SeQuant

MS Detection of Homocysteine, Methylmalonic Acid, and Succinic Acid Using HILIC Separation on a Covalently Bonded Zwitterionic Stationary Phase

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Rapid, simple, rugged, yet sensitive are prerequisites often sought for analytical techniques used in clinical laboratories. The vast amount of samples in difficult matrices, i.e. urine, plasma, serum etc., requires techniques on which uncomplicated methods can be developed and used, preferably unmanned, with little or no downtime. A typical example is the quantification of homocysteine, methylmalonic acid, and succinic acid; see Figure 1. Homocysteine and methylmalonic acid (MMA) are diagnostic markers for B_{12}-deficiency, while succinic acid is a physiologically abundant isomer to MMA that often interfere with the quantification of the former compound. Over the years, yet in vain, different approaches using various analytical techniques have been tested with the overall goal to allow quantitation of all three compounds within the same chromatographic run.

Today there is a feasible alternative, hydrophilic interaction liquid chromatography (HILIC) combined with single stage mass spectrometric detection (MS). HILIC is a separation technique suitable for polar and hydrophilic compounds, and it utilizes an eluent containing a high content of water miscible organic solvent (e.g. acetonitrile) to promote hydrophilic interactions between the analyte and a hydrophilic stationary phase. Although there are quite a few HILIC phases commercially available, none is truly comparable with the ZIC®-HILIC and ZIC®-*p*HILIC phases. The highly polar zwitterionic columns provide a unique environment particularly capable of solvating polar and charged compounds, which enables high performance HILIC separations. The zwitterionic stationary phase, see Figure 2, can interact with charged analytes via weak electrostatic interactions, and in practice, this provides the chromatographer with a larger degree of freedom when choosing among buffer salts and ionic strength in method development, thus making the column an ideal choice for LC–MS analysis. This particular application note illustrates advantages when combining efficient separation with a sensitive detection principle, and is exemplified by an isocratic HILIC separation of homocysteine, methylmalonic acid, and succinic acid.

ZIC®-HILIC and ZIC®-*p*HILIC Columns

The ZIC®-HILIC columns are silica based stationary phases with either 3.5, 5, or 10 µm particle size, while the ZIC®-pHILIC columns are polymer based stationary phases with 5 µm particle size, yet both having a sulfobetaine type zwitterionic functionality, see Figure 2.

Experimental Conditions

Column: ZIC®-HILIC 50 × 4.6 mm, 5 µm

UV

Column temp: RT
Mobile Phase: acetonitrile/ammonium acetate (100 mM, pH 6.8); 70/30 (v/v)
Flow-rate: 1.5 mL/min
Detector: UV @ 206 nm (UFS 1.0 V)
Injection volume: 5 µL of test solution in mobile phase

MS

Column temp: 30 °C
Mobile Phase: acetonitrile/ammonium acetate (100 mM, pH 6.8); 75/25 (v/v)
Flow-rate: 1.0 mL/min
Split: 100 µL/min to MS

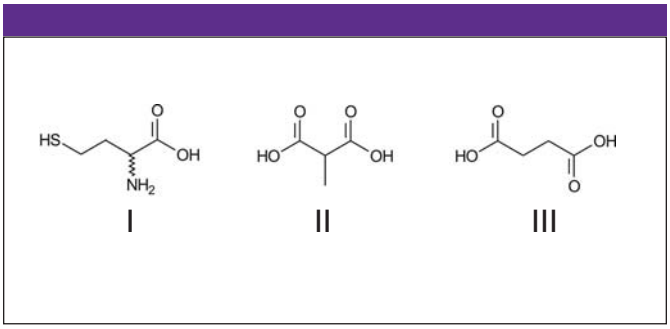

Figure 1: Structures of homocysteine (I), methylmalonic acid (II), and succinic acid (III).

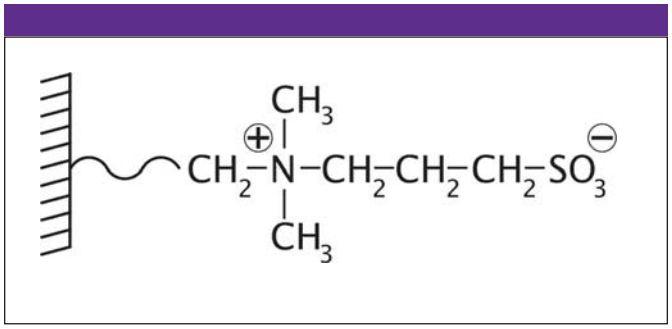

Figure 2: Schematic representation of the ZIC®-HILIC stationary phase.

Detector: MS, ESI in positive mode
Capillary Voltage: 3000 V
Fragmentor: 150 V
Mass range: 50-200 *m/z*
Injection volume: 5 µL of 0.1 mg/mL of each compound diluted in mobile phase
Sample: In elution order; homocysteine, methylmalonic acid, and succinic acid all dissolved in mobile phase.

