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Harmonization of the quantitative determination of volatile fatty acids profile in aqueous matrix samples by direct injection using gas chromatography and high-performance liquid chromatography techniques: Multi-laboratory validation study

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Abstract

The performance parameters of volatile fatty acids (VFAs) measurements were assessed for the first time by a multi-laboratory validation study among 13 laboratories. Two chromatographic techniques (GC and HPLC) and two quantification methods such as external and internal standard (ESTD/ISTD) were combined in three different methodologies GC/ESTD, HPLC/ESTD and GC/ISTD. Linearity evaluation of the calibration functions in a wide concentration range (10–1000 mg/L) was carried out using different statistical parameters for the goodness of fit. Both chromatographic techniques were considered similarly accurate. The use of GC/ISTD, despite showing similar analytical performance to the other methodologies, can be considered useful for the harmonization of VFAs analytical methodology taking into account the normalization of slope values used for the calculation of VFAs concentrations. Acceptance criteria for VFAs performance parameters of the multi-laboratory validation study should be established as follows: (1) instrument precision ($RSD_{INST} \leq 1.5\%$); (2) linearity ($R^2 \geq 0.998$; $RSD_{SENSITIVITY} \leq 4\%$; $RE_{MAX} \leq 8\%$; $RE_{AVER} \leq 3\%$); (3) precision ($RSD \leq 1.5\%$); (4) trueness (recovery of 97–103%); (5) LOD (≤ 3 mg/L); and (6) LOQ (10 mg/L).

Abbreviations: AD, anaerobic digestion; CRM, certified reference material; FDA, Food and Drug Administration (USA); GC, gas chromatography; ESTD, external standard methodology; FFAP, free fatty acid phase; FID, flame ionization detector; HPLC, high performance liquid chromatography; IS, internal standard compound; ISO, International Standard Organization; ISTD, internal standard methodology; LOD, limit of detection; LOQ, limit of quantitation; OLS, ordinary least-squares; P&T, precision and trueness; r, correlation coefficient; R, recovery rate; R², determination coefficient; RE, relative error; RF, response factor; RI, refractive index; RRF, relative response factor; RSD, relative standard deviation; RSD_{INST}, instrument RSD instrument precision; RSE, residual standard error; SS_{calib}, calibration standard solutions; SS_{Pre&Tru}, precision and trueness standard solutions; SS_{stock}, stock standard solution; SST, system suitability testing; USEPA, United States Environmental Protection Agency; VFAs, volatile fatty acids; WF/wi, weighting factor; WLS, weighted least-squares.

1. Introduction

The term volatile fatty acids (VFAs) comprises a group of aliphatic monocarboxylic acids with low-molecular weight and short chain lengths (C2–C7). They have peculiar characteristics such as relatively low volatility, high polarity and a strong hydrophilic character. In fact, they are classified as water-soluble volatile acids because they can be distilled at atmospheric pressure through co-distillation with water despite their high boiling points. The nature and concentration of these organic compounds are of interest because they are natural products from the degradation of organic matter constituting key intermediate metabolites in many biological processes. In this way, particularly, VFAs measurements have a high relevance in the anaerobic digestion (AD) research field. Therefore, monitoring the concentration of VFAs in anaerobic reactors as intermediate compounds in the metabolic pathways of fermentation and methanogenesis is viewed as a key control parameter. To consider the importance of this topic, a Scopus web search in article title/abstract/keywords using the terms “anaerobic digestion” and “volatile fatty acids” reported 2042 results in the period 1990–2015. A wide range of analytical methods is available for the determination of VFAs in various matrices, wherein GC and HPLC are the most common analytical techniques [1–3]. In fact, the scientific literature contains many papers related to different chromatographic methodologies for the determination of these organic compounds from the original work carried out by James and Martin, who reported firstly the separation of C1–C12 by GC, as early as 1952[4]. From the analytical viewpoint, it is important to note that samples can be analyzed directly [5,6], and when possible, the direct analysis is always preferable, because of its simplicity. However, also different pre-treatments such as distillation, organic extraction, derivatization and acidification increase the variability in the analytical methodology. Considering the great number of variables affecting the analytical determination of VFAs by chromatographic techniques, the standardization of these methodologies is difficult to achieve. Method validation is an important requirement in chemical analyses for testing the suitability of methods as well as the capacity of the analyst and laboratory. The results from method validation can be used to judge the quality, reliability and consistency of analytical results. Considering the importance of VFAs measurements and the numerous research groups and laboratories worldwide interested in them, the harmonization of VFAs measurements should be achieved in order to bring together different approaches, experiences and knowledge with analytical methods. In this way, it is important to note that against an in-house validation method, wherever possible and practical, a laboratory should use a method of analysis whose performance characteristics have been evaluated through a collaborative study that should conform an international protocol [7,8]. For these different reasons, the main goal of the present paper is the harmonization of VFAs results, by recognizing, understanding and explaining analytical differences among participants while taking steps to achieve worldwide uniformity in VFAs measurements. Therefore, results from a multi-laboratory validation study are presented including:

- Detailed information on the experimental validation approach.
- Performance characteristics of analytical methodologies reported by the participants.
- Information about the decision of accepting the performance characteristics of the analytical method with respect to its intended use. By this way, minimizing the risk to accept a procedure that is not sufficiently accurate or to reject a procedure that is capable of providing good results.

2. Multi-laboratory validation study

2.1. Validation of VFAs: state of the practice

Validation guidelines, in general, seldomly provide a practical approach to how validation should be carried out in a particular laboratory. There is much information about the criteria of validation to be tested, but it is frequently restricted to theoretical concepts and does not provide any experimental approach. In consequence, it is not always easy for analysts to translate the general concepts into practice considering the type of application, the method requirements and the choice of acceptance criteria. Concerning to VFAs, in spite of the many studies dealing with their measurement, only a few papers include a full study of the performance parameters that characterize the validation of the analytical methodology. On the other hand, neither ISO nor USEPA methods have been published for these organic compounds. Although the Standard Methods Committee approved the GC technique for VFAs measurements (SM 5560D) in 2005 [9], the reported methodology could be considered as inadequate. This is due to include some suggestions that can not be considered as good analytical validation practise: low number of calibration levels ($j = 4$); narrow calibration range (typically, 3.5–350 mg/L); calibration curve using the best fit through zero; acceptance criteria of linearity was based on correlation coefficient (should be higher than 0.995) and a 15–20% of deviation error for each calibration point; and finally, the precision and trueness (P&T) of the methodology were based on single-laboratory data. A long-standing objective of the AD research community has been to produce comparable results among laboratories through harmonized analytical methods. Although reliable analytical determinations of VFAs are required for the performance evaluation of anaerobic reactors, an interlaboratory study carried out recently involving laboratories working in the AD research field revealed a poor overall performance or “state of the practice” [3]. Among the causes for the poor analytical performance, human errors and inadequate analytical calibration procedures were the major problems observed. In addition, a reference methodology should be necessary to compare the VFAs results obtained by “on-line” anaerobic reactor monitoring using near-infrared spectroscopy (NIRS) technique, but unfortunately the error of prediction was too large for their accurate quantification [10]. These results showed that a good laboratory practice was complicated and a further multi-laboratory study is considered as crucial to improve the analytical reliability of VFAs measurements.

2.2. Organization

Information about this interlaboratory study was sent to laboratories and research groups working in the AD field. There was no attempt to screen participants in any manner, and therefore, all laboratories that expressed their interest to participate were welcomed. The potential candidates with interest in VFAs analysis received a first announcement of this action in October 2013. Of these, 30 laboratories, most of them members of different universities from the EU, agreed to participate in this interlaboratory study before the deadline for the distribution of the materials. The high level of positive responses can be considered as an indication of need for harmonization in the AD research field. The participating laboratories received instruction guidelines and the “validation kit” in February of 2014. Each validation kit contained 18 glass vials containing different aqueous solutions. In addition, each laboratory received the following fungible materials: volumetric flasks, vials for injection and vials to store some solutions to be prepared in the laboratory. The schedule was set to complete the

interlaboratory study within 3 months after receiving the samples. Unfortunately, only the data of 13 participating laboratories were considered as appropriate to further evaluation. Reasons for removing data of other participants included deviations from the established protocol and failure to obtain experimental results to achieve the appropriate analytical suitability of the chromatographic systems. In addition, the data from one laboratory, although adequate, were not finally considered due to being the only participant using the headspace GC technique.

