Comparison of Two Methods for the Analysis of Organic Acids

Cassandra Taylor[1], Kendon Graham[2], Ramin Jahromi[1] and Dr. David Hammond[3]

[1]Eurofins Nutrition Analysis Center (ENAC), Des Moines, IA 50321, [2] Waters Corporation, Milford, MA 01757, [3]Eurofins Scientific France, Nantes Cedex 3, F-44323

Abstract

The levels of the organic acids in a fruit juice are important to the assessment of authenticity. Two separate approaches are routinely used; HPLC linked with either UV or conductivity detection, or enzymatic procedures specific for each acid.

HPLC-UV-Vis is used widely in the US for this analysis. There are AOAC (986.13) and International Fruit Juice (IFU 65) methods available. As the acids absorb in the far UV, it is hard to get a definitive UV spectrum that makes positive identification difficult when dealing with low levels, which is a limitation.

The enzymatic methods show very good specificity and sensitivity, with detection limits typically around 10 mg/L or higher depending on the dilution used. However, only one acid can be measured at a time, which makes the procedure more time consuming to use. The kits also have limited shelf life, after opening, which increases analysis costs with limited numbers of samples.

This poster presents a comparison of the AOAC method with a new UPLC-MS/MS procedure that offers much better specificity than the UV approach and similar or better detection limits to the enzymatic methods. Using Waters UPLC technology it also significantly reduces analysis times, therefore improving sample throughput.

Comparable results were obtained for a number of juices. The repeatability was found to be similar for both methods. The MS/MS method was found to have a smaller linear range than the UV method but the improved selectivity easily offsets this limitation.

Background

The adulteration of fruit juices by producers has been a problem for years and remains so to this day. The first successful legal case taken to court in the US was in the early 80's against Beechnut. They were selling a product labeled as "apple juice" but probably contained very little juice at all. Although things have significantly improved since then, there are still cases of adulterated products appearing on the market, e.g. just recently with lemon juice.

Many different approaches have been used to try and eradicate these problems, from simple HPLC procedures to look at the individual concentrations of the sugars and organic acids, to complex isotopic methods to assess the internal ratios within the juice. The level of testing sophistication required depends on what approach the producer is using and can follow a cyclic pattern where people seem to "forget" what can be detected!

While the price of fruit juice solids are significantly higher than that of sugar and acids, users of juice must remain vigilant to ensure that they are not using adulterated product. This vigilance should include audits of suppliers and testing of their products on a regular basis. If there is no expertise within the company to carry out these audits then third parties can be contracted (such as www.Eurofinsus.com) or materials can be purchased from an IRMA (International Raw Material Assurance) approved supplier. IRMA approved companies undergo an independent check every two years to ensure they are producing acceptable products. Further information about this scheme can be found here: http://www.sgf.org/en/home/fks/halbwarenkontrolle/

As highlighted above, one of the important sets of parameters to look at are the organic acids. These can be measured by HPLC using UV-Vis detection, ion chromatography or enzymatically, but all these procedures have some limitations. This poster describes another very specific procedure by which these compounds can be quantified. This uses chromatography linked with mass spectrometry, which provides a very sensitive and specific detection system. The poster compares the results from this approach using UPLC and mass spectrometric detection with those for the conventional AOAC method 986.13.

HPLC Setup

HPLC: Waters 2695 alliance Separation Module
Detector: Waters 2996, Photodiode Array Detector
Column: Restek Allure Organic Acids 5µm, 300 x 4.6mm
Total Runtime: 50 min
Injection Volume: 20µL
Column Temperature: 30.0°C
Sample Temperature: 10.0°C

UPLC Setup

UPLC: Waters ACQUITY H-Class
Detector: Waters Xevo TQD
Column: Waters BEH C18 1.7µm, 2.1 x 100mm
Total Runtime: 12 min
Injection Volume: 1µL
Column Temperature: 30.0°C
Sample Temperature: 10.0°C

Materials and Methods

HPLC with UV-Vis detection method

Mobile Phase:	A = 50mM potassium phosphate, pH 2.5 B = 2% methanol in HPLC water C = Acetonitrile				
Gradient:	Time (min)	Flow Rate (mL/min)	% Mobile Phase A	% Mobile Phase B	% Mobile Phase C
	Initial	0.800	100	0	0
	35.00	0.800	100	0	0
	35.10	0.800	0	25	75
	40.00	0.800	0	25	75
	40.10	0.800	100	0	0
	50.00	0.800	100	0	0

Detection:	Waters 2996 PDA
Wavelength:	226nm
Sampling Rate:	0.50 points/s

UPLC with MS/MS detection method

Mobile Phase:	A = Deionized Water B = 1% Formic Acid in Water C = Acetonitrile				
Gradient:	Time (min)	Flow Rate (mL/min)	% Mobile Phase A	% Mobile Phase B	% Mobile Phase C
	Initial	0.350	50	50	0
	1.15	0.350	50	50	0
	2.30	0.350	30	30	40
	2.90	0.350	30	30	40
	2.91	0.350	0	0	100
	3.45	0.350	0	0	100
	3.46	0.350	50	50	0

