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Validation of an assay for the determination of cotinine and 3-hydroxycotinine in human saliva using automated solid-phase extraction and liquid chromatography with tandem mass spectrometric detection

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Abstract

The validation of a high-performance liquid chromatographic method for the simultaneous determination of low level cotinine and 3-hydroxycotinine in human saliva is reported. Analytes and deuterated internal standards were extracted from saliva samples using automated solid-phase extraction, the columns containing a hyper cross-linked styrene–divinylbenzene copolymer sorbent, and analysed by reversed-phase liquid chromatography with tandem mass spectrometric detection (LC–MS–MS). Lower limits of quantitation of 0.05 and 0.10 ng/ml for cotinine and 3-hydroxycotinine, respectively, were achieved. Intra- and inter-batch precision and accuracy values fell within ±17% for all quality control samples, with the exception of quality control samples prepared at 0.30 ng/ml for 3-hydroxycotinine (inter-day precision 21.1%). Results from the analysis of saliva samples using this assay were consistent with subjects' self-reported environmental tobacco smoke (ETS) exposures, enhancing the applicability of cotinine as a biomarker for the assessment of low level ETS exposure.

Keywords: Cotinine; 3-Hydroxycotinine

1. Introduction

Nicotine is the principal alkaloid in tobacco and is present as a major component of tobacco smoke. It is absorbed in measurable quantities by both active and passive smokers, the latter shown to inhale quantities of nicotine proportionally to the product of concentration, duration of exposure and respiration rate [1,2]. However, the relatively short half-life for nicotine ($t_{1/2}$≈1–2 h) precludes its use as an accurate marker for environmental tobacco smoke (ETS) exposure, or passive smoking, since assessments of low level exposure over protracted time periods are often desired. Cotinine, a primary metabolite of nicotine formed after C-oxidation via the enzyme cytochrome P450, has a much longer half-life ($t_{1/2}$≈ 18–20 h) than nicotine resulting in higher and more stable plasma concentrations and is therefore considered a more appropriate biomarker for evaluating ETS exposure [3–5].

Following exposure to nicotine, cotinine can be found in most body fluids and methods for its determination in blood (serum/plasma), urine and

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saliva are generally considered acceptable for estimating nicotine exposure [6,7]. In a review by Etzel [8] evaluating the relationship between saliva cotinine concentrations and ETS exposure, concentrations less than 10 ng/ml would usually result from ETS exposure without active smoking, although heavy passive exposure to tobacco smoke may produce levels in excess of this value. In recent times the specificity of cotinine as a biomarker has been questioned since dietary sources of nicotine have been identified (e.g., tomato, potato, cauliflower, tea) [9,10], although the contribution of dietary nicotine to serum cotinine levels is estimated to be small in comparison to ETS exposure [11]. In a recent publication by Pirkle et al. [12], reporting the findings of the Third National Health and Nutrition Examination Survey (NHANES III) in the United States, the estimated geometric mean contribution of dietary intake to serum cotinine levels was less than 0.02 ng/ml. Also reported was a median serum cotinine level of 0.526 ng/ml for adults who had reported some degree of ETS exposure either at home or at work. Hence, in order to adequately assess very low level exposure to ETS using cotinine as a biological marker, or indeed to determine the degree of any dietary contribution to cotinine levels, assay sensitivity sufficient to quantitate levels significantly lower than 0.5 ng/ml is required. To date, there are very few published methods available for the quantification of cotinine at very low concentrations.

