LC–MS/MS Analysis for Semicarbazide in Food

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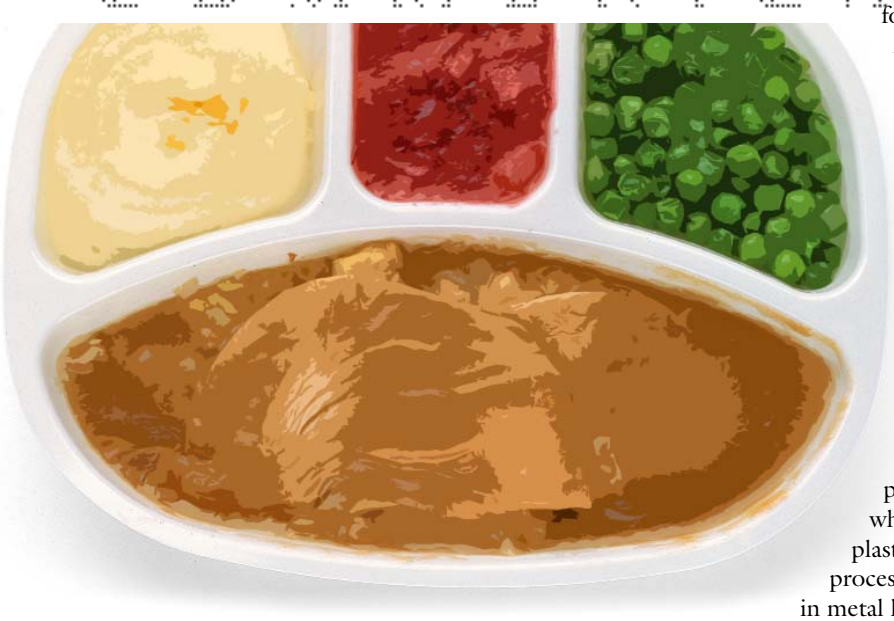
Introduction

The discovery of semicarbazide (SEM) in a number of different foodstuffs has raised many questions such as:

- Is it formed in food?
- Is it, or a precursor, deliberately added?
- Can it migrate into food from packaging?

These questions on the origin of SEM have arisen and they add to the considerable challenge of being able to accurately and quickly determine ppb (parts per billion, ug/kg) levels in a variety of different, often complex, food matrices. To answer these questions and address the concerns of industry and Government alike, the Central Science Laboratory has set about meeting the challenge of analysing SEM in complex food matrices and packaging by employing LC–MS/MS and the results are described in this article.



What is SEM and Where Does it Come From?

SEM belongs to the hydrazine family of chemicals, some members of which are known to possess carcinogenic potential. Information on the potential hazard of SEM itself is incomplete and the possibility that it is genotoxic cannot be ruled out.

SEM from nitrofurazone antibiotic usage: The first tests for semicarbazide in foods were conducted because SEM has been employed as an indicator compound for the use of nitrofurazone antibiotic.[1] Nitrofurazone is just one of a number of nitrofurans that are used as antibiotics to protect farmed poultry and aquaculture products, such as prawns and shrimps, from disease. Their use within the European Union (EU) is not permitted, but nitrofurans have been reported to be widely used in poultry and aquaculture production in the Far East and South America. Such reports led to large numbers of these products being tested by Member States prior to acceptance of the goods at the port of entry. Once administered to poultry, prawns etc. nitrofurazone is metabolized rapidly to SEM, which then binds into the muscle tissues. Thus SEM had been adopted as a marker for nitrofurazone usage and its detection in any consignment of food destined for human consumption entering the EU led to rejection of the consignment.

SEM from azodicarbonamide used to make plastics packaging materials: The discovery that SEM in food may have origins other than solely from nitrofurazone usage came about when, during routine monitoring, SEM was found in foods with no known content of animal or fish constituents. Some detective work by the food and packaging industries pinpointed the origin as the azodicarbonamide used to make plastics packaging materials and this was subsequently confirmed.[2,3]

Azodicarbonamide (ADC) has a long history of use as a blowing agent for plastic gaskets for metal lids on glass bottles and jars. ADC is authorized for use in the EU and in the USA as a blowing agent for plastics that can come into contact with food. Blowing agents such as ADC are added to plastics so that during heat-processing the ADC decomposes to liberate gases which form minute gas cells throughout the plastic, thus making it into a closed-cell foam. This process is required to produce the effective seal found in metal lids of glass jars that ensures the food inside remains airtight and is microbiologically safe.

Azodicarbonamide is structurally related to semicarbazide as shown below.

Azodicarbonamide	$H_2N–CO-N=N–CO–NH_2$
Semicarbazide	$H_2N–NH–CO–NH_2$

Processed foods packaged in bottles and jars that use these gaskets include jams, pickles, honey, baby foods and sauces.

