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INTERNAL STANDARD VERSUS EXTERNAL STANDARD CALIBRATION: AN UNCERTAINTY CASE STUDY OF A LIQUID CHROMATOGRAPHY ANALYSIS

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Traditionally, in the cigarettes industry, the determination of ammonium ion in the mainstream smoke is performed by ion chromatography. This work studies this determination and compares the results of this technique with the use of external and internal standard calibration. A reference cigarette sample presented measurement uncertainty of 2.0 μ*g/cigarette* and 1.5 μ*g/cigarette*, with external and internal standard, respectively. It is observed that the greatest source of uncertainty is the bias correction factor and that it is even more significant when using external standard, confirming thus the importance of internal standardization for this correction.

Keywords: internal and external standard calibration; measurement uncertainty; bias correction factor.

INTRODUCTION

An internal standard in analytical chemistry is a chemical substance that is added in a constant amount to samples, the blank and calibration standards in a chemical analysis.[1] This substance can be used for calibration by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte standard concentration. This is done to correct analyte losses during sample preparation.[2] The internal standard is a compound that must be show similar behaviour to the analyte.[3]

This ratio for the samples is then used to obtain their analyte concentrations from a calibration curve. The internal standard used needs to provide a signal that is similar to the analyte signal in most ways but sufficiently different so that the two signals are readily distinguishable by the instrument.

An external standard is like the internal standard (known behaviour), but is not added to the unknown. Rather it is run alone, as a sample, and usually at different concentrations, so you can generate a standard curve. Again, the peak areas are related to the known amounts of external standard run. External standards do not correct for losses that may occur during preparation of the sample, such as extraction, centrifugation, evaporation, etc. Internal standards would correct for this if added to the unknown at the beginning of the sample preparation.[4]

The measurement uncertainty based on the Guide of Uncertainty Measurement (GUM)[5] depends on the proper combination of various sources of uncertainty and therefore the mathematical model used to calculate the appropriate analyte in question, from a calibration curve with external or internal standard.[6,7]

METHODOLOGY

A certified reference material (CRM) was analyzed in 5 replicates (different days) as a sample by ion chromatography with external and internal standard calibration.[8]

Excel spreadsheets were developed to calculate the measurement uncertainty by these two methodologies, based on GUM approach.

The combined standard uncertainty is calculated from the expansion of the Taylor series based on the Law of Propagation of Uncertainties (LPU). Supposing that the output quantity $\hat{y} = f(b_1, b_2, ..., b_n)$ depends on n input quantities $b_1, b_2, ..., b_n$, where each b_i is described by a distribution of appropriate probability, the combined standard uncertainty assumes the form of Equation 1, when taking into account that the quantities are correlated among themselves:[5]

$$u_c^2(\hat{y}) = \sum_{i=1}^{n} \left[\frac{\partial f}{\partial b_i}\right]^2 u_i^2 + 2\sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \frac{\partial f}{\partial b_i} \frac{\partial f}{\partial b_j} u_i u_j r_{ij} \tag{1}$$

From the effective degrees of freedom (number of terms in a sum less the number of restrictions to the terms of the sum), the required coverage factor, k, is calculated in the t-Student table, by Equation 2:

$$\upsilon_{eff} = \frac{u_c^4(\hat{y})}{\sum_{i=1}^{n} \frac{u_i^4(\hat{y})}{\upsilon_i}} \tag{2}$$

And finally, the expanded uncertainty is given by Equation 3:

$$U(\hat{y}) = u_c(\hat{y}) \times k \text{ (for a determined level of confidence)} \tag{3}$$

Experimental data

The ammonium ion derived from the mainstream smoke of the cigarette is trapped (retained) in 4 mmol L^{-1} tartaric acid aqueous solution. Particulate matter is retained in a glass fiber filter, with a diameter of 44 mm, named as Cambridge filter. The filter is transferred to plastic flask with a trap solution, and single extraction in ultrasonic bath was performed. After, the extract is filtered in 0.45 μ*m* Millipore hydrophilic membrane and analyzed by ion chromatography equipped with a conductivity detector.

The linearity of this regression has been checked by the residuals analysis.[9] The residuals were randomly scattered above and below the y-axis.

External standard

The mathematical model is:

$$C_{NH_4^+}(\mu g/cig) = \frac{c_0 \times v}{n} \times f \tag{4}$$

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where: c_0 = result from the linear calibration curve, µg mL^{-1}; v = final volume of the extract, mL; n = number of smoked cigarettes; $f = \frac{real\ CRM}{experimental\ CRM}$ = bias correction factor.

The relevant uncertainty sources are shown in the cause and effect diagram at Figure 1.

