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Talanta 66 (2005) 1168–1174

Talanta
www.elsevier.com/locate/talanta

Estimation of the uncertainty associated with the standard addition methodology when a matrix effect is detected

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Received 16 September 2004; received in revised form 16 December 2004; accepted 12 January 2005

Abstract

If a matrix effect is detected during the validation stage of an analytical method, the standard addition methodology must be applied. To reach a good estimation of the uncertainty associated with the determination, an adequate identification and evaluation of each uncertainty source should be done. As an example to illustrate how to calculate the uncertainty in this case, the simultaneous determination of V(V) and Mo(VI) at trace levels after precolumn chelation and extraction with *N*-benzoyl-*N*-phenylhydroxylamine, has been selected. The final results show that the main contributions to the relative overall uncertainty are those closely related with the chemical aspects of the method, i.e. liquid–liquid extraction and standard addition calibration.

Keywords: Standard addition methodology; Uncertainty; Metal chelates; Liquid chromatography

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1. Introduction

Nowadays the result of a measurement must be accompanied with its uncertainty in order to evaluate the reliability of the results. The ISO [1] establishes general rules for evaluating and expressing uncertainty for a wide range of measurements, which has been interpreted for analytical chemistry by Eurachem/CITAC Guide [2], and it includes three different approaches to evaluate the uncertainty.

A slightly different strategy for the uncertainty estimation is based on grouping different sources of it whenever possible, and identifying and quantifying the most significant uncertainty components, where the utilization of data from preliminary and validation stages is recommended [2–4].

In general terms, the evaluation of the uncertainty is based on the estimation of the standard deviation associated with all the sources of variability (systematic and random components), which affect the measurement process. One of these main sources of variability arises from the calibration step. The main problem arises when there is a matrix effect and the standard addition methodology (SAM) [5] must be used, because there is, to the best of our knowledge, no general approach to the estimation of the uncertainty associated with this fact.

This paper presents a procedure to estimate the uncertainty associated with this step when a matrix effect is observed and the standard addition methodology is applied to eliminate it. In order to show the reliability of the proposed procedure it was applied to the simultaneous determination of V(V) and Mo(VI) at trace levels after precolumn chelation and extraction with *N*-benzoyl-*N*-phenylhydroxylamine [6].

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Hewlett-Packard 1050 series liquid chromatograph equipped with a variable-

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doi:10.1016/j.talanta.2005.01.021

wavelength UV–vis detector, a model 3396-A integrator (all from Agilent Technologies, Palo Alto, California) and a 7125 loop injector with a 20 μL sample loop (Rheodyne, Inc. Cotati, California). A 250 mm × 4.6 mm, 5 μm d_p Spherisorb (Phase Separations, Deeside, UK) S5 nitrile column, linked to a 10 mm × 4.6 mm Spherisorb guard column, was used for the combined determination.

A Precisterm (Selecta, Barcelona, Spain) s-137 thermostated bath was also used.

2.2. Chemical and reagents

A vanadium stock standard solution (1000 mg L^{-1}) was prepared by dissolving 0.23198 g of ammonium metavanadate (Merck, Darmstadt, Germany) in 100 mL of water. A molybdenum stock standard solution (1000 mg L^{-1}) was prepared by dissolving 0.92919 g of ammonium heptamolybdate tetrahydrate (Merck) in 500 mL of water. Working standard solutions were prepared by appropriate dilution of the above solutions.

N-benzoyl-*N*-phenylhydroxylamine (BPHA) solutions were prepared by dissolving the appropriate amount of the reagent (Merck) in chloroform (stabilized with amylene). All solutions and solvents (liquid chromatographic grade) used to prepare the mobile phase, were filtered through a 0.45 μm membrane filter and degassed before use. Doubly distilled water was used for the preparation of all aqueous solutions. All the other reagents were of analytical-reagent grade.

2.3. Procedure

The method was applied to a synthetic water sample with the composition shown in Table 1.

