Occurrence of the Tobacco Alkaloid Myosmine in Nuts and Nut Products of *Arachus hypogaea* and *Corylus avellana*

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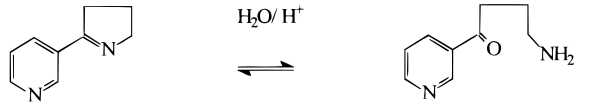
Myosmine is one of the major tobacco-specific pyridine-containing alkaloids. It is also a metabolite of the tobacco-specific nitrosamine *N*-nitrosonornicotine. In the present study it is shown that the occurrence of myosmine is not restricted to the tobacco plant. Using a newly developed method for extraction and quantitative analysis, myosmine was detected in samples of peanuts *(Arachus hypogaea)* and hazelnuts *(Corylus avellana)* in ng/g amounts. Therefore, consumption of nuts and nut products is a new as yet unknown source for human myosmine exposure. Investigations of the toxicological impact of myosmine uptake by humans are warranted.

Keywords: *Myosmine; tobacco alkaloid; peanut, hazelnut; GC-MS analysis*

INTRODUCTION

Myosmine, 3-(1-pyrroline-2-yl)pyridine, is a pyridine ring-containing tobacco alkaloid (Figure 1). Späth and Bretschneider (1928) detected 4′,5′-dihydronornicotyrine besides nicotine, when they reduced nicotyrine with Zn/HCl. This compound, commonly named myosmine, was also generated by dehydrogenation of nicotine at 570 °C over quartz (Woodward et al., 1944) and was one of the first structurally identified tobacco alkaloids besides nicotine in tobacco smoke. In samples of tobacco (*Nicotiana tabacum*) the occurrence of 0.09−0.14% myosmine was reported (Sisler, 1969; Tso and Jeffrey, 1953). Kisaki and Tamaki (1966) detected the conversion of nornicotine to myosmine in the plant species *Nicotiana glutinosa*. Mainstream and sidestream smoke of cigarettes from different tobacco varieties contained 13−33 and 73−224 μg of myosmine/cigarette (Sakuma et al., 1984). In a recent market cigarette study myosmine was detected in environmental tobacco smoke (ETS) of 50 different brands at 5.4 ± 0.8 $\mu g/m^{-3}$, with little variation according to tar and nicotine content (Martin et al., 1997). Furthermore, myosmine was detected as a major metabolite of *N*-nitrosonornicotine (NNN) in rat liver microsomes and a minor metabolite in rat urine by Chen et al. (1978).

Although small amounts of myosmine were detected in the drug-yielding plant *Duboisia hopwoodii* used by Australian aborigines (Luanratana and Griffin, 1982), up to now human uptake of myosmine was thought to be limited mostly to tobacco use. However, in the course of our analytical studies on tobacco-specific *N*-nitrosamines (TSNA; Kutzer et al. 1997), we detected myosmine in hazelnuts and peanuts using a newly developed analytical method comprising extraction, enrichment by solid-phase extraction, and identification of myosmine by GC-MS or HPLC with diode array detection (DAD).



3-(1-pyrroline-2-yl)-pyridine 4-amino-1-(3-pyridyl)-1-butanone

Figure 1. Reversible hydrolysis of myosmine under acidic conditions.

MATERIALS AND METHODS

All chemicals for synthesis of myosmine were obtained from Sigma-Aldrich (Deisenhofen, Germany). Organic solvents were of analytical grade and purchased from Merck (Darmstadt, Germany). Untreated nut samples without shell were purchased from the local market. All other samples were from commercial brands purchased at retail in 1997.

Synthesis of Myosmine. Myosmine was synthesized in accordance to the method of Brandänge and Lindblom (1976). Briefly, ethyl nicotinate (0.09 mol) and *N*-vinylpyrrolidone (0.09 mol) were added dropwise to a solution of sodium hydride (0.13 mol, 5 g of a 60% mineral oil suspension) in dry toluene (75 mL). After 1.5 h of refluxing, the solution was treated with 140 mL of a mixture of H_2O/HCl concentrated (50:90, v/v) and adjusted with 10 N NaOH at pH 4. After separation from the toluene phase, the water phase was extracted 3-fold with $CHCl_3$/EtOH (3:2, v/v) and dried over anhydrous Na_2SO_4. After concentration, the residue was treated with 125 mL of concentrated HCl and refluxed for 14 h. The reaction product was distilled under vacuum and yielded caramel colored myosmine. The physicochemical parameters of the product were in accordance with values reported in the literature: mp, 39−42 °C; UV, λ_{max} 234 nm; IR, 1618 cm^{-1} (Korte and Schulze-Steinen, 1962); MS, *m*/*z* 146, 70% (M^+), *m*/*z* 118, 100%, *m*/*z* 105, 30%, *m*/*z* 78, 20%, *m*/*z* 51, 20% (Duffield et al., 1965; Glenn and Edwards, 1978).

