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Application of Multivariate Calibration Methods in Resolution and Quantification of Drugs under Identical Profiles in HPLC

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ABSTRACT

HPLC has been used for many years to separate and determine pharmaceutical, biomedical and chemical compounds. However, for similar structures, the chromatograms are often overlapped or identical. In order to resolve the chromatographic peaks, often very time consuming method development is required. An alternative is to use chemometric methods. Taking into account the application of spectrophotometric methods in the assay of mixtures and envision a chromatogram as a spectrum, in the present study, multivariate calibration methods of classical least squares (CLS) and inverse least squares (ILS) were proposed to resolve and quantify a mixture with identical chromatograms. A mixture of trimethoprim (TMP) and phthalazine (PHZ) with identical peaks in the proposed RP-HPLC method was used as a model. The chromatograms were obtained at four-wavelengths of 235 (A), 250 (B), 260 (C), and 285 (D) nm of a dual wavelength UV-detector. From the overlapped chromatograms different binary, ternary and quaternary sets of wavelengths were used to construct the models and predict the concentration of analytic in prediction set. Using the building models, the different sets of wavelengths were compared. The proposed methods were successfully applied to the simultaneous determination of a mixture with identical peaks in RP-HPLC using multivariate calibration methods.

Keywords: Chromatography, Identical peaks, Multivariate calibration methods, Classical least squares, Inverse least squares.

1. INTRODUCTION

The analysis of complex mixtures of compounds is routinely carried out by chromatographic techniques. The major goal of analytical chromatographic methods is to produce and measure individual signal of a particular compound without any interference from the other components of the mixture. This is mainly achieved due to chromatographic resolution of the applied method. However, the resolution capability of

chromatographic methods is limited [1] and would be inappropriate for best separation, satisfactory detection and accurate quantification of all components. When samples are analyzed by chromatographic methods, it is common to encounter situations where two or more components are eluted with close retention times and, therefore, deconvolution of the overlapping peaks is one of the challenging areas in chromatographic analysis.

Some procedures such as optimization of mobile phase and changing the column have been suggested to achieve excellent separation. These ways, however, are not convenient and lead to a longer run times and higher costs than expected. To cope with these problems, different approaches have been proposed for quantification of compounds with overlapping chromatograms [2-5]. Difficulties stemming from poorly-resolved peaks may still be overcome mathematically using different chemometric methods. These methods not only assist the chromatographer in the design of experiments, the search for the best separation conditions and the analysis of the gathered data, but also provide solutions for partial (or even full) overlap of peaks. The partial separation achieved in the chromatographic domain can be completed -at least in some extent- by mathematical means. It is possible to use the combination of different chemometrics methods with hyphenated chromatographic systems to get second order data for resolving (qualitatively and quantitatively) the complex mixtures of natural compounds [6]. In second order data, each data matrix has been obtained by recording spectra at several retention times. These methods have been successfully applied for analyzing

compounds in the complex matrices using GC×GC, GC–MS, HPLC-DAD, HPLC-DAD/MS and LC–MS [6].

Several chemometrics algorithms are available for the convenient processing of second-order data under overlapping profiles: (1) alternating least-squares (ALS), such as parallel factor analysis (PARAFAC) and its variants PARAFAC2 and PARALIND (PARAFAC with linear dependencies), and multivariate curve resolution-ALS (MCR-ALS); (2) eigenvector-eigenvalue techniques, such as generalized rank annihilation; (3) direct least squares, such as bilinear least squares (BLLS); and (4) latent-structured methods, such as unfolded partial least squares (U-PLS) and multiway PLS (N-PLS) [7-8]

The existence of identical profiles for sample components, which is a special case of linear dependency, does also pose a challenge on the above mentioned second-order algorithms. A sample with two responsive components with identical profiles in one dimension will produce a data matrix with rank one; that is, the matrix will be rank-deficient, a situation also known as rank overlap. This is especially troublesome when the identical profiles correspond to a potential interfering agent and to the analyte of interest. It has been demonstrated, MCR-ALS and also PARALIND were the only algorithms that may be able to solve the situation [7-9]. Recently, another method, adapted partial least-squares/residual bilinearization has been proposed for this purpose by Lozano and et al [8].

