Heliyon 7 (2021) e06663

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Development of a novel UHPLC-MS/MS method for the determination of ochratoxin A in tea

Mariel Cina [a,b], María del Valle Ponce [a,b], Luis Dante Martinez [a,b,1], Soledad Cerutti [a,b,*]

[a] Instituto de Química de San Luis (CCT-San Luis), Facultad de Química Bioquímica y Farmacia, Universidad Nacional de San Luis, Laboratorio de Espectrometría de Masas, Bloque III, Ejército de los Andes 950, San Luis, CP5700, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, C1033AAJ, Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Ochratoxin A
Tea
DLLME-SFO
UHPLC-MS/MS
Green certificate

ABSTRACT

The mycotoxin Ochratoxin A (OTA) is responsible for producing many effects on human and animal health. In this work, the evaluation of the presence of OTA in tea beverage samples consisted of extraction and preconcentration through the solidification of a floating organic drop (DLLME-SFO) combined with an additional octadecyl silane clean-up step. The obtained extract was analyzed by UHPLC-MS/MS. Interferences from the matrix were effectively reduced and, consequently, recovery increased from 43.18% ± 4.1%–96.02% ± 2.54%. The validation assays were carried out by external calibration and spiked samples, with satisfactory recoveries. An adequate dynamic calibration range was obtained over a concentration interval between 0.5 and 70 μg mL^{-1} OTA. Capabilities of detection and quantification were 0.5 and 1.4 μg mL^{-1}. The obtained Green Certificate was compared with other techniques to establish the greenness profile of the procedure. Quantification of ochratoxin A levels in tea samples was performed.

1. Introduction

Tea is a traditional beverage, widely consumed (Toman et al., 2018). In 2013, more than 4.8 million tons of tea (black and green) were consumed, and are estimated (considering a 5% annual increase) that by 2023 more than 7 million tons will be produced (Chang, 2015). In its composition, tea contains several families of compounds associated to promote wellbeing (Rothenberg et al., 2018; Sedova et al., 2018). Also, numerous chemical contaminants and toxins can be found in tea (Cladière et al., 2018; Chen et al., 2019).

Filamentous fungi, such as *Aspergillus ssp*., *Penicillium ssp*., and *Fusarium spp*, deliver mycotoxins. Contamination with these compounds can occur at pre-and post-harvest operations, particularly when some fungi, such as *Aspergillus and Penicillium*, are capable of growth under low water activity conditions ($a_w \leq 0.85$) (Cladière et al., 2018). Through food/-feed, mycotoxins can cause a variety of adverse toxicological effects in human and animal health (Bryła et al., 2016). Aflatoxins, ochratoxin A, and fumonisins have been detected in different teas (black, red, green) (Sedova et al., 2018). Among them, ochratoxin A (OTA) is a deleterious toxicant, which is produced by diverse species of fungi (Pallarés, Font, Mañes, & Ferrer, 2017). Also, it is considered as possibly carcinogenic to humans (Wyker, 1993), with nephrotoxic, immunosuppressive, teratogenic, embryotoxic, genotoxic, and mutagenic effects (Malir et al., 2016). For these reasons, it has been necessary to establish maximum permitted limits of contaminants in food and feedstuffs. The European Commission Regulation (EC) 1881/2006 (Commission, 2006) has established accepted levels of OTA in between 5 ng mL^{-1} and 10 ng mL^{-1} in several coffee-based products. Although there is no European regulation for any mycotoxin content in tea (Jia et al., 2019), some countries such as Armenia, Belarus, Kazakhstan, Kyrgyzstan, and Russia have set a limit of 5 ug/kg for the aflatoxin B1 in unprocessed tea. In Argentina, an aflatoxin B1 threshold of 5 ug/kg has also been considered and, a total aflatoxin content of 20 ug/kg is tolerable in herbal tea infusions. However, for Asian countries limits are set only for aflatoxins in a group called "all foods", for example, in India and Japan the limits are 30 ug/kg and 10 ug/kg for aflatoxin B1, respectively, and levels between 5 and 20 ug/kg in China, depending on the kind of food (Sedova et al., 2018).

Regarding OTA determination and quantification, the tea infusion is considered a real complex, but interesting, food matrix to be analyzed, the presence of concomitant compounds, might interfere during analysis (Cladière et al., 2018). Therefore, the development of an analytical procedure for OTA analysis represents a challenge in analytical chemistry

* Corresponding author.
E-mail address: ecerutti@gmail.com (S. Cerutti).
[1] Deceased author.

https://doi.org/10.1016/j.heliyon.2021.e06663
Received 17 September 2020; Received in revised form 16 November 2020; Accepted 29 March 2021
2405-8440/© 2021 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

