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Liquid chromatography/electrospray ionization tandem mass spectrometry assay for determination of nicotine and metabolites, caffeine and arecoline in breast milk

Manuela Pellegrini[1], Emilia Marchei[1], Silvia Rossi[1], Federica Vagnarelli[2], Abhilasha Durgbanshi[3], Óscar García-Algar[4], Oriol Vall[4] and Simona Pichini[1]*

[1]Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanitá, Rome, Italy
[2]Arcispedale Santa Maria Nuova, Reggio Emilia, Italy
[3]Department of Criminology and Forensic Sciences, Dr. H.S. Gour University, Sagar, India
[4]Paediatric Service, URIE, Hospital del Mar, and Universitat Autònoma, Barcelona, Spain

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A procedure based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) is described for the determination of nicotine and its principal metabolites cotinine, *trans*-3-hydroxycotinine and cotinine-*N*-oxide, caffeine and arecoline in breast milk, using *N*-ethylnorcotinine as internal standard. Liquid/liquid extraction with chloroform/isopropanol (95:5, v/v) was used for nicotine, cotinine, *trans*-3-hydroxycotinine, cotinine-*N*-oxide and caffeine under neutral conditions and for arecoline under basic conditions. Chromatography was performed on a C_8 reversed-phase column using a gradient of 50 mM ammonium formate, pH 5.0, and acetonitrile as a mobile phase at a flow rate of 0.5 mL/min. Separated analytes were determined by electrospray ionization tandem mass spectrometry in the positive ion mode using multiple reaction monitoring. Limits of quantification were 5 μg/L for nicotine, cotinine, *trans*-3-hydroxycotinine, cotinine-*N*-oxide and caffeine, and 50 μg/L for arecoline using 1 mL human milk per assay. Calibration curves were linear over the calibration ranges for all the substances under investigation, with a minimum $r^2 > 0.998$. At three concentrations spanning the linear dynamic range of the assay, mean recoveries from breast milk ranged between 71.8 and 77.4% for different analytes. This method was applied to the analysis of analytes in human milk to assess substance exposure in breast-fed infants in relation to eventual clinical outcomes. This LC/MS/MS assay provides adequate sensitivity and performance characteristics for the simultaneous quantification of biomarkers of three of the drugs most commonly used worldwide (tobacco, caffeine and areca nut).

The major reason for drug investigation in human milk is to calculate excretion of certain compounds in this fluid and, consequently, the approximate dose ingested by breastfeeding infants.[1,2] This information is important not only to protect nursing infants from undesired effects of the maternal consumption of any licit or illicit drug, but also to allow effective pharmacological treatment of breastfeeding mothers.[3]

For many medications, the percentage of the dose received via breast milk is below 1% or between 1 and 10% maternal dose. There are, however, some for which the percentage exceeds 50% (e.g. phenobarbital and theophylline).[4]

Some drugs of abuse (e.g. cocaine, morphine, phencyclidine and amphetamine) which are weak bases have been found to be excreted in breast milk because it is slightly more acidic than plasma and this prompted the statement that mothers using those drugs should not breastfeed.[4–8]

Among other basic compounds which can be secreted in breast milk are nicotine (NIC), caffeine (CAF) and arecoline (ARECA), three of the four psychoactive drugs most consumed worldwide (Figs. 1–6).[9,10]

NIC, responsible for tobacco addiction, is the most specific component of cigarette smoke and one of its biomarkers suspected to contribute to human diseases like cardiovascular and reproductive disorders.[11,12] Although it is advised that smoking should be stopped or at least reduced during pregnancy and lactation, in many countries prevalence rates remain high.[13] NIC (half-life of about 2 h) is rapidly and extensively metabolized in humans to cotinine (COT), *trans*-3-hydroxycotinine (TRANS-3-OH-COT) and cotinine-*N*-oxide (COT-N-OX).[14] All these NIC metabolites have been measured in biological matrices to provide a better estimate of exposure to tobacco smoke.[15]

**Correspondence to*: S. Pichini, Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy.
E-mail: simona.pichini@iss.it
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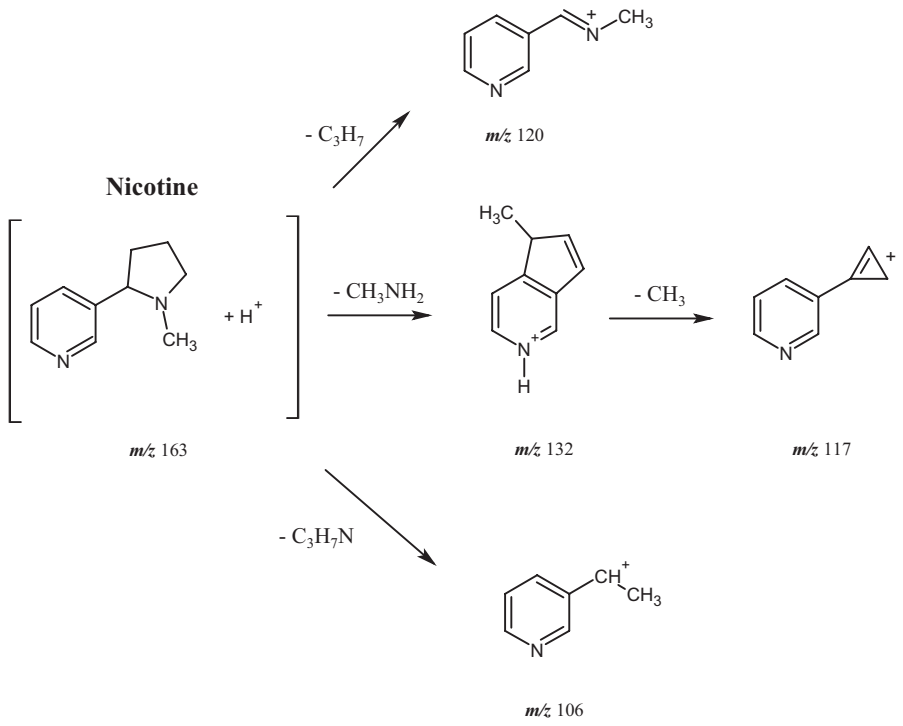

Figure 1. Proposed fragmentation pathway for $[M+H]^+$ of NIC.

