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Rapid analysis of natural sweeteners found in food and beverages using an advanced UHPLC system

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Keywords

Vanquish Flex, Syncronis HILIC, UHPLC, dulcoside, stevioside, rebaudoside, steviobioside, steviol glycosides, sweeteners

Goal

To demonstrate the development of a rapid method for the analysis of steviol glycoside based sweeteners on a Thermo Scientific™ Vanquish™ Flex system using a Thermo Scientific™ Syncronis™ HILIC, 1.7 µm column.

Introduction

Over the last decade there has been a growing interest in low-calorie alternatives to carbohydrate-based sweeteners. Recent publications have shown a dramatic increase in attention toward natural extracts such as the Stevia rebaudiana plant, not only for its sweetening effect but also for additional health benefits attributed to the plant. The major sweetening components are stevioside, rebaudioside A, rebaudioside C, and dulcoside A, each of which is over 300 times sweeter than sucrose-based sweeteners. Because of this they are widely used in beverages and foodstuffs.

The chromatographic separation of these components is difficult as they are structurally very similar (Figure 1), differing only in the number and configuration of the satellite glucose units. Because of these they are very polar, which implies that analysis by reversed-phase HPLC can be particularly challenging. The method described here demonstrates the full resolution of six steviol glycosides using an alternative HILIC-based method.

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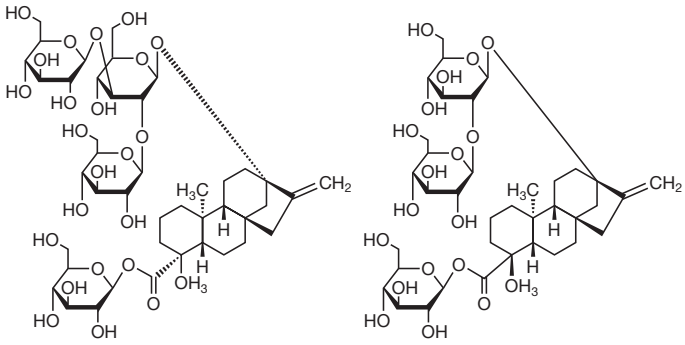

Figure 1. Chemical structure of rebaudioside A and stevioside.

One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. The Syncronis column range has been engineered to provide exceptional reproducibility due to its highly pure, high surface area silica, dense bonding, and double end-capping, all controlled and characterized through the use of rigorous testing.

The Vanquish Flex UHPLC systems have the benefit of SmartInject technology and improvements in injection system hardware synchronization. This results in superior retention time precision, providing the user with greater data confidence during method development. The Vanquish Flex systems also utilize Thermo Scientific™ LightPipe™ flow cell technology designed for the diode array detector (DAD), which provides the user with low peak dispersion due to small internal volume.

Experimental

Consumables and apparatus

- Syncronis HILIC, 100 × 2.1 mm, 1.7 µm UHPLC column (P/N 97502-102130)
- LC-MS grade 18 MΩ water from Thermo Scientific™ Smart2Pure™ system (P/N 50129845)
- Fisher Scientific™ LC-MS grade acetonitrile (P/N A955-212)
- Fisher Scientific™ Optima™ LC-MS grade formic acid (P/N A117-50)
- Fisher Scientific Optima LC-MS grade ammonium formate (P/N A115-50)
- Thermo Scientific™ Virtuoso™ 9 mm wide opening, 2 mL screw thread vial and cap kit (P/N 60180-VT400)
- Thermo Scientific™ Target2™, 30 mm, 0.45 µm, nylon syringe filter (P/N F2500-1)

Standards

The six compounds selected for use were; ducloside A, stevioside, rebaudioside A, B, C, and steviobioside

All standards were purchased from a reputable supplier.

Instrumentation

Analyses were also performed using a Vanquish Flex UHPLC System consisting of:

- Quaternary Pump F (P/N VF-P20-A)
- System Base Vanquish Flex (P/N VF-S01-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe flow cell, 10 mm (P/N 6083.0100)

Thermo Scientific™ Virtuoso™ vial identification system (P/N 60180-VT-100)

Software

Thermo Scientific™ Chromeleon™ 7.2 SR4

Sample preparation

Solutions of the six compounds were prepared by dissolving a known amount in mobile phase to produce 2 mg/mL primary solutions (rebaudioside C was prepared at 1 mg/mL). A mixed standard solution and individual working standards were used to assess method development and were prepared in mobile phase at a concentration of 0.2 mg/mL.

