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FOOD COMPOSITION AND ADDITIVES

Quantitative Analysis of Food Additives in a Beverage using High Performance Liquid Chromatography and Diode Array Detection Coupled with Chemometrics

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Abstract

Background: In many countries, the levels of synthetic food additives causing harm to humans have been determined and their use has been controlled by legal regulations. Sensitive, accurate and low-cost analysis methods are required for food additive determination.

Objective: In this study, a fast high performance liquid chromatography-diode array detection (HPLC-DAD) analytical methodology for quantification of sodium benzoate, potassium sorbate, ponceau 4R, and carmoisine in a beverage was proposed.

Methods: Partial least squares (PLS) and principal component regression (PCR) multivariate calibration methods applied to chromatograms with overlapped peaks were used to establish a green and smart method with short isocratic elution. A series of synthetic solutions including different concentrations of analytes were used to test the prediction ability of the developed methods.

Conclusions: The average recoveries for all target analytes were in the range of 98.27–101.37% with average relative prediction errors of less than 3%. The proposed chemometrics-assisted HPLC-DAD methods were implemented to a beverage successfully. Analysis results from sodium benzoate, potassium sorbate, ponceau 4R, and carmoisine in a beverage by PLS-2 and PCR were statistically compared with conventional HPLC.

Highlights: The HPLC methods coupled with the PLS-2 and PCR algorithm could provide a simple, quick and accurate strategy for simultaneous determination of sodium benzoate, potassium sorbate, ponceau 4R, and carmoisine in a beverage sample.

Different kinds of food colors and preservatives have to be admixed with processed foods to improve the color, appearance and to prevent spoilage (1–3). Since several of these additives are prepared by chemical synthesis, they pose a potential risk to human health (4), especially when they are excessively consumed (2, 5). Some studies have shown that the cumulative consumption of some food additives including colorants and preservatives can provoke allergies, obesity and metabolic disorders (6). For these reasons, laws and regulations strictly control the use of additives in foods (7). Thus, the determination of these food additives is required to ensure food safety (2).

High performance liquid chromatography with UV detection is a well-developed technology which has been frequently used for simultaneous quantitation of preservatives and colorants in food products (2, 4, 8, 9). However, almost all reported HPLC methods require an extensive clean-up process, special columns, gradient elution and long analysis time, which leads to consumption of expensive solvents such as organic modifiers (10). In addition, there are some difficulties with chromatographic data such as noise, background effect, shifts in the elution time and also overlapping peaks, which have significant effects on obtained results (11). An alternative to performing

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quantitative analysis by HPLC methods in short analysis times is to use chemometric algorithms based on the acquisition of multidimensional chromatographic data without separating the analyte peaks (12). In liquid chromatography, the use of chemometrics methods has been proposed with diode-array detection (DAD) where spectra are recorded at several retention times for each sample. The most popular multiway analysis methods are parallel factor analysis (PARAFAC), generalized rank annihilation method (GRAM), and multivariate curve resolution alternating least square (MCR-ALS) (13–15). There are also other methodologies which use latent variables for processing second order data such as partial least squares (PLS) and principal component regression (PCR) (16–21).

In this work, data collected from high performance liquid chromatography with diode array detection (HPLC-DAD) were processed by the PLS-2 and PCR algorithms. To the best of our knowledge, this is the first study to describe the simultaneous quantification of sodium benzoate, potassium sorbate, ponceau 4R, and carmoisine in a beverage using HPLC-DAD through multivariate measurements.

Materials and Methods

Reagents and Standards

Sodium benzoate (≥99.0% purity), potassium sorbate (≥99.0% purity), ponceau 4R (≥99.0% purity), and carmoisine (≥98.0% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate trihydrate, glacial acetic acid, acetonitrile (HPLC-grade) and methanol (HPLC-grade) were acquired from Merck (Darmstadt, Germany). Ultrapure water (18.25 MΩ cm) was prepared by the Milli-Q water purification system (Millipore, Milford, MA, USA). Stock solutions of sodium benzoate (BEN), potassium sorbate (SOR), ponceau 4R (PON) and carmoisine (CAR) (500, 500, 100 and 100 μg/mL, respectively) were appropriately prepared in water.

