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FLUORESCENCE DETERMINATION OF COCHINEAL IN STRAWBERRY JAM IN THE PRESENCE OF CARMOISINE AS A QUENCHER BY MEANS OF FOUR-WAY PARAFAC DECOMPOSITION

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Abbreviations[2]

Abstract

The determination of cochineal (E-120) in strawberry jam was carried out in the presence of carmoisine (E-122) using the four-way PARAFAC decomposition and excitation-emission fluorescence matrices. In the measured conditions, there was no fluorescence signal for carmoisine due to a strong quenching effect and this colorant also led to a decrease of the fluorescence signal of cochineal. The European Union has fixed a maximum residue level, MRL, for cochineal in jam (100 mg kg^{-1}). Therefore, the addition of other food colorant (carmoisine) in the jam could lead to false compliant decisions. The four-way PARAFAC decomposition avoided false compliant decisions caused by the quenching effect.

[1] Corresponding author. Telephone number: 34-947-259571. *E-mail address*: mcortiz@ubu.es (M.C. Ortiz).
[2] Capability of detection (CCβ), core consistency diagnostic (CORCONDIA), decision limit (CCα), diode array detection (DAD), European Food Safety Authority (EFSA), excitation-emission fluorescence matrices (EEMs), high-performance liquid chromatography (HPLC), International Numbering System (INS), maximum residue levels (MRLs), Parallel factor analysis (PARAFAC), Rapid Alert System for Food and Feed (RASFF), time of flight mass spectrometry (TOFMS).

Cochineal was unequivocally identified. Detection capability (CCβ) was 0.72 mg L^{-1} for probabilities of false positive and false negative fixed at 0.05. Cochineal was detected in the jam (104.63 mg kg^{-1}) above the MRL. This amount was compared with the one obtained using a HPLC/DAD method.

Keywords: Cochineal; Carmoisine; Excitation-emission fluorescence; Four-way PARAFAC decomposition; Quenching effect; Strawberry jam.

Chemical compounds studied in this article

Cochineal (PubChem CID: 14950); Carmoisine (PubChem CID: 19118).

1. Introduction

The determination of analytes in complex matrices can be carried out using multi-way analysis. Three-way data are obtained joining second-order data (a data matrix) for several samples, whereas four-way data are obtained when third-order data are added in the fourth direction for a set of samples. Third-order data are being increasingly studied (Escandar et al., 2014) and may provide richer analytical information. Examples of four-way data are obtained: i) with hyphenated instruments such as two-dimensional chromatography with diode array detection (DAD) or with time of flight mass spectrometry (TOFMS) (Parastar et al., 2011) and ii) with a single instrument recording excitation-emission fluorescence matrices (EEMs) as a function of reaction time (Carabajal et al., 2017), pH (Pagani et al., 2017), proportion of solvent (Zhang et al., 2016), volume of quencher (Rodriguez et al., 2009a), or level of dilution (Rubio et al., 2015). Four-way data can also be acquired by monitoring EEMs at different elution time points in a high-performance liquid chromatography (HPLC) procedure (Montemurro et al., 2016).

Parallel factor analysis (PARAFAC) is one of the algorithms available for processing these multi-way data (Carabajal et al., 2017; Pagani et al., 2017; Zhang et al., 2016;

Rodriguez et al., 2009a; Rubio et al., 2015). The second-order advantage in analytical chemistry enables the identification and quantification of the analytes of interest even in the presence of uncalibrated interferents. This is possible when the experimental data array is compatible with the PARAFAC model.

However, the loss of trilinearity can be due to inner-filter effects caused by high concentrations, scattering and quenching (Andersen & Bro, 2003). Fluorescence quenching represents any process that decreases the fluorescence intensity of a sample. A wide variety of molecules or ions can act as quenchers (Lakowicz, 2006). Four-way PARAFAC decomposition was useful in the fluorescence determination of tetracycline under the strong quenching effect made by tea (Rodriguez et al., 2009a) and in the study of the robustness of the procedure to determine tetracycline in milk in the presence of chlorotetracycline as quencher (Rodriguez et al., 2009b).

Screening methods are widely used to assess the quality of foodstuffs. The application of fluorescence spectroscopy together with multi-way techniques has a huge potential for the evaluation of food quality (Karoui & Blecker, 2011) and food safety due to the low cost and high sensitivity of that spectroscopy. However, there could be a quenching effect in the measured conditions of the analytes of interest which might decrease their fluorescence signal and lead to false compliant decisions in their determination. The four-way PARAFAC decomposition can be used to solve this problem.

Food colorants are used to: i) restore the original appearance of food whose color has been affected by processing, storage, packaging and distribution; ii) make food more visually appealing; and/or iii) give color to food otherwise colorless (Regulation (EC) No 1333/2008). These food additives improve the worldwide consumer demand of foodstuffs while they mask their unpleasant characteristics (Martins et al., 2016). Foodstuffs and beverages have been colored since ancient times. The evolution of the use of food colorants can be seen in (Coultate & Blackburn, 2018).

The food colorant E-120 corresponds to cochineal, carminic acid and carmines; whereas azorubine (which is also named carmoisine) is identified as E-122. The European Food Safety Authority (EFSA) Panel (EFSA, 2015a) recently proposed the modification of the specifications for E-120 to "cochineal extract, carminic acid and carmines" which would more accurately reflect the material used as food colorant.

Synthetic dyes or constituents obtained from natural sources can be used as food colorants. Plants, algae, fungi, bacteria and animals such as insects are traditional sources of natural food color additives (Solymosi et al., 2015). Carminic acid is a natural food colorant extracted from the dried bodies of female cochineal insects (*Dactylopius coccus* Costa) that are harvested from Opuntia cacti (Coultate & Blackburn, 2018) in several parts of the world. Allergic reactions induced by the ingestion of processed foodstuffs containing cochineal extract or carmine have been reported (EFSA, 2015a; Takeo et al., 2018). These cochineal extract-induced allergic reactions, which include asthma, urticaria, dyspnea and anaphylaxis, can be due to the protein impurities from the insect source that remain after extraction of the cochineal extract (Yamakawa et al., 2009). The EFSA Panel (EFSA, 2015a) considered that further indication on the proportions or percentages of the protein content, molecular weight of the key allergenic proteins, total ash, residual solvents, or insoluble matter in the commercial product of E-120 should be required.

