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Analysis of Amino Acids in Mānuka Honey

A thesis

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Abstract

Honey, a natural product produced by honeybees, has a complex matrix of sugars proteins, minerals, vitamins, enzymes and free amino acids. Amino acids, obtained from the nectar of plants, account for 50-30 mg/kg of honey, the most abundant of which is proline, a secondary amino acid originating mainly from the haemolymph of bees.

Three methods were investigated for the analysis of amino acids in honey. The first used HPLC-UV with pre-column derivatisation, the second HPLC-MS with hydrophobic interaction chromatography, and finally HPLC-MS with aTRAQ™ derivatisation.

The HPLC-UV method involves derivatisation of amino acids by OPA-MPA and FMOC. A fully automated injection program analysed seventeen primary amino acids in 19 minutes. Ultimately, the detection by UV had inadequate sensitivity, and the secondary amino acid proline could not be detected. The method was rejected for these main reasons.

ZIC-pHILIC chromatography paired with LC-MS-MS gave high-quality separation of twenty one amino acids, detected using scheduled MRM, in 10 minutes. Amino acid recovery out of vial was low for the majority, this variation originating from sample preparation. In vial loss of amino acids could not be recovered and so investigation into the last method was initiated.

The final method used an aTRAQ™ kit which labels amino acids with a Δ8 reagent for analysis and also provides Δ0 labelled internal standards for comparison. Forty eight amino acids and internal standards can be accurately detected by MRM's in 18 minutes. Sample preparation was optimised for honey and the method was validated.

The amino acid content of ten honeys were compared to values obtained from Massey University. Small differences in the majority of amino acids were observed. Mānuka and clover honeys from this data set were also compared, it was found that phenylalanine and tyrosine were at much higher concentrations in clover honey.

Seven mānuka honeys stored in different conditions, warm and cold, were analysed. Applied statistical analysis with the hypothesis that the warm honeys would have lower amino acid concentrations than the cold, found this to be true for seventeen amino acids. Glutamine and then lysine were at decreased concentrations after warm storage in the most honey samples.

The amino acid content of honey, analysed by this method can be used to investigate: botanical origin of honey, speed of honey production/harvest, effect on DHA conversion, inaccurate labelling, and sugar syrup addition.

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List of Abbreviations

Instrumentation

HPLC	High Pressure Liquid Chromatography
GC	Gas Chromatography
UV	UltraViolet
Flu	Fluorescence
MS	Mass Spectrometry
LC	Liquid Chromatography
HILIC	Hydrophobic Interaction Liquid Chromatography
TOF	Time of Flight
FID	Flame Ionisation Detection
UPLC	Ultra-High Pressure Liquid Chromatography
Upd	User-Defined Program
ODS	Octodecylsilane (C18)
MRM	Multiple Reaction Monitoring
RP	Reverse Phase (chromatography)

Statistical Terms

PCA	Principal Component Analysis
LDA	Linear Discriminate Analysis
RSD	Relative Standard Deviation
CV	Coefficient of Variation

Derivatising Agents

OPA	*o*-phthaladehyde
FMOC	Fluorenylmethyloxycarbonyl chloride
MPA	3-mercaptopropionic acid
NAC	N-acetyl-L-cysteine
MCE	2-mercaptoethanol
DEEM	Diethyl ethoxymethylenemalonate
ISTD	Internal Standard

Amino Acids

Asp	L-Aspartic Acid
Glu	L-Glutamic Acid
Ser	L-Serine
Thr	L-Threonine
Tyr	L-Tyrosine
Gly	Glycine
Pro	L-Proline
Ala	L-Alanine
Met	L-Methionine
Val	L-Valine
Phe	L-Phenylalanine
Leu	L-Leucine
MOx	L-Methionine sulfoxide
Cys	L-Cystine
His	L-Histidine
Lys	L-Lysine
Arg	L-Arginine
Nva	L-Norvaline
Ile	L-Isoleucine
Nle	L-Norleucine
Asn	L-Asparagine
Gln	L-Glutamine
Trp	L-Tryptophan
HydPro	Hydroxy -L-proline

Chapter One: Introduction

The aim of this research was to investigate amino acids in honey by creating an efficient, sensitive method of analysis.

This chapter discusses the importance of amino acid content of honeys, and many methods previously used to analyse amino acids. This research provided pathways for formulating a method of analysis.

1.1 Honey

Honey is a natural product produced by honeybees, obtained from the nectar of plants. The type of honey depends on the type of plant the nectar is obtained from and can be unifloral, originating predominantly from only one type of plant, or multifloral, originating from many different plants. Blended honeys are those that have been mixed by farmers to get a desired taste, consistency or colour. Unifloral honeys are significant, as they sell at higher prices than multifloral or blended honeys, the importance of which can be explained with the example of Mānuka honey below.

1.1.1 Mānuka Honey

Honey derived from mānuka (*Leptospermun scoparium*), a shrub native to New Zealand, exhibits unique non-peroxide antibacterial activity. All honeys contain hydrogen peroxide which inhibits bacterial growth, but mānuka also contains methylglyoxal (MGO) producing exceptionally high levels of growth inhibition[1]. The total nonperoxide antibacterial activity of mānuka honey is indicated by its unique mānuka factor (UMF), which can vary from batch to batch.

MGO is formed from the non-enzymatic conversion of dihydroxyacetone (DHA), present in mānuka nectar, during heat treatment or prolonged storage. Note that honey treatment with high temperatures, can lead to decreases in MGO production and an increase in hydroxymethylfurfuraldehyde (HMF). HMF is produced from the dehydration of fructose and is used as an indicator for heat and storage changes in honey. Often MGO, DHA and HMF are analysed in unison, to give a true indication of the honey quality.

1.1.2 Composition

Honey is a complex matrix; a supersaturated solution of sugars (e.g. glucose, fructose and small amounts of disaccharides and trisaccharides, including sucrose and maltose) with an acidic pH, also containing proteins, minerals, vitamins, enzymes and amino acids[2]. Amino acids account for 500-300 mg/100g of honey, the most abundant of which is proline (50-60%)[3]. Proline is a secondary amino acid that originates from the haemolymph of bees as well as nectar, while the other amino acids originate from plant nectars, bees, and pollen[4].

1.2 Botanical Origin

The amino acid profile represents the botanical origin of the honey; associated with the surrounding flora, rather than the site of collection. The amino acid profile from the same region can fluctuate due to many factors; availability, attractiveness to bees, seasonal variability, as well as soil and climate changes. Study of the amino acid profile of honeys can be used for controlling authenticity; by preventing fraud, inaccurate labelling, and determining if the addition of sugar syrups has occurred

Melissopalynology, the analysis of pollen in honey, is the usual method for determining the botanical origin of honey. It is a complicated and specialised field of study requiring the microscopic analysis of pollen, in addition to previous knowledge of pollen morphology, and a skilful analyst for data interpretation[5].

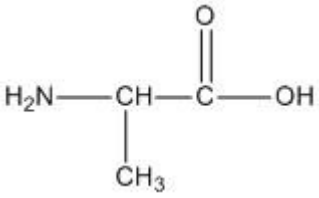
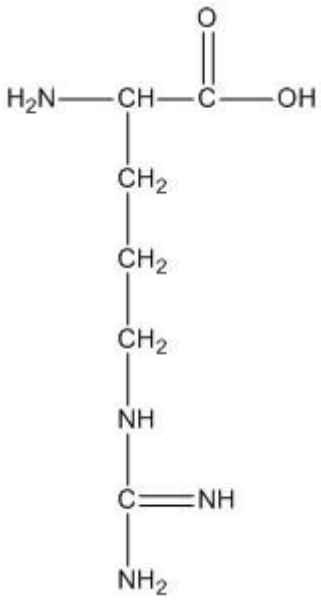
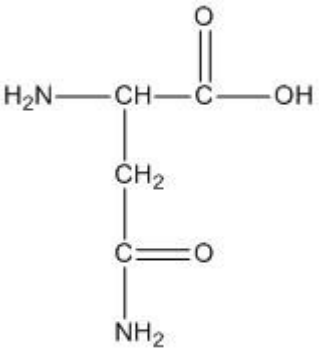
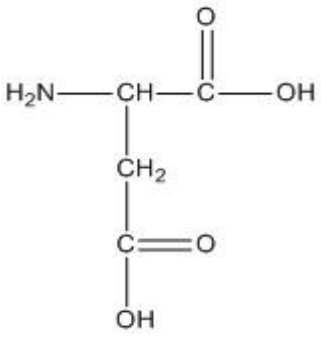
All of the analytical methods available for determining geographical and botanical origin of honey have been reviewed by Anklam[6].

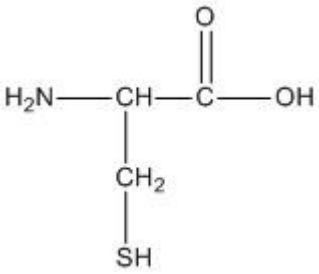
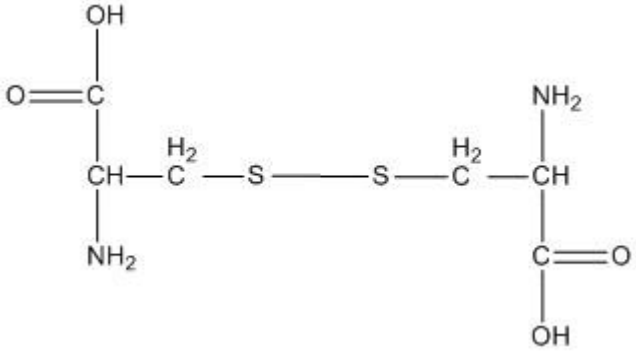
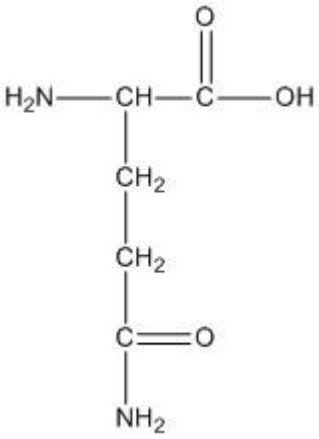
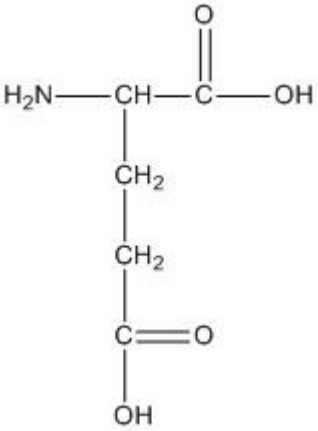
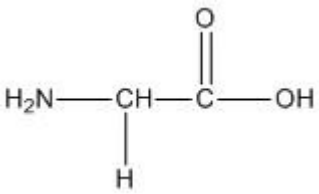
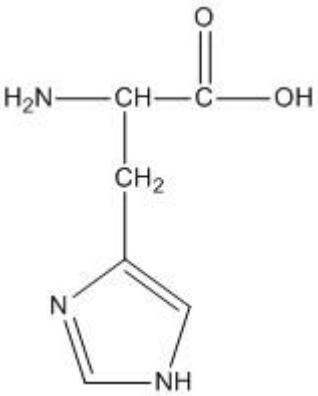
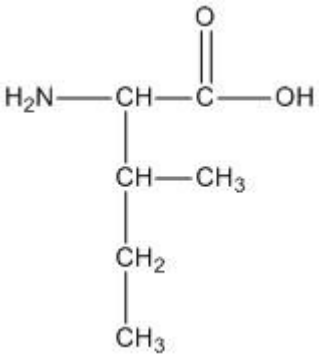
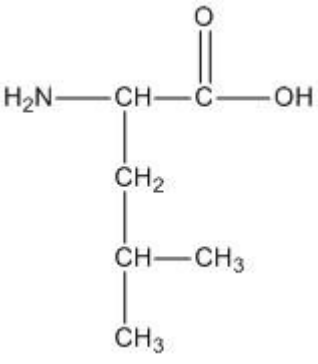
1.3 Amino Acids

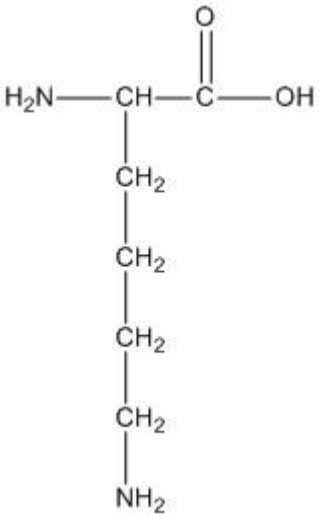
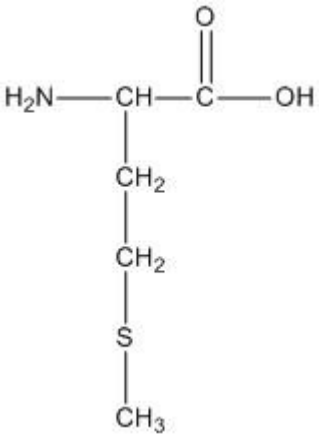
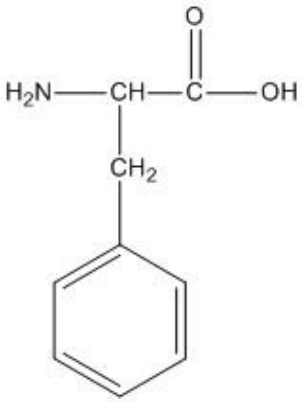
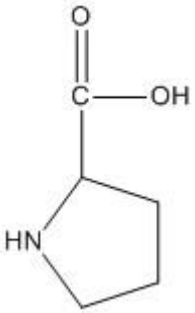
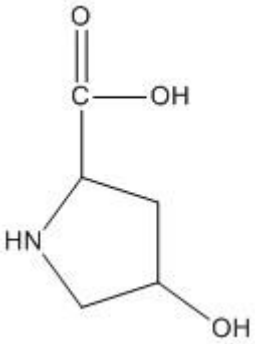
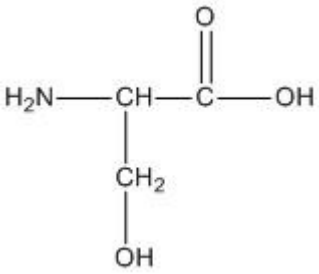
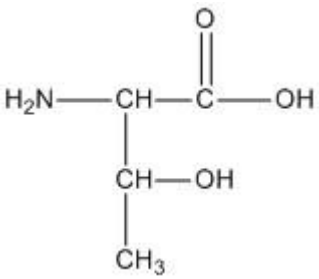
1.3.1 Structures

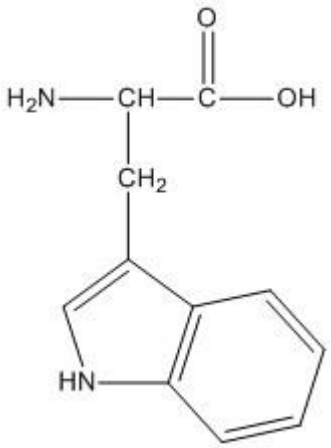
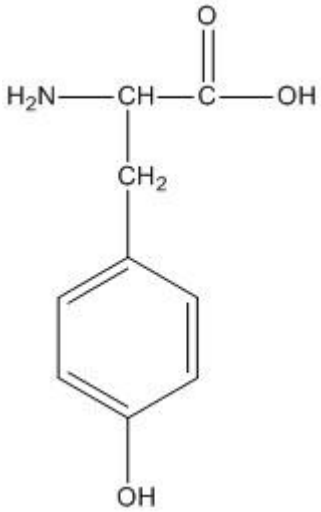
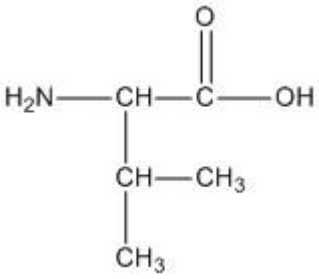
Twenty two key amino acids are depicted (Table 1.1) that show the differences in structure. While all amino acids are composed of an amine (-NH_2) and a carboxylic acid (-COOH), the differences in the side chain determines their chemistry[7].

Table 1.1: Structures of amino acids

	
Alanine	Arginine
	
Asparagine	Aspartic acid

	
Cystine	Cysteine
	
Glutamine	Glutamic acid
	
Glycine	Histidine
	
Isoleucine	Leucine

		
Lysine	Methionine	
		
Phenylalanine	Proline	Hydroxyproline
		
Serine	Threonine	

	
Tryptophan	Tyrosine
	
Valine	

1.3.2 Identification

There are several analytical methods to identify and quantify amino acids; formaldehyde titration, paper chromatography, thin-layer chromatography, and cation exchange resin; but recent reports on the use of high pressure liquid chromatography (HPLC) and gas chromatography (GC) for analysis have shown their superiority. The majority of literature on the subject of amino acids in honey involves liquid chromatography, with varying forms of detection; although there has been a small amount of gas chromatography carried out.

In the late 1940's a quantitative photometric reaction of ninhydrin and the amino group of amino acids was introduced[8]. Ion exchange chromatography was used to separate amino acid fractions, and many changes to heating times and temperatures, pH and type of buffer systems, have been since carried out to improve this method. It is still the largely suitable for routine analysis as it does not require expensive equipment, is not time consuming and has been well studied[9].

Gas chromatography requires derivatisation to produce volatile amino acids and often have faster analysis times, but usually require intense clean up procedures. HPLC can be used to analyse derivatised or underivatised amino acids, depending on the instrumentation and means of detection. The majority of HPLC analysis involves separation on reversed phase columns, in combination with UV or fluorescence detection, necessitating the amino acids to be derivatised. Mass spectrometry detection, on the other hand, does not always require derivatisation, and has higher sensitivity. Other columns such as hydrophilic interaction, monolithic, or amino acid specific columns are the newest technologies available; with fast analysis times they appear noteworthy, but, due to their novelty, they possess some idiosyncrasies.

1.3.3 Liquid Chromatography

1.3.3.1 Ion exchange chromatography with post-column ninhydrin derivatisation

Quantitation of amino acids was first carried out by Moore and Stein in the late 1940's[8]. The method involved separation by ion exchange, post-column derivatisation with ninhydrin, and photometric (UV) detection[10]. Ion exchange columns rely on ionic interactions with a strongly acidic medium, where acidic amino acids are eluted first, with neutral following, and lastly basic amino acids. This method unfortunately has low sensitivity and complications relating to post-column derivatisation, including ninhydrin degradation by exposure to light, oxygen, pH and temperature changes. Matrix interferences have also been reported. Despite these drawbacks, this method gives more repeatable results than most reversed phase liquid chromatography[11].

Recent improvements to assess heating times and temperatures, buffer systems, and solvents have been undertaken by Sun *et al.*[9] Evaluations indicated the relatively inexpensive sodium hydroxide/acetic acid buffer system was suitable, this being an improvement on the use of uncommon lithium hydroxide. Heating of the reaction time was carried out for 10 min, versus the tradition 30 min, and achieved a similar degree of colour development. These changes to the method make it more convenient, faster, and less costly; ideal for routine analysis[9].

1.3.3.2 OPA derivatisation via fluorescence detection

A simple method entails using HPLC with detection by fluorescence and two derivatising agents: *o*-phthaladehyde (OPA) and fluorenylmethyloxycarbonyl chloride (FMOC) was used to discriminate floral origin and fraud in honeys. This study[3] describes the analysis of amino acids to measure authenticity, a property that is important to be aware of in today's market. Types of honey fraud can be categorised into the addition of syrup, and inaccurate labelling of the honey. Pure honeys from seven different floral backgrounds were investigated[3]. The objective is to discriminate between authentic and adulterated honeys using principal component analysis (PCA) for statistical processing of the amino acid levels. It was found that the average phenylalanine content in lavender honeys was much higher when compared to the honeys of a different floral origin, and thus phenylalanine can be used as a marker for lavender honey. The same was found with threonine and sunflower honey. The PCA calculations could classify lavender honey, but only partially discriminate other varieties due to such dispersion of amino acid quantities within the honey types[3].

Analysis of sugar syrups determined that they did not contain amino acids, thus their addition would decrease the total concentration of amino acids in the honey[3]. Using the most discriminating parameters for the corresponding honey, leucine and glutamic acid for rape honey, an addition of 10-15% of syrup or more could be detected. While it is possible for proline to be the more reliable indicator of syrup addition, the proline content can depend on how slow or fast the harvest is, thus it is feasible for unadulterated honeys to have low proline quantity due to rapid honey production adding to confusion over the possibility of honey fraud[3].

This study[3] showed that 19 amino acids were able to be quantified, on a Hypersyl ODS (200 x 2.1 mm, 5µm) column. The analysis took 30 minutes, including equilibration time, and sample preparation was very simple, only requiring dilution with water and filtration. This method indicated a standard for what is possible with amino acid analysis.

A study by Analytical Technologies, Inc.[12] describes a similar methodology, but with varying throughput and resolution options, it presents an attractive and rapid HPLC technique. This technical note was chosen as a base method for the investigation into amino acids and is dealt with in more detail in Chapter 2.1: HPLC-UV Using Pre-column Derivatisation.

Column options provided by the study[12] consist of the following examples. A C18 column (2.1 x 50 mm, 1.8μm) put analysis time at 9 minutes, including equilibration, ideal for commercial techniques. The smaller column had less resolution than the larger options, categorised as rapid resolution high throughput, but this property is often sacrificed for commercial time constraints. Other column options included rapid resolution; 4.6 x 150 mm, 3.5 μm, with 25 min analysis time; and traditional high resolution method column of 4.6 x 250 mm, 5 μm, with an analysis time of 40 minutes. The solvents used for the 2010 method are simpler to prepare than the older 2000 method[13], including the buffer, but more solutions are necessary for derivatisation. OPA and FMOC, the derivatising agents, were detected with UV, and required the addition of 3-mercaptopropionic acid (MPA) to the OPA vial. OPA reacted in the presence of MPA with primary amino acids, forming an isoindole derivative. The OPA-MPA derivatising agent required daily preparation. Changes and optimisation of this base method is covered in Chapter 2.1: HPLC-UV Using Pre-column Derivatisation.

OPA is often the main derivatising agent used in HPLC methodologies, but in combination with different thiol-containing reducing agents, such as MPA mentioned above. A study in 2010[14] made use of OPA in combination with *N*-acetyl-L-cysteine (NAC), a bulky thiol intended to create a more stable derivative. MPA, used in the previously discussed 2010 study[3], is also considered to be bulky; this is in comparison to the older, but commonly used, 2-mercaptoethanol (MCE); a smaller thiol resulting in unstable isoindole derivatives. Preparation of the OPA-NAC derivative is more time consuming; it must be prepared on a daily basis and requires 90 minutes to stabilise, before being run. A total run time of 39 minutes also makes this method unappealing, but as the rest of its features, such as in loop derivatisation, high sensitivity, and easy sample preparation, are straightforward.

In this study[14], twenty four amino acids and biogenic amines were quantified in grapes, wine, honey and fruit. The honeys analysed were from the same region, but of five different sources. Oak honey had substantially more lysine (77 mg/kg) than isoleucine (8.2 mg/kg), compared to the other honeys, and also had the largest amount of total amino acids. Chestnut honey had the lowest amount of total amino acids. Common among all the honeys, the most abundant amino acids after proline were isoleucine, lysine, and glutamine. Also no phenylalanine was found in any of the honeys. This study[14] demonstrates a simple method for amino acid analysis, but as it was focused on wine and grape products, only a small sample of honeys were investigated. A far greater quantity of samples to analyse, in combination with PCA, could then be used for discriminatory purposes.

Reversed phase HPLC analysis of free amino acids in honey and wine[15] used fluorescence detection of OPA-MCE derivatives to quantify nineteen amino acids, along with six biogenic amines. Preparation of the derivatising agent was similar to the above method[14], but only had to be prepared every nine days. This advantage was offset by an 80 minute run time; too time-consuming in a commercial setting. This study demonstrated good limits of detection, repeatability, and recovery for the compounds investigated; aspects useful for comparison. Twelve honey samples from three different areas were analysed. The amino acids present in highest quantities were phenylalanine, glutamine, and lysine; methionine was not detected. A multifloral honey from Madeira Island had the largest amount of total amino acids, at 286 mg/L[15].

While this HPLC method[15] had high sensitivity, necessary for the response of amino acids, it did not extend to proline, cysteine, and hydroxyproline. This is because the OPA-MCE derivatising agent does not react with the secondary amino acids proline or hydroxyproline. Cysteine is likely to be present at extremely low levels, and thus unable to be quantified by the level of sensitivity of this method.

1.3.3.3 DEEM derivatisation via UV detection

Diethyl ethoxymethylenemalonate (DEEM) is an additional derivatising agent able to react with primary and secondary amino acids and detected by UV. Unfortunately the reaction of DEEM with amino acids is fast to begin with, excluding proline, and then slows, including proline; this means that analysis cannot be performed until after 24 hours reaction time, when proline is at its maximum intensity, but the other twenty two amino acids have not degraded significantly[16].

A study on the amino acid content of thirty one Spanish honeys, with five different botanical origins used DEEM as a derivatising agent[17]. The amino acids required isolation from the honey samples before derivatisation, and were filtered before being loaded onto a C18 column (300 x 3.9 mm, 4 μm) and fully eluting after 32 minutes. Twenty two amino acids were separated and quantified, the main ones being proline, phenylalanine, tyrosine and lysine. Methionine and cysteine were not found in some honeys, and only at low quantities in others.

PCA in combination with the Student-Newman-Keuls test, comparing multiple mean values, were applied to the amino acids to make a distinction between botanical origins[17]. Lavender honey is able to be distinguished from the others by its high tyrosine content; from eucalyptus honey by higher tyrosine and phenylalanine content; from rosemary and thyme honeys due to valine, alanine, and tyrosine; and lastly from orange blossom honey by noteworthy differences in valine, alanine, tyrosine, and phenylalanine. Distinctions between the remaining honeys were also found; eucalyptus honey had significant differences in valine content compared to thyme and orange blossom honeys, in addition to differing alanine and valine quantities from rosemary honey; while thyme, rosemary and orange blossom honeys had similar amino acid compositions[17].

The study commented on lack of application to multifloral honeys, since unifloral honeys only represent a small part of the market, and they were not able to distinguish between any of the multifloral honeys, only to state that the amino acid content were in range of their limits[17].

Estonian honeys were investigated in 2008, also using precolumn derivatisation with DEEM[18]. The HPLC-UV system differed from the previous study by column (Hydro-RP 250 x 4.6 mm, 4 μm), higher column temperature (45°C vs 15°C in the previous study), a longer run time of 50 minutes and no filtering step; but used a similar amino acid isolation step and the same elution solvents. A t-test was used to deduce that the differences in some amino acids; glutamine, glycine, histidine, phenylalanine, proline, serine and tryptophan; were significant. This method was later improved on in 2010, adding compatibility with MS detection[19]. A point of note from this study is the complicated analysis of cysteine. DEEM is unable to distinguish between cysteine and cystine. Cysteine is not present in Estonian honeys, so did not affect the investigation[18].

A comparative study by Bernal *et al*.[20] investigated three derivatising agents: fluorenylmethyl chloroformate (FMOC-Cl), 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) and DEEM. Samples were diluted, before derivatisation, with borate buffer and filtered and separated on a C18 column (150 x 4.6 mm, 5μm).

FMOC-Cl derivatisation was carried out with an autosampler, three solvents (acetate buffer:tetrahydrofuran:ACN [A], ACN:H_2O [B], ACN [C]) and eluted amino acids after a 60 min run with detection via fluorescence.

AQC derivatisation required more steps, including 10 minutes heating. Derivatisation, with ACQ, cannot be fully automated. Two solvents (acetate buffer [A], ACN:H_2O [B]) fully eluted amino acids after 54 min with detection via fluorescence.

DEEM derivatisation, which cannot be automated, required the most steps, including heating for 50 min, filtering and further dilutions with buffer. Two solvents (ammonium acetate [A], ACN [B]) eluted amino acids after 62 min with UV detection.

It was determined that FMOC-Cl and ACQ derivatives were better than DEEM derivatives, due to the increased sensitivity of fluorescence detection and the ability to use a (mostly) automated HPLC method. The study[20] comments that overall, the FMOC-Cl method appears to be superior.

1.3.3.4 PITC derivatisation via UV detection

A 2003 study of Argentinian honeys used a different derivatising agent, phenyl isothiocyanate (PITC)[21]. This is also known as Edman's Reagent, used for sequencing peptides[11]. Amino acids were extracted from honey samples, evaporated to dryness, derivatised, and analysed on a ODS column (4.6 x 250 mm, 5 µm) with UV detection (254 nm). Acetate buffer in water with trimethylamine and ACN made up solvent A, and H_2O:ACN (40:60) solvent B. A 25 min run eluted fifteen amino acids[21]. Detection of PITC-amino acid derivatives is approximately fifty times less sensitive than OPA or FMOC derivatised amino acids, and is not suitable for automation, making it an unfavourable choice for commercial application[11]. Cluster analysis was carried out on fifty six honey samples, determining that honeys grouped in clusters around sampling regions. PCA analysis showed that the clusters are somewhat associated with concentrations of select amino acids[21].

These HPLC-UV or HPLC–Flu methods gave a wide range of choices for column, derivatising agents, solvents and other aspects. This was very useful in choosing an ideal base method, and provided options for improvement.

1.3.3.5 MS detection

A difficulty often encountered with amino acids is the lack of resolution. Due to the similar properties of the amino acids, peaks overlap; full resolution using HPLC is rarely observed. Mass Spectrometry, on the contrary, does not need the amino acids to have high resolution for accurate detection.

Gokmen *et al.*[22] describes the analysis of twenty two underivatised amino acids using a six minute chromatographic run and MS for detection. What makes this technique unique is the use of a HILIC silica column. The HILIC, hydrophobic interaction liquid chromatography, column is superior for the analysis of small polar analytes; the low viscosity solvents allow high throughput, as well as increased sensitivity with electrospray ionisation-MS. Problems includes the large use of acetonitrile as a solvent, as well as the effect of minor changes in injection diluent and sample composition on the chromatogram, thus making it a slightly less flexible technique[23]. The few limitations of the HILIC column made this method no less attractive.

The experimental procedures in this analysis[22] included: sample preparation by tenfold dilution (ACN:H_2O) and filtering, separation on a HILIC silica column (150 x 2.1 mm, 3µm) with solvents (ACN [A], 0.1% formic acid in H_2O [B]), and detection by orbitrap MS. Validation of the method yielded good results with respect to linearity, LOD, LOQ, accuracy and precision. The analysis of amino acids was carried out on many difference matrices: juice, wine, beer, tea and honey samples, indicating the wide range of applications of this method[22].

HILIC chromatography with this application was chosen as a base method for the investigation into amino acids. Changes, optimisation and validation of this method is covered in Chapter 2.2: HPLC-MS Using Hydrophobic Interaction Chromatography.

Zwitterionic ion chromatography (ZIC), in combination with HILIC chromatography, was investigated to perceive the ability of zwitterionic sulfobetaine exchangers to separate amino acids in a study carried out by Sonnenschein *et al.*[24] This was achieved by using sodium acetate eluent, where amino acids were separated in their zwitterionic form by interacting in multiple areas with the zwitterionic stationary phase. Cation exchange was the main separation mechanism found for sulfobetaine type exchangers and a ZIC-pHILIC column, providing good separation of amino acids[24].

A 2005 study[25] depicts the use of an ion pairing reagent with an octadecasilane monolithic silica column to separate seventeen amino acids and identified them with time-of-flight (TOF) MS. Monolithic columns can be most advantageous as increased flow rates can be used, but with reduced back pressure on the HPLC system. The flow rate (2.0 mL/min) allowed separation in less than 3 minutes; this is the fastest separation time observed in the literature. The column (RP-18e 100 x 4.6 mm) with solvents (perfluoroheptanoic acid 1 mM [A], ACN [B]) is paired with a fast detector (TOF-MS) in order to maintain sufficient data acquisition for peak resolution of the mass spectrum[25].

A 2006 Agilent Technologies method[26] reports identification of twenty two underivatised amino acids, separated and detected by LC-MS. A RP narrow-bore column (100 x 2.1 mm, 3.5µM) was used to achieve separation in a very short time (7.5 minutes), in combination with an acidic mobile phase (0.01 mM acetic acid plus 0.2% formic acid in H_2O, isocratic) that increased the MS sensitivity. Positive ion mode atmospheric pressure chemical ionisation (APCI) analysed amino acids in twenty two food samples, including honey, after homogenisation and filtering. The simplicity of this method is attractive, together with the rapid analysis (including sample preparation, less than 25 minutes total) and reliable data; this method shows the superiority of LC-MS.

The Agilent Technologies method[26] was used in a separate study where twenty two amino acids were analysed in food stuffs[27]. Simple sample preparation (extraction with 0.2 mM acetic acid) was combined with fast chromatographic analysis gave adequate identification and quantification of amino acids in under 25 minutes. The amino acid content of honey was determined, demonstrating its applicability to this thesis.

Analysis of twenty amino acids in barley plant extracts was carried out by LC-MS-MS[28]. Direct analysis of hydrochloric acid-ethanol extracts was carried out by tandem MS (positive ion mode), separation in 75 minutes by a strong cation exchange column (Luna 5µ SCX 100 Å, 150 x 2 mm) with simple solvents (30 mM ammonium acetate in H_2O [A], 5% acetic acid in H_2O [B]). Matrix interferences were eliminated by the use of MRM mode, and validation was carried out determining good linearity, sensitivity, precision and accuracy[28]. While the long separation time is not ideal for commercial application, the specificity of tandem MS with MRM is ideal of amino acid analysis.

1.3.3.6 Amino acid analysis Kits

Amino acid testing kits are available as an alternative to the construction of a method, ideal for short-term testing. The aTRAQ™ Kit by AB SCIEX[29], using LC/MS/MS, can identify and quantify amino acids quickly with minimal MS experience. The aTRAQ™ kit uses Δ8 labelling of the amino acids in sample, combined with internal standards, giving accurate quantification of amino acids. The aTRAQ™ Δ8 labelling reagent and its reaction with amino acids are dealt with in greater detail in Chapter 3.1.4: Operating conditions.

The column (AAA C18 RP 150 x 4.6 mm, 5µm), solvents (0.1% formic acid, 0.01% heptafluorobutryic acid in H_2O [A], 0.1% formic acid, 0.01% heptafluorobutryic acid in MeOH [B]), and labelling agents (including sulfosalicyclic acid, labelling buffer, aTRAQ™ reagent, hydroxylamine, and aTRAQ™ internal standard) are all provided by the kit[29]. The labelling protocol involved small additions of the labelling reagents to 40 µL of sample, vortexing to mix, and centrifuging to spin contents to the bottom of the vial. Handling such small amounts may lead to accuracy problems. The internal standard should remove the need for separate calibrations, saving time and solvents. The kit provides simple and efficient testing, but is not ideal for commercial use as it creates dependence; the kit would need to be frequently purchased.

Waters AccQ-Tag Chemistry kit was used for an investigation into the amino acid content of Serbian unifloral honeys by Keckes *et al.*[30] The kit used ACQ as a derivatising agent (provided as AccQ-Fluor reagent, along with AccQ-Fluor borate buffer), an Amino Acid Analysis column (AAA, C18, 150 x 3.9 mm, 4µm), and unspecified solvents (AccQ-Tag Eluent A [A], ACN:H_2O [B]) to elute (38 minute run) and detect amino acids via fluorescence (250/395 nm).

192 Serbian unifloral honey samples were tested, and the amino acid content was analysed via PCA and linear discriminate analysis (LDA). Basil honey samples formed a well-defined cluster with phenylalanine content, while acacia, linden, sunflower and rape honeys could be reasonably separated. The main amino acids in the honeys, Pro, Ala, Ser, Val, His, and Asp, were found to be important for distinguishing botanical origin[30].

Kaspar *et al.*[31] compared amino acid analysis by iTRAQ® LC-MS, GC-MS, and post-column ninhydrin derivatisation of urinary samples.
The GC-MS method derivatised amino acids with propyl chloroformate, andseparation was carried out on Phenomenex ZB-AAA column (15 m x 0.25 mm ID, 0.1 µm film thickness) in 6 minutes.
iTRAQ® LC-MS, an older model of the aTRAQ™ Kit by AB SCIEX mentioned above, follows the same methodology.
It was found, through comparing technical error, GC-MS had higher reproducibility that iTRAQ® LC-MS. GC-MS also had sample pre-treatment that was completely automated. iTRAQ® LC-MS covered more amino acids. The study comments that both iTRAQ® LC-MS and GC-MS are better suited for high throughput analysis than post-column ninhydrin derivatisation[31].

1.3.4 Gas Chromatography

1.3.4.1 Flame Ionisation Detection

The few existing gas chromatography (GC) techniques described in the literature used either flame ionisation detection (FID) or MS, with the latter being more sensitive.

Silva *et al.*[32] analysed twenty one amino acids in jam using GC-FID with a total run time of six minutes. A solid phase extraction step was required for purification, but this may not be necessary when applied to honey which is ideal, as some losses of amino acids during the washing step occurred. Derivatisation was also essential to produce volatile amino acids for direct GC analysis; this was done by means of a rapid ethyl chloroformate reaction. Unfortunately arginine cannot be derivatised by chloroformates and required an additional reaction step if it is to be analysed. Chromatographic separation via a fused-silica column (CP-Sil 19 CB wcot, 10 m x 0.25 mm ID) was carried out at 140-280°C; this had good resolution with low reagent and instrumentation costs[32]. Rapid analysis, low detection limits (0.004-0.115 µg/mL), and accuracy makes this method ideal for analysis. Its only drawbacks being the sample preparation and the low number of analysable amino acids (arginine, cystine, and glycine are not included in this method).

1.3.4.2 MS detection

Resolution issues that occur with GC-FID analysis are avoided by the use of MS detection. The following literature examples display improved detection of amino acids by MS.

A rapid, sensitive GC-FID and GC-MS method analysing the amino acid content of seventy four honeys was carried out by Nozal *et al.*[33] Twenty two amino acids were determined where derivatisation involved a solid phase extraction step, then reaction with alky chloroformate reagent, and lastly a liquid/liquid extraction. Separation for both GC-FID and GC-MS methods were carried out on a ZB-PAAC column (10 m x 0.25 mm), with some variation to the temperature program. The GC-FID method fully eluted after 8 minutes with detection limits of 0.112-1.795 mg/L. The LC-MS method fully eluted amino acids after 5 minutes, with detection limits of 0.001-0.291 m/L using selected ion monitoring (SIM) mode. The lengthy sample preparation cannot be fully automated, not ideal in commercial applications, but the fast and sensitive analysis is decent. Classification of the seventy four honeys was carried out with discriminant analysis. The end results has sixty five honeys correctly classified corresponding to botanical origin using amino acid concentrations as variables[33].

GC-MS analysis of amino acids was carried out by Kaspar *et al.*[34] with propyl chloroformate as a derivatising agent. This allowed analysis of thirty amino acids, including those present in physiological fluids, in 30 minutes. No solid phase extraction step was required, allowing full automation of the method. Separation was carried out on ZB-AAA column (15 m x 0.25 mm ID, 0.1 µm film thickness) with good resolution. Validation of this method gave limits of detection at 0.03-12 µm, and lower limits of quantification at 0.3-30 µm[34]. While this method was only applied to biological fluids, its application to honey could be easily made. Sample preparation excluded a cation exchange clean up, and amino acids are directly derivatised, for which no changes would be required for a honey matrix.

The gas chromatography methods demonstrated quick run times with good resolution. The foremost disadvantage of gas chromatography is the essentiality of derivatisation, and the extra steps that requires in sample preparation.

1.4 Statistics

Statistical analyses carried out on amino acid content in honey have been mentioned, such as: principal component analysis, linear discriminate analysis, t-tests and the Student-Newman-Keuls test. These statistical analyses are necessary for interpreting data, to understand what values, differences, or amino acids are significant. While statistical analysis of amino acid concentration data can provide useful information, it cannot completely differentiate botanical origin by a selection of factors[30].

1.5 Aims of Present Research

The opportunity to have accurate, routine chemical analysis available to the industry is vital, and must be explored. An efficient, simple and accurate method for analysing amino acids in honey ought to be produced, in order to help understand the influence of botanical origin on honey and permit better industry standards for honey labelling.

I set out to construct and validate a method for analysis of amino acids in honeys that is reliable, reproducible, and is suitable for routine analysis.

Chapter Two: Discarded Methods for Amino acid Analysis

This chapter discusses the methods undertaken that were not successful. The samples, materials, and instrumentation are presented, in addition to modifications to the methods and why they were not successful.

2.1 HPLC-UV Using Pre-column Derivatisation

This method involved the use of HPLC with UV detection and pre-column derivatisation. The experimental procedure, results and method development were explored

2.1.1 Experimental Procedure

2.1.1.1 Instrumentation

Chromatographic separations were performed on an Ascentis® Express C18 column (10 cm x 2.1 mm, 2.7 μm) (SUPELCO Analytical). A U-HPLC system from Thermo Scientific Ultimate 3000 consisting of a pump, auto sampler, column compartment and diode array detector was used. Analysis software used was also provided by Thermo Scientific: Dionex Chromeleon™ 7.

2.1.1.2 Materials

Acetonitrile and methanol were of HPLC grade and obtained from Merck and Sigma-Aldrich, respectively. High purity L-Amino Acids; alanine, Ala; arginine, Arg; asparagine, Asn; aspartic acid, Asp; cysteine, Cys; cystine; glutamic acid, Glu; glutamine, Gln; glycine, Gly; histidine, His; hydroxyproline; isoleucine, Ile; leucine, Leu; lysine, Lys; methionine, Met; phenylalanine, Phe; proline, Pro; serine, Ser; threonine, Thr; tryptophan, Trp; tyrosine, Tyr; valine, Val; were obtained from Sigma-Aldrich. Phthaldialdehyde (OPA), sodium tetraborate decahydrate, sodium azide, sodium phosphate dibasic and mercaptopropionic acid (MPA) were supplied by Sigma-Aldrich. Hydrochloric acid (HCL) was supplied by Avantor. Sodium hydroxide pellets and neat fluorenylmethyl chloroformate (FMOC) were supplied by Merck. Deionised water, used throughout experiments, was purified by Sartorius Stedim biotech.

2.1.1.3 Analyte solutions

Separate amino acid stock solutions (1000 mg/L) were prepared with 0.1% HCL in methanol. A stock solution of the 22 amino acids (45.45 mg/L) was prepared. Further dilutions with water yielded standards with concentrations of 10, 5, 3, 2, 0.5, 0.25 and 0.1 mg/L.

2.1.1.4 Sample Preparation

Honey samples were diluted tenfold in water and shaken until dissolved.

2.1.1.5 Derivatising agents

The OPA-MPA derivatising agent was prepared by dissolving OPA (25 mg) and MPA (0.2 mg) in borate buffer (0.4 M in water, pH 10.2, 2.5 mL). This was prepared on a daily basis. Neat FMOC required no preparation.

2.1.1.6 Operating conditions

A gradient mixture of 10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$, pH 8.2: 5 mM NaN_3 (A) and acetonitrile:methanol:water (45:45:10, v:v:v) (B) was used at 40 °C with a flow rate of 0.42 mL/min. The gradient program is shown in Table 2.1.

Table 2.1: Gradient program

Time (min)	Solvent	
	%B	Curve
0	2	5
0.35	2	5
13.4	57	5
13.5	57	5
15.7	100	5
15.8	100	5
16	2	5
19	**Stop run**	

The diode array detector monitored two wavelengths, shown in Table 2.2. UV 1 monitors the OPA derivative of primary amino acids, and UV 2 monitors the FMOC derivative of secondary amino acids.

Table 2.2: Diode array detector UV signals

UV	Wavelength (nm)	Bandwidth	RefWavelength (nm)	RefBandwidth
1	338	10	390	20
2	262	16	324	8

The injection program used for derivatisation of amino acids can be found in the Appendix 1.

2.1.2 Results and Method Development

2.1.2.1 Method development

This method offered many column options, of which a 2.1 x 50 mm, 1.8µm C18 column was chosen. This was run with the recommended methodology; the same solvents, column temperature, flow rate and UV signals as mentioned earlier, but the gradient program differed to that which is described in the final operating conditions. The gradient program is illustrated in Table 2.3.

Table 2.3: Gradient program of 2.1 x 50 mm, 1.8µm C18 column

Time (min)	Solvent	
	%B	Curve
0	2	5
0.2	2	5
7.67	57	5
7.77	100	5
10	100	5
10.5	2	5
12	**Stop run**	

This mode of running generated good separation for amino acid standards, shown in Figure 2.1.

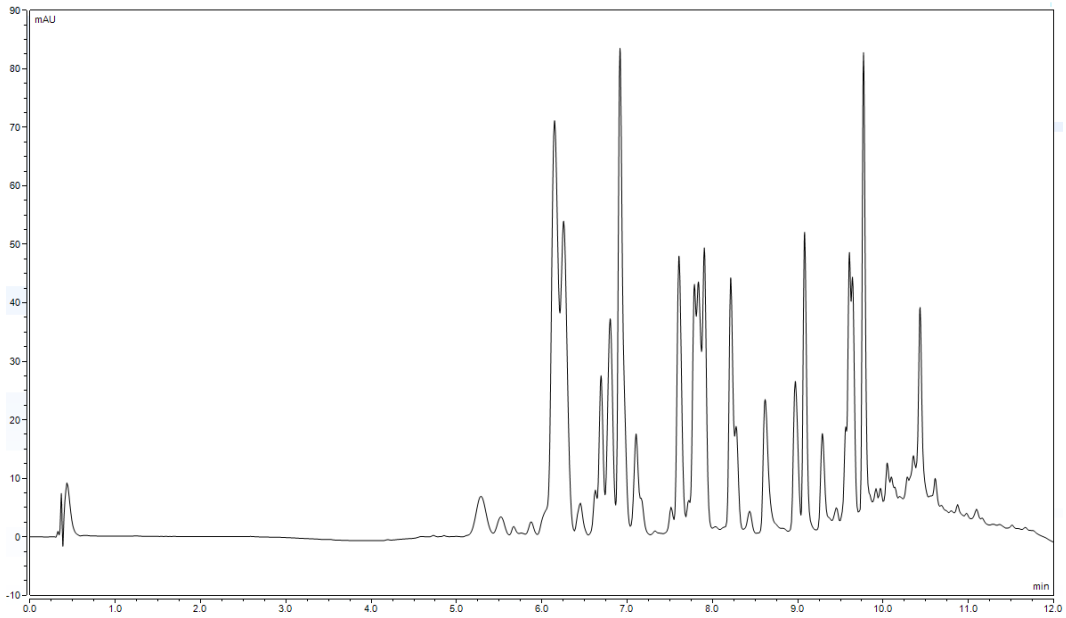


Figure 2.1: Chromatogram of 2.1 x 50 mm, 1.8µm C18 column, UV signal 1, 10 mg/L standard

Unfortunately, the instrumentation often reached the high pressure limit of the column; this is often due to the small bead size (1.8µm) leading to blockages. This occurred after a few weeks of testing. The column was cleaned by slowly running solvent B through the column backwards, resolving the problem, but only for a day. This is not ideal for routine work, especially when only clean standards had been run on the column, and it was decided that a more robust column with larger bead size would be preferred.

An Ascentis® Express C18 column (10 cm x 2.1 mm, 2.7 µm) was trialled to see the separation of amino acids. Changes had to be made to the gradient program to apply it to the longer dimensions of the column. The initial gradient program is shown in Table 2.4.

Table 2.4: Gradient program of 10 cm x 2.1mm, 2.7µm C18 column

Time (min)	Solvent	
	%B	Curve
0	2	5
0.35	2	5
13.4	57	5
13.5	100	5
15.7	100	5
15.8	2	5
16	**Stop run**	

The gradient program in Table 2.4 produced sufficient separation of the amino acids, but some changes were made to perfect this. The chromatogram for this gradient program is in Figure 2.2. The final gradient program is shown in Table 2.1.

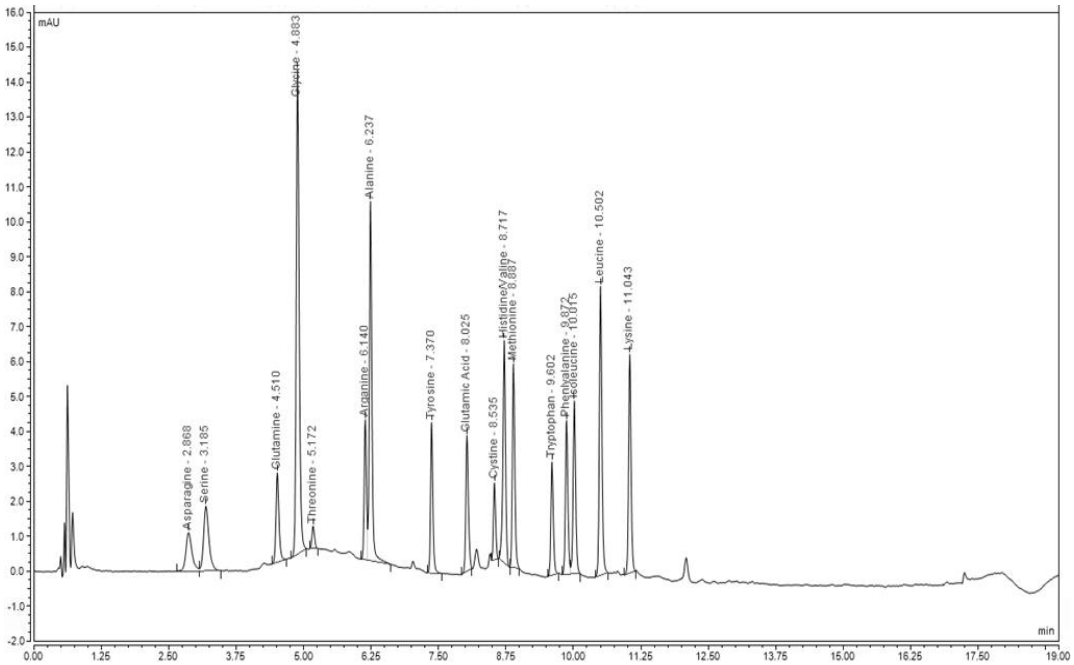


Figure 2.2: Chromatogram of 10 cm x 2.1mm, 2.7µm C18 column, UV signal 1, 10 mg/L standard

The injection peak, at around 1 min can be seen in Figure 2.2.

Aspartic acid eluted first, leading to some problems. The amino acid eluted at Vo, thus is it not retained by the column. This can be seen in Figure 2.3. Modifications to the gradient program could not force interaction of aspartic acid with the solid phase; perhaps changes to the solvents may have resolved this, but time constraints necessitated focus onto one method: HPLC-MS.

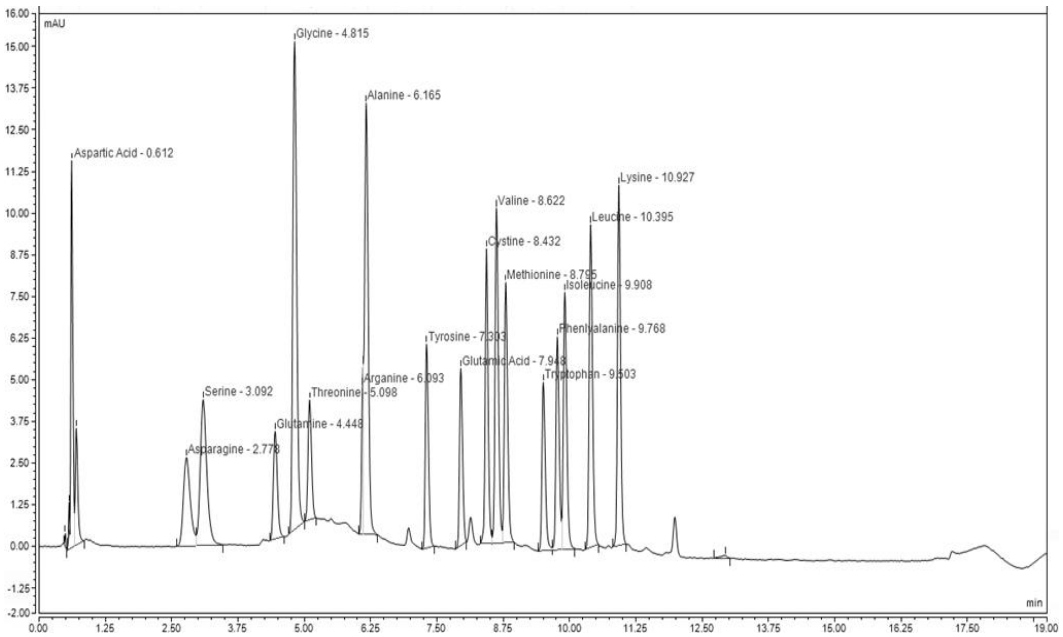


Figure 2.3: Chromatogram indicating elution time of aspartic acid peak, UV signal 1, 10 mg/L standard

Proline and hydroxyproline, the only secondary amino acids, are intended to be detected via derivatisation with FMOC on UV signal 2. This did not occur and the chromatograms of blank samples had the same signal pattern as the standards that contained the secondary amino acids. It is unlikely that the UV signal used to detect proline and hydroxyproline is incorrect, as the study employing the original method has shown their detection using the same signal. The same goes for the injection program, it is very similar to that used in the study and is unlikely to be the problem. It is possible that the preparation of the FMOC was not correct, and derivatisation could not occur. Unfortunately this was not explored further as another method was used.

2.1.2.2 Calibrations

Calibration curves were made with amino acid stock standards, containing 22 amino acids, of concentrations of 10, 5, 3, 2, 0.5, 0.25 and 0.1 mg/L. The addition of the stock standard 45 mg/L, which was run with the other standards, demonstrates a non-linearity of the calibration. This usually indicates a limit of detection (LOD), but further investigation into the LOD's was not carried out. At lower concentrations: 5, 3, 2, 0.5 and 0.25 mg/L, regularly produced good calibration curves. At 0.1 mg/L, most amino acids were not able to be detected. Figure 2.4 below shows the calibration curve of glycine using all standards.

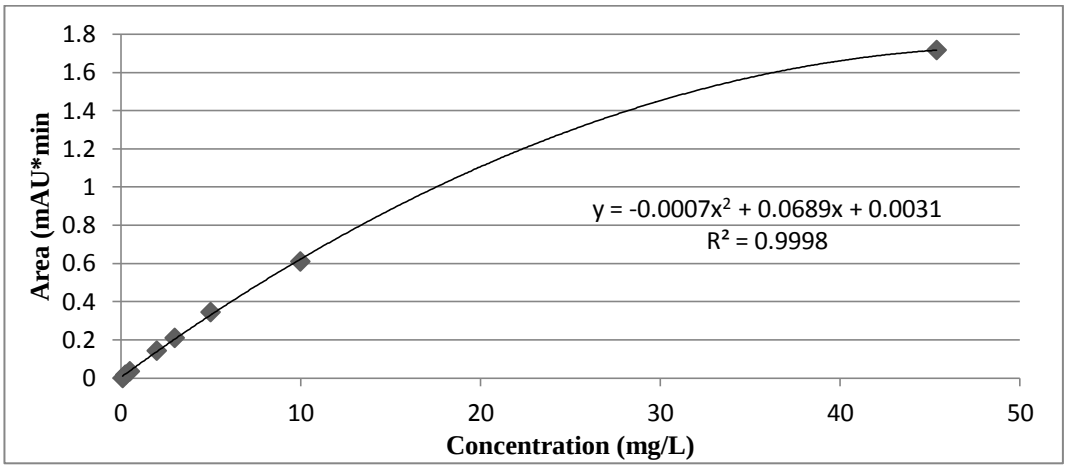


Figure 2.4: Calibration curve of glycine showing possible LOD

The levels of amino acids in honey are frequently at very low levels (between 0-20 mg/L), such that 1 in 10 dilutions were used for sample preparation, when honey samples are usually diluted 1 in 40 for routine MGO, HMF and DHA analysis. Calibration curve of glycine using the lower concentration standards is in Figure 2.5.

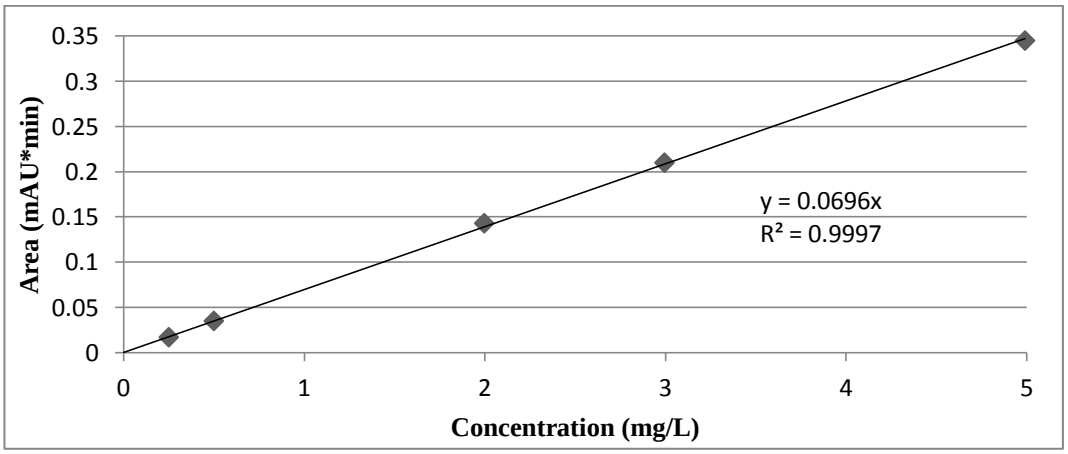


Figure 2.5: Calibration curve of glycine

2.1.2.3 Derivatising procedure

The study provides an injection program for derivatising, including the use of a borate buffer and injection diluent. As this injection program was intended for an Agilent 1100 or 1200 series HPLC system, modifications had to be carried out to meld the derivatising to the Thermo Scientific 3000 system used. The Dionex Chromeleon™ 7 software posed further complications, as specific commands were necessary; as opposed to merely draw, mix, wait, inject; Chromeleon™ requires the control of the inject valve, syringe valve and an inject marker. Another study, a technical note from 2008[35], employing the same method for analysing amino acids gives a step-by-step description of commands for the UDP for automated in-needle derivatisation. From a combination of the two studies, an injection program was produced. The following were incorporated: vials for borate buffer, OPA-MPA, FMOC, injection diluent and mixing; as well as needle wash, generation of an inject marker pulse, and the re/set of the syringe after/before injection. While the technical note suggests mixing in the injection port, an empty vial was preferred. This injection program produced good derivatisation of amino acids, as seen in Figure 2.6, but the peaks were small.

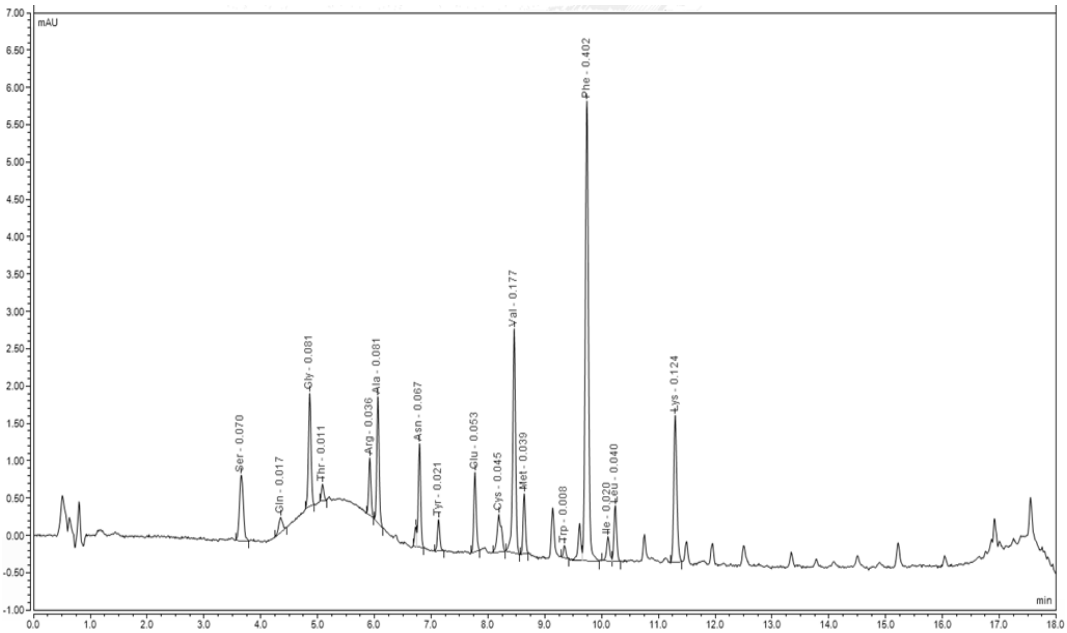


Figure 2.6: Chromatogram showing effect of injection diluent, UV signal 1, 10 mg/L standard

It was proposed that the injection diluent was not necessary; as the UV already has low sensitivity compared to MS, and the diluent was excluded from the injection program. This chromatogram, in Figure 2.7 showed improvement in peak area, but with a less defined baseline.

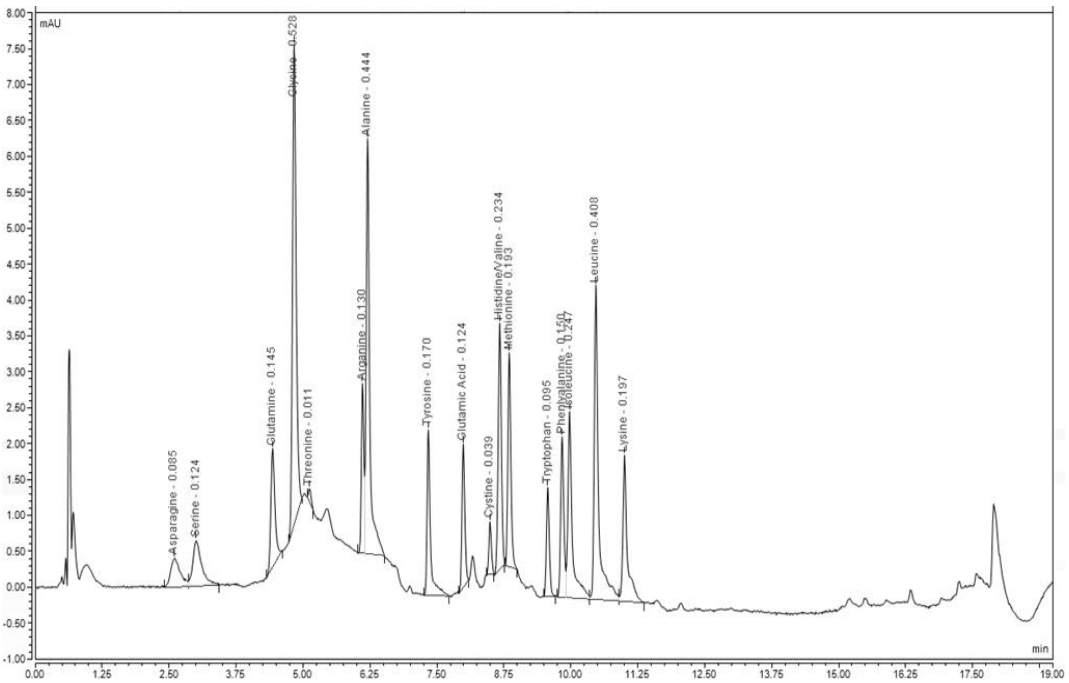


Figure 2.7: Chromatogram showing effect of no injection diluent, UV signal 1, 10 mg/L standard

Carryover was observed, especially between standards and samples; this possibly resulted from the injection program. Changes were made by adding to the injection program; air drawn for separation of reagents and additional needle washes between sample and OPA-MPA vials, and between OPA-MPA and FMOC vials. This minimised the carryover to a negligible amount. Initial changes to the draw air command were 1 µL, but this amount was too large to allow adequate mixing. This was corrected to 0.1 µL; large enough separation to avoid carryover, but small enough for derivatisation of the amino acids to occur.

Other programs suggested that solvent mixtures be injected before and after the derivatisation, in order to prepare the injection loop. This technique was attempted, but it gave poor results. This acted similarly to the injection diluent; not facilitating the derivatising procedure and decreasing the observed concentration of the peaks.

To summarise, the inability to analyse secondary amino acids (proline, hydroxyproline) and aspartic acid, low sensitivity, and long run time (25 min including injection) style this method as unfavourable for amino acid analysis.

2.2 HPLC-MS Using Hydrophobic Interaction Chromatography

This method involves the use of HILIC columns and LC-MS. The experimental procedure and method development were explored, with comment on some results obtained.

2.2.1 Experimental Procedure

2.2.1.1 Instrumentation

Chromatographic separations were performed on a SeQuant™ ZIC®-pHILIC PEEK column (150 x 2.1 mm, 5 μm polymeric beads) (Merck). A HPLC system from Agilent Technologies 1200 series consisting of a degasser, binary pump, and thermostated column compartment was used. An autosampler from Pal System, PAL HTS-xt, was employed along with an AB Sciex Triple Quad™ 4500. Analysis of ions was carried out by multiple reaction monitoring (MRM).

2.2.1.2 Materials

Acetonitrile and methanol were of HPLC grade and obtained from Merck and Sigma-Aldrich, respectively. High purity L-Amino Acids; alanine, Ala; arginine, Arg; asparagine, Asn; aspartic acid, Asp; cysteine, Cys; cystine; glutamic acid, Glu; glutamine, Gln; glycine, Gly; histidine, His; hydroxyproline; isoleucine, Ile; leucine, Leu; lysine, Lys; methionine, Met; phenylalanine, Phe; proline, Pro; serine, Ser; threonine, Thr; tryptophan, Trp; tyrosine, Tyr; valine, Val; were obtained from Sigma-Aldrich. Hydrochloric acid (HCL) was supplied by Avantor. D-(-)-fructose, sucrose, and D-gluconic acid were supplied by Sigma-Aldrich. D(+)-glucose supplied by BDH Labs, ammonium formate supplied by ACROS organics, and formic acid supplied by Merck. Deionised water, used throughout experiments, was purified by Sartorius Stedim biotech. Three mānuka honey samples (ID: 66, 78, 84) were provided fresh by Gibbs Honeybees (Masterton, NZ).

2.2.1.3 Analyte solutions

A stock solution (45.45 mg/L of each of the 22 amino acids) was diluted with water to produce a 2 mg/L standard, which was then evaporated to dryness and prepared with artificial honey solution: formate buffer:acetonitrile (25:100:875). Serial dilutions to yield concentrations of 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01 mg/L were carried out. All standard preparation above was carried out in polypropylene vials. The artificial honey was prepared combining fructose (41.4%), glucose (37.51%), sucrose (1.12%) and water (19.97%, pH 3.8-4 with gluconic acid) and mixing well. The artificial honey solution used for standards was prepared using the method for sample preparation described below. The formate buffer used for standards and samples was 500 mM ammonium formate in 0.5% formic acid.

2.2.1.4 Sample Preparation

Honey samples were diluted (1:40) with water and shaken (1 h). In polypropylene vials, honey solution (25 µL), formate buffer (100 µL) and acetonitrile (875 µL) was combined for final analysis.

2.2.1.5 Operating conditions

A gradient mixture of 20 mM ammonium formate in 0.04% formic acid (A) and 0.5% formic acid in acetonitrile (B) was used at 40 °C. The gradient program and the MRM parameters used are in Table 2.5 and Table 2.6, respectively. Additional MRM parameters include the entrance potential that was set at 10 and the collision cell exit potential set at 8, for all amino acids.

Table 2.5: Gradient program

Time (min)	**Solvent**	
	%B	Flow (µL/min)
0	90	400
0.5	90	400
2.5	85	400
5	65	500
7	40	500
8	40	500
8.5	90	500
10	90	500
10.1	90	400

Table 2.6: Scheduled MRM of the 21 amino acids analysed

Amino Acid	Q1 Mass (Da)	Q3 Mass (Da)	Retention Time (min)	Decluster Potential	Collision Energy
Asp	134	74	4.3	30	16
Glu	148	84	4.1	30	22
Ser	106	60	4.2	30	14
Asn	133	74	4.2	30	20
Thr	120	74	4	30	14
Gln	147	84	4.1	30	22
Tyr	182	136	3.8	30	18
Gly	76.1	30	4.2	35	18
Pro	116	70	3.6	30	11
Ala	90	44	4	30	16
Met	150	104	3.6	30	14
Val	118	72	3.6	30	14
Phe	166	120	3.3	30	30
Leu	132	86	3.3	30	22
Trp	205	146	3.5	30	24
Cys	241	152	4.6	30	18
His	156	110	4.6	30	19
Lys	147.001	84.001	4.7	30	22
Arg	175	70	4.7	30	30
Hydpro	132.001	86.001	4	40	18
Ile	132.002	86.002	3.4	30	22

2.2.1.6 Validation

Spike recoveries were performed by spiking selected honey samples with 100 mg/L total of 20 amino acids; Ala, Arg, Asn, Asp, Cys, cysteine, Glu, Gln, Gly, His, HydPro, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, and Val. Separate honey samples were spiked with 500 mg/L of Pro and Tyr; it has much higher concentration in honey than the other amino acids, thus requiring a larger spike concentration. Spiking experiments were carried out in triplicate.

2.2.2 Method Development

2.2.2.1 Sample Preparation

HILIC chromatography is a favourable alternative to reversed phase chromatography; it provides faster separations of polar analytes and superior peak shapes. A disadvantage in using HILIC chromatography is the reliance on acetonitrile. It is used as both a mobile phase and injection solvent, which leads to problems with analyte solubility[23].

Differing solubilities of the amino acids themselves led to problems with dissolving; acidifying the solution and vortexing the mixture improved this although cystine often took over night to dissolve fully.

Samples were initially injected as is; stock solution prepared with 0.1% HCL in methanol and then diluted with water. These samples did not yield good chromatograms; the amino acids were not well separated, likely due to the water content (Figure 2.8). The operating conditions were altered with the addition of the formate buffer to the mobile phase, requiring the samples to correspond. The samples were evaporated to dryness and reconstituted with ACN and injected. The chromatogram, seen in Figure 2.9, showed insufficient response. At this point, the separation by the HILIC column was decided to be inadequate and a new column was used.

After switching to the ZIC-pHILIC column, more changes to samples were needed to better interact with solvent conditions. Samples were dried down and reconstituted with 10%H_2O/90%ACN, hypothesising that 100% ACN was not sufficient for amino acid solubility in the vial. Figure 2.10 shows this chromatogram.

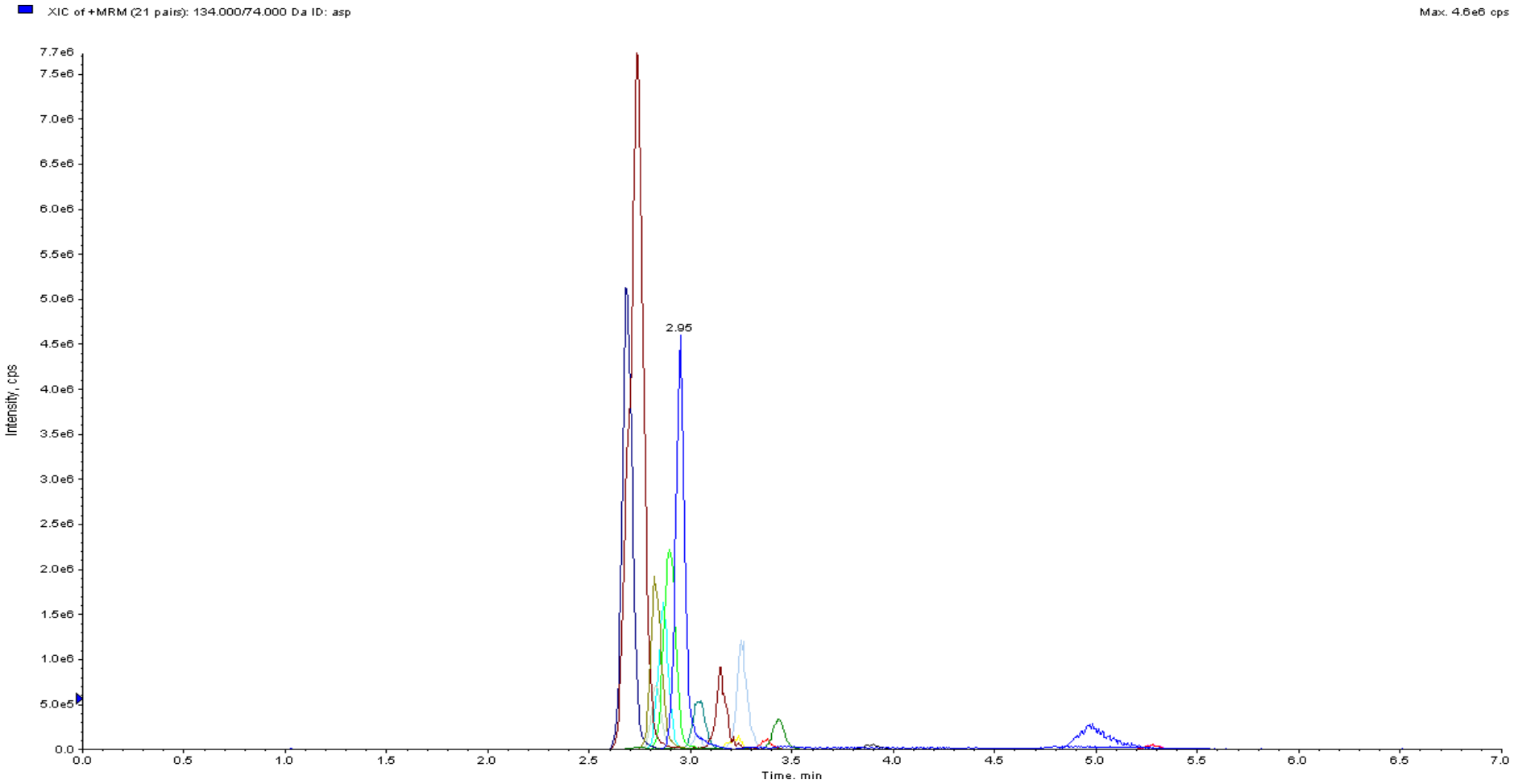


Figure 2.8: Initial chromatogram for HILIC column, samples prepared with 0.1% HCL in methanol and then diluted with water

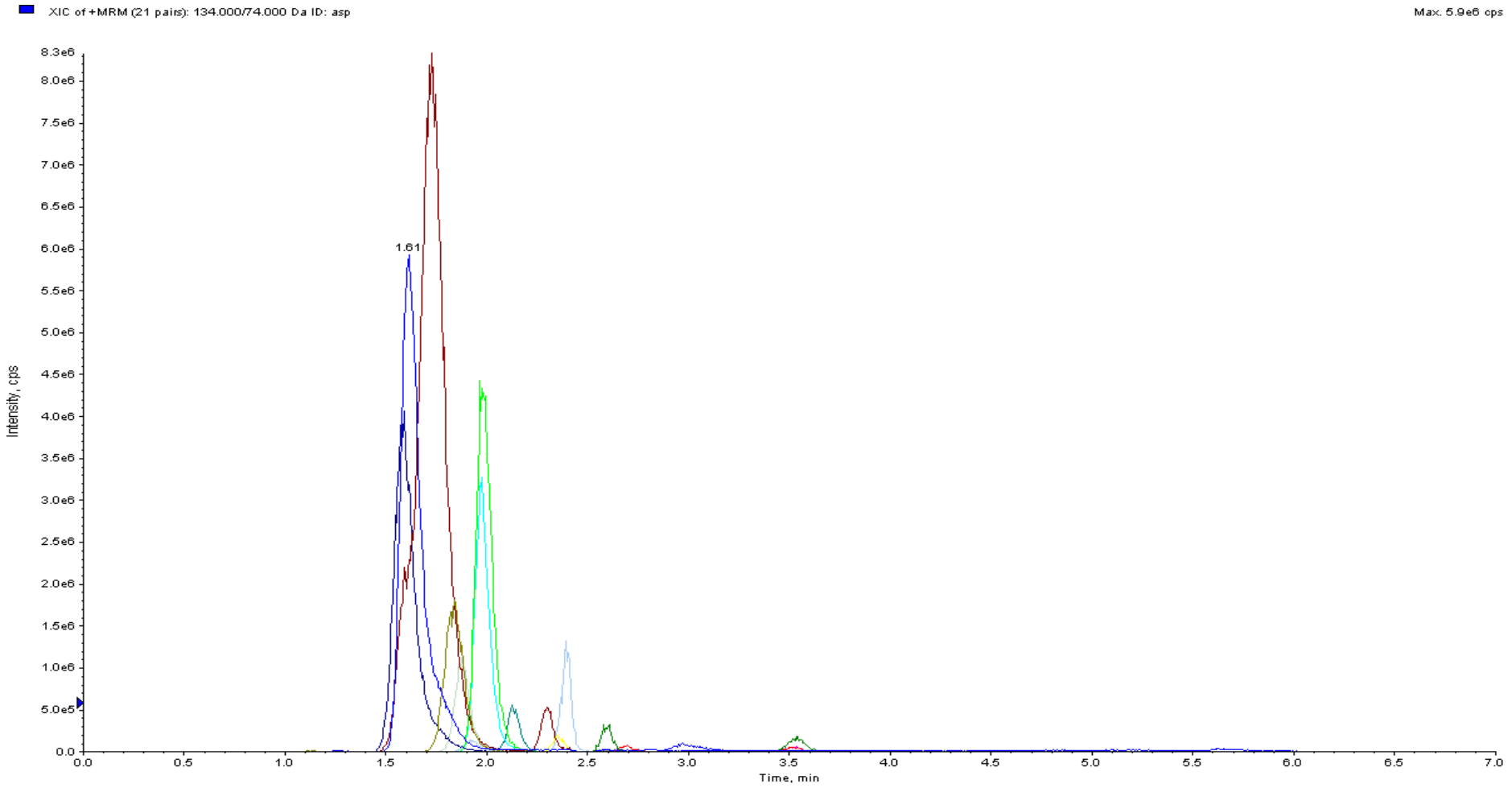


Figure 2.9: Chromatogram with new gradient for HILIC column, samples prepared with ACN

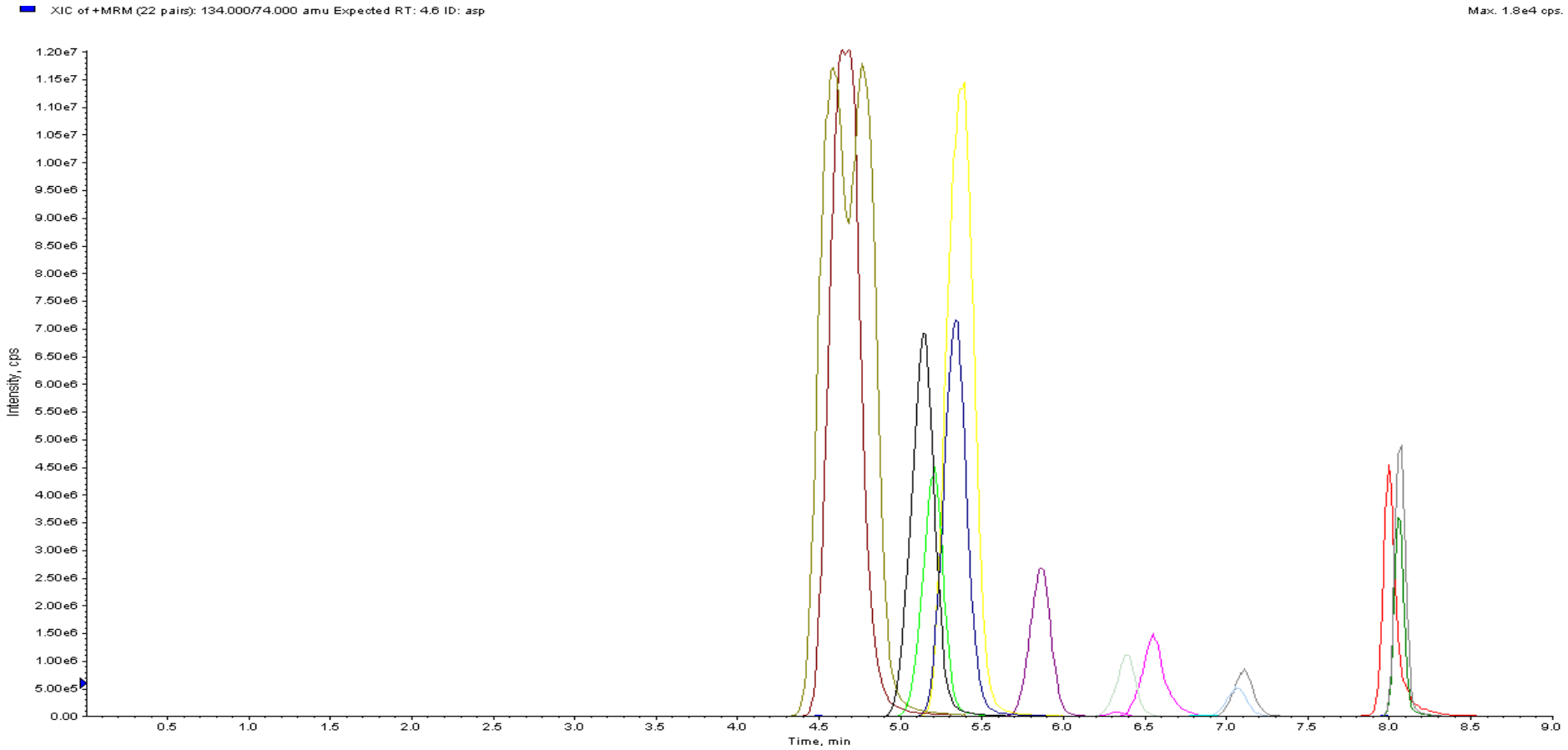


Figure 2.10: ZIC-pHILIC column, 10 ppm stock standard prepared with 10%H_2O/90%ACN

While initial chromatograms of the ZIC-pHILIC column showed sufficient separation, the amino acid response was still too low. Addition of the formate buffer to samples was carried out. The samples were reconstituted with 15% H_2O and 85% 200 mM ammonium formate in 0.5% formic acid. The initial chromatograms, Figure 2.12, were more resolved than when the HILIC column was used. Problems were still encountered with mixing with the injection solvent ACN. More changes were made to the proportions of water, buffer, and ACN in the samples; the current proportions were too water soluble, and not enough organic material to mix with the ACN solvent. The new proportions took into account the analysis of honey samples; 10% H_2O (or honey sample), 10% formate buffer, 80% ACN. This showed, Figure 2.13, an improvement in the response of the later eluting amino acids.

