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Enhanced high-performance liquid chromatography method for the determination of retinoic acid in plasma. Development, optimization and validation

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ABSTRACT

When determining endogenous compounds in biological samples, the lack of blank or analyte-free matrix samples involves the use of alternative strategies for calibration and quantitation. This article deals with the development, optimization and validation of a high performance liquid chromatography method for the determination of retinoic acid in plasma, obtaining at the same time information about its isomers, taking into account the basal concentration of these endobiotica. An experimental design was used for the optimization of three variables: mobile phase composition, flow rate and column temperature through a central composite design. Four responses were selected for optimization purposes (area under the peaks, quantity of peaks, analysis time and resolution between the first principal peak and the following one). The optimum conditions resulted in a mobile phase consisting of methanol 83.4% (v/v), acetonitrile 0.6% (v/v) and acid aqueous solution 16.0% (v/v); flow rate of 0.68 mL min^{-1} and an column temperature of 37.10 °C. Detection was performed at 350 nm by a diode array detector. The method was validated following a holistic approach that included not only the classical parameters related to method performance but also the robustness and the expected proportion of acceptable results lying inside predefined acceptability intervals, i.e., the uncertainty of measurements. The method validation results indicated a high selectivity and good precision characteristics that were studied at four concentration levels, with RSD less than 5.0% for retinoic acid (less than 7.5% for the LOQ concentration level), in intra and inter-assay precision studies. Linearity was proved for a range from 0.00489 to 15.109 ng mL^{-1} of retinoic acid and the recovery, which was studied at four different fortification levels in phuman plasma samples, varied from 99.5% to 106.5% for retinoic acid. The applicability of the method was demonstrated by determining retinoic acid and obtaining information about its isomers in human and frog plasma samples from different origins.

1. Introduction

The term 'retinoid' refers to a group of endogenous and synthetic molecules structurally similar to retinol (ROH), which is the principal form of Vitamin A, transported in the blood of most vertebrate organisms. ROH is metabolized to retinoic acid (RA) and related geometric isomers which have been detected in blood and embryonic target cells of mammals and birds [1]. These highly potent signaling molecules bind nuclear receptors evoking pleiotropic effects observed at the tissue-organism level. Retinoids have been extensively studied in birds and mammals where their imbalances are associated with multiple dysfunctions including various dermal lesions, immunosuppression, susceptibility to disease (including cancer and parasitic infections), changes in secondary sexual characteristics, inhibition of spermatogenesis, decreased embryo survival, deformities, embryonic development, and numerous other effects on reproduction [1]. There are a number of endogenous geometric isomers of RA (each of them with unique function) [2]. Many tissues and plasma have been reported containing all-trans-RA; 9,13-cis-RA; 13-cis-RA; 9-cis-RA and 11-cis-retinoids [3].

In the pharmaceutical field, both RA and 13-cis-RA are widely used in the treatment of various dermatological diseases such as acne, psoriasis, skin cancer and photoaging, regulating growth and differentiation of epithelial cell, sebum production, and collagen synthesis [2,4].

Because RA isomers are isobaric and have overlapping ultraviolet (UV) spectral profiles, mass detection and/or single wavelength

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UV detection cannot distinguish the identities of geometric isomers that co-elute. Therefore, analysis of RA requires the chromatographic separation of endogenous isomers before detection [3]. A literature search reveals that techniques like gas chromatography (GC), high performance liquid chromatography (HPLC) column-switching with and without direct injection of plasma, ultra high performance liquid chromatography (UHPLC) and capillary electrophoresis (CE), using ultraviolet, fluorescence and mass spectrometer detectors, have been mostly used in developing methods for determining RA and its isomers [5,6,7–9]. Different methods have been presented to determine these compounds, in most of them the analytes being separated with retention times close to 30 min by using C18 columns (4.6 mm × 250 mm, 5.0 μm particle size) [6–9].

Measurement of drug concentrations in biological matrices (such as serum, plasma, blood, urine, and saliva) and in pharmaceuticals is an important aspect of medicinal product development. Such data may be required to support applications for new actives substances and generics as well as variations to authorized drug products. The results of animal toxicokinetic studies and of clinical trials, including bioequivalence studies are used to make critical decisions supporting the safety and efficacy of a medicinal drug substance or product.

It is therefore paramount that the applied bioanalytical methods used are well characterized and fully validated in order to yield reliable results. Acceptance criteria wider than those defined in Guideline on Bioanalytical Method Validation of European Medicines Agency may be used in special situations, which should be prospectively defined based on the intended use of the method [10].

In this work a novel HPLC-UV method was developed, optimized and fully validated for its application in the simultaneous determination of RA and their isomers in plasma. For carrying out the objectives, three variables of the chromatographic system were studied through a central composite design to optimize four responses simultaneously in order to obtain the optimum parameters to decrease the retention time, the solvent expense and the cost of the analysis. Variables and design selection, as well as models fitting and optimization criteria to reach the global desirability are discussed.

Validation of bioanalytical methods is straightforward when analyte-free matrix and well-characterized reference standard of the analyte are available. However, the quantitative determination of endogenous (i.e. naturally occurring) compounds is more complicated because the lack of analyte-free samples of the authentic biological matrix or samples with accurately-know analyte concentrations. Therefore, the preparation of reference samples has to be addressed in a different way and, as a consequence, validation also becomes less straightforward [11]. In these cases, quantitation can be carried out by two strategies: the use of surrogate analyte in the authentic matrix or the use of authentic analyte in a surrogate matrix [11].

On the other hand, to the date, there are not official guidelines dealing with the validation of chromatographic methods for endogenous analytes and, usually, the ones existing for pharmaceuticals and xenobiotic compounds have been adapted for endogenous compounds. Thus, some authors applied method validation principles for drug assays, in particular those issued by the US FDA [12]. Whereas most authors define analytical figures of merit in the same way for xenobiotic and for endogenous compounds, Tsikas [13] pointed out that these parameters should be determined differently in both samples, as the basal concentration of endogenous ($C_{0,Ln}$) varies among biological samples, and defined the relative lower limit of quantitation (rLLOQ), which is corrected by the $C_{0,Ln}$. In addition, Schmidt et al. [14] proposed to subtract the peak areas from the corresponding unspiked blank sample to the peak area of each spiked sample, thus avoiding errors related to the calculation of $C_{0,Ln}$.

In the present work, a chromatographic method has been developed to determine the endogenous compound (RA) and obtaining information about its isomers in human and frog plasma samples in less than twelve min. Validation has been carried out using a holistic approach which considers the most relevant procedures for checking the quality parameters, as well as the estimation of robustness and measurement uncertainty. As in this case makes no sense the use of surrogate analyte in the authentic matrix because the DAD detector cannot discriminate it, the alternative approach would be the use of authentic analyte in a surrogate matrix. However, we think that to subtract the basal concentration of analyte from its signal is a more realistic option and thus, the signal corresponding to the unspiked sample was subtracted when it was suitable.

