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SHORT COMMUNICATION

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Abuse of nutmeg seeds: Detectable by means of liquid chromatography-mass spectrometry techniques?

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Abstract

Numerous case reports of intoxications with nutmeg seeds (*Myristica fragrans*, Houtt.) can be found in literature often following their abuse, as psychotropic effects were described after ingestions of large doses. The successful detection of the main ingredients of the nutmeg seeds essential oil elemicin, myristicin, and safrole, as well as their metabolites in human urine by gas chromatography coupled to mass spectrometry (GC-MS) was already described. The aim of this study was to investigate the detectability of the main ingredients of nutmeg seeds and their metabolites in human blood and urine samples using liquid chromatography coupled to linear ion trap mass spectrometry (LC-LIT-MS^n) and liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS/MS) after nutmeg seed abuse. Sample material of three individuals was retrospectively investigated after a systematic screening approach indicated an intoxication with nutmeg seeds as a likely cause of symptoms. Metabolic patterns in plasma and urine using GC-MS were comparable with those described in earlier publications. Investigations using hyphenated liquid chromatography techniques lead to the detection of myristicin and safrole, as well as further metabolites not described using GC-MS and revealed sulfation as an additional Phase II metabolic pathway. These results might help to detect or confirm future intoxications with nutmeg seeds by using LC-MS techniques.

KEYWORDS
GC-MS, LC-HRMS/MS, LC-LIT-MS^n, metabolism, nutmeg

1 | INTRODUCTION

Intoxication symptoms after the ingestion of seeds of the nutmeg tree (*Myristica fragrans*, Houtt.) have been described in literature for more than 400 years.[1] Since then, numerous cases were reported after accidental, intentional, or suicidal overdosing of the seeds, leading to symptoms such as agitation, nausea, and in severe cases to seizures.[2,3]

Recent publications even reported the occurrence of so-called "nutmeg challenges" on social media, which encourage the ingestion of high amounts of ground nutmeg and to subsequently record the effects on the user, indicating that these intoxications are still a contemporary problem.[4] Beyer et al. investigated the metabolism and detection of the main ingredients of the essential oil 1-(3,4,5-trimethoxyphenyl)-prop-2-ene (elemicin, EL), 1-(3,4-methylenedioxy-5-methoxyphenyl)-prop-2-ene (myristicin, MY), and 1-(3,4-methylenedioxyphenyl)-prop-2-ene (safrole, SA) in rat and human urine samples using gas chromatography coupled to mass spectrometry (GC-MS).[5] Their chemical structures are shown in Figure 1.

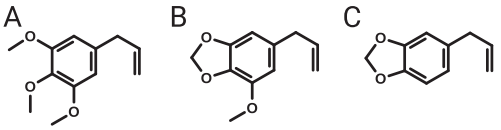

FIGURE 1 Main ingredients of the nutmeg essential oil.
A = elemicin, B = myristicin, C = safrole

However, the detection of nutmeg ingredients and their metabolites was since limited to urine screening using GC-MS, and although Neukamm et al. proposed a reporting threshold based on the detection of certain metabolites in urine, the detection of these ingredients or their metabolites in blood might facilitate an estimation of the amount that was ingested.[6] Therefore, the aim of this study was to investigate urine and blood samples after suspected nutmeg seed intake by using liquid chromatography coupled to linear ion trap mass spectrometry (LC-LIT-MS^n) and liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS/MS) in addition to GC-MS concerning the detectability of metabolites of EL, MY, and SA. These cases shall be discussed concerning the results after a systematic screening approach, including the detection of other drugs, alcohol, and the possibility of nutmeg intake being the cause of symptoms. At last, the findings of further metabolites of nutmeg seed ingredients shall be described, particularly after LC-MS analysis.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

Acetonitrile (LC-MS grade) and methanol (LC-MS grade) were obtained from VWR (Darmstadt, Germany). Ammonium formate, ammonium acetate, formic acid, and all other substances were obtained from Sigma (Taufkirchen, Germany). Water was purified with a Millipore filtration unit (18.2 Ω × cm water resistance).