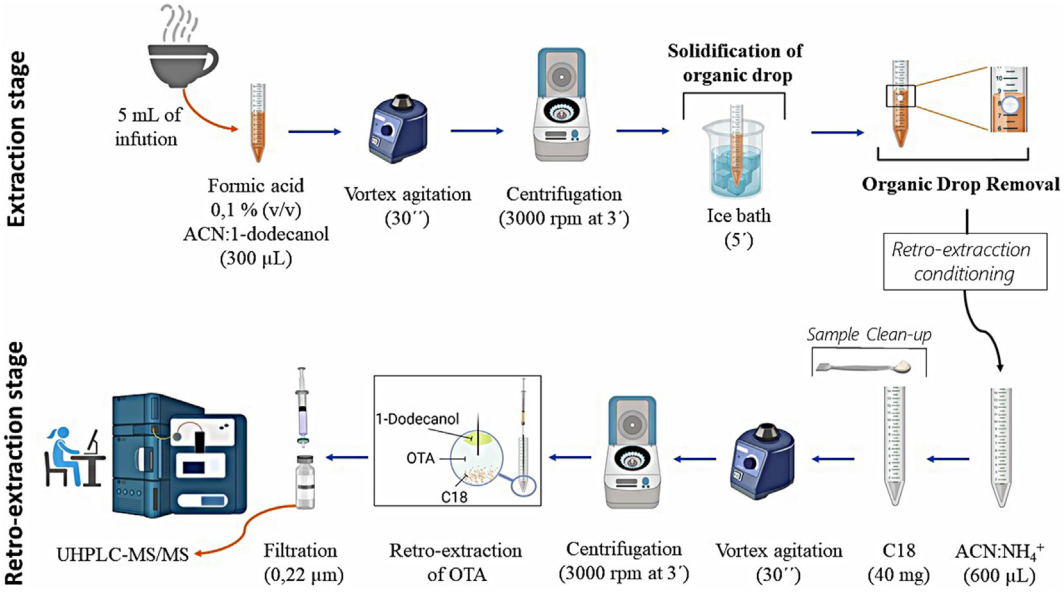

Figure 1. Determination of OTA in tea samples. Experimental DLLME-SFO procedure followed by an OTA retro-extraction step and UHPLC-MS/MS analysis.

because an effective extraction and clean-up method must be performed. Moreover, the strong matrix effect encountered in tea samples induces a signal suppression/enhancement caused by the different fragmentation of the OTA ion when it is analyzed in matrix-matched solvent compared to OTA in pure solvent (Dzuman et al., 2015; Sedova et al., 2018).

Most referenced articles have reported the presence of OTA in tea in both solid and liquid states (Malir et al., 2014; Pallarés, Font, Mañes, & Ferrer, 2017; Sedova et al., 2018; Toman et al., 2018). Several methodologies for mycotoxins extraction were used, e.g. solid phase extraction (SPE) (Duarte et al., 2020), liquid-liquid extraction (LLE) (Cladière et al., 2018), dispersive liquid-liquid extraction (DLLE) (Hacıbekiroğlu and Kolak, 2013; Pallarés, Font, Mañes, & Ferrer, 2017), dilute and shot (D & S) (Cladière et al., 2018), QuEChERS (Dzuman et al., 2015), immunoaffinity column (IAC), alone or as a combination of them (Ye et al., 2020). In general and due to strong matrix effects, the mean recovery obtained in tea has been around 80%. Thus, clean-up and pre-concentration steps should be developed to improve the analytical capability of the methods.

Therefore, a new green dispersive liquid-liquid microextraction strategy considering the solidification of a floating organic drop (DLLME-SFO) coupled to liquid chromatography and tandem mass spectrometric detection for the quantitative evaluation of OTA in tea-based beverages is proposed. Factors affecting the extraction procedure were optimized. The methodology was validated through external and standard addition calibrations, precision, and accuracy studies. The achieved analytical technique resulted to be sensitive, selective, fast, and economic, reaching a pre-concentration factor of around 7-folds. After treatment, the absence of a matrix effect was an indicator of the remarkable clean-up step efficiency. Analysis of OTA in tea bag varieties such as black, green, white, and herbal mix was performed.

2. Materials and methods

2.1. Reagents and samples

OTA (98% purity) was obtained from Fluka (Steinheim, Germany). Methanolic working and stock standard solutions (10 mg/L) were properly stored (in darkness, 4 °C). MS-grade solvents were obtained from Fisher Scientific (Fair Lawn, New Jersey, USA). Formic acid (FA) was provided by Fisher Scientific (Loughborough, UK) and ammonium hydroxide was acquired from Merck (Darmstadt, Germany). Octadecylsilane (C18) was purchased from Agilent Technology®. A Milli-Q water purification system (EASYpure, RF Barnstead, IA, USA) provided ultrapure water. Analyte's spiked infusion tea samples were used for quantification purposes. Tea bag samples were purchased from local supermarkets from San Luis, Argentina.

2.2. Instrumentation

Detection and quantification of OTA in the beverage samples was accomplished by tandem mass spectrometric analyses (Quattro Premier™ XE, Micromass MS Technologies, USA), configured with an electrospray ionization source (Z-Spray™, Waters, Milford, USA). Chromatographic separation was performed in an Ultra High-Performance LC system (Acquity™, Waters, Milford). A small particle reversed-phase column (50 × 2.1 mm i.d., 1.7µm) was used (ACQUITY UPLC®, BEH C18, Waters, Milford, USA). Also, ultrasonic cleaner (Testlab, model TB-04 TA, Buenos Aires, Argentina), centrifuge (U-320R-BOECO, Germany), and an electronic microbalance with a readability of 0.1 mg (Ohaus, model UMX2, Switzerland) were employed. The 4.1 Mass Lynx version (Waters, Milford, USA) was used for data acquisition.

2.3. Mass spectrometry and chromatography conditions

The electrospray ionization source was set in a positive polarity mode. Operational conditions were as follows: capillary voltage, 3.5 kV; extractor voltage, 1.0 kV; source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow rate, 800 L h^{-1}. Multiple reaction monitoring (MRM) mode was considered for OTA's determination. Thus, the (m/z), 404.1 $[M+H]^+$ precursor ion, the 404.1→239.2 (25 eV collision energy) quantification transition, and the 404.1→341.1 (25 eV collision energy) and 404.1→358.2 (20 eV collision energy) confirmation fragments were selected. Collision gas (Ar, 0.18 mL min^{-1}), dwell time (0.08) and cone voltage (20 V) were also optimized.

