COGENT
Bidentate C18™
Diamond Hydride™

Comparing 3 Methods to Avoid Sample Cleanup in HPLC

Histamine
Limonin
Folic Acid

Extended Application Note

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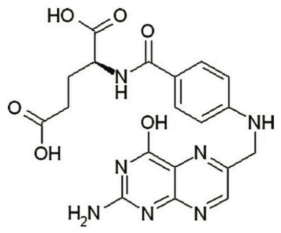
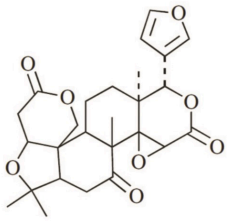
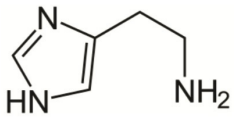


Folic Acid



Limonin



Histamine

Figure 1

Method 1:

A: DI H_2O/ 10 mM ammonium formate
B: 90% Acetonitrile/ 10% 10mM ammonium formate

Time (min.)	%B
0	100
10	90
19	50
20	100

Flow Rate: 0.5 mL/min
Detection: UV 284 nm

Introduction

Food safety in the United States is governed by numerous federal and state regulatory agencies, primarily the Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA). Determining that the specified level of a given ingredient is present in a food product is crucial for ensuring quality and consistency for the consumer.

There are several widely used analytical techniques for food and beverages. Microbiological-based assays are a common choice, but their main disadvantage is that they are specific only to one type of analyte. In chromatographic analyses on the other hand, the same HPLC column may be used to obtain specificity for a variety of different analytes of disparate structural characteristics.

With chromatographic approaches, one of the main obstacles to overcome for these types of samples is how to deal with interferences from the matrix. Unlike a pharmaceutical formulation for instance, foods and beverages are complex and contain many other compounds besides just the analyte. These compounds can present problems by building up on the column, co-eluting with analytes of interest, and so on. Often Solid Phase Extraction (SPE) or a similar sample cleanup technique is used to remove these interferences. SPE can be time-consuming though, and it is another step in the analytical process that can contribute to error.

Use of the Cogent Bidentate C18™ and Diamond Hydride™ columns can avoid the need for sample cleanup steps. In the Aqueous Normal Phase (ANP) mode, compounds that would retain strongly in reversed phase elute near the solvent front while polar analytes of interest may be retained. As another solution, a wash step can be added between runs. Finally, LC-MS can provide additional specificity through extracted ion chromatograms. These principles are discussed using analyses of folic acid in cereal, limonin in orange juice, and histamine in red wine (**Figure 1**).

Experimental

Materials

Limonin, histamine, methotrexate, and formic acid LC-MS ultra-grade were from Sigma–Aldrich (St. Louis, MO, USA). Folic acid and sodium L-ascorbate were obtained from Calbiochem–Behring Corp. (La Jolla, CA, USA). Ammonium formate (>97%) was from Matheson Coleman & Bell (Norwood, OH, USA). Deionized water (DI H_2O) was prepared on a Milli-QTM purification system from Millipore (Bedford, MA, USA). Acetonitrile (HPLC grade) was obtained from GFS Chemicals, Inc. (Powell, OH, USA).

Instrumentation

For Methods 1 and 2, a Hewlett–Packard (Palo Alto, CA, USA) 1100 HPLC system consisting of an autosampler, degasser, binary pump, and variable wavelength UV detector was used. The system was interfaced with Agilent Chemstation (Santa Clara, CA, USA) software. An Agilent (Little Falls, DE, USA) 1200SL Series LC system, including degasser, binary pump, temperature-controlled autosampler, and temperature- controlled column compartment was used for Method 3.

Method 2:

59% A: DI H_2O/ 0.1% formic acid
41% B: Acetonitrile/ 0.1% formic acid
Flow rate: 1.0 mL/min
Detection: UV 207 nm

Method 3:

A: DI H_2O/ 0.1% formic acid
B: Acetonitrile/ 0.1% formic acid

Time (min.)	%B
0	80
5	10
7	10
8	80

Flow Rate: 0.4 mL/min

The mass spectrometer system was an Agilent (Santa Clara, CA, USA) Model 6210 MSD TOF with a dual sprayer electrospray source (ESI). The analytical columns were as follows:

Columns

Method 1: Cogent Diamond Hydride™ 4µm 100Å, 4.6 x 75mm

Method 2: Cogent Bidentate C18™ 4µm 100Å, 4.6 x 150mm

Method 3: Cogent Diamond Hydride™ 4µm 100Å, 2.1 x 150mm

Samples Preparation

Method 1: A portion of fortified cereal was ground using a mortar and pestle. A 20.0 g portion of the ground cereal was added to a beaker with a stirbar. Subsequently, 500 mL of a solution consisting of DI H_2O + 10 mM ammonium formate, 0.5 mg/L methotrexate, 0.05% (w/v) sodium L-ascorbate, and 12 mM NH3 was quantitatively added to the beaker. The beaker was then covered with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). The mixture was stirred for 3 h and sonicated for 30 min. Subsequently, a portion of this mixture was centrifuged at 10,000×g for 4min. The supernatant was then collected and filtered through a 0.45µm nylon membrane HPLC filter (MicroSolv Technology Corp.) prior to HPLC-UV injections.