Calibration curves of each amino acid were evaluated, checking for carryover, good linearity, and interferences. Problematic amino acids were Asp, Glu, Asn, Gln, Lys and Arg. Asp, an acidic compound, elutes first and has a noticeable amount of carryover as seen in Figure 2.11.

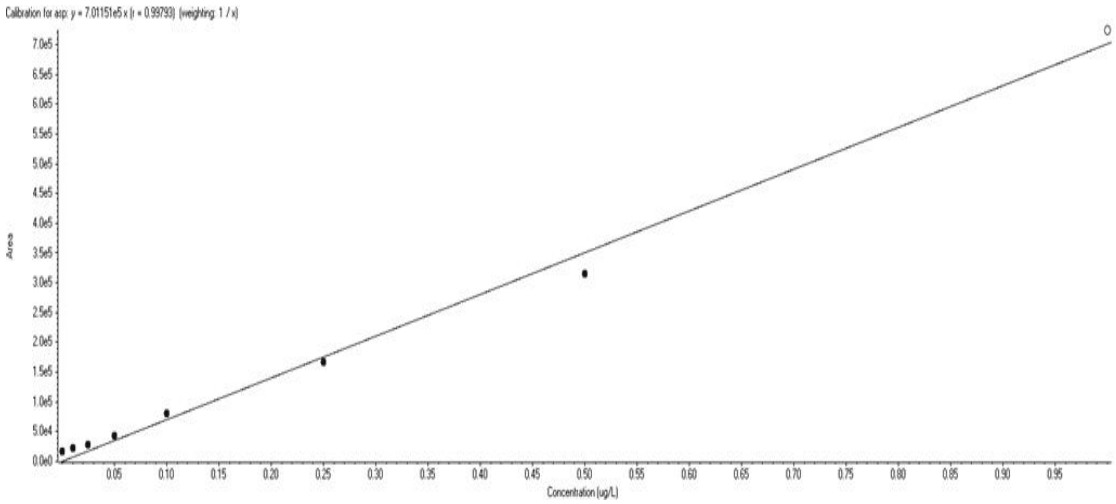


Figure 2.11: Calibration curve of aspartic acid showing carryover. y=7.01151e5x R=0.99793 weighting 1/x

Carryover is caused by insufficient washing between injections, automated by the autosampler. Small amounts of the sample stay in the injection syringe and are injected with the next sample, adding to the peak response. This can be evaluated by the calibration curve where the smaller concentration standards have larger areas than expected, with the smallest not close to zero.

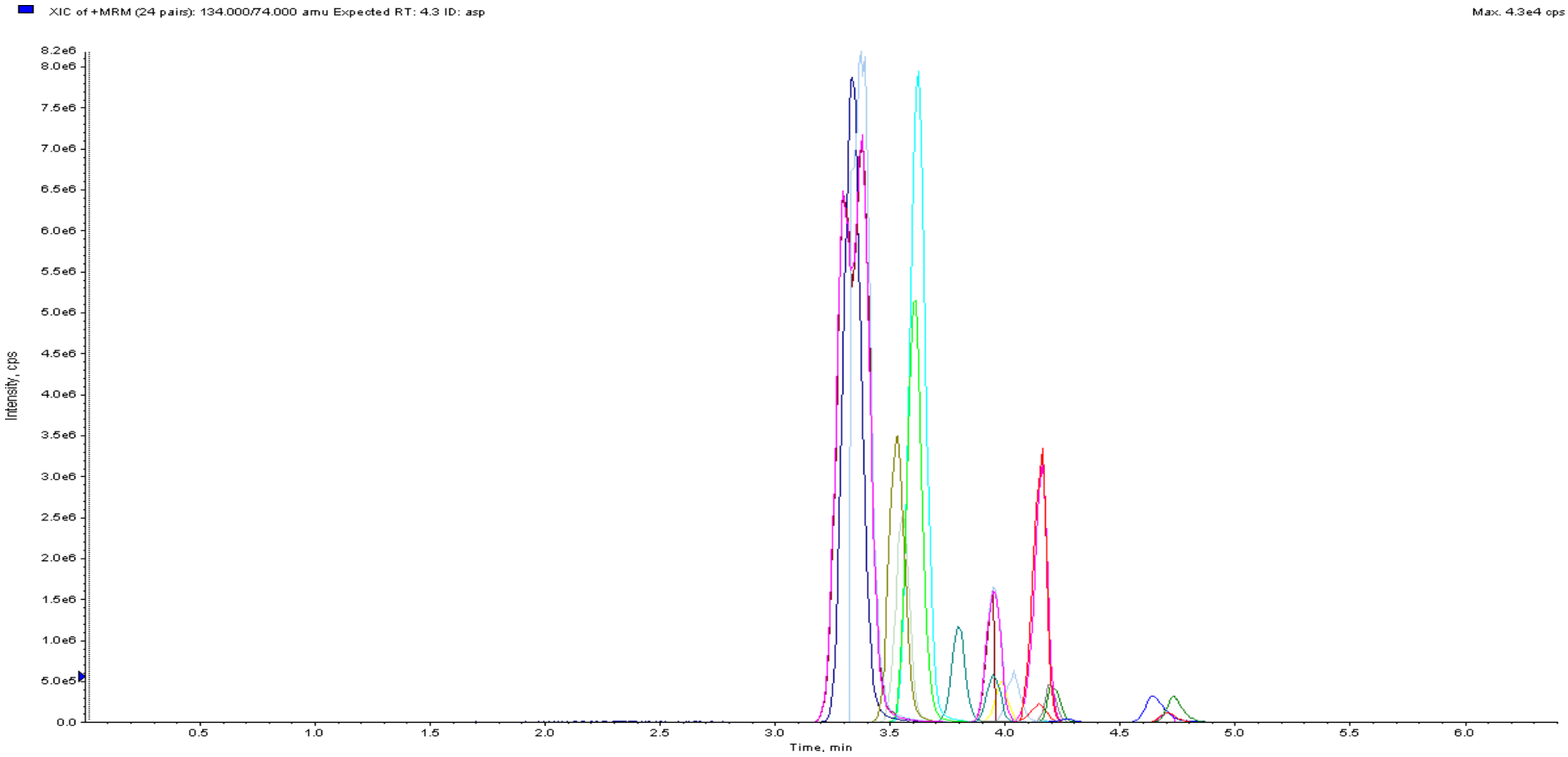


Figure 2.12: ZIC-pHILIC column, 1 ppm stock standard prepared with 15% H_2O and 85% 200 mM ammonium formate in 0.5% formic acid

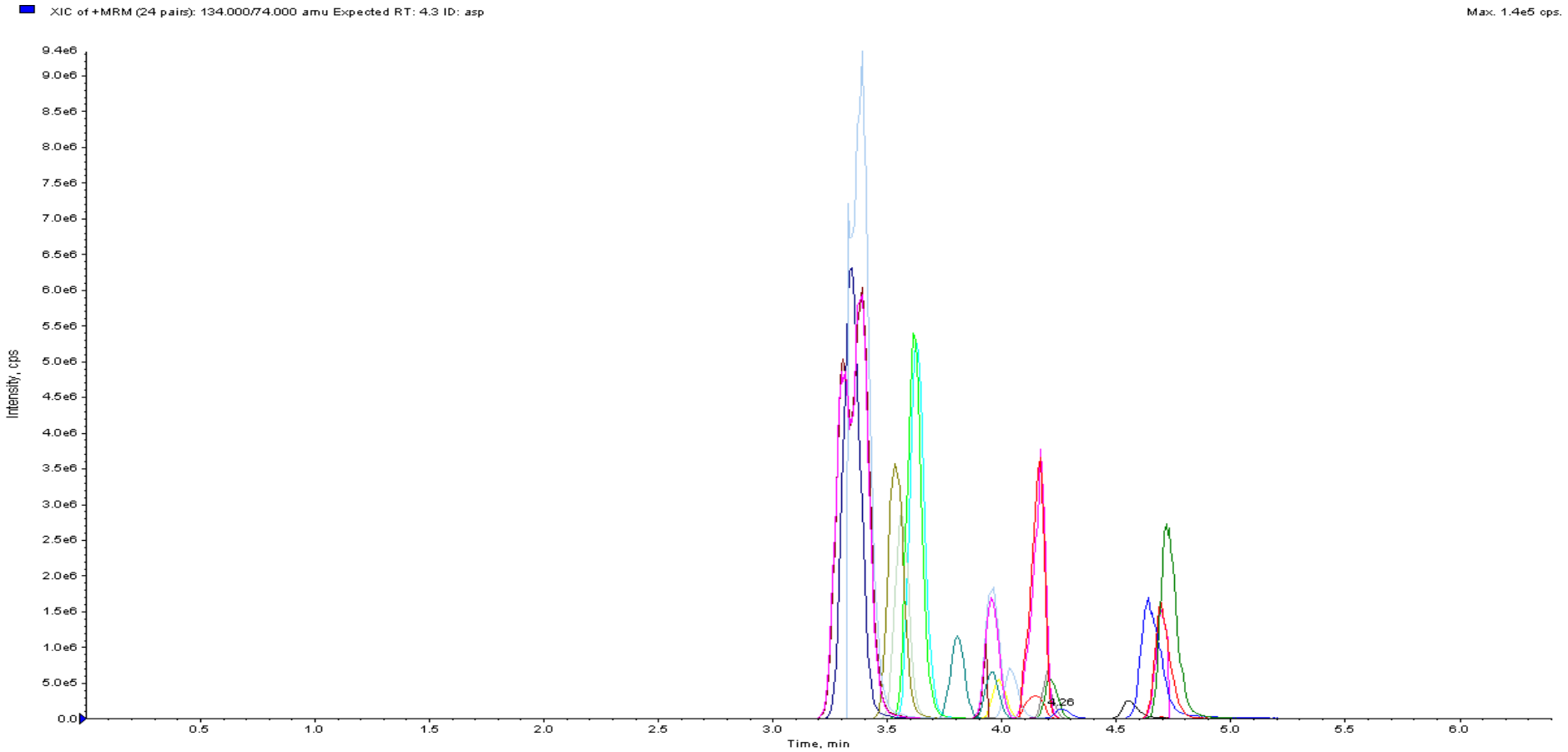


Figure 2.13: ZIC-pHILIC column, 1 ppm stock standard prepared with 10% H_2O, 10% formate buffer, 80% ACN

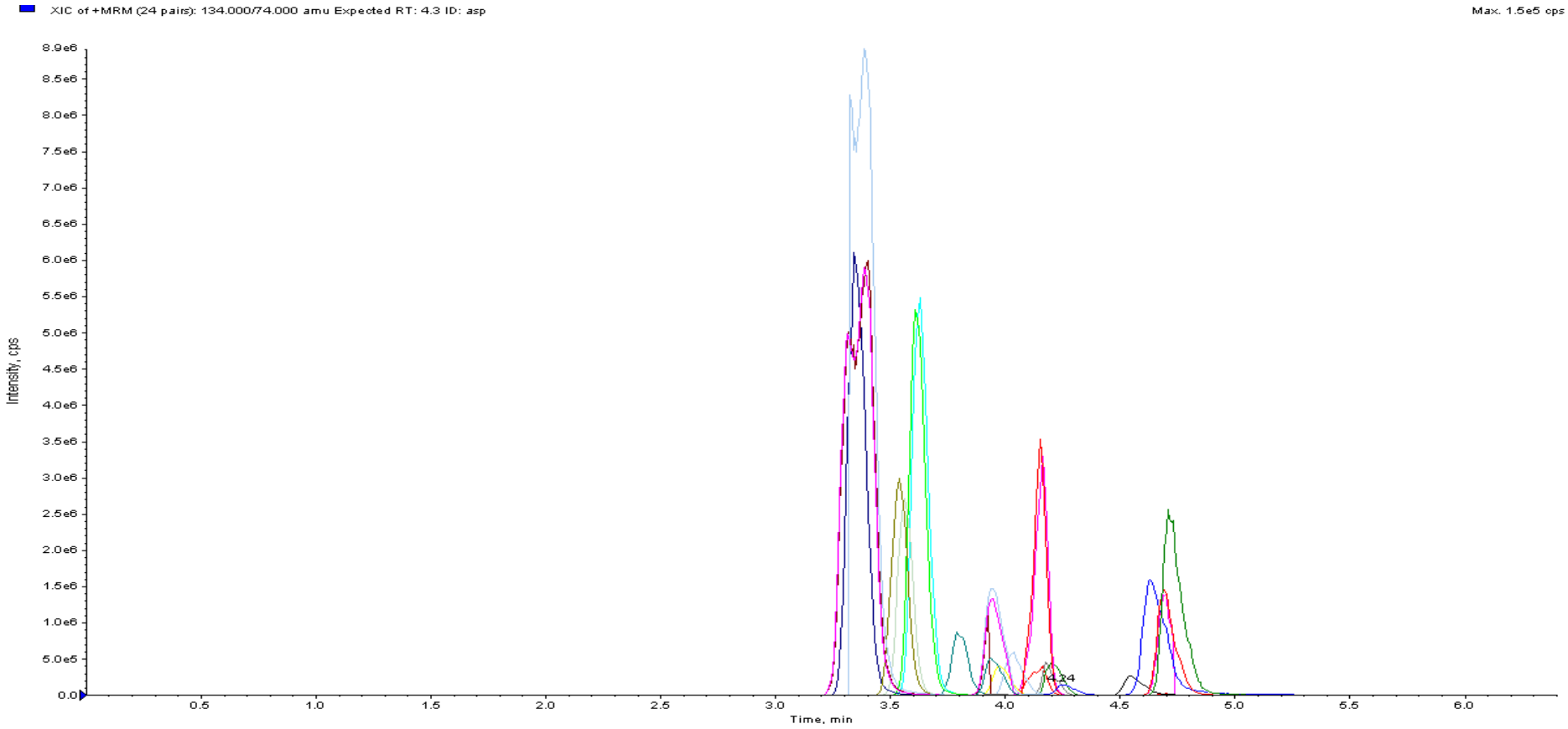


Figure 2.14: ZIC-pHILIC column, 1 ppm stock standard prepared with 500 mM ammonium formate in 0.5% formic buffer

Lys and Arg are basic amino acids, in order to increase their solubility in ACN, an extra ion pair needs to be added with a stronger buffer. Reconstitution with 500 mM ammonium formate in 0.5% formic acid buffer was trialled. Little improvement to the response of the basic amino acids was seen, as in Figure 2.14. The possibility that the glass vials (used to hold samples) may be influencing the solubility, by the compounds sticking to the glass, was theorised. The use of polypropylene vials was initiated, along with an improved needle wash to stop the carryover of acidic amino acids. The polypropylene vials decreased the loss of amino acids in vial and so their use was continued.

While carrying out spike recoveries with artificial honey and analysing honey samples, interferences from the sugars were present. The interferences can be seen in Figure 2.15, the peak shape being greatly affected.

To decrease the interference of the sugars, sample solvents were altered to reduce the honey content to 2.5% H_2O (or honey sample), 10% formate buffer, 87.5% ACN. This reduced the interferences to a satisfactory level. A comparison chromatogram of the same spiked honey sample (ID: 78 with 100 ppm amino acid stock standard) in Figure 2.15 with the new sample solvents can be seen in Figure 2.16.

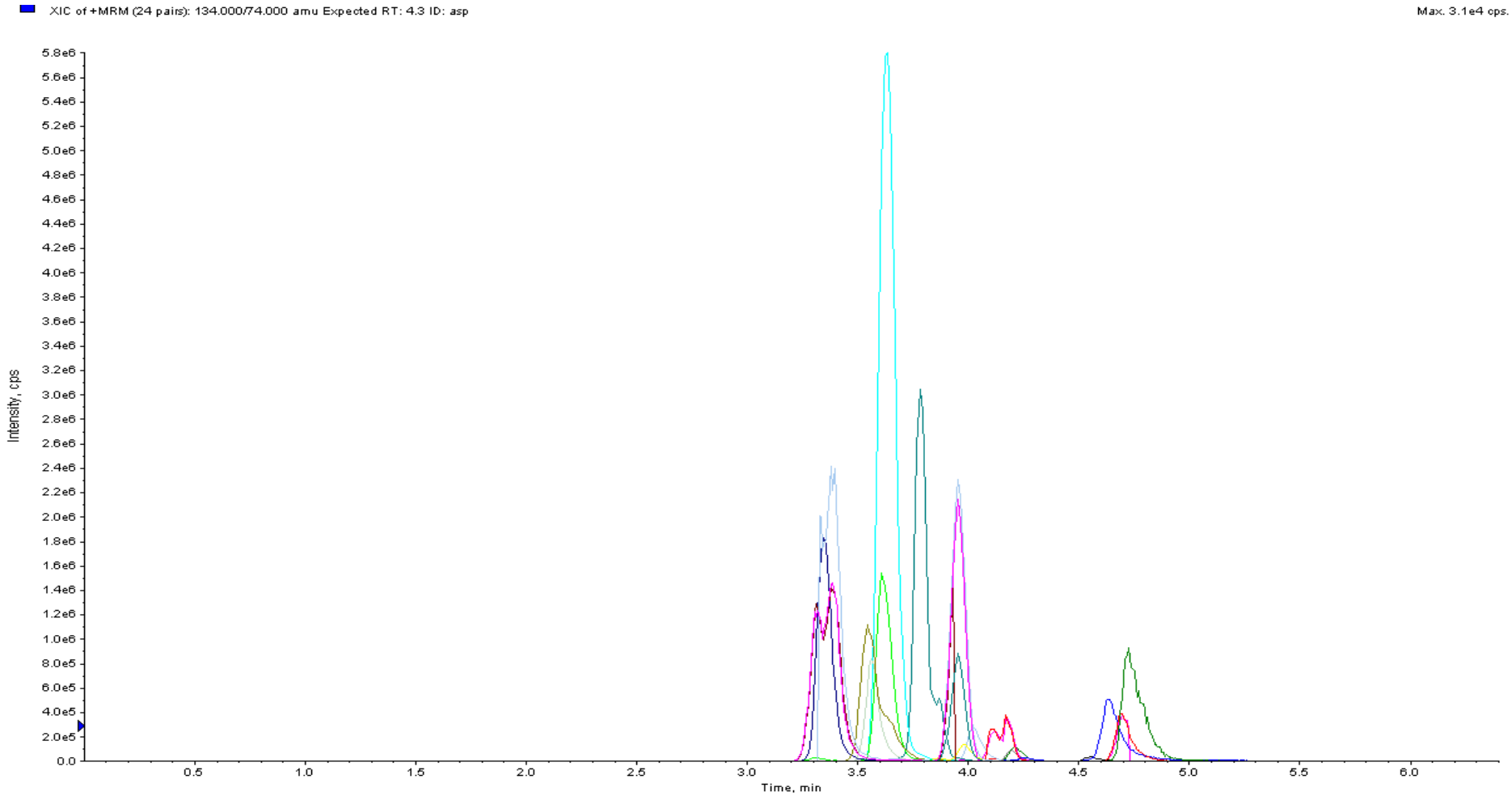


Figure 2.15: Honey sample (ID: 78) spiked with 100 ppm stock standard showing sugar interferences

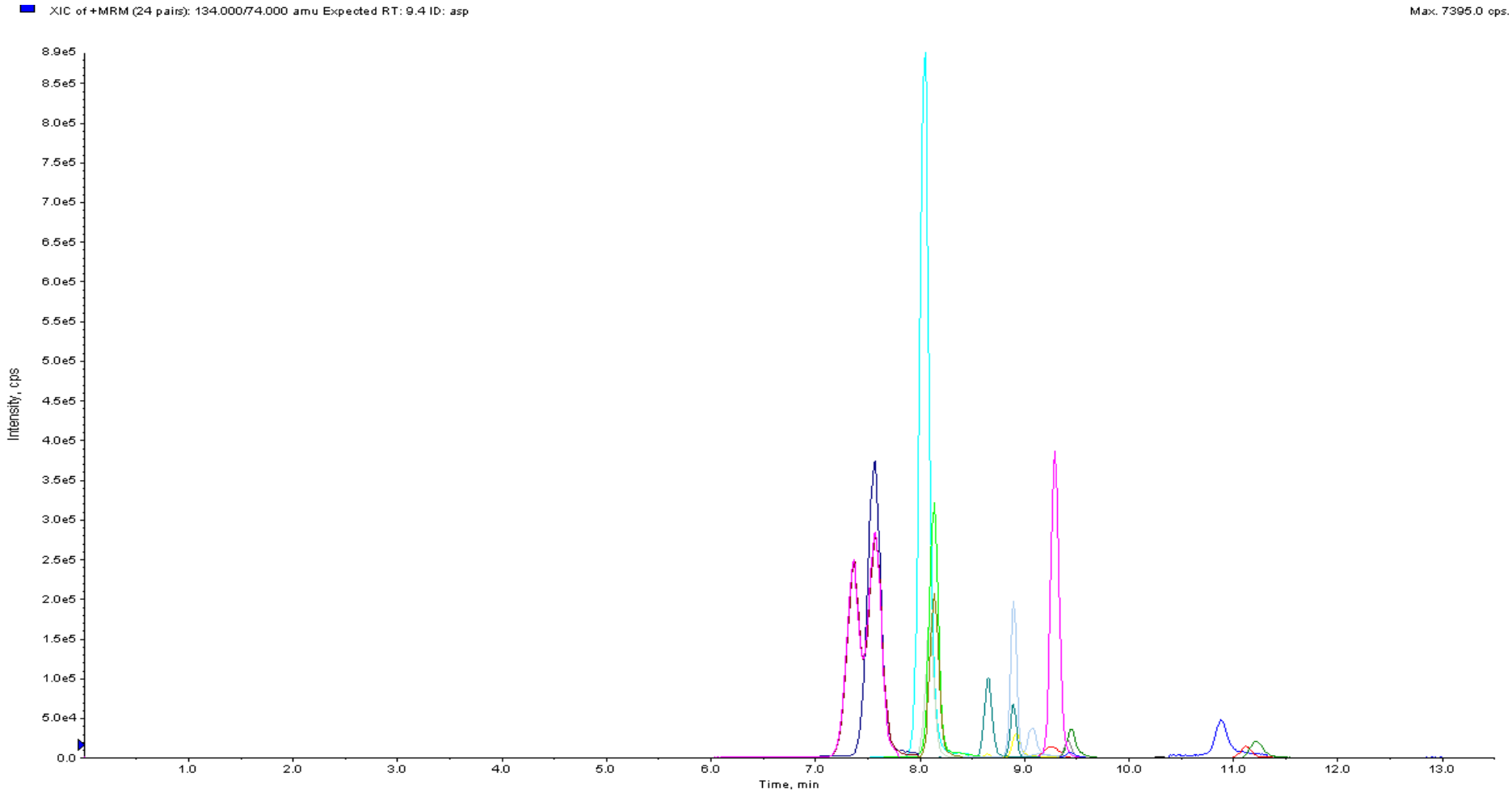


Figure 2.16: Honey sample (ID: 78) spiked with 100 ppm stock standard showing no sugar interferences

2.2.2.2 Operating Conditions

Initial conditions involved a gradient mixture of acetonitrile (A) and 0.1% formic acid in water (B) was used at 30 °C with a flow rate of 400 µL/min on a Ascentis® Express HILIC column (10 cm x 21 mm, 2.7 µm) supplied by Supelco Analytical. The gradient program is in Table 2.7. These conditions were modelled after the conditions used by Gokmen (2012)[22].

Table 2.7: Initial gradient program for HILIC column

Time (min)	%B
0	25
4	50
6	25

Later the solvents were exchanged: 0.1% formic acid in water (A) and acetonitrile (B) for convenience and the gradient program changed to reflect that. With this new set up, amino acids were not sufficiently separated and response of later eluting amino acids decreased. The chromatogram for this can be seen in Figure 2.8 on page 33.

To improve the separation, flow was increased to 600 µL/min and solvent A was changed to 0.2% formic acid in water. The gradient program used with these changes is depicted in Table 2.8. The chromatogram employing the new gradient is in Figure 2.9 on page 34. The initial gradient of 98% ACN forces interaction of the amino acids with the stationary phase. The slow addition of formic acid and water to the gradient, between 2.5 and 4 min, elutes amino acids off in sequence by increasing their interaction with the mobile phase, and this is continued by a hold of 40% B for one minute. The time between 6 and 10 min that mobile phase B is at 98% is equilibration time for the column before the injection of the next sample. All of the amino acids elute before 6 min.

Table 2.8: Improved gradient program for HILIC column

Time (min)	%B
0	98
0.75	98
2.5	98
4	40
5	40
6	98
10	98

The increased flow rate created asymmetric peaks with tailing, due to more interaction of the analytes with the solvent than the solid phase. At this point, no significant improvements were seen with the chromatogram, the problem possibly arising from the column, the stationary phase not sufficient for separation of underivatised amino acids.

HILIC is similar to normal phase chromatography but with mobile phase composition (40-97% ACN with water or buffers) better suited to MS analysis, allowing higher sensitivity. HILIC retention occurs primarily by hydrophobic partitioning where analytes elute in order of increasing polarity, but also has a second dimension of selectivity relying on electrostatic interactions. These interactions require higher buffer concentrations, which can interfere with MS detection[36].

Zwitterionic ion chromatography (ZIC) in combination with HILIC chromatography is appropriate for amino acids separation because it uses both hydrophobic partitioning and electrostatic interactions as retention mechanisms[24]. ZIC-pHILIC columns (polymethacrylate core) have a zwitterionic stationary phase, where the charged electrostatic forces are counterbalanced by ions of opposite charge, leading to weak electrostatic interactions. This allows better selectivity of the ZIC-pHILIC column with low buffer concentrations[36].

For that reason a new column with similar chemistry was tested, a ZIC-pHILIC column (150 x 2.1 mm, 5 μm polymeric beads). Solvent A was modified to 5 mM ammonium formate in 0.1% formic acid, solvent B was unchanged. The gradient program in Table 2.8 was used.

Optimisation of amino acids was carried out by continuous injection of a single amino acid into the triple quad. The Q1 mass corresponds to the molecular mass, and through MS/MS the Q3 masses were chosen by finding the optimum product ion. The decluster potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) of the ions were also optimised. The analysis process of this is tabulated in Table 2.9. The final conditions used can be found in Table 2.6.

Table 2.9: Hydroxyproline optimisation

Hydroxyproline			
Molecular mass (g/mol)	131.13	Q1	132
Product ions	68, 86	Q3	86
	Trial 1	Trial 2	Final
DP	37.7	42.4	40
CE	17.62	-	18
EP	9	10	10
CXP	5	6	6

Analysis with the mentioned conditions required further development to enhance the separation by changing the buffer conditions. The following solvents were used; 50 mM ammonium formate in 0.1% formic acid (A) and 0.5% formic acid in acetonitrile (B). The chromatogram with these mobile phases is illustrated in Figure 2.17 had some improvement. To further this a 1 M buffer was tested but with no noticeable enhancement, as seen in Figure 2.18

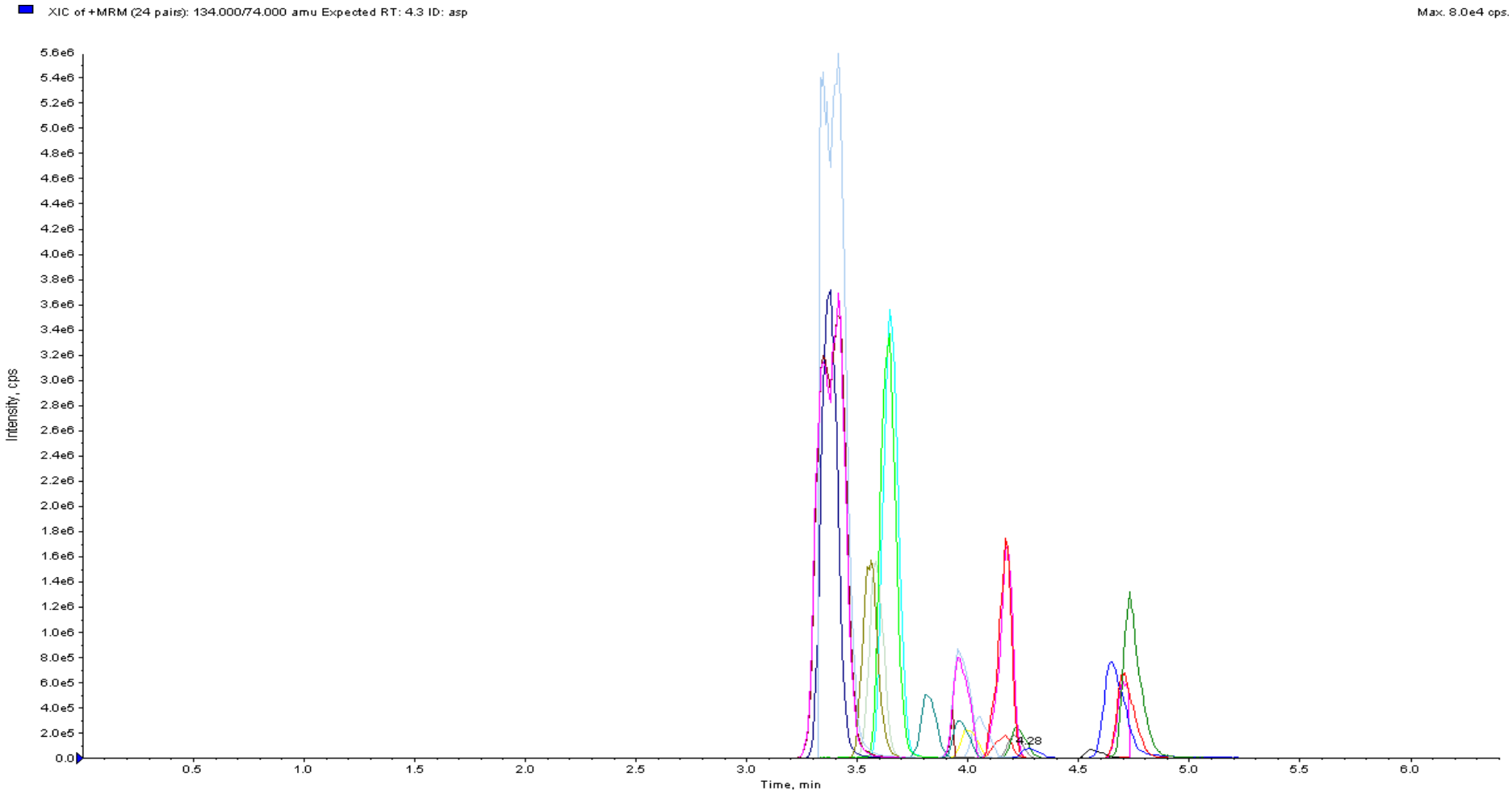


Figure 2.17: Chromatogram with 50 mM ammonium formate in 0.1% formic acid (A)

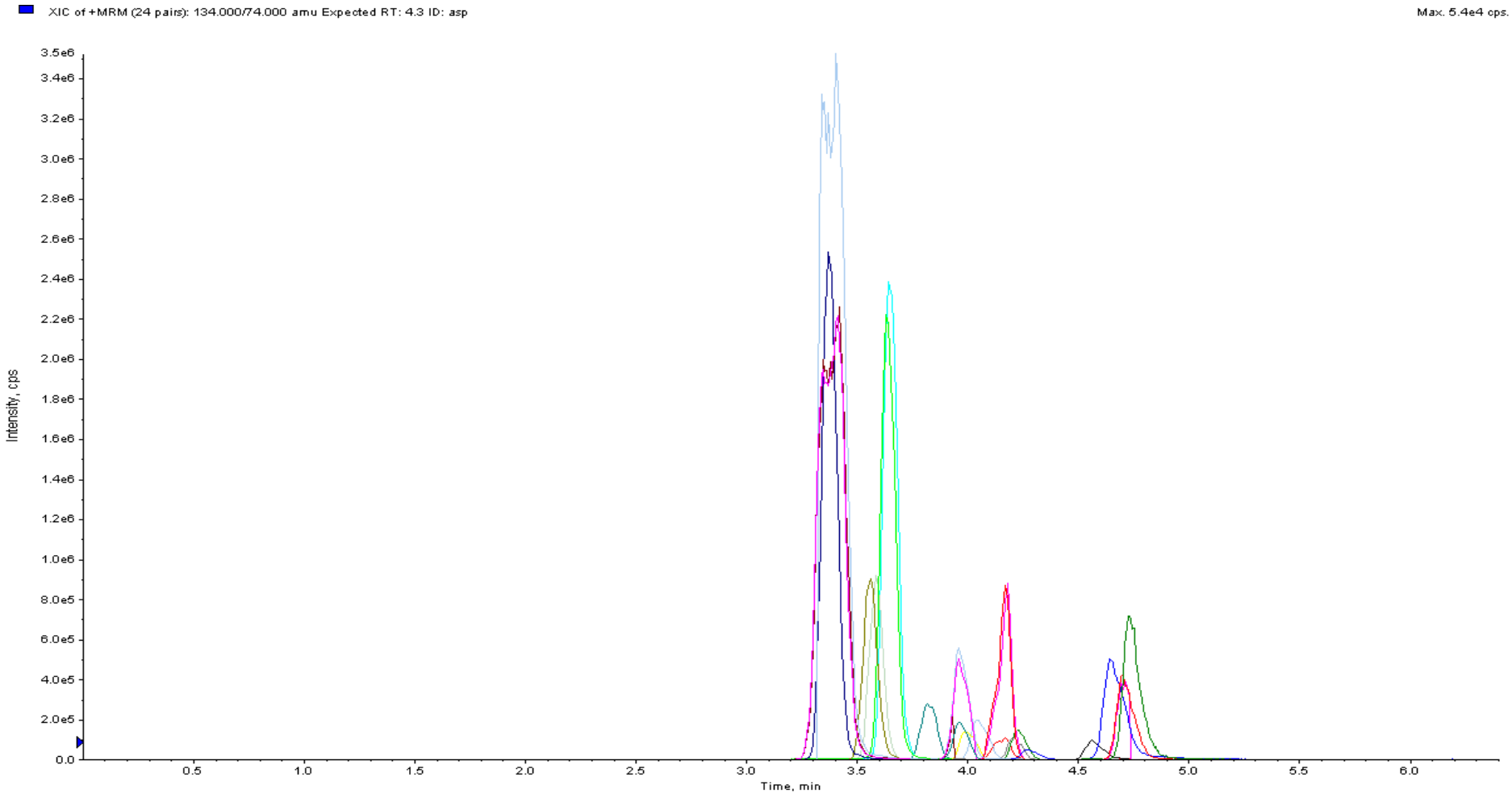


Figure 2.18: Chromatogram with 1 M ammonium formate in 0.1% formic acid (A)

Spike recoveries and testing of honey brought about new problems. Interferences from the sugars influenced the amino acids response. While changes were made to the samples, an improvement on the solvents was also required. To improve the interaction of amino acids with the organic solvent (B), was changed to; 0.5% formic acid in 5% MeOH, 95% ACN (B). The addition of the protic solvent allows basic amino acids to generate salts for ionic interaction with the column, in the same manner as water. The chromatogram for this can be seen in Figure 2.19.

The addition of methanol to solvent B did not improve interaction, so the methanol was removed from the solvent, while changes to solvent A were made to give 20 mM ammonium formate in 0.04% formic acid (A). This reduced the buffer concentration so formate suppression lessened but without preventing basic amino acids from accepting protons for ionic interaction with the column. In combination with this, a longer gradient was tested with a decreased flow in order to allow more interaction time with the stationary phase, separating the sugars that elute early from the amino acids. The long gradient is displayed in Table 2.10.

Table 2.10: Long gradient program

Time (min)	%B	Flow (µL/min)
0	90	400
0.5	90	400
2.5	85	400
5	65	500
7	40	500
8	40	500
8.5	90	500
10	90	500
10.1	90	400

The final chromatogram is in Figure 2.20.

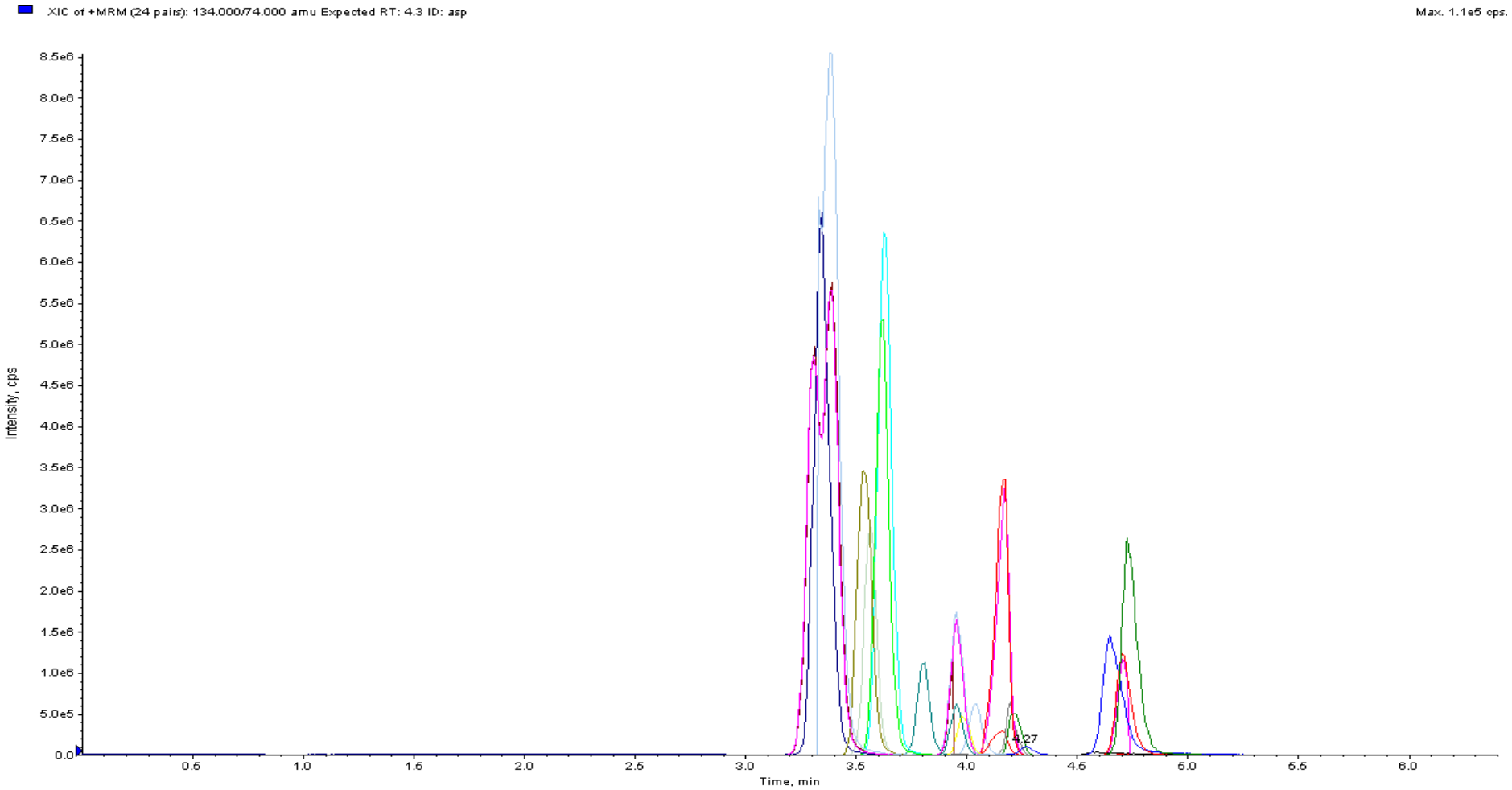


Figure 2.19: Chromatogram of 0.5% formic acid in 5% MeOH, 95% ACN (B) solvent

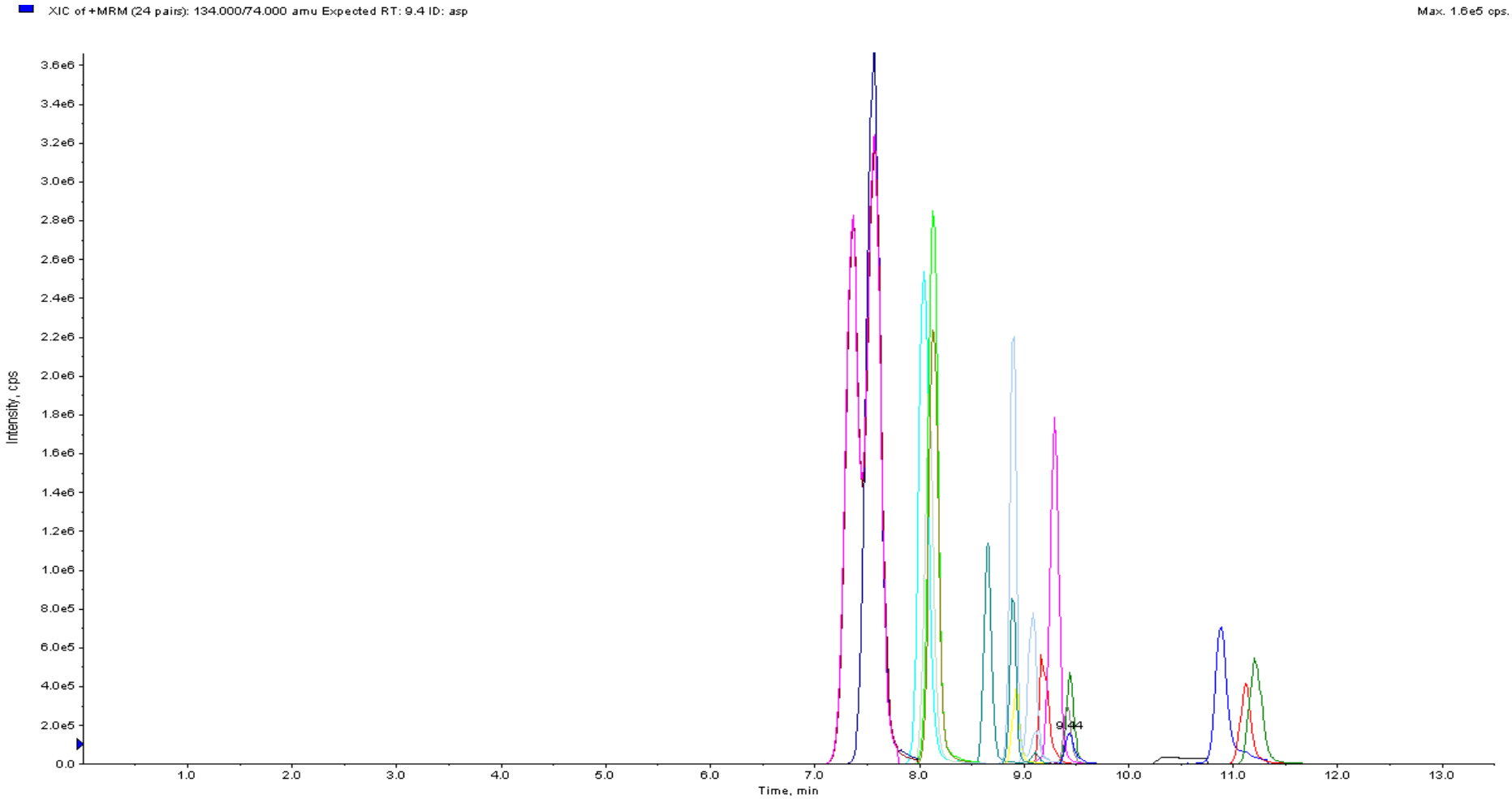


Figure 2.20: Final chromatogram of 1 ppm stock standard

2.2.2.3 Scheduled MRM

The scheduled MRM's were examined to calculate the number of concurrent MRM transitions occurring during the chromatography. The transitions that were at the same time were evaluated to see if they were happening in excessive amounts, compromising on data quality. Figure 2.21 depicts the concurrency of the scheduled MRM's, the highest number of calculations occurring at the same time being 12, a reasonably small number that allows high quality data to be recorded.

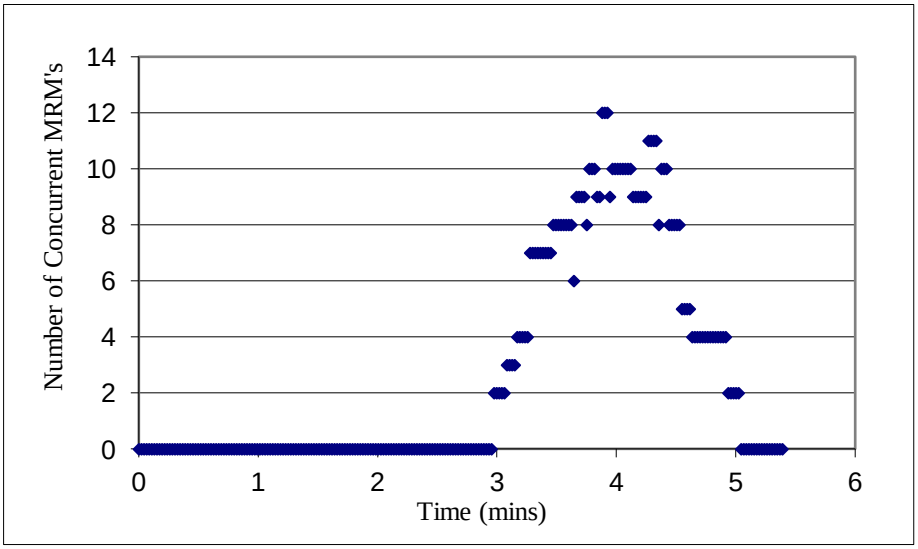


Figure 2.21: Graph depicting concurrency of scheduled MRM

2.2.3 Results

2.2.3.1 Spike recoveries

Validation of this method was carried out via spike recoveries. Artificial honey (Afh) and three honey samples (ID: 66, 78, and 84) were spiked with two different stock solutions; the first containing twenty amino acids each at 10 ppm, the second containing Pro and Tyr at 250 ppm each. The spikes for each amino acid were at approximately double the analysed concentrations in honey, Pro and Tyr being at notably larger concentrations (0-20 mg/kg for the twenty amino acids vs. 100-500 mg/kg for Pro and Tyr) than the rest. Proline is at higher concentrations because it originates from bee haemolymph and honey nectar. The high tyrosine concentration, originating from mānuka nectar and pollen, is likely to be a property of mānuka honeys. The tabulated data for these results are in Table 2.11.

Table 2.11: Spike recoveries of amino acids

Sample ID	Amino acid Spike Recovery (%)										
	Asp	**Glu**	**Ser**	**Asn**	**Thr**	**Gln**	**Tyr**	**Gly**	**Pro**	**Ala**	**Met**
66	59%	2%	82%	85%	176%	81%	854%	11%	69%	110%	148%
78	70%	7%	75%	80%	194%	71%	812%	9%	64%	126%	161%
84	70%	3%	87%	87%	180%	75%	811%	24%	73%	104%	145%
Afh	92%	19%	114%	115%	170%	144%	493%	21%	128%	118%	190%
	Val	**Phe**	**Leu**	**Trp**	**Cys**	**His**	**Lys**	**Arg**	**Hydpro**	**Ile**	
66	87%	86%	61%	190%	39%	110%	97%	206%	700%	80%	
78	101%	92%	89%	208%	42%	124%	116%	218%	756%	89%	
84	85%	88%	63%	166%	38%	118%	105%	174%	696%	77%	
Afh	151%	114%	128%	187%	39%	111%	110%	125%	339%	154%	

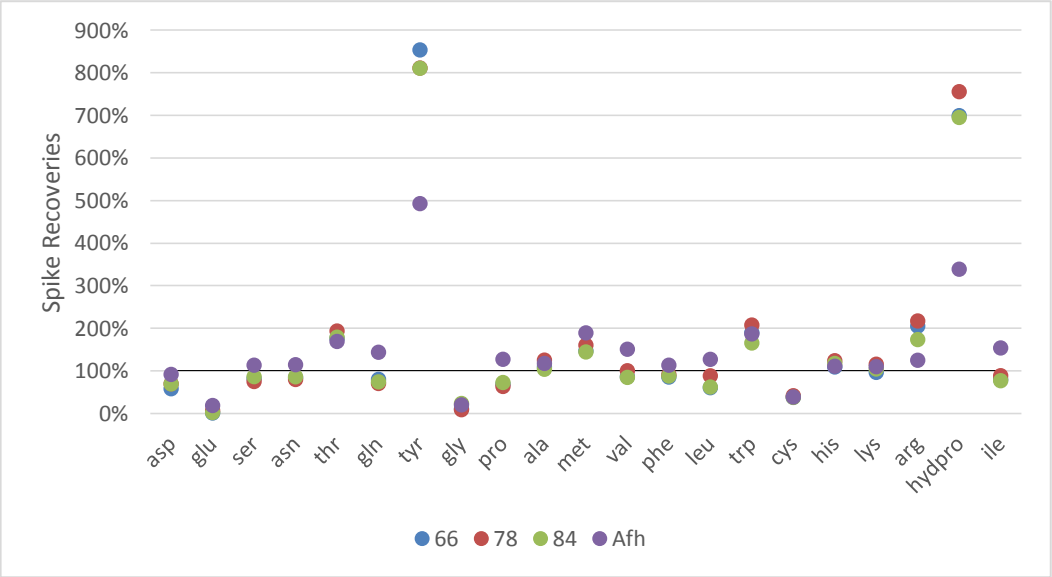


Figure 2.22: Graph showing spike recoveries of amino acids

The ideal spike recovery is at 100%, or just under that, indicating that nearly all of the spiked quantity was recovered after undergoing the processes of sampling, mixing and LC/MS analysis. Figure 2.22 shows the nearness of the results to 100%. Some amino acids, namely Asn, Val, Phe, and Lys, are at appropriate percentages of recovery; this method of analysis produces accurate results for these amino acids. The low recoveries of Glu, Gly, and Cys indicate either loss in the vial or very low responses by MS analysis; this shows that Glu, Gly, and Cys cannot be accurately determined by this method. The high recoveries such as Thr, Tyr, Met, Trp, Arg, and Hydpro are unusual; it is possible that the responses of these amino acids by MS analysis are over favoured.

Response factors were not used because it was hypothesised that the amino acid standards, in relation to their preparation, was not sufficient for full recovery. Thus it was likely that the MS detection was not showing low responses, the standard solutions had low recovery. This would lead to problems with calibrations and quality control samples, as these are prepared from the amino acid standards. An internal standard for comparison could not have worked because the internal standard itself may not give an accurate response due to lack of recovery out of the vial solution. Testing the response factors from the MS should have been carried, as the high recoveries of tyrosine and hydroxyproline are likely due to an increased MS response. It is possible that this could have been resolved if more time was available.

Due to these results, and the inability to resolve them, this method was considered unsuccessful and no further work on it was carried out.

Chapter Three: Method Procedure, Development and Validation

In this chapter the materials, instrumentation, and validation procedures for the final method are discussed. The changes undertaken to develop the method are also reviewed. The method and validation for Hill Laboratories can be found in appendix 9 and 10, respectively.

3.1 Experimental Procedure

3.1.1 Instrumentation

Chromatographic separations were performed on an Amino Acid Analyser (AAA) C18 reversed-phase column (150 x 4.6 mm, 5 μm). A HPLC system from Agilent Technologies 1200 series consisting of a degasser, binary pump, and thermostated column compartment was used. An autosampler from Pal System, PAL HTS-xt, and detection was achieved with an AB Sciex Triple Quad™ 4500. Analysis of ions was carried out by multiple reaction monitoring (MRM)[29]. Analysis software was used provided by MultiQuant.[1]

3.1.2 Materials

Acetonitrile and methanol were of HPLC grade and obtained from Merck and Sigma-Aldrich, respectively. Hydrochloric acid (HCL) was supplied by Avantor. The aTRAQ™ Starter Kit Hydrosylate provided by AB Sciex contained; the aTRAQ (derivatising) reagent Δ8, labelling buffer (borate buffer, pH 8.5), hydroxylamine (1.2% solution), isopropanol, mobile phase A (100% formic acid) and B (100% heptafluorobutyric acid), internal standard, unlabeled standard, and standard diluent (2% formic acid). The kit also supplied a certificate of analysis for the reconstituted internal standard. Deionised water, used throughout experiments, was purified by Sartorius Stedim biotech.

[1] MRM allows the user to set a quadrupole filter to select for the labelled amino acid (precursor ion Q1) which is fragmented and a second quadrupole filter to select for the cleaved aTRAQ™ Reagent label (product ion Q3) for detection. Scheduled MRM sets a window of detection around the retention time, which it monitors for the specific labelled amino acids.

Twenty four honey samples were analysed. Fourteen stored honey samples were obtained from Steens Honey Ltd. (Te Puke, New Zealand) (ID: B0 08E3, B0 14E3, B0 23E3, B0 24E3, C 463, C 887, C890).Two fresh mānuka honey samples were also obtained from Steens Honey Ltd. (ID: 946, 953). Three mānuka honey samples (ID: 66, 78, 84) were provided fresh by Gibbs Honeybees (Masterton, NZ). Four clover honeys were purchased from commercial outlets: Happy Bee clover honey (Hamilton, New Zealand), Airborne pure natural New Zealand clover honey (Leeston, Canterbury, New Zealand; batch 113411, best before 29/03/15), Katikati clover honey (Katikati, New Zealand; batch 43/11, best before 11/2016) and Holland clover honey (Timaru, New Zealand). All honeys were stored in a freezer (−20 ºC) when not used.

3.1.3 Sample Preparation

Honey samples were diluted 1:40 with water and shaken until dissolved. The labelling protocol was modified from that which was provided by the aTRAQ™ kit. Honey sample (20 µL) was added to labelling buffer (40 µL) in an (1.5 mL) Eppendorf tube, mixed five times with the pipette, vortexed (30 sec, 1000 rpm) and centrifuged (2 min, 10,000 rpm). This mixture was transferred to a new Eppendorf tube (12 µL), to which the aTRAQ™ reagent Δ8 was added (5 µL), then voxtexed and spun. After waiting (30 min), hydroxylamine (5 µL) was added to the tube, vortexed and spun. After waiting (15 min), internal standard was added (16 µL), vortexed and spun. All contents were transferred to a vial and water was added (150 µL) and mixed.

3.1.4 Operating conditions

A gradient mixture of 0.1% formic acid and 0.01% heptafluorobutyric acid in water (A) and 0.1% formic acid and 0.01% heptafluorobutyric acid in methanol (B) was used at a column temperature of 50°C with a flow rate of 800 µL/min. The gradient program is described in Table 3.1. The scheduled MRM values for mass spectra analysis is in Table 3.2. Relevant example structures of the Q1 and Q3 aspartic acid ion are in Figure 3.1.

Table 3.1: Gradient program

Time (min)	**Solvent**	
	A%	B%
0	98	2
6	60	40
10	60	40
11	10	90
12	10	90
13	98	2
18	98	2

Table 3.2: Scheduled MRM of the 24 amino acids and their corresponding internal standards

ID	Q1 Mass (Da)	Q3 Mass (Da)	Retention Time (min)	Collision Energy	Entrance Potential	Decluster Potential	Collision Cell Exit Potential
Asp	282.1	121.1	3.7	30	10	30	5
Glu	296.2	121.1	4.3	30	10	30	5
Ser	254.2	121.1	3.4	30	10	30	5
Thr	268.2	121.1	4.2	30	10	30	5
Tyr	330.2	121.1	6.8	30	10	30	5
Gly	224.1	121.1	3.6	30	10	30	5
Pro	264.2	121.1	5.3	30	10	30	5
Ala	238.2	121.1	4.3	30	10	30	5
Met	298.2	121.1	6.3	30	10	30	5
Val	266.2	121.1	6.4	30	10	30	5
Phe	314.2	121.1	7.9	30	10	30	5
Leu	280.2	121.1	7.8	30	10	30	5
MOx	314.2	121.1	3.9	30	10	30	5
Cys	537.2	121.1	5.4	30	10	50	5
His	304.2	121.1	3.9	30	10	30	5
Lys	443.3	121.1	5.6	30	10	50	5
Arg	323.2	121.1	4.7	30	10	30	5
Nva	266.2	121.1	6.7	30	10	30	5
IleISTD	272.2	113.1	7.6	30	10	30	5
Ile	280.2	121.1	7.6	30	10	30	5
Nle	280.2	121.1	8	30	10	30	5

NleISTD	272.2	113.1	8	30	10	30	5
AspISTD	274.1	113.1	3.7	30	10	30	5
GluISTD	288.2	113.1	4.3	30	10	30	5
SerISTD	246.2	113.1	3.4	30	10	30	5
ThrISTD	260.2	113.1	4.2	30	10	30	5
TyrISTD	322.2	113.1	6.8	30	10	30	5
GlyISTD	216.1	113.1	3.6	30	10	30	5
ProISTD	256.2	113.1	5.3	30	10	30	5
AlaISTD	230.2	113.1	4.3	30	10	30	5
MetISTD	290.2	113.1	6.3	30	10	30	5
ValISTD	258.2	113.1	6.4	30	10	30	5
PheISTD	306.2	113.1	7.9	30	10	30	5
LeuISTD	272.2	113.1	7.8	30	10	30	5
MOxISTD	306.1	113.1	3.9	30	10	30	5
CysISTD	521.2	113.1	5.4	30	10	50	5
HisISTD	296.2	113.1	3.9	30	10	30	5
LysISTD	427.3	113.1	5.6	30	10	50	5
ArgISTD	315.2	113.1	4.7	30	10	30	5
NvaISTD	258.3	113.2	6.7	30	10	30	5
Asn	281.2	121.1	3.4	30	10	30	5
Gln	295.2	121.1	3.7	30	10	30	5
Trp	353.2	121.1	8.5	30	10	30	5
HydPro	280.1	121.1	3.5	30	10	30	5
AsnISTD	273.2	113.1	3.4	30	10	30	5

HydProISTD	272.1	113.1	3.5	30	10	30	5
GlnISTD	287.2	113.1	3.7	30	10	30	5
TrpISTD	345.2	113.1	8.5	30	10	30	5

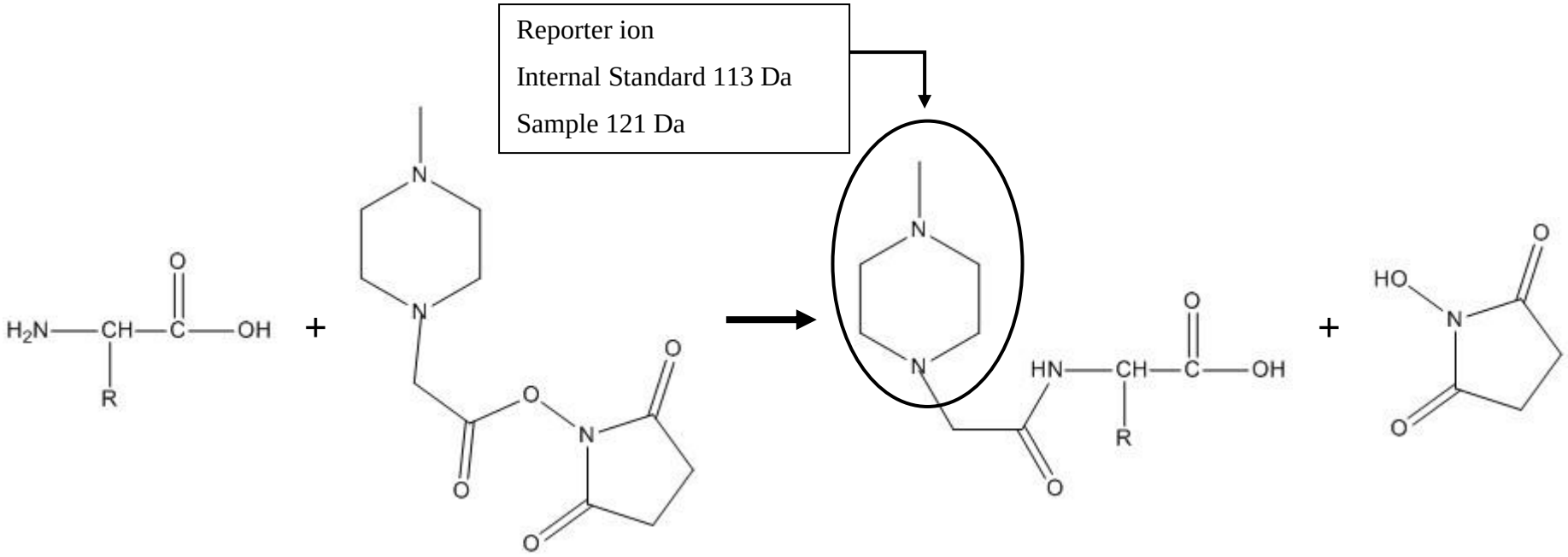


Figure 3.1: General structures of the Q1 and Q3 ions of labelled amino acid samples and standards monitored by MRM

3.2 Method Development

3.2.1 Sample Preparation

The first run of the aTRAQ™ kit followed the protocol for physiological samples. The unlabeled standard provided by the kit was derivatised as described by the protocol except for these modifications, after adding the internal standard; the volume was not reduced, and water (148 µL) was added. This was done initially because the volume was already too small and the high sensitivity of the Triple Quad™ 4500 system requires the sample to be diluted so the detector is not saturated. The protocol states that these changes may be necessary for the more sensitive systems. The results from the unlabelled standard were within the ranges expected (Figure 3.2).

Further investigation led to the deletion of the sulfosalicyclic acid step. This step precipitates protein, so both free and bounded amino acids are characterised. For the results to be relevant, only free amino acids must be characterised. This also means that no norleucine standard, an amino acid in the sulfosalicyclic acid solution that indicates recovery of precipitated protein, will show up in the analysis. Figure 3.3 and Figure 3.4 show the difference in chromatograms for analysis of honey (ID: 78).

Chromatograms showed large peak differences between the amino acids and their internal standards, and the two needed to be changed to similar levels to increase the reliability of their comparison. This was resolved by changing the following; doubling the honey sample addition, and halving the internal standard addition. This improved with all amino acids, peaks yielding at similar heights, except proline. The response for proline is approximately ten times larger than the other amino acids, and so the proline internal standard peak is smaller. This change to proline did not have a large effect on the reliability of the results. Figure 3.5 depicts the changes in the chromatogram for analysis of honey (ID: 78).

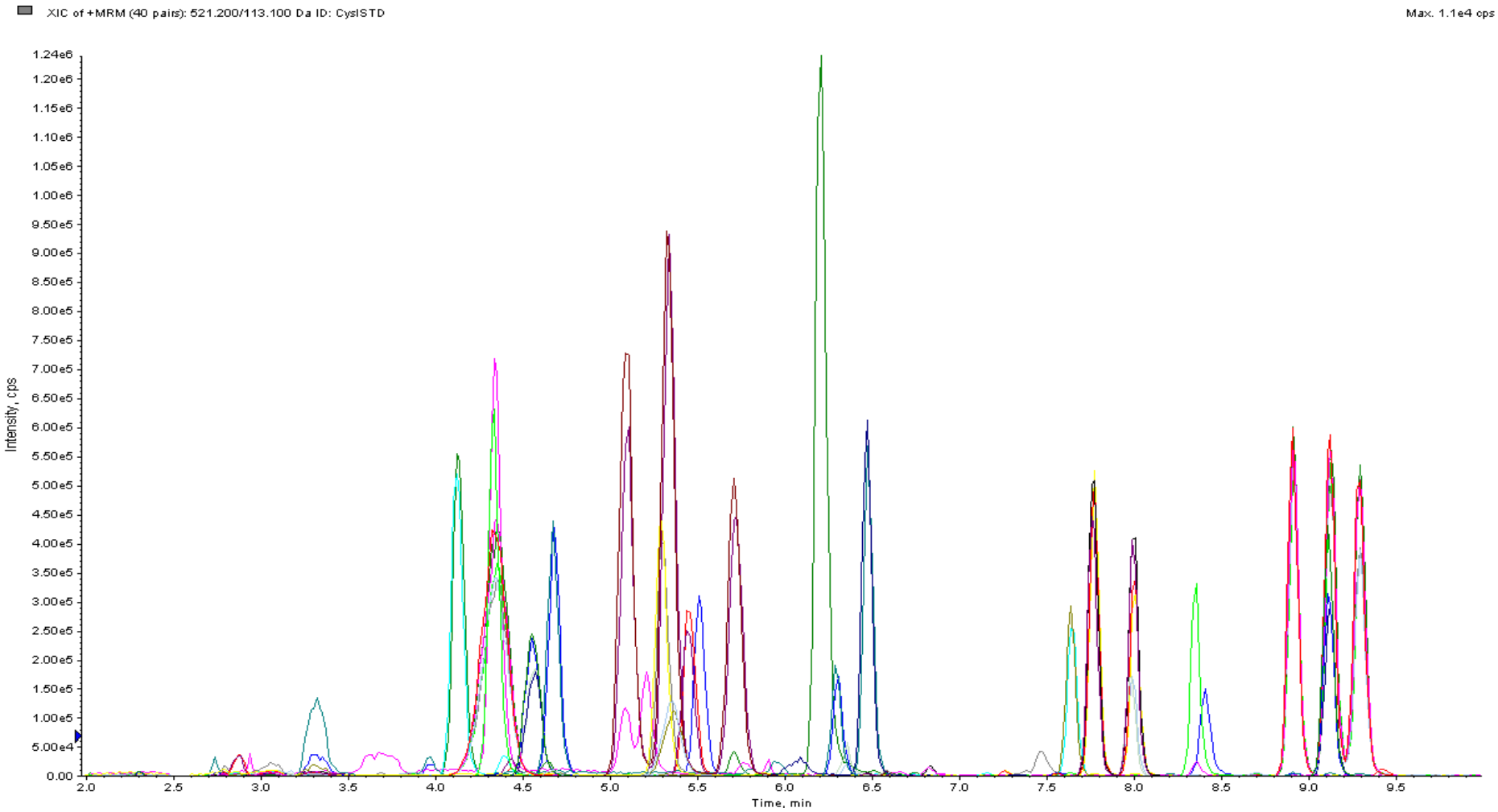


Figure 3.2: First chromatogram of unlabelled standard

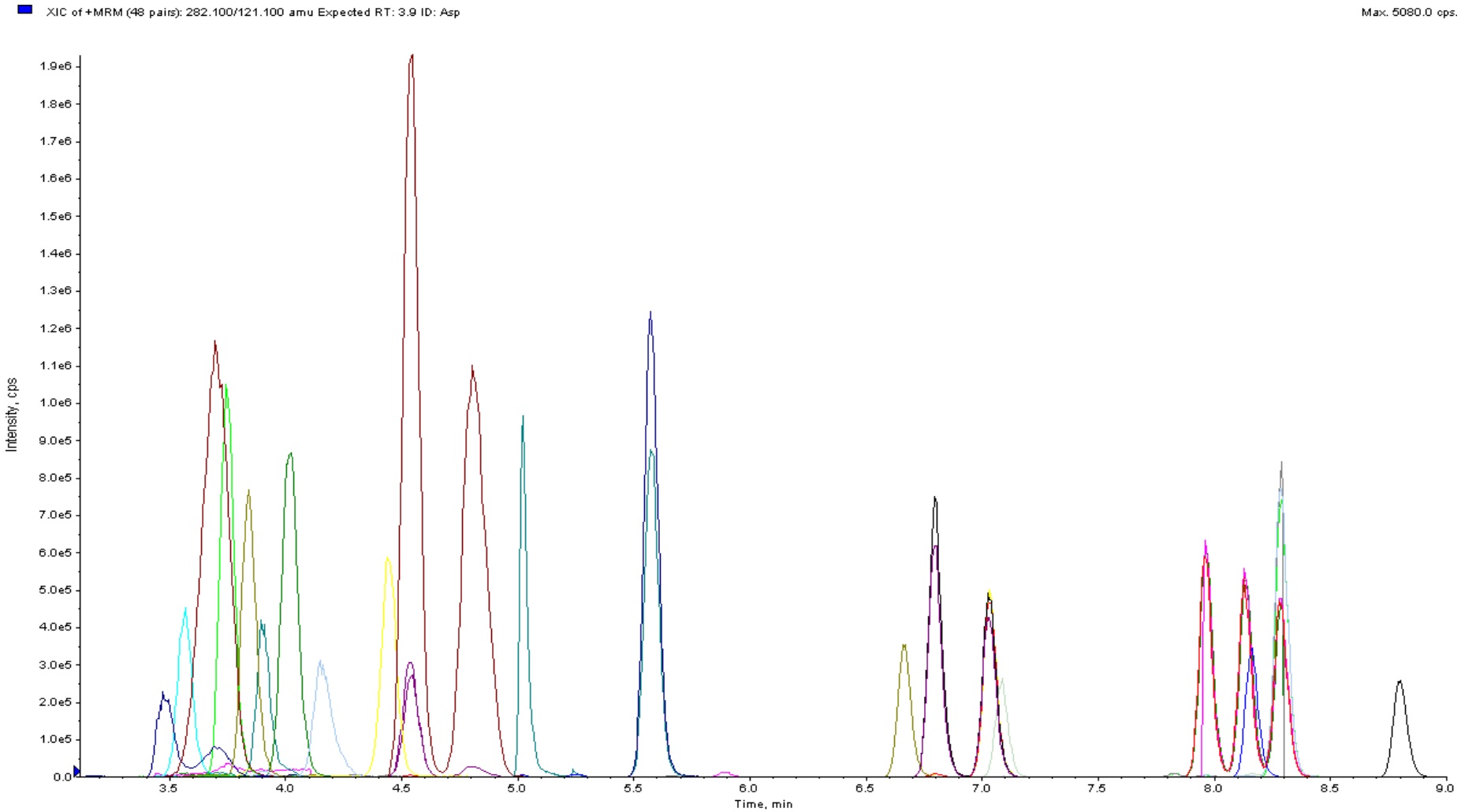


Figure 3.3: Chromatogram of honey (ID: 78)

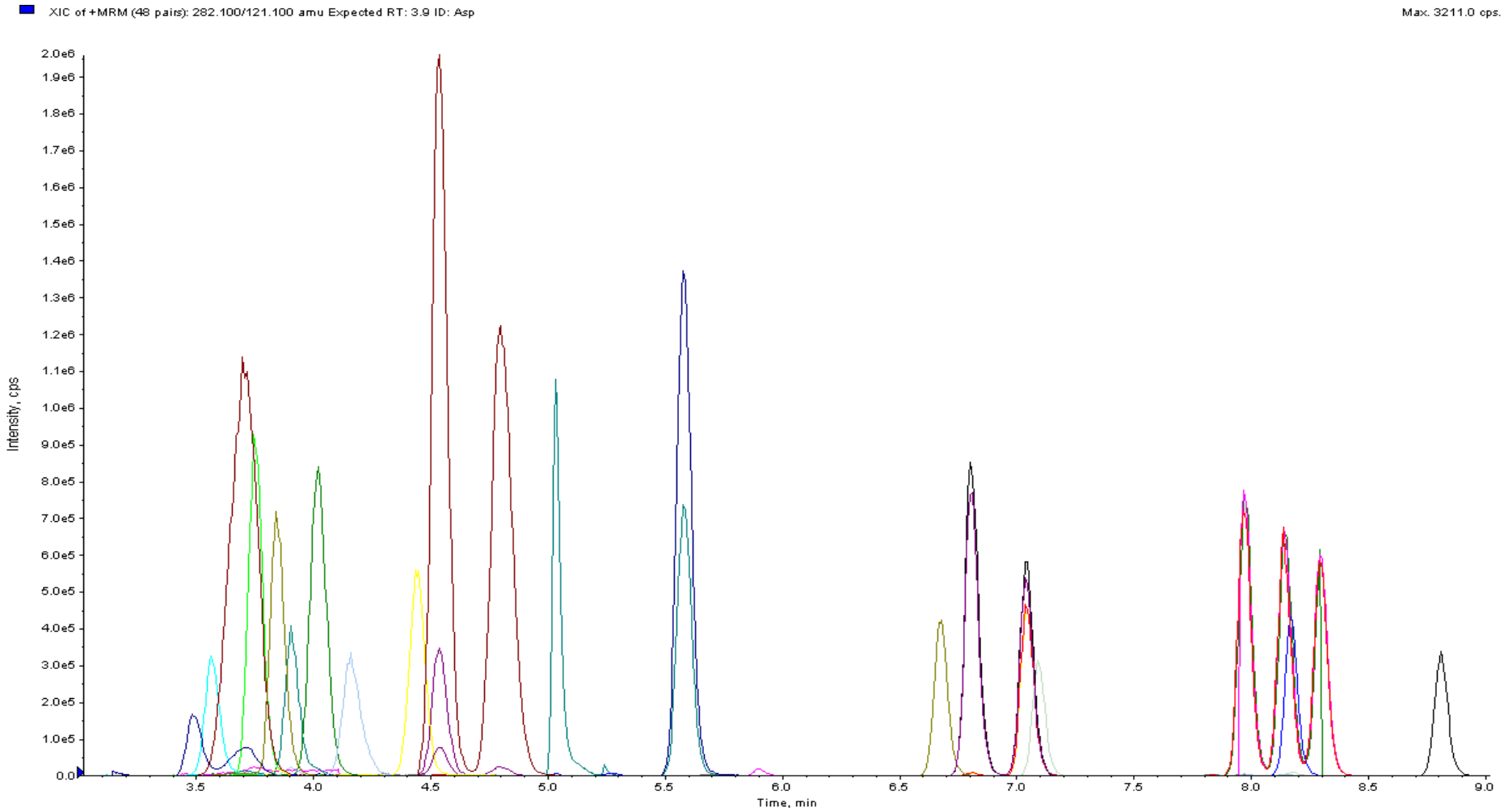


Figure 3.4: Chromatogram of honey (ID: 78) with deletion of sulfosalicyclic acid step

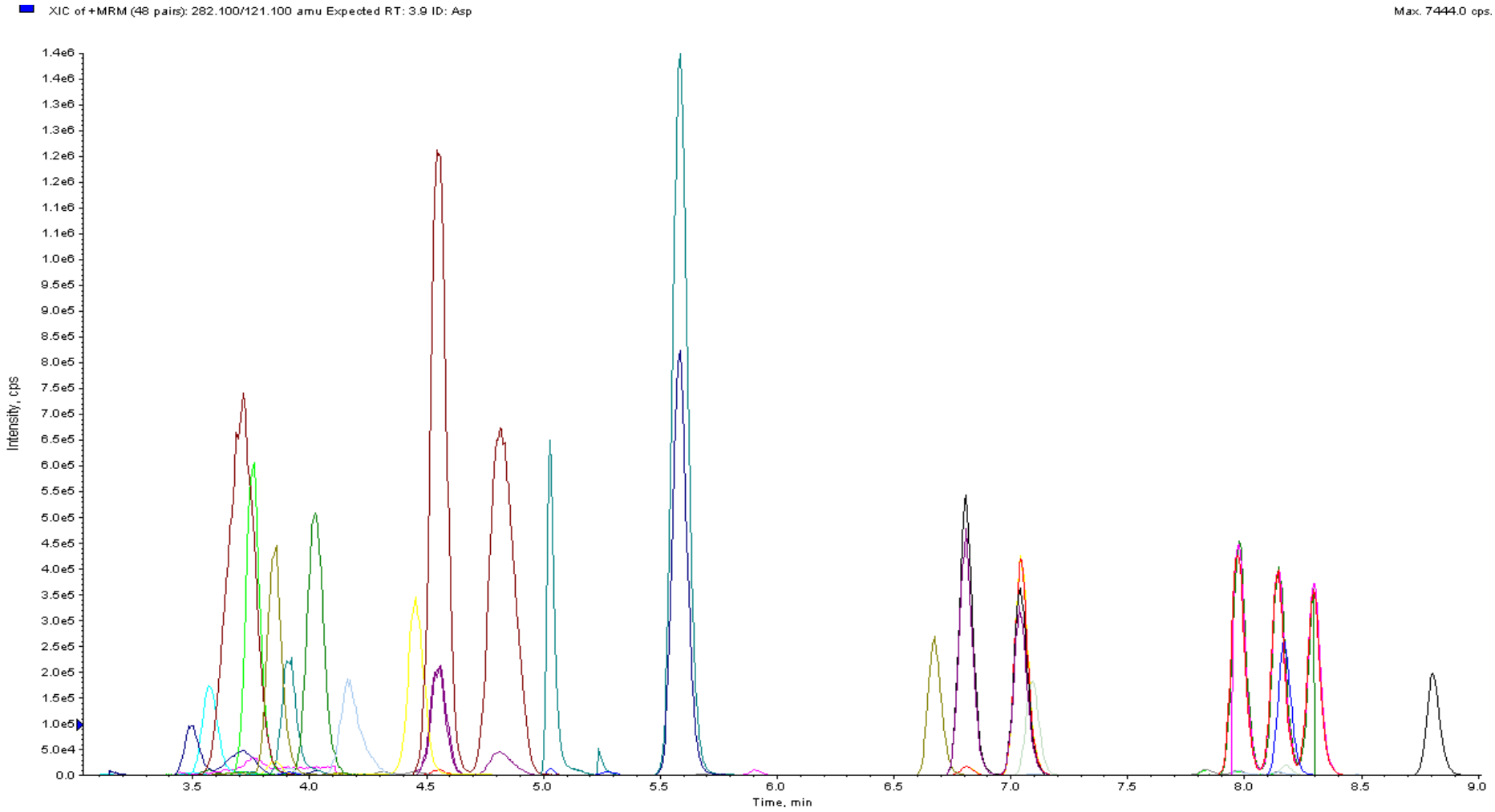


Figure 3.5: Chromatogram of honey (ID: 78) with 2x honey sample, and ½x internal standard

3.2.2 Scheduled MRM

MRM's are screened to evaluate the number of concurrent MRM transitions, in order to improve sensitivity and accuracy. Concurrency is the property of multiple computations occurring simultaneously.

When first developing an acquisition method, MRM values are monitored over the whole chromatogram, and thus a small amount of data points per peak are collected. Upon chromatographic analysis, retention times of the monitored ions are used to produce a scheduled MRM. Scheduling MRM allows large amounts of monitoring to occur without compromising on data quality by decreasing the amount of concurrent MRM transitions. This allocates collection of more data points per peak, producing more accurate quantitation.

Figure 3.6 depicts the final concurrency of the scheduled MRM's; the highest number of which is 16 calculations. Without scheduled MRM, the highest number of calculations would have been 48. This small number of MRM calculations allows for good quality data to be recovered.

