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ULUSAL METROLOJİ ENSTİTÜSÜ

Certification Report

Aflatoxin B_1, B_2, G_1, G_2 and Total Aflatoxin in Dried Fig
UME CRM 1302

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ABBREVIATIONS

ANOVA	Analysis of variance
CRM	Certified Reference Material
AF	Aflatoxin
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
CRM	Certified Reference Material
EU	European Union
FLD	Fluorescence Detection
HPLC	High Performance Liquid Chromatography
IAC	Immunoaffinity Column
IDMS	Isotope Dilution Mass Spectrometry
ISO	International Organization for Standardization
LC	Liquid Chromatography
LTS	Long Term Stability
$MS_{between}$	Mean square between-bottle from ANOVA
MS_{within}	Mean square within-bottle from ANOVA
MRL	Maximum Residue Limit
QNMR	Quantitative Nuclear Magnetic Resonance
Rel	Relative
RSD	Relative standard deviation
SI	International System of Units
STS	Short Term Stability
TRaNS	Random Stratified Sampling Software

SYMBOLS

α	Significance level
N	Number of replicates per unit
s	Standard deviation
s_{bb}	Between-bottle standard deviation
$s_{bb,rel}$	Relative between-bottle standard deviation
s_{wb}	Within-bottle standard deviation
s_{wb}	Relative within-bottle standard deviation
u_{bb}	Standard uncertainty related to possible between-bottle heterogeneity
$u_{bb,rel}$	Relative standard uncertainty related to possible between-bottle heterogeneity
u^*_{bb}	Standard uncertainty of heterogeneity that can be hidden by method repeatability
$u^*_{bb,rel}$	Relative standard uncertainty of heterogeneity that can be hidden by method repeatability
u_{char}	Standard uncertainty related to characterisation
u_{lts}	Standard uncertainty related to long term stability
u_{sts}	Standard uncertainty related to short term stability
$\bar{t}$	Mean of all time points
t_{α}	Two-tailed t-critic value (t test)
t_i	Time point for each parallel

ABSTRACT

Aflatoxins are human carcinogens and their maximum residue limits in foodstuffs are assigned by various regulations. Turkish regulations set MRLs as 8 μg/kg for B_1 and 10 μg/kg for sum of AFB_1, AFB_2, AFG_1 and AFG_2 (total aflatoxins) in Turkish Food Codex (published in Official Gazette numbered 28157 and dated 29/12/2011 with) where EU regulations set MRLs as 6 μg/kg for B_1 and 10 μg/kg for total aflatoxins [1]. Also EU regulations (EU 884/2014) increased frequency of physical and identity checks at import to 20 % for aflatoxins in dried fig exported from Turkey. In order to control and improve measurement quality, validation of analytical methods, ensuring accuracy and traceability of the measurement results for aflatoxins in dried fig, production of a certified material is inevitable.This report explains the various stages of production and certification of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) and total aflatoxin (sum of AFB_1, AFB_2, AFG_1 and AFG_2) in dried fig. These materials have been produced according to the requirements of ISO Guide 34:2009 [2]. Also, it is approved by TURKAK accreditation assessment that production of the material fulfills the requirements of ISO 17034:2016 [3]. Raw materials used in the production of dried figs were obtained as sarı lop (Calimyrna) type cultivated in the Aydın province of Turkey.

Homogenity between units and stability tests were performed according to ISO Guide 35:2006 [4]. It is approved by TÜRKAK assessment that the certification process is in accordance with the ISO Guide 35:2017 [5]. Characterization strategy was planned to utilize TÜBİTAK UME laboratories applying inhouse validated HPLC-FLD and LC-IDMS methods where extraction part was modified from BS EN 14123:2007 according to 6 g minimum sample intake.

INTRODUCTION

Ficus carica L., a deciduous tree belonging to the Moraceae family, is one of the earliest cultivated fruit trees [6]. Carica comes from the region in western Turkey, Caria. Figs are a widespread species commonly grown, especially in warm, dry climates [7]. Fig color varies from dark purple to green [8]. Leaves are used in cosmetic and medicinal industry and also as animal feed; male fruits are used in caprification of female fruit and also as jam; fresh fruits are used as table comsumption or as jam or marmalade; dried fruits are used as table consumption or as in many ways in food industry [8]. Figs are popular as dried fruit since drying prolongs their storability.

Dried fig highly health promoting crop, which is sodium, cholesterol and fat free. It comprise high amount of crude fibers where more than 28 % of it is soluble type, which promotes control of blood sugar and cholesterol [9]. It contains 17 types of amino acids and also one of the highest concentration of polyphenols among the commonly consumed fruits and beverages [8].

Although there are various types of figs cultivated in Turkey, main varieties are Bursa Black and Sarı Lop (Calimyrna). Sarı Lop is one of the best for drying due to its size, taste, fleshy tissue, light color and soft skin, high sugar and low acid content. Best suitable cultivation area for this type of fig is the Aegean region and especially province of Izmir and Aydın. Aydın by itself supplies almost 70-75 %

dried fig since ecologically highly suitable [10]. Fresh harvested figs are dried under sun for 2-4 days over wooden or plastic trays.

Due to unsuitable storage or drying conditions, growing microorganisms in figs generates changes in taste and composition and also produces secondary metabolites named as mycotoxins. Among mycotoxins most important ones are aflatoxins. Generally encountered aflatoxins are AFB_1, AFB_2, AFG_1 and AFG_2 which are produced by mainly molds named *Aspergillus flavus, Aspergillus parasiticus or Aspergillus nomius.* They are named according to their luminescence under UV light, blue color ones named as B and green color ones called G type.

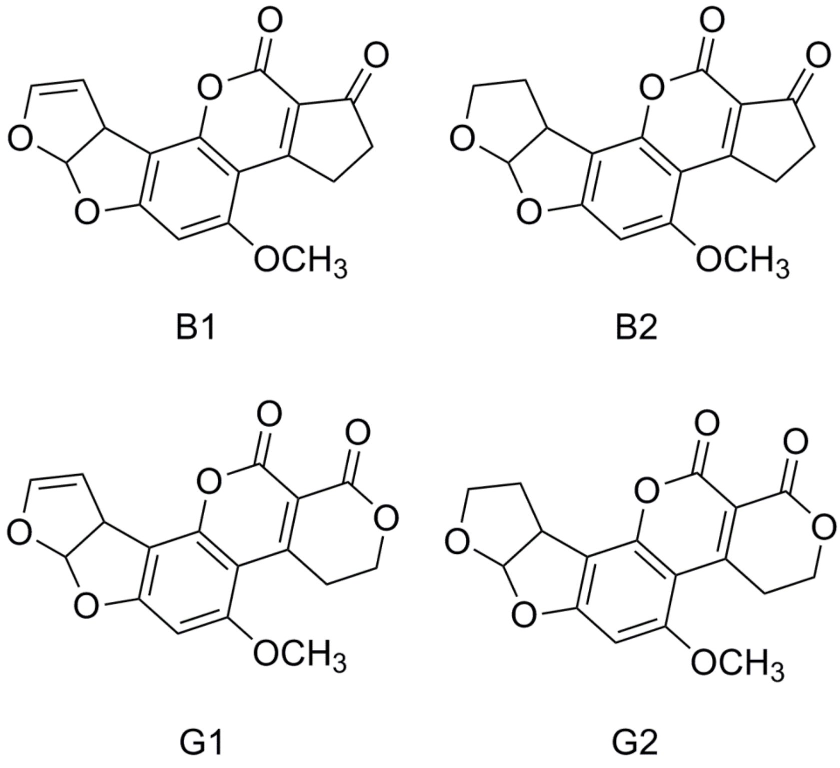

Figure 1. Molecular structures of aflatoxins

Aflatoxins are human carcinogens and may cause several toxic effects in human and animal health such as mutagenicity, immunotoxicity, teratogenicity and suppression of immune system [11]. EU MRL levels for aflatoxins are set as 5 µg/kg for AFB_1 and 10 µg/kg for sum of AFB_1, AFB_2, AFG_1 and AFG_2 (total aflatoxins).

Turkey is the biggest producer of fresh fig and also number one producer and exporter of dried fig, where it provides almost 50-60 % of world's production. From time to time dried figs are rejected from borders due to high contamination. There is no matrix CRM available for aflatoxins in dried fig in the world. The matrix certified reference materials available in the market are starting points in the validation of analytical methods, ensuring accuracy and traceability of the measurement results.

The production of UME CRM 1302, which was carried out by TÜBİTAK UME is described in this report and it is intended to be used as a quality assurance and quality control tool especially by the laboratories involved in the mandatory monitoring prescribed by relevant food legislations.

PARTICIPANTS

All production and certification processes were performed mainly in TÜBİTAK UME and TÜBİTAK MAM GIDA Enstitüsü laboratories. Raw material and bottled final product were gamma irradiated at GAMMA-PAK in Çerkezköy, Tekirdağ. Lyophilization of the product was performed at İSTANBUL PENDIK VETERİNER KONTROL ENSTİTÜSÜ in Pendik, Istanbul.

