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Challenges in Implementation of an LC/MS/MS Method for the Analysis of Pyrrolizidine Alkaloids in Food Matrices

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What are Pyrrolizidine Alkaloids?

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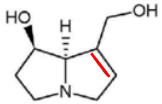
- Secondary plant metabolites
- Produced by over 6000 plant species
- More than 660 PAs are known

ASTERACEAE
(Compositae)

FABACEAE
(Leguminosae)

BORAGINACEAE

- Genotoxic and carcinogenic properties of 1,2 unsaturated PAs



- Commission Regulation (EU) 2020/2040

  enforced from **1st July 2022**

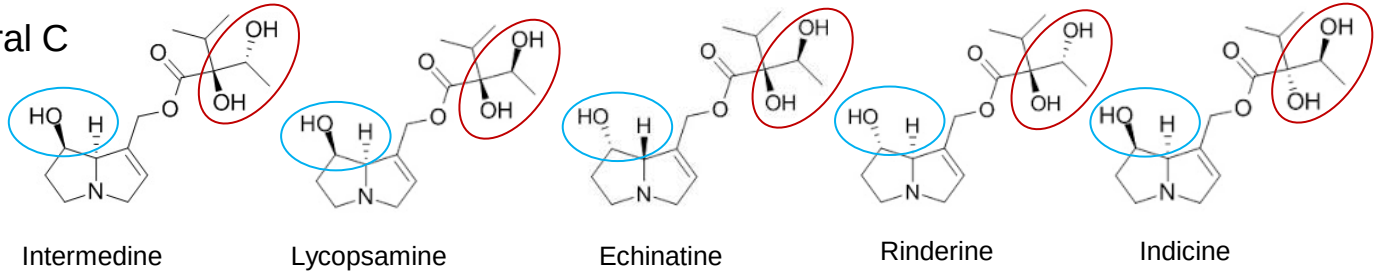
  ML set for sum of PAs in certain foodstuffs

European Commission

	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 [19] Pollen and pollen products	500
8.4.8.	Borage leaves (fresh, frozen) placed on the market for the final consumer (**)	750
8.4.9.	Dried herbs with the exception of the dried herbs referred to in 8.4.10. (**)	400
8.4.10.	Borage, lovage, marjoram and oregano (dried) and mixtures exclusively composed of these dried herbs (**)	1 000
8.4.11.	Cumin seeds (seed spice)	400

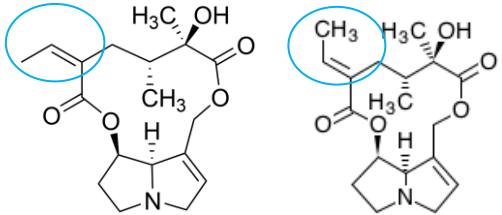
Structure of PAs - types of isomers

- STEREOISOMERS
  - R/S-configuration on chiral C



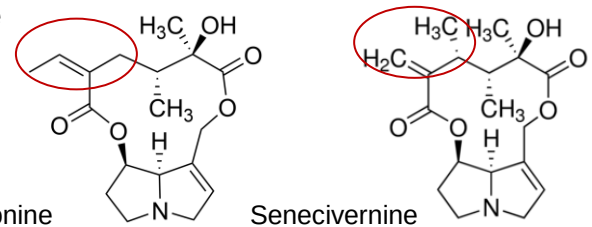
Intermedine Lycopsamine Echinatine Rinderine Indicine

  - E/Z-configuration at double bond



Senecionine Integerrimine

- STRUCTURAL ISOMERS
  - Shift of methyl group



Senecionine Senecivernine

Challenges in PAs analysis using LC-MS/MS

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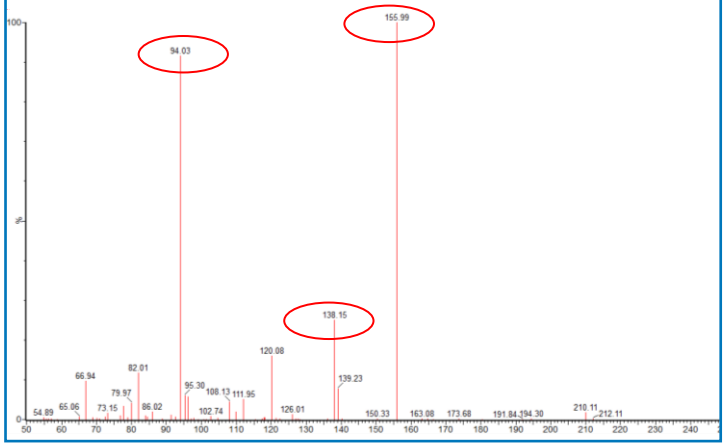
Detection

