

PROJECT A03037: LC-MS METHOD DEVELOPMENT FOR THE SCREENING OF NON-VOLATILE AND POLAR COMPOUNDS PRESENT IN PAPER AND BOARD OR PLASTIC FOOD CONTACT MATERIALS AND ARTICLES:

TESTING FOR SEMICARBAZIDE WITHOUT DERIVATISATION OR ACID HYDROLYSIS: FINAL REPORT

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Summary

This part of project A03037 is being reported rapidly as a contribution to continuing scientific work in the European Union (EU) following the reported finding of semicarbazide in some bottled foods.

LC-MS and LC-MS/MS methods have been developed at the Central Science Laboratory (CSL), for the determination of underivatized semicarbazide (SEM) in azodicarbonamide (ADC), biurea (BU) and urazole (UR). Existing methods of detecting SEM involve acid extraction and chemical derivatization. It has been proposed that this may lead to some conversion of ADC to SEM. SEM, BU and UR are potential contaminants of ADC. ADC is used as a blowing agent in plastic gaskets for 'push-on twist-off' lids of some jars.

There was good agreement between analyses using LC-MS and LC-MS/MS. Recovery was acceptable by both methods (76-86% by LC-MS and 85-95% by LC-MS/MS). Sensitivity was approximately 10 fold better with LC-MS/MS.

SEM was found in each of the compounds analysed. Due to the limited solubility of BU and ADC and the need to work at ppm levels, this work was undertaken on saturated solutions of these substances in water. UR was completely soluble in water. No firm values were put on the concentration of SEM found in BU or ADC because the efficiency of extraction from a solid powder is not known. However, based on estimates of the solubility limits of BU, ADC, and UR in plain water at room temperature, their solubilities were expected to be 20-25%, 10-15% and 100% respectively.

The same samples were analysed for SEM using an acid hydrolysis/derivatization method (the 'derivatization method'). Recovery was poor (20-30%). Whilst there was good agreement between the results with and without derivatization for the UR standards, this was less good for BU standards and very poor for ADC. The low recoveries may have played a part in this discrepancy.

A second independent laboratory, TNO Nutrition and Food Research, determined whether the work undertaken by CSL was reproducible. Although sensitivity was less on the LC-MS/MS instrument used at TNO for the no derivatization method, they found CSL's work to be reproducible. SEM was detected, by both laboratories, in BU and UR, by the no

derivatisation method and in all three chemicals by the derivatisation method. There was also reasonable agreement between the two laboratories on the levels of SEM found in ADC, BU and UR.

It can be concluded from this work that:

- SEM is present as an impurity in the chemicals ADC, BU and UR.
- The newly developed method that doesn't use derivatisation is a good qualitative one for SEM in solution. But further work would be required to establish this as a robust, quantitative method.
- Preliminary results indicate that there is reasonable agreement between the levels determined with or without derivatisation. However, this cannot be established definitively until the quantification problems with the method that doesn't use derivatisation have been resolved.
- Even using robust, validated methods, no firm values could be put on the concentrations of SEM found in ADC or BU solutions because the efficiency of extraction from a solid powder is not known.
- UR is completely soluble at the concentration used (1 mg/ml). Reasonable agreement was seen for levels of SEM in UR determined by both methods at both laboratories
- Apart from CSL results for the derivatised samples, levels of SEM found in ADC and BU by both methods are in reasonable agreement.

Background

In July 2003 the possible occurrence of SEM in bottled foods was reported. This was thought to be as a result of migration of this substance, and/or a parent compound ADC, from plastic gaskets used in the 'push-on twist-off' lids of some jars. Use of ADC as a blowing agent is permitted in EU plastics legislation (1). SEM was found in bottled foods that were not of animal origin so its presence could not be from the use of nitrofurazone in animals. SEM is a marker for such usage.

ADC when used as a blowing agent will decompose to gases, primarily nitrogen and carbon monoxide together with some carbon dioxide and ammonia. It is believed that normally the residual amounts of non-volatile substances are only small (e.g. BU is present at about 2% in ADC). However, on occasion residue levels of BU could be as high as 34%. Other possible non-volatile reaction products include urazole, cyanuric acid and cyanemide (2).