Study	Laboratory
Project management and data evaluation	TÜBİTAK UME, Ulusal Metroloji Enstitüsü, Gebze, Kocaeli, Turkey
Sampling and processing	TÜBİTAK MAM GIDA Enstitüsü, Gebze, Kocaeli, Turkey İSTANBUL PENDİK VETERİNER KONTROL ENSTİTÜSÜ GAMMA-PAK STERİLİZASYON SAN. ve TİC. A.Ş., Çerkezköy, Tekirdag, Turkey
Homogeneity study	TÜBİTAK UME, Ulusal Metroloji Enstitüsü, Gebze, Kocaeli, Turkey
Stability studies	TÜBİTAK UME, Ulusal Metroloji Enstitüsü, Gebze, Kocaeli, Turkey
Characterisation study	TÜBİTAK UME, Ulusal Metroloji Enstitüsü, Gebze, Kocaeli, Turkey

MATERIAL PROCESSING

Preliminary works for the processing of UME CRM 1302 were mainly divided into two parts. In the first part, it was aimed to obtain a powder product having the desired properties. For this purpose, laboratory and pilot scale feasibility studies were carried out. The most appropriate technique was determined in the laboratory scale studies using different processing techniques. In the second part, homogeneity and short term stability tests of obtained bottled powder products were performed and final decision about the route for large scale processing was given.

Raw materials used in the production of dried figs were obtained from the Aydın province that meet about 70-75% of the production in Turkey. 300 kg of uncontaminated dried fig and 25 kg of dried fig contaminated by aflatoxin were supplied from an exporting company in Aydın province as a starting material for the production of certified reference materials of aflatoxins in dried fig. The starting material was examined considering the visual UV findings before beginning the processing. All raw material was subjected to gamma irradiation at around 5.3 kGy to prevent any microbiological activity. Since the aflatoxin content of the starting material is known to be stable under dry, dark and cold conditions, raw material was then kept in cold storage rooms at -18 °C until the processing.

One of the most important and critical steps in the processing was the lyophilization, which is necessary to reduce moisture content of the material to minimize biological activity and improve long term storage stability. Lyophilization process was optimized for powder fig material which was obtained with the use of a blender homogenizer (Robot Coupe, Blixer 23, USA) with the addition of ~13 % moisture-retaining material. The flowchart of the production process is presented in Figure 2.

After lyophilization (loss of mass was ~14%) and blending processes, all powder material was sieved with 500 µm sieve. After homogenization with 3-D mixer (3-D MegaMix, HKTM, Turkey), material was bottled (as 160 g to each bottle) using a semi-automatic filling machine (Augapack, Vectofill, Belgium) and capped CRMs were subjected to second gamma irradiation (5.3 kGy) before storing at - 80 °C.

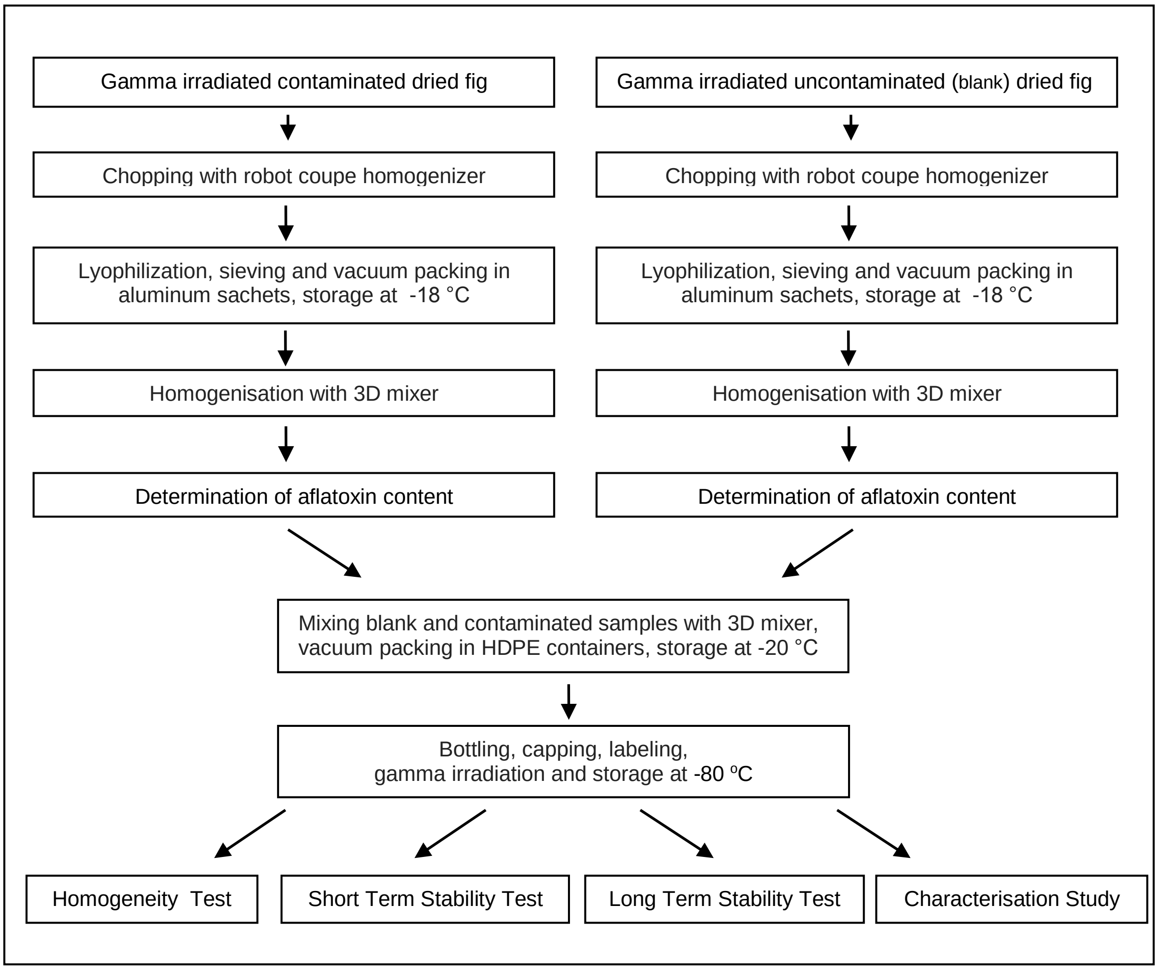

Figure 2. The flowchart of the production process of dried fig

The product obtained from the production of certified reference material was bottled as 160 g. The powder product obtained from the production was filled into light-impermeable airtight brown bottles Samples wererandomly selected with TRaNS was subjected to homogeneity, stability and characterisation tests. The results obtained by the analysis of selected units as were evaluated statistically.

HOMOGENEITY

Homogeneity study between the units is performed to show that assigned value is valid for all units within the stated uncertainty. Homogeneity study between the units is performed with number of samples representing the whole batch. In this project, 20 units (10 spare) are selected by using random stratified sampling software (TRaNS) and were reserved for the study of homogeneity between units. Homogeneity tests were carried out for all analytes of candidate CRM by measuring 3 sub-samples under the repeatability conditions. The method used for these measurements was validated and the samples to be analysed were introduced to the instrument by random order to find out any trend arising from analytical and/or filling sequences. All homogeneity measurements were carried out using HPLC-FLD method. All the data for homogeneity measurements are given in Annex 1 and the plots are given in Annex 2.

The data for all analytes were evaluated statistically by regression analysis for the presence of any trend in analytical and filling sequence. After evaluation of data, no trend was found for any analyte in CRM candidate at 95% confidence level.

Grubbs test was applied to all data for the presence of outlier at 95% confidence level. According to data obtained for each analyte in the candidate CRM, it was found that the distribution was found normal and no outlier was found (Table 1).

Table 1. Statistical Evaluation Result of Homogeneity of CRM 1302

Analyte	Is there aTrend?		Is there an Outlier?		Distribution
	Analytical sequence	Filling sequence	All data	Unit averages	All data
AFB_1	No	No	No	No	Normal/unimodal
AFB_2	No	No	No	No	Normal/unimodal
AFG_1	No	No	No	No	Normal/unimodal
AFG_2	No	No	No	No	Normal/unimodal
Total AF	No	No	No	No	Normal/unimodal

Analysis of Variance (ANOVA) is a statistical tool used to estimate the uncertainty contribution from homogeneity of the materials. All data were examined for normal data distribution using Shapiro-Wilk test and histograms before applying one way ANOVA test. All analytes (AFB_1, AFB_2, AFG_1, AFG_2 and total AF) showed normal distribution on Shapiro-Wilk test and histogram diagrams. Uncertainities of homogeneity between units were evaluated with one way ANOVA for all analytes. The equation (1) is used for the calculation of the repeatability of the method (s_{wb}) and equation (2) is used for the calculation of standard deviation between units (s_{bb}).

$$s_{wb} = \sqrt{MS_{within}} \qquad (1)$$

where

MS_{within} : mean of square of variance within the unit

s_{wb} equals to "s" of the method as long as sub samples represent the whole unit.

$$s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \tag{2}$$

where

$MS_{between}$: mean of square of variance between units

n : number of replicates per unit

$MS_{between}$ is found to be smaller than MS_{within} in conditions for which the heterogeneity of the material is smaller than heterogeneity that can be determined by the applied analytical method or measurement fluctuations that may have occurred randomly. In these cases, since s_{bb} can not be calculated, u^*_{bb} is calculated as heterogeneity contributing to uncertainty including method repeatability using equation (3).

$$u^*_{bb} = \frac{s_{wb}}{\sqrt{n}} \sqrt[4]{\frac{2}{\nu_{MSwithin}}} \tag{3}$$

where,

$\nu_{MSwithin}$: degree of freedom of MS_{within}

The uncertainity values obtained from the homogeneity study are given in Table 2.

