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An Investigation to establish the types and levels of N-nitroso compounds (NOC) in UK consumed foods

A report prepared for the Food Standards Agency

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1. Executive summary

This project was undertaken to address an Agency requirement to identify and determine constituent amounts of nitroso compounds (NOC) in foods formed as a direct result of the manufacturing process. Application of a screening method to 63 retail samples (tested as consumed), representing 10 categories of food / manufacturing processes, showed that 36 samples were at risk of NOC contamination. However, subsequent investigation of these samples for individual NOC (volatile nitrosamines) known to be harmful to human health showed that, with the exception of a dried marine product, contamination with volatile nitrosamines appeared to be minimal in foodstuffs from the UK retail market. This report details the progress in developing screening methods for NOC, methods to quantify constituent amounts of NOC in a wide range of foods / processes available in the UK retail market and recommendations for further work.

2. Contents

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3. Abbreviations

3.1 General abbreviations

BF_3-methanol	Boron trifluoride in methanol
C18	A bonded-phase silica (octadecylsilane) used for reverse-phase liquid chromatography and sample clean-up
CLD	Chemiluminescence detector
EHT	Extreme high tension
GCB	Graphitised carbon black
HS-SPME	Headspace-solid phase microextraction
MSTFA	N-Methyl-N-(trimethylsilyl) trifluoroacetamide
MRM	Multiple Reaction Monitoring (in MS/MS)
MS/MS	Tandem mass sectrometry
PICI	Positive ion Chemical Ionisation
PMT	Photomultiplier tube
PSA	Primary-Secondary Amine sorbent, offering both normal phase and anion exchange retention mechanisms, widely used for pesticide sample clean-up
SA	Sulphamic acid
SAR	Sarcosine
SPE	Solid phase extraction
SPME	Solid phase microextraction

TEA	Thermal energy analyser
z-Sep	A hybrid zirconia-coated silica sorbent developed for the removal of fatty components, widely used in dispersive SPE clean-up applications

3.2 N-nitroso and related compound abbreviations

ATNC	Apparent total nitroso compounds
NAA	N-nitrosoamino acid
NDBA	N-nitrosodibutylamine
NDBZA	N-nitrosodibenzylamine
NDEA	N-nitrosodiethylamine
NDMA	N-nitrosodimethylamine
NDELA	N-nitrosodiethanolamine
NDPA	N-nitrosodipropylamine
NDPhA	N-Nitrosodiphenylamine
NHMT	N-nitroso-2-(hydroxymethyl)thiazolidine
NHMTCA	N-nitroso-2-hydroxymethylthiazolidine-4-carboxylic acid
NHPRO	N-nitroso-4-hydroxyproline
NMAMBA	N-nitroso-N-(1-methylacetonyl)-3-methylbutylamine
NMAMPA	N-nitroso-N-(1-methylacetonyl)-2-methylpropylamine
NMEA	N-Nitrosomethylethylamine
NMOCA	N-nitroso-5-methyloxazolidine-4-carboxylic acid
NMOR	N-nitrosomorpholine
NMTCA	N-nitroso-2-methylthiazolidine-4-carboxylic acid
NMU	N-nitrosomethylurea
NPIP	N-nitrosopiperidine
NPYR	N-nitrosopyrrolidine
NPRO	N-nitrosoproline (D- & L-)
NSAR	N-nitrososarcosine
NTCA	N-nitrosothiazolidine-4-carboxylic acid
NTHZ	N-nitrosothiazolidine
NTHZCA	N-nitrosothiazolidine carboxylic acid and related homologues
NO_3	Nitrate
NO_2	Nitrite
NOC	N-nitroso compound
NOCA	N-nitroso-oxazolidine-4-carboxylic acid
NO_x	Nitrogen oxides
NOZ	N-nitroso-oxazolidinones
NVNA	Non-volatile nitrosamines
VNA	Volatile nitrosamines

4. Introduction

N-nitroso compounds (NOC) are a class of chemicals with the basic structure $R^1R^2N\text{-}N{=}O$, where the R^1 and R^2 groups attached to the amine nitrogen may range from a simple hydrogen (H) to more complex chemical substituents (including ring structures that incorporate the amine nitrogen). Under certain conditions (e.g. pH, temperature, time etc.), NOC can be easily formed from the reaction of secondary amino compounds with nitrosating agents such as nitrite salts and nitrogen oxides (NO_x). Hence NOC might be expected to occur in foods that utilise nitrite salts for preservation and colouring and / or combustion gases for drying etc. NOC most frequently found in foods have included: the dialkylnitrosamines such as N-nitrosodimethylamine (NDMA), and cyclic nitrosamines, e.g., N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP) and N-nitrosothiazolidine (NTHZ); and the group of so called non-volatile nitrosamines (NVNA) which consists mainly of N-nitrosated amino acids, such as the N-nitroso products of sarcosine (NSAR), proline (NPRO) and thiazolidine-4-carboxylic acid (NTCA). A wide range of foods and nearly all Western foods have been tested and found to contain NOC in varying amounts: Foods with some of the highest NOC were sausage, smoked meats, bacon (cooked), and luncheon meats (Stuff et al 2009). Figure 1 gives the structures of some of the nitrosamines found in foods.

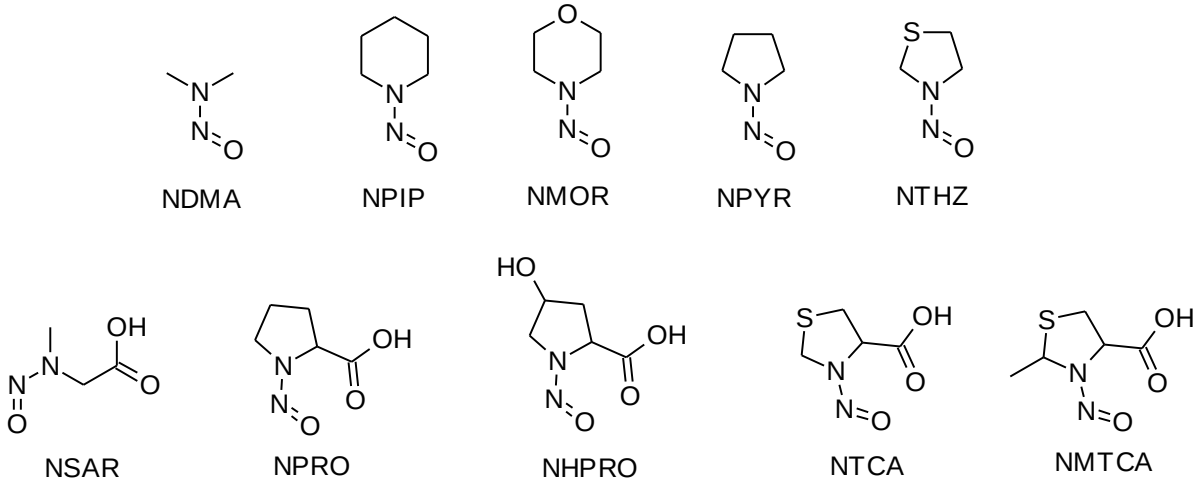


Figure 1. Structure of some volatile (VNA) and non-volatile (NVNA) nitrosamines in foods: NPIP, N-nitrosopiperidine; NMOR, N-nitrosomorpholine; NPYR, N-nitrosopyrrolidine; NTHZ, N-nitrosothiazolidine; NSAR, N-nitrososarcosine; NPRO, N-nitrosoproline; NHPRO, N-nitrosohydroxyproline; NTCA, N-nitrosothiazolidine-4-carboxylic acid and NMTCA, N-nitroso-2-methylthiazolidine-4-carboxylic acid.

4.1 Historical

In 1937 two cases of acute liver toxicity caused by exposure to N-nitrosodimethylamine (NDMA) in a laboratory were reported (Freund et al 1937): In one of these cases, the individual had cleaned up a spill in the open laboratory from a broken bottle of NDMA, became ill soon after and later died from acute liver toxicity. Later,

the carcinogenic potential of NDMA was described by Magee and Barnes (1956) who reported a high incidence of liver tumours in rats fed a diet containing 50 mg/kg NDMA. The first indication that NOC were an environmental hazard was the discovery in 1965 that NDMA in spoiled herring was responsible for an outbreak of acute liver toxicity in Norwegian sheep (Sakshaug et al 1965). The treatment of food with nitrite was subsequently suspected to be the causative factor for the formation of NOC such as nitrosamines. These findings triggered a large number of analytical investigations to discover the extent of human exposure to NOC from foods. The challenging analytical detection of NOC in food was greatly improved with the advent of the highly sensitive and specific gas chromatographic detector described by Fine and co-workers (Fine and Rounbehler 1975; Fine et al 1975), i.e. the Thermal Energy Analyser (TEA). The TEA relies on selective thermal cleavage of the N-NO bond and detection of the liberated NO radical by the chemiluminescence signal generated by its reaction with ozone. Analyses of NOC in foods reached a peak during research conducted the 1970s and 1980s.

4.2 Health risks

While many NOC are carcinogenic in various animal species including higher primates, and at a variety of sites and organs (Forman, 1987; Lijinsky, 1990; Tricker and Preussmann, 1991), some are not, and their potency varies depending on their molecular structure (Dai, 1998; Luan et al., 2005). Metabolic activation of NOC produces intermediates that can react with cellular components such as DNA, proteins and RNA, so that these NOC are carcinogenic and genotoxic. N-nitrosodimethylamine (NDMA) is a highly potent carcinogen, commonly detected in foods, and often used as an indicator compound for nitrosamines. The degree of carcinogenicity among the latter compounds can vary dramatically: N-nitrosodiethylamine (NDEA) is the most potent carcinogen among the nitrosamines, while N-nitrosodiphenylamine (NDP(h)A) is 15,000 times less potent. The carcinogenic hazard of NOC has been classified by a number of international organizations and regulatory authorities. For example, the IARC has classified NDMA and NDEA as “probably carcinogenic to humans” (Group 2A); the UN Globally Harmonised System of Classification (McGregor 2010) categorises NDMA and NDEA as category 1B (Presumed to have carcinogenic potential for humans; largely based on animal evidence); the US Environmental Protection Agency classifies both NDMA and NDEA as “probable human carcinogen (category B2)” under its 1986 carcinogen assessment guidelines. While it is important to reduce preformed NOC in the diet, it must be recognised that human exposure to NOC also occurs via lifestyle and workplace activities as well as endogenous formation from dietary precursors such as nitrite, amines and amides.

4.3 Regulations and limits

Few regulatory limits exist for NOC in foodstuffs: In the USA there are (non-federal) limits for NDMA or total nitrosamines in bacon, barley malt, ham and malt beverages while Switzerland has a limit for total N-nitrosamines of 0.5 µg/kg in beer. Regulatory guideline or assessment levels for drinking water have also been set by a number of international authorities: Health Canada (2011) has set a limit of 0.04 µg/l NDMA in drinking water (Government of Canada 2011); and the WHO has proposed a guideline level of 0.1 µg/l NDMA (WHO 2008). While there are no regulatory limits for NOC in foods in the EU, Directives limit total N-nitrosamines

to 10 µg/kg in rubber pacifiers (EC 1993) and there is a negative list for NOC in cosmetics and regulations for cosmetic ingredients to avoid NOC formation (EC 1976).

4.4 Project brief and lines of approach

This project was undertaken to address an Agency requirement to identify and determine constituent amounts of NOC in foods formed as a direct result of the manufacturing process. Based on earlier studies in the UK (Massey and Key 1989; Massey et al 1990), the Agency had identified products produced by fermentation for possible investigation. Historically, much of the data relating to NOC in UK foods have been reported as apparent total nitroso compounds (ATNC) providing no information on the types or levels of NOC present thus preventing toxicological evaluation of the risks to consumer health. Furthermore, measurements of ATNC in foods suggested that individual NOC have mostly not been identified as the ATNC measurements appeared to exceed the sum of individual NOC. However, the question of whether ATNC methods from this period were truly selective for NOC has not been answered fully.

The project was undertaken in two phases. In the first phase, a thorough review of the literature was undertaken covering known / potential unknown NOC, toxicology, analytical methods, formation mechanisms (with particular reference to microbes / enzymes used in food processing), occurrence in foods and exposure, knowledge gaps and recommendations for subsequent investigations (phase two). The data from the review was then used to identify foodstuffs, NOC and analytical methods for a "phase two" investigation. A method to measure ATNC was subsequently developed and carefully validated to provide a measure of the total NOC in target foods. Data generated from these measurements was then used to identify candidate foods for the analysis of known NOC of toxicological significance e.g. some nitrosamines. The results from the analysis of individual NOC were then critically reviewed in relation to the data obtained by ATNC. Conclusions and recommendations for further work were made.

5. Knowledge gaps, conclusions and recommendations from the pre-investigation review

A review of the literature covering the chemistry of formation, health effects, analysis, occurrence and exposure to preformed N-nitroso compounds in foods and beverages was undertaken at the outset and was on-going throughout project. A summary of the conclusions from the review together with recommendations for a "Phase 2" analytical investigation are given below.

5.1 The chemistry of NOC

Nitrosamines derived from secondary amines are relatively stable compounds found in the environment and processed foods. Nitrosamides, on the other hand are directly reactive chemicals, relatively unstable and unlikely to be abundant in foods.

In aqueous media, pH is decisive for nitrosation. Acid-catalysed nitrosation is negligible at pH >5 and hence nitrosamines in foods are more likely to be formed by exposure to atmospheric nitrogen oxides (NOx).

Nitrites, thiocyanates, formaldehydes and organic acids, added or present naturally in foods can catalyse the formation of NOC. The nitrosation reaction can also be inhibited by redox compounds such as ascorbic acid and tocopherols (e.g. vitamin E), sulphydryl compounds and phenols.

The reaction of α-amino acids, aldehydes and nitrite has been found to lead to the formation a potentially toxic group of heterocyclic nitrosamines, nitroso-oxazolidinones (NOZ). It is not known if these chemicals are formed in foods.

5.2 Preformed dietary NOC of toxicological concern

The most likely classes of preformed NOC in foods of known toxicological significance are the simple (i.e. without additional substituents) diakyl and cyclic nitrosamines (VNA). With the exception of NHPRO and NPRO the carcinogenicity of many NVNAs remains poorly elucidated (Tricker et al 1991; Herrmann et al 2015) as full toxicological evaluations cannot be completed due insufficient data. However, NSAR was reported to be a relatively weak oesophageal carcinogen in rats and not genotoxic (IARC 1978; Couch and Friedman 1976) while most other NVNA were not believed to be genotoxic and carcinogenic (Habermeyer and Eisenbrand 2009).

5.3 Method of analysis

Most of the principal methods for determining NOC relied on the conversion of NOC to NO which was detected by TEA. The conversion of NOC to NO had been achieved by pyrolysis at 450°C after GC separation (GC-TEA) and by chemical treatment of NOC with e.g. HBr. GC-TEA had been widely used for the analysis of VNA mainly because of its specificity and hence simple traces. However, GC-MS was a useful method for confirming the identity of GC-TEA responses and some recent studies favoured MS detection.

Individual NOC comprising ATNC in extracts from foods have mostly not been identified. This raises the question of whether ATNC is a reliable measure of NOC, especially since it has been reported that some chemicals such as nitrosothiols can also be determined as ATNC. Hence ATNC may be more useful in determining the potential for NOC formation in foods. Nevertheless, research is needed to determine the specificity of ATNC systems and / or the identity of ATNC in foods.

Analysis of foods reached a peak in the early 1980s and it appears that the monitoring of foods and beverages for their NOC content has slackened in recent years. However, it is essential to continue this effort by periodic analysis so that the impact of changes in e.g. agricultural or manufacturing practices on NOC can be determined.

5.4 Formation and exposure

Beer, meat and fish have previously been identified as the main sources of exposure to VNA from which previous exposure estimates to VNA were in the range 0.34 – 14 ng/kg bw/day (Penttila et al 1990; Tricker et al 1991; Lutz and Schlatter 1992; Herrmann et al 2015). Based on these data, most estimates of exposure risk in the literature, using the so called Margin of Exposure approach, were >10000 (BMDL10[1]) for NDMA / VNA

[1] Bench Mark Dose Lower Limit - the 95% lower confidence interval on a BMD for a 10% increase in tumour incidence (EFSA, 2005; Barlow et al 2006)

(Dybing et al 2008; Habermeyer and Eisenbrand 2009; Herrmann et al 2015), indicating that they would be of low concern in foods (EFSA 2005). However, preformed NOC in the diet and environmental media are not the only source of human exposure. The intake of tobacco specific nitrosamines from smoking as well as endogenous formation (from the intake of dietary substances) may also represent significant sources of human exposure to NOC.

5.5 Recommendations for analytical investigation

5.5.1 Selection of foodstuffs

Table 1 gives the products that were identified from the review for investigation on the basis that either they were known to contain NOC or they might have been subjected to nitrosating conditions during production or processing:

Table 1. Recommended foodstuffs for investigation

Foodstuff	Risk factor(s)
Bacon	Nitrite / domestic cooking
Beer / malt, especially beer imported from developing countries	Direct drying of malt (NOx)
Cheese	Nitrite
Cured meat and fish, including marine products from Asia	Nitrite accumulation / domestic cooking
Infant formulae, powdered soups, spices, tea	Direct drying (NOx), nitrite content, nitrosatable amine precursors
Food containing acid-HVP, e.g. soups, gravies, bouillons, soy sauces	Low pH processing HVP, nitrite content
Pickled foodstuffs including fish and vegetables	Low pH / nitrite accumulation from nitrate reductase enzymes (endogenous / exogenous)
Smoked (wood) cheese, meat and fish	Aldehyde catalysts in wood smoke / temperature and time / domestic cooking
Soy sauces especially from developing countries	Nitrite accumulation from nitrate reductase enzymes (microbial) / thermal treatments (pasteurization)

Since thermal processes could have a significant impact on NOC, it was proposed that foodstuffs should tested as consumed by cooking or preparing (according to manufacturers' guideline) prior to analysis.

5.5.2 Potential / unknown NOC for analysis

- ATNC should be measured in all candidate foods and beverages and used primarily as an indicator of NOC contamination
- Subject to the availability of reference standards, the following known NOC should be determined in foods as appropriate: NDBA, NDEA, NDMA, NDPA, NMEA, NMOR, NPIP, NPYR, NTHZ, NSAR and NTCA
- N-nitroso-oxazolidinones (NOZ) are a potent group of carcinogens that might be formed in foods from simple dietary chemicals (amino acids and aldehydes) in the presence of nitrosating agents. Subject to the availability of reference standards, a method should be developed to determine whether these chemicals are formed in foods.

5.5.3 Choice of analytical methods

- Most methods for ATNC have used chemical stripping (de-nitrosation) with Chemiluminescence detector (CLD) with precautions. The selectivity / specificity of the chemical stripping / CLD system should be established by careful validation to determine whether ATNC measured is representative of NOC.

- GC-TEA has been the method of choice for VNA and methods based on solvent extraction are likely to be applicable foods. More recently, a number of studies have reported using GC-MS and the use of chemical ionisation as an ion preparation technique looks promising.
- SPME is the only rapid method of analysis that has been reported for NOC (nitrosamines). However, variable recoveries can be a feature of SPME necessitating the use of isotopically labelled internal standards for accurate quantification: Isotopically labelled NDMA (NDMA-d_6) is commercially available.
- Methods for NVNA are less developed than those for VNA although GC-MS of volatile derivatives (e.g. using alkylation or silylation) appears to be robust and solvent extraction procedures used for VNA may be applicable.
- LC-MS/MS may be the detection method of choice for NOZ. No methods have been reported for NOZ in foods and the availability of reference standards may be a limiting factor.

6. Sampling

In total, 10 categories of foods were identified for investigation using the criteria defined in 5.5.1. These categories included: unsmoked bacon; beer from EU and non EU countries; cheese (unsmoked); cured meat and fish (unsmoked); dried products (i.e. infant formulae, milk powders, soups, spices, tea); malted foods (i.e. beverages and breakfast cereals); pickled products (i.e. fish and vegetables); soups, gravies and bouillons; soy sauces and wood smoked products (i.e. fish, meat and cheese). Individual samples / brands were then identified based on UK market share information (as of 2014; Mintel, London) and / or availability in the UK provided by the sampling contractor, Hallmark (Veterinary and Compliance Services, Stonehouse, Gloucester). A sampling plan (see Appendices 10.1) was constructed in conjunction with the sampling contractor and the Food Standards Agency prior to collection of 63 samples from UK supermarkets and specialist on-line retailers by Hallmark over the period Nov 2014 – Jan 2015. Samples were delivered to PAS in batches within shelf life and with consideration of storage (and transportation temperature) requirements given on retail packs. Perishable and short shelf life foods were stored on receipt at -18° until required for preparation / analysis.

6.1 Sample preparation

Since thermal processes could have a significant impact on NOC, samples requiring cooking or diluting with hot water were prepared according to manufacturers guidelines on retail packs (where given) immediately before analysis. The remainder of prepared samples were stored at -18°C. Sample preparation details can be found in 10.3 of the Appendices.

7. ATNC measurements on target foodstuffs

The method generally used for the measurement of apparent total NOC (ATNC) was developed in the late 1970s by the Walters Group (Downes et al 1976; Walters et al 1978) and was dependent on the finding that HBr, but not HCl, reacted with NOC to produce NO via a chemical denitrosation reaction (Equations I and II).

$$R^1N\,R^2(NO) + HBr \quad R^1NHR^2 + NOBr \qquad (I)$$

$$2NOBr \quad 2NO + Br_2 \qquad (II)$$

Solutions of NOC, generally but not necessarily in water, were treated with sulphamic acid (SA) under acidic conditions to destroy any nitrite present. SA also eliminated a slight response due to nitrate (Sen and Kubacki 1987). The resulting solution was reacted with HBr to liberate NO (from NOC) which was then passed to a chemiluminescence detector (CLD) for measurement. When SA was not added, the method could be used to determine nitrite (which apparently was also reduced to NO by HBr), preferably after separating the nitrite by HPLC (Sen et al 1994). The NO passed to the CLD was oxidized in an evacuated reaction chamber with ozone (O_3), producing electronically excited nitrogen dioxide (NO_2*). The excited nitrogen dioxide rapidly decayed back to its original ground state with the emission of a photon (chemiluminescence). This release of energy could be measured using a photomultiplier and was proportional to the concentration of NO.

The apparatus used for the chemical denitrosation of NOC was typically custom manufactured in glass with a system of ports (septa) to introduce reagents/samples: the NO released by chemical denitrosation was then swept to the detector under positive pressure using an inert gas. Massey et al (1984; 1987; 1989; 1990; 1991) treated prepared food extracts contained in the apparatus with the denitrosation reagent (HBr in acetic acid) directly while other workers determined extractable NOC using an organic solvent such as acetonitrile (Fiddler et al 1995) (for a review see Mirvish 2008). In the former case, it could be assumed that ATNC measured was therefore representative of all NOC as the entire food was essentially diluted with water in the apparatus. The main disadvantages of this approach were the relatively slow turn-around of samples for analysis and the potential for false positive responses from other components of the foodstuff. On the other hand, the presentation of selective food extracts to the denitrosation reagent contained in the apparatus could permit multiple analyses as the (excess) reagent was not fully consumed. While the latter approach gave some selective clean-up up of samples for analysis, extraction efficiencies appear to have been assumed as data for recoveries of known NOC had not been reported.

