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中国産飼料へのメラミン混入に関する対応について（注意喚起）

中国産の家畜用飼料及びペットフード（飼料原料を含む。以下「家畜用飼料等」という。）へのメラミン混入に関する対応については、「中国産植物性たん白の飼料への使用について（注意喚起）」（平成１９年５月７日付け１９消安第１４２４号農林水産省消費・安全局畜水産安全管理課長、生産局畜産部畜産振興課長通知）、「中国産植物性たん白を使用したペットフードに関するリコールの徹底について（注意喚起）」（平成１９年６月８日付け１９消安第２６８３号農林水産省消費・安全局畜水産安全管理課長、生産局畜産部畜産振興課長通知）、「輸入魚粉の品質管理の徹底について（注意喚起）」（平成１９年８月３１日付け１９消安第６５５４号農林水産省消費・安全局畜水産安全管理課長通知）及び「中国産牛乳が使用されている飼料原料の使用について（注意喚起）」（平成２０年９月２０日付け２０消安第６９３６号農林水産省消費・安全局畜水産安全管理課長、生産局畜産部畜産振興課長通知）により、家畜用飼料等について事前にメラミンの混入がないことを確認すること等について注意喚起をお願いしたところです。

さらに今般、中国政府は、同国内において新たにメラミンが家畜用飼料に混入されていた事実を公表しました。

このような事態にかんがみ、中国産の家畜用飼料等を輸入する場合には、事前にメラミンの混入がないことを確認することが、安全な家畜用飼料等の安定的な供給に極めて重要となっています。

つきましては、中国産の家畜用飼料等の取扱いについては下記により対応していただきますよう、貴会（組合）の会員（組合員）への周知徹底方お願いします。

記

1　中国産の家畜用飼料等の輸入及び飼料原料としての使用の実態について、再確認を行うこと。

2　１の結果、中国産の家畜用飼料等の輸入又は使用が確認された場合には、メラミン混入の有無について検査を行うとともに、その検査結果について農林水産省消費・安全局畜水産安全管理課（以下「畜水産安全管理課」という。）まで報告すること。

3　今後当分の間、中国産の家畜用飼料等を輸入する場合には、すべてのロットについてメラミン混入の有無を検査し、メラミンの混入が確認された場合には、その使用を自粛するとともに、直ちに畜水産安全管理課まで報告すること。その際、シアヌル酸の濃度についても併せて分析し、追って畜水産安全管理課まで報告すること。

4　２及び３の検査のための試験方法は、米国食品医薬品局（ＦＤＡ）が公表している方法（別添）を用いることとすること。

GC-MS Method for Screening and Confirmation of Melamine and Related Analogs

Version 2 May 7, 2007

PURPOSE:

This procedure was developed by PRLNW and FCC to screen various matrices for the presence of melamine and some related compounds at levels of 10 µg/g and above using gas chromatography/mass spectrometry. Samples are extracted using a mixture of acetonitrile/water/diethylamine and the analytes are subsequently converted to trimethylsilyl derivatives for analysis.

SCOPE:

This procedure has been evaluated using dry protein materials (wheat gluten, rice protein, corn gluten, and soy protein), wet and dry pet foods, and dry animal feeds. It is anticipated that the method will also be applicable to a variety of other matrices.

RESPONSIBILITY:

It is the responsibility of the analyst to note any modifications to or deviations from this procedure in the documentation associated with the analysis.

DEFINITIONS AND ACRONYMS:

TMS – trimethylsilyl
DEA – diethylamine

SAFETY CONSIDERATIONS:

Accepted safety measures should be employed when working with chemicals and pressurized gases. It is advisable to work in a fume hood whenever the procedure calls for the use of diethylamine and pyridine. Use caution with diethylamine as it is a volatile strong base and can cause chemical burns.

EQUIPMENT AND SUPPLIES:

1. Agilent 5975i GC-MS system equipped with a 30 m Agilent DB5-MS capillary column (or equivalent) or other mass spectrometer system.
2. Laboratory Centrifuge capable of applying 5000 g to 50 mL centrifuge tubes.
3. Pierce Reacti-therm / Reacti-Vap Sample Preparation System or other device suitable for evaporating solutions to dryness.
4. 50-mL polypropylene centrifuge tubes with screw caps.

