Neural Network Analysis of Spectroscopic Data of Lycopene and *β*-Carotene Content in Food Samples Compared to HPLC-UV-Vis

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In this study a neural network (NN) model was designed to predict lycopene and *β*-carotene concentrations in food samples, combined with a simple and fast technique, such as UV−vis spectroscopy. The measurement of the absorbance at 446 and 502 nm of different *β*-carotene and lycopene standard mixtures was used to optimize a neural network based on a multilayer perceptron (MLP) (learning and verification process). Then, for validation purposes, the optimized NN has been applied to determine the concentration of both compounds in food samples (fresh tomato, tomato concentrate, tomato sauce, ketchup, tomato juice, watermelon, medlar, green pepper, and carrots), comparing the NN results with the known values of these compounds obtained by analytical techniques (UV−vis and HPLC). It was concluded that when the MLP-NN is used within the range studied, the optimized NN is able to estimate the *β*-carotene and lycopene concentrations in food samples with an adequate accuracy, solving the UV−vis interference of *β*-carotene and lycopene.

KEYWORDS: *β*-Carotene; lycopene; chemical interferences; neural networks; UV spectroscopy; HPLC

INTRODUCTION

Linear and nonlinear algorithms have been used to provide an adequate resolution of complex spectra such as for food samples (*1−3*), to interpret voltamperometries of mixtures composed of glucose, uric, and ascorbic acids (*4*) or hazardous chemicals (*5*), or to solve the interference of UV−vis spectra to quantify the concentration of chemicals in quaternary complex mixtures composed of hydrocarbons and ionic liquids (*6*).

Despite great interest in both lycopene and *β*-carotene analysis in food products, which can be carried out by different methods, at present there is not a technique highly versatile, fast, sensitive, and selective such as needed for a reliable analysis of the complex matrices that can be found in food samples (*7−10*).

Olives Barba et al. optimized and compared an HPLC method with a spectrophotometric standard method for the determination of lycopene and *β*-carotene in vegetables (*11*). These authors indicated the overlap of lycopene and *β*-carotene spectra, near 446 nm, as the main reason of observed deviations, compared with optimized HPLC analysis data. The HPLC method showed good accuracy and sensitivity for the quantification of lycopene in fruit and vegetable samples, being more specific than spectophotometry. However, the spectrometric method can be used to rapidly assess the lycopene and *β*-carotene content of different vegetables products, but this method showed an overestimation of *β*-carotene content in lycopene-rich samples.

To solve this problem multilayer perceptron (MLP) neural networks (NNs) have been applied as a fast and reliable tool to accurately determine the *β*-carotene and lycopene concentrations in complex mixtures using a common analytical and simple technique such as UV−vis spectroscopy, avoiding interferences, overestimation, and time-consuming calibration methods (*12*).

To continue in this research area, the main goal of the present study has been to apply the previously developed NNs based on a MLP to analyze a wide range of food samples as complex matrices containing lycopene and *β*-carotene, with different carotenoid profiles in order to propose a reliable mathematical model that estimates the lycopene and *β*-carotene concentrations using the absorbance values at 446 and 502 nm obtained by a common analytical and simple technique such as UV−vis spectroscopy (solving the overlapping effect) and tested with a more complex analytical and accurate technique such as HPLC.

MATERIALS AND METHODS

Reagents, Standards, Food Samples, Instrumentation, and Analytical Methods. Standards of *all-trans*-lycopene and *β*-carotene were from Sigma (St. Louis, MO) with purity of ≥90%.

The HPLC grade solvents were purchased from Symta (Madrid, Spain) in the case of methanol and acetonitrile (ACN) and from Sigma (Steinheim, Germany) in the case of triethylamine (TEA) and tetrahydrofuran (THF) stabilized with <0.025% butylated hydroxytoluene. The reagent grade solvents used in liquid−liquid extraction were supplied by Merck (Darmstadt, Germany) in the case of *n*-hexane; diethyl ether, acetone, and ethanol were supplied by Panreac (Barcelona, Spain).

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Doubly distilled water was obtained from a Milli-Q System (Millipore, Bedford, MA).

