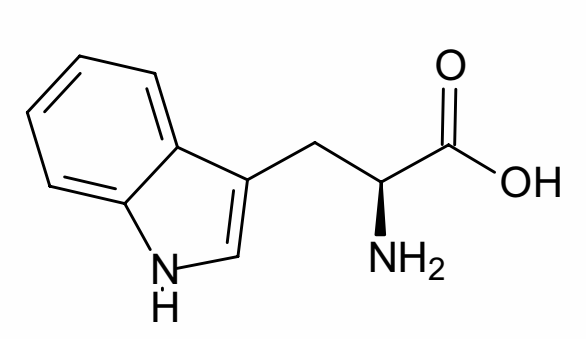
Development and Validation of LC-MS/MS Methods for the Quantification of Biomarkers Tryptophan, Kynurenine and 3-Hydroxykynurenine in Human Plasma

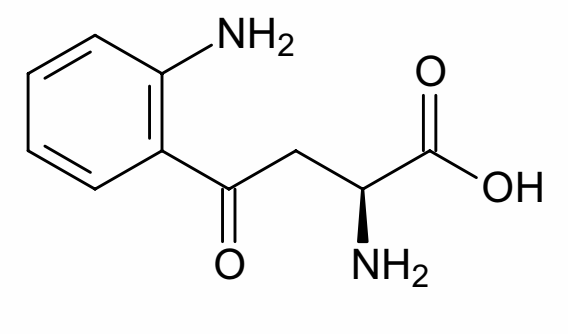
J. Anguizola PhD[1], A. Dzerk[1], C. Kafonek[1], G. James[1], S. Koshiba PhD[2], S. Horita[2], Y. Liu PhD[3], and M. Mizutani PhD, D.V.M[3]
[1]Celerion, Lincoln, NE USA, [2]Translational Research Unit, Research and Development Division, Kyowa Hakko Kirin Co.Ltd.,
Shizuoka, Japan and [3] Translational Research, Kyowa Kirin Pharmaceutical Development, Inc., NJ USA

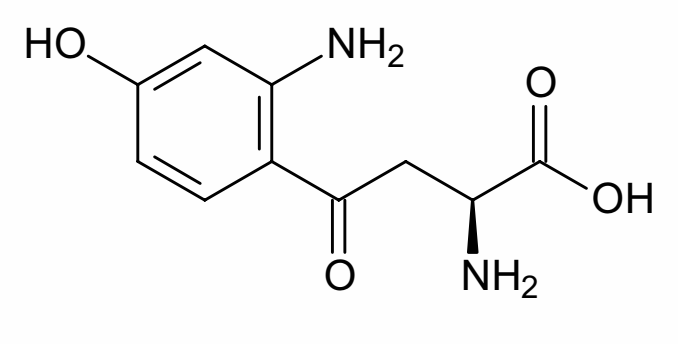
celerion
Translating Science to Medicine

INTRODUCTION

- Tryptophan is an essential amino acid known for its crucial role in protein synthesis and as the precursor for several biologically active compounds such as a kynurenine.
- Several pathologies are associated with the metabolism of tryptophan and the generation of its kynurenine and 3-OH kynurenine metabolites.
  - Cancer patients tend to have tryptophan concentrations lower than normal and kynurenine levels higher than normal due to the increasing activity of enzyme indoleamine 2,3-dioxygenase (IDO) which catalyzes the rate-limiting step of tryptophan degradation along the kynure nine pathway.
- The purpose of this work was to develop and validate reliable and accurate methods for the quantification of tryptophan, kynurenine, and 3-OH kynurenine in human plasma with limits of quantitation below the endogenous concentrations found in a healthy population.
- Quantification of abnormally low endogenous concentrations in human plasma required that calibration standards and low level QCs be prepared in surrogate matrix (ultrapure water or 4X charcoal stripped plasma).


L-Tryptophan


L-Kynurenine


3-OH Kynurenine

METHODS

- Separate analytical methods were developed for tryptophan/kynurenine and 3-OH kynurenine due to significantly different target concentration ranges.
- Processing was by protein precipitation using chilled acetonitrile, evaporation of supernatant and appropriate reconstitution for injection.
- Positive ions generated by a TurboIonSpray source were monitored in the multiple reaction-monitoring mode using AB Sciex triple quadrupole mass spectrometers.
- For tryptophan/kynurenine, analysis was performed using a PhenomenexSynergi™ Polar RP (50 x 2.0 mm) column and AB SciexAPI 4000 detector.
- For 3-OH kynurenine, analysis was performed using a Waters Acquity UPLC HSS T3 (100 x 2.1 mm) column and AB SciexAPI 4000 detector.