REAGENTS AND STANDARDS:

1. Diethylamine (DEA), SigmaUltra, Sigma Chemical Co.
2. Pyridine, Certified A.C.S. Reagent, Fisher Scientific
3. Extraction Solvent: 10 / 40 / 50 : DEA / Water / Acetonitrile

   Prepare a solution which consists of 10 parts (by volume) diethylamine, 40 parts water and 50 parts acetonitrile. Store in the dark. The solution turns yellow with time.

4. Silylating Reagent: BSTFA with 1% TMCS: bis(trimethylsilyl)trifluoroacetamide with 1% Trimethylchlorosilane (e.g. Sylon BFT, Supelco)
5. Benzoguanamine, CAS 91-76-9 99+%: Lancaster

   For use as an internal standard. Prepare a stock solution at 1.0 mg/mL in pyridine.
   For adding to sample extracts, dilute the stock solution in pyridine to a concentration of 10 µg/mL.
   Use of benzoguanamine as an internal standard is still undergoing evaluation.

6. Melamine, CAS 108-78-1, Cat. 240818-5G, Aldrich

   Prepare a stock solution of 1.0 mg/mL in a mixture of 20 / 80 : DEA / H2O (v/v)

7. Ammelide, CAS 645-93-2, Cat. A0645, TCI America

   Prepare a stock solution of 1.0 mg/mL in a mixture of 20 / 80 : DEA / H2O (v/v)
   Brief sonication may be required to solubilize this standard.

8. Ammeline, CAS 645-92-1, Cat. A0676, TCI America

   Prepare a stock solution of 1.0 mg/mL in a mixture of 20 / 80 : DEA / H2O (v/v)
   A modest amount of sonication may be required to solubilize this standard.

9. Cyanuric Acid, CAS 108-80-5, Cat. C0459, TCI America

   Prepare a stock solution of 1.0 mg/mL in a mixture of 20 / 80 : DEA / H2O (v/v)

Store stock solutions of standards in the refrigerator to retard hydrolysis. It has not been established how rapidly the solutions degrade but the potential does exist. Stock standards have sat on the benchtop at room temperature for two weeks and shown no evidence of degradation.

QC ELEMENTS:

A 1:1 (v/v) mixture of Sylon BFT and pyridine should be run at the onset of each analysis and then occasionally throughout the analysis to show that there is no carryover.

A method blank which consists of 20 mL of extraction solvent taken through the entire procedure including the addition of the internal standard should be evaluated to make sure that there is no contamination from the reagents, the nylon filter or the containers.

Condition the system at the beginning of each sample set by making two injections of the high standard (see below). These injections will not be used for semi-quantitation.

A low standard, which consists of each analyte at 0.10 µg/mL, should be analyzed at the beginning of the sample set to show that the necessary sensitivity is being attained by the instrument.

A high standard at 1.0 µg/mL of each analyte is analyzed at the beginning of the sequence and after all samples in the batch have been injected to provide a basis for semi-quantitative evaluation and to demonstrate whether the amount of drift during the analysis of the set of samples is tolerable.

A control sample which is representative of the type of samples which are being analyzed is fortified with each analyte (viz. melamine, ammelide, ammeline and cyanuric acid) at a level of 10 µg/g. Analysis of this spiked control sample must indicate that the compounds are present which serves to demonstrate effective system performance at the desired reporting level. See Sample Fortification, below.

PROCEDURE:

This procedure should be used with the GC operating in the splitless mode for greater sensitivity.

A. Extraction Procedure

Weigh out approximately 0.5 g of a representative portion of the sample into a 50mL polypropylene centrifuge tube.

Add 20 mL of extraction solvent (10:40:50 DEA : H_2O : Acetonitrile).

Mix well to thoroughly wet the entire sample.

Sonicate for 30 minutes.

Centrifuge for 10 minutes at 5000 g (or better).

Filter a portion of the supernatant using 0.45µm nylon filter discs (a two-stage or molecular weight cutoff filter may be used for difficult extracts). For example, a 2 mL filtered portion will allow 200 µL for the derivatization step and additional filtrate in the event that the derivatization needs to be repeated or if further work is necessary.

B. TMS-Derivatives

Sample Extracts:

Transfer 200 µL of filtrate from Step A to a GC vial.