Caffeine (CAF) is known to be consumed by pregnant and lactating mothers in large quantities in beverages and in prescription and non-prescription drugs despite the suggestion that its consumption should be reduced during pregnancy.[16,17] The elimination of CAF is surprisingly slow in the newborn due to an immaturity in the hepatic N-demethylation metabolic pathway, implying that the breast-fed newborns with a significant daily intake of CAF via maternal breast milk could potentially accumulate toxic concentrations of CAF with consequent adverse reactions.[18,19]

The areca nut (the fruit of the Areca catechu tree of the 'palmacae' family) is the fourth most commonly used drug in the world after tobacco, alcohol and CAF.[20] It is commonly consumed as betel quid chewing or betel nut smoking by Asian populations and Asian communities living in Europe

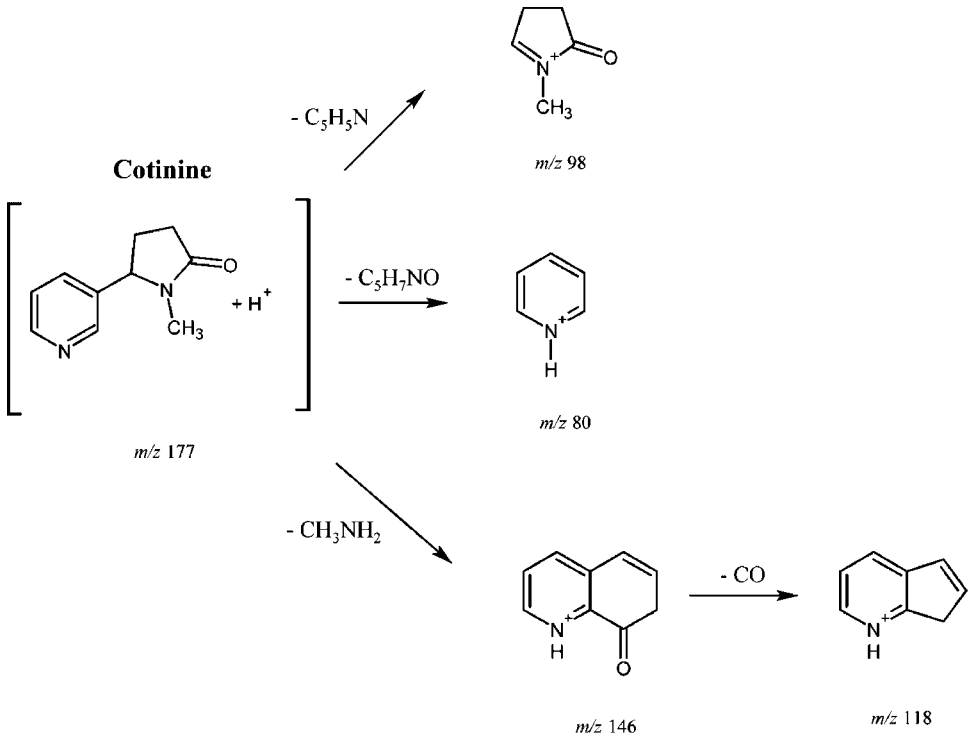

Figure 2. Proposed fragmentation pathway for $[M+H]^+$ of COT.

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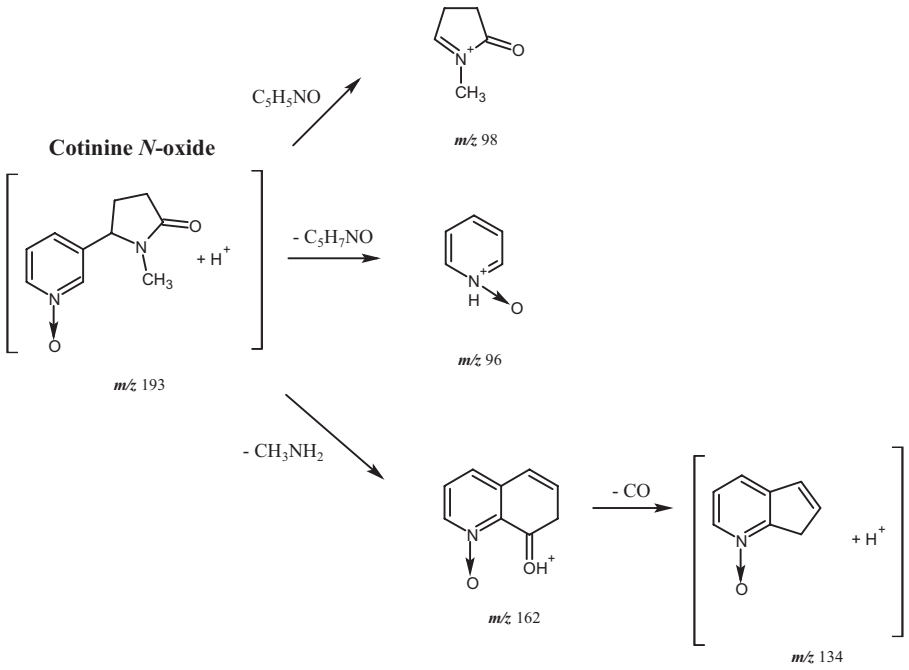

Figure 3. Proposed fragmentation pathway for $[M+H]^+$ of COT N-OX.

and North America.[10] ARECA, the main alkaloid present at up to 1% of dry weight, is a central nervous system stimulant, muscarinic agonist with psychoactive effects.[9] Because of the 'positive' effects attributed to betel quid chewing, some women continue to consume this preparation during pregnancy, especially to prevent morning sickness, with a non-negligible prevalence of adverse pregnancy outcome.[21,22]

Determinations of NIC and metabolites, CAF and ARECA in human breast milk are essentially lacking, with the exception of two papers dealing with the liquid chromatography (LC) ultraviolet detection (UV) of NIC and COT and another involving the LC-UV determination of CAF and other methylxanthines.[23–25]

Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) can provide identification, with a high grade of certainty, of minute amounts of substances contained in complex biological matrices. Coupling MS with LC as a separation technique provides a valuable mean for the determination of hydrophilic and hydroxylated compounds.[26]

We have developed an LC electrospray ionization (ESI)-MS/MS method for the determination of NIC and its