Bernert et al. [13] have recently reported the development and 'validation' of an assay for determining cotinine in human serum with a limit of detection of 0.05 ng/ml, the method utilising protein precipitation followed by liquid–liquid extraction prior to liquid chromatography with tandem mass spectrometric detection. From the data presented, this method was estimated to have a lower limit of quantitation (LLOQ) in the region of 0.17 ng/ml. The method described here utilises solid-phase extraction followed by liquid chromatography with tandem mass spectrometric detection, and has been validated for the simultaneous determination of cotinine and 3-hydroxycotinine in human saliva with LLOQs of 0.05 and 0.10 ng/ml, respectively. The automation of sample extraction reduces the opportunity for sample contamination and contributes significantly to the achievement of low level quantitation. Also of critical importance for the achievement of these LLOQs was the use of water as a surrogate matrix for the preparation of calibration standards, since analyte-free control human saliva was not available. Subsequent evaluation of the influence of any ion suppression or background noise differences between water and human saliva was performed. At present, there are no published methods available for the simultaneous quantification of both cotinine and 3-hydroxycotinine in any matrix at the LLOQs reported here. This validation was designed to fulfil the requirements outlined in the Washington consensus meeting (1990) reported by Shah et al. [14] as well as incorporating current regulatory opinion.

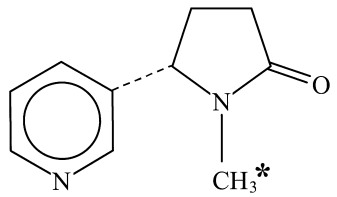
2. Experimental

2.1. Standards and reagents

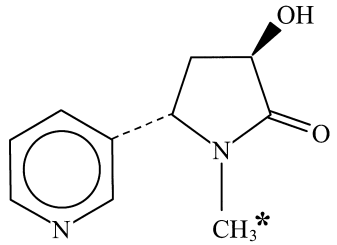
(−)-Cotinine and (±)-cotinine-d_3 (deuterated internal standard) were obtained from Sigma-Aldrich (Gillingham, UK). (±)-*trans*-3-Hydroxycotinine and d_3-methyl-(±)-*trans*-3-hydroxycotinine (deuterated internal standard) were kindly supplied by R.J. Reynolds Tobacco Company (Winston-Salem, NC, USA). The structures for these materials are presented in Fig. 1. Ammonium formate, formic acid (98% minimum), potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate dihydrate (all AnalaR grade) and water (HPLC grade) were obtained from BDH (Merck; Lutterworth, UK). Methanol (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, UK). Control human saliva was obtained in-house at Covance Laboratories.

2.2. Liquid chromatography–tandem mass spectrometry (LC–MS–MS)

A Jasco model PU-980 pump (Jasco UK, Great Dunmow, UK) was used to deliver mobile phase, consisting of methanol–30 m*M* ammonium formate–formic acid (50:50:5, v/v/v), at a flow rate of 1 ml/min through an OmniPac PCX-500 (ethylvinylbenzene/divinylbenzene polymeric core with a polymeric colloid containing sulphonic groups) 50×4.6



Cotinine



3-Hydroxycotinine

Fig. 1. Structures for cotinine and 3-hydroxycotinine. Asterisks denote the position of the deuterated label for internal standards.

mm I.D. column (Dionex Chromatography, Camberley, UK). Sample injection was performed using a Gilson 231XL autosampler (Anachem, Luton, UK) and the column eluate split at a ratio of 1:20 prior to mass spectrometric detection using a MicroMass Quattro triple quadrupole mass spectrometer equipped with a positive ion electrospray interface (MicroMass, Altrincham, UK). The source was maintained at 150°C with a spray voltage of 3.25 kV and a counter electrode voltage of 0.4 V. Nitrogen was used as the nebulizer gas at a flow rate of 15 l/h and also as the desolvation bath gas at a flow-rate of 300 l/h. The respective parent ions were selected in the first quadrupole mass analyser and transmitted into the collision cell, containing argon at a pressure of 3×10^{-4} mbar, with collision energies ranging between 40 and 45 eV. Multiple reaction monitoring of five mass/charge (m/z) transitions was performed, with an inter-channel delay of 0.02 s. Two transitions for cotinine were monitored, one used for quantification (m/z 177 $[M+H]^+ > m/z$ 80) and the other used for confirmation purposes (m/z 177 $[M+H]^+ > m/z$ 98). The remaining three transitions were used to monitor cotinine-d_3 (m/z 180 $[M+H]^+ > m/z$ 101), 3-hydroxycotinine (m/z 193 $[M+H]^+ > m/z$ 80) and 3-hydroxycotinine-d_3 (m/z 196 $[M+H]^+ > m/z$ 80). The retention times for cotinine and 3-hydroxycotinine under the chromatographic conditions employed were approximately 2.2 and 1.9 min, respectively.

