Appendix B

Measurement Equations and Measurement Uncertainty Budgets

Lab ID **BAM**

The folic acid content in the sample and its standard uncertainty were determined by qNMR according to:

$$P_X = \frac{I_X}{I_{CRM}} \cdot \frac{N_{CRM}}{N_X} \cdot \frac{M_X}{M_{CRM}} \cdot \frac{m_{CRM}}{m_X} \cdot P_{CRM}$$

$$\frac{u(P_X)}{P_X} = \sqrt{\left(\frac{u_w(P_X)}{P_X}\right)^2 + \left(\frac{u(M_X)}{M_X}\right)^2 + \left(\frac{u(M_{CRM})}{M_{CRM}}\right)^2 + \left(\frac{u(m_{CRM})}{m_{CRM}}\right)^2 + \left(\frac{u(m_X)}{m_X}\right)^2 + \left(\frac{u(P_{CRM})}{P_{CRM}}\right)^2}$$

P_X, P_{CRM}: purity of the analyte and the internal standard
I_X, I_{CRM}: integral of the signal of the analyte and the internal standard
N_X, N_{CRM}: number of H atoms contributing to the signal of the analyte and the internal standard
M_X; M_{CRM}: molar mass of the analyte and the internal standard
m_X, m_{CRM}: mass of the sample and internal standard
$u_w(P_X)$: standard deviation of the mean of replicate qNMR purity determinations

uncertainty budget:

uncertainty source		*value*	*uncertainty*	*rel. uncertainty*
P_X	[g/g]	0.90095	$2.8323*10^{-4}$	$3.1437*10^{-4}$
M_X	[g/mol]	441.404	0.0284	$6.4358*10^{-5}$
M_{CRM}	[g/mol]	168.192	0.0153	$9.095*10^{-5}$
m_X	[mg]	12.07419	$1.9191*10^{-4}$	$1.5895*10^{-5}$
m_{CRM}	[mg]	6.42576	$1.6710*10^{-4}$	$2.6005*10^{-5}$
P_{CRM}	[g/g]	0.99463	$2.1806*10^{-4}$	$2.1924*10^{-4}$
combined uncertainty	[g/g]		$3.6*10^{-4}$	$4.0*10^{-4}$
expanded uncertainty $U_{95\%}$ (k=2)	[g/g]		$7.2*10^{-4}$	

Lab ID **CENAM**

Folic Acid (mg/g) =1000 - Total related – water – inorganics

Uncertainty source	value	units	information source	Original uncertainty	units	Distribution	Combined uncertainty
water	63.97	mg/g	experimental	5.17	mg/g	A normal	5.17
related impurity A	0.432	mg/g	experimental	0.021	mg/g	A normal	0.02
related impurity D	0.014	mg/g	experimental	0.00024	mg/g	A normal	0.00024
unknown related impurity	0.006	mg/g	experimental	0.00030	mg/g	A normal	0.0003
inorganics (Na)	0.181	mg/g	experimental	0.0044	mg/g	A normal	0.004
					uc (mg/g)	=	**5.17**
					U (mg/g) k=2	=	**10.33**
					Ur (%)	=	1.10
Folic acid			=	**935.40**	**±**	**10.33**	**mg/g**

Lab ID **GLHK**

$X_{PC} = 1 - \Sigma X_{IC}$

where PC – principle component ; IC – impurities components

$U(X_{PC}) = U(\Sigma X_{IC})$

major components of $U(X_{IC})$ include purity of reference standards, precision, and estimation for unknown and undetected impurities.

The estimation for total related structure impurities contributed about 40% of the overall budget whereas uncertainty contributed by water, non-volatiles/inorganics, and residual organic solvent contributed 34%, 25% and 1% respectively.

Lab ID **HSA**

Mass fraction of folic acid (mg/g) was calculated using the equation below:

$$m = (1000 - I_{RSI}) \times (1000 - F_{Others})/1000 \quad (1)$$

Where,

I_{RSI} is the mass fraction (mg/g) of total related structure impurities determined by HPLC-DAD (assuming similar HPLC-DAD response factors and 1000 mg/g of total HPLC purity);

F_{Others} is the sum of mass fraction (mg/g) of other impurities.

$$F_{Others} = F_{VO} + F_W + F_{IR} \quad (2)$$

Where,
FVO is the mass fraction (mg/g) of residual organic solvent;
FW is the mass fraction (mg/g) of water;
FIR is the mass fraction (mg/g) of total non-volatiles/inorganics.

The reported mass fraction of total related structure impurities (FRSI) in Section 3 was calculated using the equation below:

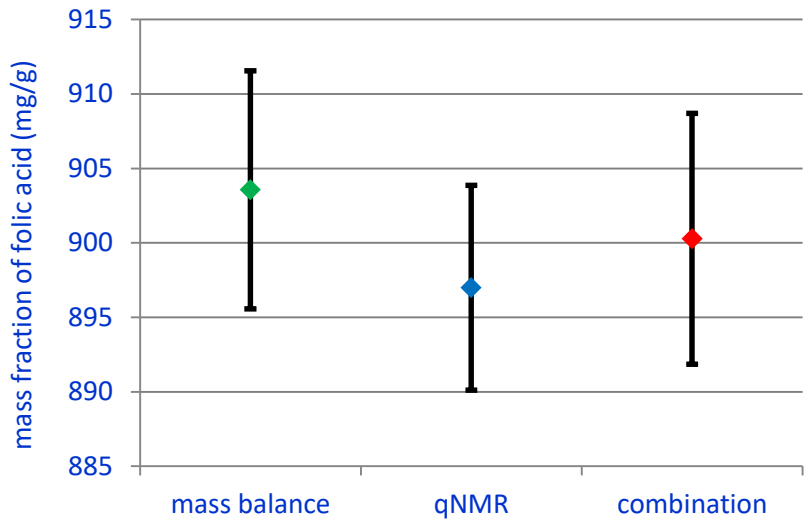
$$FRSI = (1000 - FVO - FW - FIR) \times IRSI /1000 \quad (3)$$

Lab ID **INMETRO**

The average of results obtained by qNMR (897.0 ± 6.9 mg/g, k=2) and mass balance approach (903.6 ± 8.0 mg/g, k=2) was used to assign the mass fraction of folic acid in the sample CCQM-K55.d (Figure 1). The uncertainties from both methods were combined using the following equation:

$$U_{95}(\bar{Y}) = 2\sqrt{\frac{\left(\sum_{j=1}^{N}(Y_j - \bar{Y})^2/N - 1\right) + \left(\sum_{j=1}^{N}\left(\frac{U_{95}(Y_j)}{2}\right)^2/N\right)}{N}}$$