> *Note: A smaller aliquot may be used provided that the necessary sensitivity level (10 µg/g of sample) is achieved. Reducing the amount of matrix present improves the general performance of the evaporation/derivatization step and saves wear and tear on the instrument.*

Evaporate to dryness at 70°C (a low flow stream of dry air or nitrogen may be used).

Note: Taking the filtrate completely to dryness is a critical step in the derivatization process. The presence of water prevents formation of TMS derivatives of the analytes. If the internal standard response is much lower than usual (less than 30%), there may have been problems associated with the derivatization step. In addition, if the vial warms significantly to the touch after addition of the derivatization reagents, residual water was present and a new aliquot of filtrate must be prepared.

Add 200 µL pyridine.

Add 200 µL Sylon BFT.

Add 100 µL of benzoguanamine internal standard solution at 10 µg/mL in pyridine. This produces a concentration in the extract of 2 µg/mL. If you are not using the internal standard, then add 100 µL of pyridine.

Shake well or vortex to mix.

Incubate at 70°C for 45 minutes.

Ready for Injection.

Note: If insoluble material is observed at the bottom of the vial after 45 minute incubation, transfer liquid portion to another GC vial or filter before analysis.

C. Instrument Parameters

GC Conditions:

Column	30m DB-5MS 5% phenyl 95% dimethyl-polysiloxane ID: 0.25mm Film Thickness: 0.25 microns
Inlet Temperature	280°C
Detector Temperature	290°C
Injection Mode	Splitless
Injection Volume	1 µL
Carrier Gas Flow	He at 35 cm/sec (constant flow)
Oven Program	75°C (hold 1 minute) to 320°C at 15°C/minute (hold 2.67 min) for a total run time of 20 min.

Note: Alternate GC conditions may be used provided that adequate resolution is obtained between the target analytes. Any such deviations from the method must be noted in corresponding documentation. With small volume liners, some peak splitting has been observed under the above conditions. Using a higher starting temperature (100°C) alleviated the problem.

MS Conditions (Full Scan Mode):

Tune	Autotune (to maximize sensitivity across mass range) *A +306V multiplier bump may be added after Autotuning*
Acquisition parameters	EI; scan mode, 50-450 amu
Sampling Rate	2 (scan rate at 3.58 scans/sec)
Threshold	100
Filament Delay	6 minutes
MS Temp	230°C (Source); 150°C (Quad)

If the sensitivity which is required to detect analyte spikes at 10 µg/g in the matrix cannot be achieved in full scan mode, use selected-ion monitoring parameters below.

MS Conditions (SIM Mode): Select three or four ions to track: M, M+2, M-15 and another

Group	Start Time [a] (min)	Ions [b]					
		M [c]	M + 1	M + 2	M - 15	Other	Other
Urea / Biuret from di- and tri-TMS derivatives of urea [d]	6	276 (tri-tms)			189 (di-tms) 261 (tri-tms)		
Cyanuric Acid	9	345 (100)[e]	346 (30)	347 (14)	330 (33)	188 (11)	
Ammelide	9.7	344 (100)	345 (30)	347 (14)	329 (50)	286 (7)	198 (28)
Ammeline	10.4	343 (100)	344 (30)	345 (14)	328 (115)	285 (30)	198 (27)

Melamine	11	342 (54)	343 (16)	344 (8)	327 (100)	285 (12)	197 (13)
Benzoguanamine	12	331	332	333	316	171	

[a] Start Times may need to be adjusted based upon the retention time of standards on your system.

[b] Dwell times should be adjusted to produce a cycle time of about 4 scans/sec

[c] M is the Molecular Ion for the tri - TMS derivative of the compound.

[d] Urea and Biuret are not formally part of the screen but they are related to the compounds of interest and may be detected

[e] Percent relative abundance with respect to the molecular ion from directly silylated standards under Standard Spectrum Autotune. The relative abundances should be confirmed under the conditions of use by evaluating standards.

D. Peak Identification and Results

The approximate retention times of the tri-TMS derivatives are as follows (minutes):

Cyanuric Acid	9.2
Ammelide	10.0
Ammeline	10.7
Melamine	11.2
Benzoguanamine	13.4 (di-TMS derivative)

These need to be confirmed on your system and the parameters above adjusted to fit.

