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# Determination of nicotine and cotinine in rat plasma by liquid chromatography–tandem mass spectrometry

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**Abstract**

A method was developed for the efficient determination of nicotine and cotinine in rat plasma samples originating from nicotine exposure studies. Nicotine and cotinine were extracted from plasma samples with dichloromethane and concentrated to minimum volume with nitrogen stream. The volatility of nicotine was prevented by the addition of hydrochloric acid to the organic solvent during evaporation. The samples were analysed using liquid chromatography with triple quadrupole mass spectrometry. For quantification, the deuterated internal standards were added and the most intensive MS–MS ion of the analyte and internal standards were monitored. For confirmatory analysis, two specific MS–MS ions, viz. *m/z* 132 and 106 for nicotine and *m/z* 80 and 98 for cotinine, were monitored and the ratios between the ions were calculated and compared with those of standards. The ratios have to be within the tolerances of the EU criteria. The limit of identification of the developed method was 1 μg/l. The repeatability ranged from 5 to 12% (R.S.D.) for nicotine and from 3 to 5% for cotinine at the concentration level of 1–60 μg/l ($n = 4$).


*Keywords:* Nicotine; Cotinine; Alkaloids

## 1. Introduction

Environmental tobacco smoke (ETS) is the material released into the ambient atmosphere by smoking tobacco products, which consists of a heterogeneous mixture of gases, uncondensed vapours and particulate phase. Since several years, the health risks of ETS containing lung cancer and heart diseases have been reported [1]. Nicotine and its metabolite, cotinine, in biological fluids have been used to estimate active smoking behaviour, to validate abstinence from smoking, and to evaluate the levels and significance of ETS exposure. It has been suggested that cigarette smoking is more addictive than nicotine alone due to the fact that tobacco or smoke seems to contain compounds which are addictive in their own right (e.g. acetaldehyde), or increase the addictive potency of nicotine (e.g. ammonium compounds). Another

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such adjunctive agent in smoke may be nitric oxide (NO) [1,2]. Our institute is involved in the assessment of ingredients added to cigarettes and one of the studies is about the effect of NO on the absorption of nicotine. Test animals, rats, were exposed to the inhalation of air containing nicotine with and without the addition of NO. After exposure, samples of blood were taken for the analysis of nicotine and cotinine. Due to the large number of samples to be analysed for exposure studies, efficient, selective and sensitive analytical methodology is highly desirable.

For the determination of μg/l concentrations of nicotine and cotinine in biological samples, e.g. human urine, plasma and saliva, the most frequently used method is gas chromatography–mass spectrometry (GC–MS) with or without derivatisation [3,4]. Recently, studies demonstrate the feasibility of liquid chromatography (LC)–MS and LC–MS–MS for the analysis of small ionic molecules in biological matrices [5,6]; this approach is preferable, since derivatisation is now superfluous.

This study reports on the development of an efficient method for the analysis of both nicotine and cotinine in samples of rat plasma. The analytical procedure makes use of a simple sample pre-treatment procedure followed by the analysis with LC–triple quadrupole mass spectrometry (QqQ-MS). This technique combines the quantification and confirmation in one single method. For confirmation of the identity of nicotine and cotinine, the EU criteria are applied as described in Commission Decision 2002/657/EC [7].

## 2. Materials

### 2.1. Chemicals and reagents

Nicotine, cotinine and the deuterated analogous, nicotine-$d_3$ and cotinine-$d_3$ were obtained from Sigma–Aldrich Chemie (Zwijndrecht, The Netherlands). Trichloroacetic acid (TCA), dichloromethane (DCM) and methanol were from Baker (Deventer, The Netherlands). Hydrochloric acid (12 M), sodium chloride, sodium hydroxide, ammonium acetate and ammonia (16 M) were from Merck (Amsterdam, The Netherlands).

