Manuscript after review

Access to published version: https://www.sciencedirect.com/science/article/pii/S0889157517303137

DETERMINATION OF PUNGENCY IN SPICY FOOD BY MEANS OF EXCITATION-EMISSION FLUORESCENCE COUPLED WITH SECOND ORDER CHEMOMETRIC CALIBRATION

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Abstract

Capsaicinoids are a family of compounds responsible for the pungency of spicy foods. In this work, the combination of fluorescence and chemometrics was investigated as a novel quantification method of capsaicinoids in spicy food samples. The excitation – emission matrices (EEMs) of the two major capsaicinoids (capsaicin and dihydrocapsaicin) were identical. Hence, the results were referred to the total content of capsaicinoids. The EEMs of a group of paprika, cayenne and chilli peppers, and of another group of spicy sauces were registered. The decomposition of the EEMs of each group was performed by Parallel Factor Analysis (PARAFAC), obtaining three principal components in each case. After the decomposition, the component corresponding with capsaicinoids was identified by comparison with the profile of a standard mixture of capsaicin and dihydrocapsaicin. Besides, the score values of this component were correlated with the Scoville Heat Units (SHU) calculated by means of a HPLC – FLD method. Good results of correlation were obtained in both groups (0.998 and 0.992), confirming the assignation of the component to capsaicinoids. Subsequently, a set of calibration was built to carry out the calibration in the spectrofluorimeter, using PARAFAC and U-PLS/RBL as second-order calibration algorithms. Good results for the SHU determination were obtained in both groups with both algorithms and when the fluorimetric method was validated by means of liquid chromatographic analysis the Relative Error of Prediction, REP, was less than 11.3 %.

Keywords: capsaicinoids; pungency; spicy foods; fluorescence; PARAFAC; U-PLS/RBL; Food Analysis; Food Composition

1. INTRODUCTION

The pungency of *Capsicum* fruit is due to a group of compounds called capsaicinoids, which are composed of an acid amide of vanillylamine and C9-C11 branched fatty acids (Iwai et al., 1979). Capsaicinoids are known for their pharmacological properties, as chemoprotectors against mutagenesis or tumorigenesis, as antimicrobials or as antioxidants; for their analgesic effects, and for their anticancer effect (Sganzerla et al., 2014).

Five analogues of capsaicinoids, capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin, have been reported. Of these, capsaicin and dihydrocapsaicin comprise 80 – 90 % of the capsaicinoids found in peppers. These are in concentrations of 0.1 – 1.0 %, in a ratio of 1:1 – 2:1 and they are the two most pungent capsaicinoids (Hayman and Kam, 2008).

Pungency is a sensorial parameter that is important to evaluate in several foodstuffs. For this reason, it is necessary to dispose of methods to carry out this, it in order to guarantee the quality of the products founded in markets.

The conventional method employed to evaluate this attribute of peppers and other foodstuff is through the Scoville Heat Units (SHU), developed in 1912 by Scoville (Scoville, 1912), and it consisted in an organoleptic method. In 1977, Todd et al. (Todd et al., 1977) determined the pungency of pure samples of individual capsaicinoids and established the threshold pungency values for these materials. Therefore, by combining the concentration and threshold pungency of the individual capsaicinoids, the Scoville pungency of the material can be determined. Nowadays, the way to calculate the pungency is multiplying the individual capsaicinoid content by the corresponding value of threshold pungency, 9.3 for nordihydrocapsaicin, 16.1 for capsaicin and dihydrocapsaicin and 8.1 for homodihydrocapsaicin and homocapsaicin. Then, all the values are added.

Regarding to the procedures to extract these compounds from different food matrices, different ways such us extraction by supercritical fluids (Daood et al., 2002; de Aguiar et al., 2014; De

57 Aguiar et al., 2013; Duarte et al., 2004; Fernández-Ronco et al., 2011; Gnayfeed et al., 2001;
58 Perva-Uzunalić et al., 2004; Santos et al., 2015), pressurized hot water extractor (Bajer et al.,
59 2015), microwave (Barbero et al., 2006) or ultrasounds (Barbero et al., 2008a; Boonkird et al.,
60 2008; Dawan et al., 2017), can be found (Lu et al., 2017). Besides, clean-up procedures have been
61 also employed to remove other interfering components (Attuquayefio and Buckle, 1987;
62 Juangsamoot et al., 2012; Thompson et al., 2006).

63 Usually, these compounds have been analyzed in different foods (chilies, peppers, paprika, hot
64 sauces, oleoresins, spray peppers…) employing different separative techniques. In most of the
65 cases, methods use liquid chromatography with different detectors: ultraviolet-visible (UV)
66 (Arnka et al., 2002; Juangsamoot et al., 2012); fluorescence (Barbero et al., 2008b; Collins et al.,
67 1995; Peusch et al., 1996); and mass detection (Games et al., 1984; Garcés-Claver et al., 2006;
68 Kozukue et al., 2005; Reilly et al., 2001), specially for identifying analogues of capsaicin and
69 dihydrocapsaicin.

70 Moreover, gas chromatography combined with mass detection (Iwai et al., 1979; Ramírez-maya
71 and Alvarado-suárez, 2009), capillary electrophoresis (Liu et al., 2010) or micellar liquid
72 chromatography (Chin-chen et al., 2010) have been also employed.

73 Also, in the literature, some methods appear that employ alternative techniques, such us UV
74 spectroscopy (González-Zamora et al., 2015; López et al., 1987; Perucka and Oleszek, 2000);
75 adsorptive stripping voltammetry (AdsSV) with carbon nanotubes (CNTs) (Kachoosangi et al.,
76 2008); electronic nose (Korel et al., 2002); near – infrared spectroscopy and visible and near-
77 infrared spectroscopy (VNIR)(Lee et al., 2005; Lim et al., 2015; Mo et al., 2013) have been
78 reported. Recently, colorimetric methods have been also employed to determine the total content
79 of capsaicinoids (Dawan et al., 2017; Ryu et al., 2017).

