Application Note

Determination of Limonin in Citrus Flour by Time of Flight (TOF) LC/MS

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Abstract

In recent years, citrus flour has become a popular additive to meat and dairy plant-based substitutes as it holds water, emulsifies, binds oil, improves texture and stability over time, and is vegan. Citrus flour contains high concentrations of limonin, a limonoid that results in a delayed bitter taste and is undesirable. Industry processes reduce bitter taste in citrus flour by removal or modification of limonin and other limonoids. The method presented here is useful in the quantitation of limonin in foods, food additives, juices, and plants. This method yields good accuracy, precision, linearity, and range in matrix. The use of an Agilent 6230B Time of Flight LC/MS allows limonin to be distinguished and quantitated. Congeners can also be identified and quantitated.

Introduction

The recent rise in popularity of meat and dairy plant-based substitutes have necessitated the use of citrus fiber or flour to replace starches, gums, chemical emulsifiers, and stabilizers. Citrus flour holds water, emulsifies, binds oil, and is vegan. This natural citrus flour improves texture and stability over time and is particularly useful in baked goods, beverages, dairy products, dressings, meats, sauces, frozen foods, pet foods, dairy alternatives, and plant-based meats.

The global citrus flour market size was valued at USD 427.3 million in 2022 and is projected to reach USD 726.9 million by 2032, growing at a compound annual growth rate (CAGR) of 5.7% from 2023 to 2032.[1] The citrus flour market is being driven in part by the increased focus on health and well-being throughout the world. Being a high-quality dietary fiber source, citrus flour supports satiety and digestive health. Citrus fiber is becoming a sought-after component in formulas with a health focus as consumers become more conscious of the connection between nutrition and overall health and seek goods that have beneficial nutritional qualities and taste good.

Limonin is enriched in citrus fruits and is often found in higher concentrations in seeds, pulp and peel of oranges, grapefruit, lemons, limes, pumellos, bergamots, and mandarins.[2] Citrus flour is made from leftover dried fibrous material after the fruit is juiced and can have high concentrations of limonin and other limonoids. Limonin and other limonoid compounds contribute to the delayed bitter taste of some citrus food products and is therefore undesirable.

Limonin is a limonoid: a bitter, white, crystalline substance found in citrus and other plants. It is also known as limonoate, D-ring-lactone, and limonoic acid di-delta-lactone.[3] Chemically, it is a member of the class of compounds known as furanolactones. Limonin has an exact mass of 470.19406791 g/mol.[4]

The method presented in this application note is useful in the quantitation of limonin in foods, food additives, juices, and plants. The use of a Time of Flight LC/MS allows excellent quantitation of limonin as well as the characterization of other congeners.

Limonin
$C_{26}H_{30}O_8$

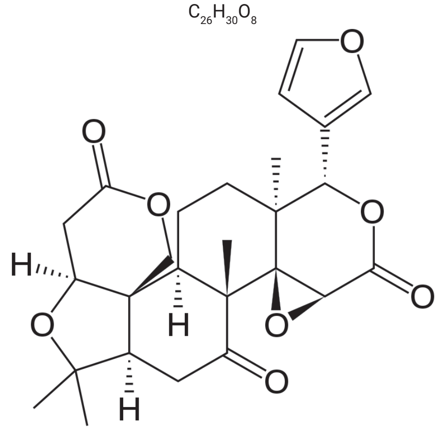

Figure 1. The structure of limonin (2aR,4aR,4bR,5aS,8S,8aS,10aR,10bR,14aS)-8-(Furan-3-yl)-2,2,4a,8a-tetramethyldecahydro-11H,13H-oxireno[2,3-c]pyrano[4'',3'':2',3']furo[3',4':5,6]naphtho[1,2-d]pyran-4,6,13(2H,5aH)-trione.[4]

Experimental

Equipment

All experiments in this study were performed using an Agilent 1290 Infinity II LC consisting of an Agilent 1290 Infinity II multisampler (G7129B), an Agilent 1290 Infinity Flexible pump (G7104A), and an Agilent 1260 Infinity II multicolumn thermostat (G7116A) coupled to an Agilent Time of Flight (G6230B) mass spectrometer. The system was controlled by Agilent Mass Hunter software, version 11. Data processing was performed using the same MassHunter software.

