

Environmental Analysis

De-iced, but not decontaminated

Due to their anti-corrosive properties, benzotriazoles are used in a wide variety of applications. These properties are based on their ability to form metal complexes that allow them to physically adhere to and form a water-resistant protective layer on copper, silver, and other metal surfaces. These advantages have increased their use as additives in aircraft de-icing fluids, dishwashing detergents, engine coolants, as well as in brake and hydraulic fluids. There is a downside, however: Benzotriazoles are classified as potentially hazardous to the health of aquatic organisms, and unfortunately, a study has found significant concentrations of these compounds in surface water, which would suggest that they are not removed by wastewater treatment plants. Benzotriazoles should therefore be monitored in natural water sources as well as in seafood. The preferred method of monitoring these compounds is to use solid phase extraction (SPE) combined with LC-MS/MS determination.

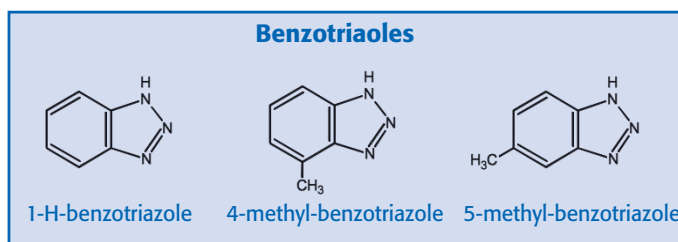
Several thousand liters of airplane de-icing fluid are needed to de-ice just one long-haul passenger aircraft. The harsher the winter weather conditions, the greater the amount of de-icing fluid needed. In recent years, many places have experienced more severe winters. The modern globalized economy cannot function without mobility and since a grounded aircraft leads to financial losses, there is no reason to expect that the use of de-icing fluids will decrease anytime soon. A well-known manufacturer of aircraft de-icing fluid recently announced plans for expanding and strengthening the supply network to reliably meet demand in Europe [1]. This in turn predictably leads to a recurrence of a phenomenon that scientists at the Helmholtz Center Geesthacht in Germany have studied in detail [2]: Come spring, large quantities of benzotriazoles are once again expected to appear in the German Bight, the Southeasternmost part of the North Sea. Benzotriazoles are transported into the North Sea by the Rhine in particular, but also via many other rivers.

According to the Helmholtz scientists' findings, many metric tons of benzotriazoles have already entered the North Sea via German wastewater flows and surface water runoff. These include 1-H-benzotriazole (BT) and the isomers 4- and 5-methyl-benzotriazole, which are known under the collective name tolyltriazoles (TT).

Benzotriazoles – how to perform the analysis?

As a contract analytical laboratory based in Bremerhaven, Germany, TeLA mainly supports local food and seafood suppliers. Our laboratory services include method development and method implementation for liquid chromatography combined with mass selective detection (LC-MS). In addition, we perform contract analysis within the fields of quality assurance and food safety monitoring. Since benzotriazoles are classified as toxic to fish and shellfish, they are of course highly relevant to our work – especially if they accumulate in fish and shellfish and pose a risk to consumers. Against this backdrop, we asked ourselves whether we could develop an automated procedure that would reliably and accurately determine benzotriazoles in water and fish tissue as per existing guidelines and

exceed the specified detection limit requirements. The chemistry of benzotriazoles and their remarkable solubility in water and polar solvents all point to HPLC as the most suit-



able technique to use for the analysis. This is also reflected in recent literature [2, 3]. We developed an automated LC/MS method (described below) based on work done by Weber et al. from the State Water Supply Association in Langenau (Germany) [3]. The Langenau scientists monitored 1-H-benzotriazole as well as 4- and 5-methyl-benzotriazole in river water without pre-concentration using LC-MS/MS based on a 100 µL sample injection into an Agilent Technologies 1200 Series HPLC system. The compounds were separated on a C18 reversed-phase column (Zorbax Eclipse XDB C18, 50 x 4.6 mm, 1.8 µm) using a water-methanol gradient with 0.1 % (v/v) formic acid. Detection was performed using an API 5000 Triple Quadrupole MS (AB SCIEX) with electrospray ionization (ESI) in positive ion mode (ESI⁺). Weber et al. specified 5 ng/L as the limit of detection (LOD).

As Wolschke et al. had done previously [2]; the Langenau scientists examined the pathways of benzotriazoles into ground and surface waters. Ultimately, their work led them to the conclusion that treated wastewater is the source. During a poster session at the 2012 Langenau Water Forum, the Langenau scientists explained that wastewater contains mainly tolyltriazoles, whereas in surface water and especially in groundwater, the 1-H-benzotriazole concentration is higher. Since the ratio between the concentrations of the two compound groups in ground and surface water is shifting towards higher tolyltriazoles concentrations, it was clear that waste water was entering the surface and ground waters.