Aliquots of sample containing less than 7.5 μg of V(V) and 20 μg of Mo(VI) (V_{S1} = 30 mL, V_{S2} = 60 mL) were transferred into 250 mL separating funnels. Then, different aliquots of intermediate standard solutions of V(V) and Mo(VI) (V_V, V_{Mo}) were added. For V_{S1} = 30 mL, 20 mL of doubly distilled water (V_1) and 36.4 mL of concentrated hydrochloric acid (V_2) were added, adjusting to a final volume of 100 mL with doubly distilled water (V_3). For V_{S2} = 60 mL, up to 3.6 mL of doubly distilled water (V_1) and 36.4 mL of concentrated hydrochloric acid (V_2) were added. After shaking for 3 min with 5 mL of a 0.1% (w/v) solution of BPHA in chloroform (V_4), the organic phases were collected, dried over anhydrous sodium sulphate and filtered through a disposable syringe filter (0.2 μm).

Table 1
Composition of synthetic water sample

Ion[a]	Content (mg L^{-1})	Ion	Content (mg L^{-1})
As(V)	0.049	Pb(II)	0.023_7
Ba(II)	0.044	Mn(II)	0.028
Be(II)	0.019	Mo(VI)	0.085
Bi(III)	0.011	Ni(II)	0.049
Br^-	0.094	Se(IV)	0.009_7
Cd(II)	0.020	Ag(I)	0.009_8
Cr(III)	0.018_6	Sr(II)	0.227
Co(II)	0.026	Tl(III)	0.008
Cu(II)	0.021_9	V(V)	0.045_2
Fe(III)	0.099	Zn(II)	0.066

[a] Added as nitrates, sulphates, chlorides or ammonium and sodium salts.

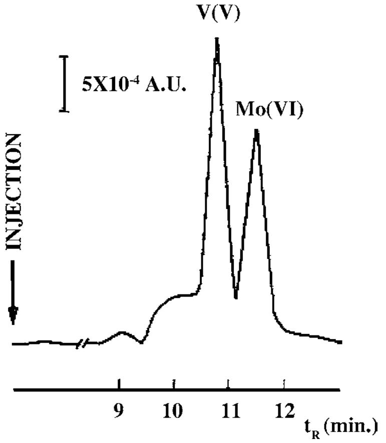

Fig. 1. Representative chromatogram of V(V) and Mo(VI)–BPHA complexes on a Spherisorb nitrile column. Mobile phase 5.9×10^{-4} M in $CHCl_3$ (stabilized with amylene).

For the chromatographic analysis, the nitrile column was equilibrated at 10 °C with a mobile phase consisting of chloroform (stabilized with amylene) containing 5.9×10^{-4} M BPHA. An aliquot of 20 μL of the organic phase was injected onto the LC system. The complexes were eluted at a flow rate of 0.6 mL min^{-1} being detected at 360 nm. Fig. 1 shows a typical chromatogram of vanadium and molybdenum complexes.

The data for internal calibration, were collected for four added concentrations ranging from 0.0 to 0.045 mg L^{-1} for V(V) and from 0.0 to 0.150 mg L^{-1} for Mo(VI) using duplicate responses at each concentration and randomized arrangements. This procedure was applied to two sample levels (30.0 and 60.0 mL), which were selected from a Youden calibration. Peak height was measured for quantitative purposes.

2.4. Theoretical aspects of the estimation of uncertainty

Traditionally, the main sources of uncertainty of an analytical method have been:

- Operational or working uncertainty, which is affected by instrumental effects, reagent purity, measurement conditions and sample handling among others,
- and those ones due to the bias error associated with the method, generally estimated only by recovery studies.

Bearing in mind that nowadays there are suitable tools available for the adequate evaluation of uncertainty, two sources associated with factors not controlled by the operator could be evaluated. The first one, *intrinsic uncertainty* ($u_{intrinsic}$), closely related to the chemical stages indicated in the procedure, depends on the chemical parameters. The second, *chemical calibration uncertainty* ($u_{\text{chem-cal}}$), is related to the chemical calibration process provoked by the transformation of the analytical signal in concentration and the acceptance of a normal distribution in the generation of the analytical signal. In a previous paper [7], these sources of uncertainty (intrinsic plus chemical calibration), which affect the analyte concentration in the sample, have been called *inherent uncertainty*.

The evaluation of $u_{\text{intrinsic}}$ is carried out by randomized replicates assays at the different stages of the analytical procedure. When $u_{\text{chem-cal}}$ is going to be estimated, the main problem arises during the validation procedure if a matrix effect is detected. In that case, the analyte concentration must be estimated by an internal calibration procedure (standard addition methodology, SAM).

