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Harmonization of strategies for the validation of quantitative analytical procedures: A SFSTP proposal Part IV. Examples of application

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ABSTRACT

A harmonized approach for the validation of analytical methods based on accuracy profile was introduced by a SFSTP commission on the validation of analytical procedure. This fourth and last document aims at illustrating this methodology and the statistics used. Therefore the validation of real case methods are proposed such as methods for the quality control of drugs, for the quantitation of impurities in drug substances, for bioanalysis or for the determination of nutriments. Furthermore, different types of analytical methods are used in order to demonstrate the applicability of the proposed approach to a wide range of methods such as liquid chromatography (LC-UV, LC–MS), spectrophotometry or ELISA.

1. Introduction

This publication consists in the last part of a guide intending to describe a new harmonized global approach of analytical method validation (intra-laboratory). The two first parts aimed at presenting the methodological and conceptual aspects of this approach, which central point is the accuracy profile as decision tool, specifying its vocabulary, as well as the minimum applicable experimental designs [1,2]. The third part presents the statistical and algorithmic aspects of the approach, and provides the reader with all the computation formulas, indispensable for practical implementation [3].

This fourth part presents a series of already operational applications in various fields: drugs control, impurities quantification in raw materials, biological analysis and food analysis. Each example has been chosen because it illustrates a specific situation, classically faced by analysts. All examples are presented in the same way. A brief reminder of the analytical procedure provides the type of analytical technique used, as well as the goals to be achieved. In general, raw data and type of applied experimental design are illustrated by a diagram representing the calibration and validation data. Then, the accuracy profile is illustrated in another figure, and allows interpreting and decision making regarding the method validation. Finally, the corresponding trueness, precision, accuracy data, as well as the higher and lower tolerance interval limits are then summarized in figures. When necessary, quantification limits have been computed. However, no detection limit has been assessed even though it is allowed by the proposed approach [1–3]. Whenever it has been necessary to apply

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a correction coefficient due to a poor recovery rate, the linearity straight line allows to understand how this coefficient has been achieved and a second accuracy profile shows whether the method can be validated after correction. Actually, it appeared to be indispensable, in various cases, to apply a correction coefficient in order to compensate a too weak recovery (or recuperation) rate. This possibility to determine a consistent correction coefficient for the whole application range illustrates this approach potency.

Moreover, it should be mentioned, from now on, that, correcting data increases, in a very visible way, the uncertainty of measurements: which is perfectly consistent with the metrological theory behind the computation of uncertainty. In addition, it has been demonstrated that the uncertainty of measurements could easily be deduced from the tolerance interval width. Any interested reader can refer to the works of Feinberg et al. [4] and González and Herrador [5,6].

This publication aims at providing the analysts with a series of case studies to help them better understand how the harmonized approach proposed by the SFSTP Commission represents an important advance for the laboratories, whatever the analytical procedures they have to validate. However, this ambition to universalism must be moderated by remembering the note 4 posted in the clause §5.4.3 of the ISO 17025 standard, stipulating that "validation is always a balance between costs, risks and technical possibilities". In addition to the presented statistical tools, as efficient as they may be in risk computing, the analysts must always consider the validation economic and practical aspects. Thus, even though a document recommends carrying out 10 trials in the same day, while the technical constraints of the method do not allow it, this does not imply that any validation is impossible. Through several examples, we have intended to demonstrate that it was always possible to apply the global approach, as long as that the risk to achieve incorrect results remains acceptable, even if greater.

2. 1992 STP pharma pratiques data

2.1. Objective and background

The data of this example that was published in 1992 have been used to illustrate the validation procedure proposed at that time [7]. Thus, it was interesting to revisit them in order to apply the new 2003 validation approach, that leads to the accuracy profile [1–3], and compare the conclusions obtained in both cases. This comparison concerns more particularly the results obtained for the trueness (named accuracy in 1992) and precision criteria (repeatability and intermediate precision).

The results were coming from a pharmaceutical development department and have been obtained during the validation of a high-performance liquid chromatography (HPLC) method aiming at determining an active ingredient within a pharmaceutical product.

2.2. Experimental designs

Referring to the protocols proposed in the recent 2003 validation approach [2], the trials carried out and illustrated in the 1992 article get close to the V4 protocol with various particularities, the most important of them being the absence of repetition for the calibration trials.

Design P1. Calibration standards without matrix, also called "samples of active ingredient alone" according to the 1992 terminology:

- five concentration levels distributed from 60 to 140% of the tested drug product nominal value;
- three series carried out in intermediate precision conditions, *i.e.* over 3 distinct days;
- these measurements are carried out without any repetition.

Design P2. Calibration standards within matrix or "reconstituted samples", according to the 1992 terminology:

- five concentration levels distributed from 60 to 140%;
- three series carried out in intermediate precision conditions;
- these measurements are carried out without any repetition.

Design P3. Validation standards within matrix:

- one single concentration level, at 100% of the nominal value;
- three series carried out in intermediate precision conditions;
- six independent repetitions by series.

The P1 and P2 designs were intended to check the response function, particularly the linearity according to the 1992 terminology, the absence of a possible matrix effect and the bias assessment and the P3 design allowed to assess the precision. Taking into account these experimental designs, it must be outlined that the computation of the variance of intermediate precision can only be done at the nominal concentration (100%), and not on the whole validation range, which, moreover, has become a requirement when ICH Q2A (now ICHQ2R1 [8]) was later issued. Fig. 1 presents a global vision of these experimental designs.

2.3. Results

Two ways of exploiting data coming from the P3 experimental design have been used. The first one consists in using only a single concentration level for the calibration, the second one is to keep all the five levels. However, given that the validation standards only include a single concentration level, both accuracy profiles can only be drawn at that 100% nominal value of the concentration level. The acceptance limit has arbitrarily been set at ±3% and the proportion of future results to be included inside the tolerance interval is 95%. Table 1 provides the various statistics illustrated in Fig. 2.

According to the 1992 methodology, the method precision was considered very good since the repeatability relative standard

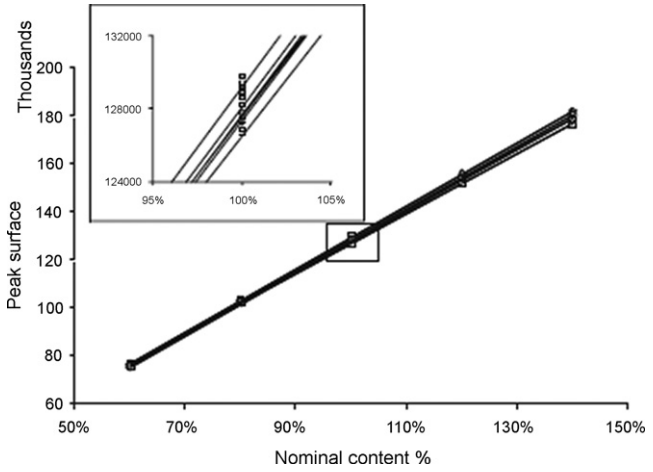

Fig. 1. 1992 SFSTP data. Experimental design graphical illustration. The six (2 × 3) calibration functions are indicated on the diagram. Zooming on the 100% area indicates the calibration and validation data position.

