The analysis of hydrophilic compounds in food

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Innovative chromatographic technologies and test methods offer the ability to develop comprehensive and sensitive methods to address unknown, unexpected emerging threats to our food supply.

The challenge is to make these methods robust enough to work in diverse matrices. Developing methods that are fast and accurate in a single matrix is rather straightforward, but in many cases, information can be lost if the samples are analysed with only one chromatographic selectivity.

Analysis of polar molecules in complex mixtures is problematic since the separation is difficult due to their inherently poor retention in traditional reversed phase chromatography. Screening methods based on complementary chromatographic selectivities, together with sensitive and specific detection techniques, can provide much more complete information.

For hydrophobic substances, reliable methods on C-18 reversed phase columns have been developed and leveraged for more than 30 years. Until recently, analysing hydrophilic compounds in a matrix such as food had been a significant undertaking, involving derivatisation and complex sample preparation procedure.

This article describes how hydrophilic interaction liquid chromatography (HILIC) in general, and in particular the bonded zwitterionic SeQuant ZIC-HILIC stationary phase (Merck Millipore, Darmstadt Germany), are used for food safety screening as well as for food constituent control. By employing these columns, food analysis laboratories can deliver more secure analytical results for polar and hydrophilic analytes whether LC-MS/MS or more traditional HPLC with detection by UV light absorption or ELSD (evaporative light scattering) is used.

HILIC technology

Analysis of polar molecules in complex mixtures is problematic since the separation is difficult due to their inherently poor retention in traditional reversed-phase liquid chromatography.

SeQuant ZIC-HILIC HPLC columns leverage buffered aqueous eluents rich in organic solvents such as acetonitrile. This mode of operation results in low column back-pressure allowing high speed separations, enhanced sensitivity when interfaced with mass spectrometry (MS), and simplified sample preparation schemes.

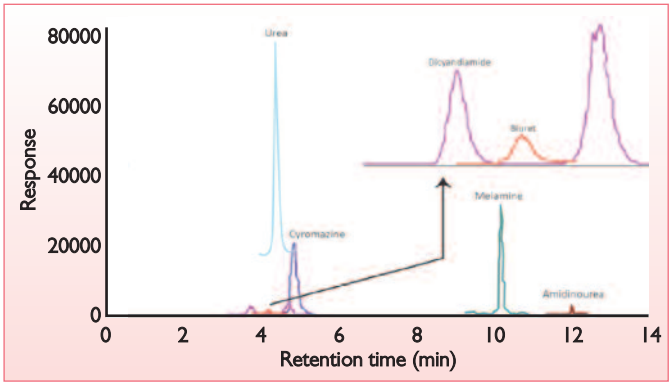
Separations are equally easy to develop on ZIC-HILIC columns as on traditional RP HPLC columns since the eluents are similar. A key difference is the effect of the water in the separation; in HILIC mode, water is the strongest solvent.

To increase analyte retention, the organic portion of the mobile phase needs to be increased, and the water or buffer portions decreased.

This will increase the hydrophilic partitioning into the water-enriched stationary phase, and thus increase the retention of the analyte.

The zwitterionic character of ZIC-HILIC with a 1:1 balanced charge, gives further possibilities for selectivity by weak electrostatic interactions between the stationary phase and the molecules that are separated. This interaction can be tuned by changing buffer type and concentration, typically in the interval 5-50 mM. Buffer pH is also an important parameter to control retention, but also here the thinking is opposite to in RP mode; more ionised compounds will be more hydrophilic and thus have more retention on ZIC-HILIC.

Fig. 1. Determination of nitrogen-rich adulterants in food using HILIC-MS/MS.



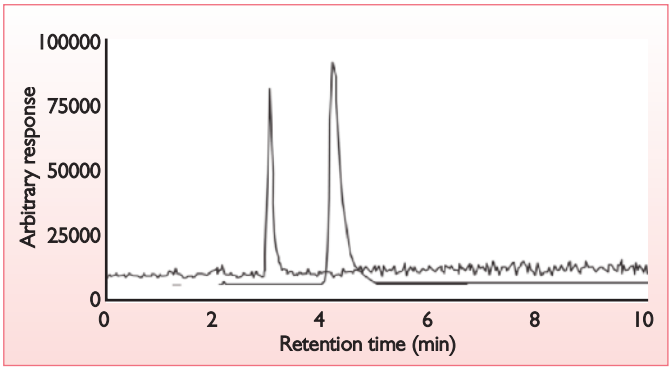


Fig. 2. Detection of mepiquat and chlormequat using HILIC-MS in positive mode.

Adulterants

The traditional standard technique for measuring protein content in food is the Kjeldahl method, a quantitative determination of total nitrogen content. The method consists of heating a substance with sulphuric acid, which decomposes the organic substance by oxidation to liberate the reduced nitrogen as ammonium sulphate.

