



Agence fédérale pour la sécurité de la chaîne alimentaire  
(Federal Agency for the Safety of the Food Chain – FASFC)

Laboratoire fédéral pour la sécurité alimentaire- Liège

### I-MET 063

**Determination and quantification by GC-MS of melamine (1 mg/kg), ammeline and cyanuric acid (20 mg/kg)**

**Date of coming into force : 28 September 2008**

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### List of revisions

Revision by/date*	Validation by / date**	Reason of revision	Amended part of text / Importance of revision
30-09-08 Pallares O.	01-10-08 Etienne-Thewissen F.	Validation : change from 20 to 2,5 mg/kg Change of IS	
06-10-08 De Tandt Th.	07-10-08 Etienne-Thewissen F.	Validation : change from 2,5 to 1 mg/kg Added : GC-MS Quad	

\* The time interval between this date and the latest revision must not exceed 5 years.

\*\* Adjust the number of revision and the year (when relevant). Upon approval, adjust the date of coming into force (taking into account the time needed to inform the staff members involved).

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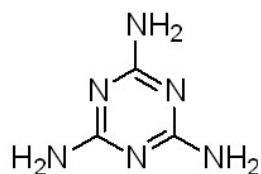
**Head of Section :** Fabian Etienne-Thewissen

# Determination and quantification by GC-MS of melamine (1 mg/kg), ammeline and cyanuric acid (20 mg/kg)

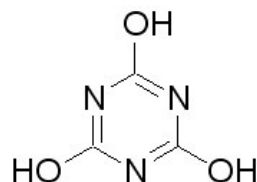
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# 1 Subject

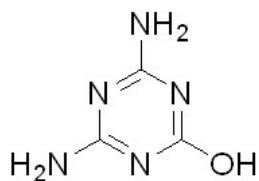
The procedure developed makes it possible to quantify and to confirm the presence of melamine at a concentration of more than 1 mg/kg and of two related compounds – cyanuric acid and ammeline - at a concentration of more than 20 mg/kg. It should be noted that we also included ammelide, a third melamine derived molecule, in this method but that due to problems with the determination, the method could not be validated for the detection and/or the quantification of that substance.



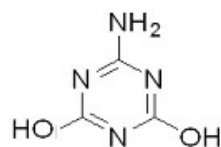
Melamine



Cyanuric acid

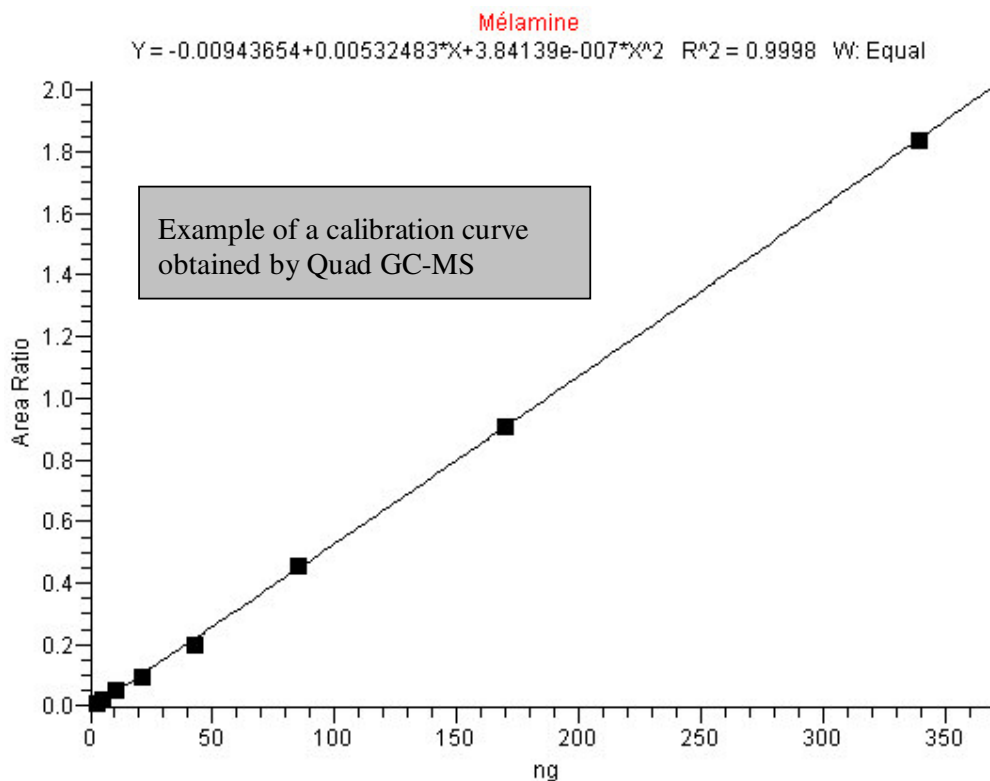


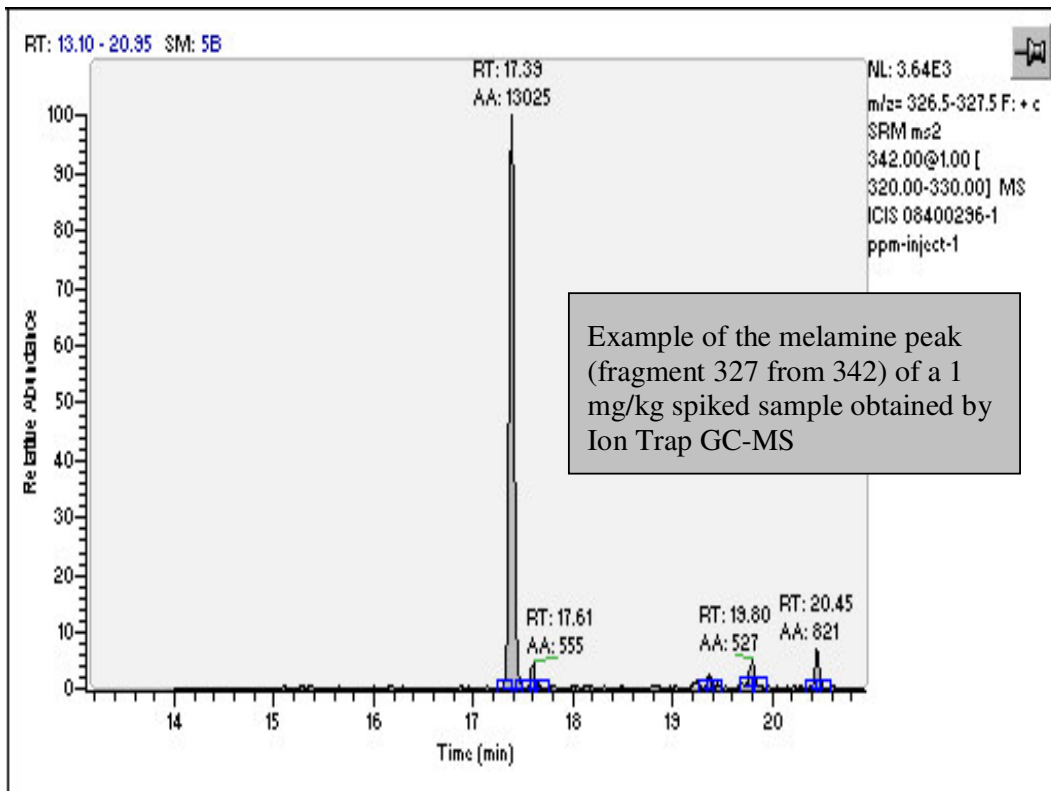
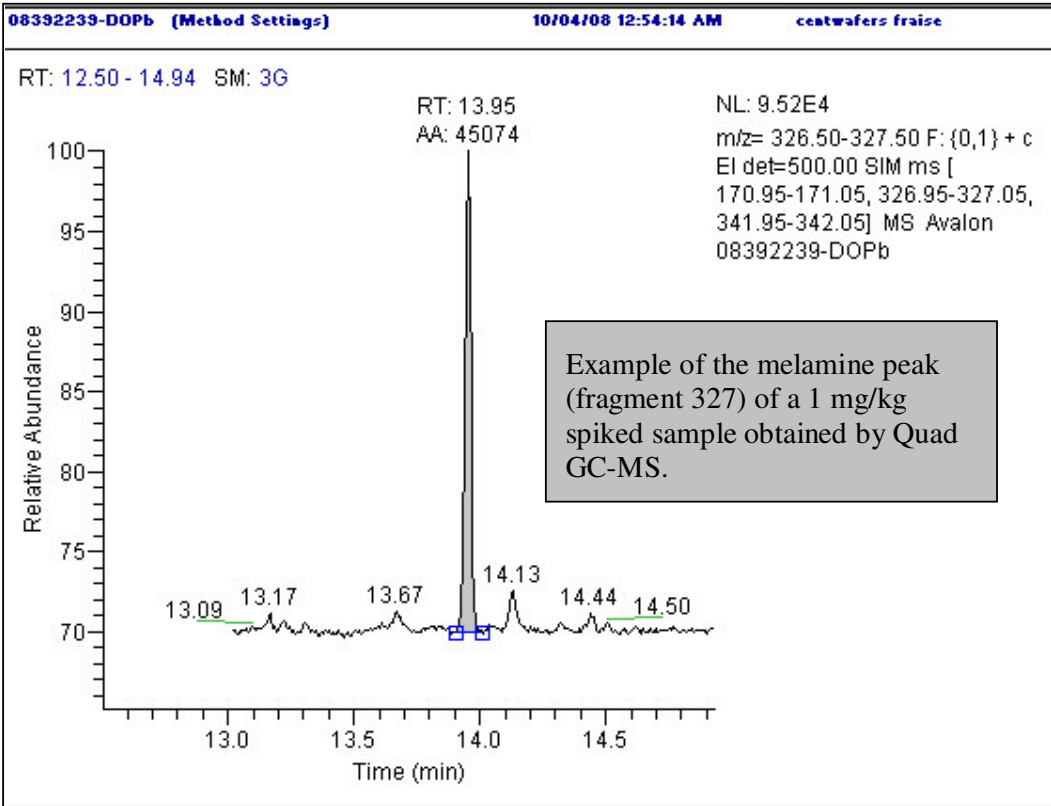
Ammeline



