MELAMINE IN FISH FEED

PROFICIENCY TESTING PROGRAM

APLAC T069

FINAL REPORT

26 August 2009

CONTENTS

Summary of Results

1. The proficiency testing program (APLAC T069) is aimed at evaluating the testing capability of participants on the quantitative analysis of melamine in fish feed.

2. A total of 52 laboratories from 20 economies registered to participate in the program and 48 of them returned results to the organizer.

3. Consensus mean and performance standard deviation were respectively determined by robust statistic and Horwitz equation. Z-score was used to assess the performance of participants in this program.

4. Summary of participants' results:

Data submitted	:	48
Data for consensus mean	:	47
Median	:	2.40 mg/kg
Consensus mean	:	2.53 mg/kg
Range	:	0.768-24.1 mg/kg
Between-laboratory variation	:	23.5%

5. Summary of participants' z-scores:

Performance	Number of participants (Percent)
$\|z\| \leq 2$	35 (74.4%)
$2 < \|z\| < 3$	2 (4.4%)
$\|z\| \geq 3$	10 (21.3%)
Total:	47 (100%)

1. Introduction

1.1. Melamine (1,3,5-triazine–2,4,6-triamine, or $C_3H_6N_6$) was first synthesized in 1834 and had later been found to have extensive uses in the industry. With high nitrogen content in the melamine molecule, the chemical could be unethically added to food products in order to increase the apparent protein content. Conventional analytical methods such as the Kjeldahl test which measures only the amount of nitrogen in samples and correlates the values as the protein content. As such, the nitrogen measurements can be misled by spiking samples with nitrogen-rich compounds like melamine. Although the toxicity of melamine is not regarded as very high, the recent incidents of melamine tainted pet food have resulted in the fatal renal failure of cats and dogs in the United States[8.1] . These incidents have strengthened our understanding on the adverse effects of melamine.

1.2. New analytical methods such as GC-MS, LC-MS/MS, CE and ELISA for the quantification of melamine in food matrices have been rapidly developed and published in the literature. However, there were very limited external quality assessments available to validate the measurement capability of those reported methods. Hence, a proficiency testing program (APLAC T069) on the quantitative analysis of melamine in fish feed was proposed to the Asia-Pacific Laboratory Accreditation Co-operation (APLAC) PT Committee in February 2009. The program was organised by Hong Kong Accreditation Service (HKAS) in collaboration with the Hong Kong Government Laboratory (HKGL), under the auspices of APLAC.

1.3. The program commenced in December 2008 and completed in September 2009 (TABLE I). A total of 52 laboratories from 20 economies registered to participate in the program and 48 of them returned their results on or before the deadline (TABLE II). Each participant was confidentially assigned with a unique laboratory code (1 to 48).

2. Objectives

2.1. The main objective of this program is to assess the performance of participants in the analysis of melamine in fish feed through inter-laboratory comparison.

2.2. Test results from participants are evaluated according to the statistical techniques outlined in ISO13528:2005[8.2] "Statistical methods for use in proficiency testing by inter-laboratory comparisons" and in the international harmonized protocol for the proficiency testing of analytical chemistry laboratories[8.3]. Z-score was used as a numerical indicator to assess individual participant's proficiency relative to the others in the program.

3. Test Material

3.1. About 10 kg of dry fish feed (in pellet form) were purchased from a local pet food shop and the sample was confirmed to contain an amount of melamine appropriate for this PT program. The pellets samples were ground to powder by a high speed blender and sieved through 200 μm sieves. All batches of fine powder (≤ 200 μm) collected were mixed in a 3-dimensional mixer for more than 7 days. The thoroughness of mixing was monitored by taking portions from the sample and checking them for homogeneity. The homogenized fine powder was then packed into cleaned and nitrogen-flush amber glass bottles, each in 15 g portion and more than 200 sample bottles were finally prepared. The sample bottles were disinfected by γ-irradiation at a dose of about 1 kGy dose and stored at room temperature before shipment.

3.2. Homogeneity (performed in February 2009) and stability (in March and June 2009) of the samples were determined in accordance with the procedures stipulated in APPENDICES I and II. Results indicated the samples were suitable to be used as the test materials for the program.

3.3. Samples together with "Instructions to Accreditation Bodies", "Receipt Form", "Instructions to Participating Laboratories", and "Result Proforma" were sent to participating accreditation bodies (AB) in May 2009. A specimen copy of these instructions was given in APPENDICES III to VII.

3.4. All AB were requested to follow the procedures stipulated in "Instructions to Accreditation Bodies" and to distribute the sample(s) and the necessary documents to their nominated laboratories as soon as possible.

3.5. Each participant was provided with one bottle of 15 g of dry fish feed powder in the program.

4. Test Required

4.1. Participants were required to determine, preferably in triplicate, the concentration of melamine in the test sample by their appropriate validated methods. Participants were asked to report the results in mg/kg to three significant figures or three decimal places, whichever was appropriate.

4.2. Participants were also asked to report the expanded measurement uncertainty and technical information of the method used.

4.3. All analytical results and information were reported in the Result Proforma provided to the participants.

5. Statistical Evaluation

5.1. Participants' test results

5.1.1. Test results received from participants are presented in TABLE III. Their methodologies and techniques employed as well as their accreditation status are tabulated in TABLES IV to V.

