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## Application and validation of a clean-up tandem assay column for the screening of ochratoxin A in cocoa powder

### Abstract

5 A rapid antibody-based assay for the detection of ochratoxin A in cocoa powder is  
described, involving sequential clean-up and visual detection of the toxin ('clean-up  
tandem assay column'). The screening test was developed to have a cut-off level of 2  $\mu\text{g}$   
10  $\text{kg}^{-1}$  and was shown to have false positive and false negative rates of 10 % and 2 %  
respectively. The analysis of six samples can be carried out in the field in approximately  
30 min by untrained workers. Ten retail cocoa powders were all found not to contain  
detectable ochratoxin A ( $< 2 \mu\text{g kg}^{-1}$ ) by the proposed rapid screening test. These samples  
were also found to be negative ( $< 2 \mu\text{g kg}^{-1}$ ) when analysed using an LC-MS/MS method.

**Keywords :** ochratoxin A, cocoa powder, immunoassay, qualitative test, validation

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15 **Introduction**

Consumption of cocoa powder can contribute to the intake of the mycotoxin ochratoxin A (OTA). In cocoa beans OTA is most notably produced by *Aspergillus carbonarius* and *Penicillium verrucosum* at a minimum water activity of 0.85. OTA is mainly formed in the initial stages of sun drying. The drying period is critical to avoid the appearance of OTA in cocoa beans, rather than the storage period (Bucheli et al. 2000).

There is no doubt that prevention of contamination at the source is the best procedure for controlling the contamination. However, despite improvements in production and storage techniques and current scientific and technical knowledge, it is not possible to entirely eliminate these moulds and consequently eliminate OTA from food. Therefore, tight EC limits are set for ochratoxin A (Commission Regulation (EC) No. 123/2005 of 26 January 2005) for a variety of food products ranging from 2-10  $\mu\text{g kg}^{-1}$ . For cocoa and chocolate regulatory limits are still under discussion, the most likely limits will be in the range 1-2  $\mu\text{g kg}^{-1}$  for raw cocoa materials and finished products respectively (Serra Bonhevi 2004).

Few conventional methods have been published for OTA determination in cocoa beans (Hurst and Martin 1983, 1998, Serra Bonhevi 2004, Amézqueta et al. 2004) and cocoa-derived products (Burdaspal and Legarda 2003, Brera et al. 2003). Clean-up of extract solutions using immunoaffinity columns (IAC) combined with high performance liquid chromatography (HPLC) provides sensitive and selective results, but are laborious, time-consuming, require sophisticated equipment and cannot be used *in situ*.

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6 Most commercial test kits for OTA make use of ELISA-methods  
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8 (<http://www.aoac.org/testkits/kits-toxins.htm>). Other rapid assays for OTA detection  
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10 described in literature are array biosensors, fluorescence polarisation immunoassays,  
11 40 screen printed electrode immunoassays, surface plasmon resonance biosensors, quartz  
12  
13 crystal microbalance biosensors (Visconti and De Girolamo 2005). Only few on-site  
14  
15 rapid assays that can be used without special laboratory equipment are available (lateral  
16  
17 flow devices, membrane-based flow-through portable immunoassays, test cards)  
18  
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21  
22 45 (Dietrich et al. 1995, De Saeger and Van Peteghem 1999, Danks et al. 2003, Zheng et al.  
23  
24 2005, Saha et al. 2006). However, application of methods with visual detection is difficult  
25  
26 for intensively coloured products such as cocoa powder. There are no commercial on-site  
27  
28 tests for this food matrix. Therefore, we developed a home-made clean-up tandem assay  
29  
30 column (patent application pending (Sibanda et al. 2001)), for the rapid detection of OTA  
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33  
34 50 in cocoa powder. In this column, clean-up and detection are performed in one step  
35  
36 enabling visual evaluation.  
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## 41 **Materials and methods**

