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# Evaluation of the uncertainty associated with the off line HPLC–GC(FID) determination of 4-desmethyl sterols in vegetable oils

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ABSTRACT

This paper discusses the estimation of the uncertainty of the chromatographic determination of 4-desmethyl sterols in vegetable oils, combining the off line HPLC fractionation of the analytes, from the unsaponifiable fraction of the samples, with their determination as TMS derivatives by GC(FID), using the data obtained from a single internal calibration (one surrogate) at one level and "bottom up" approach. The methodology used, makes possible to identify the main uncertainty contributions, find their origins, and reduce them. The final results show that the main contributions to the relative overall uncertainty are those closely related with the chemical aspects of the method, i.e. those related to derivatization reaction and quantification of the analytes, although others aspects, such as the addition of a mass of surrogate, are not negligible.



## 1. Introduction

Nowadays, it is well established that the information obtained from analytical measurements must be accompanied with its uncertainty, in order to assure the reliability of the results. The International Organization for Standardization (ISO) has established general rules for evaluating and expressing the uncertainty for a wide range of measurements, which have been applied to analytical chemistry by EURACHEM (A Focus for Analytical Chemistry in Europe) and CITAC (The Cooperation on International Traceability in Analytical Chemistry). Different authors have developed different approaches for its evaluation: the bottom-up and top-down strategies [1–5] are the most used, although there are other as, fitness-for-purpose, validation-based and robustness-based [6,7], that can also be applied.

According to the last version of the Guide for the Expression of Uncertainty in Measurement (GUM) [8], and as some authors has been pointed out [6], to determine the uncertainty of analytical results using a bottom-up approach, the following steps must be satisfied: (1) to define the measurement procedure and the measurand; (2) to establish a mathematic model from which the analyte concentration can be obtained; (3) to assign the values to all the possible parameters that could affect the final result of the analysis, as well as to determine the standard uncertainties of each of them; (4) to apply the principles of uncertainty propagation and (5) to express the final result as result ± expanded uncertainty (K factor).

Bagur et al. [9] consider that the main uncertainty sources of an analytical method are:

- Operational or working uncertainty ($u_{working}$), due to various factors such as instrumental effects, reagents purity, measurement conditions and sample handling, as more important.
- Recovery uncertainty ($u_{recovery}$), which comes from the bias error associated with the method.
- Inherent uncertainty ($u_{inherent}$), which comes from factors not controlled by the operator that affects directly to the analytical results. It has two components associated with two aspects of the chemical measurement process:
  - (i) The Intrinsic uncertainty ($u_{intrinsic}$), closely related to the chemical stages indicated in the procedure, depends on the chemical parameters.
  - (ii) The chemical calibration uncertainty ($u_{chem\text{-}cal}$), is related to the chemical calibration process provoked by the transformation of the analytical signal in concentration and the acceptance of a normal distribution in the generation of the analytical signal [10].

One of the chemical calibration methodologies most used in routine chromatographic analysis is the internal calibration (I.C.) [11], because it combines several advantages:

(i) It is possible to carry out the simultaneous quantification of several analytes with one sample portion, using an internal standard (I.S.) or surrogate which represents all the analytes.

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(ii) The analysis time is reduced, since only one analytical preparation for calibration and quantification is needed.
(iii) It makes up for the losing of analyte during sample preparation and, in a moderate way, for the matrix effect. That is why, it is advisable its use when at least one of the following circumstances is present: (a) the sample preparation process is long and complicated; (b) a long time for the measure is required and (c) there is no, or it is impossible to acquire analyte standard.

The olive oil official analytical methods of the European Union (UE) for the determination of waxes [12], sterols [13], stigmastadienes [14], and aliphatic alcohols [15] by capillary column gas chromatography, constitute examples of common application of I.C. In these methods, a one level I.S. calibration, for the simultaneous quantification of analytes belonging to the same chemical family, is used.

In our opinion, for these cases, the chemical calibration uncertainty could be redefined as "quantification uncertainty ($u_{quantif.}$)" considering that the use of one level internal calibration with a surrogate implies that the calibration is implicit in the quantification process. Thus, in the estimation of this source of uncertainty, it is necessary to consider that both analytical signal, i.e. very analyte peak area, and surrogate area, are correlated. This fact must be taken into account for uncertainty budget, mainly due to it is implicit into the equation used to estimate the concentration of the analytes, which is given by

$$C_{sterol(i)} = 1000 \times \frac{m_{surrogate}}{m_{sample}} \times \frac{A_{sterol(i)}}{A_{surrogate}} \quad (1)$$

where $C_{sterol(i)}$ is the concentration of sterol "i" in the oil sample analyzed, expressed in mg $kg^{-1}$; $m_{surrogate}$ is the mass of surrogate, expressed in mg; $m_{sample}$ is the mass of oil sample, expressed in g; $A_{sterol(i)}$ is the peak area of sterol "i", expressed in arbitrary units; $A_{surrogate}$ is the peak area of surrogate, expressed in arbitrary units; 1000 is the conversion factor to express the concentration of the analytes in mg $kg^{-1}$.

This paper presents a procedure to estimate the uncertainty associated with the determination of 4-desmethyl sterols in 24 vegetable oil samples, using a bottom-up strategy. The analytical methodology implies the fractionation of sterols from the saponification extract by HPLC and its quantification by GC(FID), using an internal calibration at one level, with 5-cholestanol as surrogate.

## 2. Experimental

### 2.1. Apparatus and software

The liquid chromatograph consisted of a Hewlett Packard 1050 series equipped with an UV–visible variable wavelength detector, Rheodyne (Rheodyne, Inc. Cotati, Ca, USA) 7125 loop injector with a 20 μl sample loop, and a 3396-A integrator. A Lichrospher 100 CN (244 × 4.5 mm i.d., 5 μm) column with a Lichrospher guard column (10 × 4.6 mm i.d.) was used for the fractionation of the unsaponifiable fraction of the oil.

The gas chromatograph used in the study, equipped with a flame ionization detector (GC-FID) and a split-splitless injector, was an Agilent 6890 system (Palo Alto, CA, USA). A fused silica capillary column 25 m long DB-5 (0.32 mm i.d., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA) was used for the analysis of sterols as trimethylsilyl ethers.

A Vortex Heidolph mixer, model Reax 2000, a BHG Fixette 2 centrifuge and a heater, model Selecta were used. Agilent ChemStation was used for data acquisition and processing.

### 2.2. Chemical and reagents

A 2 M potassium hydroxide (Panreac, Castellar del Vallès, Barcelona, Spain) solution in ethanol was prepared, adding 20 mL of distilled water to a 13 g of potassium hydroxide and, after shaking, the solution was made up to 100 mL with ethanol. This solution was kept in a well-stoppered dark glass bottle.

