Ministry for Primary Industries
Manatū Ahu Matua

Determination of Four Chemical Characterisation Compounds in Honey by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Chemistry Laboratory Method

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1 Introduction

1.1 BACKGROUND

This method was developed and validated by the National Measurement Institute, Port Melbourne, Australia as part of the Ministry for Primary Industries Mānuka Honey Science programme. The assay is one of the key tests that will be used to verify authenticity of monofloral and multifloral Mānuka honey.

The validated assay was used to determine the specificity and concentrations of the target chemicals in nectar (~500 samples) from a variety of plant species representing 17 *Leptospermum* species, 5 *Kunzea* species and 13 other plant species relevant to honey production in New Zealand. The assay has been tested on over 800 honey samples from various floral sources and geographic locations. Samples were sourced from two New Zealand flowering seasons, New Zealand industry archives (up to 5 years) and foreign sourced honeys (e.g. Australia, China, Africa, USA, Europe).

1.2 SUMMARY OF PROCEDURE

The four chemical characterisation compounds are extracted from honey with a mixture of aqueous acetonitrile and formic acid. The extract is further diluted and filtered before determination by LC-MS/MS.

1.3 APPLICABILITY

This method is suitable for the quantification of chemical characterisation compounds in honeys of various floral types at the levels listed in Table 1.

Table 1: Chemical Characterisation Compounds

Compound Common Name	**Abbreviation**	**CAS#**	**Limit of Reporting (LOR) (mg/kg)**	**Linear Range (mg/kg)**
2'-Methoxyacetophenone	2MAP	579-74-8	1	1 – 100
2-Methoxybenzoic acid	2MBA	579-75-9	1	1 – 100
3-Phenyllactic acid	3PA	828-01-3	1	1 – 100
4-Hydroxyphenyllactic acid	4HPA	306-23-0 or 6482-98-0	1	1 – 100
Forchlorfenuron (*internal standard only*)	FCF	68157-60-8	-	-

2 Equipment

Note: Equivalent equipment may be substituted.

2.1 APPARATUS

a. Balance – analytical ± 0.0001 g
b. Balance – top loading ± 0.01 g
c. Bottle – amber, glass, 10 mL

d. Beakers – 10 mL
e. Centrifuge – refrigerated, with rotor for 50 mL tubes
f. Centrifuge tubes – 15 and 50 mL conical, disposable, polypropylene with caps
g. Pipettes – Adjustable 10 – 5000 µL
h. Shaker – horizontal flatbed
i. Syringe filter, 13 mm diameter 0.45 µm PTFE
j. Vials – amber, glass 1.5 or well plate suitable for use in an auto-sampler
k. Volumetric flasks – 10, 50, 100 mL and 1 L
l. Vortex mixer – variable speed

2.2 INSTRUMENTATION

a. Waters Aquity UPLC I-Class System
b. Waters Xevo TQ-S Triple Quadrupole MS
c. HPLC Guard Column – Waters Acquity UPLC BEH C18 VanGuard Pre-column, 130Å, 1.7 mm, 2.1 mm x 5 mm, (Waters P/N 186003975)
d. HPLC Column – Waters Acquity UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm, (Waters P/N 186002352)

3 Reagents and Solutions

Note: All reagents are AR Grade, unless otherwise specified. Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependent on the expiration dates of the compounds used. The maximum length of time that a working reagent shall be used is 1 year unless the laboratory has produced extension data.

3.1 REAGENTS

a. Acetonitrile – HPLC grade
b. Formic acid – Fisher Chemical, 99.5+%, Optima™ LC/MS Grade, P/N A117-50
c. Water – Deionized, HPLC grade

3.2 SOLUTIONS

a. Extraction solution: 10:1:90 Acetonitrile/formic acid/water

Approximately half fill a 1L volumetric flask with deionised water. Measure 100 mL of acetonitrile and measure 10 mL of formic acid into the volumetric flask. Fill to volume with deionised water and invert gently to mix.

Store at room temperature. Prepare fresh weekly.

b. 1% Formic acid in acetonitrile

Approximately half fill a 100 mL volumetric flask with acetonitrile. Pipette 1 mL of formic acid into the volumetric flask. Fill to volume with acetonitrile and invert gently to mix.

Store at room temperature. Prepare fresh for each use.

c. Mobile Phase A: 0.1% Formic acid in water

Approximately half fill a 1L volumetric flask with deionised water. Pipette 1 mL of formic acid into the volumetric flask. Fill to volume with deionised water and invert gently to mix.

Store at room temperature. Prepare fresh for each analytical batch.

d. Mobile Phase B: 0.1% Formic acid in acetonitrile

Approximately half fill a 1L volumetric flask with acetonitrile. Pipette 1 mL of formic acid into the volumetric flask. Fill to volume with acetonitrile and invert gently to mix.

Store at room temperature. Prepare fresh for each analytical batch.

4 Standard(s)

Note: Equivalent standards / solutions may be substituted. Purity and counter-ions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependent on the expiration date of the components used. In-house prepared standards shall be assigned an expiration date that is no later than the expiration date of the earliest expiring component or no later than the stability stated in the method, whichever ends soonest. The maximum length of time that an in-house prepared standard shall be used is 3 months unless the laboratory has produced extension data.

4.1 STANDARD INFORMATION

a. 2'-Methoxyacetophenone – 99% pure. Catalogue No. M9203 Sigma-Aldrich
b. 2-Methoxybenzoic acid – 99% pure. Catalogue No. 169978 Sigma-Aldrich
c. 3-Phenyllactic acid – 97% pure. Catalogue No. P7251 Sigma-Aldrich
d. 4-Hydroxyphenyllactic acid – 95% pure. Catalogue No. 222023 Fluorochem
e. Forchlorfenuron (Internal Standard) – 99% pure. Catalogue No. 32974 Sigma-Aldrich

4.2 PREPARATION OF STANDARD SOLUTION(S)

Note: Adjust all standard weights for purity.

a. Stock standard and internal standard solutions (~1000/10000 mg/L)

Weigh approximately 10 mg of the 2MAP, 2MBA, 4HPA and FCF standards into its own 10 mL volumetric flask and bring to volume with acetonitrile. Weigh approximately 100 mg of the 3PA standard into its own 10 mL volumetric flask and bring to volume with acetonitrile. Record the weight to 0.1 mg and calculate the exact concentration, taking into account both the moisture, purity and salt content. Transfer to an amber, glass bottle with minimal headspace.

These standards are stable for 3 months when stored in a refrigerator at 2 – 8°C.

b. Mixed spiking standard solution (100/1000 mg/L)
Pipette ~1000 µL (adjusted for the actual stock standard concentration) of each stock standard solution into a 10 mL volumetric flask and bring to volume with 1% formic acid in acetonitrile. Transfer this solution into a 10 mL amber, glass bottle with minimal headspace.

This standard is stable for 3 months when stored in a refrigerator at 2 – 8°C.

c. Mixed spiking standard solution (10/100 mg/L)
Pipette 1000 µL of the **100/1000 mg/L** mixed spiking standard solution into a 10 mL volumetric flask and bring to volume with 1% formic acid in acetonitrile. Transfer this solution into a 10 mL amber, glass bottle with minimal headspace.

This standard is stable for 3 months when stored in a refrigerator at 2 – 8°C.

d. Mixed calibration standard solution (0.25 and 2.5 mg/L)
Pipette 250 µL of the **10/100 mg/L** mixed spiking standard solution into a 10 mL volumetric flask and bring to volume with 1% formic acid in acetonitrile. Transfer this solution into a 10 mL amber, glass bottle with minimal headspace.

This standard is stable for 3 months when stored in a refrigerator at 2 – 8°C.

e. Forchlorfenuron internal standard solution (~10 mg/L)
Pipette 500 µL of the stock FCF internal standard solution into a 50 mL volumetric flask and bring to volume with 1% formic acid in acetonitrile. Transfer this solution into a 10 mL amber, glass bottle with minimal headspace.

This standard is stable for 3 months when stored in a refrigerator at 2 – 8°C.

5 Sample Preparation

5.1 SAMPLE HOMOGENISATION

a. Ensure honey samples are brought to room temperature and thoroughly mixed before sub-sampling.

6 Analytical Procedure

6.1 PREPARATION OF CONTROLS

a. Weigh four 1 g ± 0.1 g of blank homogenised honey portions into 50 mL disposable centrifuge tubes.

Note: A honey known to contain negligible concentrations of the four characterisation compounds should be used for the control samples.

b. Fortify the positive control samples with the volumes of mixed spiking solution as described in Table 2.

Table 2: Fortification of positive control samples

Positive Control and Concentration	**Concentration of Mixed Spiking Standard Solution**	**Volume of Mixed Spiking Standard Solution (µL)**
LOR[1] (1/10 mg/kg)	10/100	100
2x LOR (2/20 mg/kg)	10/100	200
10x LOR (10/100 mg/kg)	100/1000	100

6.2 SAMPLE EXTRACTION

a. Weigh 1 g ± 0.1 g of homogenised honey samples into 50 mL disposable centrifuge tubes. Record the weight of the analytical portion to 0.01 g accuracy. Include the prepared controls in the sample set at this time.

Note: In addition to the control samples, one of the analytical samples is weighed in duplicate per batch. Also it is recommended laboratories develop and run an in-house quality control (QC) sample for batch to batch perform monitoring.

b. Add 9 mL of 10:1:90 acetonitrile/formic acid/water solution to each tube.

c. Vortex mix vigorously to ensure thorough mixing.

d. Agitate the tubes on a horizontal shaker vigorously for 10 minutes.

e. Centrifuge the tubes for 10 minutes at approximately 3500 RCF at 10°C.

f. Filter ~ 1 mL of each dilution through a 0.45 µm PTFE filter into a 1.5 mL, amber auto-sampler vial.