For chromatographic separation, the following parameters were optimized: column temperature (35 °C), injection volume (10 µL), mobile phase compositions (water with 0.1% (v/v) of formic acid (A) and acetonitrile with 0.1% (v/v) of formic acid (B)), mobile phase flow rate (0.30 mL/min), elution mode (gradient composition). The gradient started at 50% of A (1 min). Then, a linear gradient was applied until 10% of A was reached (1 min). Finally, the gradient returned to 50% of A (1 min). Under this program, OTA's retention time was 1.37 min.

2.4. Preparation of tea beverage

The study of OTA involved the analysis of tea bag samples purchased in local supermarkets from the province of San Luis, Argentina.

Following the producers' recommendations, the infusions were prepared. Thus, a measured mass of tea material (1 g) was placed into a beaker, then 200 mL of 80 °C ultrapure Milli Q water was added, the solution was stirred occasionally for 5 min, and finally cooled to room temperature. The beverage was used to extract OTA for analysis.

2.5. DLLME–SFO procedure

The analytical procedure for OTA determination consisted of two stages. The first one, named extraction, was the transfer of OTA molecules from the infusion to the rich-solvent (1-dodecanol) extraction phase and, the second stage, the retro-extraction, consisted of the transference of the analyte from the 1-dodecanol phase to the solvent mixture compatible with the detection system (an operational flowchart is illustrated in Figure 1).

2.5.1. Extraction stage

For the DLLME-SFO step, a measured volume of the beverage (5 mL) was conditioned with formic acid (0.1% (v/v)). Then, the extraction mixture (300 μL, 1:1, this ratio was selected for practical considerations to favor the drop formation and its subsequent removal from the solution) consisting of extraction (1-dodecanol) and dispersion (acetonitrile) solvents were added rapidly. After vortexing (30 s), a cloudy suspension was formed. Then, centrifugation (3 min at 3000 rpm) was applied and, due to its lower density, a small organic drop was noticed on the surface of the infusion. Considering that the melting point of the extraction solvent is 24 °C, to solidify the floating drop, the tube was placed into an ice bath (5 min). Following, the drop was extracted with a spatula and placed in a new glass tube. Finally, the retro-extraction stage was continued.

2.5.2. Retro-extraction stage

This step was based on the transfer of OTA from the 1-dodecanol rich-phase to a solvent solution suitable with the separation/detection system. Thus, the solid drop was melted at room temperature. Additionally, for sample clean-up, 40 mg of C18 sorbent was added. After that, 600 μL ACN previously conditioned to pH 9.5 with 25 mM ammonium solution was added to favor the transference of OTA from 1-dodecanol to the solvent. Vortexing (30 s) and centrifugation (3000 rpm for 3 min) were applied. A syringe was used to draw the retro-extraction solution; thus, 200 μL were transferred into proper vials through membrane filters.

2.6. Method validation

Validation of the proposed methodology for OTA quantification in tea infusions was accomplished according to the IUPAC recommendations (Currie, 1995; Olivieri, 2015).

External calibration (EC) was performed by assessing the analyte's signal (peak area) of a proper number of calibration points: blank sample, 1, 2.5, 8, 30, 50, 70 ng mL^{-1}. For matrix-matched calibration (M-MC), the following OTA standard concentrations were considered: 0.1, 0.50, 1, 2.5, 8, 10 ng mL^{-1}, in addition to the blank sample of tea infusion (a 7-fold enrichment factor should be considered). Both curves, EC and M-MC, were measured in triplicate.

Detection (LOD) and quantification (LOQ) limits were calculated using MatLab Software based on the IUPAC guidelines.

