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Novel Solid-Phase Extraction for Epimer-Specific Quantitation of Ergot Alkaloids in Rye Flour and Wheat Germ Oil

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ABSTRACT: Ergot alkaloids and their epimer-specific determination have gained increasing importance for food safety. A solid-phase extraction and cleanup method based on sodium-neutralized strong cation exchange (Na^+-SCX) was developed to quantitate 12 priority ergot alkaloids in rye flour and wheat germ oil by HPLC fluorescence analysis. Sample preparation is achieved by omitting acidic and alkaline conditions enabling minimized epimerization, which is necessary to determine ergot alkaloids according to their natural distribution in foods. Ergot alkaloids are eluted from SCX-column by forming ion pairs using a sodium hexanesulfonate containing solution which prevents epimerization for at least 96 h. Method validation yielded recoveries of 80−120% (rye flour) and 71−96% (wheat germ oil) with a maximum limit of quantitation (LOQ) of 2.0 $\mu g\ kg^{-1}$ per ergot alkaloid for both matrices. The applicability of the developed method was demonstrated by analyzing 16 samples from German retail markets: 9 rye flours (max 178 ± 5 $\mu g\ kg^{-1}$) and, reported for the first time, 7 wheat germ oils (max 56.8 ± 2.7 $\mu g\ kg^{-1}$) expressed as the sum of 12 ergot alkaloids.

KEYWORDS: *sample preparation, SCX-resin, isomerization, vegetable oil, rye flour, HPLC*

INTRODUCTION

Species of the parasitic fungus *Claviceps*, mainly, *Claviceps purpurea* (*C. purpurea*), are known to infest commercially important grains such as rye, wheat, rice, corn, millet, and oat.[1] They produce dark colored sclerotia, which contain ergot alkaloids in largely varying concentrations and alkaloid compositions. These highly toxic secondary metabolites of the fungus can cause severe diseases such as gangrene or paresis. In the Middle Ages, many people died after consuming sclerotia-contaminated cereal products in epidemic outbreaks of ergotism.[2,3] *C. purpurea* biosynthesizes many structurally related ergot alkaloids; six are formed predominantly. These substances are divided into simple lysergamides (ergometrine) and ergopeptines (ergosine, ergotamine, ergocornine, ergocryptine (mixture of α- and β-isomers) and ergocristine). All ergot alkaloids consist of an ergoline structure, differing only in their substituents in the asymmetric C8-position. Currently, 12 ergot alkaloids are considered as a priority (Figure 1). As the C8-position can be (*R*)- or (*S*)-configured, all ergot alkaloids form epimeric pairs, whereas both epimers can be transformed into each other (Figure 2).[4] While the C8-(*R*)-isomers (suffix "-ine") show a high toxicity, the C8-(*S*)-isomers ("-inines") are considered as biologically less active or not active.[1,5]

Since the first ergot alkaloid ergotamine (**11** according to numbering in Figure 1) was isolated and structurally identified in 1918,[6] this group of mycotoxins gained increasing importance in recent years. There are several activities within EU related to ergot alkaloids: A survey of ergot alkaloids in cereals for human consumption and animal feeding showed high incidences of 95% in rye food.[7] On the basis of available data, the European Food Safety Authority (EFSA) established a tolerable daily intake (TDI) of 0.6 $\mu g\ kg^{-1}$ body weight per day (sum of ergot alkaloids).[1] Recently, the development of standardized methods for the quantitation of ergot alkaloids in food and feed was mandated to CEN.[8,9] Furthermore, the establishment of chemically based maximum levels for ergot alkaloids is currently discussed. Although details of possible regulatory limits have not been assigned, so far epimer-specific quantitations of ergot alkaloids are urgently needed. Furthermore, the native ergot alkaloid constitution of food and feed samples can be assessed in a more realistic way by keeping the epimerization at a minimum. An analytical method that quantifies both epimers separately must minimize the epimerization rate during sample preparation in order to produce accurate results for each epimer. Several different procedures have been described for the extraction of ergot alkaloids from solid samples such as flour or processed cereals based on alkaline solvent mixtures.[10−14] But also extraction methods using acidic conditions are available.[15,16] An accelerated epimerization of ergot alkaloids is common to all of the extraction procedures that use acidic or alkaline conditions.[4,17] The epimerization rate of ergot alkaloids in protic solvents such as methanol or methanol−water mixtures is much higher than in aprotic solvents, for example, acetonitrile or acetone.[18] Consequently, protic solvents and pH-modifiers should be avoided for epimer-specific analysis of ergot alkaloids. An efficient cleanup is required to remove matrix components from raw extracts in order to analyze ergot alkaloids by HPLC with fluorescence detection avoiding interferences. There are several approaches ranging from adsorbent materials such as alkaline alumina columns,[13,19] dispersive solid-phase extraction (SPE) using primary or secondary amines[10,11] to MycoSepTM 150 Ergot columns.[20] Another way is to bind ergot alkaloids to functionalized particles, removing matrix components and subsequently eluting ergot alkaloids from the resin. For this,

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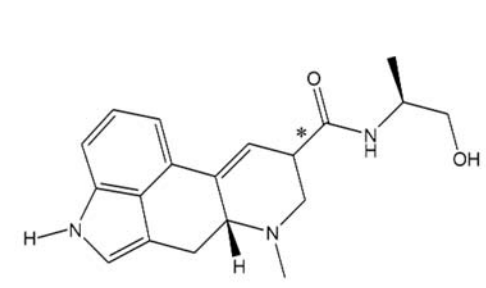
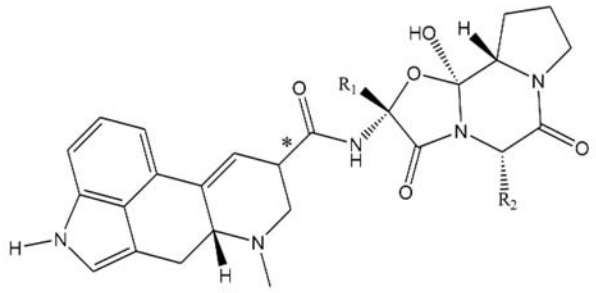