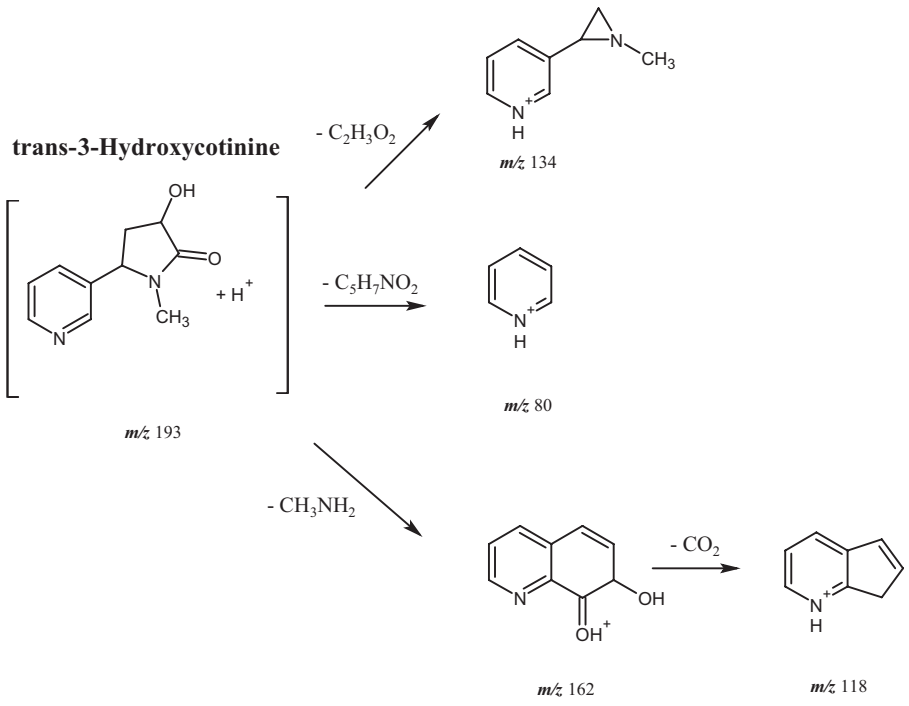

Figure 4. Proposed fragmentation pathway for $[M+H]^+$ of TRANS-3-OH-COT.

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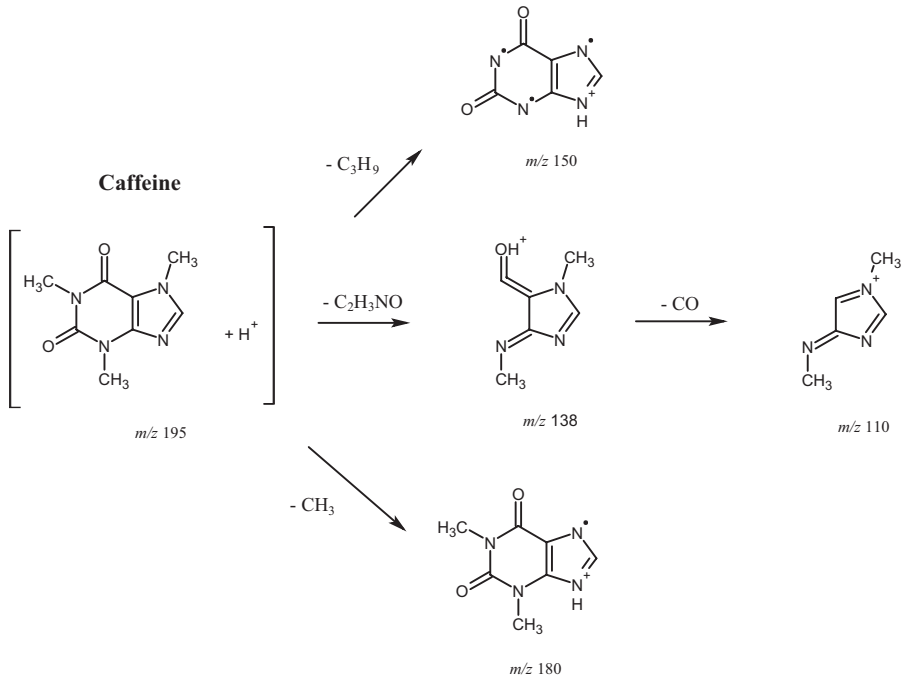

Figure 5. Proposed fragmentation pathway for $[M+H]^+$ of CAF.

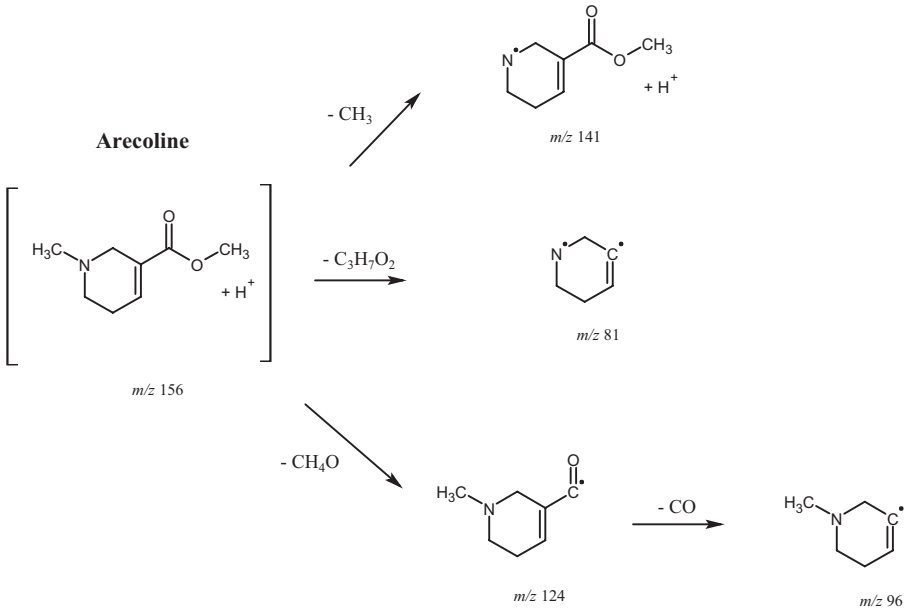

Figure 6. Proposed fragmentation pathway for $[M+H]^+$ of ARECA.

principal metabolites, CAF and ARECA in human breast milk aiming to assess infant exposure to those substances through maternal milk and to relate such exposure to adverse clinical outcomes.

EXPERIMENTAL

Materials

Standards of NIC, COT, TRANS-3-OH-COT, COT-N-OX, CAF, ARECA and *N*-ethylnorcotinine (NENC) used as internal standard (IS) were supplied by Sigma-Aldrich (Milan, Italy). Ultrapure water and all other reagents of analytical grade were obtained from Carlo Erba (Milan, Italy).

Breast milk samples

Breast milk samples came from the Neonatal Unit of Hospital del Mar, Barcelona, Spain (the fourth largest hospital of this Mediterranean city); the Neonatal Intensive Care Unit Arcispedale Santa Maria Nuova, Reggio Emilia, Italy (the only hospital in this northern Italian town); and the Neonatal Unit of Sagar Hospital (one of the largest hospitals of this city situated in the central part of India). The collection protocol was approved by local ethical committees of the different hospitals. Milk samples were collected, aliquoted and stored

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at −20°C until analysis. Lactating mothers were interviewed regarding smoking habits, consumption of caffeinated drinks and, in the case of Indian women, the use of areca nut or betel nut.

