Sensitive quantitation of 2-acetylfuran-3-glucopyranoside as a marker for rice syrup adulteration in honey

Sensitive detection of as little as 1% (w/w) adulteration of honey using the SCIEX QTRAP 6500+ system

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This technical note describes a simple dilute-and-shoot method for the quantitation of 2-acetylfuran-3-glucopyranoside (AFGP) as a chemical marker for detecting honey adulterated with rice syrup. The sensitivity of the QTRAP 6500+ system enabled the detection of AFGP with a limit of quantitation (LOQ) of 0.05 mg/kg, which is below the level required by the Food Safety and Standards Authority of India (FSSAI). This method quantified AFGP in honey artificially adulterated with as little as 1% (w/w) rice syrup and in various locally purchased honeys (Figure 1).

A recent US FDA survey reported adulteration in 10% of imported honey products,[1] while an investigation led by the European Commission found that 46% of honey samples tested were suspected to be adulterated by inexpensive sweeteners such as sugar syrups from rice, beets and cane.[2] Honey authenticity is strictly regulated in the US and Europe, which warrants the need for analytical techniques capable of detecting low-level syrup adulterants.

Rice syrup is comprised of the same oligo- and polysaccharides as honey.[3] Therefore, detecting honey adulteration is difficult. Common techniques, such as stable carbon isotopic ratio analysis (SCIRA) and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), lack the specificity to distinguish rice syrup from honey and often require laborious sample preparation. Targeted LC-MS/MS-based approaches have monitored AFGP as a distinct marker for rice syrup adulteration in honey.[3-5] The FSSAI mandates that AFGP must be absent in honey products.[6] Here, a method that meets the FSSAI minimum required performance level (MRPL) of 1 mg/kg for detecting AFGP as a rice syrup marker in honey is described.

Key features of honey authenticity testing

- The sensitivity of the QTRAP 6500+ system enabled a simple dilute-and-shoot approach for the quantitation of AFGP at a LOQ of 0.05 mg/kg, below that required by the FSSAI
- Chromatographic conditions were optimized to achieve separation of AFGP from co-eluting sugars in honey
- Acceptable ion ratios (±20%), accuracies (70–130%) and precision values (%CV <15%, *n* = 6) were achieved in quality control spikes prepared in honey and lab-adulterated honey
- Application of the method successfully detected AFGP in honey adulterated with as little as 1% (w/w) rice syrup

