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OPTIMIZATION AND VALIDATION OF A CHEAPER, SAFER, AND MORE SUSTAINABLE METHODOLOGY FOR AFLATOXINS DETERMINATION IN RICH-LIPIDIC MATRICES (PISTACHIO NUTS) USING DEEP EUTECTIC SOLVENT EXTRACTION AND UHPLC-FLD ANALYSIS

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ABSTRACT

Aflatoxins pose a major health concern and require strict monitoring in food products. Existing methods rely on hazardous organic solvents for extraction, prompting the development of a greener alternative. This study explores deep eutectic solvents (DESs) for aflatoxin extraction from pistachios, a valuable food product prone to aflatoxin contamination. The proposed method utilizes DES extraction followed by solid-phase extraction cleanup and ultrahigh-performance liquid chromatography coupled with fluorescence detector analysis. Recovery rates ranged from 85.5 to 99.1% for pistachios spiked with 1−8 ng/g aflatoxins, in compliance with EU regulations, with coefficients of variation less than 2.94%. The method demonstrates good sensitivity with limits of detection and quantification in the range of 0.02−0.22 ng/g and 0.05−0.72 ng/g, respectively. Greenness assessment using AGREEPrep and White Analytical Chemistry metrics confirms its environmental sustainability. This approach offers a promising, safer, and more eco-friendly alternative for aflatoxin extraction from complex food matrices like pistachios

KEYWORDS: DEEP EUTECTIC SOLVENT EXTRACTION, AFLATOXINS, RAW PISTACHIO NUTS, METHOD VALIDATION, UHPLC-FLD

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1. Introduction

Since their discovery in the early 1960s' in Great Britain, aflatoxins (AFs) are considered the strongest naturally occurring carcinogenic substances.[1] AFs are mycotoxins produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*; molds naturally present worldwide and practically unavoidable. These fungi's natural environments are mainly irrigated hot deserts and warm humid areas. Nevertheless, due to climate change, it is expected that areas today not affected by this threat will face temperature increases and droughts promoting AFs contamination.[1−3] It is generally reported that *A. parasiticus* can produce B-type and G-type AFs (AFB_1, AFB_2 and AFG_1, AFG_2) while *A. flavus* only B-type AFs. However, according to a recent study, some Korean strains of *A. flavus* can also produce G-type AFs.[4] These toxins can be found in different foodstuffs of daily consumption, including nuts, figs, dairy products, wheat, rice, and corn. Since the long-term intake of these contaminants can increase the possibility of developing hepatocellular carcinoma, the International Agency for Research on Cancer classified AFs in group one, hence, carcinogenic to humans.[5,6] Regulatory bodies worldwide set maximum limits of AFs in foods that vary between countries and according to the potentially contaminated goods.[7] A diverse range of commodities is prone to contamination by AFs.[8] Staple foods such as rice, wheat, and corn are associated with a higher risk of daily intake through dietary consumption.[8] Stringent regulations are in place for these commodities within the European Union (EU), where the maximum allowable limits are set at 2 ng/g for AFB_1 and 4 ng/ g for the sum of four AFs (AFB_1, AFB_2, AFG_1, AFG_2); the same limits are set for groundnuts (peanuts) since, especially the derived oilseeds, are widely used.[9] Other food commodities, such as tree nuts (almonds, pistachios, Brazilian nuts, hazelnuts, etc.), exhibit a lower daily intake compared to staple foods, requiring slightly less stringent regulatory limits. Among tree nuts, pistachios hold particular significance as a globally consumed product, either in its natural form or as a key ingredient in derivative products such as pastries, confectionery, and beverages. The European Commission sets maximum levels for AFs in pistachios, placed on the market for the final consumer or used as ingredients in food, at 8 ng/g for AFB_1 and 10 ng/g for the sum of four AFs (i.e., AFB_1, AFB_2 and AFG_1, AFG_2).[9] Pistachios can be affected by AFs contamination pre- or postharvest. Due to the elevated cost of this product, contamination can lead to a significant economic loss for the seller and/or the buyer, with the risk that a highly contaminated batch can reach the final consumers. For instance, between 2020 and 2021, the Rapid Alert System for Food and Feed received 56 notifications from different EU countries regarding pistachio batches exceeding the legal limit.[10] The scientific community has been working intensively to develop sensitive, robust, and reliable analytical procedures ever since the discovery of AFs.[11] To date, several official methods have been developed and validated.[12−15] Due to the complexity of the pistachio matrix, a sample preparation step is mandatory to obtain an extract purified from interferents and enriched in AFs.[7]

This research aims to address this challenge by exploring deep eutectic solvents (DESs) as eco-friendly alternatives for AF extraction. DESs are promoted as environmentally friendly alternatives to traditional organic solvents or ionic liquids, although comprehensive studies on their toxicological and

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biodegradable properties are still lacking. The potential for creating a vast array of DES formulations with diverse physicochemical characteristics makes them an intriguing area for further investigation, and readers seeking more information on this subject can refer to additional sources.[16,17] In this study, a novel sample preparation procedure using DESs was developed, optimized, and validated, demonstrating the potential of DESs as greener extraction media. This research contributes to the field by providing a detailed DESbased extraction methodology, highlighting its strengths and limitations. Specifically, it was shown that the DES-based method achieved analytical validity comparable to official methodologies, with the added benefit of being greener.