Chromatography Conditions and Methods

Chromatographic analyses were performed using a Shimadzu HPLC system (Kyoto, Japan) consisting of a model LC-10 AT VP pump unit, SIL-20AC autosampler, SPD-M10A VP diode array detector and an Inertsil® C18 RP column (5 μm, 250 × 4.6 mm; GL Sciences, Tokyo, Japan). The operating conditions were as follows: flow rate, 1.0 mL/min; injection volume, 20 μL; monitoring wavelength range, 200–600 nm with an interval of 1.2 nm. The simple mobile phase consisted of water and methanol (10:90, v/v) under isocratic conditions. All solutions were filtered through 0.45 μm nylon membrane filters prior to HPLC analysis.

Solutions and Sample Preparation

Five concentration levels of four analytes were constructed by multilevel multifactor design (22) for calibration with PLS-2 and PCR (Table 1). The levels correspond to values in the range of 20–40 μg/mL for BEN, 30–50 μg/mL for SOR, and 3–15 μg/mL for PON and CAR.

A commercial beverage sample was acquired in a local market in Istanbul, Turkey. A 4 mL beverage sample was transferred into a 25 mL measuring flask, and its volume was made up to the mark with water. After filtering through a 0.45 μm membrane filter, the beverage sample was injected onto the HPLC column for the analysis of the studied components.

Table 1. Concentration levels for the mixtures in the calibration set

Sample	BEN (μg/mL)	SOR (μg/mL)	PON (μg/mL)	CAR (μg/mL)
1	30	40	9	9
2	30	30	3	15
3	20	30	15	6
4	20	50	6	15
5	40	35	15	9
6	25	50	9	6
7	40	40	6	6
8	30	35	6	12
9	25	35	12	15
10	25	45	15	12
11	35	50	12	9
12	40	45	9	15
13	35	40	15	15
14	30	50	15	3
15	40	50	3	12
16	40	30	12	3
17	20	45	3	9
18	35	30	9	12
19	20	40	12	12
20	30	45	12	6
21	35	45	6	3
22	35	35	3	6
23	25	30	6	9
24	20	35	9	3
25	25	40	3	3

Multivariate Calibration Methods

Two-way PLS-2 and PCR multivariate calibration methods were applied to HPLC-DAD data for the simultaneous quantification of the target compounds. A 25 × 333 table of summed absorbances over 187 chromatographic points in time at each wavelength for each sample were produced for calibration data. A new calibration matrix was subjected to traditional and well-known PCR and PLS-2 analysis. These techniques have been fully described in the literature (23–25). All calculations were performed in Matlab 7 (The MathWorks, USA) using the PLS-2 Toolbox v5.2 (Eigenvector Technology).

Comparison with Conventional HPLC Analysis Utilizing Single-Wavelength Detection

In conventional HPLC analysis, a successful chromatographic separation of the desired peaks and optimization of the HPLC parameters are a very laborious, time-consuming and costly process because these procedures often require multiple injections. In the analysis of real samples containing a large number of analytes, a weak chromatographic separation or the existence of partially overlapped peaks prevents an accurate and reproducible result in HPLC analyses. To prove the accuracy of the chemometrics assisted HPLC-DAD methods, the conventional optimized HPLC method was performed on the same instrument as a comparison (8). In this context, for the purpose of completely separating all analytes, the gradient conditions were as follows: 0.0–5.0 min acetate buffer solution (25 mM, pH 6.0): acetonitrile (95:5, v/v); 5.01–10.0 min acetate buffer solution: acetonitrile (70:30, v/v); flow rate, 1.0 mL/min; injection volume, 20 μL; detection wavelength 230 nm. Total run time was nearly 9 min in this conventional HPLC method. The calibration curves for each additive compound were obtained by triplicate injections of five aqueous solutions of different

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concentrations. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

