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DETERMINATION OF COCHINEAL AND ERYTHROSINE IN CHERRIES IN SYRUP IN THE PRESENCE OF QUENCHING EFFECT BY MEANS OF EXCITATION-EMISSION FLUORESCENCE DATA AND THREE-WAY PARAFAC DECOMPOSITION

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Abstract

The simultaneous determination of two food colorants (cochineal (E-120) and erythrosine (E-127)) was achieved by means of excitation-emission fluorescence matrices and three-way PARAFAC decomposition together with the use of a calibration set that contained binary mixtures of both analytes. In the measured conditions, the amount of cochineal present in the sample affected the fluorescence signal of erythrosine since cochineal caused a quenching effect in the fluorescence of the other food additive. However, the signal of cochineal was not affected by the presence of erythrosine.

A calibration line for erythrosine was built for each different concentration level of cochineal. The slopes of these regressions were different depending on the amount of quencher, whereas the intercepts were statistically equal to 0 at a 95% confidence level. The quantification of erythrosine was possible using the regression "amount of cochineal" versus "the slope of the calibration line for erythrosine". Using this procedure, the mean of the absolute values of the relative errors in prediction for mixtures of both colorants were 5.86% (n = 10) for cochineal and 4.17% (n = 10) for erythrosine. Both analytes were unequivocally identified by the correlation between the pure spectra and the PARAFAC excitation and emission spectral loadings.

Pitted cherries in syrup were analyzed. Cochineal and erythrosine were detected in those cherries at a concentration of 185.05 mg kg^{-1} and 10.76 mg kg^{-1}, respectively. These concentration values were statistically equal to the ones obtained with a HPLC/DAD method.

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Graphical Abstract:
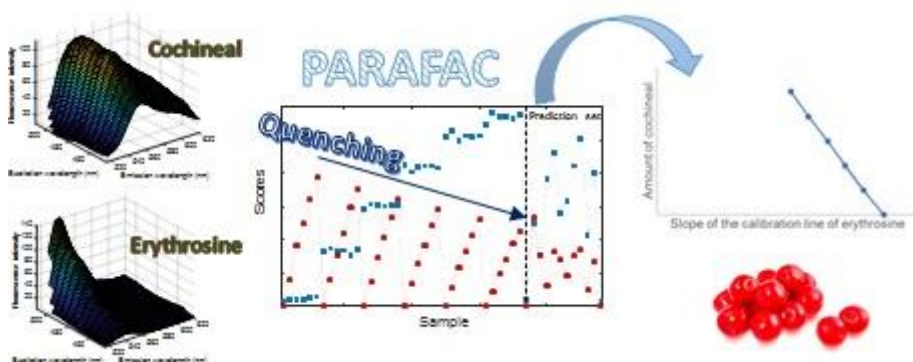
Abbreviations

CCβ, Capability of detection; CORCONDIA, core consistency diagnostic; CCα, decision limit ,DAD, diode array detection; EEMs, excitation-emission fluorescence matrices; HPLC, high-performance liquid chromatography; INS, International Numbering System; LS, linear least squares; MRL, maximum residue level; MLR, multiple linear regression; PARAFAC, Parallel Factor Analysis; PCA, principal component analysis; RASFF, Rapid Alert System for Food and Feed

Keywords: Cochineal; Erythrosine; Excitation-emission fluorescence; PARAFAC; Quenching effect; Cherries.

1. Introduction

Food colorants, which belong to one of the categories of food additives, are widely used in the food industry. Colour is one of the most important organoleptic properties of foodstuffs that directly influences consumers' selection and commercial success of the product [1]. Food industry adds natural or synthetic food colorants to: i) compensate of colour loss of natural food products due to the exposure to air, light, temperature and processing and storage conditions, ii) enhance the natural colour of the foodstuff, or iii) provide colour to foodstuff with no inherent colour to make the food more appealing to consumers [2,3]. Therefore, food colorants mask those unpleasant characteristics and improve the attractiveness of foodstuffs.

Synthetic food colorants have been associated with undesirable and harmful effects in some cases [1]. By way of example, the presence of four iodine atoms in the structure of erythrosine, a synthetic food colorant, may have a negative influence on the thyroid gland [2]. This has encouraged producers to obtain colouring alternatives from natural sources since these natural food colorants are perceived by consumers as safer [1,4,5]. However, the safety and whole effects of the natural ones need to be fully assessed. One of the natural food colorants is carminic acid which is extracted from the

dried bodies of the female cochineal insects, *Dactylopius coccus* Costa [4,6,7,8,9]. This compound is a major component of the cochineal extract. Those insects can be found on cacti from the type Opuntia in tropical and subtropical areas [10,11]. Carmine is the aluminum chelate of carminic acid [5,8]. Carminic acid has no mutagenic potential. However, some cases of severe allergic reactions have been reported after the consumption of carmine-containing foodstuffs and/or the exposure to cochineal extract [9,12,13].

The identification of food additives in the European Union is possible through a system of E-numbers which consist of the letter E (which stands for Europe) followed by the International Numbering System (INS) three-digit number [2]. The identification code as a food additive for erythrosine is E-127, whereas E-120 corresponds to cochineal, carminic acid and carmines.

Worldwide consumers want appealing foodstuffs that are at the same time safe and healthy [1]. Regulatory legislation has been established to ensure the good manufacturing practices and protect the health of consumers. European Union has established maximum residue levels (MRL) for some food additives depending on the food category [14]. Nowadays, erythrosine is permitted as a colorant in foodstuff but is limited for use only in cocktail cherries or candied cherries up to a MRL of 200 mg kg^{-1} and in Bigarreaux cherries in syrup and in cocktails up to 150 mg kg^{-1}. However, the use of cochineal is allowed in several foodstuffs such as preserves of red fruit, jam, jelly, fruit-flavoured breakfast cereals, precooked crustacean, sausages, bitter soda and some types of cheese. The MRL for cochineal has been fixed at 200 mg kg^{-1} in preserves of red fruit.

The Rapid Alert System for Food and Feed (RASFF) of the European Commission [15] has reported 115 notifications of an unauthorised use of E-127 in different foodstuffs from 22/10/2001 to 01/12/2017. In addition, 11 notifications for E-120 were transmitted to the RASFF from 13/06/2003 to 02/08/2017: 8 of them were due to an unauthorised use, whereas the rest were due to a high content of this food colorant. The values found were: 517 mg kg^{-1} in candied cherries, 356 mg kg^{-1} in smoked horse sausage and 114.9 mg kg^{-1} in soluble preparation for green tea and forest fruits flavoured soft drinks.

Accurate and reliable methods for the determination of food colorants are required for the assurance of food safety. Several analytical methods have been developed for the determination of E-120 and/or erythrosine in foodstuffs using spectrophotometric methods [16,17,18], differential pulse polarography [7], capillary electrophoresis with laser-induced fluorescence detection [19] and chromatographic methods such as high-performance liquid chromatography (HPLC) with diode array detection (DAD) [4,20], fluorescence detection [4] or with an ultraviolet detector [21], and ultra-high-performance liquid chromatography/tandem high-resolution mass spectrometry [22]. A HPLC method with a photodiode array detector to differentiate carmine from carminic acid has also been reported [6].

