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Analytical Methods

Determination of aflatoxin M1 in milk samples by means of an inductively coupled plasma mass spectrometry-based immunoassay

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ABSTRACT

An inductively coupled plasma mass spectrometry (ICP-MS)-based immunoassay has been developed to quantify aflatoxin M1 (AFM1) at ultra-trace levels in milk samples. AFM1 detection is carried out by means of a competitive immunoassay using secondary biotinylated antibodies and streptavidin-conjugated Au nanoparticles. After acid addition, nanoparticles are decomposed and Au signal is registered by means of ICP-MS. Results demonstrate that, under optimum conditions, the limit of detection of the immunoassay (0.005 μg kg^{-1}) is low enough to quantify AFM1 according to current international policies (including the more restrictive European one). Method accuracy and precision was checked by analyzing an AFM1 certified reference material and different milk samples spiked with known amounts of AFM1. AFM1 recovery values range from 80% to 102% whereas inter-assay and intra-assay precision are lower than 15%. Finally, this immunoassay methodology affords a higher dynamic working range (0.012–2.5 μg kg^{-1}) than other immunoassay methodologies described in the literature.

1. Introduction

Aflatoxins are secondary metabolites produced by different filamentous fungi (mainly *Aspergillus* species) and they are known to represent a high risk for human health due to their mutagenic and teratogenic effects. These substances could be found in different kinds of food and animal feeds (e.g. cereals, cocoa, coffee, etc.) that have been in contact with fungi through the food chain under high temperature and humidity conditions (Bhat, Rai, & Karim, 2010).

Aflatoxin M1 (AFM1) is the most significant aflatoxin in milk and dairy products. This compound is the hydroxylated form of the aflatoxin B1 (AFB1) and it is usually present in milk when animals have been fed with feedstuffs containing AFB1 (Flor-Flores, Lizarraga, López de Cerain, & González-Peñas, 2015). AFM1 has been classified as Group 2 human carcinogen by the International Agency of Research on Cancer (IARC, 2002). For this reason, and taking into account the significance of milk and milk products in human diet (especially for children), the maximum allowed levels of AFM1 are strictly regulated worldwide (Flor-Flores et al., 2015; Prandini et al., 2009). Food and Drug Administration from USA limits the concentration of AFM1 in milk and processed milk products at 0.50 μg kg^{-1} (FDA, 2005). However, European Community Legislation is even more restrictive and does not allow AFM1 levels in milk and infant formula above 0.050 and 0.025 μg kg^{-1}, respectively (EC, 2001, 2004).

AFM1 determination is usually carried out by means of high-performance liquid chromatography (HPLC) or immunoassays after an extraction treatment to reduce matrix effects and preconcentrate the analyte (Reiter, Zentek, & Razzazi, 2009; Shephard, 2008). HPLC is considered the reference method for AFM1 analysis (Aguilera-Luiz, Plaza-Bolaños, Romero-González, Martínez Vidal, & Garrido Frenich, 2011; Beltran et al., 2011; Diniz Andrade, Laine Gomez da Silva, & Dutra Caldas, 2013; Dragacci, Grosso, & Gilbert, 2001). The detection of AFM1 is generally achieved by means of both fluorescence and mass spectrometry. However, HPLC analysis requires laborious sample preparation treatments to reduce matrix effects and improve analytical figures of merit. On the other hand, immunoassays (mainly Enzyme Linked Immunosorbent Assay, i.e., ELISA) are widely used for screening purposes due to their high sample throughput, simplicity and low budget (Guan, Li, Zhang, Zhang, & Jiang, 2011; Jiang et al., 2013; Pico, 2007; Vdovenko, Lu, Yu, & Sakharov, 2014).

Inductively coupled plasma mass spectrometry (ICP-MS) is a powerful technique for inorganic analysis due to its: (i) low limits

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of detection (LoD) (usually in the μg kg^{-1}-ng kg^{-1} range), (ii) good precision; (iii) multi-element capability, (iv) high dynamic range; and (v) the possibility to obtain analyte isotopic information (Bettmer et al., 2009). Traditionally, the analysis of organic molecules by ICP-MS has been limited to those analytes containing metals, metalloids and some non-metals (e.g. P or S) because of the difficulty to utilize C, H, N and O for quantification purposes at ultra-trace levels. However, it has been demonstrated that ICP-MS can be used as a detector for all kinds of organic molecules (e.g. proteins, mRNA, DNA, etc.) after a derivatization procedure with a heteroatom or a compound containing a heteroatom (Kretschy, Koellensperger, & Hann, 2012; Tholey & Schaumlöffel, 2009). In this context, ICP-MS has been employed as a detector of proteins and biomolecules in immunoassays in view of it is quite straightforward to functionalized antibodies with elements detectable by this technique (Giesen, Waentig, Panne, & Jakubowski, 2012; Liu, Wu, Yang, Hou, & Lv, 2014). In general, antibodies (or any other species present in the immunoassay) are conjugated with elements presumably not present in biological samples, such as lanthanide based chelates or Au nanoparticles. The latter approach is especially advantageous to amplify the analytical response because of the significant number of Au atoms in each nanoparticle. The use of ICP-MS as a detector in immunoassays affords several attractive features such as: (i) specificity to heteroatom detection; (ii) compound-independent detection sensitivity; (iii) high elemental sensitivity and dynamic range; (iv) robustness (complex sample pre-treatments are not required to diminish matrix effects); and (vii) multielement capabilities, since the antibodies can be conjugated with different heteroatoms and detected in a single run. In spite of the above mentioned features, the use of ICP-MS-based immunoassays in food analysis has been limited so far. Nonetheless, these methods have been successfully applied to quantify peanut allergens (Careri, Elviri, Mangia, & Mucchino, 2007), ochratoxine A in wine (Giesen, Jakubowski, Panne, & Weller, 2010) and progesterone in milk (Montoro Bustos et al., 2012).

