SECOND-ORDER CALIBRATION IN COMBINATION WITH FLUORESCENCE FIBRE-OPTIC DATA MODELLING AS A NOVEL APPROACH FOR MONITORING THE MATURATION STAGE OF PLUMS

Olga Monago-Maraña*[1], Jaime Domínguez-Manzano[2], Arsenio Muñoz de la Peña[2,3], Isabel Durán-Merás[2,3]

[1] Nofima AS – Norwegian Institute of Food, Fisheries and Aquaculture Research, PB 210, N-1431, Ås, Norway

[2]Department of Analytical Chemistry, University of Extremadura, Badajoz 06006, Spain

[3]Research Institute on Water, Climate Change and Sustainability (IACYS), University of Extremadura, Badajoz 06006, Spain

*corresponding author: olgamonago@unex.es

Abstract

In this work, non-destructive autofluorescence of plums was employed to study the chlorophylls' concentration evolution along the maturation process. For that, excitation-emission matrices (EEMs), containing full fluorescence information, were collected with a fibre-optic, assembled to a spectrofluorometer. Data analysis was performed with several second-order multi-way algorithms, such as parallel factor analysis (PARAFAC), multi-way partial least-squares (N-PLS), unfolded partial least-squares (U-PLS), and multivariate curve resolution-alternating least-squares (MCR-ALS). Firstly, the EEMs of each plum, collected each week along the maturation process, were processed with PARAFAC. Two components were used to model the data and the excitation and emission loadings were obtained. Score values for the first PARAFAC component showed a clear evolution with time, increasing during the first five weeks, and decreasing for the last weeks. Also, the chlorophyll concentrations obtained by HPLC analysis, in the skin and the whole fruit, were compared with those obtained with different algorithms mentioned before. Best results were obtained in the case of skin for all algorithms. Similar correlation coefficients (r) were obtained in all cases (0.899 (PARAFAC); 0.940 (U-PLS); 0.936 (N-PLS) and 0.958 (MCR-ALS)). When the elliptical joint confidence region (EJCR), for the slope and intercept, were calculated, the theoretically expected values of 1 and 0, for the slope and intercept, respectively, were included in all ellipses. However, it was observed that for the skin data and U-PLS and N-PLS algorithms, the EJCR confidence region was smaller than in the other cases.

Keywords: Fibre-optic; plums; autofluorescence; second-order algorithms.

1. Introduction

Nowadays, the use of non-invasive approaches to characterize solid samples is being a successful alternative in any environment. Among others, the autofluorescence of foods obtaining fluorescence data with a fibre-optic has drawn attention, due to the fact that a previous extraction process is not necessary, and short response times and minimal instrumentation requirement are required.

The multidimensional nature of photoluminescence makes fluorescence spectroscopy an unique potential for simultaneous characterization of multiple fluorescence components in complex matrices. Nevertheless, to support this multidimensional information, chemometric techniques are necessary to process and model the fluorescence data sets, in order to extract the highest possible information content. Methods of classification, modelling, multivariate regression, similarity analysis, principal components analysis, experimental design and optimization, have been applied in different fields, such as environmental [1], food control [2] or medical and biotech processes [3–5], among others.

A common non-destructive technique widely employed for quality assessment of foods and agricultural products has been Near-Infrared Spectroscopy (NIRs). The benefit of this technique is due to the rapid, non-destructive and low-cost analysis [6]. The first-order data obtained with NIRs are mostly processed with partial least-squares regression (PLSR), being widely applied in food and in agriculture analysis [7–10]. NIR absorption spectra approximately describe the aggregate effect of absorption and scattering in food samples; they do not offer separate information on the absorption and emission properties. Hence, NIRs, in essence, is an empirical technique that relies on statistical methods to relate spectral features to the chemical or physical attributes of food samples. Because of its empiricism, conventional NIR measurements are not the most adequate for quantitative analysis. Other important trouble in NIRs analysis of solid

63 samples is that the spectral variations may be due to physical phenomena, such as dispersion,
64 and not related with sample chemical information [11]. For last, the first-order data obtained
65 with these techniques may not be enough to characterize complex matrices.

