Application Note

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Rapid and Sensitive Quantitation of Metanephrines and 3-Methoxytyramine in Human Plasma Using an Agilent Ultivo Triple Quadrupole LC/MS

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Abstract

This application note presents a highly sensitive workflow for fast measurement of metanephrine (MN), normetanephrine (NMN), and 3-methoxytyramine (3-MT) from plasma matrix using an Agilent Ultivo triple quadrupole LC/TQ system. The targets chromatographically elute under four minutes and the observed limit of detection (LOD) for MN and 3-MT was 4 ng/L, while LOD for NMN was 20 ng/L. The workflow method performance was evaluated using ChromSystems certified reference samples, and results were satisfactory. All three targets displayed excellent linearity, with R^2 >0.999, and method accuracy was within 90 to 110% across the linearity range (CV <4%). Analyte absolute peak area response showed RSD <11% (including LOQ), and retention time RSD was <0.3%. The average recovery of targets for three QC levels was within 90 to 110%, with an interday reproducibility RSD of ≤4% (n = 3). The newly developed LC/MS/MS workflow can also be deployed for simultaneous analysis of metanephrines and catecholamines.

Introduction

Metanephrine, normetanephrine, and 3-methoxytyramine are the O-methylated metabolites of the catecholamines adrenaline, noradrenaline, and dopamine, respectively. High-performance liquid chromatography combined with electrochemical detection (HPLC-ECD) or fluorescence detection (HPLC-FLD) is the typical analytical technique to determine plasma metanephrines and 3-MT. However, quantitative measurement based on LC/MS/MS analysis has a huge potential to provide specificity and high sensitivity. Among the three targets, the chromatographic separation of MN and 3-MT is critical, since these compounds share common fragments.

The LC/MS/MS workflow in this study allows sensitive quantification of all three targets from blood plasma. A solid-phase extraction (SPE) sample preparation procedure was used to remove biological interferences and to extract the analytes, while a 10-minute chromatographic method using an Agilent Pursuit 3 PFP column was used to separate all three analytes. Specific MRM transitions of targets and deuterated internal standards were included to ensure sensitive and reproducible measurement using the Ultivo LC/TQ.

Experimental

Chemicals and reagents

Targets (MN, NMN, 3-MT), deuterated standards (MN d3, NMN d3, 3-MT d4), and human plasma were purchased from Sigma-Aldrich (St. Louis, MO, USA). When not in use, all standards were stored at –20 °C. MassChrom metanephrine in plasma reference samples were purchased from ChromSystems (Graefelfing, Germany). LC/MS grade acetonitrile, methanol, formic acid, ammonium dihydrogen phosphate ($NH_4H_2PO_4$), and all other reagents were purchased from Sigma-Aldrich. Ultrapure Milli Q water was produced using an in-house water purification system (Merck Millipore, MA, USA).

Equipment and consumables

Laboratory equipment and consumables used for sample preparation were as follows:

- SampliQ WCX Polymer, 100 × 1 mL tubes, 30 mg (SPE cartridges), (part number 5982-3513)
- Positive pressure manifold, PPM-48, (part number 5191-4101)
- PPM-48 collection rack for 12 × 75 mm tubes, (part number 5191-4106)
- PPM-48 1 mL SPE cartridge rack, (part number 5191-4102)
- Hi-Recovery vial, 1.5 mL, 100/pk, (part number 5183-2073)
- Vial screw cap 100/pk, (part number 5190-7024)
- Disposable glass collection tube 12 × 75 mm tubes for 1 mL SPE
- Ultrasonic bath
- Multitube vortexer >500 rpm
- Pipettors and matching tips

Instrumentation

An Agilent 1260 Infinity II LC system was used for the analysis. The system consisted of:

- 1260 Infinity II binary pump (G7112B)
- 1260 Infinity II multisampler (G7167A)
- 1260 Infinity II multicolumn compartment (G7116A)

A 0.3 µm inline filter (part number 5067-6189) was installed between the autosampler injector valve port 6 and the multicolumn compartment. The LC conditions are listed in Table 1 and Ultivo LC/TQ source parameters, optimized using Agilent MassHunter optimizer software (version 1.2), are included in Table 2.

