Validation of Geosmin and 2-Methyl-i-Borneol Analysis by CLSA–GC–FID Method to Obtain ISO-17025 Accreditation

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Abstract

A study of the accreditation process using closed loop stripping analysis (CLSA)–gas chromatography (GC)–flame ionization detection (FID) methodology for the analysis of geosmin and 2-methyl-i-borneol (MIB) is performed, completing the instrumental validation process. Quality parameters, such as the linearity ranges, repeatability and reproducibility, efficiencies, matrix effects, and interference, are presented. The experimental work is completed with a study of the associated uncertainty using a "Bottom-up Approach Method" and a short description of a control-protocol for preserving the validation conditions as a method of quality assurance protocol. The results show that CLSA–GC–FID-MS is a very good tool for the analysis of geosmin and MIB at a low level threshold, and the working range obtained is 10–400 ppt (ng/L) for geosmin and 15–400 ppt for MIB, respectively, in both drinking and natural waters. Uncertainty was approximately 16% for both compounds; good reproducibility with precision below 10% and bias between 85–90% for the three matrices considered are obtained.

Introduction

The ISO 17025 (1) is the world normative for analytical laboratory accreditation. In the ISO 17025 normative, all the requirements to carry out the technical accreditation of the laboratory and the validation of the analytical method are compiled; different guidelines, explanations, and interpretations are available to help laboratories in the process of accreditation (2,3).

"Validation" is an internal laboratory process to check a specific method/assay or determination obtaining several reports and documents that can show a laboratory's ability to perform a method/assay or determination with accuracy and efficiency. During the validation process, it is necessary to completely check the entire method: to standardize the analytical protocol and remove imprecise material, and control subjective human errors. "Accreditation" is an external official recognition process from a competent international authorized agency to make a specific method/assay or determination with a specific technical and management requirement. In order to obtain the "accreditation", the validation has to be completed with management and performance protocols in order to establish a quality assurance (QA) system.

There are different ways to carry out validation. The first way is to adopt a standardized methodology. The second way is to compare our results with regard to another validated or standardized method. And finally, an extensive validation of our method can be performed by itself using a certified reference material (CRM) to guarantee a traceable result. Sometimes, special validations are possible from results of interlaboratory exercises or interlaboratory development projects. Regardless of the method chosen, the use of a CRM is recommended by ISO 17025 to check the methodology during the validation process and perform daily routine quality control experiments. The validation process for geosmin and 2-methyl-i-borneol (MIB) have a major problem because there are no available CRM and/or inter-laboratory exercises. In consequence, extreme care must be taken to obtain ISO17025 accreditation.

Geosmin and MIB are the two compounds more frequently identified in drinking water taste and odor episodes (4). Both compounds have a natural origin, being produced by Cyanobacteria, a blue-green algae as well as a few bacterial species (5). These compounds are a serious problem for water supply companies and water treatment plants because of their very low odor threshold (about 10 ppt), especially in those countries with chronic algae blooms. There is no correlation between organoleptic effects in water of these compounds and their toxicity (6). Vilalta et al. (7) studied the mechanism and dynamics of geosmin bloom episodes in Llobregat River (Spain); Romero and Ventura (8) studied, in the same river, the occurrence of geosmin across a water treatment plant during several episodes.

The closed loop stripping analysis (CLSA) coupled with high-

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resolution gas chromatography (GC) and flame ionization (FID) or mass spectrometry (MS) detectors has been proposed by several authors (9,10) to analyze geosmin and MIB as well as other compounds with a low odor threshold. The CLSA is a clean technique that allows the extraction of many compounds causing taste and odor problems both in drinking and natural water at trace level concentrations. This work studies the ability of the CLSA–GC–FID technique to analyze geosmin and MIB through their validation as a simple way to obtain ISO 17025 technical accreditation, keeping in mind that obtaining validation and accreditation is an easy process that makes it possible to better understand the technique and the significance of its results.

Experimental

Reagents

Geosmin and MIB, as certified concentration standard mix [100 ng/µL, 15 % relative standard deviation (RSD) for GC-analysis], were purchased from Supelco (Geneva, Switzerland), two different sets were used due to the fact that CRM for step 1 and step 2 were not available. Carbon disulfide for spectroscopy (Merck, Germany) was used as elution solvent for CLSA analysis. The 1-chloroalkanes C_5, C_6, C_{12}, C_{16}, C_8, C_{10}, C_{14}, and C_{18} (Fluka and Aldrich, Geneva, Switzerland) were the surrogates and internal standard considered. Acetone (Carlo-Erba, Italy) was bidestilled over glass and used to make patrons of the standard. Ascorbic acid (Carlo-Erba, Italy), as 0.1N solution in ultrapure water, was used to remove free chloride from drinking water samples prior analysis.