Ergot alkaloid	#	MW	Stereobond type (*)
Ergometrine	1	325.4	solid wedged
Ergometrinine	2	325.4	hashed wedged



Ergot alkaloid	#	MW	Stereobond type (*)	R_1	R_2
Ergocornine	3	561.7	solid wedged	$CH(CH_3)_2$	$CH(CH_3)_2$
Ergocorninine	4	561.7	hashed wedged	$CH(CH_3)_2$	$CH(CH_3)_2$
Ergocristine	5	609.7	solid wedged	$CH(CH_3)_2$	$CH_2C_6H_5$
Ergocristinine	6	609.7	hashed wedged	$CH(CH_3)_2$	$CH_2C_6H_5$
α-Ergocryptine	7	575.7	solid wedged	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
α-Ergocryptinine	8	575.7	hashed wedged	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
Ergosine	9	547.3	solid wedged	CH_3	$CH_2CH(CH_3)_2$
Ergosinine	10	547.3	hashed wedged	CH_3	$CH_2CH(CH_3)_2$
Ergotamine	11	581.7	solid wedged	CH_3	$CH_2C_6H_5$
Ergotaminine	12	581.7	hashed wedged	CH_3	$CH_2C_6H_5$

Figure 1. Chemical structures of the priority ergot alkaloids **1**−**12**.

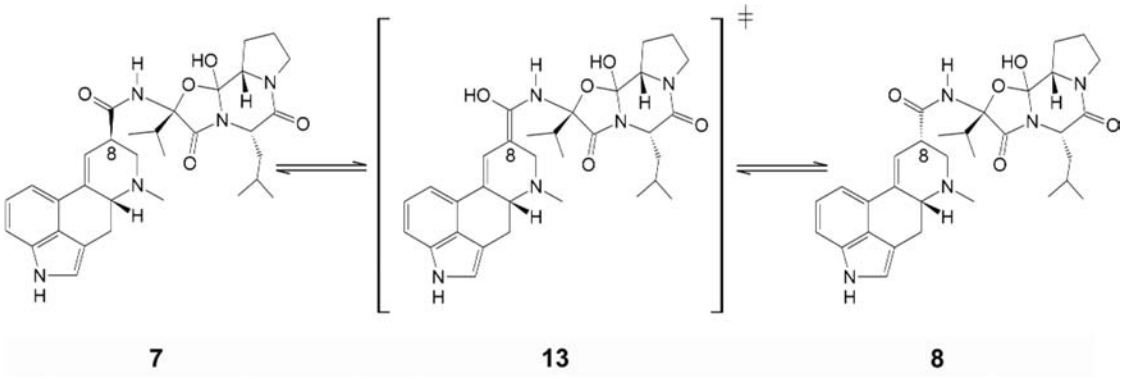


Figure 2. Epimerization reaction of ergot alkaloids at C8-position, exemplarly shown for the (*R*)-epimer **7** and the corresponding (*S*)-epimer **8** including the enol intermediate **13** at the transition state.

Chromabond C18 ec SPE cartridges,[15] Extrelut NT3 columns,[21−24] molecularly imprinted polymers (MIP),[25] and H^+-loaded strong cation exchange (H^+-SCX) columns[16] have been applied. However, all these methods use alkaline or acidic conditions posing the risk of an accelerated epimerization of the ergot alkaloids.

Many different matrices have been studied regarding their ergot alkaloid content such as flour, processed cereal products, and even vascular tissues.[10−12,15,26] To the best of our knowledge, vegetable oils that are widely appreciated for their pharmaceutical and nutritional value[27] have not been analyzed for ergot alkaloids so far. However, due to the lipophilic properties of ergot alkaloids, there is a potential risk for contaminations. The major analytical challenge consists of extraction and purification of lipophilic ergot alkaloids from the oil matrix. Size exclusion chromatography and liquid/liquid partitioning techniques are either time-consuming and/or of poor selectivity. Target-specific immunoaffinity columns are not available for ergot alkaloids and would not be applicable for oil samples without prepurification steps due to solvent incompatibility.

For the sum of reasons, the quantitative analysis of the 12 priority ergot alkaloids **1**−**12** (according to numbering in Figure 1) is focused to minimize the epimerization in order to assess the native ergot alkaloid constitution of food samples. Thus, the objective of this work was to develop a simple, fast, and efficient extraction and cleanup method and to show its suitability for solid (rye flour) and liquid (wheat germ oil) samples. The developed and validated method based on a sodium-neutralized SCX-resin was recently published as a German patent.[28] This is the first report employing a novel sample preparation method to vegetable oils which so far are have not been investigated for ergot alkaloids.

MATERIALS AND METHODS

Chemicals. All solvents were of HPLC gradient grade; standard chemicals were of analytical reagent grade. Acetonitrile and acetone were purchased from LAB-SCAN (Gliwice, Poland). Pure ergot alkaloid standard substances for calibration were obtained from Alfarma s.r.o. (Černošice, Czech Republic) and checked for purity by HPLC diode array detection (DAD) measurements. For all experiments, ultrapure water provided by a Seralpur PRO 90CN (Ransbach-Baumbach, Germany) was used. Ammonium carbamate and ammonium carbonate were supplied by Merck (Darmstadt, Germany); ammonium formate was supplied by Sigma-Aldrich (Steinheim, Germany). Macroporous H^+-SCX resin consisting of polymer-bond *p*-toluenesulfonic acid (particle size: 30−60 mesh, i.e., 250−595 μm; extent of labeling: 2.0−3.0 mol g^{-1}) was purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hexanesulfonate (IPC grade) was obtained from AppliChem (Darmstadt, Germany).