MRM optimization

MassHunter optimizer software (version 1.2) was used to obtain analyte-specific multiple reaction monitoring (MRM) transitions, fragmentor voltage, and collision energies. The MS/MS optimization was performed without chromatographic separation, using 2 µL injections of neat solutions of individual analytes at about 1,000 µg/L. The MS/MS settings used for the analysis are included in Table 3.

Table 1. Agilent 1260 Infinity II LC parameters.

Parameter	Value
Needle Wash	Standard wash, flush port, 20 s; 60/40 acetonitrile/water with 0.1% formic acid
Autosampler Temperature	6 °C
Injection Volume	20 µL
Analytical Separation	Agilent Pursuit PFP, 3.0 × 100 mm, 3 µm analytical column (p/n A3051100X030) with Agilent InfinityLab Poroshell 120 PFP, 3.0 mm, 1.9 µm UHPLC guard Column (p/n 823750-942)
Column Temperature	30 °C
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Methanol with 0.1% formic acid
Gradient	Time (min) %B Flow Rate (mL/min) 0.00 5 0.55 0.50 5 0.55 3.00 70 0.55 4.00 95 0.80 6.00 95 0.80 6.10 5 0.80 8.50 5 0.80 9.00 5 0.55

Table 2. Agilent Ultivo LC/TQ mass spectrometer configuration and parameters.

Parameter	Value
Configuration	Agilent Ultivo LC/TQ (G6465B) equipped with an Agilent Jet Stream (AJS) Electrospray ion source
Ionization Polarity	Positive
MS/MS Mode	MRM
Drying Gas Temperature	325 °C
Drying Gas Flow	11 L/min
Nebulizer Pressure	30 psi
Sheath Gas Temperature	375 °C
Sheath Gas Flow	12 L/min
Nozzle Voltage	0 V
Capillary Voltage	3,000 V
Diverter Valve to Waste	At 4.4 min

Table 3. MRM parameters: MS1 resolution: unit; MS2 resolution: unit; dwell: 50 (ms); polarity: positive.

Analyte	Precursor (*m/z*)	Fragmentor (V)	Quant		Qual	
			(*m/z*)	CE (V)	(*m/z*)	CE (V)
MN	180.1	125	165.1	16	148	16
NMN	166.1	110	134	13	106	16
3-MT	151.1	130	91	20	119	10
MN-d3	183.1	125	168	16		
NMN-d3	169.1	110	137	13		
3-MT-d4	155.1	130	95	20		

Sample preparation

SPE cleanup was performed for a sample size of 500 µL. Each 500 µL sample was mixed with an equal volume of 10 mM $NH_4H_2PO_4$ buffer and subjected to SPE cleanup. The positive pressure manifold 48 processor (PPM-48) was used to perform SPE. Figure 1 describes the optimal sample preparation procedure in detail. While performing ChromSystems reference samples, the 10 mM $NH_4H_2PO_4$ buffer was spiked with internal standards at a concentration of 400 ng/L to correct for the analyte loss during SPE sample preparation. The restricted-flow ports included in the PPM 48 ensured consistent SPE cleanup across the manifold. The pressure was adjusted at 1 to 2 psi to maintain a steady flow rate of 3 sec/drop while performing SPE loading and elution.

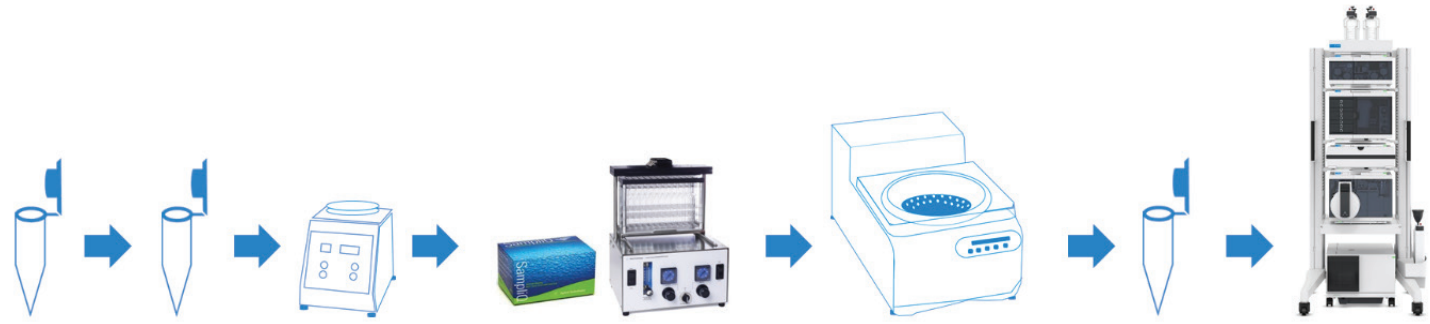

Figure 1. Agilent PPM-48 based SPE workflow for sensitive measurement of MN, NMN, and 3-MT in plasma using the Agilent Ultivo LC/TQ.

