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Pyrrolizidine Alkaloids in the Food Chain: Development, Validation, and Application of a New HPLC-ESI-MS/MS Sum Parameter Method

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ABSTRACT: Contamination of food and feed with pyrrolizidine alkaloids is currently discussed as a potential health risk. Here, we report the development of a new HPLC-ESI-MS/MS sum parameter method to quantitate the pyrrolizidine alkaloid content in complex food matrices. The procedure was validated for honey and culinary herbs. Isotopically labeled 7-*O*-9-*O*-dibutyroyl-[9,9-$^2H_2$]-retronecine was synthesized and utilized as an internal standard for validation and quantitation. The total pyrrolizidine alkaloid content of a sample is expressed as a single sum parameter: retronecine equivalents (RE). L_d/L_q for honey was 0.1 μg RE/kg/0.3 μg RE/kg. For culinary herbs, 1.0 μg RE/kg/3.0 μg RE/kg (dry weight, dw) and 0.1 μg RE/kg/0.3 μg RE/kg (fresh weight, fw) were determined, respectively. The new method was applied to analyze 21 herbal convenience products. Fifteen products (71%) were pyrrolizidine alkaloid positive showing pyrrolizidine alkaloid concentrations ranging from 0.9 to 74 μg RE/kg fw.

KEYWORDS: *Borago officinalis, culinary herbs, high-performance liquid chromatography-electrospray-tandem mass spectrometry (HPLC-ESI-MS/MS), honey*

■ INTRODUCTION

Pyrrolizidine alkaloids (Figure 1) are toxic secondary plant metabolites which are constitutively expressed as a chemical defense strategy in some plant families. So far, more than 400 structures isolated from more than 560 plant species are known.[1] In the plant kingdom pyrrolizidine alkaloids (for example **1**) and corresponding pyrrolizidine alkaloid *N*-oxides (for example **2**) are solely found in the angiosperms, and the occurrence is mainly restricted to four plant families: Asteraceae (Senecioneae and Eupatorieae), Boraginaceae, Apocynaceae, and the genus *Crotalaria* within Fabaceae.[1] Despite their rich diversity they all share a common theme; i.e., they are ester alkaloids composed of a necine base esterified to one or more necic acids. Structural features of selected pyrrolizidine alkaloids relevant for this study are summarized in Figure 1. 1,2-Unsaturated pyrrolizidine alkaloids (for example **1**−**5**) are in general toxic. They are rapidly absorbed via the gastrointestinal tract and distributed in the body.[2,3] Studies of pyrrolizidine alkaloid metabolism in different model systems (mainly rodents) revealed three main routes leading to either detoxification or formation of reactive 6,7-dihydropyrrolizine ester species (pyrrolic esters, for example **6**) inducing toxic, genotoxic, and carcinogenic effects. The pyrrolic species are considered as the most active species responsible for the toxicity of 1,2-unsaturated pyrrolizidine alkaloids via their alkylating potential toward cellular macromolecules like DNA or proteins.[4,5] So far, the carcinogenic potential was demonstrated after pyrrolizidine alkaloid treatment of rodents by detecting tumors in liver, lung, bladder, and the gastrointestinal tract of the experimental animals.[4] In vitro studies with hepatic microsomes suggest that humans may also be among the species sensitive to 1,2-unsaturated pyrrolizidine alkaloid toxicity.[4]

In 2002, a report was published suggesting a general potential health risk due to possible contamination of honey via the nectar of bee pasture plants.[6] This initiated a series of newly developed analytical methods and studies on pyrrolizidine alkaloid levels in honey and pollen products.[7−15] Recently, pyrrolizidine alkaloids were also found in honey-containing medical products used for wound care[16] and in foodstuff containing honey as an ingredient, documenting a downstream contamination in those supply chains.[17]

In light of the above, national authorities of several European countries (UK, The Netherlands, and Germany) conducted individually risk characterizations and risk assessments for the occurrence of pyrrolizidine alkaloids in food and feed.[18] In addition, in 2011, the European Food Safety Authority (EFSA) published a scientific opinion on pyrrolizidine alkaloids in food and feed.[18] On the basis of a two year carcinogenicity study of lasiocarpine (**3**) in rats,[19] the COT (Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment, UK), BfR (The Federal Institute of Risk Assessment; Bundesinstitut für Risikobewertung, Germany), and EFSA concluded concurrently that the pyrrolizidine alkaloid exposure from food should be as low as possible.[18] Because of the genotoxic and carcinogenic nature of 1,2-unsaturated pyrrolizidine alkaloids a margin of exposure (MOE) approach was

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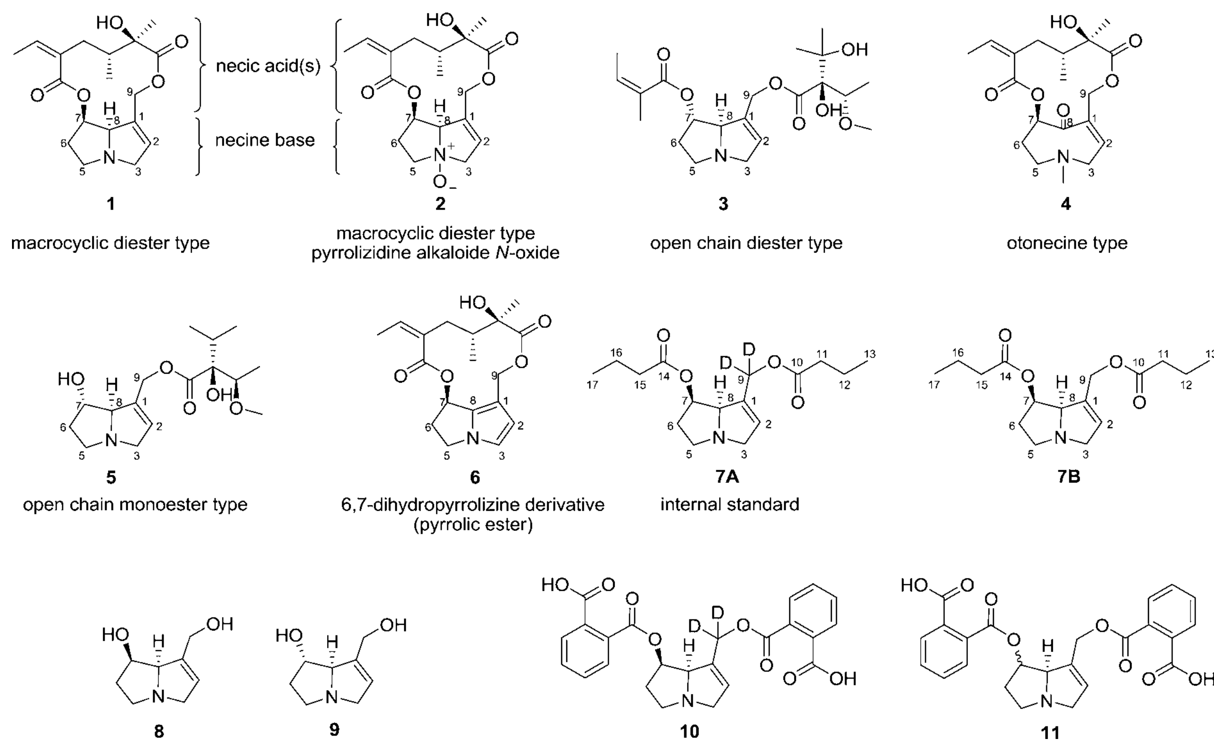

