

---

**IDENTIFICATION AND QUANTIFICATION OF MELAMINE AND ITS DEGRADATION  
COMPOUNDS IN FOOD BY GAS CHROMATOGRAPHY COUPLED TO TANDEM  
MASS SPECTROMETRY**

Document: LABERCA/08MEL-A1.7      7<sup>th</sup> revision of 20/10/08

1<sup>st</sup> issue of 09/07/07

**Research and methodological development:**

B. Veyrand, S. Elaudais, Ph. Marchand

**Scientific Manager:** J.P. Antignac

**Director:** B. Le Bizec

**WARNINGS AND SAFETY PRECAUTIONS**

**Wear gloves for handling acids and concentrated bases, organic solvents and standards (pure compounds or solutions). As a rule, derivatisation reagents and solvents must be used in a fume cupboard.**

**0 Introduction**

In September 2008, DG SANCO passed on, via RASFF, some information concerning the presence of melamine in milk destined to young children. The addition of melamine during the manufacture of dairy produce aims at illegally and artificially increasing the apparent protein content of the product. Consuming contaminated food can cause kidney trouble in young children. Several cases of death have been reported by the Chinese Authorities. The import into Europe of milk and produce made from more than 50 % of dairy produce from China is forbidden; however, it is not excluded that certain member states may consider that compound products are not affected by this ban. To date, no marketing network has been identified in France. However, considering the health risk for consumers and in particular children, there is a need to ensure that these products are absent from the French market. Considering these elements, it was deemed necessary to develop an analytical method for the screening of melamine and its degradation compounds (ammelide, ammeline, cyanuric acid).

## 1 Field of application

The method described in this note is applied to the detection of melamine and its main metabolites (ammelide, ammeline and cyanuric acid) in food by gas chromatography coupled to tandem mass spectrometry after ionisation by electronic impact (GC-(EI)-MS/MS), signal acquisition in SRM and isotopic dilution analysis.

## 2 Principle

The method comprises several steps, which main ones are as follows:

- Sample preparation
- Solid/liquid extraction
- Filtration
- Derivatisation
- Detection, identification and analysis by GC-(EI)-MS/MS
- Data handling and interpretation of results

## 3 Reagents and products

During the analysis, unless specified, all reagents must be of analytical grade quality. Only ultrapure or distilled water must be used. Suppliers references are given as examples.

### 3.1 – Reference compounds

**3.1.1** – Melamine (BCP Instruments)

**3.1.2** – Ammeline (BCP Instruments)

**3.1.3** – Cyanuric acid (BCP Instruments)

**3.1.4** – Ammelide (BCP Instruments)

**3.1.5** –  $^{13}\text{C}_3\text{-}^{15}\text{N}_3$  Melamine (CIL – CNLM-8150-1.2)

**3.1.6** –  $^{13}\text{C}_3\text{-}^{15}\text{N}_3$  Cyanuric acid (CIL – CNLM-4661-1.2)

**3.1.7** – Benzoguanamine (external standard) (BCP Instruments)

**3.1.8** – Melamine mix at 100 ng/ $\mu\text{L}$  (solution containing melamine, ammelide, ammeline and cyanuric acid)

**3.1.9** – Melamine mix at 10 ng/ $\mu\text{L}$  (solution containing melamine, ammelide, ammeline and cyanuric acid)

**3.1.10** – Internal standards mix at 1 ng/ $\mu\text{L}$  (solution containing  $^{13}\text{C}_3\text{-}^{15}\text{N}_3$  melamine and  $^{13}\text{C}_3\text{-}^{15}\text{N}_3$  cyanuric acid)

Standard melamine, ammeline, ammelide and cyanuric acid solutions are prepared in a  $\text{H}_2\text{O}$ /Diethylamine (80/20, v/v) mixture. Benzoguanamine solutions are prepared in pyridine,  $^{13}\text{C}_3\text{-}^{15}\text{N}_3$  Melamine and  $^{13}\text{C}_3\text{-}^{15}\text{N}_3$  cyanuric acid solutions in ultrapure water.

All solutions must be stored in a refrigerator ( $\leq 4^\circ\text{C}$ ).

Upon receipt and before use, these standards must be characterised at least by a GC-MS method ; sample purity and compound identity must be proven.