Table 1
1992 SFSTP data expressed in mg

Validation criteria	One calibration level	Five calibration levels
Level (% of the nominal concentration)	100	100
Mean introduced concentration	162.10	162.10
Lower β tolerance limit	161.80	161.70
Upper β tolerance limit	165.10	165.20
Lower relative β tolerance limit (%)	−0.18	−0.24
Upper relative β tolerance limit (%)	1.84	1.90
Repeatability standard deviation	0.41	0.41
Intermediate precision standard deviation	0.60	0.62
Repeatability R.S.D. (%)	0.26	0.25
Intermediate precision R.S.D. (%)	0.37	0.38
Predicted concentration	163.40	163.40
Absolute bias	1.35	1.34
Relative bias (%)	0.83	0.83
Recovery (%)	100.80	100.80

Validation results obtained according to two calibration functions: with one or five levels. R.S.D.: relative standard deviation.

deviation (R.S.D.) was 0.25% and the intermediate precision R.S.D. 0.37%. It is to be reminded that this computation was only possible at the nominal value. However, trueness did not seem to be satisfactory according to the 1992 protocol as the confidence interval of the mean recovery yield was located between 100.7 and 100.9% and thus did not include the 100% target value. This little ambiguous conclusion is already widely discussed in Ref. [1].

In Fig. 2, obtained by applying the approach proposed in 2003, both accuracy profiles demonstrate that the method could have been declared valid, whatever the calibration data used (one or five concentration levels): in both cases, the tolerance interval is entirely included inside the acceptance limits. Using the 1992 data remains a little artificial because, obviously, the used experimental designs do not match those developed in the 2003 approach. However, it was interesting to do this exercise in order to show the continuity between both approaches.

3. Active substances determination by HPLC within a tablet

3.1. Equipment and methods

This example concerns the determination, within a tablet, of two active substances, A and B, containing few mg/tablet of active. The determination is carried out by HPLC-UV with external calibration, thus allowing to simultaneously quantify both substances. The operating procedure consists in dissolving a tablet into a mixed solution of acetonitrile and water, then filtrate this solution before injection. Calibration is carried out with two independent solutions of the active ingredients references subtances, at the nominal concentration (level 100%).

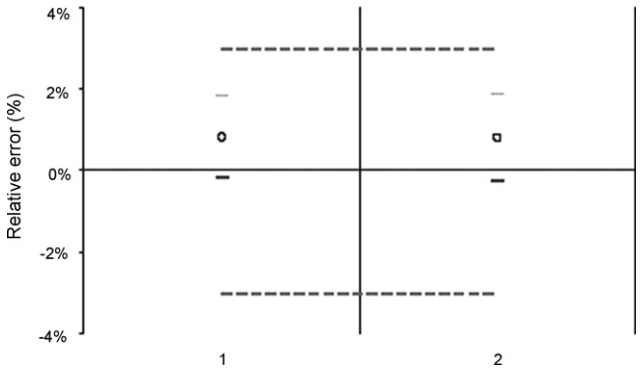

Fig. 2. 1992 SFSTP Data. Accuracy profiles obtained at the nominal concentration by using two calibration protocols out of matrix: (1) with a single level; (2) five levels (acceptability limits ±3%).

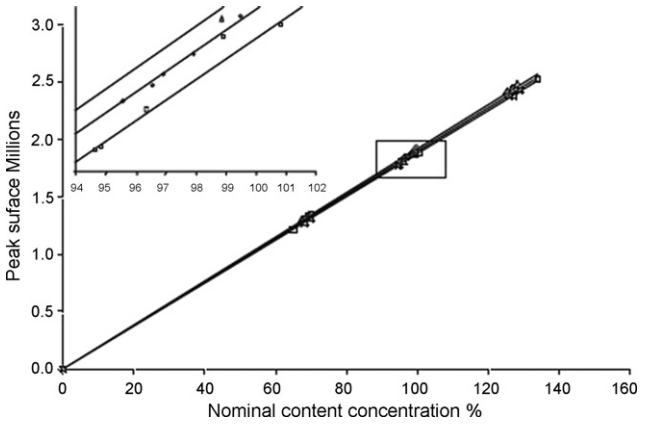

Fig. 3. Compound A determination. Data collected day 1, day 2 or day 3 are represented by squares, triangles and circles, respectively. The insert is a zoom on the diagram central part.

3.2. Experimental designs

The validation design consists in 3 days, three levels and three repetitions ($3 \times 3 \times 3$), *i.e.* 27 trials. It is a V1 protocol [2]. The chosen concentration levels are 70, 100 and 130% of the nominal concentration, in order to cover a concentrations range corresponding to the regulatory control of the tablet content uniformity, *i.e.* 75–125% of the nominal content (or ±25%). Regarding calibration data, the calibration function consists in a straight line with a single point at 100%. This choice implies that the prediction of concentrations higher than 100% are done by extrapolation. The data related to product A are illustrated in Fig. 3; the compound B follows the same protocol.

In addition, this figure shows that the solutions have been prepared independently, from independent weightings; since for an identical "level", we observe that the responses are not vertically aligned. Let us remind that the proposed validation approach includes a realignment step in order to compensate these slight differences, when existing, in the reference concentrations [3].

3.3. Results

Processing these data according to the procedure described in [3], makes it possible to build up Table 2 that contains all the performance criteria for both analytes, at each concentration level. Most of these criteria are expressed both in absolute and relative value. The relative values allow to build up Figs. 4 and 5.

The accuracy profile for the compound A, if setting a ±5% acceptance limit, allows to conclude that the method is valid, as shown in Fig. 4. Let us remind that these acceptance limits must not be confounded with the proportion of future results that was used for the profile computation, *i.e.* the 95% tolerance interval. To be reminded: an expected 95% proportion of future measurements are supposed to be included inside the tolerance interval.

On the contrary, it can be observed for substance B, that beyond 120% of the nominal value, the tolerance interval is no longer inside the ±5% acceptance limits, as shown in Fig. 5. A higher quantification limit can then be calculated, by taking the intersection point between the acceptance limit and the tolerance interval: in this case, it equals 121% of the product nominal value. As a result, it can be concluded that the method is not valid for the whole studied range, but however achieves its objective, between 60 and 121%.