66 With the object of obtaining more selective information for the evaluation of agricultural
67 products using non-invasive techniques, artificial noses, tongues and sensors based in fibre-
68 optics have been developed, and the complex information provided by these instruments only
69 can be interpreted as useful information by means of chemometric tools [12]. Hence, the
70 combination of autofluorescence data, obtaining with a fibre optic probe, with chemometrics
71 has been probed as a useful tool to characterize multiple fluorescent components in intact
72 sample, allowing on-line monitoring for an appropriate quality control.

73 In this context, numerous studies have been performed to assess fruit harvesting time and to
74 study the evolution of the pigments along the maturation process [13]. The most visible and
75 frequent change in maturing fruits is the loss of green colour due to chlorophyll degradation.
76 Plums are climacteric fruits in which the chlorophyll content decreases during ripening, and the
77 measurement of this change is an unequivocal indication of maturation [14]. The chlorophylls
78 determination involves tedious treatments of the sample such as several extraction steps, under
79 dimmed light to prevent isomerization and photodegradation of pigments. These processes are
80 time-consuming and require specialized sample preparation [15,16].

81 Few studies can be found in the literature where fluorescence has been employed for the
82 estimation of maturity. For example, this technique was used to estimate the maturity of citrus
83 using deep learning [17] or for assessment of winegrape phenolic maturity [18]. Also,
84 chlorophyll fluorescence has been employed as non-destructive method to assess maturity of
85 mango fruits [19] and the chlorophyll fluorescence was measured with a hand-held multi-
86 parametric fluorescence sensor to follow the maturation of plums [20]. However, a different

variety was used in that study. There are no studies where the full information provided by EEMs was employed for this purpose.

The objective of this work is to show the usefulness of chemometrics for the control of maturation process of plums, without treatment of the sample, using well-established chemometric tools available to any user. For that, EEMs were analysed with different purposes: exploratory analysis and quantification of chlorophylls. Different algorithms were used for that: parallel factor analysis (PARAFAC) [21] for exploratory analysis, and PARAFAC, multiway partial least-squares (N-PLS) [22], unfolded partial least-squares (U-PLS) [23], and multivariate curve resolution-alternating least-squares (MCR-ALS) [24] for quantification.

2. Materials and Methods

2.1. Reagents, solvents and standards

Chlorophylls *a* and *b* (chl *a* and chl *b*) were obtained from Sigma-Aldrich Chemical Co. and used as received. Stock solutions of chlorophylls a and b were prepared by dissolving the ampules content (1 mg of each chlorophyll) in 25.0 mL of acetone and stored at −4 °C in darkness until use. Working solutions were prepared by dilution of the appropriate aliquots with acetone. Acetone was purchased from Merck (Darmstadt, Germany) and methanol (MeOH), acetonitrile (ACN), both of HPLC-grade, were purchased from Panreac (Barcelona, Spain). A methanolic solution of 5 mM ammonium acetate was prepared by dissolving a suitable amount of ammonium acetate in methanol.

2.2. Sampling

The effect of maturity was studied with *Friar* Plums variety. Plums sampling was carried out in a cultivar located in Badajoz, Extremadura, Southwest of Spain. Fruits were harvested each

week, from last week of May to August 2018. Samples were randomly collected and, for each week along the maturation process, four fruits were analyzed. Firstly, the EEMs of each one were recorded and, after that, the skin and the whole fruit were treated to extract the chlorophylls.

2.3. Excitation-emission matrices (EEMs) collection

For each plum randomly collected each week, four EEMs were collected in four spots, in order to have an average of the whole fruits. Hence, measurements were obtained from peduncle zone, from the bottom and from other two plum faces. All measurements were obtained with direct contact between the fibre and the fruits under normal laboratory illumination with a Cary Eclipse spectrofluorimeter, where a fibre-optic was assembled (Agilent Technologies, Madrid, Spain). The equipment was connected to a PC microcomputer via an IEEE 488 (GPIB) serial interface. The Cary Eclipse 1.2 software was used for data acquisitions. EEMs were recorded as a set of fluorescence emission spectra over a range of excitation wavelengths. The excitation wavelengths ranged was from 360 to 500 nm in 5 nm increments. At each excitation wavelength, the emission spectra were recorded from 600 to 700 nm, at 1 nm intervals. The slits of excitation and emission monochromators were set at 2.5 and 5 nm, respectively. The photomultiplier tube sensitivity was 700 V and the scan rate was set at 300 nm min^{-1}. The total scanning time per sample was approximately 5 min.

