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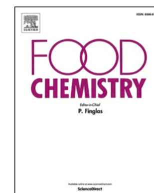

Extracting mycotoxins from edible vegetable oils by using green, ecofriendly deep eutectic solvents

Fernando Pradanas-González, Rubén Aragoneses-Cazorla, Miguel Ángel Merino-Sierra, Elena Andrade-Bartolomé, Fernando Navarro-Villoslada *, Elena Benito-Peña *, María Cruz Moreno-Bondi [1],*

Departamento de Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, E-28040 Madrid, Spain

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ABSTRACT

In this work, we developed an environmentally friendly liquid–liquid microextraction method using a natural deep eutectic solvent in combination with liquid chromatography for the simultaneous determination of four mycotoxins (deoxynivalenol, alternariol, ochratoxin A and zearalenone) in edible vegetable oils. A chemometric approach assessed the effect of the operational parameters on the mycotoxin extraction efficiency. The extracts were analyzed by HPLC coupled with a diode array and fluorescence detector. The optimum NADES composition resulted in the highest extraction recoveries, and it was applied to coextract the target mycotoxins in several types of edible vegetable oils without using hazardous solvents or requiring further clean-up. The limits of detection ranged from 0.07 to 300 μg kg^{-1}, and recoveries were close to 100%, except for zearalenone (*viz.* 35%), with relative standard deviations below 9% in all cases. The proposed method was validated following the European Commission 2002/657/EC and 2006/401/EC.

1. Introduction

Edible vegetable oils are among the essential ingredients of human nutrition and daily diet because they possess beneficial properties that arise from their compounds, such as vitamin E and unsaturated fatty acids (omega-3 and omega-6, mainly). Edible vegetable oils can be extracted from various plant species, including olive, sunflower, corn, rice bran, coconut, peanut and sesame (Eom et al., 2017); unfortunately, the oils may be contaminated with hazardous substances such as mycotoxins (Ma et al., 2016).

Mycotoxins are low-molecular-weight secondary metabolites produced by filamentous fungi. Most of these agents can be hemo-, hepato-, nephron-, dermato-, neuro- or immunotoxic; some are even carcinogenic (Bhat & Reddy, 2017). Based on data reported by the Rapid Alert System for Food and Feed (RASFF), mycotoxins generate a worrying number of alert notifications (RASFF Annual Report 2019, 2020). For example, more than 400 mycotoxins have been identified in foodstuffs (Weidenborner, 2013). According to Commission Regulation No 1881/2006, which has set the maximum tolerated levels for mycotoxins in foods (Commission Regulation (EC) No 1881/2006, 2006), the most prevalent mycotoxins are aflatoxins (AFs), fumonisins (FBs), patulin, zearalenone (ZEA), deoxynivalenol (DON) and ochratoxin A (OTA).

In recent years, the presence of ZEA, DON, and OTA in vegetable oils has been reported (Escobar et al., 2013; Qian et al., 2015), as well as alternariol (AOH) to a lesser extent (Moya-Cavas et al., 2023). This presence is especially worrisome because most mycotoxins are relatively stable over the typical range of food boiling and frying temperatures (80–120 °C) (Francesca Bosco & Chiara Mollea, 2012). The European Commission has only regulated the maximum tolerated levels of zearalenone in vegetable oils such as refined corn oil (Commission Regulation (EC) No 1881/2006, 2006) and published a recommendation on monitoring the presence of Alternaria toxins in food (Commission Recommendation (EU) No 2022/553, 2022).

Several existing analytical techniques are routinely used for mycotoxin detection, including thin-layer chromatography (TLC), capillary electrophoresis (CE) (Weidenborner, 2013) and immunoassays (Peltomaa et al., 2017). Most often, mycotoxins are determined by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) (Mohebbi et al., 2022), but liquid chromatography coupled with fluorescence and diode array detectors (HPLC–FLD/DAD) has also been

* Corresponding authors.
E-mail addresses: fenavi@ucm.es (F. Navarro-Villoslada), elenabp@ucm.es (E. Benito-Peña).
[1] The late.

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broadly used (Stojanović & Kos, 2020; Razzazi-Fazeli & Reiter, 2011).

Although these techniques provide accurate and reproducible results, they involve cumbersome sample preparation procedures, which in the case of oil samples are complicated by the fatty nature of the matrix (Razzazi-Fazeli & Reiter, 2011). The most popular methods for mycotoxin extraction and purification from vegetable oil samples are based on solid-phase extraction (SPE) (Zhao et al., 2017), matrix solid-phase dispersion (MSPD), immunoaffinity chromatography (IAC), liquid–liquid extraction or QuEChERS (Quick, Easy, Cheap, Effective, Rugged & Safe) (Zhao et al., 2017). However, most of these techniques use hazardous volatile organic solvents such as hexane, acetonitrile or methanol (Cvjetko Bubalo et al., 2015) as extractants. These solvents are well-known environmental pollutants and are dangerous to human health owing to their high toxicity and poor biodegradability. They should therefore be avoided to meet society's growing demand for more sustainable products and to encourage the use of effective and nonhazardous extractants.

