PLS Calibration to Resolve Overlapping Peaks of Lutein and Zeaxanthin in Vegetable Samples by LC

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Abstract

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The chromatographic method in combination with a Partial Least Squares (type PLS-1) chemometric tool was developed to analyse simultaneously the carotenoid compounds, lutein and zeaxanthin. Both analytes appear together in the same chromatographic peak. Different calibration matrices were done using the absorbance spectra, obtained in the peak apex. The method was successfully applied to plant material such as cabbage, broccoli, red pepper, yellow pepper, and green pepper. The higher content of lutein was found in the green leafy vegetables such as broccoli and cabbage. On the other hand, zeaxanthin was only found in pepper, with the highest concentration having been found in red pepper.

Keywords: caroteroids; Partial Least Squares; liquid chromatography; pepper; broccoli; cabbage

Carotenoids are a wide group of plant pigments that have an important bioactivity in humans and animals. The intake of foods rich in these compounds is considered to be beneficial in the prevention of some diseases, including certain cancers, atherogenesis, cardiovascular diseases, neuronal damage, and eye disease (Cantuti-Castelvetri 2000; Cooper 2004; Krinsky & Johnson 2005). The beneficial effects of carotenoids are thought to be due to their role as antioxidants. Food sources of these compounds include a variety of fruits and vegetables, in adition, egg yolk is a highly bioavailable source of lutein and zeaxanthin (Handelman *et al.* 1999).

Lutein and zeaxanthin have been suggested to be protective against certain eye diseases. The selective accumulation of lutein and zeaxanthin in the macula of the eye retina and their capability to absorb potentially damaging blue light have raised expectations that their ingestion may reduce the risk of age-related macular degeneration (AMD) and cataracts (Landrum *et al.* 1997; Vu *et al.* 2006).

In the literature are presented various analytical methods, such as spectrophotometry, colourimetry, and HPLC, for the determination of carotenoids in food products and biological samples (Schoefs 2002). The most commonly employed methods are based on liquid chromatography (LC) coupled to different detection techniques, mainly with diode-array or mass spectrometric detection.

The compounds lutein and zeaxanthin are poorly separable on a variety of LC columns and the authors have usually reported the sum of the con-

centrations of these compounds (Su *et al.* 1999; Takahashi *et al.* 2006). In these cases, the pigments could be individually quantified if chemometric calibration was applied to the chromatographic peak signal. Among the existing first-order chemometric calibration methods, Partial Least Squares (PLS) is a factor analysis method first applied to chemical analysis by Wold *et al.* (1983) and it has been widely used in analysis during the last decade. As far as we know, only few authors have reported studies using PLS in the simultaneous identification of these carotenoid pigments in plant material (Meléndez-Martínez *et al.* 2003; Pereira *et al.* 2007), but not in dynamic chromatographic signals.

In this paper, a chemometric method based on the application of partial least squares (PLS) calibration is proposed for the simultaneous determination of lutein and zeaxanthin in cabbage, broccoli, and pepper plants.

A survey of the literature shows that chemometric methods like partial least squares (PLS-1 and PLS-2) and principal component regression (PCR) have been applied to chromatographic signals of co-eluted analytes such as tocopherols and pesticide mixtures (Gil García *et al.* 1997; Galeano Díaz *et al.* 2007).

The goal of this work is the simultaneous and independent quantification of lutein and zeaxanthin by a reverse phase-HPLC method. Due to the total coincidence of the peaks, a chemometric tool (PLS-1) has been used over the absorbance spectra obtained in the peak. The developed method has been successfully applied to different vegetable samples.

MATERIAL AND METHODS

Chemicals. All solvents used were of analytical reagent quality and were purchased from Panreac (Cordóba, Spain). Lutein and zeaxanthin were purchased from Sigma-Aldrich (Madrid, Spain). Standard solutions of each compound (100 μg/ml) were prepared by dilution in acetone, avoiding the exposure to direct light, and keeping at 4°C. The working standard solutions consisted of the mixtures of the two carotenoids of different concentrations in acetone, and were used immediately after the preparation. HPLC grade water was produced from a Milli-Q system (Millipore, Miford, USA). Plant materials were purchased in local groceries.