2.3. Preparation of primary and working stock solutions

Separate primary stock solutions for cotinine (100 μg/ml) and 3-hydroxycotinine (100 μg/ml) were prepared by dissolving 2 mg of each material into 20 ml methanol. Stock solutions for the preparation of calibration standards were made from weighings independent to those used for the preparation of quality control samples. All stock solutions were stored refrigerated (nominal 4°C) in amber glass vessels for up to 2 months.

Separate primary stock solutions for cotinine-d_3 (100 μg/ml) and 3-hydroxycotinine-d_3 (100 μg/ml) were prepared by dissolving 0.5 mg of each material into 5 ml methanol. All internal standard stock solutions were stored refrigerated (nominal 4°C) in amber glass vessels for up to 2 months.

2.3.1. Preparation of calibration standards

A combined working stock solution containing both cotinine and 3-hydroxycotinine at a concentration of 10 μg/ml was prepared by diluting 1 ml of each primary stock solution to 10 ml with methanol. Dilutions of this combined calibration working stock solution and subsequent diluted working stock solutions, using a minimum number of serial dilutions, were performed in order to provide working standard solutions containing cotinine and 3-hydroxycotinine at concentrations of 400, 360, 200, 40, 20, 8, 4, 2, 1.2 and 0.8 ng/ml in methanol. Working standard solutions were stored refrigerated (nominal 4°C) in amber glass vessels for up to 2 months. Calibration standards were prepared fresh on each analysis occasion by the addition of 25 μl of each working standard solution to 1 ml of water giving a calibration range of 0.020–10.0 ng/ml.

2.3.2. Preparation of quality control samples

Separate dilutions of the primary stock solutions with methanol were performed in order to provide working stock solutions at concentrations of 1000, 100, 10 and 1 ng/ml for both cotinine and 3-

hydroxycotinine. These solutions were stored refrigerated (nominal 4°C) in amber glass vessels for up to 2 months. Aliquots of these working stock solutions were spiked into control human saliva (50 ml) to produce quality control samples containing both cotinine and 3-hydroxycotinine at nominal concentrations of 0.060, 0.150, 0.300, 4.00, 8.00 and 20.0 ng/ml, taking account of determined endogenous levels of cotinine and 3-hydroxycotinine within the control saliva pool used. Quality control samples at concentrations below determined endogenous levels in the control matrix pool were prepared by dilution of the control matrix using water to achieve the required concentration of the most abundant analyte and subsequently spiked with the least abundant analyte to the required concentration. Aliquots (2.5 ml) were stored frozen (nominal −20°C) in polypropylene tubes prior to analysis.

2.3.3. Preparation of internal standard solutions

A working stock internal standard solution containing both cotinine-d_3 (10 ng/ml) and 3-hydroxycotinine-d_3 (20 ng/ml) was prepared by diluting 100 and 200 μl of the respective primary stock solutions to 1000 ml with methanol. This solution was stored refrigerated (nominal 4°C) in an amber glass vessel for up to 2 months.