Ammelide

The calibration proposed in this method makes it possible to cover a very wide range of melamine concentrations : the lowest point of the curve represents a sample containing 0,5 mg melamine/kg whereas the highest point represents a sample containing 64 mg melamine/kg. As a consequence, it is not necessary to restart the analysis on diluted samples when samples with substantial melamine contamination must be analysed.





## 2 Scope

The method is validated on matrices such a milk powder, candy and biscuits. It should also be applicable to soy protein, gluten and other foodstuffs of plant origin for which we did not find a matrix effect.

## 3 Legislation and standards

GC-MS Method for Screening and Confirmation of Melamine and Related Analogs (Version 2 May 7, 2007). US Food and Drug Administration.

## 4 Definitions and abbreviations

BSTFA – 1% TMCS : bis(trimethylsilyl)trifluoroacetamide – 1% trimethylchlorosil.

DEA : diethylamine

ACN : acetonitrile

S/N : signal to noise ratio

SIM : selected ion monitoring

TIC - MS/MS : alternative acquisition of the signal in TIC (total signal) and MS/MS.

RSD : relative standard deviation

IS : internal standard

R123 : R followed by a three digits number represents a laboratory specific reference to a reagent.

## 5 Principle

The samples are extracted by means of a mixture of diethylamine/water/acetonitrile and the derivatised analytes (BSTFA-TMCS) are searched for by GC-MS. The method was tested with two different detectors : a simple quadrupole and an Ion Trap.

With the quadrupole, the detection and the quantification of ammeline and melamine are done in SIM mode. The tests show that cyanuric acid cannot be detected at a 20 ppm concentration by a simple quadrupole.

With the Ion Trap, the detection and the quantification of cyanuric acid, ammeline and melamine are done in TIC - MS/MS mode.

## 6 Performance indicators

### 6.1 Peak identification.

The retention times of the sample analytes must not differ more than 0,05 minute from the mean retention times of the corresponding analytes in the spiked samples of the sequence.

Moreover, as for melamine, the characteristic fragment ratios (171, 327, 342) of the sample must be within the ranges mentioned in the table below :

171	327	342
31,1 % to 40,8 %	28,2 % to 45,4 %	16,3 % to 38,2 %

These characteristic fragment ratios result from the 1ppm melamine validation file.

Finally, the signal detected and confirmed at the retention times of the analytes under detection must be at least 5 times the background noise.

The spectra of the 4 molecules are represented below for further information :

SRD5UGTIC #415-432 RT: 19.65-19.79 AV: 18 NL: 1.70E6  
F: +c Full ms [ 100.00-400.00]

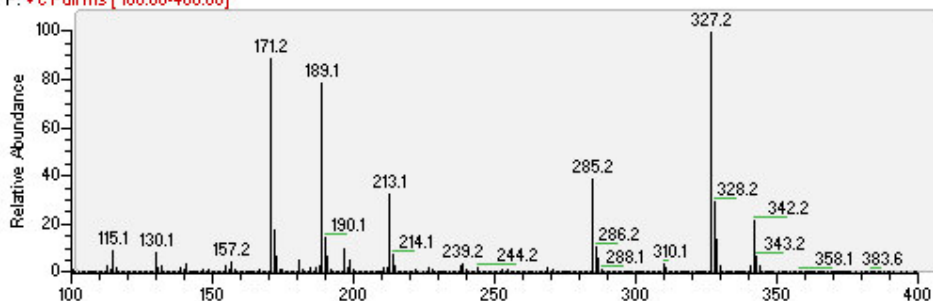


Diagram 1 : Spectrum of melamine (TMCS derived)

SRD5UGTIC #60-72 RT: 16.56-16.66 AV: 13 NL: 6.74E5  
F: +c Full ms [ 100.00-400.00]

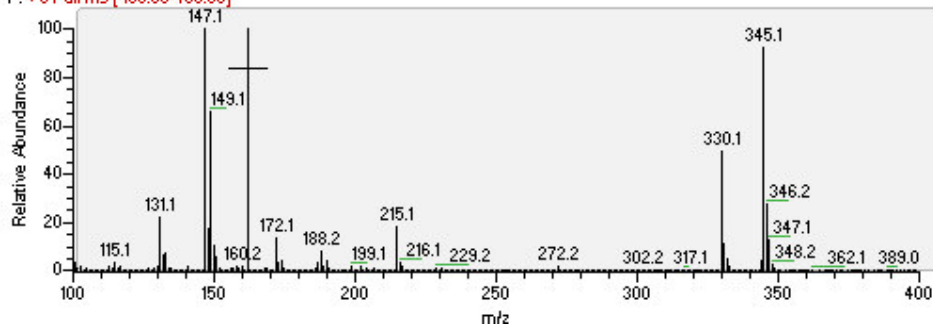


Diagram 2 : Spectrum of cyanuric acid (TMCS derived)

SRD5UGTIC #323-335 RT: 18.87-18.97 AV: 13 NL: 1.44E6  
F: +c Full ms [ 100.00-400.00]

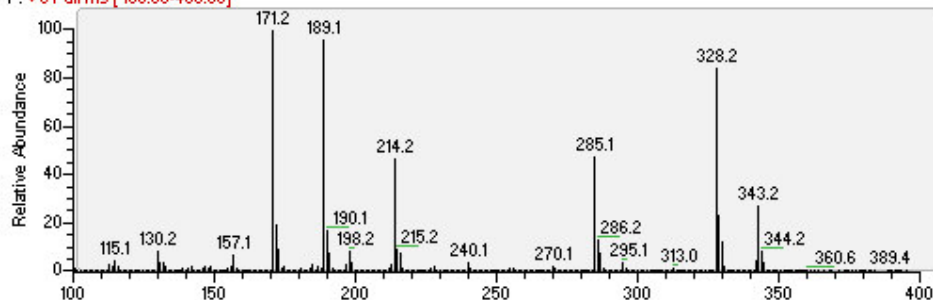


Diagram 3 : Spectrum of ammeline (TMCS derived)

