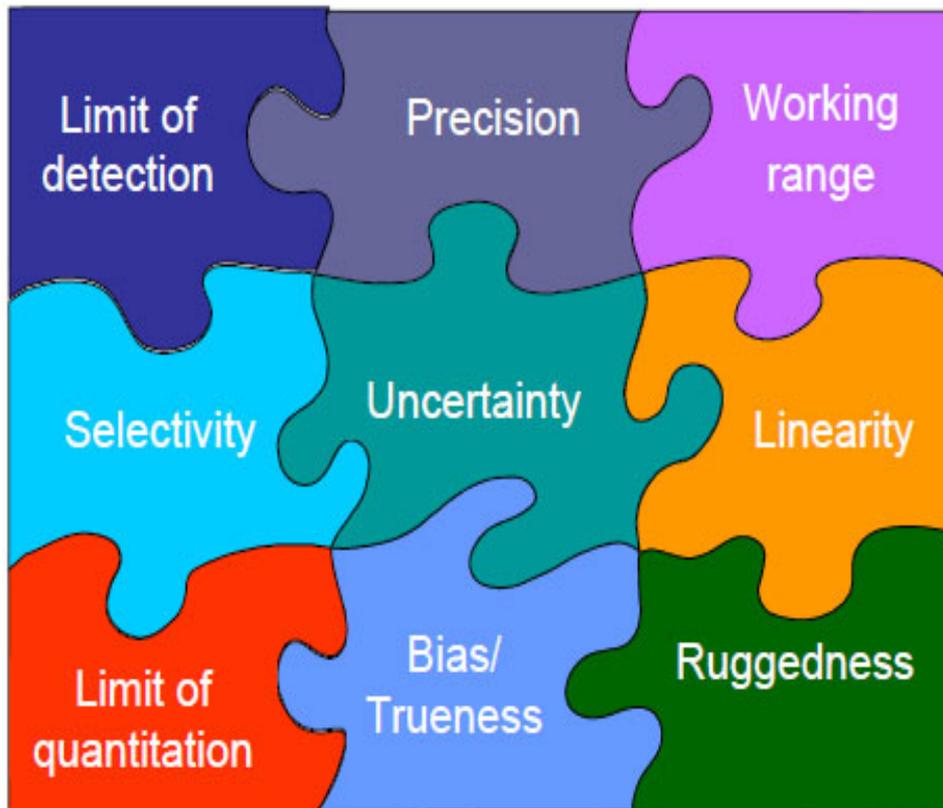
ISO 17025:2005 - General requirement for the competence of calibration and testing laboratories

The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice, ensuring the comparability between analysts/ laboratories/countries.



Validation of analytical methods is also required by most regulations and quality standards that impact laboratories.

VALIDATION



«The validation puzzle»

Selectivity/specificity

🧩 am I measuring what I think I'm measuring?
are there any interferences?

Precision (repeatability, reproducibility)

🧩 how close are the results of replicate measurements made on the same sample?

Bias, recovery

🧩 how close are the results to the 'right' answer

Working range

🧩 range of analyte concentrations that can be measured reliably
🧩 limit of detection, limit of quantitation, linearity

Ruggedness/robustness

🧩 control necessary for each stage of the procedure

Uncertainty

🧩 dispersion of the quantity values being attributed to a measurand

Natural toxins as PAs and TAs are still not included in any guideline concerning the performance of analytical methods for contaminants in food, but

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amending Regulation (EC) No 1881/2006 as regards maximum levels of tropane alkaloids in certain cereal-based foods for infants and young children

amends Regulation (EC) No. 1881/2006 setting maximum levels for some contaminants in foodstuffs. This in turn refers to

20.8.2011

EN

Official Journal of the European Union

L 215/9

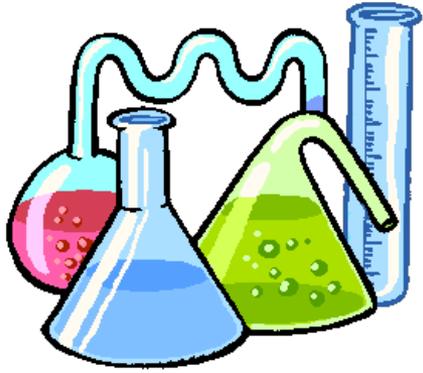
COMMISSION REGULATION (EU) No 836/2011

of 19 August 2011

amending Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs

In view of this, the validation was carried out referring to these communitary guidelines.

