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Chromatographic multivariate quality control of pharmaceuticals giving strongly overlapped peaks based on the chromatogram profile

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Abstract

In the present paper, the simultaneous quantification of two analytes showing strongly overlapped chromatographic peaks ($\alpha = 1.02$), under the assumption that both available equipment and training of the laboratory staff are basic, is studied. A pharmaceutical preparation (Mutabase) containing two drugs of similar physicochemical properties (amitriptyline and perphenazine) is selected as case of study. The assays are carried out under realistic working conditions (i.e. routine testing laboratories). Uncertainty considerations are introduced in the study. A partial least squares model is directly applied to the chromatographic data (with no previous signal transformation) to perform quality control of the pharmaceutical formulation. Under the adequate protocol, the relative error in prediction of analytes is within the tolerances found in the pharmacopeia (10%). For spiked samples simulating formulation mistakes, the errors found have the same magnitude and sign to those provoked.
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1. Introduction

A usual problem in chromatography is the presence of overlapped peaks in the chromatogram. Different strategies to solve the problem of overlapped peaks have been proposed in the literature. Unluckily, they pay little attention to uncertainty, contrary to the actual tendencies in quality assurance of testing laboratory [1,2]. Moreover, some of the proposed solutions are too complex for the staff qualification in most routine testing laboratory (quality managers and/or analysts). Finally, many papers deal with the resolution of partially overlapped peaks but the proposed strategies could fail when a higher overlapping degree is present.

The easiest way to undertake this problem consists on increasing the resolution to achieve experimentally a complete separation ('baseline resolution') of the peaks (i.e. derivatization, pH change, etc. . .). When this option is not feasible, a solution could be to take advantage of the spectral differences between the co-eluting compounds, combined with multivariate models or other algorithms to process this two-dimensional (2D) information (time and spectral domains) [3,4]. However, this option has five practical limitations: (i) The degree of spectral resolution between the analytes. (ii) The availability of a detector, which allows acquiring spectral information during the elution process. Such detectors are more expensive than the conventional ones and they could not be available in several testing laboratories. (iii) The staff qualification degree to deal with such bi-dimensional information. (iv) In quality control schemes, the mandatory accumulation of large amounts of 2D data becomes a problem. (v) The magnitude of the uncertainty contribution due to the use of 2D data is difficult to evaluate.

In many cases, it is necessary to solve this situation within the time domain [one-dimensional (1D) information]. Different approaches have been proposed in the bibliography based on signal transformation, like for example deconvolution of the peaks using a given mathematical function [5] or mathematical calculations on the chromatographic data (i.e. perpendicular drop area [6]). The first strategy implies the selection of the adequate mathematical function from the wide variety of proposals in the literature. The magnitude of

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the uncertainty contribution due to computational effect is difficult to evaluate. Finally, this approach requires a particular qualification. The second strategy may also increase the uncertainty and could be unreliable for a severe overlapping degree.

In multicomponent spectral analysis, it is possible to deal with very low spectral resolution problems by using multivariate models [i.e. principal component analysis (PCA) or partial least squares (PLS)] [7]. The robustness of a spectrum (spectral domain) is usually higher than the robustness of a chromatographic profile (time domain); consequently, comparable results cannot be expected. However, the direct multivariate treatment of the chromatographic profile offers an alternative approach for the simultaneous determination of two analytes showing chromatographic peaks with an extreme overlapping degree. This approach shows several advantages compared with those previously mentioned: (i) It is simple (there is no need of signal transformation), (ii) it is conceptually recognizable for the staff (the chromatographic signal is treated as an spectrum), (iii) it employs well-known and wide-spread calibration models (i.e. PLS) which can be used by the analysts with minimum training.

The goal of this paper is to evaluate the capability and usefulness of this methodology for the quality control of pharmaceuticals in a testing laboratory under a realistic routine testing scheme (i.e. minimum experimental effort). Using 1D information, the selectivity is the most critic aspect. In order to cover all possible situations, an extreme overlapping degree (i.e. selectivity factor, α, close to 1) was selected. In such case, a minimization of the sources of signal uncertainty as well as a strict quality control protocol is a key point to avoid errors.

2. Experimental

2.1. Instrumental and measurements

A Hewlett-Packard HP 1100 chromatograph with an isocratic pump, an UV–visible detector (variable-wavelength detector), a column thermostat and an autosampler with a 20 μl loop were used. Data acquisition and processing were performed by means of HP Vectra XM computer (Amsterdam, The Netherlands) equipped with HP-Chemstation software (A.07.01 [682]). A Kromasil C_{18} column (5 μm, 150 mm × 4.6 mm i.d.; Scharlab, Barcelona, Spain) and a guard column of similar characteristics (5 μm, 35 mm × 4.6 mm i.d.; Scharlab) were used. The mobile phase flow rate was 1.0 ml min^{-1}, the detection was performed in UV at 254 nm and the column temperature was kept at 25 °C for all assays.

2.2. Reagents and standards

A mobile phase of 0.04 M cetyltrimethylammonium bromide (CTAB) (pH 3.37) + 5% 1-butanol was prepared by dissolving CTAB (99%; Acros Organics, Geel, Belgium) in 0.05 M citrate buffer solution by means of a magnetic stirrer and after that, an appropriate amount of 1-butanol (reagent grade; Scharlab) was added. The buffer solution was prepared using sodium citrate (European Pharmacopoeia; Guinama, Valencia, Spain) and the appropriate amount of hydrochloric acid (for analysis; Merck, Darmstadt, Germany). The pH of the micellar mobile phase was adjusted to 3.37 before adding the alcohol.

