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Vesicular aggregate-based solventless microextraction of Ochratoxin A in dried vine fruits prior to liquid chromatography and fluorescence detection

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ABSTRACT

A solventless microextraction was proposed for the development of a simple, fast, low-cost and environmental friendly sample treatment for the determination of Ochratoxin A (OTA) in dried vine fruits. The objective was to offer an alternative to conventional sample treatments, which invariably involve extractions with large solvent volumes followed by clean-up with expensive, not recyclable and limited storage stability immunoaffinity sorbents. The method involved the stirring of 300 mg of dried vine fruit subsamples with 400 μL of a supramolecular solvent (SUPRAS) made up of decanoico acid/tetrabutylammonium decanoate vesicles. Then, the sample was centrifuged for 15 min and OTA was quantified in the extract by liquid chromatography/fluorescence detection against solvent-based calibration curves. Neither dilution nor further clean-up steps of extracts were needed. Quantitation of OTA was interference-free and recoveries ranged between 95% and 101%. The precision of the method, expressed as relative standard deviation (RSD), was about 3%. The limit of quantification (5.3 $\mu g\,kg^{-1}$) was below the threshold limit established for OTA in dried vine fruits by EU directives (10 $\mu g\,kg^{-1}$). Representativity of subsamples was proven. The method was successfully applied to the analysis of several dried vine fruits (sultanas and muscatels) purchased in local supermarkets in Córdoba (South of Spain). OTA was not detected in any of the analyzed samples. This solventless sample treatment allows quick and simple microextraction of OTA, while delivering accurate and precise data, and extends the range of eco-friendly methods in labs.

1. Introduction

Regulatory agencies and quality control laboratories are continuously demanding faster, simpler and cheaper methods for the analysis of trace contaminants in food. Development of more general and valuable sample preparation procedures that meet the demanding regulatory limits established, minimize the number of steps required and decrease organic solvent consumption continues as the strongest priority in food chemical analysis [1]. Some strategies intended to reduce solvent consumption (e.g. solid-phase extraction [2], miniaturization [3], accelerated solvent extraction [4] and supercritical fluids extraction [5]) play currently an important role in sample handling for the analytical control of food in labs.

Supramolecular solvents (SUPRASs) have a great potential to develop organic solvent free, single-step sample treatments in food analysis. They are water-immiscible liquids made up of supramolecular assemblies of amphiphiles dispersed in a continuous phase. Aggregation occurs through a sequential self assembly process. First, amphiphilic molecules spontaneously form three-dimensional aggregates (aqueous or reverse micelles or vesicles) above a critical aggregation concentration. Then, the generated nanostructures self-assemble in larger aggregates 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.

Two properties of SUPRASs render them ideal in analytical extractions. First, the aggregates making up SUPRASs have regions of different polarity that provide a variety of interactions for analytes. The type of interaction may be tuned varying the hydrophobic or the polar group of the amphiphile and in theory one may design the most appropriate SUPRAS for a specific application because amphiphiles are ubiquitous in nature and synthetic chemistry. Second, the concentration of amphiphiles in the solvent is very high (typically 0.1–1 $mg\,\mu L^{-1}$) which is an ideal platform for amplification of solute binding. Additional properties of SUPRASs include the use of self-assembly based synthetic procedures that are within everyone's reach and non-volatility and non-flammability which permit the implementation of safer processes. To date, SUPRASs from a variety of surfactant aggregates, i.e. non-ionic [6], zwitterionic [7], cationic [8] and anionic [9] aqueous micelles, reversed micelles [10] and vesicles [11] have been successfully used for

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the extraction of pollutants in the environment [12–19] and, more recently, in foodstuffs [20–23].