80 In spite of the fact that these compounds present fluorescent properties, to date, fluorescence
81 spectroscopy has not been employed as method of determination of capsaicinoids. However, it
82 has been used as a detection method in chromatographic (liquid chromatography) approaches. In

this work, a new method has been proposed to take advantage of their fluorescence properties and to determine the pungency of some spicy foods combining fluorescence and chemometrics. This method allows determining the total content of capsaicinoids in presence of some interferences without separating them from these interferences, which is an advantage respect to older methods.

2. EXPERIMENTAL SECTION

2.1. Chemicals, reagents and samples

Capsaicin (≥ 95%), dihydrocapsaicin (~ 90%), and the solvents employed (methanol and acetonitrile, grade HPLC) were purchased from Sigma Aldrich (Sigma-Aldrich Química, S.A. Madrid). Acetic Acid was obtained from Panreac (Panreac Química, S.A.U., Barcelona). Sep-Pak Plus cartridges of 360 mg were obtained from Waters (Waters Corp., Milford, MA, USA). The water was obtained from a Milli-Q water system (Millipore S.A.S., Molsheim, France).

The analyzed samples consisted in 28 samples of paprika, 14 hot sauces, and 2 hot dried peppers. All of them were purchased from local markets.

2.2. Treatment of samples

Capsaicinoids were extracted from paprika samples with a simple procedure. A precise weight around 0.2 g was extracted with 20 mL of methanol during 10 min in the ultrasound bath. After that, the samples were centrifuged during 5 minutes and evaporated to dryness. They were reconstituted in 15 mL of methanol:water (30:70, v/v) and 5 mL of this extract was subjected to a solid phase extraction procedure. This consisted in passing the sample through a C18 cartridge. Firstly, the cartridge was conditioned with 8 mL of acetonitrile and 8 mL of water, after that, the interfering compounds (flavonoids, tocopherols…) were removed with 4 mL of methanol:water (60:40, v/v) and capsaicinoid compounds were eluted with 4 mL of methanol:water (80:20, v/v). An aliquot of this fraction (from 0.2 to 0.6 mL, taking into account the linear range of the calibrations curves) was diluted to 3.0 mL with the mobile phase (liquid chromatography analysis) or with acetonitrile (fluorescence analysis).

In order to analyse the hot sauces, an aliquot around 1.0 g was precisely weighted, diluted to 10 mL with methanol:water (30:70, v/v), and centrifuged during 5 minutes. An aliquot of 5 mL of the supernatant was subjected to the solid phase extraction procedure described for paprika samples.

For the hot peppers, the samples were milled to obtain a powder. An amount around 0.2 g was precisely weighted and extracted in the same conditions that paprika samples.

2.3. Chromatographic analysis

The chromatographic analysis was performed by means of an Agilent Model 1260 infinity LC instrument (Agilent Technologies, Palo Alto, CA, USA), equipped with degasser, quaternary pump, autosampler, DAD detector and FLD detector (Agilent infinity II). The OpenLAB LC ChemStation software (Version A.02.14) was used to control the instrument, data acquisition and data analysis.

The mobile phase consisted in 1% acetic acid: acetonitrile 57:43, v/v, with a flow rate of 1.0 mL min^{-1}. The fluorescence detection was set at 230 nm for excitation and 310 nm for emission. A rapid resolution Zorbax Eclipse XDB-C18 column (4.6 mm x 50 mm x 1.8 μm) (Agilent Technologies) was used for the separation of both analytes.

The corresponding calibration curves were obtained with standard solutions containing mixtures of capsaicin and dihydrocapsaicin. The concentrations were comprised from 0.0 to 50.0 ng mL^{-1}. The peak values were measured using the Chemstation package. An in-house MatLab routine, ACOC (Espinosa Mansilla et al., 2005), was used to obtain the analytical figures of merit for the calibration curves.

2.4. Developing EEMs

In order to obtain the fluorescence excitation-emission matrices, a Cary Eclipse VARIAN spectrofluorimeter equipped with two Czerny-Turner monochromators, a Xenon light source and a photomultiplier tube, as detector, was employed. A 1.0 cm quartz cell was used. Data acquisition was performed with the Cary Eclipse software.

The excitation – emission matrix (EEM) of each analyzed sample was obtained registering emission spectra from 260 nm to 400 nm, each 1 nm, varying the excitation wavelength from 210 nm to 300 nm, each 5 nm. The slid widths employed were 5 nm for excitation and emission and the photomultiplier voltage used was 600 V.

Analysis of data were done using MatLab R2008a (MATLAB Version 7.6, The Marhworks, Natick, Massachusetts, 2010) and the MVC2 routine (Olivieri et al., 2009).

2.5. Second-order algorithms

Parallel factor analysis (PARAFAC) is the method of choice for three-way trilinear data. EEM data consists of measurements of fluorescence at *J* emission wavelengths and *K* excitation wavelengths for each *I* samples with the data collected into a three-way data cube X_{IxJxK}.

PARAFAC decomposes the array into sets of scores and loadings that hopefully describe the data in a more condensed form than the original data array (Bro, 1997). The input array is decomposed by minimizing the sum of squares of the residuals, $e_{ijk,}$ in the model

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \quad (1)$$

where x_{ijk} is the fluorescence intensity for sample i, at the emission wavelength j and excitation wavelength k, and e_{ijk} indicates an element of the array **E**, which collects the variability not accounted by the model. For a given component f, the elements a_{if}, b_{jf} and c_{kf} are arranged in the score vector $\mathbf{a_f}$ (whose elements are directly proportional to its concentration in each sample), and the loading vectors $\mathbf{b_f}$ and $\mathbf{c_{f,}}$, which estimate their emission and excitation profiles.

