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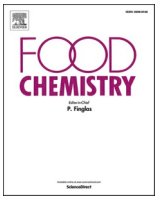

Development and validation of an analytical method for determination of citrinin in red rice and red yeast rice-based food supplements by ultra-high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Citrinin is a hepato-nephrotoxic mycotoxin produced by fungal species. The *Monascus purpureus* fungus plays a crucial role in the fermentation of red rice to produce red yeast rice-based food supplements, which represent the primary source of human exposure to citrinin. In this study, a simple and sensitive analytical method was successfully developed and validated for the citrinin determination in these products. The extraction process involved a QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) step and citrinin determination by ultra high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). The proposed method provided satisfactory linearity, percentage of recovery from 82 to 104% with relative standard deviations (RSD) lower than 14%, and limits of detection and quantification of 0.07 μg/Kg and 0.24 μg/kg, respectively. Among the 14 samples analyzed, citrinin was found in two red rice samples (0.24 and 0.46 μg/kg) and in six food supplements (from 0.44 to 87 μg/kg).

1. Introduction

Mycotoxins are toxic by-products generated by specific types of fungi. These fungi tend to infect crops, causing contamination either while the crops are still growing in the field or after harvest. Additionally, mycotoxins can contaminate human foods and animal feeds under favourable conditions, including appropriate levels of moisture, water activity, and temperature (Ojuri et al., 2018). According to data published by the Rapid Alert System for Food and Feed (RASFF, 2020), mycotoxins represent the category of toxic substances with the highest number of reported incidents.

Among mycotoxins, citrinin is generally produced after harvest by filamentous fungi belonging to the *Aspergillus, Penicillium*, and *Monascus* genera. It occurs mainly in stored grains but also in other products such as olives, apples, spices, fruit and vegetable juices, beer, cheese, infant formulae, and dry meat products such as fermented sausages. However, the primary source of human exposure to citrinin is red rice, widely used in Asia, and red yeast rice (RYR)-based food supplements (European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM), 2012), as it is produced through the fermentation of rice by the *Monascus Purpurea* fungus strain if conditions are not carefully monitored. These products can reduce blood-lipid levels in humans, such as lowering total cholesterol, low density lipoprotein (LDL), and triglycerides in the plasma of hyperlipidaemic patients, making them appropriate for the primary and secondary prevention of heart disease and other complications of atherosclerosis due to their main component, monacolin K (Nigović et al., 2013).

After ingestion, citrinin is rapidly absorbed and distributed in the body, primarily to the liver and kidneys. A recent toxicokinetic study in humans showed that 40% of ingested citrinin was excreted in the urine without metabolism (Degen et al., 2018). Researchers have detected the citrinin metabolite dihydrocitrinone (HO-CIT) in urine in multiple studies at concentrations ranging from 2 to 416 ng/L (Ali et al., 2015; Föllmann et al., 2014; Heyndrickx et al., 2015; Huybrechts et al., 2015; Vidal et al., 2018).

Regarding the toxic effects of citrinin, it interferes with the mitochondrial respiratory chain by promoting the generation of reactive oxygen species (ROS) and superoxide anions, inhibiting the flow of

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electrons at the level of the complex I (Flajs & Peraica, 2009). Additionally, these anions deactivate catalase and GSH-peroxidase, leading to an increase in hydrogen peroxide concentration within the cell, resulting in oxidative stress and the induction of cell apoptosis. This, in turn, leads to conditions such as nephropathy, hepatotoxicity, fetotoxicity, and renal adenomas (Ribeiro et al., 1997). Numerous studies have documented the correlation between citrinin exposure and cell apoptosis (Jiang et al., 2022). Furthermore, EFSA classified citrinin as a nephrotoxic substance in 2012 (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2012; López Sáncheza et al., 2017) and the International Agency for Research on Cancer (IARC) classified it as a Group III carcinogenic substance (IARC, 2023). Moreover, Li et al. (2023) published a study on the effects of citrinin in male rats, demonstrating that exposure to doses exceeding 2.5 mg/kg significantly reduced their testosterone levels by stimulating Leydig cell apoptosis.

Numerous international institutions, such as the European Commission and the World Health Organization (WHO), have recognized the potential health risks associated with citrinin exposure and have taken measures to address the issue by establishing regulatory limits. The European Commission set a maximum limit for citrinin in food supplements based on fermented red yeast *Monascus Purpurea* at 100 μg/kg (European Commission (EC), 2023a).

To ensure compliance with these food safety policies, it is imperative to employ suitable analytical techniques for citrinin determination. Various analytical methods can determine citrinin, including gas chromatography coupled with mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC) coupled with fluorescence (FD) and/or ultraviolet (UV) detection, or immunological tools such as ELISA (Atapattu & Poole, 2020; Silva et al., 2020). However, over the past decade, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has gained prominence for mycotoxin detection due to its ability to unequivocally identify substances and provide selective and sensitive detection (Kamle et al., 2022; Solfrizzo et al., 2009; Tangni et al., 2021).

In terms of sample preparation, researchers commonly extract citrinin from food using acetonitrile or methanol (Zhang et al., 2021). However, many methodologies include a purification step after extraction, mainly relying on liquid-liquid extraction (LLE) or solid-phase extraction (SPE) (Silva et al., 2020). Immunoaffinity columns (IAC) have gained importance lately because they offer extraordinary specificity for single toxins as a clean-up method (Alvito et al., 2010; Mair et al., 2021); however, they are expensive and easily affected by organic solvent, pH, ionic strength, and temperature. By contrast, researchers have utilized extraction procedures designated as “quick, easy, cheap, effective, rugged, and safe” (QuEChERS) (Anastassiades et al., 2003; Berthiller et al., 2017) due to their simplicity and minimal organic solvent requirements for the purification of complex samples. Nevertheless, organizations such as the Association of Official Agricultural Chemists (AOAC), the European Committee for Standardization (CEN), and the International Organization for Standardization (ISO) have not published any official methods for citrinin analysis in food (Bessaire et al., 2019).

