Cooperation Centre for Scientific Research Relative to Tobacco

Tobacco and Tobacco Products Analysis Sub-Group

CORESTA Recommended Method No. 105

DETERMINATION OF NICOTINE IMPURITIES AND DEGRADANTS IN NICOTINE POUCHES BY LC-MS/MS

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0. INTRODUCTION

In 2020, the CORESTA Tobacco and Tobacco Products Analytes (TTPA) Sub-Group conducted a proficiency study for the determination of nicotine and nicotine impurities and degradants (i.e., nicotine degradants) in nicotine pouches.[1,2] Nineteen laboratories participated in the nicotine analysis and fifteen laboratories participated in the nicotine degradants analysis. The objective of this proficiency study was to provide an assessment of laboratory capability for the analysis of nicotine and nicotine degradants in nicotine pouches. Laboratories determined nicotine, anatabine, anabasine, nicotine-N'-oxide, myosmine, β-nicotyrine, cotinine, and nornicotine using their in-house methodologies. The amount of nicotine determined by all labs in all samples was found to be relatively consistent despite the use of various extraction and analytical methods, but still more variable than would be expected with a single standardized method. The proficiency study showed that in many cases the participating laboratories obtained different results for the nicotine degradants which was attributed to the use of different methodologies. Results from this study were discussed at the virtual TTPA meeting held on April 22, 2020 and published in the TTPA-246-CTR technical report.[1] The TTPA Sub-Group recommended that developing a consensus standardized method would likely improve the consistency of the nicotine degradants results. Therefore, a small group was formed to develop a proposal for a draft CORESTA Recommended Method for the determination of nicotine degradants in nicotine pouches.

In 2023, the TTPA Sub-Group completed a collaborative study for the determination of the seven nicotine impurities and degradants in nicotine pouches.[3] The intent of the study was to calculate repeatability and reproducibility values for the method. Four commercial-like nicotine pouch filler samples were included in the study and 8 laboratories participated. This Recommended Method has been shown to be fit for the determination of nicotine impurities and degradants of nicotine pouches.

1. FIELD OF APPLICATION

This recommended method is applicable to the determination of nicotine degradants and impurities in nicotine pouches. Nicotine pouches, or white pouches, are oral tobacco products that contain tobacco derived nicotine, but not tobacco leaf. The nicotine degradants included in this Recommended Method are: anatabine, anabasine, nicotine-N'-oxide, myosmine, β-nicotyrine, cotinine, and nornicotine.

2. NORMATIVE REFERENCES

2.1 ISO 3696, Water for analytical laboratory use - specification and test methods

3. PRINCIPLE

After addition of deuterium isotopically labelled internal standards, the sample is extracted into an aqueous buffer and filtered. The filtrate is analyzed by liquid chromatography - triple quadrupole mass spectrometry (LC-MS/MS). The results are reported in units of micrograms per gram.

4. EQUIPMENT AND APPARATUS

Normal laboratory apparatus and equipment including the following items:

4.1 High performance liquid chromatography coupled to tandem triple quadrupole mass spectrometry (LC-MS/MS) with an electrospray ionization (ESI) source consisting of:

- 4.1.1 Binary pump
- 4.1.2 Chilled autosampler
- 4.1.3 Column oven
- 4.1.4 Tandem mass spectrometer
- 4.1.5 Data collection system

4.2 C18 HPLC column, 1,7 µm particle size, 2,1 mm × 100 mm (or 50 mm), or equivalent

4.3 Analytical balance (0,0001 g resolution)

4.4 Orbital shaker, wrist action shaker, or similar

4.5 Amber autosampler vials with PTFE lined caps

4.6 Disposable polypropylene 3 ml syringes

4.7 Syringe filter, 0,2 µm PTFE or equivalent

NOTE: Other filter materials may also be suitable; however, recovery should be evaluated before routine use.

