R&D REPORT

on

Validation of published test methods of Vitamins in identified food matrices for Revision of Indian Standards

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R&D REPORT

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FINAL REPORT

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1.0 PROJECT DESCRIPTION

a. Study Information

i. **Study Title:** Validation of published test methods of Vitamins in identified food matrices for Revision of Indian Standards

ii. **Study objective:** To undertake Collaborative method validation study using multi-lab participation for specific vitamins in food matrices, following Standard Method Performance Requirements (SMPR)

iii. **Project Leader :** Dr. Usharani Dandamudi

iv. **Study Description : (a)** Single Laboratory Validation (SLV) of Vitamin B5 (Panthothenic acid) (ISO 20639: 2015)- Nestle

(b) Single Laboratory Validation of Vitamin B12 (AOAC 2014.02)- Nestle

© Multi Laboratory Validation (MLV) of B9 (AOAC 2013.13)- Nestle

(d) Multi Laboratory Validation of Vitamin D in oils (IS 17177 : 2019/ ISO 20636 : 2018 – Envirocare

(e) Multi Laboratory Validation of Vitamin B12 AOAC 2011.10/ FSSAI.FR.16.003.2022)- CFTRI

v. **Statistical evaluation:** Validation criteria include Linearity, precision by reproducibility (RSDR), Repeatability (RSDr) and HorRat ratio

b. Materials, Method and Laboratories

i. **Matrices:**

S. No	Matrices	Analyte
1	Cereal Based Matrices, Malt based beverages, Milk Powders, Premixes, FRK, Spirulina	SLV of Vitamin B12
2	Fruits & vegetables products, Nuts and Nut products, Malt and milk based beverages, break fast cereals	SLV of Vitamin B5
3	Cereal based- Fortified wheat flour atta, Fruits and vegetables-dried peas Nut and Nut products- Ground nut	MLV of Vitamin B9
4.	Vegetable Oils	MLV of vitamin D
5	Cereal products- fortified wheat flour atta Nuts and Nut products- Almonds Meat and Meat products- Chicken	MLV of Vitamin B12

ii. **Homogeneity test:** Fortified wheat atta, dried peas and ground nut for Vitamin B9, Fortified atta and almond for Vitamin B12 and Fortified oils for vitamin D are completed and the data is enclosed in Annexure -A

iii. **Method: SLV method for** UHPLC/UV for Vitamin B12, LC-MS/MS for Vitamin B5. MLV method of Vitamin B9 (folic acid, 5-MeTHF) forms**,** Vitamin D (D2 and D3) forms and Vitamin B12 forms (Cyanocobalmin and methylcobalamin) using LC-MS/MS method. Full method details are given in Annexure -B. In brief the method is as follows.

(a) Sample Preparation of Vitamin B12 for SLV: 25 gm of powder sample was taken; 200 ml of water was mixed until suspension is homogenous. Further 60 gms of suspension was taken in 250 ml flask and added 1 ml of 1 % sodium cyanide and 0.05 amylase for starch containing matrices. Further 25 ml of sodium acetate was added mixed well and boiled with water batch for 30 min in autoclave for 100 °C. Cool and dilute the sample with water in 100 ml volumetric flask and filter solution with folded paper and analysed through HPLC UV-visible or PDA detector.

(b) Sample preparation of Vitamin B5 for SLV: 25 gm of grinded sample (blended/ non homogenous powder) was taken, 200 ml of water was added & Polytron was used for better mixing of samples and further 15 ml homogenized sample aliquot was taken in 50 ml. Spiking were done in 15 ml aliquot. Further 25 ml of 0.4 M ammonium acetate solution of pH = 3.8 added to sample aliquot. Sample extract is diluted with volume of water. After stirring for 10 min, the 20 ml portion is filtered through folded paper and analysed for Mass spectrometry.

(c) Sample Preparation of Vitamin B9 for MLV: 25gm of sample was taken in 200ml of Milli Q water and 100mg of taka diastase was added. The samples were incubated at 37 °C for 15 minutes and 15g of the suspension was taken for analysis. To the suspension, 40ml of extraction buffer was added and incubated at 90 °C for 30 minutes with shaking. Let it cool at room temperature. 2ml of 1mg/ml pepsin was added and incubated at 37 °C for 30 minutes. Make up the volume to 100 ml and consider 10 ml of the filtrate for SPE (SAX cartridge). After SPE, the samples are reconstituted to 1.5ml using Dissolution solution C containing DTT and ascorbic acid.

(d) Sample Preparation of Vitamin D for MLV: 0.5 gm of sample added with 0.5 ml of SILIS and 10 ml of ethanolic pyrogallol solution. Further sample needs to saponified with 2m of potassium hydroxide and kept in water batch at 70 °C for 1 hr and vortex every 15 min. After the sample is clear, cool and add 10 ml of isooctane and placed in horizontal shaker for 10 min. Further 20 ml of water is added 10 times and place in centrifuge at 2500 rpm for 15 min. The upper isooctane layer is transferred to 15ml tube and 5 ml is added to remove any amount of saponifiable matter by centrifugation at 12000 rpm for 5 min. To upper octane layer 75 µl of PTAG solution is added for derivatization and the sample is reconstituted with 1ml of acetonitrile. After centrifugation for 5 min collet 500 ul of upper layer and add water to make upto to 2ml and analyze the sample through LC-MS/MS.

(e) Sample Preparation of Vitamin B12 for Multi Laboratory: 3 gm of sample is homogenized in 100 ml of flask with 25 ml of water and 1 ml of 6 %-taka diastase solution. Incubate at 37 °C for 30 minutes. Add 30 ml of 0.1 M sodium acetate buffer (pH 4.5) and 100 mg of ascorbic acid and 1ml of pepsin (for almond). Samples heated at 105 °C for 60 minutes and cooled in ice bath. Make up the solution to 100 ml and filter the sample through 0.45 µm filter. 80 ml of filter is passed through 500 mg C18 SPE cartridge and collected 5 ml of solution. It is injected to LC-MS/MS analysis for various forms of Vitamin B12.

iv. **Participant laboratories**:

S. No	Analyte	Laboratory
1	SLV of Vitamin B12, SLV of Vitamin B5,	Nestle
2	MLV of Vitamin B9	Nestle (lead), Eureka, Eurofins, Microchem Skiller, CSIR-CFTRI
3	MLV of vitamin D	Envirocare (lead), Eureka, Eurofins, NDDB-CALF, CFTRI
4	MLV of Vitamin B12	CFTRI (Lead), Eureka, Eurofins, Nestle, Envirocare

c. Forms

i. Covering letters : In-house

ii. Sample receipt forms: In-house

iii. Reporting templates : Excel Sheet

2. INSTRUCTION TO PARTICIPANTS

2.1 SLV Method set up and qualification of participants: Single method performance requirements (SMPR) to be checked for the matrices for almonds, fruits and vegetables, spirulina, fortified rice kernel for vitamin B12 of AOAC 2014.02. The matrices of Nuts and Fruits and vegetables for vitamin B5 based on IS 16642 : 2018/ ISO 20639 : 2015.

2.2 Single laboratory Test Participation: Nestle Laboratory

2.3 Vitamin B9 Method set up and qualification of participants for MLV: The method identified for folic acid and natural form of vitamin B9, 5-methyl tetrahydrofolic acid is AOAC 2013.13. The matrices are fruits and vegetables, Nuts and nut products and cereals and pulses. The practice samples were sent to the participants of four different laboratories. The qualification of the participants was ensured based on the results of practice samples.

2.4 Vitamin B9 Multi laboratory Test Participation: Nestle Laboratory (Nodal), Eureka, Eurofins, Micochem and CSIR-CFTRI.

2.5 Vitamin D Method set up and qualification of participants for MLV: The method identified for vitamin D2 and D3 in vegetable oils AOAC 2016.02. The matrices include two different vegetable oils, sunflower oil and blend of sunflower oil and rice bran oil. In addition, control oil sample unfortified are validated. The practice samples were sent to the participants of four different laboratories. The qualification of the participants was ensured based on the results of practice samples.

2.6 Multi laboratory Test Participation: Envirocare (Nodal), Eureka, Eurofins, NDDB-Calf and CSIR-CFTRI.

2.7 Vitamin B12 Method set up and qualification of participants for MLV: The method identified for Vitamin B12 was FSSAI.FR.16.003.22 and AOAC 2011.10. The two methods were unable to have repeatability for fortified atta and almond samples. The initial method was reoptimized based on the repeatability and reproducibility of proposed matrices and provided the finalised method setup to the participant laboratories. One laboratory able to obtain the practice sample and the sample analysis of multi laboratory were not obtained due to limited timeline for reporting and analysis. The practice samples and analysis samples of fortified atta, almond, meat along with control sample of wheat a were sent to the participants of four different laboratories. The qualification of the participants was ensured based on the results of practice samples of one laboratory.

2.8 Multi laboratory Test Participation: CSIR-CFTRI (Nodal), Eureka, Eurofins, Envirocare, Nestle

The sample details, receipt form laboratory qualifications of MLV study are enclosed in Annexure C-E.

3.0 RESULTS

3.1 Single Laboratory Results of Vitamin B12 in Foods- HPLC-UV method

Objective was to check suitability of AOAC 2014.02 method for other food matrices. AOAC 2014.02 method has a scope for Vitamin B12 (Cyanocobalamin) in Infant Formula and Adult/Pediatric Nutritional Formula. It includes Infant Formula Powder, Partially Hydrolysed, Milk Based, Infant Formula Powder, Partially Hydrolysed Soy Based, Infant Formula Powder, milk Based, Infant Formula Powder, Soy Based, Adult Nutritional RTF, high Protein and SRM 1849a. Further Nestle laboratory has validated the method for several food matrices that includes cereals and pulses (fortified rice kernel), Spirulina, Fruits, Beverages, Nuts (Almonds) etc (Flow chart 1). For Vitamin B12, LOQ has been established at 0.12 mcg/100 g and spiking to get 0.12 mcg/100g and 0.60 mcg/100 g is verified for Nuts and juices. While fortified rice kernel spike at 6 mcg/100 g was verified. Table 1 and Table 2 shows the comparison of results for various matrices. The new matrix evaluation of vitamin B12 including data on different days to include the intermediate reproducibility on matrices and raw data is provided in Annexure -G.

Flow chart 1: Estimation of B12 in food matrices a brief outline of the method setup.

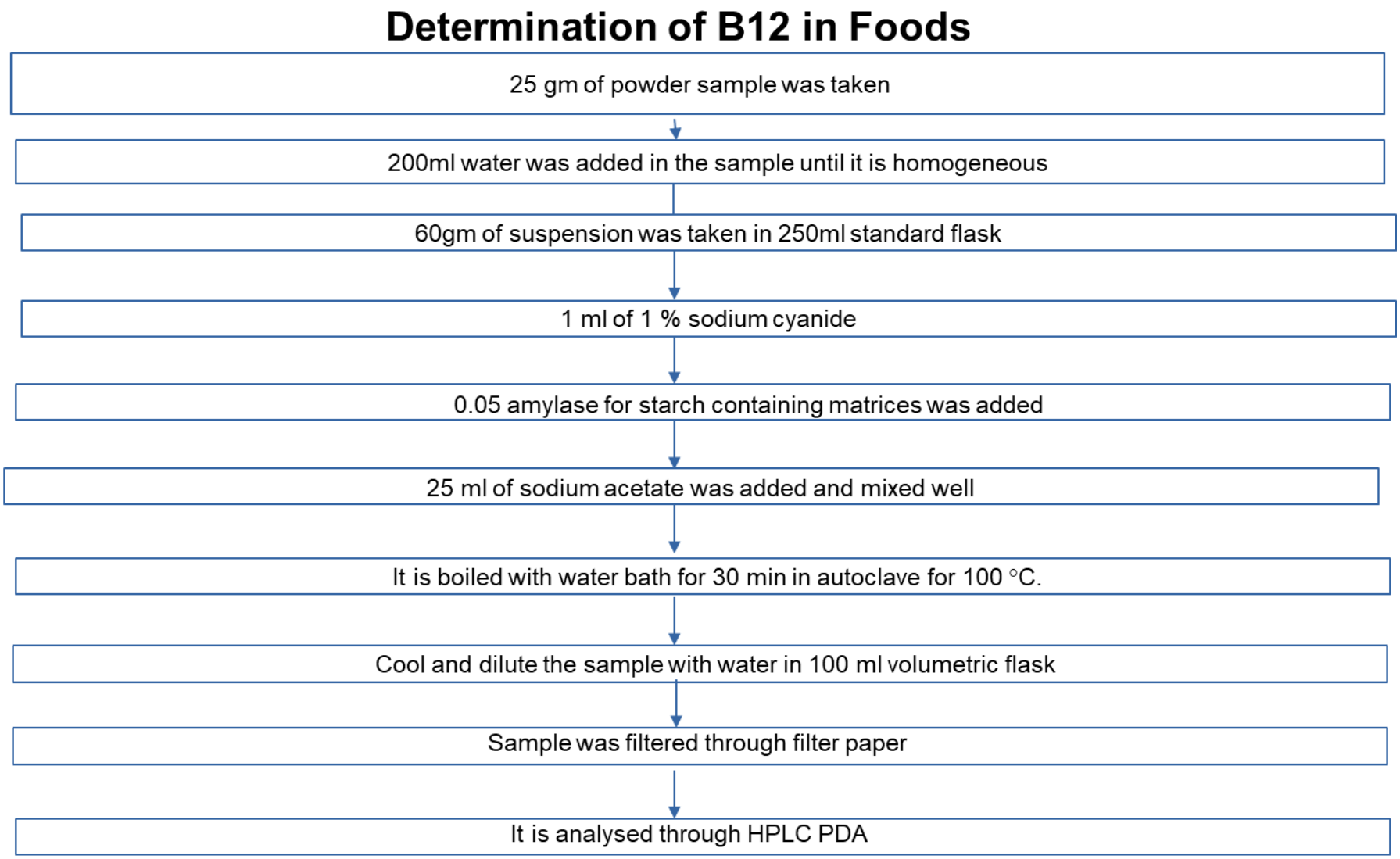


Table 1: Performance Characteristics of Vitamin B12 reported in AOAC 2014.02 and New Matrix single laboratory validation

	Matrices given in Method						NEW MATIX						
	Infant Formula Powder, Partially Hydrolysed, Milk Based	**Infant Formula Powder, Partially Hydrolysed, Soy Based**	**Infant Formula Powder, milk Based**	**Infant Formula Powder, Soy Based**	**Adult Nutritional RTF, high Protein**	**SRM 1849a Infant/Adult Nutritional Formula**	**Spirulina**	**Beverages**	**Fortified Rice Kernel**	**Cereal**	**Vitamin Premix**	**Nuts Spiked 0.12 mcg/ 100 g**	**Juice**
Mean	0.35	0.26	0.24	0.43	1.18	0.435	132.381	5.030	13.937	0.642	319.6	0.119	0.116
SD(b)	0.019	0.074	0.017	0.031	0.043	0.01	-	-	-	-	-	-	-
SDr	0.012	0.007	0.02	0.013	0.042	0.019	4.520	0.020	0.164	0.014	9.605	0.010	0.012
Cvr %	3.4	2.7	8.2	3.0	3.6	4.4	3.4%	0.4%	1.2%	2.2%	3.0%	8.3%	10.0%
SDiR	0.021	0.009	0.022	0.032	0.055	0.017	5.076	0.031	0.150	0.019	8.017	0.009	0.011
CViR %	3.5	3.3	9.0	7.4	4.6	3.8	4%	1%	1%	3%	3%	8%	9%

Table 2: Performance Characteristics of Vitamin B12 at higher spiked level as per AOAC 2014.02 method.

	Fortified Rice Kernel Spiked 6 mcg/100 g	**Nuts Spiked 0.6 mcg/100 g**	**Juice Spiked 0.6 mcg/100 g**
Mean	20.041	0.544	0.581
SD(b)	NA	NA	NA
SDr	0.250	0.020	0.029
Cvr %	1.2%	3.7%	5.1%
SDiR	0.222	0.015	0.029
CViR %	1%	3%	5%

3.2 Single Laboratory Results of Vitamin B5 in Foods- UHPLC-MS/MS method

As objectives was to check suitability of ISO 20639 for matrices other than those mentioned in the ISP 20639 carried out evaluation against ISO 20639 SMPRS.As per evaluation, SMPRs of these new matrices are quite close to the already established matrices i.e. Infant & adult Nutrition foods as per method. Slight variation at LOQ levels are there which are much lower than the means values provided in method. **ISO 20639** method has a scope for Pantothenic acid in Infant Formula and Adult/Pediatric Nutritional Formula. It includes Adult Nutritional Powder Milk protein based, Infant formula powder partially hydrolysed soy based, Adult Nutritional Powder low fat, IF powder soy based, Child formula powder, IF RTF milk based, Infant elemental powder, Adult Nutritional RTF high fat Adult Nutritional RTF High protein and SRM 1849a. Further Nestle laboratory has validated the method for food matrices such as nuts and fruits (Flow chart 2). Table 3 shows the comparison of results for various matrices and Table 4 shows the results of higher spiked levels.

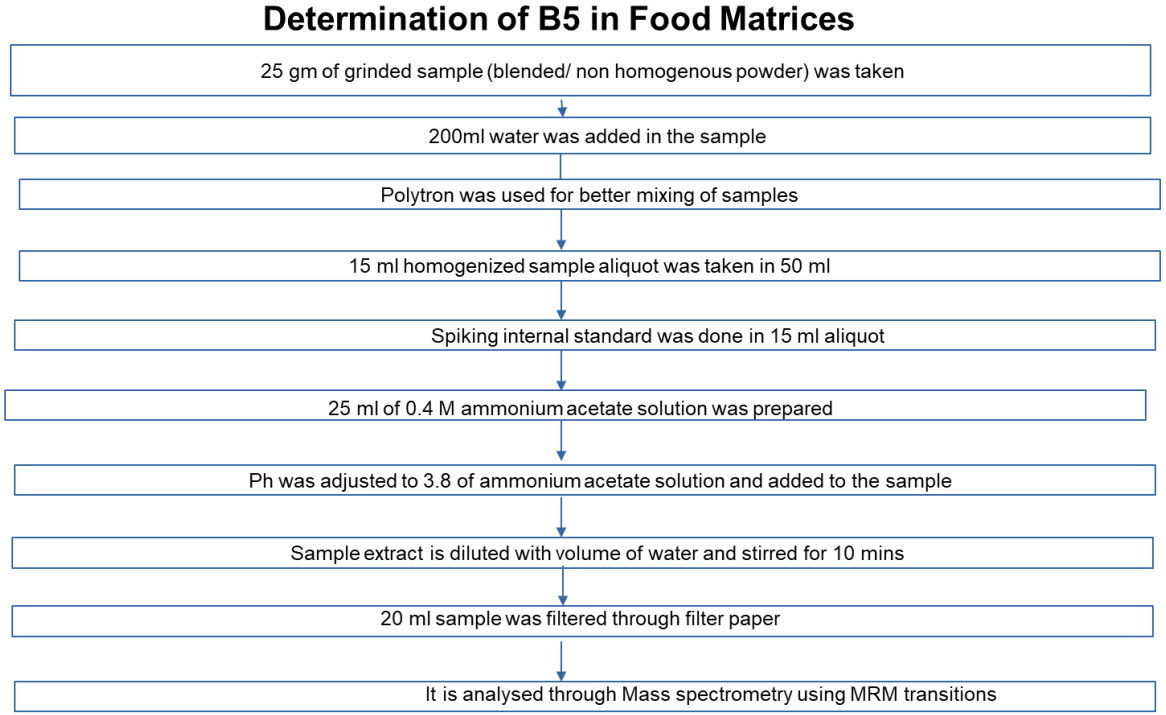


Table 3; Performance Characteristics of Vitamin B5 reported in ISO 20639 and New Matrix single laboratory validation

	Matrices given in Method										New Matrices		
Performance indicators	**Adult Nutritional Powder Milk protein based**	**Infant formula powder partially hydrolysed soy based**	**SRM 1849 a**	**Adult Nutritional Powder low fat**	**IF powder soy based**	**Child formula powder**	**IF RTF milk based**	**Infant elemental powder**	**Adult Nutritional RTF high fat**	**Adult Nutritional RTF High protein**	**Almond LOQ level at 0.8mg/100 g**	**Fruit LOQ level At 0.8mg/100 g**	**Vegetables LOQ* level 0.8mg/100 g**
Mean	2.59	3.85	6.96	8.07	5.04	5.91	0.549	6.65	2.07	1.57	0.872	0.818	0.862
SDr	0.05	0.05	0.14	0.13	0.14	0.17	0.008	0.22	0.06	0.03	0.037	0.046	0.046
SDR	0.13	0.2	0.35	0.33	0.23	0.29	0.022	0.36	0.14	0.09	0.034	0.039	0.034
Cvr	1.9%	1.3%	2.0%	1.6%	2.8%	2.8%	1.5%	3.3%	2.9%	1.7%	4.3%	5.7%	5.4%
CVR	5.0%	5.3%	5.1%	4.1%	4.7%	4.9%	4.1%	5.4%	7.0%	5.5%	4%	5%	4%
Repeatibility limit	0.14	0.14	0.39	0.36	0.39	0.48	0.02	0.62	0.17	0.08	0.104	0.129	0.130
r%	5.4%	3.6%	5.6%	4.5%	7.8%	8.1%	4.1%	9.3%	8.1%	5.4%	12.0%	15.8%	15.1%
Reproducibility limit	0.36	0.56	0.98	0.92	0.64	0.81	0.06	1.01	0.39	0.25	0.094	0.11	0.10
R%	14.1%	14.5%	14.1 %	11.4%	12.8%	13.7%	11.2 %	15.2%	18.9%	16.1%	11%	13%	11%
HorRat value	**0.51**	**0.57**	**0.60**	**0.50**	**0.53**	**0.57**	**0.33**	**0.63**	**0.69**	**0.52**	**0.24**	**0.29**	**0.24**

Table 4: Performance Characteristics of Vitamin B5 at higher spiked level as per ISO 20639 method.

Performance indicators	Cereal	Beverages	Almond LOQ*5 level 4mg/100g	Fruit LOQ*5 level 4mg/100g	Vegetables LOQ*5 level 4mg/100g
Mean	2.082	12.008	4.252	3.983	3.983
SDr	0.071	0.255	0.117	0.067	0.067
SDR	0.076	0.292	0.091	0.058	0.058
Cvr	3.4%	2.1%	2.7%	1.7%	1.7%
CVR	4%	2.4%	2%	1%	1%
Repeatibility limit	0.198	0.713	0.327	0.186	0.186
r%	9.5%	5.9%	7.7%	4.7%	4.7%
Reproducibility limit	0.213	0.713	0.326533	0.186293	0.186293
R%	10.2%	5.9%	8%	5%	5%
HORRAT Value	0.25	0.22	0.17	0.11	0.11

For Vitamin B5, LOQ has been established at 0.8 mg/100gm by considering lowest point of Calibration curve i.e.0.08 mcg/ml and it was verified at Nestle research laboratory by spiking to get 0.8 mg/100g and CV was 3.1%.). The native value 0.08 represent the limit of detection of the neat standard. The new matrix evaluation of Pantothenic acid including data on different days to include the intermediate reproducibility on matrices- Nuts, Fruits & Vegetables (Almonds, Apple Dices & Carrot Crunchies). Appendix G enclosed with the calculations of recoveries, Median, SD, CV, repeatability limit and repeatability % and table with real data, SD, RSD, Horrat.

