

Gas Chromatography – Mass Spectrometry

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The Determination of Low Levels of Nitrosamines in Beer Using the Clarus 680 GC/MS and a D-Swafer System

Introduction

Nitrosamines are a class of compounds that are often found in food and other organic products. They are highly carcinogenic and many countries apply controls on the acceptable levels of these compounds in food. Nitrosamines are formed as food is heated through the reaction of amines with nitrites, which are sometimes added as a preservative.

Malt and its derivative products are of particular concern and beer represents (along with fried bacon and tobacco) the major source of ingested nitrosamines in humans. Historically, malt was roasted over open fires and the nitrogen oxide gases in the smoke would react with amines in the malt to form nitrosamines. Modern malt production uses indirect fire roasting and the levels of nitrosamines have consequently dropped significantly – by a factor of over 50x from malt produced 20 years ago.

Nitrosamines generated during malt production will pass into beer. Examples of maximum acceptable levels of nitrosamines in beer are 5 µg/kg in the United States, 0.5 µg/kg in Italy, Switzerland and Germany and 2-15 µg/kg in Russia.

The main compound that is monitored in malt and beer is nitrosodimethylamine (NDMA). This compound and its homolog, nitrosodiethylamine (NDEA) are the compounds targeted in this application note.

To determine NDMA and NDEA at low concentrations in beer typically involves a liquid-liquid extraction followed by a multi-step extensive sample clean-up regime and determination by gas chromatography, including a highly specific and selective detector, such as a thermal energy analyzer (TEA) detector.

In this application note, we present a more efficient and rapid method of analysis. It uses a fast and simple single-step liquid-liquid extraction technique followed by direct injection of the extract into a GC/MS system for separation and quantification.

A Swafer™ heartcutting system is used to selectively transfer timed cuts of the effluent, that contain the analytes, from one GC column into the inlet of a second column with a different stationary phase. This technique eliminates the solvent and bulk of other compounds extracted from the sample matrix from the chromatography in the second column, which provides an extra level of analytical selectivity and reduces the need for the sample clean-up procedures.

A quadrupole MS detector system was used in electron ionization mode to monitor the chromatography on the second column. This approach means that more sample residue is likely to accumulate over time in the injector liner, but liner replacement is a much easier option than a multi-stage sample clean-up regime. To achieve the detection limits sought, data collection used single ion recording (SIR). This provided another degree of selectivity to overcome potential matrix interferences.

A second detector (flame ionization) was used to monitor the chromatography on the precolumn and establish the heartcut and the backflush timings.

Experimental Instrumentation

Figure 1 gives a schematic diagram of the analytical system, which is from the Swafer Utilities Software that was used to develop this method. Table 1 lists the full operating conditions for the final method.

Gas Chromatograph	PerkinElmer Clarus 680
Oven Temperature	35 °C for 1 min., then 10 °C/min. to 185 °C (16-minute run)
Injector	Programmable Split/Splitless (PSS)
Injector Temperature	35 °C for 1 min., then 200 °C/min. to 250 °C and hold until the end of the run
Carrier Gas	Helium
Injector Pressure	Initially 27 psig then 2 psig at 12.81 min. Maintained at 2 psig during oven cooldown.
Injector Split Flow Rate	Initially Off, then 25 mL/min. at 3 min.
Detector	a) Flame Ionization (FID) b) Mass Spectrometer (MS)
FID Temperature	250 °C
FID Combustion Gases	Air: 450 mL/min., Hydrogen: 45 mL/min.
FID Range	x1
FID Attenuation	x4
MS Transfer Line Temperature	200 °C
MS Filament Temperature	200 °C
MS Data Collection	a) SIR m/z 74 from 12.00 – 13.50 min. (for NDMA) b) SIR m/z 102 from 15.00 – 16.00 min. (for NDEA) Dwell Time 0.5 sec Interchannel Delay 0.02 sec
Switching/Backflush System	D-Swafer configured in D4 mode
Precolumn	30 m x 0.250 mm x 0.25 µm PerkinElmer Elite™ 1
Analytical Column	30 m x 0.250 mm x 0.25 µm PerkinElmer Elite Wax (connected to MS)
Restrictor Tubing	51 cm x 0.100 µm deactivated fused silica (connected to FID)
(Midpoint) Pressure at D-Swafer	18 psig throughout
Timed Events	PSS Pressure set to 27 psig at -1.50 min. (to raise pressure after oven cooldown) PSS Split Flow set to 0 mL/min. at -1.00 min. (for splitless mode) PSS Split Flow set to 25 mL/min. at 3.00 min. (to vent liner) Switching Valve On at 9.27 min. (to cut NDMA) Switching Valve Off at 9.48 min. Switching Valve On at 12.60 min. (to cut NDEA) Switching Valve Off at 12.80 min. PSS Pressure set to 2 psig at 12.81 min. (to backflush)
Sample Injection	Normal injection of 3.0 µL of sample dichloro-methane extract using an autosampler

