Application of Classical Least Squares, Principal Component Regression and Partial Least Squares Methods for Simultaneous Spectrophotometric Determination of Rutin and Ascorbic Acid in Their Combined Dosage Form

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Abstract: This presented paper deals with application of three multivariate calibration methods methods for simultaneous spectrophotometric determination of two active substances in combined pharmaceutical formulation, composed of rutin (RU) and ascorbic acid (AA). The multivariate methods are classical least squares (CLS), principal component regression (PCR) and partial least squares (PLS). The results showed the high performance of three methods for the analysis of the binary mixture. The optimum assay conditions were established and the proposed methods were successfully applied for the assay of the two drugs in an independent validation set and combined pharmaceutical tablets with excellent recoveries. No interference was observed from common pharmaceutical additives.

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Keywords: Rutin; Ascorbic acid; Spectrophotometry; Multivariate calibration methods; Pharmaceutical tablets.

1. Introduction

Rutin (RU, Fig.1) is chemically known as (quercetin-3-O-(6-O-rhamnosid)glucoside). It is a well-known and widely occurring flavonoid. It is present in many foods, including buckwheat, onion, apple, tea, and red wine. It is highly consumed not only in food, but also due to its pharmacological properties. Studies have shown that RU scavenges free radicals, suppresses cellular immunity, has an antioxidant and anti-inflammatory effects, as well as anti-carcinogenic and antimicrobial potential, and even antihypertensive and as an adjuvant for type 2 diabetes treatment (Deschner *et al*, 1991, Erlund *et al*, 2000, Kamalakkannan *et al*, 2006, Kandaswami *et al*, 1994, Middleton *et al*, 2000, Rotelli *et al*, 2003). RU has been used in the treatment of peripheral vascular diseases, because of its vascular-protective property e.g. acute attack of piles, metrorrhagias, circulatory disturbances and capillary fragility disorders (Erlund *et al*, 2000).

Vitamin C (ascorbic acid) (AA, Fig. 1) is chemically known as (5-methyl-2-oxo-1,3-dioxolen-4-yl)methoxy-4-(1-hydroxy-1-methylethyl)-2-propyl-1-{4-[2-(tetrazol-5-yl)phenyl]phenyl}methylimidazol-5-carboxylate. It is an essential vitamin for humans. Animals can make their own AA, but people must get this vitamin from food and other sources. Good sources of AA are fresh fruits and vegetables, especially citrus fruits. Its role as an endogenous antioxidant is well recognized. Historically, AA was used for preventing and treating scurvy. These days, AA is used most often for preventing and treating the common cold (Barrett *et al*, 2007). Moreover, supplementation of AA has been verified as an effective therapy for the treatment of certain respiratory diseases, including allergic rhinitis (Thornhill *et al*, 2000), and chronic rhinosinusitis (Helms *et al*, 2006). RU has been marketed in combination with AA in tablet dosage form (RUTA C 60® tablets). The combination of RU and AA is intended for oral administration for altering the increased fragility and permeability of capillaries.

Various methods for the individual determination of AA and RU in drugs or other samples have been reviewed. Only few methods for determining the active compounds in mixtures were reported. The simultaneous determination of RU and AA in their combined dosage forms has been achieved by UV-spectrophotometry (Hassan *et al*, 1999) electrochemical method (Deng *et al*, 2013), voltammetry (Yang *et al*, 2010), chemiluminescence (Zeng *et al*, 2013), capillary electrophoresis (Chen *et al*, 2000, He *et al*, 2002, Li *et al*, 2001, Li *et al*, 2002)

and HPLC (Abdallah *et al*, 1993, Legnerová *et al*, 2003, Sun, 2012). NIR FTIR (Du *et al*, 2000).

These methods employed intensive instrumentation (e.g. HPLC and capillary electrophoresis) or some methods require complicated instrument and skilled operator, which make them less convenient in practice. The scientific novelty of the present work is that the methods used are simple, rapid, selective, less expensive and less time consuming compared with other published HPLC methods. Furthermore, these methods have high precision and accuracy as compared with the reported spectrophotometric methods because calibration procedures depend on whole spectra. So, the aim of this work was to develop simple, sensitive and validated chemometric assisted spectrophotometric methods for the simultaneous determination of RU and AA in powdered forms, laboratory prepared mixtures and in pharmaceutical formulation. The applied chemometric methods are classical least squares (CLS), principal component regression (PCR) and partial least squares (PLS).