CHALLENGES

- 3-OH Kynurenine was shown to have serious sensitivity to light and temperature. To maintain stability, samples were:
  - Stored in brown tubes at -80°C.
  - Thawed / aliquotted on ice under yellow light.
- High variability in tryptophan data was traced to the MS/MS duty cycle (1-period method) which caused gaps between the analyte and the IS data acquisition of 80 or 160 msec.
  The much lower dwell times for the relatively more sensitive tryptophan had no effect on the kynurenine data. The ions were scanned in the order of increasing Q1 m/z, for the 1-period method:
  - Tryptophan (m/z 205)/ 40 msec dwell time
  - Kynurenine (m/z 209)/ 160 msec dwell time
  - d5-Tryptophan(m/z 210)/ 20 msec dwell time
  - d6-Kynurenine (m/z 215)/ 80 msec dwell time

	1-Period Tryptophan QC A PK Area	IS Area	Ratio	1-Period Tryptophan QC C PK Area	IS Area	Ratio
Mean	16431	157289	0.104	1119523	155976	7.18
CV %	14.8	7.2	8.8	3.9	4.8	4.6
n	6	6	6	6	6	6
	2-Period Tryptophan QC A PK Area	**IS Area**	**Ratio**	**2-Period Tryptophan QC C PK Area**	**IS Area**	**Ratio**
Mean	50077	434866	0.115	4389488	608845	7.23
CV %	35.0	35.0	2.4	13.3	15.0	2.2
n	6	6	6	6	6	6

By utilizing a 2-period method the ratio data for tryptophan at low concentrations became routinely acceptable even though the peak area variability increased.

RESULTS

- The injection cycle for each method (tryptophan/kynurenine or 3-OH kynurenine) was less than 3 minutes.
- Calibration curves were regressed with a weighted (1/concentration^2) least squares linear regression over the ranges of 1.00 – 250 µM for tryptophan, 0.100 – 25.0 µM for kynurenine and 5.00 – 1500 nM for 3-OH kynurenine.
- Intra/Inter-batch precision (C.V.%) accuracy (R.E.%) of the quality control samples met validation acceptance criteria (See Table 1).
- The selectivity and integrity of the methods were demonstrated by accurate quantitation of the analytes in multiple lots of human plasma (including hemolyzed and lipemiclots) fortified with several concentrations spread across the standard curve. See Tables 2-4 for examples of the highest and lowest concentrations fortified.
- Selectivity was demonstrated against multiple compounds (typical over-the-counter compounds and commonly prescribed medications) that could potentially interfere with quantitation.
- Stability was demonstrated for all analytes and established for stock solutions, surrogate matrix samples, plasma samples and injection solvent for all the appropriate temperatures, light, and storage conditions.

