Analysis of Limonin in Citrus Juice Using QuEChERS and LC-MS/MS

UCT Part Numbers

ECQUEU7-MP
Mylar pouch containing 4g $MgSO_4$, 1g NaCl, 1g $Na_3Cit•2H_2O$ and 0.5g $Na_2Cit•1.5H_2O$

CUMPSC1875CB2CT
2mL dSPE tube with 150mg $MgSO_4$, 50mg PSA, 50mg C18, 7.5mg GCB

SLC-18100ID21-3UM
Selectra® C18 HPLC column
100 × 2.1 mm, 3 µm

SLC-18GDC20-3UM
Selectra® C18 guard cartridge
10 × 2.1 mm, 3 µm

SLGRDHLDR
Guard cartridge holder

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Summary:

Limonin is a naturally occurring compound that contributes to the bitter flavor of citrus fruits. It is present in the fruit as the non-bitter precursor molecule limonoate A-ring lactone, which is converted to the bitter limonin by acids that are naturally present in the fruit (Figure 1). Furthermore, citrus juice can increase in bitterness when held at room temperature for an extended period of time due to the delayed conversion of the non-bitter precursor molecule to limonin. Limonin is commonly analyzed in commercial citrus juice as an indicator of overall quality.

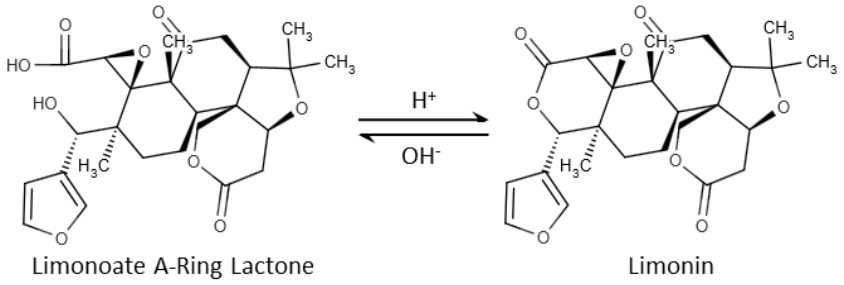

Figure 1. Formation of limonin from limonate A-ring lactone.

This application note outlines a simple, fast and cost-effective QuEChERS-based method for the determination of limonin in citrus juice. Limonin is extracted from a variety of juice samples using acetonitrile and citrate-buffered salts. The sample undergoes cleanup by dispersive-SPE (dSPE) using primary-secondary amine (PSA), C18 and graphitized carbon black (GCB) to remove unwanted matrix components, including sugars, acids and pigments, and yields a clear final extract. Analysis is performed by LC-MS/MS using a Selectra® C18 HPLC column (although HPLC-UV can also be used). A recovery study was carried out by spiking various citrus juice samples (lemon, lime, orange and grapefruit), that naturally contain limonin, at a concentration of 10 µg/mL. Matrix-matched calibration curves, ranging from 1-20 µg/mL, were used for quantitation. The mean recovery was found to be in the range of 88 to 97%, while repeatability was less than 7.5%.

QuEChERS Procedure:

Sample Extraction:

1. Allow citrus juice to come to room temperature and thoroughly mix the sample before analysis.
2. Transfer 10 mL of juice into a 50 mL polypropylene centrifuge tube.
3. Add 10 mL of acetonitrile.
4. Add the contents of the **ECQUEU7-MP** Mylar pouch and vortex or shake (manually or mechanically) for 5 minutes.
    - For this work a SPEX® SamplePrep® 2010 Geno/Grinder® was used (1500 rpm).
5. Centrifuge the samples at ≥3000 rcf for 5 minutes.

Sample Clean-Up:

1. Transfer 1 ml of supernatant to a **CUMPSC1875CB2CT** 2 mL dSPE tube.
2. Vortex the samples for 30 seconds.
3. Centrifuge the samples at ≥3000 rcf for 5 minutes.
4. Transfer 500-600 µL of purified supernatant into an autosampler vial for analysis.