### 42 *Reagents and materials*

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44  
45 55 OTA standard, casein sodium salt (casein), Tween 20 and tetramethoxysilane were  
46  
47 purchased from Sigma Chemical Co (Bornem, Belgium). Rabbit anti-mouse  
48  
49 immunoglobulins (IgG) (protein concentration: 2,7 g l<sup>-1</sup>) were supplied by  
50  
51 DakoCytomation (Heverlee, Belgium). CNBr-activated Sepharose 4B was purchased  
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53  
54  
55 from Amersham Biosciences AB (Uppsala, Sweden). NH<sub>2</sub>-derived silica (Bio-Sil NH<sub>2</sub>,  
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4 60 diameter 0.040 mm), Bond Elut SAX-columns, Bond Elut Si-columns, tubes (Bond Elut  
5 reservoir, 1 ml) and polyethylene frits (1/4 in. diameter) were supplied by Varian  
6  
7  
8 Belgium NV/SA (Sint-Katelijne-Waver, Belgium). Bakerbond<sup>TM</sup>SPE C<sub>18</sub>-columns were  
9  
10 purchased from JT Baker B.V. (Deventer, Holland). OchraTest<sup>TM</sup> immunoaffinity  
11  
12 columns were supplied by Vicam (Watertown, MA).  
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15 65  
16  
17 OTA-horse radish peroxidase (HRP)-conjugate was prepared by the Agricultural  
18  
19 Biotechnology Center, Diagnostic Laboratory, Gödöllő, Hungary. Monoclonal antibodies  
20  
21 against OTA were produced and characterized by the same Institute. The antibody was an  
22  
23 IgG1 with kappa light chains with a 9.3% cross-reaction with ochratoxin B but none at all  
24  
25  
26  
27 70 with ochratoxin  $\alpha$ , coumarin, 4-hydroxycoumarin and D,L-phenylalanine (Gyöngyösi-  
28  
29 Horváth et al. 1996). Phosphate buffered saline (PBS) 0.01 M, pH 7.4, was used to  
30  
31 prepare the wash solution (PBS-Tween 0.05%) and the assay buffer (PBS-casein 0.1%).  
32  
33 Proclin 300 (5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one)  
34  
35 was purchased from Supelco (Bellefonte, PA, USA) and was added to the buffers as an  
36  
37  
38  
39 75 antimicrobial preservative. Methanol was HPLC-grade and water was obtained from a  
40  
41 Milli-Q Gradient System (Millipore, Brussels, Belgium). The substrate chromogen  
42  
43 solution used was Colorburst<sup>TM</sup>Blue TMB/Peroxide (ALerCHEK, Inc. USA). Stock  
44  
45 solutions of OTA (1 mg ml<sup>-1</sup>) and working solutions (100 ng  $\mu$ l<sup>-1</sup>, 10 ng  $\mu$ l<sup>-1</sup>, 1 ng  $\mu$ l<sup>-1</sup>)  
46  
47 were prepared in methanol and stored at -20°C. Ederol filters No. 14, 110 mm were  
48  
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51 80 purchased from Binzer & Munktell Filter GmbH (Battenberg, Germany).  
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3 *Blank cocoa powder sample*  
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5 The OTA concentration in a commercially available cocoa powder sample, which was  
6 intended to be used for the development of the clean-up tandem assay column, was  
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10 85 determined using a liquid chromatography tandem mass spectrometry (LC-MS/MS)  
11 method with immunoaffinity clean-up.  
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18 Extraction of the cocoa powder sample (20 g) was carried out by adding 50.0 ml  
19  
20 MeOH/3% NaHCO<sub>3</sub> (80/20, v/v) to the sample and by shaking for 30 min at ± 250  
21  
22 90 revolutions per min (rpm) with an Orbital Shaker SO3 (Stuart Scientific, UK). The extract  
23  
24 was centrifuged for 10 min (3600 rpm). The supernatant (10.0 ml) was diluted with 40.0  
25  
26 ml PBS-buffer. This solution (50.0 ml) was filtered through an Ederol filter and passed  
27  
28 through the OchraTest™ column at the rate of gravity. The column was washed with 5.0  
29  
30 ml water (under gravity). OTA was eluted with 3.0 ml MeOH. The eluate was evaporated  
31  
32 95 to dryness under a N<sub>2</sub> stream and re-dissolved with 300 µl mobile phase for the LC-  
33  
34 MS/MS analysis.  
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41 A Waters Alliance 2695 XE high performance liquid chromatography (HPLC) system  
42  
43 coupled to a Micromass Quatro micro triple quadrupole mass spectrometer was used  
44  
45 100 (Waters, Milford, MA, USA). The analytical column was an Alltima C<sub>18</sub>, 5 µm, 150 x 3.2  
46  
47 mm (Alltech Associates, Deerfield, IL, USA), while the guard column was an Alltima  
48  
49 C<sub>18</sub>, 5 µm, 7.5 x 3.2 mm (Alltech Associates, Deerfield, IL, USA). Injection volume was  
50  
51 20 µl. The mobile phase consisted of variable mixtures of formic acid in acetonitrile  
52  
53 (0.3%, solvent A) and formic acid in water (0.3%, solvent B) at a flow of 0.3 ml min<sup>-1</sup>.  
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3 105 The gradient elution programme was: isocratic conditions with 10% A: 90% B for 3 min,  
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5 linear gradient from 10 to 100% A for 6.5 min and isocratic conditions with 100% A for  
6  
7 4.5 min. Then, within 1 min, the concentration of A was brought back to 10%. This was  
8  
9 maintained for 15 min. The mass spectrometer was operated in the positive electrospray  
10  
11 ionisation (ESI+) mode using multiple reaction monitoring (MRM). Capillary voltage  
12  
13 was 3.7 kV and nitrogen was used as spray gas. Source and desolvation temperatures  
14  
15 110 were set at 120 and 350°C, respectively. The precursor ion was m/z 404. The cone  
16  
17 voltage was 35 V. Product ions and collision energy were determined. Collision gas was  
18  
19 argon. For OTA product ions were at m/z 239 (collision energy 24 eV) and m/z 341  
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21 (collision energy 20 eV).  
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29 *Extraction solution*