Table 2. Homogeneity Results of UME CRM 1302

Analyte	Average value (ng/g)	$s_{bb,rel}$ (%)	$u^*_{bb,rel}$ (%)	$u_{bb,rel}$ (%)
AFB_1	5.38	2.27	2.29	2.29
AFB_2	0.60	$MS_{between}<MS_{within}$	4.05	4.05
AFG_1	2.21	7.46	4.30	7.46
AFG_2	0.18	4.00	4.57	4.77
Total AF	8.37	1.52	2.05	2.05

The values of $MS_{between}$ were found to be smaller than the values of MS_{within} for analyte AFB_2. Thus $\mathbf{u^*_{bb}}$ is calculated and used as the uncertainty contribution due to homogeneity. For the cases where both s_{bb} and u^*_{bb} can be calculated, the bigger one is taken as uncertainty contribution due to between bottles homogeneity (u_{bb}).

STABILITY

Stability studies were carried out with the simulation of conditions in the laboratory, considering environmental conditions that may occur during shipment to the user and storage conditions.

Short Term Stability Study

Stability studies were performed with isochronous design which is cited in ISO Guide 35 [4]. For the Short Term Stability (STS) test, two different temperatures (-20˚C and 4˚C) and 4 time points (1, 2, 3 and 4 weeks) were tested. 10 samples for CRM 1302 were selected by TRaNS. 8 samples were subjected to the test temperatures for the specified time intervals.

Samples were moved to -80˚C (reference temperature) after completion of the test time. All samples were analysed at the same time. Two replicate samples were prepared from each unit and were analyzed by HPLC-FLD method under the repeatability conditions for determining the mass fractions of AFB_1, AFB_2, AFG_1, AFG_2 and total AF.

The data for each temperature were first examined by single Grubbs test for both 95 % and 99 % confidence intervals to find out outliers. Number of detected outliers are given in the Table 3. Since no technical reason can be found to reject these data, all outliers were included in the STS calculations.

Values calculated for each time point were plotted against the time for the assessment of short term stability. The relationship between variables were analyzed in order to determine if any significant change exists in mass fraction values with the testing time (*regression analysis*). It was found that the slopes were not significantly different than zero for all CRMs in the 95 % confidence interval. All data related with short term stability are given in Annex 3 and plots are given in Annex 4.

Uncertainty calculations are done using equation (4). Maximum time for transfer is chosen as 2 weeks.

$$u_{sts} = \frac{RSD}{\sqrt{\sum(t_i - \bar{t})^2}}\, x\, t \qquad (4)$$

where,

RSD : relative standard deviation obtained from all data in STS

t_i : time point for each replicate

$\bar{t}$: mean of all time points

t : maximum time suggested for transfer: 2 weeks

Results obtained from short term stability are given in Table 3.

Table 3. Short Term Stability Test Results

Analyte	-20 °C $u_{sts,rel}$ (%) for 2 weeks	4 °C $u_{sts,rel}$ (%) for 2 weeks	Number of outliers in 95 % confidence interval*		Number of outliers in 99 % confidence interval*		Is there a significant trend in 95 % confidence interval?		Is there a significant trend in 99 % confidence interval?	
			-20 °C	4 °C	-20 °C	4 °C	-20 °C	4 °C	-20 °C	4 °C
AFB_1	2.41	2.42	1	-	1	-	No	No	No	No
AFB_2	2.42	2.76	-	-	-	-	No	No	No	No
AFG_1	6.38	4.81	-	-	-	-	No	No	No	No
AFG_2	5,25	4.34	-	-	-	-	No	No	No	No
Total AF	3.06	2,58	1	-	-	-	No	No	No	No

* Single Grubbs Test

Result of this study showed that the CRM can be transferred to the end users within 2 weeks time interval ensuring the temperature not to exceed + 4°C with cooling elements.

Long Term Stability Study

Shelf life of the produced CRM is determined by the long-term stability (LTS) studies. +4 °C was choosen as the test temperature for long term stability tests and totally 20 units including references (10 spare) for CRM were reserved for this study. Samples were selected by TRaNS software and kept at +4 °C for 9 months. Two units for each time point (0, 2, 4, 6, and 9 months) were stored at +4 °C and transferred to -80 °C (reference temperature) after completion of the test time. Two replicate samples were prepared from each unit and analyzed by HPLC-FLD under the repeatability conditions for determining the mass fractions of AFB_1, AFB_2, AFG_1, AFG_2 and total AF.

The data was first examined by single Grubbs test for both 95 % and 99 % confidence intervals to find out outliers. Number of detected outliers are given in the Table 4. Since no technical reason was present to reject these data, all outliers were included in the LTS calculations. All data related with long term stability are given in Annex 5 and plots are given in Annex 6.

Values calculated for each time point were plotted against the time for the assessment of LTS. The relationship between variables were analyzed in order to determine if any significant change exists in mass fraction values with the testing time (regression analysis). It was found that the slopes were not significantly different than zero for all analytes in the 95% confidence interval. Uncertainity contribution of long term stability, u_{lts}, is calculated using equation (5) for 6 months of shelf life at +4 °C.

$$u_{lts} = \frac{RSD}{\sqrt{\sum (t_i - \bar{t})^2}} \times t \qquad (5)$$

where,

RSD : relative standard deviation obtained from all data in LTS
t_i : time point for each replicate
$\bar{t}$: mean of all time points
t : shelf life suggested at +4 °C: (6 months)

Shelf life for CRM is set as 6 months after sales. Continuous post certification monitoring studies are going to be carried out in order to check the validity of the certified value over longer time.

Table 4. Long Term Stability Test Results of CRM 1302

Analyte	$u_{lts,rel}$ (%) at +4 °C for 6 months	Number of outliers in 95 % confidence interval*	Number of outliers in 99 % confidence interval*	Is there a significant trend in 95 % confidence interval?	Is there a significant trend in 99 % confidence interval?
AFB_1	5,36	-	-	No	No
AFB_2	6,66	1	-	No	No
AFG_1	6,38	1	1	No	No
AFG_2	6,30	-	-	No	No
Total AF	4,93	-	-	No	No

* Single Grubbs Test

CHARACTERIZATION

Characterization and value assignment in accordance with ISO Guide 34 can be made in four different ways [1]. In this project, characterization was performed using two methods of demonstrable accuracy in one competent laboratory in compliance with ISO Guide 34. For the characterization two different methods were used: In-house validated methods based on immunoaffinity column (IAC) clean up and reversed phase HPLC with post column derivatization and fluorescence (FLD) detection modified from BS EN 14123:2007 method for 6 g sample size and also LC-IDMS method based on same extraction and clean up. Standards used for the calibration of the methods were obtained from commercial suppliers and purity values were assigned by Q-NMR at TÜBİTAK UME, traceable to SI via UME CRM 1301 reference material.

Samples to be analyzed were selected randomly from the set of samples determined by TRaNS.

Data obtained from characterization study revealed normal distribution and measurement uncertainities were calculated according to the "Guide to the Expression of Uncertainty in Measurements (GUM)" and "EURACHEM/CITAC Guide Quantifying Uncertainty in Analytical Measurement" documents, with Equations (6) – (8) stated in the literature by M. S. Lenson et al.

$$u(B) = \frac{|X_{HPLC} - X_{LC\ ID\ MS}|}{2\sqrt{3}} \quad (6)$$

$$u(X) = \sqrt{\left(\frac{1}{2}\right)^2 u^2(HPLC) + \left(\frac{1}{2}\right)^2 u^2(LC\ IDMS)} \quad (7)$$

$$u_{char} = \sqrt{u^2(X) + u^2(B)} \quad (8)$$

where,

$u(B)$: The standard uncertainty based on the difference of results of two methods

$u(X)$: The standard uncertainty obtained by combining uncertainties of two methods

u_{char} : The standard uncertainty of characterization by two methods

Value assignment of the material performed by averaging two method results.

All data related to the characterisation study are given in Annex 7.

PROPERTY VALUE AND UNCERTAINTY ASSIGNMENT

Assigned values and uncertainities of the CRM was evaluated by applying approach in the characterization and uncertainty data that contribute to the homogeneity and stability assessments.

Data obtained in the characterization study were checked for normal distribution and outliers. Distributions were found to be normal and no outlier was detected.

Mean value of all accepted characterisation results is assigned as the property value of the reference materials. Absence of bias for the assigned value due to same extraction method in two methods used for characterization was further confirmed by dataset obtained from the supplementary comparison study "CCQM-K138 Determination of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2 and Total AFs) in Dried Fig" organized by TUBITAK UME within Organic Analysis Working Group (OAWG) of Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) [13]. Graphs of the results reported by the participating laboratories to the comparison study is given in Annex 8.

Formula (7) is used to calculate the combined expanded uncertainty of CRMs:

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2 + u_{sts}^2} \quad (9)$$

Uncertainity value on CRM certificate includes uncertainity contribution from characterization (u_{char}), homogeneity (u_{bb}) and long term stability (u_{lts}). Expansion of uncertainity value of CRMs were done with a covarege factor (*k*=2) representing 95 % confidence level. Certified values and uncertainities are given in Table 5 and relative percent contribution of each component to uncertainity is given in Table 6.