However, these chemometric techniques have a main disadvantage; the requirement to understand the often complex mathematics of second order algorithms. On the other hand, getting second order data need the high cost of hyphenated instruments. In the present work, a very simple and easy to understand method was proposed for the resolving of two analytes under identical profiles in HPLC. The proposed method is suitable for analysis of binary mixtures with identical profiles in HPLC that show a difference in their absorbance at selected wavelengths in detection step.

In the spectrophotometric method, it is possible to quantify a mixture of components using the idea that at each wavelength, the absorbance of the mixture solution is the sum of the absorbances of each component [10]. Considering the chromatogram of an analyte in HPLC as a spectrum, it was proposed to apply a less complex multivariate calibration methods of classical least squares (CLS) and inverse least squares (ILS) for the quantification of compounds with identical chromatograms. These chemometric techniques have the advantage of being relatively simple, rapid, and of low cost. Trimethoprim (TMP) and Phthalazine (PHZ) were used only as model compounds which have identical peaks in the developed HPLC condition in this study.

2. EXPERIMENTAL

2.1. Chemicals, reagents and solutions

Trimethoprim (TMP) and phthalazine (PHZ) were supplied by Aldrich (Dowel, UK). HPLC-grade acetonitrilie, methanol and all other chemicals were obtained from Merck (Darmstadt, Germany). Water was obtained by double distillation and additionally purified with a Milli-Q system. Stock solutions of 726 µg mL^{-1} of TMP and 325 µg mL^{-1} of PHZ were prepared by dissolving 18.1 mg (TMP) and 8.12 mg of (PHZ), in the mobile phase [acetonitrile-methanol-potassium dihydrogen phosphate (10 $mmol^{-1}$, pH 6.0) (v/v/v, 38:4:58) and prepared to 25 mL.

2.2. Equipment, Instrumentation and software

HPLC was performed using an Agilent-1100 liquid chromatographic system (Agilent Technologies, USA) equipped with a binary HPLC pump (Waters Model 515), a thermostatted auto sampler, and a column heater. Sykam S3240 Programmable 4-channel UV/Vis Detector (Laserchrom, UK) was used as a detector. Using this detector it is possible to obtain the chromatogram at the four wavelengths in a single injection.

The absorption spectra were recorded using a Beckman DU 640 UV-Visible spectrophotometer.

All data were saved in ASCII format and transferred to a PC computer for subsequent manipulation. Data were handled using Microsoft excel and MATLAB software (7.1 versions).

2.3. Chromatographic conditions

The original mobile phase optimization procedure was carried out based on the selectivity triangle approach using three solvents and taking into consideration the molar fraction of the solvents [11-12]. Accordingly, the optimized mobile phase was obtained as an acetonitrile-methanol-potassium dihydrogen phosphate buffer (10 mM, pH =6): acetonitrile (38:58:4 v/v/v).

The separations were performed on a Symmetry C18 HPLC column (250 mm×4.6 mm, 5 µm particle size) from Waters (USA). The flow rate was maintained at 0.9 mL min^{-1} and the injection volume was 25 µL. The mobile phase was prepared daily, filtered through a 0.45 µm membrane filter, and degassed before use.

2.4. Individual calibrations

Individual calibration curves were constructed using several points as peak height versus TMP and PHZ concentration in the range of 0.14-2.90 µg mL^{-1} and 0.06-1.30 µg mL^{-1} for TMP and PHZ, respectively, and the results evaluated by linear regression. In order to obtain the calibration curves of the compounds, peak height was measured at four different wavelengths for both compounds. The characteristics of calibration graph and the validation parameters for determination of TMP and PHZ are summarized in Table 1.

2.5. Calibration procedure for the simultaneous determination

TMP and PHZ as binary mixtures were prepared as follows: appropriate volumes of the standard solutions (in the dynamic linear range) were transferred into a 10 ml volumetric flask and made up to the mark. The chromatograms of the analyte solutions were recorded at 235, 250, 260 and 285 nm as wavelength of UV detector. These wavelengths were selected based on the spectra of the analytes (Fig. 1). The optimized calibration models were applied to calculate the concentration of each analyte in the prediction set.