2.4. Sample extraction

An aliquot of each saliva sample (1 ml), quality control sample or calibration standard (1 ml water containing 25 μl of an appropriate working standard solution) was transferred into a borosilicate glass culture tube (75×10 mm, LIP (Equipment and Services), Shipley, UK) and 100 μl of internal standard working solution (10 ng/ml cotinine-d_3; 20 ng/ml 3-hydroxycotinine-d_3) added. Buffer (1/15 *M* potassium dihydrogen orthophosphate–1/15 *M* disodium hydrogen orthophosphate, 41:59, v/v) was added (1 ml) and the tube capped, briefly vortex mixed and centrifuged at 1500 *g* for 5 min. Following centrifugation, the tube cap was removed and the tube was transferred to a Gilson ASPEC™ or ASPEC XL™ instrument (Anachem, Luton, UK) for automated solid-phase extraction. The solid-phase extraction column, Isolute ENV+100 mg/1 ml (International Sorbent Technology; Jones Chromatography, Mid Glamorgan, UK), was conditioned with methanol (1 ml) followed by water (1 ml) and the sample solution subsequently applied to the column under low positive pressure. Following sample application, the column was sequentially washed with water (1 ml) and water–methanol (70:30, v/v, 1 ml) and the analytes eluted into ASPEC™ collection tubes (45×12 mm borosilicate glass tubes; purchased from Anachem) with methanol (2.5 ml). The sample extract was then evaporated to dryness at 40°C under a gentle stream of nitrogen using a Techne Dri-Block® SC-3 sample concentrator (BDH (Merck)) and dissolved in 200 μl of 12.5 m*M* ammonium formate–methanol–formic acid (80:20:0.5, v/v/v). The tube was briefly vortex mixed and centrifuged at 1500 *g* for 5 min. Following centrifugation, the sample extract was transferred into a tapered microvial and 100 μl were taken for injection onto the column.

3. Results and discussion

3.1. Assay linearity and the limits of quantitation

For the determination of low level cotinine and 3-hydroxycotinine in human saliva, calibration standards were prepared using water since analyte-free control matrix was not available. The validity of using water in place of saliva for this purpose was investigated by comparing response functions for six calibration curves prepared using saliva from six different individuals with those for a curve prepared using water. Coefficients of determination (r^2) for all seven curves ranged between 0.9674 and 0.9987 (mean 0.9917) for cotinine and between 0.9813 and 0.9999 (mean 0.9932) for 3-hydroxycotinine. The coefficient of variation between determined gradients was 4.9% for cotinine and 5.2% for 3-hydroxycotinine, indicating that water was a suitable surrogate matrix. Linear regression of the peak height ratios for analytes and internal standards versus concentration was performed using $1/x^2$ weighting and the following linear equations are typical for calibration curves prepared using water over the validated concentration ranges: cotinine, $y=0.00188x+0.02699$, $r^2=0.99756$; 3-hydroxycotinine, $y=0.00056x+0.02237$, $r^2=0.99367$. Peak height

was used for quantitation in preference to peak area since the positioning of chromatographic baseline for height is less critical and lends itself more readily to automated chromatographic evaluation, greatly reducing the need for manual intervention for baseline positioning. Peak area would generally be used for assays where peak height did not provide a linear response over the intended concentration range, which could be due to factors such as poor peak symmetry or alteration of peak shape with increased column load.

Since endogenous levels of cotinine and 3-hydroxycotinine precluded the use of control matrix for the preparation of calibration standards, and the fact that analytical background noise associated with human saliva differed from that for water, sensitivity could not simply be defined as the lowest concentration on the standard curve that could be measured with acceptable accuracy and precision. In this instance, the LLOQ was also determined according to the procedures developed by IUPAC [15] and the American Chemical Society's Committee on Environmental Improvement [16], which were based upon an original idea by Kaiser [17]. This involved the evaluation of short-term baseline noise immediately preceding and following the peak of interest, quantified for at least 10 individual samples of control matrix, and the LLOQ expressed as the mean plus 10 times the standard deviation for these measurements. Using this regime, LLOQs of 0.050 and 0.100 ng/ml were calculated for cotinine and 3-hydroxycotinine, respectively. The precision and accuracy for quality control samples prepared in water at these concentrations were subsequently evaluated as reported below. Endogenous levels of cotinine-d_3 and 3-hydroxycotinine-d_3 were not found to be present in any of the control matrices analysed during the course of this validation. Representative chromatograms generated from the analysis of water blanks, calibration standards prepared at the LLOQ and control human saliva for cotinine and 3-hydroxycotinine are presented in Figs. 2 and 3, respectively.