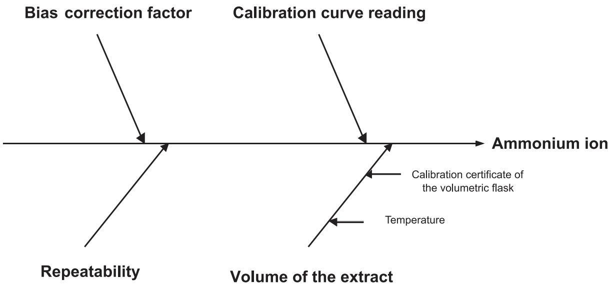

Figure 1. *Relevant uncertainty sources in ion ammonium determination by liquid chromatography using external calibration*

The five calibration standards are measured once a time each, providing the results in Table 1.

Table 1. External calibration results

Concentration, µg mL^{-1}	Signal, mV s^{-1}
0.261	109
1.003	439
2.481	1096
5.000	2216
10.004	4441

The analyte combined standard uncertainty from the linear calibration curve, u_{c_0} is:[10]

$$u_{c_0} = \frac{S}{a} \times \sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(c_0 - \bar{c})^2}{\sum_{j=1}^{n} (c_j - \bar{c})^2}} \tag{5}$$

where: S = residual standard deviation, µg mL^{-1}; a = angular coefficient, mV mL µg^{-1} s^{-1}; p = number of measurements to determine c_0; n = number of measurements for the calibration; c_0 = determined ammonium concentration, µg mL^{-1}; $\bar{c}$ = mean value of the different calibration standards (n number of measurements), µg mL^{-1}; j = index for the number of measurements to obtain the calibration curve.

Considering the non correlated quantities, the combined standard uncertainties derived from (1), when applied to Equation 4 are:

$$\left(\frac{u_{c_f}}{f}\right)^2 = \left(\frac{u_{real\ CRM}}{real\ CRM}\right)^2 + \left(\frac{u_{experiment\ al\ CRM}}{experimental\ CRM}\right)^2 \tag{6}$$

$$u^2_{c_{NH_4^+}} = \left(\frac{\partial C_{NH_4^+}}{\partial c_0} \times u_{c_0}\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial v} \times u_v\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial f} \times u_f\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial Repeatability} \times u_{Repeatabil\ ity}\right)^2 \tag{7}$$

$$u^2_{c_{NH_4^+}} = \left(\frac{v \times Repeatability \times f}{n} \times u_{c_0}\right)^2 + \left(\frac{c_0 \times Repeatability \times f}{n} \times u_v\right)^2 + \left(\frac{v \times Repeatability}{n} \times u_f\right)^2 + \left(\frac{c_0 \times v \times f}{n} \times u_{Repeatabil\ ity}\right)^2 \tag{8}$$

Internal standard

This approach uses a linear calibration curve built with an internal standard of cesium.

The mathematical model is:

$$C_{NH_4^+}(\mu g/cig) = \frac{\left(\frac{A_{ammonium}}{A_{cesium}} - b\right) \times C_{cesium} \times V_{ext}}{a \times n} \times f \tag{9}$$

where: $A_{ammonium}$ = area of ammonium in the sample, mV s^{-1}; A_{cesium} = area of cesium in the sample, mV s^{-1}; C_{cesium} = cesium concentration in extractive solution, µg mL^{-1}; V_{ext} = extractive solution volume, mL; n = number of smoked cigarettes; a = angular coefficient, mV mL µg^{-1} s^{-1}; b = linear coefficient, mV s^{-1}; $f = \frac{real\ CRM}{experimental\ CRM}$ = bias correction factor.

The relevant uncertainty sources are shown in the cause and effect diagram at Figure 2.

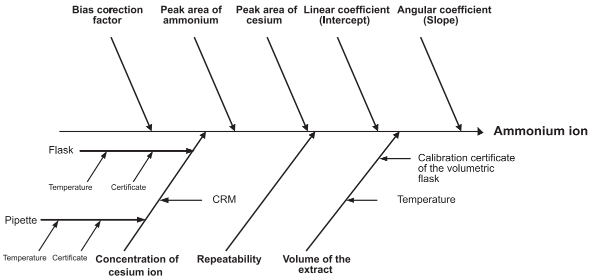

Figure 2. *Relevant uncertainty sources in ion ammonium determination by liquid chromatography using internal calibration*

The five calibration standards are measured once a time each, providing the results in Table 2.