This paper evaluates the capability of a supramolecular solvent made up of decanoic acid/tetrabutylammonium decanoate for the eco-friendly single-step extraction/clean-up of Ochratoxin A (OTA) in dried vine fruits. OTA is a widespread contaminant in both raw and processed food commodities [24] that exerts nephrotoxic, immunosuppressive and neurotoxic effects [25]. The International Agency for Research on Cancer (IARC) has considered it as a possible carcinogen to humans (Group 2B) [26]. Its toxicity along with its worldwide occurrence [27] has fostered the development of international regulations to set maximum levels for OTA in a variety of commodities [28–31].

Dried vine fruits are progressively becoming great demand products in the health food market [32]. Environmental conditions both during post-harvest storage and the whole grape drying process cause the growth of *Aspergillus carbonarius*, the main responsible for OTA production in grapes and dried vine fruits [24]. The contribution of these products to the total human dietary intake of OTA has been reported in different surveys [27,33–42]. Some maximum levels found for OTA were 35 $\mu g\,kg^{-1}$ in Swedish raisins [37], 26 and 54 $\mu g\,kg^{-1}$ in Canadian [35] and Turkish [42] sultanas, respectively and 54 $\mu g\,kg^{-1}$ in English currants [34]. The European Union has established a maximum residue level (MRLs) for OTA in dried vine fruits of 10 $\mu g\,kg^{-1}$ [28].

Few methods have been reported for the quantitation of OTA in dried vine fruits so far and, although most of them are straightforward and provide reliable results, unresolved issues remain in both sampling and sample treatment. Sampling concerns mainly derive from the random nature of fungal contamination and thus the uneven distribution of OTA in raw and processed dried fruits. Statistically based sampling plans for mycotoxins are available [43]. Samples from the bulk lot needs to be minced and slurried and then mixed for a reasonable period to obtain homogeneity prior to sub-sampling for analysis [44]. Sampling of retail products is less problematic because representative data are best obtained through analysis of large numbers of varied samples. Slurries are usually made with water [34–36] or aqueous bicarbonate [37,38].

Solvent extraction with methanol is by far the most used strategy for isolation of OTA from dried vine fruits. In order to improve recoveries samples need to be acid- [34] or alkali-extracted [37], with the latter giving better recoveries. The volume of organic solvent consumed per sample is relatively high (50–100 mL). Conventional solid-phase extraction with C_{18} was used in the past for sample clean-up however, over the past ten years, most laboratories have moved towards using antibody-based affinity column (IAC) clean-up since it provides sample extracts generally free of interferences [34–40]. A major problem of the use of IACs in routine analysis is their cost, which demands for cheaper alternatives.

Liquid chromatography coupled to 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 [34–42]. LC with mass detection (MS) provides unequivocal identification of OTA [45,46], however owing to its potential, this technique is more appropriate for multitoxin analysis. Fourier infrared spectroscopy attenuated total reflection has been also proposed for the detection of OTA in dried vine fruits, however, matrix matched calibration was required for quantitation [47].

In this paper, the supramolecular solvent-based extraction/cleanup is combined with LC-fluorescence for the determination of OTA in dried vine fruits with the aim of simplifying sample preparation while keeping method sensitivity below the legislative limits and enough selectivity. The SUPRASs made up of decanoic acid/tetrabutylammonium decanoate vesicles was selected on the basis of the different types of interactions it provides for OTA solubilization (i.e., hydrogen bonding, ionic, π–cation and dispersion forces) and the high amphiphile concentration in the solvent (around 0.96 mg μL^{-1}), all of which should give high extraction efficiencies. Parameters affecting sample representativity, extraction efficiency and detection and quantification limits were optimized and the method was successfully applied to the determination of OTA in different varieties of dried vine fruits.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent-grade and were used as supplied. Decanoic acid and tetabutylammonium hydroxide (Bu_4NOH) were obtained from Sigma-Aldrich (Barcelona, Spain), HPLC-grade acetonitrile, methanol and acetic acid glacial were supplied by Panreac (Sevilla, Spain). Ultra-high-quality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain). Ochratoxin A was purchased from Sigma (St. Louis, MO, USA). 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.

