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A simple method for point-of-need extraction, concentration and rapid multi-mycotoxin immunodetection in feeds using aqueous two-phase systems

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ABSTRACT

The rapid detection of mycotoxins in feed samples is becoming an increasingly relevant challenge for the food production sector, in order to effectively enforce current regulations and assure food and feed safety. To achieve rapid mycotoxin detection, several biosensing strategies have been published, many reaching assay times of the order of a few minutes. However, the vast majority of these rely on sample preparation based on volatile organic solvents, often comprising complex multi-step procedures and devoid of clean-up and/or concentration effects. Here, a novel sample preparation methodology based on a green, non-toxic and inexpensive polyethylene glycol-sodium citrate aqueous two-phase system is reported, providing single-step extraction and concentration of three target mycotoxins within 20 min: aflatoxin B1 (AFB1), ochratoxin A (OTA) and deoxynivalenol (DON). With point-of-need applications in mind, the extraction procedure was optimized and validated using a rapid multi-toxin microfluidic competitive immunoassay. The assay was successfully tested with spiked complex solid matrices including corn, soy, chickpea and sunflower-based feeds and limits of detection of 4.6 ng g^{-1} ± 15.8%, 24.1 ng g^{-1} ± 8.1% and 129.7 ng g^{-1} ± 53.1% (±CV) were obtained in corn for AFB1, OTA and DON, respectively. These sensitivities are fit-for-purpose at the required regulatory and recommended limits for animal feed, providing an effective and safe semi-quantitative mycotoxin analysis that can be performed in the field.

1. Introduction

The detection of analytes at the point-of-need using portable devices [1] is increasingly in demand in fields such as medical diagnostics, environmental analysis, food safety and industrial food production. In the latter two applications, the detection of mycotoxins in foods and feeds is particularly relevant [2,3]. Mycotoxins are secondary metabolites of fungi that can be produced after contamination of raw materials at multiple stages of production, storage and transport [4]. These compounds are highly toxic to both humans and animals [5,6] and particular examples of such effects are the carcinogenicity of aflatoxin B1 (AFB1) [7], the hepatotoxicity of ochratoxin A (OTA) [8] and the acute toxicity of deoxynivalenol (DON) [9]. Therefore, strict regulatory limits for their presence in food and feeds have been imposed by the European Union [10], which currently has one of the highest food standards worldwide according to the portal for the rapid alert system for food and feed (RASFF) [11]. These regulatory limits are in the range of 2–12 ng g^{-1} for AFB1, 2–10 ng g^{-1} for OTA and 200–750 ng g^{-1} for DON, considering foods for consumption by human adults [12]. Currently, the regulatory limits for mycotoxins in feeds are only being enforced for AFB1 [13], with maximum limits between 5–50 ng g^{-1}. Nevertheless the EU also recommends maximum limits for OTA (50–100 ng g^{-1}) and DON (0.9–5 μg g^{-1}), which is of particular importance in the livestock industry to prevent losses in product quality and animal productivity [14].

Currently, the challenge of analyzing mycotoxin contamination in a variety of complex matrices is typically addressed by strategically collecting and homogenizing the samples [15], followed by extraction, sample preparation and quantification in certified laboratories, which utilize highly sensitive and high performance techniques such as HPLC–MS [16,17]. Within the scope of extrac-

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tion and sample preparation, critical steps for effective mycotoxin quantification in solid feeds [18,19], several strategies are being developed and optimized to effectively extract multiple key mycotoxins prior to detection, typically comprising a variety of mixtures containing high concentrations of organic solvents (≈60–80%) including, most commonly, methanol and acetonitrile [20,21]. Extractions based on organic solvents are also being used coupled to simpler "yes-no" biosensors such as lateral flow immunoassays both at the research [3,22] and commercial (i.e. Reveal Q+ from Neogen [23] and Ochra-V [24], Afla-V [25] and DON-V [26] from Vicam) levels towards a simpler, faster and less expensive screening. However, in order to effectively enforce regulations, there is a high demand for rapid on-site screening methodologies prior to sending the samples to a certified lab. In this case, it would be highly advantageous to have a simple, non-toxic, non-volatile, stable, green, biodegradable and efficient strategy to process samples at the point-of-need, able to minimize matrix interference and provide a fit-for-purpose sensitivity at the required limits. A review of the recent literature reveals that strategies that are being developed towards this goal can be primarily divided into: (1) extraction based on dispersive liquid-liquid microextraction (DLLME) which provides a dramatic reduction in the required volumes of organic solvents [27,28]; (2) extraction using supramolecular complexes with selectivity towards small molecules [29]; (3) liquid-liquid extractions based on aqueous two-phase systems typically composed of soluble alcohols and salts [30,31]; (4) extraction based on solid-phase microextraction (SPME), a solvent free extraction process using specific sorptive solid matrices dispersed in solution [32]; (5) extractions using ionic liquids [33,34] and (6) extractions using surfactant or polymer additives [35,36]. Among these different approaches, (1), (2) and (3) require organic solvents and/or toxic reagents and multiple steps. The complexity of the procedure is also an issue for SPME (4) since the sorptive matrix has to be recovered and processed to recover the target molecule. The use of ionic liquids (5) is very promising considering the high potential for optimization due to the more than 10^6 possible binary combinations [37], combined with high extraction efficiencies/selectivities obtained by several research groups [38–41]. However, the potential toxicity and environmental impact of several ionic liquids is still poorly understood [42,43] and these are also typically expensive and complex to synthesize [44]. Finally, regarding the use of nontoxic additives such as sodium dodecyl sulphate (SDS) or polyethylene glycol (PEG) (6), while these methods proved effective regarding matrix neutralization and extraction efficiencies [35,36,45], they do not provide a concentration effect which can be highly advantageous at low mycotoxin concentrations.