Table 2. Internal calibration results

Concentration ratio	Signal ratio
0.02	0.13
0.06	0.48
0.16	1.20
0.31	2.63
0.63	5.15

Considering the non correlated quantities, the bias correction uncertainty is given by:

$$\left(\frac{u_{c_f}}{f}\right)^2 = \left(\frac{u_{real\ CRM}}{real\ CRM}\right)^2 + \left(\frac{u_{experiment\ al\ CRM}}{experimental\ CRM}\right)^2 \tag{10}$$

The combined standard uncertainty, Equations 11 and 12, is calculated considering the following correlated quantities: analyte area and internal standard area; angular coefficient and linear coefficient.

$$u^2_{c_{NH_4^+}} = \left(\frac{\partial C_{NH_4^+}}{\partial A_{NH_4^+}} \times u_{A_{NH_4^+}}\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial A_{Cs^+}} \times u_{A_{Cs^+}}\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial C_{Cs^+}} \times u_{C_{Cs^+}}\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial a} \times u_a\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial b} \times u_b\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial V_{ext}} \times u_{V_{ext}}\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial f} \times u_f\right)^2 + \left(\frac{\partial C_{NH_4^+}}{\partial Repeteability} \times u_{Repeteabil\ ity}\right)^2 + 2 \times \left(\frac{\partial C_{NH_4^+}}{\partial A_{NH_4^+}} \times u_{A_{NH_4^+}}\right) \times \left(\frac{\partial C_{NH_4^+}}{\partial A_{Cs^+}} u_{A_{Cs^+}}\right) \times r_{A_{NH_4^+} A_{Cs^+}} + 2 \times \left(\frac{\partial C_{NH_4^+}}{\partial a} \times u_a\right) \times \left(\frac{\partial C_{NH_4^+}}{\partial b} \times u_b\right) \times r_{a,b} \tag{11}$$

$$u^2_{c_{NH_4^+}} = \left(\frac{C_{Cs^+} \times \left(\frac{1}{A_{Cs^+}} - b \right) \times V_{ext} \times f \times Repeatability}{a \times n} \times u_{A_{ammonium}} \right)^2 + \left(- \frac{C_{Cs^+} \times \left(\frac{A_{NH_4^+}}{A^2_{Cs^+}} - b \right) \times V_{ext} \times f \times Repeatability}{a \times n} \times u_{A_{cesium}} \right)^2 + \left(\frac{\left(\frac{A_{NH_4^+}}{A_{Cs^+}} - b \right) \times V_{ext} \times f \times Repeatability}{a \times n} \times u_{C_{Cs^+}} \right)^2 + \left(- \frac{C_{Cs^+} \times \left(\frac{A_{NH_4^+}}{A_{Cs^+}} - b \right) \times V_{ext} \times f \times Repeatability}{a^2 \times n} \times u_a \right)^2 +$$

$$\left(- \frac{C_{Cs^+} \times V_{ext} \times f \times Repeatability}{a \times n} \times u_b \right)^2 + \left(\frac{C_{Cs^+} \times \left(\frac{A_{NH_4^+}}{A_{Cs^+}} - b \right) \times f \times Repeatability}{a \times n} \times u_{V_{ext}} \right)^2 + \left(\frac{C_{Cs^+} \times \left(\frac{A_{NH_4^+}}{A_{Cs^+}} - b \right) \times V_{ext} \times Repeatability}{a \times n} \times u_f \right)^2 + \left(\frac{C_{Cs^+} \times \left(\frac{A_{NH_4^+}}{A_{Cs^+}} - b \right) \times V_{ext} \times f}{a \times n} \times u_{Repeatability} \right)^2 + \tag{12}$$

$$2 \times \left(\frac{C_{Cs^+} \times \left(\frac{1}{A_{Cs^+}} - b \right) \times V_{ext} \times f \times Repeatability}{a \times n} \times u_{A_{ammonium}} \right) \times \left(- \frac{C_{Cs^+} \times \left(\frac{A_{NH_4^+}}{A^2_{Cs^+}} - b \right) \times V_{ext} \times f \times Repeatability}{a \times n} \times u_{A_{cesium}} \right) \times r_{A_{ammonium}, A_{cesium}} + 2 \times \left(- \frac{C_{Cs^+} \times \left(\frac{A_{NH_4^+}}{A_{Cs^+}} - b \right) \times V_{ext} \times f \times Repeatability}{a^2 \times n} \times u_a \right) \times \left(- \frac{C_{Cs^+} \times V_{ext} \times f \times Repeatability}{a \times n} \times u_b \right) \times r_{a,b}$$

RESULTS AND DISCUSSION

The results and their expanded uncertainties of ammonium ion in cigarette smoke for the certified reference material, based on Equations 1 to 3, whose reference value is 10.5 ± 0.5 µ*g / cigarette* (k = 2; 95.45%), are: external standard: 10.6 ± 2.0 µg/cigarette, k = 2.28, 95.45%; internal standard: 10.5 ± 1.5 µg/ cigarette, k = 2.04, 95.45%.

