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# Measurement uncertainty of the caffeine determination in soluble coffee by HPLC

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## Introduction

Even if caffeine analysis in regular soluble coffee has an interest for manufacturers to characterize their products, it is much more used for checking the compliance of decaffeinated soluble coffee with regulations. In Europe the anhydrous caffeine content of decaffeinated coffee may not exceed 0.1 % in green and roasted coffee, and 0.3 % in soluble coffee (dry matter basis). In the USA, the caffeine content has to be reduced to less than 3 % of its original content to label coffee as “decaffeinated”.

Official methods for caffeine analysis in coffee and tea are based on hot water extraction of the caffeine and determination by HPLC (1, 2).

Following the requirements of the ISO 17025 (3), we calculated the uncertainty associated to the determination of caffeine by HPLC in soluble coffee. The approach applied was the one suggested by the Eurachem guide (4). Using this approach, the main uncertainty sources are identified and their contribution to overall uncertainty evaluated.

## Methodology

The procedure used for the estimation follows the recommendations given in the Eurachem guide. Therefore, it is divided into six steps:

- 1) Description of the method
- 2) Specification of the measurand
- 3) Identification of all uncertainty sources
- 4) Quantification of individual uncertainty components
- 5) Calculation of combined uncertainty
- 6) Expression of final expanded uncertainty

### Step 1. Description of the method

The method used is based on the norms DIN 10777 (1) and ISO 10727 (2). After water extraction and clarification using magnesium oxide, caffeine is determined by reversed-phase chromatography with UV detection at 274 nm. Quantification is performed by comparison with external standards. A flow chart of the method is shown in figure 1.

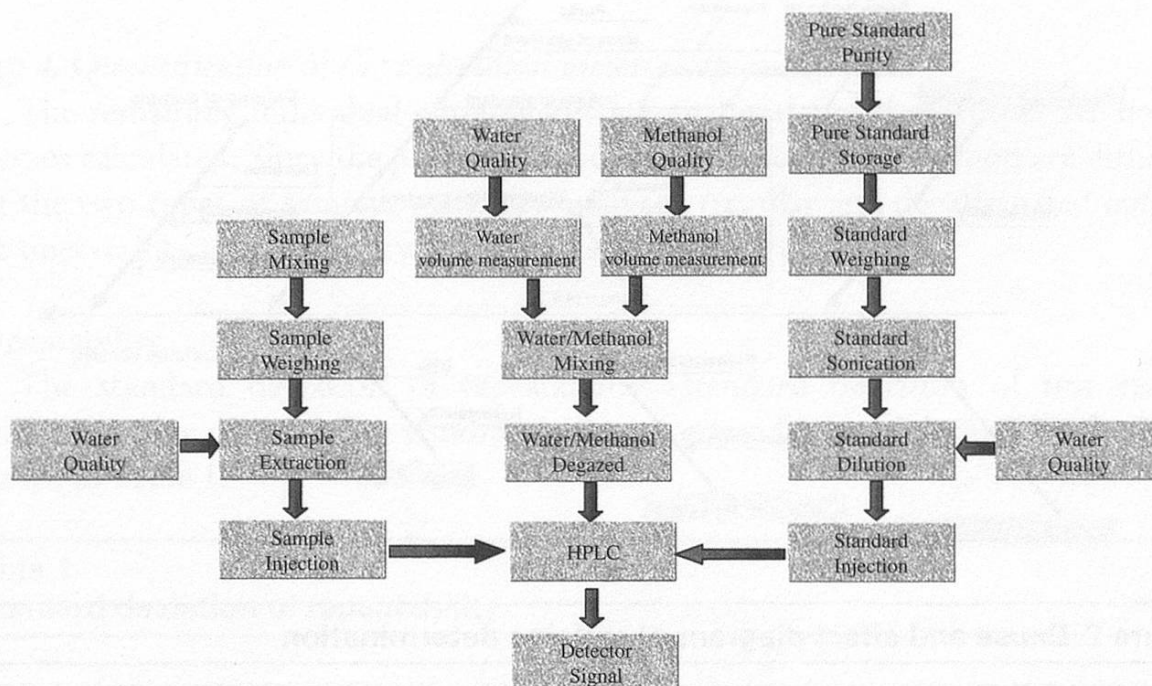


Figure 1 Flow chart of caffeine determination by HPLC

### Step 2. Specification of the measurand

The relationship between the measurand (caffeine) and the input quantities upon which it depends is given by the following equation.

