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ARTICLE

Updating of Quantitative Models in Validation and Routine Tests of Comparative Chemical Methods

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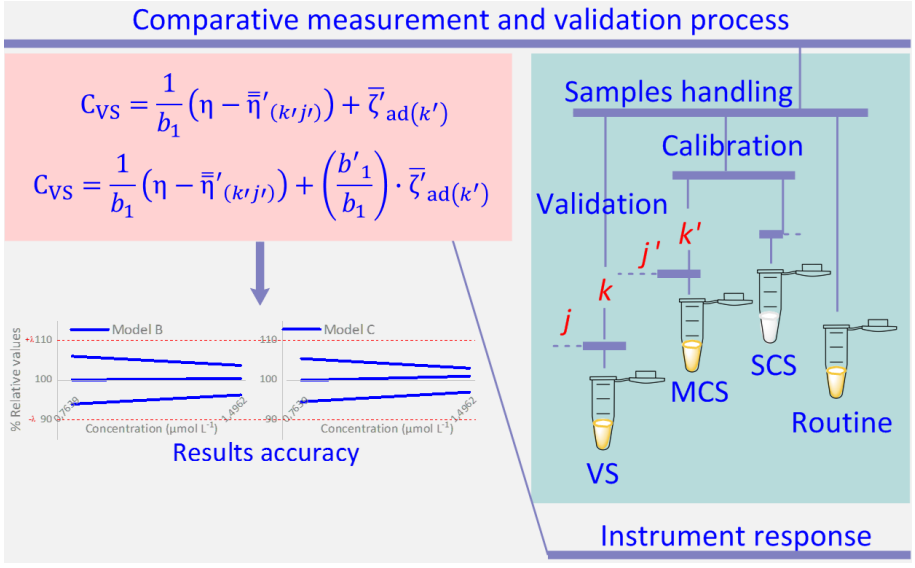

Nowadays, health and safety requirements are becoming more urgent, through normative and regulatory texts, considering the intense demands of customers from different sectors of socio-economic activities. Chemical testing must undoubtedly assume a large part of the tasks related to these concerns, despite the delay observed in their metrological concepts due to the complexity of the chemical and biological samples. Hence, technical and methodological creativity will be well supported, including theoretical revisions and updates of existing methods, in order to overcome the various encountered analytical problems and to fill some frequent lack of metrological tools. In this study, we propose hybrid quantification models, while showing their contributing effects on analytical improvement and decision-making in validation and routine testing of comparative chemical methods. To this end, external calibration plans, with or without a matrix, were established to generate and compare various quantitative models, which make it possible to determine, cleverly, validation and real samples concentrations. The obtained results shed light on the real causes leading to the poor quality that can be found in the obtained validation data. However, highlighted quantitative models show an improvement in both precision and accuracy, which reduce by 5 to 9% the uncertainty measurement. In addition, these estimates show a comparable quality for routinely tests.

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INTRODUCTION

Chemical analyses are infinitely involved in all areas of socio-economic activities. Their related results are intended to be tangible and reliable to meet the daily human needs in product control, pollution evaluation, diagnoses and medical treatments, as well as in international trade and standards establishment, etc. However, this can only be achieved by implementing increasingly creative methodological and metrological practices, although the chemical and biological complexity samples make it difficult to directly fulfill all such requirements.[1,2] Yet, efforts are still maintained by regulatory bodies concerned with chemical measurements, but often individually, with the aim to modernize practices in analytical chemistry by publishing periodically updated recommendations.[3–7] Whose intentions concur with the perspective metrology strategies of the BIPM Consultative Committee for Amount of Substance, across targeted key sectors.[8] Even though, chemical measurements are until nowadays call upon validated and acceptable metrological secondary methods, as the only tools adapted to guaranty comparable results, in order to overcome the various analytical problems.[1]