3.3 MLV of Folates in Foods:

AOAC 2013.13 method was validated at three different food matrix that includes cereals and pulses fortified atta, dried peas of fruits and vegetables and peanut from nuts and nut products. The homogeneity and stability of prepared food matrix are evaluated and the practice sample of different laboratories are acceptable (Annexure H and G). The performance characteristics of different laboratories of two matrixes that is fortified atta and dried peas in tabulated in Table 5 and 6. Peanut data is provided in Annexure -H.

s

Folic acid and 5-MeTHF for Foods

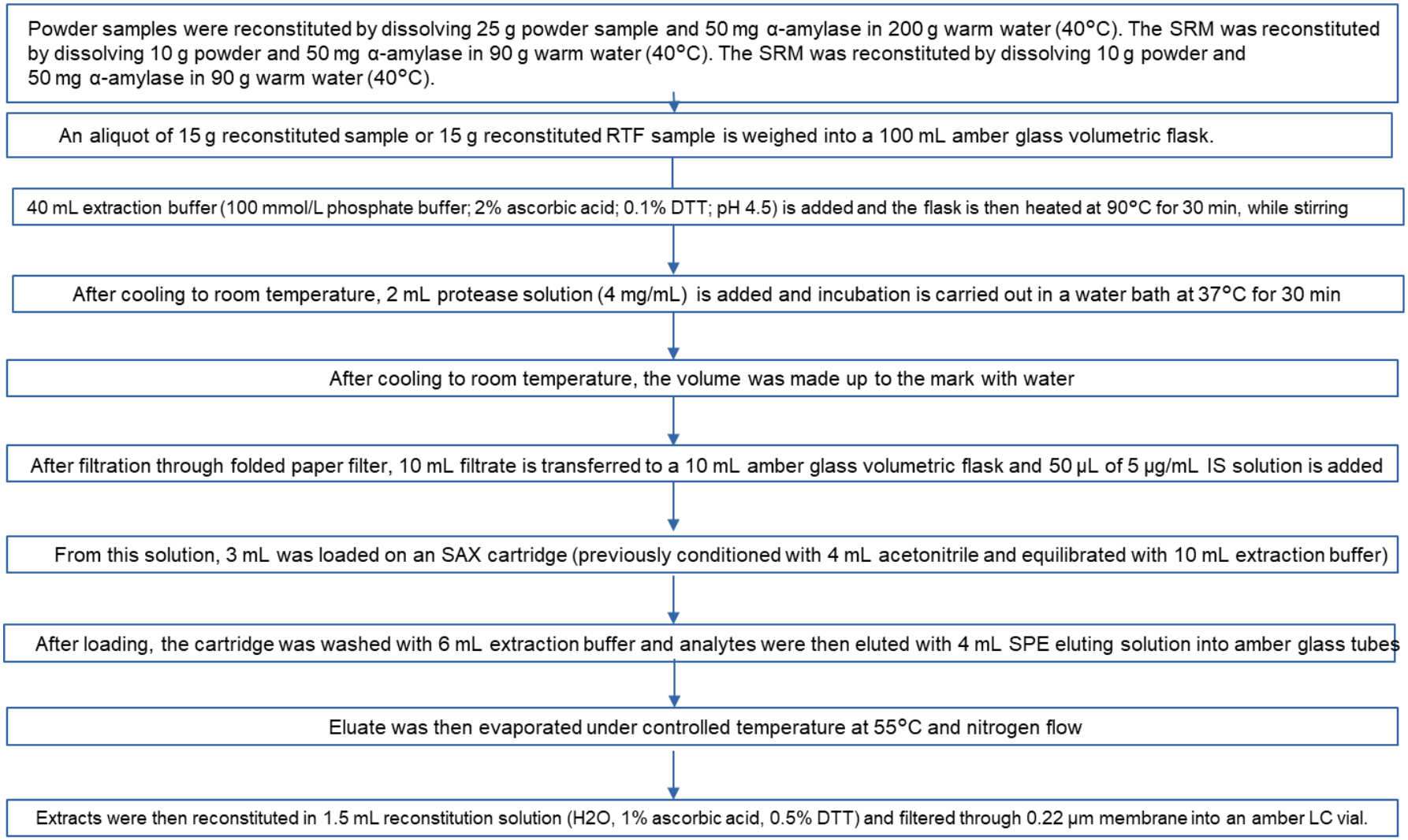
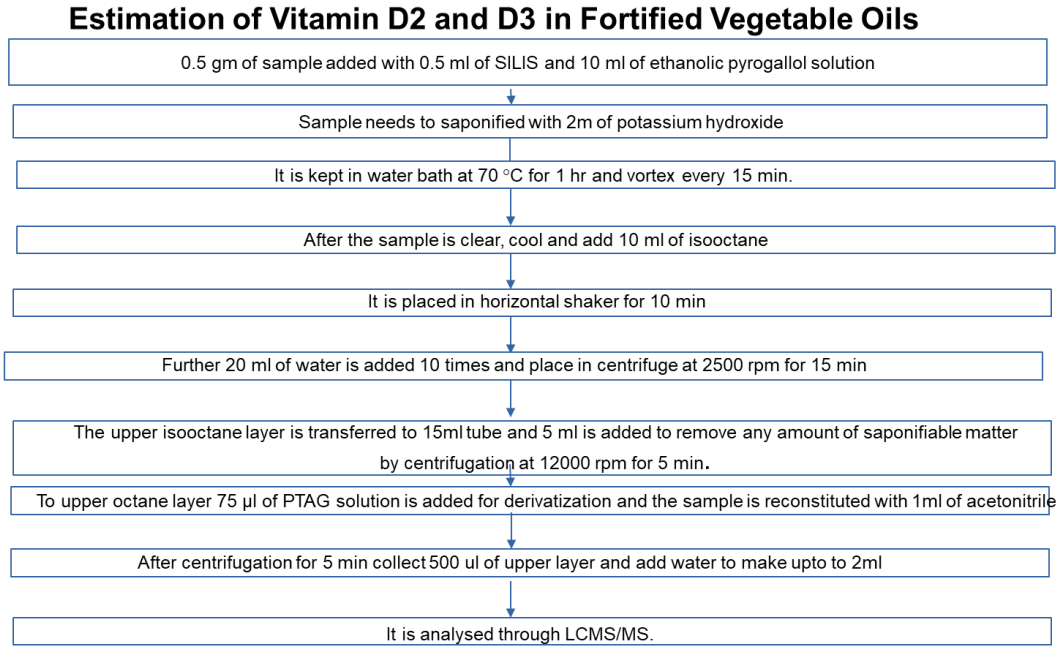


Table 5; Performance Characteristics of Folic acid in fortified atta and 5-MeTHF in dried peas based on AOAC 2013.13 Multi laboratory validation.

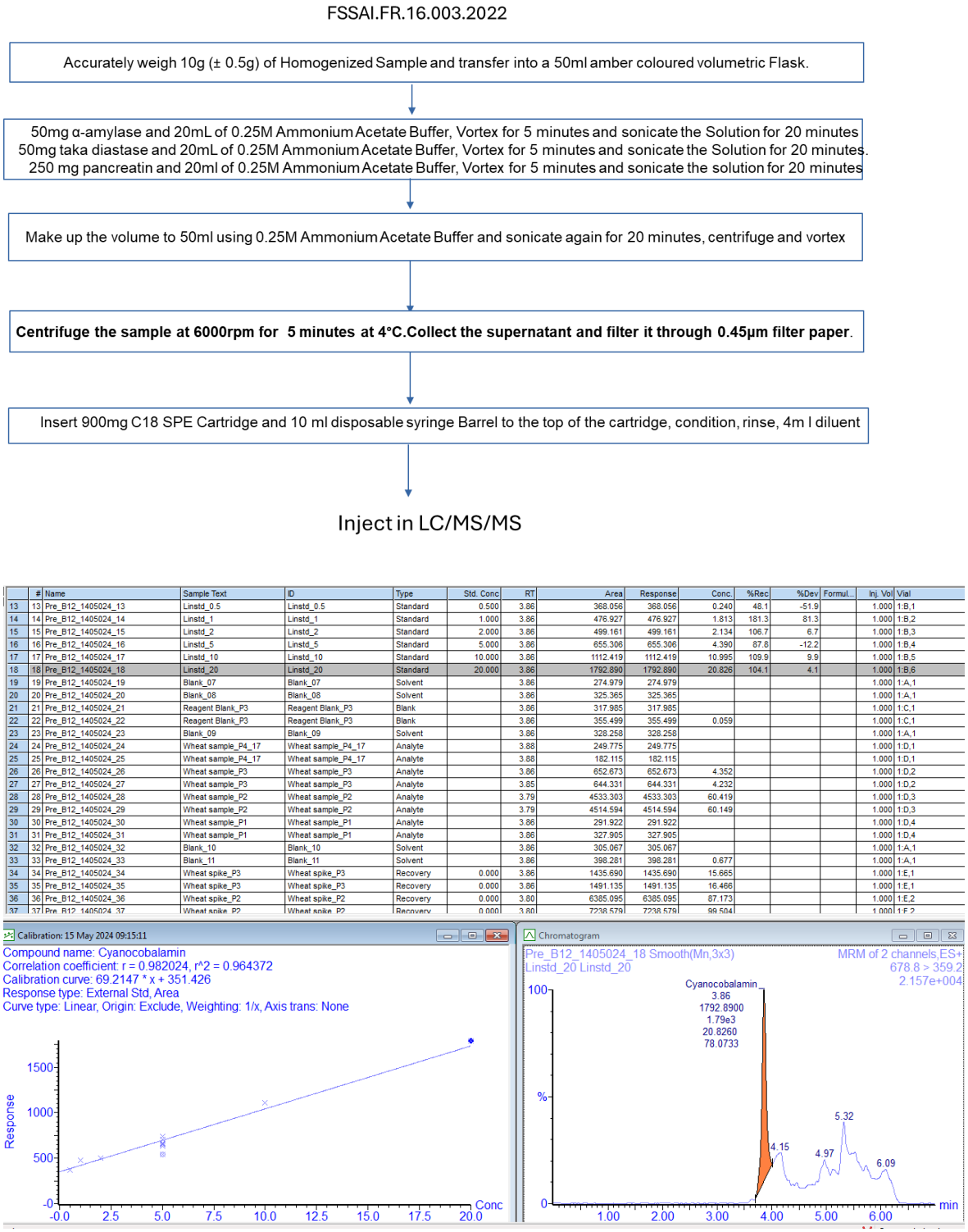
	Fortified Atta	Dried Peas
Mean	15.712	119.620
SDr	2.122	10.468
Repeatability RSDr % (Observed)	13.500	8.526
Predicted RSDr% (Horwitz)	6.975	5.160
HORRATr	**1.936**	**1.666**
SD_R	2.129	10.635
Reproducibility RSD_R % (Observed)	**13.55**	**8.89**
Predicted RSD_R% (Horwitz)	**10.57**	**7.79**
$HORRAT_R$	**1.28**	**1.14**
Remarks	**Within the Recommended value**	

3.5 MLV of Vitamin D: Method was optimised for two different vegetable oils including sunflower oil and blended sunflower oil with rice bran oil and control sample was passed the homogeneity and stability test and practice samples are also within the range of fortified oil testing and complete details are given in Annexure -H and G. The details are given in Annexure -H and G. A brief outline of the method is given below.



3.4 MLV of Vitamin B12: Method optimization of vitamin B12 for various matrices to identify the natural forms such as methylcyanocobalmin and cyanocobalmin for

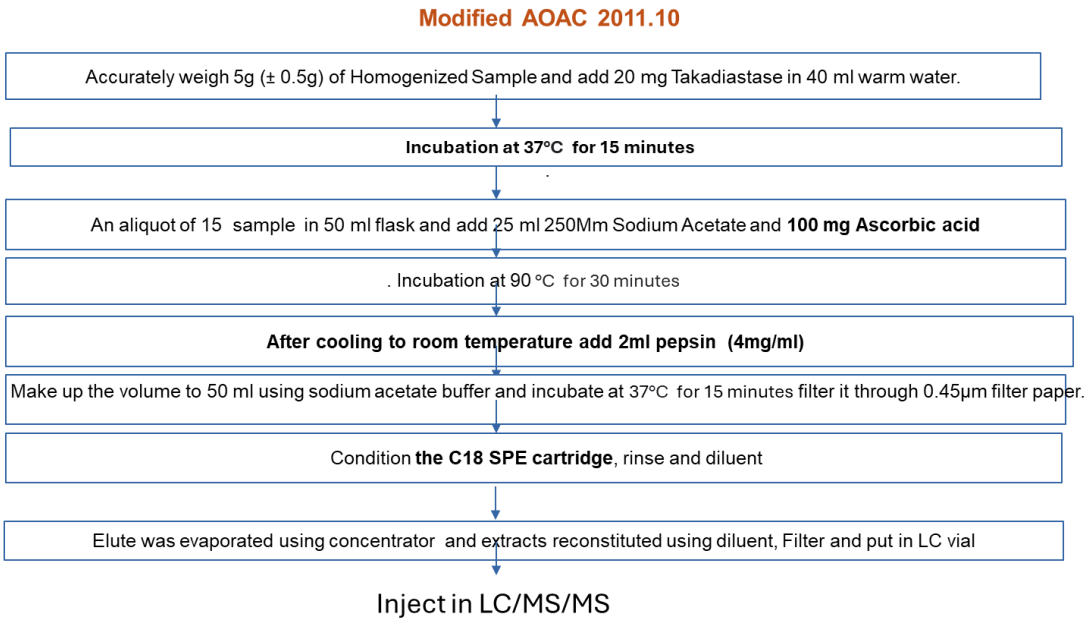
fortified atta was revalidated using FSSAI. FR. 16.03.2022. The single laboratory validation for the repeatability, reproducibility and recovery for fortified wheat flour atta, and almond powder was not achieved with FSSAI.FR. 16.003.22 and AOAC 2011.10 method. Different enzymatic treatment were considered that includes, taka diastase, pancreatin, pepsin and analyzed for recovery given below.

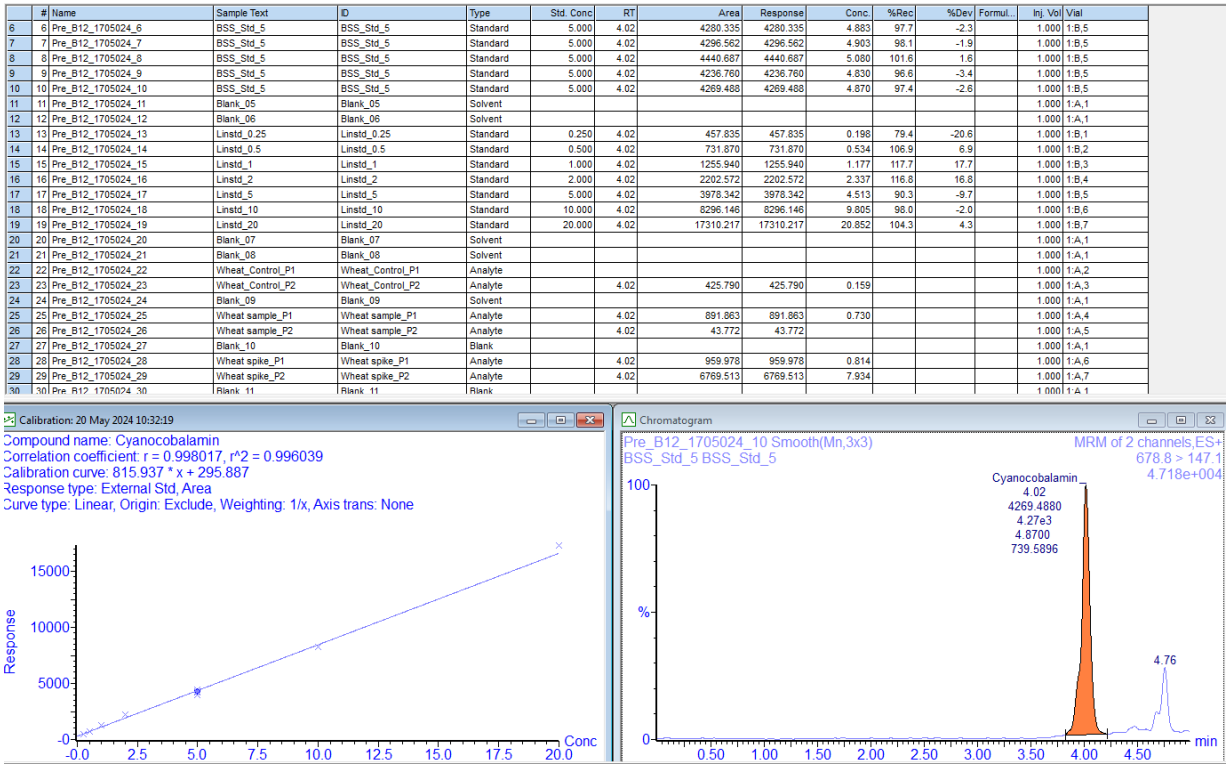


	#	Name	Sample Text	ID	Type	Std. Conc	RT	Area	Response	Conc.	%Rec	%Dev	Formul...	Inj. Vol	Vial
13	13	Pre_B12_1405024_13	Linstd_0.5	Linstd_0.5	Standard	0.500	3.86	368.056	368.056	0.240	48.1	-51.9		1.000	1:B,1
14	14	Pre_B12_1405024_14	Linstd_1	Linstd_1	Standard	1.000	3.86	476.927	476.927	1.813	181.3	81.3		1.000	1:B,2
15	15	Pre_B12_1405024_15	Linstd_2	Linstd_2	Standard	2.000	3.86	499.161	499.161	2.134	106.7	6.7		1.000	1:B,3
16	16	Pre_B12_1405024_16	Linstd_5	Linstd_5	Standard	5.000	3.86	655.306	655.306	4.390	87.8	-12.2		1.000	1:B,4
17	17	Pre_B12_1405024_17	Linstd_10	Linstd_10	Standard	10.000	3.86	1112.419	1112.419	10.995	109.9	9.9		1.000	1:B,5
18	18	Pre_B12_1405024_18	Linstd_20	Linstd_20	Standard	20.000	3.86	1792.890	1792.890	20.826	104.1	4.1		1.000	1:B,6
19	19	Pre_B12_1405024_19	Blank_07	Blank_07	Solvent		3.86	274.979	274.979					1.000	1:A,1
20	20	Pre_B12_1405024_20	Blank_08	Blank_08	Solvent		3.86	325.365	325.365					1.000	1:A,1
21	21	Pre_B12_1405024_21	Reagent Blank_P3	Reagent Blank_P3	Blank		3.86	317.985	317.985					1.000	1:C,1
22	22	Pre_B12_1405024_22	Reagent Blank_P3	Reagent Blank_P3	Blank		3.86	355.499	355.499	0.059				1.000	1:C,1
23	23	Pre_B12_1405024_23	Blank_09	Blank_09	Solvent		3.86	328.258	328.258					1.000	1:A,1
24	24	Pre_B12_1405024_24	Wheat sample_P4_17	Wheat sample_P4_17	Analyte		3.88	249.775	249.775					1.000	1:D,1
25	25	Pre_B12_1405024_25	Wheat sample_P4_17	Wheat sample_P4_17	Analyte		3.88	182.115	182.115					1.000	1:D,1
26	26	Pre_B12_1405024_26	Wheat sample_P3	Wheat sample_P3	Analyte		3.86	652.673	652.673	4.352				1.000	1:D,2
27	27	Pre_B12_1405024_27	Wheat sample_P3	Wheat sample_P3	Analyte		3.85	644.331	644.331	4.232				1.000	1:D,2
28	28	Pre_B12_1405024_28	Wheat sample_P2	Wheat sample_P2	Analyte		3.79	4533.303	4533.303	60.419				1.000	1:D,3
29	29	Pre_B12_1405024_29	Wheat sample_P2	Wheat sample_P2	Analyte		3.79	4514.594	4514.594	60.149				1.000	1:D,3
30	30	Pre_B12_1405024_30	Wheat sample_P1	Wheat sample_P1	Analyte		3.86	291.922	291.922					1.000	1:D,4
31	31	Pre_B12_1405024_31	Wheat sample_P1	Wheat sample_P1	Analyte		3.86	327.905	327.905					1.000	1:D,4
32	32	Pre_B12_1405024_32	Blank_10	Blank_10	Solvent		3.86	305.067	305.067					1.000	1:A,1
33	33	Pre_B12_1405024_33	Blank_11	Blank_11	Solvent		3.86	398.281	398.281	0.677				1.000	1:A,1
34	34	Pre_B12_1405024_34	Wheat spike_P3	Wheat spike_P3	Recovery	0.000	3.86	1435.690	1435.690	15.665				1.000	1:E,1
35	35	Pre_B12_1405024_35	Wheat spike_P3	Wheat spike_P3	Recovery	0.000	3.86	1491.135	1491.135	16.466				1.000	1:E,1
36	36	Pre_B12_1405024_36	Wheat spike_P2	Wheat spike_P2	Recovery	0.000	3.80	6385.095	6385.095	87.173				1.000	1:E,2
37	37	Pre_B12_1405024_37	Wheat spike_P2	Wheat spike_P2	Recovery	0.000	3.80	7238.579	7238.579	99.504				1.000	1:E,2

Figure 1: Workflow of FSSAI and AOAC 2011.10 for vitamin B12 and the spike recovery obtained from different methods.

Figure 1 shows the spike recovery of three methods, taka diastase (wheat sample _P2), and enzymatic treatment as p per AOAC 2013.10 (wheat sample P1) and wheat sample-P3 (FSSAI with taka diastase). Further method verification of AOAC 2011.10 is for infant food and nutritional's that was proposed in the project was verified for matrices such as fortified wheat flour atta and almonds. The method of AOAC has to be optimised for these matrices as KCN is added to obtain the most stable form of vitamin B12 that is cyanocobalmin. To retain the natural forms of vitamin B12, and obtain bound form of the vitamin B12 in almond, pepsin enzyme treatment is considered and LC-MS/MS method is optimized that could be applied for the two matrices proposed (Figure 2). This optimized method led to improved repeatability and recovery of wheat and almond samples. Further to avoid the interreferences of the column pressure noise, the gradient program and mobile phase are altered (see Appendix B). The performance characteristics of single laboratory validation is shown in Table 7. Raw data of samples, homogeneity, stability, linearity, specificity is given in appendix.





	#	Name	Sample Text	ID	Type	Std. Conc	RT	Area	Response	Conc.	%Rec	%Dev	Formul..	Inj. Vol	Vial
6	6	Pre_B12_1705024_6	BSS_Std_5	BSS_Std_5	Standard	5.000	4.02	4280.335	4280.335	4.883	97.7	-2.3		1.000	1:B,5
7	7	Pre_B12_1705024_7	BSS_Std_5	BSS_Std_5	Standard	5.000	4.02	4296.562	4296.562	4.903	98.1	-1.9		1.000	1:B,5
8	8	Pre_B12_1705024_8	BSS_Std_5	BSS_Std_5	Standard	5.000	4.02	4440.687	4440.687	5.080	101.6	1.6		1.000	1:B,5
9	9	Pre_B12_1705024_9	BSS_Std_5	BSS_Std_5	Standard	5.000	4.02	4236.760	4236.760	4.830	96.6	-3.4		1.000	1:B,5
10	10	Pre_B12_1705024_10	BSS_Std_5	BSS_Std_5	Standard	5.000	4.02	4269.488	4269.488	4.870	97.4	-2.6		1.000	1:B,5
11	11	Pre_B12_1705024_11	Blank_05	Blank_05	Solvent									1.000	1:A,1
12	12	Pre_B12_1705024_12	Blank_06	Blank_06	Solvent									1.000	1:A,1
13	13	Pre_B12_1705024_13	Linstd_0.25	Linstd_0.25	Standard	0.250	4.02	457.835	457.835	0.198	79.4	-20.6		1.000	1:B,1
14	14	Pre_B12_1705024_14	Linstd_0.5	Linstd_0.5	Standard	0.500	4.02	731.870	731.870	0.534	106.9	6.9		1.000	1:B,2
15	15	Pre_B12_1705024_15	Linstd_1	Linstd_1	Standard	1.000	4.02	1255.940	1255.940	1.177	117.7	17.7		1.000	1:B,3
16	16	Pre_B12_1705024_16	Linstd_2	Linstd_2	Standard	2.000	4.02	2202.572	2202.572	2.337	116.8	16.8		1.000	1:B,4
17	17	Pre_B12_1705024_17	Linstd_5	Linstd_5	Standard	5.000	4.02	3978.342	3978.342	4.513	90.3	-9.7		1.000	1:B,5
18	18	Pre_B12_1705024_18	Linstd_10	Linstd_10	Standard	10.000	4.02	8296.146	8296.146	9.805	98.0	-2.0		1.000	1:B,6
19	19	Pre_B12_1705024_19	Linstd_20	Linstd_20	Standard	20.000	4.02	17310.217	17310.217	20.852	104.3	4.3		1.000	1:B,7
20	20	Pre_B12_1705024_20	Blank_07	Blank_07	Solvent									1.000	1:A,1
21	21	Pre_B12_1705024_21	Blank_08	Blank_08	Solvent									1.000	1:A,1
22	22	Pre_B12_1705024_22	Wheat_Control_P1	Wheat_Control_P1	Analyte									1.000	1:A,2
23	23	Pre_B12_1705024_23	Wheat_Control_P2	Wheat_Control_P2	Analyte		4.02	425.790	425.790	0.159				1.000	1:A,3
24	24	Pre_B12_1705024_24	Blank_09	Blank_09	Solvent									1.000	1:A,1
25	25	Pre_B12_1705024_25	Wheat sample_P1	Wheat sample_P1	Analyte		4.02	891.863	891.863	0.730				1.000	1:A,4
26	26	Pre_B12_1705024_26	Wheat sample_P2	Wheat sample_P2	Analyte		4.02	43.772	43.772					1.000	1:A,5
27	27	Pre_B12_1705024_27	Blank_10	Blank_10	Blank									1.000	1:A,1
28	28	Pre_B12_1705024_28	Wheat spike_P1	Wheat spike_P1	Analyte		4.02	959.978	959.978	0.814				1.000	1:A,6
29	29	Pre_B12_1705024_29	Wheat spike_P2	Wheat spike_P2	Analyte		4.02	6769.513	6769.513	7.934				1.000	1:A,7
30	30	Pre_B12_1705024_30	Blank_11	Blank_11	Blank									1.000	1:A,1

Figure 2: Work flow for vitamin B12 and spike recovery for wheat flour atta.

Table 6: Performance characteristics of B12 in Wheat flour atta spiked LOQ based on AOAC 2011.10

	Fortified Atta Spike 0.5 mcg/kg	**Fortified atta Spike 5 mcg/kg**	**Almonds**
Mean	0.485	4.230	**0.186**
SDr	0.027	0.092	**0.006**
Repeatability RSDr % (Observed)	6.277	2.172	**3.359**
Predicted RSDr% (Horwitz)	11.986	8.608	**13.655**
HORRATr	**0.52**	**0.252**	**0.259**
SD_R	0.061	0.372	0.008
Reproducibility RSD_R % (Observed)	12.50	8.80	**4.42**
Predicted RSD_R% (Horwitz)	17.84	12.88	**20.60**
$HORRAT_R$	**0.70**	**0.68**	**0.21**
Remarks	Within Recommended value		

4.0 Conclusions: The proposed matrices for validation of published methods

(a) SLV for vitamin B12 and B5 for all the proposed results gave satisfactory results. Limit of quantification of 0.12mcg/100g and 0.8mg/100g for Vitamin B12 and Pantothenic acid respectively for food matrices.

(b) In case of Vitamin B9. Cereal Products such as fortified atta, and Fruits and Vegetable category (dried peas) has completed multi laboratory validation. The nut and nut products such as peanut data compilation suggested very less quantity of folate form and is under progress.

(c) MLV of Vitamin D: Oils samples Envirocare and multi laboraotries have completed practice samples and data compilation for samples is under progress.

(d) MLV of Vitamin B12: Method optimization of AOAC 2011.10 modification is completed. Cereal Products and Nut and Nut Products single laboratory validation showed satisfactory results with acceptable HORRAT value. While Meat and Meat Products- the method is not rugged, and robustness was not achieved.

