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Simultaneous spectrophotometric determination of ethinylestradiol and levonorgestrel by partial least squares and principal component regression multivariate calibration

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Abstract

Two spectrophotometric methods for the determination of Ethinylestradiol (ETE) and Levonorgestrel (LEV) by using the multivariate calibration technique of partial least square (PLS) and principal component regression (PCR) are presented. In this study the PLS and PCR are successfully applied to quantify both hormones using the information contained in the absorption spectra of appropriate solutions. In order to do this, a calibration set of standard samples composed of different mixtures of both compounds has been designed. The results found by application of the PLS and PCR methods to the simultaneous determination of mixtures, containing 4–11 $\mu g\ ml^{-1}$ of ETE and 2–23 $\mu g\ ml^{-1}$ of LEV, are reported. Five different oral contraceptives were analyzed and the results were very similar to that obtained by a reference liquid chromatographic method.

Keywords: Ethinylestradiol; Levonorgestrel; Spectrophotometry; Multivariate calibration; Partial least squares; Principal component regression; Contraceptives analysis

1. Introduction

Oral contraceptives have had an enormous positive impact on public health for the past three decades, and although a remarkably low incidence of troublesome side effects has been reported, there is still a need for reliable methods of analysis.

Ethinylestradiol (ETE), semi-synthetic estrogen, is a female sex hormone and Levonorgestrel (LEV) is a synthetic steroid with an extremely potent progestational steroid. The formulations of these steroids in tablets of low dosage, i.e. 30–250 μg per tablet, had presented a challenging analytical problem. A sensitive, accurate, and rapid procedure will be desired for testing content uniformity of the dosage form. The LEV structure has a characteristic Δ^4-3-keto group in A-ring, with a different chromophoric power respect to the ETE (Scheme 1).

Most oral contraceptive formulations, in current use, contain 50 μg or less of ETE and 1 mg or less of the various progestins: norethindrone (0.5–1 mg), or LEV (0.05–0.25 mg). Thus, a sensitive method, which is unaffected by a large excess of progestogen,

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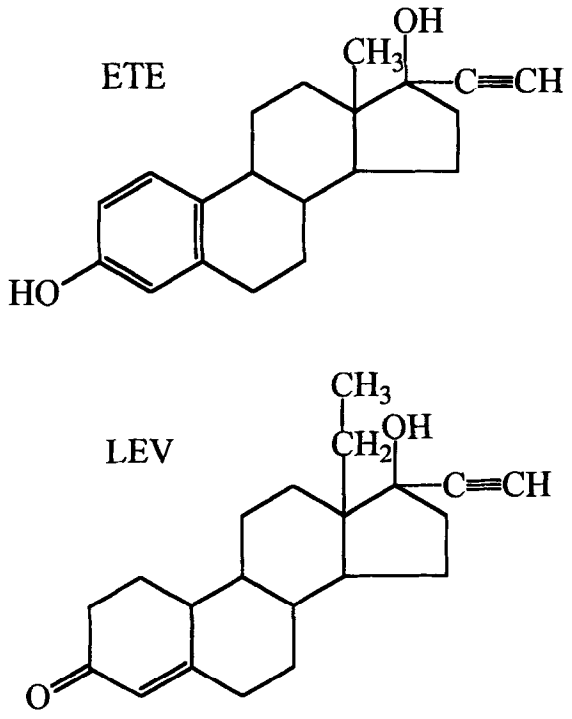

Scheme 1.

is required for the analysis of these estrogen and progestogen.

There are several descriptions in the literature [1–11] of the determination of LEV or ETE, very small amounts of which form the active substances in preparations for hormone contraception. The methods described, which include the use of radioactively labelled derivatives [1,2], dansyl or other fluorescent derivatives [3–5], spectrophotometry or photometry [6–9] or gel or column chromatography [10,11] are complicated. No references were found for the simultaneous spectrophotometric determination of ETE and LEV by multivariate calibration methods.

Partial least squares (PLS) is a multivariate calibration method based on factor analysis and PLS-1 and PLS-2 types have been described. PLS-2 differs from PLS-1 in the way used to perform the signal decomposition and the regression analysis. PLS-2 calculates the number of factors on all the components simultaneously and one weighed number of factors is optimized. PLS-1 performs the optimization of the number of factors for only one component at a time. The bibliographic data mainly refer to its application in spectroscopic techniques [12–16].