3. THORY

These approaches are based on the application of multi linear regression (MLR) to the ratio of the peak height of each analyte [13]. The matrix equation describing the system is given as follows:

$$R = CK \quad (1)$$

where, R is M N matrix and represents the peak height responses of M samples at N wavelengths, C denotes the concentrations of L for the investigated compounds in M samples, and K is L N matrix of the calibration coefficients. In calibration step, it is assumed that a series of experiments are performed in which C is known (e.g. a set of mixtures of compounds with known concentrations are recorded). C^T is the transpose of the matrix C. An estimate of K can then be obtained by:

$$K= (C^TC)^{-1} C^TR \quad (2)$$

and can be used in the prediction step to predict the concentrations in any unknown samples (Cun):

$$Cun = Run\ K\ (K^TK) \quad (3)$$

where Run is the unknown peak height response and KT represents the transpose of the matrix K.

The approach described above is a form of classical calibration, and it is also possible to envisage an inverse calibration model (Inverse Least Square, ILS) [13].

$$C = R\ B \quad (4)$$

The matrix B is given by:

$$B = (R^TR)\ R^TC \quad (5)$$

where RT is the transpose of the matrix R.

This can be extended to estimate the concentrations in any unknown sample:

$$Cun = Run\ B \quad (6)$$

This use of the inverse model is only practicable if: 1) the number of experiments and wavelengths is at least equal to the number of components in the mixture, and 2) the number of experiments is at least equal to the number of wavelengths [13].

The condition 2 requires a large number of extra experiments to be performed. There have been a number of algorithms developed for wavelength selection, enabling inverse models to be produced, but it has been proposed that there is no real advantage over classical least squares in these situations [13].

Such equations make assumptions that the concentrations of the significant analytes are all known and they work well only if this is true. In those cases that there is an application to mixtures with unknown components, it can result in serious estimation errors.

In this paper, the signals in data matrix are heights of the peak in the chromatograms at four wavelengths. So there is a vector, r, with the size 1×4 for each sample. R is column augmented matrix, each row is the vector that was obtained for each sample. Since the obtained data are first order, the mentioned simple first order methods, CLS and ILS, can be used to analyze them. The proposed method can be used not only to analyze components with overlapped chromatograms, but also to resolve the components with identical chromatograms. It is necessary to emphasis that the proposed method can be used when there isn't any linear dependency between UV spectra of two components at selected wavelengths.

4. RESULTS

The chromatograms of the TMP and PHZ standard solutions and the mixed solutions are shown in Fig. 2. This figure shows that the chromatograms are identical with different intensities. As mentioned in theory section, peak heights of chromatograms at different wavelengths for each sample can be considered as a vector. So, chemometric methods, discussed above, can be used to resolve the components in the mixtures. Thus it can be suggested that the multivariate calibration methods coupled with HPLC to determine the analytes even when there is an observed overlapping of the chromatographic peaks.

The first step in linear models of multivariate calibration methods such as CLS and ILS is checking additive property. This step is necessary in order to check that the other sources of signal variation, such as any interactions between the chemical components, changes of shape of the component peak and detector noise, exist or not. Additive property is a requirement for successful application of these quantification methods. The peak heights, UV signals, of these two compounds showed good additive properties at selected wavelengths (Fig. 3) and thus the linear models of multivariate calibration of CLS and ILS can be used for the simultaneous determination of these components. Four wavelengths in the UV region were used as detector sensors in recording chromatograms.

The first step in the simultaneous determination using the multivariate calibration methods involves constructing of the calibration matrix for the binary mixture. In the study, calibration sets were optimized with the aid of the orthogonal design method [14]. Tables 2 and 3 show the composition of the calibration and prediction samples with four concentration levels in the dynamic linear range for TMP and PHZ.

The recovery, standard error of prediction (SEP) and relative standard of prediction (REP) data obtained by application of the mixtures for TMP and PHZ by CLS and ILS have been summarized in Table 4. The statistical parameters of standard error of prediction (SEP) and recovery percentage were used to evaluate the prediction ability for the proposed models. The formula for the SEP and REP parameters were calculated by the following equations are shown in Eqs. (7) and (8).

$$SEP = \left[\frac{\sum_{i=1}^{m} (C_{pre} - C_{act})^2}{m} \right]^{1/2} \quad (7)$$

$$REP\,(\%) = 100 \times \left[\frac{\sum_{i=1}^{m} (C_{pre} - C_{act})^2}{\sum_{i=1}^{m} C_{act}^{\,2}} \right]^{1/2} \quad (8)$$

where, Cact indicates the actual concentration in the sample, Cpre is the predicted concentration and m is the number of samples in the prediction set.

