


Nitrosamine Analysis in Tobacco and Tobacco Product using LC-MS/MS

Okta Lian Atikah, S.Si | Field Application Support LC-MS/MS

SCIEX
The Power of Precision

18 February 2020.

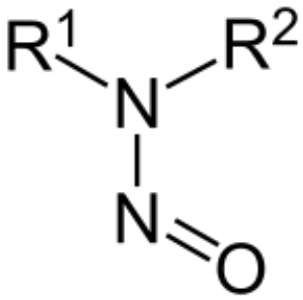
PT. LABORINDO SARANA

Outline

- Nitrosamine and Tobacco-specific Nitrosamine
- Determination of tobacco-specific nitrosamines in tobacco and tobacco products by LC-MS/MS (CRM No. 72)
- Other contaminants analysis in Tobacco using LC-MS/MS

Nitrosamine

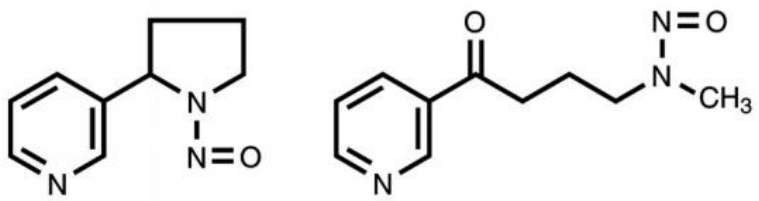
- **Nitrosamines** are organic compounds of the chemical structure R2N−N=O
- Most nitrosamines are **carcinogenic**.
- Nitrosamines can be found in water, foods, cosmetics, tobacco, packing materials and drugs.
- **Tobacco-specific nitrosamines (TSNAs)** are nitrosamine compound group that only found in tobacco products, and possibly in some other nicotine-containing products



Nitrosamine Structure

Tobacco-Specific Nitrosamine (TSNAs)

- **Tobacco-specific nitrosamines (TSNAs)** are formed from nicotine and related compounds by a nitrosation reaction that occurs during the curing and processing of tobacco
- The IARC (International Agency for Research on Cancer) has identified 8 tobacco-specific nitrosamines in tobacco and tobacco smoke.
- Two of them have been classified as Group 1 carcinogens:
  - N-nitrosonornicotine (NNN)
  - 4-methyl-N-nitrosamino-1-(3-pyridyl)-1-butanone (NNK)



NNN NNK

- In January 2017, US FDA (Food and Drug Administration) has proposed rule to establish limit of N-Nitrosonornicotine (NNN) in finished smokeless tobacco product

How to determine TSNAs in Tobacco and Tobacco Product?

CORESTA Recommended Method

CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) has released 3 recommended methods (CRM) for determination of TSNAs

- CORESTA Recommended Method (CRM) No. 63: Determination Of Tobacco Specific Nitrosamines in Cigarette Mainstream Smoke – GC-TEA Method (Newest version released January 2019)
- CORESTA Recommended Method (CRM) No. 75: Determination Of Tobacco Specific Nitrosamines in Cigarette Mainstream Smoke by LC-MS/MS (Newest version released August 2019)
  - The reproducibility data was better for LC-MS/MS than for GC-TEA
- CORESTA Recommended Method (CRM) No. 72: Determination Of Tobacco Specific Nitrosamines in Tobacco and Tobacco Products by LC-MS/MS (Newest version released July 2017)

*coresta.org

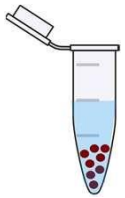
CORESTA Recommended Method No.72

Determination of tobacco-specific nitrosamines (TSNAs) in tobacco and tobacco products by LC-MS/MS

- The TSNAs determined with this method are
  - N-nitrosonornicotine (NNN)
  - 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
  - N-nitrosoanatabine (NAT)
  - N-nitrosoanabasine (NAB).

