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The contribution of dietary nicotine and dietary cotinine to salivary cotinine levels as a nicotine biomarker

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Abstract

Dietary nicotine, as a source of nicotine biomarkers such as salivary cotinine, has been a controversial topic, mainly because of limited published data on nicotine in foods. Recently, a sample preparation method for nicotine in foods and an analytical method, based on gas chromatography with mass spectrometric detection, were developed and validated in our laboratory. Nicotine was determined in fresh, cooked and processed foods of the *Solanaceae* family. In this study we have furthered this work to investigate the presence of cotinine in these vegetables, using GC–MS and HPLC–MS as independent alternative analytical techniques. A large number of samples was investigated, but cotinine was not detected in any of the samples, although very high sensitivity could be achieved. Based on the results, we report a Monte Carlo simulation of salivary cotinine levels obtained utilising dietary nicotine, food consumption data, and the variance in these parameters. In this estimate, a mean salivary cotinine concentration of 0.022 ng ml^{-1} is predicted from dietary sources. This level is below the limit of detection for traditional analytical methods, but perhaps not for newly-developed methods.

Keywords: Dietary nicotine; Salivary cotinine; Nicotine biomarkers; Nicotine; Cotinine

1. Introduction

Nicotine is reported to be found in a variety of plants. Leete (1983) reported the presence of nicotine in various plants belonging to 12 families and 24 genera, including the large family of the nightshades (*Solanaceae*). Besides the tobacco plant (*Nicotiana tobaccum*), which is cultivated because of its high nicotine content, some vegetables that are common parts of our diet belong to the nightshade family — including tomatoes (*Lycopersicon esculentum*), potatoes (*Solanum tuberosum*), eggplants (aubergines, *Solanum melonga*), and peppers (*Capsicum annum* and *Capsicum frutescens*). There has been controversy in the literature concerning the relative amount of nicotine intake from dietary sources in relation to the exposure to environmental tobacco smoke, or even active smoking of a small number of cigarettes (Castro & Monji, 1986; Davis, Stiles, De Bethizy, & Reynolds, 1991; Domino, Hornbach, & Demana, 1993; Idle, 1990; Jarvis, 1994; Repace, 1994; Sheen, 1988). Osler (1998) recently suggested that non-smokers generally consume a healthier diet than smokers and consequently have a larger consumption of raw and cooked vegetables and teas. If nicotine is present in these foods and beverages, non-smokers may have a larger intake of dietary nicotine.

Recently, Siegmund, Leitner and Pfannhauser (1999b) developed and validated an analytical method based on gas chromatography–mass spectrometry for the determination of nicotine in food substances. Siegmund, Leitner, and Pfannhauser (1999a) have reported detailed results of the determination of nicotine in a variety of vegetables. The matrices analysed include raw and cooked vegetables from the nightshade family that are frequently consumed (i.e. tomatoes, potatoes, and eggplants) and processed foods made from these vegetables such as tomato sauce, ketchup and French fries (Pommes frites). Green peppers and teas were included

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because nicotine has been reported in these materials with conflicting conclusions (Chappell & Gratt, 1996; Davis et al., 1991; Domino et al., 1993; Idle, 1990; Sheen, 1988). Siegmund et al. (1999a) reported that the nicotine concentrations for various vegetables ranged from 2–7 μg kg^{-1} for fresh ("as is") vegetables. The results provided a set of means and standard deviations of the nicotine contents for these food substances. This information was combined with food consumption data from 13 European countries and the USA to perform a Monte Carlo Simulation (Decisioneering, 1996) of the distribution of dietary nicotine intake. The mean and the 95th percentile of dietary nicotine intake based on these data are 1.4 μg day^{-1} and 2.25 μg day^{-1}, respectively.

In the mammalian system, nicotine is metabolised, whereas the most important metabolic reaction of nicotine is the formation of cotinine by oxidation (Gorrod & Schepers, 1999; Fig. 1). As a consequence of this metabolic pathway, cotinine is generally used as a biomarker of exposure to tobacco smoke and it is typically measured in blood, plasma, saliva or urine (Benowitz, 1996; Lee, 1999). Based on the fact that nicotine was also found in frequently-consumed vegetables (Siegmund et al., 1999a), it was thought that the dietary intake of nicotine would contribute significantly to cotinine levels in biological fluids. Furthermore, a decrease of nicotine concentration was observed during the ripening process of tomatoes, indicating a possible degradation or oxidation of nicotine. Based on these results, investigations were continued to determine whether cotinine is formed by oxidation of nicotine in vegetables. Should this be the case, dietary cotinine would be of potential impact on salivary cotinine levels, due to its direct supply via foods.

