

Use of Experimental Design for Calibration and Validation of Ascorbic Acid and Citric Acid Mixtures

Fuensanta Sánchez Rojas,^{1*} Catalina Bosch Ojeda,¹ Antonio Jesús Ruiz Sánchez,² and María Espinosa Bosch³

¹ Department of Analytical Chemistry, Faculty of Sciences, University of Málaga, Málaga, Spain. fsanchezr@uma.es

² Department of Organic Chemistry, Faculty of Sciences, University of Málaga, 29071, Málaga, Spain

³ Department of Pharmacy, General Hospital, University Hospital "Virgen del Rocío", Manuel Siurot s/n, 41013, Sevilla, Spain.

Received June 24, 2008; accepted October 28, 2008

Abstract. The general objective of present work has been the development and application of analytical methodologies based on the combination of UV spectra with chemometrics methods of experimental design for establishing the matrix of calibration and later analysis of the obtained results. The analytes selected for this study have been citric acid and ascorbic acid. The citric acid is a tri-carboxylic organic acid that is present in most of the fruits, mainly in citrus like the lemon and the orange. Ascorbic acid (vitamin C) is an organic acid with antioxidant properties; their importance for the human immune system and for the prevention of various diseases is a matter of common knowledge.

Keywords: Ascorbic acid, Citric acid, Experimental design, ANOVA, Multiple linear regression.

Resumen. El objetivo general de este trabajo ha sido el desarrollo y aplicación de metodologías analíticas basadas en la combinación de medidas espectroscópicas de UV con métodos quimiométricos de diseño de experimentos para establecer la matriz de calibración y posterior análisis de los resultados obtenidos. Los analitos seleccionados para este estudio han sido el ácido cítrico y el ácido tartárico. El ácido cítrico es un ácido orgánico tricarbóxico presente en la mayoría de las frutas, principalmente en cítricos como el limón y la naranja. El ácido ascórbico (vitamina C) es un ácido orgánico con propiedades antioxidantes, importante para el sistema inmunológico y para prevenir enfermedades.

Palabras clave: Ácido ascórbico, ácido cítrico, diseño de experimentos, ANOVA, regresión lineal múltiple.

Introduction

Nowadays the commercial citric acid production is made mainly by fermentation processes that use dextrose or molasses of sugar cane as raw material and *Aspergillus niger* as fermentation organism. The fermentation produces liquid citric acid that later is purified, concentrated and crystallized. It is a natural preservative and is also used to add an sour taste to foods and soft drinks. It also serves as an environmentally benign cleaning agent and acts as an antioxidant.

Ascorbic acid is an organic acid with antioxidant properties. Ascorbic acid is easily oxidized and so is used as a reductant in photographic developer solutions (among others) and as a preservative. Ascorbic acid and its sodium, potassium, and calcium salts are commonly used as antioxidant food additives. The concentration of a solution of ascorbic acid can be determined in many ways the most common ways involve titration with an oxidizing agent. The determination of ascorbic acid by spectrophotometry, chemiluminescence and fluorescence techniques, sorption-spectroscopy, and visual-test methods is revised by Zaporozhets and Krushinskaya [1].

Although many papers were published on the determination of citric acid and ascorbic acid individually, only a few works were presented on the simultaneous determination of both analytes. There are diverse methods for the simultaneous determination of ascorbic acid and citric acid including titrimetry method [2], kinetic spectrophotometric method employing the Kalman filter [3] and H-point standard method [4], dual wavelength spectrophotometric method using an artificial

neural network [5], electro-analytical [6,7], high-performance liquid chromatography [8-16] and capillary electrophoresis [17,18].

Chromatographic methods require expensive instrumentation and are relatively highly time-consuming. Although, spectrophotometric methods are simpler and faster, the simultaneous determination of both analytes is not possible by conventional direct UV absorption measurements, because of the spectral overlap.

