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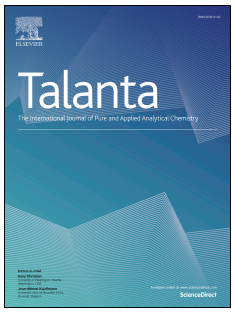

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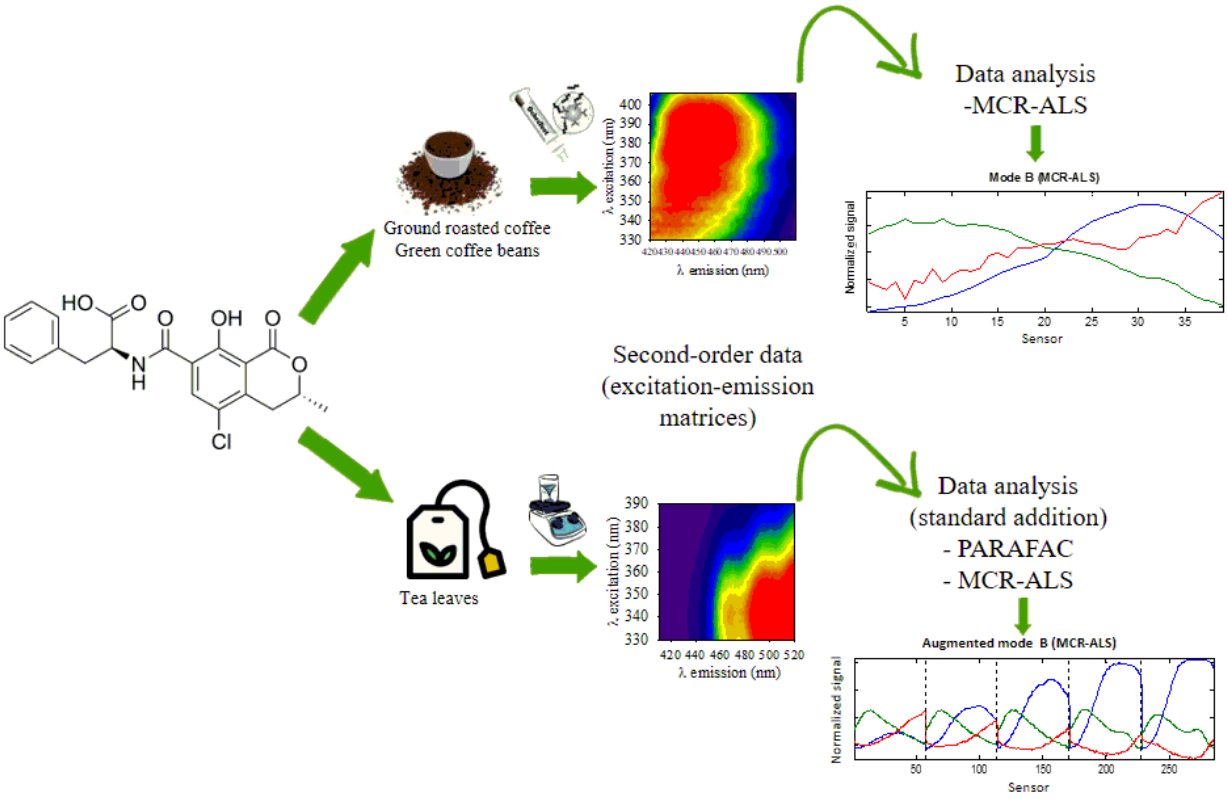

Determination of ochratoxin A in coffee and tea samples by coupling second-order multivariate calibration and fluorescence spectroscopy

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Abstract

A new method to quantify the mycotoxin ochratoxin A (OTA) in coffee and tea samples is proposed based on second-order multivariate calibration and excitation-emission fluorescence matrix (EEFM) data. Experimental conditions were optimized by studying the effect of pH and various organized media on the fluorescence signal of OTA. For each analysed matrix (coffee grains and tea leaves), several sample pretreatments and calibration methods (external or standard addition) and data processing by chemometric models (e.g., parallel factor analysis/PARAFAC and multivariate curve resolution-alternating least squares/MCR-ALS) were evaluated and discussed. The MCR-ALS algorithm provided an adequate fit to the data for both samples, while PARAFAC was satisfactory only for the tea samples. Regarding the figures of merit, the limits of detection were in the range of 0.2–0.3 ng mL^{-1}; furthermore, low relative prediction errors, between 2% and 4%, were achieved in both the fortified and real samples. Accordingly, the proposed methodology was applied to analyse fortified roasted and green coffee and real tea leaf samples. Satisfactory recoveries were achieved (ranging from 92 to 110%), and the obtained concentrations were in agreement with the values obtained by the reference method (based on high-performance liquid chromatography with fluorescence detection/HPLC-FLD). In addition, all samples contained OTA levels lower than the maximum permissible levels. Finally, the proposed strategy allows the use of green analytical chemistry principles; for instance, the use of organic solvents and the generation of waste products were significantly lower than for similar analytical methods reported in the literature.

Keywords: Ochratoxin A; Fluorescence spectroscopy; Second-order multivariate calibration; Coffee samples; Tea leaves

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by different *Aspergillus* and *Penicillium* fungal species [1,2] that presents elevated toxicity and nephrotoxic effects, with teratogenic and immunosuppressive effects in animals and humans [3–6]. Additionally, OTA has been classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic (2B group) [7]. OTA is commonly found in food and feed, including cereals, oleaginous seeds, groundnuts, spices, coffee beans and even beverages such as beer, wine and tea [8–10]. Due to the potential risk of exposure to OTA through the consumption of contaminated food, several international organizations have established control and maximum permissible levels in a wide variety of foodstuffs [11,12].

In particular, the presence of OTA has been reported in highly consumed beverages such as coffee and tea [13–15]. Coffee beans and tea leaves can be contaminated with OTA during storage and transport processes, where, in addition to the processing stages, the temperature and humidity can increase the production of OTA [16,17].

To reduce the risk of exposure, the European Commission has established a maximum permissible OTA concentration of 5 µg kg^{-1} in ground roasted coffee and 10 µg kg^{-1} in soluble coffee [12], while maximum levels in both green coffee beans and tea leaves have not been established [18]. However, OTA levels above the allowed limits are frequently found in the above types of samples [19,20]. Thus, to assess the risk of human exposure, it is necessary to determine OTA content in food and beverages with sensitive and reliable analytical methods developed for this purpose.