For using Unfolded-Partial Least-Squares (U-PLS), as it is described in the literature (Bohoyo Gil et al., 2006; Olivieri et al., 2015; Olivieri and Escandar, 2000), the first step is to convert the calibration data arrays into vectors. This would produce from a ***I x J x K*** three-way data array a ***IJK*** *x 1* vector. With all the unfolded calibration data, a new calibration matrix $\mathbf{X_{cal}}$, suitable for the application of PLS regression is built by placing all the column vectors adjacent to each other.

The $\mathbf{X_{cal}}$ matrix is subjected to the classical and well known PLS regression analysis. This involves decomposition of $\mathbf{X_{cal}}$ into the product of two matrices:

$$\boldsymbol{X_{cal} = PT^T + E_{cal}} \quad (2)$$

where **P** is called the loading PLS matrix and **T** is the score PLS matrix, while $\mathbf{E_{cal}}$ collects the residuals.

If unexpected components do not occur in the test sample, $\boldsymbol{v}$ (the vector of regression coefficients) could be employed to estimate the analyte concentration according to

$$y = \boldsymbol{v^T t} \quad (3)$$

where **t** is the test sample score vector, obtained as the projection of the unfolded data matrix for the test sample in the space defined by the calibration PLS loadings.

When unexpected constituents occur in the test data matrix $\mathbf{X_{test}}$, the sample scores t obtained by projecting $\mathbf{X_{test}}$ onto the PLS loadings are unsuitable for analyte prediction through the equation 3. One indication that this is indeed the case comes from the inspection of the U-PLS sample residuals:

$$s_{UPLS} = \|vec(\boldsymbol{X_{test}}) - \boldsymbol{Pt}\|/(JK - A)^{1/2} \quad (4)$$

where the product (**P t**) represents the best approximation of PLS to the signals of the test sample and A is the trial number of factors. In the presence of unexpected sample components, the s_{UPLS} residuals will be abnormally large in comparison with the typical instrumental noise level, because the product (**P t**) cannot successfully reconstruct the test sample vector, $vec(\boldsymbol{X_{test}})$. For a certain number of principal components (N_{RBL}), the mission of the procedure known as residual bilinearization (RBL) is the minimization of the residual error, s_{RBL}, to a level compatible with the degree of noise present in the measured signals, with s_{RBL} given by:

$$s_{RBL} = \|vec(\boldsymbol{E_{RBL}})\|/[(J - N_{RBL})(K - N_{RBL}) - A]^{1/2} \quad (6)$$

Therefore, if more than one unexpected component is considered, RBL should select the simplest model giving a residual value, which is not statistically different from the minimum one. These considerations are the basis for the estimation of the correct number of RBL components.

3. RESULTS AND DISCUSSION

3.1. Optimization of the clean-up procedure

Apart from capsaicinoids, many other compounds are present in the target samples of this study. Among all these compounds, carotenoids are included. The content of these compounds is higher than the one of capsaicinoids and might cause inner filter effect due to their strong absorption. For this reason, it is necessary to carry out a solid-phase extraction clean-up procedure before the fluorimetric analysis.

Firstly, a paprika sample was weighted, extracted with 20 mL of methanol during 10 minutes in the ultrasound bath, and evaporated to dryness. After that, the residue was reconstituted in water and was passed through the cartridge. The fraction passed through the cartridge was injected in the chromatographic system and it was observed that capsaicinoids were retained in the cartridge. Then, different methanol:water mixtures were assayed for the elution of analytes. The most relevant details are summarized in the Table 1.

From this table, it can be observed that analytes start to elute with methanol:water 60:40 (v/v). For this reason, it was decided to use methanol:water, 30:70 (v/v) to reconstitute the sample before loading in the cartridge, checking this mixture did not present risk of eluting the analytes during sample loading. In this way, other possible interfering compounds are not retained in the cartridge thus simplifying the subsequent cleaning-up of the cartridge previous to the elution of capsaicinoids.

To select the clean-up step different experiences were performed. One of them consisted in passing 5 mL of methanol:water, 50:50 (v/v) and the another one consisted in passing 4 mL of methanol:water, 60:40 (v/v). The last mixture was the most effective in eliminating interferences without compromising analyte elution (only 2-3 % of capsaicinoids were removed).

Finally, different volumes of methanol:water (70:30, v/v) and of methanol:water (80:20, v/v) were checked to elute all the capsaicinoids retained in the cartridge. When methanol:water (70:30, v/v) was employed, more than 10 mL were necessary to get recoveries > 90 % whereas 4 mL of

methanol:water (80:20, v/v) were enough. The use of pure methanol was not feasible because carotenoids were co-eluted.

Hence, the final procedure of solid phase extraction consists in only 3 quick steps: passing 5 mL of the sample extract solved in methanol:water (30:70, v/v), clean-up step with 4 mL methanol:water (60:40, v/v) (only a 2-3 % of capsaicinoids were lost with this volume), and elution with 4 mL of methanol:water (80:20, v/v). An aliquot of this last fraction (from 0.2 to 0.6 mL, taking into account the linear range calibrations curves) was diluted to 3.0 mL with the mobile phase (liquid chromatography analysis) or with acetonitrile (fluorescence analysis). The chromatograms of the different extracts corresponding to these steps for a paprika sample are shown in the Figure 1. These chromatogram were obtained according the described in section 2.3. Chromatographic analysis.

This procedure was validated with spiked samples also analysed by LC and, at the same time, a non-spiked sample was analyzed to subtract the concentration found in it and evaluate the recoveries of the procedure. A known amount of capsaicin and dihydrocapsaicin (100 μg g^{-1} of each one) was added to a paprika sample and the above procedure was performed. This was done in triplicate and the recovery values obtained were 100 ± 7 % for capsaicin and 100 ± 8 % for dihydrocapsaicin. These results claim the precision and accuracy of the procedure.

Figure 2 shows the EEMs corresponding to a paprika sample with and without applying the clean-up procedure and using in both cases the same dilution factor. As can be observed, the signal is much lower when the clean-up procedure was not employed because of the inner filter effect produced by the presence of carotenoids.