In this step, potassium sulphate is added to increase the boiling point of the medium. Chemical decomposition of the sample is complete when the medium has become clear and colourless.

The solution is then distilled with sodium hydroxide which converts the ammonium salt to ammonia. The amount of ammonia present (hence the amount of nitrogen present in the sample) is determined by back titration. The Kjeldahl method is therefore a non-direct measurement of protein, hence it is possible to artificially enhance protein concentrations by adding nitrogen-rich chemicals.

Any chemical compound having a high percentage of nitrogen, by weight, has the potential to be used in economically motivated adulteration of protein-containing food products. In 2007, several brands of pet food were recalled in the US, EU and other countries in response to reports of renal failure in pets.

Initially, the recalls were associated with the consumption of mostly wet pet foods made with wheat gluten.

A month after the first recall, rice protein was also identified as being contaminated, causing kidney failure in pets.

On April 27, 2007, United States Food and Drug Administration (FDA) detained all vegetable proteins imported from China, intended for human or animal consumption. This included wheat gluten, rice gluten, rice protein, corn gluten, soy protein, proteins (includes amino acids and protein hydrolysate), mung bean protein, and variants thereof.

It was found that melamine (MEL)

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and cyanuric acid (CYA), a hydrolysis by-product of melamine, had been deliberately added to pet food, to 'fool' the Kjeldahl method for protein determination.

Melamine and cyanuric acid can cause serious health issues in humans and pets. While each is relatively innocuous, they can form a complex (MEL:CYA) which is nearly insoluble and crystallises in kidney tubules, leading to illness or death.

The determination of MEL and CYA, and the MEL:CYA complex is quite challenging because the compounds are very polar. A number of screening methods including gas and liquid chromatography have been developed for the determination of MEL and CYA in foods in recent years. Most of these methods require complex derivatisation and/or extraction procedures, and do not allow for simultaneous quantitation or provide sufficient identification confidence for regulatory action.

A ZIC-HILIC method for the determination of melamine and cyanuric acid in animal feeds was developed by scientists at the US FDA Center for Veterinary Medicine. The protocol was successfully employed for the analysis of melamine in commercial aquaculture, fish and shrimp feed.

In September 2008, several Chinese companies were involved in a scandal involving milk and infant formula adulterated with melamine, leading to kidney stones and other renal failure, especially among young children. By December 2008, nearly 300,000 people had become ill, with more than 50,000 infant hospitalisations and six infant deaths.

Until 2007, melamine had not routinely been tested in food, except in the perspective of plastic safety or insecticide residue. In October 2008, the US FDA issued new methods for the analysis of melamine and cyanuric acid in infant formulations.

Similar recommendations have been issued by other authorities, including the Japanese Ministry of Health, Labor and Welfare, all based on liquid chromatography – mass spectrometry (LC/MS) detection after hydrophilic interaction liquid chromatography (HILIC) separation.

After the Chinese scandal, the Joint Research Centre (JRC) of the European Commission established a website about methods to detect melamine.

In 2012, the FDA published a new analytical methodology to be considered as a powerful tool against economically motivated adulteration in protein-containing products.

The method is used to determine the presence of six nitrogen-rich compounds: cyromazine, dicyandiamide, urea, biuret, triuret, and amidinourea together with melamine. The method has been validated in skim milk, skim milk powder, soy protein, wheat flour, wheat gluten, and corn gluten meal matrices at concentrations as low as 1ppm. After acidic treatment of samples, acetonitrile is added to induce precipitation of proteins. Samples are then analysed using a SeQuant ZIC-HILIC column and tandem mass spectrometry (HILIC–MS/MS) using electrospray ionisation (Fig. 1).

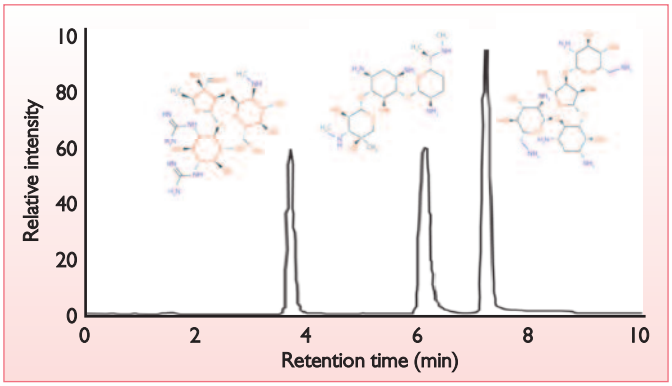


Fig. 3. Determination of streptomycin, gentamycin, and neomycin using HILIC-MS.