Table 1. *List of the Candidate DESs and Calculated $E_T(30)$ Measurements for the Common Organic Mixtures Used for AFs Extraction*[a]

candidates	DES components	mole ratio	$E_T(30)$ (kcal/mol)[b]	T_m (°C)	observation	reference
DES-1	ChCl: D-Glu	1:1	50.4		semisolid at T_r	39
DES-2	ChCl: CA	1:1	48.3	−9.7	semisolid at T_r	39
DES-3	ChCl: MA	1:2		10.0	liquid at T_r	26
DES-4	TAC: MA: H_2O	1:2:1			liquid at T_r	26,40
DES-5	ChCl: U	1:2	57.0	12.0	liquid at T_r	41,42
DES-5$_W$	ChCl: U: H_2O	1:2[c]	58.8		liquid at T_r	28
organic mixtures usually used for extraction						
MeOH			55.4			18
ACN			46.6			18
MeOH:H_2O (80:20)			56.3			this work
MeOH:H_2O (70:30)			56.5			this work
ACN:H_2O (90:10)			53.4			this work

[a]The chemicals used to obtain the reported combinations are the following: choline chloride (ChCl), D-glucose (D-Glu), citric acid (CA), malonic acid (MA), tetramethylammonium chloride (TAC), and urea (U). [b]Calculated using eq 1. T_m = melting temperature. T_r = room temperature. [c]20% (w/w) of water was added to this mixture.

2. Material and methods

2.1. CHEMICAL AND REAGENTS.

AFB_1, AFB_2, AFG_1, and AFG_2 from *A. flavus* (HPLC ≥ 98%), 1 mg each, were purchased from Sigma-Aldrich (MO, USA). Each AF was quantitatively transferred into a vial and solubilized in MeOH to have a 0.1 mg/mL solution. The obtained stock solutions were stored in the dark at −18 °C. The daily standard working solutions were obtained by diluting the stock solutions. Methanol (MeOH), acetonitrile (ACN),

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and chloroform ($CHCl_3$) were purchased from Sigma-Aldrich (Germany). Isopropanol and dichloromethane (CH_2Cl_2) were purchased from Scharlab (Spain). All the solvents used in the present study were HPLC-grade. Tetramethylammonium chloride (TAC), malonic acid (MA), choline chloride (ChCl), D-glucose (D-Glu), citric acid (CA), urea (U), sodium chloride, ammonium acetate, trisodium citrate, formic acid, magnesium sulfate, and Reichardt's dye were purchased from SigmaAldrich (Germany). Ultrapure water was obtained through a Milli-Q system (Millipore, USA).

2.2. DES FORMATION AND CHARACTERIZATION.

DESs were produced by the heating and stirring method.[17] Components in ratios reported in the literature, and summarized in Table 1, were placed in a round-bottom flask and stirred in a thermostatic bath at 70 °C until a homogeneous liquid was formed. The DES was then left to equilibrate at room temperature. In the case of DES-5_w, choline chloride, and urea were stirred at 70 °C until the formation of the DES, then the temperature was reduced to 50 °C, and 20% of water in weight was added. After 10 min of stirring at 50 °C, the clear liquid was left to equilibrate at room temperature. The additional step at 50 °C was implemented to avoid precipitation of the other components when water is added at room temperature. The $E_T(30)$ value was used to have an empirical scale of the solvent polarity.[18] This measurement is based on the high solvatochromic band shift of the 2,6-diphenyl-4(2,4,6-triphenyl-1-pyridinio)phenolate also known as Reichardt's betaine dye 30. The $E_T(30)$ is determined as the molar transition energy of the dye, typically expressed in kcal/mol at room temperature and standard pressure. This value is calculated using eq 1:

$$E_T = \frac{hcN_A}{\lambda_{max}} = \frac{28{,}591}{\lambda_{max}}$$

in which h is Planck's constant, c is the speed of light in a vacuum,

and N_A is Avogadro's constant.

Table 1 lists the candidate solvents tested. Solutions of betaine 30 at 0.1 mM were produced with each solvent. Equation 1 was used to calculate the $E_T(30)$ values. The λ_{max} was measured on a Shimadzu UV-1800 UV spectrophotometer (Shimadzu, Japan) and elaborated with UVProbe software, version 2.31.

2.3. SAMPLES AND SPIKING PROCEDURE.

Raw (unshelled, unsalted, and unroasted) pistachio samples were purchased from local markets in Belgium and Italy, and a total of 18 real samples were collected. For method optimization, a spiked sample was created. The kernels were preliminary washed with MeOH to remove residual contamination before the addition of the standard. 100 g of raw pistachios were weighed and immersed in 200 mL of MeOH. This mixture was stirred at room temperature for 30 min. After the washing step, pistachios were recollected and left to dry overnight on Petri dishes under a hood in the dark. Additionally, comparative analyses were conducted between samples that underwent the MeOH washing step and those that did not. Specifically, a portion of the sample (named Spain 2) was not subjected to the washing step. After extraction under optimized conditions, the chromatographic profiles of Spain 2 with and without MeOH washing were compared. The results showed no significant differences in the chromatographic profiles, indicating that the MeOH washing step did not affect the composition of the

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samples in a way that would influence the analytical determination of AFs. Moreover, a portion of the batch washed with MeOH was not spiked to perform a blank analysis, which confirmed the absence of AFs.