3.2. Chromatographic analysis of spicy foods

Previously to the spectrofluorimetric exam of the extracts, the chromatographic analysis of the different spicy foods was performed in the conditions of 2.3. Chromatographic analysis. The fluorimetric detection of capsaicin and dihydrocapsaicin was set at λ_{exc} 230 nm and λ_{em} 320 nm. Under these chromatographic conditions, both analytes offered well-resolved peaks and the

237 analysis was carried out in less than 7 minutes. Besides, due to the employment of a clean-up
238 procedure in the case of real samples, the column does not need to be cleaned after consecutive
239 injections, which saves analysis time.

240 After the building of the calibration curves of each analyte, by dissolving the standard in the
241 mobile phase, the analytical parameters were obtained (Table 2). The limits of detection were
242 0.29 and 0.32 ng mL^{-1} for capsaicin and dihydrocapsaicin, respectively. The limits of
243 quantification were 0.96 and 1.0 ng mL^{-1}, respectively. The evaluation of the precision was
244 performed by carrying out the analysis of several standard solutions in two levels of concentration
245 (1.0 ng mL^{-1} and 5.0 ng mL^{-1}). The analysis was done in the same day (intra-day precision) and,
246 in different days, during 10 days (inter-day precision). The results are shown in the Table 3, in all
247 cases the RSDs were less than 6 %.

248 The total repeatability of this procedure was probed by extracting a sample of paprika in triplicate
249 and performing the different steps (extraction and clean-up). The RSD values were 2.4 and 3.5 %
250 for capsaicin and dihydrocapsaicin, respectively. Therefore, it can be claimed that the procedure
251 offers good and repetitive results.

252 Twenty eight hot paprika samples, 2 hot dried peppers (cayenne and chilli peppers) and 14 hot
253 sauces were analyzed with this methodology. The total content of capsaicinoids were comprised
254 between $62 – 130 \cdot 10^1$ μg g^{-1} for paprika and chilli peppers, $713 \cdot 10^1$ μg g^{-1} for cayenne and 19 –
255 130 μg g^{-1} for sauces. Taking into account the relation stablished by Todd et al. (Todd et al., 1977)
256 between the concentration of capsaicin and dihydrocapsaicin with the SHU, these were calculated
257 multiplying the total content of both capsaicinoids by 16.1. In this way, the SHU were comprised
258 between $100 \cdot 10^1 – 210 \cdot 10^2$ for paprika and chilli peppers, $115 \cdot 10^3$ for cayenne, and $300 – 210$
259 $\cdot 10^1$ for sauces.

260 The products can be classified as extreme ($150 \cdot 10^3 – 855 \cdot 10^3$ SHU), strong ($350 \cdot 10^3 – 100 \cdot 10^2$
261 SHU) or medium ($800 \cdot 10^1 – 100 \cdot 10^1$ SHU). Most paprika samples can be classified as strong,
262 cayenne is also classified as strong and sauces are classified as medium.

3.3. PARAFAC decomposition

From the chromatographic analysis, it was obtained that the capsaicinoids content was lower in the case of sauces samples than in the solid samples. Besides, less interfering signals appear in the sauce samples EEMs compared with the paprika samples EEMs.

Taking into account these differences, the samples were divided in two groups to perform the preliminary PARAFAC analysis. On the one hand, solid samples, the paprika, cayenne, and chili pepper samples. On the other hand, the spicy sauces.

To carry out the analysis by PARAFAC, the two first steps are the determination of the number of responsive components and the identification of specific components.

For identifying capsaicinoids in paprika samples, complete EEMs, excitation from 210 to 300 nm and emission from 260 to 400 nm, were used for the analysis. The optimum number of components was established as 3 from the core consistency value versus the number of component plot (Figure 3E) and according to the core consistency criteria (Bro, 1997). The 3D loadings and the corresponding score values were obtained. As can be seen in the Figure 3, the second component (Figure 3G) is clearly corresponding with the profile of capsaicinoid compounds (capsaicin, Figure 3B, and dihydrocapsaicin, Figure 3C). Two excitation maxima appear at 230 and 280 nm, which correspond with the absorption maxima of these compounds. Excitation-emission matrices of both analytes are identical. Therefore, all results refer to the total content of both capsaicinoids in further studies.

The identification of the second component with capsaicinoids is also supported by the correlation (correlation coefficient of 0.998) between the scores values corresponding to this component and the SHU values calculated as described below by HPLC methodology (Figure 4A) .

The same strategy was followed for spicy sauces. In this case, better results were obtained with the following selected sensors: excitation from 215 to 280 nm and emission from 290 to 380 nm.

With these wavelengths, the number of components was two and the first one relates to capsaicinoids (Figure 5). Figure 4B shows the score values of component one versus the total content of capsaicinoids calculated by HPLC methodology. In this case, the correlation coefficient obtained was 0.992, clearly identifying this component with the mixture of capsaicinoids present in spicy sauces samples.

From these good results, the quantification of the C+DC content, and the evaluation of the pungency of these kind of foodstuffs, by means of combination of the three-way fluorimetric signals with the second order multivariate algorithms PARAFAC and U-PLS/RBL seems feasible, being also an interesting method for companies. This method could replace the chromatographic analysis, which needs more time, solvent and a less affordable instrumentation.

3.4. PARAFAC and U-PLS/RBL quantification

Once the identification of a specific component due to capsaicinoids was made, as described in the previous section, a set of calibration was built to obtain the absolute concentration in unknown samples.

For that, standards of different concentrations of capsaicin plus dihydrocapsaicin (0.0 – 2.0 μg mL^{-1}) were prepared in acetonitrile and their corresponding matrices were registered as it is described in the 2.4. Developing EEMs section. Different wavelengths ranges were assayed as selected sensors with both algorithms in order to obtain the best results. These were obtained in the selected sensors 290 – 380 nm for emission and 250 - 285 nm for excitation in the case of paprika, chilli pepper and cayenne samples, and in the selected sensors 290 – 380 nm for emission and 215 – 285 nm for excitation in the case of spicy sauces.