Pesticides and residues

Currently, there are over 500 pesticides listed with maximum residue limits by the EU. Many of these are difficult to analyse using traditional methods, including chlormequat and mepiquat, two very hydrophilic pesticides. Detection of both using HILIC-MS in positive mode is shown in Fig. 2. Dithiocarbamates is an important class of pesticides. They are widely used as fungicides mainly for preservation of fruit and vegetables. It is the class of pesticides that most often exceed the maximum allowed residue limits (MRL) in food imported to Europe.

Dithiocarbamates decompose instantly under acidic conditions forming carbon disulphide. The method currently used for dithiocarbamate analysis is a cumbersome hot acid digestion of the sample.

The released carbon disulphide is then analysed either by titration or UV/Vis absorbance. This procedure makes determination of individual pesticides impossible and also leads to problems when analysing crops rich in natural carbon disulphide like broccoli, cabbage, cauliflower or papaya. Much effort has been spent trying to develop LC-MS methods for dithiocarbamate determination, and for such an analysis, a pH stable column is essential since the pH has to be high during the entire analysis. A reversed phase method based on methyl iodide derivatisation has been proposed, but suffers from using a very carcinogenic derivatisation agent. A better approach is to use pH stable HILIC columns, for example SeQuant ZIC-pHILIC (pH range 2-12), where pre-column derivatisation can be omitted for separation of these hydrophilic molecules.

A ZIC-pHILIC LC-MS method was developed at the University of Hohenheim and later evaluated and validated by the German NRL for Pesticide Residues within the Federal Office of Consumer Protection and Food Safety. This method uses an eluent containing only acetonitrile and 10mM aqueous ammonia in a step gradient.

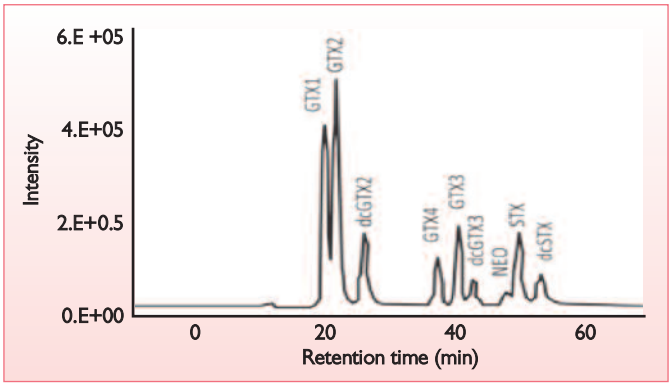
Antibiotics

Aminoglycosides are bactericidal antibiotics which have amino-modified sugar in their molecules.

This particular group of antibiotics is widely used as clinical and veterinary medicines to treat infections caused by Gram-negative or some Gram-positive bacteria, and are classified as bactericidal agents because of their interference with bacterial replication. These antibiotics can also cause varying degree of ototoxicity and nephrotoxicity. Overuse of antibiotics and exposure from the animal food are the two major routes attributed to the antibiotic resistance. Therefore, it is important to develop sensitive and reliable analytical methods for determining and monitoring aminoglycosides residuals in different sample matrices.

Aminoglycosides are normally very hydrophilic and carry several amino groups, which mean they are very positively charged at neutral pH condition. Fig. 3 shows the determination of streptomycin, gentamycin, and neomycin using HILIC-MS.

Fig. 4. Determination of paralytic shellfish toxins using HILIC.



Toxins

HILIC-based methods can also be used to assess food for the presence of toxins. With increasing sea temperatures the number of algal blooms is increasing especially in over-fertilised areas like the Baltic sea. These algae are consumed by shellfish filter feeders who accumulate toxins produced by dinoflagellates, diatoms and cyanobacteria.

Paralytic shellfish poisoning (PSP) toxins often referred to as saxitoxins, can reach lethal levels in shellfish. Detection of saxitoxin in mussels, clams and scallops frequently leads to closures of commercial and recreational shellfish harvesting.

PSP toxins are powerful sodium channel blockers that can cause respiratory insufficiency and death. An AOAC mouse bioassay used to determine PSP's only determines the total toxicity of the sample.

Chromatographic separation is used to identify individual toxins. Fig. 4 summarises an analytical method for the determination of paralytic shellfish toxins using HILIC. This method provides a sensitive and selective tool which can be employed with either a fluorescence detector or a mass spectrometric detector. The method can be used for various phytoplankton and the routine analysis of seafood.

There are a number of other toxins associated with algae and cyanobacteria such as anatoxins and β-N-methylamino-L-alanine (BMAA), where methods using the ZIC-HILIC column have been published.

During the last century methods of food production, processing and preservation have been the subject of continuous and substantial improvement. In parallel, there have been dramatic and profound developments regarding the analytical methods needed to safeguard the food supply and ensure acceptable food safety standards are met. New methods such as HILIC are enabling more effective and efficient analysis of polar compounds in complex samples. ■

References are available from the authors on request