Standards of perphenazine and thioridazine were obtained from Sigma (St. Louis, MO, USA) and amitriptyline from Guinama. Stock standard solutions (500 mg l^{-1}) were prepared by dissolving 50 mg of the analytes in 100 ml of mobile phase solution. Working solutions were prepared by dilution of the stock standard solutions using the mobile phase solution. Every working solution contained a fixed amount (20 mg l^{-1}) of thioridazine that was used as internal standard. The solutions were stored under refrigeration at 5 °C.

Barnstead E-pure, deionized water (Sybron, Boston, MA, USA) was used throughout. The mobile phase and the solutions injected into the chromatograph were vacuum-filtered through 0.45 μm nylon membranes (Micron Separations, Westboro, MA, USA).

2.3. Sample preparation

The sample selected in this study was Mutabase 2–10 (Schering-Plough, Madrid, Spain), a pharmaceutical preparation commercialised in Spain. This formulation contains 2 and 10 mg per tablet of perphenazine and amitriptyline, respectively. For the analysis of this sample, five tablets were weighed, ground in a mortar and finally an adequate amount of the solid was taken and dissolved in mobile phase solution with magnetic stirring. An appropriate amount of sample solution was taken and diluted in the mobile phase solution so that the analytes concentration in the working solutions was located in the middle of the calibration design. All sample solutions contained a fixed amount (20 mg l^{-1}) of thioridazine as internal standard.

2.4. Nomenclature

Scalars are represented by italics. Column vectors are denoted by boldface lowercase letters and all matrices are represented by boldface uppercase letters. Chromatographic data were arranged into matrices ($\mathbf{X}$) as well as the corresponding concentration of both analytes ($\mathbf{Y}$).

2.5. Experimental design and data treatment

Table 1 shows the experimental design. As can be observed, two series of experiments were performed in different days (S1 and S2). The working solutions were classified into three categories: 'Cal' and 'Ref', whose concentrations are known (there are $\mathbf{Y}_{Cal}$ and $\mathbf{Y}_{Ref}$ matrices associated with the chromatograms $\mathbf{X}_{Cal}$ and $\mathbf{X}_{Ref}$, respectively), and 'Test',

Table 1
Experimental design

Series[a]	Category[b]	Solution type	cl[c]	*Nr*[d]	Code[e]
S1	Cal	Calibration standard	1·1	2	S1-Cal-1, S1-Cal-2
			3·3	2	S1-Cal-3, S1-Cal-4
			3·1	2	S1-Cal-5, S1-Cal-6
			1·3	2	S1-Cal-7, S1-Cal-8
	Ref	Verification standard	2·2	7	S1-Ref-1 to S1-Ref-7
		Control sample	2·2	7	S1-Ref-8 to S1-Ref-14
		Spiked standard	2·3	2	S1-Ref-15, S1-Ref-16
	Test	Sample 1	(2·2)	2	S1-Test-1, S1-Test-2
		Sample 2	(2·2)	2	S1-Test-3, S1-Test-4
		Sample 3	(2·2)	2	S1-Test-5, S1-Test-6
		Spiked sample 1	(2·3)	2	S1-Test-7, S1-Test-8
		Spiked sample 2	(2·3)	2	S1-Test-9, S1-Test-10
S2	Cal	Calibration standard	1·1	2	S2-Cal-9, S2-Cal-10
			3·3	2	S2-Cal-11, S2-Cal-12
			3·1	2	S2-Cal-13, S2-Cal-14
			1·3	2	S2-Cal-15, S2-Cal-16
	Ref	Verification standard	2·2	2	S2-Ref-17, S2-Ref-18
		Control sample	2·2	2	S2-Ref-19, S2-Ref-20
		Spiked standard	2·3	2	S2-Ref-21, S2-Ref-22
	Test	Sample 1	(2·2)	2	S2-Test-11, S2-Test-12
		Sample 2	(2·2)	2	S2-Test-13, S2-Test-14
		Sample 3	(2·2)	2	S2-Test-15, S2-Test-16
		Spiked sample 1	(2·3)	2	S2-Test-17, S2-Test-18
		Spiked sample 2	(2·3)	2	S2-Test-19, S2-Test-20

[a] Working series, S1 and S2, were performed in different days.
[b] Cal: synthetic mixtures containing both analytes that are used in the calibration step to construct the models; Ref: synthetic mixtures containing both analytes that are not used in the calibration step, they are used to verify (at the beginning of a working session) and control (control sample during a working session) the method; Test: sample solutions.
[c] Concentration level coded as 1–3 corresponding to 80, 100 and 120%, respectively, of the analyte amounts in the sample declared by the manufacturer. The first cl number corresponds to amitriptyline (major component) and the second one to perphenazine (minor component). For samples, the cl (in parenthesis) was according to the declared amounts in the pharmaceutical preparation.
[d] Number of replicas (injections) of each working solution
[e] Code assigned to every injection according to its series, category and a number, *n*.

whose concentrations are unknown. In each category, different solution types can be found depending on their role in the quality control protocol. The analyte concentration levels (cl) assayed, which correspond to 80, 100 and 120% of the amounts declared by the manufacturer, agree with the recommended minimum range for drug tests in pharmaceuticals [8].

For test sample solutions, the analyte quantities declared were used as ‘target values’ (i.e. $\mathbf{Y}_{\mathrm{Test}}$ matrix). From the quality control point of view, the identification of a well-prepared sample as ‘in-control’ is as important as the detection of a badly-prepared sample as ‘out-of-control’. In order to simulate an error in the pharmaceutical preparation, one reference standard and two test sample solutions (initial cl = 2·2) were spiked with perphenazine stock standard solution so that the analytes concentrations correspond to 100% for amitriptyline and 120% for perphenazine (final cl = 2·3).