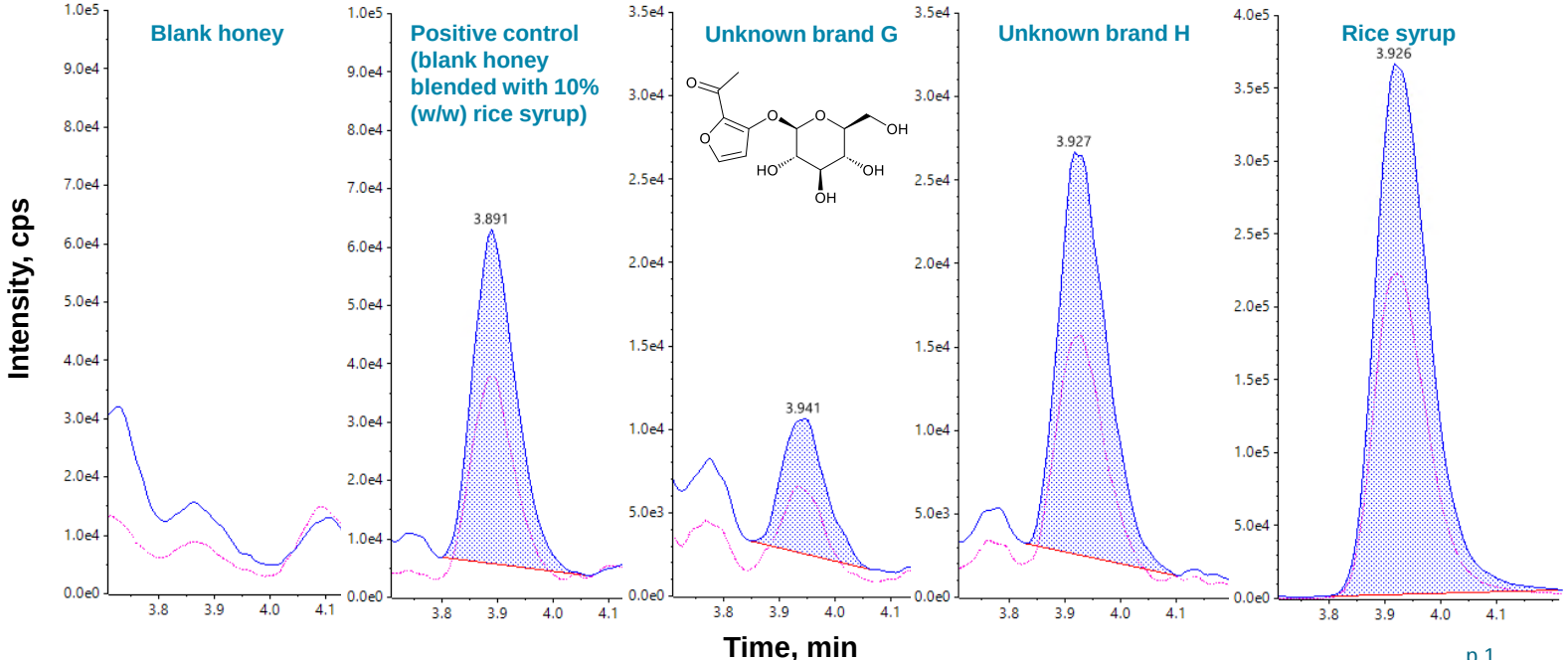

Figure 1. Comparison of AFGP extracted ion chromatograms (XICs) detected in 4 honey samples. A pure rice syrup sample and a honey sample artificially adulterated with 10% (w/w) of that rice syrup were included as positive controls. Note the y-axis for the rice syrup XIC is approximately 1 order of magnitude higher than the y-axis for the other samples.

Methods

Chemicals and samples: A neat standard of AFGP was used to prepare stock solutions in methanol. From the stock solutions, calibration standards (0.5–25 ng/mL) were prepared using an aqueous solution of 2mM ammonium formate as diluent.

Eight honey samples (brands A–H) and 1 brown rice syrup sample were locally purchased and pre-screened for AFGP. An AFGP-free honey brand was selected for the preparation of quality control (QC) samples.

Procedural recoveries in spiked honey matrices: QC matrix spikes were prepared in triplicate by spiking the AFGP-free honey at 2 different concentration levels (0.5 mg/kg and 2.5 mg/kg) before and after the sample preparation process. For the pre-spiked QC samples, AFGP was spiked directly into an aliquot of honey and then taken through sample preparation. For the post-spiked samples, spiking occurred in the final extract. Recovery was calculated as the quotient of the peak area in the pre-spiked and post-spiked QC samples. Recoveries were also evaluated by spiking AFGP at 1 mg/kg and 2.5 mg/kg in honey blended with 10% (w/w) rice syrup to assess the sample preparation performance in a lab-adulterated honey matrix.

Lab-adulteration of honey with rice syrup: Rice syrup was blended with honey at 1%, 2.5%, 5%, 10%, 20% and 30% (w/w) to simulate adulteration. Briefly, rice syrup was weighed in a 15 mL glass bottle to which honey was added based on the above ratios. To reduce viscosity, the blended mixture was incubated in a heated water bath at 60–70°C for 5–10 minutes until the sample consistency reached a homogeneous and free-flowing state.