Workflow sensitivity assessment

Due to the presence of endogenous targets in control plasma samples, method sensitivity assessments had to be evaluated using deuterated targets (internal standards) spiked in plasma at various concentrations (4, 10, 20, and 40 ng/L) and used for accurate limit of detection (LOD) and limit of quantitation (LOQ) estimations.

Verification using certified standards

ChromSystems lyophilized reference samples were reconstituted according to the manufacturer's protocol and used to characterize the workflow performance. The ChromSystems sample set includes blank control, six-level calibrators, and three QC levels: low-range QC (LQC), mid-range QC (MQC), and high-range QC (HQC), respectively.

Data acquisition and analysis

Plasma samples spiked with internal standards, control samples, two batches of calibrators, and three technical preparations of each QC level were subjected to the in-house developed sample preparation protocol. LC/TQ data was acquired in four replicates using the Ultivo equipped with the AJS source. MassHunter LC/MS Data Acquisition software (version 1.2) and MassHunter quantitative analysis software (version 10.0) were used to acquire and process the data.

Sensitivity assessment was performed using plasma samples spiked with internal standards. Method characteristics like precision, accuracy, linearity, recovery, recovery repeatability, and reproducibility were evaluated using ChromSystems certified reference standards. Recovery (%) and repeatability (%RSD) values were calculated from three intraday technical preparations of each QC level. Interday QC recovery deviation (%RSD) was calculated to assess the workflow reproducibility. Target responses from a diluent injection performed immediately after the highest calibrator, was used to compute the carryover.

Results and discussion

Workflow sensitivity

The SPE sample preparation procedure described here allowed simultaneous extraction of all three targets (MN, NMN, and 3-MT) from plasma. The Agilent Pursuit 3 PFP column offered baseline separation of all three analytes in under four minutes. The chromatographic baseline separation, together with MRM-based detection provided unambiguous identification of MN and 3-MT. To eliminate the issue of endogenous target interference, sensitivity was assessed using plasma samples spiked with deuterium-labeled targets at various concentrations. For each compound, the signal-to-noise ratio (S/N) thresholds were defined as S/N >3 for LOD, and S/N >10 for LOQ. The overlay of control plasma, LOD, and LOQ levels of all three deuterated analytes are included in Figure 2. For MN d3 and 3-MT d4, the LOD was 4 ng/L, and 10 ng/L was assigned as LOQ. For NMN d3, the LOD was 20 ng/L, and 40 ng/L exceeded LOQ requirements.

