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Estimation of the uncertainty associated with the results based on the validation of chromatographic analysis procedures: Application to the determination of chlorides by high performance liquid chromatography and of fatty acids by high resolution gas chromatography

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ABSTRACT

This article presents a model to calculate the uncertainty associated with an analytical result based on the validation of the analysis procedure. This calculation model is proposed as an alternative to commonly used *bottom-up* and *top-down* methods. This proposal is very advantageous as the validation of the procedures and the estimation of the uncertainty of the measurement are part of the technical requirements needed in order to obtain the ISO 17025:2005 accreditation. This model has been applied to the determination of chloride by liquid chromatography in lixiviates and in the determination of palmitic acid and stearic acid by gas chromatography in magnesium stearate samples.

1. Introduction

The uncertainty is the parameter associated with the result of a measure which characterizes the dispersion of the obtained values that could be reasonably attributed to the measurand [1,2]. This variability in the measured values can be caused by inaccuracy or lack of precision. The uncertainty is associated with the range within which the real value of the quantity is found, once the corrections due to known errors are made.

There are several possible options to estimate the uncertainty in a laboratory. Nevertheless, there are two procedures commonly used, known as *bottom-up* and *top-down* [3–5].

The *bottom-up* method gives an approach based on the decomposition of all analytical operations in primary activities. These are combined or grouped in common activities and give an estimation of the contribution of each activity to the value of the uncertainty of the measurement procedure. The benefit for the analyst is that this approach provides a clear comprehension of the analytical activities that notably contribute to the uncertainty and that, therefore, can be assigned as critical control points to reduce or manage the uncertainty of the measure in future applications of the method. Nonetheless, the bottom-up method can be very laborious and requires a deep knowledge of the analytical process [2,6,7].

The *top-down* method is based on the calculation of the uncertainty using the standard deviations provided by the reproducibility of an interlaboratory study. This estimation is based on the idea that the uncertainty is produced by the method with the highest variability. The method gives a reliable estimation of the execution and the uncertainty related to its application. However, this method's application is difficult, as it is not always possible to obtain the interlaboratory information and the fact that the estimation is made in collaboration between laboratories can lead to results with uncertainties that cannot be compared to those results obtained when working in a single laboratory [6,8].

In this article, an alternative method for the calculation of the uncertainty of a procedure, which is based on the validation of the analytical procedures in a laboratory, is presented. This proposal is very advantageous as the validation of the procedures and the estimation of the uncertainty of the measurement are part of the technical requirements needed in order to obtain the ISO 17025:2005 accreditation [9]. This method has been applied in the determination of chlorides by liquid chromatography in lixiviates and in the determination of palmitic

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Table 1
Models for linearity study.

Assays	Concentration range	Standards	Replicates
Richness	95–105%	3–5	3
Majority	80–120%	3–5	3
Minority[a]	50–120%	5–7	3
Wide limits	50–150%	3–7	3

[a] To minority assays is often used the limit of quantification as the lowest concentration.

and stearic acids by gas chromatography in magnesium stearate samples.

2. Validation of analytical procedures

The validation of an analytical procedure consists in the acquirement of proofs, conveniently documented, demonstrative of the fact that the studied procedure is reliable enough to obtain the expected result within the defined range. In other words, the main objective in a validation is to demonstrate that the studied procedure is adequate for the proposed use [10].

All the validation starts from an already proved and adjusted method. The validation consists in proving, with a minimum number of essays, that both the analytical method and its associated analytical system will yield results that meet requirements previously set forth. The parameters taken into account are typically: selectivity, linearity, accuracy, precision and sensitivity [11,12].

2.1. Selectivity

A method is selective if it can discern and differentiate the response from the studied analyte independently, from the other substances that form the matrix, without interferences from impurities, degradation products, related compounds or excipients present in the sample [10].

The determination of the selectivity depends on the used analytical technique and on the type of essay. Comparative essays are used in the selectivity studies, where the responses of the standard sample, the placebo, the sample itself or the added placebo are taken into account, as well as the laboratory blank.

2.2. Linearity

The linearity of an analytical procedure is the capacity of obtaining a directly proportional response to the concentration of the analyte, within a determined interval of concentrations. The essays can be made on standard dissolutions as well as on added samples [10,13].

Depending on the type of essay and on the sample, the range of concentration, the number of standards in the range of study and the number of repetitions for each standard concentration have been defined (Table 1) [12].

2.3. Accuracy

Accuracy expresses the proximity between the obtained value and a value considered true and gives information about the systematic errors of the procedure.

One of the main difficulties that arose when an accuracy study is made is to set the true, or reference, value. For that purpose, three different values can be used: the value obtained from an already validated method, the value from a reference material or the result obtained when applying the standard addition method [14].

Accuracy is determined for the whole specified range of the analytical method. It is recommended that a minimum of 9 replicates over the three concentration levels are carried out and, usually, it is expressed as recovery.

2.4. Precision

The precision of an analytical procedure represents the degree of dispersion in a series of results obtained from multiple repetitions of the same homogeneous sample under the conditions described in the method [10,13]. Depending on the major or minor degree of concordance between the different sources that introduce variability into the result, the following parameters can be characterized:

(a) *Repeatability*: It is the measure of precision between individual results, acquired under the same conditions, by the same analyst, in the same laboratory, using the same equipment and reagents, and in the course of the same series of analysis, made, usually, in a short period of time.
(b) *Intermediate precision*: It is the precision between individual results from the same sample collection, including variations within the same laboratory, for instance, different days, operational conditions, analyst or equipment.
(c) *Reproducibility*: It studies the variability of the method under different operative conditions and in different laboratories.

Precision studies are usually carried out at three levels of concentration and are expressed as the coefficient of variation (CV%) of the series of measurements.

When results from several different days are available and in order to obtain more representative results for the repeatability calculation, the values from one of the days are not the ones used. Instead, the calculation of an average standard deviation that takes into account the independent degrees of freedom is proposed, depending on the day of the analysis and the number of essays made. The coefficient of variation to determine the repeatability for more than a day is calculated according to Eq. (1) [10]:

$$\mathrm{CV}\ (\%) = \frac{\sqrt{((n_1-1)\times {s_1}^2 + (n_2-1)\times {s_2}^2 + (n_3-1)\times {s_3}^2)/((n_1-1)+(n_2-1)+(n_3-1))}}{x_{n_1,n_2,n_3}} \times 100 \qquad (1)$$

where s_1, s_2, s_3 are the standard deviations for each day; n_1, n_2, n_3 are the amount of data for each day; x_{n_1,n_2,n_3} are the average value of the results obtained during the days of essay.

2.5. Limit of quantification

The limit of quantification is defined as the minimum amount of analyte present in the sample that can be quantified, under the described experimental conditions and with an adequate precision and accuracy [10,13].

In instrumental procedures it can be calculated theoretically using the variability of the blank signal and establishing the concentration corresponding to 10 times its variability as the limit of quantification.