Sample preparation: The sample preparation procedure was the same for all sample types (QC matrix spikes, different brands of retail honey, lab-adulterated honey and rice syrup). Briefly, a 500 mg sample was combined with 20 mL of 2mM ammonium formate in water in a 50 mL tube and vortexed until completely dissolved. The final volume was made up to 50 mL with 2mM ammonium formate in water, shaken, vortexed for 30 seconds and then filtered through a 0.22 µm PVDF syringe filter. An aliquot of the sample filtrate was transferred to autosampler vials for LC-MS/MS analysis without further dilution.

Chromatography: LC separation was performed on an ExionLC AD system using a Phenomenex Gemini-NX C18 column (3 µm, 100 Å, 100 x 3 mm, P/N: 00D-4453-Y0). A flow rate of 0.35 mL/min, an injection volume of 5 µL and a column temperature of 35°C were used. The gradient is presented in Table 1.

Table 1. Chromatographic gradient.

Time (min)	Flow rate (mL/min)	A%	%B
0.0	*0.35*	*95*	*5*
2.5	*0.35*	*5*	*5*
4.0	*0.35*	*90*	*10*
5.0	*0.35*	*90*	*10*
5.2	*0.35*	*5*	*95*
6.0	*0.35*	*5*	*95*
6.1	*0.8*	*95*	*5*
9.0	*0.8*	*95*	*5*
9.1	*0.35*	*95*	*5*
12.0	*0.35*	*95*	*5*

Mobile phase A: Water with 2mM ammonium bicarbonate (v/v)
Mobile phase B: Acetonitrile

***Mass spectrometry*:** Analysis was performed using the QTRAP 6500+ system with an IonDrive Turbo V ion source in positive electrospray ionization mode. A solution of AFGP was infused into the mass spectrometer to optimize the source, gas and compound-dependent parameters, as shown in Table 2.

Table 2. Source, gas and compound-dependent parameters.

Source parameters				
Curtain gas (CUR)	*40 psi*			
Collision gas (CAD)	*Medium*			
Ion spray voltage (ISV)	*5000 V*			
Temperature (TEM)	*400°C*			
Nebulizer gas (GS1)	*60 psi*			
Heater gas (GS2)	*70 psi*			
Compound-dependent parameters				
Q1 (m/z)	***Q3 (m/z)***	***DP (V)***	***CE (V)***	***CXP (V)***
311.2	*149.3*	*131*	*22*	*18*
311.2	*185.4*	*131*	*19*	*22*

Data processing: All data were processed using SCIEX OS software, version 2.1.6.

Chromatographic optimization for AFGP

Chromatographic conditions were extensively optimized to separate AFGP from coeluting sugars present in honey by testing different LC columns, mobile phases and gradients. Different buffers and organic solvents were screened for mobile phase selection. Figure 2 shows the separation of AFGP from the coeluting background using the final optimized conditions, as described earlier.

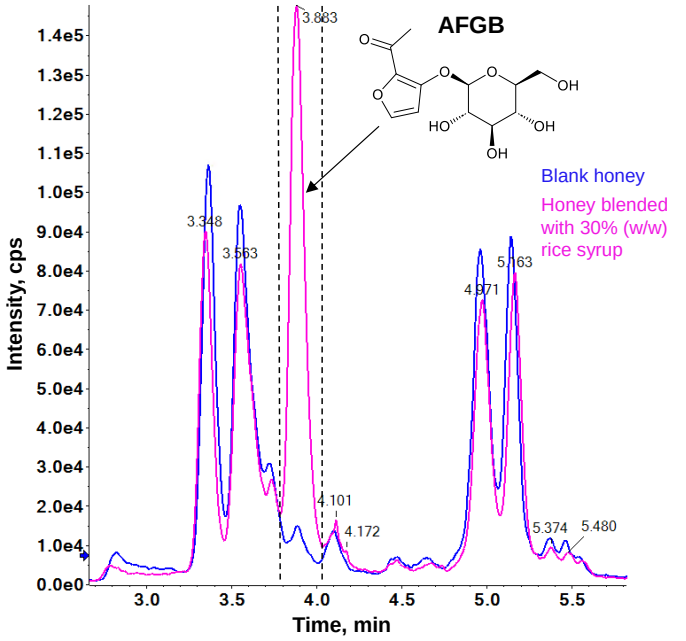

Figure 2. Chromatographic separation of AFGP in honey. The blue trace represents the XIC of an AFGP-free honey sample, while the pink trace represents the XIC of honey blended with 30% (w/w) rice syrup.

