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Reduced graphene oxide and gold nanoparticle composite-based solid-phase extraction coupled with ultra-high-performance liquid chromatography-tandem mass spectrometry for the determination of 9 mycotoxins in milk

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Zearalenone (PubChem CID: 5281576)
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ABSTRACT

A reliable solid-phase extraction (SPE) procedure using reduced graphene oxide and gold nanoparticle (rGO/Au) composite as sorbent was proposed for purification and enrichment of aflatoxin B_1 (AFB_1), aflatoxin M_1 (AFM_1), ochratoxin A (OTA), zearalenone (ZEA), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) in milk. Main parameters affecting the performance of SPE procedure were thoroughly investigated. The optimized conditions included 2% acetonitrile in water as the loading solution, 5% methanol in water as the washing solution, and methanol/acetonitrile/formic acid (50/49/1, v/v/v) as the elution solvent. Satisfactory linearity ($R^2 \geq 0.992$), high sensitivity (limit of quantification in the range of 0.02–0.18 ng mL^{-1}), adequate recovery (70.2–111.2%), and acceptable precision (RSD, 2.0–14.9%) were obtained when the optimal sample pretreatment protocol was combined with ultra-high-performance liquid chromatography-tandem mass spectrometry detection. The applicability of the validated method was further verified in real milk samples.

1. Introduction

Mycotoxins are secondary metabolites produced by various toxigenic fungi, such as *Fusarium*, *Aspergillus*, and *Penicillium* species, and they can contaminate a variety of feed products under favorable conditions (Anfossi, Giovannoli, & Baggiani, 2016; Misihairabgwi, Ezekiel, Sulyok, Shephard, & Krska, 2017). Excessive intake of mycotoxins through contaminated feeds can result in mycotoxin residues in animal products, such as meat, milk, or other animal-derived foods (Peng, Marchal, & van der Poel, 2018; Scaglioni, Becker-Algeri, Drunkler, & Badiale-Furlong, 2014). Milk is an important food that provides nutrition for the growth, development, and maintenance of human health. However, milk can also be a carrier of mycotoxins and their metabolites, causing various physiological problems in individuals (e.g.,

Abbreviations: 2D, two-dimensional; AFB_1, aflatoxin B1, AFM_1, aflatoxin M1; ELISA, enzyme-linked immune sorbent assay; ESI^-, electrospray source in negative ionization mode; ESI^+, electrospray source in positive ionization mode; FLD, fluorescence detection; GO, graphene oxide; HPLC, high-performance liquid chromatography; IAC, immunoaffinity column; LLE, liquid-liquid extraction; LOD, limit of detection; LOQ, limit of quantification; ME, matrix effect; MRM, multiple reaction monitoring; OTA, ochratoxin A; rGO, reduced graphene oxide; rGO/Au, reduced graphene oxide and gold nanoparticle; R^2, coefficients of determination; RSD, relative standard deviation; S/N, signal-to-noise; SPE, solid-phase extraction; SSE, signal suppression/enhancement; u, measurement uncertainty; U, expanded measurement uncertainty; u_c, combined measurement uncertainty; UHPLC-MS/MS, ultra-high-performance liquid chromatography–tandem mass spectrometry; UHT, ultra-high-temperature processed; ZAN, zearalanone; ZEA, zearalenone; α-ZAL, α-zearalanol; α-ZOL, α-zearalenol; β-ZAL, β-zearalanol; β-ZOL, β-zearalenol

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carcinogenic, mutagenic, teratogenic, estrogenic, neurotoxic, hepatotoxic, nephrotoxic, and cytotoxic or immunosuppression) (Alshannaq & Yu, 2017; Bennett & Klich, 2003). Additionally, mycotoxins present in milk and milk products are able to migrate into the blood and tissues of breeding mammals, and thus evoke global concern for milk safety (Degen, Partosch, Munoz, & Gundert-Remy, 2017; Giovati et al., 2015).