Vial labeling was supported by the Virtuoso vial identification system.

UHPLC conditions

Various conditions were explored as part of the method development described below. These values represent those selected for the final method.

HPLC column:	Syncronis HILIC, 1.7 µm UHPLC column, 100 mm × 2.1 mm
Mobile phase A:	10 mM Ammonium formate, pH 3.0
Mobile phase B:	Acetonitrile
	On-pump mixing, 15% A : 85% B
Flow rate:	0.8 mL/min
Column temperature:	40 °C, still air with eluent pre-heating
Injection volume:	2 µL
UV detection:	210 nm
Backpressure:	110 bar
Mixer:	50 µL capillary + 350 µL static

Results and discussion

Method development focused on column choice, mobile phase composition and mixing, buffer concentration, and column temperature to produce a method with satisfactory resolution between target analytes. Once this was established, the method throughput was investigated by increasing the flow rate.

Column chemistry

In HILIC methods the water in the mobile phase forms an aqueous-rich layer adsorbed onto the polar surface of the stationary phase. Polar analytes preferentially partition into this layer and are retained through a complex combination of hydrophilic partitioning, hydrogen bonding, and electrostatic interactions.
A number of different HILIC column types have been developed to exploit different aspects of these retention mechanisms and a key part of method development is the selection of the optimal column chemistry.[1]

Three columns with different HILIC capability were screened:

- Thermo Scientific™ Acclaim™ Trinity P1
- Thermo Scientific™ Accucore™ 150-Amide-HILIC
- Syncronis HILIC

The best retention, peak shape, and selectivity for these compounds were obtained using the Syncronis HILIC column.

Mobile phase composition

HILIC methods can be sensitive to relatively small changes in the buffer : organic ratio of the mobile phases as well as buffer concentration. This was investigated by analyzing the same standard mixture with different mobile phase compositions, allowing the pump to proportion the two mobile phase streams.

As the proportion of water increases, the retention of the standards decrease as does the resolution between the critical pair (Figure 2). A proportion of 15% buffer: 85% acetonitrile was selected.

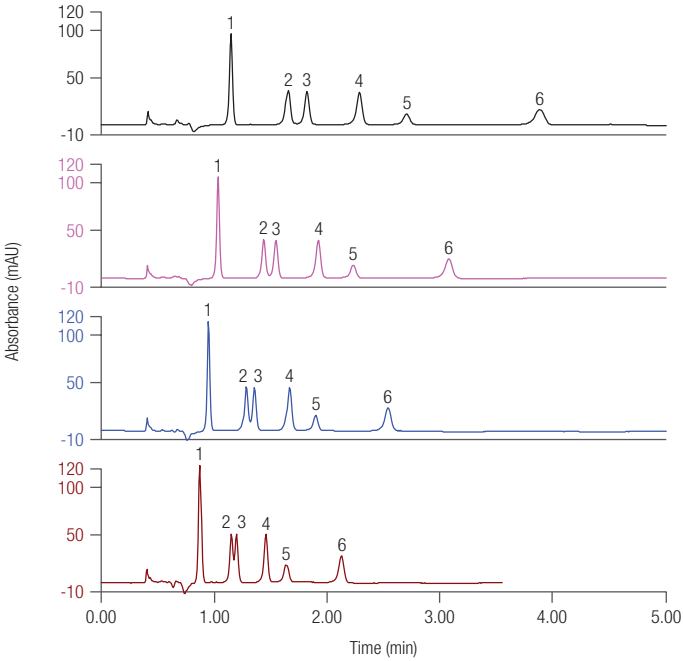

Figure 2. Chromatograms showing the effect on retention and selectivity when making 1% incremental changes to mobile phase. Upper trace 14% buffer, then 15%, 16%, and 17% in lower traces.