Linear dynamic range and sensitivity

Acceptable linear performance was achieved for the solvent-based calibration curve ranging from 0.5 to 25 ng/mL prepared in 2mM ammonium formate in water (Table 3).

Table 3. Linear range, correlation coefficient, accuracy and precision ranges across the calibration curve.

Compound	Linear range (ng/mL)	Correlation coefficient (*r*)	Accuracy range (%)	Precision range (%CV)
AFGP	*0.5 – 25*	*0.994*	*93 – 104*	*7 – 11*

The in-vial LOQ of AFGP was determined to be 0.5 ng/mL based on the lowest calibration level achieving an average accuracy of ±15%, %CV <15% and ion ratios within ±30%. Figure 3 shows example XICs of the LOQ calibration standard at 0.5 ng/mL injected in triplicate throughout the batch.

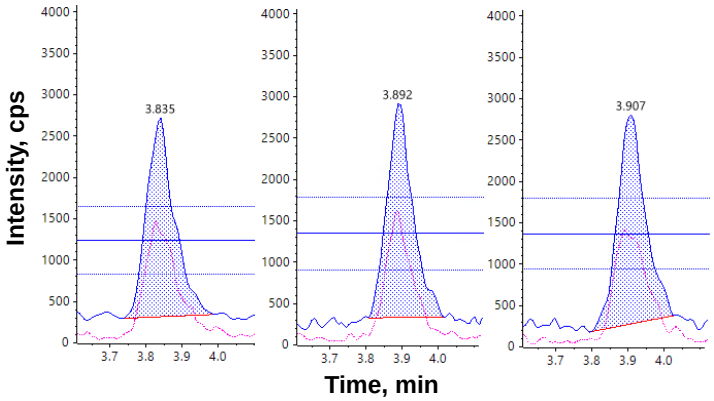

Figure 3. Example XICs of the LOQ standard. The blue and pink traces represent the quantifier and qualifier transitions, respectively.

Method performance in QC matrix spikes

A blank honey matrix and honey blended with 10% (w/w) rice syrup were spiked with AFGP before and after sample preparation to evaluate procedural recoveries at concentrations near the FSSAI MRPL of 1 mg/kg.[6] Acceptable matrix recoveries of 102–110% with %CV of 2–6% for 6 replicates at each spiking level were achieved based on the quotient of the peak areas in the pre-and post-spiked samples (Table 4). This calculation was performed directly in the results table using the calculated columns feature in SCIEX OS software, as shown in Figure 4.

Table 4. Average accuracy and %CV (*n* = 6) for the recovery of AFGP spiked in an AFGP-free honey and the same honey artificially adulterated with 10% (w/w) rice syrup.

QC spike	Spiking level (mg/kg)	Average accuracy (%)	%CV (*n* = 6)
Blank honey	*0.5*	*103*	*2.0*
	2.5	*102*	*2.4*
Lab-adulterated honey with 10% (w/w) rice syrup	*1*	*106*	*5.3*
	2.5	*110*	*6.0*

The use of solvent-based calibration was also deemed appropriate here based on the low matrix suppression (<20%) calculated from the quotient of the peak areas in the post-spikes of the blank honey matrix and standards prepared at the same concentrations. Higher suppression was observed for the lab-adulterated honey spikes likely due to the background AFGP and additional interferences from the rice syrup matrix, which can be resolved by using a mass-labeled or an appropriate surrogate internal standard to compensate for matrix effects.