Because SEM seems to be a minor breakdown product of the ADC used to make the gasket, and because the gasket may come into direct contact with food during heat processing and transport of the pack, then migration of SEM may occur into these foods. Typical migration levels appear to be 10 to 20 ppb from gaskets containing 10–30 ppm with occasional higher values.[4] The use of ADC to make plastics intended for food packaging is to be banned in the EU from August 2005.[5]

SEM from azodicarbonamide used as a flour treatment agent: As well as use as a blowing agent, ADC has also been used for many years as an additive to flour. The use of ADC as a flour improver is not permitted within the EU but it is permitted in the USA and in other countries. Usage levels up to 45 ppm of ADC in flour are permitted in these countries. Although ADC decomposes in flour mainly to the non-toxic compound biurea, a minor breakdown pathway seems to form SEM. This could be the route for its occurrence in a number of processed foods such as breaded prawns and chicken produced outside of the EU.[6] SEM has also been reported to be found in baked goods such as bread made in the laboratory using ADC-treated flour.[7] The levels of SEM in the cooked bread were in the 10 to 135 ppb range. The highest level was in the crust, which experiences the higher cooking temperatures.

SEM from the action of hypochlorite on foods: Additionally, it has been reported that the action of hypochlorite on foods and food ingredients can give rise to the formation of SEM. This came to light following the discovery of low levels of SEM in carrageenan gum. This food additive is obtained from seaweed and some processes use hypochlorite to bleach the product. Laboratory tests have also been reported[8] but these tests used rather high concentrations of active chlorine and it is not clear how much SEM, if any, could be formed using lower levels of active chlorine. Residual chlorine may persist on an incompletely rinsed food preparation surface or following washing of salad vegetables with chlorinated water, for example.

Technique for Testing Foods for SEM

SEM is extracted from food samples (e.g., poultry, prawns, baby foods) using 0.2 M hydrochloric acid (Figure 1). A known quantity of triple-labelled stable isotope ($^{13}C^{15}N_2$) SEM is added to every sample as an internal standard to allow the calculation of recovery of any SEM present. The extracted SEM is derivatized with 2-nitrobenzaldehyde and the extract is neutralized. The extract is then applied to a solid-phase extraction (SPE) cartridge and the SEM derivative (NBz-SEM) eluted from the cartridge with ethyl acetate. The eluate is concentrated and the SEM present in the concentrated extract is determined using high performance liquid chromatography (HPLC) with tandem mass spectrometric detection (MS/MS) and an electrospray interface.[1] Using the acid extraction conditions, this procedure determines the total (both free and bound) SEM present in the sample.

The derivatized SEM is separated from any co-extracted interferences by the HPLC column. The retention time is compared with that of a standard solution. The first ionization chamber of the mass spectrometer is then used to detect the SEM derivative using its mass-to-charge ratio (m/z) (for the NBz-SEM derivative a m/z of 209, and a m/z of 212 for the isotopically labelled internal standard). Thus, only molecules of the correct mass (the NBz-SEM derivatives) reach the middle section of the instrument, the collision cell where the molecules are fragmented in a controlled and highly reproducible manner. The fragment molecules (called daughter ions) then enter the second half of the mass spectrometer, and only daughter ions of the correct m/z values (134, 166, 209 and 168 for the internal standard) are allowed to reach the detector. The daughter ion of m/z 166 is used as the quantification ion and the concentration is determined by the ratio of that ion to the internal standard daughter ion of m/z 168. The 134 and 192 daughter ions are normally used only for further confirmation of identity.

LC–MS/MS analysis allows for an unambiguous identification (as described in EU Commission Directive 2002/657/EC) of any SEM present in the sample, as it must have the same retention time, mass and daughter ion fragmentation as an SEM standard.

Figure 2(a) shows the LC–MS/MS traces for the analysis of a baby food containing SEM at 10 ppb. The peak for the internal standard (isotope-labelled SEM) as well as the quantification peak and the confirmation peaks for SEM itself can be seen clearly, with good signal-to-noise ratio. Figure 2(b) shows the equivalent LC–MS/MS traces for a blank baby food. Here, only the internal standard channel shows a peak at the expected retention time and the 3 channels monitored for SEM are blank.

LC–MS/MS analysis allows for an unambiguous identification (as described in EU Commission Directive 2002/657/EC) of any SEM present in the sample, as it must have the same retention time, mass and daughter ion fragmentation as an SEM standard. The use of the stable isotopically labelled internal standard allows for accurate quantification of SEM levels by correcting for any losses during sample extraction and clean-up.

Figure 1: Analysis for bound SEM.