The $\mathbf{Y}$ matrices containing the vectors corresponding to the amitriptyline and perphenazine concentrations in the working solutions and accounting for *Nr* values were $\mathbf{Y}_{\mathrm{Cal}}(16 \times 2)$, $\mathbf{Y}_{\mathrm{Ref}}(22 \times 2)$ and $\mathbf{Y}_{\mathrm{Test}}(20 \times 2)$. Once chromatographic data were recorded, a time-window of 701 data per chromatogram containing the analytes and internal standard peaks was selected resulting in three $\mathbf{X}$ matrices, $\mathbf{X}_{\mathrm{Cal}}(16 \times 701)$, $\mathbf{X}_{\mathrm{Ref}}(22 \times 701)$ and $\mathbf{X}_{\mathrm{Test}}(20 \times 701)$.

In order to minimize the impact of particular sources of variation that can affect the results, different signal corrections (see Table 2) were tested. All possible treatment combinations (including the case of no treatment) were tested. After any correction, the signal of the internal standard was eliminated selecting a time-window (285 data), which includes the signal of the analytes. The new $\mathbf{X}_{\mathrm{Cal}}(16 \times 285)$ data were column centered, respect to the $\mathbf{X}_{\mathrm{Cal}}$ vector of means. $\mathbf{X}_{\mathrm{Ref}}(22 \times 285)$ and $\mathbf{X}_{\mathrm{Test}}(20 \times 285)$ matrices were scaled using the previously determined $\mathbf{X}_{\mathrm{Cal}}$ vector of means prior multivariate calculations. All the computational work was performed with laboratory-made Matlab subroutines (Matlab Ver. 5.3.0.10183 (R11), Mathworks, Natick, MA, USA).

Table 2
Summary of some relevant sources of uncertainty related to a multi-analyte chromatographic method with overlapped peaks

Source	Treatment in this work and comments
Baseline	Each chromatogram is moved along the signal axis so that its initial signal is zero.
Retention, i.e. retention time	Each chromatogram is moved along the time axis so that the peak maximum of the internal standard coincides with the corresponding to the S1-Cal-4[a] injection.
Signal size, i.e. peak height	The signal of each chromatogram is divided by the maximum signal of the internal standard peak.
Repeatability (inter-injections)	The influence of using $Nr = 2$ or $Nr = 7$ replicates is proved. Although low Nr values do not usually provide consistent results, testing laboratories use them for practical purposes.
Intermediate precision (inter-day[b])	The chromatograms of one series (i.e. day 2) are standardized by means DS[c] or PDS[d], to be assimilated with the chromatograms of the first series (i.e. day 1). The transference standards to obtain the transference matrix are S1-Cal-n[a] (n = 1, 3, 5 and 7). These treatments require staff's special qualification.
Computational effect (i.e. data adjusted to a function)	The chromatograms are not adjusted to a mathematical function, so the individual contribution of each chromatogram to uncertainty is eliminated. Probably, a PLS[e] model, due to its flexibility and absence of co-linearity problems, supposes an important reduction in the uncertainty respect to classic models (i.e. MLR[f]).

Note: Systematic peak changes due to coelution and peak widening could be modelled by PLS improving the selectivity, except in the case of irreproducible results. In the last case (non-controlled effects), they contribute to the sources of uncertainty related to precision (repeatability and intermediate precision) and can cause punctual out-of control situations. Other general factors should be considered in addition to these sources of uncertainty, i.e. sampling, storage (stability), sample preparation, instrumental effects (i.e. equipment calibration, temperature control device), laboratory conditions (i.e. temperature, humidity), analyte–matrix effect (i.e. matrix effect, fortification), data interpolation into a calibration model (computational effect), or operator effect.

[a] See Table 1 for code.
[b] The concept intermediate precision could also include equipment and operator changes in routine work.
[c] DS: direct standardization (multivariate standardization approach).
[d] PDS: piecewise direct standardization (multivariate standardization approach).
[e] PLS: partial least squares (multivariate regression model).
[f] MLR: multiple linear regression (multivariate regression model).

3. Results and discussion

Nowadays, quality standards recommend the inclusion of result uncertainty in the report of an assay. For some complex methods, difficulties on the uncertainty estimations have been recognized. In these cases, identifying the major components of uncertainty has been recommended [1]. Table 2 shows some sources of variation associated to the case of overlapped chromatographic peaks together with other general uncertainty sources [2]. Changes in baseline, retention, signal size, peak widening and coelution effects (inter-injections and inter-days), could greatly contribute to the repeatability and intermediate precision and therefore to the uncertainty. Therefore, prior to modelling the **X**–**Y** relationship it is convenient to identify and evaluate the impact of the sources of uncertainty affecting the **X** data.

3.1. Exploratory analysis

Fig. 1 shows the overlaid chromatograms of injections of pure standards of amitriptyline and perphenazine, as well as the chromatogram of a synthetic mixture of both analytes with internal standard. Analytes elute around 5.5 and 5.56 min while the internal standard elutes around 7.2 min. As can be seen, there is an extreme overlap between the peaks of analytes in the mixture under the chromatographic conditions assayed. The α-value, calculated from the separate injections of the analytes, was slightly lower than 1.02. Fig. 2a and b show the original data (Cal, Ref and Test) for the working sessions, S1 and S2, respectively. Fig. 2a shows three groups of chromatograms in the analytes signal, which points out their different concentration. The heterogeneity observed in the internal standard peak reveals the contribution of the inter-injection differences (repeatability effect), which could be affected by some other factors shown in Table 2. Fig. 2b shows an extreme case of signal displacement along the time axis (retention effect). Fig. 2c shows the