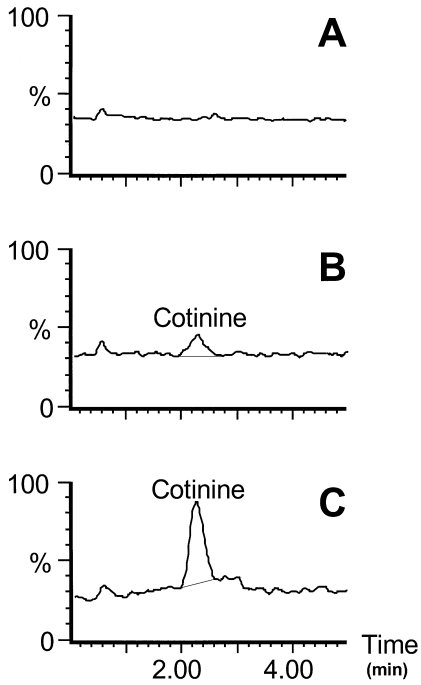


Fig. 2. Representative LC–MS–MS chromatograms from the analysis of cotinine in human saliva for (A) a water blank, (B) a calibration standard at the lower limit of quantitation (0.05 ng/ml cotinine in water) and (C) a sample of control human saliva (0.24 ng/ml).

3.2. Precision and accuracy

Intra-batch (within-run) precision and accuracy were determined by replicate analysis ($n=6$) of quality control samples (QCs) prepared at concentrations equivalent to the LLOQ (prepared with water), the upper limit of quantitation (ULOQ) and at three additional concentrations spanning the intended concentration range (low, mid and high) for each analyte. Accordingly, samples were prepared to contain both cotinine and 3-hydroxycotinine at concentrations of 0.050 ng/ml (LLOQ QC for cotinine), 0.100 ng/ml (LLOQ QC for 3-hydroxycotinine), 0.060 ng/ml (low QC for cotinine), 0.300 ng/ml (low QC for 3-hydroxycotinine), 4.00 ng/ml (mid QC), 8.00 ng/ml (high QC) and 10.0 ng/ml (ULOQ QC). Additional QC samples were also prepared at a concentration exceeding the calibration range (20.0 ng/ml) which were diluted 5-fold with water prior to analysis. For validation purposes, low QC levels are normally selected to lie between two and three times the concentration of the LLOQ. Low QC samples for cotinine were prepared at 0.060 ng/ml since the method was originally intended to have an LLOQ of

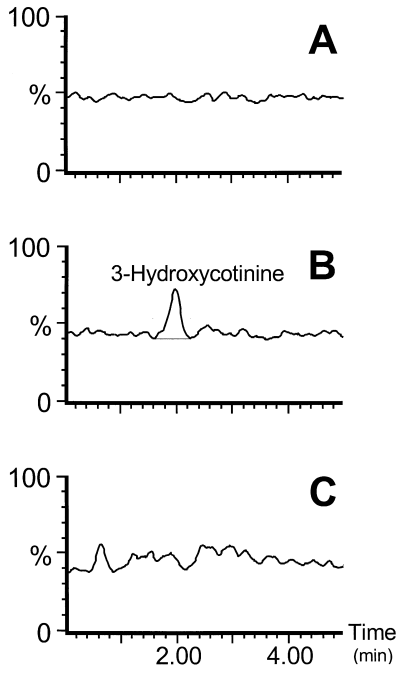


Fig. 3. Representative LC–MS–MS chromatograms from the analysis of 3-hydroxycotinine in human saliva for (A) a water blank, (B) a calibration standard at the lower limit of quantitation (0.10 ng/ml 3-hydroxycotinine in water) and (C) a sample of control human saliva.

0.020 ng/ml for this analyte, which subsequently proved to be unachievable.

Inter-batch (between-run) precision and accuracy were similarly calculated by analysing QC samples ($n=6$; excluding LLOQ and ULOQ QCs) on nine separate occasions spanning an 8-week period. Mean concentrations (±standard deviation) and the coefficient of variation for each analyte at each concentration are presented in Table 1.

These data indicate that the method is precise and accurate for the determination of cotinine over the concentration range 0.050 to 10.0 ng/ml. For 3-hydroxycotinine, assay precision at the low QC concentration (21.1% at 0.300 ng/ml) fell marginally outside the generally accepted limit of 20%. However, this variability was considered to be acceptable since poorer assay sensitivity and generally lower endogenous concentrations for 3-hydroxycotinine make this analyte less important as a marker for ETS exposure than cotinine.