Preparation and Purification of the Nut Extracts. Samples of nut material, 80−100 g, were moistened with 5−10 mL of H_2O and ground with mortar and pestle. After addition of 50 mL of 6 N H_2SO_4 the sample was stirred for 15−20 min. Subsequently, 80−120 mL of H_2O was added and the sample stirred for 10 h. After filtration of the slurry (No. $579^{1/2}$, Schleicher and Schüll) the solution was adjusted to pH 6.5−7 with 6 N NaOH and sonicated for 3 min. The extract was centrifuged at 2500 rpm for 10 min and the supernatant transferred on a preconditioned 3 mL C_{18} solid-phase extrac-

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tion tube with stainless steel frits and 500 mg of sorbent material 3CC/500 (Varian GmbH, Darmstadt, Germany). The extraction tube was washed with 3 volumes of H_2O, and after being dried by centrifugation at 1500 rpm, the tube was eluted with 4 × 250 μL $CHCl_3$/MeOH (3:2, v/v). The solution was concentrated in a vacuum centrifugal concentrator (Bachofer, Reutlingen, Germany) for analysis by GC-MS, HPLC, or TLC.

In some cases the filtered slurry was chromatographed prior to neutralization over an ion-exchange resin [Dowex 50 WX 4-50 H^+-form (Aldrich)]. The sample solution was poured in a 5 × 300 mm glass column containing 15 g of the conditioned (6 N HCl) resin. After washing of the resin with 200 mL of H_2O, the column was eluted with 6 N HCl. The sample solution was then neutralized and worked up as described above by sonication and solid-phase extraction (SPE).

Instrumentation. TLC separation was executed on analytical or preparative Kieselgel F254 plates (Merck) using $CHCl_3$/MeOH (9:1, v/v). The relevant spots were removed from the plates, eluted with the same solvent, and analyzed further by HPLC or GC-MS.

HPLC was performed on a Gynkotek HPLC-DAD system using a LiChrospher 100 RP-18 125 × 4 mm 5 μ column equipped with a 4 × 4 mm 5 μ precolumn (Merck) at a flow rate of 0.7 mL/min with a gradient using methanol and 0.05 M ammonium acetate buffer. After an initial hold for 3 min at 0% MeOH/100% buffer, methanol was linearly increased over 33 min up to 55%. Within 1 min the eluent was returned back to 0% MeOH/100% buffer and the column reconditioned for at least 5 min. The operating wavelength of the diode array system was adjusted to 270 nm with a bandwidth of 140 nm (200−340 nm). The chromatograms were recorded at an actual wavelength range of 224 and 235 nm.

Quantitation of myosmine was performed in the electron impact mode at 70 eV on a HP 5890 Series II gas chromatograph coupled to a HP 5972 quadrupole mass selective detector (Hewlett-Packard). The samples were separated on a BPX5 capillary column (60 m × 0.22 mm i.d., 0.25 μm film thickness) from SGE (Weiterstadt, Germany). The temperature program was started at 50 °C, held for 1 min, and then raised at 10 °C/min to 300 °C, where it was held for 20 min. The temperatures of the injection port, transfer line, and ion source were held at 250, 300, and 180 °C, respectively. Helium was used as the carrier gas in the constant flow mode with a head pressure of 20 kPa. The acquisition in the scan mode was executed between 50 and 250 amu. In the SIM mode the masses *m/z* 118 and 146 were recorded.

Rate of Recovery. The recovery was determined with the addition method by spiking sample material with known amounts of myosmine. Six samples of 70−80 g from one peanut charge were worked up as described above. Before addition of H_2SO_4 to the ground samples, 4.8 μg of myosmine was added to three samples; the other three samples served as unspiked controls. The recovered amount of myosmine in the spiked samples was counterbalanced to the control sample in the external standardization mode.