Some wavelengths contain collinear absorbance data (i.e. the same peak height for analytes) and some have uninformative data. To investigate the prediction ability of the resultant CLS and ILS models and to compare the effect of wavelength selection, the calibrated models were used to determine the analytes in a separate prediction set that did not have contribution in the model building steps. Four

wavelengths and different ternary and binary sets of wavelengths were used to construct the models and predict the concentration of analytes in prediction set. The advantage of the constructed model is likely to be its simplicity, if it can be used with low numbers of wavelengths. As can be shown in Tables 4 and 5, the results for the binary sets of wavelengths are as good as the result of four wavelengths.

Once the method was established the determination of figures of merit (FOM) was important for the validation of the chemometric methods. The FOM, such as sensitivity (SEN), selectivity (SEL) and limit of detection (LOD), can be estimated and used to compare analytical methods [15]. When expressing FOM for multivariate calibration methods, the part of the signal that relates to the analyte is more important than the total signal. This unique signal is termed the net analyte signal and is defined as the part of the signal that is orthogonal to the signal of the interferences present in the sample. The SEL (Eq 9) is a measure, ranging from 0 to 1, of how the unique signal of the analyte can be compared with the other species. It indicates the part of the total signal that is not lost due to spectral overlap, and can be defined in the multivariate context by resorting to NAS calculation [15]:

$$SEL = \frac{\|k_s^*\|}{\|k_s\|} \qquad (9)$$

where || || means the Euclidian norm of vector and is a signal containing analyte s at unit concentration and is its corresponding NAS [16]. On the other hand, the sensitivity measures the changes in response as a function of the concentration of a particular analyte [17]. The formula for the SEN parameter was calculated by the following equation and is given by Eq 10:

$$SEN = \|k_s^*\| \qquad (10)$$

To find the LOD, the following simple equation has been proposed for its estimation [15]:

$$LOD = \frac{3\|\varepsilon\|}{\|k_s^*\|} \qquad (11)$$

where ||ε|| is a measure of the instrumental noise. The value of ||ε|| may be estimated from the standard deviation in the NAS of several blanks.

Estimated FOM's for TMP and PHZ were determined with the CLS and ILS models for different wavelengths (Table 5). Results also indicate that there are not any significant differences between results obtained for ternary and binary wavelength sets with four wavelengths and therefore these results indicate that simple models can be used with binary wavelength sets, to give analyte prediction. But using only two wavelengths when the there are two components gives no degrees of freedom to the model. In other words, the calculated model (that is calculated from only two wavelengths), will include the measurement noise, in the same way that a calibration line fitted with only two standards includes the noise. So it is better to use three or four wavelengths to build model, it is possible to collect these data by usual UV detector without need to diode array detector.

5. CONCLUSION

HPLC as a powerful separation and analysis method is widely used for simultaneous determination of analytes. But there can be problems when two compounds have the identical chromatograms. In this work, CLS and ILS are two simple, but powerful chemometric methods which can be used for simultaneous determination of compounds with the overlapped and identical chromatograms. When it is not possible to resolve of two components by changing or modification of mobile phase, the proposed method is a good alternative. In comparison with second order algorithms the proposed method is simple, rapid and easy to understand and apply.

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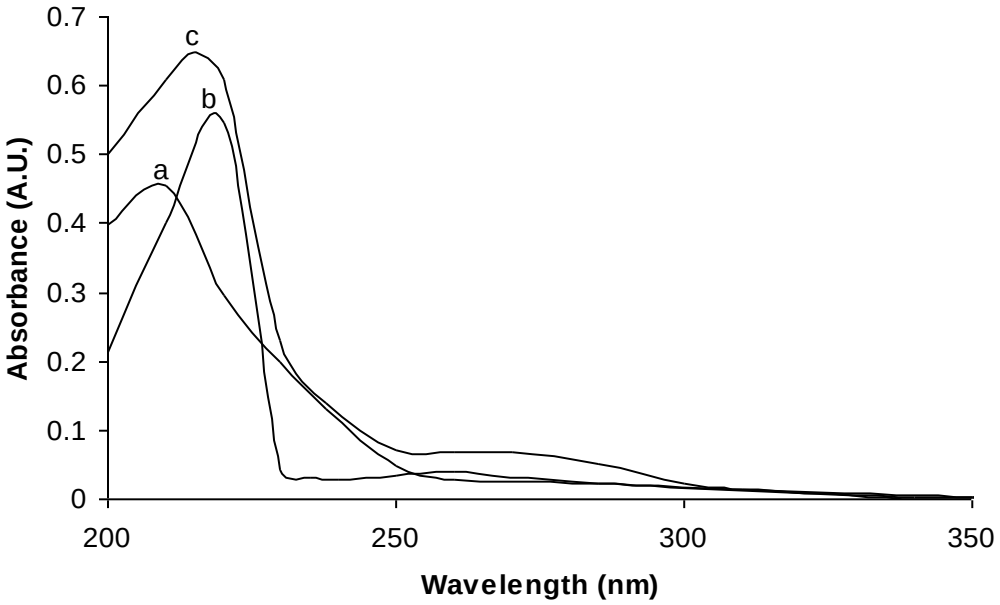

