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Supramolecular solvent-based microextraction of ochratoxin A in raw wheat prior to liquid chromatography-fluorescence determination

Sergio García-Fonseca, Ana Ballesteros-Gómez, Soledad Rubio*, Dolores Pérez-Bendito

Department of Analytical Chemistry, Facultad de Ciencias, Edificio Anexo Marie Curie, Campus de Rabanales, 14071-Córdoba, Spain

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ABSTRACT

A supramolecular solvent made up of reverse micelles of decanoic acid, dispersed in a continuous phase of THF:water, was proposed for the simple, fast and efficient microextraction of OTA in wheat prior to liquid chromatography-fluorescence determination. The method involved the stirring of 300 mg-wheat subsamples (particle size 50 μm) and 350 μL of supramolecular solvent for 15 min, subsequent centrifugation for 15 min and the direct quantitation of OTA in the extract, previous 5.7-fold dilution with ethanol/water/acetic acid (49.5/49.5/1), against solvent-based calibration curves. No clean-up of the extracts or solvent evaporation was needed. Interactions between the supramolecular solvent and major matrix components in the wheat (i.e. carbohydrates, lipids and proteins) were investigated. The reverse micelles in the extractant induced gluten flocculation but only in the coacervation region of lower analytical interest (i.e. at percentages of THF above 11%). The quantitation of OTA was interference-free. Representativity of the 300 mg-wheat subsamples was proved by analysing a reference material. OTA recoveries in wheat ranged between 84% and 95% and the precision of the method, expressed as relative standard deviation, was 2%. The quantitation limit of the method was 1.5 $\mu g\,kg^{-1}$ and was below the threshold limit established for OTA in raw cereals by EU directives (5.0 $\mu g\,kg^{-1}$). The method developed was validated by using a certified reference material and it was successfully applied to the determination of OTA in different wheat varieties from crops harvested in the South of Spain. OTA was not detected in any of the analysed samples. This method allows quick and simple microextraction of OTA with minimal solvent consumption, while delivering accurate and precise data.

1. Introduction

Ochratoxin A (OTA) is one of the most widespread and hazardous mycotoxins contaminating foodstuffs [1]. It is produced by several fungi (*Aspergillus* and *Penicillium* species) in a variety of plant products, such as cereals, pulses, coffee, wine, grape juice, dried fruits and spices [2]. OTA is considered a potent nephrotoxic and genotoxic agent [3] and has been classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer [4]. Monitoring OTA in cereals has become an important issue worldwide due to both the impact on human health and the high economic losses associated to crop production. In fact, among the most frequently contaminated food commodities, cereals are the main European dietary source of OTA (44%), increasing this value in the case of Spain (60%) [5]. The European Union has established maximum residue levels (MRLs) of OTA in raw cereals and derived products of 5 and 3 $\mu g\,kg^{-1}$, respectively [6].

Several official methods are available for surveillance of OTA residues in cereals and cereal products (e.g. barley [7–10], wheat flour [11], barley, wheat bran and corn [12] and wheat, rye and corn [9]). Liquid chromatography with fluorescence detection (LC-FL), which provides quantitation limits between 0.1 and 10 $\mu g\,kg^{-1}$, is by far the most used technique for OTA determination. Recoveries and relative standard deviations for these methods range in the intervals 65–100% and 10–20%, respectively. On the other hand, several non-official LC-FL methods, affording recoveries above 75%, have been reported [13–15]; many of them constituting a good alternative to official methods in terms of sensitivity, reproducibility and/or simplicity. Immunochemical methods, highlighting immunosensors based on enzyme linked immunosorbent assay (ELISA) [16], have been proposed as simple on-site screening methods (detection limits in the $\mu g\,kg^{-1}$ range).

Due to the complexity of food matrices, sample preparation (extraction, concentration and clean-up) is essential in OTA analysis. Solvent extraction is by far the most used strategy for isolation of OTA from cereals and derived products. A variety of solvents and solvent mixtures, including acidified chloroform [9,10,12,17], methanol–aqueous bicarbonate [15], methanol–phosphate buffer [18,19], acidified toluene–magnesium chloride

* Corresponding author. Tel.: +34 957 218644; fax: +34 957 218644.
E-mail address: qa1rubrs@uco.es (S. Rubio).