Apparatus. The HPLC system used was an Agilent 1100 Series (Agilent Technologies Inc., Palo Alto, USA) composed by a G1311A Agilent quaternary pump, a G1322A Agilent vacuum solvent delivery degasser, a G1315B Agilent UV-VIS photodiode array detector, and a G1313A ALS autosampler. The liquid chromatographic system was controlled and the data were collected and processed by the HP ChemStation for LC 3D software (Agilent Technologies Inc., Palo Alto, USA).

Chromatographic system. The analysis was performed applying the method proposed by Mínguez-Mosquera and Hornero-Méndez (1993), and modified by García *et al.* (2007). Briefly, carotenoids were extracted from the fruits and/or vegetables with acetone, saponified overnight, and the obtained extract was injected into the chromatographic system. The separation was accomplished in a reversed phase C18 column (RP-18 Lichrosorb, 10 μm, 200 mm × 4.6 mm). The initial mobile phase was acetone:water (75:25, v/v) for 5 min, raised to (95:5, v/v) over 10 min with a flow rate of 1 ml/minute. The chromatograms were recorded at 445 nm. The absorbance spectra, at the peak maximum, were collected from 380 nm to 600 nm (intervals of 2 nm).

Chemmometric software. The program MVC1 (Olivieri *et al.* 2004) written in MATLAB 6.0 (1999) (The Math Works Inc., Natick, USA) was used for PLS-1 calibration. For the experimental design, the Unscrambler was used (Unscrambler Software, Vers. 6.11 of CAMO, Trondheim, Norway).

Partial least squares approach. Partial least squares (PLS) technique based on factor analysis was first applied to chemical analysis by Wold *et al.* (1983). PLS uses the full-spectrum information and has the advantage that it is not necessary to explain the variations on the concentrations of the analytes. The PLS-1 method involves a two-step procedure: (1) calibration, where the relation between the spectra and reference component concentrations is established from a set of standard samples, and (2) prediction, in which the calibration results are employed to estimate the component concentrations in unknown samples (Martens & Naes 1989). Before calibration, the optimum number of factors should be selected in order to avoid overfitting. This has been performed by applying the cross-validation method to calculate the prediction residual error sum of

squares (PRESS). To select the optimum number of factors, the criterion proposed by Haaland and Thomas (1988) was applied.

To quantify the prediction ability of the model, the square of the correlation coefficient (R^2) was used, which is an indication of the quality of the fit of all the data to a straight line.

The estimation of analytical figures of merit, such as sensitivity (SEN), selectivity (SEL), and limit of detection (LOD), were calculated (Lorber & Kowalski 1988; Booksh & Kowalski 1994; Ferré & Faber 2003).

RESULTS AND DISCUSSIONS

Chromatographic approach

Carotenoid pigments were extracted from the plant material according to the procedure listed above and the extracts solutions were injected into the chromatographic system for their separation. Figure 1A represents the chromatogram of the carotenoids from a red pepper extract, the chromatograms of the standard solutions of lutein and zeaxanthin are represented in Figures 1B and 1C, respectively. The retention times of the studied compounds are the same, and therefore the peak Lute +

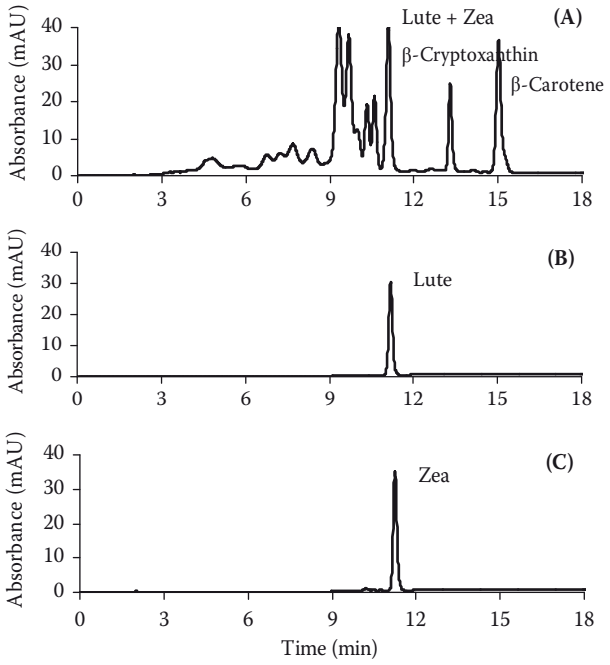

Figure 1. Chromatogram plot of red pepper extract (A), lutein standard solution (5 μg/ml) (B), and zeaxanthin standard solution (10 μg/ml) (C), recorded at 445 nm