Figure 1. Chemical structures and structural features of pyrrolizidine alkaloids: (**1**) senecionine, (**2**) senecionine *N*-oxide, (**3**) lasiocarpine, (**4**) senkirkine, (**5**) heliotrine, (**6**) 6,7-dihydropyrrolizine derivative of senecionine, (**7A**) 7-*O*,9-*O*-dibutyroyl-[9,9-$^2H_2$]-retronecine, (**7B**) 7-*O*,9-*O*-dibutyroyl-retronecine, (**8**) necine base retronecine, (**9**) necine base heliotridine, (**10**) [9,9-$^2H_2$]-retronecine-diphthalate derivative, (**11**) retronecine-diphthalate/heliotridine-diphthalate derivatives.

applied, and a $BMDL_{10}$ for excess cancer risk of 70 μg/kg of body weight (bw) was derived. Hence, pyrrolizidine alkaloid doses of up to 0.007 μg/kg bw per day would imply a MOE of at least 10 000 and are unlikely to be of concern for a cancer risk.[18]

However, besides honey there is almost no data available for other possible sources of dietary exposure to pyrrolizidine alkaloids. Therefore, the EFSA panel concluded "...that ongoing efforts should be made to collect analytical data on occurrence of pyrrolizidine alkaloids and corresponding pyrrolizidine alkaloid *N*-oxides in relevant food and feed commodities".[18]

Here, we report the development and validation of a new HPLC-ESI-MS/MS sum parameter method to quantitate low levels of 1,2-unsaturated pyrrolizidine alkaloids in complex food matrices. The objective of the new method is to replace a former GC-MS sum parameter method which was established to analyze pyrrolizidine alkaloids in honey.[11] Particular attention has been paid to optimize and simplify sample workup while increasing overall sensitivity. Finally, the new method was applied to analyze the pyrrolizidine alkaloid content of culinary herbs available on the German market to generate a first data set on pyrrolizidine alkaloid contamination of such products.

■ MATERIAL AND METHODS

General Methods. *Nuclear Magnetic Resonance (NMR).* The NMR spectra of **7B** in a $CDCl_3$ solution were measured at 399.9 MHz (^1H) and 100.6 MHz (^{13}C) on an Avance DRX-400 spectrometer (Bruker BioSpin, Rheinstetten, Germany) at room temperature. Chemical shifts were referenced to internal tetramethylsilane (^1H, δ = 0.00 ppm) and $CDCl_3$ (^{13}C, δ = 77.01 ppm). The techniques used to assign the spectra were DEPT-135, H,H-COSY, H,C-HSQC, H,C-HMBC, and H,H-NOESY. The isotopic purity of the 9,9-$^2H_2$ derivative **7A** was determined from an ^1H NMR spectrum (in $CDCl_3$) taken at 600.1 MHz on an Avance II 600 spectrometer equipped with a 5 mm TCI cryo probehead (Bruker BioSpin, Rheinstetten, Germany).

Electrospray Enhanced Product Ion Mass Spectrometry (ESI-EPI-MS). Samples of the analyte and internal standard (IST) were dissolved in 30% acetonitrile and 70% deionized water at a concentration of 1 μg/mL and directly infused into the mass spectrometer with a 2.3 mm i.d. syringe pump (Harvard, Holliston, Massachusetts, USA). The flow rate was 10 μL/min. Ionization and fragmentation were performed with the 3200 QTrap mass spectrometer (Applied Biosystem/MDS SCIEX, Darmstadt, Germany) and an ESI interface. The positive ion mode was used for the EPI-scan mode. The EPI-conditions were: source voltage 5.5 kV, ambient temperature, curtain gas at 50 psi, GS1 35 psi, GS2 45 psi, declustering potential 51 V, and collision energy 45 eV. Nitrogen was used as a curtain and auxiliary gas.

Culinary Herbs. Twenty-one different culinary herbs were purchased from several supermarkets or local farmer markets across Germany. The focus of the supermarket products was frozen culinary herb mixes (6−8 different herbs per product), lyophilized herb mixes for salads/cooking (bought in February 2013, sampled in May 2013), and herb mixes in oil (bought in June 2013, sampled in June 2013). In addition, fresh herb mixes from local farmer markets (bought in June 2013, sampled in June 2013) and convenience products (bought in February 2013, sampled in June 2013) for the preparation of "Frankfurter Grüne Soße" ("green sauce", "salsa verde") were analyzed. "Frankfurter Grüne Soße" is a dish, typically served in State of Hesse in Germany. In addition, authentic plant material of *Borago officinalis* was obtained from the "Arzneipflanzengarten der TU Braunschweig" (collected in June 2013, sampled in June 2013). All herbs were stored at −20 °C before lyophilization. Then, samples were weighed, lyophilized, weighed again, and homogenized with a coffee grinder to yield dry powders of each sample which were used for further analysis. Lyophilized powders were stored at room temperature until sample workup and analysis.

Table 1. ^{1}H and ^{13}C NMR Data of 7B[a]

position[b]	^{13}C	^{1}H[c]	HMBC[d,e]
C-10	173.25	–	9a, 11, 12
C-14	172.72	–	(7), 15, 16
C-1	133.76	–	(3a), 3b, 9a, 9b
C-2	127.11	5.74, sext, $J \approx 1.7$ Hz, 1 H	3b, 9a, 9b
C-8	75.67	4.29, m, 1 H	2, 5a, 6b, 9a
C-7	73.61	5.31, m, 1 H	5a, 6b
C-3	62.79	a) 3.93, dqi, J = 15.0, 2.1 Hz, 1 H	2, (5a), 5b
		b) 3.38, ddq, J = 15.0, 5.8, 2.0 Hz, 1 H	
C-9	60.86	a) 4.68, AB, J = 13.6 Hz, of "qi" with ΣJ = 5.8 Hz, 1 H	(2)
		b) 4.58, AB, J = 13.6 Hz, of "qi" with ΣJ = 6.3 Hz, 1 H	
C-5	53.67	a) 3.30, ≈ddd, $J \approx$ 9, 7, 2 Hz, 1 H	3b, (7)
		b) 2.63, m, ΣJ = 27.2 Hz, 1 H	
C-15	36.46	2.23, m, 2 H	16, 17
C-11	35.99	2.28, m, 2 H	12, 13
C-6	34.49	2.12–2.00, m, 2 H	(5a), 5b
C-12	18.39	1.65, sext, J = 7.4 Hz, 2 H	11, 13
C-16	18.39	1.60, sext, J = 7.4 Hz, 2 H	15, 17
C-13	13.65	0.95, t, J = 7.4 Hz, 3 H	11, 12
C-17	13.65	0.93, t, J = 7.4 Hz, 3 H	15, 16

[a]Solvent: $CDCl_3$; internal chemical shift references: tetramethylsilane (TMS, δ = 0.00 ppm) for ^{1}H, $CDCl_3$ (δ = 77.01 ppm) for ^{13}C; observation frequency 399.9 MHz. [b]For numbering of the carbon atoms, refer to the formula. [c]For CH_2 groups with diastereotopic protons a) and b) indicate the deshielded and shielded nucleus, respectively. [d]Entries in the column HMBC indicate ^{1}H nuclei showing long-range correlations with the ^{13}C chemical shift in the second column. [e]Experiment optimized for J_{CH} = 7.5 Hz. Weak cross-peaks are indicated by parentheses.