The proposed methods for OTA determination involve an extraction/clean-up process followed by analytical determination procedures. Many options have been proposed for the pretreatment of coffee beans and tea extracts for the detection of OTA, such as solid phase extraction (SPE) specifically using immunoaffinity columns (IACs) and quick, easy, cheap, effective, rugged and safe (QuEChERS); IACs are the most commonly used approach, achieving adequate selectivity, sensitivity and satisfactory recovery percentages that are commonly between 75 and 120% [8,21,22]. Regarding the quantification of OTA, thin layer chromatography (TLC) and enzyme-linked immunosorbent assays (ELISAs) have been reported; however, the most commonly used methods are high-performance liquid chromatography with fluorescence detection (HPLC-FLD) or mass spectrometry (HPLC-MS) [8,22]. Unfortunately, these techniques are time consuming and produce large amounts of organic solvent waste, which is not environmentally friendly.

Fluorescence spectroscopy appears to be an interesting strategy for OTA determination, considering that OTA is inherently fluorescent. For organic pollutants, this technique has exhibited high sensitivity and selectivity with a low consumption of toxic organic solvents, thus showing promise as a green analytical method [23,24]. However, a lack of selectivity can be problematic when a fluorometric assay is carried out [25], and in this particular case, coffee has a remarkably complex matrix that hinders the potential detection of OTA [17,21,26]. The coupling of fluorescence with modern chemometric tools such as multivariate calibration allows the extraction of analyte information from poorly selective instrumental signals, which restores the selectivity of the studied system, especially when second- or higher-order data are used [27,28]. In this context, some algorithms provide a “second-order advantage”, a property that allows the accurate quantification of one or more analytes in complex samples containing unexpected interferences not considered in the calibration set [29]. Currently, only two methods based on

fluorescence detection and multivariate calibration are reported for the determination of OTA [24,30]. In these works, OTA was determined in cereal and peanut samples. However, no similar approaches have been reported for OTA determination in highly consumed foodstuffs such as coffee and tea.

In this work, a new analytical method based on fluorescence spectroscopy coupled to second-order multivariate calibration is evaluated for OTA determination in ground roasted coffee, green coffee beans and tea leaves. Therefore, in this work, the analytical performance of some second-order multivariate algorithms, such as parallel factor analysis (PARAFAC) and multivariate curve resolution-alternating least squares (MCR-ALS), were evaluated for modelling excitation-emission fluorescence matrices (EEFMs), which were then used for a quantitative analysis of OTA in the selected matrices. Finally, after optimization of the experimental conditions, the proposed method was successfully applied to the quantification of OTA in coffee (ground roasted and green beans) and tea leaf samples after a simple pretreatment of the sample.

2. Experimental

2.1. Reagents and solutions

All experiments were performed with analytical grade reagents, which were used as received: sodium hydroxide (NaOH), sodium phosphate monobasic (NaH_2PO_4), sodium chloride (NaCl) and potassium chloride (KCl) were purchased from Merck SA (Darmstadt, Germany); sodium hydrogen carbonate ($NaHCO_3$) and ammonium chloride (NH_4Cl) were obtained from Cicarelli (San Lorenzo, Argentina); and sodium phosphate dibasic (Na_2HPO_4) was obtained from JT Baker (Waltham, MA, USA). Hexadecyltrimethylammonium bromide (HTAB), hexadecyltrimethylammonium chloride (HTAC), sodium dodecyl sulfate (SDS), Triton X 100

(TX-100) and β-methylcyclodextrin were obtained from Sigma Aldrich (Steinheim, Germany). Acetonitrile, methanol, ammonia solution (25%) and fuming hydrochloric acid (37%) were purchased from Merck KGaA (Darmstadt, Germany). Acetic acid (glacial) was obtained from Sintorgan (Buenos Aires, Argentina). All of these reagents were HPLC grade.

Ultrapure water provided by a Milli-Q purification system was used. Immunoaffinity columns (Ochratest®) were purchased from R-Biopharm (Glasgow, Scotland). Ochratoxin A, sterigmatocystin (STE) and zearalenone (ZEN) were purchased from Sigma Aldrich (Steinheim, Germany).

A stock standard solution of OTA (100.0 µg mL^{-1}) was prepared in methanol, and stock standard solutions of STE and ZEN (100.0 µg mL^{-1}) were prepared in acetonitrile. All solutions were stored in silanized amber vials at 4 °C in the dark. Working solutions were prepared immediately before use by taking appropriate aliquots of diluted methanol solutions, drying the solvent under a nitrogen stream and adding the medium for the fluorescence analysis or mobile phase (see below) for dilution to the desired concentrations.

To evaluate the effect of pH on the fluorescent response, HCl (0.1 mol L^{-1}) and NaOH (0.1 mol L^{-1}) were used. All solutions of organized media (0.025 mol L^{-1}) were prepared directly in ultrapure water. For the analysis of OTA in coffee samples, a phosphate-buffered saline solution (PBS, pH 7.4) containing NaH_2PO_4 (0.2 g L^{-1}), Na_2HPO_4 (1.2 g L^{-1}), NaCl (8.0 g L^{-1}) and KCl (0.2 g L^{-1}) was prepared and used for the extraction procedure. For the measurement of EEFMs for calibration, validation, and coffee samples, an ammonia buffer (pH = 9.3) containing NH_4Cl and NH_3 (0.020 mol mL^{-1}) was prepared.

Mycotoxin solutions were handled with extreme care (gloves and protective clothing) due to their high toxicity. After every analysis, all materials were decontaminated overnight with sodium hypochlorite solution and then washed with ultrapure water.

