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Validation of a confirmatory analytical method for the determination of aflatoxins B1, B2, G1 and G2 in foods and feed materials by HPLC with on-line photochemical derivatization and fluorescence detection

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9 **Validation of a confirmatory analytical method for the determination of**
10 **aflatoxins B₁, B₂, G₁ and G₂ in foods and feed materials by HPLC with on-line**
11 **photochemical derivatization and fluorescence detection**
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Abstract

A sensitive and selective analytical method for the simultaneous separation and quantitative determination of aflatoxins B₁, B₂, G₁ and G₂ in foodstuffs and materials for feed has been validated. The method is based on high performance liquid chromatography with on-line post-column photochemical derivatization and fluorescence detection. The chromatographic separation of aflatoxins was accomplished by using a C₁₈ column eluted with an isocratic mobile phase consisting of water, methanol and acetonitrile. The sample preparation required a simple extraction of aflatoxins with MeOH/H₂O (80:20, v/v) and a purification step by immunoaffinity column cleanup. The total analysis time, including sample preparation and chromatographic separation, did not exceed 40 min with a run time of 10 min. The on-line photochemical derivatization ensures better results in terms of simplicity, sensitivity and reproducibility with respect to chemical derivatization techniques, and provides an increase of the peak resolution and an extent of automation in comparison with the electrochemical ones. The procedure for the determination of aflatoxins in food samples and cereals for animal consumption was extensively validated following the Regulation (EC) No. 882/2004. Detection limits in wheat bran samples of 0.08 µgkg⁻¹ for AFB₁, 0.02 µgkg⁻¹ for AFB₂, 0.16 µgkg⁻¹ for AFG₁ and 0.04 µgkg⁻¹ for AFG₂ were attained. The method allows high recovery with mean values ranging from 72% to 94% and it satisfies the necessary requirements for sensitivity, linearity, selectivity, precision and ruggedness, demonstrating the conformity of the method with provisions of Regulation (EC) No. 401/2006.

Keywords: Aflatoxins, HPLC, photochemical derivatization, validation, Regulation (EC) No. 401/2006, Regulation (EC) No. 882/2004

48 Introduction

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6 Aflatoxins B₁, B₂, G₁ and G₂ (AFB₁, AFB₂, AFG₁ and AFG₂), secondary metabolites of mould
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9 50 fungi *Aspergillus flavus* and *Aspergillus parasiticus*, are a group of structurally related
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11 mycotoxins, which exhibit acute and chronic toxicity including mutagenic, carcinogenic and
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14 52 teratogenic effects in several organisms (Harwig and Munro, 1975, Bullerman, 1979, Dickens
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16 and Jones, 1963, Garner and Kingfisher, 1979). The International Agency for Research on
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18 54 Cancer (IARC) classified aflatoxins B₁, B₂, G₁ and G₂ as group 1 of human carcinogens (IARC,
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20 1993). The widespread occurrence of the *Aspergillus* mould means that several agricultural
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23 56 commodities are vulnerable to contamination with aflatoxins. The storage of these products
24
25 under high moisture and increased temperature conditions (25–30°C) enhances the natural
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28 58 occurrence of aflatoxins in a great variety of foods for animal and human consumption (Peraica
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30 et al., 1999, Eaton and Groopman, 1994), such as cereals, corn, peanuts, pistachios, almonds and
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32 60 beans.

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37 62 The European Union (EU) has set a maximum level (ML) of 2 µgkg⁻¹ for AFB₁ and 4 µgkg⁻¹ for
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39 total aflatoxins (B₁, B₂, G₁, and G₂) in nuts, dried fruit and cereals intended for direct human
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42 64 consumption (Regulation (EC) No. 1881/2006). In baby food products, more restrictive limits
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44 (0.10 µgkg⁻¹ for AFB₁) have been established (Regulation (EC) No. 683/2004). In agreement
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46
47 66 with the Directive (EC) No. 100/2003, a maximum residue limit of 20 µgkg⁻¹ for AFB₁ has been
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49 fixed in cereals for animal consumption (Italian Ministerial Decree 149/2004, Italian Official
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51 68 Journal n.139, June 2004).

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56 70 Due to the significant health risks associated with the presence of aflatoxins in foods and to
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58 satisfy the rigorous legal requirements too, it is important to have efficient techniques for the
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72 detection of aflatoxins in foods and animal feeds. Several procedures for the determination of