2.2. Apparatus

The liquid chromatographic system used consisted of a ThermoQuest spectra system (San Jose, CA, USA) furnished with a SCM 1000 vacuum membrane degasser, a P2000 binary pump, an AS3000 autosampler and a FL3000 fluorescence detector. In all experiments a PEEK Rheodyne 7125NS injection valve with a 20 μL sample loop was used (ThermoQuest, San Jose, CA, USA). The analytical column was a Hypersil ODS C_8 (5 μm 150 mm × 4.6 mm) from Analysis Vínicos (Tomelloso, Spain). A Robot300 food chopper from Taurus (Berlin, Germany), a homogenizer-disperser Ultra-Turrax T25 Basic from Ika (Werke, Germany) a vortex-shaker REAX Top equipped with an attachment (ref. 549-01000-00) for 10 microtubes from Heidolph (Schwabach, Germany) and a high speed brushless centrifuge MPW-350R equipped with an angle rotor 36 × 2.2/1.5 mL (ref. 11462) from MPW Med-Instruments (Warschaw, Poland) were used for sample preparation. A digitally regulated centrifuge Mixtasel equipped with an angle rotor 4 × 100 mL (ref. 7001326) from JP-Selecta (Abrera, Spain) was used for supramolecular solvent production.

2.3. Supramolecular solvent production

The following procedure was routinely followed for the production of the supramolecular solvent (Fig. A.1). In a 50 mL-glass centrifuge tube were placed in sequence, distilled water (42 mL), decanoic acid (2.63 g) and Bu_4NOH (5 mL of 40%, w/v). The mixture was centrifuged at 3500 rpm (1860×*g*) for 8 min to speed solvent separation up, which is less dense than water. Next, it was withdrawn using a 10 mL-syringe, transferred to a hermetically close storage glass vial and stored at 4 °C. Under these conditions, the solvent produced was stable for at least one month. The volume of solvent obtained can be adjusted at will by choosing an appropriate, constant decanoic acid/Bu_4NOH/water proportion.

2.4. Determination of OTA in dried vine fruits

2.4.1. Sample preparation

Five dried vine fruit varieties (sultana, white sultana, Málaga muscatel, muscatel and Chile muscatel) were purchased in supermarkets in Córdoba (South of Spain) and were stored at 4 °C until analysis. Sultana and Málaga muscatel were produced in