2.2. Instrumentation

Fluorescence measurements were performed on a Varian Cary Eclipse (Varian, Mulgrave, Australia) luminescence spectrometer equipped with a 7 W Xenon pulse lamp and connected to a PC microcomputer. EEFMs were measured in ranges of 330–406 nm (every 2 nm, excitation) and 410–510 nm (every 1.67 nm, emission) for the coffee analysis and 310–390 nm (every 2 nm, excitation) and 450–580 nm (every 2 nm, emission) for the tea analysis. Both the excitation and emission slit widths were 10 nm using 1.00 cm quartz cells. The photomultiplier tube (PMT) sensitivity was fixed at 720 V and 700 V for the coffee and tea analyses, respectively, and the scan rate was 1000 nm min^{-1}. The EEFMs were saved in an ASCII format and transferred to a PC for subsequent chemometric analysis.

Chromatographic runs were performed on an HP 1200 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, quaternary pump, column oven, manual six-way injection valve with a 50.0 μL fixed loop, multi-scan fluorescence detector and ChemStation software package to control the instrument and data acquisition. The Poroshell 120 EC-C18 column (Agilent Technologies, Santa Clara, CA, USA) packing had a solid 4.6 mm core with a porous 50 mm thick outer layer and a total particle size of 2.7 μm. The column temperature was set to 40 °C. The data acquisition and instrumental control were performed with an HPLC 1200 software package.

2.3 Calibration, validation and test samples

A calibration set was prepared in duplicate with seven concentrations of OTA equally spaced in a range of 0–21.0 ng mL^{-1}. A validation set of six samples was prepared employing concentrations different from those used for calibration and following a random design. To

159 evaluate the method in the presence of potential interferents, namely, ZEN and STE, which have
160 fluorescence signals overlapping with those for OTA, eight test samples were prepared
161 containing random OTA concentrations in a range of 0–20.5 ng mL^{-1} and high interferent
162 concentrations in a range of 70–150 ng mL^{-1} (see Table 1).

163 Calibration and validation solutions were prepared as follows: an adequate volume of a
164 methanol OTA solution was taken, and the solvent was evaporated to dryness under a stream of
165 nitrogen. Then, the volume was adjusted to 2.00 mL with a mixture of 0.0025 mol mL^{-1} HTAB
166 and ammonia buffer (0.020 mol mL^{-1}, pH = 9.3). For test samples, the interferences considered
167 were ZEN and STE because these mycotoxins can be found together with OTA in coffee and tea
168 samples [31–34]. Accordingly, test samples were prepared in a similar way to validation
169 solutions but with the addition of ZEN and STE solutions before evaporating the solvent and
170 reconstituting the solution with a 2.00 mL mixture of HTAB and ammonia buffer.

171

172 **2.4 Real samples**

173 *2.4.1. HPLC procedure*

174 The proposed fluorescent method was validated by a chromatographic procedure,
175 following a modified method previously proposed by Benites et al. [26]. The separation was
176 carried out in isocratic mode using a mobile phase of acetonitrile:water:acetic acid (55:44:1
177 v/v/v) and a flow rate of 0.9 mL min^{-1}. The analysis time was 10 min, and fluorescence detection
178 was performed at 333 and 445 nm excitation and emission wavelengths, respectively. A standard
179 curve was prepared in a concentration range of 0.00–25.00 ng mL^{-1}. The OTA concentration was
180 quantified by an external calibration that used an adequate dilution of the sample extracts.

181

182 *2.4.2 Ground roasted coffee and green coffee samples*

Coffee samples were prepared according to the modified procedure described by Benites et al. [26]. Briefly, 150 mL of extraction solvent, which was composed of a 50:50 (v/v) mixture of methanol and NaHCO3 (0.36 mol L-1), was added to 15.00–25.00 g of ground roasted coffee or previously ground green coffee beans. The extract was magnetically stirred for 30 min. After that, the samples were filtered through a paper disk. Then, a 10.00 mL aliquot of this extract was transferred to a flask, mixed with 10 mL of PBS (pH 7.4) and passed through an Ochratest® immunoaffinity column with a flow rate of 2–3 mL min^{-1}. The column was washed with 10 mL of ultrapure water with a flow rate of 5 mL min^{-1}, and then air was passed through the column with a plunger to remove traces of liquid. The retained analyte was eluted four times with 1.0 mL of methanol:acetic acid (98:2, v/v), and backflushing was performed three times for each portion of the solution. Finally, the acidified methanol extract was divided into two fractions of equal volume, and the solvent was evaporated to dryness under a nitrogen stream. One fraction was reconstituted with 1.00 mL of the mobile phase, vortexed for 5 min, sonicated for 5 min, and injected directly into the HPLC system. The other fraction was reconstituted with 1.00 mL of ammonium buffer (0.020 mol mL−1; pH 9.3) containing 10% HTAB (0.0025 mol mL−1) and subjected to the fluorescent method proposed here. The above method implies a preconcentration degree of 3.5; therefore, the previously described method was successfully applied for the extraction and preconcentration of trace levels of OTA.

OTA was not detected in ground roasted coffee samples, and therefore, a recovery study was performed by spiking samples with different concentrations of OTA. Thus, samples were spiked with OTA by adding the appropriate amount of methanolic stock solution to obtain concentrations above the corresponding limit of quantification in each coffee sample. Then, the slurry was stirred for approximately 5 min at room temperature for homogenization before carrying out the entire extraction procedure. All analyses were performed in duplicate.

2.4.3 Tea leaf samples

With the aim of minimizing the sample manipulation and analysis time, the IAC was not used for tea leaf analysis. However, considering the significant effect of the matrix, a standard addition method was applied. Since the evaluated tea samples did not contain detectable levels of OTA, a recovery study was carried out by spiking duplicate samples with the analyte to a final concentration range of 1.0–15.3 µg kg^{-1}. For this, approximately 1.500 g of leaf tea was weighed and placed in a 250 mL volumetric flask. The samples were then spiked with OTA by adding the appropriate amount of methanolic stock solution to obtain the concentrations mentioned above, stirred to homogenize the OTA, and allowed to stand for approximately 5 min at room temperature before the entire extraction procedure. Then, the samples were treated with 5.00 mL of extraction solvent, which was composed of a mixture of acetonitrile:acetic acid 99:1 (v/v). The extract was magnetically stirred for 15 min. Then, the mixture was sonicated for 15 min and centrifuged for 15 min. Finally, the supernatant was evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1.00 mL of acetonitrile containing 0.1 mol L^{-1} NaOH (pH 9.3). Then, a 300 µL aliquot of the sample was placed in a quartz microcell, and the EEFM was evaluated under the conditions described in Section 2.2. Subsequently, four adequate volumes of standard OTA solutions were added directly to the cell, and one EEFM was measured after every addition to carry out the standard addition method.