The literature contains a number of papers dealing with the determination of cotinine in matrices such as blood, plasma, urine or saliva using gas chromatography or liquid chromatography and a variety of detection systems (Baranowski, Pochopién, & Baranowska, 1998; Bentley, Abrar, Kelk, Cook, & Phillips, 1999; Bernert Jr. et al., 1997; Crooks & Byrd, 1999; Jacob III & Byrd, 1999; Moore, Cooper, & Kram, 1993; Skarping, Willers, & Dalene, 1988). Nevertheless, from these citations, it is not apparent which of these methods would be most suitable for the determination of cotinine in foods. In addition, no references were found on the determination of cotinine in any food matrix. In this paper we describe methods for the determination of cotinine in various food matrices using liquid chromatographic as well as gas chromatographic techniques, as well as the results from these investigations.

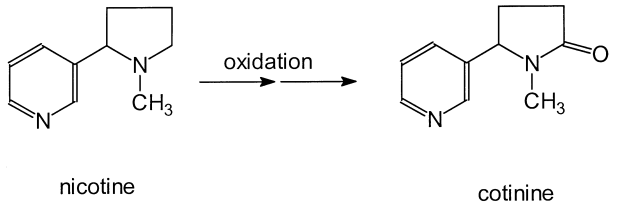

Fig. 1. Oxidation of nicotine to form cotinine.

To estimate the influence of dietary factors on cotinine levels in human biological fluids, we used the results of Siegmund et al. (1999a) to calculate a distribution of salivary cotinine concentration that might be expected from dietary intake of nicotine. Furthermore, edible nightshades were analysed for their cotinine concentrations, to determine whether there is a direct cotinine contribution to salivary cotinine via diet.

2. Materials and methods

2.1. Chemicals

Dichloromethane, ethyl acetate, sodium sulfate granular (all minimum 99.5% purity) were purchased from Promochem, Wesel, Germany. Butyl acetate (HPLC quality, 99.7%), triethylamine (purum, 99%), cotinine (purum, 98%) and deuterated cotinine ([$^2H_3$]methylcotinine, purum 98%; used as internal standard) were purchased from Sigma-Aldrich, Steinheim Germany. Sodium hydroxide (analytical-reagent grade), potassium hydroxide (analytical-reagent grade), hydrochloric acid (analytical-reagent grade), acetic acid (analytical-reagent grade) and ammonium hydroxide (25%, analytical-reagent grade) were purchased from Merck, Darmstadt, Germany. Acetonitrile and methanol (both HPLC gradient-grade quality) were purchased from Riedel-de-Haen, Seelze, Germany. Microfilters (Teflon, 0.45 μm pore width) were purchased from Pall Gelman Laboratory, Ann Arbor, Michigan, USA.

Standard solutions for the compounds cotinine and deuterated cotinine were prepared in butyl acetate/0.01% triethylamine (concentrations of the stock solutions for cotinine 101 g l^{-1} and for deuterated cotinine 1.14 g l^{-1}). These solutions were stored in the freezer at −18°C. Dilutions of the samples were prepared just before use using ethyl acetate/0.01% triethylamine.

2.2. Collection of vegetable samples

Based on the results of previous studies (Siegmund et al., 1999a, b), nicotine concentration in tomatoes was found to depend on the degree of ripening; therefore special emphasis was placed on the collection of samples. Fresh tomatoes, at various stages of ripeness, were obtained from an Austrian vegetable breeding station. The tomatoes were grown in greenhouses and harvested according to their degree of ripening. Other vegetables, such as eggplants, potatoes and tea-samples, were purchased in local supermarkets. Three greenhouse tomato varieties in four degrees of ripening each, two tomato samples, purchased in a supermarket, two

potato samples, two eggplant samples, four green pepper samples, and two tea samples, were investigated. Complete duplicate analyses were performed on each sample.