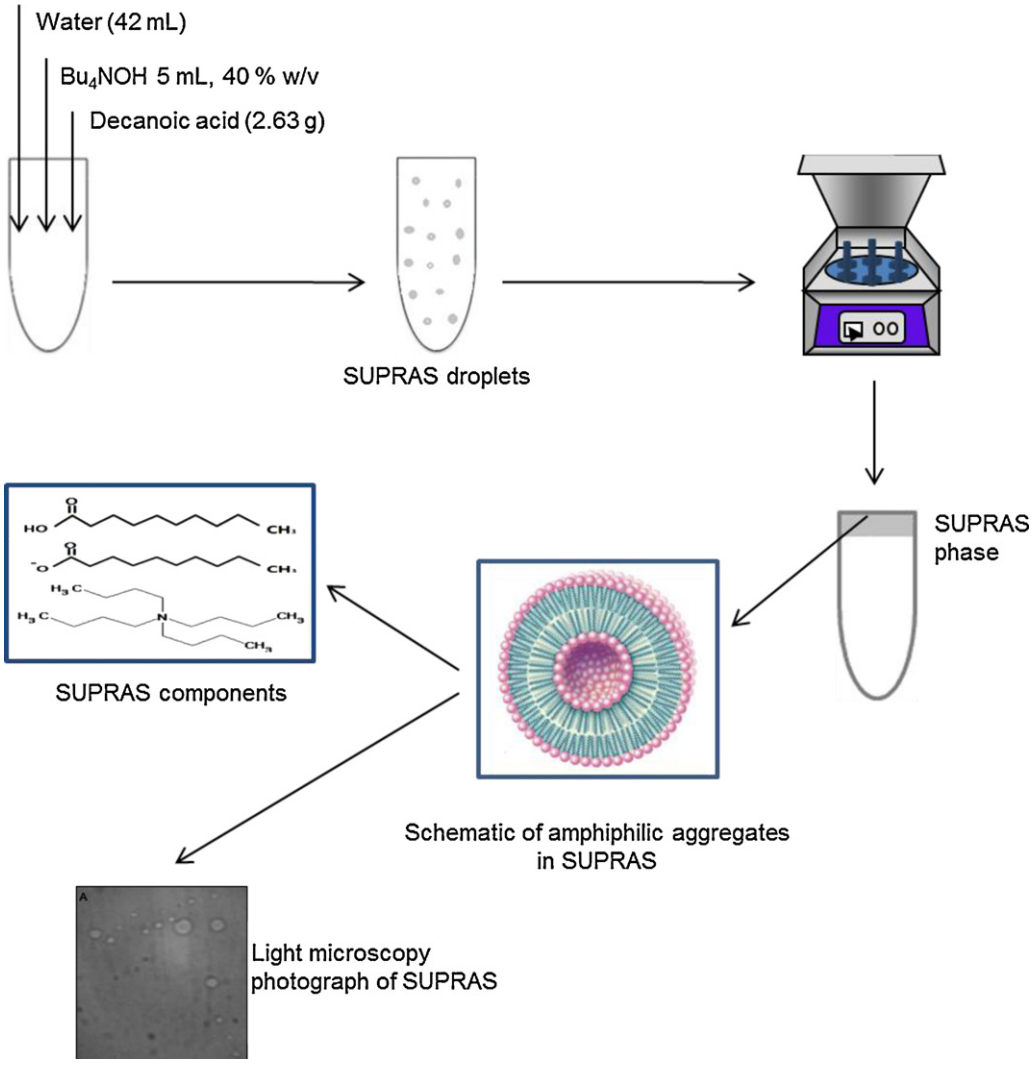

Fig. A.1. Schematic of supramolecular solvent production and composition.

the South of Spain, while the origin of muscatel, Chile muscatel and white sultana was South America. The whole fruit content in consumer size-packages was used for sample treatment (typically 250 g). Samples were subsequently chopped, mixed with aqueous hydrochloric acid (pH 4) at a water (mL):sample (g) ratio of 4:5, and homogenized with a high-speed ultraturrax for 5 min. Then, portions of 300 mg were taken for analysis and recovery experiments, which were performed in triplicate. Spiking of samples was made by adding the corresponding volume of the working standard solution (100 $\mu g\,L^{-1}$ of OTA) to the 300 mg-subsamples to give a final concentration of 10 or 20 $\mu g\,kg^{-1}$ and they were allowed to stand at room temperature for 90 min before analysis. OTA was stable in the samples during this period of time.

2.4.2. Supramolecular solvent-based microextraction

In a 2 mL-microtube Safe-Lock from Eppendorf Ibérica (Madrid, Spain) were placed 300 mg-subsample and 500 μL of the vesicular supramolecular solvent. Four little glass balls (3 mm diameter) were introduced in the microtube to favor sample dispersion during extraction, which was made by sample vortex-shaken at 2500 rpm for 10 min. Then, the mixture, thermostated at 20 °C, was centrifuged at 15,000 rpm for 15 min to separate the solvent from the solid residue. SUPRAS aliquots were taken using a microsyringe, microfiltered through 0.45 μm nylon filters (Análisis Vínicos S.L. Tomelloso, Spain) to remove possible suspended solids and injected into the liquid chromatographic system. A schematic diagram of this procedure is illustrated in Fig. A.2.