Matrices

Three different matrices have been used during this experimental work: ultrapure, natural, and drinking waters. Ultrapure water was used for all calibration, repeatability, and reproducibility experiments. The study of matrix influence was carried out with Llobregat River ground water and Barcelona drinking water. The water treatment plant of Llobregat River carries out a conventional treatment to supply drinking water to Barcelona from Llobregat River ground water.

CLSA

Analyses were carried out in a commercial CLSA apparatus (Brechbüler, Switzerland) according to the method developed by Grob (9,11). One liter of water samples was spiked with 1-chloroalkanes (C_5, C_6, C_{10}, C_{12}, C_{16}, and C_{18}) at a final concentration of 400 ppt for each compound. Filters with 1.5 mg of activated carbon trapping organic compound stripped during 1.5 h. Temperatures of 45°C and 55°C were used for water-bath and carbon filter, respectively. After stripping, the filters were spiked with C_8 and C_{14} 1-chloroalkanes (400 ppt) and extracted with carbon disulfide to obtain a final volume of 20 µL.

Instrumental conditions

CLSA extracts were analyzed on a Fisons 8560 gas chromatograph (GC) equipped with flame ionization detector (FID). A volume of 1 µL of the carbon disulfide extract was injected cold in on column mode. The chromatographic capillary column was a 50 m × 320-µm i.d. CP-Sil 19CB (0.25-µm film thickness) from Chrompack (Amsterdam, the Netherlands). The oven temperature program was 30°C (5 min) to 280°C (10 min) at a rate of 3°C/min. Helium (31 cm/s at 30°C) was the carrier gas, and nitrogen was used as make-up (125 kPa). A VG TRACE-MS equipped with a Fisons TRACE-GC 2000 working at the same chromatographic conditions as described earlier was used to confirm GC–FID results. The MS detector was operated in EI^+ at 70 eV; transfer line and ion source temperatures were 200°C and 250°C, respectively.

Results and Discussion

The "technical accreditation process" can be achieved following these four steps: (*i*) calibration process, method compression, and limitated interference; (*ii*) validation or method check, (*iii*) post-validation actions or method quality assurance; and (*iv*) estimation of uncertainty associated.

Several authors think that these four steps form part of the same process, the so called "validation", but there are many differences between the validation process and accreditation process. One of the most important is that "validation" is a specific (singular, static) process, while "accreditation" is a dynamic process because of the inclusion of a quality system with continuous checks and evaluations and it is subjected to standard judge. In the technical audit, the heavy point is the "assay quality assurance", which involves daily controls, checks, improvements, deviations, correction actions and management, and equipment maintenance. These aspects are detailed in the step 3, and this step symbolyzes the time projection.

In the first step, "calibration process", the method must be studied, with the application in mind, marking off specifications. This step should be irrespective of the accreditation or validation process because a lab should always carry out a similar study (more or less in depth) when deciding to apply an instrumental method to measure something. In this step, the "validation framework" must be fixed. The second step, "validation", is the specific validation process; the definition of validation in ISO 17025 guide says that "the validation is the confirmation using measurements and the proof with objective evidences of the compliment of requires for a specific use". The process of validation mainly consists of repeatedly checking the efficiency of the method among the framework established in the calibration step. In the third step, "post-validation", a control program must be developed to detect possible deviations in the "validation framework", establishing the corrective actions that must be taken. Moreover, the new measurement method must be introduced into a laboratory QA. Finally, the estimation of "uncertainty" is a very important requirement for ISO 17025, and it is related to accuracy of the method. The uncertainty allows one to assess the maximum mistake that can get through the "validation framework".

Step 1: calibration

A methodology based on CLSA–GC–FID as a qualitative (pre-

sumptive) and quantitative method and CLSA–GC–MS as a confirmative tool has been developed. The difference between presumption and confirmation is a requirement of ISO17025, and it is focused on guaranteeing the univocal identification of the compound. All instrumental conditions, sample processing, methodology, and criteria of this step must be clearly compiled into a comprehensive standard operative procedure (SOP) to describe the method according to ISO 17025.