Table 5. Certified values and uncertainity components

Analyte	Certified value (µg/kg)	U_{CRM} (µg/kg) (k=2)	$U_{CRM,rel}$ (%) (k=2)	$u_{char,rel}$ (%)	$u_{bb,rel}$ (%)	$u_{lts,rel}$ (%)	$u_{sts,rel}$ (%)
AFB_1	5.5	0.8	14.22	3.28	2.29	5.36	2.42
AFB_2	0.61	0.12	18.74	4.42	4.05	6.66	2.76
AFG_1	2.19	0.52	23.72	4.61	7.46	6.38	4.81
AFG_2	0.19	0.04	21.28	5.65	4.77	6.30	4.34
Total AF	8.5	1.1	12.73	2.32	2.05	4.93	2.58

Table 6. Percent contribution of each component to u_{CRM}

Analyte	$u_{char, rel}$ (%)	$u_{bb, rel}$ (%)	$u_{lts, rel}$ (%)	$u_{sts, rel}$ (%)
AFB_1	21	10	57	12
AFB_2	22	19	50	9
AFG_1	15	40	29	16
AFG_2	28	20	35	17
Total AF	13	10	60	17

ADDITIONAL INFORMATION

Moisture content of the CRM was analyzed by two methods: Coulometric Karl Fischer and oven drying with vacuum oven. Two replicate samples over 10 different units were examined during this process. Average moisture content of CRM was determined as 6.73 %.

No dry mass correction was applied to aflatoxin results.

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TRACEABILITY

Stock solutions and calibrations solutions used in the in-house validated methods were gravimetrically prepared from the solid materials for which the purity values were assigned by Q-NMR measurements in TÜBİTAK UME. Analytical balances were controlled before every use with weight sets traceable to TÜBİTAK UME national standard of mass. The assigned values of the calibrants are traceable to SI by Q-NMR via UME CRM 1301 reference material.

INSTRUCTIONS FOR USE

Storage conditions

UME CRM 1302 should be stored at + 4 °C or lower temperatures. It is recommended to use CRM as soon as possible after opening the bottle. It is recommended to store opened bottle at temperatures below – 20 °C. Division of the material into subsamples to minimize freeze-thaw cycles is recommended, however sample should not be subjected to moisture in air and light for prolonged time. TÜBİTAK UME cannot be held responsible for changes that might happen during storage of the material at the customer's premises, to noncompliance of the instructions for use, and the storage conditions given. The material can be safely dispatched where the temperature does not exceed 4 °C, and the transportation period of 2 weeks.

Minimum sample intake

Minimum sample intake amount is 6 g, which is the minimum amount used in the homogeneity study.

Safety precautions

Usual laboratory safety precautions apply. Use and dispose of materials according to existing local rules is strongly recommended. The use of face masks with dust filter, and working in a good ventilated laboratory is highly recommended. It is recommended to read the Safety Data Sheet before using the UME CRM 1302.

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REVISION HISTORY

Date	Remarks
31.08.2016	First issue.
25.11.2019	Uncertainty values are revised according to a new estimation formula and for 6 months shelf life. Information of CCQM comparison and information on conformity of material to ISO 17034 and conformity of certification to ISO Guide 35:2017 were added.

Annex 1. Homogenity Data for UME CRM 1302

Table A1.1. Raw Data of Homogenity for UME CRM 1302

		Results, ng/g				
Unit No	Unit replicates	AFB1	AFB2	AFG1	AFG2	Total AF
149	149.1	5.28	0.55	2.70	0.20	8.72
	149.2	5.27	0.73	2.83	0.22	9.05
	149.3	4.58	0.58	2.05	0.21	7.42
254	254.1	5.49	0.56	2.16	0.16	8.37
	254.2	5.21	0.59	2.32	0.19	8.30
	254.3	6.06	0.67	1.64	0.14	8.50
382	382.1	4.45	0.48	1.79	0.15	6.87
	382.2	5.86	0.64	2.30	0.20	9.01
	382.3	5.21	0.55	1.94	0.15	7.84
341	341.1	5.74	0.61	1.92	0.16	8.43
	341.2	5.63	0.57	2.07	0.22	8.49
	341.3	5.08	0.53	1.82	0.15	7.58
36	36.1	5.62	0.55	1.84	0.18	8.19
	36.2	5.50	0.68	2.27	0.23	8.69
	36.3	6.12	0.61	2.04	0.16	8.92
424	424.1	5.79	0.60	2.07	0.17	8.62
	424.2	5.93	0.72	2.08	0.20	8.93
	424.3	5.20	0.65	2.74	0.24	8.82
359	359.1	5.90	0.65	2.15	0.18	8.88
	359.2	5.15	0.55	2.09	0.16	7.95
	359.3	5.80	0.67	2.05	0.17	8.68
76	76.1	5.45	0.58	2.24	0.16	8.42
	76.2	5.32	0.60	2.04	0.16	8.13
	76.3	5.17	0.65	3.03	0.23	9.08
247	247.1	4.59	0.48	1.96	0.16	7.19
	247.2	4.75	0.52	1.88	0.16	7.31
	247.3	5.60	0.63	1.79	0.12	8.14
193	193.1	5.31	0.53	1.82	0.15	7.81
	193.2	5.63	0.60	2.54	0.18	8.96
	193.3	5.69	0.59	2.48	0.18	8.94
318	318.1	4.82	0.44	2.74	0.17	8.17
	318.2	5.20	0.79	2.76	0.19	8.95
	318.3	5.35	0.63	2.27	0.17	8.42
451	451.1	5.08	0.64	2.60	0.19	8.52
	451.2	5.29	0.71	2.25	0.18	8.43
	451.3	5.45	0.61	2.41	0.18	8.65