On the other hand, it can also be determined experimentally by the analysis of samples with decreasing concentrations of the analyte, establishing the limit of quantification as the minimum level in which acceptable values of accuracy and precision can be obtained.

3. Estimation of the uncertainty of the measurements

The result of an essay ($X_{average}$) can differ from the true value. Furthermore, due to the conceptual impossibility of determining the true value, the reference value is considered the best approximation.

The uncertainty associated with the results includes various components related to the sources of error that determine the precision and accuracy of the measurement. Therefore, the estimated value of the uncertainty (u) includes the various systematic measurement errors (accuracy) and the random measurement errors (precision). The obtained value of uncertainty is multiplied by a coverage factor to obtain the expanded uncertainty or tolerance. Usually a factor of 2 is used to obtain a confidence level of 95% [6,15].

Thus, the interval $X_{average} \pm 2u$ will include the true value, with a confidence level of 95% (Fig. 1) [11].

The uncertainty can be obtained from the data acquired during the validation of the analytical procedure, using both reference materials and added samples. The equation proposed to obtain the uncertainty consists in a quadratic addition that includes the following terms: standard uncertainty ($u_{standard}$), instrumental measurement uncertainty ($u_{instrumental\ system}$), and sample uncertainty (u_{sample}) (Eq. (2)) [12]:

$$u\ \ (\%) = \sqrt{u_{standard}{}^2\ \ (\%) + u_{instrumental\,system}{}^2\ \ (\%) + u_{sample}{}^2\ \ (\%)} \quad (2)$$

It is worth pointing out that all terms included in the calculations related to Eq. (2) must be Gaussian. In the present work, the terms corresponding to a rectangular function are conveniently normalized using a factor of $\sqrt{3}$ [1]. On the other hand, those terms obtained from the data related to tolerances ($\pm T$) should be divided by the applied coverage factor.

3.1. Uncertainty of the measurement standard

The uncertainty of the measurement standard is calculated by the quadratic addition of two terms: the uncertainty certified by manufacturer (u_{stock}) and the uncertainty corresponding to its preparation by dilution or weighting ($u_{preparation}$) (Eq. (3)):

$$u_{standard}\ \ (\%) = \sqrt{u_{stock}{}^2\ \ (\%) + u_{preparation}{}^2\ \ (\%)} \quad (3)$$

The *stock uncertainty* (u_{stock}) is calculated from a value given by the manufacturer. Depending on the nature of the given value, this uncertainty is calculated using Eqs. (4) or (5):

If the tolerance is expressed as $\pm T\%$:

$$u_{stock}\ \ (\%) = \frac{T\%}{2} \quad (4)$$

If the purity is expressed as $P\%$:

$$u_{stock}\ \ (\%) = \frac{(100 - P\%)}{\sqrt{3}} \quad (5)$$

The *uncertainty of the preparation* ($u_{preparation}$) is obtained taking into account each of the steps needed to prepare the measurement standard (weighting, dilution, etc.). Each one of them is an independent term of the quadratic addition.

The uncertainty associated with the weighting is obtained from the tolerance ($\pm T$) of the scale (Eq. (6)):

$$u_{weighting}\ \ (\%) = \frac{T\ \ (\mathrm{g})/2}{\mathrm{weighting}\ \ (\mathrm{g})} \times 100 \quad (6)$$

The uncertainty associated with a process of volume dilution is calculated from the tolerance ($\pm T$) of the volumetric material used (Eq. (7)):

$$u_{dilution}\ \ (\%) = \frac{T\ \ (\mathrm{mL})/2}{\mathrm{volume}\ \ (\mathrm{mL})} \times 100 \quad (7)$$

The uncertainty of the preparation term ($u_{preparation}$) includes the analyst's handling errors in preparing the standards because tolerance values (Eqs. (6) and (7)) are experimentally calculated. When there are independent standard preparations at each concentration level, the $u_{preparation}$ term could be eliminated. In this case, the contribution of this term is included in the $u_{precision}$ term of the instrumental equipment.

3.2. Uncertainty of the instrumental equipment

The uncertainty of the instrumental equipment includes all the sources of error related to the resolution, calibration and stability of the measurement. If the resolution is considered negligible, it can be obtained as the quadratic addition of the uncertainty associated with the precision ($u_{precision}$) and with the accuracy ($u_{accuracy}$) of the calibration, taking into account that the stability of the measurement is included in the precision term (Eq. (8)):

$$u_{instrumental}\ \ (\%) = \sqrt{u_{precision}{}^2\ \ (\%) + u_{accuracy}{}^2\ \ (\%)} \quad (8)$$

In those cases where the quantification is made by interpolation of the signal from the sample on a calibration curve, the uncertainty of the instrumental equipment ($u_{instrumental\ system}$) is considered to be the value of the measurement standard which has the most unfavorable result. When the quantification is made by response factor, the uncertainty corresponds to the quantification standard used.

The *uncertainty of the precision* ($u_{precision}$) is calculated from the coefficient of variation of the n replicates of the measurements (Eq. (9)):

$$u_{precision}\ \ (\%) = \frac{\mathrm{CV}\ \ (\%)}{\sqrt{n}} \quad (9)$$

The *uncertainty of the accuracy* ($u_{accuracy}$) when quantified by calibration curve is calculated as the value of the residual (Eq. (10)):

$$u_{accuracy}\ \ (\%) = \mathrm{residual}\ \ (\%) = \frac{\left|Y_{exp} - Y_{cal}\right|}{Y_{cal}} \times 100 \quad (10)$$

Y_{exp}: experimental measurement; Y_{cal}: value calculated by interpolating the value of the concentration of standard in the calibration curve.

If the quantification is made by response factor in order to obtain the uncertainty of the accuracy a reference measurement standard is used (Eq. (11)):

$$u_{accuracy}\ \ (\%) = \frac{\left|F_{R\,exp} - F_{R\,ref}\right|}{F_{R\,ref}} \times 100 \quad (11)$$

$F_{R\,exp}$: response factor of the experimental measurement; $F_{R\,ref}$: response factor of the reference standard.

3.3. Uncertainty associated with the sample

The uncertainty associated with the sample is obtained after a series of replicate analysis of reference material or sample and its additions. It is calculated as the sum of squares of terms relating to the preparation, precision and accuracy of the results (Eq. (12)):

$$u_{sample}\ \ (\%) = \sqrt{u_{preparation}{}^2\ \ (\%) + u_{precision}{}^2\ \ (\%) + u_{accuracy}{}^2\ \ (\%)} \quad (12)$$

The *influence of the sample's preparation* ($u_{preparation}$) is included in the precision term if it is obtained independently for each essay.

