Method development for analysis of formaldehyde in food-simulant extracts of melamine-ware by GC-MS and LC-MS/MS

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1 Executive Summary

As part of Mass Spectrometry capability building, the Organic Analysis team were requested to assist in the development of a method for the analysis of formaldehyde in food-simulant extracts of melamine-ware as part of the Government Chemist programme. The analytical techniques investigated were LC-MS/MS (Q-TOF analyser) and GC-MS (single quadrupole analyser).

2 Background

Melamine resin is a hard plastic material obtained from the polymerization of melamine and formaldehyde. Melamine resin is often used in kitchen utensils and food contact articles. Formaldehyde and melamine monomer have been found leaching from the resin after exposure to the food simulant (e.g. 3% acetic acid as the most aggressive simulant towards melamine plastics) **[1,2].** Formaldehyde can exist naturally in food (e.g. vegetable, meat and seafood) and ingestion of a small amount is unlikely to cause acute adverse health effects. However, intake of large amounts may result in severe abdominal pain, coma or renal injury.

One of the most efficient approaches for the determination of carbonyl compounds in aqueous samples is their derivatisation in acidic media with 2,4-dinitrophenylhydrazine (DNPH) to form the corresponding 2,4-dinitrophenylhydrazones. 2,4-dinitrophenylhydrazones can be analysed by reversed-phase HPLC and detected by UV–Vis at wavelengths between 349 nm and 380 nm **[3]**. However, UV–Vis detection lacks selectivity especially in complex matrices and doesn't often allow the quantification of carbonyl compounds with the required accuracy. Mass spectrometry-based methods can supply the needed selectivity and sensitivity for the detection and identification of carbonyl compounds and unknown sample constituents.

3 Objective

Compliance of food contact melamine-ware is usually checked by evaluation of the Total Specific Migration Limit (SML (T)) of formaldehyde and hexamethylenetetramine (HMTA), expressed as formaldehyde, as required by the European Commission Directive 2002/72/EC **[4]**. The SML (T) for formaldehyde is 15 mg per Kg of food simulant, equivalent to 2.5 mg/dm^2. The aim of this study was to develop a mass spectrometry-based analytical method for the detection, confirmation and quantitation of formaldehyde at the SML (T) levels in melamine-ware aqueous extracts. Both LC-MS and GC-MS approaches have been investigated, highlighting some of the main issues involved in the sample preparation procedure for the two different methods. However, further development of the LC-MS method was not pursued due to the higher suitability of the GC-MS to this specific task. In fact, the GC-MS system allowed the combined Scan/SIM acquisition mode which could guarantee sensitive quantitation and ID confirmation within one run. For this reason, efforts to obtain a finalised method were focused on the GC-MS methodology.

4 Materials and methods

4.1 Standards and reagents

Standards of formaldehyde-dinitrophenylhydrazone (FA-DNPH; Supelco 44-2597) and acetaldehyde- dinitrophenylhydrazone (AA-DNPH; Supelco 44-2434) were obtained from Sigma-Aldrich.

The derivatising reagent 2,4-dinitrophenylhydrazine (2,4-DNPH) was also purchased from Sigma-Aldrich (Fluka 42215-250mL; phosphoric acid solution). Isotopically labeled formaldehyde 2,4-diphenylhydrazone-3,5,6-d3 (d3-FA-DNPH; CDN isotopes D-7765/0.1; lot M28695; isotope purity 99.3 atom% D) as internal standard was supplied by CDN isotopes inc., Quebec, Canada via their UK distributor QMX Laboratories, Thaxted, Essex, UK.

The chemical structures of the analytes, derivatising reagent and derivatives are shown in Figure 1 below.

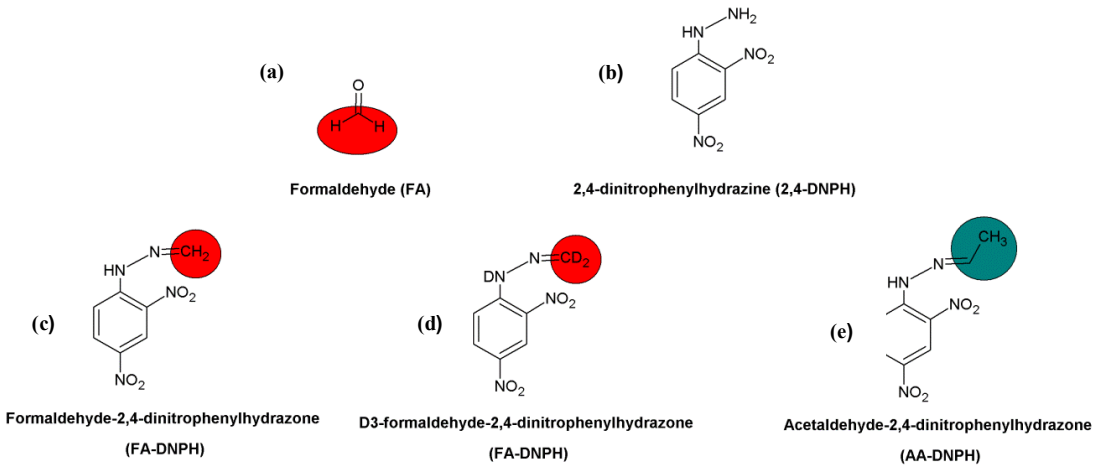


Figure 1: Structures of formaldehyde (a), 2,4-DNPH derivatising reagent (b), natural (c) and labeled (d) formaldehyde 2,4- dinitrophenylhydrazones and acetaldehyde 2,4-dinitrophenylhydrazone

4.2 Solvent standards preparation

Individual solvent standards of FA-DNPH, AA-DNPH and d3-FA-DNPH were prepared in acetonitrile (LGC Promochem Optigrade SO-9184). FA-DNPH was prepared at concentration of 1.2 mg/mL, equivalent to 0.17 mg/mL of FA. The internal standard d3-FA-DNPH was prepared at concentration 0.59 mg/mL, equivalent to d3-FA concentration of 0.08 mg/mL. AA-DNPH was also used as additional internal standard in the initial stage of the project when the labeled d3-FA-DNPH was unavailable.