SRD5UGTIC #205-216 RT: 17.84-17.94 AV: 12 NL: 7.81E4  
F: +c Full ms [ 100.00-400.00]

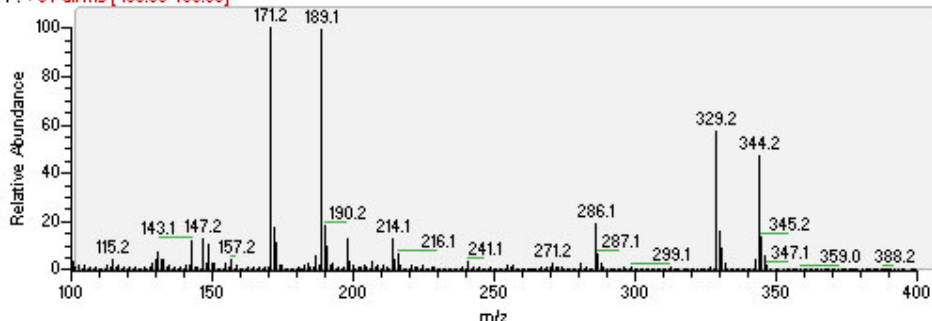


Diagram 4 : Spectrum of ammelide (TMCS derived)

## 6.2 Sequence control

The determination coefficient of the calibration curve must be above 0,99.

The blank matrix must not show any peak of more than 3 times the background noise at the retention times of the analytes under detection.

The calculated content of the spiked samples must amount to 70 to 120 % of the theoretical content.

If this is not so, start again the preparation of spiked samples and/or of the blank and/or of the calibration solutions and re-inject the sequence.

## 7 Safety instructions and particular measures

Follow the safety instructions mentioned on the safety charts of the reagents.

All solvent handling must take place under a hood.

## 8 Reagents and prepared solutions

### 8.1 Reagents

- 8.1.1 Melamine (2,4,6-Triamino-1,3,5-triazine -  $C_3H_6N_6$ ) > 99% (CAS 108-78-1 – R644)
- 8.1.2 Cyanuric acid (1,3,5-Triazine-2,4,6-triol 2,4,6-Trihydroxy-1,3,5-triazine -  $C_3H_3N_3O_3$ ) > 98% (CAS 108-80-5 – R647)
- 8.1.3 Ammeline (4,6-Diamino-2-hydroxy-1,3,5-triazine -  $C_3H_5N_5O$ ) > 99,5% (CAS 645-92-1 – R652)
- 8.1.4 Ammelide (2-Amino-4,6-dihydroxy-1,3,5-triazine -  $C_3H_4N_4O_2$ ) > 99,5% (CAS 645-93-2)
- 8.1.5 Benzamide (Benzoic amide-  $C_6H_5CONH_2$ ) 99% (CAS 55-21-0 – R424)
- 8.1.6 BSTFA – 1% TMCS – R648
- 8.1.7 HPLC – R016 grade methanol
- 8.1.8 HPLC – R472 grade acetonitrile
- 8.1.9 HPLC – R650 grade DEA
- 8.1.10 HPCL – R081 p.a. grade pyridine
- 8.1.11  $H_2O$  ultrapure – R144

## 8.2 Solutions

### 8.2.1 Extraction solution – DEA/H<sub>2</sub>O/ACN (10/40/50, v/v/v).

Prepare a stock solution with 10 parts of DEA, 40 parts of H<sub>2</sub>O and 50 parts of ACN. The solution is kept in the refrigerator and in the dark.

### 8.2.2 Internal standard solution – Benzamide.

In a volumetric flask, prepare a benzamide solution at a 4 µg/ml concentration in pyridine. Dissolve for 5 minutes in an ultrasonic bath. Prepare a sufficiently large amount of this solution (used at a rate of 100 µl per solution to be injected).

### 8.2.3 1 mg/ml melamine solution

Prepare, in a volumetric flask of 10 ml, a 1 mg/ml melamine solution in a DEA/H<sub>2</sub>O mixture (20/80, v/v). Dissolve for 15 minutes in an ultrasonic bath.

### 8.2.4 Mother solution of the 4 molecules.

Weigh 20 mg of each of the 3 derivative molecules (8.1.2, 8.1.3, 8.1.4) in a volumetric flask of 50 ml. Add 1 ml of the 1 mg/ml melamine solution (8.2.3). Make up to the mark with the DEA/H<sub>2</sub>O mixture (20/80, v/v). Dissolve for 15 minutes in an ultrasonic bath.

This solution has a melamine concentration of 0,02 mg/ml and a concentration of 0,4 mg/ml for the 3 derivative molecules.

### 8.2.5 Levels 7 and 8 calibration solution

Dilute the mother solution 6,25 times with the DEA/H<sub>2</sub>O mixture (20/80, v/v) : 4 ml in a volumetric flask of 25 ml.

This solution has a melamine concentration of 3,2 µg/ml and a concentration of 64µg/ml for the 3 derivative molecules.

### 8.2.6 Levels 4, 5 and 6 calibration solutions

Dilute the mother solution 50 times with the DEA/H<sub>2</sub>O mixture (20/80, v/v) :1 ml in a volumetric flask of 50 ml.

This solution has a melamine concentration of 0,4 µg/ml and a concentration of 8 µg/ml for the 3 derivative molecules.

### 8.2.7 Levels 1, 2 and 3 calibration solutions

Dilute the mother solution 400 times with the DEA/H<sub>2</sub>O mixture (20/80, v/v) : 2,5 ml of the levels 4, 5 and 6 (8.2.6.) calibration solution in a volumetric flask of 20 ml.

This solution has a melamine concentration of 0,05 µg/ml and a concentration of 1 µg/ml for the 3 derivative molecules.

### 8.2.8 Spiking solution

Weigh 20 mg of each of the three derivative molecules (8.1.2., 8.1.3., 8.1.4.) in a volumetric flask of 10 ml. Add 1 ml of the 1 mg/ml melamine solution (8.2.3). Make up to the mark with the DEA/H<sub>2</sub>O mixture (20/80, v/v). Dissolve for 15 minutes in an ultrasonic bath.

This solution has a melamine concentration of 0,1 mg/ml and a concentration of 2,0 mg/ml for the 3 derivative molecules.

### 8.2.9 Diluted spiking solution.

Dilute the spiking solution (8.2.8.) 20 times with the DEA/H<sub>2</sub>O mixture (20/80, v/v) : 1 ml in a volumetric flask of 20 ml.

This solution has a melamine concentration of 5 µg/ml and a concentration of 100 µg/ml for the 3 derivative molecules.

Note : Injection vials containing 5 µg of the analytes under detection were prepared and evaporated to dryness under nitrogen flow and on sand bath during the validation tests of the

method. The vials are closed hermetically and kept at – 20°C. These vials are used in a qualitative approach, particularly after a change of column or a maintenance intervention in order to check the drift of the retention times and the quality of the signal and the spectra. While in use, take the dry extract with 50 µl of solvent and evaporate under nitrogen flow in order to eliminate all traces of water. Add 200 µl of BSTFA-1%TMCS and 200 µl of pyridine into the vial. After vortex treatment, put the vial in the oven at 45 mn for 70°C. The concentrated standard solution (5 µg/vial) will then be ready for injection.