2. Experimental

2.1. Apparatus and software

All experiments were performed using an Agilent 1100 Series liquid chromatograph equipped with a quaternary pump, degasser membrane, thermostated column compartment, autosampler and (DAD) (Agilent Technologies, Waldbronn, Germany). Chromatograms were registered at 350 nm. The Chemstation version B 0103 was used for data acquisition and processing. The HPLC column was a Zorbax C18 (4.6 mm × 75 mm, 3.5 μm particle size) from Agilent. Experimental design, surface response modeling and desirability function calculations were performed using the Stat-Ease Design-Expert 8.0.0 [15].

2.2. Chemicals and reagents

RA (CAS 302-79-4) was purchased from Sigma (Sigma–Aldrich Inc, St Louis, USA). Roaccutan Isotretinoin was purchased from Roche (R.P Scherer GmbH & Co, KG Eberbach, Germany). Hexane p.a. and ethyl acetate p.a. were supplied by Anedra (San Fernando, Argentina), and tetrahydrofuran p.a. and acetic acid p.a. by Cicarelli (San Lorenzo, Argentina). Acetonitrile and methanol HPLC-grade were obtained from Merck (Darmstadt, Germany). HPLC-grade water was obtained from a Milli-Q Biocel System (Millipore SAS, Molsheim, France).

Solutions and solvents for mobile phase were always filtered through 0.45 μm nylon filters. Standards and sample solutions were also filtered through syringe 0.20 μm nylon membrane before injection in the chromatographic system.

2.3. Standard solutions and samples for validation study

A RA stock standard solution of 0.600 mg mL^{-1} was prepared by exactly weighing and dissolving the adequate amount of standard in methanol. The solution was conserved at 4 °C in light-resistant containers and was allowed to reach room temperature before use. Calibration standard solutions were prepared at the moment of use by diluting an appropriate volume of the stock standard solution in methanol yielding concentrations of RA in the range 0.00489–20.06 μg mL^{-1}.

An aliquot of 10 μL of each stock solution was diluted with 40 μL of basal human plasma to give concentrations of RA in the range 0.0074–4.954 μg mL^{-1}. Then these standard solutions were processed as samples.

A solution of isotretinoin 1.0 μg mL^{-1} was prepared by exactly weighing and dissolving the drug in methanol and was used to identify the peak of this compound.

2.4. Sample preparation

Aliquots (50 μL) of sample plasma were transferred into a 1.5 mL centrifuge tubes and 100 μL of acetonitrile were added. The samples were vortexed for 10 s and then, 300 μL of a solvent mixture composed of ethyl acetate (50%) and hexane (50%) was added. Finally, the samples were vortexed for 10 s, centrifuged at 6000 rpm for 2 min and the organic phase was transferred to glass tubes. The extraction was repeated thrice and the organic phases were collected and mixed and, finally, evaporated to dryness under a gentle stream of nitrogen gas. The residue was dissolved in 50 μL acetonitrile and 15 μL of final solution were injected into the HPLC.

Twelve plasma samples were analyzed. Six of them corresponded to human healthy patients and patients with leukemia, i.e. under metotrexate treatment, and six belonged to frog of two different regions (pristine and undergone to agrochemicals treatment).

2.5. Experimental design and optimization

Four responses were selected for optimization purposes: (a) area under the peaks, (b) quantity of peaks, (c) analysis time, and (d) resolution between the first principal peak and the following one. In addition, polar or spherical coordinates were used to overcome correlation among components of a mixture and to be able to model simultaneously then with other process variables like flow rate and column temperature.

2.6. Method validation: selectivity, limit of detection (LOD), limit of quantitation (LOQ), matrix effect, linearity, precision, accuracy, robustness and uncertainty

Selectivity was assessed by injecting ten basal human plasma samples, which were prepared by processing the plasma as previously described and by evaluating the presence of peaks at the same retention time for the analyte and the peak purity.

LODs and LOQs were calculated by using the signal to noise ratio (S/N) criterion, the IUPAC criterion, calibration curve parameters and by the EURACHEM criterion, whereas the method detection limit (MDL) was calculated following the USEPA criterion.

Matrix effect was evaluated by comparing the calibration graphs obtained by spiking basal human plasma with a volume of an adequately prepared standard solution of RA, and the calibration graph obtained from standard solutions. These calibrations graphs were prepared with concentrations of 0.007, 0.001, 0.410, 0.830, 4.130, 8.260, 10.400 and 12.390 $\mu g\,mL^{-1}$.

In order to study the linear range, calibration standards were prepared in triplicate in methanol at concentrations of 0.005, 0.100, 0.990, 4.950, 10.160, 12.630 and 15.110 $\mu g\,mL^{-1}$. On the other hand, to determinate the working range the concentrations were 0.007, 0.035, 0.100, 0.350, 0.500, 0.990, 1.650, 2.480, 3.540 and 4.950 $\mu g\,mL^{-1}$. These solutions were introduced into the instrument in a randomized way and calibration plots were built.

The within-day repeatability was assessed by repetitive measurements ($n = 6$) of standard solutions at four different concentrations (LOQ, 3 × LOQ, 50% calibration curve and 75% calibration curve) prepared by spiking basal human plasma with a volume of an adequately prepared standard working solution. The between-day precision was evaluated by performing repeated measurements of the same standard sample through 4 weeks. Then, the relative standard deviation (RSD) was calculated in both precision studies. The recovery of the analyte was evaluated by using the same solutions that for the precision study.

In order to assess the method robustness, different chromatographic parameters were varied within a realistic range and the influence of these variables on area, number of peaks, end time and resolution were evaluated. A twelve experiments Plackett–Burman design was built considering small variations in r^2, θ, flow rate and column temperature. It should be taken into account that variations in r^2 and θ involve mobile phase composition.

In order to evaluate the uncertainty of measurements, basal human plasma samples at four spiking concentration levels with eight replicates in each case were used. The uncertainty associated to the concentration of the RA in the spiked human samples, as well as the ones from the method validation (repeatability and trueness) were used and the expanded uncertainty was calculated as recommended by the EURACHEM CITAC Guide CG4 [16].

2.7. General considerations

Due to the high affinity of retinoids to silanol groups of glass surfaces, disposable glassware was utilized to minimize any risk whenever possible following recommendations provided in Ref. [14].

In addition, a stability study was performed in order to assess about the store conditions of the samples. Three portions of plasma were maintained in plastic tubes at 4 °C during three different period of time: 24 h, 7 days and 14 days. Other two portions of plasma were maintained at −20 °C and at and room temperature for the same time, and then processed as previously described.

3. Results and discussion

3.1. Optimization of the chromatographic separation

The use of experimental design in separation science has been increased in the last years [17–23]. In this context, the popularly called response surface methodology (RSM) enables to find the optimum experimental conditions to reach certain responses that assure the best system performance [24].

