# PLANT ALKALOIDS: troubleshooting in analysis of newly regulated natural toxins

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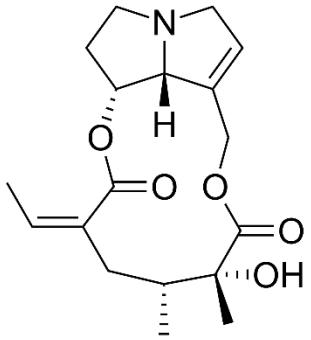
# Pyrrolizidine alkaloids (PA)

- Over 600 PAs known
- Probable genotoxicity of 1,2 unsaturated PAs
  - Margin of Exposure (MOE) 7 ng/kg bw per day
- Production by a variety of plants:



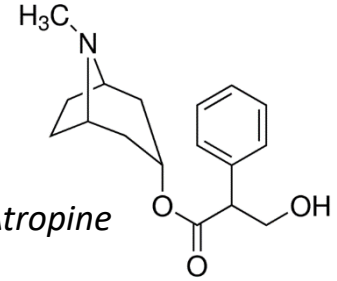

*Senecionine*

*Asteraceae*
(tribes *Senecio*, *Eupatorium*)

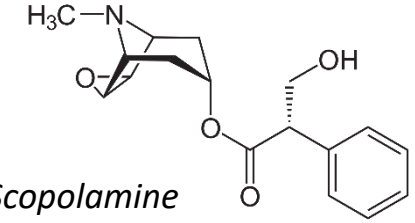
*Boraginaceae*
(most of the genera)

*Fabaceae*
(*Crotalaria* genus)

# Tropane alkaloids (TA)



*Atropine*



*Scopolamine*

- Over 200 TAs known
- Major representatives: atropine, scopolamine
  - Acute Reference Dose (ARfD) 16 ng/kg (sum of TAs)
- Production by a variety of plants:

*Brassicaceae*
*(Cochlearia sp.)*

*Convolvulaceae*
*(Convulvus arvensis)*

*Solanaceae*
*(Datura, Hyoscyamus, Mandragora g.)*

# Toxic alkaloids & EU legislation

Commission Regulation (EU) 2016/239

| FOODSTUFF | ML Atropine | ML Scopolamine |
|---|---|---|
| Processed cereal-based foods and baby foods for infants and young children, containing millet, sorghum, buckwheat or their derived products | 1 µg/kg | 1 µg/kg |

millet

sorghum

buckwheat

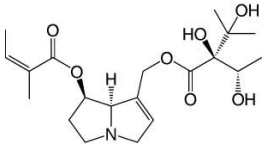
EUR-Lex

- New EU legislation for toxic alkaloids is in preparation...

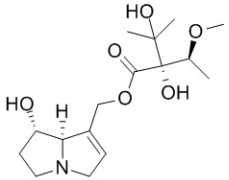
# Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs

| Foodstuffs ([1]) | Maximum level (µg/kg) | |
|---|---|---|
| **Tropane alkaloids** ([62]) | | |
| | Atropine | Scopolamine |
| Processed cereal-based foods and baby foods for infants and young children, containing millet, sorghum, buckwheat, maize or their derived products ([3]) ([29]) | 1,0 | 1,0 |
| | Sum of atropine and scopolamine | |
| Unprocessed millet and sorghum ([18]) | 5,0 as from 1 September 2022 | |
| Unprocessed maize ([18]) with the exception of<br>— unprocessed maize intended to be processed by wet milling ([37]) and<br>— unprocessed maize for popping | 15 as from 1 September 2022 | |

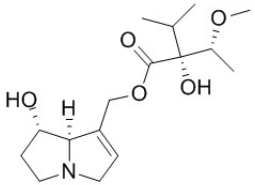
# New maximum limits



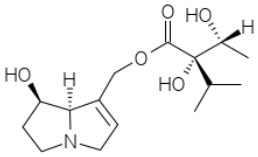
*Echimidine*



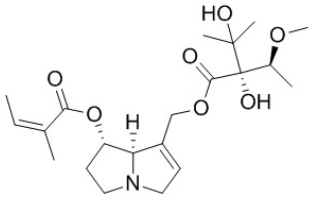
*Europine*



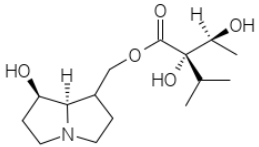
*Heliotrine*



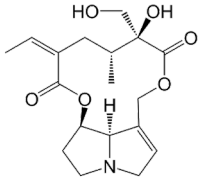
*Intermedine*



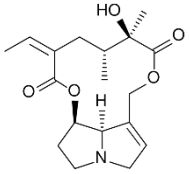
*Lasiocarpine*



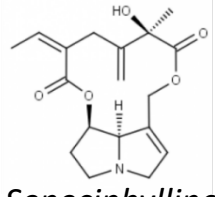
*Lycopsamine*



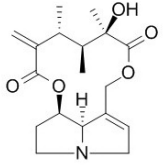
*Retrorsine*



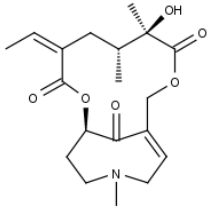
*Senecionine*



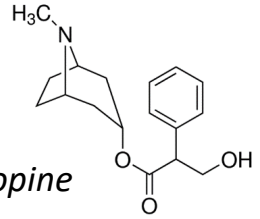
*Seneciphylline*



*Senecivernine*

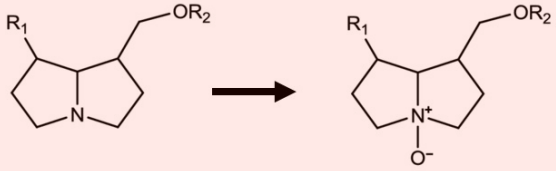


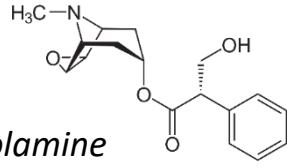
*Senkirkine*



*Atropine*

**+ N-oxides of PAs**





*Scopolamine*

# Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs

| | "Foodstuffs [1] | Maximum level (*) (µg/kg) |
|---|---|---|
| 8.4. | **Pyrrolizidine alkaloids** | |
| 8.4.1. | Herbal infusions (dried product) (**) (***) with the exception of the herbal infusions referred to in 8.4.2. and 8.4.4. | 200 |
| 8.4.2. | Herbal infusions of rooibos, anise (*Pimpinella anisum*), lemon balm, chamomile, thyme, peppermint, lemon verbena (dried product) and mixtures exclusively composed of these dried herbs (**) (***) with the exception of the herbal infusions referred to in 8.4.4. | 400 |
| 8.4.3. | Tea (*Camellia sinensis*) and flavoured tea (****)(*Camellia sinensis*) (dried product) (***) with the exception of the tea and flavoured tea referred to in 8.4.4. | 150 |
| 8.4.4. | Tea (*Camellia sinensis*), flavoured tea (****)(*Camellia sinensis*) and herbal infusions for infants and young children (dried product) | 75 |
| 8.4.5. | Tea (*Camellia sinensis*), flavoured tea (****)(*Camellia sinensis*) and herbal infusions for infants and young children (liquid) | 1,0 |
| 8.4.6. | Food supplements containing herbal ingredients including extracts (**) with the exception of the food supplements referred to in 8.4.7. | 400 |
| 8.4.7. | Pollen based food supplements [39]<br>Pollen and pollen products | 500 |
| 8.4.8. | Borage leaves (fresh, frozen) placed on the market for the final consumer (**) | 750 |