However, whatever the practice used, we are often opposed to certain unrealizable duties, such as trying to obtain certified reference material (CRM), which is non-existent in some cases, expensive, or even unavailable in time for many laboratories. Added to the unavailability of suitable sample blanks as well as the instability of certain materials whose related errors can enlarge the uncertainty, if they are not wrongly counted, in the contribution of the laboratory staff. In contrast, the replacement of such a material in the validation tests can also make the measurement uncertainty worse, as much if not more, with regard to the above-mentioned drawbacks. This also calls into question the spiking technique, which until now offers a key tool in validation process, provided showing that it is free of any ambiguity accompanying it's use.[4,9-11]

In this sense, a revision has been made in order to update the use of this technique, knowing that, in the absence of certified reference materials, suitable synthetic reference materials can be used in the calibration of comparative methods. Hence, experiments were planned to draw up external calibration plans with or without a matrix, in such a way to derive various equations to determine, mainly and cleverly, the validation standard concentrations and to calculate the validation parameters. Our thinking here is to generate and compare quantitative models, in order to avoid accumulating errors, as when considering ordinary matrices with native content in the designed validation process. In parallel, quality control routine samples were also handled and homologue models quantified their contents. Finally, to highlight results quality arising from those models, measurement uncertainty was estimated according to the single-laboratory validation approach.[12,13]

MATERIALS AND METHODS

Sample handling

Pure Sigma-Aldrich reagents (>95 %) were handled to obtain diluted ethanolic solutions of synthetic and natural retinol, with a constant amount of retinyl acetate as an internal standard (IS). On the other hand, a frozen human transfusion plasma was manipulated in accordance with the ethic committee requirements of the Research Laboratory Spectrochemistry and Structural Pharmacology of the University of Tlemcen, to reach similar ethanolic dilution. The experiments were adapted for a 70/30%, MeOH/MeCN based isocratic reversed phase HPLC analysis. The analytes were eluted at 2.0 mL min^{-1} flow rate and detected at 325 nm wavelength.[14]

Calibration plans

Different solutions were prepared daily to obtain, firstly, five points' synthetic calibration standards type (SCS) within a 0.5 – 2.5 µmol L^{-1} concentrations range. Secondly, the available natural plasma was spiked with the same standard solutions to obtain, after final treatment, on the one hand, validation standards type (VS), and on the other hand, matrix-based calibration standards type (MCS), issued from extra samples (XS) spiked at the concentration range limits.

Preparation of quality control samples

The preparation of the routine quality control (QC) samples was achieved, regarding the difference in physical proprieties of the plasma constituents, mainly, aqueous and no aqueous components. As the lipid matter trend to melt earlier during the plasma thawing process, then we can drop successively different concentrations of the lipophilic compounds until the complete defrosting of the plasma. The selected portions were combined to produce the desired samples, in content and volume, to suit within the validation interval and to allow testing over a period of more than one month.

Theory

Quantitative models

The least squares linear regression based calibration was used to designate the function that links the instrument response to the concentration of the analyte. In the absence of CRM, samples with natural content of analyte of interest are commonly used in spiking practices.[3,4,7,15] In such a situation, the determination of the VS concentration requires measuring, primarily, the endogenous quantity, which will then be subtracted from the whole calculated concentration.[11,15] Unfortunately, without knowing about its influence on the VS quantity measurement, it is even difficult to estimate its contribution to uncertainty in the case of an early standards addition determination.[16,17] While, it should be noted that at this stage the main objective is not yet to determine the native quantity, but rather to eliminate its effect on the validation measurement process. Thus, we intended by establishing the above experimental calibration plans, to address some concerns on the native content estimation of the used matrix, in validation and routine assays, by generating proper quantitation models.

Putting this in mind and assuming an internal standardization, the back calculated concentration (C_{mes}) of the analyte is given as Equation 1:

$$C_{mes} = \frac{1}{b_1}(\eta - b_0) \quad (1)$$

where the instrument response η designate the areas ratio of the analyte of interest over the internal standard and the coefficients (b_1, b_0) represent the slope and the intercept of the calibration curve, respectively.

