Agilent Application Solution

Analysis of ascorbic acid, citric acid and benzoic acid in orange juice

Application Note

Food

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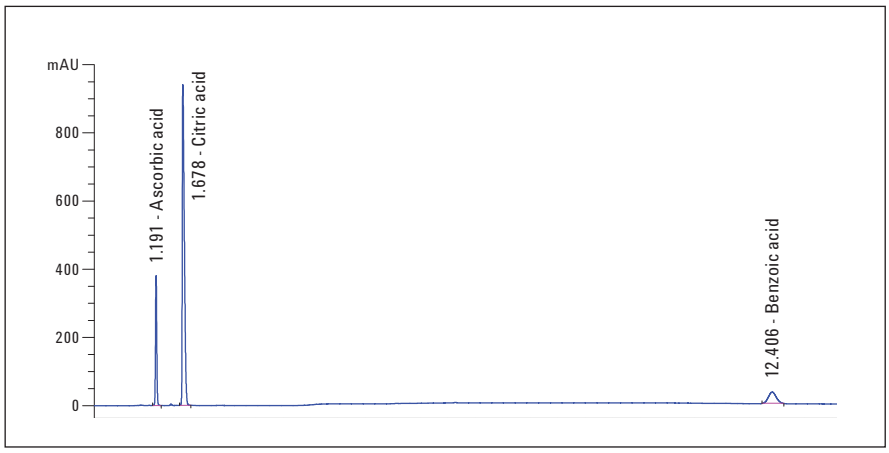

Abstract

Food additives, such as antioxidants and preservatives, are added to increase the shelf life of food items. In this Application Note, we describe a method to quantify an antioxidant (vitamin C) and preservatives (citric acid, benzoic acid) in orange juice. The method was developed on an Agilent 1260 Infinity LC system using an Agilent Poroshell EC-C18 column. Partial method validation was performed in aqueous samples to demonstrate linearity, robustness and precision in area and retention time. The limit of detection (LOD) for benzoic acid was found to be 0.2 µg/mL. During sample recovery studies, greater than 90% recovery was obtained for all three compounds. The method was effectively converted to a short ultra high performance liquid chromatography (UHPLC) method using an Agilent 1290 Infinity LC system. This new method was five times faster with the same LOD for benzoic acid. Both methods can be effectively applied by food manufacturers for quality control of food additives.

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Introduction

Antioxidants, such as ascorbic acid prevent oxidation by decreasing the available oxygen in the environment. Ascorbic acid is preferentially oxidized to the dehydroascorbic acid (DHA) form, thus preventing the oxidation of the matrix. Preservatives, such as citric acid or benzoic acid prevent or inhibit the growth of microorganisms in food. While some fruit naturally contain ascorbic acid, citric acid and benzoic acid[1], these components are added additionally added to fruit juices to increase the shelf life. Although the regulatory limit for benzoic acid in fruit juices is 400 to 600 µg/mL, concerns exist regarding the liberation of carcinogenic benzene by reaction of benzoic acid with ascorbic acid under certain conditions[2,3].

The amount of ascorbic acid is reported to diminish with time, temperature and other factors[4] to DHA. The AOAC Official Method 967.22 describes the analysis of vitamin C content by first oxidizing ascorbic acid to DHA followed by derivatization and fluorescence detection. Regarding UV based analysis, DHA has little absorbance above 220 nm, while ascorbic acid has absorbance from 244–265 depending on the pH of the buffer[5]. The AOAC Official Method 994.11 shows the UV based detection of benzoic acid in orange juice.

In this Application Note, a method is described to simultaneously quantify ascorbic acid, citric acid, and benzoic acid using UV based detection and a simple extraction procedure.

Reagent and materials

All the chemicals and solvents used were HPLC grade. Highly purified water used was from a Milli Q water purification system (Millipore Elix 10 model, USA). Acetonitrile 'super gradient' was purchased from Lab-Scan (Thailand) and potassium phosphate monobasic was obtained from Fluka (Germany). O-Phosphoric acid was purchased from Fluka (Switzerland). Standards of ascorbic acid, citric acid, and benzoic acid were from Sigma-Aldrich (India). International brand named orange juice manufactured in India were purchased.