2.3. System suitability testing (SST)

System suitability testing is commonly used by laboratories to ensure that the complete analytical system (instruments, analytical operations and samples) is suitable for the intended application. Appropriateness of the instrumental system must be checked before starting the calibration period to ensure that potentially valuable samples are not injected into an unsuitable system. Despite its importance, in many cases the SST is overlooked by analysts when they develop analytical methods. There are numerous guidelines which describe different parameters and their expected limits for typical chromatographic methods. The FDA guideline for validation of chromatographic methods [11] recommends 5 parameters to be tested such as: (i) capacity factor (k'); (ii) injection repeatability (RSD_{INST}); (iii) resolution (R_s); (iv) tailing factor (T); (v) number of theoretical plates (N). The suggested specifications are: $k' > 2$; $RSD_{INST} \leq 1\%$ (with injections number, $n \geq 5$); $R_s > 2$; $T \leq 2$; $N > 2000$. These specifications will vary depending on the conditions of the analytical method, but provide a good starting point in the early method development process. Full system suitability parameters should be self-tested by the participants and then SST factors, except instrument precision, are beyond the scope of this paper. Although the analytical protocol for this interlaboratory study clearly stated the appropriateness of SST, there were still some participants with erroneous results due to bad instrument settings, incorrect peak assignment, insufficient resolution, and high noise and drift and, therefore, their results were not considered for further evaluation.

2.4. Analytical validation: performance parameters

Analytical validation can be defined as the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application. Therefore, it is important to note that the main objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose [12,13]. Nowadays, several guidelines, conference reports and publications concerning validation can be found in the literature. Some of them are reported by several international renowned organizations, as it was summarized by Rambla-Alegre et al. [14]. Others are specific or chromatography techniques [11,15–18]. By this way, parameters for method validation have been defined in different working groups and committees at national and international levels. The different performance parameters of an analytical method that can be found in scientific literature are: selectivity/specificity, linearity/linear range, precision, trueness, limit of detection (LOD), limit of quantitation (LOQ), and robustness. A brief definition of each performance parameter is summarized in Table 1 [15].

3. Materials and methods

3.1. Chemicals

- Reagent water-HPLC or similar ultrapure grade was provided by Sigma–Aldrich (Madrid, Spain).
- Concentrated phosphoric acid (85% w/w) was provided by Pan-reac (Barcelona, Spain)
- Pure VFAs reagents (acetic, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric, iso-caproic and n-caproic acids) were purchased from Sigma–Aldrich (Madrid, Spain). The purity of all liquid chemicals was higher than 99.5%. Heptanoic acid was omitted considering the low prevalence in anaerobic reactors and their limited solubility (2.2 g/L) in water.
- 2-Ethylbutyric acid. It was selected as internal standard (IS) compound. It was provided by Sigma–Aldrich (Madrid, Spain) and their purity was higher than 99.5%.

3.2. Standard solutions

All the solutions used for the interlaboratory study were aqueous and diluted only with reagent water. Different standard solutions were necessary:

- Stock standard solution (SS_{stock}). This standard solution can be prepared gravimetrically from the individual VFAs standards of highest purity. The selected concentration of 2500 mg/L was an agreement between the requested concentration for the calibration study and the limited solubility of the VFAs with highest molecular weight. Considering the great importance of the accurate concentration in this solution, the SS_{stock} was prepared as a tailored solution by Supelco (Bellefonte, USA.) The uncertainty of the gravimetric preparation was very low and fixed at 0.5% (w/w) for all the individual components.
- Calibration standard solutions (SS_{calib}). The solutions were prepared from the SS_{stock} by dilution. In this way, different volumes of 4 mL, 20 mL, 40 mL, 100 mL, 200 mL, 300 mL and 400 mL of SS_{stock} were transferred to 1000 mL volumetric flasks and diluted with the corresponding volume of reagent water to provide the requested concentrations for the calibration study of 10 mg/L, 50 mg/L, 100 mg/L, 250 mg/L, 500 mg/L, 750 mg/L and 1000 mg/L, respectively. To follow the suggestion of independent measurements, two different solutions were prepared for each calibration standard. To avoid an excessive number of bottles to be delivered, the third calibration sequence or round was supposed to be carried out by the participating laboratories by mixing the present solutions at 50% v/v.
- Precision and trueness standard solutions ($SS_{Pre\&Tru}$). The solutions had to be prepared in the laboratory by each participant from a surplus standard solution of 1000 mg/L ready to be diluted. This solution was obtained in similar way as before (400 mL of SS_{stock} + 600 mL of ultrapure water). In fact, for independent measurements, two 1000 mg/L standard solutions were delivered and they should be used as stock standard solutions for the preparation of $SS_{Pre\&Tru}$. Each participating laboratory received detailed information about the procedure to obtain the requested concentrations for this study. By this way, the corresponding volumes of 0.8 mL, 2 mL and 6 mL of the above mentioned 1000 mg/L solutions were to be taken using a calibrated micropipette. Following they were diluted with ultrapure water in the provided 10 mL volumetric flask to obtain the three concentration levels for the study: 80 mg/L (low), 200 mg/L (medium) and 600 mg/L (high). The final solutions were to be stored in the 10/20 mL glass vials that were also provided to the participating laboratories.

- Acidification solution. This solution was prepared mixing 300 mL of concentrated phosphoric acid (85% w/w) and 700 mL of reagent water and then providing a mixture reagent of 30% v/v. This solution was checked for samples with high values of total alkalinity (15 g CaCO₃/L), giving pH < 2, assuring the organic compounds in molecular form and therefore being appropriate for GC methodology. Alternatively, the organic formic acid could be used for the acidification of samples.
- 2-Ethylbutyric acidification solution. This IS solution was pre-pared by weighting accurately 1500 mg of the pure IS and diluting it with the acidification solution to a total volume of 1000 mL. Only 10% of the volume of this IS solution had to be added to the samples to be analyzed according to the ISTD methodology. Thus, the final concentration in the vials was diluted 10 folds, to be 150 mg/L.

3.3. Instrumental devices and experimental conditions.

Considering that VFAs are volatile compounds, GC is the preferred technique for their quantitative determination [3]. Both chromatographic techniques, GC and HPLC, were previously described as specific and accurate [1,2], and as suitable for real aqueous samples by direct injection. In chromatography analysis, the experimental conditions should be the first aspect to be defined. Table 2a and b summarizes the different chromatography columns and instrumental conditions used by the participating laboratories whose data were used for further evaluation. As can be seen, some instrumental conditions in the study differed strongly among the participants. Therefore, the demonstration of robustness of the different analytical methodologies is expected to be an important step towards the harmonization of results.

3.3.1. GC system

This methodology was used by 9 of the participating laboratories that reported valid data. This number is one above the minimum of eight participants required for collaborative studies according to international agreement [7,8]. All the GC systems were equipped with flame ionization detectors (FIDs), whose limited sensitivity leads to relatively high detection limits. However, they proved to be sufficient for the determination of VFAs by direct aqueous injection technique at the fairly low level of concentration (mg/L). Capillary columns were used by the majority of participating laboratories, except one which used a packed glass column. Relating to the stationary phase for the separation of VFAs using capillary column, the most recommended one contains a polar chemically-bound film of polyethylene glycol (e.g. DB-WAX). The incorporation of an acid functional group in this phase, most often by treatment with nitroterephthalic acid, provided acid-modified polyethylene glycol columns, which have being demonstrated to be selective for VFAs [19]. They are useful and, then, widely used allowing GC resolutions with excellent peak shape (e.g. FFAP-free fatty acid phase brands and EC-1000). Participants reported the use of FFAP columns with different dimensions in terms of length (15–30 m), internal diameter (0.25–0.53 mm) and film thickness (0.25–1.20_μm). All the GC systems were automated including automatic mode injections. The reported injection systems were mainly split systems, although some participants reported the use of a splitless system. As usual, the injection volume was in the range of 0.5–1_μL. All the participants, except one, used temperature-programmed analysis, in which a controlled change of the column temperature occurred as a function of time. For this interlaboratory study, the initial and final temperatures as well as the heating rates were broadly

variable. Helium was used as common carrier gas. Although, from a chromatographic point of view N₂ is known to be a less suitable carrier gas for this type of analysis, it was reported from three laboratories. Finally, the run time of each determination was in the range of 9–17 min, with an average time around 13 min.