$$\text{Caffeine (g/100 g)} = \frac{A_s \cdot C_0 \cdot V_s \cdot 100}{A_0 \cdot M_s \cdot 10^6} \cdot \frac{1}{R}$$

Where:

$A_s$  = peak area of caffeine in the sample

$C_0$  = concentration of the standard solution (mg/l)

$V_s$  = volume of sample solution (ml)

$A_0$  = peak area of caffeine in standard solution

$M_s$  = mass of sample portion (g)

100 = conversion factor to express the final result in g/100 g

$10^6$  = conversion factor to express the concentration of the standard solution in g/ml

$R$  = correction factor for recovery



### Step 3. Identification of uncertainty sources

The relevant uncertainty sources are shown in a cause and effect diagram. The measurand is represented by the central arrow and the major diagonal arrows represent the variables from the equation (figure 2):

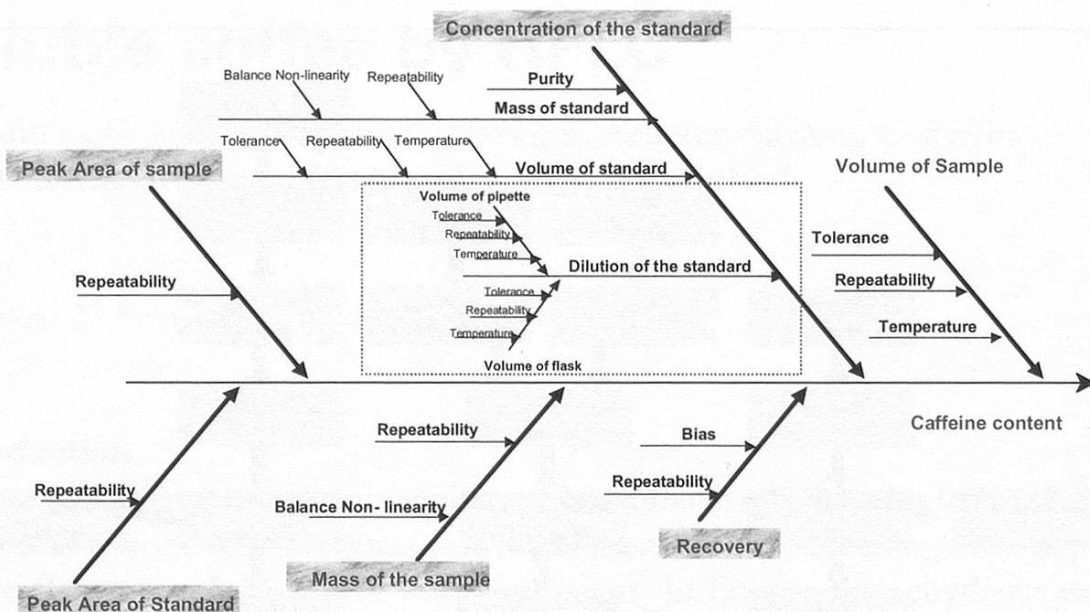


Figure 2 Cause and effect diagram of caffeine determination

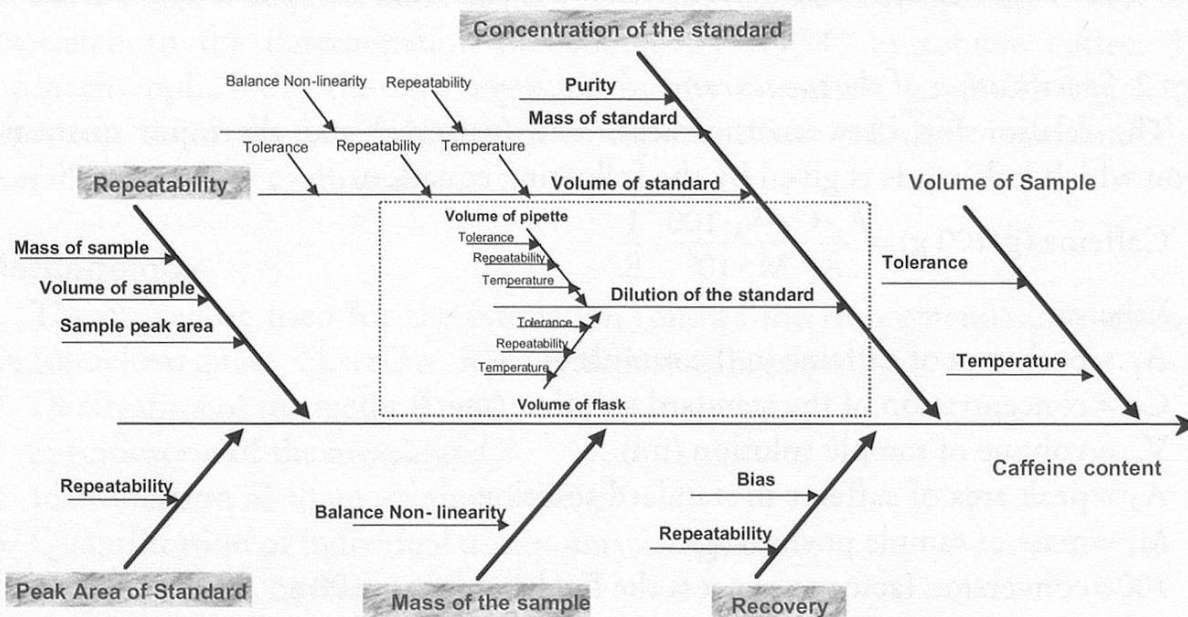


Figure 3 Refined cause and effect diagram of caffeine determination

This diagram can be refined (figure 3) taking into account the following remarks:

- 1) Samples are analysed in duplicate, the repeatability contributions from all operations regarding sample preparation and chromatography (mass, volume of sample and peak area) can be included into a unique repeatability standard deviation of the whole analytical procedure, which is known from validation studies.
- 2) A dilution of standard solution is applied only in the case decaffeinated samples are analysed.