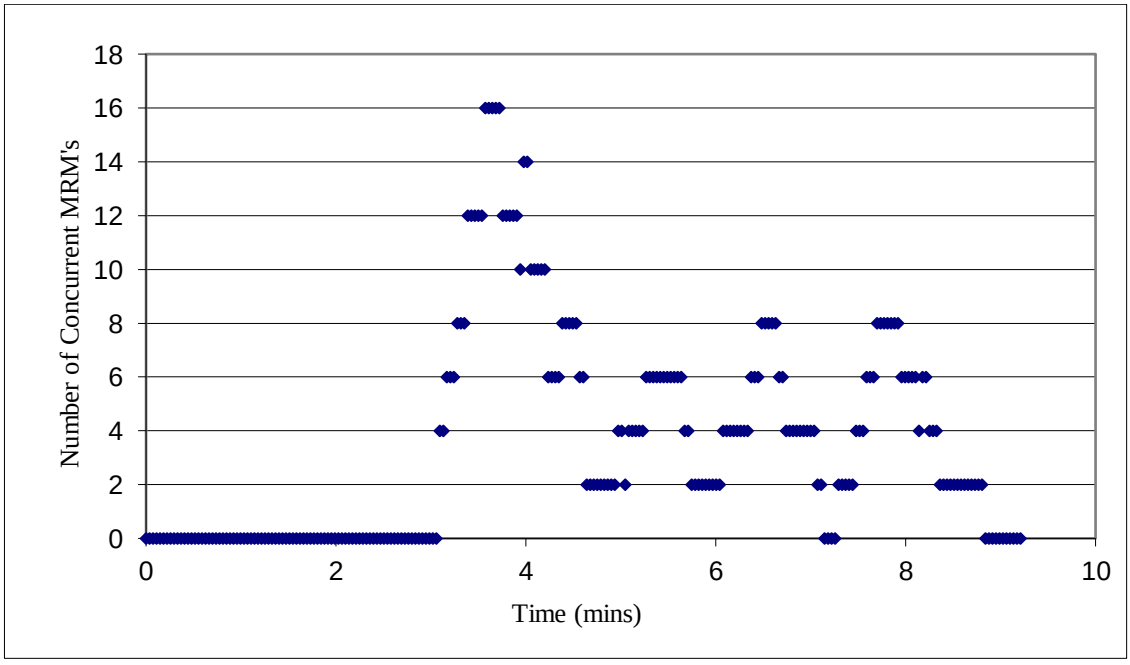


Figure 3.6: Graph depicting concurrency of scheduled MRM

3.3 Validation

3.3.1 Chromatographic Performance

To analyse the precision of the instrument, one sample was injected eight consecutive times and the chromatographic elements analysed. The amino acid content, the retention times, start and end times, and the height and area ratios of derivatised amino acids are compared to the corresponding internal standard.

The data for seven amino acids, illustrating the average value and coefficient of variation (CV) for each category, are in Table 3.3 and Table 3.4. The %CV's for all results are trivial, indicating high precision of the instrument.

Table 3.3: Precision of instrument for seven amino acids

	Amino Acid Content (mg/kg)		Retention Time (min)		Start Time of Peak (min)		End Time of Peak (min)	
	Average	**%CV[2]**	**Average**	**%CV**	**Average**	**%CV**	**Average**	**%CV**
Asp	23.49	2.36%	3.82	0.22%	3.72	0.40%	3.97	0.43%
Glu	17.78	1.99%	4.47	0.27%	4.34	0.44%	4.60	0.24%
Ser	14.61	2.36%	3.51	0.19%	3.40	0.38%	3.67	0.55%
Thr	6.37	3.49%	4.39	0.27%	4.20	0.54%	4.52	0.09%
Tyr	3.18	3.87%	6.97	0.10%	6.89	0.14%	7.06	0.24%
Gly	10.23	2.28%	3.71	0.24%	3.54	0.40%	3.89	0.82%
Pro	166.75	0.84%	5.45	0.19%	5.30	0.24%	5.62	0.08%

Table 3.4: Precision of instrument for seven amino acids continued

	Height Ratio		Area Ratio	
	Average	**%CV**	**Average**	**%CV**
Asp/AspISTD	0.22	4.33%	0.22	2.36%
Glu/GluISTD	0.17	5.02%	0.17	1.99%
Ser/SerISTD	0.18	4.02%	0.18	2.36%
Thr/ThrISTD	0.06	4.05%	0.07	3.49%
Tyr/TyrISTD	0.03	7.05%	0.02	3.87%
Gly/GlyISTD	0.15	1.58%	0.17	2.28%
Pro/ProISTD	1.80	2.24%	1.80	0.84%

[2] The coefficient of variation was calculated by: standard deviation / average x 100

3.3.2 Method Precision

To investigate the precision of the method, two honey samples were analysed in triplicate, once a week, over three weeks. A fully nested Analysis of Variance (ANOVA) was performed to examine the intra- and inter-batch variation of amino acid content (Table 3.5). Intra-batch variation refers to within batch variation, for example sub-sampling and run drift, while inter-batch is error from calibrations.

Table 3.5: Variation of amino acid content: between replicates and between days

Amino Acids	Intra-batch CV%	Inter-batch CV%
Asp	9%	22%
Glu	8%	32%
Ser	43%	22%
Thr	19%	15%
Tyr	12%	94%
Gly	45%	0%
Pro	5%	69%
Ala	11%	71%
Met	15%	21%
Val	12%	38%
Phe	5%	121%
Leu	16%	58%
MOx	7%	49%
His	12%	66%
Lys	6%	76%
Arg	49%	33%
Nva	6%	45%
Ile	13%	67%
Asn	8%	30%
Gln	6%	0%
Trp	18%	53%
HydPro	4%	107%

Phe and HydPro have the largest inter-batch variation, over 100%, while Arg has the largest intra-batch variation at 49%. The large inter-batch variation likely results from sample derivatisation and addition of the internal standard. Ways to improve this is discussed in section 3.3.6. This data shows that the output of amino acid content is stable over time, allowing samples analysed over different days to be compared.

3.3.3 Limits of Detection (LOD) and Limits of Quantification (LOQ)

The amino acid content of honeys can vary greatly, and the amino acids are often at very low levels. It is very important to define the limit of detection and the limit of quantification of each amino acid, as results can only be reliable if within these limits. These limits are calculated by comparing the signal to noise ratios (S/N) for a given honey; the variation of which is likely to be very small. The limits for each amino acid can be found in Table 3.6.

Table 3.6: The S/N, LOD, and LOQ of amino acids

Amino Acid	S/N[3]	LOD[4] (mg/kg)	LOQ[5] (mg/kg)
Asp	0.035	0.796	2.652
Glu	0.030	0.544	1.813
Ser	0.052	0.821	2.738
Thr	0.068	0.410	1.367
Tyr	0.084	0.290	0.968
Gly	0.061	0.654	2.180
Pro	0.002	0.359	1.197
Ala	0.008	0.114	0.379
Met	0.469	0.392	1.306
Val	0.018	0.101	0.337
Phe	0.063	0.336	1.120
Leu	0.102	0.253	0.843
MOx	0.232	0.406	1.355
Cys	2.770	0.309	1.029
His	0.054	0.302	1.006
Lys	0.168	2.028	6.759
Arg	0.034	0.153	0.510
Nva	0.003	0.298	0.993
Ile	0.087	0.290	0.967
Asn	0.095	0.670	2.232
Gln	0.046	0.729	2.430
Trp	0.477	0.713	2.377
HydPro	0.173	0.197	0.657

[3] S/N was calculated by: noise height x 3/ signal height.
[4] LOD was calculated by: S/N x amino acid content in mg/kg.
[5] LOQ was calculated by: LOD x 10 / 3.

Cystine has a very high signal to noise ratio; it is an extremely small peak on the chromatogram, its results are often within the same level of a blank. Other studies have had similar trouble with analysing cystine, although it is also suspected that cystine is at extremely low levels in honeys. This means that the aTRAQ method cannot accurately analyse cystine in honey; the problem being insolubility or the extremely low concentration.

This data was manually calculated on the MultiQuant software. The software automatically smooths chromatogram peaks, improving the results' precision. The smoothing function was also used in calculating the signal to noise ratio, for consistency.

The noise on the chromatogram originates from the nature of the electrospray; it is often due to background contaminants, impurities in mobile phases and degradation products of the tubing[37].

3.3.4 Carryover

The carryover of the instrument was analysed by running a blank water sample after a standard. The treatment of blanks was prepared using the same methodology as that used for standards and samples. The carryover is represented as a percentage of the amino acid content of the blank compared to the standard, and is in Table 3.7.

Table 3.7: Carryover of amino acids

Amino Acids (mg/kg)			
	Standard	**Blank**	Carryover (%)
Asp	389.55	1.05	0.27%
Glu	605.23	0.21	0.04%
Ser	351.73	3.14	0.88%
Thr	326.88	0.96	0.29%
Tyr	597.91	0.62	0.10%
Gly	334.06	3.37	1.00%
Pro	291.49	0.31	0.11%
Ala	354.56	1.22	0.34%
Met	514.91	0.01	0.00%
Val	391.42	0.45	0.12%
Phe	499.19	0.28	0.06%
Leu	472.28	0.48	0.10%
MOx	414.12	0.01	0.00%
Cys	148.50	0.16	0.11%
His	470.93	0.94	0.20%
Lys	563.46	0.56	0.10%
Arg	603.67	0.56	0.09%
Ile	452.67	0.56	0.12%
Asn	336.04	0.02	0.01%
Gln	179.82	0.12	0.07%
Trp	726.27	0.41	0.06%
HydPro	391.76	0.04	0.01%

The carryovers of all the amino acids are at extremely low levels, the highest of which is glycine at 1.00%. These levels are insignificant and do not affect the results.

3.3.5 Ruggedness

The ruggedness of a method indicates the lack of influence operational and environmental variables have on test results. Interlaboratory comparisons are often used to determine this.

The aTRAQ kit uses prederivatised internal standards which make for very accurate quantitation, compared to calibration with standards that required derivatisation. With the discarded methods tested in Chapter Two, many problems with calibration standards were encountered. Differing solubilities of the amino acids led to problems with dissolving, modifications to the samples to match eluents required for the method. Recovery out of the vial relied on buffer compounds and pH, and in one incident, the use of polypropylene vials over glass. Tailoring the solvents to match the chromatography and the amino acids to avoid loss in vials can also be difficult. Standards must be prepared on a weekly basis, to ensure fresh calibrations. These factors are considered to show that pre-derivatised internal standards make this method more rugged. Unfortunately an interlaboratory comparison was not able to be carried out, in order to show the ruggedness of this method by way of data analysis.

3.3.6 Robustness

Robustness indicates the reliability of the method during its normal usage. It is often measured by making small, deliberate variations to parameters.

Derivatising samples entails pipetting small volumes, with centrifuging between steps. The pipetting has the most room for error, and can affect the robustness of the method. The centrifuging and vortexing improves accuracy, by ensuring all sample is mixed and together. Rinsing techniques were employed to make certain that the entire honey sample was deposited and mixed with the labelling buffer. The same was carried out with the internal standard, another critical step for accuracy. To improve to robustness of this method, utilising robotics for the derivatising steps would remove all technician variation.

Chapter Four: Analysis of Honey Samples

In this chapter, the results from the honey analysis are presented and discussed. The amino acid content of ten honey samples are compared to results generated by Massey University. The effect of storage conditions of seven honeys on amino acid content is evaluated.

4.1 Comparison to Massey Data

Massey University, Nutrition Laboratory analysed 19 free amino acids of ten honey samples by RP HPLC separation using AccQ Tag derivatisation. The AccQ-Tag kit has a similar set up to aTRAQ kit. Separation was carried out on Waters AccQ-Tag ultra (2.1 x 100 mm, 1.7 µm) column. Derivatisation with borate buffer and 6-aminoquinolyl-N-hydroxysuccinimidyl carbarmate (AQC) powder reconstituted with ACN, plus a 100 pmol/µL calibration standard. Solvents are provided in the kit. Detection of derivatised amino acids is by UV[38].

The comparison data (Table 4.1 and Table 4.2) has little differences between methods for amino acids Asp, Pro, Leu, and Arg. Gly shows some problematic differences, some being a factor of ten out from the Massey University values. Honey sample 14.4 comparison (Figure 4.1) shows minor differences between amino acid content results.

Unfortunately no statistical analysis to determine if the results are statistically different can be carried out, due to the lack of information from the Massey University results.

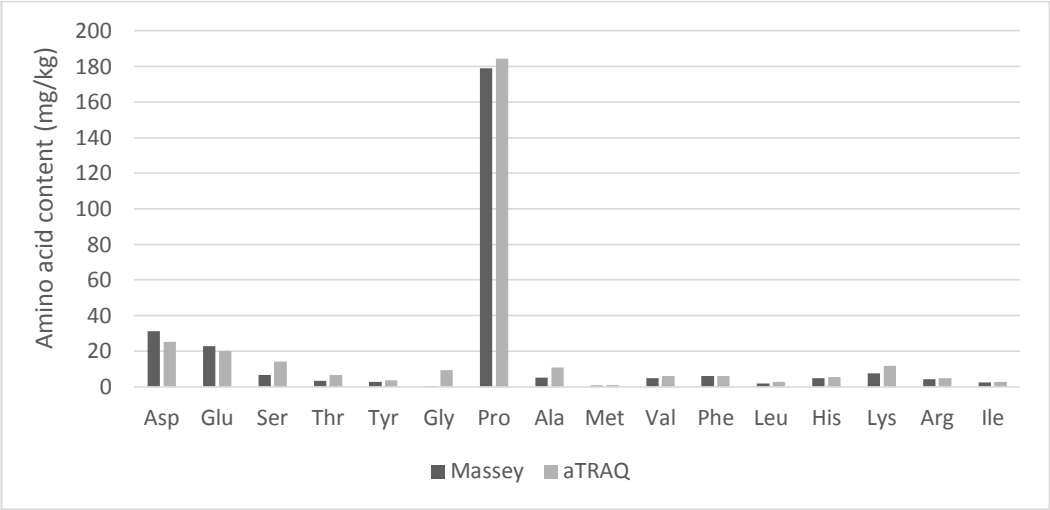


Figure 4.1: Comparison of amino acid content of honey sample 14.4

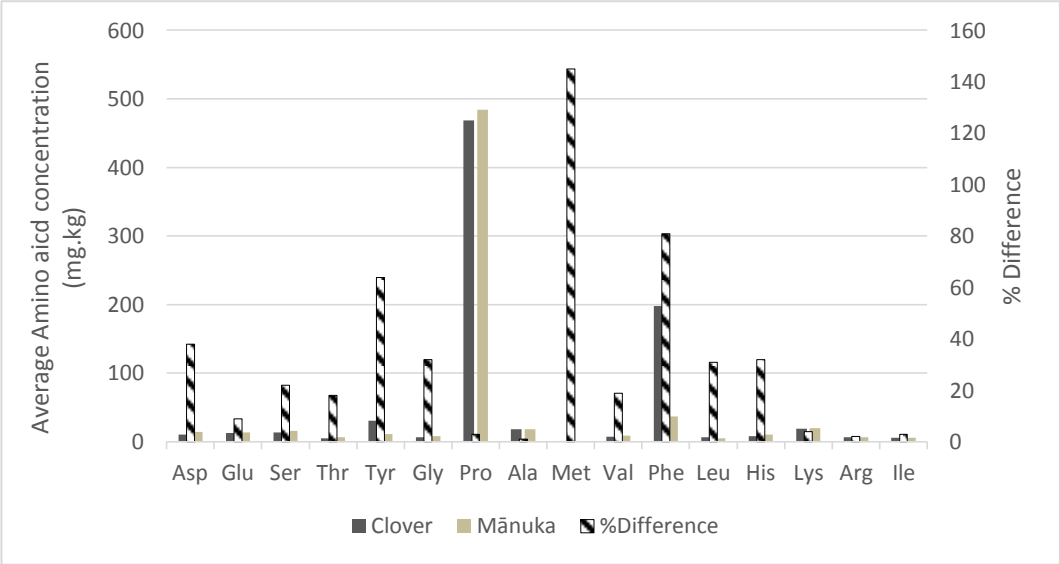


Figure 4.2: Differences of amino acid content between Clover and Mānuka honeys

Figure 4.2 shows the differences in amino acid content are between clover and mānuka honeys. Methionine is at extremely low levels in honey, between 0.02-0.82 mg/kg, but shows the largest difference between clover and mānuka honeys. Phenylalanine had the next largest difference in clover and mānuka honeys with an average of 200 mg/kg in clover and 40 mg/kg in mānuka honeys. Average tyrosine in clover (30 mg/kg) and in mānuka (11 mg/kg) gave a large difference of 64%. Smaller differences were seen with aspartic acid, serine, leucine, and histidine, averaging at 20-30% difference between the two honeys.

Proline was at very similar concentrations in both honeys, 468 mg/kg in clover and 484 mg/kg in mānuka honeys.

Table 4.1: Comparison of amino acid content of honeys, part 1

Amino acid (mg/kg)	Honey ID									
	Happy Bee		Airborne		Hollands		Katikati		14.4	
	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ
Asp	11	9.77	11.4	9.99	9	9.37	11.4	11.29	31.1	25.23
Glu	14.5	12	15.2	12.08	13.6	12.61	10	8.51	22.9	20.15
Ser	8.4	15.06	11.8	12.82	13.9	19.69	9.2	14.53	6.6	14.13
Thr	1.8	6.26	3.6	7.64	3.8	7.15	3	7.32	3.4	6.55
Tyr	7.5	33.08	12.6	8.95	65.4	57.13	33.9	24.14	2.8	3.53
Gly	2.2	11.07	3.6	8.15	3.4	7.93	3	8.66	0.17	9.25
Pro	328.1	427.09	639.7	521.49	378.5	404.47	551.9	494.89	179	184.51
Ala	7	25.29	10.8	29.4	9.6	28.8	9.7	26.28	5	10.98
Met	0.1	0.11	0.2	0.15	0.4	0.46	0.2	0.02	0.6	0.82
Val	5.1	7.5	9.2	8.39	9	9.49	1.8	7.75	4.9	6.12
Phe	27.1	174.35	47.8	37.76	480.8	569.75	135.7	110.63	5.9	6
Leu	2.7	4.63	5.8	5.11	6.3	8.45	9.6	10.77	1.7	2.87
His	5.7	9.54	9.8	10.12	6.2	8.32	5.7	8.41	4.7	5.43
Lys	9.3	23.59	24.4	31.53	12.3	18.02	14	18.67	7.5	11.72
Arg	5.2	5.49	8.1	5.54	5.3	4.36	9.4	7.13	4.3	4.84
Ile	2.6	4.72	5.6	6.48	4.5	6.84	6.1	6.97	2.3	2.88

Table 4.2: Comparison of amino acid content of honeys, part 2

Amino acid (mg/kg)	Honey ID									
	946		953		66		78		84	
	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ
Asp	20.2	21.23	16.9	18.11	4.6	8.97	5.9	8.28	4.6	7.3
Glu	15.1	14.88	14.9	13.51	9.6	9.81	10.3	11.15	8.8	9.67
Ser	16	21.37	13.8	27.33	10.4	31.01	7.1	16.04	10.9	17.93
Thr	6.9	9.86	6	10.9	2.8	8.57	1.9	4.82	1.9	8.2
Tyr	20.1	17.83	17.5	12.51	13.6	11.7	7.1	6.63	10	9.33
Gly	4.6	11.97	4.5	17.15	2.2	20.21	1.5	9.76	2.4	11.58
Pro	709.3	730.38	674.6	593.4	465.2	421.97	326.4	343.6	656.2	527.51
Ala	20.7	32.78	18.3	25.4	11.6	21.19	7.3	22.85	12.3	29.3
Met	0.5	0.66	0.5	0.73	0.2	0.58	0.2	0.29	0.4	0.55
Val	10.9	12.47	12.2	12.22	7.2	9.57	4.8	6.17	8	9.78
Phe	88.7	89.44	54.3	43.53	42.1	36.88	19.2	18.04	19.6	20.31
Leu	7.2	8.7	5	7.13	3	6.48	2	3.52	2.8	4.94
His	19.9	18.39	18.9	18.59	5.5	11.53	2.4	7.56	3.1	10.68
Lys	23.9	45.27	22.2	33.59	16.1	25.07	6.8	13.56	10.2	21.95
Arg	13.7	10.3	8.9	6.94	5.2	7.29	3.5	5.41	1.2	5.81
Ile	6.6	9.07	7.5	8.89	4.6	7.17	2.8	3.96	4.8	7

4.2 Effect of Honey Storage

To assess the effect of storage conditions on the amino acid content of honey, seven honeys were analysed. Each sample was stored in two different conditions; cold and warm, thus fourteen honey samples in total were analysed. The full storage information is in Table 4.3.

Table 4.3: ID and storage information of honeys tested[6]

Honey ID		Year of origin	Storage since
B0 08E3	Cold	2011	19/9/11
	Warm	2011	19/9/11
B0 14E3	Cold	2011	23/4/12
	Warm	2011	23/4/12
B0 23E3	Cold	2011	21/9/12
	Warm	2011	21/9/12
B0 24E3	Cold	2011	21/9/12
	Warm	2011	21/9/12
C 463	Cold	2009	Oct 2011
	Warm	2009	Oct 2011
C 887	Cold	2009	Oct 2011
	Warm	2009	Oct 2011
C 890	Cold	2009	Oct 2011
	Warm	2009	Oct 2011

The amino acid content of the stored honeys is in Table 4.4 and Table 4.5.

It is important to note that the length of time between collection of the fresh honey and storage is from 9 months to two years.

[6] Cold samples have been kept in the refrigerator (5 °C) for 2-4 years, the warm samples stored at room temperature (18 °C) for the same time.

Table 4.4: Amino acid content of stored honeys, part 1

Amino Acids (mg/kg)	Honey ID							
	08E3 Cold	08E3 Warm	890 Cold	890 Warm	14E3 Cold	14E3 Warm	23E3 Cold	23E3 Warm
Asp	6.43	8.15	7.68	6.79	7.45	6.49	10.79	8.40
Glu	2.22	2.79	3.39	2.30	3.51	2.55	4.79	3.17
Ser	8.23	12.64	6.77	8.00	11.88	8.40	19.32	8.29
Thr	2.37	3.14	2.30	2.17	3.12	2.34	6.55	2.71
Tyr	7.30	7.32	7.61	6.63	7.81	6.89	8.13	6.55
Gly	2.96	5.41	2.36	3.12	5.29	3.12	11.88	5.58
Pro	291.78	296.92	289.33	246.24	299.66	291.24	266.95	255.70
Ala	15.45	17.90	13.73	13.00	16.68	14.84	18.72	14.45
Met	0.29	0.36	0.33	0.23	0.41	0.26	0.35	0.24
Val	5.62	6.19	5.20	4.63	6.28	5.45	6.82	4.74
Phe	11.80	11.59	15.76	12.68	12.71	11.28	15.99	13.78
Leu	2.01	2.51	1.95	1.93	2.55	2.07	3.99	1.96
Mox	1.09	0.98	0.93	0.68	1.07	0.95	1.41	1.15
His	1.76	2.52	1.71	2.14	3.26	2.19	3.88	1.96
Lys	4.58	3.15	4.41	2.99	5.11	3.49	5.30	3.69
Arg	1.45	1.62	1.62	1.40	2.28	1.55	7.60	1.94
Ile	4.04	4.13	3.71	3.15	4.30	4.06	4.76	3.15
Asn	1.13	1.03	1.28	0.82	1.20	1.03	1.88	1.04
Gln	3.16	2.41	4.63	2.29	3.70	2.39	4.00	2.61
Trp	0.23	0.42	0.17	0.24	0.43	0.29	0.95	0.51
HydPro	2.99	3.36	3.16	2.78	2.95	2.88	2.75	2.45

Table 4.5: Amino acid content of stored honeys, part 2

Amino Acids (mg/kg)	Honey ID					
	24E3 Cold	24E3 Warm	463 Cold	463 Warm	887 Cold	887 Warm
Asp	7.95	7.22	8.01	5.77	8.03	7.56
Glu	3.41	2.81	2.67	2.37	3.48	2.60
Ser	7.48	6.97	15.34	7.59	9.63	12.58
Thr	2.63	2.40	3.97	2.28	3.06	3.39
Tyr	8.92	7.84	8.07	7.54	8.87	7.93
Gly	3.98	3.60	9.32	3.98	5.66	7.57
Pro	282.63	260.88	264.98	267.73	273.89	241.43
Ala	15.63	13.52	17.77	16.49	15.60	16.04
Met	0.33	0.22	0.41	0.16	0.26	0.20
Val	5.61	5.12	6.52	5.75	5.78	5.45
Phe	19.35	17.07	13.06	12.95	18.37	14.28
Leu	2.12	2.02	2.83	2.17	2.45	2.51
Mox	1.04	0.84	1.33	1.40	0.97	0.93
His	1.73	1.63	2.57	2.23	2.39	2.50
Lys	3.83	3.28	4.53	4.05	4.85	3.64
Arg	1.86	1.51	1.55	1.65	1.87	1.66
Ile	4.06	3.63	4.76	4.32	3.97	3.72
Asn	1.33	0.99	1.25	0.99	1.45	1.07
Gln	4.44	3.06	2.87	2.67	4.45	2.25
Trp	0.38	0.16	0.74	0.40	0.52	0.59
HydPro	2.79	2.67	2.94	3.03	2.66	2.43

Statistical analysis was carried out on the amino acid content (mg/kg) of the honeys. The expectation is that some of the amino acids are involved in a reaction in the honey that occurs during warm storage. Thus the hypothesis is that the mean of the (cold-warm) will be positive; that is because the amino acid content of the cold honeys should be greater than the warm. A paired t-test was used where the difference > 0. This test determines whether the means of two dependent groups differ. This is used to compare measurements made on the same item (the honey) under difference conditions (warm and cold storage). It can be said that the cold sample is greater than the warm sample if the p-value is less than alpha overall value, where alpha equals 0.05 if the confidence interval is 95%, and thus the lower bound is positive. A one sided hypothesis test is used because the warm samples cannot have a more amino acid content than the cold.

The following p-values of the t-test for each honey and amino acid are in Figure 4.3.

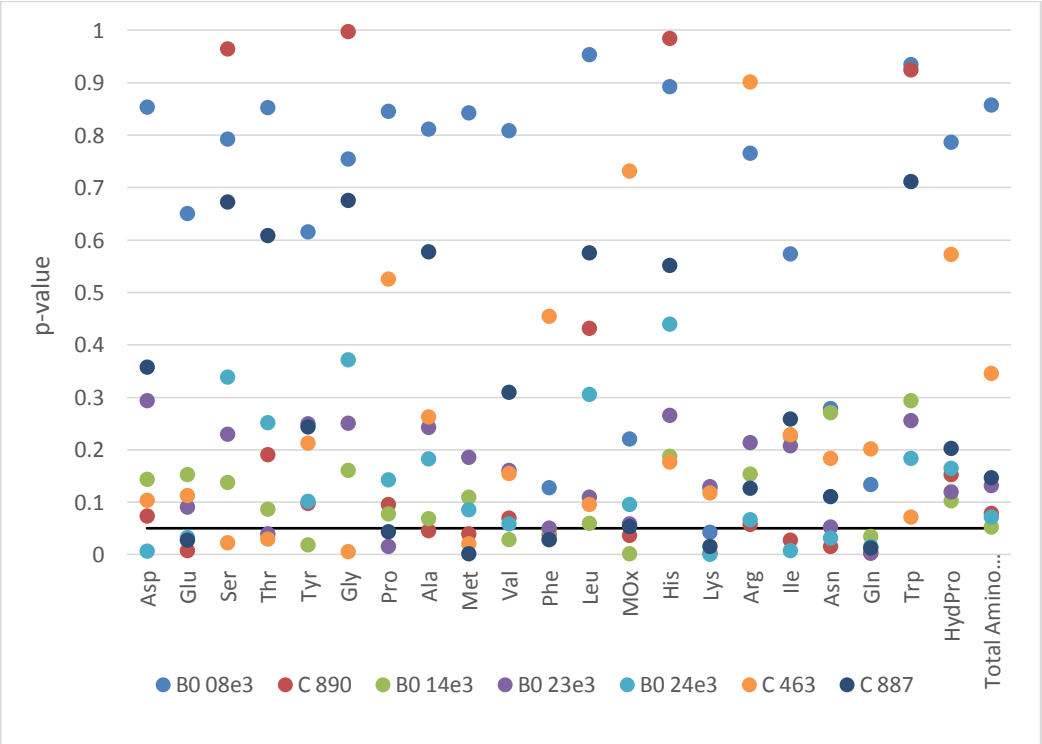


Figure 4.3: P-values of stored honeys

Figure 4.4 shows the statistically significant data points where the p-value < α, and α = 0.05.

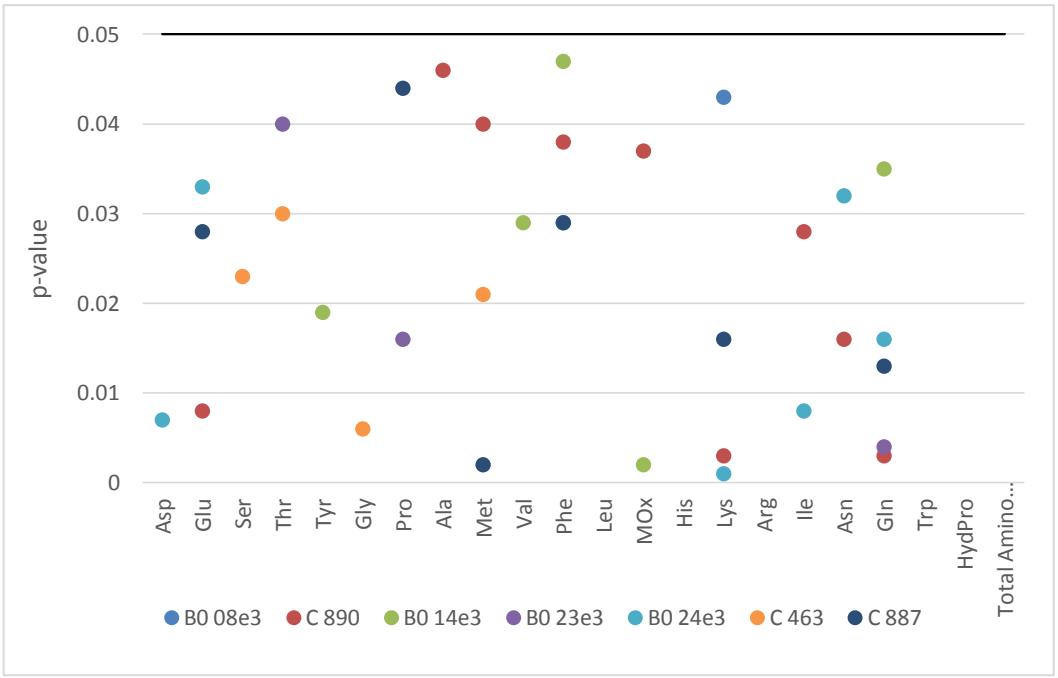


Figure 4.4: Statistically significant p-values of stored honeys

Thirty four data points were considered statistically significant. The total amino acids, Leu, His, Arg, Trp, and HydPro had no statistically significant decrease in warm conditions in any honey sample. Gln had the most statistically significant difference in five honeys; C 890, B0 14e3, B0 023e3, B0 24e3, and C 887.

Lys is in statistically lower concentration (with warm honey storage) in four honey samples: B0 24e3, C 890, C 887, and B0 08e3.
The following amino acids were at statistically significant lower concentration of warm honey samples in three honey samples: Glu, Met, and Phe.
Eleven, over half, of the amino acids were at statistically significant lower concentrations in the warm honey samples for only one or two honeys.

The C 890 honey sample had the most change in amino acids, with nine being at statistically significant lower concentrations in the warm honey samples than cold.

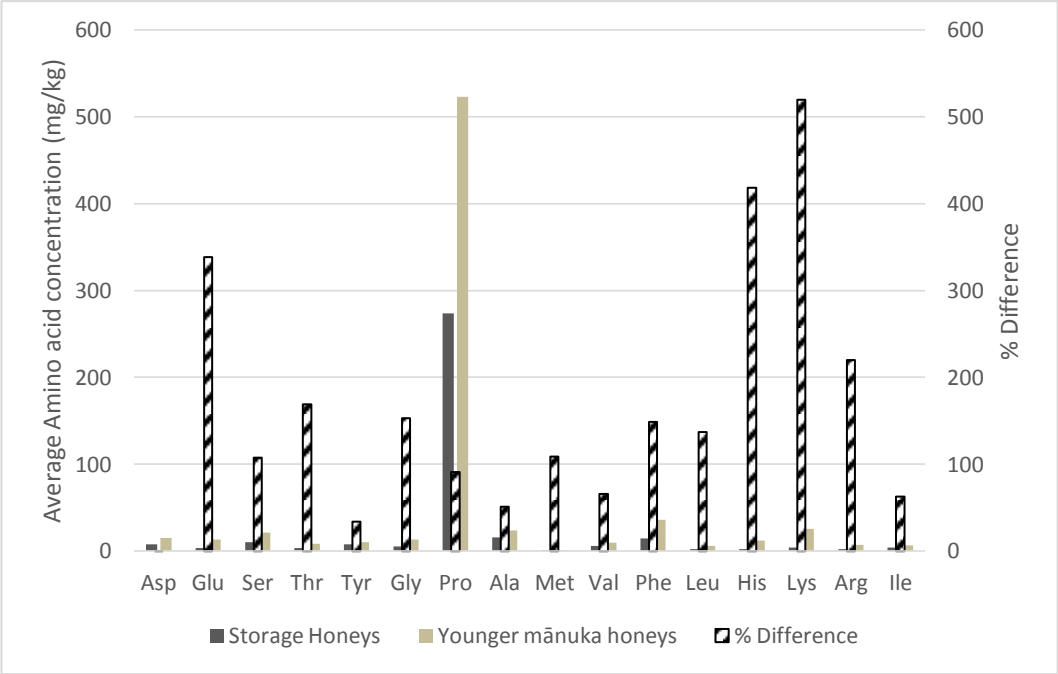


Figure 4.5: Amino acid content of stored mānuka samples vs younger mānuka samples

The results of these stored mānuka honeys were compared to younger mānuka honey samples. The younger samples contained much larger amounts of histidine (2 mg/kg vs 22 mg/kg on average), lysine (4 mg/kg vs 25 mg/kg on average), and glutamic acid (3 mg/kg vs 13 mg/kg on average). Other amino acids at notably higher concentration in younger honeys are serine, threonine, glycine, methionine, phenylalanine, leucine and arginine. Overall, all amino acids are at higher concentration, only aspartic acid has a very small difference of 1%. Proline shows a clear change, with an average of 270 mg/kg in stored honey and 520 mg/kg in younger mānuka honeys. The level of proline for the stored honeys is considered very low, indicating the old age of the honeys. The level of proline for the younger mānuka honeys ranges from 350-750 mg/kg.

In two honeys, B0 23e3 and C 887, proline is at statistically significant lower concentrations with warm storage, but this was expected to occur in more of the honeys. Alanine was at a statistically significant lower concentration with warm storage in only one honey sample, C 890.

Research into the kinetics of the conversion of DHA into MGO has investigated the effect of amino acids on this reaction[39]. Artificial honey with DHA in the presence of amines, both primary and secondary, shows an initial rapid decrease in DHA concentration. It is theorised that the primary amine alanine catalyses the conversion of DHA to MGO, while secondary amine proline has a faster side reaction with DHA. After the initial reaction, a secondary reaction occurs with slow loss of DHA, with respect to the DHA-proline system. The secondary reaction rate for the DHA-alanine system shows no further loss of DHA.

800 mg/kg of proline was added to the artificial honey with DHA, this being a similar level of proline found in fresh honey[39]. The levels of proline found in the storage honeys ranges from 250-300 mg/kg which is very low.

While only three statistical differences in total for proline and alanine occurred, that does not mean that the studied DHA-amine reactions and these observations contradict each other. The stored honeys were in fact up to two years old once they were stored, so the rapid reactions that first occur between the amino acids and DHA had already been exhausted.

Glutamine, changed in the most honeys, has an amide side chain and lysine, changed in the second most honeys, has basic chemistry. The research into the conversion of DHA into MGO showed that the addition of an amide to artificial honey containing DHA, had no effect on the DHA concentration[39].No research into the effect of different side chain chemistry of amino acids on DHA conversion was carried out. Thus it is unlikely that the conversion of DHA to MGO accounts for the amino acids changes in the storage honeys.

Honey is a very complex matrix, containing many components that may or may not interact. It is possible that amino acids, such as glutamine, are involved in small reactions within the honey over time. This could account for the lower concentration of specific amino acids in honey samples stored in warm conditions versus cold conditions.

Chapter Five: Conclusions and Recommendations for Further Work

5.1 Conclusions

Three different methods for the analysis of amino acids in honeys were investigated: HPLC-UV using pre-column derivatisation, HPLC-MS using hydrophobic interaction chromatography, and HPLC-MS using aTRAQ™ derivatisation. The first two methods were ineffective for accurate quantitation of primary and secondary amino acids, but the aTRAQ™ method was successful.

The first method, HPLC-UV, uses OPA-MPA and FMOC as derivatives, detected by UV. The method includes an injection program with fully automated derivatisation and a 19 minute run on a C18 column (100 x 2.1 mm, 2.7 µm), separating only seventeen primary amino acids. The column was initially much smaller (1.8 µm bead size), but blockages initiated a switch to the more robust column. Detection by UV was ultimately not sensitive enough. Amino acids could only accurately quantitated from 5-10 mg/kg (and up) levels while many are present in lower levels (0.1-20 mg/kg average range for twenty primary amino acids). Aspartic acid, proline and hydroxyproline were not detected by this method. For these reasons, this method was discarded.

The second method, HILIC chromatography, gave much better sensitivity. The use of a ZIC-pHILIC column (150 x 2.1 mm, 5 µm) gave high-quality separation of amino acids in 10 minutes. While full resolution was not achieved, scheduled MRM's allowed accurate detection. The buffer systems were important to separation; standards and honey samples were prepared with honey:buffer:acetonitrile (25:100:875) solvents with a formate buffer (500 mM ammonium formate in 0.5% aqueous formic acid) and polypropylene vials. Recovery of amino acids out of vial were varied, spike recoveries showed variance from 2-845%. These inconsistencies likely originated from the sample preparation, with loss of amino acids occurring in vial. No further improvements to the recovery were made and this method was discarded.

The final method, aTRAQ™ kit, labelled amino acids with a Δ8 reagent for analysis and provided Δ0 labelled internal standards for comparison. No calibration curves or response factors were necessary to implicate. Changes were made to sample preparation to better suit honey samples, and validation of the method was carried out. The validation included chromatographic performance, method precision (statistical analysis by ANOVA), limits of detection, limits of quantification, carryover, ruggedness, and robustness. Scheduled MRM of the MS was used to accurately detect forty eight amino acids and internal standards in 18 minutes.

The aTRAQ™ method was used to determine amino acid content of ten honeys analysed by Massey University. The final results were compared and small differences were observed in the majority of amino acids.

The aTRAQ™ method was applied to seven honeys that had been stored for up to two years in both warm and cold conditions. It was hypothesized that the warm honeys would have lower amino acid concentrations than the cold, and with applied statistical analysis by way of a paired t-test, this was found true for; glutamine in five of the honeys; in lysine in four of the honeys; and in Asp, Glu, Ser, Thr, Tyr, Gly, Pro, Ala, Met, Val, Phe, MOx, Ile, Asn, and Trp in one to three of the honeys. Only five amino acids had no decrease in concentration with warm storage.

5.2 Recommendations

Further investigation into more amino acids in honey can be done. The aTRAQ™ kit can analyse forty five amino acids in total, not including the corresponding internal standards. While it is possible that the majority of these excess amino acids are not present, or present in only small quantities, in honey, the analysis can be easily extended.

Application of the aTRAQ™ method to other food stuffs such as wine, jam, fruits and more can be made. Changes to sample preparation for thick or pulpy food stuffs, where particles would interfere with pipetting of the labelling agents, would have to be incurred. These modifications can be made to separate earlier procedures, so the labelling process is unchanged.

Automation of the labelling procedure should be carried out, utilising robotics. This would remove all variation between technicians and improve the robustness of this method for routine analysis.

The results from this method can be applied to many areas of honey evaluation. Investigation into the botanical origin of honey though statistical analysis utilises the amino acid content. The analysis of proline content of honeys can indicate how slow or fast the harvest was. Low quantities of proline is due to rapid honey production[3].

Amino acid content in honey, both natural and artificial, can be used to analyse the effect of amino acids on the conversion of DHA to MGO in mānuka honey.

It is possible for inaccurate labelling of honey can be determined using the amino acid content, in the same way that assigning botanical origin is concluded. Cotte *et al* [3] used PCA of amino acid content of honeys and determined adulteration by the addition of 10-15% of sugar syrups. Sugar syrups do not contain amino acids, thus their addition would decrease the total concentration of amino acids in honey[3].

In conclusion, many applications are possible for the analysis of amino acid content of honey.

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Appendix 1: Injection program

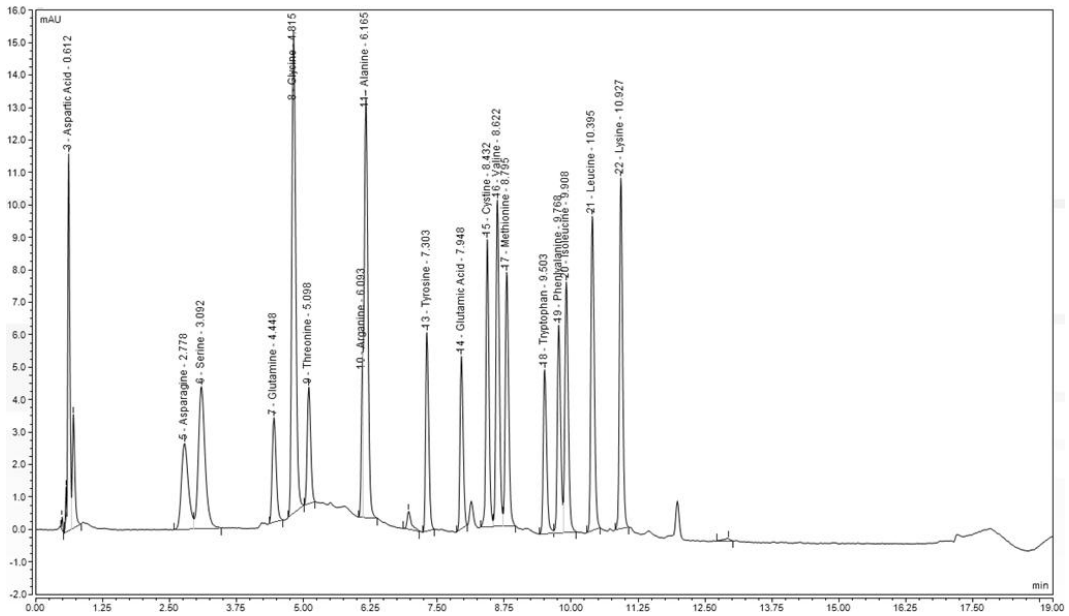
	Command	Parameters
1	UdpInjectValve	Inject
2	UdpSyringeValve	Needle
3	UdpMixNeedleWash	50 [µL]
4	UdpInjectValve	Load
5	UpdDraw	ReagentAVial, 2.5 [µL], GlobalSpeed, GlobalHeight
6	UpdDraw	Air, 0.1 [µL], GlobalSpeed, GlobalHeight
7	UpdDraw	SampleVial, 1 [µL], GlobalSpeed, GlobalHeight
8	UdpMix	ReagentDVial, 3.6 [µL], GlobalSpeed, GlobalHeight, 5
9	UdpMixWait	12 [s]
10	UpdDraw	Air, 0.1 [µL], GlobalSpeed, GlobalHeight
11	UdpMixNeedleWash	50 [µL]
12	UpdDraw	ReagentBVial, 0.5 [µL], GlobalSpeed, GlobalHeight
13	UdpMix	RegentDVial, 4.2 [µL], GlobalSpeed, GlobalHeight, 10
14	UpdDraw	Air, 0.1 [µL], GlobalSpeed, GlobalHeight
15	UdpMixNeedleWash	50 [µL]
16	UpdDraw	ReagentCVial, 0.4 [µL], GlobalSpeed, GlobalHeight
17	UdpMix	ReagentDvial, 4.7 [µL], GlobalSpeed, GlobalHeight, 10
18	UpdDraw	Air, 0.1 [µL], GlobalSpeed, GlobalHeight
19	UdpMixWait	5 [s]
20	UdpInjectValve	Inject
21	UdpInjectMarker	
22	UdpSyringeValve	Waste
23	UdpMoveSyringeHome	GlobalSpeed

Injection program vials.

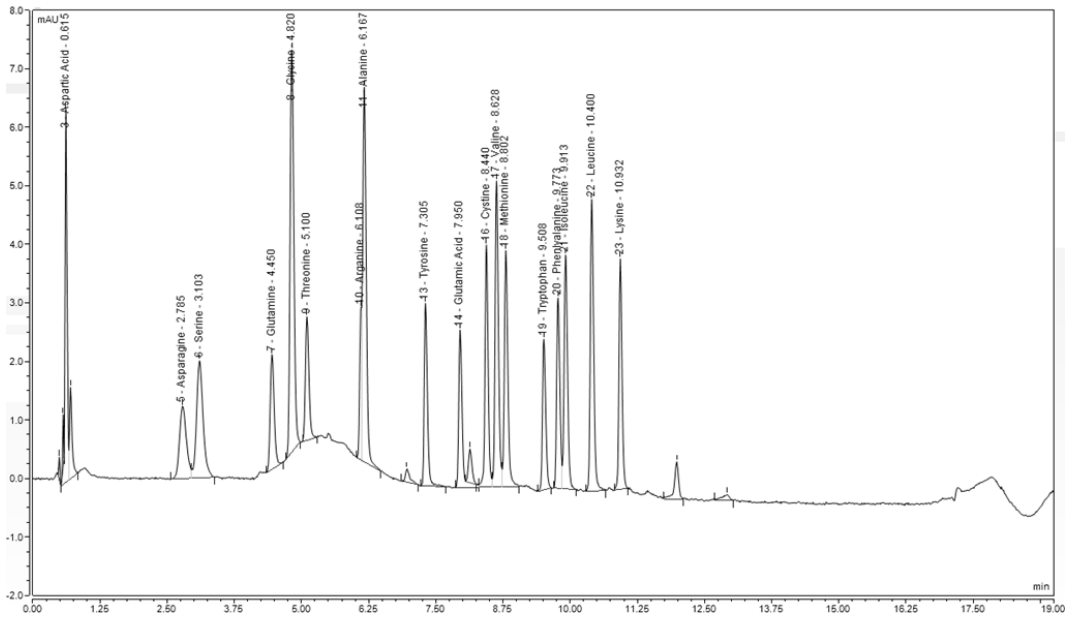
Reagent Vial	Component
A	Borate Buffer
B	OPA-MPA
C	FMOC
D	Empty (mixing vial)

Please note the injection program is specific to Dionex Chromeleon™ 7 software and will have different input depending on the system.

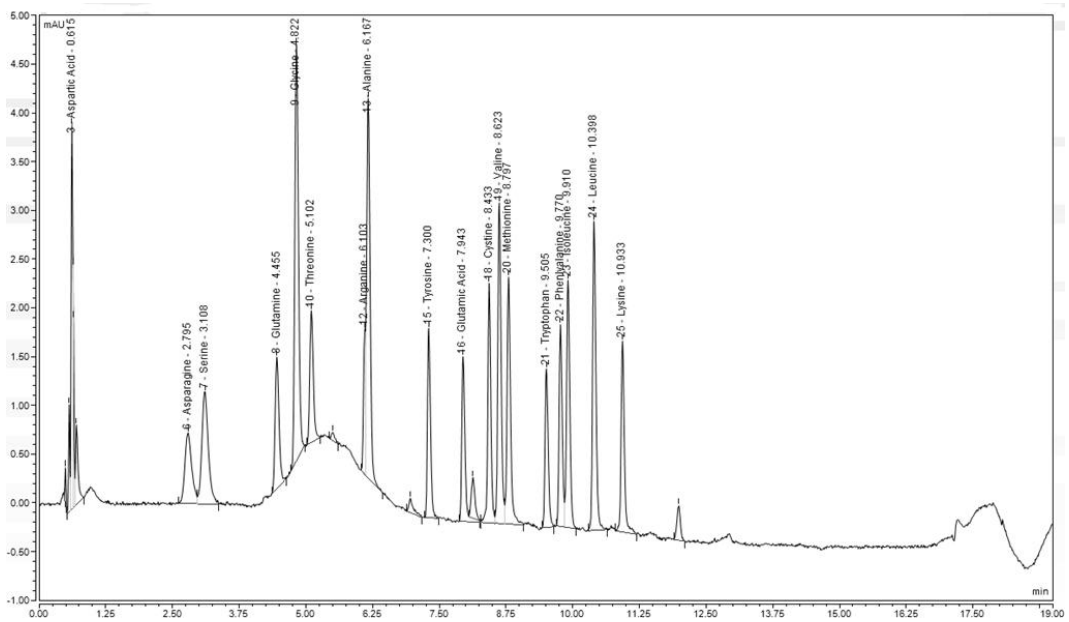
Appendix 2: Chromatograms of Standards for HPLC-UV Method



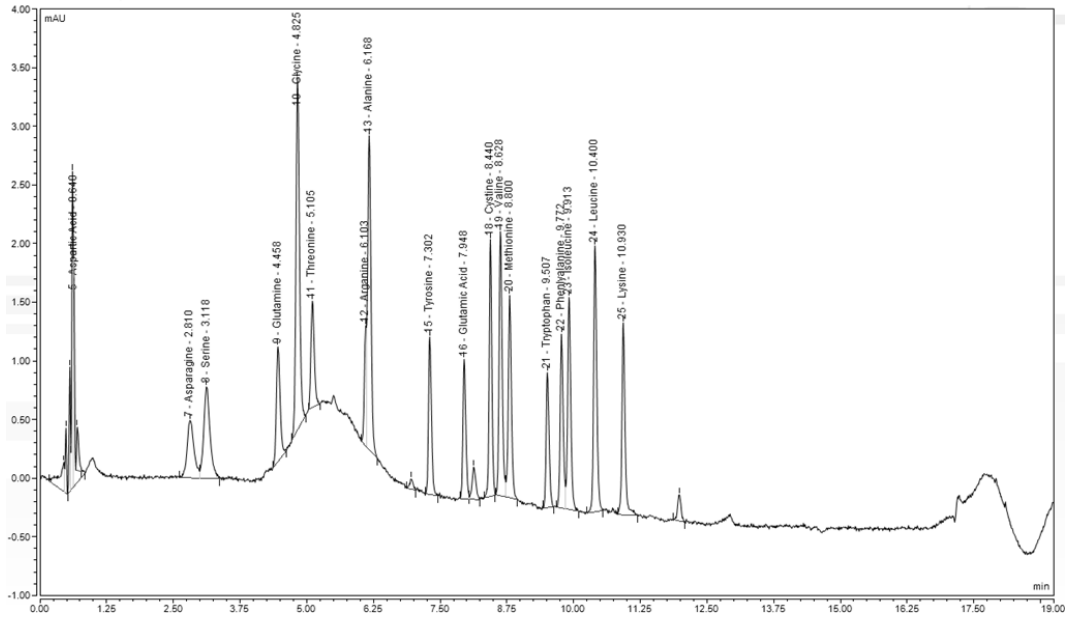
Standard 1: 10 mg/L



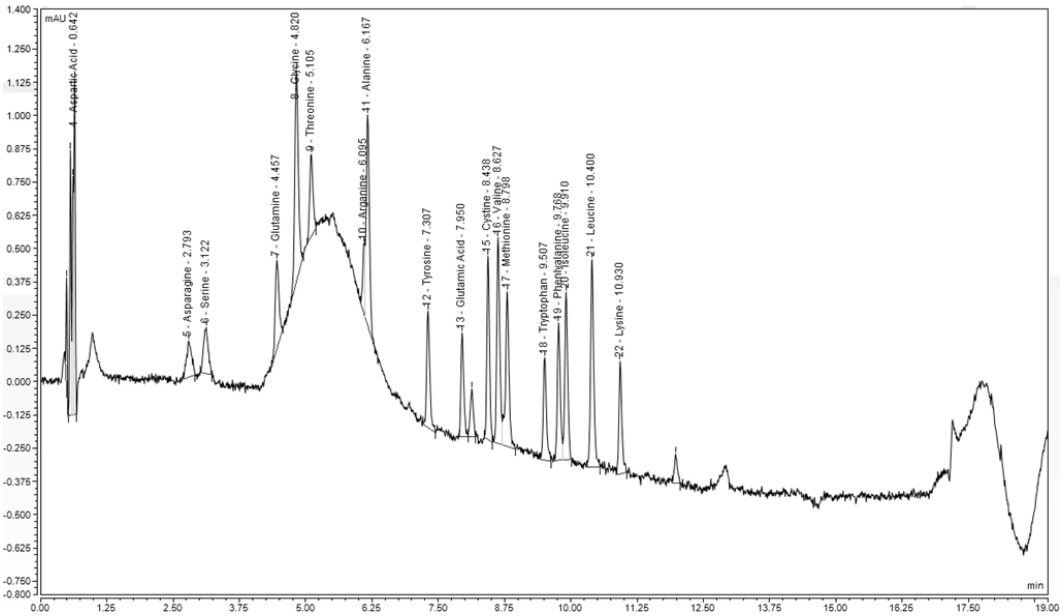
Standard 2: 5 mg/L



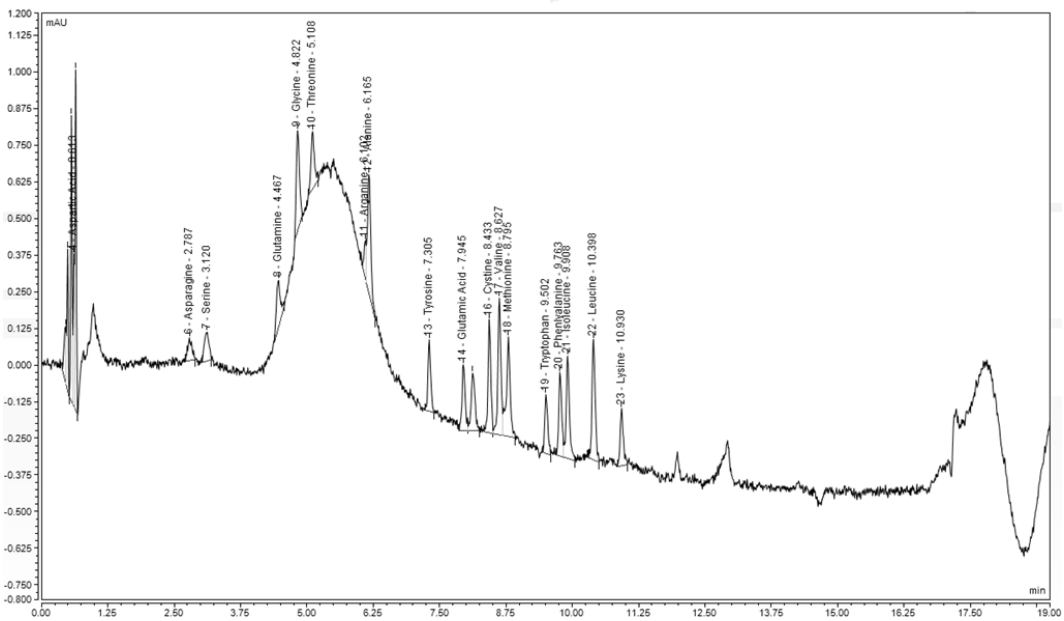
Standard 3: 3 mg/L



Standard 4: 2 mg/L

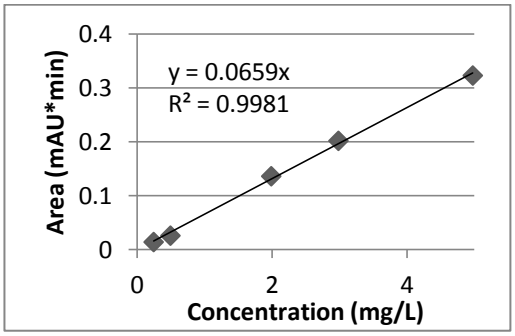


Standard 5: 0.5 mg/L

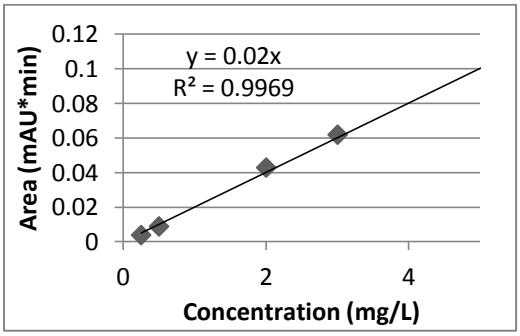


Standard 6: 0.25 mg/L

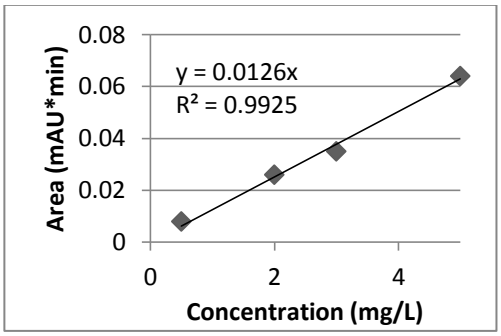
Appendix 3: Calibration Curves for HPLC-UV Method



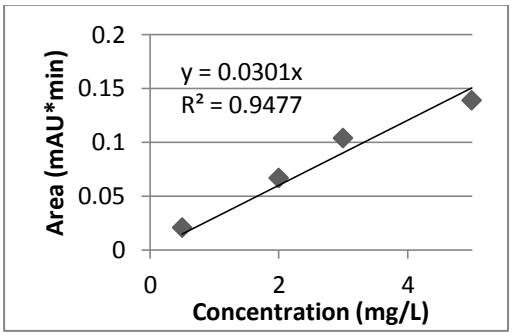
Alanine



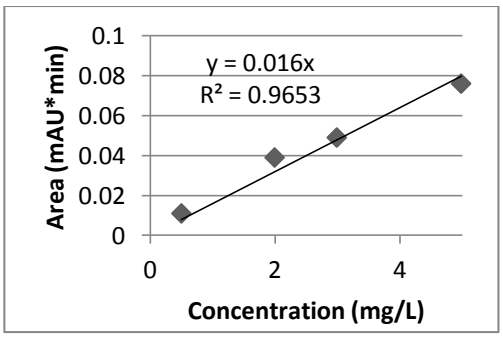
Arginine



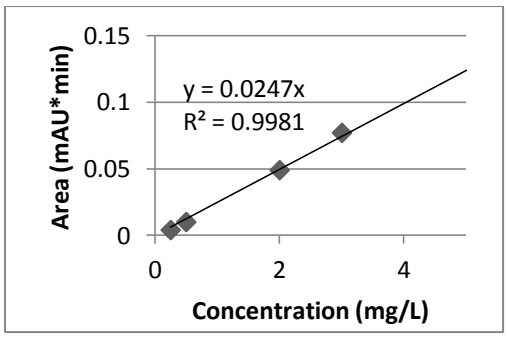
Asparagine



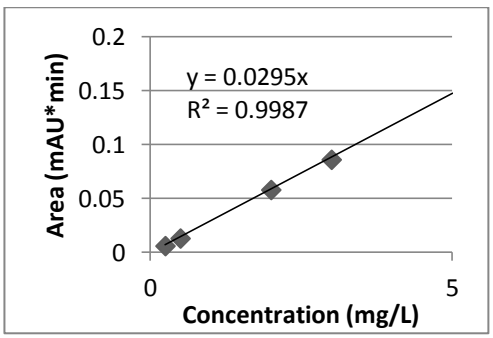
Aspartic acid



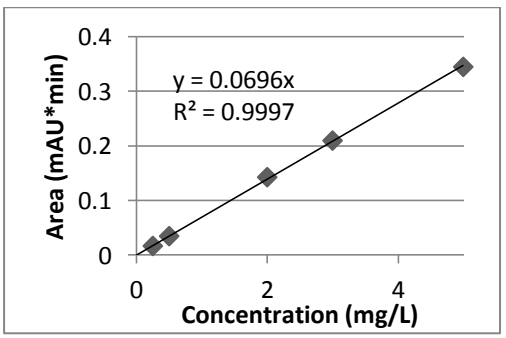
Cystine



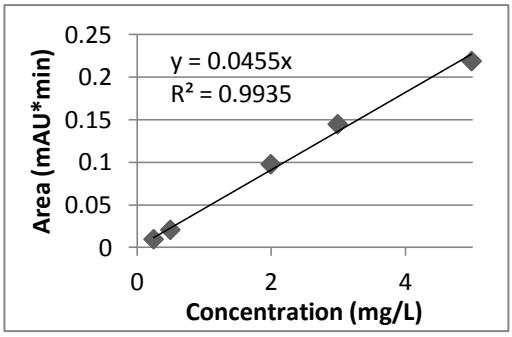
Glutamine



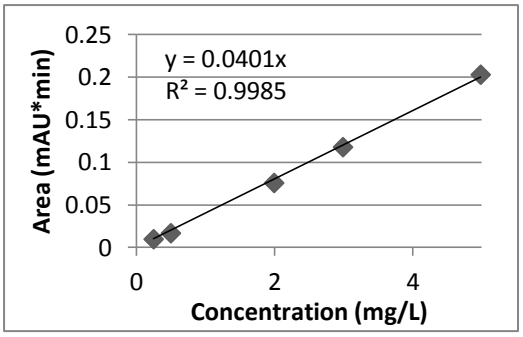
Glutamic acid



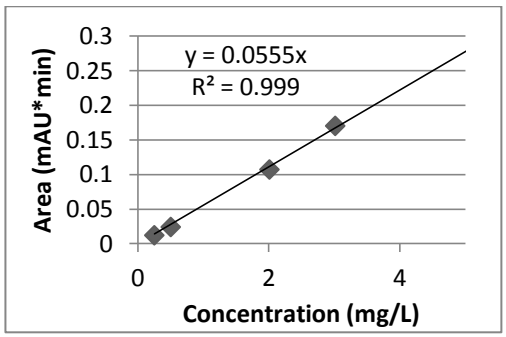
Glycine



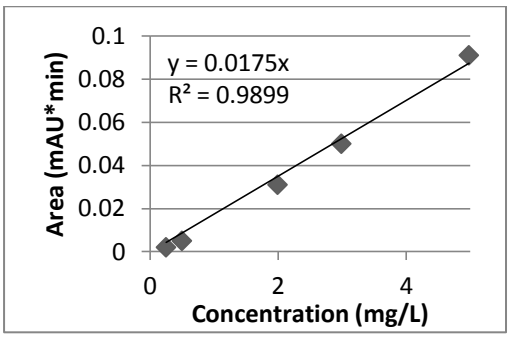
Histidine



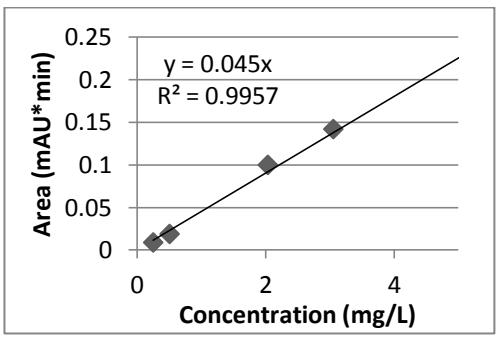
Isoleucine



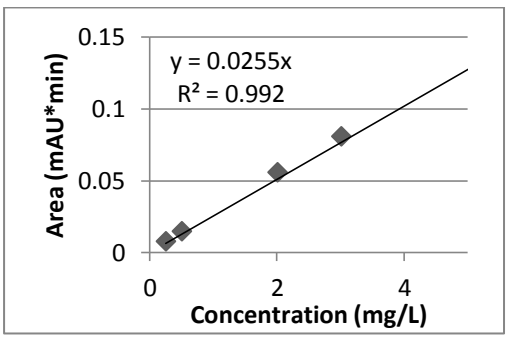
Leucine



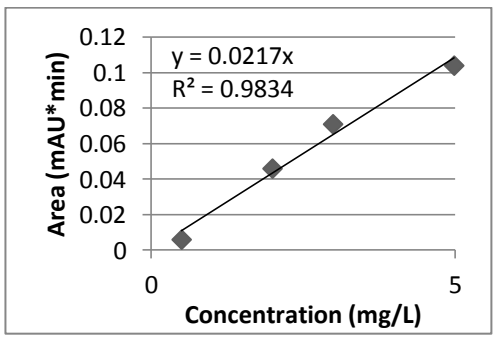
Lysine



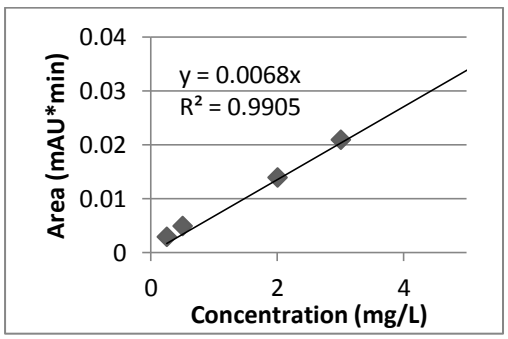
Methionine



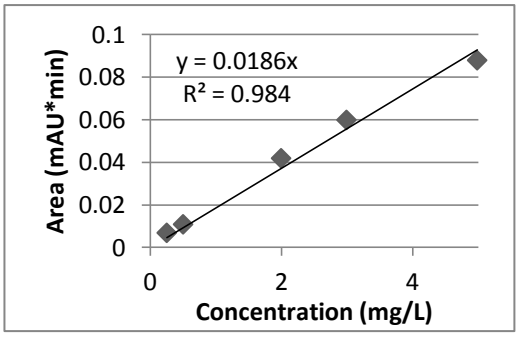
Phenylalanine



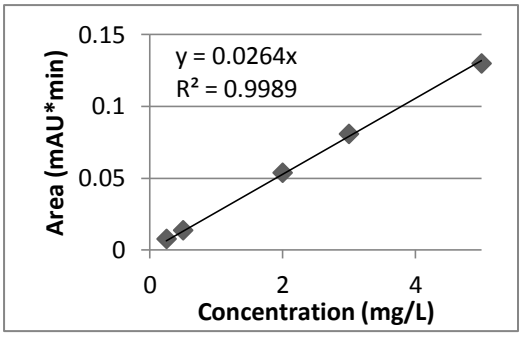
Serine



Threonine

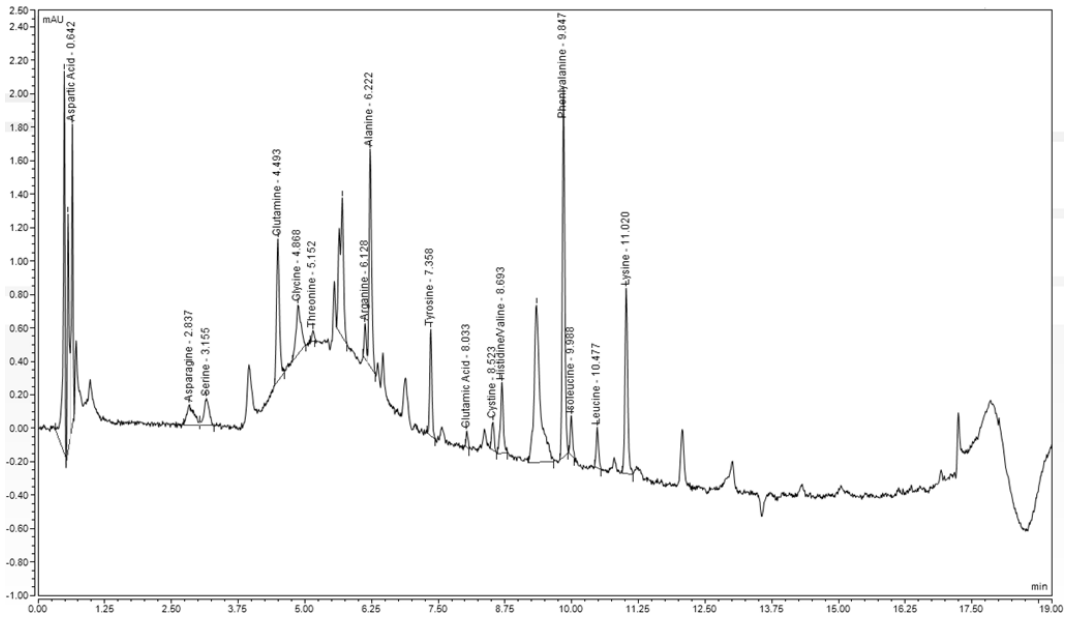


Tryptophan

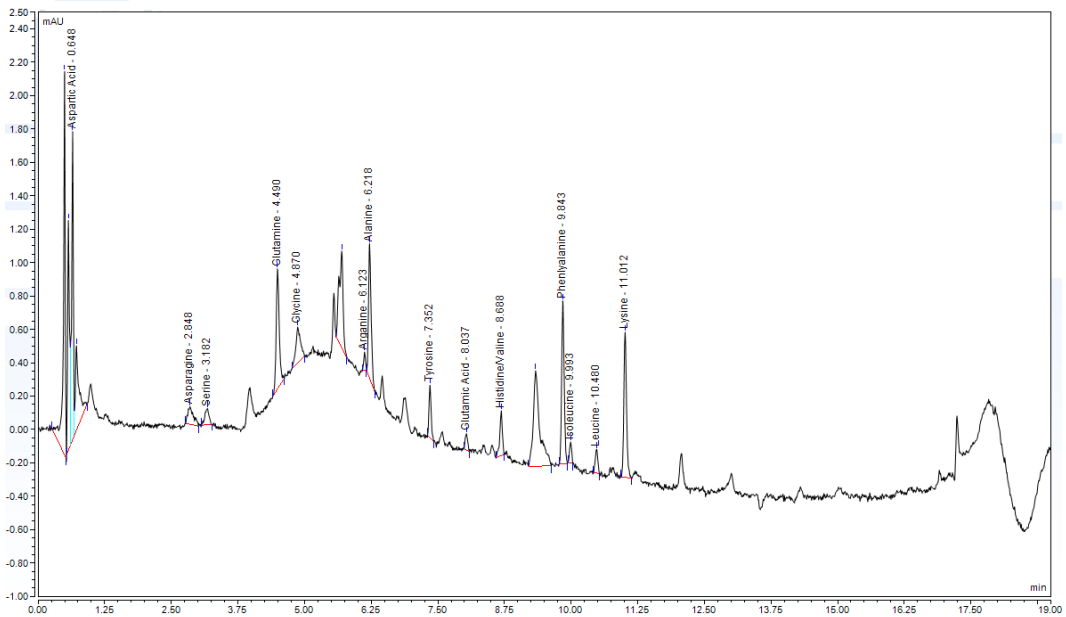


Tyrosine

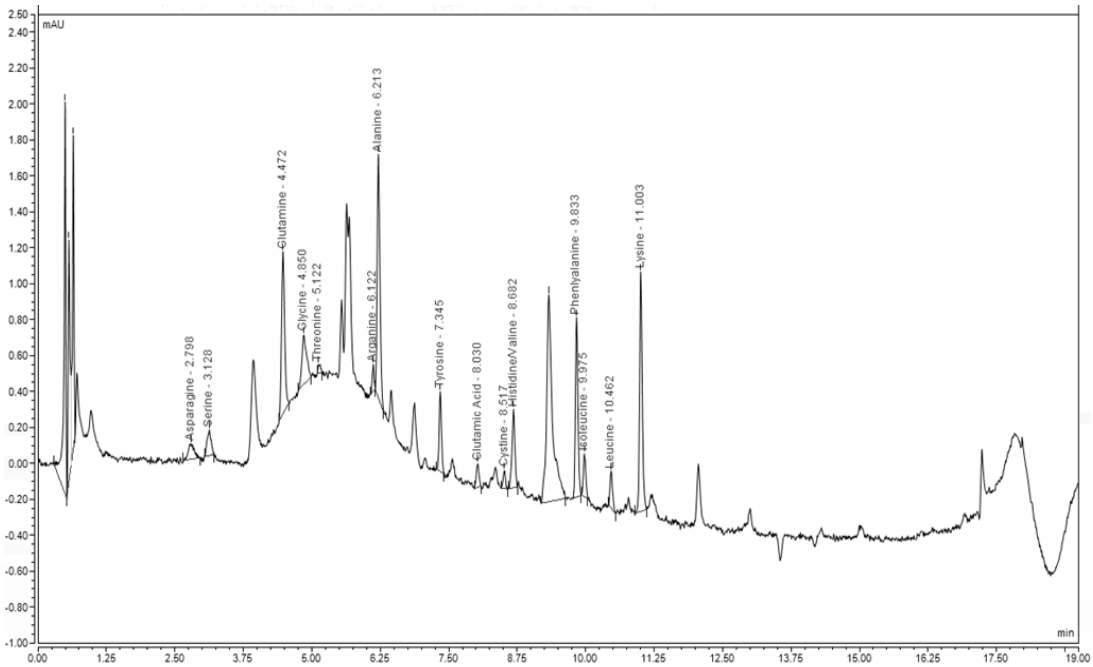
Appendix 4: Chromatograms of Honeys for HPLC-UV Method



Honey ID: 66

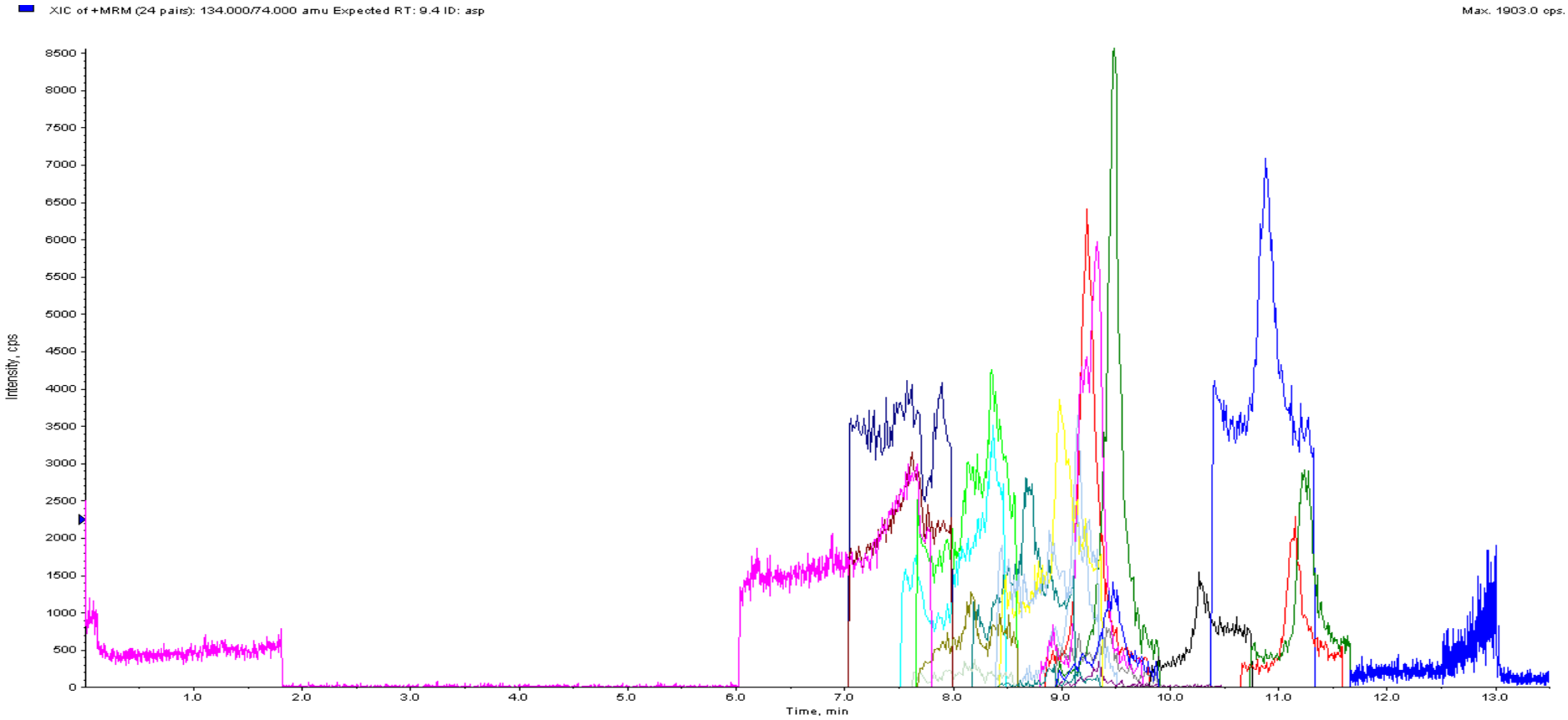


Honey ID: 78

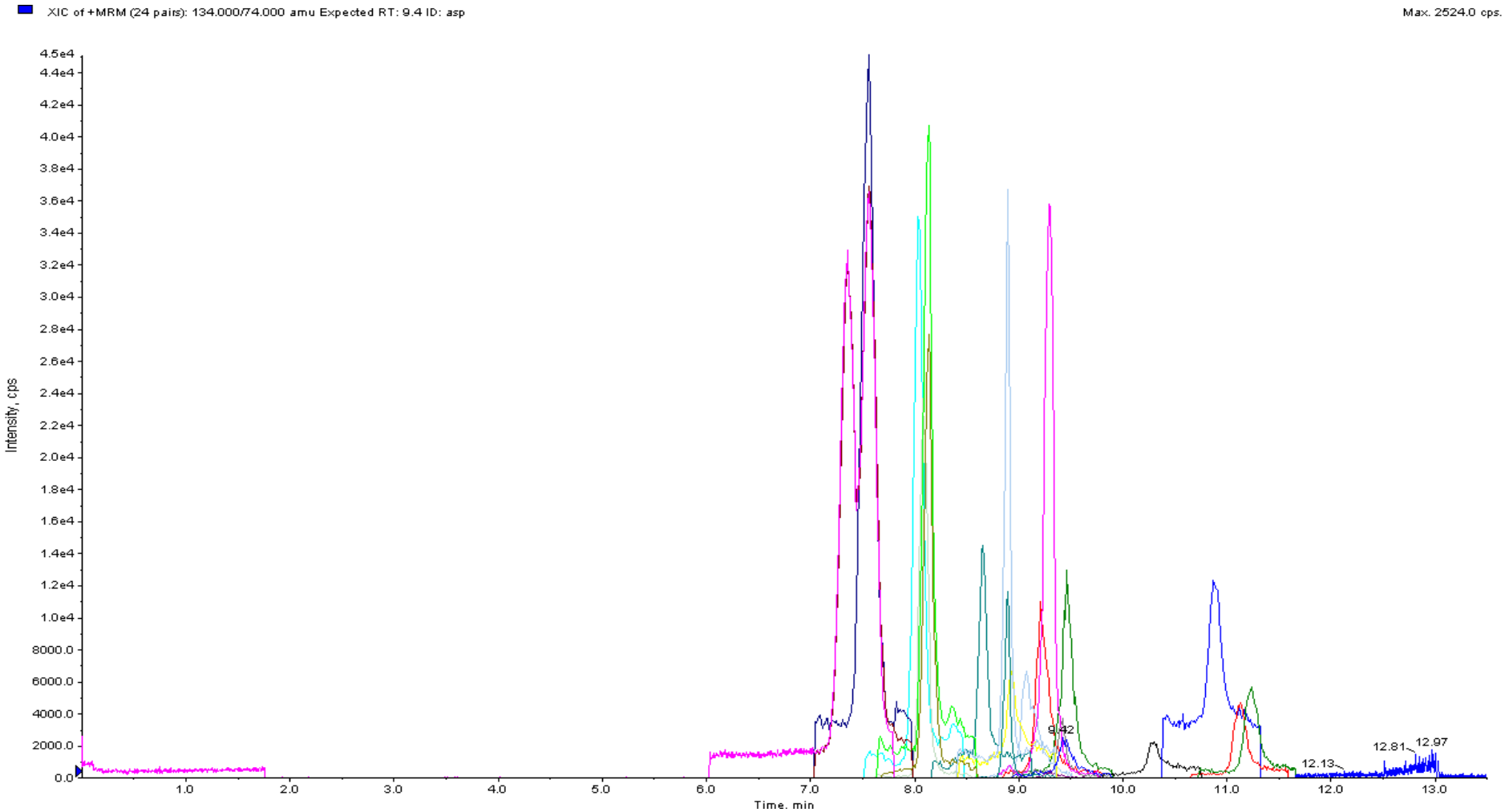

mAU
min
Asparagine - 2.798
Serine - 3.128
Glutamine - 4.472
Glycine - 4.850
Threonine - 5.122
Arginine - 6.122
Alanine - 6.213
Tyrosine - 7.345
Glutamic Acid - 8.030
Cystine - 8.517
Histidine/Valine - 8.682
Phenylalanine - 9.833
Isoleucine - 9.975
Leucine - 10.462
Lysine - 11.003