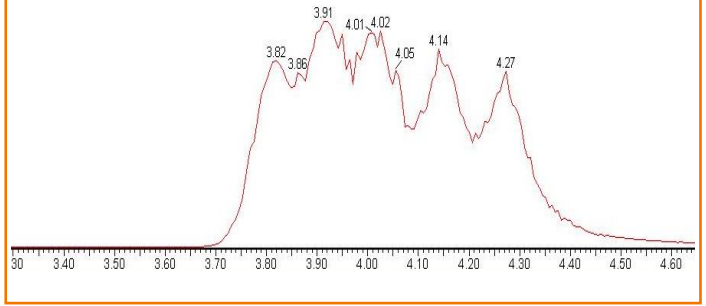
- Isomers provide identical MS/MS transitions



Chromatographic separation is needed

Separation

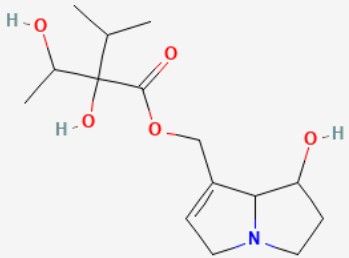
- Coelution of isomers
- 10 groups of isomers for a total of 28 compounds

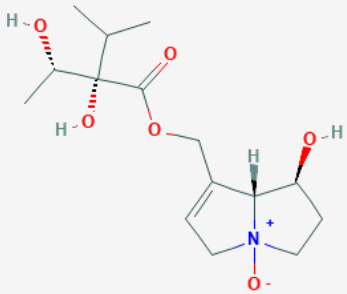


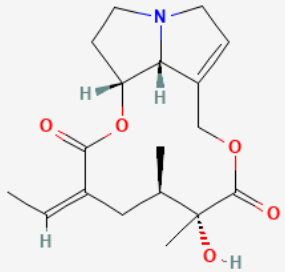
The most challenging compounds

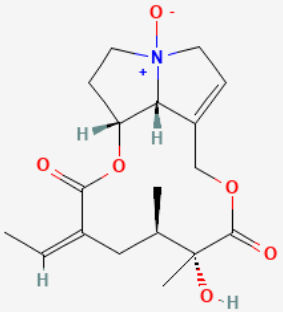
- Separation of isomers with different oxidation is no problem – retrorsine/senecionine-N-oxide
- Separation of other types of isomers is more difficult

Intermedine Group	Intermedine-N-O Group	Senecionine Group	Senecionine-N-O Group
Echinatine	Echinatine-N-Oxide	**Senecionine**	**Senecionine-N-Oxide**
Indicine	Indicine-N-Oxide	Integerrimine	Integerrimine-N-Oxide
Intermedine	**Intermedine-N-Oxide**	**Senecivernine**	**Senecivernine-N-Oxide**
Lycopsamine	**Lycopsamine-N-Oxide**		
Rinderine	Rinderine-N-Oxide		