The main problem of spectrophotometric multicomponent analysis is the simultaneous determination of two or more compounds in the same mixtures without preliminary separation. Several spectrophotometric determination methods have been used for resolving mixtures of compounds with overlapping spectra. When these methods are compared with each other, the range of application of derivative spectrophotometry is more reliable with respect to utility and sensitivity than normal spectrophotometry [19].

Computer-controlled instrumentation and multivariate calibration methods are playing a very important role in the multi-component analysis of mixtures by UV/VIS spectrophotometry. Both approaches are useful for the resolution of band overlapping in the quantitative analysis. In general, a multivariate calibration model is constructed from instrumental response data collected for a set of multicomponent samples of known concentrations with respect to the analytes of interest. Each method needs this calibration step, followed by a prediction step in which the results of the calibration are used to determine the component concentrations from the sample spectrum. The sequence diagram is shown in figure 1.

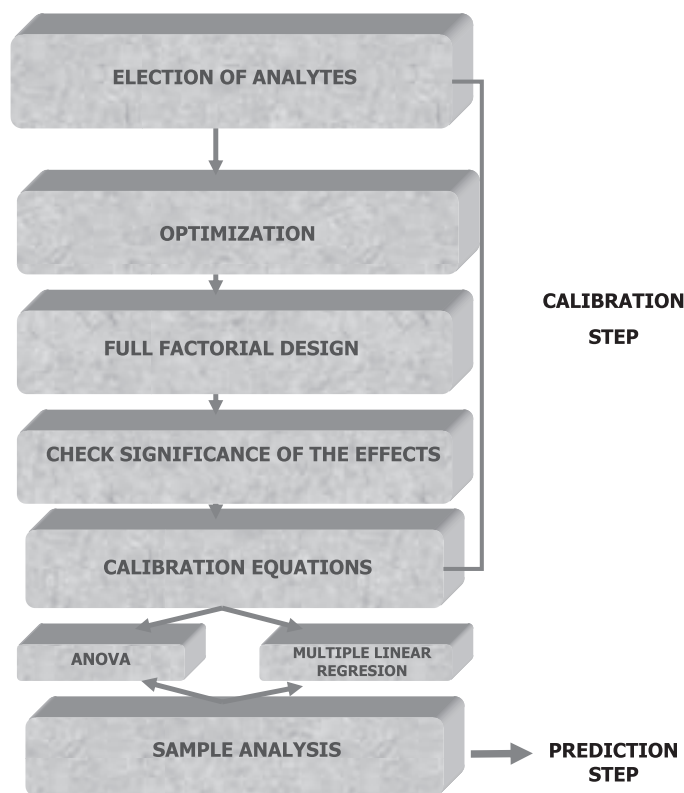


Fig. 1. Sequence diagram for calibration and prediction steps.

The objective of this work is the establishment of a calibration matrix and its validation to determine ascorbic acid and citric acid mixtures by direct and derivative UV-visible spectroscopic methods making use of the experimental design.

Experimental

Reagents and Instrumentation

Analytical reagent grade chemicals were used throughout. Standard solution 1000 $\mu\text{g/mL}$ ascorbic acid and citric acid were prepared by (Fluka). Standards of working strength were made by appropriate dilution as required, immediately prior to use. Water was deionised with a Milli-Q system.

A Varian Cary 50 Spectrophotometer was used for absorbance measurements. The spectra were obtained using a cell of 1 cm optical path length.

Statgraphics plus 5.1 programs [20] has been used for experimental design and statistical studies.

Calibration Matrix and Data Analysis

The calibration matrix was statistically designed over the concentrations ranges of 0.5–6 $\mu\text{g/mL}$ of each analyte. A fractional multi-level factorial design (2^4) has been used for the calibration matrix design. The design has been carry out in two block

and the order of experiments have been randomize for protect they from occult variables. 25 synthetic mixtures of ascorbic acid and citric acid were employed (according to Table 1). The concentration ranges were selected within the previously established linear calibration range for each compound.

Analysis of variance and multiple linear regression techniques has been applied to the data analysis obtained experimentally from absorption spectra, first, second, third and fourth derivative spectra.