5.2. Performance evaluation

5.2.1. Performance of each participant was evaluated by means of a performance index, z-score, which is calculated as:

$$z = \frac{x_i - m}{S}$$

where x_i = reported mean of individual participant
m = consensus value
S = standard deviation (in mg/kg) estimated from Horwitz equation

5.2.2. *z*-Score is interpreted as follows:

(a)	$\|z\| \leq 2$	:	Satisfactory
(b)	$2 < \|z\| < 3$	:	Questionable
(c)	$\|z\| \geq 3$	:	Unsatisfactory

5.2.3. Participants having *z*-score ≥ 3 or ≤ -3 are regarded as outliers and they may wish to conduct an investigation and to take any necessary actions. Participants having z-score(s) in the range $2 < |z| < 3$ are encouraged to review their results.

5.3. An interim report containing participants' data and the respective z-score results was issued to participants and their ABs on 14 July 2009. Participants were requested to check the correctness of their submitted analytical data and to inform the organizers of any mistakes found on or before 24 July 2009 prior to the issuance of Final Report.

6. Results and Discussions

6.1. Statistics of participants' results:

Data submitted	:	48
*Data for consensus mean	:	47
Median	:	2.40 mg/kg
Consensus mean	:	2.53 mg/kg
Range	:	0.768-24.1 mg/kg
Between-laboratory variation	:	23.5%

*One laboratory (Lab. #28) reported a value less than the reporting limit

Owing to the five extremely large results (6.554 to 24.1 mg/kg), between-laboratory variation (= 23.5%) is higher than that of the Horwitz SD at 13.8%.

6.2. z-Scores of all participants are tabulated in TABLE VI and displayed graphically in CHART I. The overall performance, in terms of z-score achievement, of participants is summarized as follow:

Performance	Number of Participant (Percent)
$\|z\| \leq 2$	35 (74.4%)
$2 < \|z\| < 3$	2 (4.4%)
$\|z\| \geq 3$	10 (21.3%)
Total:	47 (100%)

In brief, 21.3% or 10 participants (Lab. #7, 12, 15, 23, 27, 29, 38, 39, 45 and 47) were identified as having reported unsatisfactory results.

6.3. Overview of participants' results (TABLES IV and V):

6.3.1. Thirty participants used LC-MS/MS for their determination, while ten used GC-MS or GC-MS/MS. Four participants used LC-UV and the remaining four used ELISA technique.

6.3.2. For all the LC methods, majority (24 out of 34) of the users employed hydrophilic interaction liquid chromatography (HILIC) columns and the remaining used either C_8, C_{18} or amino columns for their chromatographic separation. Retention of polar molecules such as melamine in HILIC columns improves with the increased polarity of organic solvent in the mobile phase. Such chromatographic separation principle was exercised well as all LC users in this program used the mobile phase containing high proportion of acetonitrile in acidic medium. Whereas relatively polar GC columns such as DB-5 were used by most of the participants using GC-MS.

6.3.3. Melamine was mainly extracted by acetonitrile or acidified acetonitrile or acid (diluted formic acid, acetic acid, trichloroacetic acid) with shaking, sonication and agitation. Twenty nine participants used solid phase extraction (cationic exchange columns) as the sample clean up procedure. Duration of extraction varied from 1 min to 3.5 hrs. Owing to the high polarity of melamine, recovery from fish feed sample using the above extraction techniques was generally good (thirty eight participants claimed it was greater than 80%). However, three participants (Lab. #2, 28 and 43) presented a recovery of 30 to 40% although their extraction techniques were similar to other participants.

6.3.4. Unsatisfactory performance was found in all types of analytical methods being used. Two (out 30) from LC-MS/MS; two (out of 4) from LC-UV; four (out of 10) from GC-MS; one (out of four) from ELISA. The results showed that LC-MS/MS performed better in this program. Furthermore, all the seventeen participants (Lab. #1-5, 9-11, 18, 20, 21, 32-34, 36, 37 and 46) who employed isotope internal standard in their LC-MS/MS methods obtained $|z|$-score ≤ 2.

6.3.5. Amongst the twenty two participants who claimed their methods were accredited, twenty (91%) achieved satisfactory and two (9%) had unsatisfactory z-scores. For the non-accredited participants, twelve out of nineteen (63%) achieved satisfactory and seven (37%) had unsatisfactory z-scores.

6.3.6. Large variation of reported MU was found amongst participants (TABLE III) and the average reported relative MU was 15.8%. About 15% of the participants did not report the MU. This would indicate that some participants may not understand the proper procedure for estimation of the measurement uncertainties associated with their testing and therefore have reported an unreasonable MU estimate. Although MU data were

not used in the performance assessment of participants in the program, participants should be capable of properly estimating MU because ISO/IEC 17025 requires testing laboratories to have and apply procedure for estimating uncertainty of measurement. This proficiency testing programme suggests that training in estimation of MU in testing is needed and that ABs should place special emphasis on assessing the procedure of testing laboratories for estimating MU and ensure that they are competent to perform MU estimation. ABs are strongly encouraged to take note of, in addition to the test results, the MU reported by their nominated laboratories and take appropriate follow-up actions, if necessary.

7. Remarks

7.1. Contributions and efforts from all participating ABs and laboratories to this program are gratefully noted. Special thanks are also extended to the APLAC PT Committee, HKAS Executive and the Government Chemist of Government Laboratory for their support to the program.