Natural eutectic deep eutectic solvents or natural DES (NADES) are an excellent alternative to organic solvents. In the last decade, NADES have gained much attention as "green" chemicals because of their high biodegradability and biocompatibility, water miscibility, and low toxicity, along with other favorable properties (Cunha & Fernandes, 2018). In general, DES (Kalhor & Ghandi, 2019) are classified according to the nature of their constituents, exhibiting a significant freezing point depression and liquid state at room temperature. Type III DES consist of a hydrogen bond acceptor (HBA, usually the quaternary salt choline chloride) and a hydrogen bond donor (HBD) such as an amine, carboxylic acid, alcohol, carbohydrate or a mixture thereof (Marcus, 2018). Many type III and V eutectic solvents (e.g., honey, maple syrup, sugar beet) occur naturally and are referred to as NADES (Kudłak et al., 2015). NADES are physicochemically similar to synthetic ionic liquids (ILs) and are suitable for various applications (Chen et al., 2019; Zhang et al., 2012). For example, they have proven to be effective media for (bio) reactions (Smith et al., 2014) and key ingredients of cosmetic and medical products (Jeong et al., 2017). NADES are also excellent solvents for polysaccharides and lignins (Li & Row, 2016). They have been used to extract bioproducts from natural sources and contaminants from food samples (Mogaddam et al., 2022; Nemati et al., 2022; Piemontese et al., 2017). In contrast, no DES-based extraction of mycotoxins in food samples has been reported without using organic solvents (or complex processes).

In this work, we developed an environmentally green NADES-based liquid–liquid microextraction method to extract ZEA, DON, OTA and AOH from vegetable oils (Fig. 1). To the best of our knowledge, this is the first time NADES have been used to simply extract mycotoxins from such matrices and avoid harmful solvents. The extraction conditions were optimized by a fractional factorial design (FFD) to examine the influence of the selected operational variables on the extraction efficiency for each mycotoxin (i.e., sample weight, NADES volume, NADES water content, extraction temperature and extraction time). The following method was validated in various edible vegetable oils according to Commission Decisions 2002/657/EC (Commission Decision 2002/657/EC, 2002) and 2006/401/EC (Commission Regulation (EC) No 401/2006, 2006).

2. Experimental

2.1. Samples and chemicals

All reagents used were analytical grade, and solvents were LC grade. The four mycotoxins studied were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (MeCN) and methanol (MeOH) were supplied in HPLC grade by Scharlab (Barcelona, Spain), trifluoroacetic acid (TFA) in HPLC grade (99.5%) by Apollo Scientific (Manchester,

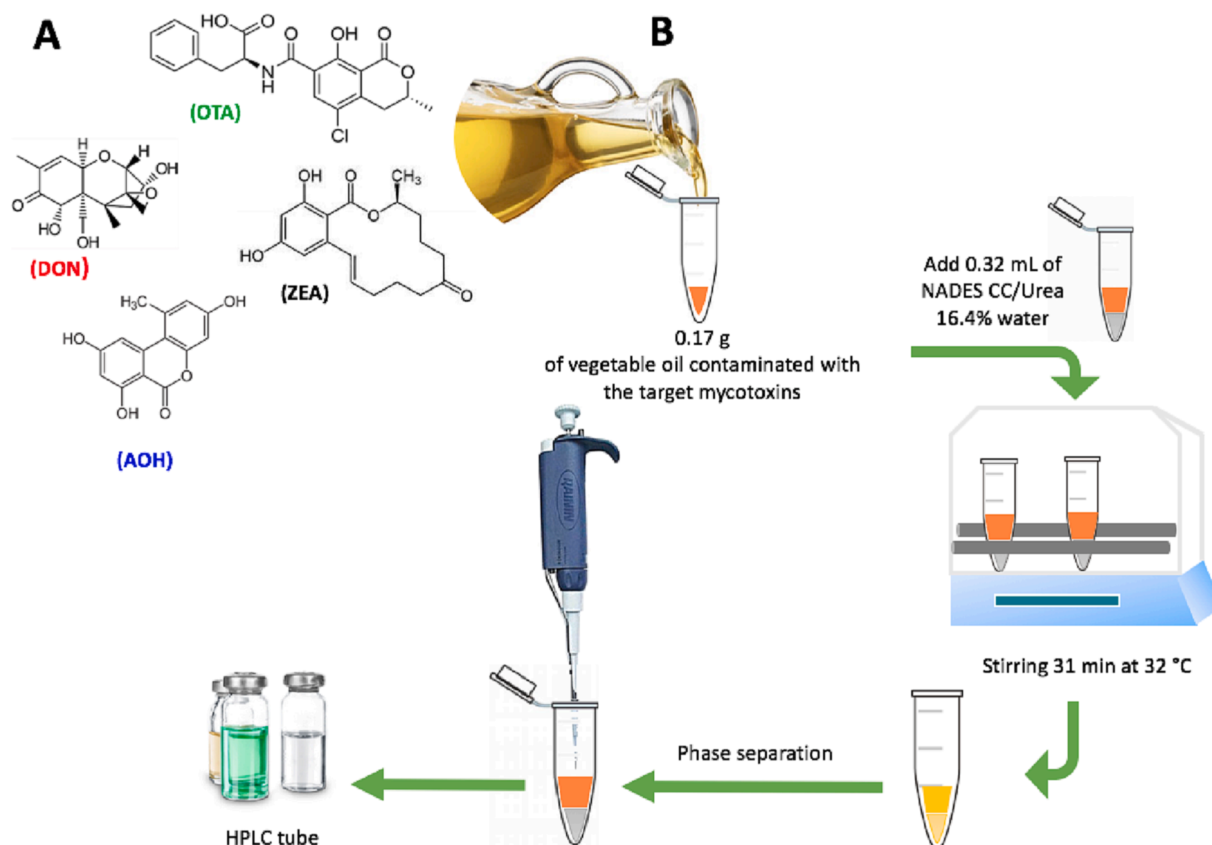

Fig. 1. (A) Chemical structure of the mycotoxins: zearalenone (ZEA), alternariol (AOH), ochratoxin A (OTA) and deoxynivalenol (DON). (B) Workflow of sample treatment.