2.5 Software and chemometric algorithms

The theory of second-order algorithms has been well documented in the literature. Several papers [35–37] are presented in the Supplementary Material to provide details about the main goals with the algorithms selected in this study for data analysis. All algorithms were applied

using an MVC2 program, which is a MATLAB graphical interface toolbox that is a new version of that already reported in the literature [38] and is freely available on the Internet [39].

2.6 Figures of merit and statistical indicators

The evaluated figures of merit were the limit of detection (LOD), limit of quantification (LOQ), root mean square error of prediction (RMSEP) and relative error of prediction (REP), which were estimated in accordance with previous publications [40]. All of the above figures of merit are integrated into the MVC2 interface and can be easily estimated.

3. Results and discussion

3.1. Fluorescence signal optimization

To improve the inherent fluorescence of OTA and thus increase the analytical performance of the proposed method, experimental variables that can influence this signal were optimized. First, different solvents were evaluated to measure the fluorescence response of OTA. As shown in Figure 1, a different-shaped emission spectrum is obtained in acetonitrile (Figure 1A) with a lower maximum intensity than in water (Figure 1B), thus evidencing a redshifted maximum emission when the most polar medium is used. These results are in accordance with previous reports [41, 42] and suggest that the polarity of solvents affects the photophysical behaviour of OTA. However, from an analytical point of view, both solvents produce similar sensitivities and appear to be potentially useful for OTA determination by fluorescence spectroscopy.

Another variable to be considered is the pH because OTA has acid-base properties ($pK_{a1} \sim 4$ and $pK_{a2} \sim 7$). At low pH, the protonated form of OTA is predominant, while the anionic form exists in basic aqueous media [43, 44]. The results presented in Figure 1A and 1B suggest that

the anionic form of OTA is more fluorescent, which is in agreement with previous reports [41–44]. Therefore, a pH of 9.3 was chosen for the subsequent experiments. Furthermore, as reported in previous work [45, 46], basic ammonia buffer can improve the fluorescent response of OTA; for this reason, this buffer solution was chosen for calibration, validation and coffee sample analysis.

Moreover, the influence of organized media on the fluorescence response of OTA was studied. The presence of methyl-β-cyclodextrin and micelles formed by SDS, TX-100 and HTAC did not produce significant changes in OTA fluorescence (see Figure S8 in the Supplementary Material). In contrast, a considerable improvement in the fluorescence was observed in the presence of HTAB (see Figure 1C), probably due to the capacity of this cationic surfactant to stabilize the OTA phenolate group [41]. Thus, HTAB was selected as an organized medium for further experiments.

On the basis of the experiments described above, the optimal experimental conditions for the spectrofluorimetric determination of OTA can be obtained in aqueous medium at pH 9.3 (ammonia buffer 0.020 mol mL^{-1}) in the presence of HTAB (10% v/v of 0.0025 mol mL^{-1}). Alternatively, an alkaline acetonitrile solution (0.1 mol L^{-1} NaOH, pH 9.3) can be used depending on the sample preparation requirements.

3.2 Calibration, validation and test samples

First, EEFMs of OTA were measured to produce calibration sets (Figure 2A). Then, a PARAFAC model was evaluated by arranging the EEFMs as a three-way array, and the number of factors required was selected by a “core consistency analysis” [47]. Additionally, a non-negativity constraint was applied in both modes and used for all analyses. According to this analysis, two components are required for an optimum data fit and can be justified considering

277 the presence of OTA and the background (see Figure S3 in the Supplementary Material). In
278 addition, the figures of merit and lower prediction errors (lower than 3%) for the validation set
279 presented in Table 1 confirm satisfactory fitting to the above-mentioned three-way data array
280 using the PARAFAC model.

281 To compare the analytical performance of different multivariate models, the MCR-ALS
282 model was also applied to predict the OTA concentration in the validation samples. The MCR-
283 ALS models obtained by using both augmentation modes were similar for synthetic samples
284 (validation set and test set). In the validation samples, the number of components was two, as
285 estimated by principal component analysis (PCA). The initial spectral profiles employed to start
286 the MCR-ALS fitting were obtained from the "purest variables" in the non-augmented mode.
287 Non-negativity in both modes was the constraint imposed during the ALS fit. As expected, MCR-
288 ALS was able to correctly retrieve the pure OTA spectral profile (see Figure S3 in the
289 Supplementary Data) and allowed satisfactory recoveries of the validation samples. In addition,
290 the obtained figures of merit were comparable to those obtained with PARAFAC (see Table 1).

291 The prediction of OTA concentration in the presence of unmodelled interferences is
292 mandatory when a real sample analysis is considered. For this reason, two additional fluorescent
293 mycotoxins, ZEN and STE, which can be present in coffee and tea samples [32, 33, 48], were
294 added to the samples (test samples) and evaluated as potential interferences (Figure 2B). These
295 samples were processed by both the PARAFAC and MCR-ALS models, and the estimated
296 number of responsive components was 4 in both cases (see Figure S4 in the Supplementary
297 Data). This number can be ascribed to the three fluorescent mycotoxins and the background
298 signal. For analysis of the test samples with MCR-ALS, an additional constraint provided by the
299 so-called correspondence was used, which indicates to the algorithm that certain components
300 (ZEN and STE) are absent in calibration samples. The recoveries and prediction results presented

in Table 1 for the test samples are quite similar for both models, suggesting that neither the accuracy and precision, as measured through RMSEP and REP, nor the sensitivity are significantly affected by the addition of these new mycotoxins.

3.3 Real matrices

The analytical performance of the proposed method must be tested with a real sample to demonstrate its analytical potential. Therefore, different Argentinian brands of coffee and tea were obtained from local stores and used as real matrices for further analysis.

3.3.1 Coffee samples

In this study, two types of coffee samples were investigated: ground roasted coffee and green coffee beans. Preliminary studies conducted on both types of coffee samples confirmed the severe inhibition of OTA fluorescence by the matrix constituents. The above effect had already been reported in a previous work [17]. Therefore, a clean-up procedure using IACs was carried out before taking the fluorescence measurements. In the case of the roasted coffee samples, OTA was not detected, and a recovery study was carried out by spiking the samples with different concentrations of the studied analyte. In contrast, the presence of low concentrations of OTA was verified in the selected green coffee. The mycotoxin may undergo a decomposition process during coffee roasting, which could explain the difference between the two types of coffee.