3.3.2. HPLC system

This technique was only used by four participants included in the data assessment. This is below the number of eight recommended for collaborative studies. Thus, the data were not large enough to develop a statistically significant prediction of this technique to take into account for the HPLC users. Therefore, this information has to be seen as provisional. However, it was still reported considering that scarce information is available in the literature for this methodology and none from the interlaboratory point of view. When compared with GC, liquid methodologies required higher sample volumes (10–50 µL, injected by valves), and longer runtimes (45–60 min). The detectors used were refractive index (RI) and conductivity. The selected mobile phase was mainly sulphuric acid solutions.

3.4. Experimental design

The goal of this experimental work was to create a consistency in the validation procedure reporting clearly the experimental set-up to study the fundamental validation parameters. A clear experimental design including two main groups of experiments, such as calibration and P&T studies, was established in order to investigate the main performance characteristics of the method in terms of linearity, precision and trueness. The calibration data were also used to evaluate the precision of the system and to calculate the LOD and LOQ. It is important to note that some performance parameters, normally included in the full validation procedures, were not considered important for this study, in detail:

- Selectivity for VFAs measurement has been previously demonstrated [1,2].
- Robustness was not checked specifically by each participating laboratory. Considering the huge amount of analytical variables, some modifications of the methodologies used for the participants were tolerated. Because of this, the overall assessment of the participating laboratories was regarded as a proof of their robustness.

In addition to the validation parameters, another important issue is the correct sequence of validation experiments. Taking into account that there are no official guidelines, the optimal sequence may depend on the analytical method itself. For this interlaboratory work, the calibration study (linearity) was treated as a matter of utmost importance considering that all other performance parameters depended strongly on the reliability of this determination.

3.4.1. Instrument precision study

The precision of chromatographic analysis includes contributions from sampling, sample preparation and the instrument. The variability of the measurement itself is addressed in the instrument precision, also termed system or injection precision. This parameter provides valuable information about the variability of the analytical system, mainly due to instrument causes. The peak-area of the separated components is one of the most important parameters for equipment qualification and the SST of

chromatographic analysis. The precision of the injector can be demonstrated by making replicate injections from a standard solution under repeatability conditions. The relative standard deviation (RSD) of the responses is then calculated to evaluate the instrument precision. For this study, precision at multiple levels was calculated using the data from the calibration study. Due to the strong concentration dependence of the variance contribution to system precision, the lowest concentration level (10 mg/L) was excluded from the overall calculation. Results were obtained individually for each VFA and then pooled as an average value for each participating laboratory.

3.4.2. Calibration study

There are three stages in the process of analytical calibration. Firstly, planning the experiments; secondly, the selection of the regression method, model and fitting technique; and thirdly the evaluation of correct linearity. In the following, each stage will be described in detail.

3.4.2.1. Planning the experiments. For an initial assessment of the calibration study in this multi-laboratory validation work the proposed scheme was:

- Working range: the calibration shall cover the concentration range in which the usual content of real samples is expected. Considering that the concentration range of VFAs in samples from anaerobic reactors is usually broad, for this study a wide calibration range of 10–1000 mg/L was selected.
- Sequences or rounds (i): taking into account the suggestions provided by different guidelines and standards for the validation of chromatographic methods, the whole study consisted of three sequences or rounds of calibration. It is important to note that the sequences should ideally be independent from each other, because replicate measurements on the same calibration standard give only partial information about the calibration variability. Therefore, each sequence should analyze “genuine” standards. Another important aspect is the schedule. The sequences must be carried out over at least 3 different weeks of analytical work to study the stability of signal response versus time.
- Number of standard levels (j): in general, the more points exist in the calibration curve the better. For this interlaboratory study, seven different concentrations covering the whole calibration range were selected. It has to be stressed that the application of calibration designs based on standard concentrations that correspond to multiples of the next concentration is strongly discouraged. Although this approach is frequently found in practice, the relatively wide spacing of the upper standards in such geometric series could mask the situation where the detector is reaching saturation and the instrument’s responses are levelling off somewhere between the last two standards. Therefore, it is preferable to use a partial arithmetic series (in this study: 10 mg/L, 50 mg/L, 100 mg/L, 250 mg/L, 500 mg/L, 750 mg/L and 1000 mg/L), where the concentrations of the upper standards differ by a constant amount (in this case 250 mg/L), not by a constant factor.
- Replicates (k): some guidelines recommend many replicate analyses but more than six do not provide big additional benefit from the statistical point of view. Therefore, three replicates at each concentration level were proposed in this study.
- Calibration mode: external/internal standard methodology (ESTD/ISTD). Which methodology should be used? This question is directly related to the quantitation purpose. In order to reply accurately, the experimental data should be previously checked [20]. Although ISTD methodology should be considered as usually beneficial for analytical instrumentation, limited information was reported in the

literature relating to its use for VFAs measurements. Considering the usefulness of ISTD relating to the harmonization of VFAs measurement, its effect in the normalization of data for linearity study was considered as a key subject for this interlaboratory study. Some important key points for ISTD methodology are:

- The selection of the IS compound. For this interlaboratory study 2-Ethylbutyric acid has been selected due to its similar structure to the target VFAs and for allowing an intermediate retention time. This compound was previously reported as accurate for the determination of VFAs by headspace GC procedures [21,22].
- The IS concentration. The amount of IS added to samples should yield a concentration close to that expected for the target analytes. In this case, the potential concentration of targets spans several orders of magnitude. Thus a concentration of 150 mg/L was regarded as reasonable.
- The volume of IS solution. Taking into account that an IS compound is usually added by a repeater pipette, small volumes (50 μ L or less) are more prone to imprecision than large ones. Therefore, higher volumes are suggested [23]. Specifically, for this study, 100 μ L (10% of total volume) was considered as appropriate to be mixed with 900 μ L of samples or standards [20].
- Time of IS injection. Slight pre-treatment of real samples is necessary for VFAs measurement. Therefore, IS can be considered as reference only for injection. To compensate the peak-area signals for quantitation purpose, this makes it necessary to add the IS compound just prior to the injection of the sample. In this study, GC users had the opportunity to select freely the method for quantitation, and both ESTD and ISTD methodologies were evaluated. For HPLC technique, only ESTD was suggested considering the variability in the instrument systems used by the participants and that no previous acidification of samples was necessary. By this way, three different configurations were assessed for this interlaboratory study: GC/ESTD, HPLC/ESTD and GC/ISTD.

3.4.2.2. Selection of regression method, model and fitting technique. The most common statistical method used is the least-squares regression, which works by finding the “best curve” through the data that minimizes the sums of squares of the residuals. There are a number of least-squares regression models such as linear, logarithmic, exponential and power. The most common measurement model is the one described by the linear function because of simplicity and ease of use. With respect to the fitting technique, ordinary least-squares (OLS) regression is often selected for a mathematical fit of the relation between concentration and instrumental response. Linear regression by OLS assumes that each data point in the calibration curve has a constant variance (homoscedasticity). However, many analytical methods produce data with increasing variance as a function of concentration (heteroscedasticity) [24]. It is important to note that a systematic error occurs if heteroscedastic calibration data are evaluated by using OLS [25]. This error should not cause problems if the concentration range is small, but if the calibration is carried out over a few orders of magnitude, this systematic error should be the cause of biased regression values (slope and intercept), then, sensitive to extreme data points. In consequence, concentration values for “unknown samples” could be unnecessarily overrated [26]. The solution for this issue is to use a weighted least-squares (WLS) regression procedure, which is similar to OLS but defines weights to the calibration data [27]. Although the general concept of WLS is mentioned in texts of statistics, the major problem with WLS is the

proper assignment of the weighting factors (WF/wi). The most reported procedure is to weight according to the inverse of the variance in the response at that point ($w_i = 1/s^2$) [22,27]. Alternatively, the variance can be modelled as a direct function of “x” ($1/x^{0.5}$; $1/x$; $1/x^2$) or “y” values ($1/y^{0.5}$; $1/y$; $1/y^2$) [28].