7.2. If participants have further comments or disagreement with the assessment, please contact the organizers as follows:

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8. References

8.1. RLM Dobson et al. Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs. *Toxicol Sci.,* **2008**, 106: 251–262.

8.2. ISO 13528:2005. Statistical methods for use in proficiency testing by interlaboratory comparisons, **2005**, Geneva, Switzerland.

8.3. M. Thompson, S.L. Ellison, R. Wood. The International harmonized protocol for the proficiency testing of analytical chemistry laboratories (IUPAC technical report), *Pure Appl. Chem.*, **2006**, 78: 146-196.

TABLE I: Timeline of the Program

EVENT	SCHEDULE
Preparation of sample	Dec 2008 – Jan 2009
Homogeneity testing	Feb 2009
Submission of proposal to APLAC PT Committee for approval	Feb 2009
Stability testing	Mar – Jul 2009
Invitation of participants	Mar – Apr 2009
Dispatch of samples	May 2009
Deadline for result submission	Jun 2009
Statistical analysis of pooled data	Jul 2009
Interim Report	Jul 2009
Preparation of Draft Final Report	Aug 2009
Submission of Draft Final Report to APLAC PT Committee for approval	Sept 2009
Distribution of Final Report	Sept 2009

TABLE II: Geographic Distribution of Participating Laboratories

No.	ECONOMIES	PARTICIPANTS ENROLLED	RETURNED RESULTS
1	Argentina	2	2
2	Australia	2	2
3	Brunei Darussalam	1	1
4	Canada	3	3
5	Chile	1	1
6	Cuba	1	0
7	Ecuador	1	1
8	Germany	3	3
9	Hong Kong	5	5
10	Indonesia	4	4
11	Israel	1	0
12	Japan	4	4
13	Korea	4	4
14	Macau	1	1
15	New Zealand	3	3
16	China	4	4
17	Singapore	3	3
18	Sri Lanka	1	1
19	USA	4	3
20	Vietnam	4	3
	Total:	**52**	**48**

TABLE III: Participants' Results for Melamine

Lab. Code	Concentration (mg/kg)				Expanded Measurement Uncertainty	
	Data #1	Data #2	Data #3	Mean	± mg/kg	Coverage Factor
1	2.14	2.29	2.28	2.24	0.25	2
2	2.0	2.1	2.2	2.1	0.5	2
3	2.54	2.42	2.53	2.50	0.2	2
4	2.36	2.48	2.36	2.40	1.92-2.88	2.015
5	2.63	2.85	2.84	2.77	0.75	2
6	2.17	2.17	2.13	2.15	0.745	2
7	3.82	3.63	3.71	3.72	0.24	2
8	2.26	2.30	2.34	2.30	0.6	2
9	2.339	2.345	2.351	2.35	0.35	2
10	2.75	2.73	2.76	2.75	0.01	2
11	2.40	2.38	2.39	2.39	0.2	2
12	1.41	1.39	1.44	1.41	0.0146	2.07
13	2.16	2.06	2.07	2.07	0.19	2.14
14	1.85	1.84	1.82	1.84	0.13	2
15	1.11	1.04	1.22	1.13	0.10	2
16	2.814	2.787	2.580	2.727	0.256	2
17	2.48	2.65	2.66	2.60	0.5	2
18	2.75	2.69	2.71	2.72	0.46	2
19	2.94	2.92	2.76	2.87	1.38	2
20	2.54	2.55	2.39	2.49	0.35	2.064
21	2.3	2.2	2.2	2.2	0.4	2
22	2.027	2.023	2.017	2.022	---	---
23	0.853	0.690	0.760	0.768	0.154	2
24	2.03	2.06	2.05	2.05	0.06	2
25	2.304	2.244	2.411	2.320	0.1636	2
26	2.34	2.44	2.33	2.37	0.121	2
27	6.162	6.874	6.625	6.554	0.425	2
28	<5	<5	<5	<5	---	---
29	6.30	7.20	6.50	6.67	0.39	2
30	2.85	2.96	3.12	2.98	0.28	2
31	2.31	2.25	2.29	2.28	0.24	2
32	2.51	2.61	2.45	2.52	0.50	2
33	2.30	2.27	2.39	2.32	---	---
34	2.10	2.27	1.96	2.11	0.310	2
35	3.034	2.903	3.089	3.009	0.19	2
36	2.47	2.42	2.51	2.47	0.684	2
37	2.28	2.32	2.32	2.31	0.22	2
38	4.29	4.38	4.34	4.34	---	---
39	24.6	24.5	23.3	24.1	---	---
40	2.51	2.74	2.90	2.72	---	---
41	1.719	1.699	1.721	1.713	---	---
42	2.42	2.45	2.51	2.46	0.6	
43	3.34	3.15	3.28	3.26	0.25	2
44	2.119	1.885	2.265	2.090	0.82	2
45	7.69	7.53	7.75	7.66	0.58	2
46	3.10	3.20	3.30	3.20	0.10	2
47	9.470	9.380	9.005	9.285	1.299	2
48	2.487	2.500	2.187	2.391	0.552	2