3.3. Analyte recoveries

Due to the presence of endogenous analytes in control saliva, recoveries for cotinine and 3-hydroxycotinine were determined using the deuterated internal standards. Analyte peak heights determined from the extraction of control saliva fortified with cotinine-d_3 and 3-hydroxycotinine-d_3 at low, mid and high QC concentrations were compared with peak heights determined for control saliva extracts fortified with equivalent amounts of cotinine-d_3 and 3-hydroxycotinine-d_3, representative of 100% recovery at these concentrations. Analyte recoveries expressed as a percent of analyte added are presented in Table 2. The detector response for deuterated analytes was found to be less sensitive than for unlabelled 'native' material, with equimolar concentrations of labelled cotinine and 3-hydroxycotinine producing detector responses equivalent to 54% and 93% of those for respective native analytes over the concentration range investigated. The poor reproducibility of peak heights and exaggerated recoveries calculated for low QC concentrations were attributed to this fact.

For 3-hydroxycotinine, this reduced sensitivity was attributed to the isotopic purity of the d_3 material which was estimated as being >90%. The reduced sensitivity observed for cotinine-d_3 was not due to its isotopic purity, which was >98%, but was attributed to the use of a transition using a different daughter ion (m/z 180>m/z 101) to that for native cotinine (m/z 177>m/z 80).

3.4. Analyte stability

The stability of cotinine and 3-hydroxycotinine in human saliva to three additional freeze-thaw cycles (four in total) and after storage for 24 h under ambient conditions of temperature and lighting was investigated at low, mid and high QC sample concentrations. The stability of analytes in sample extracts stored refrigerated (nominal 4°C) for up to 48 h was also investigated at the same concentrations. These data are presented in Tables 3 and 4 and demonstrate that the analytes were stable under the storage conditions investigated. Deviations from theoretical of 19.0 and 24.7% were observed for 3-hydroxycotinine low QC samples (0.300 ng/ml)

Table 1
Precision and accuracy data

Analyte	Actual concentration (ng/ml)	Observed concentration (mean±SD) (ng/ml)	Coefficient of variation (%)	Accuracy (%)	*n*
Intra-batch precision and accuracy					
Cotinine	0.050	0.049±0.0053	10.8	98.0	6
	4.00	4.10±0.102	2.5	102.5	6
	8.00	8.17±0.306	3.7	102.1	6
	10.0	10.0±0.37	3.7	100.0	6
	20.0[a]	21.3±0.51	2.4	106.5	6
3-Hydroxycotinine	0.100	0.093±0.0151	16.2	93.0	6
	0.300	0.325±0.0261	8.0	108.3	6
	4.00	4.31±0.172	4.0	107.8	6
	8.00	8.8± 0.203	2.3	110.5	6
	10.0	10.8±0.60	5.6	108.0	6
	20.0[a]	20.9±0.90	4.3	104.7	6
Inter-batch precision and accuracy					
Cotinine	0.060	0.057±0.0095	16.7	95.0	48
	4.00	4.00±0.400	10.0	100.0	54
	8.00	8.29±0.427	5.2	103.6	54
	20.0[a]	20.6±1.11	5.4	103.0	42
3-Hydroxycotinine	0.300	0.303±0.0638	21.1	101.0	54
	4.00	3.79±0.509	13.4	94.8	54
	8.00	8.16±0.690	8.5	102.0	54
	20.0[a]	19.4±1.27	6.5	97.0	42

[a] Analysed after a 5-fold dilution with HPLC grade water.

following storage for 24 h at room temperature and after three additional freeze–thaw cycles, respectively. However, these deviations reflected an increase in analyte concentration and determined levels were within 15% of the reported concentrations for 'untreated' low QC samples, included for the assessment of inter-batch assay variability. As such, these deviations were considered to be due to inherent assay variability for 3-hydroxycotinine at low concentrations, rather than a reflection of any analyte instability.