Among the matrix effects previously described in the literature [8], the first order matrix effect respect to the analyte amount but independent from the matrix/analyte ratio is the most insidious, because if the independence from the matrix/analyte ratio is not fulfilled, it invalidates the SAM. In order to obtain a proportional interaction effect that does not change with the matrix/analyte ratio, it is necessary to apply the SAM to two sample levels, which guarantees that the previous requirement, as pointed out by Tyson [9], has been satisfied.

Although the evaluation of the uncertainty associated with the calibration step is perfectly established when an external calibration procedure is used, no information have been reported about how to estimate the uncertainty when SAM is used.

Once a matrix effect is detected, and the SAM at two sample levels has been carried out, the associated standard deviation at each sample level is calculated by the expression:

$$u_{\text{chrom-det}} = \frac{s_{\text{resid}}}{b}\sqrt{\frac{1}{n} + \frac{\bar{y}^2}{b^2\sum(c_i - \bar{c})^2}} \tag{1}$$

where b is the slope of the calibration curve, s_{resid} the standard deviation of residuals, n the number of points used in the internal calibration, c_i the analyte concentration, $\bar{c}$ the average concentration and $\bar{y}$ is the average response in the calibration.

When each standard deviation is obtained, it is necessary to check if they are statistically different or not. In the case of a statistical significant difference, a new SAM must be carried out at two new levels in which Tyson's requirement as well as homogeneity of variances should be satisfied.

If there is no difference, the standard deviation associated with the concentration of the analyte in the sample is calculated, being the uncertainty associated with the calibration process the pooled standard deviation.

Taking into account that the uncertainty expressed as above-mentioned is given in absolute value, it must be divided by the pooled concentration to obtain the relative uncertainty.

3. Results and discussion

The different contributions to the overall uncertainty associated with the analytical method applied, are shown in a cause–effect diagram (Fig. 2).

It can be seen that the uncertainty arises mainly from:

3.1. Operational or working uncertainty: u_{op}

First, it should be mentioned that for the basic equipment the u^{rel} values were calculated from the manufacturers' specifications, taking into account that: (a) for the microbalance it was obtained directly, and, (b) for volumetric material (pipettes and volumetric flasks) it was calculated by dividing the specified tolerance by $\sqrt{6}$ [2], since a triangular distribution was considered.

The main contributions were evaluated from:

3.1.1. Preparation of the stock and intermediate solutions: $u_{\text{intermediate-sol}}$

As it can be seen in Fig. 2, $u_{\text{intermediate-sol}}$ depends on $u_{\text{stock-sol}}$ which, among others factors, depends on the purity of the standards. Since the manufacturers do not supply any uncertainty concerning this issue, the methodology based on van Look and Meyer [10] was applied here. For purities based on titration, they propose to use a rectangular distribution with a range from $P_{\min}$ (minimum grade of purity indicated by the manufacturer) to 101%. The expected purity value is the mean and the standard uncertainty of the purity $u(P)$ is the 29% of the range.

The concentration of the stock standard solution is given by the mass (m) of the solid standard weighted in the analytical balance, the final volume (V_{F1}), the purity of the substance, the molar masses of the salts used (M_{Msalt}) and ions (M_{Mion}) and the stoichiometric factor (n) which was 1 for V(V) and 7 for Mo(VI), respectively. Thus the concentration of the stock solution is given by:

$$C_{\text{stock-sol}}\,(\text{mg L}^{-1}) = \frac{m \times P \times 1000}{V_{\text{F1}}} \times \frac{M_{\text{Mion}}}{M_{\text{Msalt}}} \times n \tag{2}$$

where P is the purity of the substance given as mass fraction and 1000 is the conversion factor from mL to L.

The relative standard uncertainty for the concentration of the stock solution is calculated by:

$$u^{\text{rel}}_{\text{stock-sol}} = \sqrt{\left(\frac{u(m)}{m}\right)^2 + \left(\frac{u(P)}{P}\right)^2 + \left(\frac{u(V_{\text{F1}})}{V_{\text{F1}}}\right)^2} \tag{3}$$

where we assume that the molar masses do not have a significant influence on the uncertainty [2].