The day following the MeOH washing step, samples were completely immersed in 200 mL of CH_2Cl_2, in a round-bottom flask, and to this, a standard solution of AF was added to obtain the desired spiking samples at 1, 2, 4, 6, and 8 ng/g upon CH_2Cl_2 removal. The mixture was left to stir overnight in the dark at room temperature. CH_2Cl_2 was then removed with a rotary evaporator. Pistachios were left to stand overnight on Petri dishes, in the dark. The spiked pistachios were finely ground with a commercial coffee grinder to obtain a homogeneous powder. Spiked samples were stored at −18 °C before analysis.

2.4. AOAC OFFICIAL METHOD 991.31.

The method developed in this study was compared to the AOAC Official Method 991.31.[15] The latter was carried as follows; 25 g of the test portion was weighed in a blender jar, followed by the addition of 5 g NaCl and 125 mL of MeOH: H_2O (70:30) extraction solution. Extraction was performed by blending at high speed for 2 min. The extract was then filtered through filter paper, and 15 mL of the filtrate was then collected and diluted with 30 mL of water before another filtration through a glass microfiber filter. Subsequently, 15 mL of the diluted extract was quantitatively passed through an AflatestWB immunoaffinity column (IAC). The IAC was then washed with 10 mL of water twice, and the AF-enriched fraction was eluted with 1 mL of MeOH. This fraction was finally subjected to chromatographic separation and detection. The described official method requires a postcolumn derivatization (iodination) step to increase sensitivity. However, this step was not necessary due to the high sensitivity of the UHPLC instrument used, capable of detecting AFs at ppb (part per billion) levels.

2.5. DISPERSIVE LIQUID−LIQUID MICROEXTRACTION.

A dispersive liquid−liquid microextraction (DLLME) method was evaluated for its potential in preconcentrating and purifying AFs. This method was based on the work of Campone et al., with modifications to the solvent ratio.[19] Tests were conducted using 600 μL of MeOH and 400 μL of $CHCl_3$. To a 6 g aliquot of DES-5_w spiked at 4 ng/g for each of the four AFs, the solution containing both extracting and dispersing solvents was rapidly injected. The resulting cloudy dispersion was vortexed for 30 s and then centrifuged at 5000 rpm for 4 min. Subsequently, 50 μL of $CHCl_3$ was collected from the bottom of the test tube, dried under nitrogen, reconstituted with 200 μL of MeOH, and subjected to chromatographic separation. This DLLME approach was then applied to a spiked sample extract prepared with DES-5_w. Specifically, 2.5 g of spiked pistachios (4 ng/g for each AF) were combined with 12 g of DES-5_w in a 50 mL falcon tube and vortexed for 3 min. The suspension was centrifuged at 10,500 rpm for 5 min, and 6 g of DES were recovered. These 6 g underwent the previously described DLLME procedure. However, the precipitation of coextracted interferents after injection of the dispersion/extraction solution prevented the collection of the enriched $CHCl_3$ fraction from the bottom of the test tube, leading to the discarding of this approach.

2.6. SOLID-PHASE EXTRACTION: OPTIMIZATION.

Due to the unsuitability of the DLLME approach, a solid-phase extraction (SPE) method was developed and optimized. AFs' SPE extraction was optimized using a response surface methodology approach via a central composite inscribed (CCI) design with three variables ($k = 3$) as the design of experiment (DoE).[20] The center points for the CCI were chosen based on published works and official methods.[15,21,22] The variables considered were extraction time (2−10 min range), extraction temperature (20−40 °C range), and mass of DES (6−24 g range), the quantity of pistachios was kept fixed at 2.5 g. The model's analysis, the Pareto charts of the standardized effects, and the response surfaces were obtained using R v4.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

The optimized procedure involved grinding 25 g of raw pistachios into a homogeneous powder, from which 2.5 g were collected and placed in a round-bottom flask with 15 g of DES-5_w. The mixture was stirred in a thermostatic bath for 6 min at 30 °C. The resulting slurry mix was diluted 1:2 with water and filtered under vacuum using filter paper and a Buchner funnel.

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The filtered solution was then passed through a Discovery DSC-18 cartridge (bed width 1 g, volume 6 mL; Supelco, Germany) with the aid of an SPE vacuum manifold. The cartridge was conditioned with 4 mL of MeOH (80:20 v/v) followed by 4 mL of water. After conditioning, the entire volume of the filtered extract was loaded into the cartridge, followed by 25 mL of water at a rate of two drops per sec. The analytes were then eluted with 3 mL of MeOH. The SPE procedure was adapted from Rezaee et al.[23] The AFs-enriched methanolic extract was then filtered on a 0.2 μm nylon filter and injected into the UHPLC system.