Alike, the VS concentration (C_{VS}) added to the handled matrix can be quantified as Equation 2:

$$C_{VS} = \frac{1}{b_1}(\eta - b_0) - \zeta_{nat} \quad (2)$$

As we can see, this equation tolerates to acquire two types of data, regarding the natural quantity estimating method, either by the current validated method or from previous tests. Accordingly, we can also deduct, for each validation series, the native content (ζ_{nat}) from the XS quantities samples as Equation 3:

$$\zeta_{nat} = \frac{1}{b_1}\left(\eta'_{(k'j')} - b_0\right) - \zeta'_{ad(k')} \quad (3)$$

where, ($\eta'_{ad(k')}$) and ($\zeta'_{ad(k')}$) represent, respectively, the instrument response and the added content of the (j'th) measurement of the (k'th) XS.

Then, by considering a ($k' \times j'$) plan and combining Equations 2 and 3, we obtain Equation 4:

$$C_{VS} = \frac{1}{b_1}\left(\eta - \bar{\eta}'_{(k'j')}\right) + \bar{\zeta}'_{ad(k')} \quad (4)$$

As can be seen, instead of subtracting an earlier calculated native quantity, which we don't known about its uncertainty, the hybrid Equation 4 allows, in fact, to overcome this inconvenience and highlights only the main actual influential steps of the validation measurement process, such as calibration preparation and instrumental run.

On the other hand, we can express the native response by means of MCS data, as Equation 5:

$$\eta_{nat} = \eta' - b'_1 \zeta'_{ad} \quad (5)$$

where b'₁ designate the slope of the standard addition curve.

Now, assuming we back-calculate the native-content by means of b₁ and b₀, then we can establish, for the same (k' × j') plan, the following Equation 6:

$$\zeta_{nat} = \frac{1}{b_1}\left(\bar{\bar{\eta}}'_{(k'j')} - b_0\right) - \left(\frac{b'_1}{b_1}\right)\bar{\zeta}'_{ad(k')} \quad (6)$$

Therefore, by substituting the Equation 6 into Equation 2, we can rewrite another VS quantitative equation, as Equation 7:

$$C_{VS} = \frac{1}{b_1}\left(\eta - \bar{\bar{\eta}}'_{(k'j')}\right) + \left(\frac{b'_1}{b_1}\right)\bar{\zeta}'_{ad(k')} \quad (7)$$

As it is well noted, this equation appears more complete than the Equation 4, where the slopes ratio factor (b'₁/b₁) proves skillful to redressing the matrix effect if it occurs.

Otherwise, we can also calculate the added concentration of the validation standard by Equation 8:

$$C_{VS} = \frac{\eta - \eta_{nat}}{b'_1} \quad (8)$$

Since η_{nat} is equal to the intercept b'₀ of the standard addition curve, and then we can write, according to Equation 5:

$$\frac{\eta'}{b'_1} - \zeta_{(ad)(k)} = \frac{b'_0}{b'_1} \quad (9)$$

where the ratio (b'₀/ b'₁) recalls the classic standard addition determination of the native content.[18]

When considering the all extra experimental results, the native content can be expressed as Equation 10:

$$\zeta_{nat} = \frac{\bar{\bar{\eta}}'_{(k'j')}}{b'_1} - \bar{\zeta}'_{ad(k')} \quad (10)$$

Another approach to quantify the native content can be established by substituting $\bar{\bar{\eta}}'_{(k'j')}$ by $\left(\bar{\bar{\eta}}'_{std(k'j')} + \eta_{nat}\right)$ into Equation 3 to find Equation 11:

$$\zeta_{nat} = \frac{1}{b_1}\left(\bar{\bar{\eta}}'_{std(k'j')} + b'_0 - b_0\right) - \bar{\zeta}'_{ad(k')} \quad (11)$$