The calibration step was reduced to obtain the calibration curve as an internal standard calibration curve for geosmin and MIB, plotting the normalized response of the target compound (chromatographic area normalized with chromatographic area of the internal standard) versus the analyte concentration. A total of 10 spiked Milli Q water samples were used. Each concentration was measured by duplicate, and the internal standard was 1-chlorohexane. The advantages of internal standard calibration include the fact that it can be used to account for routine variation in the response of the chromatographic system, as well as variations in the exact volume of sample or extract introduced into the chromatographic system. With the internal standard (IS) mode, better repeatability results are obtained.

From the calibration curve, all the parameters that constitute the validation framework can be easily obtained. In the inner working range, the reliability of the results with low, uniform, and studied errors can be obtained. This fact will have to be demonstrated in the validation step. Validation framework was defined by: linearity range analysis, limit of detection (LOD), and limit of quantitation (LOQ). In most cases, the linear range must be accommodated to obtain good LOD and LOQ according to specific regulations. Table I shows the calibration parameters obtained for geosmin and MIB using the classical chromatographic criteria in comparison with statistical criteria (obtained from linearity curve). Similar results have been obtained for geosmin and MIB considering both criteria. This means that the reliability of the statistical criteria can reduce the time consumption in this first step.

Our recovery results are close to the literature (9) for geosmin and MIB using the CLSA technique, and they are quite similar to other extraction techniques, such as solid-phase microextraction and solid-phase extraction (12). Working with FID response made the method very easy and stable (robust), with acceptable LODs for our purposes; this is very important when there are no problems with overlapping peaks or peak resolution that can affect the chromatographic detection. Therefore, the LOQ (see Table I) for geosmin (15 ppt) is higher than its odor threshold (10 ppt); it is important for us to be able to measure this compound at a level producing odor episodes in real conditions, although in chlorinated drinking water, the average population odor threshold is around 15–20 ppt. Because of this fact, it was decided to decrease the LOQ to 10 ppt for geosmin; during step two, it will be proven that this change is useful, and good reproducibility and good accuracy for this low level will have to be obtained.

We define retention time (t_R) for an analyte as the time it takes after sample injection for the analyte peak to reach the detector. A stable retention time is important for correct identification of complex environmental matrices. To stabilize (or to get a more precise) t_R, the relative retention time (Rt_R) was used as a presumptive identification method. The tentative identification of a single-component analyte occurs when a peak from a sample extract falls within the Rt_R window (Rt_R ± 0.1 min.). In the Rt_R method, all the retention time peaks are related with the time of standards group (1-chloroalkanes), and it has a fixed t_R. When the analytical quantitation exceeds the LOQ value, the identification must be confirmed because FID response is non-specific for the compounds studied. GC–MS was used for univocal identification; the acceptation criteria to identify a spectrum as geosmin or MIB are good matches in a commercial library search (NIST or Wiley library) or good agreement (lower than 15% difference) in the proportion of two characteristic ions.

The quantitation process is important to obtain the real sample results just as for Uncertainty (U) estimation. According to the "IS quantitation method", the quantitation of geosmin or MIB peak was made by the internal response factor (R_f). This factor relates the analytical area response of an analyte with its real concentration, normalized with the analytical area response of a fixed concentration of the internal standard (see equation 3). The R_f is a constant value for the linear range and it can also be expressed using the calibration curve as "1/slope" value (1/a):

Table I. Calibration, Criteria, and Validation Parameters of the Geosmin and MIB Analysis*

	Criteria	Acceptance	Geosmin	MIB
Validation Framework				
Linearity	Statistical	SDa/a < 0.05	0.0094	0.0086
	Chromatographic	$r^2 > 0.99 (n > 10)$	0.998	0.999
LOD (ppt)	Statistical	3SDb/a	4.97	5.2
	Chromatographic	2S/N > 5	5	5
LOQ (ppt)	Statistical	3 LOD	14.9	15.4
	Chromatographic	3 LOD	10†	15
Working range (ppt)	Statistical	> LOQ	15–500	15–500
	Fixed	—	10†–400	15–400
Quantificaction				
Internal Standard	Chromatographic		1-Chlorohexane	1-Chlorohexane
Related factor (R_f)	Statistical	1/a	1.194	1.241
	Chromatographic	3LOD	1.208	1.225
CLSA recoveries (%)	Chromatographic	–	80–90	80–100
Identification				
RRT (min)	Chromatographic	RRT±0.1	43.64	33.45
Confirmation				
GC–QMS	Agreement	< 15%	mass spectra	mass spectra

* SDa and SDb are the SD (standard deviation) of slope "a" and independent term "b" from calibration curve. Other definitions: relative retention time (RRT), limits of detection (LOD), and limits of quantitation (LOQ), respectively.
† See explanation in the text.

