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Direct monitoring of ochratoxin A in cheese with solid-phase microextraction coupled to liquid chromatography-tandem mass spectrometry

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ABSTRACT

An *in situ* application of solid-phase microextraction (SPME) as a sampling and sample preparation method coupled to HPLC-MS/MS for direct monitoring of ochratoxin A (OTA) distribution at different locations in a single cheese piece is proposed. To be suited to the acidic analyte, the extraction phase (carbon-tape SPME fiber) was acidified with aqueous solution of HCl at pH 2, instead of the traditional sample pre-treatment with acids before SPME sampling. For calibration, kinetic on-fiber-standardization was used, which allowed the use of short sampling time (20 min) and accurate quantification of the OTA in the semi-solid cheese sample. In addition, the traditional kinetic calibration that used deuterated compounds as standards was extended to use a non-deuterated analogue ochratoxin B (OTB) as the standard of the analyte OTA, which was supported by both theoretical discussion and experimental verification. Finally, the miniaturized SPME fiber was adopted so that the concentration distribution of OTA in a small-sized cheese piece could be directly probed. The detection limit of the resulting SPME method in semi-solid gel was 1.5 ng/mL and the linear range was 3.5–500 ng/mL. The SPME–LC-MS/MS method showed good precision (RSD: ~10%) and accuracy (relative recovery: 93%) in the gel model. The direct cheese analysis showed comparable accuracy and precision to the established liquid extraction. As a result, the developed *in situ* SPME–LC-MS/MS method was sensitive, simple, accurate and applicable for the analysis of complicated lipid-rich samples such as cheese.

1. Introduction

Ochratoxin A (OTA), a secondary metabolite produced by several common storage fungi (moulds) such as *Penicillium* moulds found in temperate regions and *A. ochraceus* in tropical regions, is one of the most widely occurring nephrotoxic, carcinogenic and immunosuppressive toxins and is considered to be involved in severe pathological response from humans and animals [1–3]. High toxicity of OTA presents a major health concern, so there is an increasing need for accurate monitoring of this mycotoxin in food products [4–10]. However, traditional sample preparation approaches, such as liquid extraction (LE) and solid-phase extraction (SPE), have proven to be time-consuming and labor-intensive. Antibody-based immunoaffinity chromatography (IAC) and active protein based affinity separation have also been used extensively for monitoring of OTA [3,11,12]. However, the cost and the fragility of the non-reusable column prevent it from direct application to complicated cheese sample matrix. Therefore, the need to develop fast and low-cost sample preparation approaches for OTA analysis in semi-solid food samples such as cheese cannot be overemphasized. Although moulds are intentionally introduced to some types of cheese to form special flavors, there were few observations of OTA occurrence in mould-ripened cheese [13]. But the observation of the OTA in blue cheese indicated the significance of a quality analytical method to the monitoring of OTA in the complicated cheese sample [3].

As an effective sampling and sample preparation method, solid-phase microextraction (SPME) has gained extensive application and recognition in many areas since its introduction [14–27]. One of the key reasons is its unique capability in integrating sampling, sample preparation, and sample introduction into one single step, thus greatly simplifying the total process of chemical analysis. Although SPME method coupled with LC-UV/diode array detection (DAD) was previously applied to the analysis of mycotoxins such as cyclopiazonic acid and mycophenolic acid in cheese samples with good detection limits [18,19], the SPME fiber was only used to provide additional sample clean-up after a traditional liquid extraction of cheese sample. Therefore, the resulting procedure was still tedious and time-consuming.

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To address above problems, in the current work, the SPME fiber was directly exposed in the cheese sample in order to demonstrate the effectiveness of the technique in performing *in situ* sampling and sample preparation. In this report, *in situ* sampling refers to the fact that the sampling process was performed at specific sites within a small-size cheese sample (similar to direct sampling of a reaction mixture), and is different from the widely used term "on-site sampling" or "field sampling", which implies that the sampling is performed at a place where an event of interest occurred rather than in the laboratory.

2. Experimental

2.1. Chemicals and materials

OTA, ochratoxin B (OTB) and other chemicals were of analytical grade and ordered from Sigma–Aldrich (Oakville, Canada). Stock solutions of OTA and OTB were prepared in methanol and stored at −20 °C. Carbon-tape was obtained from TAAB Laboratories Equipment (Reading, UK). The stainless-steel wires (0.01 in.) were purchased from Small Parts (Miami Lakes, FL, USA) and cut into pieces of 5-cm length.