#### *Step 4. Quantification of the individual uncertainty components*

The remaining individual components are analysed and their respective uncertainties calculated. Since the performance characteristics of the method are different for the two types of samples generally analysed (regular and decaffeinated coffee), the uncertainty components will be analysed separately.

#### Repeatability

The standard deviation of repeatability (standard deviation of test results obtained under repeatability conditions (5) is known from validation data and it is shown in Table 1.

Table 1

#### **Standard deviation of repeatability**

	$S_r$
Soluble coffee	0.0361
Decaffeinated soluble coffee	0.0072

#### Trueness/Recovery

The method recovery,  $\bar{R}_m$ , is estimated by comparing the mean of  $n$  replicate analysis of a reference sample (6, 7) from an internal collaborative test with the consensus value,  $\bar{R}_m = \frac{\bar{c}_{obs}}{c_{ref}}$ . The uncertainty associated with this estimate,  $u(\bar{R}_m)$ , is composed of the uncertainty in the observed value (standard deviation of the  $n$  observations),  $u(c_{obs})$ , and the uncertainty in the reference value  $u(c_{ref})$  and is calculated using the following equation. Data and results are given in table 2.

$$u(\bar{R}_m) = \bar{R}_m \cdot \sqrt{\frac{1}{n} \left( \frac{u(\bar{c}_{obs})}{\bar{c}_{obs}} \right)^2 + \left( \frac{u(c_{ref})}{c_{ref}} \right)^2}$$