4.3 Samples and matrix calibration standards

Samples and matrix calibration standards were provided by FA30. Samples consisted of 3% acetic acid food simulant extract of melamine plates. The extraction was performed at 70°C for 2 hours. Matrix calibration standards were prepared in 3% acetic acid by spiking certain amounts of formaldehyde (FA) standards to achieve final concentrations in solution of 0, 1.5, 5.8, 10.2, 14.6 and 29.1 mg/mL.

4.4 2,4- DNPH derivatisation

Prior to the instrumental analysis, samples and matrix standards were derivatised by with 2,4-DNPH **[5]**. Details of the finalized derivatisation procedure are reported below.

Optimized Derivatisation Procedure (GC-MS analysis)

1. 0.5mL of sample were transferred into a 15mL falcon tube (BD falcon 352096).
2. 0.1mL of 2,4-DNPH derivatising reagent were added and the sample was vortex mixed for 30 seconds.
3. 0.175mL of aqueous NH_3 (32%, d= 0.88; VWR 153312K) were added to the mixture and the sample was vortex mixed for 20 seconds.

4. 0.1mL of internal standard solution (0.59mg/mL d3-FA-DNPH in acetonitrile) was added and the sample further vortex mixed for additional 20 seconds.
5. 1mL toluene (Acros Organics 332070025) was added to allow *in situ* liquid/liquid extraction and the mixture was then vortex mixed for 60 seconds.
6. An aliquot of the organic phase (toluene layer) was transferred to an autosampler vial and then injected into the GC system.

The LC-MS/MS preliminary study also involved derivatisation of the samples and matrix standards prior to analysis in order to generate the corresponding DNPH derivatives. However, in this specific case, the sample preparation procedure did not require the *in situ* liquid/liquid extraction step with toluene to change injection solvent (no aqueous mixture can be injected on the GC-MS system). In its place, 1mL of acetonitrile was added after the derivatising reagent and the extract was injected on the LC-MS system directly. When internal standards are available, the derivatisation procedure shown above for GCMS can be employed with substitution of step 5 for the addition of 1mL of acetonitrile.

4.5 Instrumental analysis

LC-MS/MS

LC-(+)ESI-MS analysis were performed on an Agilent 1200 HPLC system coupled to an Agilent 6530 Q-TOF mass spectrometer.

- *HPLC separation conditions*

HPLC separation was performed using an Agilent C18 Zorbax, 4.6 mm x 50 mm, particle size 1.8 µm. An acetonitrile–water binary gradient at a flow-rate of 1 mL/min, column temperature at 40 °C using the gradient reported in Table 1. The injection volume was 5 µL.

Time [min]	***% Mobile phase B [ACN]***
0	40
1	40
5	80
6	80
6.5	40
8	40

Table 1: HPLC separation gradient

- *MS detection conditions*

MS tuning (source and acquisition conditions) for the analytes of interest was optimised by post column mobile phase infusion of solvent standard solutions. Details of the condition settings are reported in Table 2.

Ion Source	Jet stream (+ESI)
Drying Gas N_2 flow [L/min]	8
Drying Gas N_2 T [°C]	325
Sheath Gas N_2 flow [L/min]	11
Sheath Gas N_2 T [°C]	350
Capillary voltage [V]	3500
Fragmentor[V]	175
Scan acquisition range [m/z]	100-1000
Scan acquisition rate [spectra/sec]	1

Table 2: Ion source tuning conditions for 2,4-DNPH derivatives solvent standards

GC-MS

Gas chromatographic analysis was carried out on an Agilent 7890 gas chromatograph coupled to a MSD 5975C inert XL EI/CI with triple-axis detector. Instrumental conditions are reported in Table 3 below.

Column:	Restek Rxi-HT 30m x 0.25mm ID x 0.25 µm df
Inlet mode:	Split
Inlet Temperature [°C]	260
Septum purge:	3mL/min
Split flow:	100 mL/min
Split ratio:	100:1
Injection mode:	Automatic, Combi PAL G6500 CTC (CTC Analytics)
Air volume:	1µL
Pre-clean with i-octane:	2µL
Pre-clean with sample:	2µL
Injection volume:	1µL
Filling speed:	2µL/s
Filling strokes:	4
Injection speed:	50µL/s
Pre-injection delay:	0 ms
Post-injection delay:	500 ms
Post-clean with acetone:	6
Carrier gas:	He, 1mL/min
Oven program:	70°C held for 1min 35°C/min to 340°C 340°C held for 2 min
Transfer line:	300°C
Solvent delay:	3min
Source:	300°C
Quad:	150°C
Acquisition mode:	Scan/SIM
SIM Ions monitored [m/z]:	152, 155, 180, 183, 198, 210, 213
Dwell time:	15 ms
Scan range [m/z]:	50 to 500
Run time [min]:	10.7

Table 3: GC-MS conditions for 2,4-DNPH derivatives analysis

5 Results

5.1 LC-MS approach

Initial work was carried out before the strength of the acid was known, this adversely affected the results from the derivatisation, leading to poor linearity and response of the analyte peaks. However a separation was achieved as is detailed in Figure 2.