Samples, standards, and consumables

- Agilent EC-C18 column, 3.0 × 100 mm, 2.7 µm (part number 695975-302)
- Agilent LC/MS grade Water (part number 5191-4498)
- Agilent Formic Acid (part number G2453-85060)
- Agilent LC/MS Grade Methanol (part number 5191-4497)
- Biorbyt limonin reference standard (part number orb322562)
- Agilent QuEChERS extract pouch (part number 5982-0650)
- Agilent Dispersive 2 mL Universal kit (part number 5982-0028)
- OmniSolv Acetonitrile LC/MS Grade (part number AX0156-6)

Limonin in acetonitrile

Table 1. Chromatographic conditions.

Parameter	Setting
Analytical Column	Agilent EC-C18, 3 × 100 mm, 2.7 µm (p/n 695975-302)
Column Oven	30.0 °C
Injection Volume	0.1 µL
Run Time	15.00 min
Post-Run Time	5.00 min
Mobile Phase Flow Rate	0.6 mL/min

Table 2. Solvent composition and gradient.

Channel	Solvent
A	Water + 0.1% formic acid
B	Methanol
C	Not used
D	Not used

Table 3. Gradient time table.

Time (min)	%A	%B
0	95	5
10	5	95

Table 4. MS source parameters.

Parameter	Value
Ion Source	Dual ESI
Polarity	Positive
Gas Temperature	350 °C
Drying Gas Flow	8 L/min
Nebulizer	60 psig
Capillary Voltage	3,500 V
Fragmentor	380
Skimmer1	140
Oct 1 RF	750
Mass Range	100 to 1,700
Acquisition Rate	1.0 spectra/sec

Standards preparation: The instrumental portion of this method was evaluated using a standard solution of limonin in acetonitrile. The calibration curves were generated using the load-on-column method to minimize propagation of pipetting errors. The calibration range was chosen to be 12.5 to 1,250 ppm.

Table 5. Limonin standards preparation.

Standard Concentrations (ppm)
12.5
25
50
62.5
100
125
250
500
625
1,000
1,250

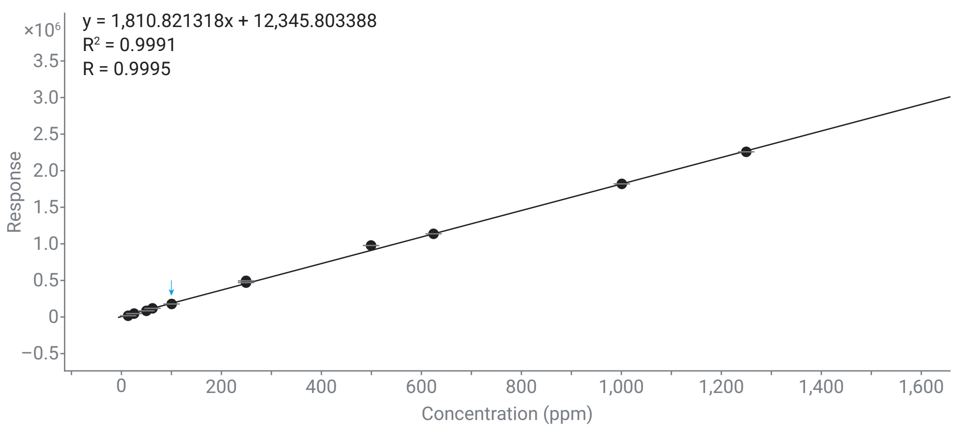

Figure 2. Limonin in acetonitrile calibration curve.

Table 6. Limonin in acetonitrile precision.

Sample	RT	Concentration (ppm)
1	7.624	126.313
2	7.626	125.750
3	7.621	125.352
4	7.624	126.015
5	7.628	125.843
6	7.626	125.087
7	7.625	125.874
8	7.623	125.981
9	7.627	124.986
10	7.625	126.078
11	7.624	124.990
12	7.626	124.412
13	7.623	126.102
14	7.623	125.047
15	7.627	124.862
Mean	**7.625**	**125.580**
STD	**0.001870**	**0.493979**
RSD (%)	**0.02453**	**0.39336**

Table 7. Limonin in acetonitrile accuracy.

Reference Standard Concentration (ppm)	Quanted	Accuracy
125	125.1067	100.0854
250	233.0621	92.22484
375	373.2743	99.53981
500	493.5971	98.71942
625	628.1149	100.4984
875	899.7894	102.8331
1,250	1,247.536	99.80291
Mean Accuracy	–	**99.2434**
STD	–	**2.94585**
RSD (%)	–	**2.968308**

Table 8. Limonin in acetonitrile linearity and range.