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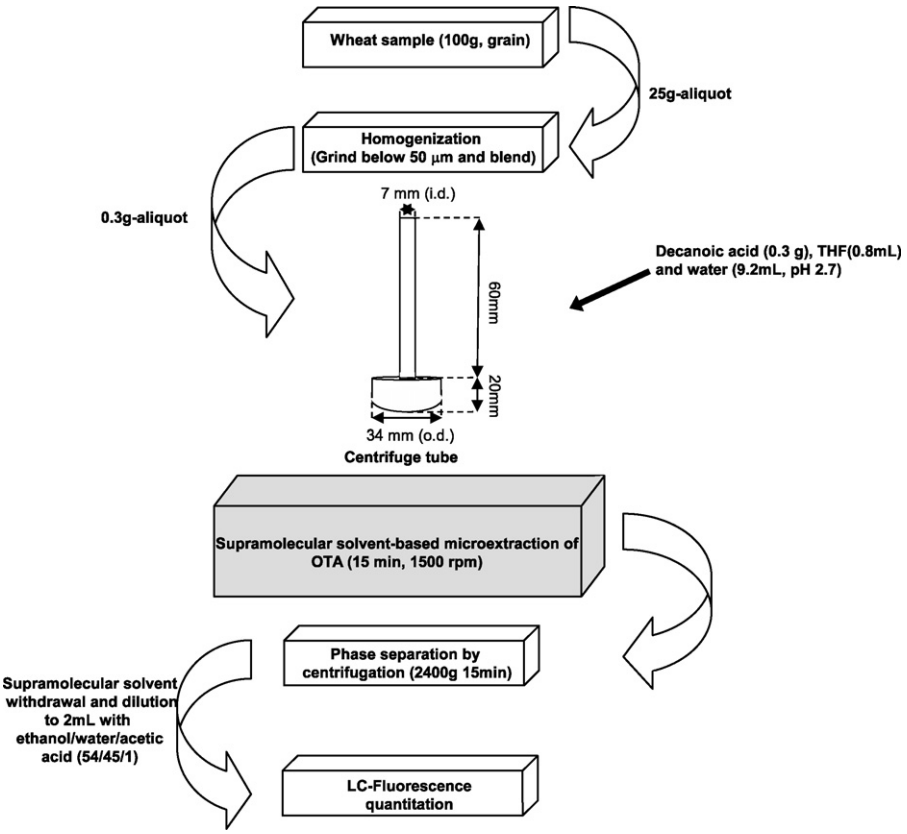

Fig. 1. Scheme of the different steps followed for the analysis of OTA in cereals.

[11] and acetonitrile–water [7,8,20], have been proposed. The volume of organic solvent consumed per sample is relatively high (50–250 mL) and after extraction, further clean-up is usually needed. Immunoaffinity chromatography (IAC) is widely used for sample clean-up [8,15,17–21], although other approaches, including purification through a silica gel column [11], defatting with n-hexane [17] or filtration through diatomaceous earth followed by back-extraction with aqueous bicarbonate and purification through C_{18} cartridges [9,10,12], are also being used. Recently, new strategies including matrix solid phase dispersion (MSDP), which combines extraction and clean-up in one step and considerably reduces solvent consumption [22], and molecularly imprinted polymers (MIP), which provide selectivity comparable to IAC without the cost of producing antibodies [23], have been reported. So far, IAC surpasses other reported clean-up methods in versatility (e.g. it is applicable to a wider range of matrices), selectivity (e.g. it provides cleaner extracts and consequently lower quantification limits) and reproducibility (e.g. usually, relative standard deviations are below 5%) [15,17]. However, IAC presents important disadvantages for routine analysis; immunoaffinity columns are expensive, not recyclable, have a limited storage time and, in some cases, show cross-reactivity with ochratoxin C [24].

According to the state of the art in this field, new methods intended to improve OTA determination in cereals should focus on simplifying sample preparation making it faster, cheaper and environmentally friendly (i.e. by reducing considerably solvent consumption) while keeping method sensitivity below the legislative limits and enough selectivity. In this context, this paper explores the suitability of supramolecular solvents to simplify sample treatment in the determination of OTA in wheat. Supramolecular solvents are water-immiscible liquids made up of supramolecular assemblies dispersed in a continuous phase. They are produced from amphiphile solutions by a sequential self assembly process occurring on two scales, molecular and nano, and constitute a valuable strategy to replace organic solvents in analytical extraction processes. First, amphiphilic molecules spontaneously form three-dimensional aggregates above a critical aggregation concentration, mainly aqueous (size 3–6 nm) or reversed (size 4–8 nm) micelles and vesicles (size 30–500 nm), depending on the structure of amphiphiles and solvent properties. Then, the generated nanostructures self-assemble in larger aggregates, with a wide size distribution in the nano and micro scale regimes, by the action of an external stimulus (e.g. temperature, electrolyte, pH, solvent) and separate from the bulk solution as an immiscible liquid by a phenomenon named coacervation. To date, supramolecular solvents from a variety of surfactant aggregates, i.e. non-ionic [25], zwitterionic [26], cationic [27] and anionic [28] aqueous micelles, reversed micelles [29] and vesicles [30] have been successfully used for the extraction of pollutants from the environment [31–34] and, more recently, from foodstuffs [35].