Index	Sample Name	Comp... Name	Mass Info	Rete... Time	Area	Ion Ratio	Ion Ratio...	*Pre-spike areas	*Pre/Post RE	*Avg RE 10 ppb	*%CV 10ppb
59	10 PPB Post-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.89	391739	0.6406	✓	408291	104	106	5.3
61	10 PPB Post-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.86	385761	0.6218	✓	377692	98	106	5.3
63	10 PPB Post-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.86	380991	0.6306	✓	435536	114	106	5.3
65	10 PPB Post-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.89	372113	0.6245	✓	407786	110	106	5.3
67	10 PPB Post-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.91	389232	0.6223	✓	404101	104	106	5.3
69	10 PPB Post-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.91	386474	0.6152	✓	413869	107	106	5.3
87	10 PPB Pre-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.87	408291	0.6454	✓				
89	10 PPB Pre-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.86	377692	0.6408	✓				
91	10 PPB Pre-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.86	435536	0.6449	✓				
93	10 PPB Pre-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.86	407786	0.6441	✓				
95	10 PPB Pre-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.86	404101	0.6396	✓				
97	10 PPB Pre-spike 10% RS Blend Matrix	AFGP_01	311.2 / 149.3	3.86	413869	0.6351	✓				

Formula name: Pre/Post RE

COUNT | MAX | STDEV | Clear
SUM | MIN | MEDIAN | (
MEAN | ABS | IF |)
GET | GETGROUP | GETSTAT | +
/ | * | - | =

Note: The "Original text" option is recommended for formulas that contain functions, such as the IF function, that compare non-numeric values to numeric values.

```
IF([Sample Name]='10 PPB Post-spike 10% RS Blend Matrix '||[Sample Name]='25 PPB Post-spike 10% RS Blend Matrix ';([Pre-spike areas]/[Area])*100;'')
```

Formula Details

Figure 4. Screenshots demonstrating custom calculations in SCIEX OS software. Top) Results table showing custom columns (denoted by asterisks) that included user-defined and calculated values, an example of which is highlighted in yellow for the procedural recoveries. Bottom) Custom formula used for calculating the procedural recovery based on the quotient of the peak areas in the pre- and post-spiked matrix samples.