The basic concept of PLS regression was originally developed by Wold [17], and the use of the PLS method for chemical analysis was pioneered by Wold et al. [18,19]. A detailed description of the mathematical principles of the PLS algorithms have been reported by Martens et al. [20].

The principal component regression (PCR) is simply a principal component analysis followed by a regression step [21]. PLS is related to PCR in that the spectral decomposition is also performed, but this decomposition step is performed differently. In PCR, the spectra are decomposed on the basis of the maximum variance between spectral data and information about the concentrations is not used, while PLS use both spectral data and concentration data in modeling.

The absorption spectra, with different concentrations of the two hormones have been used for the multivariate calibration and two methods of determination have been developed for the direct simultaneous determination of ETE and LEV in commercial oral contraceptive tablets.

The results obtained by both proposed methods have been compared with the results obtained by application of a reference LC method (spectrophotometric detection) proposed by the authors.

2. Experimental

2.1. Apparatus

A Beckman DU-70 spectrophotometer equipped with 1.0 cm quartz cells and connected to an IBM-PS 2 model 30 computer provided with Beckman Data Leader software, and an Hewlett Packard LaserJet IIIp printer was used for all the absorbance measurements.

A Shimadzu high-performance liquid chromatograph equipped with a Nova-Pak C_{18} 60A column (15×0.39 cm i.d.) and 4 μm packing, a diode array detector, a Rheodyne injection valve and connected to a computer provided with Base Line software was used.

A Crison micropH 2002 was used for the pH measurements.

The GRAMS-386 Level I Version 3.01 software package, with the PLS plus version 2.1G application software [22], connected to an EGA computer and an Hewlett Packard LaserJet IIIp, were used for the statistical treatment of the data and the application of the PLS method.

2.2. Standard solutions

All chemicals and solvents used were of analytical grade. ETE and LEV were obtained from Sigma and their stock solutions were prepared in absolute ethanol (100 μg ml^{-1}).

The commercial formulations Microgynon, Neogynona and Tryagynon were obtained from SHERING AG (Spain) and the Ovoplex and Triciclor from WYETH-ORFI, S.A. (Spain).

2.3. Procedures

2.3.1. PLS and PCR of the absorption spectra

The calibration matrix was obtained by using the absorption spectra obtained (between 250 and 315 nm with an 120 nm min^{-1} scan speed) from different ethanolic solutions of ETE and LEV mixtures (see Table 1). A number of factors of 4 for both compounds was used in the PLS-1 method and 3 in the case of PCR.

These absorption spectra have then been employed as standard samples, to optimize the calibration matrix, by using PLS and PCR multivariate calibration. The optimized calibration matrix, calculated by application of the PLS and PCR multivariate methods, was applied to analyze the spectra of eight synthetic mixtures and five pharmaceutical oral contraceptives and to determine the concentrations of ETE and LEV in the pharmaceutical samples

2.3.2. Commercial pharmaceutical preparations

Two tablets were finely powdered and diluted with 7 ml of absolute ethanol by sonication during 15 min, and shaken by mechanical means for 20 min. The mixture was filtered by a polypropylene Swinnex disc filter holder 13 mm diameter (Millipore) with a FH 0.5 μm fluoropore (PFTE) membrane into a 10 ml calibrated flask, the residue was washed two times with the same solvent and diluted to the mark.

Table 1
Training set a composition

Standard	Ethinylestradiol (μg ml^{-1})	Levonorgestrel (μg ml^{-1})
1	2.06	0.00
2	4.13	0.00
3	6.19	0.00
4	8.26	0.00
5	10.32	0.00
6	15.48	0.00
7	20.64	0.00
8	26.10	0.00
9	0.00	2.59
10	0.00	5.18
11	0.00	7.77
12	0.00	10.36
13	0.00	12.95
14	0.00	19.42
15	0.00	25.9
16	2.06	3.88
17	4.13	7.77
18	6.19	7.77
19	2.06	10.36
20	4.13	10.36
21	2.06	10.36
22	3.10	15.54
23	8.26	20.72

The absorption spectra were recorded against an ethanol absolute blank and stored in a IBM-PS computer. For determining ETE and LEV, the absorption spectra is predicted by the optimized calibration matrix of PLS and PCR.