2.2. Preparation and characterization of SPME fibers

Carbon-tape fiber was used as the extraction phase for the sampling. Except for the dimension of the fibers, the procedure for fiber preparation was exactly the same as described before [20]. The double-sided carbon-tape was cut into 1.0 mm × 1.0 mm pieces and each piece was directly immobilized onto one end of the 5 cm stainless-steel wire. The resulting carbon-tape based SPME fibers were 1.0 mm in length and 0.6 mm in thickness.

The 1% agarose gel medium was used to simulate the semi-solid cheese sample for fiber characterization. A systematic investigation was conducted to characterize the extraction behavior of the fibers including extraction time profile, desorption time profile, pH effect on the extraction efficiency, and dynamic range. All the characterization experiments were performed in gel medium to optimize the experimental conditions for cheese samples.

2.3. SPME sampling and sample preparation

All fibers were acidified with hydrochloric acid and pre-loaded with standard simultaneously by exposure into 50 mL of loading solution for 8 h. The loading solution was prepared by spiking OTB standard into 50 mL of aqueous HCl solution (0.01 M, pH 2) at 100 μg/L. The cheddar cheese was used for this project and was separated into three different sets. Set A comprised aged cheese allowed to develop mould stains while Set B had no mould stains. Set C had no mould stain, however it was less matured compared to Sets A and B. For Set A cheese with the mould stain (∼5 cm × 1 cm × 0.5 cm), two SPME fibers were introduced into two sampling sites at different distances from the mould stain. The distances between the mould stain and fiber 1 and fiber 2 were 5 and 10 mm, respectively. In the case of Set B (∼3 cm × 1.5 cm × 0.5 cm) and C (∼4 cm × 4 cm × 0.3 cm), three fibers were introduced in an equilateral triangular manner with a 10 mm distance and a fourth fiber was placed at the center of this triangle. SPME sampling was done for 20 min after which the fibers were cleaned with Kimwipes to remove any cheese residues from the surface of the coating. For desorption, 250 μL polypropylene inserts were placed into wells of a 96-well plate and filled with 150 μL of pure methanol. The fibers were placed into inserts and desorbed for 15 min with 100 rpm agitation on a mechanical orbital shaker (KV-300, Jeiotech, Seoul, South Korea). Finally, 20 μL of desorption solution was injected in LC-MS/MS system for quantification. The carryover of the fiber, which is defined as the amount of analyte that remains on the fiber after the solvent desorption, was determined by performing a second desorption in a fresh 150 μL of pure methanol for 1 h on the shaker (100 rpm).

2.4. Calibration of the SPME results

Regarding kinetic calibration, the principle and experimental procedure of the kinetic calibration were extensively described elsewhere [21–27]. Briefly, the fibers pre-loaded with OTB were introduced into the sample for extraction of OTA. The extraction of OTA from sample matrix into the fiber was calibrated by desorption of OTB into the sample matrix from the fibers based on the symmetric relation between the two processes. The calculation of the initial sample concentration, C_0, is based on the following equation [25].

$$C_0 = \frac{nq_0}{q_0 - Q} \cdot \frac{1}{k_{fs}V_f} \quad (1)$$

All the parameters including q_0, Q, and the product of K_{fs} and V_f were determined experimentally and are defined below. The average amount of standard OTB that is pre-loaded in the extraction phase, q_0, was determined by immediate desorption in methanol of 10 pre-loaded fibers (no extraction performed) followed by instrumental quantification. The amount of OTB which remained on each fiber after SPME sampling, Q, and the amount of analyte OTA extracted by the same fiber, n, were determined immediately after the sampling by performing desorption followed by LC-MS/MS analysis and calibration. The product of fiber volume, V_f, and the fiber coating/sample distribution coefficients of the analytes, K_{fs}, was obtained using equilibrium SPME and LE as discussed below. In addition, it is worth noting when calculating the OTA concentration using Eq. (1), the distribution coefficient (K_{fs}) is for the analyte OTA rather than that for the standard OTB.