Chromatographic separation of PAs

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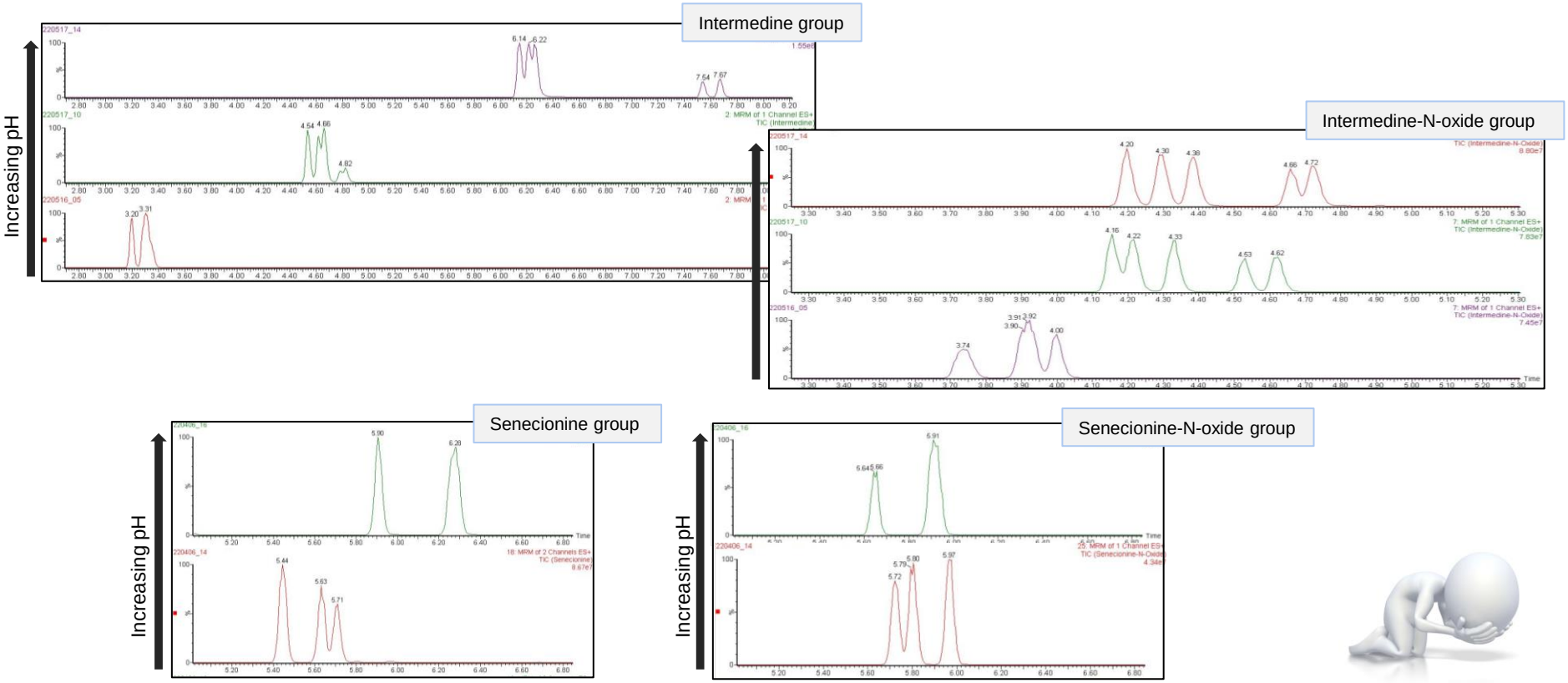
- Different types of analytical columns were tested ⟶ C18 were chosen for the final method

Combination of small particle size of stationary phase and long column is a good starting point to separate mixture of isomers and matrix interferences

