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RESEARCH ARTICLE

Analysis of myosmine, cotinine and nicotine in human toenail, plasma and saliva

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Abstract

Myosmine is a minor tobacco alkaloid with widespread occurrence in the human diet. Myosmine is genotoxic in human cells and is readily nitrosated and peroxidated yielding reactive intermediates with carcinogenic potential. For biomonitoring of short-term and long-term exposure, analytical methods were established for determination of myosmine together with nicotine and cotinine in plasma, saliva and toenail by gas chromatography–mass spectrometry (GC/MS). Validation of the method with samples of 14 smokers and 10 non-smokers showed smoking-dependent differences of myosmine in toenails (66 ± 56 vs 21 ± 15 ng g^{-1}, $p < 0.01$) as well as saliva (2.54 ± 2.68 vs 0.73 ± 0.65 ng ml^{-1}, $p < 0.01$). However, these differences were much smaller than those with nicotine (1971 ± 818 vs 132 ± 82 ng g^{-1}, $p < 0.0001$) and cotinine (1237 ± 818 vs < 35 ng g^{-1}) in toenail and those of cotinine (97.43 ± 84.54 vs 1.85 ± 4.50 ng ml^{-1}, $p < 0.0001$) in saliva. These results were confirmed in plasma samples from 84 patients undergoing gastro-oesophageal endoscopy. Differences between 25 smokers and 59 non-smokers are again much lower for myosmine (0.30 ± 0.35 vs 0.16 ± 0.18 ng ml^{-1}, $p < 0.05$) than for cotinine (54.67 ± 29.63 vs 0.61 ± 1.82 ng ml^{-1}, $p < 0.0001$). In conclusion, sources other than tobacco contribute considerably to the human body burden of myosmine.

Keywords: *Myosmine; cotinine; nicotine; toenail; plasma; saliva*

Introduction

The tobacco alkaloid 3-(1-pyrroline-2-yl)pyridine was discovered in 1933 by Wenusch and Schöller (1933) in cigar smoke. Because of its intensive smell reminding of mice it was given the trivial name myosmine. For a long time, myosmine was thought to be as specific for tobacco as nicotine. In mainstream smoke it is present at 13–33 μg per plain non-filter cigarette, about 50–100 times less than nicotine (Baker 1999). The ratio is lower in environmental tobacco smoke (ETS) with an estimated maximal 24-h time-averaged exposure in heavily polluted environments of about 20 μg myosmine, 12 times less than nicotine (Nelson et al. 1998). Although previously detected in some nicotine-containing plants other than tobacco (Smalberger et al. 1968, Halim et al. 1971, Luanratana & Griffin 1982) it was only after the detection of myosmine in nuts and many other edible plants by Zwickenpflug and co-workers (Zwickenpflug et al. 1998, Tyroller et al. 2002) that myosmine has attracted interest as an alkaloid occurring in nature independently from other tobacco alkaloids. The uptake of myosmine from these sources was estimated to be in the range of a few μg/day. However, preliminary results from our group showed the presence of myosmine in plasma and saliva from non-smokers in concentrations up to 5 ng ml^{-1} (Maier et al. 2005, Maier 2005). In view of similar toxicokinetics of myosmine and nicotine in rats (Kyerematen et al. 1988, Zwickenpflug et al. 2005, Glas et al. 2007) and assuming similar conditions in humans (Dempsey et al. 2004, Hukkanen et al. 2005), these myosmine concentrations in human saliva are not compatible with an uptake of only a few micrograms of myosmine.

The purpose of the present study was to establish biomarkers for the determination of human myosmine uptake. Metabolism studies have shown that only a very minor urinary metabolite, 3'-hydroxymyosmine, could

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be specific for myosmine. In Wistar rats this metabolite accounts for less than 3% of the dose and the extent of formation in humans is unknown (Zwickenpflug et al. 2005). The suitability of plasma and saliva as a short-term biomarker of myosmine has been demonstrated in a preliminary study (Maier et al. 2005). Recently, nicotine, cotinine and tobacco-specific nitrosamines (TSNA) in toenail have been proposed as a long-term marker for biomonitoring of human exposure to tobacco smoke (Al-Delaimy et al. 2002, 2008, Al-Delaimy & Willett 2008, Stepanov et al. 2006, 2007, Stepanov & Hecht 2008). Analysis of toenail has the advantage over hair as it is independent of melanin (Richter et al. 2007). Due to the slow growth rate of toenail of ~0.1 cm per month, concentrations of tobacco alkaloids in toenail cuttings reflects the average exposure over more than a month (Palmeri et al. 2000).