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31 The optimal composition of the extraction solution which will be used for the clean-up  
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33 tandem assay procedure was determined by high performance liquid chromatography  
34  
35 (HPLC) coupled to fluorescence detection (FLD). Blank cocoa powder samples (5 g)  
36  
37 120 were fortified ( $5 \mu\text{g kg}^{-1}$ ,  $15 \mu\text{g kg}^{-1}$  and  $25 \mu\text{g kg}^{-1}$ ,  $n = 3$  for each concentration level)  
38  
39 with OTA the night before using the afore mentioned working solutions in MeOH. They  
40  
41 were extracted with 15 ml of different ratios (100/0, 95/5, 90/10, 80/20, 75/25, 50/50,  
42  
43 v/v) of a MeOH/3% NaHCO<sub>3</sub> (v/v) solution (Lobeau et al. 2005) by shaking for 5  
44  
45 minutes at  $\pm 200$  rpm with an Orbital Shaker SO3 (Stuart Scientific, UK). The suspension  
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50 125 was filtered through an Ederol filter and the filtrate was analysed by HPLC-FLD.  
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3 Sample extracts (50  $\mu\text{l}$ ) were injected with an autosampler and separated on a Supelco  
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5 Discovery  $\text{C}_{18}$  (5  $\mu\text{m}$ , 25 cm x 4.6 mm) column (Supelco, Bellefonte, PA, USA) at room  
6  
7 temperature. The mobile phase used was acetonitrile/water/acetic acid (54/44/2) at a flow  
8  
9 rate of 1 ml  $\text{min}^{-1}$ . The HPLC system consisted of a Waters 600 Controller, a Waters 610  
10  
11 130 Fluid Unit and a Waters 474 scanning fluorescence detector (333 nm excitation  
12  
13 wavelength, 470 nm emission wavelength). Sample OTA concentrations were quantified  
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15 by comparing sample peak areas to those of standards and extraction recoveries were  
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17 determined.  
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24 *Clean-up tandem assay column: Clean-up layer*  
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27 Various solid-phase materials (aminopropyl-derived silica ( $\text{NH}_2$ -derived silica),  
28  
29 octadecyl-derived silica, silica (silanol groups), Strong Anion Exchange-silica (SAX))  
30  
31 were evaluated as to their ability to adsorb the brown colour and other matrix  
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33 interferences (Sibanda et al. 2002b), while at the same time allowing OTA to elute  
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35 140 directly.  
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42 Blank cocoa powder samples (5 g) were fortified (20  $\mu\text{g kg}^{-1}$ , 40  $\mu\text{g kg}^{-1}$ , 80  $\mu\text{g kg}^{-1}$  and  
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44 160  $\mu\text{g kg}^{-1}$ ) with OTA the night before using the afore mentioned working solutions in  
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46 145 MeOH and extracted with 15 ml MeOH/3%  $\text{NaHCO}_3$  (95/5, v/v) by shaking for 5  
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48 minutes at  $\pm 200$  rpm with an Orbital Shaker SO3 (Stuart Scientific, UK). The suspension  
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50 was filtered through an Ederol filter.  
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4 The filtrate was diluted in order to reduce the methanol concentration. This avoids the  
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6 150 denaturation of the antibodies in the *clean-up tandem assay column*. Milli-Q water and a  
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8 3% NaHCO<sub>3</sub> solution (Sibanda et al. 2002a) were evaluated as dilution solutions by  
9  
10 comparing the partition coefficients ( $K_d$ ) for the dispersion of OTA between the selected  
11  
12 solid phase (i.e. NH<sub>2</sub>-derived silica, see Results: Clean-up layer) and the MeOH/3%  
13  
14 NaHCO<sub>3</sub> (95/5, v/v) mobile phase. The filtrate (1.5 ml) was diluted with 2.1 ml Milli-Q  
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17 155 water or with 2.1 ml of a 3% NaHCO<sub>3</sub> solution. This dilution was extracted over 200 mg  
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19 of NH<sub>2</sub>-derived silica without application of pressure (Lobeau et al. 2005). The eluate  
20  
21 from the column was collected and the OTA concentration was measured by HPLC-FLD.  
22  
23 The second fraction, obtained by washing the column with 4 ml of MeOH, was  
24  
25 evaporated to dryness and redissolved in 300 µl of the mobile phase for HPLC analysis.  
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29 160 The partition coefficient ( $K_d$ ) was calculated using the formula  $K_d = [\text{OTA wash}$   
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31  $\text{fraction}]/[\text{OTA frontal elution fraction}]$  or  $K_d = [\text{OTA solid phase}]/[\text{OTA mobile phase}]$ .  
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37 *Clean-up tandem assay column: detection layer Sepharose gel*

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39 The secondary rabbit anti-mouse antibodies were coupled to the Sepharose gel following  
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41 165 the principles and methods of Pharmacia LKB Biotechnology (Pharmacia LKB  
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43 Biotechnology 1993, Lobeau et al. 2005).  
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48 *Clean-up tandem assay column: detection layer sol-gel*

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50 Encapsulation of the monoclonal mouse anti-OTA-antibody was carried out according to  
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52  
53 170 Zühlke et al. 1995. Hydrochloric acid (0.4 ml of 0.04 M), Milli-Q water (1.5 ml) and 6.8  
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55 ml tetramethoxysilane (TMOS) were mixed and sonicated under ice-cooling for 30 min.  
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3 This silica sol (1 ml) was added to 1 ml of ice-cooled PBS pH 7.6 containing the amount  
4 of the antibodies to be immobilised (10  $\mu$ l of 2 mg ml<sup>-1</sup> primary anti-OTA antibody) in a  
5 beaker. The gel was weighed and the beaker was stored at 4°C without capping. The  
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11 175 drying process was stopped after 20 hours incubation resulting in a clear glass. After a  
12 weight loss of  $\pm$  30%, the glass was ground in a mortar and packed into a column. The  
13 sol-gel glass immunoabsorber was then stored in PBS at 4°C.  
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#### 20 *Preparation of the clean-up tandem assay column*