Table 2
Validation results obtained for both hormones expressed in % of the nominal content

Validation criteria	Hormone A			Hormone B		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
Mean introduced concentration	68.21	97.27	127.60	70.47	100.50	131.50
Lower β tolerance limit	67.52	95.36	126.90	612	98.74	122.80
Upper β tolerance limit	68.83	98.97	129.80	71.37	137.80	137.80
Lower relative β tolerance limit (%)	−1.02	−1.97	−0.58	−1.91	−1.70	−6.64
Upper relative β tolerance limit (%)	0.90	1.74	1.69	1.28	1.27	4.80
Repeatability standard deviation	0.27	0.61	0.59	0.46	0.38	1.63
Intermediate precision standard deviation	0.27	0.69	0.59	0.46	0.51	2.41
Repeatability R.S.D. (%)	0.39	0.63	0.46	0.65	0.38	1.24
Intermediate precision R.S.D. (%)	0.39	0.71	0.46	0.65	0.51	1.84
Predicted concentration	68.17	97.16	128.30	70.24	130.30	130.30
Absolute bias	0.00	−0.11	0.71	−0.22	−0.22	−1.21
Relative bias (%)	0.0	−0.1	0.6	−0.3	−0.2	−0.9
Recovery (%)	99.9	99.9	100.6	99.7	99.8	99.1

The tolerance interval broadening beyond 100% can reasonably be interpreted according to the choices made for the calibration experimental design. Actually, using the single 100% level forces to compute, by extrapolating, the inverse predicted values for the higher levels. Yet, it is well known that this method tends to degrade the prediction quality [9].

One solution to get out of this situation would be to choose a 90% probability, which would result in narrowing the tolerance interval, but also increasing the risk to have routine determinations beyond the acceptance limits. Another possibility with a comparable effect would consist in reporting every result as being the mean between two independent results or more: in this case, the intermediate precision variance is reduced. This example of method validation aiming at controlling the concentration of an active ingredient in a drug product illustrates well the new proposed approach flexibility.

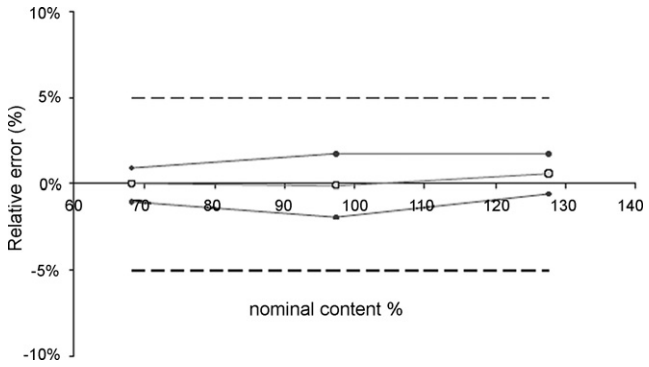

Fig. 4. Substance A accuracy profile (±5% acceptance limits).

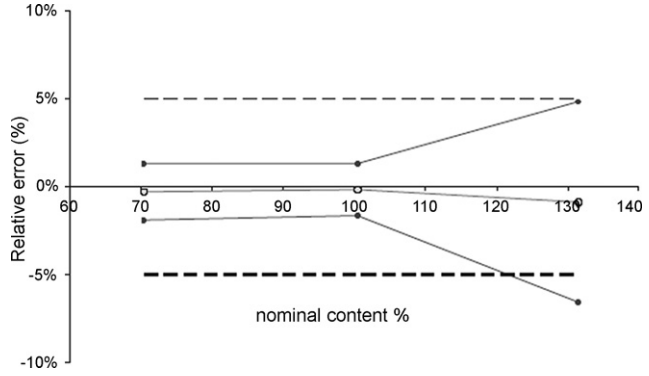

Fig. 5. Substance B accuracy profile (±5% acceptance limits).

We can almost speak of the accuracy profile "potentiality", in the sense that it integrates in a single diagram a set of performance criteria such as precision, trueness, accuracy, associated risk and indirectly the number of repetitions.

4. Influence of the calibration protocol on the accuracy profile—hydrocortisone determination by HPLC-UV in an ointment

4.1. Equipment and methods

As part of the quality control of an ointment at 0.1% of hydrocortisone, a method has been developed and validated. The determination is carried out by HPLC-UV. The operating procedure consists in extracting the active ingredient and dissolving it into the mobile phase. The calibration is carried out with an independent standard of the active ingredient reference substance. The calibration range extends from 0.8 to 1.2 mg/g of ointment.

4.2. Experimental design

The experimental design (Table 3) concerned is a V4 protocol [2] applied using three concentration levels and two repetitions per level during 3 days, *i.e.* 18 trials in total ($3 \times 2 \times 3$). The calibration standards were prepared within and without the matrix. No information was available regarding the existence of a matrix effect and the possibility to quantify the active ingredient with a single level of calibration. As a result, the safest but heaviest option in terms of number of trials has been kept. The validation standards included three levels and three repetitions during 3 days, *i.e.* 27 trials. All the validation standards (nine) are independently prepared every day of analysis.

4.3. Results

Processing collected data according to the V4 protocol makes it possible to build up various profiles by applying different calibration models. Indeed, it is possible to compute regression models by using the calibration data: (A) between 80 and 120% of the nominal concentration; (B) at 100% of this concentration; (C) at 120%. And for each case, the data obtained from the calibration standards prepared within or without the matrix can be used. In total, we can thus propose six accuracy profiles based on six calibration functions. In all the cases, the calibration model that has been kept is the straight line.

Fig. 6 gathers these diagrams and makes it possible to select the one providing the most efficient results. Finally, it appears that the best adapted profile is the one consisting in calibrating only at the

Table 3
Detail of the experimental design used for hydrocortisone determination

Type of sample	Levels	Hydrocortisone concentration (mg/g)	Number of replicates per day	Number of assays for 3 days of validation
Calibration standards without matrix	SE1	0.8	2	6
	SE2	1	2	6
	SE3	1.2	2	6
Calibration standards within matrix	SE1	0.8	2	6
	SE2	1	2	6
	SE3	1.2	2	6
Validation standards	SV1	0.8	3	9
	SV2	1	3	9
	SV3	1.2	3	9
Total				63

120% level, prepared without the matrix. Table 4 includes all these methods performance criteria, at every concentration level, in the case where the calibration model is built up with a single concentration level at 120% of the nominal value. This example of validation of a classical method intended to control an active ingredient concentration in a pharmaceutical formulation illustrates once again, the proposed approach flexibility. It expresses how the operating procedure can be finalised and the calibration procedure chosen in order to ensure optimum routine performances.

Table 4
Validation results obtained for the determination of hydrocortisone (μg/g) using a single point calibration at 120%

Validation criteria	Hormone A		
	Level 1	Level 2	Level 3
Mean introduced concentration	80.00	100.00	120.00
Lower β tolerance limit	797.80	964.70	1161.00
Upper β tolerance limit	812.90	1034.00	1034.00
Lower relative β tolerance limit (%)	−0.2	−3.4	−3.1
Upper relative β tolerance limit (%)	1.7	3.6	2.9
Repeatability standard deviation	1.87	0.98	1.66
Intermediate precision standard deviation	1.80	7.12	7.59
Repeatability R.S.D. (%)	0.2	0.1	0.1
Intermediate precision R.S.D. (%)	0.3	0.7	0.7
Predicted concentration	799.10	998.30	1198.00
Absolute bias	6.06	1.18	−1.10
Relative bias (%)	0.8	0.1	0.1
Recovery (%)	100.8	100.1	99.9

5. Pork plasma acrylamide determination by LC–MS

5.1. Equipment and methods

Acrylamide is a newly formed product when some foods are being cooked. It comes from the Maillard reaction by combining a monosaccharide (glucose), and some amino acid such as