Parameter	Value
Slope	1,810.8213
R	0.99954365
R^2	0.99908751
Range	12.5 to 1,250 ppm

Limonin in citrus flour sample preparation

Sample extraction:

1. Mix 1 g of matrix powder with 10 mL of water in a 50 mL centrifuge tube.
2. Add 10 mL of acetonitrile.
3. Add the contents of an Agilent QuEChERS extraction pouch (part number 5982-0650) and vortex or shake manually for 5 minutes.
4. Centrifuge the sample at > 3,200 rcf for 5 minutes.

Sample cleanup:

1. Transfer 1 mL of supernatant to an Agilent Dispersive 2 mL Universal kit (part number 5982-0028) tube.
2. Vortex the sample for 30 seconds.
3. Centrifuge the sample at > 3,000 rcf for 5 minutes.
4. Transfer 500 to 600 µL of purified supernatant into an autosampler vial for analysis.

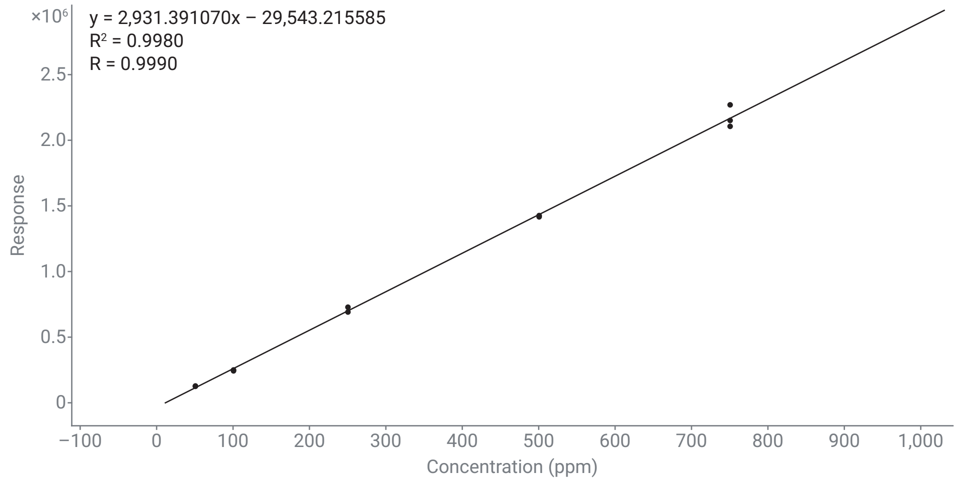

Figure 3. Limonin in citrus flour calibration curve.

Table 10. Limonin in citrus flour linearity and range.

Parameter	Value
Slope	2,931
R	0.9989
R^2	0.9979
Range	50 to 750 ppm

Table 12. Limonin in citrus flour precision statistics.

	Concentration	Retention Time
Average	259.61	8.4489
Standard Deviation	6.7519	0.008212
Relative Standard Deviation	2.6007	0.097%

Table 9. Limonin in citrus flour calibration data.

Calibration Level	Concentration (ppm)	Retention Time (min)
1	50	8.454
1	50	8.437
1	50	8.454
2	100	8.454
2	100	8.437
2	100	8.454
3	250	8.454
3	250	8.454
3	250	8.437
4	500	8.454
4	500	8.471
4	500	8.454
5	750	8.454
5	750	8.454
5	750	8.471

Table 11. Limonin in citrus flour precision.

Replicate Number	RT	Concentration (ppm)	Replicate Number	RT	Concentration (ppm)
1	8.454	245.95	11	8.454	268.31
2	8.454	263.39	12	8.454	265.76
3	8.454	258.74	13	8.454	263.86
4	8.454	256.97	14	8.437	259.77
5	8.454	263.30	15	8.454	269.66
6	8.454	257.71	16	8.454	268.98
7	8.437	263.69	17	8.437	255.30
8	8.454	251.05	18	8.454	258.75
9	8.454	259.32	19	8.437	249.96
10	8.454	249.17	20	8.454	262.61

Table 13. Limonin in citrus flour accuracy data and results.