# Project goals

Development of a sensitive instrumental LC–MS/MS based method for determination of multiple toxic plant alkaloids

- Focus on a broad spectrum of toxic plant alkaloids:
  - Pyrrolizidine alkaloids (n = 33)
  - Tropane alkaloids (n = 22)
  - Quinolizidine alkaloid (n = 1)
- Method validation and utilization for routine analyses

# Overview of analytes (n = 56)

## Tropane alkaloids (n = 22)

- nortropinone
- tropinone
- pseudotropine
- tropine
- 6β-hydroxytropinone
- scopoline
- phenylacetoxytropane
- apoatropine
- noratropine
- homatropine
- convolidine
- aposcopolamine
- norscopolamine
- atropine
- littorine
- convolvine
- fillalbine
- scopolamine
- anisodamine
- convolamine
- anisodine
- hydroxymethyl atropine

## Quinolizidine alkaloid (n = 1)

- sparteine

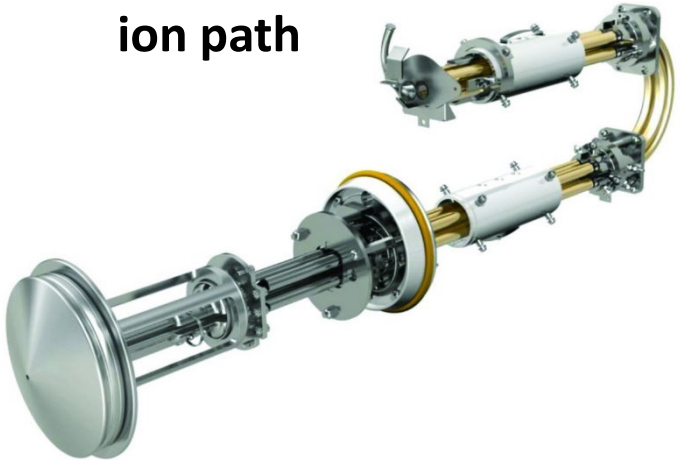
## Pyrrolizidine alkaloids & N-oxides (n = 33)

- indicine
- intermedine
- lycopsamine
- echinatine
- heliotrine
- monocrotaline
- europine
- seneciphylline
- senecionine
- senecivernine
- erucifoline
- jacobine
- retrorsine
- echimidine
- lasiocarpine
- trichodesmine
- senkirkine
- retronecine
- indicine-N-oxide
- intermedine-N-oxide
- lycopsamine-N-oxide
- echinatine-N-oxide
- heliotrine-N-oxide
- monocrotaline-N-oxide
- europine-N-oxide
- seneciphylline-N-oxide
- senecionine-N-oxide
- senecivernine-N-oxide
- erucifoline-N-oxide
- jacobine-N-oxide
- retrorsine-N-oxide
- echimidine-N-oxide
- lasiocarpine-N-oxide

# LC–MS instrumentation

- Utilization of QTRAP® 6500+ LC–MS/MS system (SCIEX)

**QTRAP® 6500+ system ion path**

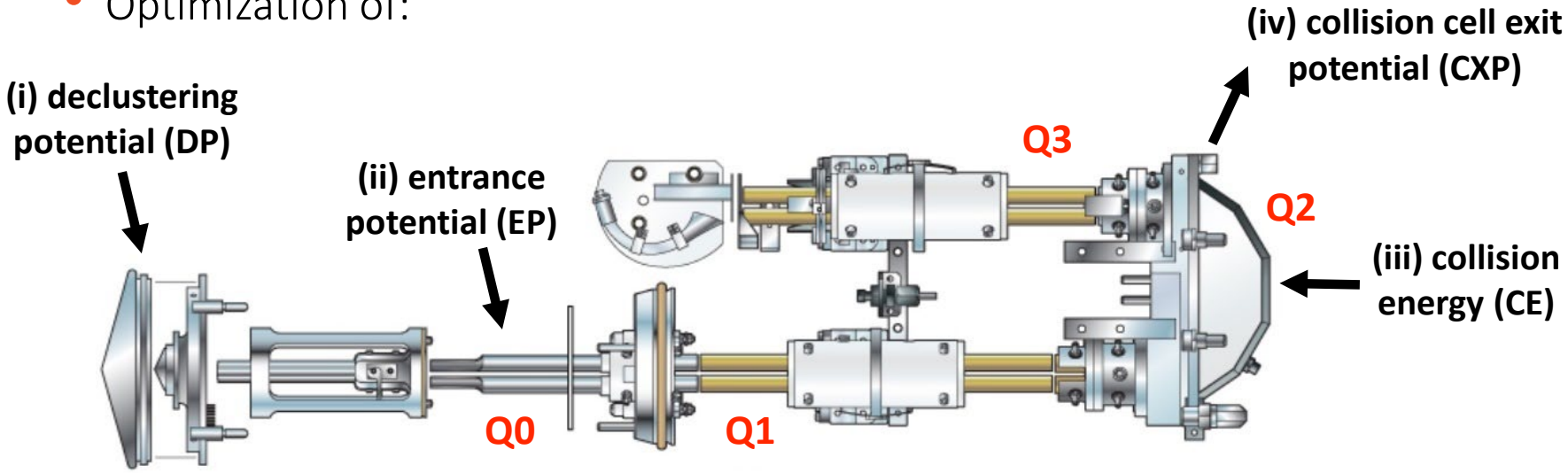


# Instrumental method development:

## 1. Optimization of compound dependent parameters

# Compound(s) optimization (1/3)

- Optimization of compound dependent parameters
  - Direct infusion of analytical standards into the ion source (ESI)
  - Optimization of:



# Compound(s) optimization (2/3)

- Optimization of compound dependent parameters
- The aim: to generate a wide set of mass transitions in order to:
  - Produce as many transitions unique for the alkaloids as possible

Achievement of confident confirmation of alkaloids within the future analyses

# Compound(s) optimization (3/3)

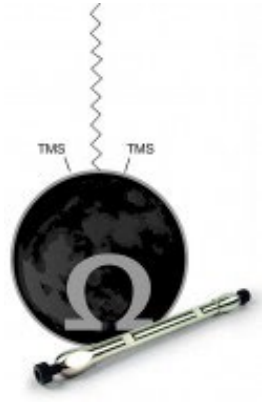
- Successful optimization of compound dependent parameters
- All included alkaloids provide the highest ionization yield in ESI(+) as protonated molecules
- Optimization of up to 8 mass transitions for each alkaloid
- Relatively high signal intensity obtained for the vast majority of the included analytes

... low LOQs can be expected?