Experimental

Instruments and Software

An Agilent 1260 Infinity Binary LC system consisting of the following modules was used:

- Agilent 1260 Infinity Binary Pump (G1312B)
- Agilent 1260 Infinity Autosampler and Thermostat (G1367E, G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity Diode Array Detector (G4212B) with 10-mm Max-Light flow cell

The UHPLC analysis was developed and performed using an Agilent 1290 Infinity LC system consisting of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler and Thermostat (G4226A, G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A) with 10-mm Max-Light flow cell

Columns:

- Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7µm (p/n 697975-302)

Software:

- Agilent ChemStation B.04.02

Chromatographic parameters

The chromatographic parameters used for reverse phase liquid chromatography and UHPLC are shown in Table 1.

Preparation of standards

Ascorbic acid, citric acid, and benzoic acid were accurately weighed out and dissolved in mobile phase A to obtain stock solutions of 5,000 µg/mL (ppm), 50,000 ppm and 100 ppm respectively. A 10 minute sonication was required to completely dissolve benzoic acid. Linearity levels were prepared by subsequent dilution from these stock solutions using mobile phase A as shown in Table 2. Mobile phase A has a pH of 2.5 which prevents the conversion of ascorbic acid to other ionization forms.

Sample preparation

An appropriate amount of o-phosphoric acid was added to 5 mL of orange juice, to adjust pH to 2.5 and vortexed. The solution was spun at 1879 x g for 5 minutes and filtered through Agilent Regenerated Cellulose Econofilter, 0.2 µm (p/n 5185-5830). The filtered solution was directly used for sample analysis.

Procedure

A 5 µL amount of mobile phase A was injected as blank, followed by each linearity level in six replicates. Area and retention time (RT) information for each level was used to calculate relative standard deviation (RSD) values. The limit of detection (LOD) and limit of quantitation (LOQ) were established from the lower linearity level injections for benzoic acid. Prior to determining the linearity level, the extracted orange juice was injected to measure the approximate concentration of ascorbic acid, citric acid, and benzoic acid. The average area of each linearity level was plotted against the concentration to obtain a linearity curve.

Chromatographic conditions

Parameter	Agilent 1260 Infinity LC system		Agilent 1290 Infinity LC system	
TCC temperature	20 °C		20 °C	
Acquisition rate	40 Hz		40 Hz	
DAD wavelength (nm)	210.0, 230.0, 243.5		210.0, 230.0, 243.5	
Flow cell	10 mm, 1 µL		10 mm, 1 µL	
Sample thermostat	4 °C		4 °C	
Mobile phase A	20 mM monobasic phosphate buffer (KH_2PO_4), pH 2.5 adjusted by o-phosphoric acid		20 mM monobasic phosphate buffer (KH_2PO_4), pH 2.5 adjusted by o-phosphoric acid	
Mobile phase B	60% methanol - 40% acetonitrile		60% methanol - 40% acetonitrile	
Gradient	Time (min)	%B	Time (min)	%B
	0	5	0	5
	2	5	0.5	5
	2.1	25	0.6	25
	13.0	25	3.0	25
	13.1	90	3.1	70
	18.0	90	3.9	70
	18.1	5	4.0	5
	25.0	5	5.0	5
Flow	1.0 mL/min		1.5 mL/min	
Injection volume	5 µL with 3.0 s flush port wash		4 µL with 5.0 s flush port wash	

Table 1
Chromatographic parameters used for the Agilent 1260 Infinity LC and Agilent 1290 Infinity LC systems.

Concentration levels	Ascorbic acid (µg/mL)	Citric acid (µg/mL)	Benzoic acid (µg/mL)
1	10	5500	0.2
2	45	6000	1
3	63	6500	2
4	90	7000	3
5	108	7500	5
6	144	8000	10
7	162	8500	20
8	180	9000	35
9	225		50

Table 2
Dilution table for the three analytes.

To perform the recovery studies, the pH of the orange juice was adjusted to 2.5. Low and high amount of vitamin C, citric acid, and benzoic acid were spiked to obtain low and high concentration spiked samples. The difference between the high and low concentration spiked samples was used for recovery calculations. To evaluate the robustness of the method, four critical method parameters were changed – flow rate ± 2%, TCC temperature ± 5%, injector ± 5%, and wavelenth ± 3%.