The $K_{fs}{\cdot}V_f$ values of the carbon-tape fiber in semi-solid cheese was determined by the following equations [26].

$$K_{fs} = \frac{C_f}{C_s} = \frac{n_e/v_f}{C_s} \quad (2)$$

Eq. (2) can be rewritten as

$$K_{fs}V_f = \frac{n_e}{C_s} \quad (3)$$

where n_e is the amount of OTA extracted by SPME at equilibrium and C_s is the concentration of OTA in cheese samples. Herein, we used the equilibrium SPME method to obtain n_e and then detected the sample concentration with traditional organic solvent (methanol) extraction. First of all, the SPME fibers were placed into the cheese sample for 10 h to ensure the extraction reached equilibrium. The traditional liquid extraction was calibrated by standard addition method to compensate for the matrix effect. Finally, the calculated $K_{fs}{\cdot}V_f$ value ranged from 0.15 to 0.22 μL for the three different semi-solid cheese samples. The effective fiber volume, V_f, was not estimated separately because carbon-tape was a porous solid coating rather than a liquid SPME coating where V_f, can be estimated by its gross volume. In addition, the carbon proportion in the carbon-tape was not disclosed by the manufacturer.

With all the parameters ($K_{fs}{\cdot}V_f$, q_0, Q, n) determined, the sample concentration was calculated by Eq. (1).

2.5. LC-MS/MS analysis

A CTC-PAL autosampler/Shimadzu 10 AVP LC/MDS Sciex API 3000 triple quadrupole MS system was used for the analysis of OTA and OTB. The column was a Waters Symmetry Shield RP18,

50 mm × 2.1 mm, 5 μm particle size (Millford, MA, USA). Gradient elution was performed with a flow rate of 0.5 mL min^{-1} for and using mobile-phase (A) acetonitrile/water (10:90) with 0.1% acetic acid, and (B) acetonitrile and acetic acid 100:0.1. The gradient started with 10% B for the first 0.1 min, followed by a linear increase to 40% B in 6 min, and then it was ramped to 100% B in 0.1 min, held for 3 min before decreasing to 10% B in 0.01 min. This provided a total chromatographic run time of 8 min including column re-conditioning. For experiments using cheese samples, a by-pass pump from Ohaus (Florham Park, NJ, USA) with a Waters switching valve was used to direct LC effluent to waste for the first 1 min of run time, to eliminate co-extracts from entering the mass spectrometer. Electrospray ionization using TurboIonSpray source and negative ion mode was used. The MS parameters were the same as described before [20]. The following m/z transitions were analyzed: m/z 402.1 ⇒ 357.9 (OTA), m/z 402.1 ⇒ 314.0 (OTA), m/z 368.0 ⇒ 133.1 (OTB). Two m/z transitions were monitored simultaneously for OTA for the confirmation of identification in real cheese samples [28,29]. The more intense 402.1 ⇒ 357.9 transition which corresponds to loss of carbon dioxide from M–H parent ion was used for quantitation. Only one m/z transition was chosen for OTB because OTB was not endogenous in cheese samples and was only used as a calibration standard. The amount of OTA and OTB (in ng) in each SPME extract was determined using OTA and OTB calibration curves obtained by direct LC-MS/MS injection of appropriate standards dissolved in pure methanol.

2.6. Conventional cheese analysis

In order to validate proposed SPME method, traditional liquid extraction with methanol was performed using the method reported previously with minor modification [18,19]. About 0.05 g of cheese sample (n = 3) was weighed in a pre-weighed 2 mL amber vial with a PTFE sealed screw cap. Pure methanol (1 mL) was added and the resulting mixture was sonicated for 30 min, followed by a 20-min centrifugation (15 000 × g). The supernatant fluid (900 μL) was divided into 3 identical 300-μL aliquots into 3 new amber vials. In order to determine the endogenous analytes in the original cheese sample accurately and compensate for the complicated matrix effect, a standard addition calibration method was used for the conventional analysis [30]. A 15-μL volume of standard solutions (0, 100, and 200 ng/mL in pure methanol) was added into the three vials separately. Afterwards the solvent in the three samples was evaporated under nitrogen gas. The residue was reconstituted with 100 μL mobile-phase A and subjected to instrumental analysis.