The methods of analysis used to detect SEM in food involve acid hydrolysis and a derivatisation step. These steps are to help extract and measure SEM bound to protein, in the testing of meat for this substance as a marker for nitrofurazone, which is rapidly metabolised to SEM. They may not be needed for the detection of SEM in free solution

¹ Commission Directive 2002/72/EC of 6 August 2002 relating to plastic materials and articles intended to come into contact with foodstuffs.

² Hunter, B.A. Chemical Foaming Agents Chemistry and Decomposition Mechanisms (<http://www.uniroyalchemical.com/celocad.htm>)

and may in theory lead to artefacts, for example the production of SEM as a result of hydrolysis of ADC or some of its impurities, during the analytical work-up.

A method of analysis that does not use acid hydrolysis or derivatisation was therefore developed, initially to look for the presence of SEM in ADC and the related chemicals biurea (BU) and urazole (UR). CSL were commissioned by the Food Standards Agency to undertake this method development as part of project A03037.

Objectives

1. Development of an independent LC-MS method for semicarbazide analysis without acid hydrolysis or derivatisation using nitrobenzaldehyde.
2. Comparison of results obtained for levels of SEM in ADC, BU and UR using this newly developed method and an existing method of acid hydrolysis and derivatisation using nitrobenzaldehyde.
3. A between-laboratory check commissioned at a second, independent laboratory, TNO. The presence of SEM in ADC, BU and UR to be determined by both the methods mentioned in objective 2 above. Results for these chemicals to be compared with those obtained from CSL. Both methods (with and without derivatisation) also to be applied to two samples of gaskets.

Methodology

Chemicals ADC, BU and UR were obtained from commercial sources.

Solutions

Separate solutions of ADC, BU and UR were prepared by shaking 5 mg of each compound with water (5 ml). The saturated solutions were shaken for approximately 2.5 hours at room temperature and then centrifuged. The supernatant was removed and analysed, either as such using the method without derivatisation for SEM or after derivatisation with 2-nitrobenzaldehyde (2NBA).

'Spiked' solutions were prepared by adding 1 microgram/ml of SEM to the supernatants obtained above.

Derivatisation method

A CSL method (PVMC SOP41) was used. This involved adding 25 ml 0.125N HCL to the sample, mixing, addition of 250 microlitres of derivatising agent 2NBA, and then incubation overnight at 37°C. This was followed by extraction and LC-MS/MS analysis as described in PVMC SOP41.

Analytical detail for SEM measurement without derivatisation

LC-MS instrument: Platform benchtop mass spectrometer (Micromass)

LC-MS-MS instrument: Perkin-Elmer PE Sciex API2000.

Ionisation mode: Positive ion electrospray.

Mobile Phase: 0.2 per cent acetic acid in 90/10 water/acetonitrile (isocratic).

Column: Supelco Discovery HS F5 (15 cm x 2 mm, 5 micrometre particle size).

Injection volume: 40 microlitre.

LC-MS ions monitored for SEM: m/z 31, 59, 76 [M+H]⁺, 117 (acetate adduct) and 151 (protonated dimer).

LC-MS ions monitored for ADC and BU: m/z 41, 43, 44, 81, 91, 100, 119 and 122.

LC-MS/MS transitions monitored: 76>59, 76>44, 76>31, 76>76*, 98.1>98.1. *

*The parent and adduct ions at m/z 76 and 98 respectively, were monitored as a transition to themselves. This is an accepted practice in MS-MS to monitor an analyte or an adduct by MS/MS without effecting fragmentation in the collision cell.

Results

Solubility

ADC and BU were poorly soluble. Acetone proved to be the best solvent but there were concerns that this may react with any SEM in the sample (carbonyl reagent) and it also caused the LC separation to fail. The same was found with an alternative solvent, dimethyl sulphoxide. During the preparation of the aqueous solutions of ADC, BU and UR it was noted that:

- Extraction of 5, 10 or 20 mg of ADC or BU with water (5 ml) gave the same concentration of SEM in the supernatant.
- Extraction of 5 mg of ADC or BU with 5, 10 or 20 ml of water gave the same concentration of SEM in the supernatant.
- Extraction of 5 mg of ADC or BU successively with 3 x 5 ml aliquots of water gave the same concentration of SEM in the 1st 2nd and 3rd extracts.
- UR, which is water soluble, behaved as expected.