Standards Mixtures. A five-level factor experimental design (25 standard mixtures) of two factors and two response variables was performed to optimize the MLP NN model (*12*). These factors were concentration values of β-carotene and lycopene working standard solutions by diluting in *n*-hexane (Merck) in a range between 0.4 and 3.2 μg mL^{-1}. The response variables were the absorbance values at 446 nm for β-carotene and at 502 nm for lycopene. Their purity was checked by calculating the concentration of the standard solution using the extinction coefficient (*13*, *14*).

Food Samples. A total of 25 vegetable food samples were used for carrying out two external validation processes of the MLP NN model used in this study. These included 10 lycopene-rich samples (tomato concentrate, tomato sauce, ketchup, tomato juice, and tomato pure), which were used for its first external validation. The remaining 15 vegetable samples (fresh tomato, carrots, watermelon fruits, green pepper, and medlar) were selected by their different carotenoid profiles (*15*, *16*) and used as a second external validation sample.

Instrumentation. A Perkin-Elmer UV−visible spectrophotometer (Lambda EZ210) was used for absorbance measurements using quartz cells of path length of 1 cm. Data acquisition and spectrometric evaluation were carried out by Perkin-Elmer software PESSW version 1.2. In all cases, a minimum of three replicate measurements of spectroscopic absorption for each sample were carried out.

The chromatographic apparatus consisted of a Micron Analítica, S.A. (Madrid, Spain) PU II isocratic pumping system; a Jasco (Tokyo, Japan) AS-1555 autosampler; a ERC-Gecko-2000 (Riemerling, Germany) column heater; and a Thermo Separation Spectra series UV100 (San Jose, CA) UV−vis detector. For data processing and analysis, Biocrom 2000 3.0 version software from Micron Analítica, S.A. was used. The analytical column was a μBondapack C18 (300 mm × 2 mm, 10 μm pore size), with a μBondapack C18 precolumn (20 mm × 3.9 mm, 10 μm pore size), both purchased from Waters (Milford, MA). A Büchi Labortechnik AG (Flawil, Switzerland) rotatory evaporator was used to obtain the dry extracts.

Analytical Methods. Samples were extracted in a mixture of hexane/acetone/ethanol (50:25:25). After 30 min in magnetic stirring, 10 mL of water was added, and the upper hexane layer was separated for spectrophotometric analysis at 446 and 502 nm. Another aliquot was evaporated to dryness and diluted in a mixture of THF/ACN/methanol (15:30:55 v/v/v) for HPLC analysis according to Olives Barba et al. (*11*). Every sample was prepared in triplicate, and then each one was monitored three times.

Using individual standard concentrations of β-carotene and lycopene and their respective absorbance values at 502 and 446 nm (UV−vis spectrophotometry), as well as their respective peak areas (HPLC), the calibration equations were obtained (**Table 1**). The calibration parameters and sensitivity of the methods applied to β-carotene and lycopene analysis are also shown in **Table 1**. The detection (LOD) and quantification (LOQ) limits for these analytical methods have been estimated following ICH Guideline Q2B (*17*), eqs 1 and 2

$$\text{LOD} = \frac{3S}{m} \quad (1)$$

$$\text{LOQ} = \frac{10S}{m} \quad (2)$$

where S and m are, respectively, the intercept and slope of fit equations.

Neural Network Model. The NN used here is based on a multilayer perceptron, which is a feed-forward and supervised network. It consists of several neurons (information-processing units) arranged in two or more layers. Each receives information on all of the neurons of the previous layer. Each connection is controlled by a weight that modules the output of the neuron before inputting its numerical content into a neuron in the following layer. The process by which the weights are optimized is called the learning or training process (*6*, *18*, *19*). The training algorithm used to optimize the weights is based on a back-propagation (BP) algorithm (*19*). The inputs of each neuron are added by activation function, and the result is transformed by a transfer function, which serves to limit the amplitude of the neuron output. When the NN parameters are adjusted, by a slight refresh of its weights, the NN is able to learn from its environment. The NN model used in this work was designed using Matlab version 7.01.24704 (R14) (*18*). The statistical analyses were carried out by SPSS version 15.0.1.