# Instrumental method development:

## 2. Optimization of chromatographic separation

# Chromatographic separation




- Toxic plant alkaloids are a mixture of medium and up to polar compounds
  - ➢ Selection of suitable reverse-phase chromatography

- Use of analytical column **Luna Omega C18** (150 x 2.1 mm; 1.6 µm)
  - Combination of a long column and small particle size stationary phase is a good starting point to separate highly complex mixtures of analytes and matrix components (focus on herbal-based samples)
  - Modified silica technology provides improved column life-time through considerable inertness and mechanical strength

# Mobile phases composition (RPLC)

- According to our experience with toxic plant alkaloids, a combination of water and methanol was used in RPLC
  - Utilization of acetonitrile results in lower backpressure, but especially slightly lower separation efficiency
  - Addition of isopropanol did not show improved separation compared to methanol, higher backpressure
  - Acidification of mobile phases and/or use of volatile buffers needed for ionization efficiency improvement

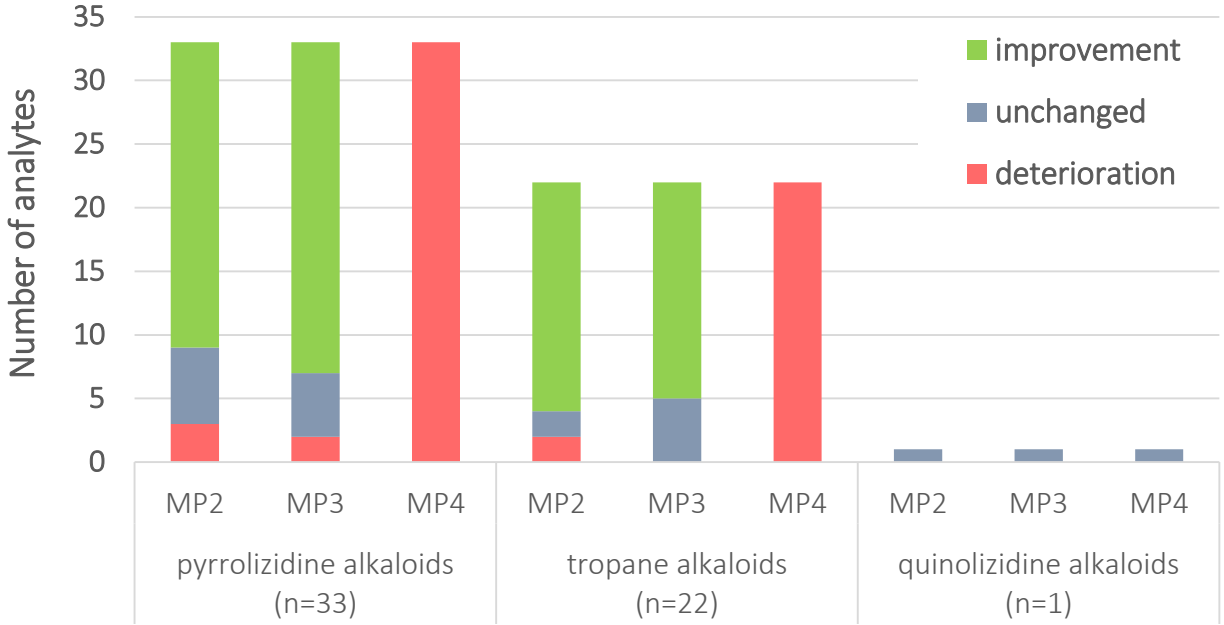
# Mobile phases composition (RPLC)

- Testing of several additional mobile phase components
  - **Ammonium acetate** – 5 mM (pH 6.75) – decreased ionization yield, poor separation of a number of alkaloids
  - **Ammonium formate** – 2 mM, 5 mM, 10 mM, 20 mM
  - **Formic acid** – 0.1 %, 0.2 %, 0.5 %, 1 % (v/v)

Addition of a combination of **formic acid (0.1 %)** and **ammonium formate (2 mM)** provides the best results

# Mobile phases composition (RPLC)

- Comparison of selected mobile phases composition




- **Mobile phase 1** (= reference)