The main advantages of fluorescence spectroscopic methods lie in their ease of use, high sensitivity, fast measurements, availability of portable instruments and low cost in

contrast to the use of chromatographic techniques. However, the signals of the analytes may be overlapped with each other, with interferents or with the fluorescent matrix constituents in complex mixtures and even present quenching effect. The problem of spectral overlapping can be resolved using chemometric methods. The specificity can be recovered using Parallel Factor Analysis (PARAFAC) with excitation-emission fluorescence matrices (EEMs).

El-Kommos et al. [23] developed a spectrofluorimetric method for the determination of some non-sedating antihistamines based on the quenching effect of those drugs on the fluorescence intensity of erythrosine B. Several works have studied the quenching effect caused by other food colorants: sunset yellow (E-110) quenched the fluorescence of acridine orange [24], amaranth (E-123) quenched the fluorescence of haemoglobin [25], and quinoline yellow (E-104) quenched the fluorescence of bovine serum albumin [26].

The physical model of the quenching effect can be modelled by a PARAFAC quadrilinear model [27] if the possible absorbance of the quencher in the same excitation spectral region of the analyte of interest does not decrease significantly its emission intensity. In other case, a correction of the fluorescence signal as a function of the quencher concentration should be done [28] to maintain the quadrilinearity. In both cases, the amount of quencher can be considered as a fourth way. The four-way PARAFAC decomposition was used for the determination of tetracycline in tea under the strong quenching effect caused by the matrix in [29] and for the determination of polycyclic aromatic hydrocarbons in the presence of humic acid as fluorescent quencher [30]. The quenching effect can also be studied using three-way PARAFAC decomposition [31,32]. The identification and quantification of carbamate pesticides in dried lime tree flowers using three-way PARAFAC decomposition and EEMs were possible through an experimental procedure that minimized the quenching effect produced by the fluorophores of the lime flowers in [33].

The purpose of the present work was to develop a new, simple, selective and inexpensive method for the simultaneous determination of cochineal and erythrosine in cherries in syrup by means of EEMs and three-way PARAFAC decomposition without any time-consuming clean-up procedures or evaporation steps prior to the analysis. In the measured conditions, the fluorescence of erythrosine decreased with increasing amounts of cochineal. Therefore, a strategy was proposed to handle the quenching effect that cochineal caused in the fluorescence signal of the other food additive. This procedure did not require the use of four-way arrays that need a broad experimentation. The concentration of both food colorants found in the cherries in syrup was also estimated with a HPLC/DAD method.

2. Material and methods

2.1. Chemicals

Cochineal powder was obtained from Panreac (Barcelona, Spain). Erythrosine B (CAS no. 16423-68-0, for microscopy), methanol (CAS no. 67-56-7) (gradient grade for liquid

chromatography LiChrosolv®) and sodium tetraborate decahydrate (CAS no. 1303-96-4) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (37%, CAS no. 7647-01-0) was from VWR International (Radnor, Pennsylvania, USA).

Deionised water was obtained by using the Milli-Q® Direct 8 water purification system from Millipore (Bedford, MA, USA).

2.2. Standard solutions

A sodium borate, hydrochloric acid buffer solution (borate buffer) at pH 8 was prepared [34]. Stock solutions of cochineal and erythrosine at 1000 mg L^{-1} were prepared individually in the buffer solution. Intermediate solutions of 10 mg L^{-1} were prepared from the stock solutions by dilution in the same solvent. All these solutions were stored at low temperature (4ºC) and protected from light.

2.3. Sample preparation procedure

A jar of pitted cherries in syrup, which label specified that cochineal and erythrosine had been added to the foodstuff during its manufacture, was purchased from a local supermarket (Burgos, Spain). These cherries were homogenized using a blender and 2 g was placed into a 50-mL polypropylene tube. Next, 10 mL of the buffer solution was added, and 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. Then, the extract was diluted 8 times in buffer solution prior to its measurement in the fluorescence spectrometer, whereas the extract was not diluted in the HPLC/DAD analysis.

To prepare the spiked extract, each 2 g of sample was fortified prior to extraction to contain 200 mg kg^{-1} of cochineal and 6 mg kg^{-1} of erythrosine. After 15 min, the sample was extracted following the same procedure described previously.

2.4. Instrumental

The pH value of the buffer solution was measured using a micropH 2002 Crison pH-meter (Barcelona, Spain). A vortex stirrer LBX Instruments V05 series (Barcelona, Spain), with speed control, was also used. The seven different filters evaluated were: a Whatman™ filter paper qualitative (110 mm diameter), two types of Whatman® glass microfiber filters (GF/A grade and GF/C grade, 47 mm diameter) which were obtained from GE Healthcare (Little Chalfont, UK), a filter paper (Albet® LabScience, 73 g/m²), a polypropylene syringe filter and a polyethersulfone syringe filter (13 mm diameter, 0.22 µm pore size) purchased from Membrane Solutions (Kent, WA, USA), and a fritted funnel made of borosilicate glass with a diameter of 47 mm (Boroglass S.L., Barcelona, Spain).

A PerkinElmer LS 50B Luminiscence spectrometer (Waltham, MA, USA) was used to register the excitation-emission fluorescence measurements.

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 excitation-emission fluorescence measurements were performed at room temperature. The excitation-emission matrices were recorded using the standard cell holder and a 10 mm quartz SUPRASIL® cell with cell volume of 3.5 mL by PerkinElmer (Waltham, MA, USA). The excitation spectra were recorded between 445 nm and 510 nm (each 5 nm), whereas the emission wavelengths ranged 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}. The photomultiplier voltage was automatically selected by the instrument as a function of the slit width of the excitation monochromator.

2.6. Chromatographic conditions

A volume of 10 µL was injected into the chromatographic system. The mobile phase consisted of 60% of methanol and 40% of water. 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 diode array detector was programmed to measure the absorbance at the following wavelengths: 470, 500, 525 and 535 nm considering in all cases a reference wavelength of 400 nm (reference bandwidth of 30 nm). The integration of the chromatographic peak areas was carried out at 500 nm for cochineal and at 535 nm for erythrosine.

2.7. Software

The FL WinLab software (PerkinElmer) was used to register the excitation-emission fluorescent signals, whereas the OpenLab CDS software was used in the HPLC/DAD. The excitation-emission data were imported to MATLAB [35] using the INCA software [36]. The Rayleigh signals were removed by inserting missing values into the matrix in the wavelengths that correspond to this effect using [36]. PARAFAC decompositions were performed with the PLS_Toolbox [37] for use with MATLAB. The regression models were fitted and validated with the statistical program STATGRAPHICS Centurion XVI [38]. Decision limit (CCα) and capability of detection (CCβ) were calculated using the DETARCHI program [39].

