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Ergot alkaloids in cereal products

Results from the Bavarian Health and Food Safety Authority

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The danger of ergot alkaloids has been well known for centuries. The origin and toxicology of these compounds have been clarified and flour mills assure us that they have mastered the problem technologically. Nevertheless, one can find food products with sometimes relatively high contents of these toxic alkaloids. Here we report on our experiences and results of an LC-MS/MS-method which we have developed to determine ergot alkaloids and which was applied to commercial products during the past two years.

Keywords: Cereal products / Ergot alkaloids / LC-MS/MS / Regulation

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1 Introduction

Ergot alkaloids produced by fungus from Clavicipitaceae family, mostly by *Claviceps purpurea*, are responsible for different toxic syndromes and poisonings after ingestion of grain that has been infested by Claviceps spp. To limit the risk for poisonings the authorities intend to minimise the ergot content of grain. According to EU regulation 824/2000 a limiting value of 0.05% w/v of ergot is valid within the EU for interventional grain [1], but not for consumption grain. (The EU has established an intervention system, in order to stabilise the markets and ensure a fair standard of living for the agricultural community in the cereals sector [2]. Through this system the EU dictates certain standards in grain for interventional affairs within the common market, which individual countries can accept also for consumption grain).

In Germany this value is accepted also for consumption grain. However, since the total alkaloid content within each single ergot shows significant variations (between 0.01 and 1% w/v), no limiting value for maximum ergot alkaloids can be derived from the maximum value for ergot. But, if one assumes an average alkaloid content of 0.2% w/v in the ergot, the legislative ergot value of 0.05% w/v in grain leads to a guidance level of about 1000 µg/kg ergot alkaloids in cereal. So, German law has no limiting value for ergot alkaloids in cereal and cereal products, while in other countries, for example Switzerland, a limiting value of 100 µg/kg ergot alkaloids in cereal has been discussed recently [3].

In 1918 ergotamine has been the first ergot alkaloid isolated by Stoll *et al.* [4, 5]. Since that time about 50 different ergot alkaloids have been found and analysed with different methods [6]. The most common used are CE [7], MS [6, 8, 9], HPLC [10 – 15] and in recent years, also LC-MS methods [16 – 19].

After several press reports and official warning messages [20, 21, 22] about strongly increased ergot alkaloid contents in rye flour and rye products we tried to established a detection method in mid-2004, which uses a simple sample preparation and facilitates a specific detection of ergot alkaloids also in cereal products. We decided to use an extraction method simplified to that of Scott *et al.* [11] and Klug *et al.* [14] and analysed these extracts with an LC-MS/MS-method in ESI-positive mode on a triple-quadrupole mass spectrometer modifying the work of Bockhorn *et al.* [18]. With this method we could determine the content of ergometrine, ergotamine, ergocristine, ergocornine and α-ergocryptine, referring to as 'sum of ergot alkaloids' or 'ergot alkaloid content' in the following, having in mind, that these are only the main alkaloids in ergot, without testing for ergovaline or other possible ergot alkaloids. Up to now 66 rye products have been examined.

2 Materials and methods

2.1 Chemicals and materials

Ergometrine, ergocornine, α-ergocryptine and ergocristine were purchased from Sigma-Aldrich (Taufkirchen bei München, Germany). Ergotamine-D-tartrate was purchased

