Enhanced Resolution of Glucosinolates using ZIC®-HILIC Chromatography

A ZIC®-HILIC column provided a significantly more robust separation of Glucosinolates from cruciferous vegetables than a RP (reversed phase) or other HILIC columns. The separation was extremely tolerant to small changes in the solvent, salt or pH concentrations and provided over 500 separations with no degradation of peak geometry, while the performance of other HILIC columns degraded after 100 injections.

Introduction

Glucosinolates are phytochemicals that are found in cruciferous vegetables and are precursors of isothiocyanates, which are believed to be anti-cancer agents. Approximately 120 glucosinolates have been isolated from a broad variety of vegetables such as broccoli, cabbage, pak choy, rapeseed and Brussels sprouts. The general structure of glucosinolates is shown in Figure 1, typical side chains are aliphatic, sulfur containing (thioalkyl), alcoholic, indolic or glycosylate.

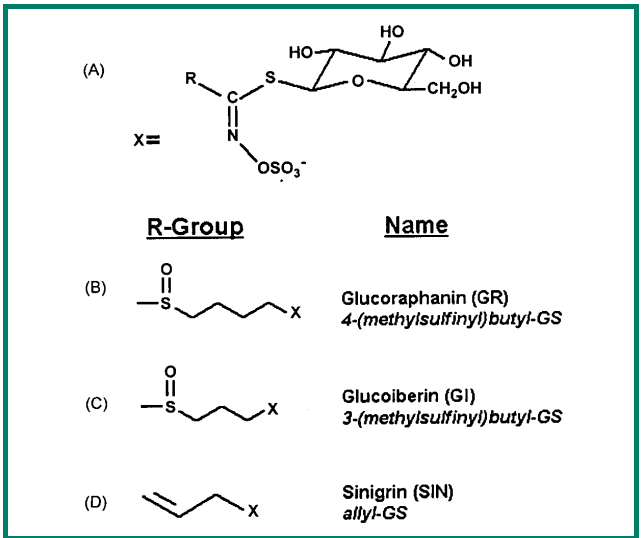

Figure 1: Structure of Glucosinolates.

The separation and identification of glucosinolates can be performed via HPLC. These compounds can be readily detected by enzymatic desulfation followed by separation on a C18 column. While this technique is useful, the separated glucosinolates are no longer biologically active (and cannot be converted to biologically active compounds) and scale up is not possible to obtain samples of separated glucosinolates.

In recent years, isocratic or gradient ion-pair techniques with PDA or MS detection have been developed for the separation of the glucosinolates. While the use of ion-pair techniques can provide useful separations, they require a desalting step before mass spectrometric detection. In addition, if more than a few glucosinolates are present in a sample, a lengthy gradient (up to an hour per injection) may be required to obtain a useful separation. An additional problem is that non target compounds frequently co-elute with the compounds of interest and MS is necessary for accurate detection.

Hydrophilic interaction liquid chromatography (HILIC) is a very powerful technique for the separation of complex mixtures of polar compounds such as glucosinolates. HILIC separates compounds using a mostly organic hydrophobic mobile phase with a hydrophilic stationary phase. The solutes elute in order of increasing hydrophilicity, which is the opposite of the elution order in reverse phase chromatography and is especially useful for the separation of polar compounds that are poorly separated by reverse phase.

ZIC®-HILIC chromatography is a unique form of HILIC that involves the bonding of zwitterionic sulfobetaine groups to a silica or polymer backbone of the stationary phase and thus allows for a significant aqueous fraction in the mobile phase. This allows greater solubility of polar analytes in the mobile phase and therefore provides greater sensitivity. Recently J.W. Fahey and co-workers[1] used ZIC®-HILIC to rapidly separate glucosinolates with baseline resolution and found that the ZIC®-HILIC approach provided for separations that were more robust than the use of other HILIC columns.

Experimental

Glucosinolate samples: Glucoraphanin and Glucoiberin were purified by counter current chromatography and Sinigrin was obtained from Sigma Chemical Co (St. Louis MO).

Instrumentation: The HPLC system included pumps (Model 616), autosampler (Model 717 Plus), Alliance System ((Model 2695), PDA detector (Model 2996) and empower software from Waters Associates, Milford MA.

HILIC separations were performed using a ZIC®-HILIC column (150 x 4.6 mm, 5 µm, 200 Å pore size with a silica support; part number 2712-155, SeQuant, Umea, Sweden) and a isocratic mobile phase consisting of 15 mM ammonium formate (pH 4.5) in CH_3 CN:H_2O as using a 0.5 mL/min flow rate.

HILIC separations were also performed using a polyhydroxy-ethyl aspartamide column (150 x 4.6 mm, 3 µm particle size, 100 Å pore size, PolyLC, Columbia MD) with an isocratic mobile phase of 30 mM ammonium formate, pH 5.4 in 85:15 (v:v) CH_3 CN:H_2O at a flow rate of 2 mL/min.

Reversed phase separations were performed with a SunFire C18 column (250 x 4.6 mm, 5 µm particle size, 200 Å pore size, Waters Associates, Milford MA) with a gradient mobile phase from 5 to 100% methanol with 0.1% glacial acetic acid at a flow rate of 1 mL/min.