LC-MS/MS Parameters:

Table 1. Instrumentation	
HPLC system	Thermo Scientific™ Dionex™ Ultimate™ 3000
MS system	Thermo Scientific™ TSQ Vantage™ (MS/MS; APCI⁺)
HPLC column	UCT Selectra® C18, 100 × 2.1 mm, 3 µm (p/n: SLC-18100ID21-3UM)
Guard column	UCT Selectra® C18, 10 × 2.1 mm, 3 µm (p/n: SLC-18GDC20-3UM)
Guard column holder	p/n: SLGRDHLDR
Column temperature	40°C
Flow rate	300 µL/min
Injection volume	1 µL

Table 2. Mobile Phase Gradient		
Time (min)	**Mobile Phase A** **Water + 0.1% formic acid**	**Mobile Phase B** **Methanol + 0.1% formic acid**
0.0	90	10
3.0	0	100
6.0	0	100
6.1	90	10
10.0	90	10

Table 3. MRM Transitions					
Compound	**t_R (min)**	**Precursor ion**	**Product ion 1**	**Product ion 2**	**Product ion 3**
Limonin	4.7	471.21	105.03	161.05	425.27

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Results and Discussion:

For this application note, analysis of limonin was performed by LC-MS/MS using a Selectra® C18 HPLC column. Atmospheric pressure chemical ionization (APCI) was found to give significantly better signal response than electrospray ionization (ESI). In APCI the most intense signal response was generated in positive mode ($APCI^+$), forming the protonated molecular ion $[M+H]^+$ at *m/z* 371, whereas in ESI the most intense signal response was generated in negative mode (ESI^-). Furthermore, in both APCI and ESI it was found that the use of methanol in the mobile phase generated a higher signal response compared to the use of acetonitrile, regardless of the mobile phase additive used. HPLC-UV can also be used for limonin analysis but it may necessitate a slightly longer gradient time to ensure adequate separation between limonin and any potential interfering matrix components. Due to the higher specificity of LC-MS/MS a much shorter run time can be used. Furthermore, since the concentration of limonin in citrus juice is traditionally high (ppm levels), the excellent sensitivity obtained with LC-MS/MS requires that only a small injection volume (1 µL) is required for analysis (alternatively, the sample can be diluted with deionized water prior to analysis and a larger injection volume used).