All vegetables were washed with hot water, cold water and finally with double-distilled water to avoid surface contamination. Then the fresh fruits were dried and homogenised using a BÜCHI homogeniser (BÜCHI mixer B400, BÜCHI Labortechnik AG, Flawil, Switzerland). The samples were transferred into carefully cleaned polyethylene boxes; the remaining gas volume was purged with nitrogen, and the samples were then stored in the freezer at −18°C until use.

2.3. Sample preparation and extraction of cotinine

Homogenised vegetable samples (50 g) were lyophilised and used for one extraction. The internal standard (45.6 ng per 50-g sample) was added before the lyophilisation step and consequently passed through the whole sample preparation procedure. One millilitre of KOH (10 N) was added to the dried material (residual water content approx. 5%), homogenised again and transferred quantitatively into a glass column (length 18 cm, inner diameter 1 cm) where it was used as a solid phase. The analyte was eluted directly from the material in the column using 200 ml CH_2Cl_2. The organic extract was reduced to 0.5 ml using the Zymark rotation evaporator (Zymark Turbo Vap 500, Hopkinton, MA, USA). This extract was used directly for the GC–MS analyses. For HPLC–MS analyses, CH_2Cl_2 was evaporated to dryness using a gentle nitrogen stream, then diluted in 1 ml of the mobile phase that is described for the HPLC analyses, and put into the ultrasonic bath for several minutes. The final extract was filtered through a Teflon microfilter and injected into the LC–MS system.

2.4. Analytical methods for the determination of cotinine

2.4.1. GC–MS

The GC analyses were performed on a Hewlett-Packard HP 5890 II Plus gas chromatograph equipped with a Hewlett-Packard mass-selective detector (HP MSD 5972). The capillary column employed was a HP 5 MS (crosslinked 5% phenyl methyl siloxane, column length 30 m, and inner diameter 0.25 mm, film thickness 0.25 μm). Helium (quality 5.0) was used as the carrier gas. The operating conditions were as follows: a split/splitless injection was used in the splitless mode with an injection volume of 1 μl; the injector temperature was 235°C. The pressure program employed a pressure pulse at the beginning of the chromatographic run: 150 kPa were held for 0.5 min, then the pressure was decreased at a rate of 500 kPa min^{-1} to 50 kPa; constant flow was used for the remaining time (0.87 ml min^{-1} or 24.1 cm s^{-1}, respectively). The following temperature program was used: 70°C for 1 min; then a temperature ramp for 25°C min^{-1} was carried out to a final temperature of 280°C, which was held for 5 min. The detector temperature was 280°C. For the mass spectrometry, electron impact ionisation (70 eV) was performed. The data were acquired in the selected ion mode (characteristic masses for cotinine m/z 98, m/z 176; [$^2H_3$]methylcotinine m/z 101, m/z 179). Special attention had to be given to the condition of the liner of the injector to obtain good peak shapes for the analytes. The treatment of the GC-injection liner is described in detail by Siegmund et al. (1999b).

2.4.2. HPLC–MS

The HPLC analyses were performed on a Hewlett Packard HP 1100 MSD. The employed mass selective detector was equipped with an atmospheric pressure ionisation electrospray (API–ES) using a fragmentation voltage of 50 V for positive ionisation of the analytes. The analytical column used was a C18 reversed phase material (Merck Purospher STAR, RP 18e, LiChroCART 55-2, column of dimensions: length 55 mm, inner diameter 2 mm, particle diameter 3 μm). The composition of the mobile phase was a mixture of 87% double-distilled water, 7% acetonitrile, 4% methanol and 2% acetic acid at pH 5.0 (adjusted by NH_4OH) with a flow rate of 0.3 ml min^{-1} for the isocratic separation. After each run it was necessary to rinse the analytical column with 100% methanol to (1) clean the analytical column carefully and (2) to recondition the column. To achieve maximum ionisation yield, a make-up solution was added just before the entrance into the MSD (acetonitrile with 0.2% acetic acid at a flow rate of 0.2 ml min^{-1}). The data were acquired in the selected ion mode (m/z 177 for cotinine and m/z 180 for the deuterated cotinine).

2.5. Monte Carlo simulation

The Monte Carlo simulation reported in this work used a PC-based program (Decisioneering, 1996). The parameters entered into the program were the mean and standard deviation of the nicotine content of each of the vegetables reported previously (Siegmund et al., 1999a), and the mean and standard deviation of the consumption of these vegetables for all of the 14 countries for which data could be found. These data were used to calculate a hypothetical population distribution of daily nicotine intake using a Monte Carlo approach. The distribution was assumed to be lognormal (Siegmund et al., 1999a).