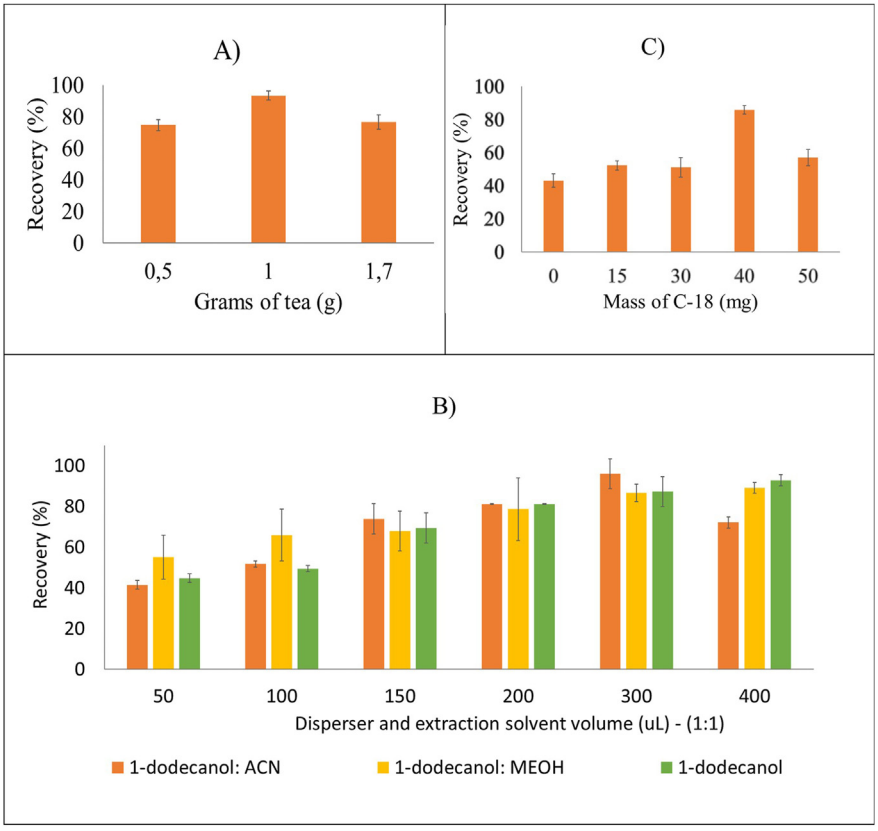

Figure 2. A) Mass sample effect over the extraction procedure B) Extraction and dispersion solvent effect C) C-18 sorbent over the sample clean-up.

2.6.1. Evaluation of matrix effect and other aspects

The ratio between the initial volume of solution (tea infusions) and the final volume obtained from the process (Eq. 1) is defined as the enrichment factor. On the other hand, relative recovery (Recovery (%)) calculation is defined in Eq. (2), the relationships between the analyte's concentrations before (C_{Real}) and after (C_{Found}) spiking the sample with a known amount of the standard and the concentration of the known amount of standard that was spiked to the real sample ($C_{Initial}$). Additionally, to evaluate the complex sample effect over the detection system, the ratio of the slopes from the EC and M-MC curves were compared according to Eq. (3); where *a* and *b* are the slopes in the M-MC and the EC plots; respectively.

$$Enrichment\ factor\ (EF) = \frac{Volume\ of\ initial\ solution}{Volume\ of\ final\ solution} \quad \text{Eq. 1}$$

$$Recovery\ (\%) = \frac{C_{Found} - C_{Real}}{C_{Initial}} \quad \text{Eq. 2}$$

$$Matrix\ effect\ (\%) = \left[\frac{a}{b} - 1\right] \times 100 \quad \text{Eq. 3}$$

2.6.2. Green certificate

In the last years, a consciousness for developing environmentally friendly analytical methodologies has arisen (Fernández et al., 2018). In this sense and to evaluate the greenness of an analytical methodology, different metrics have been created. Thus, De la Guardia and his colleagues proposed a modification of the existing Eco-scale named "Green Certificate" (Armenta, Garrigues and de la Guardia, 2015).

The Green Certificate is based on the application of two colors, green and red, together with letters, from A to G, on a scale from 100 to 0 score. This methodology subtracts the penalty points (PPs) from the highest score contemplating the volume of reagents used, an issue that is not generally considered in other green scales. The categories are divided as follows: A, the most sustainable (less than 10 PPs) and its label color is green; B (less than 20 PPs); C (21–30 PPs); D (31–45 PPs); E (46 - 60 PPs); F (61–80 PPs); and G (more than 81 PPs), having a red label (Valcárcel et al., 2017).

In this work, different parameters (reagents, energy consumed, and waste) were considered to calculate the PPs, reagent (PPr), and waste volume (PPw) penalty points were calculated by Eqs. (4) and (5):

$$PPr = (0.61 \pm 0.05) \times V^{(0.31 \pm 0.02)} \quad \text{Eq. 4}$$

$$PPw = (1.50 \pm 0.08) \times W^{(0.40 \pm 0.02)} \quad \text{Eq. 5}$$

Where V and W are reagent and waste volumes; respectively.

Additionally, energy consumption penalty points were considered following Raynie and Driver (Raynie and Driver, 2009) recommendations.

3. Results and discussion

The extraction efficiency in the proposed system can be influenced by different factors (Guiñez et al., 2017). In this work, some of them were the mass of tea infusion, the selection and the volume relationship between the disperser and the extraction solvents, the mass of C18 sorbent, and the influence of the pH on the retro-extraction stage. Therefore and taking into consideration the optimal recovery/enrichment of OTA, a careful study was performed.

3.1. Evaluation of the extraction parameters

3.1.1. Tea sample mass

A determined amount of tea from the teabag samples was used to prepared infusions of 200 mL at 80 °C. The masses evaluated were 0.5, 1, and 1.7 g. The average recoveries of OTA obtained in the infusions prepared with different masses of tea are shown in Figure 2A. According to the results, 1 g of tea clearly showed the best recovery for OTA.