2.2 | Blood and urine samples

Blood, anticoagulated using ethylenediamine tetraacetic acid (EDTA), and urine samples of each subject were submitted to the authors' laboratory for toxicologic analysis and confirmation of a suspected intake of nutmeg seeds. Subject 1 experienced initial agitation and later increasing somnolence. Subject 2 had increased phases of shaking and vomiting. Subject 3 also experienced initial agitation, followed by an increasing somnolence and finally a single seizure.

2.3 | GC-MS apparatus and systematic screening approach conditions

Apparatus conditions were set according to previously published studies.[5,7,8] Details and the conditions for ethanol quantification in plasma can be found in the supporting information.

2.4 | LC-LIT-MS^n apparatus and systematic screening approach conditions

Apparatus conditions were set according to previously published studies.[9,10] Details can be found in the supporting information.

2.5 | LC-HRMS/MS apparatus and systematic screening approach conditions

Apparatus conditions were set according to previously published studies.[11,12] Details can be found in the supporting information.

2.6 | Sample preparation for plasma screening

The blood samples were centrifuged, and 1 ml of the supernatant plasma were extracted using a published method.[13,14] The method consisted of a two-step extraction using 5 ml of a diethyl ether/ethyl acetate mixture (1:1, v/v) after addition of 100 µl methanolic trimipramine-d_3 (1 mg/L) as internal standard and 2 ml of aqueous saturated sodium sulfate solution in the first step. After phase separation by centrifugation, the upper organic extract was transferred into a flask. The aqueous residue was then basified with 0.5 ml of a 1 M aqueous sodium hydroxide solution, extracted a second time with 5 ml of the solvent mixture, the organic extract transferred to the flask containing the first residue, and at last evaporated at 60°C to dryness under reduced pressure. The combined residues were dissolved in 100 µl methanol and transferred into an autosampler vial. After preparation, 1 µl was injected into the GC-MS, and 10 µl were injected into each of the LC-MS devices.

2.7 | Sample preparation for GC-MS urine screening

Sample preparation was performed according to previous publications.[5,13] The urine samples (5 ml) were divided into two aliquots. One aliquot (2,5 ml) was refluxed with 1 ml of 37% hydrochloric acid for 15 min. After hydrolysis, the sample was mixed with 2 ml of 2.3 mol/L aqueous ammonium sulfate and 1.5 ml of 10 mol/L aqueous sodium hydroxide to obtain a pH value of 8–9. Before extraction, the other aliquot of unhydrolyzed urine was added, and this solution was extracted with 5 ml of a dichloromethane-isopropanol-ethyl acetate mixture (1:1:3; v/v/v). After phase separation by centrifugation, the organic layer was transferred into glass flasks and carefully evaporated to dryness at 75°C under vacuum. The residue was derivatized by acetylation with 100 µl of an acetic anhydride-pyridine mixture (3:2; v/v) for 5 min under microwave irradiation at about 440 W. After careful evaporation of the derivatization mixture, the residue was dissolved in 100 µl of methanol, and 1 µl of this sample was injected into the GC-MS.

2.8 | Sample preparation for LC-LIT-MS^n and LC-HRMS/MS urine screening

Sample preparation was performed according to a previous study.[9] To 0.1 ml of urine, 0.5 ml of acetonitrile was added. The mixture was shaken on a rotary shaker for 2 min at 2000 rpm. After centrifugation for 3 min at 10,000 × *g*, 0.5 ml was transferred into a glass vial and evaporated to dryness under a gentle stream of nitrogen at 50°C. The residue was dissolved in 50 µl of a mixture of eluent A and eluent B (1:1; v/v) from the LC-HRMS/MS apparatus.