Where:
N = nominally valid estimates from different analytical methods for measurand Y
Yj = the best estimate of the value from method j
$U95(Yj)$ = fully evaluated 95% expanded uncertainty (Duewer *et al.*, 2004)[1]



Lab ID **KRISS**

Equation for assigned content of folic acid and uncertainty budget is following as:

$$P_{\text{Folic acid}} = P_{\text{LC-UV}} \times (1 - P_{\text{water}} - P_{\text{residual organic solvent}} - P_{\text{non-volatile residue}})$$

where $P_{\text{Folic acid}}$ is the final folic acid content (mass fraction), $P_{\text{LC-UV}}$ is content of folic acid by the chromatographic method (LC-UV with C18 column), P_{water} is content of water by K.-F. coulometer, $P_{\text{residual } organic\ solvent}$ is content of residual organic solvent by headspace GC-MS, and $P_{\text{non-volatile residue}}$ is content of non-volatile residue by TGA. The standard uncertainties for the individual measurements including each component by chromatographic method, water content, residual organic solvent contents, and non-volatile residue were independently calculated concerning each uncertainty sources as summarized in Table 1.

Table 1. Uncertainty sources for each individual measurement

Uncertainty		Sources
$u(P_{\text{chromatography}})$		Repeatability of LC analysis
$u(P_{\text{non-chromatography}})$	$u(P_{\text{water}})$	Repeatability of K.-F. coulometer
	$u(P_{\text{residual organic solvent}})$	Repeatability of HS-GC/MS analysis Detection limit of GC/MS Uncertainty in sample weighing
	$u(P_{\text{non-volatile residue}})$	Reproducibility of TGA analysis

The uncertainties of non-chromatography impurity were calculated by by following equation:

$$u(P_{non-chromotagrphy}) = \sqrt{u^2_{P_{water}} + u^2_{P_{residual\ organic\ solvent}} + u^2_{P_{non-volatile\ residue}}}$$

Where $u(P_{\text{chromatography}})$ is the combined uncertainty of all the non-chromatographic uncertainty,

$u(P_{\text{water}})$ is the standard uncertainty of water content, $u(P_{\text{residual organic solvent}})$ is the standard uncertainty of residual organic solvents and $u(P_{\text{non-volatile residue}})$ is the standard uncertainty of non-volatile residue. The final uncertainty ($u(P)$) was calculated by final purity (P) and the relative uncertainty for purity ($u_r(P)$). And the relative uncertainty for purity, $u_r(P)$, was calculated by combining of non-chromatography, $u_r(P_{\text{non-chromatography}})$, and the relative uncertainty of folic acid by chromatography, $u_r(P_{\text{non-chromatography}})$, as following equation:

$$u_r(P) = \sqrt{u_{r\,P_{non-chromotagrphy}}^2 + u_{r\,P_{chromotagrphy}}^2}$$

$$u(P) = \mathrm{P} \times u_r(P)$$

Lab ID **LGC**

The measurement equation (Eqn. 1) to assign the purity of folic acid content of CCQM-K55.d using a mass balance approach is:

$$P_{Total} = \left[1 - \left[\frac{\%water}{100} + \frac{\%IR}{100} + \frac{\%res\ solvent}{100} + \frac{\%rel\ imp}{100}\right]\right] \times P_{org} \qquad \textbf{(Eqn. 1)}$$

Where,

P_{Total} = total purity of folic acid
IR = inorganic residue
$res\ solvent$ = residual solvent
$rel\ imp$ = related impurity
P_{org} = organic purity of folic acid

The standard uncertainty associated with the mass fraction content was calculated from equation 2 (Eqn. 2):

$$u_{Folic\ acid} = \sqrt{u^2_{UPLC\text{-}DAD} + u^2_{water} + u^2_{IR} + u^2_{res\ solvent} + u^2_{rel\ imp}} \qquad \textbf{(Eqn. 2)}$$

Where,

$u_{Folic\ acid}$ = standard uncertainty (su) of the purity value of folic acid
$u_{UPLC-DAD}$ = su of purity value of folic acid determined by UPLC-DAD
u_{water} = su of water content
u_{IR} = su of inorganic residue
$u_{res\ solvent}$ = su of residual solvent
$u_{rel\ imp}$ = su of related impurity by Q-NMR

The uncertainty budget for folic acid using a mass balance approach is summarized in Table 1 (as %m/m).

Table 1. Uncertainty budget for folic acid using mass balance approach

	UPLC	Water	Inorg Res	Res Solvent	Rel Imp
u	2.00E-01	1.61E-01	1.07E-02	2.74E-02	3.11E-03
u^4	1.60E-03	6.72E-04	1.32E-08	5.66E-07	9.34E-11
n	32	33	infinity	3	5
n-1 (d.o.f)	31	32	infinity	2	4
u^4/n-1	5.16E-05	2.10E-05	0.00E+00	2.83E-07	2.33E-11

Components	u_c	v_{eff}
	0.26	61.21

Total standard uncertainty (u_c)	0.26
Effective degrees of freedom (v_{eff})	61.21
t (effective d.o.f), 95%	2.00
Expanded uncertainty (U)	0.52

The measurement equation (Eqn. 3) to assign the purity of folic acid content of CCQM-K55.d using a direct approach is:

$$\%Purity_{Analyte} = \frac{m_{IS}}{m_{Analyte}} \times \frac{Mwt_{Analyte}}{Mwt_{IS}} \times \frac{I_{Analyte}}{I_{IS}} \times \frac{\rho_{IS}}{\rho_{Analyte}} \times 100 P_{IS} \qquad \textbf{(Eqn. 3)}$$

Where,

m_{IS} =	mass of internal standard
$m_{Analyte}$ =	mass of analyte
$Mwt_{Analyte}$ =	molecular weight of analyte
Mwt_{IS} =	molecular weight of internal standard
$I_{Analyte}$ =	integration of analyte
I_{IS} =	integration of internal standard
ρ_{IS} =	no. of nuclei for internal standard
$\rho_{Analyte}$ =	no. of nuclei for analyte
P_{IS} =	purity of internal standard

The standard uncertainty associated with the direct purity assignment of folic acid is given in equation 4 (Eqn. 4):

$$u_{P_{Analyte}} = P_{Analyte} \times \sqrt{\left(\frac{u_{\rho_{IS}}}{\rho_{IS}}\right)^2 + \left(\frac{u_{\rho_{Analyte}}}{\rho_{analyte}}\right)^2 + \left(\frac{u_{P_{IS}}}{P_{IS}}\right)^2 + \left(\frac{u_{Mwt_{Analyte}}}{Mwt_{Analyte}}\right)^2 + \left(\frac{u_{Mwt_{IS}}}{Mwt_{IS}}\right)^2 + \left(\frac{u_{m_{IS}}}{m_{IS}}\right)^2 + \left(\frac{u_{m_{Analyte}}}{m_{analyte}}\right)^2 + \left(\frac{u_{repeatability_{Analyte}}}{repeatability_{Analyte}}\right)^2} \qquad \textbf{(Eqn. 4)}$$

The uncertainty budget for folic acid using a direct approach to assign purity is summarized in Table 2 (as %m/m).