"---" denotes data/information was not provided by participants

TABLE IV: Participants' Analytical Instrument and Conditions Used

Lab Code	Analytical Instrument	Analytical Column	Mobile phase / Carrier gas	Internal standard
1	LC-MS/MS	HILIC	ACN: 20mM $AcNH_4$	$^{13}C_3{}^{15}N_3$-Melamine
2	LC-MS/MS	Polar RP	ACN: MeOH/ 5mM $AcNH_4$	$^{13}C_3{}^{15}N_3$-Melamine
3	LC-MS/MS	HILIC	ACN: H_2O: 10mmol $AcNH_4$	$^{13}C_3$-Melamine
4	LC-MS/MS	ZIC HILIC	ACN: H_2O: 10mmol $AcNH_4$	^{13}C-Melamine
5	LC-MS/MS	HILIC	ACN: H_2O: FA	$^{13}C_3{}^{15}N_3$-Melamine
6	LC-MS/MS	Amino	ACN: H_2O: 10mM AcNH4	No
7	GC-MS	HP-5MS	He	Diaminochloropyrimidine
8	GC-MS	HP-5MS	He	4-chloro-2,6-diaminopyrimidine
9	LC-MS/MS	HILIC	10 mM $AcNH_4$/ACN: 10 mM $AcNH_4$	$^{15}N_3$-Melamine
10	LC-MS/MS	HILIC	10 mM $AcNH_4$/ACN: 10 mM $AcNH_4$	$^{13}C_3{}^{15}N_3$-Melamine
11	LC-MS/MS	HILIC	ACN: $AcNH_4$	$^{13}C_3{}^{15}N_3$-Melamine
12	LC-MS/MS	HILIC	10 Mm NH_4 formate: 10 mM NH_4 formate/ACN	No
13	GC-MS/MS	DB 5	He	$^{13}C_3{}^{15}N_3$-Melamine
14	LC-MS/MS	HILIC	FA/ACN: 20mM NH_4 formate : ACN	No
15	LC-MS/MS	HILIC	0.1 % FA/ACN: 20 mM NH_4 formate/ACN	No
16	LC-MS/MS	HILIC	ACN/FA: ACN/20 mM NH_4 formate	No
17	LC-MS/MS	HILIC	20mM $AcNH_4$: ACN	No
18	LC-MS/MS	HILIC	$AcNH_4$: ACN	$^{13}C_3{}^{15}N_3$-Melamine
19	LC-MS/MS	C_{18}	ACN/FA: ACN/10mM NH_4 Formate	No
20	LC-MS/MS	HILIC	FA/$AcNH_4$:: ACN	$^{13}C_3$-Melamine
21	LC-MS/MS	C_8	ACN: 10mM $AcNH_4$	$^{13}C_3{}^{15}N_3$-Melamine
22	LC-MS/MS	C_{18}	ACN: 10 Mm acid amine	Yes
23	GC-MS	HP-5MS	He	$^{13}C_3{}^{15}N_3$-Melamine
24	LC-UV	C_{18}	1-HSA Na/citric acid: ACN	No
25	LC-MS/MS	HILIC	10 mM $AcNH_4$/ACN	No
26	GC-MS	DB-1701	He	$^{15}N_3$-Melamine
27	ELISA	NA	NA	NA
28	LC-UV	HILIC	ACN: 10 mM $AcNH_4$	No
29	GC-MS	Rxi-5ms	He	No
30	LC-MS/MS	Polar RP	AcNH4/AA: ACN	No
31	GC-MS	DB5	He	No
32	UPLC-MSMS	HILIC	ACN: 10mM $AcNH_4$	$^{13}C_3{}^{15}N_3$-Melamine
33	LC-MS/MS	HILIC	ACN: 5mmol/L NH_4 formate	$^{15}N_3$-Melamine
34	LC-MS/MS	HILIC	10mM NH_4 formate/ACN	$^{15}N_3$-Melamine
35	LC-MS/MS	ZIC HILIC	ACN:50mM $AcNH_4$	No
36	LC-MS/MS	C_{18}	ACN:20mM $AcNH_4$	$^{13}C_3{}^{15}N_3$-Melamine
37	LC-MS/MS	HILIC	ACN:20mM $AcNH_4$	$^{13}C_3{}^{15}N_3$-Melamine
38	ELISA	NA	NA	NA
39	LC-UV	C_{18}	Citric buffer: ACN	No
40	ELISA	NA	NA	NA
41	LC-UV	C_{18}	33mM OSA/PO_4 buffer : ACN	No
42	GC-MS	DB5	He	$^{13}C_3$-Melamine
43	LC-MS/MS	ZIC HILIC	ACN: 25mM NH4Ac/AA buffer	No
44	ELISA	NA	NA	Yes
45	GC-MS	DB-5MS	He	Yes
46	LC-MS/MS	HILIC	---	$^{13}C_3{}^{15}N_3$-Melamine
47	GC-MS	DB 5MS	He	2,6-Diamino-4-chloropyrimidine
48	LC-MS/MS	HILIC	ACN: 5mM NH_4 formate	No

"---" denotes information was not provided by participants; "NA" denotes not applicable
"AA" = acetic acid; "ACN" = acetonitrile; "$AcNH_4$" = ammonium acetate; "FA" = formic acid; "HSA" = heptanesulfonic acid and "OSA" = octanesulfonic acid