Figure 1. The UV spectra of 2.9 µg mL^{-1} Trimethoprim (TMP) (a), 1.3 µg mL^{-1} Phthalazine (PHZ) (b), and the mixture of these two solutions (c)

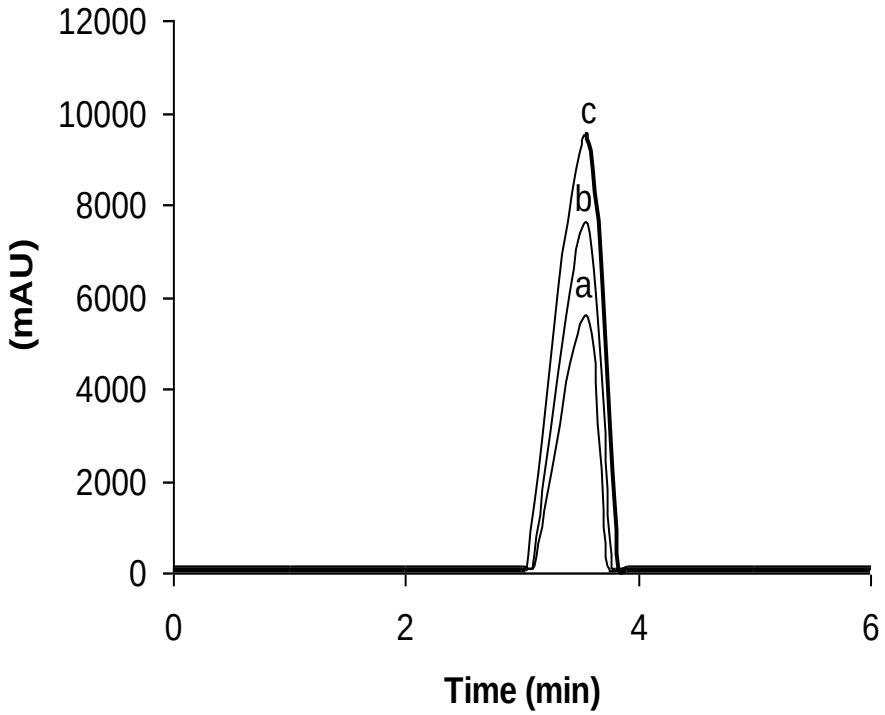

Figure 2. The chromatogram obtained from a standard solution containing 1.45 µg mL^{-1} TMP (a), 0.65 µg mL^{-1} PHZ (b), and their mixture (c)