6.2.1 200 times dilution (controls and samples)

g. Add 500 µL of the supernatant (step 6.2 f.), 100 µL of forchlorfenuron internal standard solution (10 mg/L) and 9.4 mL of 10:1:90 acetonitrile/formic acid/water solution into a 15 mL, polypropylene tube. Cap and mix thoroughly.

6.2.2 5000 times dilution (samples only)

h. Add 20 µL of the supernatant (step 6.2 f.), 100 µL of forchlorfenuron internal standard solution (10 mg/L) and 9.88 mL of 10:1:90 acetonitrile/formic acid/water solution into a 15 mL, polypropylene tube. Cap and mix thoroughly.

i. Transfer ~ 1 mL of each dilution into a 1.5 mL, amber auto-sampler vial for LC-MS/MS determination

Note 1: The dilution series above are recommended based on the instrument sensitivity achieved at the time of validation. Laboratory may need to adjust dilution ratios depending on instrument performance.

Note 2: Typically, both recommended dilution series are required to be analysed to cover the expected range of characterisation compounds found in Mānuka honey.

Note 3: In addition, a reagent blank of acetonitrile solution is prepared and injected the instrument sequence between the positive controls and the sample set.

6.3 PREPARATION OF CALIBRATION CURVE STANDARDS

a. Prepare the calibration standards using 10:1:90 acetonitrile/formic acid/water solution according to table 3 in 1.5 mL amber, glass vials. Cap and vortex mix. Prepare calibration curve standards fresh for every batch and store at 2 – 8°C.

[1] Note: The LOR referred to corresponds to 2MAP, 2MBA, and 4HPA. The spiking level for 3PA is 10 times that compound's LOR.

Table 3: Preparation of calibration curve standards

2MAP, 2MBA, 4HPA Cal. Std. Conc. (mg/L)	3PA Cal. Std. Conc. (mg/L)	2MAP, 2MBA, 4HPA Honey Conc. (mg/kg)	3PA Honey Conc. (mg/kg)	Volume of Mixed Cal. Standard (µL)	Volume of FCF Internal Standard (µL)	Volume. of Extraction Solution (µL)
0	0	0	0	0	10	990
0.0025	0.025	0.5	5	10	10	980
0.005	0.05	1	10	20	10	970
0.01	0.1	2	20	40	10	950
0.025	0.25	5	50	100	10	890
0.05	0.5	10	100	200	10	790
0.1	1	20	200	400	10	590

6.4 INSTRUMENT SETTINGS

Note: The instrument parameters may be optimized to ensure system suitability.

Table 4: HPLC Conditions:

Mobile Phase A	0.1% Formic acid in water
Mobile Phase B	0.1% Formic acid in acetonitrile
Flow Rate	0.2 mL/min
Column Temperature	40°C ± 2°C
Injection Volume	2 µL
Auto-sampler Temperature	8°C ± 5°C
Run Time	10 minutes

Table 5: HPLC Run Events – Diverter value

Event Sequence	Event Description	Time (mins)
Event 1	Flow State Waste	0.00
Event 2	Flow State LC	3.20
Event 3	Flow State Waste	5.95

Table 6: HPLC Mobile Phase Gradient Table:

Time (mins)	% Mobile Phase A	% Mobile Phase B
0.00	95	5
0.75	95	5
2.00	85	15
4.00	30	70
6.00	2	98
6.50	2	98
7.00	95	5
10.00	95	5

Table 7: Instrument Set-Up and Parameters

Negative Ion Mode (ES-) 4-Hydroxyphenyllactic acid 3-Phenyllactic acid Forchlorfenuron (internal standard)		**Positive Ion Mode (ES+)** 2'-Methoxyacetophenone Methoxybenzoic acid	
Capillary voltage	2.00 kV	Capillary voltage	3.00 kV
Cone voltage	40.00 V	Cone voltage	40.00 V
Source Temperature	150°C	Source Temperature	150°C
Desolvation Temperature	450°C	Desolvation Temperature	450°C
Cone Gas Flow	150 L/Hr	Cone Gas Flow	150 L/Hr
Desolvation Gas Flow	800 L/Hr	Desolvation Gas Flow	800 L/Hr
Collision Gas Flow	0.14 mL/min	Collision Gas Flow	0.13 mL/min
Nebuliser Gas Flow (Bar)	7	Nebuliser Gas Flow (Bar)	7
LM 1 Resolution	2.9	LM 1 Resolution	2.8
HM 1 Resolution	14.9	HM 1 Resolution	14.9
Ion Energy 1	0.4	Ion Energy 1	0.5
MS Mode Entrance	1	MS Mode Entrance	1
MS Mode Collision Energy	5	MS Mode Collision Energy	5
MSMS Mode Collision Energy	20	MSMS Mode Collision Energy	20
MS Mode Exit	1	MS Mode Exit	1
MSMS Mode Entrance	30	MSMS Mode Entrance	30
MSMS Mode Collision Energy	20	MSMS Mode Collision Energy	20
MSMS Mode Exit	30	MSMS Mode Exit	30
LM 2 Resolution	2.8	LM 2 Resolution	2.7
HM 2 Resolution	15	HM 2 Resolution	15
Ion Energy 2	0.7	Ion Energy 2	0.5
Gain	1	Gain	1

Table 8: MS/MS Parameters

Compound	Retention window (min.)	Precursor Ion† (m/z)	Product Ion† (m/z)*	Accurate Mass Observed ‡	Dwell Time† (Auto) (sec)	Cone Voltage† (Volts)	Collision Energy† (Volts)
2MAP	5.3 – 6.0	150.70 150.70	79.31 **105.32**	151.0752 79.0546 105.0700	0.029 0.029	2 2	24 20
2MBA	4.7 – 5.3	152.99 152.99	**77.07** 92.02	153.0545 77.0389 92.0259	0.019 0.019	30 30	39 19
3PA	4.6 – 5.0	165.01 165.01	**103.13** 119.12	165.0548 103.0543 119.0492	0.019 0.019	45 45	18 16
4HPA	3.8 – 4.2	181.01 181.01 181.01	73.11 119.12 **135.05**	181.0498 72.9920 119.0492 135.0441	0.025 0.025 0.025	50 50 50	14 16 18
FCF (Internal Standard)	5.4 – 5.9	245.82 245.82	90.95 **126.87**	246.0441 91.0294 127.0063	0.036 0.036	4 4	28 14

* Most abundant product ion (quantification ion) is in bold.

† The parameters shown in the table are specific to instrumentation used at the time of validation. These can vary depending on the instrumentation used and the most appropriate precursor and product ions needs to be determined by each laboratory.

‡ The observed accurate masses shown in the table were found using liquid chromatography high-resolution accurate-mass spectrometry.

6.5 INJECTION SEQUENCE/SAMPLE SET

a. Calibration Curve
b. Positive Controls
c. Matrix Blank
d. Reagent Blank
e. QC sample
f. Samples and duplicates

Calibration standards should be injected at the start of each batch and one of these standards re-injected after every 8 – 10 samples throughout the batch.

The lowest calibration standard point (0.0025/0.025 mg/L) is to be re-injected at the end of each analytical batch sequence as a book end to enable the analyst to check the consistency and stability of the instrument's response. If instrument response is found to have significantly degraded (or enhanced) then the analyst must consider the impact to the veracity of the findings and investigate potential causes.

Linearity of standards has been checked and found to be generally good in the range equivalent to sample concentrations 1 – 100 mg/kg. This can vary depending on instrumentation used and needs to be confirmed by each laboratory. Samples with

calculated concentration outside the linear range of the methods should be diluted to a suitable concentration.

7 Calculations / Identification

7.1 CALCULATIONS

a. Calculation of batch recovery

$$\text{Recovery (\%)}_{\text{Spike}} = \frac{\text{Conc.}_{\text{Spike}} - \text{Conc.}_{\text{Blank}}}{\text{Conc.}_{\text{Added}}} \times 100$$

where:
Recovery (%) $_{\text{Spike}}$ = percentage recovery of the control spike
Conc. $_{\text{Spike}}$ = calculated concentration of the control spike
Conc. $_{\text{Blank}}$ = calculated concentration of the matrix blank
Conc. $_{\text{Added}}$ = expected concentration of the control spike

The percentage recovery is found for each of the each spiking levels

b. Calculation of relative percentage difference (RPD) of duplicates

$$\text{RPD (\%)} = \frac{|\text{Conc.}_{\text{Duplicate 1}} - \text{Conc.}_{\text{Duplicate 2}}|}{\text{Mean Conc.}_{\text{(Duplicate 1 \& 2)}}} \times 100$$

where:
RPD (%) = relative percentage difference of the duplicates
Conc. $_{\text{Duplicate 1}}$ = calculated concentration of the first duplicate
Conc. $_{\text{Duplicate 2}}$ = calculated concentration of the second duplicate
Mean Conc. $_{\text{(Duplicate 1 \& 2)}}$ = arithmetic mean of the calculated concentration of the duplicates

7.2 QUANTIFICATION CALCULATION

a. Peak areas of analytes and internal standards are used for quantification.
b. The coefficient of correlation (r^2) must be ≥ 0.99.
c. Do not use the origin as a data regression point.
d. Determine sample concentrations for 2MAP, 3PA and 4HPA is typically found using a weighted linear regression (1/x) and determine sample concentrations for 2MBA is typically found using a weighted quadratic regression (1/x). This can vary depending on the instrumentation used and the most appropriate curve fitting needs to be determined by each laboratory. It is recommended curve fitting is optimised to minimise residuals for each calibration point.
e. Calculation of concentration of characterisation compounds is found as follows. (linear regression example)

$$\text{Calculated concentration (mg/kg)} = \frac{(y - c)}{m} \times d \times \frac{1}{s_w}$$

where:
y = ratio of the quantification ion peak areas of the analyte/internal standard

c = y intercept
m = gradient
d = dilution factor (typically 200 or 5000)
s_w = sample weight (g)

7.3 CONFIRMATION CRITERIA

a. The retention time of the analyte(s) must match that of the positive control or the external standard injected most recently before the relevant sample within ± 0.1 min.
b. Product ion abundance ratios must match that of the positive control or the external standard injected most recently before the relevant sample within ± 30% (relative).
c. Each ion must have a signal-to-noise ratio ≥ 3.