EXTRACTION METHOD



Quick
Easy
Cheap
Effective
Rugged
Safe

Dissolve honey (3 g) in sulphuric acid 0.1 mol/L (10 mL) and add zinc dust (0.5 g), weakly shake for 1.30 h, centrifuge



Pour supernatant into a QuEChERS extract tube with acetonitrile (10 ml)



Extraction step: add ceramic homogenizer and **QuEChERS salt EN method**, vigorously shake for 4 min, centrifuge



Purification step: supernatant (8 mL) into QuEChERS dSPE (PSA and MgSO_4), vortex 1 min, centrifuge



Dry supernatant (6 mL) under vacuum at 40°C and re-dissolve it with 1 mL of reconstitution solution

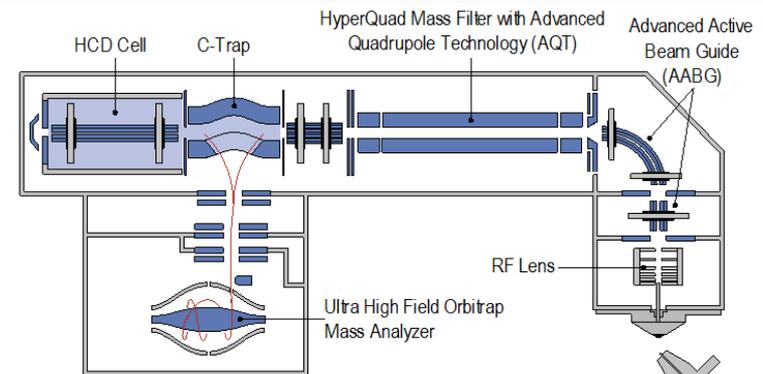
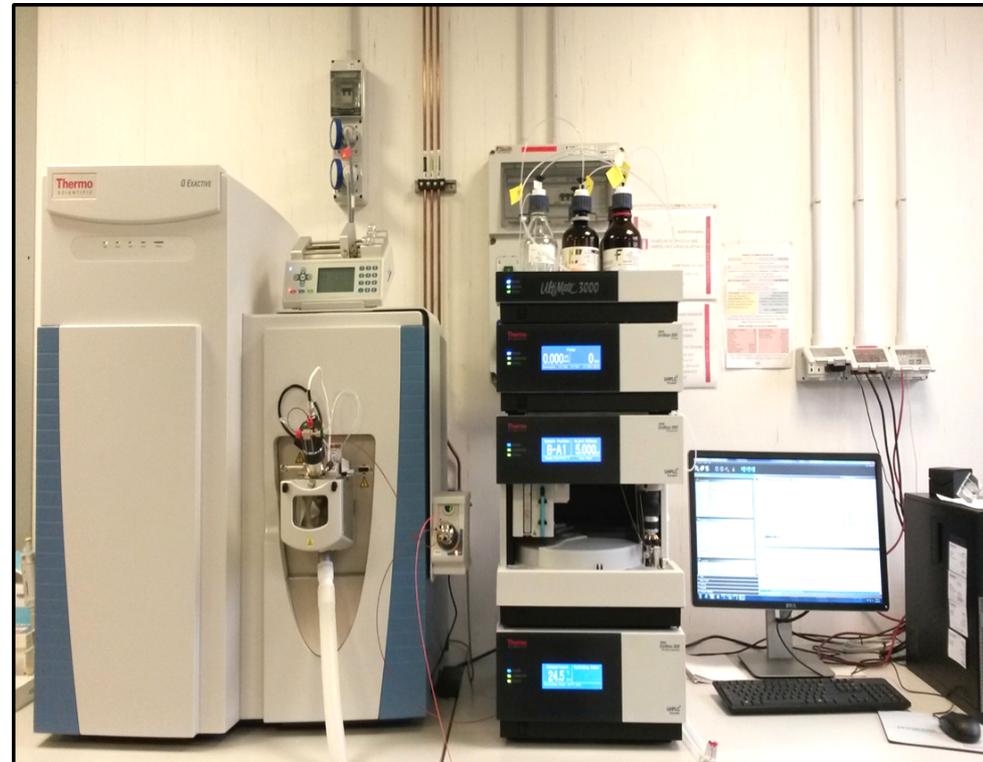
DETECTION

Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer

High resolution mass spectrometer (**HRMS**) analyzer couples the high resolution performances of the Orbitrap with the high selectivity of the quadrupole

High mass accuracy can be helpful in the analysis of complex samples such as honey where many interfering compounds co-extracted from the matrix are present, allowing separation of isobaric compounds.

The use of Full Scan-data dependent acquisition (FS-DDA) method allows to perform a further qualitative and quantitative retrospective data analysis of additional analytes.



HRMS parameters of FS and MS/MS detection

Analyte	R _T (min)	Precursor mass [M+H] ⁺	Fragment 1 (m/z)	Fragment 2 (m/z)	Fragment 3 (m/z)	NCE
Intermedine	6.4	300.18055	156.102	138.091	120.081	35
Lycopsamine	6.7	300.18055	156.102	138.091	120.081	35
Scopolamine	8.2	304.15433	156.102	138.091	121.065	40
Retrorsine	9.2	352.17546	324.180	138.091	120.081	35
Heliotrine	9.7	314.19620	156.102	138.091	120.081	35
Seneciphylline	10.0	334.16490	306.170	174.076	138.091	35
Atropine	11.0	290.17507	260.164	124.112	93.070	35
Senecionine	11.9	336.18055	138.091	120.081	94.066	45
Senkirkine	13.2	366.19111	168.102	150.091	122.060	35
Echimidine	13.3	398.21733	220.133	120.081	83.050	25
Lasiocarpine	14.7	412.23298	336.180	220.133	120.081	35

HRMS conditions

Parameters FullMS		Parameters dd-MS ² / dd-SIM	
Polarity	Positive	Resolution	17500
Resolution	70000	AGC target	2x10 ⁵
AGC target	3x10 ⁶	Maximum IT	75 ms
Maximum IT	290 ms	Loop count	10
Scan range	150 to 650 m/z	TopN	10