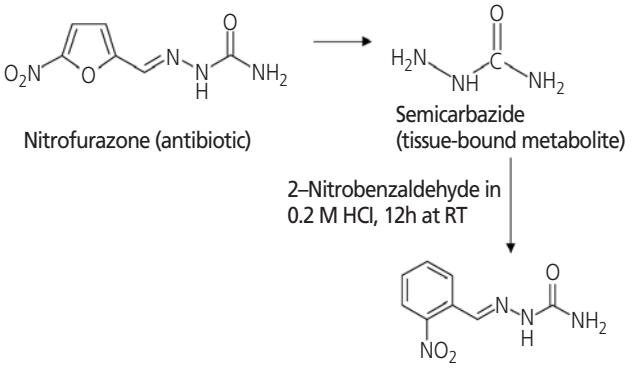


Figure 2: (a) Baby food containing 10 ppb of SEM and (b) containing no detectable SEM.

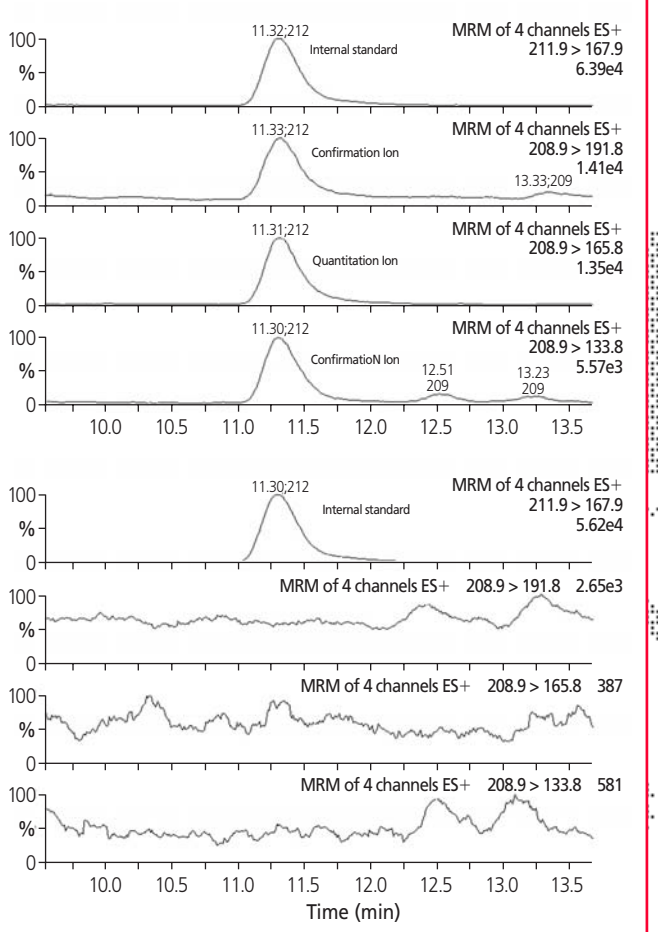
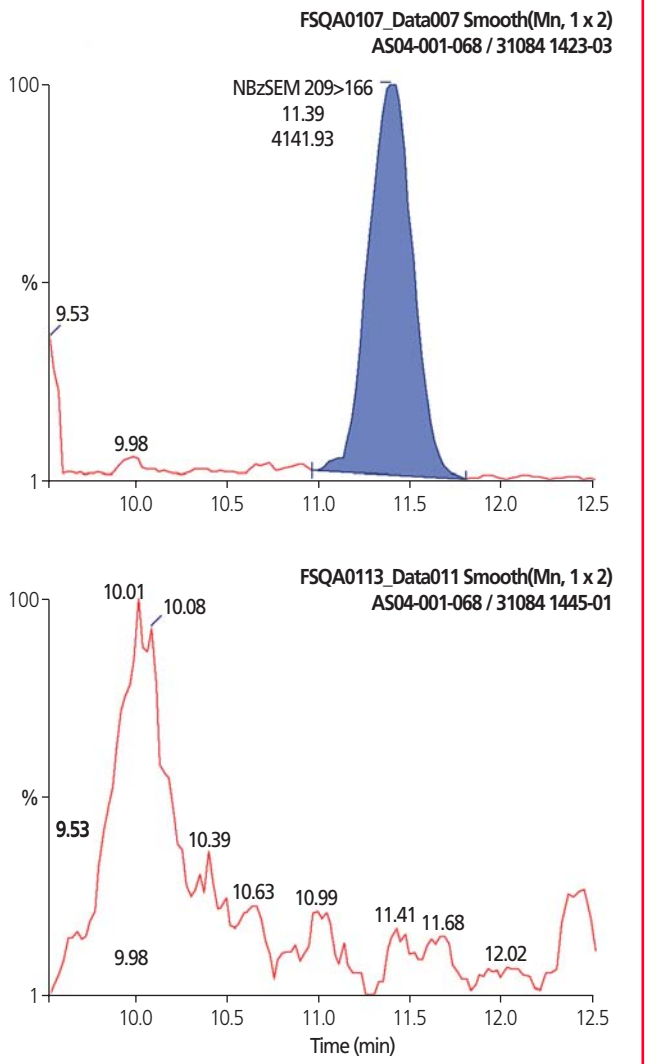


Figure 3: (a) Breaded chicken product (prior to washing step) containing SEM ~1 ppb and (b) after washing step.