Finally, intermediate stock solutions were prepared by drawing an aliquot of 1.0 mL, $V_{\text{stock-sol}}$ ($u_{V_{\text{stock-sol}}} = 0.004$ mL) from the stock solutions prepared previously into a 100 mL flask, V_{F2} ($u_{V_{\text{F2}}} = 0.033$ mL). The $u^{\text{rel}}_{\text{intermediate-sol}}$ for each an-

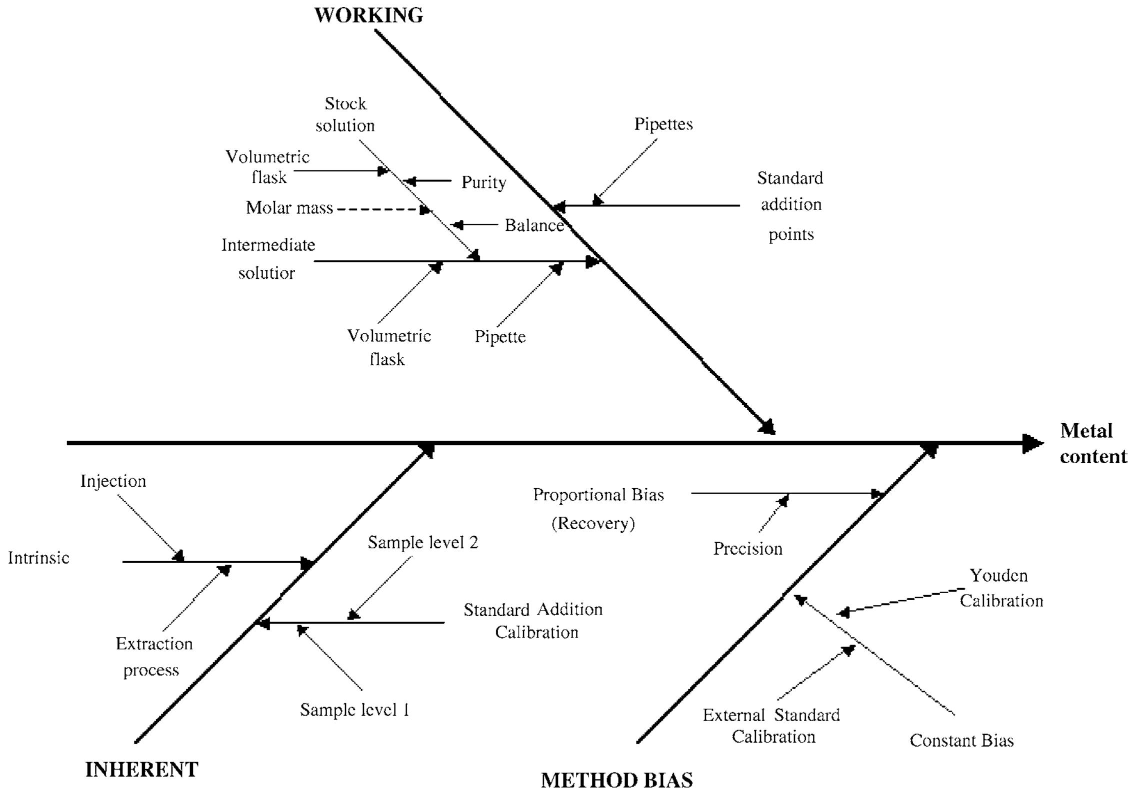

Fig. 2. Cause and effect diagram (Ishikawa diagram) for the chromatographic determination of V(V) and Mo(VI).

alyte is calculated by:

$$u^{\mathrm{rel}}_{\text{interm-sol}}(\mathrm{V}) = \sqrt{\left(\frac{u_{\text{stock-sol}}(\mathrm{V})}{C_{\text{stock-sol}}}\right)^2 + \left(\frac{u_{V_{\text{stock-sol}}}(\mathrm{V})}{V_{\mathrm{V}}}\right)^2 + \left(\frac{u_{V_{\mathrm{F2}}}}{V_{\mathrm{F2}}}\right)^2} \quad (4)$$

$$u^{\mathrm{rel}}_{\text{interm-sol}}(\mathrm{Mo}) = \sqrt{\left(\frac{u_{\text{stock-sol}}(\mathrm{Mo})}{C_{\text{stock-sol}}}\right)^2 + \left(\frac{u_{V_{\text{stock-sol}}}(\mathrm{Mo})}{V_{\mathrm{Mo}}}\right)^2 + \left(\frac{u_{V_{\mathrm{F2}}}}{V_{\mathrm{F2}}}\right)^2} \quad (5)$$

Table 2 shows the data used for the calculation of $u^{\mathrm{rel}}_{\text{intermediate-sol}}$.