Table 2. Uncertainty budget for folic acid using a direct approach

Quantity/units	Value	u	rel u (%)
$P_{analyte,\ mean}$/%	90.11	0.11	0.1180
$r_{internal\ std.}$	2	0	0.0000
$r_{analyte}$	1	0	0.0000
$P_{internal\ std.}$/%	99.8	0.08992806	0.0901
$MW_{analyte}$	441.3977	0.01537169	0.0035
$MW_{internal\ std.}$	116.0722	0.00342905	0.0030
$M_{internal\ std.}$	10.52633	0.006	0.0570
$M_{analyte}$	21.04933	0.006	0.0285
P_{mean}/%, u_c	**90.11**	**0.146**	**0.162**

Number of samples run	5
Degrees of freedom	4
k	2.78
Purity	90.11
Uncertainty at 95% C.I. ±	0.40

$$\%Purity_{Combined} = \frac{\sum_{i=1}^{N} wiXi}{\sum_{i=1}^{N} w_i} \qquad \textbf{(Eqn. 5)}$$

Where,

w_i = weight factor, $w_i = \frac{1}{U_i^2}$

U_i = expanded uncertainty

x_i = purity value of folic acid

The uncertainty associated with the combined purity assignment is given in equation 6 (Eqn. 6):

$$U_{Combined} = \frac{1}{\sqrt{w_{Direct} + w_{Indirect}}} \qquad \textbf{(Eqn. 6)}$$

Where,

$U_{Combined}$ = uncertainty of the combined purity value of folic acid

w_{Direct} = weight factor for purity value using the direct approach

$w_{Indirect}$ = weight factor for purity value using the mass balance approach

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Lab ID NIM

External method (used in most measurement)**:**

By external standard methods, the standard curve was calculated:

$$A_s=aC_s+b$$

A_s: peak area of standard solutions;
C_s: concentration of standard solutions (mg/mL);
a: slope of standard curve (mL/mg);
b: intercept of standard curve (it is zero);
The mass fraction of analyte in folic acid sample was:

$$X_x=(A_{x\text{-FA}}-b)/a/C_{FA}$$

X_x: mass fraction of analyte x in folic acid sample (g/g)
$A_{x\text{-FA}}$: the area of analyte x in folic acid solution
C_{FA}: the concentration of folic acid solution (mg/mL)
The combined relative uncertainty of mass fraction $u_r(X_x)$ was calculated by:

$$u_r(X_x)=(u_{rA}+u_{rW}+u_{rS}+u_{rL})^{1/2}$$

u_{rA}: the relative uncertainty of repeatability;
u_{rW}: the relative uncertainty of mass weighing;
u_{rS}: the relative uncertainty of purity of standard material;
u_{rL}: the relative uncertainty of linearity.
The uncertainty of mass fraction $u(X_x)$ was calculated by:

$$u(X_x)= X_x \times u_r(X_x)$$

The expanded uncertainty of mass fraction $u(X_x)$ was calculated by:

$$U(X_x)= k \times u(X_x)$$

LC-DAD Method:

For known impurities, external method was used by the area at the maximum absorption wavelength of each impurity.

a) AGA (N-(4-Aminobenzoyl)-L-glutamic acid)

A neat AGA material is bought from TCI (Tokyo Chemical Industry Co. Ltd.) which purity was determined by qNMR and is 973.9 mg/g.

The AGA in folic acid sample was determined by external method using the neat AGA material as an external standard.

b) PA (Pteroic acid)

A neat PA material is bought from Sigma-Aldrich Company, which purity was determined by mass balance method (LC-DAD and KFT) is 966.9 mg/g.

The PA in folic acid sample was determined by external method using the neat PA material as an external standard.

c) Other impurities (Unknown)

The other known impurities were determined by external method using the neat AGA, neat PA and a neat folic acid (~900 mg/g) material as three external standards. The average of three results from three standard curves was regarded as the result. And the uncertainty is combined by three uncertainties from three standard curves, and uncertainties from the difference of absorption (u_{Abs}: the difference between the areas at respective maximum absorption wavelength for each impurities and the area at 280 nm), and the uncertainty between three curve (u_{Between}:the half between the maximum and the minimum of three results).

$$u_{\mathrm{c}} = \sqrt{\left(\frac{u_{\mathrm{M1}}}{3}\right)^2 + \left(\frac{u_{\mathrm{M2}}}{3}\right)^2 + \left(\frac{u_{\mathrm{M3}}}{3}\right)^2 + u_{\mathrm{Abs}}{}^2 + u_{\mathrm{Between}}{}^2}$$

	AGA	PA	Other Impurity			Total
value(mg/g)	3.060	0.4767	11.3737			14.9101
			by FA	by AGA	by PA	
value(mg/g)			11.5893	12.8899	9.6419	
u_{rA}:	8.0%	2.0%	1.9%	1.9%	1.9%	
u_{rW}	0.1%	0.1%	0.1%	0.1%	0.1%	
u_{rS}	5.0%	5.0%	5.0%	5.0%	5.0%	
u_{rL}	0.7%	3.8%	2.0%	0.4%	0.4%	
u_{r} (X_x)	9.5%	6.6%	5.7%	5.4%	5.4%	
$u(X_x)$ (mg/g)			0.663	0.691	0.517	
u_{Abs} (mg/g)			3.639			
u_{Between} (mg/g)			1.624			
$u(X_x)$ (mg/g)	0.290	0.031	4.002			4.012
$U(X_x)$ (mg/g)	0.580	0.063	8.0033			8.025

The water content (X_{w}) of folic acid is:

$$X_{\mathrm{w}} = \frac{m'}{m} \cdot \frac{X_{\mathrm{s}}}{X_{\mathrm{s}}'}$$

m is the weight of sample (FA) (g);

m' is the detected mass of water (mg);

X_{s} is the water content of standard material (hydranal-standard sodium tartrate dihydrate from Sigma-Aldrich (156.6 mg/g));

X_{s}' is the detected water content of standard material (mg/g).

The uncertainty of X_{w} is:

$$u(X_w) = X_w\sqrt{u_A{}^2 + \left(\frac{u_r(m)}{m}\right)^2 + \left(\frac{u_r(m')}{m'}\right)^2 + \left(\frac{u_r(X_s')}{X_s'}\right)^2 + \left(\frac{u_r(X_s)}{X_s}\right)^2}$$

u_A is RSD of Karl Fisher titration.