Instrumentation

LC/MS/MS analyses were performed using an Alliance HPLC system (Waters, Etten-Leur, The Netherlands) interfaced to a Micromass Quattro micro API triple quadrupole mass spectrometer (Waters) equipped with an ESI probe. Chromatographic separation was achieved using an Eclipse XDB-C8 column (100 × 3.0 mm, 3.5 μm; Agilent Technologies, Palo Alto, CA, USA). The mobile phase was a gradient of a mixture of (A) 50 mM ammonium formate, pH 5.0, and (B) acetonitrile programmed as follows: initial 95% A maintained for 3 min, then decreased to 70% in 5 min, maintained at 70% for 3 min, and finally increased to 95% in 9 min. The flow rate was 0.5 mL/min. All chromatographic solvents were degassed with helium before use. The column temperature was set at 30°C.

MS and MS/MS spectra of the compounds under investigation were acquired as follows. The substances, dissolved in methanol at a concentration of 10 mg/L, were infused through an integrated syringe pump into the ESI probe at a rate of 10 μL/min to tune the mass spectrometer and optimize the acquisition parameters. The following optimized conditions were used: collision energy ranging from 15 and 20 eV used throughout, with capillary voltage at 3.0 kV, cone voltage at 25 V, source temperature at 120°C, and desolvation temperature at 400°C. The cone and desolvation gas flows were set at 50 and 400 L/h, respectively. The collision gas was argon at a collision cell pressure of 0.25 Pa (2.5×10^{-3} mbar).

For multiple reaction monitoring (MRM) of the individual compounds, the protonated molecule $[M+H]^+$ was selected in the first quadrupole and the collision energy was adjusted to optimize the signal for the most abundant product ions.

Calibration standards and quality control samples

Stock standard solutions (1 g/L) and working solutions (10 and 1 mg/L) of analytes were prepared in methanol and stored at −20°C until analysis. The IS working solution was used at a concentration of 10 mg/L. Calibration standards containing 1000, 500, 100, 50, 10 and 5 μg NIC, COT, TRANS-3-OH-COT, COT-N-OX; 1500, 500, 100, 50 and 5 μg CAF; and 1000, 750, 500, 250, 100 and 50 μg ARECA per liter of milk were prepared daily for each analytical batch by adding suitable amounts of methanol working solutions to 1 mL of pre-checked drug-free liquid formula milk (Formulat 1 liquido; Dicofarm S.p.A., Roma, Italy). Quality control (QC) samples of 850 μg/L (high control), 400 μg/L (medium control), 6 μg/L (low control for NIC, COT, TRANS-OH-COT, COT-N-OX and CAF) and 75 μg/L (low control for ARECA), and samples at the limit of quantification (LOQ) of each analyte were prepared in drug-free milk, pooled and stored at −20°C. They were included in each analytical batch to check calibration, accuracy and precision, and the stability of samples under storage conditions.

Sample preparation

Breast milk (1 mL): 10 μL of IS working solution was diluted with 1 mL 0.1 M phosphate buffer, pH 6.8, to extract NIC, COT, TRANS-OH-COT, COT-N-OX and CAF with two aliquots of 2.5 mL chloroform/isopropanol (95:5, v/v). The aqueous phase containing milk and phosphate buffer at pH 6.8 was added with 2 mL ammonium chloride, pH 9.5, to extract ARECA with two aliquots of 2.5 mL chloroform/isopropanol (95:5, v/v). The organic phase was evaporated to dryness under a stream of nitrogen and redissolved in 100 μL 50 mM ammonium formate, pH 5.0. A 20 μL volume was injected into the LC column.

Validation procedures

Prior to application to real samples, the method was tested in a validation protocol following the accepted criteria for bioanalytical method validation.[27,28] Selectivity, matrix effect, recovery, linearity, limits of detection and quantification, precision, accuracy and stability were determined.

Twenty different human milk samples from non-exposed, non-smoking, nursing mothers (checked by urinalysis) who did not consume any caffeinated drinks and did not chew or smoke betel nut were extracted and analyzed to assess potential interferences from endogenous substances. The apparent responses at the retention times of the analytes under investigation and the IS were compared with the response of analytes at the LOQ and IS at its lowest quantifiable concentration. Potential interferences from principal drugs of abuse such as opiates, cocaine and metabolites, cannabinoids, the most widely used benzodiazepines, and antidepressants were also evaluated by spiking 1 mL of pre-checked drug-free human milk pool with 1 μg of each of the aforementioned substances (final concentration: 1000 μg/L as the highest point of calibration curve) and carrying these through the entire procedure.

The potential for carryover was investigated by injecting extracted drug-free human milk, with added IS, immediately after analysis of the highest concentration point of the calibration and measuring the area of eventual peaks at the retention times of analytes under investigation.

Absolute analytical recoveries were calculated using four replicates for each QC sample concentration by comparing the peak areas obtained when samples were analyzed by adding the analytical reference standard and the IS in the extract of drug-free pooled milk prior to and after the extraction procedure. For the evaluation of the matrix effect, the peak areas of extracted pooled drug-free samples spiked with standards at QC concentrations after the extraction procedure were compared with the peak areas of pure diluted substances.

Calibration curves were tested over the calibration range for all the analytes. Peak area ratios between compounds and IS were used for calculations. A weighted (1/concentration) least-squares regression analysis was used for slopes and intercepts. The standard deviations of residuals (Sy/x) were also calculated.[29]

Five replicates of drug-free milk samples were used to calculate the limits of detection and quantification. The standard deviation (SD) of the mean noise level over the retention time window of each analyte was used to

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determine the detection limit (LOD = 3 SD) and the quantification limit (LOQ = 10 SD). To be accepted, the calculated LOQ had to show precision and accuracy within 20% relative SD (RSD) and relative error, respectively.

Five replicates at each of three different QC sample concentrations added to drug-free pooled samples, extracted as reported above, were analyzed for the determination of intra-assay precision and accuracy. The inter-assay precision and accuracy were determined for three independent experimental assays of the aforementioned replicates. Precision was expressed as the RSD of concentrations calculated for QC samples. Accuracy was expressed as the relative error of the calculated concentrations.

The effect of three freeze/thaw cycles (storage at −20°C) on the stability of the compounds in human milk was evaluated by repeated analysis (n = 3) of QC samples. In addition, a mid-term stability test was performed for real samples stored at −20°C. Three replicates of four samples were analyzed once a month during a 6-month period. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of the analytes both in QC and real samples.