In this paper, a supramolecular made up of decanoic acid reverse micelles, previously described by our research group [29] and applied to the extraction of OTA in liquid matrices such as wine [36], was selected for the microextraction of OTA in raw wheat prior to LC/FL determination. Selection was based on the different types of interactions (i.e. hydrogen bonding and dispersion forces) it provides for OTA solubilisation and the high amphiphile concentration in the solvent (around 0.75 mg μL^{-1}), all of which should give high extraction efficiencies. The aim was to develop a simple, low-cost and rapid method for the routine control of this contaminant in wheat matrices. Parameters affecting sample representativity, extraction efficiency and detection and quantification limits were optimised and the method was successfully applied to the determination of OTA in different varieties of wheat including bread and durum wheat and two synthetic wheat hybrids.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent-grade and were used as supplied. Tetrahydrofuran (THF), HPLC-grade acetonitrile, methanol, ethanol and glacial acetic acid were supplied by Panreac (Sevilla, Spain). Ultra-high-quality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain). Decanoic acid was purchased from Fluka (Madrid, Spain), while starch, gluten from wheat and ochratoxin A (OTA) were obtained from Sigma (St. Louis, MO, USA). The certified reference material BCR471 (an OTA free wheat) was obtained from Sigma (St. Louis, MO, USA) and the reference material OW815 batch (OTA content in wheat: 4.9 ± 1.0 ppb) was supplied by R-Biopharm (Glasgow, Scotland). A stock standard solution of 10 mg L^{-1} of OTA was prepared in methanol and stored under dark conditions at −20 °C. Working solutions were prepared by dilution of the stock solution with methanol and calibration solutions were made with a mixture of water/ethanol/acetic acid (49.5/49.5/1).

2.2. Apparatus

The liquid chromatographic system used (Spectra System SCM1000, ThermoQuest, San Jose, CA, USA) consisted of a P2000 binary pump and a FL3000 fluorescence detector. In all experiments a PEEK Rheodyne 7125NS injection valve with a 100 μL sample loop was used (ThermoQuest, San Jose, CA, USA). The stationary-phase column was a Kromasil C_8 (25 cm × 4.6 mm i.d., 5 μm) from Análisis Vínicos (Tomelloso, Spain). A Retsch MM301 mixer mill and a Mixtasel Selecta centrifuge were employed for sample preparation. Centrifuge tubes with narrow necks (Fig. 1) were designed by authors in order to make easier the measurement and collection of the supramolecular solvent after microextraction. Pobel S.A. (Madrid, Spain, web page: www.pobel.com) constructed them from commercial heavy-duty glass cylindrical centrifuge tubes with round-bottom (ref. 159050) by keeping their basic structure at the bottom (34 mm of outside diameter) but reducing the diameter from a specified height, which depended on the required tube capacity.

2.3. Determination of OTA in wheat

2.3.1. Sample preparation and preservation

The cereal samples analysed ($n = 6$) were kindly supplied by the Department of Agronomy and Genetic Improvement of the Spanish National Research Council (SNRC) of Córdoba (Spain) and consisted of two durum wheat (*Triticum durum* Desf), two bread wheat (*Triticum aestivum* L.) and two wheat hybrids, namely Triticale (*Triticosecale* spp., which is an hybrid from bread wheat and rye) and Tritordeum (*Hordeum–Triticum*, which is an hybrid from a South American wild barley and durum wheat [37]). Samples were supplied as 100 g-single bags. Aliquots of 25 g were ground and blended in a Retsch MM301 mixer mill, in two steps (2 min each), at a vibrational frequency of 28 s^{-1}. This procedure gave particle sizes below 50 μm. Aliquots of 300 mg were subsequently subjected to microextraction with the supramolecular solvent. Otherwise, the samples were vacuum-packaged in foil-laminate pouches, heat-sealed and stored at −4 °C until their analysis. Fig. 1 outlines the sequential steps followed for the determination of OTA in wheat including subsampling, microextraction and quantitation.

2.3.2. Supramolecular solvent-based microextraction

A wheat subsample (300 mg) was introduced in a specially designed 10 mL-glass centrifuge tube (Fig. 1) and then, decanoic acid (300 mg) dissolved in THF (0.8 mL) and 9.2 mL of water (pH 2.7 adjusted with hydrochloric acid) were added in sequence. The supramolecular solvent, made up of reverse micelles of decanoic acid dispersed in THF:water spontaneously formed and separated from the THF:water solution as an immiscible liquid. The mixture was stirred at 1500 rpm for 15 min to favour analyte partition and then centrifuged at 2400 × *g* for 15 min to accelerate the complete separation of the two immiscible liquids. The supramolecular solvent, which was standing at the top of the solution into the narrow neck of the tube, was withdrawn with a 100 μL-microsyringe and transferred to a 2-mL calibrated flask. Then, it was diluted to the mark with a mixture of ethanol/water/glacial acetic acid (54/45/1), filtered (0.45 μm filters) and 100 μL-aliquots were injected in the LC/FL system.