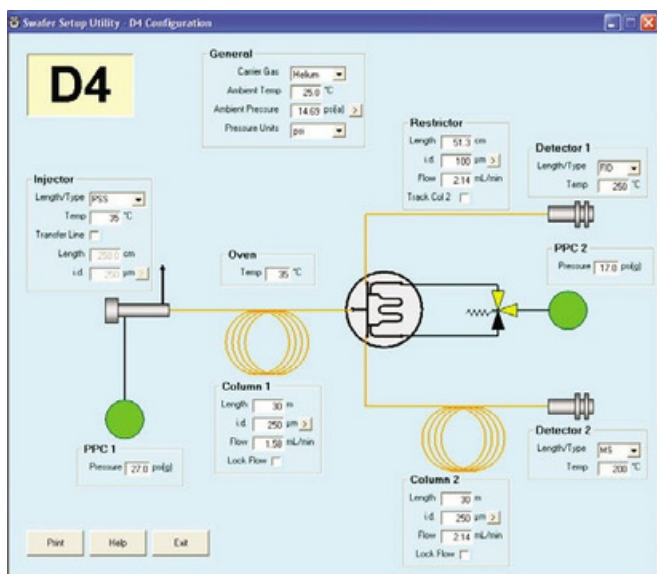


Figure 1. Swafer Utilities Software used for the determination of nitrosamines in beer.

Sample Preparation

Approximately 25 mL of the beer sample was poured into a 50 mL beaker and placed in a cool ultrasonic bath for two minutes to de-gas the sample.

10.0 ± 0.5 g of de-gassed beer were weighed to a precision of 0.01 g into a 15-mL polypropylene centrifuge tube.

1-mL of 1.0 M sodium hydroxide solution prepared in de-ionized water and 3.0 ± 0.1 g of crystalline sodium chloride were added to the sample and shaken to dissolve the salt.

1-mL of dichloromethane was added using a Grade-A bulb pipette and carefully shaken with a gentle rocking motion with the treated beer sample for 10 minutes. Vigorous shaking was avoided to minimize the formation of emulsions.

The tube was centrifuged at high speed for 20 minutes. In instances where emulsions had formed, the contents of the tube were rocked backwards and forwards a few times and then re-centrifuged (this was usually effective at breaking up the emulsion). An example of a successful extraction is given in Figure 2.



Figure 2. Example of successful beer extraction.

Using a dropper pipette, a sufficient volume of the lower organic layer was transferred directly into an autosampler vial for GC analysis. Note that it was not necessary to transfer the whole extract. Care was taken to ensure that none of the aqueous phase was transferred to the vial along with the extract. The vial was sealed with a suitable cap and refrigerated until analysis.

Method Development

Figure 3 shows a TIC of a concentrated standard mixture containing a series of nitrosamines including NDMA and NDEA. In this chromatogram, the D-Swafer has been set to direct the effluent from the precolumn into the analytical column, which is connected to the mass spectrometer. In practice we will need to see nitrosamines at a concentration below the 1 ppb level in the samples (equivalent to 10 ppb in the extracts). This is more than 1000x less than the concentration shown here.

Another point that should be mentioned is the role of the PSS injector in this analysis. The sample extracts are in dichloromethane. This is a highly volatile solvent that boils at 40 °C (at atmospheric pressure). It is not a good solvent to use for splitless injection as it is difficult to refocus at the column inlet. The injection of large volumes will easily cause injector blow-out and cause peak distortion and loss of injected sample. In this method the PSS is set to a low temperature (35 °C) during injection and then heated to vaporize the rest of the injected sample. This provides a much more controlled vaporization process and the 3 µL injection volume provides symmetrical and well separated peaks as shown in Figure 3. One concern in using the PSS at low temperatures like this is the time needed to cool the injector back to this temperature before the next run. The Clarus® 680 PSS uses an optimized heatsink and a high-speed dedicated cooling fan to achieve this cooling in less than four minutes.