Table 2

**Estimates of recovery and its uncertainty obtained from replicate analysis of reference samples**

	$C_{ref}$	$u(C_{ref})$	$n_{ref}$	$\bar{C}_{obs}$	$u(\bar{C}_{obs})$	$n_{obs}$	$\bar{R}_m = \frac{\bar{C}_{obs}}{C_{ref}}$	$u(\bar{R}_m)$
Soluble coffee	2.81	0.0186	29	2.815	0.0358	8	1.0018	0.0081
Decaffeinated soluble coffee	0.29	0.0045	29	0.272	0.0084	5	0.9379	0.0195

$C_{ref}$  = reference value (g caffeine/100 g product)

$u(C_{ref})$  = standard uncertainty on the reference value (estimated as the interlaboratory reproducibility divided by the square root of the number of laboratories)

$n_{ref}$  = number of participating laboratories

$\bar{C}_{obs}$  = mean observed concentration in the replicate analysis

$u(\bar{C}_{obs})$  = standard deviation of the observations

$n_{obs}$  = number of replicates

A significance test was used to determine whether the mean recovery was significantly different from unity. The statistic  $t = \frac{|\bar{R}_m - 1|}{u(\bar{R}_m)}$  was compared to a critical value  $t_{crit}$  (two tailed t test at 95 % confidence level with  $n-1$  degrees of freedom) and to the coverage factor ( $k=2$ ) used for the final expression of uncertainty (Table 3).

Table 3

**Significance test for recovery estimation**

	$\bar{R}_m = \frac{\bar{C}_{obs}}{C_{ref}}$	$u(\bar{R}_m)$	$t = \frac{ \bar{R}_m - 1 }{u(\bar{R}_m)}$	$t_{crit}$	
Soluble coffee	1.0018	0.0081	0.22	2.4	$\bar{R}_m$ is not significantly different from 1
Decaffeinated soluble coffee	0.9379	0.0195	3.2	2.8	$\bar{R}_m$ is significantly different from 1

For regular coffee samples, a correction is not necessary, or implicitly  $R=1$ . The standard uncertainty associated with  $R=1$  is given by

$$u(R) = \frac{t_{crit} \cdot u(\bar{R}_m)}{1.96} = 0.0099$$

For decaffeinated coffee samples,  $\bar{R}_m$  is significantly different from 1 but a correction to the final result is not applied. The standard uncertainty is then increased by using the following equation (7) to ensure that the range quoted will include the true value.

$$u(R) = \sqrt{\left(\frac{1 - \bar{R}_m}{k}\right)^2 + u(\bar{R}_m)^2} = 0.0367$$



Where  $k$  is the coverage factor that will be used in the calculation of the expanded uncertainty.

#### Peak area of standard

The response of caffeine has been found to be linear in the range of 10–210 mg/l. In this range, mean and relative standard deviation of response were calculated to be used in the uncertainty budget.

#### Concentration of standard solution

A solution is prepared by weighing 25 mg of caffeine into a 250 ml flask. The contributions to the uncertainty in the preparation of the standard solution are summarized in the uncertainty budget shown in Table 4.

Table 4

#### Estimation of uncertainty in the concentration of the caffeine standard solution ( $c_0$ )

$c_0 \text{ (mg/L)} = \frac{m \cdot P}{V}$				
	Symbol ( $x_i$ )	Value	Standard uncertainty $u(x_i)$	Relative uncertainty $\frac{u(x_i)}{X_i}$
Mass of caffeine	$m$	25 mg	0.236 mg <sup>a)</sup>	0.0094
Purity of caffeine	$P$	>99 %	$\frac{1}{\sqrt{3}} = 0.577\%$ <sup>b)</sup>	0.0058
Volume of solution	$V$	250 ml	0.203 ml <sup>c)</sup>	0.0008
$\frac{u(c_0)}{c_0} = \sqrt{(0.0094)^2 + (0.0058)^2 + (0.0008)^2} = 0.0111$				

a) The relative standard deviation (repeatability) obtained by weighing ten times a mass of 25 mg is 0.17 mg. The contribution due to the calibration (linearity) of the balance is  $\pm 0.2$  mg. This interval needs to be transformed into a standard deviation. A rectangular distribution is assumed (divide by  $\sqrt{3}$ ). This contribution is taken into account twice, once for the tara and one for the sample.