2.3.3. Liquid chromatography-fluorescence detection

Quantification of OTA was carried out by LC-FL. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both containing 1% acetic acid. The elution program was: isocratic conditions (55% of A and 45% of B) for 5 min, linear gradient from 55% to 50% in A for 10 min, then linear gradient from 50% to 0% in A for 5 min and, finally, 5 min more from 0% to 55% in A to recover initial conditions. The flow-rate was 1 mL min^{-1}. OTA was monitored at λ_{ex} 334 nm and λ_{em} 460 nm. Quantification was performed by measuring peak areas. Calibration curves for OTA in ethanol/water/glacial acetic acid (54/45/1) were constructed in the range of 0.2–10 μg L^{-1}.

3. Results and discussion

3.1. Supramolecular solvent–wheat interactions: analytical consequences

A good knowledge of the interactions between extractant and matrix is essential to prevent interferences and set up efficient extraction schemes, so before optimisation of the extraction procedure we investigated the major interactions occurring between the supramolecular solvent and wheat. Considering that most of raw and processed foods contain similar constituents (e.g. carbohydrates, lipids, proteins, etc.), the conclusions derived of this study can be probably extended to other applications involving supramolecular solvent microextractions of contaminants in food.

The supramolecular solvent selected for OTA microextraction consists of decanoic acid reverse micelles dispersed in a THF:water continuous phase [29]. It spontaneously forms in a ternary system made up of decanoic acid, THF and water, at the proportions shown in Fig. 2A, through a phenomenon named coacervation [38]. Major components of the supramolecular solvent are decanoic acid and THF; water is a minor constituent.

The volume of solvent produced depends on the amount of decanoic acid ($y = a + bx$) and the percentage of THF ($y = b_0 e^{b_1 z}$) [34]. In these equations, y is given in μL, x is the amount of decanoic acid in mg, and z the THF percentage (v/v).

Concerning wheat, carbohydrates (mainly as starch), proteins (mainly as gluten), lipids and water are the main constituents of this cereal, the relative percentages depending on wheat variety. These percentages range between around 71–75%, 10–14%, 2–2.5% and 10.5–11%, respectively, for the wheat varieties analysed [39–41].

The influence of wheat matrix components on supramolecular solvent formation and volume was investigated. Fig. 2B shows the phase diagram obtained in the presence of 300 mg of wheat, independently of the variety tested. By comparison with Fig. 2A, it is clearly observed that the upper boundary moved toward lower THF percentages (around 11%), owing to the apparition of a dense whitish precipitate that was standing at the interface between the supramolecular solvent and the bulk solution, as a very thin layer,

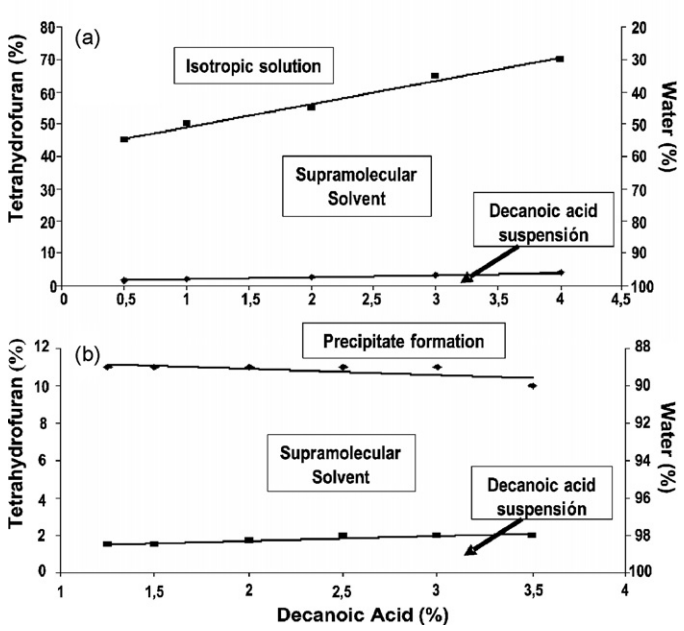

Fig. 2. Phase diagram of ternary mixtures made up of tetrahydrofuran, decanoic acid and water in the (A) absence and (B) presence of wheat (300 mg).

after centrifugation. This layer became wider as the percentage of THF in the bulk solution increased, until the entire clear upper liquid phase corresponding to the supramolecular solvent was completely adsorbed by the precipitate at around 25% THF. This behaviour was pH-dependent; precipitation being favoured at the lowest pHs (e.g. the upper boundary in the phase diagram moved around 8% of THF at pH 2).

