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Aqueous two-phase systems for enhancing immunoassay sensitivity: Simultaneous concentration of mycotoxins and neutralization of matrix interference

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ABSTRACT

Immunoassays have a broad application range, from environmental and food toxicology to biomedical analysis, providing rapid and simple methods for analyte quantification. Immunoassays, however, are often challenging at nM and sub nM concentrations and are affected by detrimental matrix interference effects, as is the case of the detection of ochratoxin A (OTA) and Aflatoxin B1 (AFB1). These are widespread mycotoxins found in food and feed, with serious potential implications for human health. This work demonstrates the use of polymer–salt aqueous two phase systems (ATPSs) for the simultaneous concentration of mycotoxins and neutralization of matrix interference. In particular, polyethylene glycol (PEG)-phosphate salt ATPSs were used to enhance the detection sensitivity of OTA and AFB1 in wines and beer by an indirect competitive ELISA. Using this methodology it was possible to quantify both analytes spiked in red wine with limits-of-detection (LoD) down to 0.19 ng/mL and 0.035 ng/mL, respectively, with results comparable to those obtained using solutions of toxins in phosphate buffered saline (PBS) buffer (0.7 ng/mL and 0.009 ng/mL, respectively). Furthermore, a very low matrix-to matrix variability was observed, with LoD and half inhibitory concentration (IC_{50}) values of 5.17 ± 1.08 and 33.2 ± 3.5 ng/mL (±SD) obtained in the detection of OTA spiked in red and white wines, beer or PBS buffer. These results indicate the potential of ATPS as a fast and simple concentration step and in providing matrix-independent analyte quantification for enhanced immunoassay sensitivity below regulatory levels.

1. Introduction

Immunoassays are powerful bioanalytical techniques that are used extensively in medical diagnostics [1], allowing a simpler multiplexing using a different label for each target analyte (label resolved methods) [2] or an array of spots, with each spot targeting a given analyte (space resolved methods) [3,4]. Immunoassays have also been successfully broadened to environmental analysis [5,6], with a significant and increasing share being targeted to mycotoxin quantification in feeds and foodstuff [7–9]. However, immunoassays suffer from significant matrix interference problems in samples such as blood [10], urine [11], water [5] and food extracts [12–15]. This interference is responsible for a pronounced decrease in sensitivity and inter-assay reproducibility.

Two mycotoxins, ochratoxin A (OTA) and aflatoxin B1 (AFB1) were selected as environmentally relevant model analytes for testing with immunoassays. Both are of high importance due to confirmed multiple toxic effects in animals and potential toxic effects in humans such as carcinogenicity and hepatotoxicity [16,17], together with their widespread prevalence in foods and feeds [18]. The detection of OTA and AFB1 using competitive immunoassays has been well explored in literature [19–25] and is gaining an increasing amount of interest due to rapid assay times and low-cost when compared to conventional chromatographic methods [26,27]. This detection is particularly relevant in both

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wines and beer [28–30], with regulatory limits set as low as 2 ng/mL for OTA in wines and 2 ng/mg for AFB1 in cereal based products [31]. However, these matrices result in pronounced interference effects, resulting in a sharp decrease in analysis sensitivity. This is generally related to the high concentration of polyphenols such as tannins, anthocyanins and gallic acid [32–34], which are reported to reversibly or irreversibly bind to proteins [35,36]. Hence, the existing immunoassays for mycotoxin quantification in wines, for instance, are often dependent on some type of matrix clean-up procedure such as solid phase extraction [20,21] or matrix neutralization by the addition of $NaHCO_3$ and polyethylene glycol (PEG), for example [22]. While the former provides simultaneous matrix clean-up and analyte concentration, it increases the costs and complexity of the assay. On the other hand, while being simple, the latter implies sample dilution, which is obviously undesirable when high sensitivity is demanded.

In this paper, we use a simple aqueous two phase system (ATPS) for rapid and low-cost analyte concentration and matrix neutralization, prior to performing an indirect competitive ELISA (icELISA). ATPS is a type of liquid–liquid separation phenomenon that occurs when certain compounds are mixed in an aqueous solution above a particular critical concentration [37]. This solution is then capable of splitting into two or more spontaneously generated phases, with different chemical compositions, but both comprised mainly of water. There are many pairs of such compounds that are capable of generating biphasic interfaces like polymer–polymer [38] systems, polymer–salt systems [39], thermosensitive polymer–water systems [40], alcohol-salt systems [41] and polymer- or salt-ionic liquid systems [42,43]. Among these, the PEG-NaH_2PO_4/K_2HPO_4 pair, which has been widely used by our research group for protein purification [44–46], is chosen here as the model matrix clean-up and toxin concentration system. The choice of this ATPS pair allows the exploitation of the high electrochemical gradient between the two water-rich phases, inherent to this system, resulting in a pronounced uneven molecular partition behavior [47]. This allows the optimization of conditions to effectively concentrate a target analyte by manipulating the phase volumes, by increasing or decreasing the final volume ratio (V_r) of the system, defined as the volume of top phase divided by the volume of bottom phase. Thus, we propose a novel combination of PEG-salt ATPS-based extraction with a well plate icELISA for simultaneous analyte concentration and screening of matrix interference, leading to enhanced immunoassay performance.