3. Results and discussion

3.1. Dietary cotinine

For the determination of nicotine in food matrices, GC–MS provided a very selective and sensitive method

(Siegmund et al., 1999b). Cotinine is a more polar compound than nicotine. In the course of these investigations it was found that cotinine could not be determined using GC–MS with stable performance of the system. The decrease in performance manifested itself as strongly tailing peaks and a decrease of sensitivity, which was mainly dependent on the condition of the injection system and the analytical column. A similar behaviour had been found before for the determination of nicotine. With a special treatment of the injection liner (including special cleaning and deactivation steps) that had to be performed frequently, this problem had been solved for nicotine. The conditions that were used are described in detail in Siegmund et al., 1999b. In the case of cotinine, this phenomenon was even more pronounced. Even a small number of injections into the freshly set-up system led to strong tailing of the cotinine peaks that made quantification impossible. Consequently, GC–MS could not be used for routine quantification of cotinine for a high number of food samples. Nevertheless, using a freshly cleaned and deactivated injection liner, as well as a new analytical column, the GC–MS method was sensitive enough to be used as an independent method for verification of results obtained by liquid chromatography.

HPLC, coupled with an atmospheric pressure ionisation electrospray-mass spectrometer (HPLC–API–ES–MS, henceforth HPLC–MS), was found to be a viable analytical technique for the determination of cotinine with consistent performance. The use of a small analytical column (length 55 mm, inner diameter 2 mm) permits rapid analysis (retention time of the analyte 2.8 min). Linear calibration models in the concentration range 10–60 ng ml^{-1} were prepared. Linear regression was obtained using four replicates for the lowest and highest concentrations and duplicates for the middle two concentrations versus the peak areas that yielded correlation coefficients of $R=0.9929$ for cotinine and $R=0.9955$ for the internal standard (IS). The linearity of the calibration curves was examined by linearity tests according to Mandel (Funk, Damman, & Donnevert, 1992). The suitability of the calibration model was verified by analysis of the residuals; the test for homogeneity of the variances was performed according to an *F*-test, based on the 95 and 99% confidence interval. The calculations of the lower limits of detection (LOD) and lower limits of quantification (LOQ) were performed according to DIN 32645 (1994). The values for LOD and LOQ are shown in Table 1. Recoveries of 85±2% for tomatoes, 103±6% for potatoes, 103±18% for eggplants, 105±5% for peppers, and 72±16% for tea were obtained with consistent values at different concentration levels.

A large number of samples was analysed to determine the cotinine concentrations. For those samples in which the presence of cotinine could not be excluded unambiguously, GC–MS was used as an alternative analytical technique to avoid false positive results. Cotinine was not detected in any of the samples investigated at a concentration larger than 0.1 μg kg^{-1} fresh vegetable. As a consequence, the calculation of salivary cotinine derived from dietary sources can be based only on dietary nicotine according to data published by Siegmund et al. (1999a).

3.2. Conversion of nicotine intake to salivary cotinine concentration

Benowitz (1996) has reviewed the use of cotinine as a biomarker for nicotine intake from environmental tobacco smoke (ETS) exposure. Although the bioavailability and pharmacokinetics of dietary nicotine may differ from that absorbed from ETS, relationships described by Benowitz (1996) provide a useful beginning to describe the conversion of ingested nicotine to salivary cotinine concentration. Benowitz (1996) has suggested that the intake of nicotine in 24 h can be related to the blood serum cotinine concentration according to Eq. (1), where C_B is the concentration of cotinine in blood.

$$\text{Intake of nicotine (mg/24-h)} = 0.08 \times C_B(\text{ng ml}^{-1}) \quad (1)$$

Curvall, Elwin, Kazemi-Valla, Warholm, and Enzell (1990) have reported that the ratio of salivary cotinine concentration (C_S) to blood cotinine concentration is approximately 1.2. Substituting this relation in Eq. (1) yields Eq. (2).

$$\text{Intake of nicotine (mg/24-h)} = 0.067 \times C_S(\text{ng ml}^{-1}) \quad (2)$$

Table 1
LOD and LOQ values for cotinine and the internal standard (IS)

Compound	LOD (pg)	LOQ (pg)	LOD (μg kg^{-1}) wm[a]	LOQ (μg kg^{-1}) wm[a]	S.D. (pg)[b]
Cotinine	15	45	0.1	0.3	6.3
IS	15	45	0.1	0.3	6.6

[a] LOD and LOQ are related to an amount of 50 g fresh fruit (wm wet mass).
[b] S.D. is the standard deviation of the *y*-intercepts of the regressions lines describing the standard deviation of the whole calibration model.