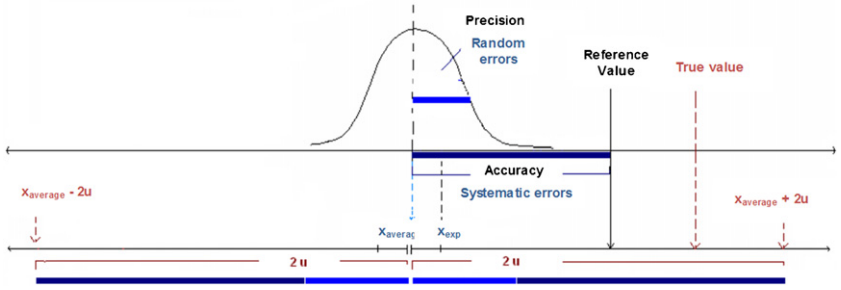

Fig. 1. Measurement uncertainty.

The *uncertainty of the precision* ($u_{precision}$) corresponds to the coefficient of variation of the *n* replicates of the measurement made under conditions of repeatability or intermediate precision (Eq. (13)):

$$u_{precision}\ (\%) = \frac{CV\ (\%)}{\sqrt{n}} \tag{13}$$

The *uncertainty of accuracy* ($u_{accuracy}$) is calculated from % of bias obtained in the recovery of the added samples or the reference material. This value could be obtained considering either the average or the worst bias divided by the square root of three, according to the following expression (Eq. (14)):

$$u_{accuracy}\ (\%) = \text{Average(Bias(\%))} \text{ or } \frac{\text{Max(Bias(\%))}}{\sqrt{3}} \tag{14}$$

4. Application to chlorides determination by HPLC in lixiviate samples

4.1. Analytical procedure

The chlorides determination in lixiviate samples is made by ionic exchange liquid chromatography and a conductivity detector. The quantification is made by interpolation on a calibration curve obtained by injecting chlorides standards of 5, 10, 20 and 50 mg/L. The concentration interval in the samples ranges from 5 to 10,000 mg/L [12].

4.2. Reagents and standards

The reagents and standards used in the analysis and the validation are acetonitrile for HPLC from Merck, sodium gluconate synthesis from Merck, boric acid from Riedel-de-Haën, sodium tetraborate decahydrate crystallized from Riedel-de-Haën, glycerine from Panreac, standard of Cl^-, SO_4^{2-}, NO_3^- of 1000 mg/L from Merck and Milli-Q water.

Working solutions of 5, 10, 20 and 50 mg/L are obtained from the standard chlorides solution of 1000 mg/L. Ultrapure water (Milli-Q) is taken as blank. A borate-gluconate buffer, used as mobile phase, is prepared weighing: 16.0 g of sodium gluconate, 18.0 g of boric acid, 25.0 g of sodium tetraborate, 250 mL of glycerine and diluting to 1 L with Milli-Q water [16].

4.3. Equipment and chromatographic conditions

The equipment used were a liquid chromatograph Waters ILC-1 and a scale Mettler Toledo AB204 (min = 10 mg; max = 210 g; *e* = 1 mg; *d* = 0.1 mg). The chromatographic conditions are detailed in Table 2.

4.4. Sample acquisition and conditioning

The analysis requires three samples obtained from lixiviates. Each aliquot is filtered using a 0.45 μm nylon filter and is collected in an injection vial. When the concentration of the sample is higher than 50 mg/L, the necessary dilutions must be made (max: 1:200).

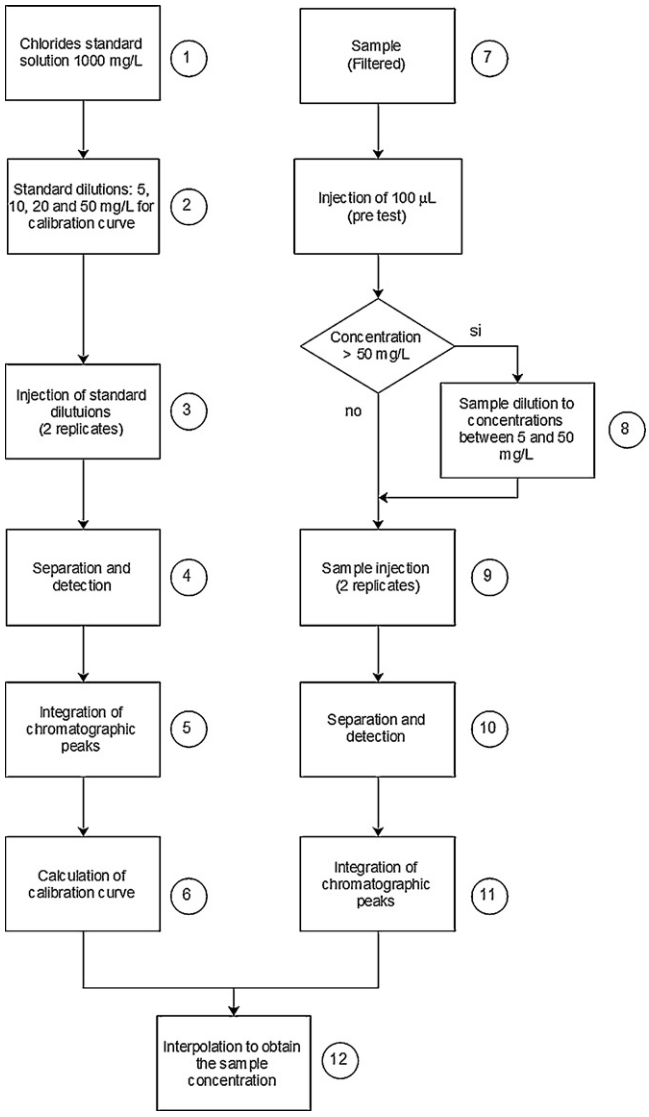

Fig. 2. Flowchart of chlorides determination.