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3 aflatoxins have been developed, comprising immunological methods, such as enzyme linked
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6 74 immunosorbent assays (Trucksess and Stack, 1994, Park et al.,1989), and chromatographic
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8 methods based on RP-HPLC and fluorescence detection (FLD) with chemical pre- or post-
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11 76 column (Wilson and Romer, 1991, Torres Espinosa et al., 1995, Roch et al., 1992, Saad et al.,
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13 1995, Garcia-Villanova et al., 2004, Davis et al., 1981, Tuinstra and Haasnoot, 1983,
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15 78 Niedwetzki and Lach, 1994, Ahmed and Robinson, 1998, Holcomb and Thompson, 1991,
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17 Garner et al., 1993, Chan et al., 2004, Va'zquez et al., 1999, Cepeda et al., 1996) or on-line
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19
20 80 electrochemical (Kok et al., 1986, Kussak et al., 1995, Dorner et al., 1993, Scholten and Spanjer,
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22 1996, Tavčar-Kalcher et al., 2007) derivatization of analytes.
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27 Although ELISA methods are extensively used for rapid qualitative screenings of aflatoxins,
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30 84 they are not useful in providing a definitive confirmation of the toxins and an accurate
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32 quantitative determination. The present trend is the use of HPLC for the determination of
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34 86 aflatoxins because of characteristics of specificity, high sensitivity and simplicity of operation.
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36 Additionally, the chromatographic determination allows quantifications of each toxin
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39 88 individually, obtaining more information than methods that give only a total quantification of
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41 aflatoxins. This is particularly important for food analysis where the determination of AFB₁ is
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44 90 required.
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49 92 The highly conjugated and rigid aflatoxin moieties give rise to native fluorescence
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51 characteristics, therefore small structural modifications can have a strong influence on the
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54 94 fluorescence properties; for instance aflatoxins G₂ and B₂ are more fluorescent than B₁ and G₁
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56 that are required to be converted into more highly fluorescent derivatives. Several methods of
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59 96 derivatization are available, including pre-column treatment with trifluoroacetic acid (TFA)
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(Garcia-Villanova et al, 2004) and post-column derivatization with iodine (Davis et al., 1981,

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3 98 Tuinstra et al., 1983, Niedwetzki et al., 1994, Ahmed et al., 1998, Holcomb et al., 1991),
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5 cyclodextrins (Va'zquez et al., 1999, Cepeda et al., 1996) and pyridinium hydrobromide
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7
8 100 perbromide (Garner et al., 1993, Chan et al., 2004). However, these methods are laborious and
9
10 present a number of disadvantages, such as handling of toxic reagents, instability of the
11
12 derivatives, a low day-to-day reproducibility. An alternative approach is based on post-column
13 102 electrochemically generated bromine (Cepeda et al., 1996, Kok et al., 1986, Kussak et al., 1995,
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16
17 104 Dorner et al., 1993, Scholten et al., 1996) that do not give a good chromatographic peak
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19 resolution and allows a less extent of automation, since any accidental interruption of the mobile
20
21 phase flux determines a serious damage to the electrochemical cell. Nowadays, an on-line
22 106 photochemical derivatization system is available (Joshua, 1993, Papadopoulou-Bouraoui et al.
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27 108 2002, Sobolev and Dorner, 2002, Sobolev, 2007, Waltking et al., 2006, Arranz et al., 2006,
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29 Zang and Lim, 2005), but its potential has not fully investigated. It has been reported that the
30
31 on-line photochemical derivatization coupled to fluorescence detection guarantees an
32 110 enhancement of efficiency and performances of the HPLC methods (Papadopoulou-Bouraoui et
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36 112 al., 2002, Zang et al., 2005). Differently from chemical pre- and post-column derivatization
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38 techniques, the photochemical derivatization does not involve any additional chemicals and,
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40 consequently, no auxiliary pump is required, ensuring better results without any control of the
41 114 derivatization reaction. Nevertheless, to the best of our knowledge chromatographic methods for
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46 116 the determination of aflatoxins, exploiting the photochemical derivatization and fluorescence
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48 detection, have been never validated according to the recent European Commission guidelines.
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53 In the present work, the procedure for the determination of aflatoxins B₁, B₂, G₁ and G₂ in foods
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55 and materials for feed, based on immunoaffinity column sample cleanup and liquid
56 120 chromatography coupled to photochemical derivatization and fluorescence detection, has been
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58 submitted to the validation procedure following the Regulation (EC) No. 882/2004. The
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3 validation of the proposed analytical method is required in order to provide accurate and
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6 124 reproducible results within- and inter-official control laboratories, which are involved in the
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8 monitoring and risk-assessment studies, as well as in official controls. Through validation
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11 126 procedure, linearity, selectivity, recovery, precision, detection and quantification limits (LODs,
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13 LOQs), ruggedness of the method and measurement uncertainty have been evaluated.
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15 128 Furthermore, laboratory performances were tested by taking part in a proficiency test round. The
16
17 results of the validation procedure demonstrate the conformity of the method of aflatoxins
18
19 analysis in foods and animal feeds with provisions of Regulation (EC) No. 401/2006, which, as
20 130 underlined by European Commission Sanco 0895/2007, establishes more specific performance
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22 criteria than Decision 657/2002/EC for methods of analysis to be used for official control of
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24 mycotoxins in foodstuffs.
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31 **Materials and Methods**