On the other hand, the safety of synthetic food colorants has also been widely evaluated. Several analytical methods have been developed for the analysis of azo dyes commonly used in food industries of different parts of the world (Yamjala et al., 2016). Carmoisine is a synthetic azo colorant authorized as a food additive in the European Union. Regulation (EC) No 1333/2008 (Regulation (EC) No 1333/2008) establishes that the labelling of food containing carmoisine shall include the following warning information on the packaging: "may have an adverse effect on activity and attention in children". McCann et al. (2007) reported that the intake of mixtures of four

synthetic colorants (containing carmoisine) together with sodium benzoate as preservative increased hyperactivity in 3-years old and 8- to 9-years old children. However, EFSA Panel concluded that it is not possible to relate the findings of that study to any of the individual compounds since the hazards of individual food additives cannot be evaluated using mixtures (EFSA, 2008). EFSA Panel has also reported a refined exposure assessment for carmoisine as a food additive (EFSA, 2015b).

In the European Union, the maximum residue levels (MRLs) for some food colorants have been set depending on the food category in Commission Regulation (EU) No 1129/2011 to ensure good manufacturing practices and health security of consumers. Several notifications have been transmitted to the Rapid Alert System for Food and Feed (RASFF) of the European Commission (RASFF Portal, 2018) by different EU countries due to an unauthorised use or a too high content of E-120 and E-122. From 13/06/2003 to 20/06/2018, 11 notifications for E-120 and 63 for E-122 were transmitted.

A wide revision about the methodology used for the determination of cochineal/carminic acid/carmine in foods from 1986 to 2011 can be seen in (Scotter, 2011). In 2015, a re-evaluation of cochineal, carminic acid, carmines (E-120) as food additives, which was carried out by EFSA Panel (EFSA, 2015a), reported several methods for the determination of these colorants. The most used techniques are spectrophotometric techniques (Samari et al., 2010) or HPLC with UV-Vis or fluorescence detectors. The electrochemical procedures have been less used due to the lack of selectivity caused by the high dependence of external factors such as pH, temperature or the presence of organic interferences. Ultra-high-performance liquid chromatography/tandem high-resolution mass spectrometry analysis has been recently used for that task since it is a sensitive and accurate technique (Gossetti et al., 2015). Yamjala et al. (2016) reported several methods to determine azo dyes employed in food industry such as carminic acid and carmine in wine and soft drink. Ordoudi et al.

(2018) details several procedures based on spectroscopic and chromatographic techniques and the recoveries found with them for a specific concentration level.

Although these methods are well-proven and widely accepted, there are some problems (relatively expensive equipment, requirement of advanced technical expertise, extensive and time-consuming sample preparations) that prevent them from developing and expanding their application. Therefore, the development of a fast, cost-effective and sensitive method for the quantitative determination of cochineal is needed. A suitable candidate would be EEM fluorescence, which is a highly sensitive technique, coupled with PARAFAC to avoid extraction steps.

In this work, the determination of cochineal in strawberry jam was carried out in the presence of carmoisine, which acted as a quencher, using molecular fluorescence spectroscopy (with excitation-emission data) and four-way PARAFAC decomposition. Nowadays, the MRL for cochineal has been fixed at 100 mg kg^{-1} in fruit jam, jellies and marmalades (Commission Regulation (EU) No 1129/2011). As far as the authors are aware, this is the first time that the analysis of samples that contained these two food colorants has been performed using PARAFAC which avoids the use of time-consuming clean-up procedures. The amount of cochineal found in the jam was also estimated with a HPLC/DAD method.

2. Material and methods

2.1. Chemicals

Cochineal powder was obtained from Panreac (Barcelona, Spain). Chromotrope FB (carmoisine, CAS no. 3567-69-9) (dye content 50%) was purchased from Sigma-Aldrich (Steinheim, Germany). Sodium tetraborate decahydrate (CAS no. 1303-96-4) and methanol (CAS no. 67-56-7) (gradient grade for liquid chromatography

LiChrosolv®) were obtained from Merck (Darmstadt, Germany). Hydrochloric acid

(37%, CAS no. 7647-01-0) was supplied by VWR International (Radnor, Pennsylvania,

USA).

The Milli-Q® Direct 8 water purification system from Millipore (Bedford, MA, USA) was

used to obtain deionised water.

2.2. Standard solutions

A stock solution of cochineal at 1000 mg L^{-1} and of carmoisine at 500 mg L^{-1} were

prepared individually in a sodium borate, hydrochloric acid buffer solution (borate

buffer) at pH 8 (Perrin & Dempsey, 1974). Intermediate solutions were prepared from

the stock solutions by dilution in the borate buffer. All the solutions were stored at low

temperature (4ºC) and protected from light.

The samples for the standard addition method were prepared by adding 1 mL of the

test sample or the final extract (depending on the case study) and the appropriate

volume of the cochineal and/or carmoisine solutions into 5-mL volumetric flasks and

completing to the mark with buffer solution.

2.3. Sample preparation procedure

A jar of strawberry jam, whose label specified that cochineal was contained as food

colorant, was purchased from a local supermarket (Burgos, Spain). 2 g of this jam was

placed into a 50-mL polypropylene tube and extracted with 10 mL of the buffer solution.

The mixture was stirred for 2 min using a vortex mixer. The extract was filtered through

a Whatman™ filter paper qualitative and the filtrate was collected in a 10-mL volumetric

flask and completed to the mark. To perform the standard addition method, this

procedure was repeated, and the resulting extracts were combined to eliminate the

variability.

For the recovery study, each 2 g of sample was fortified prior to extraction to contain 187.5 mg kg^{-1} of cochineal.

2.4. Instrumental

A micropH 2002 Crison pH-meter (Barcelona, Spain) was used to measure the pH value of the buffer solution. A vortex stirrer LBX Instruments V05 series (Barcelona, Spain), with speed control, was also used. The Whatman™ filter paper qualitative (110 mm diameter) was obtained from GE Healthcare (Little Chalfont, UK). The polypropylene syringe filters (13 mm diameter, 0.22 μm pore size) used to filter the samples prior to the HPLC/DAD analysis were purchased from Membrane Solutions (Kent, WA, USA).

Fluorescence measurements were performed at room temperature on a PerkinElmer LS 50B Luminiscence spectrometer (Waltham, MA, USA). A standard cell holder and a 10 mm quartz SUPRASIL® cell with cell volume of 3.5 mL by PerkinElmer (Waltham, MA, USA) were used.

An Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) that consisted of a quaternary pump (G1311C), a standard autosampler (G1329B), a thermostatted column compartment (G1316A) and a diode array detector (G7117C) was also used. The chromatographic column was a Kinetex EVO C18 (150 mm × 4.6 mm, 5.0 μm particle diameter) (Phenomenex, Torrance, CA, USA).

2.5. Fluorescence measurements

The corresponding excitation-emission matrices were recorded in the following ranges: excitation between 445 nm and 510 nm (each 5 nm), whereas the emission wavelengths varied from 515 nm to 620 nm (each 1 nm). The excitation and emission

monochromator slit-widths were both set to 10 nm and the scan speed was 1500 nm min^{-1}.