4.8 Amber glass extraction containers, 50 ml - 100 ml, with PTFE screw cap liners

4.9 Graduated cylinders

4.10 Volumetric flasks, Class A

4.11 Volumetric pipette, calibrated pipette or equivalent

5. REAGENTS

Use reagents of recognized analytical grade and solvents of HPLC-grade or better.

5.1 Water, Grade 1 (refer to ISO 3696)

5.2 Acetonitrile (HPLC-grade)

5.3 Methanol (HPLC-grade)

5.4	Ammonium hydroxide (6 M)	(≥ 98 %)
5.5	Ammonium acetate	(≥ 98 %)
5.6	Ammonium formate	(≥ 98 %)
5.7	Acetic acid (1 M)	(≥ 98 %)
5.8	Formic acid	(≥ 98 %)
5.9	Anabasine, CAS-No 13078-04-1 (RS) or 494-52-0 (L)	(≥ 98 %)
5.10	Anatabine, CAS-No 2743-90-0 (RS)	(≥ 95 %)
5.11	Cotinine, CAS-No 486-56-6	(≥ 98 %)
5.12	Nornicotine, CAS-No 5746-86-1	(≥ 94 %)
5.13	Myosmine, CAS-No 532-12-7	(≥ 98 %)
5.14	β-Nicotyrine, CAS-No 487-19-4	(≥ 97 %)
5.15	Nicotine-N'-Oxide, CAS-No 63551-14-4	(≥ 98 %)
5.16	Anabasine-d4, CAS-No 1020719-08-7	(≥ 98 %)
5.17	Anatabine-d4, CAS-No 1020719-11-2	(≥ 98 %)
5.18	Cotinine-d3, CAS-No 110952-70-0	(≥ 98 %)
5.19	Nornicotine-d4, CAS-No 66148-18-3	(≥ 97 %)
5.20	Myosmine-d4, CAS-No 66148-17-2	(≥ 98 %)
5.21	β-Nicotyrine-d3	(≥ 98 %)
5.22	Nicotine-N'-Oxide-d3	(≥ 98 %)

NOTE: Reference materials should be purchased with the highest purity available in analytical quality.

6. SOLUTIONS PREPARATION

6.1 Reagents

6.1.1 Extraction Solution

Prepare 2 litres of 100 mM ammonium formate buffer (pH 3) by adding 12,61 g of ammonium formate to a 2000-ml graduated cylinder volumetric flask containing approximately 1800 ml of Grade 1 water. Adjust the pH to 3,0 ± 0,1 with approximately 13 ml formic acid and then dilute to volume using Grade 1 water. Store the solution at room temperature in a 2-l glass bottle.

NOTE: This solution has been shown to be stable for 14 days at room temperature.

6.1.2 Mobile Phase A, 10 mM Ammonium Acetate Buffer

Add approximately 900 ml of Grade 1 water to a 1000-ml graduated cylinder. Add 10 ml of 1 M acetic acid. Add 13,0 ml of 6 M ammonium hydroxide. Adjust the pH to 10,0 ± 0,1 with 6 M ammonium hydroxide or 1 M acetic acid. Dilute to 1000 ml with Grade 1 water. Mix well and store in 1-l glass bottle at room temperature. This solution has been shown to be stable for up to 1 month at room temperature.

6.1.3 0,05 % Ammonium Hydroxide Solution for Calibration Standards

Add 0,2 ml of ammonium hydroxide (25 %) to a 100-ml graduated cylinder. Dilute to 100 ml with Grade 1 water. This solution must be prepared at each time calibration standards are prepared.

6.2 Standards

All standard solutions shall be prepared and stored in amber, or light-protected glassware and stored at approximately -20 °C, except the calibration standards shall be stored in a refrigerator. All standards shall be stored in amber glass bottles with PTFE screw caps. Mixed standard solutions have been shown to be stable for one year. The calibration standards have been shown to be stable for 6 months. Laboratories should determine the stability of the standards under their conditions of use.