### 2.2. Solutions

Stock solutions containing 1 mg/ml of analytes were prepared in methanol and stored at −20 °C. Working solutions were prepared by sequential 10-fold dilutions of the stock solutions to a single series of appropriate standard solutions. These solutions were stored in the dark at about 4 °C (range: 1–10 °C) for a period of maximum 6 months. Before analysis, the PTFE tubes were thoroughly rinsed with a solution of 1 M sodium hydroxide followed by a wash step with DCM.

### 2.3. Samples

The test animals (rats) were exposed to 10 mg/m$^3$ nicotine tartrate aerosol for 3–100 min, with and without 100 μg/l NO. Within 10 min after exposure, blood sample were collected in EDTA-containing tubes and centrifuged immediately at 1000 × $g$. The plasma supernatant was collected and frozen at −20 °C until analysis.

### 2.4. Equipment

For analysis a LC–QqQ-MS system (triple quadrupole system; Micromass Quatro Ultima, Almere, The Netherlands) in the positive electrospray ionisation (ESI(+)) mode and an Allicance (Waters, Etten-Leur, The Netherlands) pump and autosampler were used. Separations were obtained at 30 °C under isocratic conditions using a Waters XTerra 100-2.1 mm, RP-18, 3.5 μm particles with an 1 cm pre-column and methanol–20 mM ammonium acetate in water (5:95, v/v) at 0.25 ml/min as eluent. Acquisition parameters were: capillary voltage, 3.0 kV; cone voltage, 30 V; source temperature, 120 °C; desolvation temperature, 400 °C; cone gas flow, 150 l/h; desolvation gas flow, 400 l/h; ions were detected in the ESI(+) mode. For quantification, the most intensive MS–MS ion of the analyte and internal standards were monitored. For confirmation of the identity of the analyte, two MS–MS ions were monitored and the ratios between the ions were calculated and compared with those of standards. Acquisition parameters were optimised by 20 μl/min infusion of 1 ng/μl standard working solutions. Table 1 presents the analytes in combination with the parent ion and the corresponding MS–MS ions monitored.

Table 1
Ions used for LC–ESI(+)–MS–MS screening and confirmation

| Analyte | Parent ion | MS–MS screening ion | | MS–MS confirmation ion | |
|---|---|---|---|---|---|
| | *m/z* | Collision energy (eV) | *m/z* | Collision energy (eV) | *m/z* |
| Nicotine | 163 | 16 | 132 | 17 | 106 |
| Nicotine-$d_3$ | 166 | 16 | 132 | | |
| Cotinine | 177 | 27 | 80 | 24 | 98 |
| Cotinine-$d_3$ | 180 | 27 | 80 | | |

## 3. Methods

### 3.1. Extraction procedure

To 0.5 ml rat plasma, 10 ng of each of the deuterated internal standards were added together with 50 μl of 4 M HCl and 0.6 ml of 1 M NaCl, for the precipitation of the plasma proteins. The mixture was washed with 2 ml of DCM after centrifugation and the aqueous phase was transferred to an empty tube. Next, 100 μl of a 20% NaOH solution was added for neutralisation of the analytes. The (neutral) analytes were extracted by the addition of an other 2 ml of DCM. After centrifugation, the aqueous layer was removed and the DCM layer was transferred and evaporated on a heating block at 50 °C under a stream of nitrogen. To prevent vaporisation of the analytes during evaporation of the DMC, 50 μl of 6 M HCl was added. After evaporation of the DCM layer, the analytes were neutralised now by the addition of 50 μl of 16 M $NH_3$. $NH_3$ evaporated very quickly on a heating block at 50 °C under a stream of nitrogen and the obtained residue was re-suspended in 100 μl water. Finally, 25 μl of the extract was injected in the LC–MS system.