2.3.3. Liquid chromatography (LC) in commercial formulations

After the preparation of the ethanolic solution of the two tablets (as described before), an aliquot of 2.5 ml of this solution and was transfered into a 10 ml calibrated flask and water was added. This solution was injected in the LC system. The mobile phase was a deaerated mixture of acetonitrile : methanol : water in a relation 3.5 : 1.5 : 4.5 and spectrophotometric detection was performed at 215 nm. The flow rate is about 1 ml min^{-1}. The described method is a slight modification of the one proposed by the Pharmacopeia [23].

3. Results and discussions

3.1. Influence of chemical variables

The influence of pH on the absorption spectra of ETE and LEV was studied in solutions with a total content of ethanol of 25%. The best analytical results were obtained in the range pH 4.0–9.5. The samples prepared with a percentage of 100% of ethanol showed spectra very close to those obtained at the optimized pH.

Samples were prepared in absolute ethanol solution. Diluted solutions of ETE and LEV were stable for at least 12 h. The use of absolute ethanol permits the best recovery of the hormones in the oral contraceptive tablets.

3.2. PLS and PCR multivariate calibration

In Fig. 1, the spectra of ETE and LEV in the 210–315 nm wavelength range are shown. It can be seen that the absorption spectrum of LEV strongly overlaps with the ETE spectrum. The direct determination of ETE directly seems to be easy, at the start, but the small content of this steroid in the commercial tablets, its low molar absorptivity and the high content of LEV (relation ETE : LEV is normally 1 : 4 or 1 : 5) in oral contraceptives may cause problems.

The solutions of real filtered samples after dissolution of the hormones look slightly cloudy, not perceptible at the beginning, but the spectrum shows a small displacement of the absorbance along the Y-axis due to the cloud. This behavior is shown when the spectrum of an artificial binary mixture was compared with the spectrum of a solution of real contraceptive tablets of similar concentration (Fig. 2). Thus, the direct ETE determination in contraceptives was not possible because there is a contribution of other components (excipients). If the concentration of one or more component is omitted, the predicted absorbance will be incorrect. For these reasons the Classical Least Squares (CLS) is not recommended in this case. Therefore, PLS or PCR

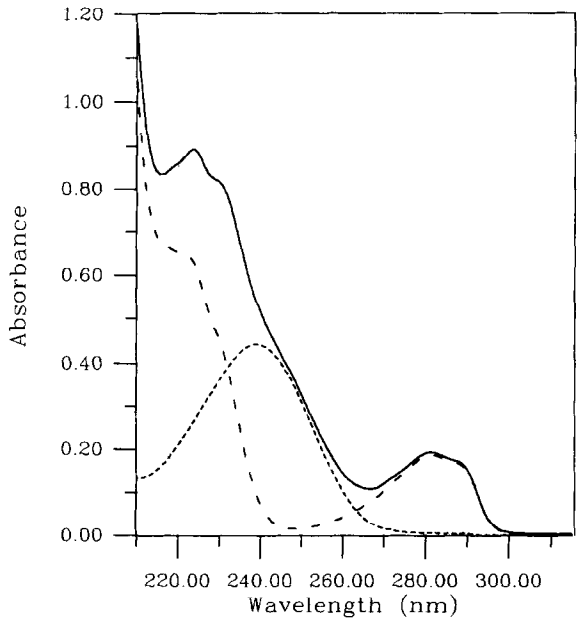

Fig. 1. Absorption spectra of Ethinylestradiol (26.01 μg ml^{-1}) (– – –), Levonorgestrel (7.17 μg ml^{-1}) (- - -) and their mixture (———).