RESULTS

The calibration curve for the quantification of myosmine by GC-MS was linear over a range from 47 to 6000 pg with a correlation coefficient of 0.997. Figure 2 shows GC-MS chromatograms and scans of a myosmine standard and a peanut extract. Myosmine was readily detected in the SIM mode with a retention time of 24.4 ± 0.1 min. In the scan mode the relative abundancy of the characteristic mass fragments *m/z* 146 and 118 were identical in the standard and the sample extract (Figure 3). The detection limit in the SIM mode was 100 ± 15 pg, and the rate of recovery from peanut matrix was 89 ± 5%. Due to the lower limit of detection in HPLC-DAD, 50 ± 10 ng, compared to GC-MS, the determination of myosmine by HPLC is possible only with larger sample amounts. However, other unknown substances

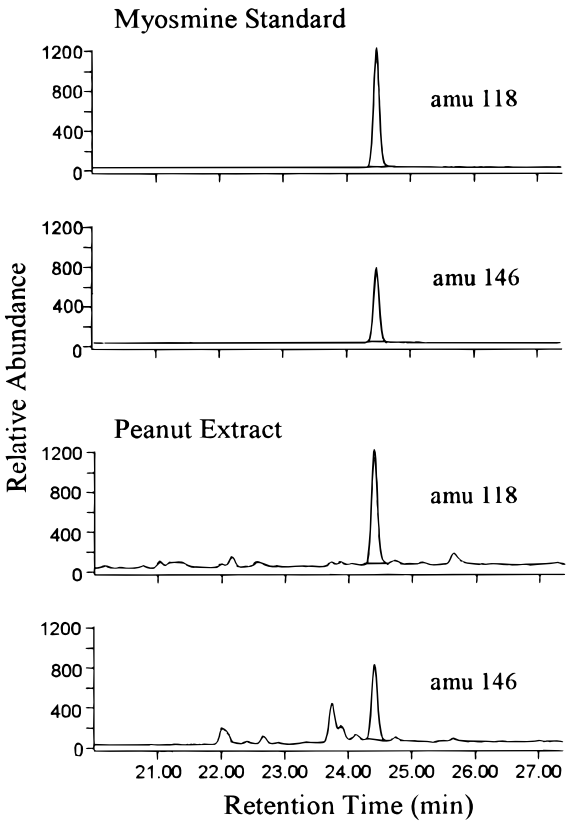

Figure 2. GC-MS chromatograms of myosmine standard (upper trace) and peanut sample extract (lower trace). In the SIM mode the significant ions *m/z* 118 and 146 indicating myosmine were detected at a retention time of 24.4 min.

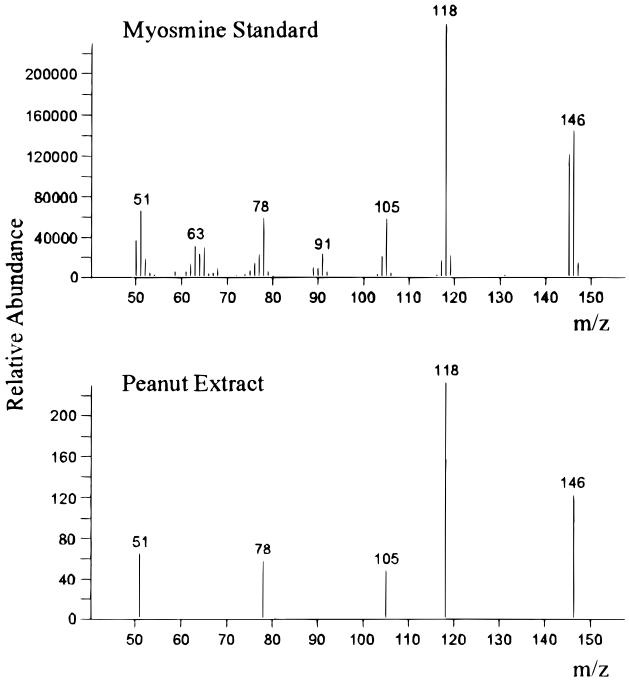

Figure 3. Mass spectrum of myosmine standard (upper trace) and peanut sample extract (lower trace) representing the relevant ions *m/z* 51, 78, 105, 118, and 146.

eluted from the HPLC column in the retention time window of 34 ± 2 min (data not shown).

Table 1 shows the amounts of myosmine found in eight different nut samples. Myosmine levels ranged from 0.2 to 2.0 ng/g. The highest concentration of

Table 1. Myosmine in Different Brands of Nut Samples

sample no.	sample description	wt of sample (g)	myosmine concn (ng/g)
I	peanut, roasted (oil), salted	100	0.3
II	hazelnut, untreated	100	0.7
III	peanut, untreated	80	0.4
IV	peanut, roasted (oil), salted	100	1.8
V	peanut, roasted (oil), salted	100	0.8
VI	peanut, roasted (oil), salted	100	0.2
VII	peanut, roasted	100	2.0
VIII	peanut, roasted, salted	100	0.4

myosmine was present in a unsalted peanut sample roasted without oil.

