

Simultaneous Determination of Melamine and Cyanuric Acid in Animal Feed via ZIC®-HILIC Chromatography - Mass Spectrometry

Melamine and Cyanuric Acid can be readily determined by ZIC®-HILIC chromatography over the 0.5 - 50 µg/g range using a single procedure. The assay does not require derivatization and can confirm the presence of the compounds for regulatory purposes.

Introduction

Melamine (MEL) and cyanuric acid (CYA) can cause serious health issues in humans and pets. While each is relatively innocuous, they can form a complex (M:C) which is nearly insoluble and crystallizes in kidney tubules, leading to illness or death. MEL and CYA were added to pet foods, presumably to increase the observed nitrogen content.

In recent years, a number of screening methods including GC and LC methods have been developed for the determination of MEL and CYA in foods. These require complex derivatization and/or extraction procedures, and do not allow for simultaneous quantitation or provide sufficient identification confidence for regulatory action. The isolation and separation of MEL, CYA and the M:C complex is quite challenging because the compounds are very polar.

HILIC (Hydrophilic Interaction Liquid Chromatography) is a powerful alternative for the separation of polar compounds that uses a hydrophobic mobile phase and a hydrophilic stationary phase. Solutes elute in order of hydrophilicity, the opposite order from RP, thus being especially useful for the separation of compounds that are poorly retained by RP.

ZIC®-HILIC chromatography is a unique form of HILIC where a bonded zwitterionic sulfobetaine group acts as the interactive layer. The low reactivity and zwitterionic properties of the group makes the ZIC®-HILIC a logical choice for the separation of polar compounds. In this note, we describe the development and validation of an analytical method for the determination of MEL and CYA in animal feeds developed by scientists at the US FDA Center for Veterinary Medicine.

Experimental

Samples, Reagents and Eluants: Reagents and eluants were obtained from commercial sources and used as received. Powdered feeds were used as is, and pellet feeds were ground to a coarse powder with a food blender.

Sample Preparation: Aqueous Formic Acid (2.5% v/v, 14 mL per 2 g powder) was added to the sample and homogenized to a suspension. The suspension was sealed, sonicated and centrifuged. After centrifugation, the aqueous extract was forced through a 0.22 µm PVDF filter. 50 µL of the supernatant was mixed with 950 µL of acetonitrile and centrifuged before analysis.

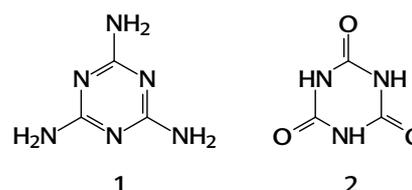


Figure 1: Melamine [MEL] (1), Cyanuric Acid [CYA] (2)

Separation/Detection: Separation was performed via an Acquity HPLC (Waters, Milford MA) with a ZIC®-HILIC column, 150 x 2.1 mm, 5 µm (Merck SeQuant, Umeå, Sweden), A gradient mobile phase was employed (A=acetonitrile +0.1% aqueous formic acid (19+1), B= acetonitrile + 20 mM aqueous ammonium formate (1+1) from 100 % A to 25% A in 9 min at a flow rate of 0.4 mL/min with a total run time of 14 min. Detection was performed with a Quattro micro triple quadrupole mass spectrometer (Waters) using the parent ions, the parent ion transitions 128→85 and 128→42 (-ESI) for CYA and the 127→85 and 127→68 (+ESI) transitions for MEL.

Results

Figure 2 presents an overlay of chromatograms from wheat flour. The control flour was fortified at 1 µg/g, equivalent to standards at 7 ng/mL actual. Similar chromatograms were obtained for swine feed, pelleted fish feed and other grain based animal feeds.

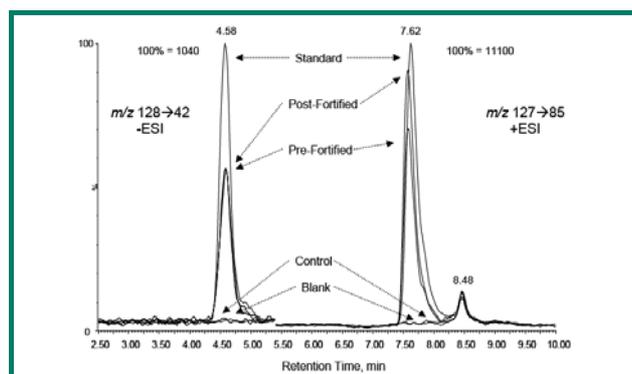


Figure 2: Overlay of five analyses of wheat flour: solvent blank, control flour, control flour fortified at 1 µg/g MEL and CYA, extract of control flour post-fortified at 1 µg/g equivalent, and standards in solvent at a concentration equivalent to 1 µg/g in flour (7 ng/mL actual). (reprinted from reference with permission from John Wiley Sons Ltd.)

Validation

Applicable concentration range

The concentration range for which the analytical protocol is valid is the limit at which MEL and CYA remains fully dissolved in solvent A (the injection solvent). Figure 3 presents standard curves for MEL and CYA combined in solvent A. The response falls above approximately 750 ng/mL for both compounds in the mixture, probably due to the formation of the M:C complex. Small peaks (with a S/N <5) were observed in some extracts at the retention time for CYA, but did not match the product ion ratio of the standard.