Annex 2. Homogeneity Plots

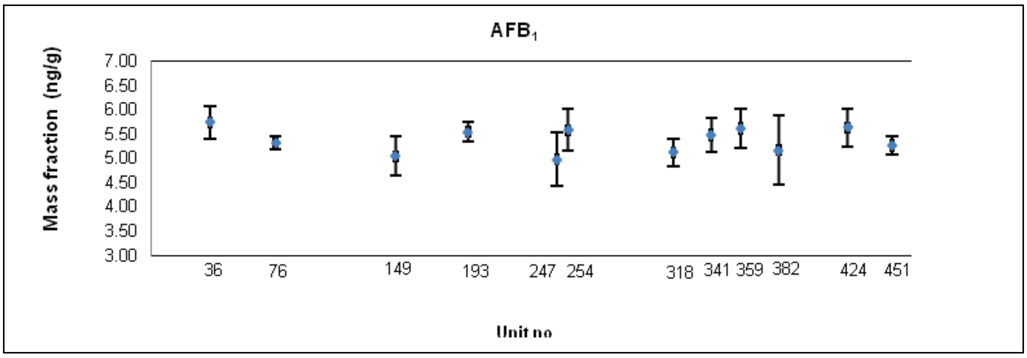


Figure A2.1. AFB_1, Homogeneity plot

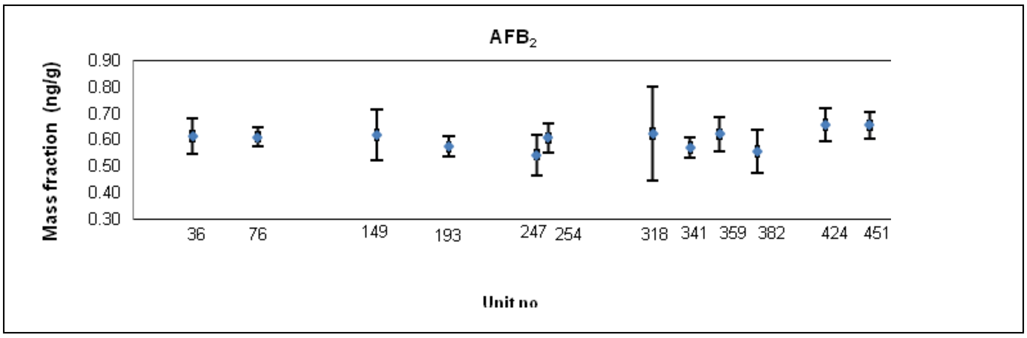


Figure A2.2. AFB_2, Homogeneity plot

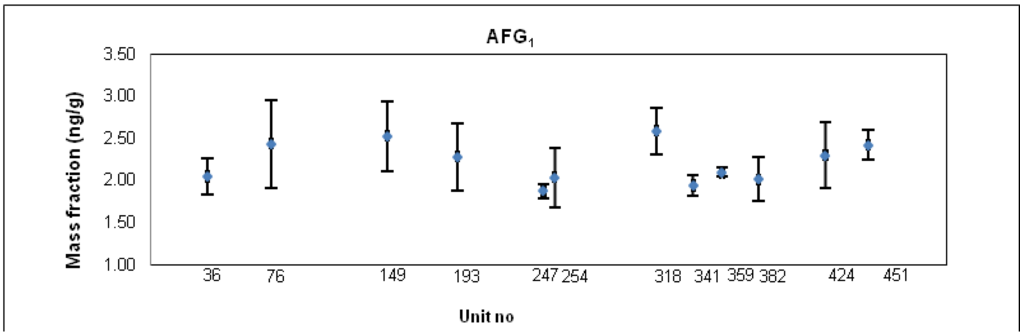


Figure A2.3. AFG_1, Homogeneity plot

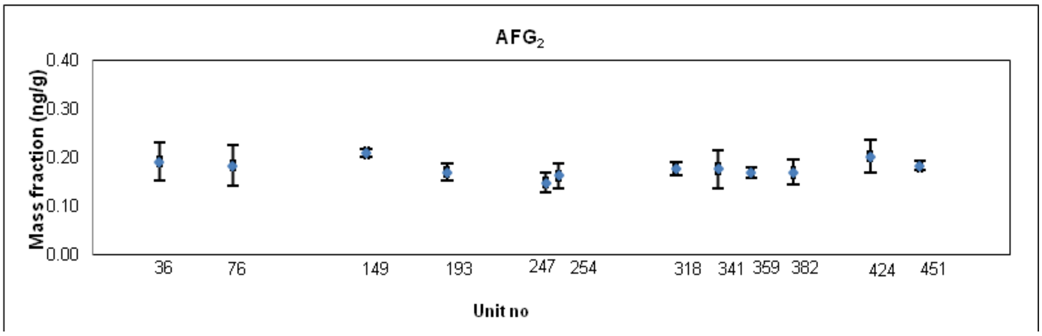


Figure A2.4. AFG_2, Homogeneity plot

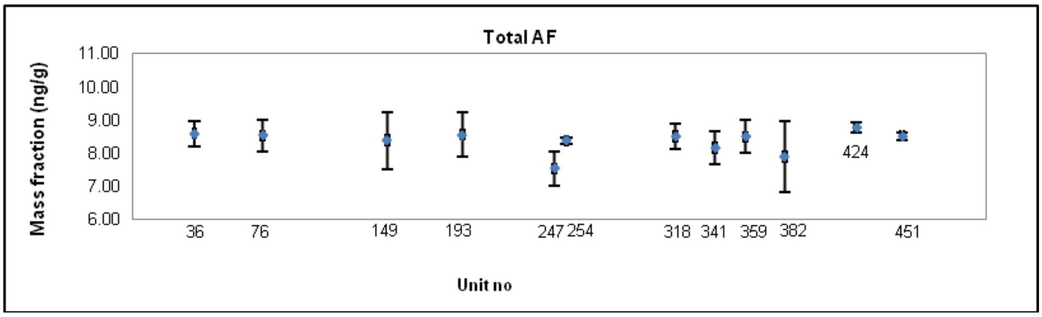


Figure A2.5. Total AF, Homogeneity plot

Annex 3. Short Term Stability (STS) Data for UME CRM 1302

Table A3.1. STS Data of AFB1 for UME CRM 1302, at (+4 and -20) ºC

Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g	Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g
12	108	0	-80	5.46	12	108	0	-80	5.46
10	311	0	-80	5.46	10	311	0	-80	5.46
15	108	0	-80	4.97	15	108	0	-80	4.97
23	311	0	-80	5.44	23	311	0	-80	5.44
22	22	1	-20	5.69	16	7	1	4	6.24
13	388	1	-20	6.71	2	262	1	4	5.17
32	22	1	-20	5.36	25	7	1	4	5.88
9	388	1	-20	5.20	34	262	1	4	5.75
18	33	2	-20	5.25	17	44	2	4	4.75
5	415	2	-20	5.37	33	297	2	4	5.01
35	33	2	-20	5.09	24	44	2	4	5.78
11	415	2	-20	5.62	4	297	2	4	5.23
20	86	3	-20	5.67	3	63	3	4	5.21
27	206	3	-20	5.80	19	319	3	4	5.65
26	86	3	-20	5.08	6	63	3	4	5.56
7	206	3	-20	5.79	1	319	3	4	5.59
36	121	4	-20	5.01	29	104	4	4	4.75
21	361	4	-20	5.26	30	495	4	4	5.15
28	121	4	-20	5.82	31	104	4	4	5.94
14	361	4	-20	5.06	8	495	4	4	5.73

Table A3.2. STS Data of AFB2 for UME CRM 1302, at (+4 and -20) ºC

Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g	Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g
12	108	0	-80	0.64	12	108	0	-80	0.64
10	311	0	-80	0.76	10	311	0	-80	0.76
15	108	0	-80	0.72	15	108	0	-80	0.72
23	311	0	-80	0.76	23	311	0	-80	0.76
22	22	1	-20	0.70	16	7	1	4	0.86
13	388	1	-20	0.86	2	262	1	4	0.77
32	22	1	-20	0.68	25	7	1	4	0.70
9	388	1	-20	0.74	34	262	1	4	0.75
18	33	2	-20	0.75	17	44	2	4	0.65
5	415	2	-20	0.72	33	297	2	4	0.69
35	33	2	-20	0.69	24	44	2	4	0.73
11	415	2	-20	0.71	4	297	2	4	0.76
20	86	3	-20	0.80	3	63	3	4	0.71
27	206	3	-20	0.79	19	319	3	4	0.84
26	86	3	-20	0.68	6	63	3	4	0.73
7	206	3	-20	0.84	1	319	3	4	0.82
36	121	4	-20	0.67	29	104	4	4	0.64
21	361	4	-20	0.76	30	495	4	4	0.68
28	121	4	-20	0.75	31	104	4	4	0.74
14	361	4	-20	0.77	8	495	4	4	0.80

Table A3.3. STS Data of AFG1 for UME CRM 1302, at (+4 and -20) ºC

Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g	Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g
12	108	0	-80	1.29	12	108	0	-80	1.29
10	311	0	-80	1.53	10	311	0	-80	1.53
15	108	0	-80	1.76	15	108	0	-80	1.76
23	311	0	-80	1.40	23	311	0	-80	1.40
22	22	1	-20	2.18	16	7	1	4	1.83
13	388	1	-20	2.14	2	262	1	4	1.36
32	22	1	-20	2.03	25	7	1	4	2.11
9	388	1	-20	1.15	34	262	1	4	1.79
18	33	2	-20	2.02	17	44	2	4	1.28
5	415	2	-20	1.54	33	297	2	4	1.34
35	33	2	-20	1.34	24	44	2	4	1.54
11	415	2	-20	1.89	4	297	2	4	1.27
20	86	3	-20	1.74	3	63	3	4	1.71
27	206	3	-20	1.70	19	319	3	4	1.18
26	86	3	-20	1.21	6	63	3	4	1.58
7	206	3	-20	1.51	1	319	3	4	1.37
36	121	4	-20	1.88	29	104	4	4	1.46
21	361	4	-20	1.22	30	495	4	4	1.53
28	121	4	-20	1.90	31	104	4	4	1.61
14	361	4	-20	1.44	8	495	4	4	1.55

Table A3.4. STS Data of AFG2 for UME CRM 1302, at (+4 and -20) ^oC

Injection no	Unit No	Time (week)	Temp. (^oC)	ng/g	Injection no	Unit No	Time (week)	Temp. (^oC)	ng/g
12	108	0	-80	0.13	12	108	0	-80	0.13
10	311	0	-80	0.18	10	311	0	-80	0.18
15	108	0	-80	0.20	15	108	0	-80	0.20
23	311	0	-80	0.15	23	311	0	-80	0.15
22	22	1	-20	0.23	16	7	1	4	0.20
13	388	1	-20	0.23	2	262	1	4	0.17
32	22	1	-20	0.15	25	7	1	4	0.23
9	388	1	-20	0.15	34	262	1	4	0.20
18	33	2	-20	0.22	17	44	2	4	0.16
5	415	2	-20	0.18	33	297	2	4	0.14
35	33	2	-20	0.15	24	44	2	4	0.17
11	415	2	-20	0.22	4	297	2	4	0.16
20	86	3	-20	0.20	3	63	3	4	0.20
27	206	3	-20	0.19	19	319	3	4	0.14
26	86	3	-20	0.14	6	63	3	4	0.17
7	206	3	-20	0.17	1	319	3	4	0.15
36	121	4	-20	0.19	29	104	4	4	0.16
21	361	4	-20	0.14	30	495	4	4	0.17
28	121	4	-20	0.19	31	104	4	4	0.17
14	361	4	-20	0.17	8	495	4	4	0.18

Table A3.5. STS Data of Total AF for UME CRM 1302, at (+4 and -20) ^oC

Injection no	Unit No	Time (week)	Temp. -20 ^oC	ng/g	Injection no	Unit No	Time (week)	Temp. 4 ^oC	ng/g
12	108	0	-80	7.51	12	108	0	-80	7.51
10	311	0	-80	7.92	10	311	0	-80	7.92
15	108	0	-80	7.64	15	108	0	-80	7.64
23	311	0	-80	7.75	23	311	0	-80	7.75
22	22	1	-20	8.79	16	7	1	4	9.12
13	388	1	-20	9.94	2	262	1	4	7.46
32	22	1	-20	8.22	25	7	1	4	8.90
9	388	1	-20	7.23	34	262	1	4	8.48
18	33	2	-20	8.23	17	44	2	4	6.83
5	415	2	-20	7.80	33	297	2	4	7.18
35	33	2	-20	7.26	24	44	2	4	8.21
11	415	2	-20	8.44	4	297	2	4	7.42
20	86	3	-20	8.40	3	63	3	4	7.82
27	206	3	-20	8.46	19	319	3	4	7.80
26	86	3	-20	7.10	6	63	3	4	8.03
7	206	3	-20	8.30	1	319	3	4	7.92
36	121	4	-20	7.74	29	104	4	4	7.01
21	361	4	-20	7.37	30	495	4	4	7.52
28	121	4	-20	8.66	31	104	4	4	8.46
14	361	4	-20	7.43	8	495	4	4	8.25