### 3.2. LC–QqQ-MS for quantification

The final extract was analysed by LC–QqQ-MS. Calibration curves for nicotine and cotinine were constructed by the injection of six standard solutions corresponding with 1–100 μg/l of nicotine and cotinine and 20 μg/l of the deuterated internal standards, [$^2H_3$]nicotine (nicotine-$d_3$) and [$^2H_3$]cotinine (cotinine-$d_3$). The most intense MS–MS ion (screening ion) of analyte and deuterated analogous were monitored and the calibration curve was constructed by plotting the ratios of heights of the ions of analyte/deuterated analogous against the concentration. For the samples of rat plasma, the same ratio was calculated and by using linear regression method, the concentrations of nicotine and cotinine in the sample were estimated. Calibration samples, blank samples of rat plasma fortified with standards and internal standard solutions at concentration levels between 1 and 100 μg/l were analysed together with every series of samples. See Table 1 for the specific ions monitored.

### 3.3. LC−QqQ-MS for confirmation

For confirmation of the identity of the analytes, the samples were re-analysed according to the method described for the quantification but without the addition of the internal standards. For confirmation, the intensities of two MS–MS ions selected from the MS–MS spectra were monitored (Table 1). The ratio between these ions was calculated. The ratios obtained for the samples were compared with those of the calibration standards. The ratios have to be comparable within the tolerances of the EU criteria [7].

### 3.4. Method validation

The limit of identification was at the concentration at which both MS–MS ions originating from the analyte show signal-to-noise (S/N) responses of S/N $\geq$ 3.

For the determination of the repeatability and the recoveries, a sample fortified with each of the analytes at 5–6 μg/l (low control) and one at 50–60 μg/l (high control) were analysed four times, together with the analysis of the ‘blank’ (non-fortified) sample. From the results obtained, the repeatability, recovery and accuracy were calculated.

## 4. Results and discussion

### 4.1. Introduction

The analytical procedure described is based on the strategy of screening/quantification and confirmation, two methods which are defined according to the definitions of the EU criteria as described in Commission Decision 2002/657/EC [7]. Screening/quantification is used to detect, and quantify, the presence of an analyte or class of analytes at the level of interest. Confirmation should provide full or complementary information enabling the analyte to be identified unequivocally at the level of interest. The identity of the present target analytes were verified by using the EU criteria, i.e. they were treated as 'illegal compounds'.

### 4.2. Sample preparation

For sample pre-treatment, the first step was the precipitation of the proteins. It is important to avoid the use of glassware because nicotine from the air or from the sample absorbed to it. Initially, protein precipitation was performed by the addition of TCA as described in literature [8]. By switching from TCA to a solution of HCl and NaCl as described by Jung et al. [4], higher absolute recoveries were observed. The protein precipitation was followed by a DCM wash step. After the addition of NaOH, DCM was added for analytes extraction. Finally, the aqueous layer was removed and the DCM was evaporated. As described by Nakajima et al. [5], the evaporation of the DCM is a critical step concerning the recovery due to the volatility of nicotine. Improvement of nicotine recovery was observed by the addition of HCl to the DCM extract before the DCM was evaporated. The addition of an acid resulted in the protonation of the nicotine and cotinine and so vaporisation was prevented. After evaporation of the DCM, neutralisation of the extract was necessary in order to prevent excessive band broadening during LC analysis as a consequence of the protonated analytes.

### 4.3. LC analysis and quantification

A XTerra RP-18 LC column and a mobile phase containing methanol–50 mM ammonium acetate (95:5, v/v) provided adequate retention. The retention obtained for nicotine and cotinine were, respectively, 4.2 min ($k' = 1.8$) and 6.9 min ($k' = 3.6$). The R.S.D. of the reproducibility of the method based on the multiple analysis of control samples ($n = 5$) were for both nicotine and cotinine at the concentration range from 1 to 80 μg/l (≤10%), the only exception was nicotine at the concentration between 1 and 10 μg/l which showed a R.S.D. from 34 to 46%.