2.4.3. Liquid chromatography/fluorescence detection

OTA was separated from matrix components by liquid chromatography using isocratic elution. The mobile phase consisted of water/acetonitrile (45:55), both containing 1% glacial acetic acid, and was pumped at a constant flow rate of 1 mL min^{-1}. The volume of injection of samples and standards was 20 μL. OTA was monitored at λ_{ex} 334 nm and λ_{em} 460 nm. Under these conditions the analyte was eluted at approximately 10 min. Calibration curves were run from standards dissolved in methanol. No differences were detected in peak areas or retention times for the analyte injected in the SUPRASs or organic solvent. Quantitation was performed by measuring peak areas. Calibration curves for OTA were constructed in the range 2.5–30 $\mu g\,L^{-1}$. The precision of the method was evaluated by extracting eleven independent samples of sultana ($n = 5$) and muscatel ($n = 6$), fortified with 10 $\mu g\,kg^{-1}$ of OTA. The decision limit (CCα, the limit at and above which it can conclude with an error probability of α that a sample is not-compliant) was established by analyzing 20 blank sultana samples fortified with OTA at the permitted limit (i.e. 10 $\mu g\,kg^{-1}$), and it was calculated from the concentration at the permitted limit plus 1.64 times the standard deviation of the blank samples measurements ($\alpha = 5\%$).

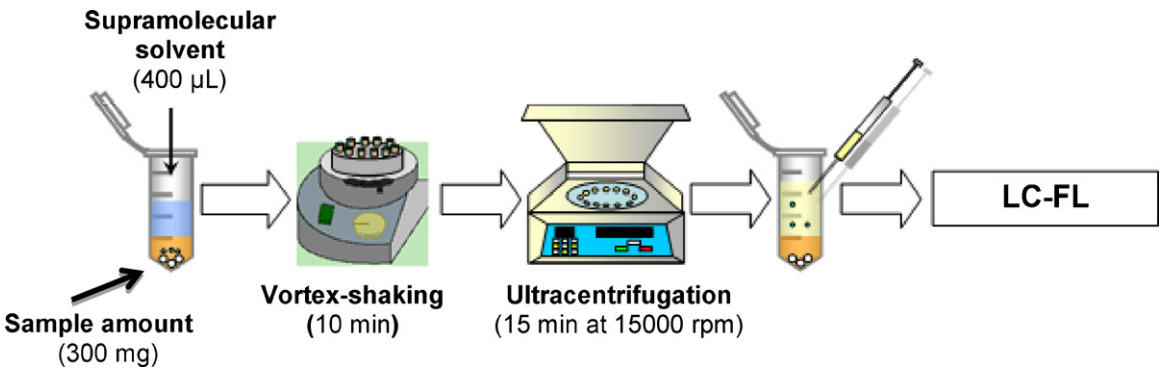

Fig. A.2. Schematic of the method proposed for OTA determination.

3. Results and discussion

3.1. Sample homogenization

The great difficulty in preparing suitably homogeneous analytical samples of dried vine fruits has been identified by different researchers [34,35]. Inhomogeneity primarily arises from the random nature of fungal contamination and is of particular importance in compliance testing where the analysis of a sample may result in the acceptance or refusal of a large, valuable lot of dried fruits. Analytical control of fruit contamination in consumer-size packages is easier because their whole content is usually taken for analysis.

Because of microextraction methods rely on the analysis of minute amounts of subsamples (e.g. 300 mg are here proposed instead of the 10–50 g-subsamples usually analyzed) thorough sample homogenization studies were carried out to determine if these subsamples were representative of the whole sample. To our knowledge, no certified reference materials for OTA in dried vine fruits are commercially available.

Formation of proper slurries was found essential for sample homogenization. Slurries were obtained by mixing the minced sample (250 g) with 0.01 M aqueous hydrochloric acid (at solution (mL):sample (g) ratios from 1:5 to 5:5) and OTA (at the level of 10 or 20 $\mu g\,kg^{-1}$) using a high-speed ultraturrax. Subsamples (300 mg) were analyzed in triplicate and recoveries, along with their corresponding standard deviations, found.

