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

E. Pérez-Castaño, D. Gázquez Evangelista, M. Sánchez-Viñas, M.G. Bagur-González *

Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain

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A B S T R A C T

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.

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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.

* Corresponding author. Tel.: +34 958243327; fax: +34 958243328.
E-mail addresses: mgbagur@ugr.es, hellobrigui09@hotmail.com (M.G. Bagur-González).

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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.

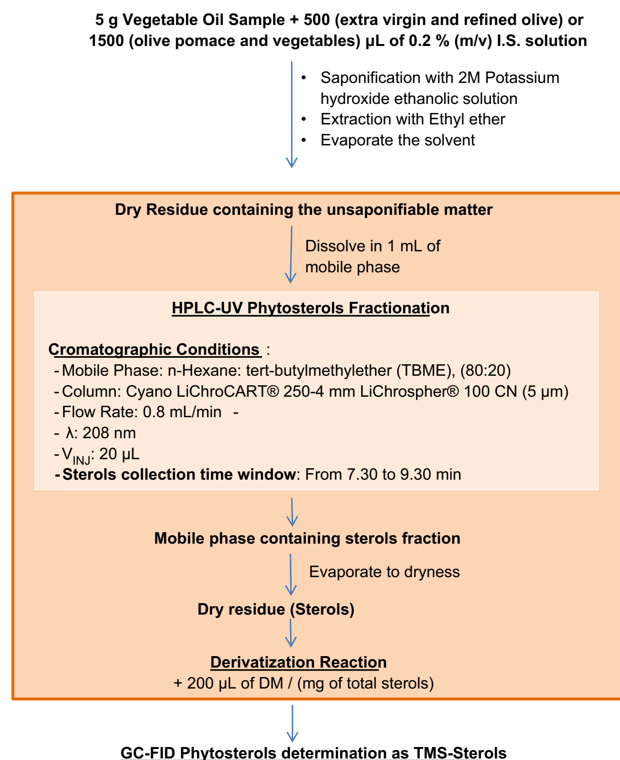

Fig. 1. Procedure for the off line HPLC–GC(FID) determination of 4-desmethyl sterols in vegetable oils. The boxes include the changes with respect the method of COI.

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