Using Eq. (2) and nicotine content and food consumption data reported by Siegmund et al. (1999a), a Monte Carlo simulation was performed to estimate the distribution of salivary cotinine concentration in saliva that results from dietary intake of nicotine.

3.3. Monte Carlo simulation

In its simplest terms, a Monte Carlo simulation makes individual random selection of values of parameters in a mathematical expression and uses these values to compute a result. The values that are selected are governed by the mean and standard deviation of the parameters, the type of distribution (normal, lognormal, etc.) that the parameters follow, and upper and lower boundaries of values, based on prior knowledge. Upon repeating these computations a large number of times, a probability distribution of the result is obtained. Because of the lack of sufficient data to determine whether the variation of the nicotine content in particular vegetables follows a normal, lognormal or any other type of distribution, a lognormal distribution was selected as appropriate for these simulations.

Fig. 2 shows the computed distribution of salivary cotinine concentration based on the analytical results and the food consumption data. The mean is 0.022 ng ml^{-1} with a standard deviation of ± 0.007 ng ml^{-1}. The 95th percentile value is 0.035 ng ml^{-1}. These values fall between the LOD and LOQ of analytical methods developed recently for the determination of salivary cotinine (Bentley et al., 1999).

A number of assumptions must be made when computing the predicted distribution of salivary cotinine. Some of these assumptions may work to cause overestimation of the salivary cotinine levels, whereas some may cause the opposite result. We have assumed the bioavailability of the dietary nicotine to be similar to that of inhaled nicotine and, consequently, conversions applied to nicotine inhaled from ETS (Benowitz, 1996). Lindell, Lunnell, and Graffner (1996) report that nicotine administered by transdermal nicotine systems collects in the acidic gastric juices of the stomach. Compton et al. (1997) have demonstrated that delayed release oral nicotine has reduced systemic bioavailability, likely resulting from first-pass hepatic metabolism rather than poor mucosal absorption. These reports suggest the possibility that the bioavailability of nicotine ingested by means other than inhalation may not be the same as inhaled nicotine. How these considerations will affect salivary cotinine levels for a given amount of nicotine ingested by different routes is not clear. However, analytical methods now exist to permit investigations of these questions.

A second limitation is the scope and validity of the food consumption data used by Siegmund et al. (1999a). These data were reported by each of the 13 European countries (TNO, 1997) or by the US Department of Agriculture (USDA, 1997). There is a possibility that information used by the individual countries is not consistent, and little information is available on the consumption of processed foods such as tomato sauce, ketchup and canned vegetables as well as for tea.

Acknowledging these limitations, the level of predicted salivary cotinine from dietary nicotine intake may be compared with that expected from exposure to ETS and to the analytical capability to detect salivary cotinine. Benowitz (1996) has reported that, with light activity breath ventilation rates (approximately 1 m^3 h^{-1}), approximately 71% of inhaled ETS nicotine is absorbed (Iwase, Aiba, & Kira, 1991). Based on personal monitoring studies in 16 US cities, Jenkins and Counts (1999) report that the median 24-h time-weighted average (TWA) exposure of subjects that are exposed to ETS in the workplace, but not away from