UK), and dimethyl sulfoxide by VWR (Radnor, PA, USA). Choline chloride, urea, malonic acid and glucose were obtained from Thermo Fisher Scientific (Waltham, MA, USA), and ethylene glycol was purchased from Scharlab (Barcelona, Spain). Ultrapure water (18.2 MΩ cm^{-1}) supplied by a Milli-Q system from Millipore (Bedford, MA, USA) was used throughout. The edible vegetable oils studied included rice bran, wok, seed, peanut, sunflower, and olive oils purchased from local markets in Madrid. All the samples were processed as received.

A standard stock solution of each mycotoxin containing a 1 mg mL^{-1} concentration in dimethyl sulfoxide (DMSO) was prepared and stored at –20 °C in the dark until use. As needed, these stock solutions were diluted with acetonitrile to prepare working-strength solutions.

2.2. Preparation of natural deep eutectic solvents

Typically, NADES are prepared by mixing an HBA with an appropriate amount of a HBD. In this work, HBA chlorine chloride (ChCl) was mixed with different HBDs, namely, urea, ethylene glycol, malonic acid or glucose, in different molar ratios. Each mixture was placed in 50 mL screw cap centrifuge tubes, vortexed for 20 s, and shaken vigorously in an orbital shaker at 80 °C for 2 h. Then, the homogeneous colorless liquids obtained were supplemented with a variable amount of water from 15% to 30% (w/w) and kept at room temperature. Kinematic viscosity was measured at 45 °C using an Ubbelohde 0B viscometer from Comecta S.A. (Barcelona, Spain). The temperature was controlled by an external water bath and circulator from JP Selecta (Barcelona, Spain). NADES pH values were measured at 25 °C with a GLP21 pH meter from Crison (Barcelona, Spain), and densities were determined by weighing 25 mL of the mixture on a Model 125 A analytical balance from Precisa (Dietikon, Switzerland). Experimental densities and kinematic viscosities were used to calculate the dynamic viscosity of the mixtures. Table 1 illustrates the composition, viscosity and hydrophobicity (as the logarithm of the *n*-octanol/water partition coefficient) of the four different NADES prepared.

2.3. Preparation of spiked samples and extraction protocol

Oil samples were checked to ensure that they contained none of the target mycotoxins at quantifiable concentrations for recovery assessment. Then, the samples were spiked with a solution containing variable concentrations of the analytes ranging from 3 to 2500 μg kg^{-1}. Following the workflow depicted in Fig. 1, aliquots of 0.17 g were transferred to 2 mL centrifuge tubes and vortex-mixed with 0.32 mL of ChCl/urea mixture in a 1:2 mol ratio supplemented with 16.4% (w/w) Milli-Q water. The mixture was then shaken at 32 °C in an IKA KS 4000i incubator shaker (Staufen, Germany) for 31 min. After extraction, the samples were allowed to stand at room temperature until phase separation for 10 min, and 50 μL of supernatant was collected and diluted 1:1 with Milli-Q water for chromatographic analysis.

The working conditions for extracting the mycotoxins were optimized using statistical experimental design methodology as described in Sections 2.5 and 3.3.

2.4. HPLC–FLD/DAD methodology

Samples were analyzed by using a chromatographic system consisting of an HP-1100 Series high-performance liquid chromatograph (HPLC) from Agilent Technologies (Palo Alto, CA, USA) equipped with a quaternary pump, online degasser, autosampler, automatic injector and column thermostat and coupled to a diode-array (DAD) and a fluorescence (FLD) detector.

HPLC separation was performed on a C18 Zorbax Eclipse Plus RRLC analytical column (150 × 4.6 mm, 3.5 μm film thickness) from Agilent (Santa Clara, CA, USA) and gradient elution with water + 0.1% TFA (A), MeCN + 0.1% TFA (B) and methanol (C). A constant proportion of C (5%) was held. The initial condition was 2% B for 5 min, followed by a linear gradient to 90% B at 17 min, 10 min hold isocratic at 90% B, and 5 min at initial conditions for re-equilibration. The flow rate was 0.8 mL min^{-1}, and the injection volume was 50 μL. DON was detected by DAD at 219 nm, whereas OTA, ZEA and AOH were detected by FLD at 330, 236 and 258 nm excitation wavelengths, respectively, and 450 nm emission wavelength for the three mycotoxins.

2.5. Experimental design

The influence of selected independent operational variables on the extraction efficiency of the target mycotoxins was assessed using a screening fractional factorial design (FFD). The variables included in the two-level FFD were sample weight (*S*), NADES volume (*V*), NADES water content (*W*), extraction temperature (*T*) and extraction time (*t*). The influence of the variables was examined over the following ranges: 0.1–0.3 g (*S*), 0.15–0.35 mL (*V*), 15–330% (*W*), 25–75 °C (*T*) and 15–45 min (*t*). The target response was the recovery of each mycotoxin from rice bran oil. A total of 16 runs were conducted in duplicate and five replicates at the experimental design center. Therefore, 37 runs were performed in a randomized sequence to perform the FFD screening design.

A response surface design, i.e., a central composite design (CCD), was used to build an empirical model relating mycotoxin recovery to the operational variables. These empirical models were then used to determine the optimum operating conditions for the simultaneous quantitative extraction of the target mycotoxins. The experimental domain to be examined for each operational variable in the CCD design was established from the levels leading to optimal extraction with the FFD design, namely, 0.1–0.2 g (*S*), 0.25–0.35 mL (*V*), 15–20% (*W*), 25–50 °C (*T*) and 20–40 min (*t*). A total of 90 runs were used to develop

Table 1
Composition, pH, log *P* and viscosity of the studied NADES, log P or log D (pH 3/7) and pK_a of the target mycotoxins. Recovery of the target mycotoxins on NADES of variable composition from a rice bran oil sample spiked with DON and AOH at 2.5 μg g^{-1}, ZEA at 0.2 μg g^{-1} and OTA at 0.003 μg g^{-1}.