2.6. HPLC/DAD conditions

The mobile phase consisted of 60% of methanol and 40% of water. The injection volume was set at a volume of 10 µL. The flow rate was set to 1 mL min^{-1} and the temperature of the column compartment was 20ºC. The total elution time was 4 min except for the extracts in which 10 min was considered to maintain the cleanliness of the system. The chromatograms were fragmented around the retention time of cochineal considering only 100 elution times (Section 4.2.3). The diode array detector was programmed to measure the absorbance in a range between 350 nm and 600 nm (each 2 nm) for each elution time.

2.7. Software

The FL WinLab software (PerkinElmer) was used to register the fluorescent signals, whereas the OpenLab CDS software was used in the HPLC/DAD. The data were imported to MATLAB (2017) using the INCA software (Andersson, 2018) which removes the Rayleigh signals into the matrix in the wavelengths that correspond to this effect. PARAFAC decompositions were performed with the PLS_Toolbox (Wise et al., 2017) for use with MATLAB. The regression models were fitted and validated with STATGRAPHICS Centurion XVI (2010). Decision limit (CCα) and capability of detection (CCβ) were calculated using the DETARCHI program (Sarabia & Ortiz, 1994).

3. Theory

3.1. Four-way PARAFAC decomposition

The quenching effect in fluorescence spectroscopy can be described by means of multi-way approaches (Leurgans & Ross, 1992). When the level of quencher is considered, a four-way array $\underline{\mathbf{X}}$ with dimension ($I \times J \times K \times L$) can be built with the fluorescence intensity of the sample i-th at the emission wavelength j-th, excitation wavelength k-th and l-th level of quencher. In this case, the quadrilinear PARAFAC model obtained through the decomposition of $\underline{\mathbf{X}}$ is the following:

$$x(i,j,k,l) = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} d_{lf} + \varepsilon_{ijkl}, \quad i = 1,..., I, j = 1,..., J, k = 1,..., K, l = 1,..., L \qquad (1)$$

where F is the number of factors; a_{if}, b_{jf}, c_{kf} and d_{lf} are the elements of the loading matrices $\mathbf{A}$ ($I \times F$), $\mathbf{B}$ ($J \times F$), $\mathbf{C}$ ($K \times F$) and $\mathbf{D}$ ($L \times F$), respectively; and e_{ijkl} is the residue that is not explained by the model. In this work, the vectors $\boldsymbol{a}_f = (a_{if})$, $\boldsymbol{b}_f = (b_{jf})$, $\boldsymbol{c}_f = (c_{kf})$ and $\boldsymbol{d}_f = (d_{lf})$ are the sample, emission, excitation and quencher profiles of the f-th fluorophore, respectively.

If the level of quencher is not taken into account, the trilinear PARAFAC model for a three-way data array with dimension ($I \times J \times K$) is used:

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk}, \quad i = 1, 2,..., I; \quad j = 1, 2,..., J; \quad k = 1, 2, ..., K \qquad (2)$$

The elements of the loading vectors for each factor in Eq. (2) have the same meaning as in Eq. (1).

A sample should be considered as an outlier in a profile of the PARAFAC decomposition when the Q and Hotelling's T^2 statistics exceed their threshold value at a certain confidence level. In that case, the PARAFAC model should be estimated again without that sample.

Data are quadrilinear or trilinear when the experimental data array is compatible with the structure of Eq. (1) and Eq. (2), respectively. In this case, the profile estimations are

unique. The degree of trilinearity of the experimental data array can be measured through the core consistency diagnostic (CORCONDIA) (Bro & Kiers, 2003) which should be close to 100%. The uniqueness property enables the unequivocal identification of analytes by means of the excitation and emission spectra (Bro, 1997). Several European regulations (Commission Decision (EC) No 2002/657/EC) allow the unequivocal identification of the analytes with a validated method when two independent modes are provided for the identification (for example, the excitation and emission profiles obtained through the excitation-emission fluorescence matrices).

4. Results and discussion

4.1. Reference spectra

In the measured conditions of this work, there was no fluorescence signal for carmoisine due to a strong quenching effect as can be seen in the EEM landscapes of Fig. 1. A sample containing 5 mg L^{-1} of carmoisine in buffer solution (Fig. 1 (b)) decreased the fluorescence intensity of the buffer solution (Fig. 1 (a)), whereas the stock solution of carmoisine at 500 mg L^{-1} in buffer solution (Fig. 1 (c)) did not show fluorescence.

On the other hand, Fig. 2 shows the EEM landscapes of some samples that contained a fixed amount of cochineal and increasing concentrations of carmoisine. In this case, the presence of carmoisine caused the decrease of the fluorescence signal of cochineal without a modification in the shape of the signal. Therefore, it could be concluded that carmoisine acted as a strong quencher.

The experimental reference spectra for cochineal were obtained from the EEM recorded of a reference sample in buffer solution. The reference emission spectrum for this analyte (Fig. 3 (a)) was taken at the excitation wavelength that provided the maximum fluorescence intensity in the recorded region (475 nm), whereas the

reference excitation spectrum (Fig. 3 (b)) was obtained at the emission wavelength of 558 nm. These emission and excitation spectra were used as reference for the unequivocal identification of cochineal in the following stages of this work. This identification was carried out through the correlation between the reference spectra and the spectral loadings estimated from the corresponding PARAFAC model. The spectra of Fig. 3 had been normalized to compare them with the PARAFAC spectral loadings in each case.

4.2. Determination of cochineal in the presence of carmoisine

Five standards for cochineal (concentration range: 0-20 mg L^{-1}) were prepared at four different levels of carmoisine (quencher, concentration range: 0-1.5 mg L^{-1}) in buffer solution. The EEMs of all these 20 samples were arranged in a three-way array $\underline{\mathbf{X}}_1$ (20 x 106 x 14). The first dimension of this array corresponds to the number of samples, whereas the second and third ones correspond to the number of emission and excitation wavelengths recorded, respectively. The three-way PARAFAC decomposition of this array (no constraints imposed, explained variance of 99.78%) indicated the existence of one single factor corresponding to cochineal. The loadings of the three profiles of this PARAFAC model are included in Fig. 4. Each five loadings of Fig. 4 (a) corresponded to the five increasing levels of cochineal for a fixed concentration level of carmoisine. As can be seen in this figure, the loadings related to a same amount of cochineal decreased when the quantity of carmoisine contained in the sample was higher, so the values of the slope for different levels of quencher would be different. This fact could lead to false compliant decisions in the determination of cochineal according to the MRL established in (Commission Regulation (EU) No 1129/2011) so it is important to solve this problem to ensure food safety. Therefore, the use of this three-way PARAFAC model was not adequate.

