# Rapid Gradient and Elevated Temperature UHPLC of Flavonoids in Citrus Fruit

Application Note

General Chromatography, Food Industry


## Authors

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## Abstract

Citrus fruits were analyzed by gradient UHPLC for naringin and other flavonoids. Gradient reversed phase HPLC with long (150 mm) sub-2-µm columns is a powerful technique for analyzing complex matrices with numerous analytes such as grapefruit juice. Sufficiently flushing the column with strong solvent and re-equilibration with weak solvent starting conditions is necessary but time consuming, possibly impinging on overall lab productivity. An Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse Plus C18, 2.1 mm × 150 mm, 1.8 µm column was able to resolve over 70 peaks in grapefruit juice in seven minutes. The column was then properly flushed with strong solvent and re-equilibrated in the next three minutes by operating at a high flow rate (0.8 mL/min). Orange and lime fruit were similarly analyzed.


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## Introduction

Plant extracts such as lime juice and peel are ideal candidates for gradient analysis due to the wide range of polarity of the compounds found in the matrix; from hydrophilic small organic acids (citric acid) and sugars (fructose) to moderately polar polyphenols to the hydrophobic essential oil of lime. An isocratic method cannot accommodate the wide range of peak retention in a reasonable time whereas a gradient method can.

Gradient profiles of plant extracts, or fingerprints, are often used for identification, authentication and quality control. Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns are designed for 1200 bar operation making them ideal for fast or high-resolution gradient separations, and a reliable tool for the food and herbal medicine industries.

High resolution chromatographic profiles or fingerprints of three types of citrus fruit were created using UHPLC conditions, highlighting several flavonoids. Citrus flavonoids are thought to have health benefits, based on antioxidant activity. These benefits include anticancer, antiviral, and anti-inflammatory properties. Some citrus flavonoids provide a bitter or sweet taste.

Gradient analyses often take longer than isocratic analyses due to flushing and re-equilibrating the column. One way to reduce analysis time is to increase the flow rate.The analyses in this application note take advantage of high flow rates.

## Experimental

An Agilent 1290 Infinity LC System comprised of the following was used to obtain all data:

- Agilent 1290 Infinity Diode Array Detector (DAD) and 10 mm, 1 µL Max-Light flow cell
- Agilent 1290 Infinity Thermostatted Column Compartment (TCC)
- Agilent 1290 Infinity Automatic Liquid Sampler (ALS)
- Agilent 1290 Infinity Binary Pump with Jet Weaver V35 mixer

UHPLC columns and chromatographic conditions were:

| | |
|---|---|
| Columns: | Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm, 1.8 µm p/n 959759-902<br>Agilent ZORBAX RRHD StableBond C18, 2.1 mm × 150 mm, 1.8 µm p/n 859700-902 |
| Mobile phase: | Solvent A: Water (0.1% formic acid)<br>Solvent B: Acetonitrile (0.1% formic acid) |
| Flow rate and gradient: | 0.8 mL/min |

| Time | %B |
|---|---|
| 0 | 0 |
| 0.5 | 0 |
| 7 | 40 |
| 7.1 | 100 |
| 8.6 | 100 |
| 8.7 | 0 |
| 10 | end |

| | |
|---|---|
| Column temperature: | 40 °C or 90 °C |
| Detection: | UV 276 nm, 4 nm, Ref. OFF |
| Injection volume: | 1.0 µL |

### Samples

Rinds were peeled from the fruit flesh and promptly ground with a food processor until grounds were about 1–3 mm in diameter. Two hundred milligrams of ground rind were then extracted with 10 mL MeOH (0.1% KOH) in an ultrasonic bath for 10 min, then 0.45-µm syringe filtered. Fruit flesh was hand squeezed and the collected juice was 0.45 µm filtered with a 0.45 µm filter into amber autosampler vials.

