

LC/MS Analysis of Various Hydrophilic Compounds Using a Polymer-Based Amino Column - Shodex™ HILICpak™ VG-50 2D

Introduction

Components of pharmaceutical and food products often include highly polar compounds. The compounds are slightly retained under reversed phase mode. To analyze these samples, methods using pre-column derivatization or the addition of an ion-pair reagent to the eluent are often applied. Drawbacks include a complicated and long process of the derivatization and increased background noise caused by the ion-pair reagent residues on the column and the flow-lines.

HILIC columns with amino functional groups are often used to prevent the separation of anomers; however, one drawback of the amino functional group is the low recovery rate of reducing sugars, such as glucose and mannose. Reducing sugars are able to adhere to the packing material through the formation of a Schiff base, and must be hydrolyzed with acid for removal. The base of Shodex HILICpak VG-50

packing material is polyvinyl alcohol with a modified tertiary amine (Fig. 1). Due to the novel packing material, this column series is able to achieve a high recovery rate of saccharides. Moreover, column bleeding (elution of column packing material and related debris that is often observed with silica-based amino columns is rarely found with the HILICpak™ VG-50 series columns, and consequently the related problems of increased background and/or ion suppression in MS are

less likely to occur. Another advantage of this series over silica-based amino columns is the HILICpak™ VG-50 series columns can be used under alkaline conditions (pH range of 2-13). Allowing for high sensitivity analysis of saccharides and acids using negative mode in ESI-MS. Anionic compounds, such as organic acids, tend to be retained in the column when previously available methods are used. Using alkaline conditions with the HILICpak™ VG-50, anionic compounds easily elute and make it possible to analyze organic acids.

This application introduces the results of not only saccharides, but the simultaneous analysis of saccharides, organic acids, and amino acids using a semi micro size column, Shodex™ HILICpak™ VG-50 2D, with LC/MS alkaline gradient conditions.

Experimental

Shimadzu Nexera / LCMS-8030 Plus system was used with Shodex™ HILICpak™ VG-50 2D (2.0 mm I.D. x 150 mm; particle size 5 µm; pore size 100 Å). High pressure linear gradient with eluents of (A) either 0.1% or 0.5% ammonia water and (B) acetonitrile were used. The flow rate was set at either 0.2 or 0.3 mL/min. The column temperature was set at 30, 40, or 60 °C. ESI was used as a means of ionization and SIM or MRM mode was used for the detection. Specific analytical conditions used for each analysis will be mentioned with their results. It should be noted that the pH of 0.5% ammonia water is about 11.5. The LC/MS system used in the experiment was durable against the alkaline condition up to pH 13.

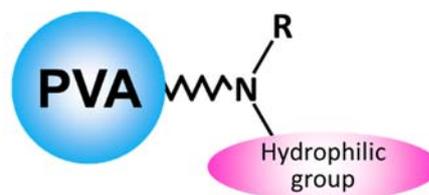


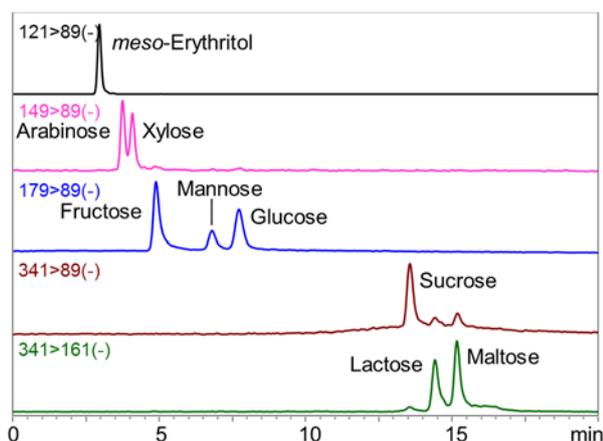
Fig.1 A schematic diagram of VG-50 base material

Results and Discussion

1. LC/MS analysis of sugars

1-1. Neutral saccharides

Fig. 2 shows the chromatograms for *meso*-erythritol, arabinose, xylose, fructose, mannose, glucose, sucrose, lactose, and maltose. Gradient elution of 0.1% ammonia water / acetonitrile was used. The neutral saccharides analyzed in this experiment can also be separated using water / acetonitrile and results with similar resolution. However, the addition of ammonia (i.e., analyzing under alkaline conditions) promotes deprotonation during ESI, increasing the sensitivity of negative ion detection. The peak height observed using ammonia water / acetonitrile eluent was three times higher than that of using water / acetonitrile eluent. The pH of 0.1% ammonium is about 11, which means that most silica-based LC columns cannot sustain use under these conditions. This emphasizes an advantage of VG-50 2D, packed with polymer-based packing material, as it well-tolerates against the high pH eluent like the one used in this experiment.