Irrespective of the limitations of ATNC, the technique has enabled foods that contain relatively high levels of NOC to be identified for further investigation although care must be taken when interpreting data from ATNC measurements.

7.1 Method development

7.1.1 Target method performance criteria

At the project outset the performance criteria for the ATNC method were defined in relation to the Agency project brief, in particular the requirement for rapid methods. The following target method performance criteria were identified:

- A method detection limit of circa 10 µg NNO / kg foodstuff
- A method capable of multiple determinations without the requirement to clean and recharge the apparatus
- A method capable of selective extraction of NOC permitting:
    - reduced cleanup / removal of e.g. nitrite
    - defined recoveries for different classes of known NOC

7.1.2 Selection / sourcing of the ATNC system

Early systems used for ATNC were based mainly on the commercial TEA manufactured by Thermo Electron interfaced to custom built chemical denitrosation systems. The TEA comprised a flash catalytic pyrolyzer, for the thermal formation of NO from N-NO linked to a chemiluminescence detector. For ATNC measurements the TEA was operated without the catalytic pyrolyser as NO was generated by chemical denitrosation. While the Thermo Electron TEA units were no longer available, a development of these systems was currently in production with Ellutia in the UK. Furthermore, a commercial "Chemical Stripping System" had been developed for use with the Ellutia TEA based on established chemical denitrosation chemistry. An application produced by Ellutia for the determination of total nitrosamines (Langdon 2012) indicated that the system could provide the basis for a method to measure ATNC in foods. No further manufacturer of TEA systems could be identified and a system was subsequently purchased from Ellutia and installed in May 2014.

7.1.2.1 The Elutia TEA and the chemical stripping system

A schematic of the Ellutia 810 TEA system is shown in Figure 2 while images of the actual system can be seen in Figure 3. The reaction vessel, (5) shown in Figure 3 D), comprised a two-neck round bottomed flask: the first neck was fitted with a PTFE backed septum for sample introduction and a fitting for the nitrogen carrier gas; the second neck was fitted with reflux condenser exiting at the top with connection to pass the carrier gas via PTFE tube to a cold trap. An immersion chiller provided the cooling to the reflux condenser via a peristaltic pump to and from the coolant reservoir. The cold trap situated after the reflux condenser was placed within the coils of the immersion chiller in the coolant reservoir to provide a localised temperature of -18° at the cold trap. A needle valve, located after the cold trap, was used to adjust the flow of nitrogen to the 810 TEA. In the "chemical stripping" mode, the pyrolyser unit of the TEA was bypassed (via a 3-port valve) and NO liberated from NOC in the reaction vessel was swept directly to the CLD under nitrogen flow.

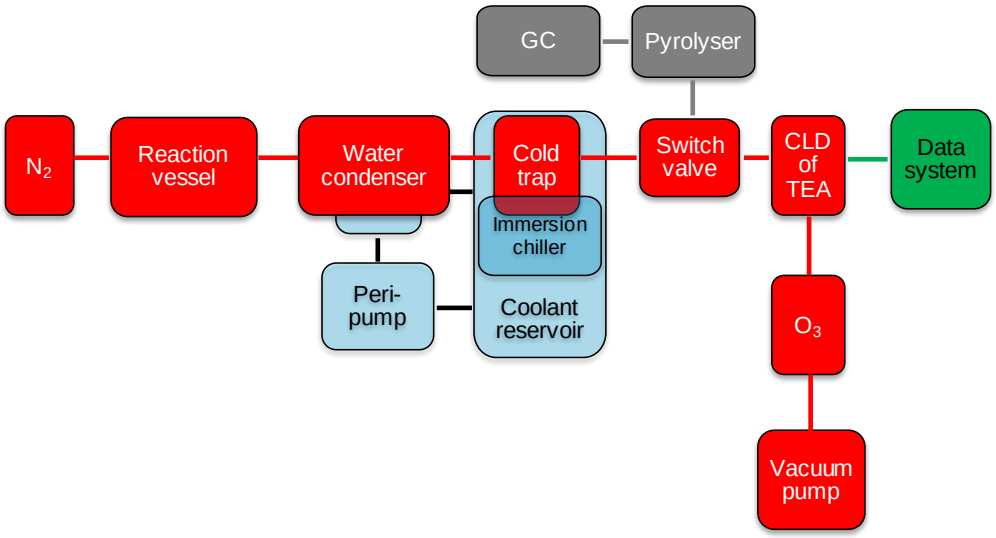
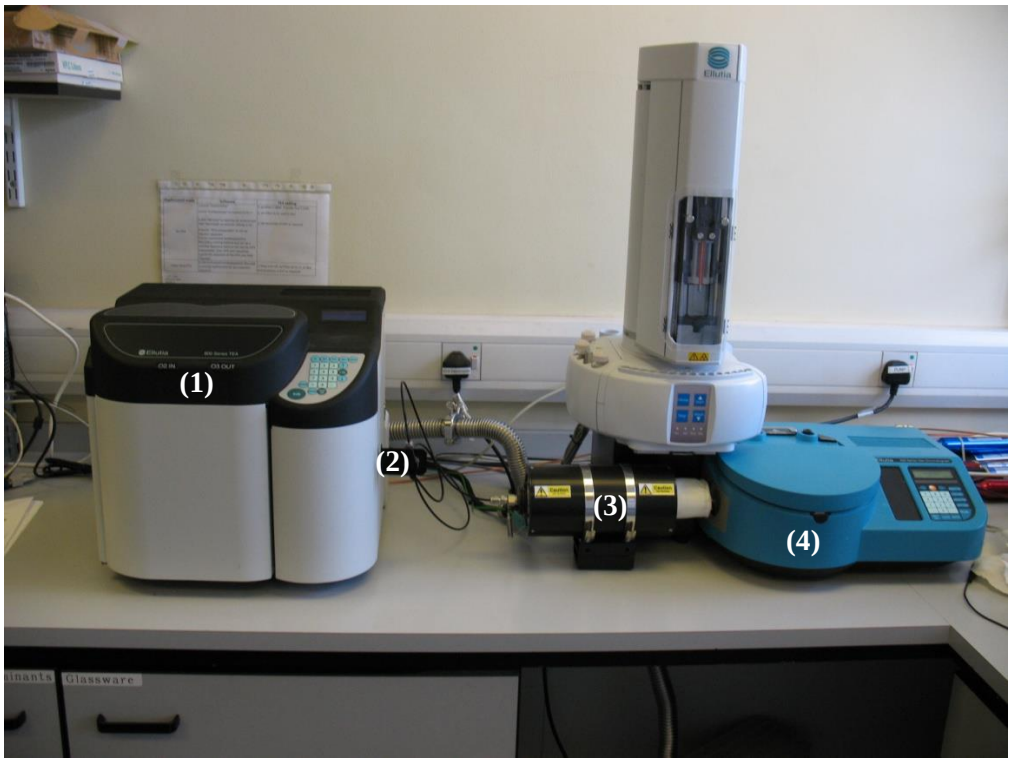


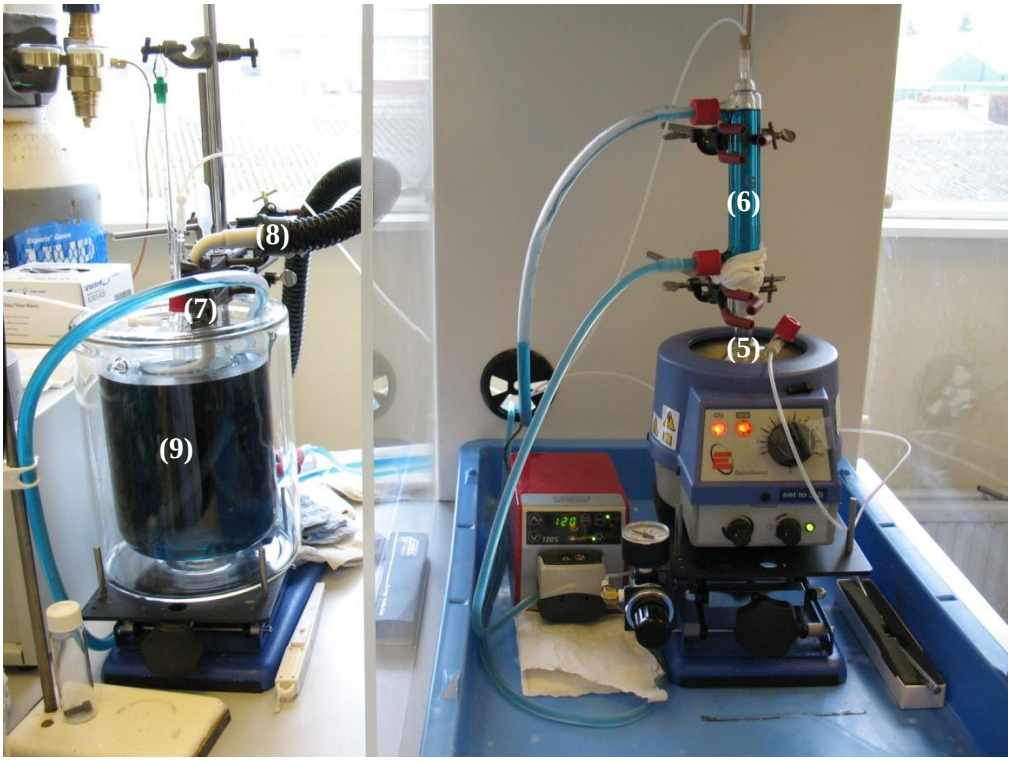
Figure 2. Schematic of Ellutia 810 TEA system operating in chemical stripping mode (red pathway)

A)

B)



C)



D)

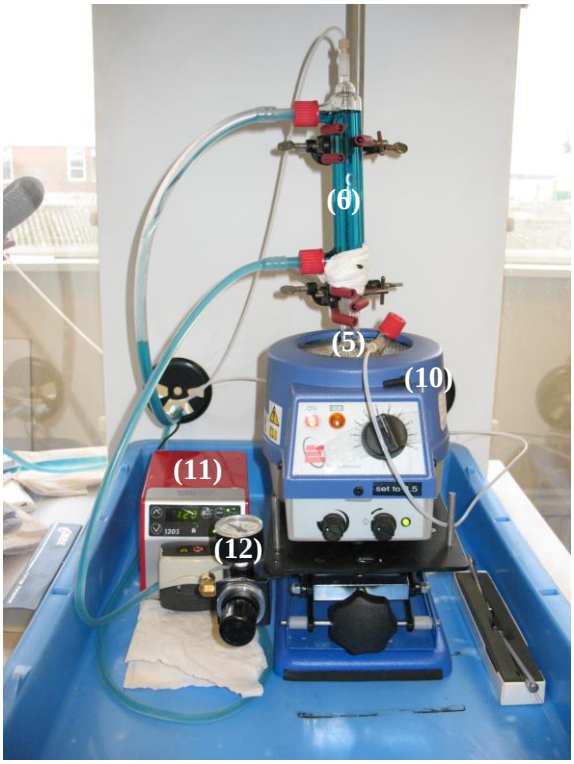


Figure 3. The Ellutia TEA system showing: A) The complete system; B) The 810 TEA (1), switching valve (2), pyrolyser (3) and GC (4); C) the Chemical Stripping reaction vessel (5), water condenser (6), cold (solvent) trap (7), immersion chiller (8) and reservoir (9); D) close up of reaction vessel (5), heater / magnetic stirrer (10), water condenser (6), peristaltic pump (11) and nitrogen pressure regulator (12).

7.1.3 Reference standards

A total of nine VNA (NDMA, NMEA, NDEA, NDPA, NDBA, NPIP, NPYR, NMOR, NDPhA) together with their isotopically labelled analogues (for later use, see section 8) were obtained from Fisher Scientific (Loughborough, UK) and CDN Isotopes (Quebec, Canada) respectively. A total of six NVNA (NSAR, D- and L- NPRO, NTCA, NMTCA and NHPRO) were supplied by Toronto Research Chemicals (Ontario, Canada). NTHZ and NOZ, identified in the pre-investigation review, were not commercially available and hence methods to determine these NOC were not progressed. Details of all reference standards can be found in 10.2 of the Appendices.

Stock solutions of VNA (in dichloromethane) were serially diluted with ethyl acetate as required. Stock solutions of NVNA in water were serially diluted with methanol as required. Mixed standards and standards for sample addition were prepared in methanol.

7.1.4 Initial enabling and sustained performance

The Ellutia 610 TEA / Chemical Stripping system was installed in May of 2014 and following a period of familiarisation, operational and data handling training was undertaken in August 2014.

In the chemical stripping mode, the system was arranged according to the diagram in Figure 2 and the images shown in Figure 3. The default operation conditions provided by the manufacturer are given in Table 2.

Table 2. TEA / Chemical Stripping System: default operational conditions (manufacturer)

TEA CONDITIONS		COMMENTS
Sensitivity	213	
Oxygen Flow (ml/min)	3.4	Used to generate O_3 (within TEA)
Vacuum (Torr)	1.78	Operation vacuum during Chemical Stripping
Pump	Edwards RV3	Exhaust fitted with a 610 ozone trap
CHEMICAL STRIPPING SYSTEM		
Carrier gas	Nitrogen	
Carrier gas pressure (psi)	5	
Carrier gas flow (ml/min)	50	
Coolant (ethylene glycol)	50% (w/w)	
Cold trap temperature (°C)	-25 ±5	
Reaction vessel	Hydrobromic acid (5 ml) in ethyl acetate (15 ml)	Magnetic stir bar used

With the apparatus assembled and the carrier gas and O_3 needle valves closed, the vacuum at the TEA was approximately 0.2 Torr. The O_3 needle valve was opened to give a vacuum of approximately 0.9 Torr: The carrier gas needle valve was then opened until a carrier flow of approximately 50 ml/min (at a pressure of 5 psi) was attained. Under these conditions, the vacuum at the TEA was circa 2 torr. Hydrobromic acid (5 ml) and

ethyl acetate (15 ml) were added to the reaction vessel using a syringe (through the reaction vessel septum port) and the solution stirred and heated until a steady reflux of the solvent was observed. The system was allowed to equilibrate for approximately 20 minutes prior to making injections (initially 50 µl) of standard solutions of NDPA.

Figure 4 shows the typical chemical stripping-TEA chromatograms obtained for standard solutions of NDPA under the conditions described in the text above. A typical sequence of injections comprising solvent blank / standard / solvent blank showed no extraneous peaks (in the blank injections), no carry over of standards and full recovery of the baseline after each injection. Although the denitrosation reagent was replenished prior to operation on a daily basis, this did not appear to be a limiting factor for repeated injections of standards (n≤50).

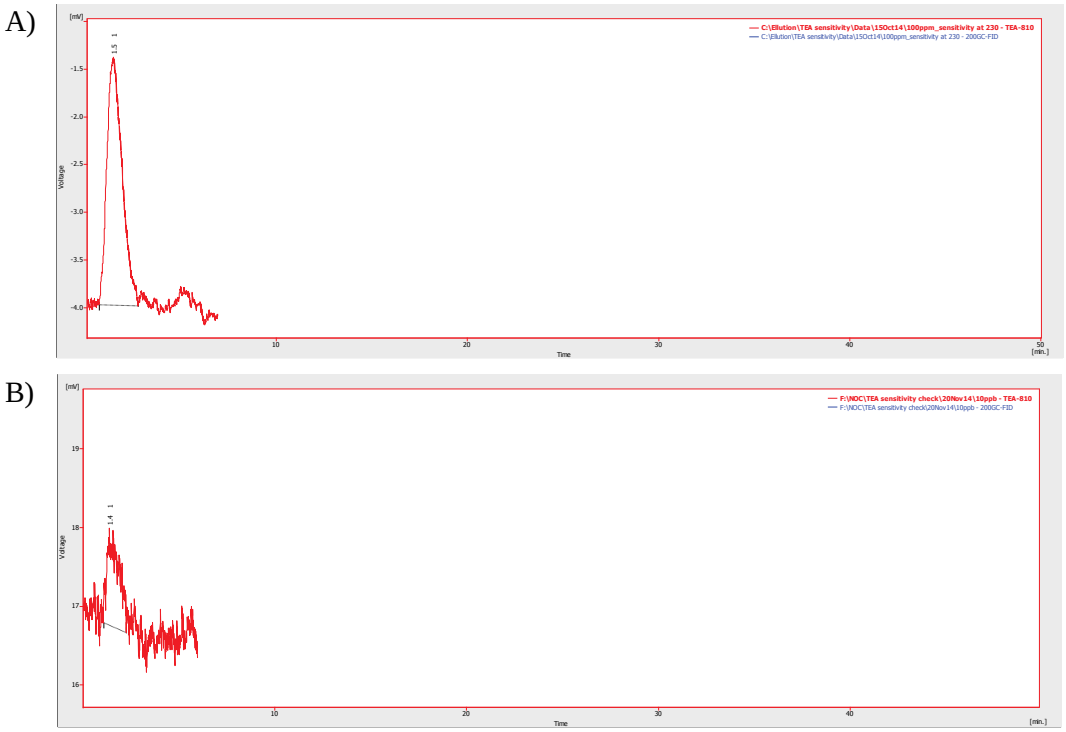


Figure 4. Chemical stripping-TEA chromatograms from initial ATNC performance trials: A) 100 µg/l NDPA; B) 10 µg/l NDPA

Optimum signal to noise ratios (S/N) of circa 26 and 2 were obtained for repeated injections (100 µl) of NDPA at 100 µg/l and 10 µg/l respectively but using an enhanced sensitivity setting of 230 (compared to a specification of 213) indicating that the sensitivity of the system did not meet the manufacturers specification (see 10.4 of the Appendices). There then followed a prolonged dialog with the manufacturer and an exchange of reference standards to confirm the lack sensitivity, neither of which resolved the inadequate performance. During this period the system had been operated "on demand" i.e. it was shut down completely post operational, on a daily basis. A chance finding after leaving the system operational over a weekend indicated that the system required prolonged equilibration under vacuum (days) for maximum sensitivity, and this was much improved by leaving the TEA under constant vacuum. Under these conditions, a limit of detection of 8 µg/l NNO (S/N=3) could be

attained although this required 100 µl injections of standard solutions of NDPA at a sensitivity setting of circa 220 (see Figure 5). Note, the ATNC system was calibrated in NNO equivalents, obtained by multiplying the concentration of NOC in solution by the mol fraction (i.e. 44/molecular weight of the reference NOC).

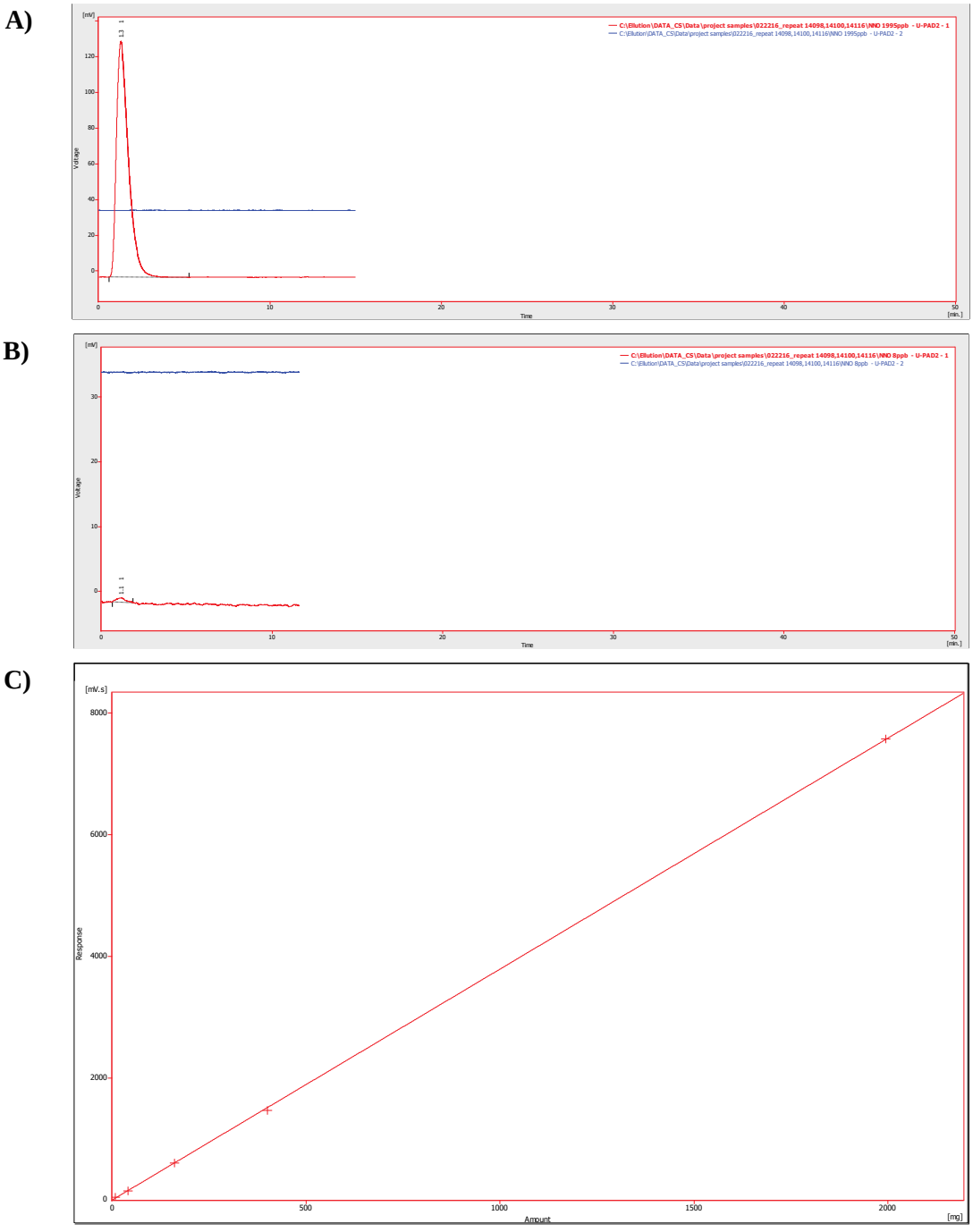


Figure 5. Calibration of the ATNC system: A) ATNC chromatogram from a 1995 µg/l NNO reference standard; B) ATNC chromatogram from a 8 µg/l NNO reference standard; C) calibration curve (y axis = detector response; x axis = µg/l NNO) for the concentration range 0-1995 µg/l NNO.