Possible explanations for these observations are that SEM is at its solubility limit, that it is in equilibrium in solution with ADC or BU or that the water is simply dissolving away only the surface layer of the chemicals, which are at their solubility limits.

Method without derivatisation for SEM

LC separation was the key to the analysis because the parent compounds have the potential to decompose in the heated source of the mass spectrometer to form semicarbazide as an artefact. Several HPLC columns were evaluated but only one of these gave any useful separation. This column resulted in elution of the parent compounds with the solvent front at a retention time of approximately 2 minutes, whilst the semicarbazide was well resolved eluting at approximately 4.6 minutes.

0.2% Acetic acid was required in the mobile phase. Omission of this resulted in poor ionisation and chromatography. This was a far milder treatment than the acid hydrolysis step used in the derivatisation method (pH 3.5 as opposed to pH 2 and exposure for only the 5 minutes it takes from injection to elution).

The LC-MS method had rather poor sensitivity and using LC-MS/MS analysis on a more modern instrument provided further additional sensitivity. The identification of SEM using LC-MS/MS rested on the LC retention time, on three mass ions, and on the ratios of these ions. Figure 1 shows the chromatograms for m/z 76>59, 76>76 and 98>98 ion transitions for a blank solution and a 0.05 microgram/ml standard solution of SEM. Figures 2-4 show chromatograms for the SEM peak in solutions of ADC, BU and UR, obtained from both 'unspiked' and 'spiked' solutions. The detection limit using the LC-MS/MS approach was ca. 4 ng/ml for the m/z 76>59 transition.

The solubility of ADC, BU and UR was found to be 10-15%, 20-25% and 100% respectively. 'Spike' recovery using the method without derivatisation ranged from 76-86% by LC-MS and 85-95 % by LC-MS-MS.

Analysis with derivatisation

The samples prepared for analysis without derivatisation were also tested for SEM by the acid hydrolysis/derivatisation with 2NBA method for comparison of results. Recoveries were low - in the range of 20-30%.

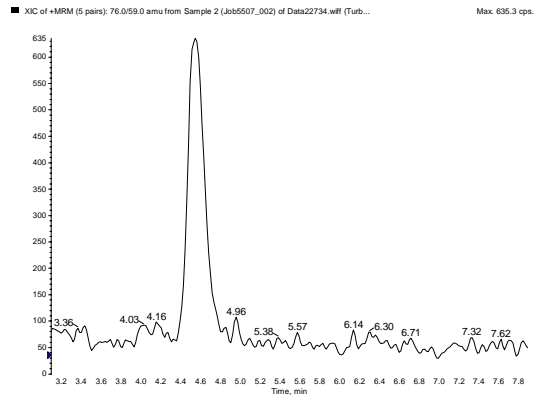
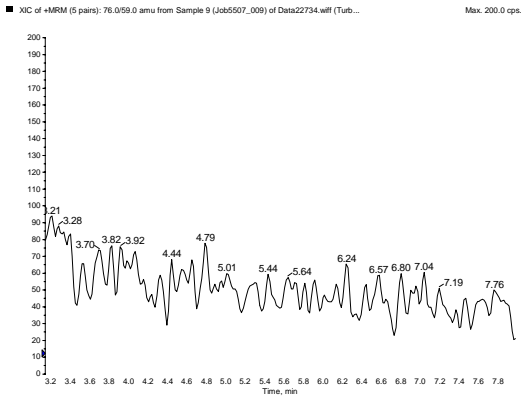
Table 1. Comparison of results obtained by the no derivatisation and derivatisation methods. Calculated on a mg SEM per kg chemical tested basis after correction for analytical recovery and solubility.

Sample	LC-MS No derivatisation (mg/kg)	LC-MS/MS No derivatisation (mg/kg)	LC-MS/MS Derivatisation (mg/kg)
ADC	<LOD* (n=3)	121 (n=3)	2583 [#] (n=2)
BU	718 (n=4)	641 (n=4)	1727 [#] (n=3)
UR (sample 1)	481 (n=6)	436 (n=6)	670 [#] (n=2)
UR (sample 2)	457 (n=5)	482 (n=5)	Not determined