Method 2: Orange juice was spiked with 50.0 ppm limonin. A portion was centrifuged at 7,000 g for 10 min using a DuPont (Newton, CT, USA) Sorvall® GLC-2B centrifuge. Then the supernatant was extracted and filtered through a 0.45µm nylon syringe filter into an autosampler vial.

Method 3: Red wine sample was filtered with 0.45µm nylon syringe filter and diluted 1:5 with 50/50 solvent A/solvent B diluent.

Results and Discussion

Method 1: Folic acid is an important B vitamin which is fortified in cereals, juices, and other products. It is very polar though and analysis by reversed phase is complicated by interferences from the sample. In contrast, this method for cereal extracts shows how use of ANP can produce excellent retention of both folic acid (1) and a spiked internal standard methotrexate (2) with no other peaks nearby. SPE was not required here as most of the sample peaks eluted near the solvent front (**Figure 2**).

Peaks:

1. Folic Acid
2. Internal Standard

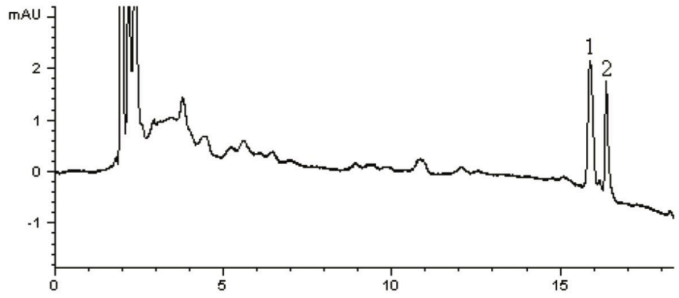


Figure 2

Method 2: Limonin is a bitter compound that should be present below a certain threshold in citrus juices to ensure satisfactory taste for consumers. It is relatively hydrophobic and therefore reversed phase may be called for in this case rather than ANP. Consequently this analysis used the Bidentate C18™ column to achieve separation.

Here the main issue was that strongly hydrophobic compounds in the orange juice samples would build up on the column and slowly elute in subsequent runs as extremely broad bands (peak width ~4–5 min). To avoid the use of SPE sample cleanup in this case, a wash step was incorporated into the injection sequence. The wash consisted of strongly eluting reversed phase conditions (95% acetonitrile/ 5% DI water/ 0.1% formic acid) to get the contaminants to come off of the column rapidly.

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It was observed that the wash did not need to be performed after every run; rather, every six injections was found to be sufficient. Data is shown for runs from the injection sequence with the wash step added (**Figure 3A**) and with the washing omitted (**Figure 3B**). This ghost peak could potentially interfere with quantitative integration for analyte peaks.

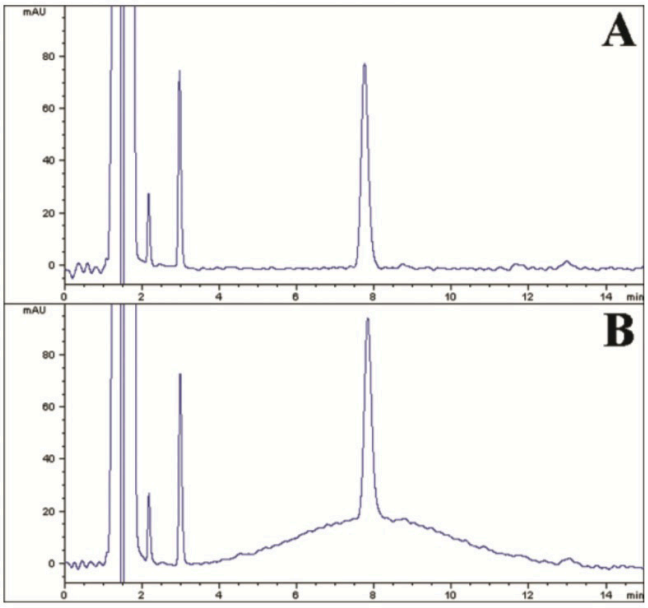

Figure 3

Method 3: A third way that sample cleanup steps can be avoided is through the use of more sophisticated detection methods such as LC-MS. In this method using the Diamond Hydride™, peaks that may co-elute chromatographically with the analyte of interest can be resolved by their unique m/z values in the Extracted Ion Chromatograms (EIC). This is shown with analysis of histamine in red wine, where there is a major difference between the complex Total Ion Chromatogram (TIC, **Figure 4A**) and the clean EIC for histamine (**Figure 4B**).

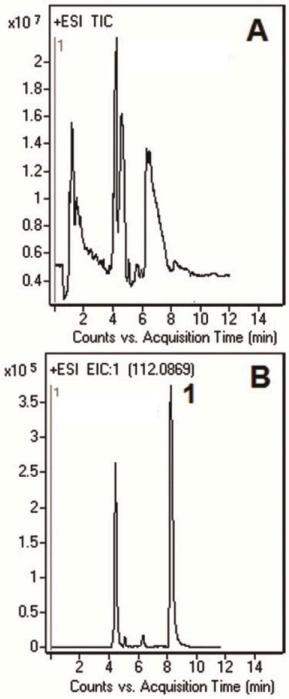

Figure 4

1. Histamine

Conclusion

The Bidentate C18™ and Diamond Hydride™ columns offer excellent separation applicability for various analytes in food and beverage products. Complicated sample prep techniques such as SPE can be avoided using ANP chromatography, wash steps, and/or LC-MS technology.