Results and Discussion

Calibration Graphs

The absorption spectra of ascorbic acid and citric acid in the same mixture are strongly overlapping in the wavelength range of 190–300 nm. Therefore, a new approach by using conventional spectrometry and derivative spectrophotometry (DS) was developed for the simultaneous determination of the above-mentioned compounds. The concentration ranges were tested for the preparations of calibrations for both compounds and the working range between 0.5 and 6 $\mu\text{g/mL}$ was selected. Good linearity was found in this concentration range for ascorbic acid and citric acid in all cases.

Table 1. Calibration matrix.

Row	Ascorbic acid ($\mu\text{g/mL}$)	Citric acid ($\mu\text{g/mL}$)
1	1	2.7
2	2.7	6
3	4.3	6
4	6	6
5	6	2.7
6	1	4.3
7	2.7	2.7
8	4.3	4.3
9	6	4.3
10	1	1
11	1	6
12	2.7	1
13	6	2.7
14	6	6
15	4.3	4.3
16	6	4.3
17	1	6
18	4.3	1
19	1	1
20	6	1
21	2.7	4.3
22	2.7	6
23	2.7	1
24	4.3	6
25	2.7	2.7

Fractional Factorial Design

The main class of statistical design techniques is the so-called factorial methods. Such designs are straightforward to implement and their results can be very easily interpreted; these methods are created to measure the additive effects on a response for each of the input factors. In addition, the effects of interactions between factors can also be investigated. Two level factorial designs have many advantages in analytical procedures. In this method, there are comparisons available for each main effect on the experimental results. The total number of runs in factorial design is much less as compared to the “one variable at a time” approach. Estimation of the interaction of effects is an additional advantage over the “one variable at a time” approach. In addition, fractional factorial designs can be used to reduce the size of the design matrix. In this work, a fractional multi-level factorial design, involving 25 runs were used to calibration. The factors and their levels are shown in Table 1.

The significance of the effects was checked by analysis of the variance (ANOVA) and using *P*-value significance levels. This value represents the probability of the effect of a factor being due solely to random error. Thus, if the *P*-value is less than 5%, the effect of the corresponding factor is significant. Table 2 shows the results obtained of these studies for the all selected wavelengths. As can be seen from this table the best results are achieved when data are obtained at 265 nm in absorbance measure and third and fourth derivatives. Also, the ANOVA results produced the graphs showing the influence of main effects represented in figure 2, interaction plot in figure 3 and standardized Pareto chart in figure 4.

On the other hand, the results obtained fit the equations display in Table 3 for each selected signal. With respect to the linear regression model, the results obtained were similar to the ANOVA after to eliminate the no significant effects. For this reason R-square values are lightly lower than the corresponding ANOVA values. In Table 4 are showed the linear equations for this study.

Table 2. Analysis of variance.

Selected λ	<i>P</i> -value					<i>R</i> -square (%)
	Source					
	A: Ascorbic acid	B: Citric acid	AA	AB	BB	
Absorbance at 265 nm	0.0000	0.0000	0.0540	0.0103	0.2989	94.7829
Absorbance at 195 nm	0.0000	0.0001	0.1938	0.1134	0.2249	89.6915
1 st derivative at 194-196 nm	0.0053	0.0001	0.2830	0.0738	0.4472	74.3713
2 nd derivative at 195 nm	0.0051	0.0001	0.2782	0.0720	0.4547	74.4713
2 nd derivative at 265 nm	0.0000	0.0000	0.5598	0.0039	0.0539	93.425
3 rd derivative at 194-196 nm	0.0050	0.0001	0.2756	0.0707	0.4224	74.6806
3 rd derivative at 264-266 nm	0.0000	0.0000	0.7099	0.0019	0.0294	95.1864
4 th derivative at 195 nm	0.0061	0.0001	0.2780	0.0749	0.4492	74.1349
4 th derivative at 265 nm	0.0000	0.0000	0.7108	0.0020	0.0277	95.2524