Figure 1. Kynurenine LLOQ (Surrogate Matrix) Overlay with Control Blank

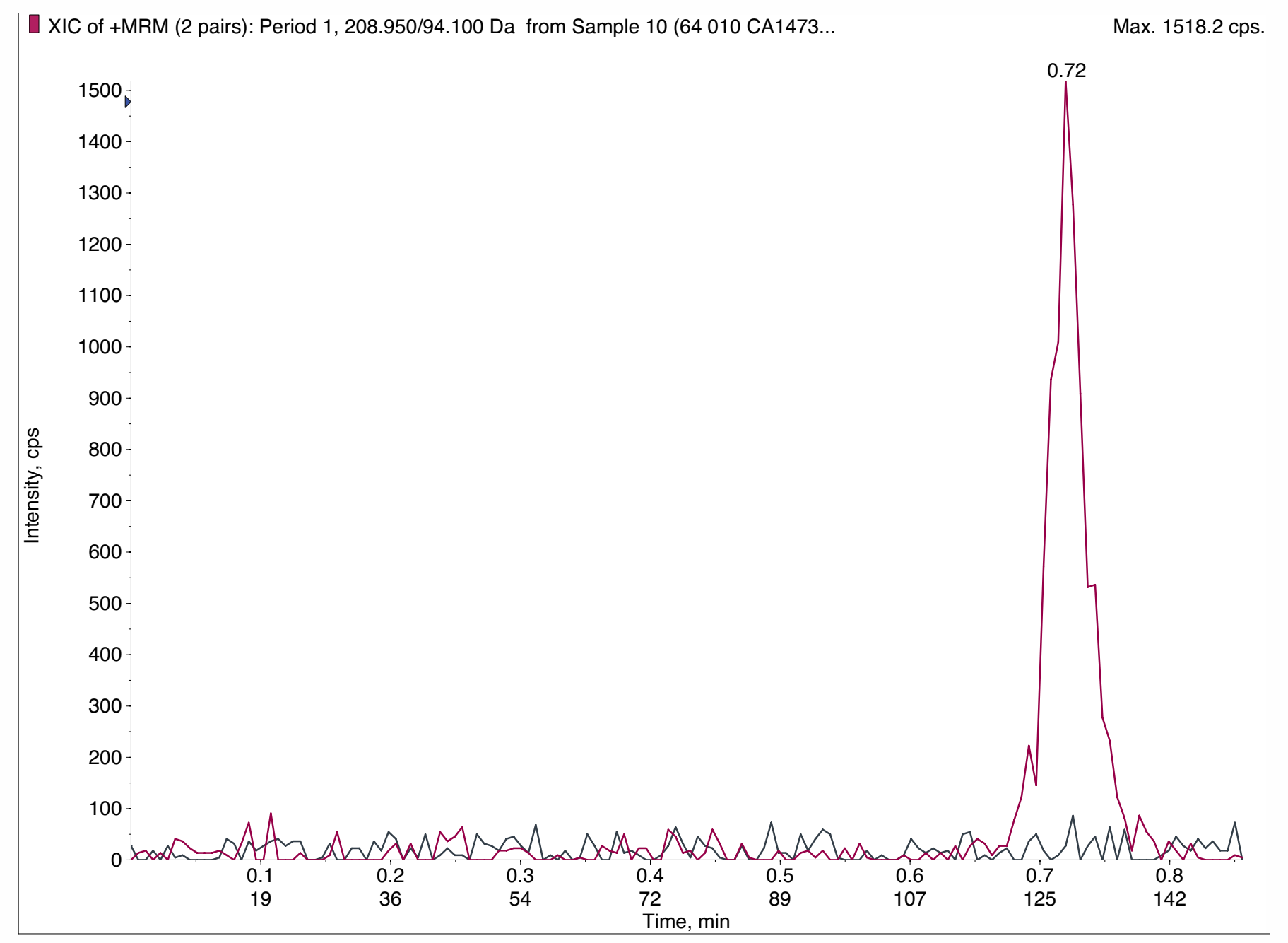


Figure 2. Kynurenine Low Plasma QC

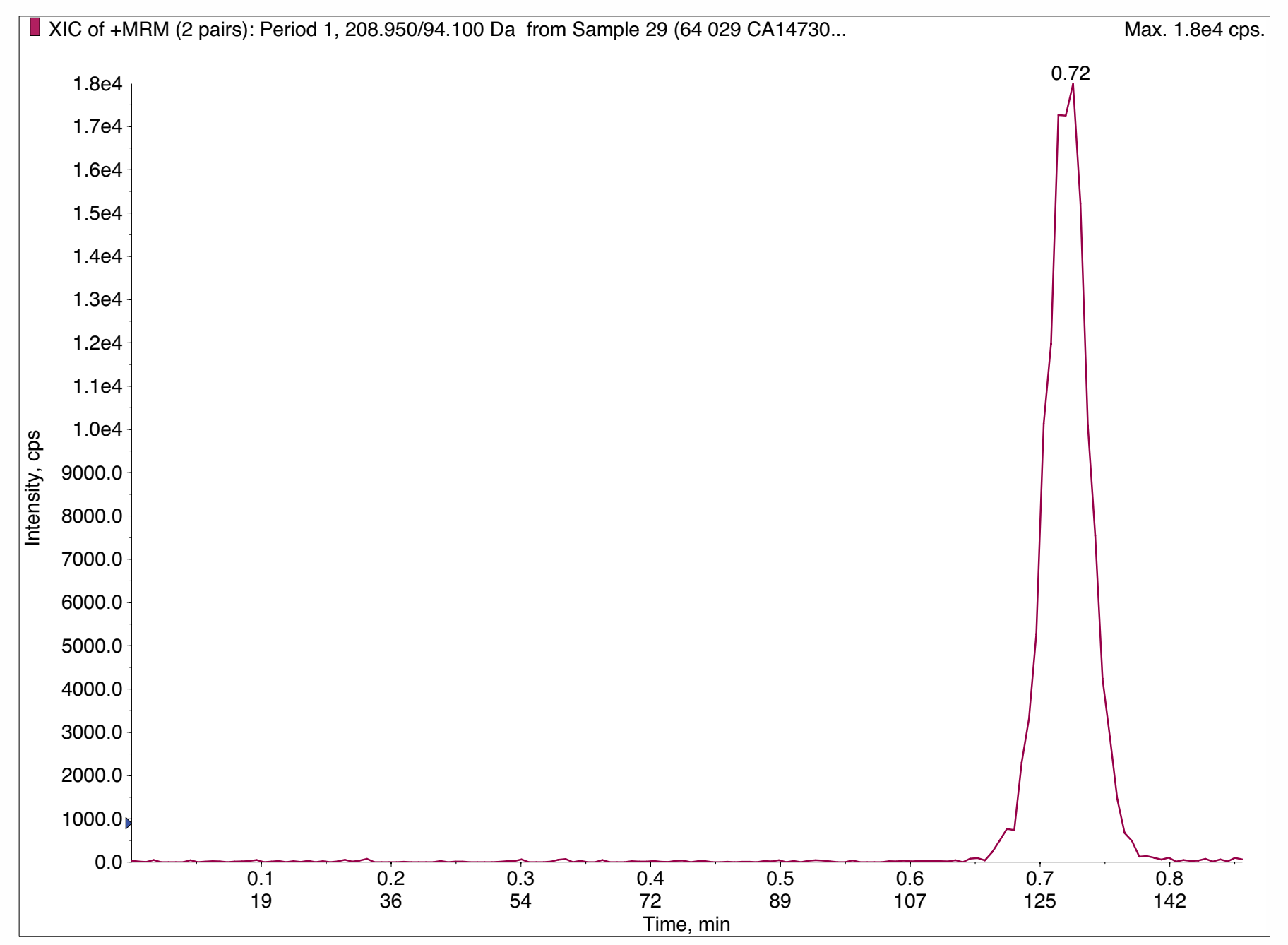


Figure 3. Tryptophan LLOQ (Surrogate Matrix) Overlay with Control Blank

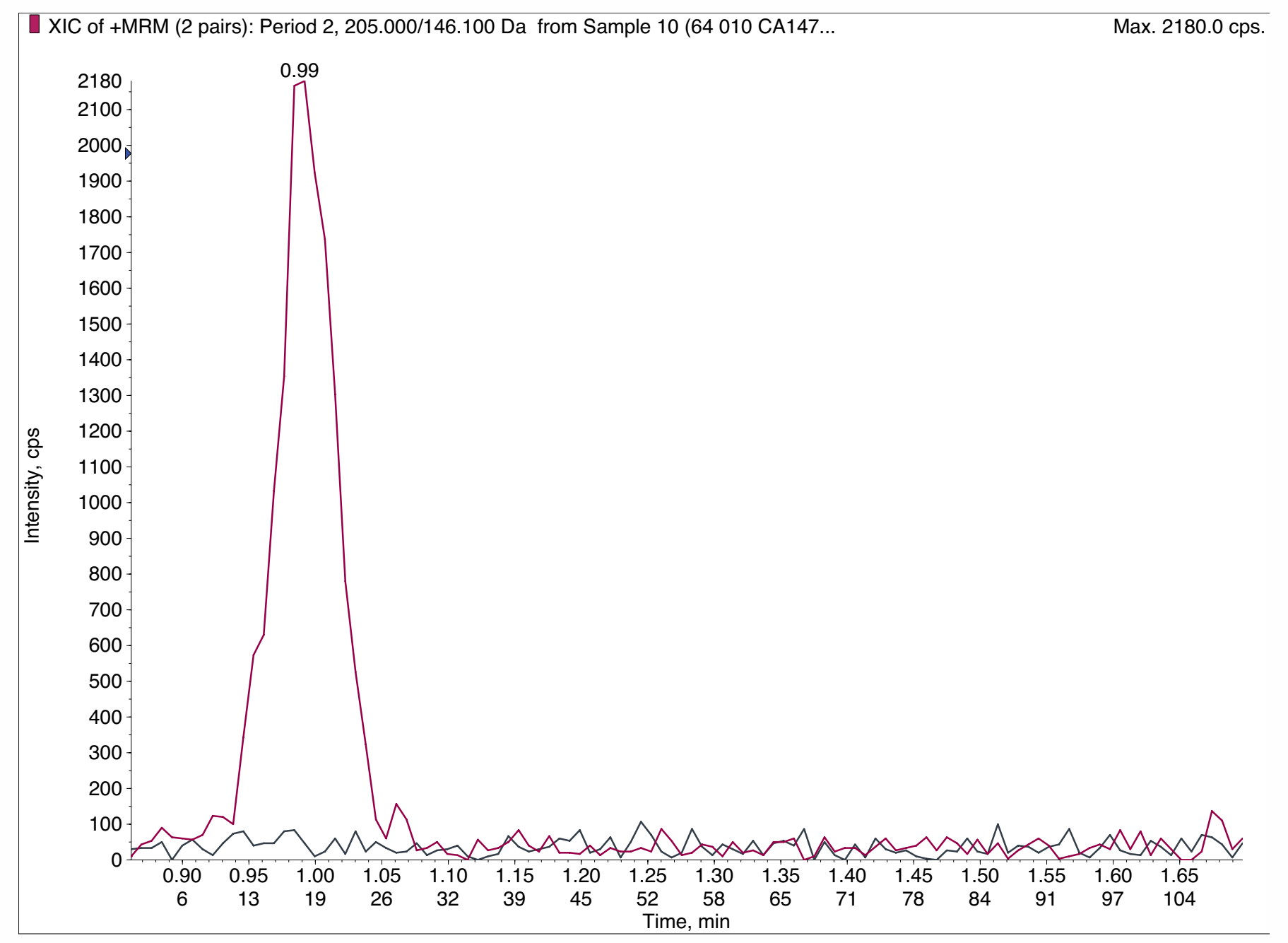


Figure 4. Tryptophan Low Plasma QC

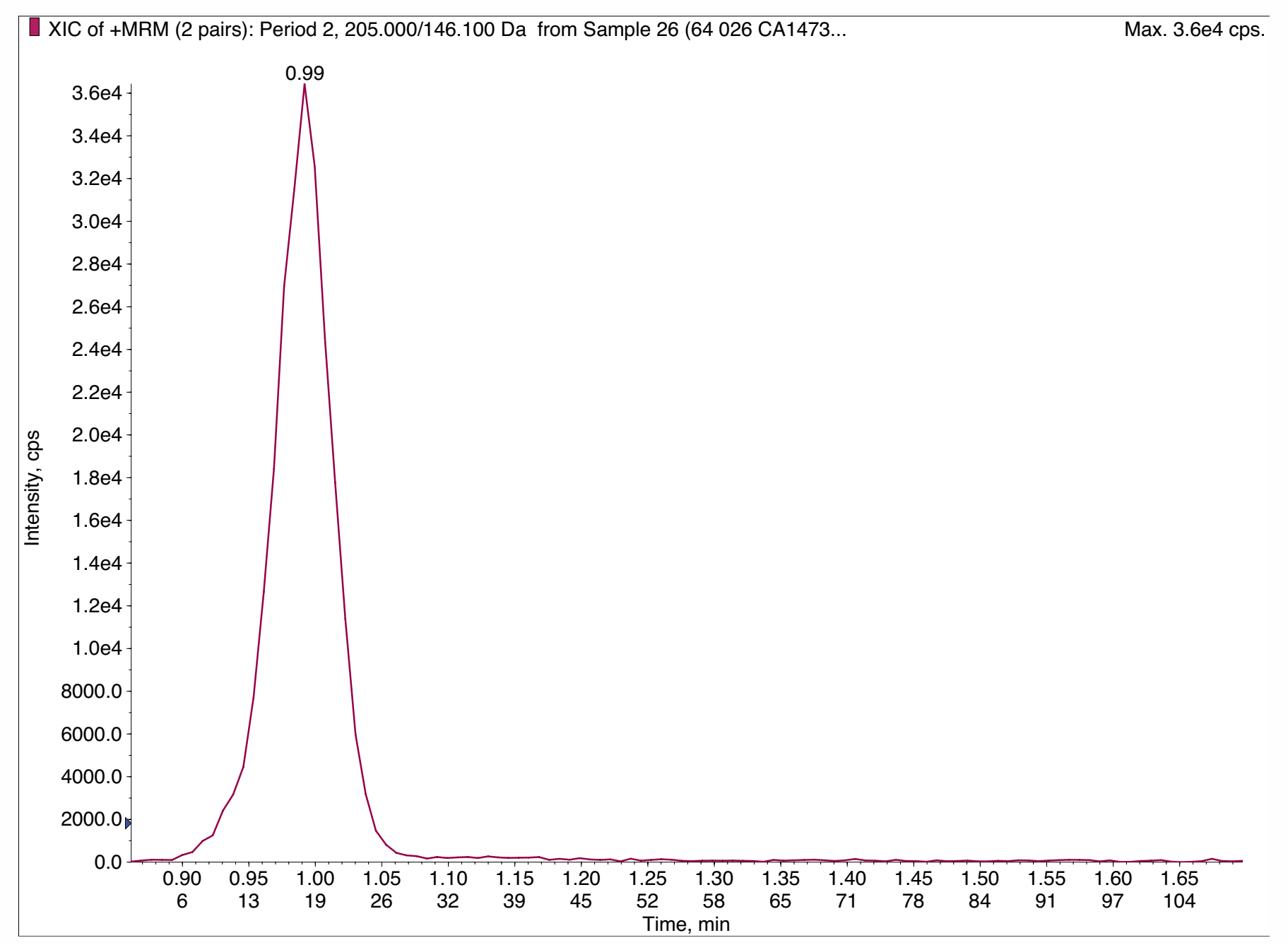


Figure 5. 3OH-Kynurenine LLOQ (Surrogate Matrix) Overlay with Control Blank