Food Samples. Nine whole rye flour samples and seven wheat germ oils were purchased in German retail markets in 2012/2013, stored at room temperature, and analyzed without further processing (e.g., milling, sieving, filtration, etc.).

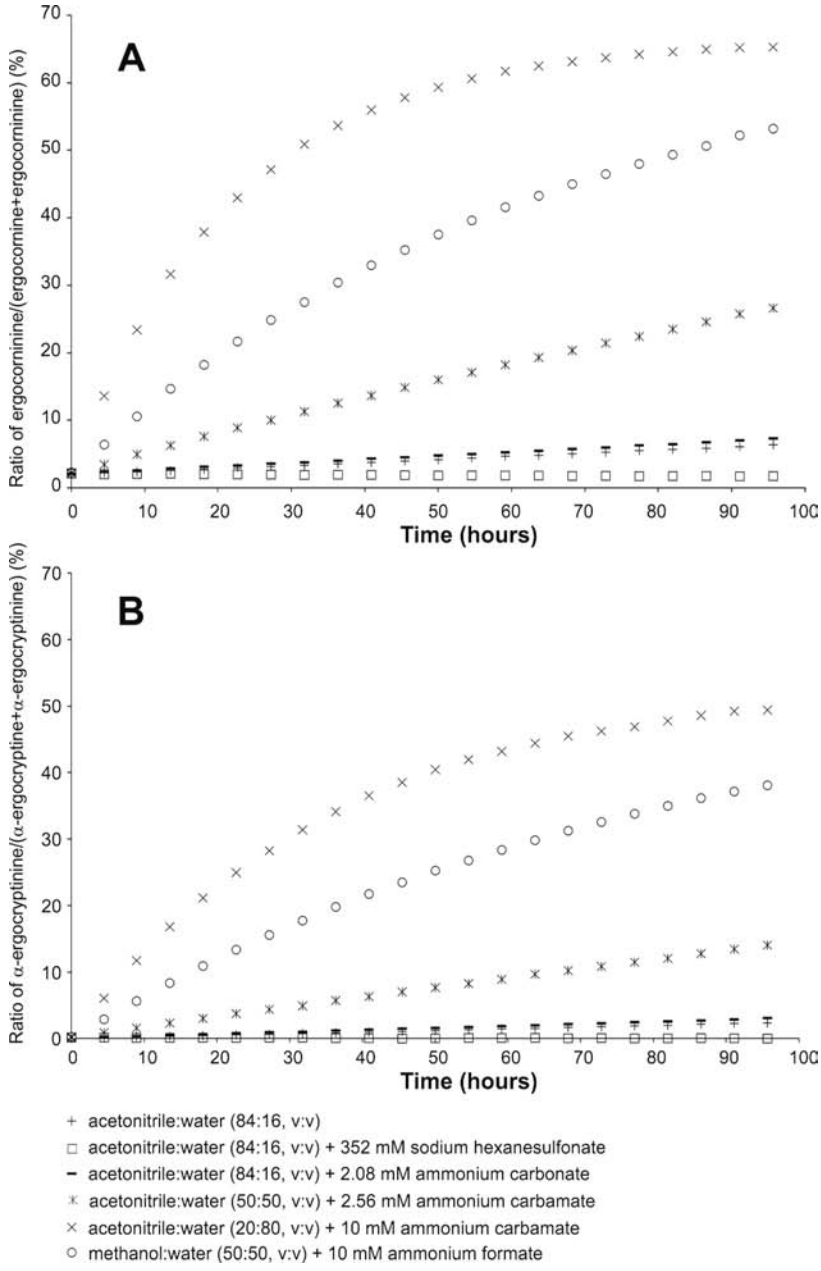


Figure 3. Epimeric shift from (*R*)- to (*S*)-epimer in different solvents over 96 h at room temperature for (A) ergocornine **3** and (B) α-ergocryptine **7**.

A contaminated rye flour material was prepared for method validation using sclerotia from private sources, which were ground by a centrifugal mill (ZM 1000, Retsch GmbH, Haan, Germany) under cooled conditions (liquid nitrogen) to a particle size of <250 μm. An amount of 3.4 g of the milled, homogenized, and analyzed sclerotia was mixed with 1 kg of blank rye flour (<250 μm) in an overhead shaker at 30 rpm for 12 h to yield a content of about 1600 μg kg^{-1} expressed as the sum of ergot alkaloids **1**−**12**. The sclerotia-contaminated rye flour was tested for homogeneity and used to investigate the extraction efficiency and the method precision.

Na^+-SCX Column for Cleanup. Prior to SPE cleanup of rye flour and wheat germ oil sample extracts, the macroporous H^+-SCX resin was Na^+ loaded for neutralization by treating with sodium hydroxide solution (4%, w/w), neutralized by washing with water, and finally washed with acetone. After drying of Na^+-SCX resin was ground by mortar and pestle and sieved to obtain a particle size fraction of 100−200 μm. A mini SPE column was prepared by filling glass wool and 200 ± 2 mg of Na^+-SCX resin in a 1 mL plastic cartridge.

Ergot Alkaloid Analysis. *Rye Flour.* 50 mL of acetonitrile/water (84:16, v:v) was added to 10 g of rye flour and shaken for 1 h using a horizontal shaker (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 300 min^{-1}. Afterward, the mixture was centrifuged (centrifuge model 6K 15, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 2605*g* for 10 min at 20 °C. An aliquot of 4 mL was loaded onto a Na^+-SCX SPE cartridge that was preconditioned with 1 mL of acetonitrile/water (84:16, v:v). After the loaded resin was washed with 5 mL of acetonitrile/water (84:16, v:v), the ergot alkaloids were finally eluted from the mini column using 4 mL of an elution mix prepared by dissolving 66.3 mg (0.352 mmol) of sodium hexanesulfonate in 1 mL of acetonitrile/water (84:16, v:v). A flow rate of approximately 0.3 mL min^{-1} was used for all SPE steps. After SPE cleanup, the purified solution was filtered through a 0.2 μm PTFE syringe filter and stored in a 2 mL amber glass vial at −20 °C prior to HPLC fluorescence analysis. Samples for method validation were analyzed six times, whereas real samples were analyzed three times.