3.4.2.3. Linearity evaluation. Once the calibration function is established, it should be tested for absolute conformance to the model. Considering that the majority of the analytical methods use a linear relationship, examination of the calibration function for linearity before using it is important. Linearity assessment, as a required performance in method validation, has always been subject to different interpretations and definitions by various guidelines, protocols and papers [29–32]. In addition, there are very limited applicable implementation procedures that can be followed by analysts for assessing linearity. By this way, the acceptance criteria for linearity were not specified in ICH, AOAC, USP and IUPAC international guidelines for analytical method validation [33]. In any case, the first step for linearity assessment is to plot the paired data, and to check the linearity by visual observation of the calibration plot. Similarly, the y-residuals plot with values fairly distributed between positive and negative values has been suggested as indicator of the deviation in relation to the linearity assumption [34]. Unfortunately, linearity cannot be demonstrated over a given working range by simple visual observation of calibration and residuals plots because both processes are subjective. The FDA validation guideline indicates that “standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical test for goodness of fit” [35]. For the statistical evaluation, the quality of fit must be evaluated using statistical values that describe the line that best fits a set of points. The correlation (r) and determination (R^2) coefficients are statistical parameters commonly used when performing analytical calibration assessments as indicators for how well a linear regression equation fits the model. However, it is important to note that correlation or response variability and linearity are only loosely related and, in consequence, their values are frequently misinterpreted. Thus, it is important to consider that these statistical parameters are misleading in the context of testing the linearity [29,31]. In spite of this, the linearity evaluation of the majority of chromatographic methods reported in the literature for VFAs measurement relies only on these parameters. By this way, all the previous methods that reported values of r or R^2 close to 1 were considered as appropriate without further evaluation. This interlaboratory validation study was planned as a way to establish a practical approach to evaluate the linearity range applied to an in-house validated method for the determination of VFAs. Some statistical parameters have been checked for linearity assessment to facilitate comparisons of analytical methodologies and were proposed as acceptance criteria among diverse configurations for VFAs measurements. In particular, two different parameters have been considered:

(1) Sensitivity/response or relative response factors (%RSD_{S-RF}/%RSD_{S-RRF})

The sensitivity of the analytical instrumental was suggested to be checked in terms of the linearity of the calibration curves [36,37]. In this method, the sensitivity is calculated as the individual response values divided by their corresponding concentration. By this way, the linear range is defined as the range of concentration over which the sensitivity (slope of calibration curve), expressed in the form of response factor (RF) for ESTD or the relative response factor (RRF) for ISTD, is constant within a

defined tolerance. A tolerance limit of $\pm 5\%$ is generally used in the literature to evaluate the results. In this Interlaboratory study, tolerance limits and expectations regarding the slope consistency have been used for the first time to evaluate the suitability of a linear model for VFAs measurements.

(2) Relative error (%RE)

In order to assess the linearity of calibration models, the relative error of the estimated regression line should be evaluated appropriately. In this way, it is suggested to estimate the concentration at each “y-experimental” value, and to determine the “x-residuals” by comparing it with the theoretical or nominal values of the used calibration standards [28, 36]. Then, the %RE is a simple way to become aware of the error contribution from the linear regression:

$$\%RE = [(x_{exp} - x_{theo}) / x_{theo}] \cdot 100 \quad (1)$$

This parameter can be useful as a plot, showing %RE versus concentration values using log units [38]. In addition, further values were evaluated as novelty in this interlaboratory study: (i) $\%RE_{MAX}$, the maximum specific error at any point of the calibration curve; (ii) $\%RE_{AVER}$, the overall error from the whole calibration curve.

3.4.3. Precision and trueness study

This study must be carried out after finishing the calibration step, once the linear regression model and fitting technique have been evaluated and considered as appropriate to avoid obtaining biased values. For this interlaboratory study, P&T were considered together because they are interdependent and the primary criteria in assessing the quality and acceptance of an analytical method. Specifically, P&T were assessed by repeated analysis of standards that must be different to the ones employed in the calibration study. Spiked standards at three different levels of concentration were selected to represent low (80 mg/L), medium (200 mg/L), and high (600 mg/L) concentrations of the calibration curve.

3.4.3.1. Precision study. Precision depends only on the distribution of random errors and does not relate to the true value or the specified value. Precision may be considered at three levels such as repeatability, intermediate precision and reproducibility. However, for this interlaboratory study, the precision of reported analytical data were evaluated calculating only two components such as repeatability and reproducibility. The dispersion of results for this multi-laboratory validation study was evaluated considering two different factors such as the type of analyte and the concentration level. In order to make the comparability of the results easier, the precision parameters were expressed as relative standard deviations (%RSD). Therefore, the following parameters were used to evaluate the precision of different methodologies:

- RSD_{r-min} : minimum value of repeatability precision reported among participating laboratories.
- RSD_{r-max} : maximum value of repeatability precision reported among participating laboratories.
- RSD_R : reproducibility (average value) precision reported among participating laboratories.

3.4.3.2. Trueness study. The determination of trueness allows estimating the extent to which systematic errors affect a particular method. Thus, it has been highlighted as the most crucial aspect that any analytical method should address. For a proper assessment of the trueness of the results the use of a CRM is suggested. There is a commercial CRM standard but unfortunately the selected concentration unit is 10 mM, and, thus, it is not possible to obtain uniformity for all the individual VFAs if concentration levels are expressed as mg/L. Considering the lack of VFAs material from the market, the use of spiked or fortified solutions was proposed as alternative method. In this interlaboratory study the measure of trueness was expressed using the recovery factor:

$$R(\%) = (C_{\text{measured}}/C_{\text{spiked}}) * 100 \quad (2)$$

3.4.4. LOD/LOQ assessment

These parameters have caused controversy among the analytical community due to the different ways to calculate them [39,40]. It is important to note that for VFAs measurement in the AD research field, LOD/LOQ are not key parameters because VFAs compounds can be found at relatively high concentration (mg/L) in biological reactors. In addition, samples can be pre-treated to decrease the LOD to values as low as a few ng/L [41]. Taking into account that a well-designed calibration study was carried out prior to this study, for this interlaboratory work a calibration curve approach over the whole range of quantitation was selected. Therefore, the raw data from the calibration study were needed, to obtain the ratio between the relative standard error ($RSE_{(w)}$) and slope values ($b1_{(w)}$) from the WLS fitting technique. These ratio values were multiplied by 3.3 and 10 to calculate LOD and LOQ, respectively.

3.5. Analytical procedure

The analytical procedure refers to the way of performing the analysis of samples. The procedure followed the method designed for raw samples. The raw sample was centrifuged and later filtrate through a glass-fiber filter. For HPLC user's, the sample could be injected in the chromatograph directly. For GC user's, a volume of 900 μ L of filtrate was introduced in a 1.5 mL polypropylene microtube and mixed with 100 μ L of the acidification reagent for ESTD, or alternatively with 100 μ L of the IS solution for ISTD [20]. In any case, due to the precipitation of some organic compounds from the acidified samples, the supernatant had to be collected from the microtube with a micropipette and introduced in the glass vial to be used for the chromatographic determination. Taking into account that no real samples were analyzed in this multi-laboratory validation study, the samples analyzed (SS_{calib} and $SS_{\text{Pre\&Tru}}$) were nor centrifuged nor filtered, but processed as received following the given specifications

3.6. Statistical treatment of data

OLS regression was conducted by MS Excel[®] and WLS by Statgraphic[®] Plus 5.0 software package. Linearity assessment study was carried out using Excel spreadsheets. Statistical significance was established at the probability level of ≤ 0.05 .

4. Results and discussion

To date a multi-laboratory validation study has not been reported for VFAs measurement. It seems to be important that the analysts know how to obtain the maximal performance of the instrumental system and how the system reacts to certain setting changes. The overall results from this interlaboratory study are explained in the next sections.

4.1. Instrument precision study

High precision requires absolute constancy of the separation conditions. Table 3 summarizes the results reported by the different participating laboratories. To reflect the performance of the instruments, the full design of the calibration study was not taken into consideration. By this way, the variance for the smallest available reference standard concentration (10 mg/L) was rather large, and therefore, removed from the overall calculations.

4.1.1. GC/ESTD

For GC's users the values of peak-area comparison, expressed as RSDINST, ranged from 0.4% to 3.6%, with an average value of 1.4%. Only two laboratories (2 and 3) reported higher levels of imprecision, with average values of 3.6% and 3.0%, respectively. For technical reasons, RSDINST mainly reflects the ability of the injector to draw the same amount of sample in replicate injections. Therefore, if the variability of the sample/standard amount being injected into the column is not controlled tightly, the basic principle of ESTD quantitation is seriously compromised.