This paper outlines the development and validation of a simple and sensitive methodology for analyzing citrinin in red rice and red yeast rice-based food supplements. The methodology uses QuEChERS-based extraction and ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS), offering a rapid and straightforward procedure. The determination of citrinin is carried out by external calibration using the isotopically labelled citrinin internal standard, citrinin-$^{13}C_{13}$. Validation follows the criteria set in Commission Implementing Regulation (EU) 2023/2782 of 14 December 2023, which lays down the methods of sampling and analysis for the official control of mycotoxins levels in foodstuffs and repeals Regulation (EC) No 401/2006 (European Commission (EC), 2023b), as well as the Monitoring Document for the determination of mycotoxins in food and feed by the General Directorate of Health and Food Safety (SANTE) (European Commission DG-SANTE, 2016).

2. Material and methods

2.1. Reagents and chemicals

Acetonitrile, methanol, and water, all MS-grade, were supplied by VWR International (Barcelona, Spain). Additionally, ammonium acetate (HPLC grade) and formic acid (MS-grade), from Panreac (Barcelona, Spain) were used.

EN 15662 QuEChERS extraction kits (4 g de $MgSO_4$, 1 g NaCl, 1 g $Na_3Citrate{\cdot}H_2O$, and 0.5 g $(Na_2Hcitrate)_2{\cdot}3H_2O$) and ceramic homogenizers for QuEChERS extraction were obtained from Agilent Technologies (Santa Clara, CA, USA). For sample preparation, QuEChERS d-SPE 15 mL tubes DisQuE™ (1200 mg $MgSO_4$, 400 mg PSA, 400 mg C_{18}, 400 mg GCB; 750 mg $MgSO_4$, 250 mg PSA, 150 mg C_{18}, 150 mg alumina; 1200 mg $MgSO_4$, 400 mg PSA; 1200 mg $MgSO_4$, 400 mg C_{18}) from Waters™ (Barcelona, Spain) and d-SPE 15 mL tubes (900 mg $MgSO_4$, 150 mg PSA, 150 mg C_{18}) from Teknokroma (Barcelona, Spain) were used.

2.2. Standards and solutions

Citrinin (CAS 518–75-2) and citrinin-$^{13}C_{13}$ (CAS 2802898–41-3) high-purity analytical standards (>99.9%) were purchased from Romer Labs Diagnostics GmbH (Getzersdorf, Austria) as solutions in acetonitrile at concentrations of 100.2 ± 1.2 mg/L and 10.17 ± 0.12 mg/L, respectively. Working intermediate solutions (1000, 10 and 0.1 μg/L of citrinin and 100 and 10 μg/L of citrinin-$^{13}C_{13}$) were prepared in 10 mL glass volumetric flasks by diluting these solutions in acetonitrile. Finally, calibration standard solutions containing 0.025 to 25 μg/L of citrinin and 1 μg/L of citrinin-$^{13}C_{13}$ were prepared in 10 mL glass volumetric flasks by diluting the individual intermediate solutions with acetonitrile/water (80/20, *V/V*). Stock, intermediate and calibration solutions were stored in the freezer at −20 °C. Working intermediate and calibration standard solutions were assigned expiration dates of six months from the date of preparation.

2.3. Instruments

An AB SCIEX™ ExionLC coupled with an AB SCIEX™ QTRAP® 6500+ (Foster City, CA, USA) equipped with an electrospray ionization source (ESI) was used for UHPLC-MS/MS analyses. Chromatographic separation was achieved on a Waters Acquity C_{18} column (50 × 2.1 mm, 1.7 μm) from Barcelona, Spain.

Additionally, an Advanced IR vortex mixer from VELP Scientifica (Usmate Velate, Italy), an Allegra X-15R centrifuge from Beckman Coulter (Indianapolis, IN, USA), an AGYTAX SR2 shaker from Agytax Lab (Madrid, Spain), and an Eppendorf™ 5424 microcentrifuge (Hamburg, Germany) were used.

2.4. Sampling, collection, and storage

The sampling procedure followed the guidelines outlined in Section L of the Annex I of Commission Implementing Regulation 2023/2782, which lays down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (European Commission (EC), 2023b). During the period from January to November 2023, two samples of white rice, four samples of red rice, and eight samples of RYR dietary supplements were collected. The samples were obtained from local stores in the province of Valencia (Spain), and trademarks were not disclosed for confidentiality reasons. The samples were stored at room temperature until analysis.

2.5. Sample preparation

The entire contents of the red rice samples were extensively ground using a laboratory mill M20-grinder and homogenized with an Ultra-

Turrax T-25, both from Ika Werke (Staufen, Germany). For the dietary supplements, all the tablets in a container were ground and homogenized. In the case of capsules, all the capsules in the package were completely removed from their shells, and the contents were homogenized.