3. Theory

3.1. PARAFAC decomposition

PARAFAC is a decomposition method which can be considered partly as a generalization of bilinear principal component analysis (PCA) to n-way data [40].

The PARAFAC model corresponds to the physical model for fluorescence data [41]. The trilinear PARAFAC model for a three-way data array **X** with dimension ($I \times J \times K$) which contains the fluorescence intensity of sample i-th at the excitation wavelength k-th and emission wavelength j-th, is:

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

where F is the number of factors; a_{if}, b_{jf} and c_{kf} are the elements of the loading matrices **A** ($I \times F$), **B** ($J \times$ F) and **C** ($K \times$ F), respectively; and e_{ijk} is the residue that is not explained by the model. The vectors $\boldsymbol{a_f} = (a_{if})$, $\boldsymbol{b_f} = (b_{jf})$ and $\boldsymbol{c_f} = (c_{kf})$ are the sample, emission and excitation profiles of the f-th fluorophore, respectively. The PARAFAC model is found by minimizing the sum of squares of the residuals and constraints (unimodality, non-negativity, etc) could be imposed in the profiles if they are necessary to develop an adequate model. These constraints should be applied considering the specific knowledge of the raw data [40]. For instance, the excitation and emission spectra and the concentration profile must always be positive.

The outliers can be identified in every profile through the Q and Hotelling's T^2 statistics. The Q residual index indicates the difference or residual between the value of the sample and its projection on the subspace of the model. A sample is influential in the PARAFAC model if its Mahalanobis distance, T^2, to the center of the data is large. When both indices exceed the threshold value at a certain confidence level in a sample, the PARAFAC model should be estimated again without that sample since it is an outlier.

Data are trilinear when the experimental data array is compatible with the structure of Eq. (1). The core consistency diagnostic (CORCONDIA) [42] is an index that measures the degree of trilinearity of the experimental data array. A trilinear model has a value of CORCONDIA index close to 100%. If the data are trilinear and the appropriate number of factors has been chosen to fit the model, the PARAFAC decomposition provides unique profile estimations. Therefore, it is possible to identify and quantify the analyte of interest in the presence of unknown interferents because the interferent(s) appear as new factor(s) without affecting the rest due to the second-order advantage [43]. The uniqueness property can be used for the unequivocal identification of analytes by means of the excitation and emission spectra [40].

3.2. Decision limit and capability of detection

According to the ISO norm 11843 [44], the decision limit is "*the value of the net concentration the exceeding of which leads, for a given error probability α, to the decision that the concentration of the analyte in the analyzed material is larger than that in the blank material*". Decision 2002/657/EC [45] accepts this definition as CCα or decision limit, whereas it is named as L_C by the International Union of Pure and Applied

Chemistry (IUPAC) [46]. On the other hand, the capability of detection or minimum detectable net concentration has been defined, for a given probability of false positive α, as "*the true net concentration of the analyte in the material to be analyzed which will lead, with probability 1-β, to the correct conclusion that the concentration in the analyzed material is different from that in the blank material*". This definition has also been accepted by IUPAC [46]. The minimum detectable net concentration is named CCβ in [45], L_D in [46], whereas in the ISO standard 11843 is x_d.

The capability of detection can be estimated using the following equation:

$$CC\beta = \frac{\Delta(\alpha,\beta)\omega_{x_0}\hat{\sigma}}{\hat{b}} \qquad (2)$$

where Δ is the parameter of a non-central Student's t-distribution, ω_{x_0} is a function of the standard concentrations, and $\hat{\sigma}$ and $\hat{b}$ are the residual standard deviation and the slope of the regression "calculated concentration" *versus* "true concentration", respectively.

4. Results and discussion

4.1. Reference spectra of cochineal and erythrosine

The experimental reference spectra were obtained from the EEMs of the pure analytes. Figs. 1 (a) and (e) show the EEM landscapes obtained for the reference sample of cochineal and erythrosine, respectively, prepared in buffer solution. As can be seen in the contour plots of the pure analytes (see Fig. 1 (b) for cochineal and Fig. 1 (f) for erythrosine), the spectra of both compounds were highly overlapped; especially in the excitation range from 470 to 510 nm and in the emission range from 530 to 570 nm. In addition, erythrosine exhibited more intense fluorescence than cochineal.

The maximum fluorescence intensity in the emission spectra appeared at 553 nm for cochineal and 541 nm for erythrosine. The excitation spectrum was taken in those wavelengths for each analyte (see Fig. 1 (c) for cochineal and Fig. 1 (g) for erythrosine). On the other hand, the maximum excitation intensities were obtained at 495 nm for cochineal and 510 nm for erythrosine. The emission spectrum was considered in each of these wavelengths (see Fig. 1 (d) for cochineal and Fig. 1 (h) for erythrosine).

The selection of an excitation and emission wavelength to obtain the emission and excitation spectra, respectively, can be avoided using a PARAFAC model. In this case, the corresponding spectra are independent of the concentration of the reference samples. Two three-way data arrays that contained the EEM of six reference samples for cochineal and erythrosine at different concentrations, respectively, were built. The excitation and emission profiles of each analyte will be used as reference "profiles" (subrogated spectra). The details of this approximation can be found in the Supplementary Material (section S1). The correlation between the PARAFAC reference

profiles and the PARAFAC excitation and emission loadings obtained in the rest of the analyses of this work were equal to the correlations obtained using the reference spectra (see Table S1 of the Supplementary Material).

In contrast to the reference profiles, the excitation and emission spectra obtained from a reference sample are experimental data without any preprocessing. Therefore, spectra were used as reference for the unequivocal identification of the analytes in the following stages of this work. The identification of each analyte was carried out through the correlation between the reference spectra and the spectral loadings estimated from the corresponding PARAFAC model.

4.2. Determination of cochineal and erythrosine

4.2.1. Calibration using pure standards as calibration set

Seven standards of cochineal were prepared within the concentration range of 0-30 mg L^{-1} and measured. The concentration range for other seven standards of erythrosine was 0-1 mg L^{-1}. In addition, four buffer blanks were measured throughout the experimentation to check the cleanliness of the cell. Three binary mixtures of both analytes were also measured. The EEMs of these samples were arranged to build a three-way array of dimension 21 × 106 × 14. The first mode corresponds to the number of samples (21), whereas 106 and 14 are the number of emission and excitation wavelengths recorded, respectively. The PARAFAC decomposition of this array needed three factors (CORCONDIA index of 94%), where the non-negativity constraint was imposed for the three profiles as both the excitation and emission spectra must always be positive. The explained variance was 99.96% and no outlier data were detected considering the Q and Hotelling's T^2 indices. The first factor of this model was associated to cochineal, the second factor was erythrosine, while the last one was related to the background. The analytes were unequivocally identified since the correlation coefficients for the excitation and emission profiles, regarding the reference spectra (see Section 4.1), were 0.995 and 0.998 for cochineal and 0.999 and 0.994 for erythrosine, respectively. The sample scores of the background remained constant in all the samples.