Existing analytical methods for the determination of nicotine and cotinine in plasma, saliva and toenails (Moyer et al. 2002, Stepanov et al. 2006) were adapted for the determination of myosmine. For the control of tobacco-dependent uptake of myosmine, human saliva and plasma were analysed additionally for cotinine, and samples of toenail for cotinine and nicotine.

Material and methods

Chemicals

The tobacco alkaloids myosmine, cotinine and nicotine (Figure 1) and their associated deuterium-labelled standards d_4-myosmine, d_3-nicotine, and d_3-cotinine were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Keratin powder was from MP Biomedicals Europe (Illkirch, France). Solvents were of gas chromatography (GC) quality (Suprasolv; Merck, Darmstadt, Germany) and water was from a $MilliQ_{PLUS}$ system (Millipore, Königsstein, Germany). All other chemicals were of analytical grade and purchased from Merck.

Extraction of myosmine, cotinine and nicotine from toenail

Toenails were digested as described by Stepanov et al. (2006) (Figure 2). Briefly, 20–30 mg of toenail clippings were weighed into 2.0 ml Eppendorf cups, washed for 2 h in 1.5 ml CH_2Cl_2 at room temperature and, after removal of CH_2Cl_2, digested at 50°C overnight in 0.5 ml 1 N NaOH. After addition of 8 µl each of the internal standards d_4-myosmine (0.5 ng µl^{-1}), d_3-cotinine (1.35 ng µl^{-1}) and d_3-nicotine (1.0 ng µl^{-1}), the analytes were extracted three times with 0.5 ml CH_2Cl_2. The combined extracts were dried over Na_2SO_4, reduced under N_2 to ≈100 µl and, after addition of 1 ml hexane, reduced under N_2 to a final volume of 20 µl. Aliquots of 1 µl were injected into the GC/mass spectrometer (MS).

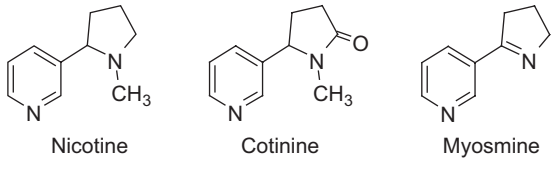

Figure 1. Structures of compounds analysed in human toenails.

Extraction of myosmine, cotinine and nicotine from plasma and saliva

Aliquots of 2–3 ml of saliva and plasma were subjected to solid-phase extraction (SPE) on Strata X-C columns (Cation Mixed Polymer 60 mg/3 ml; Phenomenex, Aschaffenburg, Germany) (Figure 2). The columns were conditioned on a vacuum manifold with 3 × 2 ml of elution medium (56 ml methanol + 20 ml 25% ammonia and 24 ml H_2O), 3 ml of methanol and 3 ml of 0.1 M acetic acid. After addition of 8 µl each of the internal standards d_4-myosmine (0.5 ng µl^{-1}) and d_3-cotinine (1.35 ng µl^{-1}) and 4–6 ml of acetic acid, the samples were applied to the SPE column. The column was washed with 3 ml 1.0 M HCl and twice with 3 ml methanol before drying under vacuum. The analytes were extracted from the SPE column with 2 ml of elution medium. After addition of 100 µl 4 M NaOH, the samples were reduced under N_2, at 50°C for 15 min. Concentrated extracts were transferred into Eppendorf cups and extracted three