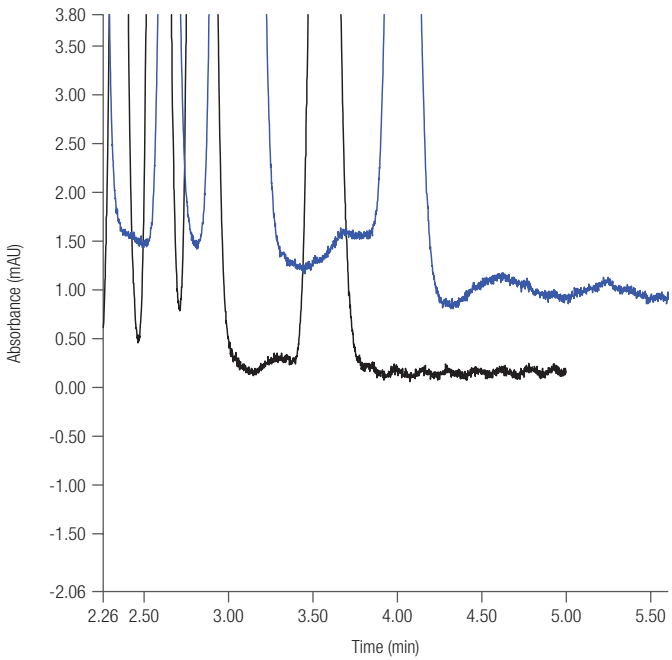

Figure 3. Baseline comparison with different mixer volumes. Upper trace 200 µL total mixing volume, lower trace 400 µL total mixing volume.

In these experiments, the proportion of each component was mixed on the Vanquish Flex quaternary pump at low pressure through a proportioning valve controlled by the Chromeleon software. This is of significance when transferring methods between different instruments and laboratories. If the method is transferred to a different pump or mixer type, or if the mobile phase is mixed offline and delivered from a single channel, there will be differences in observed retention. Further information on effective mixing for UHPLC methods is available in a technical note.[2]

Mobile phase mixing

Due to the low detection wavelength, the UV baseline is sensitive to small changes in proportioning from the mixing of the two channels as the buffer and acetonitrile do not absorb equally. Static mixer configurations of 150 µL and 350 µL were trialed, in addition to the installed 50 µL capillary mixer.

Reduction in baseline ripple was seen with the larger mixer configuration (Figure 3). Also observed was a slight shift to a shorter retention time, indicating that the lower volume configuration had not achieved adequate mixing of the two components.

Using a pre-mixed mobile phase that is pumped through a single channel will further decrease the noise seen on the UV channel, however the convenience of on-pump proportioning will be lost. It has been shown that even small changes in mobile phase composition can have a significant impact on retention, so manual mixing of mobile phase will need to be prepared accurately and consistently. Whatever process is selected, it is good practice to include the detail in the method protocol.

Buffer concentration

The strength of the buffer can also have an effect on peak retention and resolution. Results were compared when using a 10 mM and a 50 mM buffer. With the stronger buffer, there is a general increase in retention and a slight improvement in peak resolution between the dulcoside A and rebaudioside B peak. Both were detrimental to the goal of a rapid method (Figure 4).

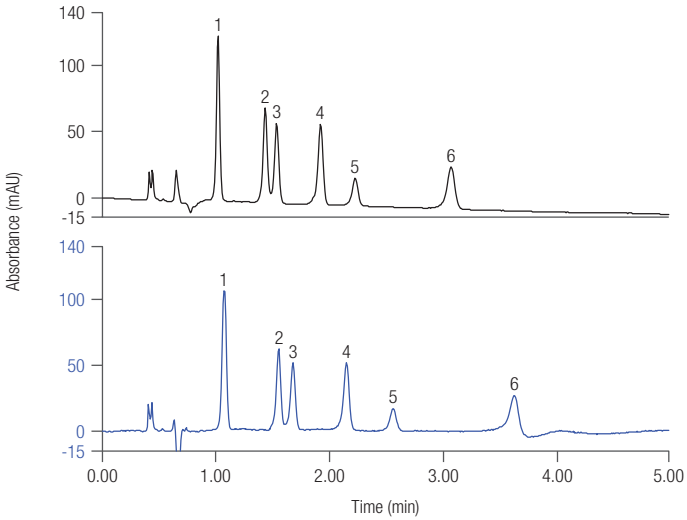

Figure 4. Impact of buffer strength on analyte retention. Upper trace with 10 mM buffer, lower trace with 50 mM buffer.