Performance of the LC–MS/MS Approach

We have been participating in a European project dealing with, amongst other issues, the validation of a method for determining SEM residues in baby food. For this we have produced various reference materials for other laboratories to test. These have included rice-based, meat-based and fruit-based foods. We have demonstrated the ability to determine levels of SEM in such baby foods in the range 0.25 to 40 ppb with excellent linearity. Higher levels can be determined by dilution of the final extract. For a sample fortified at 10 ppb, on 20 replicate extractions we found an average level of 9.3 ppb with a relative standard deviation of 1.9%. From the performance data obtained we were able to demonstrate that no false positives or false negative results were obtained because of the very high level of selectivity afforded by use of LC–MS/MS. The method can be used effectively for the determination of SEM in a wide variety of processed foodstuffs with minimal method re-optimization.

Free or Bound SEM?

The current methods of analysis used to measure food all involve acid hydrolysis and a derivatization step with 2-nitrobenzaldehyde (2NBA). The hydrolysis releases any SEM bound to protein and the derivatization helps to improve the sensitivity of the MS/MS measurement. As described above, methods were originally developed for the testing for SEM in meat and fish products, as a marker for nitrofurazone usage. However, hydrolytic methods may not be needed for the detection of free SEM, and may actually cause the formation of artefacts, for example the production of SEM, if ADC or similar SEM precursors are present in the test sample.

There are currently no methods for measuring free SEM[2] at ppb levels in food matrices. One suggestion to distinguish between nitrofurazone and other sources of SEM has been to measure the ratio of free SEM to tissue-bound SEM in the food sample. This can be achieved by removing the free SEM present in the sample by including a number of washing steps in the method prior to acid hydrolysis and derivatization. Figure 3(a) shows the LC–MS/MS trace for the 209 >166 quantification transition for SEM, for the analysis of a breaded chicken product. The product was analysed as such (i.e., with the coating included), and it was found to contain approximately 1 ppb of SEM. Using the washing procedure

described above, we found that the SEM could be washed out (Figure 3(b)) and so was not tissue-bound. The conclusion for this sample was that the SEM had originated from the breadcrumb coating rather than from the illegal use of nitrofurazone. In general, both physical removal of any coating before extraction, along with use of the pre-washing procedure, is recommended.

However, interpretation of results is not always so clear-cut. We have observed that when some samples are laboratory spiked with SEM, only between 65 to 85% of this free SEM is removed by subsequent washing. We have also analysed a number of breaded poultry and seafood products found to contain SEM, by using the pre-washing method. Between 15 to 35% of the SEM found in the original product was still present after washing and could be liberated using the acid hydrolysis step. The per cent removal depended on the type of sample being analysed. For example, a higher percentage of SEM was washed from raw chickens and prawns than from their processed equivalents (cooked and coated with breadcrumbs).

Furthermore, following exposure to hypochlorite, it has been reported that some food products give rise to a high fraction of SEM present in an extractable form whereas other food products give rise to SEM in a non-extractable tissue-bound form.[9]

A Minimum Required Performance Reporting Level (MRPL) of 1 ppb for nitrofuran metabolites, including SEM, has been adopted by the European Commission for use in the testing of poultry meat. However, because nitrofurans are banned substances, any detectable level of SEM could be used as an indicator of misuse. Thus it is essential that the method used to determine SEM in samples must be able to distinguish between the free form and the bound metabolite. Given that SEM is a reactive molecule, reacting with carbonyl compounds for example, then interactions with macromolecules such as proteins are likely *in vivo* but also *in vitro*. Consequently it will be difficult, if not impossible, to reliably distinguish SEM arising from nitrofurazone usage from other sources of SEM by using the ratio of free:bound. In fact, the identification and use of other, less ambiguous indicator compounds for nitrofurazone misuse, is desirable.[9]

Conclusions

Low levels of SEM in a variety of raw and processed foods can be identified and measured with high analytical reliability, using LC–MS/MS. However, given that SEM in food may originate from several sources, the results of testing foods must be interpreted with great care.

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