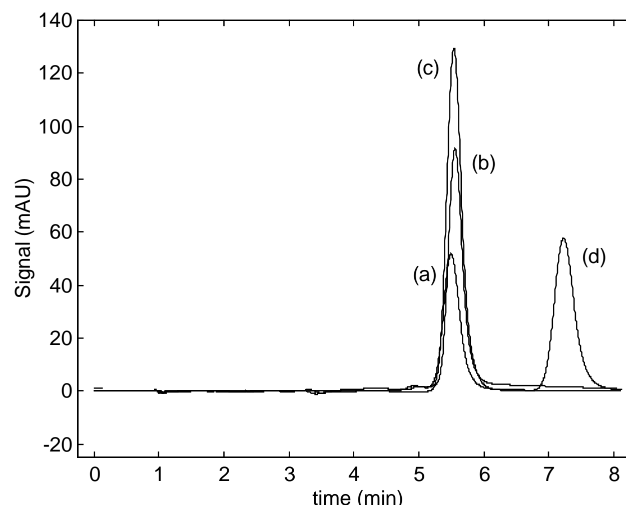

Fig. 1. Overlaid chromatograms of injections of pure standards of (a) $10\,mg\,l^{-1}$ perphenazine, (b) $50\,mg\,l^{-1}$ amitriptyline and (c) a synthetic mixture of both analytes at the above concentrations with (d) $20\,mg\,l^{-1}$ of thioridazine (internal standard).

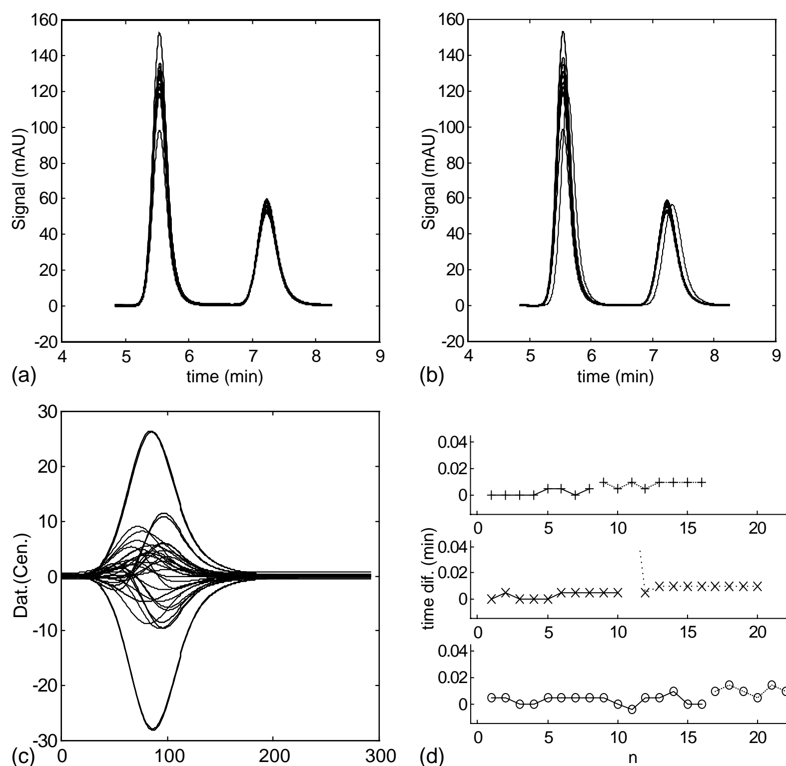

Fig. 2. (a) S1 and (b) S2 original chromatographic data after selecting a time-window of 701 data per chromatogram. (c) S1 column mean centered data, Dat. (Cen.), after removing the signal corresponding to the internal standard, resulting in 285 variables, Var., per chromatogram. (d) Differences between the retention times, time dif., of the internal standard in series S1 (—) and S2 (- - -), for Cal (+), Ref (×) and Test (○) chromatograms, respect to S1-Cal-4 chromatogram. n indicates the number used in the code (Table 1).

S1 column centered data after removing the internal standard peak in Fig. 2a. As can be observed, there are retention displacements mainly for the central group of chromatograms. It also reveals differences in the chromatogram baselines, which also contributes to uncertainty. Fig. 2d shows the differences in the internal standard retention time of all the chromatograms respect to the corresponding one in S1-Cal-4 chromatogram. These differences are higher for the S2 chromatograms (particularly for S2-Test-11, 0.097 min) than for S1, which points out the contribution of the intermediate precision (inter-day effect) to uncertainty.

Fig. 3a shows the concentrations of amitriptyline (Analyte 1) and perphenazine (Analyte 2) in the Cal and Ref (as well as the spiked Ref) working solutions. It reflects the structure of the **Y** data (experimental design; Table 1). Fig. 3b shows the chromatograms of some S1-Cal solutions. The chromatograms related to a proportional increase in the concentration of both analytes (cl = 1·1 and 3·3) have signal sizes markedly different ('overall concentration' effect). The signal differences between the chromatograms related to a change in the relative concentration of analytes (cl = 1·3 and 3·1) are less important ('selectivity' effect). However, small displacements along the time axis between chromatograms of cl = 1·3 and 3·1 probably benefits selectivity.