All solutions shall be prepared using appropriately sized Class A volumetric pipettes (or calibrated pipettes) and volumetric flasks.

NOTE: For standard solutions stored in the freezer, they must be allowed to equilibrate to room temperature before use.

6.2.1 Internal Standard Stock Solutions

Individually weigh approximately 10 mg anatabine-d_4, anabasine-d_4, nicotine-N'-oxide-d_3, myosmine-d_4, β-nicotyrine-d_3, cotinine-d_3, and nornicotine-d_4 into individual 10-ml volumetric flasks. Record the exact weight to 0,1 mg. Dilute to volume with methanol. The concentration in each solution is approximately 1000 µg/ml.

6.2.2 Mixed Internal Standard Solution

Using a Class A volumetric pipette (or calibrated pipette), transfer 1,00 ml of each of the Internal Standard Stock Solutions into a single 10-ml volumetric flask and dilute to volume with methanol, mix well. The concentration of all internal standards is approximately 100 µg/ml.

6.3 Calibration Standard Solutions

6.3.1 Nicotine Degradants Stock Solutions

Individually weigh approximately 10,0 mg anatabine, anabasine, nicotine-N'-oxide, Myosmine, β-nicotyrine, cotinine, and nornicotine into individual 10-ml volumetric flasks. Record the exact weight to 0,1 mg. Dilute to volume with methanol. The concentration in each solution is approximately 1000 µg/ml.

6.3.2 Primary Mixed Nicotine Degradants Standard Solution

Using a Class A volumetric pipette (or calibrated pipette), transfer 1,00 ml of each of the single Nicotine Degradants Stock Solutions into a single 10-ml volumetric flask and dilute to volume with methanol and mix well. The concentration is approximately 100 µg/ml of all seven nicotine degradants.

6.3.3 Secondary Mixed Nicotine Degradants Standard Solution

Using a Class A volumetric pipette (or calibrated pipette), transfer 1,00 ml of the Primary Mixed Nicotine Degradants Standard solutions into a single 10-ml volumetric flask and dilute to volume with methanol and mix well. The concentration is approximately 10 µg/ml of all seven nicotine degradants.

6.3.4 Nicotine Degradants Calibration Standards

Prepare 7 working calibration standard solutions that cover the concentration range of interest. An example of calibration standard preparation is provided in Table 1. The nicotine degradants calibration standards are prepared in seven separate 10-ml volumetric flasks. Briefly, add 2 ml of 0,05 % ammonium hydroxide solution to each volumetric flask. Add 20,0 µl of the Mixed Internal Standard Solution (100 µg/ml) to each of the seven volumetric flasks. Next, add the appropriate volume of the primary and secondary nicotine degradants standard solutions specified in Table 1. Finally, each of the seven flasks is diluted to volume with 0,05 % ammonium hydroxide solution and mixed well. Calculate the exact concentrations for each calibration standard.

NOTE: Stock solutions of the individual nicotine degradants and deuterated internal standards in methanol can be purchased at the required concentrations.

NOTE: The linearity range should be determined for each lab/instrument to fit the instruments capabilities and the range of samples usually being measured in that laboratory. Samples can be diluted in 0,05 % ammonium hydroxide solution containing internal standards if necessary.

Table 1. Concentration and preparation of nicotine calibration standards

Calibration Standard	Flask (ml)	Volume of Primary Mixed Nicotine Degradants Standard Solution (100 µg/ml) (µl)	Volume of Secondary Mixed Nicotine Degradants Standard Solution (10 µg/ml) (µl)	Volume of Mixed Internal Standard Solution (100 µg/ml) (µl)	Internal Standard Final Concentration (µg/ml)	Calibration Standard Concentration (µg/ml)
1	10	NA	25	20	0,20	0,025
2	10	NA	100	20	0,20	0,10
3	10	25	NA	20	0,20	0,25
4	10	50	NA	20	0,20	0,50
5	10	100	NA	20	0,20	1,00
6	10	250	NA	20	0,20	2,50
7	10	500	NA	20	0,20	5,00

*NA = Not applicable

7. PROCEDURES

7.1 Sampling

Sampling is conducted such that the laboratory test sample is representative of the population received for analysis.