Honey ID: 84

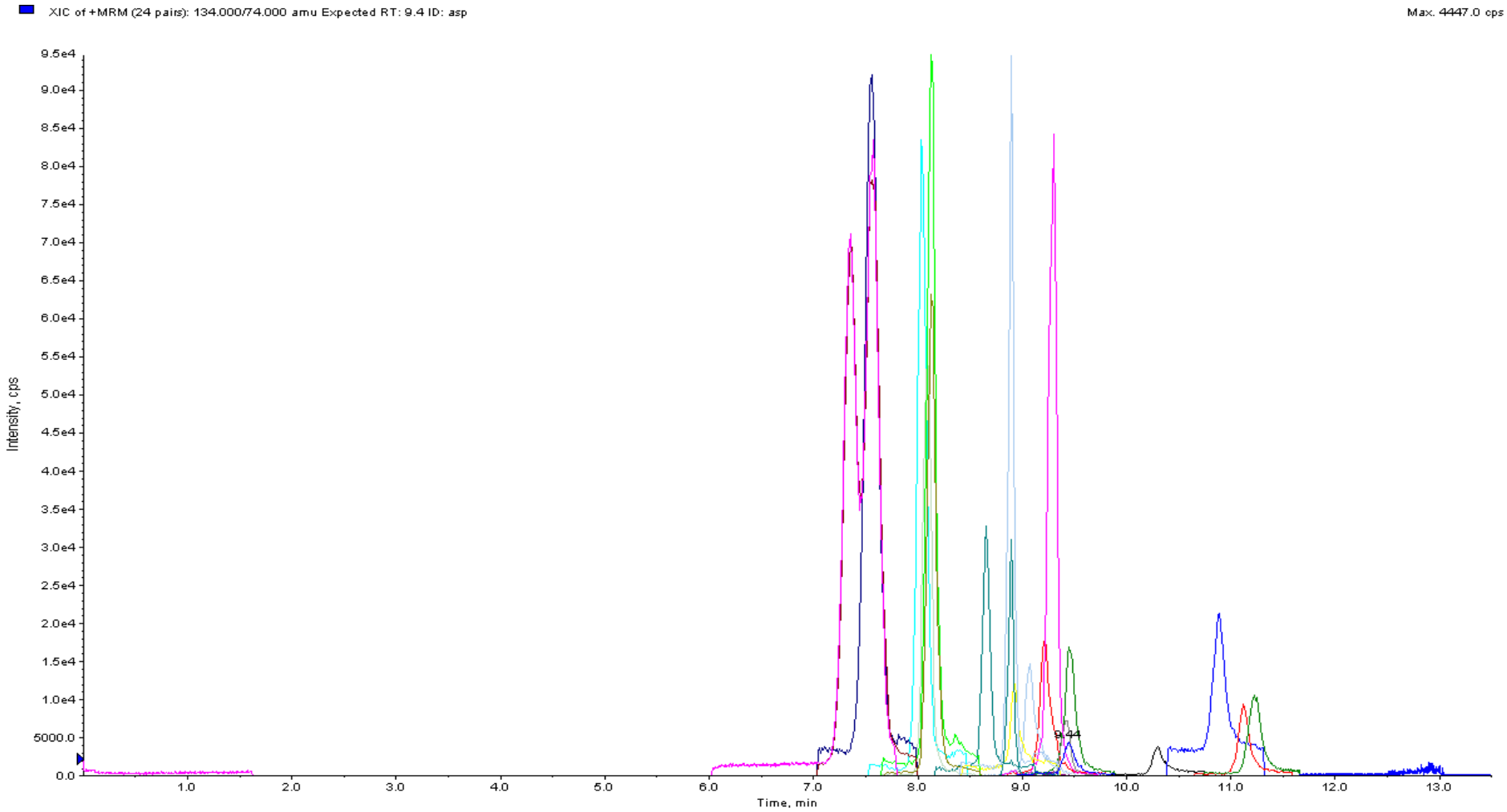
Appendix 5: Chromatograms of Standards for HILIC Method



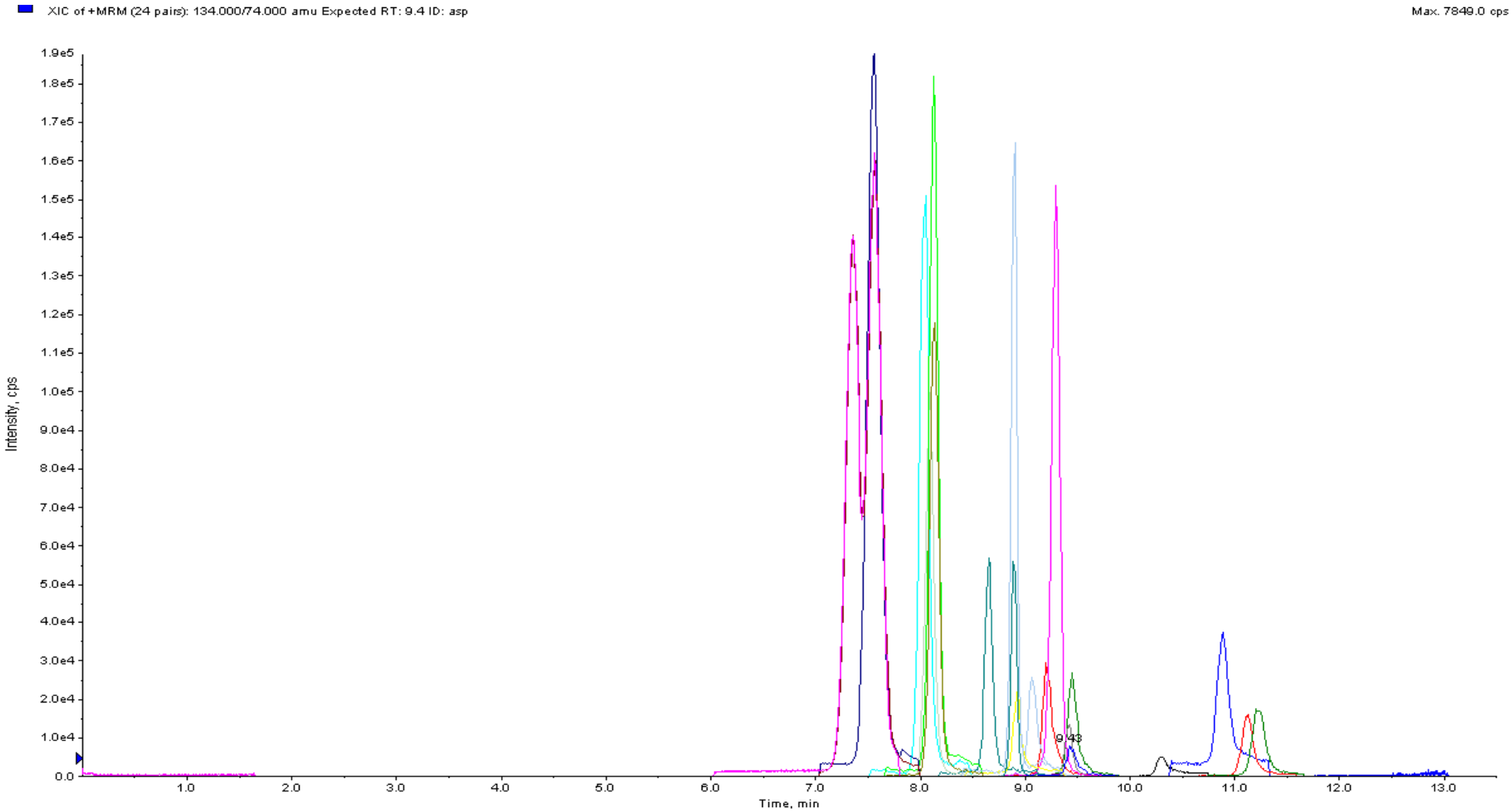
Standard 1: 0 ppm



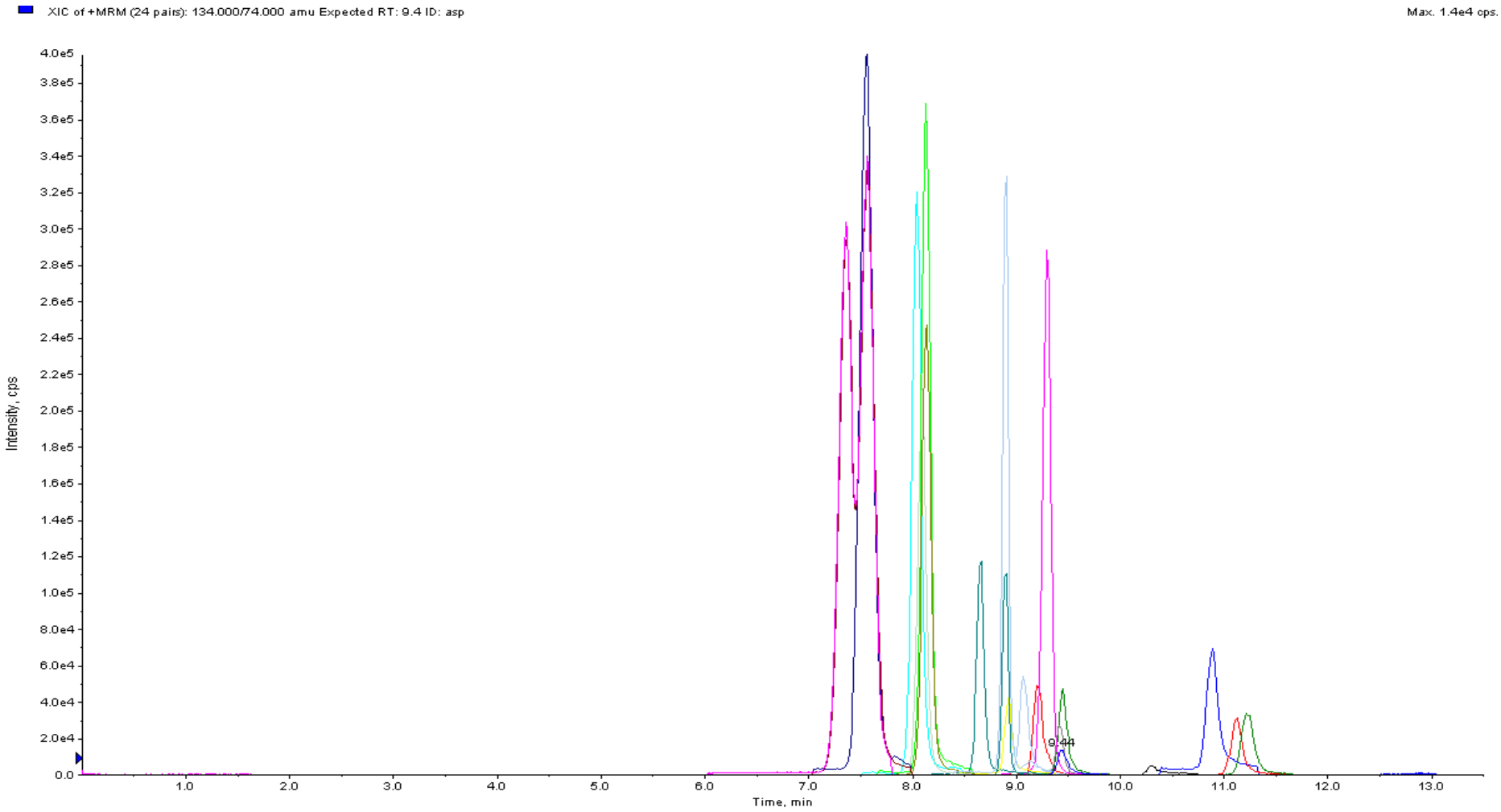
Standard 2: 0.01 ppm



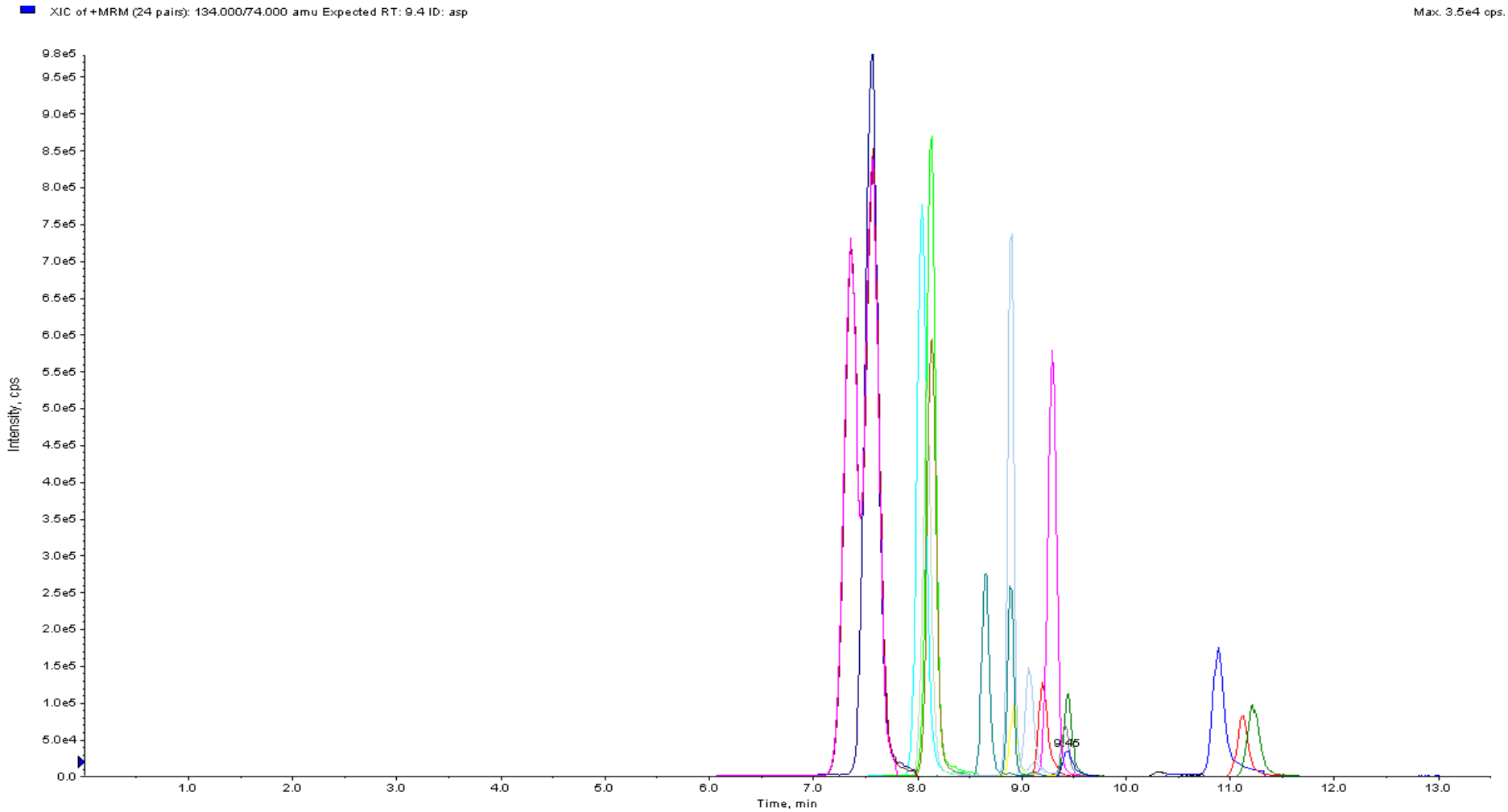
Standard 3: 0.025 ppm



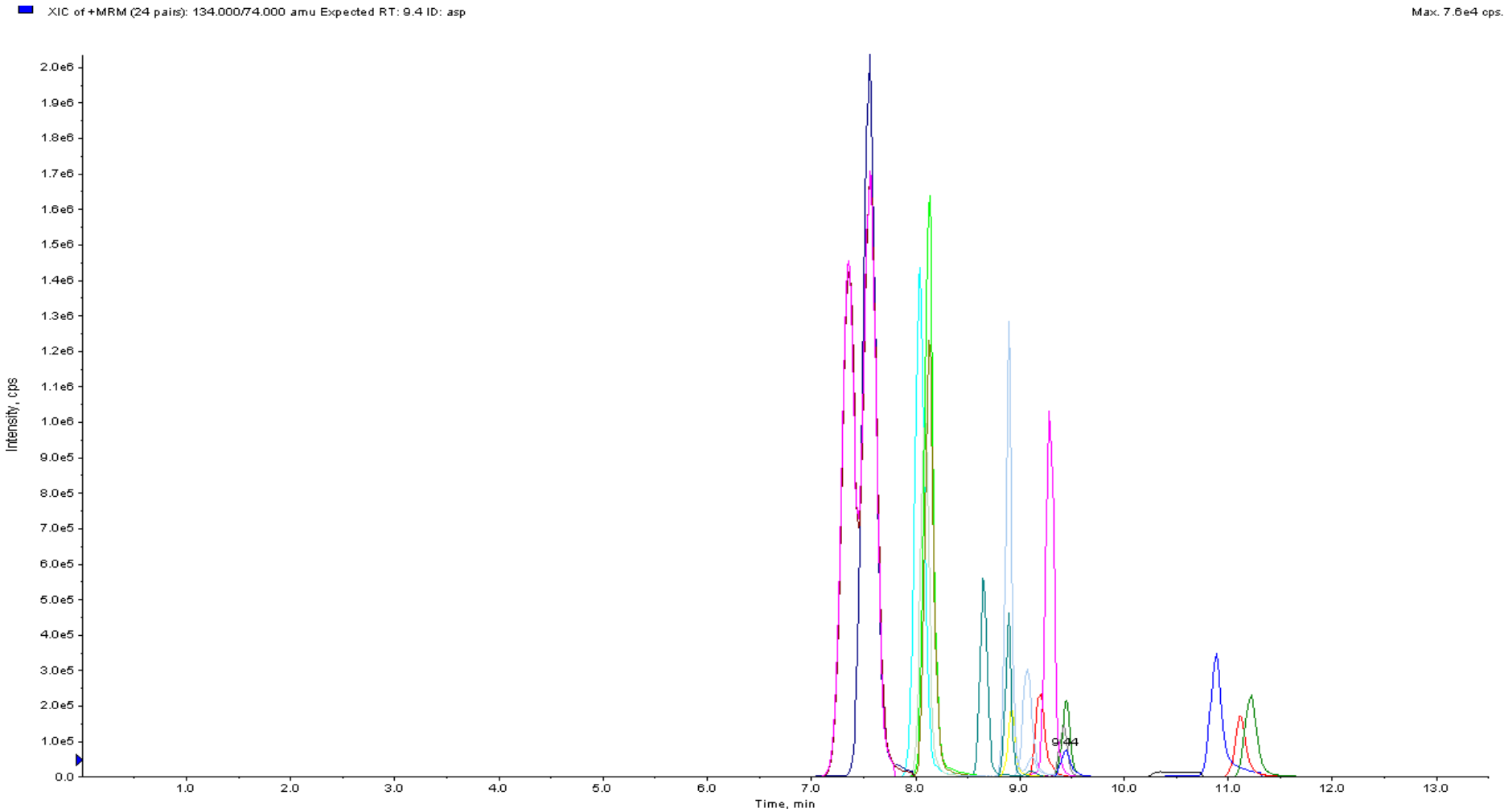
Standard 4: 0.05 ppm



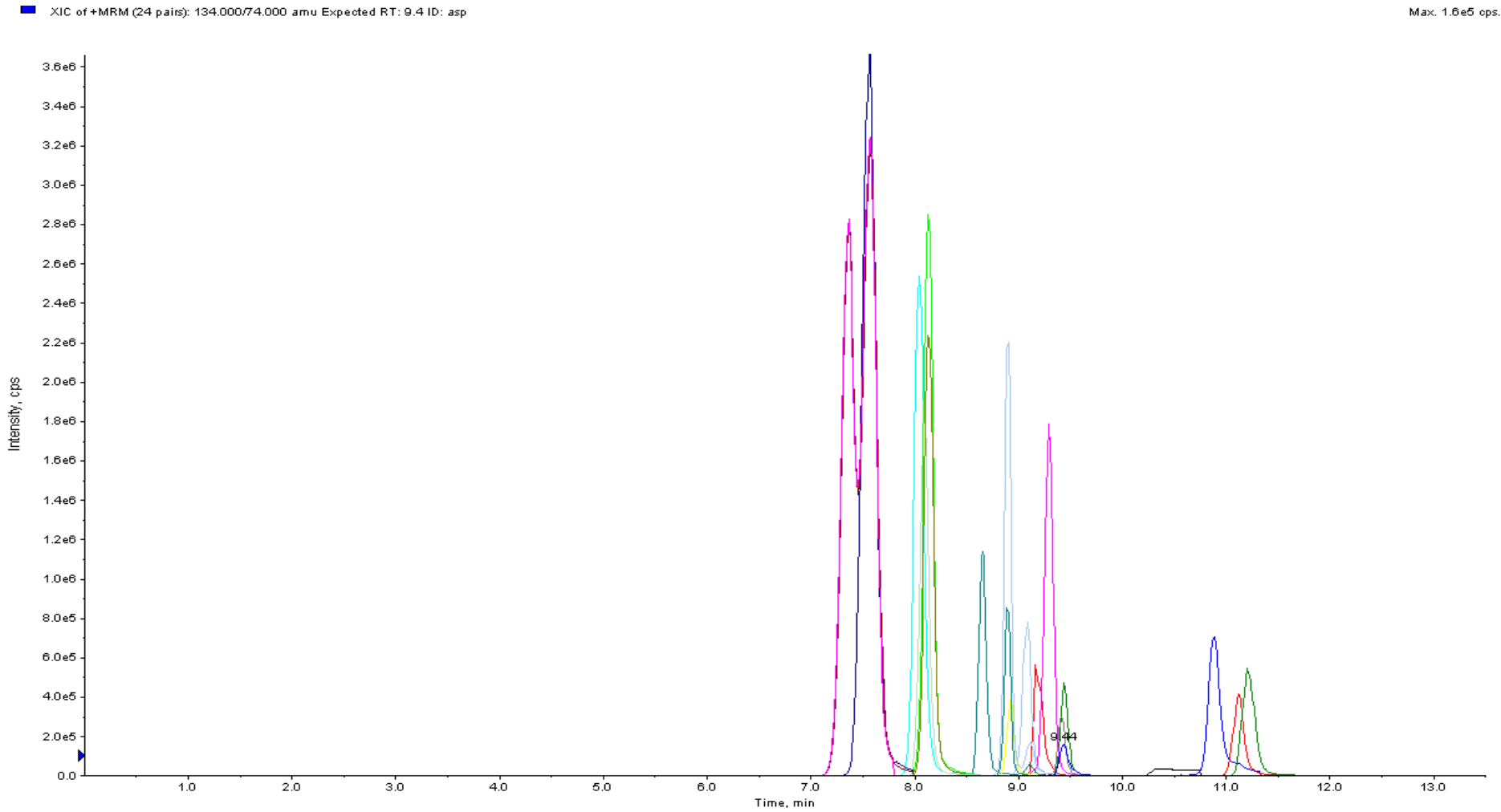
Standard 5: 0.1 ppm



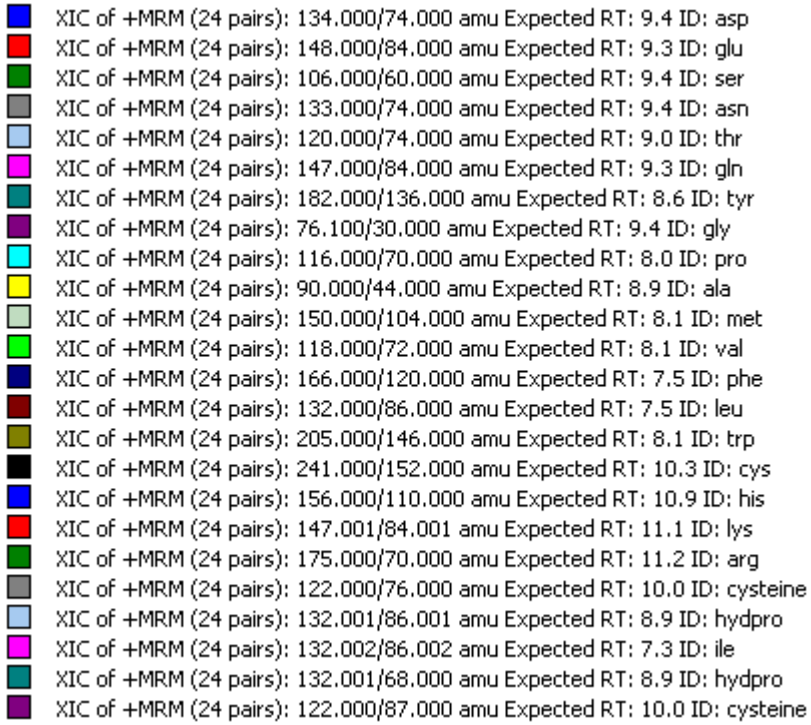
Standard 6: 0.25 ppm



Standard 7: 0.5 ppm

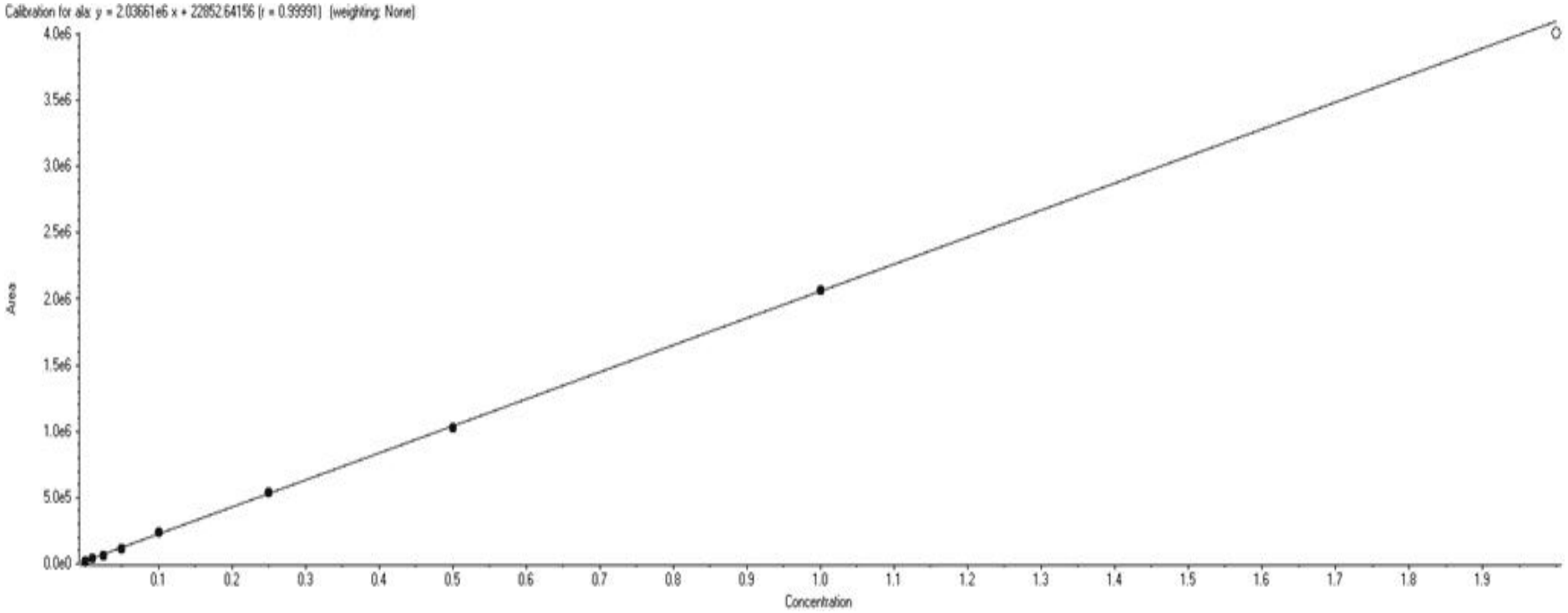


Standard 8: 1 ppm

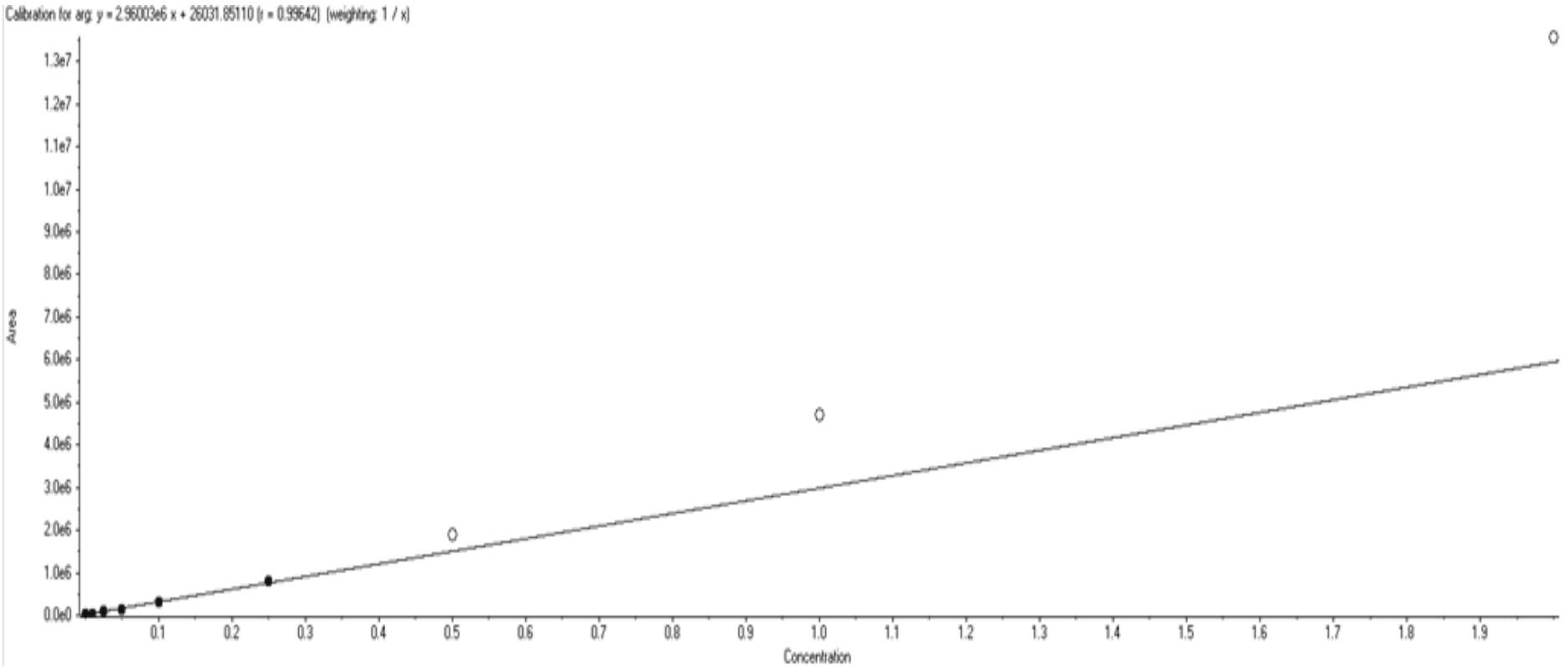


Legend for HILIC chromatograms

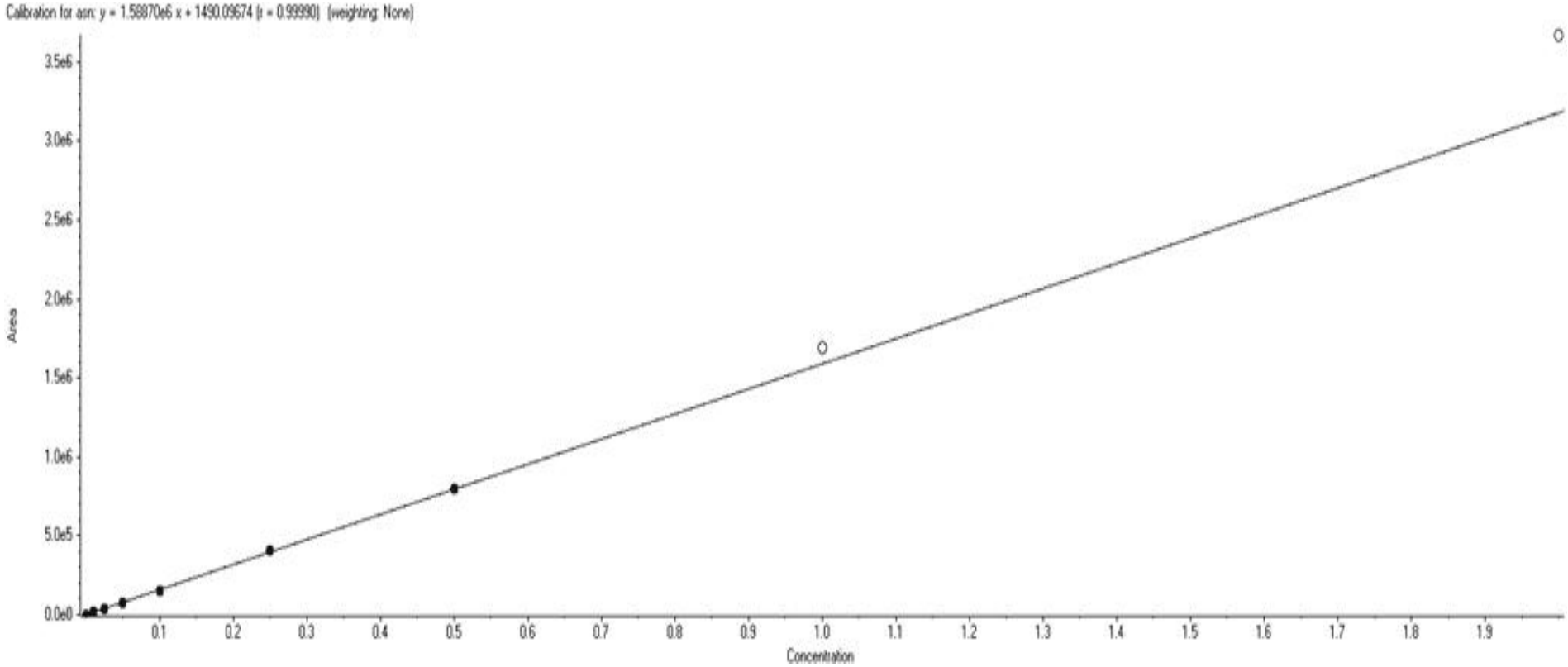
Appendix 6: Calibrations Curves for HILIC Method



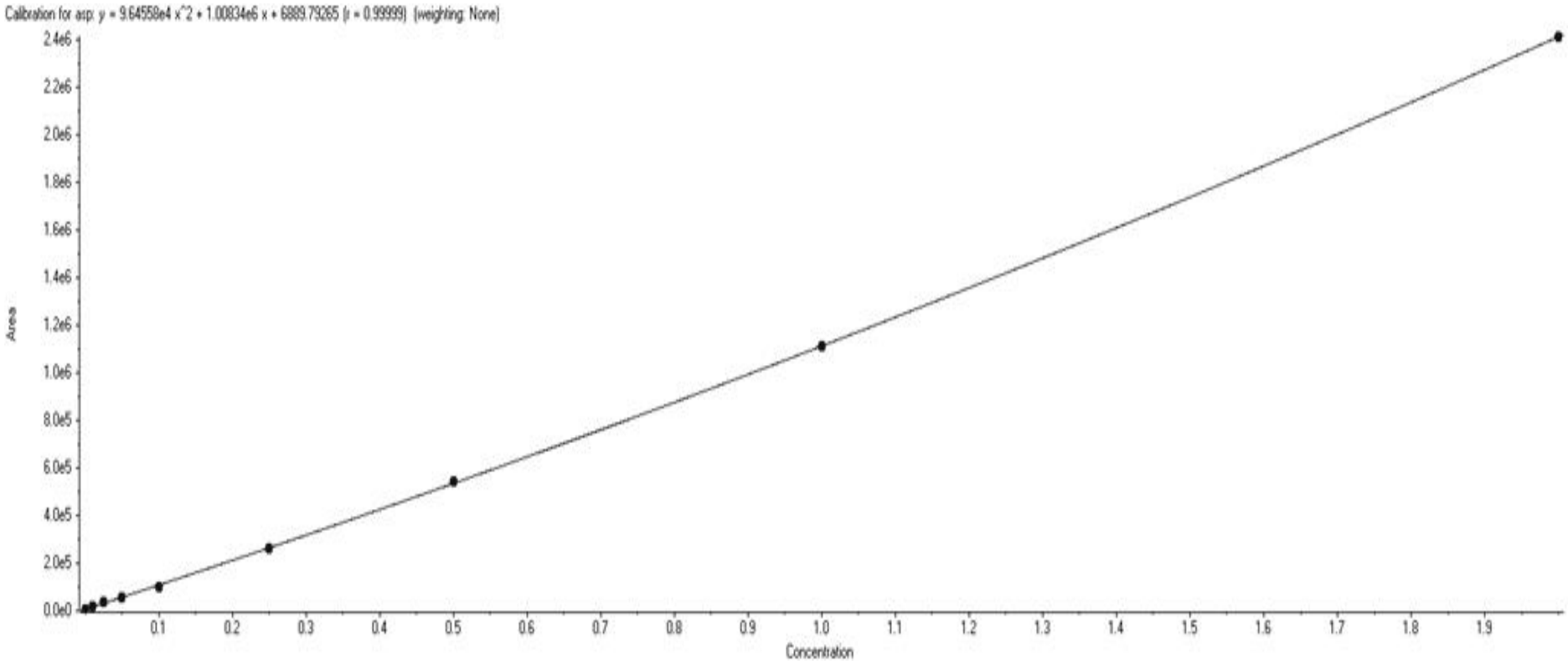
Alanine y = 2.03661e6 x + 22852.64156 (r = 0.99991)



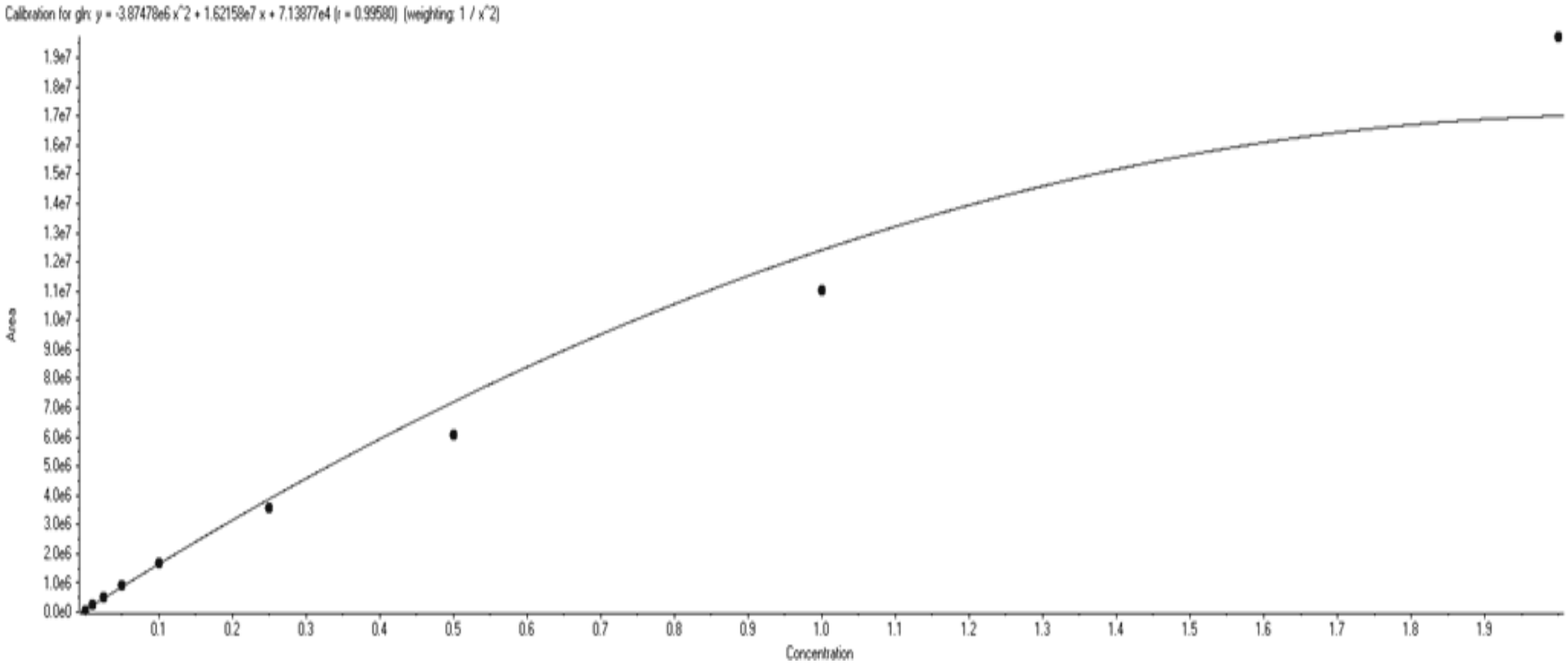
Arganine y = 2.96003e6 x + 26031.85110 (r = 0.99642) (weighting: 1 / x)



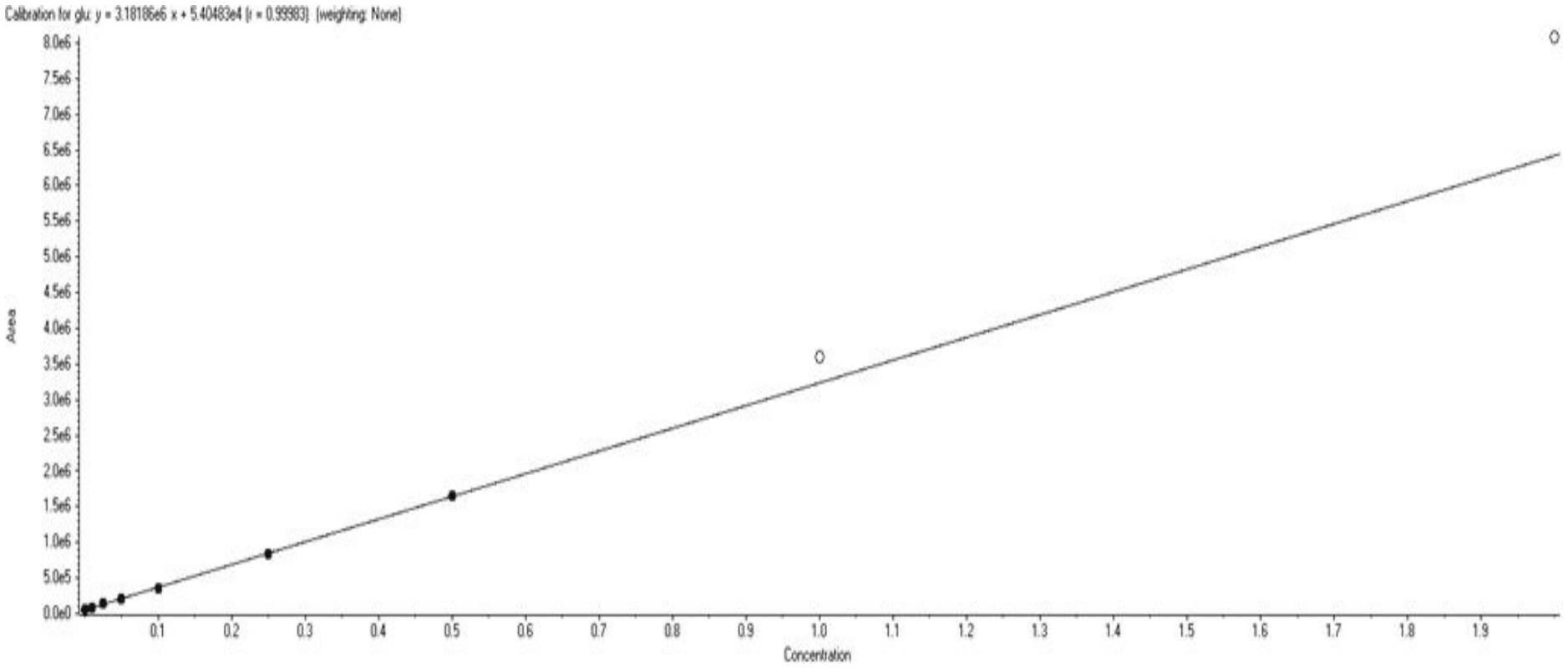
Asparagine y = 1.58870e6 x + 1490.09674 (r = 0.99990)



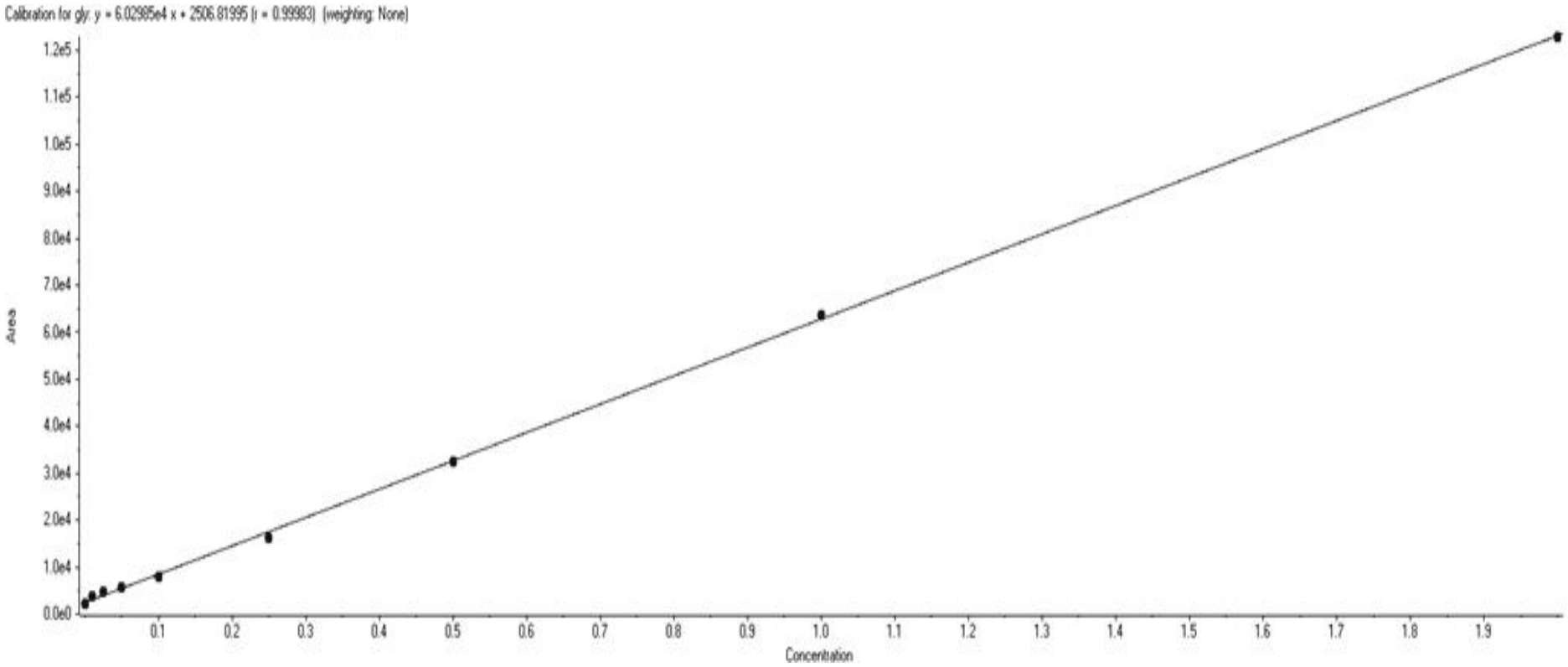
Aspartic acid y = 9.64558e4 x^2 + 1.00834e6 x + 6889.79265 (r = 0.99999)



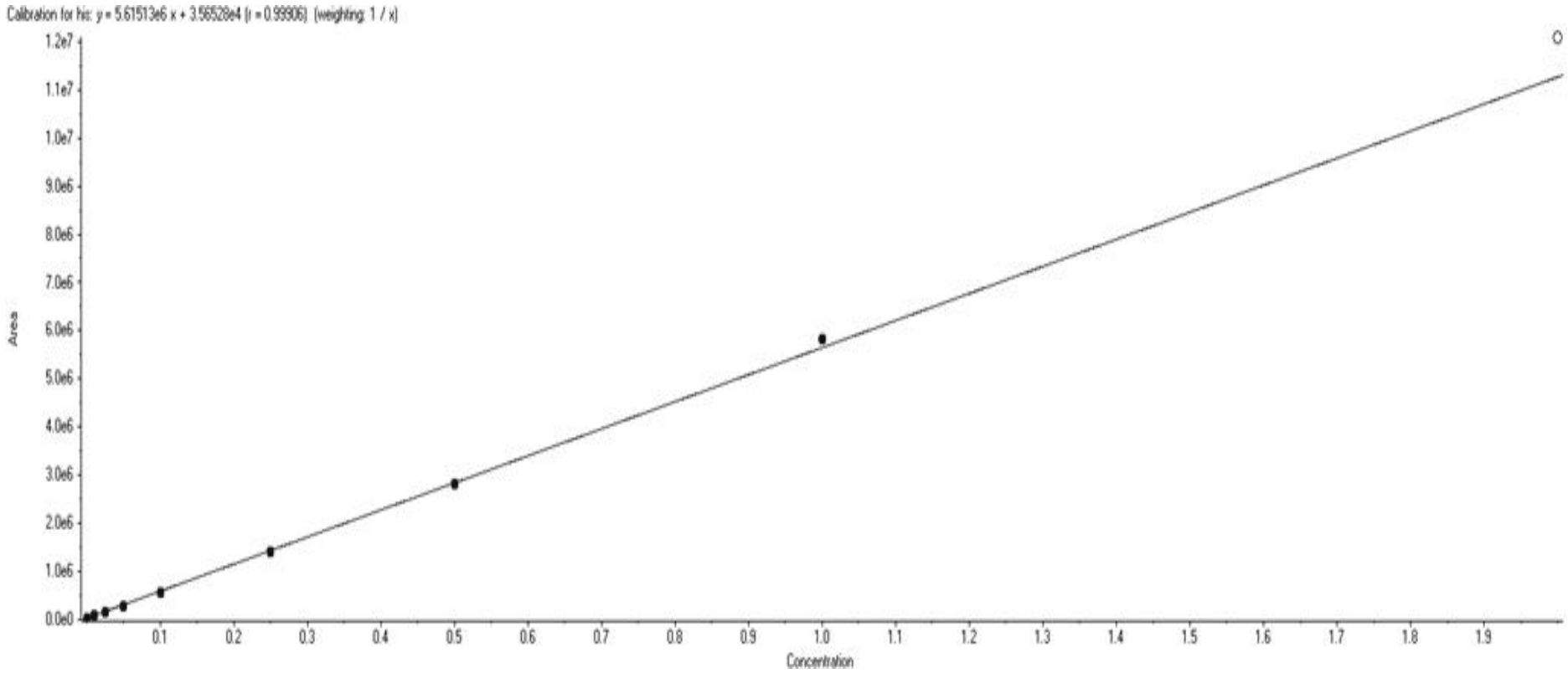
Glutamine y = -3.87478e6 x^2 + 1.62158e7 x + 7.13877e4 (r + 0.99580) (weighting: 1 / x^2)



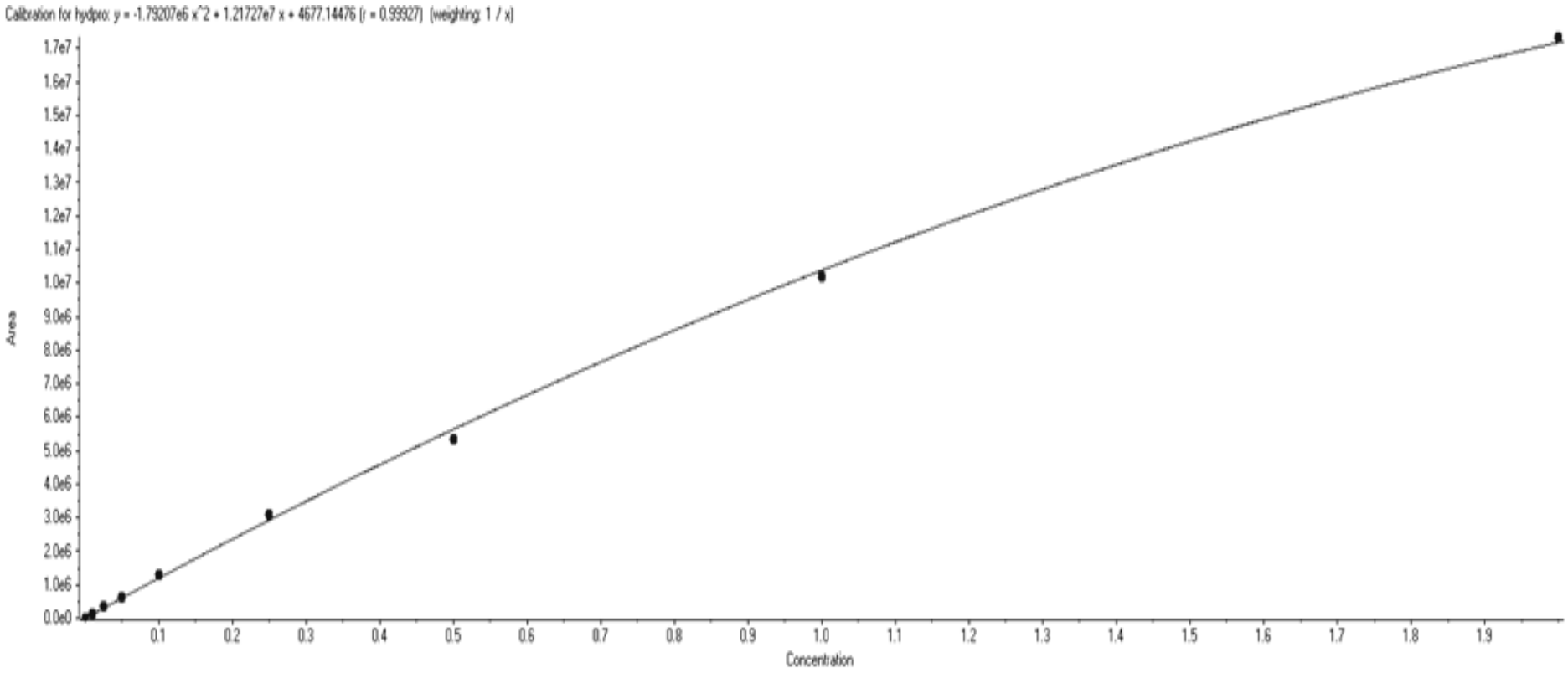
Glutamic acid y = 3.18186e6 x + 5.40483e4 (r = 0.99983)



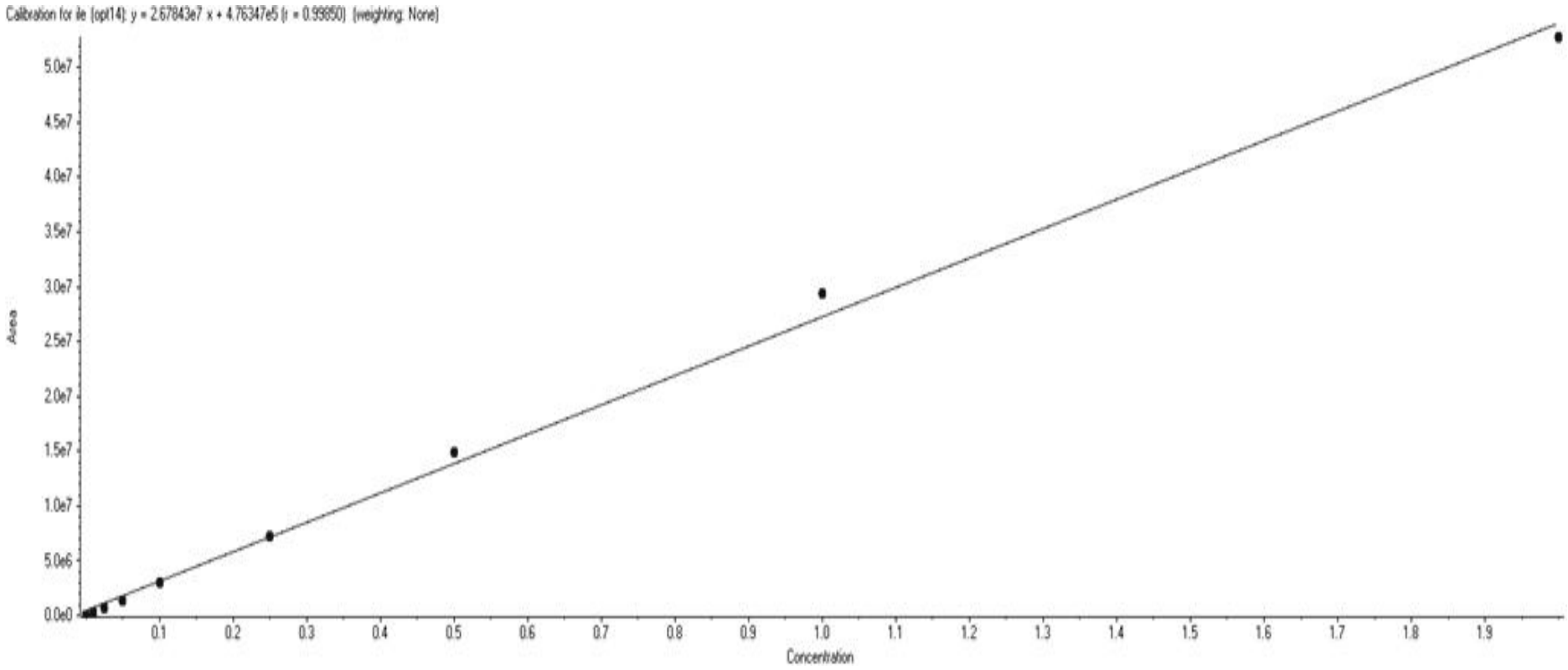
Glycine y = 6.02985e4 x + 2506.81995 (r = 0.99983)



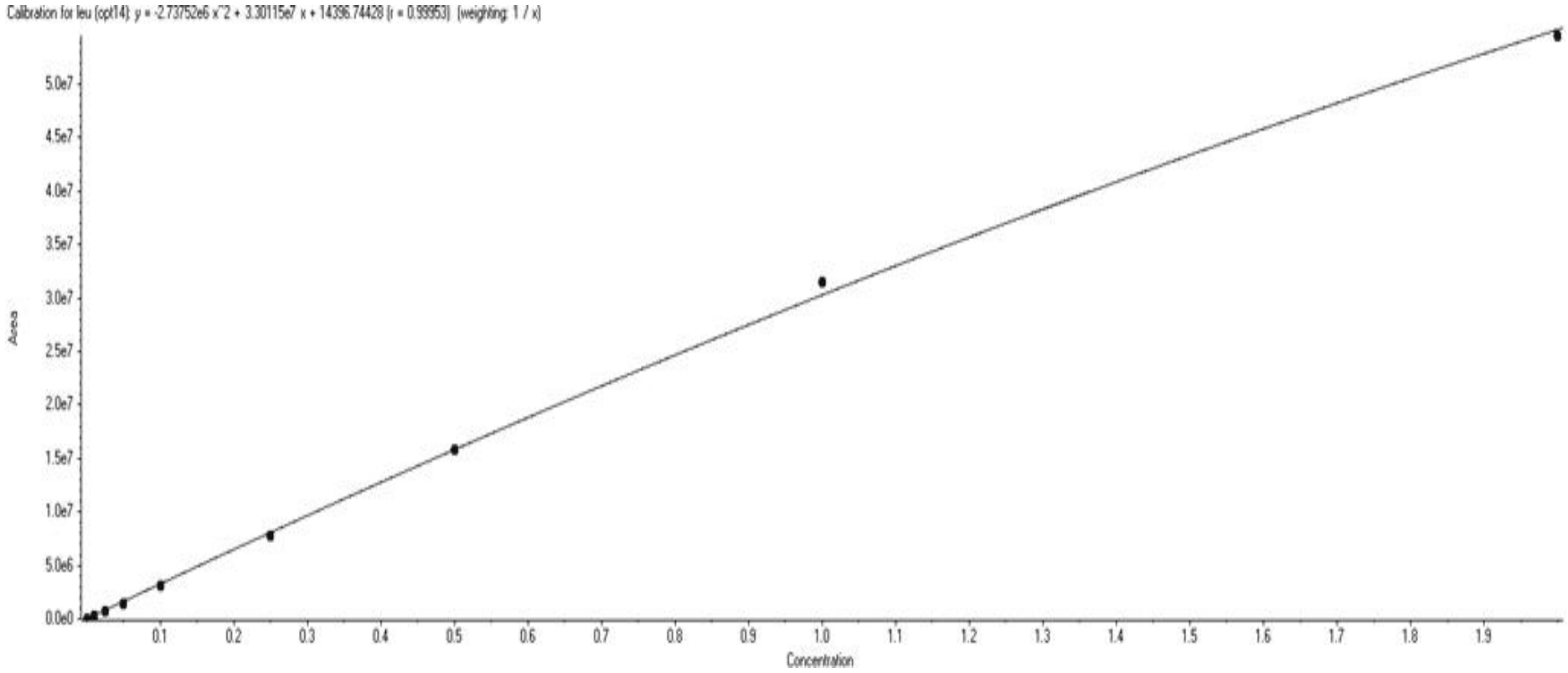
Histidine y = 5.61513e6 x + 3.56582e4 (r = 0.99906 (weighting: 1 / x)



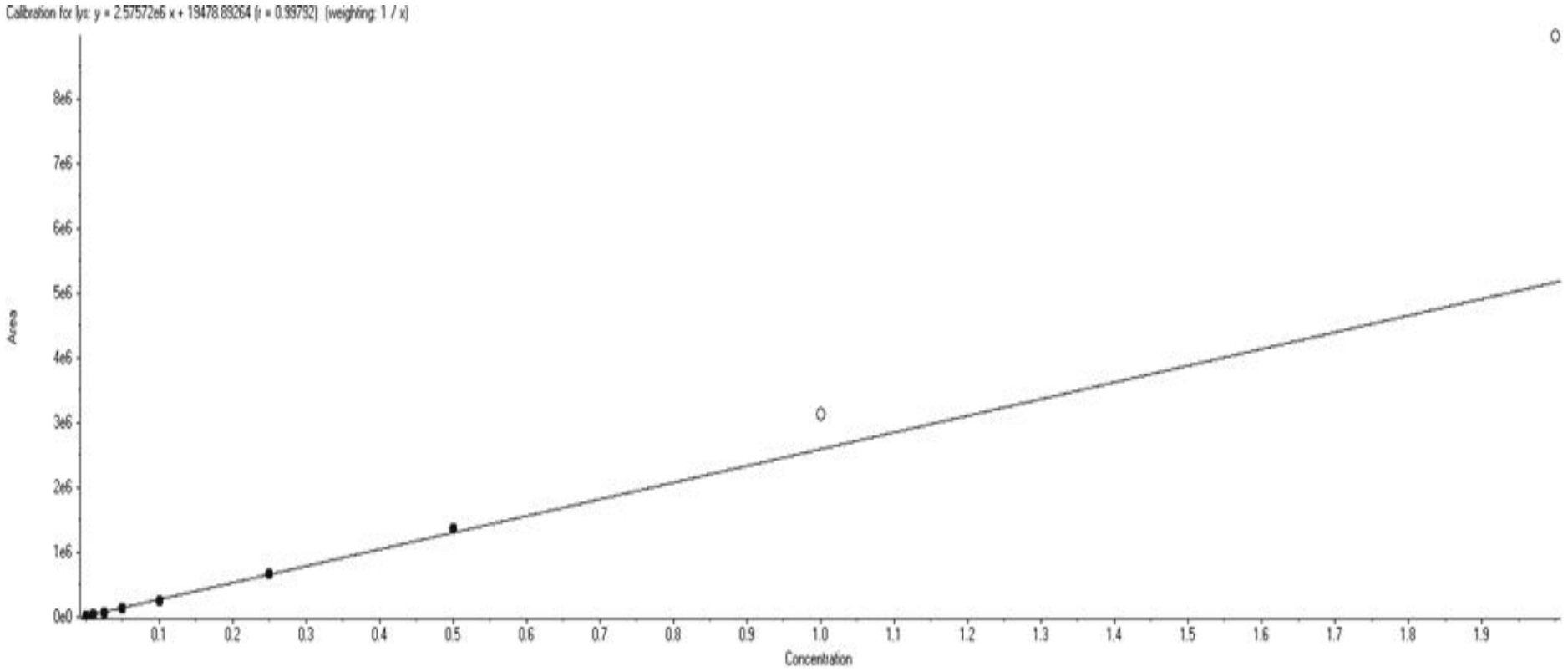
Hydroxyproline y= -1.79207e6 x^2 + 1.21727e7 x + 4677.14476 (r = 0.99927) (weighting: 1 / x)



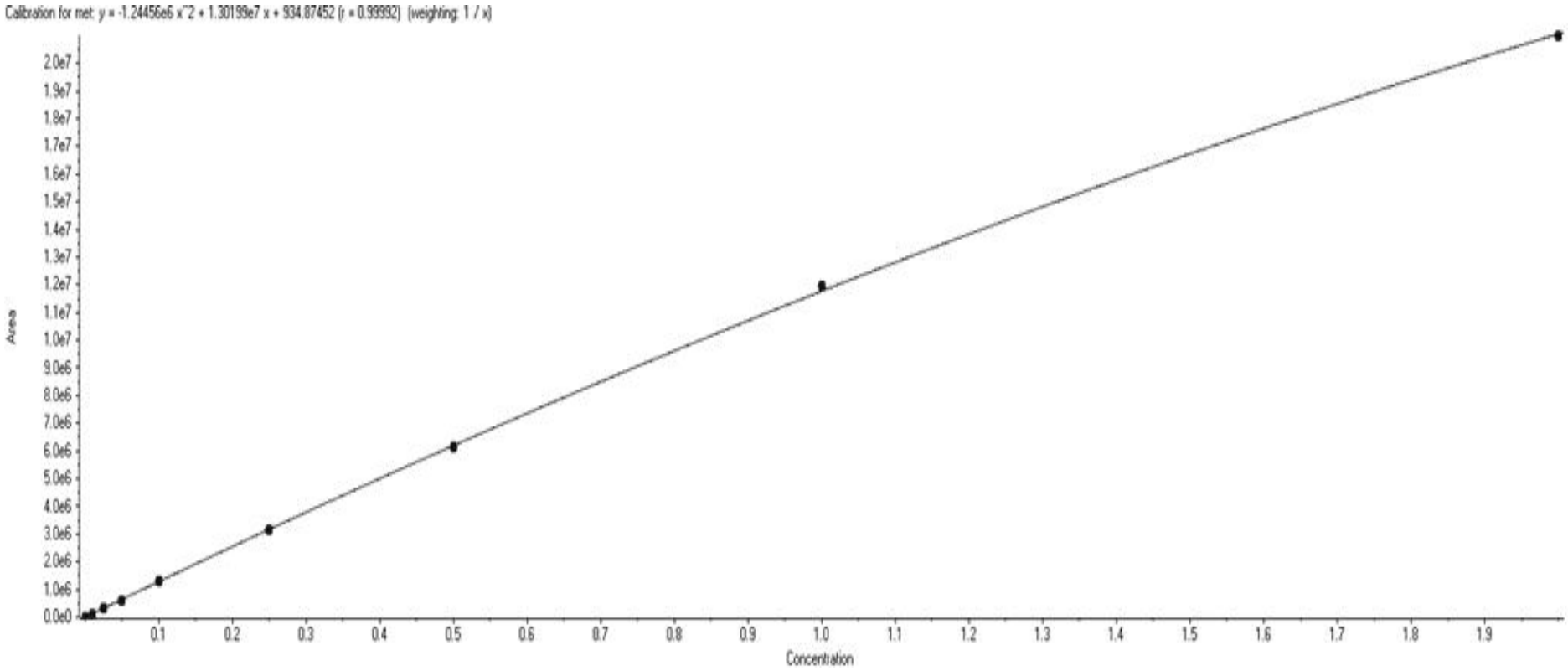
Isoleucine y = 2.67843e7 x + 4.76347e5 (r = 0.99850)



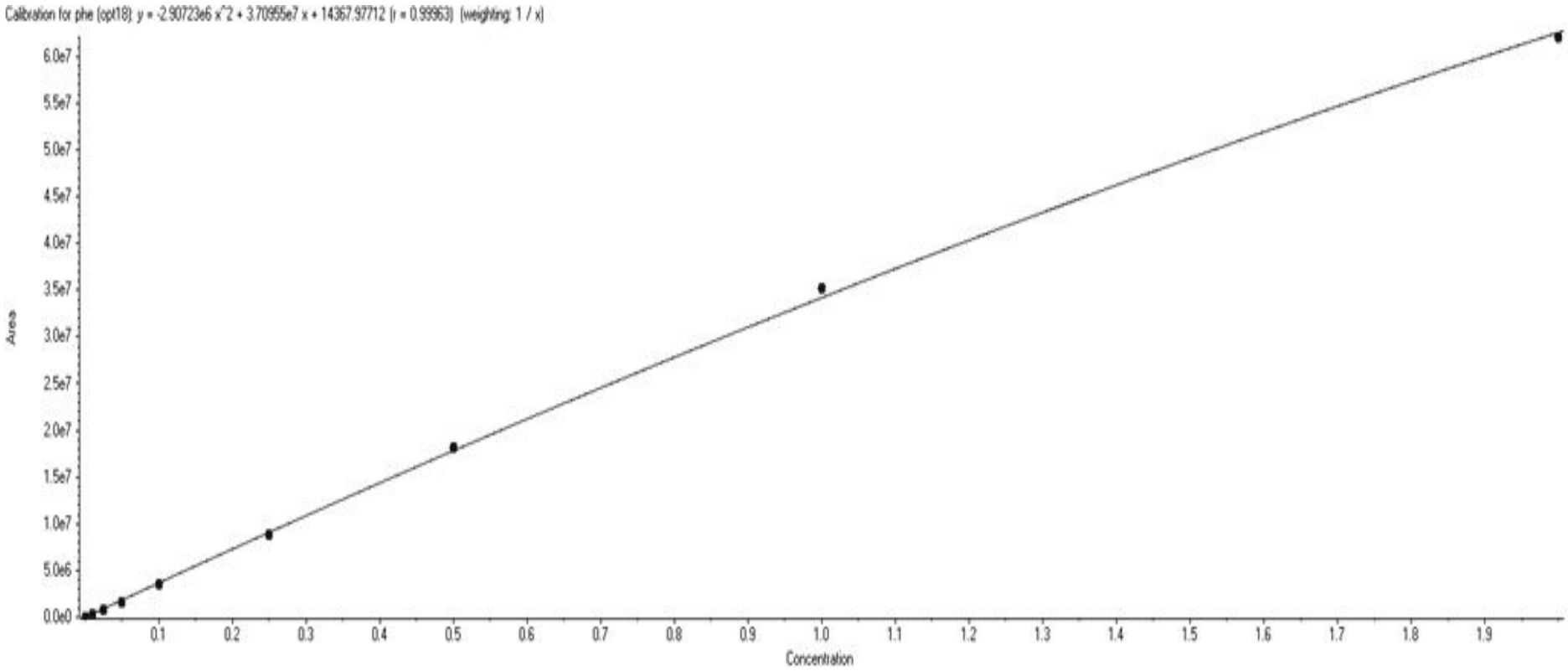
Leucine y = -2.73752e6 x^2 + 3.30115e7 x + 14396.74428 (r = 0.99953) (weighting: 1 / x)



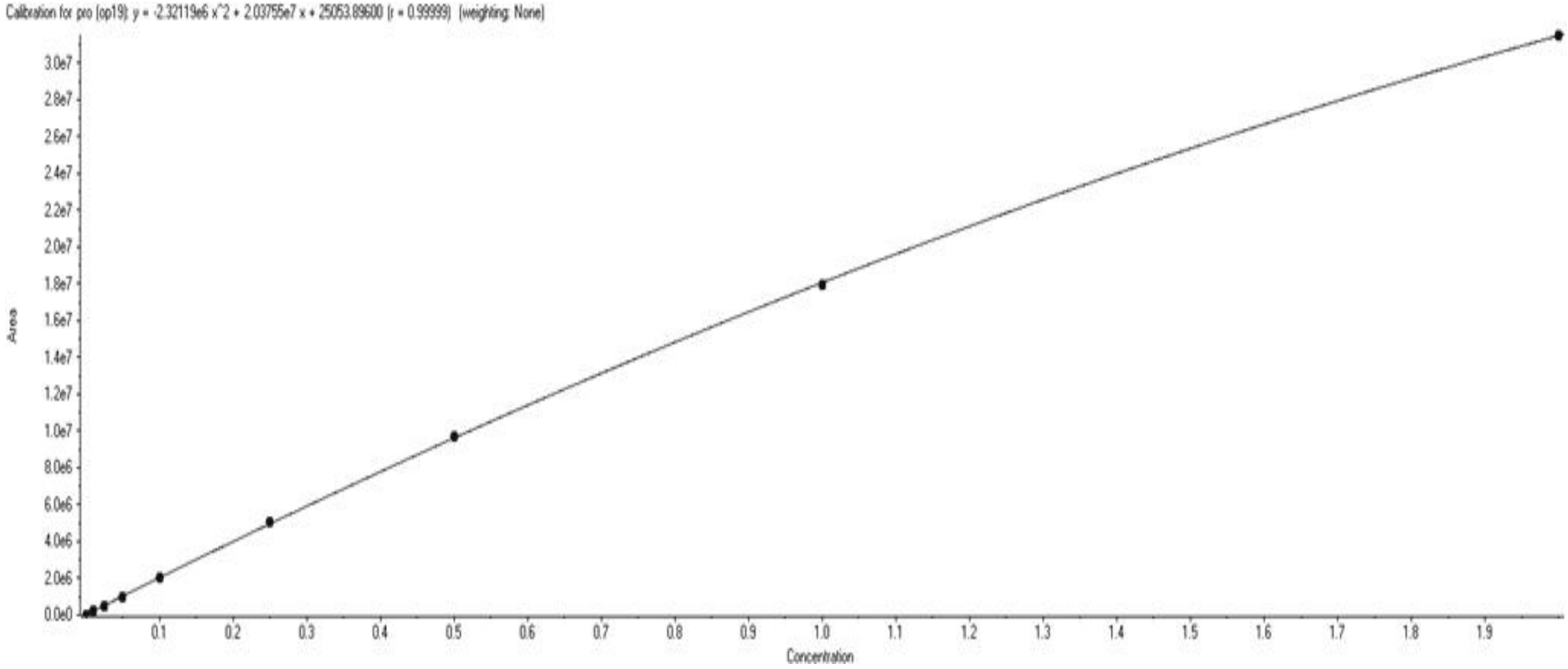
Lysine y= 2.57572e6 x + 19478.89264 (r = 0.99792) (weighting: 1 / x)



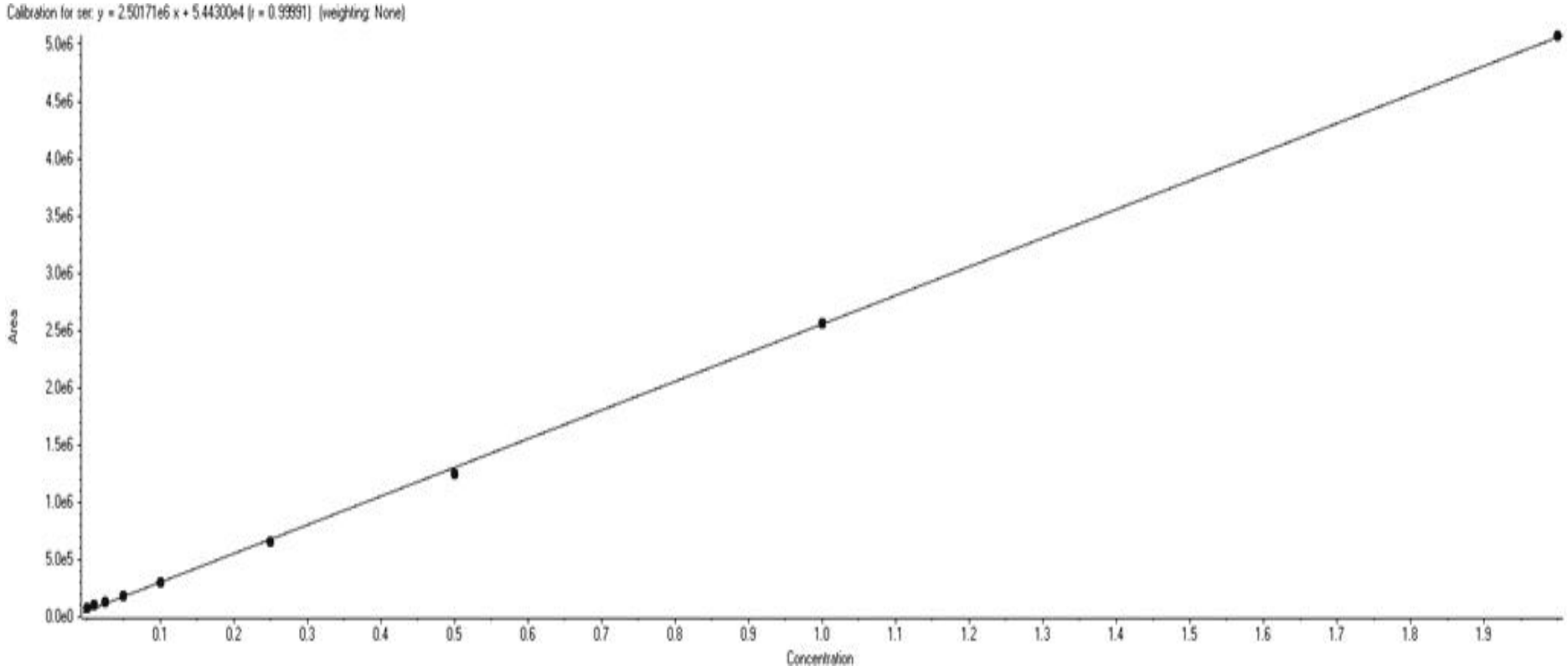
Methionine y = -1.24456e6 x^2 + 1.30199e7 x + 934.87452 (r = 0.99992) (weighting: 1 / x)