Firstly, when using PARAFAC for the group of paprika, cayenne and chili pepper, the core consistency criteria applies for the selection of the optimal number of factors for each sample. The core value drops below 50 with a number of factors higher than three, for most of the samples, although, in some cases, this core value drops below 50 when the factors are two. Once obtained the content of capsaicin plus dihydrocapsaicin in all samples, the values of SHU, compared with

314 the corresponding to the HPLC analysis are in Table 4. Twenty-four out of thirty samples were
315 well-predicted. The statistical parameters were evaluated for those well-predicted samples
316 through the relative error of prediction (REP %) and the root mean square error of prediction
317 (RMSEP). These values were satisfactory being 71.5 μg g^{-1} and 11.3 %, respectively.

318 This result is reinforced by the elliptical join confidence region (EJCR) (González et al., 1999)
319 test, which computes the joint confidence interval for the intercept and the slope of the found vs.
320 nominal concentration plot, and check if the ideal values of 0 and 1 are within the ellipse (Figure
321 6A). The result of EJCR test offered a good correlation between both methods as it complies the
322 test.

323 Secondly, when applying U-PLS/RBL for this group, the number of factors is given by the
324 Haaland and Thomas criterion (Haaland and Thomas, 1988) and the optimum number of factors
325 is given by a PRESS value statistically no different to the minimum PRESS value (F-ratio
326 probability falling below 0.75), founding that two factors are enough. In this case, the selection
327 of the optimum number of components is performed with standards and without considering
328 samples to be analyzed. When applying U-PLS/RBL to the real samples, it was necessary to assess
329 the number of unexpected components to be employed in the RBL procedure. This depends on
330 the sample that it want to be analyze (Olivieri et al., 2011).The number of unexpected components
331 were a single new factor besides those required for calibration; however, in some cases the results
332 were better with two factors. The number of unexpected components was assessed by comparing
333 the final residuals with the instrumental noise level until it stabilizes at a value compatible with
334 the experimental noise (Bortolato et al., 2007). This approach predicts well the capsaicinoids
335 content of twenty-seven out of thirty-one samples. Table 4 shows the results. In this case, the REP
336 % and the RMSEP were 59.2 μg g^{-1} and 9.9 %, respectively. These values were a bit better than
337 in the case of PARAFAC quantification. The result of EJCR test also offered a good correlation
338 between both methods as it complies the test (Figure 6A).

339 The correlation coefficients obtained between results by and PARAFAC and U-PLS/RBL
340 methods and by the HPLC method were 0.994 for both cases. These results also probe the good
341 accuracy and precision of the developed method.

342 For spicy sauces, when PARAFAC was applied in the indicated selected sensors, three
343 components were the optimum number in all cases, and the first one was related with
344 capsaicinoids. Twelve out of fourteen samples were predicted correctly. Table 4 also summarizes
345 these results. The statistical parameters were evaluated for those well-predicted samples through
346 the relative error of prediction (REP %) and the root mean square error of prediction (RMSEP).
347 These values were satisfactory being 6.55 μg g^{-1} and 10.8 %, respectively.

348 For U-PLS/RBL analysis, the optimum number of factors was three and the unexpected
349 components was zero in most cases and one single factor for two samples. This approach predict
350 correctly all samples, and Table 4 summarizes the results. The statistical parameters were
351 evaluated for those well-predicted samples through the relative error of prediction (REP %) and
352 the root mean square error of prediction (RMSEP). These values were satisfactory being 5.18 μg
353 g^{-1} and 9.15 %, respectively. Besides, the results of EJCR are shown in Figure 6B.

354 The correlation coefficient between results by and PARAFAC and U-PLS/RBL methods and by
355 the HPLC method were 0.987 and 0.993, respectively. These results also probe the good accuracy
356 and precision of the developed method.
357 It can be said that in both cases U-PLS/RBL offers better results according to the RMSEP, which
358 is lower when U-PLS/RBL is used. Also, in the Figure 6, it can be observed that the data
359 dispersion in the EJCR test is lower in the case of U-PLS/RBL.
360

361 **4. CONCLUSIONS**

362 The quantifying of capsaicinoids in spicy food has been addressed for the first time by a new
363 method based on fluorescence and chemometrics. This methodology allows determining the
364 Scoville Heat Units of spicy food.

To carry out the fluorimetric analysis, the development of a solid phase extraction procedure was necessary obtaining good recoveries and the isolation of the capsaicinoid compounds from the food matrix. This clean-up procedure presents the advantage to remove some interfering compounds, such us carotenoids, which present a strong inner filter that avoid quantify capsaicinoids by fluorescence.

Then, combining fluorescence total signals and second order algorithms (PARAFAC and U-PLS/RBL) the total content in capsaicinoids expressed as Scoville Heat Units was predicted in foodstuffs of different pungency. When comparing these results with those provided by chromatographic analysis good agreements occurs for a total of 25 samples of paprika, 1 sample of cayenne, 1 sample of chilli pepper, and 12 spicy sauces. Correlation coefficients were higher than 0.987 in all cases assayed.

This method can be presented as a useful tool for the industries that have to determine the pungency of spicy food. It is faster, easier, more affordable and more respectful with the environment than older classical methods, including the chromatographic ones.

Acknowledgments

Financial support was provided by the Junta de Extremadura (Ayuda GR15090-Research Group-FQM003 and Project IB16058) and Ministerio de Economía, Industria y Competitividad of Spain (Project CTQ2017-82496-P), both co-financed by the European FEDER funds. Olga Monago Maraña is grateful to the Ministerio de Educación, Cultura y Deporte of Spain for a FPU grant (Resolución de 18 de noviembre de 2013, de la Secretaría de Estado de Educación, Formación Profesional y Universidades, BOE nº 279, de 21/11/13, reference number FPU13/00249.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Optimization of the clean-up procedure. Results about presence or not of capsaicinoids in the different fractions assayed.