The four-way PARAFAC decomposition was proposed to handle the quenching effect. So, the same EEMs contained before in the three-way array were now arranged in a four-way array $\underline{X}_2$ of dimension 5 x 106 x 14 x 4. In this case, the first dimension corresponds to the levels of cochineal, the second and the third ones are the same as in the previous array and the fourth dimension corresponds to the levels of quencher. The PARAFAC model, which was estimated with no constraints, needed only one factor (explained variance of 99.77%) and no outlier data were found once Q and Hotelling's T^2 statistics were applied. The CORCONDIA index cannot be calculated in the PARAFAC decomposition with only one factor. The sample, emission, excitation and quencher profiles of this one-factor model are shown in Fig. 5 (figures on the left). Cochineal was unequivocally identified since the correlation coefficients between its reference emission and excitation spectra (Fig. 3 (a) and (b) respectively) and the PARAFAC emission and excitation profiles (Fig. 5 (b) and (c), respectively) were 0.97 and 0.95, respectively.

The quencher profile (Fig. 5 (d)) was coherent since the loadings of cochineal decreased with increasing concentrations of carmoisine. On the other hand, only a sample profile for cochineal was obtained for all the levels of quencher due to the use of a four-way PARAFAC model (see Fig. 5(a)). Therefore, false compliant decisions in the determination of cochineal were avoided using this model.

A quadratic regression model "sample loading *versus* true concentration" was fitted and validated for cochineal. The second column of Table 1 collects the parameters of this regression and of the corresponding accuracy line, that is, the regression "predicted concentration *versus* true concentration". The mean of the absolute value of the relative errors in calibration was 1.17% (n = 4). As can be seen in this table, the p-values of the hypothesis test for the slope (b_1) and for the intercept (b_0) of the accuracy line were higher than 0.05 so the intercept and the slope were significantly not different

from 0 and 1, respectively. Therefore, the method had not constant or proportional bias at a 95% confidence level.

The values of decision limit (CCα) and capability of detection (CCβ) for cochineal were 0.38 and 0.72 mg L^{-1} with the probabilities of false positive (α) and false negative (β) fixed at 0.05.

4.2.1. Determination in a test sample

The determination of cochineal in a test sample was performed using the same standard addition method at 5 different levels of the quencher so a four-way analysis can be carried out. The concentration ranges were 0-25 mg L^{-1} for cochineal (6 standards) and 0-4 mg L^{-1} for carmoisine. The test sample was prepared in the laboratory at a known concentration of cochineal and carmoisine in buffer solution being 7.5 mg L^{-1} and 0.5 mg L^{-1} the final concentration, respectively. The 30 samples used in the standard addition method were prepared as explained in Section 2.2.

The four-way array $\underline{X}_3$ (6 x 106 x 14 x 5), which contained the EEMs of these samples, was built. Two buffer blanks were measured at the beginning and at the end of the experimentation to check the cleanliness of the cell, but these samples were not included in the array.

A four-way PARAFAC decomposition of the array $\underline{X}_3$ was carried out and the four profiles of the one-factor model obtained (no constraints imposed and explained variance of 99.7%) are shown in Fig. 5 (figures on the right). No outliers were detected in this model. The loadings were coherent with the experimental knowledge as can be seen in Fig. 5. The correlation coefficients were 0.99 for the emission and excitation profiles (Fig. 5 (f) and Fig. 5 (g), respectively) when these were compared to the reference spectra of cochineal (Fig. 3) so this analyte was unequivocally identified.

Table 1 (third column) collects the parameters of the calibration model "sample loading *versus* added concentration" and of the accuracy line for cochineal performed with the data obtained from this standard addition method. In the case of the calibration model, the corresponding quadratic regression model was significant. There was neither proportional nor constant bias according to the p-values of the hypothesis tests for the slope and the intercept (see Table 1, third column). As can be seen in this table, the CCα and CCβ values were nearly the same as the ones obtained in the previous Section 4.2.

The concentration of cochineal in the test sample was obtained in $y = 0$ due to the use of the standard addition method. The relative error in this determination was -16%.

4.2.2. Determination in strawberry jam

The predicted concentration of cochineal in the strawberry jam was calculated following the same previous strategy as in the determination of the test sample. Therefore, the same standard addition method as in Section 4.2.1 was performed at 5 different levels of the quencher. In addition, the strawberry jam was spiked only with cochineal at the beginning of the procedure to assess the recovery of the method. So, the standard addition method was also repeated using the spiked extract. Therefore, the two four-way arrays built were $\underline{\mathbf{X}}_4$, which contained the data of the standard addition method performed with the non-spiked extract, and $\underline{\mathbf{X}}_5$, which contained the data of the spiked extract. The dimension of both arrays was 6 x 106 x 14 x 5. The corresponding extracts were obtained following the sample preparation procedure detailed in Section 2.3, whereas the samples for the standard addition method were prepared as explained in Section 2.2. Some buffer blanks were also measured during the analysis as in Section 4.2.1, but they were not included in those arrays. The explained variance of the one-factor PARAFAC models obtained with $\underline{\mathbf{X}}_4$ was 99.28% and 99.44% with $\underline{\mathbf{X}}_5$ (no constraints imposed in any case). No outlier data were detected at a 95% confidence

level and the loadings of the four profiles were coherent with the experimental knowledge in both cases. However, the models were not coherent when an additional factor was considered, so there was not another fluorophore related to the matrix. The correlation coefficients for the emission and excitation profiles, regarding the reference spectra of cochineal (Fig. 3), were 0.98 and 0.93 for the data obtained from the non-spiked extract, and 0.99 and 0.95 for the data obtained from the spiked extract, respectively.

The parameters of the calibration models "sample loading *versus* added concentration" and of the accuracy line for cochineal obtained in both cases are collected in the two last columns of Table 1. As can be seen in this table, the absence of bias of the analytical procedure was verified at a 95% confidence level.

The recovery percentage was 65.47%, whereas the amount of cochineal found in the strawberry jam was 104.63 mg kg^{-1} which was above its MRL (Commission Regulation (EU) No 1129/2011). The confidence interval for that amount of cochineal found was (92.25, 116.75) mg kg^{-1} at a 95% confidence level.

4.2.3. Comparison of the determination of cochineal in the jam with a reference method

The amount of cochineal in the strawberry jam was also estimated with a HPLC/DAD method in order to compare the results with the ones obtained in Section 4.2.2. Therefore, ten standards of cochineal were prepared in buffer solution within the concentration range of 10-100 mg L^{-1}. In addition, the same non-spiked extract and the spiked extract measured in the previous Section 4.2.2 were also injected. However, a standard addition method was not performed, and those extracts were not diluted in this case. All the standards and the extracts were filtered through a polypropylene syringe filter (13 mm diameter, 0.22 μm pore size) before injection in the chromatographic system. A buffer blank was also measured at the beginning and at the end of the analytical sequence.