In order to explain the observed phenomenon, phase diagrams were constructed in the presence of starch and gluten, as representative of carbohydrates and proteins, respectively; at the maximal concentrations these constituents are usually found in wheat (i.e. 225 mg of starch and 42 mg of gluten per 300 mg of sample). The pH in these experiments ranged between 1.5 and 4. Starch did not influence phase diagrams but gluten flocculated in the presence of decanoic acid reverse micelles and phase diagrams were identical to those obtained in the presence of wheat (Fig. 2B). Flocculation of proteins by reverse micelles has been previously reported in the literature [42–44]. From an analytical point of view, it is worth noting that the effect of wheat proteins on the supramolecular solvent phase diagram is not significant for extraction purposes, since analytical applications are usually carried out near the lower phase boundary in order to use the minimal amount of THF for solvent production [29,34]. With the aim of avoiding gluten flocculation, and having into account that the supramolecular solvent forms at pH below 4, extraction of OTA in wheat should be carried out at pHs between about 2.5 and 4.

To study the influence of wheat constituents on the volume of solvent produced, a set of solutions containing 300 mg of bread wheat, amounts of decanoic acid between 125 and 350 mg, THF percentages between 4% and 11% and water (96–89%) were prepared. The volumes of supramolecular solvent obtained were measured with a digital calliper and the relationships between these volumes and the amount of decanoic acid and THF were investigated. Tables 1 and 2 show the results obtained.

As expected, the volume of supramolecular solvent produced was linearly dependent on the amount of surfactant used (Table 1), but the slopes of these linear relationships were around 10% below those obtained in the absence of wheat [34], probably due to the adsorption of some decanoic acid on matrix components.

Table 1
Figures of merits of the linear relationship ($y = a + bx$) between the coacervate volume (y, μL) and the amount of decanoic acid (x, mg) at different THF percentages.

THF (%)	$b \pm S^a$ (μL mg^{-1})	$a \pm S^a$ (μL)	$R^{2\,b}$
4	1.15 ± 0.03	−61 ± 6	0.998
6	1.30 ± 0.02	−66 ± 4	0.9992
8	1.39 ± 0.02	−65 ± 5	0.9990
9	1.42 ± 0.03	−46 ± 8	0.998
11	1.55 ± 0.06	−44 ± 13	0.995

[a] Standard deviation.
[b] Correlation coefficient; $n = 6$.

Accordingly, the volume of solvent produced decreased in around 20–40 μL in the interval of decanoic acid tested. The y-intercepts were negative and different from zero, thus indicating that more initial amount of surfactant was needed in bulk solutions containing wheat to start producing the supramolecular solvent.

The relationship between the volume of supramolecular solvent and the THF percentage was exponential (Table 2). Similar to water, the parameter b_0 was linearly related to the amount of decanoic acid. However, the parameter b_1, which describes how rapidly the volume of coacervate increases as the THF (%) does, did not keep constant (mean value for b_1 in water 0.046 ± 0.001 [34]) and it was exponentially related to the decanoic acid amount, thus corroborating the interaction of the surfactant with matrix components.

Because of supramolecular solvent volumes in the presence of wheat were reproducible and related to decanoic acid and THF through clear relationships, the reduction in around 10% compared to the volumes obtained in the absence of wheat is irrelevant for analytical extraction processes. So, although the reverse micelles in the supramolecular solvent are expected to flocculate proteins from food, the effects produced (i.e. reduction of the proportions THF:water at which the supramolecular solvent forms and decrease in solvent volume) should have no effect in extraction processes.

3.2. Sample representativity study

OTA is distributed irregularly in raw cereals due to the random nature of fungal contamination [2] and this makes sampling and subsampling a major issue. Because of subsamples of at least 25 g are recommended for analysis of cereals, reliable microextraction methods, based on the analysis of minute amounts of sample, require a thorough sample homogenization prior to subsampling in order to achieve representativity.

With the aim of obtaining homogeneous 25 g-samples and thus representative 300 mg-subsamples, the influence of reducing the particle size on the accuracy of OTA microextraction was investigated. For this purpose, the reference material CRM OW 815 (OTA concentration: 4.9 ± 1.0 μg kg^{-1}, particle size < 1 mm) was used as raw material. Experiments were carried out by extracting 300 mg-subsamples ($n = 11$) at three ranges of particle size (x), namely:

Table 2
Figures of merits of the exponential relationships ($y = b_0 e^{b_1 z}$) between the coacervate volume (y, μL) and the concentration of THF (z, %) at different amounts of decanoic acid.

Decanoic acid (mg)	$b_0 \pm S^a$ (μL)	$b_1 \pm S^a$	$R^{2\,b}$
125	55 ± 4	0.092 ± 0.009	0.97
150	86 ± 5	0.069 ± 0.007	0.97
200	127 ± 5	0.062 ± 0.004	0.990
250	179 ± 6	0.059 ± 0.004	0.990
300	224 ± 9	0.057 ± 0.005	0.98
350	277 ± 6	0.056 ± 0.003	0.995

[a] Standard deviation.
[b] Correlation coefficient; $n = 5$.