2.4. Softwares for data modelling

All calculations were carried out in Matlab (Matlab R2007b, version 7.5.0.342). Routines for PARAFAC were available on the internet thanks to Bro (http://www.models.kvl.dk/source/). MVC2, a useful Matlab graphic interface (http://www.iquir-conicet.gov.ar/descargas/mvc2.rar) was used for PARAFAC, U-PLS, N-PLS and MCR-ALS calculations [25,26].

133 *2.5. Extraction and HPLC analysis of chlorophylls*

134 For samples picked each week, the skin and whole fruit were independently analysed. The skin
135 of plums from fresh fruits was quickly removed and immediately treated. For the analysis of
136 whole plums, seeds were removed just before homogenization during 20 s with a mill. In all
137 cases, adequate weights (4 g of skin or 10 g of whole fruit) were extracted three times with 15
138 mL of THF:MeOH (1:1, v:v), in presence of BHT 0.1%, and 1 g of magnesium carbonate, with
139 continuous stirring. The supernatants were filtered under vacuum through quantitative filter
140 paper nº 1242 (Filter-Lab, Anoia, Barcelona, Spain) on a Büchner funnel. The combined
141 supernatants were re-extracted three times with 15 mL of petroleum ether and 15 mL of 10%
142 NaCl. The combined organic phases were evaporated to dryness (35 ºC in a rotatory
143 evaporator), and the residue dissolved in 5.0 mL of THF:MeOH (1:1, v:v). The measurements
144 of chlorophylls were carried out by diluting each aliquot (1:250). The extracts were filtered
145 with 0.25 μm diameter Chromafil filters (Düren, Germany), prior to the injection into the HPLC
146 system (UFLC Shimadzu Prominence LC-AD) and using a modification of the method
147 proposed by Orazem et al [27]. The analytical column was a Kinetex C18 (150 x 4.6 mm, 5
148 μm), with an analytical temperature of 30 ºC. The injection volume was 5 μL. The mobile phase
149 consisted on acetonitrile:methanolic solution of ammonium acetate (5 mM), 95:5 (v/v), with a
150 flow-rate of 1.0 mL min^{-1}. A fluorimetric detector was used, and 660 and 420 nm were set for
151 emission and excitation wavelength, respectively. External standard calibration, based on peak
152 areas, was used for quantification, and the concentrations of chlorophyll pigments were
153 determined in whole fruit and in the skin.

154 **3. Results and discussion**

155 *3.1. Excitation-emission matrices description of intact plums*

In order to obtain the fluorescence fingerprints from intact plums, EEMs were collected with a fibre-optic along nine weeks from May to August 2018. The advantage of using a fibre-optic is that it allows obtaining fluorescence information of fruits directly on the tree or in the packaging conveyor belt. The conditions employed to collect the EEMs were as described in section 2.3.

In Figure 1, the EEMs of samples harvested the first and the last sampling weeks and some pictures of samples from these weeks are illustrated. As can be observed, during the first weeks, samples exhibited high fluorescence signals at emission wavelengths between 670 and 700 nm, with high excitation signals between 400 and 500 nm. This fluorescent signal decreases as plums maturation takes place, and it is not appreciated practically after the week number eight. Visually, the change of colour is also appreciated in the pictures.

The advantage of using fluorescence signals as fingerprint of samples is related with its high selectivity and sensitivity. Three-dimensional map of samples offers a huge information of complex samples as foods are. Furthermore, the obtention of the EEMs with a fibre-optic avoids the sample treatment and allows on-line monitorization of samples for an appropriate quality control.

3.2. Qualitative study about the maturation process of plums

Firstly, a previous qualitative study about the maturation process was performed. For that, EEMs of the plums, recollected each week, during a period of nine weeks, were separately examined. A different 3D data set for each week was built, resulting 12 EEMs, corresponding to three plums samples and, for each plum, four EEMs obtained in different faces of the fruit.