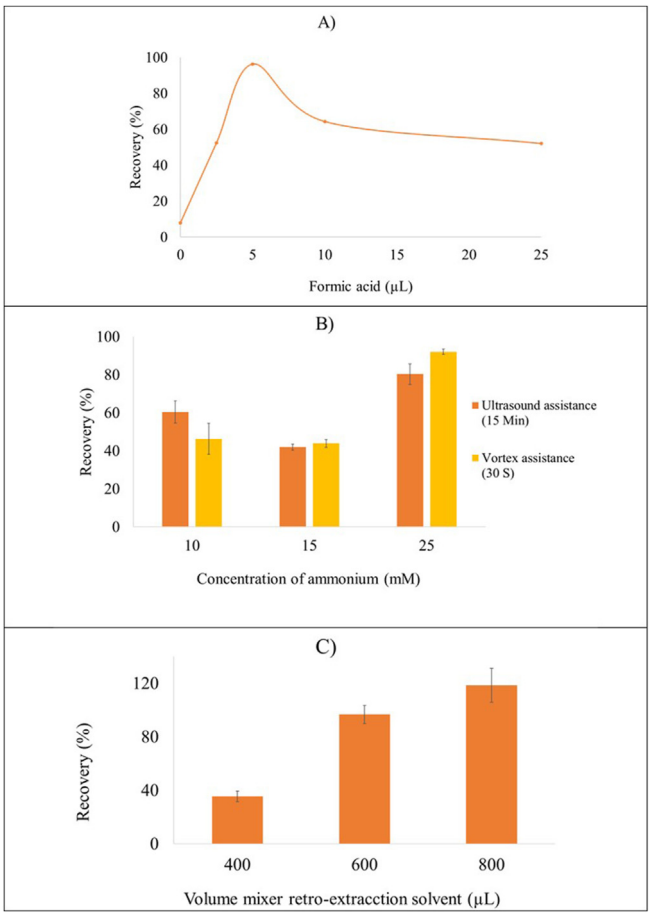

Figure 3. A) Formic acid influence on OTA extraction B) Effect of ammonium concentration over OTA retro-extraction (ultrasound assistance and vortex were kept constant at 15 min and 30 s; respectively) C) Effect of ACN:NH_4^+ mixture over OTA retro-extraction.

3.1.2. Optimization extraction stage. Nature and solvent volumes

As mentioned by Guiñez et al., the extraction solvent for DLLME-SFO must fulfill some needs (Guiñez et al., 2017). Due to its melting point, several studies report the use of 1-dodecanol as a suitable extractant for organic compounds (Viñas et al., 2015). Thus, 1-dodecanol was selected for this purpose. Moreover, the miscibility of the disperser solvent into the extraction solvent and the aqueous solution (sample infusions) is a critical aspect of the DLLME-SFO process. Thereby, ACN and MeOH were chosen to disperse the 1-dodecanol.

To optimize the extraction and disperser solvent (1:1) volumes, different solutions were prepared: 50, 100, 150, 200, 300, and 400 µL, as well as 1-dodecanol without the addition of disperser solvent.

Recoveries ranged from 41.5 – 96.0 % using ACN as disperser solvent, from 55.0 to 89.0% when MeOH was considered, and from 44.7 - 92.8 % without any disperser solvent. According to the results, the use of a disperser solvent in the infusion is necessary to produce the emulsion. As it was mentioned, the matrix under study is quite complex and the fact that acetonitrile has a lower polarity than methanol, better miscibility between the tea infusion and 1-dodecanol can be achieved using this solvent. For this reason, the selected mixture consisted of 1-dodecanol (extraction solvent) and acetonitrile as the disperser agent.

The obtained results revealed that a volume of 300 μL allowed to achieve the highest extraction efficiency (96.0%), in comparison to 200 μL (81.2%) and 400 μL (72.2%), the obtained results are illustrated in Figure 2B.

3.1.3. Effect of C18 sorbent

Although the specific steps of the DLLME-SFO methodology were evaluated and optimized, it was not enough to overcome the OTA's signal reduction caused by the complex composition of the tea infusions. Therefore, octadecylsilane (C18) was used as a sorbent in the retro extraction stage to achieve a further clean-up of the sample. The amount of C18 added was evaluated (0, 15, 30, 40, 50 mg) for this purpose. The recoveries for OTA (triplicate measurements) were 43.2 ± 4.1, 52.3 ± 2.9, 85.9 ± 2.5, and 57.1 ± 5.0, respectively. As it can be observed, OTA recoveries improved significantly as the C18 content increased up to 40 mg, demonstrating a positive effect on the extraction efficiency. Corresponding experimental results are presented in Figure 2C.

As mentioned, the matrix effect in the analysis of tea infusions was important. The inclusion of a sorbent in the developed methodology revealed the need for a further clean-up step in this type of sample. The addition of 40 mg of C18 was the optimized amount to retain undesired interferences.

3.1.4. Effect of pH

Different factors such as type of tea, contact time, and pH may be responsible for the transfer of OTA into the prepared infusions. The moieties of this molecule confer diverse pKa values (4.2–4.4 for the carboxyl group and 7.0–7.3 for the phenolic hydroxyl group), for the carboxyl group and the phenolic hydroxyl group, respectively. Consequently, OTA is likely to be more soluble in an aqueous solution at pH values is above 7 (Malir et al., 2014; Toman et al., 2018). In this work, to favor transference of OTA from the infusion to 1-dodecanol, it was necessary to decrease the pH of the prepared infusions (the pH of the black tea infusion was approximately 6.8). Conditioning of the beverages with different volumes-concentrations- of formic acid (0, 2.5, 5, 10, and 25 μL) was carried out. The satisfactory effect of the 0,1 % (v/v) of formic acid, which corresponded to a minimum volume of 5 μL and a pH value equal to 3, was significant on the extraction of ochratoxin A (Figure 3A).