## 9 Equipment

- 9.1 GC (Trace 2000) with an Ion Trap (Polaris ®) detector and GC (Trace 2000 ®) with a simple quadrupole detector (Finnigan).
- 9.2 DB-5MS column of 30 m x 0,25 mm ; 0,1 µm phase (tested brand : J&W ®).
- 9.3 Thermostatised oven at 70°C.
- 9.4 Evaporation system at 60°C under nitrogen flow.
- 9.5 Analytical balance with a 0,0001 g precision.
- 9.6 Vortex.
- 9.7 Crusher with which candy can be reduced to powder (type IKA ®).
- 9.8 Mortar to crush biscuits.
- 9.9 Centrifuge to treat tubes of the Falcon ® type of 50 ml at 2500 r/min.
- 9.10 Ultrasonic bath.
- 9.11 Orbital shaker.
- 9.12 Micropipettes of different volumes (from 50 to 200 µl and from 100 to 1000µl)
- 9.13 Usual laboratory glassware.
- 9.14 Disposable plastic syringes, type Luer-Lock ®.
- 9.15 Polyamide Chromfil ® filters, 0,2 µm porosity.
- 9.16 Falcon ® tubes of 50 ml.

## 10 Operating mode

### 10.1 Chromatographic conditions

10.1.1 Parameters for Ion Trap GC-MS (details : see Annex A) :

Injection volume : 1 µl

Injector temperature : 180°C

Splitless injection (1 mn)

Split flow : 40 ml/mn

Gas : Helium - 2 ml/mn

Oven parameters :

Initial temperature : 70 °C for 2 mn

Rate : 10 °C / mn

Final temperature : 250 °C for 5 mn

Detector parameters : Annex A gives the parameters for the detection of 1 mg/kg melamine : MS/MS : 342->327. If cyanuric acid is under detection, add window 345->330 and for ammeline, add window 343->328.

Analysis time : 25 mn

10.1.2 Parameters for quadrupole GC-MS (details : see Annex B) :

Injection volume : 1 µl

Injector temperature : 210 °C

Splitless injection (0,2 mn)

Split flow : 40 ml/mn

Gas : Helium - 1 ml/mn

Oven parameters :

Initial temperature : 80 °C for 2 mn

Rate : 10 °C / mn

Final temperature : 250 °C for 2 mn

Detector parameters : Annex A gives the parameters for the detection of 1ppm melamine : SIM window : 327, 342 and 171. If ammeline is under detection, add window SIM : 343 and 328.

Analysis time : 21 mn

## 10.2 Preparing the calibration curve

This curve has 8 points with increasing analytes concentrations.

Take from levels 1, 2 and 3 calibration solution (8.2.7) volumes of 50 µl, 100 µl and 200 µl and pour them into vials. These vials are referenced L1 to L3.

Take from the levels 4, 5 and 6 calibration solution (8.2.6) volumes of 50 µl, 100 µl and 200 µl and pour them into vials. These vials are referenced L4 to L6.

Take from the levels 7 and 8 calibration solution (8.2.5) volumes of 50 µl and 100 µl and pour them into vials. These vials are referenced L7 and L8.

The vials are evaporated under a light nitrogen flow in an oven thermostatised at 60°C. After evaporation to dryness, add 100 µl of the internal standard solution ( 8.2.2), 200 µl of pyridine and 200 µl of BSTFA-1%TMCS.

Screw the cover on the vial, shake briefly in the vortex and put the vial in an oven at 70°C for 45 minutes. The **calibration solutions** are then ready for injection.

Theoretical amount of analyte for each level prepared in this manner :

Amount (ng)	Melamine	Derivative molecules
level 1 :	2,5	50
level 2 :	5,0	100
level 3 :	10,0	200
level 4 :	20,0	400
level 5 :	40,0	800
level 6 :	80,0	1600
level 7 :	160,0	3200
level 8 :	320,0	6400

Table 1 : amount of analyte (ng) / level

## 10.3 Preparing the sample

Weigh in a Falcon tube, to the nearest 0,0001 g, approximately 0,5 g of homogenised sample of a representative sample portion. Add 20 ml of the extraction solvent mixture (8.2.1) with a pipette. Shake vigorously (by hand or with the vortex). Put the Falcon tubes in the ultrasonic bath for 15 minutes. Then, put the tubes on the orbital shaker for 15 minutes. Centrifuge at 2500 r/min for 5 minutes. Filter a portion of the supernatant on a Chromafil filter (0,2 µm).

Transfer 200 µl of the filtrate to a vial and evaporate under a light nitrogen flow in an oven thermostatised at 60 °C. After evaporation to dryness, add 100 µl of the internal standard solution (8.2.2), 200 µl of pyridine and 200 µl of BSTFA-1%TMCS.

Note : the filtrate must be entirely evaporated because the presence of water will prevent the derivatisation of the analytes under detection.

Screw the cover on the vial, shake briefly in the vortex eand put the vial in an oven at 70 °C for 45 minutes. The sample is then ready for injection.

Note : If insoluble material is found to be present at the bottom of the vial, transfer the supernatant solution to another vial prior to injection.

A flow chart of this procedure is given in Annex C.

## 10.4 Preparing spiked samples.

Per analysis sequence is spiked a blank matrix that is representative of the nature of the sample to be analysed.

### 10.4.1 Low concentration spiked sample

To achieve this, follow the procedure for preparing samples (10.3) and add 100 µl of the diluted spiking sample (8.2.9) to the test sample. The blank matrix prepared in this manner has a melamine content of 1 mg/kg.

### 10.4.2 High concentration spiked sample

To achieve this, follow the procedure for preparing samples (10.3) and add 200 µl of the spiking solution (8.2.8) to the test sample. The blank matrix prepared in this manner has a melamine content of 40 mg/kg.

## 10.5 Preparing a blank matrix

Per analysis sequence is prepared a blank matrix that is representative of the nature of the sample to be analysed. This is done according to the procedure for preparing samples (10.2).

# 11 Quality control and chromatographic analysis

## 11.1 Checking the status of the chromatographic system

Inject one of the vials containing 5 µg of analytes (note 8.2) in order to check the working order of the chromatographic system (peaks at the right retention times and signal of the same amplitude as in former tests).

## 11.2 Injecting solutions

Sequence of injection :

Calibration solution : L1→L9 (1×)

Blank matrix solution (1×)

Spiked sample solution (1×)

Sample solution : no more than 6 samples

Spiked sample solution (1×)

Sample solution : no more than 6 samples

Spiked sample solution 1 (1×)

...

## 12 Calculating results

### 12.1 Calculation

Draw the calibration curve on the basis of the values mentioned in Table 1 (corrected by means of accurate weighings and the purity rates of the standards used) for the calibration levels. The curve must be calculated for at least 5 levels. Neither the lowest nor the highest level may be eliminated.

Calculate the analyte content with the following formula :

$$\text{Analyte content (mg/kg)} = Q / (10 \times P)$$

with

Q = Amount found in the 200 µl sample solution (ng) : value supplied by the software on the basis of the calibration line obtained.

P = Test sample (g)

### 12.2 Reporting

If an analysis reveals the presence of one of the analytes under detection, the report will mention for each of the analytes involved :

« Presence of "name of the analyte" at a concentration of X mg/kg ».

If no signal has been detected, the report will mention for each of the analytes involved :  
« "name of the analyte" not detected at the limit of 1 mg/kg ».

The file of each analysis sequence shall contain :

- The applications for analysis ;
- The weighing data ;
- The implementation reports (F543) ;
- The sequence injected ;
- The executive Excel file (raw data and calculations).