A typical EEFM of one roasted ground coffee spiked with OTA after the IAC treatment is shown in Figure 2C. The presence of strong interference is evident. Additionally, it is necessary to highlight the fact that although the sample was passed through the IAC, the presence of interferences is still observed, which makes it difficult to use a univariate calibration. Therefore,

the need to use a second-order multivariate calibration is confirmed because the cleaning of the sample is not complete.

When PARAFAC was applied to these samples, poor predictions were obtained, and the predictions were independent of the selected component numbers. This fact may be explained by the spectral similarity between the analyte and the constituents in the coffee spectra, which hinders the decomposition of the three-way data array to physically reasonable profiles and scores. Therefore, MCR-ALS was applied, which in principle is able to circumvent the problem of spectral similarity. The number of responsive components obtained in the coffee samples was four and could be explained by considering the presence of OTA, interferences and background signals. For MCR-ALS modelling, the augmentation was made on the emission wavelength dimension, non-negativity in both modes, and the correspondence between the components and samples were the constraints used during the ALS fit. This last constraint was applied considering that the analyte (component 1) is present in both calibration and coffee samples, but the interferences (components 2 and 3) are present only in coffee samples. Table 2 presents the MCR-ALS results obtained for the roasted and green coffee samples.

To demonstrate the accuracy of the proposed method for real coffee samples, a chemometric model was compared with a reference method based on HPLC-FLD. Both methods were compared through a paired Student's t-test, and the obtained values (t= 0.28 for roasted ground coffee and t=1.00 for green coffee beans, see Table 2) could be favourably compared with the tabulated values for *n*-1 degrees of freedom at a 95% significance level ($t_{crit\ (0.05,5)}$=2.77 and $t_{crit\ (0.05,3)}$=4.30, respectively); thus, the results suggested that the obtained values were statistically comparable to those provided by the reference method. The statistical equivalence among the obtained values demonstrates the capacity of MCR-ALS to cope with interferences from concomitants in the real samples.

348 It is also important to mention that although the European Commission has not
349 established maximum levels for green coffee samples, the LOD obtained with the proposed
350 method allowed the quantification of OTA at the $\mu g\,kg^{-1}$ level in the samples.
351
352 *3.3.2 Tea leaves*
353 Three types of tea leaf samples (black, red and white tea) were considered in this study.
354 Preliminary assays of the above samples showed no detected levels of OTA, and a recovery study
355 was therefore carried out; the samples were spiked with different concentrations of the analyte of
356 interest. This analysis was performed without previous clean-up and separation steps. Only an
357 extraction step was conducted using acidified acetonitrile, which is able to efficiently extract
358 OTA in similar matrices, such as herbal medicines [34]. After performing the procedure
359 described in Section 2.5.3, the signal of one real tea sample (Figure 3B) showed the presence of
360 significant fluorescent interferences that could hinder the quantification of OTA (Figure 3C). In
361 addition, preliminary results showed a significant matrix effect on the fluorescent response of
362 OTA. An alternative for the above problem was the use of the standard addition method coupled
363 with second-order calibration to ensure appropriate selectivity and the successful quantification
364 of OTA in these samples. Figure 4A shows the signal after successive additions of an OTA
365 standard solution, demonstrating the positive response to the OTA standard addition method.
366 Consequently, when PARAFAC and MCR-ALS were used to estimate the concentration of OTA
367 in spiked tea samples, both displayed satisfactory prediction results, which agreed with the
368 nominal values (see Table 3). For PARAFAC, recovery was between 90 and 110%, whereas for
369 MCR-ALS, the recovery was between 95 and 110%, suggesting that the proposed methodology
370 could overcome the problem of interactions with the background and the presence of unexpected
371 compounds (see Figure 4B).

372

373 *3.3.3 Comparison of the analytical methods*

374 Finally, the proposed method to determine OTA in matrices of interest is summarized and
375 compared with previously reported methods [17, 19, 26, 49–55]. As shown in Table 4, the
376 advantages and analytical potential of the proposed methods are evident. The proposed methods
377 are well suited for OTA analysis in complex matrices with the use of very simple equipment.
378 Additionally, the experimental time is minimal, and the procedures do not require significant
379 amounts of organic solvents, thus complying with the principles of green chemistry.

380

381 **4. Conclusions**

382 The analytical performance for the determination of ochratoxin A in coffee and tea
383 samples by matrix fluorescence spectroscopy and second-order multivariate calibration was
384 demonstrated. The applied chemometric models showed low prediction errors and adequate
385 figures of merit. Therefore, the successful results indicate that this method provides a useful and
386 reliable methodology for the satisfactory determination of OTA in complex samples such as
387 coffee because it was possible to quantify the analyte even in the presence of concomitant
388 interferences because the chemometric tools efficiently achieved the “second-order advantage”.
389 In the case of tea leaf samples, the determination was carried out without the need for sample
390 pretreatment; instead, a standard addition method was used.

391 In conclusion, the proposed methods are sensitive and selective and require minimal
392 experimental time; additionally, the methods are environmentally friendly because the volumes
393 of the employed organic solvents are minimal.

394 **Acknowledgements**

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FIGURE CAPTIONS

Fig. 1. (A) Fluorescence emission spectra for OTA in acetonitrile at different pH values. Profiles obtained for λ_{exc} = 345 nm. (B) Fluorescence emission spectra for OTA in water at different pH values. Profiles obtained for λ_{exc} = 390 nm. (C) Fluorescence emission spectra for OTA in a mixture of 10:90 HTAB/water at different pH values. Profiles obtained for λ_{exc} = 390 nm.

Fig. 2. Contour plots of the EEFMs for (A) a calibration sample containing 21.0 ng mL^{-1} OTA, (B) a test sample containing 20.5 ng mL^{-1} OTA, 100.0 ng mL^{-1} ZEN and 75.0 ng mL^{-1} STE, and (C) a ground roasted coffee sample spiked with 25.0 ng mL^{-1} OTA.