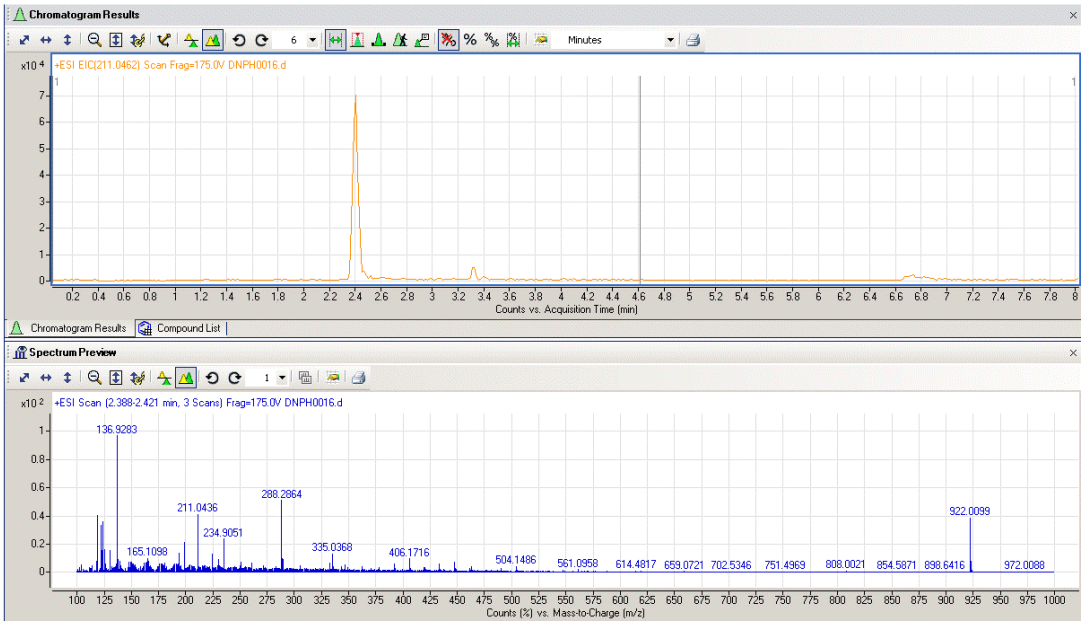


Figure 2: Extracted Ion Current Chromatogram and Extracted Spectra of FA-DNPH

Due to the promise shown by the GC-MS approach, the investigation into the quantitative LC-MS approach was suspended. However, with the knowledge gained on the sample and its derivatisation from the GC method development it is probable that the method could be further developed rapidly.

5.2 GC-MS approach

Derivatisation procedure optimisation

During the optimisation process, some issues were encountered which needed to be overcome in order to generate a successful finalised method. The derivatising reagent 2,4-DNPH is supplied as 60% H_3PO_4 solution which significantly decreases the pH of the reagent mixture. Initially, an additional step of pH neutralisation by NH_3 addition was added to the procedure in order to avoid degradation of the GC column stationary phase due to the high acidity of the extract. However, further experiments proved that the high acidity of the extract was also causing hydrogen-deuterium exchange (HDX) on the d3-FA-DNPH, producing natural FA-DNPH and consequently affecting the ratios between natural and labelled in the tested calibration curves. Figure 3 shows the comparison between results obtained with and without NH_3 neutralisation. Therefore, the neutralisation step was confirmed to be essential to guarantee quality data from the GC-MS analysis.