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34 136 **Chemicals.** A standard solution of aflatoxins B₁, B₂, G₁ and G₂ with a total certified
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36 concentration of 5 µg mL⁻¹ corresponding to a concentration of 2.0 µg mL⁻¹ each for B₁ and G₁
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38 and of 0.5 µg mL⁻¹ each for B₂ and G₂, was supplied by Riedel-de Haën (Sigma-Aldrich
39 138 Laborchemikalien, Seelze, Germany). Water, methanol and acetonitrile of HPLC grade were
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41 purchased from Baker (Deventer, Holland). Sodium chloride (≥99.0%), Na₂HPO₄·2H₂O
42
43 (≥99.0%), NaH₂PO₄·H₂O (≥99.0%) and TWEEN 20® were purchased from Sigma-Aldrich. All
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46 chemicals were of ACS grade. A 500 µg L⁻¹ stock solution of aflatoxins standard, containing 200
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48 µg L⁻¹ of AFB₁, 50 µg L⁻¹ of AFB₂, 200 µg L⁻¹ of AFG₁ and 50 µg L⁻¹ of AFG₂, were prepared in
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51 acetonitrile and stored in amber flask at -20°C up to 6 months. Working standard solutions were
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53 prepared by dilution in mobile phase and stored at -20°C when not in use; these solutions were
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56 stable for at least 4 weeks. Phosphate buffer salinum (PBS) was prepared by dissolving 9.0 g of
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58 sodium chloride, 2.85 g of Na₂HPO₄·2H₂O, 0.55 g of NaH₂PO₄·H₂O and 1.0 mL of TWEEN
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4 148 20 μ L in 990 mL of water. The pH value was adjusted to 7.2 with concentrated hydrochloric acid
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6 or NaOH 1M and the volume made to 1L with water.
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9 150 **Instrumentation.** Chromatographic separations were performed on a HPLC system, Agilent
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11 Technologies SL 1200 Series (Waldbronn, Germany) composed of a binary pump equipped with
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13 152 micro vacuum degasser, thermostated autosampler, column compartment and fluorescence
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15 detector (model G1321A). On-line photochemical derivatization was performed using a
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17 commercially available system UVETM LCTech GmbH (Dorfen, Germany) placed between the
18 154 separation column and the fluorescence detector, which consisted of a 254 nm low-pressure
19
20 mercury lamp and a 1 mL knitted reaction coil, fitted around the UV lamp. All the separations
21
22 156 were performed by using a ZORBAX Eclipse XDB C₁₈ column (150 \times 4.6 mm i.d., particle size
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24 5 μ m, Agilent Technologies) operating at a flow-rate of 1.0 mLmin⁻¹ in isocratic elution with a
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26 mixture of water, methanol and acetonitrile (55:15:30, v/v/v). The injection volume was 20 μ L
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28 158 and the column temperature was set at 40°C. The fluorescence detection was carried out at a λ_{exc}
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30 and λ_{em} of 365 and 435 nm, respectively. The system was interfaced, via network
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32 160 chromatographic software (Agilent ChemStation), to a personal computer for instrumentation
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34 control, data acquisition and processing.
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42 164 **Sample preparation.** Samples of wheat bran, almonds and pistachios were bought in local
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44 stores; durum wheat, maize and oats were supplied by farms chosen among the major cereal
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46 166 producers in the province of Foggia (Italy). Both sample extraction and cleanup procedure were
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48 those recommended by LCTech and slightly modified in order to simplify the analytical
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50 protocol and to reduce reagents and chemical wastes. The method did not require vacuum or
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52 168 pumping devices ensuring better recoveries and reproducibility. For each matrix, from a sample
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54 weight of 1 kg, an amount of 15 g of ground sample was suspended in 30 mL of 80 % methanol
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56 170 and vortexed for few min. For the validation measurements, samples were spiked, prior to the
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58 extraction, with the proper amounts of mixed aflatoxin standard solutions to give required
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3 concentrations. After filtration with paper (Whatman No. 40), 2 mL of the mixture were added
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6 174 to 8 mL of PBS solution and passed through the immunoaffinity columns Aflaclean™ (LCTech
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8 GmbH, Dorfen, Germany) for sample clean-up. After washing with 10 mL of water, the fraction
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10 containing the aflatoxins was slowly eluted with 2 mL of methanol by gravity at a flow rate
11 176 lower than of 0.5 mL min⁻¹. The eluate was evaporated to dryness at 40 °C under nitrogen
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13 stream (Dubnof Bath BSD/D). Finally, the residue was solubilized in 2 mL of the mobile phase,
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15 178 filtered by an Anotop 10 LC (0.2 µm, 10 mm, Whatman) and then injected. The analytical
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17 procedure resulted in a 2-fold dilution of aflatoxins B₁, B₂, G₁ and G₂, evaluated against a
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19 known concentration added to a blank real sample. The stability of purified extracts was high
20 180 enough to allow autosampler overnight injections. No significant change in concentration or
21
22 purity of aflatoxins within 8 hours of analysis was observed.
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29 184 **Validation procedure.** In consequence of different legal limits for aflatoxins in foodstuffs and
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31 cereals for animal consumption, different sets of standard solutions and spiked samples were
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33 used for foodstuffs (wheat bran, almonds and pistachios) and materials for feed (durum wheat,
34 186 maize and oats). The linearity test in foodstuffs was performed by three series of analyses on
35
36 three different days, by injecting four standard solutions of aflatoxins B₁ and G₁ each at
37
38 concentrations of 0.25, 0.5, 1.0 and 2.0 µgL⁻¹ and aflatoxins B₂ and G₂ each at concentrations of
39 188 0.0625, 0.125, 0.25 and 0.5 µgL⁻¹. Likewise, method linearity in feed materials was determined
40
41 by using working standard solutions of AFB₁ at concentration of 2.5, 5.0, 10 and 20 µgL⁻¹. The
42
43 method selectivity was tested by the analysis of 20 independent blank samples of foods (7 of
44 190 wheat bran, 7 of almonds and 6 of pistachios) and 20 samples of cereals (7 of durum wheat, 7 of
45
46 maize and 6 of oats), found negative by ELISA screening. The absence of interfering peaks in
47
48 the retention time-window of interest was checked for each analyte within the ± 2.5% retention
49 192 time range. Precision, recovery and both detection and quantification limits of the method were
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51 determined by performing tests on three sets of blank wheat bran samples (six replicates each)
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4 198 fortified with AFB₁ at concentration of 0.5, 1.0 and 1.5 times the ML in foodstuffs (i.e., 1.0, 2.0
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6 and 3.0 µgkg⁻¹) and aflatoxins B₂, G₁ and G₂ at concentration of 0.625, 1.25 e 1.875 times the
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9 200 ML of the sum of aflatoxins B₁, B₂, G₁ and G₂ (i.e., 1.0, 2.0 and 3.0 µgkg⁻¹ for B₁ and G₁, and
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11 0.25, 0.5 and 0.75 µgkg⁻¹ for B₂ and G₂). As far as the analysis of feed materials is concerned,
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14 202 only two sets of blank durum wheat samples (six replicates each) fortified with AFB₁ at
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16 concentration of 0.5, 1.0 and 1.5 times the ML in cereals for animals (i.e., 10, 20 and 30 µgkg⁻¹)
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19 204 were analysed. The experiments, corresponding to a total number of 54 and 36 for food samples
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21 and animal feed materials, respectively, were performed in different days with the same
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23 206 instruments but different operators.