The same extraction procedure was used for both red rice and RYR supplements. 5 ± 0.01 g of sample was weighted into a 50 mL polypropylene conical-bottom tube (Falcon™). Then, 20 mL of acetonitrile/water (80/20, *V/V*) with 0.1% formic acid and the contents of a QuEChERS extraction packet (4 g de $MgSO_4$, 1 g NaCl, 1 g Na_3Citrate·H_2O, and 0.5 g $(Na_2Hcitrate)_2{\cdot}3H_2O$), were added to the sample. The mixture was vigorously shaken manually for 20 s and stirred with a vortex mixer for 1 min, followed by an additional 15 min (amplitude 100 mm, speed 2 m/s and acceleration 50 m/s^2) in a vertical shaker (Agytax SR2) to reduce the agglomeration of salts and to ensure proper interaction between the sample and the extraction buffer. The sample tube was then centrifuged for 5 min at 7600 x*g*. Subsequently, 0.1 mL of the supernatant was mixed in a vial with 0.1 mL of a citrinin-$^{13}C_{13}$ working solution of 10 μg/L and 0.8 mL of acetonitrile/water (80/20, *V/V*) with 0.1% formic acid. The resulting solution was transferred to a 2 mL Eppendorf™ microtube with a 0.22 μm PTFE filter (Millipore, Bulington, MA, USA) and filtered by microcentrifugation for 2 min at 10700 x g before being placed in a 1.5 mL amber glass vial for subsequent UHPLC-MS/MS analysis.

2.6. UHPLC-MS/MS analysis

Ten microliters of the standards or sample extracts were injected into the UHPLC-MS/MS system. The column temperature was set at 40 °C, and the mobile phase flow rate was 300 μL/min. The mobile phase consisted of a mixture of 0.1% of formic acid and 5 mM ammonium acetate in water (A), and 0.1% of formic acid and 5 mM ammonium acetate in methanol (B). The gradient was as follows: 0 min 10% B, 4 min 90% B, 6 min 10% B, and 10 min 10% B.

For mass spectrometry, acquisition was performed using multiple reaction monitoring (MRM) and ESI was employed for the ionization of target analytes, operating in negative polarity mode. The optimized MS parameters in ESI- mode, set after tuning, were as follows: electrospray voltage −4500 V, source temperature 500 °C, nitrogen flow rate 35 mL/min, and collision gas flow rate 6 mL/min. Identification and quantification of citrinin and citrinin-$^{13}C_{13}$ by MRM were performed employing the transitions with mass-to-charge ratio (*m/z*) 280.8 > 248.9, 280.8 > 204.9 and 293.8 > 261.9, 293.8 > 217.9, respectively. According to the guidance document on identification of mycotoxins and plant toxins in food and feed (European Commission (EC), 2023c), the confirmation was based on three criteria: (i) No fewer than two product ions for each analyte: one for quantification and one for confirmation (see Table 1); (ii) the retention time (RT) of the citrinin in the unknown samples should coincide with the RT in the calibration standards with a maximum tolerance of ±0.1 min in the same batch (a difference of ±0.05 min in the case of the internal standard), and (iii) the relative ion ratio from both product ions in the samples should be within ±30% of the average of the calibration standards from the same working sequence.

Table 1
MS-MS parameters employed in the determination of citrinin and citrinin-$^{13}C_{13}$ (internal standard) in negative electrospray ionization.

Compound	Q1 (*m/z*)	Q3 (*m/z*)[a]	DP (V)	EP (V)	CE (V)	CXP (V)
Citrinin	280.8	**248.9**	25	10	22	11
		204.9	25	10	32	31
Citrinin-$^{13}C_{13}$ (IS)	293.8	**261.9**	40	10	36	21
		217.9	40	10	30	15

Abbreviations: CE, collision energy; CXP, collision cell exit potential; EP, entrance potential; DP, declustering potential; IS, internal standard; Q1, parent ion; Q3, product ion.

[a] In bold quantification ion.

2.7. Method validation

To validate the developed method, parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effect, recovery, and precision were evaluated to ensure compliance with the criteria established in European Commission Implementing Regulation 2023/2782 (European Commission (EC), 2023b). Linearity was assessed using internal standard calibration curves with concentration levels ranging from 0.025 to 25 μg/L citrinin, prepared in acetonitrile/water (80/20, *V/V*). Analysis of variance (ANOVA), *p*-value for the F-test, and R^2 value were utilized to assess the validity of the linear model. Additionally, goodness-of-fit coefficients (%) were calculated, taking into account the difference between nominal value of the calibration curve and the calculated concentration, which should be <20%. LOD and LOQ were determined based on the signal-to-noise ratio (S/N) of 3 and 10, respectively. Accuracy assessment was conducted through recovery studies, involving the analysis of three replicates of red rice and RYR supplements samples spiked at four different levels (1, 10, 100, and 1000 μg/kg citrinin) over three different days. The precision of the developed method was evaluated with spiked samples through intraday and interday studies ($n = 3$), reported as % relative standard deviation (RSD).

The analytical performance of the analytical method was also verified using reference test material from the Fapas® proficiency test 17,244, which contained red yeast rice containing citrinin at a concentration of 90.8 μg/kg.