The chromatograms obtained from these 14 samples were fragmented around the retention time of cochineal. Figs. S1 (a) and (b) in the Supplementary Material show the chromatograms obtained at each wavelength for a standard containing 50 mg L^{-1} of cochineal and for the spiked extract, respectively. The first derivative of every spectrum at each elution time was carried out using a Savitzky–Golay method with a window of 15 points and a second-order polynomial for each sample. The derivative spectra at each elution time of a standard containing 50 mg L^{-1} of cochineal and of the spiked extract can be seen in Figs. S1 (c) and (d) of the Supplementary Material, respectively. A three-way array of dimension 100 × 126 × 14 was built with the matrices obtained with the preprocessing procedure. The first dimension corresponds to the number of elution times considered (see Fig. S1 (a) of the Supplementary Material), the second dimension is the number of wavelengths and the third one corresponds to the number of samples. The PARAFAC2 decomposition (Bro et al., 1999) was needed due to the shifts in the retention time in the samples. A two-factor PARAFAC2 model was estimated (CORCONDIA of 100%, explained variance of 97.49%, no outliers found) after a non-negativity constraint had been laid down only on the sample profile. The chromatographic, spectral and sample profiles of this model are shown in Fig. S2 in the Supplementary Material. As can be seen in this figure, the PARAFAC2 decomposition provides a chromatographic profile of each factor for each sample and a unique spectral profile for each factor that is common to all the samples. When Fig. S1 and Fig. S2 (a) and (b) of the Supplementary Material are compared, it is clear that one of the factors corresponded to cochineal, whereas the other one was related to the matrix together with the baseline drift. A reference sample containing 50 mg L^{-1} of cochineal was used to calculate the permitted tolerance interval for the retention time of this analyte. The retention time of cochineal obtained through the chromatographic profile was within that tolerance interval. In addition, the correlation coefficient between the reference derivative spectrum and the derivative spectrum obtained through the

PARAFAC2 spectral loadings was 0.99. Therefore, cochineal was unequivocally identified.

The calibration line "PARAFAC2 sample loading *versus* true concentration" for cochineal was $y = -2.29 \cdot 10^{-1} + 2.64 \cdot 10^{-1} \, x$ ($R^2 = 99.89\%$, $s_{yx} = 0.31$). The values of CCα and CCβ with the probabilities of false positive (α) and false negative (β) fixed at 0.05 were 2.51 and 4.89 mg L^{-1}, respectively. In this case, the amount of cochineal found in the strawberry jam was 98.43 mg kg^{-1}.

5. Conclusions

There was no fluorescence signal for carmoisine due to a strong quenching effect in the measured conditions and this food colorant also caused the decrease of the fluorescence signal of cochineal which could lead to false compliant decisions in the determination of cochineal. However, the four-way PARAFAC decomposition ensured food safety since it overcame that problem.

Cochineal was unequivocally identified and quantified even in the presence of carmoisine as a quencher using the proposed strategy based on the four-way PARAFAC decomposition and excitation-emission matrices together with the use of the standard addition method. In addition, cochineal was detected in the strawberry jam above its MRL (fixed at 100 mg kg^{-1}). The results have been compared with the ones obtained with a HPLC/DAD method.

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Author information

The authors declare no competing financial interest.

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FIGURE CAPTIONS

Fig. 1   EEM landscapes recorded for: (a) the buffer solution, (b) 5 mg L^{-1} of
carmoisine in buffer solution, and (c) the stock solution of carmoisine (500
mg L^{-1}) in buffer solution. (For interpretation of the references to color in this
figure legend, the reader is referred to the web version of the article).

Fig. 2   EEM landscapes of some samples that contained 20 mg L^{-1} of cochineal in
buffer solution (a) and: (b) 0.5 mg L^{-1} of carmoisine; (c) 1 mg L^{-1} of
carmoisine; and (d) 1.5 mg L^{-1} of carmoisine. (For interpretation of the
references to color in this figure legend, the reader is referred to the web
version of the article).

Fig. 3   Normalized emission (a) and excitation (b) reference spectra obtained for
cochineal with the reference sample (100 mg L^{-1} of cochineal in buffer
solution).

Fig. 4   Loadings of the three-way PARAFAC model with one factor (cochineal)
obtained with the data array $\underline{X}_1$ (20 x 106 x 14) for the: (a) sample profile,
(b) emission profile and (c) excitation profile.

Fig. 5   Loadings of the four-way PARAFAC model with one factor (cochineal)
obtained with the data array $\underline{X}_2$ (5 x 106 x 14 x 4, figures on the left) and
with the data array $\underline{X}_3$ for the determination of the test sample (6 x 106 x 14
x 5, figures on the right) for the: (a) and (e) sample profile, (b) and (f)
emission profile, (c) and (g) excitation profile, and (d) and (h) quencher
profile.

Fig. 1 (L. Rubio)

(a)

(b)