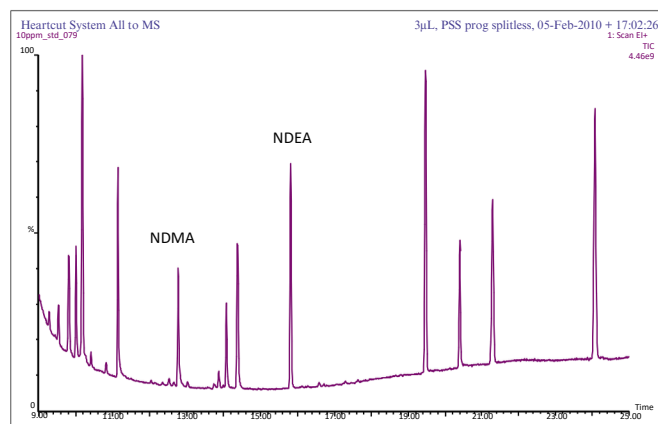


Figure 3. Total ion chromatogram (m/z 35 to 150) from a 3 µL injection of a 10 ppm standard mixture of nitrosamines with the precolumn effluent directed to the second column and the MS detector.

The concern regarding detection limits is illustrated in Figure 4. This shows a TIC obtained from a beer extract run under the same conditions as used for Figure 3. The chromatogram is plotted with an expanded time scale but with a similar response scale. Expected elution times of the two nitrosamines are indicated. We need to see peaks at less than 1000x the size of those shown in Figure 3 without significant interference from co-eluting peaks from the sample matrix. This is clearly a challenge using this type of method as much better sensitivity and much better selectivity are needed.

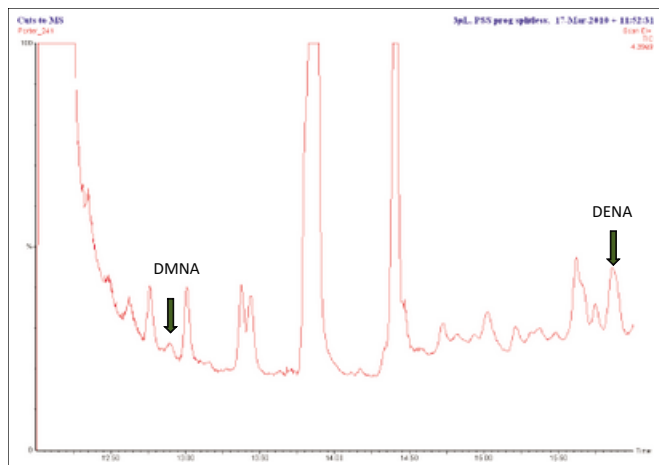


Figure 4. Total ion chromatogram of an extract taken from an American porter ale sample with the precolumn effluent directed to the second column and the MS detector.

Better sensitivity and selectivity are easily obtained on a quadrupole mass spectrometer by operating it in the SIR mode.

Figure 5 and Figure 6 give the structures and mass spectra of the two nitrosamines being monitored. These figures were taken from the NIST® Mass Spectral Search Program v. 2.0 supplied with the Clarus 680 MS system. In each case there is a strong molecular ion at m/z 74 and m/z 102 for NDMA and NDEA respectively. These ions were used in a SIR MS method to collect and process the data.

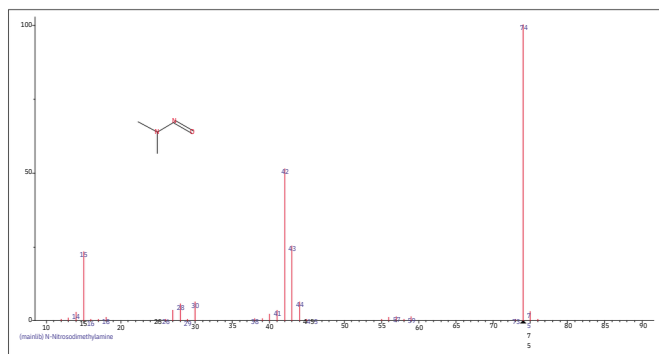


Figure 5. Structure and mass spectrum of nitrosodimethylamine.