Annex 4. STS Plots for UME CRM 1302 at at (+4 and -20) °C

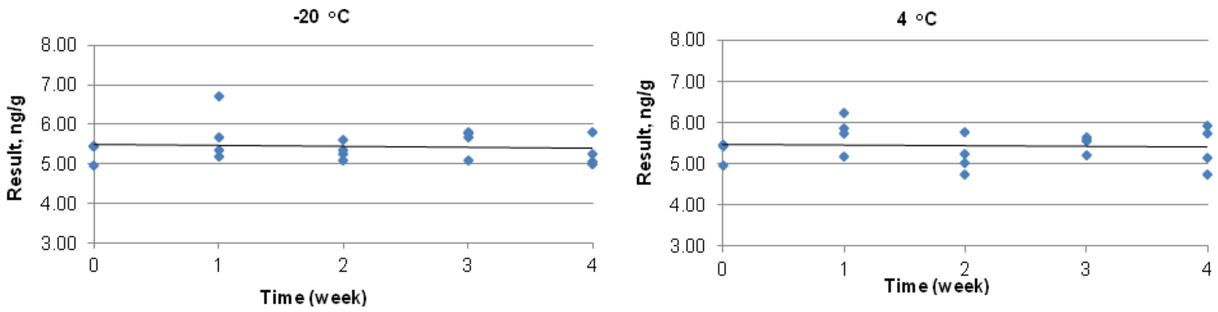


Figure A4.1. UME CRM 1302 AFB1 Short Term Stability Plots

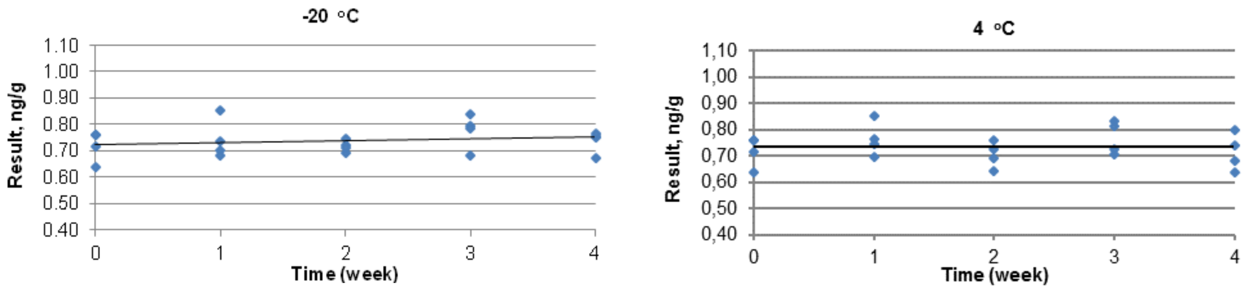


Figure A4.2. UME CRM 1302 AFB2 Short Term Stability Plots

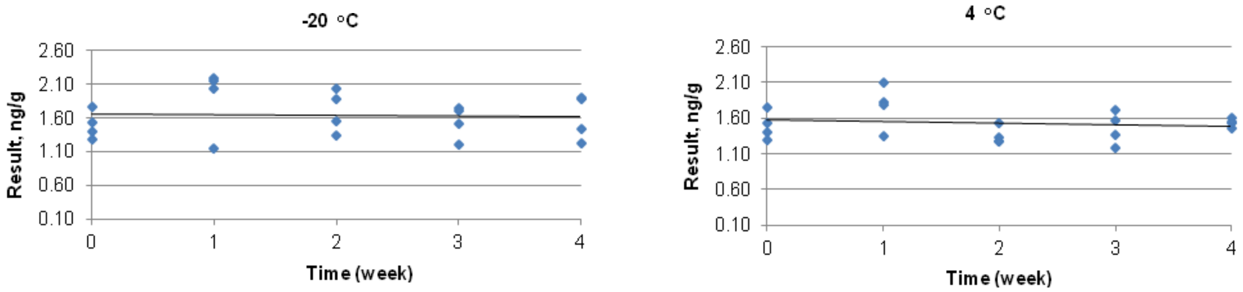


Figure A4.3. UME CRM 1302 AFG1 Short Term Stability Plots

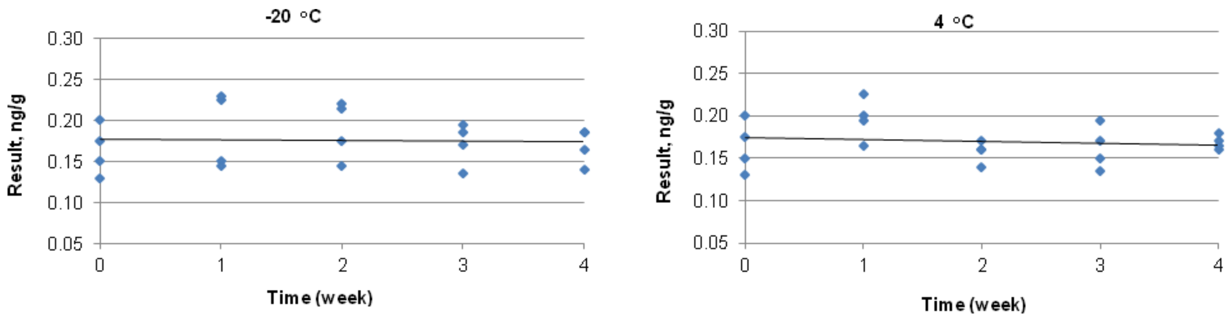
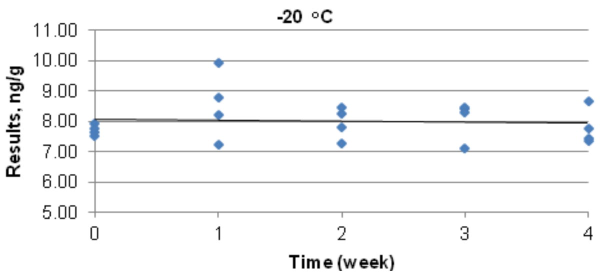


Figure A4.4. UME CRM 1302 AFG2 Short Term Stability Plots



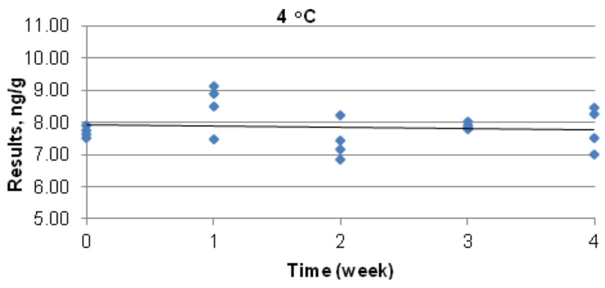


Figure A4.5. UME CRM 1302 Total AF Short Term Stability Plots

Annex 5. Long Term Stability (LTS) Data for UME CRM 1302

Table A5.1. LTS Data for UME CRM 1302, AFB_1

Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g
8	24	0	-80	5.48
17	439	0	-80	5.64
29	24	0	-80	4.64
19	439	0	-80	5.19
26	128	2	4	6.92
27	397	2	4	4.95
36	128	2	4	5.19
10	397	2	4	6.47
35	166	4	4	6.75
1	423	4	4	5.39
6	166	4	4	6.54
32	423	4	4	5.39
11	70	6	4	6.76
14	323	6	4	5.78
22	70	6	4	6.03
9	323	6	4	5.42
20	240	9	4	5.38
24	469	9	4	5.66
5	240	9	4	5.30
31	469	9	4	7.28

Table A5.2. LTS Data for UME CRM 1302, AFB_2

Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g
8	24	0	-80	0.54
17	439	0	-80	0.55
29	24	0	-80	0.52
19	439	0	-80	0.57
26	128	2	4	0.72
27	397	2	4	0.55
36	128	2	4	0.56
10	397	2	4	0.67
35	166	4	4	0.88
1	423	4	4	0.59
6	166	4	4	0.78
32	423	4	4	0.61
11	70	6	4	0.62
14	323	6	4	0.61
22	70	6	4	0.62
9	323	6	4	0.58
20	240	9	4	0.61
24	469	9	4	0.54
5	240	9	4	0.57
31	469	9	4	0.78

Table A5.3. LTS Data for UME CRM 1302, AFG_1

Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g
8	24	0	-86	1.96
17	439	0	-86	1.64
29	24	0	-86	1.74
19	439	0	-86	1.79
26	128	2	4	2.81
27	397	2	4	2.38
36	128	2	4	2.04
10	397	2	4	2.13
35	166	4	4	1.98
1	423	4	4	1.73
6	166	4	4	1.85
32	423	4	4	1.81
11	70	6	4	2.41
14	323	6	4	2.04
22	70	6	4	1.80
9	323	6	4	1.72
20	240	9	4	1.80
24	469	9	4	1.80
5	240	9	4	2.04
31	469	9	4	2.02

Table A5.4. LTS Data for UME CRM 1302, AFG_2

Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g
8	24	0	-80	0.13
17	439	0	-80	0.12
29	24	0	-80	0.14
19	439	0	-80	0.14
26	128	2	4	0.19
27	397	2	4	0.21
36	128	2	4	0.16
10	397	2	4	0.16
35	166	4	4	0.15
1	423	4	4	0.15
6	166	4	4	0.14
32	423	4	4	0.14
11	70	6	4	0.18
14	323	6	4	0.18
22	70	6	4	0.13
9	323	6	4	0.16
20	240	9	4	0.16
24	469	9	4	0.14
5	240	9	4	0.18
31	469	9	4	0.18