$u_r(m)$ is determined by the uncertainty of the balance;

$u_r(m')$ is determined by the uncertainty of limit of detection (LOD);

$u_r(X_s)$ is from the certificate of standard material (the difference between the certified value and actual detected value is included);

$u_r(X_s')$ is determined by the RSD of titration of standard material;

Value (mg/g)	78.70
u_A is RSD of Karl Fisher titration	0.97%
$u(m)$ is determined by the uncertainty of the balance	0.06%
$u(m')$ is determined by the uncertainty of limit of detection (LOD)	0.10%
$u(X_s)$ is from the certificate of standard material	4.04%
$u(X_s')$ is determined by the RSD of titration of standard material	1.01%
u_r	4.28%
u (mg/g)	3.367
U (mg/g)	6.733

4) Non-volatiles/ inorganics -ICPMS

	72 Elements	Cl element	Na element
value(mg/g)	1.180	0.142	0.184
u_{rA}: repeatability	5.2%	5.5%	5.0%
u_{rW}: weighing;	0.1%	0.1%	0.1%
u_{rS}: purity of standard material;	1.0%	1.0%	1.0%
u_{rL}: linearity. (ICPMS for 72 elements is (Semi-quantification)	50.0%	1.0%	1.0%
$u_r(X_x)$	50.3%	5.7%	5.2%
$u(X_x)$ (mg/g)	0.593	0.008	0.010

5) Mass balance result

$$P=1-X_R-X_W-X_N-X_V$$

where P is the mass fraction of folic acid;

X_R is the total related structure impurity content determined by LC-DAD;

X_w is the moisture content determined by Karl Fisher titration;

X_N is the non-volatile content determined by ICPMS;

X_V is the volatile impurities content determined by HSGC.

The combined uncertainty $u(P)$ can be calculated as follows:

$$u(P) = \sqrt{u^2(X_R) + u^2(X_W) + u^2(X_N) + u^2(X_V)}$$

Lab ID **NIMT**

Measurement equation

$W_{\text{Folic acid}}$ = 1000- *impurities*

$W_{\text{Folic acid}} = 1000 - [W_{Rel.Subst} + W_{Water} + W_{Non\ Vol.} + W_{Org.\ Solv.}]$

$W_{\text{Folic acid}}$ = Mass fraction of folic acid in CCQM-K55.d sample

$W_{\text{Rel. Subst.}}$ = Mass fraction of folic acid–related structure impurities in CCQM-K55.d sample

W_{Water} = Mass fraction of water in CCQM-K55.d sample

$W_{\text{Non Vol}}$ = Mass fraction of non-volatile impurities in CCQM-K55.d sample

$W_{\text{Org. Solv.}}$ = Mass fraction of volatile organic solvent impurities in CCQM- K55.d sample

Uncertainty estimation

$$u_{\text{wfolic acid}} = \sqrt{(u_{\text{wRel.Subst}})^2 + (u_{\text{wWater}})^2 + (u_{w\text{Non Vol}})^2 + \left(u_{\text{wOrg.Solv.}}\right)^2}$$

a. Summarise the relative contributions of the major components of the overall uncertainty budget.

Uncertainty Budgets

Source of uncertainty	x_i (mg/g)	$u(x_i)$ (mg/g)	Degree of Freedom
Folic acid related impurities	16.69	0.17	5
Water	71.83	5.15	4
Non-volatile impurities	0	0.52	8
Volatile Organic solvent	Not detected	Not detected	Not detected
Total impurities	88.52		
Folic acid content	911.47	5.18	
Expanded uncertainty, k=2	10.36		

Lab ID **NIST**

For the $^{1}H\ qNMR_{IS}$ method, the purity of the K55.d folic acid Test sample $Purity_T$ was estimated using the measurement equation:

$$Purity_T = \left(\frac{N_{IS}}{N_T}\right)\left(\frac{MW_T}{MW_{IS}}\right)\left(\frac{Area_T}{Area_{IS}}\right)\left(\frac{mass_{IS}}{mass_T}\right)Purity_{IS}$$

where: N_{IS} is Multiplicity of the signal of the Internal Standard,
N_T is Multiplicity of the signal of the Test sample,
MW_{IS} is Molecular weight of the Internal Standard,
MW_T is Molecular weight of the Test sample,
$Area_{IS}$ is the integrated signal area of the Internal Standard,
$Area_T$ is the integrated signal area of the Test sample,
$mass_{IS}$ is the mass of the Internal Standard,
$mass_T$ is the mass of the Test sample, and
$Purity_{IS}$ is the purity of the Internal Standard.

Mass buoyancy effects were taken into account, and the uncertainty was considered negligible for this exercise.

The uncertainty evaluation was accomplished using the Observation equation approach as follows:

a. Re-write the measurement equation into:

$$\left(Purity_T \frac{mass_T}{MW_T}\right)\frac{1}{Area_T} = \left(Purity_{IS} \frac{mass_{IS}}{MW_{IS}}\right)\frac{1}{Area_{IS}} = K$$

b. Use the observations of $Area_{IS}$ to obtain a probability distribution of K.
c. Use the observations of $Area_T$, and the probability distribution of K to obtain a probability distribution of $Purity_T$. The computation was accomplished using Markov Chain Monte Carlo coded in OpenBUGS.

The mass balance approach accounts for all the observable impurity components (ICs) via the generic model (1000 - $\sum$ ICs) mg/g. The reported mass fractions and expanded uncertainties (U_{95}) were transformed into probability distributions (i.e., normal, triangular, uniform) for each individual IC. The distributions were used to generate random draws via a Markov Chain Monte Carlo model of (1000 - $\sum$ ICs) mg/g and produce a probability density for the folic acid purity.

For mass fraction assignment of the total related structure impurities, each of seven impurities were combined via a Markov Chain Monte Carlo model, analogously to the complete mass balance approach (above). The distributions for the seven impurities included two Gaussian, two triangular, and three uniform (described in more detail below.)