Description of Learning, Verification, and Validation Samples (Database). Considering that the NN used is based on a supervised algorithm, to optimize the matrix of weights it is necessary to use input and output data that adequately characterize the process to be modeled. In this work, the data are organized in four rows (absorbance at 446 and 502 nm and their respective β-carotene and lycopene concentration values) and one column for each sample measured. For learning and verification purposes standard mixture data were randomly distributed into the learning (80%) and verification (20%) samples.

For validation, two sets of samples have been considered. The first validation sample is composed of β-carotene and lycopene concentrations (obtained by UV−vis spectroscopy) from tomato concentrate, tomato sauce, ketchup, tomato juice, and tomato pure (10 lycopene-rich food samples). The second validation set of samples includes β-carotene and lycopene concentrations of fresh tomato, carrots, watermelon fruits, green pepper, and medlar (15 samples with different carotenoid profiles) obtained by HPLC.

Neural Network Model Optimization and Verification Process. The MLP model here used consists of three layers (input, hidden, and output), a topology widely used to model systems with similarly complex levels (*6*). In particular, the input layer consists of two nodes to input the absorbance values at 446 and 506 nm. The output layer consists of two neurons to estimate the lycopene and β-carotene concentrations. The neurons in the hidden layer or hidden neurons number (HNN) should be fixed by optimization techniques (vide infra).

As the absorbance values ranged between 0 and 1, the sigmoid function (bounded in the same range) has been selected to be used as the MLP transfer function (*18*, *19*).

The NN training is done by the application of the BP algorithm, which is based on the Bayesian regularization (trainBR) training function. It was selected because the generalization power of trainBR is higher than those of other training functions and, in addition, it avoids overfitting and overtraining when the small learning sample is used (*18*). TrainBR minimizes a linear combination of squared errors and weights by the computation of the Hessian matrix of this combination (*18*, *20*). In addition to the weights (vide supra), its parameters are the learning coefficient (Lc), learning coefficient decrease (Lcd), and learning coefficient increase (Lci). The Lc parameter is similar to "*h*" in Newton's method (often called the Newton−Raphson method). Lcd and Lci control the value of Lc depending on the MLP model performance. To avoid an overfitting of the NN model, the learning process was repeated while the verification mean square error (MSE), defined by eq 3, was decreased. A

Table 1. Calibration Parameters and Sensitivity of the UV−Vis and HPLC Methods Applied to Lycopene and β-Carotene[a]

	equation	range (μg mL^{-1})	R^2	σ intercept	σ slope	LOD[b] (μg)	LOQ[c] (μg)
			UV−Vis				
lycopene	Abs$_{502}$ = 0.200 [C μg mL^{-1}] − 0.001	0.4−3.2	>0.999	0.002	0.001	0.04	0.11
β-carotene	Abs$_{446}$ = 0.252 [C μg mL^{-1}] − 0.001	0.4−3.2	>0.999	0.003	0.002	0.04	0.13
			HPLC				
lycopene	area = 103521 [C μg mL^{-1}] + 3003.2	2−9	>0.999	1008.39	157.21	0.087	0.29
β-carotene	area = 105641 [C μg mL^{-1}] − 325.8	0.25−5	>0.999	5368.00	2129.96	0.099	0.03

[a] R^2, correlation coefficient; σ, standard deviation; LOD, limit of detection; LOQ, limit of quantification. [b] Equation 1. [c] Equation 2.

detailed description of the calculation process is described in the literature ([5]).

$$\text{MSE} = \frac{1}{N}\sum_{k=1}^{N}(r_k - y_k)^2 \qquad (3)$$

In eq 3, N, y_k, and r_k are, respectively, the number of data sets of the database, the response of each output neuron, and its respective real output response. The HNN and NN parameters are optimized by an experimental design based on the Box−Wilson Central Composite Design 2^4 + Star Points, commonly called a central composite design. This design contains an imbedded factorial design with center points that is enlarged with a group of star points that allows the estimation of curvature ([21, 22]). The experimental factors analyzed were Lc (between 1 and 0.001), Lcd (between 1 and 0.001), and Lci (between 2 and 100) ([23]). Taking the learning sample size into account, the HNN range was selected (between 1 and 10) ([24]). The responses of the experimental design were the mean prediction error (MPE, eq 4) and the correlation coefficient (predicted vs real values, R^2). Both indices are easily computed and provide a good description of the predictive performance of the NN model ([25]).