Table A5.5. LTS Data for UME CRM 1302, Total AF

Injection no	Unit No	Time (week)	Temp. (ºC)	ng/g
8	24	0	-80	8.11
17	439	0	-80	7.95
29	24	0	-80	7.05
19	439	0	-80	7.69
26	128	2	4	10.64
27	397	2	4	8.10
36	128	2	4	7.95
10	397	2	4	9.43
35	166	4	4	9.76
1	423	4	4	7.85
6	166	4	4	9.30
32	423	4	4	7.95
11	70	6	4	9.97
14	323	6	4	8.62
22	70	6	4	8.58
9	323	6	4	7.89
20	240	9	4	7.95
24	469	9	4	8.15
5	240	9	4	8.09
31	469	9	4	10.26

Annex 6. Long Term Stability (LTS) Plots for UME CRM 1302

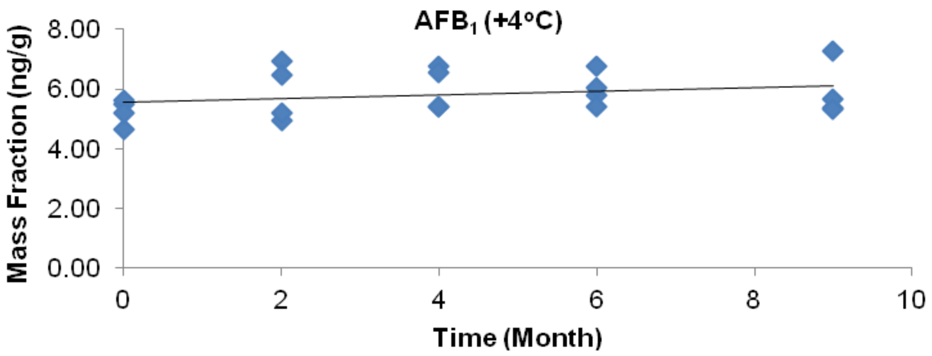


Figure A6.1. UME CRM 1302 AFB_1 Long Term Stability Plots

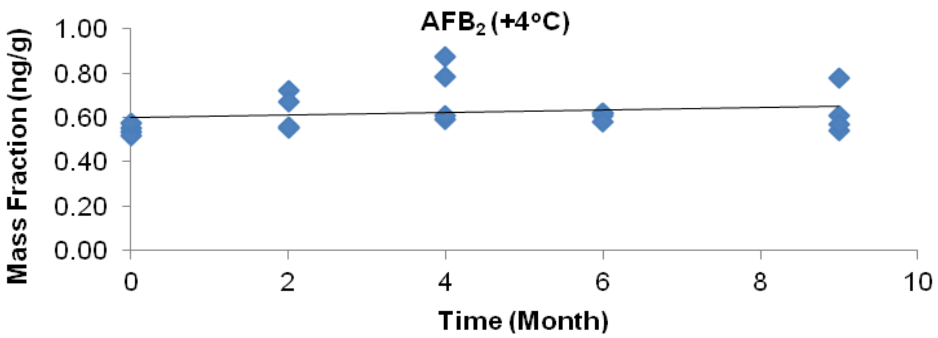


Figure A6.2. UME CRM 1302 AFB_2 Long Term Stability Plots

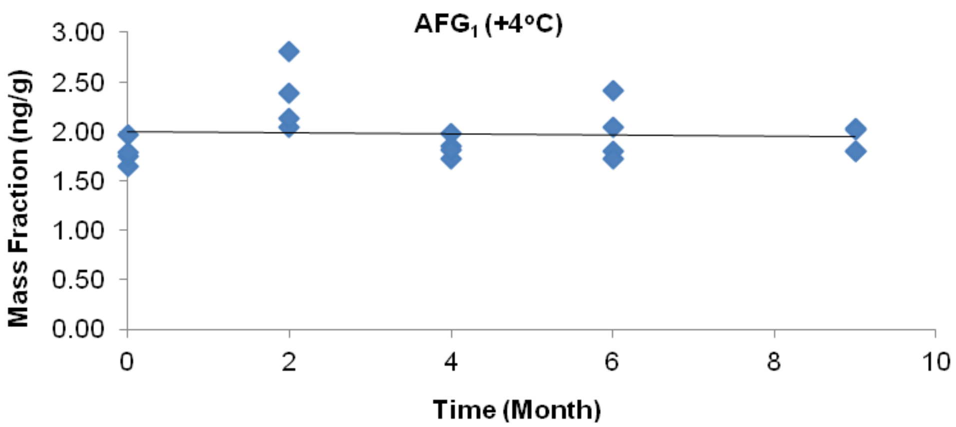


Figure A6.3. UME CRM 1302 AFG_1 Long Term Stability Plots

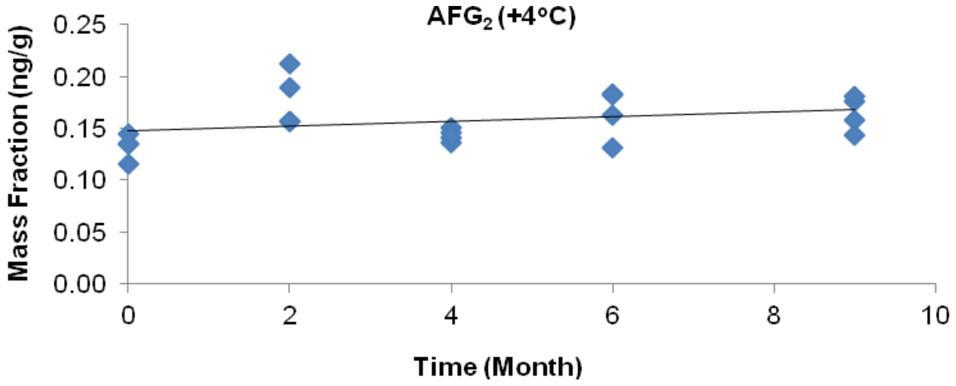


Figure A6.4. UME CRM 1302 AFG$_2$ Long Term Stability Plots

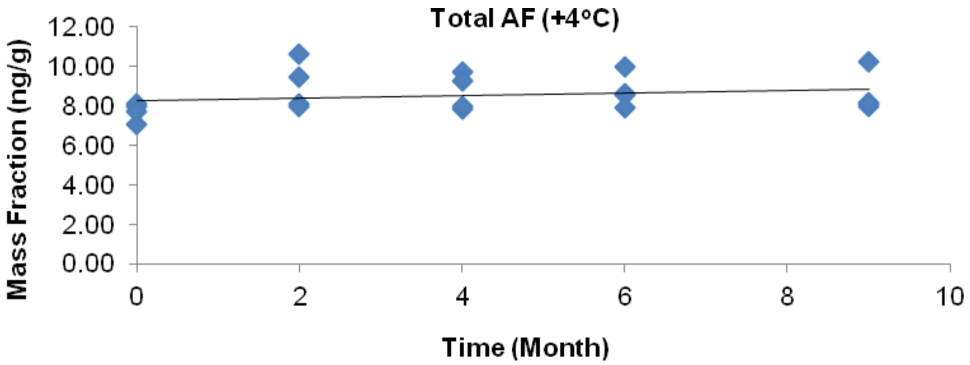


Figure A6.5. UME CRM 1302 Total AF Long Term Stability Plots

Annex 7. Characterization Data for UME CRM 1302

Table A.7.1. Characterization results of LC-IDMS for UME CRM 1302

Unit-Replicate No	AFB1	AFB2	AFG1	AFG2	Total AF
141-1	5.37	0.70	1.98	0.17	8.23
141-2	5.03	0.62	2.07	0.17	7.90
141-3	5.92	0.61	1.95	0.20	8.67
359-1	5.06	0.60	2.15	0.21	8.02
359-2	5.05	0.59	2.44	0.24	8.41
359-3	5.69	0.68	2.27	0.22	8.86
359-4	5.74	0.58	2.44	0.20	8.96
141-1	5.36	0.62	2.26	0.21	8.45
141-2	5.28	0.55	1.87	0.17	7.87
141-3	5.63	0.62	1.99	0.18	8.42
141-4	5.55	0.65	2.08	0.21	8.48
359-1	6.01	0.71	2.42	0.23	9.38
359-2	5.41	0.63	2.13	0.24	8.40
359-3	5.03	0.56	2.07	0.20	7.85
359-4	5.15	0.70	2.27	0.21	8.32

Table A.7.2. Characterization results of HPLC-FLD for UME CRM 1302

Unit-Replicate No	AFB1	AFB2	AFG1	AFG2	Total AF
149-1	5.54	0.57	2.77	0.21	9.25
149-2	5.46	0.61	2.90	0.20	8.66
149-3	4.85	0.60	2.13	0.22	7.80
254-1	5.73	0.58	2.23	0.17	8.72
254-2	5.46	0.61	2.39	0.20	8.66
254-3	6.31	0.69	1.73	0.15	8.87
382-1	4.72	0.50	1.87	0.16	7.26
382-2	5.71	0.59	2.11	0.19	8.60
382-3	5.29	0.55	2.02	0.17	8.12
341-1	6.37	0.61	1.92	0.16	9.05
341-2	5.31	0.56	2.28	0.19	8.35
341-3	5.07	0.53	1.81	0.15	7.56
36-1	5.61	0.55	1.83	0.18	8.17
36-2	5.64	0.61	2.36	0.16	8.31
36-3	6.11	0.61	2.03	0.15	8.91
424-1	5.78	0.60	2.06	0.17	8.61
424-2	5.98	0.61	2.12	0.20	8.91
424-3	5.88	0.65	2.05	0.23	8.81
359-1	5.47	0.61	2.51	0.18	8.77
359-2	5.25	0.55	2.09	0.15	8.04
359-3	5.89	0.66	2.05	0.16	8.77
76-1	5.61	0.61	2.35	0.15	8.72
76-2	5.42	0.60	2.04	0.16	8.22
76-3	5.44	0.65	2.38	0.17	8.64
247-1	4.68	0.47	2.28	0.16	7.60
247-2	5.28	0.52	2.56	0.18	8.53
247-3	5.70	0.63	1.79	0.18	8.29
193-1	5.29	0.53	1.95	0.21	8.52
193-2	5.61	0.60	2.53	0.19	8.93
193-3	5.94	0.59	2.47	0.18	9.18
318-1	5.07	0.50	2.66	0.16	8.39
318-2	5.37	0.58	2.75	0.19	8.89
318-3	5.33	0.63	2.26	0.17	8.39
451-1	5.23	0.61	2.14	0.20	8.18
451-2	5.95	0.58	2.24	0.18	8.95
451-3	5.43	0.61	2.40	0.18	8.61