7.4 QUANTIFICATION CRITERIA

a. The sample peak retention time must be within ± 0.1 min of the positive control or the external standard injected most recently before the relevant sample.
b. The quantification ion must have a signal to noise ratio of ≥ 10.
c. The additional ion(s) must be present in sample with a signal to noise ratio of ≥ 3.
d. The coefficient of determination (r^2) for the calibration curve must be ≥ 0.99

Note: Quantification criteria are required only for analytes that are to be quantified in the sample set.

8 Safety Information and Precautions

8.1 REQUIRED PROTECTIVE EQUIPMENT

a. Safety glasses and/or face shield
b. Disposable gloves
c. Laboratory coat

8.2 HAZARDS

Table 9: Hazards

Procedure Step	Hazard	Recommended Safe Procedures
Acetonitrile	Flammable and poisonous	Use reagents in an efficient fume hood away from all electrical devices and open flames. Wear gloves and protective eyewear.
Formic acid	Acid burns	Wear protective equipment and avoid contact with skin.

9 Quality Assurance Plan

9.1 PERFORMANCE STANDARD

a. Positive controls are positive for all analytes using the criteria in Section 7.
b. The positive control recovery at the LOR[1] spiking level is between 80 – 110% for all analytes that will be quantified. For any recovery <80% or >110%, the data should be reviewed and/or the batch repeated.

c. The calculated amounts for each of the compounds of the QC sample should be in an acceptable range (mean ± 2 standard deviations) determined by using a control chart.
d. The relative percent difference (RPD) between duplicates should be <20%.
e. Relative response factors for standards injected through the analytical run should vary by no more than 15%.

9.2 CRITICAL CONTROL POINTS AND SPECIFICATIONS

Record	***Acceptable Control***
Sample weight of honey	1.0 ± 0.1 g

9.3 INTER-LABORATORY CHECK SAMPLES

a. System, minimum contents.
   i. Frequency: As available, per analyst when samples tested.
   ii. Records are to be maintained.
b. Acceptability criteria.
   Refer to 9.1.
   If unacceptable values are obtained, then:
   i. Investigate following established procedures.
   ii. Take corrective action as warranted.

9.4 CONDITION UPON RECEIPT

a. Room temperature, no evidence of spoilage, leakage or container damage.

9.5 REPORTING

a. Each characterisation compound is reported in units of mg/kg to two significant figures.

10 Appendix

10.1 SAMPLE CHROMATOGRAMS AND CALIBRATION CURVES

Following are a series of chromatograms obtained for analysis of honey prepared and analysed during the initial validation of the method.