Results

Method development is commonly carried out using UV detection, due to its ease of use and robustness, but the technique often lacks the sensitivity needed to allow quantification at relevant physiological levels. Herein it is illustrated that provisional optimal separation conditions can be established via UV-detection, see Figure 3a, and then easily transferred, and slightly modified to better fit MS detection in order to gain sensitivity. Baseline separation for all compounds can be achieved within 90 s, and that the compounds elute with a k' between 1.4 and 3. Worth noting is the dip in between homocysteine and methylmalonic acid. The rationale for the phenomena is a combination of low detection wavelength and a slight mismatch in buffer concentration between the sample and the mobile phase. When transferring the separation from the LC–UV to the LC–MS instrumental set-up, it became evident that the ionic strength was compromising the detection, and that a higher efficiency was needed in order to compensate for additional extra-column band-broadening effects between the column and the detector. Lowering the flow rate (from 1.5 to 1.0 mL/min) and the aqueous portion in the mobile phase (from 30 to 25 volume-%), and slightly decreasing the ionic strength, sufficient separation efficiency was reached, as seen in Figure 3b. An overall ionic strength of 25 mM ammonium acetate is typically too high for optimised MS sensitivity, yet for the particular application it is a necessity from a chromatographic perspective. Using the provisional MS compatible conditions, clinical relevant concentration levels can easily be quantified, however by switching to selected ion monitoring and optimising typical mass spectrometer parameters such as, capillary voltage, nebulizer gas flow, drying gas pressure and drying gas temperature, the sensitivity may improve by a factor of 10–50 depending on the fragmentation.

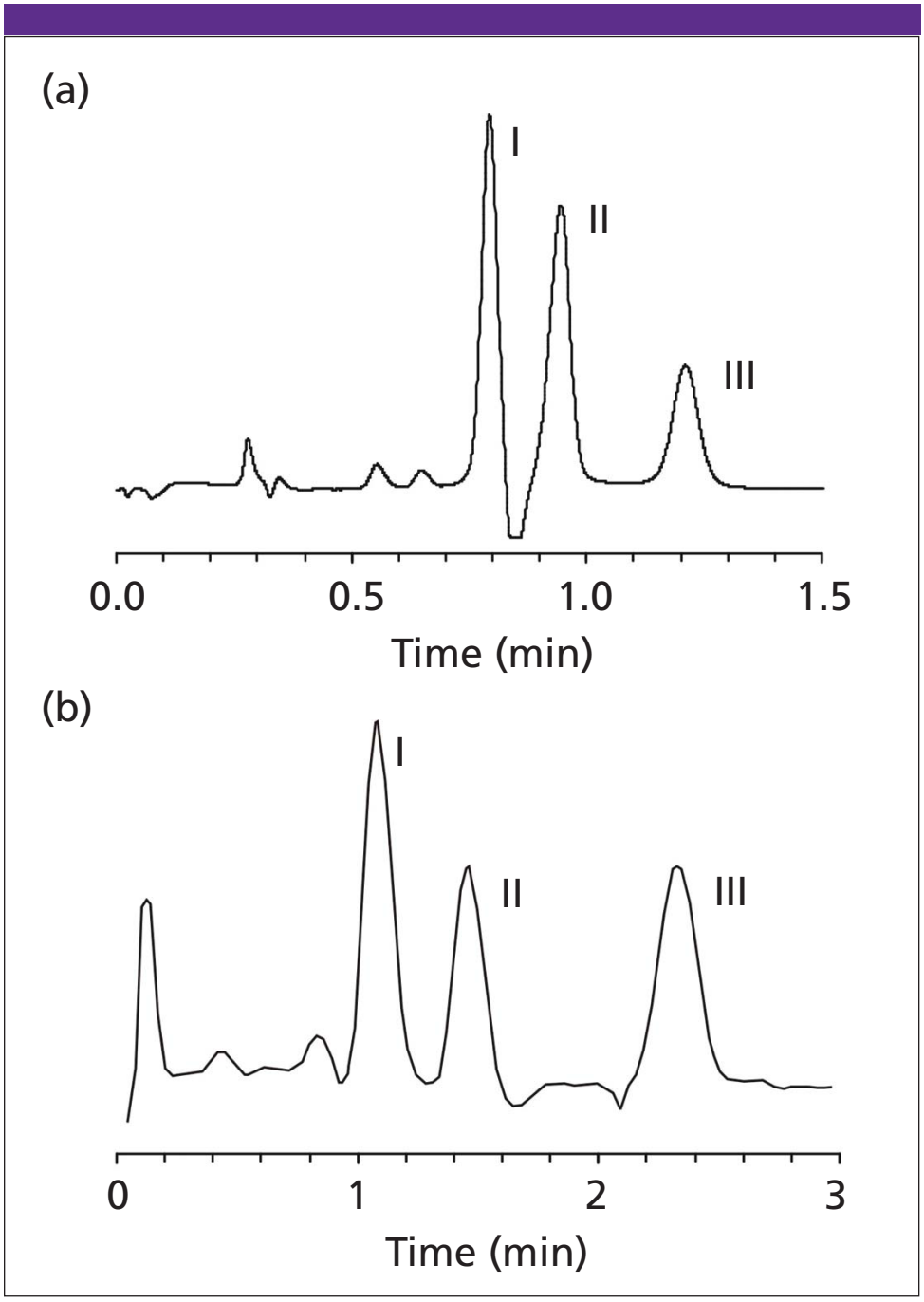

Figure 3: Chromatograms of the separation of (in elution order) homocysteine, methylmalonic acid, and succinic acid, using detection by (a) UV and (b) MS, respectively.

Conclusion

The ZIC®-HILIC column is indeed a suitable tool for separation of homocysteine, methylmalonic acid, and succinic acid. Combined with MS detection, physiological relevant concentration can easily be quantified with the possibility of processing up to 20 samples per hour.

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