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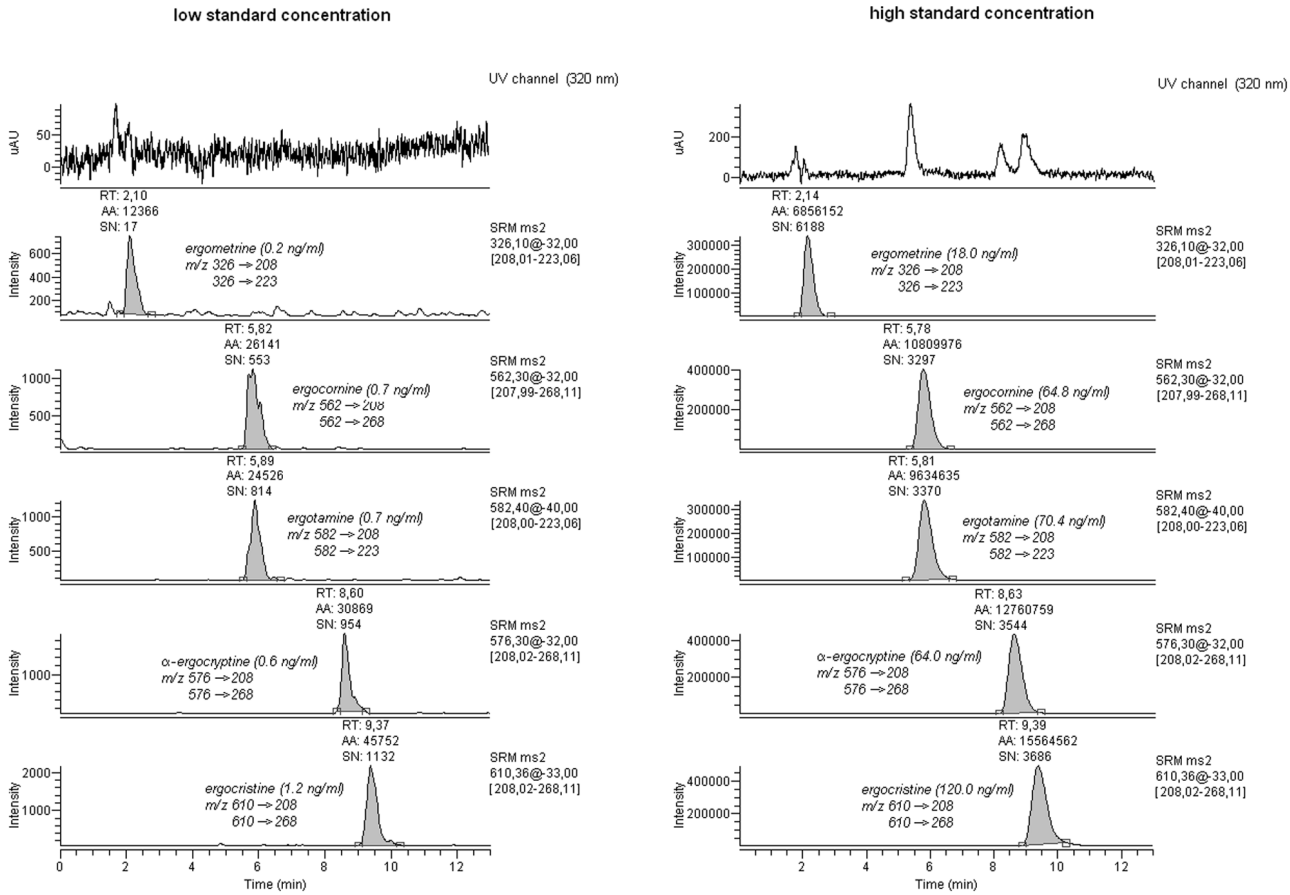


Figure 1. LC-MS/MS chromatograms of two standard mixtures each containing five ergot alkaloids in ESI-positive mode (the signal sum of two fragments is recorded, *cf.* Table 1). First trace showing the UV-channel at 320 nm; second trace: ergometrine, 0.2 resp. 18.0 ng/mL; third trace: ergocornine, 0.7 resp. 64.8 ng/mL; fourth trace: ergotamine, 0.7 resp. 70.4 ng/mL; fifth trace: α-ergocryptine, 0.6 resp. 64.0 ng/mL; sixth trace: ergocristine, 1.2 resp. 120.0 ng/mL (RT, retention time (min); AA, automatically integrated area; MA, manually integrated area; SN, signal to noise ratio at the peak maximum; NA, not available, SRM ms2 326.10@32.00 [208-01-223-06] = fragmentation of precursor ion 326.10 amu in selected reaction mode with a collision energy of 32 eV into two fragments with a mass of 208.01 and 223.06 amu).

from Fluka (Buchs SG, Switzerland). Formic acid, ammonium acetate and formiate (25%) were purchased from Riedel-de-Haen (Fluka). Methanol was purchased from Merck (Darmstadt, Germany). Dichloromethane and ethyl acetate were purchased from Roth KG (Karlsruhe, Germany). All chemicals were in purris. p.a. grade.