For each variation, a standard spike mix concentration of 108 ppm of vitamin C, 7,000 ppm of citric acid, and 5 ppm of benzoic acid were injected in seven replicates. Three different brands of orange juices were analyzed to determine the concentration of the three acids.

The method was then effectively transferred to an UHPLC method. LOD, LOQ and linearity of each standard was evaluated and precision of the method was established by Area and RT RSD.

Results and Discussion

Separation and detection

The separation of vitamin C, citric acid, and benzoic acid were tested on various columns. Samples used were standards spiked into orange juice and standards dissolved in mobile phase A to determine matrix interference. Agilent's phenyl-hexyl and Poroshell EC-C18 column showed good separation for aqueous standards.

Agilent Poroshell EC-C18 was used for further experiments. A low temperature of the TCC, 60% methanol –40% acetonitrile in mobile phase B gave better separation of standards from the matrix peaks. Ascorbic acid has the maximum peak absorbance at 243.5 nm in its acidic form at pH 2.5. Ascorbic acid is easily quantifiable since there are less absorbing matrix peaks at this wavelength. Citric acid was monitored at 210.0 nm while benzoic acid was monitored at 230.0 nm. Since ascorbic acid is stable at low temperatures, the autosampler was maintained at 4 °C during the analysis.

Margolis et al.[6], reported a significant drop in ascorbic acid concentration when stored in autosampler vials for 22 hrs. A loss of 89% in the concentration of ascorbic acid was shown in different lots of autosampler vials, however vials cleaned by a base-acid wash procedure described by Margolis showed a maximum loss of only 4%.

In this study, three different vials were tested:

- MS verified vials (p/n 5190-2280)
- ALS vials (p/n 5182-0716)
- ALS vials cleaned by base-acid wash procedure

Since the calibration standards were stored in buffer at 4 °C in thermostatted ALS, after 16 hours, ascorbic acid showed 3% loss in area, while citric acid and benzoic acid showed less than 1% loss in area in all three vials. This study suggests that any of the vials can be used in this analysis. For this application, MS verified vials were used. Figure 1 shows the chromatogram of ascorbic acid, citric acid, and benzoic acid separated using an Agilent 1260 Infinity LC system. A step gradient to 25% mobile phase B was necessary to elute out benzoic acid away from the matrix peak. A hold at higher percentage organic beyond 13 minutes was necessary to remove matrix peaks from orange juice.

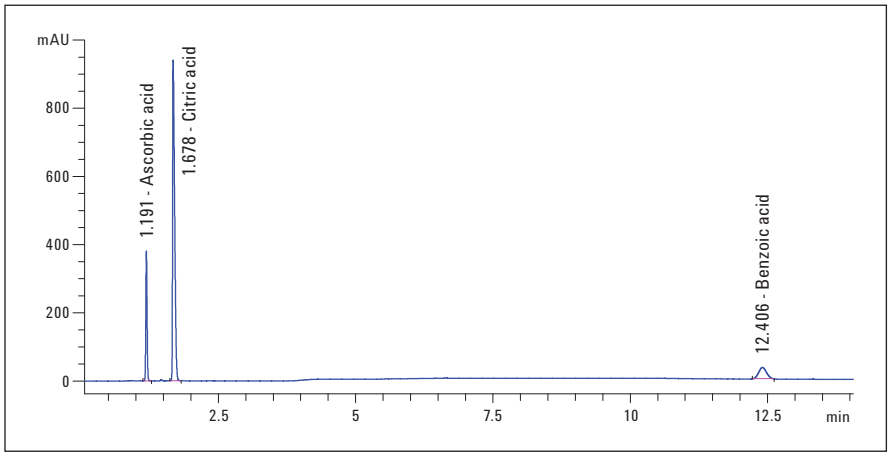

Figure 1
Separation of three standards ascorbic acid, citric acid and benzoic acid using an Agilent Poroshell 120 EC-C18 column. The chromatogram was collected at 230 nm.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The analyte concentration that provides a signal to noise ratio (S/N) of > 3 was considered as LOD and analyte concentration with S/N > 10 was considered as LOQ. Peak to peak method was used to calculate noise and compared with analyte peak height to obtain S/N values. In this application note, benzoic acids' LOD and LOQ were measured. LOD was 0.05 µg/mL with S/N = 3 and LOQ was 0.2 µg/mL with S/N = 16.