The goal of this work is to develop a new procedure to quantify AFM1 in milk samples by ICP-MS at the security levels required by the current international policies with accuracy and precision. The proposed methodology is based on a competitive immunoassay using secondary biotinylated antibodies and streptavidin-Au nanoparticles followed by Au detection by ICP-MS.

2. Experimental

2.1. Reagents and materials

All solutions were prepared using ultrapure water (Milli-Q water purification system, Millipore Inc., Paris, France).

Sodium carbonate, sodium hydrogen carbonate, monosodium phosphate, disodium phosphate, sodium chloride, biotinylated goat anti-rabbit IgG secondary antibody, AFM1 from Aspergillus flavus, AFM1-Bovine Serum Albumin conjugate (AFM1-BSA), streptavidin conjugated 40 nm Au nanoparticles from *Streptomyces avidinii*, polyethylene glycol sorbitan monolaurate (Tween 20) and HPLC-grade acetonitrile were purchased from Sigma-Aldrich (Steinheim, Germany). Bovine serum albumin (BSA) was obtained from Biowest (Nuaillé, France) whereas anti-AFM1 primary rabbit polyclonal antibody was obtained from Agrisera (Vännas, Sweeden). Iridium 1000 mg L^{-1} stock solution was provided by Merck (Darmstadt, Germany). Thiourea, 69% w w^{-1} nitric acid and 35% w w^{-1} hydrochloric acid were purchased from Panreac (Barcelona, Spain).

F16 maxisorp polystyrene microtiter plates were obtained from Thermo-Scientific (Roskilde, Denmark).

2.2. Buffers and solutions

Standard stock solution of AFM1 (10 μg L^{-1}) was prepared in pure acetonitrile in an amber vial. AFM1-BSA was dissolved in 2 mL of phosphate buffer solution (PBS, 10 mol L^{-1} monosodium phosphate, 2 mmol L^{-1} disodium phosphate, 154 mmol L^{-1} sodium chloride, pH 7.6) for a final concentration of 500 μg mL^{-1}. Both solutions were kept at −20 °C. Primary rabbit polyclonal antibody was dissolved in 500 μL ultrapure water and kept at 4 °C.

The following solutions were employed in the ICP-MS-based immunoassay: (a) carbonate/bicarbonate buffer solution (15 mmol L^{-1} sodium carbonate and 35 mmol L^{-1} sodium hydrogen carbonate, pH 9.6); (b) 1% w V^{-1} BSA in a PBS solution for plate blocking; (c) 1% w V^{-1} BSA and 0.05% V V^{-1} Tween 20 in PBS as incubation media; (d) 0.05% V V^{-1} Tween 20 in PBS for washing microtiter plate wells and (d) 4% V V^{-1} nitric acid and 12% V V^{-1} hydrochloric acid for Au-nanoparticles digestion.

2.3. Immunoassay procedure

The analysis of AFM1 by ICP-MS is based on a competitive immunoassay (Giesen et al., 2010) in which varying amounts of free AFM1 inhibit the binding of specific antibodies to the solid phase coated with AFM1-BSA conjugate using secondary biotinylated antibodies and Strep-Au for ICP-MS detection (see Fig. 1). First of all, the polystyrene microtiter plate wells were coated with 100 μL of the appropriate AFM1-BSA concentration in carbonate-bicarbonate buffer (step 1a). After a 1 h incubation at room temperature, wells were washed three times and blocked with 1% w V^{-1} BSA in PBS for 1 h at room temperature. Simultaneously, samples or AFM1 standards were mixed with the anti-AFM1 antibody solution (step 1b). The mixture was incubated 1 h at room temperature and then 100 μL of it were transferred to the plate wells for another incubation step of 2.5 h at room temperature (step 2). After washing -three times in order to eliminate the antigen-antibody complexes present in the solution as well as the free antibody-, the microwell plates were sequentially incubated with 100 μL of a secondary biotinylated goat anti-rabbit IgG solution (step 3) and then with 100 μL of the Strep-Au solution (step 4). The incubation time for the previous steps was 1 h at room temperature followed by three washing steps. Finally, before ICP-MS analysis, Au nanoparticles were digested (step 5) with 150 μL of the digestion acid mixture and spiked with 50 μL of a 1.0% w V^{-1} thiourea solution containing 2.5 μg L^{-1} Ir. All the AFM1 standards were analyzed in triplicate wells whereas the samples containing unknown AFM1 amounts in quintuplicate wells.

2.4. ICP-MS instrumentation

According to the immunoassay procedure described above, AFM1 is quantified by means of ICP-MS using the $^{197}Au^{+}$ signal. Despite the poor Au ionization in the plasma because of its high ionization potential (9.23 eV) (NIST atomic spectradatabase, 2016), the use of Au nanoparticles is especially advantageous to amplify the analytical response due to the high number of atoms present in each nanoparticle. ICP-MS measurements were performed by means of a 7700x quadrupole-ICP-MS system (Agilent, Santa Clara, USA). Operating conditions were daily optimized to maximize $^{197}Au^{+}$ signal following the instrument user's guide (Table 1).

On account of the limited volume of sample available in the immunoassay (200 μL), a micronebulizer (OneNeb, Ingeniatrics, Sevilla, Spain) coupled to a double pass quartz spray chamber (Agilent, Santa Clara, USA) was selected as the sample introduction system. Using this configuration, $^{197}Au^{+}$ sensitivity for a sample