27 208 **Results and Discussion**

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30 **Method Validation.** The proposed method has been validated according to Regulation (EC) No.
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32 210 882/2004, a document about official controls performed to ensure the verification of compliance
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34 with feed and food laws, animal health and animal welfare rules, which demonstrates the
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36 conformity of the analytical performances with criteria established in Regulation (EC) No.
37 212 401/2006. Therefore, through validation procedure, linearity, selectivity, recovery, precision,
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41 214 detection and quantification limits, ruggedness of the method and measurement uncertainty have
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43 been evaluated.
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47 216 **Selectivity towards interferences.** Figure 1 shows, as an example, the fluorescence enhancement
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49 attained by on-line photochemical derivatization for a wheat bran sample fortified with AFB₁
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51 and AFG₁ at 3.0 µgkg⁻¹ and AFB₂ and AFG₂ at 0.75 µgkg⁻¹. Through the post-column UV
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53 irradiation, AFB₁ and AFG₁ are converted by the reaction with water contained in the mobile
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56 220 phase to fluorescent hemiacetals, which are similar compounds to those obtained by
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58 derivatization with TFA (Garcia-Villanova et al., 2004). This photochemical modification
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222 determines an increase of the response sensitivity of AFB₁ and AFG₁, while a very slight

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4 modification of signal intensities has been observed for AFB₂ and AFG₂. Use of both the
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6 224 immunoaffinity sample cleanup and post-column photochemical derivatization permitted an
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8 efficient elimination of possible endogenous matrix interfering substances. In pre-validation
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10 experiments the probable interfering compounds, ochratoxin A and fumonisins B₁ and B₂,
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12 common contaminants of cereals, nuts and dried fruit, have been tested. These compounds
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14 showed retention times significantly different from those of aflatoxins or undetectable signals
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16 under the adopted experimental conditions. Chromatograms of Figure 2, obtained for blank and
17
18 spiked samples with aflatoxins B₁, B₂, G₁ and G₂, demonstrate the selectivity of the method
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20 230
21 toward other naturally present compounds, since at the retention times of the aflatoxins no
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23 interfering peaks were observed.
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27 **Calibration curves and limits of detection and quantification.** The goodness-of-fit of the data
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29 to the calibration curve is obtained in terms of response factor distribution (signal-to-
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31 concentration ratio, y_i/x_i) whose reference range is $y/x_{\text{mean}} \pm 10\%$. Furthermore, any systematic
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33 instrumental bias can be ruled out since the intercept includes the zero value at 95% confidence
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35 level. The calibration parameters evaluated for each aflatoxin by using standard solutions are
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37 reported in Table 1. Limits of detection and quantification were calculated according to the
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39 238 following equations (Miller and Miller, 1993): $\text{LOD} = 3.3 s_a/b$ and $\text{LOQ} = 10 s_a/b$, where s_a is the
40
41 standard deviation of the intercept and b is the slope of the regression line, obtained from the
42
43 calibration curve. The limit of quantification for AFB₁ of $0.11 \mu\text{gL}^{-1}$ corresponding to a value of
44 240
45 $0.22 \mu\text{gkg}^{-1}$ in matrix is satisfactory with respect to the allowed maximum content of $2 \mu\text{gkg}^{-1}$
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47 (Regulation (EC) No. 1881/2006). Fit of the data to the calibration curve of AFB₁ in cereals for
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49 242 feeds gave the linear regression equation $y = (1.002 \pm 0.006) x + (0.09 \pm 0.06)$ with a correlation
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51 coefficient of 0.9999, where y is the peak area expressed in luminescence unit (LU) and x is the
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53 244 concentration of AFB₁ in μgL^{-1} . The obtained limit of quantification of $1.2 \mu\text{gkg}^{-1}$ is noticeably
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4 lower than the maximum content of $20 \mu\text{gkg}^{-1}$ set for feed materials (Italian Ministerial Decree
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6 248 149/2004, Italian Official Journal n.139, June 2004).