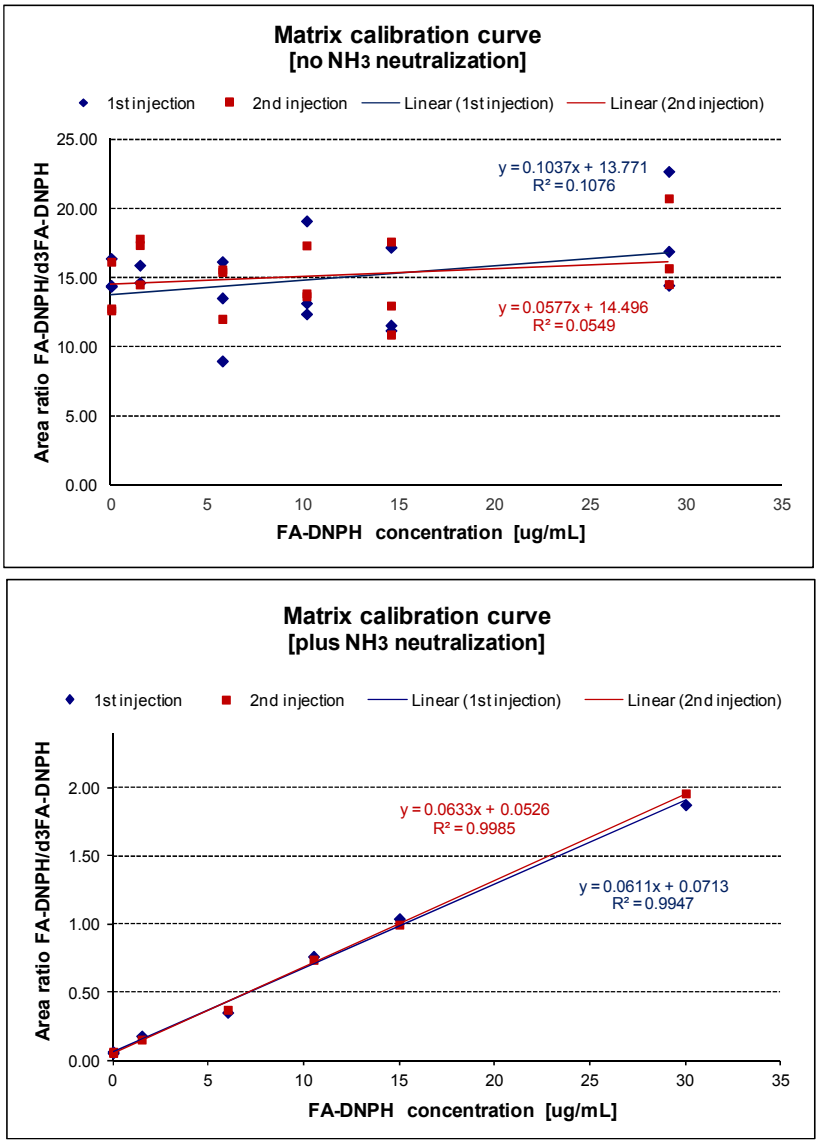


Figure 3: NH_3 neutralisation effect on matrix calibration curves for FA-DNPH

Analyte Scan/SIM characterisation

The use of the combined Scan/SIM acquisition mode permits selective and sensitive quantitation (SIM mode) acquiring simultaneously full scan data which can be helpful for ID confirmation purposes. Scan/SIM chromatograms for a solvent standard solution 1 µg/mL and a matrix standard solution 1.5 µg/mL are reported in Figure 4 and Figure 5, respectively.

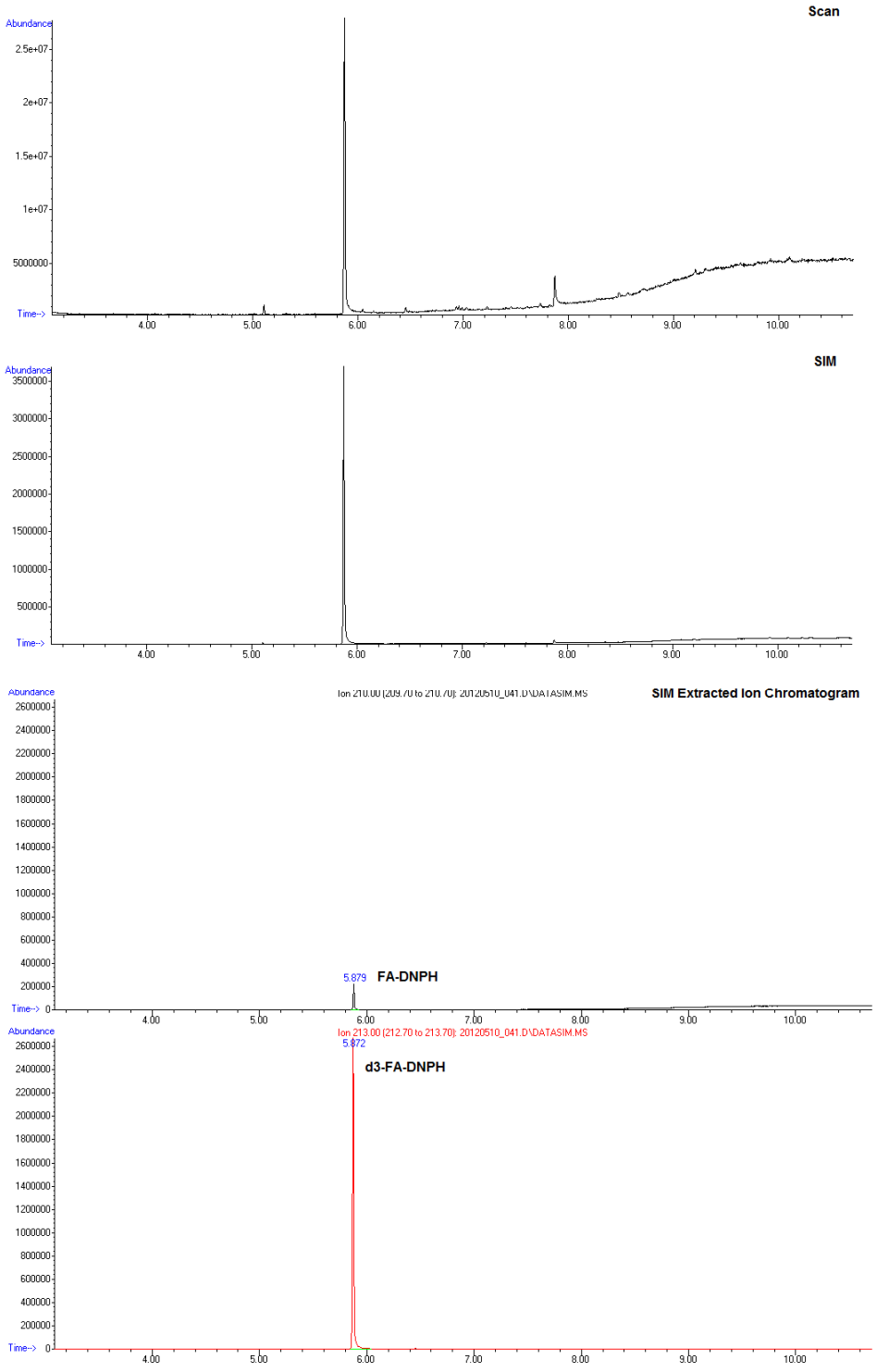


Figure 4: Scan/SIM chromatogram for a FA-DNPH solvent standard solution 1 µg/mL (IS d3-FA-DNPH 15 µg/mL)