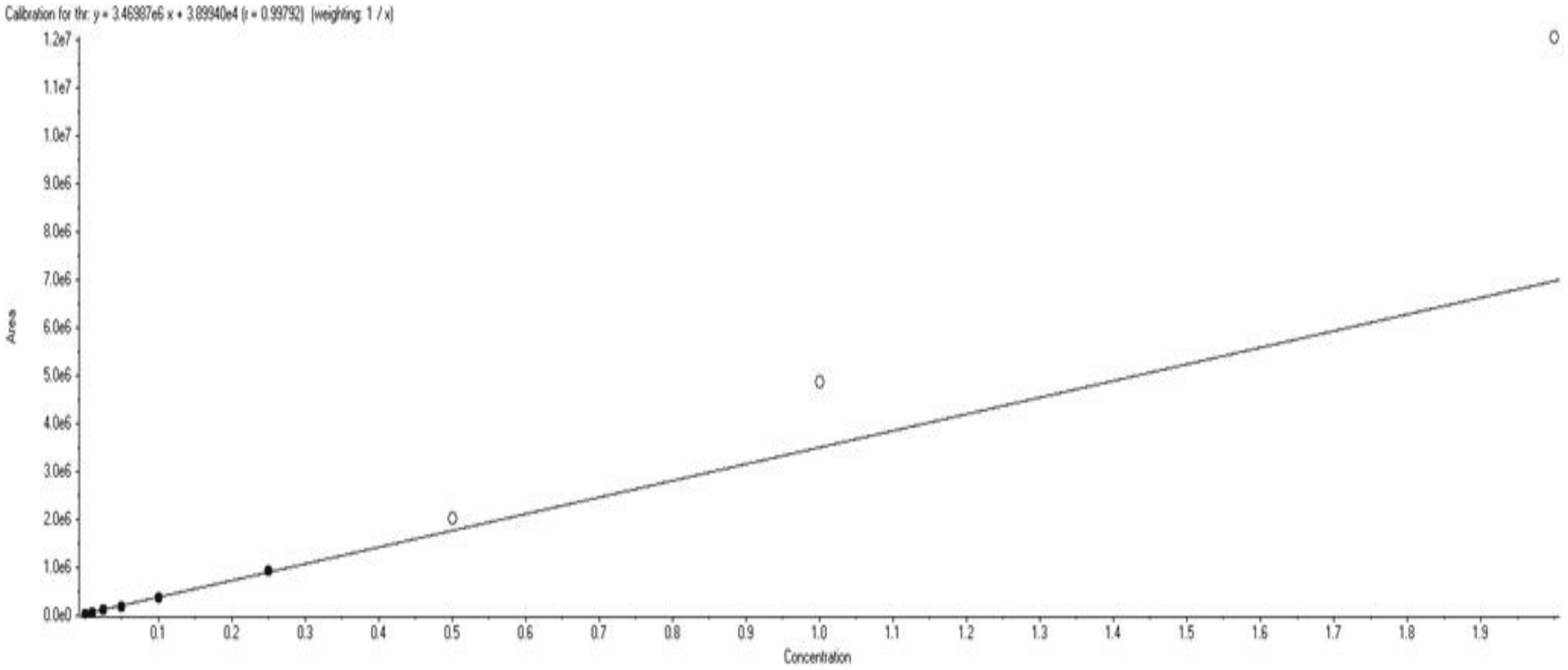
Phenylalanine y = -2.90723e6 x^2 + 3.70955e7 x + 14367.97712 (r = 0.99963) (weighting: 1 / x)



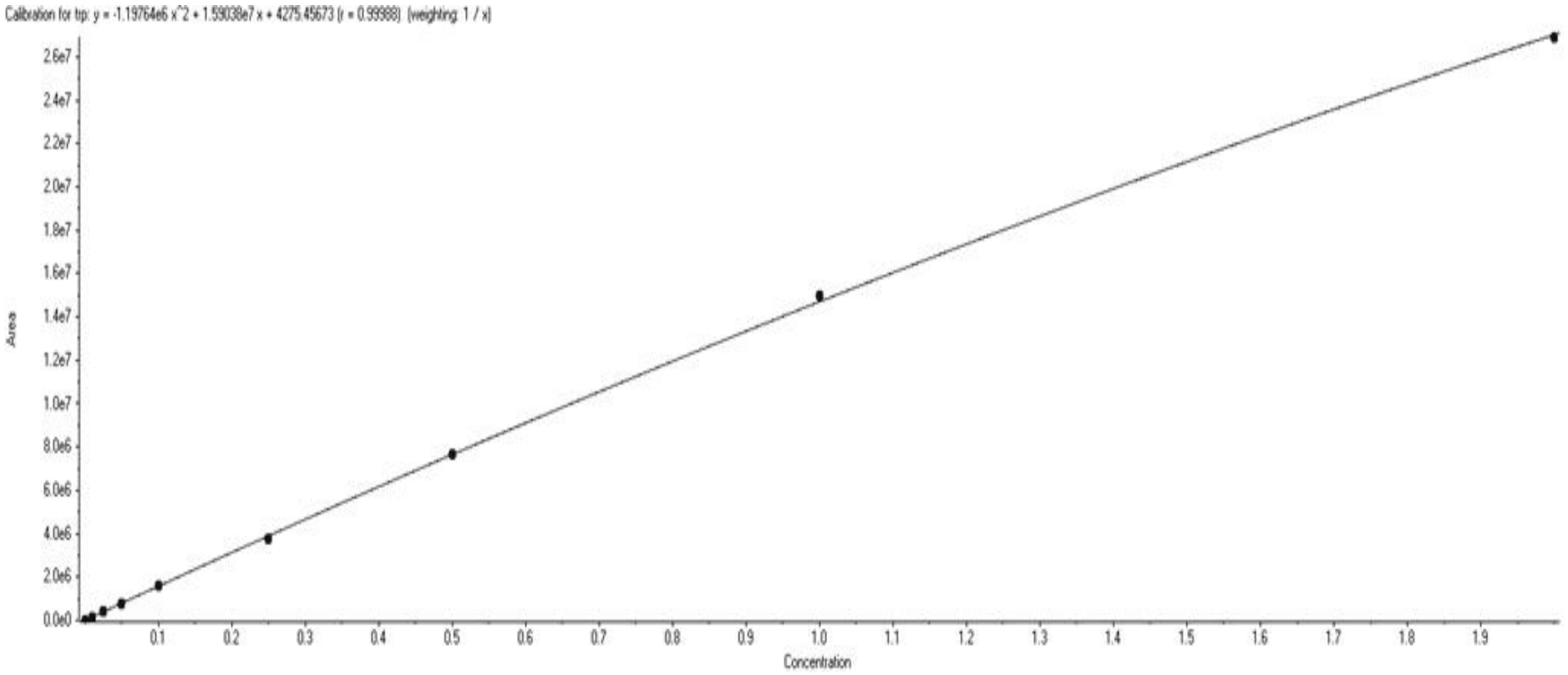
Proline y = -2.32119e6 x^2 + 2.03755e7 x + 25053.896 (r = 0.99999)



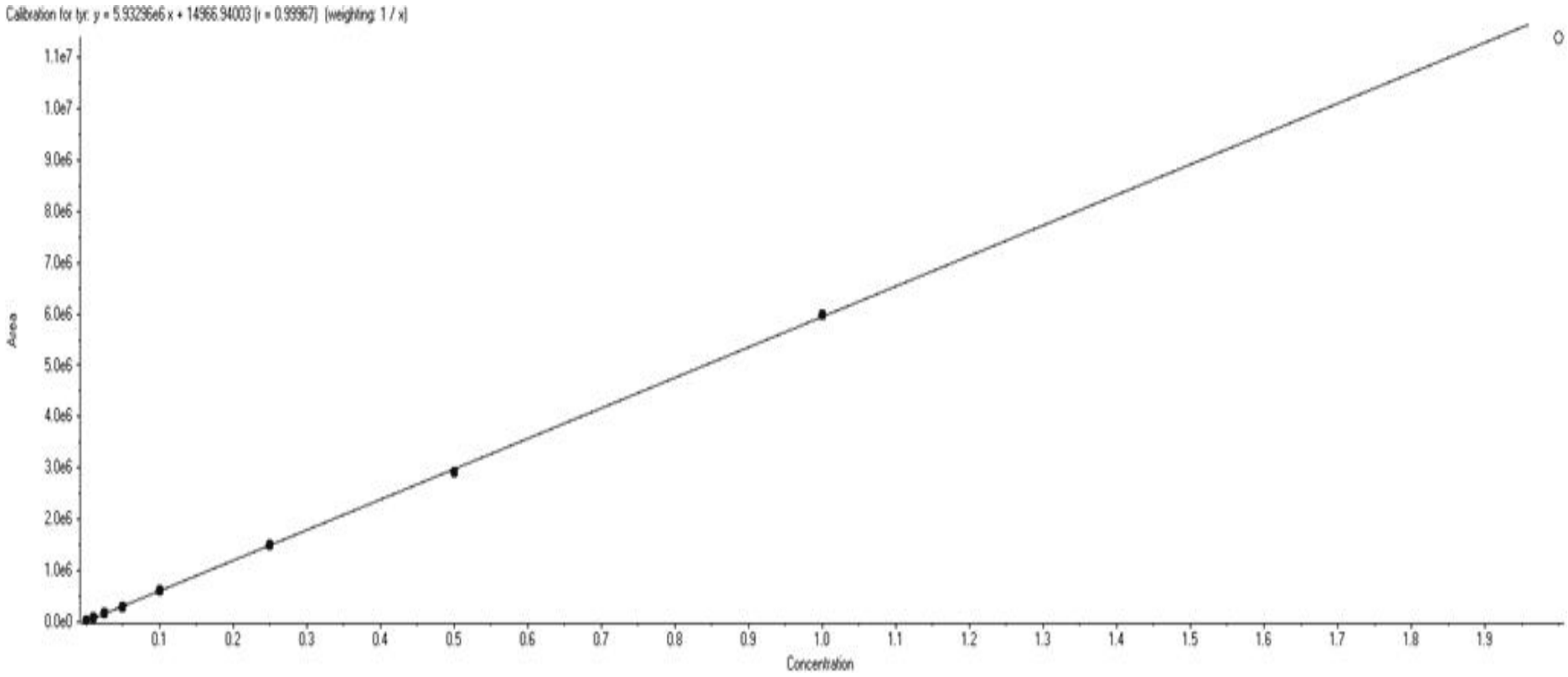
Serine y = 2.50171e6 x + 5.44300e4 (r = 0.99991)



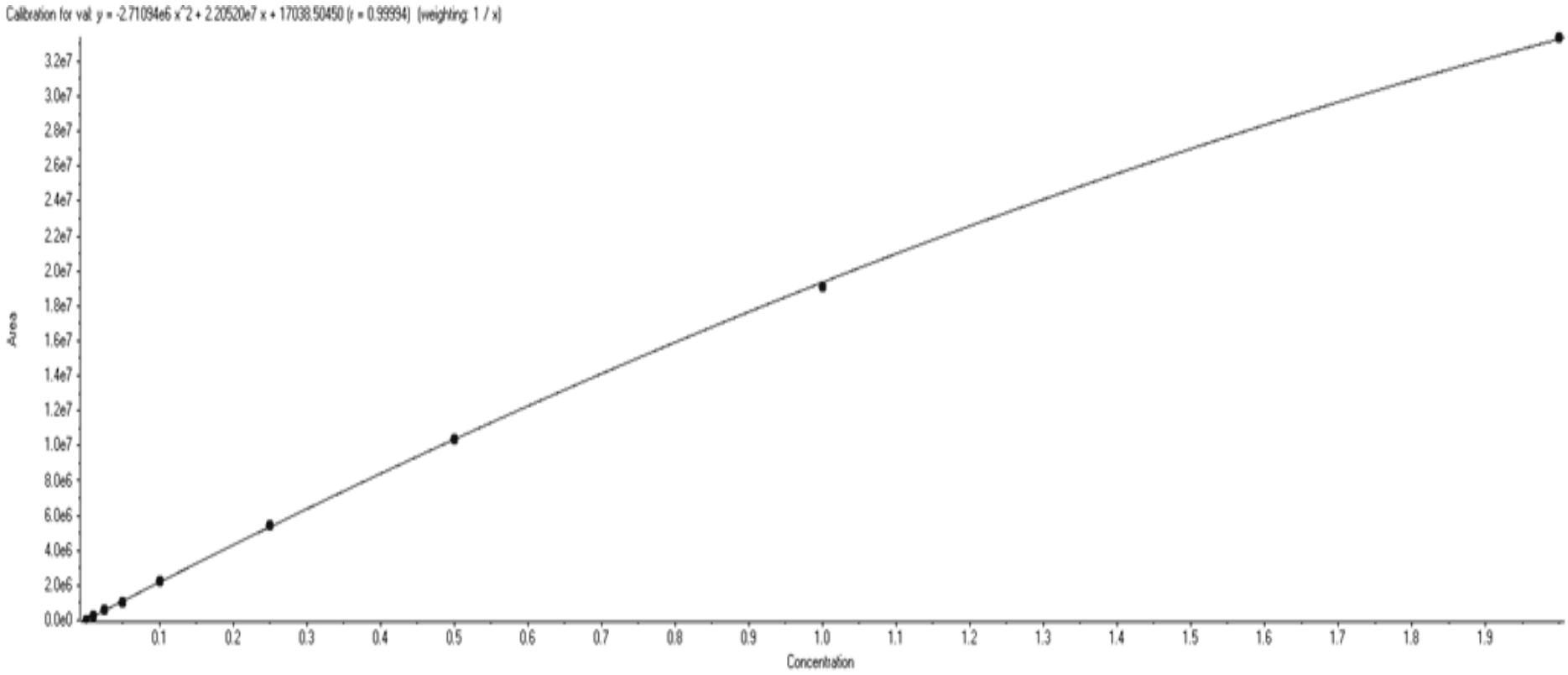
Threonine y= 3.46987e6 x + 3.89940e4 (r = 0.99792) (weighting: 1 / x)



Tryptophan y = -1.19764e6 x^2 + 1.59038e7 x + 4275.45673 (r = 0.99988) (weighting: 1 / x)

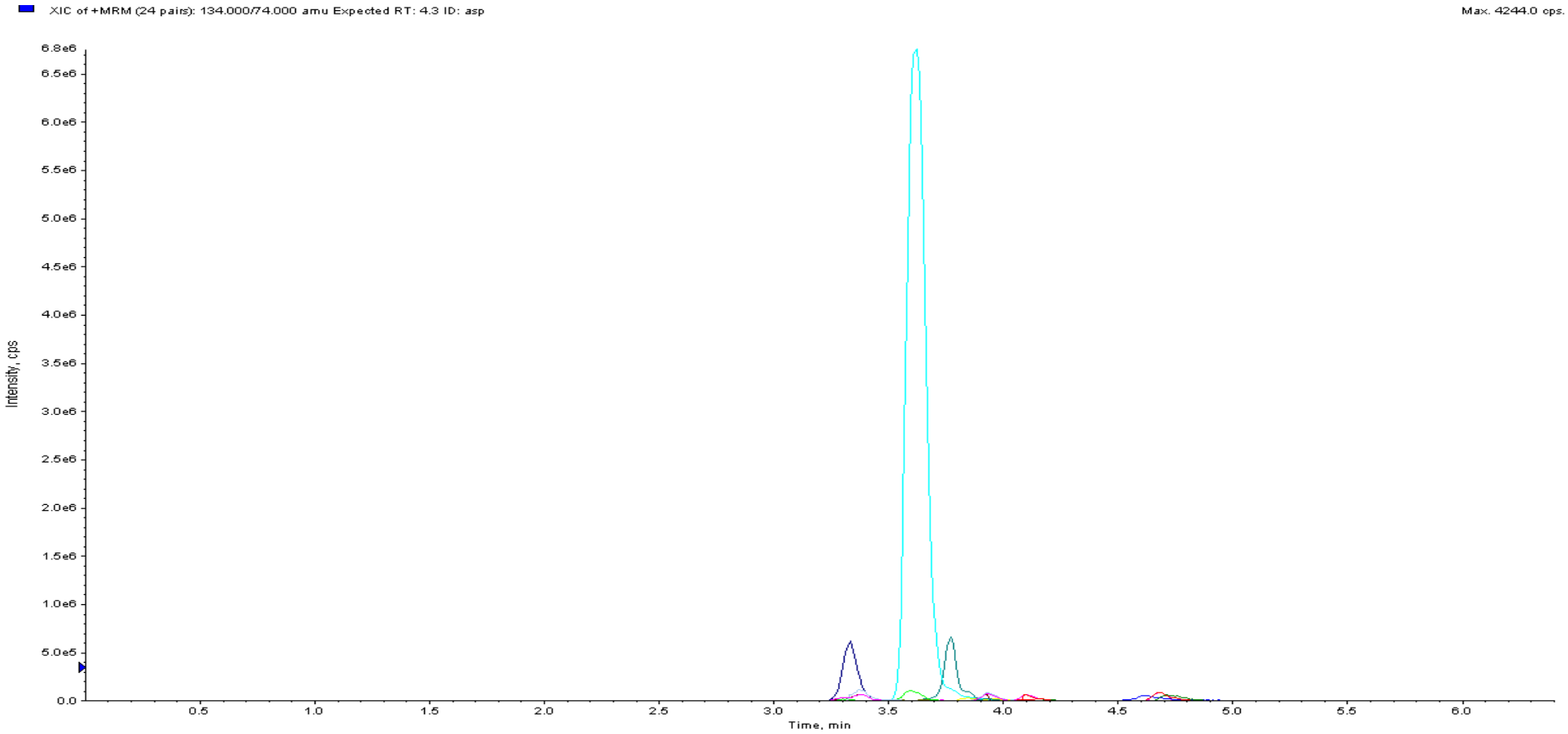


Tyrosine y = 5.93296e6 x + 14966.94003 (r = 0.99967) (weighting: 1 / x)

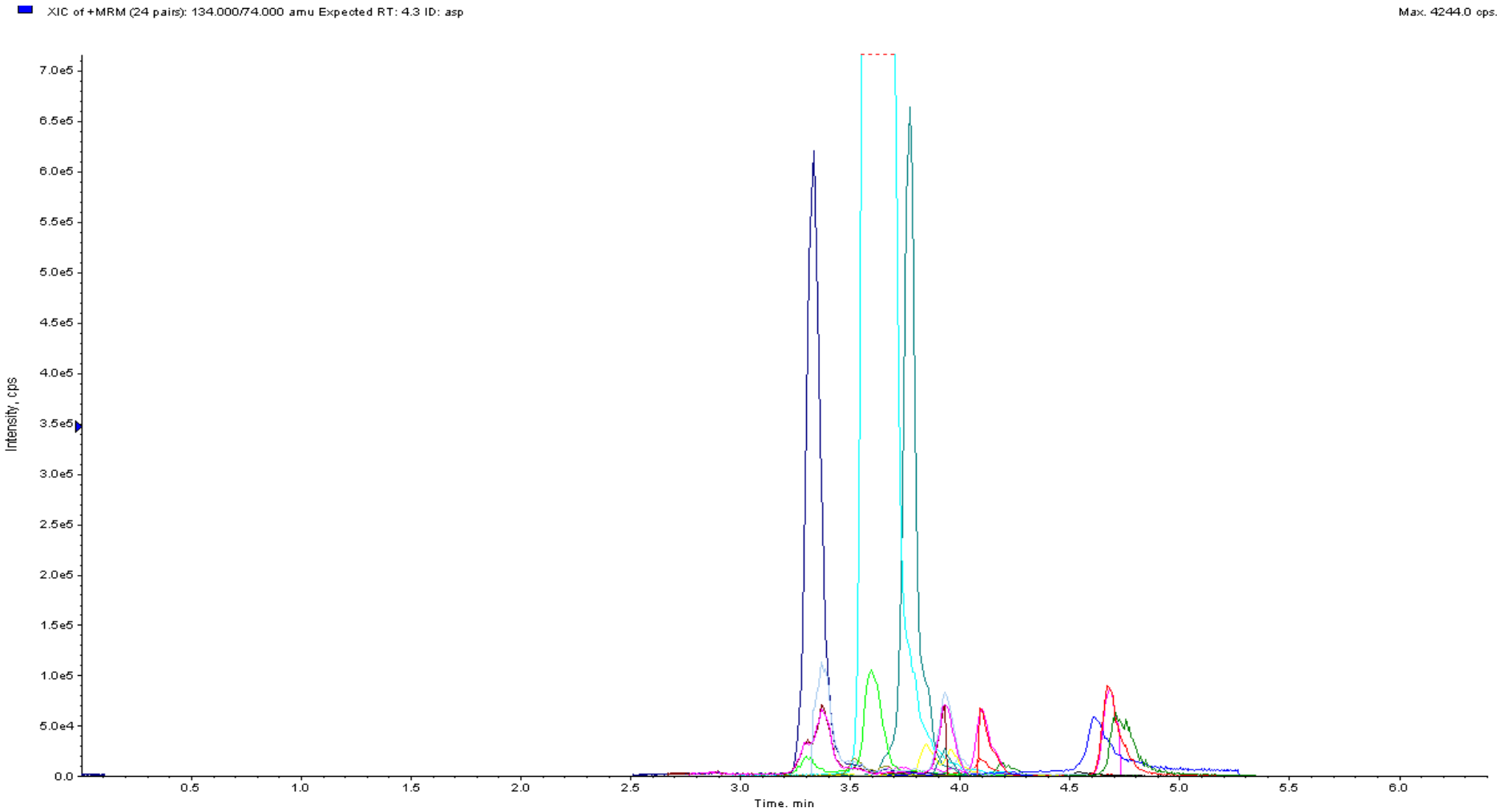


Valine y = -2.71094e6 x^2 + 2.20520e7 x + 17038.50450 (r = 0.99994) (weighting: 1 / x)

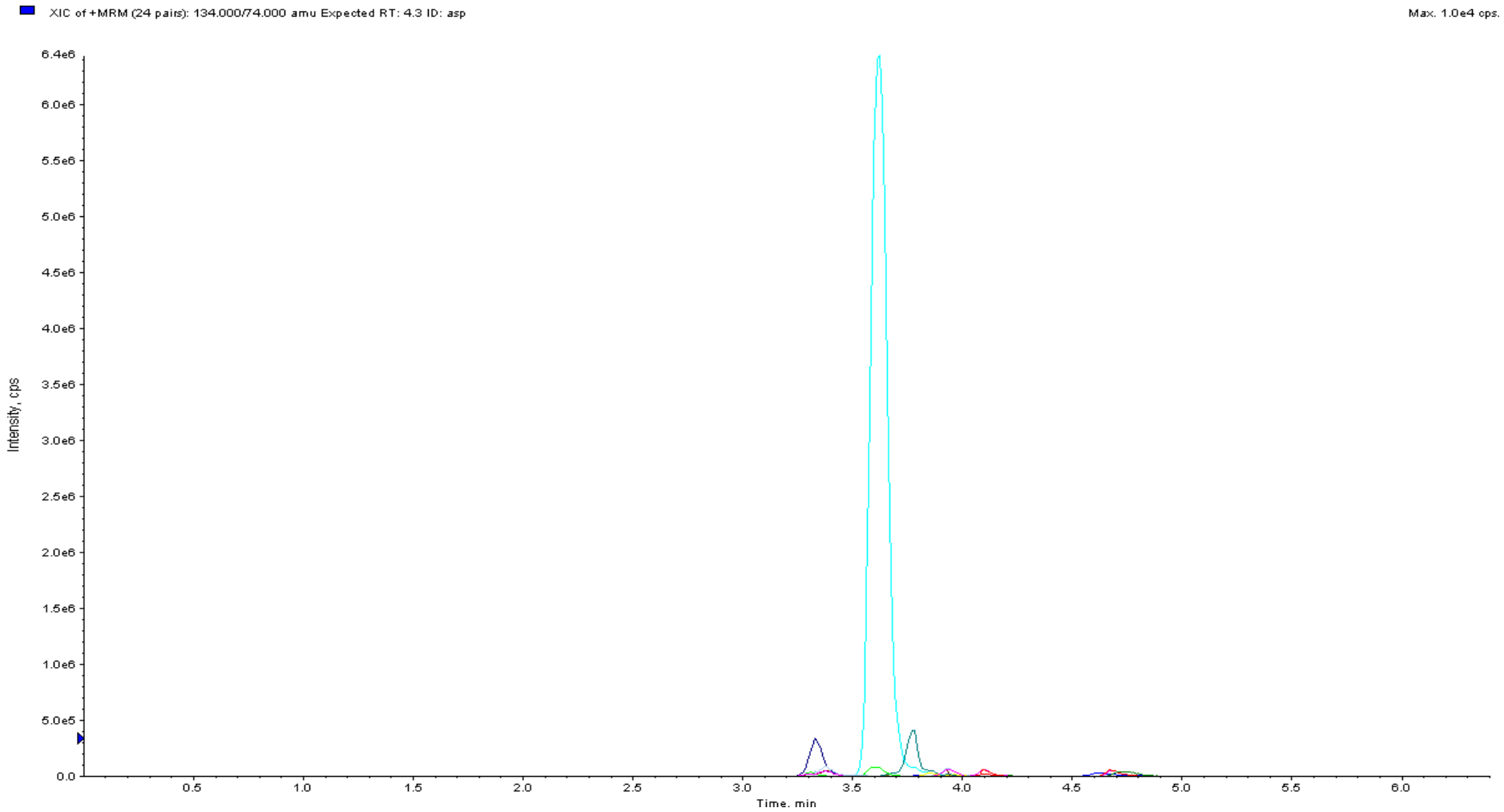
Appendix 7: Chromatograms of Honeys for HILIC Method



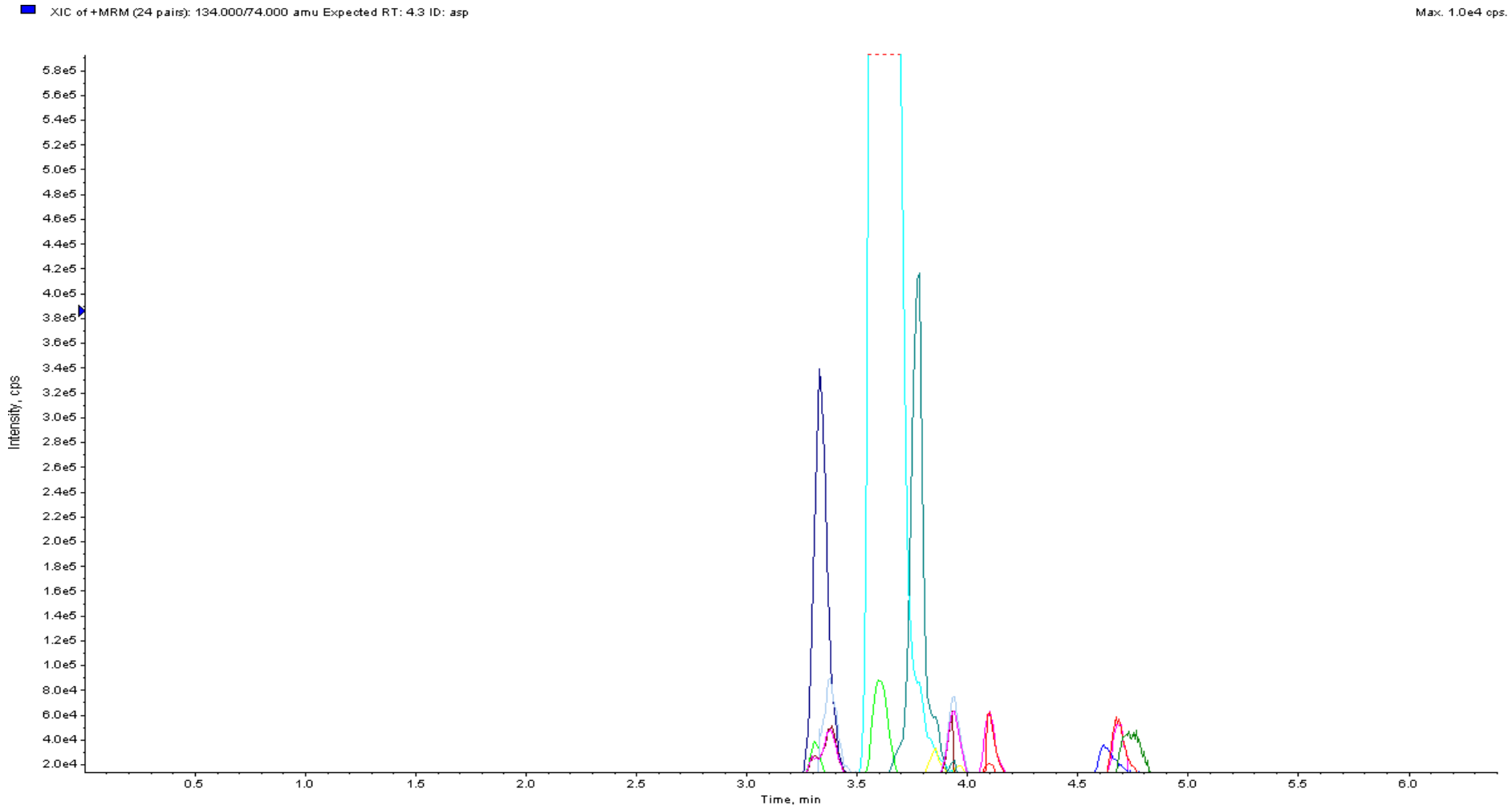
Honey ID 66



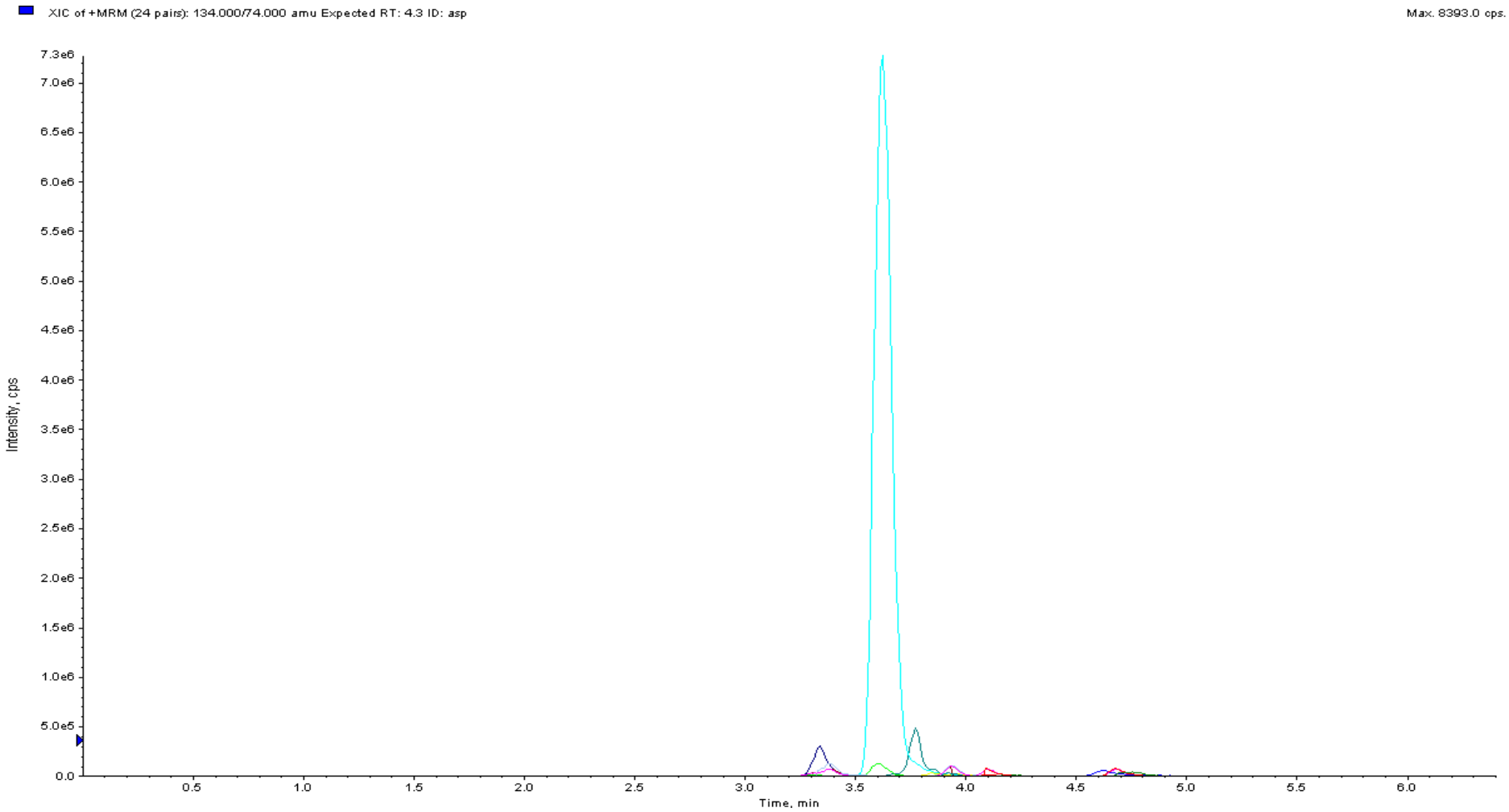
Honey ID 66 (zoomed in)


XIC of +MRM (24 pairs): 134.000/74.000 amu Expected RT: 4.3 ID: asp
Max. 1.0e4 cps.
Intensity, cps
6.4e6
6.0e6
5.5e6
5.0e6
4.5e6
4.0e6
3.5e6
3.0e6
2.5e6
2.0e6
1.5e6
1.0e6
5.0e5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
Time, min

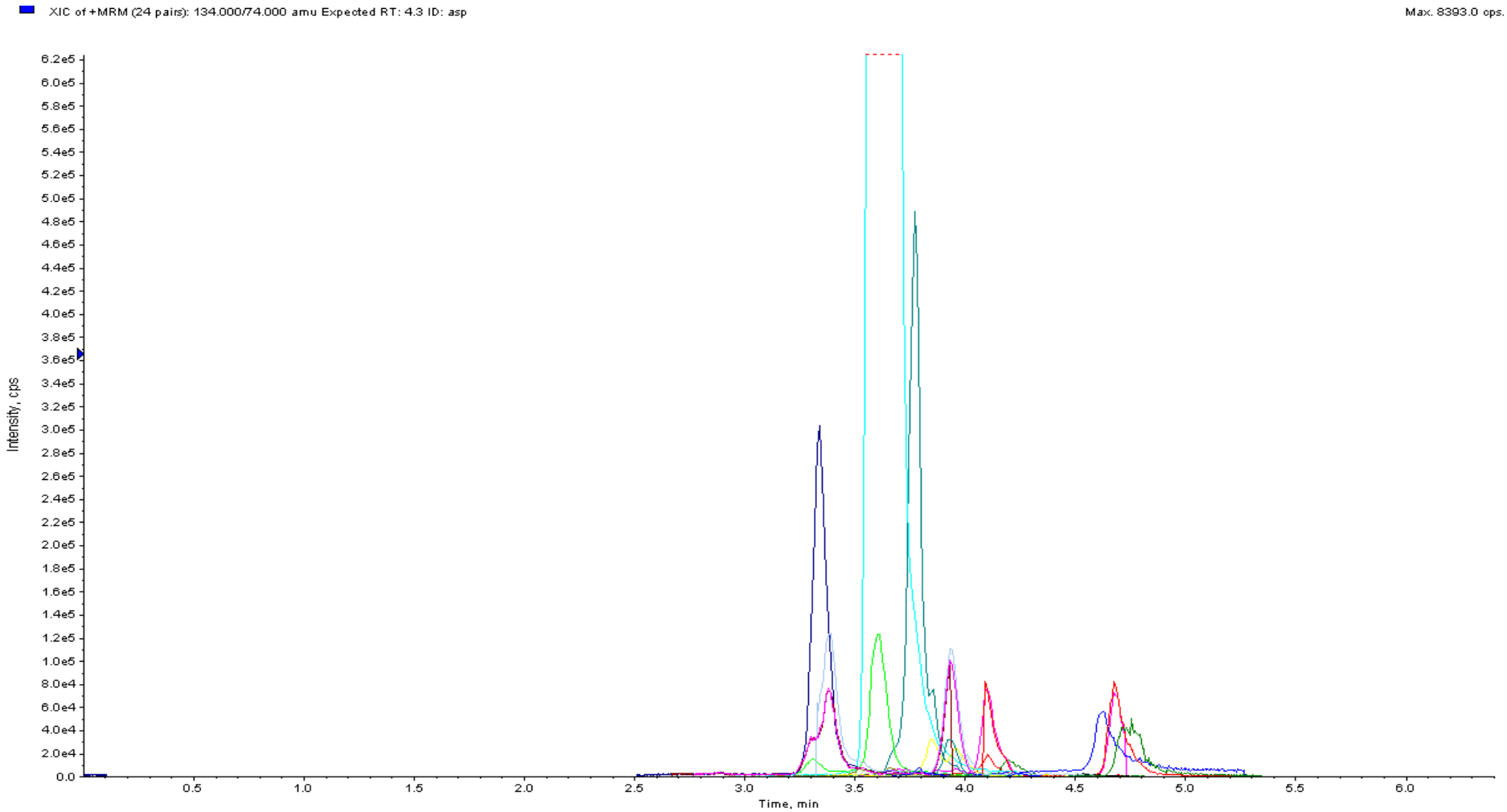
Honey ID 78



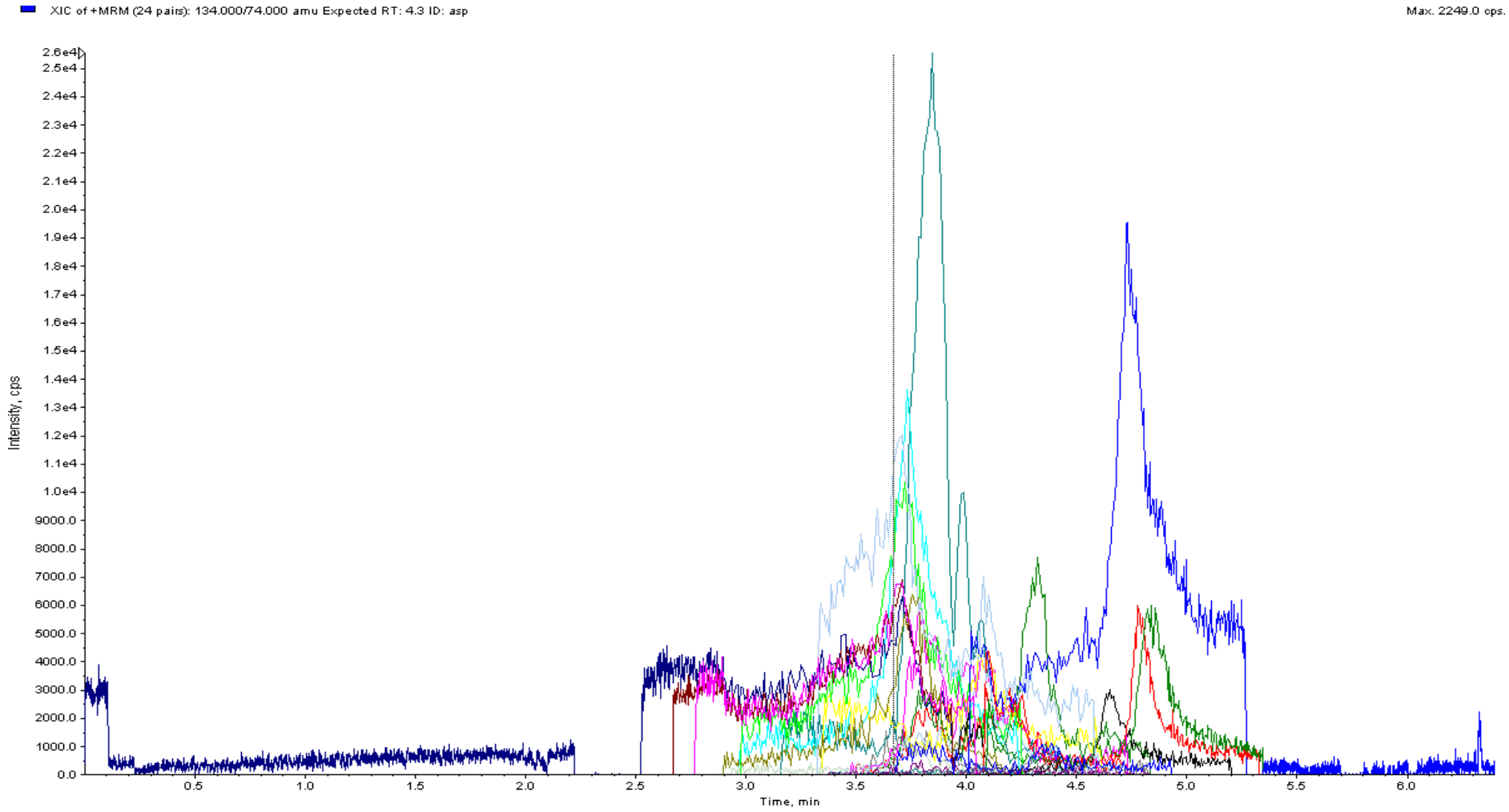
Honey ID 78 (zoomed in)


XIC of +MRM (24 pairs): 134.000/74.000 amu Expected RT: 4.3 ID: asp
Max. 8393.0 cps.
Intensity, cps
7.3e6
7.0e6
6.5e6
6.0e6
5.5e6
5.0e6
4.5e6
4.0e6
3.5e6
3.0e6
2.5e6
2.0e6
1.5e6
1.0e6
5.0e5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
Time, min

Honey ID 84

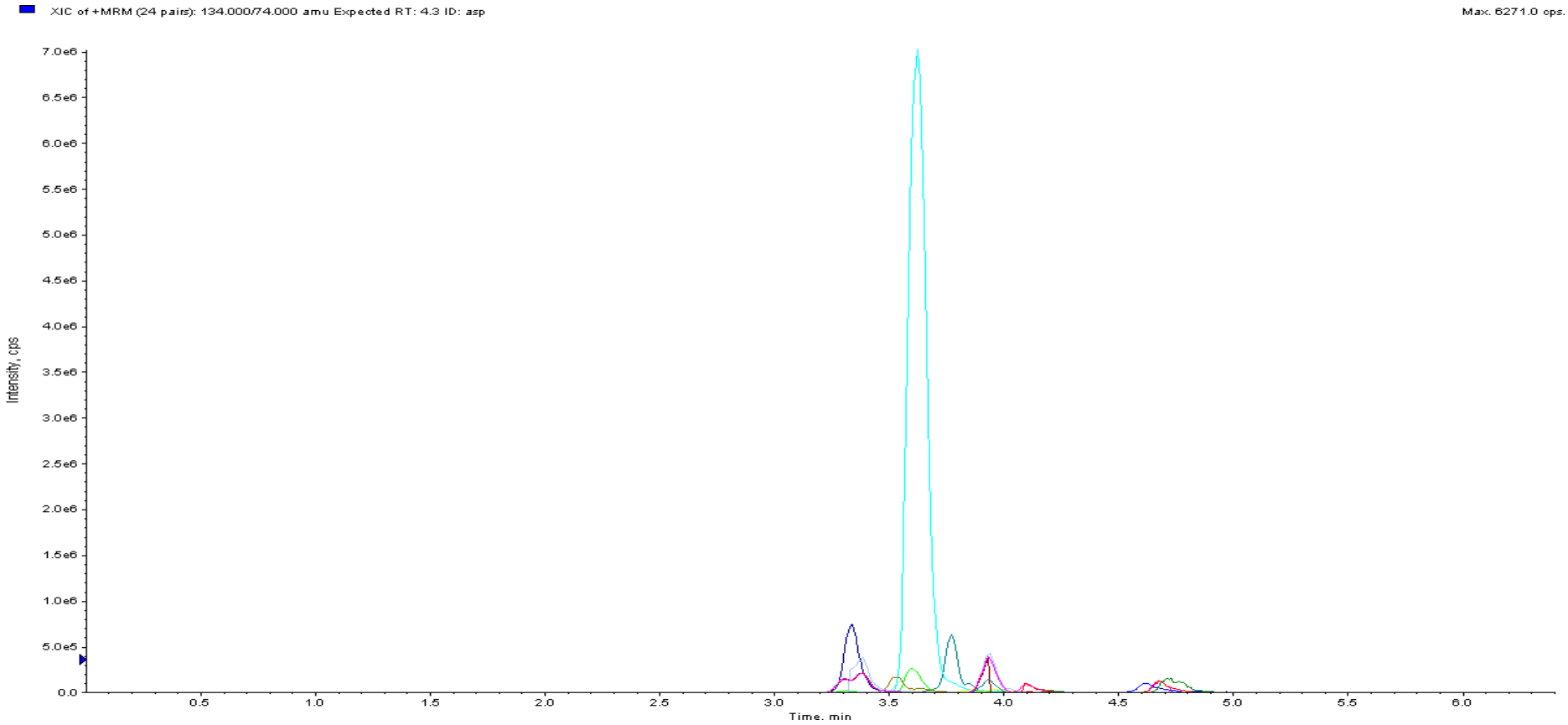


Honey ID 84 (zoomed in)

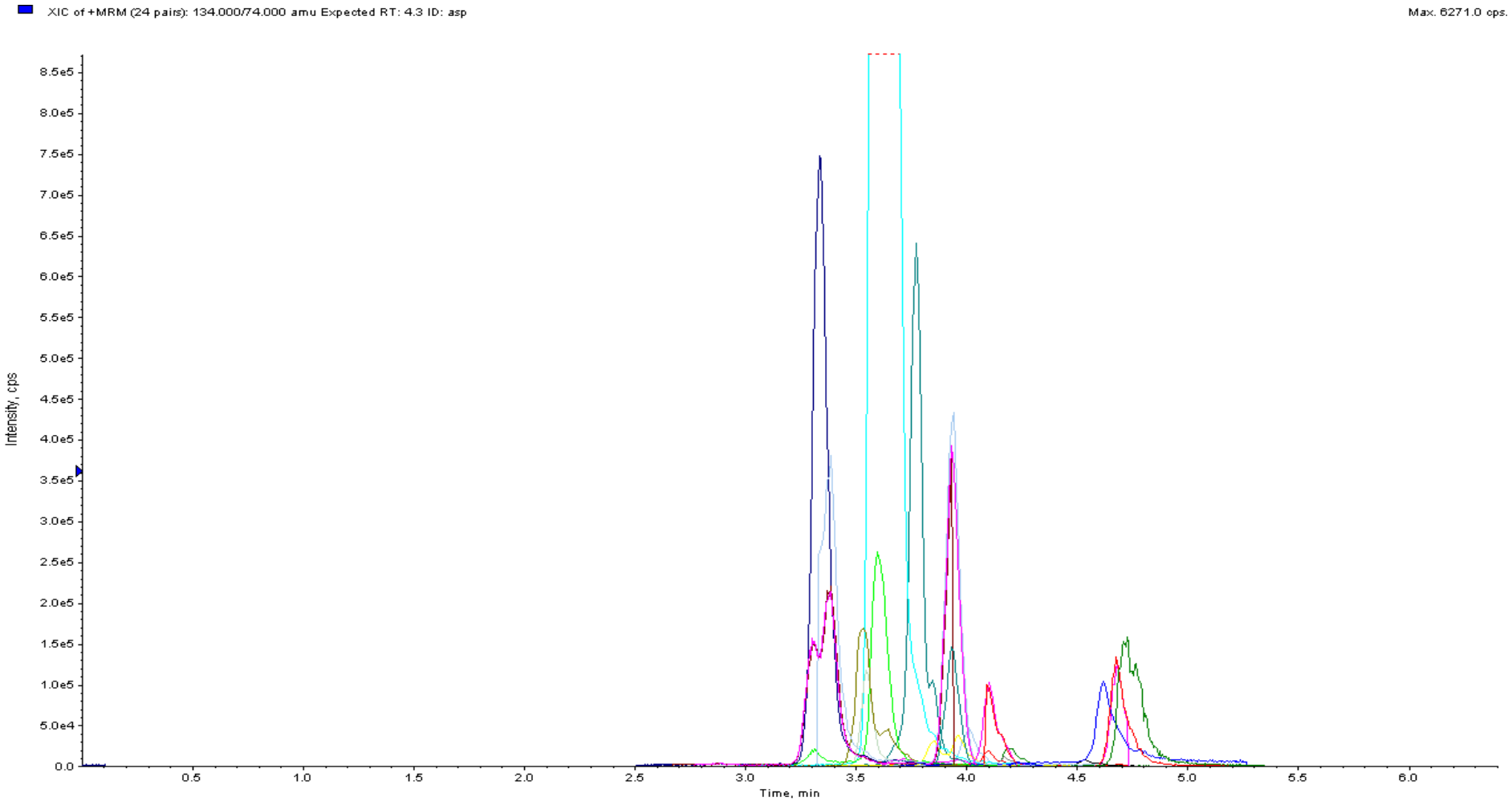


Artificial Honey (no spike)

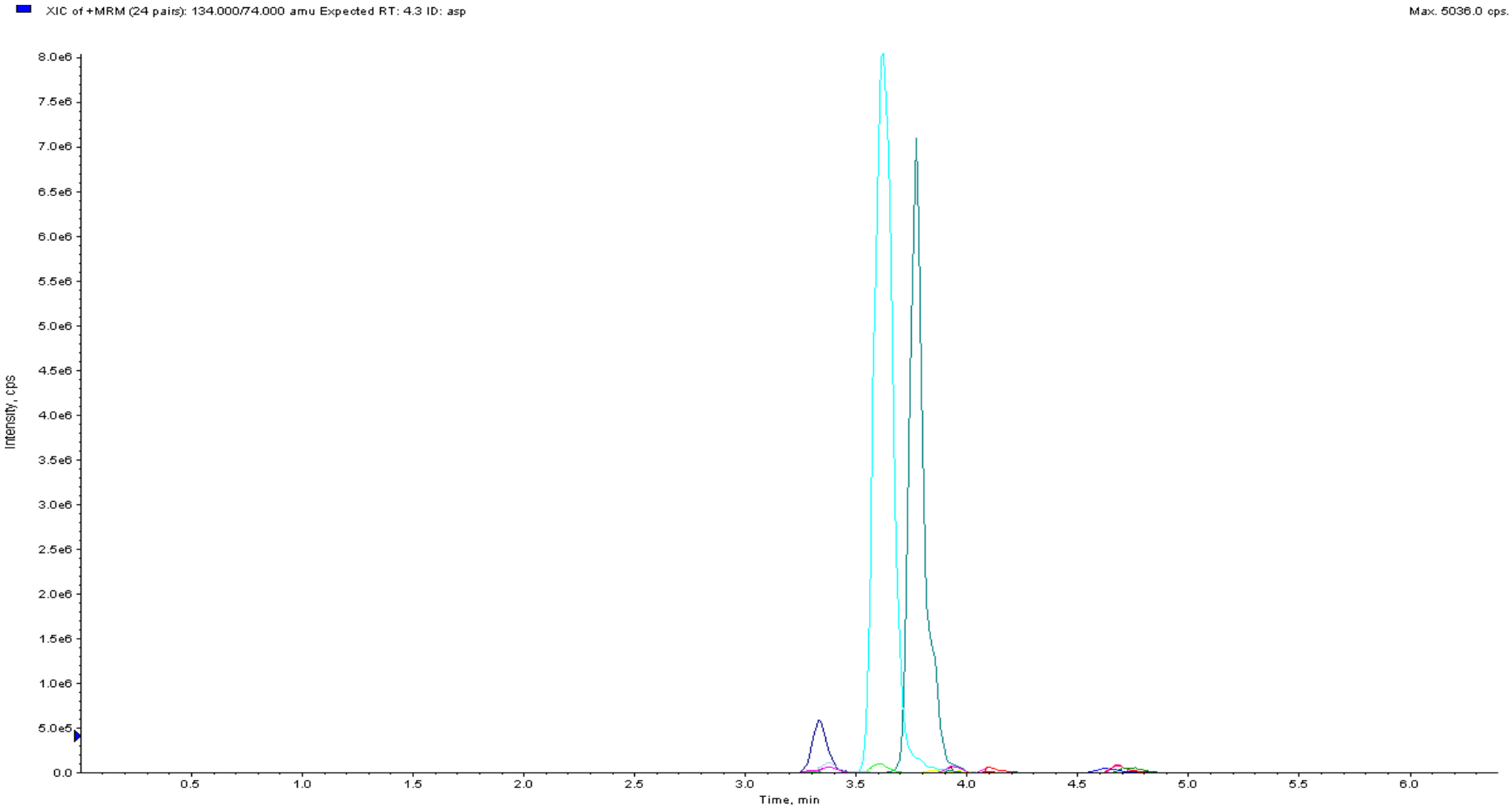
Appendix 8: Chromatograms of Spike Recoveries for HILIC Method



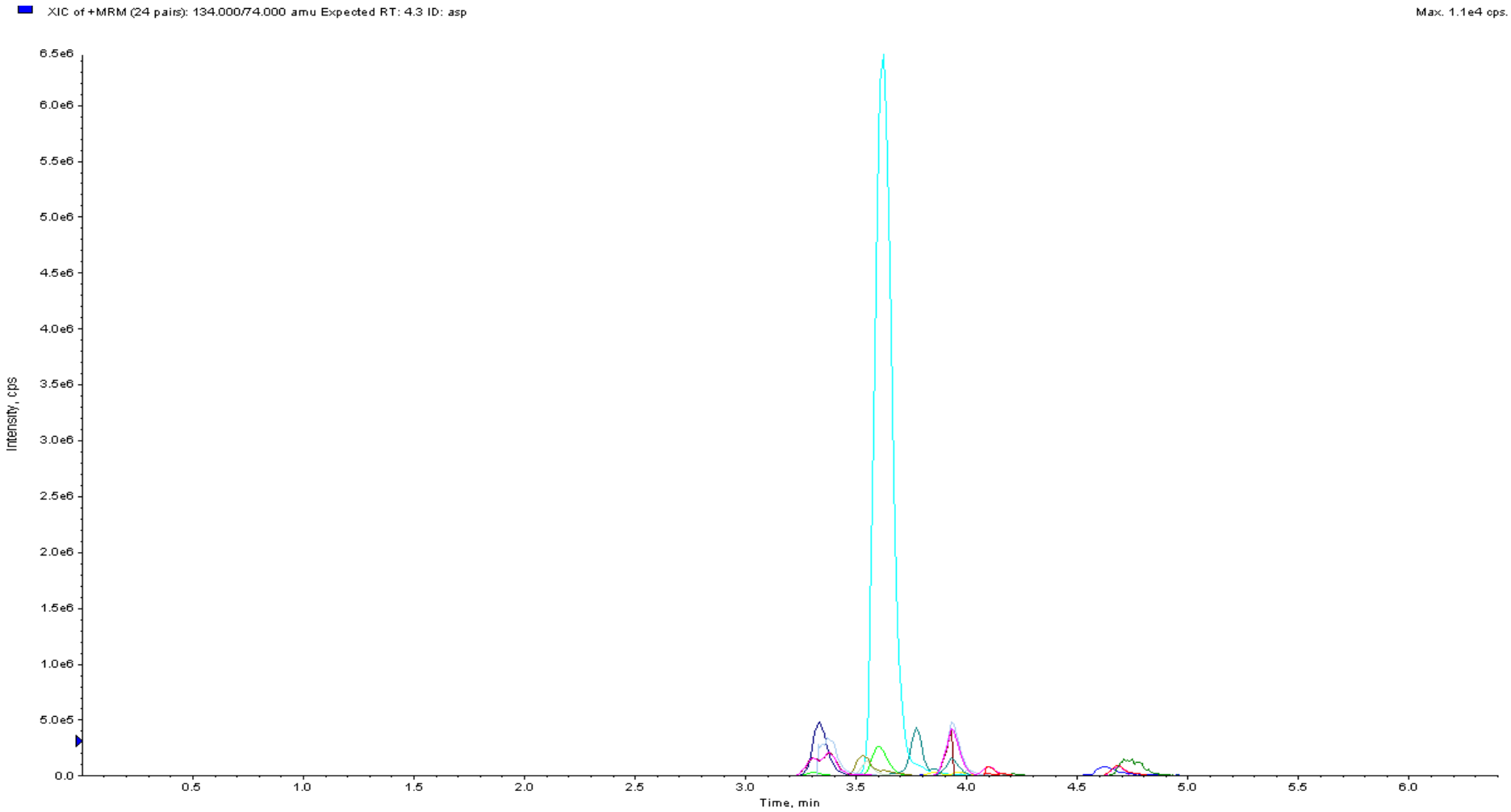
Honey ID 66, 20 amino acid spike 100 mg/L total



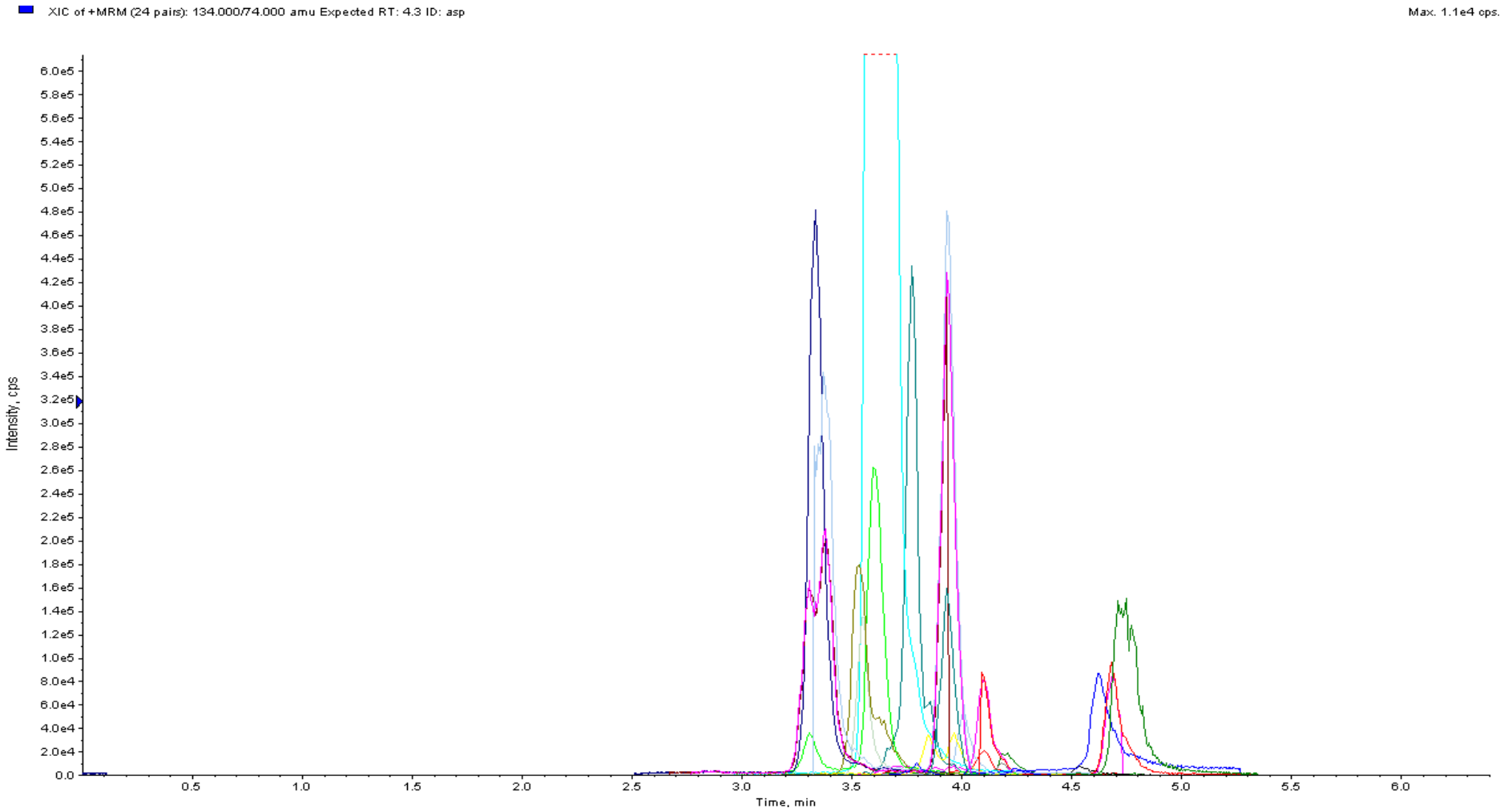
Honey ID 66, 20 amino acid spike 100 mg/L total (zoomed in)



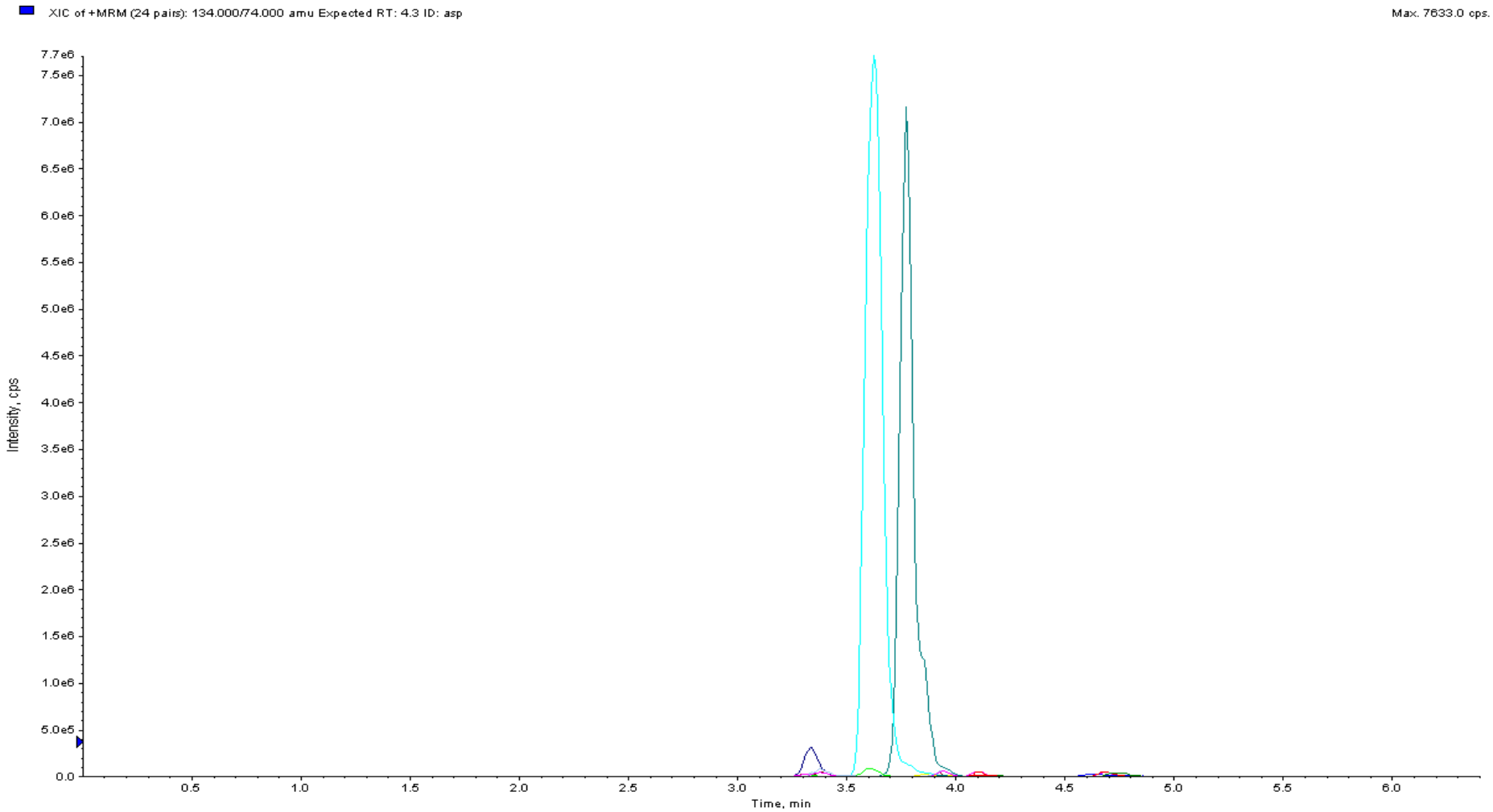
Honey ID 66, proline/tyrosine spike 500 mg/L total



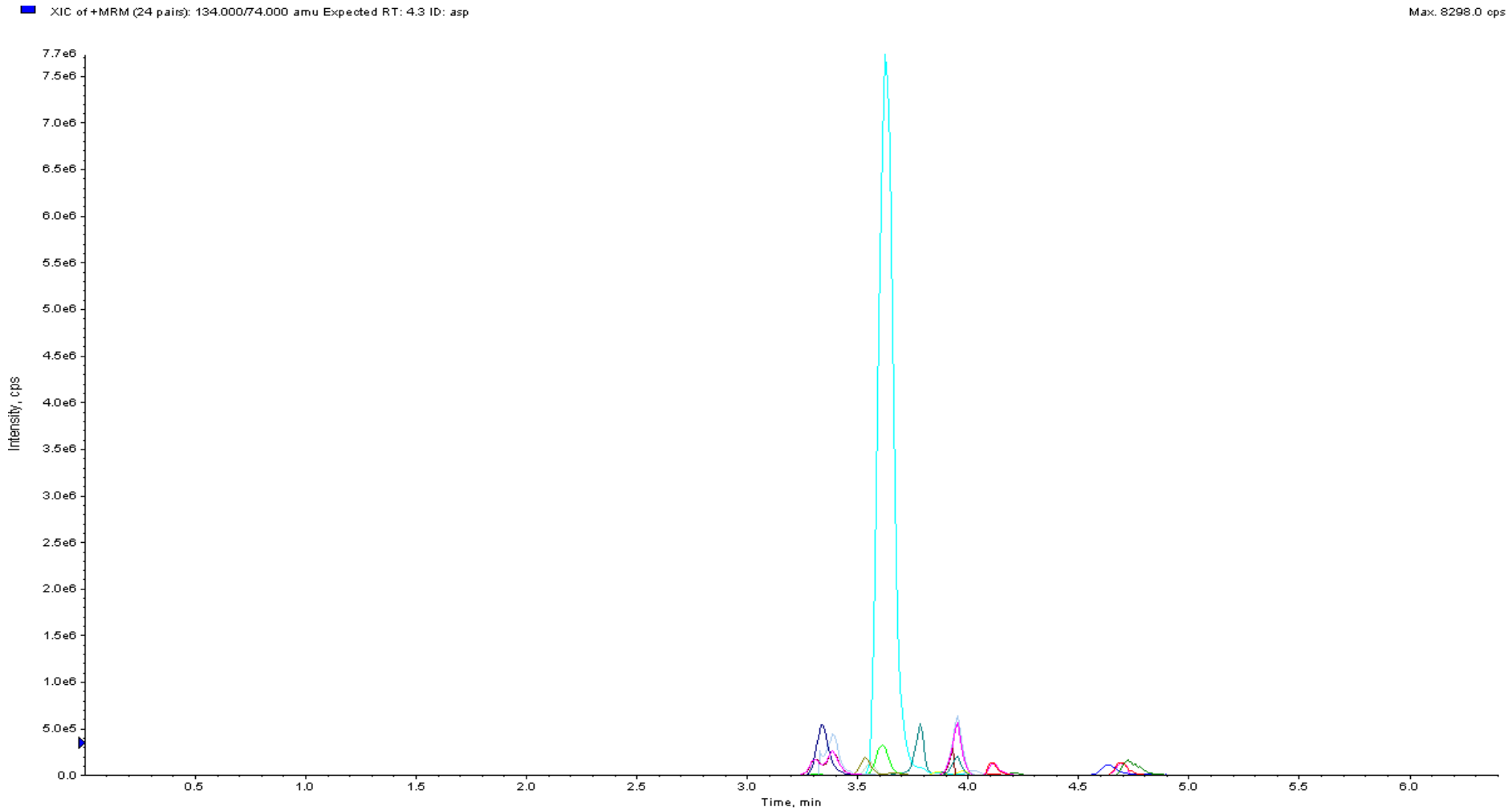
Honey ID 78, 20 amino acid spike 100 mg/L total



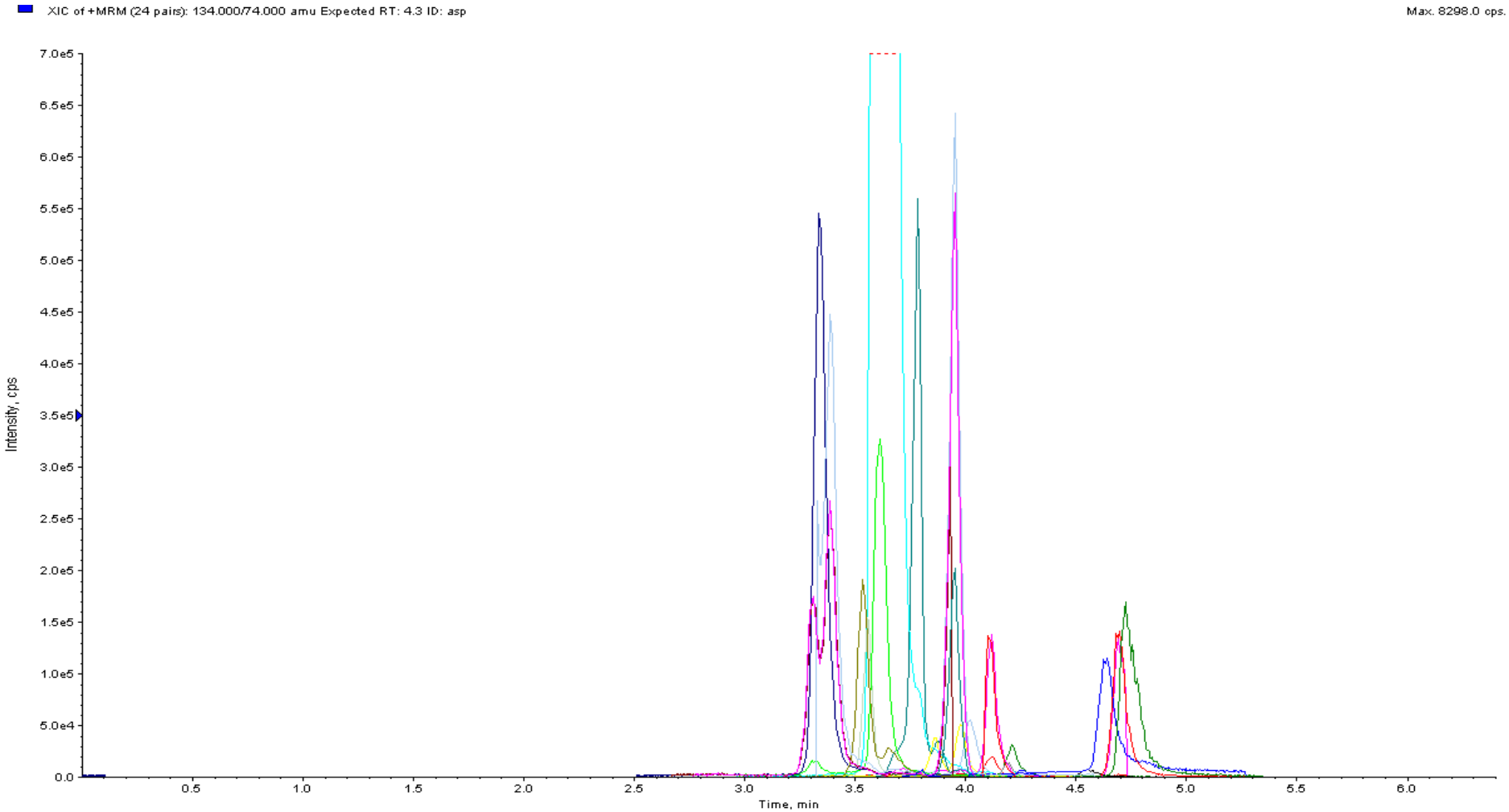
Honey ID 78, 20 amino acid spike 100 mg/L total (zoomed in)



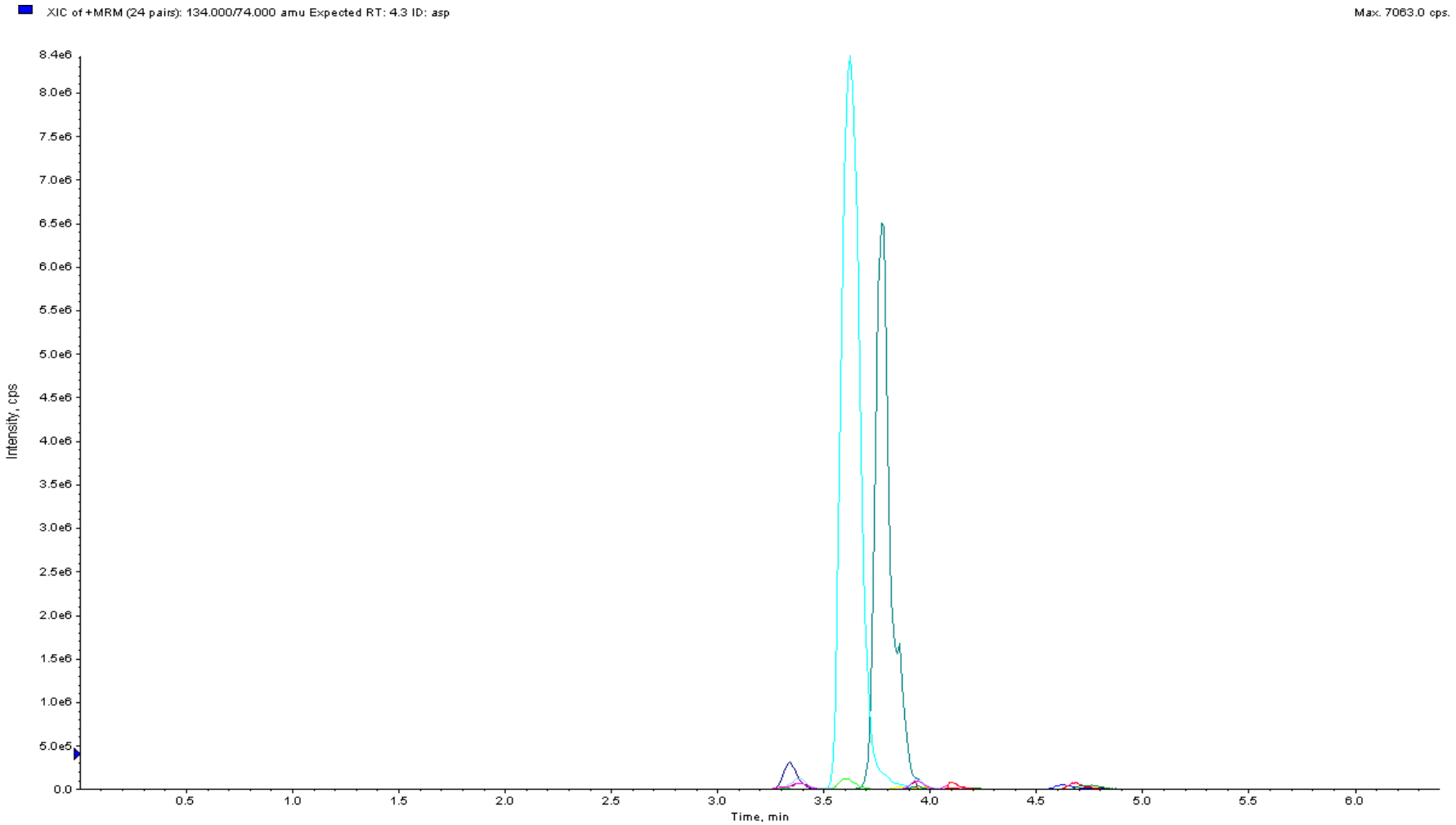
Honey ID 78, proline/tyrosine spike 500 mg/L total



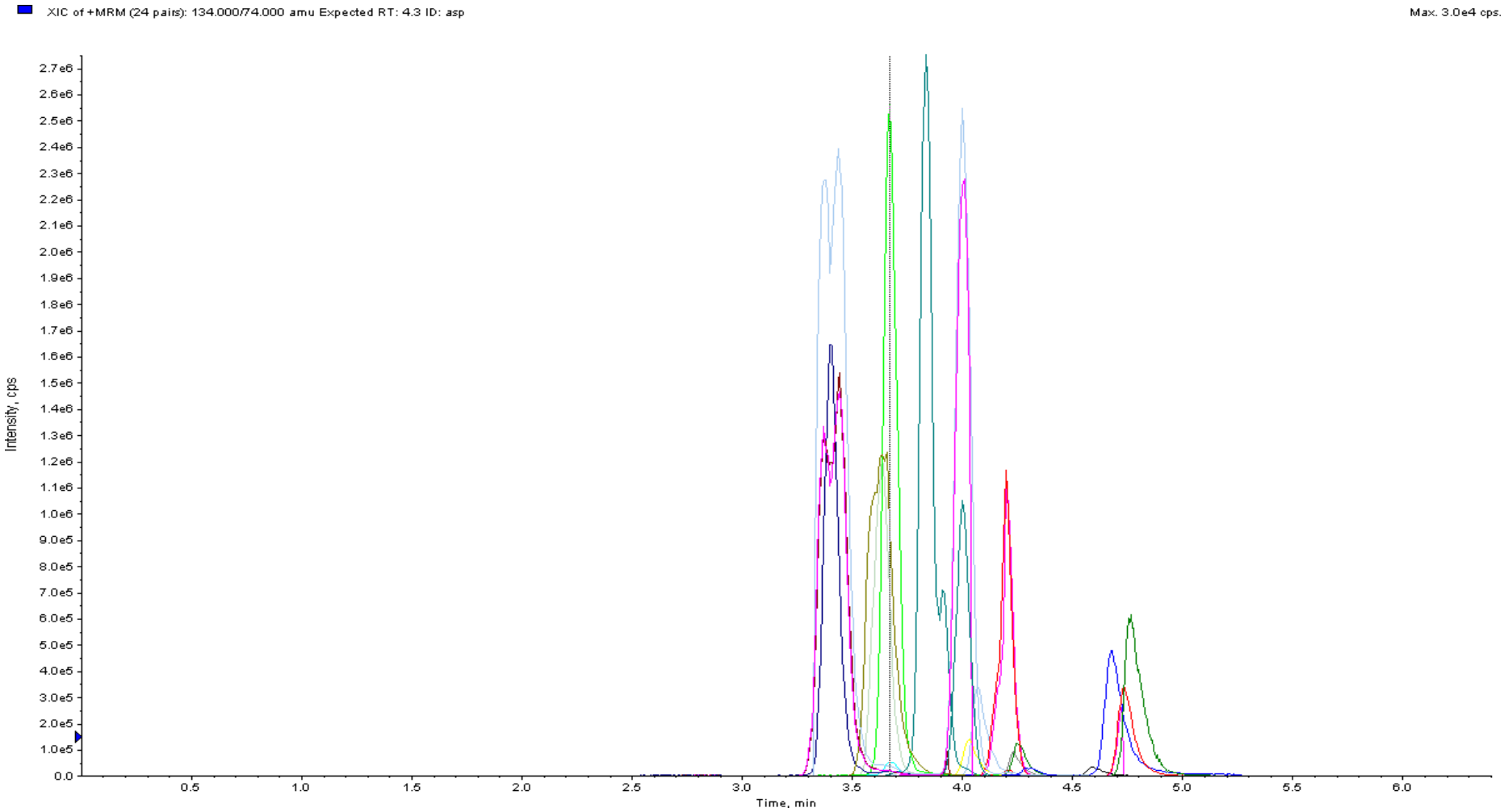
Honey ID 84, 20 amino acid spike 100 mg/L total



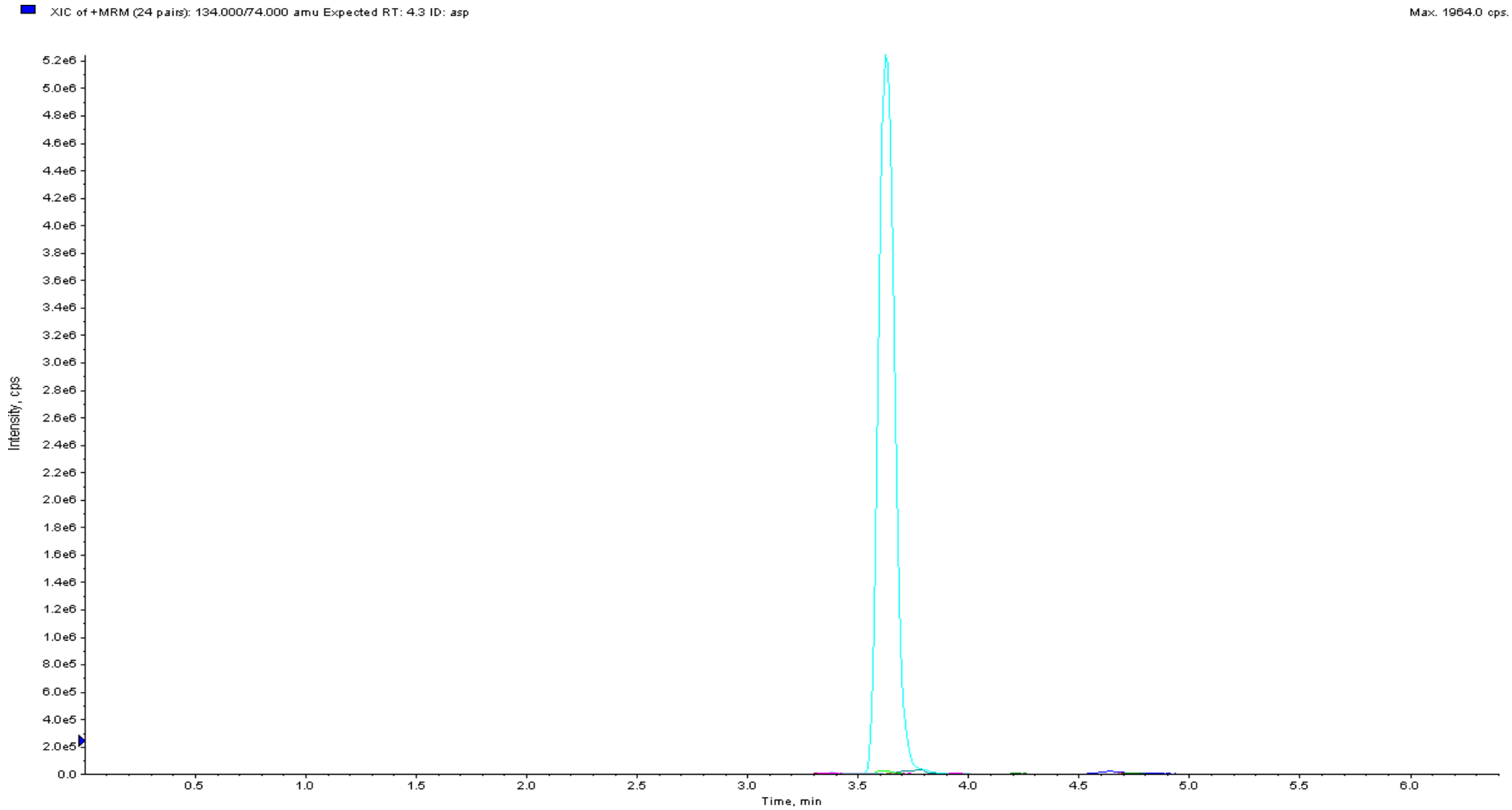
Honey ID 84, 20 amino acid spike 100 mg/L total (zoomed in)



Honey ID 84, proline/tyrosine spike 500 mg/L total



Artificial Honey, 20 amino acid spike 100 mg/L total



Artificial Honey, proline spike 250 mg/L

Appendix 9: Method Hill Laboratory

Analysis of Amino Acids in Honey by LC-MS/MS

Method Code:

1. Principles and Purpose of Test

The purpose of this method is to analyse 22 amino acids in honey. The method uses an aTRAQ™ kit from AB Sciex to derivatise amino acids in aqueous honey solutions. The aTRAQ™ kit provides aTRAQ™ reagent Δ8 for labelling samples and a mixture of Δ0-labelled amino acids as internal standards. Samples are prepared by pipetting; mixing diluted honey with the Δ8 derivatising agent and adding the Δ0 internal standard. Samples are then analysed by LC-MS/MS with MRM, and quantitation is achieved by comparing the Δ8-labelled peak areas from the sample to the equivalent Δ0-labelled internal standard peak areas. A certificate of internal standard concentrations is provided with each kit for quantitative calculations.