(eq. 1)		(eq. 2)
Calibration curve:	vs	Quantitation expression:
[Aan (IS/Ais)] = amount ($1/R_f$)	vs	amount = [Aan (IS/Ais)] R_f
(Y = aX + b)	vs	(X = [Y – b] 1/a)

Where Aan is the chromatographic area of analyte; Ais is the chromatographic area for internal standard; IS is the internal standard concentration (fixed); "amount" is de analyte concentration in the sample, and R_f is the Internal Response factor value.

R_f value can be also calculated in a manual way, using different and independent CLSA–GC–FID experiments by the expression:

$$R_f = (IS/Ais)\ (Aan/amount) \qquad \text{Eq. 3}$$

The coincidence between R_f -manual and R_f -statistical is dependent on the linearity degree and proximity to 0.0 value in the calibration curve. Very close values in manual and statistical methods (see Table I) have been obtained, but it is better to use the manual method because this estimation has been performed in real conditions, giving importance to "b" value, and also because more information to calculate the Uncertainty with more precision was obtained. The independent experiments can be used to plot the calibration curve, so R_f will be an average value (with RSD) of an independent group of R_f that has been calculated at different concentrations throughout the total working range. According to quality criteria from EPA Standard Method (9), if the %RSD of the calibration factors (R_f) is lower than 20% over the working range, then linearity through the origin can be assumed, and the average R_f can be used instead of a calibration curve.

Step 2: validation

According to the ISO guides, all non-normalized methods must be validated, when either designed or developed internally in our lab or used outside of previous application, including enlargements and modifications to verify that they are for use. This validation process must be as deep as possible to clear any doubt about the correct use of our method. Due to the lack of availability of CRM and interlaboratory exercises for geosmin and MIB, extreme care was taken to obtain ISO 17025 accreditation by using certified standard of geosmin and MIB. The most useful way is to perform "n" determinations of several different concentrations in order to calculate the repeatability, reproducibility, and accuracy of the method throughout the working range. Three different concentrations were chosen: high, medium, and low. The low concentration corresponded to the LOQ, and the high concentration is the other extreme of the working range without dilutions; an intermediate point of the working range was added as the medium concentration.

Table II shows the validation results obtained for geosmin and MIB of independent experiments under repetitive and reproducible conditions. The experiments have been performed using Milli-Q as water matrix, which was spiked with a commercial certified standard (or dilution) of geosmin and MIB (different from the one used in the calibration step) to get the final concentration. For repeatability, Table II shows the results obtained for identical experiments performed on the same day; for reproducibility, Table II shows the results obtained for independent experiments in several days (so called intra-laboratory reproducibility or intermediate precision) (17). Normally, an instrumental GC method is considered to have high reproducibility and repeatability when good precision and good accuracy (Bias) values are obtained. The guidelines established by the *Journal of Chromatography B* required precision to be within 10% of the relative standard deviation (RDS) at normal concentrations, and 20% for concentration at LQ level (13). RDSs (Table II) around 10% for all cases were obtained. This means a low measure of imprecision and a quantitative method without variations in the measurement intra and inter-daily in the inner of the working range. The variation in these duplicates (measured

Table II. Validation Results of Intraday and Multiday Analysis of Geosmin and MIB for Precision and Accuracy in Ultrapure Milli-Q Water as Matrix