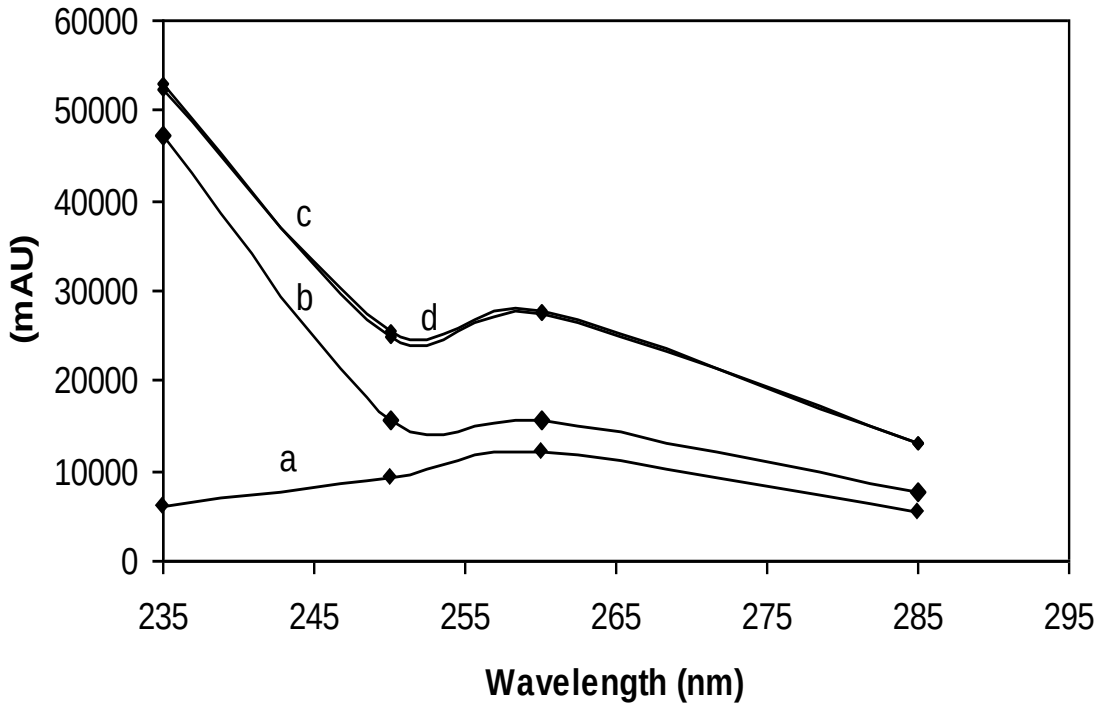

Figure 3. The UV spectra of PHZ (a), TMP (b), a mixture solution of PHZ and TMP(c), and the spectrum obtained from the sum of PHZ and TMP signals at 235-285 nm (d)

Table1. Linear regression analysis, limit of determination (LOD), and limit of quantification (LOQ) for determination of Trimethoprim (TMP) and Phthalazine (PHZ) by HPLC

Drug	λ (nm)	Regrassion equation	R	LOD (μgml^{-1})	LOQ (μgml^{-1})
TMP	235	$A=1.62\times10^{3}C_{TMP}+266$	0.9999	0.002	0.008
	250	$A=5.41\times10^{3}C_{TMP}+48.7$	0.9997	0.003	0.010
	260	$A=5.36\times10^{3}C_{TMP}+107$	0.9999	0.0034	0.011
	285	$A=2.69\times10^{3}C_{TMP}-94.3$	0.9998	0.004	0.014
PHZ					
	235	$A=4.59\times10^{3}C_{PHZ}+33.43$	0.9998	0.017	0.060
	250	$A=7.11\times10^{3}C_{PHZ}-44.1$	0.9997	0.016	0.056
	260	$A=9.43\times10^{3}C_{PHZ}-232$	0.9999	0.015	0.054
	285	$A=4.24\times10^{3}C_{PHZ}-153$	0.9997	0.019	0.061

Table 2. The composition of the calibration samples with four different concentration levels in the dynamic linear range for Trimethoprim (TMP) and Phthalazine (PHZ) obtained from the orthogonal design method

Experiment	TMP	PHZ
1	1.45	0.65
2	1.45	1.30
3	1.45	0.13
4	1.45	0.06
5	2.90	0.65
6	2.90	1.30
7	2.90	0.13
8	2.90	0.06
9	0.29	0.65
10	0.29	1.30
11	0.29	0.13
12	0.29	0.06
13	0.14	0.65
14	0.14	1.30
15	0.14	0.13
16	0.14	0.06

Table 3. The composition of the prediction samples with three different concentration levels in the dynamic linear range for Trimethoprim (TMP) and Phthalazine (PHZ) selected from the calibration sample set[a] for the chemometric analysis

Experiment	TMP	PHZ
1	1.45	0.65
2	1.45	0.13
3	1.45	0.06
4	0.29	0.65
5	0.29	0.13
6	0.29	0.06
7	0.14	0.65
8	0.14	0.13
9	0.14	0.06

[a] From Table 2.