$$\text{MPE} = \frac{1}{N}\sum_{k=1}^{N}\frac{|r_k - y_k|}{r_k} \times 100 \qquad (4)$$

Due to the fact that the main goal is to have a NN that predicts carotenoid concentrations with the highest accuracy possible, the consideration taken into account to analyze the experimental design was to obtain the least MPE with the highest values of the correlation coefficient (estimation of lycopene and β-carotene concentrations by NN versus their respective experimental concentration values).

Neural Network Model Validation Process. To test the optimized NN model, two external validation processes were carried out. The lycopene and β-carotene concentrations of food samples were estimated by the NN model using as inputs the absorbance values at 446 and 502 nm. Once the estimation process had finished, the values obtained (outputs) were statistically compared with the experimental ones (UV−vis and HPLC).

RESULTS AND DISCUSSION

Neural Network Model Optimization and Verification. The NN optimized (**Table 2**) consists of two nodes to input the absorbance values at 446 and 506 nm. The output layer consists of two neurons to estimate the lycopene and β-carotene concentrations and five hidden neurons number

Using the verification sample, the β-carotene and lycopene were estimated, and these were statistically compared with the experimental concentration values. The mean correlation coefficient was >0.99, and MPE values were less than 1 and 2.1%, respectively. In **Table 3**, the main statistical results corresponding to these NN estimations versus experimental concentrations are shown.

Neural Network Model Validation. *First Validation Process.* Using the validation sample, which is composed of lycopene and β-carotene concentration present in fresh tomato, tomato concentrate, tomato sauce, ketchup and tomato juice (10 samples of tomato products chosen due to their high lycopene content together with the presence of β-carotene), the optimized NN model has been tested. The optimized NN was used to estimate the concentrations of lycopene and β-carotene in the aforementioned food samples by their absorbance values at 446 and 502 nm. Then, these estimations and their respective concentration values measured by the UV−vis technique values (vide supra) were statistically compared (**Table 4**). Correlation coefficients of estimated versus experimental values of lycopene and β-carotene are >0.99, and the MPE values were less than 2.02 and 3.61%, respectively. The slopes of their linear fits are close to 1 (0.984 and 1.103 for lycopene and β-carotene cases, respectively).

Table 2. Parameters and Characteristics of the Optimized Neural Network (NN) Model

NN model characteristics	
transfer function	sigmoid function
training function	trainBR
optimized parameters of the NN model	
input neurons number	2
hidden neurons number (HNN)	5
output neurons number	2
learning coefficient	0.40
learning coefficient decrease	0.60
learning coefficient increase	62

Table 3. Main Statistical Results of the Neural Network Model Corresponding to Verification Sample

	β-carotene	lycopene
mean prediction error (MPE, %)	1.0	2.1
correlation coefficient, R^2	>0.99	>0.99
standard deviation (μg mL^{-1})	0.011	0.025

Table 4. Validation of Optimized Neural Network Model To Estimate Carotenoid Concentration in Food Samples Measured by UV−Vis

| food	samples	lycopene (μg mL^{-1}) UV−vis	estimated	β-carotene (μg mL^{-1}) UV−vis	estimated
tomato concentrate	1	1.955[a]	1.969	1.004[a]	1.032
	2	2.798[a]	2.781	1.536[a]	1.643
tomato sauce	1	1.711[a]	1.689	0.881[a]	0.941
	2	1.591[a]	1.639	0.909[a]	0.889
ketchup	1	1.706[a]	1.739	0.964[a]	0.970
	2	1.461[a]	1.501	0.778[a]	0.731
tomato juice	1	1.401[a]	1.457	0.699[a]	0.679
	2	1.192[a]	1.155	0.675[a]	0.700
tomato puree	1	1.272[a]	1.305	0.663[a]	0.665
	2	2.818[a]	2.826	1.536[a]	1.594
statistical results					
mean prediction error (MPE, %)		2.028		3.607	
correlation coefficient, R^2		>0.99		>0.99	

[a] Reference *12*.