RESULTS AND DISCUSSION

Mass spectral results

The ESI-MS/MS analysis of the compounds is summarized in Table 1, which reports the quantification and confirmation transitions chosen for each compound.

Under the conditions used, the protonated molecule of NIC, $[M+H]^+$, at *m/z* 163 fragmented in MS/MS to give a major product ion at *m/z* 132, corresponding to the loss

Table 1. MS characteristics of tested compounds

Compound	Segment of MS experiment	MRM transition, *m/z*	Collision energy, eV	Retention time, min
NIC	I	**163 → 132**[a]	15	7.8
		163 → 106		
COT	I	**177 → 80**	18	11.8
		177 → 146		
COT-N-OX	I	**193 → 96**	18	7.9
		193 → 134		
TRANS-3-OH-COT	I	**193 → 80**	18	10.6
		193 → 134		
CAF	I	**195 → 138**	20	12.4
		195 → 180		
ARECA	I	**156 → 81**	18	6.4
		156 → 124		
NENC	I	**191 → 120**	20	12.8
		191 → 148		

[a]The selected quantification transitions are in bold.

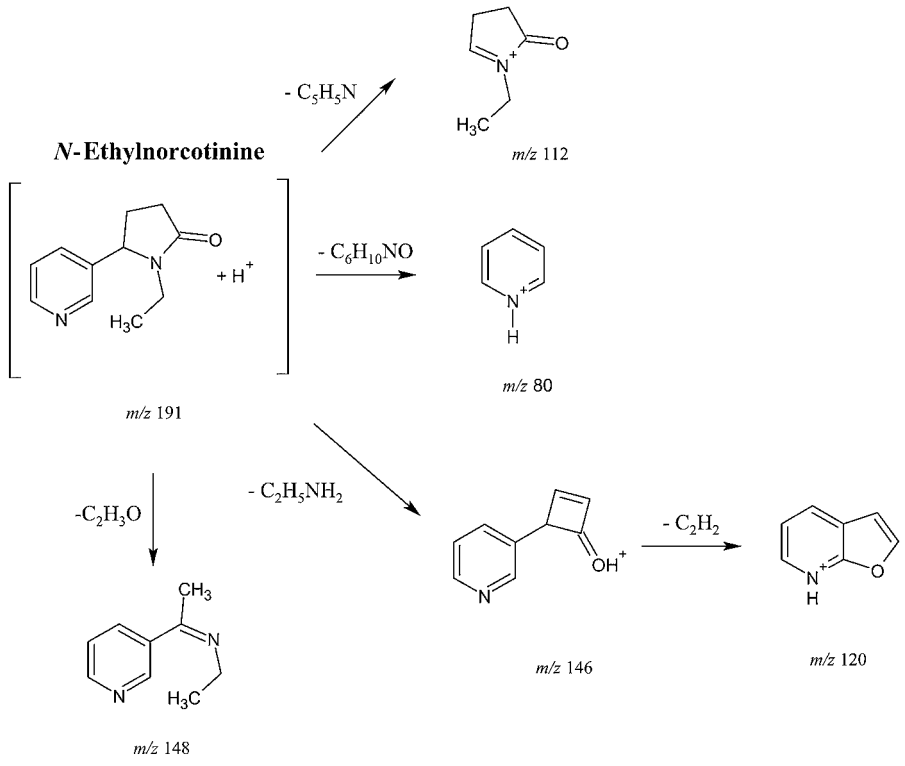

Figure 7. Proposed fragmentation pathway for $[M+H]^+$ of NENC.

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of CH_3NH_2. Two other fragmentations occurred through the losses of C_3H_7 and C_3H_7N from *m/z* 163 to yield, respectively, *m/z* 120 and 106; the latter was chosen as a confirmation transition (Fig. 1).

For COT, the major product ion at *m/z* 80 corresponded to the loss of the methylpyrrolidinone ring from the protonated molecule $[M+H]^+$ at *m/z* 177. Other fragmentation pathways included the loss of pyridine to produce protonated methylpyrrolidinone at *m/z* 98 and the loss of CH_3NH_2 to produce an ion at *m/z* 146 (Fig. 2).

For COT-N-OX, the product ion spectrum of the $[M+H]^+$ ion at *m/z* 193 included the protonated pyridine *N*-oxide at *m/z* 96 formed by loss of the methylpyrrolidinone ring. Other fragmentation pathways included the loss of CH_3NH_2 to produce an ion at *m/z* 162, followed by the further loss of CO to give *m/z* 134. The loss of pyridine *N*-oxide to produce protonated methylpyrrolidinone at *m/z* 98 was another competing pathway (Fig. 3).

The protonated molecule $[M+H]^+$ at *m/z* 193 of TRANS-3-OH-COT gave a product ion at *m/z* 80 corresponding to loss of the methylhydroxypyrrolidinone ring. The loss of $C_2H_3O_2$ produced a second characteristic ion at *m/z* 134. Another fragmentation pathway was the loss of CH_3NH_2 to produce an ion at *m/z* 162, followed by the further loss of CO_2 to give *m/z* 118 (Fig. 4).

The major product ion at *m/z* 138 of protonated CAF at *m/z* 195 corresponded to loss of C_2H_3NO. *m/z* 138 then lost CO to produce an ion at *m/z* 110. The loss of CH_3 from the protonated molecule yielded *m/z* 180 and the concerted loss of three CH_3 groups produced *m/z* 150 (Fig. 5).

For ARECA, the product ion at *m/z* 81 was hypothesized as being formed by the loss of CH_3 and $C_2H_4O_2$ from the