With the developed method, more than 200 rat plasma samples taken during 'nicotine exposure studies' were analysed. The concentrations found ranged from 2 to 50 μg/l for nicotine and from <1 to 100 μg/l for cotinine. Fig. 1 shows an example of the LC–QqQ-MS screening chromatogram of rat plasma fortified with 58 μg/l nicotine and 56 μg/l cotinine. In Table 2, the method characteristics are presented. Absolute recoveries of >50% are for this type of analysis, low concentrations of small molecules in complex matrices, satisfactory. However, the concentration of nicotine monitored in the blank is a point of concern. The question is if the nicotine concentration is endogenous or if during sample pre-treatment the sample—or laboratory instruments used—are contaminated with nicotine. Research is going on at this moment on the feasibility of on-line extraction by the use of restricted access materials (RAM) in LC. Separation columns packed with RAM provide efficient separation of target analytes (small molecules) but exclude large molecular size compounds, e.g. proteins, from retention. This makes RAM columns very attractive for the direct processing of plasma, thus eliminating or reducing the time of sample pre-treatment and so reducing the risk of contamination of the sample with nicotine from glassware, chemicals, air, etc. [9].

### 4.4. Confirmation

To be sure that no other interfering compound was responsible for the nicotine response—observed in the chromatogram of the blank plasma sample—confirmatory analysis was performed. For the confirmation of nicotine and cotinine in rat plasma, the criteria for identification as described in [7] were applied. Although the EU criteria are primarily used in the field of identification of veterinary drugs and specific contaminants in animals and fresh meat, the approach is universal applicable to the identification of organic residues and contaminants [10].

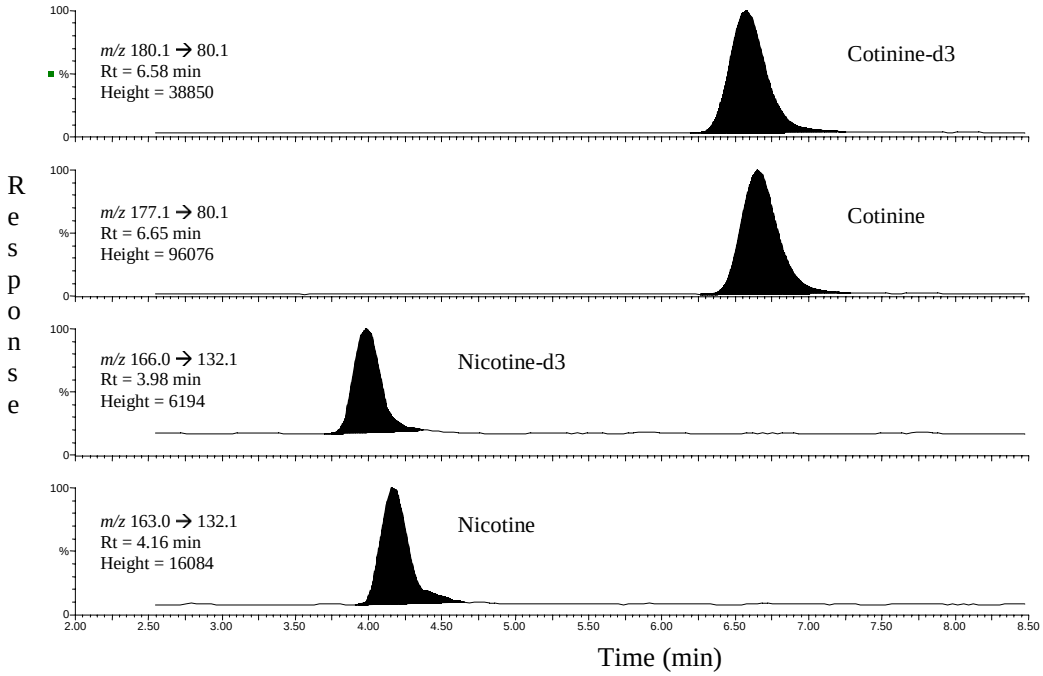


Fig. 1. LC–QqQ-MS screening chromatogram of fortified sample of rat plasma at the level of 58 μg/l for nicotine (*m/z* 163 → 132) and 56 μg/l cotinine (*m/z* 177 → 80) and the internal standards at the level of 10 μg/l for nicotine-$d_3$ (*m/z* 166 → 132) and cotinine-$d_3$ (*m/z* 180 → 80).