3.5. Application

The method as described was used to measure concentrations of cotinine and 3-hydroxycotinine in saliva samples collected from subjects with a range of self-reported recent ETS exposure histories. Determined levels for subjects reporting no recent exposure, some recent exposure, living with a smoker, being an occasional smoker and being a smoker are reported in Table 5. Subjects reporting no recent ETS exposure had lower mean saliva cotinine

Table 2
Analyte recoveries

Analyte	Concentration added (ng/ml)	Mean peak height (*n*=3) of extracted QC (CV%)	Mean peak height (n=3) spiked QC extract (CV%)	Recovery (%)
Cotinine	0.060	655 (15.7)	380 (–)[a]	172.4
	4.00	19188 (1.1)	22617 (1.2)	84.8
	8.00	39584 (0.6)	46147 (1.7)	85.8
3-Hydroxycotinine	0.300	1645 (12.9)	1449 (4.6)	113.5
	4.00	15283 (1.9)	18039 (2.3)	84.7
	8.00	31001 (2.1)	36374 (2.3)	85.2

[a] *n*=2.

Table 3
Room temperature and freeze–thaw stability of cotinine and 3-hydroxycotinine in human saliva

Analyte	Actual concentration (ng/ml)	Mean concentration observed (CV%) (ng/ml)	Deviation from actual (%)	*n*
Storage for 24 h at room temperature				
Cotinine	0.060	0.064 (3.7)	6.7	4
	4.00	4.08 (4.0)	2.0	6
	8.00	8.35 (9.5)	4.4	6
	20.0[a]	19.6 (4.5)	2.0	6
3-Hydroxycotinine	0.300	0.357 (9.9)	19.0	6
	4.00	4.01 (4.0)	0.3	6
	8.00	7.77 (3.9)	2.9	6
	20.0[a]	19.3 (5.5)	3.5	6
3 additional freeze-thaw cycles				
Cotinine	0.060	0.061 (14.8)	1.7	6
	4.00	4.19 (6.1)	4.8	6
	8.00	7.83 (4.6)	2.1	6
	20.0[a]	19.6 (3.2)	2.0	6
3-Hydroxycotinine	0.300	0.374 (26.4)	24.7	6
	4.00	3.95 (5.6)	1.3	6
	8.00	7.86 (10.8)	1.8	6
	20.0[a]	19.3 (5.8)	3.5	6

[a] Analysed after a 5-fold dilution with HPLC grade water.

concentrations (0.145 ng/ml) than those reporting some exposure (0.689 ng/ml) or living with a smoker (1.28 ng/ml). However, there was an overlap in the range of cotinine values reported for all of these ETS exposure groups. This was possibly due to nicotine intake derived from sources other than ETS (e.g., dietary intake) or, more likely, a reflection of subjects' abilities to accurately assess any recent ETS exposure. A less noticeable trend was apparent for 3-hydroxycotinine, with a far greater overlap in the ranges of determined values, which was attributed to the poorer assay sensitivity for this analyte and the fact that saliva concentrations were found to be in the region of 40% of the corresponding cotinine levels. Representative chromatograms, generated from the analysis of saliva samples from subjects reporting varying degrees of ETS exposure for cotinine and 3-hydroxycotinine, are presented in Figs. 4 and 5 respectively.

The results from this preliminary investigation indicate that saliva cotinine levels, and, to a lesser extent, 3-hydroxycotinine levels, determined over

Table 4
Stability of cotinine and 3-hydroxycotinine in human saliva extracts

Analyte	Actual concentration (ng/ml)	Mean concentration observed (CV%) (ng/ml)	Deviation from actual (%)	*n*
Extracts stored refrigerated for 48 h				
Cotinine	0.060	0.068 (4.5)	13.3	6
	4.00	4.08 (3.6)	2.0	6
	8.00	8.18 (4.3)	2.3	6
	20.0[a]	21.5 (4.1)	7.5	6
3-Hydroxycotinine	0.300	0.274 (5.6)	8.7	6
	4.00	3.56 (1.2)	11.0	6
	8.00	7.51 (2.4)	6.1	6
	20.0[a]	18.3 (3.2)	8.5	6

[a] Analysed after a 5-fold dilution with HPLC grade water.