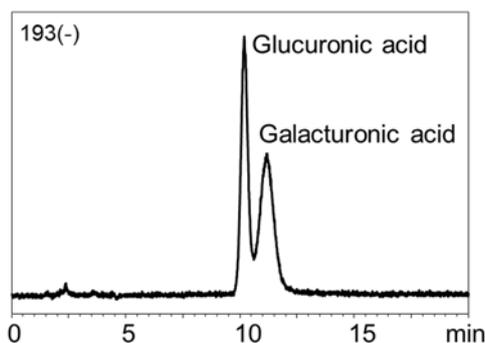


Sample: 50 ng/mL each in H₂O/CH₃CN=1/9, 5 μ L
 Column: Shodex HILICpak VG-50 2D
 Eluent: (A) 0.1% NH₃ aq. / (B) CH₃CN
 Linear gradient; B%=95%(0-5 min) \rightarrow 80%(15-20 min)
 Flow rate: 0.3 mL/min
 Detector: ESI-MS MRM (-)
 Column temp.: 60°C

Fig. 2 Chromatograms of various neutral saccharides

1-2. Acidic saccharides

Water / acetonitrile eluent cause the acidic saccharides to be retained in the column due to ionic adsorption. However, the use of an alkaline eluent will prevent the dissociation of amino functional groups on the stationary phase, and thus saccharides will not be retained in the column. Fig. 3 shows the chromatograms of glucuronic acid and galacturonic acids. The degree of separation achieved here was better than that of previously available method using ion-exclusion chromatography. Using a high acetonitrile ratio is also advantageous for achieving a high sensitivity MS result.

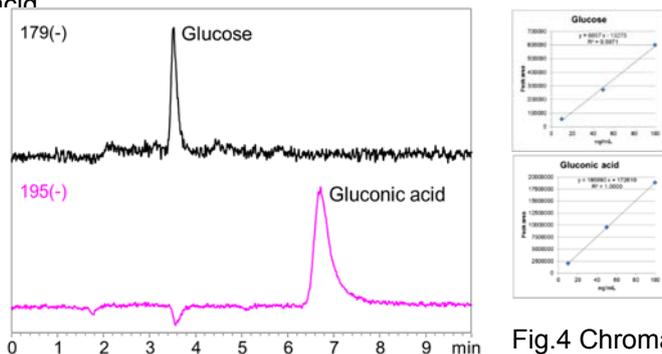


Sample: 10 ng/mL each in H₂O/CH₃CN=1/4, 5 μ L
 Column: Shodex HILICpak VG-50 2D
 Eluent: (A) 0.5% NH₃ aq. / (B) CH₃CN = 25 / 75
 Flow rate: 0.2 mL/min
 Detector: ESI-MS SIM (-)
 Column temp.: 30°C

Fig.3 Chromatograms of two acidic saccharides

1-3. Glucose and gluconic acid

The production of gluconic acid is generally completed through the conversion from glucose, requiring the simultaneous analysis of both compounds. However, previously available methods using ion-exclusion chromatography did not provide an effective separation. Fig.4 shows chromatograms demonstrating a clear separation of the two compounds. Acidic conditions will lactonize the gluconic acid which causes the peak tailing, but analyzing under alkaline condition will not let the lactonization, and so tailing will not be observed. This is another advantage of the method as it is an improved quantification method for the gluconic acid

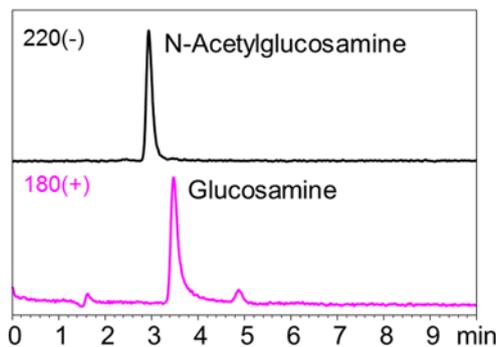


Sample: 10 ng/mL each in H₂O/CH₃CN=1/4, 5 μL
 Column: Shodex HILICpak VG-50 2D
 Eluent: (A) 0.5% NH₃ aq. / (B) CH₃CN = 25 / 75
 Flow rate: 0.2 mL/min
 Detector: ESI-MS SIM (-)
 Column temp.: 40°C

Fig.4 Chromatograms of glucose and gluconic acid

1-4. Amino acids

Fig. 5 shows chromatograms of N-acetylglucosamine and glucosamine. Since glucosamine contains an amino functional group, higher sensitivity was achieved by monitoring protonated compound than monitoring its deprotonated compound. Amino sugars and their acetylated metabolite counterparts can also be analyzed under the alkaline condition (data not shown).