Fig. 3. Contour plots of the EEFMs for (A) a standard sample containing 20.0 ng mL^{-1} OTA in acetonitrile at pH 9.3, (B) a blank red tea sample (without OTA), and (C) a red tea sample spiked with 15.8 ng mL^{-1} OTA.

Fig. 4. (A) Augmented mode of the MCR-ALS profile for the addition standard method of a spiked black tea sample and (B) emission spectra of the spiked black tea sample: OTA (blue line), interference (green line) and baseline (red line).

TABLE CAPTIONS

Table 1. Prediction and statistical results for samples with OTA (validation set) and samples with OTA in the presence of potential interferences (test set) using a micellar medium (HTAB) and second-order multivariate calibration[a].

Table 2. OTA concentrations (µg kg^{-1}) in different samples of green coffee.

Table 3. Recovery study of OTA (µg kg–1) for spiked tea samples applying the standard addition method[a].

427 **Table 4**. Analytical performance of recently reported selective methods for OTA in coffee and
428 tea samples.

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Table 1. Prediction and statistical results for samples with OTA (validation set) and samples with OTA in the presence of potential interferences (test set) using a micellar medium (HTAB) and second-order multivariate calibration[a].

Validation set			**Test set[b]**		
Nominal	**PARAFAC**	**MCR-ALS**	**Nominal**	**PARAFAC**	**MCR-ALS**
0	-0.1 (0.1)	-0.2 (0.1)	0	0.0 (0.1)	-0.2 (0.1)
1.5	1.4 (0.1)	1.6 (0.1)	0.5	0.6 (0.2)	0.6 (0.2)
3.0	2.9 (0.1)	2.7 (0.3)	1.0	0.8 (0.2)	1.0 (0.1)
5.0	5.1 (0.1)	5.2 (0.2)	4.0	4.3 (0.1)	3.9 (0.3)
12.0	12.3 (0.1)	12.6 (0.1)	8.0	8.0 (0.1)	7.5 (0.1)
20.0	19.1 (0.1)	19.6 (0.1)	15.0	15.6 (0.1)	15.1 (0.1)
			18.0	18.2 (0.1)	19.2 (0.3)
			20.5	20.4 (0.1)	20.3 (0.9)
LOD[c]	0.2	0.2		0.2	0.2
LOQ[d]	0.5	0.5		0.6	0.5
RMSEP[e]	0.4	0.3		0.3	0.5
REP[f]	4	3		3	5

[a] Values are given in ng mL^{-1}. The found values are mean of duplicates. Standard deviation between parentheses.

[b] Samples containing random concentrations of ZEN and STE in the range of 70–150 ng mL^{-1}.

[c] LOD (ng mL^{-1}): limit of detection calculated as reference [40]

[d] LOQ (ng mL^{-1}): limit of quantification calculated as LOD×3

[e] RMSEP (ng mL^{-1}): root mean square error of prediction. RMSEP was calculated in accordance with $RMSEP = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)2}{n}}$, where $\hat{y}_i$ and y_i are the nominal and predicted concentrations for a given analyte in the *i*th sample, and n the total number of test samples.

[f] REP (%): relative error of prediction. REP was calculated in accordance with $REP\% = \frac{100 \times RMSEP}{\bar{y}_{cal}}$, where $\bar{y}_{cal}$ is the mean calibration concentration for the analyte of interest.

Table 2. OTA concentrations (µg kg–1) in different samples of green coffee.

A) Green coffee samples

	Found (HPLC)[b]	Found (MCR-ALS)[b]
Green coffee beans, brand X	1.05 (0.08)	1.0 (0.1)
Green coffee beans, brand Y	0.31 (0.01)	0.37 (0.04)
Green coffee in little bags	0.32 (0.01)	0.31(0.05)

B) Spiked ground roasted coffee samples

	Taken	Found (HPLC)[a]	Found (MCR-ALS)[a]	% Recovery[c]
With sugar, brand A	3.2	3.1 (0.1)	3.2 (0.2)	100
With sugar, brand B	10.0	10.2 (0.1)	10.9 (0.1)	109
Without sugar, brand C	6.0	5.9 (0.1)	5.7 (0.2)	95
Without sugar, brand D	13.0	11.8 (0.1)	12.0 (0.2)	92
Decaffeinated without sugar	25.0	25.1 (0.1)	25.2 (0.7)	101

[a] The found values are mean of duplicates. Standard deviation between parentheses.
[b] Not spiked. Preconcentration factor = 3.5 (see text).
[c] Recoveries, were calculated in relation to the added concentrations.

Table 3. Recovery study of OTA (µg kg–1) for spiked tea samples applying the standard addition method[a].

	Nominal	PARAFAC	Recovery (%)	MCR-ALS	Recovery (%)
Black tea	0	ND	–	ND	–
	5.5	5.8 (0.4)	105	5.8 (0.3)	105
	9.8	8.8 (0.1)	90	9.8 (0.1)	100
	15.3	15.0 (0.7)	98	15.2 (0.1)	99
Red tea	0	ND	–	ND	–
	1.0	0.8 (0.3)	80	1.1 (0.1)	110
	7.9	7.7 (0.6)	97	7.9 (0.2)	100
	11.7	11.7 (0.9)	100	11.9 (0.3)	102
White tea	0	ND	–	ND	–
	2.0	2.2 (0.1)	110	2.0 (0.2)	100
	3.9	4.2 (0.1)	108	4.0 (0.1)	102
	9.8	9.9 (0.5)	101	9.3 (0.1)	95
LOD[b]		0.3			0.2
LOQ[c]		0.8			0.5
RMSEP[d]		0.4			0.2
REP[e]		4			2

[a] Mean of duplicates. Standard deviation between parentheses. ND, not detected.
[b] LOD (ng mL^{-1}): limit of detection calculated as reference [40]
[c] LOQ (ng mL^{-1}): limit of quantification calculated as LOD×3
[d] RMSEP (ng mL^{-1}): root mean square error of prediction.
[e] REP (%): relative error of prediction.

Table 4. Analytical performance of recently reported selective methods for OTA in coffee and tea samples.