Reference Standard Concentration (ppm)	Quanted	Accuracy (%)
40	40.541	101.35
60	59.012	98.35
80	77.189	96.49
200	215.055	107.53
400	400.486	100.12
600	593.019	98.84
800	812.998	101.62
Mean Accuracy		**100.61**
STD		**3.53**
RSD (%)		**3.51**

Evaluation of matrix effects:

$\% ME = Slope_{Matrix\ Matched} / Slope_{Solvent} \times 100$

$\% ME = (2,931/1,810) \times 100$

$\% ME = 161.9\%$

Results and discussion

The method presented here for the determination of limonin in citrus flour by time-of-flight (TOF) LC/MS was shown to be linear across typical industrial concentration ranges, both in solvent and in matrix. Significant matrix effects required that a matrix-matched calibration curve be used. This method was found to be accurate and precise, with a precision relative standard deviation (RSD) of 0.097% and an average accuracy of 100.61%. A scan of masses shows many congeners or matrix components (Figure 4).

While limonin is a major component causing a delayed bitter taste, other congeners of interest may also be extracted and quantitated or excluded from consideration. The chromatogram in Figure 5 shows a compound with a similar molecular mass but with a different exact mass. These two peaks are separated by approximately one minute in retention time. While it would not be possible to distinguish this other compound from limonin using a HPLC SIM, the Time of Flight LC/MS spectrometer clearly shows that this second peak is not limonin.

Likewise, desired analytical targets can be distinguished and quantitated. Nomilin is another compound found in citrus products causing a bitter taste (Figure 6).

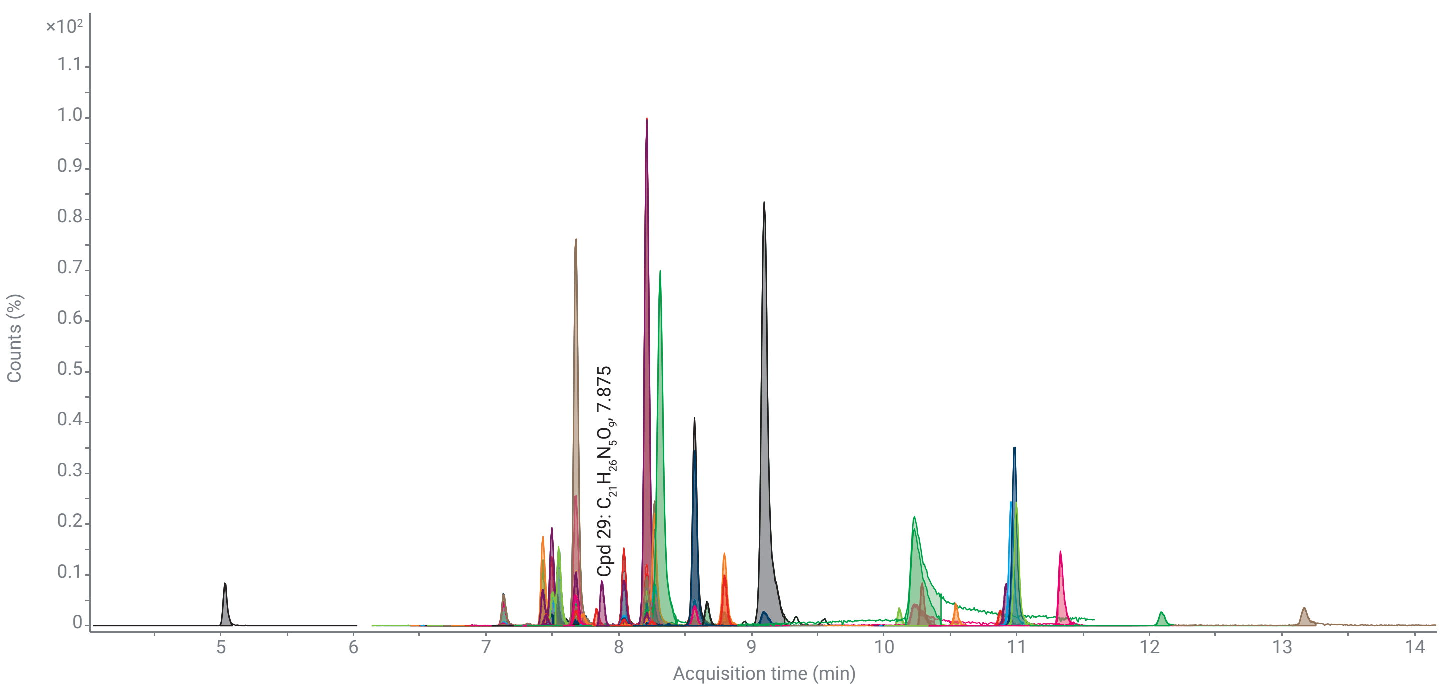

Figure 4. Other matrix components in citrus flour.