Next, calibration models "sample scores *versus* true concentration" were fitted and validated for each analyte. The standard at the highest concentration was detected as an outlier in the calibration model for erythrosine since it had a standardized residual greater than 3 in absolute value. Therefore, it was removed being 0-750 µg L^{-1} the new concentration range and a new linear least squares (LS) fitting was performed and validated with the remaining data. In all cases, the regression models were significant. The accuracy lines were also performed, that is, the regressions "predicted concentration *versus* true concentration". Table 1 shows the parameters of the calibration models and of the corresponding accuracy lines. The method had not proportional or constant bias since the p-values of the hypothesis test for the slope (b_1) and for the intercept (b_0) were higher than 0.05 (see Table 1), and thus, the intercept and the slope were significantly not different from 0 and 1, respectively. The values of

decision limit (CCα) and capability of detection (CCβ) are also listed in Table 1, being the probabilities of false positive (α) and false negative (β) fixed at 0.05. The CCβ values were 6.37 mg L^{-1} for cochineal and 47.35 µg L^{-1} for erythrosine. In addition, the mean of the absolute values of the relative errors were below 10% in calibration for both analytes (see Table 1) and in prediction for cochineal in the three mixtures measured. All the results were satisfactory except for the relative errors in prediction obtained for erythrosine which were high for the mixtures (data not shown). In those two mixtures, the predicted concentration for erythrosine was below the true concentration due to a quenching effect (see Section 4.2.2).

4.2.2. Calibration performed including binary mixtures of the analytes

In this case, the calibration set consisted of a blank, five standards for cochineal, five standards for erythrosine together with 25 binary mixtures of both analytes. Each analyte was at six concentration levels (including the blank) and the concentration range was 0-25 mg L^{-1} for cochineal and 0-750 µg L^{-1} for erythrosine. This distribution of concentrations was chosen following the experimental plan of a complete factorial design. In addition, 10 binary mixtures were prepared within the concentration ranges of both food colorants and used as prediction set. Two buffer blanks were also measured at the end of the measurement of the calibration and prediction samples, respectively. Table 2 shows the concentration of these samples prepared in buffer solution.

A three-way array of dimension 48 × 106 × 14, which contained the EEMs of the samples placed in the order shown in Table 2, was built. The PARAFAC decomposition was applied to this array with the non-negativity constraint imposed for the three profiles and a two-factor PARAFAC model was chosen (CORCONDIA index of 100% and explained variance of 99.92%). The PARAFAC model was not coherent when a new factor was considered so there was no other interferent or artefact that could be modelled. There were more binary mixtures in this three-way array than in the one of Section 4.2.1 so the variance due to those samples was higher and the contribution of the signal associated to the background was lower. The factor corresponding to the background signal in the PARAFAC model of Section 4.2.1 only explained a 0.80% of the variance of the model, whereas its contribution in this new array was relatively lower. This is the reason why the background did not contribute as a third factor in the PARAFAC model in this case. The scores of the sample profile and the loadings of the emission and excitation profiles of the two-factor model are included in Fig. 2. Cochineal and erythrosine were unequivocally identified since the correlation coefficients between their reference emission and excitation spectra (see Fig. 1) and their PARAFAC emission and excitation profiles (Fig. 2(b) and Fig. 2(c), respectively) were 0.999 and 0.991 for cochineal, respectively, and 0.999 in both cases for erythrosine. As can be seen in Table 2, each six samples of the calibration set corresponded to the six increasing levels of erythrosine for a fixed concentration level of cochineal. The PARAFAC sample scores of cochineal in the calibration set remained constant for each concentration level (see Fig. 2(a)) as expected. The sample scores of erythrosine in the calibration set increased with the concentration of this analyte but

this increase varied depending on the amount of cochineal present in the standard (see Fig. 2(a)). The score of erythrosine in samples at the same concentration of this analyte was lower when the quantity of cochineal was higher as can be seen in this last figure. Therefore, the amount of cochineal present in the sample affected the fluorescence signal of erythrosine in the measured conditions. In addition, no shift of the emission wavelength was observed on the signals. It can be concluded with the chosen distribution of concentrations that cochineal caused a quenching effect in the fluorescence of the other food additive. This is the reason why the predicted concentration for erythrosine was below the true concentration in the mixtures measured in Section 4.2.1 which could led to false compliant decisions for this analyte. Therefore, the calibration using pure standards as calibration set is not adequate if the quantification of both food colorants is pursued.

The signal of cochineal was not affected by the presence of erythrosine, so the quantification of cochineal was carried out using the calibration line fitted and validated for this analyte. The parameters of this regression model are included in the first row of Table 3 (columns 3 and 5).

$$SCORE_{COCHINEAL} = b_0 + b_1\ CONCENTRATION_{COCHINEAL} \tag{3}$$

However, the fluorescence signal of erythrosine depends on the amount of cochineal which acts as a quencher as explained before.

A first approximation would be to consider a calibration using a multiple linear regression (MLR) with the scores of both analytes. This approximation is explained in Section S2 (Calibration of binary samples by means of a multiple regression on the PARAFAC scores) of the Supplementary Material. As can be seen in Table S2 of the Supplementary Material, the MLR approach to obtain the concentration of erythrosine increased the standard deviation of the regression which affects all the figures of merit (CCα, CCβ, confidence intervals for the concentration of the test sample and intermediate precision of the procedure).

However, in this work, the quantification of erythrosine in the mixtures of the prediction set was possible using the following procedure (second procedure):

i) A regression line "sample score of erythrosine" versus "true concentration of erythrosine" was built for each different concentration level of cochineal. Fig. 3 shows the six regression lines fitted in each case where the slopes were different depending on the amount of quencher. Table 3 (rows 2 to 7, columns 3 to 5) contains the parameters of these calibration models. In all cases, the regression models were significant, and no outliers were detected. It must be checked if the intercepts of those regression lines are significantly equal to zero. As can be seen in the fourth column of Table 3, the p-values of the hypothesis test for the intercept of those regressions were higher than 0.05 so these intercepts were statistically equal to 0 at a 95% confidence level.

ii) Then, a linear regression is built with the pair of data (amount of cochineal$_i$, slope$_i$) i=1,….,6, to estimate the slope associated with any concentration of the quencher within the calibration range.

$$\text{CONCENTRATION}_{\text{COCHINEAL}} = m_0 + m_1 \text{ SLOPE} \tag{4}$$

In this case, the equation of this regression was: $y = -23.5\,x + 60.99$ ($R^2 = 99.93\%$).

iii) The predicted amount of cochineal in the corresponding sample was calculated with Eq. (3) and the slope of the regression model used to obtain the predicted concentration of erythrosine was obtained taking the equation in step ii) into account. The predicted concentration of erythrosine was finally estimated with the corresponding score and that slope using the following equation:

$$\text{CONCENTRATION}_{\text{ERYTHROSINE}} = \text{SCORE}_{\text{ERYTHROSINE}}/\text{SLOPE} \tag{5}$$

The MLR model (see Section S2 of the Supplementary Material) "averages" the impact that the concentration of cochineal causes in the slope of the calibration line for erythrosine, that's why there were differences in the results. The results obtained with the MLR would be the ones obtained with a common slope to all the samples equal to 2.18 in equation (3) which is an intermediate value of the range [1.54,2.61] obtained for those slopes according to the quencher concentration (see Table 3). This is the reason why the procedure based on a MLR model has not been used.