Linearity

Linearity curves with different concentration ranges were plotted for the three compounds. For benzoic acid, the linearity level was established starting from the LOQ level. Each linearity solution was injected six times and its average was used to construct the calibration curve. The linearity ranges cover the compounds' amount in orange juice. The linearity level for ascorbic acid is displayed in Figure 3. LOD and LOQ values, along with the linearity results are included in Table 3.

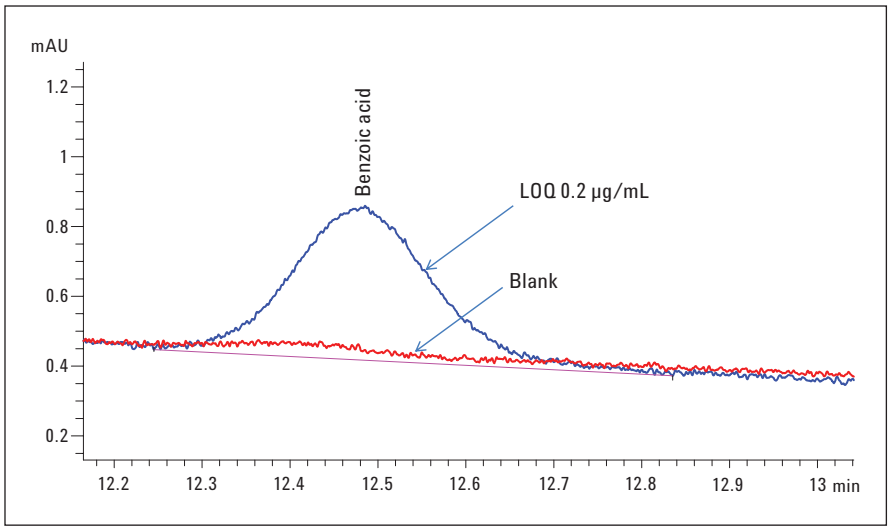

Figure 2
A 0.2 µg/mL (1 ng on column) solution of benzoic acid (at LOQ level) overlaid with blank injection. S/N ratio obtained at this concentration was 16.

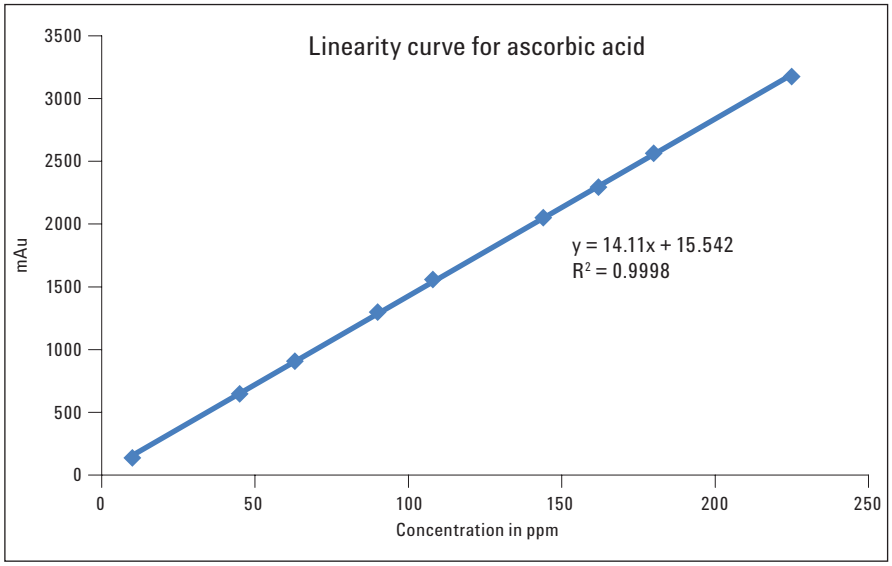

Figure 3
Linearity level of ascorbic acid from 10 µg/mL to 225 µg/mL showing the coefficient value.

Sl No:	Name	LOD µg/mL	LOD S/N	LOQ µg/mL	LOQ S/N	Linearity range (µg/mL)	R^2 value	Levels, replicate = 6	Accuracy
1	Ascorbic acid	–	–	–	–	10–225	0.9998	9	L1=87% (98%–100%)
2	Citric acid	–	–	–	–	5500–9000	0.9995	8	99%–101%
3	Benzoic acid	0.05	3	0.2	16	0.2–50	1	9	L1 = 106% 96–101%

Table 3
Linearity levels for ascorbic acid, citric acid and benzoic acid. The linearity range tested covers the compounds' content in orange juice.