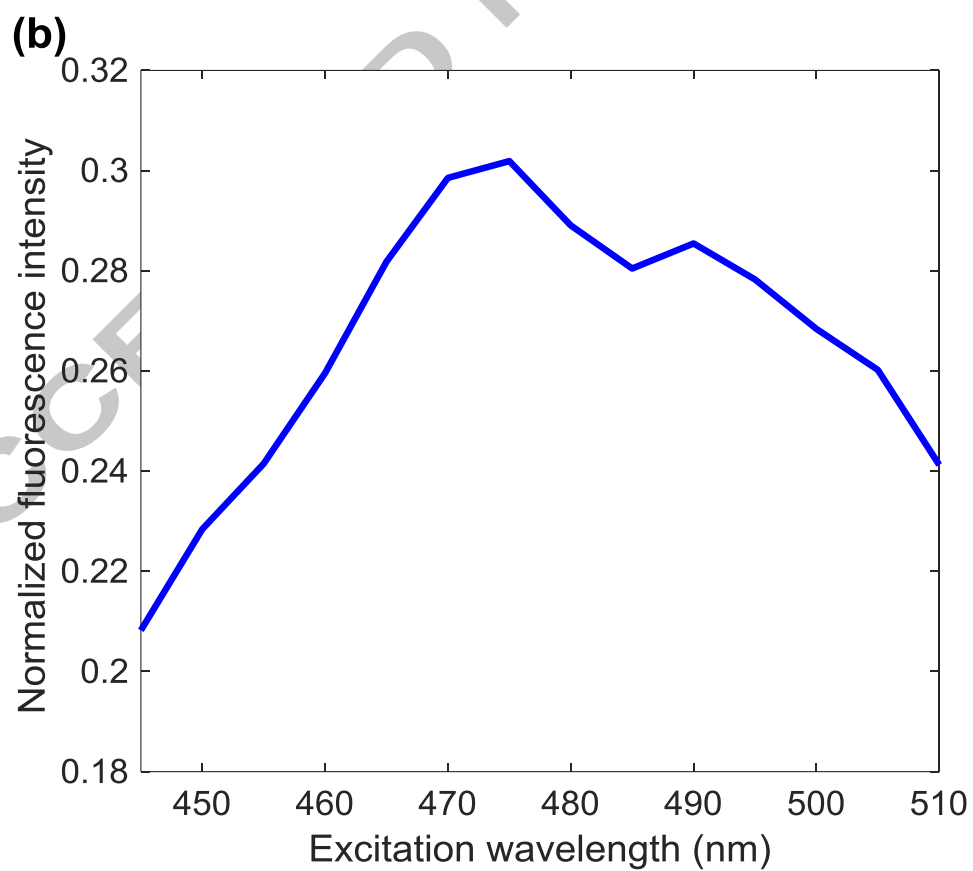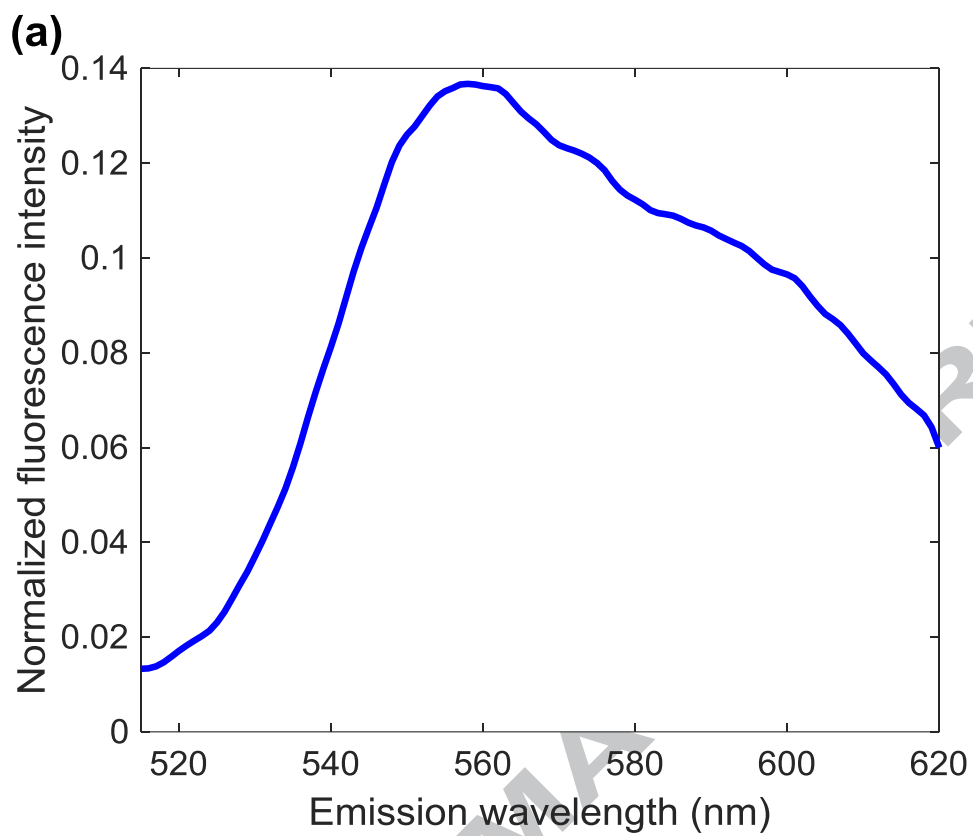**(c)**

(d)

Fig. 2 (L. Rubio)

(a)

(b)

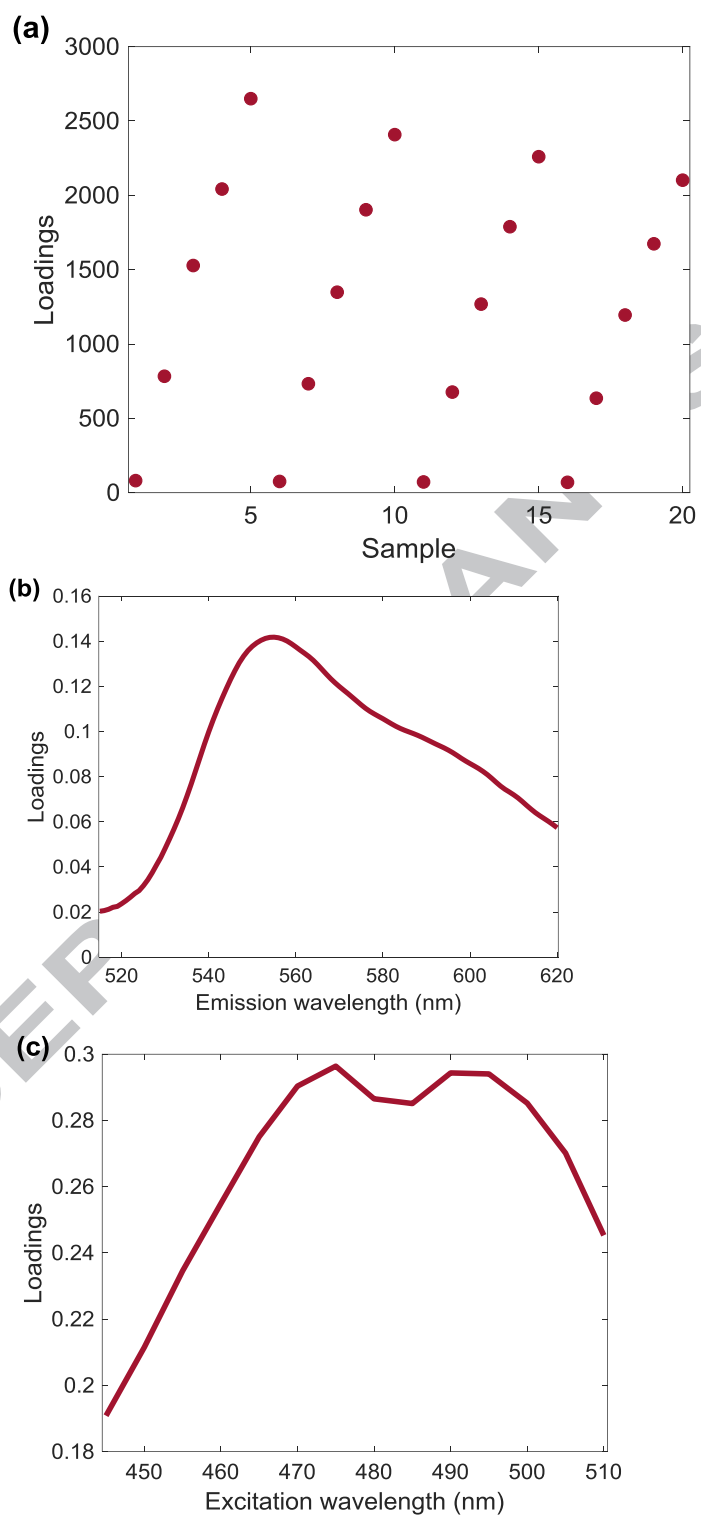**Fig. 3 (L. Rubio)**

(a)