1.1 Summary of Modifications to Standard or Reference Method

The following changes were made to the reference method.

After adding the internal standard; the volume was not reduced, and water (148 µL) was added. This was done initially because the volume was already too small and the high sensitivity of the Triple Quad™ 4500 system requires the sample to be diluted so the detector is not saturated.

Deletion of the sulfosalicyclic acid step. This step precipitates protein, so both free and bound amino acids are characterised. For the results to be relevant, only free amino acids must be characterised. This also means that no norleucine standard, an amino acid in the sulfosalicyclic acid solution that indicates recovery of precipitated protein, will show up in the analysis.

Chromatograms showed large peak differences between the amino acids and their internal standards, and the two needed to be changed to similar levels to increase the reliability of their comparison. This was resolved by changing the following; doubling the honey sample addition, and halving the internal standard addition. This improved all amino acids, with peaks yielding similar heights, except for proline, which is present at relatively high concentrations in honey. The response for proline is approximately ten times larger than for the other amino acids, and so the proline internal standard peak is relatively smaller. This change to proline did not have a large effect on the reliability of the results.

2. Scope and Limitations

The purpose of this method is to analyse 22 amino acids in Honey. The amino acids analysed are: alanine, Ala; arginine, Arg; asparagine, Asn; aspartic acid, Asp; glutamic acid, Glu; glutamine, Gln; glycine, Gly; histidine, His; hydroxyproline; isoleucine, Ile; leucine, Leu; lysine, Lys; methionine, Met; phenylalanine, Phe; proline, Pro; serine, Ser; threonine, Thr; tryptophan, Trp; tyrosine, Tyr; valine, Val; methionine sulfoxide, MOx.

The amino acids content of honey can be used to indicate floral origin. All amino acids are within the range of 0.5-20 mg/kg, with the exception of proline that can be 200-500 mg/kg.

Minimum sample size would be 5 g of honey.

3. Interferences

Instrument chromatographic interferences are minimised due to the specificity provided by LC-MS/MS. The aTRAQ™ reagent derivatises acid groups of amino acids, and potential interferences from other organic acids are avoided as they would have different MRMs.

4. Quality Control

QC Type	QC Name	Frequency analysed	Use of QC and frequency reviewed	QC data location
Blanks	Blank	Per worksheet	Reagents contamination, per WS	LabSys
Replicates	Replicate	Per worksheet	UoM, per WS	LabSys
Spikes	N/A			
Reference Materials	N/A			
In-house QC	QC honey	Per worksheet	Accuracy/bias, per WS	LabSys
ILCPs	If available		Accuracy/bias	
Surrogates	Norvaline	Per sample	Monitors derivatisation	LabSys

Note: The pass/fail criteria should be defined in the QC data location and the relevant worksheet. The actions to take in the event of QC failure should be defined in the "Procedure" section below.

5. Health and Safety

5.1 Reagents (including Solvents) and Standards

Reagent	MSDS KBI	Hazards	Handling
Heptafluorobutyric acid			Avoid breathing vapours. Protect eyes and skin.
Formic acid	6882		**Can form explosive vapour/air mixtures above 69°C.** Avoid breathing vapors. Protect eyes and skin.
Methanol	6941		Protect eyes.
aTRAQ reagent Δ8			Toxic. Protect eyes and skin.
Isopropanol			Protect eyes.

5.2 Equipment

Refer to LC-MS/MS user manuals.

Beware of moving parts on the autosamplers.

Equipment	SOP KBI	Hazard	Handling
Centrifuge			Follow manufacturer's instructions.

5.3 Samples

Samples may contain chemical residues. Gloves should be worn when handling samples.

6. Reagents

6.1 Supplied.

Equivalent reagents may be used in place of any of these items at the discretion of the Method Expert.

Methanol. LiChrosolv grade (Merck 1.06018.2500).

Type-1 water. From Sartorius, Arium 611 water purification system.

Formic Acid. Analysis grade (BDH, 101145D).

The aTRAQ™ Starter Kit Hydrosylate provided by AB Sciex contained; the aTRAQ (derivatising) reagent Δ8, labelling buffer (borate buffer, pH 8.5), hydroxylamine (1.2% solution), isopropanol, mobile phase A (100% formic acid) and B (100% heptafluorobutyric acid), internal standard, unlabeled standard, and standard diluent (2% formic acid).

7. Standards

The aTRAQ™ Starter Kit Hydrosylate contains the internal standard. The kit supplied a certificate of analysis for the reconstituted internal standard.

8. Apparatus, Equipment and Glassware

Air displacement micropipette, centrifuge, Eppendorf tubes (1.5 mL and 50 mL), chopsticks and vortex. aTRAQ™ kit must be frozen (stored at < 15 °C) when not in use.

9. Procedure

9.1 Sampling and Sample Storage

Honey must be stored frozen at all times possible. Before sub-sampling, warm to room temperature, then mix thoroughly with a chop-stick.

9.2 Worksheeting (Batching/Sequencing)

A batch should comprise 2 injections of the Blank, followed by the QC honey, then the sequence of samples and replicates.

9.3 Sample Preparation

Honey samples are diluted 1:40 with water and shaken on a flat-bed shaker until dissolved. Honey solution sub-samples (20 µL) are added to labelling buffer (40 µL) in a 1.5 mL Eppendorf tube, then the pipette tip rinsed five times with the solution in the tube, vortexed (30 sec, 1000 rpm) and centrifuged (2 min, 10,000 rpm). 12 µL of this mixture is transferred to a new 1.5 mL Eppendorf tube, to which 5 µL of the aTRAQ™ reagent Δ8 is added, then vortexed and spun. After waiting (30 min), hydroxylamine (5 µL) is added to the tube, vortexed and spun. After waiting (15 min), 16 µL of internal standard is added, vortexed and spun. All contents are transferred to a 2-mL glass vial and 150 µL of water is added and mixed by vortexing.

9.4 Disposal of Reagents and Samples

No special disposal is required.

9.5 Instrument Set-up

Chromatographic separations are performed on an Amino Acid Analyser (AAA) C18 reversed-phase column (150 x 4.6 mm, 5 µm). A HPLC system from Agilent Technologies 1200 series consisting of a degasser, binary pump, and thermostated column compartment is used, with an autosampler from Pal System, PAL HTS-xt, and detection with an AB Sciex Triple Quad™ 4500. Analysis of ions is carried out by multiple reaction monitoring (MRM).

9.6 Analysis and Instrumental Parameters

A gradient mixture of 0.1% formic acid and 0.01% heptafluorobutyric acid in water (A) and 0.1% formic acid and 0.01% heptafluorobutyric acid in methanol (B) is used at a column temperature of 50°C. The gradient program is described in Table 1. The scheduled MRM values for mass spectra analysis is in Appendix 1 along with the relevant structure in Appendix 2.

Table 1: Gradient program

Time	**Solvent**		
(min)	Flow rate (µL/min)	A%	B%
0	800	98	2
6	800	60	40
10	800	60	40
11	800	10	90
12	800	10	90
13	800	98	2
18	800	98	2

9.7 Calibration

Internal standards for each amino acid are provided with the kit and used for calibration. A certificate with concentrations for the internal standards comes with each kit, and needs to be used to calculate analyte concentrations.

9.8 Data Processing

Analysis software is provided by MultiQuant.[1]
Amino acid peak areas are determined in MultiQuant and exported to Excel where calculations take place.

9.9 Data Verification and Approval

Amino ISTD peak areas should be compared with those from a reference chromatogram to check instrument sensitivity.

.

9.10 Method QC Assessment (including limits and actions on QC failure)

The blank should have levels of amino acids 10-fold lower than the detection limits, or 10-fold lower than the lowest level found in samples in the batch.

The QC honey should fall with action limits.

Replicates should be within 40% of each other, for all amino acids found above the detection limits.

The surrogate (norvaline) should be within action limits, showing that the derivatisation has been successful, and dilutions accurate.

[1] MRM allows the user to set a quadrupole filter to select for the labelled amino acid (precursor ion Q1) which is fragmented and a second quadrupole filter to select for the cleaved aTRAQ™ Reagent label (product ion Q3) for detection. Scheduled MRM sets a window of detection around the retention time, which it monitors for the specific labelled amino acids.

10. Calculations (including the Reporting and Expression of Results).

1) Concentration of AA in-vial (µmol/L) = peak area of AA from sample/ISTD peak area × conc of ISTD (µmol/L)/2

2) Conversion to µg/L = conc (µmol/L) × M_r (g/mol)

3) Derivatisation dilution factor = 4/1.6 = 2.5

4) Honey solution concentration (g/L) = weight of honey (g)/0.04 (L)

5) AA concentration in honey (mg/kg) = AA conc. in-vial (ug/L) / (Honey Solution Concentration [g/L] * Deriv. dilution factor)

Example calculations for honey 946, for the amino acids aspartic acid and glutamic acid, are in Appendix 3. Final calculations and results of this are given in Table 2 and Table 3, respectively.

Table 2: Final calculations of data

Sample Name	Amino acids (mg/kg)	
	Asp	Glu
946a	18.28	12.65
946b	23.99	14.26
946c	20.76	12.80
Average	21.01	13.24
SD	2.863496	0.892016
CV	0.136304	0.06739
%CV	14%	7%

Table 3: Final results

Sample Name	Amino acids (mg/kg)	
	Asp	Glu
946	21.01	13.24
%CV	14%	7%

11. Performance Characteristics and Criteria

Detection limits (LOD and LOQ) are given in Table 4 and measurement precision and uncertainty in Table 5.

Table 4: The S/N, LOD, and LOQ of amino acids

Amino Acid	S/N[1]	LOD[2] (mg/kg)	LOQ[3] (mg/kg)
Asp	0.035	0.796	2.652
Glu	0.030	0.544	1.813
Ser	0.052	0.821	2.738
Thr	0.068	0.410	1.367
Tyr	0.084	0.290	0.968
Gly	0.061	0.654	2.180
Pro	0.002	0.359	1.197
Ala	0.008	0.114	0.379
Met	0.469	0.392	1.306
Val	0.018	0.101	0.337
Phe	0.063	0.336	1.120
Leu	0.102	0.253	0.843
MOx	0.232	0.406	1.355
Cys	2.770	0.309	1.029
His	0.054	0.302	1.006
Lys	0.168	2.028	6.759
Arg	0.034	0.153	0.510
Nva	0.003	0.298	0.993
Ile	0.087	0.290	0.967
Asn	0.095	0.670	2.232
Gln	0.046	0.729	2.430
Trp	0.477	0.713	2.377
HydPro	0.173	0.197	0.657

[2] S/N was calculated by: noise height x 3/signal height.
[2] LOD was calculated by: S/N x amino acid content in mg/kg.
[3] LOQ was calculated by: LOD x 10 / 3.

Table 5: Method precision data for an mānuka honey

Amino Acids (mg/kg)	Mean (mg/kg)	SD	% SD
Asp	19.15	4.37	23%
Glu	13.34	2.39	18%
Ser	21.60	8.74	40%
Thr	9.23	2.34	25%
Tyr	16.38	2.70	16%
Gly	11.58	5.14	44%
Pro	616.37	166.63	27%
Ala	28.32	6.30	22%
Met	0.67	0.10	14%
Val	11.36	2.05	18%
Phe	77.98	17.88	23%
Leu	7.68	2.16	28%
His	16.13	3.67	23%
Lys	40.86	6.88	17%
Arg	8.86	2.25	25%
Ile	8.36	1.21	14%
Asn	12.28	1.77	14%
Gln	20.58	2.47	12%
Trp	3.33	0.76	23%
HydPro	9.53	1.13	12%

12. References

Reference	KB Item # / Location
AB Sciex aTRAQ method. http://www.absciex.com/Documents/Downloads/Literature/4445543C.pdf	

13. Notes

N/A

14. Appendices

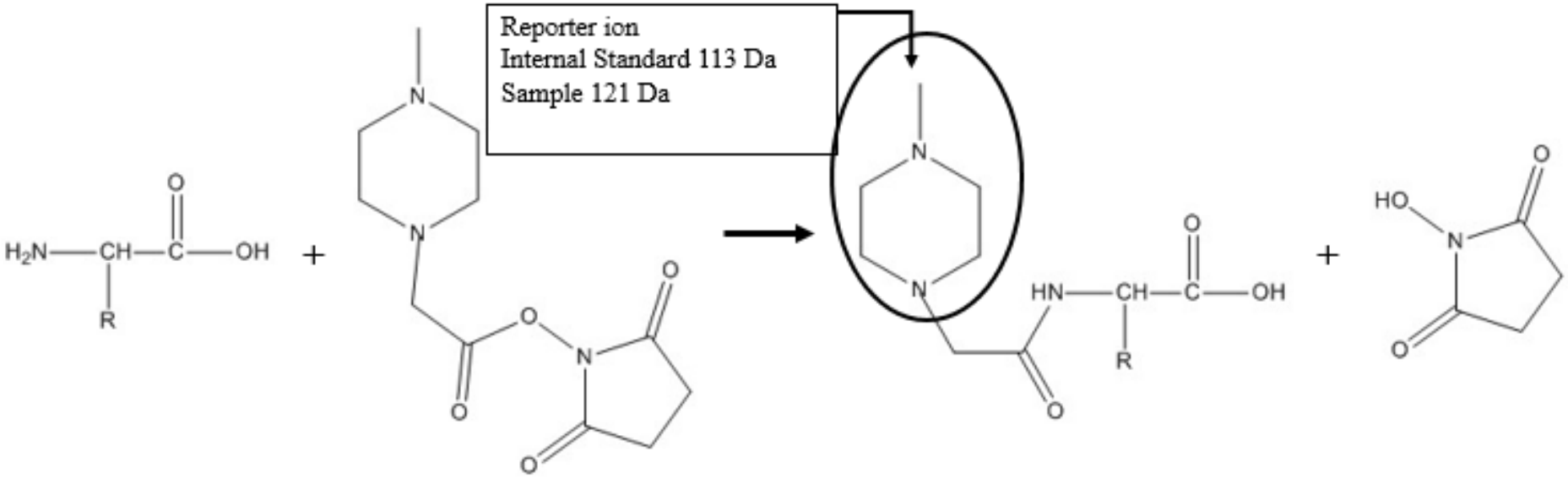
Appendix 1: Scheduled MRM of the 24 amino acids and their corresponding internal standards

ID	Q1 Mass (Da)	Q3 Mass (Da)	Retention Time (min)	Collision Energy	Entrance Potential	Decluster Potential	Collision Cell Exit Potential
Asp	282.1	121.1	3.7	30	10	30	5
Glu	296.2	121.1	4.3	30	10	30	5
Ser	254.2	121.1	3.4	30	10	30	5
Thr	268.2	121.1	4.2	30	10	30	5
Tyr	330.2	121.1	6.8	30	10	30	5
Gly	224.1	121.1	3.6	30	10	30	5
Pro	264.2	121.1	5.3	30	10	30	5
Ala	238.2	121.1	4.3	30	10	30	5
Met	298.2	121.1	6.3	30	10	30	5
Val	266.2	121.1	6.4	30	10	30	5
Phe	314.2	121.1	7.9	30	10	30	5
Leu	280.2	121.1	7.8	30	10	30	5
MOx	314.2	121.1	3.9	30	10	30	5
Cys	537.2	121.1	5.4	30	10	50	5
His	304.2	121.1	3.9	30	10	30	5
Lys	443.3	121.1	5.6	30	10	50	5
Arg	323.2	121.1	4.7	30	10	30	5
Nva	266.2	121.1	6.7	30	10	30	5

IleISTD	272.2	113.1	7.6	30	10	30	5
Ile	280.2	121.1	7.6	30	10	30	5
Nle	280.2	121.1	8	30	10	30	5
NleISTD	272.2	113.1	8	30	10	30	5
AspISTD	274.1	113.1	3.7	30	10	30	5
GluISTD	288.2	113.1	4.3	30	10	30	5
SerISTD	246.2	113.1	3.4	30	10	30	5
ThrISTD	260.2	113.1	4.2	30	10	30	5
TyrISTD	322.2	113.1	6.8	30	10	30	5
GlyISTD	216.1	113.1	3.6	30	10	30	5
ProISTD	256.2	113.1	5.3	30	10	30	5
AlaISTD	230.2	113.1	4.3	30	10	30	5
MetISTD	290.2	113.1	6.3	30	10	30	5
ValISTD	258.2	113.1	6.4	30	10	30	5
PheISTD	306.2	113.1	7.9	30	10	30	5
LeuISTD	272.2	113.1	7.8	30	10	30	5
MOxISTD	306.1	113.1	3.9	30	10	30	5
CysISTD	521.2	113.1	5.4	30	10	50	5
HisISTD	296.2	113.1	3.9	30	10	30	5
LysISTD	427.3	113.1	5.6	30	10	50	5
ArgISTD	315.2	113.1	4.7	30	10	30	5
NvaISTD	258.3	113.2	6.7	30	10	30	5
Asn	281.2	121.1	3.4	30	10	30	5
Gln	295.2	121.1	3.7	30	10	30	5

Trp	353.2	121.1	8.5	30	10	30	5
HydPro	280.1	121.1	3.5	30	10	30	5
AsnISTD	273.2	113.1	3.4	30	10	30	5
HydProISTD	272.1	113.1	3.5	30	10	30	5
GlnISTD	287.2	113.1	3.7	30	10	30	5
TrpISTD	345.2	113.1	8.5	30	10	30	5

Appendix 2: General structures of the Q1 and Q3 ions of labeled amino acid samples and standard monitored by MRM



Appendix 3: Calculations for data processing

Sample Name	**Peak Area of AA from Sample**		
	Asp	***Glu***	
946a	144413.3904	119212.037	area SAMPLE
946b	201706.1673	130673.257	
946c	161810.9451	104979.997	
	Concentration of ISTD from certificate with kit (umol/L)		
	AspISTD	***GluISTD***	
	123.6	107.6	
	61.8	53.8	stnd amount / 2
	Peak Area of ISTD		
	AspISTD	***GluISTD***	
946a	1031175.136	1183882.32	area ISTD
946b	1007857.286	1056769.38	
946c	1004910.476	1017429.42	
	AA Concentration from Sample (umol/L)		
	Asp	***Glu***	
946a	8.654928937	5.41743675	area SAMPLE / area ISTD * (stnd amount / 2)
946b	12.36826019	6.65255956	
946c	9.951052009	5.55117012	
	Molar Mass (g/mol)		
	133.1	147.13	
	AA Conc. converted to ug/L		
946a	1151.971042	797.06747	AA Conc in umol/L *molar mass
946b	1646.215431	978.791088	

946c			1324.485022	816.74366	
			Dilution consideration		
			Derivatisation Dilution Factor	Is 2.5	= 4 / 1.6
			Honey solution concentration (g/L)		Weight of honey (g)/0.04 (L)
			AA Concentration in Sample (mg/kg)		
	Weight Honey (g)	Prep factor (1 / Honey (g))	***Asp***	***Glu***	
946a	1.0084	0.99166997	18.27800145	12.646846	AA conc. in ug/L / (Honey Solution Concentration [g/L] * Deriv. dilution factor)
946b	1.098	0.91074681	23.9885673	14.2628938	
946c	1.0209	0.97952787	20.75791983	12.8003708	

<End>

Appendix 10: Validation Hill Laboratory

Method Validation: Determination of Amino Acids in Honey by LC-MS/MS

1.. Introduction

This report summarises the method performance and resulting fitness for purpose for the method "Analysis of Amino Acids in Honey by LC-MS/MS". The validation work has been carried out according to the EURACHEM Guide and the requirements outlined in the QC Manual, Chapter 5, Validation (KB Item: 2124).

This validation covers the;

Validation of a new method

The source of the method is;

A modified method from that supplied by an instrument company

1.1 Changes from the Previous Version of this Report

N/A

2.. Context of the Method Validation

2.1 Method Purpose and Scope

The purpose of this method is to analyse 22 amino acids in Honey. The amino acids analysed are: alanine, Ala; arginine, Arg; asparagine, Asn; aspartic acid, Asp; glutamic acid, Glu; glutamine, Gln; glycine, Gly; histidine, His; hydroxyproline; isoleucine, Ile; leucine, Leu; lysine, Lys; methionine, Met; phenylalanine, Phe; proline, Pro; serine, Ser; threonine, Thr; tryptophan, Trp; tyrosine, Tyr; valine, Val; methionine sulfoxide, MOx.

The amino acids content of honey can be used to indicate floral origin, or to predict formation of MGO from DHA. All amino acids are within the range of 0.5-20 mg/kg, with the exception of proline that can be 200-500 mg/kg.

2.2 Principles of the Method

The aTRAQ™ Starter Kit Hydrosylate provided by AB Sciex is used. The free amino acids are derivatised by the aTRAQ Δ8 reagent and compared to the internal standards supplied, pre-labelled by the aTRAQ Δ0 reagent. This is analysed by LC-MS/MS on a triple quad system in MRM mode. The HPLC separation is carried out in a C18 column at 50°C, with HFBA ion pairing. Quantitation is by comparison of amino acid and internal standard peak areas, using the certified concentration of each internal standard supplied with the kit.

2.3 Analytical Performance Requirements

The method should provide a robust and reproducible analysis for the amino acids present in honey. Detection limits need to be below the levels commonly found in New Zealand honeys.

2.4 Test Method Procedure

Honey samples are diluted 1:40 with water and shaken on a flat-bed shaker until dissolved. Honey solution sub-samples (20 µL) are added to labelling buffer (40 µL) in a 1.5 mL Eppendorf tube, then the pipette tip rinsed five times with the solution in the tube, vortexed (30 sec, 1000 rpm) and centrifuged (2 min, 10,000 rpm). 12 µL of this mixture is transferred to a new 1.5 mL Eppendorf tube, to which 5 µL of the aTRAQ™ reagent Δ8 is added, then vortexed and spun. After waiting (30 min), hydroxylamine (5 µL) is added to the tube, vortexed and spun. After waiting (15 min), 16 µL of internal standard is added, vortexed and spun. All contents are transferred to a 2-mL glass vial and 150 µL of water is added and mixed by vortexing.
Samples are injected (2 µL) onto an LC-MS/MS instrument (AB Sciex Triple Quad 4500), fitted with an Amino Acid Analyser (AAA) C18 reversed-phase column (150 x 4.6 mm, 5 µm). Mobile phase A is 0.1% formic acid and 0.01% heptafluorobutyric acid in water and mobile phase B is 0.1% formic acid and 0.01% heptafluorobutyric acid in methanol.

2.5 Validation Methodology

To analyse the precision of the instrument, one sample was injected eight consecutive times and the chromatographic peaks analysed.

To investigate the precision of the method, one honey was analysed in triplicate, once a week, over four weeks. The variation of the amino acid content, as standard deviations, is given.

The limit of detection and the limit of quantification of each amino acid are calculated by comparing the signal to noise ratios (S/N) for a given honey.

The carryover of the instrument was analysed by running a blank water sample after a standard. The treatment of blanks was prepared using the same methodology as that used for standards and samples.

3.. Method Performance Characteristics

3.1 Identification of the Measurand

3.1.1 Confirmation of Identity, Selectivity, Specificity and Interferences

Instrument chromatographic interferences are minimised due to the specificity provided by LC-MS/MS, and the MRM transitions used. Amino acids are identified by their MRMs and retention times matching those of the equivalent Δ0 labelled internal standards. The aTRAQ™ reagent derivatises acid groups of amino acids, and potential interferences from other organic acids are avoided as they would have different MRMs.

3.2 Working and Calibration Range

Calibration is provided by the internal standards for each amino acid, added during sample preparation. Calibration range is between the instrument detection limits 1×10^7 peak height, above which saturation starts to occur, giving non-linearity.

3.3 Accuracy

3.3.1 Precision (Standard Deviation)

Instrument precision

For 8 consecutive instrument injections of the same vial, the amino acid levels, the retention times, start and end times, and the height and area ratios of derivatised amino acids are compared to the corresponding internal standard.

The data for all amino acids, illustrating the mean values and coefficients of variation (CVs) for each category, are shown in Tables 1 and 2.

Table 1: Precision of instrument (8 consecutive injections) for all amino acids, showing: concentration of amino acids, retention time, start and end time of peaks.

Amino Acid	Concentration in honey		Amino Acid	Retention Time (min)		Start Time of Peak (min)		End Time of Peak (min)	
	Mean(mg/kg)	CV		Mean	CV	Mean	CV	Mean	CV
Asp	23.49	2.36%	Asp	3.821	0.22%	3.720	0.40%	3.973	0.43%
Glu	17.78	1.99%	Glu	4.469	0.27%	4.338	0.44%	4.601	0.24%
Ser	14.61	2.36%	Ser	3.506	0.19%	3.403	0.38%	3.675	0.55%
Thr	6.37	3.49%	Thr	4.388	0.27%	4.196	0.54%	4.516	0.09%
Tyr	3.18	3.87%	Tyr	6.975	0.10%	6.893	0.14%	7.058	0.24%
Gly	10.23	2.28%	Gly	3.710	0.24%	3.541	0.40%	3.889	0.82%
Pro	166.75	0.84%	Pro	5.446	0.19%	5.301	0.24%	5.617	0.08%
Ala	14.18	1.02%	Ala	4.508	0.29%	4.378	0.29%	4.620	0.29%
Met	0.75	7.29%	Met	6.521	0.14%	6.422	0.35%	6.612	0.11%
Val	5.57	1.93%	Val	6.641	0.12%	6.525	0.25%	6.759	0.11%
Phe	5.45	8.39%	Phe	8.079	0.07%	7.978	0.20%	8.203	0.14%
Leu	2.48	7.26%	Leu	8.026	0.08%	7.946	0.15%	8.107	0.16%
His	6.47	5.23%	MOx	4.024	0.22%	3.920	0.51%	4.144	0.21%
Lys	11.53	6.44%	His	4.080	0.34%	3.972	0.37%	4.208	0.34%
Arg	4.40	2.54%	Lys	5.818	0.25%	5.751	0.30%	5.907	0.17%
Ile	3.17	5.58%	Arg	4.804	0.16%	4.754	0.19%	4.909	0.29%
Asn	7.13	5.24%	Nva	6.876	0.13%	6.760	0.13%	7.016	0.02%
Gln	15.99	2.05%	IleISTD	7.846	0.10%	7.749	0.09%	7.939	0.11%
HydPro	0.97	13.48%	Ile	7.844	0.10%	7.773	0.11%	7.937	0.16%

	AspISTD	3.818	0.22%	3.726	0.19%	4.013	0.01%
	GluISTD	4.466	0.26%	4.310	0.43%	4.611	0.16%
	SerISTD	3.504	0.19%	3.390	0.46%	3.665	0.51%
	ThrISTD	4.386	0.28%	4.200	0.46%	4.518	0.01%
	TyrISTD	6.973	0.11%	6.863	0.11%	7.107	0.12%
	GlyISTD	3.709	0.25%	3.579	0.39%	3.906	0.29%
	ProISTD	5.446	0.20%	5.307	0.33%	5.616	0.08%
	AlaISTD	4.505	0.30%	4.384	0.27%	4.615	0.30%
	MetISTD	6.517	0.13%	6.411	0.20%	6.618	0.04%
	ValISTD	6.639	0.12%	6.511	0.22%	6.774	0.13%
	PheISTD	8.076	0.08%	7.955	0.17%	8.212	0.07%
	LeuISTD	8.023	0.09%	7.937	0.11%	8.112	0.08%
	MOxISTD	4.014	0.22%	3.775	0.52%	4.206	0.31%
	HisISTD	4.080	0.34%	3.970	0.37%	4.217	0.01%
	LysISTD	5.816	0.22%	5.731	0.31%	5.913	0.08%
	ArgISTD	4.801	0.16%	4.740	0.18%	4.902	0.16%
	NvaISTD	6.875	0.13%	6.774	0.15%	7.014	0.06%
	Asn	3.464	0.22%	3.372	0.20%	3.588	1.20%
	Gln	3.798	0.23%	3.686	0.54%	3.951	0.40%
	HydPro	3.653	0.27%	3.512	0.49%	3.775	0.78%
	AsnISTD	3.462	0.21%	3.375	0.20%	3.555	0.26%
	HydProISTD	3.652	0.23%	3.357	0.32%	3.896	0.45%
	GlnISTD	3.797	0.22%	3.646	1.97%	3.969	0.47%
	TrpISTD	8.756	0.06%	8.678	0.08%	8.817	0.02%

Table 2: Precision of instrument (8 consecutive injections) for all amino acids showing: the height and area ratios of derivatised amino acids compared to the corresponding internal standard

	Height Ratio		Area Ratio	
	Mean	**%CV**	**Average**	**%CV**
Asp/AspISTD	0.22	4.33%	0.22	2.36%
Glu/GluISTD	0.17	5.02%	0.17	1.99%
Ser/SerISTD	0.18	4.02%	0.18	2.36%
Thr/ThrISTD	0.06	4.05%	0.07	3.49%
Tyr/TyrISTD	0.03	7.05%	0.02	3.87%
Gly/GlyISTD	0.15	1.58%	0.17	2.28%
Pro/ProISTD	1.80	2.24%	1.80	0.84%
Ala/AlaISTD	0.19	2.08%	0.19	1.02%
Met/MetISTD	0.01	11.10%	0.01	7.29%
Val/ValISTD	0.06	1.67%	0.06	1.93%
Phe/PheISTD	0.05	10.14%	0.05	8.39%
Leu/LeuISTD	0.03	9.16%	0.03	7.26%
MOx/MOxISTD	0.01	7.86%	0.01	6.54%
His/HisISTD	0.06	7.85%	0.06	5.23%
Lys/LysISTD	0.11	13.09%	0.11	6.44%
Arg/ArgISTD	0.03	2.57%	0.03	2.54%
Nva/NvaISTD	1.74	2.67%	1.75	1.09%
Ile/IleISTD	0.03	6.86%	0.03	5.58%
Asn/AsnISTD	0.07	4.25%	0.07	5.24%
Gln/GlnISTD	0.13	4.47%	0.13	2.05%
HydPro/HydProISTD	0.01	10.84%	0.01	13.48%

The %CV's for the amino acid concentrations in the honey sample used, were all below 10%, except for hydroxyproline (13.48%), present at low level (0.97 mg/kg), with a small peak, where noise started to affect peak shape and area reproducibility (Table 1). This indicates high precision of the instrument injection, and effective internal standard correction. Retention times were stable (%CV <0.5%), and relative peak heights between amino acids and internal standards reproducible (Table 2), indicating Gaussian peak shapes were maintained over 8 injections.

Method Precision.

For method precision, a mānuka honey was analysed in triplicate, once a week, over four weeks. Mean and standard deviation results are given in Table 3. These results indicate % standard deviations are less than 30% for all amino acids except for serine and glycine (40 and 44% respectively). For these two amino acids, there appeared to be a small amount of instrument suppression or enhancement to the left of the peak (around 3.4 minutes), possibly due to honey matrix, and in spite of using internal standards with identical retention times, this affected quantitation variability.

Table 3: Method precision data for a mānuka honey.

Amino Acids (mg/kg)			
mg/kg	**Mean (mg/kg)**	**SD**	**% SD**
Asp	19.15	4.37	23%
Glu	13.34	2.39	18%
Ser	21.60	8.74	40%
Thr	9.23	2.34	25%
Tyr	16.38	2.70	16%
Gly	11.58	5.14	44%
Pro	616.37	166.63	27%
Ala	28.32	6.30	22%
Met	0.67	0.10	14%
Val	11.36	2.05	18%
Phe	77.98	17.88	23%
Leu	7.68	2.16	28%
His	16.13	3.67	23%
Lys	40.86	6.88	17%
Arg	8.86	2.25	25%
Ile	8.36	1.21	14%
Asn	12.28	1.77	14%
Gln	20.58	2.47	12%
Trp	3.33	0.76	23%
HydPro	9.53	1.13	12%

Carryover

The carryover of the instrument was analysed by running a blank water sample after a standard. The treatment of blanks was prepared using the same methodology as that used for standards and samples. The carryover is represented as a percentage of the amino acid content of the blank compared to the standard, and is in Table 4.

Table 4: Carryover of amino acids

	Amino Acids (mg/kg)		
	Standard	**Blank**	Carryover (%)
Asp	389.55	1.05	0.27%
Glu	605.23	0.21	0.04%
Ser	351.73	3.14	0.88%
Thr	326.88	0.96	0.29%
Tyr	597.91	0.62	0.10%
Gly	334.06	3.37	1.00%
Pro	291.49	0.31	0.11%
Ala	354.56	1.22	0.34%
Met	514.91	0.01	0.00%
Val	391.42	0.45	0.12%
Phe	499.19	0.28	0.06%
Leu	472.28	0.48	0.10%
MOx	414.12	0.01	0.00%
His	470.93	0.94	0.20%
Lys	563.46	0.56	0.10%
Arg	603.67	0.56	0.09%
Ile	452.67	0.56	0.12%
Asn	336.04	0.02	0.01%
Gln	179.82	0.12	0.07%
Trp	726.27	0.41	0.06%
HydPro	391.76	0.04	0.01%

The carryover percentages of all the amino acids were at ≤ 1%, acceptable for the analysis of honey samples. The highest carryover was for glycine at 1.00%. These levels would have insignificant effect on results.

3.3.2 *Trueness (Bias and Recovery)*

The aTRAQ kit is supplied with a certificate of analysis for the concentrations of amino acids in the reconstituted internal standard. These are used to quantitate amino acids in samples. As long as pipetting of the internal standard and honey solution is accurate, bias will be minimal, if derivatisation has gone to completion (monitored by the SMC, norvaline).

Bias was assessed by comparing data from 10 samples analysed at Massey University, Nutrition Laboratory and by the method being validated (Tables 5.1 and 5.2). Massey University used RP HPLC separation after AccQ Tag derivatisation. The Waters AccQ-Tag kit derivatises amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in borate buffer, and has a 100 pmol/µL pre-derivatised calibration standard. Separation is carried out on Waters AccQ-Tag ultra (2.1 x 100 mm, 1.7 µm) column. Detection of derivatised amino acids is by UV. Problems with co-elution were apparently encountered and they had to use two different separation gradients.

Comparison of data (Table 5.1 and Table 5.2) shows little differences between methods for amino acids Asp, Pro, Leu, and Arg. Gly, Phe and Ala show the largest differences, some being a factor of ten out from the Massey University values. However this is not the case for all of the honeys, and aTRAQ results are all higher than Massey results. Whether this indicates incomplete derivatisation of these amino acids by the AccQ-Tag reagent with some honeys, would require further study, using the AccQ-Tag kit. Representative honey sample 14.4 comparison shows minor differences between amino acid content between the two methods, indicating low method bias.

Table 5.1:Comparison of amino acid content of honeys, part 1

Amino acid (mg/kg)	Honey ID									
	Happy Bee		Airborne		Hollands		Katikati		14.4	
	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ
Asp	11	9.77	11.4	9.99	9	9.37	11.4	11.29	31.1	25.23
Glu	14.5	12	15.2	12.08	13.6	12.61	10	8.51	22.9	20.15
Ser	8.4	15.06	11.8	12.82	13.9	19.69	9.2	14.53	6.6	14.13
Thr	1.8	6.26	3.6	7.64	3.8	7.15	3	7.32	3.4	6.55
Tyr	7.5	33.08	12.6	8.95	65.4	57.13	33.9	24.14	2.8	3.53
Gly	2.2	11.07	3.6	8.15	3.4	7.93	3	8.66	0.17	9.25
Pro	328.1	427.09	639.7	521.49	378.5	404.47	551.9	494.89	179	184.51
Ala	7	25.29	10.8	29.4	9.6	28.8	9.7	26.28	5	10.98
Met	0.1	0.11	0.2	0.15	0.4	0.46	0.2	0.02	0.6	0.82
Val	5.1	7.5	9.2	8.39	9	9.49	1.8	7.75	4.9	6.12
Phe	27.1	174.35	47.8	37.76	480.8	569.75	135.7	110.63	5.9	6
Leu	2.7	4.63	5.8	5.11	6.3	8.45	9.6	10.77	1.7	2.87
His	5.7	9.54	9.8	10.12	6.2	8.32	5.7	8.41	4.7	5.43
Lys	9.3	23.59	24.4	31.53	12.3	18.02	14	18.67	7.5	11.72
Arg	5.2	5.49	8.1	5.54	5.3	4.36	9.4	7.13	4.3	4.84
Ile	2.6	4.72	5.6	6.48	4.5	6.84	6.1	6.97	2.3	2.88

Table 5.2:Comparison of amino acid content of honeys, part 2

Amino acid (mg/kg)	Honey ID									
	946		953		66		78		84	
	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ	Massey	aTRAQ
Asp	20.2	21.23	16.9	18.11	4.6	8.97	5.9	8.28	4.6	7.3
Glu	15.1	14.88	14.9	13.51	9.6	9.81	10.3	11.15	8.8	9.67
Ser	16	21.37	13.8	27.33	10.4	31.01	7.1	16.04	10.9	17.93
Thr	6.9	9.86	6	10.9	2.8	8.57	1.9	4.82	1.9	8.2
Tyr	20.1	17.83	17.5	12.51	13.6	11.7	7.1	6.63	10	9.33
Gly	4.6	11.97	4.5	17.15	2.2	20.21	1.5	9.76	2.4	11.58
Pro	709.3	730.38	674.6	593.4	465.2	421.97	326.4	343.6	656.2	527.51
Ala	20.7	32.78	18.3	25.4	11.6	21.19	7.3	22.85	12.3	29.3
Met	0.5	0.66	0.5	0.73	0.2	0.58	0.2	0.29	0.4	0.55
Val	10.9	12.47	12.2	12.22	7.2	9.57	4.8	6.17	8	9.78
Phe	88.7	89.44	54.3	43.53	42.1	36.88	19.2	18.04	19.6	20.31
Leu	7.2	8.7	5	7.13	3	6.48	2	3.52	2.8	4.94
His	19.9	18.39	18.9	18.59	5.5	11.53	2.4	7.56	3.1	10.68
Lys	23.9	45.27	22.2	33.59	16.1	25.07	6.8	13.56	10.2	21.95
Arg	13.7	10.3	8.9	6.94	5.2	7.29	3.5	5.41	1.2	5.81
Ile	6.6	9.07	7.5	8.89	4.6	7.17	2.8	3.96	4.8	7

3.3.3 *Uncertainty of Measurement*

Due to lack of ILCPs or reference materials to determine bias, method precision results (Table 3) should be used for UoMs.

3.4 *Detection and Reporting Limits*

The amino acid content of honeys can vary greatly, and the amino acids are often at very low levels. The limits for each amino acid can be found in Table 6.

Table 6: The S/N, LOD, and LOQ of amino acids

Amino Acid	S/N[1]	LOD[2] (mg/kg)	LOQ[3] (mg/kg)
Asp	0.035	0.796	2.652
Glu	0.030	0.544	1.813
Ser	0.052	0.821	2.738
Thr	0.068	0.410	1.367
Tyr	0.084	0.290	0.968
Gly	0.061	0.654	2.180
Pro	0.002	0.359	1.197
Ala	0.008	0.114	0.379
Met	0.469	0.392	1.306
Val	0.018	0.101	0.337
Phe	0.063	0.336	1.120
Leu	0.102	0.253	0.843
MOx	0.232	0.406	1.355
Cys	2.770	0.309	1.029
His	0.054	0.302	1.006
Lys	0.168	2.028	6.759
Arg	0.034	0.153	0.510
Nva	0.003	0.298	0.993
Ile	0.087	0.290	0.967
Asn	0.095	0.670	2.232
Gln	0.046	0.729	2.430
Trp	0.477	0.713	2.377
HydPro	0.173	0.197	0.657

[1] S/N was calculated by: noise height x 3/ signal height.
[1] LOD was calculated by: S/N x amino acid content in mg/kg.
[1] LOQ was calculated by: LOD x 10 / 3.

This data was manually calculated on the MultiQuant software. The software automatically smoothes chromatogram peaks, improving the results' precision. The smoothing function was also used in calculating the signal to noise ratio, for consistency.

The noise on the chromatogram originates from the nature of the electrospray; it is often due to background contaminants, impurities in mobile phases and degradation products of the tubing.

3.5 *Ruggedness and Robustness*

The ruggedness of a method indicates the lack of influence operational and environmental variables have on test results. Interlaboratory comparisons are often used to determine this.
The aTRAQ kit uses pre-derivatised internal standards which make for very accurate quantitation, compared to calibration with external standards that require derivatisation. Problems with calibration standards can be encountered, due to poor solubilities of the amino acids leading to problems with dissolving them, then losses on sitting in instrument vials. Recovery out of the vial can require buffer to give a low pH (protenating the acid), or addition of ammonia to ion pair the amine groups, or the use of polypropylene vials over glass, to prevent interaction with silanols. Standards must be prepared on a weekly basis, to ensure fresh calibrations. These factors are considered to show that pre-derivatised internal standards make this method more rugged. Unfortunately an interlaboratory comparison was not available, in order to show the ruggedness of this method by way of data analysis.

Robustness indicates the reliability of the method during its normal usage. It is often measured by making small, deliberate variations to parameters. Derivatising samples entails pipetting small volumes, with centrifuging between steps. The pipetting has the most room for error, and can affect the robustness of the method. The centrifuging and vortexing improves accuracy, by ensuring all sample is mixed together at the bottom of the tube. Rinsing techniques were employed to make certain that the entire honey sample was deposited and mixed with the labelling buffer. The same was carried out with the internal standard, another critical step for accuracy. To improve to robustness of this method, utilising robotics for the derivatising steps would remove all technician variation.

4.. Conclusions and Fitness for Purpose

The precision data, limits of detection and comparison with Massey University analyses indicate that this method is suitable for the analysis of amino acids in honey samples, for the purpose of identifying floral origin, or used to predict catalysis of DHA to MGO by amino acids.

5.. References

Reference	KB Item # / Location
The Fitness for Purpose for Analytical Methods; A Laboratory Guide to Method Validation and Related Topics (EURACHEM Guide)	4725
Add further references if required	

<End>

Appendix 11: Precision of Instrument

Precision of instrument for all amino acids showing: the height and area ratios of derivatised amino acids compared to the corresponding internal standard.

	Height Ratio		Area Ratio	
	Average	**%CV**	**Average**	**%CV**
Asp/AspISTD	0.22	4.33%	0.22	2.36%
Glu/GluISTD	0.17	5.02%	0.17	1.99%
Ser/SerISTD	0.18	4.02%	0.18	2.36%
Thr/ThrISTD	0.06	4.05%	0.07	3.49%
Tyr/TyrISTD	0.03	7.05%	0.02	3.87%
Gly/GlyISTD	0.15	1.58%	0.17	2.28%
Pro/ProISTD	1.80	2.24%	1.80	0.84%
Ala/AlaISTD	0.19	2.08%	0.19	1.02%
Met/MetISTD	0.01	11.10%	0.01	7.29%
Val/ValISTD	0.06	1.67%	0.06	1.93%
Phe/PheISTD	0.05	10.14%	0.05	8.39%
Leu/LeuISTD	0.03	9.16%	0.03	7.26%
MOx/MOxISTD	0.01	7.86%	0.01	6.54%
Cys/CysISTD	0.01	55.20%	0.00	72.96%
His/HisISTD	0.06	7.85%	0.06	5.23%
Lys/LysISTD	0.11	13.09%	0.11	6.44%
Arg/ArgISTD	0.03	2.57%	0.03	2.54%
Nva/NvaISTD	1.74	2.67%	1.75	1.09%
Ile/IleISTD	0.03	6.86%	0.03	5.58%
Asn/AsnISTD	0.07	4.25%	0.07	5.24%
Gln/GlnISTD	0.13	4.47%	0.13	2.05%
HydPro/HydProISTD	0.01	10.84%	0.01	13.48%

Precision of instrument for all amino acids showing amino acid content.

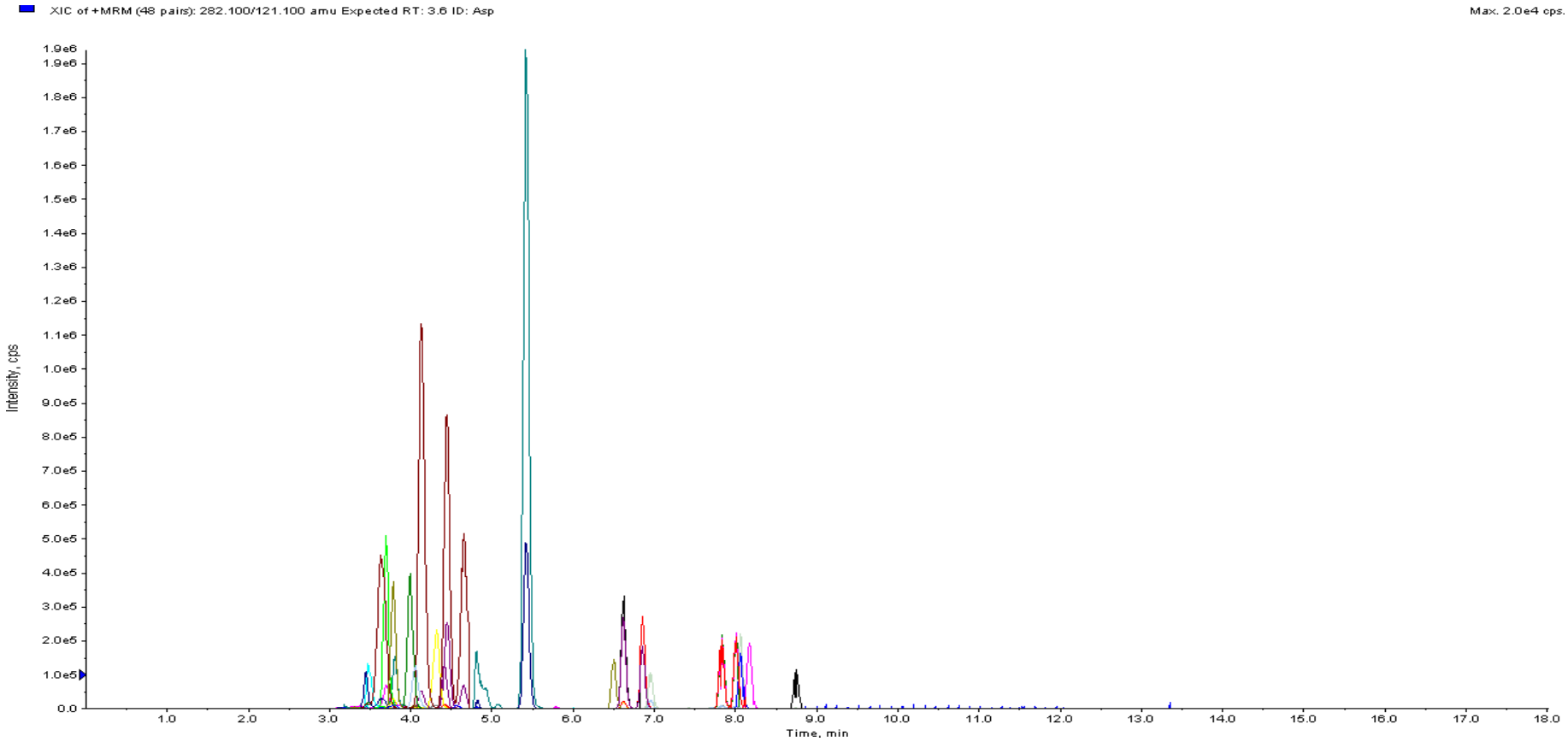
	Amino Acids mg/kg	
	Average	%C.V.
Asp	23.49	2.36%
Glu	17.78	1.99%
Ser	14.61	2.36%
Thr	6.37	3.49%
Tyr	3.18	3.87%
Gly	10.23	2.28%
Pro	166.75	0.84%
Ala	14.18	1.02%
Met	0.75	7.29%
Val	5.57	1.93%
Phe	5.45	8.39%
Leu	2.48	7.26%
Cys	0.10	72.96%
His	6.47	5.23%
Lys	11.53	6.44%
Arg	4.40	2.54%
Ile	3.17	5.58%
Asn	7.13	5.24%
Gln	15.99	2.05%
HydPro	0.97	13.48%

Precision of instrument for all amino acids showing: retention time, start and end time of peak.

	Retention Time		Start Time of Peak		End Time of Peak	
	Average	%C.V.	Average	%C.V.	Average	%C.V.
Asp	3.82	0.22%	3.72	0.40%	3.97	0.43%
Glu	4.47	0.27%	4.34	0.44%	4.60	0.24%
Ser	3.51	0.19%	3.40	0.38%	3.67	0.55%
Thr	4.39	0.27%	4.20	0.54%	4.52	0.09%
Tyr	6.97	0.10%	6.89	0.14%	7.06	0.24%
Gly	3.71	0.24%	3.54	0.40%	3.89	0.82%
Pro	5.45	0.19%	5.30	0.24%	5.62	0.08%
Ala	4.51	0.29%	4.38	0.29%	4.62	0.29%
Met	6.52	0.14%	6.42	0.35%	6.61	0.11%
Val	6.64	0.12%	6.53	0.25%	6.76	0.11%
Phe	8.08	0.07%	7.98	0.20%	8.20	0.14%
Leu	8.03	0.08%	7.95	0.15%	8.11	0.16%

MOx	4.02	0.22%	3.92	0.51%	4.14	0.21%
Cys	5.33	0.77%	5.30	0.78%	5.36	0.73%
His	4.08	0.34%	3.97	0.37%	4.21	0.34%
Lys	5.82	0.25%	5.75	0.30%	5.91	0.17%
Arg	4.80	0.16%	4.75	0.19%	4.91	0.29%
Nva	6.88	0.13%	6.76	0.13%	7.02	0.02%
IleISTD	7.85	0.10%	7.75	0.09%	7.94	0.11%
Ile	7.84	0.10%	7.77	0.11%	7.94	0.16%
Nle	8.20	0.38%	8.17	0.37%	8.24	0.37%
NleISTD	8.19	0.08%	8.11	0.08%	8.31	0.05%
AspISTD	3.82	0.22%	3.73	0.19%	4.01	0.01%
GluISTD	4.47	0.26%	4.31	0.43%	4.61	0.16%
SerISTD	3.50	0.19%	3.39	0.46%	3.66	0.51%
ThrISTD	4.39	0.28%	4.20	0.46%	4.52	0.01%
TyrISTD	6.97	0.11%	6.86	0.11%	7.11	0.12%
GlyISTD	3.71	0.25%	3.58	0.39%	3.91	0.29%
ProISTD	5.45	0.20%	5.31	0.33%	5.62	0.08%
AlaISTD	4.51	0.30%	4.38	0.27%	4.62	0.30%
MetISTD	6.52	0.13%	6.41	0.20%	6.62	0.04%
ValISTD	6.64	0.12%	6.51	0.22%	6.77	0.13%
PheISTD	8.08	0.08%	7.95	0.17%	8.21	0.07%
LeuISTD	8.02	0.09%	7.94	0.11%	8.11	0.08%
MOxISTD	4.01	0.22%	3.78	0.52%	4.21	0.31%
CysISTD	5.57	0.21%	5.47	0.34%	5.69	0.21%
HisISTD	4.08	0.34%	3.97	0.37%	4.22	0.01%
LysISTD	5.82	0.22%	5.73	0.31%	5.91	0.08%
ArgISTD	4.80	0.16%	4.74	0.18%	4.90	0.16%
NvaISTD	6.87	0.13%	6.77	0.15%	7.01	0.06%
Asn	3.46	0.22%	3.37	0.20%	3.59	1.20%
Gln	3.80	0.23%	3.69	0.54%	3.95	0.40%
HydPro	3.65	0.27%	3.51	0.49%	3.78	0.78%
AsnISTD	3.46	0.21%	3.37	0.20%	3.55	0.26%
HydProISTD	3.65	0.23%	3.36	0.32%	3.90	0.45%
GlnISTD	3.80	0.22%	3.65	1.97%	3.97	0.47%

Appendix 12: Chromatograms of Honeys for aTRAQ Method



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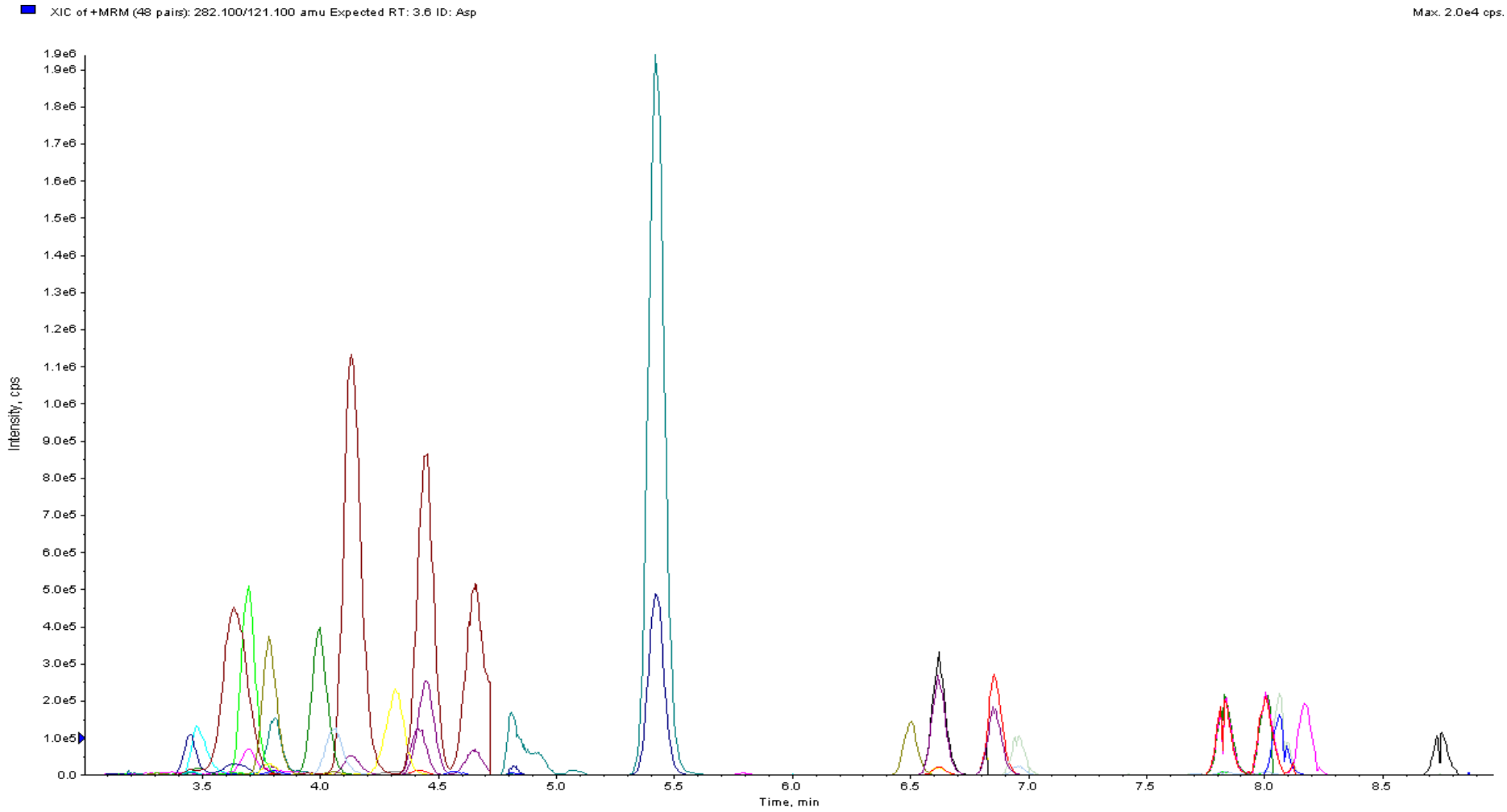

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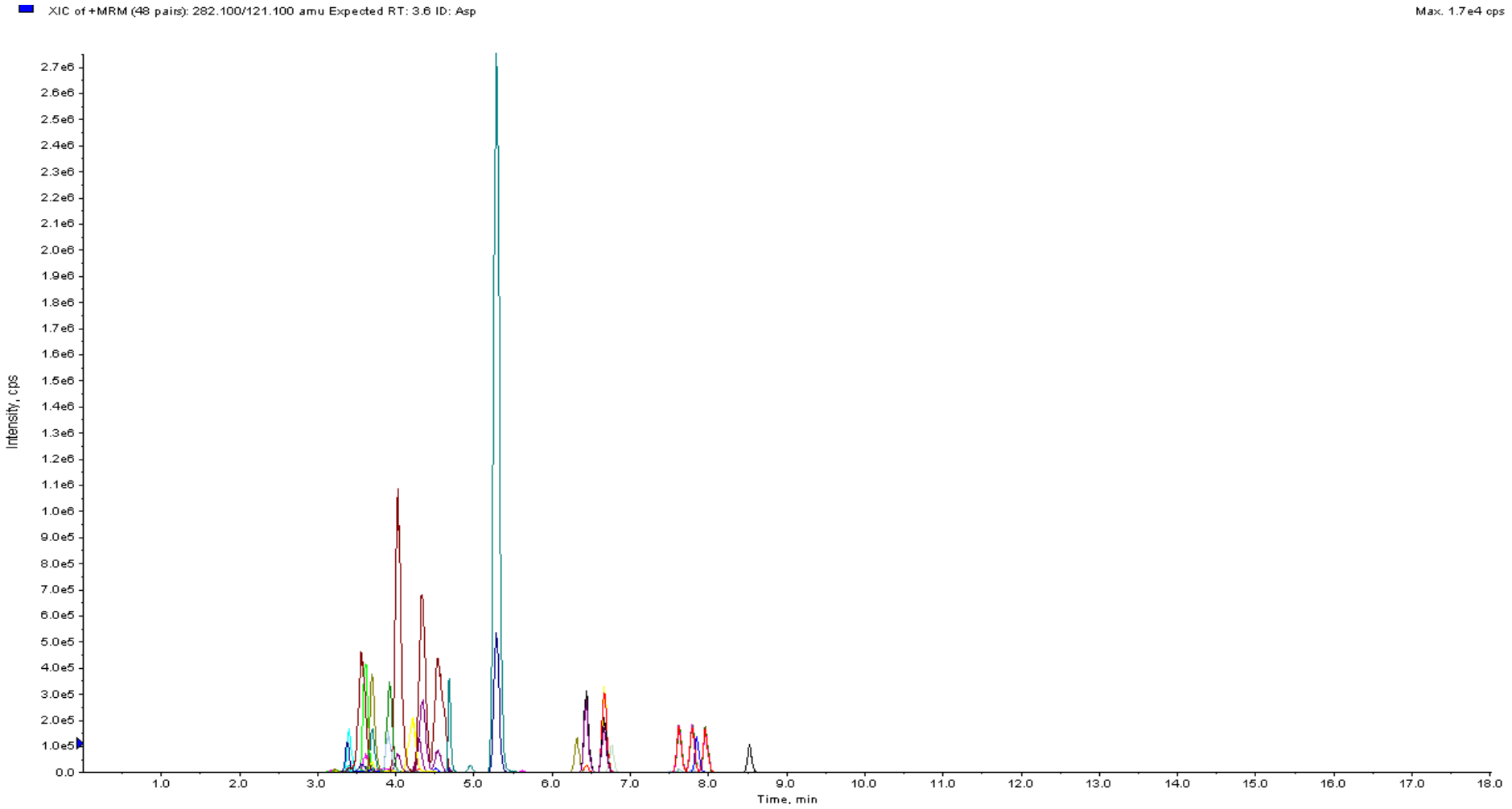
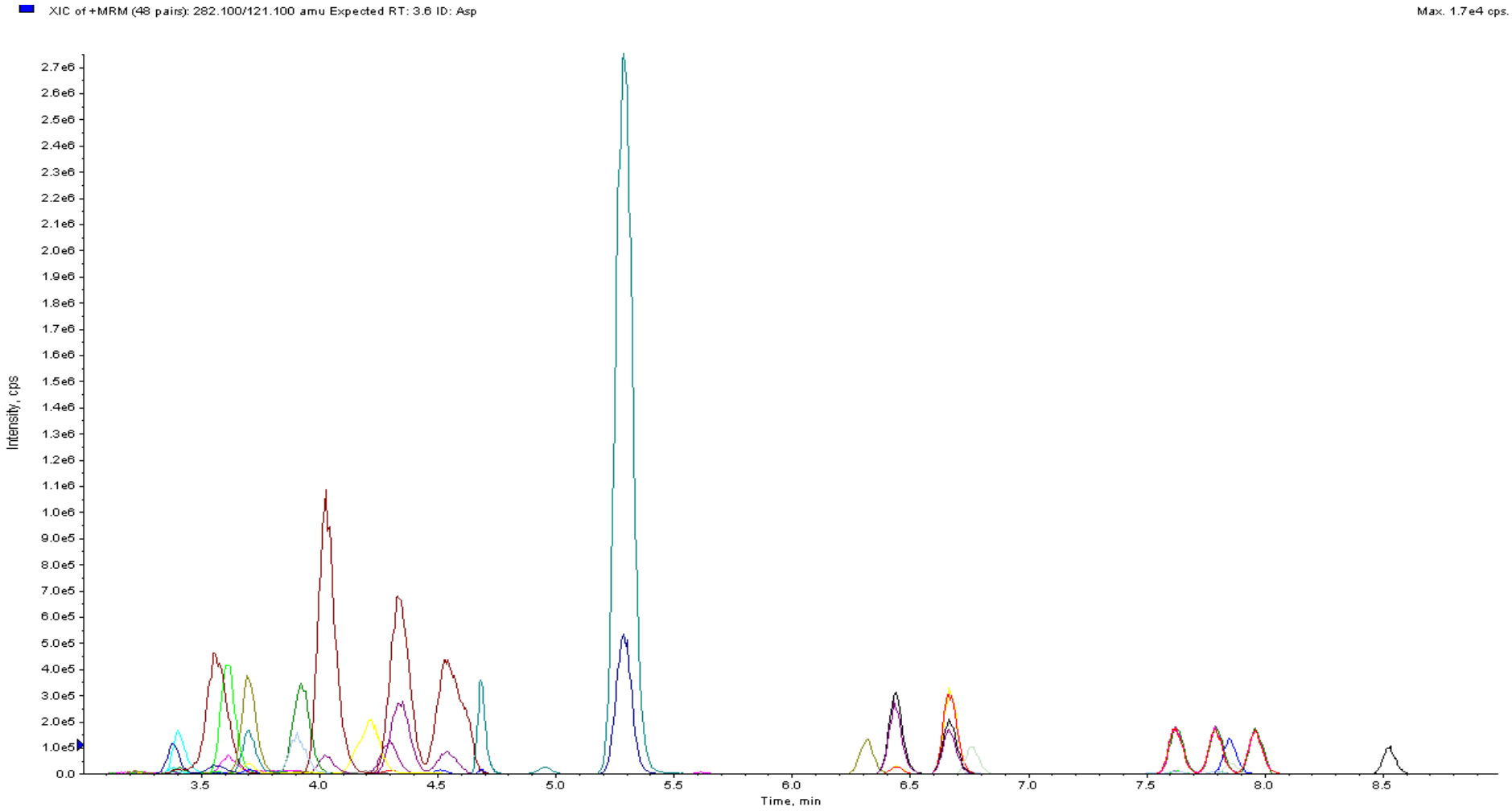
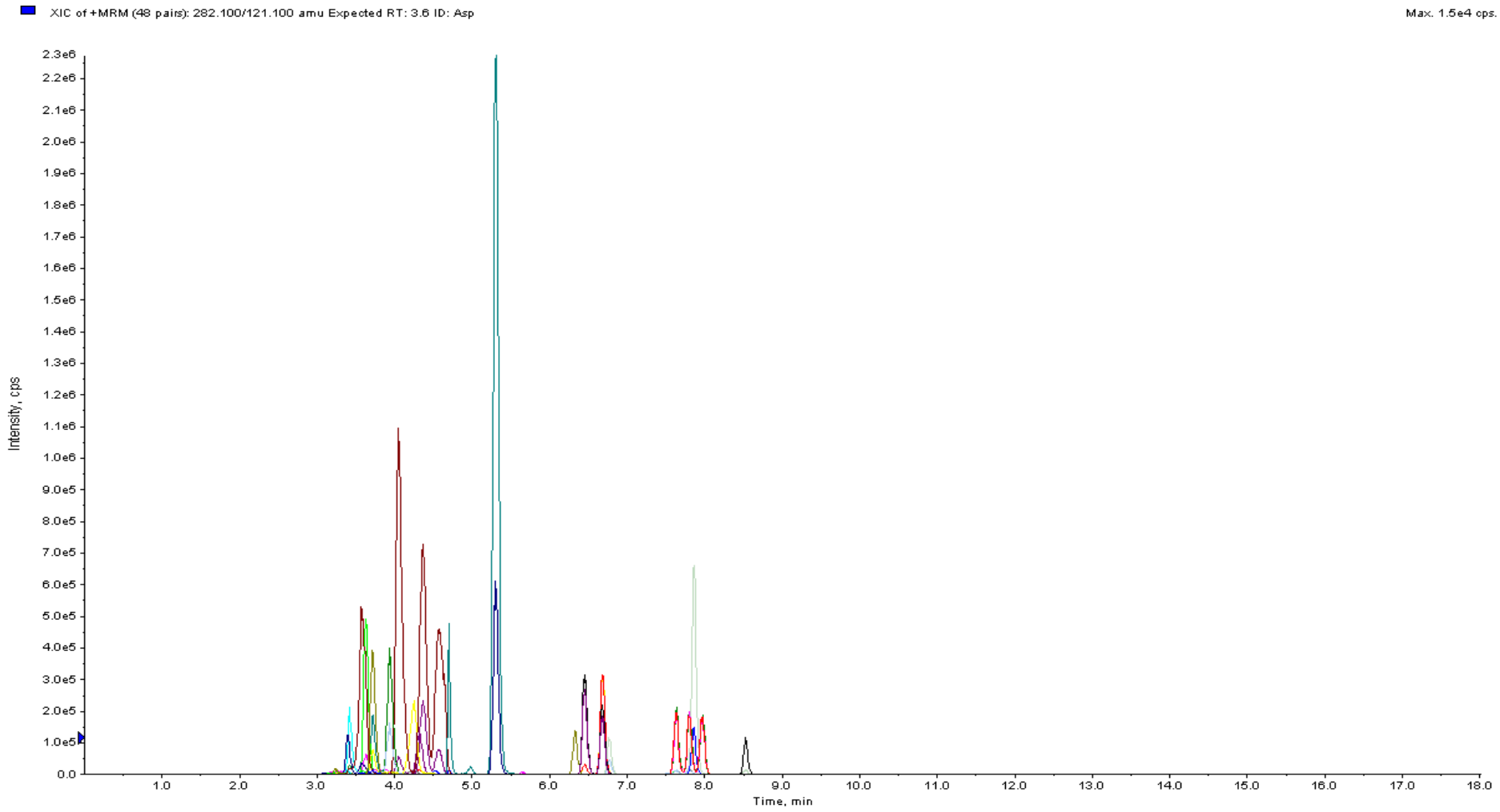


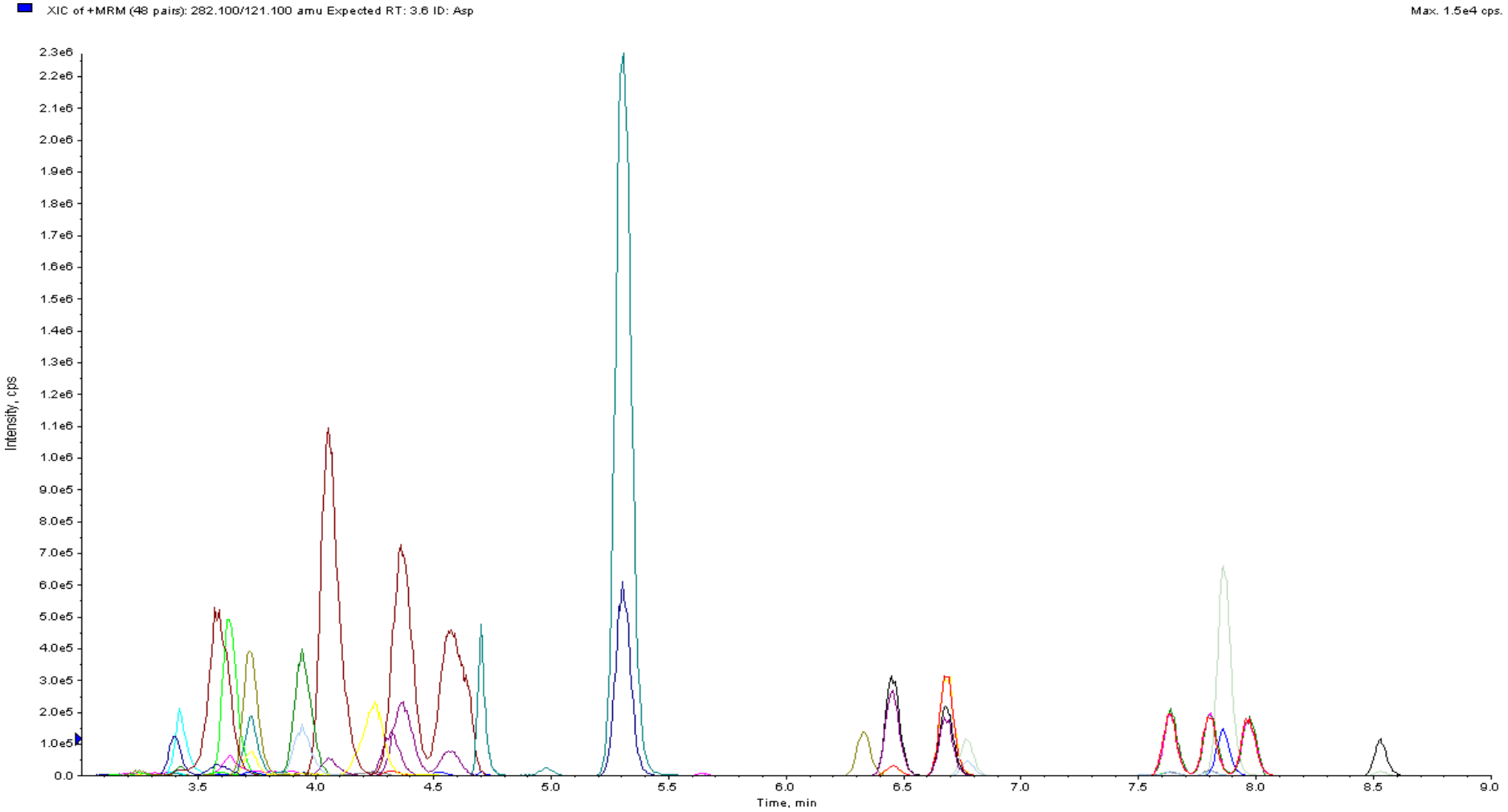
Honey ID Airborne



Honey ID Airborne (zoomed in)



Honey ID Hollands



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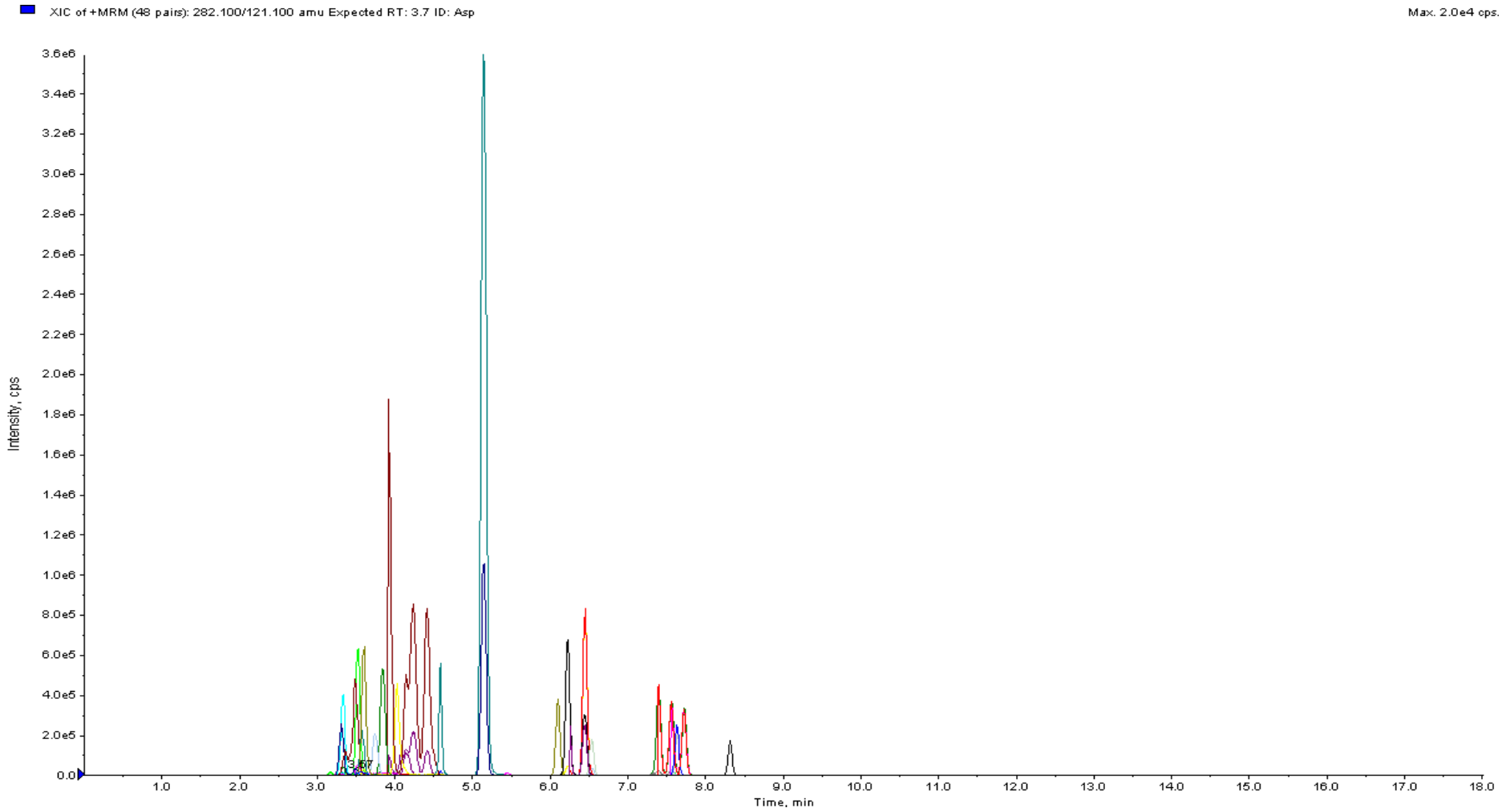
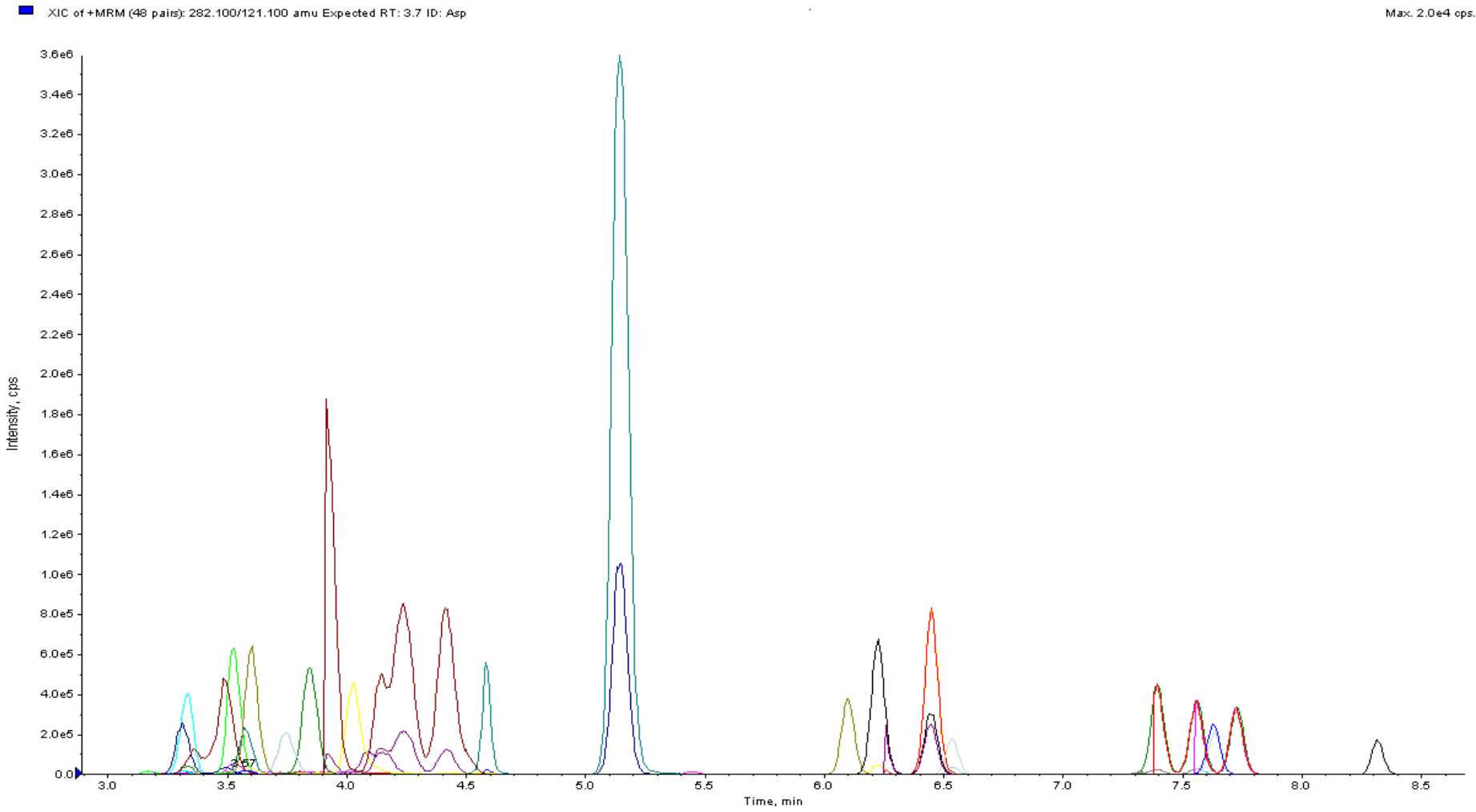