3. Results and discussion

3.1. UHPLC-MS/MS method development

3.1.1. Selection of the ionization mode and optimization of MS/MS conditions

Researchers proposed electrospray ionization mode for the MS/MS determination of citrinin, as used in previous studies (Fernandez-Lopez et al., 2022; Meerpoel et al., 2018). ESI is widely employed because it is the most reliable and soft ionization technique for polar compounds, preserving the original structure of the precursor ion to a great extent. However, ionization efficiency (IE) in ESI depends highly on the solvent and ionization mode (Cech & Enke, 2001), and better IEs enable improved sensitivity and lower detection limits. Therefore, we evaluated positive (ESI+) and negative (ESI-) modes by directly infusing of a 100 μg/L citrinin standard solution into the mass spectrometer, scanning in the first quadrupole MS (Q1) to identify of the precursor ions. We identified the citrinin-methanol adduct $[M + MeOH\text{-}H]^-$ (280.8 *m/z*) and citrinin $[M + H]^+$ (251.2 *m/z*) as precursor ions for ESI- and ESI+ modes, respectively. To follow the identification criteria established in the guidance document on the identification of mycotoxins and plant toxins in food and feed (European Commission, 2023c) for MS/MS analysis, we chose the precursor ions and two fragments to produce two MRM transitions for qualitative and quantitative analysis of citrinin. After directly infusing of a 100 μg/L citrinin standard solution into the mass spectrometer and scanning in the third quadrupole MS (Q2), we selected the fragment *m/z* 248.9, corresponding to the elimination of methanol $[M\text{-}H]^-$, as quantification ion, and the fragment *m/z* 204.9, as qualifier ion, in negative mode. Similarly, we selected the fragment *m/z* 233.2, as quantification ion, and the fragment *m/z* 205.2 as qualifier ion, in positive mode. We selected equivalent ions for the determination of the internal standard. The SCIEX™ OS v. 2.0.0 software automatically optimized ion source settings such as declustering potential, entrance potential, collision energy, and collision cell exit potential to maximize the intensity of the fragments. Fig. 1 shows the chromatograms obtained from a 100 ng/L standard solution after injection in positive and

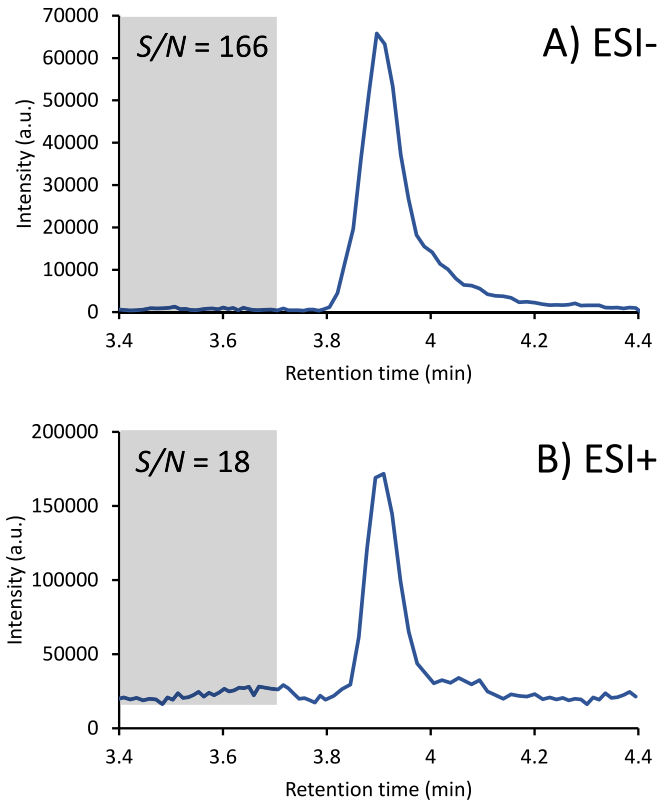

Fig. 1. UHPLC-MS chromatogram for citrinin in negative (A) and positive (B) electrospray modes.

negative ESI. Both ESI modes provided detectable signals for citrinin, with ESI- being the most sensitive, showing a significantly higher S/N (166) than the positive mode (S/N 18) in the measurement of a 100 μg/L citrinin standard. Thus, we selected ESI- mode for MS/MS acquisitions. Table 1 shows the detector settings employed for the determination of citrinin and citrinin-$^{13}C_{13}$ in ESI- mode.

3.1.2. Selection of the UHPLC parameters

3.1.2.1. Mobile phase. Researchers typically carry out chromatography separation of citrinin using reverse-phase partition chromatography, employing methanol and acetonitrile as organic solvents in combination with an aqueous buffered solution to improve analyte separation and minimize the matrix effect. We used a Waters™ ACQUITY UPLC BEH C18 column for the separation of citrinin with a gradient of mobile phases made of methanol and deionized water. Moreover, we added 0.1% of formic acid and 5 mM ammonium acetate as a buffer to both organic and aqueous mobile phases to control the ionization of the analytes and achieve reproducible chromatography (Atapattu & Poole, 2020; Freitas-Silva et al., 2011). The obtained retention time for citrinin, using the proposed gradient conditions, was 3.83 ± 0.03 min. Additionally, we evaluated the signal intensity of citrinin using acetonitrile and methanol as organic solvents in the mobile phase. The response, intensity, and peak shape of citrinin were better when using methanol in the mobile phase compared to acetonitrile, and it provided a more stable baseline. Thus, we selected methanol as solvent in the mobile phase.

3.1.2.2. Selection of the solvent for the preparation of the calibration standards. We prepared two calibration curves, ranging from 0.025 to 25 μg/L citrinin, in acetonitrile/water (80/20, *V/V*) and methanol/water (80/20, *V/V*), both with 0.1% formic acid. Using a mobile phase consisting of a mixture of 0.1% of formic acid and 5 mM ammonium acetate in water (A) and 0.1% of formic acid and 5 mM ammonium acetate in methanol (B) to favor the formation of the citrinin-methanol adduct, we found that the slope for the calibration curve prepared in acetonitrile was three times higher than that obtained from methanol standards. Additionally, the methanol calibration curve lost linearity at citrinin concentration around 10 μg/L. Therefore, we proposed acetonitrile/water (80/20, *V/V*) with 0.1% formic acid as solvent for the preparation of calibration standards.