ACQUITY UPLC and XBridge HPLC/UHPLC Columns	Particle/Ligand	Ligand Density	Carbon Load	Endcapped	USP Class No.	pH Range	Temperature Limits	Surface Area
BEH C_{18} UPLC: 1.7 µm UHPLC: 2.5 µm *XP* HPLC: 3.5, 5, 10 µm	MAXPEAK PREMIER	3.1 µmol/m²	18%	Yes	L1	1–12	Low pH = 80 °C High pH = 60 °C	185 m²/g
		Performance Benefits: General purpose column ideally suited for method development due to extreme pH stability and applicability to the broadest range of compound classes.					**Bonding**: Trifunctional C_{18}, fully endcapped, bonded to an Ethylene Bridged Hybrid (BEH) substrate.	
BEH Amide UPLC: 1.7 µm UHPLC: 2.5 µm *XP* HPLC: 3.5, 5 µm	MAXPEAK PREMIER	7.5 µmol/m²	12%	No	L68	2–11	Low pH = 90 °C High pH = 90 °C	185 m²/g
		Performance Benefits: Rugged HILIC stationary phase designed to separate a wide range of very polar compounds. Especially good at separating carbohydrates (saccharides) using high concentrations of organic modifier, elevated temperature, and high pH. Compatible with all modern detectors including MS, ELSD, UV, and fluorescence.					**Bonding**: Trifunctional amide bonded to an Ethylene Bridged Hybrid (BEH) substrate.	
BEH Phenyl UPLC: 1.7 µm UHPLC: 2.5 µm *XP* HPLC: 3.5, 5 µm	MAXPEAK PREMIER	3.0 µmol/m²	15%	Yes	L11	1–12	Low pH = 80 °C High pH = 60 °C	185 m²/g
		Performance Benefits: Excellent method development column for alternate selectivity, particularly in regard to polyaromatic compounds. Povides unique level of pH stability for a phenyl bonded phase.					**Bonding**: Trifunctional C_6 phenyl, fully endcapped, bonded to an Ethylene Bridged Hybrid (BEH) substrate.	
CSH C_{18} UPLC: 1.7 µm UHPLC: 2.5 µm *XP* HPLC: 3.5, 5, 10 µm	MAXPEAK PREMIER	2.3 µmol/m²	15%	Yes	L1	1–11	Low pH = 80 °C High pH = 45 °C	185 m²/g
		Performance Benefits: General purpose reversed-phase column that offers excellent pH stability and rapid mobile-phase re-equilibration for method development. Charged Surface Hybrid (CSH™) Technology enables superior peak shape and increased loading capacity for basic compounds.					**Bonding**: Trifunctional C_{18}, fully endcapped, bonded to a Charged Surface Hybrid (CSH) substrate.	
HSS PFP UPLC: 1.8 µm UHPLC: 2.5 µm *XP* HPLC: 3.5, 5 µm	MAXPEAK PREMIER	3.2 µmol/m²	7%	No	L43	2–8	Low pH = 45 °C High pH = 45 °C	230 m²/g
		Performance Benefits: A general purpose column designed to maximize selectivity differences for Lewis bases through pi-pi interactions. The rigid aromatic ring provides additional selectivity based on shape, dipole moment, and hydrogen bonding interactions.					**Bonding**: Trifunctional pentafluoro-phenyl, non-endcapped, bonded to a High Strength Silica (HSS) substrate.	