2 mM ammonium formate
+ 0.5 % formic acid

- **Mobile phase 2**

2 mM ammonium formate
+ 0.2 % formic acid

- **Mobile phase 3**

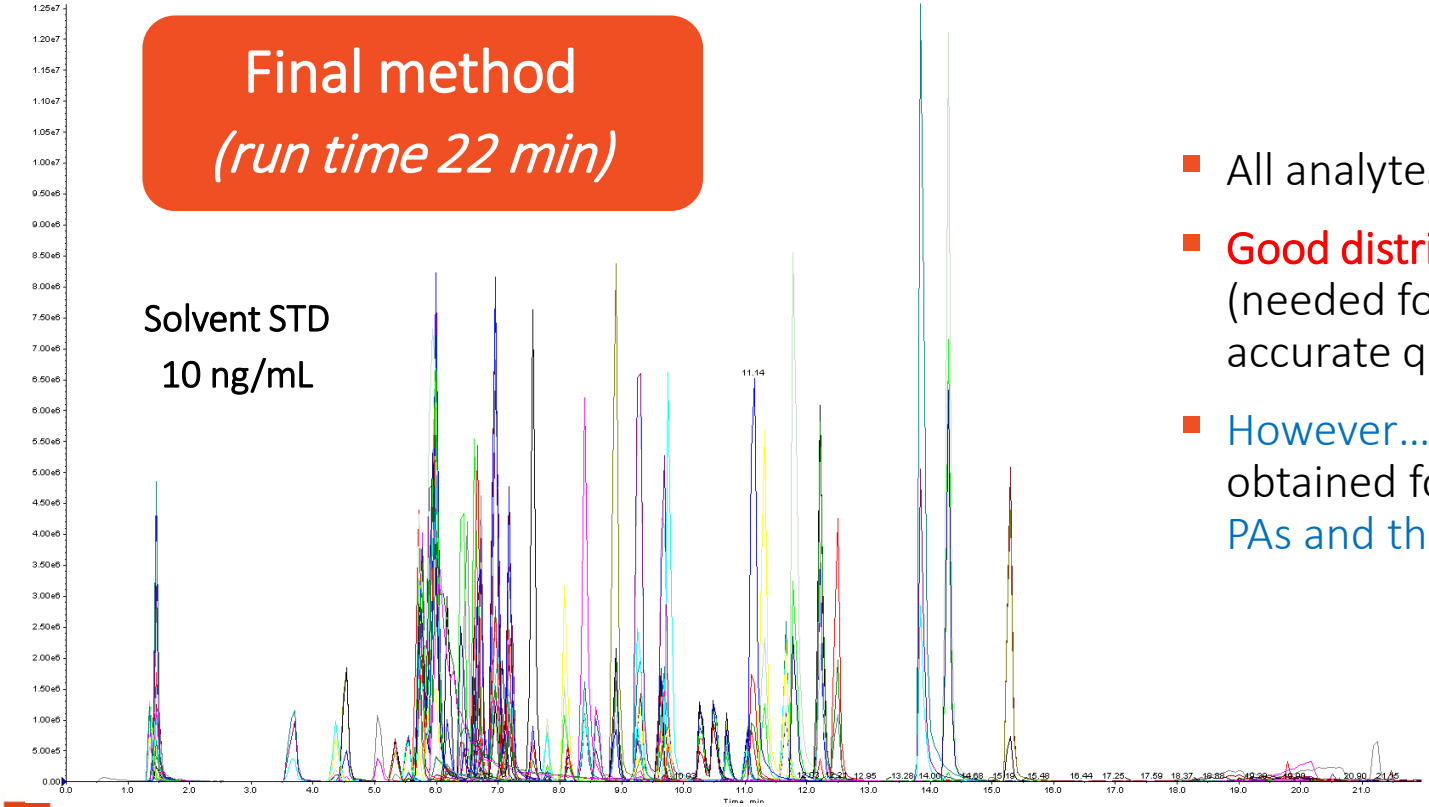
2 mM ammonium formate
+ 0.1 % formic acid

- **Mobile phase 4**

5 mM ammonium acetate
+ 0.1 % formic acid

# Example of a chromatogram (RPLC)

**Final method**
*(run time 22 min)*

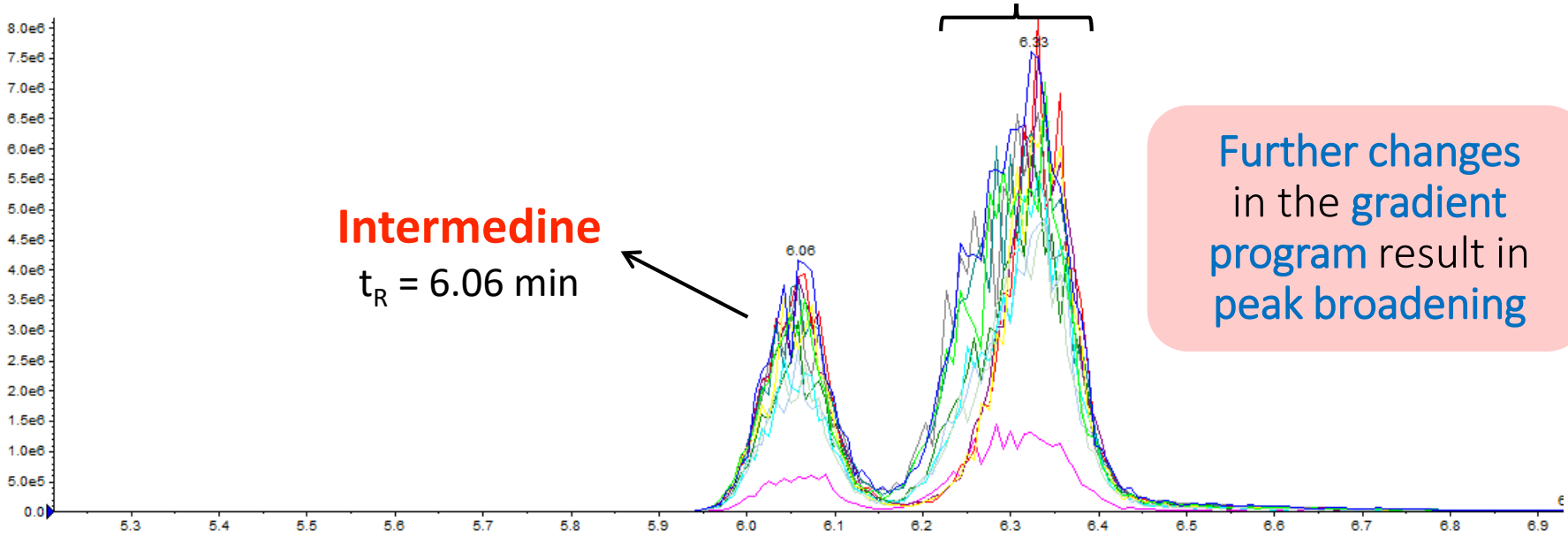



- All analytes detectable in one run
- Good distribution of elution (needed for peak plotting & accurate quantification)
- However… insufficient separation obtained for several isomers of PAs and their N-oxides (2 groups/5 PAs)