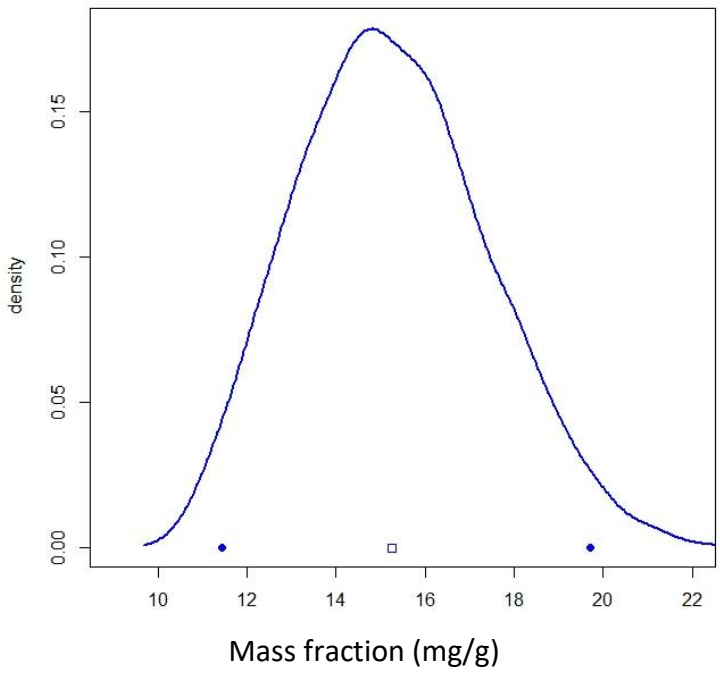


Figure 2. Estimate of mass fraction (mg/g) for structurally-related impurities. Distribution of the sum of the organic impurities was obtained via Monte Carlo method by generating random draws from the probability distributions of the seven observed impurities.

For the mass fraction assignments of water, and non-volatiles/inorganics, a standard GUM approach assuming a coverage factor of 2 was used to combine the individual data and determine the expanded uncertainty.

Lab ID **NMIA**

The measurement equation used to derive the assigned purity value for folic acid, in mg/g, is shown in equation [1].

$$Purity = (1000 - I_{HPLC}) \times (1000 - I_{OT}) \quad \textbf{[1]}$$

Where I_{HPLC} is the mass fraction of all organic impurities of similar structure to the main analyte (folic acid), as determined by HPLC chromatography with PDA detection at 282 nm (λ_{max} of folic acid). Raw HPLC peak areas are converted to mass fractions through consideration of the molar UV response factor (Ri) relative to folic acid, for which R_{FA} is assigned a value of 1, and the respective molecular weights of each component i.

$$I_{HPLGall} = \left(\frac{\sum_{1}^{i} \frac{A_i}{R_i} xMW_i}{\frac{A_{FA}}{R_{FA}} xMW_{FA} + \sum_{1}^{i} \frac{A_i}{R_i} xMW_i} + I_{NR} + I_{ND} \right) x1000 \qquad \textbf{[2]}$$

Where

A_i = Normalised UV area of minor component i

A_{FA} = Normalised UV area of folic acid

The main source of uncertainty is derived from ANOVA of 7 sub-samples analysed in duplicate, using the normalised UV area of folic acid. Uncertainties associated with the molecular weights of individual components are determined using established atomic mass uncertainties [see a) Dolan, J.W., LCGC North America (2009) 27, 472-479, b) IUPAC, Coplen, T.B., Pure and Applied Chemistry 1996, 68, 2339-2359, c) Pure and Applied Chem. 2011, 83(2) 359-396 and d) Pure and Applied Chemistry. 2003, 75(6) 683-800].

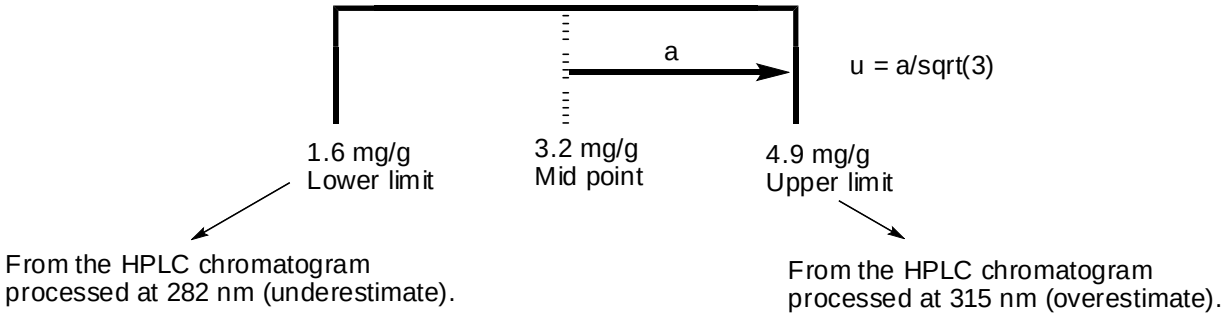
Components shown to have identical chromophores to folic acid are assigned a relative UV response factor (Ri) of 1 with zero uncertainty. For components possessing different chromophores the relative UV response factor (Ri) and associated uncertainty is determined from calibration studies of individual components or direct comparison with molar ratios determined by quantitative NMR spectroscopy.

The mass fraction (mg/g) of water (Karl Fischer analysis), common organic solvents (thermogravimetric analysis and ^{1}H NMR under quantitative condition) and non-volatile residue (e.g. inorganic salts) is summed to provide a value for I_{OT}.

All uncertainties are combined using the square root of the sum of the squares approach, using standard uncertainties or relative standard uncertainties as appropriate.

The major components of the uncertainty budget are:

- ANOVA derived variance between sub sample and within sub-sample determined from 7 sub samples run in duplicate.
- The uncertainty related to quantification of Isomer 2 which was based on the estimated lowest of highest possible value and a rectangular distribution between the two. This is shown schematically below (water content not taken into account).



- The standard deviation of n sub samples analysed for water content by Karl Fischer analysis.

Lab ID **NMIJ**

Table 3 Uncertainty budget of $C_{\text{related imp1}}$

Standard uncertainty component $u(x_i)$	Source of uncertainty	Value of component x_i		Value of standard uncertainty $u(x_i)$		c_i $(= \partial f / \partial x_i)$	$u_i(C_{\text{related imp1}}) = \lvert c_i \rvert u(x_i)$ / (m/g)
$u(C_{\text{imp1 mea}})$	Mesurement standard deviation	4.06	mg/g	0.06	mg/g	1	0.056
$u(A_{\text{related imp1}})$	Measurement variation	135		included $u(C_{\text{imp1 mea}})$		-	-
$u(b)$	Slope of calibration curve	27		0.013		0.0005	6.11×10^{-6}
$u(m_{\text{K55.d sample solution}})$	Balance used	2.999	g	4.95×10^{-6}	g	1.65×10^{-6}	8.17×10^{-12}
$u(a)$	Intercept of calibration curve	-2.94×10^{-6}		1.92×10^{-5}		-6.53	0.0001
$u(m_{\text{K55.d sample}})$	Balance used	3.072	mg	0.005	mg	0.002	7.98×10^{-6}

$u_c(C_{\text{related imp1}}) = 0.06$ mg/g

This result was validated by the comparison of data obtained with LC-UV (280 nm).