Elution	Presence of capsaicin	Presence of dihydrocapsaicin
5 mL H_2O	-	-
5 mL methanol: water (30:70)	-	-
5 mL methanol: water (50:50)	-	-
2 mL methanol: water (60:40)	+	+
2 mL methanol: water (70:30)	++	++
2 mL methanol: water (80:20)	++	++
2 mL methanol	++	++

549

Table 2. Chromatographic analysis. Analytical figures of merit

Analyte	Linear range (ng mL^{-1})	Intercept ± SD	Slope ± SD (mL ng^{-1})	Determination coefficient (R^2)	Linearity (%)	LOD[a] (ng mL^{-1})	LOQ[b] (ng mL^{-1})
Capsaicin	1.0 – 50.0	153 ± 22	58.1 ± 0.9	0.9958	98.4	0.29	0.96
Dihydrocapsaicin	1.0 – 50.0	0 ± 10	48 ± 28	0.9991	99.1	0.32	1.0

550 SD: Standard Deviation
551 [a]LOD: Limit of detection, calculated as SD of a standard of 0.05 ng mL^{-1} (n = 11)·3/Slope
552 [b]LOQ: Limit of quantification, calculated as SD of a standard of 0.05 ng mL^{-1} (n = 11)·10/Slope
553

Table 3. Chromatographic analysis. Relative Standard Deviation (%)

Analyte	Intra-day[a]				Inter-day[b]			
	1.00 ng mL^{-1} (n = 10)		5.00 ng mL^{-1} (n = 10)		1.00 ng mL^{-1} (n = 10)		5.00 ng mL^{-1} (n = 10)	
	tR	PA	tR	PA	tR	PA	tR	PA
Capsaicin	0.21	2.5	0.17	2.5	1.2	6.0	0.93	5.6
Dihydrocapsaicin	0.32	5.5	0.14	1.6	1.6	5.7	1.2	3.0

554 tR: time of retention
555 PA: peak area

Table 4. Correlation between results of the fluorimetric developed method and HPLC method

Sample	SHU in paprika, cayenne and chilli peppers · 10^{-2}				
	PARAFAC	components	U-PLS/RBL	RBL	HPLC
1	38.5	3	37.8	1	27.9
2	198	3	197	1	224
3	162	3	161	1	176
4	53.6	3	522	1	61.3
5	47.2	3	47.3	1	48.9
6	16.3	3	16.6	1	19.2
7	34.1	3	34.6	1	31.4
8	7.08	3	6.92	1	9.98
9	50.4	3	49.3	1	53.1
10	51.5	2	53.1	2	53.1
11	72.8	3	53.9	2	54.4
12	38.8	3	38.6	1	33.5
13	50.9	3	50.7	1	44.3
14	127	2	131	1	130
15	181	3	181	1	201
16	40.3	3	40.7	1	37.4
17	139	2	143	1	148
18	57.2	3	38.0	2	38.3
19	94.0	2	75.2	2	78.3
20	82.3	2	60.4	2	69.1
21	137	2	142.2	1	143
22	135	2	138	2	120
23	138	2	120	2	117
24	123	2	127	1	123
25	67.3	3	66.7	1	75.8
26	74.7	3	73.0	1	67.1
27	867	2	878	1	1148
28	164	3	163	1	126
29	197	2	201	1	209
30	186	2	190	2	195
	SHU in spicy sauces · 10^{-2}				
	PARAFAC	components	U-PLS/RBL	RBL	HPLC
1	17.4	3	17.4	0	16.4
2	22.2	3	22.2	0	19.8
3	3.38	3	3.38	0	30.6
4	13.4	3	13.5	0	13.9
5	14.7	3	14.7	0	14.7
6	2.90	3	3.06	0	30.6
7	12.4	3	13.2	0	13.9
8	13.4	3	13.9	0	14.7
9	4.35	3	4.35	0	4.35
10	3.70	3	4.99	1	4.35
11	3.38	3	4.67	1	4.35
12	4.83	3	4.35	0	3.86
13	4.67	3	4.19	0	3.86
14	4.51	3	3.86	0	3.86

Figure captions

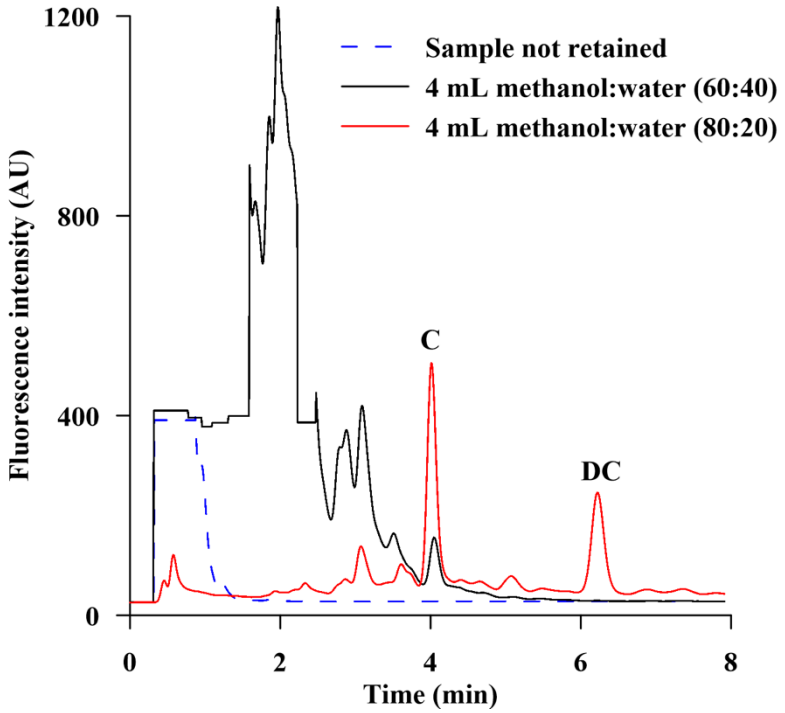


Figure 1. Chromatograms for a paprika sample corresponding to the different steps of the solid phase extraction procedure, capsaicin (C) and dihydrocapsaicin (DC). Fraction corresponding to the elution (methanol:water, 80:20, v/v) is diluted to avoid the saturation of the detector.