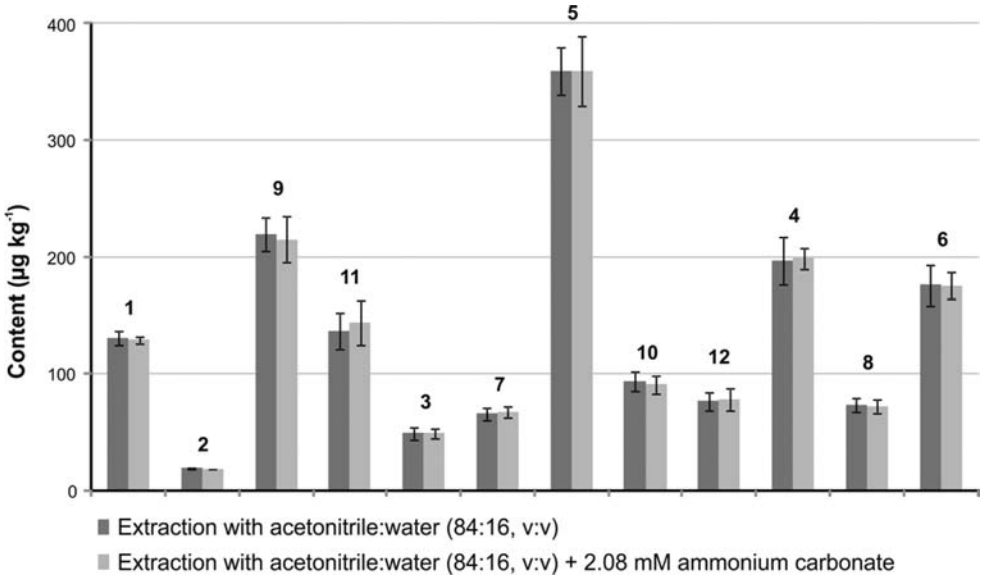


Figure 4. Extraction of sclerotia-contaminated rye flour by horizontal shaking (1 h) using acetonitrile/water (84:16, v:v) with and without the alkaline modifier ammonium carbonate. Error bars represent the standard deviation of six replicates (n = 6). Numbering of ergot alkaloids according to Figure 1.

Wheat Germ Oil. Five milliliters of acetone was added to 1 mL of wheat germ oil (weighed) and thoroughly mixed for 20 s by vortex (IKA Werke GmbH & Co. KG, Staufen, Germany). This mixture was loaded onto the Na^+-SCX SPE cartridge and washed with 5 mL of acetone. Afterward, the resin was dried for 5 min in a vacuum using a manifold assembly (Avantor Performance Materials, Griesheim, Germany). After the dried resin was conditioned with 3 mL of acetonitrile/water (84:16, v:v) the ergot alkaloids were finally eluted from the resin with the elution mix and further processed as the rye flour; see above.

HPLC Fluorescence Analysis. The HPLC system consisted of an Agilent 1200 series HPLC equipped with an autosampler, degasser, binary pump, column oven (thermostatted at 30 °C), and fluorescence detector set at λ_{ex} = 330 nm and λ_{em} = 415 nm. For experiments concerning epimerization in different solvent mixtures, the chromatographic method of Krska et al.[10] was adopted using a Phenomenex Gemini C18 column (2.1 × 150 mm, particle size 3 µm), supplemented by a guard column of the same material (4 × 2 mm). Chromatographic run parameters were as follows: injection volume: 10 µL, flow rate: 0.3 mL min^{-1}, solvent A: water modified with 200 mg L^{-1} ammonium carbonate, solvent B: acetonitrile. The following gradient was used: 0−4 min 15−36% B, 4−20 min 46−60% B, 20−22 min 60−100% B, 22−30 min 100% B, and 30−40 min 15% B (re-equilibration). For ergot alkaloid analysis after SPE cleanup, the chromatographic method of Müller et al.[13] was slightly modified using a Phenomenex Luna phenyl-hexyl column (250 × 4.6 mm, particle size 5 µm) including a Luna phenyl-hexyl guard column (4 × 3 mm). The following chromatographic parameters were applied: injection volume: 20 µL, solvent A: water modified with 200 mg L^{-1} ammonium carbamate, solvent B: acetonitrile. A flow gradient with isocratic conditions A:B 50:50 (v:v) was used: 0−17 min, 0.8 mL min^{-1} and 17−37 min, 1.5 mL min^{-1}. The applied HPLC conditions are suited to separate all analyzed ergot alkaloids **1**−**12**.

Calibration and Quantitation. A standard mix stock solution was prepared by dissolving the pure ergot alkaloids 1−12 in acetonitrile (10 mg kg^{-1} for each ergot alkaloid in solvent). For spiking experiments, the standard mix stock solution was diluted by acetonitrile, forming different spiking solution with ergot alkaloid contents of 59, 90, 878, 1456, 1501, and 2217 µg kg^{-1}. For an intermediate standard mix solution containing 100 µg kg^{-1} of each ergot alkaloid, an aliquot of the standard mix stock solution was diluted using acetonitrile. All standard and calibration solutions were prepared gravimetrically and stored at −25 °C. For quantitation measurements, an aliquot of the intermediate standard mix solution was evaporated to dryness and reconstituted in the elution mix to generate an external 10-point calibration curve (linear regression model) with contents ranging from 2 to 50 µg kg^{-1} per ergot alkaloid. Linearity in this range was demonstrated through Mandel's fitting test with a significance level of 0.05. A calibration curve was measured within each sequence. Statistical analyses were performed using the Microsoft Excel-based computer program Statist 2.0 (Statist version 2.0, Rev.4c, 2000−2003 Georg Schmitt/Michael Herbold (from 2001 ARVECON GmbH), Walldorf, Institut für Rechtsmedizin der Universität Heidelberg).