Challenges: In view of method verification of proposed FSSAI. FR.16.003.2022 and AOAC 2011.10 the food matrix proposed were unable to be determined. The method validation of Modified AOAC 2011.10 method and obtaining the MLV results has limited the time due to unforeseen hurdles of participating laboratories such as LC-MS/MS instrument down time and preassigned work of analysis.

To summarise, homogeneity, stability of proposed matrices of MLV samples of vitamin B9, B12 and D have satisfactory results. Moreover, the multi laboratory validation for Vitamin B9 and Vitamin D for proposed matrices are completed and vitamin B9 for fortified atta and dried peas have satisfactory results with recommended value of HORRATr and $HORRAT_R$ values. Single laboratory validation of B12, B5 have led to extend the scope of AOAC 2014.02 and ISO -20639 method with accepted recommended values of $HORRAT_R$. The Modified AOAC 2011.10 method of AOAC 2011.10 enabled to quantify the methylcobalmin and cyanocobalmin to 0.5 mcg/Kg. Similarly, Folic acid and 5-MeTHF using AOAC 2013.13 led to quantify up to 0.33 mcg/100g. Limit of Quantification for Vitamin D is 25mcg/kg.

Annexure -A : List of Materials-Table form list of practice samples, MLV samples of B12

Sl No	Participating Laboratory and contact details	Sample & Code	Standards
1	Dr. Jyoti, Assistant Director - Laboratory Eurofins Analytical Services India Pvt. Ltd. 540/1, Doddanekundi Industrial Area 2, Graphite India Road, Hoodi, Whitefield, 560048, Bengaluru, India	Wheatflour Control-C18 63 8 35 24 50 Almonds 100 11 13 28 64 5 Meat 1 13 27 23	Methylcobalamin (100ppm)-PHR3410 Hydroxycobalamin (100ppm)- PHR3186 Pepsin Takadiastase
2	Dr. Gouri Ray, Attn: Dr. Hemalatha B Head corporate Quality, Eureka Analytical Services Pvt Ltd # 617, AB SQUARE, 5th Main, OMBR LAYOUT Banaswadi, Bengaluru 560043, Karnataka	Wheat flour Control-C13 41 11 28 49 12 Almonds 60 56 72 96 35 41 Meat 18 11 22	Methylcobalamin (100ppm)-PHR3410 Hydroxycobalamin (100ppm)- PHR3186

		32	
3.	Dr. Priti Amritkar Director Labs Envirocare labs Enviro House, A7-A8, MIDC Main Road, Wagle Estate, Thane Maharashtra, India 400604	Wheat flour Control-C2 32 21 3 9 61 Almonds 42 77 4 33 43 80 Meat 8 12 4 21	Methylcobalamin (100ppm)-PHR3410 Hydroxycobalamin (100ppm)- PHR3186 Pepsin Takadiastase Ascorbic acid
4.	Dr. Amrit Kaur, Nestle India Limited Laboratory Services, NQAC Moga, PB No 11, Ludhiana-Ferozepur Road Moga, Punjab state, India -142001 ,	Wheat flour Control-C15 34 20 45 38 14 Almonds 44 68 32 71 91 25 Meat 5 35 7 10	Methylcobalamin (100ppm)-PHR3410 Hydroxycobalamin (100ppm)- PHR3186

Received **samples**

Sl No	Laboratory	Sample and sample code
1	Dr. Amrit Kaur, Nestle India Limited Laboratory Services, NQAC Moga, PB No 11, Ludhiana-Ferozepur Road Moga, Punjab state, India -142001 Tel- +91 1636512041,	Wheat 19 17 23 21
2.	Dr. Amrit Kaur, Nestle India Limited Laboratory Services, NQAC Moga, PB No 11, Ludhiana-Ferozepur Road Moga, Punjab state, India -142001 Tel- +91 1636512041,	Peas- 9 19 4 25
3.	Dr. Amrit Kaur, Nestle India Limited Laboratory Services, NQAC Moga, PB No 11, Ludhiana-Ferozepur Road Moga, Punjab state, India -142001 Tel- +91 1636512041, M: 97799408	Peanuts- 27 19 6
4.	Dr. Priti Amritkar Director Labs Envirocare labs Enviro House, A7-A8, MIDC Main Road, Wagle Estate, Thane Maharashtra, India 400604 M: +91 9167232001	Oil samples 1 Practice sample 2 for analysis

ANNEXURE B- Method- Full method

VITAMIN B12 -AOAC 2014.02 (SLV)

APPARATUS AND MATERIALS

(a) Balances—With readability of 0.1 mg and 0.01 g.

(b) Sonicator.

(c) In-line water bath—With magnetic stirrers or autoclave.

(d) pH meter.

(e) Rotary shaker for biochemistry—Labnet International (Edison, NJ, USA) or Stuart LB3 (Barloworld, Bibby Sterilin Ltd, Staffordshire, UK), or equivalent.

(f) Heating block—With nitrogen evaporation.

(g) Vortex.

(h) Homogenizer—Polytron® PT3000 (drive unit), Aggregate PT-DA 3012 (Kinematica, Lucerne, Switzerland), or equivalent.

(i) Volumetric flasks—Amber glass; 10, 50, 100, 200, 250; clear glass, 2000 mL.

(j) Graduated cylinders—50, 100, and 1000 mL.

(k) Beakers—Amber glass, 250 mL.

(l) Flat-bottom round flasks or Erlenmeyers—Amber glass, 250 mL.

(m) Folded paper filters—602H 1/2 or 597 1/2 (Whatman Inc., Maidstone, UK), or equivalent.

(n) Amber vials—Screw top, 7 or 4 mL (Supelco Inc., Bellefonte, PA, USA).

(o) Micro LC vials—Amber.

(p) Pipets—Graduated glass, 10 mL, or volumetric glass, 9 mL.

(q) Electronic digital pipet—Variable volume, 200–1000 μL.

(r) Syringes—Disposable, 20 mL, equipped with a perforated rubber stopper attached to the tip.

(s) Immunoaffinity columns—EASI-EXTRACT® VITAMIN B12 LGE (R-Biopharm AG; Product Code P88).

(t) Immunoaffinity column rack—R-Biopharm AG, Product Code CR1.

(u) Chromatographic system—HPLC or UHPLC system equipped with a quaternary or binary pump, sample injector, UVVIS detector (or optionally a PDA detector), degassing system, and data software.

(v) Analytical column—Depending on the chromatographic system available, use HPLC or UHPLC columns.

(1) UHPLC column—Waters Acquity UPLC® BEH C18, 1.7 μm, 2.1 × 100 mm (Waters, Milford, MA, USA), or equivalent.
(2) HPLC column—Nucleosil 100-3 C18 HD, 125 × 3.0 mm (Macherey-Nagel, Inc., Oesingen, Switzerland), C18 ACE 3AQ, 150 × 3.0 mm (ACE, Aberdeen, Scotland, UK), or equivalent.

REAGENTS AND STANDARDS

(a) Methanol—HPLC grade.
(b) Acetonitrile—HPLC grade.
(c) Acetic acid, glacial.
(d) Milli-Q water—Millipore (Bedford, MA, USA).
(e) Sodium cyanide puriss—Fluka (Buchs, Switzerland), or equivalent.
(f) Sodium acetate trihydrate p.a.—Merck (Darmstadt, Germany), or equivalent.
(g) Sodium hypochlorite—Technical grade.
(h) TFA—Merck, or equivalent.
(i) Vitamin B12 (cyanocobalamin)—Purity >99%; Sigma-Aldrich (St. Louis, MO, USA), or equivalent.

SOLUTION AND STANDARD PREPARATION

(a) Sodium acetate solution 0.4 M, pH 4.0.—Into a 2000 mL volumetric flask, weigh 108.8 g sodium acetate trihydrate. Add about 1800 mL water. Dissolve. Add 50 mL acetic acid and adjust pH to 4.0 with acetic acid. Dilute to volume with water.

(b) Sodium cyanide solution, 1% (w/v)—Weigh 0.5 g sodium cyanide into a 50 mL amber glass volumetric flask. Dilute to volume with water. Any excess of 1% sodium cyanide solution must be destroyed by adding 1.5 mL of a 15% solution of sodium hypochlorite per 1 mL sodium cyanide solution. Let it react for 2 days in a fume hood.

(c) Mobile phase A.—To 1000 mL water, add 250 μL TFA. Mix well.

(d) Mobile phase B.—To 1000 mL acetonitrile, add 250 μL TFA. Mix well.

(e) Sample dilution solvent—Mix 90 mL mobile phase A with10 mL mobile phase B.

(f) Vitamin B12 stock standard solution (100 μg/mL)—Accurately weigh 20.0 mg cyanocobalamin into a 200 mL amber glass volumetric flask. Add about 150 mL water. Dissolve by sonication and stirring for a few minutes. Dilute to volume with water. This solution is stable for ≥6 months at –20°C.

(g) Vitamin B12 intermediate standard solution (400 ng/mL)—Pipet 1 mL vitamin B12 stock standard solution into a 250 mL amber glass volumetric flask. Make up to volume with water.

(h) Vitamin B12 working standard solutions for calibration (2,10, 20, 40, 60, 100 ng/mL)—Pipet into six separated 10 mL amber glass volumetric flasks 50, 250, 500,

1000, 1500, and 2500 μL vitamin B12 intermediate standard solution. Dilute to volume with sample dilution solvent, D(e).

SAMPLE PREPARATION AND EXTRACTION PROCEDURE

(a**) Sample reconstitution for powder samples**—Weigh 25.0 g (W1) of sample into a 250 mL beaker. Add 200 g (W2) water at 40 ± 5°C. Mix with a glass rod until suspension is homogeneous or homogenize with a Polytron®. Proceed as described in E(d).

(b) **Sample reconstitution for amino acid based products**—Weigh 25.0 g (W1) of powder sample into a 250 mL beaker. Add 190 g (W2) of water at 40 ± 5°C and 10 g (W3) skimmed milk powder. Mix with a glass rod until suspension is homogeneous or homogenize with a Polytron. In parallel, run a blank by replacing the sample by water (215 g water + 10 g skimmed milk powder). Dilute both, the reconstituted sample and the blank, twice in water (e.g., 50 g reconstituted sample or blank + 50 g water). Proceed as described in E(d).

(c) **Sample preparation for liquid samples—**Mix well to ensure homogeneity of the sample portion. Proceed as described in E(d). In the case of high-fat nutritional products, if recovery is low, samples can be diluted in water (e.g., 50 g sample + 50 g water) before extraction to improve recovery.

(d) **Extraction**—Weigh 60.0 g (m) sample suspension E(a),E(b), blank E(b), or liquid sample E(c) into a 250 mL flat-bottom amber glass flask or Erlenmeyer with ground glass neck. Add 1 mL of 1% sodium cyanide solution D(b). If the sample contains starch, add about 0.05 g α-amylase and mix thoroughly. Stopper the flask and incubate 15 min at 40 ± 5°C. Add 25 mL sodium acetate solution D(a). Mix well. Place flask in a boiling water bath for 30 min (or autoclave 30 min at 100°C). Cool flask in ice bath or let stand at room temperature. Quantitatively transfer content of flask to a 100 mL (V1) amber glass volumetric flask. Dilute to volume with water. Filter solution through folded paper filter.

(e) **Immunoaffinity cleanup**—Let immunoaffinity columns warm to room temperature by removing them from refrigeration at least 30 min before use. Place each immunoaffinity column on the rack. Open caps and let storage buffer drain by gravity. Close the lower cap. Load column with 9 mL (V2) of clear filtrate and close the upper cap. Place column in a rotary shaker and mix slowly for 10–15 min. Return column to the support and let stand for a few minutes. Open the caps to let liquid drain by gravity. Wash column with 10 mL water. With a syringe, insert about 40 mL air to dry the column. Elute with 3 mL methanol, and collect the eluate in a 4 or a 7 mL amber glass reaction vial. Rinse column with 0.5 mL methanol, and with a syringe, insert about 20 mL air to collect all the methanol in the same vial. Evaporate at 50°C under a stream of nitrogen. Reconstitute sample in 0.3 mL (V3) sample dilution solvent D(e). Mix on a vortex mixer. Transfer to a micro amber vial.

RESULT CALCULATION

1. Quantitation (liquid and powder samples)—Calculate the concentration of vitamin B12 in μg/100 g of product as follows:

$$\frac{(\mathrm{A}-1)\times(W1+W2)\times V1\ \times V3\times 100}{\mathrm{S}\times \mathrm{W1}\times \mathrm{m}\times \mathrm{V2}\times 100}$$

where A = response (height or area) of the peak obtained for the sample solution, I = intercept of the calibration curve, S = slope of the calibration curve, W1 = weight of powder sample used for reconstitution (25 g), W2 = weight of water used for reconstitution (200 g), m = weight of sample suspension (60 g), V1 = volume of the test solution (volume used to dissolve the test portion) in mL (100 mL), V2 = volume of the aliquot of the sample solution loaded onto the affinity column (9 mL), and V3 = volume in which the aliquot of the sample solution is reconstituted after immunoaffinity cleanup (0.3 mL).

2. Quantitation (amino-acid-based products)—Calculate the concentration of vitamin B12 in the sample in μg/100 g of product as follows:

$$C\ sample = [(Ablank - A\ sample) - 1]\frac{(\mathrm{W1}+\mathrm{W2}+\mathrm{W3})\times\times V1\ \times V3\times D\times 100}{\mathrm{S}\times \mathrm{W1}\times \mathrm{m}\times \mathrm{V2}\times 1000}$$

where Ablank = response (height or area) of the peak in the blank, Asample= response (height or area) of the peak in the sample, I = intercept of the calibration curve, S = slope of the calibration curve, W1 = weight of sample used for reconstitution (25 g), W2 = weight of water used for reconstitution (190 g), W3 = weight of skimmed milk powder used for reconstitution (10 g), m = weight of sample suspension (60 g), V1= volume of the test solution (volume used to dissolve the test portion) in milliliters (100 mL), V2 = volume of the aliquot of sample solution loaded onto the affinity column (9 mL), V3 = volume in which the aliquot of sample solution is reconstituted after immunoaffinity cleanup (0.3 mL), and D = dilution factor

INSTRUMENT CONDITION

Instrument	WATERS XEVO TQ-XS
Detector	Mass Detector
Column	C18,1.7 μm, 2.1 × 100 mm
Run time	7 minutes

Column Temperature	40 °C
Flow Rate	0.25 ml
Injection Volume	5 µL- 20 µl
Mobile phase A	Tetrahydro formic acid in water
Mobile phase B	Tetrahydro formic acid in Acetonitrile
Desolvation Temperature	200 °C
CE	40eV
CV	20 V
Source	ESI +ve

GRADIENT PROGRAM:

TIME,Min	% A	%B
0.0	90	10
1.7	90	10
2.5	75	25
2.9	10	90
3.9	10	90
4.0	90	10
8.0	90	10

VITAMIN B5 1SO 20639:2015 (SLV)

APPARATUS

1. Balances, with readability of 0,1 mg, capacity 210 g; with readability of 0,1 g, capacity 4 100 g.
2. pH-meter, with readability of 0,01 pH unit.
3. Homogenizer
4. Stir plate with magnetic stirrers.
5. Filters. Syringe filters, 0,22 µm pore size, 33 mm internal diameter, Millex-GV PVDF (Millipore) membrane disc filters, 0,45 µm pore size (Millipore)3) or equivalent.
6. UHPLC-MS/MS system, UPLC column, e.g. ACQUITY UPLC®3) coupled with triple quadrupole detector equipped with electrospray ionization (ESI) source and T3 column (1,8 µm, 100 mm × 2,1 mm internal diameter; Waters Corp.)3) or equivalent.

REAGENTS AND STANDARDS

1. Acetonitrile, LC grade or equivalent.
2. Ammonium acetate, ACS grade, > 98 % (Fluka 9690)1).
3. Acetic acid, ACS grade.
4. Formic acid, ACS grade.
5. 1 % Formic acid in water, ACS grade.
6. Calcium D-pantothenate, Sigma1) or equivalent CAS 137-08-6.
7. Calcium pantothenate-[13C16, 15N2], IsoSciences1) or equivalent CAS 356786-94-2.
8. α-Amylase, Sigma A31761), from porcine pancreas, about 25 U/mg or equivalent.

PREPARATION OF STANDARD SOLUTIONS

1) **Pantothenic acid (PA) stock solution,** ϱ = 250 μg/ml. Weigh 54,5 mg of calcium pantothenate into a 200 ml volumetric flask (take into account the moisture content given in the supplier's certificate or dry to constant mass at 105 °C) and dilute to volume with water. Store aliquots at −20 °C.

2) **Pantothenic acid intermediate solution,** ϱ = 10 μg/ml. Transfer 1 ml of PA stock solution into a 25 ml volumetric flask and dilute to volume with water. Store aliquots at –20 °C.

3) **Calcium pantothenate-[13C6, 15N2] solution [IS (Internal Standard)] stock solution,** ϱ = 20 μg/ml. Weigh 5,0 mg of calcium pantothenate-[13C6, 15N2] into a 250 ml volumetric flask and dilute to volume with water. Store aliquots at –20 °C.

4) **Solutions for the five-level standard curve**. Transfer appropriate volumes of the PA intermediate solution (10 μg/ml) (4.8.2) into 10 ml volumetric flasks to obtain five different concentrations of PA (0,08 μg/ml, 0,16 μg/ml, 0,32 μg/ml, 0,64 μg/ml and 1,2 μg/ml). Add 500 μl of the IS stock solution

5) (20 μg/ml) (4.8.3) and dilute to volume with water. The concentration of IS in each standard solution is 1 μg/ml. Store aliquots of these solutions at –20 °C for no longer than one month before use.

6) **Ammonium acetate solution,** c = 400 mmol/l, pH = 3,8 (used for sample extraction). Into a 500 ml beaker, add (30,8 ± 0,10) g ammonium acetate. Add

about 300 ml water and stir to dissolve with a magnetic stirrer. Adjust to pH = 3,8 ± 0,1, carefully adding glacial acetic acid (about 150 ml is needed). Transfer into a 1 000 ml volumetric flask and make up to volume with water. This solution is stable for one month at 4 °C.

SAMPLE PREPARATION

1. General

If the product contains starch, add 50 mg α-amylase to the suspensions and incubate for 15 min at 40 °C to decrease viscosity and facilitate handling. Mix liquid samples well to ensure homogeneity and
continue directly to extraction. If the powder sample homogeneity is unknown, assume that it is non homogenous and proceed with 6.1.2.

2. Dry blended powder samples

For dry blended/non-homogenous powder samples, accurately weigh approximately 25,0 g (m1). Add 200,0 g (m2) water at 40 °C before mixing until a homogeneous suspension is obtained. A homogenizer can be used when necessary. Accurately weigh approximately 15,0 g (m3) aliquot of homogenized sample suspension into a 50 ml volumetric flask. Calculate the sample mass (ms is the powder equivalent) using Formula (1):

$$ms = \frac{m1 \times m3}{\mathrm{m1}}$$

Where,
m1 is the mass of sample weighed, in g;
m2 is the mass of water added before mixing, in g;
m3 is the mass of homogenized sample suspension, in g.

3. Wet blended powder samples

For wet blended homogenous powder samples, accurately weigh approximately 2,0 g of sample (ms) into a 50 ml volumetric flask. Add 14 g of water at 40 °C. Mix until a homogeneous suspension is obtained.

4. Liquid samples

For liquid sample samples, accurately weigh approximately 20,0 g (ms) into a 50 ml volumetric flask.

EXTRACTION

Using the prepared sample, add a 25 ml volume of a 0,4 mol/l ammonium acetate solution, pH = 3,8. Dilute the sample extract to volume with water. Add a stir bar and stir for 10 min. Filter a 20 ml portion
through folded paper (Grade 597½). Run chromatographic analysis.

ANALYSIS

Chromatographic analysis

Transfer a 1,0 ml aliquot of the filtrate obtained in 6.2 into a 15 ml polypropylene tube containing 500 µl of the IS stock solution. It is critical to use the same IS solution as used in the preparation of the standard curve. Dilute the solution to 10 ml with water, cap and mix. Filter through a 0,22 µm syringe filter (5.5). Inject into the UHPLC-MS/MS system.

CALCULATION

Calculate for each standard the peak area ratio between PA and IS. Establish a 5-point calibration curve (ranging from 0,16 ng to 2,4 ng on column) by plotting peak area ratio (y-axis) versus PA concentration
(x-axis). Calculate the linear regression. It is recommended to use a weighed regression curve (1/x).

Calculate the slope (S) and the intercept (I) of the calibration curve.
Calculate the PA mass fraction, w, in mg/100 g, using Formula (2):

$$w = \frac{(\mathrm{A} - 1) \times \mathrm{V1} \times \mathrm{V3} \times 100}{\mathrm{S} \times \mathrm{m} \times \mathrm{V2} \times 1000}$$

Where,
A is the peak area ratio PA/IS in the test solution;
I is the intercept of the calibration curve;
S is the slope of the calibration curve;
V1 is the volume of the of sample extract, in ml (= 50);
V2 is the volume of the filtrate pipetted, in ml (= 1);
V3 is the final volume of the of the test solution, in ml (= 10);
m is the mass of the test portion, in g;
100 is the conversion to 100 g basis;
1 000 is the conversion from µg to mg.

Instrument	WATERS XEVO TQ-XS
Detector	Mass Detector

Column	C18 1.7μm, 2.1*100mm
Run time	7 minutes
Column Temperature	30 °C
Flow Rate	0.45 ml/MIN
Injection Volume	2 μl
Mobile phase A	0.1% (v/v) formic acid in water
Mobile phase B	Acetonitrile
Desolvation Temperature	350 °C
Desolvation gas flow	40 1/h
CE	40eV
CV	25V
Source	ESI +ve

GRADIENT PROGRAM:

TIME	Mobile phase A%	Mobile phase B%
0	92	8
2.2	80	20
2.4	50	50
4.0	50	50
4.1	92	8
7.0	92	8

VITAMIN B9 – AOAC 2013.13 (MLV)

APPARATUS

(a) Column—UHPLC HSS T3, 1.8 μm; 2.1×150 mm (Waters Corp., Milford, MA) or equivalent.

(b) Liquid chromatograph—Agilent 1290 Infinity (Agilent Technologies, Santa Clara, CA) or equivalent.

(c) Detector—Agilent 6460 MS in positive electrospray ionization (ESI+) mode operating at unit resolution, or equivalent.

d) Amber glassware—Standard laboratory Class A.

(e) Micropipet—Adjustable (volumes from 2 to 20 μL) and disposable tips.

(f) Micropipet—Adjustable (volumes from 10 to 100 μL) and disposable tips.

(g) Micropipet—Adjustable (volumes from 100 to 1000 μL) and disposable tips.

(h) Multipette® plus—Eppendorf (Hamburg, Germany), or equivalent.

(i) Analytical balance—Precision 0.1 mg.

(j) Homogenizer—Polytron 3100 (Kinematica, Lucerne, Switzerland), or equivalent.

(k) pH meter—Mettler-Toledo (Columbus, OH), or equivalent.

(l) Water bath (up to 90°C)—With magnetic stirrers (Labotech; DWB 16) or equivalent.

(m) Folded filters—S&S 597½ (diameter 185 mm; Whatman, Piscataway, NJ), or equivalent.

(n) Solid phase extraction (SPE) cartridges SAX.—500 mg bed weight, 6 mL column volume, Supelco DSC-SAX (Supelco, St. Louis, MO) or Thermo HyperSep SAX (Thermo Scientific, Waltham, MA).

(o) Disposable plastic syringe—10 mL (Becton Dickinson, Franklin Lakes, NJ), or equivalent.

(p) Disposable plastic syringe—2 mL (Becton Dickinson), or equivalent.

(q) Syringe-driven filter unit—0.22 μm, Millipore Millex GP (Bedford, MA), or equivalent.

(r) HPLC amber vials—2 mL (Agilent Technologies), or equivalent.

REAGENTS

(a) L-Ascorbic acid—Sigma (St. Louis, MO) A4544, or equivalent.

(b) Ammonium acetate p.a.—Merck (Darmstadt, Germany), or equivalent.

(c) DTT—VWR (Radnor, PA), or equivalent.

(d) Disodium hydrogen phosphate powder—VWR, or equivalent.

(e) α-Amylase from porcine pancreas—Type VI, >10 units/mg (Sigma A3176), or equivalent.

(f) α-Amylase from Bacillus subtilis—Approximately 50 units/mg (Fluka 10070; Buchs, Switzerland), or equivalent.

(g) Protease from Streptomyces griseus—Type IV, >3.5 units/mg (Sigma P5147), or equivalent

SOLUTIONS AND STANDARD PREPARATION

(a) Formic acid p.a.—Merck, or equivalent.

(b) Acetic acid glacial p.a.—Merck, or equivalent.

(c) Sodium hydroxide solution—1 M (Merck), or equivalent.

(d) Hydrochloric acid—1 M (Merck), optional

(e) Hydrochloric acid—37% p.a. (Merck), or equivalent.

(f) Ortho-phosphoric acid—85% (Merck), or equivalent.

(g) Folic acid—Schirck Laboratories (Jona, Switzerland) 59-30-3, or equivalent.

(h) (6R, S)-5-Me THF acid calcium salt—Schirck Laboratories 151533-22-1, or equivalent.

(i) [13C5]-Folic acid—Merck, or equivalent.

(j) [13C5] -(6S)-5-Me THF calcium salt—Merck, or equivalent.