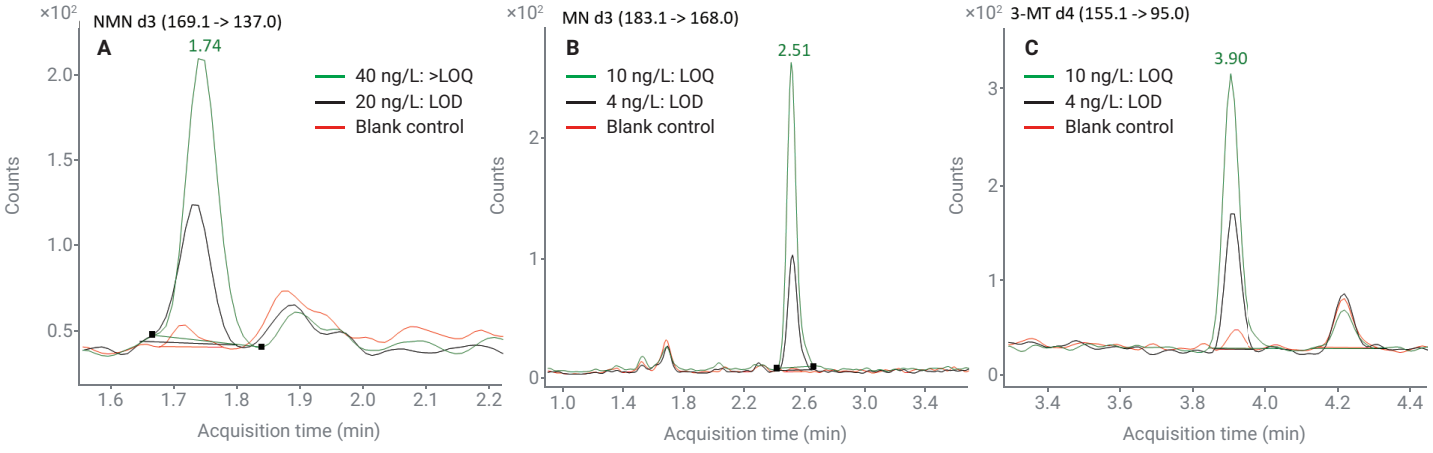

Figure 2. MRM trace overlay of control plasma (red trace), LOD (black trace), and LOQ (green trace) for NMN d3 (A), MN d3 (B), and 3-MT d4 (C).

Calibration curve linearity using reference calibrators

Two batches of signal linearity curves for each analyte were constructed using six calibrator reference levels with internal standard correction. When compared with the control matrix, the analyte peak for the lowest calibrator level was significant, thus ensuring easy target identification and sensitive detection (Figure 3). All three targets displayed a linear response with R^2 values >0.999 (calibration model type: linear; origin: ignore; weight: 1/x). The linear regression coefficient and slopes from both batches were consistent, and thus confirmed the assay reproducibility in the given analytical range.

Precision and accuracy

Using four replicate injections of the calibration levels, precision was determined by calculating the %RSD of the target retention time (RT) and response ratio. Satisfactory RT and response ratio precision values for all analytes were obtained for both batches, with %RSD <0.3% and <6%, respectively. The average accuracy value for each plasma calibration-level was calculated from replicate injections. Accuracy for all three analytes across the calibration range was within 90 to 110%, with %RSD <4%.

Recovery

In this experiment, the impact of sample preparation on target recovery was assessed using three preparations of three levels of QC samples (LQC, MQC, and HQC). Each preparation was injected into the LC/TQ in four replicates. Sample preparation recovery (%) was calculated using respective calibration curve equations (Table 4). The intraday recovery repeatability was measured as %RSD of average recovery values, calculated using technical preparations. Recoveries for overall analytes were within 90 to 110%, with intraday %RSD ≤5%.