Figure 1: **Blank in positive and negative ion**

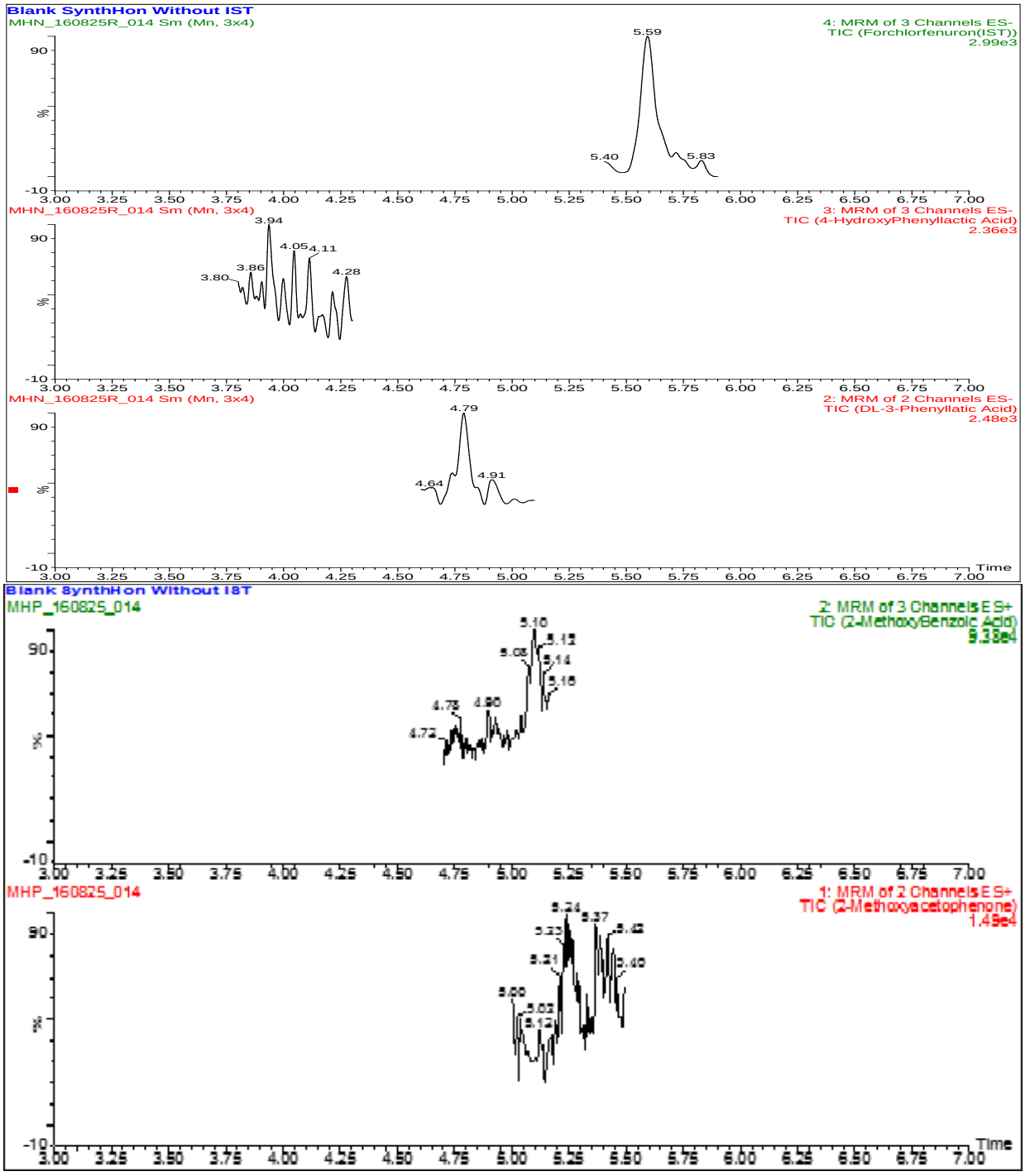


Figure 2: **0.002 mg/L Matrix standards**

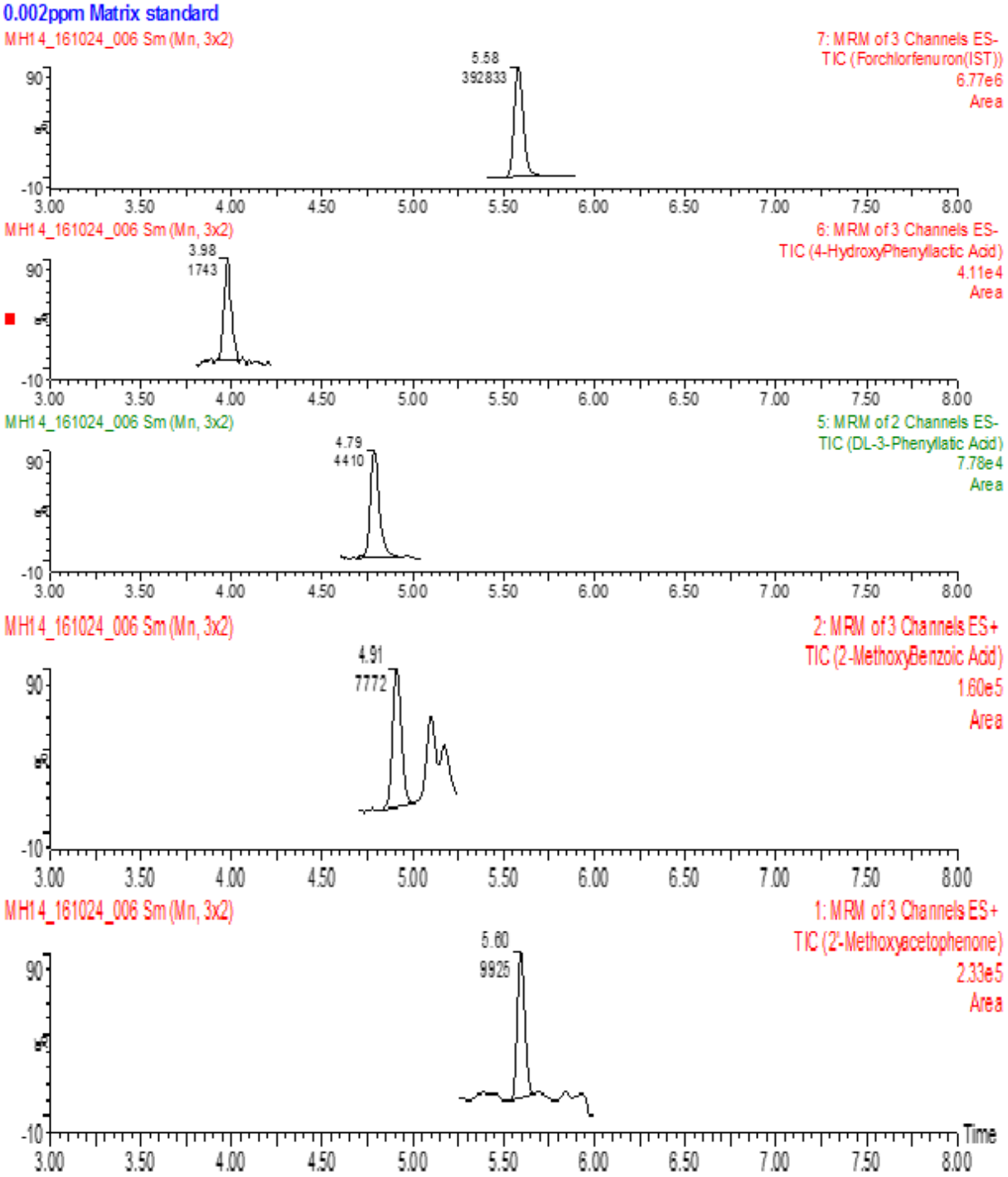


Figure 3: **1.0 mg/L Matrix standards**

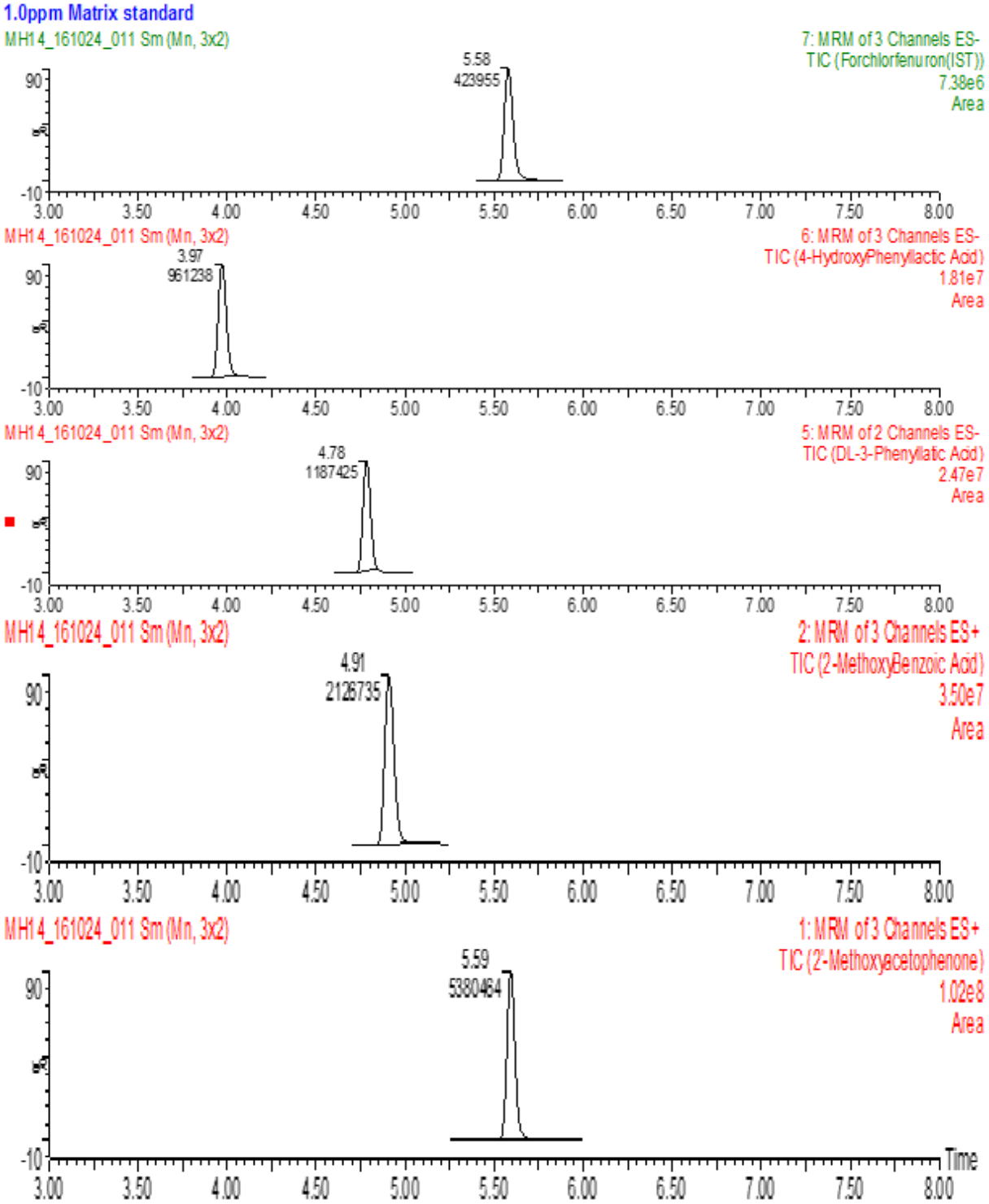


Figure 4: **1 mg/kg Honey spike**

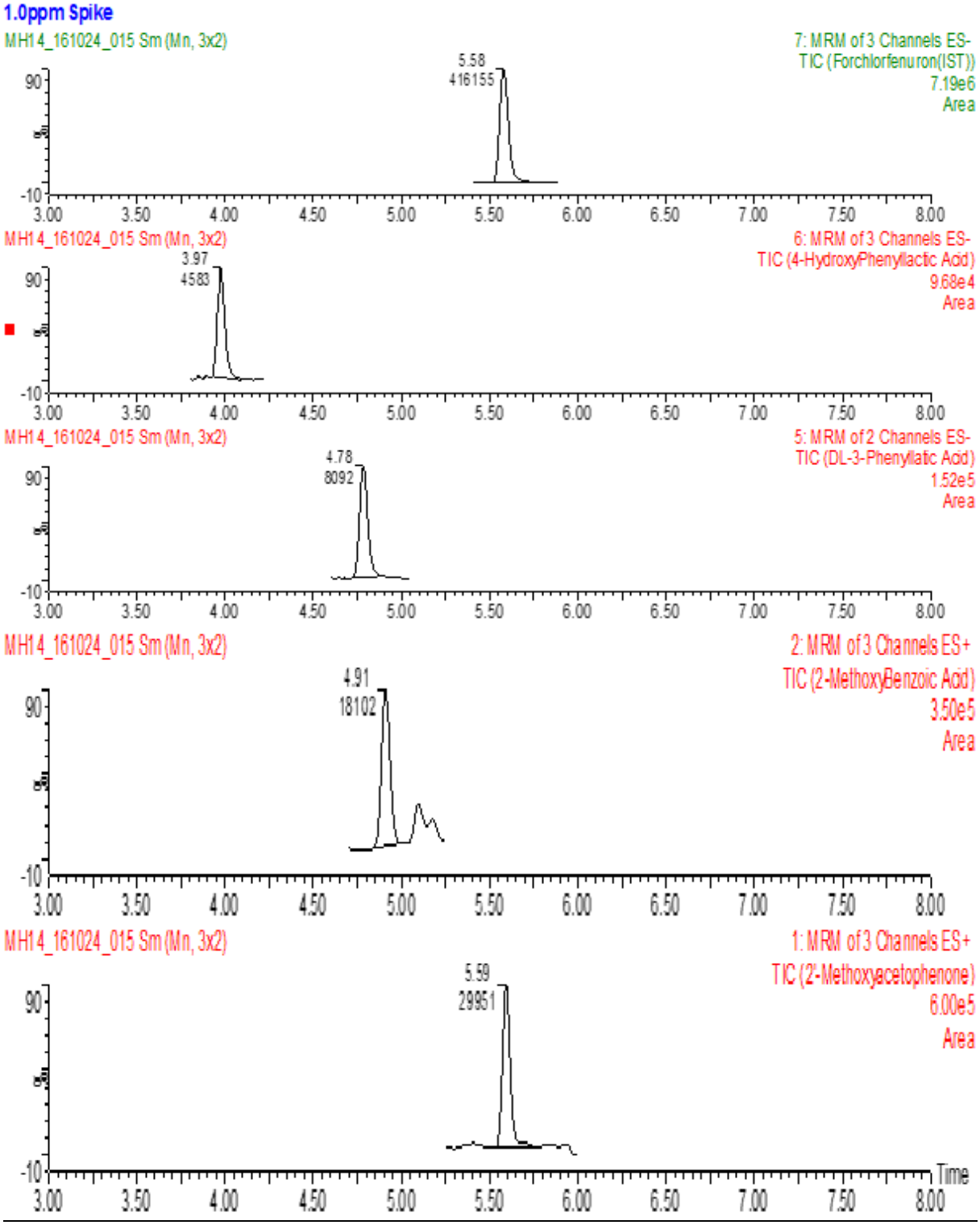