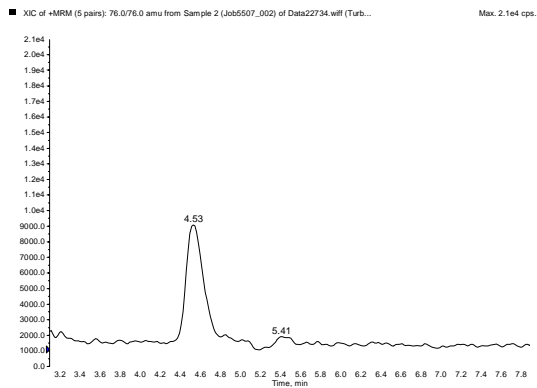
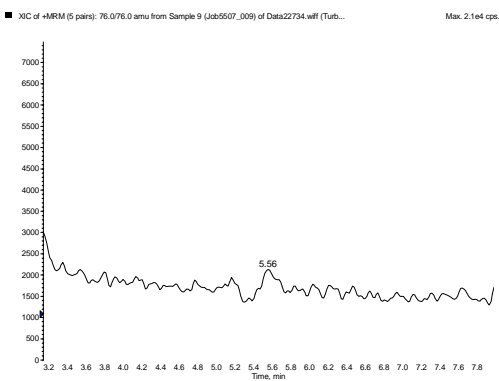
*LOD = 160 mg/kg allowing for solubility

[#] Recovery outside acceptable limits, therefore value given for information only

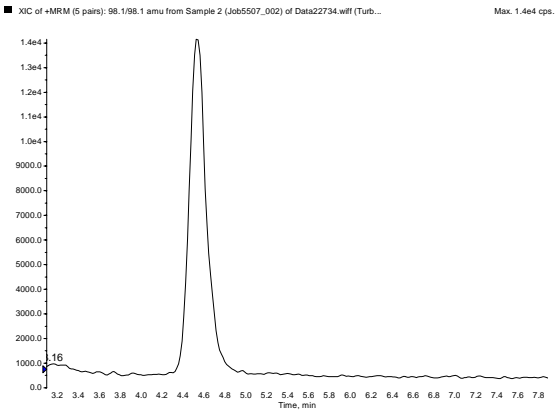
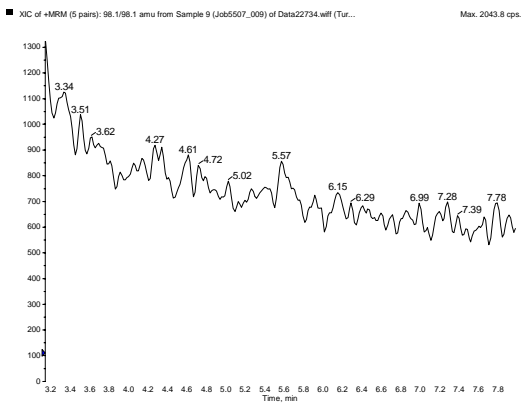
Transition 76>59



Transition 76>76



Transition 98>98

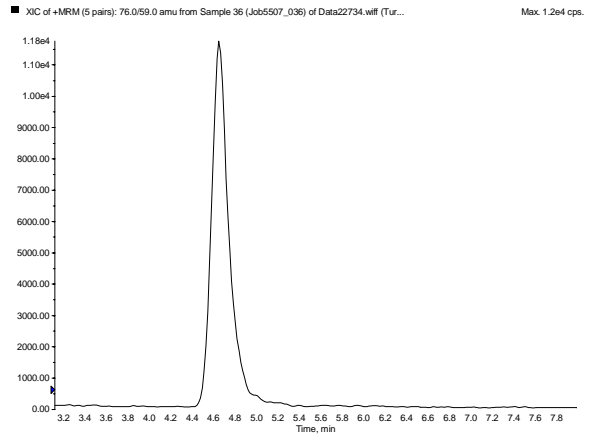
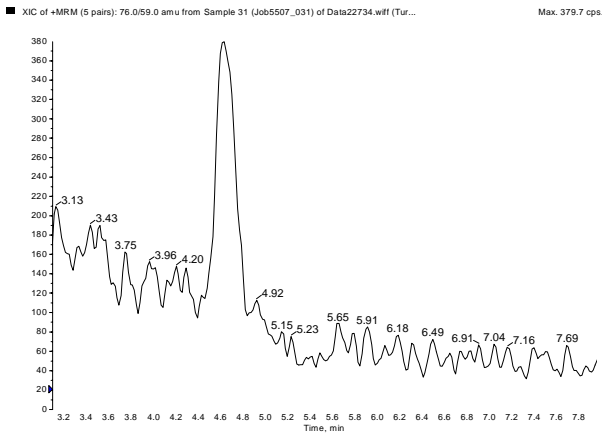