Precision of retention time (RT) and area

The area precision was measured as RSD (%) across the linearity levels. The maximum RSD value of 1.9% for level 1 (L1) is obtained for benzoic acid as shown in Figure 4. Similarly, RT precision calculation showed a maximum RSD value of only 0.13%. The low RSD values for area and RT show acceptable reproducibility and precision of the method. Graphical representation of area RSD values is shown in Figure 4.

Robustness

To test the robustness of the method, a standard mix solution containing 108 µg/mL of ascorbic acid, 7,000 µg/mL of citric acid and 5 µg/mL of benzoic acid was used. Four critical method parameters (flow, TCC temperature, injector volume, and wavelength) were tested and data was collected in seven replicate injections. Analyte response areas from the last six replicates were used for the analysis. Allowed deviation for the area and retention time was set to ± 5% and ± 3% respectively.

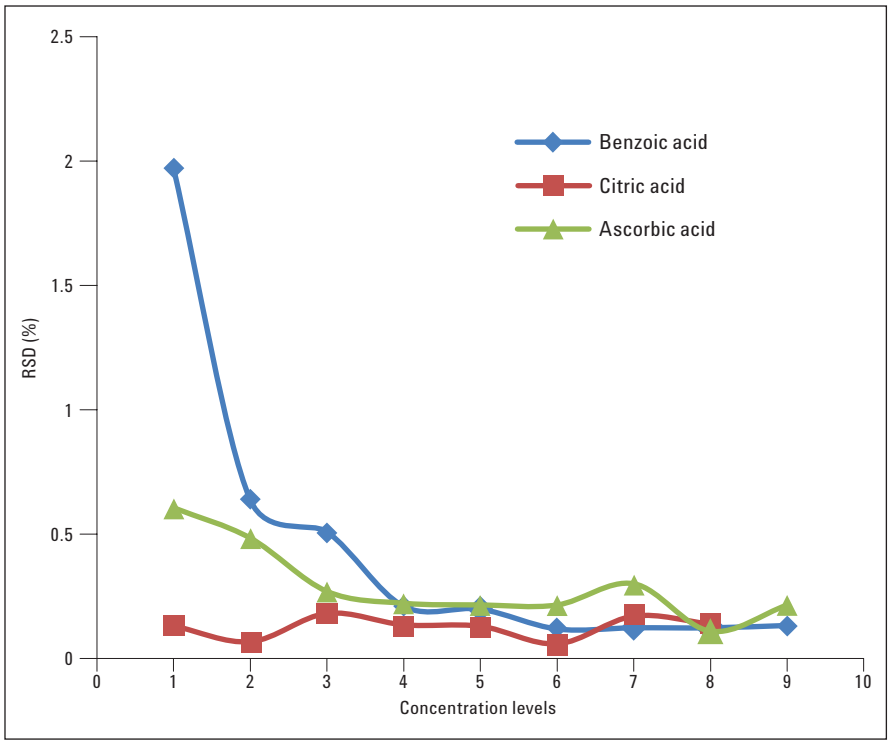

Figure 4
Area precision measured as RSD (%) for six replicates at each concentration level.

Parameters	Changes	Ascorbic acid % area	Ascorbic acid % RT	Resolution of citric acid at 230 nm % resolution	Citric acid % area	Citric acid % RT	Benzoic acid % area	Benzoic acid % RT
Flow: 1.0 mL/min ± 2%	High: 1.02 mL/min	6.2	2.1	0.0	2.2	2.2	3.4	1.8
	Low: 0.98 mL/min	3.3	1.8	0.6	1.2	1.3	0.5	1.7
TCC: 20 °C ± 5%	High: 21 °C	7.6	0.4	0.1	0.1	0.6	2.0	1.4
	Low: 19 °C	18.1	0.5	0.7	0.4	0.9	2.7	1.5
Injector: 5 µL ± 5%	High: 5.25 µL	2.4	0.2	1.6	5.6	0.3	4.3	0.9
	Low: 4.75 µL	8.2	0.0	0.5	5.0	0.2	6.1	0.2
Wavelength: 210.0, 230.0, 243.5 nm ± 3 nm	High: 213.0, 233.3, 246.5 nm	2.5	0.1	0.1	5.6	0.2	5.2	0.5
	Low: 207.0, 227.0, 240.5 nm	3.3	0.1	0.8	3.3	0.1	3.4	0.3

Table 4
Robustness test method results. The red numbers in Table 4 indicate that the allowed deviation was exceeded more than the allowed limit compared to the standard method.