2.7. UHPLC-FLD ANALYSIS.

A Shimadzu Nexera LC-40B x3 UHPLC instrument (Shimadzu, Japan), equipped with a SIL-40c x3 autosampler (Shimadzu, Japan) and an RF-20A xs fluorometric detector (FLD) (Shimadzu, Japan), was used for the AFs' separation and detection. Chromatographic separation was performed on an Ascentis Express C18 (100 mm L × 2.1 mm I.D., 2 μm particle size) column (Supelco, USA). The mobile phase consisted of water (phase A) and MeOH (phase B) set with the following gradient program: from 30% B to 60% B in 7.5 min, followed by re-equilibration at 30% B from 7.51 to 8.5 min. The flow rate was set at 0.45 mL/min; with 2 μL injection volume, and the column oven was set at 35 °C. To prevent carryover, the syringe needle was washed with MeOH before and after each injection. The FLD, equipped with two acquisition channels, was set as follows. Channel 1: excitation wavelength 365 nm, emission wavelength 430 nm; Channel 2: excitation wavelength 365 nm, emission wavelength 450 nm.

2.8. QUANTIFICATION AND METHOD VALIDATION.

Three sets of calibration curves were produced: an external standard, a matrixmatched, and a spiked procedural blank calibration curve, the latter to account for the matrix effect. Standard solutions, containing all four AFs, were prepared diluting stock solutions with appropriate volumes of MeOH. The external standard calibration curves were built using six levels of concentration from 0.5 to 20 ng/g, each analyzed in triplicate. Matrix-matched calibration curves were obtained spiking AFs-free pistachios to obtain five concentration levels: from 1 to 8 ng/ g (8 ng/g is the legal limit imposed by EU for AFB_1), four replicates for each level. The spiked samples underwent the entire optimized analytical workflow. The spiked procedural blank calibration curves were generated by adding known concentrations of the analytes (ranging from 1 to 8 ng/g, five concentration levels) to blank extracts that underwent the entire optimized analytical procedure.

A lack-of-fit test verified the calibration curves' linearity (Table S5). Recoveries and matrix effects were evaluated by comparing the slopes of the external standard, matrix-matched, and spiked procedural blank calibration curves. Quantification and validation were based on matrix-matched calibration curves, while recoveries were calculated using the procedural blank spiked calibration curve.

Trueness and repeatability were determined by analyzing spiked samples at 1, 2, 4, 6, and 8 ng/g in triplicate on 2 different days. Recovery was calculated using the mycotoxins' peak area and interpolating this with the calibration curve obtained through spiked procedural blank calibration. Limits of detection (LOD) and quantification (LOQ) were calculated as follows:

$$\mathrm{LOD} = 3 \times \frac{\sigma}{\sqrt{\mathrm{n}}}$$

$$\mathrm{LOQ} = 10 \times \frac{\sigma}{\sqrt{\mathrm{n}}}$$

where *n* is the number of replicates observations and σ is the estimated standard deviation of *n* single runs at a near-zero concentration.[24]

Statistical analyses were performed using Excel (Microsoft Office, version 16.75.2), and R v4.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

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3. Results and discussion

3.1. DES CHARACTERIZATION AND SELECTION.

The initial phase of this study involved identifying the most promising solvent for extracting AFs from pistachios. Given the moderately polar nature of AFs, conventional methods involve using MeOH: H_2O or ACN: H_2O mixtures for their extraction from pistachios and similar matrices.[7,25] The preliminary selection criteria were based on the polarity of DESs, referencing their $E_T(30)$ values (if available) in comparison to MeOH or ACN or their mixture, and their applications for mycotoxins' extraction regardless of the matrix. The list of the preliminary candidate DESs is reported in Table 1.

Among the candidates selected from the literature, further selection was made based on the following considerations:

1. DES-1 and DES-2 were semisolid and extremely viscous at room temperature, manipulation and any practical operation were difficult. Due to their high viscosity and low manageability, these solvents were excluded.
2. DES-3 has been used in the literature to extract AFs from rice, obtaining recoveries between 70 and 95%.[26] However, preliminary tests and literature review indicated that DESs based on choline chloride and carboxylic acids are unstable due to ester formation.[27] Consequently, DES-3 was excluded.
3. DES-4 was also used for extracting AFs from rice, exhibiting recoveries ranging from 78.93 to 113.64%.[26] However, preliminary tests revealed a decrease in chromatographic areas for AFB_1 and AFG_1 in AF solutions prepared with DES-4 and stored before chromatographic analysis. Solutions stored at 5 °C for 6 h before injection exhibited an 80% reduction of the AFB_1 and AFG_2 chromatographic areas compared to freshly prepared and immediately injected solutions. Solutions stored at −18 °C for 3 days resulted in a 50% decrease in chromatographic area (Figure S1). This output was attributed to potential degradation of the analytes and/or a significant interaction between the solvent and analytes, leading to signal suppression over time, thus lacking robustness (Figure S1). Consequently, DES-4 was also excluded from further investigations.
4. DES-5 results in a clear and slightly viscous liquid. However, the viscosity of DES-5 raised concerns about its suitability as an extraction medium. To reduce viscosity, 20% water in weight was added, following Piemontese et al.'s method for reducing DES-5 viscosity to extract mycotoxins from wheat.[28] The addition of 20% water resulted in a less viscous and more manageable solvent, although the $E_T(30)$ value increased to 58.8 kcal/mol. Despite the increased polarity, DES-5 with water (DES-5_w) was tested as an extraction medium.