Parameter computation

For a considered (i series × j duplications) validation plan, which agrees an inter-series variability (s^2B) and an intra-series repeatability (s^2r), accuracy profiles were calculated according to the Mee approximations.[19] As for a given average concentration level $\bar{\bar{C}}_{VS\_c(k)}$, the β-expectation tolerance interval (βETI) was expressed by Equation 12:

$$\bar{\bar{C}}_{VS(k)} \pm K \cdot s_{TI} \quad (12)$$

where the factor K represents the Student's β-quantile for a 95% confidence interval, using the degrees of freedom ν of Satterthwaite,[20] as Equation 13:

$$\nu = \frac{\left(\frac{s_B^2}{s_r^2} + 1\right)^2}{\frac{\left(\frac{s_B^2}{s_r^2} + \frac{1}{j}\right)^2}{i-1} + \frac{1-\frac{1}{j}}{ij}} \quad (13)$$

Finally, the standard deviation of the tolerance interval (s_{TI}), itself a function of the intermediate precision standard deviation (s_{TP}), with ($s^2_{TP} = s^2_B + s^2_r$), can be written as Equation 14:

$$s_{TI} = s_{IP} \times \left(\frac{(i+1)js_B^2 + 2s_r^2}{ijs_B^2 + s_r^2}\right)^{\frac{1}{2}} \quad (14)$$

RESULTS AND DISCUSSION

Validation

Experiments were accomplished according to an (i = 3, j = 3 and k = k' = 2) validation plan, to inspect a low level validation standard (LLVS) and a high level validation standard (HLVS). Figure ESI-1 of the electronic supporting information data provides an illustration of the experimental plan and shows the arrangement of these levels relative to the other calibration standards within the desired concentration range. However, validation parameters were calculated using a 95% probability tolerance. The results obtained are summarized in Table I. Models A-1 and A-2 correspond to the Equation 2, in which the natural quantity was estimated daily using the current applied method and when it was estimated from previous tests, respectively. Furthermore, model B agrees with Equations 3 and 4, model C with Equations 6 and 7, model D be in accord with the Equations 8 and 10 and model E links with Equation 11.

As we can see, precision was found to be inferior to 5% for all models, but with rising values for model A-2. On the other hand, the recovery results, which were quantified as the ratio of the mean calculated concentration over the analyte added amount in the plasma samples, give indication on the suitability of the extraction efficiency.[3,21] Nevertheless, they also point out a high bias for the A-2 model.

Table I. Validation and uncertainty parameters estimation results obtained from the different discussed models

	Validation samples										Routine samples				
	LLVS					HLVS									
	Model A-1	Model A-2	Model B	Model C	Model D	Model A-1	Model A-2	Model B	Model C	Model D	Model A	Model B	Model C	Model D	Model E
Sets (I)	3	3	3	3	3	3	3	3	3	3	5	5	5	5	5
Replicates (J)	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Concentration (μmol L^{-1})															
Added	0.7639	0.7639	0.7639	0.7639	0.7639	1.4962	1.4962	1.4962	1.4962	1.4962	--	--	--	--	--
Calculated	0.7723	0.7935	0.7635	0.7714	0.7642	1.4916	1.4745	1.5110	1.5075	1.5026	1.1270	1.1157	1.1144	1.1151	1.1161
Precision															
CV intra-series	2.1010	2.0448	2.1251	2.1034	2.0886	1.2413	1.2557	1.1910	1.1938	1.2276	1.0989	0.7849	0.7858	0.7899	0.5354
CV inter-series	0.2862	2.7576	0.5357	1.2020	1.0235	0.8901	2.6225	0.2361	0.3052	0.6927	1.0458	0.7249	0.5456	1.0611	1.1479
CV (IP)	2.1204	3.4330	2.1915	2.4226	2.3259	1.5274	2.9077	1.2142	1.2321	1.4096	1.5170	1.0684	0.9567	1.3228	1.2666
%Recovery	101.10	103.88	99.95	100.98	100.05	99.69	98.55	100.99	100.76	100.43	--	--	--	--	--
S(TI)	2.2392	3.8451	2.3238	2.6158	2.4988	1.6638	3.3049	1.2847	1.3067	1.5213	1.6126	1.1348	1.0079	1.4201	1.3737
Uncertainty															
%RSU	5.212	11.730	5.440	6.394	6.022	4.217	12.032	2.998	3.060	3.714	3.685	2.584	2.234	3.402	3.511
K	2.328	3.051	2.341	2.444	2.410	2.534	3.641	2.334	2.342	2.441	2.285	2.277	2.217	2.395	2.556