After several periods of prolonged operation (mths) of the chemical stripping system a gradual increase in the operation vacuum at the TEA was observed. After circa nine mths, the operational vacuum increased from a baseline value of circa 2 torr to 8 torr which also resulted in a corresponding loss of sensitivity (peak area counts reduced by circa 60% for a 1995 µg/l calibration standard). Inspection of the high vacuum pump connected to the TEA showed that solvent (ethyl acetate) was condensing in the pump and compromising the vacuum. While the manufacturer had specified a target cold trap temperature of -25°C this was not attained on our system where the temperature recorded by mercury thermometer at the trap was typically -20°C. It would appear that prolonged operation of the chemical stripping system may result in deterioration of the high vacuum pump and that this should be inspected / maintained regularly for optimum performance of the TEA. Early TEA instruments were normally used with an ice bath to trap evaporated ethyl acetate followed by a second trap filled with a mixture of liquid nitrogen with solvent (e.g. acetone, ethanol or iso-pentane) to maintain a temperature close to -100°C, which in theory trapped all but NO (Crews 2017, personal communication).

Following replacement of the pump a series of intermittent spikes were observed in TEA chromatograms. These were believed to result from the presence of moisture in the photomultiplier tube (PMT) while the instrument had been shut down for pump servicing. As the PMT was maintained at -20°C, water vapour released from trapped ice was causing the resultant spikes in chromatograms. The spikes gradually abated after prolonged pumping and the instrument was subsequently left under vacuum permanently while the extreme high tension (EHT) and ozone were turned off when not in use.

After operation of the TEA system in this way for a further 4-6 weeks no response could be obtained from the detector following start-up using standard solutions of NOC at any concentration. When the ozone sensitivity setting was increased significantly (from 213 to 245) detector response was restored. While minimising the sensitivity of the ozone generator was beneficial in maintaining the longevity of the unit, it would appear that there was a relatively narrow band in which generation could occur and that the setting required optimising (increasing) regularly. Beyond a maximum setting of 255, the unit required replacement and was a high cost item.

7.1.5 Development and optimisation of the ATNC extraction procedure

While ATNC methods are well documented in the literature, there was little or no data relating to the extraction efficiency and selectivity of these systems with respect to NOC. This may be due in part to the method by which samples have been presented to the ATNC system for analysis as typically the foodstuff was homogenised with an aqueous inhibitor (to prevent NOC formation during analysis) prior to treatment with a chemical stripping reagent. This "in-situ" approach had assumed that as all NOC were present in the sample (i.e. the sample has essentially been diluted) they would be released by treatment with the chemical stripping reagent. Although this was a reasonable assumption, it did not consider the impact of matrix components on the efficacy of the chemical stripping reagent. Furthermore, it was noted that the latter approach could also contribute a significant background response at the detector prior to chemical denitrosation, and this response was subtracted from

results after making measurements with and without the chemical stripping reagent. As each measurement required cleaning and recharging of the apparatus with freshly prepared samples, the approach was very time consuming.

The objective of this task was to develop a simplified and selective procedure, using only basic laboratory equipment with minimal solvent usage, to prepare sample extracts for injection into the chemical stripping system. This approach could permit multiple determinations to be made without the requirement to clean / recharge the system after each measurement. The challenge in developing such a procedure was to find an extraction system that was applicable to the wide range of NOC physical properties and sample compositions:

- NOC physical properties encompassing non-polar, polar, ionic (basic and acidic) and neutral
- Sample compositions with varying amounts of moisture, fat, carbohydrate, protein, sodium chloride
- Variable nitrate / nitrite content (NO_x) and potential artefact (NO) formation
- Very complex matrices (spices)

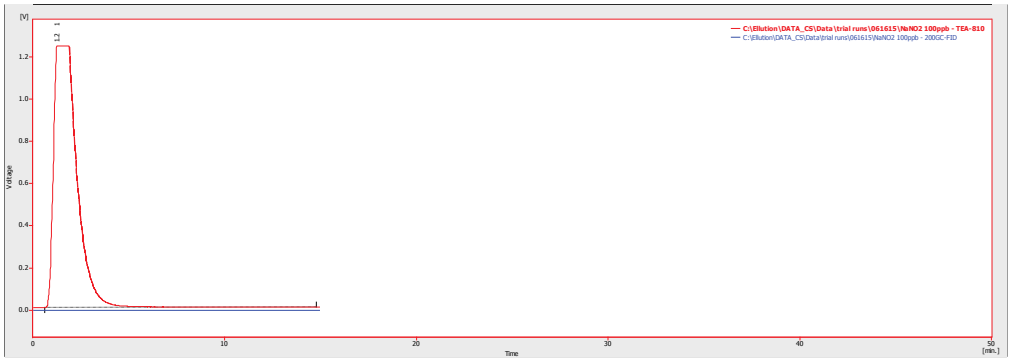
Throughout the development, the efficiency of a range of extraction methods was evaluated by measuring the spike recovery of VNA (9-standard mix), and NVNA (NSAR and NTCA).

7.1.5.1 Selectivity

Solutions of HBr, used to liberate NO from NOCs for detection by TEA, can also react with nitrite (added or present in foods) to release NO (for a review see Mirvish 2008). Sulphamic acid can suppress the formation of NO from nitrite (nitrite reacts with SA to give nitrogen, sodium sulphate and water) without interfering in the chemical denitrosation of NOC, and its effectiveness was initially evaluated using reagent blank samples spiked at the equivalent of 100 mg/kg nitrite on a sample basis (i.e. half the maximum limit for bacon).

Figure 6 shows that in the absence of SA, solutions of nitrite subjected to ATNC produced a saturated response at the detector; the addition of 1 ml of a solution of 180 g/l SA to solutions of nitrite prior to ATNC completely eliminated any response at the detector (<1µg NNO/kg expressed on a sample basis).

A)



B)

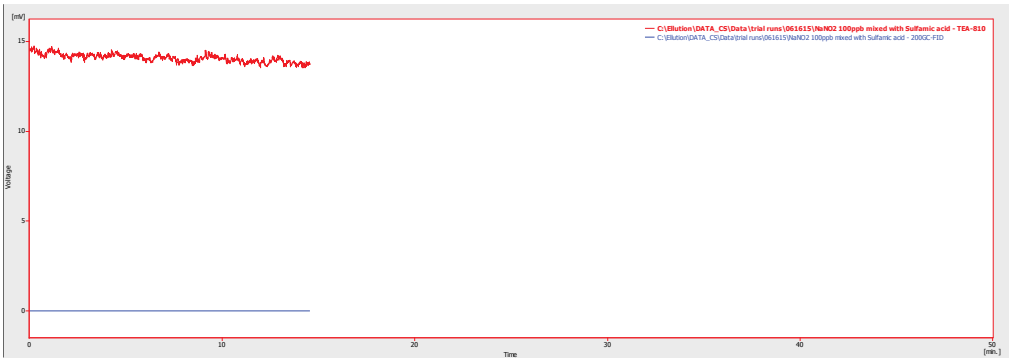


Figure 6. ATNC Chemical stripping / TEA chromatograms for reagent blank samples spiked with nitrite at the equivalent of 100 mg/kg (sample basis): A) without SA; B) with the addition of SA

The effectiveness of SA on artefact formation was subsequently evaluated during the course of method development for individual samples and is described in the text that follows.

7.1.5.2 Evaluation of solid phase extraction methods

Liquid-liquid extractions of aqueous samples such as beer, soy sauce and milk powders (as consumed) using e.g. diethyl ether resulted in emulsions making phase separation difficult and this was could not be resolved using the addition of salts such as ammonium sulphate.

To overcome this, the use of preparative solid phase extraction (SPE) was evaluated using diatomateous earth (Extrelut®) as the sorbent which could then be packed into glass columns for elution with an organic solvent. As large volumes (circa 100 ml) of solvent were used with this technique, the requirement to reduce this by circa 10 fold prior, e.g. using a Kuderna-Danish evaporator, prior to the final determination placed restrictions on the choice of elution solvents. Both dichloromethane and diethyl ether had been used by other workers and were reasonably volatile although the literature suggested that the solubility of some target NOC could be higher in the latter solvent. Furthermore, it was anticipated that the use of SPE would minimise the co-extraction of nitrite (as the latter would not be expected to partition into organic solvent) and hence avoid the use of SA during sample extraction.

When samples of beer, soy sauce and made up milk powder were prepared by SPE, initial trials demonstrated that the approach was practical for beer and soy sauces but not milk powders as plugging of the SPE column occurred during elution with organic solvent. Hence the SPE development was progressed initially for beer described below.

Samples of beer (15 g) were weighed accurately into each of seven glass beakers (labelled 1-7): Aliquots of standard solutions of VNA were added to beakers 2 – 5 to give NNO sample concentrations in the approximate range 70 – 1400 µg/kg; aliquots of NTCA were added to beakers six and seven to give NNO sample concentrations at 110 and 1100 µg/kg respectively. A sachet of Extrelut 20® was added to each beaker and mixed thoroughly using a glass rod. A set of seven 20 mm i.d. chromatography columns with PTFE rotary taps (each containing a 2 cm bed of anhydrous sodium sulphate at the bottom frit) were labelled and packed with each of the seven prepared samples. The columns were eluted with 3 portions of solvent (acetone:diethyl ether, 1:9 v/v, 20 ml x 2 + 15 ml x 1) into a 40 ml Kuderna-Danish (K-D) flask. The eluate was concentrated to 1 - 1.5 ml using the K-D apparatus (water bath @ 56°C) prior to transferring to a graduated glass test tube. The K-D flask was rinsed with acetone and combined with the solvent in the graduated test tube. The final volume was adjusted to 3 ml using acetone and the prepared solutions stored at -18°C until required for ATNC analysis by Chemical Stripping/TEA. The procedure was repeated for spiked and unspiked samples of soy sauce.

Figure 7 shows that a near quantitative recovery of reference VNA could be obtained from spiked samples of beer and soy sauce while the recovery of the NTCA was significantly lower.

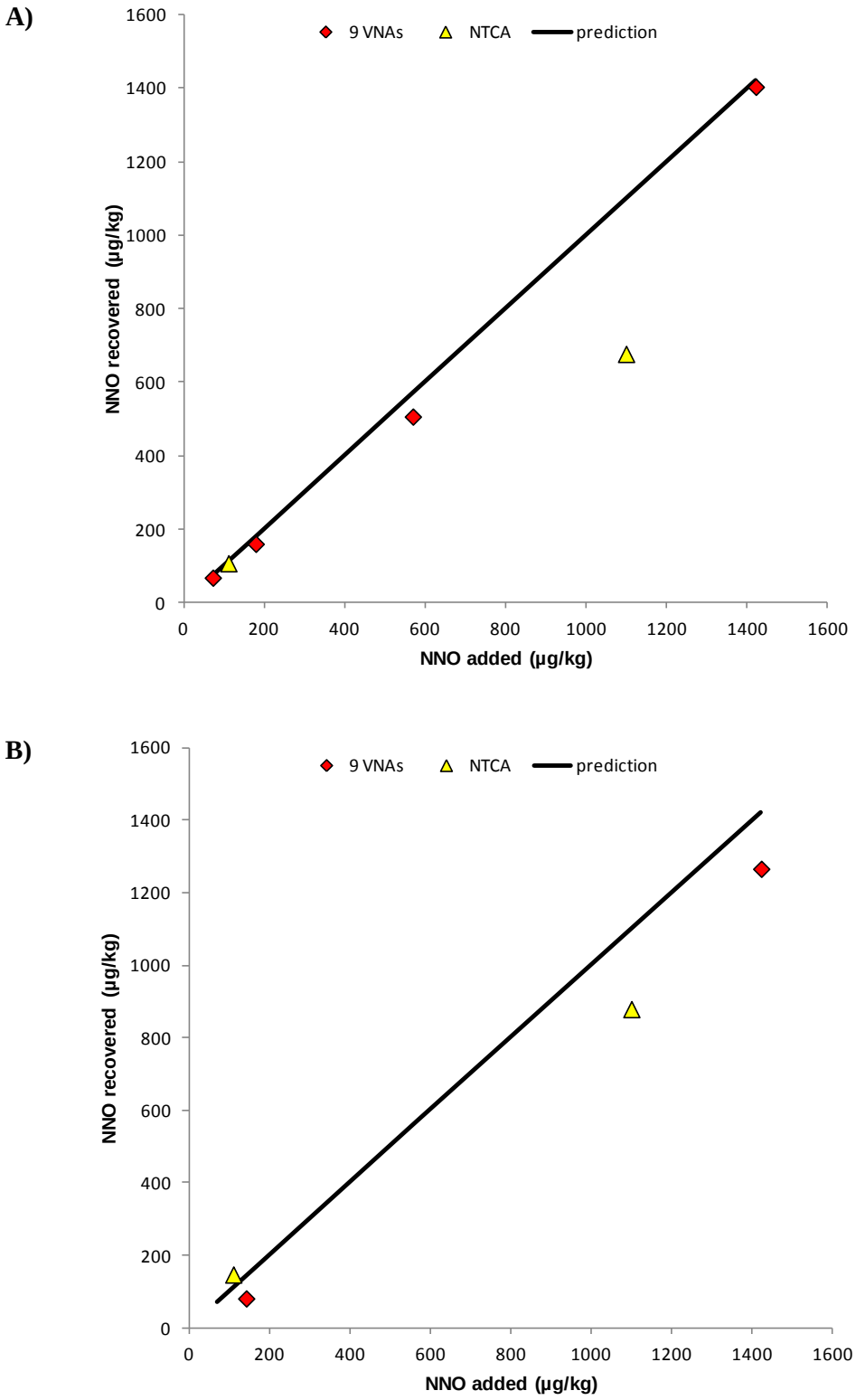


Figure 7. ATNC recovery trials for aqueous samples prepared by SPE: A) Beer; B) soy sauce

The data shown in Figure 7 were corrected for the unspiked values obtained for NNO in the beer and soy sauce of 50 and 675 µg/kg respectively. The results from the unspiked samples appeared to be relatively high

compared to values reported in the literature and this was attributed to nitrite in the samples which may not have been removed as expected during the Extrelut SPE step: Nitrite can release NO by reaction with HBr during chemical stripping step (see 7.1.5.1) and or react with nitrosatable amines to form NOC.

The SPE method was then extended to include malted cereals as this matrix was representative of the dry product samples (as consumed) that had been obtained for investigation: NSAR was also included in subsequent recovery determinations as the pre-investigation review had indicated that it may be weakly carcinogenic (IARC 1978), unlike most NVNA which were not believed to be mutagenic and not carcinogenic (Habermeyer and Eisenbrand 2009).

Malted grain samples and beer (15-20 g) were spiked with VNA or NSAR: the grain was extracted with a solution of 5% methanol in water (V/V) prior to adsorption on Extrelut 20®; beer was applied directly to the Extrelut 20®. The water / methanol extraction solvent was selected to accommodate the different solubility characteristics of both VNA and NVNA. SPE columns packed with the pre-adsorbed samples were then eluted using dichloromethane and the final extracts were concentrated to 1.5 – 2.0 ml using the K-D apparatus.

Repeated ATNC analyses of the unspiked grain and beer gave variable results: trial 1) gave NNO values of 35 and 3 µg/kg respectively; while trial 2) gave 25 and <1 µg/kg indicating a possible lack of homogeneity in the malted grain or possible artefact formation during analysis (e.g. from co-extracted nitrite). In the case of the latter, artefact formation may also have occurred from reactions of nitrosatable amines in the samples with environmental NO_x present (adsorbed) onto the diatomaceous earth matrix indicating that SA addition may be required prior to sample extraction.

A quantitative recovery of VNA was subsequently obtained from spiked samples of the grain and beer but NSAR was not recovered from either sample type. To understand where the loss of NSAR had occurred, a solution of water / methanol was then spiked with NSAR and prepared by the Extrelut SPE method. Again, NSAR was not recovered under these conditions possibly due to its high solubility in water (Herrmann 2014) / methanol and / or binding to Extrelut®. Furthermore, if the properties of NSAR were similar to the parent amino acid sarcosine, then the solubility in water / methanol might be expected to increase either side of the isoelectric point (assuming its zwitterionic around neutral pH), i.e. it may require complex buffering to be recovered from aqueous solutions into organic solvents.

Table 4 shows the results obtained from the further analysis of beer and soy sauces samples following the addition of SA (0.2 ml of 180 g/ml) prior to extraction. While blank values below the LOD and consistent results could be obtained, the results also confirm the relatively poor recovery of NVNA such as NTCA, particularly in soy sauces (10% recovery).

Table 3. ATNC results from the analysis of beer and soy sauce samples using SPE

Sample code	Description	µg NNO/kg		Recovery (%)
		Added	recovered	
-	Beer procedural blank	-	<1, <1	-
14050	UK/EU beer		2.3	-
14050	UK/EU beer	7.5[a]	6.5	57
14051	UK/EU beer	-	2.2	-
14052	UK/EU beer	-	3.0	-
14053	Imported beer	-	1.7	-
14054	Imported beer	-	2.3	-
14054	Imported beer	5.0[a]	4.7	49
14055	Imported beer	-	0.9	-
14056	Imported beer	-	1.6	-
14057	Imported beer	-	4.0, 4.5	-
	Soy Sauce procedural blank	-	<1, <1	-
14089	Dark Soy Sauce		0.5	-
14090	Soy Sauce		0.8	-
14091	Dark Soy Sauce		1.0	-
14092	Dark Soy Sauce		1.7	-
14093	Dark Soy Sauce		1.7	-
14094	Dark Soy Sauce		5.3, 6.1	-
14094	Dark Soy Sauce	10.0[a]	6.2	10
14094	Dark Soy Sauce	7.1[b]	13	97

[a] NTCA; [b] 9 standard VNA mix

7.1.5.3 Evaluation of direct solvent extraction methods

Herrmann (2014) recently described a LC/MS method for the analysis of NA in a range of meat products including bacon. The author reported good recoveries of both VNA and NVNA from cooked bacon using acetonitrile and hence the extraction procedure was evaluated for ATNC sample preparation.

Fresh, raw bacon rashers (smoked and unsmoked), obtained from a local retail outlet (High Wycombe), were cooked directly on a cast iron hot plate (equilibrated for 5 min on the hob of a domestic cooker, high setting) for 1 min each side. Cooked bacon (28 g) was immediately diced on a clean domestic china plate using a chef's kitchen knife before grinding under liquid nitrogen until homogeneous in a motor and pestle. Aliquots of the prepared samples (3 g) were transferred to a 50 ml of centrifuge tube together with 0.25 ml of 180 µg/ml SA_{aq} (omitted for artefact trial), acetonitrile (9 ml) and a ceramic stir bar. The tubes were capped and vortex mixed until uniform then tumble mixed for 10 min. The samples were then centrifuged at 3500 g_{av} for 10min and the liquid decanted to a second centrifuge tube. The mixing and centrifuge steps were repeated using 4 ml of acetonitrile and the liquid layers combined. The volume of the extracts was reduced to 3 ml under nitrogen stream prior to ATNC analysis by Chemical Stripping / TEA.

Table 4 shows the results obtained from the analysis of the cooked bacon samples. The results obtained from the analysis of smoked bacon at day 0 demonstrate the impact of SA on artefact formation with values of 1981 µg NNO/kg and 959 µg NNO/kg obtained from samples prepared without and with the addition of SA respectively. Repeat analysis of the smoked bacon after storage for 1 day at 4°C appeared to confirm the initial result (day 0) however the value obtained from the bacon after storage for 7 days at 4°C more than doubled. The amount of NNO measured in the unsmoked bacon (also prepared with SA addition) was significantly less than that in the smoked product and this also increased after storage for 7 days at 4°C.

Table 4. ATNC results obtained from the direct solvent extraction of cooked and stored bacon

cooked bacon trial	µg NNO/kg storage time at 4°C (days):		
	0	1	7
smoked, without artefact control	1981	-	-
smoked, with artefact control	959	918	2082
unsmoked, with artefact control	-	329	504

These results suggest that nitrosating agents, present in and/or generated during cooking may continue to produce NOC during the storage of cooked bacon. Although the data was limited, the results also indicate a requirement to record elapsed time / storage conditions post domestic preparation and prior to analysis, i.e. to facilitate interpretation of results. The ATNC results from this initial trail were all within the wide range of values reported in the literature for cooked bacon (Massey et al 1991) although it is interesting to note that details for elapsed time / storage between cooking and analysis of bacon samples were not given.

The direct solvent extraction method based on acetonitrile was extended to dried products such as soups / gravies / bouillon. In this instance, samples were extracted directly with acetonitrile without the addition of SA: it was assumed that any nitrite present would not be soluble in the solvent and there unlikely to be co-extracted. Analysis of these samples (3 g), extracted directly with acetonitrile, showed that four of the five samples gave a response for ATNC in the concentration range 25-104 µg/kg NNO, and that a quantitative recovery (101%) of VNA/NVNA added to a stock cube could be obtained (see Table 5). A further trial extraction of a stock cube using acetonitrile / diethyl ether appeared to give a slightly higher concentration of NNO while the recovery of NNO added to the sample was lower (82%): Diethyl was added to facilitate separation of aqueous and organic phases (acetonitrile is miscible with water) should the method be extended to these samples prepared as consumed, i.e. with the addition of hot water.

Table 5. ATNC results from the direct solvent extraction of dry soups, gravies and bouillon

Sample code	Description	µg NNO/kg		Recovery (%)
		Added	recovered	
-	Procedural blank	-	<1	-
14062[a]	Gravy powder	-	27	-
14063[a]	Soup mix	-	45	-
14069[a]	Gravy powder	-	<1	-
14070[a]	Beef stock cube	-	25	-
14071[a]	Beef stock cube	-	104	-
14071[a]	Beef stock cube	399	239	101.4
14071[b]	Beef stock cube	-	119	-
14071[b]	Beef stock cube	399	228	82.4

[a] acetonitrile extraction solvent; [b] acetonitrile / diethyl ether (3:2) extraction solvent

7.1.5.4 Evaluation of direct aqueous injection methods

Dried samples were prepared "as consumed" according to the conditions in Table 24. Prepared samples (10 ml), with or without the addition of SA (0.2 ml of 180 g/l), were centrifuged 4500rpm for 10 min and passed through a 0.45um syringe filter prior to injection into the chemical stripping system.

The results given in Table 6 show that, in the absence of SA addition, ATNC obtained by direct injection of the prepared samples ranged from samples NOC determined by ATNC ranged from 8-95 µg NNO/kg: The addition of SA to prepared samples of stock cubes gave a significant reduction in ATNC values. When the results for prepared samples were expressed on a dry weight basis, comparison with the data given in Table 5 for the samples analysed as received, suggests that the ATNC values may have increased as a consequence of the domestic sample preparation. A direct comparison may not valid due to the different extraction methods used and the assumption that artefact formation was controlled during the preparation of samples using solvent extraction (SA omitted). However, the possibility of nitrosation during domestic preparation using hot water (from nitrite in samples) and / or the presence of polar (water soluble) NOC in the aqueous samples cannot be discounted.