NADES						Mycotoxin				
HBA	**HBD**	**HBA:HBD mole ratio**	**pH**	**log *P*[(a)]**	**Viscosity at 45 °C (mPa·s)**		**DON**	**AOH**	**ZEA**	**OTA**
						log *P* or log D[(b)]	–1.41 (pH 3) –1.41 (pH 7)	3.30 (pH 3) 3.06 (pH 7)	3.83 (pH 3) 3.72 (pH 7)	4.41 (pH 3) 1.10 (pH 7)
						pK_a[(b)]	11.91	7.16	7.41	3.29/-2.20
							Recovery, % (RSD, %)			
Choline chloride (ChCl)	Malonic acid	1:1	0.16	–1.26	7.47		52 (1)	78 (4)	nd	nd
	Glucose	1:1	2.53	–	22.58		68 (2)	54 (7)	nd	nd
	Ethyleneglycol	1:2	3.77	–1.36	4.55		52 (1)	72 (4)	nd	39 (3)
	Urea	1:2	9.53	–2.11	4.22		**72 (5)**	**83 (2)**	**23 (2)**	**75 (4)**

(a) Xu M., Ran L., Chen N., Fan X., Ren D., & Yi L. (2019), *Food Chem.*, *297*, 124970. https://doi.org/10.1016/j.foodchem.2019.124970.
(b) Apfelthaler. E., Bicker W., Lämmerhofer M., Sulyok M., Krska R., Lindner W., & Schuhmacher R. (2008), *J. Chromatogr. A*, *1191*(1–2), 171–181. https://doi.org/10.1016/j.chroma.2007.12.067.

the CCD, of which 32 corresponded to the factorial design repeated twice, 10 to axial points repeated twice and 6 to replicates of the central point. The experimental designs, response surfaces, and screening and optimization results were all generated and analyzed with the statistical package Design Expert 10 (Stat-Ease, MN, USA).

2.6. Validation of the proposed method

Extracts of rice bran oil samples spiked with the target mycotoxins were used for method validation due to the lack of certified reference materials, and the sample was purchased in a local supermarket. The NADES-HPLC–FLD/DAD method for determining mycotoxins in vegetable oils was validated in terms of specificity, linearity and apparent recovery, repeatability (within-day precision, RSD_r) and reproducibility (intermediate precision, RSD_R) in compliance with the criteria of Commission Decisions 2002/657/EC (Commission Decision 2002/657/EC, 2002), 2006/401/EC (Commission Regulation (EC) No 401/2006, 2006). The specificity of the targeted mycotoxins was assessed by analyzing ten representative blank oil samples, and their absence was confirmed by liquid chromatography.

All validation parameters were calculated from the peak area of each analyte. Linearity was evaluated in triplicate for each mycotoxin at seven different concentration levels spanning the range 0.025 to 0.225 μg mL^{-1} (equivalent to 45–425 μg kg^{-1} sample) for AOH and ZEA, 0.0005 to 0.01 μg mL^{-1} (equivalent to 0.9–19 μg kg^{-1}) for OTA and 0.5 to 5 μg mL^{-1} (equivalent to 940–9400 μg kg^{-1}) for DON. Standard calibration curves were obtained by plotting the chromatographic peak areas against the mycotoxin concentrations. All determination coefficients (R^2) were greater than 0.99 (Table S1, Supplementary material).

The limits of detection (LOD) and quantification (LOQ) were estimated in triplicate from the calibration curves and calculated as 3 and 10 times the residual standard deviation of the regression line (*viz.* standard deviation of the response) divided by the slope, respectively, using a linear regression model constructed in Microsoft Excel 2016.

The decision limit (CC_α) and detection capability (CC_β) were estimated following the procedure described in the European Commission Decisions 2002/657/EC (Commission Decision 2002/657/EC, 2002) and 2006/401/EC (Commission Regulation (EC) No 401/2006, 2006). Briefly, CC_α was calculated as the concentration corresponding to the intercept plus 2.33 times the lowest calibration level's within-laboratory standard deviation (i.e., intermediate precision). CC_β was determined as CC_α plus 1.64 times the within-laboratory standard deviation at the CC_α level. The calibration curves for those substances with no established tolerated level were run from samples spiked at and above the minimum required performance level (*viz.*, LOQ for each mycotoxin) (SANCO/2004/2726, 2008).

Apparent recoveries, RSD_r and $RSD_{R,}$ were assessed by analyzing blank samples spiked at 1, 1.5 and 2 times the LOQ for each mycotoxin on the same day ($n = 6$, RSD_r) and on three different days ($n = 18$, RSD_R). Recoveries were determined using standard calibration curves to calculate measured-to-spiked concentration ratios (Sulyok et al., 2006).

3. Results and discussion

3.1. Calibration of the proposed method

The target mycotoxins were quantified against standard calibration curves based on $n = 7$ points and spanning the analyte concentration ranges from 0.025 to 0.225 μg mL^{-1} (equivalent to 45–425 μg kg^{-1} sample) for AOH and ZEA, from 0.0005 to 0.01 μg mL^{-1} (equivalent to 0.9–19 μg kg^{-1}) for OTA and from 0.5 to 5 μg mL^{-1} (equivalent to 940–9400 μg kg^{-1}) for DON. Standard and matrix-matched calibration curves were obtained by adding appropriate volumes of each mycotoxin working solution to the NADES or a blank matrix solution obtained by extracting blank rice bran oil with NADES.