With the objective of developing an improved, simpler and greener extraction methodology for point-of-use mycotoxin analysis, a novel single-step extraction procedure comprising the use of polyethylene glycol-salt aqueous two-phase systems (ATPS) is reported. As a case study, this sample processing methodology was used to simultaneously extract and concentrate three target mycotoxins, namely aflatoxin B1 (AFB1), ochratoxin A (OTA) and deoxynivalenol (DON), from multiple raw feeds and was subsequently combined with a point-of use-compatible microfluidic multi-mycotoxin immunoassay platform, previously developed and optimized by our group [46,47].

2. Experimental methods

2.1. Chemicals and biologicals

Polyethylene glycol (PEG) with molecular masses of 3.5 kDa, 8 kDa and 20 kDa, sodium phosphate monobasic, potassium phosphate dibasic, sodium citrate tribasic dehydrate, sodium bicarbonate, phosphate buffered saline (PBS) tablets (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7), 1,1′-Carbonyldiimidazole (CDI), acetone (99%), anhydrous ethanol, methanol and acetonitrile, bovine serum albumin (BSA), ochratoxin A (OTA), aflatoxin B1 (AFB1), deoxynivalenol (DON), OTA-BSA conjugates and AFB1-BSA conjugates were purchased from Sigma-Aldrich. DON-BSA conjugates were prepared in-house using CDI chemistry [47]. The stock solutions of mycotoxins were prepared as follows: OTA was dissolved in 33% methanol in water (100 μg mL^{-1}), AFB1 was dissolved in anhydrous ethanol (200 μg mL^{-1}) and DON was dissolved in anhydrous methanol (1 mg mL^{-1}). Affinity purified anti-OTA rabbit polyclonal antibodies conjugated with horse radish peroxidase (HRP) were purchased from Immunechem Pharmaceuticals (Burnaby, Canada), anti-AFB1 mouse monoclonal antibodies (AFA-1) were purchased from Abcam (Cambridge, UK) and anti-DON mouse monoclonal antibodies were purchased from Biotez (Berlin, Germany). The anti-AFB1 and anti-DON antibodies were conjugated with HRP using an HRP labeling kit, purchased also from Abcam, and performing the procedure provided by the supplier. Luminol was purchased as a SuperSignal™ West Femto Maximum Sensitivity Substrate, supplied by Thermo Fischer Scientific. All solutions were prepared using ultrapure water obtained from a MilliQ® system from EMD Millipore. All feeds under study were kindly supplied by EWOS® Innovation (Dirdal, Norway) within the scope of the project DEMOTOX (FP7-SME-2013- 604752).

2.2. Spectrofluorometric and spectrophotometric quantification of AFB1, OTA and DON in the top phase of PEG-phosphate and PEG-citrate ATPSs

The quantification of each toxin in the PEG-rich top phase was performed according to a methodology previously reported by our group [48]. Each ATPS was prepared in duplicate by mixing 1.2 mL of the respective salt solution with a certain volume of PEG (50%), ranging from 55 to 100 μL according to Table S1, in order to generate a top PEG-rich phase with approximately 80 μL. Then, the mycotoxins were spiked in one of the ATPS duplicates, henceforth referred to as "sample tube" for a final concentration of 1 μg mL^{-1} for OTA and AFB1 and 10 μg mL^{-1} for DON. In the other ATPS duplicate, henceforth referred to as "calibration tube" the same volume of solution without mycotoxins was added. Both tubes for each condition were then vigorously mixed in a vortex mixer for 30 s and subsequently centrifuged at 2000*g* for 1 min to separate the phases. The concentrations of AFB1 and OTA were measured through their intrinsic fluorescence (λ_{ex} = 369 nm; λ_{em} = 439 nm for AFB1 and λ_{ex} = 333 nm; λ_{em} = 446 nm for OTA) using a Varian Cary Eclipse plate reader spectrofluorimeter (800 V, 5 nm slits) and DON was measured by spectrophotometry (λ = 230 nm) in a SpectraMax Plus 384 Microplate Reader from Molecular Devices. To perform the measurement of the mycotoxin concentration in the top phase of each system, 20 μL of each top phase from the sample tubes were mixed with 80 μL of water and added to a well and compared in terms of fluorescence/absorbance with two other wells containing (1) 20 μL of the top phase of the calibration tube plus 80 μL of water (blank, used to subtract the background) and (2) the same composition as the blank plus 2 μg mL^{-1} AFB1 or OTA and 20 μg mL^{-1} of DON (single point calibration). The plates used were white Corning® polystyrene 96 well-plates for fluorescence measurements and Greiner UV-Star® 96 well plates for absorbance measurements. All calculated values related to logP, logD and microspecies concentrations were calculated using the Chemicalize tool by ChemAxon [49]. All partition coefficient (*Kp*) and volume ratio (*Vr*) values are defined as the quotient between the concentration of each mycotoxin in the top and bottom phases and volume of top and bottom phases, respectively.