Insufficient homogenization was obtained at solution:sample ratios below 4:5 as inferred from both sample appearance (it was heterogeneous in texture and included visible particles of different color) and standard deviations values. Although mean values for recoveries kept practically constant in the range evaluated, the corresponding standard deviations clearly increased as the volume of solution decreased. Thus, they were 14, 10 and 7% for solution:sample ratios of 1:5, 2:5 and 3:5, respectively. As previously found by other authors [34,35], mixing four parts of aqueous solution with five parts of dried fruit was enough to reach subsampling representativity (standard deviations between 0.7 and 3%). The ultraturrax allowed a thorough blending of the aqueous solution and dried vine fruits and the slurry was homogenous enough for taking representative 300 mg subsamples.

3.2. Supramolecular solvent-based microextraction of OTA

3.2.1. Solvent description

The supramolecular solvent used for OTA microextraction consisted of unilamellar vesicles of decanoic acid/tetrabutylammonium decanoate dispersed in a continuous phase (Fig. A.1) [11]. This water immiscible liquid was less dense than water and its viscosity was 97.5 mPa s at 25 °C.

The solvent was produced through a sequential self-assembly process involving two steps. Firstly, decanoic acid and decanoate formed vesicles in an aqueous solution. The amount and stability of these aggregates were maximal at decanoic acid/decanoate molar ratios of ca. 1 since, in addition to hydrophobic forces, hydrogen bonds between the polar groups of carboxylic and carboxylate molecules were the major force driving their self-assembly. Secondly, aggregate growth was promoted by reduction of ionic head group repulsion with the counterion tetrabutylammonium. The aggregates thus produced separated from the bulk solution through coacervation.

From a practical point of view, the sequential self-assembly process was simplified by the addition of tetrabutylammonium hydroxide (Bu_4NOH) to aqueous decanoic acid suspensions, at a Bu_4NOH/decanoic acid molar ratio of 0.5. In this way, tetrabutylammonium decanoate and decanoic acid, at a molar ratio of ca. 1 were produced, and the vesicular coacervate formed instantaneously without the need for sonication. Since the amount of vesicles, and accordingly of supramolecular solvent, is maximal in the pH range 7 ± 1, where 7 is the (pK_a) apparent of decanoic acid molecules as inserted in the vesicle, extractions should be carried out at neutral or slightly acid or basic pH values.

The volume of SUPRAS produced linearly depended on the amount of decanoic acid initially present in the bulk solution, thus indicating that its composition kept constant. The value of the slope of this linear relationship gave the microliters of solvent obtained per mg of surfactant ($1\ mg\,\mu L^{-1}$).

3.2.2. Optimization

Optimization was carried out by extracting 300 mg of sultana blank samples fortified with 20 $\mu g\,kg^{-1}$ of OTA. Experiments were made in triplicate. Selection of the optimal conditions was based on the recoveries (R) and the method quantitation limits (MQLs) obtained. MQLs were calculated from the instrumental quantitation limits, the volume of SUPRASs used for extraction, the recoveries obtained and the sample weight used for analysis. The variables investigated were: volume of extractant (200–700 µL), pH for the sample slurry (1.5–5.5), time required to reach equilibrium conditions (1–60 min) and time of centrifugation necessary to obtain free-particle extracts (1–30 min). After sample centrifugation, three phases were always observed; namely, the insoluble sample matrix components at the bottom; an intermediate aqueous solution arising from the slurry and probably containing very polar matrix components, and the SUPRASs extract containing OTA and other solutes from the sample.

The pH of the aqueous solution used for the formation of the sample slurry influenced both OTA recoveries and the precision of the results, as shown in Table B.1. Values of pH below 3.5 affected SUPRAS stability (the apparent pK_a for decanoic acid in the vesicular structure is around 7) provoking some destruction of the vesicular

Table B.1
Mean percent recoveries and standard deviations obtained for OTA microextraction as a function of the pH of the aqueous solution used for slurry formation.

pH	Recovery ± SD[a] (%)
2.5	76 ± 10
3	86 ± 16
3.5	87.8 ± 0.4
4	90 ± 3
4.5	91 ± 4
5.7	66.5 ± 0.7

Fortified with OTA at a concentration of 20 μg kg^{-1}.
[a] Standard deviation; n = 3; liquid:solid sample proportion: 4:5.