In full scan mode, the criteria for identification of target analytes include the agreement of the retention times with those of the standards to within 0.05 minute. Also, the mass spectra need to correspond to those of the standards with no significant peaks absent. There may be additional peaks present due to overlap with other components but this should be examined carefully.

In SIM mode, the criteria for identification of target analytes include the agreement of the retention times with those of standards to within 0.05 minute. Mass spectral confirmation of an analyte is based upon the ratios of the integrated areas for selected ions to the integrated area of the most abundant of the selected ions being tracked. The criterion is that each ratio (as a percentage) corresponds to that observed for a standard to within 10 units. Using melamine as an example, track the ions at 342 (M), 327 (M-15) and 343 (M+1), use the data in the table above as representative of a standard. For a peak nominally identified as melamine based upon retention time, the ratio of the area of peak (at 11.2 min) from m/z = 342 (M) to that of the area of the peak at m/z = 327 (M-15) needs to be between 44% and 64% while that of the ratio of the peak at m/z = 343 (M+1) to that of the area of the peak at m/z = 327 (M-15) needs to be between 6% and 26%.

Figure 1 is a standard chromatogram showing the cyanuric acid tri-TMS derivative (9.2 minutes), ammelide tri-TMS derivative (10.0 minutes), ammeline tri-TMS derivative (10.7 minutes) and melamine tri-TMS derivative (11.2 minutes). Figures 2 through 5 represent the mass spectra of each of these compounds.

E. Sample Fortification and Mixed Standard Preparation

A Mixed Standard Spiking solution is prepared by mixing portions of the stock standard solutions with 10/40/50 DEA/Water/Acetonitrile to create a solution which is 100 µg/mL of each analyte.

Spike Preparation

Weigh a 0.5 g portion of the matrix of interest into a 50 mL centrifuge tube. A previously analyzed, representative "blank" matrix may be used. If a representative matrix is not available, select one of the samples to be analyzed to use for spiking.

Add 50 µL (Low Spike) of Mixed Standard Spiking solution directly to the representative control sample and proceed with the method. The Low Spike must be observed to declare negatives as less than 10 µg/g. If it is not then negative samples will need to be re-analyzed.

A High Spike may also be prepared by adding 250 µL of Mixed Standard Spiking to another 0.5 g portion of the control. The High Spike provides an additional check in the event the Low Spike is not observed. The absence of the High Spike would serve as an indication of a major problem either with the sample preparation or the instrument.

High Standard

Dilute the Mixed Standard Spiking Solution to 10 µg/mL. Place 50 µL of this solution in a vial and take it through the derivatization reaction including the addition of the internal standard. This produces a High Standard at a concentration of 1.0 µg/mL.

Low Standard

Dilute the Mixed Standard Spiking Solution to 1 µg/mL. Place 50 µL of this solution in a vial and take it through the derivatization reaction including the addition of the internal standard. This produces a Low Standard at a concentration of 0.10 µg/mL.

F. Reporting

In the event that the analytes were observed in the representative control sample that was fortified at 10 µg/g, and no analyte signals were observed in the samples at levels which approach those in the fortified control, then the samples are not contaminated with melamine, ammeline, ammelide or cyanuric acid at levels in excess of 10 µg/g.

When it is close (within 50%), consider preparing additional portions from those samples along with a couple of additional fortified controls.

If it is clear that one or more of the analytes are present in the samples (based on the identification criteria above) and at levels in excess of 10 µg/g, then a semi-quantitative estimate may be obtained by comparing analyte responses to those from standards as described below.

G. Obtaining a Semi-quantitative Estimate of Target Analytes

Choose an ion to use for estimating the amount of the target analyte (usually the molecular ion). Using the High Standard (1.0 µg/mL) which was run at the beginning of the set of samples and the High Standard that was run at the end of the set of samples, calculate the average peak area for the selected ion. Apply the following formula:

Concentration in Derivatized Test Sample Extract (µg/mL) = Area (Test Sample) / Average Area (Standard) X 1.0 µg/mL.