Chromatographic conditions

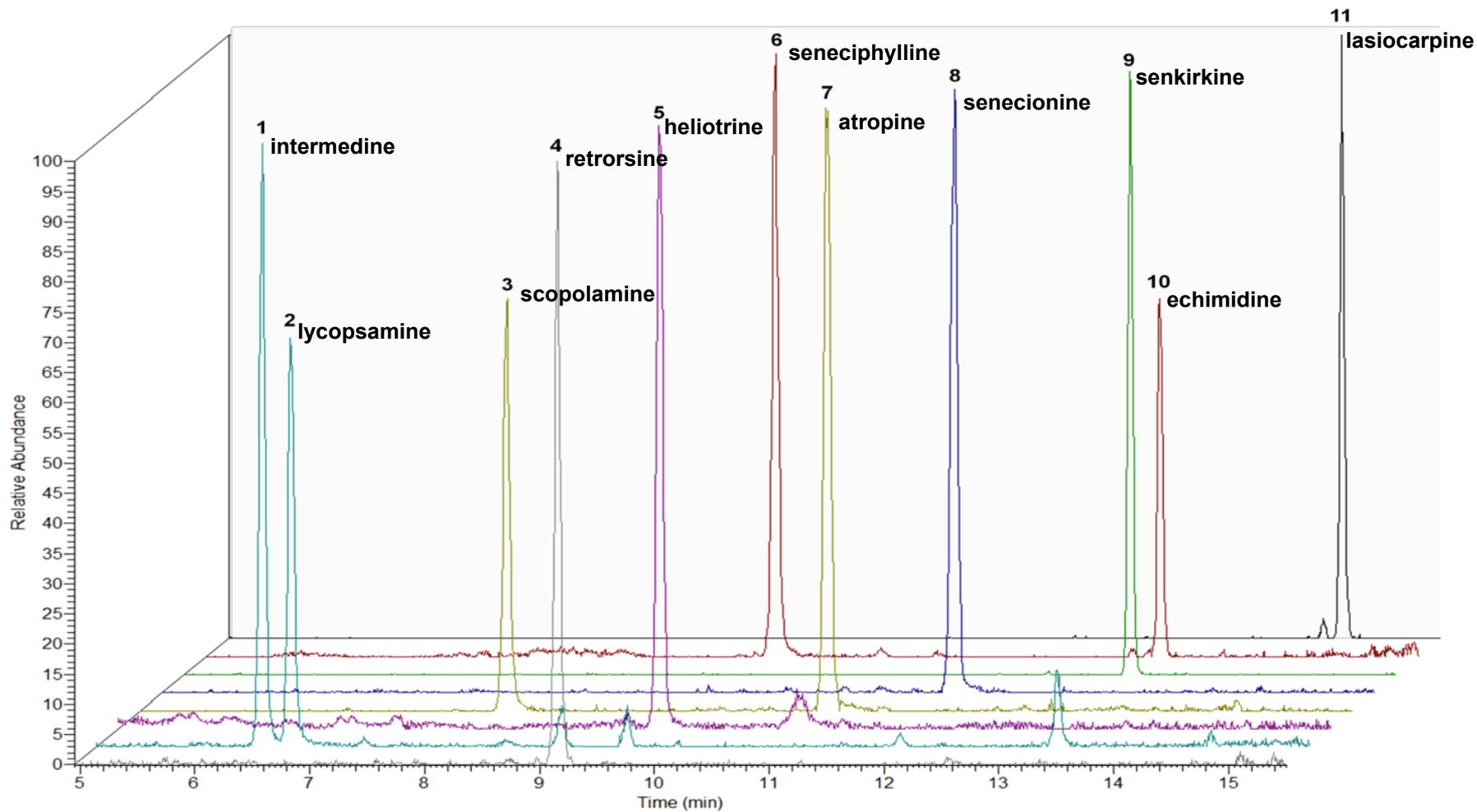
Time [min]	% solvent A	% solvent B	Flow [ml/min]
0	95	5	0.3
9	80	20	0.3
16	50	50	0.3
17	5	95	0.3
20	5	95	0.3
20.5	95	5	0.3

Solvent A: Formic Acid 0.1% v/v

Solvent B: Methanol/Acetonitrile 1:1 v/v

Injection volume: 5 µl - Column heater: 35°C

UPLC column: Ascentis Express C8 2.7µm x 3x150xmm



Chromatogram of the targeted TAs and PAs in a fortified honey sample at the lowest concentration level (2 µg/kg) spiked for validation, acquired in DDA mode.