Statistical comparisons ($\alpha = 0.05$) of the slopes of the standard and matrix-matched calibration models confirmed the presence of no matrix effect for the four mycotoxins (Andrade & Estévez-Pérez, 2014) (Table S1, Supplementary material). Additionally, the absence of chromatographic peaks at times identical to those of retention for the target mycotoxins in the analysis of blank samples confirmed that the method was interference-free and hence specific for them (Figure S1, Supplementary material).

3.2. Optimization of NADES composition

The best composition of type III DES for extracting the target mycotoxins was established from combinations of the hydrogen bond acceptor (HBA) chlorine chloride (ChCl) (Espino et al., 2016) and four natural HBDs, including an amine (urea), an alcohol (ethylene glycol), a carboxylic acid (malonic acid) and a carbohydrate (glucose). Their differences in composition give rise to NADES with different viscosities, polarities and extraction capacities (Espino et al., 2016). In addition, the selected NADES must be sufficiently polar to not be miscible with the oil samples.

Rice bran oil samples were spiked with 2.5 μg DON g^{-1}, 0.2 μg AOH g^{-1}, 0.2 μg ZEA g^{-1} and 0.003 μg OTA g^{-1} for extraction with the four types of mixtures consisting of ChCl:malonic acid (1:1) mole ratio, ChCl: glucose (1:1) mole ratio, ChCl: ethylene glycol (1:2) mole ratio, and ChCl:urea (1:2) mole ratio, all containing 20% (v/v) water. The temperature was set at 60 °C, and the extraction time was 45 min. Although the presence of water in DES mixtures weakens DES-DES interactions, it has been reported that the presence of water in a low molar ratio retains the DES nanostructure network while decreasing DES viscosity and hindering mass transfer from targets to the solution (Pradanas-González et al., 2021, Hammond et al., 2017). The recoveries obtained (Table 1) suggested a strong dependence of extraction yields on the nature of the HBD present in the NADES (Xu et al., 2019).

For example, the lowest recoveries were obtained with the solvent ChCl:malonic acid, possibly due to its low pH, significantly hindering the DES-mycotoxin interaction. Extraction yields increased for all mycotoxins while increasing the basicity of NADES extractants. Thus, a high pH could facilitate electrostatic and hydrogen bonding interactions while increasing the solubility of mycotoxins considering their pKa values.

For example, OTA extraction was strongly influenced by NADES pH. Log *P* for this mycotoxin is pH dependent. Thus, OTA behaves as a less polar analyte at pH 3 (log $P = 4.40$) than at pH 7 (log $P = 1.10$). Because the malonic acid and glucose-based NADES had pH < 3, OTA could not be extracted with them owing to its hydrophobicity under such acidic conditions. The ethylene glycol-based NADES has a pH of 3.77, so the log *P* of the OTA is still high to allow effective extraction (Table 1). OTA recovery was maximal with the urea-based NADES, which had pH 9.53 and thus facilitated extraction of this analyte thanks to its high polarity above pH 7.

Interestingly, ZEA was only extracted by the urea-based NADES. This mycotoxin was the least polar and had high log *P* values at pH 3 and 7. Because the urea-based NADES had pH 9.53, a higher value than the pK_a for ZEA (7.41), it caused the mycotoxin to be deprotonated, thereby enhancing electrostatic interactions with the NADES components and facilitating its extraction. Finally, DON and AOH, the two most polar analytes, were successfully extracted by the four NADES.

Polarity and viscosity are other factors that may influence the solubilization capacity of NADES. However, although extraction yields increased with the polarity of DES, no relationship was observed with the polarity of mycotoxins (i.e., ZEA vs. AOH) (Table 1). Moreover, as discussed above, low viscosity could improve target diffusion in eutectic solvents. Nevertheless, the moderate viscosity of malonic acid (7.47 mPa·s at 45 °C) suggests that this variable weakly influences extraction efficiency.

Based on the previous results and considering the flexibility of

NADES uses regarding their physicochemical properties, the ChCl:urea mixture was chosen for further testing because it provided the highest extraction yields for all mycotoxins.

3.3. Screening and optimization of the extraction conditions

The operational variables of the extraction process were screened and optimized using statistical experimental design methodology. The target operational variables were sample weight (*S*), NADES volume (*V*), NADES water content (*W*), temperature (*T*), and time (*t*).

As mentioned above, the amount of water alters the hydrogen bonding interactions that maintain the eutectic mixture by changing the polarity, density, viscosity, melting point, conductivity, and solubilization capacity of the extractant, even at low hydration levels (Dai et al., 2015). In fact, above a water content of 50% (v/v), the system was akin to an aqueous solution (one of ChCl and urea) (Hammond et al., 2017).

3.3.1. Screening design

The target extraction variables were screened using a two-level fractional factorial design (FFD). The results of 16 runs performed in duplicate, plus 5 replicates at the central point of the design, were used for analysis.

The standardized effect of each variable on the extraction of the mycotoxins is illustrated through Pareto charts in Figure S2 (Supplementary material). Those variables with *t* values exceeding the critical threshold were deemed statistically significant. With ZEA, however, the Box–Cox method suggested applying a natural log transformation to the observations to stabilize the variance.

According to the results, the effect of each variable on mycotoxin extraction and their significance differed. Thus, sample weight was the most influential variable on AOH and ZEA extraction, with small amounts of the sample resulting in increased AOH and ZEA recoveries. NADES volume was also a significant variable, but its effect differed among mycotoxins and was particularly important for ZEA and OTA. Increasing NADES volumes led to increasing ZEA and OTA recoveries. As expected, the NADES water content had a crucial effect on extracting the target mycotoxins. As discussed below, the substantial favorable outcome of high water contents on DON extraction and the substantial adverse impact on ZEA and AOH extraction may have resulted from structural differences among the mycotoxins (Fig. 1).