Although factorial and response surface designs such as the central composite design (CCD) possess many advantages, when working with solvent systems, high and low levels generates sums exceeding 100%. To solve this problem an alternative is to use a spherical coordinates rather than rectangular coordinates to represent the mobile phase composition. This solves the problem of representing the mobile phase composition for HPLC when the amount of one solvent is depending on another [25]. Thus, the amount and composition of organic modifiers in the mobile phase can be expressed by $(r^2 = r^2 \sin\theta + r^2 \cos\theta)$ for a ternary system. Each term in this equation represents one of the organic solvents (A and B) in the mobile phase and r^2 represents the total amount of organic solvents in the mobile phase. Consequently, the latter equation, which describes a circle in the xy plane, represents the relationship between the solvents in the mobile phase using two parameters r and θ, the circle radius and the angle, respectively, indicating the position of a point on the circle.

According to that, the composition of a ternary phase can be calculated with: $Water = 1 - r^2$, $A = r^2 \cos^2\theta$ and $B = r^2 \sin^2\theta$. The coordinates θ and r^2 can be used as factors (k) for the mobile phase composition in a central composite design. The use of polar or spherical coordinates overcome the "volume interdependency" of the solvents in the mobile phase, allowing the composition and the other factors such as pH, flow rate, column temperature and gradient.

For carrying out the experimental design, (A) was defined as portion of methanol, (B) as portion of acetonitrile and $(1 - r^2)$ as acid aqueous solution. The range levels for each factor were 0.8–0.9 for r^2, 5–85 for θ, 0.7–1.3 ($mL\,min^{-1}$) for flow rate and 22–38 °C for the column temperature. Table SM1 (see Supplementary Material) shows the experimental combinations and responses for the

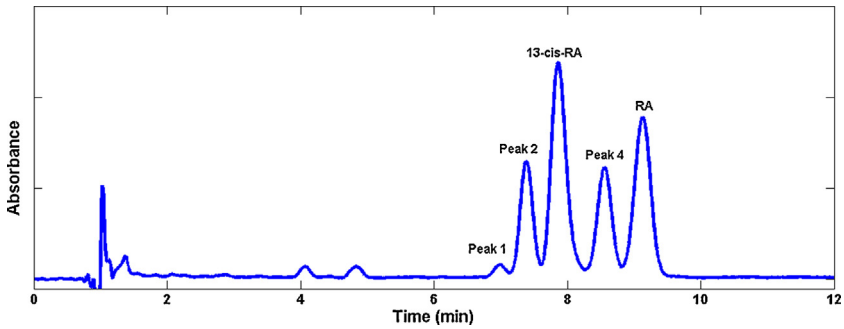

Fig. 1. Chromatogram corresponding to a standard solution of 0.992 μg mL^{-1} using the optimized conditions (flow rate of 0.68 mL min^{-1}, column temperature of 37.10 °C and mobile phase: methanol 83.4 percent, acetonitrile 0.6 percent and acid aqueous solution 16.0 percent. Recorded at 350 nm).

30 experiments, which were performed in a randomized order to assure the independence of the results, minimizing the effects of uncontrolled factors.

The resulting models were evaluated by ANOVA and a backward regression procedure to eliminate not significant factors ($\alpha = 0.10$). Thus, simplified models including only significant terms and those necessary to maintain hierarchy were obtained and undergone to ANOVA for model significance and lack of fit [26].

Owing to the large number of factors and responses, the Derringer desirability function was used [27–29]. Finally, values of the design variables that maximize D were chosen as the optimal experimental conditions, resulting in 0.84 of r^2, 5.00 of θ, flow rate of 0.68 mL min^{-1} and a column temperature of 37.10 °C. Thus, the mobile phase was comprised of methanol 83.4 percent, acetonitrile 0.6 percent and acid aqueous solution 16.0 percent. The suggested optimal conditions were then experimentally corroborated, obtaining chromatographic runs like the one presented in Fig. 1, which corresponds to one standard solution of 0.99 μg mL^{-1} and then processed as described above. In addition, Fig. 2 shows the spectra corresponding to every substance separated in the chromatogram displayed in Fig. 1.

To determine the retention time and the spectrum of 13-cis-RA, a solution of isotretinoin 1.0 μg mL^{-1} was prepared and used to identify the peak of this compound, which corresponded to the third eluted peak. Moreover, according to Gundersen et al. [30] the other peaks can be assigned as follows: peak one, 11-cis-RA; peak two, 9,13-di-cis-RA and peak four, 9-cis-RA.

3.2. Method validation

Method validation was carried out following the holistic approach proposed by González et al. [31] in combination with

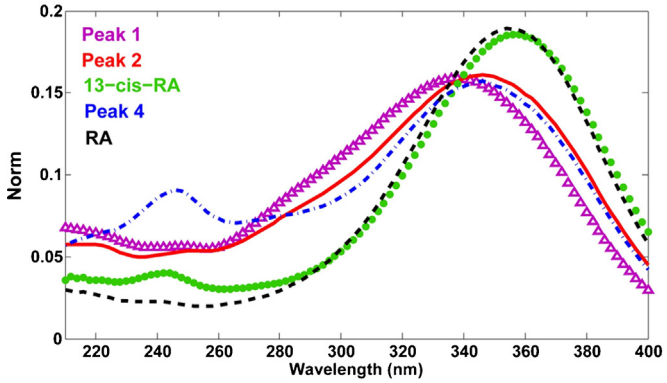

Fig. 2. Spectra (scaled, i.e. divided by the standard deviation) corresponding to every substance separated in the chromatogram displayed in Fig. 1 (peak 1: violet triangle, peak 2: red solid line, 13-cis-RA: green dots, peak 4: blue dash-dotted line, and RA: black dashed line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the recommendations by other authors for quantitative analysis of endogenous compounds in biological systems [13,14], who considered the basal concentration of analytes ($C_{0,Ln}$), except for the method detection limit which can be calculated using samples containing the target compound. In this way, Tsikas [13] pointed out that endogenous analytes occur at varying $C_{0,Ln}$ levels in biological samples and, therefore, the actual LOQ and recovery values may depend upon the extent of $C_{0,Ln}$. Hence, they subtracted $C_{0,Ln}$ when calculating accuracy and proposed a new limit of quantitation (the relative lower limit of quantitation, rLLOQ) which is corrected by the $C_{0,Ln}$, thus avoiding the above mentioned dependence. However, this strategy involves to calculate $C_{0,Ln}$, which is the goal of this work, using a reliable alternative method. To deal with this drawback, we have used the proposal by Schmidt et al. [14] and the peak areas from the corresponding blank unspiked sample were subtracted from the peak area of each spiked sample, thus avoiding errors related to the calculation of $C_{0,Ln}$.