3.1.2. *Preparation of the calibration solutions for SAM: $u_{\text{cal-sol}}$*

The different calibration points were prepared applying the described procedure in which the different aliquots of intermediate stocks solutions, ranged from 0.00 to 0.45 mL (V_{V}) ($u_{V_{\mathrm{V}}} = 0.004$ mL), and from 0.00 to 1.5 mL (V_{Mo}) ($u_{V_{\mathrm{Mo}}} = 0.004$ mL). The relative uncertainty associated with this step can be obtained from Eqs. (6)–(9). It should be noted that for the estimation of the uncertainty when the standards are not added (“zero” addition), the second and third terms of these equations, are zero

$$u^{\mathrm{rel}}_{\text{cal-sol}}(\mathrm{V}, V_{\mathrm{S1}}) = \sqrt{\left(\frac{u_{V_{\mathrm{S}}}}{V_{\mathrm{S}}}\right)^2 + (u^{\mathrm{rel}}_{\text{intermediate-sol}})^2 + \left(\frac{u_{V_{\mathrm{V}}}}{V_{\mathrm{V}}}\right)^2 + \left(\frac{u_{V1}}{V_1}\right)^2 + \left(\frac{u_{V2}}{V_2}\right)^2 + \left(\frac{u_{V3}}{V_3}\right)^2 + \left(\frac{u_{V4}}{V_4}\right)^2} \quad (6)$$

$$u^{\mathrm{rel}}_{\text{cal-sol}}(\mathrm{Mo}, V_{\mathrm{S1}}) = \sqrt{\left(\frac{u_{V_{\mathrm{S}}}}{V_{\mathrm{S}}}\right)^2 + (u^{\mathrm{rel}}_{\text{intermediate-sol}})^2 + \left(\frac{u_{V_{\mathrm{Mo}}}}{V_{\mathrm{Mo}}}\right)^2 + \left(\frac{u_{V1}}{V_1}\right)^2 + \left(\frac{u_{V2}}{V_2}\right)^2 + \left(\frac{u_{V3}}{V_3}\right)^2 + \left(\frac{u_{V4}}{V_4}\right)^2} \quad (7)$$

$$u^{\mathrm{rel}}_{\text{cal-sol}}(\mathrm{V}, V_{\mathrm{S2}}) = \sqrt{\left(\frac{u_{V_{\mathrm{S}}}}{V_{\mathrm{S}}}\right)^2 + (u^{\mathrm{rel}}_{\text{intermediate-sol}})^2 + \left(\frac{u_{V_{\mathrm{V}}}}{V_{\mathrm{V}}}\right)^2 + \left(\frac{u_{V1}}{V_1}\right)^2 + \left(\frac{u_{V2}}{V_2}\right)^2 + \left(\frac{u_{V4}}{V_4}\right)^2} \quad (8)$$

$$u^{\mathrm{rel}}_{\text{cal-sol}}(\mathrm{Mo}, V_{\mathrm{S2}}) = \sqrt{\left(\frac{u_{V_{\mathrm{S}}}}{V_{\mathrm{S}}}\right)^2 + (u^{\mathrm{rel}}_{\text{intermediate-sol}})^2 + \left(\frac{u_{VP_{\mathrm{Mo}}}}{V_{P_{\mathrm{Mo}}}}\right)^2 + \left(\frac{u_{V1}}{V_1}\right)^2 + \left(\frac{u_{V2}}{V_2}\right)^2 + \left(\frac{u_{V4}}{V_4}\right)^2} \quad (9)$$

Table 2
Relative standard uncertainty related to the preparation of the stock and intermediate solution[a]

Parameter[a]	V(V)	Mo(VI)
Purity	0.99	0.99
$u(P)$	0.0058	0.0058
Mass (mg)	229.66	920.11
$u(m)$	0.156	0.156
V_{F1} (mL)	100	500
$u(V_{F1})$	0.033	0.061
M_{Msalt}	116.979	1235.858
M_{Mion}	50.942	95.940
n	1	7
$C_{\text{stock-sol}}$ (mg L^{-1})	990	990
$u^{\mathrm{rel}}_{\text{stcok-sol}}$	0.0059	0.0059
$u^{\mathrm{rel}}_{\text{intermediate-sol}}$	0.0072	0.0072

[a] The units of each uncertainty are the same that its source.