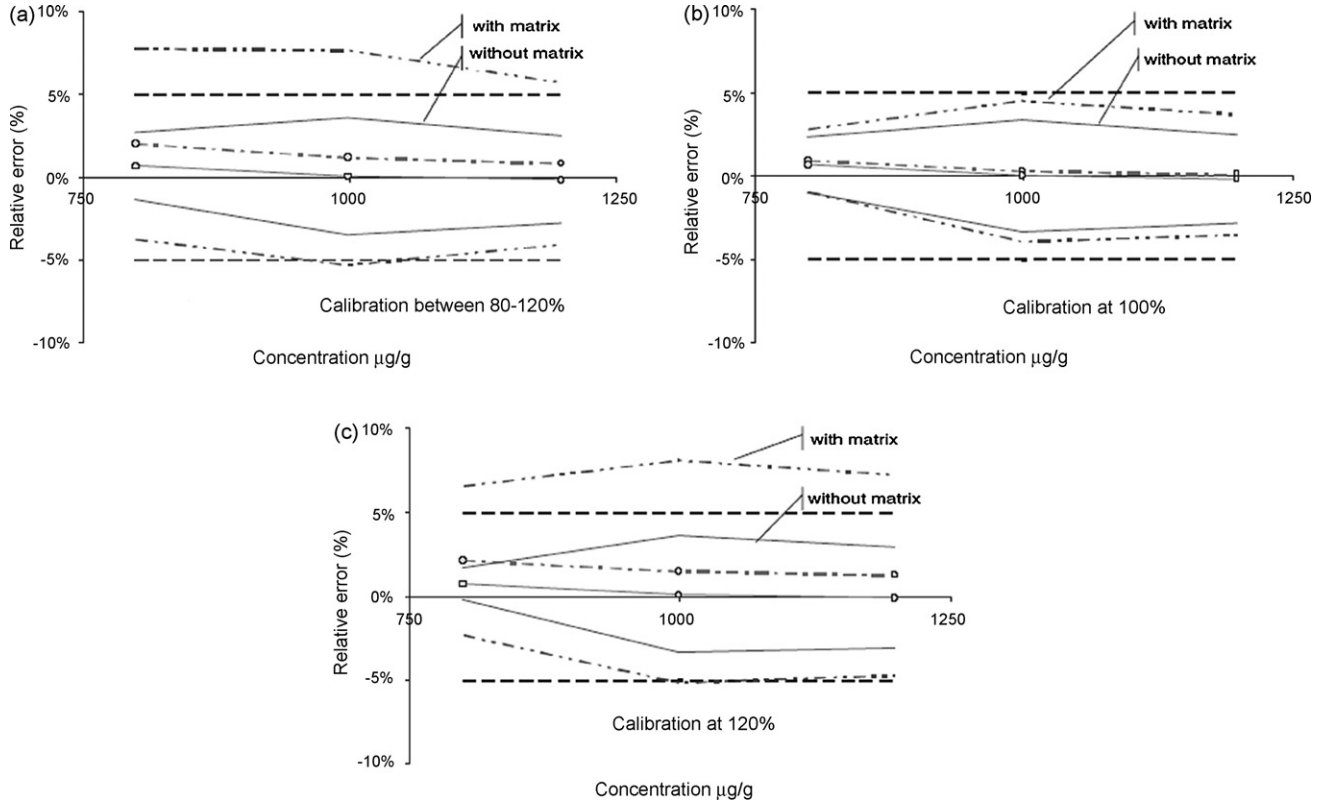

Fig. 6. Hydrocortisone in an ointment. Accuracy profiles for calibration standards prepared within and without matrix at different concentration levels (±5% acceptance limits). (a) Three concentration levels: 80, 100 and 120%. (b) One level at 100%. (c) One level at 120%.

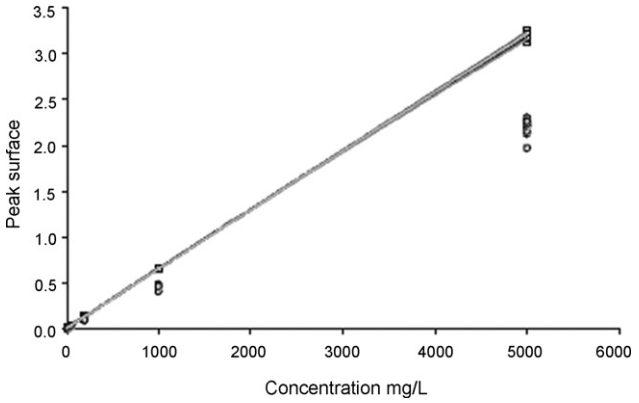

Fig. 7. Acrylamide determination (non-corrected raw data in mg/L). The results are obtained over 5 days, six levels and two repetitions by level.

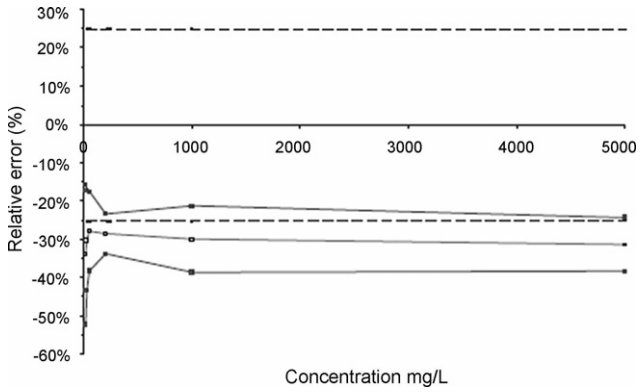

Fig. 8. Acrylamide determination in mg/L. Accuracy profile carried out with the raw data and showing that the method is not valid as it is. The results are obtained with a weighted ($1/X$) quadratic regression in order to model the response function (acceptance limits at ±25%).

asparagine. If the food acrylamide concentration is documented by various studies, yet, few ones have allowed to quantify acrylamide absorption after ingestion. A pharmacokinetic study, carried out on pork, is likely to make it possible to determine the acrylamide bioavailability. Beforehand, an analytical method is necessary to quantify the acrylamide concentrations within the pork's plasma. The selected method to carry out this assay uses HPLC combined with a mass spectrometry detector (LC–MS). Validation is then carried out on a very broad range, extending from 10 to 5000 mg/L, chosen because information is lacking on plasmatic levels observable after ingesting foods containing acrylamide.

The lack of reference materials led us to use spiked amounts on control pork plasma. Two sets of standards were prepared: calibration standards and spiked plasma samples (validation standards). The calibration range is carried out in a 0.01 M ammonia acetate solution, adjusted at pH 6 with formic acid.

The sample preparation consists in adding into 200 μL of plasma, 100 μL of saturated ZnSO4 solution, 1000 μL of acetonitrile and then 100 μL of D5 labelled acrylamide used as internal standard. After stirring and centrifugating, the supernatant is evaporated. The residue is processed again with ammonium acetate 200 μL, 0.001 M, pH 6. The injection volume is 50 μL. The used analytical conditions are a 0.2 mL/min flowrate; the chromatographic column is a Hypercarb column (50 mm × 2.0 mm i.d.; particle size: 5 μm) and the MS detection is performed on the acrylamide 72 molecular ion.