Pyridine (99.5% purity) from Panreac, hexamethyldisilazane and trimethylchlorosilane (97% purity) from Sigma Chemical Co. (St. Louis, MO, USA), were used. In order to form TMS derivatives a combination of pyridine: hexamethyldisilazane: trimethylchlorosilane (9:3:1, v/v/v) was used as derivatization mixture (DM).

A 0.2% (m/v) 5α-cholestan-3β-ol (cholestanol) (Sigma Chemical Co. (USA)) solution was prepared by adding 10 mL of ethyl acetate to 20 ± 0.01 mg of cholestanol and shaking with the Vortex mixer until complete dissolution.

### 2.3. Procedure

To 5 g of sample, 500 μL (for extra virgin and refined olive oils) or 1500 μL (for olive pomace and vegetable oils) of the cholestanol solution (I.S) are added, and the saponification of the sample is made according to the COI procedure [13]. Then, the separation of the sterols family is made by HPLC, following the next steps: the dry residue containing the insaponifiable matter is dissolved in 1 mL of a n-hexane:tert-butylmethylether (TBME) (80:20) mobile phase and 20 μL are injected in an HPLC-UV system, using the conditions described in Fig. 1. The sterols are collected in the time interval indicated in the figure and derivatized using 200 μL of D.M./mg sterols. Finally, the analytes are determined by GC-FID using the conditions established in the official procedure.

This procedure could be considered as a possible alternative to the official analytical procedure for sterols determination [13], in which, the time-consuming and the tedious stage of TLC is replaced for an off line HPLC stage.

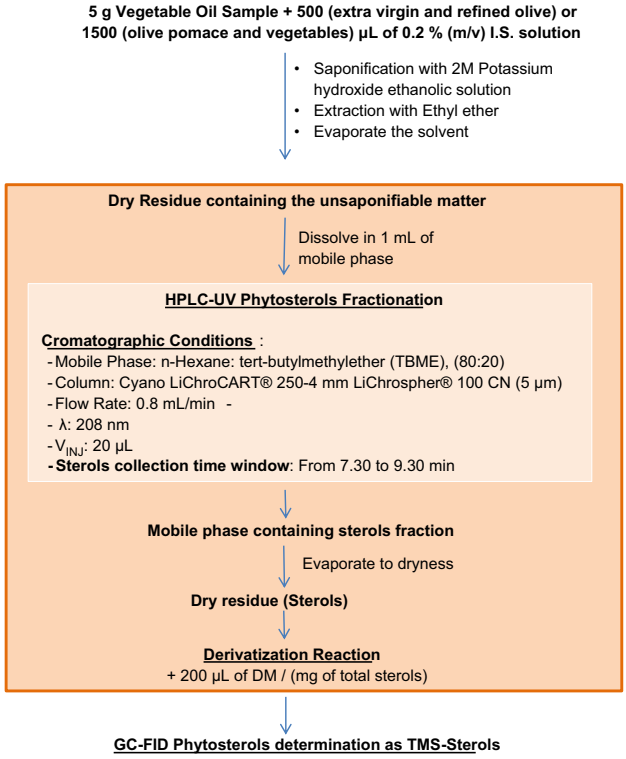


**Fig. 1.** Procedure for the off line HPLC–GC(FID) determination of 4-desmethyl sterols in vegetable oils. The boxes include the changes with respect the method of COI.

**Table 1**
Types of vegetable oils analyzed and 4-desmethyl phytosterols determined.

| Vegetable oils ($n$)[a] | Code | Analyte | Code |
|---|---|---|---|
| | | cholesterol | (1) |
| Extra virgin olive (3) | (EVOO) | brassicasterol | (2) |
| Refined olive (1) | (ROO) | 24-methylen-cholesterol | (3) |
| Pomace olive (3) | (POO) | campesterol | (4) |
| Sunflower (1) | (SFO) | campestanol | (5) |
| High oleic Sunflower (2) | (SFO(o)) | stigmasterol | (6) |
| Rapeseed (2) | (RO) | $\Delta^7$-campesterol | (7) |
| Canola (2) | (CanO) | $\Delta^{5,23}$-stigmastadienol | (8) |
| Soybean (2) | (SyO) | clerosterol | (9) |
| Corn (2) | (CO) | β-sistosterol | (10) |
| Peanut (2) | (PeaO) | sitostanol | (11) |
| Grapeseed (2) | (GO) | $\Delta^5$-avenasterol | (12) |
| Sesame (2) | (SesO) | $\Delta^{5,24}$-stigmastadienol | (13) |
| | | $\Delta^7$-stigmastenol | (14) |
| | | $\Delta^7$-avenasterol | (15) |

[a] Number of samples analyzed.

### 2.4. Samples

The method was applied to twenty four trade mark edible vegetable oils, from different origins, Spain, France, Mexico and USA, purchased from local market or gourmet shops. The type of oil and the different 4-desmethyl phytosterols analyzed are shown in Table 1. Fig. 2 shows typical GC(FID) chromatograms obtained for two of the samples analyzed: (a) extra virgin and (b) pomace olive oil.

## 3. Results and discussion

The different contributions to the overall uncertainty associated with the proposed method, are shown in a cause-effect diagram, also called Ishikawa diagram (Fig. 3)

It can be seen that the uncertainty arises mainly from:

### 3.1. Working or operational or uncertainty: $u_{working}$

For the basic equipment the $u^{rel}$ values were calculated considering the manufacturers' specifications, taking into account that, for the microbalances and pipettes used for the addition of surrogate to extra virgin and refined olive oil samples, a verification process was developed in the laboratory, and, for the rest of volumetric material (pipettes and volumetric flasks), were calculated from the data shown in Table 2(b).