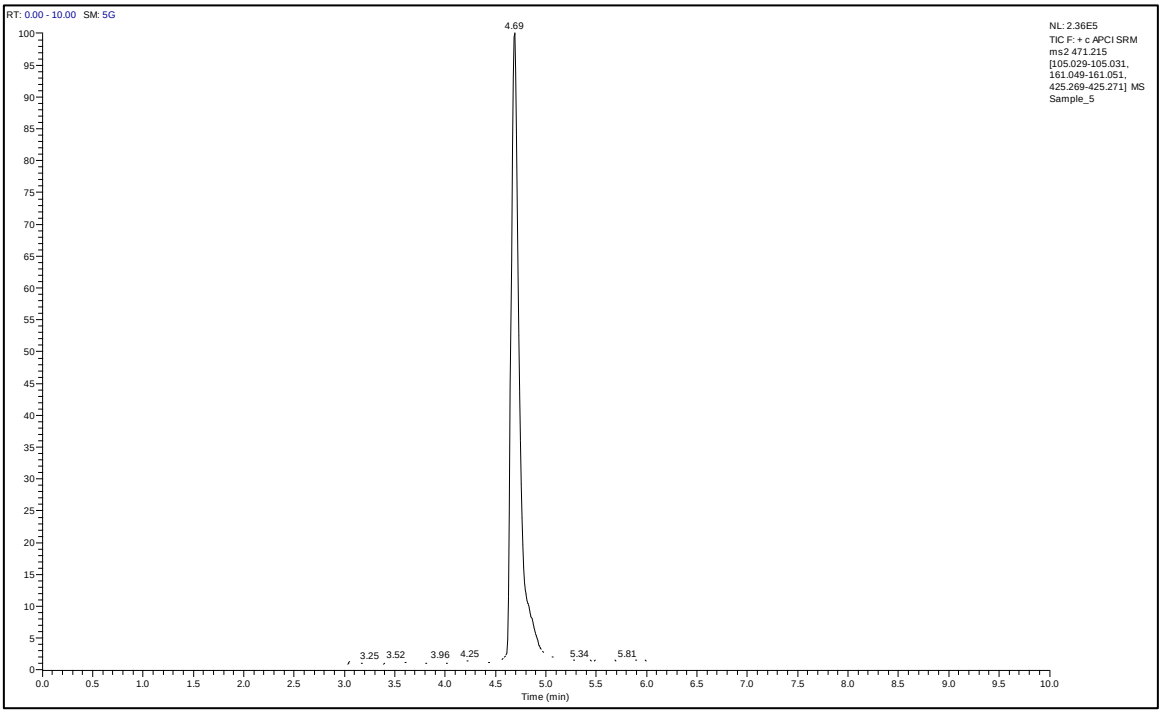

Figure 2. Chromatogram of an extracted grapefruit juice sample fortified at 10 µg/mL.

Limonin is generated from limonoate A-ring lactone in an acidic environment, although it is possible for limonin to convert back to limonoate A-ring lactone in an alkaline environment. Since citrus fruits (and juices) are naturally acidic, pH adjustment is generally not required during sample extraction. Nevertheless, for this study buffered QuEChERS salts were used to normalize the sample pH during extraction and reduce any variability that could be encountered due to the differences in pH of the various citrus juices tested. For sample cleanup, dSPE using a combination of PSA, C18 and GCB generated a clean extract by removing unwanted matrix components, including sugars, acids and pigments (Figure 3).

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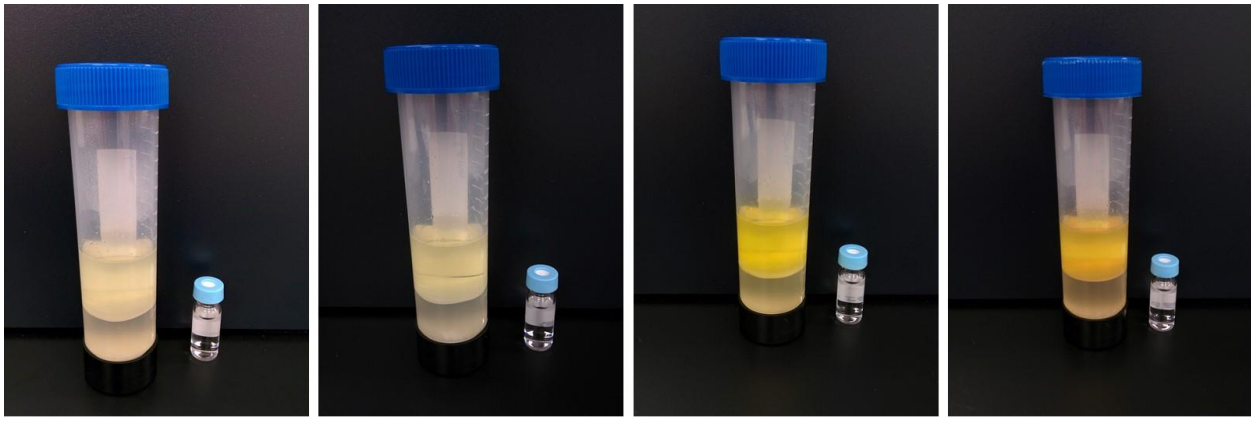

Figure 3. Photographs of the four citrus juice samples in 50 mL centrifuge tubes after QuEChERS extraction (left), and the purified sample extracts in autosampler vials after undergoing dSPE cleanup (right).

Since citrus juice naturally contains limonin it is not possible to obtain a "true" blank sample for conducting recovery experiments. Instead, thoroughly homogenized citrus juice was fortified with known concentrations of limonin standard and the recovery generated using matrix-matched standards generated from the same juice sample containing the equivalent background concentration of limonin (i.e. standard addition).

- Samples were fortified with limonin at 10 µg/mL (50 µL of a 2 mg/mL acetonitrile standard).
- Six-point matrix-matched calibration curves were prepared at 1, 2, 5, 10, 15 and 20 µg/mL. Non-fortified juice samples were extracted according to the QuEChERS procedure described above and 1 mL of purified extracts were fortified with 5, 10, 25, 50, 75 and 100 µL of a 200 µg/mL acetonitrile standard.