For the analysis of beer, samples (with or without the addition of SA, 0.2 ml of 180 g/l) were passed through a 045 um syringe filter without any further preparation prior to injection into the chemical stripping system.

The results from the analysis of beer samples prepared with and without the addition of SA are given in Table 7. Surprisingly, the addition of SA appeared to give higher ATNC and overall the results obtained by direct injection were higher than those obtained using SPE, which could be due to the presence of higher concentrations of soluble (polar) NOC in the aqueous samples.

Table 6. ATNC results from the direct injection of soups, gravies and bouillon prepared as consumed and comparison with results tested as received (by solvent extraction)

Sample code	Description	µg NNO/l as consumed	
		Wet weight	Dry weight
-	Procedural blank	<1	-
14062[a]	Gravy powder	32	453
14063[a]	Soup mix	53	708
14069[a]	Gravy powder	8	118
14070[a]	Beef stock cube	70, 95	3242, 4391
14070[b]	Beef stock cube	<1, 17	<1, 782
14071[a]	Beef stock cube	38, 47	1189, 1465
14071[b]	Beef stock cube	15, 24	464, 730

[a] without the addition of SA; [b] with the addition of SA

Table 7. ATNC results obtained from the analysis of beer using direct injection into the chemical stripping system and comparison with results obtained by SPE sample preparation.

Sample code	Description	µg NNO/l		
		Direct injection		SPE (Extrelut®)
		30/12/15	19/01/16	
14050	UK/EU beer	0.0[a]	0.0 [b]	2.3
14051	UK/EU beer	0.0[a]	10.0 [b]	2.2
14052	UK/EU beer	0.0[a]	0.0 [b]	3.0
14053	Imported beer	0.0[a]	24.4 [b]	1.7
14054	Imported beer	0.0[a]	17.8 [b]	2.3
14055	Imported beer	0.0[a]	23.2 [b]	0.9
14056	Imported beer	44.2[a]	-	-
14056	Imported beer	67.4[b]	84.7 [b]	1.6
14057	Imported beer	175.3[a]	-	4.0
14057	Imported beer	180.7[b]	247.6 [b]	4.5

[a] without the addition of SA; [b] with the addition of SA

7.1.6 The QuEChERS method

To overcome some of the difficulties and limitations identified from the development trials, in particular the requirement for sample specific methods and extraction solvents for different sample types, an alternative approach to ATNC sample preparation was researched.

The QuEChERS-method (quick, easy, cheap, effective, rugged, and safe) was developed for the analysis of veterinary drugs (anthelmintics and thyreostats) in animal tissues but after realising its great potential in the extraction of polar and particularly basic compounds it was subsequently published as a pesticide residue method (Anastassiade et al, 2002; 2003). QuEChERS has since become a European standard method for pesticides and has found many applications in contaminants analyses. The compatibility of QuEChERS with NOC extraction solvents (e.g. acetonitrile) favoured by researchers (Herrmann 2014, Fiddler and Pensabene 1996), tolerance to water, high extraction efficiency for polar compounds and speed of analysis made this a strong candidate for a universal sample preparation method and initial trials were conducted according the method described below.

Samples (2.5 - 10 g) were dispersed with acetonitrile (10 ml + 5 ml) in the presence of aqueous SA (0.25 ml of 180 g/l) and anhydrous magnesium sulphate (4-6 g) in a 50 ml centrifuge tube. Combined acetonitrile extracts were concentrated under nitrogen stream to a final volume that was equivalent to the initial sample weight prior to analysis by chemical stripping / TEA.

For the latter step, trials with spiked solutions of acetonitrile showed that a quantitative recovery of NOC could be attained as long as the final extract was not taken to dryness. The recovery of VNA and NVNA added to a range of aqueous and non-aqueous sample types is shown in Table 8. Overall, the recovery of VNA ranged from 74 - 121% (mean 104%) and the recovery of NVNA (NSAR and NTCA) ranged from 13 – 137 % (mean 73%). The recovery of the latter still appeared to be sample dependent, particularly for smoked fish and soy sauce, but was an overall improvement on initial methods using liquid-liquid and solid phase extraction. A detailed description of the method including the preparation of samples can be found in 10.5 of the Appendices.

Table 8. ATNC spike recovery measurements for samples prepared by the QuEChERS-method

sample code	sample description	test date	NNO added		NNO recovered			
			VNA (µg/kg)	NVNA (µg/kg)	VNA (µg/kg)	VNA (%)	NVNA (µg/kg)	NVNA (%)
14057	Beer	07/01/2016	-	80	-	-	70	88
14050	Beer	18/01/2016	-	80	-	-	64	80
14062	Gravy	15/01/2016	-	160	-	-	219	137
14062	Gravy	15/01/2016	162	-	175	108	-	-
14068	Chicken Soup	15/01/2016	-	80	-	-	94	118
14071	Stock cube	20/01/2016	-	80	-	-	78	97
14081	Muesli	21/01/2016	-	200	-	-	144	72
14081	Muesli	21/01/2016	178	-	171	96	-	-
14084	Beverage	27/01/2016	-	80	-	-	22	28
14089	Soy Sauce	26/01/2016	-	80	-	-	74	93
14094	Soy Sauce	26/01/2016	-	160	-	-	43	27
14094	Soy Sauce	17/02/2016	-	80	-	-	25	31
14094	Soy Sauce	17/02/2016	57	-	47	82	-	-
14098	Smoked bacon	19/02/2016	1620	-	1960	121	-	-
14115	Bacon	25/02/2016	-	399	-	-	235	59
14100	Smoked cheese	18/02/2016	-	40	-	-	12	30
14100	Smoked cheese	22/02/2016	114	-	131	115	-	-
14102	Smoked fish	25/02/2016	142	-	105	74	-	-
14102	Smoked fish	25/02/2016	-	200	-	-	26	13
14116	Cured meat	17/02/2016	-	399	-	-	343	86
14116	Cured meat	17/02/2016	285	-	316	111	-	-
14064	Tea	26/02/16	100	-	-	-	102	102
14064	Tea	26/02/16	-	71	80	113	-	-
14087	Beetroot	27/02/2016	712	-	819	115	-	-
14087	Beetroot	27/02/2016	-	998	-	-	1032	103
		min	57	40	47	74	12	13
		max	1620	998	1960	121	1032	137
		mean	371	201	423	104	162	73

When the method was applied to samples of beetroot and spices (paprika, cardamon) a very broad peak (circa 12 min at the baseline) and rising baseline was observed in the TEA chromatogram post injection. Figure 8 shows the response obtained for a sample of pickled beetroot and was typical of the responses also observed for cardamom and paprika. When a VNA or NVNA reference standard was co-injected with the sample a relatively narrow peak and quantitative response (Figure 8 B and C) was obtained indicating that the denitrosation chemistry for the standards was not compromised by the sample. Fiddler et al (1995) reported broad peaks some classes of NOC determined as ATNC which appeared to be a related to the denitrosation chemistry / conditions used. Broad peaks and rising baselines can also be an indication of pressure pulses in the chemical stripping apparatus, e.g. from chemical reactions occurring between components of the sample and reagents used and malfunctions in the vacuum system. Repeat injections demonstrated that the vacuum at the TEA remained stable during analysis and hence the origin of the broad peaks remained unknown. These examples illustrate the

complexity of ATNC measurements using chemical denitrosation and the care that should be exercised when interpreting results.

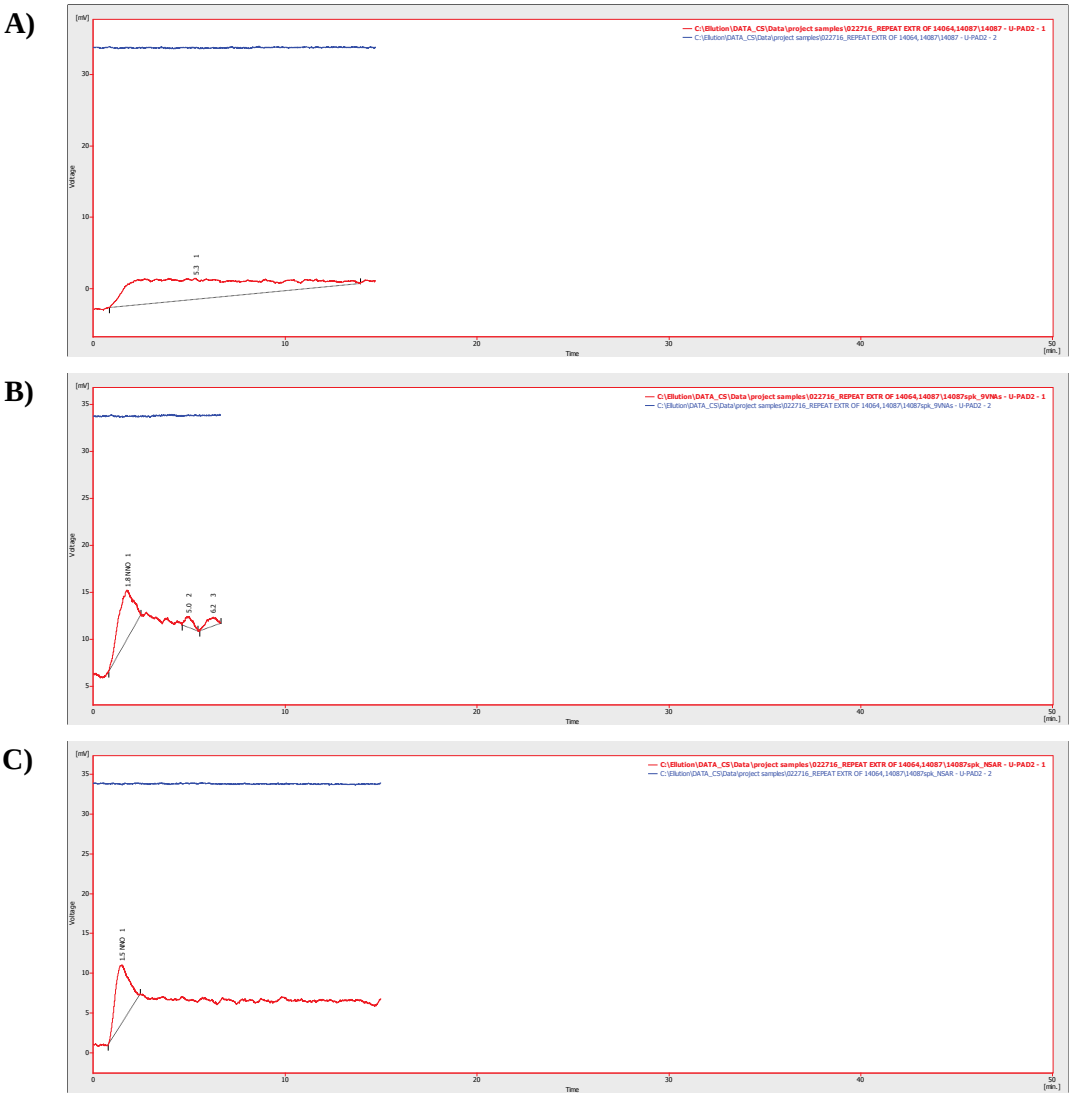


Figure 8. ATNC chromatograms from the analysis of pickled beetroot: A) sample extract; B) sample extract + co-injection of VNA at 80 µg NNO/kg; C) sample extract + co-injection of NVNA at 102 µg NNO/kg

To assess the reproducibility of the method, samples representative of the different sample matrices were subjected to repeat determinations over a short period of time (≤1 mth): individual stock cubes and aliquots of soy sauce were taken from stored stock samples of the same batch (homogeneity assumed); bulk samples were prepared for the cheese, cured fish and meat and smoked bacon to ensure homogeneity. The latter was stored at -18°C between analyses.

Table 9 shows the results obtained from repeat analyses of selected samples under conditions of intermediate reproducibility (same analyst, different date). With the exception of the dried shrimp sample, all repeat analyses showed reasonable agreement (RSD 8-22%) over a period of ≤ 1 mth for prepared samples stored at -18ºC. No

explanation can be given for the increase in ATNC with time for the dried shrimp product although additional testing carried out over the extended period 17/02/2016 – 27/04/2016 appeared to confirm the increase.

Table 9. ATNC reproducibility measurements for samples prepared by the QuEChERS-method

Sample code	Sample description	Test Date	NNO (µg/kg)					
			individual	mean	min	max	SD	SE
14071	Stock cube	20/01/2016	15	16	15	18	1.4	0.70
14071	Stock cube	16/02/2016	16					
14071	Stock cube	18/02/2016	15					
14071	Stock cube	19/02/2016	18					
14093	Soy Sauces	26/01/2016	20	19	16	21	2.4	1.2
14093	Soy Sauces	16/02/2016	16					
14093	Soy Sauces	18/02/2016	20					
14093	Soy Sauces	19/02/2016	21					
14094	Soy Sauces	26/01/2016	16	17	16	18	-	-
14094	Soy Sauces	17/02/2016	18					
14100	Smoked Cheese	11/02/2016	22	17	14	22	3.9	2.2
14100	Smoked Cheese	18/02/2016	16					
14100	Smoked Cheese	22/02/2016	14					
14107	Dried shrimp	08/02/2016	689	1052	689.0	1400	262.0	117.0
14107	Dried shrimp	17/02/2016	971					
14107	Dried shrimp	18/02/2016	1029					
14107	Dried shrimp	19/02/2016	1173					
14107	Dried shrimp	27/04/2016	1400					
14116	Cured Meat	08/02/2016	406	347	303	406	42.8	21.4
14116	Cured Meat	17/02/2016	335					
14116	Cured Meat	18/02/2016	303					
14116	Cured Meat	22/02/2016	344					
14098	Smoked bacon	05/02/2016	2822	2620	2426	2822	198.2	99.10
14098	Smoked bacon	19/02/2016	2426					
14098	Smoked bacon	22/02/2016	2610					

7.2 Preparation and analysis of samples

Samples requiring domestic preparation were cooked / prepared according the guidelines on the retail packs immediately before analysis (see 10.3 of the Appendices). Sample extracts were prepared using the QuEChERS-method (see 7.1.6) prior to chemical denitrosation and CLD described in 10.5 of the Appendices.

Table 10 shows the ATNC results obtained from the analysis of the 63 samples collected for investigation. A total of 36/63 samples tested as consumed gave a positive response for ATNC. Figure 9 shows that samples with the highest ATNC (>200 µg NNO/kg) included all cooked bacon, cured meats (Chorizo, Salami) and the dried shrimp. Products with the lowest ATNC (≤20 µg NNO/kg) included cheese (smoked / unsmoked), malted foodstuffs (cereals & beverages), most beers, most bouillons gravies and soups, most dried products (infant formulae, milk powders, soups / gravies, tea), most pickled products and soy sauces.

ATNC values for cooked unsmoked bacon (mean 428 µg NNO/kg; range 240 – 571 µg NNO/kg,) were significantly lower than the smoked product (mean 1646 µg NNO/kg; range 944 - 2853 µg NNO/kg). The mean ATNC values for the cooked bacon were however lower than those reported in a previous UK study (Massey

et al 1991) but within the overall range of values of 430-6800 µg NNO/kg (*ibid.*). ATNC concentrations in the cured meats and fish ranged from 21-2315 with highest amounts found in Italian Salami (2315 µg NNO/kg), Spanish Chorizio (1646, 1947 µg NNO/kg) and dried shrimp from China (677 µg NNO/kg, measured immediately after preparation). While literature data for ATNC in the latter was lacking, the relatively high values obtained were consistent with reports that similar foods were major dietary sources of NOC (Stuff et al 2009; Tricker and Preussmann 1991). ATNC measured in the Pepperoni (406 µg NNO/kg) was in the range reported by Fiddler et al (1995) for similar products from the USA (mean 400 µg NNO/kg; range µg NNO/kg 61-764). ATNC measured in the dried and salted fish from the UK was significantly lower (16-21 µg NNO/kg) than that reported elsewhere (Hoarah et al 2001) for salted, dried fish from China, South Africa and USA (mean 255 µg NNO/kg, range 79-915 µg NNO/kg).

ATNC was detected in four of eight beer samples in the range 12-95 µg NNO/kg. These were all imported speciality beers (from China, Australia, USA, Japan) and the values were within the ranges (mean 20 µg NNO/kg; range <20-125 µg NNO/kg) reported elsewhere (Massey et al 1990). ATNC was below the method detection limit of 8 µg NNO/kg in a single beer from Thailand and the three beers produced in the UK / EU. The latter findings were in contrast to amounts reported in 1990 (*ibid*.) for UK retail products which were in the range <20-569 µg NNO/kg (mean 54 µg NNO/kg).

ATNC in cheese (five from UK and one from Germany; three unsmoked / three smoked) ranged from 12-20 µg NNO/kg (mean 17 µg NNO/kg) and was consistent with the previous findings of Massey and Key (1989) that similar products were all less than the method detection limit (<20 µg NNO/kg). A sample of pickled red cabbage and smoked salmon gave ANTC values of 130 and 70 µg NNO/kg respectively. Although literature data for ATNC in similar products was not available, individual NOC are known to be associated with smoked fish and picked vegetables (Tricker and Preussmann 1991; Sen et al (1986).

Table 10. ATNC results in samples tested as consumed

Sample No.	Sample description	Category for project	ATNC (µg NNO /kg)
Unsmoked meats			
14C-14113	Unsmoked bacon	1	240, 366
14C-14114	Unsmoked bacon	1	388, 449
14C-14115	Unsmoked bacon	1	571, 556
Beer			
14C-14050	UK/EU beer	2	<8
14C-14051	UK/EU beer	2	<8
14C-14052	UK/EU beer	2	<8
14C-14053	Imported beer	2	<8
14C-14054	Imported beer	2	20
14C-14055	Imported beer	2	12
14C-14056	Imported beer	2	31
14C-14057	Imported beer	2	95, 74
Cheese unsmoked			
14C-14095	Cheese Unsmoked	3	16
14C-14096	Cheese Unsmoked	3	15
14C-14097	Cheese Unsmoked	3	16
Cured fish and meat (excluding smoked products)			
14C-14107	Dried Shrimp	4	677
14C-14108	Dried Fish	4	21
14C-14109	Salted Mackerel	4	16, 17
14C-14111	Chorizo	4	1646, 1947
14C-14112	Italian Salami	4	2315
14C-14116	Pepperoni	4	406
Dried products			
14C-14058	Infant formulae	5	<8
14C-14059	Infant formulae	5	<8, <8
14C-14060	Milk powder	5	19
14C-14061	Milk powder	5	17, 16
14C-14063	French Onion soup powder	5	54, 48
14C-14064	Loose leaf tea	5	<8, <8
14C-14065	Loose leaf tea	5	<8
14C-14067	Mushroom soup powder	5	<8, <8
14C-14085	Paprika	5	NQ
14C-14110	Cardamon Pods	5	NQ
Malted foodstuffs			
14C-14079	Malted Cereal	6	<8
14C-14080	Malted Cereal	6	<8
14C-14081	Malted Cereal	6	<8
14C-14082	Malted beverages	6	11
14C-14083	Malted beverages	6	<8
14C-14084	Malted beverages	6	22
Pickled products			
14C-14086	Silverskin onions in vinegar	7	<8
14C-14087	British beetroot In vinegar	7	NQ
14C-14088	British red cabbage in vinegar	7	130
14C-14117	Rollmop herrings (canned)	7	<8
14C-14118	Mussels in vinegar	7	18
14C-14119	Cockles in vinegar	7	<8

Table 10 continued...

Sample No.	Sample description	Category for project	ATNC (µg NNO /kg)
Bouillons, gravies and soups			
14C-14062	Gravy Powder	8	23, 27
14C-14069	Gravy powder	8	<8
14C-14066	Oxtail soup	8	20
14C-14068	Chicken Soup	8	<8
14C-14070	Beef stock cube	8	<8, <8
14C-14071	Beef stock cube	8	16, 13
Soy sauces			
14C-14089	Soy Sauce	9	<8
14C-14090	Soy Sauce	9	<8
14C-14091	Soy Sauce	9	<8
14C-14092	Soy Sauce	9	<8
14C-14093	Soy Sauce	9	20
14C-14094	Soy Sauce	9	16
Wood smoked products			
14C-14098	Smoked bacon	10	2853, 2792
14C-14099	Smoked bacon	10	945, 944
14C-14104	Smoked bacon	10	1215, 1124
14C-14101	Smoked mackerel	10	28
14C-14102	Smoked salmon	10	70
14C-14103	Smoked salmon	10	<8, <8
14C-14100	Smoked cheese	10	20
14C-14105	Smoked cheese	10	17, 20
14C-14106	Smoked cheese	10	12

NQ = not quantified

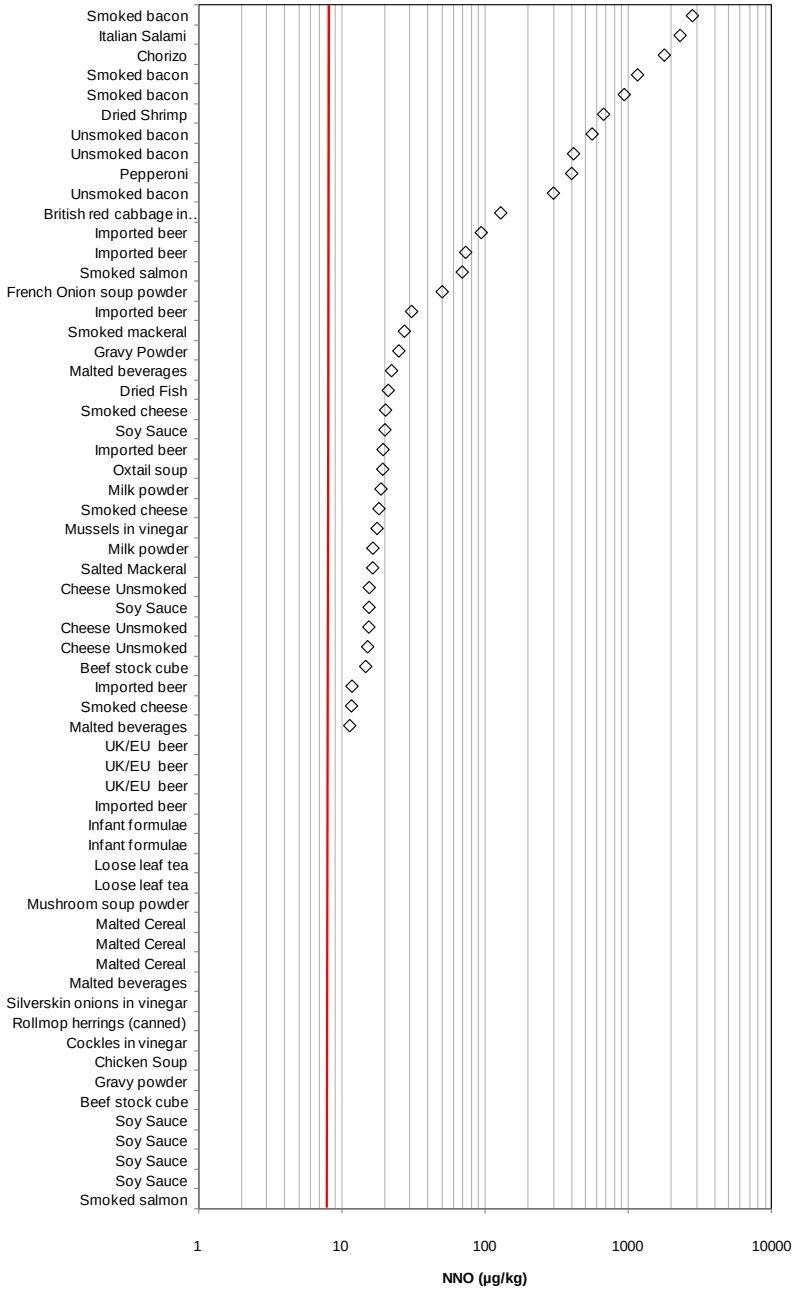


Figure 9. ATNC results in order of increasing concentration: log scale; red line denotes method LOD of 8 µg NNO/kg

8. Measurement of nitrosamines in target foodstuffs

8.1 Volatile nitrosamines

Recently, Lehotay et al (2015) reported the use of the QuEChERS-method and CG/MS for the analysis of VNA in bacon. While this and our own experience with the use of the QuEChERS-method had demonstrated good

recoveries of VNA measured as ATNC, co-extracted fats were a feature of this extraction method. As co-extracted fats were known to be detrimental to GC performance, the main analytical challenge was to develop a system to minimise their extraction whilst maintaining a relatively simple / rapid sample preparation procedure. Furthermore, we proposed to use GC in conjunction with chemical ionisation MS/MS to provide a highly selective and sensitive detection of VNA in a wide range of matrices.