The range for quantitation range for routine analyses was limited to 3.6-360 ng/mL in the injection solvent (equivalent to 0.5 to 50 µg/g in feed samples), to avoid the higher concentration where the M:C complex might form. Higher concentrations were diluted into the working range.

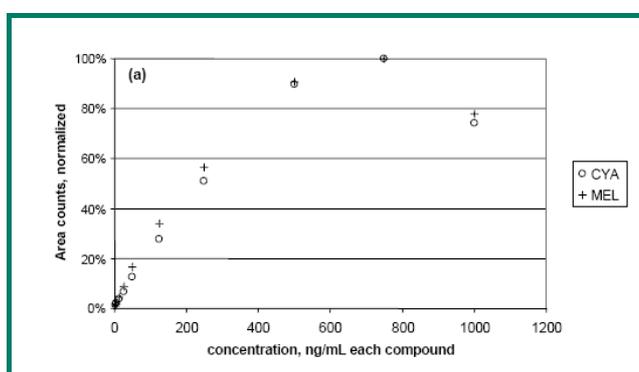


Figure 3: Standard Curves for CYA and MEL in Solvent A (reprinted from reference with permission from John Wiley Sons Ltd.)

The method was applied to a broad range of grain-based feed types and ingredients, including wheat flour, wheat gluten pelletized hog feed, granular poultry feed, granular swine feed, pelletized fish feed (trout, tilapia), pelletized shrimp feed. The recovery was found to be excellent (Table 1). The low recovery of MEL in flour is believed to be due to the relatively low salt level in flour, which led to broader peaks with tailing. This observation suggests that the interaction of MEL on the column varies with the ionic strength of the mobile phase.

The method was used to identify MEL in commercial fish and shrimp feeds and found levels ranging from 1.0 ppm to >100 ppm. No sample contained > 0.5 ppm CYA.

Feed	CYA			MEL		
	% Recovery	SD	cv	% Recovery	SD	cv
gluten	99	7.5	7.6	95	3.3	7.9
hog	100	3.0	3.0	95	2.9	6.2
flour	100	4.7	4.7	76	1.2	5.0
swine	99	5.2	5.3	92	2.7	7.0
poultry	96	2.6	2.7	91	2.3	7.7
fish	95	4.7	4.9	92	4.6	1.8

Table 1 Recovery of CYA and MEL from Various Feeds

Conclusions

A zwitterionic ZIC®-HILIC column and a triple quadrupole mass spectrometer were employed to provide a validated analytical protocol for the determination of MEL and CYA acid in a broad range of animal feeds. The zwitterionic stationary phase, which is designed to separate very polar compounds is an superb approach to separate the compounds of interest as it enables the analyst to perform the desired separation without the need to derivatize the compounds of interest.

The protocol was successfully employed for the analysis of spiked compounds of interest in animal feeds in the range from 0.5 to 50 µg/g and was able to detect MEL in commercial aquaculture, fish and shrimp feed. The method was developed by FDA scientists for regulatory analysis of animal feed, and it appears that the same method is applicable to analysis of powdered infant formula and other dairy-containing food products or ingredients, *see hyperlink below*.

About ZIC®-HILIC Chromatography

The ZIC®-HILIC stationary phase is based on the covalently bonded permanent zwitterionic sulfobetaine group shown in Figure 4. It is available with a silica support in 3.5, 5 and 10 µm particle sizes in various column dimensions from capillary to semi-preparative (75 µm up to 20 mm ID). In addition, it is available with a polymeric support in 5 µm particles (ZIC®-pHILIC). Merck SeQuant also publishes the tutorial booklet *A Practical Guide to HILIC*, which is available free.

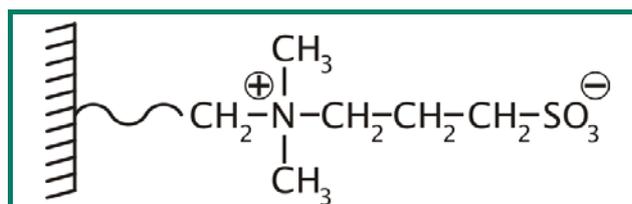


Figure 4: The Bonded Zwitterionic Sulfobetaine Group of ZIC®-HILIC.

About Merck SeQuant

Merck SeQuant is a Swedish company that develops a broad range of innovative products for separation and purification of complex samples via chromatography. The company was founded in 1987 by researchers from Umeå University. Products include ZIC®-HILIC columns for HPLC, Membrane Suppressors and Suppressor Regeneration Systems for Ion Chromatography, which are marketed and supported around the world by a series of distributors.

Reference

This application note is condensed from the paper "Simultaneous determination and confirmation of melamine and cyanuric acid in animal feed by zwitterionic hydrophilic interaction chromatography and tandem mass spectrometry" by Heller, D. N., and Nochetto, C.B. .; published in Rapid Communications in Mass Spectrometry (2008), 22, 3624-32. Figure 1 and 2 are reprinted with permission from John Wiley Sons Ltd.

<http://www.cfsan.fda.gov/~frf/lib4421.html>

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