Honey as a Matrix for Spiking Experiments. Honey was obtained from a local supermarket. The absence of pyrrolizidine alkaloids and pyrrolizidine alkaloid *N*-oxides was tested and verified before it was used as a matrix in method development.

Culinary Herbs as a Matrix for Spiking Experiments. Lyophilized culinary herbs were purchased from a local supermarket. The absence of pyrrolizidine alkaloids and pyrrolizidine alkaloid *N*-oxides was verified before using the herbs as a matrix in method development.

Chemicals. All chemicals used were purchased from Roth (Karlsruhe, Germany) and Sigma-Aldrich (Seelze, Germany) and of HPLC grade purity or redistilled before use. Lithium aluminum hydride solution (1 M) in THF and pyridine both in AcroSeal quality were acquired from ACROS Organics (New Jersey, USA). For spiking experiments and method validation, certified reference materials of senecionine (**1**), senecionine-*N*-oxide (**2**), and heliotrine (**5**) were purchased from PhytoLab (Vestenbergsgreuth, Germany) and Latoxan (Valence, France), respectively.

Preparation of the Isotopically Labeled Internal Standard 7-*O*,9-*O*-Dibutyroyl-[9,9-$^{2}H_2$]-retronecine (7A). Starting from monocrotaline, **7A** was synthesized according to Kempf (2009).[20] The diesterification final step was modified to reduce byproduct formation and to facilitate purification to obtain a high-purity product and is described in detail here.

Diesterification. For esterification, 66 mg of deuterated retronecine was dissolved in 5 mL of dry acetone, and a mixture of 660 μL of butyric acid anhydride in 10 mL of dry acetone was added and stirred for 5 h at 90 °C. The mixture was then dried under a gentle stream of nitrogen, redissolved in 1 mL of sodium hydroxide solution (1.25 mM, pH 8), and extracted with dichloromethane (6 × 1 mL). Phases were separated by centrifugation (90 s, 13 400 rpm), and the organic phases were combined and dried under a gentle stream of nitrogen.

The residue was purified by silica gel column chromatography using methanol as solvent. Fractions were collected and analyzed by thin-layer chromatography (TLC) and detected by iodine vapors. Target fractions were further checked for purity and identity by ESI-MS/MS (direct infusion). Combined fractions were purified further on Chromabond $C18_{ec}$ solid phase extraction cartridges (6 mL/1000 mg) (Macherey Nagel, Düren, Germany). The cartridges were conditioned by rinsing with 6 mL of methanol followed by 12 mL of deionized water. The sample was loaded in 1 mL of methanol:water (1:10, v/v), and the elution was performed by applying a step gradient starting at 100% water to 100% methanol in 10% steps. **7A** was detected in fractions containing 30−20% water. Pure target fractions of high purity were combined and lyophilized yielding 9 mg of **7A**. Full structural characterization, purity, and the degree of deuteration were determined by NMR and ESI-EPI-MS/MS.

ESI-EPI-MS m/z (relative intensity %): 298 $(M + H)^+$, 228 (6), 210 (7), 192 (7), 140 (10), 122 (100), 96 (8).

EI-MS: m/z (relative intensity %): 297 (5), 227 (30), 210 (44), 183 (5), 138 (48), 122 (39), 121 (49), 96 (43), 95 (100), 82 (11), 71 (24).

RI (DB-1MS): 1972

NMR: Full assignment of the NMR signals of **7B** is given in Table 1.

HPLC-ESI-MS/MS. Analysis was performed using a 1200 Series HPLC (Agilent, Waldbronn, Germany) in combination with ESI-MS/MS detection. The chromatographic separation was achieved using a 150 mm × 2.1 mm, 3 μm, Discovery HS F5 column (Sigma Aldrich, Seelze, Germany) including a precolumn cartridge of the same material. The following gradient was applied at a flow rate of 250 μL/min (solvent A: 0.1 M formic acid in water, solvent B: acetonitrile): 0−3 min (95% A), 3−13 min (95−0% A), 13−18 min (0% A), 18−19 min (0−95% A), 19−29 min (re-equilibration 95% A).

Ionization and fragmentation were performed in the ESI-positive mode, and quantitation was achieved in the multireaction monitoring (MRM) mode using the following MS parameters: curtain gas, 20 psi; ionization voltage, 5500 V; ion source temperature, 475 °C; gas1, 50 psi; gas2, 70 psi; collisionally activated dissociation (CAD) gas, high; declustering potential (DP), 71 V; entrance potential (EP), 8.5 V; collision cell exit potential (CXP), 4.0 V. Blank determinations were performed at regular intervals.

Four transitions per compound (analyte and internal standard) were selected.

For the analyte (retronecine-diphthalate): 452.00 → 94.00 Da (cell entrance potential (CEP), 18.000; collision energy (CE), 50), 452.00 → 120.00 Da (CEP, 18.000; CE, 36), 452.00 → 149.00 Da (CEP, 18.000; CE, 55), and 452.00 → 304.00 Da (CEP, 23.957; CE, 40).

For the internal standard ([9,9-$^{2}H_2$]-retronecine-diphthalate): 454.00 → 96.00 Da (CEP, 18.000; CE, 50), 454.00 → 122.00 Da