Table 5 Uncertainty budget of $C_{\text{related imp2}}$

Standard uncertainty component $u(x_i)$	Source of uncertainty	Value of component x_i		Value of standard uncertainty $u(x_i)$		c_i $(= \partial f / \partial x_i)$	$u_i(C_{\text{related imp2}}) = \lvert c_i \rvert u(x_i)$ / (m/g)
$u(C_{\text{imp2 mea}})$	Measurement standard deviation	3.17	mg/g	0.10	mg/g	1	0.097
$u(A_{\text{related imp2}})$	Measurement variation	4		included $u(C_{\text{imp2 mea}})$		-	-
$u(b)$	Slope of calibration curve	12		0.0002		1.77×10^{-5}	3.69×10^{-9}
$u(m_{\text{K55.d sample solution}})$	Balance used	2.999	g	4.95×10^{-6}	g	1.65×10^{-6}	8.17×10^{-12}
$u(a)$	Intercept of calibration curve	-0.0004		8.36×10^{-6}		-0.020	1.68×10^{-7}
$u(m_{\text{K55.d sample}})$	Balance used	3.072	mg	0.005	mg	0.002	7.98×10^{-6}

$u_c(C_{\text{related imp2}}) = 0.10$ mg/g

Total related structure impurities ($C_{\text{total related imp}}$) were calculated by adding $C_{\text{related imp1}}$ and $C_{\text{related imp2}}$ (= 7.24 mg/g).

Its standard uncertainty was estimated by combining $u(C_{\text{related imp1}})$ and $u(C_{\text{related imp2}})$ (= 0.11 mg/g).

Especially, $C_{\text{related imp2}}$ and $u(C_{\text{related imp2}})$ were used for calculation of final purity mass fraction as mentioned in section 9.

7-2. Water analysis

Mass fraction of water was evaluated from the following equation.

$$C_{\text{water}} = \frac{(Q_{\text{sample,meas}} - dQ_{\text{sample}} \cdot t_{\text{sample}}) - Q_{\text{blank}}}{m}$$

$$Q_{\text{blank}} = Q_{\text{blank,meas}} - dQ_{\text{blank}} \cdot t_{\text{blank}}$$

where,

C_{water}: Mass fraction of water (μg/mg)

$Q_{\text{sample,meas}}$: Mass of water detected in the sample measurement (μg)

dQ_{sample}: Background drift for the sample (μg/min)

t_{sample}: Titration time of the sample (min)

Q_{blank}: Mass of water for the blank (μg)

$Q_{\text{blank,meas}}$: Mass of water detected in the blank measurement (μg)

dQ_{blank}: Background drift for the blank (μg/min)

t_{blank}: Titration time of the blank (min)

m: Weighed mass of the sample (mg)

Table 9 Uncertainty budget of water on K55.d sample

Standard uncertainty component $u(x_i)$	Source of uncertainty	Value of component x_i	Value of standard uncertainty $u(x_i)$	c_i $(=\partial f/\partial x_i)$	$u_i(C_{\text{water}})=\lvert c_i \rvert u(x_i)$ / (μg/mg)	Degrees of freedom
$u(C_{\text{water,rep}})$	Repeatability of water content	79.81 μg/mg	0.14 μg/mg	1	0.14	4
$u(Q_{\text{sample,meas}})$	End point of titration for sample	1710 μg	18.94 μg	$1/m$	0.95	large
$u(\text{d}Q_{\text{sample}})$	Difference between drifts of background for sample	4.96 μg/min	0.18 μg/min	$-t_{\text{sample}}/m$	0.05	large
$u(t_{\text{sample}})$	Titration time of sample	5.17 min	-	$-\text{d}Q_{\text{sample}}/m$	-	-
$u(Q_{\text{blank}})$	Water amount of blank	90.24 μg	18.95 μg	$1/m$	0.95	large
$u(m)$	Amount of sample	19.97 mg	0.004 mg	$-C_{\text{water}}/m$	0.01	large

$u_c(C_{\text{water}})$ = 1.35 μg/mg (= 1.35 mg/g), U = 2.70 μg/mg (= 2.70 mg/g, k = 2)

Mass fraction of residue on ignition (C_{residue}) is evaluated by the following equation.

$$C_{\text{residue}} = \frac{m_{\text{residue}} - m_{\text{bl,after}}}{m_{\text{sample\_res}} - m_{\text{bl,before}}}$$

where m_{sample} and $m_{residue}$ are mass of sample and mass of sample after ignition, respectively. $m_{bl,before}$ and $m_{bl,after}$ are mass of blank before and after ignition, respectively. The results of TGA are tabulated in the following table.

However $m_{residue}$ are smaller than quantification limit of mass loss (4 μg), so the quantification limit is assumed to be an expanded uncertainty with rectangular distribution, and the uncertainty of m_{sample} was negligible. Therefore, standard uncertainty of $C_{residue}$ was estimated as shown below.

$$u(C_{residue}) = (4/\sqrt{3})\ \mu g / 5.4157\ mg$$
$$= 0.43\ mg/g$$

Purity mass fraction by qNMR of K55.d sample was evaluated by using the following equation.

$$P_{qNMR} = \frac{S_a}{S_s}\frac{N_s}{N_a}\frac{m_s}{m_a}\frac{M_a}{M_s}P_{IS}$$

where, P_{qNMR} (kg/kg) is the purity determined by qNMR, P_{IS} (kg/kg) is the purity of IS, S is the integral value of a signal, N is a number of protons generating the signal, M (g/mol) is the molar mass, m (g) is the weighed mass. Indexes a and s correspond to analyte, and IS, respectively. P_{qNMR} was determined to be (0.9018 ± 0.0104) kg/kg (k = 2). Standard uncertainty of the purity mass fraction was estimated by the combination of standard uncertainty of each parameter in above equation. Uncertainty budget of purity determination by qNMR is shown in the following table.

Table 14 Uncertainty budget for purity determination of K55.d sample

Standard uncertainty component $u(x_i)$	Source of uncertainty	Value of component x_i		Value of standard uncertainty $u(x_i)$		$c_i = \partial f/\partial x_i$	$u_i(P_{qNMR}) = \lvert c_i \cdot u(x_i)\rvert$ / (kg/kg)
NMR experiments	ANOVA (sample preparation, peak deviation and repeateability of NMR)	0.9018	kg/kg	0.0022	kg/ kg	1	0.00222
Balance	Mass, (sample + tare)	28.2747	mg	0.00350	mg	$-P_{qNMR}/m_a$	0.00031
	Mass, tare	24.8621	mg	0.00350	mg	P_{qNMR}/m_a	0.00031
	Mass, (std + tare)	36.385	mg	0.00350	mg	P_{qNMR}/m_s	0.00180
	Mass, tare	24.589	mg	0.00350	mg	$-P_{qNMR}/m_s$	0.00180
NMR, Area	Area, sample	5260.6		Included in P_{qNMR} (ANOVA)		P_{qNMR}/S_a	—
	Area, std	31178.9		Included in P_{qNMR} (ANOVA)		$-P_{qNMR}/S_s$	—
NMR, relaxation	NMR peak saturation, sample	1		0.00003		P_{qNMR}/R_a	0.00003
	NMR peak saturation, std	1		0.00003		$-P_{qNMR}/R_s$	0.00003
H-1 nucleus	^{1}H Natural abundance, sample	1		0.00004		$-P_{qNMR}/N_a$	0.00004
	^{1}H Natural abundance, std	9		0.00040		P_{qNMR}/N_s	0.00004
Molar mass	Molar mass, sample	441.404	g/mol	0.017	g/mol	P_{qNMR}/M_a	0.00004
	Molar mass, std	224.35	g/mol	0.01	g/mol	$-P_{qNMR}/M_s$	0.00004
Purity, Internal standard	Internal std	0.9230	kg/kg	0.00400	kg/kg	P_{qNMR}/P_s	0.00391
					Combined standard uncertainty :		**0.0052**