The results of the robustness tests are summarized in Table 4. The red numbers indicate combinations where the allowed deviation was exceeded. A flow rate change of 2% results in a change in both area and RT for ascorbic acid. However, the peak area exceeds the allowed limit of 5% while the RT remained within the acceptable limits. Similarly, for ascorbic acid, the peak area is also found to have the greatest deviation caused by small variations in TCC temperature. These results show the importance of maintaining the column temperature during the analysis. Area reproducibility for citric acid and benzoic acid were found to be affected most by injector precision and wavelength accuracy. It is important that UV DAD is appropriately calibrated and passes accuracy tests. Robustness results indicate that the method is reliable for normal usage and to a great extent the performance remains unaffected by deliberate change in parameters. However, some parameters are critical and must be carefully controlled.

Recovery from sample matrix

As the blank matrix was not available, the recoveries of the three analytes were tested by spiking experiments. A low concentration standard spike solution contained ascorbic acid (300 µg), citric acid (4,000 µg) and benzoic acid (20 µg) spiked into orange juice (pH adjusted to 2.5 using o-phosphoric acid). Another high concentration spiking mix containing ascorbic acid (600 µg), citric acid (8,000 µg), and benzoic acid (40 µg) was also spiked into a separate orange juice sample. The analytes were extracted from the orange juice sample as described above. Using the aqueous linearity curve (see paragraph "Linearity" and Figure 3 on page 5), the area was converted to concentration values. The low concentration spiking mix concentration was subtracted from the high concentration value and the difference was compared with the difference in spiking amounts to obtain recovery values. This difference method would account for the degradation of any compound/matrix during analysis. The recovery experiment was performed in triplicate and the results are shown in Table 5. Ascorbic acid shows excellent recovery because of its unique absorbance maximum at 243.5 nm where background absorbance was minimum. Greater than 90% recovery for all three analytes were observed.

Compound name	Recovery (%)
Ascorbic acid	100 ± 3
Citric acid	91 ± 12
Benzoic acid	98 ± 6

Table 5
Recovery values results from spiking experiment performed in triplicates.

Sample analysis

In this study, the content of ascorbic acid, citric acid, and benzoic acid in orange juice was determined using the extraction procedure and the developed chromatographic method. Orange juices, labeled here as O-juice 1, O-juice 2, and O-juice 3, were analyzed using three different aliquots from each orange juice. Samples were prepared as described above. The results of the analysis were compared against the linearity equation to obtain the concentration values (Table 6) and show that the amount of the three analytes varies with the brand of orange juice. The O-juice 1 nutrition label claims 111 µg/mL of ascorbic acid. The actual amount found was 145 µg/mL. This is probably because ascorbic acid is also naturally present in orange juice thereby increasing the content level, or due to a batch variation in the product. O-juice 1 also claims to add no preservatives however, a trace amount of benzoic acid was found. O-juice 3 claims to add (without specifying the amount) antioxidant E300 and acid regulator E330 which corresponds to ascorbic acid and citric acid respectively. Both of these compounds were detected and quantified using this method. However, 2.25 µg/mL of benzoic acid was also detected in this sample as shown in Figure 5.

Orange juice samples	Ascorbic acid µg/mL	Citric acid µg/mL	Benzoic acid µg/mL
O-juice1	145 ± 2	8895 ± 21	0.62 ± 0.07
O-juice 2	93 ± 3	8188 ± 43	0 ± 0
O-juice 3	35.4 ± 0.3	3160 ± 8	2.25 ± 0.06

Table 6
Amount of ascorbic acid, citric acid and benzoic acid present in three different brands of orange juice. (Note that the added amount of most of these compounds are not specified on the nutrition label).