PREPARATION OF SOLUTIONS

(a) (1) Mobile phase A.—Acetic acid 0.5% (v/v) in water. Into a 1000 mL volumetric flask, add 5.00 mL acetic acid. Add about 800 mL water. Mix well. Make up to volume with water. This solution remains stable for 1 week at room temperature.
(2) Mobile phase B.—Acetonitrile.
(b) Needle wash solvent—Water–acetonitrile–isopropanol (5+2+3) + 2% (v/v) formic acid. Into a 1000 mL bottle with cap, mix 500 mL water, 200 mL acetonitrile, and 300 mL isopropanol. Add 18 mL formic acid. Mix well. This solution remains stable for 1 month at room temperature. Note: Needle wash solvent is instrument-dependent. Solution to minimize carryover should be studied on each analytical system.
(c) Extraction buffer—Sodium phosphate buffer 100 mmol/L, ascorbic acid 2% (w/v), DTT 0.1% (w/v), pH 4.5. Into a 1000 mL beaker, weigh 14.20 g disodium hydrogen phosphate (Na2HPO4), 20.0 g ascorbic acid, and 1.0 g DTT. Add about 800 mL water, dissolve, and adjust to pH 4.5 with ortho-phosphoric acid 85%. Transfer into a 1000 mL volumetric flask and make up to volume with water. This solution remains stable for 2 weeks at 4°C.
(d) Protease solution—4 mg/mL in water. Into a 100 mL volumetric flask, weigh 400 mg protease. Dissolve and make up to volume with water. Prepare this solution fresh on the day of use.
(e) SPE eluting solution—Acetonitrile–extraction buffer– acetic acid (6+3+1). Into a 250 mL bottle with cap, mix 150 mL acetonitrile, 75 mL extraction buffer, and 25 mL acetic acid using a measuring cylinder. This solution remains stable for 2 weeks at 4°C.
(f) (1) Dissolution solution A.—Sodium hydroxide 0.1 mol/L 5% (v/v)–ethanol 20% (v/v). Into a 100 mL volumetric flask containing about 50 mL water, mix 5.0 mL sodium hydroxide solution 1 mol/L and 20 mL ethanol. Make up to volume with water. This solution remains stable for 2 weeks at 4°C.
(2) Dissolution solution B.—Ammonium acetate 10 mmol/L, ascorbic acid 10% (w/v), DTT 2% (w/v)–methanol (1 + 3). Into a 50 mL beaker, weigh 38.5 mg ammonium acetate, 5.0 g ascorbic acid, and 1.0 g DTT. Add about 40 mL water, dissolve, and make up to volume with water. Mix 50 mL of this solution with 150 mL methanol. This solution remains stable for 2 weeks at 4°C.
(3) Dissolution solution C.—Ascorbic acid 1% (w/v), DTT 0.5% (w/v). Into a 1000 mL volumetric flask, weigh 10.0 g ascorbic acid and 5.0 g DTT. Add about 800 mL water, dissolve, and make up to volume with water. This solution remains stable for 2 weeks at 4°C.
PREAPARATION OF STANDARDS
(a) Folic acid stock standard solution—About 100 μg/mL. Into a 50 mL amber glass volumetric flask, weigh 5.00 ± 0.20 mg folic acid and record the mass to 0.01 mg.

Dissolve and make up to volume with dissolution solution A. Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

(b) 5-Me THF stock standard (approximately 100 μg/mL)— Into a 50 mL amber glass volumetric flask, weigh 5.00 ± 0.20 mg 5-Me THF acid calcium salt and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution B. Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

(c) Standard Mix 1 (intermediate solution, 5000 ng/mL)— Into a 10 mL amber glass volumetric flask, transfer by pipetting the calculated amount of folic acid stock solution and the calculated amount of 5-Me THF (free form) stock solution to obtain an exact final concentration of folic acid and 5-Me THF in its free form of 500 ng/mL. Make up to volume with dissolution solution C. Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

(d) Standard Mix 2 (intermediate solution, 75 ng/mL)—Into a 10 mL amber glass volumetric flask, transfer by pipetting 150 μL of standard Mix 1. Make up to volume with dissolution solution C. Store in aliquots flushed with N2. This solution remains stable for 3 months at –20°C.

(e) [13C5]-Folic acid stock solution (approximately 200 μg/mL)—Into a 10 mL amber glass volumetric flask, weigh 2.00 ± 0.20 mg [13C5]-folic acid and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution A. Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

(f) [13C5] -(6S)-5-Me THF IS stock solution (approximately 200 μg/mL)—Into a 10 mL amber glass volumetric flask, weigh 2.00 ± 0.20 mg [13C5] -(6S)-5-Me THF calcium salt and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution B. Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

(g) IS mix working solution (5000 ng/mL)—Into a 10 mL amber glass volumetric flask, transfer by pipetting the calculated amount of folic acid IS stock solution and the calculated amount of 5-Me THF IS (free form) stock solution to obtain an exact final concentration of folic acid and 5-Me THF IS in its free form of 500 ng/mL. Make up to volume with dissolution solution C.

SAMPLE PREPARATION

Sample reconstitution—Powder samples were reconstituted by dissolving 25 g powder sample and 50 mg α-amylase in 200 g warm water (40°C). The SRM was reconstituted by dissolving 10 g powder and 50 mg α-amylase in 90 g warm water (40°C). The samples were digested at 40°C for 15 min to let the enzyme work

EXTRACTION PROCEDURE

(a) An aliquot of 15 g reconstituted sample or 15 g reconstituted RTF sample was weighed into a 100 mL amber glass volumetric flask.

(b) 40 mL extraction buffer (100 mmol/L phosphate buffer; 2% ascorbic acid; 0.1% DTT; pH 4.5) was added and the flask was then heated at 90°C for 30 min, while stirring.

(c) After cooling to room temperature, 2 mL protease solution (4 mg/mL) was added and incubation was carried out in a water bath at 37°C for 30 min.

(d) After cooling to room temperature, the volume was made up to the mark with water.

(e) After filtration through folded paper filter, 10 mL filtrate was transferred to a 10 mL amber glass volumetric flask and 50 µL of 5 µg/mL IS solution was added.

(f) From this solution, 3 mL was loaded on a SAX cartridge (previously conditioned with 4 mL acetonitrile and equilibrated with 10 mL extraction buffer).

(g) After loading, the cartridge was washed with 6 mL extraction buffer and analytes were then eluted with 4 mL SPE eluting solution into amber glass tubes.

(h) Eluate was then evaporated under controlled temperature at 55°C and nitrogen flow.

(i) Extracts were then reconstituted in 1.5 mL reconstitution solution (H2O, 1% ascorbic acid, 0.5% DTT) and filtered through 0.22 µm membrane into an amber LC vial.

RESULT CALCULATION:

$$W1 = C \times \frac{(m1+m2)\times V1\times V3\times 100}{m1\times m3\times V2\times 1000}$$

where C = concentration in the test solution (ng/mL) of FA or 5-Me THF, calculated using the dedicated calibration curve; m1=mass of the sample weight for slurry, in g (= 25 g); m2 = mass of water weight to prepare the slurry, in g = 200 g); m3 = mass of the test portion, in g (= 15 g); V1=volume of the of sample extract, in mL (= 100 mL); V2=volume of sample loaded on SPE, in mL (= 3.0 mL); V3=volume of the reconstituted sample, in mL (= 1.5 mL); 100 = conversion to 100 g basis; 1000 = conversion from ng to µg.

INSTRUMENT CONDITION

Instrument	WATERS XEVO TQ-XS
Detector	Mass Detector
Column	HSS T3 2.5µm, 2.1*100mm
Run time	10minutes
Column Temperature	40 °C
Flow Rate	0.25 ml
Injection Volume	5 µL- 20 µl
Mobile phase A	Acetic acid 0.5% (v/v) in water.
Mobile phase B	Acetonitrile
Desolvation Temperature	400 °C

CE	30eV
CV	20 V
Source	ESI +ve

GRADIENT PROGRAM

TIME	FLOW (ML/Min)	% A	%B
0.0	0.25	100	0.0
0.50	0.25	100	0.0
0.60	0.25	90	10
2.00	0.25	90	10
5.50	0.25	75	25
5.60	0.25	1.0	99
7.50	0.25	1.0	99
7.60	0.25	100	0.0
10.0	0.25	100	0.0

MRM TRANSITION:

Time Range min	**Analyte**	**Q1**	**Q3**	**Fragment or Voltage V**	**Collision energy, eV**
2.0-5.0	5- Me THF (Q)	460.2	313.1	108	14
2.0-5.0	5- Me THF (q)	460.2	180.0	108	42
2.0-5.0	5- Me THF IS	465.2	313.1	120	15
5.0-8.0	Folic Acid(Q)	442.2	295.1	90	10
5.0-8.0	Folic Acid(q)	442.2	176.0	90	40
5.0-8.0	Folic Acid IS	447.1	295.0	92	10

VITAMIN D – AOAC 2016.05 (MLV)

APPARATUS

(a) Triple-quadrupole mass spectrometer —Triple Quad 6500 (Sciex, Framingham, MA)

or equivalent tandem MS (MS/MS) instrument.

(b) Column — Agilent Eclipse plus C18, 100 × 2.1 mm, 1.8 μm or equivalent

(c) UV spectrophotometer —Digital readout to three decimal places.

(d) Centrifuge tubes —Polypropylene, 15 mL

(e) Boiling tubes —Glass, 60 mL

(f) Water baths —Cold 20°C, hot 70°C.
(g) Disposable syringes —1 mL
(h) Syringe filters —PTFE, 0.2 μm, 13 mm.
(i) Centrifuge —Suitable for 60 mL boiling tubes and 15 mL centrifuge tubes.
(j) Pipets —0.1 ml, 1.0 ml, 5.0 ml adjustable.
(k) Horizontal shaker.
(l) Eppendorf vials —2 mL.
(m) Filter membranes —0.45 μm nylon.
(n) Cryogenic vials —2 mL
(o) Schott bottles —1 L, 100 mL
(p) vials, septa, and caps.

REAGENTS AND CHEMICALS:
(a) Vitamin D2 (Ergocalciferol) —CAS No. 50-14-6, purity: ≥99%.
(b) Vitamin D3 (Cholecalciferol) —CAS No. 67-97-0, purity: ≥99%.
(c) d6-Vitamin D2 — (d6 ergocalciferol), CAS No. 1311259-89-8, enrichment: ≥99%, purity: ≥99%.
(d) d6-Vitamin D3 — (d6 cholecalciferol), CAS No. 118584-54-6, enrichment: ≥99%, purity: ≥99%.
(e) PTAD —Reagent grade (store in desiccator at 2–8°C).
(f) Formic acid —LC–MS grade.
(g) Potassium hydroxide —Reagent grade.
(h) Magnesium chloride anhydrous —Reagent grade.
(i) Pyrogallol —Reagent grade.
(j) Ethanol —LC grade.
(k) Methanol —LC–MS grade.
(l) Isooctane (2,2,4-trimethylpentane) —LC grade.
(m) Acetone —LC grade.
(n) Acetonitrile —LC–MS grade.
(o) Water —Reagent grade (≥18 MΩ).
STANDARD PREPARATION:
Vitamin D is sensitive to light, perform all steps under UV-shielded lighting. If vitamin D3 is exclusively required for analysis, then standards pertaining to vitamin D2 need not be used and vice versa.
(a) Stable isotope-labeled vitamin D2 or vitamin D3 stock standard (SILD2SS or SILD3SS; ~10 μg/mL).
1. Dispense the contents of a 1 mg vial of d6-vitamin D2 or a 1 mg vial of d6-vitamin D3 into separate 100 mL volumetric flasks.
2. Dissolve in ~90 mL ethanol. To promote dissolution, sonicate if necessary. Mix

thoroughly; dilute to volume with ethanol.
3. Immediately dispense aliquots of SILD2SS or SILD3SS (~1.3 mL) into cryogenic vials and freeze at ≤15°C.
a. Stable isotope-labelled internal standard (SILIS; ~1 μg/mL).—Make fresh daily.—
(1) Prepare an adequate volume of SILIS for the daily sample numbers. For every 15 samples (or part thereof) in an analytical run, remove one
1. Pipet 1.0 mL each of SILD2SS and SILD3S into the same 10 mL volumetric flask (use a separate 10 mL volumetric flask for each set of 15 samples). Dilute to volume with acetonitrile and mix thoroughly.
2. Pool all 10 mL volumetric flasks together and mix thoroughly
3. Nonlabeled vitamin D2 or vitamin D3 stock standard (NLD2SS or NLD3SS; ~1 mg/mL)
4. Accurately weigh approximately 50 mg vitamin D2 or vitamin D3 into separate 50 mL volumetric flasks.
5. Dissolve in ~40 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol. Store in a freezer at ≤15°C for a maximum of 3 months.
a. Nonlabeled vitamin D2 or vitamin D3 purity standard (NLD2PS or NLD3PS; ~10 μg/mL) —Make fresh daily
i. Pipet 1.0 mL NLD2SS or NLD3SS into separate 100 mL volumetric flasks. Dilute to volume with ethanol.
6. Measure the absorbance of an aliquot of each solution at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Record the absorbance and calculate the concentration. Use this concentration for making the working standards.
7. Non labeled working standard (NLWS; ~1 μg/mL) —Make fresh daily —Pipet 1.0 mL NLD2PS and 1.0 mL NLD3PS into a single 10 mL volumetric flask. Dilute to volume with acetonitrile.
8. While preparing the solvent/matrix-matched calibration-10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml, follow the below steps
a. Calibration Standard 1: Pipette 250 μl NLWS from 1 μg/ml and 250 μl SILIS from 1 μg/ml into 25 ml vol. flask
b. Calibration Standard 2: Pipette 625 μl NLWS from 1 μg/ml and 250 μl SILIS from 1 μg/ml into 25 ml vol. flask
c. Calibration Standard 3: Pipette 1250 μl NLWS from 1 μg/ml and 250 μl SILIS from 1 μg/ml into 25 ml vol. flask
d. Calibration Standard 4: Pipette 2500 μl NLWS from 1 μg/ml and 250 μl SILIS from 1 μg/ml into 25 ml vol. flask
e. Calibration Standard 4: Pipette 5000 μl NLWS from 1 μg/ml and 250 μl

SILIS from 1 μg/ml into 25 ml vol. flask
f. Then add 5 mL acetonitrile and 75 μL PTAD solution in all the calibrations; shake to mix.
g. Leave the calibration standards in the dark for 5 min.
h. Add 6.25 mL water to each calibration standard and then dilute to volume with acetonitrile; shake to mix.
i. Transfer ~1 mL of each calibration standard to an amber vial ready for analysis.
REAGENT PREPARATION:
1) PTAD solution (10 mg mL-1): - To a 5 mL volumetric flask, add 50 mg PTAD, then add
4 mL acetone, and dissolve; dilute to volume with acetone. Expiry: 1 day.
2) Potassium hydroxide solution (50%, w/v): - Dissolve 100 g potassium hydroxide in 200 mL water. Expiry: 1 month.
3) Ethanolic pyrogallol solution (1%, w/v): - Dissolve 5 g pyrogallol in 500 mL ethanol. Expiry: 1 day.
4) Mobile phase A (formic acid; 0.1%, v/v) :- To 500 mL water, add 0.5 mL formic acid. Expiry: 1 week.
5) Mobile phase B (methanol; 100%, v/v) :- 500 mL methanol, expiry: 1 month
EXTRACTION AND DERIVATIZATION:
1) 1g sample, add 0.5 mL SILIS, add 10 mL ethanolic pyrogallol solution and vortex mixture.
2) Add 2 mL potassium hydroxide solution to the boiling tube; cap and vortex mix.
3) Place the boiling tube in a water bath at 70°C for 1 hr; vortex mixes every 15 min.
4) Place the boiling tube in a water bath at room temperature until cool
5) Add 10 ml isooctane to the boiling tube; cap the boiling tube tightly and place on a horizontal shaker for 10 min.
6) Add 20 mL water to the boiling tube and invert the tube 10 times; place in a centrifuge at 2500 rpm for 15 min
7) Transfer a 5 ml aliquot of the upper isooctane layer into a 15 mL centrifuge tube using a micro pipette, taking care not to transfer any of the lower layer.
8) Add 5 mL water to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 12000 rpm for 5 min.
9) Transfer 4-5 ml upper isooctane layer to a new 15 mL disposable centrifuge tube using a micro pipette, taking care not to transfer any of the lower layer.
10)Add 75 μL PTAD solution to the centrifuge tube; cap and immediately vortex mix.
11)Allow to stand in the dark for 5 min to allow the derivatization reaction to complete.
12)Add 1 mL acetonitrile to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 12000 rpm for 5 min

13)Using a variable volume pipette, transfer 500 µL lower layer into 2 ml centrifuge tube, taking care not to transfer any of the upper layer.
14)Add 167 µL water into 2 ml centrifuge tube; cap and vortex mix.
15)Using a syringe filter, transfer an aliquot from the 2 ml centrifuge tube to an amber vial; then cap.
CALIBRATION CURVE:
Calibration curve shall be prepared either by using matrix matched method or matrix-based method. If matrix matched calibration is being used, use the matrix matched linearity points as follows - 10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml.

The preparation of these calibration points is explained sr.no. 8 under 'Standard Preparation'.
When using the matrix based or procedural standards fortify the non-fortified edible oil blank samples at following levels 25 µg/kg, 50 µg/kg, 100 µg/kg and 200 µg/kg.
1. Calibration Standard 1: Pipette 25 µl NLWS from 1 µg/ml and 500 µl SILIS from 1 µg/ml into 25 ml vol. flask
2. Calibration Standard 2: Pipette 50 µl NLWS from 1 µg/ml and 500 µl SILIS from 1 µg/ml into 25 ml vol. flask
3. Calibration Standard 3: Pipette 100 µl NLWS from 1 µg/ml and 500 µl SILIS from 1 µg/ml into 25 ml vol. flask
4. Calibration Standard 4: Pipette 200 µl NLWS from 1 µg/ml and 500 µl SILIS from 1 µg/ml into 25 ml vol. flask
Each of this matrix-based calibration point is then processed as a 'sample' and steps 1 to
16 mentioned under the title 'Extraction and Derivatization' are carried out. The resulting vials are named as 25 µg/kg, 50 µg/kg, 100 µg/kg and 200 µg/kg procedural calibration points.
QUANTIFICATION AND RESULT CALCULATION:
While selecting the quantification method in commercial software of LC MS/MS vendors, select 'Internal standard' method.
Concentration of unknown is found out using the following formula
Vitamin D (µg/kg) = ((Area of Analyte x IS Concentration)/IS Area) - ± Intercept x DF
Slope
where DF is the Dilution factor of the method
In case of matrix matched calibration, dilution factor as appropriate is selected whereas for matrix-based calibration curve, dilution factor is selected as 1.
The final results obtained using matrix-based calibration include internal standard and recovery correction.

INSTRUMENT CONDITION

Instrument	WATERS XEVO TQ-XS
Detector	Mass Detector
Column	C18 1.7µm, 2.1*100mm
Run time	7 minutes
Column Temperature	40 °C
Flow Rate	0.5 ml
Injection Volume	5 µL- 20 µl
Mobile phase A	0.1% (v/v) formic acid in water
Mobile phase B	Methanol
Desolvation Temperature	500 °C
CE	40eV
CV	20 V
Source	ESI +ve

GRADIENT PROGRAM:

TIME	FLOW (ML/Min)	% A	%B
0.01	0.5	30	70
1	0.5	30	70
3	0.5	00	100
5	0.5	00	100
7	0.5	30	70
10	0.5	30	70

MRM TRANSITION:

		Precursor ion, (m/z)	**Product ion, (m/z)**
Vitamin D3	Analyte quantifier	560.50	298.30 280.10
Vitamin D3-D6	Internal Standard quantifier	566.60	298.20 280.30
VitaminD2	Analyte quantifier	572.50	298.20 298.10
Vitamin D2-D3	Internal Standard quantifier	578.20	298.10 280.20

Limit of Quantification: 25.00 µg/kg

VITAMIN B12 -AOAC 2011.10 (MLV)

APPARATUS AND MATERIALS

a) Instrument- UHPLC with Triple-quadrupole mass spectrometer
b) Column- C18 1.7 μm, 2.1*150 mm or 100 mm
c) Oven—Capable of maintaining temperatures of 95 ± 5 °C and 105 ± 5 °C.
d) PH meter—With calibration buffer.
e) Analytical balance—Capable of weighing 0.00001 g.
f) Beakers—Glass, assorted sizes.
g) Filter paper—Whatman 2V or equivalent.
h) Funnels—suitable to use with filter paper.
i) Disposable syringes —3 mL
j) Syringe filters —PTFE, 0.2 μm, 13 mm.
k) Pipets —Variable volume, 100–1000 μL
l) Eppendorf vials —2 mL.
m) Filter membranes —0.45 μm nylon.
n) Cryogenic vials —2 ML
o) Test tubes
p) LC vials, septa, and caps.

REAGENTS AND STANDARDS

(a) Taka-diastase- Sigma 86247
(b) Sodium acetate anhydrous or sodium acetate trihydrate-ACS.
(c) Milli Q Water
(d) Methanol- LC/MS Grade
(e) Ascorbic acid- Sigma A92902
(f) Pepsin- Sigma P7000
(g) Ammonium Formate - LC/MS Grade
(h) Vitamin B12 (Cyanocobalamine)- CRM PHR1234
(i) Vitamin B12 (Hydroxycobalamine)- CRM PHR3186
(j) Vitamin B12 (Methylcobalamine)- CRM PHR3410

SOLUTION AND STANDARD PREPARATION

All solutions can be scaled up or down for convenience provided good laboratory practices are observed. Solutions can be stored at 2–30 °C in tight, inert containers unless otherwise noted.

(a) Mobile phase A- 20 Mm Ammonium formate in water
(b) Mobile phase B- Methanol
(c) 0.1 M sodium acetate buffer—Dissolve 16.4 g sodium acetate anhydrous or 27.2 g sodium acetate trihydrate in approximately 1800 mL Milli Q. Adjust pH to 4.50

with concentrated acetic acid. Dilute to 2000 mL with laboratory water. Expiration 3 months.

(d) 6% Taka-diastase—Dissolve 0.6 g taka-diastase in 10 mL water. Prepare fresh immediately before use.

(e) Pepsin- 1 mg/ml

(f) Standards—Prepare all standards under UV shielded fluorescent lights and store at 2–8 °C in tightly stoppered volumetric flasks

1. Stock solution (1000ppm):

Accurately weigh 10 mg of the standard and transfer it into a 10 ml amber coloured volumetric flask. Add 300µl of 0.1 N NH4OH to dissolve it & make up the rest of the volume with Milli Q water and vortex for 2 minutes. Store the solution at 4°C in a light protected area.

2. Intermediate Stock solution 1 -ISS 1(100ppm):

Pipette out 1.0 ml of stock solution to a 10 ml amber coloured volumetric flask containing 2 ml of Milli Q water. Make up the rest of the volume with diluent (25% Methanol) and vortex the solution for 2 minutes

3.Intermediate Stock solution 2 -ISS 2(10ppm): Pipette out 1.0 ml of ISS 1 to a 10 ml amber coloured volumetric flask containing 2 ml of Milli Q water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes.

4.Intermediate Stock solution 2 -ISS 3(1ppm): Pipette out 1.0 ml of ISS 2 to a 10 ml amber coloured volumetric flask containing 2ml of Milli Q water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes.

5.Intermediate Stock solution 2 -ISS 4(100ppb): Pipette out 1.0 ml of ISS 3 to a 10 ml amber coloured volumetric flask containing 2 ml of Milli Q water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes.

6. Bracketing standard solution/ Standard stock solution 4: Pipette out 0.5 ml of ISS 4 to a 10 ml amber coloured volumetric flask containing 2 ml of Milli Q water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes.

EXTRACTION PROCEDURE

(a) Sample preparation

(1) Sampling.

Mix all products thoroughly before sampling. Weigh 3 g of sample. Add 25 mL Milli Q and mix until all of the powder dissolves.

Add 1 mL of 6% taka-diastase if samples contain significant levels of starch. Allow taka-diastase to react with samples for at least 30 minutes before continuing with the extraction.

(2) Extraction.Add 30 mL 0.1 M sodium acetate buffer with 100mg ascorbic acid (pH 4.5), adjust PH with acetic acid to each sample and swirl to mix and add additionally 1 ml pepsin in case of almond (1mg/ml)

Heat samples in a 105 °C oven for at least 60 min, but for no more than 120 min. (Oven temperature will drop when the door is opened. Start timing when oven temperature returns to 105 °C.)

After at least 60 min, remove samples from oven and immediately cool in ice bath.

Make up the volume to 100 ml with Milli Q. Mix well.

Filter samples through Whatman 2V filter paper and transfer liquid layer to funnels lined with filter paper

(3) Sample concentration.

For each sample that will be cleaned up and concentrated, insert a 500 mg SPE cartridge onto the stopcock of the vacuum

Condition each cartridge with at least 20 ml Methanol and rinse each cartridge with at least 10 mL laboratory water. Add 80 ml sample

If necessary, apply enough vacuum so that the samples drip steadily through the cartridges. Discard eluant.

After all of the sample filtrate has passed through the cartridge, rinse each cartridge with 10 mL laboratory water and discard eluant. Air-dry each cartridge by pulling a vacuum until no more effluent is observed. Close each stopcock. Collect 10 ml aliquot

Filter an aliquot of each standard and prepared sample through a 0.22 μm syringe filter into an autosampler vial.