PARAFAC was applied independently in the 12 EEMs of each week. The data were arranged in 3D arrays with dimensions 12 x 101 x 29 (samples (3 plums x 4 faces of each plum) x number of emission wavelengths x number of excitation wavelengths). These arrays were decomposed

by PARAFAC [21], applying the core consistency diagnostic (CORCONDIA) [28], analysing the residuals [29], and evaluating the shape profile of the loadings, for optimization of the number of components [26]. The core consistency analysis consists on studying the structural model based on the data and the estimated parameters of gradually augmented models. A model is considered to be appropriate if adding other combinations of components does not improve the fit considerably [28]. On the other hand, the analysis of residuals considers the residual fit of the PARAFAC model as a function of increasing number of factors. The appropriate model is the one which is not statistically different from the model leading to the minimum residual fit [26,29]. For the 3D array of each week, different numbers of components were assayed (from 1 to 5). In all cases, non-negative constraints, for the resolved profiles in all modes, were applied, with the purpose of obtaining a realistic solution, because concentrations and spectral values cannot be negative.

In Table 1, core consistency values, explained variance and the standard deviation of residuals with the number of components are shown. The optimum number of components for each week are bold remarked. As appreciated, during the first six weeks, and using the core consistency criterion, the optimal number of components was two. This optimization was performed considering that the optimal number of components is selected as the largest tested value for which the core consistency is larger than ≈50%. For the first six weeks, when the number of components goes from two to three, the core consistency percentage falls from 100% to about 40%. The SD residual values decrease from 3 to 1, approximately, when the number of components goes from one to two and then, the value was stable from two to five. Hence, with this criterion, the optimal number of components was also two. For the explained variance, it is appreciated that in all cases is higher than 99 % when two components are used as optimal.

In the Figures 2A, 2B and 2C it can be seen the excitation-emission profiles retrieved from PARAFAC for different weeks (week 2, week 4 and week 7). The excitation and emission loadings of the first components show very similar profiles along the weeks sampling. The first component shows a very clear and defined shape with an emission maximum at 685 nm, and two excitation maxima at 440 and 485 nm. Regarding to the second component, its emission profile does not show relevant information, however, the excitation profile is well-defined with a maximum at 395 nm and it slightly increases when the maturation process does it.

From seventh week, when PARAFAC was applied to the recorded EEMs, the number of components increased as indicated by the core value, which falls from 60% to 20% (approximately) when the number of components goes from three to four (Table 1). Also, increasing the number of components the value for the explained variance is similar to previous weeks. The excitation and emission profiles of the three components are represented in Figure 2C. The shape of the two first components are similar to those obtained along the first six weeks, and the third component shows a well-defined excitation maximum at 400 nm, and two emission maxima at 600 nm and 650 nm. However, it was difficult the identification of this component.

After this, taking into account the variability of different EEMs along the maturation process, different 3D arrays were obtained with the objective of reducing that variability. The first 3D array was from plums (average of four measurements in different faces) of each week, resulting a structure with dimensions 27 x 101 x 29 (samples (3 plums x 9 weeks) x number of emission wavelengths x number of excitation wavelengths). As in previous case, different criteria were used to select the optimal number of components and two were enough to explain 99.4 % of the variance. Figure 3A shows the score values for the first and second components with the time. Score values for the first component increase during the first weeks and then decrease

from the sixth week. Scores values for the second component appear quite constant along the time, a clear trend is not observed in this case.

Secondly, another data set was obtained from the average of all EEMs from each week. In this case, the number of samples was reduced to 9, corresponding with the number of weeks that samples were recollected. As appreciated in Figure 3B, the evolution of score values for the first component shows that it increases along the three first weeks, after that, it is constant for two weeks and, then, it starts to decrease until it is almost zero.

According to the excitation and emission wavelengths of the loading profiles for the first component, this component could be related with the chlorophyll compounds present in plums. In the literature, the excitation/emission maxima for the chlorophyll are 458/653 nm when the spectra were register in acetone/water medium [30]. The position of the maximum can shift to different wavelengths when spectra are obtained from intact solid samples rather from a solution due to the variation that molecules could suffer, as we proved in another study with other compounds [31].

For this reason, a quantification of these compounds was performed by HPLC-FLD, as detailed in the next section. Taking into account that the best results were obtained when using the average of samples from each week, these data were used in the following section.