Table B.2
Mean percent recoveries, along with their respective standard deviations, and quantitation limits obtained for OTA as a function of the volume of SUPRAS used for microextraction.

Supramolecular solvent (μL)	Recovery ± SD[a] (%)	Quantitation limits (μg kg^{-1})
200	72 ± 4	3.7
300	88 ± 3	4.5
400	100 ± 1	5.3
500	103.5 ± 0.7	6.4
600	105 ± 3	7.5
700	104.5 ± 0.7	8.8

[a] Standard deviation, n = 3; fortified with OTA at a concentration of 20 μg kg^{-1}.

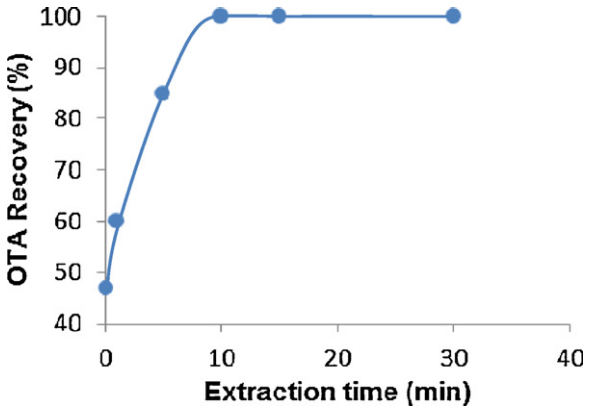

Fig. A.3. Dependence of OTA recovery as a function of the extraction time.

structures and consequently some solubilization of the solvent into the aqueous phase. This resulted in lower recoveries and irreproducible results (see Table B.1). Maximal recoveries were obtained at pHs around 4, which were selected as optimal. This result indicated that the neutral form of OTA was preferentially extracted (pK_a for the OTA acid group = 4.4). Major interactions expected to be the driving forces for extraction were: (1) hydrophobic interactions between the hydrocarbon chain of decanoic acid and the aromatic rings of OTA (the octanol–water partition coefficient, log K_{ow}, for OTA is 4.74), (2) hydrogen bonds between the carboxylate/carboxylic acid groups of vesicles and the hydrogen bond donors and acceptors of the mycotoxin, and (3) π–cation interactions between the aromatic rings of OTA and the quaternary ammonium group of Bu_4N^+.

Table B.2 shows the recoveries obtained, along with their respective standard deviations, as a function of the volume of SUPRASs used for microextraction. Quantitative recoveries were obtained from SUPRAS volume (μL)/sample amount (mg) ratios above 1.3. So, a volume of 400 μL was selected as optimal because it provided quantitative recoveries and limits of quantitation below the threshold limit established for OTA by EU directives (10 μg kg^{-1}).

The time used for extraction of samples (vibration motion = 1500 rpm) influenced the recoveries obtained for OTA (Fig. A.3). Equilibrium conditions were reached after 10 min of vortex shaking-assisted extraction and this time was selected as optimal. The minimal centrifugation time required to achieve an effective separation of the supramolecular extract from sample particles was 15 min.

3.3. Analytical performance

Calibration curves were run using standard solutions in methanol. No differences in peak areas or retention times were observed for the analyte injected in the SUPRAS or methanol. Correlation between peak areas and OTA concentration (2.5–30 μg L^{-1}) was determined by linear regression and was 0.991, indicating a good fit. The slope of the calibration curve was 77 ± 4 absorbance units L μg^{-1} (n = 6). 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 2.2 and 0.7 μg L^{-1}. The method LOD and LOQ were estimated from the respective instrumental LOD and LOQ taking into account the actual amount of sample analyzed (166 mg), the recoveries obtained (~100%) and the volume SUPRAS used for extraction (400 μL). The value of LOD was 1.7 and LOQ 5.3 μg kg^{-1}. The precision of the method, expressed as relative standard deviation (RSD), was about 3%. The decision limit (CCα) obtained for OTA was 10.25 μg kg^{-1} which proves the suitability of the proposed method for establishing compliance with the legislation for OTA in dried vine fruits [48].