3.2.1. Selectivity

According to Taverniers et al. [32], specificity and selectivity give an idea of the reliability of the analytical method. The term "specific" generally refers to a method that produces a response for a single analyte only, while the term "selective" is used for a method producing responses for different analytes which can be distinguished from each other or producing a target response which is distinguished from all other ones. Therefore, in our case we have used the term "selectivity", as different isomers and metabolites are extracted along with RA.

The selectivity of this method depends mainly on the fact that few other biological compounds significantly absorb light in the wavelength range of 310–370 nm [33]. Thus, the photodiode array signal was used to evaluate homogeneity of the chromatographic peaks corresponding to the analyte present in the ten plasma samples, obtaining in all cases peak purity values higher than 0.99 (Fig. 3).

3.2.2. Limit of detection and limit of quantitation

The LOD was calculated by using standard solutions prepared in solvent and in basal human plasma, applying different criteria.

Firstly, the LOD was calculated as the concentration of analyte giving a signal three times the noise level (S/N = 3), using standard solutions prepared in solvent. Otherwise, this parameter was estimated in basal human plasma by the IUPAC criterion [34] using the expression:

$$Y_{LOD} = Y_{blank} + 3.3 s_{blank} \quad (1)$$

where Y_{LOD} is the response generated by the LOD, Y_{blank} is the average of the blank signal and s_{blank} is its corresponding standadard deviation. The Y_{LOD} value was converted to concentration through the calibration function using the slope b. When working with endogenous compounds, Y_{LOD} value is determined by

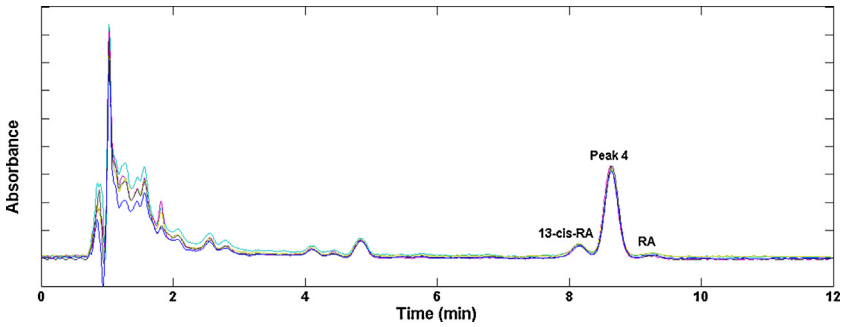

Fig. 3. Chromatograms of ten basal human plasma processed under optimized conditions (flow rate of 0.68 mL min^{-1}, column temperature of 37.10 °C and mobile phase: methanol 83.4 percent, acetonitrile 0.6 percent and acid aqueous solution 16.0 percent. Recorded at 350 nm). Three peaks could be identified (13-cis-RA, peak 4 and RA) and no co eluting interferents were observed.

assuming that Y_{blank} value corresponds to zero and the Y_{LOD} is calculated as from the value obtained by s_{blank} analyzing basal human plasma [13].

Additionally, the LOD was computed from the calibration curve using the standard deviation of the regression (s_y) through the expression [14]:

$$\mathrm{LOD} = \frac{3.3 S_y}{b} \quad (2)$$

Finally, method detection limit (MDL) was determined using a statistical approach established by the USEPA [35], which defined it as "the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero and it is determined from analysis of a sample in a given matrix containing the analyte".

Following the USEPA protocol, firstly, the detection limit was estimated as the concentration value corresponding to an instrument S/N between 2.5 and 5, resulting in 1.8 ng mL^{-1} of RA. As the $C_{0,\mathrm{Ln}}$ was less than the estimated detection limit, seven replicates of basal plasma were spiked with RA at 3.6 ng mL^{-1}, i.e. 2 times the estimated MDL, in such a way that its total level was between one and five times the estimated detection limit, and they were processed through the entire analytical method. Then, the standard deviation (s) of the replicate measurements was obtained (1.59 ng mL^{-1}) and the MDL was calculated as follows:

$$\mathrm{MDL} = t_{(n-1,1-a=0.99)} \times s \quad (3)$$

where $t_{(n-1,1-\alpha=0.99)}$ is the student's t value appropriate for a 99% confidence level and $n-1$ degrees of freedom.

In this way, the MDL obtained was 5.9 ng mL^{-1}. To verify the reasonableness of the estimate of the MDL, seven replicates were spiked at the calculated MDL, i.e. at 5.9 ng mL^{-1} and processed as described above, yielding a standard deviation (s) 1.305 ng mL^{-1}. Then, the s^2 obtained from current MDL calculation and the s^2 obtained from the previous MDL calculation were compared by a F test. As the computed F-ratio $= s_A^2/s_B^2 = 1.644$ was lower than the F-ratio tabulated (5.820), there was not significant differences among both variances and a pooled standard deviation was calculated as:

$$s_{pooled} = \left[\frac{6s_A^2 + 6s_B^2}{12}\right]^{1/2} \quad (4)$$

and it was used to calculate the final MDL according to the following equation:

$$\mathrm{MDL} = t_{[12,(1-a)=0.99]} \times s_{pooled} \quad (5)$$

The MDL thus calculated was 3.8 ng mL^{-1}. The same procedure was followed to calculate the MDL using standard solutions.

The LOD and MDL values obtained by these criteria are displayed in Table 1, where it can be seen that when working with basal plasma, the LOD value obtained by the IUPAC criterion (3.5 ng mL^{-1}) is in the same order than the MDL (3.8 ng mL^{-1}) and they are slightly lower than the LOD calculated from the linear regression parameters (4.5 ng mL^{-1}). On the other hand, the value based on the S/N (10.6 ng mL^{-1}) is higher than the ones obtained by the others criterions. As for the use of standard, the value yielded from of the linear regression parameters (4.0 ng mL^{-1}) is higher than the MDL (1.9 ng mL^{-1}) and the LOD based on the S/N (1.3 ng mL^{-1}). Finally, the values obtained when working with basal plasma are higher than with standard, as expected.

Firstly, it was calculated as the concentration of analyte giving a signal ten times the noise level (S/N = 10) using standard solutions prepared in solvent. Otherwise, this parameter was estimated in basal human plasma by the IUPAC criterion [34] using the expression $Y_{LOD} = Y_{blank} + 10\, s_{blank}$.

Additionally, the LOQ was computed from the linear regression analysis using the standard deviation of the regression (s_y) as was done for the LOD but using a factor equal to 10. Also, according with EURECHAM guide, the LOQ was calculated as the concentration of analyte which provides a standard deviation (RSD %) equal to a value determined by the analyst. In this work this value was set at 10% [36].