	Geosmin						MIB					
	Repeatability			Reproducibility			Repeatability			Reproducibility		
	10 ppt	100 ppt	400 ppt	10 ppt	100 ppt	400 ppt	10 ppt	100 ppt	400 ppt	10 ppt	100 ppt	400 ppt
	10.1	114.8	412.5	10.2	114.8	406.6	8.2	100.6	385.6	10.3	104.4	397.7
	9.9	118.1	401.7	9.4	96.7	393.6	7.8	111.3	390.9	9.7	91.5	389.5
	9.1	112.2	434	10.7	94.3	381.1	8	105.6	424	11.5	102.2	372.1
	9.2	94.2	402.8	8.9	86.8	412.5	8.7	97.8	374.6	9.5	85.7	385.6
	11.1	114.7	411.1	10.1	111.4	404.2	8	100.5	390.8	8.1	111.9	380.1
	10.2	118	398.3	9.8	106.6	395.3	7.6	98.4	390.5	10.7	106.4	381.5
				9.9	105.7	397.2				10.2	105.9	351.6
Average	9.9	112	410.1	9.9	102.3	398.6	8.1	102.4	392.7	10	101.1	379.7
SD	0.67	8.218	11.84	0.537	9.295	9.507	0.345	4.716	15.107	0.987	8.529	13.657
Precision (%)	6.74	7.34	2.89	5.45	9.08	2.38	4.29	4.61	3.85	3.87	8.43	3.6
Repetitions (n)	6	6	6	7	7	7	6	6	6	7	7	7
Bias (%)	99.33	112	102.52	98.57	102.33	99.66	80.5	102.37	98.18	100	101.14	94.93
Error (%)	0.7	12	2.5	1.4	2.3	0.3	19.5	2.4	1.8	0	1.1	5.1

as RDS) incorporates variation attributable to sample analysis portion (i.e., homogeneity) and precision (random error). Barwick (14) studied and identified the main sources of measurement error associated with GC analyses, the review is intended as a source of documentation for analysts to reduce errors when improving the method by a better knowledge of the GC system.

It is not typical that some of the reproducibility values of precision were lower than the repeatability ones, but if the results of repeatability are analyzed, the third value for 400 ppt analysis (the same for geosmin and MIB) are higher than expected, which deforms the distribution of results probably due to a sample preparation problem. If these values were removed, a lower precision for repeatability than for reproducibility will be achieved. It is not necessary to remove any results, especially in repeatability, to hold the integrity of the set results in real conditions, especially when the results obtained are so good. Moreover, in most cases, the validations only consider the reproducibility intra-laboratory, and this problem is not raised.

The accuracy of a measurement method is the ability of a measurement method to give responses close to a true value, this is sometimes termed trueness (17). Normally, when accuracy is applied to a set of test results it involves a combination of random error components and systematic error components, in a context of quality. The systematic error is expressed as a deviation of the mean value of a series of measurements from the accepted reference value measured in percentage (Bias), and random error can be defined as closeness between independent test results measured in RSD (precision), which is a measure of the distribution of the random error. However, the sum of Bias and precision are laborious and then the accuracy is used as a qualitative term or it is often used to describe only the systematic error component (i.e., in the sense of bias) (17).

It is very important to know the Bias throughout the working range, because a method with low Bias (high systematic error) made it unviable for quantitative purposes without further correction of results and continuous deviation checking. Normally, a chromatographic method has good accuracy when bias is higher than 85% (80% in LQ), according to Conference Report criteria (15). In drinking water, Spanish regulations for Bias and precision of 75% and 25%, respectively, are allowed for organic compounds in GC determinations (16). Our method agrees with all criteria requirements, and no corrections are needed because an accuracy higher than 90% has been obtained.

Interference and matrix effects are one of the major problems in environmental analysis. The security of a result is "trueness", with specificity, when it is free of interference that could be masking the real truth. To study these problems, short experiments were made using two matrices, and the results were compared with the ones obtained with interference-free milli-Q water. Table III shows the results obtained for drinking water and natural water (Llobregat River water). Only drinking water and river water were used because geosmin and MIB are only available these types of water samples. The Llobregat river has an annual algae bloom with geosmin production (7,8), and the matrix influence study for Llobregat river samples had to be carried out in July because in March it was impossible to reach 10 ppt level with the high concentration of geosmin in the river, but this was not the case for MIB.

No matrix influence or organic compound interference has been observed in the chromatographic profiles; the results obtained for precision and accuracy are good and close to interference-free matrix experiments. On the other hand, the Uncertainty calculated (step 4) is higher than the RSD. Reasonably, all the results obtained included the reference value in the inner of interval: result ± Uncertainty.

Table III. Effect of Matrix Interferences in the Analysis of Geosmin and MIB

	Geosmin			MIB			
Conc. (ppt)	Average	Precision	Error (%)	Average	Precision	Error (%)	*n*
Raw Water*							
10	10.3	7.6	3.3	9.4	12.2	6.5	4
100	110	7.9	10.5	102	9.3	2.4	4
400	397	5.8	0.9	373	8.9	6.8	5
Tap Water†							
10	11.4	11.5	13.7	11	5.9	10.5	4
100	107	6.5	13.1	110	9.2	9.5	4
400	402	1	1	365	3.3	8.7	4

* Llobregat River: total organic carbon (TOC): 6mg C/L; ammonia 0.5–15 mg N_2/L; conductivity 1600–1400 µs/cm²; turbidity 10–20 nephelometric turbidity units (NTU); sulfates 160–200 ppm; UV 254 10–15.