Table 4
Standard deviations and uncertainty values obtained in calibration process for V(V) and Mo(VI) determination

	Sample volume		s_{pooled}	c_{p} (mg L^{-1})	$u^{\mathrm{rel}}_{\text{chem-cal}}$
	$s_{\mathrm{s}}(\mathrm{S}_1)$	$s_{\mathrm{s}}(\mathrm{S}_2)$			
V(V)	0.00491	0.00356	0.00429	0.044	0.0975
Mo(VI)	0.00756	0.00387	0.00601	0.086	0.0701

Finally the results obtained for operational relative uncertainty are shown in Table 3.

It can be seen that $u^{\mathrm{rel}}_{\mathrm{op}}$ depends on the sample volume to which SAM is applied and the volumes of analyte intermediate stock solutions added. That is why, the $u^{\mathrm{rel}}_{\mathrm{op}}$ values selected to estimate the overall uncertainty have been, 0.0299 for V and 0.0149 for Mo.

3.2. Inherent uncertainty: $u_{inherent}$

As it has been mentioned previously, the main contributions to this uncertainty are due to:

3.2.1. Intrinsic uncertainty: $u_{intrinsic}$

These main effects are produced by: (A) Liquid–liquid extraction ($u_{\text{extract-chem}}$). Its contribution was evaluated as the R.S.D. values obtained when the extraction process was replicate m times and then, each extract was injected. (B) The chromatographic separation ($u_{\text{chrom-sep}}$), estimated replicating the injection of one extract. The $u^{\mathrm{rel}}_{\mathrm{intrinsic}}$ is calculated according to the next equation:

$$u^{\mathrm{rel}}_{\mathrm{intrinsic}} = \sqrt{(u^{\mathrm{rel}}_{\text{extract-chem}})^2 + (u^{\mathrm{rel}}_{\text{chrom-sep}})^2} \qquad (10)$$

The obtained results were 0.0558 for V(V) and 0.0418 for Mo(VI), respectively.

3.2.2. Chemical calibration uncertainty: $u_{chem\text{-}cal}$

The matrix effect in the synthetic water sample was detected from three calibration procedures, external calibration, internal calibration and a calibration with different sample sizes (Youden calibration) [5,11].

To prove that the proportional interactive effect did not change with the matrix/analyte ratio for the extraction of both analytes, the determination was carried out by applying SAM to two sample levels selected from Youden calibration ($V_{S1} = 30$ mL, $V_{S2} = 60$ mL). The results obtained, $u^{\mathrm{rel}}_{\text{chem-cal}}$ values, are shown in Table 4.

Once the homogeneity of variances has been satisfied for both elements, it permits to use the s_{pooled} to evaluate the uncertainty associated with the chemical calibration. In order to calculate the relative value of uncertainty, the pooled concentration obtained from the two levels assayed has been used.

Thus, $u^{\mathrm{rel}}_{\mathrm{inherent}}$ was calculated according to:

$$u^{\mathrm{rel}}_{\mathrm{inherent}} = \sqrt{(u^{\mathrm{rel}}_{\text{chem-cal}})^2 + (u^{\mathrm{rel}}_{\mathrm{intrinsic}})^2} \qquad (11)$$

The relative inherent uncertainties were 0.1123 for V(V) and 0.0816 for Mo(VI).

3.3. Contribution of bias to uncertainty (u^{rel}_{Bias}): method bias

The definition of uncertainty [12] indicates that the presentation of results must be free from systematic errors, so sometimes, it is necessary to correct the results considering the two bias components: the proportional and the constant bias. Although the method bias was not significant, its uncertainty (method bias uncertainty), should be considered in the uncertainty budget.