According to Weber et al., the chromatographic determination of tolyltriazoles has shown that 4-methyl benzotriazole, enters the environment via waste water at concentration levels two to ten times higher than those of 5-methyl benzotriazoles. However, in surface water, the isomers were present in the same concentration range. Further, the

scientists pointed out that benzotriazoles can be removed from water quite efficiently using ozonation and filtration over activated carbon. However, benzotriazoles are not biodegradable [2]. Their health-related limit value is set to 100 ng/L (0.1 ng/mL).

Analytical Method

We developed our HPLC-MS/MS method for determining benzotriazoles using a 1290 UHPLC system and a 6460 Triple Quadrupole mass spectrometer – both from Agilent Technologies. Initially, spiked water samples were analyzed and method applicability was then evaluated by analyzing real samples (surface water, seawater and fish). Our goal was to improve the detection limit of 5 ng/L achieved by Weber et al. while maximizing sample preparation efficiency.

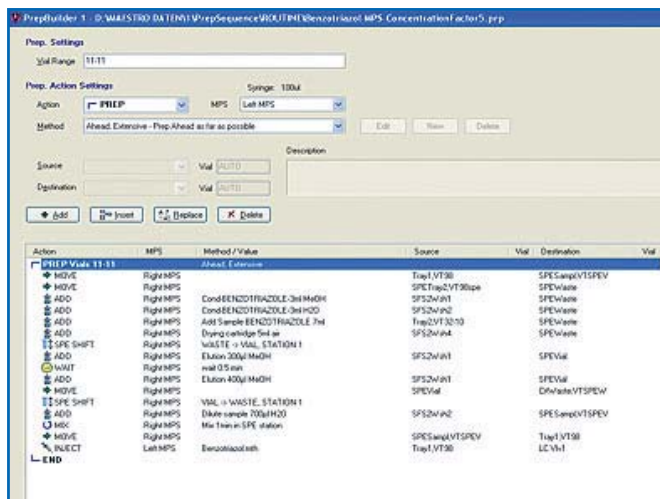
Sample Preparation

The analytes were extracted from water samples using automated solid phase extraction (SPE). SPE is used to clean-up samples in order to eliminate interfering matrix, which is often present in surface water, seawater, or food. The SPE process was automated using the GERSTEL SPE option configured on the GERSTEL MultiPurpose Sampler (MPS) – dual-head version. The dual-head version of the MPS features two separate robotic towers that can move independently. Having two robotic towers allows for the complete automation of both sample preparation and injection by having one tower equipped with the tools necessary for sample preparation and the other tower able to perform the sample injections. In this case, one tower was equipped with a gripper for moving SPE cartridges and at the same time allowed for accurate handling of large volumes of liquid while the second tower was equipped with a syringe suitable for injection of the final extracts into the HPLC system. (The sample preparation steps and Prepbuilder method are shown on page 12.)

LC-MS/MS Analysis

The LC-MS/MS analysis run time was approximately 20 minutes. Analytes were separated on a C18 reversed-phase column (Macherey & Nagel Nucleodur Sphinx C-18, 250 x 2.1 mm, 5.0 µm) using an eluent gradient consisting of 50 mM formic acid in

The MPS-SPE-LC-MS/MS analysis system used by TeLA GmbH for the determination of benzotriazoles.



SPE clean-up of surface water, seawater and fish samples was automated using the GERSTEL MPS (PrepBuilder method shown above, sample preparation steps below). The entire sample preparation and analysis took 20 minutes to perform. Parallel processing of sample preparation and LC-MS/MS analysis ensures maximum throughput. Using the MAESTRO software, the complete system including LC-MS/MS is conveniently operated using one integrated sequence table in combination with the LC MassHunter Software (Agilent Technologies).

water (A) and acetonitrile (B): 0.0-6.0 min 30 % B, 6.0-12.0 min 60 % B, 12.0-14.0 min 70 % B, 14.0-14.1 min 30 % B. Following the LC gradient, the column was then re-equilibrated to the starting conditions. An isocratic flow rate of 0.3 mL/min was used and the column temperature was controlled at 50 °C. Analytes

were detected using electrospray ionization (ESI) in positive ionization mode. Nitrogen at 320 °C was used as sheath gas in the Jet Stream ion source and the nebulizer gas pressure was set to 45 psi. A nozzle voltage of 500 V and a Jet Stream ion source temperature of 300 °C were maintained. Automated SPE sample preparation was synchronized with the LC-MS/MS analysis using the