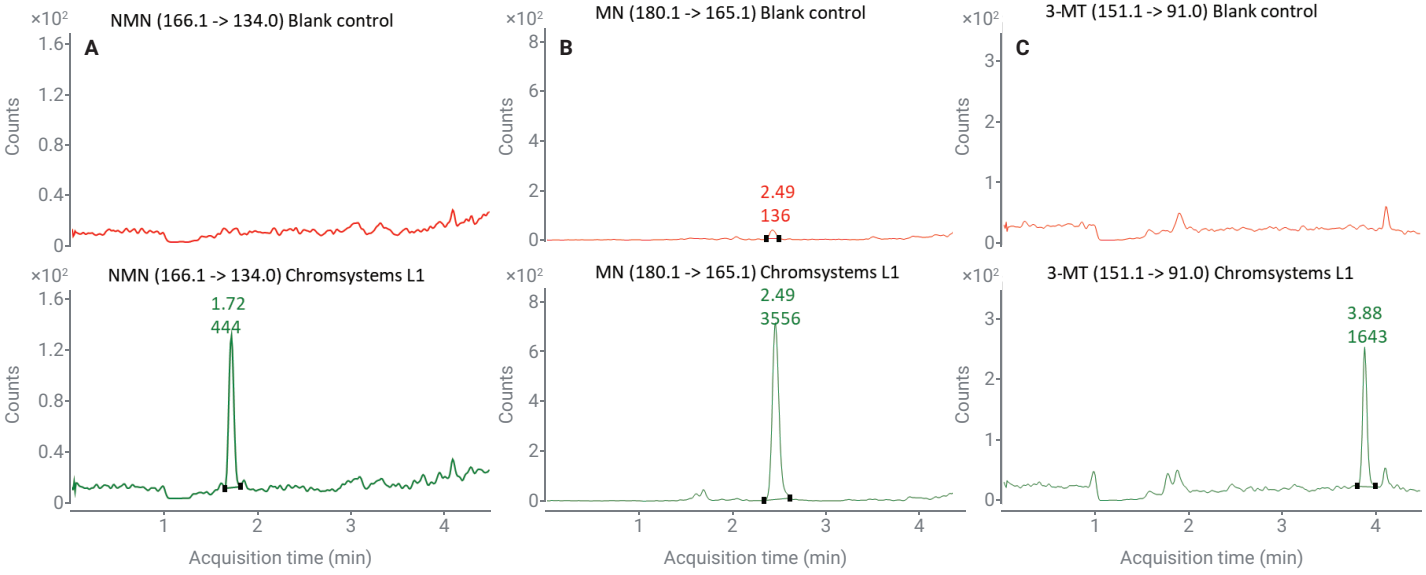

Figure 3. MRM trace overlay of ChromSystems control plasma (red trace) and level 1 calibrator (green trace) for NMN at 29.8 ng/L (A), MN at 24.5 ng/L (B), and 3-MT at 14.8 ng/L (C).

Table 4. Intra-day recovery results using three technical preparations of three QC levels.

QC	Technical Preparation	MN			NMN			3-MT		
		Expected Concentration (ng/L)	Calculated Concentration (ng/L)	Recovery (%)	Expected Concentration (ng/L)	Calculated Concentration (ng/L)	Recovery (%)	Expected Concentration (ng/L)	Calculated Concentration (ng/L)	Recovery (%)
LQC	1	58.4	58	99	127.0	124	98	22.7	23	101
	2		63	109		136	107		23	102
	3		61	105		128	101		24	105
MQC	1	182.0	175	91	279.0	281	101	92.8	87	94
	2		177	92		287	103		90	97
	3		183	95		283	101		92	99
HQC	1	950.0	921	97	1,499.0	1477	99	878.0	822	94
	2		937	99		1553	104		836	95
	3		916	96		1527	102		807	92

Interday reproducibility

Average recovery results from three consecutive days were compared, and %RSD was calculated to assess interday workflow method reproducibility. The observed average recovery values for all three targets across three days were within 93 to 106%, and interday reproducibility was within 4 %RSD (Figure 4). These results confirm the consistency of SPE extraction and LC/TQ methodology for routine quantitative analysis of MN, NMN, and 3-MT.

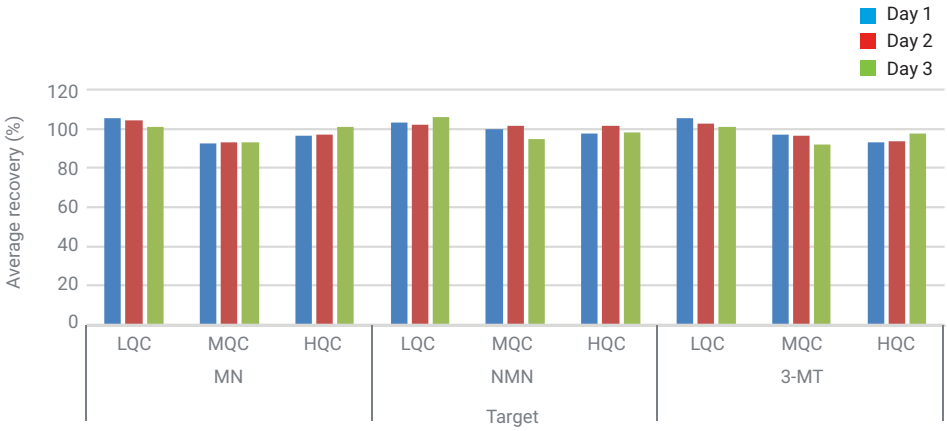

Figure 4. Recovery reproducibility of MN, NMN, and 3-MT across 3 days for all three targets.

Carryover analysis

Target response from a solvent blank injection immediately after the highest calibration level was compared against that from calibrators to assess percent carryover. The observed carryover in the solvent injection was <0.02% when compared with the level 6 calibrator, and < 2% when compared with the level 1 calibrator.

Simultaneous separation of metanephrines and catecholamines

The LC/MS/MS method described here was also extended to the separation of norepinephrine (NEN), epinephrine (EN), and dopamine (Dopa). The method offered good separation of all six targets (Figure 5).