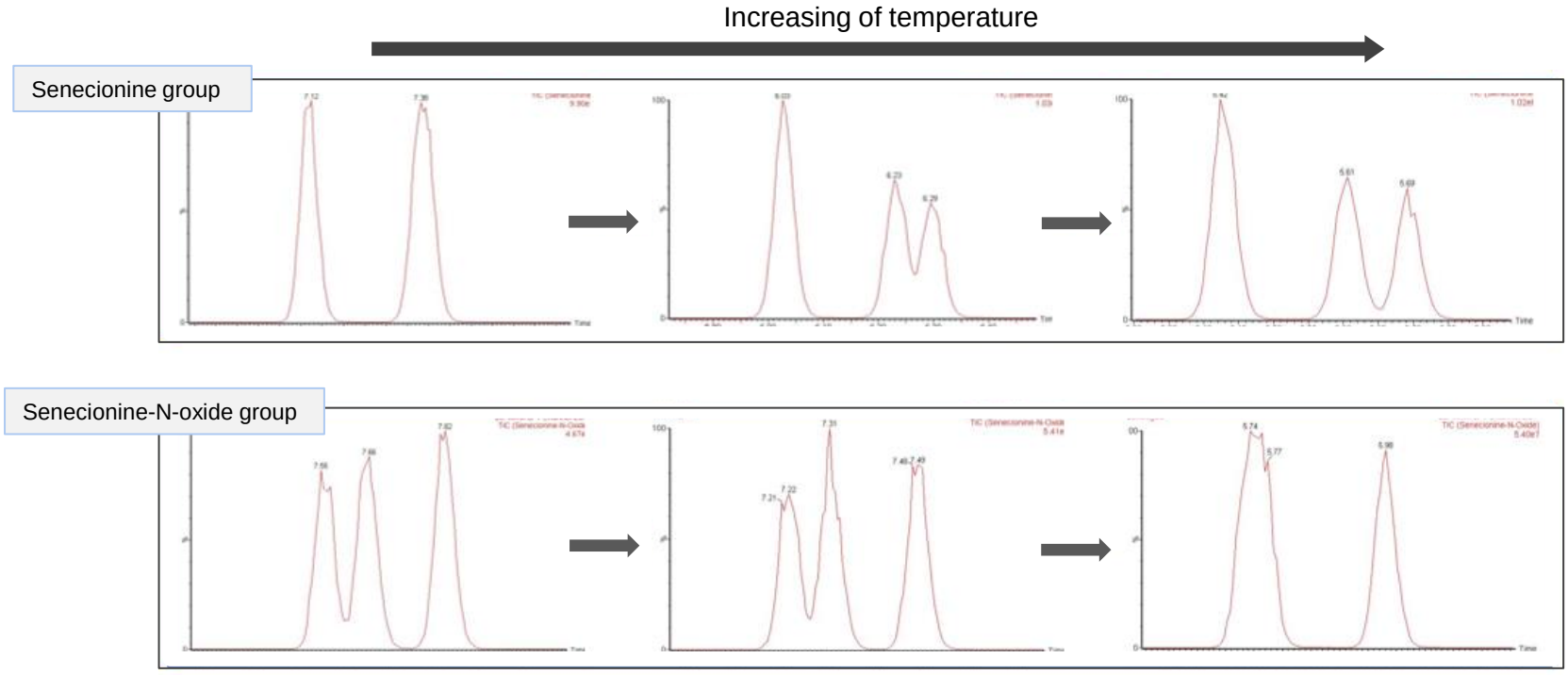
Chromatographic separation of PAs

- pH of mobile phase – critical part of successful separation



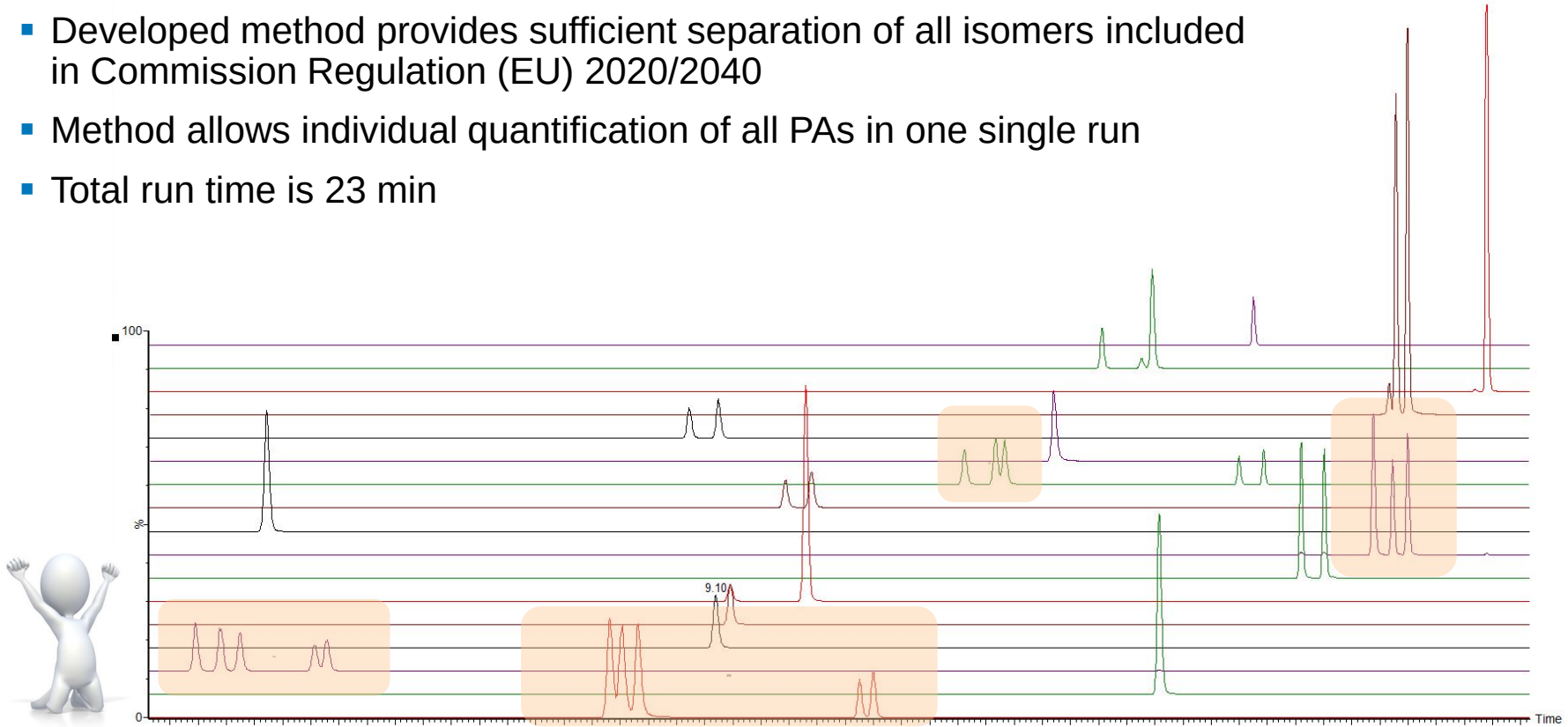
Chromatographic separation of PAs

- Column temperature



Chromatographic separation of PAs

- Developed method provides sufficient separation of all isomers included in Commission Regulation (EU) 2020/2040
- Method allows individual quantification of all PAs in one single run
- Total run time is 23 min



Extraction method - QuPPe