Using the second procedure, the mean of the absolute values of the relative errors in prediction were 5.86% (n = 10) for cochineal and 4.17% (n = 10) for erythrosine. Therefore, the proposed strategy enabled to handle the quenching effect that cochineal caused in the fluorescence of the other food additive.

Table 3 also includes the parameters of the accuracy lines together with the values of CCα and CCβ obtained in each case. There was neither constant nor proportional bias considering the p-values of the hypothesis test for the intercept and the slope (see columns 7 and 8 of Table 3, respectively).

4.2.3. Determination of both food colorants in cherries in syrup

Initial studies using the seven different filters specified in Section 2.4 were carried out to select the most adequate filter to perform the analysis in cherries in syrup. So, standards prepared in buffer solution and the extracts obtained from the cherries in syrup were filtered. Although the lowest errors in prediction for the standards were achieved using the fritted funnel, this filter was rejected since the recovery for erythrosine in matrix was very low. A Whatman$^{\text{TM}}$ filter paper qualitative was finally chosen as the best option.

Five non-spiked extracts and five spiked extracts were prepared as explained in Section 2.3. In this analysis, the calibration set consisted of the 24 first standards of Table 2. Therefore, cochineal was at four concentration levels since the highest concentration considered was 15 mg L^{-1}, whereas erythrosine was at the same six

concentration levels as in Section 4.2.2. A three-way array of dimension 37 × 106 × 14 was built with these 24 standards, the non-spiked extracts, the spiked extracts together with three buffer blanks. The PARAFAC decomposition of this array yielded a two-factor model (CORCONDIA index of 100%, explained variance of 99.81% and no outliers were detected), where the non-negativity constraint was imposed for the three profiles. The correlation coefficients for both food colorants were greater than 0.996 for the emission and excitation profiles when these were compared with the reference spectra, so both analytes were unequivocally identified. A fluorophore related to the matrix was not present in this analysis since the PARAFAC model was not coherent when a new factor was considered.

The same previous strategy explained in Section 4.2.2 to handle the quenching effect that cochineal caused in the fluorescence signal of erythrosine was applied to the determination of these food colorants in the cherries in syrup. Table 4 collects the parameters of the calibration lines and the corresponding accuracy lines of cochineal and of erythrosine at each concentration level of cochineal. The method had not proportional or constant bias (see Table 4). The intercepts of each regression line "sample score" *versus* "true concentration of erythrosine" were statistically equal to 0 at a 95% confidence level as can be seen in Table 4. In this case, the equation of the regression "amount of cochineal" *versus* "the slope of the calibration line for erythrosine" was: $y = -21.51\ x + 55.48$ ($R^2 = 99.82\%$).

The average recovery percentages were 122.4% (n = 5, s = 13.11) for cochineal and 48.8% (n = 5, s = 7.50) for erythrosine. Cochineal and erythrosine were found in those cherries at an average concentration of 185.05 mg kg^{-1} and 10.76 mg kg^{-1}, respectively. The found concentration for erythrosine was below its MRL, whereas the found concentration for cochineal was above or below the MRL depending on the variety of the cherries. The label of the product did not specify the variety.

4.2.4. Comparison of the determination in cherries in syrup with a HPLC/DAD method

The concentration of both food colorants in the cherries in syrup was also estimated with a HPLC/DAD method in order to compare the results with the ones obtained in Section 4.2.3. Therefore, ten standards of cochineal and erythrosine were prepared in buffer solution within the concentration range of 10-100 mg L^{-1} and 0.25-2.5 mg L^{-1}, respectively. In addition, the same five non-spiked extracts and the five spiked extracts measured in the previous Section 4.2.3 were injected. A buffer blank was also measured. 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.

Then, the integration of the corresponding peak areas of cochineal and erythrosine was carried out at 500 and 535 nm, respectively, and the calibration models "peak area" *versus* "true concentration" were fitted and validated for both food colorants. The parameters of these regressions are included in Table 5. In this case, the average concentration of cochineal and erythrosine found in the cherries in syrup was 174.35 mg kg^{-1} (n = 5) and 10.18 mg kg^{-1} (n = 5), respectively. The confidence interval (at a

95%) in each case is listed in the last row of Table 5. The amount found of both food colorants in the cherries in Section 4.2.3 was within these confidence intervals.

5. Conclusions

Cochineal acted as a quencher in the fluorescence signal of another food colorant (erythrosine) in the measured conditions. However, the fluorescence signal of cochineal was not affected by the presence of erythrosine.

The determination in mixtures was possible by means of the three-way PARAFAC decomposition of EEM fluorescent signals together with the use of a calibration set that contained binary mixtures of both analytes. The proposed methodology enabled the unequivocal identification and quantification of cochineal and erythrosine in cherries in syrup despite the quenching effect and the high overlapping signals. In addition, both food colorants were detected in the cherries in syrup analysed.

6. Acknowledgements

Author information

The authors declare no competing financial interest.

Table 1 Parameters of the calibration model "sample score *versus* true concentration" and accuracy line for cochineal and erythrosine obtained with the calibration performed using pure standards (Section 4.2.1).

		Cochineal	Erythrosine
Calibration model		$y = 107.90\,x + 146.43$	$y = 2.63\,x + 3.55$
	Residual standard deviation, s_{yx}	157.72	28.38
	R^2 (%)	98.55	99.88
	Number of outliers removed	0	1
	$\overline{\|e_r\|}$ calibration (%)[a]	6.61[b] (n = 6)	7.11 (n = 6)
Accuracy line		$y = 0.99\,x + 1.15 \cdot 10^{-3}$	$y = 0.99\,x + 0.00$
	p-value b_1[d]	0.99	0.92
	p-value b_0[e]	0.99	0.99
	Residual standard deviation, s_{yx}	1.46	10.79
	CCα ($x_0 = 0$) (mg L^{-1})	3.30	$2.47 \cdot 10^{-2}$
	CCβ ($x_0 = 0$)[c] (mg L^{-1})	6.37	$4.74 \cdot 10^{-2}$

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

[b] Samples with calculated concentration lower than CCα were excluded

[c] α = β = 0.05

[d] Slope

[e] Intercept

Table 2 Concentration of cochineal and erythrosine in each of the samples contained in the three-way array built in Section 4.2.2.