For the confirmation analysis, a set of three samples containing different concentrations of the analytes inclusive a blank sample were re-analysed without the addition of the internal standard. For both analytes, two MS–MS ions were monitored (see Table 1) and the ratio between these ions were calculated. Fig. 2 shows the MS–MS spectra of nicotine (a) and cotinine (b) and the proposed structures of the MS–MS ions monitored. Together with the samples, six standards solutions were injected in the concentration range of 2–50 μg/l. Fig. 3 shows a LC−QqQ-MS chromatogram for the confirmation analysis of a sample of rat plasma at

Table 2
Method characteristics

| | Accuracy[a] (%, $n = 2$) | Average[b] (μg/l, $n = 4$) | Repeatability[b] (R.S.D., $n = 4$) | Recovery[a,b] (%, $n = 4$) |
|---|---|---|---|---|
| Nicotine | | | | |
| Blank | | 4.4 | 11 | |
| Low control (5.8 μg/l) | 107 | 7.5 | 5 | 53 |
| High control (58 μg/l) | 120 | 36.5 | 8 | 55 |
| Cotinine | | | | |
| Blank | | <1 | | |
| Low control (5.6 μg/l) | 100 | 3.8 | 5 | 68 |
| High control (56 μg/l) | 104 | 37 | 3 | 66 |

[a] Corrected for blank.
[b] Calculated using external standard.