Blank solution

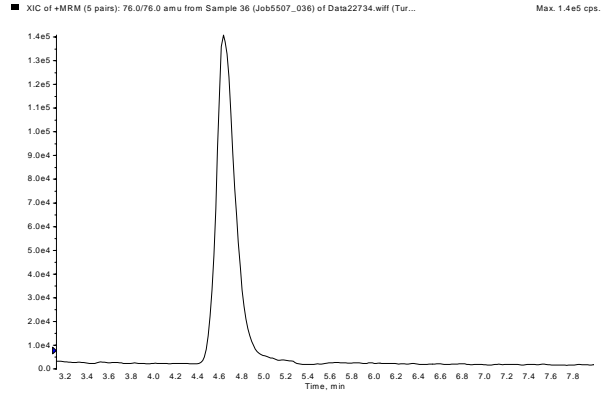
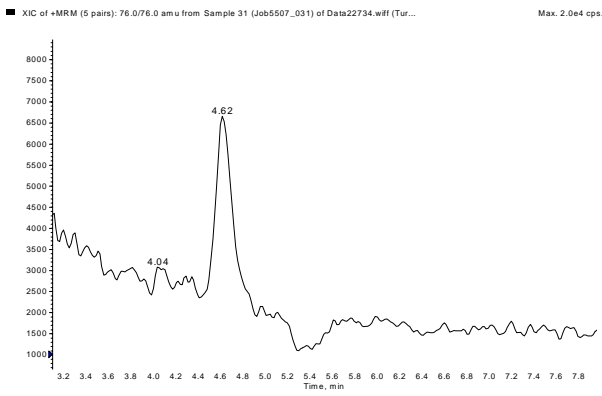
Standard SEM solution

Figure 1. Selected ion chromatograms of a blank solution and a 0.05 microgram/ml standard solution of SEM (HCl)

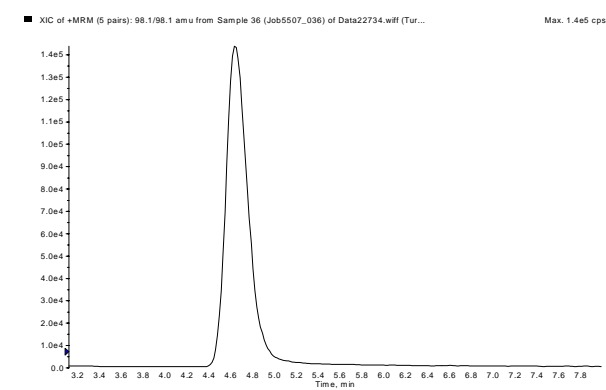
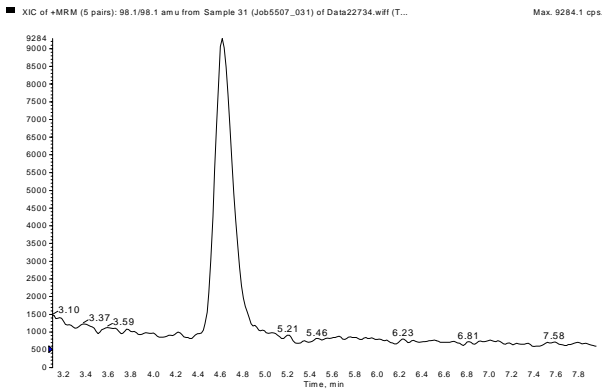
ADC001: transition 76>59