Final purity mass fraction ($P_{\text{K55.d sample}}$) was calculated by subtracting mass fraction of tautomerism isomer obtained with LC-CAD ($C_{\text{related imp2}}$) as mentioned in section 7-1 from P_{qNMR}.

$P_{\text{K55.d sample}}$ was calculated using the following equation.
P_{qNMR} = 0.9018 kg/kg = 901.8 mg/g

$$P_{\text{K55.dsample}} = P_{\text{qNMR}} - C_{\text{relatedimp2}} = 901.8 - 3.17 = 898.6 \text{ mg/g}$$

Associated uncertainty was estimated using $u(P_{\text{qNMR}})$, $u(C_{\text{related imp2}})$, and a difference between the final purity and purity mass fraction determined by mass balance approach ($P_{\text{mass balance}}$) as a method variation.
$P_{\text{mass balance}} = 1000 - (C_{\text{total related imp}} + C_{\text{water}} + C_{\text{residual solvent}} + C_{\text{residue}})$

$= 1000 - (7.24 + 79.81 + 0.05 + 0.35) = 912.6$ mg/g

In this study, uncertainty of the method variation, $u(V_{\text{method}})$, was calculated using a triangular distribution from 912.6 mg/g to 898.6 mg/g because the final purity based on qNMR considered the effect of the unknown impurity. The $u(V_{\text{method}})$ was estimated using the following equation.
$u(V_{\text{method}}) = (912.6 - 898.6) / \sqrt{6} = 5.7$ mg/g
The $u(P_{\text{K55.d sample}})$ was estimated using the following equation.
$u(P_{\text{qNMR}})$ = 0.0052 kg/kg = 5.2 mg/g
$u(C_{\text{related imp2}})$ = 0.10 mg/g
$u(V_{\text{method}})$ = 5.7 mg/g
$u(P_{\text{K55.d sample}}) = \sqrt{(5.2)^2 + (0.10)^2 + (5.7)^2} = 7.7$ mg/g

Lab ID **NMISA**

Measurement Equation:

$$W_{Folic\ acid} = 1000 - (w_{imp\ HPLC\text{-}UV} + w_{RS} + w_{H2O} + w_{nv})$$

Parameter	Description
$W_{Folic\ acid}$	Mass fraction of Folic acid in K55d sample (mg/g)
$w_{imp\ HPLC\text{-}UV}$	Moisture-corrected mass fraction of the sum of organic impurities determined by external calibration by HPLC-UV and %peak area response (mg/g)
w_{RS}	Mass fraction of residual solvent (mg/g) determined by HS-GC-FID
w_{H2O}	Mass fraction of water in K55d sample (mg/g) determined by KF coulometry (direct insertion & oven transfer) and TGA
w_{nv}	Mass fraction of inorganic/ non-volatile residue (mg/g) determined by TGA

Structurally related impurities uncertainty contributors (similarly for acetone):

4-ABGA		3.84	mg/g		
Uncertainty parameter	**Source**	**x**	**u/x**	**vi**	**TYPE**
Mass balance	From certificate	0.005	0.0019	10000	**B**
Precision	ESDM of repeat analysis	3.838452	0.009964	4	**A**
Error on est.	Regression analysis	3.838452	0.032144	7	**A**
			0.0011361	uc/x	
Regression			**0.13**	**uc**	
LOD (mg/g)	0.49		**0.26**	**U (k=2)**	1.72E+09 *veff*
LOQ (mg/g)	1.63		**6.7**	**% Rel U**	

Example of uncertainty contributor for unknown impurity 1 (similarly for impurity 2-6 and residual solvent 1)

Uncertainty parameter	**Source**	**x**	**u(x)**	**u(x)/x**	**vi**	**TYPE**
Precision	ESDM of repeat analysis	0.084942	0.002759	0.032481	5	**A**
Response factor error	deviation between min and max response/ sqrt n	0.037067	0.010823	0.291994	10000	**A**
					0.024956	uc/x
					0.050	U = 2 x u
					59	%Urel

TGA determination uncertainty contributors:

Uncertainty parameter	**Source**	**x**	**u(x)**	**u(x)/x**	**vi**	**TYPE**
Precision	ESDM of repeat analysis	3.21	0.594	0.185023	4	A
Accuracy (CaOX)	Recovery against Calcium Oxalate Ref. Std	0.9853	0.00091	0.000924	4	A
				0.185026	uc/x	
				0.59	uc	
				4.00	veff	
				2.78	k	
				1.6	U (k=2.78)	
				51.4	%Urel	

Moisture content determination uncertainty contributors:

KF Oven transfer	x	u(x)	u(x)/x	vi	type A/B	
Precision	78.75585	0.817558	0.00734	5	A	
Accuracy (NIST SRM2890)	1.004068	0.002657	0.002647	4	A	
Accuray Oven transfer std	0.992279	0.005209	0.005249	4	A	
			0.009404	uc/x		
			0.740651	uc		
			3.367832	veff		
			3.182446	k		
			2.357081	U (k=2.4)	3.0	%Urel
KF direct insertion	x	u(x)	u(x)/x	vi	type A/B	
Precision	82.61413	0.735852	0.006298	5	A	
Accuracy (NIST SRM2890)	1.043466	0.005035	0.004826	4	A	
			0.007934	uc/x		
			0.655499	uc		
			3.148451	veff		
			3.182446	k		
			2.086091	U (k=2.4)	2.5	%Urel
TGA	x	u(x)	u(x)/x	vi	type A/B	
Precision	75.69	0.154434	0.00204	4	A	
Accuracy (CaOX)	1.020902	0.001274	0.001248	4	A	
			0.002392	uc/x		
			0.181039	uc		
			7.553836	veff		
			2.364624	k		
			0.428088	U (k=2)	0.57	%Urel
Combined KF oven transfer, direct insertion and TGA results and UoM:						
	x	u(x)	U (k=2)			
	79.0	2.1	4.1			

