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

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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 spp., Penicillium spp., 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 ≤ 0.85) (Cladière et al., 2018). Through food/feed, mycotoxins can cause a variety of adverse toxicological effects in human and animal health (Bryla 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 (Pallarès, Font, Manés, & 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 μg/kg for the aflatoxin B1 in unprocessed tea. In Argentina, an aflatoxin B1 threshold of 5 μg/kg has also been considered and, a total aflatoxin content of 20 μg/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 μg/kg and 10 μg/kg for aflatoxin B1, respectively, and levels between 5 and 20 μg/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.
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; Pallarès, Font, Manès, & 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 (DLLLE) (Hacbebekiroglu and Kolak, 2013; Pallarès, Font, Manès, & 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 methodology was validated through external and standard addition calibrations.

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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., 1.7 μm) 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_initial) 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.

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

\[
\text{Recovery (\%)} = \frac{C_{\text{found}} - C_{\text{real}}}{C_{\text{initial}}} \quad \text{Eq. 2}
\]

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

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 – 40 PPs); D (41– 60 PPs); E (46 - 60 PPs); F (61–80 PPs); and G (more than 81 PPs), having a red label (Valcarcel 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):

\[
PPr = (0.61 \pm 0.05) \times W^{0.31 \pm 0.02} \quad \text{Eq. 4}
\]

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

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 (Guínez 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.

3.1.2. Optimization extraction stage. Nature and solvent volumes

As mentioned by Guínez et al., the extraction solvent for DLLME-SFO must fulfill some needs (Guínez et al., 2017). Due to its melting point, several studies report the use of 1-dodecanol as a suitable extractant for organic compounds (Vinas 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 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 Guínez et al., volume of retro extraction solvent was an crucial factor to consider (Guínez, Bazan, Martínez and Cerutti, 2018; Guínez, Canales, Martínez 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 unsatisfactory 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.

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### Table 1. Analytical figures of merit and recovery study.

| Figure of merit | 0.5 - 74 |
|-----------------|---------|
| LOD (ng mL⁻¹)   | 0.5     |
| LOQ (ng mL⁻¹)   | 1.4     |
| Intra-day precision RSD%, (n = 3) | 6.5 |
| Inter-day precision 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) | RR (%) | RSD (%) | n = 3 | 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    |      |    |

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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.
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

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

| Sample   | Tea variety | Concentration added (ng mL\(^{-1}\)) | 1EF | OTA Concentration determined\(^1\) (ng mL\(^{-1}\)) | 2RR (%) | 3RSD (%) | 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      |

\(^1\) The expressed concentration considers the spiked amount of OTA and the enrichment factor.

\(^2\) EF: Enrichment Factor.

\(^3\) 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 |
|-------------|------------------------|--------|---------------------|-------|-------|----------|-----------------|----------|
|             |                        |        |                     |       |       |          | LOD             | LOQ      |
| SPE (raw material) | NaCl/H\(_3\)PO\(_4\) | 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   | 0     | 0        | 0               |          |          |            |                |
|             | NaHCO\(_3\)             | 5      | 1                   | 1.0   | 0     | 0        | 0               |          |          |            |                |
|             | Formic acid            | 0.5    | 6                   | 3.0   | 0     | 0        | 0               |          |          |            |                |
|             | Buffer solution        | 17     | 0                   | 0.0   | 0     | 0        | 0               |          |          |            |                |
|             | Methanol               | 7.5    | 6                   | 6.8   | 0     | 0        | 0               |          |          |            |                |
| SPE (Beverages) | Methanol               | 13.8   | 6                   | 8.3   | 1     | 0        | 7               | 84 B     |          |            |                |
|             | Buffer solution        | 50     | 0                   | 0.0   | 0     | 0        | 0               |          |          |            |                |
| DLLEM       | NaCl (g)               | 1      | 0                   | 0.0   | 0     | 0        | 0               |          |          |            |                |
|             | Acetonitrile           | 0.95   | 4                   | 2.4   | 0     | 0        | 0               |          |          |            |                |
|             | Ethyl acetate          | 0.62   | 4                   | 2.1   | 0     | 0        | 0               |          |          |            |                |
|             | Methanol               | 1.45   | 6                   | 4.1   | 0     | 0        | 0               |          |          |            |                |
|             | Chloroform             | 1.12   | 2                   | 1.3   | 0     | 0        | 0               |          |          |            |                |
| LLE         | Acetonitrile           | 3      | 4                   | 3.4   | 0     | 0        | 0               |          |          |            |                |
|             | Salts (g)              | 3      | 0                   | 0.0   | 0     | 0        | 0               |          |          |            |                |
| LLE (Leaves) | Acetonitrile           | 4.7    | 4                   | 3.9   | 3     | 0        | 2               | 89 B     |          |            |                |
|             | Methanol               | 0.5    | 6                   | 3.0   | 0     | 0        | 0               |          |          |            |                |
| D&S         | Acetonitrile           | 2      | 4                   | 3.0   | 3     | 0        | 4               | 90 A     | 10 μg/kg  |            |                |
|             | Methanol               | 0.06   | 6                   | 1.5   | 0     | 0        | 0               |          |          |            |                |
| QaEChERS    | 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   | 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   | 0     | 0        | 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   | 0     | 0        | 0               |          |          |            |                |

1 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.
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 assay. 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 Martínez: 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.

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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.

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