3. Results and discussion

Most SPME methods developed to date were performed on liquid and homogenous samples. In order to successfully develop an *in situ* SPME method for direct extraction of an analyte such as OTA from lipid-rich, semi-solid heterogeneous matrix such as cheese, three main challenges needed to be addressed. *In situ* analysis in a small-sized cheese piece required the miniaturization of SPME fiber in order to enable probing of local concentrations in the sample. Any such reduction in fiber dimensions (and consequently fiber volume) also results in the reduction in the amount of analyte extracted by SPME. Therefore, in order to achieve adequate analytical sensitivity, it was necessary to select a fiber coating with good extraction efficiency for the analyte of interest. Secondly, pH modification of matrix is not feasible when sampling from semi-solid matrices, so an alternative approach using acidification of extraction phase prior to sampling is proposed in current work. Finally, the kinetics of mass-transfer in solid-phase is very slow, which precludes the use of equilibrium SPME for this type of application. This was addressed by the use of on-fiber-standardization approach in order to keep sampling times as short as possible and still enable accurate quantitative analysis.

3.1. SPME method development: selection of extraction phase and miniaturization of SPME fibers

Previous studies showed that the carbon-tape SPME fiber had significant affinity for OTA [20]. It exhibited high extraction efficiency compared to the commercial fibers. For *in situ* analysis in small-sized sample, the spatial resolution of the SPME fiber was improved by a reduction in the size of the fibers. Therefore, in the current study the dimensions of SPME extraction phase were reduced to 1 mm × 1 mm.

However, the reduction of the coating volume reduces the amount of analyte extracted by SPME and thus decreases the overall method sensitivity, so it was important to ensure that the amount extracted by the miniaturized fiber was still sufficient for this analysis. The results showed that the limits of detection (LOD) and quantification (LOQ) for the 1 mm fiber in gel matrix were 1.5 (S/N = 3) and 3.5 ng/mL (S/N = 10) respectively, which was sufficient for determination of OTA in real cheese samples.

3.2. SPME method development: acidification of extraction phase prior to sampling

OTA is a weak acid with the carboxylic group on the phenylalanine moiety (pK_a = 4.4) and thus shows a strong dependence of extraction yield on sample pH. All the previous SPME experiments for the analysis of OTA were conducted around pH 3 by the adjustment of matrix pH in homogenous and acidified liquid samples [18–20]. For *in situ* monitoring of OTA in semi-solid matrix such as cheese, the direct adjustment of matrix pH was not feasible, so a new strategy was proposed on the basis of the acidification of SPME extraction phase prior to sampling.

To improve the extraction performance, the fibers were acidified prior to sampling and tested in gel matrix. A pH series (pH 1, 2, 3, 4, 7) of aqueous HCl solutions were prepared and the carbon-tape fibers (n = 3) were immersed in 10 mL of each of the acidic solutions in a 40-mL vial for 8 h at room temperature. Afterwards, the fibers were used for extraction in agarose gel containing 10 ng/mL of OTA. As presented in Fig. 1, the results showed the strong pH dependence of the extracted amount in both gel matrix and in

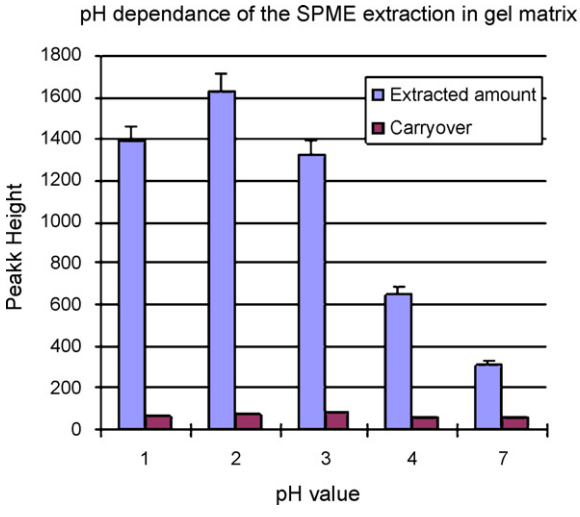

Fig. 1. The pH dependence of OTA extraction using the miniaturized carbon-tape SPME fibers in agarose gel matrix (1%) (n = 3).

the cheese. In addition, this data showed that the carryover for all extractions was similar, so the different extraction performance was due to the different affinity of the fibers to the analyte rather than the different carryover on the fibers. For subsequent experiments, aqueous solution of HCl at pH 2 was used to acidify fibers prior to sampling.