# Separation of isomers (1/3)

**Lycopsamine** & **Echinatine** & **Indicine**

**$t_R$ = 6.25–6.33 min**




**Intermedine**

$t_R$ = 6.06 min

Further changes in the gradient program result in peak broadening

# Separation of isomers (2/3)

**Intermedine-N-oxide** & **Indicine-N-oxide**

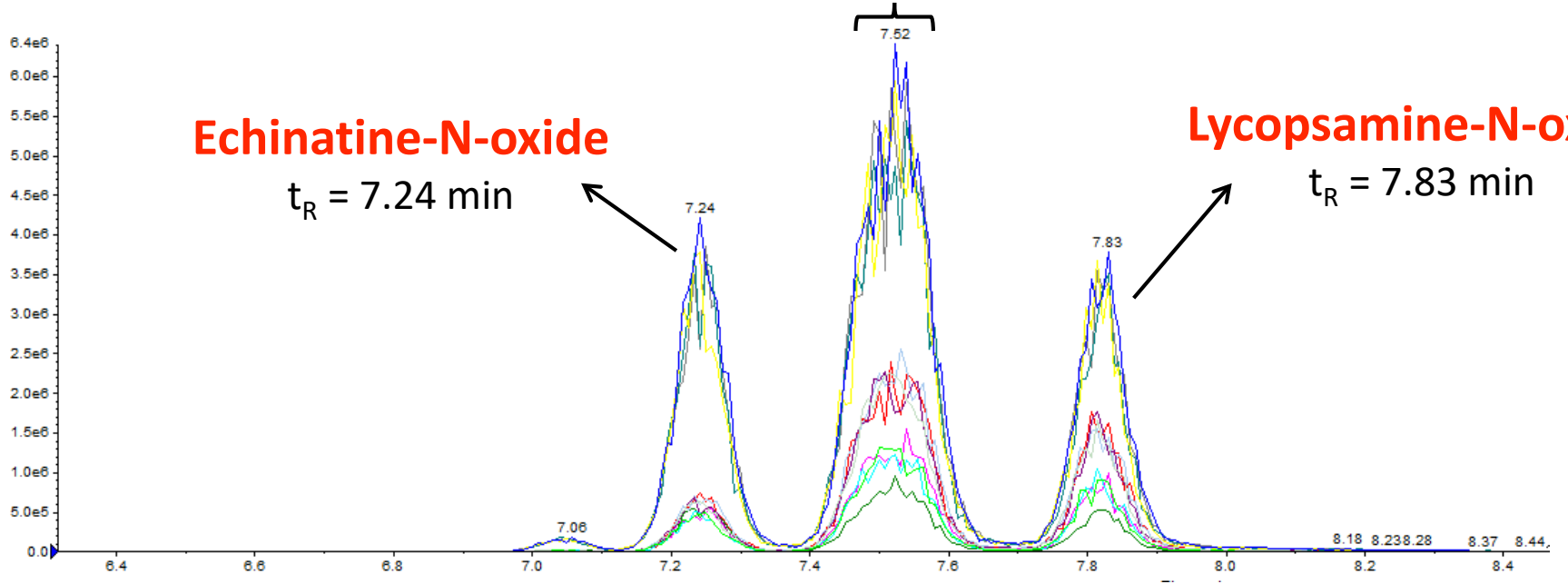
$t_R$ = 7.52 min (the same $t_R$ !!)

**Echinatine-N-oxide**

$t_R$ = 7.24 min

**Lycopsamine-N-oxide**

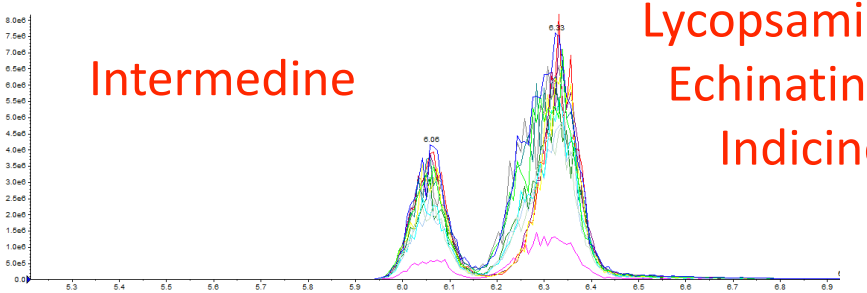
$t_R$ = 7.83 min



# Additional HRMS experiment

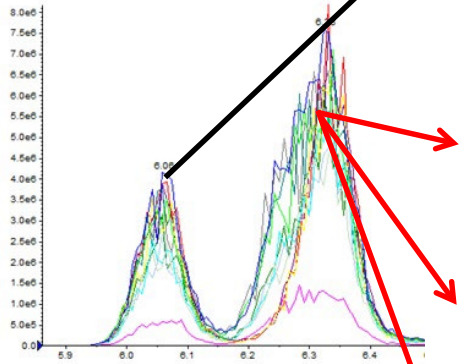
- Exploration of HRMS/MS spectra of selected isomers
  - Measurement of individual analytical standards
- Utilization of TripleTOF® 6600 LC–MS/MS system (Q-TOF, SCIEX)

Intermedine

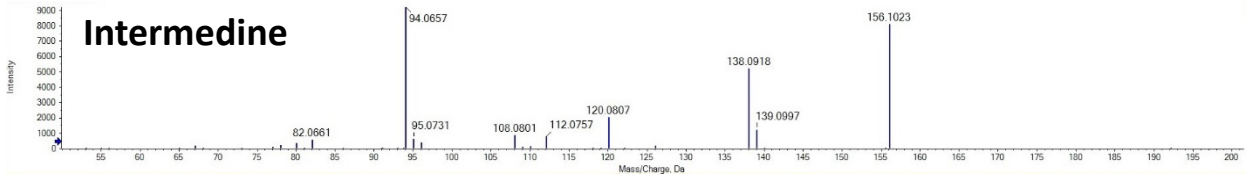


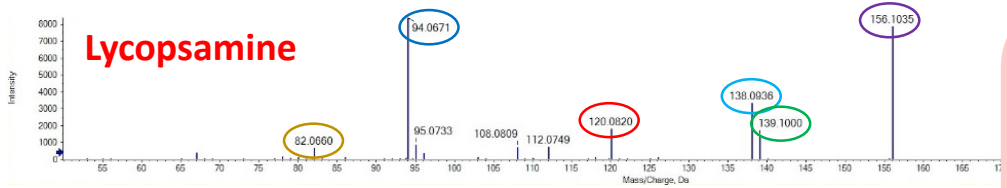
Lycopsamine &
Echinatine &
Indicine

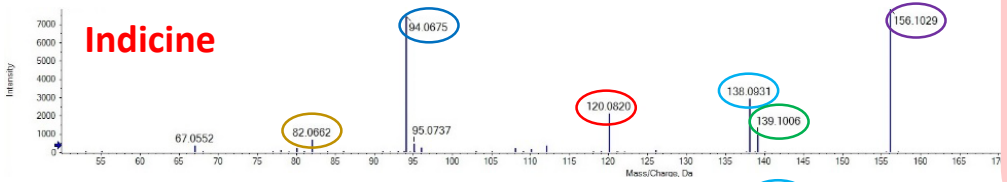
# Selected plant alkaloids $MS^2$ spectra

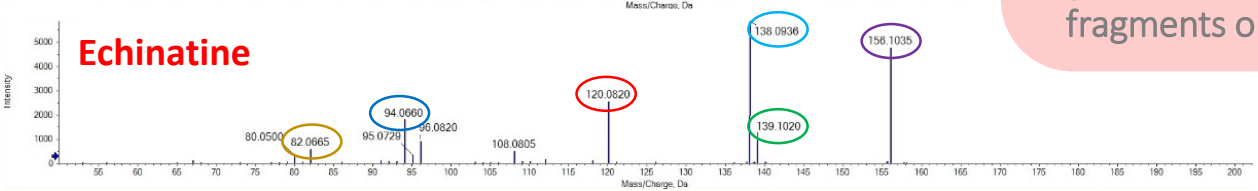


Co-eluted alkaloids




- ✓ No specific fragment ions found for the co-eluted alkaloids
- ✓ Echinatine has slightly different fragmentation pattern (but the same fragments observed…)

# Separation of isomers (3/3)

- As outlined at the beginning, it is very complicated to achieve successful separation of all problematic isomers of pyrrolizidine alkaloids using reverse-phase chromatography
  - Several combinations of mobile and the additives tested
  - Tens of elution gradient programs tested – including several experimental methods with very long run time (≥ 30 min)
  - Either consensus on reporting sum(s) of isomeric alkaloids...
    - Sum 1: *lycopsamine + echinatine + indicine*
    - Sum 2: *intermedine-N-oxide + indicine-N-oxide*
  - ➢ OR additional testing using different chromatography settings?