Figure 5: **100 mg/kg Honey spike**

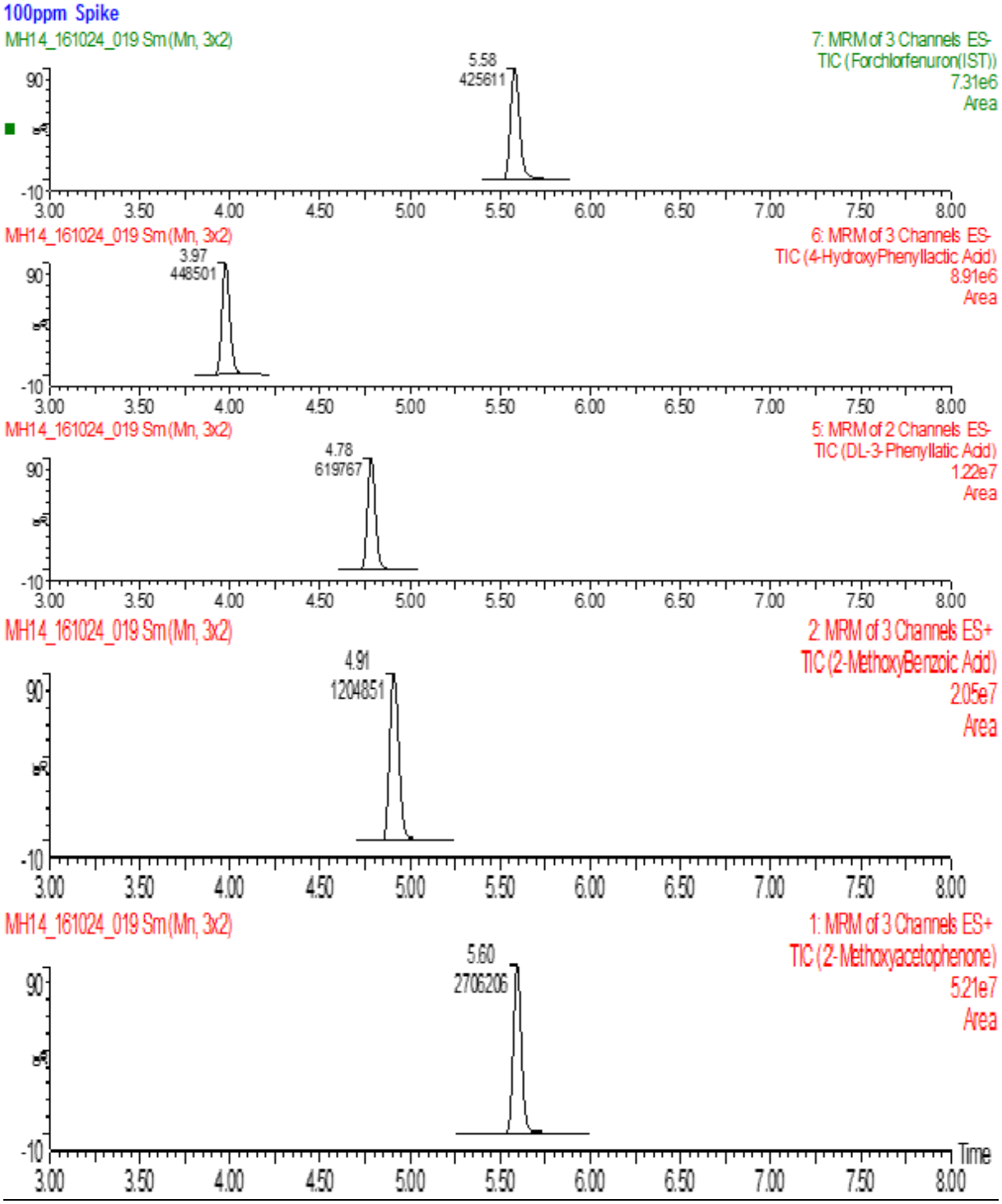


Figure 6: **MH2 - Commercial source honey in negative ion (TIC and MRM)**

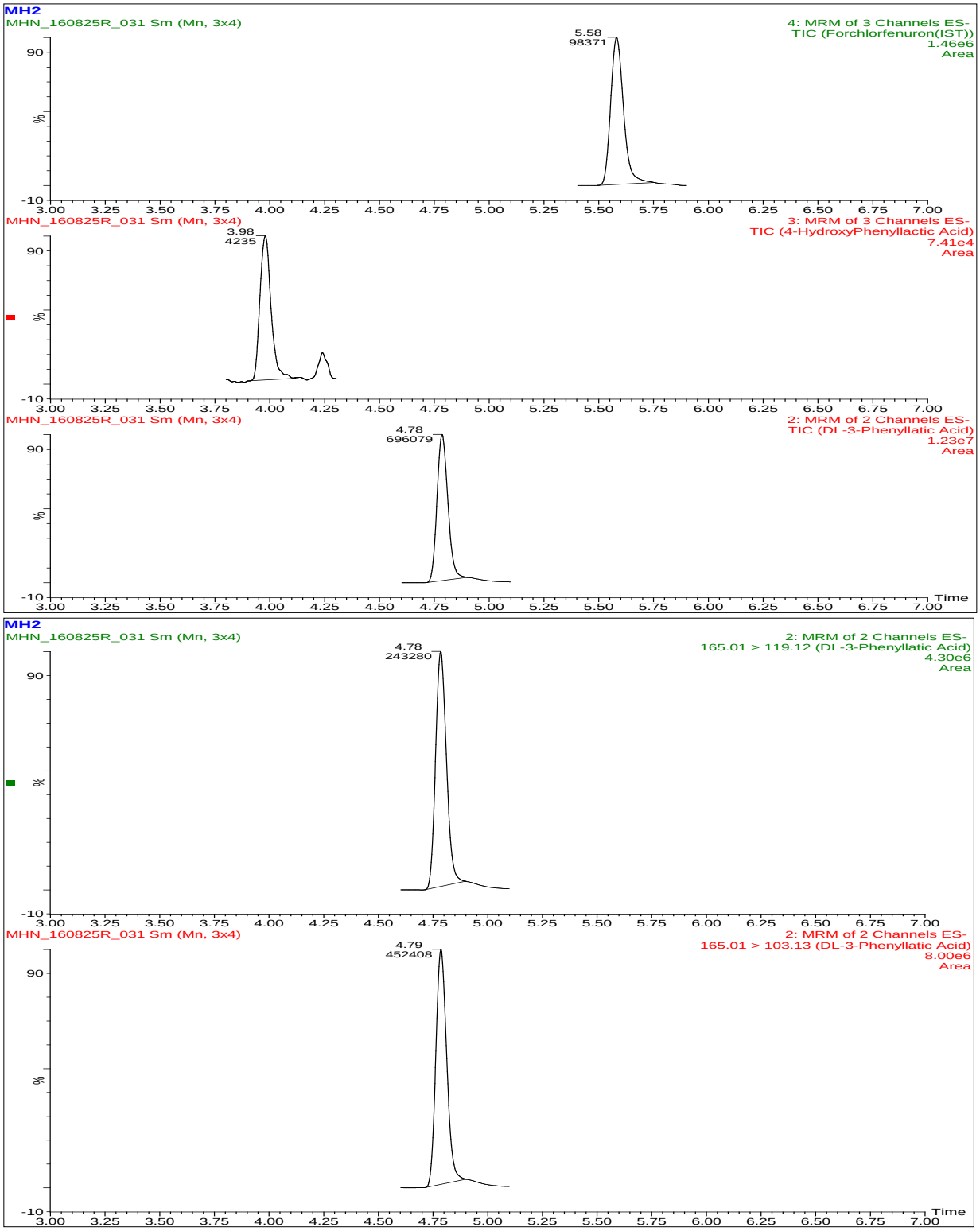


Figure 7: **MRM of 2 Channels ES- for 3-phenyllactic acid of 1.0 mg/kg honey spike and 0.05 mg/kg matrix standard**