† Treatment water from Llobregat River: TOC: 3.2 mg C/L; free chlorine 1.5 Cl/L; conductivity 1600–1400 µs/cm²; turbidity < 1 NTU; Sulfates 160–200 ppm; UV 254 4–5.

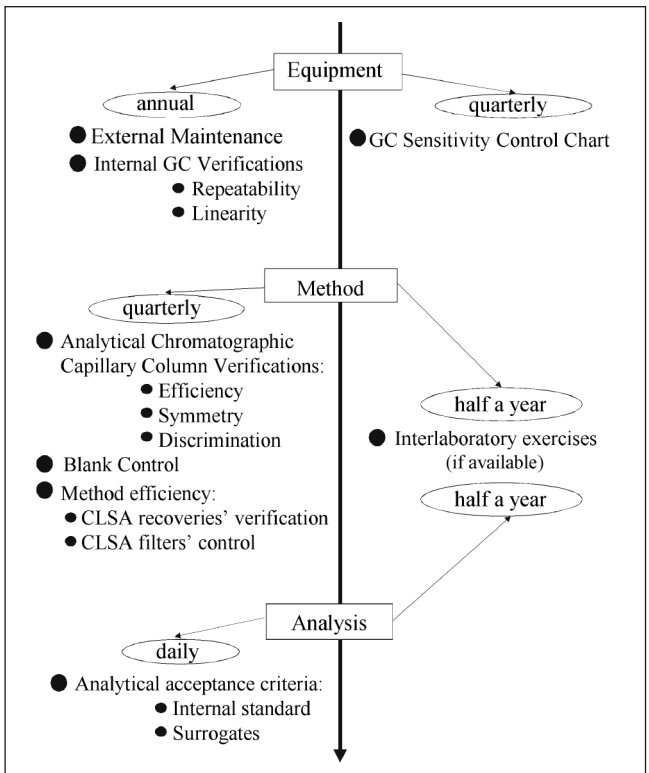

Figure 1. Annual model of tasks to be performed at different time intervals for equipment and method maintenance.

Step 3: quality assurance methodology

The new measurement method must be introduced into our quality assurance system. This process involves several actions in the way of modified general quality laboratory documents and to apply to the new method all the requirements of our quality system. Each laboratory has different quality systems. In this step, a set of specific actions related with the measurement method, the technical competence, and to ensure the quality of the measurement results are shown.

A model of actions to schedule throughout the year are show (Figure 1). These actions are focussed on checking the correct operation of equipment and materials used in the analysis. If the response of equipment that can affect the analytical result is held invariable, the validation conditions and the analytical results will be within the accreditation. This practice reduces the number of controls in the multianalysis systems because the same control is valid for different methods (for the analysis of different families of organic compounds), as it uses the same equipment and components (i.e., analytical column).

Each of these controls has to be clearly explained in various laboratory documents [i.e., in standard operation procedure (SOP) documents]. In these SOPs, an explanation should be given to each control: how to do them, what the frequency is, the criteria or values to pass the check, where the results should remain registered, and what should be done if the tolerance set fails. It is possible to reduce the frequency of a control that usually does not give problems when sufficient information is available or to increase the frequency of a more problematic parameter.

A good practice in the inner of accreditation mark is post-validation short studies. Regardless of the improvement achieved from internal and external technical audits, every two or three years a short validation should be performed to expand or to improve several aspects: from the initial validation, from uncertainty, or from a procedure of the methodological technique by using the experience in the systematic analysis obtained during the previous years.

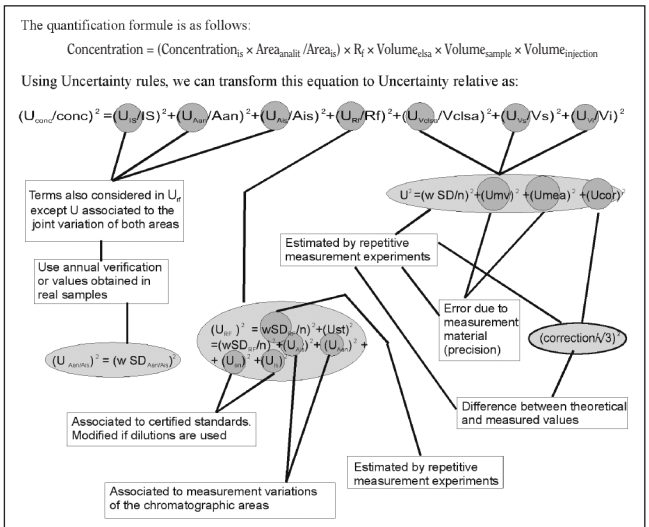

Figure 2. Development of the Uncertainty estimation using "Bottom-up Approach Method" for CLSA–GC–FID analysis from the quantitation way. (The symbols are explained in the text, step 4).