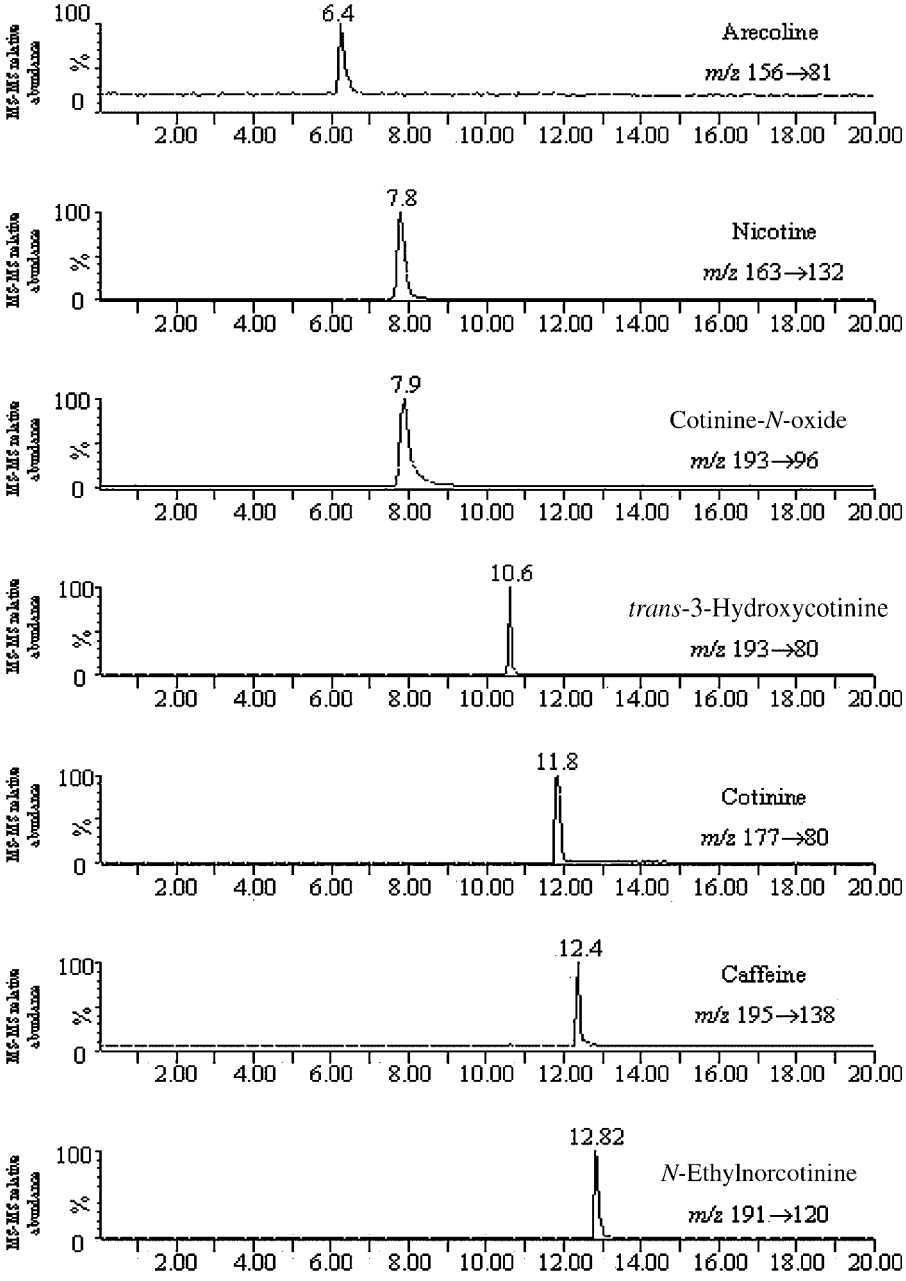

Figure 8. LC/MS/MS chromatogram of an extract of 1 mL drug-free pooled sample spiked with 50 ng NIC, COT, COT-N-OX, TRANS-3-OH-COT, CAF, ARECA and 100 ng IS.

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$[M+H]^+$ ion at *m/z* 156. The loss of CH_4O from *m/z* 156 produced an ion at *m/z* 124 and further loss of CO yielded *m/z* 96. Another fragmentation pathway involved the loss of CH_3 to produce *m/z* 141 (Fig. 6).

For the IS, NENC, the $[M-H]^+$ ion at *m/z* 191 gave the products ions at *m/z* 148, through loss of C_2H_3O, and at *m/z* 146 through loss of $C_2H_5NH_2$. The latter ion further rearranged and lost C_2H_2 to give an ion at *m/z* 120 (Fig. 7).

The transitions obtained for the ESI-MS/MS analysis of the compounds were in agreement with those previously reported for NIC and its metabolites and for CAF in other biological matrices.[14,30–33] On the basis of what had been reported for these substances, we hypothesized similar pathways for ARECA.

Chromatographic results

A representative extracted ion chromatogram obtained following the extraction of 1 mL drug-free pooled sample spiked with 50 ng NIC, COT, TRANS-3-OH-COT, COT-N-OX, CAF, ARECA and 100 ng IS is shown in Fig. 8. Separation of the compounds and the IS was completed in 13 min. A 7-min equilibration time was necessary at the end of each run for the elution of endogenous compounds.

Validation results

No additional peaks due to endogenous substances that could have interfered with the detection of compounds of interest give signals in the time scale of the experiment. Similarly, none of the drugs of abuse or aforementioned medications, carried through the entire procedure, interfered with the assay. Although the different drugs of abuse might be expected in breast milk in different concentrations, a quite high concentration was used to avoid any doubt of possible drug interferences.

Table 2. Recovery of analytes under investigation

Analyte	n	Concentration (μg/L)	Mean recovery (%)	SD
NIC	4	6	73.2	1.8
		400	77.4	5.3
		850	72.5	2.7
COT	4	6	77.3	2.9
		400	71.8	6.6
		850	75.3	2.4
COT-N-OX	4	6	75.5	1.8
		400	82.7	5.2
		850	81.6	2.9
TRANS-3-OH-COT	4	6	74.4	2.5
		400	80.5	3.4
		850	80.6	2.1
CAF	4	6	78.5	1.9
		400	85.7	5.8
		850	80.6	4.9
ARECA	4	75	76.8	1.5
		400	84.7	4.2
		850	82.6	3.4

Table 3. Method calibration in human milk

Analyte	Calibration line slope[a]	Calibration line intercept[a]	SD of residuals (Sy|x)	Determination coefficient[a] (r^2)	LOD (μg/L)	LOQ (μg/L)
NIC	0.0009 ± 0.0004	0.012 ± 0.008	0.0004	0.998 ± 0.003	1.6[b]	5.0[b]
COT	0.003 ± 0.015	0.017 ± 0.002	0.0015	0.996 ± 0.003	1.6[b]	5.0[b]
COT-N-OX	0.003 ± 0.0008	0.022 ± 0.026	0.0015	0.997 ± 0.006	1.6[b]	5.0[b]
TRANS-3-OH-COT	0.002 ± 0.0003	0.015 ± 0.02	0.0009	0.995 ± 0.003	1.6[b]	5.0[b]
CAF	0.00014 ± 0.0001	0.0013 ± 0.00022	0.00007	0.998 ± 0.002	1.6[b]	5.0[b]
ARECA	0.00004 ± 0.00007	0.0004 ± 0.0005	0.0002	0.999 ± 0.0003	16.0[b]	50.0[b]

[a] Mean ± SD of three replicates.
[b] LOD and LOQ calculated from SD of the mean noise level of drug-free milk samples over the retention time window of each analyte.