Possible interferences from matrix components that could co-elute with OTA were assessed by comparison of the slopes of the calibration curves (n = 7) obtained from standard solutions with those obtained from dried vine fruits fortified with known amounts of OTA (1–12 ng to 300 mg subsamples) and run using the whole procedure. The difference between both slopes (77 ± 4 for standard solutions and 71 ± 4 for spiked samples) was found to be not statistically significant by applying an appropriate Student's t-test [49]. The calculated t-value (1.21) was below the critical t-value (2.20), being significance established at the 0.05 level. Therefore matrix components were not expected to interfere in the determination of OTA.

3.4. Analysis of dried vine fruits

Five types of dried vine fruits were analyzed 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 B.3 shows the recoveries obtained after spiking the samples at two levels of concentration (10 and 20 μg kg^{-1}). Recoveries were expressed

Table B.3
Mean percent recoveries and standard deviations obtained in the determination of OTA in different varieties of fortified dried vine fruits.

Ochratoxin A (μg kg^{-1})	Recovery ± SD[a] (%)				
	Sultana	White sultana	Muscatel	Chile muscatel	Málaga muscatel
10	98.7 ± 0.6	101 ± 2	100 ± 4	98 ± 1	95 ± 3
20	100 ± 1	98 ± 1	99 ± 2	97 ± 2	97 ± 2

[a] Standard deviation; n = 3.

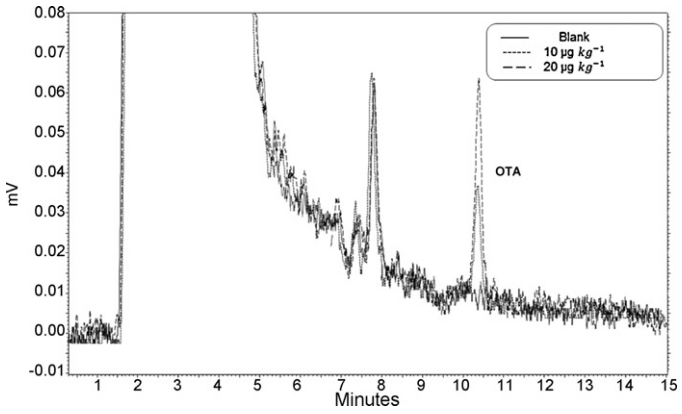

Fig. A.4. Chromatograms obtained from a muscatel blank sample and a muscatel sample fortified at 10 and 20 μg kg^{-1}.

as the mean value of three independent determinations along with their corresponding standard deviations. Their values were between 95 and 101% with standard deviations ranging from 0.6 to 4%. No interference from matrix components was detected for any of the samples analyzed. Fig. A.4 shows the chromatograms obtained for a blank and two-level fortification muscatel samples.

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, a supramolecular solvent made up of decanoic acid/tetrabutylammonium decanoate vesicles is proposed as a valuable tool for the microextraction of OTA from dried vine fruits. The sample treatment proposed offers a simple, inexpensive and rapid alternative to conventional sample preparation methods, which combine high solvent consumption with the need for immunoaffinity column-based clean-up. Valuable assets of the proposed sample treatment are: it takes about 30 min and several samples can be simultaneously treated; it requires a low sample amount (0.3 g fruit), which is made representative of the bulk by strong homogenization, and a low eco-friendly SUPRAS volume (400 μL); it features low cost and conventional equipment in labs is used. The method can be used for the routine control of OTA in dried vine fruits below the tolerance level permitted by the European Directives.

Acknowledgments

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