In the same way, the pH was modified in the retro-extraction stage to favor the transfer of OTA from 1-dodecanol to retro-extraction solution because the extraction solvent was not compatible with the separation/detection system. Therefore, buffer ammonium at different concentrations (10, 15, and 25 mM) was added to raise the pH. The results are illustrated in Figure 3B. The obtained recoveries using different concentrations of ammonia to form the retro-extraction mixture with acetonitrile were 60.4, 41.9, and 80.3% with ultrasonic stirring and, 48.0, 47.8, and 94.8% with vortex-assisted stirring; the latter mixing approach was selected. The results demonstrated that low concentrations of ammonia were not sufficient to create a buffer effect and raise the pH of acetonitrile to a value near 9. This feature demonstrates the importance of pH in the extraction and retro-extraction stages. In the first stage, it is necessary to lower the pH of the solution (tea infusion) and, in the second stage, it is necessary to increase it to promote the transfer of the analyte from 1-dodecanol to the retro-extraction solution. Vortex agitation was used to speed and improve the process. The use of an ultrasound agitation could favor the subsequent extraction of other compounds present in tea suppressing the OTA signal. Consequently, 25 mM of ammonia buffer and vortex-assisted agitation were selected for the OTA retro extraction step.

Additionally, as mentioned by Guiñez et al., volume of retro extraction solvent was an crucial factor to consider (Guiñez, Bazan, Martinez and Cerutti, 2018; Guiñez, Canales, Martinez and Cerutti, 2018). To achieve this purpose, different volumes of the retro-extraction solution were tested: 400, 600, and 800 μL. The results revealed that, with a volume of 600 μL, the best recovery efficiency (96.7%) was achieved in comparison to 200 μL (35.5%) and 800 μL (118.5%). Recovery resulted insufficient with a retro extraction solvent volume of 200 μL (Figure 3C). Meanwhile, at higher volumes (600 and 800 μL), transfer of OTA from 1-dodecanol to the retro-extraction solution increased, resulting in major phase separation and extraction efficiency. In the case of 800 μL, an increment of the standard deviation was observed. Therefore, a volume of 600 μL was selected.

3.2. Matrix effect and analytical performance

As mentioned, the matrix effect was assessed. Thus, calibration curves of both, spiked matrix and retro extraction solvent mixture solutions

Table 1. Analytical figures of merit and recovery study.

Figure of merit					
[1]LR (ng mL^{-1})					0,5 - 74
[2]LOD (ng mL^{-1})					0,5
[3]LOQ (ng mL^{-1})					1,4
Intra-day precision [4]RSD%, (n = 3)					6,5
Inter-day precision [4]RSD%, (n = 3)					7,8
r2					0,9985
Recovery study for the analysis of spiked infusion tea samples after applying the proposed methodology					
Sample concentration (ng mL^{-1})	Concentration added (ng mL^{-1})	Concentration found (ng mL^{-1})	[5]RR (%)	RSD (%) n = 3	[6]EF
N/D*	0	—	—	—	7
*	1	5,1	76,1	3,8	
*	5	31,9	95,6	5	
*	8	49,5	92,8	2,8	
*	10	64,5	96,7	4,2	

[1] LR: linear range.
[2] LOD: Limit of Detection.
[3] LOQ: Limit of Quantitation.
[4] RSD: Relative Standard Deviation.
[5] RR: Relative Recovery.
[6] EF: Enrichment Factor, N.D.: not detected.

Table 2. Application of the proposed methodology to different varieties of tea samples.

Sample	Tea variety	Concentration added (ng mL^{-1})	[1]EF	OTA Concentration determined* (ng mL^{-1})	[2]RR (%)	[3]RSD (%) n = 3
Sample 1	Black	5	7	32,9	98,6	3,4
Sample 2	Black	5		31,1	93,3	1,7
Sample 3	Green	5		33,9	101,7	0,8
Sample 4	White	5		35,6	106,7	5,8
Sample 5	Boldo	5		34,4	103,3	2,5
Sample 6	Herbal mix	5		30,0	90,0	3,7
Sample 7	Linden	5		29,8	89,3	3,9

* The expressed concentration considers the spiked amount of OTA and the enrichment factor.
[1] EF: Enrichment Factor.
[2] RR: Relative Recovery.
[3] RSD: Relative Standard Deviation.

Table 3. Green certificate calculation for the proposed OTA methodology in comparison other referenced works.

*Methodology	Penalty point reagents				Energy	Occupational Hazard	Waste	Total	Category	OTA Sensitivity		Reference
	Reagents type	Reagents amount (mL)	Hazard	Subtotal						LOD	LOQ	
SPE (raw material)	$NaCl/H_3PO_4$	10	1	1,2	1	0	5	79	C	0,1 μg/kg	0,35 μg/kg	(Malir et al., 2014)
	Chloroform	15	2	2,8								
	$NaHCO_3$	5	1	1,0								
	Formic acid	0,5	6	3,0								
	Buffer solution	17	0	0,0								
	Methanol	7,5	6	6,8								
SPE (Beverages)	Methanol	13,8	6	8,3	1	0	7	84	B			
	Buffer solution	50	0	0,0								
DLLEM	NaCl (g)	1	0	0,0	3	0	3	84	B	5 μg/L	17 μg/L	(Pallarés, Font, Manˆes, & Ferrer, 2017)
	Acetonitrile	0,95	4	2,4								
	Ethyl acetate	0,62	4	2,1								
	Methanol	1,45	6	4,1								
	Chloroform	1,12	2	1,3								
LLE	Acetonitrile	3	4	3,4	3	0	4	90	A	10 μg/kg		(Cladière et al., 2018)
	Salts (g)	3	0	0,0								
LLE (Leave)	Acetonitrile	4,7	4	3,9	3	0	2	89	B			
	Methanol	0,5	6	3,0								
D&S	Acetonitrile	2	4	3,0	3	0	4	90	A			
QuEChERS	Acetonitrile	10	4	5,0	3	0	4	86	B	0,27 μg/kg	0,83 μg/kg	(Reinholds et al., 2019)
	Methanol	0,06	6	1,5								
	Salts (g)	1,5	0	0,0								
SPE	Ethyl acetate	20	4	6,2	3	0	4	78	C	0,07 μg/kg	0,24 μg/kg	(Reinholds et al., 2019)
	Methanol	3	6	5,1								
	Acetonitrile	5	4	4,0								
DLLME-SFO (Beverages)	Acetonitrile	0,6	4	2,1	3	0	4	91	A	0,48 μg/L	1,38 μg/L	***This work***
	1-dodecanol	0,15	1	0,3								