Step 4: uncertainty estimation

Uncertainty can be defined as a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurement (3). No commercial CRM for MIB and geosmin in water are available. It is not possible to use the standard method based on overall precision and bias to calculate the Uncertainly with repetitive analysis experiments with CRM (black box method).

To resolve the problem, the Uncertainty must be calculated throughout the analytical process, estimating the Uncertainty of each step as a component of the overall Uncertainty or "Bottom-up Approach Method" (3). Figure 2 shows a proposed model for the bottom-up approach Uncertainty in this case. This approach uses plain partial derivatives, is long, complex, and unpleasant, but it provides great knowledge of the analytical procedure, and it will allow the detection of systematic mistakes and improve the total Uncertainty by reducing the Uncertainty of the specific component that increases it. Moreover, partial derivative expressions can be disregarded with a simple transformation to convert absolute uncertainty to relative uncertainty mode.

From a generic equation:

$$Y = f(X_1,,,X_n) \qquad \text{Eq. 4}$$

its Uncertainty is:

$$(Uy)^2 = \Sigma_{i=1-n}[(\delta f(x_i)/dxi)\ U_{xi})]^2 = (Y/Y)^2\ \Sigma_{i=1-n}[(\delta f(x_i)/dxi)U_{xi})]^2 = (Y)^2\ \Sigma\ (U_{xi}/Xi)^2$$

or

$$(Uy\text{-relative})^2 = (Uy/Y)^2 = \Sigma(U_{xi}/Xi\ )^2 = \Sigma(U_{xi\text{-relatives}})^2 \qquad \text{Eq. 5}$$

In our case, for Uncertainty estimation throughout the analytical process, the function used to calculate the final concentration must be know because it contains all the factors that affect final uncertainty; for CLSA–GC–FID analysis according to equation 2:

$$\text{amount} = \{IS\ (Aan\ /Ais)\}\ R_f\ (Ve\ Vs\ Vi) \qquad \text{Eq. 6}$$

where "amount" is the final concentration in ppt; IS is the internal standard amount injected in the GC in ng; Ais is the chromatographic area obtained for the internal standard; Aan is the chromatographic area obtained for the analyte to quantify; R_f is the internal response factor according to equation 3 (step 1); Vs is the sample volume analyzed in liters; Ve is the extract of CLSA; and Vi is the volume injected to GC in µL.

And the "Uncertainty relative" estimation in accordance with equation 5 is:

$$(Uamount/amount)^2 = (U_{IS}/IS)^2 + (U_{Aan}/Aan)^2 + (U_{Ais}/Ais)^2 + (U_{Rf}/Rf)^2 + (U_{Ve}/Ve)^2 + (U_{Vs}/Vs)^2 + (U_{Vi}/Vi)^2 \qquad \text{Eq. 7A}$$

With this equation, it is possible to calculate the Uncertainty of the CLSA–GC–FID analysis of MIB and geosmin. Only the method for obtaining each U component is explained because detailing the whole system process would be very lengthy.

Further information is available in the Uncertainty estimation of the Eurachem Guide (3).

U_{vs}, U_{ve}, and U_{vi} are obtained by SD of repetitive volume measurements of each component. Then the following equation was used:

$$(U_{vs,\ ve,\ vi})^2 = (w\ SD/\sqrt{n})^2 + (U_{mv})^2 + (U_{mea})^2 + (U_{cor})^2 \qquad \text{Eq. 8}$$

where n is the number of the repetitions; SD is the standard deviation of the repetitions; U_{mv} is the U for the measurement volumetric material (normally it is specified by the manufacturer); U_{mea} means the U associated to personal measurement (a good practice used for this purpose is half value of the scale division of volumetric material, split by √3-quadratic distribution-). U_{cor} is the U associated to correction between the obtained result in repetitive experiments and theoretical value. If the final result is not corrected, the correction as Uncertainty must be included by the following equation:

$$U_{cor} = \text{correction}/\sqrt{3} = \text{theoretical} - \text{obtained}/\sqrt{3} \qquad \text{Eq. 9}$$

The U_{vi} does not need to be calculated because it depends on the area variation, and then it is already included in another term.