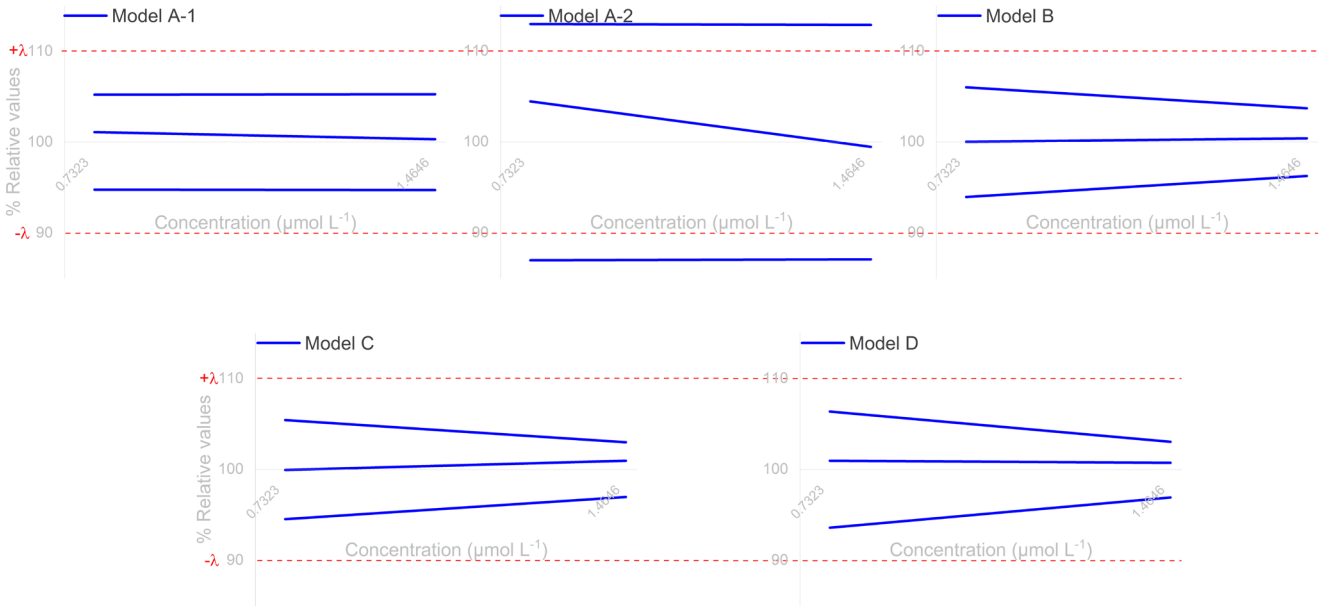

Figure 1. Accuracy profiles relating to the relevant quantitative models obtained by plotting of the relative mean percent values against the introduced concentration.

Figure 1 shows the models-related accuracy profiles obtained by plotting the percent relative values of the interval tolerance limits from either side of the recovery horizontal line. As can be seen, the classic A-2 diagram model shows an excessive variability, which pushes the tolerance interval outside a (λ = ± 10%) predefined acceptance limits and therefore rejects the validity of the results.[22-25] Indeed, this model reveals serious trouble when determining VS concentrations by subtracting the previous defined native content from the actual measured concentration. This fall is also evoked in cases where native quantities are determined by consensus, even if they are provided with an estimate of their uncertainties, moreover, which are considered poor according to this spiking technique.[26] Indeed, such a practice implies a systematic error, which masks wrongly the variability claimed to expose the sample to the random effects of the intermediate precision conditions, while it is requested to eliminate this known error. In this case, the sample must be examined at least for each validation series, as is done when using model A-1. Over and above that, the highlighted hybrid models of Equations 4 and 7 completely exclude this ambiguity, given that the native quantity does not appear in the expression of the VS concentration. This demonstrates that the spiking technique is not responsible for the poor quality that can be found in the investigated validation data. However, the similarity observed of their relating s_{TI}, especially for HLVS indicate a closeness of the calibration curve slopes b_1 and b'_1 and thereby neglected the existence of any matrix effect. Moreover, a two-way ANOVA test was performed using Origin software to support this conclusion. This assessment consists to examine the slope dependency on the curve nature (factor A) and the series (day) variation (factor B). Table II recaps 15 generated values for each slope's type by assuming a min-max calibration points with two replicate each and considering one to two replicates, each time, from one level-point to the other.