High extraction temperatures had a substantial adverse impact on AOH extraction. Nevertheless, the temperature interacted synergistically with the other variables and boosted the extraction of the other mycotoxins. Finally, long extraction times had a substantial favorable effect on DON extraction. Overall, the five variables examined considerably influenced the extraction of the target mycotoxins and were considered for further optimization with response surface methodology based on a central composite design.

3.3.2. Response surface modeling

Mycotoxin extraction with the NADES was optimized by using response surface-based methodology based on a central composite design (CCD) whereby the variance of the response predicted by the empirical model was constant at a given distance from the central point of the design —which was thus a rotatable design (Zahran et al., 2003).

The experimental domain was defined in terms of the results previously obtained with the FFD design. Table S2 outlines the experimental design, where maximum and minimum levels were selected regarding the distance of the axial points. Mycotoxin recoveries in the experimental domain were between 63% and 112%, averaging approximately 100%, except for ZEA, with an average of 23%. As noted earlier, such a low recovery may have resulted from a polarity mismatch between ZEA and the NADES-based extractant.

A total of 90 experiments were performed, including 32 factorial points and 10 axial points (both in duplicate) plus 6 replicates of the central point. Mycotoxin recoveries were estimated using a statistical model including interactive and polynomial terms. All possible interactions between the five factors were initially considered. Additionally, the significant coefficients of each empirical model were selected in terms of stepwise regression and *p–values*, using a threshold of 0.1 for adding or removing coefficients. An Analysis of Variance (ANOVA) of the response surface model estimated by the stepwise regression for each mycotoxin gave *p values greater than 0*.05 in the lack of fit test, so the lack of fit of the estimated models was not significant relative to the pure error. On the other hand, the Box–Cox method suggested applying a natural log transformation to the ZEA observations to stabilize the variance.

Least-squares methodology was used to estimate the regression coefficients of the multiple linear regression model for each mycotoxin. All models were significant at $p\ value < 0.0001$, and their goodness of fit was assessed by an analysis of variance (ANOVA). The ability of the models to explain mycotoxin extraction performance and predict a recovery value from new observations was estimated by calculating the coefficient of multiple determination adjusted for the number of parameters in the model relative to that of runs in the design (Adj R^2), as well as the predicted coefficient of determination (Pred R^2) (Table S3, Supplementary material). Invariably, Pred R^2 was reasonably consistent with Adj R^2 (the difference between the two was always <0.2). Hence, the data fitted the models well and accurately estimated the system's response.

The accuracy of the empirical models was additionally assessed by using three different types of diagnostic tools: (*a*) normal probability plot of studentised residuals (Boylan & Cho, 2012); (*b*) studentised residuals against predicted values; and (*c*) predicted versus actual responses. The normal probability plots of studentised residuals resemble a straight line, consistent with normally distributed errors (Figure S3, Supplementary material). On the other hand, the plots of studentised residuals versus predicted values exhibited no unusual data or trends, all randomly distributed around zero (Figure S4, Supplementary material). As noted earlier, ZEA observations were subjected to natural log transformation to stabilize the variance. Although the plot for the OTA model was shaped like an outward-opening funnel and thus suggested potential variability in the variance, the Box–Cox method confirmed that no data transformation was necessary.

A scatter plot of predicted versus actual recovery for each mycotoxin revealed good consistency between the two datasets (Figure S5, Supplementary material). Therefore, none of the three types of plots exposed any inadequacies in the models for the target mycotoxins. Perturbation plots constructed using the central point of the experimental design as a reference showed that, overall, NADES-based mycotoxin extraction performance was influenced by the sample weight, i.e., the greater the amount of sample was, the lower the recovery of ZEA excepted. Judging by its variation, ZEA recovery strongly depended on NADES water content and increased with decreasing content (Figure S6, Supplementary material).

Mycotoxin recoveries were simultaneously optimized by using the overall desirability function. The process merely involves identifying the levels of the different operational variables leading to optimal analytical performance. For this purpose, each response (mycotoxin recovery) was converted into an individual desirability function *d* spanning the range from zero to unity, with $d = 1$ if the response was at its target and $d = 0$ if it fell outside the acceptable region. Design parameters were chosen in such a way as to maximize the overall desirability function.

$$D = (d_1 \times d_2 \times d_3 \times \ldots . \times d_m)^{1/m} \quad (1)$$

where *m* is the number of responses. If any individual responses are undesirable, then $D = 0$ (Montgomery, 2017).

In the built-in optimization step, the desired goal for each factor was chosen within the studied range. The response target (*viz.*, mycotoxin recovery) was maximal. Contour plots for the overall desirability function in the experimental domain showed that the conditions maximizing

Table 2
Predicted and experimental recoveries (±confidence interval at the 95% confidence level) for each sample ~~used to validate the prediction ability of the model~~. Extraction conditions: 0.32 mL DES containing 16.4% water, 0.17 g sample, 31 min and 32 °C. Samples spiked with 2500 μg kg^{-1} DON, 200 μg kg^{-1} AOH and ZEA and 30 μg kg^{-1} OTA.

Analyte	Predicted recovery (Predicted interval) (%)	Experimental recovery (%) (n = 3)			
		Rice bran oil	Wok oil	Seed oil	Peanut oil
DON	101 (94 – 107)	103 ± 8	91 ± 2	101 ± 1	96 ± 3
AOH	109 (103 – 115)	108 ± 5	110 ± 10	98 ± 1	107 ± 6
OTA	99 (91 – 108)	100 ± 7	91 ± 3	82 ± 2	95 ± 4
ZEA	35 (30 – 41)	36 ± 3	37 ± 2	37 ± 1	42 ± 3

Table 3
Linear dynamic range and estimated LOD, LOQ, CC_{α} and CC_{β} for each mycotoxin.