Sample	Cochineal (mg L^{-1})	Erythrosine (μg L^{-1})
1[a]	0	0
2	0	150
3	0	300
4	0	450
5	0	600
6	0	750
7	5	0
8	5	150
9	5	300
10	5	450
11	5	600
12	5	750
13	10	0
14	10	150
15	10	300
16	10	450
17	10	600
18	10	750
19	15	0
20	15	150
21	15	300
22	15	450
23	15	600
24	15	750
25	20	0
26	20	150
27	20	300
28	20	450
29	20	600
30	20	750
31	25	0
32	25	150
33	25	300
34	25	450
35	25	600
36	25	750

37[a]	0	0
38[b]	10	600
39[b]	5	300
40[b]	15	150
41[b]	20	450
42[b]	10	300
43[b]	12	200
44[b]	18	400
45[b]	22	500
46[b]	8	100
47[b]	16	350
48[a]	0	0

[a] Buffer blank
[b] Samples for the prediction set

Table 3 Parameters of the calibration model "sample scores *versus* true concentration" and of the accuracy line for cochineal and for erythrosine at each concentration level of cochineal obtained with the calibration performed including binary mixtures of the analytes (Section 4.2.2)

Analyte	Amount of cochineal present	Calibration line			Accuracy line			CCα (x_0 = 0) (mg L^{-1})	CCβ (x_0 = 0) [b] (mg L^{-1})
		Model (R^2, s_{yx})	p-value b_0	$\overline{|e_r|}$ calibration[a] (%)	Model (R^2, s_{yx})	p-value b_0	p-value b_1		
Cochineal	0-25 mg L^{-1}	$y = 111.63 x + 261.65$ (97.83 %, 146.21)	0.00	7.14 (n = 30)	$y = 1.00 x - 0.00$ (97.83 %, 1.31)	0.998	0.998	2.31	4.59
Erythrosine	0 mg L^{-1}	$y = 2.61 x + 41.44$ (99.55 %, 54.91)	0.36	4.27 (n = 5)	$y = 0.99 x - 3.81 \cdot 10^{-3}$ (99.55 %, 21.04)	0.999	0.983	5.59 $\cdot 10^{-2}$	1.07 $\cdot 10^{-1}$
	5 mg L^{-1}	$y = 2.37 x +$	0.28	2.30	$y = 0.99 x +$	0.99	0.96	4.04	7.70

		$|\overline{e_r}|$						
	32.86 (99.77%, 35.97)		(n = 5)	$3.33\cdot10^{-3}$ (99.77%, 15.18)	9	6	$\cdot10^{-2}$	$\cdot10^{-2}$
10 mg L^{-1}	$y = 2.16 x + 35.30$ (99.83%, 28.12)	0.16	2.10 (n = 5)	$y = 0.99 x + 3.81\cdot10^{-3}$ (99.83%, 13.02)	0.99 9	0.97 0	3.46 $\cdot10^{-2}$	6.60 $\cdot10^{-2}$
15 mg L^{-1}	$y = 1.96 x + 23.37$ (99.87%, 22.13)	0.22	1.93 (n = 5)	$y = 1.00 x + 0.00$ (99.87%, 11.29)	1.00 0	0.91 0	2.97 $\cdot10^{-2}$	5.67 $\cdot10^{-2}$
20 mg L^{-1}	$y = 1.74 x + 13.03$ (99.84%, 21.77)	0.45	2.95 (n = 5)	$y = 1.00 x - 1.43\cdot10^{-3}$ (99.84%, 12.51)	0.99 9	0.99 6	3.29 $\cdot10^{-2}$	6.28 $\cdot10^{-2}$
25 mg L^{-1}	$y = 1.54 x - 3.98$ (99.85%, 18.66)	0.78	3.58 (n = 5)	$y = 1.00 x + 1.90\cdot10^{-3}$ (99.85%, 12.11)	0.99 9	0.97 0	3.19 $\cdot10^{-2}$	6.08 $\cdot10^{-2}$

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

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

Table 4 Parameters of the calibration model "sample scores *versus* true concentration" and of the accuracy line for cochineal and for erythrosine at each concentration level of cochineal obtained for the determination of both food colorants in cherries in syrup (Section 4.2.2.1)

Analyte	Amount of cochineal present	Calibration line			Accuracy line			CCα ($x_0 = 0$) (mg L^{-1})	CCβ ($x_0 = 0$) [b] (mg L^{-1})
		Model (R^2, s_{yx})	p-value b_0	$\overline{\|e_r\|}$ calibration [a] (%)	Model (R^2, s_{yx})	p-value b_0	p-value b_1		
Cochineal	0-15 mg L^{-1}	$y = 134.93 x + 146.57$ (99.58%, 51.20)	0.00	3.80 (n = 18)	$y = 0.99 x + 0.00$ (99.58%, 0.38)	0.995	0.991	0.70	1.38
Erythrosine	0 mg L^{-1}	$y = 2.59 x + 41.58$ (99.54%, 54.96)	0.35	4.30 (n = 5)	$y = 0.99 x + 1.43 \cdot 10^{-3}$ (99.54%, 21.22)	0.999	0.973	$5.64 \cdot 10^{-2}$	$1.07 \cdot 10^{-1}$
	5 mg L^{-1}	$y = 2.33 x + 22.41$ (99.81%, 32.23)	0.39	1.78 (n = 5)	$y = 1.00 x - 2.86 \cdot 10^{-3}$ (99.81%, 13.83)	0.999	0.994	$3.64 \cdot 10^{-2}$	$6.94 \cdot 10^{-2}$
	10 mg L^{-1}	$y = 2.11 x + 14.16$ (99.91%, 19.62)	0.38	1.91 (n = 5)	$y = 0.99 x + 1.90 \cdot 10^{-3}$ (99.91%, 9.30)	0.999	0.922	$2.47 \cdot 10^{-2}$	$4.72 \cdot 10^{-2}$
	15 mg L^{-1}	$y = 1.89 x - 2.25$ (99.95%, 13.57)	0.83	2.06 (n = 5)	$y = 1.00 x - 1.90 \cdot 10^{-3}$ (99.95%, 7.18)	0.999	0.886	$1.89 \cdot 10^{-2}$	$3.61 \cdot 10^{-2}$

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

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

Table 5 Parameters of the calibration model "peak area *versus* true concentration" for cochineal and erythrosine and the final concentration found of each analyte in the cherries in syrup (together with the corresponding confidence interval) obtained with the HPLC/DAD

	Cochineal	Erythrosine
Calibration model	$y = 1.22\,x - 3.88$	$y = 54.20\,x - 0.89$
Residual standard deviation, s_{yx}	1.60	1.05
R^2 (%)	99.83	99.94
Sample concentration (mg kg^{-1}) (n = 5)	174.35	10.18
Interval	(160.69,187.59)	(9.24,11.08)
(at a 95% confidence level)		