3.3.1. Uncertainty associated to proportional bias (u^{rel}_{R})

Proportional bias is calculated using recovery factors (R), from spiked samples. When the recovery has been estimated, this type of bias must be checked to see whether it is statistically significant or not. This is done by seeing whether this parameter is statistically different from one, within the limit

Table 3
Operational relative uncertainty for the two sample levels selected

Vanadium(V)			Molybdenum(VI)		
C (mg L^{-1})	$V_{\mathrm{S}} = 30$ mL	$V_{\mathrm{S}} = 60$ mL	C (mg L^{-1})	$V_{\mathrm{S}} = 30$ mL	$V_{\mathrm{S}} = 60$ mL
0.000	0.0101	0.0077	0.000	0.0101	0.0077
0.015	0.0299	0.0292	0.050	0.0149	0.0134
0.030	0.0184	0.0172	0.100	0.0132	0.0115
0.045	0.0154	0.0140	0.150	0.0129	0.0113

of its uncertainty, performing a significance test of the form:

$$\frac{|R-1|}{u_R} \leq t_{\alpha/2,\nu} \tag{12}$$

where $t_{\alpha/2,\nu}$ is the two sided t tabulated value for the effective degrees of freedom associated with u_{MPB}, which is the uncertainty on the estimated recovery [4].

For this purpose a recovery study was carried out for both analytes. The application of the t-test, explained previously, was not significant. The u_R^{rel} obtained were 0.0225 and 0.0115 for V(V) and Mo(VI), respectively.

3.3.2. *Uncertainty associated to constant bias* (u^{rel}_{MCB})

Bearing in mind that the analytes are already presents in the samples, this type of bias denoted as MCB, is calculated from two calibration experiments (Youden calibration (YC) and external calibration (EC)). The procedure used is based on the one proposed by Maroto et al. [13], in which:

$$u_{\mathrm{MCB}} = \sqrt{s_a^2(\mathrm{YC}) + u^2_{\mathrm{conditions}} + \frac{s_a^2(\mathrm{EC})}{b^2(\mathrm{EC})} + \frac{s_b^2(\mathrm{EC})a^2(\mathrm{YC})}{b^2(\mathrm{EC})} - \frac{2a_{\mathrm{YC}}}{b^2(\mathrm{EC})}\,\mathrm{cov}(a(\mathrm{EC}), b(\mathrm{EC}))} \tag{13}$$

where $s_a(\mathrm{YC})$ represents the standard deviation of the Youden calibration when concentration is plotted against the amount of sample, $u_{\mathrm{conditions}}$ denotes the uncertainty associated with how the amount of samples and the standards of the external calibration are analyzed. If they are analyzed under intermediate conditions, $u_{\mathrm{conditions}} = 0$. $s_a(\mathrm{EC})$ represents the standard deviation of the intercept of the external calibration, $s_b(\mathrm{EC})$ is the standard deviation of the slope of the external calibration, $\mathrm{cov}(a, b)$ denotes the covariance of the intercept and the slope of the external calibration and $a(\mathrm{YC})$ represents the intercept of the Youden calibration.

Finally, when the constant bias has been estimated, it must be checked to see whether it is statistically significant or not. This is done by seeing whether this parameter is statistically different from one, within the limit of its uncertainty, performing a significance test of the form:

$$\frac{|\mathrm{MCB}|}{u_{\mathrm{MCB}}} \leq t_{\alpha/2,\nu} \tag{14}$$

where $t_{\alpha/2,\nu}$ is the two sided t tabulated value for the effective degrees of freedom associated with u_{MCB}, which is the uncertainty of the estimated constant bias.

The application of this test was not significant. The $u^{\mathrm{rel}}_{\mathrm{MCB}}$ obtained were 0.0113 and 0.0099 for V(V) and Mo(VI), respectively.

The total uncertainty associated to the bias $u^{\mathrm{rel}}_{\mathrm{Bias}}$ can be expressed as:

$$u^{\mathrm{rel}}_{\mathrm{Bias}} = \sqrt{(u_R^2)^2 + (u^2_{\mathrm{MCB}})^2} \tag{15}$$

In this case, the final results are 0.0252 and 0.0152 for V(V) and Mo(VI), respectively.