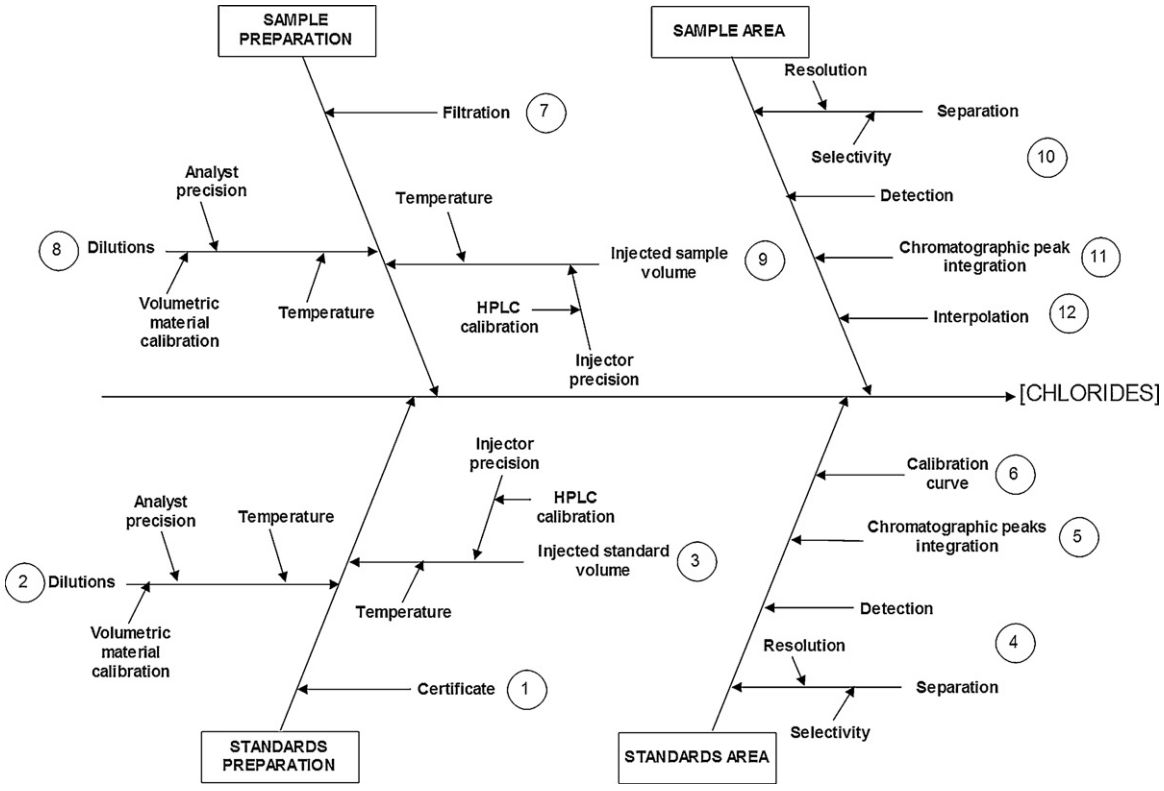

Fig. 3. Cause–effect diagram of chlorides determination in lixiviate samples.

4.5. Suitability test

Injecting a blank twice tests the absence of interference peaks. Moreover, the variation of the response factor when injecting twice each of the measurement standards is also verified and must be less than 5%.

4.6. Quantification

The method of the least squares is used to generate the calibration curve, using the areas and concentrations corresponding to each measurement standard. The concentration is obtained by multiplying the inverse of the dilution factor of the sample and the result of the interpolation of the area value on the calibration curve. A flowchart of the analytical procedure is shown in Fig. 2.

Fig. 3 gathers all the different sources of uncertainty of the analytical procedure. Each analytical stage contributes to the global uncertainty. All sources of error are also shown in the diagram. These can be systematic sources, like the calibration of the volumetric material, or random sources of error such as the precision of the analyst.

Table 2
Chromatographic conditions for the determination of chlorides by HPLC.

HPLC chromatographic conditions		
Injector	Type	Waters 717
	Injected volume	100 μL
	Draw rate	5.00 μL/s
	Syringe volume	250 μL
	Loop volume	200 μL
Column	Type	IC-Pak Anion 10 μm, 4.6 mm × 50 mm
	Flow	1.2 mL/min
	Mobile phase	86% Milli-Q water 12% ACN 2% borate-gluconate buffer
Detector	Type	Waters 430
	Temperature	ON
	Range	500 μS
	Polarity	+

4.7. Validation

The results of the linearity, accuracy and precision parameters are presented. These parameters are later used in the uncertainty calculation.

4.7.1. Linearity

The linearity is studied analyzing three times chlorides measurement standards at six concentration levels: 5, 10, 20, 40, 50 and 80 mg/L. The average area is plotted versus the concentration (Fig. 4) performing a least squares adjustment and obtaining a coefficient r^2 of 0.99992.

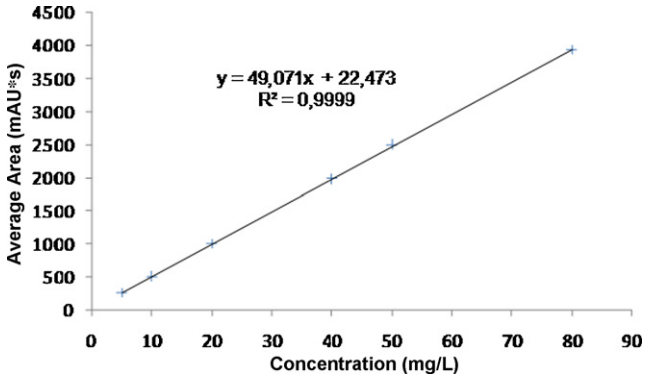

Fig. 4. Chlorides calibration curve.

Table 3
Linearity study.

Concentration (mg/L)	A (mUA s)	F_R (mg/(L mUA s))	CV (%)	Residual (%)
5	261	0.0192	0.38	2.6
10	509	0.0197	0.10	0.81
20	1008	0.0199	0.21	0.38
40	1983	0.0202	0.20	0.12
50	2498	0.0200	0.28	0.90
80	3935	0.0203	0.029	0.32

In Table 3, the average area (A), the response factors (F_R), the repeatability of the response factors (CV%) and the residual error (residual) are presented for each concentration of measurement standard.

4.7.2. Accuracy and precision

In order to study the accuracy and precision of the method samples at three levels of concentration are prepared. These chloride samples of 50, 500 and 6000 mg/L cover the range of the procedure. There are nine determinations available for each level of concentration, as three preparations are made for three days (3 levels × 3 days × 3 preparations).

The accuracy (studied as recovery) is obtained from the nine determinations made for each level of concentration, bearing in mind the sample concentration and in the added sample (Fig. 5).

The repeatability for each level of concentration is calculated as the average of the three coefficients of variation for the three results of each day (Eq. (1)). On the other hand, the intermediate precision is obtained taking the set of nine determinations used in the calculation of the variation coefficient.

The obtained average results of recovery, repeatability and intermediate precision for each concentration level are shown in Table 4.

4.8. Uncertainty

Measurement standard uncertainty ($u_{standard}$ %) is obtained from the uncertainty certified by the manufacturer of the measurement standard, and the uncertainty associated with its preparation by dilution.

The uncertainty value related to the certificate from the manufacturer can be obtained from the nominal value. The chlorides measurement standard used is 995–1005 mg/L. Therefore, the tolerance introduced by the measurement standard (±0.5%) is divided by two obtaining an uncertainty value of 0.25%.

The contribution of the dilution process to the uncertainty value is calculated for the worst-case scenario. In this case, the calculation is made for the preparation of the 50 mg/L measurement standard, due to the volumetric flask and the pipette used, it is the one with the highest uncertainty in its preparation process. The 5 mL pipette and the 100 mL volumetric flask have an uncertainty of 0.12% and 0.046%.

With the quadratic addition of the contributions of the certified measurement standard and the diluting process, a value of a 0.28% of uncertainty in the measurement standard is obtained.

Table 4
Study of accuracy and precision.

Concentration (mg/L)	Recovery (%)	CV repeatability (%)	CV intermediate precision (%)
50	98.3	0.26	0.29
500	99.8	1.4	1.3
6000	99.4	1.4	2.3

Table 5
Instrumental system uncertainty.