TABLE V: Other Technical Information

Lab Code	Extraction Method	Extraction Solvent	Clean-up Procedure	% Recovery	Method Accredited
1	Shaking	50% ACN	MCX	101	Yes
2	Liq-solid extraction	2.5% FA	No	30	Yes
3	Ultrasonic	2.5% FA in ACN	No	80	Yes
4	Agitation	50% ACN	MCX	111	Yes
5	Agitation	50% ACN in 1N HCl	MCX	106	No
6	Homogenization	50% ACN	PSA + SCX	80.5	No
7	Agitation + Ultrasonic	DEA/H_2O/ACN	---	98.5	Yes
8	Shaking + Ultrasonic	1% TCA	MCX	106	No
9	Agitation	50% ACN	PSA + SCX	97.4	Yes
10	Ultrasonic	0.2M $HClO_4$	MCX	104	Yes
11	Shaking + Ultrasonic	ACN	No	95	Yes
12	Shaking + Sonication	50% ACN	MCX	119	Yes
13	Sonication	DEA/H_2O/ACN	---	98	Yes
14	Shaking + Sonication	H_2O/ACN	MCX	85	Yes
15	Shaking + Ultrasonic	50% ACN	MCX	87	No
16	Agitation	2.5% FA in ACN	Centrifuge +0.22µm filter	90	Yes
17	Agitation + Shaking + Ultrasonic	ACN	MCX	92	No
18	Ultrasonic	ACN in H_2O	No	120	No
19	Shaking	2.5% FA	SPE	69	No
20	Liq-solid extraction	ACN in H_2O	None	70	Yes
21	Ultrasonic	ACN	MCX	96.5	No
22	Ultrasonic	20% MeOH	0.2µm filter	95.9	---
23	SPE	1 % TCA/PbAc/MeOH pyridine, BSTFA	SPE	89.6	Yes
24	Ultrasonic	10g/L TCA	MCX	80.7	Yes
25	Agitation	50% ACN	MCX	94.64	No
26	Ultrasonic	ACN/H_2O/DEA	---	-	No
27	Centrifugation	---	---	101.4	No
28	SPE	50% ACN	MCX	40	No
29	Ultrasonic	DEA/ACN, pyridine, BSTFA	No	---	No
30	Agitation	ACN in 2% HCl	MCX	85.3	No
31	Agitation	TCA	MCX	96.7	Yes
32	Ultrasonic	50% ACN	No	101	Yes
33	Agitation	1% TCA	MCX	95.2	Yes
34	Agitation	50% ACN	MCX	100.8	Yes
35	Ultrasonic	50% ACN	Dilution	116	No
36	Shaking	ACN in 1N HCl	MCX	93	Yes
37	SPE	50% ACN in HCl	MCX	99.1	No
38	---	70% MeOH	---	---	No
39	SPE	Citric buffer pH 3.0	SCX	96.5	No
40	Agitation	MeOH/1N HCl	SPE	85	No
41	SPE	TCA/ACN	MCX	101	No
42	Shaking + Ultrasonic	MeOH/H_2O/FA	SCX	80-110	Yes
43	Shaking	10% FA	Centrifuge + filtration	31	Yes
44	Shaking + Ultrasonic	70% MeOH	No	---	Yes
45	SPE	ACN	SPE	91.76	No
46	Ultrasonic	0.2M PCA	Cartridge X-C strate	---	---
47	Ultrasonic	DEA/H_2O/ACN	0.45µm filter	70-110	Yes
48	SPE	2% TCA	---	82	No

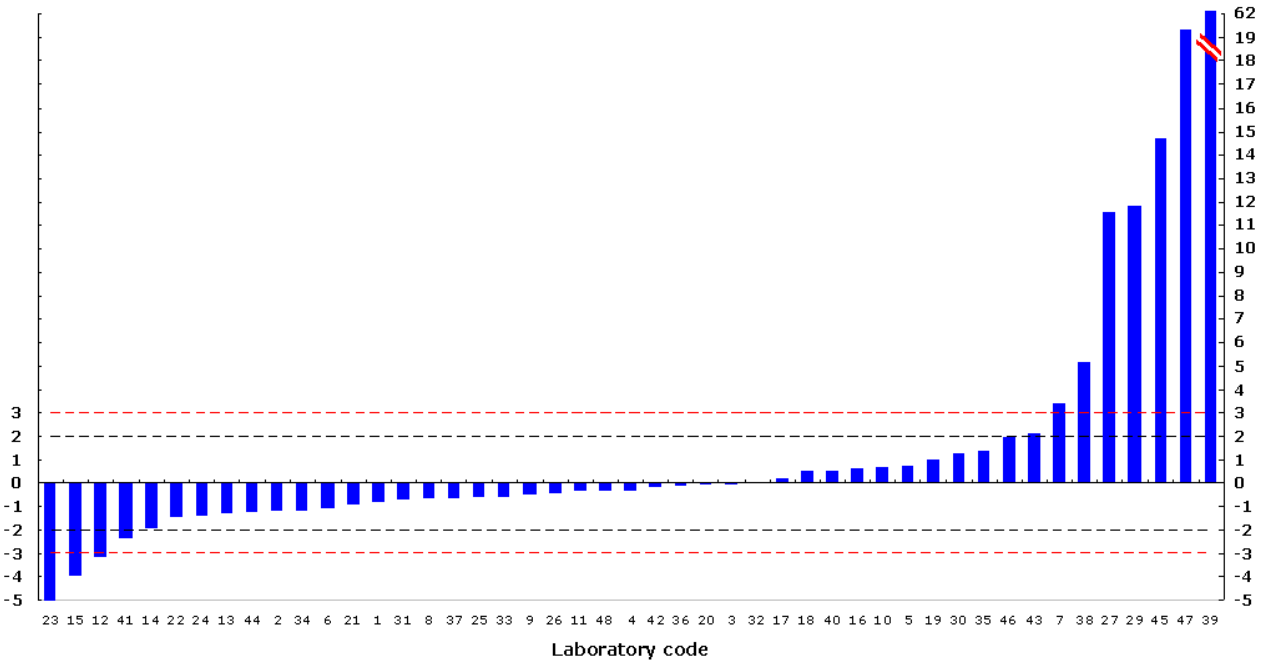
"---" denotes information was not provided by participants; "NA" denotes not applicable; "ACN" = acetonitrile; "BSTFA" = N.O-Bis(trimethylsilyl)trifluoro acetamide; "DEA" = dimethylamine; "FA" = formic acid; "MeOH" = methanol; "PCA" = perchloric acid and "TCA" = trichloroacetic acid; "MCX, SCX" = cationic exchange columns; PSA: amines column