2. Experimental methods

2.1. Materials

PEG with 2, 6 or 10 kDa average molecular weight (MW), NaH_2PO_4, K_2HPO_4, OTA, AFB1, phosphate buffered saline (PBS) tablets, methanol (99.9%), OTA-bovine serum albumin (BSA) conjugate, AFB1-BSA conjugate, Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB) ready-to-use liquid substrate (super-slow) for ELISA and BSA were purchased from Sigma Aldrich. Ultrapure water was obtained from a Milli*Q* purification system from Millipore (Billerica, MA, USA). OTA and AFB1 stock solutions were prepared with an equal concentration of 100 μg/mL in 25% (v/v) and 50% (v/v) methanol in PBS, respectively. The anti-OTA IgG and anti-AFB1 IgG were purchased from Abcam (Cambridge, UK) as monoclonal mouse antibodies (3C5 and AFA-1, respectively). Horseradish peroxidase (HRP) conjugated polyclonal goat anti-mouse IgG (whole molecule) was purchased also from Abcam. Generic Portuguese red wine (11% v/v ethanol), white wine (11% v/v ethanol) and beer (5.2% v/v ethanol), were purchased from a local store.

2.2. ATPS preparation for evaluation of analyte partition

The ATPSs were prepared for a total of 1 g in 1.5 mL microtubes. Aqueous solutions of PEG 50% (w/w) (2000, 6000 or 10,000 Da average MW and NaH_2PO_4/K_2HPO_4 40% (w/w) (adjusted to a final pH of 6, 7 or 8 by changing the ratio of monosodic to dipotassic salts added) were added depending on the conditions chosen and adjusted to the total mass of the system using ultrapure water. Then, each tube was agitated in a vortex for 10 to 15 s. Finally, to obtain clear separated phases the microtubes were centrifuged at 6000 × *g* for 2 min. The analytical balance used was a Mettler Toledo (Columbus, OH, USA) XS205 Dual Range. The statistical analysis of the results was performed using the computer software Statistica 7 from StatSoft (Tulsa, OK, USA).

2.3. Fluorimetric quantification of the analytes

Analyte quantification in both phases of 1 μg/mg analyte spiked ATPS was performed using a Varian (Palo Alto, CA, USA) Cary Eclipse plate reader spectrofluorimeter. All experiments were performed using a high voltage (800 V) setting for the photomultiplier tube and the excitation/emission slits were set at 5 nm. The readings were averaged from 5 consecutive measurements with a 0.5 s interval in white polystyrene Corning® (Corning, NY, USA) 96 well plates. The excitation and emission wavelengths for OTA and AFB1 quantification were set at 333/446 nm and 370/439 nm, respectively. A single point calibration of the fluorescence emission was performed by adding a known amount of analyte (2.5 μg/mL) to both top and bottom phases of a second ATPS prepared for each condition. This addition was performed after separating each phase to individual microtubes. A third ATPS was also prepared to subtract the background fluorescence value for each phase. The validity of this approach is supported by the linear dependence of the fluorescence emission as a function of the analyte concentration (Fig. A-1).

2.4. ATPS preparation for analyte immunodetection

Undiluted red wine, white wine and beer were first adjusted to pH 6 using 10 M NaOH before any further processing. Then NaH_2PO_4/K_2HPO_4 were added at a mass ratio of 1.27 or 0.43 for a final pH of 6 or 7, respectively, and weighed together with wine or beer to the intended final mass concentration. This mixture was then agitated in a vortex for 20–30 s for complete salt dissolution. Then, 1881 μL of this solution were transferred to individual 2 mL microtubes and spiked with 19 μL of an OTA or AFB1 solutions prepared in PBS, at a concentration 100 times higher than the intended final concentration in wine. For the reference solutions, 19 μL of PBS were added instead of the OTA or AFB1. A 50% (w/w) aqueous solution of the second phase forming component (PEG 1000, 8000 or 20,000) was then added, 4–5 mg at a time, with intermittent agitations for 20–30 s in a vortex and 2 min of centrifugation at 6000 × *g*. This process was stopped when a collectable amount of top phase was obtained and was only performed the first time a new system was used or a new top phase volume was required. The collected top phase was then diluted with a PBS solution containing anti-OTA or anti-AFB1 IgG, yielding the final IgG concentration of 50 or 100 ng/mL, respectively. Depending on the experiment, in cases where polyphenol precipitation occurred after the dilution step, the solution was centrifuged a second time under the above mentioned conditions. This final solution was then used as SUA (sample under analysis) for the wellplate icELISA.

2.5. Indirect competitive ELISA

MaxiSorp® flat-bottom 96 well microtiter plates from Nunc (Roskilde, Denmark) and Bio-one PS medium bind ELISA plates from