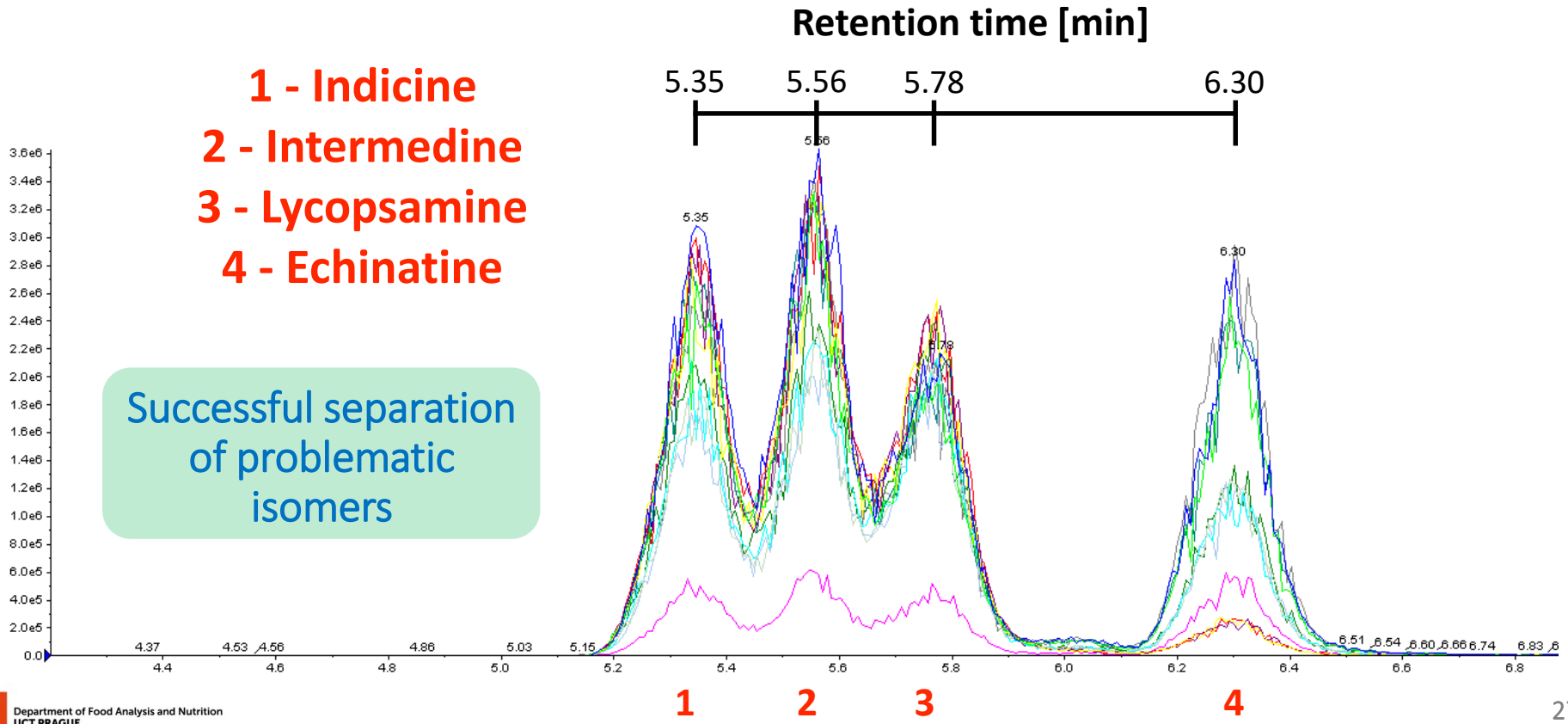
# Instrumental method development:

## 3. Optimization of chromatographic separation / complementary instrumental method (HILIC)

# Separation of isomers – HILIC (1/2)

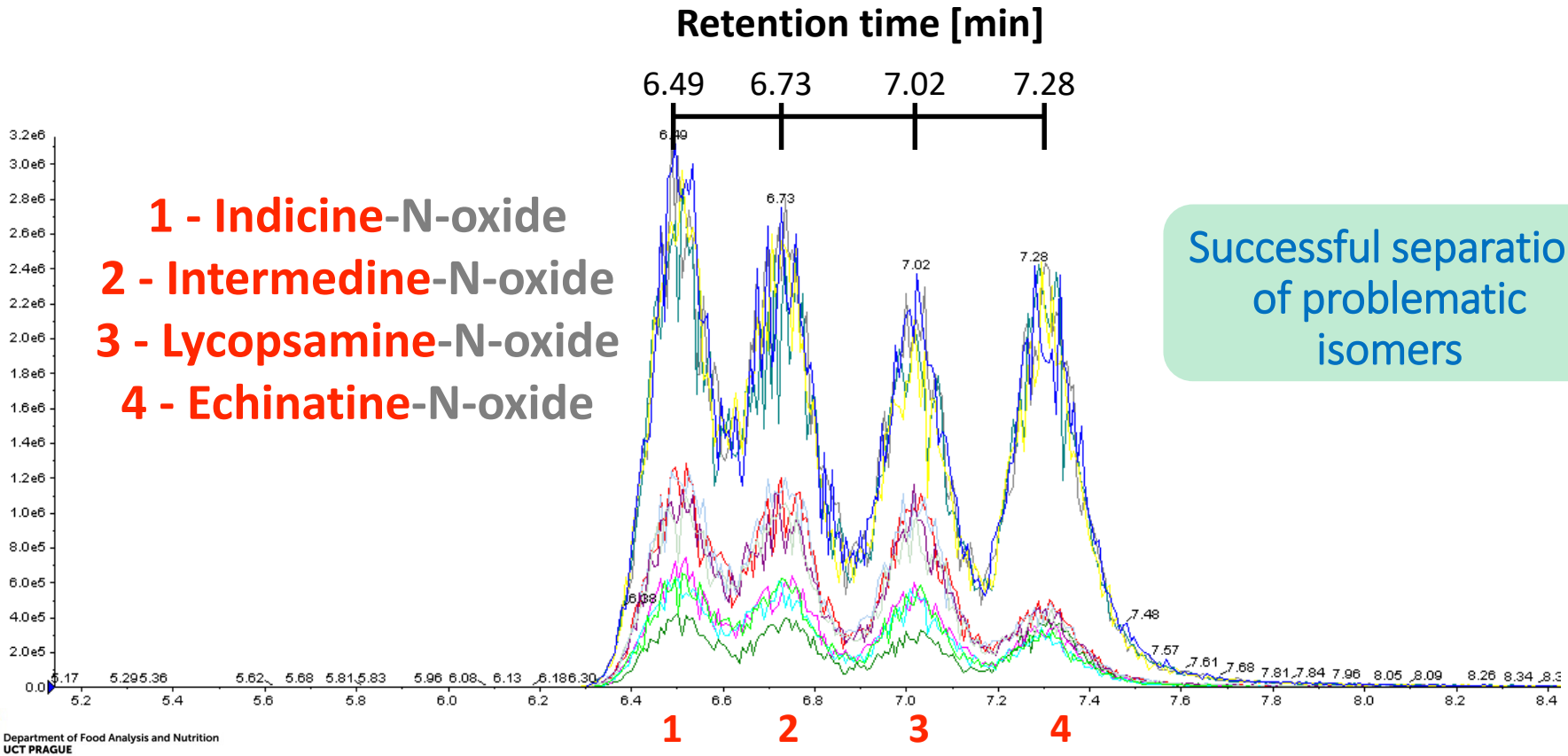
1 - Indicine
2 - Intermedine
3 - Lycopsamine
4 - Echinatine