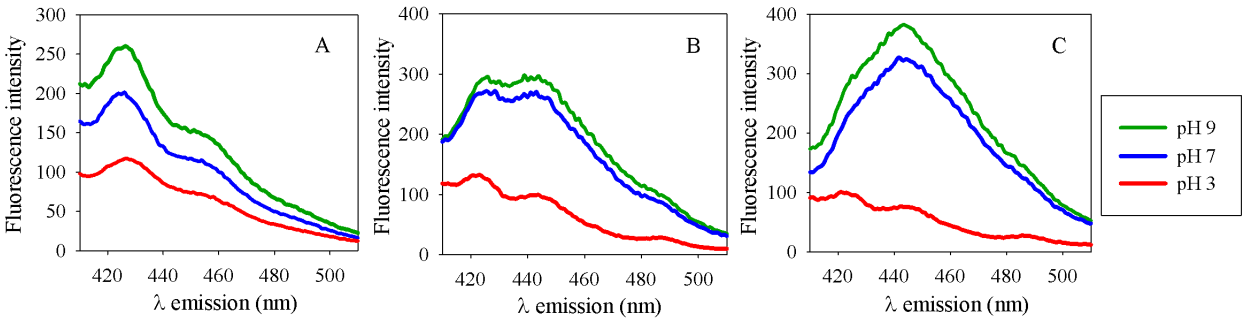
Sample preparation	Analytical method	Medium	LOD[a]	Accuracy / Precision[b]	REF
A) Coffee samples					
Step 1: S-L extraction **Step 2**: SPME clean up	**Analytical technique**: HPLC-FD **Column**: C18 (150 x 4,6 mm; 5µm) **Mobile phase**: acetonitrile/acetic acid 2% (44:56 v/v) **Run time**: 20 min	Organic	0.3	**RSD**: 3.3-4.1	[17]
Step 1: S-L extraction **Step 2**: IAC clean up	**Analytical technique**: HPLC-FD **Column**: Hypersil/BDS (125 x 4,0 mm; 5µm) **Mobile phase**: acetonitrile/water/acetic acid (421.5:570:8.5 v/v/v) **Run time**: 10 min	Organic	0.02	**REC**: 76.68-104 **RSD**: 1.54-8.20	[19]
Step 1: S-L extraction **Step2**: IAC clean-up	**Analytical technique**: HPLC-FD **Column**: Inertsil (150 x 4,6 mm; 5µm) **Mobile phase**: acetonitrile/water/acetic acid (50:49:1 v/v/v) **Retention time**: 9.5-10.8 min	Organic	0.07	**REC**: 83 **RSD**: 2.53	[49]
Step 1: S-L extraction **Step2**: IAC clean-up	**Analytical technique**: HPLC-FD **Column**: C18 (250 x 4,6 mm; 5µm) **Mobile phase**: acetonitrile/methanol/ water/acetic acid (35:35:29:1 v/v/v/v) **Run Time**: 10 min	Organic	0.09	**REC**: 88.8-90.6 **RSD**: 1.5-2.3	[50]
Step 1: S-L extraction **Step2**: IAC clean-up	**Analytical technique**: HPLC-FD **Column**: Zorbax Eclipse XDB-C18 (150 x 4,6 mm; 5µm) **Mobile phase**: acetonitrile/water/acetic acid (50:50:0.3 v/v/v) **Run Time**: 10 min	Organic	0.08	**REC**: 68.4-99.3 **RSD**: 7.41	[51]
Step 1: S-L extraction **Step2**: IAC clean-up	**Analytical technique**: HPLC-FD **Column**: Zorbax Eclipse plus-C18 (250 x 4,6 mm; 5µm) **Mobile phase**: acetonitrile/water/acetic acid (51:47:2 v/v/v) **Run Time**: 20 min	Organic	0.26	**REC**: 95.5-109.8 **RSD**: 1.5	[26]
Step 1: S-L extraction **Step2**: IAC clean-up	**Analytical technique**: FD **Analysis Time** : 4 min	Aqueous	0.20	**REC**: 92-109 **REP**:3-5	This work
B) Tea Samples					
Step 1: S-L extraction	**Analytical technique**: ELISA	Aqueous	0.02	**NR**	[52]
Step 1: S-L extraction **Step2**: IAC clean-up	**Analytical technique**: HPLC-FD **Column**: C18 (150 x 4,6 mm; 3µm) **Mobile phase**: acetonitrile/water/acetic acid (47:53:1 v/v/v)	Organic	**NR**	**REC**: 85	[53]

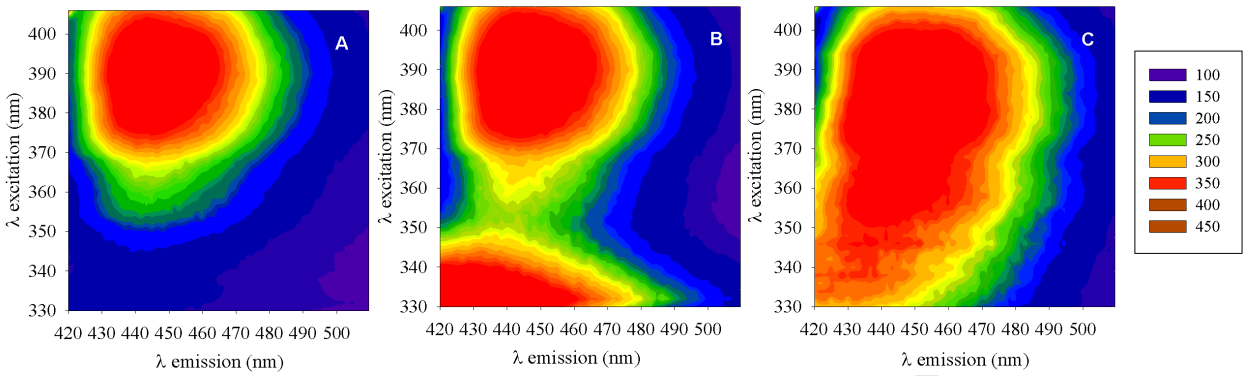
	Analysis Time: 20 min			**RSD**: 2.3	
Step 1: S-L extraction **Step2**: IAC clean-up	**Analytical technique**: HPLC-FD **Column**: C18 (300 x 4,6 mm; 10µm) **Mobile phase**: methanol/acetonitrile/ 0.05 mM sodium acetate/acetic acid (300:300:400:14 v/v/v/v) **Retention time**: 7.1 min	Organic	0.10	**REC**: 75-85 **RSD**: 1.3-3.7	[54]
Step 1: DLLME	**Analytical technique**: HPLC-MS/MS **Column**: Gemini-NX C18 (150 x 4,6 mm; 5µm) **Mobile phase A**: water/5mM ammonium formate, 0.1% formic acid) **Mobile phase A**: methanol/5mM ammonium formate, 0.1% formic acid) **Retention time**: 10.2 min	Organic	5.00	**REC**: 66 **RSD**: 3.0	[55]
Step 1: S-L extraction	**Analytical technique**: FD **Analysis Time**:4 min	Organic	0.2-0.3	**REC**: 95-110 **REP**:3-4	This work