Results and Discussion

HPLC-DAD Analysis Coupled with Chemometrics

The advantages of the proposed chemometrics assisted HPLC-DAD methodologies in comparison with traditional HPLC are evident in the simultaneous determination of the analytes in real samples: there is no requirement for full resolution using a complex solvent gradient elution, ease of preparation of mobile phase (water is generally used without adjusting pH), no need to use a special column for substances that are very similar, a reduction in the elution time and consequently in the solvent consumption, which makes the analysis low-cost, eco-friendly and rapid. In this study, based on the retention times and the characteristics of the peaks, it was decided to use a mobile phase consisting of only water and methanol. Chemometric techniques are capable of solving the overlapping of the analyte retention times. Since optimum mobile phase proportion was able to be determined, different compositions of water-methanol and water-acetonitrile were tested. The mobile phase used for all chromatographic runs was water: methanol (10:90, v/v), delivered at a flow rate of 1.0 mL/min at room temperature with a chromatographic system operating under isocratic mode. A three-dimensional plot and liquid chromatography profile of mixed standard solution acquired under the optimum HPLC conditions are displayed in Figure 1a–c.

The data matrices were collected using wavelengths ranging from 200 to 600 nm each 1.2 nm, and times from 1.0 to 3.0 min every 0.64 s. In this way, a three-dimensional matrix with dimensions of 187 × 333 × 25 was created, respectively, where the number 25 corresponds to 25 calibration samples in the prediction set (Table 1). This matrix was then converted to a two-dimensional matrix consisting of absorption by wavelength. The obtained data matrix was analyzed by the PCR and PLS-2 methods. To test the suitability of the calibration data, the calculated root mean square of difference (RMSD), the relative error of prediction (REP%), R^2 and the optimum number of factors are shown in Table 2.

Table 2. Statistical parameters of PLS-2 and PCR calibrations

	BEN		SOR		PON		CAR	
Parameters[a]	PLS-2	PCR	PLS-2	PCR	PLS-2	PCR	PLS-2	PCR
Factor	6	6	6	6	6	6	6	6
PRESS	24.01	43.56	48.30	49.70	5.52	7.56	8.70	9.61
RMSD	0.98	1.32	1.39	1.41	0.47	0.55	0.59	0.62
REP%	3.27	4.40	3.47	3.52	5.22	6.11	6.55	6.89
R^2	0.9808	0.9651	0.9613	0.9602	0.9877	0.9832	0.9807	0.9786
LOD (μg/mL)	0.18	0.19	0.21	0.23	0.15	0.16	0.17	0.18
LOQ (μg/mL)	0.60	0.63	0.70	0.77	0.50	0.53	0.57	0.60

[a] $\text{PRESS} = \sum_1^m (c_{act} - c_{pred})^2$ $\text{RMSD} = \left[\frac{1}{m}\sum_1^m (c_{act} - c_{pred})^2\right]^{1/2}$ $\%\text{REP} = \frac{100}{\bar{c}}\left[\frac{1}{m}\sum_1^m (c_{act} - c_{pred})^2\right]^{1/2}$ and $R^2 = 1 - \left(\sum_1^m (c_{act} - c_{pred})^2\right)/\left(\sum_1^m (c_{act} - \bar{c})^2\right)$ $\bar{c}$ is the average component concentration in the m mixtures. PRESS: prediction error sum of squares.