In order to analyse the structure of the **X** data, a PCA model was built with the S1-Cal-n data. Table 3a shows the explained variance (EV) by the principal components (PCs). Fig. 3c shows the score plot (t_2 versus t_1) for the first two PCs. S1-Cal scores (+) show a distribution into the t_2 versus t_1 space related to the concentration map of Fig. 3a, which implies that **X** data are related to **Y** data. In Fig. 3c, the points '+1' and '+3' (cl = 1·1 and 3·3, respectively) define t_1-axis, therefore t_1 (EV = 97.9%) mainly represents the 'overall concentration' factor. On the other hand, the points '+5' and '+7' (cl = 3·1 and 1·3, respectively) define t_2-axis, therefore and t_2 mainly represents the 'selectivity' factor. The variance associated with t_2 (EV = 2.09%) suggests little information of **X** related to the selectivity. In addition, repeatability effect can also be observed in PC2 (i.e. duplicate S1-Cal scores (+) do not coincide). Fig. 3c also shows the inter-day effect (intermediate precision) by comparing S1-Cal (+) and the interpolated S2-Cal (×) scores. S2-Cal scores show a distortion in the latent structure respect to S1-Cal scores and also a larger dispersion between duplicate scores.

Fig. 3d shows the Q–T^2 plot [7] for the 2 PCs-PCA model calculated using S1-Cal data (+), where S2-Cal data have been interpolated (×). As can be observed, all the S1-Cal (and some S2-Cal) points are within the Q–T^2 space at the 95% confidence level. In contrast, S2-Cal-n (n = 11, 13, 14, 15 and 16) points are above this limit, pointing out the relative importance of the inter-day effect.

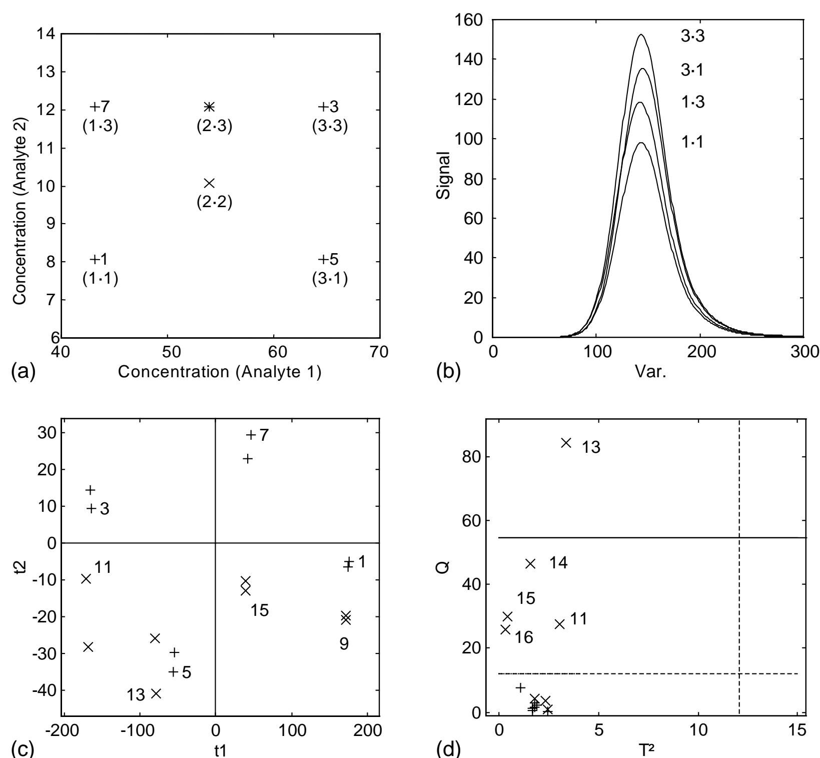

Fig. 3. (a) Concentration values of analytes 1 and 2 (ppm, in the working solutions) corresponding to the Cal (+; $n = 1, 3, 5$ and 7), Ref (×) and spiked Ref standards (✱). The corresponding cl values (Table 1) are indicated between brackets. (b) Chromatograms showing the region of elution of analytes with cl 1·1, 1·3, 3·1 and 3·3 (S1-Cal-n; $n = 1, 7, 5$ and 3, respectively). (c) Score plot for the first two PCs of the PCA model calculated with S1-Cal (+) data where S2-Cal values (×) have been interpolated. For clarity, only odd n labels are shown. (d) Q vs. T^2 plot for a 2PCs-PCA model calculated with the S1-Cal Data (+), where S2-Cal values (×) have been interpolated. Confidence levels: 95% (- - -) and 99.7% (—).

S2-Cal-13 and S2-Cal-14 show the highest Q residual, which could be related to the higher displacement in the t_1-axis respect to S1-Cal-5 and S1-Cal-6 data (see Fig. 3c).

The results of the exploratory analysis suggest the need of including some correction in **X** data, as those proposed in Table 2, prior to model their relation to **Y** data via PLS. They also indicate that, as expected, the intermediate precision contribution is higher than the repeatability contribution. For this reason, we established an approach based on the accumulation of the data obtained from different series to build a unique model (incorporating the inter-day effect) instead of using each series to build independent models. This strategy also allows the control of the calibration standards in routine testing, since the calibration standards of one series (day) can be compared with those obtained in other series (but in the same model, not after interpolation).

Table 3a shows the results obtained when both S1-Cal-n and S2-Cal-n data where used together to construct the PCA model, which can be compared with those obtained by the PCA model based on S1-Cal-n data. After incorporating the S2-Cal-n information, some changes in the explained variance of t_1, t_2 and t_3 were observed. These differences can be attributed to the intermediate precision contribution.