Table II. ANOVA-test results on generated slopes data

Factor A	Factor B			Mean	SD
	day1	day2	day3		
b_1	4.1015	4.0375	4.1137		
	4.1350	4.0449	4.1343		
	4.0680	4.0300	4.0932		
	4.0874	4.0623	4.1261		
	4.1156	4.0126	4.1014	4.07113	0.07065
b'_1	4.1046	4.0027	4.1060		
	4.1270	3.9916	4.1394		
	4.0822	4.0139	4.0727		
	4.0411	4.0825	4.0336		
	4.1682	3.9229	4.1785	4.08424	0.03955
Mean	4.0306	4.02011	4.10988		
SD	0.03613	0.04390	0.03946		

ANOVA	df	SS	MS	F Value	P Value
(factor A)	1	0.00129	0.00129	0.77451	0.38755
(factor B)	2	0.04995	0.02498	15.0152	5.90E-5
Interaction	2	0.00190	9.50E-4	0.57137	0.57225
Model	5	0.05314	0.01063	6.38954	6.62E-4
Error	24	0.03992	0.00166	--	--
Corrected Tot	29	0.09306	--	--	--

The test results show that there is no significant difference between the populations averages for factor A (P-value > 0.05), at 95% confidence level. However, there is a significant difference between the populations of the factor B, which exposes a day effect, but without any influence on factor A and does not cause any interaction. Accordingly, it is confirmed at those concentration levels, intended to cover the validation plan, that no matrix effect will occur and the slopes b_1 and b'_1 are very close to each other.

Routine assays

The results shown in Table I give comparable values for all analogous designated models, which appear consistent with those found for the studied validation concentration range, always with a respected headway for the B and C models. However, model E shows that Equation 11 allows us to detect any possible effects linked to the injected media variation, as it seems to be of the negative drift due to the rapid elution of the polar compounds in the chromatogram of the plasma solution of Figure ESI-2. Indeed, the related results attest that there is no significant difference when it comes to quantify the native concentration using the response of the aqueous solutions of the matrix-based standards or that of the organic standard solutions. Furthermore, which may point out the influencing factor magnitudes, whether qualitatively or quantitatively, during the sample handling process and peaks integration of analytes of interest in the final phase of the chromatographic run.

Uncertainty estimation

The measurement uncertainty was estimated in accordance with the Guide for the Expression of Uncertainty in Measurement (GUM).[12,13] Indeed, the quantitative equations terms normally cover all the potential sources of errors relating to the relevant steps of the measurement process, in particular, the above prospected effects. However, the evaluation was based on the statistical TI's calculations,[26] where the combined uncertainty of the measured sample concentration is defined as the sTI for the I days × J repetitions plan, and whose expanded form leads us back to the ± term of the Equation 12, as Equation 15:

$$U(C_{spl}) = K \times s_{IP} \times \left(\frac{(I+1)Js_B^2 + 2s_r^2}{IJs_B^2 + s_r^2}\right)^{\frac{1}{2}} \qquad (15)$$

As we can see, this estimation seems to be rigorously higher by an effective number times, even if it is close to unity (1.04 – 1.15) in our case, compared to that which can be obtained simply by expanding the s_{IP}. Table I recaps the expanded measurement uncertainty results for all models and indicates that high values always appear at low concentrations, also with clear improvement for highest levels, by decreasing to less than 3% for models B and C. Except, for the model A-2, which shows great variability regardless the examined concentration level, by reaching up to 12% uncertainty.