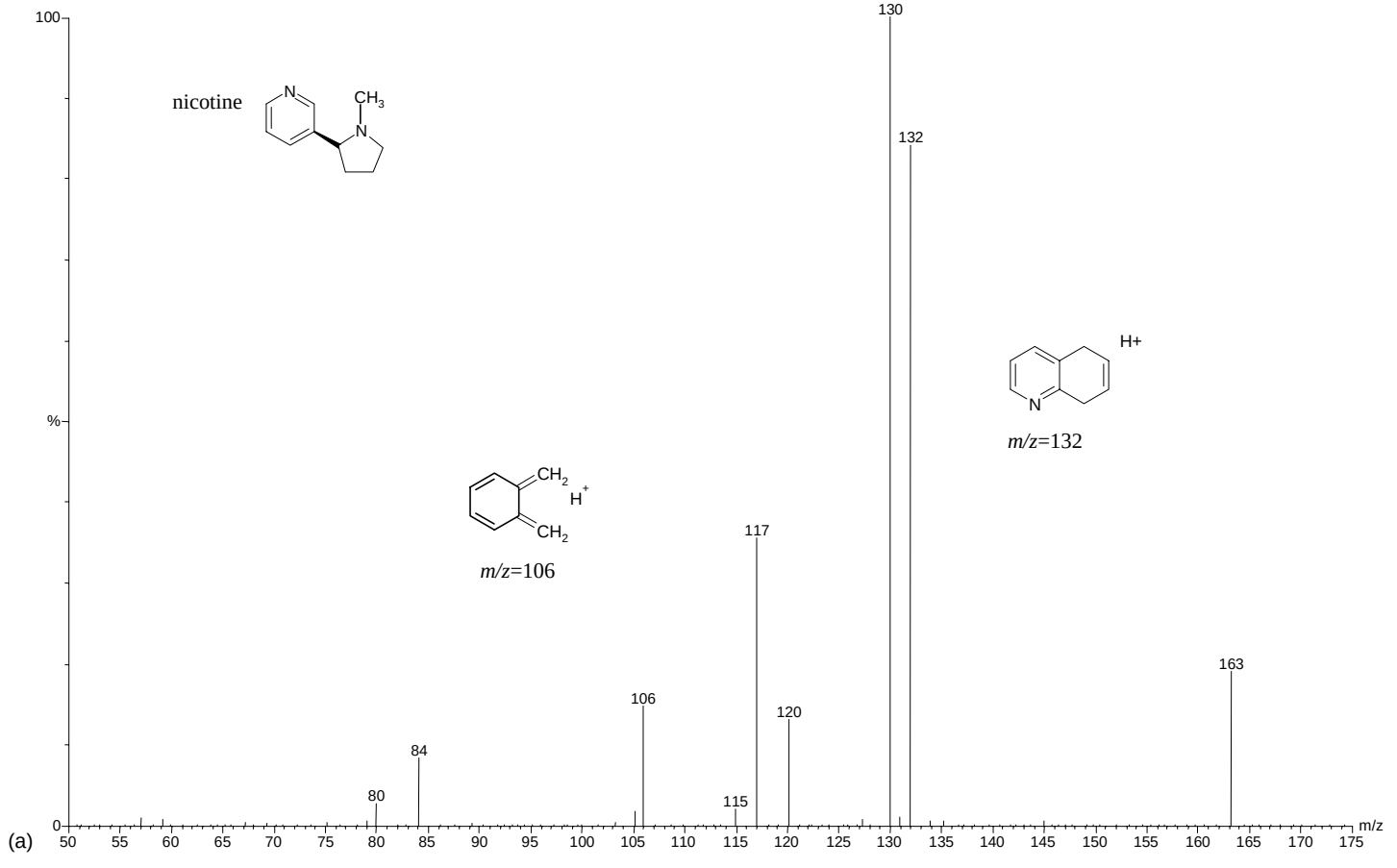


Fig. 2. Full MS–MS spectra recorded for nicotine (a) and cotinine (b) on a LC–QqQ-MS system and proposed structures of MS–MS ions. For experimental details, see text.