Sample: 100 ng/mL each in H₂O/CH₃CN=1/4, 5 μL
 Column: Shodex HILICpak VG-50 2D
 Eluent: (A) 0.5% NH₃ aq. / (B) CH₃CN = 25 / 75
 Flow rate: 0.2 mL/min
 Detector: ESI-MS SIM (+/-)
 Column temp.: 40°C

Fig. 5 Chromatograms of N-Acetylglucosamine and glucosamine

2. Simultaneous LC/MS analysis of saccharides, organic acids, and amino acids

As [T1] the developed method was effective for separating acidic saccharides under alkaline conditions, it can be expected that the method can be extended for the separation of organic acids and amino acids. Fig. 6 shows the chromatograms of a mixture containing 14 saccharides, 9 organic acids, and 20 amino acids. A gradient method was used for the analysis.

The results show the HILICpak VG-50 column's capability of analyzing organic acids. The elution order of the organic acids was mono, di and tribasic acids. It required ca. 0.5% ammonia to make the citric acid (a tribasic acid) to elute. Oxalic acid and citric acid are not retained well by ion-exclusion chromatography. However, the method developed here retains those acids well, and this helps them less likely to be affected by early eluting impurity peaks.

It also demonstrated the feasibility of analyzing amino acids. The chromatograms showed hydrophobic amino acids have tendencies of eluting earlier while acidic amino acids tend to elute later.

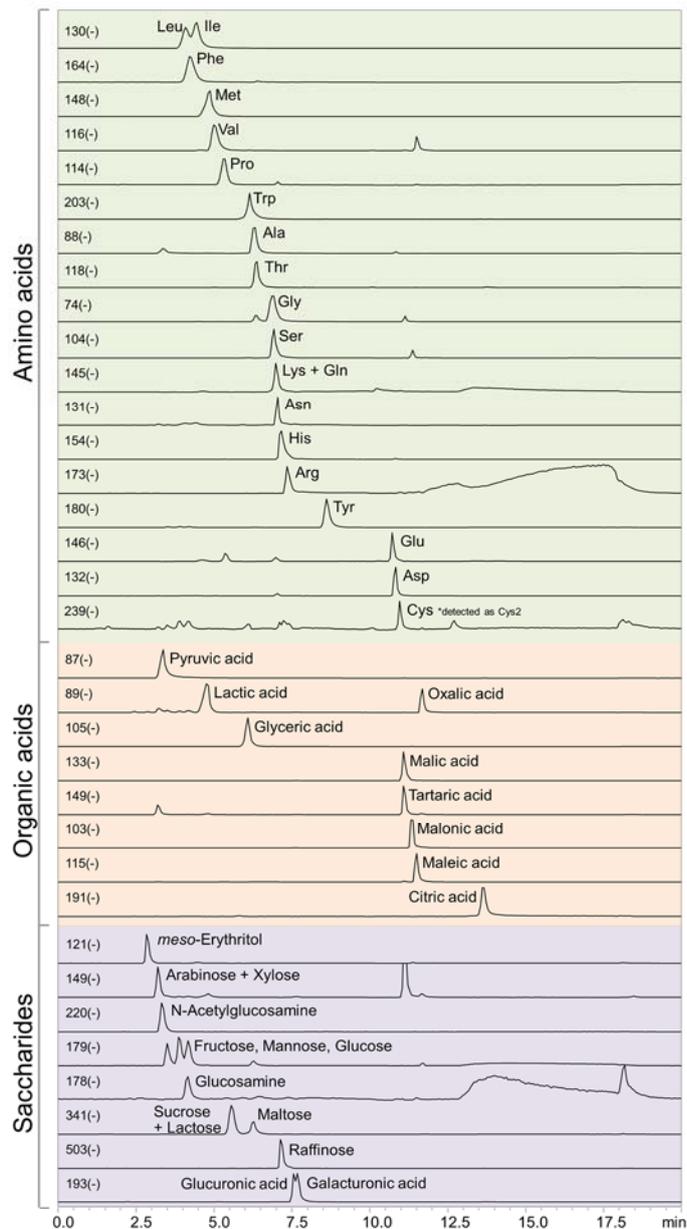


Fig. 6 Chromatograms of the simultaneous LC/MS analysis of a mixture containing saccharides, organic acids, and amino acids