TABLE VI: z-Scores of Participants

Lab Code	Reported Mean	z-Score
1	2.24	-0.83
2	2.1	-1.2
3	2.50	-0.08
4	2.40	-0.37
5	2.77	0.69
6	2.15	-1.1
7	3.72	**3.4**
8	2.30	-0.66
9	2.35	-0.51
10	2.75	0.63
11	2.39	-0.40
12	1.41	**-3.2**
13	2.07	-1.3
14	1.84	-2.0
15	1.13	**-4.0**
16	2.727	0.57
17	2.60	0.20
18	2.72	0.55
19	2.87	1.0
20	2.49	-0.11
21	2.2	-0.94
22	2.022	-1.5
23	0.768	**-5.0**
24	2.05	-1.4
25	2.320	-0.60
26	2.37	-0.46
27	6.554	**12**
28	<5	NA
29	6.67	**12**
30	2.98	1.3
31	2.28	-0.71
32	2.52	-0.03
33	2.32	-0.60
34	2.11	-1.2
35	3.009	1.4
36	2.47	-0.17
37	2.31	-0.63
38	4.34	**5.2**
39	24.1	**62**
40	2.72	0.55
41	1.713	-2.3
42	2.46	-0.20
43	3.26	2.1
44	2.090	-1.3
45	7.66	**15**
46	3.20	1.9
47	9.285	**19**
48	2.391	-0.40

z-Scores ≥ 3 or ≤ -3 are bolded ; NA denotes not applicable

CHART I: Participants' *z*-scores



APPENDIX I: Homogeneity Test

a. A total of 12 bottles were randomly selected for homogeneity test in February 2009. Determination of melamine was conducted using a validated LC-MS/MS method for two sub-samples (0.5 g each) from each bottle.

b. Results of melamine concentration in the 24 sub-samples were as follows:

Sample Code	Melamine concentration (mg/kg)	
	Data 1	Data 2
14	1.97	1.96
29	2.00	2.00
38	1.96	1.99
47	1.98	1.96
61	1.95	1.95
83	2.06	2.02
99	2.00	2.02
122	2.06	2.00
141	2.09	2.01
163	1.97	2.08
187	1.99	1.99
190	2.06	1.96
Mean ($\bar{x}$)	2.00 mg/kg	
SD (S_x)	0.033 mg/kg	
RSD	1.64 %	

c. According to ISO13528:2005, the samples are considered to be homogeneous if:

$S_s \leq 0.3\sigma_R$ where S_s = Between-sample standard deviation; σ_R = Performance standard deviation

S_s is calculated as:

$\sqrt{S_x^2 - (S_w^2/2)}$ where S_x = Standard deviation of sample averages; S_w = Within-samples standard deviation

S_w is calculated as:

$\sqrt{\sum w_t^2/(2n)}$ where w_t = Absolute difference of the duplicate sample; n = Number tested samples

APPENDIX I: Homogeneity Test (Cont'd)

σ_R (in percent) is estimated using Horwitz equation of 2 $C^{-0.15}$ where C was the mean concentration in mass fraction of melamine from homogeneity test:

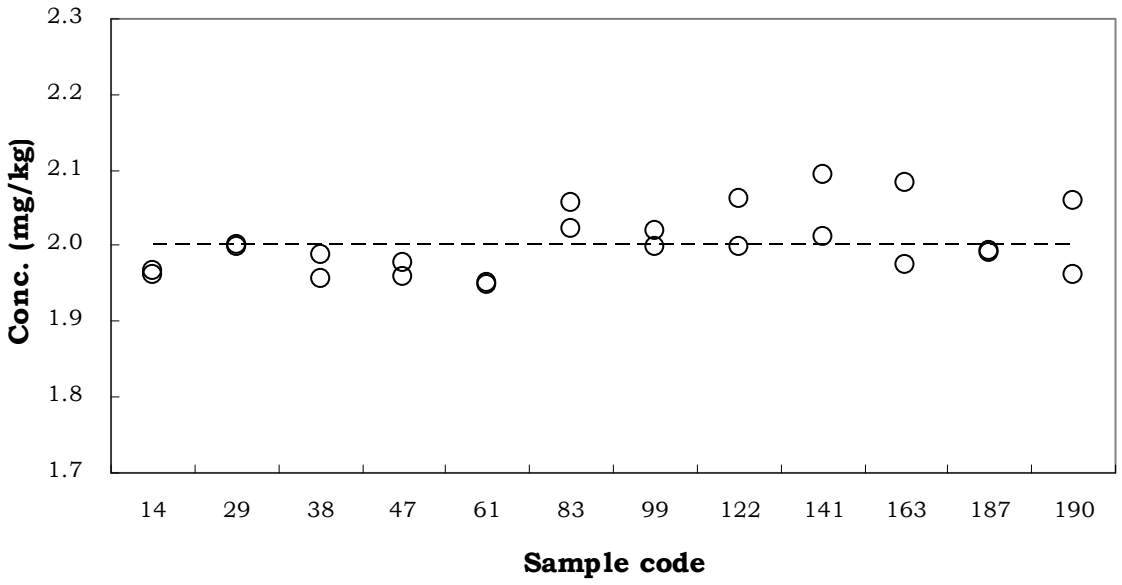
$\bar{x}$ (mg/kg)	:	2.00
σ_R (%)	:	14.3
$0.3\sigma_R$ (%)	:	4.30
$0.3\sigma_R$ (mg/kg)	:	0.0859