3.1.3. Citrinin extraction

The extraction of citrinin from target the matrix depends on various factors, including sampling, sample amount, extraction solvent, and the use of partitioning salts.

3.1.3.1. Sample amount. Determining mycotoxins in food samples is complex due to the heterogeneous distribution of mycotoxins within the sample. Literature studies typically use sample aliquots ranging from 0.03 to 20 g (see Table 2). Thus, we proposed using 5 g of red rice or RYR, striking a balance between sample representativity and reagent consumption.

3.1.3.2. Selection of the extraction solvent. Researchers typically use methanol/water (from 70/30 to 100/0 *V/V*) as the main solvent for extracting citrinin from red rice samples, although other organic solvents like acetonitrile, ethanol, acetone, and ethyl acetate are also proposed (see Table 2). We selected acetonitrile/water (80/20, *V/V*) with 0.1% formic acid as extraction solvent due to its high performance in the UHPLC-MS/MS measurements of citrinin. Moreover, this solvent is commonly used in the extraction of mycotoxins in multianalyte determinations in food samples (Agriopoulou et al., 2020) and is also recommended in the EN 15662:2018 multiresidue QuEChERS-based methodology (European Standards, 2018).

3.1.3.3. Evaluation of the matrix effect. During the development, validation, and regular application of an LC-MS/MS method, addressing the matrix effect is crucial. The matrix effect refers to changes in ionization efficiency caused by simultaneous presence of other substances during analysis. Although these effects may not be visibly apparent in the chromatogram, they can significantly impair the accuracy and sensitivity of the analytical method. We spiked five grams of red rice and a RYR supplement sample with 10, 100, and 1000 μg/Kg citrinin, extracting them using 20 mL acetonitrile/water (80/20, *V/V*) with 0.1% formic acid, without using partition or clean-up salts, and shaking for 15 min. The obtained standard addition curves were much lower than the calibration curve prepared in acetonitrile/water (80/20, *V/V*) with 0.1% formic acid, indicating a very high signal suppression. The acceptance criteria of matrix effect, calculated as explained in **Eq. 1**, is set in the 80–120% range. The obtained signals were almost negligible, with matrix effects values of 14 and 5.4%, for red rice and RYR supplement samples, respectively. This result evidenced that the extraction procedure must be modified to avoid the very high signal suppression due to matrix effect.

$$ME(\%) = \frac{Slope_{standard\ addition}}{Slope_{external\ calibration}} 100 \quad (1)$$

3.1.3.4. Selection of the calibration curve. To compensate the matrix effect, we evaluated the use of internal standard calibration with the ^{13}C-labelled citrinin isotope ($^{13}C_{13}$-citrinin). We proposed a 1:10 dilution step by adding 100 μL of internal standard (10 μg/L) and 800 μL acetonitrile/water (80/20, *V/V*) with 0.1% formic acid to 100 μL the sample extract. The internal standard adequately compensated for the suppression effect observed in red rice spiked extracts, providing quantitative recoveries from 95 to 110%. However, the matrix effect of RYR supplement samples (48%) was not compensated by the internal standard due to their complex composition, indicating a need to modify the sample treatment.

Table 2
Analytical properties of selected methodologies for the determination of citrinin in red rice and RYR supplement samples.

Sample amount (g)	Extraction	Purification	Determination	LOD (μg/kg)	Recovery (%)	References
1	Ethanol/water (75/25 *V/V*), shaking 30 min	–	HPLC-FD	–	–	Wu et al., 2011
1	Methanol/water (70/25 *V/V*)	SPE	HPLC-FD LC-(ESI+)MS/MS	0.8	–	Li et al., 2012
1	Methanol/water (80/20 *V/V*), vortex 3 min, UAE 60 min	–	MECK-DAD	0.3	98–103	Nigović et al., 2013
1	Ethanol/water (70/30 *V/V*), UAE 10 min	–	HPLC-FD	2	95–101	Wang et al., 2014
1	Methanol, vortex 3 min, UAE 30 min	–	HPLC-FD	1	75–103	Ji et al., 2015
20	Methanol/water (75/25 *V/V*), blender 2 min	IAC-SPE	LC-FD	10	80–110	Marley et al., 2016
4	Ethyl acetate/acetonitrile/acetic acid (75/24/1 *V/V*), shaking 1 h, partition with $MgSO_4$/NaCl	–	UHPLC-MS/MS	0.1	64–86* 87–102**	Kiebooms et al., 2016
0.03	Acetone/water (60/40 *V/V*), vortex 30 s		HPTLC-UV/FD	4000	101–120	Klingelhöfer & Morlock, 2019
10	Methanol/water (70/25 *V/V*), stirring 3 min, UAE 20 min	IAC-SPE	HPLC-FD	1	75–80	Gu et al., 2019
0.5	Methanol/water (70/25 *V/V*), UAE 30 min	MIP-SPE	HPLC-DAD	75	76–91	Lhotská et al., 2019
0.5	Acetonitrile/water/acetic acid (79/20/1, *V/V/V*) UAE 30 min	SPE	LC-(ESI+)MS/MS	–	91–111	Vuković et al., 2019
2.5	Methanol/water (70/30 *V/V*), NaCl, shaking 30 min	–	Immunosensor	0.6	89–110	Jiang et al., 2020
10	Methanol/water (70:30 *V/V*)	IAC-SPE	HPLC-FD	0.2	85–90	Twarużek et al., 2021
2.5	Methanol/water (70/30 *V/V*), NaCl, shaking 30 min	–	Immunosensor	0.1	83–90	Zong et al., 2021
5	QuEChERS (acetonitrile), vortex 1 min, shaking 15 min	–	UHPLC-(ESI-)MS/MS	0.07	82–104	Our method