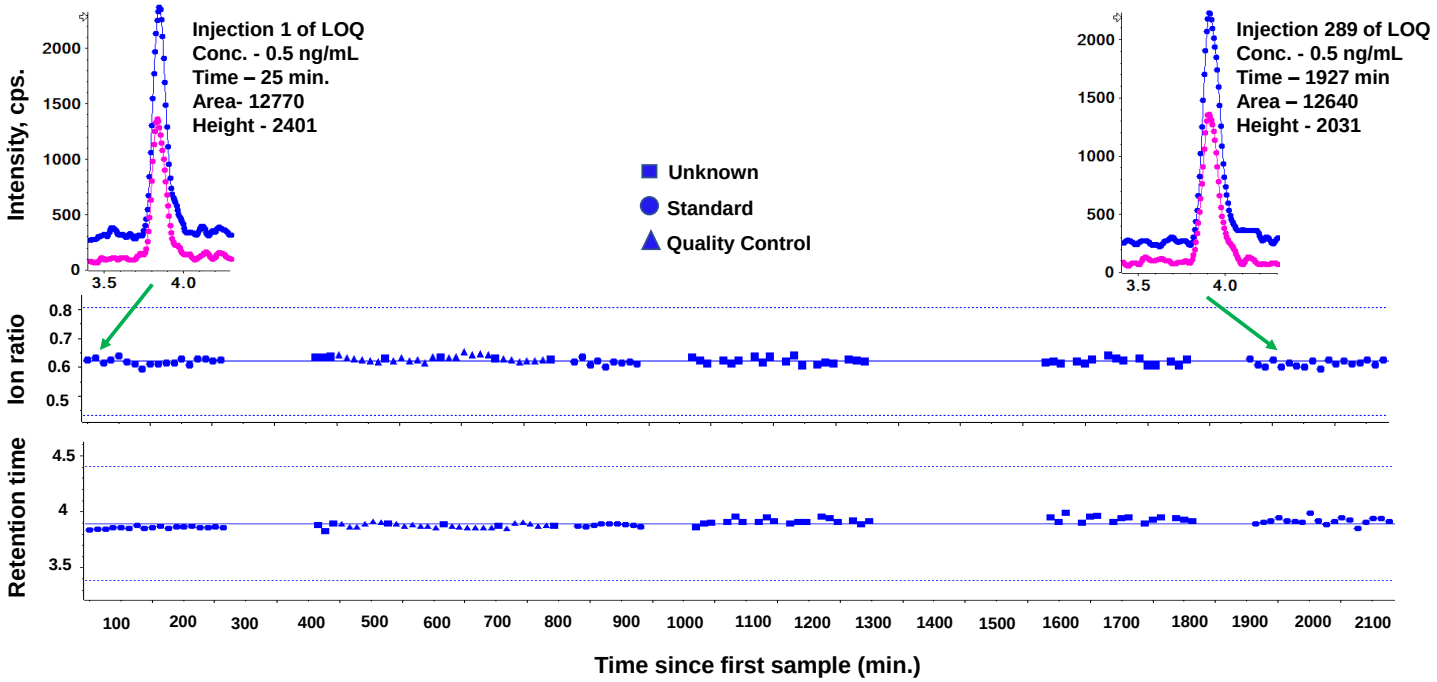

Figure 5. Metric plots of ion ratios and retention times for all samples within a 35-hour acquisition batch. A comparison of the XICs for the LOQ calibration standard injected at the beginning and end of the batch showed similar peak area responses.

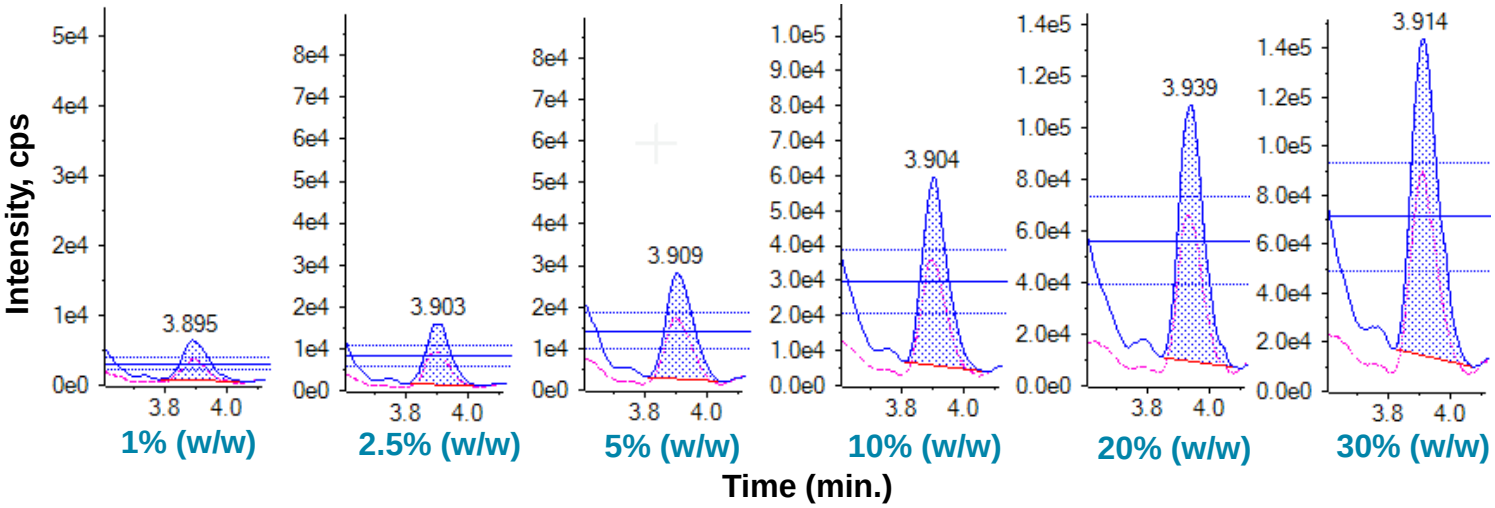

Figure 6. Representative XICs of lab-adulterated honey with 1%–30% (w/w) rice syrup.