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8 **Precision and recovery.** Precision results have been previously processed by the Shapiro–Wilk
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10 test to verify the normal distribution. Afterwards, ANOVA one way test was performed in order
11 250 to verify the homogeneity of the concentration mean values evaluated on three different days. In
12
13 Table 2 and 3 the results of repeatability (RSD_r) and within-laboratory reproducibility (RSD_R)
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15 252 are given for each aflatoxin at the three contamination levels in spiked wheat bran samples.
16
17 Furthermore, Table 4 shows the results of repeatability and within-laboratory reproducibility
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19 254 determined for AFB_1 in spiked durum wheat (cereal for feeds). Regulation (EC) No. 401/2006
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21 issued that the recommended values of experimental RSD for each concentration level must be
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23 256 lower or equal than value derived by Horwitz equation, which provides the expected $\text{RSD}\%$
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25
26 lower or equal than value derived by Horwitz equation, which provides the expected $\text{RSD}\%$
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28 258 only on the basis of the concentration, independently of the matrix and analytical method used.
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30 Nevertheless, for mass fractions lower than $120 \mu\text{gkg}^{-1}$, the $\text{RSD}\%$ can be predicted in a more
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32 260 restrictive manner by the Thompson equation (Thompson, 2000), which provides a
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34 recommended value of RSD_r of 15 % and a reference inter-laboratory reproducibility of 23%.
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36 As can be seen in Table 2 and 4, RSD_r % values are in agreement with the calculated
37
38 262 repeatability by Thompson equation. In absence of within-laboratory reproducibility reference
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40 values, our experimental data have been compared with the repeatability maximum permitted
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42 264 limit of 30%, which has been calculated as 2 fold the RSD_r recommended value, by using a
43
44 similar approach proposed for the calculation of the maximum permitted value of Horwitz RSD_R
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46 (Regulation (EC) No. 401/2006). As shown in Table 3, all RSD_R values resulted well below of
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48 266 30%, and even in agreement with the repeatability recommended values of 15%, apart from
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50 AFG_2 .
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52 In Table 5 mean recoveries of aflatoxins evaluated at each fortification level in spiked wheat
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54 268 bran and durum wheat samples are reported. As can be noticed, the recovery values obtained for
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4 272 food products ranged from 76.4 to 94.6% for aflatoxins B₁ and G₁ and from 79.5 to 92.8% for
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6 aflatoxins B₂ and G₂. These results are in agreement with Regulation (EC) No. 401/2006 that
7
8 274 establishes recoveries in the range 70-110% and 50-120% for concentrations in the range 1-10
9
10 μgkg^{-1} and less than 1 μgkg^{-1} , respectively. In the analysis of cereals for animal feeds, mean
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13 276 recovery data higher than 72.6 % were obtained. It was not possible to compare experimental
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15 mean recoveries with reference values since so far no legal criteria have been set for these
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18 278 matrices.

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20 Assessment of LODs and LOQs in real samples has been calculated for each analyte by mean
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22
23 280 the fit of the data to calibration curves obtained by plotting the fluorescence signals of 18 spiked
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25 wheat bran samples (six replicates evaluated in the three different working sessions at each
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27 282 validation level) as a function of the nominal fortification level. LOD and LOQ values, which
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29 have been calculated by using the same equations previously described, ranged from 0.06 to 0.3
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32 284 μgkg^{-1} and from 0.2 to 0.8 μgkg^{-1} , respectively, with LOD and LOQ for AFB₁ of 0.2 μgkg^{-1} and
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34 0.6 μgkg^{-1} , respectively. As expected, taking into account the loss of analyte during extraction
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37 286 and cleanup steps, LODs and LOQs are higher than those determined by calibration curves of
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39 standard solutions, but still considerably lower than legal limits.

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42 288 **Ruggedness (major changes).** The method ruggedness under conditions of major changes was
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44 evaluated by using the Youden experimental design as reported elsewhere (Muscarella et al.,
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46 290 2007). Two independent Youden tests were performed for different sets of real samples:
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48 almonds and pistachios (foodstuffs) and maize and oats (feed materials) spiked at the AFB₁
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51 292 maximum level. The seven factors chosen as variables for the Youden test were the matrix and
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53 six fictitious factors; the use of a fictitious variable means no variation of analysis conditions. In
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55
56 294 this case the Youden experimental design requires eight independent experiments: four with the
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58 matrix of validation (e.g. wheat bran and durum wheat) and four with each testing matrices
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296 (almonds, pistachios, maize and oats). Analysis of foods for human consumption gave a