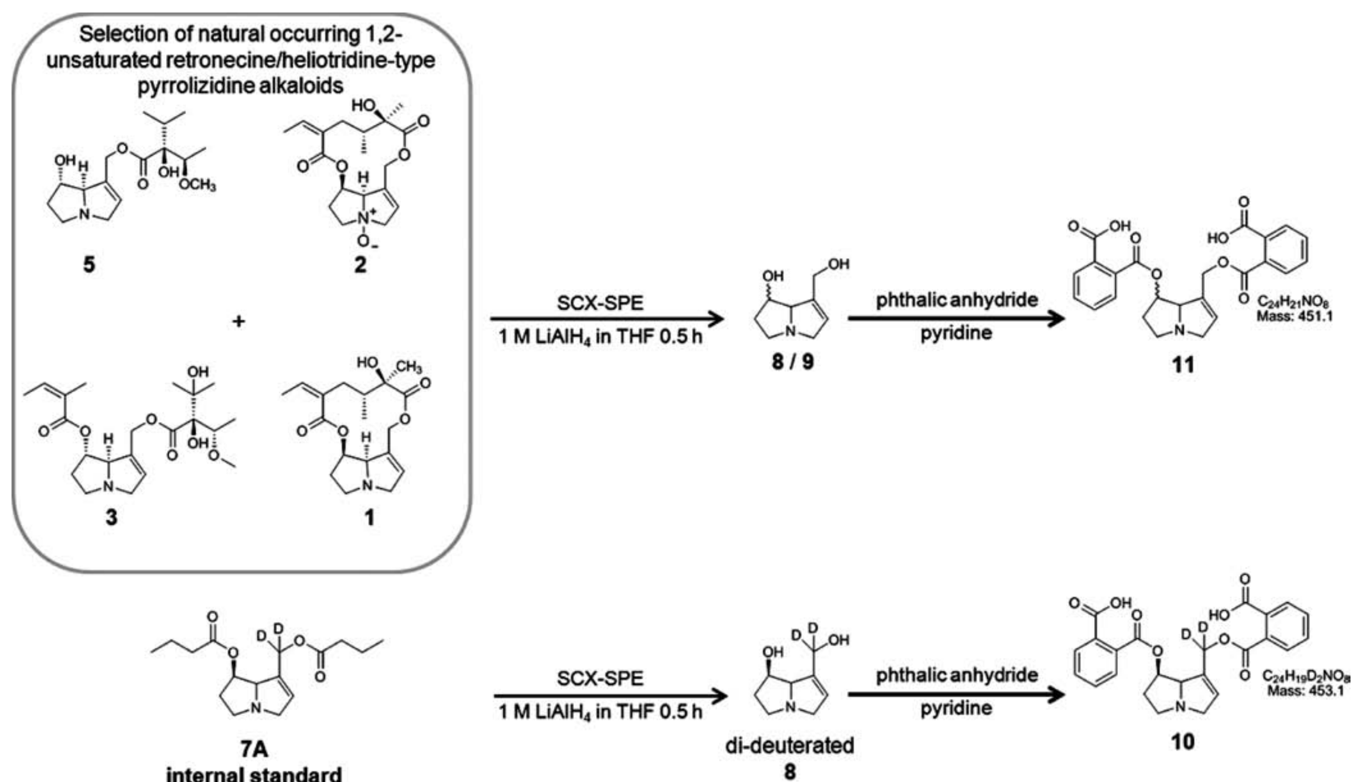

Figure 2. Scheme of the improved sample preparation for 1,2-unsaturated pyrrolizidine alkaloid *N*-oxides and pyrrolizidine alkaloids. Below, by analogy, the procedure for the internal standard **11**.

(CEP, 18.000; CE, 36), 454.00 → 149.00 Da (CEP, 24.015; CE, 55), and 454.00 → 306.00 Da (CEP, 24.015; CE, 40). Data analysis and integration were achieved with Analyst 1.4.2 Software (Applied Biosystems MDS Sciex, Darmstadt, Germany).

Optimizing derivatization Parameters. Time dependency of the derivatization of the procedure was optimized. Retronecine (2 μg) was added to a mixture of 60 mg of phthalic anhydride in 1 mL of pyridine. The sample was sealed and kept at 85 °C. At 5, 15, 30, 60, 120, 180, 240, and 300 min, 50 μL (corresponding to 100 ng of retronecine) were taken out and cooled to room temperature (RT) for 2 min, and 0.25 mL of acetonitrile and 0.7 mL of water were added to stop the reaction. The mixture was briefly mixed and analyzed by HPLC-ESI-MS/MS.

In addition, the optimum combination of phthalic anhydride and pyridine was tested. Test assays of 100 ng of retronecine containing six different concentrations of phthalic anhydride ranging from 10 to 180 mg of phthalic anhydride in 0.3 mL of pyridine were treated and analyzed as described above.

Investigation of Linearity. Linearity of the new HPLC-ESI-MS/MS method was determined. Eleven different concentrations of **1** and **7A** ranging from 0.25 ng to 1000 ng/mL underwent reduction with $LiAlH_4$ and derivatization using the phthalic anhydride/pyridine procedure and were analyzed by HPLC-ESI-MS/MS. Linear regression analysis was performed by plotting the respective peak areas against the concentration of the analyte in nanograms per milliliter using Excel. Linearity is expressed by the correlation coefficient r, and the goodness of fit is expressed by the coefficient of determination r^2.

Sample Preparation. Sample preparation was oriented at the recent GC-MS sum parameter method,[11] but major modifications were implemented to save time and simplify the procedure. The modified procedure is described here.

An amount of 0.1−0.4 g of the sample was dissolved or suspended in 0.05 M sulphuric acid in 2 or 6 mL for honey or culinary herbs/plant material, respectively. **7A** was added as internal standard (40−80 ng per sample) from a 2 μg/mL stock solution in methanol. The resulting mixture was homogenized and mixed by shaking (3 min). In the case of plant material/culinary herbs the mixture was centrifuged (10 min at 12 096*g*, 22 °C), and the extraction was repeated two more times each time using 6 mL of 0.05 M sulphuric acid. The resulting (combined) aqueous acidic solution was filtered through glass wool onto preconditioned 6 mL/500 mg strong cation exchanger solid phase extraction cartridges (SCX-SPE) (Phenomenex, Aschaffenburg, Germany). Preconditioning was accomplished with 6 mL of methanol followed by 6 mL of 0.05 M sulphuric acid.

The loaded cartridges were first washed with 12 mL of water followed by 12 mL of methanol. The elution (flow: 1 mL/min) was performed by applying three times 6 mL of ammoniacal methanol (6 mL conc. aq. NH_4OH in 100 mL of methanol). The eluates were combined and dried under a stream of nitrogen. The resulting residue was redissolved with 50 μL of methanol, and 600 μL of $LiAlH_4$ solution (1 M in THF) was added. The mixture was vortexed and kept at 4 °C for 30 min. The reduction reaction was stopped by adding 1 mL of dichloromethane and 200 μL of aqueous sodium hydroxide solution (10%, w/w). Sodium sulfate (0.5 g) was added, and the mixture was briefly vortexed. The organic phase was separated using a Pasteur pipet and filtered through glass wool. The extraction was repeated three more times (1 mL of dichloromethane each), and the organic fractions were combined and dried under a stream of nitrogen gas. The residue was redissolved in a mixture of 0.06 g of phthalic anhydride in 0.3 mL of pyridine, in tightly sealed 2 mL autosampler vials, and kept at 85 °C for 60 min for derivatization. Derivatization reaction was stopped after a 2 min cool-down period by the addition of 0.7 mL of water. The resulting mixture was briefly shaken and directly used for HPLC-ESI-MS/MS analysis. Pyrrolizidine alkaloid content of the individual samples was calculated using eq 1.

$$c\ [\text{ng/kg}] = [\text{amount (IST) [ng]}\cdot 0.53(\text{RE factor})\cdot \text{area (analyte diphthalate derivative)}] / [\text{area (IST diphthalate derivative)}\cdot \text{amount (culinary herb) [kg]}] \quad (1)$$

Evaluation of Intraday and Interday Stability. For intraday stability 8 ng of pyrrolizidine alkaloids (mixture of 4 ng of **1** and 4 ng of **2**) was reduced with $LiAlH_4$ and derivatized with phthalic anhydride and pyridine. The sample was analyzed by HPLC-ESI-MS/MS. Quantitation was performed by 80 ng of **7A**. The same sample was measured 10 times during one day. After the sixth injection, a 12 h break was taken to check the yield of the analyte after this time break. The recovery rate was determined in comparison to the first injection (100%).

For interday stability three different concentrations (20, 100, and 500 ng of pyrrolizidine alkaloids (1:1 mixture of **1** and **2**)) were prepared in duplicates as described for the intraday stability. The internal standard was added at 20, 100, and 500 ng, respectively. All samples were analyzed by HPLC-ESI-MS/MS, then frozen for 23 h at −20 °C. Before the next injection the samples were thawed for 1 h at room temperature. This procedure was performed for 8 days, and the recovery rate was determined in comparison to the first injection for each concentration level (100%).