Analyte	Matrix matched calibration curve				Standard calibration curve			
	slope	intercept	R ²	recovery (%)	slope	intercept	R ²	recovery (%)
Atropine	0.179	0.021	0.999	104.5	0.170	0.010	1.000	107.9
Scopolamine	0.127	0.018	0.999	96.8	0.124	0.005	1.000	96.2
Echimidine	1.437	0.126	0.998	103.9	1.138	0.270	0.995	148.2
Heliotrine	2.022	0.398	0.998	105.0	1.737	0.222	0.999	119.0
Intermedine	2.193	0.368	0.991	106.7	1.871	0.147	0.999	122.9
Lasiocarpine	0.114	0.022	0.998	85.4	0.091	0.011	0.999	102.8
Lycopsamine	1.878	0.006	0.996	101.0	1.740	0.190	0.999	112.0
Retrorsine	1.507	0.125	0.997	97.0	1.148	0.156	0.999	129.4
Senecionine	2.704	0.458	0.999	104.1	2.400	0.206	0.999	113.1
Seneciphylline	2.311	0.246	0.999	102.0	2.155	0.121	0.999	108.7
Senkirkine	2.701	0.193	1.000	99.0	2.422	0.213	0.998	109.5

Linearity and calibration studies (recoveries calculated at concentration of 5 µg/kg, n = 6).

Analyte	Trueness (% recovery)			Repeatability (RSD _r %)			Reproducibility (RSD _R %)			LOD	LOQ
	level 1	level 2	level 3	level 1	level 2	level 3	level 1	level 2	level 3		
Atropine	103.7	100.9	101.4	2.7	0.9	1.0	3.5	1.4	1.1	0.1	0.5
Scopolamine	108.6	98.0	96.0	3.4	3.1	2.5	8.7	5.7	4.9	0.2	0.5
Echimidine	110.3	112.6	114.8	15.1	13.5	11.6	15.6	15.5	14.5	0.1	0.2
Heliotrine	107.3	102.7	102.4	6.6	6.1	5.7	9.5	7.2	6.4	0.1	0.3
Intermedine	100.9	104.6	104.4	5.0	4.1	4.5	5.0	4.0	4.5	0.2	0.7
Lasiocarpine	100.7	99.2	92.3	11.2	5.4	13.9	15.1	13.2	15.1	0.04	0.1
Lycopsamine	97.6	99.8	99.7	4.1	3.3	5.0	5.0	5.0	5.0	0.2	0.6
Retrorsine	98.5	97.6	97.4	6.0	5.6	3.1	7.1	5.7	3.2	0.1	0.4
Senecionine	107.0	103.7	101.2	7.4	4.4	1.5	7.7	4.8	2.7	0.1	0.4
Seneciphylline	107.2	101.9	97.0	3.4	4.7	5.2	5.1	4.4	5.9	0.1	0.3
Senkirkine	105.8	101.8	99.3	6.0	4.7	5.2	6.3	5.4	5.2	0.1	0.2

(level 1 corresponding to 2 µg/kg spiking, level 2 corresponding to 5 µg/kg, level 3 corresponding to 10 µg/kg)



Specificity



20 blank honey samples of different botanical origin



No interfering peaks



Uncertainty



calculated with Horwitz-Thompson equation,
recognized by Reg. No 333/2007

The method resulted to be accurate and very sensitive, with LOQ lower than 1 $\mu\text{g}/\text{kg}$, the lowest limit fixed for TAs, and in particular equal to 0.5 $\mu\text{g}/\text{kg}$ both for atropine and scopolamine.



The characteristics of the method are in agreement with **Commission recommendation (EU) 2015/976** stating that LOQ for atropine and scopolamine should be preferably below:

- ✓ 5 $\mu\text{g}/\text{kg}$ and not higher than 10 $\mu\text{g}/\text{kg}$ for agricultural commodities, ingredients, food supplements and herbal teas
- ✓ 2 $\mu\text{g}/\text{kg}$ for finished foods (e.g. breakfast cereals)
- ✓ 1 $\mu\text{g}/\text{kg}$ for cereal-based foods for infants and young children

and with **Regulation (EU) 2016/239** stating that the maximum levels of atropine and of scopolamine in certain cereal-based foods for infants and young children should be 1.0 $\mu\text{g}/\text{kg}$.

A future perspective is the monitoring of the presence of PAs and TAs (the latter never analyzed in this matrix) in honeys present on national market.

**Thank you for
your attention!**

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