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22 180 A polyethylene frit (grid) was first put in the 1 ml tube, followed by the addition of 200  
23  $\mu$ l of the detection layer Sepharose gel suspended in PBS or 50 mg of sol-gel powder.  
24 Another grid separated the detection layer and 200 mg clean-up layer above. A third grid  
25 was placed above the clean-up layer (Lobeau et al. 2005).  
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#### 34 185 *Clean-up tandem assay procedure*

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36 The clean-up tandem assay procedure was the same as mentioned in Lobeau et al. 2005.  
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38 In the clean-up tandem assay, each reagent was applied in sequence at the inlet or at the  
39 outlet means of the tube at a rate of  $\pm$  1 drop/s. These reagents were respectively primary  
40 mouse anti-OTA antibodies (100  $\mu$ l), wash solution (3 ml), diluted sample extract (3.6  
41 ml), wash solution (3 ml), OTA-HRP conjugate (200  $\mu$ l), wash solution (3 ml), colour  
42 substrate (50  $\mu$ l) (see: Reagents and Materials). For columns filled with sol-gel powder,  
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46 190 the application of the diluted cocoa powder extract (3.6 ml) onto the column was the first  
47 step.  
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3 195 The test was developed to have a visual cut-off level of  $2 \mu\text{g OTA kg}^{-1}$  cocoa powder (EC  
4 proposed limit for cocoa powder) by optimising the dilutions of the primary mouse anti-  
5 OTA antibodies and the OTA-HRP conjugate. In this way the absence of a developed  
6 blue colour indicated a positive result with an OTA content above  $2 \mu\text{g kg}^{-1}$ .  
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15 200 *OTA analysis of 10 different commercial cocoa powder samples*  
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17 Ten cocoa powder samples commercially available on the Belgian market were analysed  
18 in triplicate using the clean-up tandem assay column. The results were compared with  
19 these using the above mentioned LC-MS/MS method with immunoaffinity clean-up.  
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26  
27 205 **Results and discussion**  
28

29 *Blank cocoa powder sample*  
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31 The OTA concentration in the tested cocoa powder sample was found to be less than  $0.1$   
32  $\mu\text{g kg}^{-1}$  ( $0.0955 \mu\text{g kg}^{-1}$ ) with the LC-MS/MS method. The clean-up tandem assay  
33 column was further developed with this essentially-blank sample.  
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41 *Extraction solution*  
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43 Lobeau et al. 2005 used  $\text{MeOH}/3\% \text{NaHCO}_3$  (80/20, v/v) as solvent for the extraction of  
44 OTA from roasted coffee. For OTA extraction from cocoa powder different ratios of  
45  $\text{MeOH}/3\% \text{NaHCO}_3$  (v/v) were examined and recoveries were determined using the  
46 described HPLC-FLD method. Large amounts of  $3\% \text{NaHCO}_3$  in the extraction solution  
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50 215 resulted in dark filtrates. Less than 20% of a  $3\% \text{NaHCO}_3$  solution was necessary to avoid  
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3 this interfering coloration. The best recovery ( $100.2 \pm 7.1 \%$ ) was found with MeOH/3%  
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5 NaHCO<sub>3</sub> (95/5, v/v).  
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10 220 *Clean-up layer*

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12 Only SAX-silica and NH<sub>2</sub>-derived silica were found to be able to adsorb most of the  
13 colour of the cocoa powder. Recoveries were comparable for both. NH<sub>2</sub>-derived silica  
14 was chosen because of the easier availability.  
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22 225 Dilution of the methanolic filtrate was necessary to avoid denaturation of the antibodies  
23 in the column. Milli-Q water and a 3% NaHCO<sub>3</sub> solution were tested as dilution solvent  
24 and compared by calculating the partition coefficients ( $K_d$ ) for the dispersion of OTA  
25 between the NH<sub>2</sub>-derived silica solid phase and the MeOH/3% NaHCO<sub>3</sub> (95/5, v/v)  
26 mobile phase. OTA should elute directly to the detection layer, so, the partition  
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34 230 coefficient should be as low as possible.  
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39 Partition coefficients are shown in Table I. On average lower coefficients ( $K_d = 0.4828 \pm$   
40  $0.086$ ) were obtained using 3% NaHCO<sub>3</sub> than with Milli-Q water ( $K_d = 0.9806 \pm 0.488$ ).  
41  
42 Therefore, 1.5 ml of the sample extraction filtrate was diluted with 2.1 ml of a 3%  
43  
44  
45  
46 235 NaHCO<sub>3</sub> solution (Lobeau et al. 2005). This was a reduction of the MeOH content to  $\pm$   
47  
48 40%.  
49