7.2 Sample Preparation

7.2.1 The target aliquot weight for each replicate analysis is approximately 1,0 g.

7.2.2 Unit pouches shall be analyzed and include both the paper and filler. Extract a sufficient number of pouches to come as close to the target weight as possible. Cut the pouch into two halves and add the filler and pouch paper directly into the extraction flask.

7.3 Sample Extraction

7.3.1 Using an analytical balance, weigh approximately 1,0 g (note the exact weight with 4 decimals) of sample into the extraction vessel.

7.3.2 Add 60,0 µl of the 100 µg/ml Mixed Internal Standard Solution (using a calibrated pipette (or equivalent).

7.3.3 Add 30,0 ml of extraction solution (100 mM ammonium formate (pH 3)) and cap the flask.

7.3.4 Shake vigorously the sample(s) for 40 min ± 5 min at a rate to ensure sufficient mixing and allow samples to settle for 5 minutes then proceed to the next step.

7.3.5 Filter each sample using a 0,2 µm PTFE syringe filter directly into amber vials and cap each vial.

NOTE: Samples may be extracted in a centrifuge tube instead of glass vials and be centrifuged after shaking.

NOTE: Samples that exceed the calibration range may be diluted using the 0,05 % ammonium hydroxide solution containing internal standards.

7.3.6 The extract is ready for injection into the LC-MS/MS system.

NOTE: The stability of the prepared samples in the refrigerator (4–6 ºC) was investigated by analyzing samples immediately after preparation and after 3, 7 and 14 days. The samples were stored in vials with perforated and unperforated septa. The results revealed that the samples were stable for at least seven days in vials with unperforated septa and only three days in vials with perforated septa.

8. DETERMINATION

Set up and operate the LC-MS/MS system in accordance with the manufacturer's instructions. Equilibrate the system prior to use. The instrument conditions given below have been shown to produce suitable results. Depending on the instrument used, it may be necessary to modify the instrument conditions to provide equivalent results.

8.1 Suggested HPLC Parameters

The following are recommended conditions for the LC system and may be modified to achieve acceptable performance:

- Column temperature: 45,0 °C
- Injection volume: 1,0 µl
- Flow rate: 0,4 ml/min
- Autosampler temperature: 10 ºC
- Mobile phase A: 10 mM ammonium acetate, pH 10,0
- Mobile phase B: acetonitrile

Depending on the HPLC column that is used, it may be necessary to adjust the HPLC gradient provided in Table 2.

Table 2. HPLC gradient

Time (min)	Flow (ml/min)	% Eluent A	% Eluent B	Gradient type
Initial	0,40	95	5	Initial
3,0	0,40	45	55	linear
3,1	0,40	10	90	linear
4,0	0,40	10	90	linear
4,5	0,40	95	5	linear
5,5	0,40	95	5	linear

8.2 MS/MS Parameters

Setup the triple quadrupole mass spectrometer in positive electrospray mode using multiple reaction monitoring (MRM). It is necessary that the triple quadrupole mass spectrometer has been carefully optimized for sensitivity of each analyte before analysis can occur. The dwell times need to be optimized to achieve accurate quantification and the number of data points across each peak should be 15 to 20. Once optimized, the same LC-MS/MS conditions must be used for the analysis of all standards and samples.

8.2.1 Quantification and Qualification Transitions

The most sensitive ion precursors listed in Table 3 are used for the quantification.