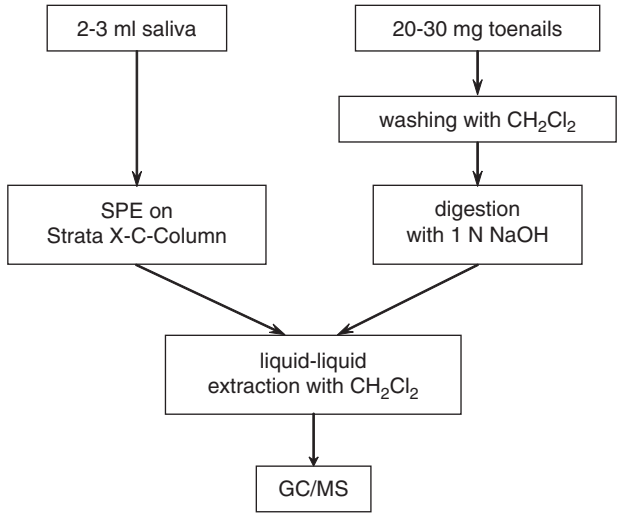

Figure 2. Procedure for myosmine, nicotine and cotinine analysis in human toenails and for myosmine and cotinine in saliva. For details see Materials and methods. SPE, solid-phase extraction; GC, gas chromatography; MS, mass spectrometry.

times with 0.5 ml CH_2Cl_2, and reduced to a final volume of 20 µl as described for toenail analysis.

Determination of myosmine, cotinine and nicotine by GC/MS

Analysis of myosmine, cotinine and nicotine by GC/MS with selected-ion monitoring (EI^+) was performed with a model TRIO 1000 Benchtop GC/MS (Fisons Instruments, Mainz-Kastel, Germany). The samples were injected in the splitless mode on a DB5-MS (30 m × 0.250 mm, 0.25 µm film thickness) capillary column (J&W Scientific, Folsom, CA, USA) operated with helium as carrier gas at a pressure of 80 kPa. The temperatures of the injection port and the source were set to 200°C, and the transfer line to 220°C. The temperature programme for the column oven was: 50°C for 1 min, then an increase of 7.5°C min^{-1}, 170°C followed by an increase of 15°C min^{-1} to 300°C, which was then kept constant for 5 min. Selective-ion monitoring was in the EI^+ mode at 70 eV with 84 *m/z* and 87 *m/z* for nicotine and the internal standard d_3-nicotine, 176 *m/z* and 179 *m/z* for cotinine and d_3-cotinine and at last 118 *m/z* and 122 *m/z* for myosmine and the internal standard d_4-myosmine, respectively.

Subjects

Ten non-smokers and 14 smokers were recruited for analysis of toenail and saliva. Toenails were collected from both feet of each subject and cut into small segments. Saliva was collected using salivettes (Sarstedt, Nümbrecht, Germany). All samples were stored frozen in polypropylene tubes at -20°C. Plasma samples were obtained in the early morning from 84 fasting patients undergoing gastro-oesophageal endoscopy at the Department of Surgery, Klinikum rechts der Isar, Technical University Munich, Germany. Patients gave written consent and filled in a detailed questionnaire on sociodemographic data, dietary habits, drinking and smoking in the course of a multicentre study 'Basic Research on Risk-adapted Therapy of Barrett Carcinoma' approved by the local ethics committee.

Statistical analyses

The results are presented as means ± SD. For samples below the limit of quantification (LOQ) a value of half of the LOQ was taken whereas samples below the limit of detection (LOD) were calculated as zero values. With the exception of nicotine concentrations all values were non-normally distributed as determined by the Shapiro–Wilk test for normality. Therefore, statistical analyses of differences between smokers and non-smokers were performed by the Mann–Whitney test. Correlations were analysed with the Spearman rank test. *p*-Values were two-sided. All data analyses were performed using Prism 4 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results

Validation of analytical methods for saliva and toenail

Calf serum and keratin powder were used as sample matrices for plasma/saliva and toenail, respectively. For determination of the LOD in plasma/saliva, calf serum was spiked with 0.01–2.0 ng ml^{-1} of myosmine and 0.2–3 ng ml^{-1} of cotinine, whereas keratin powder was spiked with 1.0–2000 ng mg^{-1} of myosmine and 20–5000 ng mg^{-1} of cotinine and nicotine. The LOD was calculated from the calibration lines according to the German standard method DIN 32645 (Anon 1994) resulting in LOD for myosmine, and cotinine in serum/saliva of 0.0012 and 0.05 ng ml^{-1}, respectively, and myosmine, nicotine and cotinine in toenail of 10, 20 and 35 ng g^{-1}, respectively. The LOQ was specified with all three analytes as the triple value of the LOD. For values below the LOD, a value of zero was used. For values within the LOQ and the LOD, half of the LOQ was used. In the same series of analyses recovery of the internal standards were 91–93% from serum and 97–102% from keratin.