Evaluation of Recovery Rates for Pyrrolizidine Alkaloids/Pyrrolizidine Alkaloid *N*-Oxides in Honey and Culinary Herbs. Pyrrolizidine alkaloid free honey (0.4 g) or pyrrolizidine alkaloid free culinary herb mix matrix were each spiked with three to four pyrrolizidine alkaloid levels (1:1 ratio **1** and **2**) at 0.8 (honey only), 8, 80, and 800 ng and 80 or 40 ng internal standard (**7A**) each. All samples underwent full sample preparation. The recovery was calculated as the rate of the true value (added pyrrolizidine alkaloids) per kilogram of honey or culinary herbs, respectively.

Determination of L_d and L_q. An amount of 0.4 g of honey or culinary herbs as matrix was each spiked with the low concentrations of **1** and **2** plus the internal standard (**7A**). After sample preparation and analysis the limit of detection (L_d) and limit of quantitation (L_q) were calculated with S/N = 3/1 and S/N = 7/1, respectively.

RESULTS AND DISCUSSION

Improvement of the Analytical Method. The existing GC-MS sum parameter method, which we used in the past to quantitate pyrrolizidine alkaloid content in honey[11] and bee products requires usually 20 g of sample material and uses an elaborate sample preparation to achieve a L_q of 10 μg of retronecine equivalents (RE)/kg. Hence, our main goals for the sample preparation of the new HPLC-ESI-MS/MS method were simplification including time savings but without sacrificing sensitivity.

The former study showed the advantageous use of SCX-SPE to reduce matrix load and concentrating pyrrolizidine alkaloids from complex matrices. Hence, this part of the sample workup remained in the new procedure. In the former methods 20 g of honey was diluted with 30 mL of dilute acid (ratio approximately 1:1.5). Nevertheless, certain honeys tend to clog the SPE columns, requiring special SPE columns or elevated temperature (40 °C) during sample loading.[11,21] We decided to reduce the amount of honey (0.4 g) while adding 2 mL of water (ratio approximately 1:5) which results in smaller sample volumes and lower viscosity. Those solutions could be extracted more rapidly without any clogging problems at room temperature.

A major improvement of the new approach is the simultaneous reduction of pyrrolizidine alkaloid *N*-oxides and pyrrolizidine alkaloid ester functionalities using $LiAlH_4$ to yield the corresponding necine base diols (**8**, **9**; Figures 1 and 2). The former method[11] used a consecutive approach, first reduction of the pyrrolizidine alkaloid *N*-oxides using zinc dust in acidic solution, followed by SPE and subsequent chemical reduction of the ester pyrrolizidine alkaloids using $LiAlH_4$. The new approach was optimized by analyzing a test series of pyrrolizidine alkaloid mixtures and showed that the former three steps/7.95 h procedure could be improved by increasing the amount of reducing reagent ($LiAlH_4$) to a two steps/4.80 h procedure (Figure 2).

The elution volume for the SPE needed to be increased by a factor of three which resulted in some extra time for elution/concentration, but this was necessary to maintain high recovery rates of the analyte.

Stable Isotope Labeled Internal Standard. In the GC-MS sum parameter approach[11] heliotrine **5** (Figure 1) was used as IST. This was due to the fact that heliotrine-type pyrrolizidine alkaloids rarely occur in honey samples.[18] Hence, the GC-MS sum parameter approach resulted in one peak for retronecine-di-TMS (**8**-di-TMS derivative) representing all retronecine-type (**8**) pyrrolizidine alkaloids (analytes), while heliotridine-di-TMS (**9**-di-TMS derivative) was representing the added IST (**5**). Both compounds are diastereomers and could be separated on regular DB-1 or DB-5-type GC-columns, allowing a rapid quantitation of the pyrrolizidine alkaloid content.[11] However, all samples had to be analyzed in duplicates to exclude false positive quantitation if natural occurring heliotridine-type pyrrolizidine alkaloids would have been present in the sample. Although it never occurred in our study,[11] co-occurrence of retronecine- and heliotridine-type (**8** and **9**) pyrrolizidine alkaloids would prevent the quantitation of such samples in the GC-MS sum parameter approach. To compensate this problem, we decided to synthesize a stable isotope labeled standard **7A** (Figure 1). **7A** is now used as an internal standard replacing **5**. It is added at the beginning of the sample workup procedure to cover for recovery losses and as the main advantage double workup becomes obsolete.

The ^1H and ^{13}C NMR chemical shifts and assignments of the nondeuterated 7-*O*,9-*O*-dibutyroylretronecine (**7B**) including HSQC and HMBC correlations are given in Table 1. The ^1H NMR spectrum of **7A** showed the almost complete absence of the 9-H signals. Integration of the corresponding region proved the isotopic enrichment to be better than 97%. The following differences in signal multiplicities were noted between the 9,9-$^1H_2$ and the 9,9-$^2H_2$ isotopologues. Only the signals of 2-H, 3-H_a, 3-H_b, and 8-H are affected. While 2-H in **7B** gives a sextet-like multiplet with ΣJ = 8.6 Hz, it is simplified in **7A** to a quartet-like absorption with ΣJ = 5.7 Hz; i.e., the two allylic couplings J(2,9a) and J(2,9b) with a sum of 2.9 Hz have disappeared. Similarly, 3-H_a in **7B**, a doublet (15.0 Hz) of quintet-like multiplets (ΣJ = 8.0 Hz), is converted to a doublet (15.0 Hz) of triplet-like multiplets (ΣJ = 4.6 Hz) in **7A**; i.e., the two homoallylic couplings J(3a,9a) and J(3a,9b) with a sum of 3.4 Hz are missing. In **7B** 3-H_b gives a doublet (J = 15.0 Hz) of doublets (J = 5.7 Hz) of quartet-like multiplets (ΣJ = 6.0 Hz) which later in **7A** turn into doublets with J = 1.9 Hz, indicating also the loss of two homoallylic couplings, J(3b,9a) and J(3b,9b), the sum of which is 4.1 Hz. Finally the signal of 8-H, a broad, little-structured multiplet in **7B**, changes to a multiplet of the same total width but of eight now relatively sharp lines in **7A**. Here the small unresolved couplings J(8,9a) and J(8,9b) over four single bonds are eliminated. Iterative band shape analysis showed the 8-H multiplet in **7A** to be a dddd with J = 5.7, 3.9, 2.6, and 1.9 Hz (half-height line width 1.3 Hz, R = 0.03%).