### Citrus flavonoid standards

The following standards included rutin, naringin, hesperidin, quercetin, and naringenin. The calibration samples were made by diluting standard stock solutions with water to the 10–1000 ppm range. Most of the standard stock solutions were aqueous, but some were made in methanol/$H_2O$ due to solubility issues. A chromatogram of the standard mixture including the elution order of the flavonoids, and two calibration curves are shown in Figure 1.

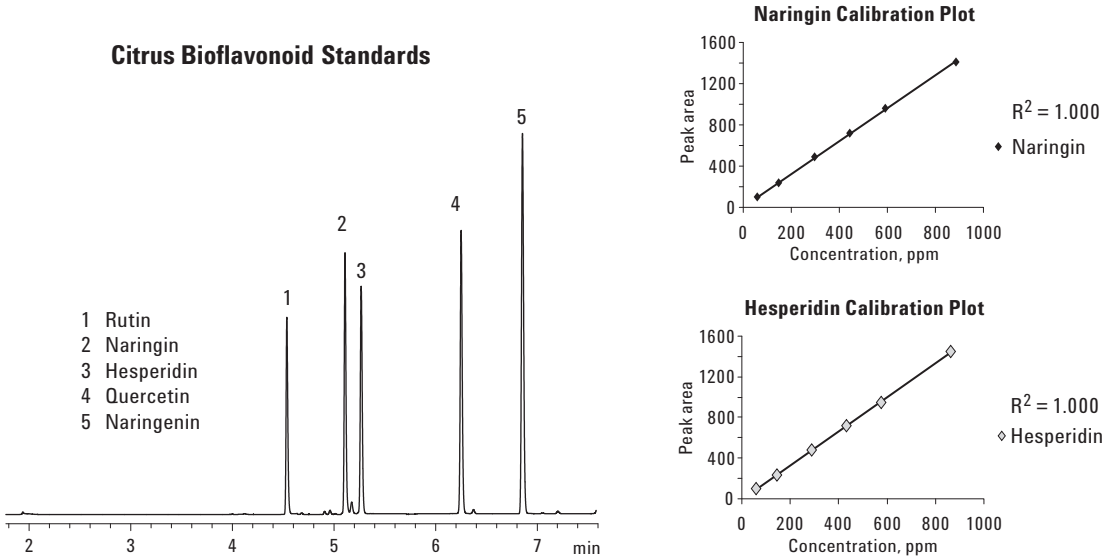


*Figure 1. Chromatogram of the flavonoid standard mixture and calibration curves for naringin and hesperidin. Chromatographic conditions are listed in experimental section.*

## Results and Discussion

Higher mobile phase linear velocity (flow rate) using sub-2-µm columns has less of an adverse effect on the height of a theoretical plate (H) than larger particle columns allowing faster flow without significant resolution loss (Figure 2). This small loss in efficiency at high flow rate, an RRHD column characteristic, is welcome in gradient analysis because an increase in flow rate when holding k* constant, results in a decrease in gradient time:

Equation 1: $t_G = k^* V_m/F$

Where

- $k^*$ is the gradient retention factor
- $t_G$ is the gradient time
- F is the flow rate
- $V_m$ is the column volume, assumed to be the same between the two columns

Increasing the number of column volumes per unit time (flow rate) in gradient analysis also reduces the other gradient segments including the isocratic hold, strong solvent flush and re-equilibration, and consequently reduces analysis time.

Narrow bore (2.1 mm id) columns are often used over larger bore columns for their compatibility with MS detectors, increased sensitivity, and reduced solvent usage.