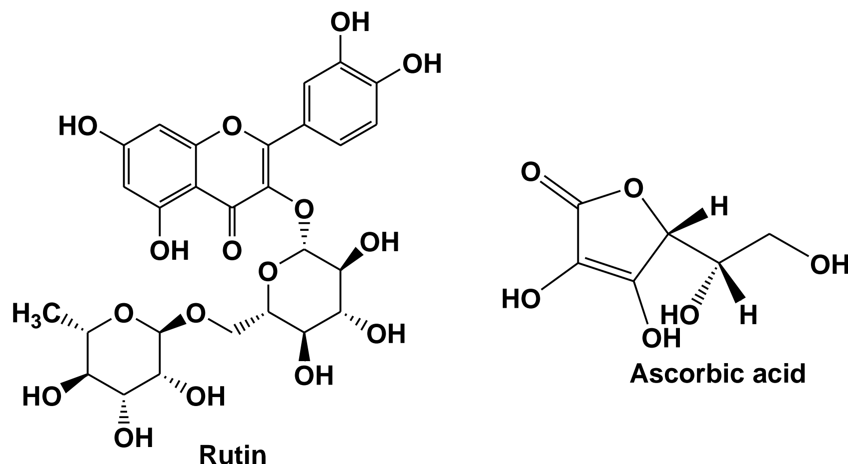

Figure1: Chemical structures of rutin and ascorbic acid.

2. Experimental

Apparatus

A double-beam UV–visible spectrophotometer (shimadzu, japan) model uv-1650 pc with quartz cell of 1 cm path length, connected to an IBM-compatible computer.

The spectral bandwidth was 2 nm and wavelength-scanning speed 2800 nm/min. A uv lamp with a short wavelength (254 nm).

All recorded spectra converted to ASCII format by UV-prob personal spectroscopy software version 2.21.

Software

All chemometric methods were implemented in Matlab® 7.1.0.246 (R14). PCR and PLS were carried out by using PLS-Toolbox software version 2.1. ANOVA test was performed using Microsoft® Excel. All calculations were performed using intel® core ™ i5-2400, 3.10 GHz, 4.00GB of RAM under Microsoft Windows 7.

Materials

All chemicals for the optimisation procedures and final determination of AA and RU were of analytical grade and they were used without further purification. AA and RU

(Sigma–Aldrich, USA), methanol (Chromasolv®, for HPLC, Sigma–Aldrich) were used. Ruta C 60® tablets (Kahira Pharm.Chem.Ind.Co., Cairo-Egypt) are labeled to contain 60 mg of RU and 160 mg of AA (Batch number 1210864).

Preparation of RU and AA standard solutions

Stock solutions of RU (800 μg mL^{-1}) and AA (800 μg mL^{-1}) were prepared by dissolving 20 mg of RU and 20 mg of AA, separately in 25 mL methanol. Stock solutions were stable for at least two weeks when stored refrigerated at 4 °C. Working solutions (200 μg mL^{-1}) of the mentioned stock solutions were prepared by suitable dilution in methanol

Preparation of pharmaceutical tablets sample solutions

Ruta C 60 ® tablets were weighed and finely powdered. An accurately weighed portion of the powder equivalent to 60 mg of RU and 160 mg of AA was extracted twice into methanol with the aid of sonication for 20 minutes and the extract was filtered. The filtrate was diluted with methanol to obtain final concentrations of 60 and 160 μg mL^{-1} for RU and AA, respectively. 500 μL of Ruta C 60 ® tablet solution were transferred into a 5 mL volumetric flask and diluted to the mark with methanol to get a final concentration of RU (6 μg mL^{-1}) and AA (16 μg mL^{-1}). Spectral acquisition and the calculations were

performed in the same manner as described in **"Multivariate Calibration procedures".**