2.9 | Sample preparation for ethanol quantification using GC-MS

For the quantification of ethanol, 100 µl plasma was mixed with 100 µl of tert-butanol as internal standard and subsequently transferred to a headspace autosampler vial. Calibration samples were prepared accordingly at concentrations of 0.2, 0.5, 1.0, 2.0, 3.0, and 4.0 g/L. Quality control samples were prepared at concentrations of 0.5 and 3.0 g/L. The mixtures were injected into the GC-MS system as described in the supporting information.

3 | RESULTS AND DISCUSSION

3.1 | Systematic screening approach of blood and urine samples

In total, blood and urine samples of three subjects were analyzed. Screening of sample material from Subject 1 did not reveal any drugs or ethanol. Merely metabolites of EL, MY, and SA were detected after urine screening using GC-MS. The screening of Subject 2 revealed the consumption of ethanol with a plasma concentration of 1.1 g/L. Further drugs were not found but metabolites of EL, MY, and SA after urine analysis using GC-MS. Subject 3 showed baclofen traces in urine, and ethanol was found with a plasma concentration of 1.4 g/L. Again, metabolites of EL, MY, and SA were found after urine analysis using GC-MS. From a toxicological point of view, these screening results made intoxications with nutmeg seeds the most likely cause of symptoms in each of the three subjects.

3.2 | Tentative identification of nutmeg metabolites using GC-MS

In addition to the systematic screening approach above, a specific search for metabolites using GC-MS was conducted as proposed by Beyer et al. using the ions with *m/z* 150, 165, 180, 194, 252, and 266 for the tentative identification of metabolites of EL, MY, and SA.[5] Corresponding to the findings of Beyer et al., no metabolites of EL, MY, or SA were found in plasma samples of the three subjects using GC-MS. However, urine samples displayed almost the same metabolic pattern as described in the corresponding publication. Merely the metabolite formed after demethylation of EL or demethylenation and following methylation of MY (10 in Figure 2) was not detected in urine, though two further metabolites were identified. The first metabolite was the result of a formation of an oxo and a hydroxy group at the side chain of SA (9 in Figures 2 and S1). The second additional metabolite was formed after demethylation of EL, as well as further hydroxylation in benzylic position of the side chain (11 in Figures 2 and S1). EI spectra of all other metabolites were the same as described by Beyer et al. and are thus not included in the supporting information.

3.3 | Tentative identification of nutmeg metabolites using LC-LIT-MS^n and LC-HRMS/MS

Each analysis using LC-LIT-MS^n or LC-HRMS/MS was retrospectively searched for EL, MY, and SA, as well as their metabolites. The results of these investigations are summarized in Figure 2, displaying the extended metabolic pathways of EL, MY, and SA in human. A summary of detectable targets after using GC-MS and each of the LC-MS techniques can be found in Table 1, a detailed table containing information about which analyte was detected in which subject can be found in Table S1. All corresponding spectra, as well as proposed structural formulas and fragmentation patterns, can be found in Figures S2–S4. Plasma analyses using LC-LIT-MS^n revealed three detectable metabolites. The first metabolite and its isomer were formed either after bisdemethylation of EL or demethylenation of MY (5 and 6 in Figures 2 and S2). The second metabolite was either formed after demethylation of EL or demethylenation and subsequent methylation of MY (10 in Figures 2 and S2). The last metabolite was formed after oxidation and further hydrolysis of the double bond at the side chain of MY (15 in Figures 2 and S2). In addition to these metabolites, urine analyses using LC-LIT-MS^n revealed the detection of MY itself (8 in Figures 2 and S2), a metabolite formed after hydroxylation of EL (12 in Figures 2 and S2), as well as the metabolite formed after demethylenation, subsequent methylation and hydroxylation of SA (22 in Figure 2). Glucuronides or sulfates were not detected using LC-LIT-MS^n.