50 “[Insert Table I about here]”  
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55 *Clean-up tandem assay column set-up*  
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3 240 The reason for binding secondary rabbit anti-mouse antibodies to the Sepharose gel was  
4 that direct binding of the capture protein (the primary monoclonal anti-OTA antibody)  
5 gave no binding results probably due to conformational changes that reduced the anti-  
6 OTA antibody's affinity for the analyte (Wild 2001, Cichna et al. 2001).  
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15 245 In most cases the immobilisation reaction can only be applied using highly purified  
16 ligands and often leads to conformational changes of bioligands and therefore decreases  
17 the affinity for the analyte. This problem can be avoided when ligands are immobilised in  
18 the pores of a sol-gel glass matrix (Cichna et al. 2001). This method was compared with  
19 the Sepharose method. The preparation of the sol-gel was found to be less labour  
20 intensive than the Sepharose method. However, to apply it as a gel in the clean-up tandem  
21 assay column, it was not found to be applicable. The colour substrate seemed to react  
22 non-specifically with polar silane groups in the gel and colour development immediately  
23 appeared only on the side where the substrate made the initial contact with the gel. This  
24 result was observed for both negative and positive samples.  
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41 An example of a serial of results for the clean-up tandem assay column using Sepharose  
42 gel is given in Figure 1. The dilution of the primary mouse anti-OTA antibodies and the  
43 OTA-HRP conjugate were respectively 1/300 and 1/500 in assay buffer. The intensity of  
44 the colour development decreased with increasing concentrations of OTA. No blue colour  
45 developed at a concentration of  $2 \mu\text{g kg}^{-1}$ . Therefore, this was the cut-off level of the  
46 assay.  
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50 260  
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54  
55 “[Insert Figure 1 about here]“  
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5 The analysis of six samples can be carried out in the field in approximately 30 min by  
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7 265 untrained workers.  
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11 Dilution of the filtrate with 3% NaHCO<sub>3</sub> resulted after a few min in precipitation and  
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13 gelation of the proteins in the cocoa powder due to the alkaline pH. Because this could  
14  
15 compromise the flow of the sample through the column, dilution with Milli-Q water was  
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18 270 found to be more appropriate, contrary to the results obtained in Results: Clean-up layer.  
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22 *Validation of the clean-up tandem assay method using fortified cocoa powder samples*  
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25 An intra-laboratory validation was performed with blank cocoa powder samples fortified  
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27 at 0, 1, 2, 3, 4 and 5 µg OTA kg<sup>-1</sup> the night before using the afore mentioned working  
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29 275 solutions in MeOH. The following performance characteristics were determined:  
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31 precision (in terms of false positive and false negative results), sensitivity, specificity,  
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33 positive and negative predictive values and unreliability region (Trullols et al. 2004a,  
34  
35 Trullols et al. 2004b).  
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41 280 For this qualitative screening test, precision was expressed as the number of false positive  
42  
43 and false negative results as can be seen in Table II. Based on these results, the sensitivity  
44  
45 i.e. the ability to detect a true positive result, and the specificity i.e. the ability to detect a  
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47 true negative result, were respectively 98 and 90%. The predictive value of positive  
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49 results i.e. fraction of positive results that are true positives, was 93 % and the predictive  
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53 285 value of negative results i.e. fraction of negative results that are true negatives, was 97%.  
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55 The unreliability region, the region in which false results were obtained, was between 1  
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3 and  $2 \mu\text{g kg}^{-1}$ . These results are shown in Table III. For mycotoxins criteria for screening  
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5 methods are not available but according to Commission Decision 2002/657/EC the  
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7 requirement for a screening method is the following: only those analytical techniques, for  
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11 290 which it can be demonstrated in a documented traceable manner that they are validated  
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13 and have a false compliant rate of  $< 5 \%$  at the level of interest shall be used for screening  
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15 purposes. In case of a suspected non-compliant result, this result shall be confirmed by a  
16  
17 confirmatory method.  
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20 “[Insert Tables II and III about here]”  
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22 295

23  
24 *Performance characteristic curve*  
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27 Performance characteristics were also estimated by a curve (Trullols et al. 2004b). Blank  
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29 cocoa powder samples were fortified at 0, 0.5, 1, 1.5, 2, 3, 4 and  $5 \mu\text{g OTA kg}^{-1}$  the night  
30  
31 before using the afore mentioned working solutions in MeOH. At each concentration  
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34 300 level, the percentage of positives,  $P(X)$  and the percentage of negatives,  $N(X)$  was  
35  
36 calculated. With these data, a graph of positives’ percentage versus the concentration  
37  
38 levels tested was plotted. The performance characteristic curve was obtained by fitting  
39  
40 the experimental results from Table IV to a sigmoidal function that minimized the root  
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42 mean square of the residuals (Figure 2). To obtain the performance parameters, the  
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45 305 probabilities of committing false-positive ( $\alpha$ ) and false-negative ( $\beta$ ) errors that could be  
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47 accepted had to be fixed. Usually, these values are fixed at 5% and correspond to the  
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49 horizontal lines plotted on Figure 2,  $\alpha = 5\%$  and  $100 - \beta = 95\%$  (Commission Decision  
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51 657/2002). Once the probabilities of error were fixed, all performance parameters were  
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55 calculated. This was possible using the equation of the sigmoidal curve:  
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3 310  $y = y_0 + (a/(1+e^{-(x-x_0)/b}))$  with:

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6  $a = 100.4838, b = 0.2642, x_0 = 1.3449, y_0 = -1.0662$

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11 “[Insert Table IV and Figure 2 about here]”