## 13 List of reference documents

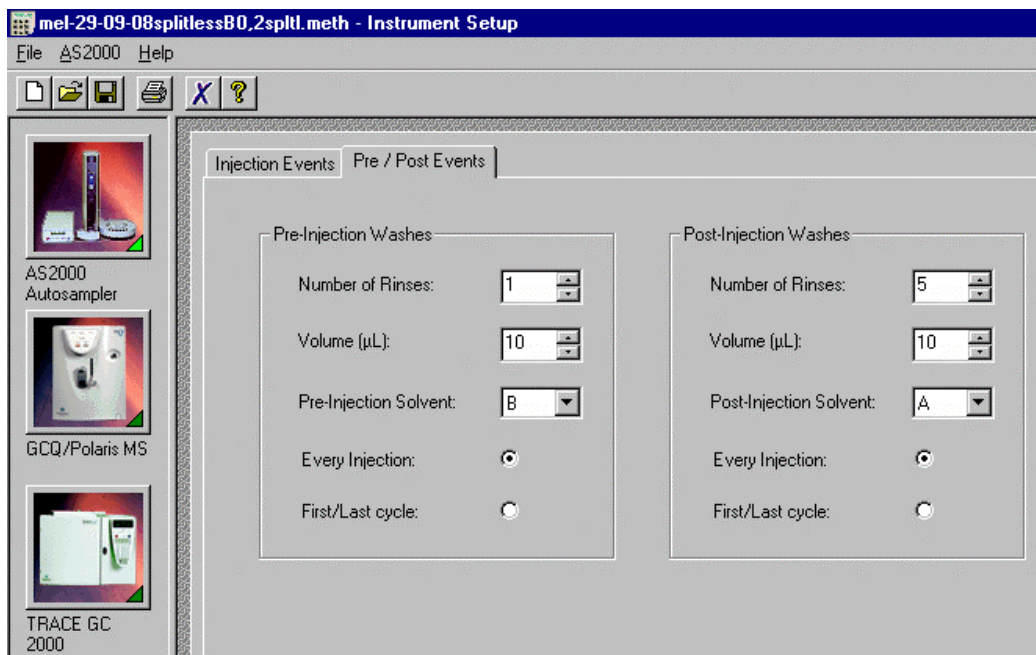
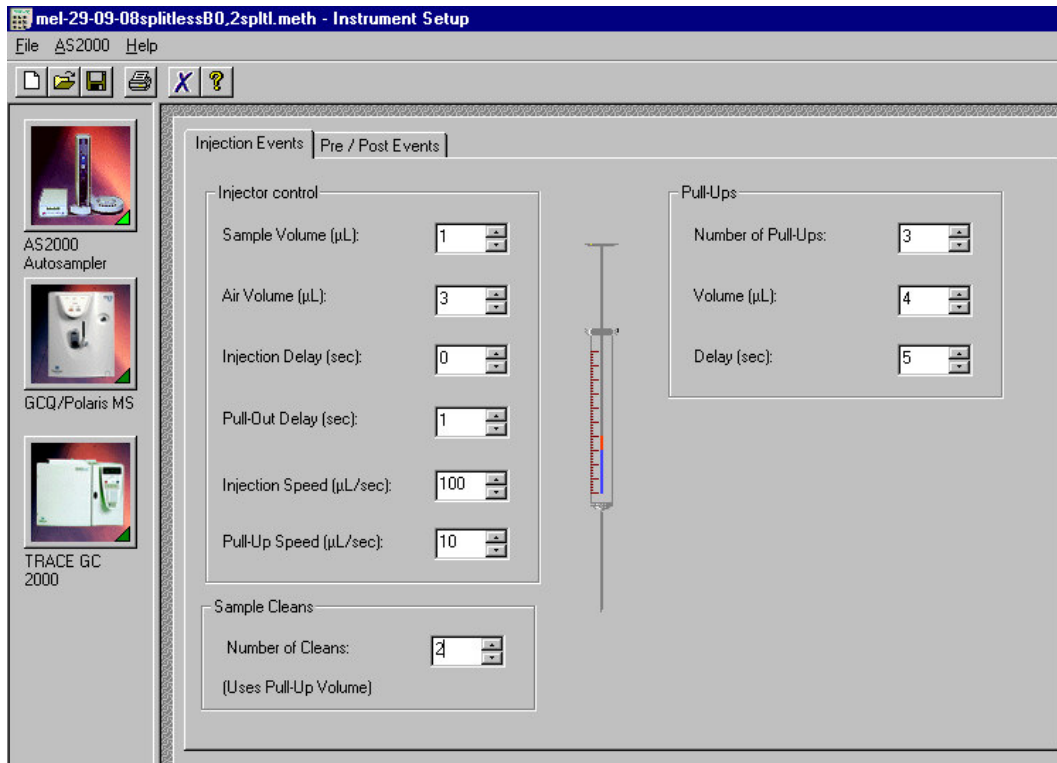
### 13.1 Form

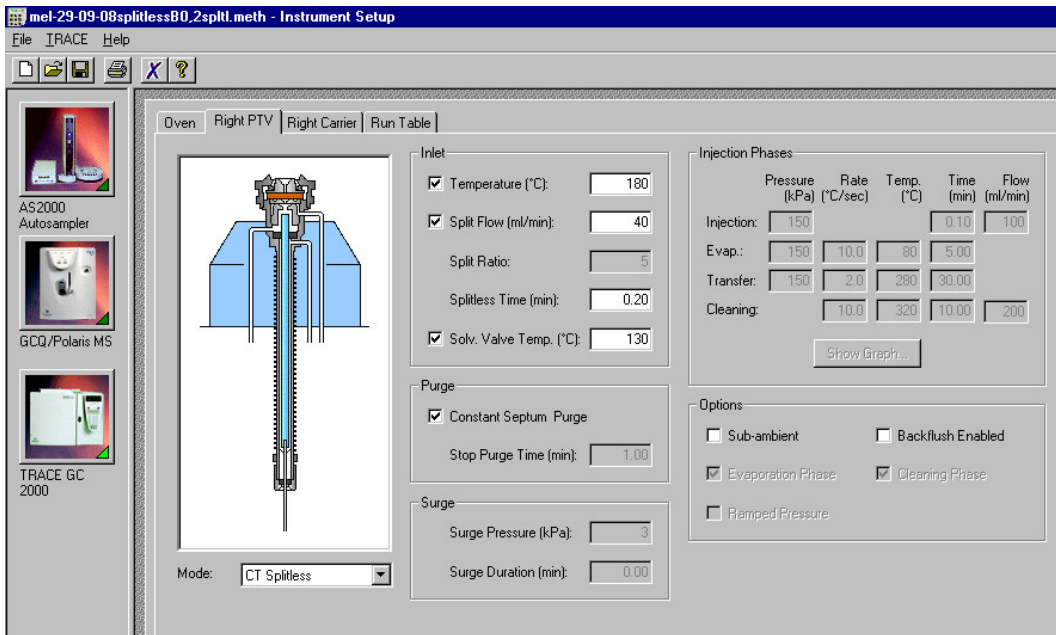
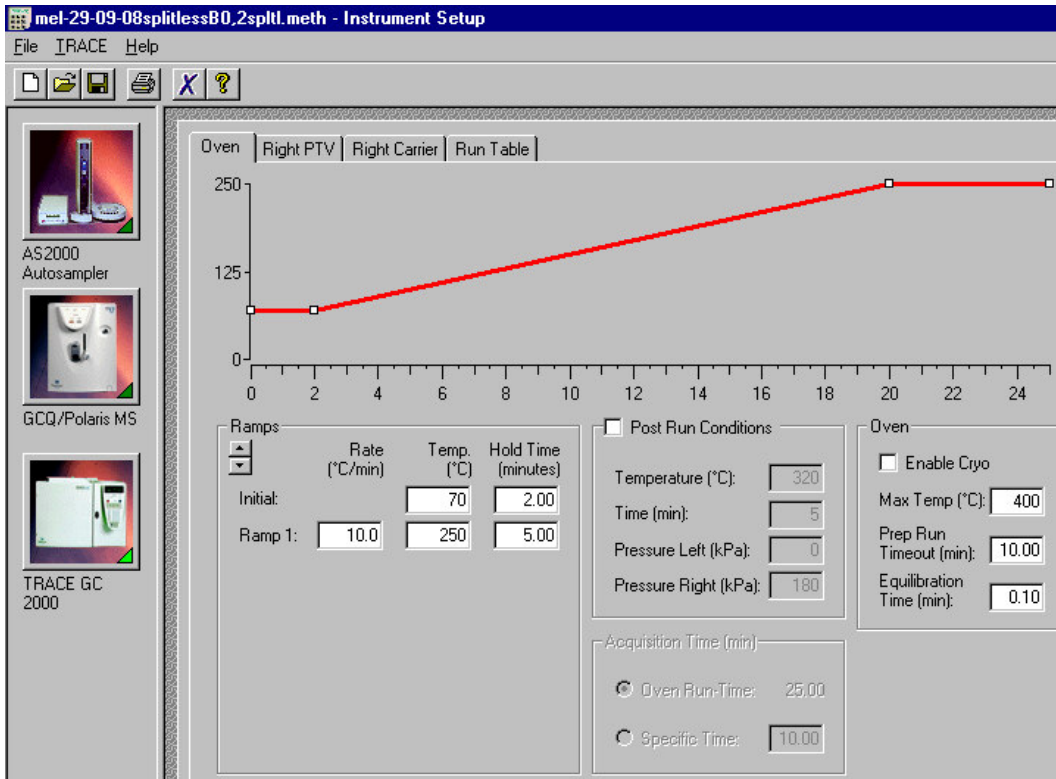
F543                      Implementation report