Purified, deionised water was produced using a Milli-Q gradient system (Millipore, Eschborn, Germany).

2.2 Sample preparation and extraction

The samples were ground with a commercial mixer (Moulinette, Moulinex France). Ground sample (2.5 g) was transferred into a centrifuge tube with screw cap. Twenty microlitres extraction solution (dichloromethane:ethyl acetate:methanol:ammonia (25% w/v; 50 : 25 : 5 : 1 v/v/v/v) was added according to Scott *et al.* [11] and Klug *et al.* [14]. The extraction solution was mixed for 1 h on a horizontal shaker (150 rpm) and subsequently centrifuged for 5 min at 3500 rpm. The supernatant was separated and 0.5 mL of the supernatant was transferred into a vial. The solvent was removed with a prewarmed gentle stream of nitrogen (60°C). The residue was reconstituted in 1.0 mL of methanol : 0.01 M aqueous ammonium formiate (1 : 1 v/v) and filtered into a brown glass autosampler vial through a 0.45 μm filter (Pall minispike, GHP-membrane).

2.3 LC-MS/MS analysis

The LC-MS/MS analysis was carried out on a Thermo Electron Surveyor HPLC with quarternary narrowbore MS-pump and a Thermo Electron Triple-Quadrupole Mass Spectrometer (TSQ Quantum Discovery), fitted with an