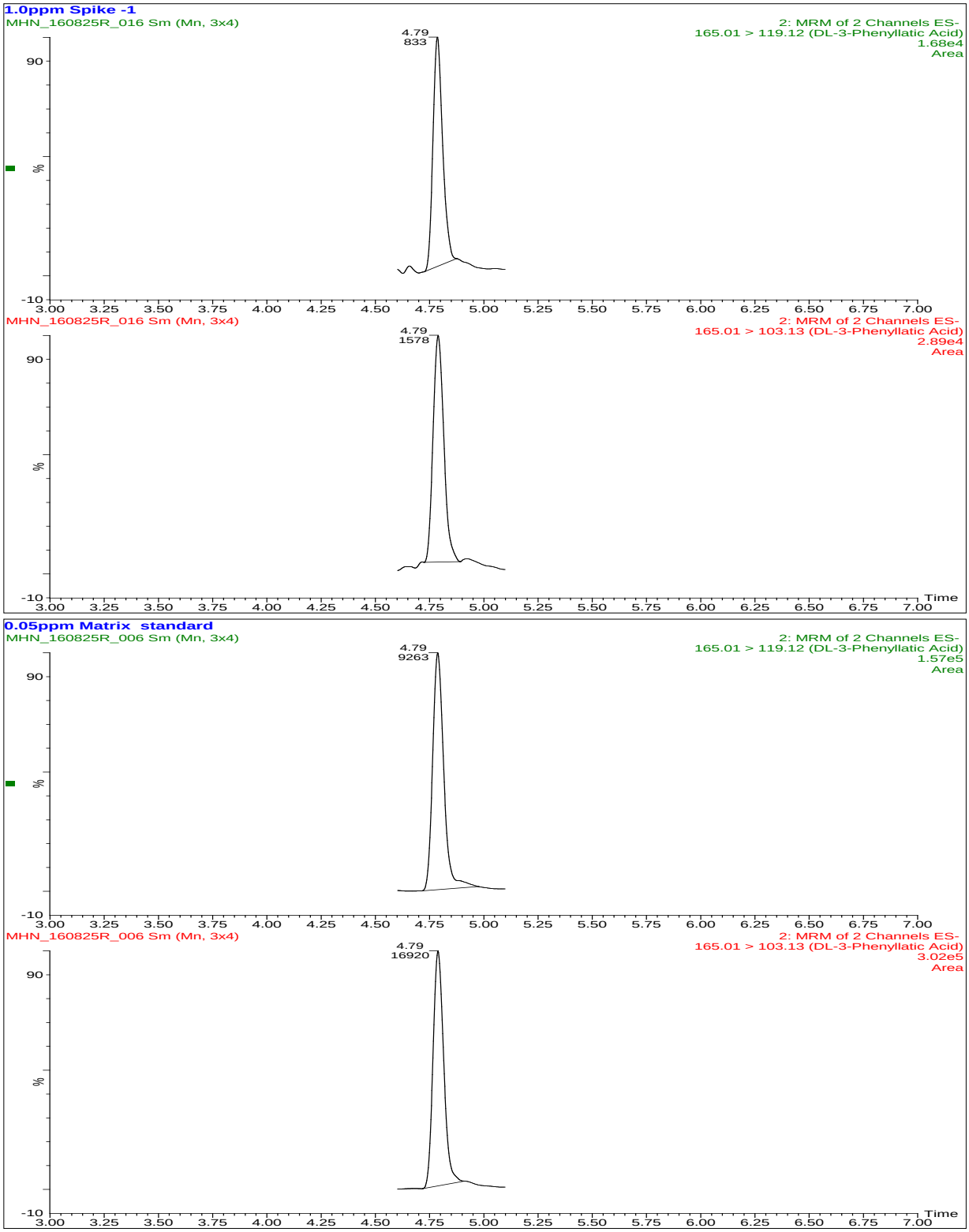


Figure 8: **MH2- Commercial source honey in positive ion (TIC and 3MRM ES+ 2-methoxybenzoic acid)**

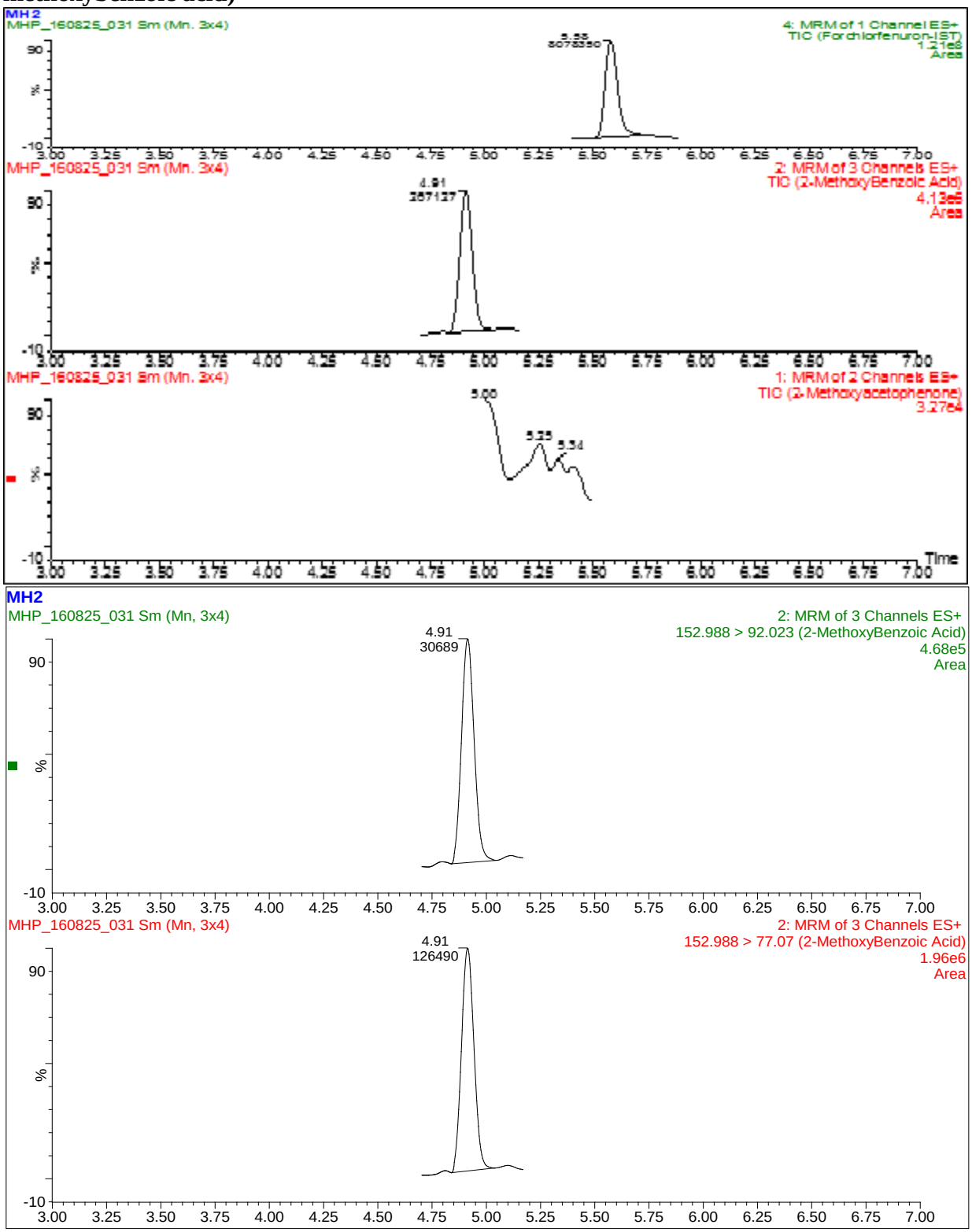


Figure 9: **MRM of 3 Channels ES+ for 2'-Methoxyacetophenone of 1.0mg/kg honey spike and 1.0 mg/kg matrix standard**