Those analyses using LC-HRMS/MS revealed the detection of a wide range of additional metabolites. Concerning Phase I metabolites, several combinations of hydroxylation and dealkylation were detectable for each of the investigated nutmeg ingredients. Three Phase I metabolites (4, 8, and 14 in Figures 2 and S3) and five Phase II metabolites (17, 18, 20, 21, 23, and 24 in Figures 2 and S4) were detected in plasma samples, as well as MY itself. Concerning urine analysis, MY, SA, and eight Phase I metabolites (2, 3, 5–8, 10, 12, 14, and 15 in Figures 2 and S3), as well as eight Phase II metabolites (17–19 and 20–24 in Figures 2 and S4) were detectable. All detected Phase II metabolites were formed after sulfate conjugation and except for the one formed after either bisdemethylation of EL or demethylenation of MY and subsequent sulfation (18 in Figures 2, S3, and S4), all sulfate metabolites were only detectable using negative ionization mode. It is

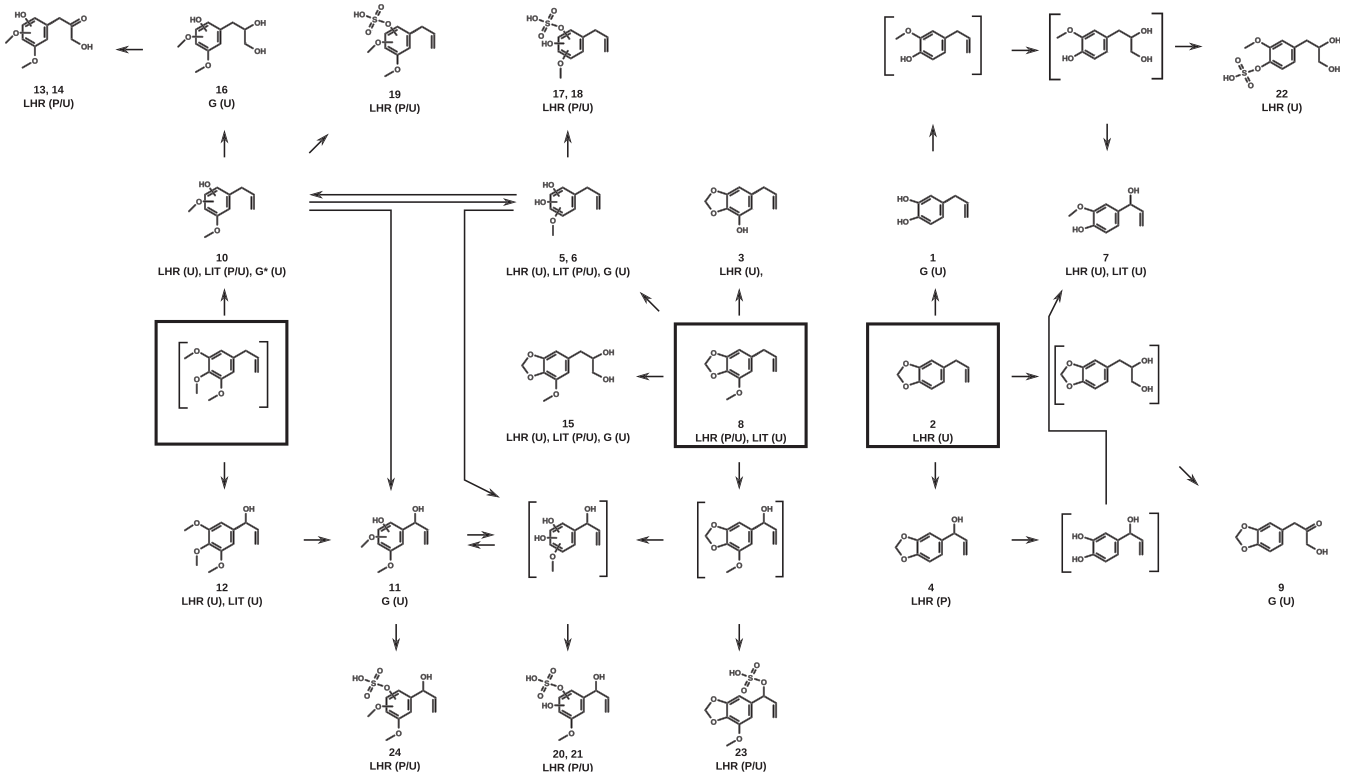

FIGURE 2 Extended metabolic pathways of elemicin (left), myristicin (middle), and safrole (right) in humans after biosamples analysis using GC- and LC-MS techniques. Parent compounds are indicated by a black rectangle; compounds in brackets are suspected intermediate metabolites or parent compound that were not detected in the current study; numbers correspond to the metabolites ordered by mass and retention time after detection using LC-HRMS/MS. Markush bonds refer to either meta or Para position and never to ortho position. G = detected using GC-MS, LIT = detected using LC-LIT-MS^n, LHR = detected using LC-HRMS/MS, P = detected in plasma, U = detected in urine, * = only detected by Beyer et al.[5]

TABLE 1 Number of detectable targets in each of the applied hyphenated mass spectrometry techniques including those found by Beyer et al.[5]

	Plasma	Urine
GC-MS	0	7
LC-LIT-MS^n	4	7
LC-HRMS/MS	9	17

Abbreviations: GC-MS, gas chromatography coupled to mass spectrometry; LC-LIT-MS^n, liquid chromatography coupled to linear ion trap mass spectrometry; LC-HRMS/MS, liquid chromatography coupled to high resolution mass spectrometry.

most likely that those Phase II metabolites would have also been detectable using LC-LIT-MS^n in negative ionization mode. Because our standard screening approach does not contain such a method, a confirmation was not performed. These results underline the importance of negative ionization mode in combination with urine sample analysis for the detection of metabolites of the investigated nutmeg ingredients, because they appear to often underlie sulfation. This is not surprising giving the fact that cytosolic sulfotransferases appear to have a high affinity for substrates containing aromatic nucleophiles.[15]