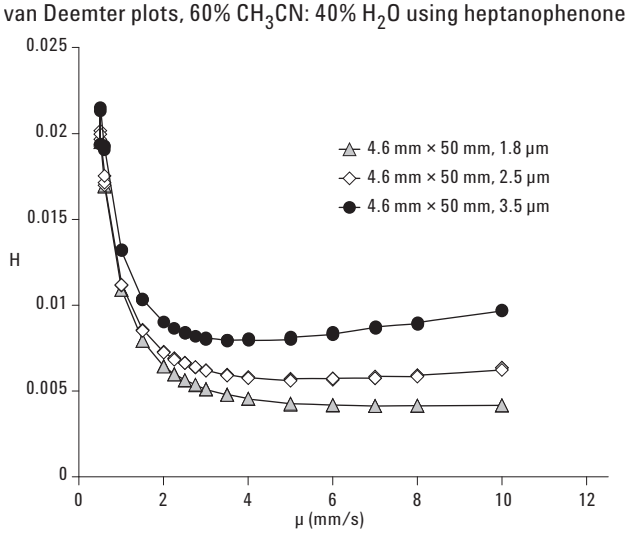


*Figure 2 Plots of plate height versus velocity. Plate heights for sub-2-µm columns remain relatively constant compared to larger particle sized columns as mobile phase velocity (flow rate) increases.*

Conventional 2.1 mm × 150 mm columns packed with 5 µm particles typically use flow rates of about 0.2 mL/min. The 0.2 mL/min flow rate combined with the relatively large column size and extra column volume contributes to long analysis times. For example, re-equilibrating the column to starting conditions with four column volumes of mobile phase at starting conditions takes at least six min. This does not include flushing out any delay volume (the volume of the flow path from the gradient formation point to the head of the column). Column re-equilibration time is calculated from a number of column volumes divided by the flow rate:

Equation 2: $n ( \pi r^2 L (0.6)) / F = t$

or for the 2.1 × 150 mm column (F = 0.2 mL/min) example

$4 (\pi 0.105^2 15 (0.6)) / 0.2 = 6.3$ min

Where:

- n is the number of column volumes
- r is column internal radius (cm)
- L is column length (cm)
- 0.6 is the void fraction of the packed column
- F is the flow rate (mL/min)
- t is the re-equilibration time in minutes

Operating under UHPLC conditions, and using a faster flow rate (0.8 mL/min.) with 1.8 µm particles, column re-equilibration time is only 1.6 min, which is four times faster. The gradient and pressure profiles, and column volumes are shown graphically with a lime juice chromatogram in Figure 3. Only four column volumes were necessary for re-equilibration. This

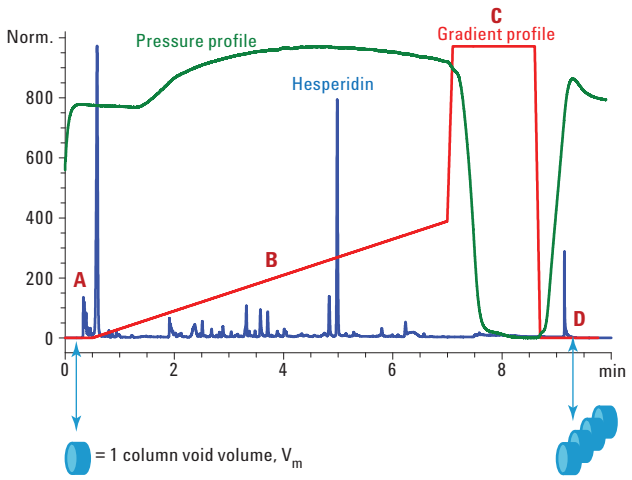


*Figure 3. UHPLC of grapefruit juice with pressure and gradient profiles, and column volumes needed for re-equilibration. Segments of the gradient include A: initial isocratic hold, B: linear elution ramp, C: strong solvent column flush, D: re-equilibration.*

was proven by injecting samples repeatedly without any change in k*, $\alpha$, or resolution (Figure 4).