DISCUSSION

The present analytical method is based on the conversion of myosmine into its amino ketone form by ring opening under acidic conditions as shown in Figure 1. The pyrrolidine ring opening of nicotine derivatives has been the subject of intense studies throughout many years. Blau (1893) and Pinner (1893) were the first to postulate octahydronicotine derivatives with opened pyrrolidine rings in the course of their studies on reduction of nicotine with sodium in alcoholic solution and on bromination of nicotine, respectively. Brandänge and Rodriguez (1983) definitely confirmed the ring−chain tautomerism of myosmine using ^{1}H and ^{13}C NMR. The first analytical application of the amino ketone from myosmine was reported by Sisler (1969) using SCX exchange chromatography in acidic milieu to extract the compound from tobacco plant samples. However, this method is not applicable to nut samples with its totally different biological matrix including a lot of lipophilic substances. After conversion to the amino ketone form myosmine is transferred into the acidic aqueous phase and can be separated from the bulk of lipophilic matrix by simple filtration of the slurry. Reconversion of myosmine to its lipophilic imino form by adjustment of the extraction solution to pH 7 allows further cleanup by SPE. Triglycerides, fatty acids, and etheric oils not separated from myosmine by filtration potentially reduce the retention capacity of RP-18 SPE columns for myosmine. However, using up to 100 g of nut material the present method allows nearly quantitative recovery, 89 ± 5%, of added myosmine by GC-MS. Traces of the relevant ions at *m/z* 118 and 146 did not show any compounds interfering with myosmine. Therefore, additional cleanup by SCX and/or HPLC fractionation which was tested during method development proved to be not necessary for determination of myosmine by GC-MS. However, these methods may be useful when larger quantities of nut material have to be analyzed by the less specific and less sensitive HPLC-DAD method.

The origin of myosmine in nuts remains speculative. With the analytical method used, nicotine and nornicotine, possible precursors of myosmine (Bush et al. 1993), were not detected in nut extracts (data not shown). Therefore, consumption of nut and nut products may be an important source of myosmine distinct from nicotine, which has been detected in small amounts in several foods including eggplants, potatoes, and tomatoes. However, Benowitz (1996) in his recent review concluded that food represents an insignificant source of nicotine exposure in nonsmokers as compared to the involuntary uptake of the tobacco alkaloid with environmental tobacco smoke (ETS). This may not be true for myosmine. No direct measurements of myosmine uptake by humans are available. From the 24 h time weighted average airborne concentrations reported by Jenkins et al. (1996) ranging between 0.004 and 0.185 μg myosmine/m^3 a daily myosmine uptake of 0.01−2.2 μg can be estimated for nonsmokers with low or high ETS exposure, assuming a typical ventilation rate for an adult during light activity of 1 m^3/h and a 71% absorption rate equal to nicotine (Benowitz, 1996). According to our results consumption of 250 g of peanuts or hazelnuts would result in a comparable myosmine uptake of 0.05−0.5 μg. Furthermore, the uptake of myosmine from other food sources, e.g. peanut butter, chocolate products, or nut oil based food additives, should be taken into account.

The metabolism of myosmine was studied by Hecht et al. (1981) in the course of their studies on NNN metabolism. In F344 rats given myosmine the major metabolite detected by HPLC with UV detection was 4-oxo-4-(3-pyridyl)butyric acid, which is also a major metabolite of the tobacco-specific nitrosamines NNN and NNK. This has to be considered when this metabolite is proposed as an urinary marker of NNK/NNN metabolic activation (Hecht and Trushin, 1997). We are not aware of any studies on the toxicity of myosmine. From the metabolism study of Hecht et al. (1981) when rats tolerated an oral dose of 100 mg/kg body weight it may be concluded that myosmine is of rather low acute toxicity. However, from its chemistry myosmine may be much more easily nitrosated than nicotine and could give rise to endogenous nitrosation as shown for nornicotine (Carmella et al. 1997). Therefore, studies on the chronic toxicity and genotoxic potential of myosmine are warranted.

In conclusion, a method for the detection of myosmine in nut material is presented. The possibility of a significant uptake of myosmine from food and food products clearly requires more research.

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