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Istituto Zooprofilattico Sperimentale del Lazio e della Toscana *M. Aleandri*

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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

efsa
European Food Safety Authority

**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*.

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# Pyrrolizidine alkaloids (PAs)

*Borago officinalis*

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*).

*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.**

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# 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.*

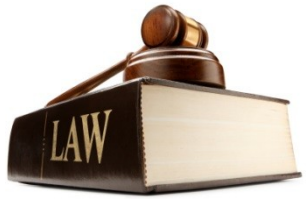
***Atropa belladonna***

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 Official Journal of the European Union L 45/3

**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.**

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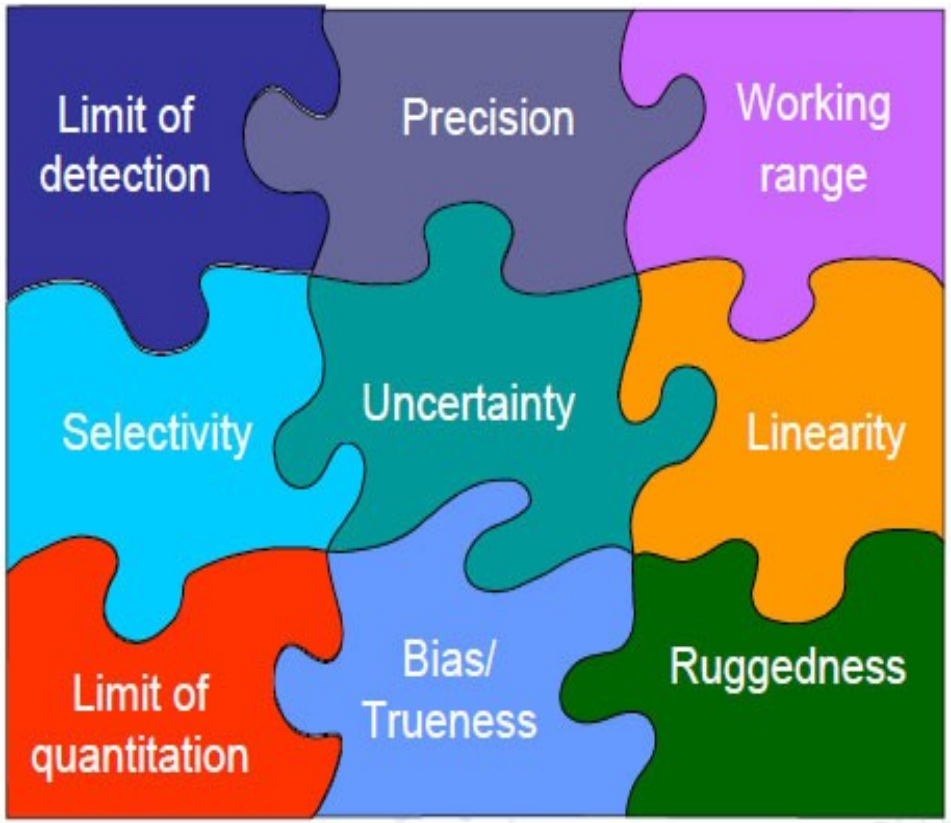
# VALIDATION




*«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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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

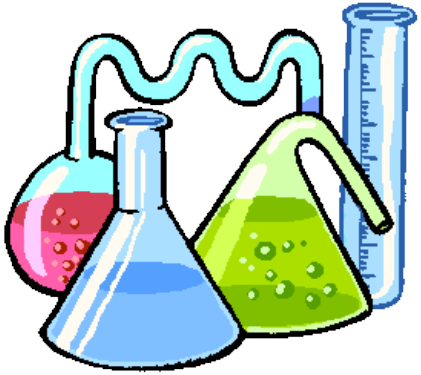
**COMMISSION REGULATION (EU) No 836/2011**