For determination of precision under repeated conditions, pooled samples of toenail from non-smokers were homogenized by fine grinding. Six samples each of pooled toenail and pooled saliva were analysed either within 1 day (intraday precision) or once a week (between-day precision). The percentage SD (intraday/between-day) was 14.5/8.0, 13.3/8.7 and 4.4/8.0 for myosmine, cotinine and nicotine in toenail, respectively, and 14.8/17.9 and 7.5/3.7 for myosmine and cotinine in saliva.

Analysis of toenail and saliva from smokers and non-smokers

The analytical method for toenail and saliva was tested in a small pilot study with samples of 10 non-smokers and 14 smokers (Table 1). In toenail from smokers, all three alkaloids could be determined. Only two toenail samples had myosmine concentrations below the LOQ, but above the LOD. In toenail from non-smokers, myosmine was not detectable in one sample and below the LOQ in six samples. Whereas cotinine was not detectable in toenail from non-smokers, nicotine was detectable in 8 of 10 samples. In saliva, myosmine was detectable in all samples from smokers and non-smokers and cotinine in all smokers but in only three samples from non-smokers.

Concentrations of myosmine were significantly higher in toenail samples from 14 smokers (66 ± 56 ng g^{-1}) compared with 10 non-smokers (21 ± 15 ng g^{-1}, p <0.01). This 3.1-fold difference in myosmine concentrations between smokers and non-smokers was clearly less than the 15-fold difference in nicotine concentrations (1 971 ± 818 vs 132 ± 82 ng g^{-1}, p <0.0001) and less than the 35-fold difference in cotinine which was detectable only in toenail of smokers (1237 ± 818 vs < 35 ng g^{-1}).

In saliva samples taken in parallel with toenail, the 3.5-fold difference between 14 smokers and 10 non-smokers was again smaller for myosmine (2.54 ± 2.68 vs 0.73 ± 0.65 ng ml^{-1} p <0.01) than the 52-fold difference for cotinine (83.14 ± 54.30 vs 1.85 ± 4.50 ng ml^{-1}, p <0.0001).

Analysis of plasma from smokers and non-smokers undergoing gastro-oesophageal endoscopy

In the course of clinically indicated gastro-oesophageal endoscopy, plasma was obtained in the morning from 84 fasted patients, 44 males and 40 females. For definition of the actual smoking status a cut-off value of 10 ng cotinine ml^{-1} plasma was applied (Tricker 2006) giving 25 smokers, 15 men and 10 women. The results are summarized in Table 2. Myosmine was below the detection limit in 10 of 59 non-smokers and in only one of 25 smokers. The differences between smokers and non-smokers were again much smaller for myosmine (0.296 ± 0.349 vs 0.155 ± 0.183 ng ml^{-1}, 1.9-fold, p < 0.05) than for cotinine (54.67 ± 29.63 vs 0.61 ± 1.82 ng ml^{-1}, 90-fold, p < 0.0001). There was no significant correlation between the concentrations of myosmine and cotinine in plasma (Spearman's r = 0.1249, p = 0.258). Male subjects had about 2-fold higher concentrations of myosmine in plasma compared with female subjects (significant in the whole group and in non-smokers, p<0.01). Frequent drinking of alcohol, more than two drinks a week, increased the plasma concentrations of myosmine by more than 2-fold (significant in the whole group, p < 0.01, and in non-smokers, p < 0.05). Frequent drinking was more prevalent in men than in women (42% vs 13%). However, gender differences of myosmine in plasma remained significant in infrequent drinkers, 0.170 ± 0.172 ng ml^{-1} in 22 men compared with 0.121 ± 0.192 ng ml^{-1} in 34 women (p <0.05).