### **3.2 – Necessary chemical products and reagents**

- 3.2.1 - Diethylamine (Sigma-Aldrich – D0806-1L)
- 3.2.2 - Pyridine (Aldrich – 27,097-P)
- 3.2.3 - Acetonitrile (SDS – 00637G21)
- 3.2.4 - MSTFA (Fluka – 69479)

### **3.3 - Gaz**

- 3.3.1 - Nitrogen (nitrogen generator) and water trap (Merck - 6107)
- 3.3.2 - Alpha 1 Helium (Air liquide)

## **4 Equipment**

*Suppliers' references are given as examples.*

### **4.1 - Automatic pipettes**

### **4.2 – Centrifuge**

### **4.3 – Agitator** ("Vortex" type)

### **4.4 – Heated blocks, evaporators**

- 4.4.1 - For flasks with nitrogen facility (C.I.L. – Therme-vap)
- 4.4.2 - For flasks, derivatisation reactions only (Pierce - 18780)
- 4.4.3 - For hemolysis tubes, with nitrogen facility:
  - thermostated hot plates,
  - Evaporation banks (Mini-vap Supelco - 2.2971),
  - Diameter 16 racks: H92612 (for glass tubes (4.10)),
  - Plastic stopcocks for nitrogen flow regulation (Prolabo - 07487007).

### **4.5 – Derivatisation flasks**

- 4.5.1 - Derivatisation flasks and caps (Chromacol – 09 CTV and 11AC7)
- 4.5.2 - Hand crimper (Chromacol – CR-11)
- 4.5.3 - Decapper (Chromacol – DCR-11)

### **4.6 – Disposable glass tubes** (CML TVU 16) **and their caps** (CML – 0BA13)

### **4.7 - Grinder** (blender type)

### **4.8 – Teflon 0.45 µm filters** (CIL – L605806)

## **4.9 - GC-MS/MS Equipment**

- 4.9.1-** Gaz chromatographer equipped with a capillary column, a split/splitless injector, a programmable oven up to 300°C (Hewlett Packard – 6890) and an automatic injector (Hewlett Packard – 7683).  
Non polar-phase capillary column with low bleeding (DB-5MS type)  
Length = 30 m  
Internal diameter = 0.25 mm  
Film thickness = 0.25 µm
- 4.9.2 -** Quadripole-type Mass Spectrometer (Micromass – Quattro Micro type) allowing the analysis with electronic impact of molecules with a molecular weight of up to 1000 u.
- 4.9.3 -** Computer workstation
- 4.9.4 -** Printer

## **5 Samples**

- 5.1** – Sample size must be large enough to allow the repetition of the analysis once at least.
- 5.2** – Samples must be stored and processed so as to allow proper identification in the laboratory.
- 5.3** – Transport and storage conditions must maintain the integrity of the sample and must not hinder the results. The sample will be preferably stored in a dark place.

## **6 Screening analysis**

*NB: When the matrix is not easy to grind and homogenise, take 0.5 g of sample so that the sample is representative of the batch. The necessary quantities of internal standards and extraction solvent need to be adjusted accordingly.*

### **6.1 – Sample preparation**

Grind each sample in a perfectly clean grinder (4.7) until a fine powder is obtained.

#### **6.1.1 - Sample processing**

Weigh 100 mg ( $\pm$  10 mg) of sample and place it directly in a glass tube (4.6). Add 200 ng of standard mix (200 µL of the « Standards Mix » solution at 1 ng/µL (3.1.10)).

#### **6.1.2 - Blank sample**

Weigh 100 mg ( $\pm$  10 mg) of food containing no melamine or its degradation products. Add 200 ng of the standard mix (200 µL of the « Standards Mix » solution at 1 ng/µL (3.1.10))

#### **6.1.3 - Supplemented blank sample (2.5 ppm)**

Weigh 100 mg ( $\pm$  10 mg) of food containing no melamine or its degradation products. Add 200 ng of the standard mix (200  $\mu$ L of the « Standards Mix » solution at 1 ng/ $\mu$ L (3.1.10)) and 250 ng of the melamine mix (25  $\mu$ L of the « Melamine Mix » solution at 10 ng/ $\mu$ L (3.1.9)).

## **6.2 – Solid/liquid extraction**

- 6.2.1 - Add to the previously prepared samples 4 mL of a H<sub>2</sub>O/Acetonitrile (50/50, v/v) mixture
- 6.2.2 - Agitate in vortex for 30 s.
- 6.2.3 - Sonicate for about 30 min.
- 6.2.4 - Centrifuge for about 10 min at 2000g.