RESULT CALCULATION:

$Cp = Ci \times D1 \div SS \times D2 \div V$

where Cp = product concentration in μg/kg; Ci = vitamin B12 concentration of the injected sample preparation extrapolated from standard curve in μg/L; D1= volume of the first dilution in mL (100 mL); ss = sample size in g; D2= volume of the second (final) dilution in mL (10 ml); V = volume of filtrate loaded onto the cartridge in mL (80ml)

INSTRUMENT CONDITION

Instrument	WATERS XEVO TQ-XS
Detector	Mass Detector
Column	C18 1.7μm, 2.1*100mm
Run time	7 minutes
Column Temperature	40 °C
Flow Rate	0.2 ml
Injection Volume	5 μL- 20 μl

Mobile phase A	0.1% (v/v) formic acid in water
Mobile phase B	Methanol
Desolvation Temperature	200 °C
CE	40eV
CV	20 V
Source	ESI +ve

GRADIENT PROGRAM:

TIME	FLOW (ML/Min)	% A	%B
0.00	0.2	90	10
2.00	0.2	90	10
4.00	0.2	10	90
5.00	0.2	90	10
7.00	0.2	90	10

MRM TRANSITION:

		Precursor ion, (m/z)	**Product ion, (m/z)**	**Cone (V)**	**Collision (eV)**
Methylcobalamine	Analyte quantifier	673.1000	147.1300 359.1400 665.5300	10 10 10	38 18 18
Cynacobalamine	Analyte quantifier	678.8000	147.1000 359.2000	20 30	40 25
Hydroxycobalamine	Analyte quantifier	664.7000	147.3000 359.1000	6 2	55 24

Limit of Quantification: 0.5 µg/kg

Annexure -C List of participating laboratories- List of participants

Sl No	Name and Address	Name of the concerned person
1	Department of Food Safety And Quality Control Laboratory CSIR-Central Food Technological Research Institute Cheluvamba Mansion Mysore- 570020	Dr. Usharani.D
2	Nestle India Limited Laboratory Service, NQAC Moga PB No 11, Ludhiana- Ferozepur Road Mogu, Punjab State, India Pin code- 142001 Tel: +911636512041 (Extn: 2041/2023)	Dr. Amrit Kaur
3	Envirocare Labs Pvt.Ltd Enviro House, A7- A8 MIDC Main Road, Wagle Industrial Estate, Thane Maharashtra, India 400604 Tele: +912225838286-89	Dr. Priti Amritkar
4	Assistant Director -Laboratory Eurofins Analytical Services India Pvt. Ltd. 540/1, Doddanekundi Industrial Area 2, Graphite India Road, Hoodi, Whitefield, 560048, Bengaluru, India	Dr. Jyoti Sindhu
5	Head corporate Quality, Eureka Analytical Services Pvt Ltd # 617, AB SQUARE, 5th Main, OMBR LAYOUT Banaswadi, Bengaluru 560043, Karnataka	Dr. Gouri Ray c/o Hemalatha B

Annexure D- Cover letters to participants- if sent can provide

1. **B9 MLV- Nestle Covering Letter**

As per previous communication on BIS study for Folate analysis by AOAC 2013.13 in various matrices, you will be receiving fortified wheat flour samples.
Please find attached the flowchart for simplified overview on method, chemicals &instrument conditions.
You can further refer to AOAC 2013.13 for detailed instructions, sample preparation, chemicals, MRMs & standard preparation.
Analysis & Handling of Sample:-
You will receive 4 packs of homogenized wheat flour; each has been coded.
Please keep the samples intact & at controlled lab temperature & RH conditions.
Please acknowledge when you receive the samples.
One pack can be used for practice before actual reporting of results.
You need to analyse 2 packs on 2 different days in duplicate for study of method performance characteristics i.e. repeatability & intermediate repeatability.
You will have one surplus pack, please keep it with you.
Reporting of Results: Results to be reported as per enclosed format within 15 days of sample receipt.
Results to be reported for practice as well as study samples keeping track of their codes.
Please share the Instrument conditions as well as MRMs.
Please share the chromatograms for standards as well and samples.
Please share the Format with all records to me within 15 days of sample receipt.

2. **B12 MLV- CSIR-CFTRI Covering Letter**
3.

We have dispatched the samples to the labs. Please note that two matrices are sent (a) fortified wheat flour atta (b) Almond. We sent two practice and three samples to obtain enough practice and data point for repeatability due to very low content of B12.
For your reference here are the blue dart details

Sl No	Address	Tracking ID	Samples
1	Nestle India, Dr Amrit Kaur B12 samples	20638233663	Wheat flour – control -2 practice and 3 samples – total = 6 Almonds – 2 practice and 3 samples- total =5
2	Nestle India, Dr Amrit Kaur-	20638237546	Return of Peas and Wheat flour sample 3Kgs- Ground nut powder sample for B9 100 gms-FRK sample

3	Eureka Analytical Services PL Dr Gouri Ray C/O Hemalatha B	20638219836	Wheat flour –control -2 practice and 3 samples – total = 6 Almonds – 2 practice and 3 samples- total =5 Pepsin and taka diastase
4	Envirocare Labs, Dr Priti Amritkar	20638223034	Wheat flour – control -2 practice and 3 samples – total = 6 Almonds – 2 practice and 3 samples- total -5 Return of Oil sample
5	Eurofins Analytical Services, Dr Jyoti,	20638229953	Wheat flour – control -2 practice and 3 samples – total = 6 Almonds – 2 practice and 3 samples- total =5

I would like to discuss the Multi-Lab Validation (MLV) project initiated by BIS for vitamin B12. Thank you for your support to participate in this collaborative study. The project proposal included two methods FSSAI.FR.16.003.2022 and AOAC 2011.10. We have shared earlier a flow chart for FSSAI.FR.16.003.2022. Initial single laboratory validation, homogeneity study and repeatability were not obtained for fortified wheat flour attta based on FSSAI. FR. 16.003.2022 method. While AOAC 2011.10, or infant formula and nutritionals, involves the use of KCN, making it challenging to obtain natural forms of vitamin B12 in meat and almond samples. Thus we have to modify the method to obtain natural bound forms of vitamin B12. A Modified AOAC 2011.10 method is optimised in our laboratory. We need Taka diastase and pepsin enzymes in addition to sodium acetate and C18 cartridges.Please provide the contact person who will analyse and lead the team for further discussion and dispatching of samples.

4. VITAMIN D -MLV- Envirocare

Thank you for participating in the multi-lab validation project of vitamin D analysis in edible oil.
As per our discussion, we have dispatched the following edible oil samples and internal standards as a part of this project, through courier on 16th May, 2024. Will share the dispatch details by tomorrow morning.
1. Practice sample
2. Sample I (actual study sample I)
3. Sample II (actual study sample II)
4. Sample for transport stability
5. Non fortified / blank edible oil sample for matrix based /procedural calibration
6. Separate vials of isotope labelled internal standard of vitamin D2 and D3 – 1 ml of 100

mg/l each.
Sample for transport stability is to be sent back to Envirocare Labs for assessing the stability of oil samples during transport.
Instructions for analysis of edible oil samples-
1. The practice sample is to be analysed in duplicates and results to be reported to Envirocare Labs.
2. You are requested to complete the analysis of practice sample preferably before 27th May.
3. If the results of practice sample are within the satisfactory range, analysis of Study Sample I and Sample II are to be initiated. Each study sample is to be analysed in duplicates on two different days.
4. The range of testing for all the oil samples and reporting template will be shared one mail very soon.

Annexure E: Practice and MLV samples receipt forms-

CSIR-Central Food Technological Research Institute

SAMPLE ACKNOWLEDGEMENT FORM

Name of the Laboratory	
Sample codes received	
No. of. Samples received	
Sample details (*On Arrival)*	
Date & Time of sample received	
Whether packaging satisfactory (Put "√" mark)	YES/NO
Whether seal intact (Put "√" mark)	YES/NO
Whether sample containers condition satisfactory (Put "√" mark)	YES/NO
Comments (if any):	

Note: The samples should be kept in a stability chamber at 27°C in dark and the enzymes should be stored at 2-4°C in a refrigerator.

Name & Designation of Authorized person: Signature, stamp &Date

Annexure F: Reporting templates- format

MLV Study Reporting templates
Determination of Vitamin B12 in Almonds samples

Name of the concerned person in lab	
Name of Laboratory	
Instrument Model and Make**	

Practice samples:-

1. Calibration Curve for Methylcobalamin (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

2. Calibration Curve for Methylcobalamin (CC2)

	Concentration (ng/ml)	**Peak Area* (Quantifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

3. Calibration Curve for Hydroxycobalamin (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

4. Calibration curve for Hydroxycobalamin (CC2)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**

		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

Total Vitamin B12 results **Methylcobalamin results**

	Sample details		**Practice sample result**	
	Code	**Sample wt (g)**	**Methylcobalamin**	**Total Results (Sum) (mcg/100g)**
Practice Sample 1 Dup.1				
Practice Sample 1 Dup.2				
Practice Sample 2 Dup.1				
Practice Sample 2 Dup.2				

*Supporting chromatograms of calibration and samples to be provided with the results
** Instrumental conditions to be provided

MRM Details & Column used	
Methylcobalamin-Qualifier	
Methylcobalamin-Quantifier	
Hydroxycobalamin- Qualifier	
Hydroxycobalamin- Quantifier	
LC column description	

Multi laboratory Validation (MLV) Samples: Raw Data & Results

Name of the concerned person in lab	
Name of Laboratory	
Instrument Model and Make**	

Calibration Curves Day 1

1. **Calibration Curve for Methylcobalamin Qualifier (CC1)**

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

2. Calibration Curve for Methylcobalamin Quantifier (CC2)

	Concentration (ng/ml)	**Peak Area* (Quantifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

3. Calibration Curve for Hydroxycobalamin (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

4. Calibration curve for Hydroxycobalamin (CC2)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

MLV Study samples

Methylcobalamin results

	Sample details		**MLV sample results**		
	Code	**Sample wt (g)**	**Peak area* (Qualifier)**	**Peak area* (Quantifier)**	**Results (mcg/100g)**
MLV Sample 1 Dup.1					
MLV Sample 1 Dup.2					
MLV Sample 2 Dup.1					
MLV Sample 2 Dup.2					

Supporting chromatograms of calibration and samples to be provided with the results

** Instrumental conditions to be provided

Calibration Curves Day 2

1. Calibration Curve for Methylcobalamin Qualifier (CC1)

	Concentration (ng/ml)	Peak Area* (Qualifier)	Regression parameters	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

2. Calibration Curve for Methylcobalamin Quantifier (CC2)

	Concentration (ng/ml)	Peak Area* (Quantifier)	Regression parameters	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

3. Calibration Curve for Hydroxycobalamin (CC1)

	Concentration (ng/ml)	Peak Area* (Qualifier)	Regression parameters	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

4. Calibration curve for Hydroxycobalamin (CC2)

	Concentration (ng/ml)	Peak Area* (Qualifier)	Regression parameters	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

MLV Study samples

Methylcobalamin results

	Sample details		MLV sample results		
	Code	**Sample wt (g)**	**Peak area* (Qualifier)**	**Peak area* (Quantifier)**	**Results (mcg/100g)**
MLV Sample 1 Dup.1					
MLV Sample 1 Dup.2					
MLV Sample 2 Dup.1					
MLV Sample 2 Dup.2					

Limit of Quantification (LOQ)	-
Range of Testing (optional)	
Measurement Uncertainty (optional)	

*Supporting chromatograms of calibration and samples to be provided with the results

** Instrumental conditions to be provided

MLV Study Reporting templates
Determination of Cyanocobalamin in Fortified Wheat Flour

Name of the concerned person in lab	
Name of Laboratory	
Instrument Model and Make**	

Practice samples:-

5. Calibration Curve for Cyanocobalamin (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Cyanocobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

6. Calibration Curve for Cyanocobalamin (CC2)

	Concentration (ng/ml)	**Peak Area* (Quantifier)**	**Regression parameters**	
		Cyanocobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

Cyanocobalamin results

	Sample details		Practice sample result		
	Code	Sample wt (g)	Peak area* (Qualifier)	Peak area* (Quantifier)	Results (mcg/100g)
Practice Sample 1 Dup.1					
Practice Sample 1 Dup.2					
Practice Sample 2 Dup.1					
Practice Sample 2 Dup.2					

*Supporting chromatograms of calibration and samples to be provided with the results
** Instrumental conditions to be provided

MRM Details & Column used	
Cyanocobalamin-Qualifier	
Cyanocobalamin-Quantifier	
LC column description	

Multi laboratory Validation (MLV) Samples: Raw Data & Results

Name of the concerned person in lab	
Name of Laboratory	
Instrument Model and Make**	

Calibration Curves Day 1

5. Calibration Curve for Cyanocobalamin-Qualifier (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Cyanocobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

6. Calibration Curve for Cyanocobalamin Quantifier (CC2)

	Concentration (ng/ml)	**Peak Area* (Quantifier)**	**Regression parameters**	
		Cyanocobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

MLV Study samples
Cyanocobalamin results

	Sample details		MLV sample results		
	Code	Sample wt (g)	Peak area* (Qualifier)	Peak area* (Quantifier)	Results (mcg/100g)
MLV Sample 1 Dup.1					
MLV Sample 1 Dup.2					
MLV Sample 2 Dup.1					
MLV Sample 2 Dup.2					

Supporting chromatograms of calibration and samples to be provided with the results
** Instrumental conditions to be provided

Calibration Curves Day 2

1. **Calibration Curve for Cyanocobalamin Qualifier (CC1)**

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Cyanocobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

2. **Calibration Curve for Cyanocobalamin Quantifier (CC2)**

	Concentration (ng/ml)	**Peak Area* (Quantifier)**	**Regression parameters**	
		Cyanocobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

MLV Study samples

Cyanocobalamin results

	Sample details		MLV sample results		
	Code	Sample wt (g)	Peak area* (Qualifier)	Peak area* (Quantifier)	Results (mcg/100g)
MLV Sample 1 Dup.1					

MLV Sample 1 Dup.2					
MLV Sample 2 Dup.1					
MLV Sample 2 Dup.2					

Limit of Quantification (LOQ)	0.025µg per 100g- 0.25 ug/Kg
Range of Testing (optional)	
Measurement Uncertainty (optional)	

*Supporting chromatograms of calibration and samples to be provided with the results
** Instrumental conditions to be provided

MLV Study Reporting templates
Determination of Vitamin B12 in Almonds samples

Name of the concerned person in lab	
Name of Laboratory	
Instrument Model and Make**	

Practice samples:-

7. Calibration Curve for Methylcobalamin (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

8. Calibration Curve for Methylcobalamin (CC2)

	Concentration (ng/ml)	**Peak Area* (Quantifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

9. Calibration Curve for Hydroxycobalamin (CC1)

	Concentration (ng/ml)	Peak Area* (Qualifier)	Regression parameters	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

10. Calibration curve for Hydroxycobalamin (CC2)

	Concentration (ng/ml)	Peak Area* (Qualifier)	Regression parameters	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

Total Vitamin B12 results **Hydroxycobalamin results**

	Sample details		Practice sample result	
	Code	Sample wt (g)	Hydroxycobalamin	Total Results (Sum) (mcg/100g)
Practice Sample 1 Dup.1				
Practice Sample 1 Dup.2				
Practice Sample 2 Dup.1				
Practice Sample 2 Dup.2				

*Supporting chromatograms of calibration and samples to be provided with the results
** Instrumental conditions to be provided

MRM Details & Column used	
Methylcobalamin-Qualifier	
Methylcobalamin-Quantifier	
Hydroxycobalamin- Qualifier	
Hydroxycobalamin- Quantifier	
LC column description	

Multi laboratory Validation (MLV) Samples: Raw Data & Results

Name of the concerned person in lab	
Name of Laboratory	
Instrument Model and Make**	

Calibration Curves Day 1

7. Calibration Curve for Methylcobalamin Qualifier (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

8. Calibration Curve for Methylcobalamin Quantifier (CC2)

	Concentration (ng/ml)	**Peak Area* (Quantifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

9. Calibration Curve for Hydroxycobalamin (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

10. Calibration curve for Hydroxycobalamin (CC2)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

MLV Study samples

Hydroxycobalamin results

	Sample details		MLV sample results		
	Code	**Sample wt (g)**	**Peak area* (Qualifier)**	**Peak area* (Quantifier)**	**Results (mcg/100g)**
MLV Sample 1 Dup.1					
MLV Sample 1 Dup.2					
MLV Sample 2 Dup.1					
MLV Sample 2 Dup.2					

Supporting chromatograms of calibration and samples to be provided with the results
** Instrumental conditions to be provided

Calibration Curves Day 2

5. Calibration Curve for Methylcobalamin Qualifier (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

6. Calibration Curve for Methylcobalamin Quantifier (CC2)

	Concentration (ng/ml)	**Peak Area* (Quantifier)**	**Regression parameters**	
		Methylcobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

7. Calibration Curve for Hydroxycobalamin (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				

Calib. Level 7 (optional)			

8. Calibration curve for Hydroxycobalamin (CC2)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Regression parameters**	
		Hydroxycobalamin	R2	
Calib. Level 1			Slope:	
Calib. Level 2			Intercept:	
Calib. Level 3			**MRM details**	
Calib. Level 4				
Calib. Level 5				
Calib. Level 6(optional)				
Calib. Level 7 (optional)				

MLV Study samples

Hydroxycobalamin results

	Sample details		**MLV sample results**		
	Code	**Sample wt (g)**	**Peak area* (Qualifier)**	**Peak area* (Quantifier)**	**Results (mcg/100g)**
MLV Sample 1 Dup.1					
MLV Sample 1 Dup.2					
MLV Sample 2 Dup.1					
MLV Sample 2 Dup.2					

Limit of Quantification (LOQ)	-
Range of Testing (optional)	
Measurement Uncertainty (optional)	

*Supporting chromatograms of calibration and samples to be provided with the results

** Instrumental conditions to be provided

MLV Study Reporting templates
Determination of Folates in Fortified Wheat Flour by AOAC 2013.13

Method name:	
Name of the concerned person in lab	
Name of Laboratory	
Instrument Model and Make**	

Practice samples:-

11. Calibration Curve for Folic Acid-Qualifier (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Area (IS)**	**Regression parameters**	
		Folic Acid	**Folic Acid IS**	R2	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3				**MRM details**	
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

12. Calibration Curve for Folic Acid- Quantifier (CC2)

	Concentration (ng/ml)	**Peak Area (Quantifier)**	**Area (IS)**	**Regression parameters**	
		Folic Acid	**Folic Acid IS**	R2	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3				**MRM details**	
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

13. Calibration Curve for 5-Methyl Tetra hydro Folic acid-Qualifier (CC3)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Area (IS)**	**Regression parameters**	
		5 Me- THF	**5 Me- THF IS**	**R2**	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3				**MRM details**	
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

14. Calibration Curve for 5 -Methyl Tetra hydro Folic acid – Quantifier (CC4)

	Concentration (ng/ml)	Peak Area (Quantifier)	Area (IS)	Regression parameters	
		5 Methyl THF	5 Methyl THF	R2	

Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3				**MRM details**	
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

Practice sample results

Folic acid results

	Sample details		**Practice sample result**			
	Code	Sample wt (g)	Peak area* (Qualifier)	Peak area* (Quantifier)	Area (IS)	Results (mcg/100g)
Practice Sample 1 Dup.1						
Practice Sample 1 Dup.2						

5 -Methyl Tetra hydro Folic acid:-

	Sample details		**Practice sample result**			
	Code	Sample wt (g)	Peak area* (Qualifier)	Peak area* (Quantifier)	Area (IS)	Results (mcg/100g)
Practice Sample 1 Dup.1						
Practice Sample 1 Dup.2						

Total Folates results: (Sum of Folic acid +5 -Methyl Tetra hydro Folic acid)

	Sample details		**Practice sample result**		
	Code	**Sample wt (g)**	**Folic acid**	**5 -Methyl THF**	**Total Results (Sum) (mcg/100g)**
Practice Sample 1 Dup.1					
Practice Sample 1 Dup.2					

*Supporting chromatograms of calibration and samples to be provided with the results
** Instrumental conditions to be provided

MRM Details & Column used	
Folic Acid-Qualifier	
Folic Acid-Quantifier	
5 -Methyl Tetra hydro Folic acid – Qualifier	
5 -Methyl Tetra hydro Folic acid – Quantifier	
LC column description	

Multi laboratory Validation (MLV) Samples: Raw Data & Results

Method name:	
Name of the concerned person in lab	
Name of Laboratory	
Instrument Model and Make**	

Calibration Curves Day 1

11. Calibration Curve for Folic Acid-Qualifier (CC1)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Area (IS)**	**Regression parameters**	
		Folic Acid	**Folic Acid IS**	R2	
Calib. Level 1				Slope:	

Calib. Level 2				Intercept:	
Calib. Level 3					
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

12. Calibration Curve for Folic Acid- Quantifier (CC2)

	Concentration (ng/ml)	**Peak Area (Quantifier)**	**Area (IS)**	**Regression parameters**	
		Folic Acid	**Folic Acid IS**	R2	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3					
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

13. Calibration Curve for 5-Methyl Tetra hydro Folic acid-Qualifier (CC3)

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Area (IS)**	**Regression parameters**	
		5 Me- THF	**5 Me- THF IS**	**R2**	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3					
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

14. Calibration Curve for 5 -Methyl Tetra hydro Folic acid – Quantifier (CC4)

	Concentration (ng/ml)	Peak Area (Quantifier)	Area (IS)	Regression parameters	
		5 Methyl THF	5 Methyl THF	R2	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3					
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

MLV Study samples

Sample 1

Folic acid results

	Sample details		**MLV sample results**			
	Code	Sample wt (g)	Peak area* (Qualifier)	Peak area* (Quantifier)	Area (IS)	Results (mcg/100g)
MLV Sample 1 Dup.1						

MLV Sample 1 Dup.2						

5 -Methyl Tetra hydro Folic acid:-

	Sample details		Practice sample result			
	Code	Sample wt (g)	Peak area* (Qualifier)	Peak area* (Quantifier)	Area (IS)	Results (mcg/100g)
MLV Sample 1 Dup.1						
MLV Sample 1 Dup.2						

Total Folates results: (Sum of Folic acid +5 -Methyl Tetra hydro Folic acid)

	Sample details		**Practice sample result**		
	Code	**Sample wt (g)**	**Folic acid**	**5 -Methyl THF**	**Total Results (Sum) (mcg/100g)**
MLV Sample 1 Dup.1					
MLV Sample 1 Dup.2					

Supporting chromatograms of calibration and samples to be provided with the results
** Instrumental conditions to be provided

Calibration Curves Day 2

3. **Calibration Curve for Folic Acid-Qualifier (CC1)**

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Area (IS)**	**Regression parameters**	
		Folic Acid	**Folic Acid IS**	R2	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3					
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

4. **Calibration Curve for Folic Acid- Quantifier (CC2)**

	Concentration (ng/ml)	**Peak Area (Quantifier)**	**Area (IS)**	**Regression parameters**	
		Folic Acid	**Folic Acid IS**	R2	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3					
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

5. **Calibration Curve for 5-Methyl Tetra hydro Folic acid-Qualifier (CC3)**

	Concentration (ng/ml)	**Peak Area* (Qualifier)**	**Area (IS)**	**Regression parameters**

		5 Me- THF	5 Me- THF IS	R2	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3					
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

6. Calibration Curve for 5 -Methyl Tetra hydro Folic acid – Quantifier (CC4)

	Concentration (ng/ml)	Peak Area (Quantifier)	Area (IS)	Regression parameters	
		5 Methyl THF	5 Methyl THF	R2	
Calib. Level 1				Slope:	
Calib. Level 2				Intercept:	
Calib. Level 3					
Calib. Level 4					
Calib. Level 5					
Calib. Level 6(optional)					
Calib. Level 7 (optional)					

MLV Study samples

Sample 2

Folic acid results

	Sample details		MLV sample results			
	Code	Sample wt (g)	Peak area* (Qualifier)	Peak area* (Quantifier)	Area (IS)	Results (mcg/100g)
MLV Sample 2 Dup.1						
MLV Sample 2 Dup.2						

5 -Methyl Tetra hydro Folic acid:-

	Sample details		Practice sample result			
	Code	Sample wt (g)	Peak area* (Qualifier)	Peak area* (Quantifier)	Area (IS)	Results (mcg/100g)
MLV Sample 2 Dup.1						
MLV Sample 2 Dup.2						

Total Folates results: (Sum of Folic acid +5 -Methyl Tetra hydro Folic acid)

	Sample details		Practice sample result		
	Code	**Sample wt (g)**	**Folic acid**	**5 -Methyl THF**	**Total Results (Sum) (mcg/100g)**
MLV Sample 2 Dup.1					
MLV Sample 2 Dup.2					

Limit of Quantification (LOQ)	
Range of Testing (optional)	
Measurement Uncertainty (optional)	

*Supporting chromatograms of calibration and samples to be provided with the results

** Instrumental conditions to be provided

Annexure G: Raw data MLV samples for homogeneity and stability

Table A1: Homogeneity studies of Folic acid in Fortified atta based on AOAC 2013.13 method

HOMOGENEITY WORK SHEET					
Parameter	Folic Acid			**Sample ID**	Wheat flour
Sample ID	**test portion xt,1**	**test portion xt,2**	**Sample average, $x_{t,.}$**	**between-test-portion ranges, w_t**	**Wt^2**
1	12.30	13.26	12.78	0.96	0.92160
2	12.92	14.25	13.59	1.33	1.76890
3	12.80	13.24	13.02	0.44	0.19360
4	12.43	15.64	14.04	3.21	10.30410
5	13.33	12.82	13.08	0.51	0.26010
20	13.72	12.19	12.96	1.53	2.34090
22	14.45	14.09	14.27	0.36	0.12960
43	15.33	13.99	14.66	1.34	1.79560
45	12.10	12.83	12.47	0.73	0.53290
50	14.86	12.57	13.72	2.29	5.24410
Sum of Wt^2	23.4914				
g	10				
X	13.45600				
s_x	0.71153				
s_w	1.08378				
s_s	0.00000				
Sd	1.12000				
0.3 x Sd	0.33600				
Ss $\leq$ 0.3 x Sd	**Yes**				
Assessment	**Passed**				

Table A2: Homogeneity studies of Folic acid in Dried Peas based on AOAC 2013.13 method

HOMOGENEITY WORK SHEET					
Parameter	Folic Acid			**Sample ID**	Peas
Sample ID	**test portion xt,1**	**test portion xt,2**	**Sample average, $x_{t,.}$**	**between-test-portion ranges, w_t**	**Wt^2**
29	125.15	124.98	125.07	0.17	0.02890
34	129.81	127.02	128.42	2.79	7.78410
43	130.26	132.99	131.63	2.73	7.45290
44	126.47	128.6	127.54	2.13	4.53690
42	120.44	119.71	120.08	0.73	0.53290
27	128.26	119.85	124.06	8.41	70.72810
30	130.48	126.69	128.59	3.79	14.36410
32	133.12	127.63	130.38	5.49	30.14010
36	132.1	125.48	128.79	6.62	43.82440
39	129.38	130.76	130.07	1.38	1.90440
Sum of Wt^2	181.2968				
g	10				
X	127.45900				
s_x	3.47174				
s_w	3.01079				
s_s	0.00000				
Sd	3.97000				
0.3 x Sd	1.19100				
Ss ≤ 0.3 x Sd	**Yes**				
Assessment	**Passed**				