8.1.1 Method development

8.1.1.1 Preparation of standards

Mixed calibration standard solutions were prepared in methanol by serial dilution of nitrosamine reference standards (see 7.1.3) covering the range of 1 – 60 µg/l and 2-100 µg/l, each with a corresponding deuterium labelled nitrosamine internal standard at 40 µg/l.

8.1.1.2 Materials

Malted grain (Laboratory code 16C-02178), used for method development, was obtained from a commercial supplier.

Supel™ QuE PSA/ENVI-Carb tubes (55230-U), Supel™ QuE PSA/C18 tubes (55229-U), Supel™ QuE z-Sep/C18 tubes (55284-U), Supel™ QuE z-Sep+ tubes (55486-U), were all obtained from Supelco UK. HyperSep™ Dispersive SPE tubes, 400 mg Primary/Secondary Amine, Graphitized Carbon Black (GCB) 10639685, were obtained from Fisher UK. Chem Elut SLE cartridges were obtained from Agilent, UK.

8.1.1.3 GC/CI-MS/MS

Initial trials were carried out using a Varian 1200 GC/MS/MS system operating in the positive ion chemical ionisation mode (PICI) with ammonia as the reagent gas. When the mass spectrometer was operated at a source temperature of 140°C and CI reagent gas pressure of 3 torr, abundant protonated molecular ions $[MH]^+$ and ammonium adduct ions $[M+NH_4]^+$ were readily obtained for all target VNA. The ability of ammonia to generate adduct ions in PICI permitted the selection of abundant high mass precursor ions for subsequent selective MS/MS detection.

Splitless injections made into a range of 30 m x 0.25 mm i.d. capillary GC columns (VF-5, Varian Inc, California, USA; ZB-XLB-HT Inferno GC column, Phenomenex, Macclesfield; DB Wax, J&W, USA) with the MS operating in scanning mode showed that optimum separation of nine VNAs (NDMA, NMEA, NDEA, NDPA, NDBA, NPIP, NPYR, NMOR, NDPhA) could be attained using a carbowax capillary column. High bleed was a known feature of this column phase particularly at the relatively high temperature required to elute NDPhA from GC column indicating that analysis of the latter VNA might be detrimental to long term method performance. However, GC columns with low bleed variants of the Carbowax phase were also known to be available and low bleed MS version was subsequently obtained. Furthermore, NDPhA was not a common contaminant of foods and the option to exclude this VNA from the method could be considered at a later stage. When the (temperature programmable) GC injector was switched to large volume injection mode, a significant

increase in response at the MS was observed indicating that a target method detection limit in the low µg/kg range would be readily attainable. The GC/MS conditions are given in Table 11.

Precursor ammonium adduct ions $[M+NH_4]^+$ corresponding to each VNA were then selected in Q1 of the triple quadrupole MS while Q3 was scanned for repeated incremental collision energies at Q2 using argon collision gas at 1.8 mtorr. The selected multiple reaction monitoring (MRM) parent and product ion combinations for each VNA are given in Table 12. Figure 10 shows the ammonia GC/PICI-MS/MS chromatogram for eight VNA (NDMA, NMEA, NDEA, NDPA, NDBA, NPIP, NPYR, NMOR) each at a concentration of 10 µg/l and five isotopically labelled VNA internal standards (NDMA-d6, NDPA-d14, NPIP-d10, NDBA-d18, NMOR-d8). A calibration typical curve (NDMA) is shown in Figure 11: the curve was linear over the concentration range 1-60 µg/l.

Table 11. Initial conditions for the analysis of VNA using GC/MS detection (scanning mode)

Carrier gas:	Helium flow at constant flow of 1.5 ml/min
GC column:	30 m x 0.32mm ID x x 0.5 µm TR-WAXMS
Injector:	1079; large volume injection mode; 37°C (hold for 0.1min) to 230°C at 200°C/min (hold for 6min)
Injection volume (µ):	5
GC oven:	45°C (hold for 2.5min) to 180°C at 10C/min then increase to 250C at 35 C/min (hold for 5 min)
Ammonia CI (torr):	3
Source temperature (°C):	140

Table 12. VNA product and precursor ions for ammonia CI MRM

Chromatogram segment	VNA	Q1 mass	Q3 mass	Collision energy	Dwell time (s)
1	NDMA	75	75	3	0.1
1		92	75	3	0.1
1	NDMA-d6	81	81	3	0.1
1		98	81	3	0.1
1	NMEA	89	61	11	0.1
1		106	89	3	0.1
1	NDEA	103	103	3	0.1
1		120	103	3	0.1
2	NDPA	131	131	3	0.1
2		148	131	5	0.1
2	NDPA-d14	145	145	2.5	0.1
2		162	145	3	0.1
3	NPYR	101	101	3	0.1
3		118	101	4	0.1
3	NPIP	115	69	15	0.1
3		132	115	3	0.1
3	NPIP-d10	125	125	8	0.1
3		142	125	8	0.1
3	NDBA	159	57	10	0.1
3		176	159	4.5	0.1
3	NDBA-d18	177	66	10	0.1
3		194	177	6	0.1
4	NMOR	117	117	3	0.1
4		134	117	4	0.1
4	NMOR-d8	125	125	3	0.1
4		142	125	7	0.1

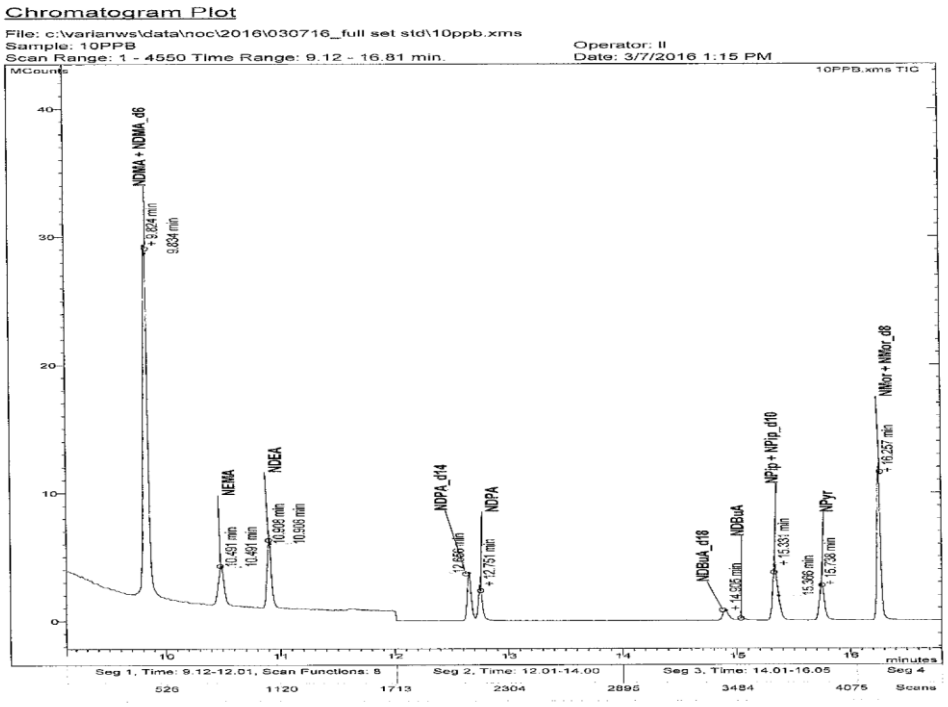


Figure 10. GC/PICI-MS/MS chromatogram for a 13 component VNA mix: the concentration of each non-labelled VNA was 10 µg/l; the reagent gas was ammonia.

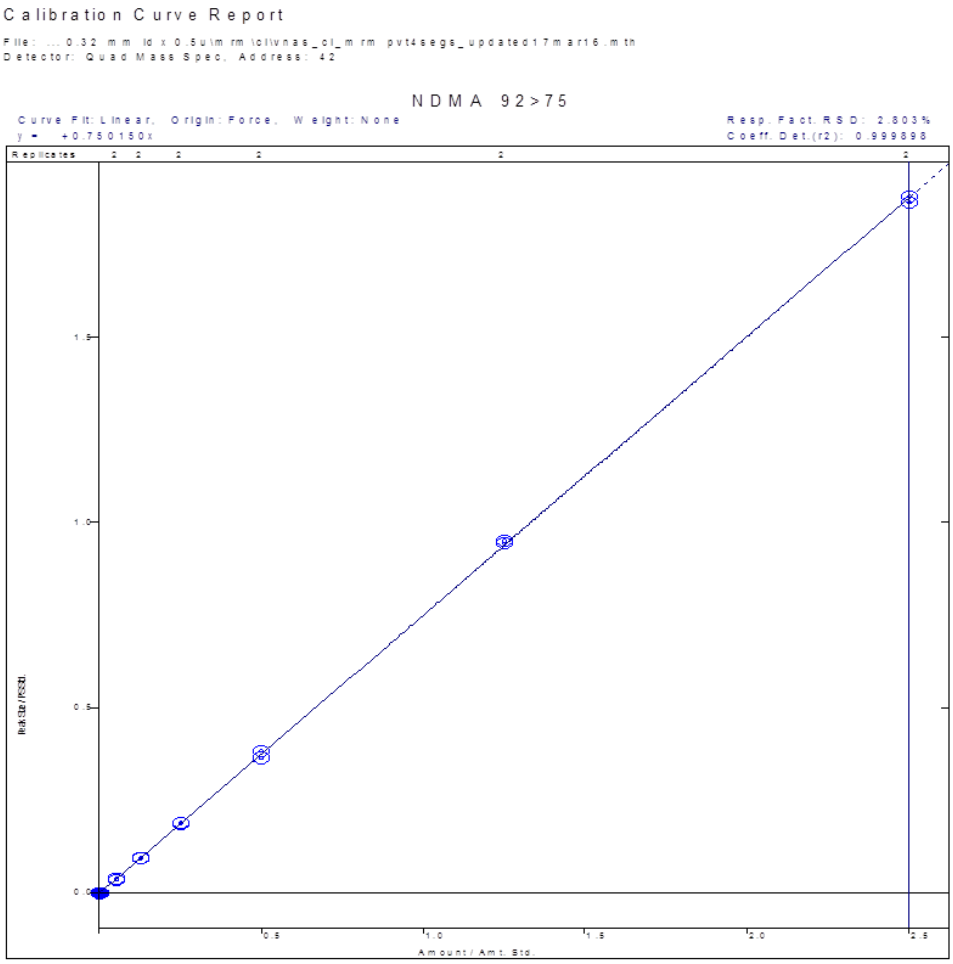


Figure 11. GC/CI-MS/MS calibration curve for NDMA over the concentration range 1-60 µg/l

8.1.1.4 Development and optimisation of analytical methods

Lehotay et al (2015) recently reported a dispersive SPE clean-up procedure for the analysis of VNA in bacon based on the QuEChERS method of extraction. The authors reported the use a GC "backflushing" technique to improve the ruggedness of the GC method for fatty sample extracts, presumably as fat was (partially) extracted with the solvents used (acetonitrile). As the "backflushing" technique was not available in our laboratory, we elected to optimise the selection of extraction solvents and sorbents to minimise the co-extraction of sample fats using this approach. Advances in dispersive sorbent technology for QuEChERS had seen the development of new hybrid zirconia-coated silica for fat removal and the effectiveness of these developments would be assessed in conjunction with conventional column SPE trials during method development.

8.1.1.4.1 Aqueous methanol extraction

Aqueous methanol has been used for the extraction of VNA by a number of workers and the conventional column SPE method of Sannino and Bolzoni (2013) was modified to evaluate the extraction efficiency of VNA:

Aqueous SA was used in place of aqueous sodium hydroxide to inhibit artefact (VNA) formation during sample preparation and the SPE fat removal step using Florisil® mini-columns was omitted as the zirconia-coated silica sorbents would be evaluated for this task during method development. Initial trials were therefore carried out using a dried malted grain matrix that was relatively low in lipid. Samples (5 g) were weighed accurately into a 50 ml centrifuge tubes and internal standard solution (50 µl of 4 µg/ml NDMA-d6, NDPA-d14, NPIP-d10, NDBA-d18, NMOR-d8) and SA solution (0.25 ml) were added. Spiked samples were prepared by adding 10 – 500 µl of a 0.4 µg/ml mixed solution of 8-VNA to each tube. Water (15 ml) and methanol (5 ml) were added, the tubes were capped and vortex mixed for 15 s then tumble mixed for 15 min. The tubes were then centrifuged at 3500 g_{av} for 10min and the upper layer decanted onto a Supported Liquid Extraction cartridge (Chem-Elut®, Agilent Santa Clara, USA). After equilibration (15 min) the cartridges were eluted with 25 ml and 2 x 5 ml of DCM into a 40 ml of KD evaporator flask and the solvent reduced to 2 ml at 55°C. The concentrated solution was transferred to an amber 4 ml vial, the KD apparatus was rinsed with 2 ml of ACN and the solvent combined in the amber vial and mixed well. Prepared extracts were stored at -18°C until required for GC/CIMS.

The results given in Table 13 show that the VNA could be readily recovered from the malted grain using this relatively simple procedure although an over-recovery was likely for some VNA added at concentrations below 10 µg/kg: The non-spiked sample gave a positive response for NPYR of 5.8 µg/kg. The estimated LOD and LOQ using this method were 1 and 2 µg/kg respectively.

Table 13. Recoveries of VNA from a malted cereal product using aqueous methanol extraction and GC/CI-MS/MS detection.

	Recovery (%)							
added (µg/kg)	NDMA	NMEA	NDEA	NDPA	NDBA	NPIP	NPYR	NMOR
2.0	114	95	99	94	146	76	135	139
10.0	118	102	107	97	147	107	132	153
20.0	103	81	93	93	127	98	92	124
40.0	99	85	92	95	121	94	85	116

8.1.1.4.2 QuEChERS dispersive SPE

Initial trials with the QuEChERS dispersive SPE clean-up procedure using the Supel™ QuE range of PSA (Primary-Secondary Amine (PSA, for removal of polar compounds, sugars and acids)/C18 (for removal of lipids and other non-polar compounds) were carried out under procedural blank conditions to evaluate background responses from the sorbents used. Figure 12 shows that a relatively high background signal occurred in segment 1 of the GC/MS chromatogram from the sorbent procedural blank and that this was mainly due to interferences in the precursor ion selections / transitions for NDMA and NMEA, in particular *m/z* 106->89 (see Table 12 for MRM transitions).

Following optimisation of the MRM transitions (see Table 14), application of the standard QuEChERS extraction method (Lehotay et al 2015) to the malted grain sample (prepared according to the conditions given in Table 15) resulted in a deterioration of chromatographic performance at the GC/MS after only two injections.

The dispersive SPE step was carried out with both Supel™ QuE PSA/C18 and Supel™ QuE z-Sep /C18. While the z-Sep appeared to give fewer extraneous peaks in the chromatogram than the PSA/C18 sorbent, interpretation was not conclusive due to the unacceptable loss of chromatographic peak shape (see Figure 13). The loss of chromatographic performance was consistent with the findings of other workers (Lehotay et al 2015) and it was evident that further development / optimisation of the GC system, extraction solvents and clean-up sorbents would be required for sustained performance using dispersive SPE clean-up.

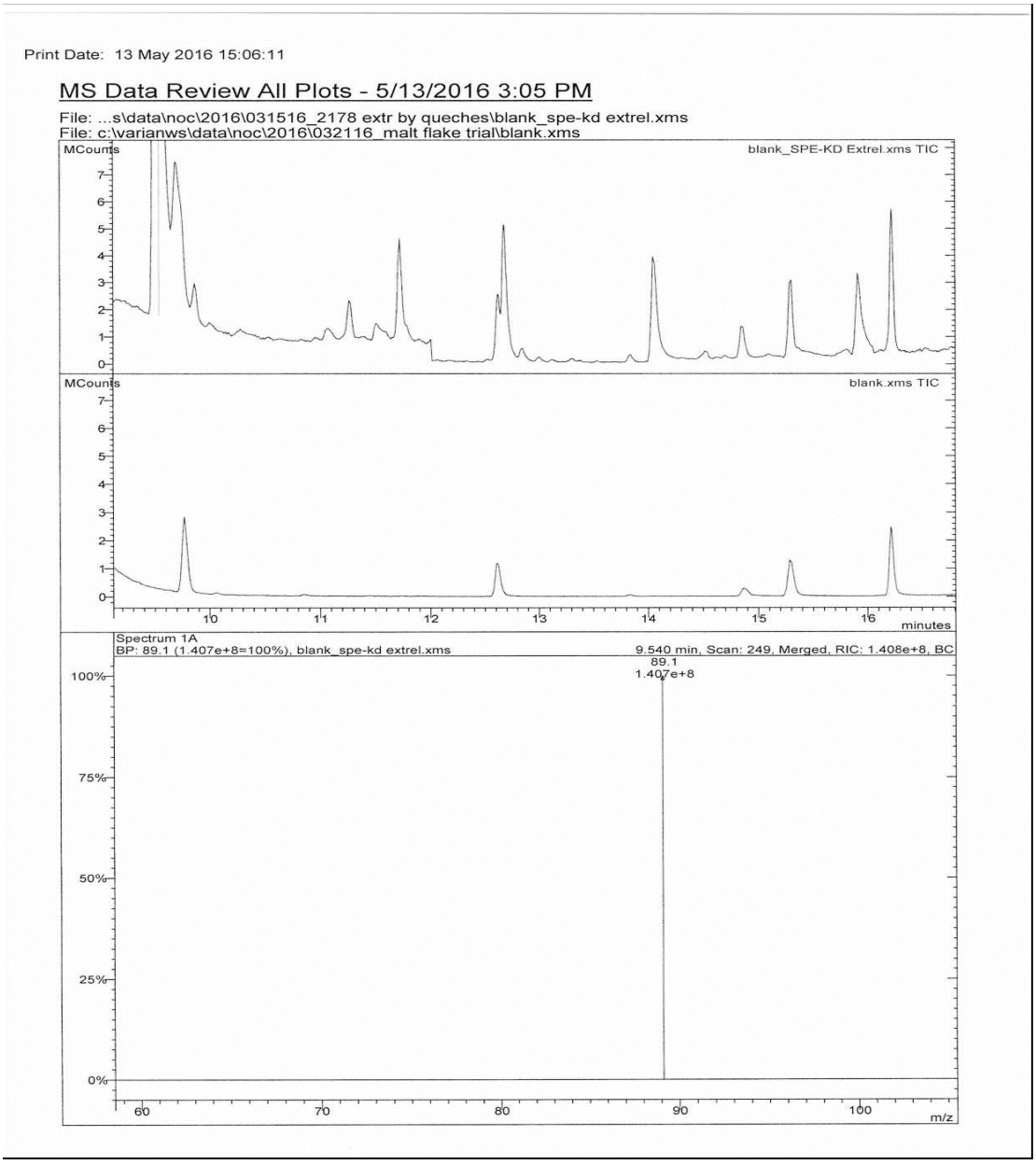


Figure 12. VNA GC/PICI-MS/MS chromatograms from the analysis of procedural blank samples: Upper chromatogram obtained from QuEChERS method; lower chromatogram obtained from initial SPE trial; the mass chromatogram (taken from the baseline at 9.54 min) shows that the high baseline in segment 1 of the upper chromatogram was due to interferences (mainly on the transition *m/z* 106->89, NMEA).