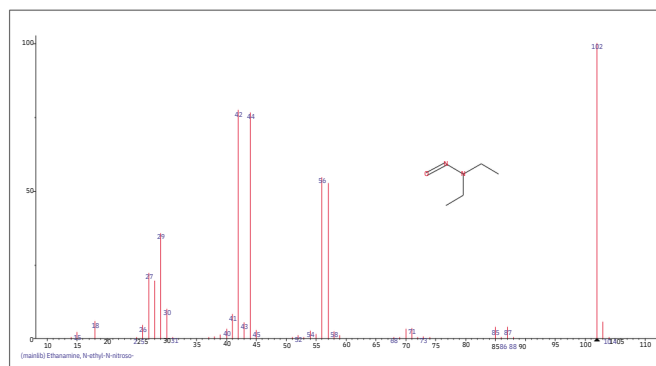


Figure 6. Structure and mass spectrum of nitrosodiethylamine.

Figure 7 shows chromatography of a much more dilute standard mixture of nitrosamines under the same conditions as used for Figure 3 and Figure 4. These peaks would represent nitrosamine concentrations of 1 ppb in the samples, and clearly indicate the potential to detect nitrosamines at levels below 1 ppb in the samples.

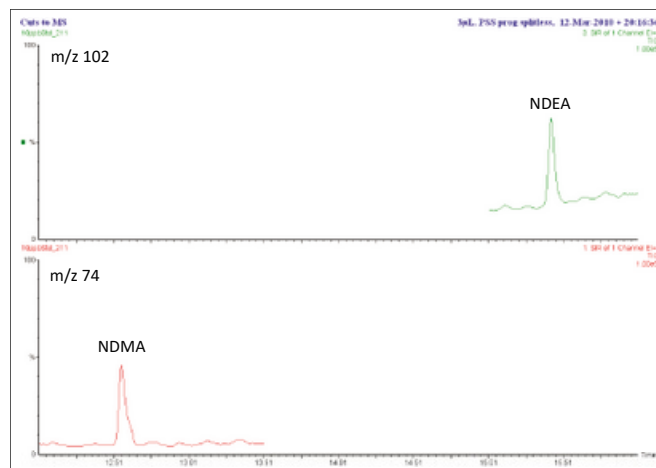


Figure 7. SIR chromatographic traces at m/z 74 and 102 showing expected elution times of NDMA and NDEA respectively from an injection of a 10 ppb stand mixture of nitrosamines (equivalent to 1 ppb in samples).

However, when a beer extract is chromatographed as shown in Figure 8 under the same conditions as used for Figure 7, there are significant interferant peaks in the chromatography that would obscure the nitrosamine peaks at the required levels. This chromatogram has the same scaling as Figure 7. Some further improvements to the selectivity are required.

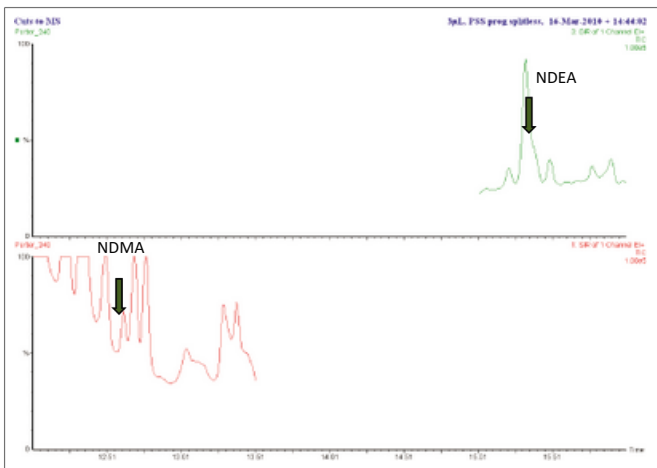


Figure 8. SIR traces at m/z 74 and 102 showing expected elution times of NDMA and NDEA respectively.

The use of the D-Swafer in the D4 heartcutting configuration provides an additional high degree of selectivity by transferring narrow cuts around the elution times of the nitrosamines from the precolumn on to the analytical column. This way, the solvent and the bulk of the extracted sample matrix are eliminated completely from the chromatography being monitored. Because the analytical column stationary phase is very polar, peaks that would co-elute with the nitrosamines on the non-polar precolumn and cut with them to the analytical column, would become separated by the different stationary phase. Figure 9 shows chromatography of the concentrated nitrosamine standard mixture indicating the regions around the eluting nitrosamines that are to be heartcut to the analytical column.