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Weigh homogenized sample into 50 mL PP tube

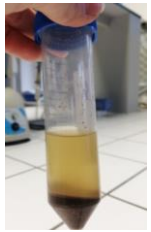
Add 10 mL H2O

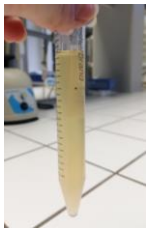
Add 10 mL MeOH containing 1% FA

Shake for 15 minutes

Centrifugation for 5 minutes

Filtration into vial





Method validation – Recovery

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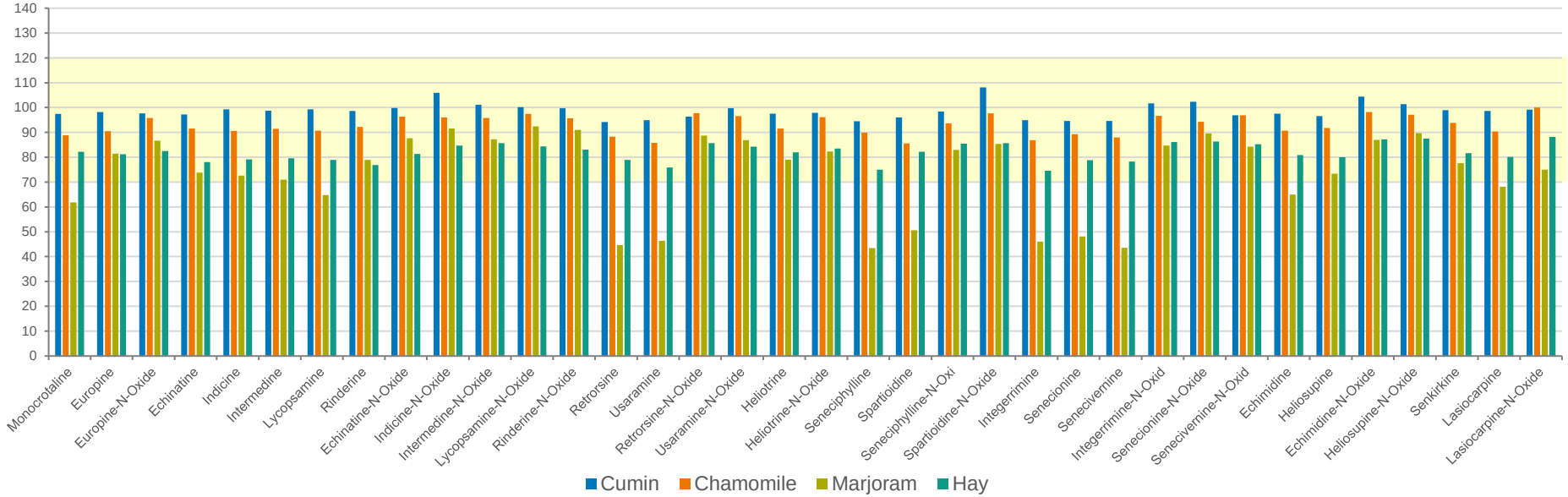
- Average recovery for all PAs together across all spike levels:
  - Cumin seed – 99%
  - Chamomile – 93%
  - Marjoram – 74%
  - Hay– 82%