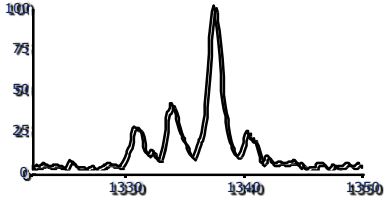
Method Workflow – Targeted

Determination of tobacco-specific nitrosamines (TSNAs) in tobacco and tobacco products by LC-MS/MS



Sample and Standard Preparation

Sample Analysis Using LC-MS/MS



Data Process

Calibration Standard Preparation

- Stock solution for standard and IS is diluted in ACN
- Mixed TSNA std. Nr.2: The concentration is approximately 400 ng/ml of NNN, NNK, NAT and 100 ng/ml of NAB in ACN:Water (30:70)
- Internal std spiking solution 2000 ng/mL diluted in ACN.

Cal. Std.	Volume of Mixed TSNA std. Nr. 2 (ml)	Volume of Internal std spiking solution 2000 ng/ml (ml)	Volume acetonitrile (ml)	Conc. NNN (ng/ml)	Conc. NNK (ng/ml)	Conc. NAT (ng/ml)	Conc. NAB (ng/ml)
Cal 1	0,125	1,00	22	0,5	0,5	0,5	0,125
Cal 2	0,250	1,00	22	1,0	1,0	1,0	0,250
Cal 3	0,50	1,00	22	2,0	2,0	2,0	0,50
Cal 4	1,00	1,00	22	4,0	4,0	4,0	1,00
Cal 5	2,00	1,00	22	8,0	8,0	8,0	2,00
Cal 6	5,00	1,00	21	20	20	20	5,00
Cal 7	25,0	1,00	15	100	100	100	25,0

- Each of the cal std is diluted to volume 100 mL with 100 mM ammonium acetate, and mixed well

Sample Preparation

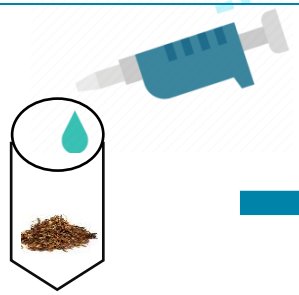
Samples	Preparation
 Tobacco and tobacco products	Tobacco and tobacco products are grounded Grinding procedure shall not generate heat or cause sample degradation
 Smokeless tobacco products	Shall be analyzed together with their pouch (paper) and shall be cut into two halves directly into the extraction flask
  Cigar and Cigarette Filler	Cigar and cigarette filler are grounded. Testing may also involve the analysis of the entire cigar where the wrapper and filler are ground together.

*The test samples shall be stored protected from light

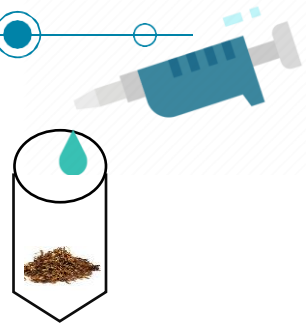
Sample Extraction

weigh samples approximately 1,000 g

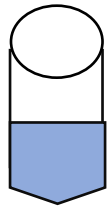
Add sample to extraction vessel



Add 0.3 mL internal standard spiking solution



Add 30 mL of 100 mM ammonium acetate and cap the conical flask



Shake the sample(s) for 40 min ± 5 min

Filter each sample using a 25 mm, 0,45 µm PTFE syringe filter directly into vials

Ready to be injected into LC-MS/MS

SCIEX Answers for Science. Knowledge for Life.™

PT. LABORINDO SARANA

LC Conditions

- Mobile Phase:
  - Mobile phase A: Water
  - Mobile phase B: 0,1 % (v/v) acetic acid in methanol
- Column: C18 HPLC Column, 2,5 µm particle size, 2,1 mm × 50 mm, or equivalent
- Column Temperature: 60,0 °C
- Injection Volume: 10 µL
- LC Gradient