However, for endogenous compounds the LOQ has been defined as the lowest concentration $C_{\mathrm{LOW+}}$ of the synthetic reference analyte which, upon addition to the biological sample that contains the endogenous substances at the measured basal $C_{0,\mathrm{Ln}}$, can be experimentally measured in the spiked sample with suitable precision and accuracy (e.g. RSD $\leq$ 20% and recovery = 100 ± 20%), i.e., it can be distinguished from the basal analytes concentration $C_{0,\mathrm{Ln}}$ [13].

The LOQ values obtained when applying the mentioned strategies are reported in Table 1. It can be observed that when working with basal human plasma, the lower LOQ value corresponded to the

Table 1
LOD and LOQ values computed according to different criteria (see References).

Guide	Standard (ng mL^{-1})[a]	Basal plasma (ng mL^{-1})[b]	Reference
LOD			
Calibration curve	4.0	4.5	[14]
MDL[c]	1.9	3.8	[34]
S/R	1.3	1.3	[14,33,36]
IUPAC[d]	–	3.5	[33,36]
LOQ			
S/R	4.4	3.2	[14,33,36]
IUPAC[d]	–	10.5	[31,33,36]
Calibration curve	11.2	12.0	[14]
EURACHEM	4.9	7.4	[5]

[a] Value obtained when processing pure standard samples.
[b] Value obtained when processing basal plasma spiked pure standard samples.
[c] Method detection limit.
[d] Y_{blank} used in Eq. (6) (see text) cannot be computed for pure solvent.

one based on the S/N (3.2 ng mL^{-1}). The value obtained by the IUPAC criterion (10.5 ng mL^{-1}) was slightly lower than the one obtained using the linear regression parameters (12.0 ng mL^{-1}), whereas the LOQ obtained by the EURACHEM criterion is in the same order but somewhat lower (7.4 ng mL^{-1}). On the other hand, when using standard the LOQs obtained follow the same pattern, being lower than those obtained using basal human plasma.

In addition, it should be pointed out that the LOQs obtained using the EURACHEM guide complies with the requirements mentioned above for endogenous compounds, as the RSD established by us was 10% (≤20%) and the recovery value obtained at the LOQ concentration level in the precision study was 102.0% (100 ± 20%).

3.2.3. Matrix effect

The matrix effect was evaluated by comparing the slopes of the calibration graphs obtained when processing pure standard solutions and basal human plasma samples spiked with standards at the same concentration levels, i.e. the slopes of an aqueous calibration line the standard addition line, by using of a *t*-test [37]. This comparison provided $p > 0.1$, which means that matrix effect is absent.

3.2.4. Linear and working ranges

The linear range was established for RA, the lower limit being the LOQ calculated by using the EURECHAM guide and the upper limit the concentration for which the signal deviates from the linearity by 3–5% [36]. Calibration curves were obtained with seven standards covering the whole linear range and each point in triplicate. They showed a good linear relationship ($r^2 > 0.9999$) between 0.0049 and 15.110 μg mL^{-1}, the calibration parameters being listed in Table SM2 (see Supplementary Material). However, for assessment of the linearity of an analytical method, linear regression calculations are not enough [34] and, therefore, the goodness of fit was tested by comparing the variance of the lack of fit against the pure-error variance as was published by different authors [31,38,39]. The adequacy of the model was estimated by a *F*-test which uses the relationship between the pure error variance (SS_{PE}/ν_{PE}) and the variance of the lack of fit (SS_{LOF}/ν_{LOF}), where SS_{PE} is the sum of squares corresponding to pure error, SS_{LOF} is the sum of squares corresponding to the lack of fit, $\nu_{LOF} = \nu_R - \nu_{PE}$ and ν_{PE} and ν_R are the degrees of freedom for estimating the sum of squares of pure error and residuals, respectively.

The calibration model is considered suitable if F_{exp} is less than the one-tailed tabulated value $F_{tab}(\nu_R - \nu_{PE}, \nu_{PE}, p)$ at a p confidence level. In our case, the calibration model can be considered adequate as the F_{exp} (1.188) was lower than $F_{tab}(\nu_R - \nu_{PE}, \nu_{PE}, 0.01)$ (4.314), which is corroborated by the *p*-value obtained (0.229), the confidence level (0.01) (Table SM2).

However, because the concentrations in the commonly investigated samples are in the lowest interval of the linear range, and following the USPH and the IUPAC guidelines [35] which establish the LOQ as the lower limit of the linear range and a 150% of the target level for the analyte as the upper limit, the latter was reduced to 4.950 μg mL^{-1} with excellent results regarding the goodness of fit (Table SM2) and this new linear range was used for next experiments. In this case, F_{exp} (1.517) was lower than F_{tab} (3.1853) with a *p*-value 0.1179.

On the other hand, homoscedasticity was checked by applying the Bartlett' statistic test, which showed no significant difference between variances of the different standard concentrations ($p > 0.05$), indicating homoscedasticity of the data [40]. In addition, homoscedasticity was checked by calculating the residual values as the differences between the actual value y and the y_{pred} value predicted from the regression curve, and plotting them against the actual concentrations of calibration standards. Homoscedasticity was confirmed as residual values were randomly distributed about the regression line without any trend [40].

3.2.5. Accuracy study

The accuracy of results was studied by considering both systematic and random errors, being therefore studied as an entity with two components: precision and trueness.

3.2.5.1. Repeatability and intermediate precision. The golden rules of the validation establish that the analytical procedure should be validated for each matrix, covering the full range of analyte concentrations. Accordingly, the sample matrix (human plasma) and 4 concentration levels (LOQ = 0.007 μg mL^{-1}, 3 × LOQ = 0.015 μg mL^{-1}, 50% of calibration curve = 2.48 μg mL^{-1} and 75% of calibration curve = 3.72 μg mL^{-1}) covering the dynamic working range, and corresponding to the low, medium and high levels, were considered with 6 replicates at each concentration. Two conditions were studied: repeatability or intra-assay variations and intermediate precision or inter-assay variations. The latter was assessed performing analysis during 4 consecutive weeks. The values obtained for these parameters were compared with the theoretical relative reproducibility and repeatability values obtained from the Horwitz function, which consist in an empirical relationship between the precision of an analytical method and the concentration of analyte. As can be expected, higher variability was found as the analyte levels were lower (Table 2).

The repeatability and the intermediate precision (intra- and inter-assay precision) were calculated as the RSD (%) of replicate samples at four concentration levels of RA as described in Section 2. On the other hand, in order to further evaluate the inter-assay precision, a two-way analysis of variance was performed for the whole recoveries obtained for every concentration during the four weeks, in such a way that both the within-condition and between-condition variances were taken into account. The *p*-value obtained (equal to 0.476) which is greater than 0.05, allowed us to conclude that there is not a statistically significant difference between the mean recoveries for each level in the four different weeks studied with a confidence level of 0.05.

Table 2 shows that both intra- and inter-assay precisions increases as the concentration level decreases, being 7.6% and 7.0% for the LOQ concentration level and lower than these values for the other ones.