Table 4. Recovery, standard error of prediction (SEP) and relative standard of prediction (REP) data obtained by application of the mixtures for (TMP) and (PHZ)

TMP											
HPLC-CLS											
	A,B,C,D	B,C,D	A,C,D	A,B,D	A,B,C	C,D	B,D	B,C	A,D	A,C	A,B
Recovery(%)	106.12	108.8	106.36	104.51	106.15	106.8	100.87	109.5	106.03	106.34	104.7
SEP(%)	0.18	0.24	0.19	0.14	0.18	0.14	0.20	0.76	0.24	0.19	0.09
REP	0.026	0.027	0.032	0.026	0.028	0.032	0.038	0.073	0.041	0.037	0.025
HPLC-ILS											
	A,B,C,D	B,C,D	A,C,D	A,B,D	A,B,C	C,D	B,D	B,C	A,D	A,C	A,B
Recovery(%)	104.1	104.68	106.91	103.56	103.55	106.95	102.62	108.87	106.85	106.46	104.84
SEP(%)	0.09	0.10	0.14	0.09	0.10	0.14	0.19	0.73	0.23	0.19	0.13
REP	0.036	0.042	0.037	0.032	0.037	0.014	0.038	0.075	0.043	0.037	0.031
PHZ											
HPLC-CLS											
	A,B,C,D	B,C,D	A,C,D	A,B,D	A,B,C	C,D	B,D	B,C	A,D	A,C	A,B
Recovery(%)	102.1	100.2	98.77	109.1	102.71	99.52	110	94.60	94.82	99	108.8
SEP(%)	0.16	0.073	0.07	0.77	0.20	0.05	3.32	0.11	0.93	0.06	1.67
REP	0.009	0.008	0.009	0.023	0.012	0.020	0.066	0.052	0.038	0.036	0.034
HPLC-ILS											
	A,B,C,D	B,C,D	A,C,D	A,B,D	A,B,C	C,D	B,D	B,C	A,D	A,C	A,B
Recovery(%)	97.05	97.01	99.1	101.86	97	99.67	109.80	95.23	96.14	99.14	109.02
SEP(%)	0.05	0.05	0.06	0.37	0.05	0.06	2.95	0.10	0.82	0.07	0.96
REP	0.015	0.011	0.010	0.033	0.015	0.023	0.070	0.056	0.042	0.037	0.036

(235 (A**)**, 250 (B**)**, 260 (C), 285 (D**)** nm).

Table 5. Estimated FOM for TMP and PHZ were determined with the CLS and ILS models for different wavelength

TMP											
HPLC-CLS											
	A,B,C,D	B,C,D	A,C,D	A,B,D	A,B,C	C,D	B,D	B,C	A,D	A,C	A,B
LOD (μgml^{-1})	0.007	0.016	0.007	0.006	0.005	0.020	0.015	0.016	0.006	0.005	0.004
Sensitivity	0.76	0.70	0.71	0.75	0.70	0.72	0.65	0.65	0.71	0.68	0.65
Selectivity	0.08	0.09	0.10	0.08	0.09	0.13	0.08	0.07	0.09	0.08	0.10
HPLC-ILS											
	A,B,C,D	B,C,D	A,C,D	A,B,D	A,B,C	C,D	B,D	B,C	A,D	A,C	A,B
LOD (μgml^{-1})	0.007	0.018	0.009	0.008	0.005	0.021	0.016	0.017	0.008	0.006	0.005
Sensitivity	0.79	0.71	0.76	0.82	0.76	0.75	0.77	0.79	0.73	0.70	0.75
Selectivity	0.13	0.11	0.10	0.10	0.15	0.15	0.13	0.12	0.10	0.13	0.10
PHZ											
HPLC-CLS											
	A,B,C,D	B,C,D	A,C,D	A,B,D	A,B,C	C,D	B,D	B,C	A,D	A,C	A,B
LOD (μgml^{-1})	0.014	0.013	0.015	0.018	0.012	0.014	0.01	0.01	0.02	0.01	0.015
Sensitivity	0.81	0.78	0.82	0.78	0.76	0.83	0.66	0.67	0.71	0.80	0. 82
Selectivity	0.03	0.06	0.03	0.03	0.02	0.04	0.05	0.03	0.04	0.05	0.07
HPLC-ILS											
	A,B,C,D	B,C,D	A,C,D	A,B,D	A,B,C	C,D	B,D	B,C	A,D	A,C	A,B
LOD (μgml^{-1})	0.016	0.015	0.017	0.019	0.014	0.016	0.011	0.05	0.03	0.013	0.017
Sensitivity	0.82	0.78	0.85	0.80	0.79	0.83	0.67	0.67	0.73	0.80	0.83
Selectivity	0.04	0.07	0.03	0.04	0.02	0.05	0.05	0.05	0.05	0.06	0.07

(235 (A)**,** 250 (B)**,** 260 (C), 285 (D**)** nm).