$$u(m) = \sqrt{(0.17)^2 + 2 \cdot \left(\frac{0.2}{\sqrt{3}}\right)^2} = 0.236 \text{ mg}$$

b) The given purity of the caffeine standard is >99%. 100% is taken as the center of a rectangular distribution, the interval being  $100 \pm 1\%$ . The value must be divided by square root of 3 to be transformed into a standard deviation.

c) The uncertainty of a volume has three components: the tolerance of the measuring instrument, the repeatability of the filling and the uncertainty due to the temperature effects. The tolerance of the glassware used is  $\pm 0.15$  ml and a triangular distribution is assumed. The repeatability observed by filling to the mark 10 times a 250 ml flask was 0.07 ml. The working temperature interval in the laboratory was  $\pm 5^\circ\text{C}$ . The volume expansion of water is  $2.5 \times 10^{-4}^\circ\text{C}^{-1}$ , the temperature effect is then  $\pm (250 \times 5 \times 2.5 \times 10^{-4}) = \pm 0.313$  ml. The total uncertainty of the volume is calculated by combining these three effects:

$$u(V) = \sqrt{\left(\frac{0.15}{\sqrt{6}}\right)^2 + (0.07)^2 + \left(\frac{0.313}{\sqrt{3}}\right)^2} = 0.203 \text{ ml}$$

This solution is diluted ten times when analysing decaffeinated samples. The uncertainty due to the dilution factor (see Table 5) needs to be added.

Table 5

**Estimation of uncertainty in the dilution factor of standard solution for analysis of decaffeinated samples (D)**

$D = \frac{V_{1 \text{ ml}}}{V_{10 \text{ ml}}}$				
	Symbol	Value	Standard uncertainty	Relative uncertainty
	$(x_i)$		$u(x_i)$	$\frac{u(x_i)}{x_i}$
Volume of pipette	$V_{1 \text{ ml}}$	1 ml	0.0044 ml	0.0044
Volume of flask	$V_{10 \text{ ml}}$	10 ml	0.019 ml	0.0019
$\frac{u(D)}{D} = \sqrt{(0.0044)^2 + (0.0019)^2} = 0.0048$				

**Mass of sample portion**

The uncertainty in the mass of sample portion depends on the calibration (linearity) of the balance, which corresponds to an interval of  $\pm 0.2$  mg. To transform into standard deviation it is divided by square root of 3 (assuming rectangular distribution). This component uncertainty is counted twice, one for the tara and one for the sample:

$$u(M) = \sqrt{2 \cdot \left(\frac{0.2}{\sqrt{3}}\right)^2} = 0.16 \text{ mg}$$

**Step 5. Calculation of the combined uncertainty**

The combined uncertainties are calculated for the two different types of samples by using the uncertainty budgets in Table 6 and Table 7. The individual contributions to the combined uncertainty are shown in a graphic form in Figure 4.

Table 6

**Uncertainty budget for a soluble coffee sample containing 2.81 g caffeine/100 g**

	Value	Standard uncertainty	Relative uncertainty
		$u(x)$	$\frac{u(x)}{x}$
Repeatability	2.81 g/100 g	0.036 g/100 g	0.013
Recovery	1.00	0.0081	0.0081
Peak area of standard	4243	40.3	0.0095
Concentration of standard solution	100 mg/l	1.11 mg/l	0.0111
Mass of sample	0.5 g	0.00016 g	0.0003
Volume of sample	0.25 l	0.00019 l	0.0008
$\frac{u(\text{caffeine})}{\text{caffeine}} = \sqrt{(0.013)^2 + (0.0081)^2 + (0.0095)^2 + (0.0111)^2 + (0.0003)^2 + (0.0008)^2} = 0.0212$			
$u(\text{caffeine}) = 2.81 \cdot 0.021 = 0.060 \text{ g caffeine/100 g}$			