Table 5
Comparison of saliva cotinine and 3-hydroxycotinine concentrations determined for subjects with varying degrees of self-reported environmental tobacco smoke (ETS) exposure

Self-reported ETS exposure	Mean concentration (ng/ml)	Range of concentrations (ng/ml)	*n*
Cotinine			
No exposure	0.145	0.025–0.613[a]	18
Some exposure	0.689	0.196–1.29	6
Live with smoker	1.28	0.360–2.61	10
Occasional smoker	12.7	2.78–23.4	4
Regular smoker	94.0	35.3–174.5	6
3-Hydroxycotinine			
No exposure	0.080	0.050–0.225[a]	18
Some exposure	0.357	0.050–0.675[a]	6
Live with smoker	0.397	0.050–1.01[a]	10
Occasional smoker	5.68	0.402–11.5	4
Regular smoker	23.5	9.46–44.1	6

[a] Determined levels falling below the limit of quantitation (LOQ) were assigned a value of 0.025 ng/ml, cotinine and 0.050 ng/ml, 3-hydroxycotinine ($\frac{1}{2}$LOQ) for the calculation of mean concentrations.

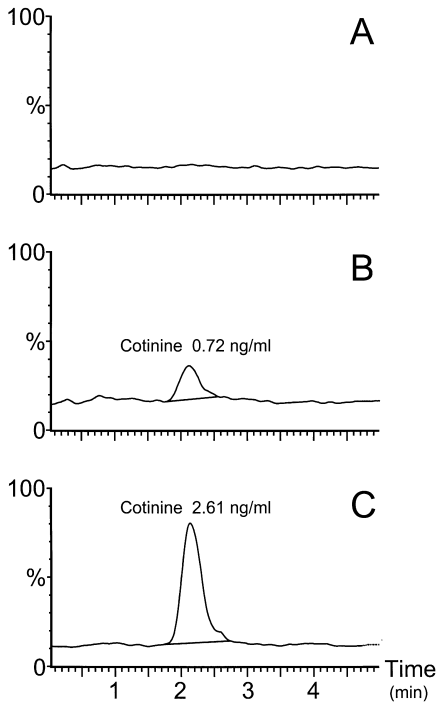


Fig. 4. Representative LC–MS–MS chromatograms from the analysis of cotinine in human saliva samples from subjects reporting (A) no ETS exposure (<0.05 ng/ml), (B) some ETS exposure and (C) to be living with a smoker.

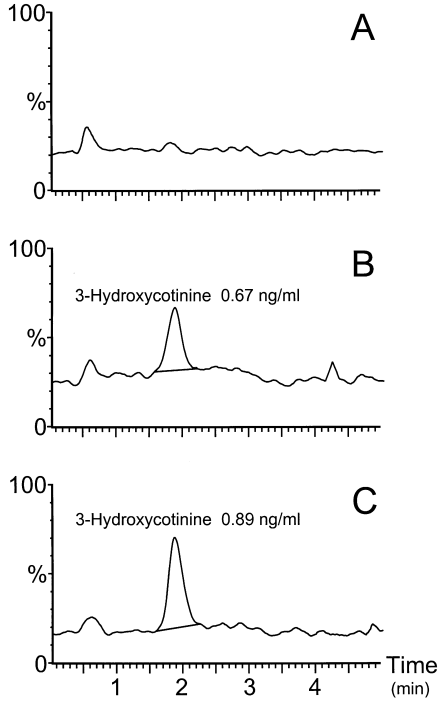


Fig. 5. Representative LC–MS–MS chromatograms from the analysis of 3-hydroxycotinine in human saliva samples from subjects reporting (A) no ETS exposure (<0.10 ng/ml), (B) some ETS exposure and (C) to be living with a smoker.

these validated concentration ranges will have increased significance as a biomarker for ETS exposure.

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