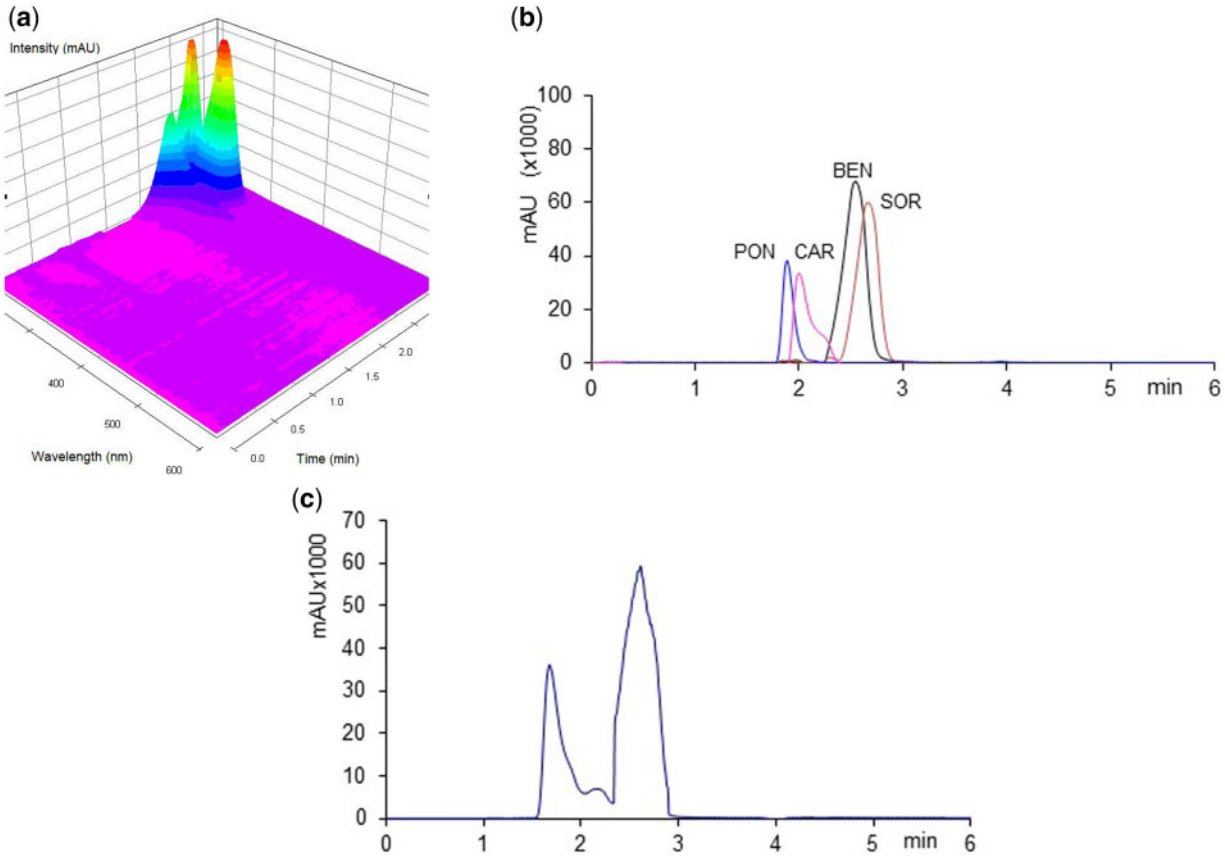


Figure 1. (a) The raw 3-dimensional plot of absorbance, wavelength, and time corresponding to a quaternary mixture of analytes in the region of 200–600 nm by DAD, (b) Time profiles of the target compounds were recorded at 210 nm for BEN, SOR, PON, and CAR, (c) Chromatogram of the standard mixture solution (30 μg/mL of BEN, 40 μg/mL of SOR, 9 μg/mL of PON and 9 μg/mL of CAR) at 210 nm. mAU is milli absorbance unit on the UV detector, which is used generally to show peak intensity.

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Table 3. Recovery results for BEN, SOR, PON and CAR in the synthetic mixtures

	PLS-2												PCR											
	BEN (μg/mL)			SOR (μg/mL)			PON (μg/mL)			CAR (μg/mL)			BEN (μg/mL)			SOR (μg/mL)			PON (μg/mL)			CAR (μg/mL)		
Added	25	30	35	35	40	45	6	9	12	6	9	12	25	30	35	35	40	45	6	9	12	6	9	12
Found	25.29	30.18	35.08	34.13	39.28	44.69	6.02	9.01	11.66	6.48	8.88	12.1	25.98	29.3	35.24	33.98	38.92	44.84	5.94	9.16	11.85	6.52	8.77	11.97
	25.50	30.47	35.12	34.36	38.88	44.75	5.98	9.82	12.27	6.37	8.95	12.04	26.4	29.38	35.48	34.96	38.56	44.32	6.18	9.63	11.78	6.32	8.48	11.81
	25.68	30.76	35.17	34.66	38.93	44.19	6.14	9.24	11.97	6.24	9.01	11.98	26.76	29.42	35.67	34.46	39.03	44.72	5.96	9.52	11.65	6.43	8.62	11.76
RMSD	0.43			0.75			0.32			0.22			0.94			0.82			0.32			0.35		
REP%	1.44			1.87			3.59			2.49			3.13			2.05			3.56			3.92		
Rec%	101.20			98.30			101.37			101.30			101.34			98.27			100.83			99.60		

In the PLS-2 and PCR modelling, the number of factors was estimated by a cross-validation procedure. First, six factors were selected to build these calibration models for four compounds, corresponding to the minimum RMSD values which were between 0.47 and 1.41 for BEN, SOR, PON and CAR.