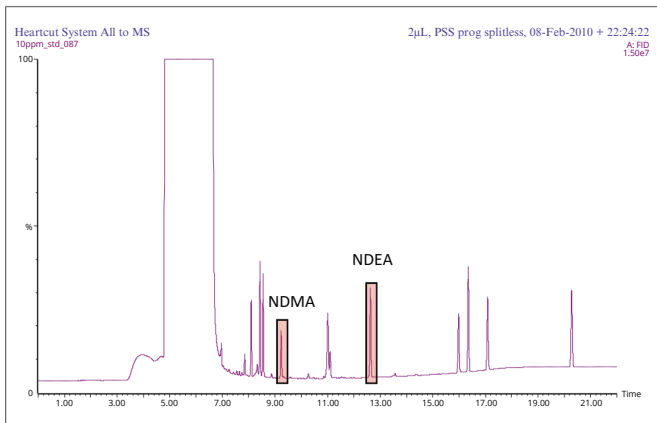


Figure 9. Precolumn chromatogram of 10 ppm nitrosamine standard mixture. This chromatogram was produced with the precolumn effluent switched to the D-Swafer outlet restrictor and the FID. The regions to be heartcut are highlighted.

Figure 10 shows a chromatogram from the same beer extract shown in Figure 4 and Figure 8, which was run under the same conditions used for Figure 9 but with the heartcutting switching applied. The heartcut regions are indicated by the drop in signal from the FID. After the NDEA peak has been heartcut, the pressure at the GC injector is reduced to a low value to initiate backflushing of the precolumn. This is indicated by the absence of chromatography beyond the last heartcut. Backflushing helps keep heavy sample material out of the Swafer and the detector, eliminates the need for extended temperature programming to elute heavy sample material from the system and reduces the analysis time.

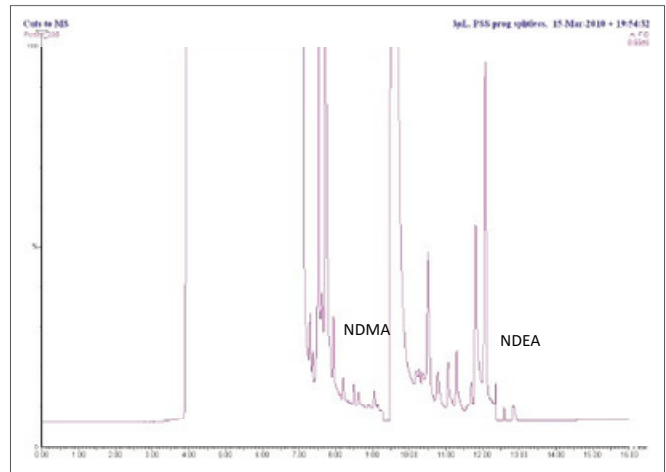


Figure 10. FID precolumn chromatogram of an American porter ale extract showing regions that have been heartcut to the analytical column.

Figure 11 shows the corresponding SIR chromatography of the heartcuts directed to the analytical column illustrated in Figure 10. Now the traces are very clean and the nitrosamine peaks, which are 0.39 ppb and 0.11 ppb for NDMA and NDEA respectively are seen and quantified with confidence. Compare these chromatograms against those in Figure 8 to see the improvements in selectivity brought about by the D-Swafer heartcutting technique.

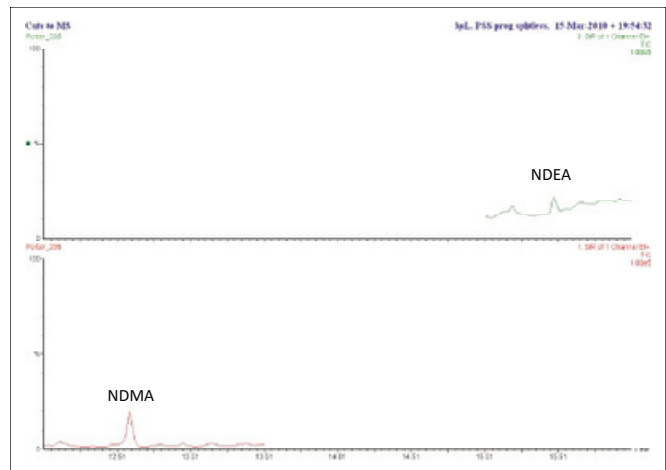


Figure 11. Analytical column SIR chromatography of an American porter ale extract.