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3.2. UHPLC TROUBLESHOOTING.

In several publications involving the use of DESs, the direct injection of the solvent itself (enriched with the extracted analytes) into HPLC, or the dilution of the DES extract followed by injection into the chromatographic system, is reported.[26,28−30] Since this procedure seemed quite common, the same approach was attempted in the early stage of this study. Unfortunately, this resulted in overpressure issues (pressure >1300 bar) and consequent system errors after a few analyses. These issues occurred with both DES-4 and DES-5_w, even when diluted 1:2 with water and filtered through a 0.2 μm nylon filter to create AF standard solutions for a calibration curve. Despite the dilution and reduction in viscosity, overpressure persisted. Specifically, after several injections of 20 ng/g AFs' standard solution prepared with DES-4, back-pressure increased, and a carryover of the four AFs was observed. To mitigate carryover, a sequential rinsing pre- and postinjection, as reported by Tamura et al., was implemented.[31] Although this addressed the carryover issue, high back pressure remained a problem.

The overpressure was attributed to the specific characteristics of the chromatographic system and column used, which were incompatible with the viscosity of the DESs. Notably, chromatographic analyses that involve the direct injection of DES are predominantly performed using HPLC systems rather than UHPLC systems. Additionally, columns used for DES injection typically have particle sizes ranging from >3.0 to 5 μm.[26,28−30] The column selected for this investigation had a smaller particle size of 2 μm. Such columns are known to enhance efficiency and resolution compared to their largerparticle counterparts, allowing for a much faster analysis while also mitigating back pressure relative to sub-2 μm columns. Therefore, the direct injection of DESs (or diluted DESs) was abandoned, and a preconcentration approach that could bring the analytes from the DES to an organic solvent suitable for the UHPLC system was investigated. Moreover, a purification step was required to eliminate interferences, improving the accuracy and the LOQ of the proposed method. It is noteworthy to report that operating in isocratic conditions, H_2O: MeOH 60:40 at a 0.45 mL/min flow rate, was possible to achieve the separation of the four AFs in less than 4 min when injecting standard solutions. However, the implemented extraction and purification step further discussed, extracted a series of polar interferents that eluted in the same chromatographic frame of the analytes. Implementing a gradient elution was therefore necessary to achieve proper resolutions of the four peaks of interest by shifting the elution of more polar compounds closer to the dead time, resulting in satisfactory separation of the AFs in less than 5 min (Figure S2).

3.3. PRECONCENTRATION APPROACHES.

Initially, a preconcentration and purification strategy employing DLLME was tested. This method injects microliter volumes of dispersing and extracting solvents into the target matrix, rapidly forming a cloudy suspension due to the immiscibility of the extracting solvent. The dispersing solvent acts as a bridging agent, facilitating analyte partitioning into small droplets of the extracting solvent. After vortexing and centrifugation, phase separation occurs, enabling the retrieval of the analyte-enriched

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extracting solvent. $CHCl_3$ and MeOH were used as the extracting and dispersing solvents, respectively, as per Campone et al.'s method for preconcentrating AFs from cereal product extracts.[19] In this study, a different solvent ratio was used. Tests were run using 600 μL of MeOH and 400 μL of $CHCl_3$. Despite achieving recoveries above 89% for all four AFs in preliminary tests, with spiked solutions of DES-5_w at 4 ng/g, the application of DLLME to pistachio extracts resulted in the precipitation of coextracted interferents, making the collection of the enriched fraction, from the bottom of the vial, challenging. For this reason, DLLME was discarded and SPE was evaluated as an alternative sample preparation technique. SPE proved to be a valid alternative since C18 SPE cartridges can strongly retain AFs and polar interferents can be eluted with water, recollecting AFs with a minimum amount of MeOH.

3.4. OPTIMIZATION OF THE EXTRACTION CONDITIONS USING THE SELECTED DES.

Following the optimization of the chromatographic method and preconcentration/purification steps, attention was directed toward optimizing the solid− liquid extraction using the selected DES-5_w. A response surface methodology was implemented using a CCI. The design revealed distinct extraction behaviors for the four AFs studied. All four models demonstrated sufficient explanatory power, with R-squared values ranging from 0.9023 (AFG_1) to 0.9605 (AFG_2), indicating that the models accounted for a large proportion of the variability in the extraction efficiencies.

All models were statistically significant ($p < 0.05$). The quantity of DES emerged as a consistently significant factor across all four models, followed by its quadratic term, indicating a nonlinear relationship between DES quantity and extraction efficiency. According to the Pareto Pareto chart (Figures S3−S6), extraction time and temperature showed less consistent effects across the four AFs. The lack-of-fit tests were nonsignificant for all models, supporting their adequacy in describing the experimental data ($p > 0.05$). The ANOVA results revealed that first-order terms were significant for AFB_1 and AFB_2, while pure quadratic terms were significant for AFG_1 and AFG_2, suggesting different dominant effects for B and G-type AFs. The stationary points identified by the models provide potential optimal extraction conditions for each AF, with DES quantity ranging from 15.23 to 16.97 g, temperatures from 30.08 to 32.90 °C, and extraction times from 4.77 to 6.43 min (Table 2).