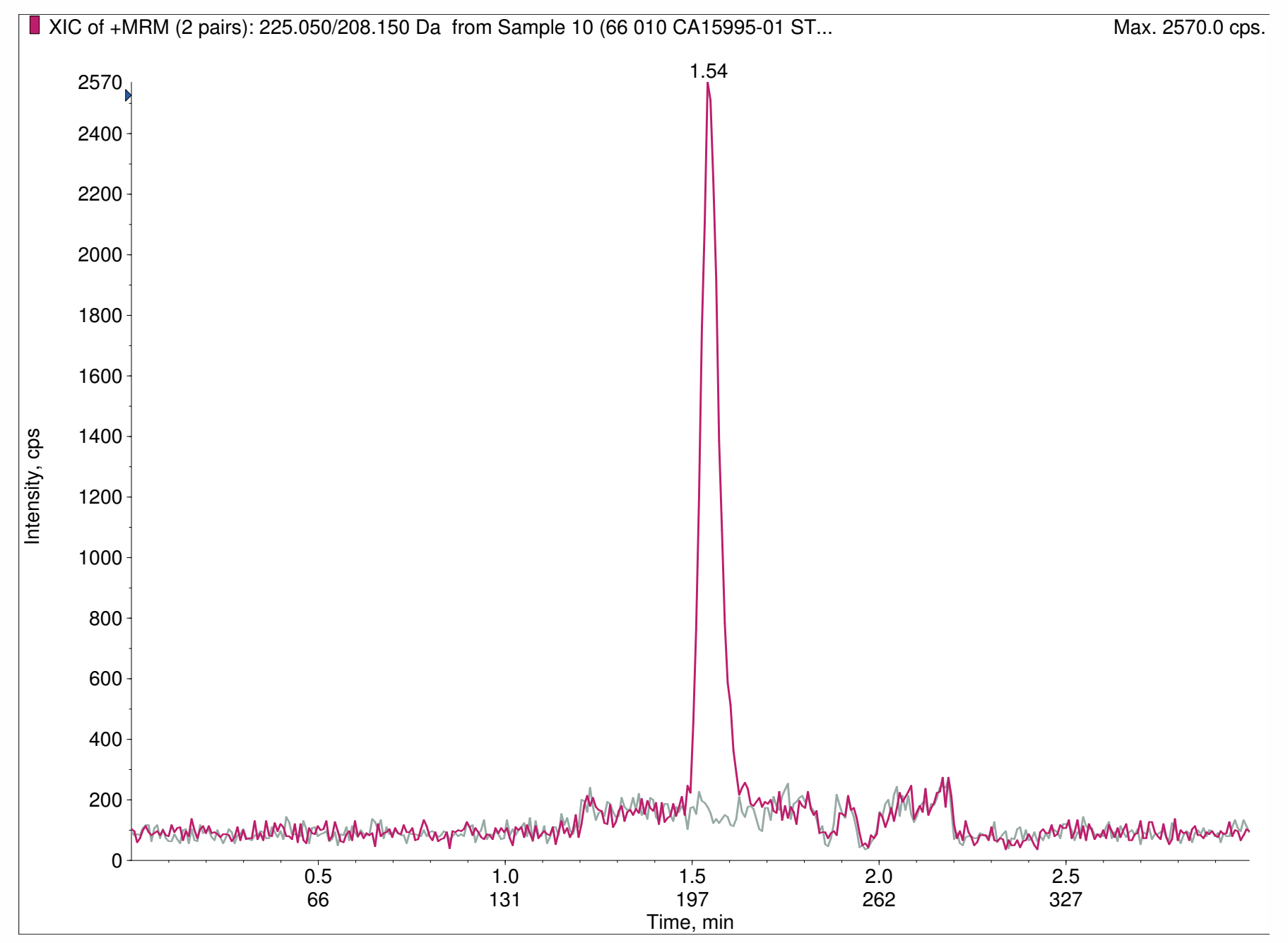


Figure 6. 3OH-Kynurenine Low Plasma QC

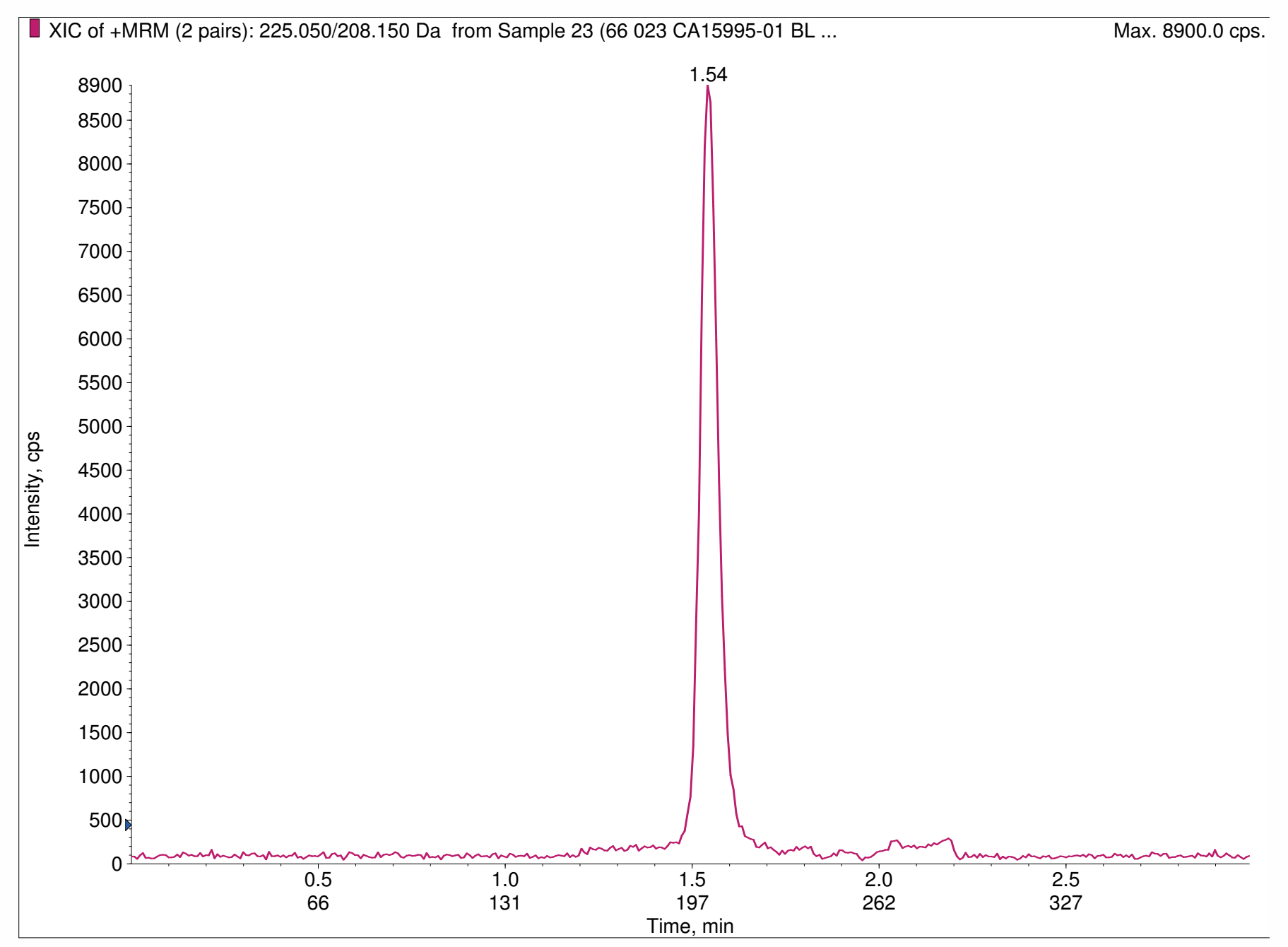


Table 1

	Kynurenine	Tryptophan	3-OH Kynurenine
QC Intra-Batch Precision Range (% CV)	1.7 to 10.9%	1.1 to 5.6%	1.7 to 16.2%
QC Intra-Batch Accuracy Range (% Bias)	-14.4 to 7.2%	- 4.7 to 3.0%	-13.8 to 2.6%
QC Inter-Batch Precision Range (% CV)	4.3 to 11.8%	2.8 to 5.1%	4.7 to 8.6%