Performance

Using these conditions, the performance of the system was checked using standard mixtures of known concentrations. Figure 12 and Figure 13 show the calibrations for NDMA and NDEA respectively over a range of effective sample concentrations from 0.05 ppb or less to 2.0 ppb. Excellent linearity for this method is demonstrated for both analytes.

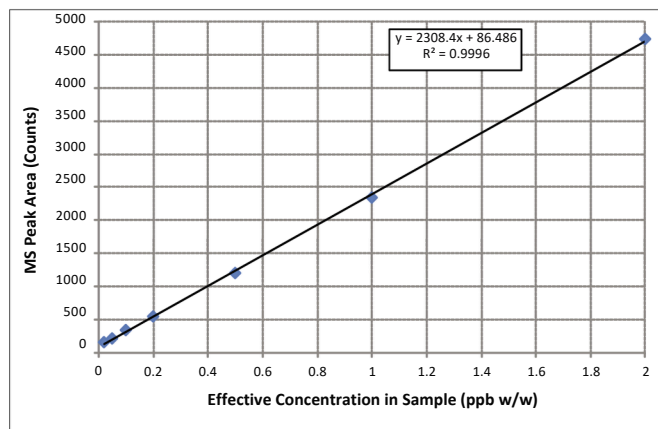


Figure 12. Calibration data for NDMA.

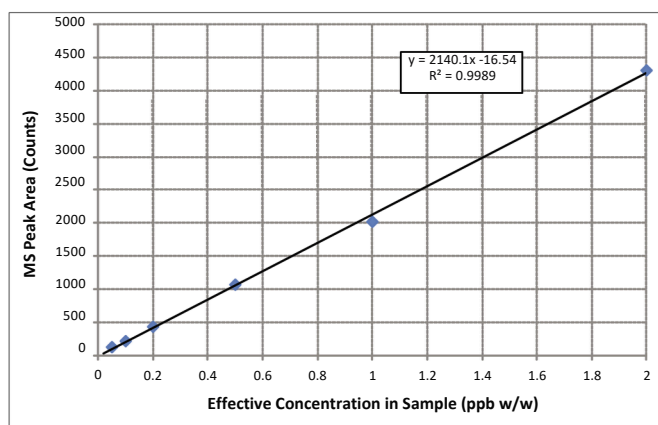


Figure 13. Calibration data for NDEA.

Results from Beer Samples

A variety of commercial beers were purchased from a local store and samples of home brewed beer (all grain recipes) were extracted and chromatographed using this method. The results are given in Table 2. All results are well within the USA FDA guideline of 5.0 ppb (CPG 510.600).

Table 2. Results obtained from various beer samples.

Sample	NDMA (ppb)	NDEA (ppb)
Oktoberfest (Germany)	<0.02	<0.0
IPA (USA)	0.09	5
English Bitter (Home Brew)	0.08	<0.0
Pilsner Lager (Home Brew)	<0.02	5
Imperial Stout (USA)	0.25	<0.0
Coffee Porter (USA)	0.39	5
IPA-B (USA)	0.08	<0.0
Pale Ale (Home Brew)	0.16	5
Paler Ale (USA)	0.14	<0.0

Making the Method More Robust

The use of an internal standard would improve the robustness of the method by reducing errors that result from partitioning the solvent into the sample or evaporation of the solvent during sample handling.

A deuterated form of NDMA was obtained and added at the 50 ppb level (equivalent to 5 ppb in the sample) to the dichloromethane extraction solvent. This NDMA-D6 compound had a primary (molecular) ion of m/z 80 in its mass spectrum so the SIR method was adjusted to also monitor the signal at this mass.

This deuterated compound is a perfect internal standard as it is chemically equivalent to the NDMA being determined and is subjected to the same sources of error.

Figure 14 shows chromatography of a standard mixture that included the internal standard.