3.2. Regression analysis

A regression analysis of the **X**–**Y** data based on a PLS2 model (the concentrations of two analytes are modelled at the same time) was performed using the S1-Cal and S2-Cal data together. A priori, the number of factors (nf) expected is two (the same PLS latent variables (LVs) as analytes). However, when the data from more than one series are jointly used, a large nf value could be expected, due to the inter-series effect. Table 3b shows the results obtained when the original Cal data (without any signal correction) were used. Some relevant observations can be obtained: (i) the $EV_i(\mathbf{X})$ values obtained with PLS are the same than those found with PCA, which implies that the latent structure of **X** directly reflects the structure of **Y**. (ii) Although the $EV_i(\mathbf{X})$ values are very low for nf $= 4$ and 5, the minimum cumulative predicted residual error sum of squares (PRESS) was found at nf $= 5$, suggesting a lack of robustness of this PLS model. Table 3c shows the results obtained when baseline and retention corrections were performed according to Table 2. In this case, the minimum cumulative PRESS was obtained at nf $= 4$ and it was almost stabilized at nf $= 3$, showing a higher robustness of the new PLS model.

These results are related to the calibration step (Cal data). At this point, it would be convenient to evaluate the prediction step (Ref and Test data). The interpolation of Ref and

Table 3
Explained variances, individual (EV_i) or accumulated (EV_t) for different PCA and PLS2 models as a function of the number of factors (nf; PCs and LVs, respectively) used

nf	**X** (S1-Cal-*n*)		**X** (S1-Cal-*n* + S2-Cal-*n*)	
	EV_i (%)	EV_t (%)	EV_i (%)	EV_t (%)
(a) PCA results obtained using S1-Cal-*n* data or S1-Cal-*n* and S2-Cal data together				
1	97.9	97.9	97.53	97.53
2	2.09	99.99	2.42	99 95
3	0.01	100.00	0.04	99.99
4	0.00	100.00	0.01	100.00
5	0.00	100.00	0.00	100.00
	X		**Y**	
nf	EV_i (%)	EV_t (%)	EV_i (%)	EV_t (%)
(b) PLS2 results obtained using S1-Cal-*n* and S2-Cal data together as calibration data[a]				
1	97.53	97.53	77.23	77.23
2	2.42	99.95	10.64	87.88
3	0.04	99.99	4.71	92.59
4	0.01	100.00	3.02	95.61
5	0.00	100.00	3.50	99.11
(c) As in (b) with baseline and retention correction of **X** data[b]				
1	98.37	98.37	76.79	76.79
2	1.59	99.96	14.37	91.16
3	0.03	99.99	6.63	97.79
4	0.01	100.00	0.83	98.61
5	0.00	100.00	0.61	99.22
(d) As in (c) after removing S2-Cal-13 and S2-Cal-14 data from the model[c]				
1	98.35	98.35	78.13	78.13
2	1.62	99.97	17.82	95.95
3	0.02	100.00	2.87	98.82
4	0.00	100.00	0.60	99.42
5	0.00	100.00	0.13	99.56

In all cases, column mean centered data have been used.
[a] Minimum cumulative PRESS at nf = 5.
[b] Minimum cumulative PRESS at nf = 4 (virtually stabilized at nf = 3).
[c] PRESS stabilized at nf = 3.

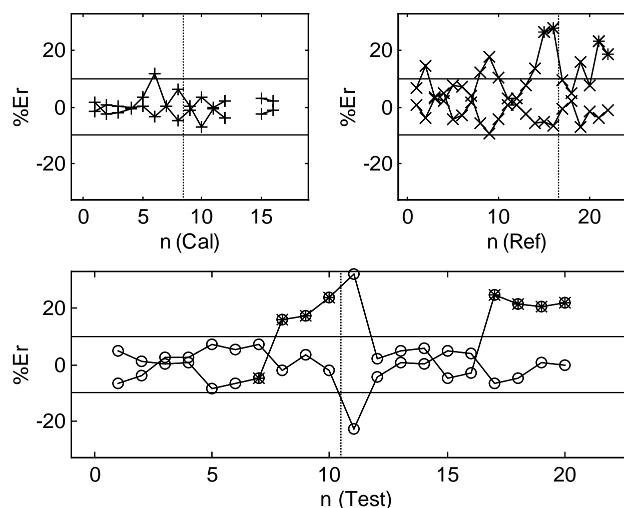

Fig. 4. PLS control chart for S1 and S2 data (separated by a vertical dashed line) in Table 3d conditions. 'Cal-chart' (+) data were used to construct the model. 'Ref-chart' (×) and 'Test-chart' (○) data were interpolated into the model. For the spiked data the symbol (✱) was superimposed.

Test chromatograms into the PLS2 model allows the prediction of the concentrations of analytes in the corresponding solutions. In order to accommodate the results to a quality control scheme, it is convenient to compare the predictions with some kind of control limits.

3.3. Multivariate quality control

The use of legal limits could be preferable to statistical control limits. For instance, the USP XXIII Pharmacopoeia [9] establishes tolerances (±10%) in terms of relative error in percentage (%Er). Therefore, %Er values were computed for all predictions based on PLS. Fig. 4 shows a particular control chart based on PLS2 results in Table 3d conditions, where %Er is used as control variable. When new series are incorporated to the PLS model, the 'Cal-chart' can be used to check if any standard (or replica) should be removed. The 'Ref-Chart' can be used to control the method performance, at the beginning (verification standards) and during the working session (control standards). In addition, the spiked standard (here with perphenazine; the minority analyte) also, checks the ability of the method to detect out-of-control situations. The 'Test-chart' can be used to check the pharmaceutical formulation preparation (real sample). Out-of-control situations can be regarded primarily as an error in sample preparation, particularly if the method has been declared globally in-control based on Ref results.