Temperature

Column temperature can play an important role in method development as temperature can have an impact on peak retention and selectivity and can modify column backpressure. The method was assessed at six temperatures ranging from 25 to 45 °C in 5 °C intervals. As the temperature increased the peak symmetry improved (Figure 5). Also, the retention time of all peaks was reduced and the resolution between peaks 2 and 3 also increased (Figure 6).

A temperature of 40 °C (experiment 5) was selected as a good compromise to provide sufficient peak resolution and improved peak symmetry.

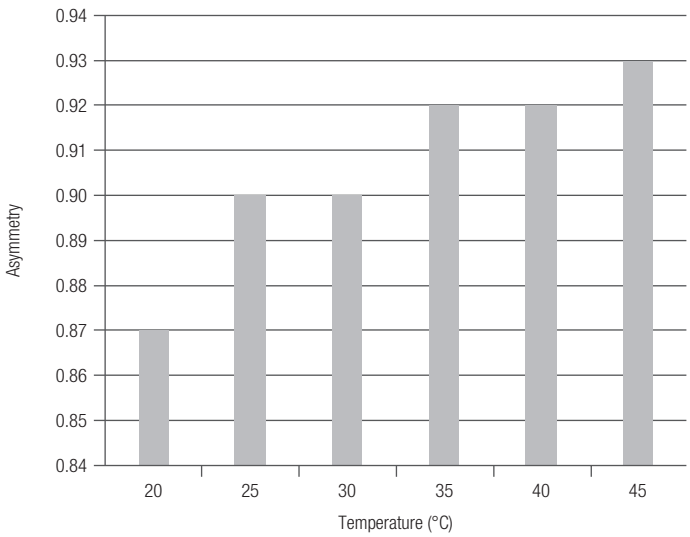

Figure 5. Asymmetry values for peak 2 at different column temperatures.

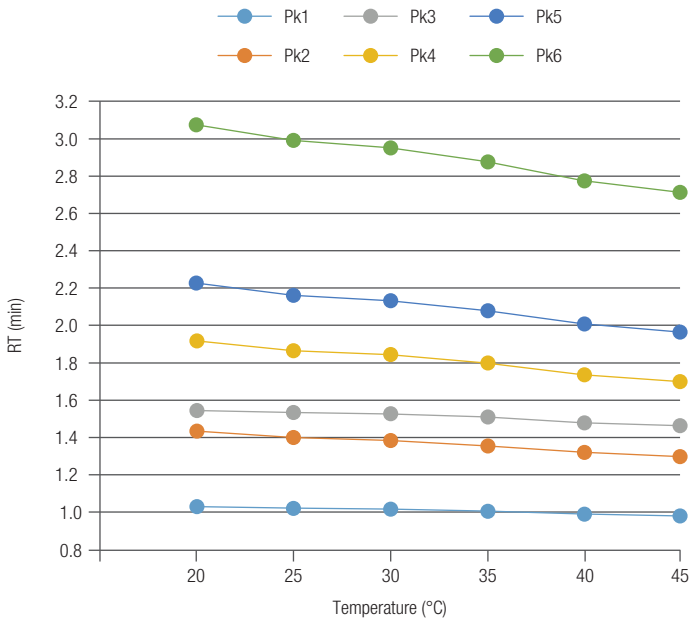

Figure 6. Plot of peak retention times against column temperature. Experiment 1 was with a column temperature of 20 °C. The temperature was increased incrementally by 5 °C in subsequent experiments to 45 °C. Resolution is represented by the spacing between adjacent lines.

Flow rate

Small particles provide good column efficiency over a range of flow rates. This was investigated by increasing the flow rate from 0.5 mL/min to 0.8 mL/min in 0.1 mL/min increments (Figure 7). Resolution between critical pairs is maintained with only a small loss in column efficiency. Full resolution of all six sweeteners was achieved within 2 minutes.