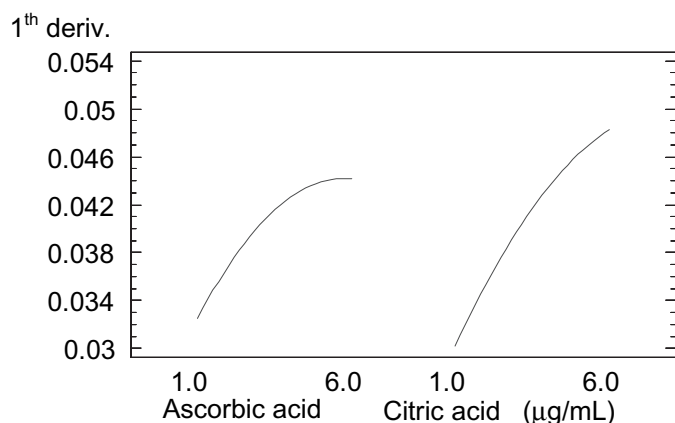


Fig. 2. Graphic of main effects for first derivative ($\lambda = 194-196$ nm).

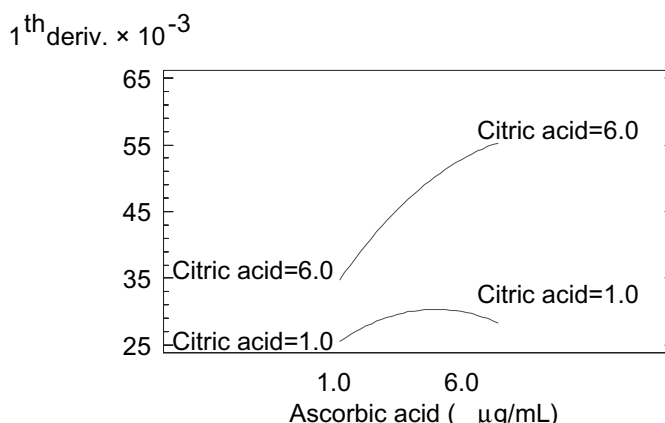


Fig. 3. Graphic of interaction factors for first derivative ($\lambda = 194-196$ nm).

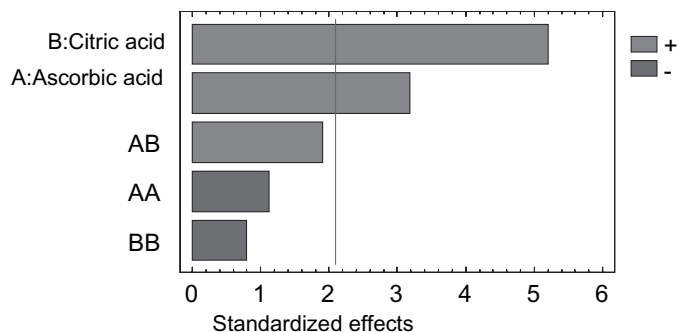


Fig. 4. Pareto diagram for first derivative ($\lambda = 194 - 196$ nm).

Validation of procedure

In the prediction step, the constructed calibration models were applied to the fifteen mixtures of analytes in the working concentration range and their results were given in Table 5. From this table can be seen some final results are not too good, but other are acceptable due some models show small differences in certain condition.

Table 3. Regression equations for different adjusted models.