Honey ID Katikati



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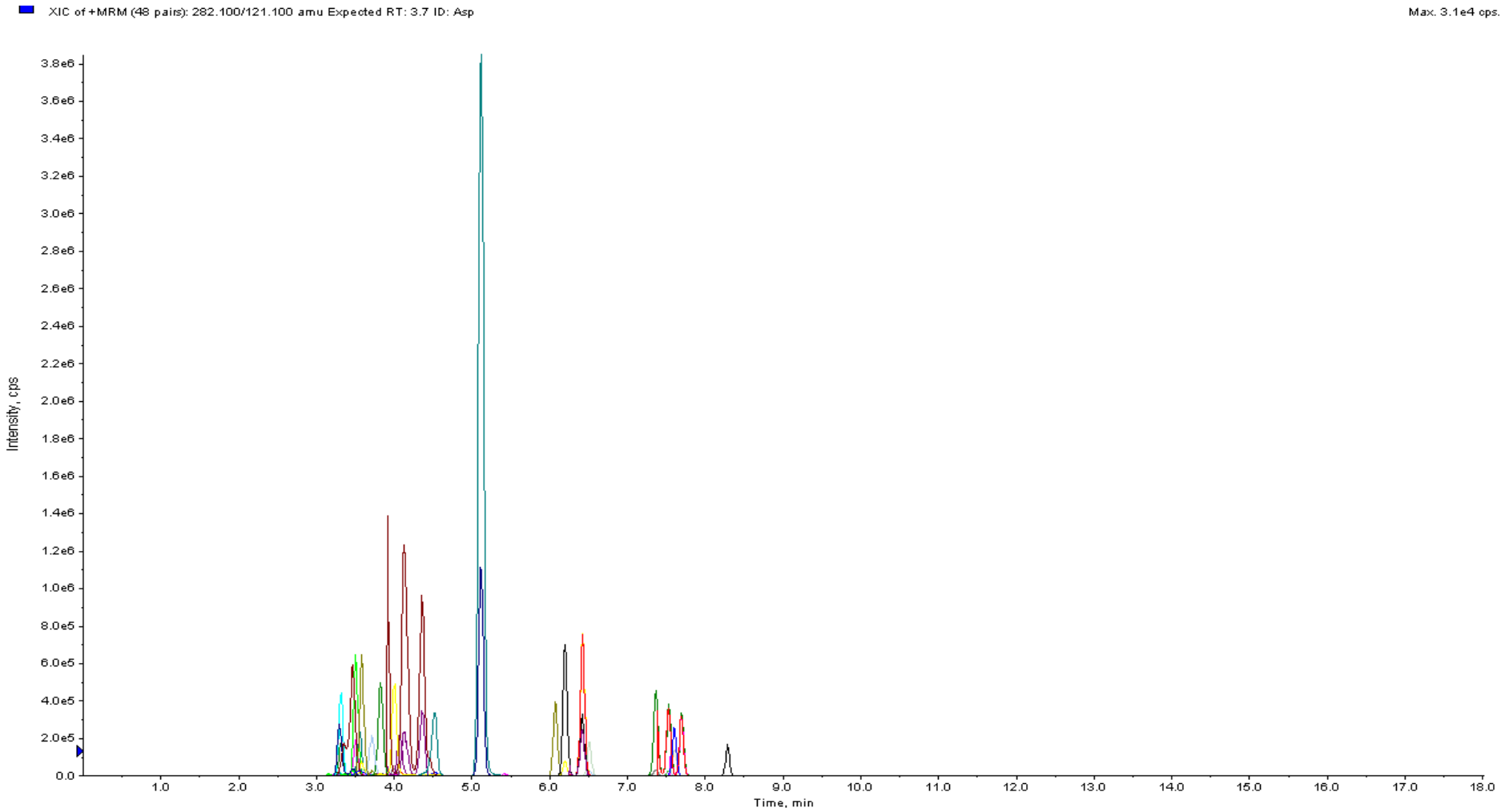
XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 3.1e4 cps.
Intensity, cps
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3.6e6
3.4e6
3.2e6
3.0e6
2.8e6
2.6e6
2.4e6
2.2e6
2.0e6
1.8e6
1.6e6
1.4e6
1.2e6
1.0e6
8.0e5
6.0e5
4.0e5
2.0e5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
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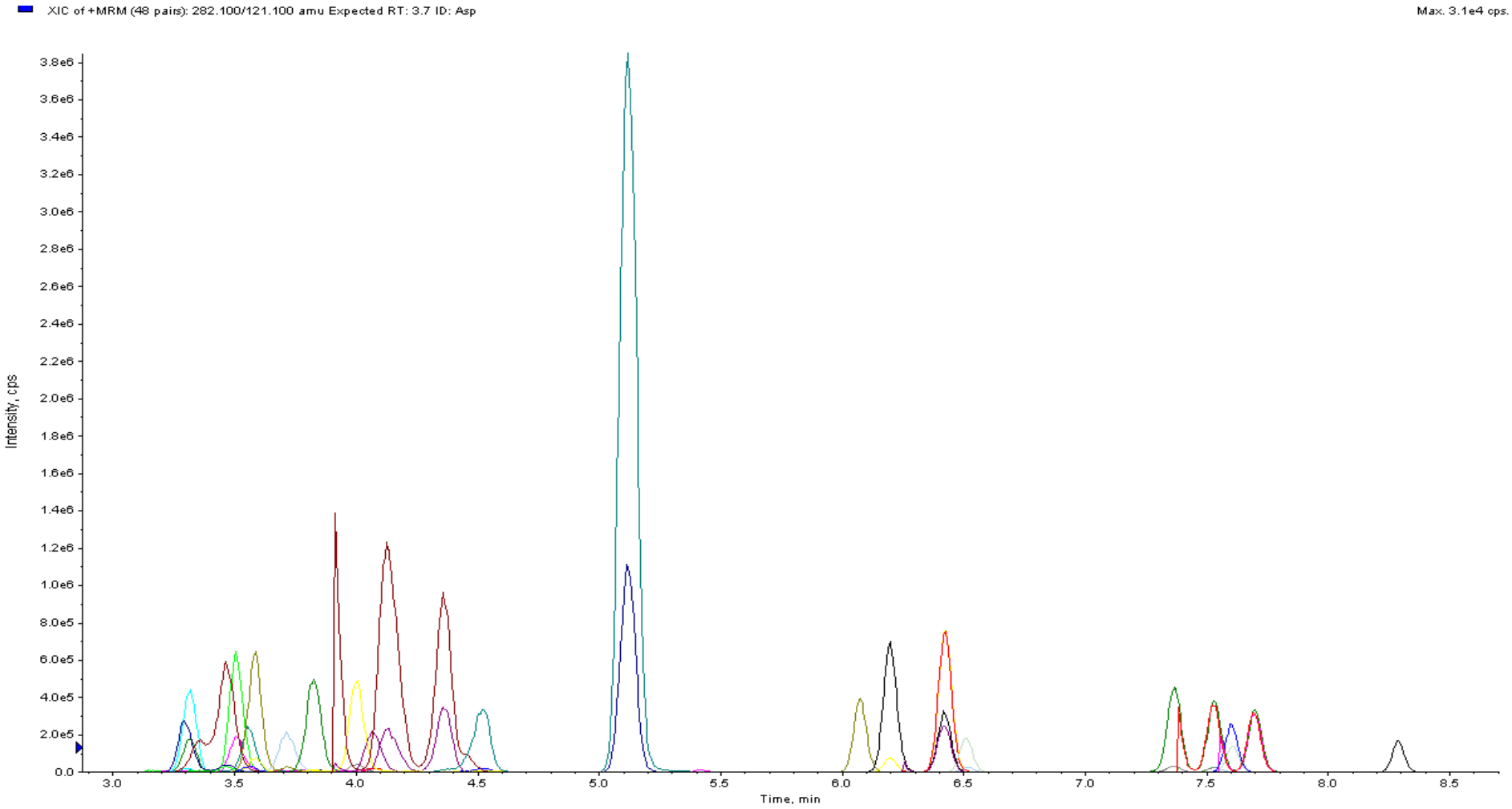
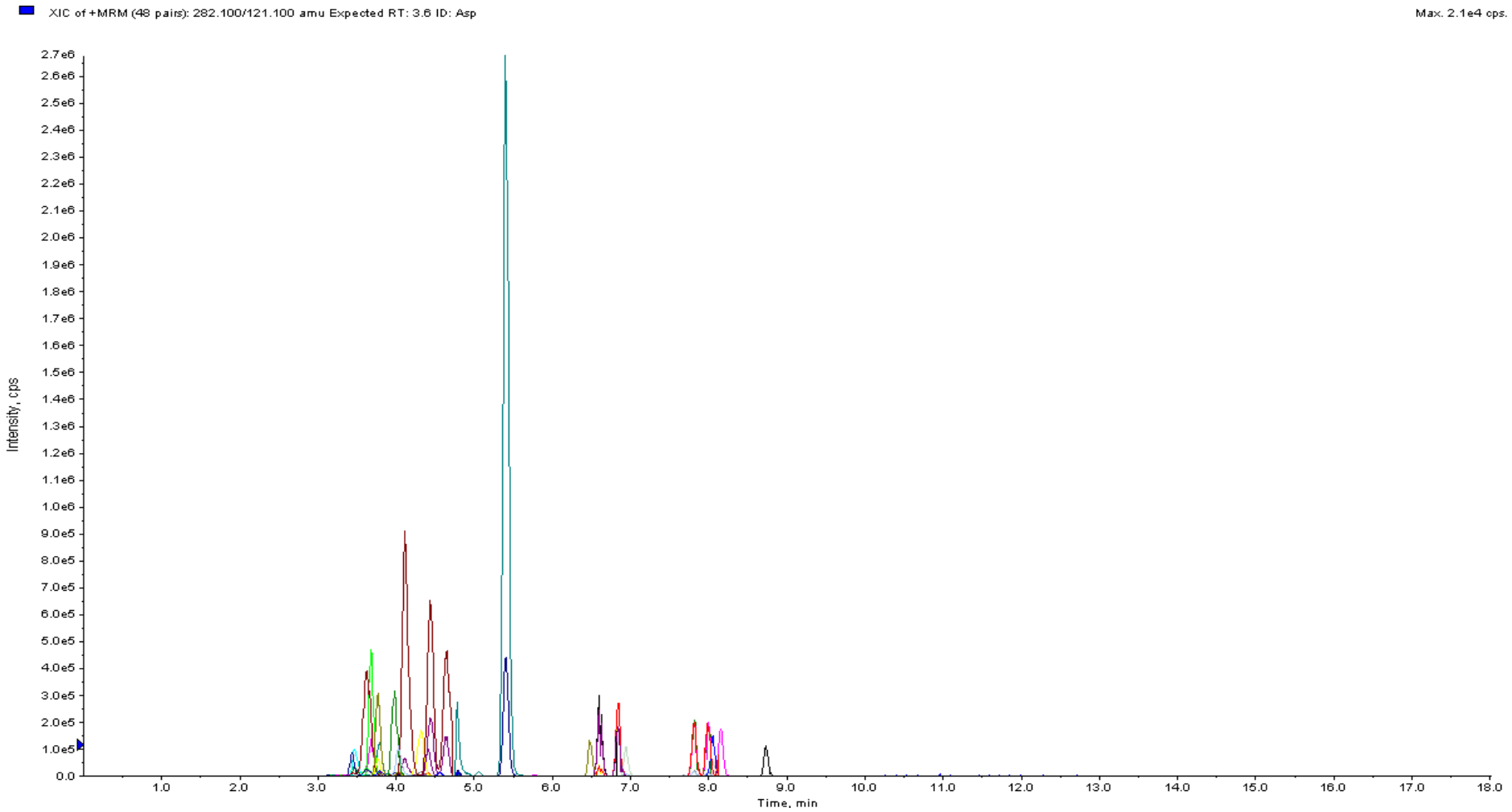
15.0
16.0
17.0
18.0
Time, min

Honey ID 946



Honey ID 946 (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.6 ID: Asp
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Intensity, cps
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2.4e6
2.3e6
2.2e6
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1.9e6
1.8e6
1.7e6
1.6e6
1.5e6
1.4e6
1.3e6
1.2e6
1.1e6
1.0e6
9.0e5
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7.0e5
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
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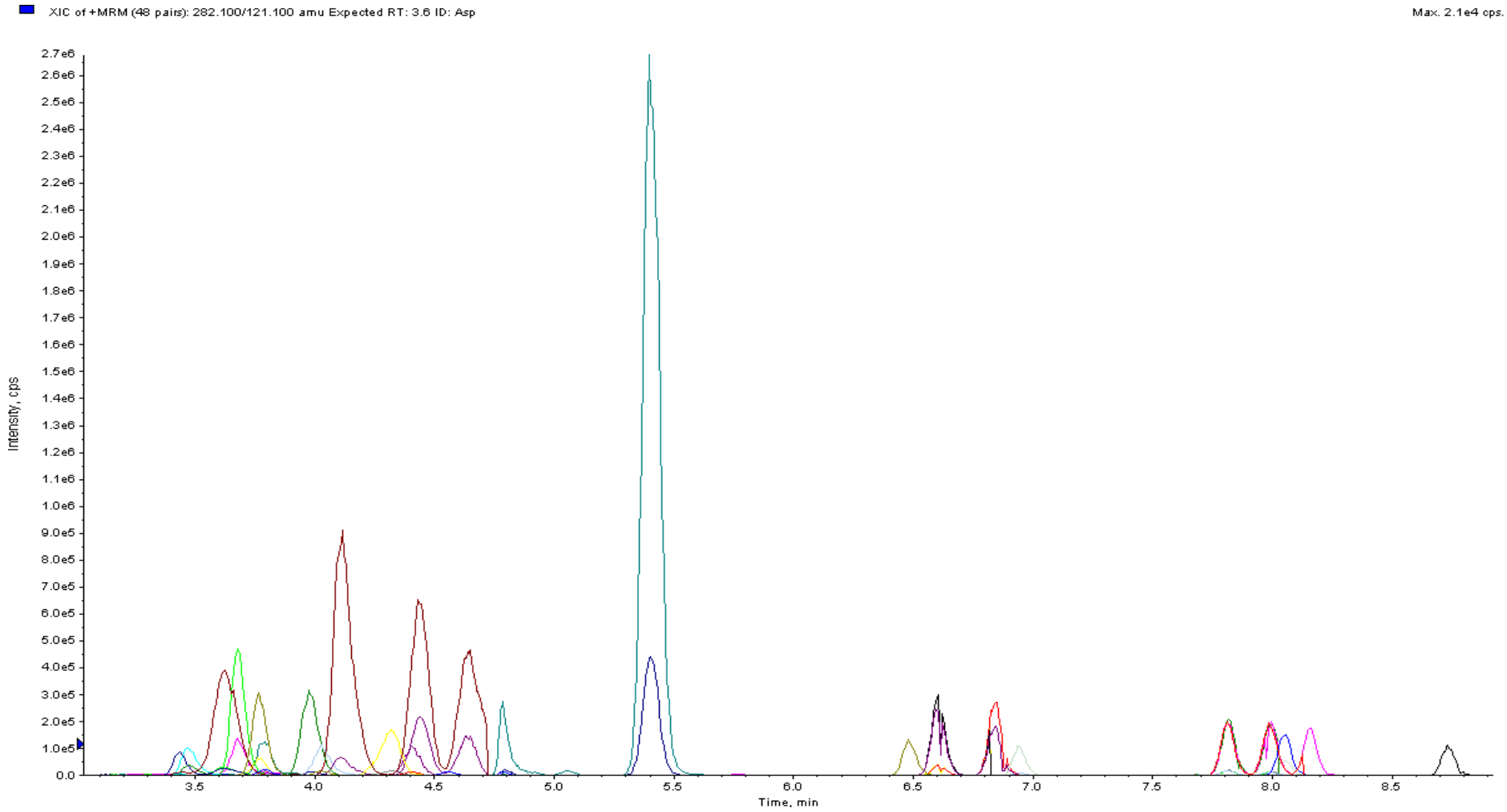
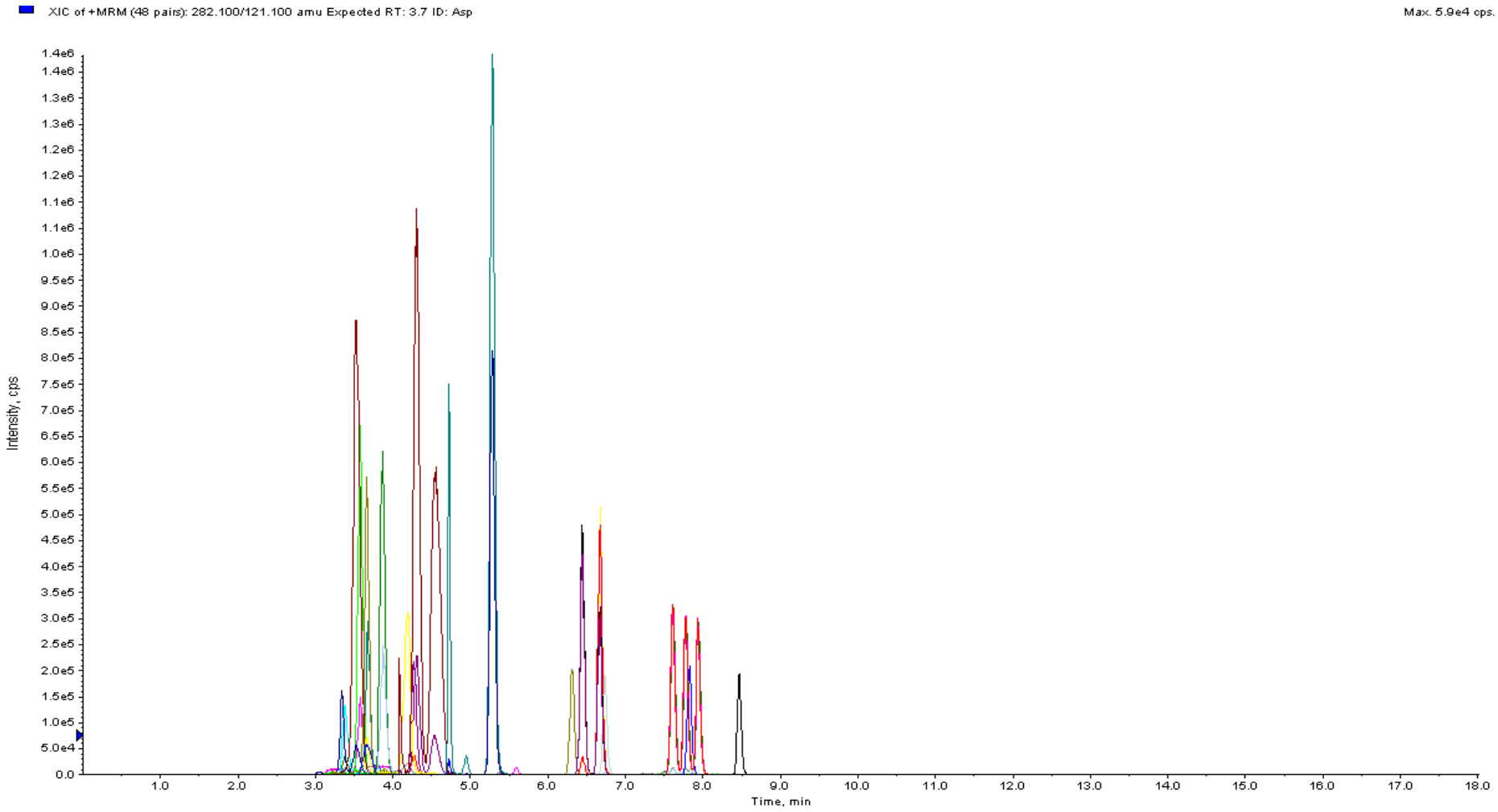
15.0
16.0
17.0
18.0
Time, min

Honey ID 953



Honey ID 953 (zoomed in)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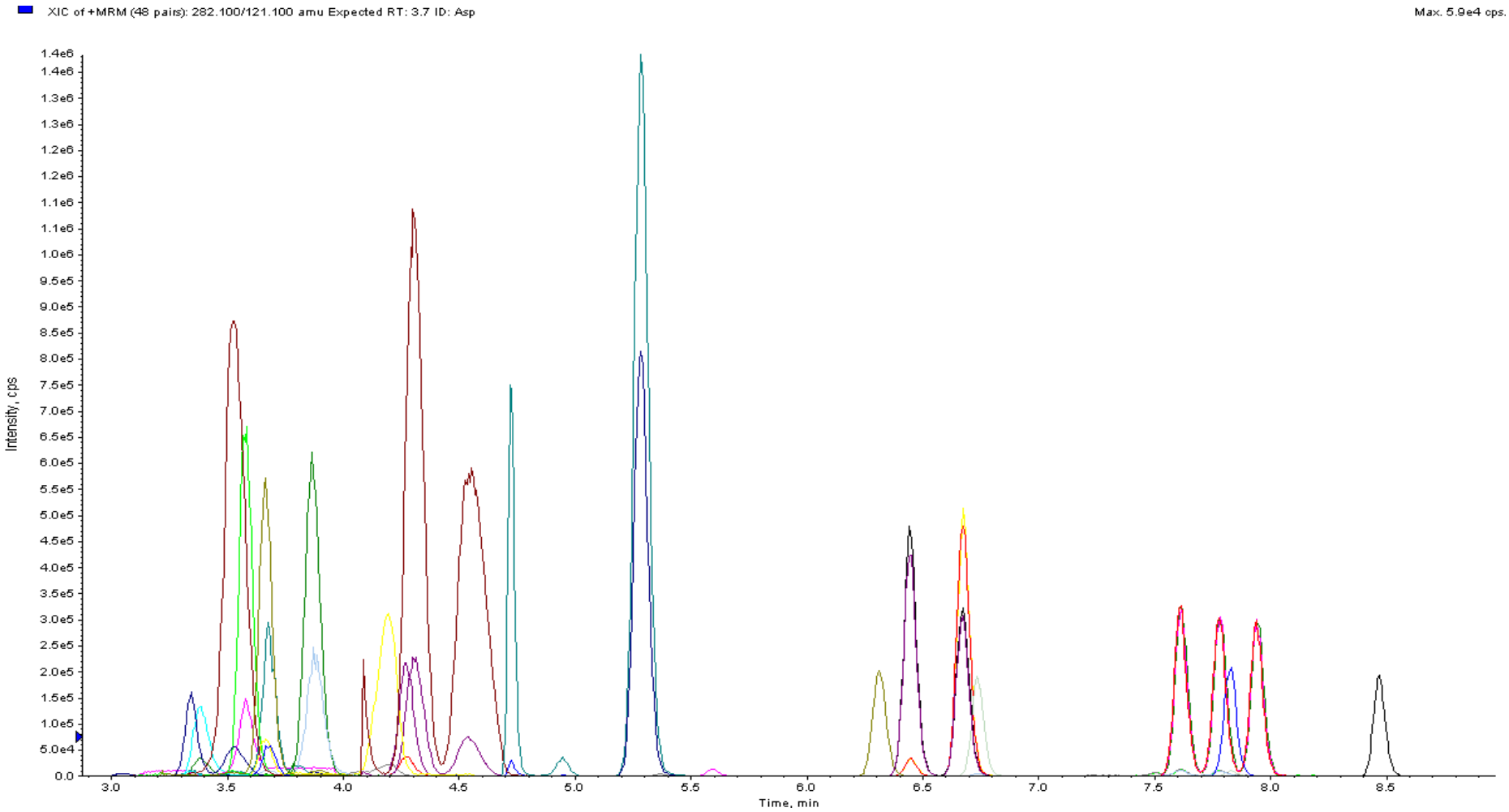
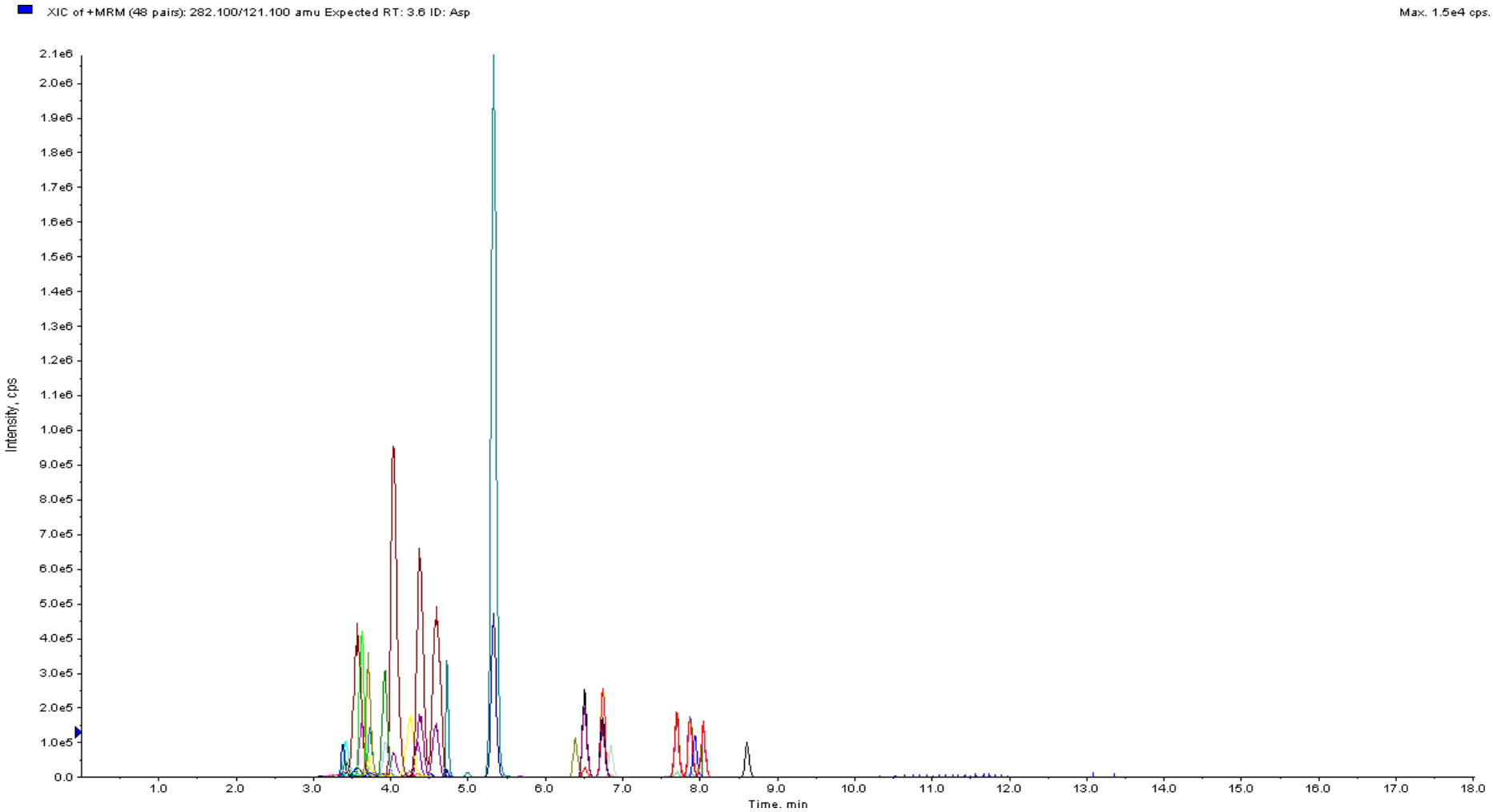
Max. 5.9e4 cps.
Intensity, cps
Time, min

Honey ID 14.4



Honey ID 14.4 (zoomed in)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Intensity, cps
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1.9e6
1.8e6
1.7e6
1.6e6
1.5e6
1.4e6
1.3e6
1.2e6
1.1e6
1.0e6
9.0e5
8.0e5
7.0e5
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
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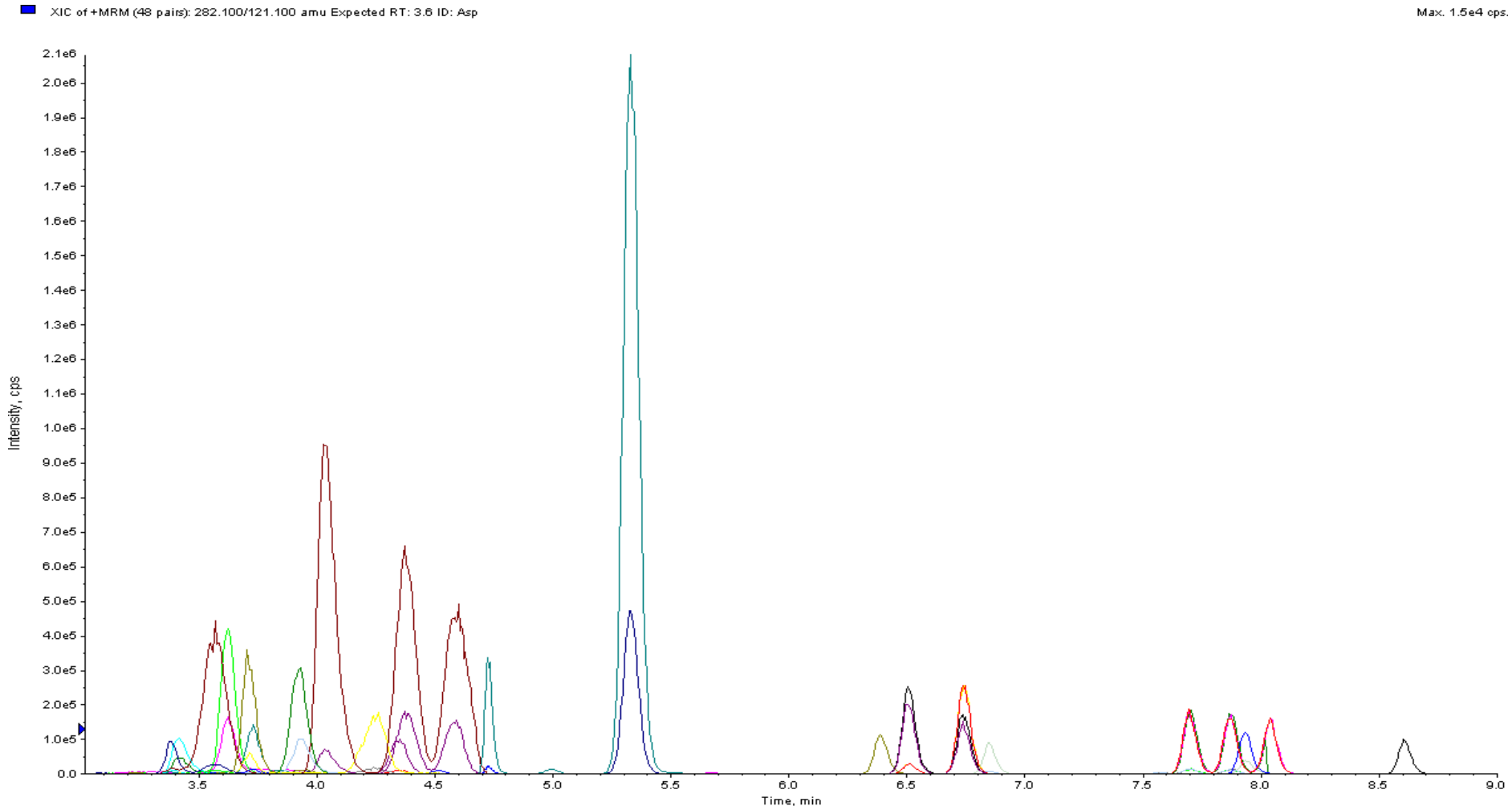
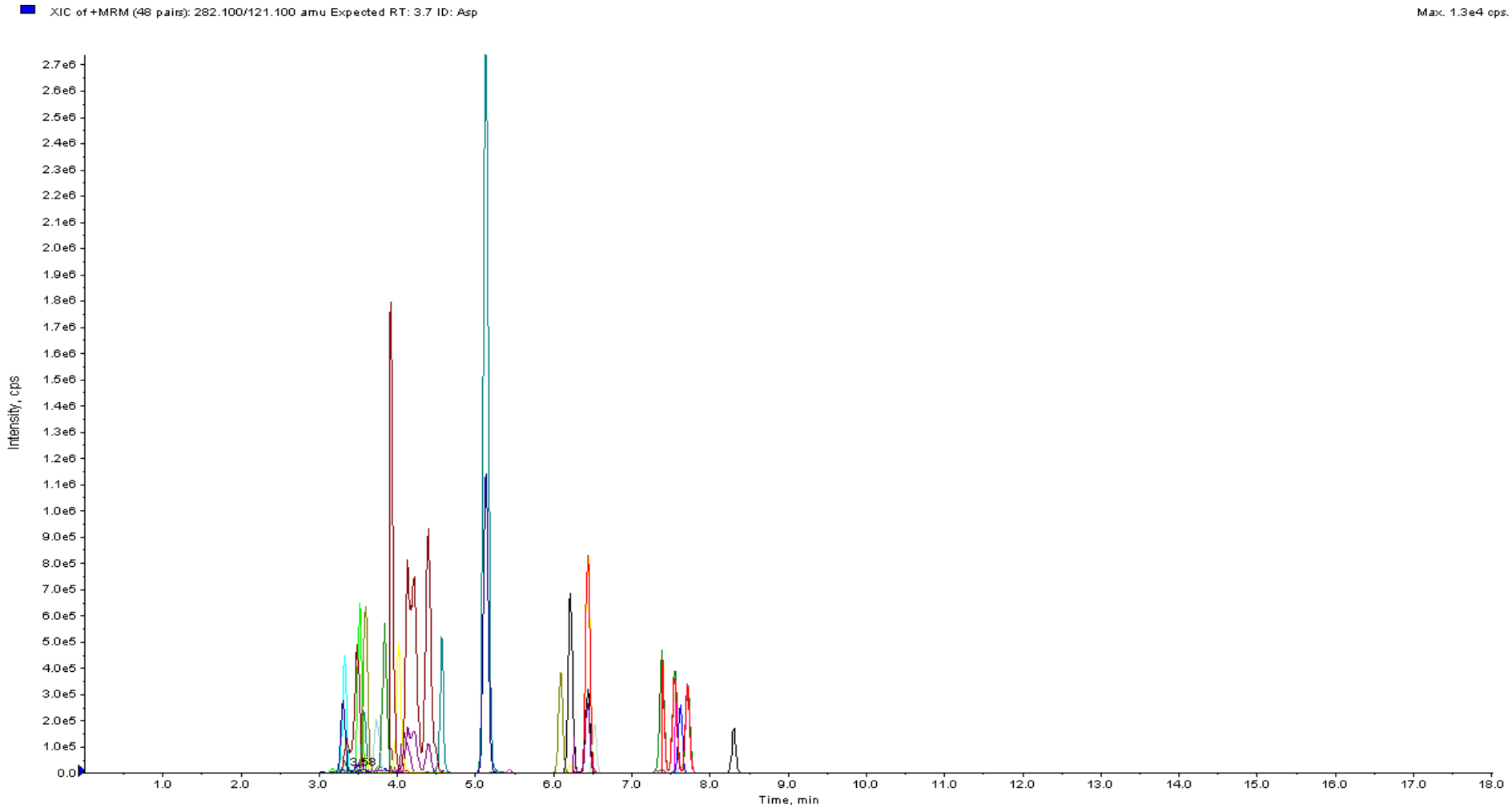
15.0
16.0
17.0
18.0
Time, min

Honey ID 66



Honey ID 66 (zoomed in)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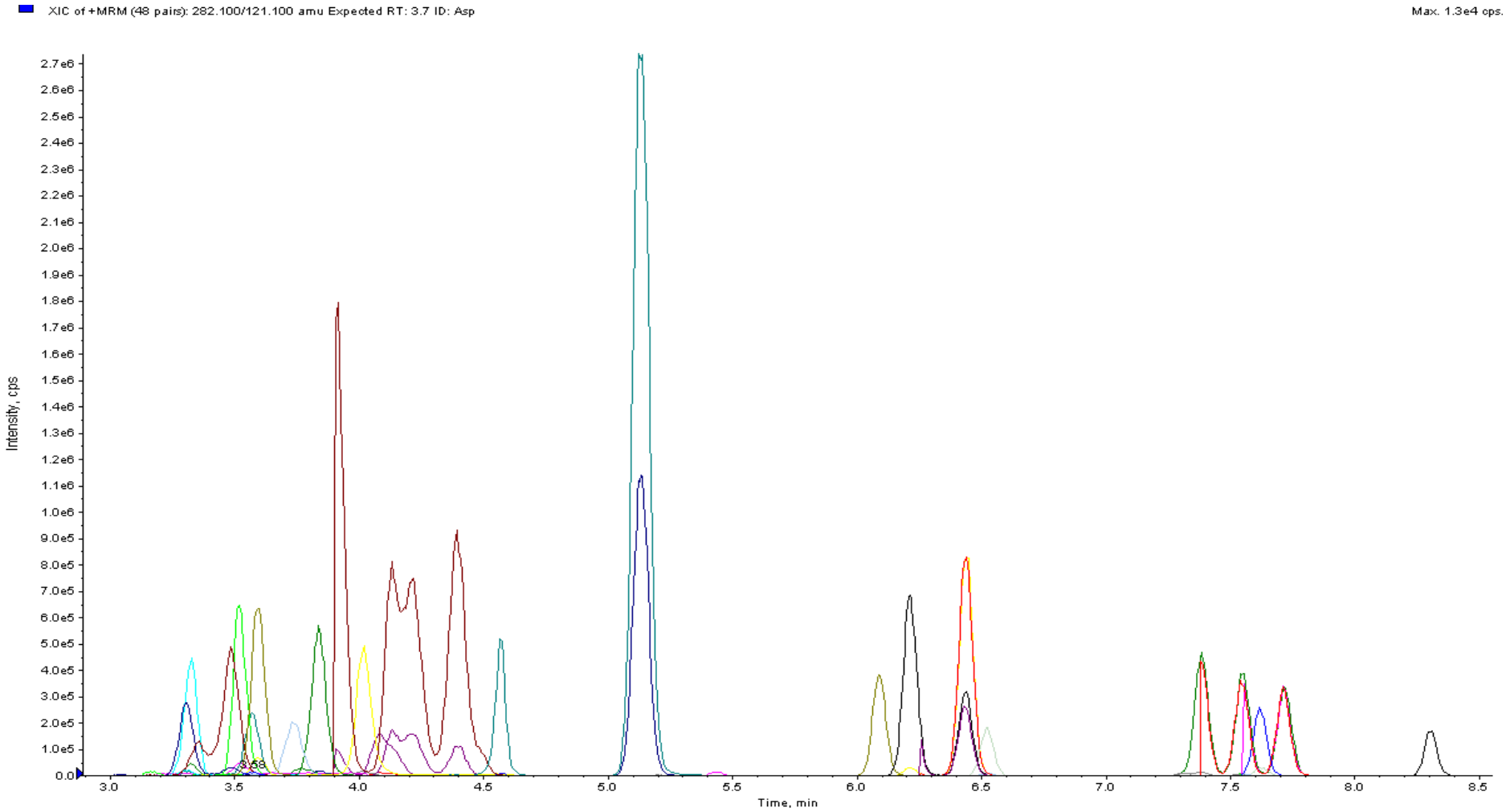
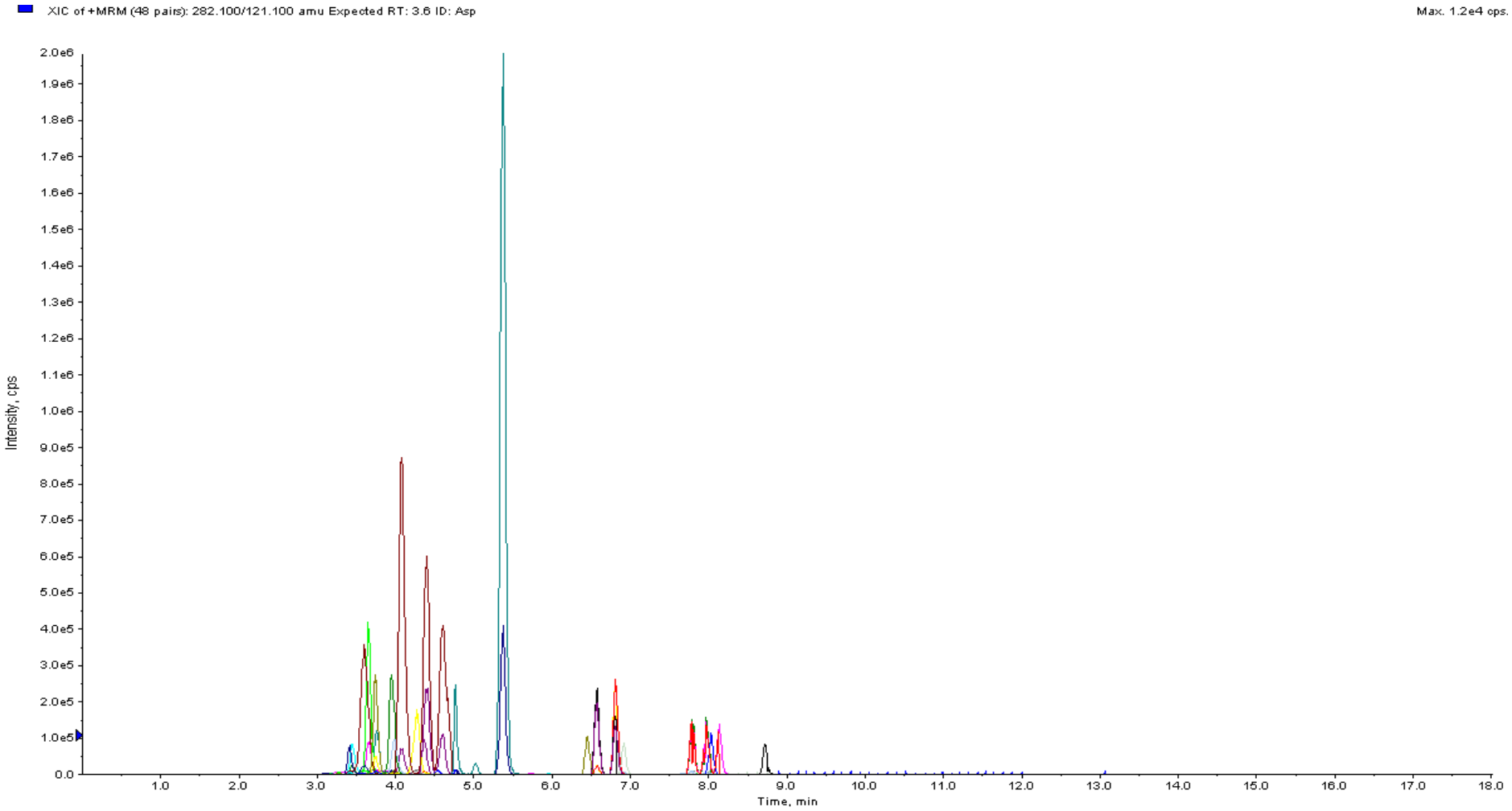
Intensity, cps
Time, min

Honey ID 78



Honey ID 78 (zoomed in)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Intensity, cps
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1.8e6
1.7e6
1.6e6
1.5e6
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1.3e6
1.2e6
1.1e6
1.0e6
9.0e5
8.0e5
7.0e5
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
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12.0
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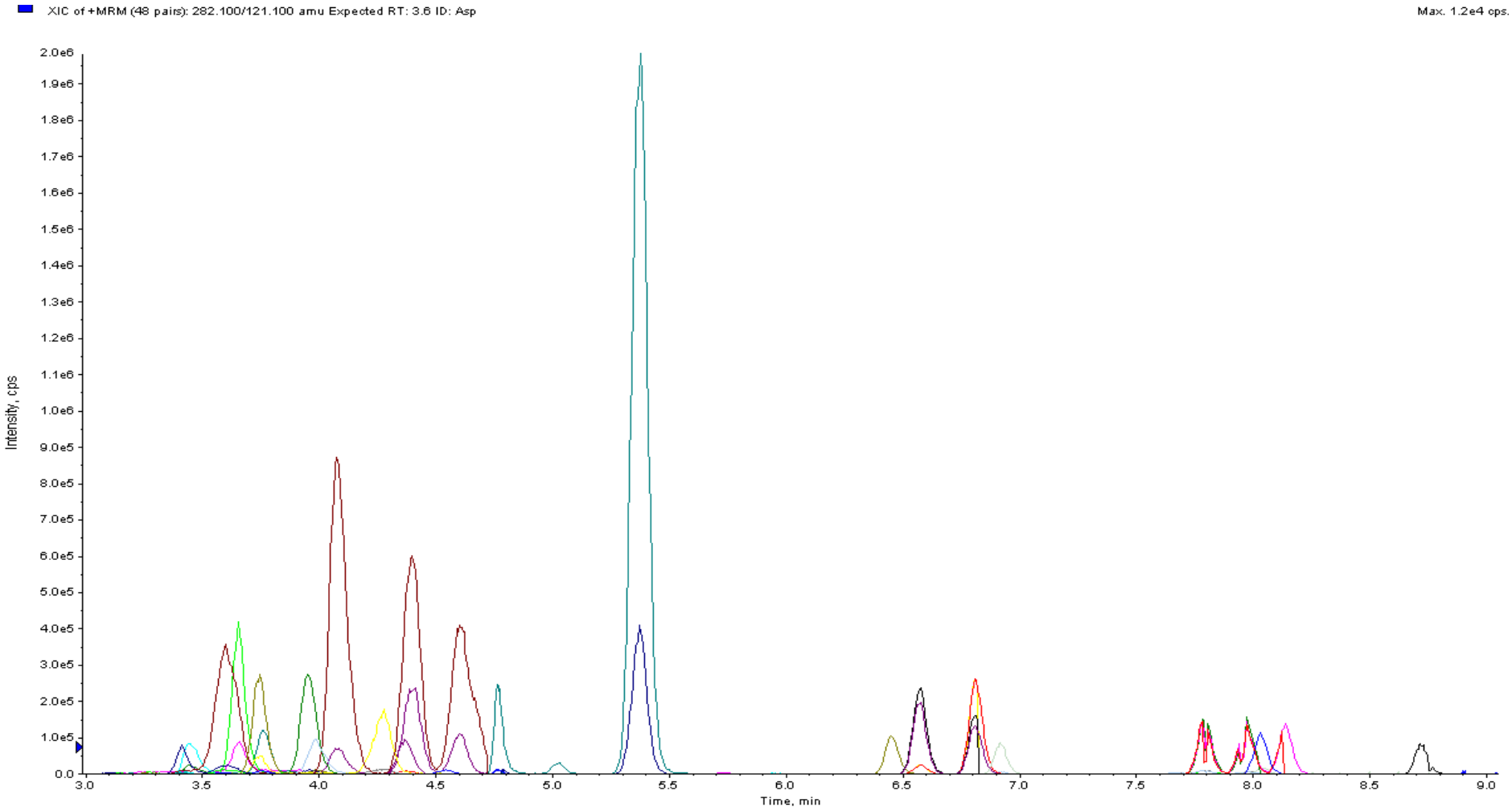
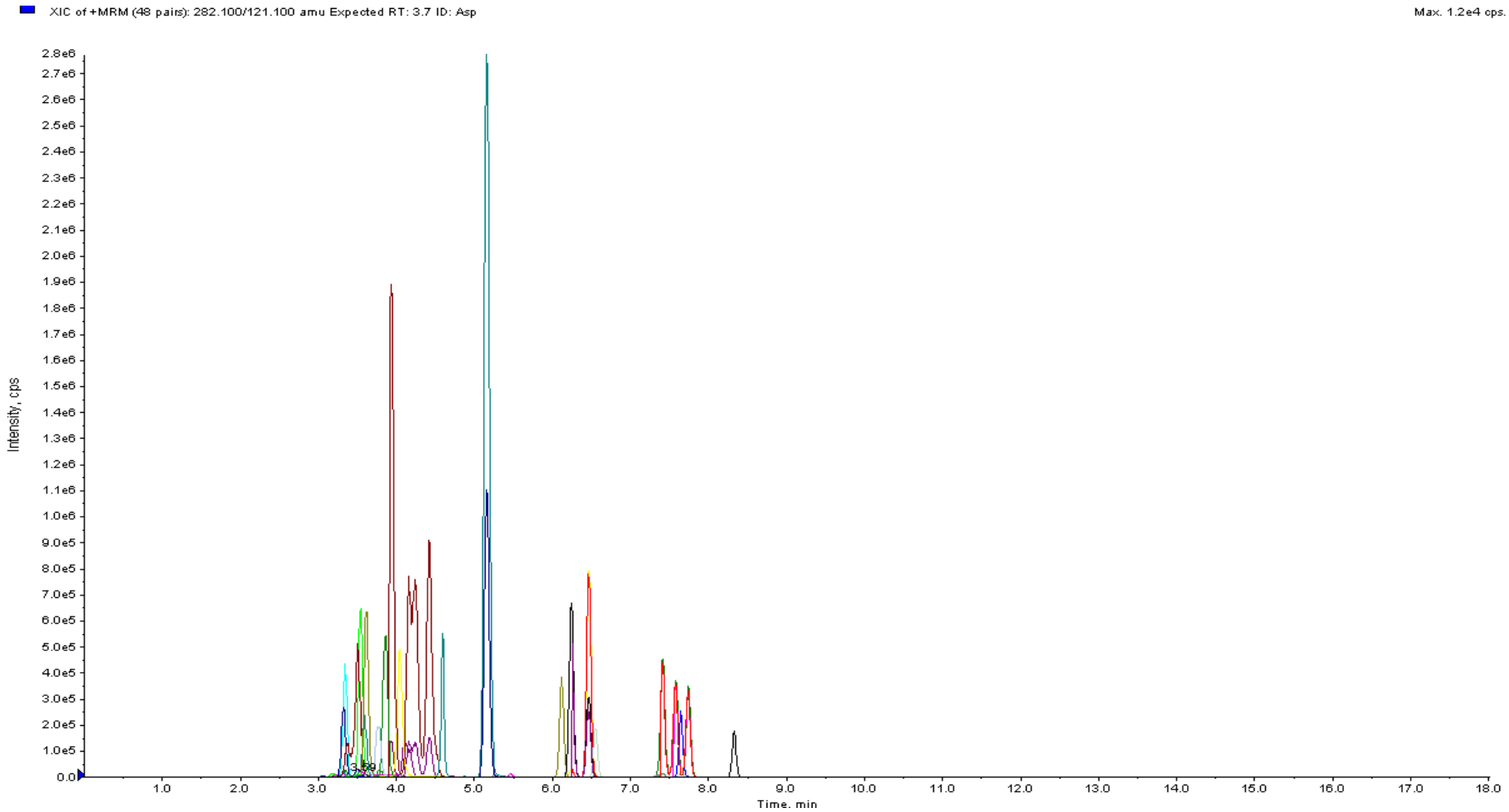
16.0
17.0
18.0
Time, min

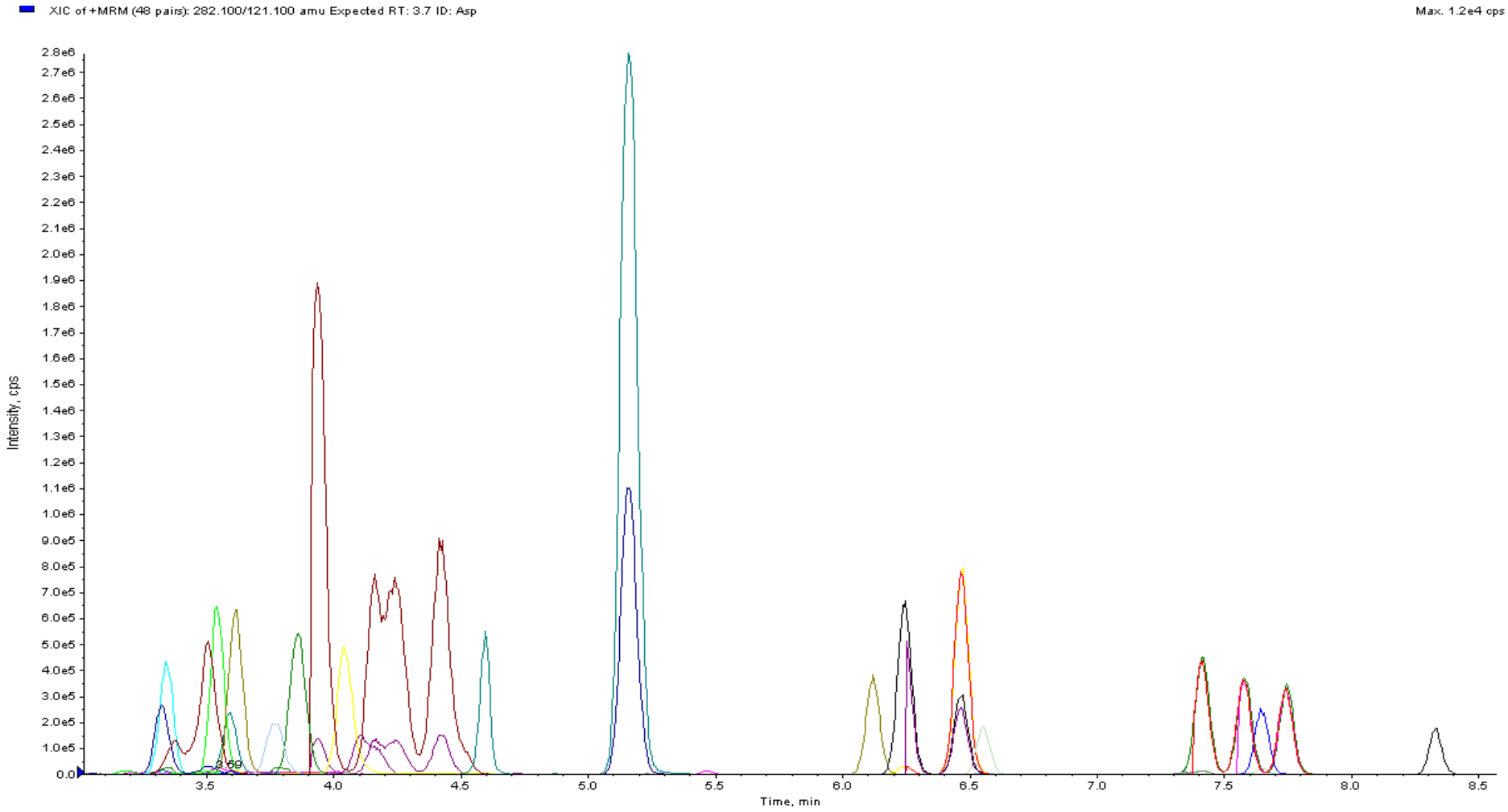
Honey ID 84



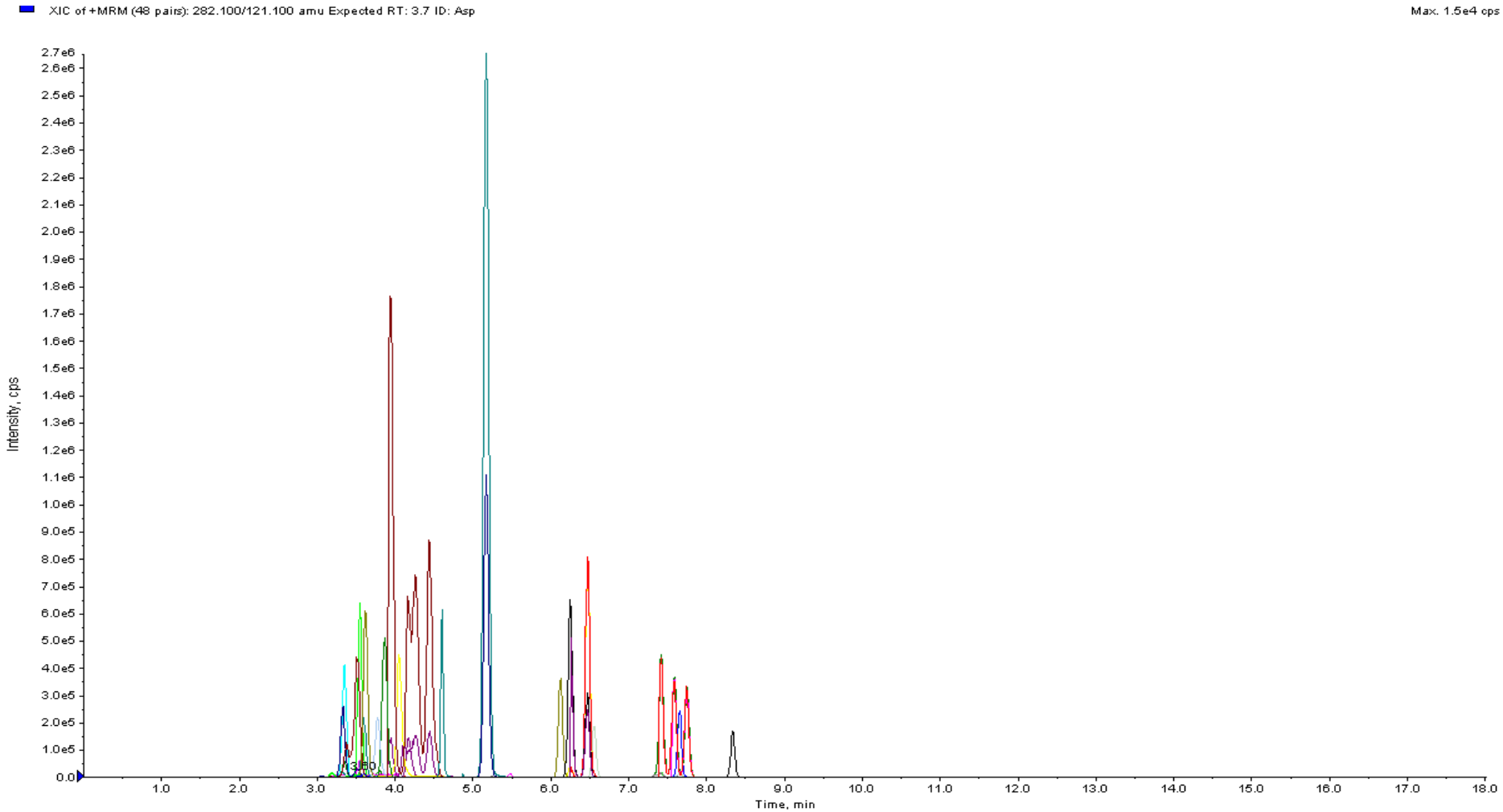
Honey ID 84 (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.2e4 cps.
Intensity, cps
Time, min

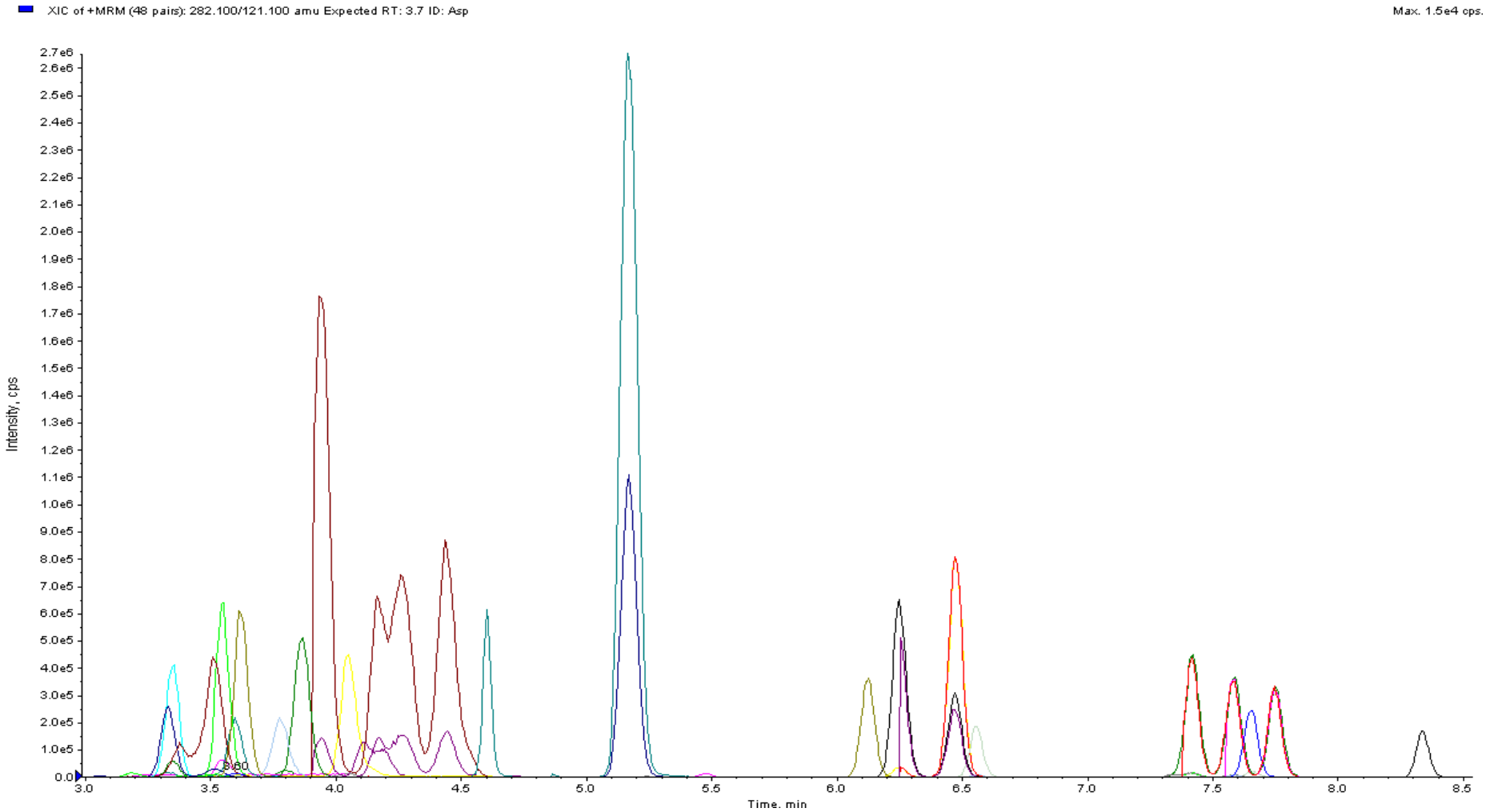
B0 08E3 Cold



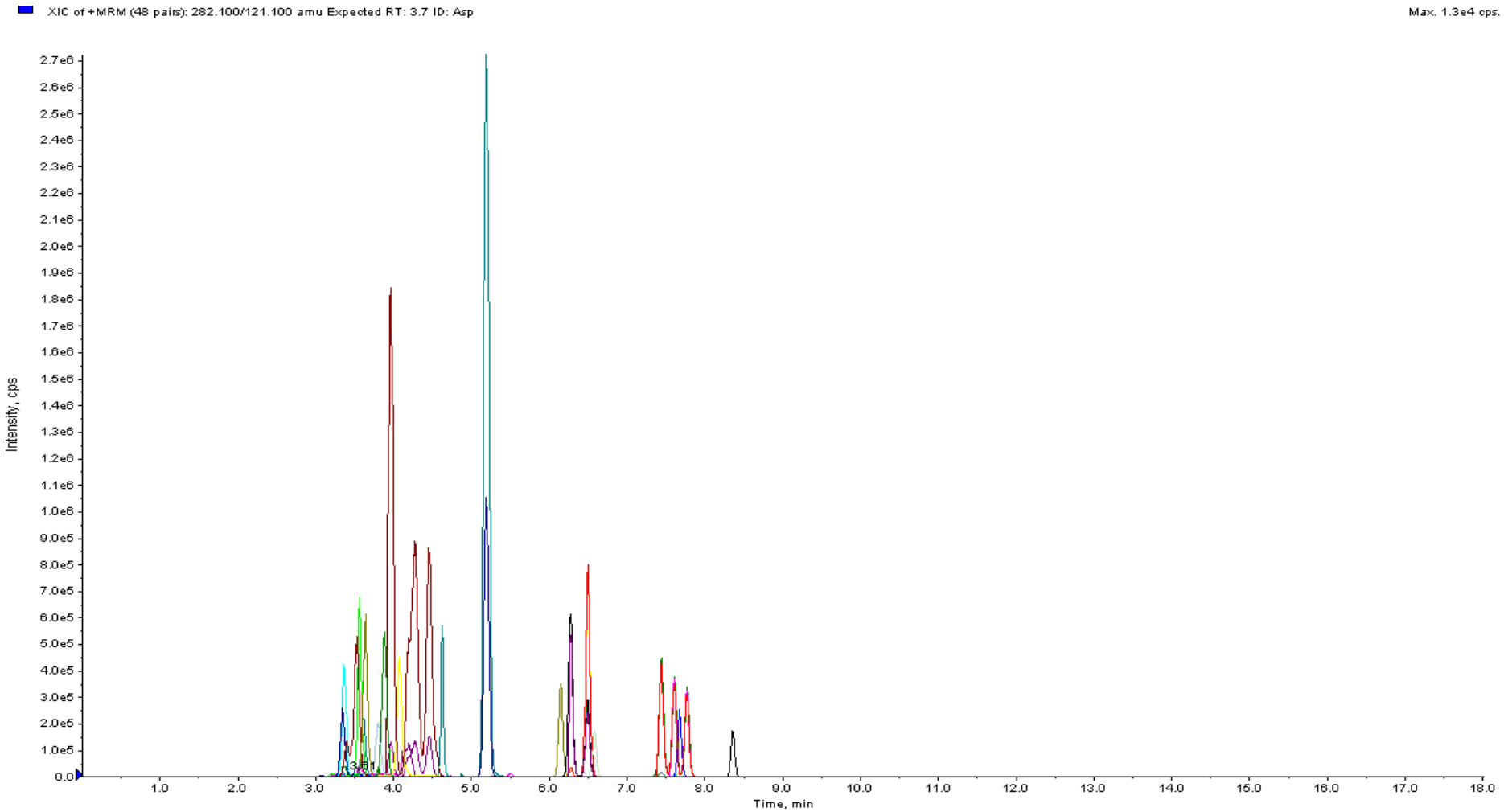
B0 08E3 Cold (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.5e4 cps
Intensity, cps
2.7e6
2.6e6
2.5e6
2.4e6
2.3e6
2.2e6
2.1e6
2.0e6
1.9e6
1.8e6
1.7e6
1.6e6
1.5e6
1.4e6
1.3e6
1.2e6
1.1e6
1.0e6
9.0e5
8.0e5
7.0e5
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
Time, min

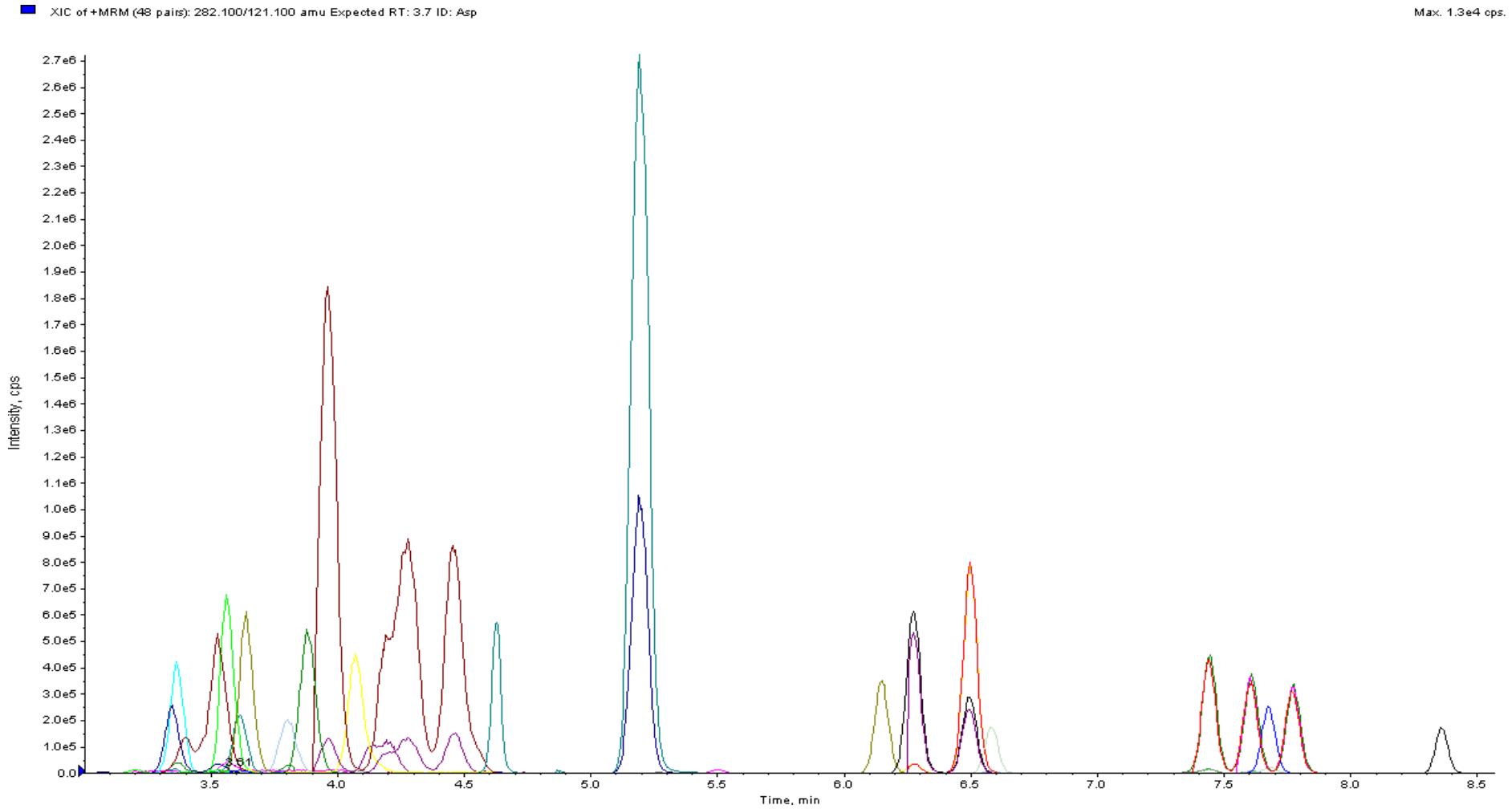
B0 08E3 Warm



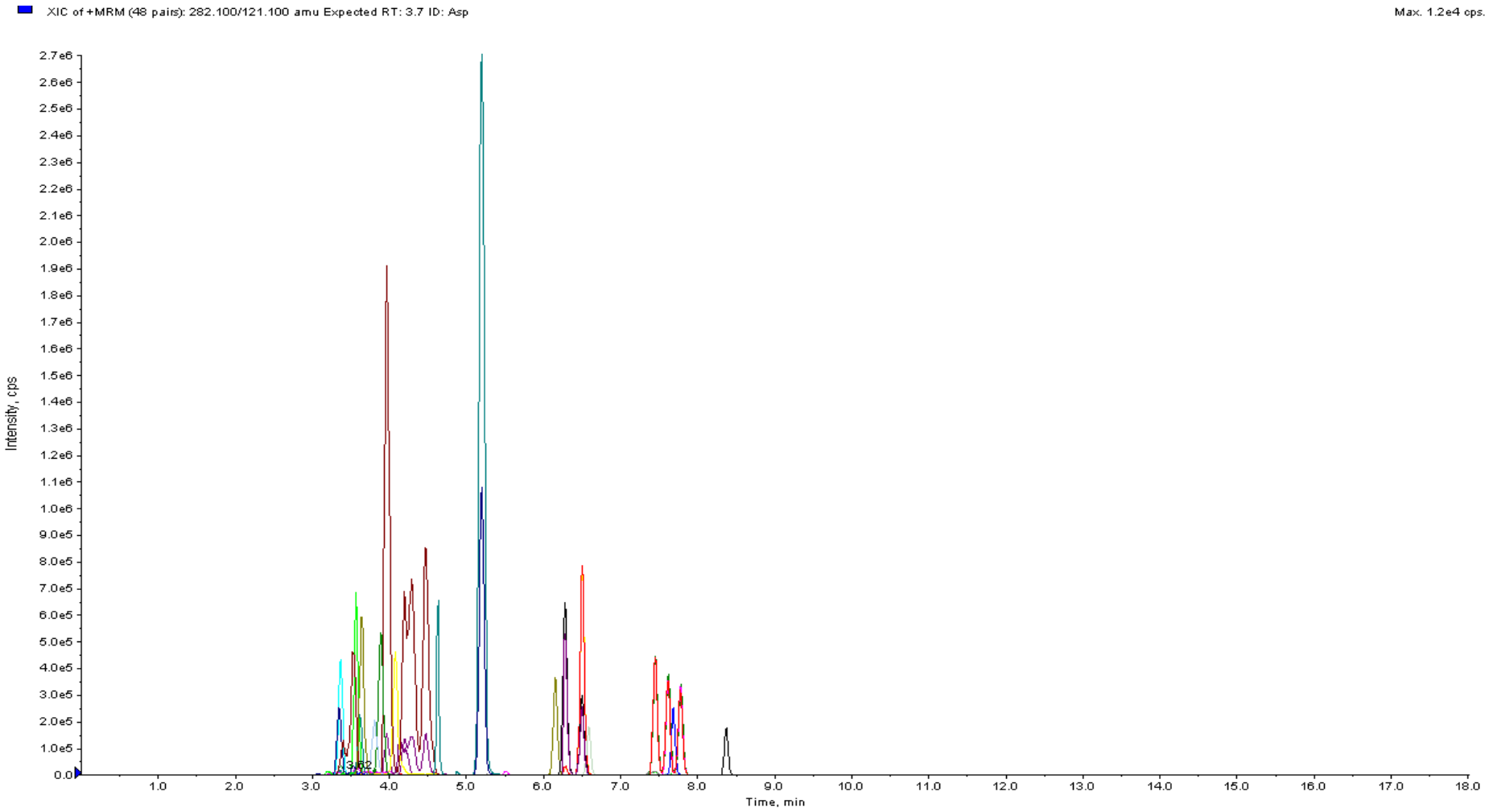
B0 08E3 Warm (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.3e4 cps.
Intensity, cps
2.7e6
2.6e6
2.5e6
2.4e6
2.3e6
2.2e6
2.1e6
2.0e6
1.9e6
1.8e6
1.7e6
1.6e6
1.5e6
1.4e6
1.3e6
1.2e6
1.1e6
1.0e6
9.0e5
8.0e5
7.0e5
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
Time, min

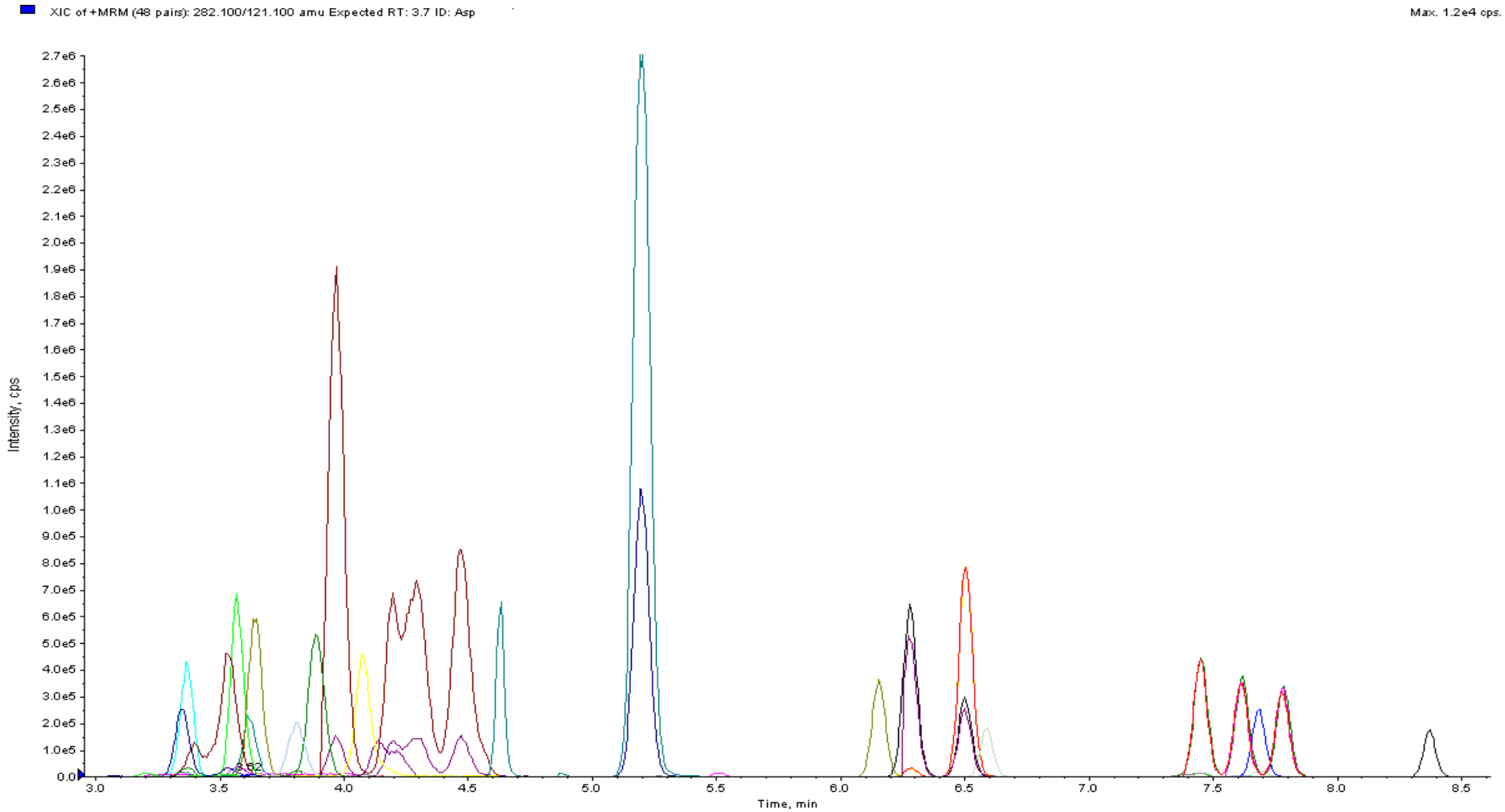
B0 14E3 Cold



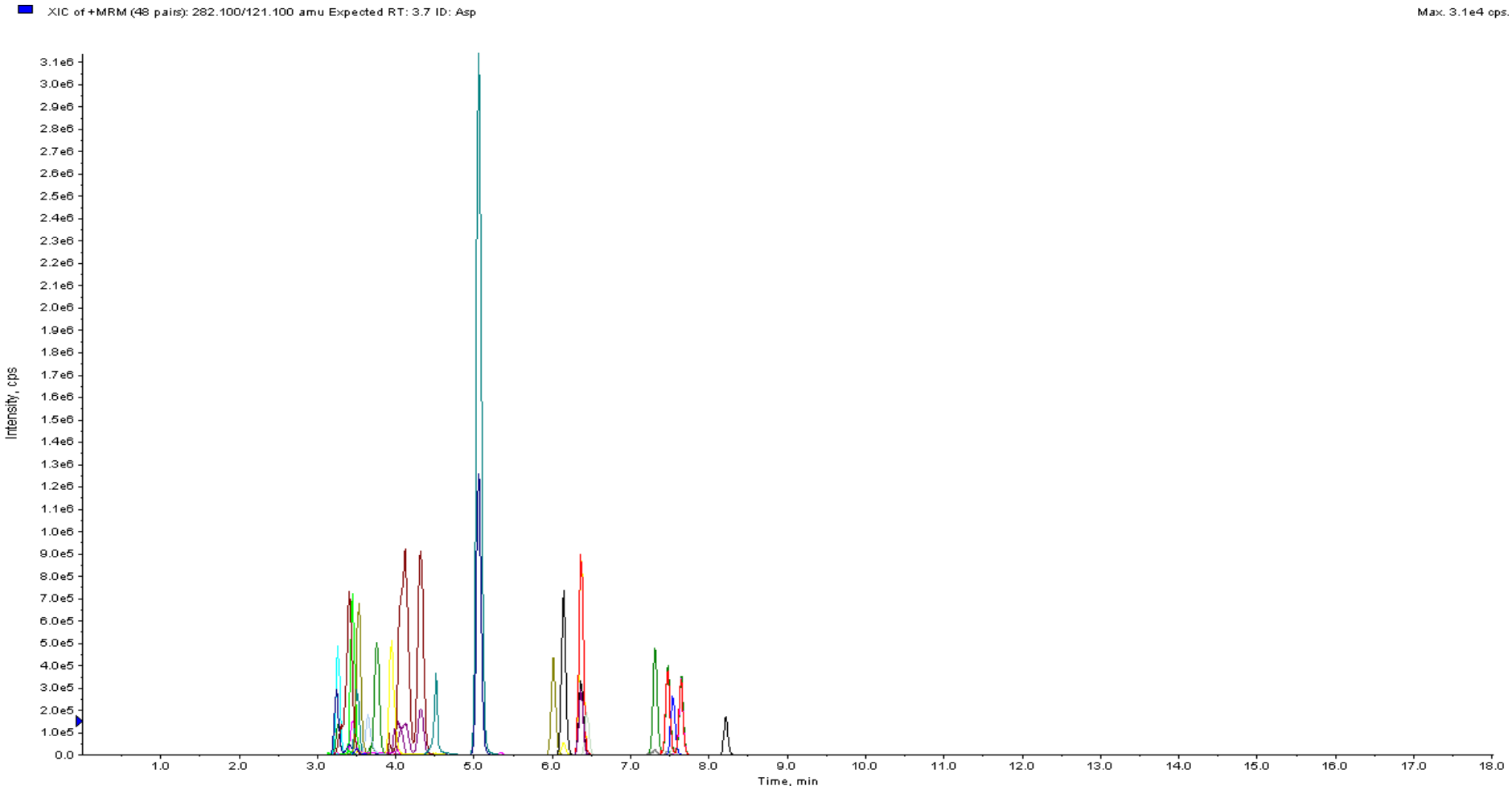
B0 14E3 Cold (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.2e4 cps.
Intensity, cps
Time, min
3.682