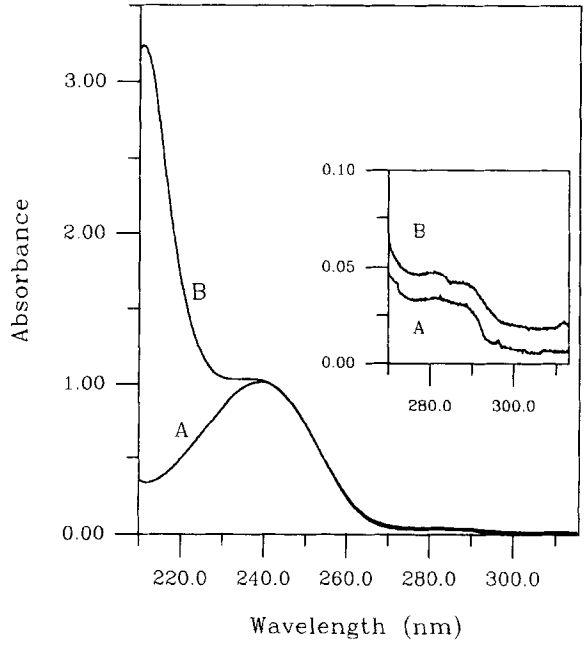

Fig. 2. Absorption spectra of (A) synthetic mixture of ETE (5.00 μg ml^{-1})–LEV (25.00 μg ml^{-1}) and (B) solution of the commercially available Ovoplex pills, containing the same concentration of ETE and LEV.

calibration methods were necessary due to the presence of interferences or matrix effects.

3.2.1. Experimental design of the calibration matrix

A training set of 23 samples was taken from different binary mixtures (Table 1). The evolution of the absorption spectra with the concentrations of the two hormones was monitored under the chemical conditions mentioned above. An absorption spectrum was scanned for each standard sample. In the selected region, the spectral information in the range of 250 and 315 nm was used for the calibration. The selection of the wavelength range was done according with the spectrum of a solution of real contraceptive tablets. The range of the spectrum between 210–250 nm was rejected due to the differences between the artificial mixture spectra and the pharmaceutical contraceptive spectra (Fig. 2). These differences could be due to the other components of the tablets as the excipients, saccharose, lactose, polyvinylpyrrolidone and so on.

To select the number of factors for PLS methods, the cross-validation method, leaving out one sample at a time, has been used. This process was repeated 22 times, until each sample had been left out once. The predicted and actual composition of the samples are compared. PRESS (prediction error sum of squares) is expressed as

$$\text{PRESS} = \sum_{i=1}^{N}\sum_{j=1}^{m}(\hat{x}_i - x_i)^2,$$

(where $\hat{x}_i$=predicted concentration and x_i=standard concentration, N=total number of samples and m=total number of components used in the prediction set), and is a measure of how well a particular PLS model fits the concentration data. Another good criterion for selecting the optimum number of factors involves the comparison of PRESS from models (h models) with the model which involves the number of factors yielding the minimum PRESS (h^* model). This criterion has been selected by us, and also the F-Snedecor statistic and the Haaland and Thomas [24] criteria were used. The number of factors for the first PRESS value whose F-ratio probability drops below 0.75 was selected as optimum.

In our particular case, a number of factors of 4 was obtained as optimum for the LEV and ETE components of the mixtures by the PLS-1 method. Also, a PCR model was optimized by using the same set of standard samples. A number of 3 factors was found to be optimum. In Fig. 3, the PRESS obtained