5.2. Experimental designs

The experimental design consists in 5 days, six concentration levels and two repetitions ($5 \times 6 \times 2$), *i.e.* 60 experiments for calibration and validation standards. According to the proposed typology, it is a V2 protocol according to [2], with a modification of the number of repetitions (two instead of three) and of concentration levels (six instead of three). Fig. 7 illustrates this experimental design as well as the whole set of responses obtained for the calibration standards and the spiked validation standards.

5.3. Results

The data have been processed in three steps:

- raw data analysis and accuracy profile drawing;
- analysis to determine the matrix correction coefficient from the recovery yield;
- accuracy profiles computation after correcting the concentrations.

5.3.1. Raw data analysis

In this example, the most adequate response function allowing to describe the relationship between the concentrations and the response is a weighted quadratic regression with a $1/X$ weighting factor, where X represents the introduced concentration. Fig. 8 represents the accuracy profile obtained by using this regression model and a 95% tolerance interval. The diagram shows a gap between the accuracy profile and the acceptance limits that have been set at ±25%. This gap is due to the matrix effect.

5.3.2. Correction coefficient computation

In order to correct this matrix effect, a correction coefficient has been computed from the linearity equation slope linking the introduced theoretical concentrations ($\text{Conc}_{\text{Added}}$) to the recovered concentrations ($\text{Conc}_{\text{Reco}}$) computed by inverse prediction. The applied correction coefficient corresponds to the inverse of the slope achieved with the validation standards. Fig. 9 illustrates these computations. The equation of the straight line is

$$\text{Conc}_{\text{Reco}} = 1.9829 + 0.686\,\text{Conc}_{\text{Added}}$$

The correction coefficient (Fc) to be applied is thus of 1/0.686, *i.e.* 1.457.

5.3.3. Accuracy profile computation after correction

A new accuracy profile computation is carried out taking the correction coefficient into account. The correction is carried out on the validation data. Fig. 10 shows this new accuracy profile. As

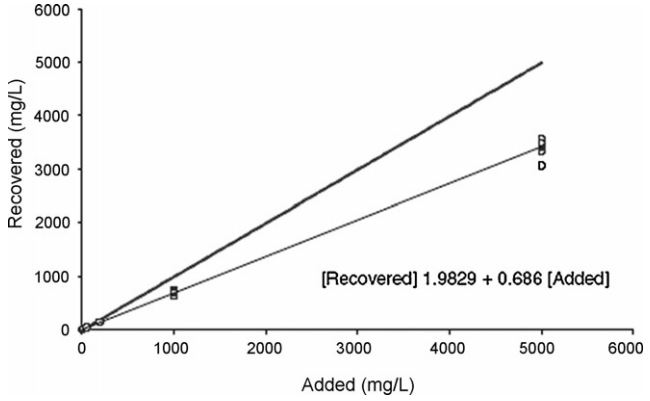

Fig. 9. Acrylamide determination in mg/L regression straight line between the introduced concentrations and the predicted concentrations.

Table 5
Validation results obtained for the determination of acrylamide (mg/L) after raw data correction

Validation criteria	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Mean introduced concentration	10	20	50	500	1000	5000
Lower β tolerance limit	6.98	16.52	44.93	192.50	894.80	4482.00
Upper β tolerance limit	12.26	24.16	60.07	223.00	1145.00	5515.00
Lower relative β tolerance limit (%)	−30.2	−17.4	−10.1	−3.8	−10.5	−10.4
Upper relative β tolerance limit (%)	22.6	20.8	20.2	11.5	14.5	10.3
Repeatability standard deviation	0.61	0.43	1.62	5.78	32.26	136.70
Intermediate precision standard deviation	0.99	1.31	2.81	6.32	48.01	199.20
Repeatability R.S.D. (%)	6.1	2.1	3.3	2.9	3.2	2.7
Intermediate precision R.S.D. (%)	9.9	6.6	5.6	3.2	4.8	4.0
Predicted concentration	9.62	20.34	52.50	207.70	1020.00	4999.00
Absolute bias	−0.38	0.34	2.50	7.73	19.78	−1.35
Relative bias (%)	−3.8	1.7	5.0	3.9	2.0	0.0
Recovery (%)	96.2	101.7	105.0	103.9	102.0	100.0

illustrated by the accuracy profile obtained after applying the correction coefficient, the method is only valid on a part of the studied application range. Indeed, the accuracy profile lower limit is beyond the acceptance limit set at ±25% for the concentration level equal to 10 mg/L. The lower and higher quantification limits computation gives 14.05 and 5000 mg/L, respectively. Thus, this method fits adequately to the objective that has been defined, as the LOQs are compatible with the concentrations usually met. Table 5 summarizes the results obtained for the whole set of concentration levels.

This study demonstrated that, when a weighted quadratic regression model is used, the method is perfectly valid for an application range set between 14.05 and 5000 μg/mL. It is also shown that the matrix effect is systematic and that, in order to correct it, a 1.457 correction coefficient must be applied.

6. Determination of a neurological disease biomarker protein by an ELISA test

6.1. Goals and methods

A "sandwich" type ELISA test has been developed in order to exactly quantify a protein likely to be a biomarker and used for a neurological disease-related therapeutic project. ELISA test consists in incubating samples on plates coated with specific antibodies with a view to capturing the protein of interest. This phase is followed by an immunologic detection of the protein specific linkage by a conjugated enzyme and a measurement of the coloured product by optical densitometry.

In order to validate this test, the calibration standards and the validation standards were prepared in appropriate matrices, from protein stock solutions by serial dilution [10].

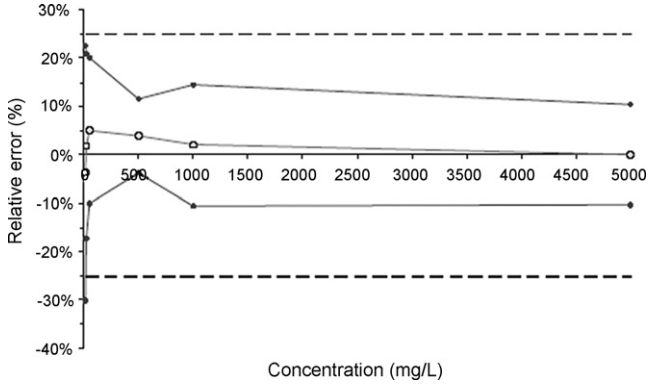

Fig. 10. Acrylamide determination in mg/L. Accuracy profile obtained with the corrected data. The results are obtained with a weighted quadratic regression ($1/X$ type) to model the response function (acceptance limits ±25%).

6.2. Experimental designs

This procedure has been carried out on four independent series, with two plates by trial, over 4 days. All the trials – either for calibration or validation standards – have been conducted in triplicates.