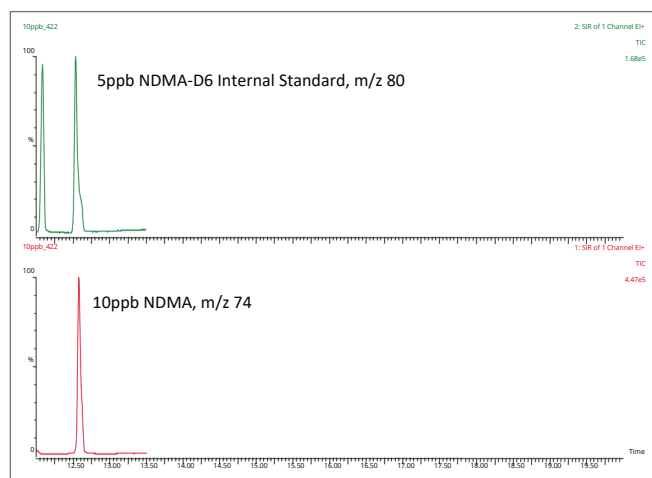


Figure 14. Chromatography of a standard mixture showing peaks for both NDMA and NDMA-D6 internal standard. Concentrations reflect those in the sample.

Figure 15 shows a calibration profile for the response ratio versus the concentration ratio for NDMA versus NDMA-D6. Excellent linearity is demonstrated at levels down to 0.1 ppb.

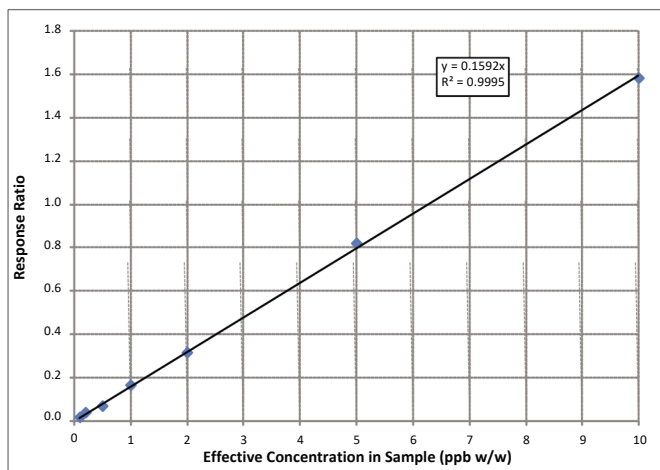


Figure 15. Calibration data for NDMA based on ratio to internal standard response.

Table 3 shows the instrumental precision obtained for multiple injections of the same standard mixture of NDMA and NDMA-D6. A relative standard deviation of 2.5% for the quantitative precision is excellent for this type of analysis – low level analytes in a very dirty sample matrix.

Table 3. Precision data obtained for NDMA using NDMA-D6 internal standard.

n	10
Mean Result (ppb)	2.27
RSD %	2.46

Another set of beer samples was selected for analysis. These were strong beers (apart from the American cream ale) with final alcohol concentrations of 7 to 10 ABV% so significant amounts of malt were used in their production. This is a severe test for the method as interferences from the sample matrix will be higher.

Figure 16 shows chromatography of an extract taken from one of the strong beer samples and Table 4 summarizes the results for all the samples tested.

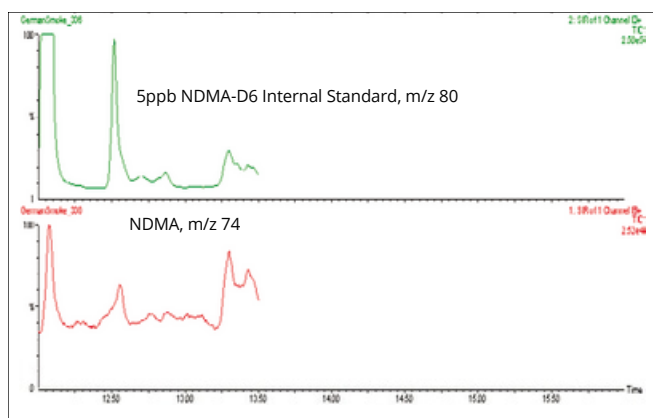


Figure 16. Chromatography of extract taken from Czech dark lager sample using DMA-D6 internal standard.

Table 4. Results from various beer samples.

Beer Sample	Polish	NDMA (ppb)
Porter	German	0.4
Rauchbier	Russian	3
Porter	Italian Birra	0.3
Blonde	USA Cream	2
Ale	Czech Dark	0.8
Lager		0

Extending the Method to Other Nitrosamines

Although the initial work focused on two nitrosamines, this method may be easily adapted to monitor other nitrosamines.

Figure 17 through Figure 19 shows how the method was extended to include nitrosopyrrolidine (NPYR) by simply adding another heartcut and extending the run time.