Table 4. Intra-day (n = 5) and inter-day (n = 15) precision (RSD) and accuracy (Error %)

Analyte	Concentration	Estimated mean ± SD	Precision	Accuracy	Estimated mean ± SD	Precision	Accuracy
NIC	6	6.3 ± 0.5	8.5	5.0	6.6 ± 0.5	6.7	10.2
	400	396.8 ± 21.5	5.4	−0.8	363.8 ± 30.3	8.3	−9.0
	850	811.6 ± 24.3	2.9	−4.5	821.5 ± 35.9	4.4	−3.3
COT	6	6.6 ± 0.7	10.6	10.0	5.6 ± 0.5	8.9	−6.6
	400	409.6 ± 22.9	5.5	2.4	391.1 ± 22.8	5.4	−2.2
	850	835.9 ± 36.4	4.3	−1.6	842.6 ± 33.5	3.9	−0.8
COT-N-OX	6	6.1 ± 0.1	1.6	1.6	5.3 ± 0.2	5.7	−11.6
	400	398.3 ± 20.1	5.0	−0.4	381.1 ± 22.8	5.9	−4.7
	850	839.9 ± 28.4	3.3	−1.1	842.6 ± 33.5	3.4	−0.8
TRANS-3-OH-COT	6	6.5 ± 0.6	9.2	8.3	5.8 ± 0.2	3.4	−3.3
	400	390.3 ± 28.1	7.1	−2.3	390.1 ± 22.8	5.8	−2.4
	850	841.5 ± 27.4	3.2	−1.0	846.6 ± 38.5	4.4	−0.4
CAF	6	6.4 ± 0.5	7.8	6.6	5.9 ± 0.8	14.2	−1.6
	400	401.3 ± 0.8	5.6	0.3	385.1 ± 21.6	5.6	−3.7
	850	845.9 ± 30.4	3.5	−0.5	848.2 ± 33.5	3.9	−0.2
ARECA	75	74.2 ± 1.2	1.6	−1.1	76.1 ± 0.2	0.2	1.4
	400	395.3 ± 25.1	6.3	−1.1	395.1 ± 24.8	6.2	−1.2
	850	844.5 ± 28.4	3.3	−0.6	851.1 ± 31.5	3.7	0.1

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Blank samples injected after the highest point of the calibration curve did not present any traces of carryover. With respect to the matrix effect, the comparison between peak areas of analytes spiked in extracted blank milk samples and those for pure diluted standards showed less than 10% analytical signal suppression due to coeluting endogenous substances. Absolute analytical recoveries (mean ± standard deviation, SD) obtained after liquid/liquid extractions at two different pHs (neutral for NIC and metabolites and CAF; basic for ARECA) were always better than 70% and showed that there were no relevant variations at different concentration levels for the analytes under investigation (Table 2).

Linear calibration curves showed determination coefficients (r^2) higher than 0.99 in all cases. LOD and LOQ values, calculated from the SD of the mean noise level over the retention time window of each analyte, were adequate for the purposes of the present study (Table 3). The calculated LOQ values, tested for precision and accuracy, presented coefficient of variations always better than 20%. The intra-assay and inter-assay precision and accuracy values, always better than 15%, satisfactorily met the internationally established acceptance criteria[27,28] (Table 4).

No relevant degradation was observed after any of the three freeze/thaw cycles, with differences in the initial concentration of less than 10%. Similar results (differences from the initial concentration always lower than 10%) were obtained in the mid-term stability test, assuring the validity of stored samples analysis.

Analysis of breast milk samples

The method presented here is routinely used to assess fetal exposure to NIC and its metabolites, CAF and ARECA in breast-fed infants when specifically requested as a diagnostic support to the neonatal units involved in this study.

Table 5 shows the results obtained for 20 breast milk samples analyzed during assay development. The self-reported smoking status was confirmed in nursing mothers. NIC and its metabolites were absent in samples from non-smokers showing an apparent non-exposure to environmental tobacco smoke. COT was the most prevalent biomarker of tobacco smoke in the breast milk of smoking mothers, while NIC was present only in samples of smoking mothers who had declared recent active smoking (within 2 h before sample collection, 73% of total smoking mothers). NIC showed concentrations higher than those of COT in three out of the four samples from Indian women consuming betel quid with an extremely high value of NIC in sample 20 (Fig. 9). One plausible hypothesis is that betel quid, which contains cut tobacco, is normally placed in the mouth and held against the mucosa of the buccal cheek and molar teeth to be episodically chewed to extract juice, releases NIC continuously. TRANS-3-OH-COT and COT-N-OX, detected for the first time in breast milk, appeared as minor metabolites in this biological matrix being quantified only in four and two of the eleven samples from smoking mothers, respectively. CAF was found in 75% of the breast milk samples of both smoking and non-smoking mothers, showing the widespread consumption of this substance during pregnancy. Similarly to TRANS-3-OH-COT and COT-N-OX, ARECA was for the first time identified in breast milk. Of the four samples from betel quid consumers, only two presented quantifiable amounts of the compound, while the other two contained only traces of the analyte (concentration values between ARECA LOD and LOQ).

Unfortunately, the low number of analyzed samples and the lack of information regarding time elapsed since drug consumption and sample collection in many cases did not allow any hypothesis regarding eventual correlation between concentration values in breast milk and amount

Table 5. Analytes concentration in breast milk samples from nursing mothers

Sample	NIC (μg/L)	COT (μg/L)	TRANS-3-OH-COT (μg/L)	COT-N-OX (μg/L)	CAF (μg/L)	ARECA (μg/L)
1 (non-smoking mother)	neg*	neg	neg	neg	315.4	neg
2 (non-smoking mother)	neg	neg	neg	neg	2827.8	neg
3 (smoking mother)	neg	20.5	neg	neg.	47.1	neg
4 (non-smoking mother)	neg	neg	neg	neg	602.6	neg
5 (non-smoking mother)	neg	neg	neg	neg	602.0	neg
6 (smoking mother)	40.0	173.9	14.4	18.4	1166.2	neg
7 (non-smoking mother)	neg	neg	neg	neg	391.5	neg
8 (non-smoking mother)	neg	neg	neg	neg	1380.1	neg
9 (smoking mother)	neg	27.1	neg	neg	162.8	neg.
10 (smoking mother)	neg	128.5	neg	neg	neg	neg
11 (non-smoking mother)	neg	neg	neg	neg	neg	neg
12 (smoking mother)	240.5	36.1	neg	neg	110.0	neg
13 (smoking mother)	142.3	81.6	17.3	5.0	neg	neg
14 (non-smoking mother)	neg	neg	neg	neg	609.8	neg
15 (non-smoking mother)	neg	neg	neg	neg	554.9	neg
16 (smoking mother)	44.1	3.7**	neg	neg	1343.0	neg
17 (betel quid consumer)	100.1	344.8	neg	neg	263.4	18**
18 (betel quid consumer)	55.6	2.2**	neg	neg	498.5	22**
19 (betel quid consumer)	69.2	12.7	3.8	neg	neg	50.7
20 (betel quid consumer)	513.5	172.3	15.2	neg	neg	152.9

* Negative sample, no substance detection.