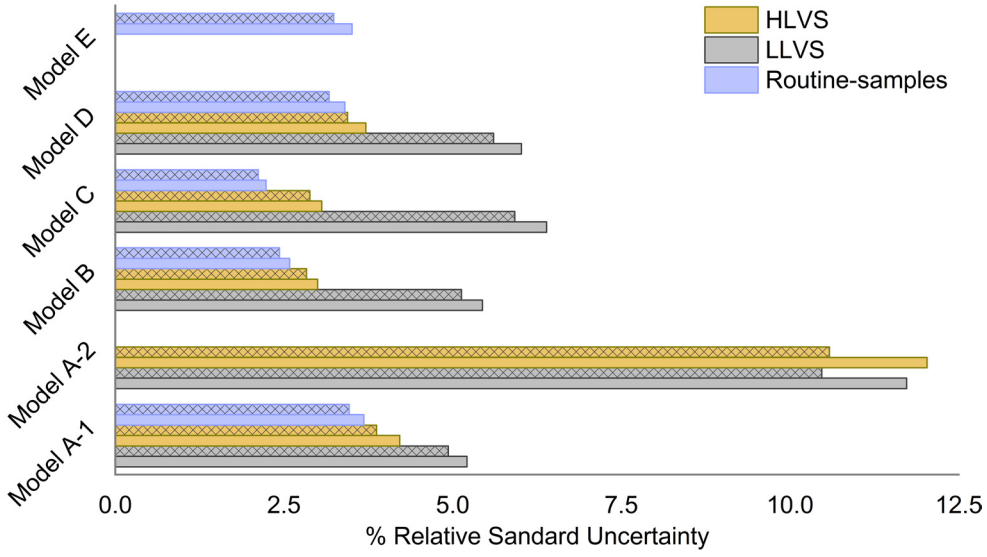

Figure 2. Comparison of the uncertainty estimation results.

Equivalently, these estimates look quite similar for routine samples, not exceeding 4% and with the same advance seen for models B and C. This, when compared to the high validation range results, can be attributed to the high repetitions numbers, which are inherent in the calculating equations of the validation and uncertainty parameters. On the other hand, Figure 2 illustrates these estimations in double, using sTI simple bars and sIP dashed bars. As can be seen, their relative magnitudes are quite similar, whether for HLVS or LLVS, ranging from a deviation of 0.2 to 0.5%, which tolerates a peaceful estimation for somewhat delicate concentration levels. Except in the case of quantification with the A-2 model, which can lead, falsely, to an underestimation of uncertainty of 1.4%. Likewise, these observations are also drawn, by comparing the routine sample results.

CONCLUSIONS

The applied experimental plan has helped to produce different quantitative models that can be used in comparative analysis, such as HPLC-based methods. The valuation of these models was well carried out by determining the validation standard concentrations during the computation of validation parameters, when no certified reference material is available. In addition, this evaluation demonstrates that the spiking technique is not responsible for the poor quality that can be found in the validation data, but rather it is its utilization mode that must be called into question, mostly, when using ordinary matrix with natural content. Indeed, the proposed hybrid models show a clear improvement of this approach, by overcoming errors relating to the native analyte, and by focusing only on the actual influencing factors of the analytical method. Furthermore, these models provide same satisfactions for the determination of the analyte content in routine tests, compared to the results relating to the classical determination model. This supports to open promising perspectives for the validation of comparative chemical methods, which present a shortage for metrological tool and even to promote justified quantitative models for testing and controlling the analytes of interest.

Conflicts of interest

There are no conflicts to declare.