Table A3: Homogeneity studies of Folic acid in Peanuts.based on AOAC 2013.13 method

Sample ID	test portion xt,1	test portion xt,2	Sample average, $x_{t,.}$	between-test-portion ranges, w_t	Wt^2
10	10.41	9.92	10.17	0.49	0.24010
16	9.50	11.46	10.48	1.96	3.84160
30	16.31	13.44	14.88	2.87	8.23690
35	14.40	14.38	14.39	0.02	0.00040
41	16.11	9.36	12.74	6.75	45.56250
1	9.80	8.00	8.90	1.80	3.24000
8	9.22	9.60	9.41	0.38	0.14440
17	9.32	12.70	11.01	3.38	11.42440
20	14.47	12.91	13.69	1.56	2.43360
29	13.99	14.28	14.14	0.29	0.08410
Sum of Wt^2	75.2080				
g	10				
X	11.97900				
s_x	2.23367				
s_w	1.93918				
s_S	1.76326				
Sd	1.12000				
0.3 x Sd	0.33600				
Ss $\leq$ 0.3 x Sd	No				
Assessment	Failed				

Table A4: Homogeneity studies of Vitamin D2 and D3 in Vegetable oil based on AOAC 2016.02

HOMOGENEITY WORK SHEET					
Parameter	Vitamin D2			**Sample ID**	MLV Sample 1
Sample ID	**test portion xt,1**	**test portion xt,2**	**Sample average, $x_{t,.}$**	**between-test-portion ranges, w_t**	**Wt^2**
H24D- 01	0.157	0.160	0.16	0.003	0.00001
H24D- 02	0.157	0.156	0.16	0.001	0.00000
H24D- 03	0.155	0.159	0.16	0.004	0.00002
H24D- 04	0.157	0.152	0.15	0.005	0.00003
H24D- 05	0.157	0.154	0.16	0.003	0.00001
H24D- 06	0.156	0.158	0.16	0.002	0.00000
H24D- 07	0.158	0.155	0.16	0.003	0.00001
H24D- 08	0.159	0.163	0.16	0.004	0.00002
H24D- 09	0.164	0.166	0.17	0.002	0.00000
H24D- 10	0.165	0.165	0.17	0.0000	0.00000
Sum of Wt^2	0.0001				
g	10				
X	0.15865				
s_x	0.00377				
s_w	0.00216				
s_S	0.00345				
Sd	0.01600				
0.3 x Sd	0.00480				
Ss ≤ 0.3 x Sd	**Yes**				
Assessment	**Passed**				

Table A5: Homogeneity studies of Vitamin D2 and D3 in Vegetable oil based on AOAC 2016.02

HOMOGENEITY WORK SHEET					
Parameter	Vitamin D2		**Sample ID**		MLV Sample 2
Sample ID	**test portion xt,1**	**test portion xt,2**	**Sample average, $x_{t,.}$**	**between-test-portion ranges, w_t**	**Wt^2**
H24D01- 01	0.098	0.096	0.097	0.002	0.00000
H24D01- 02	0.095	0.096	0.096	0.001	0.00000
H24D01- 03	0.104	0.103	0.104	0.001	0.00000
H24D01- 04	0.102	0.103	0.102	0.001	0.00000
H24D01- 05	0.109	0.109	0.109	0.000	0.00000
H24D01- 06	0.096	0.097	0.096	0.000	0.00000
H24D01- 07	0.096	0.098	0.097	0.002	0.00000
H24D01- 08	0.104	0.105	0.105	0.001	0.00000
H24D01- 09	0.102	0.103	0.103	0.001	0.00000
H24D01- 10	0.096	0.098	0.097	0.002	0.00000
Sum of Wt^2	0.00001				
g	10				
X	0.10054				
s_x	0.00450				
s_w	0.00086				
s_S	0.00446				
Sd	0.01600				
0.3 x Sd	0.00480				
Ss ≤ 0.3 x Sd	**Yes**				
Assessment	**Passed**				

Table A6: Homogeneity studies of Vitamin B12 in Wheat Flour atta based on Modified AOAC 2011.10

HOMOGENEITY WORK SHEET					
Parameter	Vitamin B12			Sample ID	Fortified Atta
Sample ID	**test portion $x_{t,1}$**	**test portion $xt_{,2}$**	**Sample average, $x_{t,.}$**	**between-test-portion ranges, w_t**	**Wt^2**
29	0.344	0.332	0.34	0.01	0.00014
47	0.328	0.328	0.33	0.00	0.00000
73	0.206	0.219	0.21	0.01	0.00017
26	0.251	0.355	0.30	0.10	0.01082
65	0.375	0.348	0.36	0.03	0.00073
6	0.336	0.316	0.33	0.02	0.00040
36	0.286	0.276	0.28	0.01	0.00010
74	0.222	0.213	0.22	0.01	0.00008
1	0.257	0.253	0.26	0.00	0.00002
33	0.252	0.247	0.25	0.01	0.00003
Sum of Wt^2	0.0125				
g	10				
X	0.28720				
s_x	0.05213				
s_w	0.02498				
s_s	0.00104				
Sd	0.05500				
0.3 x Sd	0.01650				
Ss ≤ 0.3 x Sd	**Yes**				
Assessment	**Passed**				

Table A7: Homogeneity studies of Vitamin B12 in Almonds based on Modified AOAC 2011.10

HOMOGENEITY WORK SHEET					
Parameter	Vitamin B12			Sample ID Almonds	
Sample ID	**test portion $x_{t,1}$**	**test portion $xt_{,2}$**	**Sample average, $x_{t,.}$**	**between-test-portion ranges, w_t**	**Wt^2**
113	0.08015	0.10334	0.09	0.02	0.00054
52	0.13913	0.07322	0.11	0.07	0.00434
97	0.09698	0.10553	0.10	0.01	0.00007
78	0.08638	0.41368	0.25	0.33	0.10713
108	0.51509	0.33300	0.42	0.18	0.03316
110	0.52900	0.51500	0.52	0.01	0.00020
114	0.20000	0.37500	0.29	0.18	0.03063
19	0.38333	0.45000	0.42	0.07	0.00444
12	0.39167	0.44167	0.42	0.05	0.00250
90	0.41600	0.42500	0.42	0.01	0.00008
Sum of Wt^2	0.1831				
g	10				
X	0.30366				
s_x	0.15964				
s_w	0.09568				
s_S	0.00220				
Sd	0.05800				
0.3 x Sd	0.01740				
Ss $\leq$ 0.3 x Sd	**Yes**				
Assessment	**Passed**				

Table A8: Stability studies of 5-MeTHF in Dried Peas based on AOAC 2013.13

STABILITY STUDIES WORK SHEET				
Parameter	Vitamin		**Sample ID**	Peas
Week	**Sample ID**	**Replicate 1**	**Replicate 2**	**Sample average**
Initial	29	125.15	124.98	125.07
	34	129.81	127.02	128.42
transit	25	125.43	125.38	125.41
				0.00
final day	35	129.93	137.84	133.89
	38	134.09	136.87	135.48
Overall average	129.65000			
Difference from homogeneity mean	2.10850			
0.3 x Sd	3.21000			
Diffeence ≤ 0.3 x Sd	Yes			
Assessment	**Passed**			

Table A9: Stability studies of Vitamin D2 and D3 in Vegetable oil based on AOAC 2016.02

STABILITY STUDIES WORK SHEET				
Parameter	Vitamin		**Sample ID**	xxx
Week	**Sample ID**	**Replicate 1**	**Replicate 2**	**Sample average**
Initial 21.05.2024	Sample B1_Marico	0.097	0.097	0.097
	Sample B1_Marico	0.092	0.096	0.094
transit 31.05.2024	Eurofins	0.104	0.100	0.102
	CFTRI	0.105	0.101	0.103
final day 31.05.2024	Sample B1_Marico	0.092	0.093	0.093
	Sample B1_Marico	0.094	0.095	0.095
Overall average	0.09717			
Difference from homogeneity mean	0.00337			
0.3 x Sd	0.00480			
Diffeence ≤ 0.3 x Sd	Yes			
Assessment	**Passed**			

Table A10: Stability studies of Vitamin B12 in in Wheat Flour atta based on AOAC 2011.10

STABILITY STUDIES WORK SHEET				
Parameter	Vitamin B12		**Sample**	Fortified Atta
Week	**Sample ID**	**Replicate 1**	**Replicate 2**	**Sample average**
Initial	1	0.26	0.25	0.26
	33	0.25	0.25	0.25
One month	26	0.25	0.36	0.30
	65	0.38	0.35	0.36
final day (five months)	29	0.34	0.33	0.34
	47	0.33	0.33	0.33
Overall average	0.30583			
Difference from homogeneity mean	0.28000			
0.3 x Sd	0.33600			
Diffeence $\leq$ 0.3 x Sd	Yes			
Assessment	**Passed**			

Table A11 Stability studies of Vitamin B12 in Almond based on AOAC 2011.10

STABILITY STUDIES WORK SHEET				
Parameter	Vitamin 12		**Sample**	Amonds
Week	**Sample ID**	**Replicate 1**	**Replicate 2**	**Sample average**
Initial	113	0.08	0.10	0.09
	52	0.14	0.07	0.11
One month	108	0.52	0.33	0.42
	110	0.53	0.52	0.52
final day (Four months)	12	0.39	0.44	0.42
	90	0.42	0.43	0.42
Overall average	0.33019			
Difference from homogeneity mean	0.30366			
0.3 x Sd	0.33600			
Diffeence $\leq$ 0.3 x Sd	Yes			
Assessment	**Passed**			

Annex H: Raw data practice samples- table with real data, SD, RSD, Horrat etc.

Table 01: Method Characteristics of Vitamin B12 in Spirulina using AOAC 2014.02

Vitamin B12 by AOAC 2014.02, Data extension:Spirulina

Method Performance Criteria for Vitamin B12 in Spirulina

Element	Vitamin B12	0.66*22 IF Conc. <0.12 ppm,
Units	mcg/100g	0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	**Result (Native)**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**
14.06.2024 Day 1 - Rep 1	133.63	134.470	0.627	**0.466**	**5.049**	**0.092**	Within Recommended value
14.06.2024 Day 1 - Rep 2	134.87						
14.06.2024 Day 1 - Rep 3	134.36						
14.06.2024 Day 1 - Rep 4	135.02						
15.06.2024 Day 2 - Rep 1	137.74	136.200	1.352	**0.992**	**5.039**	**0.197**	Within Recommended value
15.06.2024 Day 2 - Rep 2	136.93						
15.06.2024 Day 2 - Rep 3	135.08						
15.06.2024 Day 2 - Rep 4	135.05						
20.06.2024 Day 3 - Rep 1	122.35	126.473	4.520	**3.574**	**5.096**	**0.701**	Within Recommended value
20.06.2024 Day 3 - Rep 2	124.92						
20.06.2024 Day 3 - Rep 3	125.72						
20.06.2024 Day 3 - Rep 4	132.9						

Mean	**132.381**
SD	5.076
Reproducibility RSD_R % (Observed	**3.83**
Predicted RSD_R% (Horwitz)	**7.67**
$HORRAT_R$	**0.50**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Table 02: Method Characteristics of Vitamin B12 in Beverages using AOAC 2014.02

Vitamin B12 by AOAC 2014.02, Data extension: Beverrages

Method Performance Criteria for Vitamin B12 in Beverrages

Element	Vitamin B12
Native	~3 mcg/100g
Spiked level mcg/100g	2

0.66*22 IF Conc. <0.12 ppm,
0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	**Result (Native +spiked)**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**	**Native**	**Recovered Values**	**Recovery %**
25.05.2024 Day 1 - Rep 1	5.07	5.053	0.020	**0.389**	**8.273**	**0.047**	Within Recommended value	3.06	2.01	101%
25.05.2024 Day 1 - Rep 2	5.03								1.97	99%
25.05.2024 Day 1 - Rep 3	5.04								1.98	99%
25.05.2024 Day 1 - Rep 4	5.04								1.98	99%
25.05.2024 Day 1 - Rep 5	5.06								2	100%
25.05.2024 Day 1 - Rep 6	5.08								2.02	101%
27.05.2024 Day 2 - Rep 1	5.02	5.007	0.020	**0.393**	**8.285**	**0.047**	Within Recommended value	3	2.02	101%
27.05.2024 Day 2 - Rep 2	5								2	100%
27.05.2024 Day 2 - Rep 3	5.02								2.02	101%
27.05.2024 Day 2 - Rep 4	4.99								1.99	100%
27.05.2024 Day 2 - Rep 5	4.98								1.98	99%
27.05.2024 Day 2 - Rep 6	5.03								2.03	102%

Mean	**5.030**
SD	0.031
Reproducibility RSD_R % (Observed)	**0.61**
Predicted RSD_R% (Horwitz)	**12.54**
$HORRAT_R$	**0.05**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	**Recommended Value**
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 03: Method Characteristics of Vitamin B12 in Fortified Rice kernels using AOAC 2014.02

Vitamin B12 by AOAC 2014.02, Data extension: FRK

Method Performance Criteria for Vitamin B12 in Fortified Rice Kernels

Element	Vitamin B12	0.66*22 IF Conc. <0.12 ppm,
Units	mcg/100g	0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	**Result (Native)**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**
07.08.2024 Day 1 - Rep 1	14.17	13.990	0.164	**1.175**	**7.098**	**0.165**	Within Recommended value
07.08.2024 Day 1 - Rep 2	14.07						
07.08.2024 Day 1 - Rep 3	13.8						
07.08.2024 Day 1 - Rep 4	13.77						
07.08.2024 Day 1 - Rep 5	14.08						
07.08.2024 Day 1 - Rep 6	14.05						
08.08.2024 Day 2 - Rep 1	13.79	13.883	0.125	**0.902**	**7.106**	**0.127**	Within Recommended value
08.08.2024 Day 2 - Rep 2	13.85						
08.08.2024 Day 2 - Rep 3	13.76						
08.08.2024 Day 2 - Rep 4	13.82						
08.08.2024 Day 2 - Rep 5	14.03						
08.08.2024 Day 2 - Rep 6	14.05						

Mean	**13.937**
SD	0.150
Reproducibility RSD_R % (Observed)	**1.08**
Predicted RSD_R% (Horwitz)	**10.76**
$HORRAT_R$	**0.10**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	**Recommended Value**
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 04: Method Characteristics of Vitamin B12 in Fortified Rice kernels (spike) using AOAC 2014.02

Vitamin B12 by AOAC 2014.02, Data extension: FRK

Method Performance Criteria for Vitamin B12 in Fortified Rice Kernels

Element	Vitamin B12
Units	mcg/100g
Spiled level(mcg/100g)	6

0.66*22 IF Conc. <0.12 ppm,
0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	Result (Native)	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	$HORRAT_r$	Remarks $HORRAT_r$<2	Native	Recovered Values	Recovery %
08.08.2024 Day 2 - Rep 1	20.25	19.997	0.250	**1.248**	**6.726**	**0.186**	Within Recommended value	13.88	6.37	106%
08.08.2024 Day 2 - Rep 2	19.9							0.00	6.02	100%
08.08.2024 Day 2 - Rep 3	19.94							0.00	6.06	101%
08.08.2024 Day 2 - Rep 4	20.35							0.00	6.47	108%
08.08.2024 Day 2 - Rep 5	19.71							0.00	5.83	97%
08.08.2024 Day 2 - Rep 6	19.83							0.00	5.95	99%
09.08.2024 Day 2 - Rep 1	20.32	20.085	0.204	**1.015**	**6.722**	**0.151**	Within Recommended value	14.02	6.3	105%
09.08.2024 Day 2 - Rep 2	20.02								6	100%
09.08.2024 Day 2 - Rep 3	19.89								5.87	98%
09.08.2024 Day 2 - Rep 4	20.17								6.15	103%
09.08.2024 Day 2 - Rep 5	20.28								6.26	104%
09.08.2024 Day 2 - Rep 6	19.83								5.81	97%

Mean	**20.041**
SD	0.222
Reproducibility RSD_R % (Observed)	**1.11**
Predicted RSD_R% (Horwitz)	**10.19**
$HORRAT_R$	**0.11**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	Recommended Value
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Vitamin B12 by AOAC 2014.02, Data extension: Breakfast Cereal

Method Performance Criteria for Vitamin B12 in Cereal_Breakfast

Element Vitamin B12

Units mcg/100g

0.66*22 IF Conc. <0.12 ppm,
0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	Result (Native)	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	$HORRAT_r$	Remarks $HORRAT_r$<2
17.06.2019 Day 1 - Rep 1	0.61	0.620	0.014	**2.281**	**11.345**	**0.201**	Within Recommended value
17.06.2019 Day 1 - Rep 2	0.63						
16.07.2019 Day 1 - Rep 1	0.64	0.640	0.000	**0.000**	**11.291**	**0.000**	Within Recommended value
16.07.2019 Day 1 - Rep 2	0.64						
22.07.2019 Day 2 - Rep 1	0.62	0.633	0.013	**1.989**	**11.311**	**0.176**	Within Recommended value
22.07.2019 Day 2 - Rep 2	0.63						
22.07.2019 Day 2 - Rep 3	0.63						
22.07.2019 Day 2 - Rep 4	0.65						
05.08.2019 Day 2 - Rep 1	0.67	0.663	0.010	**1.445**	**11.233**	**0.129**	Within Recommended value
05.08.2019 Day 2 - Rep 2	0.65						
05.08.2019 Day 2 - Rep 3	0.67						
05.08.2019 Day 2 - Rep 4	0.66						

Mean	**0.642**
SD	0.019
Reproducibility RSD_R % (Observed)	**2.96**
Predicted RSD_R% (Horwitz)	**17.10**
$HORRAT_R$	**0.17**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	Recommended Value
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 06: Method Characteristics of Vitamin B12 in vitamin premix using AOAC 2014.02

Vitamin B12 by AOAC 2014.02, Data extension: Premix

Method Performance Criteria for Vitamin B12 in Vitamin Premix

Element	Vitamin B12	
Units	mcg/100g	0.66*22 IF Conc. <0.12 ppm,
Assigned Value	320	0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	Result (Native)	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	$HORRAT_r$	Remarks $HORRAT_r$<2	Recovery
30.09.2023 Day 2 - Rep 1	326	325.500	0.707	**0.217**	**4.420**	**0.049**	Within Recommended value	102%
30.09.2023 Day 2 - Rep 2	325							102%
18.04.2024 Day 3 - Rep 1	329	327.500	2.121	**0.648**	**4.416**	**0.147**	Within Recommended value	103%
18.04.2024 Day 3 - Rep 2	326							102%
29.04.2024 Day 4 - Rep 1	315	314.000	1.414	**0.450**	**4.444**	**0.101**	Within Recommended value	98%
29.04.2024 Day 4 - Rep 2	313							98%
30.05.2024 Day 5 - Rep 1	314	311.000	4.243	**1.364**	**4.451**	**0.307**	Within Recommended value	98%
30.05.2024 Day 5 - Rep 2	308							96%
25.09.2023 Day 1 - Rep 1	313	319.750	9.605	**3.004**	**4.432**	**0.678**	Within Recommended value	98%
25.09.2023 Day 1 - Rep 2	328							103%
25.09.2023 Day 1 - Rep 3	310							97%
25.09.2023 Day 1 - Rep 4	328							103%

Mean	**319.583**
SD	8.017
Reproducibility RSD_R % (Observed)	**2.51**
Predicted RSD_R% (Horwitz)	**6.72**
$HORRAT_R$	**0.37**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	Recommended Value
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 07: Method Characteristics of Vitamin B12 in Nuts (spike level 1) using AOAC 2014.02

Vitamin B12 by AOAC 2014.02, Data extension: Nuts

Method Performance Criteria for Vitamin B12 in Nuts

Element	Vitamin B12
Spiked level mcg/100g	0.12

0.66*22 IF Conc. <0.12 ppm,
0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	**Result (Native +spiked)**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**	**Native**	**Recovered Values**	**Recovery %**
01.06.2023 Day 1 - Rep 1	0.13	0.118	0.010	**8.309**	**14.520**	**0.572**	Within Recommended value		0.13	108%
01.06.2023 Day 1 - Rep 2	0.11								0.11	92%
01.06.2023 Day 1 - Rep 3	0.12								0.12	100%
01.06.2023 Day 1 - Rep 4	0.13								0.13	108%
01.06.2023 Day 1 - Rep 5	0.11								0.11	92%
01.06.2023 Day 1 - Rep 6	0.11								0.11	92%
02.06.2023 Day 2 - Rep 1	0.13	0.120	0.009	**7.454**	**14.526**	**0.513**	Within Recommended value		0.13	108%
02.06.2023 Day 2 - Rep 2	0.12								0.12	100%
02.06.2023 Day 2 - Rep 3	0.13								0.13	108%
02.06.2023 Day 2 - Rep 4	0.11								0.11	92%
02.06.2023 Day 2 - Rep 5	0.11								0.11	92%
02.06.2023 Day 2 - Rep 6	0.12								0.12	100%

Mean	**0.119**
SD	0.009
Reproducibility RSD_R % (Observed)	**7.56**
Predicted RSD_R% (Horwitz)	**22.00**
$HORRAT_R$	**0.34**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	**Recommended Value**
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 08: Method Characteristics of Vitamin B12 in Nuts (spike level 2) using AOAC 2014.02

Vitamin B12 by AOAC 2014.02, Data extension: Nuts

Method Performance Criteria for Vitamin B12 in Nuts

Element	Vitamin B12
Spiked level mcg/100g	0.60

0.66*22 IF Conc. <0.12 ppm,
0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	**Result (Native +spiked)**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**	**Native**	**Recovered Values**	**Recovery %**
07.06.2023 Day 1 - Rep 1	0.55	0.548	0.008	**1.373**	**11.557**	**0.119**	Within Recommended value		0.55	92%
07.06.2023 Day 1 - Rep 2	0.55								0.55	92%
07.06.2023 Day 1 - Rep 3	0.55								0.55	92%
07.06.2023 Day 1 - Rep 4	0.54								0.54	90%
07.06.2023 Day 1 - Rep 5	0.54								0.54	90%
07.06.2023 Day 1 - Rep 6	0.56								0.56	93%
08.06.2023 Day 2 - Rep 1	0.53	0.540	0.020	**3.704**	**11.584**	**0.320**	Within Recommended value		0.53	88%
08.06.2023 Day 2 - Rep 2	0.53								0.53	88%
08.06.2023 Day 2 - Rep 3	0.58								0.58	97%
08.06.2023 Day 2 - Rep 4	0.53								0.53	88%
08.06.2023 Day 2 - Rep 5	0.53								0.53	88%
08.06.2023 Day 2 - Rep 6	0.54								0.54	90%

Mean	**0.544**
SD	0.015
Reproducibility RSD_R % (Observed)	**2.77**
Predicted RSD_R% (Horwitz)	**17.53**
$HORRAT_R$	**0.16**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	**Recommended Value**
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 08: Method Characteristics of Vitamin B12 in Fruits (spike level 1) using AOAC 2014.02

Vitamin B12 by AOAC 2014.02, Data extension: Fruit Juice

Method Performance Criteria for Vitamin B12 in Fruit Juice

Element	Vitamin B12
Spiked level mcg/100g	0.12

0.66*22 IF Conc. <0.12 ppm,
0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	**Result (Native +spiked)**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**	**Native**	**Recovered Values**	**Recovery %**
13.06.2023 Day 1 - Rep 1	0.11	0.110	0.000	**0.000**	**14.520**	**0.000**	Within Recommended value	0	0.11	92%
13.06.2023 Day 1 - Rep 2	0.11								0.11	92%
14.06.2023 Day 2 - Rep 1	0.12	0.117	0.012	**9.910**	**14.520**	**0.683**	Within Recommended value		0.12	100%
14.06.2023 Day 2 - Rep 2	0.12								0.12	100%
14.06.2023 Day 2 - Rep 3	0.11								0.11	92%
14.06.2023 Day 2 - Rep 4	0.11								0.11	92%
14.06.2023 Day 2 - Rep 5	0.12								0.12	100%
14.06.2023 Day 2 - Rep 6	0.13								0.13	108%
14.06.2023 Day 2 - Rep 7	0.13								0.13	108%
14.06.2023 Day 2 - Rep 8	0.10								0.10	83%
14.06.2023 Day 2 - Rep 9	0.10								0.10	83%
14.06.2023 Day 2 - Rep 10	0.13								0.13	108%

Mean	**0.116**
SD	0.011
Reproducibility RSD_R % (Observed)	**9.36**
Predicted RSD_R% (Horwitz)	**22.00**
$HORRAT_R$	**0.43**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	**Recommended Value**
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 09: Method Characteristics of Vitamin B12 in Fruits (spike level 2) using AOAC 2014.02

Method Performance Criteria for Vitamin B12 in Fruit Juice

Element	Vitamin B12
Spiked level mcg/100g	0.60

0.66*22 IF Conc. <0.12 ppm,
0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	**Result (Native +spiked)**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**	**Native**	**Recovered Values**	**Recovery %**
16.06.2023 Day 1 - Rep 1	0.62	0.581	0.029	**5.057**	**11.457**	**0.441**	Within Recommended value	0	0.62	103%
16.06.2023 Day 1 - Rep 2	0.60								0.60	100%
16.06.2023 Day 1 - Rep 3	0.57								0.57	95%
16.06.2023 Day 1 - Rep 4	0.57								0.57	
16.06.2023 Day 1 - Rep 5	0.61								0.61	
16.06.2023 Day 1 - Rep 6	0.53								0.53	88%
16.06.2023 Day 1 - Rep 7	0.62								0.62	103%
16.06.2023 Day 1 - Rep 8	0.55								0.55	92%
16.06.2023 Day 1 - Rep 9	0.57								0.57	95%
16.06.2023 Day 1 - Rep 10	0.55								0.55	92%
16.06.2023 Day 1 - Rep 11	0.60								0.60	100%
16.06.2023 Day 1 - Rep 12	0.58								0.58	97%

Mean	**0.581**
SD	0.029
Reproducibility RSD_R % (Observed)	**5.06**
Predicted RSD_R% (Horwitz)	**17.36**
$HORRAT_R$	**0.29**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	**Recommended Value**
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 10: Method Characteristics of Vitamin B5 in Nuts (spike level 1) using ISO-20639

Pantothenic Acid by ISO-20639:2015, Data extension: Nuts, Spiking at 0.8 mg/100g

Method Performance Criteria for Pantothenic Acid in Nuts (Almonds)

Element Pantothenic Acid

Spike Conc. 0.8 mg/100g

All values in mg/100g

0.66*22 IF Conc. <0.12 ppm, 0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	Result	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	HORRAT T_r	Remarks HORRAT T_r<2
Day 1 - Rep 1	0.90	0.873	0.037	**4.264**	**10.775**	**0.396**	Within Recommended value
Day 1 - Rep 2	0.90						
Day 1 - Rep 3	0.80						
Day 1 - Rep 4	0.88						
Day 1 - Rep 5	0.88						
Day 1 - Rep 6	0.88						
Day 2 - Rep 1	0.88	0.870	0.033	**3.777**	**10.781**	**0.350**	Within Recommended value
Day 2 - Rep 2	0.88						
Day 2 - Rep 3	0.83						
Day 2 - Rep 4	0.89						
Day 2 - Rep 5	0.91						
Day 2 - Rep 6	0.83						

Mean	**0.872**
SD	0.034
Reproducibility	**3.85**
Predicted RSD_R	**16.33**
$HORRAT_R$	**0.24**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm, 2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc.