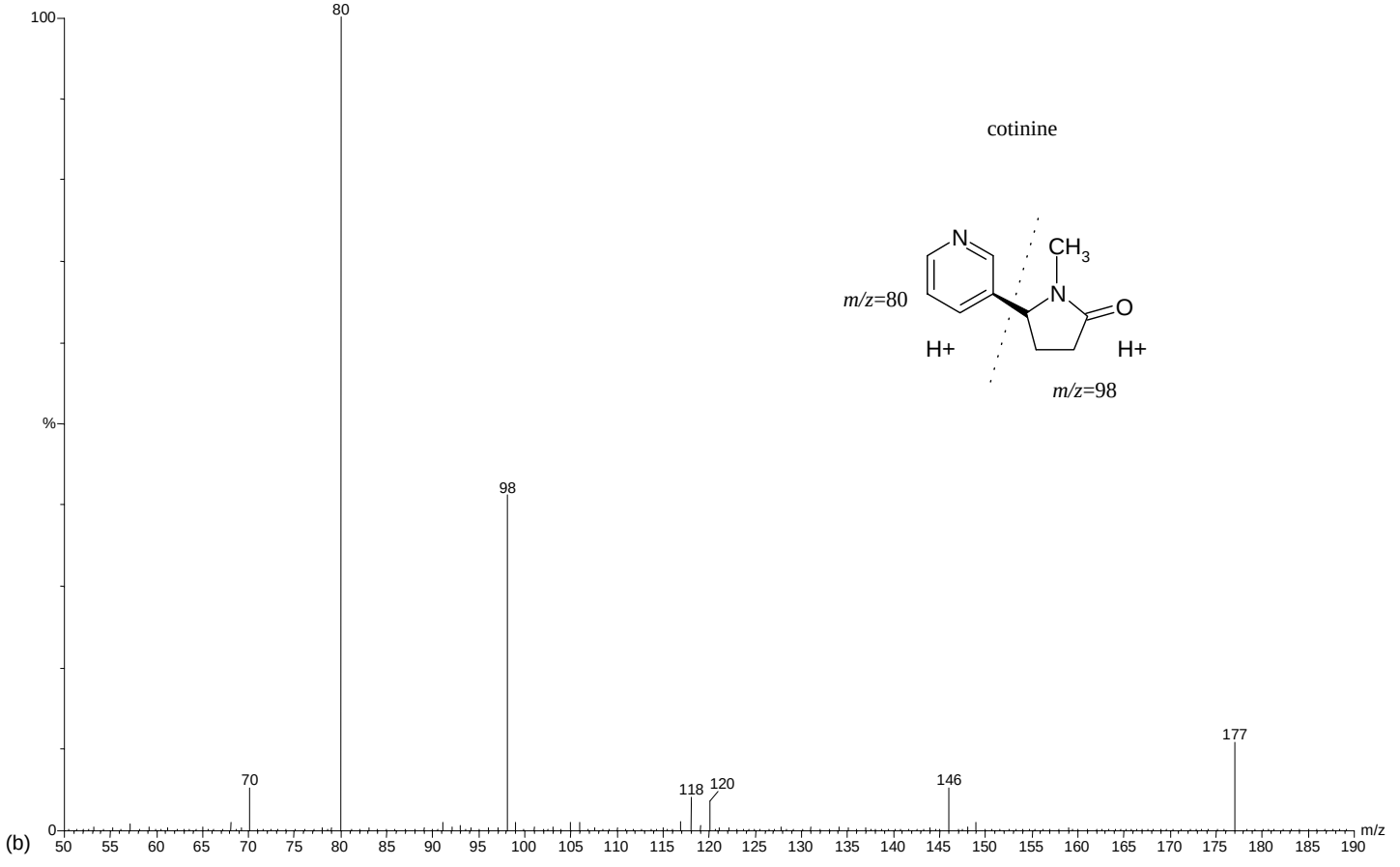


Fig. 2. (*Continued*).