Given that the most stringent legal limit is based on the presence of AFB_1, it was considered appropriate to utilize the optimial extraction conditions closer as possible to this AF. This decision was further supported by the observation that in natural samples, the presence of AFB_2, AFG_1, and AFG_2 is linked to the presence of AFB_1; the others are generally not found in the absence of AFB_1.[8] Therefore, the optimal conditions retained were 6 min, 15 g of DES, and 30 °C.

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Table 2. *Optimal Extraction Conditions Found for Each AF Running the DoE (k = 3)*[a]

optimal conditions DoE (k = 3)			
analyte	time (min)	DES (g)	temperature (°C)
AFB_1	6.28	15.23	30.17
AFB_2	4.77	16.97	31.14
AFG_1	5.14	15.39	30.08
AFG_2	6.43	16.59	32.9
chosen condition for the extraction of a sample containing the four AFs			
AFB_1; -B_2; -G_1; -G_2	6	15	30

[a]The conditions used as a compromise to maximize the extraction of AFB_1 are reported in the bottom part of the table.

3.5 METHOD VALIDATION AND ANALYSIS OF REAL-WORLD SAMPLES

The proposed method was validated following the Eurachem guidelines.[24] External calibration curves (6 concentration levels from 0.5 to 20 ng/g) were built for the four AFs, analyzing the same mixtures over 3 days and obtaining regression coefficients (R^2) above 0.999. Spiked procedural blank and matrix-matched calibration curves were obtained using five concentration levels, from 1.0 to 8.0 ng/g, over 3 days, obtaining regression coefficients above 0.995 and 0.998, respectively. The linearity of all calibration curves was assessed using lack-of-fit tests. All analytes showed a nonsignificant lackof-fit ($p > 0.05$), confirming the adequacy of the linear model.

More details about the lack-of-fit results are reported in Supporting Information, Table S5.

Matrix effects and recoveries were evaluated by comparing slopes of external, matrix-matched, and procedural blank spiked calibration curves using t tests. Significant differences ($p < 0.05$) were found between external and matrix-matched curves for all AFs. The differences can be due either to a loss of analytes during the sample preparation procedure, a matrix effect at the detector, or a combination of the two effects.

Matrix-matched and spiked procedural blank curves showed significant differences for AFB_1 and AFB_2 ($p < 0.05$) but not for AFG_1 and AFG_2 ($p > 0.05$). Consequently, quantification and validation were based on matrix-matched calibration curves, while recoveries were calculated using the procedural blank spiked calibration curve. Details regarding the matrix effect (%) calculated between external calibration and procedural blank spiked for each analyte are reported in Table S4.

Recoveries were calculated by comparing slopes of matrixmatched and procedural blank calibration curves at five concentration levels (1, 2, 4, 6, and 8 ng/g). The method demonstrated good recoveries: 86.8% for AFB_1, 83.0% for AFB_2, 99.1% for AFG_1, and 89.1% for AFG_2 (Table 3). Detailed recovery data for each spiked level are provided in Table S6.

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The method exhibited good precision, with intraday coefficients of variation (CV%) below 2.2% and interday CV % values below 3% for all AFs. LOD and LOQ were established at low ng/g levels. The LOD for AFB_1, AFB_2, AFG_1, and AFG_2 were 0.22, 0.02, 0.12, and 0.03 ng/g, respectively, and the LOQ values were 0.72, 0.05, 0.41, and 0.11 ng/g. Details are reported in Table 3 and described in the Material and Methods section. Method specificity was evaluated considering the signal ratio obtained in the two FLD channels (where different excitation and emission wavelengths were used). No significant differences were observed for these ratios in any of the calibration curves considered, confirming the specificity of the overall procedure.[32]

The validated method was applied to 18 real-world samples, with results compared to those obtained using the reference method AOAC 991. One sample showed detectable levels of AFB_1 and AFB_2, while the remaining 17 samples had AF levels below the LOD. The contaminated sample results are reported in Table 4. Quantitation of AFB_1 and AFB_2 showed no significant differences between the developed and the reference methods ($p > 0.05$), supporting the validity of the new approach.

3.6. GREEN ANALYTICAL CONSIDERATIONS.

The DES-based method proposed in this study allowed for a significant reduction in the volume of organic solvents needed and relative waste. Moreover, the sample size was reduced, and no derivatization step was required, leading to an overall safer procedure for the operator. The aspects have been evaluated compared to the official reference methods, i.e., AOAC Official Method 991.31. For comparing the greenness improvements, different metrics were used, i.e., the AGREEPrep metric and the White Analytical Chemistry (WAC)−RGB metric.[33−35] Using different metrics allowed a broader overview of the proposed method's impact on green and analytical performances compared to the reference method.