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FIGURE CAPTIONS

Fig. 1 The left-hand side figures correspond to the reference sample of cochineal (20 mg L^{-1}) in buffer solution, while the right-hand side figures correspond to the reference sample of erythrosine (500 µg L^{-1}) in buffer solution: (a) and (e) EEM landscape, (b) and (f) contour plot, (c) and (g) excitation reference spectrum (considered at the emission wavelength of 553 nm for cochineal and 541 nm for erythrosine), and (d) and (h) emission reference spectrum (considered at the excitation wavelength of 495 nm for cochineal and 510 nm for erythrosine). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

Fig. 2 PARAFAC model with two factors obtained with the array built in Section 4.2.2 that contained the samples shown in Table 2. Scores of the: (a) sample profile, and loadings of the (b) emission profile and (c) excitation profile. Cochineal is in blue and erythrosine is in red. In the sample profile, cochineal is also represented by squares and erythrosine by dots. In the emission and excitation profile, cochineal is represented by dashed line and erythrosine by continuous line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

Fig. 3 Calibration models "sample score *versus* true concentration" fitted for erythrosine for each different concentration level of cochineal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).
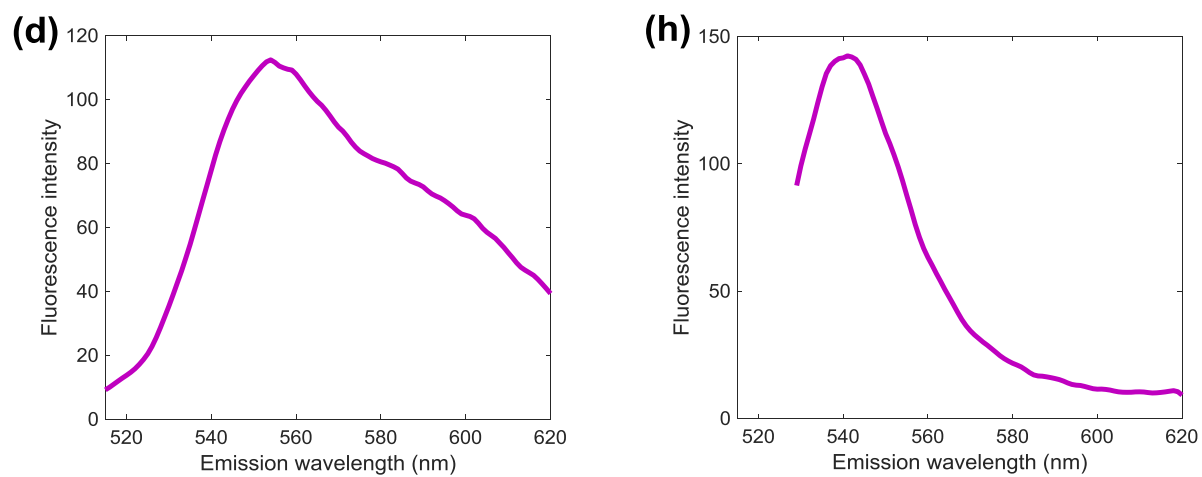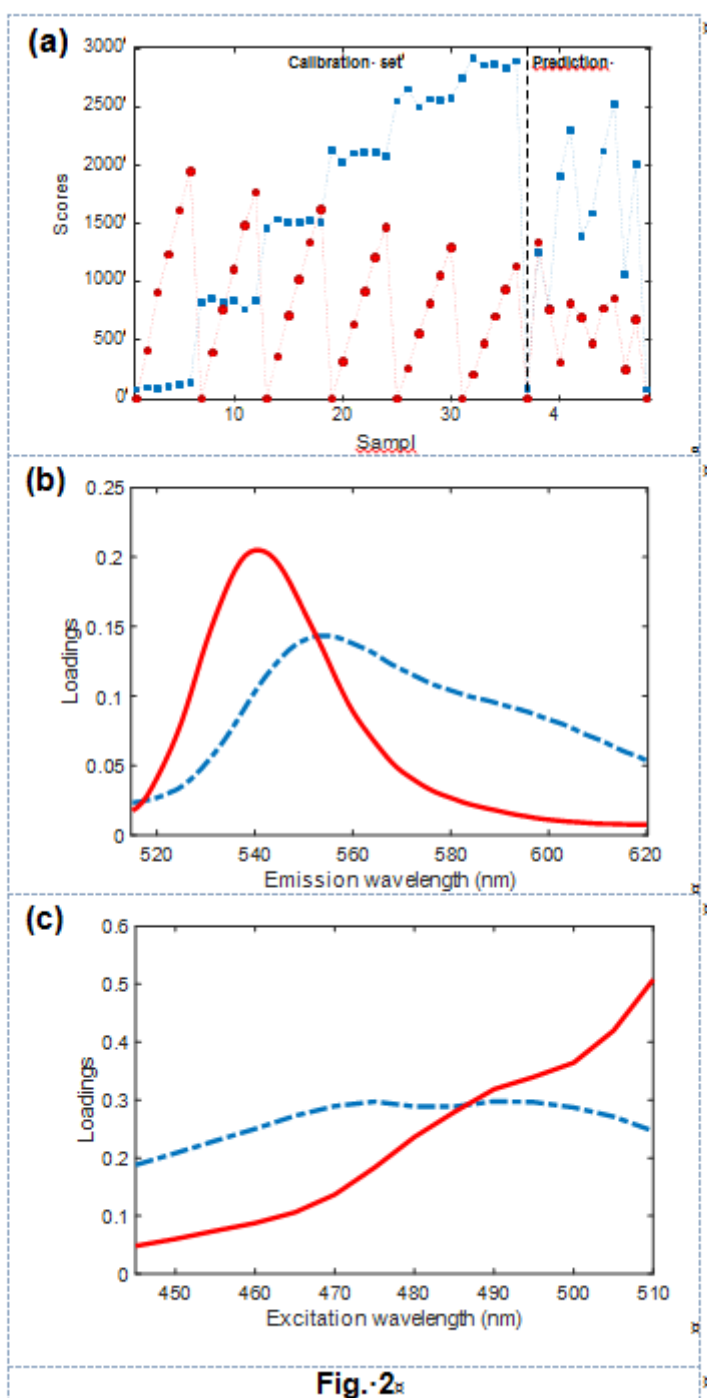
Cochineal

Erythrosine

(a)

(e)

(b)

(f)

(c)

(g)