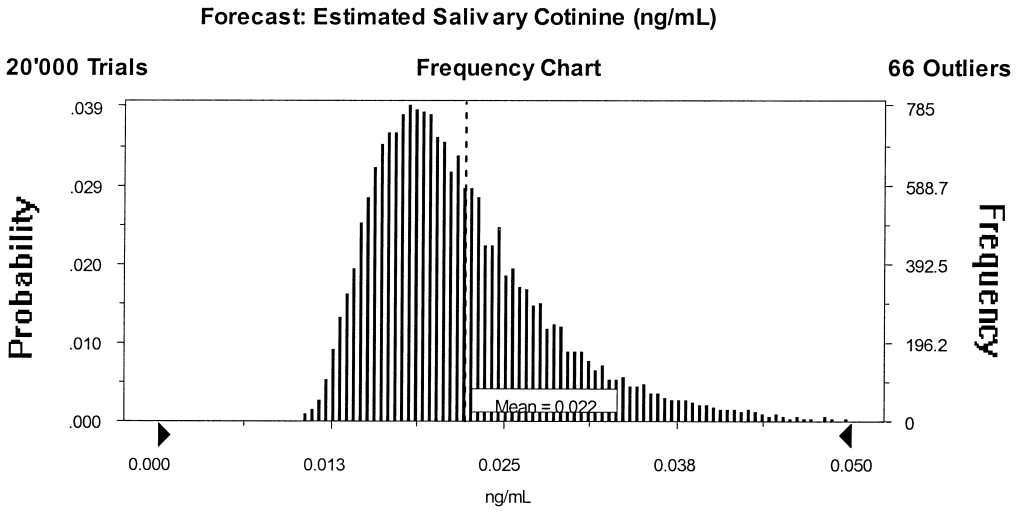

Fig. 2. Predicted distribution of salivary cotinine concentration resulting from dietary intake of nicotine.

work is 0.11 μg m^{-3}. Using these data, a median daily nicotine intake of 1.9 μg of nicotine from ETS exposure at work only is calculated. This value can be compared directly with the mean daily dietary nicotine intake of 1.4 μg reported by Siegmund et al. (1999a). Substituting daily intake of 1.9 μg into Eq. (2), an estimate of median salivary cotinine concentration of 0.028 ng ml^{-1} is obtained which can be compared with 0.022 ng ml^{-1} estimated from dietary intake. Subjects reporting ETS exposure at work and away from work had a median 24-h TWA of 1.55 μg m^{-3} (Jenkins & Counts, 1999), which corresponds to a daily intake of 26 μg. For those who report no ETS exposure at home or at work a 24-h TWA exposure of 0.03 μg m^{-3} was reported (Jenkins & Counts, 1999), which corresponds to a daily nicotine dose of 0.05 μg. This level is below that which is predicted from dietary intake of nicotine and corresponds to a predicted salivary cotinine level of <0.01 ng ml^{-1}. Jenkins and Counts (1999) report that the median of the measured saliva cotinine concentration is 9.9 times that estimated from the relationships given above.

In the past, the analytical methodology for the determination of cotinine was designed for use, principally to distinguish smokers from non-smokers. The lower limit of detection (LOD) and lower limit of quantification (LOQ) of these methods were inadequate for cotinine levels in non-smokers who were exposed to low levels of ETS. Approximately 65% of the salivary cotinine levels in one study (Jenkins, Palausky, Counts, Bayne, Dindal, & Guerin, 1996) were found to be below the typical detection limits of 0.5–1.0 ng ml^{-1}, available at the time the studies were conducted (Jenkins, R. A. 1998, personal communication). Improved methods (Bentley et al., 1999; Bernert Jr et al., 1997; Pirkle, Flegal, Bernert, Brody, Etzel, & Maurer, 1996), based on liquid chromatography coupled with atmospheric pressure mass spectrometry (LC/API/MS–MS), have lowered the LOD to approximately 0.02 ng ml^{-1} and the LOQ to approximately 0.05 ng ml^{-1}. Frozen samples, that have been analysed using the new method, show perhaps 10% that are below the new LOD (Jenkins, R. A. 1999, personal communication). The new analytical capability will provide increased opportunity to investigate the relationships between salivary cotinine concentration at low levels of ETS exposure and dietary nicotine intake.

4. Conclusions

In contrast to nicotine that was found in fresh vegetables at the μg kg^{-1} range, its main human metabolite, cotinine, could not be detected in any edible nightshades or products thereof in concentrations higher than 0.1 μg kg^{-1}. Consequently, the estimation of the influence of diet on the nicotine and/or cotinine level in biological fluids such as saliva is based only on dietary nicotine.

The calculations presented here show that dietary intake of nicotine is not significant in comparison with nicotine intake of smokers or persons who are exposed to high levels of environmental tobacco smoke (ETS). However, for those with confirmed exposure to low levels of ETS, dietary nicotine is a contributing factor to salivary cotinine levels. To fully understand the metabolism and disposition of dietary nicotine, the use of improved analytical methodology with detection and quantification in a range below 0.05 ng ml^{-1} is required.

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