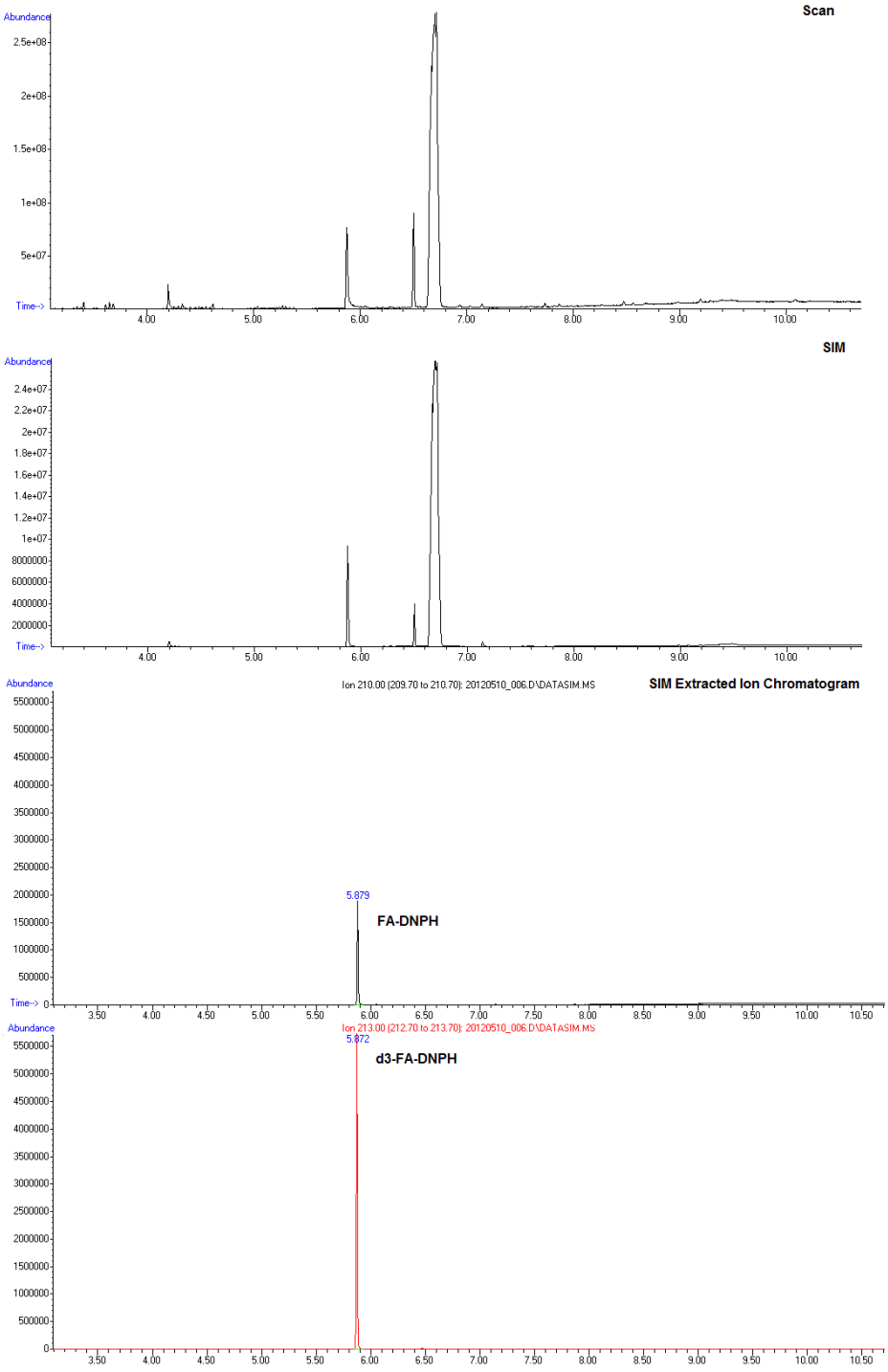


Figure 5: Scan/SIM chromatogram for a FA-DNPH matrix standard solution 1.5 µg/mL (IS d3-FA-DNPH 15 µg/mL

Full scan spectra of FA-DNPH and d3-FA-DNPH are shown in Figure 6 and Figure 7 below.