Then, Concentration in Sample (µg/g) = Concentration in Derivatized Test Sample Extract (µg/mL) X 0.50 mL X 20.0 mL / 0.20 mL X 1 / Sample Wt. (g)

If the internal standard areas are very different (> 20%) between the test sample and the standard then adjust the area of each analyte response by dividing by the integrated area of the internal standard (from its molecular ion). Use these ratios in place of the integrated areas above. Consider re-analysis if the difference is extremely large since that may indicate a partially clogged syringe or other instrumental problem.

Finally, if the signal is more than 25 X larger than the signal from the standard, then prepare a new extract using 0.25 g of sample and 40 mL of the extraction solvent since the solubility limit for ammeline and ammelide in 10/40/50 DEA/water/acetonitrile is being approached.

As a general rule-of-thumb, the closer the analyte signal from the unknown is to the analyte signal from the standard, the better the estimate of the unknown concentration. So if better numbers are required, prepare additional standards and re-analyze the samples in question.

Table 1: Feasibility Study with Analytes Spiked At and Near the Action Level

		Average Percent Recovery (N=2, Range in Parentheses)			
Matrix	**Spiking Level (µg/g)**	**Cyanuric Acid**	**Ammelide**	**Ammeline**	**Melamine**
Wheat Gluten (a)	10	84 (16)	90 (20)	86 (23)	79 (10)
	50	87 (13)	88 (16)	86 (13)	74 (11)
Rice Protein (a)	10	98 (2)	101 (4)	96 (3)	96 (2)
	50	107 (1)	108 (3)	108 (1)	101 (0)
Wet Pet Food (a)	10	106 (1)	103 (5)	114 (13)	105 (6)
	50	107 (3)	107 (0)	111 (7)	106 (5)
Corn Gluten (b)	10	139 (86)	166 (96)	172 (105)	152 (93)
	50	103 (4)	103 (3)	98 (5)	90 (4)
Soybean Meal (b,c)	10	120 (44)	118 (8)	164 (150)	152 (76)
	50	97 (7)	90 (12)	90 (10)	88 (11)

Dry Cat Food Salmon Flavor (b)	10	158 (41)	142 (33)	134 (44)	126 (26)
	50	81 (3)	82 (4)	74 (5)	77 (5)

Notes:

(a) 0.5 g extracted into 20 mL of 10/40/50 DEA/H2O/CH3CN (v/v/v). 0.20 mL extract to 0.45 mL silylated preparation. Analysis using scan mode on a 5975i MSD. Extracted ion chromatograms were used to determine area response for molecular ions.

(b) 0.5 g extracted into 10 mL of 10/40/50 DEA/H2O/CH3CN (v/v/v). 0.20 mL extract to 0.45 mL silylated preparation. Analysis by SIM on a well-worn Agilent 5973 MSD.