The representative $u^{rel}_{working}$ of this source of uncertainty is given by

$$u^{rel}_{working} = \sqrt{\left(u^{rel}_{mass\text{-}surrogate}\right)^2 + \left(u^{rel}_{mass\text{-}sample}\right)^2} \quad (2)$$

#### 3.1.1. Mass surrogate ($u_{mass-surrogate}$):

As it can be seen in Fig. 3, $u_{mass\text{-}surrogate}$ is affected by the uncertainty of the stock solution of surrogate and the uncertainty associated to the volume of this solution added to the oil sample, before the saponification step. Expressed as relative uncertainty, it is given by the next equation:

$$u^{rel}_{mass\text{-}surrogate} = \sqrt{\left(u^{rel}_{mass\text{-}surrogate\ stock\ solution}\right)^2 + \left(u^{rel}_{pipette}\right)^2} \quad (3)$$

The relative uncertainty associated to the preparation of the surrogate stock solution, among others factors, depends on the purity of the surrogate (PUR) used as internal standard. Since the manufacturers do not supply any uncertainty concerning this

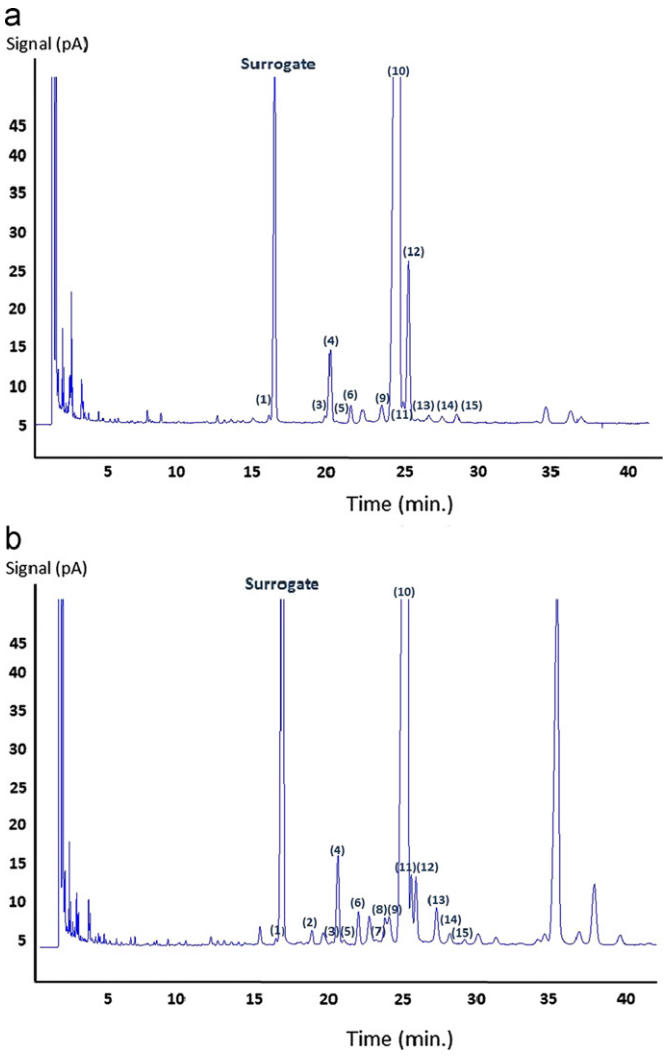


**Fig. 2.** GC(FID) chromatograms obtained for two of the samples analyzed: (a) Extra virgin olive oil (EVOO-1), and (b) Pomace olive oil (POO-1). The analytes are coded as in Table 1.

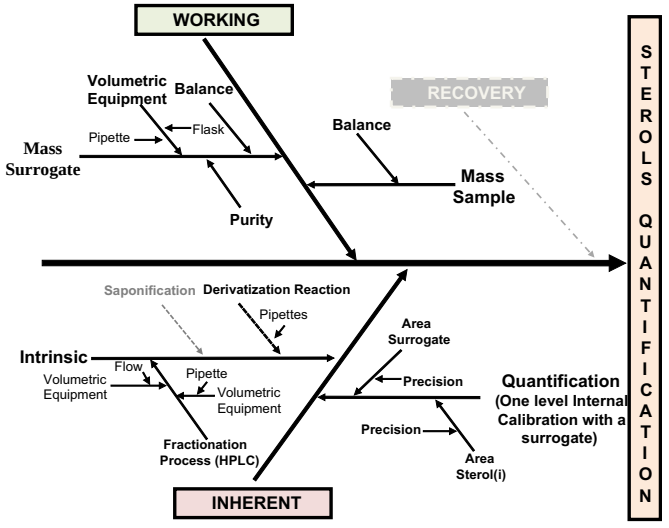


**Fig. 3.** Cause and effect diagram (Ishikawa diagram)showing the different uncertainty sources associated to the off line HPLC–GC(FID) determination of 4-desmethyl sterols in vegetable oils.

**Table 2**
Uncertainty values for basic equipment used in the procedure.

| **(a)** Equipment verified in the laboratory using an internal quality management plan | | | | |
|---|---|---|---|---|
| Microbalances | **Precision (g)** | **Mass (g)** | **Correction (g)** | **U[a](g)** |
| | 0.0001 | 1 | 0.0001 | 0.0003 |
| | 0.001 | 10 | Not needed | 0.002 |
| Mechanic pipettes | **Range (μL)** | **Volume (mL)** | **Correction (mL)** | **U (mL)** |
| | 100–1000 | 0.1 | 0.000 | 0.002 |
| | 100–1000 | 0.2 | −0.001 | 0.002 |
| | 100–1000 | 0.3 | −0.001 | 0.002 |
| | 100–1000 | 0.5 | 0.001 | 0.002 |
| | 100–1000 | 0.9 | −0.005 | 0.002 |
| | 100–1000 | 1.0 | −0.005 | 0.002 |
| **(b)**Uncertainty values given by the manufacturers[b] | | | | |
| Pipettes | | **Volume (mL)** | | **u[c]for organic solvents (mL)** |
| | 2 | | 0.0092 | |
| Flasks | **Volume (mL)** | | **u for organic solvents(mL)** | |
| | 10 | | 0.025 | |

[a] Overall uncertainty.
[b] LGC/VAM/2000/053. V.J. Barwick et al., Evaluating Confidence in Analytical Measurement. Part (d): Studies of uncertainty in laboratory operations. VAM.LGC.2000.
[c] Standard uncertainty.

issue, it has been estimated considering the last significant digit of the value of stated purity. Besides, it has been assumed that the molar mass of cholestanol does not have a significant influence on the uncertainty. The relative standard uncertainty for the concentration of the stock solution arises from:

$$u^{rel}_{surrogate\ stock\ solution} = \sqrt{\frac{u^2(m_{surrogate\ stock})}{m^2_{surrogate\ stock}} + \frac{u^2(PUR)}{PUR^2} + \frac{u^2(V_{flask})}{V^2_{flask}}} \quad (4)$$

Considering the analytical procedure used, each term (expressed as secondary relative uncertainty) has been calculated by Eqs.(A.I.1–A.I.4) included in Annex I.