Lab ID **NRC**

$$w = \frac{I_{an}}{I_{cal}} \cdot \frac{\rho_{cal}}{\rho_{an}} \cdot \frac{M_{an}}{M_{cal}} \cdot \frac{m_{cal}}{m_{an}} \cdot \frac{V_{an}}{V_{cal}} \cdot P_{cal}$$

Where for analyte (an) and calibrator (cal):
P = purity
I = integrated signal area
ρ = number of protons integrated
M = molar mass (g/mol)
m = weighed mass (g)
n = amount of substance (mol)
V = volume by mass (g) - for external standards only
w = mass fraction of folic acid (mg/g)
Combined uncertainty for qNMR:

$$u_{cnmr} = P_{an}\sqrt{\left(\frac{u(I_{an}/I_{cal})}{I_{an}/I_{cal}}\right)^2 + \left(\frac{u(M_{an})}{M_{an}}\right)^2 + \left(\frac{u(M_{cal})}{M_{cal}}\right)^2 + \left(\frac{u(m_{an})}{m_{an}}\right)^2 + \left(\frac{u(m_{cal})}{m_{cal}}\right)^2 + \left(\frac{u(V_{cal})}{V_{cal}}\right)^2 + \left(\frac{u(V_{an})}{V_{an}}\right)^2 + \left(\frac{u(P_{cal})}{P_{cal}}\right)^2}$$

The major contribution to the uncertainty resides in the determination of Ian/Ical as a result of uncertainties in sample preparation, repeatability, instrument tuning and shimming, manual phasing, baseline correction and integration. The uncertainty due to these components was estimated by the standard deviation of the measurement of the purities of four independently prepared and measured replicates of folic acid using maleic acid as either an internal or external calibrator. The maleic acid was independently value assigned using the same methodology using benzoic acid (NIST 350b) as the internal standard. The uncertainties arising from the molar masses, weighings and calibrator purity proved insignificant relative to the NMR determination of Ian/Ical. A minor correction for related substances underlying the folic acid resonance was applied.

Lab ID **SIRIM**

Assigned value of folic acid purity was obtained by qNMR. Below are the details of the method that has been carried out.

Equation1

$$P_{Ana} = \frac{I_{Ana}}{I_{Std}} \cdot \frac{N_{Std}}{N_{Ana}} \cdot \frac{M_{Ana}}{M_{Std}} \cdot \frac{m_{Std}}{m_{Ana}} \cdot P_{Std}$$

with

Ana	analyte (folic acid)
Std	internal standard (1,2,3,4-tetrachloro-3-nitrobenze)
P	purity
I	integral peak area
N	number of magnetically equivalent protons
M	molecular mass (g/mol)
m	weighed mass

Table 1: Determinaton of content of folic acid

	M	N	Purity content in %
Folic Acid	440.400	3	
Int. Std	260.890	1	**99.72**

$\overline{P_w}$ **- mean (10 samples)**	**90.22**
SD	0.56457
$u_w(P_{analyte})$	0.17853
rel. uncertainty	**0.00198**

Table 2: Uncertainty budget - applying equation 1

uncertainty value of		*value*	*uncertainty*	*rel. uncertainty*
rel. purity $\overline{P_w}$ (integration)	**%**	90.22	0.178531	0.001979
mol. mass folic acid		440.400	0.0154275	0.000035
mol. Mass Int std		260.890	0.006034	0.000023
weight folic acid (mean of 10)	**mg**	22.3712	0.000116	0.000005
weight Int std (mean of 10)	**mg**	13.8878	0.000116	0.000008
content Int Std.	**%**	99.72	0.085000	0.000852
uncertainty			0.194428	0.0021551
uncertainty U95 (k=2)			0.388855	

Purity Folic acid:
902 mg/g ± 4 m
90.2 % ± 0.4 %
with 95% confidence level (k=2).

Lab ID **UME**

a. Mass balance Purity- Measurement Equation

$$w_A = m_A / m_A + \sum m_x = n_A * M(A) / m_A + \sum m_x$$

w_A mass fraction of main component *A* in the material
m_A mass of *A* in an aliquot of the material
Σm_x summed mass of minor components (impurities) in the same aliquot
n_A moles of *A* in an aliquot of the material
M(A) Molar mass of A

$$W_A = 1000 - (W_{RS} + W_W + W_{VOC} + W_{NV})$$

wRS = mass fraction of related structure impurities in the material
wW = mass fraction of water in the material
wVOC = mass fraction of residual solvent (volatile organics) in the material
wNV = mass fraction of non-volatile compounds in the material

The uncertainty of the result of folic acid was mainly affected by the following sources:

- Sample preparation, sample weight
- Repeatability

Table 2. Parameters and their values taken into account in the calculation of uncertainty of the results

Parameter	***Value(X)***	**u(x)**	**u(x)/X**
Sample weight	5.000	0.00002774	0.00000555
Repeatability	100.000	0.397	0.004
Relative Combined Uncertainty			0.004
Result (mg/g)	911.365		
Standart Combined Uncertainty		3.620	
Expanded Uncertainty (k=2)		7.239	
Reported Value Expanded Uncertainty (k=2)	911.365	±	7.239

$$P_A = \frac{I_s}{I_{Std}} \frac{n_{Std}}{n_s} \frac{M_s}{M_{std}} \frac{m_{Std}}{m} P_{std}$$

p_{std}: mass fraction of internal standard.

m_{std}: weight of internal standard.

M_{std}: molecular weight of internal standard.

n_{std} : number of hydrogen of the quantification peak of internal standard.

I_{std}: peak area of quantification peak of internal standard.

m_s: weight of folic acid sample.

n_s: number of hydrogen of the quantification peak at the common structure part of homologues of folic acid sample.

I_s: peak area of quantification peak of folic acid sample.

p_A: mass fraction of sample(Folic acid)

Lab ID **VNIIM**

$w_{FA} = 1000 - (w_{rel.sabst.} + w_{water} + w_{inorg} + w_{VOCs.})$

$w_{FA.}$ - mass fraction of folic acid;

$w_{rel.sabst.}$ - mass fraction of total related structure impurities;

$w_{rel.sabst.} = w_{imp.A} + w_{imp.D} + \Sigma w_{(unident.\ impurity)i}$

w_{water} - mass fraction of water;

w_{inorg} - mass fraction of inorganic impurities;

w_{VOCs} - mass fraction of VOCs

$u_{FA} =$ 

components	u, mg/g	Relative contribution, %
Imp. A	0,146	7,07
Imp.D	0,016	0,77
Σ unident. impurities	1,50	72,64
water	0,39	18,89
Inorganics	0,0035	0,17
VOC	0,0096	0,46