Successful separation of problematic isomers



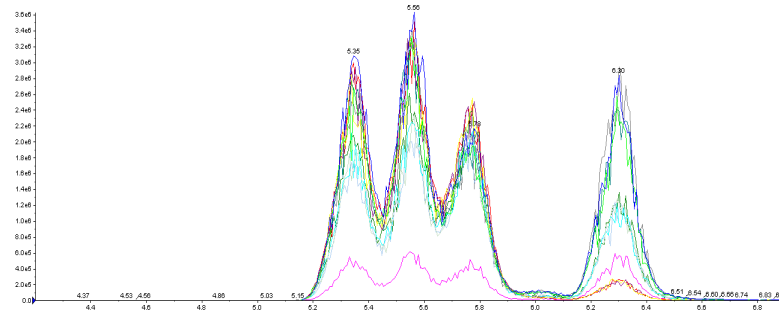
# Separation of isomers – HILIC (2/2)

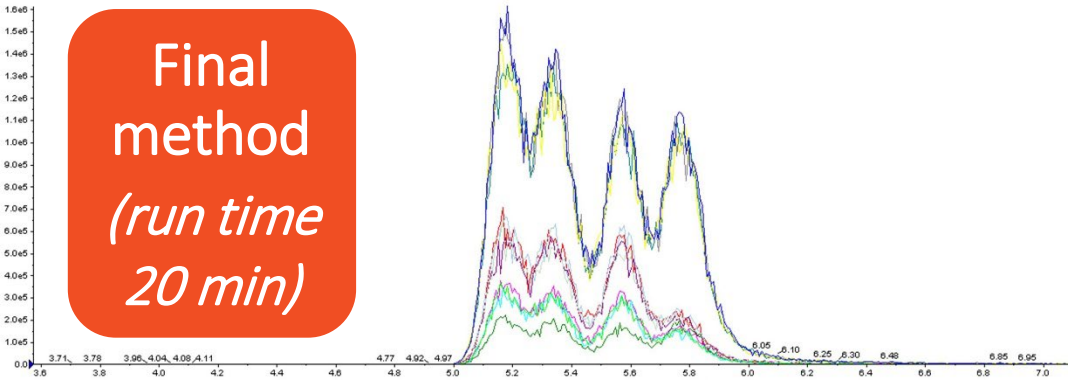
1 - Indicine-N-oxide
2 - Intermedine-N-oxide
3 - Lycopsamine-N-oxide
4 - Echinatine-N-oxide




Successful separation of problematic isomers

# Separation of isomers – HILIC (3/3)





Final method *(run time 20 min)*

- Mobile phases:

A: Acetonitrile : water (95:5, v/v)

B: Water (Milli-Q)

Mobile phase additives:

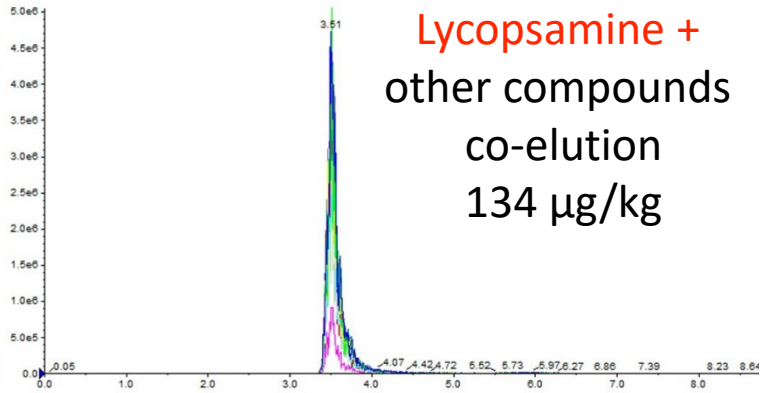
5 mM ammonium formate

0.1 % formic acid (v/v)

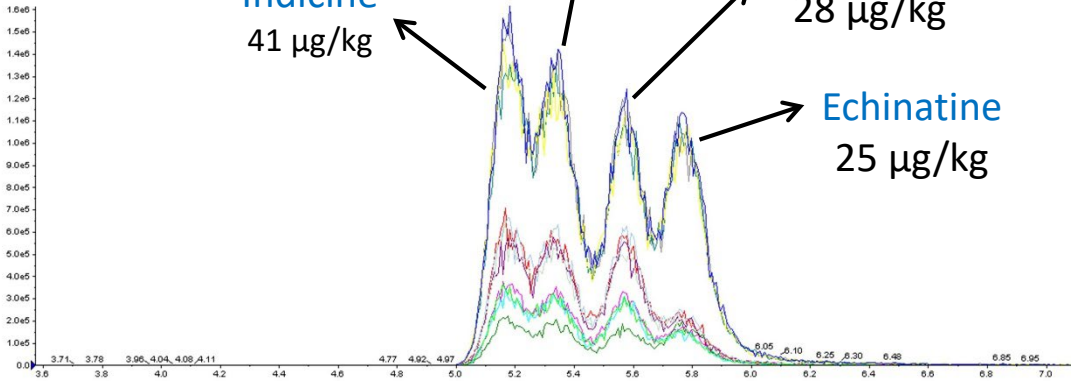
Analytical column
**ACQUITY UPLC BEH Amide**
(100 x 2.1; 1.7 µm)