Validation. The limits of detection (LODs) and limits of quantitation (LOQs) were determined as analyte's contents corresponding to a mean +3-fold standard deviation (3 SD) of the signal area (LOD) and a mean +10 SD of the signal area (LOQ) measured for blank rye flour and vegetable oil samples. In order to check the trueness of the proposed method, recovery experiments were conducted by spiking blank rye flour and vegetable oil at three levels of ergot alkaloids using spiking solutions (n = 6 per spiking level), leading to a final ergot alkaloid content in the sodium hexanesulfonate extract solution of 2, 30, and 50 µg kg^{-1} per analyte. The solvent of the spiking solutions was allowed to evaporate for 70 min in a gentle stream of nitrogen before further sample treatment as described above. Precision measurements were performed using sclerotia-contaminated rye flour and spiked blank vegetable oil with a content of 30 µg kg^{-1} per analyte in the sodium hexanesulfonate solution as described above for recovery experiments. Both matrices were analyzed (n = 6) on five consecutive days.

RESULTS AND DISCUSSION

Method Development. *Extraction.* The first step of method development was to compare different solvent mixtures to find a solvent that prevents epimerization as much as possible. Therefore, solvents that are known from the literature for extraction, cleanup, and analysis of ergot alkaloids[10,13,14,29] were examined over a time period of 96 h at room temperature using the ergot alkaloids **3** and **7** (Figure 3). No epimerization was observed in acetonitrile/water (84:16, v:v) containing 352 mM sodium hexanesulfonate. This solution is used as elution mix for the elution step from SPE and storage of ergot alkaloid extracts as described in section Cleanup. Therefore, the solvent

yielding the second lowest epimerization rate (acetonitrile/water (84:16, v:v) was selected as candidate for extraction suitable for epimer-specific analysis. All other solvents cause considerable changes in the epimeric ratio after prolonged storage. Subsequently, the extraction efficiency was evaluated. As pK_a values of ergot alkaloids are between 4.8 to 6.2,[30] they appear uncharged at higher pH values and can then be extracted by organic solvents.[31] For this reason, most solvents used for ergot alkaloid extraction contain alkaline modifiers. The influence of a commonly used alkaline modifier, ammonium carbonate,[10−12] on the extraction efficiency was investigated for acetonitrile/water (84:16, v:v) that appeared suitable regarding minimized epimerization. For this purpose, the sclerotia-contaminated rye flour was extracted using both solvent-mixtures (Figure 4). As no significant differences were observed for all ergot alkaloids (n = 6; Student's t-test, significance level α = 0.05), further extractions were performed without an alkaline modifier to reduce the epimerization rate.

Cleanup. Pretests revealed the general suitability of H^+-SCX-resins to quantitatively bind ergot alkaloids and to remove matrix components as shown by Ware et al.[16] In order to avoid epimerization, the acidic H^+ form of the *p*-toluenesulfonic acid SCX-resin was replaced by neutral Na^+. As further optimization, the particle size of the macroporous Na^+-SCX-resin was reduced to 100−200 μm in combination with an optimized flow rate of about 0.3 mL min^{-1}. Matrix compounds could then be easily washed down from a mini SPE-column showing that 5 mL of acetonitrile/water (84:16, v:v) for rye flour (5 mL of acetone for vegetable oil) is sufficient. For elution of ergot alkaloids from the resin, the solvent−mixture should meet the following requirements: (i) quantitative elution of ergot alkaloids, (ii) minimized/no epimerization of ergot alkaloids, and (iii) direct compatibility with the HPLC system. Taking into account the prerequisites of (ii) and (iii) acetonitrile/water (84:16, v:v) was considered to be suitable as an elution mix. However, in order to quantitatively elute ergot alkaloids from the SCX-resin, another agent is necessary. For that, linear sodium alkanesulfonates were attempted because this group of anionic surfactants shows a high affinity to form ion pairs with ergot alkaloids and a sufficient solubility in acetonitrile/water mixtures as well. As these alkanesulfonates are not fluorescent, solutions can be directly measured by HPLC fluorescence analysis. After several homologous compounds of sodium alkanesulfonates were tested, a solution of acetonitrile/water (84:16, v:v) containing 352 mM of sodium hexanesulfonate yielded the best recovery results for ergot alkaloids.

Because of their nonpolar matrix, vegetable oils are principally able to dissolve lipophilic organic contaminants such as pesticides and mycotoxins in considerable amounts, for example, the *Fusarium* mycotoxin zearalenone.[32] Thus, the sample preparation described here was developed not solely for rye flour but also for vegetable oils, especially wheat germ oil. In order to avoid interactions of phosphatidylcholin contained in wheat germ oil constituent[33−35] with the SCX-resin, the method was slightly modified. Considering that phosphatidylcholin is insoluble in acetone, this compound was precipitated by diluting the oil with acetone prior to loading it onto the SCX-resin. Furthermore, for practical reasons, the viscosity of the oil was reduced by diluting. As the epimerization rate for **7** in acetone is comparably low with an epimeric shift of 1% after 2 h[18] acetone can be used for epimer-specific ergot alkaloid analysis. The mixture of acetone and vegetable oil (5:1, v:v) was directly loaded onto the Na^+-SCX column. Afterward, the oil matrix was removed through washing with acetone followed by conditioning with acetonitrile/water (84:16, v:v). For eluting ergot alkaloids, the same elution mix as for rye flour was employed.