Time (Min)	Flow rate (mL/min)	Conc. A (%)	Conc. B (%)
0	0.22	100	0
3.0	0.22	10	90
4.0	0.22	10	90
5.0	0.22	0	100
6.0	0.22	100	0
10.0	0.22	100	0

MS conditions – MRM Scan Mode

Compound Name	Quantitation Transition (m/z)	Qualification Transition (m/z)	Internal Standard Reference
NNK	208 > 122	208 > 79	NNK-d4
NNK-d4	212 > 126	n/a	n/a
NNN	178 > 148	178 > 105	NNN-d4
NNN-d4	182 > 152	n/a	n/a
NAT	190 > 160	190 > 79	NAT-d4
NAT-d4	194 > 164	n/a	n/a
NAB	192 > 162	192 > 133	NAB-d4
NAB-d4	196 > 166	n/a	n/a

- Quantitation Transition will be used for Quantitation analysis in other to determine concentration of TSNAs in samples
- Qualification Transition will be used for confirmation using ion ratio

US FDA – Determination NNN in Smokeless Tobacco and Tobacco Filler

US FDA has adapted CRM No.72 with some modifications to determine NNN in smokeless tobacco and tobacco filler by LC-MS/MS

The same extraction process based on CRM No. 72 with modifications to the calibration ranges and instrumental approach.

LABORATORY INFORMATION BULLETIN

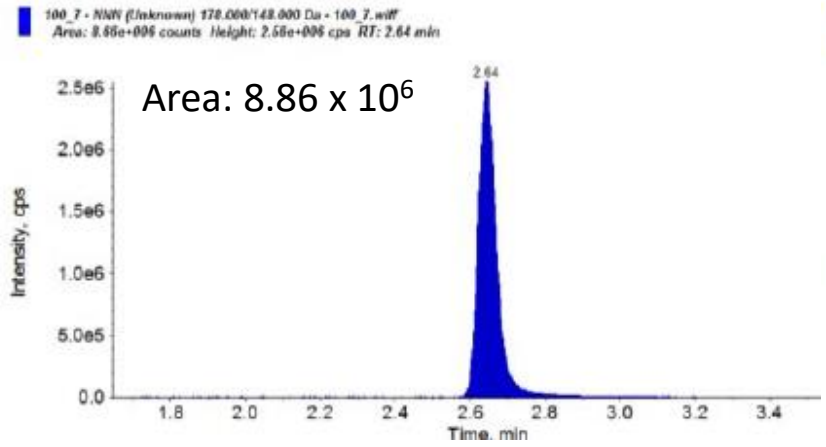
Determination of *N*-nitrosonornicotine (NNN) in Smokeless Tobacco and Tobacco Filler by HPLC-MS/MS[1]

John A. Mathis, Ph.D., Rayman A. Stanelle, Ph.D., and Jean-Marie D. Dimandja, Ph.D.
U.S. Food and Drug Administration, Office of Regulatory Affairs, Southeast Regional Laboratory, Tobacco Branch, 60 8th St. Atlanta, GA 30309. John.Mathis@fda.hhs.gov

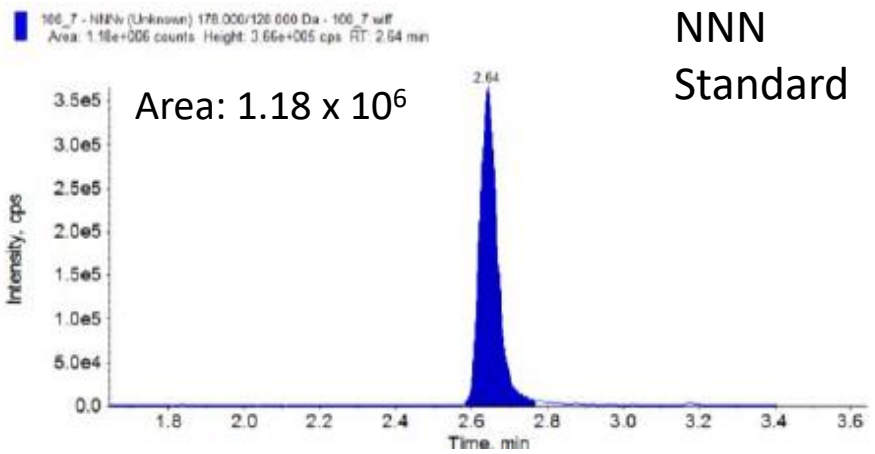
Note: The Laboratory Information Bulletin is a tool for the rapid dissemination of laboratory methods (or information) which appears to work. It does not report complete scientific work. The user must assure himself/herself by appropriate validation procedures that LIB methods and techniques are reliable and accurate for his/her intended use. Reference to any commercial materials, trade names, equipment, or procedure does not necessarily constitute approval, endorsement, or recommendation by the Food and Drug Administration or the U.S. Department of Health and Human Services.