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4 calculated standard deviation of difference S_{D_i} ($0.09 \mu\text{gkg}^{-1}$ for almonds and pistachios) lower
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6 298 than the estimated method precision at the ML ($0.21 \mu\text{gkg}^{-1}$). Similar considerations are valid
7
8 for maize sample whose standard deviation of difference S_{D_i} ($1.40 \mu\text{gkg}^{-1}$) resulted slightly
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10 300 lower than method precision ($1.45 \mu\text{gkg}^{-1}$). In the case of oats matrix, test F (performed at n-1
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12 degrees of freedom and 95% confidence level) demonstrated that S_{D_i} ($2.36 \mu\text{gkg}^{-1}$) was not
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14 statistically different from the reproducibility standard deviation ($1.45 \mu\text{gkg}^{-1}$) at the ML,
15 302 therefore the variation of the matrix has no effect on the method performances.
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20 304 **Measurement uncertainty.** The evaluation of uncertainty of analytical results is compulsory for
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22 laboratories accredited according to ISO 17025 (UNI CEI EN ISO/IEC 17025/2000), and
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24 several methods for the determination of this parameter have been proposed (EURACHEM
25 306 Guide, 1995; EURACHEM/CITAC GUIDE CG 4, 2000; Nordic Committee on Food Analysis,
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27 Guide, 1995; EURACHEM/CITAC GUIDE CG 4, 2000; Nordic Committee on Food Analysis,
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29 NMKL, 2003). In this work we have used the bottom-up method together with validation data
30 308 obtained from each step of the analytical procedure (Hund et al., 2001). On the basis of
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32 uncertainties propagation law, for each aflatoxin the concentration relative uncertainty has been
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34 310 calculated by the equation:
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$$38 \quad \bar{u} = \sqrt{(\bar{u}(C))^2 + (\bar{u}(V_f))^2 + (\bar{u}(w))^2}$$

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41 where \bar{u} indicates the relative uncertainty, C is the analyte concentration in the sample, V_f is the
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43 volume of the final extract, and w is the sample weight. The determination of \bar{u} has been
44 314 performed by considering four sources of uncertainty: (a) preparation of the standard; (b)
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46 method reproducibility; (c) method recovery; (d) calibration curve.
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51 A relative expanded measurement uncertainty was calculated by using a coverage factor k of 2,
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53 corresponding approximately to a 95% confidence level (Nordic Committee on Food Analysis,
54 318 NMKL, 2003) obtaining values of 8.4%, 12.9 %, 9.9 % and 14.2 % for aflatoxins B₁, B₂, G₁ and
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56 G₂, in foodstuffs. In the validation on cereals for animal feeds, the relative expanded
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58 320 measurement uncertainty calculated for AFB₁ was 7.6 %. These results were compared with the
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4 322 uncertainty function (Uf), reported in the Regulation (EC) No. 401/2006, to evaluate if the
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6 method of analysis is “fit-for-purpose”. This function, which gives the acceptable maximum
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8 324 uncertainty level of the method, is calculated by the following equation:

$$Uf = \sqrt{\left[\left(\frac{LOD}{2}\right)^2 + (\alpha C)^2\right]}$$

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15 326 where C is the concentration of interest and α is a C dependent constant factor. Measurement
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17 uncertainties evaluated for AFB₁ in food samples and feed materials (8.4% and 7.6%,
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19 respectively) are considerably lower than corresponding Uf values (20% at C = 2 μgkg^{-1} , $\alpha =$
20 328 0.2 and 21 % at C = 20 μgkg^{-1} , $\alpha = 0.2$, respectively). The above results confirm that method is
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25 330 “fit-for-purpose”.

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28 **Proficiency test round.** The proficiency test round was organised by Milk Standard Laboratory
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30 332 of A.I.A. (Italian Association of Breeders) in April 2007. Three unknown maize flour samples
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32 were analyzed in duplicate and the results were assessed in terms of Z-score, as calculated by the
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34 proficiency test organizer. The Z-scores obtained in the A.I.A. ring test performed on unknown
35 334 maize flour samples ($Z_1=-0.849$, $Z_2=-0.043$, $Z_3=-0.040$) and the value of the laboratory Z-score
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37 ($Z_{\text{LAB}}=-0.322$) were all below the reference value of 1, proving the technical competence of the
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39 336 laboratory and the method reliability.
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46 Conclusions

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49 340 A fast, reliable and automated method for the simultaneous determination of aflatoxins in a
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51 variety of foods and animal feed materials, based on HPLC-FLD with post-column
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53 photochemical derivatization, has been validated. Photochemical derivatization results
54 342 reagentless, no time consuming and easy to use, ensuring high sensitivity and reproducibility
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56 without any control of the derivatization step with respect to chemical derivatization systems,
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58 344 and it provides a better peak resolution and an extent of automation in comparison with the
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4 346 electrochemical ones. The accurate and highly sensitive proposed method offers several
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6 advantages in terms of simplicity, rapidity and efficiency. In fact, the results of the method
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8 348 validation, performed according to Regulation (EC) No. 882/2004, demonstrate the method
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10 conformity with provisions of Regulation (EC) No. 401/2006 and indicate that the procedure
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12 can be successfully applied for accurate confirmation analyses. The expanded measurement
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14 uncertainties and excellent results achieved in a proficiency test round confirmed the laboratory
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16 technical competence and the method reliability in the determination of aflatoxins in real
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18 352 samples, with the accuracy level corresponding to the requirements of the recent European
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20 regulation. Therefore, the proposed method is well suited to satisfy the demands for accurate
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22 354 and sensitive detection of aflatoxins with minimal sample preparation and cleanup steps.
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38 assistance.
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FIGURE CAPTIONS