Table 14. Optimised VNA product and precursor ions for ammonia CI MRM when using the QuEChERS method of sample preparation

Chromatogram segment	VNA	Q1 mass	Q3 mass	Collision energy	Dwell time (s)
1	NDMA	75	43	19.5	0.1
1	NDMA	92	75	3	0.1
1	NDMA-d6	81	46	19	0.1
1	NDMA-d6	98	81	3	0.1
1	NMEA	89	61	11	0.1
1	NMEA	106	61	16	0.1
1	NDEA	103	103	3	0.1
1	NDEA	120	103	3	0.1
2	NDPA	148	131	5	0.1
2	NPDA	131	131	3	0.1
2	NDPA-d14	145	145	2.5	0.1
2	NDPA-d14	162	145	3	0.1
3	NDBuA	159	57	10	0.1
3	NDBuA	176	159	4.5	0.1
3	NDBuA-d18	177	66	10	0.1
3	NDBuA-d18	194	177	6	0.1
3	NPYR	101	55	15.5	0.1
3	NPYR	118	101	4	0.1
3	NPIP	115	69	15	0.1
3	NPIP	132	115	3	0.1
3	NPIP-d10	125	125	8	0.1
3	NPIP-d10	142	125	8	0.1
4	NMOR	117	117	3	0.1
4	NMOR	134	117	4	0.1
4	NMOR-d8	125	125	3	0.1
4	NMOR-d8	142	125	7	0.1

Table 15. VNA dispersive SPE (QuEChERS) trial conditions using malted grain

Trial	**1**	**2**
Sample weight (g)	5	5
Added water (ml)	10	10
Volume of 5 µ/ml IS (µl)	50	50
Vortex mix time (s)	4	4
Volume acetonitrile (ml)	10	10
Vortex mix time (s)	4	4
$MgSO_4$ added (g)	4	4
Vortex mix time (s)	60	60
Centrifuge time @ 4500 rpm (min)	5	5
Dispersive SPE sorbent	Supel™ QuE PSA/C18	Supel™ QuE z-Sep
Volume of acetonitrile extract taken (ml)	4.5	4.5
Vortex mix time (s)	4	4
Centrifuge time @ 4500 rpm (min)	5	5
Supernatant final volume, nitrogen stream (ml)	2	2

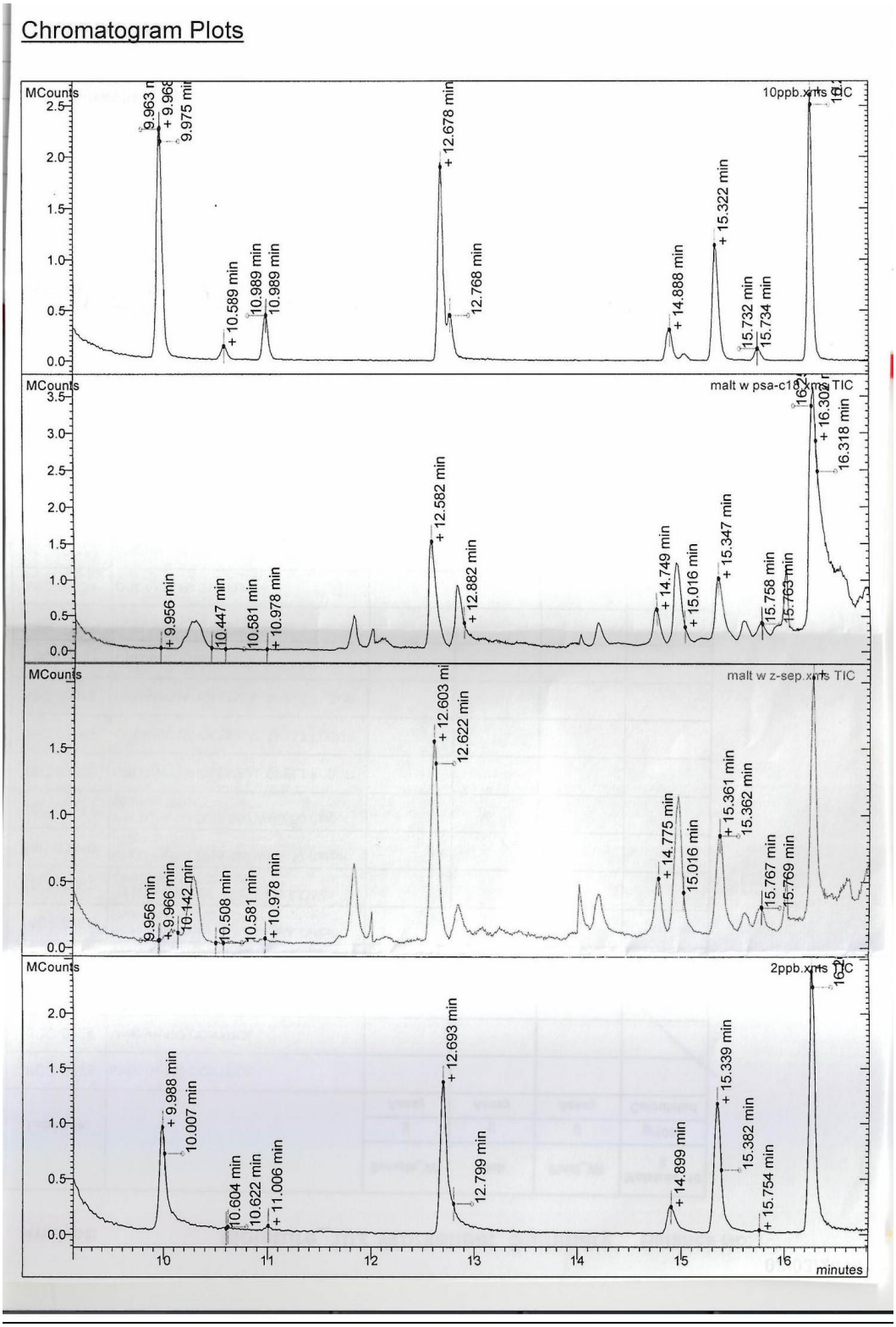


Figure 13. VNA GC/PICI-MS/MS chromatograms from the analysis of malted grain extracts prepared by QuEChERS using different dispersive SPE sorbents: A) 10 ppb VNA standard mix; B) extract prepared using PAS-C18; C) extract prepared using Z-sep. Loss of chromatographic performance (peak tailing) occurred after the injection of the two sample extracts, chromatogram D), 2 ppb mixed VNA standard.

8.1.1.4.3 GC optimisation

To further improve the robustness of the GC system, some of factors limiting sustained performance were considered, such as the relatively low maximum operating temperature of the polyethylene glycol GC column phase and / or the use of a pre-column. The latter had been added prior to the analysis of the malted grain samples prepared by dispersive SPE and, in some instances, can cause loss of GC performance due to imperfect connections to the GC column (regions of so called "activity"). To overcome these limitations, a mid-polar GC column phase was selected (DB-1701, 14% Cyanopropyl-phenyl 86% dimethyl polysiloxane) was selected for further evaluation and the use of a pre-column omitted.

Careful optimisation of the sample injection solvent (acetonitrile, methyl t-butyl ether, 4-methylpentane-2-one, dichloromethane, ethyl acetate), sample injection speed, injector split vent timings, injector temperature programme, GC column oven programme and carrier gas flow using a 60 m x 0.32 mm I.D. x 1 µm DB1701 (Hewlett Packard, UK) showed that good chromatographic performance could be attained for standard solutions of all nine VNA according to the conditions given in Table 16.

Table 16. Optimised GC conditions for the analysis of VNA using CI-MS/MS

Parameter	**Condition**	
GC column	60 m x 0.32 mm I.D. x 1 µm	
Carrier / flow	Helium @ 1.5 ml / min (vacuum flow)	
Injection solvent	Ethyl acetate	
Injection volume	5 µl	
Injection speed	9 µl/s	
Split vent programme	Time (min)	Flow (ml)
	0	20
	0.1	0
	2.6	100
	5	20
Injector temperature program	45°C, hold for 0.1 min, increase to 230°C at 200°C/min, hold for 7 min	
Col temp program:	30°C, hold for 2.5 min, increase to 250°C at 10°C/min, hold for 5 min	

8.1.1.4.4 Dispersive v column SPE

To evaluate the effectiveness of the SPE clean-up technique on samples analysed by GC/CI-MS/MS, a further trial was conducted with VNA standard solutions using both dispersive (QuEChERS) and conventional column SPE. Supel™ QuE PSA/ENVI-Carb (55230-U, Supelco, UK), a sorbent comprised of magnesium sulphate, graphitised carbon and PSA, was chosen for the evaluation as this was widely used for many QuEChERS applications. The mixed VNA standard solutions were prepared according to the steps below:

Step	Dispersive SPE	Column SPE
1	Add 2ml of 40 µg/ml 9 VNA solution to a Supel™ QuE PSA/ENVI-Carb (55230-U) tube	Pack a SPE tube with 2 x contents of a Supel™ QuE PSA/ENVI-Carb (55230-U) tube
2	Vortex mix for 30 s	Condition column with 4 ml ethyl acetate
3	Centrifuge for 30 s	Load SPE tube with 2ml of 10 µg/ml 9 VNA solution and elute to waste
4	Transfer supernatant to autosampler vial for analysis	Load SPE tube with further 2ml of 10 µg/ml 9 VNA and collect eluate in autosampler vial

Figure 14 shows the chromatograms obtained from the analysis of VNA standard solutions by dispersive and conventional SPE using GC/CI-MS/MS operating according to the conditions given in Table 16. Comparison with the chromatogram for the 10 µg/ml 9 VNA standard solution (Figure 14 C), analysed under the same conditions, shows that the dispersive SPE sorbent gave a number of extraneous peaks in the mass chromatograms: Presumably the latter were eluted to waste when the VNA standard mix was retained on the sorbent using column SPE.

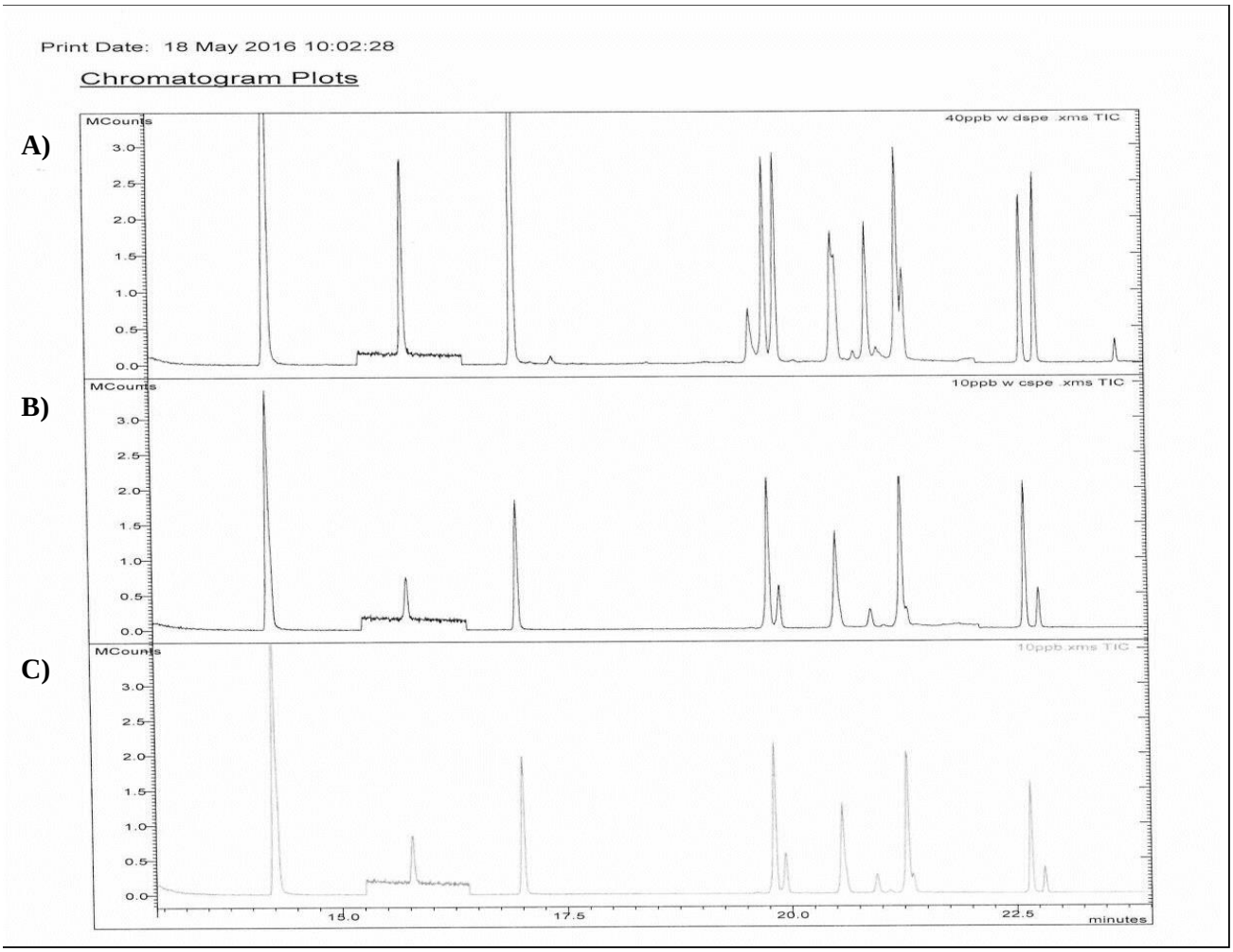


Figure 14. CG/CI-MS/MS chromatograms for VNA standard solutions prepared by A) dispersive SPE (QuEChERS); B) conventional SPE: C) no SPE preparation (9 VNA standard mix at 10 µg/ml)

On the basis of these findings, the column SPE technique afforded cleaner extracts and the method was subsequently extended to the malted grain sample used previously and a dried shrimp sample that had given a relatively high ATNC (see Table 10). Aqueous methanol was chosen for subsequent sample extractions to mitigate the co-extraction of compounds such as fats.

8.1.1.4.5 Column SPE clean-up developments

Samples of malted grain and dried shrimp (15C-14107), 5 g each, were weighed accurately into 50 ml centrifuge tubes and 50 ul of 4 µg/l ITSD solution and 0.25 ml of SA solution were added. Blank and spiked samples were prepared in the same way: 50 ul of the 0.4 µg/l nine VNA standard was added to spiked samples. Aqueous methanol (methanol:water 1:3 v/v), 25 ml, was added to each tube which was then capped and vortex mixed for 15 s and tumble mixed for 15 min. Tubes were then centrifuged at 4500rpm for 10 min and the supernatant decanted onto a Chem Elut cartridge. After equilibration for 15 min the cartridge was eluted with 25 ml + 2 x 15 ml of a solution of 2% (V/V) ethyl acetate in dichloromethane and collected in a 40 ml Kuderna Danish flask. The extract was concentrated in the Kuderna Danish apparatus to circa 3 ml at 55ºC and the concentrated extract quantitatively transferred to a 4 ml amber using 1 ml of ethyl acetate and mixed well (final volume circa 4 ml). An empty glass SPE tube (15 ml) was packed with the contents of two Supel™ QuE dSPE (55230-U) tubes and the packed sorbent conditioned with 4 ml of ethyl acetate. The sample extract was loaded onto the SPE column in two applications: a) 2 ml extract followed by elution to waste: b) a further 2 ml extract followed by collection in an autosampler vial.

Table 17 shows the results obtained from the analysis of the malted grain and dried shrimp samples using aqueous methanol extraction and conventional SPE clean-up with Supel™ QuE (magnesium sulphate, graphitised carbon and PSA): NPYR and NDMA were found in the malted grain and dried shrimp respectively. The result for NPYR in the malted grain was derived from *m/z* 101->55 as there was an interference on the MRM transition used for the quantification of NPYR in the malted grain. This result was later confirmed after optimisation of the MRM transition (from *m/z* 118->101 to *m/z* 118->55) and reinjection of the sample into the GC/MS. The GC/CI-MS/MS chromatograms are shown in Figure 15.

Table 17. Recoveries of VNA from malted grain and dried shrimp using column SPE packed with Supel™ QuE PSA/ENVI-Carb sorbent. Values in parentheses were obtained after optimisation of the MS/MS transition for NPYR due to an interference.

VNA	Malted grain		Recovery (%)	Dried shrimp		Recovery (%)
	unspiked	Spiked [a]		unspiked	Spiked [a]	
NDMA	<1 (<1)	4.8 (4.8)	120.0 (120)	11.6 (11.5)	14.4 (14.7)	70 (80)
NMEA	<1 (<1)	3.5 (4.2)	88 (105)	<1 (<1)	3.9 (4.5)	98 (113)
NDEA	<1 (<1)	3.4 (3.7)	85 (93)	<1 (<1)	3.6 (3.8)	90 (95)
NDPA	<1 (<1)	5.1 (4.1)	128 (103)	<1 (<1)	3.8 (3.7)	95 (93)
NMOR	<1 (<1)	5.1 (4.8)	128 (120)	<1 (<1)	4.4 (4.3)	110 (108)
NPYR	9.9 [b] (8.0)	11 [b] (12.8)	28 (120)	<1 (<1)	3.9 (3.9)	98 (98)
NPIP	<1 (<1)	3.5 (5.3)	88 (133)	<1 (<1)	3.4 (4.5)	85 (113)
NDBA	<1 (<1)	3.2 (3.7)	80 (93)	<1 (<1)	4.1 3.4)	103 (85)

[a] spike level was 4 µg/kg; [b] result derived from *m/z* 101->55 due to interference on quantification transition (*m/z* 118->101)

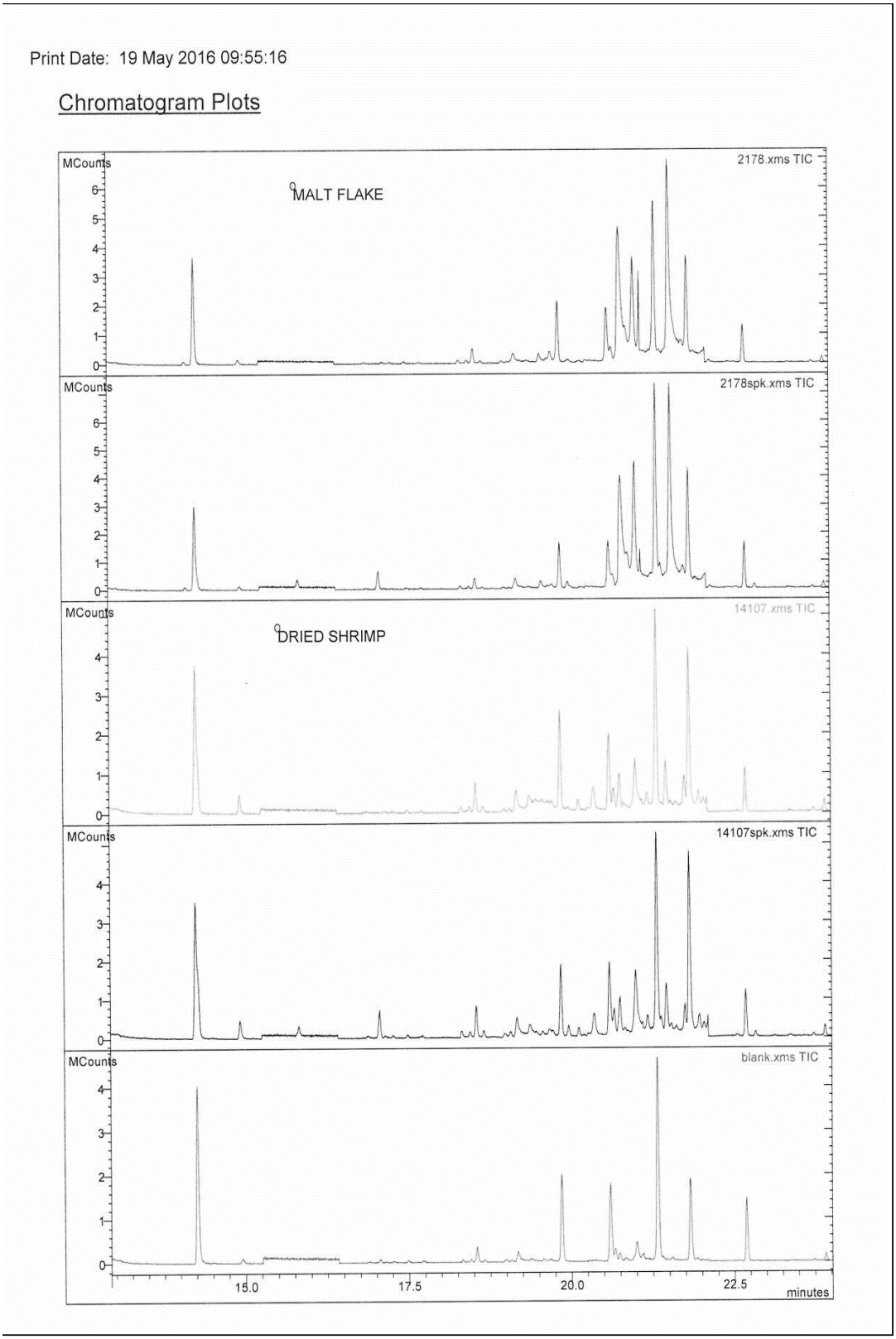


Figure 15. CG/CI-MS/MS chromatograms from the analysis of malted grain and dried shrimp using column SPE clean-up (packed with Supel™ QuE PSA/ENVI-Carb)

While sustained GC performance and recovery of VNA could now be obtained from relatively low fat samples such as malted grain and dried marine products using column SPE clean-up on PSA/ENVI-Carb, consideration was given to the clean-up of higher fat samples, such as bacon and salami. The use of Supel™ QuE z-Sep+ was evaluated as the sorbent had been developed for the clean-up of samples containing >15% fat. To assess the impact of Supel™ QuE z-Sep+ on the recovery of VNA a trial was first conducted using an aqueous methanolic extract prepared from the malted grain (prepared as previously) and a low level calibration standard mix of VNA. Sample extracts and calibration standards were then cleaned-up using both Supel™ QuE PSA/ENVI-Carb and Supel™ QuE z-Sep+ using the conditions descried previously.

The chromatograms in Figure 16 shows that visibly cleaner extracts were obtained when using the z-Sep+ than compared to PSA/ENVI-Carb. However, closer inspection of the mass chromatograms for the VNA standard solution showed that individual VNA were much reduced in response when z-Sep+ was used for clean-up.