Selected λ	Equations for dependent variable (Y)
Absorbance at 195 nm	$Y = 0.636575 + 0.0549132[\text{ascorbic acid}] + 0.0334065 [\text{citric acid}] - 0.00370452[\text{ascorbic acid}]^2 + 0.00361386[\text{ascorbic acid}][\text{citric acid}] - 0.00354931[\text{citric acid}]^2$
Absorbance at 265 nm	$Y = 0.0569959 + 0.108445[\text{ascorbic acid}] - 0.0205055[\text{citric acid}] - 0.00464405[\text{ascorbic acid}]^2 - 0.00511164[\text{ascorbic acid}][\text{citric acid}] + 0.00248018[\text{citric acid}]^2$
1 st derivative at 194-196 nm	$Y = 0.0184553 + 0.00350229[\text{ascorbic acid}] + 0.00378071[\text{citric acid}] - 0.000522316[\text{ascorbic acid}]^2 + 0.000708966[\text{ascorbic acid}][\text{citric acid}] - 0.00037743[\text{citric acid}]^2$
2 nd derivative at 195 nm	$Y = 0.00693539 + 0.00131266[\text{ascorbic acid}] + 0.00138783[\text{citric acid}] - 0.000196184[\text{ascorbic acid}]^2 + 0.000265416[\text{ascorbic acid}][\text{citric acid}] - 0.000137969[\text{citric acid}]^2$
2 nd derivative at 265 nm	$Y = 0.000810802 + 0.000631801[\text{ascorbic acid}] - 0.000334917[\text{citric acid}] - 0.0000108648[\text{ascorbic acid}]^2 - 0.0000479738[\text{ascorbic acid}][\text{citric acid}] + 0.0000388067[\text{citric acid}]^2$
3 rd derivative at 194-196 nm	$Y = 0.0148812 + 0.0029787[\text{ascorbic acid}] + 0.00328446[\text{citric acid}] - 0.000445[\text{ascorbic acid}]^2 + 0.000601929[\text{ascorbic acid}][\text{citric acid}] - 0.000334463[\text{citric acid}]^2$
3 rd derivative at 264-266 nm	$Y = 0.00177614 + 0.0013352[\text{ascorbic acid}] - 0.000726127[\text{citric acid}] - 0.0000131028[\text{ascorbic acid}]^2 - 0.0000998509[\text{ascorbic acid}][\text{citric acid}] + 0.0000844393[\text{citric acid}]^2$
4 th derivative at 195 nm	$Y = 0.0150364 + 0.00290103[\text{ascorbic acid}] + 0.00311561[\text{citric acid}] - 0.000436837[\text{ascorbic acid}]^2 + 0.000584219[\text{ascorbic acid}][\text{citric acid}] - 0.000310843[\text{citric acid}]^2$
4 th derivative at 265 nm	$Y = 0.00118536 + 0.000883086[\text{ascorbic acid}] - 0.000484882[\text{citric acid}] - 0.00000860042[\text{ascorbic acid}]^2 - 0.0000652533[\text{ascorbic acid}][\text{citric acid}] + 0.0000562933[\text{citric acid}]^2$

Table 4. Linear regression models.

Selected λ	Equations for dependent variable (Y)
Absorbance at 195 nm	$Y = 0.656384 + 0.0424964[\text{ascorbic acid}] + 0.0202544 [\text{citric acid}]$
Absorbance at 265 nm	$Y = 0.138076 + 0.0565451[\text{ascorbic acid}] - 0.019823[\text{citric acid}]$
1 st derivative at 194-196 nm	$Y = 0.0180457 + 0.002287[\text{ascorbic acid}] + 0.0035647[\text{citric acid}]$
2 nd derivative at 195 nm	$Y = 0.00675981 + 0.000853895[\text{ascorbic acid}] + 0.00132969[\text{citric acid}]$
2 nd derivative at 265 nm	$Y = 0.00115386 + 0.00037192[\text{ascorbic acid}] - 0.000217806[\text{citric acid}]$
3 rd derivative at 194-196 nm	$Y = 0.014663 + 0.00193674[\text{ascorbic acid}] + 0.00300211[\text{citric acid}]$
3 rd derivative at 264-266 nm	$Y = 0.00237791 + 0.00085728[\text{ascorbic acid}] - 0.00045416[\text{citric acid}]$
4 th derivative at 195 nm	$Y = 0.0147576 + 0.00185063[\text{ascorbic acid}] + 0.0029411[\text{citric acid}]$
4 th derivative at 265 nm	$Y = 0.00157002 + 0.000570176[\text{ascorbic acid}] - 0.000299197[\text{citric acid}]$

Table 5. Results for validation of procedure.