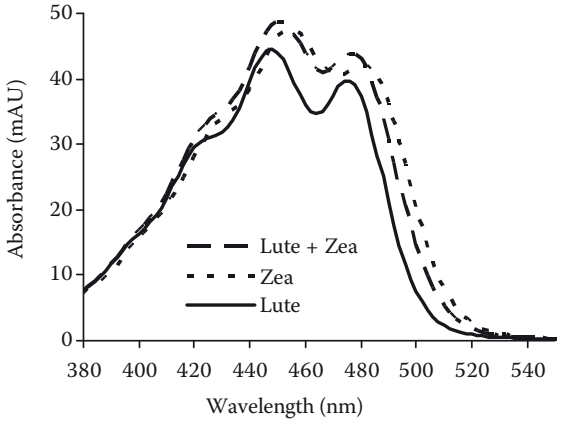

Figure 2. UV spectra of lutein standard solutions of lutein (5 μg/ml), zeaxanthin (10 μg/ml), and a mixture of both pigments

Zea in chromatogram 1A corresponds to the sum of both analytes. As both compounds are co-eluted, a PLS approach was proposed for their simultaneous determination using UV spectra recorded in the peak apex. The proposed chromatographic separation allowed us to quantify the identified carotenoids (peaks 1, 3, and 4), however, for lutein and zeaxanthin single quantification further chemometric process needs to be applied to the recorded signals. Figure 2 illustrates the UV spectra extracted from the chromatographic peaks of lutein and zeaxanthin and from their mixture. However, the overlap observed between the absorption spectra of both compounds indicates that univariate methods may not be directly applied to the resolution of the mixtures of these two analytes. For this reason, multivariate methods, such as PLS, may resolve the overlapping bands without physical separations and therefore the chromatographic analysis coupled to the chemometric analysis may resolve all the studied compounds in a single analysis.

PLS optimisation

The PLS method involves the calibration step in which the relation between bi-dimensional emission spectra and analyte concentrations is estimated from a set of reference samples (calibration set), and a prediction step in which the results of the calibration are used to estimate the component concentrations in unknown samples (prediction set). The calibration set was constructed with 9 calibration samples according to a central composite design. Lutein and zeaxanthin concen-

Table 1. Optimum number of factors and calibration statistical parameters in applying PLS algorithm to resolve the mixture of lutein and zeaxanthin using UV spectra

	Lutein	Zeaxanthin
Statistical parameters		
Factors	2	2
PRESS*	1.49	1.21
R^2	0.990	0.997
Figures of merit		
SEL*	10.813	8.6887
SEN[1]*	0.17852	0.17737
LOD[2]*	0.266	0.331

[1]expressed in absorbance ml/µg; [2]expressed in µg/ml; *PRESS – prediction residual error sum of squares; SEL – selectivity; SEN – sensitivity; LOD – limit of detection

trations ranged from 0–14 µg/ml and 0–19 µg/ml, respectively. The absorption spectra were registered between 380 nm and 600 nm, and digitised each 2 nm. In order to determine the correct number of the loading vectors to be used for data modelling, the Haaland and Thomas (1988) criteria were used and cross-validation calculations were performed to calculate the PRESS error. Moreover, different spectra ranges were studied in order to establish the optimum wavelength range for each compound determination. No significant difference was observed while using the whole or part of the wavelength spectra range.

The PLS optimised model is summarised in Table 1, and as can be observed, the optimum number of factors calculated for both pigments was 2, achieving in these conditions a correlation coefficient (R^2) of 0.990 and 0.997 for lutein and zeaxanthin, respectively.

The analytical figures of merit (LOD, SEL, and SEN) were calculated for the optimised model according to the previously published methods (Booksh & Kowalski 1994; Ferré & Faber 2003), and the obtained values are summarised in Table 1; according to them, the optimised PLS method is adequate for these pigments quantification in vegetable samples.

Validation of PLS model

To test the quality of the optimised model, the PLS model was applied to a data set of varied concentrations of lutein and zeaxanthin mixtures in order to perform its validation. The samples analysed for the validation set were random mixtures of variable amounts of both pigments whose concentrations were calculated using the optimised PLS method. The analytes concentrations were in the calibration set range. In Figure 3, a box-plot diagram of the mean recovery values for each analyte is plotted. It can be observed that recoveries around 100% are found for both carotenoid pigments.