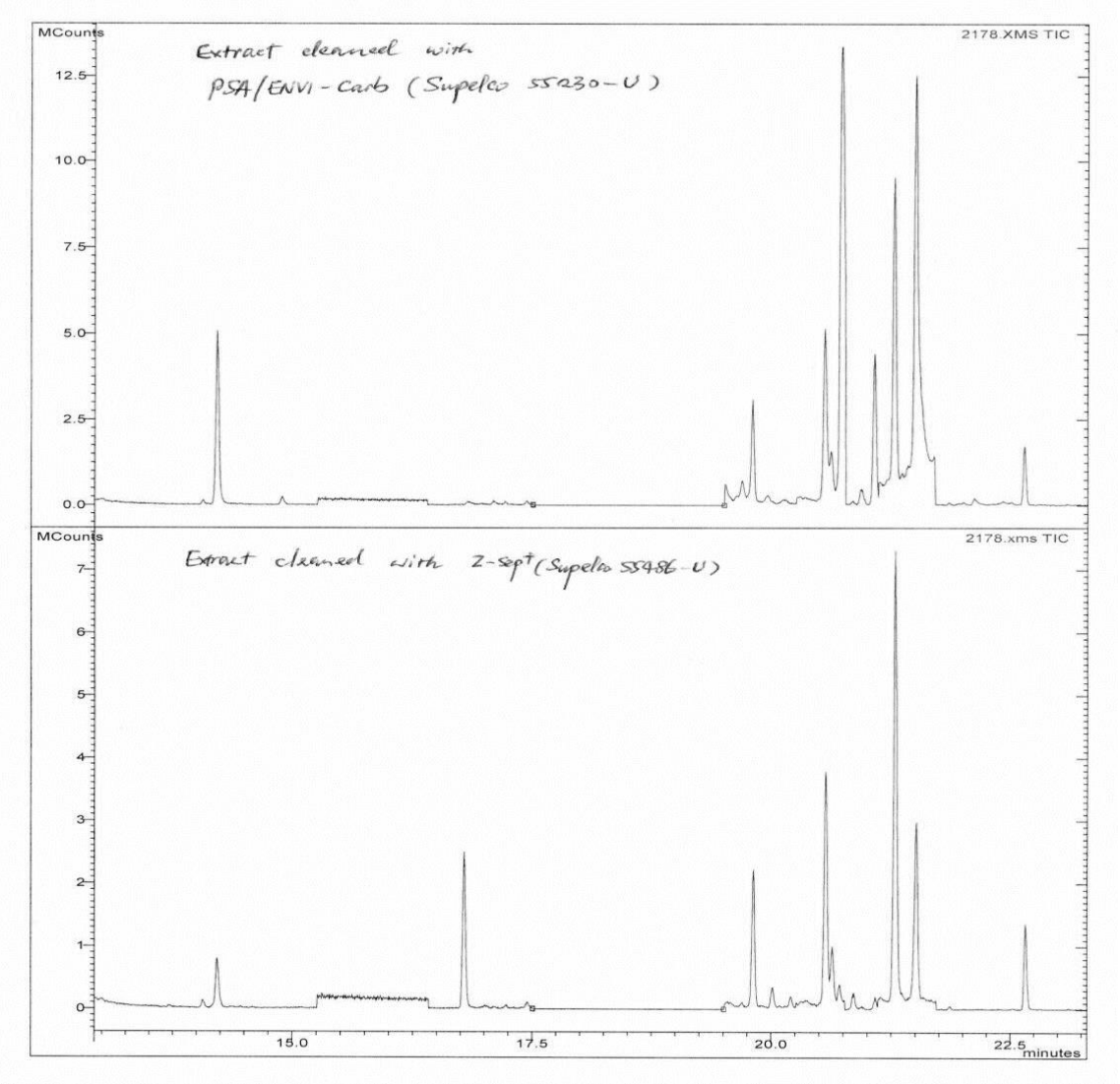


Figure 16. GC-/CI-MS/MS chromatograms of a malted grain extract comparing clean-up using Supel™ QuE PSA/ENVI-Carb and Supel™ QuE z-Sep+

The impact of the reduced recovery of VNA using z-SEP+ can be seen in the results shown in Table 18. The VNA most affected included NPYR and NDMA / NDMA-d6: NMEA and NDEA appeared to be over recovered in the malted grain and 5 µg/ml standard solutions cleaned-up using Z-Sep+ as both of these VNAs were quantified using NDMA_d6 as internal standard; responses of both NDMA / NDMA-d6 were reduced equally with Z-Sep+ and the quantified result for the 5 µg/ml standard solution was less affected although the response was poor. Consequently Z-Sep+ was not extended to the high fat samples and further trials were carried out using PSA/GCB sorbents.

Table 18. Comparison of VNA recoveries from a malted grain extract and a calibration standard after clean-up using PSA/ENVI-Carb and z-Sep+.

VNA	Procedural blank PSA/ENVI-Carb	Malted grain with PSA/ENVI-Carb	Procedural blank Z-Sep+	Malted grain with Z-Sep+	5 µg/l standard	5 µg/l standard with Z-Sep+
NDMA	<1	<1	<1	<1	4.6	4.9
NMEA	<1	<1	<1	1.4	5.1	9.9 [b]
NDEA	<1	<1	<1	<1	4.6	11.4 [b]
NDPA	<1	<1	<1	<1	5.1	5.1
NMOR	<1	1.1 [a]	<1	1.4	4.1	4.3
NPYR	<1	7.9	<1	<1 [c]	4.0	1.0 [c]
NPIP	<1	2.5 [a]	<1	<1	3.9	3.9
NDBuA	<1	<1	<1	<1	4.1	4.3

[a] interference on quantification transition; [b] over recovery due to partial loss of ISTD; [c] due to partial loss on Z-Sep+

A PSA/GCB sorbent was available from Fisher pre-packed into SPE tubes, thus avoiding the requirement to transfer and pack SPE tubes for analysis, and this product was subsequently evaluated using the malted grain and the higher fat bacon and salami samples identified earlier.

Samples of malted grain, smoked bacon (15C-14104) and salami (14C-14112), 5 g each, were weighed accurately into 50 ml centrifuge tubes and 50 ul of 4 µg/l ITSD solution and 0.25 ml of SA solution were added. Blank and spiked samples were prepared in the same way: the malt was spiked at 4 and 20 µg/kg while the bacon and salami were spiked at 4 µg/kg using nine VNA standard mix. Aqueous methanol (methanol:water 1:3 v/v, 25 ml for the malted grain and 20 ml for the bacon and salami) was added to each tube which was then capped and vortex mixed for 15 s and tumble mixed for 15 min. Tubes were then centrifuged at 4500rpm for 10 min and the supernatant decanted onto a Chem Elut cartridge. After equilibration for 15 min the cartridge was eluted with 25 ml + 2 x 15 ml of a solution of 2% (V/V) ethyl acetate in dichloromethane and collected in a 50 ml centrifuge tube (wrapped in foil to exclude light). The extract was transferred in aliquots to a Kuderna Danish apparatus (40 ml) and reduced to a volume of circa 2 ml at 55ºC. The concentrated extract was quantitatively transferred to a 4 ml amber vial using 2x1 ml of ethyl acetate and mixed well (final volume circa 4 ml). A HyperSep™ SPE tube packed with 400 mg PSA/GCB (Fisher UK, 10639685) was conditioned with 5

ml of ethyl acetate. The sample extract was loaded onto the SPE column in two applications: a) 2 ml extract followed by elution to waste: b) a further 2 ml extract followed by collection in an autosampler vial.

The responses obtained from the injection of sample extracts and standard solutions showed good peak shape and no loss of chromatographic performance was indicated. The results given in Table 19 show that recoveries of VNA obtained for all samples were within an acceptable range: some interferences were found on the MRM transitions for NMOR (malted grain and salami) and NPIP (smoked bacon) although these could be overcome by optimisation of the MRM transitions.

Table 19. Recovery trial for VNA additions to malted grain, smoked bacon and salami using column SPE clean-up on HyperSep™ SPE PSA/GCB.

VNA	Malted grain			Smoked bacon		Salami	
	Unspiked (µg/kg)	Spiked recovery (%) at 4 (µg/kg)	Spiked recovery (%) at 20 (µg/kg)	Unspiked (µg/kg)	Spiked recovery (%) at 4 (µg/kg)	Unspiked (µg/kg)	Spiked recovery (%) at 4 (µg/kg)
NDMA	<1	107	104	<1	86	<1	70
NMEA	<1	106	87	<1	102	<1	67
NDEA	<1	122	105	<1	87	<1	61
NDPA	<1	105	103	<1	78	<1	89
NMOR	1.1[a], <1[b]	130	133	<1	130	1, <1[b]	121
NPYR	6.3	116	95	<1	83	<1	76
NPIP	<1	119	80	10[c], 1.6[d]	88	1	61
NDBA	<1	88	99	3	64	<1	108

[a] interference on quantification transition *m/z* 134->117; [b] value obtained using *m/z* 117->117; [c] interference on quantification transition *m/z* 132->115; [d] value obtained using *m/z* 115->69

A summary of all spiked recovery data obtained from the analysis of a wide range of foodstuffs, over a three month period, is given in Table 20. Individual recoveries of NEMA (soup), NDEA (soup, malted beverage, malted grain), NDPA (smoked bacon, beer), NMOr (beer) and NDBA (beer) were just above acceptable limits defined by Codex (FAO/WHO 2013) indicating that some additional refinement of clean-up and / or MS/MS transitions may be required. Mean recoveries for all VNA added to samples at 4 µg/kg were in the range 89-105%; n=15); the precision of the method, determined under intra-laboratory conditions (3 mth period), was within accepted limits (*ibid.*) for each VNA (Horratt values <2, Horwitz / Thompson equation).

Procedural blanks routinely contained low levels of NDEA (0.1-1.4 µg/kg, mean 0.5 µg/kg), NMOR (0.3-0.6 µg/kg, mean 0.4 µg/kg), NPYR (0.0-0.5 µg/kg, mean 0.2 µg/kg), NPIP (0.0-1.0 µg/kg, mean 0.3 µg/kg), NDBA (0.1-0.6 µg/kg, mean 0.2 µg/kg) necessitating subtraction of the blank values from the test results. The latter were consistent with the findings of Lehotay et al (2015), who also reported low levels of some VNA (NDEA, NPYR) in both reagent blanks and solvent based calibration used, and hence the requirement to subtract blank measurements from test results. For this method however, solvent based calibrations curves were all linear over

the concentration range 0.0-100 µg/kg and passed through the intercept, indicating that the VNA found in the blanks did not originate from the solvent. An estimate of the method LOD, based on the mean response of VNA in reagent blanks (n=8) + 3sd was 0.7 µg/kg.

Despite the requirement for the use of Kuderna Danish apparatus to minimise VNA losses at the sample extract concentration stage, the relatively small volumes of solvent from the SPE step were evaporated efficiently and did not limit overall sample throughput. This method was subsequently used for the analysis of retail samples (see 8.1.2) and is described in 10.6 of the Appendices.

Table 20. Spike recovery for VNA from different matrices

Matrix	VNA added (µg/kg)	VNA recovered (%)							
		NDMA (92>75)	NEMA (106>61)	NDEA (120>103)	NDPA (148>131)	NMOR (134>117)	NPYR (101>55)	NPIP (132>115)	NDBA (176>159)
Malted grain	20	104	87	105	103	74[a]	95	80	99
Malted grain	4	107	106	122	104	86[a]	116	119	88
Smoked bacon	4	86	102	87	78	84[a]	83	87[b]	64
Smoked bacon	4	101	101	97	128	113	112	86	98
Salami	4	70	67	61	89	75[a]	76	61	108
Unsmoked bacon	4	103	101	92	119	112	111	98	108
Beer	4	104	98	113	134	144	81	96	144
Soy sauce	4	101	97	107	106	107	75	88	106
Smoked fish	4	112	112	111	99	53	90	70	94
Dried fish	4	104	111	103	94	95	110	94	84
Dry fermented sausage	4	107	106	105	106	98	92	74	127
Smoked cheese	4	98	86	106	95	77	80	88	106
Pickled red cabbage	4	94	108	114	74	80	94	89	109
Gravy powder	4	93	107	107	97	101	87	93	105
French onion soup	4	88	122	121	106	115	86	90	103
Malted cereal beverage	4	90	118	125	99	114	91	107	105
Mean	4	97.2	102.9	104.6	102.0	96.8	92.3	89.4	103.3
min	4	69.8	66.8	60.6	74.3	53.0	75.3	61.3	64.1
max	4	111.9	122.0	125.4	134.4	144.0	115.6	119.2	144.1
Rsu	4	2.8	3.4	3.9	4.0	5.9	3.7	4.0	4.4
Horrat	4	1.2	1.4	1.6	1.7	2.5	1.6	1.7	1.9

[a] Interference, mz/ 117->117 used for quantification; [b] interference, m/z 115->69 used for quantification

8.1.2 Analysis of samples

8.1.2.1 Sample selection

The ATNC results obtained from the analysis of the 63 retail samples (Table 10) were used to inform the selection of samples for the analysis of individual VNA. Application of a selection threshold of circa 20 µg/kg NNO (circa 3x ATNC LOD) gave a total of 23 samples together with a pickled product and a spice (for which ATNC could not be quantified) representing 9 of the original 10 categories for investigation.

8.1.2.2 Sample preparation and analysis

Samples were tested as consumed and prepared immediately before analysis according to the conditions given in 10.3 of the Appendix. Prepared samples were extracted using the SPE procedure based on the HyperSepTM SPE PSA/GCB (see 8.1.1.4.5) with GC-CI/MS/MS detection (see 10.6 of the Appendices for details of the method).

8.1.2.3 Results

The results from the analysis of VNA in target foodstuffs, tested as consumed, are given in Table 21. Detectable (>0.7 µg/kg) VNA were found in only two of the 25 samples tested. These included NDMA in the dried shrimp (9.4 µg/kg, confirmed by GC-TEA) and NPIP in the pepperoni (1.3 µg/kg), present in amounts that were consistent with historical data for these products (Tricker and Preussmann 1991). Analysis of the paprika using GC/PICI-MS/MS gave significant interferences on the quantification transitions for NMOR, NYR and NPIP (equivalent to 8.3, 2.6 and 124 µg/kg respectively). A secondary analysis of the extracts using GC-TEA detection did not confirm the presence of any VNA in the paprika above the system LOD of 4 µg/kg showing that the analysis of spices posed a challenge to be addressed.

Table 21. VNA results in selected foods measured as consumed and corresponding ATNC

Sample No.	Sample description	Category for project	ATNC (µg NNO /kg)	VNA	Concentration (µg/kg)
Unsmoked meats					
14C-14113	Unsmoked bacon	1	240, 366	None	<0.7
14C-14114	Unsmoked bacon	1	388, 449	None	<0.7
14C-14115	Unsmoked bacon	1	571, 556	None	<0.7
Beer					
14C-14054	Imported beer	2	20	None	<0.7
14C-14056	Imported beer	2	31	None	<0.7
14C-14057	Imported beer	2	95, 74	None	<0.7
Cured fish and meat (excluding smoked products)					
14C-14107	Dried Shrimp	4	677	NDMA	9.4 [a]
14C-14108	Dried Fish	4	21	None	<0.7
14C-14111	Chorizo	4	1646, 1947	None	<0.7
14C-14112	Italian Salami	4	2315	None	<0.7
14C-14116	Pepperoni	4	406	NPIP	1.3
Dried products					
14C-14063	French Onion soup powder	5	54, 48	None	<0.7
14C-14085	Paprika	5	NQ	None	<4 [b]
Malted foodstuffs					
14C-14084	Malted beverages	6	22	None	<0.7
Pickled products					
14C-14087	British beetroot In vinegar	7	NQ	None	<0.7
14C-14088	British red cabbage in vinegar	7	130	None	<0.7
Bouillons, gravies and soups					
14C-14062	Gravy Powder	8	23, 27	None	<0.7
Soy sauces					
14C-14093	Soy Sauce	9	20	None	<0.7
Wood smoked products					
14C-14098	Smoked bacon	10	2853, 2792	None	<0.7
14C-14099	Smoked bacon	10	945, 944	None	<0.7
14C-14104	Smoked bacon	10	1215, 1124	None	<0.7
14C-14101	Smoked mackerel	10	28	None	<0.7
14C-14102	Smoked salmon	10	70	None	<0.7
14C-14100	Smoked cheese	10	20	None	<0.7
14C-14105	Smoked cheese	10	17, 20	None	<0.7

[a] confirmed by GC-TEA; [b] detection using GC-TEA (LOD circa 4 µg/kg)

The most recent surveys of bacon, from products sourced in the USA, Denmark and Belgium, have all reported low but detectable amounts of VNA: Lehotay et al (2015) found combinations of VNA in all 48 samples of bacon from the USA retail market (NDMA mean 0.5 µg/kg; NDBA mean 1.1 µg/kg; NPYR mean 3.1 µg/kg; NPIP mean 0.2 µg/kg; NDEA <0.1 µg/kg); while Herrmann et al (2015) reported similar results for samples of Danish & Belgium bacon (NDMA 1.2-2.6 µg/kg; NPYR & 1.4-2.2 µg/kg; NPIP 0.07-0.2 µg/kg; NDEA was

undetected). The absence of detectable VNA in the cooked bacon and cured meats from this study was therefore unexpected although most of the bacon samples (5/6) obtained were of UK origin, for which recent data were not available for comparison. Similarly, De Mey et al (2014) found detectable amounts of VNA in 47/100 dry fermented sausages from the retail market in Belgium: the most abundant VNA were NMOR (22% had detectable amounts ranging from 0.6 – 1.6 µg/kg); and NPIP (28% had detectable amounts ranging from 0.8-12.3 µg/kg although most were below the LOQ of 2.5 µg/kg).

Although the samples for this investigation had been stored at -18°C prior to preparation and analysis, considerable time had elapsed since their original purchase (≤18 mths). To determine whether the latter had affected the results obtained for VNA, further samples of smoked and unsmoked UK back bacon and a cured meat (Pepperoni, Danish) were obtained local retail outlets. These samples were stored at 3°C and prepared and analysed within three days of purchase: bacon was cooked immediately before analysis according to the conditions given in 10.3.

NPIP was found in the unsmoked bacon and Pepperoni at 1.4 and 1.6 µg/kg respectively: no other VNA were detected (see Table 22). The result for NPIP in the Pepperoni was consistent with that from the analysis of the Dec 2014 sample (Lab code 14C-14116; 1.3 µg/kg) whereas no VNA were detected in any of the Dec 2014 cooked bacon samples. The concentration of NPIP in the 2016 bacon sample was within the range of values reported in recent surveys (Lehotay et al 2015) and historical data (Tricker and Preussmann 1991). The analysis of additional samples would be required to determine any trend for NPIP in cooked UK bacon.

Table 22. Concentrations of VNA found in additional bacon and cured meat samples purchased in 2016

Sample No.	Sample description	Category for project	VNA	Concentration (µg/kg)
16C-14498	Smoked Bacon	10	None	<0.7
16C-14499	Unsmoked Bacon	1	NPIP	1.6
16C-14500	Pepperoni	4	NPIP	1.4

8.2 Progress in method developments for non-volatile nitrosamines (nitrosaminoacids)

Methods for the analysis of NVNA in foodstuffs are not so well developed. Nitrosaminoacids (NAA) have mostly been determined as methyl esters after treatment with diazomethane (Ohshima and Bartsh 1981) or BF_3-methanol (Stillwell et al 1991). However, in view of restrictions with the use of highly toxic diazomethane and reports of instability of some NAA towards BF_3-methanol, an alternative derivatisation method based on silylation chemistry was investigated.

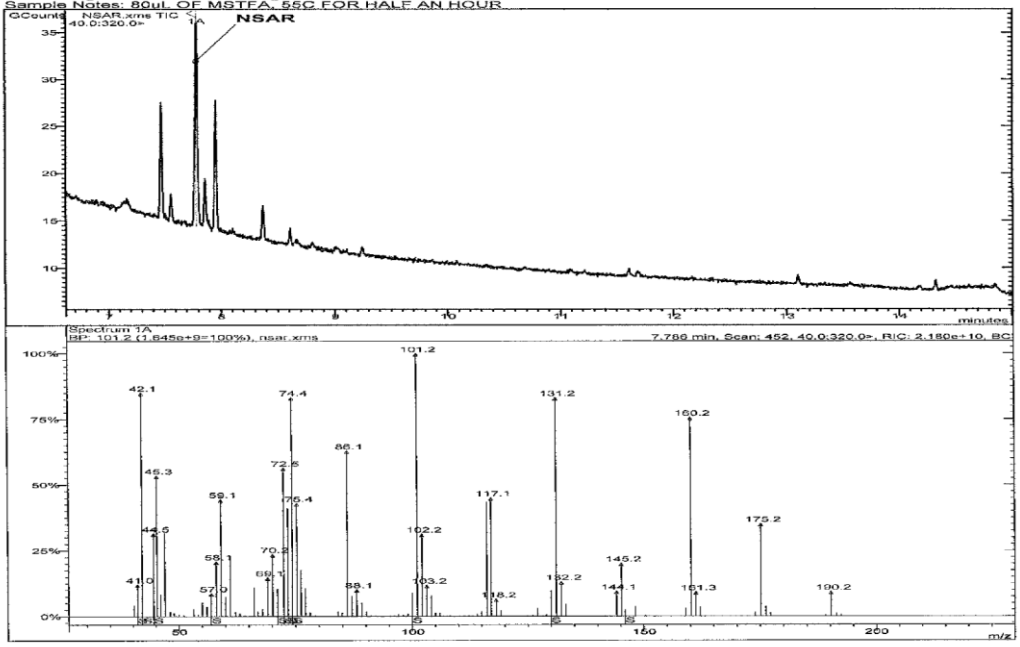
The silylation method of Eisenbrand et al (1975) for N-nitrosoamino acids based on the use of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and GC/MS was selected as this had been used previously for the analysis of NSAR, NPRO & NHPRO in processed foods. Aliquots of solutions of NPRO (200 µl of 50 µg/ml), NHPRO (200 µl 50 µg/ml), NSAR (1000 µl 4 µg/ml), and NTCA (1000 µl 4 µg/ml) were transferred to a 5 ml screw cap vial and blow to dryness under a nitrogen stream. N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, 80 µl) was added and the vial quickly capped and vortex mixed prior to incubation vial at 55°C for 30 min. When cool, 200 µl of ethyl acetate was added, vortex mixed and 1 µl injections (split mode) were made into a 30 m x 0.25 mm I.D. x 0.25µm ZB-XLB-HT Inferno GC column (Phenomenex, Macclesfield) using a Varian 1200 GC/MS system operating in the scanning mode.

Figure 17 shows the GC/MS chromatograms and electron impact (EI) mass spectra obtained from the derivatisation reactions of the NVNA with MSTFA: as expected, trimethylsilyl (TMS) derivatives of NSAR (*m/z* 190 is molecular ion), NPRO (*m/z* 216 molecular ion) and NHPRO (*m/z* 304 molecular ion) were readily formed from treatment with MSTFA; however, the corresponding -TMS derivative of NTCA was not obtained, indicating that further development would be required. Note, when NHPRO was treated with MSTFA, both the –OH and $–CO_2H$ groups were derivatised with TMS (MW 304).