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Results

Typical chromatograms of the glucosinolates are presented in Figure 2. It can be seen that the retention times and the resolution increase with an increase in the concentration of the organic modifier. At 70% acetonitrile, R_s is 1.21; and it increases to 1.51 when 75% acetonitrile was used and to 2.20 when the concentration of the organic phase is increased to 80% although the time for the separation was nearly doubled. The time for the separation could be reduced (albeit with some loss of resolution) at the highest organic content by doubling the flow rate. At the higher flow rate, the backpressure on the column was between 400 and 500 psi, so raising the flow rate is not a problem. With the polyhydroxyaspartic column the most favorable conditions led to an R_s of 0.90.

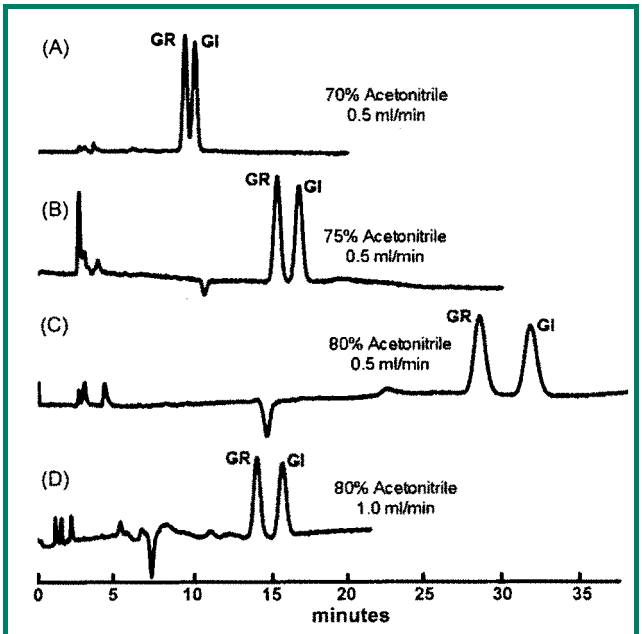

Figure 2: Separation of GR and GI, using a 150 x 4.6 mm ZIC®-HILIC column, and mobile phases consisting of 15 mM ammonium formate (pH 4.5) and different proportions of acetonitrile and water (reprinted from reference 1 with permission of Elsevier Publishing).

The developed assay using a ZIC®-HILIC column was quite reproducible for the three glucosinolates tested and the retention time RSD's are presented in Table 1. While the RSD for the retention time for SIN on the polyhydroxyaspartic column appears to be quite good, it was noted that the retention time for GC varied over a wide range, and thus the column was not acceptable for further studies. Wade and coworkers believe that this range is due to the steep gradient of solvent effects range with the mobile phase that was used. An additional issue with the polyhydroxyaspartic column was that the performance degraded after 100 injections, while the ZIC®-HILIC column provided more than 500 injections with no loss of peak geometry.

Calibration curves for the three glucosinolates, were constructed from analyses performed on three separate days, showed excellent linearity (r^2>0.999) over three orders of magnitude ranging from 81 pmol to 72 nmol with the ZIC®-HILIC column.

Table 1: Retention Time RSD for Glucosinolates on Various Columns

Column	GR	GI	SIN
ZIC®-HILIC	0.5	1.7	2.0
Polyhydroxyaspartic	a		1.3
C18			20.0

a) The retention time ranged from 12.5 to 14.5 min

Conclusions

The ZIC®-HILIC column provided an excellent separation for the glucosinolates, much better than other HILIC columns. It was found that the flexibility of the other column was quite low, by that we mean that small changes in the solvent, salt or pH concentrations had a significant effect. In contrast, the ZIC®-HILIC column was much more tolerant of changes in the mobile phase concentration, hence more robust than other HILIC columns. The data presented here clearly indicates that ZIC®-HILIC is a very powerful tool for the separation of complex mixtures of polar samples.

References

1: This application note is condensed from the scientific paper *Improved hydrophilic interaction chromatography method for the identification and quantitation of glucosinolates* by K.L. Wade, I.J. Garrard and J.W. Fahey, *J. Chromatogr. A*, 1154 (1-2), 469-472 (2007).

About ZIC®-HILIC Chromatography

The ZIC®-HILIC stationary phase is based on the covalently bonded zwitterionic sulfobetaine group indicated in Figure 3. It is available with a silica support in 3.5, 5 and 10 µm particle sizes in various column dimensions on glass lined stainless steel for capillary separations, as well as PEEK and stainless steel for analytical and preparative separations, In addition, it is available with a polymeric support in 5 µm particles (ZIC®-*p*HILIC).

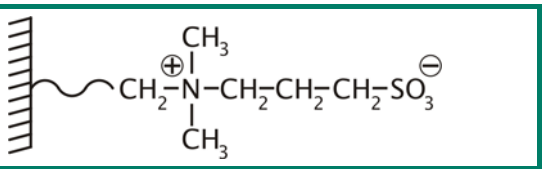

Figure 3: The Bonded Zwitterionic Sulfobetaine Group of ZIC®-HILIC.

About SeQuant

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 research workers from Umeå University. Products include silica based and polymer based ZIC®-HILIC columns for HPLC, Membrane Suppressors and Suppressor Regeneration Systems for Ion Chromatography, Proteomic Calibration Kits and Post Column Reaction Delay and Mixing Coils. These products are marketed and supported around the world by a series of distributors.

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