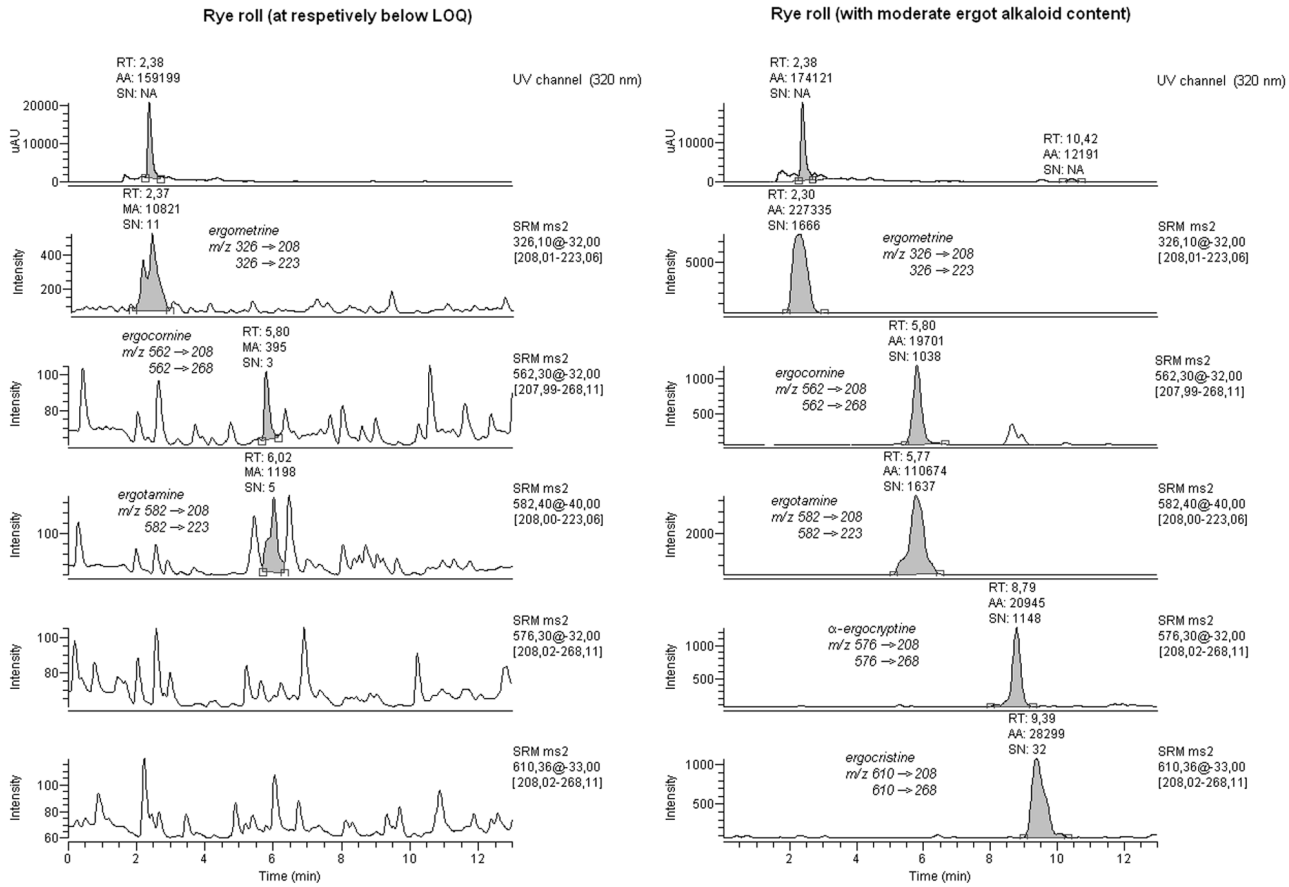


Figure 2. LC-MS/MS chromatogram of a rye roll test having a sum of ergot alkaloids of 30.7 μg/kg (right side) compared to a rye roll test showing signals of ergot alkaloids below the LOQ with the exception of ergometrine (0.4 μg/kg) (left side).

ESI source. The separation was achieved with an RP C18–phase (Phenosphere Next, 150 mm × 2 mm, 3 μm) using a guard column (10 mm × 2 mm) filled with the same material, both Phenomenex (Aschaffenburg, Germany). The mobile phase consisted of 50% methanol, 50% H_2O + 0.1% HCOOH v/v/v, run isocratic for 13 min with a flow of 0.2 mL/min. The injection volume was 2 μL.

The LC-MS/MS worked in ESI-positive mode under the following experimental conditions: capillary temperature 365°C, sheath gas flow (N_2) 0.6 L/min, auxillary gas flow (N_2) 4.5 L/min, spray voltage 3500 V and collision gas pressure (Ar) 1.5 mTorr. The dwell time was set to 0.1 s and the collision energies were set between 32 and 40 eV as shown in Figs. 1, 2.

Fragmentation chromatograms as shown in Fig.1 were obtained by operating in MS/MS mode measuring the sum of two transitions for each alkaloid (*e.g.*, ergometrine: *m/z* 326.1 → 208.0 together with *m/z* 326.1 → 223.1). Each alkaloid was identified according to the corresponding mass transitions and retention time (see Table 1 and Figs. 1, 2). The scan width was set to ±0.01 amu and the retention time was matched to ±0.5 min (except ergometrine, where retention time was matched to ±0.25 min of the corresponding standard).

The standard calibration curve (Fig. 3) was achieved from standard mixtures according to Table 2.

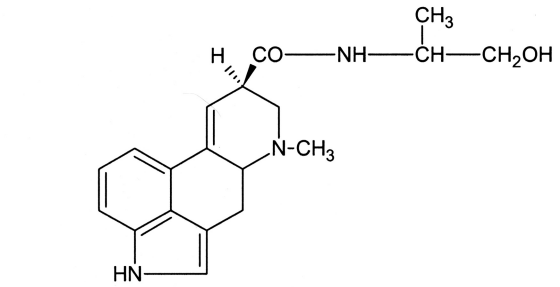
The LOQs were determined between 0.1 and 1.0 μg/kg with this method according to Table 3. They were marked when the S/N exceeded a value of ten measured with sample matrix.

