Instruction of the Clean-up Process Using SENSI*Column* IAC Ergot Alkaloids 3ml

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Clean-up of Commodity Extracts of Food and Feed Samples containing 12 Priority Ergot Alkaloids Via Immunoaffinity Chromatography and Subsequent HPLC Analysis with Fluorescence Detection

1. Target of 12 priority Ergot Alkaloids:

The most abundant ergot alkaloides produced by fungus *Claviceps purpurea* comprise six pairs of epimeric ergot alkaloides which can be classified as priority ergot alkaloides[1]. They form the target of **SENSI*Column* IAC Ergot Alkaloids 3ml** column.

C8-(*R*)-Epimers (toxic)	C8-(*S*)-Epimers (not toxic)
Ergometrine	Ergometrinine
Ergotamine	Ergotaminine
Ergosine	Ergosinine
Ergocornine	Ergocorninine
Ergocryptine	Ergocryptinine
Ergocristine	Ergocristinine

Because both epimers can change to each other under certain conditions, e.g. under acidic or basic influence, in nature and/or during sampling/extraction/analysis, the non-toxic C8-(*S*)-epimers have to be included in determination as well.

2. Principle:

This instruction of priority ergot alkaloid determination in food and feed focuses on the enrichment step of extract using immunoaffinity column (IAC) and quantification with HPLC.

Accepted laboratory extraction and IAC treatment methods could essentially be maintained. Full performance of the IAC column is given if pronounced criteria of organic solvent tolerance, elution process and working range of **SENSI*Column* IAC Ergot Alkaloids 3ml** column is followed.

Many pretreatment methods of ergot alkaloids determination in food and feed show low sensitivity because of interfering substances if problematic matrices are applied.

This method of content determination of ergot alkaloids combines the high selectivity of an immunoaffinity column (IAC) with its potential to concentrate analytes and additional step of purification by HPLC column.

Please notice that this instruction focuses on the handling with the IAC column. For the commodity extraction step a literature method is given. The given apparatus (e.g. HPLC system) might serve as an example among other possibilities.

3. Protocol:

3.1. Extraction (Literature method given):

The procedure of Krska et al.[2] is used in a modified version as follows.
25g sample, e.g. ground, rye flour etc., is extracted with 100ml acetonitrile/water (84/16 v/v) for 30min in an horizontal shaker at room temperature and the extract is filtered through Whatman No. 54 filter paper.

3.2. Enrichment Step IAC:

4ml filtered extract (see above, contains the quantity of aflatoxins of 1g sample) are diluted with 36ml 50mM PBS (pH=7.4) and then applied in a reservoir on top of the **SENSI*Column* IAC Ergot Alkaloids 3ml** column.

To maintain full performance of the column, please take care that proportion of dilution buffer in the solution on top of the column is not to small. A proportion of 8% acetonitrile, resulting in this example enrichment, does not affect column performance.

Organic solvent content of diluted extract to be applied on the column may not exceed 15%!

Caution! Be aware that no big air bubbles are neither in the gel nor between gel and luer lock outlet of column

If organic solvent proportion lies above these limits, recovery rates are diminished. Increase of diluted extract volume by diluting extract with additional PBS, on the other hand, has almost no consequences to column performance.

If samples are to be prepared simultaneously, manifold of J.T. Baker for 12 samples has proven of value. Rate of flow through the affinity gel is 1 to 3 ml/min. In case of problematic matrices rate of flow should lie below 2ml/min.
Depending on application and on expected contents, larger or smaller extract aliquots can be applied. In such cases the sample calculation (see below) must be adapted.

3.3. Wash:
After whole sample has passed through the gel the latter is washed with 5ml of 10mM PBS/Methanol (90/10 v/v). Remaining liquids in the gel are removed by applying either pressure from top of the column or underpressure from bottom.

3.4. Elution:
Sample reservoir on top of the **SENSI*Column* IAC Ergot Alkaloids 3ml** column is removed and an appropriate vial is placed below the affinity column. The bound toxins are eluted by using a total of 2ml of methanol as elution solvent. The elution process is performed in two steps to ensure complete release of analytes. First, a volume of 1ml elution solvent is applied. After that volume has passed through column half a minute is waited before the second portion of 1ml of elution solvent is eluted through the column. The flow rate of the elution process should not exceed 1ml/min very much. Remaining solvent solutions should be eluted by application of slight under- or overpressure. Both methanolic fractions are unified to give the column eluate.

The column eluate may be injected into the HPLC directly after diluting with water 1:1.

Alternatively, in case contents are low (<20ng/g), the column eluate may be concentrated by evaporation (e.g. using VLM evaporator at 50°C under a permanent stream of nitrogen).

The residue then is redissolved in HPLC solvent (e.g. 1ml) and an aliquot is finally injected into the system.

4. IAC Column Characteristics:

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4.1. Working Range and Recovery Rates of SENSI*Column* IAC Ergot Alkaloids 3ml Column:

Working Range of Column:	upto 1200ng Ergot Alkaloids per IAC
Zero Contamination of Column:	<2ng (LOD of HPLC-FLD method)
Guaranteed Recovery Rates(*) within the Working Range:	
Ergot Alkaloids (12 compounds):	70%
Ergometrine	100%
Ergometrinine	8%
Ergotamine	96%
Ergotaminine	67%
Ergosine	76%
Ergosinine	66%
Ergocornine	84%
Ergocorninine	63%
Ergocryptine	51%
Ergocryptinine	65%
Ergocristine	95%
Ergocristinine	68%

(*) Recovery rates are confined to the IAC separation procedure only (not including recovery rates of extraction process) and to organic solvent contents of diluted extract below 15% methanol or 10% acetonitrile in the manner as described in this instruction

4.2. Cross Reactivities(**) of SENSI*Column* IAC Ergot Alkaloids 3ml Column:

Ergometrine	100%
Ergometrinine	7%
Ergotamine	81%
Ergotaminine	44%
Ergosine	74%
Ergosinine	53%
Ergocornine	59%
Ergocorninine	58%
Ergocryptine	39%
Ergocryptinine	60%
Ergocristine	71%
Ergocristinine	63%

(**) Recovery rate of the 12 priority ergot alkaloids divided by recovery rate of ergometrine if a total of 1.2µg ergot alkaloids with equimolar ratio of each compound is analysed per column obeying the iac procedure described hereto.