24/27

(d)
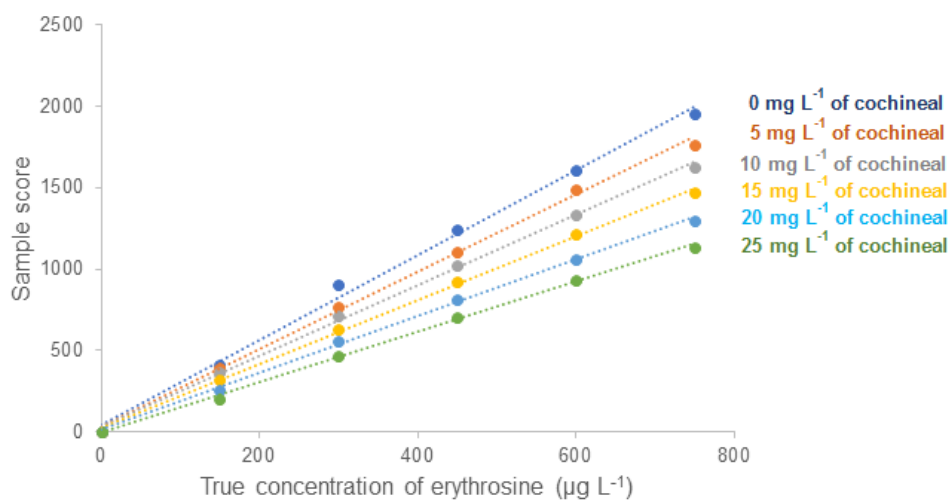

(h)

Fig. 1

Fig. 2
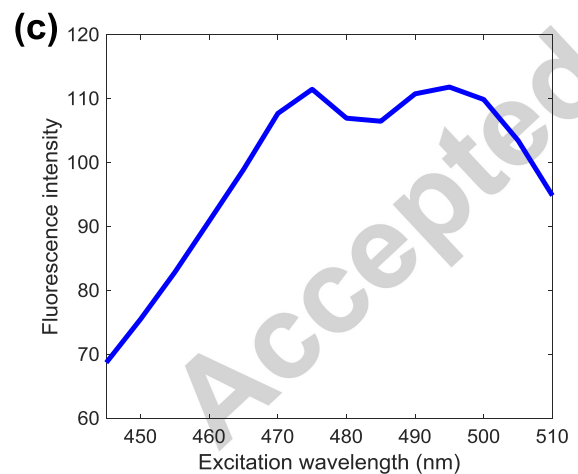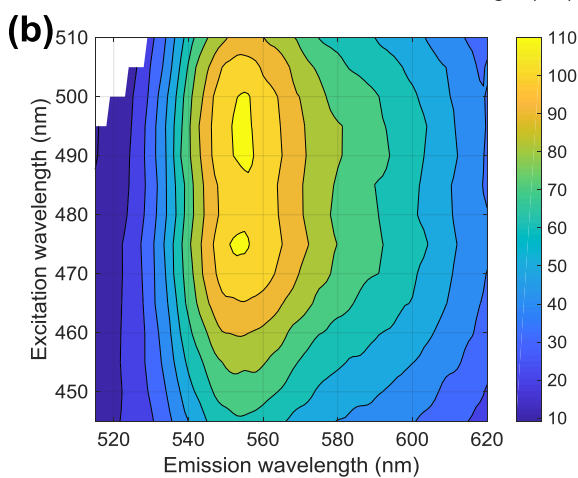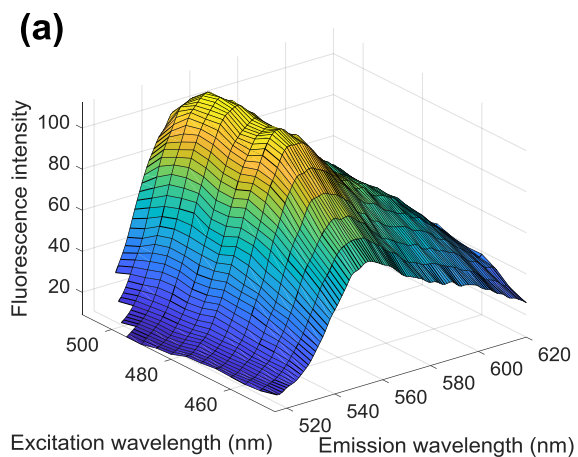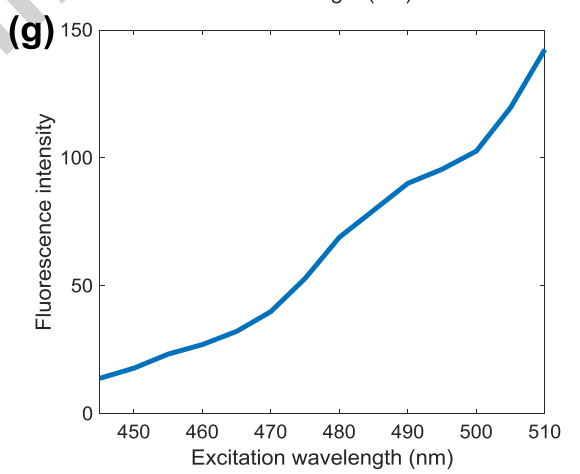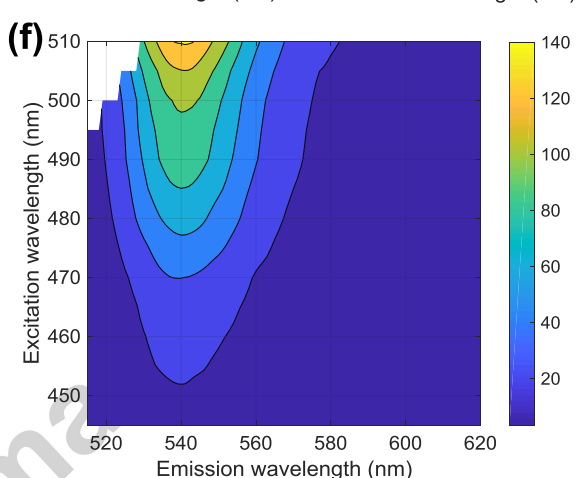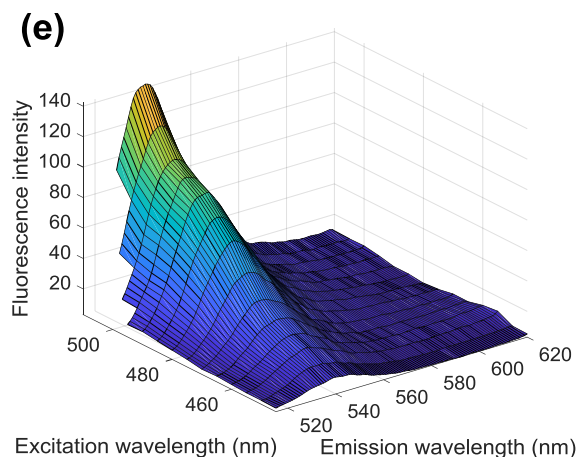
Fig. 3

HIGHLIGHTS

- Determination of two food colorants in cherries in syrup by EEM and three-way PARAFAC

- Cochineal was a quencher when erythrosine was measured by molecular fluorescence

- The fluorescence signal of cochineal was not affected by the presence of erythrosine

- A strategy was proposed to handle the quenching effect using three-way techniques