QC Inter-Batch Accuracy Range (% Bias)	-7.7 to 0.0%	- 1.2 to -0.3%	-6.7 to -1.3%

CONCLUSION/NOVEL ASPECT

Abnormally low concentrations of tryptophan, kynurenine and 3-OH kynurenine in human plasma can be measured accurately and precisely using robust and sensitive LC-MS/MS methods.

Table 2

		Kynurenine Low Conc. Spike 0.100 µM			Kynurenine High Conc. Spike 15.0 µM		
Lot #	Mean Basal Level µM	Mean Basal Level + Spike Conc. µM	Measured Conc. µM	% Dev.	Mean Basal Level + Spike Conc. µM	Measured Conc. µM	% Dev.
Plasma 1	1.74	1.84	1.93	+4.9	16.7	18.5	+10.5
Plasma 2	3.27	3.37	3.34	-0.9	18.3	19.6	+7.3
Plasma 3	1.05	1.15	1.13	-1.7	16.1	17.0	+5.9
Plasma 4	1.29	1.39	1.34	-3.6	16.3	17.5	+7.4
Plasma 5	1.14	1.24	1.21	-2.4	16.1	17.1	+5.9
Plasma 6	1.06	1.16	1.16	+0.0	16.1	17.0	+5.9
Plasma 7	1.30	1.40	1.42	+1.4	16.3	17.3	+6.1
Plasma 8	1.47	1.57	1.58	+0.6	16.5	17.8	+8.1
Plasma 9	2.16	2.26	2.23	-1.3	17.2	18.3	+6.6
Plasma 10	1.76	1.86	1.88	+1.1	16.8	18.2	+8.6
HemolyzedPlasma 1	1.21	1.31	1.38	+5.3	16.2	16.6	+2.4
HemolyzedPlasma 2	1.92	2.02	2.00	-1.0	16.9	17.0	+0.5
HemolyzedPlasma 3	1.61	1.71	1.79	+4.7	16.6	17.1	+3.0
LipemicPlasma 1	1.57	1.67	1.79	+7.2	16.6	17.2	+3.8
LipemicPlasma 2	1.13	1.23	1.28	+4.1	16.1	16.3	+1.1
LipemicPlasma 3	0.613	0.713	0.729	+2.2	15.6	15.5	-0.7