6.3. Results

A four-parameter logistic regression (4PL) has been adjusted on the data (by trial and by plate) by the maximum likelihood method, by using, as weighting, an exponential function of the observed levels (POM, power of the mean):

$$Y = \alpha + \frac{\delta - \alpha}{1 + (\gamma/X)^{\beta}}$$

where δ, α, γ and β are respectively the higher asymptote, the lower asymptote, the concentration corresponding to the curve inflexion point and the slope at this point, respectively. Graphically, for a series of measurements carried out on 4 days, the calibration curves appear like in Fig. 11. The validation standards estimated concentrations have been computed by inversing the response function, *i.e.*:

$$x_{ijk,\mathrm{calc}} = \frac{\hat{\gamma}_i}{(((\hat{\delta}_i - \hat{\alpha}_i)/(y_{ijk} - \hat{\alpha}_i)) - 1)^{1/\hat{\beta}_i}}$$

Knowing the samples theoretical contents by dilution, trueness, precision and tolerance interval have been computed for each concentration level of the validation standards (Table 6). The accuracy profile is represented in Fig. 12. The 95% tolerance interval is included inside the ±30% acceptance limits, at every concentra-

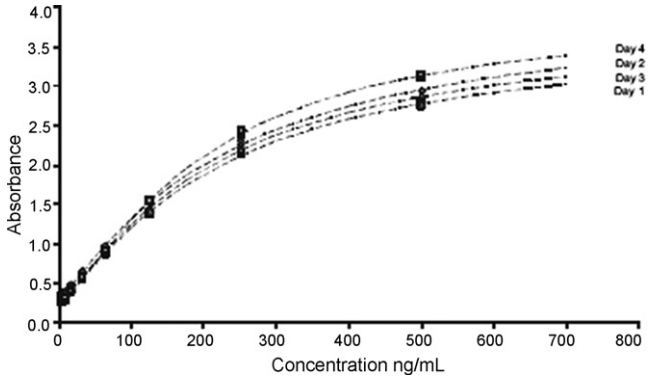

Fig. 11. ELISA assay in ng/mL. Adjustment of a four-parameter logistic model for the series of calibration data collected during the 4-day study.

Table 6
Validation results obtained with the ELISA assay (ng/mL)

Validation criteria	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7	Level 8
Level	3.5	7	14.1	28	56	113	225	450
Mean introduced concentration	3.5	7	14.1	28	56	113	225	450
Mean predicted concentration	4.019	8.184	14.98	29.3	57.82	116.6	239.2	445.6
Absolute bias	0.5187	1.184	0.877	1.301	1.818	3.607	14.16	−4.367
Relative bias (%)	14.82	16.91	6.225	4.647	3.246	3.192	6.294	−0.9705
Recovery (%)	114.8	116.9	106.2	104.6	103.2	103.2	106.3	99.03
Repeatability standard deviation	1.344	1.193	1.899	1.187	1.774	3.606	9.106	23.33
Intermediate precision standard deviation	1.344	1.588	2.004	2.304	3.556	7.127	12.82	40.55
Repeatability R.S.D. (%)	38.41	17.04	13.47	4.238	3.168	3.191	4.047	5.185
Intermediate precision R.S.D. (%)	38.41	22.69	14.21	8.229	6.349	6.307	5.696	9.01
Lower β tolerance limit	1.179	4.395	10.67	22.78	47.64	96.3	207.7	335.9
Upper β tolerance limit	6.859	11.97	19.28	35.82	68	136.9	270.6	555.3
Lower relative β tolerance limit (%)	−66.33	−37.22	−24.3	−18.64	−14.93	−14.78	−7.678	−25.35
Upper relative β tolerance limit (%)	95.97	71.05	36.75	27.93	21.42	21.16	20.27	23.41

tion level, except at the levels lower than 28 ng/mL (Fig. 12). The quantification limit is estimated at 24 ng/mL. Below this limit, by definition, the method does no longer offer sufficient guarantees to obtain accurate results.

On the basis of these validation experiments, we can conclude that the method will provide results at less than ±30% of the real value, in at least 95% of the cases, when the concentration stands between 24 and 450 ng/mL

7. Impurities determination by HPLC-UV in a pharmaceutical product

7.1. Goals and methods

As part of a quality control aiming at releasing a batch, a method has been developed for the determination of the impurities level in two pharmaceutical preparations having different concentration levels of active ingredient. This method is based on reversed phase high-performance liquid chromatography, coupled with a UV detector working at a 259 nm wavelength. Impurities are identified according to their retention time.

Sample preparation is carried out as follows: 20 mg of active ingredient are extracted from a 20 tablets crushed residue and then dissolved in methanol 20 mL. Then, the mobile phase, composed of acetonitrile, ammonium acetate and glacial acetic acid, is added. The injection volume is 40 μL. This validation study aims at guaranteeing that, in 95% of the cases, the result is at less than ±10% of the true value. Moreover, this validation should allow to assess the quantification limits.

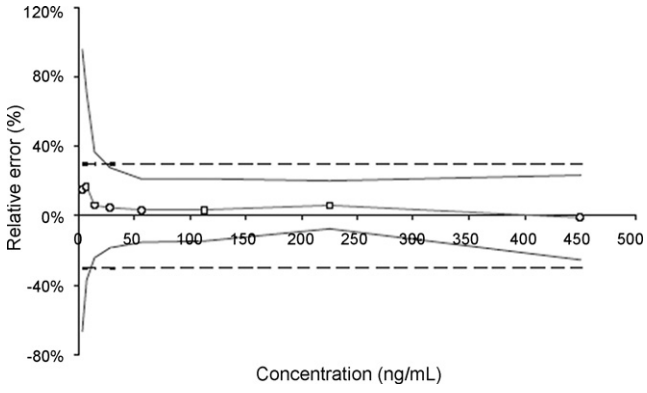

Fig. 12. ELISA determination of a biomarker protein. Accuracy profile (±30% acceptance limits).

7.2. Experimental designs

Different variability sources have been considered for this validation: operators, equipments and days. Considering two levels for each of these factors, the full factorial design includes eight combinations. A fractional factorial design has been selected and is described in Table 7. For each series, calibration and validation standards have been prepared. Calibration standards have been prepared out of the matrix, at four concentration levels: 0.05% (100 ng/mL), 0.50% (1000 ng/mL), 1.25% (2500 ng/mL) and 2.00% (4000 ng/mL), with two independent preparations at each concentration level.

As neither impurities nor excipient were available as pure product, the validation standards have been prepared from the active ingredient alone, at five concentration levels: 0.01% (20 ng/mL), 0.05%, 0.25% (500 ng/mL), 0.50% and 1.25%, with three independent preparations for each concentration level.

Impurities were thus supposed to have a similar response as the active ingredient one: samples have been prepared from the pharmaceutical formulation to simulate a 0.50% impurity level (compared to the active substance theoretical quantity) that could be found in real unknown samples. Sample preparations consisted in crushing a fixed number of tablets, extracting a determined quantity to be diluted in order to get the required concentration level. This example has been carried out in a more pedagogical than practical purpose.

7.3. Results

Among the various tested models, the linear regression forced through 0, is the one that has provided results complying with the objectives. This type of regression model has, consequently, been adjusted for every series, considering a single concentration level at 2% as calibration curve. The estimated concentrations of the validation standards have been computed by simply dividing the signal by the estimated slope of the series regression straight line. Knowing the sample theoretical concentrations, trueness, precision and tolerance interval have been computed for each concentration level (Table 8). The accuracy profile is represented in Fig. 13.