In addition to the grapefruit, lime and orange extracts were also analyzed with the method, each producing a distinct chromatographic profile. Several flavonoids were identified using retention time matching of standards as shown in Figures 5,6,7. Hesperidin or Narginin were quantified using an

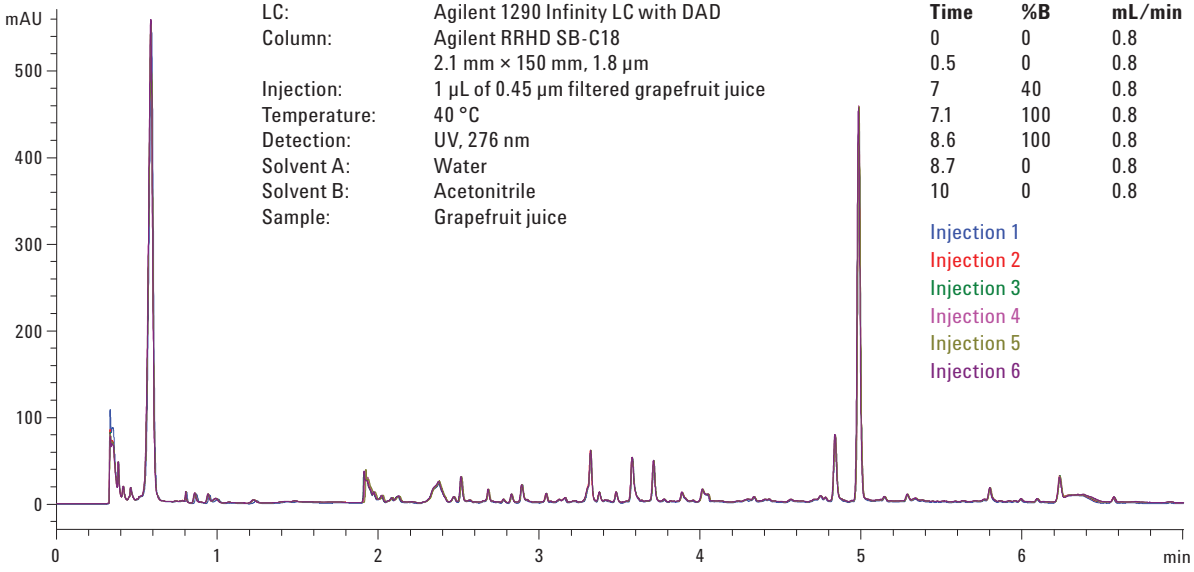


*Figure 4. Overlay of six analyses with no variability in retention, selectivity or resolution, indicating sufficient column flushing and re-equilibration.*