** 2 mL milk were analyzed to quantify these samples where analytes showed concentrations between LOD and LOQ.

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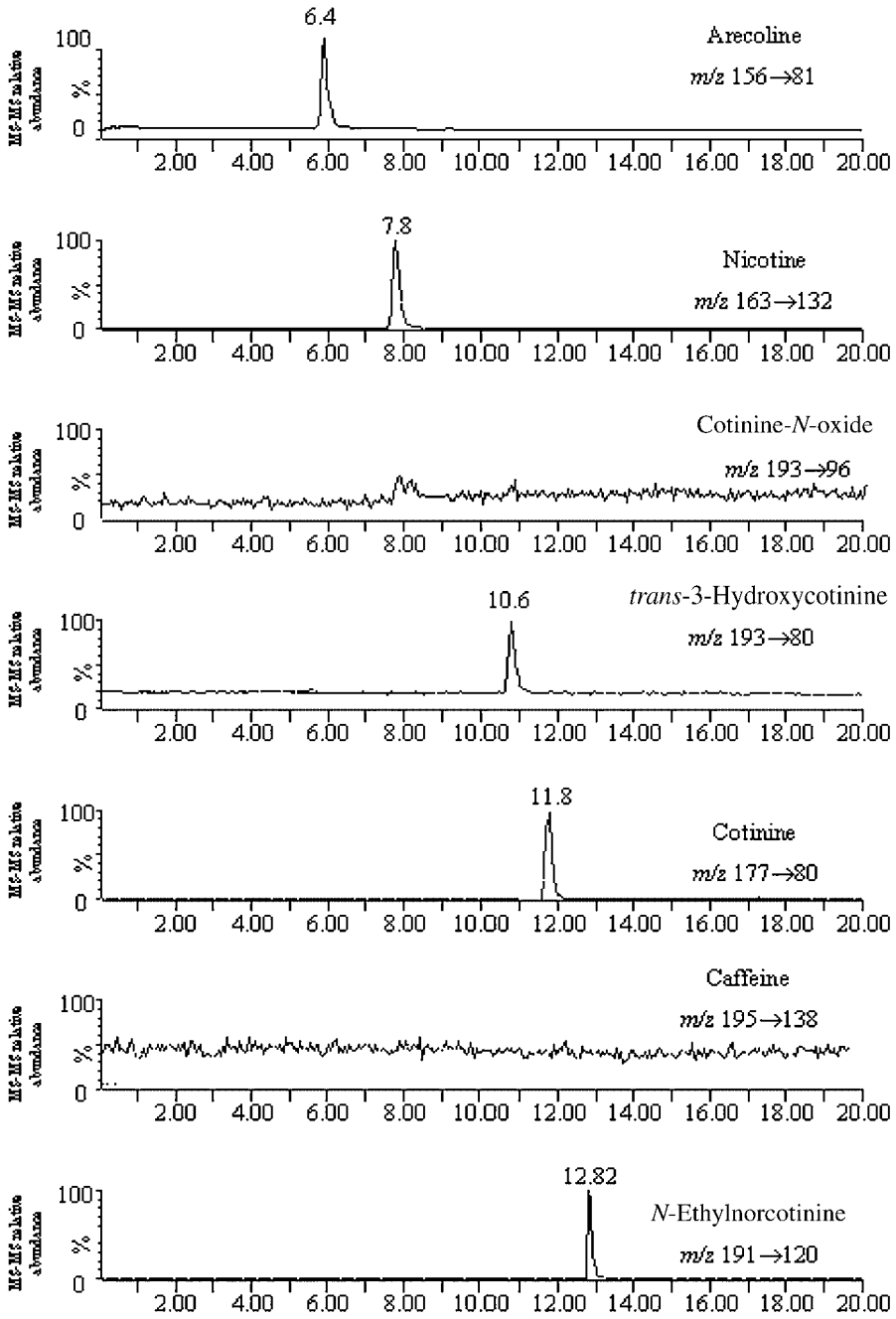

Figure 9. LC/MS/MS chromatogram of an extract of sample 20 containing 513.5 μg/L NIC, 172.3 μg/L COT, 15.2 μg/L TRANS-3-OH-COT and 152.9 μg/L ARECA.

of consumed substances. Nonetheless, this validated assay is being used as a diagnostic tool to assess substance exposure in breast-fed infants in relation to eventual adverse clinical outcomes.

A first reported case was that of an infant prenatally exposed to heavy cigarette smoke born with a severe neonatal NIC withdrawal syndrome and with spontaneous tremors while being breast-fed. The presence of non-negligible amounts of NIC and COT in breast milk showed that the mother did not stop smoking after delivery and this behaviour might have led to a postnatal withdrawal syndrome. On the basis of the analysis of breast milk, the mother was reminded of the consequences of smoking during lactation and advised to stop the habit.[34]

A similar case was that of a premature newborn from a mother reporting the drinking of mate (the infusion of Yerba Mate dry leaves with high CAF content) during pregnancy who presented with severe CAF neonatal abstinence syndrome. Intermittent irritability was still present when the infant was discharged at 24 days of age due to fluctuating CAF content measured in different breast milk feeds. The mother was strongly recommended to reduce her consumption of mate to a maximum of two cups a day during the whole breastfeeding period.[35]

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Finally, we recently reported ARECA measurement in neonatal biological matrices (e.g. cord blood, meconium, neonatal urine) accounting for acute and chronic fetal exposure to this substance[36,37] in association with adverse neonatal outcomes. ARECA measurement in breast milk was used to prove postnatal exposure and to provide advice to consumer mothers against betel quid chewing not only during pregnancy, but also during lactation.

CONCLUSIONS

NIC, CAF and ARECA are three of the four most widely consumed drugs worldwide. These drugs are often consumed in poor countries (eg. some countries from Asia and South America), where breastfeeding interruption can lead to a serious health risk for the infant. Breast-feeding mothers are often reluctant to admit to consumption of the above-mentioned drugs or they may not even be aware that they are consuming a drug, sometimes in non-negligible amounts. The accurate assessment of both acute and chronic postnatal exposure to the above-reported drugs in breast-fed infants through the objective measure of their biomarkers in maternal milk is of major importance since it provides the basis for appropriate maternal counseling, immediate infant treatment and eventual medical follow-up.

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