3.3. Quantification of chlorophylls with second-order algorithms

Once a previous identification of PARAFAC components on the basis of the fluorescence spectrum was done, it was interesting to confirm this assumption evaluating the relationship between score values obtained by PARAFAC for the first component and the concentration of chlorophyll. The chlorophyll concentrations were obtained by HPLC-FLD in extracts of plums recollected along the nine weeks, in both, skin and the whole fruit. Firstly, in the case of skin

chlorophyll content, when the score values of the first PARAFAC component were related with chlorophyll concentration values, a correlation coefficient of 0.8998 was found. In the case of whole fruit, this coefficient was lower (r = 0.8626).

In order to compare these results with those obtained with other algorithms, MCR-ALS, U-PLS and N-PLS were applied. In all cases, the data were arranged in a 3D array with dimensions 9 x 101 x 29 (samples (average values in each of the 9 weeks) x number of emission wavelengths x number of excitation wavelengths). The first step when using MCR-ALS was to obtain the augmented matrices in the excitation wavelength direction. The number of components in each augmented matrix was estimated by principal component analysis (PCA), and justified taking into account the presence of the corresponding analytes, possible interferences, and background signals. Non-negativity restriction was applied in both modes, emission and excitation spectroscopic spectral data, and unimodality restriction was applied only to the signals corresponding to the analytes, and not to the background signals. After ALS optimization for each sample, and with the aid of the corresponding pseudounivariate calibration curves, the constituents were identified and quantified. The optimal number of components was 3, which explained the 99.7 % of variance. In Figure 4, excitation profiles retrieved by MCR-ALS and the emission spectra for each component are shown. A comparison of the profiles of the first component with those obtained by PARAFAC, indicates that the excitation and emission maxima are very similar (Pearson regression coefficients of 0.9890 and 0.9637, for emission and excitation, respectively, and with 95 % of level confidence were calculated). The correlation coefficient between the score values of the first component and the HPLC data was 0.9581, when the score values of the first component *versus* the measured HPLC content of chlorophyll in the skin, were plotted, and 0.8851 for the whole fruit content.

Furthermore, U-PLS and N-PLS were assayed. In these cases, the Haaland and Thomas criterion [32,33] was employed to select the number of optimal components, which are those given a PRESS value statistically no different to the minimum PRESS value (F-ratio probability falling below 0.75). The optimum number of latent variables was three. When concentrations of chlorophyll were predicted by both algorithms, the correlation coefficients between predicted and true concentration were 0.9400 and 0.9360 for U-PLS and N-PLS, respectively, for the concentration in the plums' skin, and 0.8770 and 0.9091, respectively for concentration in whole fruits. Figures 5A and 5C show the plots obtained for all algorithms between true and predicted concentrations.

In Table 2, all figures of merit are included. It can be appreciated that the best results were obtained in the skin and for the U-PLS and N-PLS algorithms because the root mean square error of predictions (RMSEPs) and the relative error of predictions (REPs) are lower in these cases.

Finally, the elliptical joint confidence region EJCR test (at 95% confidence level) [34] was applied for the different algorithms to evaluate the slope and intercept, corresponding to the linear regression of predicted concentrations for the algorithms vs the HPLC concentrations. The corresponding ellipses are shown in Figures 5B and 5D, for the skin and whole fruit results, respectively. Note that in all cases the critical point (1,0) is included, which proved the accuracy between true and predicted concentrations for the different algorithms. However, in the case of the skin analysis, the ellipses for U-PLS and N-PLS are smaller in size, suggesting higher precision for these algorithms.

As conclusion, it can be said that the results were quite similar for all algorithms, and better in the case of the skin results. From the results obtained in this study, it can be highlighted that second-order algorithms are a powerful tool for the characterization of agronomic processes.

296 Furthermore, the combination with a non-destructive technique, such as a fibre-optic, and using
297 a selective signal, as autofluorescence of the sample, is another step in the possible
298 automatization of the maturation process.

299

300 **4. Conclusions**

301 Non-destructive characterization of fresh plums was performed for the first time, employing
302 fibre-optic data with second-order calibration. Results showed that chlorophyll content could
303 be a good indicator of maturation process. Taking into account that timing of fruit picking
304 (harvest maturity) significantly impacts in the postharvest handling systems, especially when
305 international deliveries are performed, it is very important to dispose non-destructive
306 techniques that allow deciding the harvest date. Moreover, fluorescence fingerprints in
307 combination with second-order calibrations can be a powerful tool for determination of
308 chlorophyll content in plums.