The application of **7A** allowed the simultaneous quantitation of all 1,2-unsaturated retronecine/heliotridine-type pyrrolizidine alkaloids in complex matrices in the form of a single sum parameter. During sample preparation this compound is converted into [9,9-$^2H_2$]-**8** and subsequently into **10** (Figures

2 and 3) which showed cochromatography with both possible equivalents of natural occurring retronecine/heliotridine-type

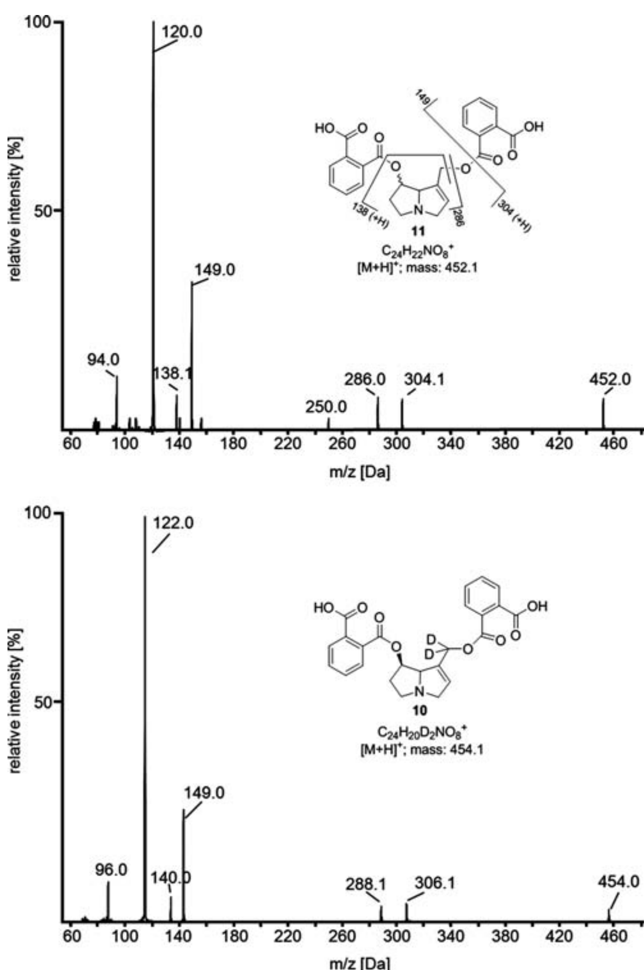

Figure 3. ESI-MS/MS spectra and proposed fragmentation in EPI-mode for the analyte derivatives **11** and internal standard derivative **10**.

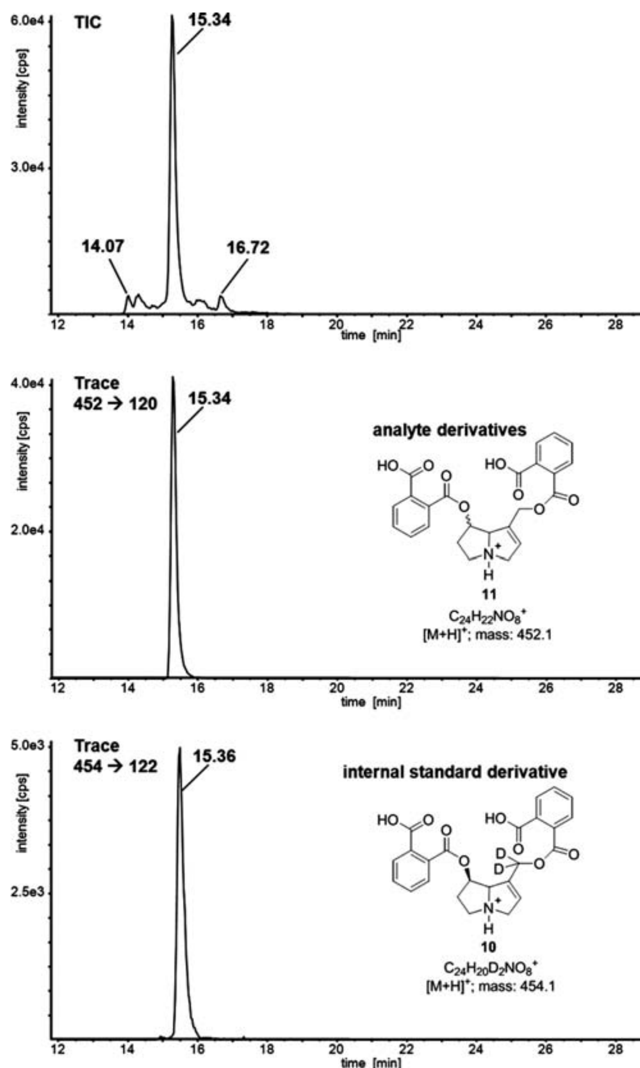

Figure 4. Chromatogram of the pyrrolizidine alkaloid positive herbal mix sample F_4. TIC and both quantifier XICs of the analyte derivatives **11** (452 → 120) and the internal standard derivative **10** (454 → 122) are shown.

pyrrolizidine alkaloids retronecine-diphthalate/heliotridine-diphthalate (**11**) (Figure 4), hence perfectly covering for any occurring matrix effects in HPLC-MS/MS.

HPLC-ESI-MS/MS analysis without derivatization of the necine base backbones (**8**, **9**, or [9,9-$^2H_2$]-**8**) was not possible since these compounds showed inadequate chromatographic and ionization behavior. Hence, an HPLC-MS compatible esterification procedure using phthalic anhydride in pyridine was established. The total derivatization procedure was balanced for overall time consumption (max. 1 h) but the highest possible signal intensity (sensitivity) using a series of test samples and varying test conditions. In those test series, the phthalic anhydride/pyridine ratio and temperature were optimized to result in high conversion rates. For practical reasons, in this case solubility of the solid phthalic anhydride in pyridine, the best results were obtained for 0.06 g of phthalic anhydride in 0.3 mL of pyridine. Similarly, 85 °C was chosen to maintain the stability and tightness of the plastic septa of the sample vials. In addition, particular emphasis was placed on a high ratio of diesterification compared to the monoderivative. Finally, the corresponding MS/MS spectra and the proposed fragmentation of the esterified retronecine/heliotridine-diphthalates derivative (**11**) and the IST-diphthalate derivative (**10**) are shown in detail in Figure 3.

Method Validation and Determination of L_d/L_q. The validation of the here described parameters was performed according to the guidelines of the German Society of Toxicological and Forensic Chemistry (GTFCh).[22] Linearity of derivatization and analysis procedure was checked by establishing 11-point calibration curves at concentration levels ranging from 0.12 ng to 500 ng RE/mL which reflects a pyrrolizidine alkaloid concentration in real samples ranging from 0.625 to 2500 μg/kg (r = 0.9981 and r^2 = 0.9963 for the analyte-diphthalate derivative).

Next, the recovery rates for the newly established method and derivatization were determined for three different concentrations (low, medium, high) for both matrices (honey/herb mix). Honey and herbs showed comparable recoveries ranging from 104 to 69% and 85 to 72%, respectively. These results were similar to those obtained for the GC-MS sum parameter approach.[11]

To further validate the method, intra- and interday stability of the derivatives and analysis were established. Intraday

stability of 10 consecutive measurements over one day showed a standard deviation of ±5.2 when compared to the first injection (100%).