The results are summarized as follows:

Σw_t^2 (mg^2/kg^2)	:	0.0348
S_w (mg/kg)	:	0.0381
S_x (mg/kg)	:	0.0328
S_s (mg/kg)	:	0.0187

Since the between-sample standard deviation (S_s) in the homogeneity test for melamine is less than the corresponding value of $0.3\sigma_R$, the materials are considered homogeneous and should be adequate to be used in the PT program.

d. A plot of the 12 random samples in homogeneity test:



APPENDIX II: Stability Test

a. Stability of melamine was monitored in March and June 2009.

b. Samples selected for the stability test were stored at (i) a room temperature of 25 °C and (ii) an elevated temperature of 37 °C.

c. One sample from each temperature set was randomly selected and analyzed in triplicate under the same operational conditions as those in homogeneity test.

d. Stability of samples was evaluated from the absolute difference between the mean value obtained in homogeneity test ($\bar{x}$) and the mean value ($\bar{y}_i$) of each stability test. According to ISO13528:2005, the absolute difference should not be more than $0.3\sigma_R$.

ie. $|\bar{x} - \bar{y}_i| \leq 0.3\sigma_R$

e. Results of stability test at 25°C and 37°C were summarized as follows:

Period		Melamine concentration (mg/kg)					$\lvert\bar{x} - \bar{y}_i\rvert$	$0.3\sigma_R$	Status
		#1	#2	#3	$\bar{y}_i$	$\bar{x}$			
Mar 2009	25°C	2.02	2.04	1.96	2.01	2.00	0.01	0.09	Pass
	37°C	2.00	2.04	1.98	2.01	2.00	0.01	0.09	Pass
Jun 2009	25°C	2.01	1.85	2.09	1.98	2.00	0.02	0.09	Pass
	37°C	2.02	2.07	1.96	2.02	2.00	0.02	0.09	Pass

f. Stability of melamine was found to be satisfactory over the entire period of the PT program.

APPENDIX III: Instructions to Accreditation Bodies

INSTRUCTIONS
(FOR ACCREDITATION BODIES)

1. ORGANIZATION

The PT program on "Melamine in Fish Feed (APLAC T069)" is organized by the Hong Kong Government Laboratory (HKGL) in collaboration with Hong Kong Accreditation Service (HKAS) under the auspices of the Asia-Pacific Laboratory Accreditation Cooperation (APLAC). The main objectives of this proposal are to provide an analytical forum for participants in the quantitative analysis of melamine in fish feed sample and to evaluate the measurement capability of participating laboratories that providing such testing services.

2. SAMPLE

Accreditation bodies (AB) should receive the PT sample(s) and instruction information (hard copy and soft copy) from the organizers. The sample(s) that each contains about 15g of dried fish feed (≤ 200 μm) are properly packaged in amber bottles. Upon receipt, AB should carefully check the samples (number of bottles, physical conditions, etc.) and shall promptly acknowledge the HKGL by returning the "Receipt Form (for Accreditation Bodies)" electronically to T69@govtlab.gov.hk. New sample(s) will be replaced for any missing and damaged bottles.

AB shall promptly distribute the samples to their nominated laboratories. The samples are recommended to store in a secure environment at room temperature (around 25°C) if immediate dispatch is not possible.

AB shall ask their nominated laboratories check the sample carefully upon receipt and promptly acknowledge the HKGL by returning the "Receipt Form (for Participating Laboratories)" electronically to T69@govtlab.gov.hk

3. RESULTS

AB shall inform their respective participating laboratories complete Part I and II of the "Result Proforma" and submit electronically to the HKGL on or before **30 June 2009** to T69@govtlab.gov.hk. (Notes: 1. Unless with special reasons, results submitted after the deadline might not be accepted by the organizers. 2. To avoid poor transmission quality, results by fax are generally not accepted.)

4. ENQUIRIES

Please contact the HKGL for further enquiries at T69@govtlab.gov.hk

APPENDIX IV: Receipt Form for Accreditation Bodies

RECEIPT FORM
(FOR ACCREDITATION BODIES)

From: _____
(Name of AB)

_____
(Contact Person)

Email : _____

Date: .....

To: Dr. Yiu-chung WONG
The PT Advisory Board
HKGL,
Hong Kong.

Email: T69@govtlab.gov.hk

I hereby acknowledge the receipt of _____ # sample(s) for the APLAC T069 programme.