Application to Synthetic Mixtures

In order to test the accuracy of the proposed methods, synthetic mixtures containing different concentrations of the related analytes were analyzed by these methods. The composition of mixtures and the predictions of these synthetic samples are shown in Table 3. With respect to the results provided by PLS-2 and PCR on the synthetic sample set, as can be seen in Table 3, good recovery results in percentage recovery (Rec%) (in the range of 98–102%) were obtained for the analysis of BEN, SOR, PON and CAR (Relative error of prediction (REP%) <4%).

Application to Beverage Samples

The developed HPLC-PLS-2 and HPLC-PCR methods were implemented in the simultaneous chromatographic determination of BEN, SOR, PON and CAR in beverage sample (pomegranate juice). The quantitative analysis results of BEN, SOR, PON and CAR in the beverage sample achieved by HPLC-PLS-2, HPLC-PCR and the conventional HPLC (8) calibrations are summarized in Table 4. The standard deviations (SD) and relative standard deviations (RSD) are also listed in this table, which are the average of five analytical results. It can be seen that the results obtained by different calibration methods are compatible with each other. Furthermore, the results obtained by the proposed methods were statistically compared with the conventional HPLC method by applying Student's t-test for accuracy and F-test for precision. There was no significant difference between the improved and conventional methods at the 95% confidence level with respect to accuracy and precision (the calculated t- and F- values were less than the tabulated value in all cases; Table 4). Moreover, *P*-values of statistical tests were found to be higher than $P = 0.05$, namely, these methods were not statistically significantly different. Consequently, the above results show that the newly developed methods can be successfully applied to the simultaneous determination of BEN, SOR, PON and CAR in beverage samples.

Conclusions

Sodium benzoate, potassium sorbate, ponceau 4R, and carmoisine, four commonly used additives in food, have been determined in a beverage by the chemometrics-assisted HPLC-DAD method. PLS-2 and PCR calibration models were applied to the experimental data. The results showed that both PLS-2 and PCR methods combined with HPLC-DAD are powerful tools in order to analyze a complex mixture of analytes without the necessity of a complete chromatographic separation in a short analysis time (within 3 min). Additionally, these methods did not require troublesome sample preparation, extra consumption of organic solvents or optimization of chromatographic conditions. In order to evaluate the performance of proposed methodologies, their results were compared to those of a conventional HPLC method, and a good outcome supported through statistical analysis revealed. In conclusion, the established HPLC-DAD method combined with chemometric analysis, as proposed in this study, proved to be an effective, reliable, and alternative

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Table 4. Results of the determination of BEN, SOR, PON and CAR in beverage samples using improved and conventional HPLC methods

	HPLC-PLS				HPLC				HPLC-PCR			
$n1=n2=5$	BEN	SOR	PON	CAR	BEN	SOR	PON	CAR	BEN	SOR	PON	CAR
Mean, μg/mL	144.12	251.60	84.06	26.50	142.65	252.16	83.24	26.05	145.15	250.20	84.53	26.66
SD	1.60	2.57	1.30	0.48	2.83	1.11	0.66	0.42	1.82	2.53	1.43	0.51
RSD (%)	1.11	1.02	1.54	1.88	1.98	0.44	1.79	1.61	1.25	1.01	1.69	1.91
t-test	1.01	0.80	1.26	1.09	$t_8^{0.05}{}_{\text{critical}} = 2.31$ $F_{4,4}^{0.05}{}_{\text{critical}} = 6.39$				1.28	1.59	0.83	0.11
P-value	0.34	0.45	0.24	0.31					0.24	0.15	0.10	0.30
F-test	3.13	5.36	3.88	1.31					2.42	5.19	4.69	1.47
P-value	0.15	0.07	0.11	0.40					0.21	0.07	0.08	0.36

$n1=n2=5$ is the number of samples of the compared methods. Mean is average results of 5 samples ($n=5$).

method for simultaneous quantification of sodium benzoate, potassium sorbate, ponceau 4R, and carmoisine in beverages.

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Conflicts of interest: We have no conflict of interest.

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