Multivariate calibration procedures

Five level, two factor calibration design (Brereton, 1997) was used for construction of 25 samples by transferring different volumes of RU and AA from their standard working solutions into 5 mL volumetric flasks and the solutions were diluted to the volume with methanol and mixed well (Table 1). 15 samples were used to build the multivariate calibration models (training set) while 10 samples were used to test the predictive ability of the proposed models (validation set). The concentrations chosen for each compound in 25 samples were based on the calibration range of each of the two drugs, the ratio of RU: AA in the Ruta C 60 tablets (3:8 respectively). The absorption spectra of the 25 samples were scanned from 200 - 300 nm against methanol as a blank (Fig. 2) and transferred to Matlab for subsequent calculations. The noisy region from 220-230 nm accounted for the rejection of this part from the spectra. The 2D Scores plot for the first two PCs of the whole concentration matrix was obtained to confirm the well position of the mixtures in space, orthogonality, symmetry and rotatability (Brereton, 1997) as indicated in Fig. 3. Mean centering of the data proved to be the best preprocessing method for getting the optimum results.

Optimisation of number of latent variables for the PCR and PLS models

Cross validation (CV) (Kramer, 1998) was applied to predict how many are the optimum number of PLS latent variables. CV involves repeatedly dividing the data into two sets, a training set used to determine a model and a test set to determine how well the model performs so that each sample (or portion of the data) is left out of the training set once only.

Leave one out (LOO) CV is used in our study for optimizing the number of PLS components, by building the model using *I*-1 samples set (training set consisting of 14 samples) to predict the one sample left (validation sample). The root mean square error of CV (RMSECV) is calculated as

$$\mathrm{RMSECV} = \sqrt{\frac{1}{\mathrm{I}}\sum_{\mathrm{i}=1}^{\mathrm{I}}\left(c_i - \hat{c}^A_{i_cv}\right)^2} \qquad \hat{c}^A_{i_cv}$$

where I is the number of objects in the calibration set, c_i is the known concentration for sample i and is the predicted concentration of sample i using A components. Mean centering was performed on the training set each time successive samples were left out.

Table 1: The 5 level 3 factor experimental design of the training and validation set mixtures shown as concentrations of the mixture components in µg mL^{-1}.

Mix No.	RU	AA	Mix No.	RU	AA
1	12	12	14	12	20
2	12	4	15	20	20
3	4	4	16	20	4
4	4	20	17	4	16
5	20	8	18	16	4
6	8	20	19	4	12
7	20	12	20	12	16
8	12	8	21	16	16
9	8	8	22	16	8
10	8	16	23	8	4
11	16	20	24	4	8
12	20	16	25	8	12
13	16	12			

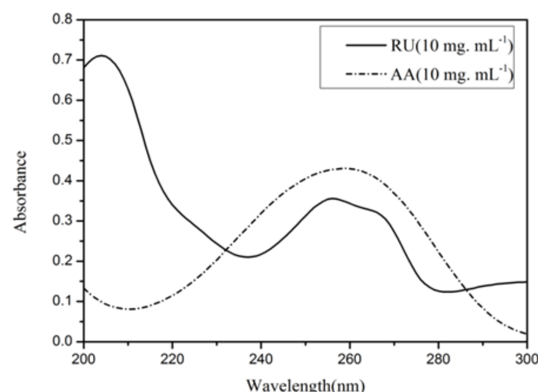

Figure 2: Absorption spectra for RU and AA against methanolas a blank (10 µg mL^{-1} each

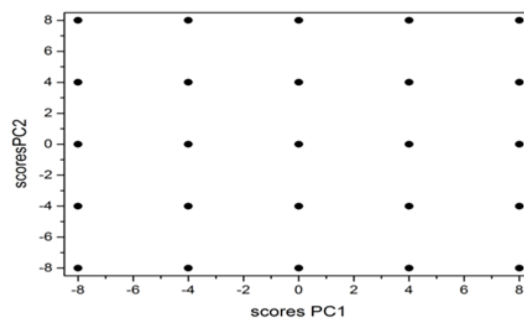

Figure 3: Scores plot for the mean centred 25 samples concentration matrix of the five level two component experimental design.