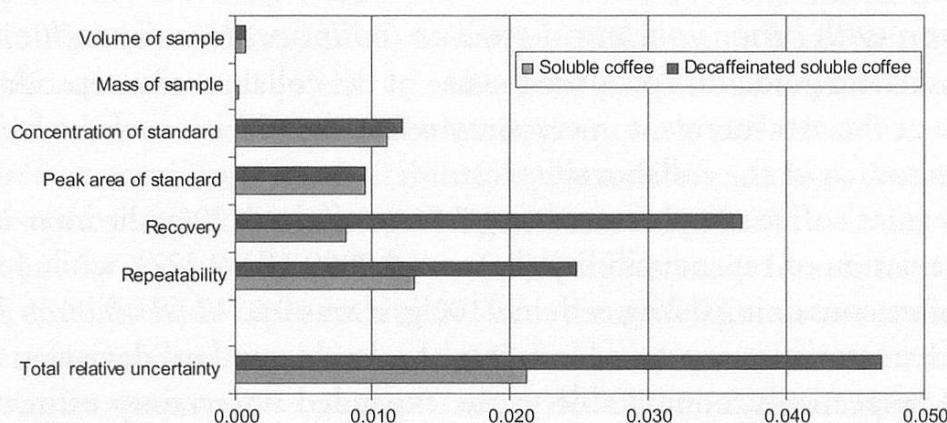
Table 7

**Uncertainty budget for a decaffeinated soluble coffee sample containing 0.29 g caffeine/100 g**

	Value	Standard uncertainty $u(x)$	Relative uncertainty $\frac{u(x)}{x}$
Repeatability	0.29 g/100 g	0.0072 g/100 g	0.0248
Recovery	1.00	0.0367	0.0367
Peak area of standard	424	4.03	0.0095
Concentration of standard solution	10 mg/l	0.121 mg/l	0.0121
Mass of sample	4 g	0.00016 g	0.00004
Volume of sample	0.25 l	0.00019 l	0.0008

$$\frac{u(\text{caffeine})}{\text{caffeine}} = \sqrt{(0.0248)^2 + (0.0367)^2 + (0.0095)^2 + (0.0121)^2 + (0.0004)^2 + (0.0008)^2} = 0.0469$$

$$u(\text{caffeine}) = 0.29 \cdot 0.047 = 0.014 \text{ g caffeine/100 g}$$


**Figure 4 Uncertainty components in the determination of caffeine by HPLC in regular and decaffeinated coffee samples**
**Step 6. Expression of final expanded uncertainty**

The final uncertainty ( $U$ ) is expressed as an interval. To transform the standard uncertainty, it is multiplied by a coverage factor of 2 as suggested by the Eurachem guide. The expression of the analytical result is then reported as Caffeine content: Result  $\pm U(x)$ , where the reported uncertainty is an expanded uncertainty calculated using a coverage factor of 2, which gives a level of confidence of approximately 95 %.

Table 8

**Summary of estimated measurement uncertainties**

	Standard uncertainty	Relative uncertainty	Standard expanded uncertainty	Relative expanded uncertainty
	$u(x)$ (g/100 g)	$\frac{u(x)}{x}$	$U(x)$ (g/100 g)	$\frac{U(x)}{x}$
Soluble coffee	0.060	0.0212	0.12	0.0424
Decaffeinated soluble coffee	0.014	0.0469	0.03	0.0938

**Results and discussion***Comparison with in-house development and validation studies*

As it can be seen in figure 4, the main contributions to the final uncertainty are the precision (repeatability), the bias (recovery) and the calibration. The mass portion and sample volume are negligible for both regular and decaffeinated coffee.

*Comparison with other validation studies (collaborative reproducibility)*

The Eurachem guidelines propose the use of the collaborative reproducibility as an estimate of the measurement uncertainty when the precision of the laboratory is comparable to that of the collaborative test.

For a regular coffee sample containing 2.81 g caffeine/100 g, the inter-laboratory standard deviation of reproducibility ( $S_R$ ) was 0.0999 ( $R=0.277$ ), while for a decaffeinated coffee containing 0.29 g caffeine/100 g, it was 0.0242 ( $R=0.067$ ). These performance characteristics expressed in terms of relative standard deviation are 0.0355 and 0.0834 respectively, comparable to the expanded uncertainty estimated in the present study of 0.0424 (Table 6) and 0.0938 (Table 7). This shows that these values could have been used if in-house validation studies had not been available.