Table 7
Experimental design used for the validation of impurities in a pharmaceutical formulation

Series	Days	Operator	Equipment
1	1	1	1
2	1	2	2
3	2	1	2
4	2	2	1

Table 8
Validation results for the determination of the impurities in a pharmaceutical formulation (ng/mL)

Validation criteria	Level 1	Level 2	Level 3	Level 4	Level 5
Level (%)	0.01	0.05	0.25	0.50	1.25
Mean introduced concentration	20.15	100.8	503.6	1008	2519
Mean predicted concentration	26.83	102.3	507.6	1004	2480
Absolute bias	6.674	1.488	4.036	−4.098	−39.1
Relative bias (%)	33.12	1.48	0.80	−0.41	−1.55
Recovery (%)	133.10	101.50	100.80	99.59	98.45
Repeatability standard deviation	4.644	1.546	6.713	7.764	18.74
Intermediate precision standard deviation	5.214	1.758	6.713	9.803	20.87
Repeatability R.S.D. (%)	23.04	1.534	1.333	0.7704	0.7439
Intermediate precision R.S.D. (%)	25.87	1.75	1.33	0.97	0.83
Lower β tolerance limit	14.39	98.05	490.7	979.1	2431
Upper β tolerance limit	39.26	106.5	524.5	1028	2530
Lower relative β tolerance limit (%)	−28.58	−2.71	−2.56	−2.84	3.52
Upper relative β tolerance limit (%)	94.81	5.66	5.66	2.03	0.42

The tolerance interval is included within the ±10% acceptance limits, at all the concentration levels, except for the 0.01% level. The limit of quantification is estimated at 97 ng/mL (Fig. 13), *i.e.* a little lower than 0.05%. This validation gives enough guarantees that the method will provide results at ±10% of the true value in at least 95% of the cases, when the concentration stands between 97 and 2500 ng/mL.

8. Plasma phosphate determination by a colorimetric method

8.1. Goals and methods

Sodium dihydrogen phosphate is added into some therapeutic plasma to keep them at neutral pH and stabilizing the coagulation factors. However, phosphate concentration must stay at around 5 mmol/L to avoid any hyperphosphatemia. Thus, the herein developed determination method by spectrophotometry in the visible light aims at making this control possible. The measurement is performed at 710 nm, on a stannous chloride reduced phosphomolybdic complex. Proteins are previously eliminated by trichloracetic acid precipitation and centrifugation. The calibration range extends from 0.28 to 0.70 μmol/mL.

8.2. Experimental designs

The protocol is a V4 protocol realized over 3 days [2]. Calibration standards include four levels and two repetitions, respectively with and without matrix, or 24 trials. The validation standards include three levels and three repetitions, or 27 trials (Table 9). For each day all the calibration (8) and validation (9) standards are independent. Responses expressed in optical density (OD) units for the calibration standards are presented in Fig. 14. No matrix effect is virtually observable and the regression equations calculated for every data set are very close. In addition, it can also be observed that the responses are not perfectly aligned. This is due to the differences between the concentration levels, as the spiking and standard solutions are obtained through accurate weighing (independent samples). A data realignment step through linear interpolation makes it possible to compensate these slight differences as proposed in [3].

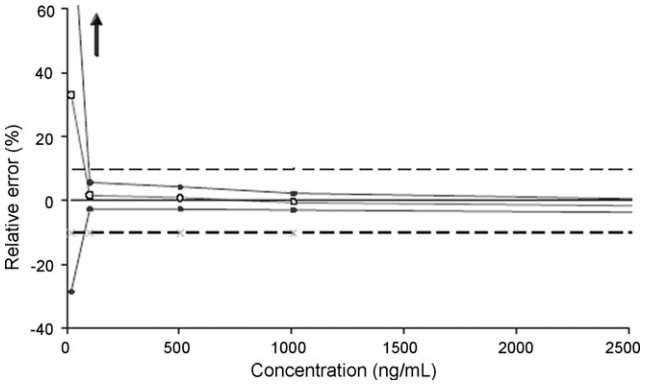

Fig. 13. Impurities determination ng/mL. Accuracy profile (acceptance limits ±10%).

8.3. Results

The most adequate response function is the linear regression weighted by the $1/X$ weighting factor. The concentrations predicted from the validation standards by the weighted linear inverse function show there is a slight matrix effect. Indeed, the linearity equations obtained from the relationship between the introduced concentration ($Conc_{Added}$) and the recovered concentration ($Conc_{Reco}$) are respectively:

- calibration without the matrix: $Conc_{Reco} = 0.9589 \times Conc_{Added} + 5.901$;
- calibration with the matrix: $Conc_{Reco} = 0.9671 \times Conc_{Added} + 5.359$.

However, it has been decided not to apply any correction coefficient, as it proved to be useless as shown in Figs. 15 and 16. These two accuracy profiles have been obtained using a ±15% acceptance limits and a 90% tolerance interval. Examining the accuracy profiles (Figs. 15 and 16) leads to the following observations:

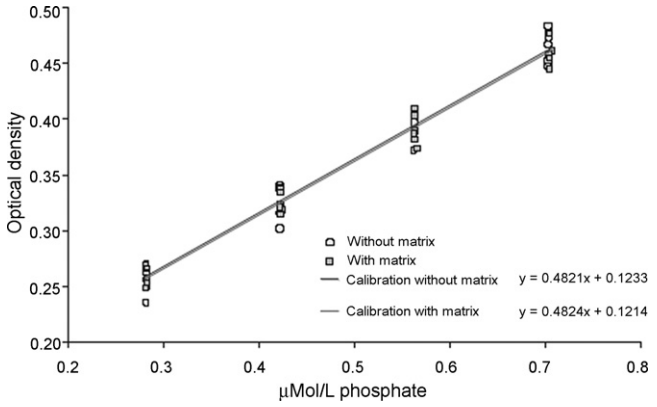

Fig. 14. Plasma phosphate in μmol/L. Calibration standards with and without matrix.

Table 9
Experimental design used for the validation of the plasma phosphate assay

Type of samples	Level	Quantity of Na_2HPO_4, $2H_2O$ (nmol)	Number of replicates per day	Total number of analyses
Calibration standards without matrix	T1	280	2	6
	T2	420	2	6
	T3	560	2	6
	T4	700	2	6
Calibration standards within matrix	A1	280	2	6
	A2	420	2	6
	A3	560	2	6
	A4	700	2	6
Validation standards	Low	280	3	9
	Mid	420	3	9
	High	560	3	9
Total				75

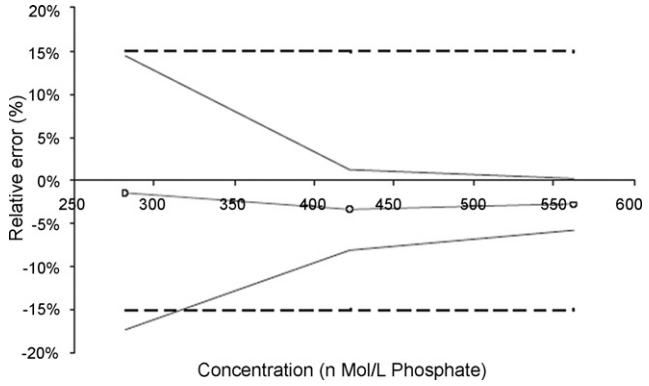

Fig. 15. Plasma phosphate in nmol/L. Accuracy profile for a calibration without matrix (acceptance limits ±15%).