*White rice; **Red yeast fermented rice-based food supplements; Abbreviation: ESI, electrospray ionization; DAD, diode array detector; FD, fluorescence detector; HPLC, high pressure liquid chromatography; HPTLC, high-performance thin-layer chromatography; IAC, immunoaffinity column; LOD, limit of detection; MEKC, micellar electrokinetic chromatography; MIP, molecularly imprinted polymer; MS-MS, tandem mass spectrometry; SPE, solid-phase extraction; UAE, ultrasound-assisted extraction; UHPLC, ultra-high pressure liquid chromatography; UV, ultraviolet.

3.1.3.5. Selection of the extraction procedure. To address the matrix effect, we need to modify the extraction procedure to prevent significant signal suppression. Immunoaffinity columns have extensively cleaned up extracts for mycotoxins determination, including citrinin (Gu et al., 2019; Marley et al., 2016; Twarużek et al., 2021). Employing SPE with immunoaffinity columns selectively extracts citrinin, suitable for HPLC-FD determinations with moderate selectivity, but it is costly for routine analysis. Modern highly selective instruments, such as MS/MS detectors, now enable generic sample treatments based on QuEChERS methodologies. Thus, we proposed employing an EN 15662 QuEChERS methodology to extract and clean citrinin from the evaluated matrices due to its high efficacy, versatility, and ease of use (European Standards, 2018). Furthermore, QuEChERS-based methodology has been used in previous literature to extract citrinin from red rice and RYR supplements (Kiebooms et al., 2016). We spiked 5 g of RYR with 100 and 1000 μg/Kg citrinin and extracted with 20 mL acetonitrile/water (80/20, *V/V*) with 0.1% formic acid and a QuEChERS extraction kit, containing 4 g $MgSO_4$ anhydrous, 1 g NaCl, 1 g trisodium citrate dihydrate, and 0.5 g of disodium hydrogen citrate sesquihydrate. Extraction involved shaking with a vortex for 1 min followed by a vertical shaker for 15 min. After centrifugation, filtration, and 10-fold dilution with the internal standard solution, we obtained quantitative recoveries ranging from 97 to 101%, indicating that buffered acetonitrile provided a clean extract, effectively mitigating any matrix effects.

Additionally, we evaluated the use of dispersive solid-phase extraction with conventional sorbents ($MgSO_4$, PSA, C_{18}, Al_2O_3, and GCB) to avoid compromising the instrument and to assess further enrichment factors. We treated the previous extract with Waters DisQuE™ and Teknokroma dispersive kits, containing PSA (1200 mg $MgSO_4$ + 400 mg PSA), C_{18} (1200 mg $MgSO_4$ + 400 mg C_{18}), PSA + C_{18} (900 mg $MgSO_4$ + 150 mg PSA + 150 mg C_{18}), PSA + C_{18} + Al_2O_3 (750 mg $MgSO_4$ + 250 mg PSA + 150 mg C_{18} + 25 mg Al_2O_3), and PSA + C_{18} + GCB (1200 mg $MgSO_4$ + 400 mg PSA + 400 mg C_{18} + 400 mg graphitized carbon black), followed by centrifugation and filtering. The use of these conventional dispersive solid sorbents provided significantly reduced citrinin recoveries, primarily due to PSA's retention of citrinin via its carboxylic acid moiety and C_{18} non-polar to its non-polar section (González-Jartín et al., 2019). Fig. 2 illustrates the recovery values obtained for PSA and C_{18} sorbents, at 34% and 31%, respectively. Combining PSA and C_{18} sorbents yielded recoveries lower than 10%, even lower when using other sorbents likes GCB and Al_2O_3, suggesting that all evaluated solid sorbents retained citrinin from the acetonitrile phase. Consequently, no dispersive solid-phase extraction is recommended for extracting citrinin from red rice and RYR supplements. Proposed QuEChERS methodology effectively removes the matrix effect from red rice and RYR supplements. However, we propose using an

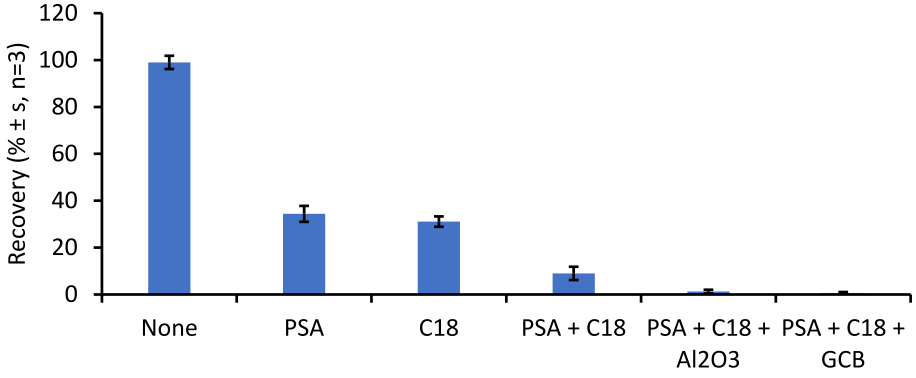

Fig. 2. Evaluation of QuEChERS dispersive sorbents in the clean-up of RYR supplement (sample 10) spiked with 100 and 1000 μg/Kg citrinin.

internal standard for quality control to ensure accurate citrinin determination by UHPLC-MS/MS and to detect any anomalies in sample analysis.