The recovery with this method was estimated in the range of 62–97% with different matrices. Mean values were between 65 and 82%, always showing the poorest values at ergometrine and ergocristine. For example see Table 4.

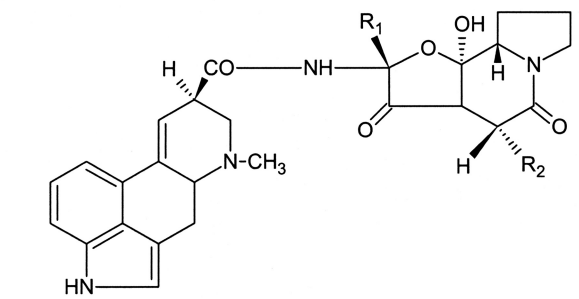
3 Results

None of the analysed samples showed an ergot alkaloid content of more than 1000 μg/kg (*cf.* Figs. 4, 5).

Table 1. Structure of ergot alkaloids and their specific fragments



I: **ergometrine**



II:

Ergot alkaloids	Empirical formula	$[M + H]^+$	Measured fragments (*m/z*)	Referring to structure	
				I	
				II	
				R_1	R_2
Ergometrine	$C_{19}H_{23}N_3O_2$	326.1	208.0 223.1		
Ergocornine	$C_{31}H_{39}N_5O_5$	562.3	208.0 268.1	$-CH(CH_3)_2$	$-CH(CH_3)_2$
α-Ergocryptine	$C_{32}H_{41}N_5O_5$	576.3	208.0 268.1	$-CH(CH_3)_2$	$-CH_2-CH(CH_3)_2$
Ergotamine	$C_{33}H_{35}N_5O_5$	582.3	208.0 223.1	$-CH_3$	$-CH_2$-phenyl
Ergocristine	$C_{35}H_{39}N_5O_5$	610.3	208.0 268.1	$-CH(CH_3)_2$	$-CH_2$-phenyl

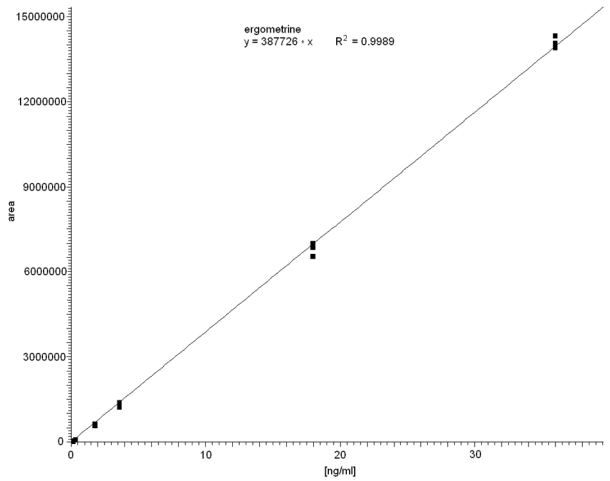


Figure 3. Example calibration curve for ergometrine.

The highest contents were recorded in the group of 23 rye breads. Here ergot alkaloid contents greater than 10 µg/kg were detected in 14 of 23 samples (>50%; Fig. 4). Up to now a mixed-grain bread (Roggenmischbrot) showed the highest value with 258 µg/kg (Fig. 5).