Sample Preparation

Sample preparation steps including SPE performed using the GERSTEL MPS:

Condition the SPE cartridge with 3 mL of methanol

Rinse the SPE cartridge with 3 mL of water

Add 7 mL of sample to the SPE cartridge

Dry the SPE cartridge by applying positive displacement using 5 mL of air

Elute analytes using 300 µL of methanol

Wait for 30 seconds

Elute the analytes using an additional 400 µL of methanol

Discard the SPE cartridge

Combine the two eluates

Add 700 µL of water

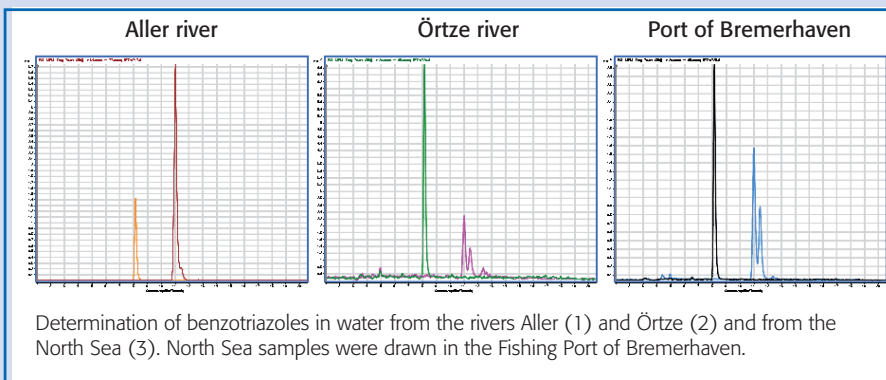
Mix the eluate-water mixture for one minute

Inject 50 µL of the eluate-water mixture into the LC-MS/MS system

All steps are performed automatically, over a period of approximately 15 minutes.

Determination of 1-H-benzotriazole in fish

	Sample 1	Sample 2	Sample 3	Recovery
Results using SPE Clean-up				
Cod, 50 µg/kg (spiked)	48.5	50.1	48.9	97 / 100 / 98 %
Cod, 20 µg/kg (spiked)	19.5	19.5	19.9	98 / 98 / 100 %
Cod, 10 µg/kg spiked	9.8	9.7	10.2	98 / 97 / 102 %
Cod, blank	< 0.1	< 0.1	< 0.1	
Results from direct injection of raw extracts				
Cod, 50 µg/kg (spiked)	41.8	41.5	45.3	84 / 84 / 91 %
Cod, 20 µg/kg (spiked)	16.0	16.8	18.4	80 / 84 / 92 %
Cod, 10 µg/kg spiked	6.7	6.6	7.0	67 / 66 / 70 %
Cod, blank	< 0.1	< 0.1	< 0.1	



Benzotriazole concentration (µg/L) in various German waters (Jan. 2012).

Sample	1-H-benzotriazole	4-methyl- and 5-methyl-benzotriazole
Aller	1639	4889
Örtze	59	22
North Sea Port	302	220

MAESTRO PrepAhead feature. This feature enables the GERSTEL MPS to begin the preparation of the next sample five minutes into each LC-MS/MS run to ensure that a sample is prepared and ready to be injected as soon as the LC-MS/MS system is ready for the next run. Using this „just-in-time“ strategy means that the LC-MS/MS is constantly analyzing samples and producing data, as opposed to having to wait for each sample to be prepared prior to injection.

Results and Conclusion

The automated SPE-LC-MS/MS method enabled us to determine benzotriazoles (1-H-benzotriazole) and tolyltriazoles (the isomers 4- and 5-methyl-benzotriazole) in

spiked surface water, river water, and North Sea saltwater samples. Analysis of benzotriazole calibration standards at concentrations of 2, 10, 20, and 200 ng/L in tap water resulted in linearity of > 0.995 and, with a moderate concentration factor of 5, a limit of quantitation (LoQ) of < 1 ng/L was realized. After SPE clean-up, no matrix effects were observed in any of the water samples investigated. We were thoroughly satisfied with the results. Since we achieved our first goal of significantly reducing the limit of detection reported by Weber et al., we subsequently analyzed samples taken in January 2012 from the Aller and Örtze rivers in the German state of Lower Saxony as well as North Sea saltwater samples from Bremerhaven harbor to precisely quantify the levels of benzotriazole contamination. Residues of the three benzo-

triazoles were found to be present in all three bodies of water.