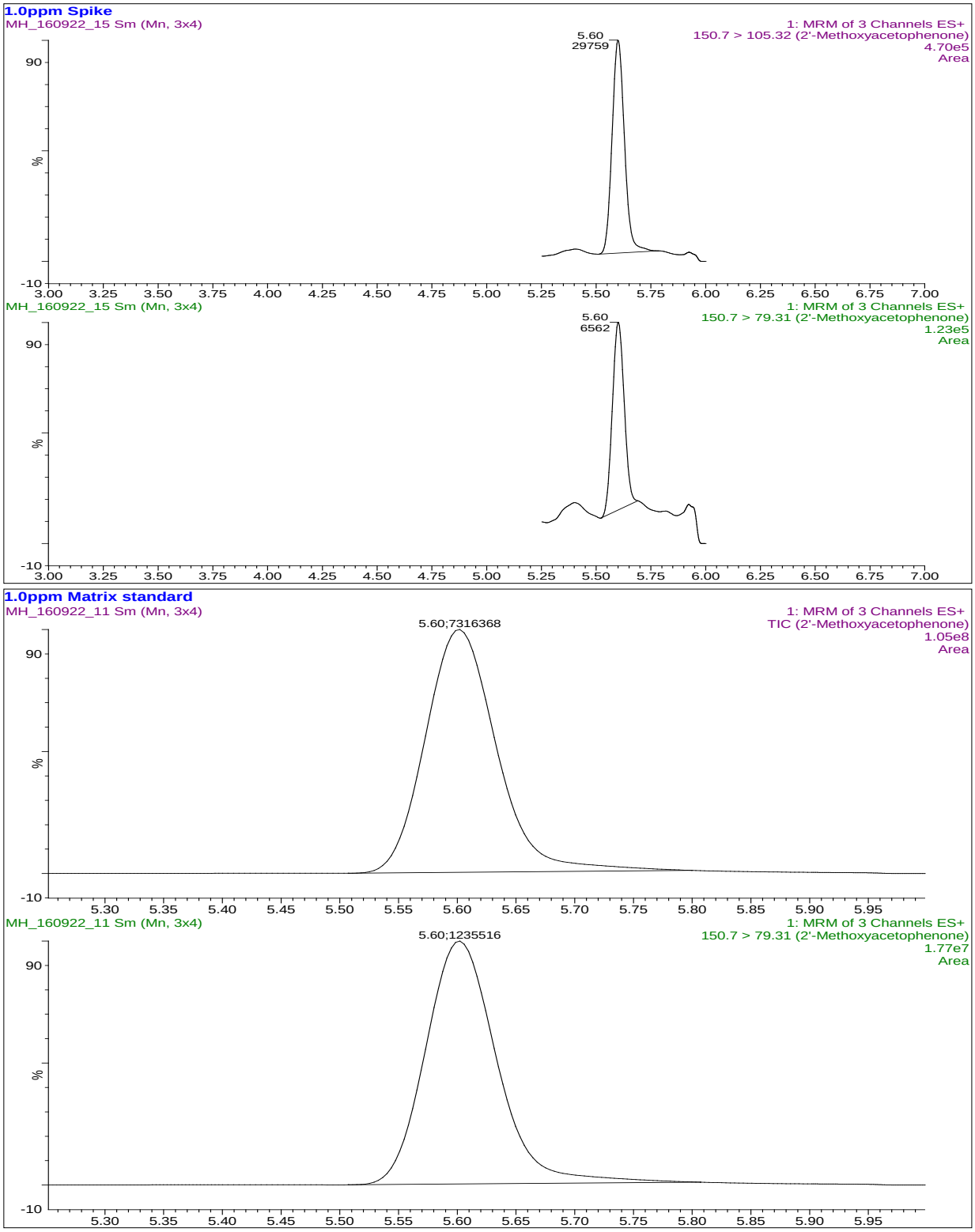
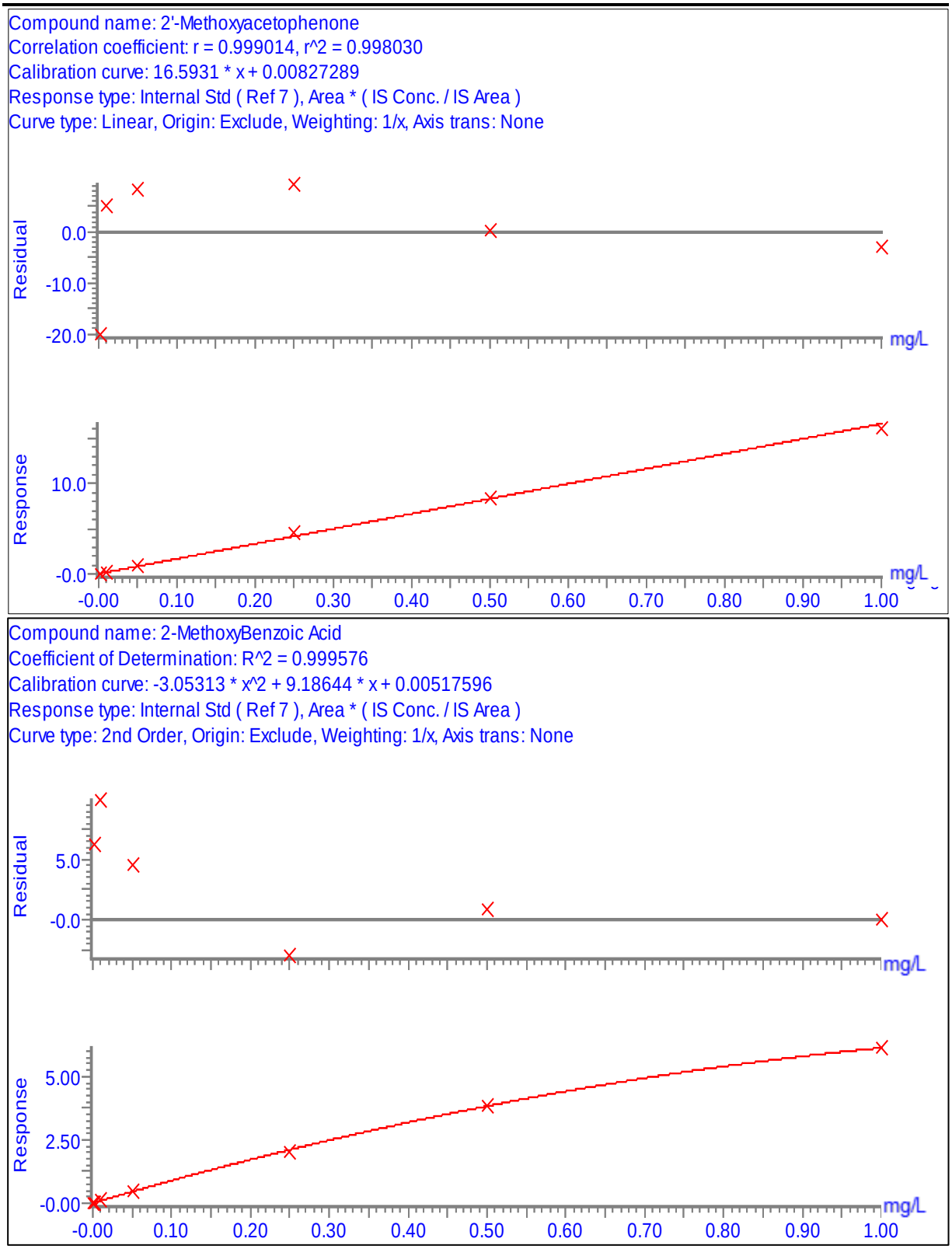
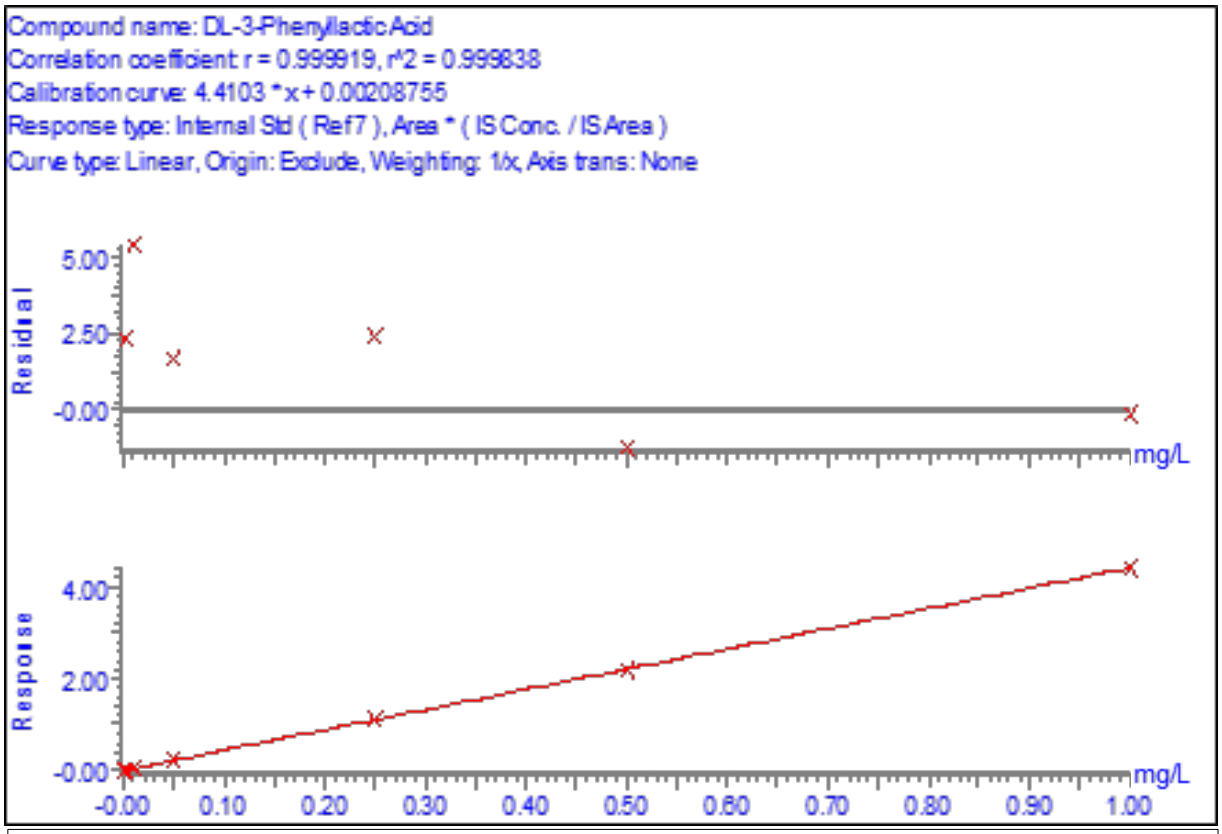
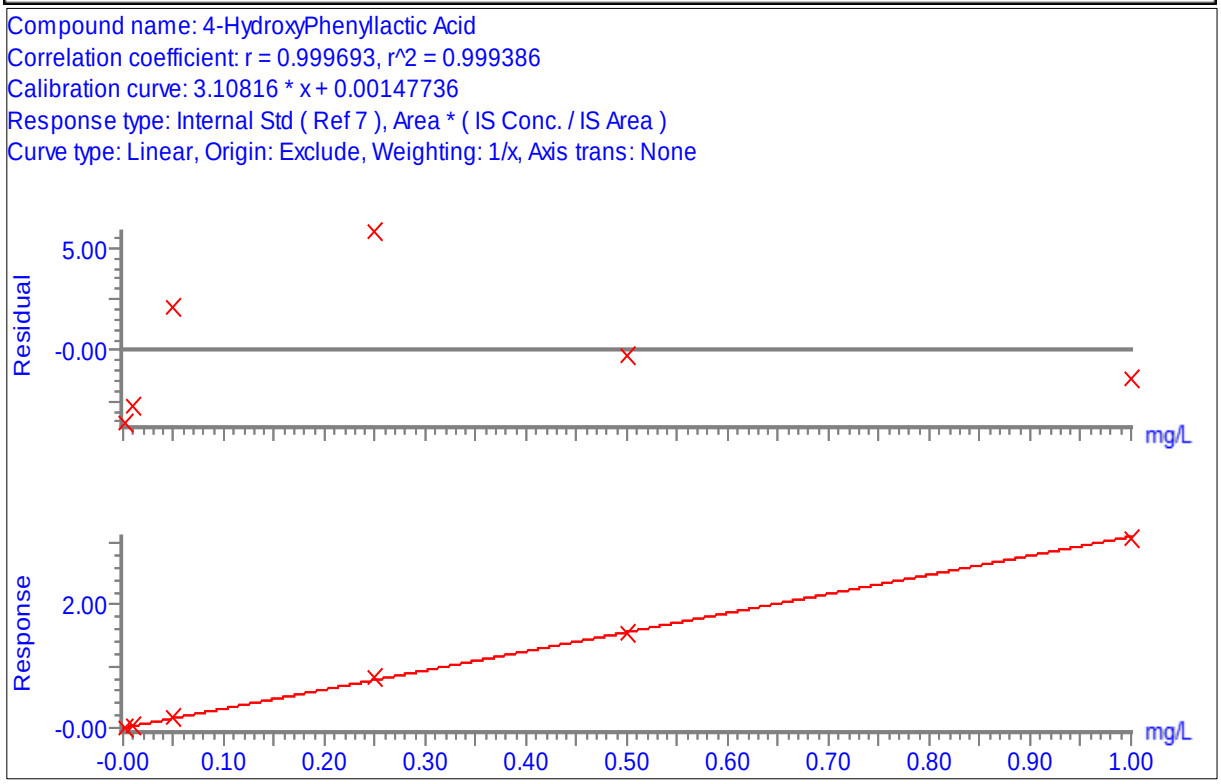