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15 315 The unreliability region was defined by its upper and lower limits. The upper limit  
16 corresponded to the concentration at which the  $100-\beta$  line crossed the  $P(X)$  curve ( $2.16$   
17  $\mu\text{g kg}^{-1}$ ). Therefore,  $2.2 \mu\text{g kg}^{-1}$  was the cut-off as well as the detection limit of our assay,  
18 corresponding to the one visually observed. The lower limit corresponded to the  
19 concentration at which the  $\alpha$  line crossed the  $P(X)$  curve ( $0.62 \mu\text{g kg}^{-1}$ ). Therefore the  
20 unreliability region was between  $0.6$  and  $2.2 \mu\text{g kg}^{-1}$ . The sensitivity rate at  $2.2 \mu\text{g kg}^{-1}$   
21 was equal to 95%, as it corresponded to the point where  $P(X) = 100-\beta$ . The false negative  
22 rate at  $2.2 \mu\text{g kg}^{-1}$  was equal to 5% as  $fn = 100-P(X)$ . Similarly, the specificity rate at  $0.6$   
23  $\mu\text{g kg}^{-1}$  was equal to 95%, as it corresponded to the point where  $N(X) = 100-P(X)$ . The  
24 false positive rate at this concentration level was also 5% because  $fp = P(X)$ . Gel  
25 preparation was probably a source of error and gave the false negative result at the  $3 \mu\text{g}$   
26  $\text{kg}^{-1}$  concentration level. Preparing the gel in larger volumes would favour the  
27 repeatability and minimize false negative results.  
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48 *OTA analysis of 10 different commercial cocoa powder samples*

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50 330 All ten commercially available cocoa powder samples were found to be negative (less  
51 than  $2 \mu\text{g kg}^{-1}$ ) using the clean-up tandem assay column. These qualitative results were  
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3 compared with the quantitative results obtained using the above mentioned LC-MS/MS  
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5 method. Results are shown in Table V.  
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10 335 “[Insert Table V about here]“  
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### 14 15 **Conclusion**

16  
17 A clean-up tandem assay column for the detection of OTA in cocoa powder was  
18 developed and the method was validated by the determination of its qualitative  
19 performance parameters. The column comprised two superposed layers; clean-up and  
20 340 detection were performed on one single column device. The result of the assay can be  
21 binary only: yes/no response indicating whether OTA is present or not above the cut-off  
22 level. The test was calibrated to a cut-off level of 2  $\mu\text{g}$  OTA  $\text{kg}^{-1}$  cocoa powder.  
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24 Establishing this parameter by means of a performance characteristic curve resulted in a  
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34 345 cut-off level of 2.2  $\mu\text{g}$  OTA  $\text{kg}^{-1}$  cocoa powder. Therefore, the described clean-up tandem  
35 assay column can be used as a rapid screening tool for OTA in cocoa powder.  
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44  
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### 50 **References**

51  
52  
53  
54  
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56  
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59  
60

1  
2  
3 Amézqueta S, González-Peñas E, Murillo M, López de Cerain A. 2004. Validation of a  
4  
5 355 high-performance liquid chromatography analytical method for ochratoxin A  
6  
7  
8 quantification in cocoa beans. Food Additives and Contaminants 21:1096-1106.  
9

10  
11  
12 AOAC International [internet]. Available from: [http://www.aoac.org/testkits/kits-](http://www.aoac.org/testkits/kits-toxins.htm)  
13  
14 [toxins.htm](http://www.aoac.org/testkits/kits-toxins.htm). Accessed 2006 May 24.  
15  
16

17 360

18  
19 Brera C, Grossi S, de Santis B, Miraglia M. 2003. High performance liquid  
20  
21 chromatographic method for the determination of ochratoxin A in cocoa powder. Journal  
22  
23 of Liquid Chromatography and Related Technologies 26:585-598.  
24  
25  
26

27  
28  
29 365 Bucheli P, Kanchanomai C, Meyer I, Pittet A. 2000. Development of ochratoxin A during  
30  
31 Robusta (*Coffea canephora*) coffee cherry drying. Journal of Agricultural and Food  
32  
33 Chemistry 48:1358-1362.  
34  
35  
36

37  
38  
39 Burdaspal PA, Legarda TM. 2003. Occurrence of ochratoxin A in samples of different  
40  
41 370 types of chocolate and cocoa powder, marketed in Spain and fifteen foreign countries.  
42  
43 Alimentaria October:143-153.  
44  
45

46  
47  
48 Cichna M, Markl P, Knopp D, Niessner R. 2001. On-line coupling of sol-gel-generated  
49  
50 immunoaffinity columns with high-performance liquid chromatography. Journal of  
51  
52 375 Chromatography A 919:51-58.  
53  
54  
55

1  
2  
3 Commission Decision 657/2002 of 12 August 2002 implementing Council Directive  
4 96/23/EC concerning the performance of analytical methods and the interpretation of  
5 results. Official Journal of the European Communities L 221/8, (August 17).  
6  
7  
8  
9

10 380

11  
12 Commission Regulation (EC) No. 123/2005 of 26 January 2005 amending Regulation  
13 (EC) No. 466/2001 as regards Ochratoxin A. Official Journal of the European Union  
14 2005, L25:3.  
15  
16  
17  
18  
19

20  
21  
22  
23 385 Danks C, Ostoja-Starzewska, Flint J, Banks JN. 2003. The development of a lateral flow  
24 device for the discrimination of OTA producing and non-producing fungi. Mycotoxins in  
25 Food Production Systems. Aspects of Applied Biology 68:21-28.  
26  
27  
28  
29