Method validation – Matrix effect

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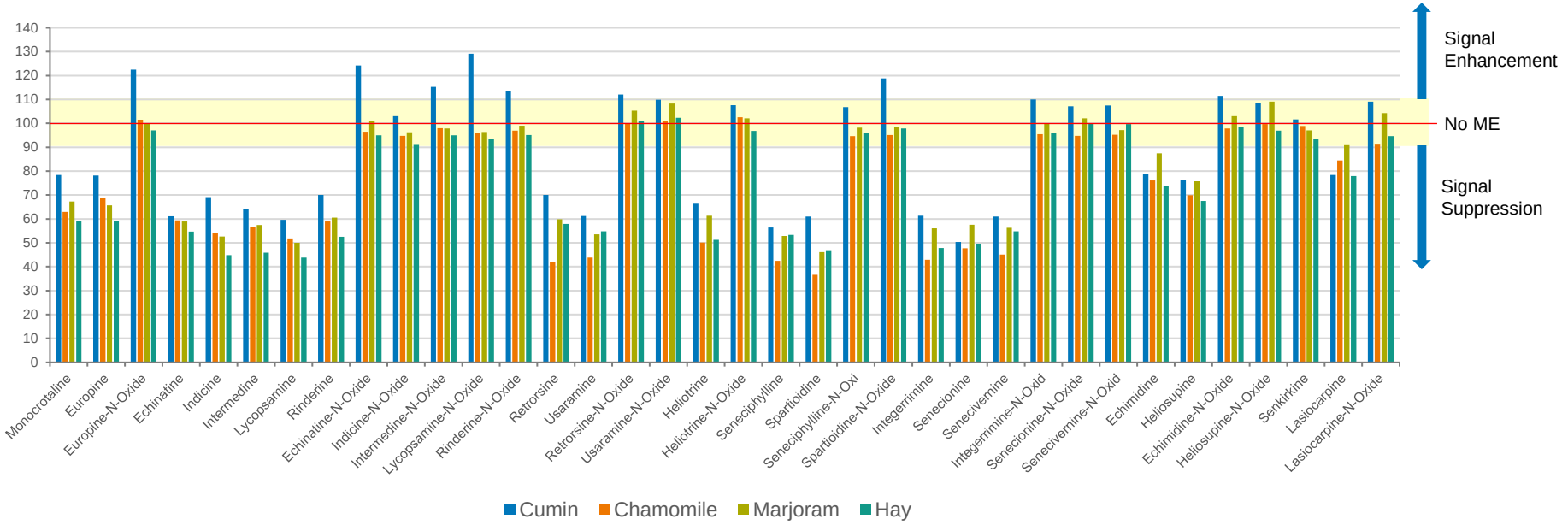
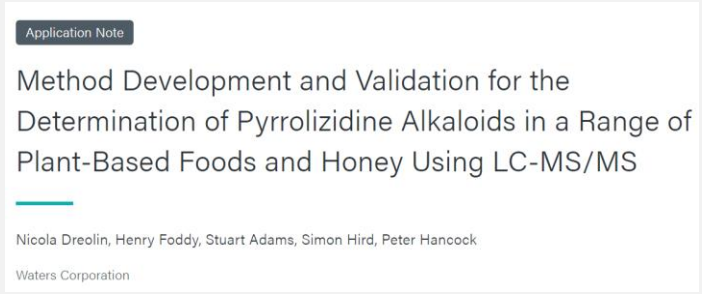


Conclusion

- Developed method allows total separation of all isomers included in EU Regulation in one single chromatographic run
- Method was successfully validated for several matrices – cumin, chamomile, marjoram, hay
- Generally low LOQ were obtained

Current Application note



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Thank you for your attention!