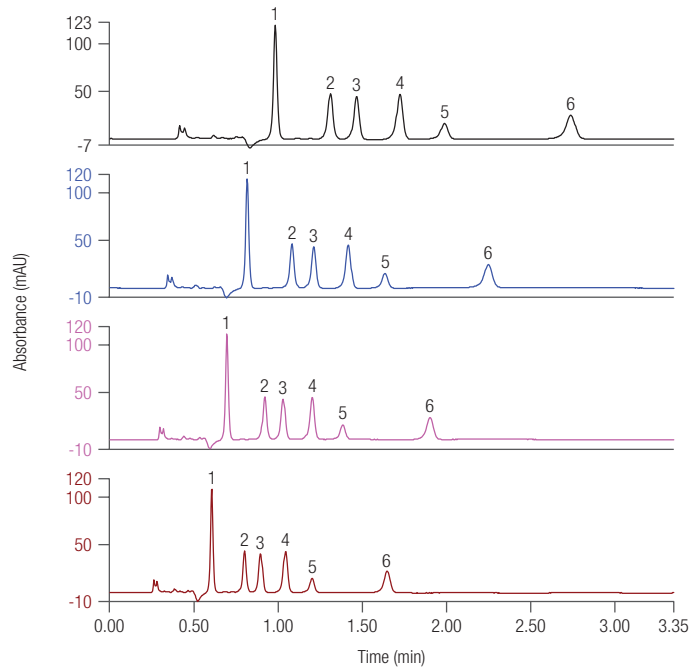

Figure 7. Chromatograms of test standards at different flow rates. Upper trace is at 0.5 mL/min and the flow rate increases in 0.1 mL/min increments in the lower traces.

A legacy method for this analysis used a longer column with a flow rate of 0.5 mL/min and a method time of 25 minutes.[2] Shifting to this shorter method increases throughput more than twelve-fold with an eight-fold decrease in mobile phase costs (Figure 8).

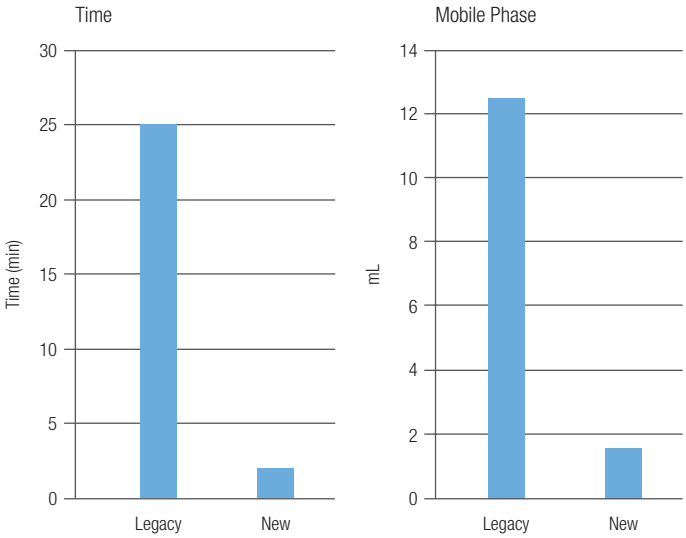

Figure 8. Relative differences between legacy method and new method for (a) method time and (b) mobile phase consumption per sample.

Retention time stability is a good measure of both pump flow rate and mixing consistency. To examine this, 24 replicate injections of the steviol standard mixture were made. The RSD% of the retention time for all peaks was less than 0.1%, equivalent to less than 0.5 s difference in retention time (Table 1).

Table 1. %RSD data for 24 replicate injections.