3.1.4. Validation of the procedure

Method validation is essential whenever creating a new method for routine analysis. The primary objective of validation is to verify that the developed method operates according to its intended purpose and consistently produces reliable and reproducible results. We evaluated the analytical performance of the developed method for the sensitive determination of citrinin in red rice and RYR supplement samples by UHPLC-MS/MS using the following parameters. We evaluated the linearity of three calibration curves from 0.025 to 25 μg/L ($n = 10$) on three different days, using 1/x weighting internal standard calibration, providing a coefficient of determination (R^2) of 0.9992 and goodness-of-fit coefficients (%) lower than 7% (see calibration curve in **Figure SM1** of the supplementary material file). The p-value for the F-test <0.05, and the results from the ANOVA test confirmed model's linearity. Instrumental LOD and LOQ were 0.0018 and 0.006 μg/L, respectively. Considering the 40-fold dilution factor of sample treatment, the proposed methodology provided a LOD of 0.07 μg/Kg and a LOQ of 0.24 μg/Kg. This methodology provides a very highly sensitive method for citrinin determination with a LOD value lower than those obtained in the literature (see Table 2).

We assessed method accuracy and precision by analyzing the recovery and RSD values obtained in the analysis of citrinin-spiked samples. Table 3 shows the recoveries and precision of red rice (sample 4) and RYR supplement (sample 8) spiked with citrinin at 1, 10, 100, and 1000 μg/Kg concentration levels. Recoveries were quantitative in all cases, ranging from 82 to 92% for red rice and from 90 to 104% for RYR supplements. The relationship between spiked and found concentrations was linear, with regression curves of $y = (-3 \pm 7) + (0.923 \pm 0.015)$ x ($R^2 = 0.99990$, F-test <0.05) for red rice and $y = (-1 \pm 3) + (0.941 \pm 0.006)$ x ($R^2 = 0.99998$, F-test <0.05) for RYR supplements. The obtained slope values were close to 1 and, although their confidence intervals did not contain 1.0, they were considered acceptable because they provided values in the 80–120% range. An adequate precision was obtained, with RSD values from 2.2 to 15.6%. We evaluated intraday ($n = 3$) and interday (n = 3) repeatability was evaluated as the RSD of the determination of spiked samples at 100 μg/Kg, obtaining values of 6.5 and 8.6% for red rice and of 2.4 and 12.5% for RYR supplement, respectively. We calculated the matrix effect as **Eq. 1** for the proposed method, providing adequate values of 92 and 94% for red rice and RYR supplements determination, respectively. Therefore, the developed procedure is a very sensitive, accurate, and precise method for determining citrinin in red rice-based products and fulfills the criteria established in the Commission Implementing Regulation (EU) 2023/2782 (European Commission (EC), 2023b). Fig. 3 shows the UHPLC-MS/MS chromatogram obtained for a red rice sample (sample 5) and RYR supplement (sample 7) extracted by the proposed methodology. **Figure SM2** of the supplementary material file shows additional UHPLC-MS/MS chromatograms obtained for other analyzed samples. As shown, the proposed methodology provided very clean extracts with interfering peaks around the retention time, allowing for the sensitive and selective determination of citrinin at trace levels.

Table 3
Accuracy and precision obtained in the analysis of citrinin in red rice and red yeast rice (RYR) supplement spiked samples.

[Spiked] (μg/kg)	Recovery (% ± s)	
	Red rice (n = 3)	RYR supplement (n = 3)
1	83 ± 13	104 ± 8
10	88 ± 6	96 ± 14
100	82 ± 7	90 ± 2
1000	92 ± 5	94 ± 10
Intraday precision (%RSD, n = 3)	6.5	2.4
Interday precision (%RSD, n = 3)	8.6	12.5

s: standard deviation.