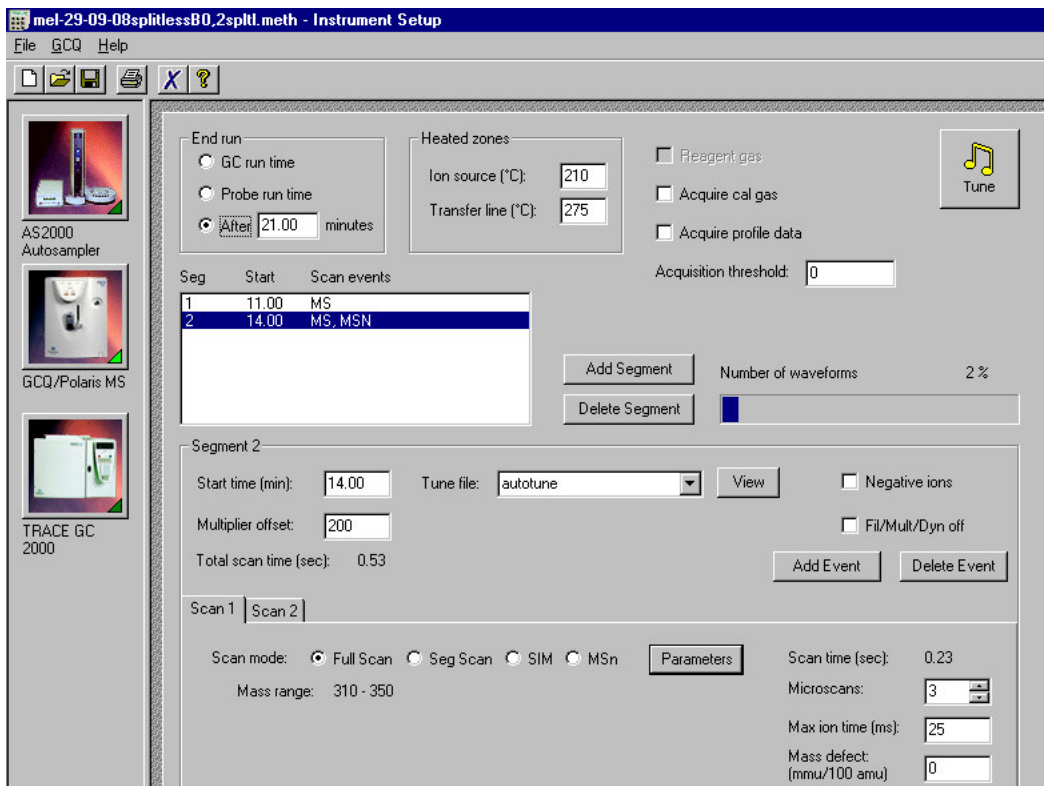
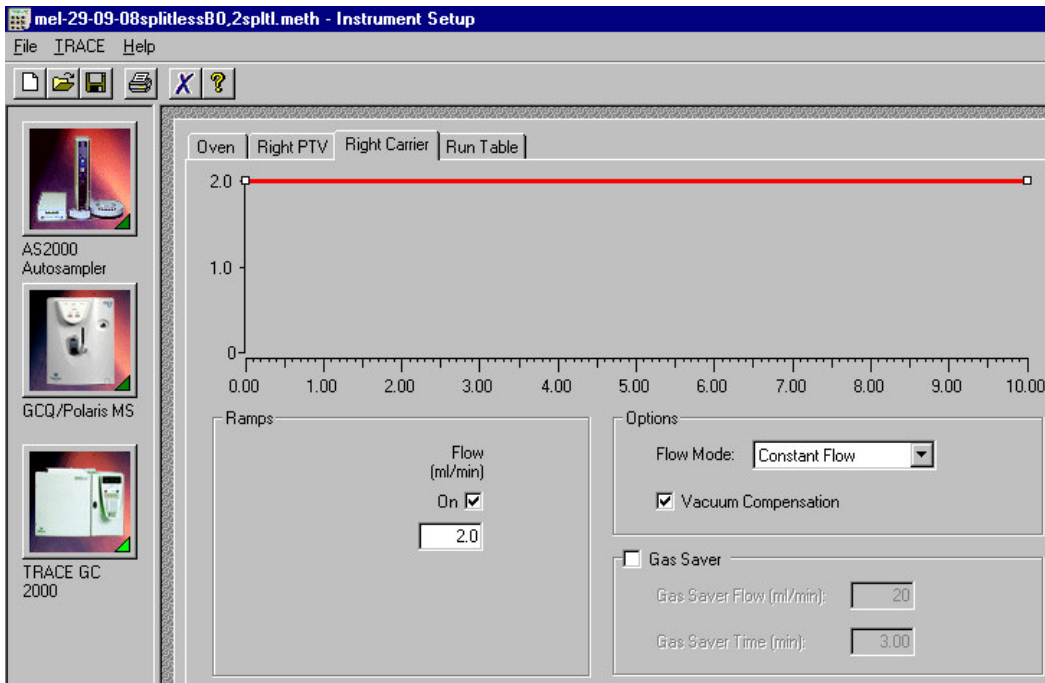
### 13.2 Annexes

Annex A                      Parameters for Ion TrapGC-MS  
Annex B                      Parameters for quadrupoles GC-MS  
Annex C                      Flowchart of sample preparing procedure

# Annex A : Parameters for Ion Trap GC-MS







mel-29-09-08splitlessB0\_2splll.meth - Instrument Setup

File GCQ Help

AS2000 Autosampler  
GCQ/Polaris MS  
TRACE GC 2000

End run:  
 GC run time  
 Probe run time  
 After 21.00 minutes

Heated zones:  
 Ion source (°C): 210  
 Transfer line (°C): 275

Reagent gas  
 Acquire cal gas  
 Acquire profile data  
 Acquisition threshold: 0

Seg	Start	Scan events
1	11.00	MS
2	14.00	MS, MSN

Add Segment    Number of waveforms: 2 %  
Delete Segment

Segment 2:  
 Start time (min): 14.00    Tune file: autotune    View     Negative ions  
 Multiplier offset: 200     Fil/Mult/Dyn off  
 Total scan time (sec): 0.53    Add Event    Delete Event

Scan 1    Scan 2

Scan mode:  Full Scan     Seg Scan     SIM     MSn    Parameters  
 Precursor ions: 342

Scan time (sec): 0.31  
 Microscans: 3  
 Max ion time (ms): 25  
 Mass defect (mmu/100 amu): 0

MSn Parameters

MSn Order: 2     Plus Options

Isolation:  
 Precursor Ion: 342.0    Width: 2.0

Excitation:  
 Voltage: 1.00  
 Maximum Excitation Energy:  
 Low: .225    Med: .30    High: .45