SCIEX 6500 QTRAP

Confirmation using ion ratio



Extracted Ion Chromatogram of Quantitation Ion



NNN Standard

Extracted Ion Chromatogram of Qualification Ion

$$Ion\ Ratio = \frac{Area\ MRM\ 2}{Area\ MRM\ 1}$$

$$Ion\ Ratio = \frac{1.18 \times 10^6}{8.66 \times 10^6}$$

= 0.136

= 0.136 ± 20%

= 0,1088 – 0,1632

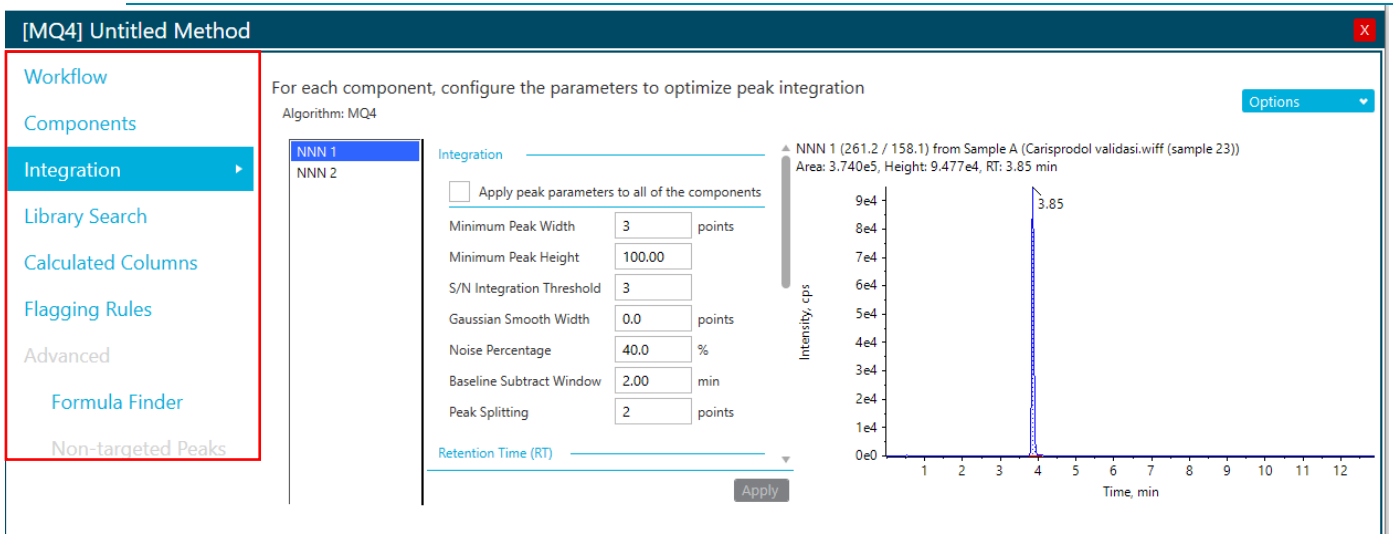
CRM No. 72 reqirement: The ion ratios must be within ± 20% of the average of the calibration standards