(c) Rubbery precipitate in some final extracts that can clog the syringe

Figure 1: Standard chromatogram showing the TMS derivatives of target compounds

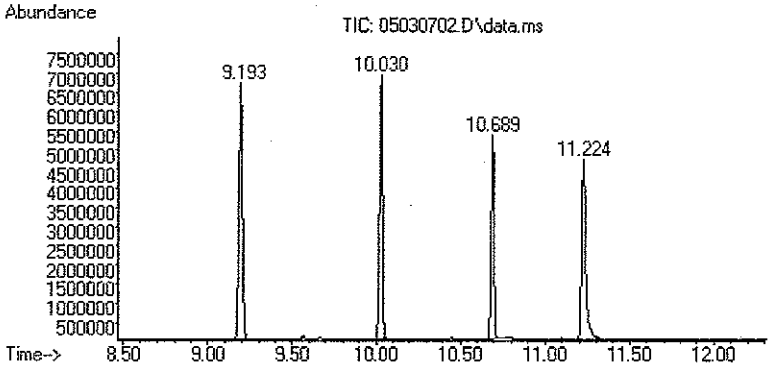


Figure 2: Cyanuric Acid tri-TMS derivative mass spectrum

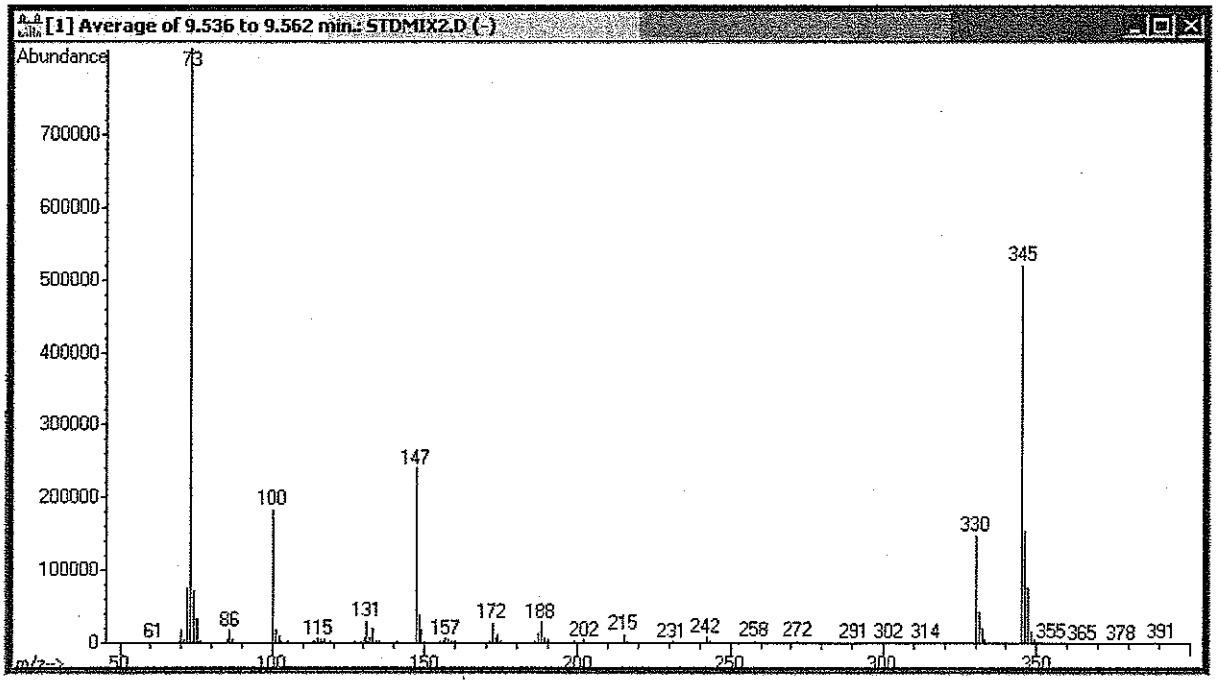


Figure 3: Ammelide tri-TMS derivative mass spectrum

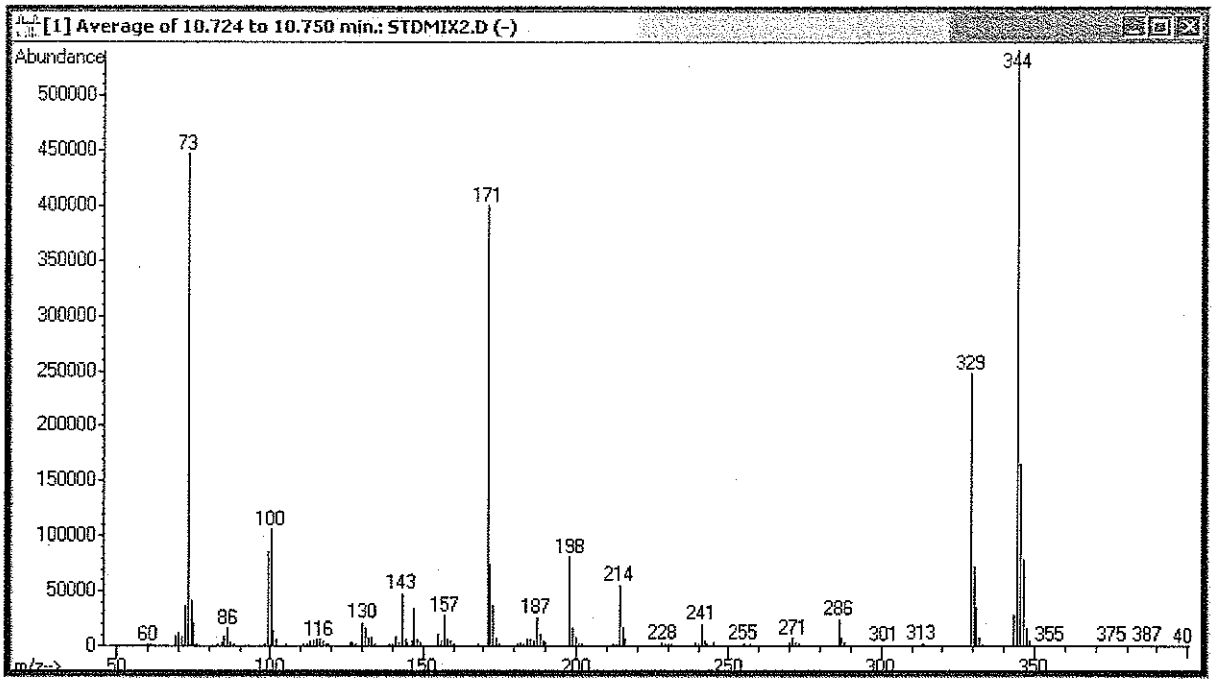


Figure 4: Ammeline tri-TMS derivative mass spectrum

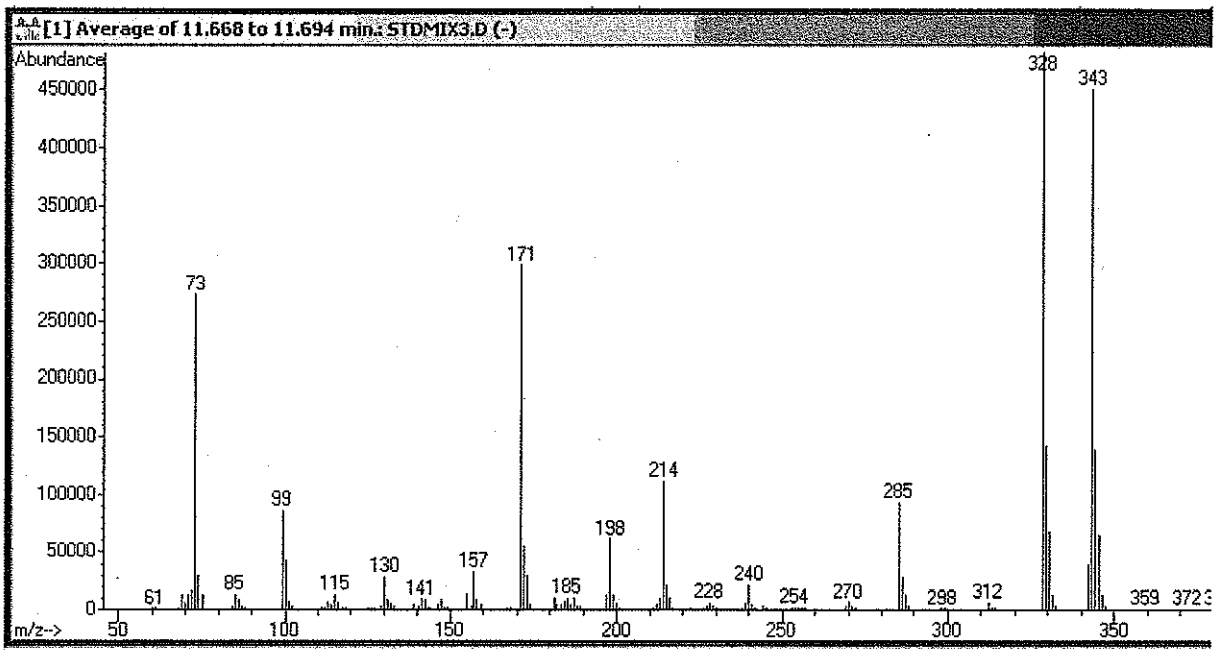