***Table 3**. Figures of Merit Are Calculated on the Matrix-Matched Calibration Curve*

	recovery% (mean ± SD)[a]	R^2	LOD (ng/g)	LOQ(ng/g)	repeatability (CV%)		A Ch1/A Chi (mean ± SD)
					intraday (n = 3)	interday (n = 6)	
AFB_1	86.8 ± 0.05	0.998	0.22	0.72	1.24	2.10	0.85 ± 0.05
AFB_2	83.0 ± 0.05	0.999	0.02	0.05	1.57	2.94	0.83 ± 0.06
AFG_1	99.1 ± 0.05	0.999	0.12	0.41	2.19	1.64	1.45 ± 0.12
AFG_2	89.1 ± 0.03	0.997	0.03	0.11	1.94	1.93	1.77 ± 0.09

[a]Recoveries are calculated at five concentration levels (1, 2, 4, 6, and 8 ng/g) on the spiked procedural blank calibration curve.

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Table 4. *Comparison of Results Obtained Applying AOAC Official Method 991.31 anti the Developed Method on Pistachios Contaminated by AFB_1, and AFB_2*

	AOAC official method 991.31 (n = 3)		developed method (n = 3)	
analyte	Concentration (ng/g) mean ± SD (C.V.%)	A_{Ch1}/A_{Ch2}(mean ± S.D.)	Concentration (ng/g) mean ± SD (C.V.%)	A_{Ch1}/A_{Ch2}(mean ± S.D.)
AFB_1	5.34 ± 0.20 (3.76)	0.83 ± 0.04	5.67 ± 0.27 (4.78)	0.85 ± 0.03
AFB_2	0.08 ± 0.01 (8.27)	0.84 ± 0.02	0.09 ± 0.01 (9.70)	0.78 ± 0.06

The WAC metric comprises 12 principles categorized into three groups: red (R) for analytical efficiency, green (G) for key aspects of green analytical chemistry, and blue (B) for practical and economic considerations. These principles, organized in three groups of four, form the basis of the RGB 12 algorithm. Each principle is rated on a scale of 0 to 100, with higher scores indicating better performance. Individual scores for the primary attributes (R(%), G(%), B(%)) are presented, and an overall whiteness score (%) is calculated as the average of the three groups. Since the user gives the scores, it is essential to provide objective and critical scores for accurate estimation.[35]

The methods' efficiency, practical aspects, and evaluation through green chemistry were assessed using the RGB algorithm. Comparable scores were assigned to both methods concerning the R1 principle (scope of application), given that both methods target and quantify the same analytes. Scores of 100 were assigned to both the reference and presented methods for the R2 principle (LOD and LOQ). For example, in the case of AFB_1, the reference method has a LOD of 0.15 ng/g and a LOQ of 0.52 ng/g,[36] whereas the proposed method exhibited values of 0.22 and 0.72 ng/g for LOD and LOQ, respectively. Regarding the R3 principle (precision), the reference method performed well for both intraday and interday precision. Consequently, both methods received a score of 100 since repeatability is satisfactory in both scenarios. Similar performances were observed for both methods concerning accuracy (R4 principle). Trueness, expressed as the average bias in percentage, showed bias% values of 2.7 (AFB_1), 3.4 (AFB_2), 5.3 (AFG_1), and 4.4 (AFG_2) for the developed method, and 2.9% (AFB_1), 2.3% (AFB_2), 2.2% (AFG_1), and 2.8 (AFG_2) for the reference method.[36] The R4 principle also evaluates recovery (%), and recoveries calculated for the developed method were 86.6% (AFB_1), 83.0% (AFB_2), 99.1% (AFG_1), and 89.1% (AFG_2). In comparison, the recoveries reported by the manufacturer of the IAC, using the AOAC 991.31 methods, were 103% (AFB_1), 100% (AFB_2), 95% (AFG_1), and 101% (AFG_2). Due to the similarities in the criteria assessed by the R4 principle (accuracy), both methods received a score of 100. A complete overview of the R principles comparison is reported in the Supporting Information (Table S1). Regarding the G principles (green chemistry), the proposed method demonstrated a fair decrease in organic solvent usage (6.2 mL of MeOH against the 101 mL used in the reference method), lowered waste production, and minimized exposure to hazardous chemicals for the operator (Table S2). Lastly, concerning the B principles (practical considerations), opting for SPE instead of IAC reduces the costs associated with each analysis. Additionally, using smaller sample sizes enhanced operational

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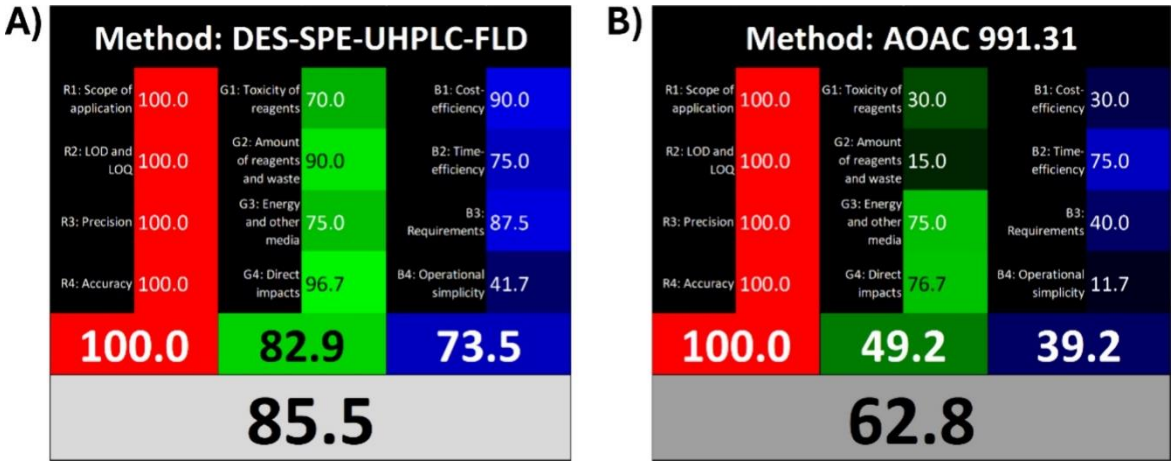
simplicity and reduced overall sample consumption (Table S3). The overall comparison of the two methods is reported in Figure 1.