*US FDA adapted CRM No.72 for determination NNN in Smokeless Tobacco and Tobacco Filler using SCIEX 6500 QTRAP

SCIEX Answers for Science. Knowledge for Life.™

PT. LABORINDO SARANA

SCIEX OS – Data Processing

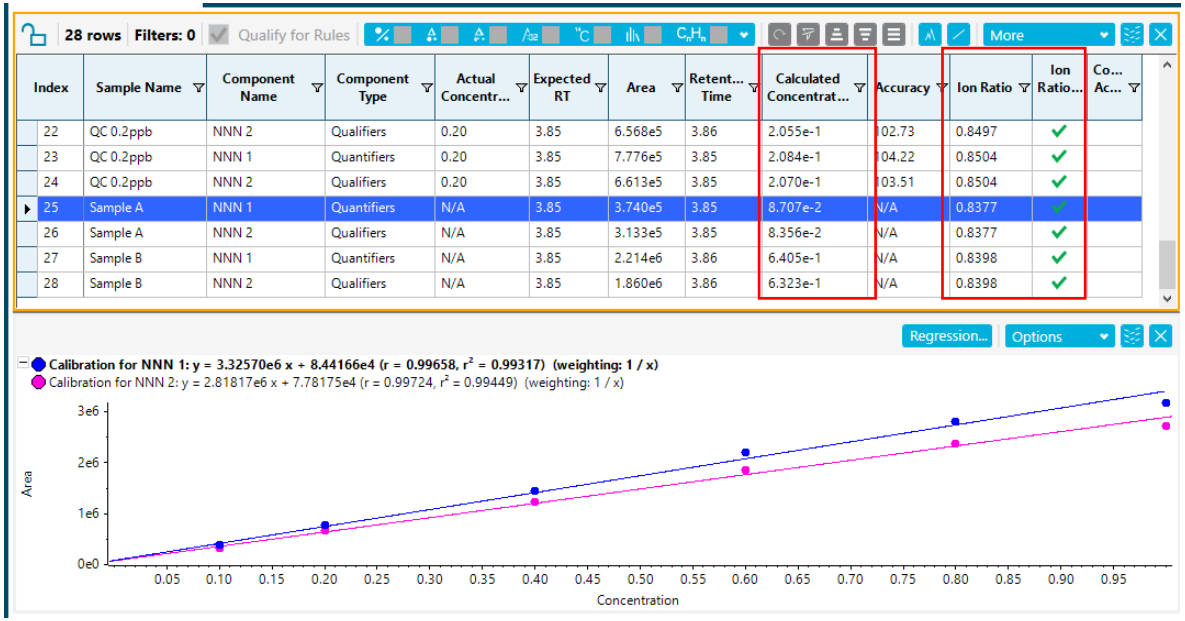


Build Processing Method

- Components
- Integration
- Ion Ratio acceptance
- Accuracy acceptance
- etc

Result Table

- Area
- Retention time
- Calculated Concentration
- Ion Ratio
- Ion Ratio Acceptance
- Calibration Curve



SCIEX
Answers for Science. Knowledge for Life.™

Which other contaminant can be analyzed using LC-MS/MS?