Detection:	Water Xevo TQD
Source, Mode:	Electrospray, Negative Ion
Capillary Voltage:	1kV
Dwell Time:	0.020s

Channel	Cone	Coll. Energy	Compound
1: 133 > 71	24.0	14.0	Malic Acid (Qualitative)
2: 133 > 115	24.0	10.0	Malic Acid (Quantitative)
3: 149 > 73	28.0	16.0	Tartaric Acid (Qualitative)
4: 149 > 87	28.0	12.0	Tartaric Acid (Quantitative)
5: 191 > 85	40.0	22.0	Quinic Acid (Quantitative)
6: 191 > 87	26.0	18.0	Citric Acid (Qualitative)
7: 191 > 93	40.0	22.0	Quinic Acid (Qualitative)
8: 191 > 111	26.0	10.0	Citric Acid (Quantitative)

Sample Preparation

All samples used in this study were prepared in duplicate at ENAC. The duplicate set was shipped overnight to Milford, MA and the original kept at ENAC.

Fruit Name	Sample Name	Brix *	Source
Apple	1	11.5	Apple concentrate #1
	2	11.5	Apple concentrate #2
	3	11.5	Commercial apple juice
	4	11.5	0.10230g malic + 0.05361g tartaric + 0.05351g quinic + apple concentrate #1
Lemon	1	7.0	Organic Lemon concentrate
	2	7.0	Commercial lemon juice
	3	7.0	Lemon juice from concentrate
	4	7.0	1.00578g citric + 0.10179g malic + organic lemon
Orange	1	11.8	Orange concentrate
	2	11.8	Organic orange concentrate
	3	11.8	Commercial orange juice, pulp-free
	4	11.8	0.10058g citric + 0.10803g malic + orange
Cranberry	1	7.5	Organic cranberry concentrate
	2	7.5	Commercial cranberry juice, 100% pure
	3	7.5	Commercial cranberry cocktail
	4	7.5	0.10762g quinic + 0.50368g citric + 0.10528g
White Grape	1	16.0	White grape concentrate
	2	16.0	Organic white grape concentrate
	3	16.0	Fresh squeezed white grape juice
	4	16.0	0.10568g tartaric + 0.05262g malic + 0.05375g
Pomegranate	1	16.0	Organic pomegranate concentrate
	2	16.0	Pomegranate concentrate
	3	16.0	Commercial pomegranate juice, 100% pure
	4	16.0	0.10166g citric + 0.10169g malic + organic

Sample Preparation

Each of the 24 samples were diluted and filtered at the two respective laboratories in Des Moines and Milford.

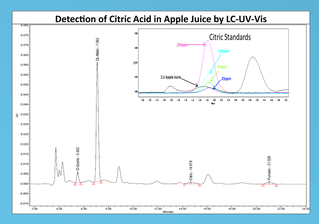
HPLC-UV-Vis		UPLC-MS/MS	
Solvent	HPLC grade water	Solvent	Deionized water
Dilutions	1:1, 1:10 & 1:50	Dilutions	1:100, 1:1000 & 1:10000
Filtration	After dilution by 0.45µm syringeless filter device	Filtration	Prior to dilution by 0.45µm membrane

Standard Preparation

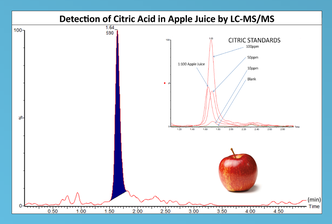
Organic Acid Standards for HPLC-UV-Vis Method		
Name	**Stock (ppm)**	**Dilution Series (ppm)**
L-Tartaric	2048.5	1000, 500, 250, 100, 50, 25
D-Quinic	2033.4	1000, 500, 250, 100, 50, 25
L-Malic	4061.8	3000, 2000, 1000, 500, 250, 100
Citric	2034.2	1500, 1000, 500, 250, 100, 50, 25
Fumaric	533.2*	15, 10, 5, 2, 1
*not injected due to high UV response; only serial dilutions used		
All standards were purchased from ACROS, except L-Tartaric is from TCI.		

Organic Acid Standards for UPLC-MS/MS Method					
Individual Stocks 10000 ppm				**Individual Stocks 1000 ppm**	
Citric	Isocitric	Malonic	Succinic	Benzoic	
Glutaric	Lactic	Quinic	Tartaric	Fumaric	
Isoascorbic	Malic	Shikimic			
A 1000 ppm standard mixture was prepared from stocks above and dilution series was made from 10ppm down to 0.1ppm					

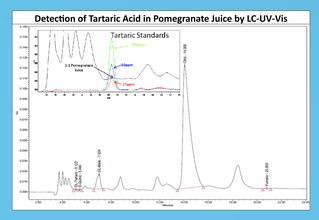
Detection of Citric Acid in Apple Juice by LC-UV-Vis