Another aspect for characterizing the acidified fiber was to study its extraction kinetics. The extraction time profile of acidified fibers in a non-acidified sample matrix (1% agarose gel) was compared with that of acidified/non-acidified fibers in acidified gel matrix (1% agarose gel, pH 3). The three profiles were consistent, though the precision for the acidified fibers in a non-acidified sample matrix (RSD ~10%, $n = 3$) was somewhat larger than sampling in the acidified gel (acidified fibers: RSD 5.6%; non-acidified fibers: RSD 6.2%; $n = 3$). The agreement of results indicated that acidifying fibers was equivalent to acidifying sample in terms of kinetic behavior (indicated by no change in time required to reach equilibrium, ~4.5 h) and thermodynamics (indicated by no change in the amount of OTA extracted at equilibrium, ~500 pg, 0.05% extracted).

Finally, the linear relationship between the sample concentrations and the instrumental response was established by performing SPME experiments in 1% agarose gel with OTA concentrations ranging from 3.5 to 500 ng/mL. The resulting calibration curve ($n = 3$ standards) showed good linearity ($y = 3162x$, $R^2 = 0.9936$), which indicated the feasibility of using the acidified fibers for quantitative analysis.

3.3. SPME calibration using non-isotopically labeled standard

For *in situ* analysis of a cheese sample with SPME, none of the three traditional calibration methods including external calibration curve, standard addition method and internal standard method, was applicable. However, the on-fiber-standardization method, or kinetic calibration, provides a solution for accurate on-site analysis since it compensates for the agitation effect and matrix effect [21,22]. As a specific case of on-site sampling, *in situ* sampling is appropriate for on-fiber-standardization.

Typical kinetic calibration is based on the use of isotopically labeled compounds as standards, which restricts the application of kinetic calibration in the cases when the isotopically labeled standards are not commercially available or unaffordable for routine use. Recently, a dominant desorption kinetic calibration method was developed, which uses the analyte itself to calibrate the analyte extraction [31]. In this approach, several fibers must be deployed simultaneously to obtain the desorption curve, which makes the method suitable for homogeneous samples where analyte is uniformly distributed in the sample matrix. The concentration of OTA in a small-sized heterogeneous cheese piece is not uniform, thus making dominant desorption method unsuitable for this application.

In the current work, we choose OTB as the standard for OTA. A proof of principle experiment was conducted in gel matrix to further test the validity of using OTB to calibrate OTA. The calculated relative recoveries were around 93% (5–500 ng/mL), which demonstrated the accuracy of the method. Generally, 93% recovery using the non-isotopically labeled standard for kinetic calibration was comparable to the results using deuterated standards [21–25].

This work revealed that the analogue (OTB) could serve as the desorption standard for the extraction of the analyte (OTA) as long as they had very similar mass-transfer kinetics during the sorption and desorption. Therefore, the main purpose for addition of OTB was to calibrate the extraction of OTA. However, the presence of OTB during all sample preparation steps also simultaneously corrected for any errors in sample volume, adsorptive losses, evaporative losses of solvent and/or differences in LC-MS injection volume, thus also partially serving as an internal standard. However, because the retention time of OTB was different from OTA, OTB did not correct for any ionization suppression effects.

Experimentally, to streamline SPME procedure the acidification of extraction phase and standard loading were combined into one single step which improved the time-effectiveness. The experimental results showed that there was no significant difference between performing the two steps separately and simultaneously. Therefore the carbon-tape fibers were loaded from acidic OTB aqueous solution (pH 2) in all subsequent experiments.

Table 1
Summary of *in situ* SPME results for OTA occurrence in three cheese samples ($n = 3$). Set A: the aged Cheddar cheese with a mould stain. Set B: the aged Cheddar cheese without mould stains. Set C: Immature cheese.

Cheese type	Sampling site	[a]S-conc. (ng/mL)	SD	[b]L-conc. (ng/mL)	SD
A	[c]1 (close)	42	5.2	39	3.7
	[d]2 (far)	20	3.5	18	2.1
B	1	11	1.4	13	1.3
	2	12	1.6	12	1.5
	3	11	0.8	10	1.1
	4	12	1.5	11	1.2
C	1	[e]nd		nd	
	2	nd		nd	
	3	nd		nd	
	4	nd		nd	

[a] Sample concentration obtained by SPME technique.
[b] Sample concentration obtained by liquid extraction technique.
[c] The #1 sampling site that is close (5 mm) to the mould stain in the cheese.
[d] The #2 sampling site that is further than #1 site (10 mm) to the mould stain in the cheese.
[e] Non-detectable.