Discussion

Nicotine concentrations in toenail of smokers and non-smokers from the present study are in good agreement with the results reported by Al-Delaimy et al. (2002, 2008) and somewhat lower than the concentrations reported for smokers by Stepanov et al. (2006, 2007). Cotinine concentrations in toenail of smokers in this study are also within the range reported in the literature (Stepanov et al. 2006, 2007). According to the results of these two groups, tobacco alkaloids in toenail are suitable long-term markers of smoke exposure. The concentrations of nicotine and cotinine correlate well with other specific biomarkers for smoking such as the tobacco-specific nitrosamines 4(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and *N'*-nitrosonornicotine (NNN) in toenail as well as NNAL and its glucuronides in urine and cotinine and *trans*-3'-hydroxycotinine in plasma (Stepanov et al. 2006, 2007, Stepanov & Hecht 2008). Toenail nicotine levels have also been shown to be a good predictor of coronary heart disease in the Nurse's Health study (Al-Delaimy et al. 2008) and to provide important additional information on tobacco smoke

Table 1. Concentrations of tobacco alkaloids in human toenails and saliva.

	Toenails (ng g^{-1})			Saliva (ng ml^{-1})	
	Myosmine	Cotinine	Nicotine	Myosmine	Cotinine
Non-smoker (n = 10)	21.2 ± 14.9	n.d.	132 ± 82	0.73 ± 0.65	1.85 ± 4.50
Smoker (n = 14)	65.9 ± 56.4[a]	1237 ± 853	1971 ± 818[b]	2.54 ± 2.68[a]	97.43 ± 84.54[b]

Data were analysed by the Mann–Whitney test, presented as mean ± standard deviation; n.d., not detected.
[a,b]Significantly different from non-smokers, p <0.01[a] and p <0.0001[b].

Table 2. Concentrations of myosmine and cotinine in human plasma.

	Myosmine (ng ml^{-1})			Cotinine (ng ml^{-1})		
	All	Non-smoker	Smoker	All	Non-smoker	Smoker
All	84, 0.197 ± 0.251	59, 0.155 ± 0.183	25, 0.296 ± 0.349[b]	84, 16.70 ± 29.57	59, 0.609 ± 1.824	25, 54.67 ± 29.63[c]
Male	44, 0.259 ± 0.285	29, 0.208 ± 0.207	15, 0.358 ± 0.385	44, 18.46 ± 31.51	29, 0.553 ± 1.655	15, 53.09 ± 33.10[c]
Female	40, 0.129 ± 0.187[d]	30, 0.104 ± 0.141[d]	10, 0.204 ± 0.280	40, 14.76 ± 27.55	30, 0.663 ± 2.002	10, 57.04 ± 25.03[c]
Frequent alcohol[a]	21, 0.336 ± 0.318	14, 0.287 ± 0.207	7, 0.433 ± 0.478	21, 20.39 ± 38.15	14, n.d.	7, 61.17 ± 44.07
Infrequent alcohol	56, 0.140 ± 0.184[f]	41, 0.122 ± 0.161[e]	15, 0.192 ± 0.235	56, 14.27 ± 25.53	41, 0.877 ± 2.142	15, 50.88 ± 24.23[c]

Data were analysed by the Mann–Whitney test, presented as n, mean ± standard deviation; n.d., not detected.
[a]More than two units of beer/wine/liquor a week; [b,c]significantly different from non-smokers, p <0.05[b] and p <0.0001[c]; [d]significantly different from males, p <0.01; [e,f]significantly different from frequent drinkers, p <0.05[e] and p <0.01[f].

exposure not captured by reported history (Al-Delaimy & Willett 2008).

In the present study we extended the determination of tobacco alkaloids in toenail to myosmine. The results clearly show that myosmine is not as specific for tobacco smoke exposure as cotinine and nicotine. This is further supported by our results for saliva and plasma. In all three matrices the difference between myosmine concentrations in smokers and non-smokers was below 3.5-fold (Tables 1 and 2) and considerably lower than the differences for nicotine (15-fold in toenail) and cotinine (about 50-fold in toenail and saliva and 90-fold in plasma). However, concentrations in saliva and plasma may be less reliable markers of myosmine exposure than toenail. Myosmine has a short half-life of about 1 h in the plasma of rats (Glas et al. 2007) similar to nicotine (Kyerematen et al. 1988). Therefore, large intra- and interindividual variations are to be expected in human saliva and plasma.