R_f is obtained by arithmetic mean of the individual point used in the linearity experiment after deciding what the working range is, then U_{Rf} is:

$$(U_{Rf})^2 = (w\ SD_{Rf}/\sqrt{n})^2 + (U_{st})^2 \qquad \text{Eq. 10}$$

in equation 10, another term explaining daily variation is not necessary because the R_f calculating mode that was used contains all the possible variations (included Vi, CLSA efficiency, and GC–FID response). And then, U_{Rf} depends on U associated with commercial certified standard and GC measurements (repeatability):

$$(Ust)^2 = (U_{Ais})^2 + (U_{Aan})^2 + (U_{an})^2 + (U_{is})^2 \qquad \text{Eq. 11}$$

Table IV. Uncertainty Estimation for Geosmin and MIB Analysis Using CLSA–GC–FID

Components		Geosmin	MIB
$U_{Aan}/A_{is}/(Aan/Ais)$		0.00300	0.00300
U_{RF}/RF	$(w\ SD_{RF}/\sqrt{n})/Rf$	0.01619	0.02077
	U_{ais}/A_{is}	0.02800	0.02800
	U_{Aan}/A_{an}	0.02800	0.02800
	U_{an}/an* (an = 10 ng/L)	0.02792	0.02773
	U_{is}/is (is = 400 ng/L)	0.04996	0.04996
U_{Ve}/Ve (Ve = 20 mcl)		0.01969	0.01969
U_{Vs}/Vs (Vs = 1 L)		0.00818	0.00818
U_{vi}/Vi (Vi = 1 mcl)		0.02880	0.02880
Urela		0.08001	0.08100
Urela (%)		8	8.1
Urel_exp (k = 2)		16	16

* It has been calculated at LOQ level. The symbols are explained in the text, step 4.

Uncertainty is associated with Ais and Aan. These were due to variations in the GC measurement. It can be estimated using the SD from repetitive injection in annual verification process. U_{an} is associated to the certified patron used in R_f calculation; this value is always contained in the manufacturer's certificate of analysis. Finally, U_{is} depends on internal standard preparation added to the samples, the purity of the standard used must be known, and precision of the volumetric material used.

Uncertainty, associated to Ais and Aan in equation 7A are included in U_{Rf}, only the U associated to Aan/Ais together must be studied. The annual GC verification with two patrons allow for the SD to be obtained for Aan/Ais coefficient. $U_{Aan/Ais}$ is calculated using the following equation:

$$(U_{Aan/Ais})^2 = (w\ SD_A)^2 \qquad \text{Eq. 12}$$

U_{is} is associated with the internal standard concentration. It is already calculated as U_{Rf}. After this, equation 7A can be rewritten as

$$(U_{amount}/amount)^2 = (U_{Aan/Ais}/(Aan/Ais))^2 + (U_{Rf}/Rf)^2 + (U_{Ve}/Ve)^2 + (U_{Vs}/Vs)^2 + (U_{Vi}/Vi)^2 \qquad \text{Eq. 7B}$$

Table IV shows the application of equation 7B to the Uncertainty estimation for geosmin and MIB analysis. To obtain the Final Uncertainty with a probability higher than 95% or Uncertainty Expanded, the U-relative had to be corrected with a statistical value (k) that, for 95% of confidence, is "2".

The estimation of Uncertainty Expanded for geosmin and MIB analysis has been of 16%; this is a very good result for an organic analytical instrumental method that usually has a value approximately 20% or between 20–30%. As confirmation of the estimation of Uncertainty, an approximation of total Uncertainty, was calculated using step 2 results and "black box method" (data not shown), the commercial standard of geosmin and MIB was considered such as a CRM. The "black box Uncertainty" agreed with "bottom-up Uncertainty" with a maximum of 20% and average of 14%. Moreover, two year of CLSA recovery verifications and filter controls according to step 3 and Figure 1 agreed with the bottom-up Uncertainty estimated.

The final conclusion is that CLSA–GC–FID is an easy methodology, very reliable, robust, and with low error in routine analyses of geosmin and MIB from drinking and natural water samples.

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Manuscript received June 2, 2006;
revision received December 1, 2006.