Various mycotoxins have been found in milk and animal products. Among those aflatoxin M_1 (AFM_1) is the most frequently occurring mycotoxin (Milicevic et al., 2017; Scaglioni et al., 2014; Zhou et al., 2018). During 2014 and 2015, 88.6% and 59.6% of ultra-high-temperature processed (UHT) milk samples collected from China were contaminated by AFM_1, respectively (Li et al., 2017). AFM_1 is thermally stable and degraded only at temperatures above 250 °C (Ellis, Smith, Simpson, & Oldham, 1991), thus if raw milk is contaminated by AFM_1, it is highly likely that the final product will also be contaminated (Sulzberger, Melnichenko, & Cardoso, 2017). Because of the adverse effects of mycotoxins in milk, a maximum permissible limit of 50 ng kg^{-1} has been set for AFM_1 in consumable milk by Commission Regulation (EC, 2007). In addition to AFM_1, aflatoxin B_1 (AFB_1) may also be detected in milk. The concept that AFB_1 is completely converted to AFM_1 has been refuted by some studies. In pasteurized milk and UHT milk samples from Brazil, AFB_1 was detected with the average contamination levels of 1.476 μg L^{-1} and 0.690 μg L^{-1}, respectively (Scaglioni et al., 2014). Other important mycotoxins, such as ochratoxin A (OTA), zearalenone (ZEA) and α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL), also have been detected in milk in low concentrations, and these compounds pose potential health risks to humans (Flores-Flores & Gonzalez-Penas, 2015; Huang et al., 2014).

The widespread occurrence of mycotoxins in milk has promoted the development of rapid, sensitive, and accurate detection methods. An enzyme-linked immune sorbent assay (ELISA) has been widely used for rapid mycotoxin detection (Bellio et al., 2016). However, ELISA methods suffer from pseudo-positive and inaccurate results, which limit their applications (Ren et al., 2007). To compensate, some confirmatory and quantification methods have been developed, such as high-performance liquid chromatography (HPLC) coupled with fluorescence detection (FLD) and mass spectrometry (MS) (Iqbal, Rabbani, Asi, & Jinap, 2014; Muscarella, Lo Magro, Palermo, & Centonze, 2007). The ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) method for multi-mycotoxin detection in milk has attracted much attention because of its ability to provide full or complementary information for the unambiguous identification of all analytes (Campone et al., 2016; Huang et al., 2014; Moreno-Gonzalez, Hamed, Garcia-Campana, & Gamiz-Gracia, 2017).