Figure 1. *Comparison of (A) DES-SPE method and (B) AOAC 991.31 official method using the RGB algorithm*



The AGREEPrep metric was also employed to compare the two methods and provide a more comprehensive assessment of the impact of the sample preparation procedure alone.

The AGREEPrep metric evaluates the greenness of the sample preparation method itself, assigning a score for each of the ten criteria of the green sample preparation principles. The result is a pictogram that illustrates the final score given for the procedure. Each criterion has a default weight that can be adjusted according to the analytical goal providing the justification.[37] Default weights for each criterion were used, except for criteria 5 (sample size, amount of chemicals and materials) and 10 (safe procedures for the operator). Indeed, the primary goals were reducing operator exposure to toxic solvents and their overall volume reduction. The weights on these criteria were adapted for the following reasons:

1. Criterion 5: Weight 5 instead of 2 was used. The sample size was reduced by 1:10 compared to the official method, demonstrating comparable performances. The reduction in sample size led to a lower volume of extraction solvent and further reduced waste. For these reasons, weight 5 instead of 2 was set for this criterion.

2. Criterion 10: weight 5 instead of 3. The developed method employed only MeOH as a hazardous chemical. Notably, the toxicology of the used DES has already been evaluated and proved to be *practically nontoxic* for marine wildlife.[38] In contrast, the official method requires using nitric acid and potassium bromide for derivatization (in addition to MeOH for extraction),[15] elevating the operator's exposure to hazardous chemicals. Due to these considerations, greater significance was assigned to criterion 5, as adopting fewer and less toxic chemicals was a primary objective in the proposed method.

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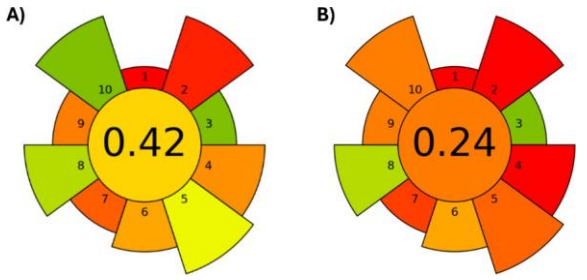
Reducing the amount of organic solvent was a key point of this study. Therefore, weight 5 for criterion 2 (use of safer solvents and reagents) was left as default. Despite the considerable reduction of organic solvent used in the overall procedure, criterion 2 assigned a score of 0.07 for the DESbased method (6.2 mL of MeOH) and 0 for the official one (101 mL MeOH). A score of 0.07 negatively affected the final output of the metric despite the noteworthy difference between the volume of MeOH between the two methods. The pictograms obtained for the two methods from the AGREEPrep tool are reported in Figure 2.

Figure 2. *Comparison of the greenness of (A) DES-SPE method and (B) AOAC 991.31 official method using the AGREEPrep tool.*



Despite a seemingly lower score obtained evaluating the proposed method using the AGREEPrep metric, the RGB algorithm confirms the overall efficiency and greenness of the DES-based method in the WAC evaluation. This demonstrates good analytical efficiency and the advantage of using a DES as a sustainable alternative to classic organic solvents, which minimizes MeOH consumption. The DES-based method offers a comprehensive, sustainable approach with reduced organic solvents, cost efficiency, and enhanced operator safety compared to the AOAC method.

The presented study has successfully introduced an ecofriendly method for extracting AFs using DES and water as alternatives to conventional organic solvents (e.g., MeOH, ACN, hexane) employed in official and routine methodologies. This DES/water mix solvent is environmentally friendly, as it is composed of biodegradable components, reduces potential health risks associated with exposure to harmful organic solvents, and contributes to safer laboratory practices. This approach eliminates the need for IAC for concentration and AF-halogen-derivatization to enhance fluorometric detection. This aligns with regulatory expectations, improving working conditions for researchers engaged in AFs extraction and detection. Method validation followed the Eurachem guidelines, proving effective, environmentally friendly, and costefficient, adhering to official guidelines for accuracy, precision, and reproducibility. Furthermore, a comparison between the developed method and the official AOAC method 991.31 was performed, demonstrating comparable results. The developed method not only fulfilled the requirements of the EU for the extraction and quantification of AFs in contaminated pistachios but also proved to be a greener alternative to standard methods in this field according to two relevant metrics.

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Associated content

SUPPORTING INFORMATION

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c05094.

Chromatograms, response surfaces, Pareto charts for each analyte, and parameters tables used for the WACRGB metric calculation (PDF)

Author information

NOTES

The authors declare no competing financial interest.

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