Concentration (mg/L)	$u_{precision}$ (%)[a]	$u_{accuracy}$ (%)[b]	$u_{instrumental\ system}$ (%)
5	0.22	2.6	2.6
10	0.058	0.81	0.81
20	0.12	0.38	0.40
40	0.12	0.12	0.17
50	0.16	0.90	0.91
80	0.017	0.32	0.32

[a] CV%/SQR(n) (n = 3).
[b] Residual (%).

The *uncertainty of the instrumental system* ($u_{instrumental\ system}$ %) is obtained from the data acquired in the linearity test (see Table 3). The obtained results are shown in Table 5.

The uncertainty of the instrumental system is established as 2.6%, the worst of the values obtained.

The *uncertainty related to the sample* (u_{sample} %) is calculated using the data obtained in the precision test (intermediate precision) and the accuracy referred to as bias (see Table 4). The results are shown in Table 6.

Table 7 summarizes the uncertainty u (%) values obtained for each level of concentration, calculated from the quadratic addition of the contributions from the measurement standard, the instrumental system and the sample.

Fig. 6 gathers the various contributions in each term of Eq. (2). As shown in the figure, these three terms include all sources of uncertainty of the analysis made.

5. Application to the determination of palmitic and stearic acids by GC in magnesium stearate samples

5.1. Analysis procedure

The analysis of palmitic and stearic acid in a magnesium stearate sample is performed by gas chromatography, with a previous methylation of the fatty acids. The quantification is carried out using internal normalization. According to European Pharmacopoeia's specifications, the sample must contain a minimum of 40% stearic acid, and the minimum percentage of both stearic and palmitic acid must be of 90% [17].

5.2. Reagent and measurement standards

The reagents used in the analysis and the validation are the following: boron trifluoride in methanol (CH_3OH/BF_3) from Sigma–Aldrich, heptane from Fluka, sodium chloride from Panreac, anhydrous sodium sulfate from Panreac, palmitic acid from Sigma–Aldrich, stearic acid from Sigma–Aldrich, methyl palmitate from Fluka, methyl stearate from Sigma–Aldrich, methyl laureate from Sigma–Aldrich, methyl myristate from Sigma–Aldrich, methyl oleate from Sigma–Aldrich, and methyl arachidate from Fluka.

To carry out the suitability test, a solution is prepared by dissolving 50 mg of methyl palmitate and 50 mg of methyl stearate in 10 mL of heptane.

Table 6
Uncertainty associated with sample.

Concentration (mg/L)	$u_{precision}$ (%)[a]	$u_{accuracy}$ (%)[b]	u_{sample} (%)
50	0.10	1.7	1.7
500	0.44	0.19	0.48
6000	0.76	0.61	0.97

[a] CV%/SQR(n) (n = 9).
[b] Average(Bias(%)).

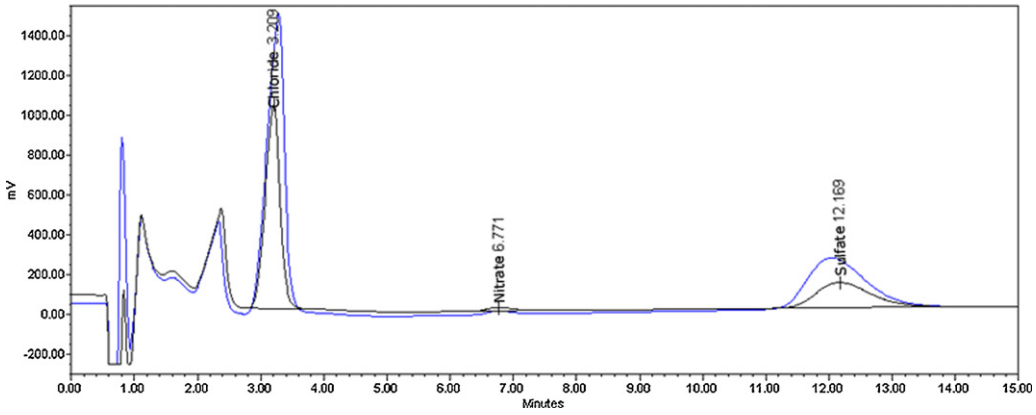

Fig. 5. Chromatograms of the sample (black) and added sample with 50 mg/L of chlorides (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 7
Uncertainty and tolerance.

Concentration (mg/L)	$u_{standard}$ (%)	$u_{instrumental\ system}$ (%)	u_{sample} (%)	u (%)	Tolerance (%)	Concentration limits (mg/L)
50	0.28	2.6	1.7	3.1	±6.2	47–53
500	0.28	2.6	0.48	2.6	±5.2	474–526
6000	0.28	2.6	0.97	2.8	±5.5	5670–6330

5.3. Equipment and chromatographic conditions

The equipment used were a HP 6890 Series GC System gas chromatograph with a flame ionization detector (FID) and a scale Mettler Toledo AB204 (min = 10 mg; max = 210 g; e = 1 mg; d = 0.1 mg). The chromatographic conditions that have been used, which differ from European Pharmacopoeia's ones, are shown in Table 8.

5.4. Sample preparation

The sample is prepared by methylation of the fatty acids using boron trifluoride in methanol (CH_3OH/BF_3) as the methylating agent. To carry out this methylation, 0.10 g of the sample of magnesium stearate is dissolved in 5 mL of boron trifluoride in methanol, and the solution is refluxed for 10 min. Then, 4 mL of heptane is added, and the solution is boiled again for 10 more minutes. After that, let it cool down and add 20 mL of saturated sodium chloride solution. Shake the mixture and allow the aqueous and organic phases to separate. 2 mL of the organic phase is taken and dried with 0.2 g of anhydrous sodium sulfate. Take 1 mL of the solution and make up to 10 mL [18].

Table 8
Chromatographic conditions for the fatty acids determination by HRGC.

HRGC chromatographic conditions			
Injector	Injected volume	1 μL	
	Carrier gas	Helium	
	Type injection	Spliter	
	Spliter flow	20 mL/min	
	Spliter relation	1:25	
	Temperature	250 °C	
Column	Type	Supelco SP 2380 Capillary Column	
	Dimensions	60 m × 250 μm × 0.20 μm	
	Flow (constant)	0.8 mL/min (P ~ 24 psi)	
	Time (min)	Temperature (°C)	Ramp (°C/min)
Oven	0–1	150	–
	1–28.5	150–260	4
	28.5–38.5	260	–
Detector FID		Air	450 mL/min
		Hydrogen	40 mL/min
		Auxiliary gas (N_2)	20 mL/min
		Temperature	250 °C

5.5. Suitability test

Through the injection of the solution prepared to carry out the suitability test, it is verified that the relative retention time between methyl palmitate and methyl stearate is around 0.88, and the resolution between these two peaks is higher than 5 [17] (Fig. 7).