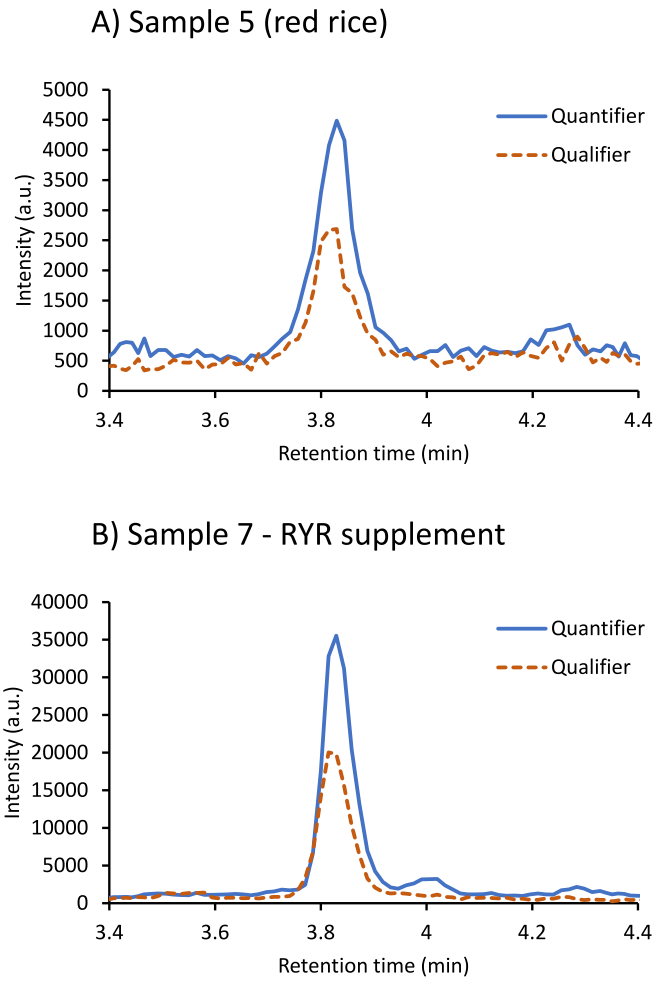

Fig. 3. UHPLC-MS/MS chromatogram obtained for red rice sample 5 (0.24 μg/Kg citrinin) and RYR supplement sample 7 (1.0 μg/Kg citrinin). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The outcome from analyzing the reference test material from the proficiency test 17,244 (red rice) was 89 μg/kg, indicating a satisfactory z-score of −0.1. **Figure SM3** of the supplementary material file shows the UHPLC-MS/MS chromatogram of the employed reference material.

4. Citrinin determination in commercial samples

We evaluated the performance of the developed method was by analyzing field samples obtained from local stores. We analyzed a total of 14 commercial samples (2 white rice, 4 red rice, and 8 RYR supplements) to measure their citrinin content (see Table 4). As expected, we did not detect citrinin in the white rice samples. However, we found citrinin in two red rice samples, with concentrations of 0.24 and 0.46 μg/Kg for samples 5 and 6, respectively. On the other hand, we detected citrinin in six of the RYR supplement samples, with concentrations ranging from 0.44 to 87 μg/kg, all below the maximum limits of 100 μg/kg established by the European Commission (European Commission (EC), 2023a). In any case, we can consider the citrinin content in the analyzed samples low, especially compared to the high concentrations found in samples analyzed in countries with less stringent legislation, which range from 230 to 20,650 μg/Kg ($n = 50$) (Samsudin & Abdullah, 2013) and from 16 to 5253 μg/Kg ($n = 59$) (Li et al., 2012). This

Table 4
Citrinin concentration found in white rice, red rice, and red yeast rice (RYR) supplement samples.

Sample	Type	[Citrinin] (μg/kg)
1	White rice	< LOQ [a]
2	White rice	< LOQ [a]
3	Red rice	< LOQ [a]
4	Red rice	<LOQ [a]
5	Red rice	0.24 ± 0.04
6	Red rice	0.46 ± 0.06
7	RYR supplement	1.02 ± 0.12
8	RYR supplement with black garlic bulb	0.44 ± 0.06
9	RYR supplement with coenzyme Q_{10}	0.76 ± 0.08
10	RYR supplement with coenzyme Q_{10}	87.01 ± 2.03
11	Guggul resin supplement with RYR	<LOQ [a]
12	Red vine supplement with RYR	<LOQ [a]
13	RYR supplement with coenzyme Q_{10}	79.36 ± 0.08
14	Yeast supplement with RYR	2.04 ± 0.08

[a] Citrinin concentration lower than limit of quantification (0.24 μg/Kg).

comparison demonstrates the strength of the food system in Europe.

5. Strengths and limitations of the developed methodology

The overview of the analytical properties of selected methodologies for determining citrinin in red rice and RYR supplement samples is shown in Table 2. The present method offers a quick, simple, sensitive, accurate, and precise approach for determining citrinin in red rice-based products, validated according to the current criteria established in Commission Implementing Regulation (EU) 2023/2782 (European Commission (EC), 2023a) and the guidance document on the identification of mycotoxins and plant toxins in food and feed (European Commission (EC), 2023b). As observed in Table 2, the proposed methodology offers the lowest LOD reported to date, set at 0.07 compared to 0.1 μg/kg reported by Kiebooms et al. (2016) using the same analytical technique, but without the use of complex solvent mixtures in the extraction step. Furthermore, it eliminates the need for immunoaffinity and molecularly imprinted polymer SPE sorbents, as reported by Marley et al. (2016), Gu et al. (2019), Lhotská et al. (2019), and Twarużek et al. (2021). Additionally, the rapid sample processing allows for higher sample throughput with an extraction time of <30 min per sample, compared to over 1 h reported by Kiebooms et al. (2016) and Gu et al. (2019). Moreover, the use of MS/MS enables unequivocal identification of citrinin.

The proposed procedure has been developed for use in the official control of citrinin in red rice and RYR supplements, with a maximum limit of 100 μg/kg established by European Commission (European Commission (EC), 2023a). However, the QuEChERS-based extraction method is a generic procedure that may be applied to different food matrices and even the multiresidue determination of mycotoxins. Therefore, the applicability of the proposed methodology to the determination of citrinin in various food samples will be evaluated in future validation studies.

6. Conclusions

This study presents the development and validation of a simple and sensitive analytical method for determining citrinin in red rice and food supplements based on red yeast rice, offering a rapid and straightforward procedure. The experimental procedure involves QuEChERS extraction followed by UHPLC-MS/MS determination of citrinin, with no need for using immunoaffinity sorbents, thus reducing both time and cost associated with the analytical procedure. The proposed method was validated in terms of linearity, precision, and accuracy, complying with the performance criteria established by the European Commission for the analysis of citrinin in food samples. Therefore, it is a useful methodology for routine use in official control purposes. The achieved LOD and LOQ of this methodology are considerably lower than those obtained in the literature.

CRediT authorship contribution statement

Paula Ponz-Perelló: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Francesc A. Esteve-Turrillas:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Miguel Ángel Cortés:** Validation, Supervision, Methodology, Investigation, Conceptualization. **Julia Herranz:** Validation, Resources, Formal analysis. **Olga Pardo:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.139941.

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