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SUPPLEMENTARY MATERIAL

Electronic Supporting Information Data

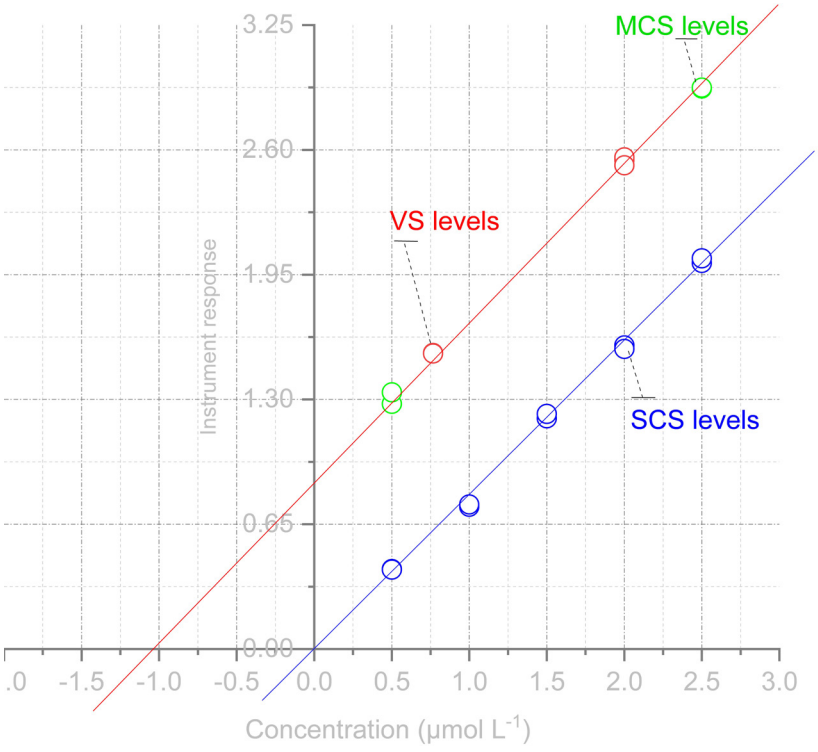

Figure ESI1. Example of a daily constructed experimental plan, showing the arrangement of the different calibration standards in the external standard calibration curve (bleu) and the standard addition calibration curve (red).

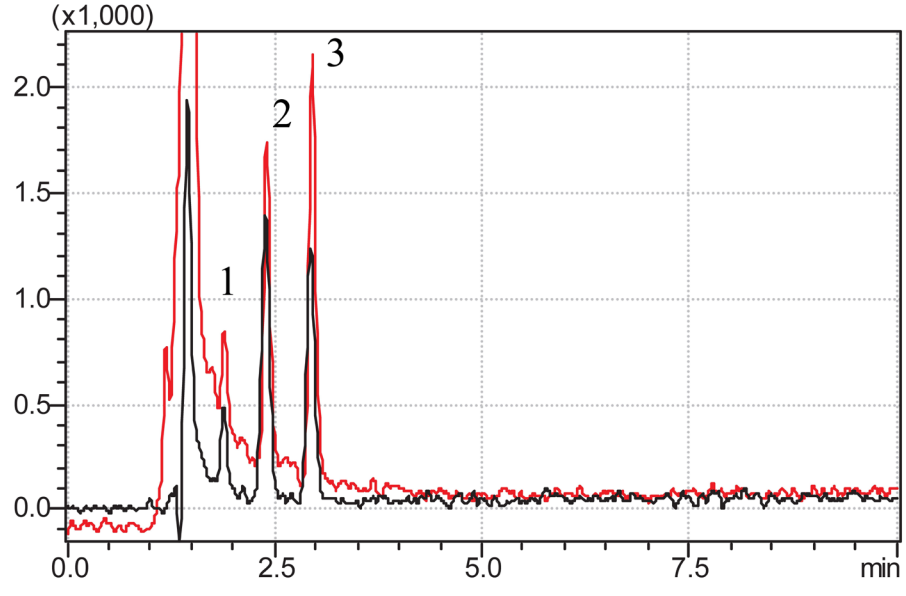

Figure ESI2. HPLC-UV responses of the injected solutions, a synthetic standard solution (black chromatogram) and plasma-sample solution (red chromatogram). Identified peaks: 1) BHT, 2) retinol, 3) retinyl acetate.

Negative drift observed in plasma aqueous medium chromatogram that is due to the rapid elution of the polar compounds, which explains the behavior of the analytical column towards the injected fluid.