Product Ions:  
 First Mass: 320.0    Last Mass: 330.0

Usable Mass Range:  
 10 - 1000 m/z  
 342  
 171 - 1000  
 320 - 330

OK    Cancel    Help

## Annex B : Parameters for Quadrupoles GC-MS

Melamine-QUAD-SIM.meth - Instrument Setup

File AS2000 Help

AS2000 Autosampler  
TRACE GC 2000  
TRACE MS

Injection Events Pre / Post Events

Injector control

Sample Volume (µL): 1

Air Volume (µL): 3

Injection Delay (sec): 0

Pull-Out Delay (sec): 1

Injection Speed (µL/sec): 100

Sample Cleans

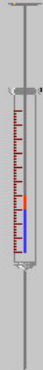
Number of Cleans: 2

Pull-Ups

Number of Pull-Ups: 3

Volume (µL): 4

Delay (sec): 5



Melamine-QUAD-SIM.meth - Instrument Setup

File AS2000 Help

AS2000 Autosampler  
TRACE GC 2000  
TRACE MS

Injection Events Pre / Post Events

Pre-Injection Washes

Number of Rinses: 1

Volume (µL): 10

Pre-Injection Solvent: A

Every Injection:

First/Last cycle:

Post-Injection Washes

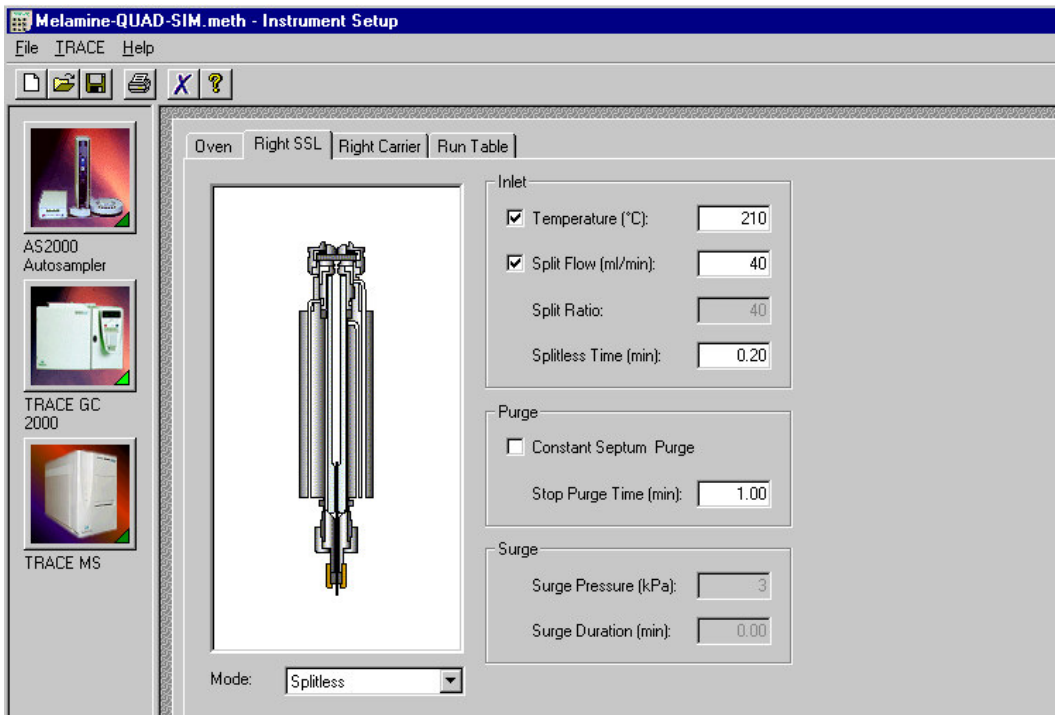
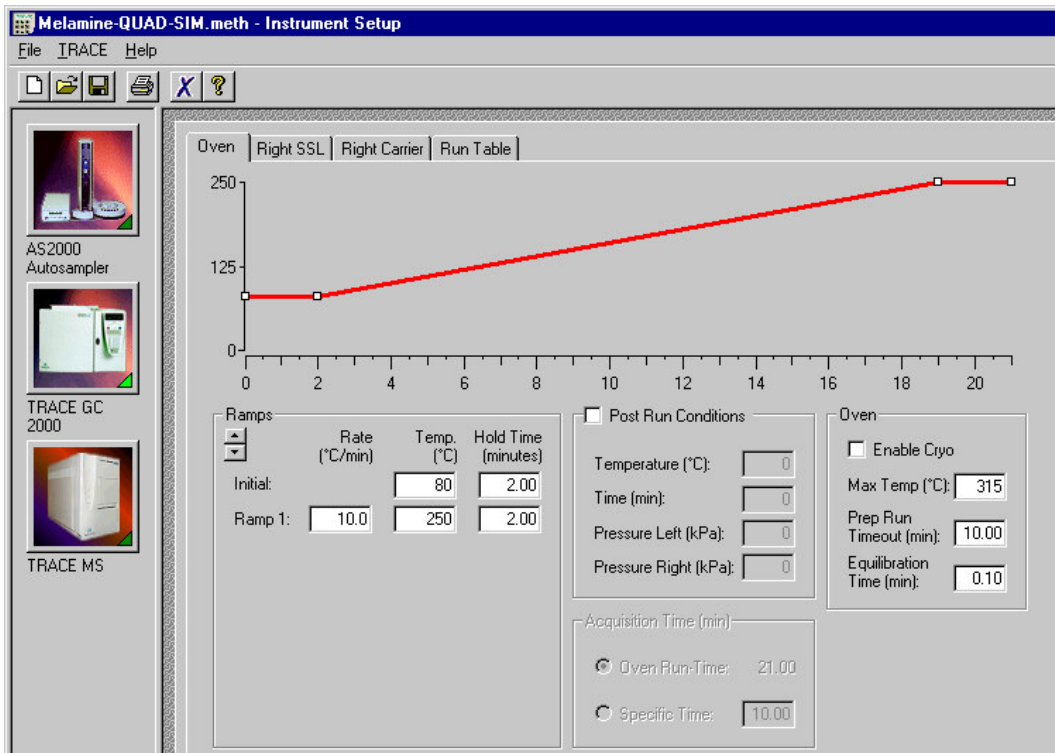
Number of Rinses: 5