Finally, as the volume of the stock solution added to the oil sample depends of the type of oil analyzed, its contribution to the uncertainty has been calculated using the next equations:

(a) Addition of 500 μL of surrogate (Extra virgin and refined olive oils):

$$u^{rel}_{pipette(500)} = \sqrt{\frac{\left(maximum\ error_{verificacion\ process}/\sqrt{3}\right)^2}{V^2_{pipette(500)}}} \quad (5)$$

(b) Addition of 1500 μL of surrogate (Pomace olive and vegetable oils):

$$u^{rel}_{pipette(1500)} = \sqrt{\frac{u^2_{standard}}{V^2_{pipette(1500)}}} \quad (6)$$

### 3.1.2. Mass sample ($u_{mass-sample}$):

The uncertainty associated to the mass of oil sample weighted, expressed as relative uncertainty, was estimated by

$$u^{rel}_{mass\text{-}sample} = \sqrt{\frac{\left(balance\ maximum\ error_{verification\ process}/\sqrt{3}\right)^2}{m^2_{sample}}} \quad (7)$$

Table 3(a) shows the influence of each term (expressed as percentage) over the relative operational uncertainty of two oil samples (extra virgin and pomace olive), selected as example of calculations. As it can be seen, in both samples, the preparation and addition of the surrogate stock solution represents the major influence, being negligible the uncertainty associated with the addition of different volumes of surrogate.

## 3.2. Contribution of bias to uncertainty: $u_{recovery}$

According to ISO [16], the definition of uncertainty indicates that the presentation of results must be free from systematic errors (proportional and constant bias). In general terms, the evidence of proportional bias is assayed by recovery studies in which, prior to the sample treatment, it is spiked with known quantities of analyte, normally at different levels. Nevertheless sometimes, as in this case, these studies cannot be carried out due to, as has been quoted previously, it is not possible to acquire analyte standards and a one level internal calibration with surrogate is used for quantification purposes. That is why, this term represented in the Ishikawa diagram, has not been included in the uncertainty budget.

## 3.3. Inherent uncertainty: $u_{inherent}$

As it has been mentioned previously, the main contributions to this uncertainty are the intrinsic and quantification uncertainties, and it is calculated by the equation:

$$u^{rel}_{inherent} = \sqrt{(u^{rel}_{intrinsic})^2 + (u^{rel}_{quantif.})^2} \quad (8)$$

### 3.3.1. Intrinsic uncertainty: $u_{intrinsic}$

The intrinsic uncertainty associated to the quantification of 4-desmethyl sterols in vegetable oils depends on the following chemical steps: saponification of the sample, fractionation of the phytosterols by off-line HPLC and derivatization reaction of analytes as trimethyl-sylilsterols for GC-FID quantification purposes. From all of them, the uncertainty arising from saponification of the sample has not been considered, mainly due to the reagents and solvents used in this step are in great excess respect to surrogate and analytes, which involves a lot of chemical processes not being "under metrological control". The intrinsic uncertainty has been

**Table 3**
Contribution of different factors to the: (a) ($u^{rel}$working.) and (b) ($u^{rel}$intrinsic.) in the determination of 4-desmethyl sterols in two of the samples analyzed. (c) Contribution of $u_{intrinsic}$ and $u_{quantif.}$ to the relative inherent uncertainty ($u^{rel}$inherent) for four analytes.

| | | EVOO1 | POO1 |
|---|---|---|---|
| | | **(a) Relative working uncertainty ($u^{rel}_{working}$)** | |
| **Mass surrogate ($u_{mass\text{-}surrogate}$)** | | 99.97 | 99.97 |
| **Mass sample ($u_{mass\text{-}sample}$)** | | 0.03 | 0.03 |
| | | **(b) Relative intrinsic uncertainty ($u^{rel}_{intrinsin}$)** | |
| **HPLC fractionation of 4-desmethyl sterols ($u_{HPLC\ fractionation}$)** | | 11.1 | 11.1 |
| **Derivatization reaction ($u_{derivatization\ reaction}$)** | | 88.9 | 88.9 |
| **Analyte**[a] | | **(c) Relative inherent uncertainty ($u^{rel}_{inherent}$)** | |
| **(4)** | $u_{intrinsic}$ | 89.9 | 42.9 |
| | $u_{quantif}$ | 10.1 | 57.1 |
| **(6)** | $u_{intrinsic}$ | 88.0 | 94.8 |
| | $u_{quantif.}$ | 12.0 | 5.2 |
| **(10)** | $u_{intrinsic}$ | 88.5 | 99.4 |
| | $u_{quantif.}$ | 11.5 | 0.6 |
| **(13)** | $u_{intrinsic}$ | 95.6 | 96.4 |
| | $u_{quantif.}$ | 4.4 | 3.6 |

[a] Analytes are coded as in Table 1.

estimated according the next equation

$$u^{rel}_{intrinsic} = \sqrt{(u^{rel}_{HPLC\ fractionation})^2 + (u^{rel}_{derivatization\ reaction})^2} \quad (9)$$

On one hand for the estimation of the uncertainty associated to the HPLC fractionation of 4-desmethyl sterols ($u_{HPLC\ fractionation}$) it has been considered that, the final residue obtained from saponification is dissolved in 1 mL of mobile phase (added with a pipette) and 20 μL of this solution are introduced in the liquid chromatograph. The fraction of eluate containing the sterols is collected, in the time window indicated in the procedure, in a graduated test tube for ulterior GC(FID) analysis. The uncertainty associated to the injection has been considered negligible. Therefore,

$$u^{rel}_{HPLC\text{-}fractionation} = \sqrt{\frac{u^2(V_{pipette})}{V^2_{pipette}} + \frac{u^2(V_{eluted})}{V^2_{eluted}}} \quad (10)$$

The uncertainty associated to the $V_{eluted}$ has been estimated considering the mass of the volume collected during the "time window" (2 min), using a flow rate of 0.8 mL/min. The collected volume (1.54 mL) was determined from the mass and density of the mobile phase (0.6675 kg/L). Thus,

$$\frac{u^2(V_{eluted})}{V^2_{eluted}} = \frac{s^2_{\Delta m}}{\Delta m^2} \quad (11)$$

where $s_{\Delta m}$ is the standard deviation of the mass of the collected volume containing the 4-desmethyl sterols; Δm is the difference between the masses of the test tube with the eluted fraction and the test tube empty.

The equation used to calculate the uncertainty associated with the addition of 1 mL of mobile phase to the final residue of the saponification is included in Annex I (Eq. (A.I.5)).