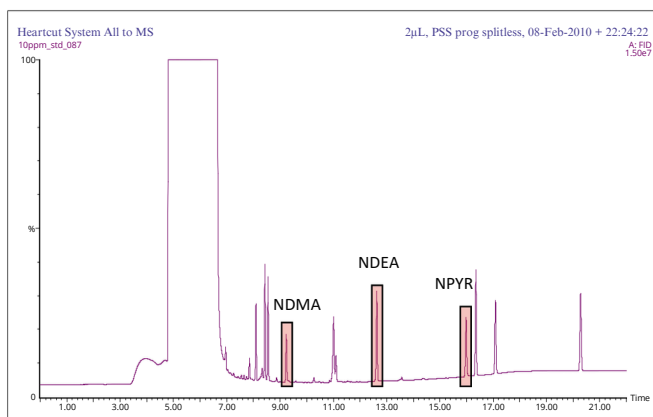


Figure 17. Precolumn FID chromatogram of 10 ppm standard mixture showing elution of NPYR and suggested additional heartcut times.

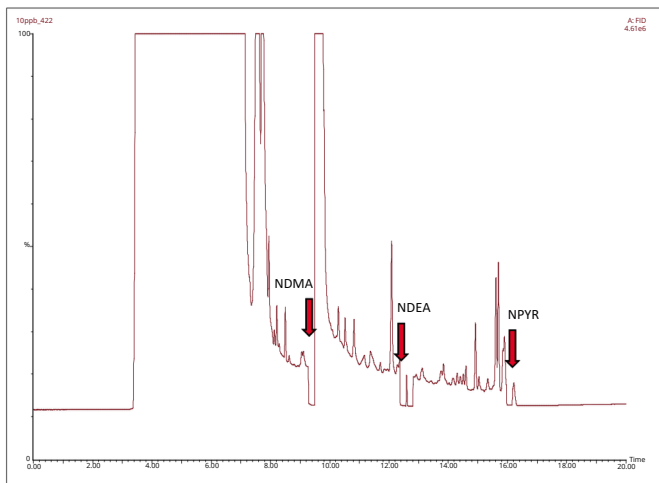


Figure 18. Precolumn FID chromatogram of beer extract showing cuts transferred to analytical column. Temperature program was extended to 225 °C and chromatographic run time was extended to 20 minutes.

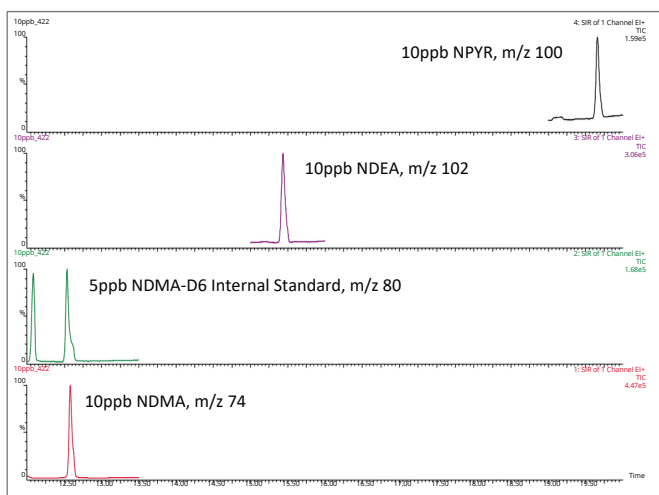


Figure 19. Analytical column SIR chromatography of spiked beer sample showing three analytes and the internal standard.

Conclusion

The need for methods to determine the levels of nitrosamines in beer has been a requirement for many years. Over time, a succession of different methods were developed yet need extensive sample clean-up, concentration procedures and exotic detection systems. The method presented in this application note uses a very simple and straightforward single-step extraction procedure with a minimum of solvent (1 mL). Sample extracts are much ‘dirtier’ than those produced in other methods but the combination of the heartcutting technique with SIR data collection on a mass spectrometer delivers the necessary selectivity without compromising the detection limits.

The combination of the heartcut technique and the fast cooling oven and PSS injector of the Clarus 680 enables the total chromatographic cycle time to be reduced to less than 20 minutes.

Examples have been shown in which nitrosamines in beer are seen at levels of 0.1 ppb or even lower.

Although this method is targeted towards beer analysis, it can be applied to the analysis of nitrosamines in other sample types.