Conclusion

A sensitive workflow was developed for the quantitation of metanephrine, normetanephrine, and 3-methoxytyramine in human plasma. The SPE sample cleanup protocol efficiently removed biological interferences. A short gradient using an Agilent Pursuit 3 PFP column helped to chromatographically resolve the targets, and provide selectivity for unambiguous measurement. The interday recovery reproducibility assessment using multiple QC levels illustrated the workflow reliability for confident day-to-day operation. The newly developed LC/MS/MS workflow for simultaneous measurement of metanephrines and catecholamines from plasma offers a convenient alternative and fast method to the standard practice of analyzing these targets from urine samples.

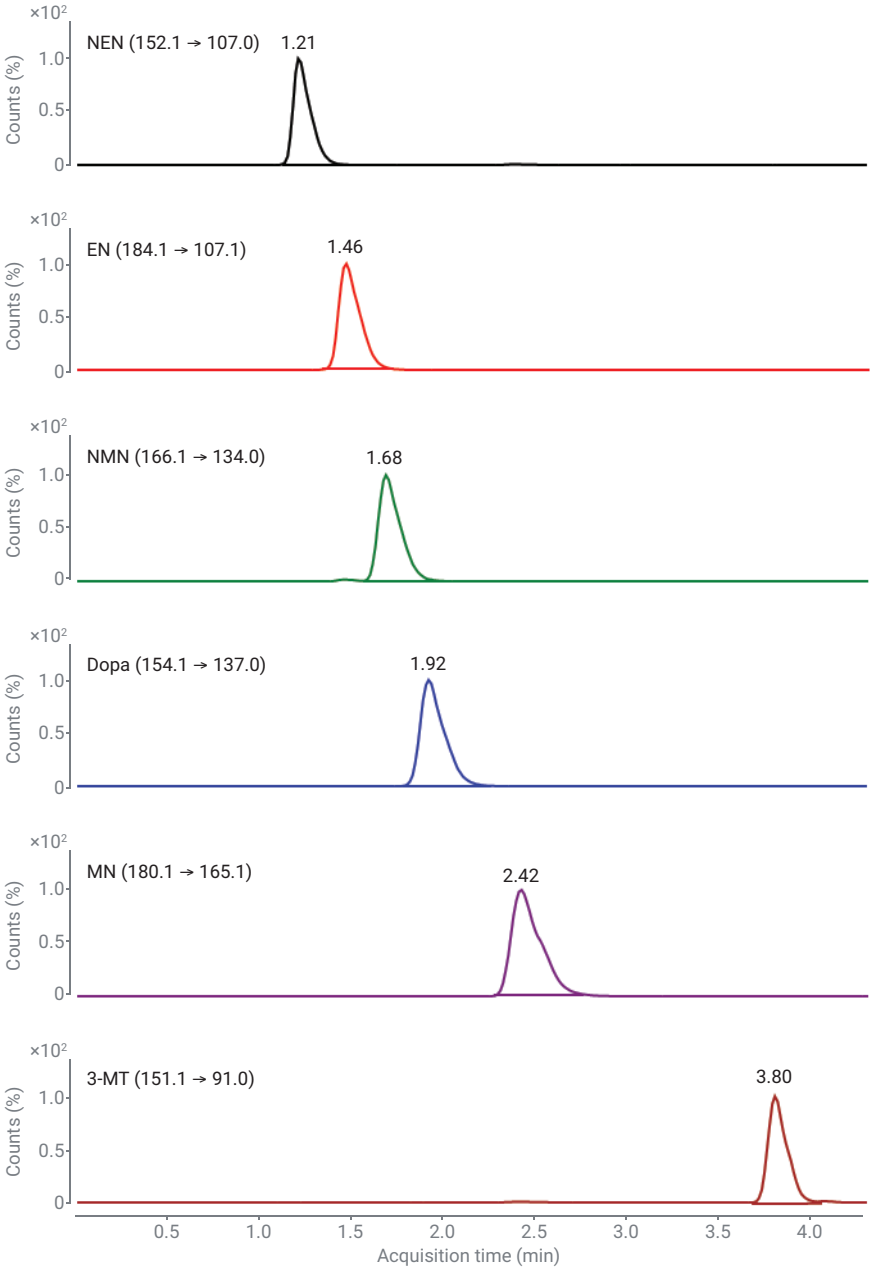

Figure 5. MRM trace of simultaneous separation of metanephrines (MN, NMN, 3-MT) and catecholamines (NEN, EN, Dopa). Sample concentration: 20 ng/mL in diluent.

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