Figure 5: Melamine tri-TMS derivative mass spectrum

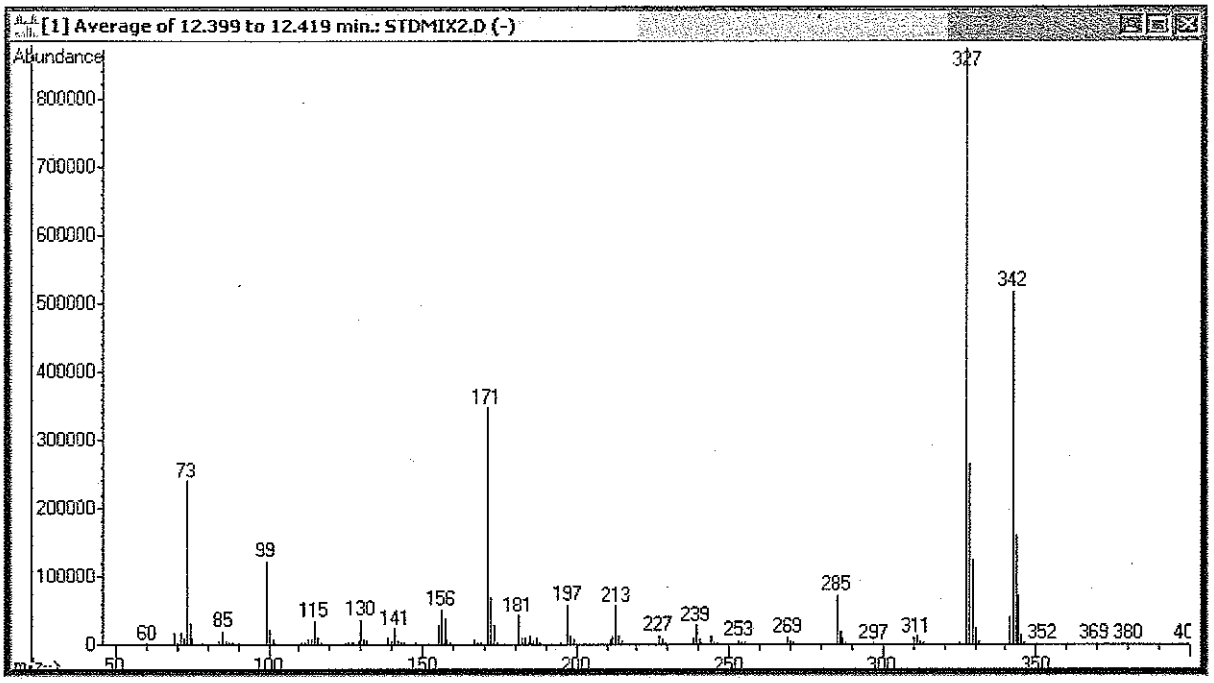
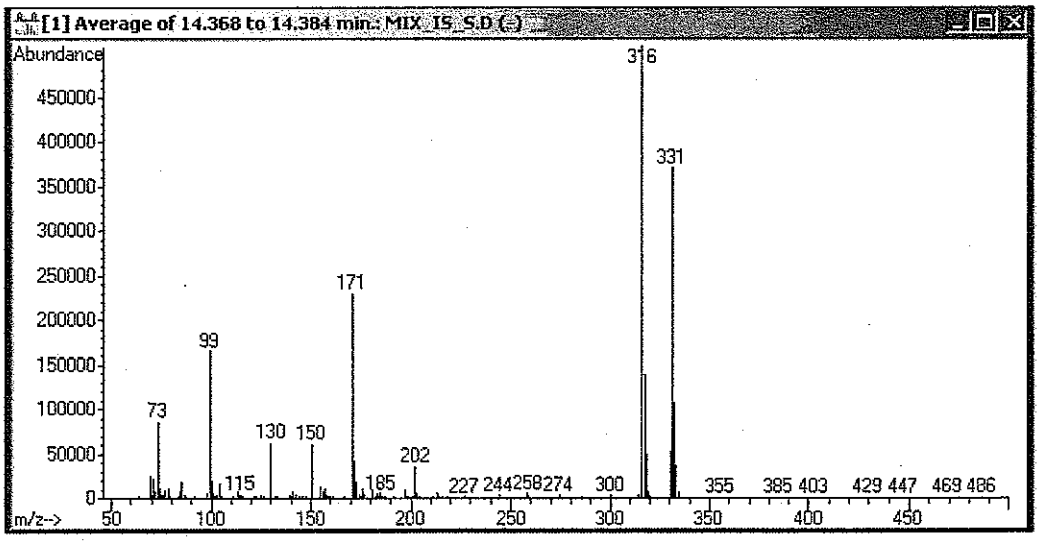


Figure 6: Benzoguanamine di-TMS derivative mass spectrum



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