On the other hand, the uncertainty associated with the derivatization reaction ($u_{derivatization\ reaction}$) comes mainly from the use of pipettes in the preparation and addition of the derivatization mixture to the dry residue obtained previously. This component of uncertainty is given by

$$u^{rel}_{Derivatization\ Reaction} = \sqrt{\left(u^{rel}_{Derivatization\ mixture(D.M.)}\right)^2 + \frac{u^2(V_{pipette})}{V^2_{pipette}}} \quad (12)$$

The equations used to estimate both terms of this expression, are included in Annex I (Eqs. (A.I.5) and (A.I.6)).

Table 3(b) shows that, for the two selected samples, the relative intrinsic uncertainty is mainly due to the preparation and addition of the derivatization mixture, although the HPLC fractionation also contributes.

### 3.3.2. Quantification uncertainty: $u_{quantif.}$

In this case, the relative uncertainty associated to the quantification, depends on the peak area of analyte ($A_{sterol(i)}$)/peak area of surrogate ($A_{surrogate}$) ratio. Thus, applying the principles of uncertainty propagation, it should be calculated according to the next equations.

$$u^{rel}_{quantif.} = \sqrt{\frac{u^2\left(A_{sterol(i)}/A_{surrogate}\right)}{\left(A_{sterol(i)}/A_{surrogate}\right)^2}} = \sqrt{\frac{u^2(A_{REL})}{(A_{REL})^2}} \quad (13)$$

$$u^2(A_{REL}) = \left(\frac{\partial A_{REL}}{\partial A_{sterol(i)}}\right)^2 u^2\left(A_{sterol(i)}\right) + \left(\frac{\partial A_{REL}}{\partial A_{surrogate}}\right)^2 u^2\left(A_{surrogate}\right) + 2 \times r_{\left(A_{sterol(i)},\ A_{surrogate}\right)} \times \left(\frac{\partial A_{REL}}{\partial A_{sterol(i)}}\right) \times \left(\frac{\partial A_{REL}}{\partial A_{surrogate}}\right) \quad (14)$$

$$\frac{u^2(A_{REL})}{A^2_{REL}} = \frac{s^2\left(A_{sterol(i)}\right)}{A^2_{sterol(i)}} + \frac{s^2\left(A_{surrogate}\right)}{A^2_{surrogate}} - 2xr_{\left(A_{sterol(i)},\ A_{surrogate}\right)} \times \frac{s\left(A_{sterol(i)}\right)}{A_{sterol(i)}} \times \frac{s\left(A_{surrogate}\right)}{A_{surrogate}} \quad (15)$$

where $s(A_{sterol(i)})$ is the standard deviation of the peak area of sterol(i), obtained from "*n*" GC-FID chromatograms run in repeatability conditions; $s(A_{surrogate})$ is the standard deviation of the surrogate peak area, obtained from "*n*" GC-FID chromatograms run in repeatability conditions; $A_{sterol(i)}$ is the arithmetic mean of the sterol(i) peak areas, obtained from "*n*" chromatograms; $A_{surrogate}$ is the arithmetic mean of the surrogate peak areas, obtained from "*n*" chromatograms; $r_{(A_{(sterol(i))},\ A_{(surrogate)})}$ is the correlation coefficient of the areas, which is 1 for multivariate probability distributions.

The equations used for the estimation of the quantification uncertainty are shown in Annex I (Eqs. (A.I.7)–(A.I.10)), bearing in mind that, depending on the type of oil, the number of replicates ranged from 7 to 9.

Table 4 shows the values of the $u^{rel}_{quantif.}$ of 4-desmethyl sterols determined in the two selected samples. In this case, each analyte has its own quantification uncertainty, coming from the areas ratio calculated from the GC(FID) chromatograms.

As it can be seen, the relative quantification uncertainty ($u^{rel}_{quantif.}$) is, in most of the cases, higher for the sterols found in

pomace olive oil, apart from, campesterol, stigmasterol, β-sistosterol and $\Delta^{5,24}$-stigmastadienol in the extra virgin olive oil. This fact could be explained on the basis of the analytes resolution.

**Table 4**
Relative uncertainty values ($u^{rel}$quant.) for the quantification of 4-desmethyl sterols in two of the samples analyzed.

| Analyte[a] | EVOO1 | POO1 |
|---|---|---|
| | $u^{rel}_{quant.}$ | $u^{rel}_{quant.}$ |
| **(1)** | 0.014 | 0.017 |
| **(2)** | – | 0.015 |
| **(3)** | 0.01 | 0.020 |
| **(4)** | 0.0058 | 0.00028 |
| **(5)** | 0.0048 | 0.020 |
| **(6)** | 0.0063 | 0.0040 |
| **(7)** | – | 0.016 |
| **(8)** | – | 0.016 |
| **(9)** | 0.0012 | 0.020 |
| **(10)** | 0.0037 | 0.0013 |
| **(11)** | 0.00048 | 0.011 |
| **(12)** | 0.0036 | 0.0068 |
| **(13)** | 0.0037 | 0.0033 |
| **(14)** | 0.0077 | 0.020 |
| **(15)** | 0.0015 | 0.0096 |

[a] Analytes are coded as in Table 1.

Finally, Table 3 (c) shows the influence of quantification and relative intrinsic uncertainties on the inherent uncertainty, for campesterol (4), stigmasterol (6), β-sistosterol (10) and $\Delta^{5,24}$-stigmastadienol (13). It can be observed that, in most of the cases, the main contribution is due to the $u^{rel}_{intrinsic}$, especially from the derivatization reaction. The quantification process predominates over the chemical factors only for campesterol, in POO-1.

### 3.4. Relative overall uncertainty

The relative overall uncertainty was obtained according to the Eq. (16), where C(sterol(i)) is the estimated result:

$$u^{rel}_{Overall}(sterol(i)) = \frac{u_{Overall}\,(sterol(i))}{C(sterol(i))} = \sqrt{(u^{rel}_{operational})^2 + (u^{rel}_{inherent})^2} \quad (16)$$

The contribution of the different uncertainty sources to the relative overall uncertainty, for the two selected samples, is shown in Fig. 4. It can be seen that in the pomace olive oil, the relative overall uncertainty values are higher or of the same order of magnitude as in extra virgin olive oil, being in all the cases the relative inherent uncertainty the predominant. In relation with this source of uncertainty, although it varies depending on the 4-desmethyl sterol, it could be said that, in the case of the extra virgin olive oil, it is mainly affected by the contribution of