30  
31  
32  
33 De Saeger S, Van Peteghem C. 1999. Flow-through membrane-based enzyme  
34 immunoassay for rapid detection of ochratoxin A in wheat. Journal of Food Protection  
35 390 62:65-69.  
36  
37  
38  
39

40  
41  
42 Dietrich R, Schneider E, Usleber E, Martlbauer E. 1995. Use of monoclonal antibodies  
43 for the analysis of mycotoxins. Natural Toxins 3:288-293.  
44  
45  
46

47 395

48  
49 Gyöngyösi-Horváth Á, Barna-Vetró I, Solti L. 1996. A new monoclonal antibody  
50 detecting ochratoxin A at the picogram level. Letters in Applied Microbiology 22:103-  
51 105 .  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 400 Hurst WJ, Martin Jr. RA. 1983. High-performance liquid chromatographic determination  
4 of ochratoxin A in artificially spiked cocoa beans. *Journal of Chromatography* 265:353-  
5  
6 356.  
7  
8

9  
10  
11  
12  
13 Hurst WJ, Martin Jr. RA. 1998. High-performance liquid chromatographic determination  
14  
15 405 of ochratoxin A in artificially contaminated cocoa beans using automated sample clean-  
16  
17 up. *Journal of Chromatography A* 810:89-94.  
18  
19

20  
21  
22 Lobeau M, De Saeger S, Sibanda L, Barna-Vetró I, Van Peteghem C. 2005. Development  
23  
24 of a new clean-up tandem assay column for the detection of ochratoxin A in roasted  
25  
26 410 coffee. *Analytica Chimica Acta* 538:57-61.  
27  
28

29  
30  
31 Pharmacia LKB Biotechnology, *Affinity Chromatography, Principles and Methods* 1993,  
32  
33 24-30.  
34  
35

36  
37  
38 415 Saha D, Acharya D, Dhar TK. Method for homogeneous spotting of antibodies on  
39  
40 membranes: application to the sensitive detection of ochatoxin A. *Analytical and*  
41  
42 *Bioanalytical Chemistry* DOI: 10.1007/s00216-006-0484-4.  
43  
44  
45

46  
47  
48 Serra Bonhevi J. 2004. Occurrence of ochratoxin A in cocoa products and chocolate.  
49  
50 420 *Journal of Agricultural and Food Chemistry* 52:6347-6352.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 Sibanda L, De Saeger S, Van Peteghem C. 2001. International patent application No.  
4  
5 PCT/EP02/01496: Device and method for detecting the presence of an analyte.  
6  
7  
8  
9  
10  
11 425 Sibanda L, De Saeger S, Van Peteghem C. 2002a. Optimization of solid-phase clean-up  
12 prior to liquid chromatographic analysis of ochratoxin A in roasted coffee. Journal of  
13 Chromatography A 959:327-330.  
14  
15  
16  
17  
18  
19  
20 Sibanda L, De Saeger S, Barna-Vetró I, Van Peteghem C. 2002b. Development of a  
21  
22 430 solid-phase cleanup and portable rapid flow-through enzyme immunoassay for the  
23 detection of ochratoxin A in roasted coffee. Journal of Agricultural and Food Chemistry  
24 50:6964-6967.  
25  
26  
27  
28  
29  
30  
31  
32 Trullols E, Ruisánchez I, Rius FX. 2004a. Validation of qualitative analytical methods.  
33  
34 435 Trends in Analytical Chemistry 23:137-145.  
35  
36  
37  
38  
39 Trullols E, Ruisánchez I, Rius FX. 2004b. Qualitative method for determination of  
40 aflatoxin B1 in nuts. Journal of AOAC International 87:417-423.  
41  
42  
43  
44  
45  
46 440 Visconti A, De Girolamo A. 2005. Fitness for purpose – Ochratoxin A analytical  
47 developments. Food Additives and Contaminants, Supplement 1:37-44.  
48  
49  
50 Wild D. 2001. Introduction to immunoassay principles. In: The Immunoassay Handbook,  
51 second edition, Nature Publishing Group, New York. p. 32-33.  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 445 Zheng MZ, Richard JL, Binder J. 2006. A review of rapid methods for the analysis of  
4  
5 mycotoxins. *Mycopathologia* 161:261-273.  
6  
7  
8

9  
10 Zühlke J, Knopp D, Niessner R. 1995. Sol-gel glass as a new support matrix in  
11  
12 immunoaffinity chromatography. *Fresenius Journal of Analytical Chemistry* 352:654-  
13  
14 450 659.  
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**Figure captions**

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12 455 Figure 1: Clean-up tandem assay: a serial of results: the detection layer of each column is  
13 shown and the colour can be visually evaluated. Above the detection layer a small part of  
14 the clean-up layer is visible. The OTA concentrations ( $\mu\text{g kg}^{-1}$ ) of the cocoa powder  
15 samples are mentioned below the tubes.  
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24 460 Figure 2: Experimental performance characteristic curve. Probability of positive  
25 responses,  $P(X)$ , were plotted versus concentration levels tested. (1)  $f_p = P(X)$ ; (2)  $X_{0.05}$   
26 where specificity =  $N(X) = 100 - P(X)$ ; (3)  $X_{0.95}$ , cut-off limit, detection limit; (4)  $f_n =$   
27  $100 - P(X)$ ; (5) sensitivity =  $P(X) = 100 - \beta$ .  
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**Table I.** Calculated partition coefficients for the distribution of OTA between the aminopropyl solid phase and the MeOH/3% NaHCO<sub>3</sub> (95/5, v/v) mobile phase\* .