Method Validation. The developed sample preparation method is suited to remove matrix components from rye flour and wheat germ oil very effectively as can be seen from the chromatograms (Figure 5), leading to low LOQs of 0.7−2.0 μg

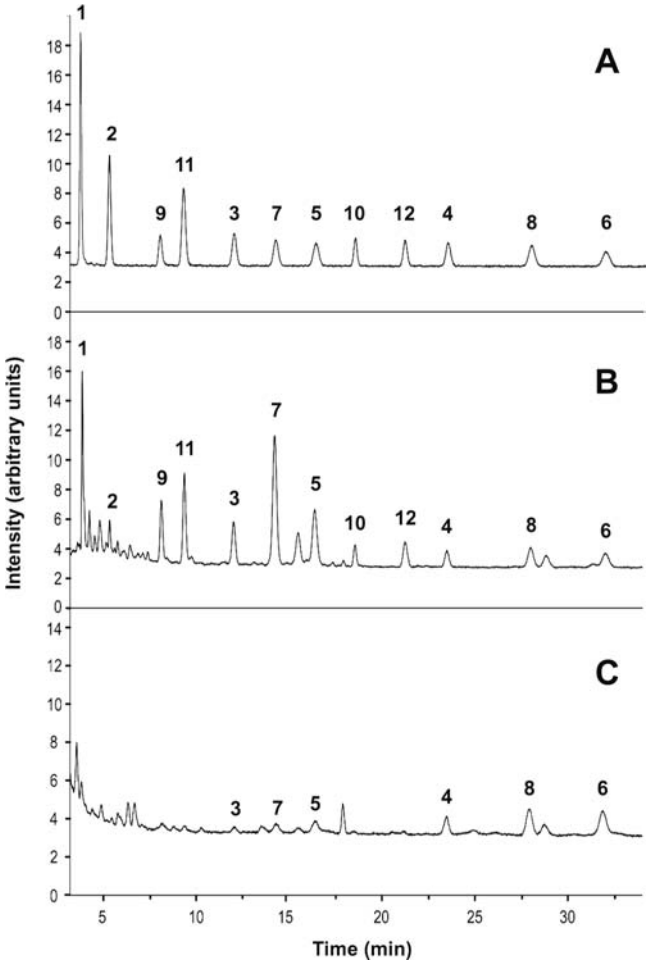


Figure 5. HPLC fluorescence chromatograms of (A) standard mix containing 5 μg ergot alkaloid per kg solvent (except **11**: 10 μg kg^{-1}), (B) rye flour sample no. 5: 178 ± 5 μg kg^{-1} (sum of ergot alkaloids **1**−**12**), and (C) wheat germ oil sample no. 5: 56.8 ± 2.7 μg kg^{-1} (sum of ergot alkaloids **1**−**12**). Numbering of ergot alkaloids according to Figure 1.

kg^{-1} for both matrices (Tables 1 and 2), which are comparable to other epimer-specific methods[13,29] using HPLC fluorescence analysis. The calibration curves were linear (Mandel's fitting test, α = 0.05) in the range of 2−50 μg ergot alkaloid per kg solvent (R^2 > 0.994) and thereby cover a range of approximately 8−212 μg kg^{-1} rye flour and 8−200 μg kg^{-1} wheat germ oil, respectively.

For recovery determination, blank rye flour and vegetable oil were spiked with ergot alkaloids in acetonitrile at three different levels (2, 30, 50 μg ergot alkaloids per kg solvent) corresponding to 8, 127, and 212 μg kg^{-1} rye flour and 8, 120, and 200 μg kg^{-1} oil. The recoveries were between 80 and 120% for rye flour (Table 1) and between 71 and 96% for oil (Table 2). Recovery rates for rye flour of previously published

Table 1. Validation Data for the Determination of Ergot Alkaloids in Rye Flour

	(μg kg^{-1})		calibration		recovery[a] (%)			precision[b] (%)	
ergot alkaloid	LOD	LOQ	R^2	s_y (a.u.)[c]	8 μg kg^{-1}	127 μg kg^{-1}	212 μg kg^{-1}	within-day	between-day
1	0.6	1.8	0.996	18.52	114.4 ± 1.6	100.9 ± 1.3	90.9 ± 0.8	6.9	2.4
2	0.5	1.7	0.994	29.79	106.4 ± 3.9	97.5 ± 4.0	87.3 ± 1.5	5.1	0.8
3	0.3	1.0	0.997	19.65	100.5 ± 1.5	86.7 ± 2.5	80.3 ± 1.2	10.5	2.6
4	0.8	1.9	0.998	2.33	97.6 ± 2.1	83.8 ± 1.1	91.0 ± 1.2	8.2	9.2
5	0.5	1.4	0.999	2.92	82.9 ± 4.2	81.4 ± 2.7	85.5 ± 1.5	7.9	2.4
6	0.8	2.0	0.998	6.38	111.8 ± 5.2	111.0 ± 1.9	104.8 ± 1.7	7.1	2.5
7	0.5	1.2	0.998	6.12	95.1 ± 5.3	89.7 ± 2.5	85.1 ± 1.1	7.1	3.7
8	0.4	1.1	0.998	6.14	103.6 ± 2.8	113.2 ± 1.5	102.6 ± 1.2	8.8	12.8
9	0.4	1.4	0.998	5.08	111.5 ± 2.0	91.8 ± 2.9	86.4 ± 1.6	8.5	3.4
10	0.7	1.9	0.998	3.60	117.5 ± 8.2	95.6 ± 2.4	97.5 ± 1.5	6.8	11.8
11	0.3	1.1	0.998	13.77	104.7 ± 1.6	86.9 ± 3.5	81.3 ± 2.0	9.0	1.4
12	0.3	0.7	0.998	7.33	107.6 ± 4.7	120.2 ± 2.3	105.6 ± 1.7	8.9	8.0

[a]Mean recoveries and their corresponding standard deviations of six independent replicates (n = 6) at three spiking levels. [b]Precision is expressed as relative standard deviation based on six independent replicates (n = 6) per day on five consecutive days. [c]s_y Residual standard deviation of the calibration curve in arbitrary units (a.u.); expression of the precision of the linear regression line.