(a) $x < 1$ mm, as supplied by the distributor; (b) $50 < x < 250$ μm, obtained after grounding 25 of the reference material with a ball crusher (Retsch MM301 mixer mill; vibrational frequency 21.6 s^{-1} for 1 min, twice); and (c) $x < 50$ μm, obtained by increasing the vibrational frequency to 28 s^{-1} for 2 min, twice. Relative standard deviations (RSD) were calculated as a measurement of homogeneity.

The results obtained for the analysis of <1 mm-material (RSD = 96%) stood out the irregular distribution of the mycotoxin, most of the amount of OTA determined being concentrated in just 2 of the 11 aliquots analysed. The aspect of this material was heterogeneous in colour and texture and included clearly visible dark particles corresponding to the wheat bran. The homogeneity moderately increased for particle sizes in the interval $50 < x < 250$ μm although the presence of outliers was still observed (RSD = 34%). A sharp decrease of the RSD value (3%) was found for the material with particle size <50 μm, which also became uniform in colour. So, the later conditions were selected for OTA microextraction with the supramolecular solvent.

3.3. Optimisation of the supramolecular solvent-based microextraction of OTA

OTA is a hydrophobic compound (its octanol–water partition coefficient, $\log K_{ow}$ is 4.74) and, because of its pK_a values (4.4 for the acid group and 7.1 for the alcohol one), it is neutral at pH values below 4, at which the solvent is produced. The sum of hydrogen donors and acceptors in OTA is 10. So, the expected driving forces for OTA microextraction in the supramolecular solvent are Van der Waals interactions between the hydrocarbon chains of the decanoic acid and the OTA aromatic framework, and hydrogen bonds between the acceptor and donor groups of the analyte and the polar head-groups of the surfactant.

Optimisation was carried out by extracting 300 mg of a blank certified reference material (BCR471 wheat) fortified with 10 μg kg^{-1} of OTA under a variety of experimental conditions (125–350 mg decanoic acid; 4–11% THF; stirring time 0–60 min; pH between 2 and 4). Experiments were made in triplicate. Selection of the optimal conditions was based on recoveries (R) in order to obtain the lower quantification limits for the method, since concentration factors were maintained constant by diluting the supramolecular solvent to 2 mL with a solvent mixture composition similar to that of the mobile phase; ethanol/water/acetic acid (54/45/1). Spiking of samples was made by adding 150 μL of OTA (20 μg L^{-1}) in methanol and left to stand for 1.5 h to allow solvent evaporation at room temperature and favour analyte–matrix interactions. Equilibration between OTA and wheat matrices for a longer period of time (i.e. overnight) did not change the extraction efficiency.

Table 3 shows the recoveries obtained along with their respective standard deviations as a function of the main constituents of the supramolecular solvent (i.e. decanoic acid and THF). Maximal recoveries were always obtained for THF percentages around 8%. The amount of OTA extracted increased as the decanoic acid did and was maximal for 300 mg (i.e. at sample/decanoic acid ratios of 1). Further studies were carried out with the aim of determining the interval of sample/decanoic acid ratios at which recoveries were above 90%. For this purpose, amounts between 100 and 2000 mg of BCR471 wheat and between 150 and 300 mg of decanoic acid were combined to give wheat/decanoic acid ratios from 0.33 to 13. Fig. 3 shows the results obtained. Recoveries around 94% were obtained for wheat/decanoic acid ratios below 1.2. From this value, recoveries progressively decreased until they kept constant at around 25% for ratios above 2.5. Gluten precipitated from ratios around 13. According to these results, the procedure can be resized at will provided that the amount of decanoic acid is similar to that of wheat, although the representativity of wheat aliquots lower than 300 mg should be thoroughly investigated before their use for OTA determination. We selected 300 mg of both sample and decanoic for further studies.

Table 3
Mean percent recoveries ($n = 3$) and standard deviations obtained for ochratoxin A in wheat (300 mg) as a function of the amount of decanoic acid and percentage of THF used for supramolecular solvent production.

Decanoic acid (mg)	Recoveries ± standard deviations (%)				
	THF (%)				
	4%	6%	8%	9%	11%
125	49 ± 2	61 ± 1	63 ± 3	57 ± 4	57 ± 3
150	59 ± 2	67 ± 2	70 ± 4	60 ± 2	63 ± 2
200	66 ± 4	72 ± 2	75 ± 6	69 ± 5	70 ± 2
250	73 ± 3	77 ± 6	80 ± 2	74 ± 5	75 ± 4
300	77 ± 6	86 ± 3	94 ± 2	87 ± 2	79 ± 6
350	79 ± 1	80 ± 2	81 ± 2	76 ± 6	Precipitate