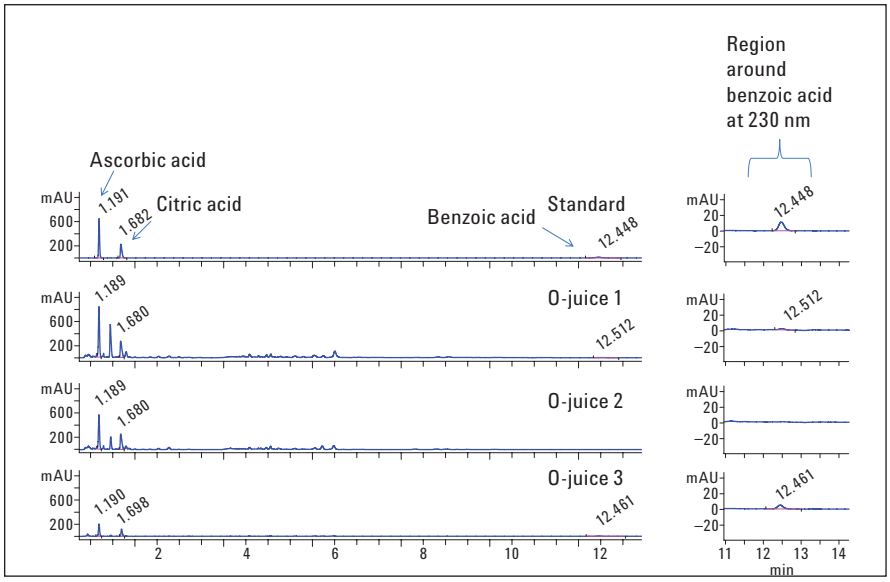

Figure 5
Three different orange juice brands analyzed at 230 nm for benzoic acid.

UHPLC Method

The HPLC method was transferred to an UHPLC method on an Agilent 1290 Infinity LC system. It was developed to provide a shorter and faster method using the same mobile phase but a shorter column. The resulting UHPLC method is only five minutes long as shown in Figure 6 compared to 25 minutes in the HPLC method. This 5× increase in speed results in 68% of solvent saving. A quick method also overcomes the possible loss of ascorbic acid during sample analysis. Note that the maximum percentage of solvent B in the UHPLC method goes up to 70% rather than 90%. Both 90% and 70% were tested and each can be used for the Agilent 1290 LC Infinity method. The LOD for benzoic acid is 0.05 µg/mL while the LOQ is 0.2 µg/mL (S/N =16), which is similar to that obtained by the Agilent 1260 Infinity LC method. The calibration curve for ascorbic acid was found to be linear as shown in Figure 7.

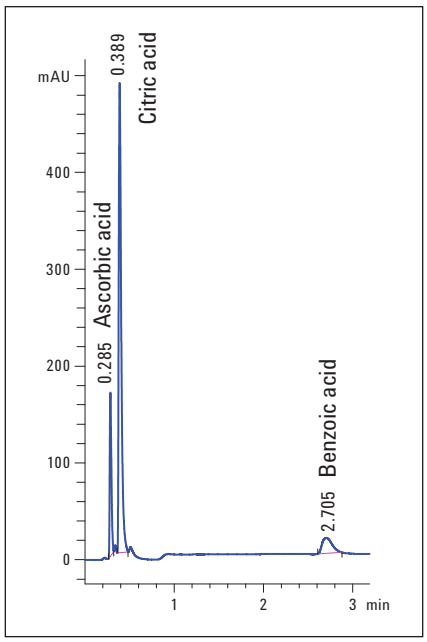

Figure 6
An UHPLC method separating the standards for ascorbic acid, citric acid, and benzoic acid on an Agilent Poroshell 120 EC-C18 column 3.0 × 75 mm, packed with 2.7-µm particles.