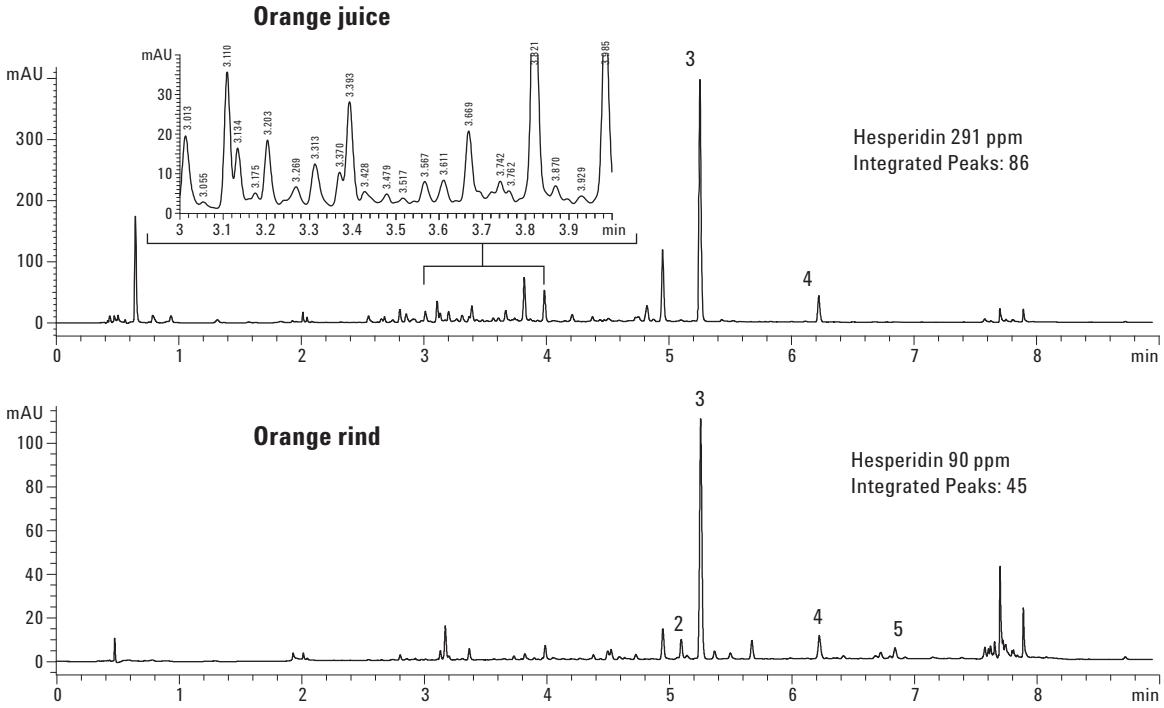


*Figure 5. UHPLC fingerprint of orange juice and rind analyzed by an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm (p/n 959759-902). Chromatographic conditions are listed in experimental section.*

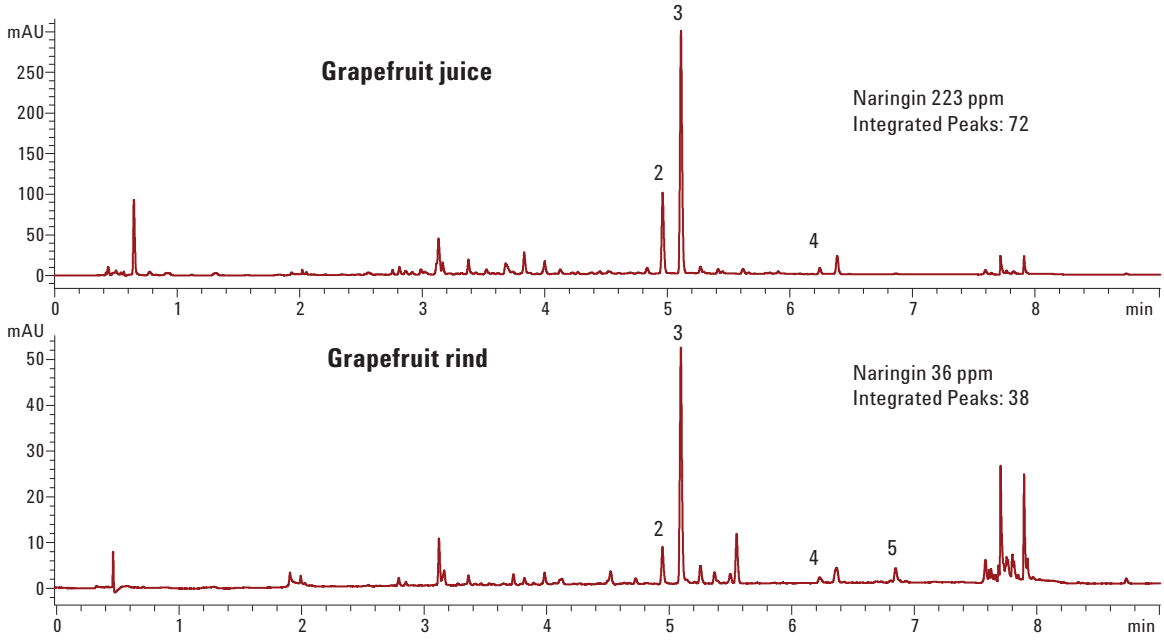


*Figure 6. UHPLC fingerprint of grapefruit juice and rind analyzed by an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm (p/n 959759-902). Chromatographic conditions are listed in experimental section.*