Sample: 1 µg/mL each in H₂O/CH₃CN=1/4, 5 µL
Column: Shodex HILICpak VG-50 2D

Eluent: (A) 0.5% NH₃ aq. / (B) CH₃CN

Linear gradient;

B%=80%(0-2 min) → 10%(12-15 min) → 80%(15.01-20 min)

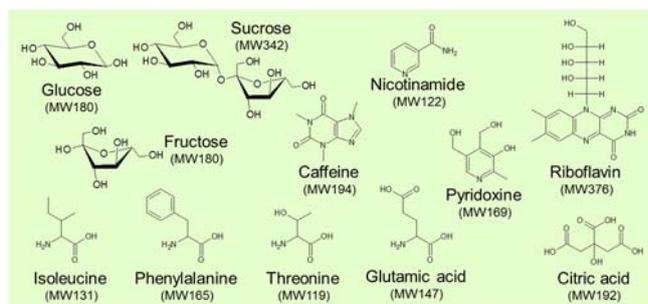
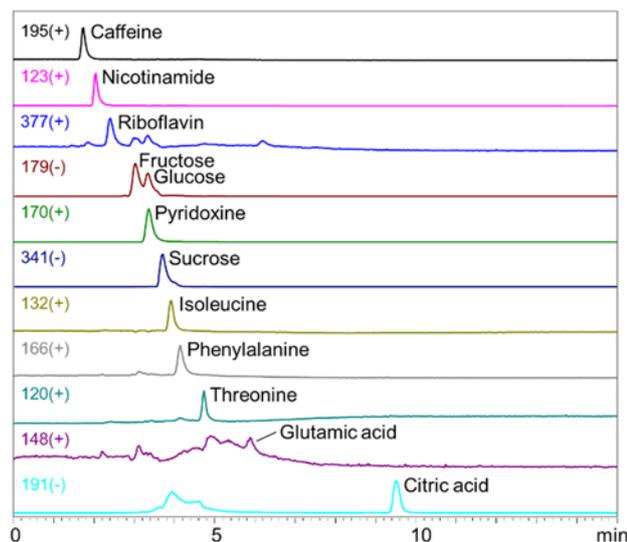
Flow rate: 0.2 mL/min

Detector: ESI-MS SIM (-)

Column temp.: 40°C

3. Application of the method for analyzing commercial energy drink

Fig. 7 shows LC/MS analysis result of a commercially available energy drink. It demonstrated an effective simultaneous analysis of saccharides (fructose, glucose, sucrose), citric acid, and amino acids (isoleucine, phenylalanine, threonine, glutamic acid). The method was also deemed feasible for the analysis of caffeine and water-soluble vitamins (nicotinamide, riboflavin, pyridoxine) present in energy drinks.



Sample: Commercial energy drink x100 dilution in H₂O/CH₃CN=1/1, 2 μL

Column: Shodex HILICpak VG-50 2D

Eluent: (A) 0.5% NH₃ aq. / (B) CH₃CN

High pressure linear gradient; B%=70%(0 min) → 10%(5-15 min)

Flow rate: 0.2 mL/min

Detector: ESI-MS SIM (+/-)

Column temp.: 40°C

Fig.7 LC/MS analysis of a commercial energy drink

Conclusions

A polymer-based amino column, Shodex™ HILICpak™ VG-50 2D, provides many advantageous analytical features when used under alkaline conditions. LC/MS with ammonia water / acetonitrile gradient elution is effective in providing good separation and high sensitivity analysis of various hydrophilic compounds. The method is feasible analyzing saccharides, organic acids, and amino acid simultaneously which was difficult and time consuming by previously available methods. This can be achieved without using pre-column derivatization nor addition of ion-pairing reagents. The alkaline conditions promote deprotonation of saccharides, making it possible to monitor negative ions and contributes to the enhanced high sensitivity detection. The developed method showed its effective analysis of a commercial energy drink. Initially the method monitored saccharides, citric acid, and amino acids, the method further demonstrated its ability to monitor caffeine and water soluble vitamins simultaneously.