The control charts results (not shown) obtained in Table 3b conditions (no signal correction) were unsatisfactory, which agrees with conclusions obtained during the exploratory analysis and confirms the need of introducing signal corrections prior to PLS regression. The results (not shown) obtained after applying two of the corrections (baseline and retention) proposed in Table 2 (Table 3c conditions), confirmed the benefits of signal correction. However two standards (S2-Cal-13 and S2-Cal-14) used in model calibration, had %Er values around 15%. This suggests either, that their preparation or their signals are not consistent with the other Cal standards. These results are consistent with those found in Fig. 3c and d. Since the Ref and Test results depend on the quality of the calibration standards, it could be convenient to remove them and recalculate the PLS model to check their influence on the results. This procedure can be performed without affecting the calibration design structure (i.e. Fig. 3a) since equivalent solutions to those eliminated are used in the calibration step from S1 series (S1-Cal-5 and S1-Cal-6). This fact is an intrinsic advantage of the accumulative series approach proposed.

Table 3d shows the EV values obtained for the PLS2 model recalculated after eliminating S2-Cal-13 and

Table 4
Report and incidences respect to the results of Fig. 4

Solution type[a]	Report[b]	Incidences
Calibration standard	Method calibration: correct	One Cal standard (S1-Cal-6) showed Er ∼10% (its replica, S1-Cal-5 is in-control)
Verification standard	Method verification: correct	One (S1-Ref-2) of the seven (S1-Ref-1 to S1-Ref-7) replicas showed Er > 10%
Control sample	Control of the method: globally in-control	Three (S1-Ref-8, S1-Ref-9 and S1-Ref-14) of the seven injections showed Er > 10% (punctual out-of-control situations). One (S2-Ref-19) of the two injections showed Er > 10% (punctual out-of-control situation)
Spiked standard	Error detection (fortification of analyte 2): positive	No incidences
Samples (1–3)	Control of samples: correct	One (S2-Test-11) of the two replicas of sample 1 showed |Er|>10% for both analytes. This result may be affected by the reported out-of-control situation (S2-Ref-19)
Spiked samples (1 and 2)	Error detection (fortification of analyte 2): positive	One (S1-Test-7) of the two replicas of spiked sample 1 showed an Er < 10% (error not detected). This result may be affected by the reported out-of-control situation (S1-Ref-14)

[a] See Table 1.
[b] Decisions based on global results (particular exceptions are included in incidences).

S2-Cal-14 data. As can be observed, the most important changes were an increase of $EV_i(\mathbf{Y})$ for $nf = 2$, a decrease of $EV_i(\mathbf{Y})$ for nf $= 3$ and the stabilization of the PRESS statistic at nf $= 3$, indicating an increase of the quality of the model. Fig. 4 shows the control charts obtained after applying baseline and retention correction and eliminating the anomalous Cal standards (Table 3d conditions). Table 4 shows a possible report based on these results. The global conclusions indicate that the results are satisfactory respect to the objectives proposed, despite the intrinsic difficulties of the case study proposed here. However, some particular observations (incidences) deserve more attention. For instance, three out-of-control situations in the series of seven injections of the control sample (S1 series; $Nr = 7$) could be considered acceptable from the internal criteria of a routine testing laboratory, but could cause problems in the case of using just $Nr = 2$ (as in S2 series). Other punctual incidences reported in Table 4 (i.e. S1-Cal-6 or S1-Ref-2 cases) indicate that occasional errors (probably due to the chromatographic signal) can be expected under the present working conditions.

A practical solution for a routine laboratory could be to perform duplicate measurements (a minimum experimental effort planning), increasing the measurements (replicas or new standards or control solutions) only in the case of unexpected results, before declaring the method as out-of-control (this practice is common in internal quality control protocols used by routine testing laboratories).

A significant result of Table 4 is the positive error detection of spiked standards (Ref) and samples (Test). In addition, the %Er values of analyte 2 were around +20% (excepting for S1-Test-7), which coincides with the introduced error. This fact is very important from the point of view of a satisfactory quality control of pharmaceuticals.

3.4. Final remarks

Table 3d conditions summarizes the optimal conditions to perform multivariate quality control in the present study based on a 3LVs-PLS2 model. In order to evaluate the information accounted by these three latent variables, the PLS score plots for Cal data (t_2 versus t_1 and t_3 versus t_1; Fig. 5)

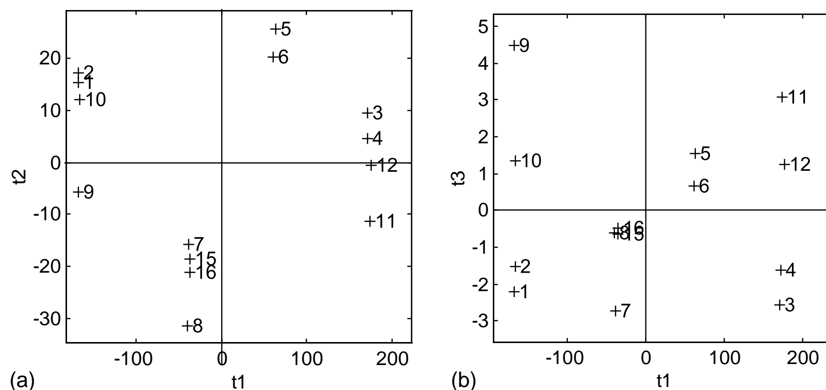

Fig. 5. Score plots of the PLS2 model for the first three LVs (Table 3d conditions). (a) t_2 vs. t_1 and (b) t_3 vs. t_1 plots.

were examined. These plots indicate that t_1 corresponds to the 'overall concentration' of the analytes in the pharmaceutical preparation, t_2 primarily represents the 'selectivity' of the method for both analytes and t_3 mainly represents the inter-series effect, associated with the simultaneous use of the S1 and S2 data in the calibration step. These results suggest that the sources of variation contributing to the success of the present strategy are incorporated into the model while most of the uncontrolled variation has been filtered out due to signal correction and latent variables selection in PLS modellization. They also suggest that the main sources of uncertainty have been sufficiently controlled.