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6 488 **Figure 1.** Chromatograms of a wheat bran sample spiked with AFB₁ and AFG₁ at 3.0 µgkg⁻¹
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8 and AFB₂ and AFG₂ at 0.75 µgkg⁻¹. A) with and B) without on-line photochemical derivation.
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10 Separation carried out by a ZORBAX Eclipse XDB C₁₈ column eluted with a mixture of water,
11 490 methanol and acetonitrile 55:15:30 (v/v) at a flow rate of 1.0 mLmin⁻¹; column temperature
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13 40°C; injected volume: 20 µL; λ_{exc} 365 nm and λ_{em} 435 nm.
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21 494 **Figure 2.** Chromatograms of a) blank samples and b) spiked samples of almonds (A), pistachios
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23 (B), maize (C) and oats (D). Almonds and pistachios samples have been fortified with AFB₁ and
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25 AFG₁ at 2.0 µgkg⁻¹, and AFB₂ and AFG₂ at 0.5 µgkg⁻¹. Maize and oats samples have been
26 496 fortified with AFB₁ and AFG₁ at 20 µgkg⁻¹, and AFB₂ and AFG₂ at 5 µgkg⁻¹. Experimental
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28 conditions as reported in Figure. 1.
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Validation of a confirmatory analytical method for the determination of aflatoxins B₁, B₂, G₁ and G₂ in foods and feed ...

Table 1. Performance parameters for aflatoxins in foodstuffs.

$y = a + bx^a$					
Aflatoxin	$b \pm SD$	$a \pm SD$	r^b	LOD ^c	LOQ ^c
B₁	0.87 ± 0.01	0.02 ± 0.01	0.9998	0.04	0.11
B₂	2.31 ± 0.04	0.02 ± 0.01	0.9996	0.01	0.04
G₁	0.368 ± 0.009	0.014 ± 0.009	0.9991	0.08	0.24
G₂	0.87 ± 0.01	0.005 ± 0.004	0.9996	0.02	0.05

^a y is the signal in luminescence unit (LU); x is the value of concentration in µg L⁻¹. ^b Correlation coefficient. ^c Values expressed in µg L⁻¹.

50 **Table 2.** Repeatability for the determination of aflatoxins in spiked wheat bran samples.

Aflatoxin	Operator	Contamination Level ($\mu\text{g kg}^{-1}$)	Determined Concentration Mean \pm SD (n = 6)	RSD _r ^a (%)
B₁	1	1	1.05 \pm 0.04	3.5
	2		1.0 \pm 0.1	9.8
	3		1.05 \pm 0.06	5.5
	1	2	1.9 \pm 0.1	7.1
	2		2.0 \pm 0.2	10
	3		1.9 \pm 0.1	4.9
	1	3	3.05 \pm 0.14	4.8
	2		3.00 \pm 0.08	2.7
	3		3.1 \pm 0.3	9.5
B₂	1	0.25	0.27 \pm 0.06	11
	2		0.25 \pm 0.02	9.1
	3		0.26 \pm 0.03	10
	1	0.50	0.46 \pm 0.03	7.0
	2		0.49 \pm 0.05	9.8
	3		0.47 \pm 0.03	5.9
	1	0.75	0.77 \pm 0.06	7.4
	2		0.75 \pm 0.02	3.0
	3		0.76 \pm 0.07	9.6
G₁	1	1	1.10 \pm 0.08	7.7
	2		1.0 \pm 0.1	10
	3		1.0 \pm 0.1	9.2
	1	2	1.8 \pm 0.2	11
	2		2.0 \pm 0.2	9.0
	3		1.9 \pm 0.1	6.2
	1	3	3.1 \pm 0.2	7.0
	2		3.0 \pm 0.1	4.4
	3		3.0 \pm 0.3	9.2
G₂	1	0.25	0.27 \pm 0.02	8.8
	2		0.24 \pm 0.04	15
	3		0.27 \pm 0.04	15
	1	0.50	0.46 \pm 0.06	13
	2		0.51 \pm 0.07	14
	3		0.47 \pm 0.05	11
	1	0.75	0.77 \pm 0.07	9.0
	2		0.74 \pm 0.05	6.6
	3		0.77 \pm 0.08	11

^a Relative standard deviation under repeatability conditions.

Validation of a confirmatory analytical method for the determination of aflatoxins B₁, B₂, G₁ and G₂ in foods and feed ...

58 **Table 3.** Within-laboratory reproducibility for the determination of
 59 aflatoxins in spiked wheat bran samples.

Aflatoxin	Contamination Level ($\mu\text{g kg}^{-1}$)	Determined Concentration RSD _R ^a	
		Mean \pm SD (n = 18)	(%)
B ₁	1	1.0 \pm 0.1	9.9
	2	1.9 \pm 0.2	11
	3	3.0 \pm 0.2	6.8
B ₂	0.25	0.26 \pm 0.03	12
	0.50	0.47 \pm 0.05	11
	0.75	0.76 \pm 0.06	7.6
G ₁	1	1.05 \pm 0.15	14
	2	1.9 \pm 0.3	15
	3	3.0 \pm 0.2	8.1
G ₂	0.25	0.26 \pm 0.05	20
	0.50	0.48 \pm 0.09	19
	0.75	0.76 \pm 0.08	10

62 ^a RSD_R, relative standard deviation under within-laboratory reproducibility conditions.