# Literature method for plant alkaloids

- Utilization of a rapid RP-LC method with run time < 10 min
  - Insufficient LC separation of lycopsamine resulting in co-elution
  - Demonstration on internal contaminated sample:



Lycopsamine +
other compounds
co-elution
134 µg/kg



# RPLC + HILIC methods utilization

- According to our results, < 20 % of samples are positive for the presence of toxic PAs and TAs
  - Only a minor part of contaminated samples are actually positive for the alkaloids not entirely separated under RPLC conditions
  - < 5 % of samples needed to run under both RPLC and HILIC
- In case of reporting sum(s) of alkaloids, presented RPLC method is fully sufficient for their analysis
  - Compromise between the analysis costs (instrument time, solvents) and accuracy of quantification must be taken into account

# Instrumental method development:

# 4. Optimization of ion source conditions

# Ion source parameters optimization (1/3)

- Optimization of ion source conditions – follows the successful chromatographic separation
  - Ion source gas 1 (nebulizer gas)
  - Ion source gas 2 (heater gas)
  - Curtain gas
  - Heater temperature
  - Ion spray voltage

*+ Collisionally activated dissociation (CAD) gas pressure optimized within this step*

**Turbo V™ Ion Source** (SCIEX)

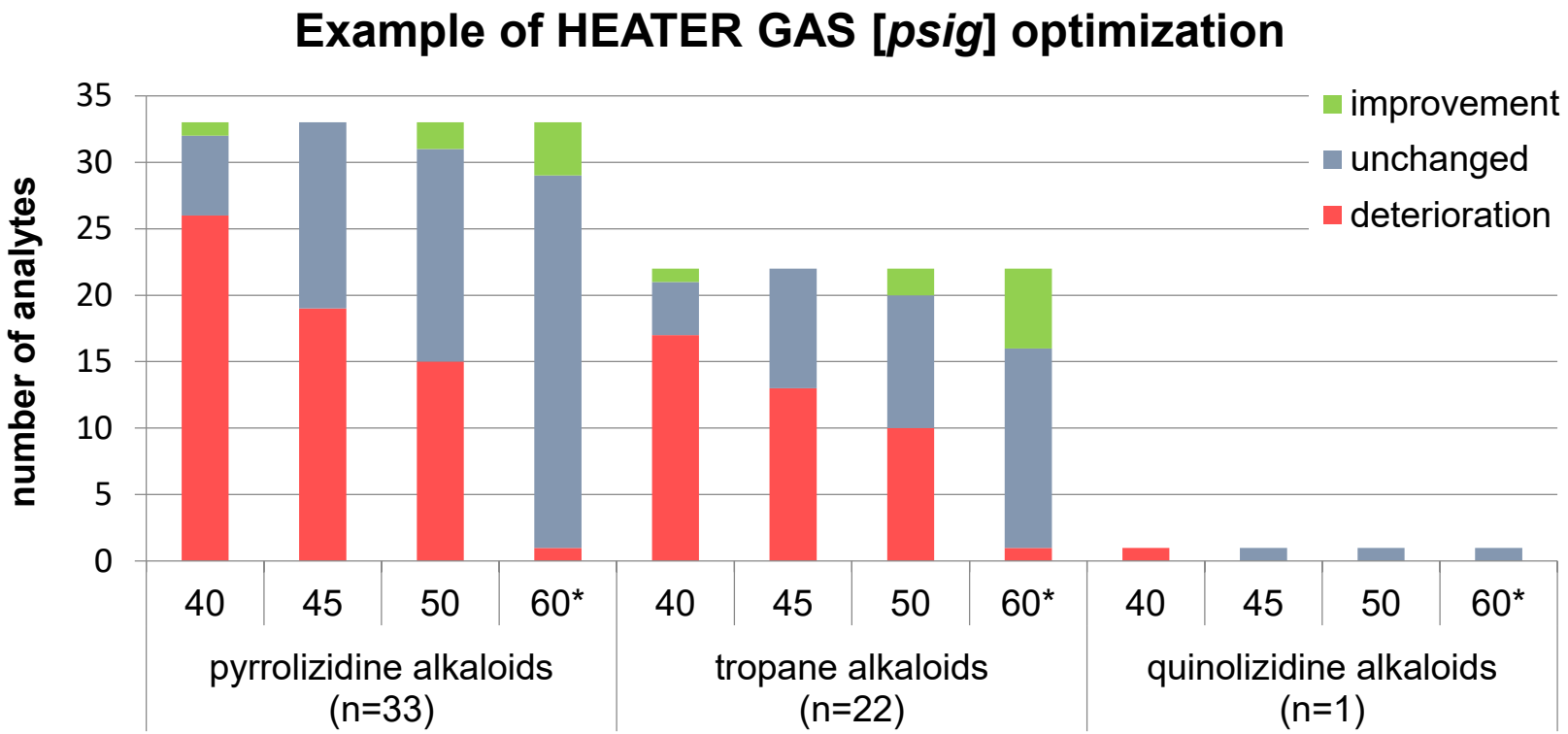
# Ion source parameters optimization (2/3)

- Optimization of ion source conditions & CAD
  - Reference parameters selected either as commonly used or recommended by the instrument manufacturer

| Parameter | Units | Reference settings | Tested settings |
|---|---|---|---|
| Nebulizer gas (gas 1) | psig | 55 | 40/45/50/55*/60 |
| Heater gas (gas 2) | psig | 55 | 40/45/50/55*/60 |
| Curtain gas | psig | 40 | 35/40*/45/50 |
| Temperature | °C | 300 | 200/250/300*/350/400/450/ /500/550/600/650 |
| Ion spray voltage | kV | 5.5 | 1.5/2.0/2.5/3.0/3.5/4.0/4.5/5.0/5.5* |
| CAD gas | psig | LOW | Low*/medium/high |

* reference settings

# Ion source parameters optimization (3/3)



# Conclusion

- New instrumental method for determination of multiple toxic plant alkaloids was developed
  - And validated for matrices herbal tea and cereals
  - Generally low detection limits were obtained (0.1 – 2 µg/kg)
  - Analytes isolation of was briefly discussed within the RAFA main program & **poster N31** (*Petra Jonatova, UCT Prague*)
- Special attention was focused on chromatographic separation of a number of isomers (mainly PAs)

# Further plans & ideas

- Additional testing of ion mobility separation potential (SelexIon, SCIEX)
- Analysis of samples from the market
- Publication in preparation...

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