* SPE-solid phase extraction, DLLE-dispersive liquid-liquid extraction, LLE-liquid-liquid extraction, D&S-dilute and shot, QuEChERS-Quick, Easy, Cheap, Effective, Rugged and Safe, DLLME–SFO– dispersive liquid-liquid microextraction based on the solidification of a floating organic drop.

were obtained, slope ratios served to account for the matrix effect. From recoveries, enhancement or suppression was not observed for OTA after applying the DLLME-SFO approach with the sample clean-up step. Consequently, external quantitation was considered in the analysis of real samples.

Figures of merit for the proposed method are summarized in Table 1. Samples consisted of three blanks and three replicates spiked at concentration levels of 1, 5, 8, and 10 ng mL^{-1}. Linearity of calibration curves resulted to be sufficient, a determination coefficient (R^2) of 0.9985 was achieved. The F-test tested the linear regression model, the lack of fit analysis yielded a *p*-value of 0.3474. The obtained LOD and LOQ values were 0.5 and 1.4 ng mL^{-1} (Table 1).

Recovery of OTA from tea infusion samples was assayed. Blank and OTA's spiked samples were considered. The relative recovery was between 76.1% and 96.7%, as shown in Table 1. As observed, good precision and satisfactory recoveries were achieved.

3.3. Real samples analysis

Although not determined in this work, it has been shown that ochratoxin A can be moved from herbs into black tea infusions (approximately 35%) (Malir et al., 2014), being this percentage lower in the case of fruit tea infusions.

Analysis by UHPLC-MS/MS and verification by fluorescence detection, with a good statistical agreement of the results, was performed. The

most consumed types of tea bags (commercial variety) were analyzed: Black (2), Green (1), White (1), Boldo (1), Herbal mix (1), and Linden (1). Levels of OTA in analyzed samples were not detectable (Table 2), these results might mean that the commercially available samples under study were not naturally contaminated. Future studies include a larger survey of samples to study OTA transference to infusions and to develop a risk assessment study. A comparison of the analytical performance of the herein presented method with other works dealing with OTA determination in tea samples (raw or infusions) is presented in Table 3.

In summary, the extraction method demonstrated to be robust since the OTA content can be evaluated in different types of tea infusion.

3.4. Green metric. Green certificate

As mentioned in section 2.6.2, the green metric estimation was applied to obtain the green certificate of the extraction procedure (Valcárcel et al., 2017).

A comparison of different techniques (LLE, SPE, QuEChERS, D&S) used for the extraction of OTA in tea samples (infusion or raw material) is shown (Table 3). As can be seen, the proposed methodology represents an advantage with the low volumes of reagents used, which resulted in lower PPr and PPw values. This is in concordance with a green certificate of category A. On the other hand, the sensitivity by the proposed methodology is like the ones reported by other techniques.

4. Conclusion

An analytical extraction and a clean-up strategy together with LC-(+) ESI-MS/MS analysis for the quantitative evaluation of OTA in tea bag samples, was developed. In this study, a rapid, sensitive, and selective a. Furthermore, the proposed DLLME-SFO methodology can be considered eco-friendly due to its category A of the calculated green certificate. The efficient extraction and clean-up stages were achieved, which allowed the determination of vestiges of ochratoxin A in widely consumed beverages. Also, the proposed methodology was demonstrated to be robust enough to evaluate different tea varieties. This work provides a valuable tool for the affordable and routine analysis of OTA in tea samples.

Declarations

Author contribution statement

Mariel Cina: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

María del Valle Ponce: Performed the experiments; Analyzed and interpreted the data.

Luis Dante Martinez: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Soledad Cerutti: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by Universidad Nacional de San Luis (Project PROICO 2-1816), CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) [Project PIP 2015 N° 0605 and PUE 0113] and ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica) [Project PICT 2016-1776].

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

This work is entirely dedicated to our mentor, friend, and co-worker Luis Dante Martinez. His teachings, stories, and encouraging words will always remain in our memory. You left implanted in all of us the love and respect for the chemical sciences.

References

Armenta, S., Garrigues, S., de la Guardia, M., 2015. The role of green extraction techniques in Green Analytical Chemistry. Trac. Trends Anal. Chem. 71, 2–8.

Bryła, M., Waśkiewicz, A., Podolska, G., Szymczyk, K., Jędrzejczak, R., Damaziak, K., Sułek, A., 2016. Occurrence of 26 mycotoxins in the grain of cereals cultivated in Poland. Toxins 8 (6), 160.

Cladière, M., Delaporte, G., Le Roux, E., Camel, V., 2018. Multi-class analysis for simultaneous determination of pesticides, mycotoxins, process-induced toxicants and packaging contaminants in tea. Food Chem. 242, 113–121.