Second Validation Process. To complete the validation of the prediction capability of the optimized NN, a new validation sample was performed based on the β-carotene and lycopene concentration values in different vegetable food samples (fresh tomato, watermelon, green pepper, carrots, and medlar), which were determined also by HPLC. The mathematical procedure followed was similar to the validation process described above. Both R^2 of estimated versus experimental values are >0.99, and MPE values in the cases of lycopene and β-carotene are less than 0.29 and 2.97%, respectively (**Table 5** and **Figure 1**). The slopes of their linear fits are close to 1 (0.999 and 1.002 for β-carotene and lycopene cases, respectively). The mean MPE in the verification process (1.55%) is less than that in the first (2.81%) and second (1.63%) validation processes. This result is in accordance with the complexity of the samples studied. In light of these results, for both compounds analyzed, the optimized NN model presents reliable results (carotenoids concentrations) when compared to the HPLC values.

Therefore, for the concentration range studied (0.4−3.2 μg mL^{-1}), the interference problems between β-carotene and lycopene can be adequately solved by the optimized models based on MLP algorithms.

To conclude, the NN model applied is an adequate tool to determine accurately the β-carotene and lycopene concentration

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Table 5. Application of Optimized Neural Network Model To Estimate Carotenoid Concentration in Food Samples Measured by HPLC

		lycopene (μg mL^{-1})		β-carotene (μg mL^{-1})	
food	samples	HPLC	estimated	HPLC	estimated
fresh tomato	1	1.246	1.228	0.212	0.225
	2	1.216	1.205	0.202	0.208
	3	1.207	1.232	0.191	0.170
carrot	1	0	0	2.748	2.748
	2	0	0	3.071	3.039
	3	0	0	2.972	2.998
watermelon	1	2.192	2.197	0.388	0.388
	2	2.04	2.043	0.317	0.317
	3	2.533	2.544	0.377	0.377
green pepper	1	0	0	0.106	0.113
	2	0	0	0.068	0.064
	3	0	0	0.104	0.110
medlar	1	0	0	0.553	0.561
	2	0	0	0.349	0.352
	3	0	0	0.461	0.452
statistical results					
mean prediction error (MPE, %)		0.290		2.973	
correlation coefficient, R^2		>0.99		>0.99	
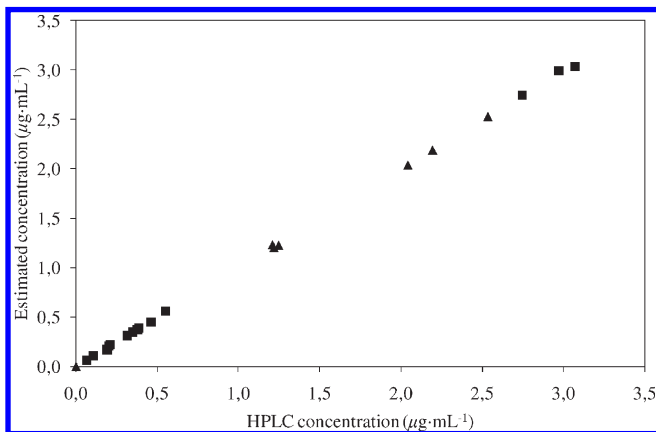
Figure 1. Application of optimized NN model to estimate β-carotene (■) and lycopene (▲) concentration in food samples (HPLC concentrations) ($R^2 > 0.99$).

in the tested food samples. This improvement in results interpretation will be very valuable for its application to a fast and reliable β-carotene and lycopene evaluation without using complex analytical techniques as HPLC. Even more, due to these mean predictive errors and the sample preparation time of this tool, this model could be appropriate to determine the concentration of these chemical compounds on line for quality and process control in the food industry, with minimum pretreatment of samples.

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