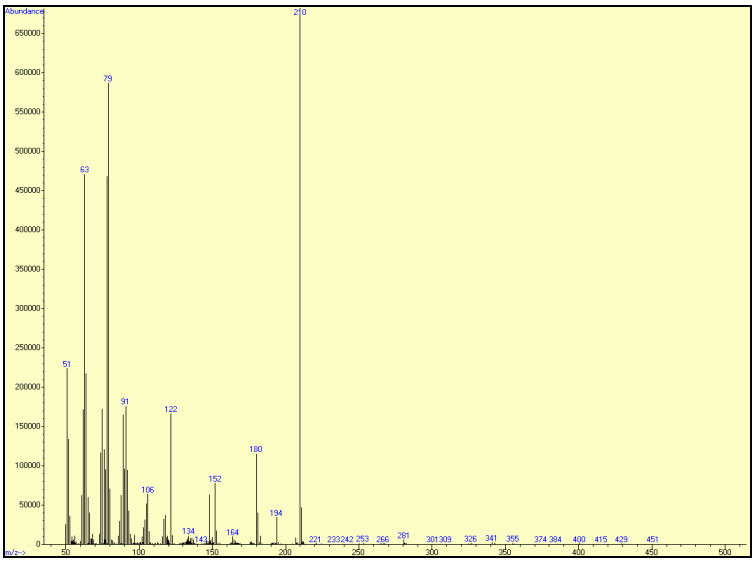


Figure 6: EI spectrum of FA-DNPH

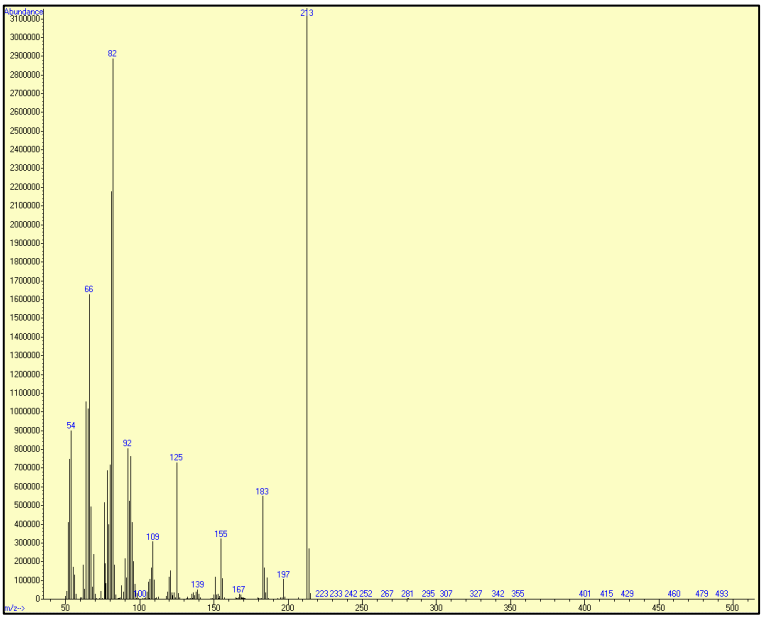
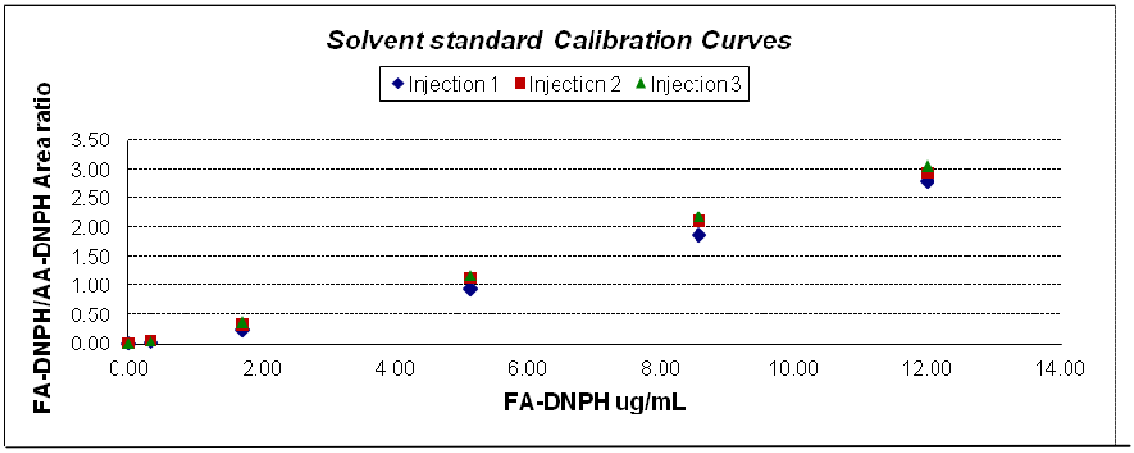