In addition, we tested 20 pumpernickels, which were requested due to a communication of increased ergot alkaloid levels in these products [23]. Apart from three samples,

Table 2. Reference standard dilution (in: methanol:0.01 M aqueous ammonium formiate (1 : 1 v/v))

	Mix 1 (ng/mL)	Mix 2 (ng/mL)	Mix 3 (ng/mL)	Mix 4 (ng/mL)	Mix 5 (ng/mL)	Mix 6 (ng/mL)
Ergometrine	0.2	0.4	1.8	3.6	18.0	36.0
Ergocornine	0.7	1.3	6.5	13.0	64.8	129.6
Ergotamine	0.7	1.4	7.0	14.1	70.4	140.8
α-Ergocryptine	0.6	1.3	6.4	12.8	64.0	128.0
Ergocristine	1.2	2.4	12.0	24.0	120.0	240.0

all the other achieved values of pumpernickel were at the LOQs, which were determined to be between 0.1 and 1.0 µg/kg by this method (*cf.* Table 3, the LOQs were marked when the S/N ratio exceeded a value of 10). The highest alkaloid content within the pumpernickel samples was measured as 47 µg/kg and with that far under the contents in the warning message (stated to be 2294 µg/kg).

The results in rye-crispbread were similarly inconspicuous. Only a single sample with 28 µg/kg (out of 14 tested samples) showed a slightly increased sum of ergot alkaloids.

Till date we examined nine rye-bread rolls. Three of them had striking contents (11, 31 and 91 µg/kg). But here also the predominant part of the samples was proved to be almost free from ergot alkaloids.

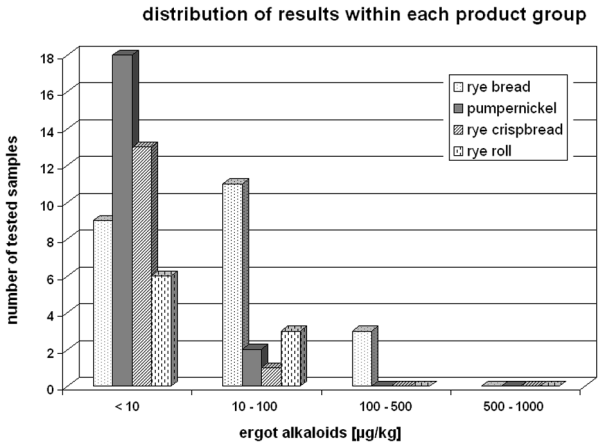


Figure 4. Distribution of ergot alkaloids in each tested product group.

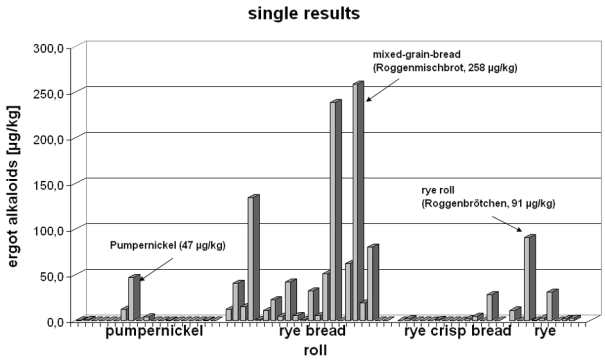


Figure 5. Grouped single results of all samples.

Table 3. LOQ of ergot alkaloid determined in pumpernickel and crisp bread

	LOQ (µg/kg)
Ergometrine	0.3
Ergocornine	0.1
Ergotamine	0.5
α-Ergocryptine	0.5
Ergocristine	1.0

Table 4. Recovery with different matrices

	Recovery (%)				
	Ergo-metrine	Ergo-cornine	Ergo-tamine	α-Ergo-cryptine	Ergo-cristine
Rye bread	74	78	86	77	71
Rye bread	90	97	92	89	87
Pumpernickel	62	87	77	93	69

4 Concluding remarks

To recapitulate, among 66 examined products there was no extremely conspicuous sample to be mentioned, but especially the product group of the rye breads showed a remarkable high percentage of samples with measurable ergot alkaloids, indicating that there is still a need to control the market.

To date we have analysed only a single rye product which had to be baked before consuming it. We specifically tested a rye roll before and after the baking process. As might be expected, there was a 25% decrease in ergot alkaloid content during the heating process. Future efforts will have to concern with this effect. We plan to compare the alkaloid content of raw dough and baked bread or rolls made of the tested dough and additionally compare it to the basic alkaloid content of the rye flour. The method of extraction has to be improved.

5 References

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