4.1.2. HPLC/ESTD

For HPLC users the values of peak-area precision ranged from 1.0% to 2.2%, with only one of four laboratories reporting a higher value. The average value of 1.3%, was similar to GC/ESTD. Therefore, although high differences are found in the volume of sample injected in both types of chromatographic techniques, the same system precision was obtained.

4.1.3. GC/ISTD.

Theoretically, the choice of the ISTD method for quantitation purpose should increase the risk of instrument imprecision due to IS reagent must be mixed with the sample prior its injection. Therefore, the successful use of an IS depends on the existence of a high correlation between the peak-areas of the analytes and IS for the complete analytical procedure. Considering that the peak-area of the IS compound is normally not controlled, the knowledge of the IS variance contribution can also be used as a starting point for method harmonization. For this study, to clarify to what extent IS responses are acceptable; a 21-injection sequence with a concentration of 150 mg/L was assessed during the calibration period. The RSDINST ranged from 0.7% to 4.5% with an average value of 2.7%. The results of the participants were distributed equally around this mean value. It must be pointed out the general remark about the importance of using automatic pipettes appropriately calibrated and disposable plastic tips for handling the IS solution. On the other hand, despite the mean for repeated analyses of the IS compound was larger than that for the analytes of interest, the instrument precision for ISTD was slightly better when compared to ESTD methodology, with values ranging from 0.3% to

3.1% and an average value of 1.1%. As an extreme case, it was observed that the peak-area precision of laboratory 3 improved from RSD_{INST} 3.0% to 1.0% when ISTD methodology was applied.

4.2. Calibration Study

4.2.1. Selection of regression method, model and fitting technique

For this interlaboratory study, the concentration range includes more than one order of magnitude, and as expected the variances obtained by participating laboratories were nonconstant (F-test). However, unweighted and weighted regression models were applied to calibration data. Weighted regression models according to Cruwys et al. [22] provided better results for all the experiments evaluated in this study. Specifically, the calibration function judged as most appropriate for this study was obtained using least-squares regression, linear model and WLS ($w_i = 1/x^2$) as fitting technique. These results are in agreement with Gu et al., who considered that $1/x^2$ should always be used as weighting factor for all bioanalytical chromatographic assays [42].

4.2.2. Linearity assessment

For this interlaboratory study the evaluation of linearity was considered of vital importance considering its influence on the rest of validation parameters. The corresponding results for the participating laboratories dealing to linearity have been compiled in two tables for each laboratory. The first one includes the traditional regression parameters such as the slope, intercept and the respective variances that are used to construct the equation function to predict the sample concentrations. The second table shows complementary statistical parameters to be considered as objective and unambiguous decision tool for an appropriate testing of the linearity of the calibration curves. For example, Table 4 represents the data obtained by the laboratory 1 using GC/ESTD. The results obtained for all the participating laboratories in terms of linearity have been included as supplementary information (Tables S1a–S22b).

4.2.2.1. GC/ESTD. Firstly, the parameters usually considered to evaluate the calibration curves were evaluated:

- The values for $b_1(w)$ were found in a wide range because the results depended on the specific integration system. For example, the values for C2 ranged from 0.04 to 2227 counts L mg^{-1} whereas the values of nC6 ranged from 0.11 to 5691 counts L mg^{-1} . It is important to consider that for a FID detector the intensity of the signal is proportional to the mass of the compound measured.

Thus, the slopes obtained for each sequence of standard injected were proportional to the molecular weights of VFAs.

- The values of $b_0(w)$ were also very variable. Linear regression should never be forced through zero to avoid the skewed effect and gaining the accurate information from the natural intercept. Constraining the calibration curve should be justifiable from the statistical point of view [32], however, the practical consequence is to increase the prediction error [34].

- The average R^2 values for the VFAs were generally close to 1.0. This fact, although erroneously, has been very frequently considered as proof of goodness for linearity of calibration curves.

Secondly, the alternative parameters proposed for linearity assessment were also considered:

- %RSD_{S-RF}: the average values for the different VFAs ranged between 2.8% (C3) and 3.6% (nC6). In addition, nearly every individual value was lower than 5%, a limit that was reported previously as a good indicator for the linearity of detectors.
- %RE: it was evaluated using two values from the linear regression, named specifically as the RE_{MAX} (5.6%) and RE_{AVE} (2.2%). Both values showed a great improvement when compared with SM5560D suggestions of 15–20% of deviation from regression curve as acceptance criterion for linearity [9].

4.2.2.2. HPLC/ESTD. Considering that only four participating laboratories used HPLC/ESTD methodology, the results will be summarized briefly. Firstly, the traditional parameters:

- The values of $b_{1(w)}$ were widely variable. In addition, no clear trend can be reported for the variation of this parameter versus the molecular weight of VFAs.
- Values for $b_{0(w)}$ were widely variable and mainly negative. The same treatment of the non-zero intercept was considered for calculations from the regression equations.
- The R^2 values reported were generally 0.990 or greater. It is important to keep in mind that this is not a very demanding test concerning the acceptance criterion for linearity assessment.

Secondly, the alternative parameters for linearity were considered:

- %RSD_{S-RF}: the average values obtained were lower than the $\pm 5\%$ reported in the literature as empirical data for good linearity.
- %RE: results were compared to the GC methodology. In this sense, %RE_{MAX} values (6.7%) were higher, whereas %RE_{AVER} values were similar (2.1%)

4.2.2.3. GC/ISTD. The use of ISTD methodology has the great advantage of normalization concerning the results among the participating laboratories. In this way, the linearity studies can provide very useful information about the results reported, independently of the instrument used, to carry out the VFAs measurements.

Firstly, the parameters usually considered to evaluate the calibration curves were assessed:

- Similarly as reported for ESTD methodology, the values of $b_{1(w)}$ were proportional to the molecular weight of the organic compounds analyzed. For this methodology, it is important to consider the high precision in average values reported by the different laboratories. This fact can be considered as a benchmark, helping to check appropriately the calibration curves independently of the instrument used.
- The values of $b_{0(w)}$ were not normalized by the use of ISTD. They varied widely and must always be considered as a non-zero intercept to achieve accurate calculations.
- The values of R^2 were always higher than 0.995. However, other approaches are necessary to evaluate appropriately the linearity of a calibration function.

Secondly, the alternative parameters for linearity were also considered:

- %RSD_{S-RRF}: the average values were around 3.0% and thus, lower than the 5% reported in the literature as empirical limit for good linearity. Similarly to %RSD_{SLOPE}: The VFAs with a higher

variability in this parameter were C2 (3.7%) and nC6 (3.4%). This can be interpreted as a slight difficulty for accurate measurement.

•%RE_{MAX}: the calibration curves were evaluated considering the extreme values (10–15%) and average values (3–5%). These values are lower than those usually reported in the literature as acceptance criterion for the assessment of linearity (15–20%). In general, the obtained linearity results can be considered as robust taking into account the high variability in the experimental conditions applied (e.g. GC apparatus, columns, carrier gas).

4.3. Precision study

The average values of precision results reported by the participating laboratories are summarized in Table 5.

4.3.1. Results obtained by the GC/ESTD method

As can be seen, in spite of the high number of data evaluated from different GC instruments, the variability of average RSDR values was not so high. With respect to the variability among the three concentration levels analyzed, the precision did not vary significantly from one concentration level to the next. Specifically, a similar precision (1.2% and 1.3%RSDR) was obtained for VFAs concentrations of 80 mg/L and 200 mg/L, respectively. Small differences were reported at the highest concentration level (600 mg/L), with 0.6%RSDR, which indicates a slightly better precision. Regarding the precision obtained for the different VFAs, no significant differences were obtained. In this way, within a laboratory, the difference among triplicate determinations gave values of RSD_{r-min} that ranged between 0.2 and 0.4% whereas RSD_{r-max} ranged between 1.7 and 3.0%. On the other hand, the average RSDR among VFAs showed a small range of 1.0–1.2%. It is important to note that the differences among concentration levels and among specific VFAs were not statistically significant, which demonstrates that the different methodologies gave robust results with a high precision. By this way, using all the data reported for the three concentration levels to determine the variability of the precision for these methodologies, an average value as low as 1.1%RSDR was obtained. This value was similar to instrument precision, confirming that, for this methodology, the main source of precision is the injection stage. It is essential to consider that this average value is lower than some values of %RSD_{r-max} reported for participants with less precision. In view of that, these results could be used to set benchmarks for the target precision obtained in single-laboratory validation procedures. Reflecting a worst-case scenario, a value of 1.5% for overall precision, including all the organic compounds, could be proposed for fitting purpose. In any case, these results showed a very good precision, which was much better than that reported previously in the SM 5660D for GC/ESTD methodology, in which duplicate analysis of C2 and C3 produced RSD_r values of 5.8% and 4.6%, respectively [9].