## **6.3 – Filtration**

The liquid phase (6.2.4) is filtered through a 0.45  $\mu$ m nylon filter (4.8) in order to obtain a minimum of 50  $\mu$ L of filtrate.

## **6.4 - Derivatisation**

*During this step, the following critical recommendations must be followed:*

- ♦ *work under anhydrous conditions: the slightest trace of water would hydrolyse silylated compounds: it can come from a badly evaporated vial, a badly rinsed syringe, a prolonged contact with ambient air.*
  - ♦ *do not breathe the reagents vapours. Work under a fume cupboard.*
- 6.4.1 - Take 50  $\mu$ L of previous filtrate (6.3) and place it in a vial (4.5.1).
  - 6.4.2 - Add 25  $\mu$ L of a benzoguanamine solution at 0.1 ng/ $\mu$ L (3.1.4).
  - 6.4.3 - Evaporate dry under nitrogen stream.
  - 6.4.4 - Add 50  $\mu$ L of MSTFA (3.2.4) and encapsulate.
  - 6.4.5 - Vortex for at least 30 seconds.
  - 6.4.6 - Place the vials in a heated block (4.4) and heat at 60 ( $\pm$  5) $^{\circ}$  C for 45 ( $\pm$  10) min.

**6.5 – Detection by GC-MS/MS****6.5.1 – Chromatographic conditions**

o Carrier gaz

Helium

Constant flow: about 1 mL.min<sup>-1</sup>

o Injection

Splitless, draining time 1 min

Temperature: 280°C

Draining flow: 20 mL/min

Injected volume: 2 µL

o Column

Compounds are split on a non polar column: DB – 5 MS: 30 m x 0.25 mm (i.d.), 0.25 µm (film thickness).

• Temperature programme

120°C (1 min) - 10°C/min until 320°C (2 min)

**6.5.2 - Spectrometric conditions**

EI Ionisation + (positive mode electronic impact)

Ionisation energy: 70 eV

Below are the transitions followed in MRM mode and the approximate retention times of the various analytes:

**Table 1:** List of the analytes and their spectrometric conditions  
(EE: External standard; EI: Internal standard; AR: Screened analyte; \* : indicative RT)

Molecules	Type	TR* (min)	Transitions	Collision energy (eV)
<sup>13</sup> C <sub>3</sub> <sup>15</sup> N <sub>3</sub> Cyanuric acid	EI	8.34	351>336	5
Cyanuric acid	AR	8.35	345>330 345>188	5 5
Ammelide	AR	9.59	344>329 344>286	5 15
Ammeline	AR	10.60	343>328 343>285	5 10
<sup>13</sup> C <sub>3</sub> <sup>15</sup> N <sub>3</sub> Melamine	EI	11.35	348>333	5
Melamine	AR	11.36	342>327 342>285 342>171	5 15 20
Benzoguanamine	EE	14.54	331>316 331>171	5 20

## 7 Confirmation analysis

A sample declared as suspect at the end of the first analysis must be extracted again, before its non compliancy can be definitely declared (identification and quantification according to the criteria defined in Decision 2002/657/EC).

### **7.1 – Sample preparation**

Grind each sample in a perfectly clean grinder (4.7) until a fine powder is obtained.

#### **7.1.1 - Sample processing**

Weigh 100 mg ( $\pm$  10 mg) of sample and place them directly in a glass tube (4.6). Add 200 ng of the standard mix (200  $\mu$ L of the « standard mix » solution at 1 ng/ $\mu$ L (3.1.10)).

#### **7.1.2 - Blank sample**

Weigh 100 mg ( $\pm$  10 mg) of food containing no melamine or its degradation compounds. Add 200 ng of the standard mix (200  $\mu$ L of the « standard mix » solution at 1 ng/ $\mu$ L (3.1.10))

#### **7.1.3 - Range of supplemented samples**

Weigh 5 x 100 mg ( $\pm$  10 mg) of food containing no melamine or its degradation products. Add 200 ng of the standard mix (200  $\mu$ L of the « standard mix » solution at 1 ng/ $\mu$ L (3.1.10)) to each sample, then successively insert in each tube :