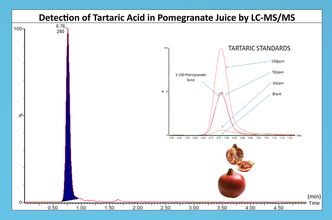
Detection of Citric Acid in Apple Juice by LC-MS/MS



Detection of Tartaric Acid in Pomegranate Juice by LC-UV-Vis



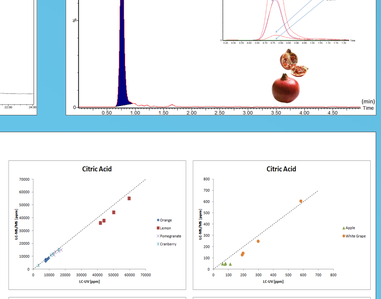
Detection of Tartaric Acid in Pomegranate Juice by LC-MS/MS



Data

Precision, Apple (n=3)	Quinic		Malic		Citric	
	LC-UV	LC-MS/MS	LC-UV	LC-MS/MS	LC-UV	LC-MS/MS
Mean Concentration (ppm)	259	260	2720	2763	105.7	44
Standard Deviation	7.3	12	80	91	5.7	1.1
%RSD	2.8	4.8	2.9	3.3	5.4	2.4

Precision, Apple (n=6)	Quinic		Malic		Citric	
	LC-UV	LC-MS/MS	LC-UV	LC-MS/MS	LC-UV	LC-MS/MS
Mean Concentration (ppm)	259	266	2738	2826	106.5	44.9
Standard Deviation	9.0	11.4	76	115	4.7	1.0
%RSD	3.5	4.3	2.8	4.1	4.4	2.2



PROS & CONS

HPLC-UV Method	
PROS	**CONS**
Low dilutions (i.e. 1:1, 1:10, 1:50) therefore smaller dilution errors	Low sensitivity
Large linear range for UV detector	Long run times (50 min)
Established method, AOAC 986.13	Large injection volume (20 µL)
HPLC column can be re-conditioned to improve separation and peak shapes	LOD significantly degrades below 25ppm (except fumaric acid because of strong UV absorbance)
	Peak shape is variable from sample to sample
	Limited number of analytes (≤ 8) can be analyzed in one 50 minute run
	λ_{max} values for organic acids are too low (200-220nm) to use PDA for confirmation of analytes
	Poor chromatographic separation and non-selective detection raises the chances of interferences e.g. malic acid in pomegranate juice
	Large amount of mobile phase is required (i.e. 500mL per 10 samples), which creates more hazardous waste
	Phosphate buffer degrades instrument parts causing constant maintenance

UPLC-MS Method	
PROS	**CONS**
High sensitivity	High dilutions (i.e. 1:100, 1:1000, 1:10000) create greater possibility for dilution errors
Short run times (12 min)	Smaller linear range than seen with UV detection
Small injection volume (1 µL)	Further method development required to resolve interference associated with quinic acid
Screen ≥ 10 analytes in one 12 min run	
Multiple MRM's can be used to confirm analyte's identity	
Greater separation efficiency effected by UPLC	
LOD <<10ppm	

Discussion

Overall, a good correlation between the two methods was seen. As expected, data provided by LC-MS/MS demonstrated slightly lower calculated concentrations owing to the higher specificity of the technique. One notable exception was the calculated concentration of quinic acid in citrus juices (orange and lemon) where an obvious interference results in a positive bias to reported values. LC-MS/MS is more accurately able to detect lower concentrations of some organic acids such as citric acid in apple juice and tartaric acid, detected in one specimen of pomegranate juice. Also, notably lower values for malic acid in pomegranate juice were reported by LC-MS/MS likely indicating an interference in the LC-UV-Vis method. Recoveries for the organic acids ranged from 71 –160%, with the majority of calculated recoveries lying in the range 90-105%. Analytical precision based upon replicate injections (n=6) of the same apple juice demonstrated nominally higher values for LC-MS/MS owing to the lack of an internal standard, which may be easily corrected.

Conclusions

1) The LC-UV-Vis method has higher linearity than UPLC-MS/MS procedure (requiring fewer sample dilution steps for an "on scale" result).
2) The LC-UV-Vis method is a well established procedure and a recognized method, AOAC 986.13.
3) UPLC offers better resolution than conventional HPLC by reducing the chance of matrix-borne interferences.
4) UPLC-MS/MS is faster; > sample throughput = > productivity.
5) UPLC-MS/MS has better sensitivity than the LC-UV-Vis method allowing the detection of trace levels of organic acids in juices.
6) UPLC-MS/MS is more specific than the LC-UV-Vis method and multiple MRM channels offer greater analytical confidence in quantitative analyses.
7) LC-MS/MS represents a promising alternative to LC-UV-Vis for the quantification of organic acids in fruit juices.

Contacts: Cassandra Taylor—CassandraTaylor@eurofinsus.com, Kendon Graham—Kendon_Graham@waters.com, Ramin Jahromi—RaminJahromi@eurofinsus.com, David Hammond—DavidHammond@eurofins.com