To avoid an excessive monitoring of all of the metabolites found in the context of this study, laboratories may focus on the most abundant ones when suspecting nutmeg seed intoxication. Concerning analyses using LC-LIT-MS^n, the main metabolite was analyte no. 15 in plasma, as well as 15 and 12 in urine samples. Concerning analyses using LC-HRMS/MS and positive ionization mode, analytes no. 13, 14, and 8 are well suited for monitoring in plasma samples, as well as 10, 5, and 8 in urine samples. When using LC-HRMS/MS and negative ionization mode, laboratories should focus on metabolite no. 18 and 19 in plasma samples, as well as 18 and 20 in urine samples. All analyte numbers correspond to those found in Figures 2 and S4. The order of their enumeration indicates the decreasing of their relative abundance and starts with the most abundant analyte.

It should be noted that this study suffers from certain limitations. First of all, the amount and the time point of the intake of nutmeg was not determinable. This may influence the detectability of certain metabolites in other samples where different amounts of nutmeg were ingested. Additionally, this study did not investigate samples from regular (low dose) food intake of nutmeg when used as a spice, leaving the discrimination of intoxications and the consumption of non-toxic amounts of nutmeg to further studies. At last, it should be pointed out that this study only included three positive cases of nutmeg seed intoxications. However, the detectability of MY, as well as

several metabolites in blood plasma by LC-MS techniques, might help future studies to determine laboratory alert levels.

4 | CONCLUSIONS

Systematic screening approach of different plasma and urine samples indicated an intoxication with nutmeg seeds. Manual investigation of the urine samples using GC-MS according to Beyer et al. revealed a comparable metabolic pattern as described before.[5] One metabolite was missing, whereas two further metabolites were detected. Manual investigation of the subjects' blood and urine samples using LC-LIT-MS^n and LC-HRMS/MS revealed eight additional metabolites formed after sulfate conjugation, which appeared to be the main Phase II metabolic pathway. Negative ionization mode was required for the detection of these metabolites. These results may facilitate the detection or confirmation of nutmeg intoxications in future cases after using a non-targeted systematic screening approach, particularly when only applying LC-MS. Limitations of this study are the unknown time point and amount of the ingestion of nutmeg and the absence of samples after intake of non-toxic amounts of nutmeg.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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