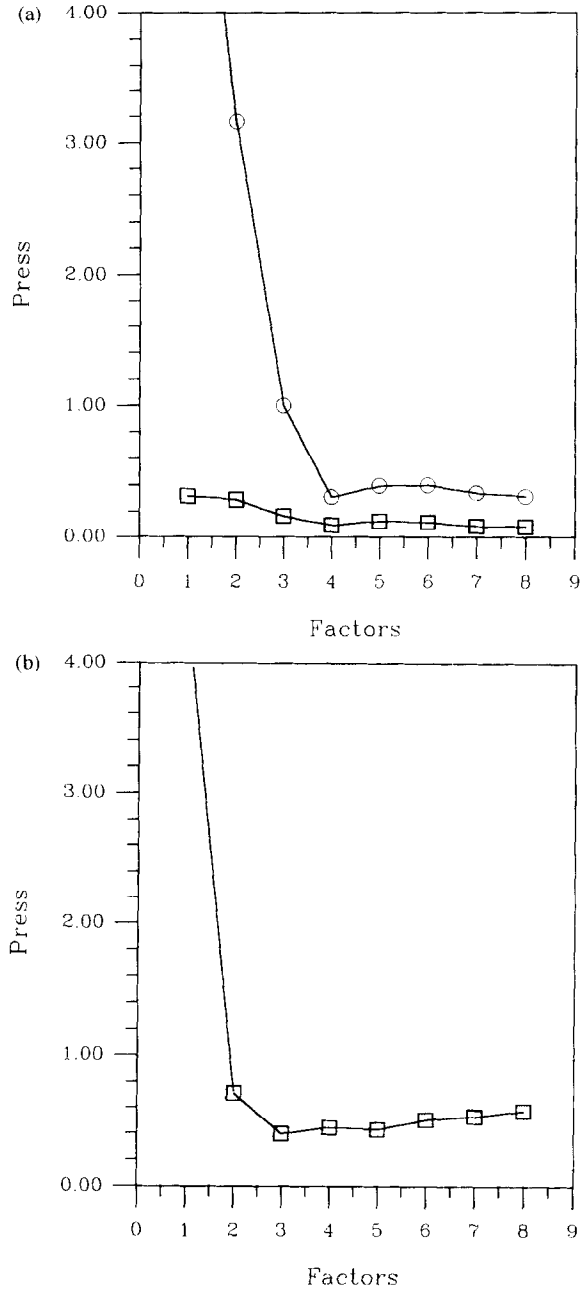

Fig. 3. Obtained PRESS values, by using the (a) PLS-1 (□ LEV; ○ ETE) and (b) PCR (□ LEV and ETE) methods, for the different tried number of factors.

Table 2
Cross validation results

Component	PLS-1				PCR			
	Factors	RMSD	SEP	R^2	Factors	RMSD	SEP	R^2
Ethinylestradiol	4	0.0626	0.0640	0.99993	3	0.1167	0.1193	0.9997
Levonorgestrel	4	0.1158	0.1184	0.99971	3	0.0593	0.0606	0.9999

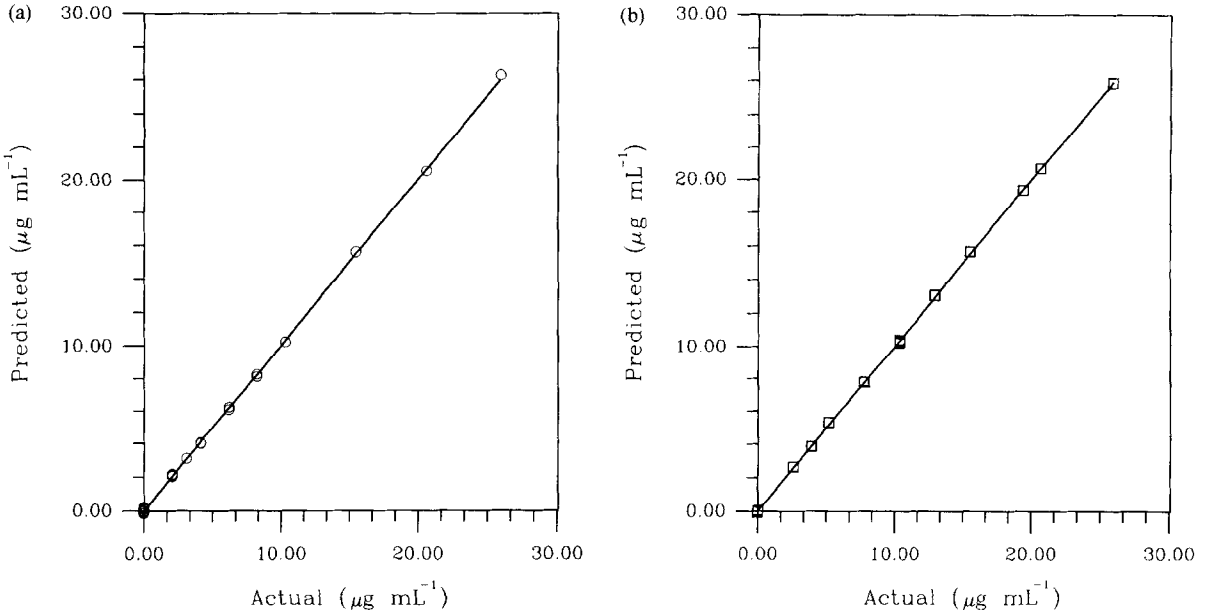

Fig. 4. Predicted versus actual concentration plots for the training set data by using the optimized matrix, (A) ETE (○) and (B) LEV (□).

by optimizing the calibration matrix of the absorption spectra with the PLS-1 and PCR methods are shown. In Table 2, the statistical root mean square difference (RMSD) and the square of the correlation coefficient (R^2) parameters obtained for the PLS-1 and PCR optimized models are summarized. Satisfactory R^2 values are obtained for both components. The predicted vs. actual concentration plots for both components are shown in Fig. 4.