Table 3. Quantification and qualification transitions for nicotine degradants

Analyte	Retention time (min)	Quantification Transition (m/z)	Qualification Transition (m/z)	Internal Standard Reference
Nicotine-N'-Oxide	~1,12	179,0 > 132,1	179,1 > 130	Nicotine-N'-Oxide-d_3
Nicotine-N'-Oxide-d_3	~1,12	182,1 > 130,1	182,1 > 132	
Nornicotine	~1,98	149,1 > 130,1	149,1 > 117; 149,1 > 132	Nornicotine-d_4
Nornicotine-d_4	~1,97	153,1 > 136,1	153,1 > 121	
Cotinine	~1,62	177,0 > 80	177,1 > 98	Cotinine-d_3
Cotinine-d_3	~1,62	180,1 > 80	180,1 > 101	
Anabasine	~2,33	163,1 > 92	163,1 > 146; 163,1 > 94	Anabasine-d_4
Anabasine-d_4	~2,31	167,1 > 96	167,1 > 122	
Anatabine	~2,09	161,1 > 107	161,1 > 144	Anatabine-d_4
Anatabine-d_4	~2,08	165,1 > 148,1	165,1 > 111	
Myosmine	~2,10	147,1 > 105	147,1 > 130	Myosmine-d_4
Myosmine-d_4	~2,09	151,1 > 81	151,1 > 109	
β-Nicotyrine	~2,96	159,1 > 144	159,1 > 117	β-Nicotyrine-d_3
β-Nicotyrine-d_3	~2,95	162,1 > 144	162,1 > 117	

NOTE: The transitions provided in Table 3 are for guidance purposes only and the actual optimized values may vary from instrument to instrument. The performance of the system should be sufficient to achieve MRM chromatograms similar to those given in Appendix I.

8.3 System Suitability

The system performance must be evaluated for sensitivity, chromatographic performance, carry over and any other criteria necessary to ensure optimization of the LC-MS/MS system.

8.4 Calibration

Analyse the initial calibration consisting of at least 7 consecutive standard concentrations per analyte. Set the quantitation method to perform an internal standard quadratic calibration with 1/x weighting. The regression should have a coefficient of determination (r^2) of at least 0,995. The calibration curve is a response of the area ratio of each analyte to the corresponding internal standard. Do not force the quadratic correlation through the origin. Inject all calibration standards and then proceed to the samples.

The initial calibration standards are acceptable if they are within 20 % of their assigned values for the lowest standard (LOQ) and within 15 % for all other standards. If the calibration does not meet these criteria, check the instrument for problems; analyse fresh standard aliquots and/or fresh standards prepared from stock solutions.

8.5 Calculations

8.5.1 The concentration of the target analyte in a sample (µg/g) is determined using the concentration obtained from the instrument with the following equation:

$$C_{ND} = \left(\frac{C \times V}{W}\right)$$

Where

C_{ND} is the nicotine degradants concentration in µg/g

C is the nicotine degradants concentration obtained from calibration curve in µg/ml

V is the extraction volume in ml

W is the sample weight in g

8.5.2 The concentration of the target analyte in a sample (µg/portion) is determined using the calculated µg/g concentration with the following equation:

$$C_P = C_{ND} \text{ x } N$$

Where

C_P is the nicotine degradants concentration in µg/portion

C_{ND} is the nicotine degradants concentration in µg/g

N is the portion weight in g

8.6 Quality Control

Each laboratory should perform quality control procedures per their quality system requirements.

9. SUGGESTED SPECIAL PRECAUTIONS

9.1 Experience has shown that the nicotine pouch matrix might lead to contamination of the ion source resulting in poor response and elevated background noise. One way to decrease contamination of the ion source is to use a switch between the column and the ion source to divert the flow prior to the analytes eluting from the column. A guard column might also be used to decrease contaminations.

10. REPEATABILITY AND REPRODUCIBILITY

In 2023, an international collaborative study involving 7 laboratories was conducted using nicotine pouch dry and wet cigarette filler. Results were analyzed according to ISO 5725-2:2019. After removal of outlying data, the final repeatability (r) and reproducibility (R) results were calculated. The r&R results for the study are presented in Table 4. It is worth mentioning that some analytes are often not detected in nicotine pouches or are below the limit of quantitation. Hence, r&R results are not calculated in all sample types for some analytes.