3.4. *Relative overall uncertainty*

The relative overall uncertainty ($u^{\mathrm{rel}}_{\mathrm{overall}}$) was obtained accordingly to:

$$u^{\mathrm{rel}}_{\mathrm{overall}} = \frac{u_{\mathrm{overall}}}{c} = \sqrt{(u^{\mathrm{rel}}_{\mathrm{op}})^2 + (u^{\mathrm{rel}}_{\mathrm{inherent}})^2 + (u^{\mathrm{rel}}_{\mathrm{Bias}})^2} \tag{16}$$

where c is the estimated result.

The relative overall uncertainties were 0.1189 and 0.0843 for V(V) and Mo(VI), respectively. The contribution of the different sources to uncertainty is shown in Fig. 3.

The influence of operational and method bias uncertainties is negligible. The dominant effect is related to chemical

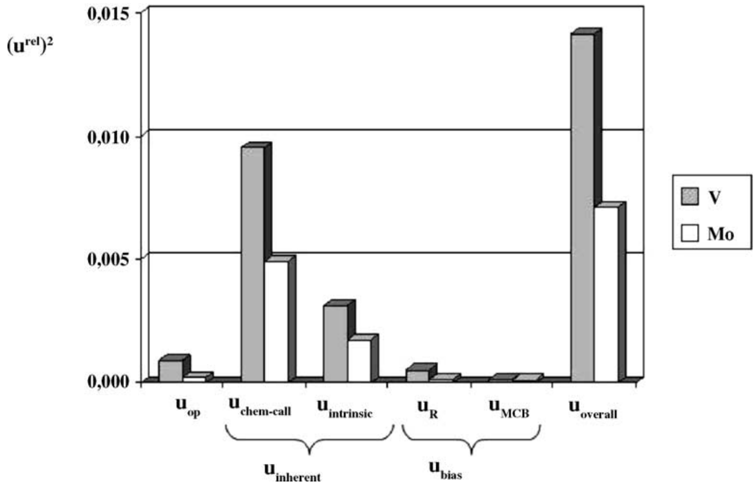

Fig. 3. Contribution of the different sources to the relative uncertainty for the chromatographic determination of V(V) and Mo(VI).

aspects of the method, which are included in the term $u_{\text{inherent}}^{\text{rel}}$. Of the two components of this source of uncertainty $u_{\text{chem-cal}}^{\text{rel}}$ is greater than $u_{\text{intrinsic}}^{\text{rel}}$ for both elements. This fact can be attributed to the necessity of applying SAM to achieve an unbiased result. The use of SAM to obtain the analytical concentration as the intercept on the abscise by extrapolating to analytical response equal to zero, implies an increase of the uncertainty associated with chemical calibration unlike the use of external calibration in which the analytical concentration is calculated by interpolating.

3.5. Expanded overall uncertainty

In order to provide a 95% level of confidence for the final results, the expanded overall uncertainties are obtained by multiplying the overall uncertainties by a coverage factor $k = 2$, so the final results for the sample analyzed, would be expressed as 0.044 ± 0.011 and $0.086 \pm 0.014\ \text{mg L}^{-1}$ for V(V) and Mo(VI), respectively. Finally, after applying a *t*-test, we have sufficient grounds [14] to accept the analytical results, as being free from interactive bias, because Tyson‘s requirement is satisfied.

4. Conclusions

This paper discusses the estimation of the uncertainty of the chromatographic determination of V(V) and Mo(VI) using the data obtained from in-house validation and “bottom-up” approach when SAM must be applied in the calibration step of the analytical procedure. In this case, the main uncertainty source is associated with the chemical aspects of the method (inherent uncertainty), being the chemical calibration the most important contribution. The estimation of $u_{\text{chem-cal}}$ should be done from two sample levels in which Tyson’s requirements have been fulfilled and it is represented by the pooled standard deviation of the sample levels assayed.

On the other hand, as u_{op} depends on the sample volume used and the volume of a intermediate analyte stock solution added, it could be estimated from the calibration point represented by the first addition of analyte at the lower level sample assayed.

Acknowledgments

The authors are grateful to the Consejería de Educación y Ciencia de la Junta de Andalucía for financial assistance (Research Groups FQM 0232 and RNM 0179, Research Project BTE2000-1493).

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