On the other hand, the residual analysis [25] permits the determination of an amount that is not explained by the model for each data point $\varepsilon_i = y_i - \hat{y}_i$ being $\hat{y}_i = b_i x_i$ where y_i is the data value and $\hat{y}_i$ is the predicted value for the model; b_i is a regression parameter and x_i is the independent variable. In Fig. 5, the spectral residual values for the two hormones are represented. No statistical differences between the mean spectral residuals values (MSR) obtained for both compounds are observed.

3.2.2. *Determination of ETE and LEV in synthetic mixture*

One set of eight artificial samples were predicted by applying the PLS-1 and PCR calibration models. Binary mixtures covering ranges between 1 : 5 and 1 : 1 ETE : LEV ratios were resolved. Mean values for two independent replicate samples for each problem were obtained. In Table 3 recoveries and standard deviation values found by PLS-1 and PCR

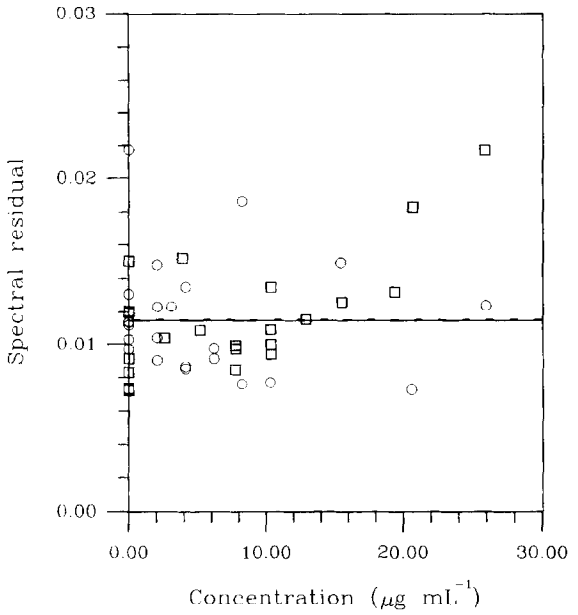

Fig. 5. Spectral residual vs. concentration plots for the two components by PLS-1 model. (- - -) mean spectral residual for ETE (○); (———) mean spectral residual for LEV (□).

calibrations have been summarized. These results show that both methods are effective for the two drugs in synthetic mixtures.

3.2.3. Statistical study

The most characteristic statistical data obtained from the reproducibility for ten different standards of 10.4 μg ml^{-1} ETE and 10.6 μg ml^{-1} LEV are given in Table 4. The reproducibilities were evaluated over two days by performing ten absorption spectrophotometric records each day.

The results show that the repeatability for both hormones on each day is satisfactory. The comparison of average concentration with the Snedecor test did not show any significant difference at a confidence level of 95%. No significant differences were found between the two proposed methods.

3.2.4. Analysis of oral contraceptives by the PLS-1 and PCR methods

The optimized matrix has been applied to the resolution of five different contraceptives formulations (three modern low-dose monophasic oral contraceptives: Ovoplex, Neoginona, Microgynon and two of them triphasic pills: Triagynon and Triciclor). The five commercial contraceptives contain ETE and LEV in ratios ETE : LEV of 1 : 1.5 until 1 : 5.

In Table 5, the obtained results by application of matrix are summarized and compared with those obtained by the LC method. The obtained recoveries were between 98% and 107% for the LEV determination in all the cases. In the same way, the recoveries of ETE were comprised between 97% and 105% in all commercial formulations except for the Triciclor.

In all the cases the recoveries were calculated with respect to results obtained by the LC method. The indicated value is the mean of two different determinations of the same commercial batch.