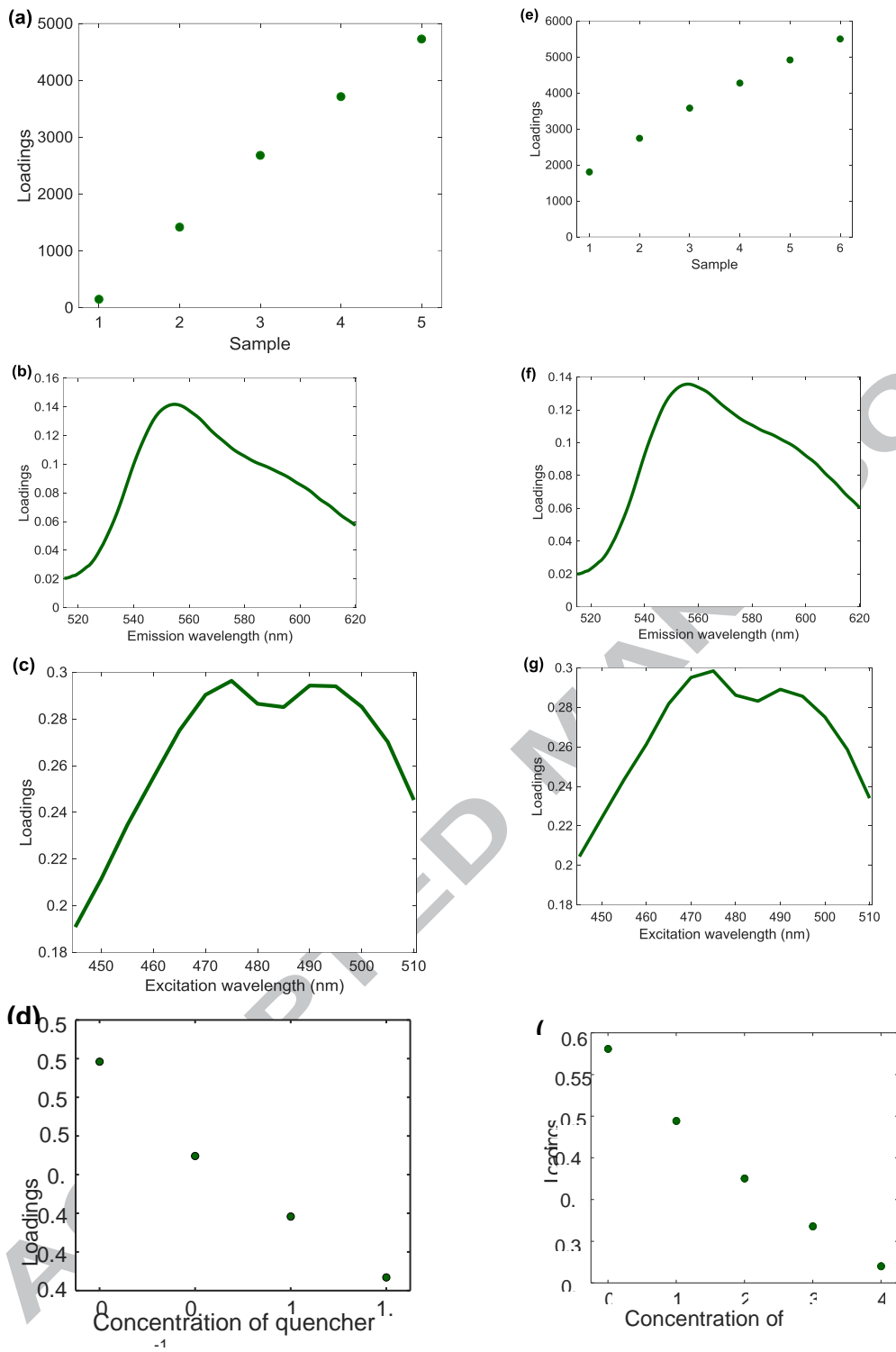
(b)