Intraday stability was performed over an 8 day period (freeze/thaw between each day) at three different concentrations prepared in duplicate representing 20, 100, and 500 ng of pyrrolizidine alkaloids/mL (1:1 mixture of **1** and **2**). The test showed standard deviations of ±18.2, ±6.5, and ±4.7, respectively, when compared to the first injection of each concentration level (100%). These results demonstrated the ruggedness of the new method and the stability of the derivatized samples over a period of 8 days. In addition, freeze/thaw cycles following the derivatization were trouble-free, allowing a separation in time for sample preparation and analysis, which was not possible with the existing method.[11]

Finally, the limit of detection (L_d) and the limit of quantitation (L_q) for both matrices were established by spiking a pyrrolizidine alkaloid free honey or herb mix with a series of known pyrrolizidine alkaloid concentrations (1:1 mixture of of **1** and **2**). From these matrix matched analytical runs L_d and L_q were calculated using a signal-to-noise (S/N) ratio = 3 and S/N ratio = 7, respectively. The L_d/L_q for honey was 0.1 μg RE/kg and 0.3 μg RE/kg and for culinary herbs 1.0 μg RE/kg and 3.0 μg RE/kg (dry weight) and 0.1 μg RE/kg and 0.3 μg RE/kg herbs (fresh weight).

These L_d/L_q's represent the total amount of retronecine/heliotridine-type pyrrolizidine alkaloids of a sample and are expressed as a single sum parameter in the form of retronecine equivalents (REs). This analytical value represents the main core structural element of the toxic natural occurring 1,2-unsaturated pyrrolizidine alkaloids.[11] Compared to our previous GC-MS method this is a gain in sensitivity of a factor of 30 while reducing the sample amount by a factor of 50. In addition the total turnaround time was reduced from 9.2 to 6.2 h allowing a convenient sample preparation during a regular working day.

Compared to existing targeted LC-MS/MS methods analyzing individual pyrrolizidine alkaloids in honey, food, feed, or plant materials the reported L_q's were usually in the same range of 0.5−2.0 μg/kg per individual pyrrolizidine alkaloid, with a few pyrrolizidine alkaloids showing higher L_q's.[13,14,18] In complex plant matrices like hay or compound feeds usually higher L_q's were observed (L_d of 4.5 μg/kg).[18] A most recent method published by the Bundesinstitut für Risikobewertung (BfR, Germany) reported L_q's of 2.9−151.7 μg/kg (average: 23 μg/kg) for a plant matrix used to analyze plant material used for herbal infusions.[23]

Comparison of Different Analytical Methods. Our sum parameter method has two limitations: (i) otonecine-type pyrrolizidine alkaloids are not detected and (ii) the result is calculated in the form of a single sum parameter representing the backbone of toxic 1,2-unsaturated pyrrolizidine alkaloids and pyrrolizidine alkaloid *N*-oxides. Concerning the first issue, so far otonecine-type pyrrolizidine alkaloids (for example **4**) were rarely found in honey and feed samples. This is reflected in the EFSA report[18] where senkirkine (**4**) was only detected in 3% of the honey samples (n = 14 604) at levels below 2 μg/kg (99th percentile; L_d = 0.5 μg/kg). In addition, 315 feed samples across different categories were analyzed. Here, **4** was detected in 282 samples (90%) but again at levels at or below 5 μg/kg (99th percentile; L_d = 4.5 μg/kg). Current available methods used for food and feed analysis monitor **4** as the only representative for otonecine-type pyrrolizidine alkaloids. In those studies, if **4** was present, usually the biosynthetic precursor retronecine-type pyrrolizidine alkaloids (**1** and **2**) were present as well, usually at higher concentrations.[13,18] With this currently available data in mind, missing the otonecine-type pyrrolizidine alkaloids of a sample would be reflected in slightly lower values, but the sample would be still analyzed as pyrrolizidine alkaloid positive. Therefore, in our opinion, the sum parameter is a very good approximation of the total content of 1,2-unsaturated pyrrolizidine alkaloids compared to existing methods used to monitor pyrrolizidine alkaloids in food and feed. On one hand we are missing the otonecine-type pyrrolizidine alkaloids, but on the other hand this sum parameter method covers for all other 1,2-unsaturated pyrrolizidine alkaloids including yet unknown pyrrolizidine alkaloids (and/or *N*-oxides) or pyrrolizidine alkaloids (and/or *N*-oxides) without available reference compounds which cannot be detected or quantitated with targeted MS/MS approaches.

The second issue relates to the loss of structural details of the corresponding pyrrolizidine alkaloids during sample preparation (Figure 2). The sum parameter method does not allow drawing conclusions regarding which individual pyrrolizidine alkaloids were present in the starting material. However, for quantitation reasons, this can be addressed if one considers that the measured RE (M_w = 155 g/mol), which comprises approximately half of any common pyrrolizidine alkaloid molecule (M_w = 299 to 349, representing lycopsamine and erucifoline) yielding a conversion factor ranging from 1.9 to 2.3. Hence, multiplying the RE value by an average factor of 2.1 gives a close approximation of the total pyrrolizidine alkaloid content. In terms of the loss of structural information it is true to say that at the present time detailed toxicological data of only a very limited number of pyrrolizidine alkaloids are available.[18] Since all classes of 1,2-unsaturated pyrrolizidine alkaloids (**1**−**5**) undergo the same metabolic activation to yield reactive pyrrolic intermediates (for example **6**) and form a common set of dihydro-pyrrolizine−DNA adducts in animal model systems, these findings suggest that a genotoxic/carcinogenic mechanism is applicable for all 1,2-unsaturated pyrrolizidine alkaloid esters and their *N*-oxides. Therefore, EFSA based its risk characterization on the effects of the well-studied lasiocarpine and applied it on the total sum of all 1,2-unsaturated pyrrolizidine alkaloids.[18] Hence, multiplying the RE result by a factor of 2.1 gives a close approximation of the total pyrrolizidine alkaloid content which could be used instead for such calculations. In addition, concerning risk characterization, the calculation of upper-bound values is strongly affected by the L_d/L_q's of the corresponding method. Hence our approach, using a single sum parameter, results in only small differences between lower-bound and upper-bound values (one analyte; L_d = 1 μg/kg RE), while with an analytical method, analyzing individual pyrrolizidine alkaloids, the sum of all L_d/L_q's should be considered (e.g., the sum of all L_q's for the 17 measured pyrrolizidine alkaloids of the most recent method results in 395 μg/kg).[23]

Pyrrolizidine Alkaloid Quantitation in Culinary Herb Products and *Borago officinalis*. The newly developed and validated method was applied to culinary herb mixes available as convenience products like frozen foods, lyophilized, conserved in vegetable oil, or as fresh products but prepacked and ready for food preparation and consumption. Those products are usually used to refine salads and other dishes. These products were purchased from supermarkets and farmer markets across Germany. Special attention was paid to products