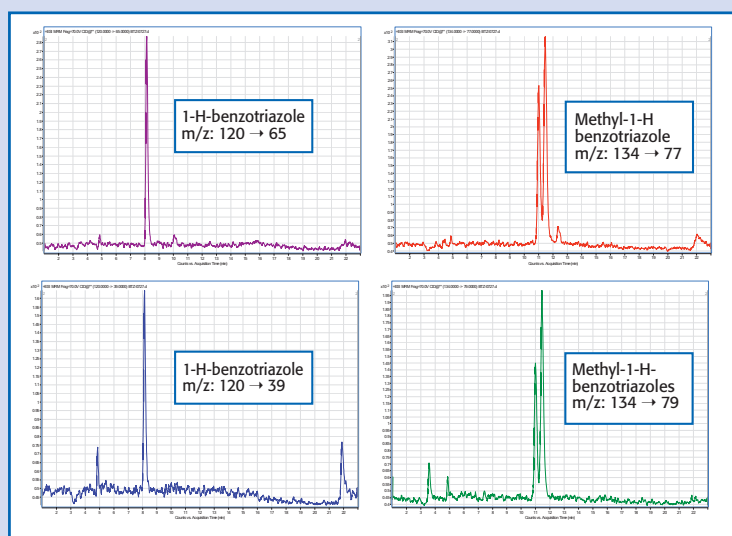
Following the water analysis, we subsequently determined 1-H-benzotriazole in spiked fish samples (cod) using two different methods:

1. SPE-LC-MS/MS, using automated SPE as a clean-up step and
2. Direct LC-MS/MS, by directly injecting raw sample extract.

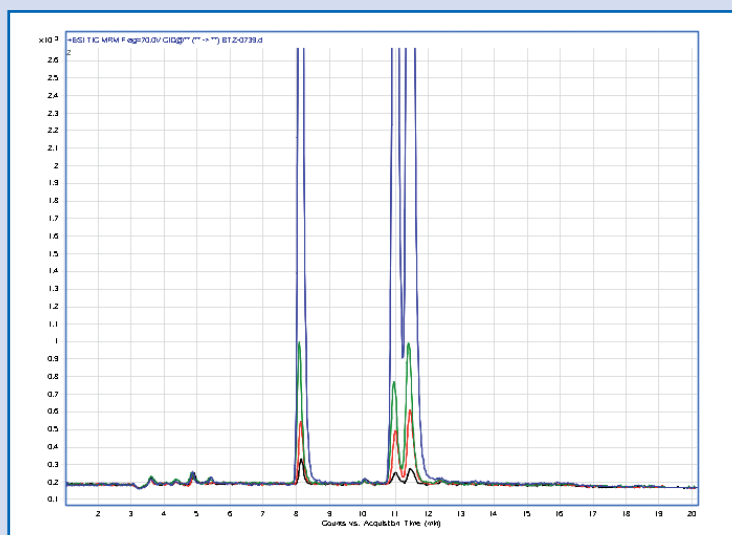
The results spoke for themselves: Sample clean-up using the SPE method produced analyte recoveries of almost 100 % with no indication of matrix effects. Direct analysis of raw sample extracts resulted in significant matrix effects and these matrix effects were shown to increase with decreasing benzotriazole concentrations.

Recovery rates ranged from 60 to 80 percent in the raw extract analysis. In summary, the study confirmed that the developed SPE-LC-MS/MS method works well in practice and is suitable for aqueous and food matrices. The limit of detection in water was < 1 ng/L. It was moreover shown that some rivers in Lower Saxony contain very high concentrations of benzotriazoles. The scope of this study unfortunately did not provide an opportunity to investigate the causes of this significant contamination.

Finally, the investigation demonstrated that automated SPE clean-up is highly suitable for determining benzotriazoles in complex food matrices – in this case cod – with high recovery rates and good linearity. A follow-up study designed to examine contamination in fish is currently underway.



Benzotriazole MS/MS traces, resulting from an aqueous standard solution (10 ng/L).



Chromatograms resulting from SPE clean-up and injection of tap water spiked with 2, 10, 20 and 200 ng/L benzotriazoles, respectively. Recovery: 100 %; coefficient of correlation (R^2) for the calibration curves: > 0.995; concentration factor: 5; limits of quantification (LOQ): < 1 ng/L.

Literature

- [1] CHEManager 16 (2011) 23-24
- [2] H. Wolschke, Z. Xie, A. Möller, R. Sturm, R. Ebinghaus: Occurrence, distribution and fluxes of benzotriazoles along the German large river basins into the North Sea. *Water Research* 45 (2011) 6259-6266
- [3] W. H. Weber, W. Seitz, W. Schulz: Poster presentation at the Langenauer Wasserforum 2012

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