4.3.2. Results obtained by the HPLC/ESTD method.

The overall precision for HPLC methodology was also very high, with a %RSDR ranging from 0.3% to 1.1%, and an average value of 0.8%. Inconsistent results can be found in the literature for C2, C3 and nC4. Dias et al. [43] reported similar values of around 1% for 1 mM standard solution, whereas Rodriguez-Cerqueira et al. reported 5–6% for a standard solution of 60 mg/L [44]

4.3.3. Results obtained by the GC/ISTD method

Similarly to the ESTD methodology, the ISTD showed that no significant differences were obtained for individual VFAs. Within a laboratory, the difference among triplicates determinations gave values for RSD_{r-min} that ranged between 0.2 and 0.4%, where as RSD_{r-max} ranged between 0.9 and 2.5%. In the same way, considering the variability among the three concentration levels analyzed, the precision did not vary significantly from one concentration level to the next. But the trend was different than for ESTD. For ISTD, the precision followed the normal trend of improvement with an increase in concentration. Specifically, %RSDR values of 1.2%, 0.6% and 0.5% were obtained for VFAs concentrations of 80 mg/L, 200 mg/L, and 600 mg/L, respectively. In summary, the differences among concentration levels and specific VFAs also were not statistically significant, demonstrating that the diverse chromatographic methodologies used by the participants had acceptable levels of precision. Taking into consideration all the data reported to study the variability of the precision for these methodologies, an average value of 0.8%RSDR was obtained. For setting specifications and taking into account the worst-case scenario, the laboratory mean values for all analyzed organic compounds were always lower than 1.5%. When comparing the overall precision results of both methodologies (ISTD and ESTD), as usual, the use of IS improved the variability of results obtained, although in this case only slightly (0.8 vs 1.1% RSD_R). This improvement can be considered as one of little advantages to justify the use of the proposed ISTD methodology.

4.4. Trueness study

The average values of recovery results reported by the participating laboratories are summarized in Table 6.

4.4.1. Results obtained by the GC/ESTD method

With respect to the trueness study, the differences in recovery rates for individual VFAs were not statistically significant, ranging from 96–98%. In the same line, no significant variation in recovery rates was evident when the analyte concentration level changed. The results showed no trends for none of the VFAs analyzed at three concentration levels. Specifically, the mean recovery rates were 97%, 96% and 97% for 80 mg/L, 200 mg/L and 600 mg/L, respectively. Therefore, the values of the multi-laboratory trueness study showed the lack of significant differences between the experimental values and theoretical content of the SSP&T. The mean recovery calculated for the laboratories was $97 \pm 5\%$. In conclusion, the different methodologies can be considered as robust also from the recovery point of view. These results are better when compared with previously reported values of the SM 5660D. The mean values reported in SM were around 87% for concentrations ranging between 120–232 mg/L, with a clear trend of lower recoveries for VFAs with a higher molecular weight.

4.4.2. Results obtained by the HPLC/ESTD method

The results reported by the three laboratories, which carried out this determination, and achieved reliable results showed an average recovery of $98 \pm 1\%$. Similarly, Dias et al. reported recovery values (97.2–98.3%) just below the expected concentrations for C2, C3 and nC4 [43]. Maximum recovery rates were also reported by Rodrigues-Cerqueira et al. [44]. These results confirm that both

chromatographic techniques, HPLC and GC, can be useful to obtain results of similar trueness, as it was previously reported [45].

4.4.3. Results obtained by the GC/ISTD method

Similarly to precision, no statistical difference was observed for the different VFAs analyzed at three calibration levels. The main characteristic of this methodology was the improvement of results reported for all the individual VFAs. The overall results ($100 \pm 4\%$), correspond to the maximum potential recovery rate. All the laboratories except one (lab 5) reported values of 95–105% for the three spiked concentrations. It is worth to mention that the use of ISTD methodology generally improved the trueness and can, therefore, be considered as another little improvement of the ESTD methodology. The improvements were higher for laboratories 1 and 4, which overall recovery increased from 89% to 104% and from 91% to 96%, respectively. A similar trend was reported for a 250 mg/L standard solution of C2 by Cruwys et al. [22]. However, considering that the ESTD methodology also permits to obtain accurate results, the most important characteristic of using the ISTD methodology is the incorporation of quality control (QC) in this routine determination. In this way, the peak-area values for IS can be considered as important data in terms of analytical performance evaluation. In addition, the presence of this IS compound in each chromatogram can also serve to confirm the suitability of the methodology.

4.5. LOD/LOQ assessment

Tables 7 and 8 summarize the results of LOD and LOQ, obtained respectively for all the VFAs by the participating laboratories. This approach was based on the calibration study because multiple experimental data were easily available. For LOD, similar overall values of 2.7 mg/L, 3.5 mg/L and 2.5 mg/L were obtained for GC/ESTD, HPLC/ESTD and GC/ISTD, respectively. In relation to LOQ, in the same line, also similar values were obtained for GC/ESTD, HPLC/ESTD and GC/ISTD, with average values of 7.8 mg/L, 10.8 mg/L and 7.1 mg/L, respectively. From the practical point of view, it has no sense to report LOQ values outside the calibration curve. Thus, for GC technique the lowest concentration of 10 mg/L can be considered as the LOQ. Similar values of LOD/LOQ were reported by Cruwys et al. for headspace-GC using the blank approach and OLS model, while the values were smaller for the WLS model [22]. Similarly, Mesquita et al., who used HPLC technique and the signal/noise ratio approach, reported values of 5 mg/L and 15 mg/L for LOD and LOQ, respectively [46].

4.6. Validation of VFAs measurements: comparative study of methodologies

Taking into account the huge amount of data reported by the participants and the different values of performance parameters obtained, a comparative evaluation of the three analytical methodologies should be helpful for the overall understanding of this interlaboratory study. Table 9 summarizes the results obtained.

4.7. Validation of VFAs measurements: acceptance criteria.

The large number of experimental data reported is useful to suggest the acceptance criteria for VFAs measurement as a way to obtain good analytical performance:

- Instrument precision: $RSD_{INST} \leq 1.5\%$ ($\leq 3\%$ for IS)

- Linearity:

$$R^2 \geq 0.998$$

$$RSD_{SENSITIVITY} \leq 4\%$$

$$RE_{MAX} \leq 8\%; RE_{AVER} \leq 3\%$$

- Precision: $\leq 1.5\%$
- Trueness: 97–103%
- LOD: ≤ 3 mg/L
- LOQ: 10 mg/L

In addition, for GC/ISTD methodology, other statistical parameters could be used as starting point for acceptance linearity criteria. It is important to consider that the sensitivity/slope for different VFAs can be useful to the normalization of results. Therefore, the following slope values, with a tolerance of 4%, should be obtained from the calibration curves and used for appropriate calculation of unknown samples.

- C2 – 0.002260; C3 – 0.003781
- iC4 – 0.004846; nC4 – 0.004728
- iC5 – 0.005414; nC5 – 0.005294
- iC6 – 0.005800; nC6 – 0.005743

5. Conclusions

Based on the results of this multi-validation laboratory study for VFAs measurement the following conclusions can be drawn:

- Appropriateness of the instrumental system must be checked before starting the analysis of standards or real samples.
- Linearity assessment of calibration curves using appropriate statistical parameters is of utmost importance. By this way, $\%RSD_{SENSITIVITY}$ and $\%RE$ were proposed as useful statistical parameters.
- The three methodologies studied GC/ESTD, HPLC/ESTD and GC/ISTD gave similar values of instrument precision, accuracy and LOD/LOQ.
- GC/ISTD methodology should be considered as advantageous due to the normalization of slopes for calibration functions and the incorporation of the QC concept.