A)

MS Data Review Active Chromatogram and Spectrum Plots - 3/10/2016 12:42 PM

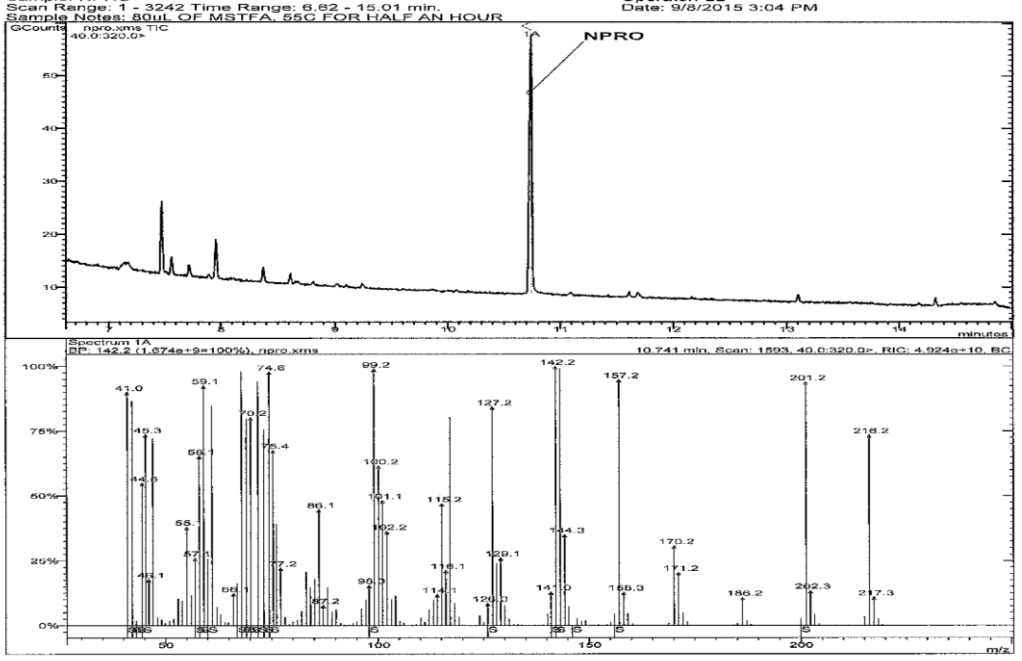
File: ...erws\data\nitrosoamino acids\2015\090815_repeat mstfa deri\nsar.xms
Sample: NSAR
Operator: LL
Scan Range: 1 - 3242 Time Range: 6.62 - 15.01 min.
Date: 9/8/2015 3:27 PM
Sample Notes: 80uL OF MSTFA, 55C FOR HALF AN HOUR



B)

MS Data Review Active Chromatogram and Spectrum Plots - 3/10/2016 12:44 PM

File: ...erws\data\nitrosoamino acids\2015\090815_repeat mstfa deri\npro.xms
Sample: NPRO
Operator: LL
Scan Range: 1 - 3242 Time Range: 6.62 - 15.01 min.
Date: 9/8/2015 3:04 PM
Sample Notes: 80uL OF MSTFA, 55C FOR HALF AN HOUR



C)

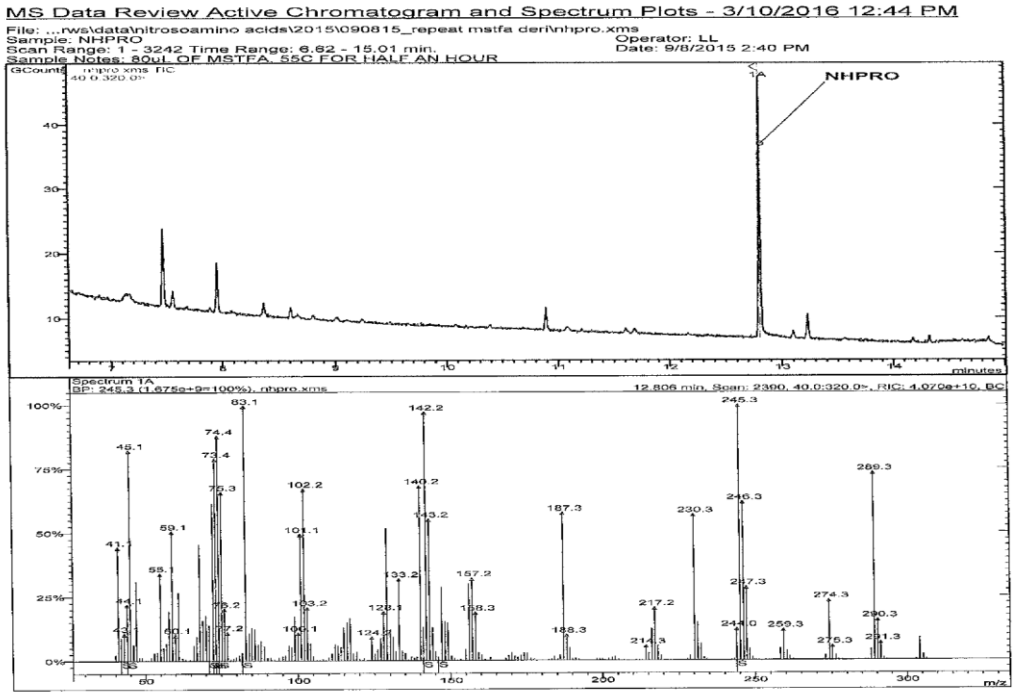


Figure 17. GC/MS data for the TMS derivatives of NSAR, NPRO and NHPRO: A) NSAR, [$M^{+\cdot}$] = *m/z* 190; B) NPRO, [$M^{+\cdot}$] = *m/z* 216; C) NHPRO, [$M^{+\cdot}$] = *m/z* 304

9. Discussion of results, conclusions and recommendations

Many NOC are known to mankind and their carcinogenic potency varies widely. However, it would seem that the simple (i.e. without additional substituents) diakyl and cyclic VNA are the most likely known class of preformed dietary NOC of toxicological significance: With the exception of NSAR, which is a relatively weak carcinogen, most NVNA are not believed to be mutagenic and carcinogenic (IARC 1978; Habermeyer and Eisenbrand 2009). However, full toxicological evaluations of most NVNA were still incomplete due to insufficient data.

Against this background, a rapid and selective (by extraction) ATNC method for screening retail foods was developed and validated with respect to the known dietary NOC of toxicological concern. During the course of development it was noted that these methods, which rely on semi-selective chemical denitrosation reactions can, in some instances, give false positive results and should therefore be considered as potential indicators of NOC contamination. Application of the ATNC method to target foods showed that 36 / 63 samples gave a positive result for ATNC.

To understand the nature of individual NOC comprising these positive samples, a method to identify and quantify individual VNA in a wide range of retail foods was developed. Developments in sample clean-up sorbents showed that a relatively rapid and selective sample preparation method could be developed when

coupled to a GC/MS system operating in PICI-MS/MS mode. Application of this method to the analysis of VNA in target samples that were positive by ATNC showed that contamination by VNA in products available in the UK retail market was minimal (4/36 positive). These included unsmoked cooked bacon (NPIP, 1.6 µg/kg), dry fermented sausages (NPIP, 1.3-1.4 µg/kg) and dried shrimp (NDMA 9.4 µg/kg).

The development and application of a method to determine other known NOC such as individual NAA was progressed to the stage of characterisation of reference standards but not taken further (see 8.2) as much of the allocated budget for this project was consumed by ATNC method developments.

Whilst data for the occurrence of individual VNA was reassuringly low, it would appear that ATNC was not a reliable indicator of known toxicologically significant NOC contamination i.e. the VNA, at least for the limited number of products tested in this study. Inspection of historical data for studies reporting individual NOC and ATNC indicates that the latter may have been a more reliable indicator for VNA formed by thermal processes (Massey et al 1991). For example, ATNC and NTHZ showed a reasonable correlation (R^2=0.7) (*ibid.*) indicative of the known thermal decarboxylation of NTCA (Tricker and Kubacki 1992). Massey et al (1990) found no correlation between VNA (NDMA) and ATNC measured in beers and suggested that the latter were likely to be influenced by fermentation compared to thermal process for the VNA. It would seem that most ATNC are likely to be highly polar species (*ibid.*), for example, circa 50% of ATNC measured in fried bacon was associated with the insoluble protein fraction in adipose tissue (Tricker et al 1985).

The results from this investigation provide reassuring information concerning the absence of NOC known to be harmful to human health. Although sample numbers were limited, the selection of samples was targeted at foods / processes previously known to be associated with NOC contamination. However, recent data on individual NOC in UK foods is still lacking and these findings should be supported by a more comprehensive survey of VNA in retail foodstuffs.

10. Appendices

10.1 Sampling plan

	Sample numbers in product category																				
Category for project	1. Bacon	2. Beer		3. Cheese, unsmoked	4. Cured meat and fish products (excluding smoked)		5. Dried products					6. Malted foodstuffs		7. Pickled products		8. Soups, gravies, bouillons	9. Soy sauces	10. Wood smoked products			Totals
	unsmoked	UK/EU	imported		fish	meat	infant formulae	milk powders	soups	spices	tea	beverages	breakfast cereals	fish	vegetables			fish	meats	cheese	
Month																					
Nov -14		3	5				2	2	2		2					6					22
Dec -14	3				3	3							3	3				3	3		21
Jan - 15				3						2		3			3		6			3	20
Totals	3	3	5	3	3	3	2	2	2	2	2			3	3	6	6	3	3	3	**63**

10.2 Reference standards obtained

Name	Abbreviation	CAS No	MW	Supplier	Cat No.	Lot no
Volatile nitrosamines						
N-Nitrosodimethylamine	NDMA	62-75-9	74	Fisher	8270-AF-C	T1120906015
N-nitrosodimethylamine-d6		17829-05-9	80	CDN	D-2937	
N-Nitrosomethylethylamine	NMEA	10595-95-6	88	Fisher	8270-AF-C	T1120906016
N-nitrosomethylethylamine-d3		69278-54-2	91	CDN	D-6874	
N-Nitrosodiethylamine	NDEA	55-18-5	102	Fisher	8270-AF-C	T1120906017
N-nitrosodiethylamine-d10		1219794-54-3	112	CDN	D-4107	
N-Nitrosodipropylamine	NDPA	621-64-7	130	Fisher	8270-AF-C	T1120906018
N-nitrosodi-n-propylamine-d14		93951-96-3	144	CDN	D-2938	
N-Nitrosodibutylamine	NDBA	924-16-3	158	Fisher	8270-AF-C	T1120906019
N-nitrosodi-n-butylamine-d18		1219798-82-9	176	CDN	D-6711	
N-Nitrosopiperdine	NPIP	100-75-4	114	Fisher	8270-AF-C	T1120906020
N-nitrosopiperidine-d10		960049-21-2	124	CDN	D-4139	
N-Nitrosopyrrolidine	NPYR	930-55-2	100	Fisher	8270-AF-C	T1120906021
N-nitrosopyrrolidine-d8		1219802-09-1	108	CDN	D-6521	
N-Nitrosomorpholine	NMOR	59-89-2	116	Fisher	8270-AF-C	T1120906022
N-nitrosomorpholine-d8		1219805-76-1	124	CDN	D-4091	
N-Nitrosodiphenylamine	NDPhA	86-30-6	198	Fisher	8270-AF-C	T1120906023
N-nitrosodiphenylamine-d6		93951-95-2	204	CDN	D-2311	
Non-volatile nitrosaminoacids						
N-Nitrosoarcosine	NSAR	13256-22-9	118	TRC	N546650	6-NSR-68-4
N-Nitroso-D-proline	NPRO	42022-03-7	144	TRC	N545451	18-WG-54-1
N-Nitroso-L-proline	NPRO	7519-36-0	144	TRC	N545450	1-HGS-139-2
N-Nitrosothiazolidine-4-carboxylic acid	NTCA	88381-44-6	162	TRC	N546750	6-JQW-87-1
N-Nitroso-2-methylthiazolidine-4-carbosylic acid	NMTCA	103659-08-1	176	TRC	N529970	6-JQW-111-2
N-Nitroso-L-hydroxyproline	NHPRO	30310-80-6	160	TRC	N528600	9-MDB-184-2

10.3 Sample preparation details

Table 23. Domestic cooking conditions for meat samples tested “as consumed”

Category 1. Unsmoked bacon

Sample code	Product description	Cooking conditions			
		Quantity	Method	Time (s)[a]	Comments
14C-14113	Dutch back bacon, rind on	2 rashers	Hot plate	80	Rashers cooked individually on equilibrated cast iron plate, 40 s each side, no oil
14C-14114	unsmoked back bacon, rind on	2 rashers	Hot plate	80	Rashers cooked individually on equilibrated cast iron plate, 40 s each side, no oil
14C-14115	unsmoked back bacon, rind on	2 rashers	Hot plate	80	Rashers cooked individually on equilibrated cast iron plate, 40 s each side, no oil
16C-14499	unsmoked back bacon rashers, rind on	2 rashers	Hot plate	80	Rashers cooked individually on equilibrated cast iron plate, 40 s each side, no oil

[a] total time per rasher

Category 10. Smoked products

Sample code	Product description	Cooking conditions			
		Quantity	Method	Time (s)[a]	Comments
14C-14098	oak smoked dry cured back bacon rashers, rind on	2 rashers	Hot plate	80	Rashers cooked individually on equilibrated cast iron plate, 40 s each side, no oil
14C-14099	oak smoked cured outdoor bred British bacon rashers, rind on	2 rashers	Hot plate	80	Rashers cooked individually on equilibrated cast iron plate, 40 s each side, no oil
14C-14104	British dry cured oak smoked rindless back bacon	2 rashers	Hot plate	80	Rashers cooked individually on equilibrated cast iron plate, 40 s each side, no oil
16C-14498	British oak smoked dry cured back bacon rashers	2 rashers	Hot plate	80	Rashers cooked individually on equilibrated cast iron plate, 40 s each side, no oil

[a] total time per rasher

Table 24. Domestic preparation details for samples tested “as consumed”

Sample code	Category	Sample description	Preparation procedure [a]		
			Sample weight (g)	Volume of water (ml)	Comments
14C-14058	5	Infant milk powder	31.6	210	freshly boiled water, cooled slightly, agitated
14C-14059	5	Infant milk powder	31.5	210	freshly boiled water, cooled slightly, agitated
14C-14060	5	Dried skimmed milk Powder	20.0	200	freshly boiled water, cooled slightly, agitated
14C-14061	5	Dried skimmed milk Powder	20.0	200	freshly boiled water, cooled slightly, agitated
14C-14067	5	Mushroom soup powder	26.0	200	Freshly boiled water, stir with glass rod
14C-14063	5	French Onion Soup powder	44.8	600	cold water, bring to boil gently, stir with a glass rod
14C-14064	5	Loose leaf tea	3.1	250	boiling water
14C-14065	5	Loose leaf tea	3.1	250	boiling water
14C-14082	6	Malted drink	25.1	200	freshly boiled water, cooled slightly
14C-14083	6	Malted drink	25.0	200	freshly boiled water, cooled slightly
14C-14084	6	Malted drink	25.0	200	freshly boiled water, cooled slightly
14C-14086	7	Silverskin onions in vinegar	-	-	drained prior to analysis
14C-14087	7	British beetroot In vinegar	-	-	drained prior to analysis
14C-14088	7	British red cabbage in vinegar	-	-	drained prior to analysis
14C-14117	7	Rollmop herrings (canned)	-	-	drained prior to analysis
14C-14118	7	Mussels in vinegar	-	-	drained prior to analysis
14C-14119	7	Cockles in vinegar	-	-	drained prior to analysis
14C-14062	8	Gravy Powder	20.1	280	cold water, bring to boil gently, stir with glass rod
14C-14069	8	Gravy Powder	20.0	280	cold water, bring to boil gently, stir with glass rod
14C-14066	8	Oxtail Soup	-	-	contents of can transferred to a stainless steel saucepan and brought to boil gently
14C-14068	8	Chicken Soup	-	-	contents of can transferred to a stainless steel saucepan and brought to boil gently
14C-14070	8	Beef Stock Cube	9.7	450	Freshly boiled water, stir with glass rod
14C-14071	8	Beef Stock Cube	6.1	190	Freshly boiled water, stir with glass rod

[a] prepared according to the guide on the retail pack, unless otherwise stated all preparations were made in a Pyrex® glass beake

10.4 Chemical stripping / TEA system: manufacturer's performance specification

800 Series TEA

Application Note

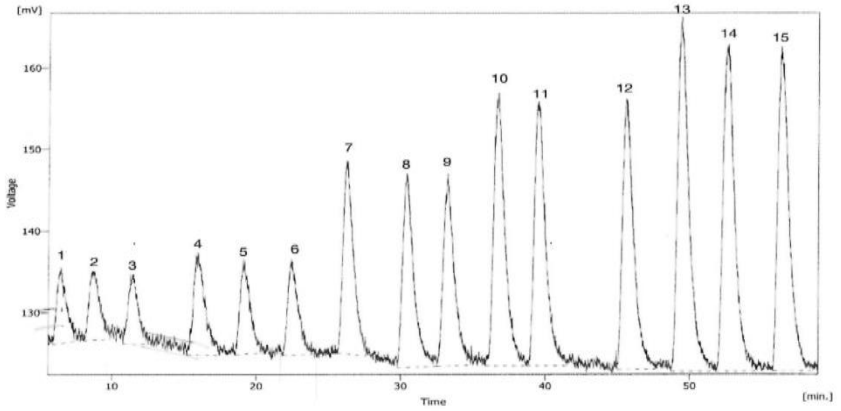
Chemical Stripping System - NDPA in Methanol Calibration

Application Number: 007

Ellutia

1-3	10 µg l^{-1} NDPA in Methanol	**10-12**	70 µg l^{-1} NDPA in Methanol
4-6	20 µg l^{-1} NDPA in Methanol	**13-15**	100 µg l^{-1} NDPA in Methanol
7-9	50 µg l^{-1} NDPA in Methanol		

Injection Volume	50 µl
Cold Trap	-19°C
TEA Vacuum	1.73 Torr
N_2 Flow	50 ml min^{-1} (under vac) 35 (no vac)
TEA Sensitivity	213 (230)



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Chromatography Solutions

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Email: info@ellutia.com **Website:** http://www.ellutia.com

10.5 The ATNC QuEChERS-method

10.5.1 Chemicals and reagents

Acetonitrile (HPLC grade), anhydrous magnesium sulphate ($MgSO_4$), ethyl acetate (high purity), hydrobromic acid (47-49% certified ACS), methanol (HPLC grade) and sulphamic acid (AR) were obtained from Fischer Scientific (Loughborough, UK). High purity water was prepared in-house (NANOpure® DIamond™, Thame, UK). Sulphamic acid solution for sample addition was prepared by dissolving 180 g sulphamic acid in 1 l water. Standard solution of 9 VNA and NSAR (see 7.1.3) for calibration and sample addition were prepared by serial dilution in methanol.

10.5.2 Sample preparation / homogenisation

Where required, samples were prepared and immediately tested "as consumed" according to the conditions given in 10.3 of the Appendices.

Cooked samples were allowed to cool. Bacon (diced), cured meats and fish, dried shrimp were homogenised using a Robo Coup Blixer 4 food processor (Isleworth, UK) under liquid nitrogen; cheese was cooled under liquid nitrogen prior to grating using a domestic (stainless) grater; dry products (as consumed) and picked vegetables were homogenised at ambient temperature using a Robo Coup Blixer 4 food processor (Isleworth, UK); soups, gravies, soy sauce and beer were mixed well to ensure homogeneity before sampling for extraction.

10.5.3 Extraction

Homogeneous samples (2.5 – 10 g) from 10.5.2 above were weighed accurately into a 50 ml polyprolylene centrifuge tube (Fisher, Loughborough) and SA, water, acetonitrile (10 ml) and $MgSO_4$ were added according to the quantities given in Table 25. Any remaining homogeneous sample was stored at -18 °C. The centrifuge tube was capped and vortex mixed for 30 s prior to centrifuging at 3500 g_{av} for 5 min. The acetonitrile layer was removed to a graduated glass tube, a further aliquot of acetonitrile was added to the centrifuge tube and the vortex mixing and centrifugation steps repeated. Combined acetonitrile extracts were reduced under nitrogen stream to the final volumes given in Table 25. Prepared extracts were stored at -18°C until required for analysis.

10.5.4 Chemical stripping/TEA

All measurements were made using an Ellutia chemical stripping system linked to a 610 TEA (Ely, UK) operating under the conditions given section 7.1.4 and Table 26 below.

Each batch of samples (≤12) comprised a procedural blank and a spiked sample (one for each different sample matrix). The denitrosation reagent, hydrobromic acid (5 ml) in ethyl acetate (15 ml), was replenished for each batch of samples. System performance was determined by injection of solvent blanks and standards (nine standard mix, see 7.1.3) prior to calibration over the concentration range 8 – 1995 µg NNO/l. A typical sequence of injections was as follows: procedural blank / sample / verification standard.

Table 25. Sample weights and reagent additions for the extraction of foodstuffs by the ATNC QuEChERS-method

Sample type	Sample weight (g)	SA (ml)	water (ml)	Acetonitrile (ml)	$MgSO_4$ (g)	Final volume (ml)
Bacon, cured meats, dried shrimp	2.5	0.25	7.5	10+5	4.0	15
Dried products tested as received	4.0	0.25	10.0	10+5	4.0	4
Cheese	5.0	0.25	5.0	10+5	4.0	5
Fish	5.0	0.25	5.0	10+6	4.0	5
Dried products tested as consumed	5 - 10	0.25	5 - 0	10+5	4.0	5 - 10
Soy sauce	5 - 10	0.25	0 - 5	10+5	4.0	5 - 10
Beer	10.0	0.25	-	10+5	6.0	10
Beverages tested as consumed	10.0	0.25	-	10+5	4.0	10
Pickled vegetables	10.0	0.25	-	10+5	4.0	10
Soup (canned)	10.0	0.25	-	10+5	4.0	10

Table 26. TEA / Chemical Stripping System: typical operating conditions

Parameter	Value or condition	Comments
TEA		
Sensitivity	220	Related to ozone generation
Oxygen Flow (ml/min)	3.4	Used to generate O_3 (within TEA)
Vacuum (Torr)	2	Operation vacuum during Chemical Stripping
Pump	Edwards RV3	Exhaust fitted with a 610 ozone trap
CHEMICAL STRIPPING SYSTEM		
Carrier gas	Nitrogen	
Carrier gas pressure (psi)	5	
Carrier gas flow (ml/min)	50	
Coolant (ethylene glycol)	50% (w/w) in deionised water	
Cold trap temperature (°C)	-20 ±1	
Reaction vessel	Hydrobromic acid (5 ml) in ethyl acetate (15 ml)	Magnetic stir bar used
Injection volume (µl)	100	

10.6 SPE method for the analysis of volatile nitrosamines using GC-CI/MS/MS detection

Samples (5 g) were weighed accurately into 50 ml centrifuge tubes and 50 ul of 4 µg/l ITSD solution and 0.25 ml of SA solution were added. Blank and spiked samples were prepared in the same way: 50 ul of 0.4 µg/l VNA (9 standard mix) was added to spiked samples. Aqueous methanol (methanol:water 1:3 v/v), 25 ml for the dry cereal products, 20 ml for all other samples, was added to each tube which was then capped and vortex mixed for 15 s and tumble mixed for 15 min. Tubes were then centrifuged at 4500 rpm for 10 min and the supernatant decanted onto a Chem Elut cartridge. After equilibration for 15 min the cartridge was eluted with 25 ml + 2 x 15 ml of a solution of 2% (V/V) ethyl acetate in dichloromethane and collected in a 50 ml centrifuge tube (wrapped in foil to exclude light). The extract was transferred in aliquots to a Kuderna Danish apparatus (40 ml) and reduced to a volume of circa 2 ml at 55ºC. The concentrated extract was quantitatively transferred to a 4 ml amber vial using 2x1 ml of ethyl acetate and mixed well (final volume circa 4 ml). A HyperSep™ SPE tube packed with 400 mg PSA/GCB (Fisher UK, 10639685) was conditioned with 5 ml of ethyl acetate. The sample extract was loaded onto the SPE column in two applications: a) 2 ml extract followed by elution to waste: b) a further 2 ml extract followed by collection in an autosampler vial. The conditions of analysis using GC/PICI-MS/MS are given in Table 14 and Table 16.

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