Ascorbic acid (µg/mL)	Citric acid (µg/mL)	Selected wavelength (nm)	Absorbance 195			Absorbance 265			1 st derivative 194-196			2 nd derivative 195		
			Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression
1	1	0.740	0.721	0.719	0.109	0.137	0.174	0.026	0.026	0.024	0.0097	0.0096	0.0089	
1	2	0.725	0.747	0.739	0.097	0.119	0.154	0.026	0.026	0.027	0.0100	0.0108	0.0102	
1	3	0.727	0.766	0.759	0.091	0.106	0.135	0.027	0.031	0.031	0.0104	0.0117	0.0116	
2	2	0.771	0.798	0.781	0.175	0.203	0.211	0.028	0.032	0.029	0.0100	0.0120	0.0111	
3	3	0.810	0.868	0.844	0.220	0.255	0.248	0.031	0.039	0.035	0.0110	0.0144	0.0133	
2	4	0.784	0.837	0.822	0.157	0.172	0.171	0.028	0.038	0.036	0.0104	0.0142	0.0137	
3	6	0.840	0.905	0.905	0.194	0.214	0.189	0.035	0.046	0.046	0.0130	0.0172	0.0172	
2	1	0.758	0.768	0.761	0.174	0.227	0.231	0.030	0.028	0.026	0.0114	0.0106	0.0098	
3	1	0.796	0.808	0.804	0.236	0.307	0.287	0.030	0.030	0.028	0.0113	0.0112	0.0107	
1	4	0.770	0.779	0.779	0.095	0.098	0.115	0.031	0.033	0.034	0.0117	0.0124	0.0129	
1	5	0.743	0.748	0.800	0.077	0.094	0.095	0.035	0.034	0.038	0.0131	0.0129	0.0142	
1	6	0.718	0.782	0.820	0.079	0.096	0.076	0.031	0.035	0.041	0.0115	0.0130	0.0155	

Ascorbic acid (µg/mL)	Citric acid (µg/mL)	Selected wavelength (nm)	2 nd derivative 265			3 rd derivative 194-196			3 rd derivative 264-266			4 th derivative 195		
			Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression
1	3	0.00015	0.00063	0.00087	0.0228	0.0260	0.0256	0.00034	0.00138	0.00187	0.0227	0.0258	0.0254	
2	2	0.00083	0.00132	0.00146	0.0220	0.0266	0.0245	0.00192	0.00288	0.00318	0.0227	0.0264	0.0243	
3	3	0.00140	0.00152	0.00161	0.0256	0.0230	0.0294	0.00340	0.00335	0.00358	0.0255	0.0316	0.0291	
2	4	0.00110	0.00093	0.00102	0.0226	0.0316	0.0305	0.00260	0.00204	0.00228	0.0225	0.0312	0.0302	
3	6	0.00072	0.00113	0.00096	0.0280	0.0383	0.0384	0.00168	0.00254	0.00222	0.0274	0.0378	0.0379	
2	1	0.00157	0.00163	0.00167	0.0250	0.0232	0.0215	0.00362	0.00355	0.00363	0.0249	0.0231	0.0214	
3	1	0.00180	0.00216	0.00205	0.0246	0.0246	0.0235	0.00416	0.00472	0.00449	0.0245	0.0244	0.0233	
1	4	0.00033	0.00052	0.00065	0.0254	0.0276	0.0286	0.00077	0.00114	0.00141	0.0254	0.0273	0.0283	
1	5	0.00022	0.00048	0.00043	0.0288	0.0285	0.0316	0.00051	0.00107	0.00096	0.0285	0.0282	0.0313	
1	6	0.00012	0.00053	0.00021	0.0252	0.0287	0.0346	0.00027	0.00118	0.00051	0.0250	0.0285	0.0342	

Table 5. (cont.) Results for validation of procedure.