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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.2015.08.008>

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Table 1. Performance parameters to be considered for validation of analytical methods

Performance	Short description
Specificity	Ability to determine the analyte of interest in presence of other compounds
Linearity	Ability of the method to obtain test results proportional to concentration
Range	Interval of concentration within the method can be considered suitable
Precision	Random error of the method
Repeatability	Precision measured under the best conditions (short period, one analyst...)
Intermediate precision	Precision within-laboratory variation (days, analyst...)
Reproducibility	Precision between laboratories (interlaboratory studies)
Trueness	Systematic error of the method
Limit of detection	Lowest sample concentration that can be detected
Limit of quantification	Lowest sample concentration that can be accurately quantified

Table 2a. Summary of chromatography conditions used by participating laboratories using GC methodology

Laboratory	GC Instrument		Column			Injector			More INFO		
	Brand	Model	Brand	Model	Dimensions: l/id/ft (m) x (mm) x (μm)	Mode	System	Sample Vol. (μL)	Oven Temp (°C)	Carrier Gas	Time (min)
1	HP/Agilent	6890N	HP/Agilent	DB-FFAP	30 x 0.25 x 0.25	Aut	Split	1	100/150/240	He	17
2	Varian	CP-3800	Varian	TRB-FFAP	15 x 0.53 x 0.5	Aut	Split	1	40/95/125/215	He	9
3	Perkin Elmer	Clarus-580	Grace-Econo	EC-1000	15 x 0.53 x 1.20	Aut	Splitless	0.5	80 to 120	N2	17
4	Varian	3900	Grace-Econo	EC-1000	15 x 0.53 x 1.20	Aut	Splitless	0.5	80 to 120	N2	17
5	HP/Agilent	7890A	Machery-Nagel	Permabond-FFAP	30 x 0.32 x 0.5	Aut	Split (1:20)	1	80/210	He	10
6	HP/Agilent	7890A	HP/Agilent	FFAP	25 x 0.32 x 0.50	Aut	Split (1:25)	1	80/120/180	H2	11
7	HP/Agilent	5890	Packed-glass	10% fluorad 431 on supelco-port	2 x 6 x 2	Aut		1	130	N2+formic acid	10
8	Thermo Electron	Focus GC	Grace-Econo	EC-1000	15 x 0.53 x 1.2	Aut	Split	1	90/175/250	He	14
9	HP/Agilent	6890 PLUS	J & W SCIENTIFIC	DB-WAX	30 x 0.53 x 1	Aut	Split (1:20)	1	80/200	He	11

Table 2b. Summary of chromatography conditions used by participating laboratories using HPLC methodology

Laboratory	HPLC Instrument		Column			Injector			More INFO			
	Brand	Model	Brand	Model	Dimensions: l/id/ft (m) x (mm) x (μm)	System	Technique	Sample Vol (μL)	Temp (°C)	Detector	Mobil Phase	Time (min)
10	Thermo	ICS-1100	Thermo	Dionex IonPac TM ICE-AS1	4 x 20	Aut	Valve	25	38	Conducti vity	Water + HFBA	45
11	Agilent	Infinity-1260	Agilent	HI-PLEX H	7.7 x 300 x 8	Aut	Valve	20	55	RI	H2SO4	58
12	Jasco	2000	Transgenomic	ICE ORH-801	0.3 x 6.5	Aut	Valve	10	35	RI	H2SO4	15
13	Waters	2996/2414	Transgenomic	ICSep ICE-ion-300	0.3 x 6.5	Aut	717 plus	50	40	RI/UV	H ₂ SO ₄	45

Table 3. Instrument precision reported by participating laboratories

Laboratory	ESTD (%RSD)	ISTD (%RSD)	IS (%RSD)
1	1.5	1.2	2.1
2	3.6	3.1	4.5
3	3.0	1.0	2.6
4	0.4	0.3	0.7
5	1.4	1.2	3.7
6	0.5	0.5	2.6
7	0.7	1.2	1.3
8	1.1	0.8	3.4
9	1.0	0.7	3.5
GC-Total	1.4	1.1	2.7
10	2.2		
11	1.0		
12	1.2		
13	1.0		
LC-Total	1.3		

Table 4a. Traditional parameters of the linear regression fit. Laboratory 1

Laboratory 1	SLOPE $[b_{I(w)}]$	EST. ERROR $[SE_{bI(w)}]$	INTERCEPT $[b_{0(w)}]$	EST. ERROR $[SE_{b0(w)}]$	COEF. DET. $[R^2]$
C2	0,149361	0,001385	0,143081	0,035732	0,99837
C3	0,245999	0,001442	0,104362	0,037195	0,99935
iC4	0,306137	0,002113	0,097728	0,054527	0,99910
nC4	0,303536	0,001412	0,121553	0,03644	0,99959
iC5	0,343228	0,001374	-0,026079	0,035461	0,99969
nC5	0,337260	0,001245	0,088187	0,032125	0,99974
iC6	0,368416	0,001513	0,016959	0,039032	0,99968
nC6	0,367216	0,00117	0,170713	0,030185	0,99981

Table 4b. Alternative parameters of the linear regression fit. Laboratory 1

Laboratory 1	%RSD _{SLOPE}	%RE _{MAX}	%RE _{AVER}
C2	0,93	10	2,5
C3	0,59	6,0	1,5
iC4	0,69	7,8	1,6
nC4	0,47	4,4	1,3
iC5	0,40	3,3	1,2
nC5	0,37	3,1	1,1
iC6	0,41	4,0	1,2
nC6	0,32	2,9	1,0

Table 5a. Precision study (%RSD) for GC-ESTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
1	1,1	1,0	1,3	1,0	1,2	1,0	1,3	1,3	1,1
2	1,4	1,3	1,5	1,4	1,7	1,6	1,8	2,0	1,6
3	2,7	2,8	1,7	1,9	1,4	2,9	1,4	1,3	2,0
4	0,4	0,3	0,3	0,5	0,2	0,4	0,5	0,5	0,4
5	1,6	2,5	1,5	2,0	1,7	1,8	1,6	3,0	2,0
6	0,5	0,5	0,3	0,3	0,4	0,3	0,4	0,4	0,4
7	0,7	0,8	0,8	0,9	0,9	0,9	0,9	0,8	0,8
8	0,9	0,9	0,8	0,8	0,8	0,9	0,8	0,8	0,8
9	0,6	0,3	0,3	0,7	1,3	1,2	0,3	0,3	0,6
AVER-vfa	1,1	1,1	1,0	1,1	1,1	1,2	1,0	1,2	1,1

Table 5b. Precision study (%RSD) for HPLC-ESTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
10	1,0	0,8	0,9	1,0	1,1	1,2	1,7	1,3	1,1
11	0,1		0,2	0,3	0,2	0,2	0,5	0,5	0,3
12	0,8	0,9	1,2	0,4	0,7	1,5			0,9
13	0,3	0,4	0,4	0,9	0,6	1,1	1,9	1,6	0,9
AVER-vfa	0,6	0,7	0,7	0,7	0,6	1,0	1,4	1,2	0,8

Table 5c. Precision study (%RSD) for GC-ISTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
1	1,1	0,5	0,4	0,7	0,6	0,7	0,6	0,5	0,6
2	1,3	0,6	0,4	0,4	0,4	0,3	0,5	0,6	0,6
3	1,3	2,1	0,9	1,0	1,0	2,5	1,0	1,2	1,4
4	0,6	0,5	0,5	0,6	0,5	0,7	0,6	0,6	0,6
5	0,7	1,7	0,9	1,2	1,1	1,2	1,2	2,2	1,3
6	1,2	1,1	0,8	0,9	0,7	0,8	0,7	0,7	0,9
7									
8	0,4	0,3	0,4	0,2	0,3	0,2	0,3	0,3	0,3
9	0,5	0,2	0,3	0,6	1,0	1,1	0,2	0,2	0,5
AVER-vfa	0,9	0,9	0,6	0,7	0,7	0,9	0,6	0,8	0,8