5.6. Quantification

The quantification of the percentage of palmitic and stearic acids and of the total percentage of both acids is carried out using internal normalization. For this purpose, in each chromatogram of

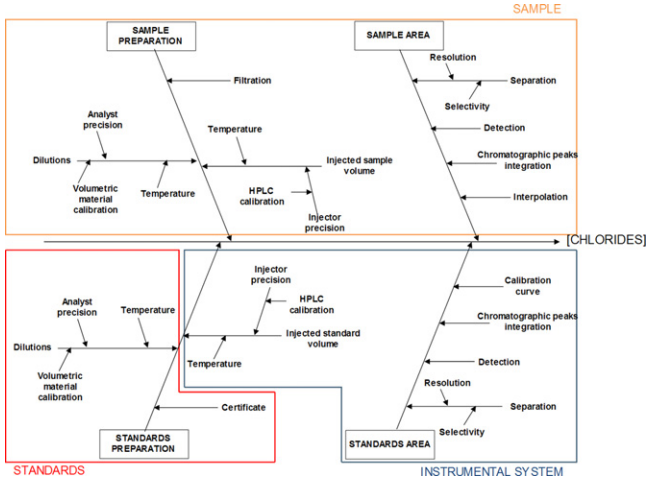

Fig. 6. Cause–effect diagram of chlorides determination in lixiviate samples grouped by source of uncertainty.

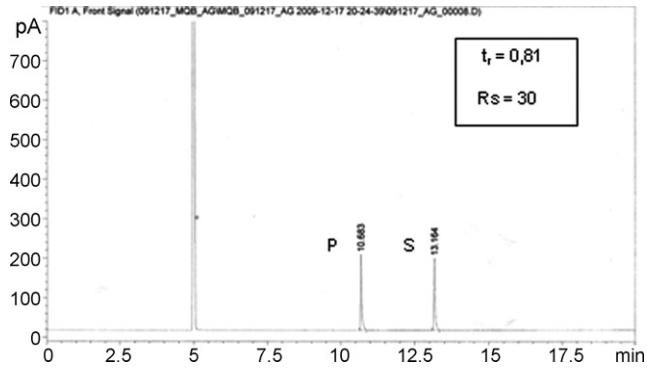

Fig. 7. Chromatogram of the reference solution P = methyl palmitate; S = methyl stearate.

the sample solution (Fig. 8), the area percentage of the peaks of methyl palmitate and methyl stearate is calculated with respect to the total area, without taking into account the peak of the solvent. Thus, the percentage of methyl esters is directly assimilated to the percentage of their corresponding fatty acids. A flowchart of the analytical procedure is shown in Fig. 9.

In Fig. 10, a diagram with the uncertainty sources of the analytical procedure is presented. In this case, the terms which correspond to the standards (preparation and area) do not appear because, as the method used is internal normalization, they do not intervene in the calculation of the percentage of fatty acids. That is why the uncertainty sources come exclusively from the sample preparation and the quantification of the various methyl esters.

5.7. Validation

The parameters of accuracy and precision are presented, as these are the ones that intervene in the calculation of the uncertainty in this case.

5.7.1. Accuracy

The accuracy is studied by the methylation of standards of stearic and palmitic acids. Solutions with a percentage higher and lower than 40% of stearic acid, specified by European Pharmacopoeia, are prepared.

In particular, standards are prepared twice with nominal proportions of 35–65, 40–60, 50–50 and 60–40% of stearic acid and palmitic acid, respectively. These standards are methylated and analyzed following the same procedure as the one used for magnesium stearate. The accuracy is calculated as the percentage of recuperation with respect to the theoretical proportion of esters.

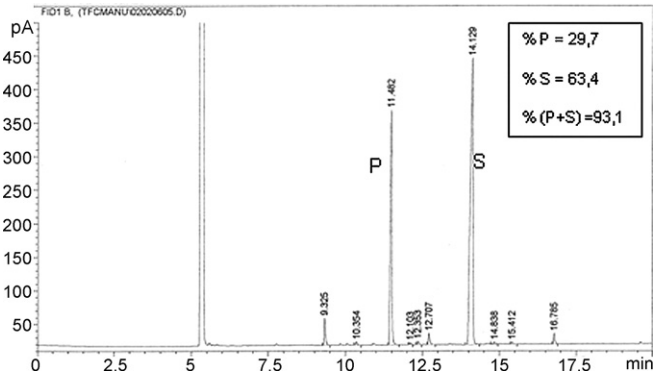

Fig. 8. Chromatogram of the sample solution P = methyl palmitate; S = methyl stearate.

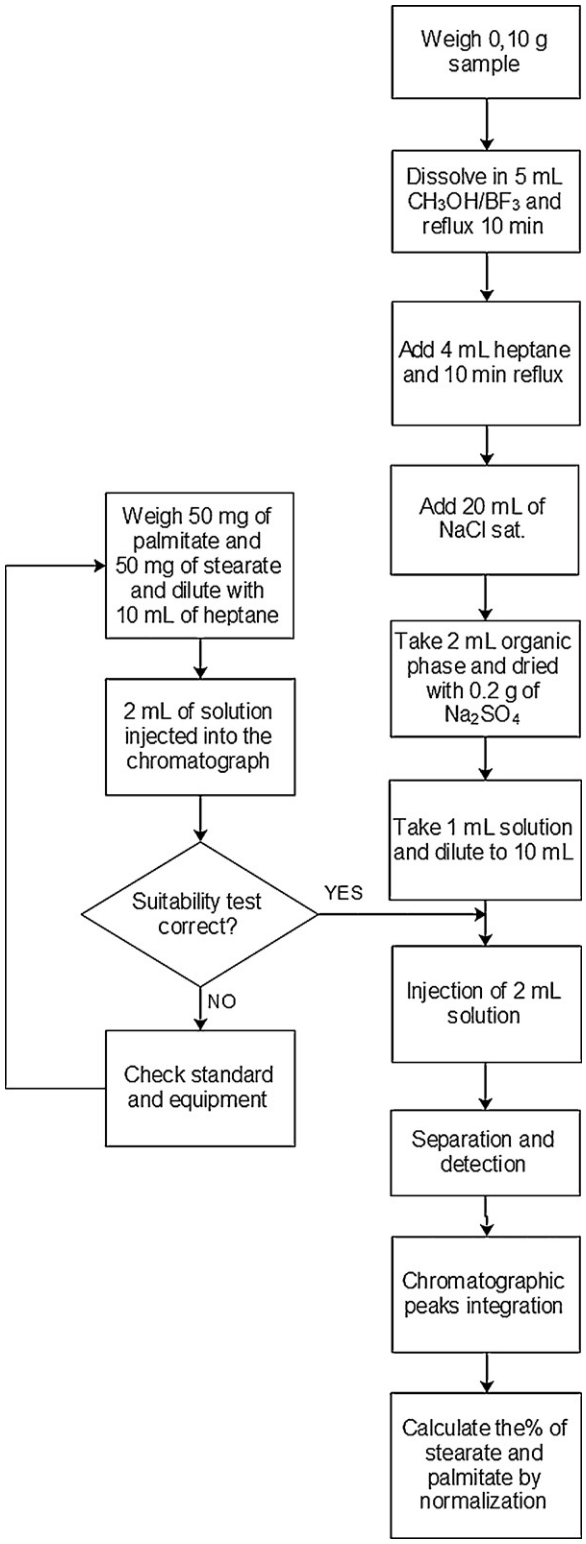

Fig. 9. Flowchart of fatty acids determination in magnesium stearate samples.