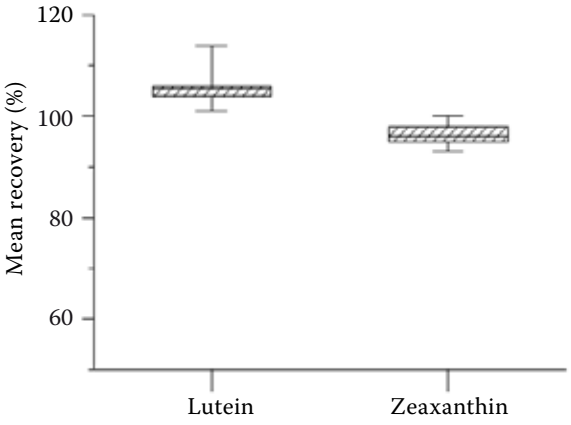

Figure 3. Diagrammatic plot of the mean recovery values calculated by the application of PLS in the analysis of synthetic mixtures of lutein and zeaxanthin

To determine the accuracy of the method, an intraday assay was made using 5 samples containing 5.00 µg/ml and 9.00 µg/ml of lutein and zeaxanthin, respectively. The concentrations were calculated by applying the proposed PLS model. A good intraday repeatability was observed in all cases, with Relative Standard Deviation (RSD%) 1.94 and 2.00 for lutein and zeaxanthin, respectively.

Table 2. Analysis of lutein and zeaxanthin pigments in different plant materials using the optimised chromatographic-chemometric method

Sample	Flesh colour	Concentration (µg/100 g FW)[1]	
		lutein	aeaxanthin
Cabbage	green	641 ± 67	traces
Broccoli	green	1204 ± 73	traces
Pepper	green	497 ± 47	46 ± 6
Pepper	red	96 ± 7	277 ± 17
Pepper	yellow	503 ± 49	53 ± 5

[1]mean of four analysis

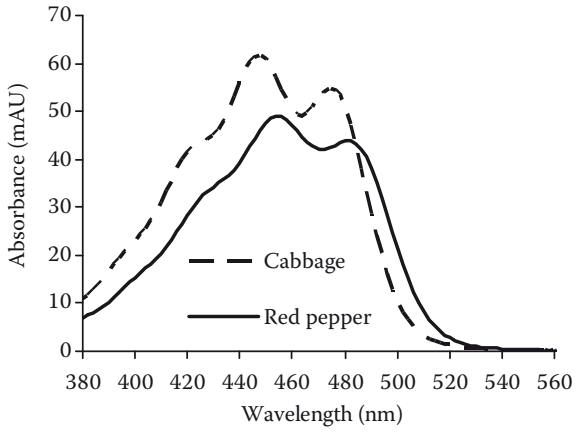

Figure 4. UV spectra of red pepper and cabbage extracts

Lutein and zeaxanthin quantification in vegetables samples

The optimised method was applied to the determination of both pigments in different commercial plant materials, such as cabbage, broccoli, and three types of pepper (green, red, and yellow). The results of the analyses are summarised in Table 2. A higher content of lutein was found in the green leafy vegetables such as broccoli and cabbage. On the other hand, zeaxanthin was only found in pepper, with the highest concentration having been found in red pepper. No amount of this pigment was found in the green leafy plants. Similar results were previously reported (Perry *et al.* 2009). To illustrate these results, UV spectra of red pepper and cabbage plants are represented in Figure 4 recorded at the maxima of the chromatographic peaks. In this figure, it can be observed that both spectra are different due to that in cabbage plant only lutein is present while in red pepper zeaxanthin is the main compound. The difference in these compounds spectra allows to make PLS methodology suitable for the simultaneous determination of both pigments.

CONCLUSIONS

The proposed chromatographic-multivariate method allows us to determine simultaneously lutein and zeaxanthin in plant extracts; due to the total overlapping of their chromatographic peaks PLS multivariate method has been used for the resolution. The model has been validated by the analysis of a set of synthetic samples containing different amounts of both pigments in different ranges of concentrations. In addition, interday and intraday assays have been carried out in order to establish the analytical robustness of the proposed method, and it has been demonstrated that the model can be applied for multiple analysis in the same and different days without any loss of accuracy. This method has been satisfactorily applied to vegetable samples, such as cabbage, broccoli, red pepper, yellow pepper, and green pepper.

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