- 50 ng of melamine mix (50  $\mu$ L of the « melamine mix » solution at 1 ng/ $\mu$ L)  
*Sample « G 0,5 »*
- 100 ng of melamine mix (100  $\mu$ L of the « melamine mix » solution at 1 ng/ $\mu$ L)  
*Sample « G 1 »*
- 200 ng of melamine mix (20  $\mu$ L of the « melamine mix » solution at 10 ng/ $\mu$ L)  
*Sample « G 2 »*
- 500 ng of melamine mix (50  $\mu$ L of the « melamine mix » solution at 10 ng/ $\mu$ L)  
*Sample « G 5 »*
- 1  $\mu$ g of melamine mix (100  $\mu$ L of the « melamine mix » solution at 10 ng/ $\mu$ L)  
*Sample « G 10 »*

*NB: when the sample is suspected during first analysis, at a concentration higher than 10 ppm, the calibration range must be adapted accordingly.*

### **7.2 – Solid/liquid extraction**

**7.2.1 -** Add to the previously prepared samples 4 mL of a H<sub>2</sub>O/Acetonitrile mix (50/50, v/v)

**7.2.2 -** Vortex for 30 s.

**7.2.3 -** Sonicate for about 30 min.

**7.2.4 -** Centrifuge for about 10 min at 2000g.

### **7.3 – Filtration**

The liquid phase (7.2.4) is filtered through a 0.45  $\mu$ m (4.8) nylon filter in order to obtain a minimum of 50  $\mu$ L filtrate.

## **7.4 - Derivatisation**

*During this step, the following critical recommendations must be followed:*

- ♦ *work under anhydrous conditions: the slightest trace of water would hydrolyse silylated compounds: it can come from a badly evaporated vial, a badly rinsed syringe, a prolonged contact with ambient air.*
- ♦ *do not breathe the reagents vapours. Work under a fume cupboard.*

- 7.4.1** - Take 50 µL of the previous filtrate (7.3) and place them in a vial (4.5.1).
- 7.4.2** - Add 25 µL of a benzoguanamine solution at 0.1 ng/µL (3.1.4).
- 7.4.3** - Evaporate dry under a nitrogen stream.
- 7.4.4** - Add 50 µL of MSTFA (3.2.4) and encapsulate.
- 7.4.5** - Vortex for at least 30 seconds.
- 7.4.6** - Place the vials in a heated block (4.4) and heat at 60 (± 5)° C for 45 (± 10) min.

## **7.5 – Detection by GC-MS/MS**

### **7.5.1 – Chromatographic conditions**

- o Carrier gaz
  - Helium
  - Constant flow: about 1 mL.min<sup>-1</sup>
- o Injection
  - Splitless, draining time 1 min
  - Temperature: 280°C
  - Draining flow: 20 mL/min
  - Injected volume: 2 µL
- o Column
  - Compounds are split on a non polar column: DB – 5 MS: 30 m x 0.25 mm (i.d.), 0.25 µm (film thickness).
  - Temperature programme
    - 120°C (1 min) - 10°C/min until 320°C (2 min)

### **7.5.2 - Spectrometric conditions**

- EI Ionisation + (positive mode electronic impact)
- Ionisation energy: 70 eV

Below are the transitions followed in MRM mode and the approximate retention times of the various analytes:

**Table 2:** List of analytes and their spectrometric conditions  
(EE : external standard; EI : internal standard; AR: Screened analyte; \* : indicative RT)

Molecules	Type	TR* (min)	Transitions	Collision energy (eV)
<sup>13</sup> C <sub>3</sub> <sup>15</sup> N <sub>3</sub> Cyanuric acid	EI	8.34	351>336	5
Cyanuric acid	AR	8.35	345>330 345>188	5 5
Ammelide	AR	9.59	344>329 344>286	5 15
Ammeline	AR	10.60	343>328 343>285	5 10
<sup>13</sup> C <sub>3</sub> <sup>15</sup> N <sub>3</sub> Melamine	EI	11.35	348>333 348>173	5 20
Melamine	AR	11.36	342>327 342>285 342>171 342>213	5 15 20 10
Benzoguanamine	EE	14.54	331>316 331>171	5 20

## 8 Interpretation of results and strategy of analysis

### 8.1 - Identification of the analytes

The result of the blank sample analysis must be taken into account. Corresponding diagnostic chromatograms must be analysed in detail and compared to the samples. Internal and external standards alone must be detected.