3. Results and discussion

Ruta C 60® tablets are combined dosage form containing RU and AA. It has been used for alterering the increased fragility and permeability of capillaries. The ratio of RU: AA in Ruta C 60 tablets is 3: 8 respectively. This study was designed to develop simple, robust and accurate multivariate methods for

the simultaneous determination of RU and AA in Ruta C 60® tablets. Because of the practical simplicity, and wide availability of spectrophotometry in quality control laboratories, it was attempted in this study. Multivariate calibration methods are very useful in spectral analysis because the simultaneous inclusion of many spectral wavelengths instead of using a single wavelength greatly improves the precision and predictive ability of these methods.

CLS model

The training set was used for constructing CLS model or (K) matrix (i.e. absorptivity at different wavelengths). The CLS method requires that all the components in the calibration samples must be known. Unlike CLS, PCR and PLS methods could be used to determine the components under investigation even in the presence of unknown components (interfering substance) which gave these two methods an advantage over CLS (Thomas *et al*, 1990). The absorbance matrix of the calibration samples (15x81) and their corresponding concentration matrix (15x2) were used to find the absorptivity matrix (k-matrix). Then, the obtained k-matrix was further used for the calculation of the predicted concentration of the two components in both the validation and pharmaceutical formulation samples.

PCR and PLS models

In order to apply PCR and PLS to the data, the raw data of the calibration samples were mean centered (Brereton, 2000) as a preprocessing step and the cross validation method, leaving out one sample at a time and RMSECV was calculated as mentioned above, was used to select the optimum number of factors (Kramer, 1998). The selection of the optimum number of latent variables was a very important pre-construction step: if the number of factors retained was more than required, more noise would be added to the data; if the number retained was too small, meaningful data that could be necessary for the calibration might be discarded. The maximum number of factors used to calculate the optimum RMSECV was selected to be eight. The method described by Haland and Thomas (Haaland *et al*, 1988) was used for selecting the optimum number of factors. After the PCR and PLS models have been constructed, it was found that the optimum number of LVs described by the developed models was three factors for both PCR and PLS methods as shown in Fig. 4.

After optimization of parameters and calibration (training) step, all models were applied successfully for analysis of RU and AA in training set (Table 2) and in validation set (Table 3). The recoveries mean recoveries, standard deviation, root mean square of calibration (RMSEC), root mean square of prediction (RMSEP) values are summarized in Tables 2 and 3. RMSEC and RMSEP were calculated as the same manner as RMSECV for calibration and validation set, respectively. RMSEP was used as a measure for performance of the proposed models (Fig. 5) showing that the three methods predicted AA and RU successively in their binary mixtures. However PLS the efficient one for RU determination as was indicated by decreasing S.D of RU results in validation set (Table 3).The proposed methods were then applied for the simultaneous determination of the two analytes in Ruta C 60 tablets (Table 4).

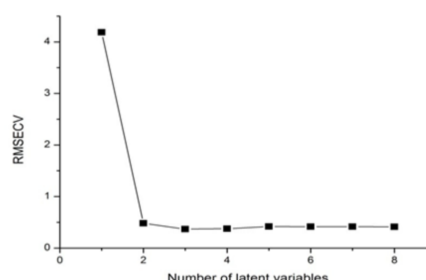

Figure 4: RMSECV plot of the cross validation results of the calibration set as a function of the number of latent variables used to construct the PLS or PCR models.

It was clear from table 4 that all models were accurate and precise for both RU and AA determination. Also ANOVA test was computed (Table 4) indicating that there is no significant difference between the three multivariate calibration methods.

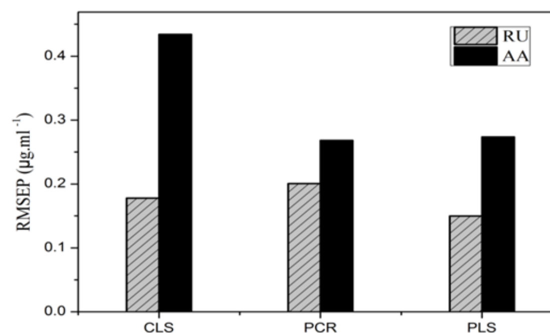

Figure 5: Bar plots for comparison of the RMSEP values obtained by application of the proposed multivariate calibration methods for the analysis of validation set

Table 2: Determination of RU and AA in calibration set by the proposed methods.