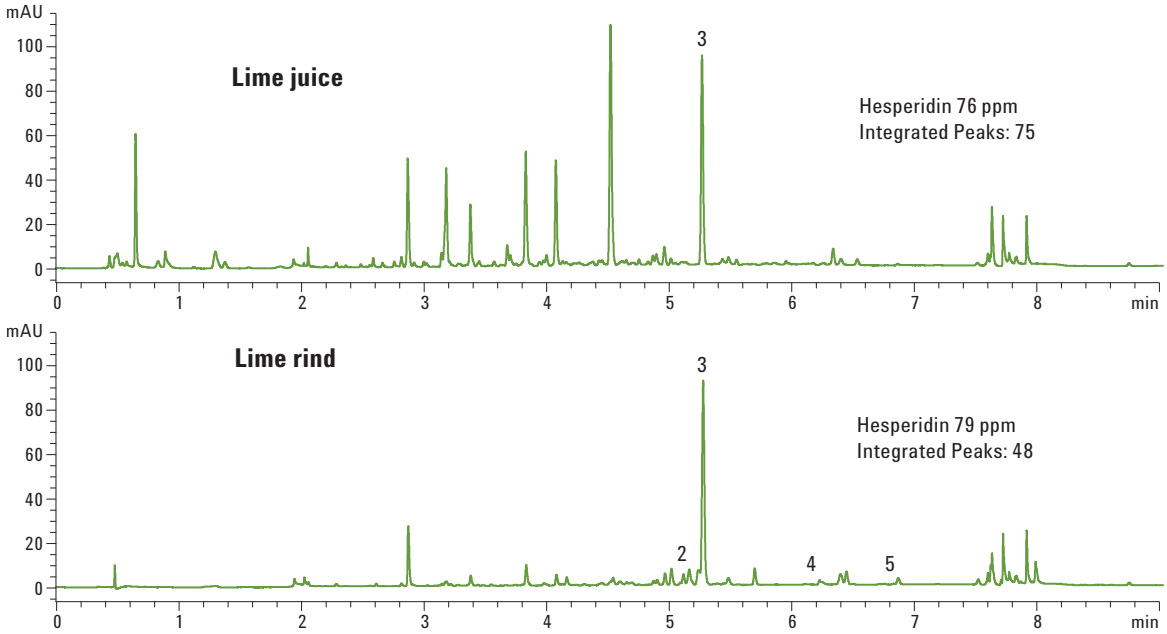


*Figure 7. UHPLC fingerprint of lime juice and rind analyzed by an Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm × 150 mm (p/n 959759-902). Chromatographic conditions are listed in experimental section.*

external standard six point calibration curve and concentrations listed in the figures. A subsection of the orange juice chromatogram is expanded to more clearly show the number of compounds present in the sample.

When using 2.1 mm × 150 mm RRHD columns, the high flow rate mitigates the additional analysis time inherent in gradient methods associated with isocratic holds, strong solvent flushing and re-equilibration. Sensitivity gains from narrower peaks however, are still preserved. Figure 8 indicates signal-to-noise