Each chromatographic peak, in order to be taken into account, needs to comply with the criteria set out in Decision 2002/657/CE, ie.:

- Internal and external standards are present ( S/N ratio > 3),
- Ratio between analyte retention time and internal standard retention time, ie. Analyte relative retention time, is equal to that of the standard analyte with a tolerance of  $\pm 0.5$  %,
- At least two analyte diagnostic transitions are detected (S/N ratio > 3),
- Intensity ratio of the two diagnostic transitions must correspond to the value obtained for the standard analyte, with a tolerance as indicated in the Table below.

**Table 3:** Tolerated variability for the intensity ratios between two diagnostic signals obtained by GC-MS/MS  
(reference: 2002/657/EC Decision).

Ratio between the measured transition and the most intense transition	Tolerated variability
> 50 %	20 %
20-50 %	25 %
10-20 %	30 %
< 10 %	50 %

## 8.2 - Quantification

- During the first screening analysis, quantification is made based on the supplemented blank sample:

The relative areas (analyte area / internal standard area) are compared and a first concentration is estimated based on the following calculation:

Example of calculation for the determination of melamine concentration:

$$\text{Concentration}_{\text{sample}} \text{ (in ppm)} = 2.5 \times [\text{Area}_{342>327} / \text{Area}_{348>333}]_{\text{sample}} / [\text{Area}_{342>327} / \text{Area}_{348>333}]_{\text{addition}}$$

Quantification during the screening step can be made on the Excel sheet provided by LABERCA.

- During the confirmation analysis, quantification is made based on the calibration curve: from the equation obtained with the calibration curve, the concentration is calculated by transferring the relative area on the curve.

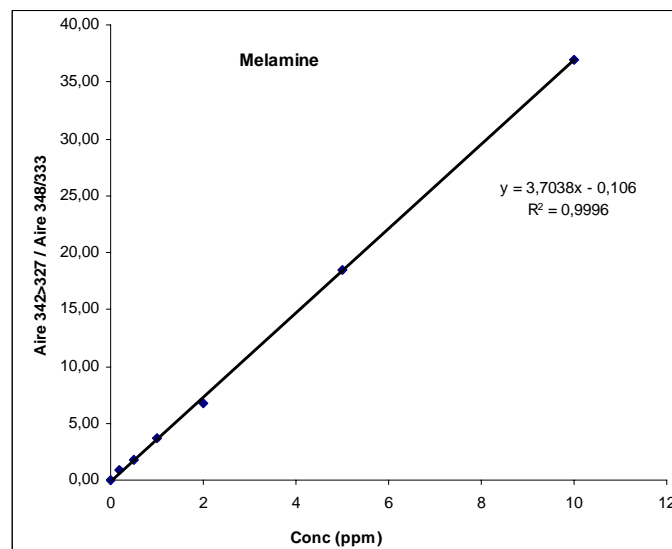


Figure 1: Example of calibration curve obtained for melamine

## 8.3 – Expression of the results

During the first screening analysis, if melamine is identified and detected at a concentration level below  $1.25 \text{ mg.kg}^{-1}$ , the sample is declared as **compliant**. Above this value, the sample is declared as suspect and the analysis is repeated according to the confirmation protocol (Paragraph 7).

During the second confirmation analysis, the sample is declared as **non-compliant** if the concentration measured is higher than the decision limit ( $CC\alpha$ ) as defined during method validation.

If the concentration is lower than the  $CC\alpha$ , the sample is declared as compliant.

In all cases, enter the estimated concentrations in an Excel file (source: LABERCA) summing up the identification sheet of each sample.

The analysis report must show the following information:

• If a sample is compliant (melamine concentration < CC $\alpha$ ):

« **Sample is compliant – Reference document: EC Decision 2008/798/EC** »

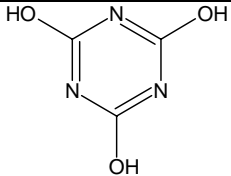
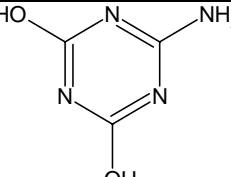
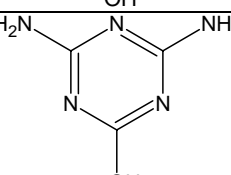
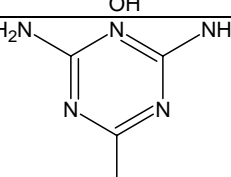
• If a sample is non-compliant (melamine concentration  $\geq$  CC $\alpha$ ):