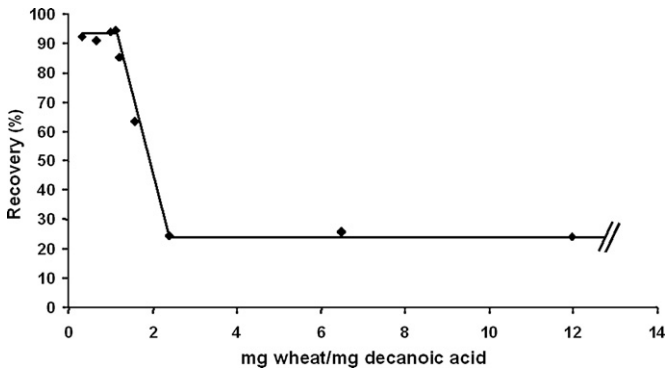

Fig. 3. Recoveries for OTA at different wheat (mg)/decanoic acid (mg) ratios.

The pH did not influence recoveries in the range 2.5–4, which is logical considering the neutral character of OTA in this interval and the type of interactions governing its solubilisation in the extractant. The extraction time (stirring rate 1500 rpm) to reach equilibrium conditions was around 15 min and this time was selected as optimal.

The high capability of the supramolecular solvent for the microextraction of OTA in wheat was consequence of both the

Table 4
Mean recoveries and standard deviations obtained for OTA in the analysis of wheat samples using the proposed method.

Concentration (μg kg^{-1})	Recovery ± standard deviation (%)[a]					
	Samples					
	M1	M2	M3	M4	M5	M6
3	90 ± 4	95 ± 3	86 ± 4	89 ± 2	87 ± 5	87.7 ± 0.3
8	88 ± 3	90 ± 1	92 ± 2	92 ± 2	84 ± 3	95 ± 3
20	91 ± 2	90 ± 1	89.1 ± 0.3	94 ± 4	90 ± 1	90.1 ± 0.1

Wheat varieties: M1 and M2, *Triticum durum*; M3, *Triticosecale*; M4 and M6, *Triticum aestivum*; M5, *Hordeum–Triticum*. Sample amount = 0.3 g.
[a] $n = 3$.

types of analyte–extractant interactions established and the special structure of the aggregates making it up. Thus, recoveries for the extraction of OTA (10 μg kg^{-1}) from wheat (BCR471 blank sample) with reverse micelles of decanoic acid in THF, at a decanoic acid concentration similar to that found in the supramolecular solvent (e.g. 1400 mM), was only around 40%. THF and water were also bad extractants for OTA with recoveries around 26% and 50%, respectively.

3.4. Analytical performance

Calibration curves for OTA were run using standard solutions prepared in ethanol/water/acetic acid (49.5/49.5/1). Because of the similar polarity of the injection solvent and the mobile phase, injection of 100 μL of sample extract did not cause broadening of the chromatographic peak for OTA. No differences in peak areas or retention times were observed for the analytes injected in the standard solutions and the supramolecular solvent (~350 μL) diluted to 2 mL with a mixture of ethanol/water/acetic acid (54/45/1). Correlation between peak areas and OTA concentration (0.2–10 μg L^{-1}) was determined by linear regression and was 0.9991, indicating a good fit. The slope of the calibration curve was 500 ± 14 L μg^{-1} (n = 8). The instrumental quantitation (LOQ) and detection (LOD) limits were calculated from blank determinations by using a signal-to-noise ratio of 10 and 3, respectively, and were 20 pg and 6.5 pg.

The method LOD and LOQ were estimated from the respective instrumental LOD and LOQ and considering the amount of wheat (300 mg), the recoveries under optimum conditions (~94%) and the final extract volume (2 mL), were 0.5 and 1.5 μg kg^{-1}, respectively. Since detection and quantitation limits calculated in this way are often optimistic, the practical LOD and LOQ were calculated from six independent complete determinations of blank wheat samples fortified with OTA in the range of 0.5–3 μg kg^{-1} [45]. The practical LOQ and LOD were equal to those previously estimated, thus indicating the low matrix influence in the method proposed.

The possible interference of matrix components that could elute with OTA was assessed by the comparison of the slopes of the calibration curves (n = 8) obtained from standards in distilled water with those obtained from wheat (BCR471) fortified with known amounts of OTA (1.5–40 μg kg^{-1}) and run using the whole procedure. The difference between both slopes (519 ± 12 L μg^{-1} for water and 491 ± 12 L μg^{-1} for wheat) was found to be not statistically significant by applying an appropriate Student's t-test [45]. The calculated t-value (1.74) was below the critical t-value (2.21), being significance established at 0.05 levels. Therefore, matrix components were not expected to interfere in the determination of the target compounds.

The precision of the method was evaluated by extracting seven independent samples (BCR471) fortified with 8.0 μg kg^{-1} of OTA. Fortification was carried out as it has been described for optimisation studies (Section 3.3). The value, expressed as relative standard deviation (RSD), was about 2%.