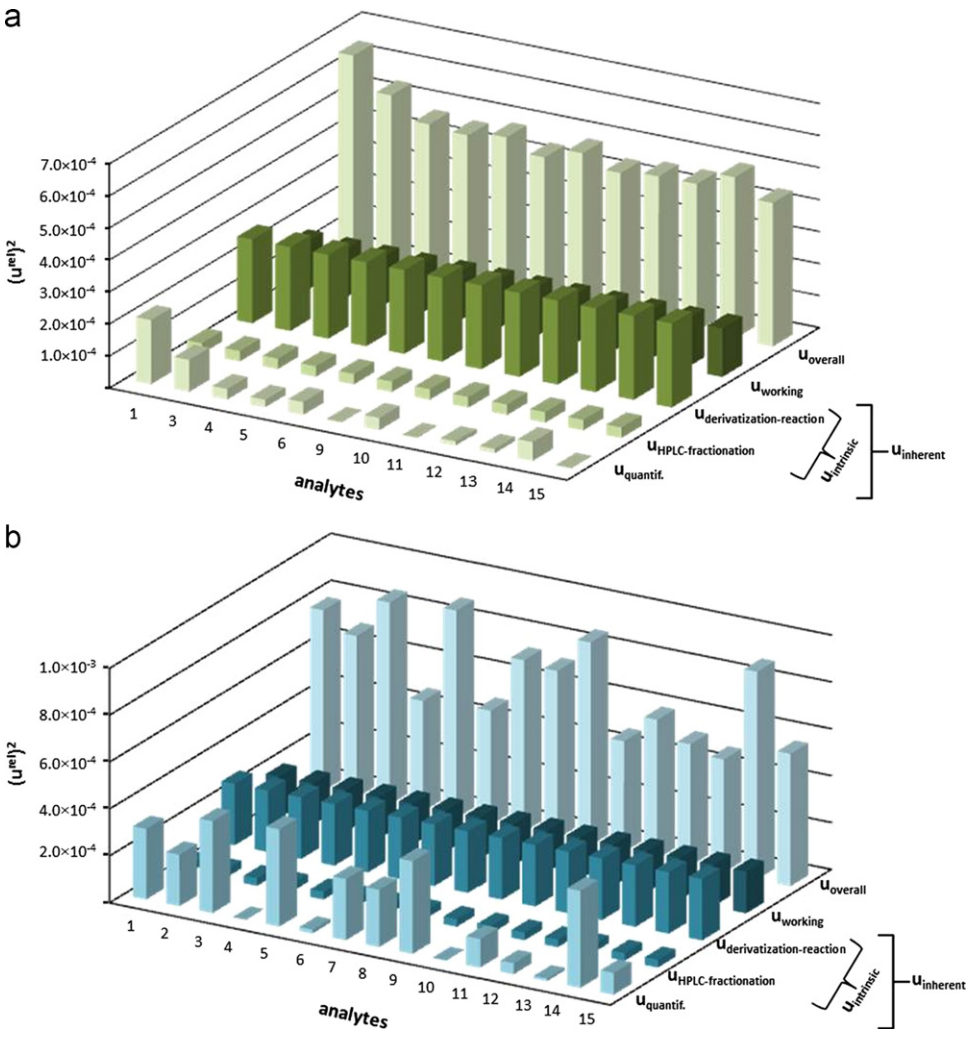


**Fig. 4.** Contribution of the different uncertainty sources to the relative overall uncertainty for the off line HPLC-GC(FID) determination of 4-desmethyl sterols in vegetable oils. (a) Extra virgin olive oil (EVOO-1), (b) Pomace olive oil (POO-1).

**Table 5**
Results obtained in the analyzed olive oil samples (the results are expressed as R ± U).

| Analyte[a] | Extra-virgin olive[b] | | | Refined Olive[b] | Pomace olive[b] | | |
|---|---|---|---|---|---|---|---|
| | EVOO-1 | EVOO-27 | EVOO-28 | ROO-1 | POO-1 | POO-2 | POO-3 |
| (1) | 1.9 ± 0.1 | 2.1 ± 0.1 | 2.0 ± 0.1 | 2.9 ± 0.1 | 5.4 ± 0.3 | 330 ± 20 | 10.3 ± 0.6 |
| (2) | < L.O.D | < L.O.D | < L.O.D | < L.O.D | 2.3 ± 0.1 | 5.7 ± 0.3 | 3.9 ± 0.2 |
| (3) | 1.8 ± 0.1 | 3.3 ± 0.2 | 3.1 ± 0.1 | 1.8 ± 0.1 | 4.2 ± 0.2 | 4.9 ± 0.3 | 2.9 ± 0.2 |
| (4) | 46 ± 2 | 49 ± 2 | 61 ± 3 | 42 ± 2 | 112 ± 5 | 91 ± 4 | 81 ± 4 |
| (5) | < L.O.D | 2.2 ± 0.1 | 1.2 ± 0.1 | 1.5 ± 0.1 | 6.7 ± 0.4 | 4.5 ± 0.3 | 3.6 ± 0.2 |
| (6) | 8.0 ± 0.4 | 17 ± 11 | 7.5 ± 0.3 | 11.2 ± 0.5 | 31 ± 1 | 29 ± 1 | 38 ± 2 |
| (7) | < L.O.D | < L.O.D | < L.O.D | < L.O.D | 6.7 ± 0.4 | 3.8 ± 0.2 | 7.6 ± 0.4 |
| (8) | < L.O.D | < L.O.D | < L.O.D | < L.O.D | 27 ± 1 | 26 ± 1 | 7.6 ± 0.4 |
| (9) | 13.9 ± 0.6 | 13.2 ± 0.6 | 11.4 ± 0.5 | 12.6 ± 0.5 | 34 ± 2 | 31 ± 2 | 27 ± 2 |
| (10) | 1250 ± 50 | 1010 ± 40 | 890 ± 40 | 940 ± 40 | 2000 ± 90 | 1600 ± 70 | 2200 ± 95 |
| (11) | 13.0 ± 0.5 | 15.2 ± 0.6 | 9.0 ± 0.4 | 9.0 ± 0.4 | 50 ± 2 | 41 ± 2 | 48 ± 2 |
| (12) | 56 ± 2 | 101 ± 4 | 69 ± 3 | 76 ± 3 | 62 ± 3 | 38 ± 2 | 82 ± 4 |
| (13) | 6.0 ± 0.3 | 8.7 ± 0.4 | 6.6 ± 0.3 | 12.6 ± 0.5 | 45 ± 2 | 53 ± 2 | 22 ± 1 |
| (14) | 5.2 ± 0.3 | 6.9 ± 0.3 | 3.9 ± 0.2 | 7.7 ± 0.4 | 18 ± 1 | 9.5 ± 0.6 | 17 ± 1 |
| (15) | 4.5 ± 0.2 | 6.1 ± 0.3 | 4.3 ± 0.2 | 6.9 ± 0.3 | 7.9 ± 0.4 | 5.2 ± 0.2 | 4.9 ± 0.2 |

[a] Analytes are coded as in Table 1.
[b] The content of each sterol is expressed in mg of sterol/kg of oil sample.