ADC001: transition 76>76



ADC001: transition 98>98

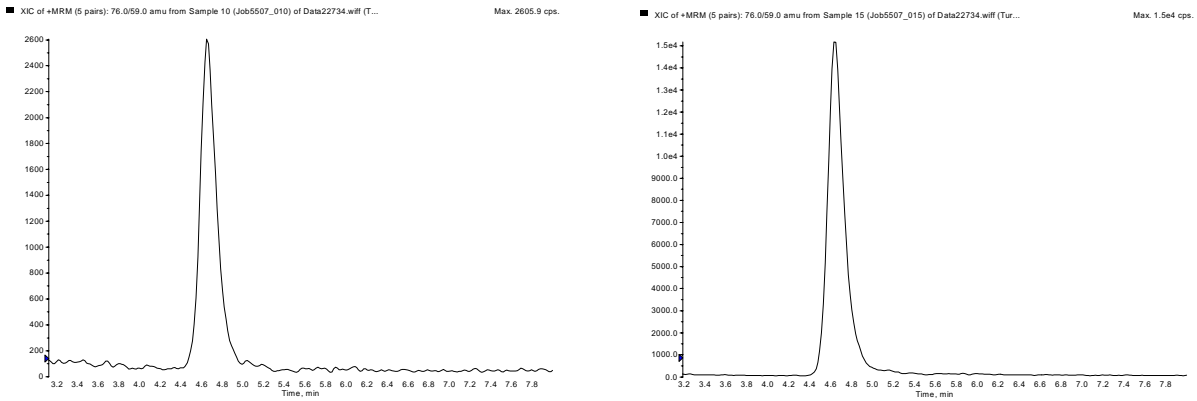


'Unspiked' sample.

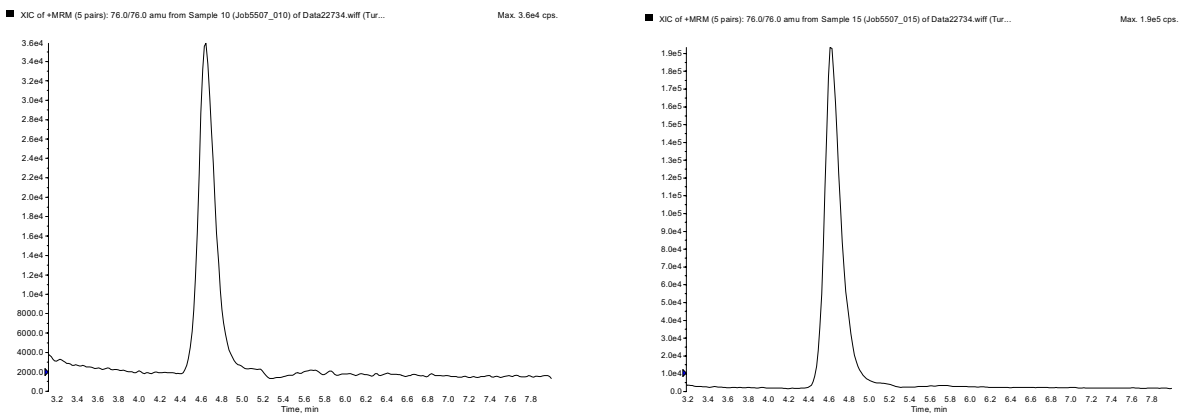
'Spiked' at 1 microgram/ml

Figure 2 Extract of ADC: 5 mg shaken with 5 ml of water. 'Unspiked' and 'spiked' at 1.0 microgram/ml.