64 **Table 4.** Repeatability and within-laboratory reproducibility for the determination of
 65 aflatoxin B₁ in spiked durum wheat samples.

Aflatoxin	Contamination Level ($\mu\text{g kg}^{-1}$)	Operator	Determined Concentration RSD ^a	
			Mean \pm SD	(%)
<i>Repeatability (n = 6)</i>				
B ₁	10	1	10 \pm 1	10
		2	10.0 \pm 0.8	7.9
	20	1	20 \pm 1	6.1
		2	20.0 \pm 1.5	7.4
	30	1	29.8 \pm 1.6	5.2
		2	30 \pm 2	5.7
<i>Within-laboratory Reproducibility (n = 12)</i>				
B ₁	10		9.9 \pm 0.9	9.6
	20		20.2 \pm 1.5	7.2
	30		29.9 \pm 1.6	5.5

68 ^a RSD, relative standard deviation under repeatability (RSD_r) and within-laboratory reproducibility
 69 (RSD_R) conditions.

Table 5. Recovery of aflatoxins in spiked wheat bran (foodstuffs) and durum wheat samples (feed materials).

Aflatoxin	Contamination Level ($\mu\text{g kg}^{-1}$)	Recovery \pm SD (%)	Recommended Range (%)
<i>Foodstuffs (n = 18)</i>			
B₁	1	76.4 \pm 6.5	70-110
	2	83.0 \pm 9.8	
	3	92.2 \pm 8.3	
B₂	0.25	79.5 \pm 11.6	50-120
	0.50	82.1 \pm 9.9	
	0.75	92.8 \pm 8.0	
G₁	1	80.4 \pm 9.6	70-110
	2	83.4 \pm 11.1	
	3	94.6 \pm 8.2	
G₂	0.25	89.2 \pm 16.9	50-120
	0.50	82.0 \pm 10.7	
	0.75	87.7 \pm 10.1	
<i>Feed materials (n = 12)</i>			
B₁	10	75.7 \pm 6.3	N.A. ^a
	20	74.3 \pm 4.7	
	30	72.6 \pm 3.9	

^a N.A., Not Available; a legal value has not been established.

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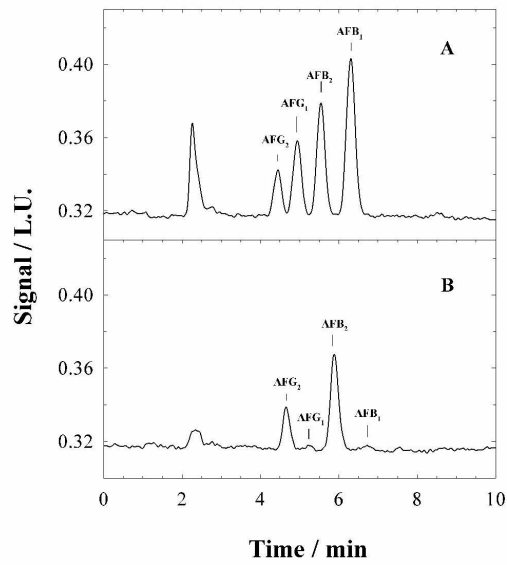


Figure 1

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Figure 1
839x1187mm (150 x 150 DPI)

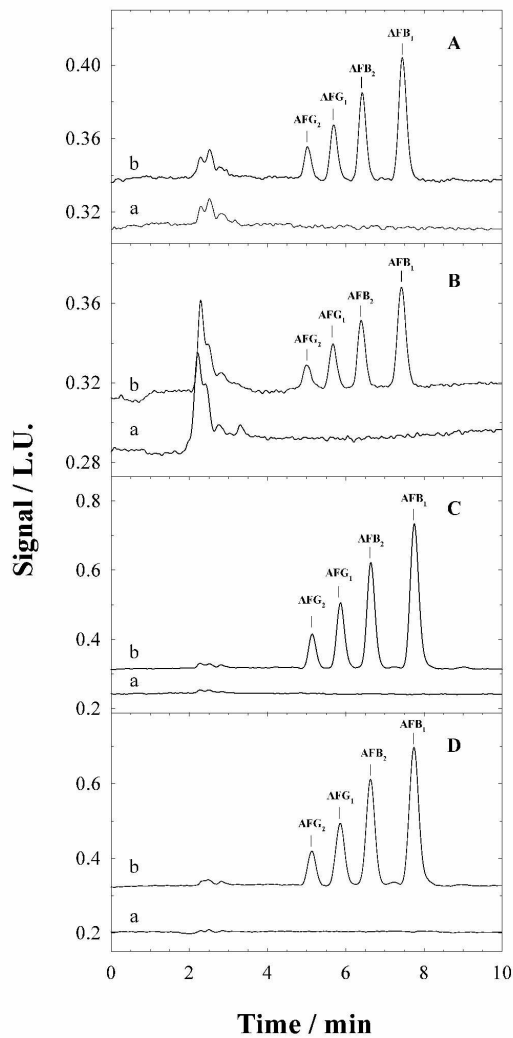


Figure 2

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Figure 2
839x1187mm (150 x 150 DPI)