**Table 6**
Results obtained in the analyzed vegetable oil samples (the results are expressed as R ± U).

| Analyte[a] | Sunflower[b] | Sunflower high oleic acid[b] | | Rapeseed[b] | | Canola[b] | | Soybean[b] | |
|---|---|---|---|---|---|---|---|---|---|
| | SFO-1 | SFO(o)-1 | SFO(o)-2 | RO-1 | RO-2 | CanO-1 | CanO-2 | SyO-1 | SyO-2 |
| (1) | 3.4 ± 0.2 | 2.4 ± 0.1 | 4.1 ± 0.2 | 24 ± 1 | 28 ± 1 | 26 ± 1 | 27 ± 1 | 9.0 ± 0.5 | 11.0 ± 0.6 |
| (2) | < L.O.D | 1.0 ± 0.1 | 1.8 ± 0.1 | 710 ± 40 | 840 ± 40 | 700 ± 40 | 710 ± 40 | 6.7 ± 0.4 | 7.6 ± 0.4 |
| (3) | 9.0 ± 0.5 | 4.8 ± 0.3 | 7.0 ± 0.4 | 17 ± 1 | < L.O.D | 57 ± 3 | < L.O.D | 31 ± 2 | 23 ± 1 |
| (4) | 280 ± 10 | 190 ± 8 | 250 ± 10 | 2600 ± 100 | 3100 ± 100 | 2300 ± 100 | 2600 ± 100 | 580 ± 30 | 520 ± 20 |
| (5) | 7.2 ± 0.4 | 13.4 ± 0.8 | 6.2 ± 0.4 | 7.9 ± 0.5 | 6.7 ± 0.4 | 11.6 ± 0.7 | 11.7 ± 0.7 | 24 ± 1 | 22 ± 1 |
| (6) | 240 ± 10 | 160 ± 7 | 218 ± 10 | 22 ± 1 | 28 ± 1 | 31 ± 1 | 32 ± 1 | 440 ± 20 | 470 ± 20 |
| (7) | 87 ± 5 | 66 ± 4 | 83 ± 4 | 80 ± 4 | 79 ± 4 | 119 ± 6 | 104 ± 6 | < L.O.D | 22 ± 1 |
| (8) | 27 ± 1 | 9.6 ± 0.5 | < L.O.D | 22 ± 1 | 20 ± 1 | 46 ± 2 | 39 ± 2 | < L.O.D | < L.O.D |
| (9) | 30 ± 2 | 20 ± 1 | 35 ± 2 | 42 ± 2 | 51 ± 3 | 64 ± 4 | 68 ± 4 | 18 ± 1 | 22 ± 1 |
| (10) | 1720 ± 70 | 1090 ± 50 | 1430 ± 60 | 3400 ± 100 | 4000 ± 200 | 3610 ± 200 | 3700 ± 200 | 1070 ± 50 | 1400 ± 60 |
| (11) | 22 ± 1 | 35 ± 2 | 20 ± 1 | 12.3 ± 0.6 | 12.3 ± 0.6 | 12.4 ± 0.6 | 10.8 ± 0.5 | 34 ± 2 | 44 ± 2 |
| (12) | 75 ± 3 | 49 ± 2 | 88 ± 4 | 206 ± 9 | 240 ± 10 | 160 ± 7 | 260 ± 10 | 46 ± 2 | 72 ± 3 |
| (13) | 79 ± 3 | 40 ± 2 | 47 ± 2 | 52 ± 2 | 59 ± 3 | 105 ± 5 | 87 ± 4 | 20.9 ± 0.9 | 30 ± 1 |
| (14) | 400 ± 20 | 300 ± 20 | 370 ± 20 | 12.5 ± 0.7 | 9.2 ± 0.5 | 26 ± 1 | 20 ± 1 | 25 ± 1 | 92 ± 5 |
| (15) | 140 ± 7 | 112 ± 5 | 151 ± 7 | 7.4 ± 0.4 | 7.0 ± 0.3 | 11.2 ± 0.5 | 12.6 ± 0.6 | 17.6 ± 0.8 | 53 ± 3 |

| Analyte[a] | Corn[b] | | Peanut[b] | | Grape seed[b] | | Sesame[b] | |
|---|---|---|---|---|---|---|---|---|
| | CO-1 | CO-2 | PeaO-1 | PeaO-2 | GO-1 | GO-2 | SesO-1 | SesO-2 |
| (1) | 12.4 ± 0.7 | 16.2 ± 0.9 | 9.0 ± 0.5 | 8.6 ± 0.5 | 5.0 ± 0.3 | 4.1 ± 0.2 | 8.6 ± 0.5 | 4.3 ± 0.2 |
| (2) | 4.7 ± 0.2 | 2.2 ± 0.1 | 8.7 ± 0.5 | < L.O.D | 4.7 ± 0.2 | 5.6 ± 0.3 | 3.5 ± 0.2 | 1.9 ± 0.1 |
| (3) | 65 ± 4 | 81 ± 5 | 10.3 ± 0.6 | 14.0 ± 0.8 | 4.9 ± 0.3 | 5.5 ± 0.3 | 114 ± 7 | 97 ± 6 |
| (4) | 1080 ± 50 | 1290 ± 60 | 330 ± 10 | 350 ± 20 | 193 ± 8 | 220 ± 10 | 9230 ± 40 | 910 ± 40 |
| (5) | 145 ± 9 | 81 ± 5 | 7.1 ± 0.4 | 7.6 ± 0.5 | 11.3 ± 0.7 | 10.5 ± 0.6 | 10.3 ± 0.6 | 15.3 ± 0.9 |
| (6) | 430 ± 20 | 480 ± 20 | 190 ± 8 | 214 ± 9 | 196 ± 9 | 220 ± 10 | 360 ± 20 | 380 ± 20 |
| (7) | < L.O.D | < L.O.D | 5.0 ± 0.3 | 6.8 ± 0.4 | 14.2 ± 0.8 | 20 ± 1 | 27 ± 1 | 29 ± 2 |
| (8) | < L.O.D | < L.O.D | < L.O.D | < L.O.D | 9.0 ± 0.5 | 8.7 ± 0.5 | < L.O.D | < L.O.D |
| (9) | 83 ± 5 | 63 ± 4 | 20 ± 1 | 22 ± 1 | 24 ± 1 | 24 ± 1 | 53 ± 3 | 54 ± 3 |
| (10) | 4440 ± 200 | 4300 ± 200 | 1400 ± 60 | 1400 ± 60 | 1400 ± 60 | 1600 ± 70 | 3100 ± 100 | 3100 ± 100 |
| (11) | 360 ± 20 | 190 ± 9 | 15.1 ± 0.7 | 14.3 ± 0.7 | 82 ± 4 | 73 ± 4 | 20 ± 1 | 16.4 ± 0.8 |
| (12) | 290 ± 10 | 290 ± 10 | 180 ± 8 | 220 ± 10 | 38 ± 2 | 46 ± 2 | 660 ± 30 | 680 ± 30 |
| (13) | 85 ± 4 | 47 ± 2 | 20.7 ± 0.9 | 23 ± 1 | 39 ± 2 | 38 ± 2 | 76 ± 3 | 81 ± 4 |
| (14) | 190 ± 10 | 51 ± 3 | 13.5 ± 0.8 | 14.7 ± 0.9 | 63 ± 4 | 89 ± 5 | 35 ± 2 | 63 ± 4 |
| (15) | 119 ± 6 | 58 ± 3 | 23 ± 2 | 25 ± 1 | 17.6 ± 0.8 | 28 ± 1 | 60 ± 3 | 71 ± 3 |