« **Sample is non-compliant, melamine concentration: X,Y ppm – Reference document: EC Decision 2008/798/EC** »

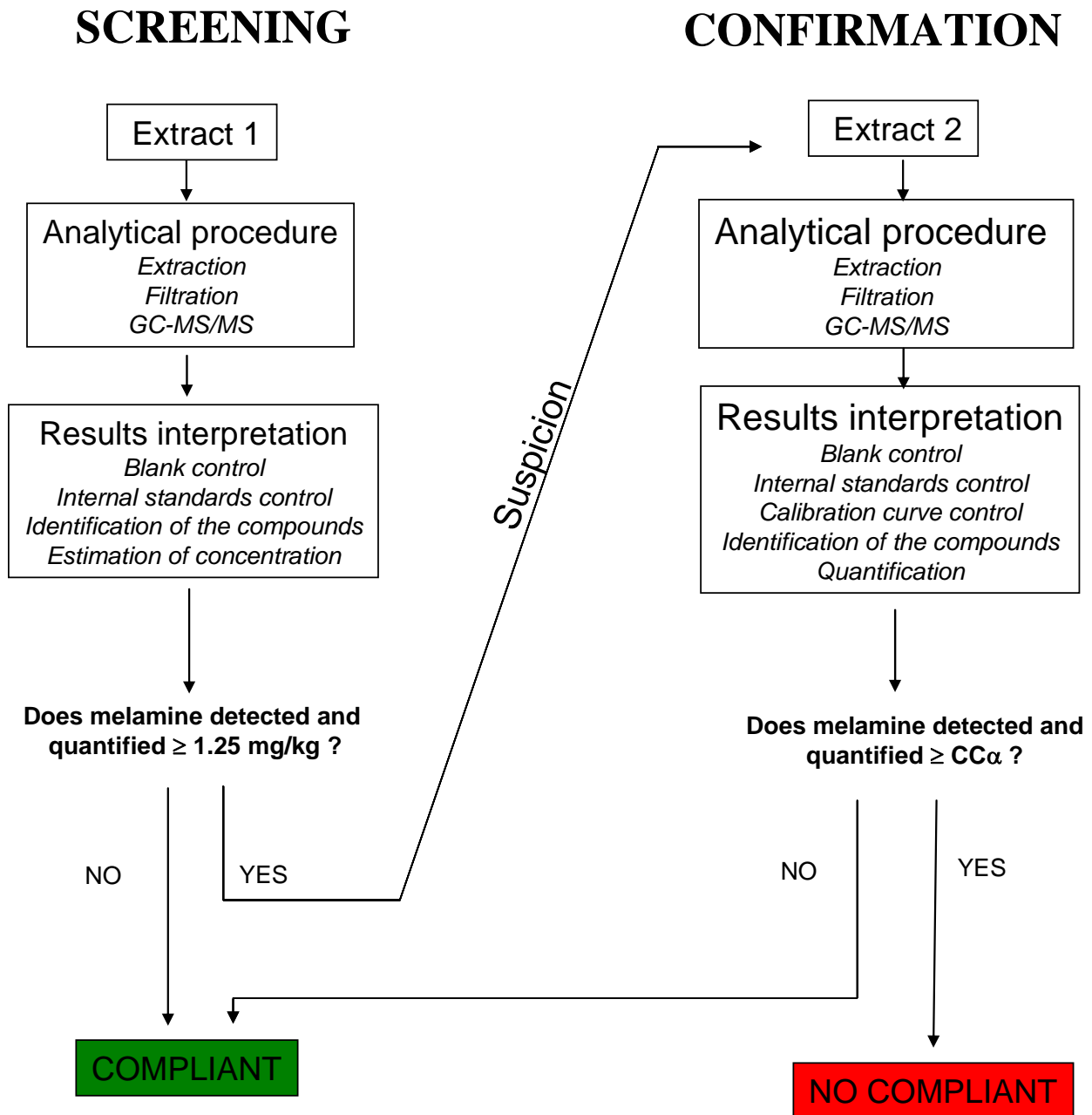
## 9 References

- 1 - “GC-MS Method for screening and confirmation of melamine and related analogs”, US EPA method, Version 2 May 2007 (<http://www.fda.gov/cvm/GCMSscreen.htm>).
- 2 - Commission Decision 2002/657/EC, Official J. European Communities, No. L221/8..
- 3 – Commission Decision 2008/798/EC of 14 October 2008, imposing special conditions governing the import of products containing milk or milk products originating in or consigned from China, and repealing Commission Decision 2008/757/EC.