Table 2. Validation Data for the Determination of Ergot Alkaloids in Vegetable oil

	(μg kg^{-1})		calibration		recovery[a] (%)			precision[b] (%)	
ergot alkaloid	LOD	LOQ	R^2	s_y (a.u.)[c]	8 μg kg^{-1}	120 μg kg^{-1}	200 μg kg^{-1}	within-day	between-day
1	0.3	1.0	0.996	18.52	80.1 ± 6.4	78.1 ± 2.3	87.1 ± 2.3	2.1	4.4
2	0.2	0.7	0.994	29.79	70.7 ± 2.7	75.3 ± 3.7	82.5 ± 2.2	3.6	2.9
3	0.3	0.8	0.997	19.65	72.7 ± 3.5	75.7 ± 1.9	79.5 ± 2.4	2.6	1.7
4	0.7	1.8	0.998	2.33	95.7 ± 4.1	89.6 ± 2.4	84.8 ± 0.8	2.7	1.8
5	0.7	2.0	0.999	2.92	72.7 ± 6.0	72.2 ± 0.9	76.5 ± 1.7	2.1	3.1
6	0.8	2.0	0.998	6.38	77.5 ± 6.2	86.6 ± 1.7	87.9 ± 1.4	2.3	2.2
7	0.7	1.9	0.998	6.12	78.0 ± 7.5	78.5 ± 2.1	82.0 ± 4.1	5.0	2.6
8	0.5	1.4	0.998	6.14	89.4 ± 5.9	84.7 ± 1.8	86.5 ± 1.1	2.3	1.5
9	0.6	2.0	0.998	5.08	75.8 ± 3.1	74.7 ± 2.2	79.1 ± 2.3	2.9	2.6
10	0.6	1.8	0.998	3.60	82.3 ± 3.5	84.8 ± 1.2	86.5 ± 0.7	2.0	0.8
11	0.3	0.9	0.998	13.77	75.7 ± 2.4	71.9 ± 2.0	78.4 ± 2.9	2.8	3.3
12	0.3	0.8	0.998	7.33	78.0 ± 5.5	84.2 ± 1.5	85.5 ± 1.0	1.5	1.0

[a]Mean recoveries and their corresponding standard deviations of six independent replicates (n = 6) at three spiking levels. [b]Precision is expressed as relative standard deviation based on six independent replicates (n = 6) per day on five consecutive days. [c]s_y Residual standard deviation of the calibration curve in arbitrary units (a.u.); expression of the precision of the linear regression line.

epimer-specific methods were in a range of 89.3−99.8% (only (*R*)-epimers without **9**)[13] and 64−97%,[21] respectively. It was apparent that the mean ergot alkaloid recovery determined for rye flour (97%) is significantly higher than that for vegetable oil (81%) as confirmed by Student's *t*-test (α = 0.05). This effect could be due to the change of solvent during the sample preparation procedure. However, optimization experiments revealed that drying of the resin, that is, removing acetone before applying acetonitrile/water (84:16 v:v) is essential. Without removal of acetone, recoveries were quite low (approximately 22%).

The method precision (within- and between-day) was determined using sclerotia-contaminated rye flour and spiked blank oil. These matrices were analyzed on five consecutive days with six replicates per day yielding a within-day precision of 5−10% and a between-day precision of 1−13% for rye flour (Table 1) and 2−5% for within-day and 1−4% for between-day precision for the oil matrix (Table 2). The slightly lower within and between-day precision values found for vegetable oil could be caused by better homogeneity of ergot alkaloids in liquid oil compared to the sclerotia-contaminated rye flour even in case of a thorough homogenization.

Method Application to Commercial Food Samples. In order to demonstrate the suitability of the method intended to analyze solid and liquid samples as well, commercial food samples (nine rye flours and seven wheat germ oils) were purchased in German retail markets to be analyzed for their ergot alkaloid contents. The maximum content found for rye flour sample no. 5 (178 ± 5 μg kg^{-1} given as sum of ergot alkaloids **1**−**12**; Table 3) is comparable to previously reported data from a sample survey in 2004 (260 μg kg^{-1})[24] but significantly lower than results from German harvest in 2003 (818 μg kg^{-1}).[24] However, statistical conclusions about the occurrence of ergot alkaloids in rye flour depending on the harvest year cannot be drawn due to the limited number of samples used in the present study, which rather show the applicability of the new method. In a recent interlaboratory comparison study for reference material certification, conducted by BAM and the Federal Institute for Risk Assessment (BfR), rye flour sample no. 7 was used as "blank flour" to determine the ergot alkaloid recoveries of the methods used at a participant's site. Most of the participants applied the standard procedure according to the German food law[36] based on the method of Müller et al.[13] The preliminary mean value of rye flour sample no. 7 from the interlaboratory comparison study

Table 3. Ergot Alkaloid Contents (μg kg^{-1}) in Nine Rye Flours and Seven Wheat Germ Oil Samples from German Retail Markets[a]