3.5. Method validation

The method proposed was validated by analysing 300 mg-aliquots (n = 11) of a reference material (RM OW 815, R-Biopharm) consisting in a wheat sample with an OTA content of 4.9 ± 1.0 μg kg^{-1}. The reference material was previously grounded to give particles sizes below 50 μm, as specified in Section 2.3.1. The average concentration found (4.1 ± 0.2 μg kg^{-1}) was included in the interval of concentration certified by the manufacturer, indicating the accuracy of this method for the determination of OTA in wheat.

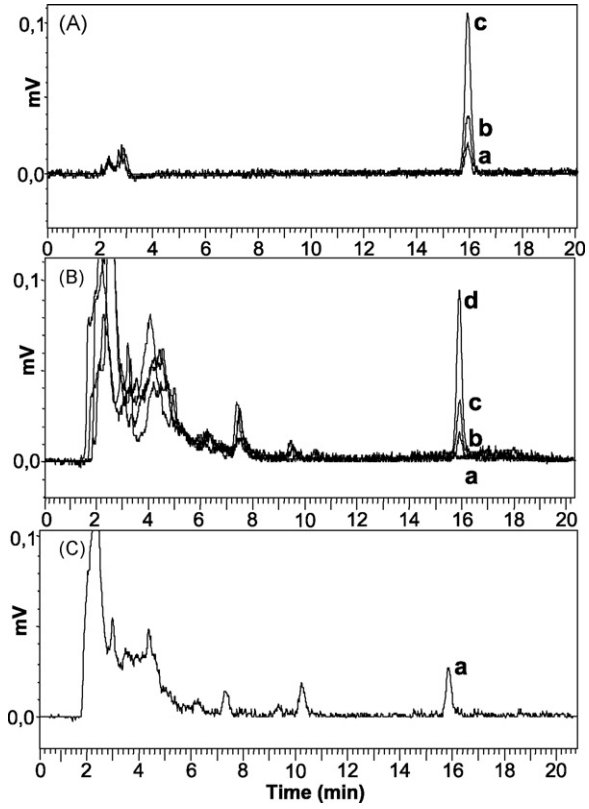

Fig. 4. LC/fluorescence chromatograms obtained from: (A) standard solutions of OTA in water/ethanol/acetic acid (49.5/49.5/1); (a) 0.5 μg L^{-1}, (b) 1 μg L^{-1} and (c) 3 μg L^{-1}; (B) a 300 mg-aliquot of *Triticum aestivum* spiked with OTA; (a) blank sample, (b) 3 μg kg^{-1}, (c) 8 μg kg^{-1} and (d) 20 μg kg^{-1}; and (C) a 300 mg-aliquot of *Triticum durum* spiked with (a) 8 μg kg^{-1} of OTA.

3.6. Analysis of wheat samples

Six wheat samples, including four varieties, were analysed in order to prove the suitability of the proposed method for the routine control of OTA. None of them contained OTA at detectable levels. Table 4 shows the recoveries obtained after spiking the samples at three levels of concentration (3, 8 and 20 μg kg^{-1}). Fortification was carried out by following the same procedure than that specified in Section 3.3. Recoveries were expressed as the mean value of three independent determinations along with their corresponding standard deviations. Recoveries were between 84% and 95% with relative standard deviations ranging from 0.1% to 5%.

The chromatograms obtained from three different concentrations of OTA in water/ethanol/acetic acid (49.5/49.5/1), a sample of *T. aestivum*, fortified at three levels of concentration (3, 8 and 20 μg kg^{-1}), and a sample of *T. durum*, fortified with 8 μg kg^{-1} of OTA are shown in Fig. 4A, B and C, respectively. No interference from matrix components was detected for any of the samples analysed.

4. Conclusions

Supramolecular solvents consist of amphiphilic nanostructures that provide multiple binding sites and regions of different polarity. These outstanding properties make them suitable to extract a variety of analytes with high efficiency and render them ideal for microextractions. In this research, supramolecular solvents made up of reverse micelles of decanoic acid in THF are proposed as valuable tools for the microextraction of OTA from wheat, offering a simple, inexpensive and rapid alternative to conventional sample preparation methods, which combine high solvent consumption with the need of clean-up and solvent evaporation steps.

The extraction procedure takes about 30 min; several samples can be simultaneously extracted; requires a low sample amount (0.3 g wheat), which is made representative of the bulk by reducing the particle size to <50 μm, and a low supramolecular solvent volume (350 μL); conventional equipment in labs is used for extraction and features low cost. The method developed can be used for the routine control of OTA in raw wheat below the tolerance level permitted by the European Directives (5 $\mu g\,kg^{-1}$) [6]. Because most of raw and processed foods contain similar constituents than wheat (e.g. carbohydrates, lipids, proteins, etc.), the microextraction procedure here proposed could be widely applicable to food residues with ability to establish hydrogen bonding and dispersion interactions with the supramolecular solvent.

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