Nicotine analysis in tobacco using LC-MS/MS

STUDIA UNIVERSITATIS BABEŞ-BOLYAI, PHYSICA, L, 4b, 2005

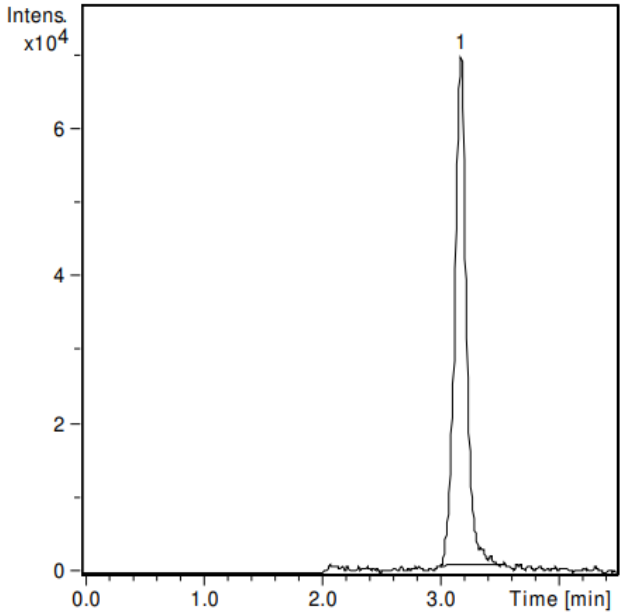
DETERMINATION OF NICOTINE FROM TOBACCO BY LC-MS-MS

Laurian Vlase[1], Lorena Filip[2], Ioana Mîndruţău[3], Sorin E. Leucuţa[1]

Faculty of Pharmacy, University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca
[1]Department of Pharmaceutical Technology
[2]Department of Environmental Chemistry
[3]Department of Physical Chemistry

ABSTRACT

A new high performance liquid chromatography coupled with mass spectrometry method (LC/MS/MS) for quantification of nicotine from tobacco was elaborated. It was utilized an Atlantis HILIC, 100 mm x 3.0 mm i.d., 3 µm column with a mobile phase containing acetonitrile/ solution 0.2% formic acid in water. The ionization was optimized using ESI(+) and enhanced selectivity was achieved using tandem mass spectrometric analysis. The precursor to product ion transitions of m/z 163 -> (105.8, 131.8) were used to measure the nicotine concentrations. The quantification was made using the external standard method. The calibration curves were made on range 0.04-4 µg/ml. Nicotine content was determined in 40 brands of cigarettes available in Romania. With our methodology, we obtained values of nicotine in tobacco between 7.5 and

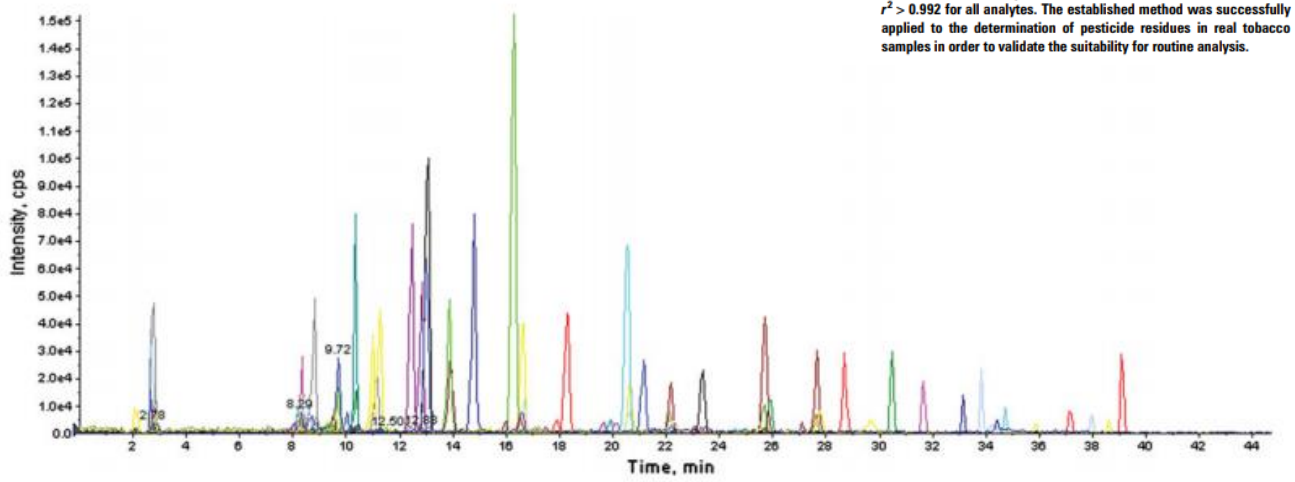


Chromatogram result of Nicotine in Tobacco Leaf Using LC-MS/MS

Which other contaminant can be analyzed using LC-MS/MS?