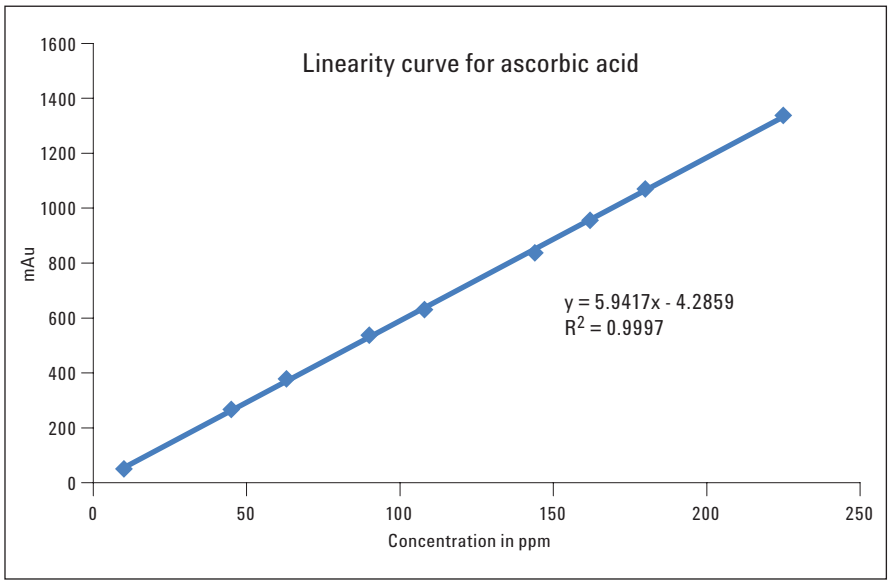

Figure 7
Linearity level of ascorbic acid from 10 µg/mL to 225 µg/mL (50 ng to 1125 ng on column) showing the coefficient value on an Agilent 1290 Infinity LC System.

RSD(%) deviation on area and RT was calculated for concentration levels same as in HPLC method. The results show that RSD(%) on area deviation was less than 2.5% for all concentration levels, as shown in Figure 8 while RSD on RT was less than 0.5%.

Conclusion

Ascorbic acid, citric acid and benzoic acid were separated and quantified using Agilent Poroshell 120 EC-C18 columns. A partially validated 25 minute method was developed. This method quantifies ascorbic acid, citric acid and benzoic acid in various orange juices with little or no matrix interference as seen by >90% recovery values. A method transfer to an Agilent 1290 Infinity LC system was effectively carried out by increasing the flow rate and using a smaller length column resulting in a 5 minute UHPLC method. Both the methods are linear and give precise results. These methods can therefore be applied to determine ascorbic acid, citric acid and benzoic acid in quality control of orange juices.

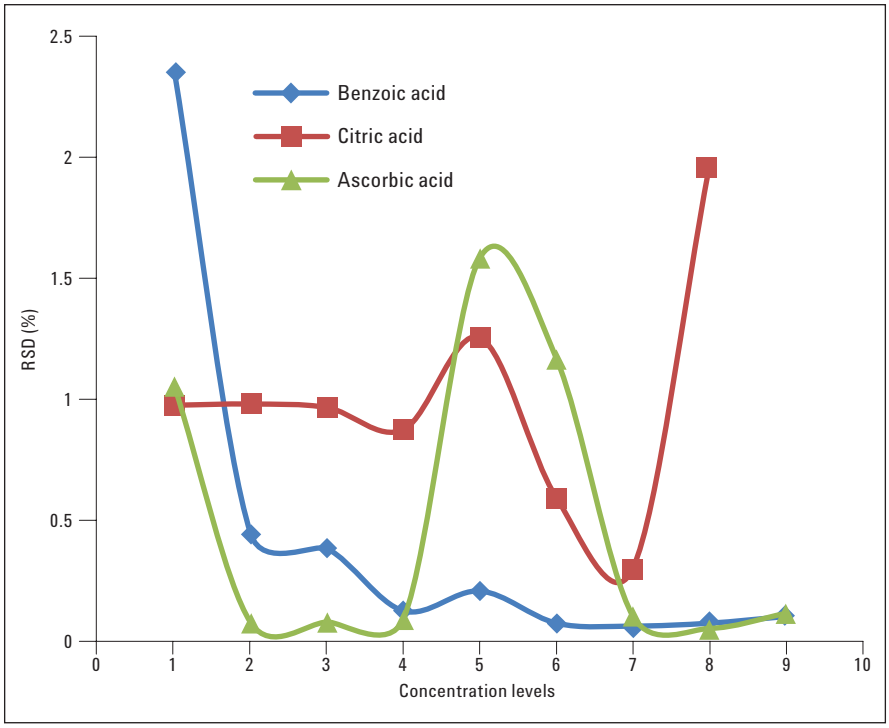

Figure 8
An UHPLC method showing area precision measured as RSD (%) for three different compounds, six replicates for each concentration level.

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