Myosmine concentrations were about 30-fold higher in toenail compared with simultaneously analysed saliva (Table 1). Therefore, myosmine can be reliably determined in much smaller samples of toenail compared with saliva or plasma. Similar to saliva and in contrast to plasma or serum, toenails can be obtained non-invasively and are easier to store than larger samples of body fluids. The analytical procedure for toenail is simpler and does not require additional clean-up by solid– or liquid–liquid extraction (Figure 2). Although the mechanism of alkaloid accumulation in the keratin matrix of nail is not well understood (Palmeri et al. 2000), nail may be superior to hair because one major confounder, melanin pigmentation of the hair is lacking in nail. Taking toenail instead of fingernail or hair also largely eliminates the problem of external contamination.

For this study plasma samples were obtained in the course of an investigation on myosmine as a risk factor for oesophageal carcinogenesis (Heppel et al. 2009). Myosmine is rapidly nitrosated under simulated gastric conditions where it forms not only the TSNA NNN (Zwickenpflug 2000, Wilp et al. 2002), an oesophageal carcinogen in rats (Hecht 1998) which is classified as 'carcinogenic to humans' by the International Agency for Research on Cancer (IARC 2007), but to an even greater extent, a reactive intermediate giving rise to pyridyloxobutylation (POB) and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) after reaction with water. HPB is also a major product of myosmine peroxidation (Zwickenpflug & Tyroller 2006). POB DNA adducts formed after metabolic activation of NNN and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are proposed to account for the carcinogenic activity of these TSNA (Hecht 2003). However, in biomonitoring studies neither POB haemoglobin nor DNA adducts releasing 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) after alkaline or acid hydrolysis, respectively, showed the expected specificity for exposure to TSNA from tobacco (Carmella et al. 1990, Falter et al. 1994, Branner et al. 1998, Atawodi et al. 1998, Hölzle et al. 2007, Schlöbe et al. 2008, Heppel et al. 2009).

The genotoxic potential of myosmine was confirmed by detection of DNA damage in the comet assay using freshly isolated human lymphocytes and nasal mucosa cells (Kleinsasser et al. 2003) as well as human oesophageal adenocarcinoma cell lines (Vogt et al. 2006). Myosmine is also mutagenic in human lymphocytes in the HPRT gene mutation assay (Havla et al. 2009). Under nitrosation conditions myosmine showed covalent binding with DNA *in vitro* (Wilp et al. 2002). A preliminary study with rats showed increased *in vivo* formation of HPB-releasing DNA and haemoglobin adducts by dietary myosmine (Richter et al. 2002). These findings suggest that myosmine should be seen not only as a minor tobacco alkaloid but also as a possible tobacco-independent dietary carcinogenic risk factor. The presence of high concentrations of POB DNA adducts in the mucosae of human oesophagus and cardia close to the gastro-oesophageal junction which are independent of smoking status (Schlöbe et al. 2008, Heppel et al. 2009) led us to hypothesize that myosmine could play a role in the aetiology of tumours at this location for several reasons. Firstly, adenocarcinoma of the gastro-oesophageal junction have shown a steep rise in incidence in Western industrialized countries while smoking has declined over the same time period (Bollschweiler et al. 2001). Secondly, the incidence of tumours is higher in males than in females (Cook et al. 2005) and this may be attributed to the male type of adipositas which is rising in these countries (Edelstein et al. 2007). Thirdly, endogenous nitrosation mediated by both acid catalysis and inflammatory responses which is thought to play an important role is especially effective within the gastro-oesophageal junction and therefore myosmine in addition to bile acids may be an important initiator of carcinogenesis (Iijima et al. 2003, Terasaki et al. 2008, Kuroiwa et al. 2008). In this context it is an important finding of this study that the concentration of myosmine is higher in men compared with women.

In conclusion, an analytical method has been established for simultaneous determination of myosmine, cotinine and nicotine in toenail, saliva and plasma from smokers and non-smokers. Results of a pilot study confirm the suitability of toenail for biomonitoring of long-term exposure to tobacco alkaloids. Much smaller differences of myosmine compared with nicotine and cotinine concentrations in toenail, saliva and plasma in dependence of smoking status confirm the importance of sources other than tobacco for human myosmine exposure.

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