		ergot alkaloid					
sample	#	**1**	**2**	**3**	**4**	**5**	**6**
rye flour	1	3.2 ± 0.4	n.d.	1.4 ± 0.4	n.d.	2.5 ± 0.4	n.d.
	2	(1.1 ± 0.2)	n.d.	(0.7 ± 0.2)	n.d.	3.8 ± 1.1	n.d.
	3	8.7 ± 0.1	3.5 ± 0.1	6.6 ± 1.0	18.7 ± 3.1	24.8 ± 2.7	10.4 ± 0.4
	4	2.3 ± 0.4	n.d.	n.d.	n.d.	4.4 ± 0.2	n.d.
	5	8.5 ± 0.9	3.3 ± 0.4	13.1 ± 0.1	8.4 ± 0.3	24.5 ± 1.2	12.1 ± 0.6
	6	2.3 ± 0.1	2.3 ± 0.1	4.2 ± 0.1	3.0 ± 0.1	7.6 ± 0.8	4.7 ± 0.4
	7	4.0 ± 0.1	n.d.	3.8 ± 0.1	n.d.	n.d.	3.3 ± 0.8
	8	n.d.	n.d.	n.d.	4.3 ± 1.5	7.5 ± 1.2	5.4 ± 0.5
	9	5.0 ± 0.2	3.3 ± 0.1	7.9 ± 0.9	5.4 ± 0.3	11.3 ± 1.4	(1.9 ± 0.6)
wheat germ oil	1	n.d.	n.d.	(0.9 ± 0.1)	n.d.	n.d.	n.d.
	2	n.d.	n.d.	1.0 ± 0.1	18.3 ± 0.6	n.d.	5.0 ± 0.3
	3	n.d.	n.d.	n.d.	12.0 ± 0.7	n.d.	7.4 ± 0.4
	4	n.d.	n.d.	1.5 ± 0.3	7.8 ± 0.9	n.d.	6.3 ± 0.2
	5	n.d.	n.d.	2.6 ± 0.1	9.0 ± 0.1	6.9 ± 0.7	18.6 ± 1.1
	6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	1.8 ± 0.1	n.d.	4.3 ± 0.4	n.d.	6.1 ± 0.5

		ergot alkaloid					
sample	#	**7**	**8**	**9**	**10**	**11**	**12**
rye flour	1	n.d.	n.d.	4.2 ± 0.8	n.d.	2.7 ± 0.4	n.d.
	2	n.d.	n.d.	n.d.	n.d.	2.0 ± 0.5	n.d.
	3	12.7 ± 0.4	8.4 ± 1.6	20.4 ± 1.8	10.3 ± 0.9	16.6 ± 0.5	2.7 ± 0.7
	4	n.d.	n.d.	3.4 ± 0.6	n.d.	3.2 ± 0.3	n.d.
	5	39.0 ± 0.4	5.4 ± 0.7	18.6 ± 1.1	5.6 ± 0.8	23.0 ± 0.8	16.3 ± 0.8
	6	4.6 ± 0.4	n.d.	7.9 ± 1.0	(1.9 ± 0.5)	8.1 ± 0.7	24.9 ± 0.7
	7	3.5 ± 0.1	(0.6 ± 0.4)	5.6 ± 0.3	(0.9 ± 0.1)	5.7 ± 0.1	9.2 ± 0.3
	8	n.d.	n.d.	6.0 ± 0.6	6.8 ± 1.4	4.0 ± 0.4	6.0 ± 0.3
	9	6.5 ± 0.5	(1.1 ± 0.2)	16.3 ± 1.3	4.7 ± 0.5	8.5 ± 0.5	15.0 ± 0.3
wheat germ oil	1	2.4 ± 0.2	n.d.	n.d.	n.d.	n.d.	(0.5 ± 0.2)
	2	2.5 ± 0.1	4.9 ± 0.3	n.d.	n.d.	n.d.	n.d.
	3	2.5 ± 0.2	3.3 ± 0.1	n.d.	2.5 ± 0.6	n.d.	1.4 ± 0.1
	4	2.2 ± 0.2	3.2 ± 0.3	n.d.	n.d.	n.d.	n.d.
	5	5.2 ± 0.1	14.5 ± 0.3	n.d.	n.d.	n.d.	n.d.
	6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	3.4 ± 0.2	n.d.	n.d.	n.d.	n.d.

[a]The mean values and their corresponding standard deviations are given based on three independent replicates (n = 3). n.d.: not detectable, that is, lower than LOD as stated in Tables 1 and 2. ( ): detectable, that is, higher than LOD, but not quantifiable, that is, lower than LOQ.

(30.2 ± 7.9 μg kg^{-1}) confirms the result obtained by the novel SCX-resin method (35.1 ± 1.9 μg kg^{-1}) expressed as sum of ergot alkaloids **1**−**12**.

The results of ergot alkaloids in commercial wheat germ oils (Table 3) indicate lower contents and fewer incidences compared to rye flour. The maximum level of 56.8 ± 2.7 μg kg^{-1} given as the sum of ergot alkaloids **1**−**12** found for wheat germ oil sample no. 5 is about one-third of the highest contaminated rye flour sample. A more detailed assessment of the current results will be possible when future data on the occurrence and distribution of ergot alkaloids are available for vegetable oils.

Significantly different distributions of the ergot alkaloids in rye flour and wheat germ oil samples could be observed (Table 3). In contrast to rye flour, an obvious shift toward the more lipophilic compounds of the ergot alkaloids was detected for the nonpolar wheat germ oil samples. Analyte's lipophilic properties correspond to rising retention times using a reversed-phase HPLC column (Figure 5). So in wheat germ oil sample no. 5 the highest content was analyzed for **6**, the most lipophilic compound indicated by the longest retention time (and the highest molecular weight).

A novel analytical procedure was developed for the quantitation of ergot alkaloids in rye flour and wheat germ oil based on a recently patented solid-phase (Na^+-SCX-resin) extraction and cleanup method. The sample preparation is achieved by omitting acidic or alkaline modifiers during extraction and cleanup enabling minimized epimerization rates and epimer-specific analysis of ergot alkaloids. By analyzing the ergot alkaloids (*R*)- and (*S*)-epimers according to their natural distribution in foods, an important contribution is given to evaluate the occurrence of ergot alkaloids as a basis for an improved risk assessment. The developed sample preparation method was seen to be applicable for rye flour as the most significant matrix for ergot alkaloid analysis, and for wheat germ oil, a complex matrix that has not been analyzed for ergot alkaloids before.

In conclusion, the issue of epimer-specific ergot alkaloid quantitation gains increasing importance in view of current

activities to setup maximum levels, to establish standardized methods, and to develop certified reference materials.

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Notes

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ABBREVIATIONS USED

SCX, strong cation exchange; SPE, solid-phase extraction

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