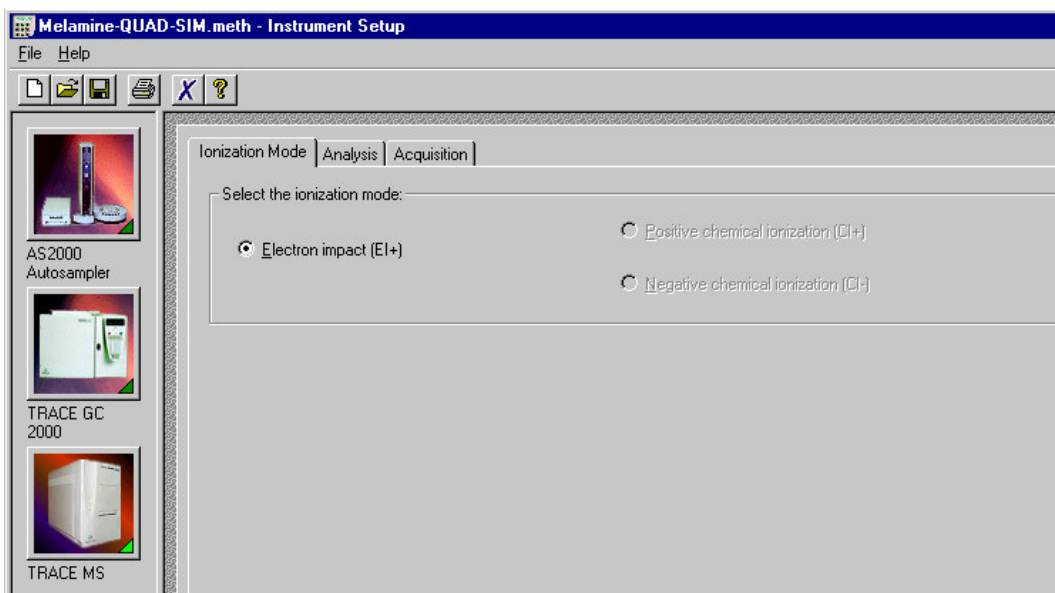
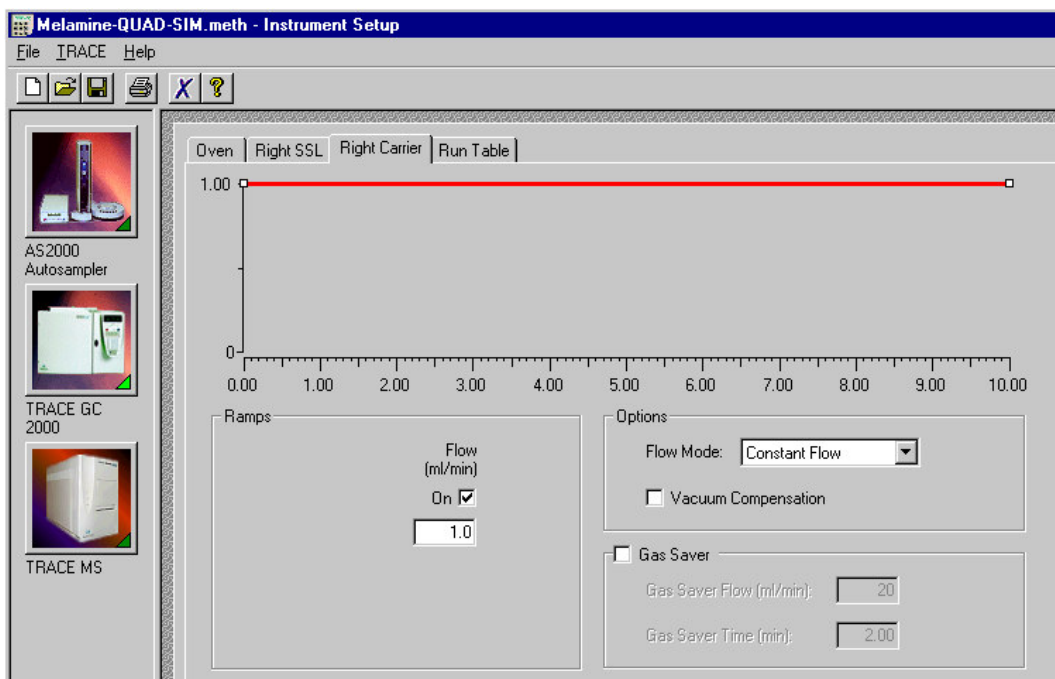
Volume (µL): 10

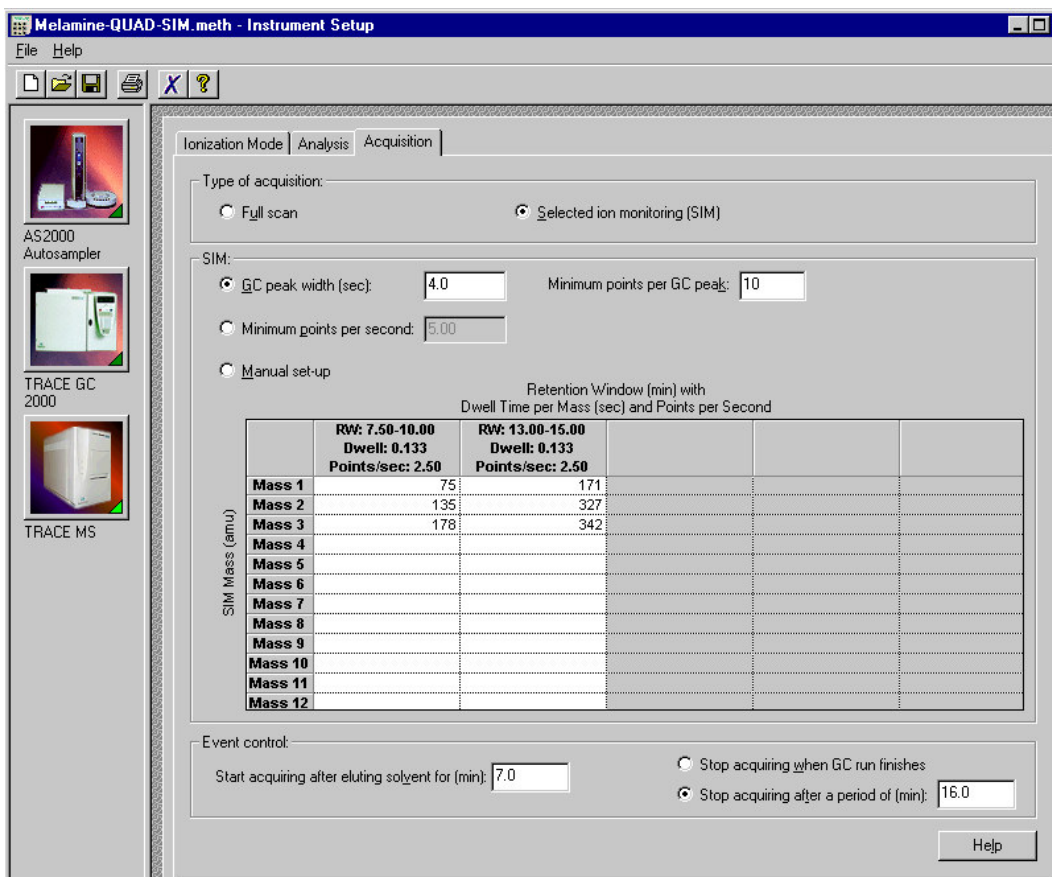
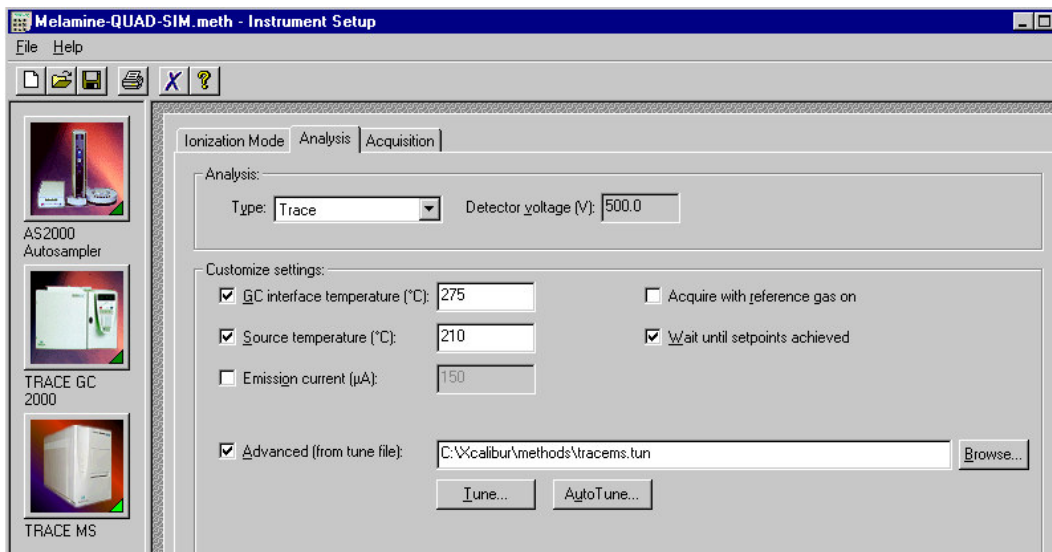
Post-Injection Solvent: B

Every Injection:

First/Last cycle:







# Annex C : Flowchart of sample preparing procedure