4.3. Dynamic Capacity (***) of SENSI*Column* IAC Ergot Alkaloids 3ml Column:

Maximum Column Capacity:	2.3µg Ergot Alkaloids

(***) An excess of ergot alkaloids, e.g. a quantity of 6µg, with equimolar ratio of each is analysed per column following the IAC procedure described hereto.

5. HPLC Method:

5.1. HPLC Analytical Method:

HPLC: Shimadzu; Column: Thermo Scientific BDS Hypersil C18, 3µm, 100x4.6mm with guard column; Mobile Phase A: acetonitrile / water (90/10 v/v); Mobile Phase B: acetonitrile / 2mM ammonium carbonate (20/80 v/v); Gradient: 0.01 min B 100 %; 2 min B 50 %; 20 min B 20 %; 20.1 min B 100 %; Flow Rate: 1ml/min; Time of Analysis: 30min; Injector Volume: 100µl; Fluorescence-Detection: λ_{EX} [nm]: 362nm; λ_{EM} [nm]: 440nm. Temperature: Machine and eluents are at room temperature. Eluents are degassed with helium gas.

5.2. HPLC Method Characteristics:

The characteristics of the HPLC Method are summarized in the following table.

Example of HPLC Analytical Parameters for 12 ergot alkaloids with the described system

Ergot Alkaloid	Sensitivity [AREA/ng]	$t_{Ret.}$ [min]	LOD (S/N=3) [ng/g]	Linearity R^2 (5-100ng ea/g)
Ergometrine	32356	3.903	2,3	0,9937
Ergometrinine	76131	7.193	1,0	0,9912
Ergotamine	69284	8.976	1,1	0,9952
Ergotaminine	80729	11.372	0,9	0,9861
Ergosine	63333	8.705	1,2	0,9896
Ergosinine	65592	10.712	1,2	0,9935
Ergocornine	73877	9.511	1,0	0,9821
Ergocorninine	61550	11.919	1,2	0,9961
Ergocryptine	72586	9.985	1,0	0,9921
Ergocryptinine	124157	12.981	0,6	0,9966
Ergocristine	121407	10.248	0,6	0,9916
Ergocristinine	103144	13.612	0,7	0,9973

6. Example Sample Calculation of Ergometrine content:

(Calculation of other ergot alkaloid contents is analogous)

A) Calculation of Sample Gramm Equivalents per HPLC injection:

$$\frac{\text{25g Sample}}{\text{100ml Extraction Solvent}} \times \frac{\text{4ml Extract}}{\text{1ml}} \times \text{0.1ml injector volume} = \text{0.1g Sample Equivalents}$$

B) Calculation of Ergometrine contamination of examined commodity in ng/g:

$$\frac{\text{\# ng injected Ergometrine}}{\text{Sample Equivalents [g]}} = \text{ng/g Ergometrine in e.g. rye flour}$$

7. Materials:

10mM PBS-Buffer, pH 7.4 (=PBS):

0.25 g Potassium dihydrogen phosphate
1.45 g Dipotassium hydrogen phosphate
8.76 g Sodium chloride

Dissolve salts in 1 L deionized water. If necessary, adjust pH at 7.4 (± 0.2) with 1M NaOH or 1M HCl

Ammonium carbonate-buffer:

2mM ammonium carbonate, pH=9:

Dissolve 200 mg ammonium carbonate in 1 L deionized water. Control pH 9.0

Elution Solvent: Methanol:

LC-Eluent:

Mobile Phase A: Acetonitrile / water (90/10, v/v):

Mix 900mL acetonitrile, 100mL water and degas

Mobile Phase B: Acetonitrile / 2mM ammonium carbonate (20/80, v/v):

Mix 800mL 2mM ammonium carbonate, 200mL acetonitrile and degas with helium

Chemicals:

- Acetonitrile, analytical grade
- Methanol, analytical grade
- Acetic acid, analytical grade
- Deionized water
- Dipotassium hydrogen phosphate, >98 %
- Potassium dihydrogen phosphate, >98 %
- Sodium chloride
- Ammonium carbonate, analytical grade

Gas:

- Nitrogen for the evaporation of IAC-eluates
- Helium as degasser

Consumables:

- **SENSI*Column* IAC Ergot Alkaloids 3ml**

8. References:

[1]"Novel Solid-Phase Extraction for Epimer-Specific Quantitation of Ergot Alkaloids in Rye Flour and Wheat Germ Oil" R. Köppen, T. Rasenko, S. Merkel, B. Mönch, and M. Koch, *J. Agric. Food Chem.* **2013**, 61 (45), pp 10699–10707

[2]"Simultaneous determination of six major ergot alkaloids and their epimers in cereals and foodstuffs by LC–MS–MS" R. Krska, G. Stubbings, R. Macarthur, C. Crews, *Anal Bioanal Chem* **2008** 391:563–576

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