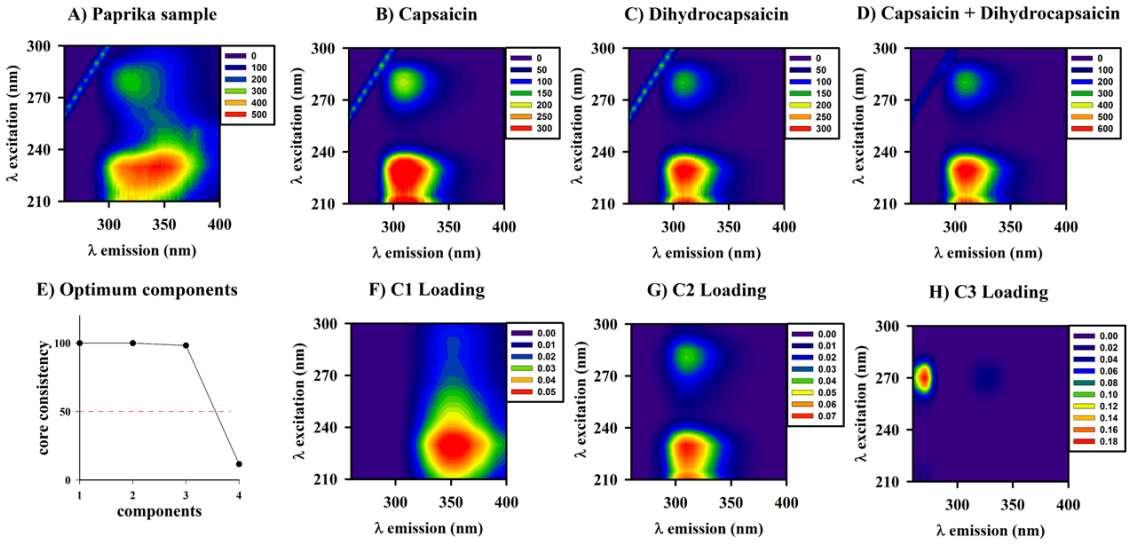
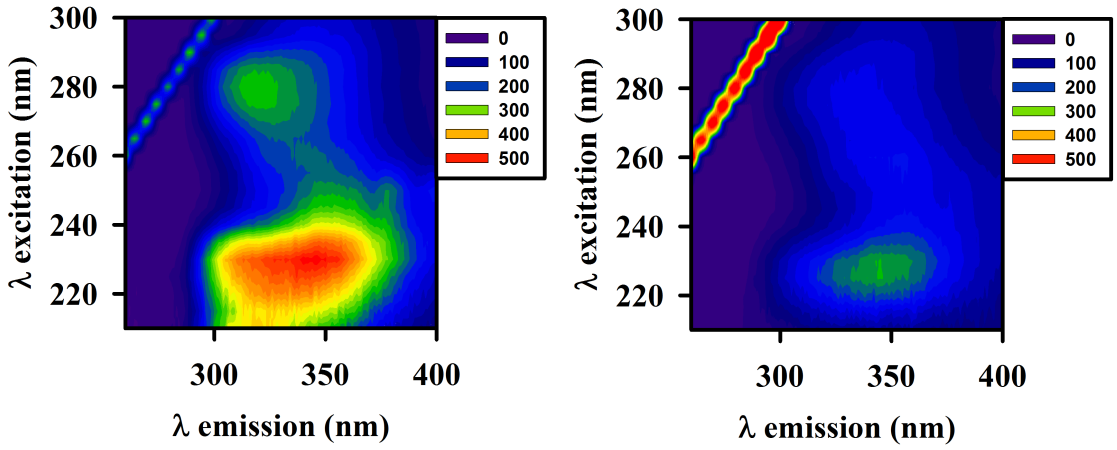
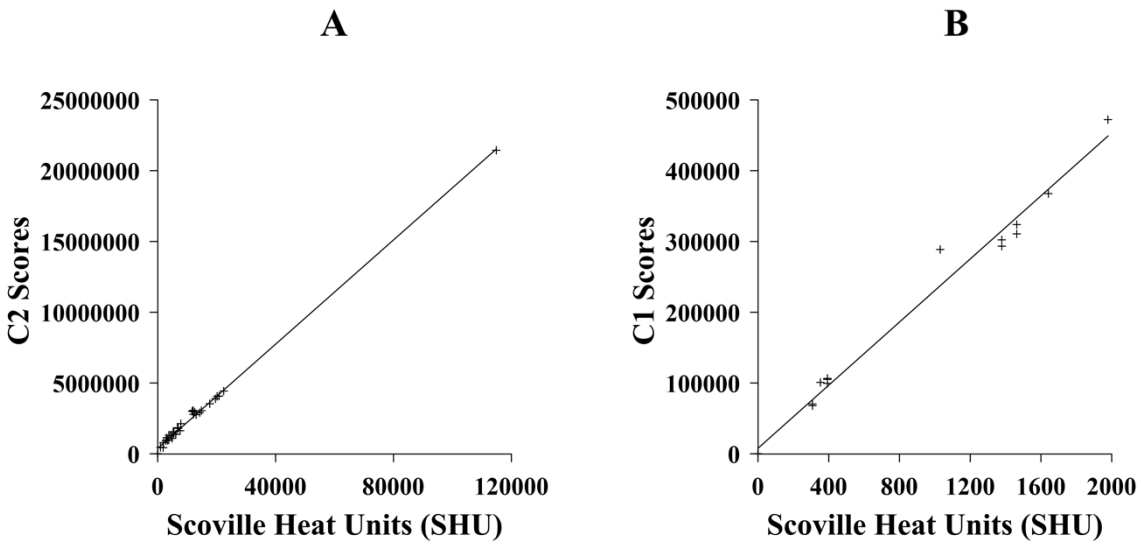


Figure 2. Excitation – emission matrix of a paprika sample after (left) and before (right) solid phase extraction procedure.