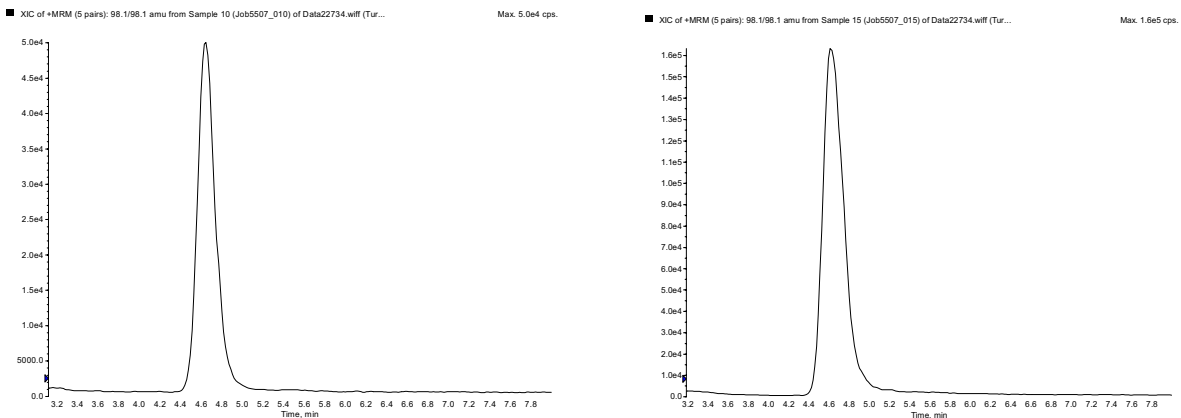
BU001: transition 76>59



BU001: transition 76>76



BU001: transition 98>98

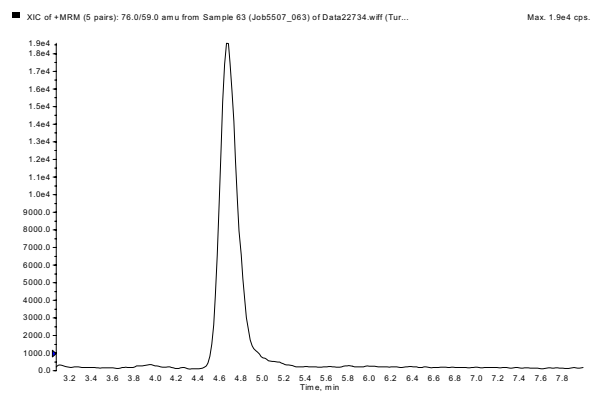
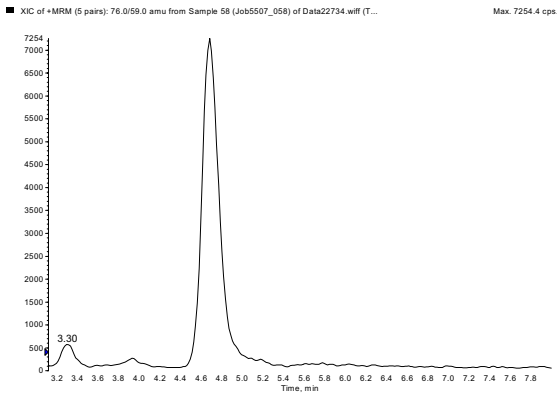


'Unspiked' sample.

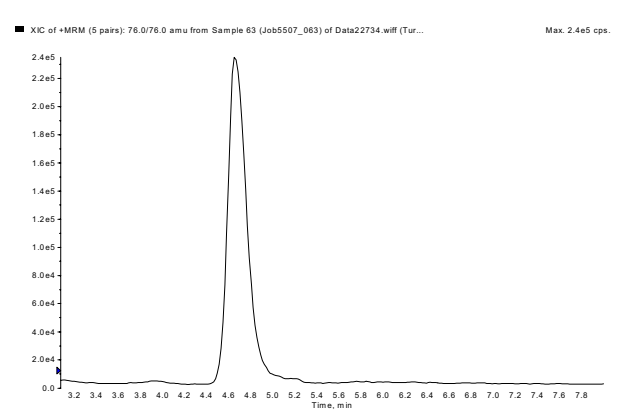
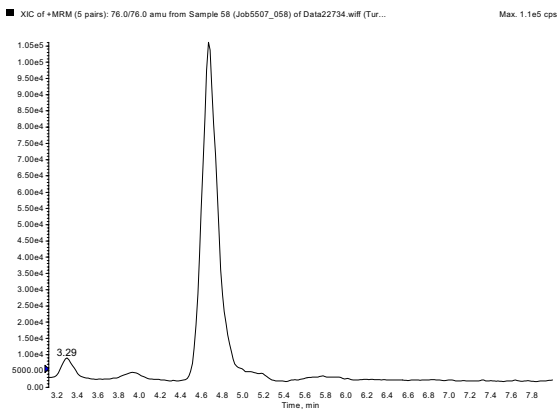
'Spiked' at 1 microgram/ml

Figure 3. Extract of BU: 5 mg shaken with 5 ml of water. 'Unspiked' and 'spiked' at 1.0 microgram/ml.