Criteria as per	Recommended Value
Repeatability (1	$HORRAT_r$ less than 2
Reproducibility	$HORRAT_R$ less than 2

Table 11: Method Characteristics of Vitamin B5 in Nuts (spike level 2) using ISO-20639

Pantothenic Acid by ISO-20639:2015, Data extension: Nuts, Spiking at 4.0 mg/100g

Method Performance Criteria for Pantothenic Acid in Nuts (Almonds)

Element	Pantothenic Acid	0.66*22 IF Conc. <0.12 ppm,
Spike Conc.	4 mg/100g	0.66x2xpower(C,-0.1505) if Conc.
	All values in mg/100g	>0.12ppm

Day - Rep.	Result	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	$HORRAT_r$	Remarks $HORRAT_r$<2
Day 1 - Rep 1	4.160	4.253	0.067	**1.564**	**8.491**	**0.184**	Within Recommended value
Day 1 - Rep 2	4.270						
Day 1 - Rep 3	4.210						
Day 1 - Rep 4	4.310						
Day 1 - Rep 5	4.340						
Day 1 - Rep 6	4.230						
Day 2 - Rep 1	4.100	4.250	0.117	**2.744**	**8.492**	**0.323**	Within Recommended value
Day 2 - Rep 2	4.140						
Day 2 - Rep 3	4.280						
Day 2 - Rep 4	4.250						
Day 2 - Rep 5	4.420						
Day 2 - Rep 6	4.310						
Mean	**4.252**						
SD	0.091						
Reproducibility RSD_R % (Observed)	**2.13**						
Predicted RSD_R% (Horwitz)	**12.87**	22% If Avg. Conc. <0.12 ppm, 2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm					
$HORRAT_R$	**0.17**						
Remarks	Within Recommended value						

Criteria as per 333/2007/EC	Recommended Value
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 12: Method Characteristics of Vitamin B5 in Fruits (spike level 1) using ISO-20639

Pantothenic Acid by ISO-20639:2015, Data extension: Fruit, Spiking at 0.8 mg/100g

Method Performance Criteria for Pantothenic Acid in Fruits(Apple Dices)

Element	Pantothenic Acid	0.66*22 IF Conc. <0.12 ppm,
Spike Conc.	0.8 mg/100g	0.66x2xpower(C,-0.1505) if Conc.
	All values in mg/100g	>0.12ppm

Day - Rep.	Result	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	$HORRAT_r$	Remarks $HORRAT_r$<2
Day 1 - Rep 1	0.86	0.837	0.018	**2.093**	**10.845**	**0.193**	Within Recommended value
Day 1 - Rep 2	0.84						
Day 1 - Rep 3	0.81						
Day 1 - Rep 4	0.85						
Day 1 - Rep 5	0.83						
Day 1 - Rep 6	0.83						
Day 2 - Rep 1	0.79	0.798	0.046	**5.790**	**10.922**	**0.530**	Within Recommended value
Day 2 - Rep 2	0.78						
Day 2 - Rep 3	0.74						
Day 2 - Rep 4	0.88						
Day 2 - Rep 5	0.81						
Day 2 - Rep 6	0.79						

Mean	**0.818**
SD	0.039
Reproducibility RSD_R % (Observed)	**4.76**
Predicted RSD_R% (Horwitz)	**16.49**
$HORRAT_R$	**0.29**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	Recommended Value
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 13: Method Characteristics of Vitamin B5 in Fruits (spike level 2) using ISO-20639

Pantothenic Acid by ISO-20639:2015, Data extension: Fruit, Spiking at 4.0 mg/100g

Method Performance Criteria for Pantothenic Acid in Fruits (Apple Dices)

Element	Pantothenic Acid	0.66*22 IF Conc. <0.12 ppm,
Spike Conc.	4 mg/100g	0.66x2xpower(C,-0.1505) if Conc.
	All values in mg/100g	>0.12ppm

Day - Rep.	Result	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	$HORRAT_r$	Remarks $HORRAT_r$<2
Day 1 - Rep 1	3.950	3.988	0.053	**1.331**	**8.573**	**0.155**	Within Recommended value
Day 1 - Rep 2	3.980						
Day 1 - Rep 3	3.940						
Day 1 - Rep 4	4.080						
Day 1 - Rep 5	4.020						
Day 1 - Rep 6	3.960						
Day 2 - Rep 1	3.920	3.977	0.067	**1.673**	**8.577**	**0.195**	Within Recommended value
Day 2 - Rep 2	3.890						
Day 2 - Rep 3	3.960						
Day 2 - Rep 4	4.020						
Day 2 - Rep 5	4.000						
Day 2 - Rep 6	4.070						

Mean	**3.983**
SD	0.058
Reproducibility RSD_R % (Observed)	**1.45**
Predicted RSD_R% (Horwitz)	**12.99**
$HORRAT_R$	**0.11**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	Recommended Value
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 14: Method Characteristics of Vitamin B5 in cereals using ISO-20639

Pantothenic Acid by ISO-20639:2015, Cereal

Method Performance Criteria for Pantothenic Acid in Cereals

Element Pantothenic Acid

Assigned Value (QC Sample) 2.06

All values in mg/100g

0.66*22 IF Conc. <0.12 ppm,
0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

Day - Rep.	Result 1	Result 2	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	$HORRAT_r$	Remarks $HORRAT_r$<2
29/01/2024	2.08	2.16	2.12	0.06	2.62	9.40	0.28	Within Recommended value
04-02-2024	2.06	2.05	2.055	0.01	0.34	9.48	0.04	
04-08-2024	2	1.92	1.96	0.06	2.95	9.57	0.31	
19/04/2024	2.01	2.03	2.02	0.01	0.70	9.49	0.07	
23/05/2024	1.95	1.95	1.95	0.00	0.00	9.55	0.00	
06-04-2024	2.15	2.11	2.13	0.03	1.34	9.44	0.14	
17/06/2024	2.17	2.07	2.12	0.071	3.42	9.46	0.36	Within Recommended value
07-02-2024	2.1	2.1	2.1	0.00	0.00	9.44	0.00	
19/07/2024	2.16	2.16	2.16	0.00	0.00	9.40	0.00	
26/07/2024	2.14	2.04	2.09	0.071	3.47	9.48	0.37	

Mean	**2.082**
SD	0.076
Reproducibility RSD_R % (Obse	**3.65**
Predicted RSD_R% (Horwitz)	**14.33**
$HORRAT_R$	**0.25**
Remarks	Within Recommended value

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Criteria as per 333/2007/EC	Recommended Value
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 15: Method Characteristics of Vitamin B5 in Beverages using ISO-20639

Pantothenic Acid by ISO-20639:2015, Data extension: Beverrages

Method Performance Criteria for Pantothenic Acid Beverrages

Element	Pantothenic Acid	0.66*22 IF Conc. <0.12 ppm,
Spike Conc.(mg/100g)	5	0.66x2xpower(C,-0.1505) if Conc.
	All values in mg/100g	>0.12ppm

Day - Rep.	Result (spike +Native)	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	$HORRAT_r$	Remarks $HORRAT_r$<2	Native	Recovered Values	Recovery %
24.11.2016 Day 1 - Rep 1	12.27	12.200	0.099	**0.811**	**7.246**	**0.112**	Within Recommended value	7.04	5.23	105%
24.11.2016 Day 1 - Rep 2	12.13								5.09	102%
25.11.2016 Day 2 - Rep 1	12.18	12.203	0.138	**1.132**	**7.245**	**0.156**		6.94	5.24	105%
25.11.2016 Day 2 - Rep 2	12.34								5.4	108%
25.11.2016 Day 2 - Rep 3	12.27							6.81	5.46	109%
25.11.2016 Day 2 - Rep 4	12.02								5.21	104%
02/12/2016 Day 3 - Rep 1	11.71	11.530	0.255	**2.208**	**7.307**	**0.302**	Within Recommended value	6.64	5.07	101%
02/12/2016 Day 3 - Rep 2	11.35								4.71	94%
03/12/2016 Day 4 - Rep 1	11.98	11.845	0.191	**1.612**	**7.278**	**0.221**		6.94	5.04	101%
03/12/2016 Day 4 - Rep 2	11.71								4.77	95%
05/12/2016 Day 5 - Rep 1	12.19	12.070	0.170	**1.406**	**7.257**	**0.194**		7.09	5.1	102%
05/12/2016 Day 5 - Rep 2	11.95								4.86	97%
Mean	**12.008**									
SD	0.292									
Reproducibility RSD_R % (Observed)	**2.43**									
Predicted RSD_R% (Horwitz)	**11.00**	22% If Avg. Conc. <0.12 ppm, 2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm								
$HORRAT_R$	**0.22**									
Remarks	Within Recommended value									

Criteria as per 333/2007/EC	Recommended Value
Repeatability (RSD_r)	$HORRAT_r$ less than 2
Reproducibility (RSD_R)	$HORRAT_R$ less than 2

Table 16: Method Characteristics of Vitamin B12 in Fortified wheat atta (spike level 1) based on Modified AOAC 2011.10

Method Performance Criteria for Cyanocobalamin in Fortified Wheat flour atta										
Element	Cyanocobalamin									
Spike Conc.	0.5 mcg/kg				0.66*22 IF Conc. <0.12 ppm,					
	All values in mcg/kg				0.66x2xpower(C,-0.1505) if Conc. >0.12ppm					
Day - Rep.	**Result**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**	**Native value**	**Recovered Values**	**Recovery %**
Day 1 - Rep 1	0.55	0.540	0.011	**2.078**	**11.583**	**0.179**	Within Recommended value	0	0.55	110%
Day 1 - Rep 2	0.55							0.00	0.55	110%
Day 1 - Rep 3	0.53							0	0.53	106%
Day 1 - Rep 4	0.53							0	0.53	106%
Day 1 - Rep 5	0.55							0	0.55	110%
Day 1 - Rep 6	0.53							0	0.53	106%
Day 2 - Rep 1	0.47	0.430	0.027	**6.277**	**11.986**	**0.524**	Within Recommended value	0	0.47	94%
Day 2 - Rep 2	0.44							0.00	0.44	88%
Day 2 - Rep 3	0.44							0	0.44	88%
Day 2 - Rep 4	0.40							0	0.4	80%
Day 2 - Rep 5	0.40							0	0.4	80%
Day 2 - Rep 6	0.43							0	0.43	86%
Mean	**0.485**									
SD	0.061									
Reproducibility RSD_R % (Observ	**12.50**									
Predicted RSD_R% (Horwitz)	**17.84**	2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm								
$HORRAT_R$	**0.70**									
Remarks	Within Recommended value									
Criteria as per 333/2007/EC	**Recommended Value**									
Repeatability (RSD_r)	$HORRAT_r$ less than 2									
Reproducibility (RSD_R)	$HORRAT_R$ less than 2									

Table 17: Method Characteristics of Vitamin B12 in Fortified wheat atta (spike level 2) based on Modified AOAC 2011.10

Method Performance Criteria for Cyanocobalamin in Wheat flour atta										
Element	Cyanocobalamin									
Spike Conc.	5 mcg/kg				0.66*22 IF Conc. <0.12 ppm,					
	All values in mcg/kg				0.66x2xpower(C,-0.1505) if Conc. >0.12ppm					
Day - Rep.	**Result**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**	**Native value**	**Recovered Values**	**Recovery %**
Day 1 - Rep 1	4.740	4.577	0.092	**2.019**	**8.398**	**0.240**	Within Recommended value	0	4.74	95%
Day 1 - Rep 2	4.604							0.00	4.60	92%
Day 1 - Rep 3	4.600							0	4.6	92%
Day 1 - Rep 4	4.510							0	4.51	90%
Day 1 - Rep 5	4.510							0	4.51	90%
Day 1 - Rep 6	4.500							0	4.5	90%
Day 2 - Rep 1	4.040	3.883	0.084	**2.172**	**8.608**	**0.252**	Within Recommended value	0	4.04	81%
Day 2 - Rep 2	3.818							0.00	3.81	76%
Day 2 - Rep 3	3.810							0	3.81	76%
Day 2 - Rep 4	3.850							0	3.85	77%
Day 2 - Rep 5	3.900							0	3.9	78%
Day 2 - Rep 6	3.880							0	3.88	78%
Mean	**4.230**									
SD	0.372									
Reproducibility RSD_R % (Observed)	**8.80**									
Predicted RSD_R% (Horwitz)	**12.88**	2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm								
$HORRAT_R$	**0.68**									
Remarks	Within Recommended value									

Table 18: Method Characteristics of Vitamin B12 in Almonds based on Modified AOAC 2011.10

Method Performance Criteria for Methylcobalamin in Almonds							
Element	Methylcobalamin				0.66*22 IF Conc. <0.12 ppm,		
LOQ	0.05 mcg/100g				0.66x2xpower(C,-0.1505) if Conc.		
	All values in mcg/100g				>0.12ppm		
Day - Rep.	**Result**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**
Day 1 - Rep 1	0.20	0.192	0.006	**3.359**	**13.539**	**0.248**	Within Recommended value
Day 1 - Rep 2	0.19						
Day 1 - Rep 3	0.19						
Day 1 - Rep 4	0.19						
Day 1 - Rep 5	0.19						
Day 1 - Rep 6	0.19						
Day 2 - Rep 1	0.18	0.181	0.006	**3.534**	**13.655**	**0.259**	Within Recommended value
Day 2 - Rep 2	0.19						
Day 2 - Rep 3	0.19						
Day 2 - Rep 4	0.18						
Day 2 - Rep 5	0.17						
Day 2 - Rep 6	0.17						
Mean	**0.186**						
SD	0.008						
Reproducibility RSD_R % (Observed)	**4.42**						
Predicted RSD_R% (Horwitz)	**20.60**	2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm					
$HORRAT_R$	**0.21**						
Remarks	Within Recommended value						

Table 19: Method Characteristics of Vitamin B9 in Fortified wheat atta (based on AOAC 2013.13).

Folic Acid by AOAC 2013.13, Data extension: Wheat flour							
Method Performance Criteria for Folic Acid in Wheat flour							
Element		**Folic Acid**					
						0.66*22 IF Conc. <0.12 ppm,	
		All values in mcg/100g				**0.66x2xpower(C,-0.1505) if Conc. >0.12ppm**	
Lab ID	**Result**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**
Lab 1-Day 1	14.904	15.715	2.122	13.500	6.975	1.936	Within Recommended value
Lab 1-Day 1	19.467						
Lab 2-Day 1	16.5						
Lab 2-Day 1	16.4						
Lab 3-Day 1	12.85						
Lab 3-Day 1	14.17						
Lab 4-Day 1	14.07						
Lab 4-Day 1	13.7						
Lab 1-Day 2	14.631	15.976	2.020	12.642	6.958	1.817	Within Recommended value
Lab 1-Day 2	18.543						
Lab 1-Day 2	15.402						
Lab 1-Day 2	16.077						
Lab 2-Day 2	16.1						
Lab 2-Day 2	15.1						
Lab 3-Day 2	12.83						
Lab 3-Day 2	11.65						
Lab 4-Day 2	16.32						
Lab 4-Day 2	17.32						
Lab 2-Day 3	16	16.842	2.269	**13.471**	**6.902**	**1.952**	Within Recommended value
Lab 2-Day 3	16.3						
Lab 1-Day 1	19.605						
Lab 1-Day 1	19.278						
Lab 4-Day 3	13.54						
Lab 4-Day 3	16.33						
Mean		**15.712**					
SD		2.129					
Reproducibility RSD_R % (Observed)		**13.55**					
Predicted RSD_R% (Horwitz)		**10.57**	22% If Avg. Conc. <0.12 ppm, 2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm				
$HORRAT_R$		**1.28**					
Remarks		Within Recommended value					

Table 20: Method Characteristics of Vitamin B9 in Dried Peas (based on AOAC 2013.13).

Method Performance Criteria for Folic Acid in Vegetables								
Element		**Folic Acid**				**0.66*22 IF Conc. <0.12 ppm,**		
						0.66x2xpower(C,-0.1505) if Conc. >0.12ppm		
		All values in mcg/100g						
Lab ID	**Sample Code**	**Result**	**Mean**	**SD**	**Repeatability RSD% (Observed)**	**Predicted RSD_r% (Horwitz)**	**$HORRAT_r$**	**Remarks $HORRAT_r$<2**
Lab 1-Day 1	19	99.22	122.772	10.468	8.526	5.119	1.666	Within Recommended value
Lab 1-Day 1	19	99.22						
Lab 2-Day 1	17	118.31						
Lab 2-Day 1	17	118.31						
Lab 3-Day 1	27	128.26						
Lab 3-Day 1	27	119.85						
Lab 3-Day 1	30	130.48						
Lab 3-Day 1	30	126.69						
Lab 3-Day 1	32	133.12						
Lab 3-Day 1	32	127.63						
Lab 3-Day 1	36	132.10						
Lab 3-Day 1	36	125.48						
Lab 3-Day 1	39	129.38						
Lab 3-Day 1	39	130.76						
Lab 4-Day 1	13	117.53						
Lab 4-Day 1	13	121.85						
Lab 5-Day 1	11	136.60						
Lab 5-Day 1	11	130.58						
Lab 1-Day 2	19	95.52	116.338	9.143	7.859	5.160	1.523	Within Recommended value
Lab 1-Day 2	19	115.35						
Lab 2-Day 2	22	117.20						
Lab 2-Day 2	22	119.15						
Lab 3-Day 2	25	125.43						
Lab 3-Day 2	25	125.38						
Lab 4-Day 2	18	122.63						
Lab 4-Day 2	18	116.92						
Lab 5-Day 2	6	110.76						
Lab 5-Day 2	6	120.04						
Lab 5-Day 2	16	107.92						
Lab 5-Day 2	16	109.57						
Lab 5-Day 2	21	104.59						
Lab 5-Day 2	21	103.00						
Lab 5-Day 2	11	120.13						
Lab 5-Day 2	11	128.14						
Mean		**119.620**						
SD		10.635						
Reproducibility RSD_R % (Observed)		**8.89**						
Predicted RSD_R% (Horwitz)		**7.79**	22% If Avg. Conc. <0.12 ppm, 2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm					
$HORRAT_R$		**1.14**						
Remarks		Within Recommended value						

Table 21: Method Characteristics of Vitamin B9 in Dried Peas (based on AOAC 2013.13).

Folic Acid by AOAC 2013.13, Data extension: Peanuts

Method Performance Criteria for Folic Acid in Nuts

Element: Folic Acid

All values in mcg/100g

0.66*22 IF Conc. <0.12 ppm,
0.66x2xpower(C,-0.1505) if Conc. >0.12ppm

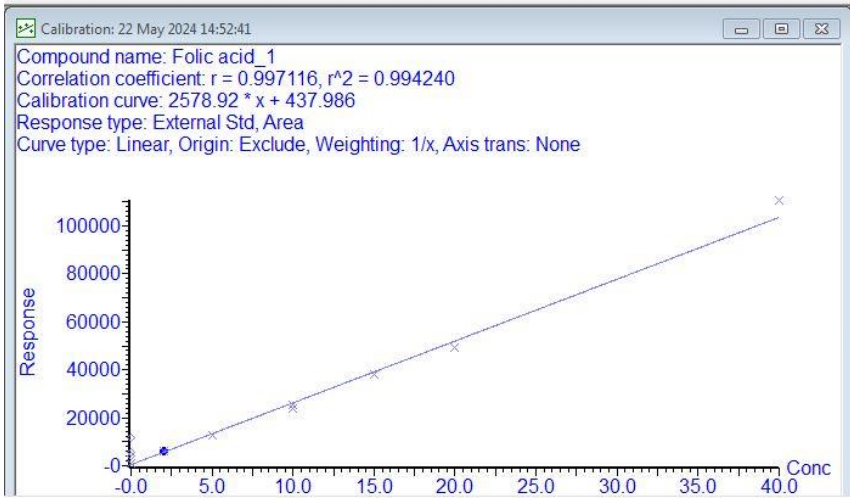
Lab ID	Sample Code	Result	Mean	SD	Repeatability RSD% (Observed)	Predicted RSD_r% (Horwitz)	$HORRAT_r$	Remarks $HORRAT_r$<2
Lab 1-Day 1	19	3.67	22.118	32.540	147.123	6.625	22.207	Fail
Lab 1-Day 1	19	3.59						
Lab 1-Day 1	27	3.99						
Lab 1-Day 1	27	3.75						
Lab 2-Day 1	15	84.14						
Lab 2-Day 1	15	83.09						
Lab 3-Day 1	4	10.96						
Lab 3-Day 1	4	9.26						
Lab 5-Day 1	26	8.79						
Lab 5-Day 1	26	9.93						
Lab 1-Day 2	19	4.00	33.186	35.501	106.976	6.233	17.164	Fail
Lab 1-Day 2	19	3.99						
Lab 2-Day 2	27	85.30						
Lab 2-Day 2	27	83.16						
Lab 3-Day 2	39	13.30						
Lab 3-Day 2	39	9.36						
Lab 5-Day 2	18	8.40						
Lab 5-Day 2	18	7.86						
Mean		**24.253**						
SD		32.948						
Reproducibility RSD_R % (Observed)		**135.85**						
Predicted RSD_R% (Horwitz)		**9.90**						
$HORRAT_R$		**13.72**						
Remarks		Fail						

22% If Avg. Conc. <0.12 ppm,
2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm

Annex I: Graphical representation of data

Figure 1: Wheat flour atta folic acid Qualifier Day 1

	#	Name	Sample Text	ID	Type	Std. Conc	RT	Area	Response	Conc.	%Rec	%Dev	Formul...	Inj. Vol	Vial
1	1	Lin_B9_0805024_14	LS1_2	LS1_2	Standard	2.000	3.90	3123.457	6246.914	2.252	112.6	12.6		1.000	1:A,2
2	2	Lin_B9_0805024_15	LS2_5	LS2_5	Standard	5.000	3.90	6324.975	12649.950	4.735	94.7	-5.3		1.000	1:A,3
3	3	Lin_B9_0805024_16	LS3_10	LS3_10	Standard	10.000	3.90	12568.204	25136.408	9.577	95.8	-4.2		1.000	1:A,4
4	4	Lin_B9_0805024_17	LS4_15	LS4_15	Standard	15.000	3.90	19126.785	38253.570	14.663	97.8	-2.2		1.000	1:A,5
5	5	Lin_B9_0805024_18	LS5_20	LS5_20	Standard	20.000	3.90	24601.439	49202.878	18.909	94.5	-5.5		1.000	1:A,6
6	6	Lin_B9_0805024_19	LS6_40	LS6_40	Standard	40.000	3.90	55341.445	110682.890	42.748	106.9	6.9		1.000	1:A,7
7	7	Lin_B9_0805024_20	Blank_06	Blank_06	Standard	0.000	3.89	388.800	777.600	0.132				1.000	1:C,1
8	8	Lin_B9_0805024_21	BSS_Std_10	BSS_Std_10	Standard	10.000	3.91	11971.774	23943.548	9.114	91.1	-8.9		1.000	1:A,4
9	9	Lin_B9_0805024_22	Blank_07	Blank_07	Solvent		3.90	504.078	1008.156	0.221				1.000	1:C,1
10	10	Lin_B9_0805024_23	Blank_07	Blank_08	Solvent		3.91	475.503	951.006	0.199				1.000	1:C,1
11	11	Lin_B9_0805024_24	ReagentBlank	ReagentBlank	Blank		3.91	1460.589	2921.178	0.963				1.000	1:D,1
12	12	Lin_B9_0805024_25	QCcontrol1	QCcontrol1	QC	0.000	3.90	2906.440	5812.880	2.084				1.000	1:D,2
13	13	Lin_B9_0805024_26	QCcontrol2	QCcontrol2	QC	0.000	3.90	5792.289	11584.578	4.322				1.000	1:D,3
14	14	Lin_B9_0805024_27	Wheat_Control	Wheat_Control	QC	0.000	3.91	1910.425	3820.850	1.312				1.000	1:D,4
15	15	Lin_B9_0805024_28	Sample_17	Sample_17	Analyte		3.90	8877.055	17754.110	6.714				1.000	1:D,5
16	16	Lin_B9_0805024_29	Sample_17_D	Sample_17_D	Analyte		3.90	8194.554	16389.108	6.185				1.000	1:D,6
17	17	Lin_B9_0805024_30	Sample_23	Sample_23	Analyte		3.91	6990.586	13981.172	5.251				1.000	1:D,7
18	18	Lin_B9_0805024_31	Sample_23_D	Sample_23_D	Analyte		3.91	8500.635	17001.270	6.423				1.000	1:D,8