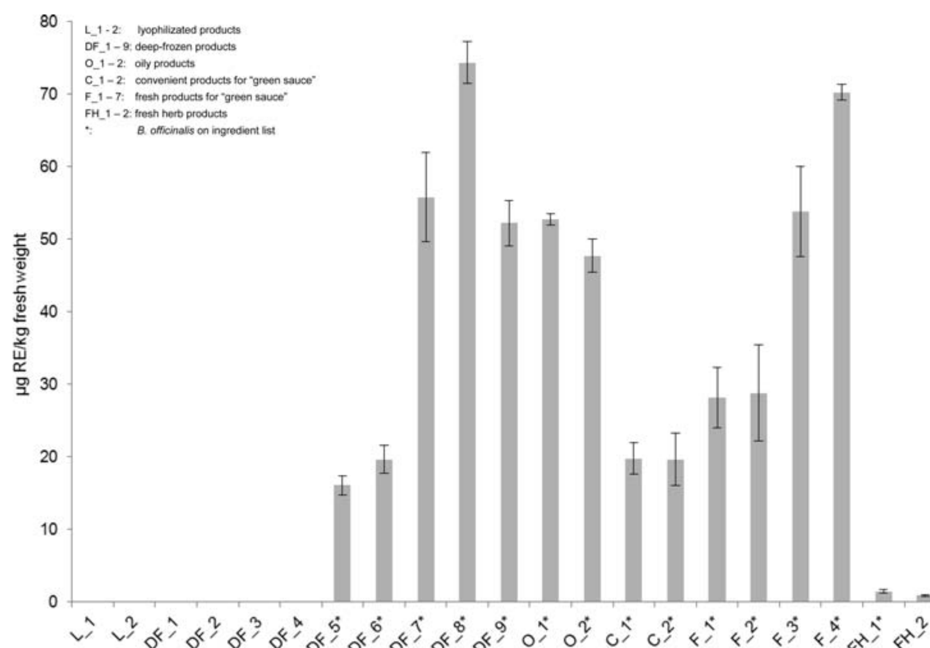

Figure 5. Overview of the pyrrolizidine alkaloid content for 21 different commercial products from different producers or brands, indicated as retronecine equivalents (RE) per kilogram fresh weight analyzed using the new HPLC-ESI-MS/MS sum parameter method (n = 3).

for a regional specialty known as "Frankfurter Grüne Soße" ("salsa verde") typically served around Frankfurt in the state of Hesse (Germany). This dish is made from seven fresh herbs including *B. officinalis*, a known pyrrolizidine alkaloid containing plant. In addition, fresh *B. officinalis* leaves and flowers (used as edible decoration for salads or other dishes) were analyzed. In total 21 different commercial products (all from different producers or brands) were investigated. An example for the ESI-MS/MS result of the positive sample F_4 is given in Figure 4.

Fifteen out of these products (71%) were pyrrolizidine alkaloid positive showing pyrrolizidine alkaloid concentrations ranging from 0.9 to 74 μg RE/kg fresh weight (fw). The average concentration in this sample group (pyrrolizidine alkaloid positive) was 36 μg/kg fw and 26 μg/kg fw if calculated for all analyzed products. The analytical results of all commercial samples are summarized in Figure 5.

As a matter of fact, all *B. officinalis* containing samples (according to the list of ingredients of the package) were pyrrolizidine alkaloid positive, while one sample (FH_2) showed trace amounts of pyrrolizidine alkaloids, without listing *B. officinalis* as an ingredient. *B. officinalis* is a known pyrrolizidine alkaloid plant containing lyopsamine-type (1,2-unsaturated retronecine backbone), supinidine-type (1,2-unsaturated but missing 7-hydroxy functionality), and isoretronecanol-type pyrrolizidine alkaloids (1,2-saturated but missing 7-hydroxy functionality).[24,25] Hence, the presence of this herb is reflected in the analytical results of these samples. All other samples (with the one exemption of FH_2) did not list *B. officinalis* (borago) as an ingredient and were analyzed as pyrrolizidine alkaloid nondetected.

Assuming the following scenario: Making a dish "Frankfurter Grüne Soße" for four persons using one serving of a *B. officinalis* containing convenience product or herb mix (C_1/2 or F_1 to F_4; Figure 5, average weight approximately 200 g) with a content of 20−70 μg RE/kg would result in approximately 1−3.5 μg of RE per person, corresponding to 1.9 to 6.7 μg of pyrrolizidine alkaloid per person (assuming a conversion factor of 1.9 for converting RE into lycopsamine). Most of these dishes are prepared without cooking and served cold, so no correction of food processing needs to be considered. In fresh leaves of *B. officinalis* an average pyrrolizidine alkaloid content of 133 μg RE/kg (fw) was detected (corresponding to 253 μg of pyrrolizidine alkaloid/kg (fw), which explains the pyrrolizidine alkaloid positive findings and the observed concentrations of all commercial products containing *B. officinalis*. In addition we analyzed the flowers of *B. officinalis*. Flowers of *B. officinalis* are sometimes used in cuisine as edible decorations. Here, we analyzed the pyrrolizidine alkaloid content of single flower heads. In average 0.07 μg of RE per flower was measured (corresponding to 0.13 μg of pyrrolizidine alkaloids per flower).

The successful transfer of our existing GC-MS pyrrolizidine alkaloid sum parameter method to HPLC-ESI-MS/MS offers several advantages. By simplifying the procedure, we were able to reduce the time necessary for sample preparation and derivatization by 30%, while at the same time achieving lower L_q's. Of course, the sum parameter approach has some drawbacks, but on the other hand it also offers some main advantages. It allowed us to introduce an isotopically labeled internal standard, which cannot be achieved for methods where individual pyrrolizidine alkaloids are analyzed. In addition, it is also an important issue in toxicological calculations that the addition of many individual L_q's can lead to significant spreading of upper bound data, while here only one L_q (1 μg/kg) needs to be considered.

The new method was validated for honey and culinary herb mixtures and applied to investigate pyrrolizidine alkaloid content of commercial products. For honey a vast amount of data are available. The recent risk characterization by EFSA[18] has pointed out the need for more information of pyrrolizidine alkaloids in the food chain, which includes in particular more information on possible routes of exposure. So far, no data on the pyrrolizidine alkaloid content of culinary herb mixes were

available. In this preliminary study, it was demonstrated that the ingredient *B. officinalis* leaves used in the preparation of such products results in 0.9−74 μg RE/kg fw. These values are unlikely to cause acute negative health effects, but since pyrrolizidine alkaloids are considered as genotoxic/cancerogenic compounds a general avoidance or minimization of exposure is the recommended goal.[18] This is especially true nowadays since more and more potential pyrrolizidine alkaloid entries in the food chain are discovered. At the moment, the risk characterization by EFSA[18] could be only based on honey because of the exclusive availability of data for honey, but as demonstrated in the past pyrrolizidine alkaloid containing plants are found as contaminants in feed,[18] in salads (especially rocket salad),[26] and most recently in tea and herbs used for the preparation of herbal infusions.[27] Therefore, additive effects of several sources of pyrrolizidine alkaloid in the food chain need to be analyzed and need to be addressed in the future.

While the pyrrolizidine alkaloid content of honey, salads, or teas occurs as an unintended contamination through coharvesting of pyrrolizidine alkaloid plant material, this issue and active pyrrolizidine alkaloid reduction is difficult to achieve for consumers and needs to be addressed during growth, harvesting, and production of these foods. The situation is quite different looking at the here-analyzed culinary herbs/herb mixes. An efficient pyrrolizidine alkaloid reduction seems easily achievable by removing the ingredient *B. officinalis* from the recipes. Of course, it is always a matter of taste and tradition, but eliminating *B. officinalis* (leaves and flower heads) should be the lesser of the two evils, particularly since *B. officinalis* does not stand out as a flavor-intense aromatic delicacy.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

$BMDL_{10}$, benchmark dose lower confidence limit; bw, body weight; COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; EPI, enhanced product ion spectrum; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single quantum correlation; MOE, margin of exposure; NOESY, nuclear Overhauser effect spectroscopy; RE, retronecine equivalents

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