Mycotoxin	**Linear dynamic range** (μg kg^{-1})	**R^2**	**LOD** (μg kg^{-1})	**LOQ** (μg kg^{-1})	**CC_{α} (α = 0.05)** (μg kg^{-1})	**CC_{β} (β = 0.05)** (μg kg^{-1})
DON	940–9400	0.9932	100	300	200	800
AOH	45–425	0.9994	1	3	1	2
OTA	0.9–19	0.9971	0.07	0.2	0.07	0.1
ZEA	45–425	0.9995	10	30	8	20

overall desirability fell in a relatively narrow region with NADES water content and extraction temperature at their lowest levels in the experimental design (Figure S7, Supplementary material). In contrast, the sample weight and NADES volume were set at their highest levels. Despite the interactions of extraction time with the other factors, the overall desirability was reasonably constant throughout the time range. Thus, the optimum working conditions, predicted mycotoxin recovery, and 95% prediction interval (Montgomery, 2017) were established in Table 2. The conditions ensuring quantitative extraction (recovery greater than 90%) of AOH, OTA, and DON from the oil samples were 16.4% water in the NADES mixture, 0.17 g of sample, 0.32 mL of NADES, 32 °C, and 31 min, with an overall desirability of 0.79. Under such conditions, however, ZEA was recovered by only approximately 35% owing to its poor solubility in the NADES.

The conclusions drawn from the optimization process were validated by further testing of different vegetable oils. As shown in Table 2, the experimental results also matched the predictions to extract the target analytes from rice bran, wok, seed and peanut oils (Table 2). In addition, it provided near-quantitative recoveries ($n = 3$) for all mycotoxins and met the general guideline ranges set by Commission Decision 2006/401/EC at the concentration levels tested (Commission Regulation (EC) No 401/2006, 2006). As expected, ZEA was extracted by only 36–42%, but it was fully acceptable in ensuring reproducibility ($RSD_r < 8\%$ compared with its recommended $RSD_r < 25\%$). The results confirm that the response surface regression models developed in the design space afforded correct interpretation and prediction for OTA, AOH and DON.

3.4. *Validation of the proposed NADES-based mycotoxin extraction method*

The suitability of the proposed method for accurately quantifying mycotoxins in edible vegetable oils was evaluated by analyzing six different products, including rice bran, wok, seed and peanut. None of them contained the target analytes above their limit of detection.

The limit of detection (LOD) and quantification (LOQ) in oil samples were calculated to span the ranges 1–100 and 3–300 μg kg^{-1}, respectively (Table 3). Decision limits (CC_{α}) and detection limits (CC_{β}) were also estimated from the calibration curves, following the guidelines of Commission Decision 2002/657/EC (Commission Decision 2002/657/EC, 2002) and the recommendations of document SANCO 2004/2726 (SANCO/2004/2726, 2008) for substances with no set maximum levels. Table 3 lists each mycotoxin's estimated LOD, LOQ, CC_{α}, and CC_{β} values.

Although the levels of the target mycotoxins in the types of vegetable oils studied have not been regulated, the LOQ of ZEA (30 μg kg^{-1}) was lower than its maximum tolerated level in a similar matrix (*viz.*, 200 μg kg^{-1} in refined corn oil). The LOQ for AOH (3 μg kg^{-1}) is also below the maximum recommended level established in another vegetable oil matrix (i.e., 10 μg kg^{-1} in sunflower oil). The LOQs were below the maximum residue levels for the respective mycotoxins in different matrices for OTA and DON. The LODs and LOQs obtained are on par with those of other methods using more sensitive detection techniques (Table S4). This work is a breakthrough in extracting mycotoxins from foodstuffs, as it allows conventional organic solvents to be replaced with similarly efficient but more environmentally friendly alternatives. Our method is simple, environmentally friendly, cost-effective, and relatively fast since it does not require preconcentration or clean-up steps.

The proposed method was further validated regarding precision (*viz.* repeatability, RSD_r, and intermediate precision, RSD_R) by applying it to real samples. Thus, rice bran oil samples were spiked with the four mycotoxins to obtain three concentrations equivalent to 1, 1.5 and 2 times their corresponding LOQs. The RSD_r parameter was evaluated by analyzing six replicates on the same day, and the RSD_R parameter was calculated from an equal number of replicates analyzed on three different days by different personnel.

Commission Decision 2002/657/EC recommends that RSD_r and RSD_R values, for analyses performed under reproducible conditions, not exceed the values calculated from the Horwitz equation (Commission Decision 2002/657/EC, 2002). As shown in Table 4, the experimental RSD_r and RSD_R figures ranged from 1.4 to 7.4% and 3.3 to 8.9%, respectively. However, the RSD_r and RSD_R values calculated from the Horwitz-Thompson equation expand from 25 to 27% and 18 to 54%, respectively. Therefore, these results attest to the method's precision for the proposed objective.

The NADES-based method was compared with a traditional method based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged & Safe) (Hidalgo-Ruiz et al., 2019). The recoveries obtained with both methodologies at different concentration levels are listed in Table 5. Quantitative and statistically comparable (α = 0.05) concentrations were obtained for DON and OTA. The NADES-based extraction method allowed recoveries of approximately 100% for AOH; however, the QuEChERS-based extraction failed in the quantitative recovery of AOH with recoveries below 65%. As described in Section 3.3., our methodology provides low recoveries of ZEA (36–42%), but this was not improved by the traditional extraction method, which results in poorer recoveries (<16%). Therefore, comparable and even higher recoveries were obtained with the developed methodology based on more

Table 4
Precision-related parameters and recoveries from rice oil samples spiked at levels equal to 1, 1.5 and 2 times the LOQ of each mycotoxin.