OTA concentration ( $\mu\text{g kg}^{-1}$ )	OTA concentration solid phase ( $\mu\text{g kg}^{-1}$ )	OTA concentration mobile phase ( $\mu\text{g kg}^{-1}$ )	Partition coefficient ( $K_d = \text{solid/mobile}$ )
dilution with a 3% NaHCO <sub>3</sub> solution			
20	3.99	6.68	0.5974
40	8.40	18.41	0.4563
80	18.23	46.55	0.3915
160	44.93	92.43	0.4861
average			<b>0.4828 ± 0.086</b>
dilution with Milli-Q water			
20	2.79	7.34	0.3806
40	10.15	11.92	0.8519
80	28.07	18.30	1.534
160	42.54	36.80	1.156
average			<b>0.9806 ± 0.488</b>

\* n = 3

**Table II.** Contingency table with numerical values as performed with the clean-up tandem assay column for the detection of ochratoxin A in cocoa powder.

	Samples fortified at 2 $\mu\text{g kg}^{-1}$ OTA or more	Samples fortified at less than 2 $\mu\text{g kg}^{-1}$ OTA	Total
Positive (no colour)	55 (tp <sup>*</sup> )	4 (fp <sup>†</sup> )	59
Negative (blue colour)	1 (fn <sup>‡</sup> )	36 (tn <sup>§</sup> )	37
Total	56	40	96 (N <sup>§</sup> )

\* tp = true positive, † fp = false positive, ‡ fn = false negative, § tn = true negative,

§ N = total amount of samples

**Table III.** Qualitative performance parameters for the detection of ochratoxin A in cocoa powder using the clean-up tandem assay column.

False positive rate <sup>*</sup>	10 %
False negative rate <sup>†</sup>	2 %
Sensitivity <sup>‡</sup>	98 %
Specificity <sup>¶</sup>	90 %
Positive predictive value <sup>§</sup>	93 %
Negative predictive value <sup>#</sup>	97 %
Unreliability region	1-2 $\mu\text{g kg}^{-1}$

\* false positive rate =  $(\text{fp}/\text{tn}+\text{fp})\times 100$ , † false negative rate =  $(\text{fn}/\text{tp}+\text{fn})\times 100$ , ‡ sensitivity =  $(\text{tp}/\text{tp}+\text{fn})\times 100$ , ¶ specificity =  $(\text{tn}/\text{tn}+\text{fp})\times 100$ , § positive predictive value =  $(\text{tp}/\text{tp}+\text{fp})\times 100$ , # negative predictive value =  $(\text{tn}/\text{tn}+\text{fn})\times 100$ .

**Table IV.** Concentration levels of ochratoxin A tested, with probabilities of positive, P(X), and negative, N(X), results calculated for each concentration level.

Concentration, $\mu\text{g kg}^{-1}$	Number of samples	P(X)	N(X)
0	20	0	100
0.5	12	0	100
1	20	25	75
1.5	12	58	42
2	14	100	0
3	14	93	7
4	14	100	0
5	14	100	0

**Table V.** OTA analysis of 10 different commercial cocoa powder samples available on the Belgian market.

Number of commercial sample	Qualitative result <sup>*</sup> (clean-up tandem assay column)	Quantitative result ( $\mu\text{g kg}^{-1}$ )(LC-MS/MS method)
1	--- <sup>†</sup>	0.0678
2	---	0.0831
3	---	0.0579
4	---	0.0553
5	---	0.0781
6	---	0.0654
7	---	0.0622
8	---	0.0532
9	---	0.0774
10	---	0.0402

<sup>\*</sup> n=3

<sup>†</sup> + = positive sample,  $\geq 2 \mu\text{g kg}^{-1}$

- = negative sample,  $< 2 \mu\text{g kg}^{-1}$

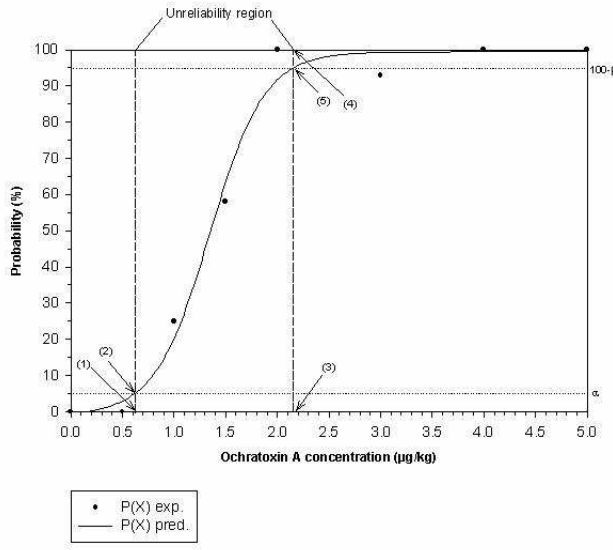
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