3.2.5.2. Trueness. Trueness is usually expressed in terms of bias or percentages of error [40–42]. As no certified reference materials were available, recoveries were examined by spiking basal human plasma with known amounts of standard solutions at the beginning of the sample preparation procedure. After extraction and analysis, the peak area in basal human plasma was subtracted from the peak

Table 2
Precision and accuracy results.

Parameter	LOQ	Level[a]		
		3 × LOQ	50% of the calibration curve	75% of the calibration curve
Intra-assay precision RSD[b] (%)	7.6	4.3	3.4	1.9
Inter-assay precision RSD[b] (%)	7.0	4.2	3.4	3.2
Accuracy				
Recovery (%)	102.0	106.8	99.5	99.9
Bias (%)	2.0	6.8	−0.5	−0.1

[a] "LOQ" = 0.007 μg L^{-1}, "3 × LOQ" = 0.015 μg L^{-1}, "50% of calibration curve" = 2.48 μg. L^{-1} and "75% of calibration curve" = 3.72 μg L^{-1}.
[b] Acceptance criteria: RSD ± 20%.

Table 3
Expanded uncertainty (in %) and its principal contributions, calculated at different concentration levels.

	Spiked plasma1	Spiked plasma2	Spiked plasma3	Spiked plasma 4
RA concentration ($\mu g\,mL^{-1}$)	0.0074	0.029	2.027	3.089
$u_{r(sample)}$ [a]	0.0023	0.0023	0.0023	0.0023
$u_{r(rep)}$ [b]	0.0309	0.0175	0.0140	0.0077
$u_{r(true)}$ [c]	0.0248	0.0148	0.0150	0.0121
u_c [d]	0.0397	0.0231	0.0206	0.0145
U (%)[e]	7.9	4.6	4.1	2.9

[a] $u_{r(sample)}$: uncertainty associated to the extraction of RA from the human plasma samples.
[b] $u_{r(rep)}$: uncertainty associated to the precision of the analytical procedure $u_{r(rep)} = RSD_{results}/\sqrt{n}$, where $RSD_{results}$ is the standard deviation derived from the precision study and n is the number of replicates.
[c] $u_{r(true)}$: uncertainty associated to the recovery of the analytical procedure, where RSD_R is the relative standard deviation derived from the recovery study.
[d] $u_c = x_{sample}\sqrt{(u_{r(sample)})^2 + (u_{r(true)})^2 + (u_{r(rep)})^2}$, where x_{sample} is the mean concentration of RA obtained for the spiked plasma $u_{r(sample)} = \sqrt{(u_{r(Vsample)})^2 + (u_{r(Vextract)})^2}$, is the relative uncertainty associated to the volume of plasma extracted and $u_{r(Vextract)}$ is the uncertainty associated to the volume of final extract for injection in the chromatographic system.
[e] U (%) see the text. For details see Ref. [45].

area of the spiked plasma sample as suggested by Schmidt et al. [14] an then, recoveries were calculated by interpolation of these the new signals on the calibration graph. Four levels were evaluated (six replicates), and the results are displayed in Table 2. It can be observed that recoveries were excellent (between 99.5 and 106.8%), even though at the LOQ level.

3.2.6. Robustness

By combining changes in conditions and performing a set of experiments, one can determine which factors have a significant or even critical influence on the analytical results [43,44]. The experiments performed to evaluate robustness and the results obtained are shown in Table SM3 (see Supplementary Material). An ANOVA test was applied to the experimental data employing the effects of dummy variables to obtain estimates of standard errors. The ANOVA allowed us to conclude that small variations in flow rate, mobile phase composition and column temperature have significant effect in two responses: end time, which varies from 8.0 to 11.6 min, and resolution between 13-cis-RA and peak 4, which varies from 1.52–1.65 ($p < 0.05$, confidence level). It should be taken into account that according to experimental design, acetonitrile concentration was varied between 0.6 and 0.7% and (this variation is considered in the variation of r^2 in Table SM3). However, besides the effect that small variations on factors exert on the responses, the analyte can be quantitated with acceptable accuracy. Nevertheless, these variables are an important issue to be considered when quantifying RA in serum and should be maintained as fixed as possible.

3.2.7. Uncertainty

Uncertainty characterizes the dispersion of the values that could reasonably be attributed to the measurand [45] as established the EURACHEM/CITAC Guide CG 4 [16] in its Guide for Quantifying Uncertainty in Analytical Measurement.

For most purposes in analytical chemistry, an expanded uncertainty U, should be used, which provides an interval within which the value of the measurand is believed to lie with a higher level of confidence. U is obtained by multiplying $u_c(y)$, the combined standard uncertainty, by a coverage factor k, which choice is based on the level of confidence desired. For an approximate level of confidence of 95%, k is usually set to 2.

In this work, to complete the validation of the method, the global uncertainty associated to the results obtained was calculated for four different concentration of retinoic acid in the human plasma samples ($0.007\,\mu g\,mL^{-1}$, $0.029\,\mu g\,mL^{-1}$, $2.027\,\mu g\,mL^{-1}$ and $3.090\,\mu g\,mL^{-1}$) and eight replicates were analyzed for each sample. This global uncertainty was expressed as expanded uncertainty U, which was calculated following the above mentioned EURACHEM/CITAC Guide CG4.

The results obtained for each individual input of uncertainty source, the combined uncertainty u_c and the expanded uncertainty U calculated as $U = k\,u_c$, using a coverage factor $k = 2$ (95% confidence level) are summarized in Table 3. The uncertainties associated to the extraction of RA from the plasma samples ($u_{r(sample)}$) ($0.023\,\mu g\,mL^{-1}$) were no dependent upon the spiked concentration of the RA in the sample. The precision term ($u_{r(rep)}$) gave results ranging from $0.0309\,\mu g\,mL^{-1}$ to $0.0077\,\mu g\,mL^{-1}$, increasing as the concentration decreased. Finally, the uncertainty associated to the

Table 4
Concentration of retinoic acid and relative areas of its isomers in frog and human plasmas.

Sample[a]	Peak 1[b]	Peak 2[b]	13-cis-RA[b]	Peak 4[b]	RA ($ng\,mL^{-1}$)
Frog plasma 1	–	–	60.0	300.0	17.5
Frog plasma 2	–	–	–	–	N.D.
Frog plasma 3	–	–	71.1	184.2	12.0
Frog plasma 4	11.1	26.7	94.4	32.2	62.3
Frog plasma 5	2.1	19.0	88.3	29.4	89.3
Frog plasma 6	3.9	39.5	111.8	134.2	53.9
Human plasma 1	–	–	302.1	2108	20.6
Human plasma 2	–	–	432.0	3016	14.4
Human plasma 3	–	–	580.0	3510	10.8
Human plasma 4	861.7	51.9	167.9	443.2	111.3
Human plasma 5	953.2	49.4	161.0	514.3	105.4
Human plasma 6	736.3	62.5	163.8	465.0	109.8

N.D.: not detectable.
[a] Samples Frog plasma 1–3: frogs of a pristine region. Samples Frog plasma 4–6: frogs of a region undergone to agrochemicals. Samples Human plasma 1–3: healthy patients. Samples Human plasma 4–6: patients with leukemia and under methotrexate treatment.
[b] Percentage of area relative to the RA area peak.