The results in Table 9 show the average result of injecting each standard twice.

5.7.2. Precision

The precision of the method is calculated studying the repeatability of preparing 5 independent samples of magnesium stearate.

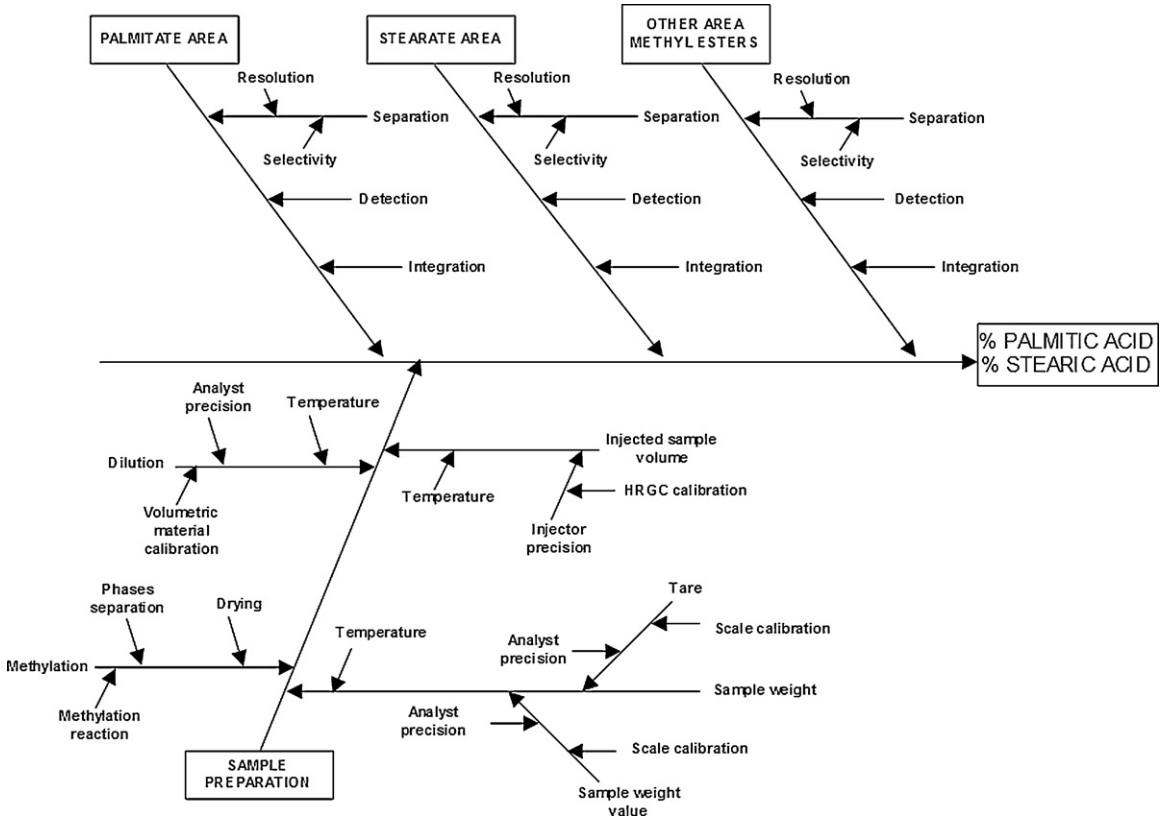

Fig. 10. Cause-and-effect diagram of fatty acids determination in magnesium stearate samples.

Table 9
Accuracy.

Nominal relation	Stearic acid			Palmitic acid		
	% theoretical	% experimental	% recovery	% theoretical	% experimental	% recovery
35–65	35.1	35.5	101.0	64.9	64.6	99.5
	36.0	36.9	102.4	64.0	63.1	98.6
40–60	41.0	42.2	103.0	59.0	57.8	97.9
	40.4	40.7	100.8	59.6	59.3	99.5
50–50	50.1	51.5	102.7	49.9	48.5	97.3
	50.3	50.3	99.9	49.7	49.7	100.1
60–40	60.4	61.6	102.0	39.6	38.4	97.0
	60.0	61.1	101.8	40.0	38.9	97.3
		% Average	101.7		% Average	98.4
		CV (%)	1.0		CV (%)	1.2

Table 10 shows the average percentage of palmitic and stearic acid of each of the samples injected three times.

5.8. Uncertainty

The uncertainty of the method is calculated according to Eq. (2). In this case, the term $u_{standard}$ is eliminated because the concentration is calculated by internal normalization.

The term $u_{instrumental\ system}$, whose error sources come from the resolution, calibration and stability of the measurement, is considered negligible. The resolution is included in the precision, the contribution of the calibration is not taken into account because the standards are not used to calculate the concentration of the sample, and the stability of the measurement is negligible because it

Table 10
Repeatability of the sample.

	% methyl palmitate area	% methyl stearate area	% palmitate and stearate area
Sample 1	29.6	63.3	92.9
Sample 2	29.6	63.3	93.0
Sample 3	29.8	63.8	93.6
Sample 4	29.8	63.7	93.4
Sample 5	29.6	63.5	93.1
Average	29.7	63.5	93.2
s	0.081	0.22	0.29
CV (%)	0.27	0.35	0.31

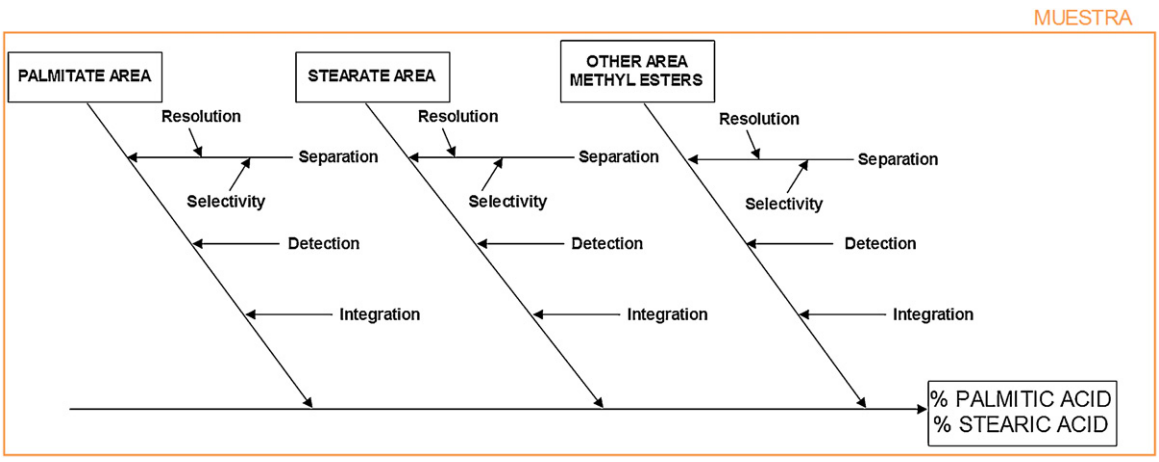

Fig. 11. Cause-and-effect diagram of fatty acids determination in magnesium stearate samples without the contribution of the sample preparation.