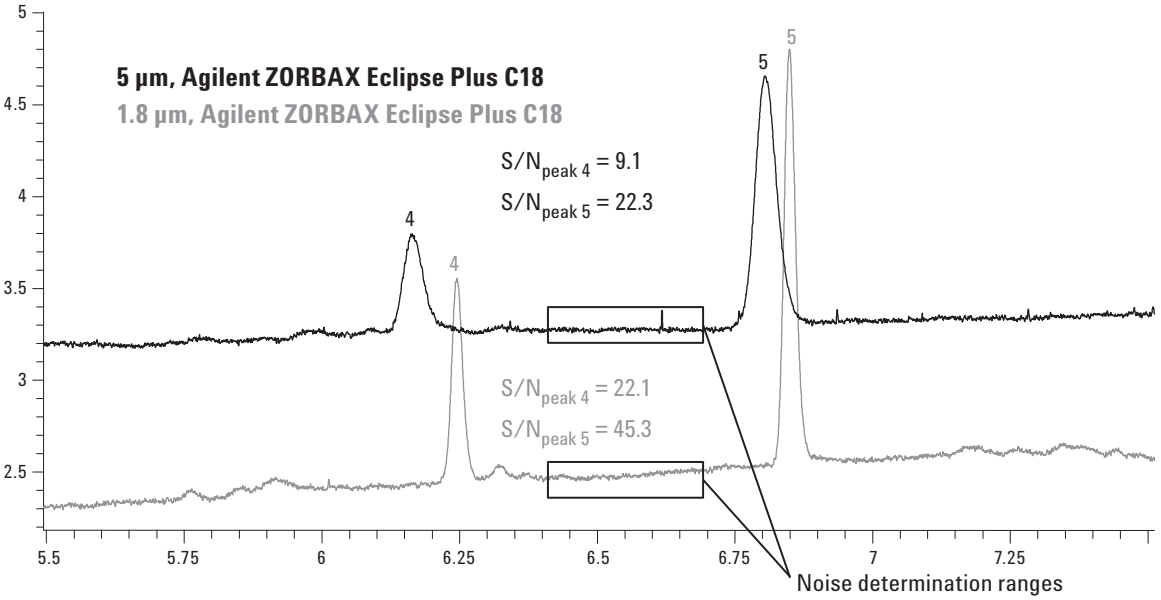


*Figure 8. Signal-to-noise comparison of 5 and 1.8 µm Agilent ZORBAX Eclipse Plus C18 columns, 2.1 mm × 150 mm using a 1 ppm each quercetin and naringenin standard. Chromatographic conditions are listed in the experimental section.*