**Conclusions**

The uncertainty in the chromatographic determination of caffeine in soluble coffee has been calculated. Individual sources of uncertainty have been identified, quantified and combined into a relative standard uncertainty for regular and decaffeinated soluble coffee.

The expanded uncertainty was about 4% of the caffeine content for regular soluble coffee while it was about 10% for decaffeinated soluble coffee. The main contributions to the higher relative uncertainty for decaffeinated coffee are the repeatability and the bias.

The relative uncertainties have also been compared with available data from collaborative studies. The relative standard uncertainties obtained from the reproducibility of the collaborative studies were found comparable to the calculated rela-



tive uncertainties. This means that the reproducibility obtained from a collaborative study can be used to estimate the uncertainty of the method, in the case in-house validation data are not available.

## Summary

The measurement uncertainty of caffeine determination by HPLC, in regular and decaffeinated soluble coffee, has been estimated according to Eurachem guidelines. The expanded uncertainty ( $U$ ) was about 4 % of the caffeine content for regular soluble coffee and about 10 % for decaffeinated soluble coffee. The main contributions to the higher relative uncertainty for decaffeinated coffee are the repeatability and the uncertainty of the bias (measured by recovery studies). These uncertainties were compared with precision data from collaborative studies. The relative reproducibilities from collaborative studies were found comparable to the calculated relative uncertainties, and could be used when no in-house validation data are available.

## Résumé

Cette étude présente l'estimation de l'incertitude de mesure pour l'analyse de la caféine par HPLC-UV. La procédure suivie est basée sur celle décrite dans le guide Eurachem. L'incertitude élargie ( $U$ ) est d'environ 4 % pour la mesure de la caféine dans le café normal alors qu'elle est d'environ 10 % pour le café décaféiné. Les contributions majeures à l'incertitude de mesure pour le café décaféiné sont la fidélité (mesurée en conditions de répétabilité) et l'incertitude du biais (estimée par des tests de récupérations). Les incertitudes de mesures estimées à l'aide des données de validation ont été comparées aux valeurs de fidélité obtenues lors de tests inter laboratoires en conditions de reproductibilité. Les valeurs relatives étant comparables, la fidélité obtenue en condition de reproductibilité est donc une bonne estimation de l'incertitude. En conséquence, si des données de validation devaient manquer pour l'estimation de l'incertitude de mesure pour cette méthode, la reproductibilité peut être utilisée comme incertitude de mesure.

## Zusammenfassung

Die vorliegende Arbeit beschreibt die rechnerische Bestimmung der Messunsicherheit der HPLC-Bestimmung von Coffein in Kaffee-Extrakt (Instantkaffee). Hierbei wurden die Richtlinien der Eurachem/CITAC zugrunde gelegt. Die erweiterte Messunsicherheit ( $U$ ) für Coffein beträgt demnach etwa 4 % des Messwertes in normalem Kaffee-Extrakt und ungefähr 10 % in entcoffeiernem Kaffee-Extrakt. Die Hauptbeiträge zur Messunsicherheit in entcoffeiernem Kaffee-Extrakt stellen die Wiederholbarkeit und der Bias dar. Die bestimmten Messunsicherheiten wurden mit statistischen Daten verglichen, die im Rahmen von Ringtests erhalten wurden. Es stellte sich heraus, dass die in Ringtests bestimmten relativen Standardabweichungen der Reproduzierbarkeit in etwa den hierin berechneten Standardabweichungen der Messunsicherheit entsprechen. Somit können diese Reproduzier-



barkeits ersatzweise zur Abschätzung der Messunsicherheit dieser Methode benutzt werden, sollten ausreichende Validierungsdaten im eigenen Labor nicht zur Verfügung stehen.

### Key words

Measurement uncertainty, validation, caffeine, soluble coffee

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