Annex 8. CCQM-K138 International Comparison Graphs

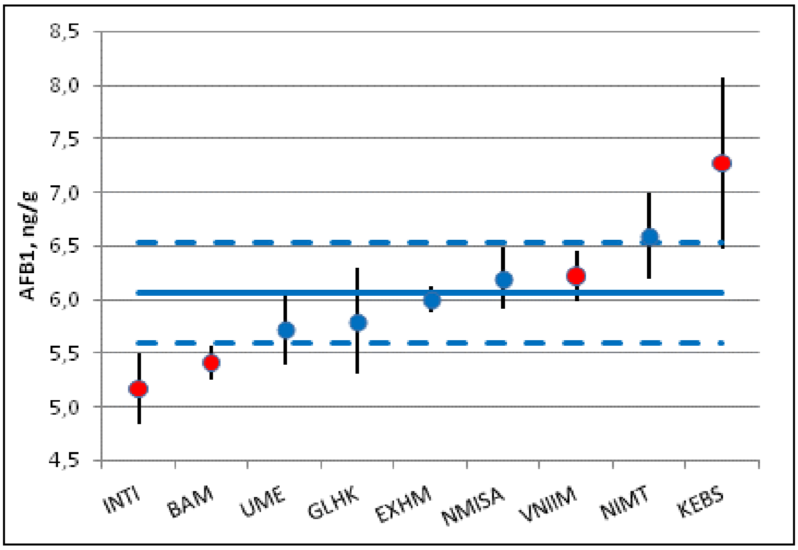


Figure A.8.1. AFB_1 results. Reference value 6.06 ng/g (solid blue line), standard uncertainty ± 0.47 ng/g (blue dashed line). Results represented by red dots were not included for evaluation.

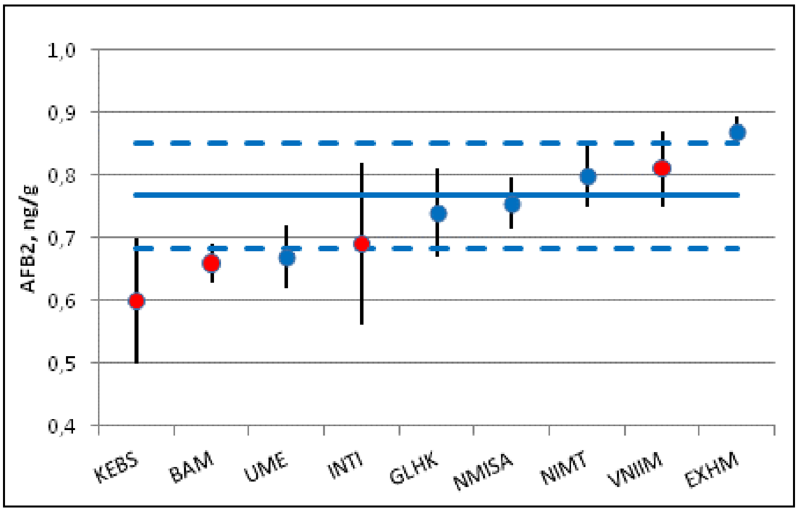


Figure A.8.2. AFB_2 results. Reference value 0.766 ng/g (solid blue line), standard uncertainty ± 0.083 ng/g (blue dashed line). Results represented by red dots were not included for evaluation.

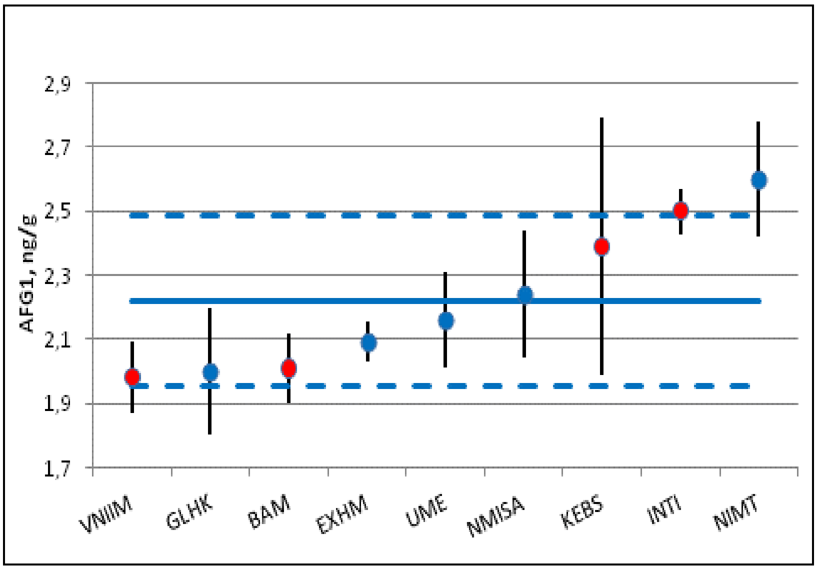


Figure A.8.3. AFG_1 results. Reference value 2.22 ng/g (solid blue line), standard uncertainty ± 0.265 ng/g (blue dashed line). Results represented by red dots were not included for evaluation.

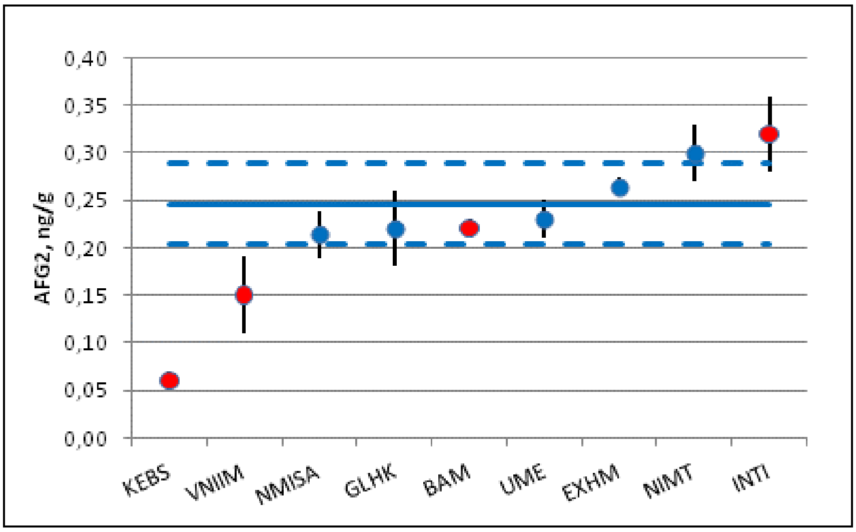


Figure A.8.4. AFG_2 results. Reference value 0.246 ng/g (solid blue line), standard uncertainty ± 0.042 ng/g (blue dashed line). Results represented by red dots were not included for evaluation.

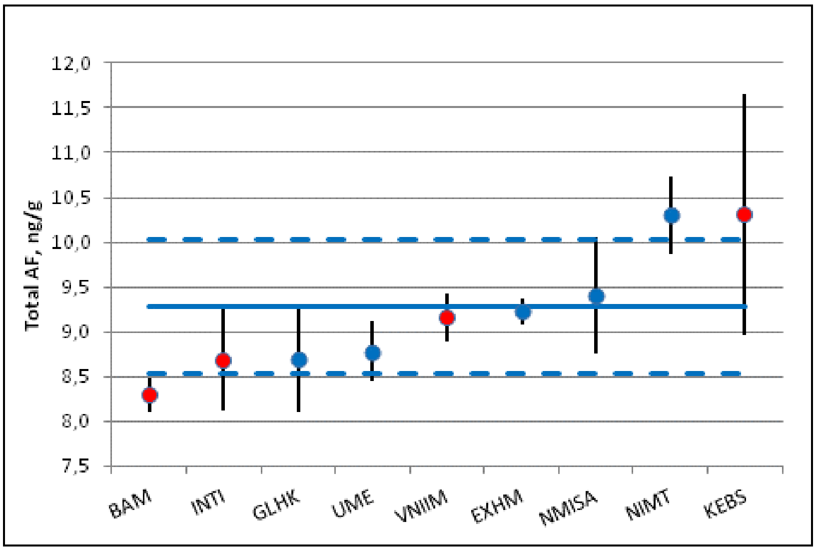


Figure A.8.5. Total AF results. Reference value 9.28 ng/g (solid blue line), standard uncertainty ± 0.742 ng/g (blue dashed line). Results represented by red dots were not included for evaluation.