[a] Analytes are coded as in Table 1.
[b] The content of each sterol is expressed in mg of sterol/kg of oil sample.

operational and chemical aspects, such as the use and preparation of surrogate, and those related to the derivatization reaction, respectively. For pomace olive oil sample, it is mainly affected for the quantification stage.

### 3.5. Expanded overall uncertainty

In order to provide a 95% level of confidence for the final results, the expanded overall uncertainties ($U_{overall}$ (sterol(i)))

were obtained by multiplying the overall uncertainties by a coverage factor, $k=2$. As an application of the previous equations to calculate the uncertainty, Tables 5 and 6 show the final results obtained for all the samples analyzed.

It can be check that, in most cases, the relative uncertainty ranged from 4% to 6%, regardless of the type of sample, the analyte and its concentration.

## 4. Conclusions

After the evaluation of the different uncertainty sources associated with the off line HPLC–GC(FID) determination of 4-desmethyl sterols in vegetable oils, it can be concluded that, the main contribution to the overall uncertainty is due to the chemical aspects of the method (inherent uncertainty). Depending on the type of oil, as well as the 4-desmethyl sterol considered, the quantification step can play an important role in the overall relative uncertainty.

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## Annex I. Equations used for the estimation of the secondary uncertainties

*Operational or working uncertainty (uop)*

*Mass surrogate ($u_{mass-surrogate}$):*

(a)

$$u^{rel}_{surrogate\ stock}=\sqrt{\frac{\left(balance\ maximum\ error_{verification\ process}/\sqrt{3}\right)^2}{m^2_{surrogate\ stock}}} \tag{A.I.1}$$

where balance maximum error$_{\text{verification process}}$ is the maximum error of the balance used to weight the surrogate, obtained from the internal quality management plan of the equipment (Table 2) and it is calculated by the expression:

$$\text{Balance maximum error}_{\text{verification process}} = |\text{Correction}| + \text{Expanded overall uncertainty} \tag{A.I.2}$$

– $m_{surrogate\ stock}$ is the mass of surrogate necessary to prepare 10 mL of a 0.2 % (m/v) surrogate stock solution.

(b)

$$u^{rel}_{PUR}=\sqrt{\frac{\left(order\ of\ last\ decimal\ PUR\ specified\ by\ manufacturer/2\sqrt{3}\right)^2}{PUR^2}} \tag{A.I.3}$$

where PUR is the purity of the surrogate provided by the manufacturer.

(c)

$$u^{rel}_{flask}=\sqrt{\frac{u^2_{standard}}{V^2_{flask}}} \tag{A.I.4}$$

where $u_{standard}$ is the standard uncertainty of the flask used to prepare the surrogate solution (Table 2); $V_{flask}$ is 10 mL.

*Inherent uncertainty: $u_{inherent}$*

(1) *Intrinsic uncertainty: $u_{intrinsic}$*

(i) HPLC fractionation of 4-desmethyl sterols ($u_{HPLC\ fractionation}$)

$$u^{rel}_{pipette}=\sqrt{\frac{\left(maximum\ error_{verification\ process}/\sqrt{3}\right)^2}{V^2_{pipette}}} \tag{A.I.5}$$

where maximum error$_{\text{verification process}}$ is the maximum error of the pipette used to add 1 mL of mobile phase to the residue of the saponification process (see Eq. (A.I.2)); $V_{pipette}$: 1 mL;

(ii) Derivatization reaction for GC(FID) analysis

$$\left(u^{rel}_{Derivatization\ Mixture\ (D.M.)}\right)^2=\frac{u^2(V_{pipette}(1))}{V^2_{pipette}(1)}+\frac{u^2(V_{pipette}(2))}{V^2_{pipette}(2)}+\frac{u^2(V_{pipette}(3))}{V^2_{pipette}(3)} \tag{A.I.6}$$

where $V_{pipette}$ (1) is the volume added of pyridine (900 μL); $V_{pipette}$ (2) is the volume added of hexamethyldisilazane (300 μL); $V_{pipette}$ (3) is the volume added of trimethylchlorosilane (100 μL). As the pipettes had been checked in the laboratory, the equation used in all the cases is analogous to Eq. (A.I.5).In the same fashion, the uncertainty associated to the addition of the D.M. is also evaluated according to the Eq. (A.I.5)

(2) *Quantification uncertainty:* $u_{quantif}$

$$\frac{s^2(A_{sterol(i)})}{A^2_{sterol(i)}}=\frac{(s_{repet}(A_{sterol(i)})/\sqrt{n})^2}{A^2_{sterol(i)}} \tag{A.I.7}$$

$$\frac{s^2(A_{surrogate})}{A^2_{surrogate}}=\frac{(s_{repet}(A_{surrogate})/\sqrt{n})^2}{A^2_{surrogate}} \tag{A.I.8}$$

$$\frac{s(A_{sterol(i)})}{A_{sterol(i)}}=\sqrt{\frac{(s_{repet}(A_{sterol(i)})/\sqrt{n})^2}{A^2_{sterol(i)}}} \tag{A.I.9}$$

$$\frac{s(A_{surrogate})}{A_{surrogate}}=\sqrt{\frac{(s_{repet}(A_{surrogate})/\sqrt{n})^2}{A^2_{surrogate}}} \tag{A.I.10}$$

In all the cases "*n*" is the number of GC(FID) injections replicated.

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