The results in Fig. 4 and Table 4 have been obtained after applying two of the corrections (baseline and retention) proposed in Table 2. With the actual data, the retention has been really the important factor, improving noticeably the global results respect to those obtained with the original data, although it was not sufficient to adjust completely the extreme time displacement in the S2-Test-11 case (i.e. Figs. 2b, d and 4), as reported in Table 4. The baseline correction did not markedly affect the actual results but we recommend its inclusion to prevent future situations related to baseline displacements. On the contrary, the signal size correction proposed in Table 2 provided worse PLS results, for this reason it is not recommended. Finally, the multivariate standardization techniques (DS or PDS) provided non-controlled effects, improving the %Er values in some cases but worsening them in others. In addition, the use of these tools is difficult and requires a special staff qualification. Therefore, we do not recommend these treatments in the present case.

3.5. Quality control protocol

The present study permits to suggest a correct protocol for quality control, which incorporates the control on the uncertainty components:

(I) Initial study:
- (I1) Prepare calibration, reference and test (real sample) solutions (i.e. Cal, Ref and Test solutions as in S1 Series in Table 1).
- (I2) Obtain the corresponding chromatograms. Store original 1D data according to the current standard operating protocol (SOP) to assure traceability. Select a time-window that contains analytes signal and internal standard peak as working data.
- (I3) Correct baseline (i.e. as in Table 2).
- (I4) Correct retention time (i.e. as in Table 2).
- (I5) Select a time-window including the analytes signal (eliminating the internal standard peak; i.e. the limits can be automatically selected at time values in which the signal is 1000 times lower than the maximum signal of one of the chromatograms).
- (I6) Apply mean column centering to the $\mathbf{X}_{\text{Cal}}$ data and scale $\mathbf{X}_{\text{Ref}}$ and $\mathbf{X}_{\text{Test}}$ data (respect to the $\mathbf{X}_{\text{Cal}}$ vector of means).
- (I7) Relate $\mathbf{X}_{\text{Cal}}$–$\mathbf{Y}_{\text{Cal}}$ data by PLS2. Optionally, two checks are recommended: verify that two or three LVs give stabilization of PRESS and that t_1 versus t_2 for Cal samples (i.e. Fig. 5a) reflects the concentration map associated with the experimental design (i.e. Fig. 3a).
- (I8) Predict Cal, Ref and Test $\mathbf{Y}$ values based on PLS model and compute the %Er values.
- (I9) Plot 'Cal-chart', 'Ref-chart' and 'Test-chart' (if a target value for the sample is available) and decide if the system is in-control (applying current laboratory rules), otherwise, verify equipment and method. If the system is declared as in-control:

(II) Control phase (i.e. for new sessions):
- (II1) Repeat steps (I1) and (I2) with new solutions (i.e. as in S2 Series in Table 1).
- (II2) Update $\mathbf{X}_{\text{Cal}}$, $\mathbf{X}_{\text{Ref}}$ and $\mathbf{X}_{\text{Test}}$ data with new data.
- (II3) Repeat steps from (I3) to (I8).
- (II4) Plot actual 'Cal-chart', 'Ref-chart' and 'Test-chart' (if a target value for sample is available) and decide if the system is in-control (applying current laboratory rules). If the system is declared as out-of-control consider eliminating inadequate Cal data (control of the calibration standards; i.e. 'Cal-chart' in Fig. 4) or replicate Ref analysis. Otherwise, verify the equipment and method. If the system is declared as in-control continue as in step (II) with new series.

4. Conclusions

In this paper the use of a chemometric strategy for the simultaneous quantification of two analytes showing an extreme chromatographic overlapping degree is evaluated. This 1D strategy is based on the direct use of the chromatographic signal in PLS regression. The procedure can be applied to verify the content of manufactured products, such as drugs in a pharmaceutical preparation. The application to unknown samples requires i.e. previous 2D information to recognize the two coeluting compounds and the identification of analytes. After that, the use of the 1D strategy can be advantageously implemented if those samples have to be controlled routinely. In the present case, the concentration ratio in real samples is 1/5, but the minor component exhibits higher molar absorptivity than the other component, so it partially compensates the signal ratio. The lower concentration (or signal) ratio the worse results would be expected. Since this is a limitation of the present approach, its performance has to be tested in the current laboratory conditions and sample under study.

All the study is conducted to fit for a purpose: the possible approach implementation for the quality control of pharmaceuticals in testing laboratories even when equipment and staff qualification are basic. For the case study evaluated here, the results obtained satisfy this requirement: (i) The

methodology is simple, since it does not require signal transformation and uses well-known and available multivariate models such as PLS. However, software to automate the process of treating a new chromatogram (analytes time-window selection, baseline and retention time corrections) and interpolating it into the model, as well as updating the models by incorporating new data series, will simplify the routine work. (ii) The protocol based on updating the models, permits the effective control of future samples, but also the control of the calibration standards in routine working. This fact permits the elimination of possible outliers with little impact on the model. (iii) The relative error in prediction is within the tolerances found in the pharmacopeia (10%), and for spiked solutions simulating preparation errors, the %Er values found had the same magnitude and sign to those provoked. (iv) The results shown in this work have been obtained for a 'worst case' (an extreme overlapping degree), which warrants an improvement in the efficiency of this protocol for better separation conditions (i.e. $\alpha > 1.02$).

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