Table 3

		Tryptophan Low Conc. Spike 1.00 µM			Tryptophan High Conc. Spike 150 µM		
Lot #	Mean Basal Level µM	Mean Basal Level + Spike Conc. µM	Measured Conc. µM	% Dev.	Mean Basal Level + Spike Conc. µM	Measured Conc. µM	% Dev.
Plasma 1	63.1	64.1	64.4	+0.5	213	208	-2.4
Plasma 2	65.5	66.5	64.9	-2.4	216	201	-6.7
Plasma 3	40.2	41.2	40.9	-0.7	190	173	-9.0
Plasma 4	55.2	56.2	53.7	-4.4	205	186	-9.4
Plasma 5	48.1	49.1	48.0	-2.2	198	192	-3.1
Plasma 6	49.8	50.8	51.0	+0.4	200	185	-7.4
Plasma 7	54.8	55.8	54.7	-2.0	205	192	-6.3
Plasma 8	63.7	64.7	64.3	-0.6	214	201	-5.9
Plasma 9	55.6	56.6	55.3	-2.3	206	196	-4.7
Plasma 10	51.2	52.2	51.0	-2.3	201	190	-5.6
HemolyzedPlasma 1	51.3	52.3	53.9	+3.1	201	199	-1.1
HemolyzedPlasma 2	54.6	55.6	54.1	-2.7	205	194	-5.2
HemolyzedPlasma 3	51.5	52.5	52.8	+0.6	202	199	-1.2
LipemicPlasma 1	55.0	56.0	55.5	-0.9	205	203	-1.0
LipemicPlasma 2	43.1	44.1	43.8	-0.7	193	190	-1.6
LipemicPlasma 3	63.5	64.5	65.3	+1.2	214	208	-2.6

Table 4

		3OH-Kynurenine Low Conc. Spike *=5.00 nM;$^{\#}$=10.0 nM;$^{@}$=12.0 nM;$^{\wedge}$=12.5 nM			3OH-Kynurenine High Conc. Spike 1130nM		
Lot #	Mean Basal Level nM	Mean Basal Level + Spike Conc. nM	Measured Conc. nM	% Dev.	Mean Basal Level + Spike Conc. nM	Measured Conc. nM	% Dev.
Plasma 1*	13.9	18.9	19.2	+1.8	1144	1110	-3.0
Plasma 2*	20.8	25.8	24.7	-4.3	1151	1040	-9.6
Plasma 3*	4.71	9.71	9.26	-4.6	1135	1050	-7.5
Plasma 4$^{\#}$	18.4	28.4	29.1	+2.3	1148	1020	-11.2
Plasma 5$^{\#}$	17.3	27.3	27.4	+0.5	1147	1020	-11.1
Plasma 6$^{\#}$	13.7	23.7	24.1	+1.8	1144	969	-15.3
Plasma 7$^{\#}$	18.1	28.1	28.5	+1.3	1148	1090	-5.1
Plasma 8$^{\#}$	18.0	28.0	29.7	+5.9	1148	994	-13.4
Plasma 9$^{\wedge}$	17.4	29.9	29.6	-1.1	1147	1060	-7.6
Plasma 10$^{\wedge}$	12.3	24.8	25.7	+3.5	1142	1380	+20.8
HemolyzedPlasma 1$^{\#}$	23.3	33.3	34.3	+3.1	1153	1250	+8.4
HemolyzedPlasma 2$^{\#}$	17.3	27.3	27.0	-1.2	1147	1270	+10.7
HemolyzedPlasma 3$^{\#}$	24.6	34.6	34.6	+0.0	1155	1110	-3.9
LipemicPlasma 1*	0.00	5.00	5.58	+11.6	1130	1220	+8.0
LipemicPlasma 2$^{@}$	35.0	47.0	47.2	+0.4	1165	1177	+1.0
LipemicPlasma 3*	3.20	8.20	7.46	-9.1	1133	1138	+0.4