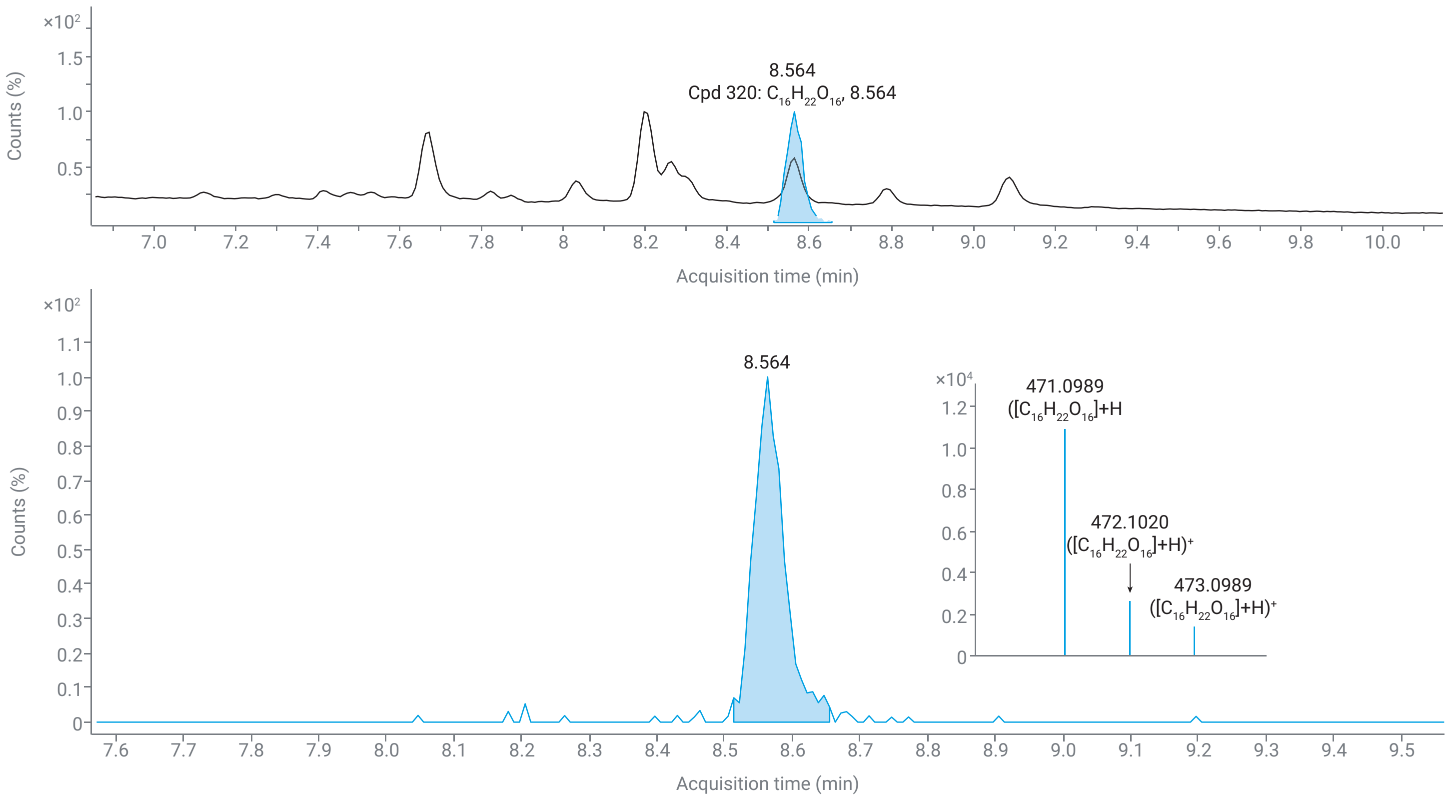

Figure 5. A compound ($C_{16}H_{22}O_{16}$ *m/z* = 471.0942) of similar molecular mass to limonin ($C_{26}H_{30}O_8$ *m/z* = 471.0989) was detected using an Agilent Time of Flight LC/MS.