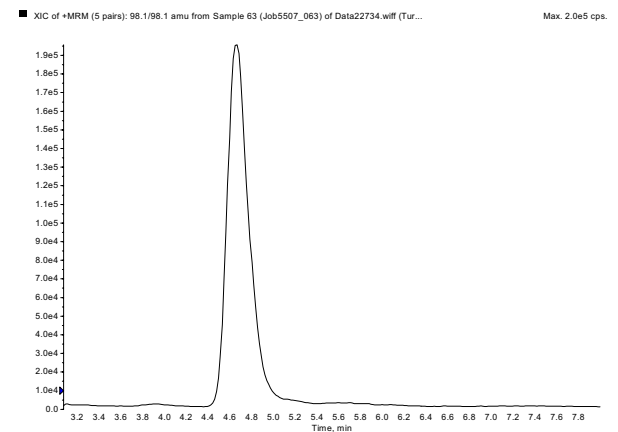
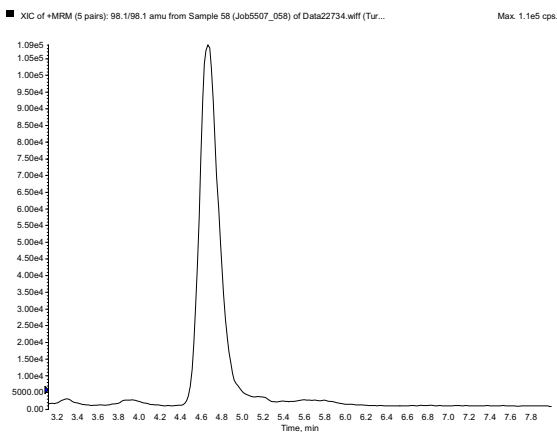
URZ001: transition 76>59



URZ001: transition 76>76



URZ001: transition 98>98



'Unspiked' sample.

'Spiked' at 1 microgram/ml

Figure 4. Urazole: 5 mg dissolved in 5 ml of water. 'Unspiked' and 'spiked' at 1.0 microgram/ml.

TNO's work on gaskets and check on CSL's work

All no derivatisation method samples were analysed using a LCQ Deca system using electrospray ionisation in the positive mode. All derivatised samples were analysed using a TSQ 7000 LC-MS system using positive ion atmospheric pressure chemical ionisation.

'Spike' recovery using the no derivatisation method ranged from 52 to 123 per cent by LC-MS/MS. 'Spike' recovery using the derivatisation method was only obtained on a solution of ADC 'spiked' with SEM. The recovery was 118%. This value was used to correct all the results on derivatised solutions.

Table 2. Comparison of check results obtained by no derivatisation and derivatisation methods (calculated on a mg SEM per kg chemical tested basis after correction for analytical recovery and solubility)

Sample	LC-MS/MS No derivatisation' (mg/kg)	LC-MS/MS Derivatisation (mg/kg)
ADC	<LOD	183 (n=3)
BU	1009 (n=3)	818 (n=3)
BU (after storage)*	877 (n=3)	Not determined
UR 1	512 (n=3)	448 (n=3)
UR 1 (after storage)*	562 (n=3)	Not determined
UR 2	486 (n=3)	Not determined

LOD = 830 mg/kg allowing for solubility

* Test solutions after 2 weeks refrigerated storage

Samples were provided to TNO by CSL. For the method without derivatisation, sample preparation and analysis were carried out according to the CSL method. TNO used their own 2-NBA derivatisation method. The table below summarises the results (mg/kg) obtained using the same chemicals by both CSL and TNO.

Table 3. Comparison of CSL and TNO's results

Sample	CSL No derivatisation LC-MS (mg/kg)	CSL No derivatisation LC-MS/MS (mg/kg)	TNO No derivatisation LC-MS/MS (mg/kg)	CSL Derivatisation LC-MS/MS (mg/kg)	TNO Derivatisation LC-MS/MS (mg/kg)
ADC	<LOD*	121	<LOD [∇]	2583 [#]	183
BU	718	641	1009	1727 [#]	818
UR 1	481	436	512	670 [#]	448
UR2	457	482	486	Not determined	Not determined

*LOD = 160 mg/kg [∇]LOD = 830 mg/kg (Both LODs quoted allow for solubility)

Unacceptable recovery data (outside acceptable limits)

TNO tested two gasket samples. Both (ca. 50 mg) were dissolved/dispersed in 3 ml tetrahydrofuran, from which a 200 microlitre aliquot was used for derivatisation. There was insufficient sensitivity (LOD 0.10 microgram/ml corresponding to 100 mg/kg) to monitor SEM in the gaskets by the no derivatisation method. By the method with derivatisation the levels found in three replicate extracts of each sample were 5.5 ± 0.4 and 6.2 ± 0.5 mg/kg. The 'spike' recoveries on blank solutions (n=3) 'spiked' with SEM were $87 \pm 4\%$.