(c)
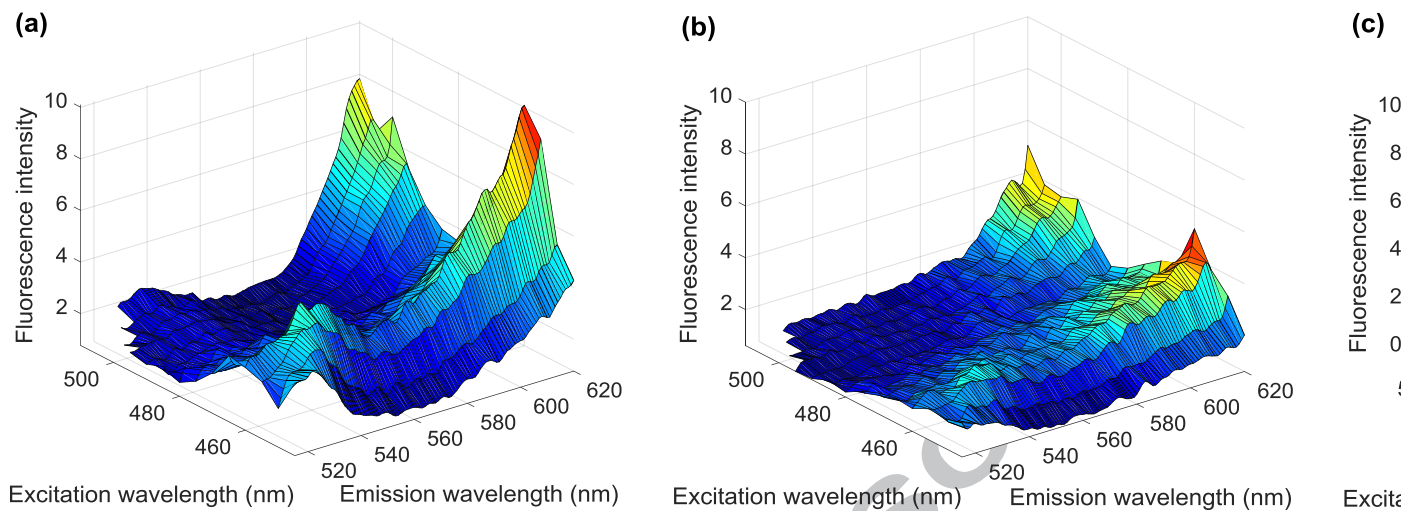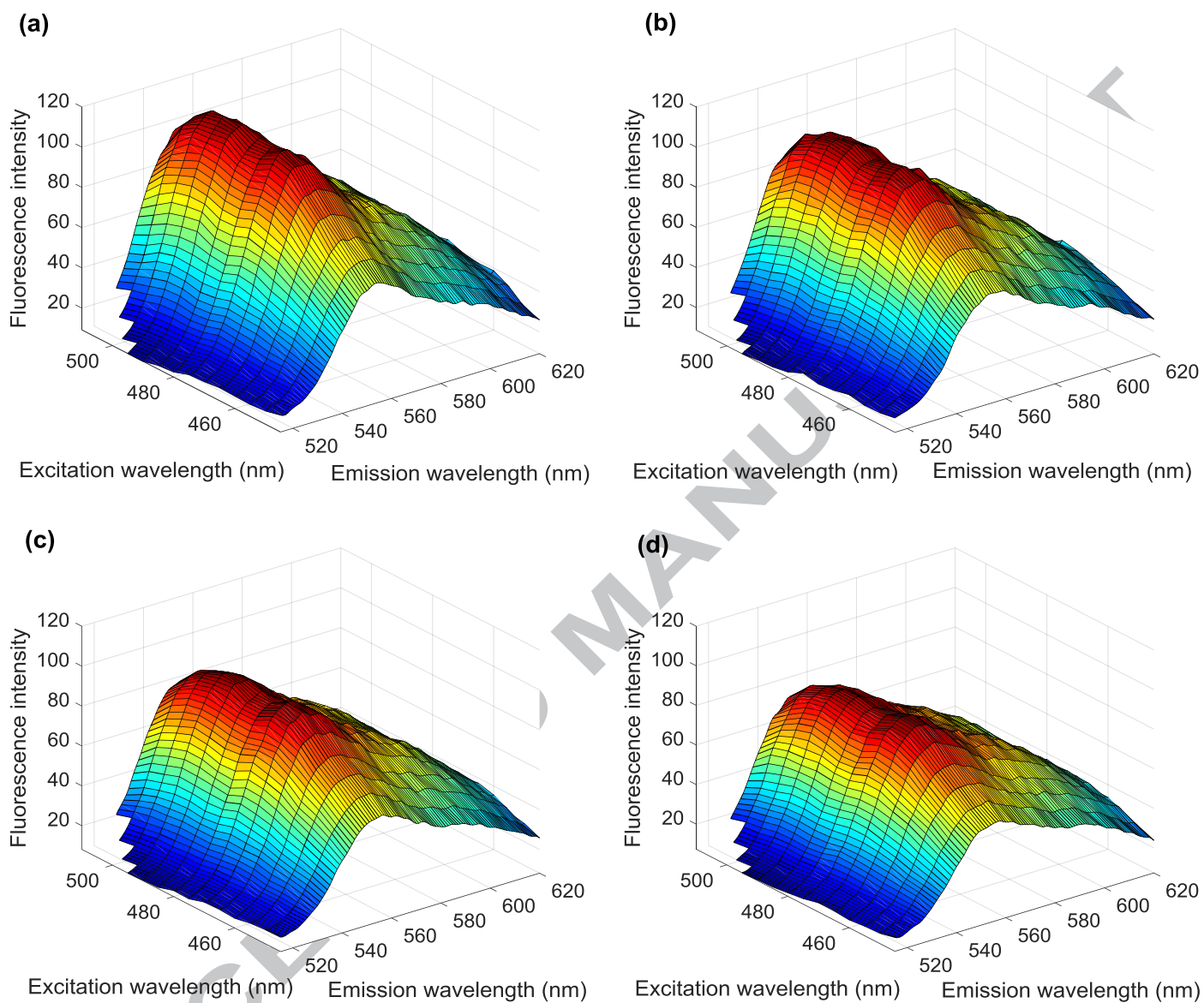
Fig. 4 (L. Rubio)

Fig. 5 (L. Rubio)

Table 1 Parameters of the calibration models ("sample loading *versus* true concentration" (second column) or "sample loading *versus* added concentration" in the rest of the cases) and accuracy line for cochineal obtained in the different stages of this work.

	Determination using standards (Section 4.2)	Determination in a test sample (Section 4.2.1)	Determination in strawberry jam (non-spiked extract, Section 4.2.2)	Determination in strawberry jam (spiked extract, (Section 4.2.2)
Calibration model	$y = 141.94 + 271.34\,x - 2.12\,x^2$	$y = 1814.86 + 192.46\,x - 1.82\,x^2$	$y = 1180.5 + 419.33\,x - 3.98\,x^2$	$y = 3042.18 + 370.29\,x - 3.57\,x^2$
Residual standard deviation, s_{yx}	36.86	21.80	57.53	59.21
R^2 (%)	99.98	99.99	99.98	99.97
$\overline{|e_r|}_{\text{calibration}}$ (%)[a]	1.17% (n = 4)	0.90% (n = 5)	1.24% (n = 5)	1.21% (n = 5)
Accuracy line	$y = 0.00 + 1.00\,x$	$y = -2.86 \cdot 10^{-3} + 1.00\,x$	$y = 4.29 \cdot 10^{-3} + 1.00\,x$	$y = -7.14 \cdot 10^{-3} + 1.00\,x$
p-value b_1	0.98	0.97	0.92	0.96
p-value b_0	1.00	0.98	0.97	0.97
Residual standard deviation, s_{yx}	0.13	0.14	0.15	0.21
CCα ($x_0 = 0$) (mg L^{-1})	0.38	0.37	0.41	0.56
CCβ ($x_0 = 0$)[b] (mg L^{-1})	0.72	0.70	0.77	1.06

[a] $\overline{|e_r|}$ is the mean of the absolute value of the relative error

[b] $\alpha = \beta = 0.05$

HIGHLIGHTS

Determination of cochineal in strawberry jam by EEM and four-way PARAFAC model

Carmoisine acted as a quencher in the fluorescence signal of cochineal

Four-way PARAFAC avoids false compliant decisions in the determination of cochineal

Cochineal was unequivocally identified and quantified in the presence of carmoisine

Cochineal was detected in the strawberry jam above its maximum residue level