Pesticide Analysis using LC-MS/MS

SCIEX LC-MS/MS allow us to analyze hundred of analytes in one run



Chromatogram of pesticides

Journal of Chromatographic Science 2014;52:788–792
doi:10.1093/chromsci/bmt112 Advance Access publication July 25, 2013

Article

Analysis of 118 Pesticides in Tobacco after Extraction With the Modified QuEChRS Method by LC–MS-MS

Fei Yang[1], Zhaoyang Bian[1,2]*, Xiaoshui Chen[1], Sansan Liu[1], Yang Liu[1] and Gangling Tang[1]

[1]China National Tobacco Quality Supervision & Test Center, Zhengzhou 450000, PR China, and [2]No.2, Fengyang Street, Zhengzhou New & Hi-Tech Industries Development Zone, Henan, China

*Author to whom correspondence should be addressed. Email: zybian@126.com

Received 3 February 2013; revised 10 June 2013

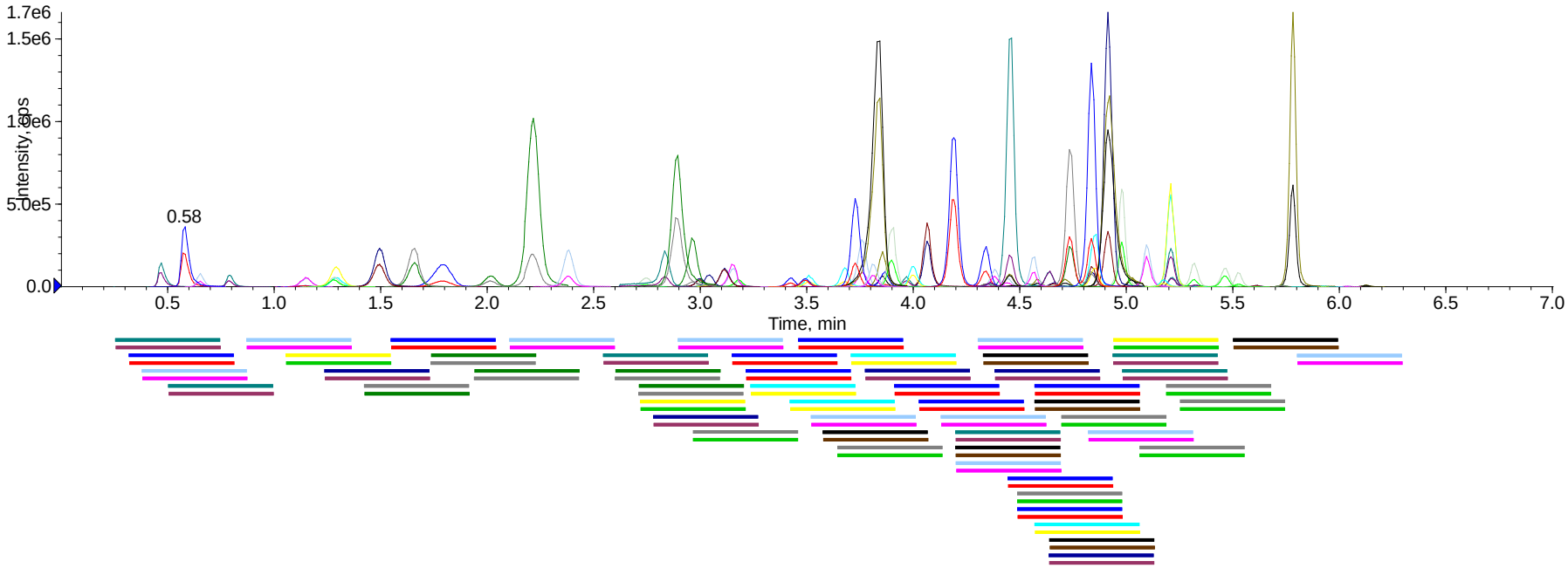
A liquid chromatography–tandem quadrupole mass spectrometry (LC–MS-MS) multi-residue method for the simultaneous target analysis of a wide range of pesticides in tobacco has been developed. Gradient elution has been used in conjunction with positive mode electrospray ionization tandem mass spectrometry to detect up to 118 pesticides in tobacco. The recoveries obtained for each pesticide ranged between 70 and 118% at two spiked concentration levels. Good linear relationships were observed with correlation coefficients $r^2 > 0.992$ for all analytes. The established method was successfully applied to the determination of pesticide residues in real tobacco samples in order to validate the suitability for routine analysis.