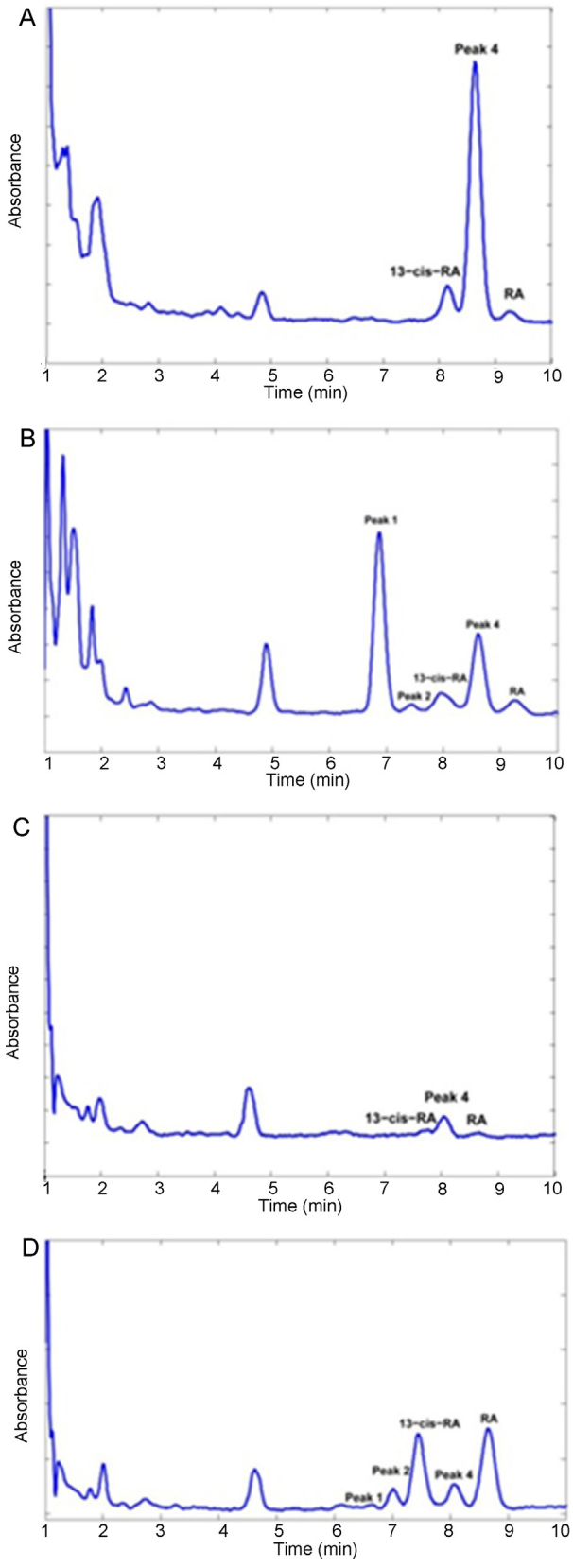

Fig. 4. Chromatograms for plasma samples corresponding to healthy person (A), patient with leukemia (under methotrexate treatment) (B), frog of a pristine region (C) and frog of a region undergone to agrochemicals (D) (flow rate of 0.68 mL min^{-1}, column temperature of 37.10 °C and mobile phase: methanol 83.4 percent, acetonitrile 0.6 percent and acid aqueous solution 16.0 percent. Recorded at 350 nm).

extraction recovery ($u_{r(true)}$) ranged between 0.0248 μg mL^{-1} and 0.0121 μg mL^{-1}, showing a slight increase as the concentration decreased. In summary, the principal uncertainty contributions were the ones associated to the precision and extraction recovery and the global uncertainty (expanded uncertainty) was in all cases was lower than 8%.

3.3. Stability study

In the study of stability, all of samples which were kept at 4 °C and −20 °C did not present significant differences ($p > 0.05$) in the concentration of RA. In addition, the chromatographic profiles obtained with these samples matched well with the chromatograms obtained for basal human plasma samples (13-cis-RA, peak 4 and RA). On the other hand, in samples which were at room temperature it was observed a decrease in the concentration of RA, appearing the peaks 1 and 2. For this reason we concluded that samples should be safe stored at −20 °C.

3.4. Applications

Finally, two sets of real samples (six of them from frogs and six from humans) (see Section 2) were analyzed with the validated method. Table 4 shows the values obtained by processing the six frog plasmas and six human plasmas. Frog plasma samples 1–3, corresponding to three frogs of a pristine region and frog plasma samples 4–6, corresponding to three frogs of a region undergone to agrochemicals, whereas human plasma samples 1–3 correspond to three healthy patients and human plasma samples 4–6 correspond to three patients with leukemia and under methotrexate treatment. Fig. 4A–D shows the chromatograms of four different samples, everyone corresponding to one of the mentioned groups. It can be observed that differences between treatments, as peaks 1 and/or 2 are absent in samples from a healthy persons and from a frogs living in a pristine region. This pattern was the same for the other samples.

Finally, a comparison with other methods published in the literature was made in order to show the advantages of the presented method. Table SM4 shows different approaches and their main characteristics, as well as their figures of merit. As can be appreciated, one of the most important achievement concerns with the reduction of analysis time when HPLC-UV–Vis methods are compared (ca. 70% of reduction). When the new method is compared with CE, although the analysis time is similar, the LOD was reduced from 60 to 2 ng mL^{-1}, i.e. a high improvement in sensitivity was reached with the implementation of the new method.

4. Conclusions

A chromatographic method has been developed to determine the endogenous compound retinoic acid in human and frog plasma samples using DAD detection. The plasma samples were undergone to an extraction procedure with ethyl acetate (50%) and hexane (50%) and as consequence the retinoic acid was split giving four peaks in addition to the one of RA and, therefore, the standards used were also undergone to the same extraction procedure. Validation was carried out using a holistic approach which considering the most relevant procedures for checking the quality parameters, as well as the estimation of robustness and measurement uncertainty.

The basal concentration of RA in plasma samples was taken into account in the validation studies by subtracting the peak area of RA present in the corresponding real sample from the peak area from the spiked samples. Matrix effect was not found and, therefore, quantitation was carried out by using standard of RA undergone to the extraction procedure.

As regards the robustness study, an ANOVA test allowed us to conclude that small variations in flow rate, mobile phase composition and temperature have significant effect in end time and resolution, although this effect is not important considering the predictive ability.