Table 11
Response factors for the mixture of methyl esters.

	Carbons	$t_{retention}$ (min)	F_R ((mg/(L mUA s))	CV (%)
Methyl laureate	C13	7.2	1.00	2.5
Methyl myristate	C15	8.6	1.02	4.5
Methyl palmitate	C17	10.6	1.04	5.6
Methyl stearate	C19	13.1	1.00	6.1
Methyl oleate	C19 (=)	13.7	0.96	6.0
Methyl arachidate	C21	15.8	0.95	6.3

Table 12
Hypothesis of response factors for methyl stearate and methyl palmitate.

Nominal proportion	Methyl stearate		Methyl palmitate	
	F_R (mg/(L mAU s)	CV (%)	F_R (mg/(L mAU s)	CV (%)
35–65	1.00	1.8	1.02	1.4
40–60	1.00	0.64	1.01	0.62
45–55	1.00	0.26	1.00	0.42

has been proved that the response factors of the different analytes found in the samples are statistically identical.

To prove this affirmation, a mixture of standards of methyl esters in heptane, has been injected in the chromatograph. ANOVA statistic proof shows that there are no significant differences between the response factors of the methyl esters from 13 to 21 carbon atoms [19]. Table 11 shows the response factors obtained when injecting twice the mixture prepared of standards of methyl esters, normalized with respect to methyl stearate.

On the other hand, ANOVA statistic proof shows that the response of methyl stearate and methyl palmitate does not depend on the proportion the two analytes in the sample. Different mixtures of methyl stearate and methyl palmitate have been prepared, in concentrations lower and higher than 40% of methyl stearate, dissolving them in heptane and performing six injections. Table 12 shows the response factors normalized with respect to methyl stearate.

For the calculation of the uncertainty, the only term whose contribution has to be taken into account is u_{sample} (Eq. (15)):

$$u\ (\%) = \sqrt{u_{standard}^{\ 2}\ (\%) + u_{instrumental\ system}^{\ 2}\ (\%) + u_{sample}^{\ 2}\ (\%)} \quad (15)$$

(0)

Nevertheless, since the quantification is carried out calculating the percentage of the areas of the peaks of methyl palmitate and methyl stearate with respect to the total area, the preparation of the sample will not be taken into account either (Eq. (16)):

$$u\ (\%) = u_{\ sample}\ (\%) = \sqrt{u_{preparation}^{\ 2}\ (\%) + u_{precision}^{\ 2}\ (\%) + u_{accuracy}^{\ 2}\ (\%)} \quad (16)$$

Thus, the expression of the calculation of the uncertainty is simplified according to Eq. (17):

$$u\ \ (\%) = u_{sample}\ \ (\%) = \sqrt{u_{precision}{}^{2}\ \ (\%) + u_{accuracy}{}^{2}\ \ (\%)} \quad (17)$$

Table 13
Uncertainty.

	$u_{precision}$ (%)[a]	$u_{accuracy}$ (%)[b]	u (%) = u_{sample} (%)	Tolerance (%)
Methyl stearate	0.12	1.7	1.7	±3.4
Methyl palmitate	0.16	1.6	1.6	±3.2

[a] CV%/SQR(n) (n = 5).
[b] Average(Bias(%)).

In Table 13, a summary of the obtained results of uncertainty is presented.

So, as can be observed in Fig. 11, all the sources of uncertainty of the analysis only come from the analysis of the sample.

6. Conclusions

The model designed to calculate the uncertainty can take advantage of the results obtained during validation of analytical procedures. This approach allows the optimization of resources and obtaining measures of uncertainty that have proven suitable for the intended purpose.

Cause–effect diagrams are a fundamental tool to identify all sources of error in the calculation of uncertainty. Different sources of error are grouped into contributions of the standard, the instrumental system and the sample.

The estimated uncertainty in the determination of chlorides by liquid chromatography in lixiviates includes the three terms considered (standard, instrumental system and sample) and evaluates the contribution of each one of them.

The estimated uncertainty in the determination of fatty acids by gas chromatography is reduced to the contribution of the sample term. This simplification is justified due to the quantification is performed by internal standardization.

References

[1] EURACHEM/CITAC, Guide CG 4 Quantifying Uncertainty in Analytical Measurement, 2000.
[2] Evaluation of Measurement Data—Guide to the Expression of Uncertainty in Measurement (Gum), 2008.
[3] P. Konieczka, J. Namiesnik, J. Chromatogr. A 1217 (2010) 882.
[4] CAC/GL 59, Guidelines on Estimation of Uncertainty of Results, 2006.
[5] A. Maroto, R. Boque, J. Riu, F.X. Rius, Quim. Anal. 19 (2000) 85.
[6] V.R. Meyer, J. Chromatogr. A 1158 (2007) 15.
[7] S. Leito, K. Molder, A. Kunnapas, K. Herodes, I. Leito, J. Chromatogr. A 1121 (2006) 55.
[8] Analytical Methods Committee, The Royal Society Of Chemistry, Uncertainty of Measurement: Implications of its Use in Analytical Science, 1995, p. 2303.
[9] Norma UNE-EN-ISO 17025:2005, Requisitos Generales Para La Competencia De Los Laboratorios De Ensayo Y Calibración, 2009.
[10] Asociación Española De Farmacéuticos De La Industria (Aefi), Validación De Métodos Analíticos, Monografía De Aefi, Gispert, La Bisbal (Girona), 2001, p. 71, 72, 84, 122, 288, 328.
[11] L.E. Vanatta, D.E. Coleman, J. Chromatogr. A 1158 (2007) 47.
[12] J. Báguena-Polo, G. Gotor-Navarro, F. Broto-Puig, M. Blanco-Roca, Afinidad 533 (2008) 65.
[13] USP 34-NF 29, Validation on Compendial Procedures, 2011, p. 749.
[14] Food and Drug Administration Center for Veterinary Medicine, Guidance for Industry, Validation of Analytical Procedures: Methodology, 20th ed., American Public Health Association, Washington, DC, 1999.
[15] V.J. Barwick, J. Chromatogr. A 849 (1999) 13.
[16] A.D. Eaton, L.S. Clesceri, A.E. Greenberg, Standard Methods for the Examination of Water and Wastewater, 20th ed., Water Environment Federation, Washington, DC, 1998, p. 4.
[17] European Pharmacopoeia 7.0, Magnesium Stearate, 2010, p. 2418.
[18] European Pharmacopoeia 7.0, Fatty Acids, Composition by Gas Chromatography Method C, 2010, p. 119.
[19] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, 2nd ed., Prentice Hall, New York, 2002.