	DON	AOH	OTA	ZEA
Repeatability (within-day)				
Spiked level(μg kg^{-1})	300/450/600	3/4.5/6	0.2/0.3/0.4	30/45/60
Level found (μg kg^{-1})	287.1/454.7/576.3	3.0/4.7/6.1	0.20/0.31/0.42	10.0/14.9/20.1
Recovery (%)	95.7/101.0/96.0	100.6/103.2/102.2	99.6/102.4/104.8	33.2/33.1/33.5
RSD_r (%) ($n = 6$)	3.3/1.8/2.2	6.5/3.4/2.9	6.9/4.6/5.5	7.4/1.4/3.4
Intermediate precision (between-day)				
Spiked level (μg kg^{-1})	300/450/600	3/4.5/6	0.2/0.3/0.4	30/45/60
Level found (μg kg^{-1})	294.5/446.3/574.6	3.1/4.7/6.1	0.20/0.30/0.40	10.6/16.7/20.0
Recovery (%)	98.2/99.6/95.7	104.7/104.4/103.1	99.9/99.6/100.5	35.2/37.2/33.4
RSD_R (%) ($n = 18$)	4.7/3.3/4.3	6.9/5.0/5.4	6.6/8.9/8.6	8.6/6.8/5.5

Table 5
Comparison of the developed extraction method based on NADES with a traditional method based on QuEChERS (n = 6).

Analyte		NADES methodology			QuEChERS methodology		
	Spiked level (μg kg^{-1})	**Level found (μg kg^{-1})**	**Recovery (%)**	**RSD (%)**	**Level found (μg kg^{-1})**	**Recovery (%)**	**RSD (%)**
DON	300	287.1	95.7	3.3	284.8	94.6	6.6
	450	454.7	101.0	1.8	432.0	96.0	2.5
	600	576.3	96.0	2.2	556.1	92.7	5.9
AOH	3	3.0	100.6	6.5	1.3	42.0	2.7
	4.5	4.7	103.2	3.4	2.9	64.4	3.5
	6	6.1	102.2	2.9	2.6	43.0	5.9
OTA	0.2	0.20	99.6	6.9	0.21	103.8	5.6
	0.3	0.31	102.4	4.6	0.32	105.5	3.8
	0.4	0.42	104.8	5.5	0.42	104.1	2.6
ZEA	30	10.0	33.2	7.4	4.2	13.9	4.6
	45	14.9	33.1	1.4	6.7	14.9	2.7
	60	20.1	33.5	3.4	9.3	15.5	4.8

environmentally friendly solvents (NADES) than with the widely used dispersive solid phase extraction (dSPE) method using QuEChERS.

Finally, the methodology was applied to analyze different real samples of commercially available vegetable oils (Table S5). Several of them showed no natural content of any of the target mycotoxins. We have also analyzed additional edible vegetable oils suspected to contain AOH or ZEA (Moya-Cavas et al., 2023). As expected, AOH was found in sunflower oil at a concentration level of 146 μg kg^{-1}, higher than the EU recommended maximum level in that matrix (10 μg kg^{-1}). This mycotoxin was also present in two olive oil samples at lower concentrations (3.5 and 3 μg kg^{-1}). Last, an organic corn oil tested positive for ZEA with a concentration of 38 μg kg^{-1}. Due to the nonquantitative recovery of ZEA, it is impossible to give a real concentration in the sample with accuracy. By assuming a recovery of 36% for ZEA, the concentration of ZEA can be estimated to be 106 μg kg^{-1} (below the maximum tolerated level of this analyte in corn samples, 200 μg kg^{-1}).

4. Conclusions

A new simple and straightforward chromatographic method based on NADES was optimized for the environmentally friendly extraction and determination of DON, AOH, OTA and ZEA in edible vegetable oils. The application of a response surface experimental design allowed the optimization of the extraction conditions (*viz.*, 0.17 g of sample, 0.32 mL of ChCl:urea (1:1) containing 16.4% water, 32 °C and 31 min) to achieve the coextraction of four mycotoxins from edible vegetable oils. Recoveries ranged from 100% to 103% except for ZEA (35%) in rice bran oil samples. The proposed NADES-HPLC-FLD/DAD method was successfully used to determine the target contaminants in eight different types of edible vegetable oils. The method has been validated according to the criteria set in European Commission Decision 2002/657/EC and 2006/401/EC and applied to the analysis of rice bran, wok, seed, peanut, corn, sunflower and olive oil samples. The relative standard deviation was always <8.9%, thus meeting EU requirements. According to the results, the proposed alternative is operationally simpler and replaces 100% of the use of organic solvents in favor of an environmentally friendly method. The results were compared favorably to those obtained using more sophisticated techniques, such as UPLC–MS/MS.

CRediT authorship contribution statement

Fernando Pradanas-González: Conceptualization, Methodology, Investigation, Validation, Writing – original draft. **Rubén Aragoneses-Cazorla:** Investigation. **Miguel Ángel Merino-Sierra:** Investigation. **Elena Andrade-Bartolomé:** Investigation. **Fernando Navarro-Villoslada:** Formal analysis, Methodology, Validation, Writing – review & editing. **Elena Benito-Peña:** Supervision, Conceptualization, Methodology, Validation, Writing – review & editing. **María Cruz Moreno-Bondi:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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