Figure 7: EI spectrum of d3-FA-DNPH

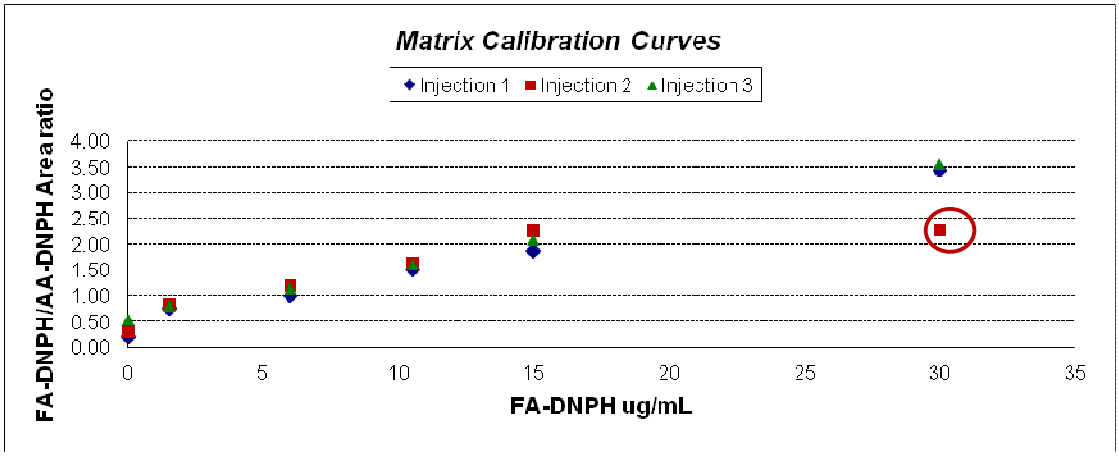
Internal standards evaluation

Preliminary experiments assessed the relative performance of different internal standards for this analysis. Initially, AA-DNPH was evaluated for use as internal standard. Calibration solutions for the internal standard method evaluation were prepared in acetonitrile at different FA-DNPH concentrations (0, 1, 5, 10, 15, 25 and 30 μg/mL) and at constant internal standard concentration of 15 μg/mL. Matrix standards and samples were derivatised as described in details in the Materials and Methods Section 4.4. Calibration curves were obtained plotting on the y-axis the peak area ratios of FA-DNPH (*m/z* 180) to of AA-DNPH (*m/z* 180) and on the x-axis the FA-DNPH concentration. Figure 8 shows the solvent standard calibration curves obtained from 3 repeated injections of each standard solution.



Injection 1	y = 0.2325x - 0.0992
	R² = 0.9915
Injection 2	y = 0.2472x - 0.0528
	R² = 0.9981
Injection 3	y = 0.2583x - 0.0543
	R² = 0.9981

Figure 8: Solvent standard calibration curves using AA-DNPH as internal standard



Injection 1	y = 0.1018x + 0.4
	R² = 0.9865
Injection 2	y = 0.0629x + 0.763
	R² = 0.7799
Injection 3	y = 0.0994x + 0.5889
	R² = 0.9987

Figure 9: Matrix calibration curves using AA-DNPH as internal standard

Calibration curves for 3 repeated injections of derivatised matrix standards are reported in Figure 9. Linearity and repeatability appeared satisfactory, with the exception of one outlier in the second injection dataset. However, background interference or contamination was noticed in the blank matrix standard (0 ug/mL FA-DNPH) as clearly revealed by intercept value above zero.

Figure 10 shows the comparison between the 0 ug/mL FA-DNPH (*m/z* 210) matrix standard and 0 ug/mL FA-DNPH solvent standard using d3-FA-DNPH (*m/z* 213) as internal standard. Clearly the matrix standard shows the presence of the natural FA-DNPH due to contamination whereas the same peak is absent in the solvent standard solution.

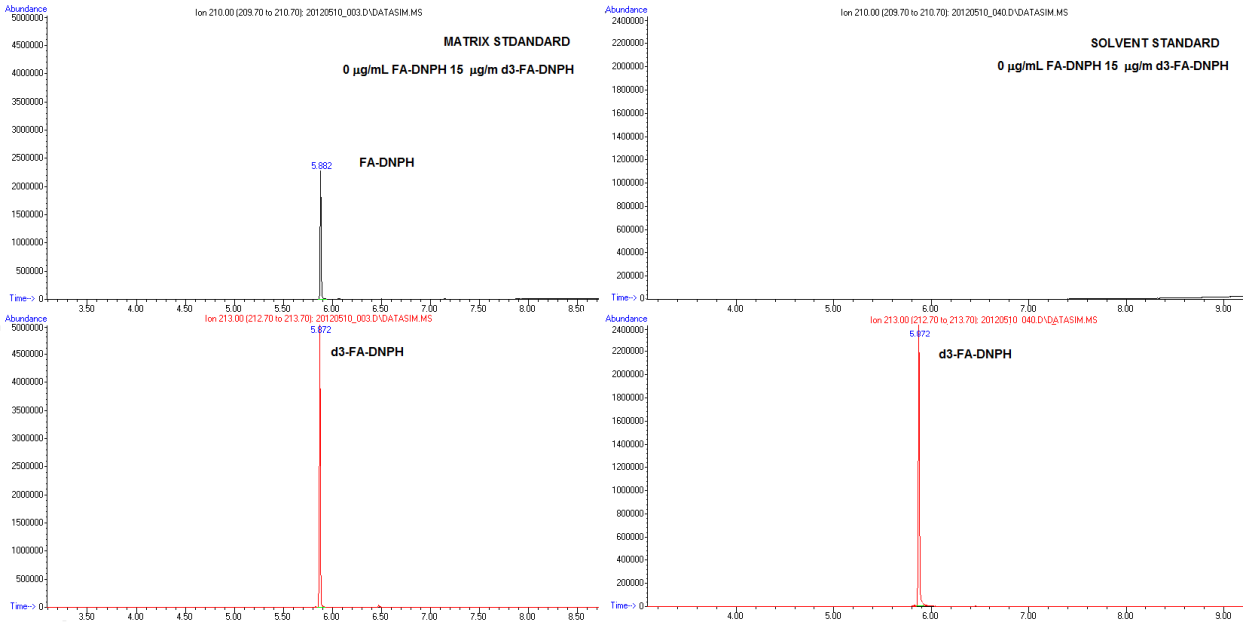


Figure 10: Matrix standard contamination effect

The isotopically labelled d3-FA-DNPH was tested as internal standard as well. As previously mentioned in the Material and Methods sections, HDX issues were observed before the addition of the neutralisation step to the sample preparation methodology. The HDX caused the inter-conversion between natural and labelled consequently affecting the performance of d3-Fa-NDPH as internal standard. However, once the methodology had been optimised, d3-FA-DNPH proved to be a good internal standard for the purposes of this study as shown in the solvent and matrix calibration curves in Figure 11. Both solvent and matrix calibration curves showed good linearity and repeatability of the results. RSD% ranged between 1 and 30 %.