Method robustness

The method robustness was assessed by an acquisition batch comprised of >300 injections which corresponded to >35 hours of runtime. The metric plots generated by SCIEX OS software indicated that the ion ratios were consistent and the retention times were within ±30% of the mean throughout the batch (Figure 5). The LOQ standard injected at the start and the end of the batch demonstrated similar peak area responses even after the injection of 300 samples of variable complexity, such as solvent blanks, honey and lab-adulterated honey blended with rice syrup. These results highlight the robust performance of the method over a long period of time, even when analyzing complex honey matrices.

Analysis of lab-adulterated honey

Previous investigations of honey authenticity have typically used a spiking range of 10–50% (w/w) rice syrup to artificially simulate honey adulteration.[3-5] Here, adulteration was assessed by blending honey with rice syrup at levels ranging from 1% to 30% (w/w) (Figure 6), with the intention of mimicking real-world fraudulent practices of honey manufacturers. The AFGP response demonstrated a linear increase (r = 0.996) with the level of adulteration. The concentrations of AFGP ranged from 0.14 mg/kg to 3.2 mg/kg. The method easily quantified AFGP at the lowest blend percentage of 1% (w/w) with consistent ion ratios (±20%) and signal-to-noise (S/N ≥20) for both the quantifier and qualifier transitions. This suggests the current dilute-and-shoot method provides sufficient sensitivity to quantify AFGP below levels typically used for adulteration (≥10%).[3,4]

Analysis of retail honey

Based on the acceptable matrix recoveries and matrix effects discussed earlier, the in-vial LOQ selected from the lowest solvent calibration standard of 0.5 ng/mL was used to determine the mass-based LOQ in honey. By back-calculating through the sample preparation process to account for dilution volume and the mass of honey extracted, the mass-based LOQ was determined to be 0.05 mg/kg, which is below the minimum FSSAI MRPL of 1 mg/kg.[6] The same calculation was applied to convert the in-vial concentrations of AFGP in the extracts to the original mass extracted in the following retail samples.

The method was applied to screen for the presence of AFGP in a brown rice syrup sample and 8 locally purchased honey brands. Table 5 summarizes the AFGP concentrations and precision based on injection replicates of the samples tested, while Figure 1 shows the example XICs of 2 honey brands (G and H) and the brown rice syrup with detectable levels of AFGP.

Table 5. AFGP concentrations (mg/kg) in different retail honeys.

Brand	AFGP concentration		%CV (*n* = 3)
	In vial (ng/mL)	*In honey (mg/kg)*	
LOQ	***0.5***	***0.05***	***N/A***
A – F	*<LOQ*	*Not detected*	*N/A*
G	*1.9*	*0.19*	*6.6*
H	*5.9*	*0.59*	*6.4*
Rice syrup	*98.6*	*9.86*	*1.2*

Conclusions

- The sensitivity of the QTRAP 6500+ system enabled a robust dilute-and-shoot LC-MS/MS method for the analysis of AFGP as a rice syrup marker to test for honey authenticity
- Acceptable method performance was achieved with calibration linearity of *r* >0.99, accuracies within ±30%, precision %CV <15% and ion ratios within ±30%
- The use of solvent-based calibration produced acceptable recoveries in the QC matrix spikes (102–110%) and matrix suppression (<20%) in the blank honey spikes, although the use of internal standards is highly recommended to combat matrix effects in more complicated matrices, such as adulterated honey.
- Calculations can be directly performed in SCIEX OS software without the need to export the data elsewhere
- The current method achieved a LOQ of 0.05 mg/kg, which is 20x below the FSSAI MRPL of 1 mg/kg for the analysis of AFGP as a marker for rice syrup adulteration in honey

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