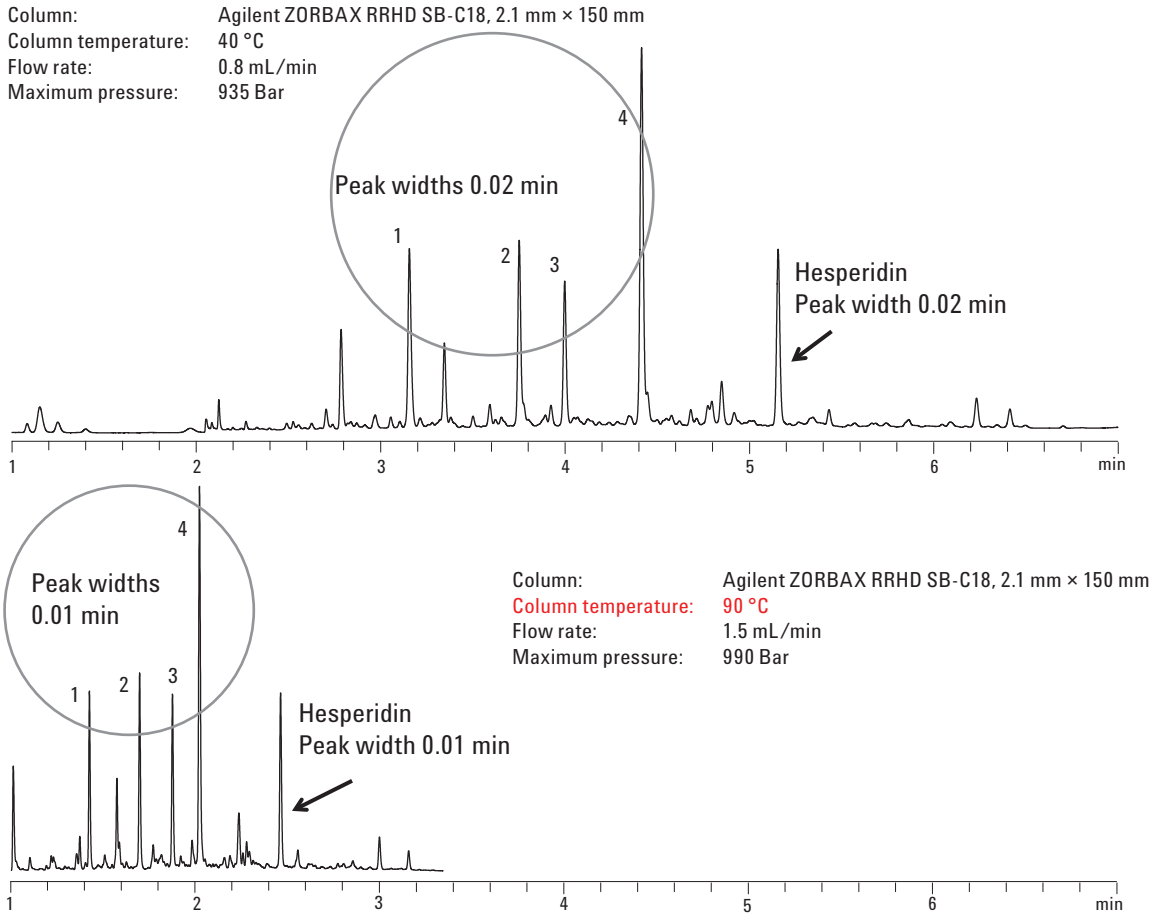


*Figure 9. UHPLC of lime juice including high temperature and faster flow rates further reduce analysis time using an Agilent ZORBAX RRHD SB-C18 column. Chromatographic conditions listed in experimental section.*

ratios of an Agilent ZORBAX RRHD Eclipse Plus C18 2.1 mm × 150 mm, 1.8 µm column is 50% to 60% greater compared to an Agilent ZORBAX Eclipse Plus C18, 2.1 mm × 150 mm, 5 µm column. The limit of quantification of quercetin is 1 ppm for the 5 µm column, and 0.5 ppm for the RRHD column.

Narrower peaks were achieved by using a faster flow rate. The temperature was raised to lower mobile phase viscosity, and the Eclipse Plus C18 phase was replaced with StableBond-C18 to withstand the extreme temperature. The temperature limit of the SB-C18 column is 90 °C, while the temperature limit of the Eclipse Plus C18 column is 60 °C. The flow rate could be increased to 1.5 mL/min at 90 °C. Gradient times were decreased by a factor of 1.875 to maintain k* for the flow rate change:

Equation 3: $k^* = (t_G \times F) \div V_m$

The 90 °C temperature, and 1.5 mL/min. rate produced 0.01 min wide peak widths at half height, which is half as wide as the 40 °C method. It also reduced analysis time proportionally to 5.3 minutes (Figure 9). RRHD column ruggedness allows these columns to operate at pressures up to 1200 bar, making them a perfect fit for the Agilent 1290 Infinity LC system. All RRHD columns can be used with other UHPLCs, however, the flow rate must be adjusted downward to allow pressure (or flow rate) to be in the operating range of the other vendor's UHPLC.

## Conclusion

Complex natural products such as fruit juices and their rinds were separated into their many components in a relatively short time using gradient elution, high flow rate, and long (150 mm) highly efficient RRHD Eclipse Plus sub-2-µm columns on a UHPLC system – the Agilent 1290 Infinity LC system.

The benefit of the long column length allows challenging separations to be accomplished with high resolution and moderate analysis times. The benefit of 1.8 µm particles is that higher mobile phase flow rates can be used with little loss in efficiency. Detection sensitivity also is intrinsically gained when converting from larger particle columns to 1.8 µm columns. Higher temperature decreased mobile phase viscosity, permitting an even higher flow rate and consequently an even faster analysis.

Agilent's ZORBAX RRHD columns are ideal for fast or high-resolution gradient separations. They contain 1.8 µm particles for high resolution and are designed for stability up to 1200 bar for high flow rates on the Agilent 1290 Infinity LC system or other vendor UHPLC's.

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