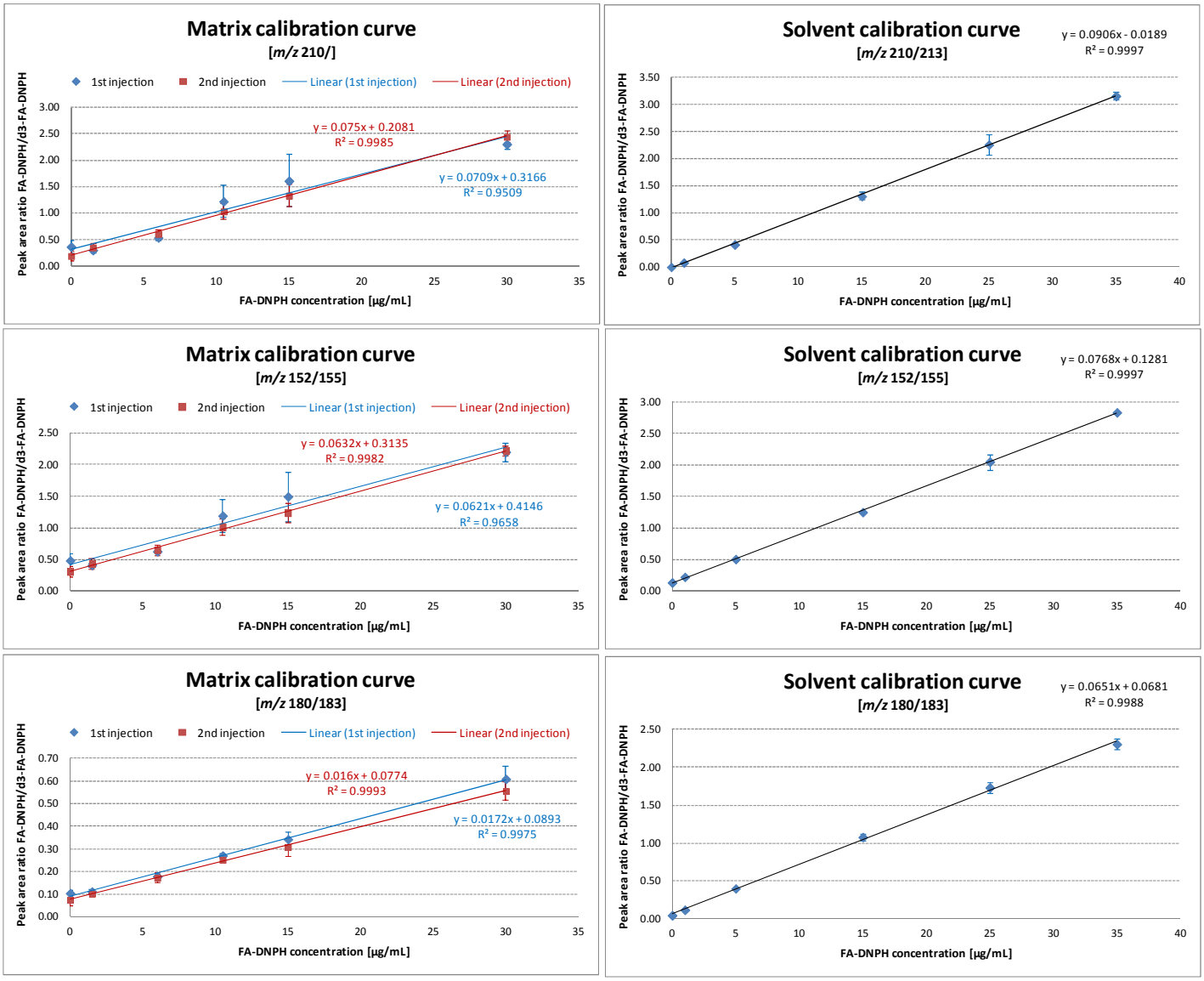


Figure 11: Solvent and matrix standard calibration curves for natural and d3-labeled FA-DNPH

6 Conclusions

Two mass spectrometry-based analytical methods were developed for the determination of formaldehyde in food-simulant extracts of melamine-ware. A derivatisation procedure was optimised for the detection of formaldehyde as 2,4-dinitrophenylhydrazone. An LC-MS approach was preliminarily evaluated but not further developed due to the superiority of GC-MS methodology in this case. Two different internal standards were tested for this study, both of them showing good results in terms of linearity and repeatability in solvent and matrix calibration curves. A fully quantitative procedure by GC-MS was developed using the labeled d3-formaldehyde-2,4-dinitrophenylhydrazone as internal standard.

The method developed is applicable to food-simulants, however there is scope for application to other aqueous extractions or formulations, such as cosmetics, with further investigation into matrix effects during the analysis stage.

7 Recommendations for further work

Investigation into the source of background contamination and methods for its reduction or elimination would be beneficial since the 0 µg/mL matrix calibration solutions showed significant levels of the analyte FA-DNPH. Optional clean-up procedures might be investigated to reduce the contamination of the GC-MS system and deliver higher robustness throughout the analysis.

8 References

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