Table 4: Repeatability (r) and Reproducibility (R) Results

Analyte	Product	Mean (µg/g)	N Labs*	Eff, N Labs#	R (µg/g)	r %	R (µg/g)	R %	HT Equation R %
Fortified Filler-1	Anabasine	9,98	5	16	0,950	9,5 %	1,55	15,5 %	31,7 %
Fortified Filler-1	Anatabine	10,32	5	8	1,110	10,7 %	2,19	21,2 %	31,5 %
Filler-2	β-Nicotyrine	0,97	7	11	0,205	21,0 %	0,43	43,7 %	45,0 %
Filler-3	β-Nicotyrine	1,13	6	7	0,203	18,0 %	0,41	36,5 %	44,0 %
Fortified Filler-1	β-Nicotyrine	9,74	5	6	0,957	9,8 %	3,37	34,6 %	31,8 %
Filler-1	Cotinine	1,10	7	9	0,142	12,8 %	0,55	49,5 %	44,1 %
Filler-2	Cotinine	3,42	7	15	0,241	7,1 %	1,49	43,4 %	37,2 %
Filler-3	Cotinine	2,12	7	10	0,245	11,6 %	0,92	43,5 %	40,0 %
Filler-4	Cotinine	6,01	7	7	0,494	8,2 %	2,17	36,2 %	34,2 %
Fortified Filler-1	Cotinine	11,10	5	11	0,932	8,4 %	1,67	15,0 %	31,2 %
Filler-2	Myosmine	1,96	7	19	0,200	10,2 %	0,65	33,2 %	40,5 %
Filler-3	Myosmine	1,12	7	10	0,208	18,5 %	0,51	45,2 %	44,0 %
Filler-4	Myosmine	1,34	7	8	0,176	13,1 %	0,54	40,0 %	42,9 %
Fortified Filler-1	Myosmine	10,56	5	11	0,866	8,2 %	1,63	15,5 %	31,4 %
Filler-1	Nicotine-N'-Oxide	10,50	7	10	1,19	11,3 %	5,21	49,6 %	31,4 %
Filler-2	Nicotine-N'-Oxide	435,6	7	9	30,5	7,0 %	222,8	51,1 %	17,9 %
Filler-3	Nicotine-N'-Oxide	52,71	7	9	6,35	12,0 %	35,07	66,5 %	24,7 %
Filler-4	Nicotine-N'-Oxide	354,0	7	9	25,8	7,3 %	159,5	45,0 %	18,5 %
Fortified Filler-1	Nicotine-N'-Oxide	20,37	5	5	3,41	16,8 %	6,06	29,8 %	28,5 %
Filler-1	Nornicotine	2,62	7	11	0,384	14,6 %	2,02	76,9 %	38,7 %
Filler-2	Nornicotine	12,03	7	12	1,083	9,0 %	5,82	48,3 %	30,8 %
Filler-3	Nornicotine	11,33	7	14	1,484	13,1 %	5,27	46,5 %	31,1 %
Filler-4	Nornicotine	6,05	7	13	0,637	10,5 %	4,67	77,2 %	34,2 %
Fortified Filler-1	Nornicotine	12,62	5	13	1,023	8,1 %	3,41	27,0 %	30,6 %

***This is the number of laboratory data sets with reported values after removal of outliers.**

The "effective" number of labs is an approximation to the equivalent number of labs involved taking into account the additional information coming from having multiple days of testing within each lab. This was calculated using the Satterthwaite approximation. The calculated values are fractions but were rounded to integers.

The Horwitz equation in this range of concentrations is $5{,}6 \cdot C^{-0{,}1505}$, where C is the analyte concentration. It is an equation predicting the reproducibility standard deviation of the analytical method and can be used as a rough benchmark for analytical variation.