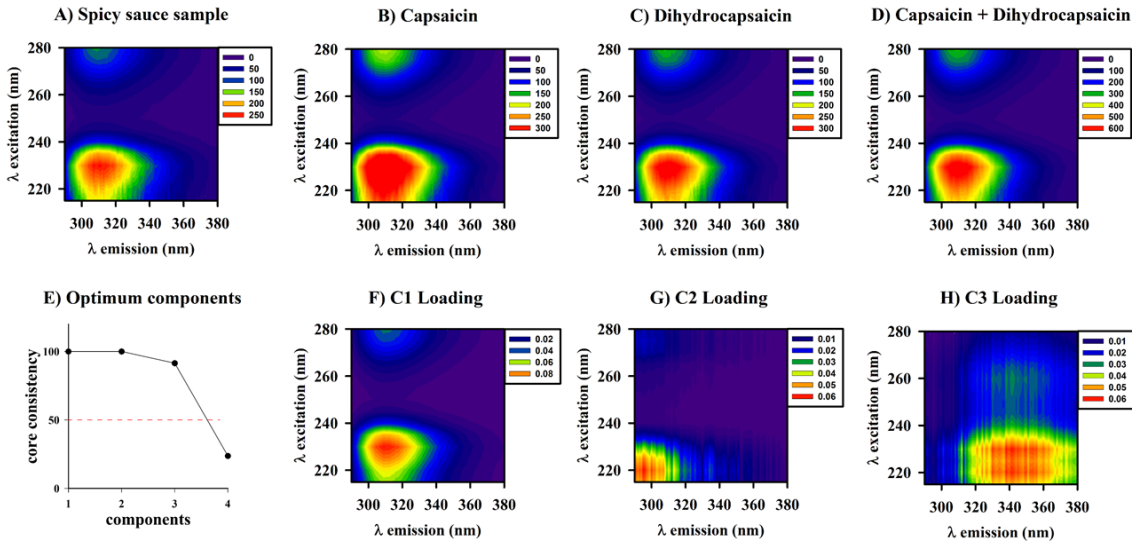
569

570 **Figure 3.** Excitation – emission matrices corresponding to a paprika sample (A), capsaicin
571 standard (B), dihydrocapsaicin (C) and the sum of capsaicin and dihydrocapsaicin standards (D).
572 Plot of core consistency value versus optimum number of components (E). Contour plots of the
573 different components obtained by PARAFAC decomposition for the group of paprika and pepper
574 samples (F, G, H).



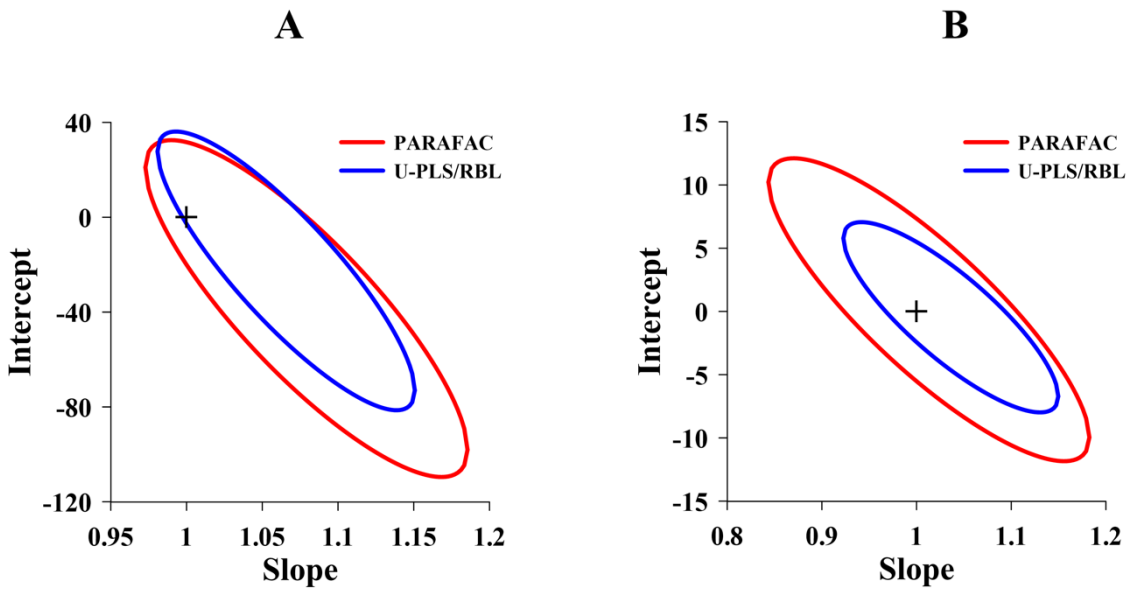
575

576 **Figure 4.** A) Correlation between the score values of component 2 and the concentration
577 calculated by HPLC for paprika and pepper samples. B) Correlation between the score values of
578 component 1 and the concentration calculated by HPLC for spicy sauces.



579

580 **Figure 5.** Excitation – emission matrices corresponding to a sauce sample (A), capsaicin standard
581 (B), dihydrocapsaicin (C) and the sum of capsaicin and dihydrocapsaicin standards (D). Plot of
582 core consistency value versus optimum number of components (E). Contour plots of the different
583 components obtained by PARAFAC decomposition for the group of spicy sauces samples (F, G,
584 H).



585

586 **Figure 6.** Elliptical join confidence region (EJCR, 95 % confidence level) for the slope and
587 intercept of the regressions of the concentration predicted by the different algorithms and those
588 calculated by HPLC. A (paprika samples and peppers) and B (spicy sauces).