The sample(s) is/are found *(intact / damaged) and should be *(suitable / not suitable) for laboratory testing.

Remark(s) : .....
.....
.....
.....
.....

Number of samples
* Please circle as appropriate

APPENDIX V: Instruction to Participating Laboratories

INSTRUCTIONS
(FOR PARTICIPATING LABORATORIES)

1. ORGANIZATION

The PT program on "Melamine in Fish Feed (APLAC T069)" is organized by the Hong Kong Government Laboratory (HKGL) in collaboration with Hong Kong Accreditation Service (HKAS) under the auspices of the Asia-Pacific Laboratory Accreditation Cooperation (APLAC). The main objectives of this proposal are to provide an analytical forum for participants in the quantitative analysis of melamine in fish feed sample and to evaluate the measurement capability of participating laboratories that providing such testing services.

2. SAMPLE

(a) Participating laboratories will be given an amber sample bottle that containing about 15 g of dried fish feed powder (≤ 200 μm) from their respective accreditation bodies (AB) or the organizers.

(b) Upon receipt of the sample, participating laboratory should carefully inspect the sample for any physical damages and defects. If so, please contact the HKGL at "T69@govtlab.gov.hk". New sample will be replaced for any damaged claim.

(c) If sample is physically satisfactory, participating laboratory shall promptly acknowledge the receipt of the sample by returning the "Receipt Form (for Participating Laboratories)" electronically to the HKGL at "T69@govtlab.gov.hk".

(d) Intact sample is recommended to be stored in a secure environment at room temperature (around 25°C) before use. Sample should be immediately capped after use and be kept in a dry box when not in use.

3. ANALYSIS

(a) Participating laboratory is required to determine the mass fraction (in **mg/kg**) of melamine in the sample. The approximate mass fraction was in the range of 0.1 to 5 mg/kg. Determination is recommended to be conducted in triplicate.

(b) Participating laboratory should use their preferable test method (either accredited, in-house, modified, ad hoc method, etc.) which is normally used to quantify melamine for his customers' samples. Details of the analytical method used should be given on the result sheet provided.

4. RESULTS

(a) Report all the triplicate data and the arithmetic mean in Part I of the "Results Proforma".

(b) Estimate and report the expanded uncertainty (± mg/kg).

(c) Fill in the information of your performed analytical method in Part II of the "Results Proforma".

(d) Submit the results electronically to the HKGL on or before **30 June 2009** to T69@govtlab.gov.hk using the soft copy of the "Result Proforma" provided. (Notes: 1. Unless with special reasons, results submitted after the deadline might not be accepted by the organizers. 2. To avoid poor transmission quality, results by fax are generally not accepted.)

5. ENQURIES

Please contact the HKGL at T69@govtlab.gov.hk for further enquiries concerning the program.

APPENDIX VI: Receipt Form for Participating Laboratories

RECEIPT FORM
(FOR PARTICIPATING LABORATORIES)

From: _____
(Name of Participating Laboratory)

_____
(Contact Person)

Email : _____

Date: .....

To: Dr. Yiu-chung WONG
The PT Advisory Board
Hong Kong Government Laboratory,
Hong Kong.

Email: T69@govtlab.gov.hk

I hereby acknowledge the receipt of ONE sample for the APLAC T069 program.

The sample(s) is/are found (damaged / satisfactory)* and should be (suitable / not suitable)* for laboratory testing.

Remark(s) : .....

.....

.....

.....

.....

* Please circle as appropriate

APPENDIX VII: Result Proforma

RESULTS PROFORMA

<Both Part I & II **MUST** be completed by Participating Laboratory>

Name of Participating Laboratory: _____

_____

Contact Person / Email: _____

Part I: Analytical Results

Analyte	Concentration (in **mg/kg**, based on as-received weight)				Measurement Uncertainty	
	Data 1	Data 2	Data 3	Mean	Expanded Uncertainty (mg/kg)	Coverage Factor (*k*)
Melamine						

Note: If the concentration is less than your limit of quantification (LOQ), please specify the LOQ, eg. < 1.0 mg/kg

§ Report values to 3 significant figures or 3 decimal places, whichever is appropriate

- **It is the responsibility of the participants to avoid collusion and falsification of result so as to ensure a reliable assessment to be made in this proficiency testing programme**
- Please submit this "Result Proforma" electronically to the HKGL at T69@govtlab.gov.hk

To be continued …

APPENDIX VII: Result Proforma (Cont'd)

Part II: Method Information

1. *Analytical Instrument(s): LC-UV / LC-MS / LC-MS/MS / GC-MS (*delete as appropriate)
Others: (please specify)

2. Chromatographic column: *eg. C_{18} (150mm x 2.1mm, 5 m) or DB 5 (30m x 0.25mm x 0.25 m)*

3. (i) Mobile phase(s):
or Carrier gas
(ii) Flowrate

4. Internal standard: YES (pls. specify) ; NO

5. Extraction solvent(s):

6. *Extraction technique: Liq-solid extraction (*Agitation / Shaking/ Ultrasonic); Soxhlet;
ASE; MSPD ; etc.
Others: (please specify)

7. Extraction duration: hrs. min.

8. Clean-up procedure: SPE (*MCX, SCX, C_{18}, Florisil, Alumina, others)
Others: (please specify)

9. Recovery (%)

10. *Correction for recovery: YES; NO

11. *Method accreditation: YES; NO

12. Other additional information:

* Please circle as appropriate