Ascorbic acid ($\mu\text{g/mL}$)	Citric acid ($\mu\text{g/mL}$)	Selected wavelength (nm)		
		4 th derivative 265		
		Exp. value	Adjusted value ANOVA	Adjusted value Regression
1	1	0.00120	0.00156	0.00184
1	2	0.00078	0.00118	0.00154
1	3	0.00023	0.00091	0.00124
2	2	0.00128	0.00191	0.00211
3	3	0.00226	0.00222	0.00238
2	4	0.00173	0.00136	0.00151
3	6	0.00112	0.00169	0.00148
2	1	0.00242	0.00236	0.00241
3	1	0.00277	0.00313	0.00298
1	4	0.00051	0.00075	0.00094
1	5	0.00034	0.00071	0.00064
1	6	0.00018	0.00078	0.00034

Conclusions

Citric acid and ascorbic acid are very interesting compounds from the point of view of their applications. The individual citric acid and ascorbic acid calibrations have been obtained, using signals in absorbance, and first, second, third and fourth derivatives.

This work is focused on the development of an UV analytical protocol for the establishment of calibration matrix and its validation. The proposed method was based on the development of calibration models with samples prepared by mixtures of ascorbic acid and citric acid.

Analysis of variance and multiple linear regression techniques have been applied to the data analysis obtained experimentally from absorption spectra, first, second, third and fourth derivative spectra. The validation of the procedure has been carried out by means of analysis ANOVA and of multiple regression for each one of the collected data, this it is absorbance, first, second, third and fourth derivative.

References

- Zaporozhets, O. A.; Krushinskaya, E. A. *J. Anal. Chem.* **2002**, 57, 286-297.
- Sigmann, S. B.; Wheeler, D. E. *J. Chem. Educ.* **2004**, 81, 1479-1481
- Sultan, S.; Walmsley, A. *Analyst* **1997**, 122, 1601-1604.
- Zarei, K.; Atabati, M.; Karimian, N. *Indian J. Chem. Technol.* **2007**, 14, 417-422.
- Zarei, K.; Atabati, M.; Karimian, N. *J. Anal. Chem.* **2008**, 63, 145-150.
- Volotovskiy, V.; Kim N. *Sensor Actuat. B-Chem.* **1998**, B49, 253-257.
- Akhond, M.; Tashkhourian, J.; Hemmateenejad, B. *J. Anal. Chem.* **2006**, 61, 804-808.
- Yu, X.; Xu, B.; Sawamura, M. *J. Jpn. Soc. Hortic. Sci.* **2004**, 73, 293-298.
- Chinnici, F.; Spinabelli, U.; Riponi, C.; Amati, A. *J. Food Comp. Anal.* **2005**, 18, 121-130.
- Alcázar, A.; Fernández-Cáceres, P. L.; Martín, M. J.; Pablos, F.; González, A. G. *Talanta* **2003**, 61, 95-101.
- Pundlik, M. D. *J. Indian Chem. Soc.* **2004**, 81, 721-723.
- Zhanguo, C.; Jiuru, L. *J. Chromatogr. Sci.* **2002**, 40, 35-39.
- Oderiz, M. L. V.; Blanco, M. E. V.; Hernandez, J. L.; Lozano, J. S.; Rodrigues, M. A. R. *J. AOAC Int.* **1994**, 77, 1056-1059.
- Guo, D. H.; Xia, L. *Se Pu* **2001**, 19, 276-278.
- Chinnic, F.; Spinabelli, U.; Riponi, C.; Amati, A. *J. Food Comp. Anal.* **2005**, 18, 121-130.
- Scherer, R.; Poloni Rybka, A. C.; Teixeira Godoy, H. *Quim. Nova*, **2008**, 31, 1137-1140.
- Wu, C. H.; Lo, Y. S.; Lee, Y. H.; Lin, T. I. *J. Chromatog. A*, **1995**, 716, 291-301.
- Mato, I.; Huidobro, J. F.; Simal-Lozano, J.; Sancho, M. T. *Anal. Chim. Acta*, **2006**, 565, 190-197.
- Bosch Ojeda, C.; Sánchez Rojas, F. *Anal. Chim. Acta*, **2004**, 518, 1-24.
- Statgraphics Plus version 5.1, Reference Manual; Manugistics: Rockville, MD, 1992.