Table 3, external standardization, shows that the most relevant uncertainty source is bias correction factor, 62.12%. However, this contribution decreases to 21.36%, when internal standardization is used, Table 4, confirming that this latter approach minimizes losses during the analysis.

Table 3. Uncertainty values in ion ammonium determination by liquid chromatography using external calibration

Uncertainty Evaluation of Ammonium Ion - External Standard										
Quantity	Quantity value	Uncertainty value	Unity	Divisor	Distribuition	Degree of freedom	Sensitivity coefficient, Ci	Standard uncertainy, u(yi) = Ci* ui	Uncertainty u(yi)^2	Contribution
Calibration curve	2.3156	0.0002	*µg/mL*	1.00	Normal	3	4.586404586	0.000722512	5.22024E-07	0.00%
Extract volume – Reading	20.00	0.02	mL	1.00	Normal	infinite	0.531020841	0.010620417	0.000112793	0.02%
Temperature		2	*ºC*	1.73	Rectangular	infinite	0.00230463	0.002661158	7.08176E-06	0.00%
Repeatability	1	0.05	-	1.00	Normal	infinite	10.62041681	0.531020841	0.281983133	37.86%
Bias correction factor	1.146601147	0.073433174	-	1.00	Normal	4	9.262520664	0.680176293	0.46263979	62.12%
					Normal	10	Combined standard uncertainty, u c(C)		0.862985122	100.00%
Number of cigarettes	5						Coverage factor, k		2.28	
							Expanded uncertainty, U(C)		1.97	
Ammonium	10.62		*µg/cig*							
Result: C = (10.6 ± 2.0) *µg/cig*									Uncertainty: 18.6%	

The expanded uncertainty is reported based on a combined standard uncertainty, multiplied by a coverage factor of k = 2.00, providing a confidence level of approximately 95%.

Table 4. Uncertainty values in ion ammonium determination by liquid chromatography using internal calibration

Uncertainty Evaluation of Ammonium Ion - Internal Standard										
Quantity	Quantity value	Uncertainty value	Unity	Divisor	Distribuition	Degree of freedom	Sensitivity coefficient, Ci	Standard uncertainy, u(yi) = Ci* ui	Uncertainty u(yi)^2	Contribution
$A_{ammonium}$	1022.0000	5	*count*	1.00	Normal	5	0.010100565	0.050502823	0.002550535	0.49%
A_{cesium}	733.5000	13	*count*	1.00	Normal	5	-0.014073316	-0.182953105	0.033471839	6.48%
b	-0.0297	0.0331	*count*	1.00	Normal	5	-7.408764193	-0.244945584	0.059998339	11.61%
a	8.3123	0.1028	*cig/mg*	1.00	Normal	5	-1.268317994	-0.130425183	0.017010728	3.29%
C_{cesium}	16.00	0.24	*µg/mL*	1.00	Normal	infinite	0.658911845	0.157206671	0.024713937	4.78%
$V_{extract}$	20.00	0.0287	*mL*	1.00	Normal	infinite	0.527129476	0.015149615	0.000229511	0.04%
Temperature		2	*ºC*	1.73	Rectangular	infinite	0.002287742	0.002641657	6.97835E-06	0.00%
Repeatability	1	0.044	-	1.00	Normal	infinite	10.54258952	0.463873939	0.215179031	41.65%
Bias correction factor	0.962243402	0.03032	-	1.00	Normal	4	10.95626066	0.332192814	0.110352066	21.36%
Correlation $A_{ammonium}$ & A_{cesium}								-0.010794844		10.28%
Correllation a & b								0.063879482		
					Normal	66	Combined standard uncertainty, u c(C)		0.718747246	100.00%
Number of cigarettes	5						Coverage factor, k		2.04	
							Expanded uncertainty, U(C)		1.47	
Ammonium	10.54		*µg/cig*							
Result: C = (10.5 ± 1.5) *µg/cig*									Uncertainty: 13.9%	

The expanded uncertainty is reported based on a combined standard uncertainty, multiplied by a coverage factor of k = 2.00, providing a confidence level of approximately 95%.

The uncertainty quantification for internal standardization is collected in Table 4.

CONCLUSIONS

This work confirms that the internal standardization is a more effective technique than external standardization, because lower measurement uncertainty values were obtained, once that the internal standardization corrects more effectively the greater uncertainty source - bias correction factor.

Generally, in order to make easier, for uncertainty evaluation, the quantities are considered non-correlated, which is not always true, as this study shows.

It also observed that the bias correction factor should be considered in cases where it can not be corrected, because otherwise they will calculate the measurement uncertainty in a wrong way.

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