Table 4. Accuracy and Precision Data

	Lemon Juice	Lime Juice	Orange Juice	Grapefruit Juice
Sample 1	96.47	93.40	91.13	90.90
Sample 2	86.54	95.90	84.29	83.47
Sample 3	99.67	91.66	90.95	95.35
Sample 4	94.57	105.85	78.15	98.60
Sample 5	87.80	*	94.59	92.00
Mean	**93.01**	**96.70**	**87.82**	**92.07**
RSD	**6.08**	**6.56**	**7.48**	**6.16**

*One outlier was omitted from the final result.

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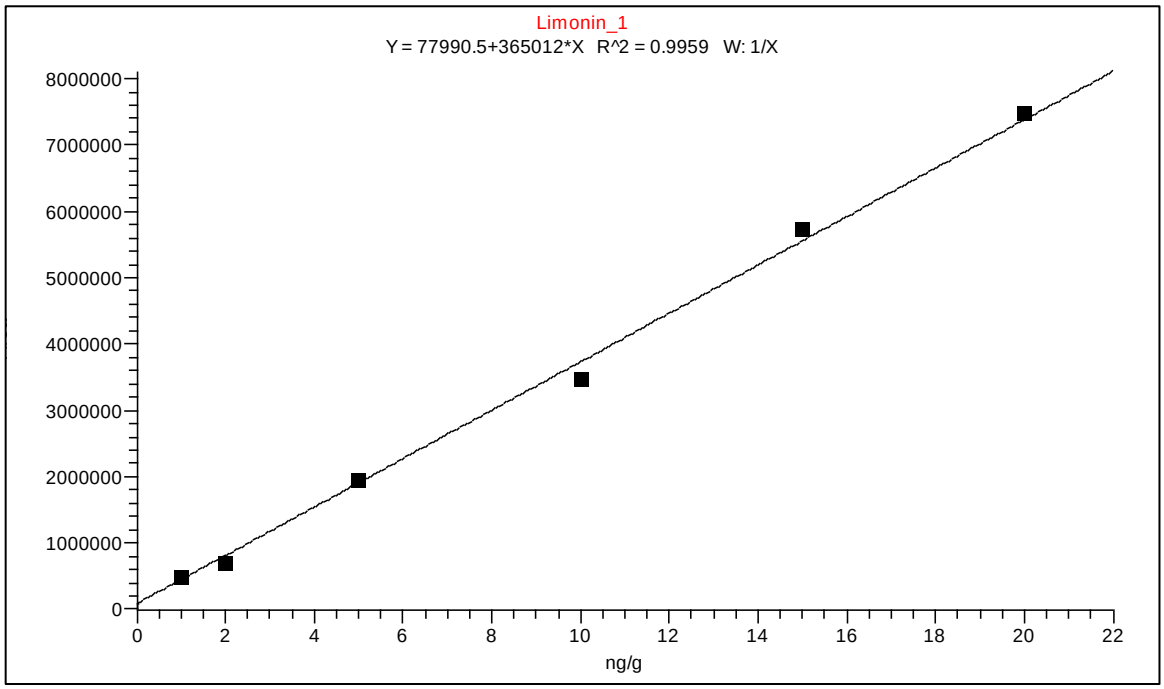

Figure 4. Example of a matrix-matched (orange juice) calibration curve (1, 2, 5, 10, 15 and 20 µg/mL).

Conclusion:

This application note outlines a simple, fast and cost-effective QuEChERS-based method for the determination of limonin in citrus juice. Citrus juice samples are extracted with citrate-buffered QuEChERS salts followed by dSPE cleanup of the supernatant using PSA, C18 and GCB, yielding a clear extract. Analysis is performed by LC-MS/MS using a Selectra® C18 HPLC column. Various citrus juices (lemon, lime, orange and grapefruit) were evaluated for recovery and repeatability using fortified samples. Excellent recovery (88 to 97%) and repeatability (≤ 7.5%) were obtained using the outlined method.

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