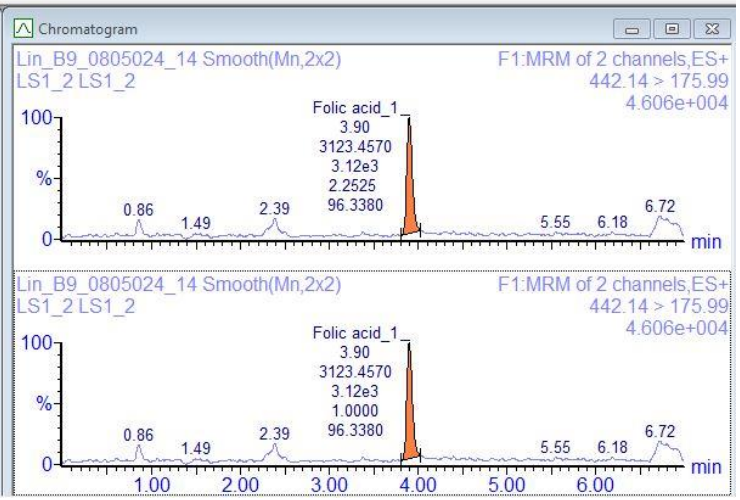


Figure 3: Dried peas 5-METHF Quantfier Day 2

	#	Name	Sample Text	ID	Type	Std. Conc	RT	Area	Response	Conc.	%Rec	%Dev	Formul...	Inj. Vol	Vial
1	1	Pre_B9_2805024_14	LS1_2	LS1_2	Standard	2.000	2.46	92766.000	116615.484	1.699	85.0	-15.0		1.000	1:A,2
2	2	Pre_B9_2805024_15	LS2_5	LS2_5	Standard	5.000	2.47	151888.016	190050.157	4.714	94.3	-5.7		1.000	1:A,3
3	3	Pre_B9_2805024_16	LS3_10	LS3_10	Standard	10.000	2.46	267567.094	334271.196	10.635	106.4	6.4		1.000	1:A,4
4	4	Pre_B9_2805024_17	LS4_15	LS4_15	Standard	15.000	2.47	365333.500	458481.664	15.735	104.9	4.9		1.000	1:A,5
5	5	Pre_B9_2805024_18	LS5_20	LS5_20	Standard	20.000	2.46	461365.375	577069.563	20.604	103.0	3.0		1.000	1:A,6
6	6	Pre_B9_2805024_19	LS6_40	LS6_40	Standard	40.000	2.46	789105.688	983397.969	37.286	93.2	-6.8		1.000	1:A,7
7	7	Pre_B9_2805024_20	Blank_06	Blank_06	Solvent		2.46	763.751	1577.076					1.000	1:C,1
8	8	Pre_B9_2805024_21	BSS_Std_10	BSS_Std_10	Standard	10.000	2.47	280448.031	351086.484	11.326	113.3	13.3		1.000	1:A,4
9	9	Pre_B9_2805024_22	Blank_06	Blank_06	Solvent		2.46	673.862	906.758					1.000	1:C,1
10	10	Pre_B9_2805024_23	Blank_07	Blank_07	Solvent		2.46	618.073	1034.899					1.000	1:C,1
11	11	Pre_B9_2805024_24	QC	QC	QC	0.000	2.46	129702.938	161482.264	3.541				1.000	1:B,1
12	12	Pre_B9_2805024_25	QC1	QC1	QC	0.000	2.46	131165.844	163894.936	3.640				1.000	1:B,1
13	13	Pre_B9_2805024_26	Blank_08	Blank_08	Solvent		2.46	683.589	1381.607					1.000	1:C,1
14	14	Pre_B9_2805024_27	Peas_09_A	Peas_09_A	Analyte		2.47	605984.875	759545.969	28.096				1.000	1:B,2
15	15	Pre_B9_2805024_28	Peas_09_B	Peas_09_B	Analyte		2.46	655316.063	822970.344	30.700				1.000	1:B,3
16	16	Pre_B9_2805024_29	Peas_19_A	Peas_19_A	Analyte		2.47	676688.938	850781.844	31.841				1.000	1:B,4
17	17	Pre_B9_2805024_30	Peas_19_B	Peas_19_B	Analyte		2.46	807438.500	1011729.156	38.449				1.000	1:B,5
18	18	Pre_B9_2805024_31	Blank_09	Blank_09	Analyte		2.46	728.645	995.608					1.000	1:C,1
19	19	Pre_B9_2805024_32	Peas_09_AO	Peas_09_AO	Analyte		2.47	524898.625	654907.945	23.800				1.000	1:C,2
20	20	Pre_B9_2805024_33	Peas_09_BO	Peas_09_BO	Analyte		2.46	539629.688	677710.813	24.736				1.000	1:C,3
21	21	Pre_B9_2805024_34	Peas_19_AO	Peas_19_AO	Analyte		2.46	642006.188	804716.063	29.950				1.000	1:C,4
22	22	Pre_B9_2805024_35	Peas_19_BO	Peas_19_BO	Analyte		2.47	611685.938	769512.532	28.505				1.000	1:C,5

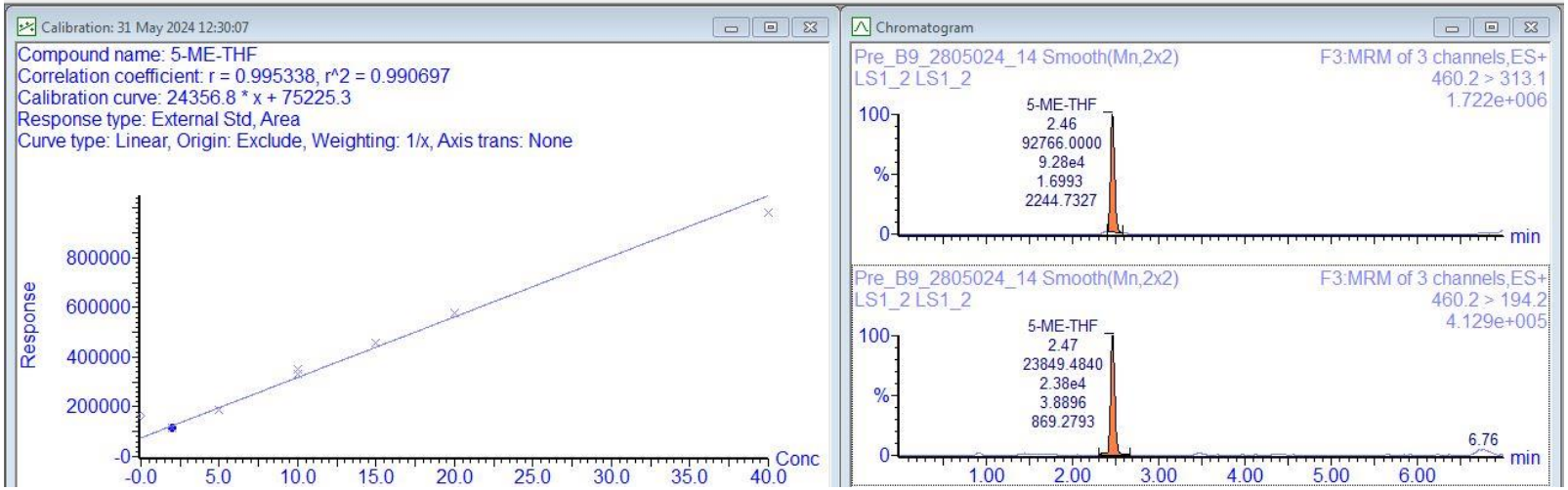


Figure 4: Vitamin D in Vegetabale oils Day 2

	#	Name	Sample Text	ID	Type	Std. Conc	RT	Area	Response	Conc.	%Rec	%Dev	Formul...	Inj. Vol	Vial
8	8	Lin_D2_D3_3007024_27	Blank_11	Blank_11	Solvent		4.74	64.227	64.227					1.000	1:A,1
9	9	Lin_D2_D3_3007024_28	Sample 1D1	Sample 1D1	Analyte		4.66	1002.196	1002.196					1.000	1:B,7
10	10	Lin_D2_D3_3007024_29	Sample 1D2	Sample 1D2	Analyte		4.67	867.130	867.130					1.000	1:B,8
11	11	Lin_D2_D3_3007024_30	Sample 2D1	Sample 2D1	Analyte		4.82	2196.003	2196.003					1.000	1:C,1
12	12	Lin_D2_D3_3007024_31	Sample 2D2	Sample 2D2	Analyte		4.81	1378.650	1378.650					1.000	1:C,2
13	13	Lin_D2_D3_3007024_32	Blank_13	Blank_13	Solvent		4.73	216.397	216.397					1.000	1:A,1
14	14	Lin_D2_D3_3007024_33	SS4_Std_1	SS4_Std_1	Standard	1.000	4.65	12433.261	12433.261	0.975	97.5	-2.5		1.000	1:A,2
15	15	Lin_D2_D3_3007024_34	SS4_Std_10	SS4_Std_10	Standard	10.000	4.65	124396.391	124396.391	134.192	1341.9	1241.9		1.000	1:A,4
16	16	Lin_D2_D3_3007024_35	Blank_11	Blank_11	Solvent		4.64	362.340	362.340					1.000	1:A,1
17	17	Lin_D2_D3_3007024_36	Blank_12	Blank_12	Solvent		4.62	31.370	31.370					1.000	1:A,1
18	18	Lin_D2_D3_3007024_37	Practice_1	Practice_1	Analyte		4.66	908.103	908.103					1.000	1:C,3
19	19	Lin_D2_D3_3007024_38	Practice_2	Practice_2	Analyte		4.65	520.650	520.650					1.000	1:C,4

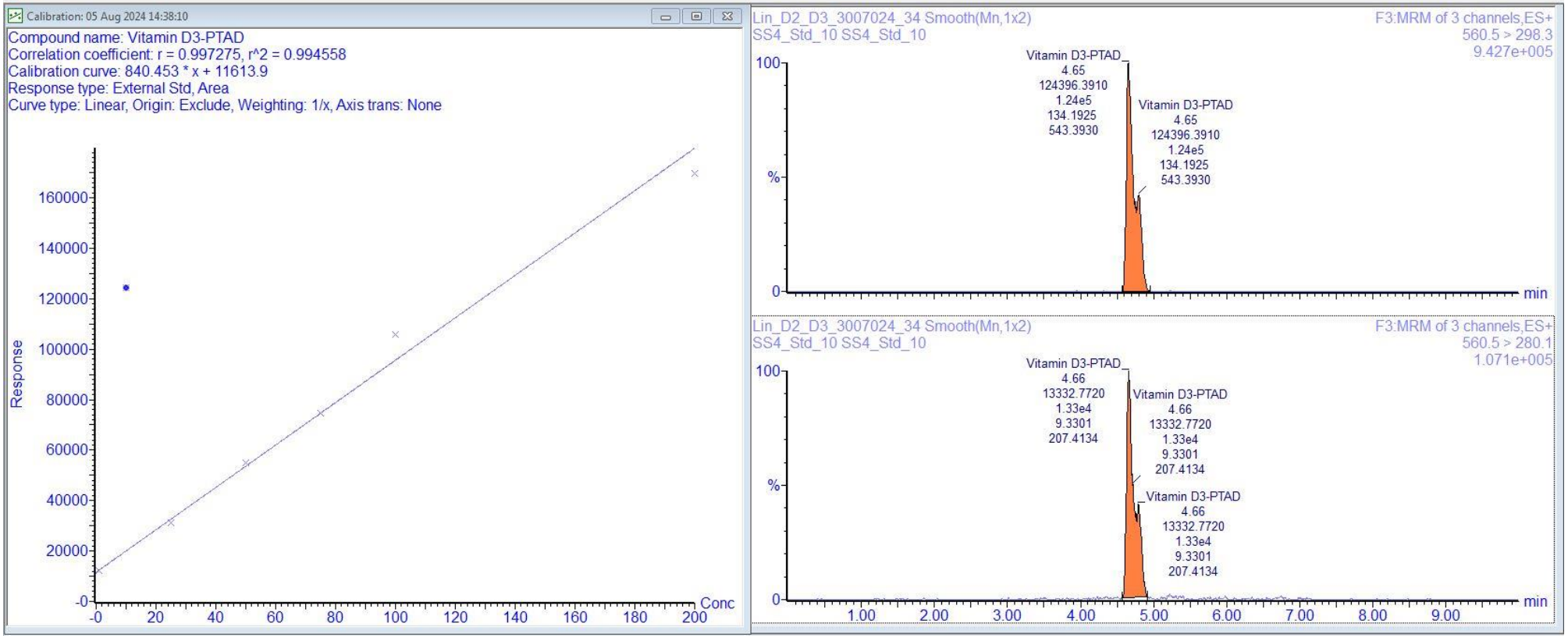
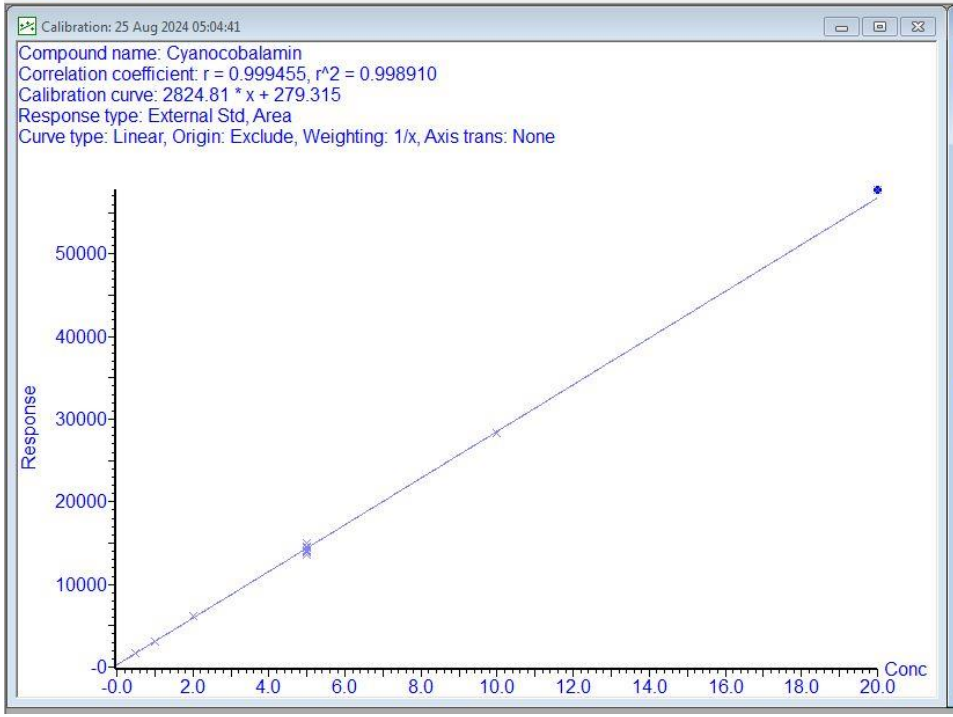


Figure 5: Cyanocobalamin in Wheat flour atta based on Modified AOAC 2011.10

	#	Name	Sample Text	ID	Type	Std. Conc	RT	Area	Response	Conc.	%Rec	%Dev	Formul...	Inj. Vol	Vial
14	14	Accu_B12_250824_14	LS3_2	LS3_2	Standard	2.000	1.68	6103.639	6103.639	2.062	103.1	3.1		1.000	1:A,5
15	15	Accu_B12_250824_15	LS4_5	LS4_5	Standard	5.000	1.68	14967.653	14967.653	5.200	104.0	4.0		1.000	1:A,6
16	16	Accu_B12_250824_16	LS5_10	LS5_10	Standard	10.000	1.68	28277.975	28277.975	9.912	99.1	-0.9		1.000	1:A,7
17	17	Accu_B12_250824_17	LS6_20	LS6_20	Standard	20.000	1.68	57753.074	57753.074	20.346	101.7	1.7		1.000	1:A,8
18	18	Accu_B12_250824_18	Blank_05	Blank_05	Solvent		1.72	41.078	41.078					1.000	1:A,1
19	19	Accu_B12_250824_19	Blank_06	Blank_06	Solvent									1.000	1:A,1
20	20	Accu_B12_250824_20	Control spike_0..5	Control spike_0..5	Recovery	0.000	1.68	16168.225	16168.225	5.625				1.000	1:B,3
21	21	Accu_B12_250824_21	Control spike_0..5	Control spike_0..5	Recovery	0.000	1.68	15271.111	15271.111	5.307				1.000	1:B,3
22	22	Accu_B12_250824_22	Control spike_0..5	Control spike_0..5	Recovery	0.000	1.68	15267.098	15267.098	5.306				1.000	1:B,3
23	23	Accu_B12_250824_23	Control spike_0..5	Control spike_0..5	Recovery	0.000	1.68	14274.582	14274.582	4.954				1.000	1:B,3
24	24	Accu_B12_250824_24	Control spike_0..5	Control spike_0..5	Recovery	0.000	1.68	14025.944	14025.944	4.866				1.000	1:B,3
25	25	Accu_B12_250824_25	Control spike_0..5	Control spike_0..5	Recovery	0.000	1.68	15218.015	15218.015	5.288				1.000	1:B,3



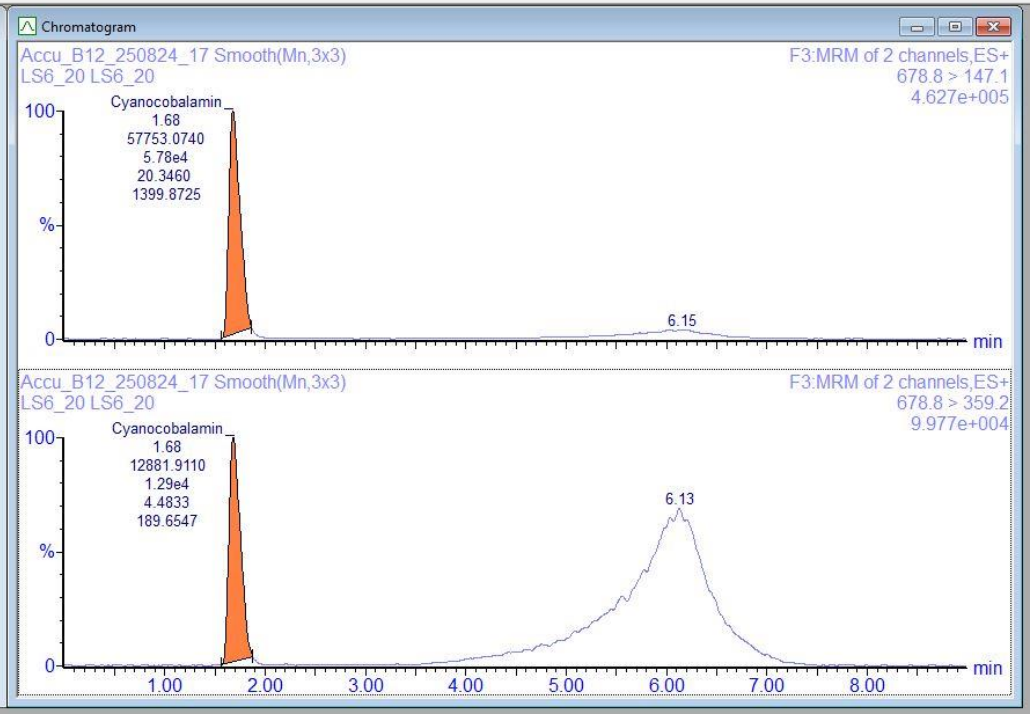
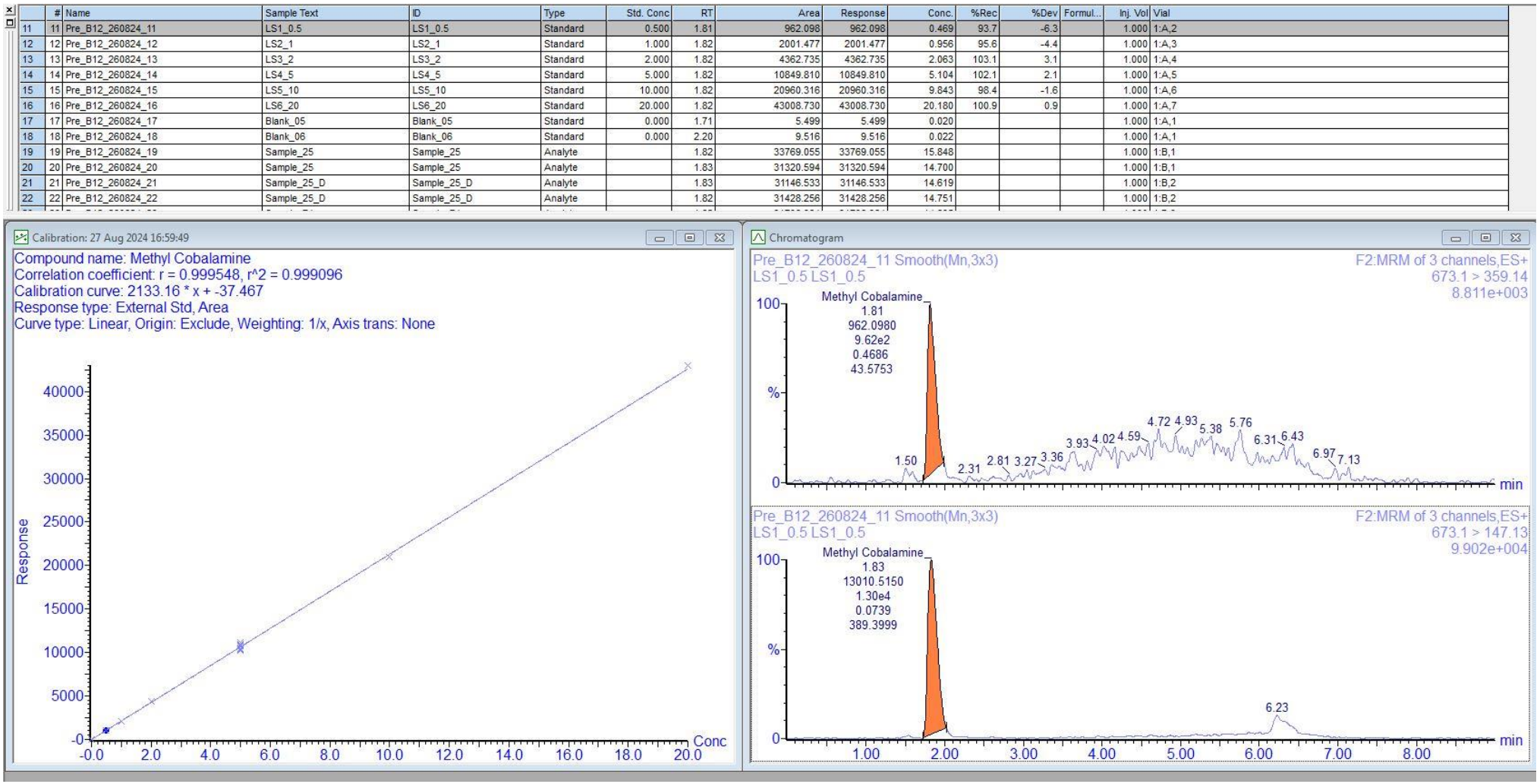


Figure 5: Methylcobalamin in Almonds based on Modified AOAC 2011.10



	#	Name	Sample Text	ID	Type	Std. Conc	RT	Area	Response	Conc.	%Rec	%Dev	Formul...	Inj. Vol	Vial
11	11	Pre_B12_260824_11	LS1_0.5	LS1_0.5	Standard	0.500	1.81	962.098	962.098	0.469	93.7	-6.3		1.000	1:A,2
12	12	Pre_B12_260824_12	LS2_1	LS2_1	Standard	1.000	1.82	2001.477	2001.477	0.956	95.6	-4.4		1.000	1:A,3
13	13	Pre_B12_260824_13	LS3_2	LS3_2	Standard	2.000	1.82	4362.735	4362.735	2.063	103.1	3.1		1.000	1:A,4
14	14	Pre_B12_260824_14	LS4_5	LS4_5	Standard	5.000	1.82	10849.810	10849.810	5.104	102.1	2.1		1.000	1:A,5
15	15	Pre_B12_260824_15	LS5_10	LS5_10	Standard	10.000	1.82	20960.316	20960.316	9.843	98.4	-1.6		1.000	1:A,6
16	16	Pre_B12_260824_16	LS6_20	LS6_20	Standard	20.000	1.82	43008.730	43008.730	20.180	100.9	0.9		1.000	1:A,7
17	17	Pre_B12_260824_17	Blank_05	Blank_05	Standard	0.000	1.71	5.499	5.499	0.020				1.000	1:A,1
18	18	Pre_B12_260824_18	Blank_06	Blank_06	Standard	0.000	2.20	9.516	9.516	0.022				1.000	1:A,1
19	19	Pre_B12_260824_19	Sample_25	Sample_25	Analyte		1.82	33769.055	33769.055	15.848				1.000	1:B,1
20	20	Pre_B12_260824_20	Sample_25	Sample_25	Analyte		1.83	31320.594	31320.594	14.700				1.000	1:B,1
21	21	Pre_B12_260824_21	Sample_25_D	Sample_25_D	Analyte		1.83	31146.533	31146.533	14.619				1.000	1:B,2
22	22	Pre_B12_260824_22	Sample_25_D	Sample_25_D	Analyte		1.82	31428.256	31428.256	14.751				1.000	1:B,2