Mix. no.	Mix. Composition (μg.ml $^{-1}$)		R%					
			CLS		PCR		PLS	
	RU	AA	RU	AA	RU	AA	RU	AA
1	4	4	93.96	104.17	96	101.35	98.23	99.99
2	8	4	100.38	101.26	98.1	102.05	97.31	101.14
3	12	4	102.74	106.57	101.97	103.52	102.34	101.52
4	16	4	100.92	109.57	99.81	101.94	99.68	99.15
5	20	4	100.55	103.66	98.41	102.22	98.04	99.17
6	4	8	104.08	101.74	105.33	98.86	105.89	98.95
7	8	8	101.39	101.86	103.65	99.35	103.63	99.29
8	12	8	98.73	102.03	101.32	98.97	102.18	98.03
9	16	8	100.52	94.82	102.04	101.42	102.14	102.13
10	20	8	100.42	101.61	100.54	100.86	100.4	101.08
11	4	12	98.73	103.64	99.13	100.74	98.16	100.4
12	8	12	101.25	99.07	103.1	97.65	103.49	97.46
13	12	12	99.62	100.35	101.84	102.13	102.63	101.65
14	16	12	99.33	96.93	98.41	98.14	98.6	98.47
15	20	12	100.19	98.03	99.72	98.72	99.56	98.91
RMSEC (μg.ml $^{-1}$)			0.1426	0.2477	0.2097	0.1426	0.2393	0.1329
Mean			100.19	101.69	100.62	100.53	100.82	99.82
S.D			2.223	3.703	2.457	1.776	2.560	1.429

Table 3: Determination of RU and AA in validation set by the proposed methods.

Mix. no.	Mix. Composition (μg.ml $^{-1}$)		R%					
			CLS		PCR		PLS	
	RU	AA	RU	AA	RU	AA	RU	AA
1	4	16	105.85	100.08	101.78	101.57	101.45	101.69
2	8	16	103.25	100.87	101.57	101.99	101.72	102
3	12	16	99.04	100.74	98.18	101.49	97.93	101.65
4	16	16	98.71	99.12	98.51	99.17	98.49	99.17
5	20	16	100.65	103.06	100.79	102.46	100.92	102.31
6	4	20	102.42	99.74	102.11	99.89	102.01	99.9
7	8	20	102.57	100.62	105.3	99.16	99.41	98.88
8	12	20	98.4	94.65	99.37	100.69	100.28	100.76
9	16	20	101	99.79	98.48	101.39	98.83	101.61
10	20	20	99.58	103.27	100.1	102.24	100.3	102.08
RMSEP (μg.ml $^{-1}$)			0.1778	0.4342	0.2006	0.2683	0.1499	0.2738
Mean			101.15	100.19	100.62	101.01	100.13	101.01
S.D			2.377	2.377	2.191	1.222	1.423	1.259

Table 4: Determination of RU and AA in Ruta C 60 tablets (Batch No. 1210864) by the proposed multivariate calibration methods.

Method		CLS		PCR		PLS	
RU	**AA**	**RU**	**AA**	**RU**	**AA**	**RU**	**AA**
True (μg ml $^{-1}$)		R%	R%	R%	R%	R%	R%
6	16	104.46	101.70	104.32	102.93	104.28	102.95
6	16	103.71	99.12	101.24	101.27	101.20	101.29
6	16	99.67	96.33	98.04	97.71	98.00	97.73
6	16	100.27	97.17	95.61	100.10	95.57	100.11
6	16	103.43	97.95	101.12	99.93	101.08	99.94
Mean (%)		102.31	98.45	100.07	100.39	100.03	100.40
S.D		2.178	2.085	3.337	1.917	3.337	1.918
ANOVA		0.947	1.611	0.947	1.611	0.947	1.611

F critical for ANOVA: single factor, 3 ° of freedom is 3.885

Conclusion

The proposed multivariate calibration methods were simple, rapid, sensitive and precise and could be easily applied in quality-control laboratories for the simultaneous determination of RU and AA in pure bulk powders. Moreover, these methods could be applied for dosage form analysis as well as in pure powder form without any preliminary separation step.

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