3.4. In situ cheese analysis

The application of the proposed *in situ* SPME approach to real sample analysis was demonstrated by analyzing three different semi-solid cheese samples as described in Section 2, in which both the spatial concentration distribution and the concentration change over time were studied. The results are presented in Table 1. It was found that for Set A with a mould stain, the OTA concentration had an inverse relation with distance between the sampling site and mould stain. However, the amount of OTA detected was directly proportional to storage time for mouldy cheese (Set A), as shown in Fig. 2. This data indicated presence of live fungi in the cheese, which was consistent with previous investigation [3]. If the fungi were not intentionally inoculated into the cheese during manufacturing, it could be safely speculated that the cheese was contaminated

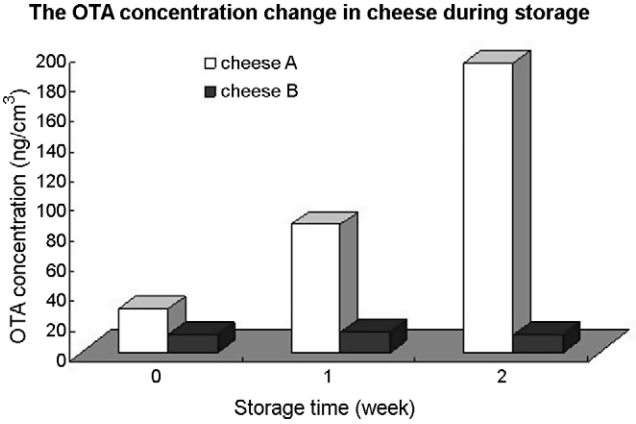

Fig. 2. The OTA concentration change in cheddar cheese during two-week storage refrigerated at 4 °C. Cheese 1: the aged cheddar cheese with a mould stain. Cheese 2: the aged cheddar cheese without mould stains.

during transportation, storage or manufacturing, for example, from packaging flaws. For the Set B without mould stains, the uniform distribution of the OTA concentration was observed; moreover, the OTA concentration did not change during two weeks of study. This result indicated that there were no active OTA-producing fungi in the cheese, which implies that the raw materials were the likely origin of contamination. In the case of Set C cheese sample, there was no detectable OTA. Based on these results, it can be concluded that the *in situ* SPME is a simple and fast approach to monitor OTA concentration changes over time and help determine if OTA-producing moulds are present or absent in a given cheese sample.

The SPME results obtained using kinetic calibration were consistent with those obtained by traditional liquid extraction that was calibrated by standard addition, as shown in Table 1, thus confirming the validity of the *in situ* SPME analysis. The accuracy of the LE was verified by the linearity of the signals versus the extracts from the three sample fractions with different amount of OTA standard ($R^2 = 0.994$). Generally, the sampling process of SPME method was simpler and faster than using LE. It must be admitted that the latter is necessary to obtain K_{fs} value for SPME analysis, but much less analyses were needed since the K_{fs} value should be the same for the whole sample; on the contrary, the traditional LE needs to be performed for every sampling site in each sample. In addition, SPME showed acceptable precision in cheese analysis (7–17% RSD, $n = 3$), which was comparable to LE (7–13% RSD, $n = 3$). In comparison to the previous studies [18,19], this work provides a simpler but more effective means to conduct direct analysis of ionizable analytes in semi-solid food matrix.

4. Conclusions

The performance and reliability of *in situ* miniaturized SPME with enhanced spatial resolution for the analysis of OTA in cheese was demonstrated. The *in situ* sampling was achieved by the acidification of the extraction phase to improve the extraction efficiency for a weakly acidic analyte and decrease sample preparation time. On-fiber kinetic calibration was developed by using OTB as a standard for OTA, which opened the possibility of using non-deuterated compound for accurate calibration. The main limitations of this calibration approach are the need to verify that proposed non-deuterated compound is not endogenously present in any of the samples under study and the need to establish that mass-transfer kinetics of the proposed standard are similar to that of the analyte. All these aspects are not only novel from theoretical perspective but also have broad implications such as the quality control in food industry or *in vivo* sampling for biomedical studies.

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