Figure10: Standard calibration curves for four chemical characterisation compounds of honey in the range of 0.002 mg/L - 1 mg/L.

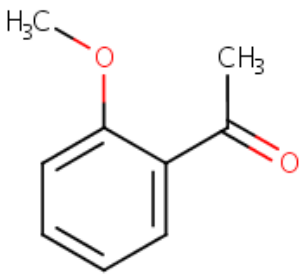
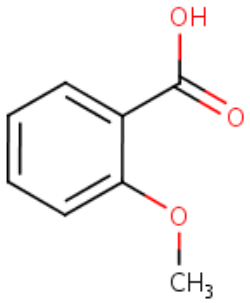
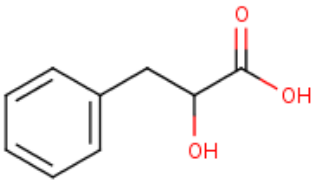
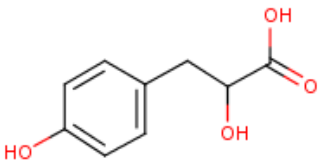



Compound name: DL-3-Phenyllactic Acid
Correlation coefficient: r = 0.999919, r^2 = 0.999838
Calibration curve: 4.4103 * x + 0.00208755
Response type: Internal Std (Ref 7), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None
Residual
5.00
2.50
-0.00
mg/L
Response
4.00
2.00
-0.00
mg/L
-0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00


Compound name: 4-HydroxyPhenyllactic Acid
Correlation coefficient: r = 0.999693, r^2 = 0.999386
Calibration curve: 3.10816 * x + 0.00147736
Response type: Internal Std (Ref 7), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None
Residual
5.00
-0.00
mg/L
Response
2.00
-0.00
mg/L
-0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00

10.2 STRUCTURES

Table 10: Characterisation compounds detail

Compound	**CAS No.**	**Molecular Formula**	**Structure**
2'-Methoxyacetophenone	579-74-8	$C_9H_{10}O_2$	
2-Methoxybenzoic acid	579-75-9	$C_8H_8O_3$	
3-Phenyllactic acid	828-01-3	$C_9H_{10}O_3$	
4-Hydroxyphenyllactic acid	306-23-0 / 6482-98-0	$C_9H_{10}O_4$	

10.3 VALIDATION DATA

a. Spiked recoveries at LOR, 2xLOR, 5xLOR, 50xLOR, 100xLOR, were carried out between three technicians from 01/08/2016 – 25/08/2016.

Table 11: Mean recoveries of honey spiked with the four characterisation compounds (1 mg/kg – 100 mg/kg)

Compound	**n**	**Recovery (%)**	**Standard Deviation (SD) (%)**	**Coefficient of Variation (CV) (%)**	**Acceptable Range ($\bar{X}$ ± 2SD) (%)**
2'-Methoxyacetophenone	40	102	7.9	7.8	86 – 117
2-Methoxybenzoic acid	40	102	6.8	6.7	88 – 115
3-Phenyllactic acid	40	90	6.9	7.7	76 – 104
4-Hydroxyphenyllactic acid	40	91	7.2	7.9	77 – 105

Table 12: Summary of individual recoveries at all levels for honey spiked with the four characterisation compounds

Compound	**Spike Level (mg/kg)**	**n**	**Recovery (%)**	**Range (%)**	**SD (%)**	**CV (%)**
2'-Methoxyacetophenone	1	8	98	86 – 110	5.9	6.0
	2	8	94	84 – 104	8.1	5.4
	5	8	107	92 – 121	7.3	6.9
	50	8	108	92 – 123	7.9	7.3
	100	8	102	94 – 111	4.2	4.1
2-Methoxybenzoic acid	1	8	106	94 – 118	5.9	5.6
	2	8	105	89 – 120	7.9	7.5
	5	8	102	92 – 112	5.1	5.0
	50	8	99	89 – 108	4.7	4.7
	100	8	98	83 – 112	7.2	7.4
3-Phenyllactic acid	1	8	90	79 – 101	5.3	5.8
	2	8	83	75 – 91	3.8	4.6
	5	8	91	80 – 102	5.3	5.8
	50	8	93	78 – 108	7.5	8.1
	100	8	92	75 – 109	8.6	9.4
4-Hydroxyphenyllactic acid	1	8	97	82 – 112	7.6	7.9
	2	8	87	78 – 96	4.3	5.0
	5	8	92	79 – 105	6.3	6.9
	50	8	90	76 – 104	7.2	8.0
	100	8	90	74 – 106	7.9	8.8

b. Measurement uncertainty determination method

The combined standard uncertainty was determined by considering the following major sources of measurement uncertainty; sample recovery, sample homogeneity, purity and linearity of analytical standard. A coverage factor of 2 (K=2) was applied to all combined

standard uncertainties to give a 95% confidence interval (CI) for the % measurement uncertainty estimates.

Homogeneity: Relative Standard Deviation (RSD) of duplicate analyses of 22 honey samples for each of the characterisation compounds.

Table 14: Relative standard deviation (RSD) for homogeneity estimates

Compound	**n**	**RSD**
2'-Methoxyacetophenone	22	0.0177
2-Methoxybenzoic acid	22	0.0184
3-Phenyllactic acid	22	0.0185
4-Hydroxyphenyllactic acid	22	0.0105

Method Recovery: The RSD of all recoveries from 3 batches carried out by three different analysts for concentrations from 1 to 100 mg/kg.

Calibration: The RSD of the calibration curve (NMI calculation template). This may be a slight overestimation as it has been determined using non-weighted concentrations whereas the calibration used by the instrument software has a $\frac{1}{x}$ weighting.

Standard Mass: The RSD of the standard mass for preparation of the stock standards.

Other sources: The total from all other sources estimated using scientific judgement.

Results are corrected for recovery. Therefore a normal distribution can be assumed for the remaining combined uncertainties.

c. The measurement uncertainty was estimated to be as follows for honey matrices
The resulting combined uncertainties are displayed in the tables below:

Table 14: Measurement uncertainties

Compound	**LOD (mg/kg)**	**LOR (mg/kg)**	**Linear Range (mg/kg)**	**Measurement uncertainty at the 95% confidence level and concentration at which MU was established**
2'-Methoxyacetophenone	0.5	1	1 – 100	± 20% at 5 mg/kg
2-Methoxybenzoic acid	0.5	1	1 – 100	± 25% at 5 mg/kg
3-Phenyllactic acid	0.5	1	1 – 100	± 20% at 5 mg/kg
4-Hydroxyphenyllactic acid	0.5	1	1 – 100	± 20% at 5 mg/kg

10.4 SYNTHETIC HONEY SUBSTITUTE FOR CONTROLS

If no naturally blank honey is available, a synthetic honey substitute can be used as an alternative blank matrix.
Note: The use of nature honey as blank matrix is recommended where possible.

10.4.1 Equipment

a. Beaker, glass, 50 mL
b. Hotplate, stirrer

10.4.2 Reagents

a. D-(–)-Fructose, ≥99% – ACS reagent grade, Sigma-Aldrich F0127
b. D-(+)-Glucose, ≥99.5% – ACS reagent grade, Sigma-Aldrich G8270
c. D-(+)-Maltose, ≥99% – ACS reagent grade, Sigma-Aldrich M5885
d. Sucrose, ≥99.5% – ACS reagent grade, Sigma-Aldrich S0389
e. Water, deionised

10.4.3 Method

Into a 50 mL beaker weigh 8.4 g ± 0.1 g of fructose, 6.6 g ± 0.1 g of glucose, 0.26 g ± 0.01 g of maltose, 0.12 g ± 0.01 g of sucrose and 4.6 g ± 0.1 g of deionised water. Place on a hotplate stirrer and warm gently while mixing until all sugars are completely dissolved. Remove from heat and allow to cool. Transfer to an air-tight container. Stable for 6 months when stored in a refrigerator at 2 – 8°C.

11 Approvals and Authorities

11.1 APPROVALS ON FILE

a. Issuing Authority: Manager, Chemical and Microbiological Assurance