## ANNEX I: Chemical structure of the screened compounds

Name	Molecules	Raw formula	Molecular weight (g.mol <sup>-1</sup> )	Mass after derivatisation
Cyanuric acid		C <sub>3</sub> H <sub>3</sub> N <sub>3</sub> O <sub>3</sub>	129	345
Ammelide		C <sub>3</sub> H <sub>4</sub> N <sub>4</sub> O <sub>2</sub>	128	344
Ammeline		C <sub>3</sub> H <sub>5</sub> N <sub>5</sub> O	127	343
Melamine		C <sub>3</sub> H <sub>6</sub> N <sub>6</sub>	126	342

ANNEX II: ANALYTICAL STRATEGY



ANNEX III : EXAMPLE OF AN INTERPRETATION SHEET



FEUILLE DE CRITERES D'IDENTIFICATION (2002/657/CE) POUR LES ANALYSES DE CONFIRMATION PAR CHROMATOGRAPHIE COUPLEE A LA SPECTROMETRIE DE MASSE  
Version 2

Date	17/10/2008	GC-EI-MS	GC-CI-MS	GC-HR-MS	GC-MS/MS XXXXXXXXXXXX	LC-MS/MS
------	------------	----------	----------	----------	--------------------------	----------

Analyte :	Melamine		Ajout	Echantillon	Echantillon	Echantillon	Echantillon	Echantillon	Echantillon
Matrice :									
Références Echantillons			Aj 2,5 ppm	Ech 1	Ech 2	Ech 3			
Tr Etalon			11,250	11,350	11,350	11,350			
Tr Analyte			11,270	11,360	11,360	11,360			
Tr Analyte / Tr Etalon			1,002	1,001	1,001	1,001			
Tolérance Tr +/- (%)	0,5		0,997   1,007	IN	IN	IN			
Abondance Etalon			3134	1248	1109	1188			
Abondance signal 1 (PB)	342	>	327	13881	157	1080	3904		
Abondance signal 2	342	>	285	1312	87	412			
Abondance signal 3	342	>	171	4423	71	323	1212		
Abondance signal 4		>							
Rapport 1 (signal 2 / signal 1)			0,095		0,081	0,106			
Tolérance Rapport 1 +/- (%)	50		0,047   0,142		IN	IN			
Rapport 2 (signal 3 / signal 1)			0,319	0,452	0,299	0,310			
Tolérance Rapport 2 +/- (%)	25		0,239   0,398	OUT	IN	IN			
Rapport 3 (signal 4 / signal 1)									
Tolérance Rapport 3 +/- (%)									
Points d'identification				2,5	5,5	5,5			
Conclusion			<del>X</del>	PRESENCE NON CONFIRMEE	PRESENCE CONFIRMEE	PRESENCE CONFIRMEE			
Concentration estimée	Unité :	ppm	2,50	0,07	0,55	1,85			
Commentaires									

  Informations générales à saisir
   Signaux à saisir
   Calcul/Conclusion automatique

Visa Operateur	
----------------	--

Visa Responsable	
------------------	--

## ANNEX IV: CRITICAL POINTS

Critical points	Identification of the criteria					Checks		Corrective action
	Parameter	Target value	Tolerance	Equipment	Danger	Control	Record	
<b>Sample preparation</b>								
Grinding / Sampling	Sample representability			Grinder	Measurement is not representative of the sample	Visual	None	Redo sampling
Weighing step	Weighing	X mg	1 decimal reading	Weighing scale	Screening is not correct	Weighing scale check	Metrology report	Redo analysis
<b>Derivatisation</b>								
Evaporation	Nitrogen flow and rotavapor	Flow and vacuum have been controlled		Evaporation ramp and rotavapor	Presence of water residues	Visual check	None	Redo analysis
<b>Detection</b>								
Sensitivity/Specificity	S/N	> 5 for internal external standards		Spectrometer	Loss in sensitivity	Addition	Injection sheet	Change column, insert or septum Clean source