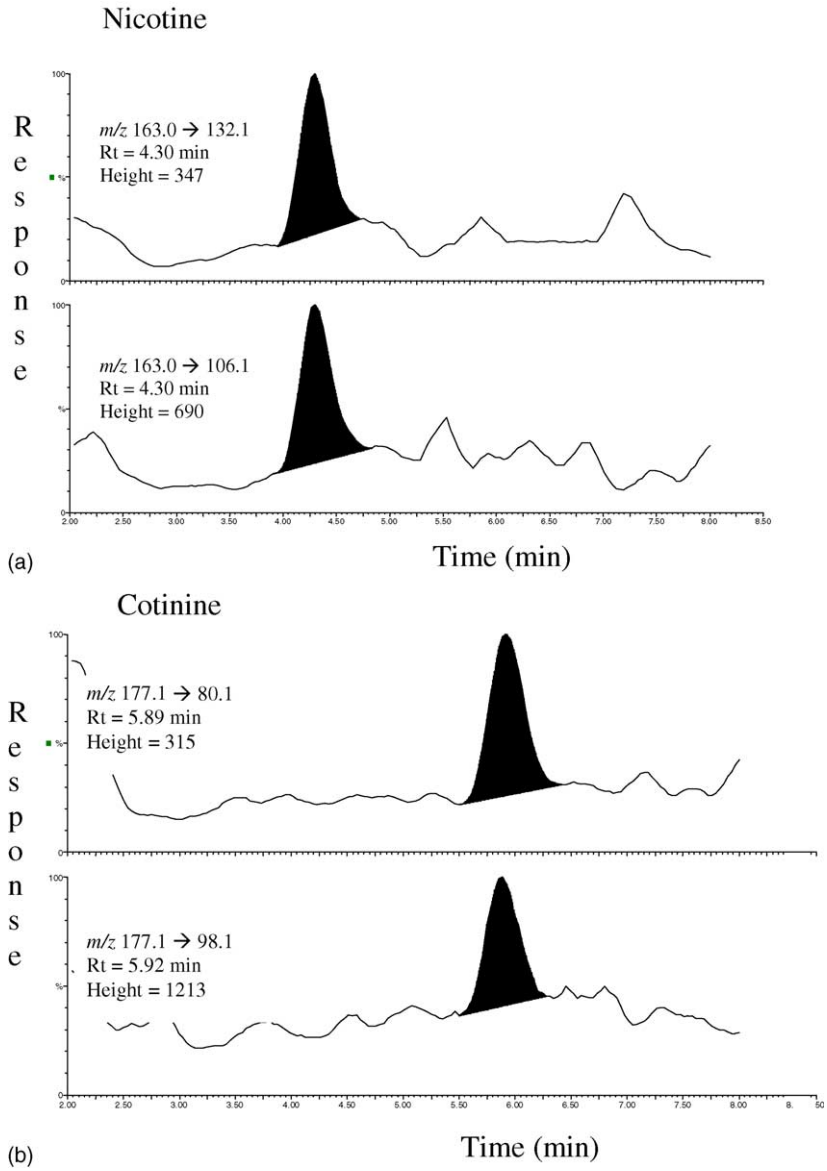


Fig. 3. LC–QqQ-MS chromatogram of confirmation of nicotine and cotinine in a blank sample ($t = 0$ min) of rat plasma with 5 μg/l nicotine and <1 μg/l cotinine. See text for LC–MS–MS conditions; see Table 1 for selected diagnostic ions and Table 3 for results.

$t = 0$ min (blank). The average ion ratios obtained for the standards and the ion ratios obtained for the samples were compared. From the results presented in Table 3, it was concluded that the ratios of all samples were within the tolerance intervals defined by the EU with one exception, cotinine in the blank was not confirmed; in other words, the blank sample of rat plasma did not contain cotinine. The limit of identification was 1 μg/l for both nicotine and cotinine.

Table 3
Results of LC–QqQ-MS confirmation of nicotine and cotinine in rat plasma

| Analyte | Concentration (μg/l) | Reference ratio[a] | Tolerance[b] (%) | Ratio in sample[c] |
|---|---|---|---|---|
| Nicotine | 5 | 0.47 | 25 (0.35–0.58) | **0.50** |
| | 10 | | | **0.55** |
| | 30 | | | **0.56** |
| Cotinine | <1 | 0.37 | 25 (0.28–0.46) | 0.26 |
| | 10 | | | **0.39** |
| | 60 | | | **0.37** |

The values given in parentheses indicate range.
[a] Abundance (confirmation ion/screening ion); reference standards in 2–50 μg/l range ($n = 6$).
[b] According to EU criteria [7].
[c] The bold values indicate that the result is confirmed.

## 5. Conclusions

The use of LC in combination with mass selective detection is a very powerful technique for the analysis of low concentration of relatively small molecules in complex matrices. The use of the LC–QqQ-MS makes it possible to combine the quantification of the analytes and the confirmation of the identity in one single method. With this method, simultaneous analysis of nicotine and cotinine in samples of rat plasma at the concentration level of 1–80 μg/l is possible. Involving a simple liquid/liquid extraction as sample pre-treatment, the method has a high sample throughput of approximately 40 samples per day.

The observed relatively high levels of nicotine (1–5 μg/l) in the 'blank' samples of rat plasma are not fully understood yet. Further research is necessary to find out at which step in the experiment from 'animal to analysis' the 'background of nicotine' is collected. The identity of nicotine in the blank is confirmed by applying the EU criteria described in 2002/657/EC, demonstrating the universal applicability of these criteria.

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