11. TEST REPORT

The test report shall state the amount of analyte per gram and per portion (as received or wet weight) and shall include all conditions not specified in this Recommended Method which may affect the results. The report shall also give all details necessary for the identification of the test samples.

Moisture content may be determined on separate sample aliquots if it is necessary to present the results on a dry-weight basis. The determination of moisture is detailed in CORESTA Recommended Method No. 76: Determination of Moisture Content (Oven Volatiles) of Tobacco and Tobacco Products [4].

12. BIBLIOGRAPHY

[1] CORESTA Technical Report, 2020 Nicotine and nicotine degradants proficiency study, September 2020 [TTPA-246-1-CTR].

[2] Avagyan, R.; Spasova, M.; Lindholm, J. Determination of Nicotine-Related Impurities in Nicotine Pouches and Tobacco-Containing Products by Liquid Chromatography–Tandem Mass Spectrometry. Separations 2021, 8, 77. https://doi.org/10.3390/separations8060077

[3] CORESTA Technical Report, Collaborative Study for the determination of Nicotine Impurities and degradants in Nicotine Pouches by LC-MS, December 2023 [TTPA-246-2-CTR].

[4] CORESTA Recommended Method No. 76: Determination of Moisture Content (Oven Volatiles) of Tobacco and Tobacco Products.

APPENDIX I – Example Chromatograms

Figure 1 - Example of a MRM-chromatogram for a nicotine degradants standard (5 µg/ml)

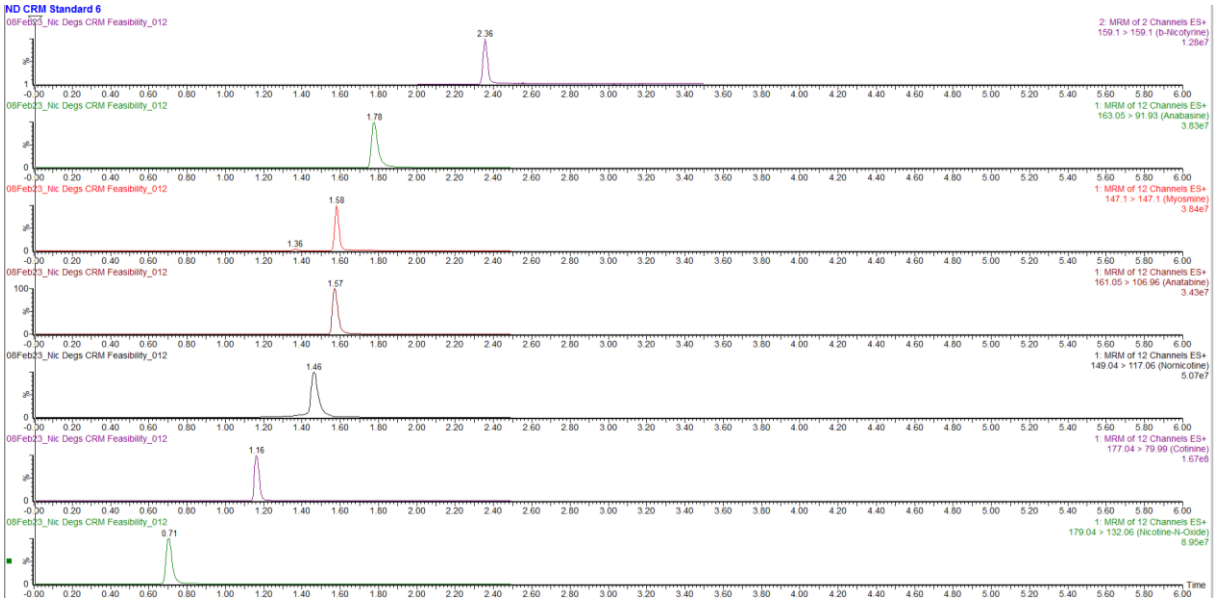

Figure 2 - Example of a MRM-chromatogram for a nicotine pouch sample