**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



**Q**uick
**E**asy
**Ch**eap
**E**ffective
**R**ugged
**S**afe

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

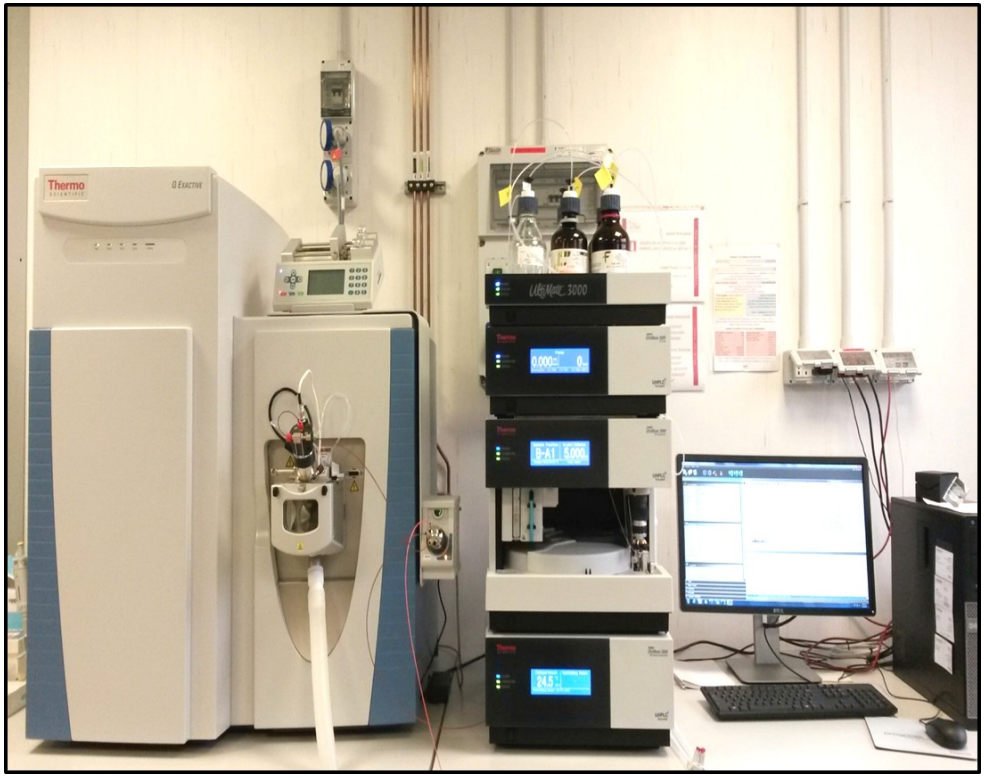
# DETECTION

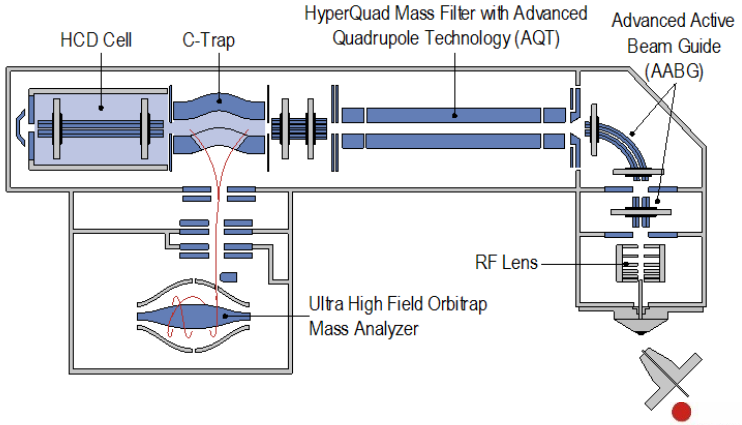
## Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer

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.