based on a liquid partitioning with acetonitrile followed by a dispersive solid-phase extraction (d-SPE) clean-up with primary secondary amine (PSA). Modifications to the original method to ensure efficient extraction of pH-dependent compounds (by using different buffer solutions) (14, 15) or addition of water to dry samples in order to obtain the necessary moisture (16, 17) have been introduced. The QuEChERS method is particularly popular for determination of polar, middle polar and non-polar pesticide residues in various food matrices (17–21) because of its simplicity, inexpensiveness, amenability to high throughput and relatively high-efficiency results with a minimal number of steps.

In this work, a simple liquid chromatography–tandem quadru

Scheduled MRM™ Algorithm

Combined with fast polarity switching



- Adjusts detection windows automatically depending on retention time
- Optimizes dwell times for each analyte and cycle time
  - Allows detecting many more MRM transitions
  - Allows using faster LC
  - Gives best S/N, accuracy and reproducibility

Related method reference

Nitrosamines Analysis

- Mathis, et al. Determination of N-nitrosonornicotine (NNN) in Smokeless Tobacco and Tobacco Filler by HPLCMS/MS. (US FDA)
- Li, et al. 2015. Simultaneous Determination of Alkaloids and Their Related Tobacco-Specific Nitrosamines in Tobacco Leaves Using LC–MS-MS. Yunnan Academy of Tobacco Agricultural Sciences
- Guo, et al. 2018. Rapid Analysis of Genotoxic Nitrosamines by HPLC-MS/MS. (SCIEX)

Pesticide Analysis

- CORESTA Guide N° 5 Technical Guide for Pesticide Residues Analysis on Tobacco and Tobacco Products
- Development of a Fast and Cost-Effective Multi-Residue Method to Determine Pesticides in Tobacco by LC/MS/MS
- Dr. Hans Rainer Wollseifen. Determination of Pesticides in Tobacco Automated with FREESTYLE QuEChERS and LC-MS/MS.

Nicotine Analysis

- McGuffrey, et al. 2014. Validation of a LC-MS/MS Method for Quantifying Urinary Nicotine, Six Nicotine Metabolites and the Minor Tobacco Alkaloids—Anatabine and Anabasine—in Smokers' Urine

Conclusion

- Determination of TSNAs can be done using LC-MS/MS according to CRM No. 72 and has adapted by US FDA
- Data processing can be done using SCIEX OS Software
- Other potent carcinogenic contaminants, such as nicotine and pesticide can also analyzed by LC-MS/MS
- ScheduleMRM allow us to analyze hundred of analyte in one run

Reference

- CORESTA Recommended Method No. 72 Version 4. July 2017
- Mathis, et al. Determination of *N*-nitrosonornicotine (NNN) in Smokeless Tobacco and Tobacco Filler by HPLCMS/MS. US FDA
- Vlase, et al. 2005. Determination of Nicotine from Tobacco Using LC-MS/MS
- Yang, et al. 2013. Analysis of 118 Pesticides in Tobacco after Extraction With the Modified QuEChRS Method by LC-MS/MS

THANK YOU FOR LISTENING!

SCIEX
Answers for Science.
Knowledge for Life.™

Email: okta.atikah@laborindo.com

Learn more about LC-MS/MS through **www.sciex.com**