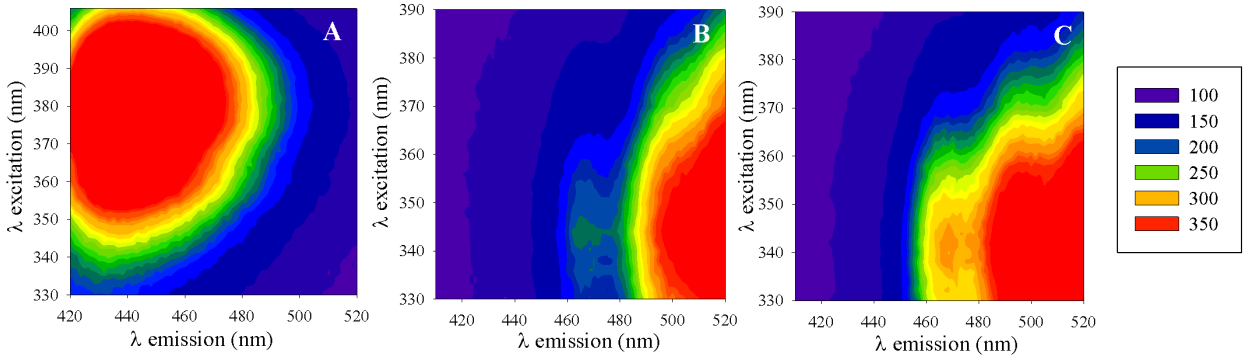
HPLC: high performance liquid chromatography. FD: Fluorimetric detection. MS: Mass spectrometry detection. MS/MS: Tandem Mass Spectrometry.

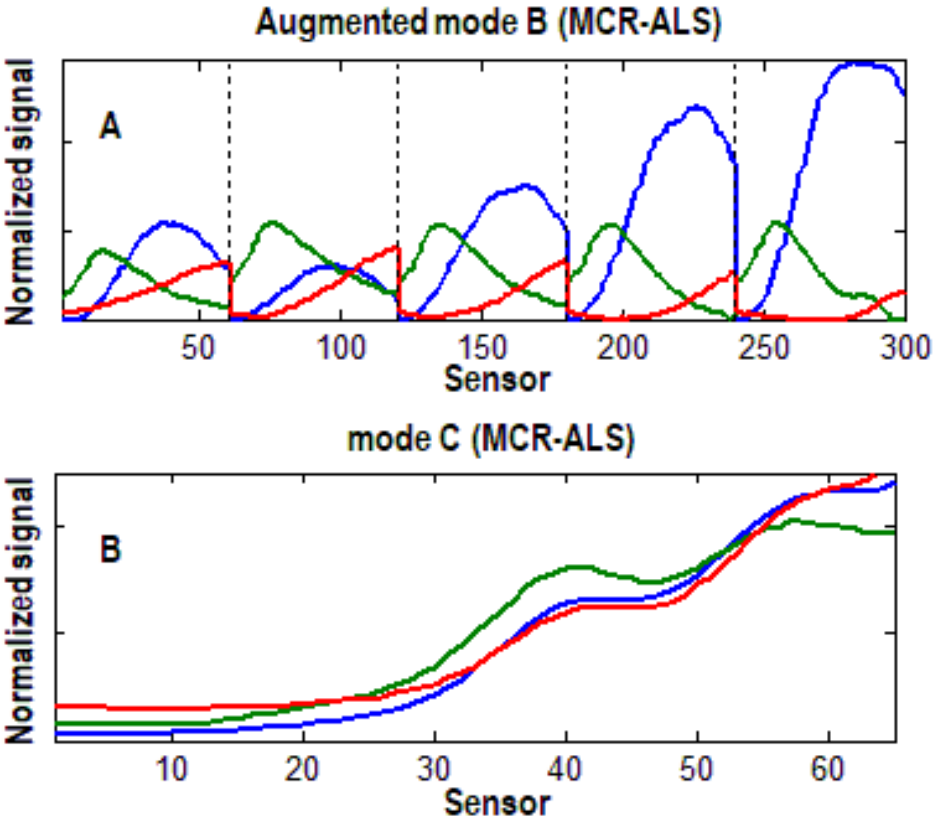
[a] For comparison, concentration units were unified to $\mu g \cdot Kg^{-1}$. Not Reported (NR)

[b] Recovery (REC), Relative standard deviation (RSD) and Relative error of prediction (REP), all in percentage. S-L, solid-liquid; IAC, inmunnoafinitty columns; DLLME, dispersive liquid-liquid microextraction, SPME, solid-phase microextraction.








Augmented mode B (MCR-ALS)
A
Normalized signal
50
100
150
200
250
300
Sensor
mode C (MCR-ALS)
B
Normalized signal
10
20
30
40
50
60
Sensor

A new analytical method based on second-order multivariate calibration was proposed for Ochratoxine A determination

The second-order advantage allowed to quantify Ochratoxine A in presence of unexpected interferences

The proposed method was applied to analysis of coffee and tea leave samples, demonstrating its analytical potential

The authors declare that there is no conflict of interest regarding the publication of this article

CRediT authorship contribution statement

Albani L. Gonzalez: Investigation, Writing-Original Draft, Conceptualization. **Valeria A. Lozano**: Investigation, Methodology. **Graciela M. Escandar**: Resources-Funding Acquisition, Supervision, Writing-review and editing. **Manuel Bravo**: Resources-Funding Acquisition, Supervision, Writing-Review and editing.