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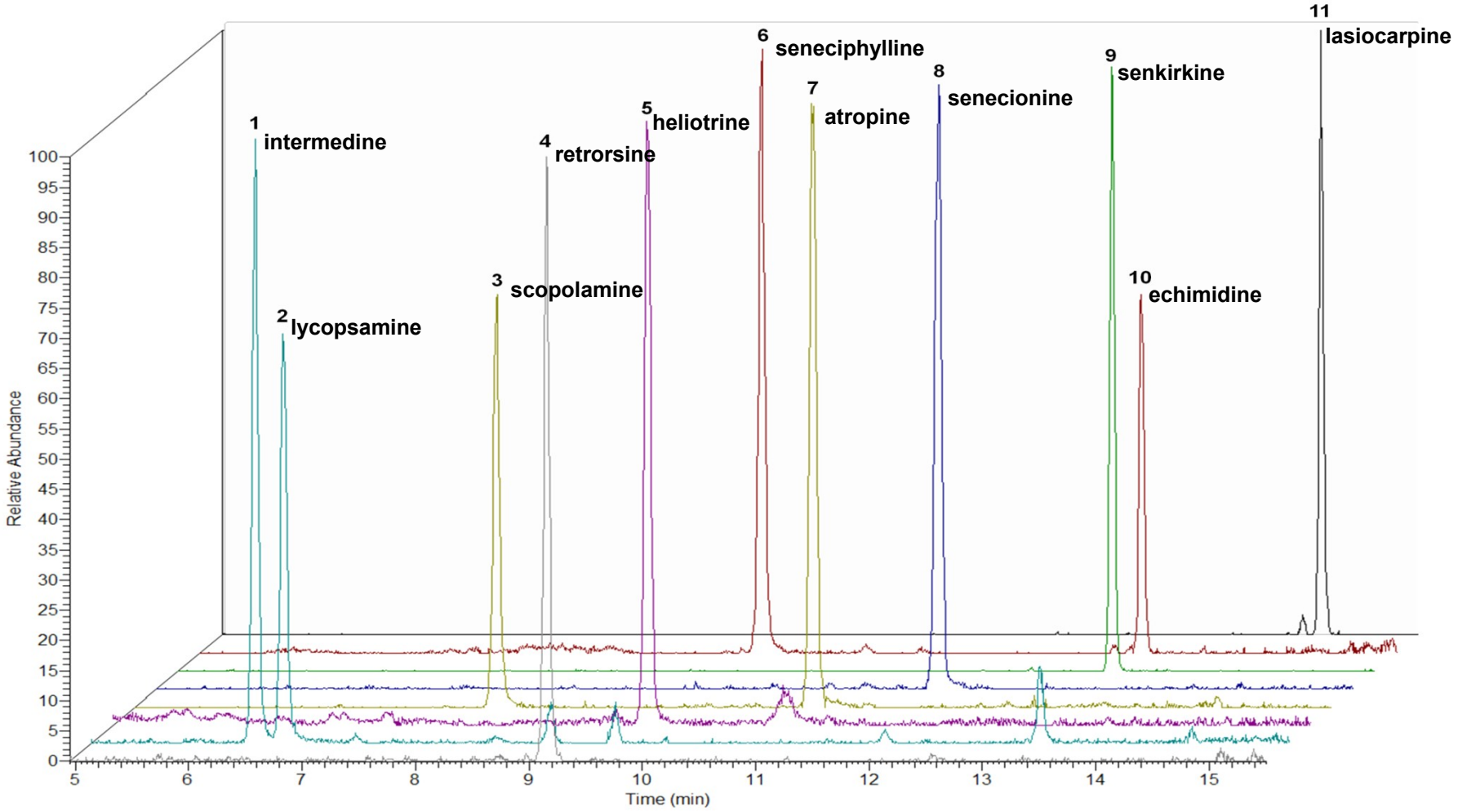
| HRMS parameters of FS and MS/MS detection | | | | | | |
|---|---|---|---|---|---|---|
| Analyte | $R_T$ (min) | Precursor mass $[M+H]^+$ | Fragment 1 (m/z) | Fragment 2 (m/z) | Fragment 3 (m/z) | NCE |
| Intermedine | 6.4 | 300.18055 | 156.102 | 138.091 | 120.081 | 35 |
| Lycopsamine | 6.7 | 300.18055 | 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 2.7µmx3x150xmm




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 µg/kg, the lowest limit fixed for TAs, and in particular equal to 0.5 µg/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 µg/kg and not higher than 10 µg/kg for agricultural commodities, ingredients, food supplements and herbal teas
- ✓ 2 µg/kg for finished foods (e.g. breakfast cereals)
- ✓ 1 µg/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 µg/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.

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# Thank you for your attention!

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