Compound	Peak Number	% RSD		
		RT	Peak Area	Width at Half Height
Steviobioside	1	0.09	0.53	0.25
Dulcoside	2	0.08	0.45	0.24
Rebaudioside B	3	0.09	0.45	0.27
Stevioside	4	0.07	0.52	0.24
Rebaudioside C	5	0.08	1.20	0.34
Rebaudioside A	6	0.08	1.44	0.26

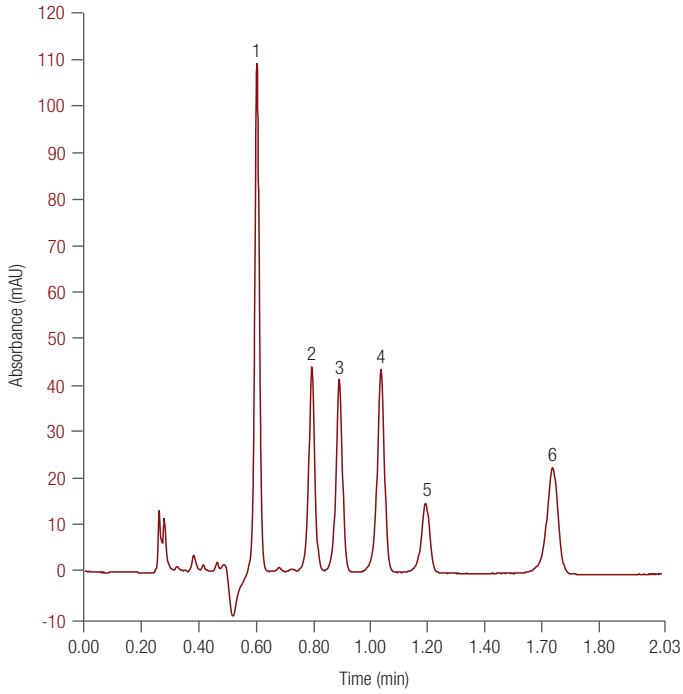

Figure 9. Chromatogram showing the separation of six sweeteners on a Syncronis HILIC column.

The final method produced the chromatogram shown in Figure 9.

Consumer product testing

Commercial sweeteners from three different steviol-containing products were qualitatively analyzed using the developed method. Samples (nominally 100 mg) were dissolved in 10 mL of mobile phase and filtered if required.

The chromatograms are shown in Figure 10. It can be seen that Brand N contained stevioside, rebaudioside A and C. The other two brands were mostly rebaudioside A, though they did have trace amounts of other steviols.

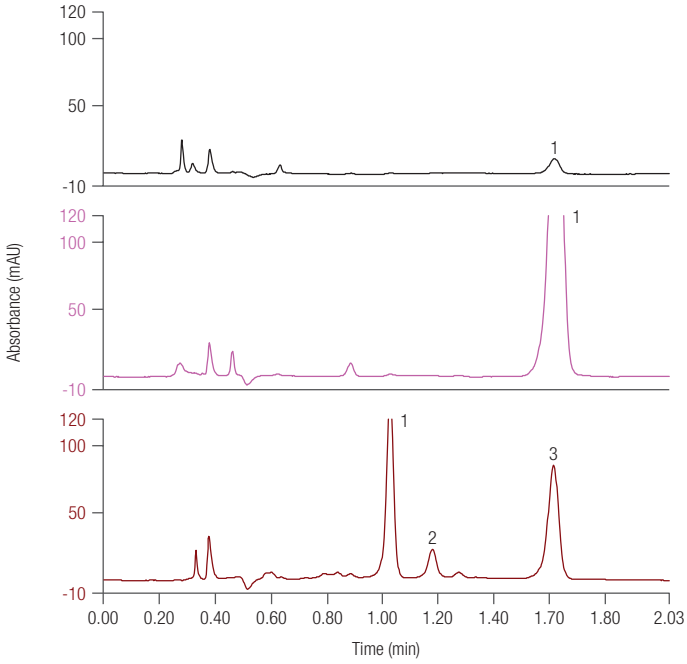

Figure 10. Chromatogram showing the analysis of three brands of steviol-containing products. Upper trace: brand T, middle trace: brand M, and lower trace brand N.

Conclusions

A high-throughput application has been developed for the analysis of steviol-based sweeteners.

This application demonstrates the following:

- Small changes in mobile phase composition can have significant effect on retention.
- Adequate mixing of mobile phase is required to improve retention stability and control baseline noise.
- Increased method throughput has been achieved with a significant reduction in cost per sample, when compared to legacy applications.

References

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