309 Although the best results are obtained with U-PLS and N-PLS in the skin of plums, as indicate
310 the smaller size of EJCR regions, it is interesting the possibility that PARAFAC and MCR-ALS
311 offer to be able to obtain spectral information about the fluorescence components.

312 The obtained results seem very promising and which could be used as references of maturation
313 in situ, employing a fibre-optic probe with a portable system. However, more samples will be
314 necessary to expand the calibration data set to develop a robust prediction model that can be
315 used in practice.

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Figure captions

Figure 1. Fluorescence contour plots of the EEMs obtained with a fibre-optic, and pictures from samples corresponding to the first week of sampling (May 2018) and last week of sampling (August 2018).

Figure 2. Excitation and emission PARAFAC profiles of samples belonging to different maturation weeks: A) week 2; B) week 4; C) week 7.

Figure 3. Evolution of the score values for the first and second components retrieved by PARAFAC, considering the average of each plum measurement (A), and considering the average of each week (B), along the time (in weeks).

Figure 4. (A) Excitation profiles retrieved by MCR-ALS analysis for different weeks of recollection. (B) Emission spectra retrieved by MCR-ALS. Dashed lines correspond to excitation profiles and emission spectra retrieved by MCR-ALS for unknown compounds and background signals. Continuous lines correspond to excitation and emission spectra, retrieved by MCR-ALS, for chlorophyll.

Figure 5. Plots of chlorophyll predicted concentrations, for the different algorithms, as a function of the true concentrations obtained by HPLC (A and C). Corresponding elliptical joint regions (at 95 % confidence level), for the slopes and intercepts of the regressions. The theoretical point (intercept = 0; slope = 1) is marked in the figure by the black cross (B and D).

Table 1. Core, SD values and explained variance obtained along the nine weeks studied.

	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8		Week 9	
Components	**Core**	**SD**	**Core**	**SD**	**Core**	**SD**	**Core**	**SD**	**Core**	**SD**	**Core**	**SD**	**Core**	**SD**	**Core**	**SD**	**Core**	**SD**
1	100	2.3	100	2.3	100	2.7	100	3.1	100	3.3	100	3.1	100	2.4	100	3.1	100	2.0
2	**100**	**0.97**	**100**	**0.94**	**99**	**1.1**	**99**	**1.3**	**100**	**1.4**	**99**	**1.3**	100	1.1	99	1.4	97	1.2
3	48	0.86	43	0.80	38	0.90	33	1.1	38	1.1	33	1.1	**58**	**0.89**	**57**	**1.0**	**57**	**0.64**
4	24	0.75	9	0.71	7.7	0.74	15	1.0	23	1.0	15	1.0	18	0.81	26	0.87	4.9	0.58
5	6	0.86	3	0.64	-0.94	0.66	0.46	0.87	0.74	0.87	0.5	0.87	1.0	0.72	2.3	0.79	3.0	0.55
Explained variance (%)*	99.5		99.4		99.3		99.4		99.3		98.9		99.3		99.1		98.9	

*for optimal number of components

Table 2. Figures of merit for different algorithms assayed.

		Skin							Whole fruit						
Algorithm	**Components**	**Slope**	**Intercept**	**R^2**	**Sr**	**Test of significance**	**RMSEP (mg/g)**	**REP (%)**	**Slope**	**Intercept**	**R^2**	**Sr**	**Test of significance**	**RMSEP (mg/g)**	**REP (%)**
PARAFAC	3	1.0	-0.001	0.8097	0.2	s	0.2	26	1.0	-1e-7	0.7440	0.1	s	0.1	37
U-PLS	3	0.93	0.03	0.8842	0.1	s	0.1	20	0.67	0.09	0.7691	0.2	s	0.1	24
N-PLS	3	0.94	0.03	0.8767	0.1	s	0.1	21	1.0	-0.09	0.8256	0.2	s	0.1	38
MCR-ALS	3	1.3	-0.31	0.9180	0.8	s	0.2	44	1.1	-0.15	0.7835	0.4	s	0.1	74

s: Pearson correlation significative test (p-value < 0.05).