The interval around the estimated value within which the value is considered true was obtained through the uncertainty calculated for spiked serum samples at four different concentration levels with acceptable values.

Finally, the applicability of the method was demonstrated by determining RA in frog and human plasma from different origins, with results coherent for each group.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.01.013.

References

[1] V.E. Bérubé, M.H. Boily, C. DeBlois, N. Dassylva, P.A. Spear, Aquat. Toxicol. 71 (2005) 109.
[2] G. Ioele, E. Cione, A. Risoli, G. Genchi, G. Ragnoa, Int. J. Pharm. 293 (2005) 251.
[3] M.A. Kane, Biochim. Biophys. Acta 1821 (2012) 10.
[4] A.F. Ourique, A. Melero, C. de Bona da Silva, U.F. Schaefer, A.R. Pohlmann, S. StanisçuaskiGuterres, C.M. Lehr, K.H. Kostka, R.C. Ruver Beck, Eur. J. Pharm. Biopharm. 79 (2011) 95.
[5] D.A. El-Hady, H.M. Albishri, J. Chromatogr. B 911 (2012) 180.
[6] C. Wu, V. Njar, A. Brodie, M. Borenstein, I. Nnane, J. Chromatogr. B 810 (2004) 203.
[7] M. Miyagi, H. Yokoyama, H. Shiraishi, M. Matsumoto, H. Ishii, J. Chromatogr. B 757 (2001) 365.
[8] R. Wyss, F. Bucheli, J. Chromatogr. B 700 (1997) 31.
[9] S. Hartmann, O. Froescheis, F. Ringenbach, R. Wyss, F. Bucheli, S. Bischof, J. Bausch, U.-W. Wiegand, J. Chromatogr. B 751 (2001) 265.
[10] EMEA/CHMP/EWP/192217/2009, Committee for Medicinal Products for Human Use (CHMP), 2012.
[11] N.C. Van de Merbel, Anal. Chem. 27 (2008) 924.
[12] U.S. Department of Health and Human Services, Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation, 2014, http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf
[13] D. Tsikas, J. Chromatogr. B 877 (2009) 2244.
[14] C.K. Schmidt, A. Brouwer, H. Nau, Anal. Biochem. 315 (2003) 26.
[15] Design Expert 8.0.5, Stat-Ease Inc., Minneapolis, USA, 2010.
[16] EURACHEM/CITAC Guide CG 4, Quantifiying Uncertainty in Analytical Measurement.
[17] R. Leardi, Anal. Chim. Acta 652 (2009) 161.
[18] L.R. Snyder, J.W. Dolan, J. Chromatogr. A 1302 (2013) 45.
[19] A. Herrero, M.C. Ortiz, L.A. Sarabia, J. Chromatogr. A 1288 (2013) 111.
[20] A. Andrade-Eiroa, P. Diévart, P. Dagaut, Talanta 81 (2010) 265.
[21] P. Iuliani, G. Carlucci, A. Marrone, J. Pharm. Biomed. Anal. 51 (2010) 46.
[22] M. Fourdinier, S. Bostyn, R. Delépée, H. Fauduet, Talanta 81 (2010) 1281.
[23] B.J. Stojanović, T. Rakić, B. Slavković, N. Kostić, A. Vemić, A. Malenović, J. Pharm. Anal. 3 (2013) 45.
[24] R.H. Myers, D. Montgomery, C.M. Anderson-Cook, Response Surface Methodology: Process and Product Optimization Using Designed Experiments, third ed., Ed. Wiley, New Jersey, 2009.
[25] V. Morris, J.H.P. Marriot, J. Chromatogr. A 1008 (2003) 43.
[26] D.C. Montgomery, Design and Analysis of Experiments, fifth ed., Arizona State University, Jhon Wiley & Sons, Inc. New York, 2000.
[27] G. Derringer, R. Suich, J. Qual. Technol. 12 (1980) 214.
[28] M. Almeida Bezerra, R. Erthal Santelli, E. Padua Oliveira, L. Silveira Villar, L.A. Escaleira, Talanta 76 (2008) 965.
[29] L. Vera Candioti, J.C. Robles, V.E. Mantovani, H.C. Goicoechea, Talanta 69 (2006) 140.
[30] T.E. Gundersen, R. Blomhoff, J. Chromatogr. A 935 (2001) 13.
[31] A.G. González, M.A. Herrador, Trends Anal. Chem. 26 (2007) 227.
[32] I. Taverniers, M. De Loose, E. Van Bockstaele, Trends Anal. Chem. 23 (2004) 535.
[33] V. Van Merris, E. Meyer, K. DeWasch, C. Burvenich, Anal. Chim. Acta 468 (2002) 237.
[34] L.A. Currie, Anal. Chim. Acta 391 (1999) 105.
[35] CFR Appendix B to part 136. Definition and procedure for the determination of the method detection limit in USEPA Protocol for EPA Approval of New Methods for Organic and Inorganic Analytes in Wastewater and Drinking Water, U.S. Environmental Protection Agency, Washington, DC, 1999 http://www.gpo.gov/fdsys/granule/CFR-2011-title40-vol23/CFR-2011-title40-vol23-part136-appB/content-detail.html
[36] Guide to Quality in Analytical Chemistry. Prepared jointly by CITAC (The Cooperation on International Traceability in Analytical Chemistry) and EURACHEM (A Focus for Analytical Chemistry in Europe), Edition 2002.
[37] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of Chemometrics and Qualimtrics: Part A, Elsevier, 1997, pp. 413.
[38] M. Mcloun, J. Militky, M. Forina, Chemometrics for Analytical Chemistry, vol. 2, Ellis Horwood, Chichester, West Sussex, UK, 1994, pp. 64.
[39] P. Araujo, J. Chromatogr. B 877 (2009) 2224.
[40] M.C. Ortiz, M.S. Sánchez, L.A. Sarabia, Comprehensive Chemometrics. Chemical and Biochemical Data Analysis, Elsevier, Amsterdam, 2009.
[41] J. Inczédy, T. Lengyel, A.M. Ure, A. Gelencsér, A. Hulanicki, Compendium of Analytical Nomenclature IUPAC, 3rd ed., Baltimore, Pergamon Press. Inc, 2000, pp. 50.
[42] J. Fleming, B. Neidhart, H. Albus, W. Wegscheider, Accredit. Qual. Assur. 1 (1996) 135.
[43] Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, J. Pharm. Biomed. Anal. 24 (2001) 723.
[44] C. Von Holst, A. Müller, E. Björklund, E. Anklam, Eur. Food Res. Technol. 213 (2001) 154.
[45] Guide to the Expression of Uncertainty in Measurement, ISO, Geneva, 1993, ISBN 92-67-10188-9, Reprinted 1995: Reissued as ISO Guide 98-3 (2008), available from http://www.bipm.org as JCGM 100:2008.