Commission, E., 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union 364, 324–365.

Currie, L.A., 1995. Nomenclature in evaluation of analytical methods including detection and quantification capabilities (IUPAC Recommendations 1995). Pure Appl. Chem. 67 (10), 1699–1723.

Chang, K., 2015. World tea production and trade Current and future development. Food Agri. Org. United Nat. Rome.

Chen, X., Du, Y., Wu, L., Xie, J., Chen, X., Hu, B., Wu, Z., Yao, Q., Li, Q., 2019. Effects of tea-polysaccharide conjugates and metal ions on precipitate formation by epigallocatechin gallate and caffeine, the key components of green tea infusion. J. Agric. Food Chem. 67 (13), 3744–3751.

Duarte, S.C., Salvador, N., Machado, F., Costa, E., Almeida, A., Silva, L.J., Pereira, A.M., Lino, C., Pena, A., 2020. Mycotoxins in teas and medicinal plants destined to prepare infusions in Portugal. Food Contr. 107290.

Dzuman, Z., Zachariasova, M., Veprikova, Z., Godula, M., Hajslova, J., 2015. Multi-analyte high performance liquid chromatography coupled to high resolution tandem mass spectrometry method for control of pesticide residues, mycotoxins, and pyrrolizidine alkaloids. Anal. Chim. Acta 863, 29–40.

Fernández, M., Boiteux, J., Espino, M., Gomez, F.J., Silva, M.F., 2018. Natural deep eutectic solvents-mediated extractions: the way forward for sustainable analytical developments. Anal. Chim. Acta 1038, 1–10.

Guiñez, M., Bazan, C., Martinez, L.D., Cerutti, S., 2018a. Determination of nitrated and oxygenated polycyclic aromatic hydrocarbons in water samples by a liquid–liquid phase microextraction procedure based on the solidification of a floating organic drop followed by solvent assisted back-extraction and liquid chromatography–tandem mass spectrometry. Microchem. J. 139, 164–173.

Guiñez, M., Canales, R., Martinez, L.D., Cerutti, S., 2018b. Solvent-based de-emulsification dispersive liquid–liquid microextraction coupled with UPLC-MS/MS for the fast determination of ultratrace levels of nitrated and oxygenated polycyclic aromatic hydrocarbons in environmental samples. Analytical Methods 10 (8), 910–919.

Guiñez, M., Martinez, L.D., Fernandez, L., Cerutti, S., 2017. Dispersive liquid–liquid microextraction based on solidification of floating organic drop and fluorescence detection for the determination of nitrated polycyclic aromatic hydrocarbons in aqueous samples. Microchem. J. 131, 1–8.

Hacıbekiroğlu, I., Kolak, U., 2013. Aflatoxins in various food from Istanbul, Turkey. Food Addit. Contam. B 6 (4), 260–264.

Jia, W., Shi, L., Zhang, F., Fan, C., Chang, J., Chu, X., 2019. Multiplexing data independent untargeted workflows for mycotoxins screening on a quadrupole-Orbitrap high resolution mass spectrometry platform. Food Chem. 278, 67–76.

Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Malir, J., Toman, J., 2016. Ochratoxin A: 50 years of research. Toxins 8 (7), 191.

Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Toman, J., Bazin, I., Roubal, T., 2014. Transfer of ochratoxin A into tea and coffee beverages. Toxins 6 (12), 3438–3453.

Olivieri, A.C., 2015. Practical guidelines for reporting results in single-and multi-component analytical calibration: a tutorial. Anal. Chim. Acta 868, 10–22.

Pallares, N., Font, G., Manes, J., Ferrer, E., 2017. Multimycotoxin LC–MS/MS analysis in tea beverages after dispersive liquid–liquid Microextraction (DLLME). J. Agric. Food Chem. 65 (47), 10282–10289.

Raynie, D., Driver, J., 2009. 13th green chemistry and Engineering conference. Washington, DC.

Reinholds, I., Bogdanova, E., Pugajeva, I., Bartkevics, V., 2019. Mycotoxins in herbal teas marketed in Latvia and dietary exposure assessment. Food Addit. Contam. B 12 (3), 199–208.

Rothenberg, D.O.N., Zhou, C., Zhang, L., 2018. A review on the weight-loss effects of oxidized tea polyphenols. Molecules 23 (5), 1176.
Sedova, I., Kiseleva, M., Tutelyan, V., 2018. Mycotoxins in tea: occurrence, methods of determination and risk evaluation. Toxins 10 (11), 444.
Toman, J., Malir, F., Ostry, V., Kilic, M.A., Roubal, T., Grosse, Y., Pfohl-Leszkowicz, A., 2018. Transfer of ochratoxin A from raw black tea to tea infusions prepared according to the Turkish tradition. J. Sci. Food Agric. 98 (1), 261–265.
Valcárcel, M., Cárdenas, S., Lucena, R., 2017. Analytical microextraction techniques: bentham science publishers.
Viñas, P., Campillo, N., Andruch, V., 2015. Recent achievements in solidified floating organic drop microextraction. Trac. Trends Anal. Chem. 68, 48–77.
Wyker, A., 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (56).
Ye, Z., Wang, X., Fu, R., Yan, H., Han, S., Gerelt, K., Cui, P., Chen, J., Qi, K., Zhou, Y., 2020. Determination of six groups of mycotoxins in Chinese dark tea and the associated risk assessment. Environ. Pollut. 261, 114180.