Table 6a. Trueness study (%Recovery) for GC-ESTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
1	94,7	90,8	87,9	89,2	87,4	88,0	86,8	87,3	89,0
2	101,0	100,6	101,8	101,2	113,5	101,1	101,4	100,0	102,6
3	95,3	96,1	95,3	95,7	95,0	96,5	94,8	95,1	95,5
4	91,6	91,0	91,6	91,3	91,4	91,4	91,7	91,3	91,4
5	90,7	80,6	94,1	95,6	99,3	93,7	98,6	94,5	93,4
6	103,1	102,7	102,1	102,7	101,8	102,5	102,4	102,5	102,5
7	97,9	95,2	95,0	94,9	94,7	94,6	94,2	94,5	95,1
8	98,8	99,4	99,0	99,3	99,2	99,3	98,9	98,7	99,1
9	101,6	99,5	101,0	100,4	100,3	100,5	100,7	100,3	100,5
AVER-vfa	97,2	95,1	96,4	96,7	98,1	96,4	96,6	96,0	96,6

Table 6b. Trueness study (%Recovery) for HPLC-ESTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
10	106,8	82,4	92,4	111,6	95,9	101,6	84,6	100,8	97,0
11	98,7		99,7	101,0	98,2	101,5	100,9	96,3	99,5
12									
13	99,2	99,3	99,0	98,8	97,9	98,5	98,1	98,4	98,7
AVER-vfa	101,6	90,9	97,0	103,8	97,3	100,5	94,5	98,5	98,4

Table 6c. Trueness study (%Recovery) for GC-ISTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
1	110,5	105,9	102,5	104,0	101,8	102,6	101,2	101,9	103,8
2	104,5	104,1	105,4	104,7	105,5	105,1	104,9	103,5	104,7
3	98,4	99,3	98,4	97,9	98,2	99,9	97,9	98,2	98,5
4	96,7	95,5	96,1	95,8	95,9	95,8	96,2	95,7	96,0
5	90,7	79,9	93,8	95,6	99,4	93,7	98,6	94,6	93,3
6	100,3	100,0	99,4	99,9	99,5	99,3	99,7	99,8	99,7
7									
8	102,2	102,7	102,4	102,7	102,5	102,7	102,3	102,0	102,5
9	103,4	101,2	102,7	102,2	102,0	102,3	102,4	102,1	102,3
AVER-vfa	100,8	98,6	100,1	100,3	100,6	100,2	100,4	99,7	100,1

Table 7a. LOD (mg/L) for GC-ESTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
1	3,1	2,0	2,3	1,6	1,3	1,2	1,4	1,1	1,7
2	3,1	3,0	3,3	3,1	3,1	3,4	3,8	5,0	3,5
3	3,8	3,6	2,4	2,4	2,7	3,4	4,1	5,6	3,5
4	4,0	2,2	2,1	2,2	2,1	2,1	1,8	1,7	2,3
5	2,7		9,2	3,4	6,2	3,0	2,5	5,9	4,7
6	3,7	3,2	1,8	1,9	2,5	1,9	3,3	2,8	2,6
7	2,3	0,9	1,0	2,8	1,1	0,9	1,1	1,0	1,4
8	3,4	1,7	1,5	1,5	1,5	1,4	1,3	1,6	1,7
9	3,2	3,4	1,5	1,6	3,5	3,5	1,8	1,8	2,5
AVER-vfa	3,3	2,5	2,8	2,3	2,7	2,3	2,3	2,9	2,7

Table 7b. LOD (mg/L) for HPLC-ESTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
10	2,1	4,4	7,8	7,8	1,1	1,8	2,4	4,1	3,9
11	2,4		3,1	4,0	7,0	8,4	4,7	5,5	5,0
12	4,2	3,0	2,6	1,7	4,9	4,1			3,4
13	1,6	1,7	1,9	1,1	1,5	1,8	2,3	2,4	1,8
AVER-vfa	2,6	3,0	3,8	3,7	3,6	4,0	3,2	4,0	3,5

Table 7c. LOD (mg/L) for GC-ISTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
1	3,7	1,3	1,7	1,1	0,8	1,1	1,4	0,9	1,5
2	2,8	2,3	2,4	2,3	2,3	2,3	2,4	3,3	2,5
3	2,6	2,3	1,2	3,5	2,0	2,2	3,0	4,9	2,7
4	4,2	2,4	2,3	2,3	2,3	2,2	2,0	6,1	3,0
5	4,1		11,5	2,9	1,8	2,5	2,6	3,0	4,1
6	3,6	3,1	1,2	1,4	2,4	1,5	3,4	2,6	2,4
7	2,6	1,2	1,3	1,3	2,8	1,3	1,4	1,5	1,7
8	3,2	1,7	1,4	1,6	1,5	1,8	1,8	1,7	1,8
9	3,0	3,4	2,7	2,3	4,3	2,0	2,6	2,7	2,9
AVER-vfa	3,3	2,2	2,9	2,1	2,2	1,9	2,3	3,0	2,5

Table 8a. LOQ (mg/L) for GC-ESTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
1	9,4	6,0	7,0	4,7	4,1	3,8	4,2	3,2	5,3
2	9,5	8,9	10,0	9,5	9,5	10,2	11,7	15,1	10,5
3	11,7	10,9	7,2	7,4	8,0	10,3	12,4	16,8	10,6
4	12,2	6,7	6,5	6,6	6,4	6,3	5,5	5,1	6,9
5	8,3			10,4	18,8	9,0	7,6	17,8	12,0
6	11,1	9,6	5,6	5,7	7,6	5,9	10,1	8,4	8,0
7	7,0	2,8	3,0	8,5	3,2	2,8	3,3	3,2	4,2
8	10,3	5,1	4,5	4,4	4,5	4,4	4,0	4,8	5,2
9	9,7	10,4	4,5	5,0	10,8	10,5	5,4	5,5	7,7
AVER-vfa	9,9	7,5	6,0	6,9	8,1	7,0	7,1	8,9	7,8

Table 8b. LOQ (mg/L) for HPLC-ESTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
10	6,3	13,2	23,8	23,7	3,2	5,6	7,3	12,3	11,9
11	7,2		9,3	12,2	21,3	25,3	14,3	16,5	15,2
12	12,7	9,2	7,8	5,2	14,8	12,3	9,0	14,9	10,7
13	4,8	5,3	5,7	3,4	4,5	5,4	7,0	7,4	5,4
AVER-vfa	7,8	9,2	11,6	11,1	10,9	12,2	9,4	12,8	10,8

Table 8c. LOQ (mg/L) for GC-ISTD method

LABORATORY	C ₂	C ₃	i-C ₄	n-C ₄	i-C ₅	n-C ₅	i-C ₆	n-C ₆	AVER-lab
1	11,1	3,9	5,1	3,3	2,3	3,4	4,2	2,8	4,5
2	8,6	7,0	7,2	6,9	7,1	7,0	7,3	10,1	7,7
3	8,0	7,1	3,6	10,7	6,1	6,7	9,0	14,9	8,3
4	12,8	7,1	6,9	7,1	6,9	6,7	6,1	18,5	9,0
5	12,4			8,8	5,5	7,5	8,0	9,1	8,5
6	10,9	9,5	3,7	4,3	7,3	4,5	10,2	8,0	7,3
7	7,8	3,7	4,0	3,9	8,4	4,0	4,2	4,6	5,1
8	9,8	5,2	4,3	4,8	4,5	5,3	5,3	5,1	5,5
9	9,0	10,3	8,3	7,0	13,1	6,0	7,8	8,1	8,7
AVER-vfa	10,0	6,7	5,4	6,3	6,8	5,7	6,9	9,0	7,1

Table 9. Performance parameters for GC/ESTD, HPLC/ESTD and GC/ISTD method

Performance Parameters	Analytical Methodologies		
	(1) GC/ESTD	(2) HPLC/ESTD	(3) GC/ISTD
1. Instrument precision (% RSD)	1.4	1.3	1.1
2. Linearity			
• R^2 *	≥ 0.998	≥ 0.998	≥ 0.998
• RSD _{SENSIBILITY} (%)	3.2	3.3	3.0
• RE _{MAX} (%)	5.6	6.7	5.3
• RE _{AVER} (%)	2.2	2.1	2.0
3. Precision (% RSD)	1.1	0.8	0.8
4. Trueness (% recovery)	97±5	98±1	100±4
5. LOD (mg/L)	2.7	3.5	2.5
6. LOQ (mg/L)	7.8	10.8	7.1

* Statistical parameters with low significance in the linearity assessment