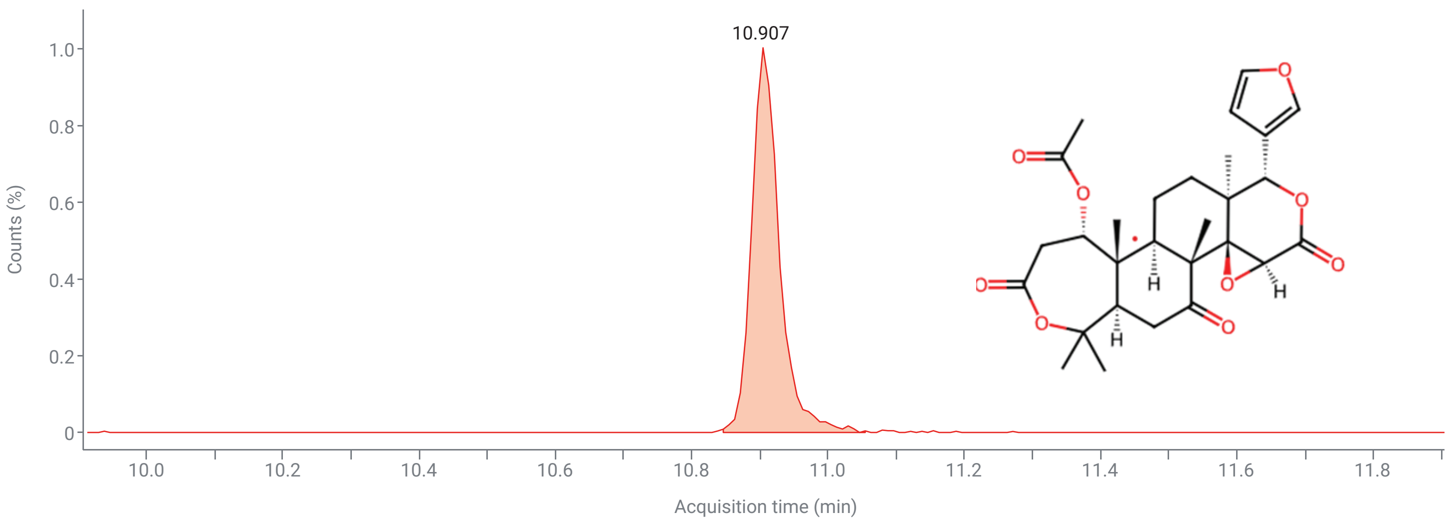

Figure 6. Nomilin in citrus flour.

Screening and data mining

The Time of Flight LC/MS allows the data mining and screening of compounds months and years after chromatographic data were initially obtained.

Phosphodiesterase type 5 (PDE-5) inhibitors are a class of drugs that are used to treat conditions such as erectile dysfunction (ED) and pulmonary hypertension. Certain companies are requiring that natural products and foods be screened for PDE-5 inhibitors, as they are prohibited over-the-counter ingredients. Four months after collecting the data for this study of limonin in citrus flour, the existing data were mined for the PDE-5 inhibitors (Table 14). None were found in the citrus flour sample.

Table 14. PDE-5 inhibitors screened for in citrus flour.

Compound Name	Formula
Acetaminotadalafil	$C_{23}H_{20}N_4O_5$
Acetildenafil	$C_{25}H_{34}N_6O_3$
Avanafil	$C_{23}H_{26}ClN_7O_3$
Homosildenafil	$C_{23}H_{32}N_6O_4S$
Hydroxyacetildenafil	$C_{25}H_{34}N_6O_4$
Hydroxyhomosildenafil	$C_{23}H_{32}N_6O_5S$
Hydroxythiohomo sildenafil	$C_{23}H_{32}N_6O_4S_2$
Lodenafil carbonate	$C_{47}H_{62}N_{12}O_{11}S_2$
Mirodenafil	$C_{26}H_{37}N_5O_5S$
Propoxyphenyl homohydroxysildenafil	$C_{24}H_{34}N_6O_5S$
Sildenafil	$C_{22}H_{30}N_6O_4S$
Tadalafil	$C_{22}H_{19}N_3O_4$
Thiohomosildenafil	$C_{23}H_{32}N_6O_3S_2$
Udenafil	$C_{25}H_{36}N_6O_4S$
Vardenafil	$C_{23}H_{32}N_6O_4S$

Conclusion

The method described in this application note for the determination and quantitation of limonin in citrus flour using an Agilent Time of Flight LC/MS has been shown to be linear, accurate, and precise over typical industrial concentrations. The use of Agilent Time of Flight LC/MS technology also allows the characterization and quantization of congeners and other matrix components. Post-run screening for other compounds is easily accomplished.

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