The development of efficient sample pretreatment methods that enrich target analytes, eliminate sample interferences and matrix effects is always the bottleneck for establishing an accurate and sensitive analytical method. Various methodologies based on liquid-liquid extraction (LLE) (Campone et al., 2016; Flores-Flores & Gonzalez-Penas, 2017), QuEChERS (acronym of Quick, Easy, Cheap, Effective, Rugged, and Safe) approach (Zhou et al., 2018) and immunoaffinity columns (IACs) have been developed for the pretreatment of dairy products. Among these, IACs are frequently used for the ability to selectively isolate mycotoxins from milk (Shuib, Makahleh, Salhimi, & Saad, 2017; Siddappa, Nanjegowda, & Viswanath, 2012). However, IACs are expensive, laborious and time consuming. Another promising purification and enrichment method is solid-phase extraction (SPE). It is a more convenient and cost-saving procedure with less organic solvent consumption and handling, high enrichment factor and good separation (Dong et al., 2015). Commercial cartridges such as Oasis HLB (Campone et al., 2016; Huang et al., 2014; Winkler et al., 2015), macropore-silica gel (Wang & Li, 2015) and C_{18} Sep-Pak (Aguilera-Luiz, Plaza-Bolanos, Romero-Gonzalez, Vidal, & Frenich, 2011) are most commonly used for the enrichment and purification of mycotoxins in milk. However, most of the commercial cartridges are too expensive to be used for high throughput screening of mycotoxins. Recently, graphene, a two-dimensional (2D) carbon nanomaterial discovered in 2004, has been reported to display a high adsorption capacity primarily because of its exceptional thermal and mechanical properties, good chemical stability, high surface area (calculated value of 2630 $m^2 g^{-1}$), and excellent electrical conductivity (Geim, 2009; Huang et al., 2011; Novoselov et al., 2004). However, the application of graphene in analytical chemistry are still limited because of its irreversible aggregation in solution by van der Waals and π-π stacking interactions leading to non-specificity, poor reliability and repeatability (Gu et al., 2016; Stankovich et al., 2007). To overcome this bottleneck, a second component acting as a nanospacer and conductor is necessary to be introduced to increase the graphene interlayer distance and minimize the agglomeration (Si & Samulski, 2008). With the oxidation of graphene and the addition of some metal nanoparticles, new synthesized materials have been applied successfully in the detection of metallic ions, sulfonamides, colorants, pesticides and aflatoxins (Table S1, supplementary data), proving to be novel effective adsorbents for sample preparation. Based on the above considerations, current study is performed in which reduced graphene oxide and gold nanoparticle (rGO/Au) composite was synthesized and used as SPE sorbents for simultaneous purification and enrichment of AFB_1, AFM_1, OTA, ZEA, α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL in milk, and analyzed by UHPLC-MS/MS to reveal the real contamination situations in milk collected from China.

2. Materials and methods

2.1. Reagents, chemicals and materials

All chemicals and reagents were HPLC or analytical grade. Acetone, acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Ammonium acetate, formic acid and aqueous ammonia were provided by Aladdin Co. (Shanghai, China). L-(+)-Ascorbic acid (> 99%) and sodium citrate ($Na_3C_6H_5O_7$, 99%) were purchased from Alfa Aesar (Ward Hill, MA, USA). Water used in the experiments was Milli-Q quality water (Milli-pore, Billerica, MA, USA). Graphene oxide (GO, 99%) was purchased from XF Nano Materials Tech Co. Ltd. (Nanjing, China). Gold (III) chloride trihydrate ($HAuCl_4$, ≥49.0%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The standards of AFB_1, AFM_1, OTA, ZEA, α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in acetonitrile to prepare 10 μg mL^{-1} of stock solutions. The stock solutions were stored at −20 °C in the dark. Milk samples were collected from dairy product factories in Shanghai and Jiangsu Province and stored at −20 °C in the dark.

2.2. Synthesis of rGO/Au composite

The rGO/Au composite was synthesized according to the previously published method (Wu, Lu, Fu, Wu, & Liu, 2017). First, 0.5 mL of $HAuCl_4$ (10 mg mL^{-1}) and 2.5 mL of ascorbic acid (4 mg mL^{-1}) were added into 50 mL water under stirring for 2 min. Then, 0.5 mL of sodium citrate (10 mg mL^{-1}) was poured and stirred to cease the chemical reaction. The mixture was centrifuged (13000 rpm, 5 min) for three times. The precipitate was re-dissolved in 20 mL of water (solution A, gold nanoparticle solution). Next, 25 mg of GO was dispersed in 50 mL of water, and the suspension was adjusted to pH = 10 with NaOH solution (8 mol L^{-1}). Afterwards, 29 mg of ascorbic acid was added, and the mixture was heated to 80 °C with stirring for 24 h. The suspension was centrifuged at 13000 rpm for 5 min, and the precipitate was re-dissolved in 20 mL of water (solution B, rGO dispersion). Finally, 0.5 mL of solution A and 1.5 mL of solution B were mixed and stirred for 30 min. This last step was performed for five times. The final solution was stored overnight at −20 °C and cryodesiccated. The composite was gained and stored at 4 °C for subsequent use.