Table 3
Results obtained for the analysis of binary mixtures by PLS and PCR methods (average of two determinations)

Sample	Ethinylestradiol					Levonorgestrel				
	Actual (μg ml^{-1})	Found (μg ml^{-1})		Recovery %		Actual (μg ml^{-1})	Found (μg ml^{-1})		Recovery %	
		PLS-1	PCR	PLS-1	PCR		PLS-1	PCR	PLS-1	PCR
1	5.47	5.36	5.36	98	98	2.07	2.09	2.07	101	100
2	5.47	5.52	5.47	101	100	15.52	15.68	15.68	101	101
3	4.38	4.42	4.38	101	100	16.56	16.73	16.73	101	101
4	6.56	6.56	6.56	100	100	4.14	4.18	4.18	101	101
5	4.38	4.20	4.12	96	94	24.84	25.09	25.09	101	101
6	6.56	6.36	6.30	97	96	20.70	20.70	20.70	100	100
7	10.93	10.71	10.71	98	98	10.35	10.45	10.35	101	100
8	5.47	5.47	5.42	100	99	23.18	23.41	23.41	101	101

Table 4
Precision for concentrations on different days (n=10 determinations on each day)

	PLS-1						PCR					
	Ethinylestradiol			Levonorgestrel			Ethinylestradiol			Levonorgestrel		
	Average μg ml^{-1}	S.D	R.S. D.%	Average μg ml^{-1}	S.D	R.S .D	Average μg ml^{-1}	S.D	R.S .D. %	Average μg ml^{-1}	S. D.	R. S. D. %
Day A	10.38	0.122	1.17	10.65	0.043	0.408	10.40	0.112	1.08	10.66	0. 050	0.467
Day B	10.36	0.122	1.18	10.71	0.072	0.674	10.35	0.052	0.508	10.71	0. 072	0.6 71

Table 5
Simultaneous determinations in five commercial formulations

Commercial formulation	Ethinylestradiol					Levonorgestrel				
	Found (mg per tablet)			Recovery (%)		Found (mg per tablet)			Recovery (%)	
	LC	PLS	PCR	PLS	PCR	LC	PLS	PCR	PLS	PCR
Ovoplex	0.0460	0.0445	0.0420	97	91	0.2300	0.2314	0.2305	101	101
Microgynon	0.0295	0.0286	0.0274	97	93	0.1444	0.1456	0.1458	101	101
Neogynona	0.0461	0.0477	0.0498	103	108	0.2383	0.2360	0.2359	99	99
Triagynon A*	0.0287	0.0303	0.0278	105	97	0.1223	0.1226	0.1223	100	100
Triagynon B*	0.0369	0.0368	0.0372	100	101	0.0708	0.0760	0.0736	107	104
Triagynon C*	0.0285	0.0295	0.0302	103	106	0.0467	0.0497	0.0495	106	106
Triciclor A*	0.0247	0.0273	0.0272	110	110	0.1271	0.1245	0.1283	98	101
Triciclor B*	0.0315	0.0347	0.0318	110	101	0.0681	0.0708	0.0695	104	102
Triciclor C*	0.0252	0.0296	0.0275	117	109	0.0488	0.0495	0.0507	101	104

In general, the obtained recoveries by PCR for LEV are similar to the PLS-1 method, but for ETE slightly different values compared to those obtained with PLS-1 are found by application of the PCR method.

4. Conclusions

Chemometrics have generated much interest in analytical molecular spectroscopy Ultraviolet/visible spectra contain non-specific data, which can be converted into useful information by multivariate calibration methods. Clear explanations of the different chemometric methods and properly designed user-friendly software should provide a bridge between chemometricians/mathematicians and potential spectroscopic users, enabling them to make successful use of these powerful tools.

The PLS and PCR approaches used in this work are simple to perform, with adequate software support, and provides a clear example of the high resolving power of this technique. According to these results we conclude that one possibility for resolving overlapping absorption spectra of these hormones by means of the assayed multicomponent analysis method, is the application of PLS and PCR to the absorption spectra as such.

The resolution of synthetic mixtures by the application of the studied methods gives rise to acceptable recovery values in both cases. However, according to the results with oral contraceptives we conclude that the better approach for resolving these drugs is PLS-1 analysis.

The proposed method can be used without any preconcentration and/or separation process in commercial oral contraceptives exhibiting low amounts of these hormones.

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