- using calibration standards without matrix is preferable, it allows to obtain accurate results in a wider concentration range (Fig. 15);
- however, under these conditions, the accuracy profile (Fig. 15) shows a high increase of the results dispersion at the lower concentration level (at 280 nmol/L).

The validation results obtained with the accuracy profile computed using the calibration standards prepared without matrix can be found in Table 10. The validation shows that 90% of the future results given by the proposed method will be included inside the ±15% acceptance region around the true value [11], when the concentration is included between 320 and 560 nmol/L. Recovery yield is higher than 96%, thus it was decided unnecessary to apply a correction coefficient considering the selected acceptance limits.

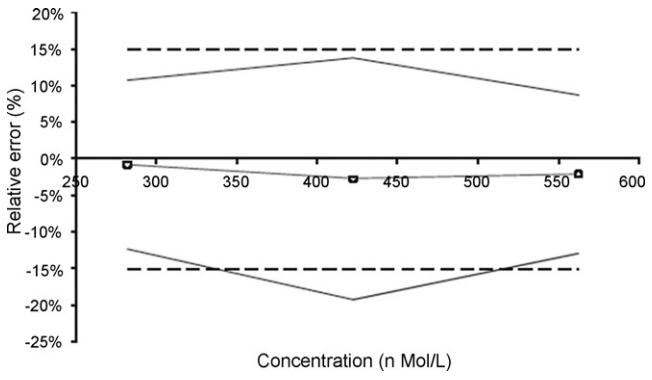

Fig. 16. Plasma phosphate. Accuracy profile for a calibration in the matrix (acceptance limits ±15%).

Table 10
Validation results obtained for the determination of phosphate in plasma (nmol/L)

Validation criteria	Low	Middle	High
Mean introduced concentration	281.60	421.90	562.30
Lower β tolerance limit	232.70	387.90	529.90
Upper β tolerance limit	322.10	427.10	563.10
Lower relative β tolerance limit (%)	−17.4	−8.1	−5.7
Upper relative β tolerance limit (%)	14.4	1.2	0.2
Repeatability standard deviation	8.63	9.95	8.43
Intermediate precision standard deviation	14.25	0.00	0.00
Repeatability R.S.D. (%)	3.1	2.4	1.5
Intermediate precision R.S.D. (%)	5.9	2.4	1.5
Predicted concentration	277.40	407.50	546.50
Absolute bias	−4.19	−14.39	−15.75
Relative bias (%)	−1.5	−3.4	−2.8
Recovery (%)	98.5	96.6	97.2

9. Conclusions

The harmonized validation approach developed by the SFSTP commission has already been given many applications. Other examples of applications can be found in the literature such as liquid chromatographic methods using different kinds of detectors such as UV, fluorescence or masse spectrometry [12–29], infra-red spectrophotometric [30–32], colorimetric [32,33] or Raman [34] methods, capillary electrophoresis [35,36], high-performance thin layer chromatography [37] or gas chromatography [38]. Herein, we have presented a selection of applications, chosen according to different situations an analyst may come upon every day. Several conclusions can be drawn from these studies.

1. Globally speaking, should we consider the validation definition as provided by ISO 17025 standard, *i.e.* "validation is the confirmation, by examination and effective evidence, that the particular prescriptions regarding a foreseen, determined utilization are respected", the new proposed approach very exactly matches this definition. Actually, we must: (1) start by clearly setting out the objectives to be fulfilled in terms of acceptance limits, (2) collect the effective evidences, thanks to various experimental designs and, finally, (3) examine the data in order to statistically and graphically confirm or refute validity of the method.
2. Computation of figures of merit level by level for establishing the accuracy profile makes it possible to cover a very wide concentration range. Thus, the approach is applicable to methods which scope of application may include several decades. Then it is possible to validate methods which variances are not homogenous according to the concentration level. This point is very important because, for many other approaches, the variances homogeneity

hypothesis over the whole application field is indispensable. It can be reminded that this dependency between concentration and uncertainty is a general phenomenon, which evidence has clearly been showed and modelled by Horwitz and Albert [39].

3. Different and even non-linear calibration models, like a four-parameter logistic function, may be used. Actually, according to this approach, the calibration function linearity hypothesis can totally be dropped when conducting the validation. In addition, weighted regression techniques may be used, making it possible, here again, to process data which variances are not homogenous. Similarly, we could see how to select the best regression model, in the case of the very classical example of controlling a drug substance between 80 and 120% of its nominal value.
4. Conflicts between trueness and precision can be simply settled. Classic validation approaches are generally addressing separately trueness and precision. This can then lead to ambiguous conclusions, should only one of these two criteria be satisfactory. The accuracy profile method makes it possible to simultaneously represent, on a same graph, both criteria (or combinations of these criteria). For this reason, this approach is much more flexible and complies with the uncertainty and accuracy definitions that always must combine trueness and precision.
5. Typically, classical validation approaches consist in testing the conformity of performance criteria with respect to reference values. Statistically speaking, this means testing the null hypothesis only on these parameters. Conversely, the proposed approach directly takes the results in the way they will be provided to end-users: this better corresponds to the new quality insurance guidelines requirements that put emphasis on "customer satisfaction". Accuracy profile consists in setting the minimum expected proportion of future results that must fall between the a priori settled acceptance limits. However, a complementary approach consisting in computing on these statistical bases the customer risk could be possible. Let us remind that this risk can be expressed as the possibility to get a result out of the acceptance limits.
6. It stands out from the presented examples that it was possible to calculate a correction coefficient that can possibly be deduced from the inverse of an averaged recovery rate. This point gave rise to many discussions, especially in the field of environmental analysis, in order to know whether measurements have or not to be corrected, when the recovery rate is very different from 100%. The answer provided by the accuracy profile is unambiguous: recovery yield may and must be taken into account. In addition, it has been possible to experimentally demonstrate that, taking this into account leads to increasing uncertainty: which is perfectly in harmony with metrologists' recommendations.

In spite of the numerous practical answers provided by the accuracy profile method, some of them are still pending. For example, to which extent does it apply to microbiological countings that are highly used in food hygiene or clinical biology? In addition, validating an alternative method by comparing it to a so called "reference" method is a classical demand for accredited laboratories. In this case, the fact that the theoretical value is known with uncertainty must also be taken into account in the computations. It is thus necessary to carry out a number of further applications in order to better assert the accuracy profile method universality.

Finally, it seems to be indispensable to remind that validation must always occur after the method development. Trying to carry out trials with a method still badly known, may probably lead to serious disappointments and could then lead to conclude to its inefficiency.

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