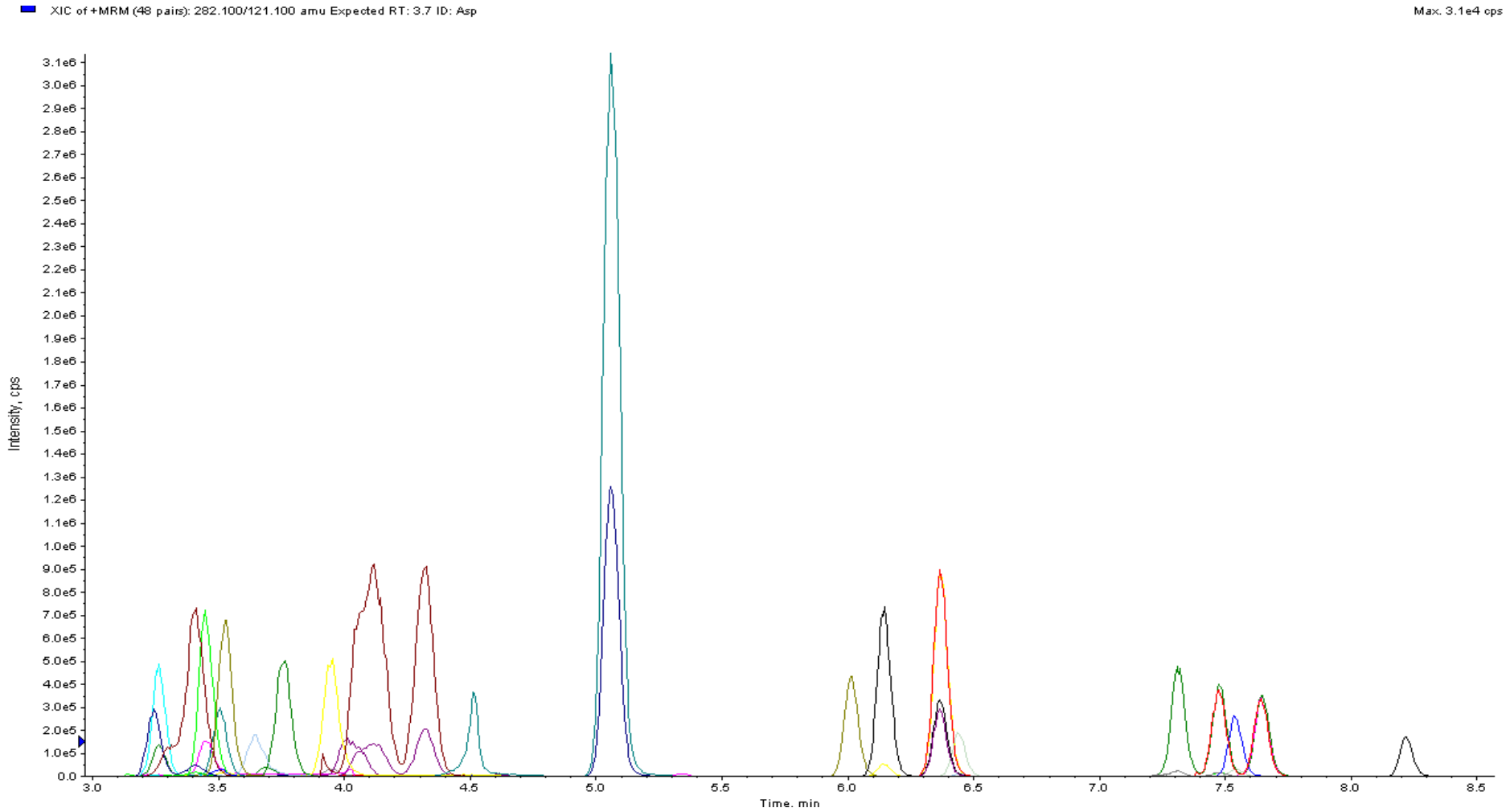
B0 14E3 Warm



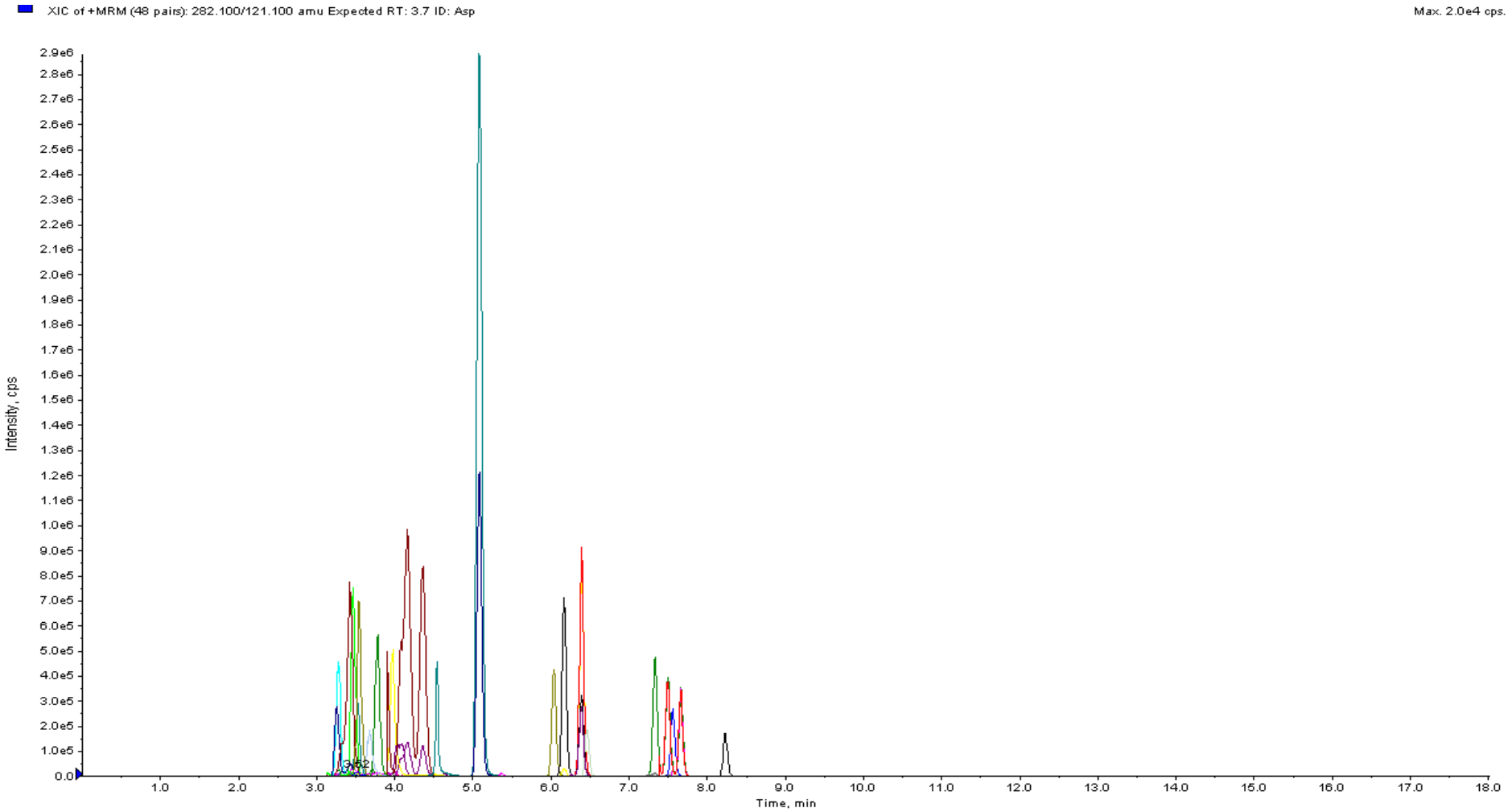
B0 14E3 Warm (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 3.1e4 cps.
Intensity, cps
Time, min

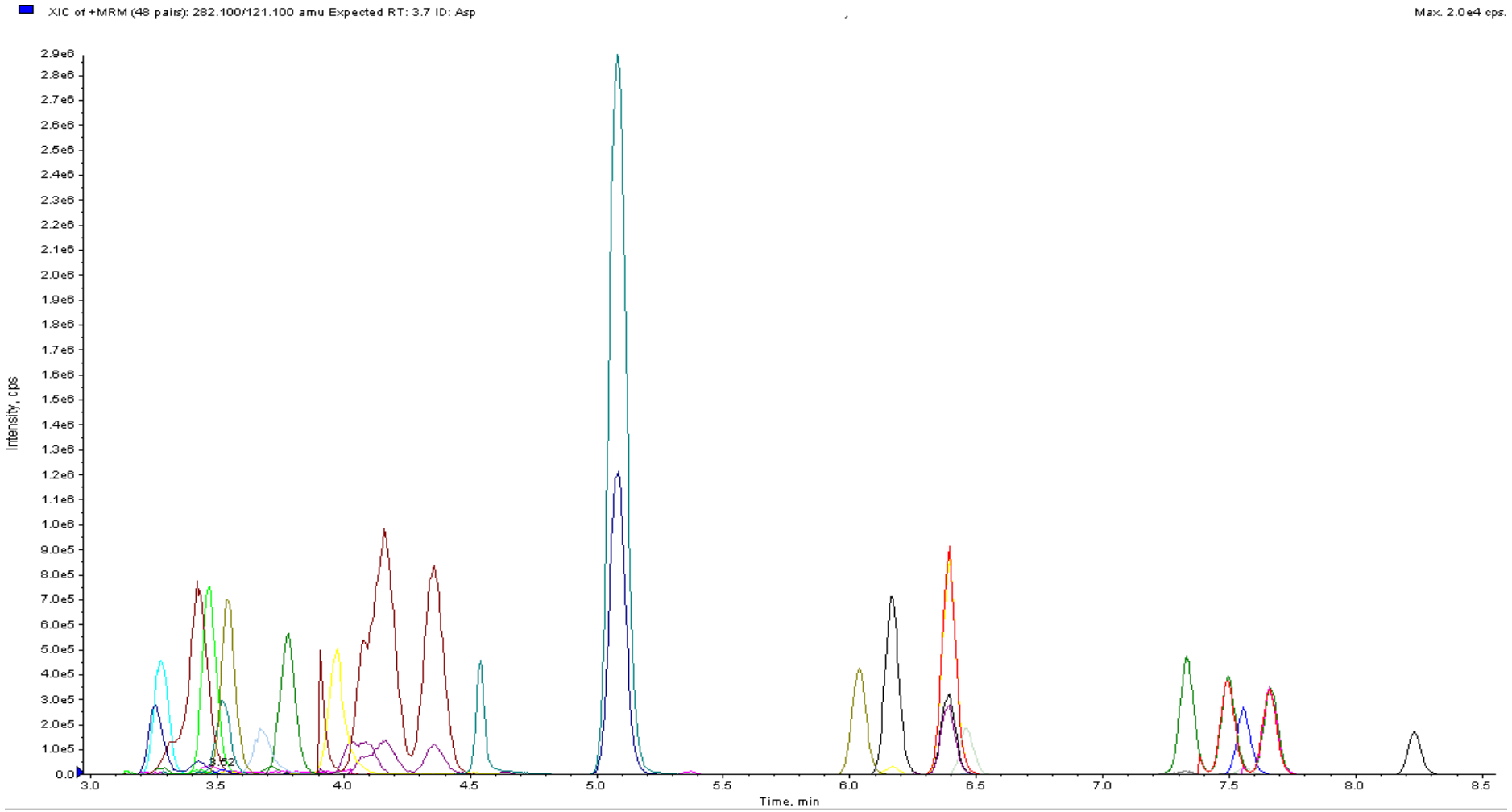
B0 23E3 Cold



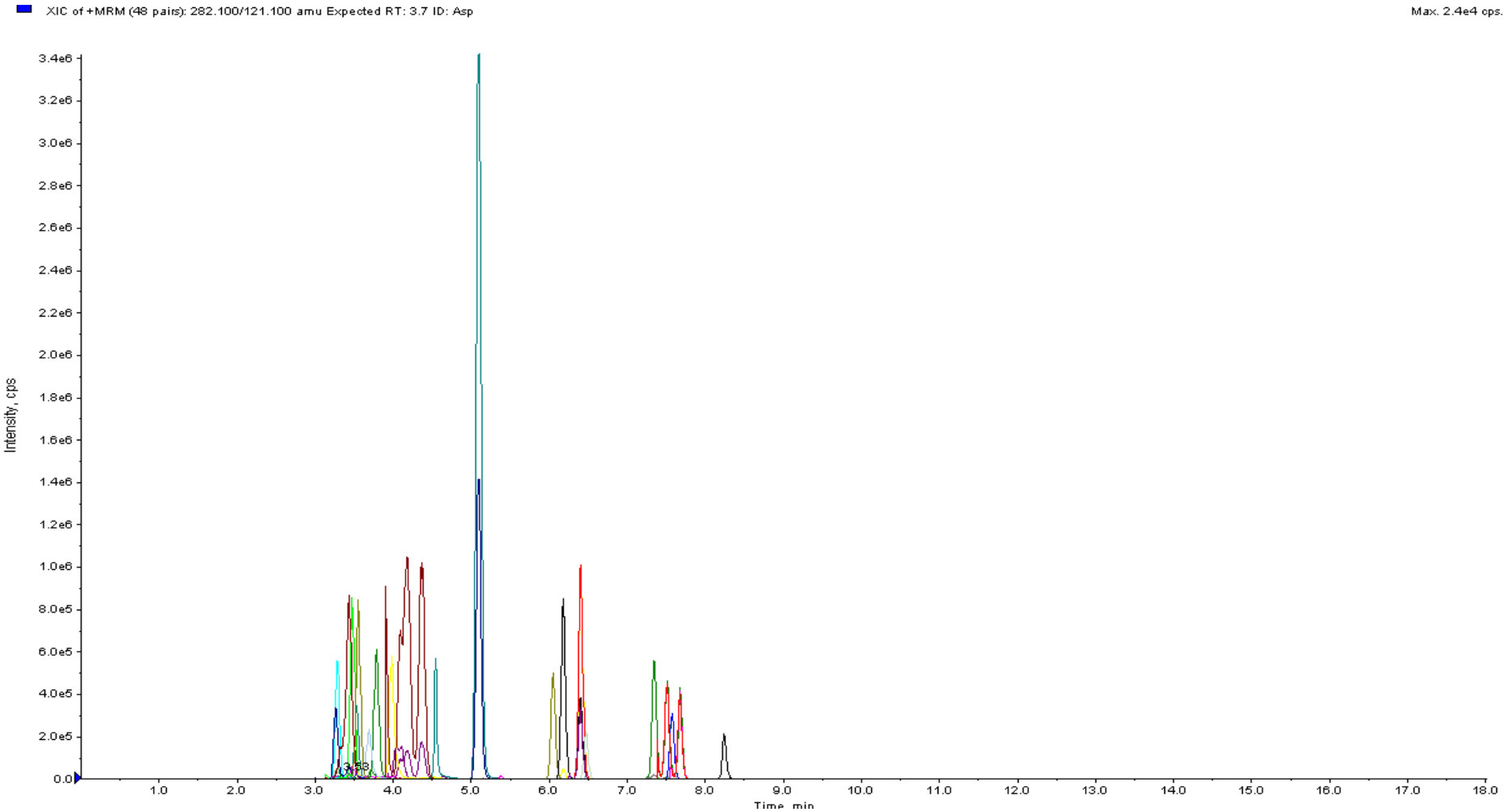
B0 23E3 Cold (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 2.0e4 cps.
Intensity, cps
Time, min

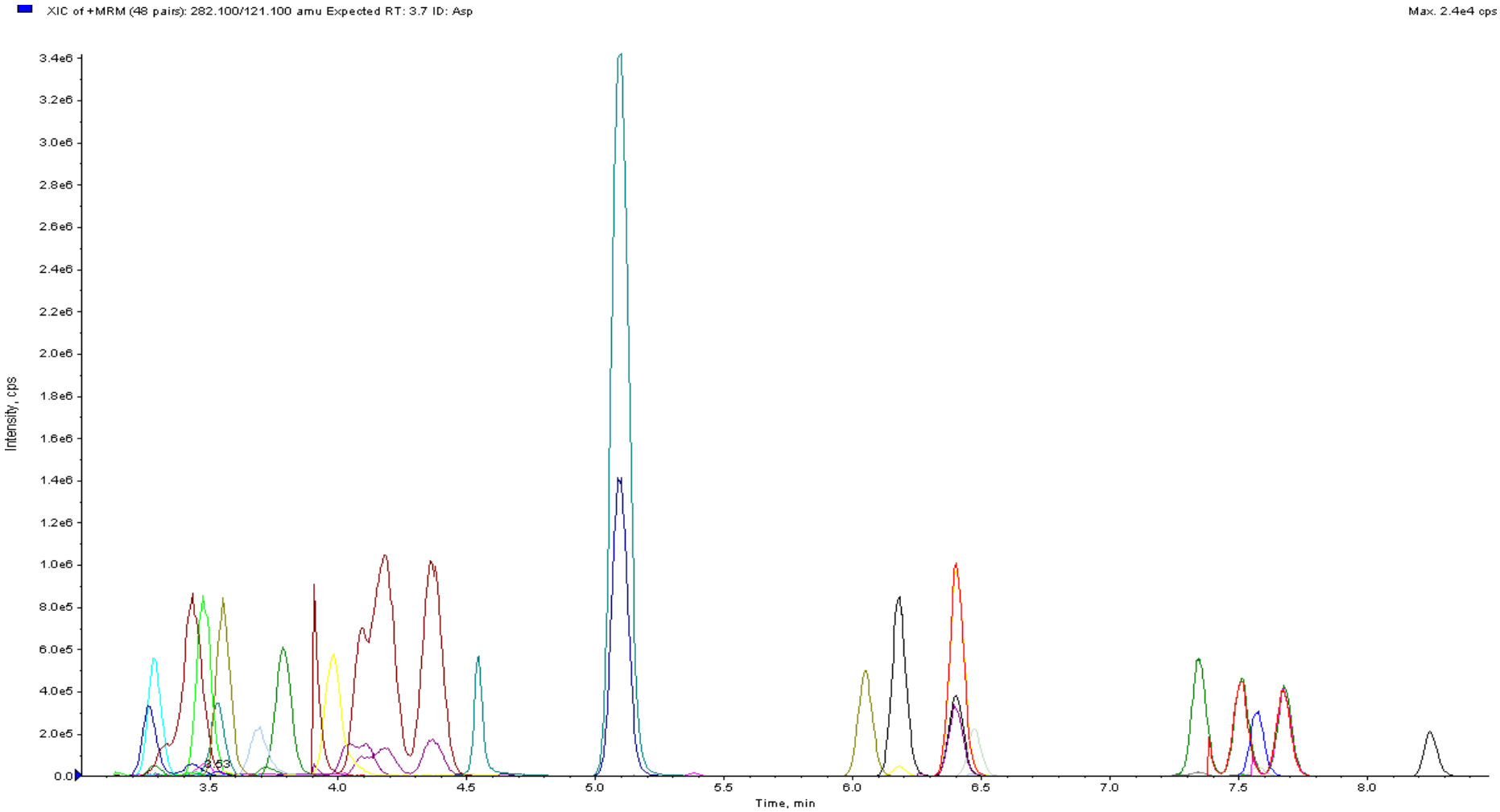
B0 23E3 Warm



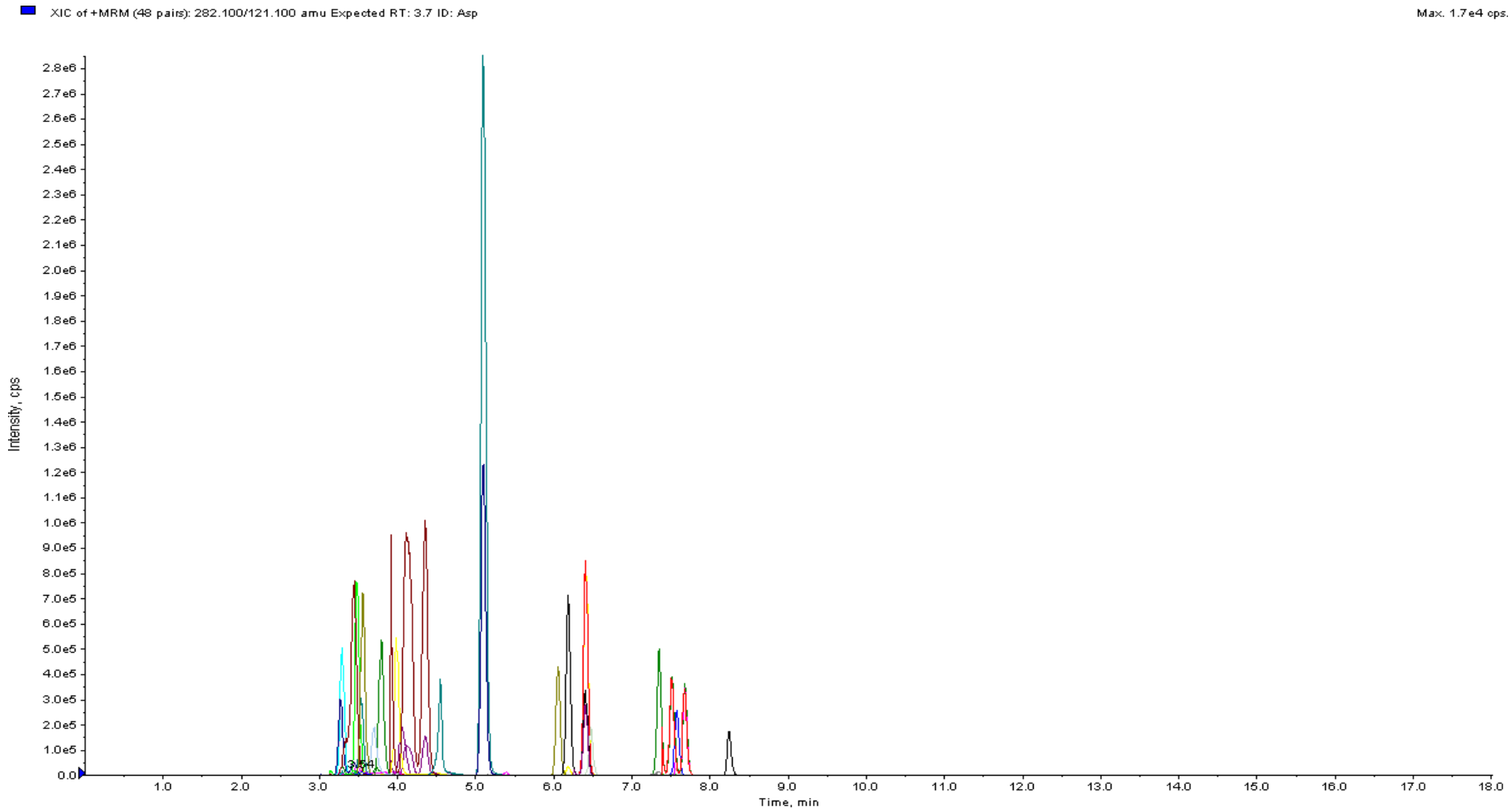
B0 23E3 Warm (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 2.4e4 cps.
Intensity, cps
3.4e6
3.2e6
3.0e6
2.8e6
2.6e6
2.4e6
2.2e6
2.0e6
1.8e6
1.6e6
1.4e6
1.2e6
1.0e6
8.0e5
6.0e5
4.0e5
2.0e5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
Time, min

B0 24E3 Cold



B0 24E3 Cold (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.7e4 cps.
Intensity, cps
2.8e6
2.7e6
2.6e6
2.5e6
2.4e6
2.3e6
2.2e6
2.1e6
2.0e6
1.9e6
1.8e6
1.7e6
1.6e6
1.5e6
1.4e6
1.3e6
1.2e6
1.1e6
1.0e6
9.0e5
8.0e5
7.0e5
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0.0
1.0
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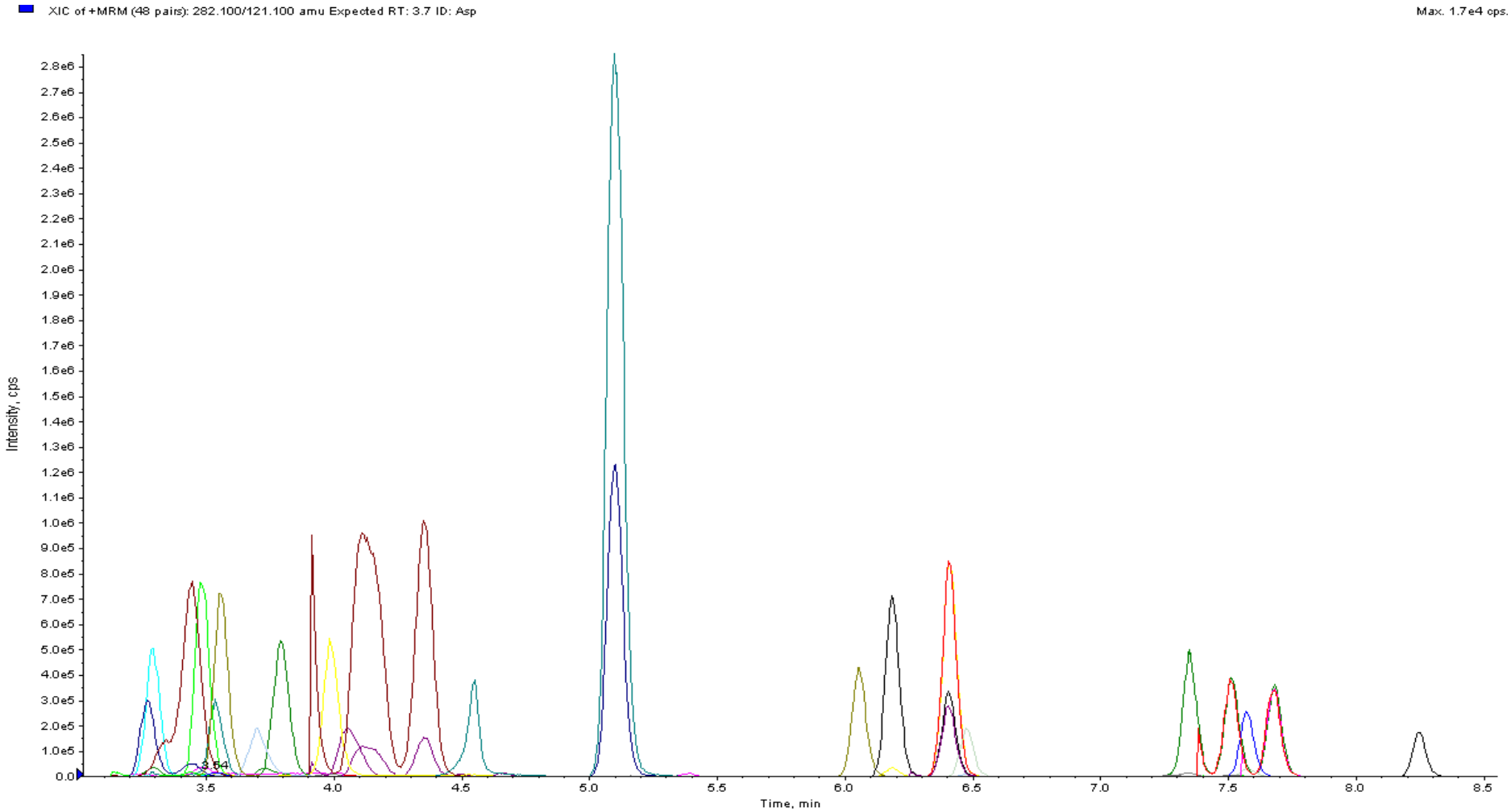
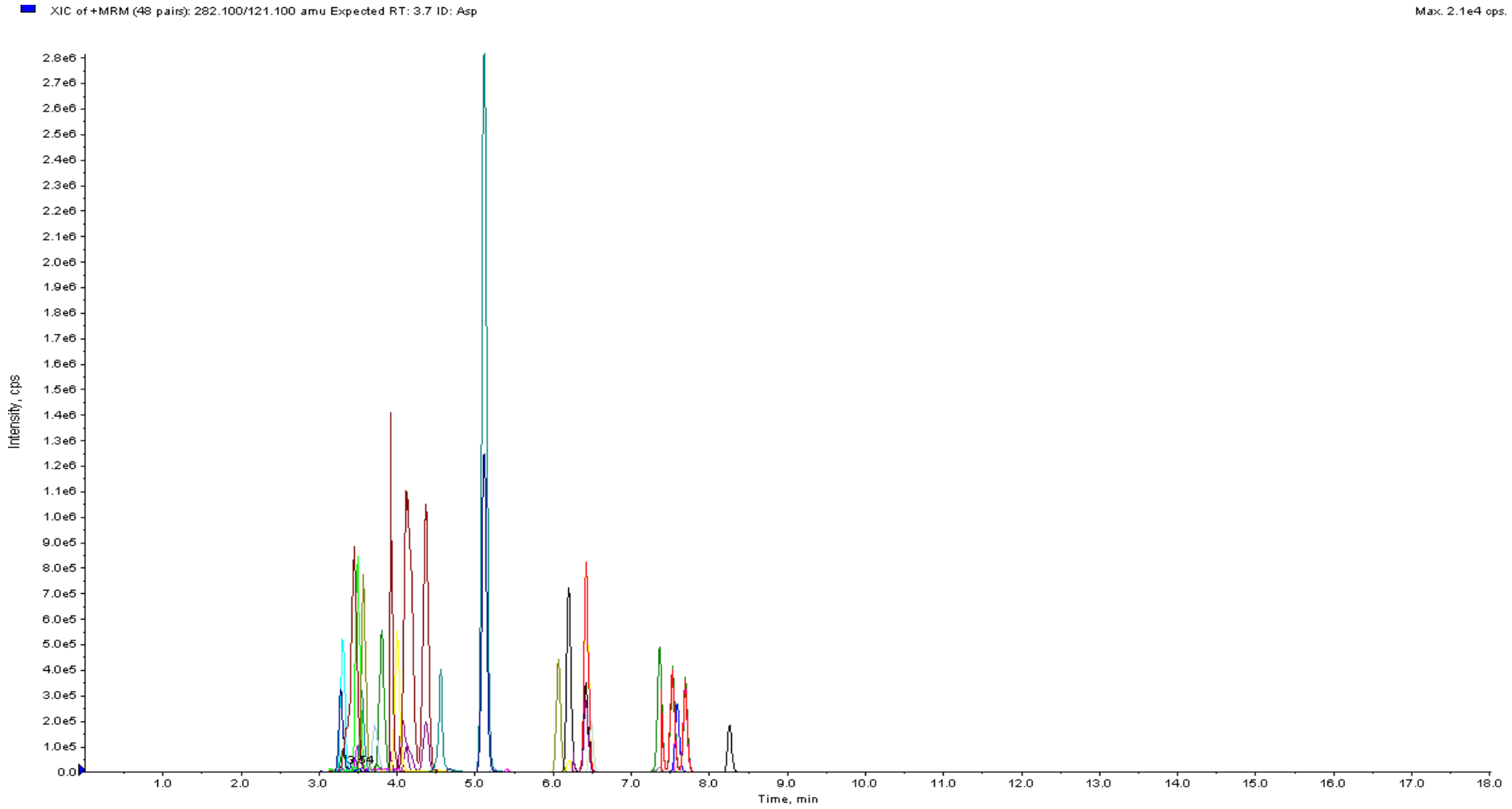
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
Time, min

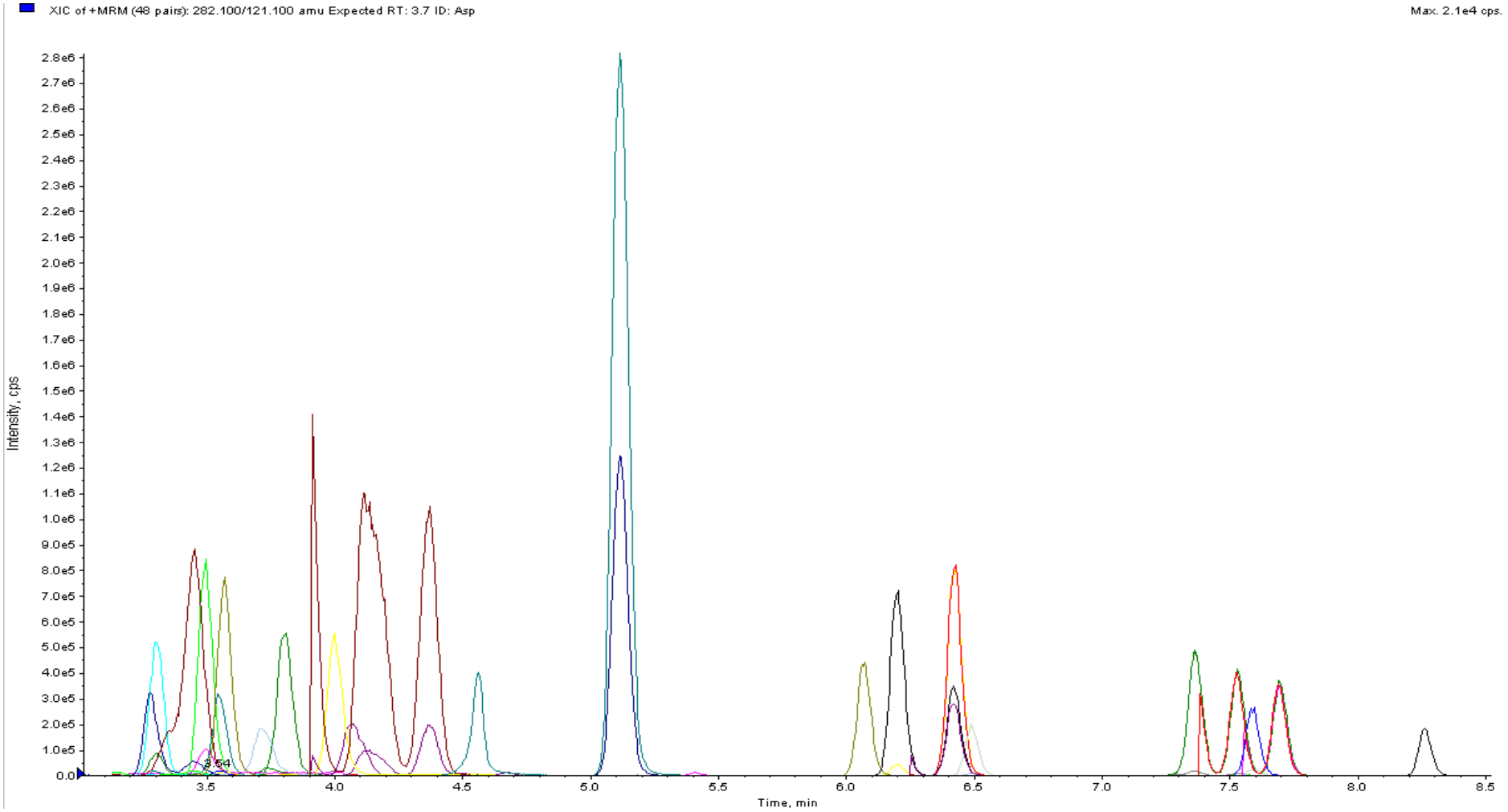
B0 24E3 Warm



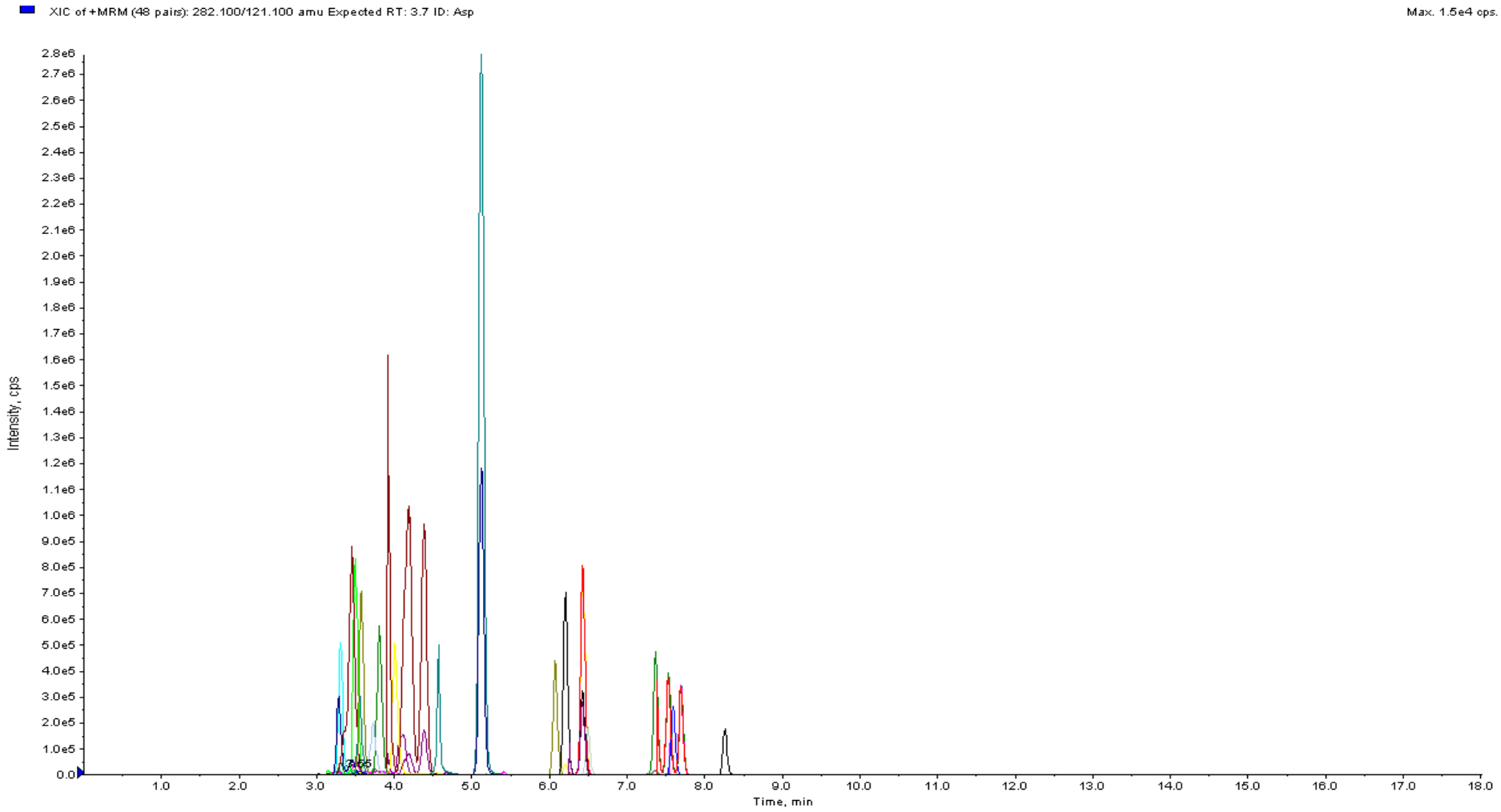
B0 24E3 Warm (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 2.1e4 cps.
Intensity, cps
Time, min

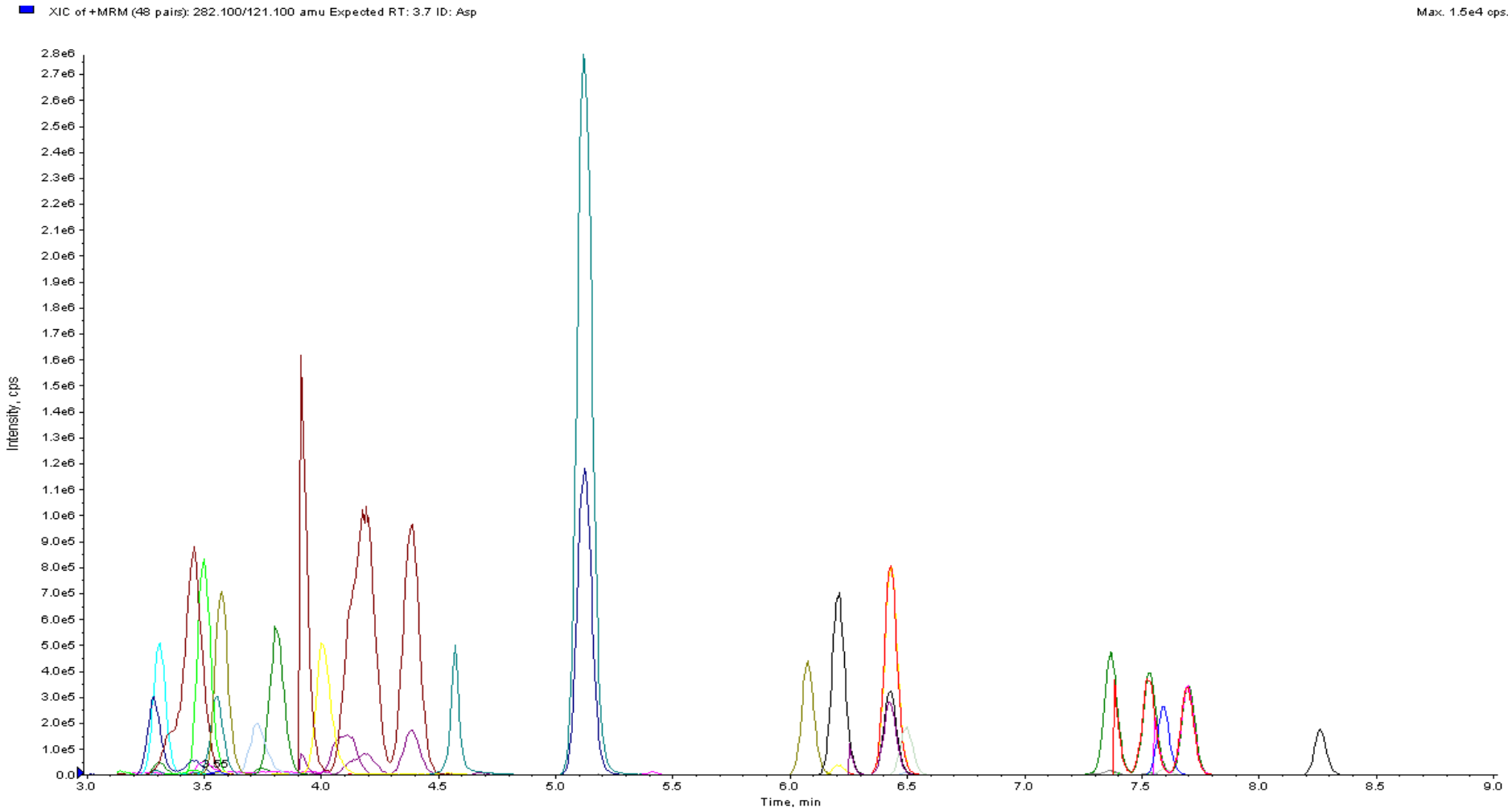
C 463 Cold



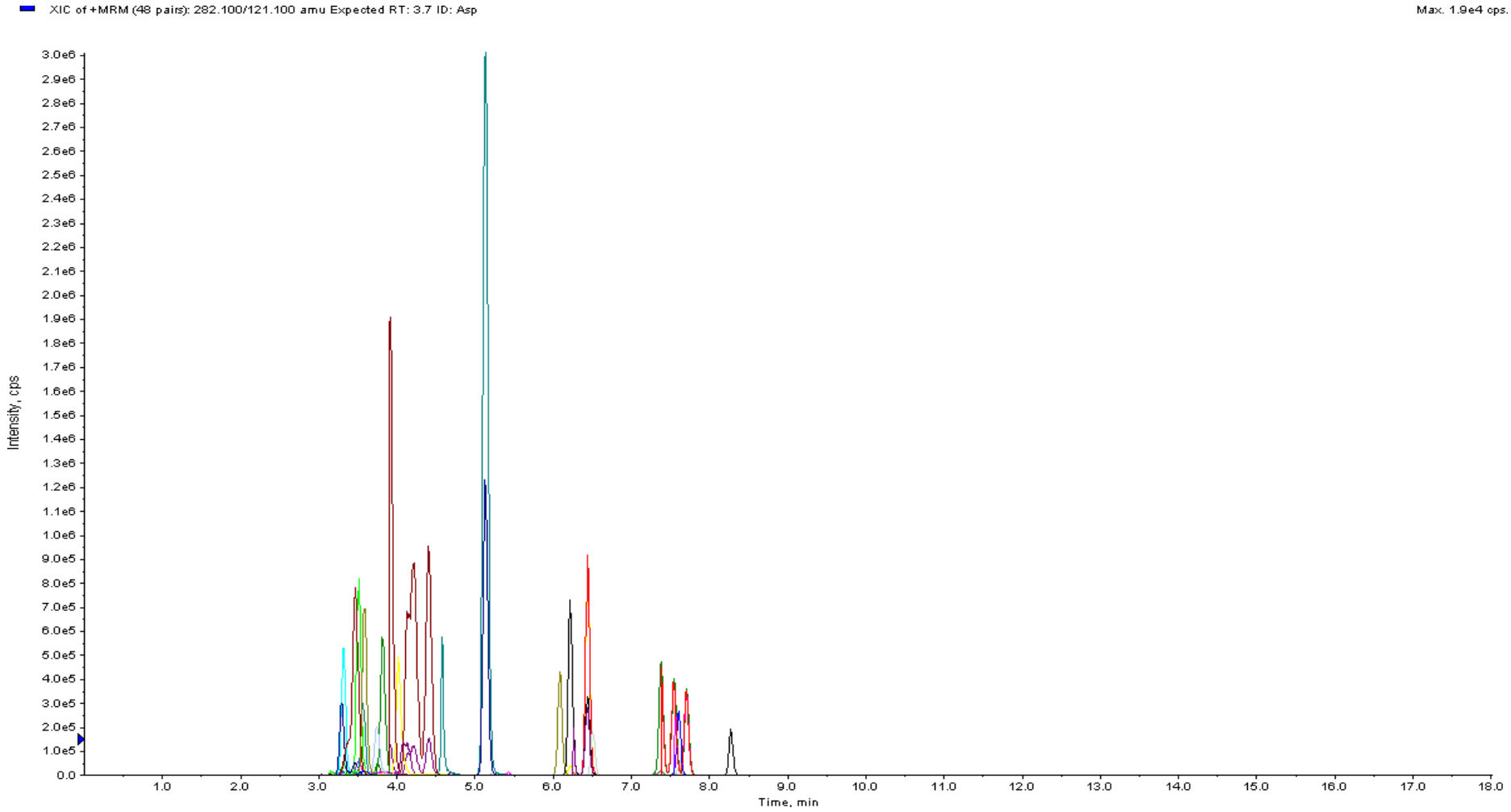
C 463 Cold (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.5e4 cps.
Intensity, cps
Time, min

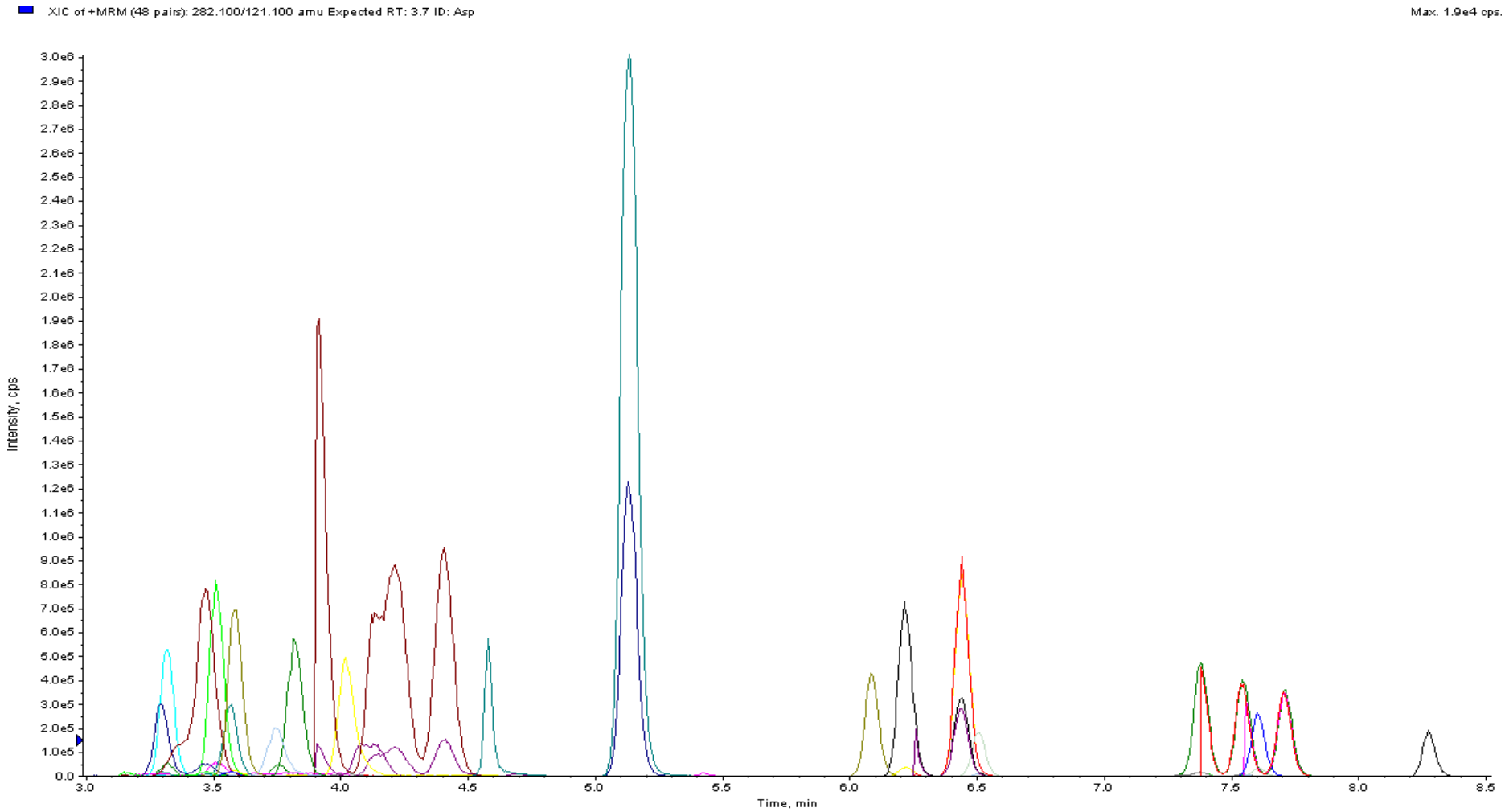
C 463 Warm



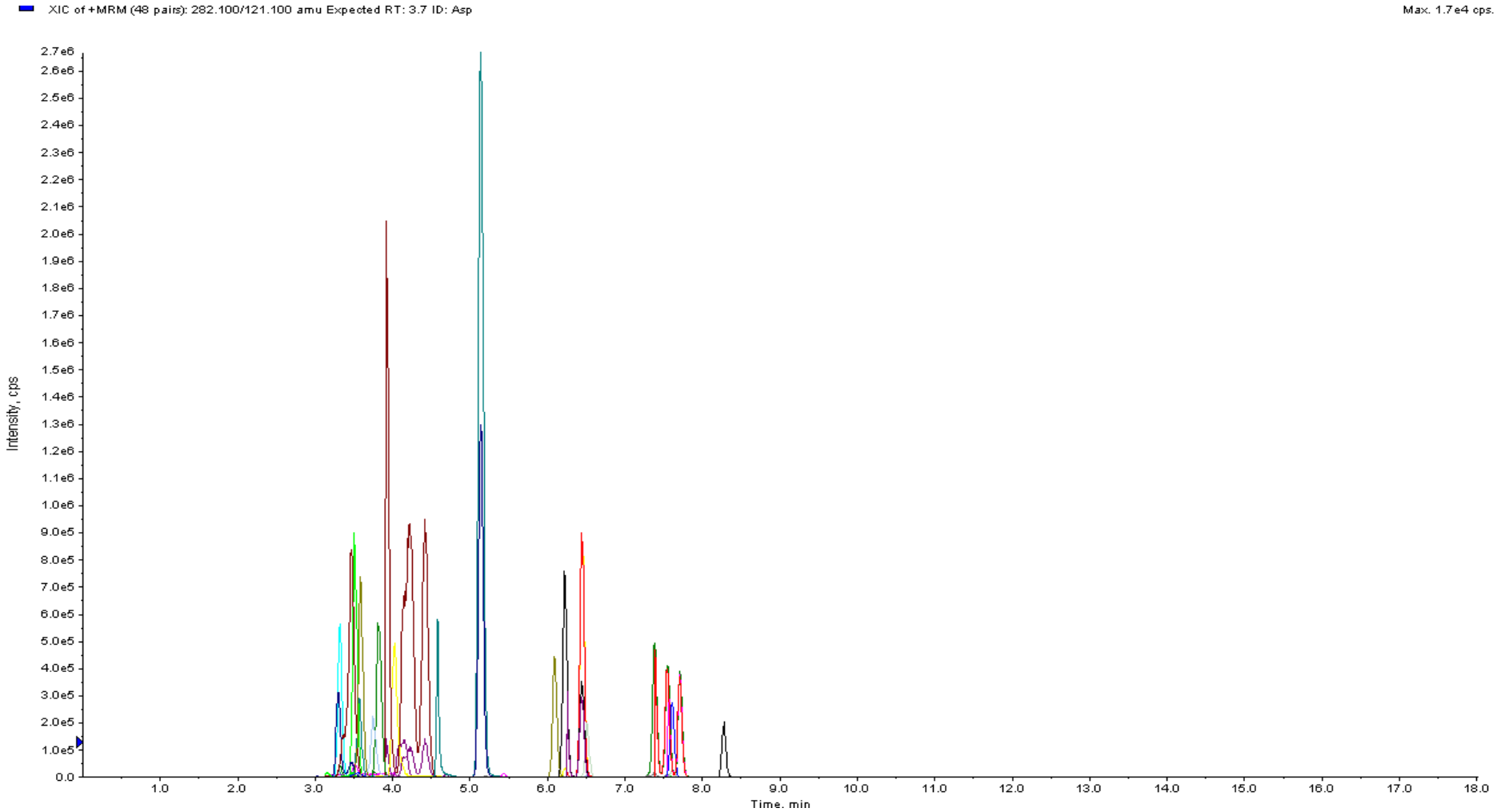
C 463 Warm (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.9e4 cps.
Intensity, cps
3.0e6
2.9e6
2.8e6
2.7e6
2.6e6
2.5e6
2.4e6
2.3e6
2.2e6
2.1e6
2.0e6
1.9e6
1.8e6
1.7e6
1.6e6
1.5e6
1.4e6
1.3e6
1.2e6
1.1e6
1.0e6
9.0e5
8.0e5
7.0e5
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
Time, min

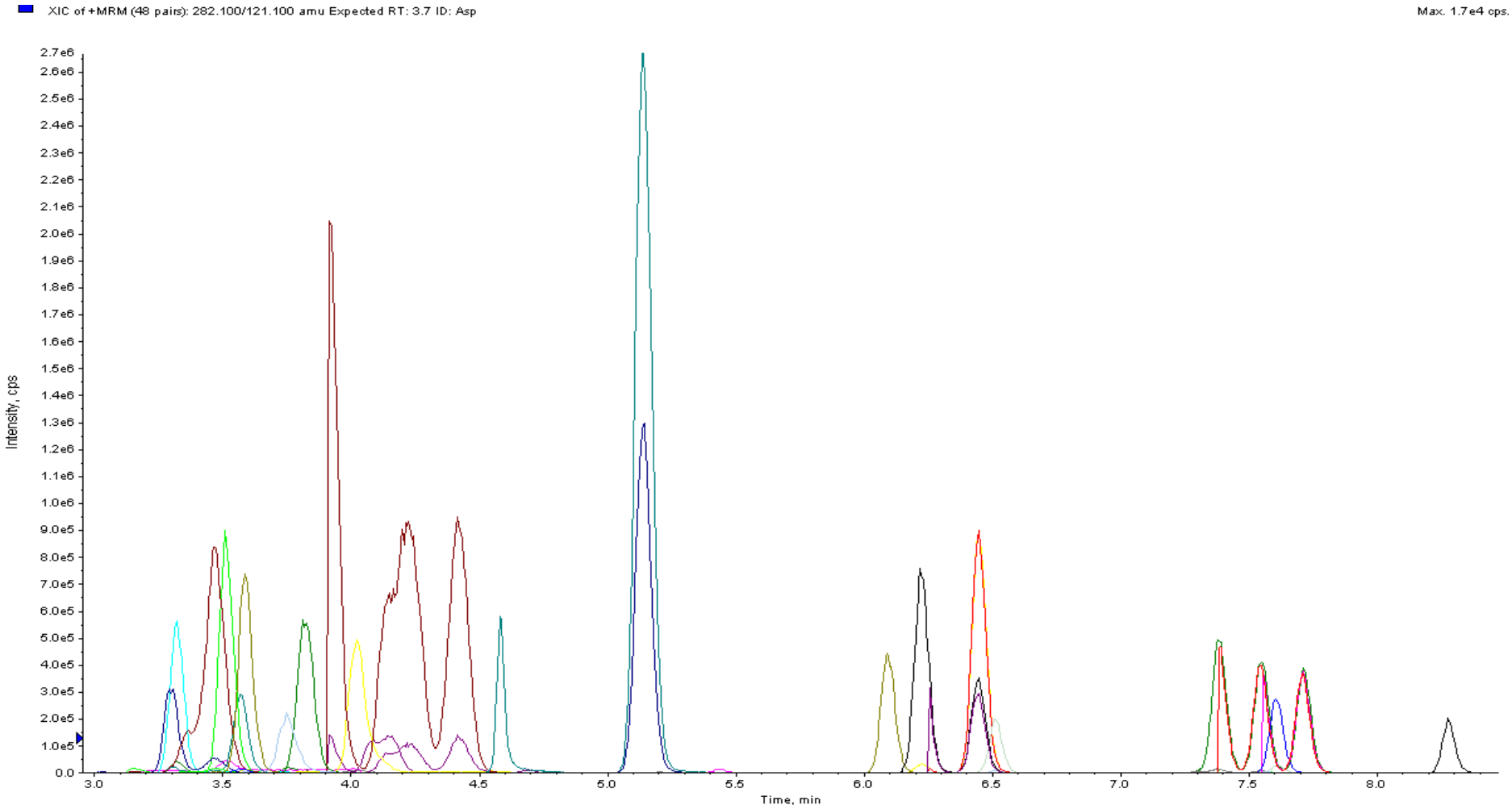
C 887 Cold



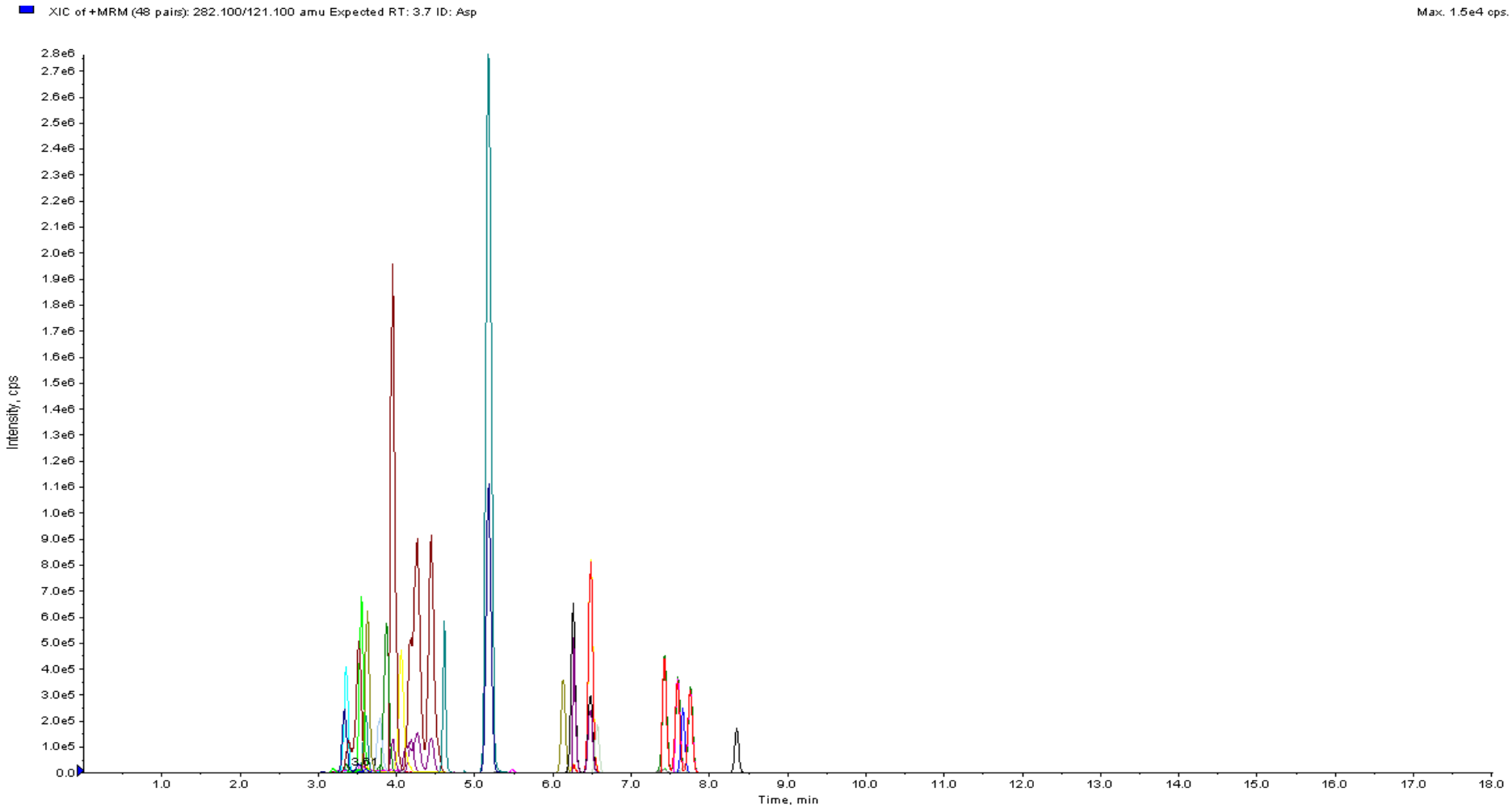
C 887 Cold (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.7e4 cps.
Intensity, cps
Time, min

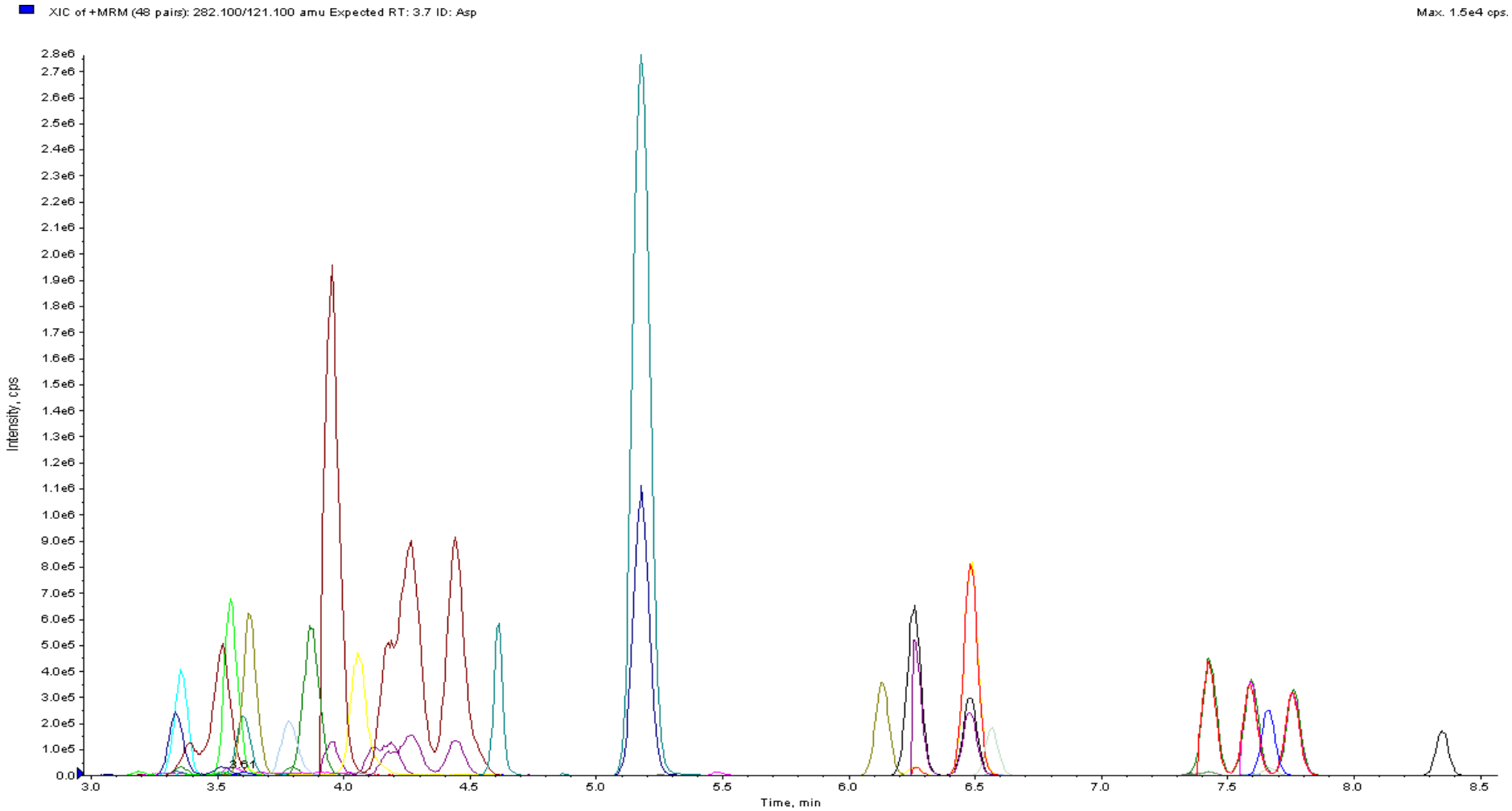
C 887 Warm



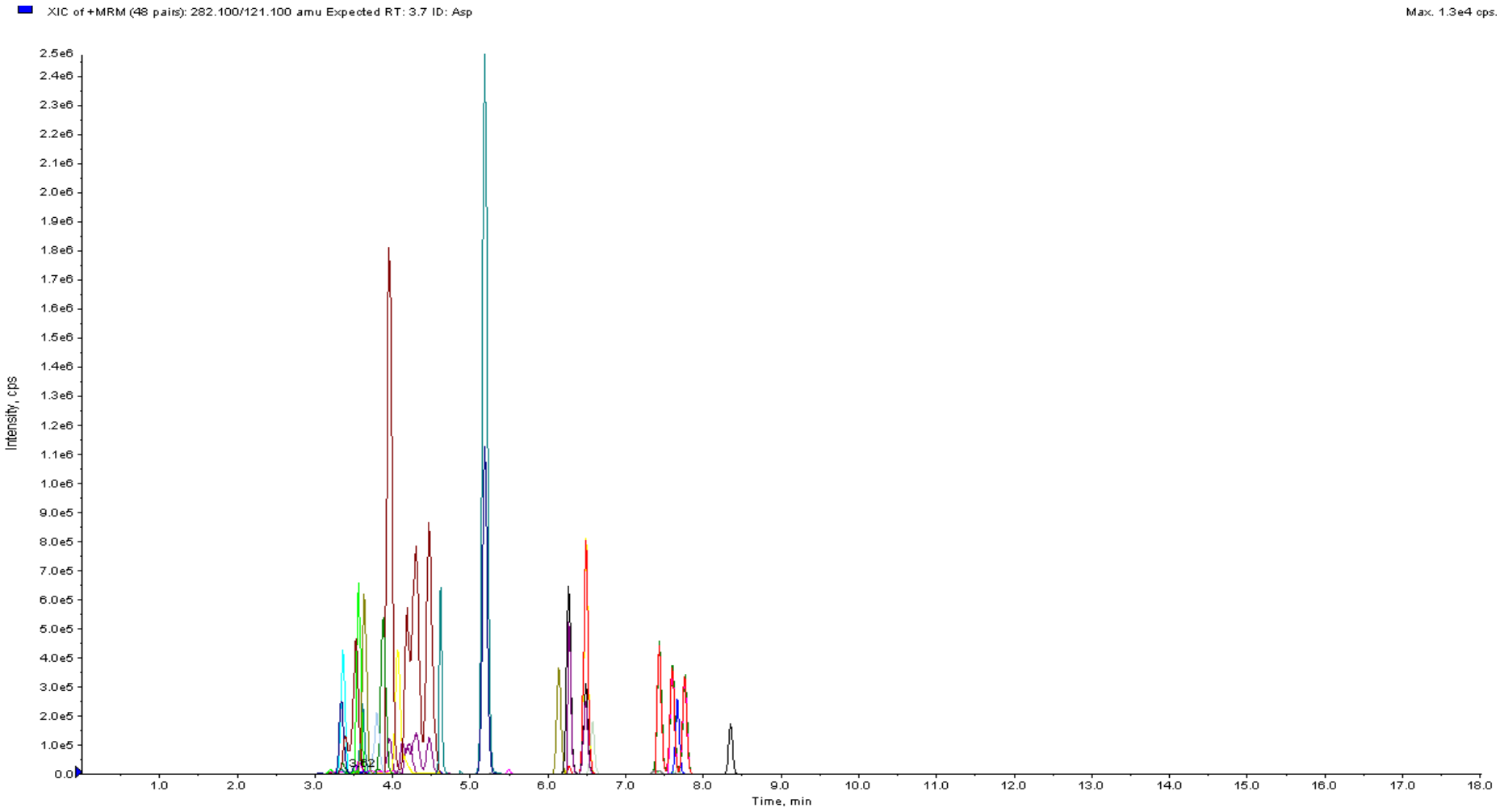
C 887 Warm (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.5e4 cps.
Intensity, cps
Time, min
3.81

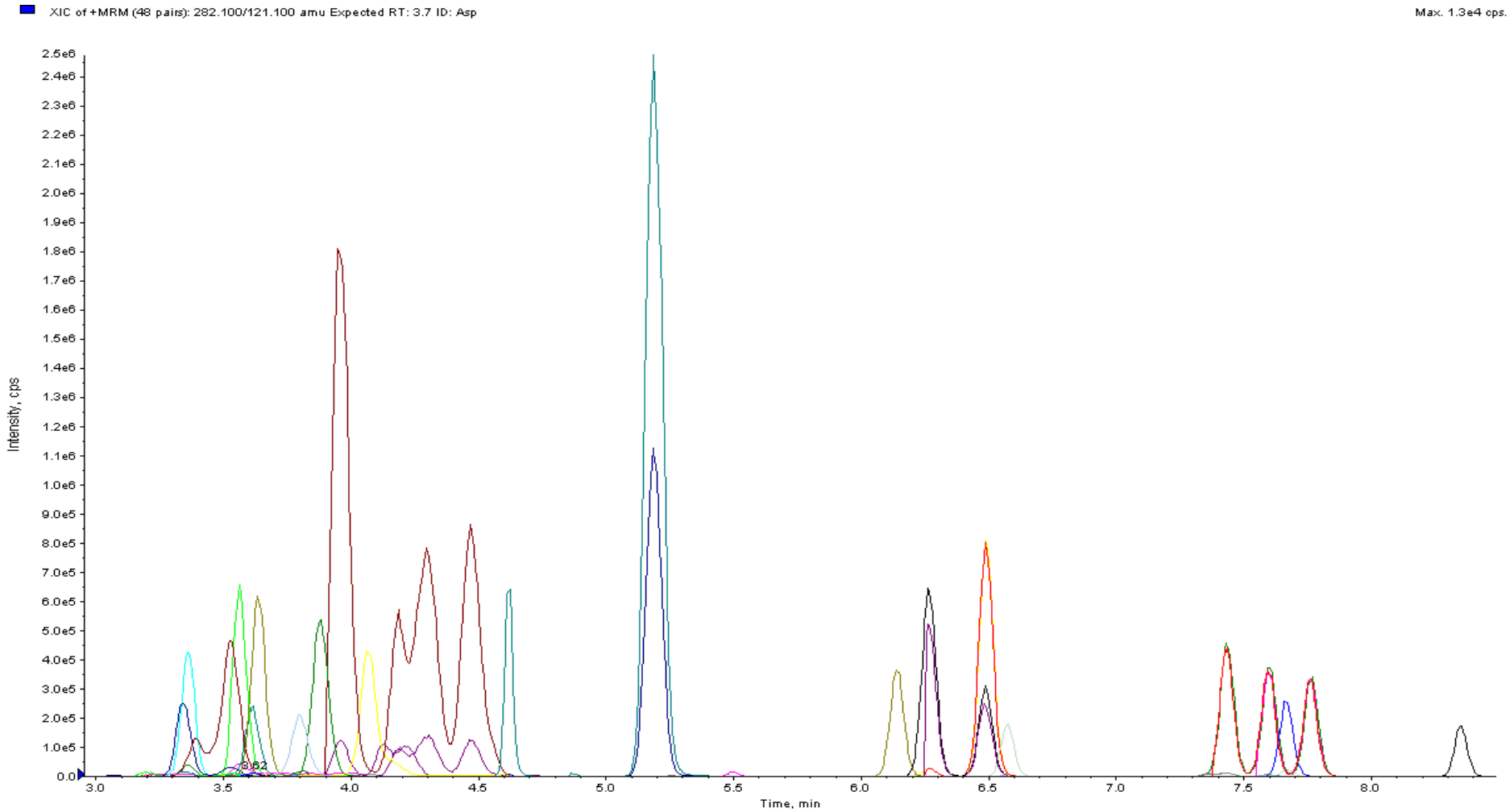
C 890 Cold



C 890 Cold (zoomed in)


XIC of +MRM (48 pairs): 282.100/121.100 amu Expected RT: 3.7 ID: Asp
Max. 1.3e4 cps.
Intensity, cps
Time, min

C 890 Warm



C 890 Warm (zoomed in)

Legend for aTRAQ chromatograms

Appendix 13: Massey University Analysis Report

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Nutrition Laboratory
Institute of Food, Nutrition and Human Health
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Email: F.S.Jackson@massey.ac.nz
http://nutritionlab.massey.ac.nz

TO:	**Megan Grainger**		AT:	**Waikato University**	
SUBJECT:	**Analysis Report**	*Client Reference:*	Honey	DATE:	**26/05/2014**
TRIAL:	**TN14-253**	*SAMPLES RECEIVED:*	***9/05/14***		

Number of pages in this report: 2 Results are on an as received basis

TN14-253

AMINO ACIDS	Happy Bee	Airborne	Hollands	Katikati	946	953	14/4	66	78	84
Aspartic Acid	1.10	1.14	0.90	1.14	2.02	1.69	3.11	0.46	0.59	0.46
Threonine	0.18	0.36	0.38	0.30	0.69	0.60	0.34	0.28	0.19	0.19
Serine	0.84	1.18	1.39	0.92	1.60	1.38	0.66	1.04	0.71	1.09
Glutamic Acid	1.45	1.52	1.36	1.00	1.51	1.49	2.29	0.96	1.03	0.88
Proline	32.81	63.97	37.85	55.19	70.93	67.46	17.90	46.52	32.64	35.62
Glycine	0.22	0.36	0.34	0.30	0.46	0.45	0.17	0.22	0.15	0.24
Alanine	0.70	1.08	0.96	0.97	2.07	1.83	0.50	1.16	0.73	1.23
Cystine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Valine	0.51	0.92	0.90	0.81	1.09	1.22	0.49	0.72	0.48	0.80
Methionine	0.01	0.02	0.04	0.02	0.05	0.05	0.06	0.02	0.02	0.04
Isoleucine	0.26	0.56	0.45	0.61	0.66	0.75	0.23	0.46	0.28	0.48
Leucine	0.27	0.58	0.63	0.96	0.72	0.50	0.17	0.30	0.20	0.28
Tyrosine	0.75	1.26	6.54	3.39	2.01	1.75	0.28	1.36	0.71	1.00
Phenylalanine	2.71	4.78	48.08	13.57	8.78	5.43	0.59	4.21	1.92	1.96
Histidine	0.57	0.98	0.62	0.57	1.99	1.89	0.47	0.55	0.24	0.31
Lysine	0.93	2.44	1.23	1.40	2.39	2.22	0.75	1.61	0.68	1.02
Arginine	0.52	0.81	0.53	0.94	1.37	0.89	0.43	0.52	0.35	0.12
Taurine	0.20	0.39	0.31	0.29	0.35	0.40	0.20	0.22	0.19	0.28
Tryptophan	0.06	0.05	0.95	0.01	0.11	0.02	0.02	0.02	0.02	0.41
Units	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g	mg/100g

ND = None Detected

Methodology

Free Amino acids: RP HPLC separation using AccQ Tag derivatization. AOAC 994.12

NB: Sample type is outside the scope of the typical sample types analysed for amino acids by the Nutrition Laboratory. Samples were run on two different HPLC systems to resolve co-elution of some peaks.

Please note, although the University has taken all due care in preparing this information in a proper manner, it shall not be liable for any loss or damage incurred by the use of this opinion by persons or organisations.

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Appendix 14: P-values for stored honeys

Sample ID	p-value										
	Asp	**Glu**	**Ser**	**Thr**	**Tyr**	**Gly**	**Pro**	**Ala**	**Met**	**Val**	**Phe**
B0 08e3	0.854	0.651	0.793	0.853	0.616	0.755	0.846	0.812	0.843	0.809	0.128
C 890	0.074	0.008	0.965	0.191	0.098	0.998	0.096	0.046	0.04	0.07	0.038
B0 14e3	0.144	0.153	0.138	0.087	0.019	0.161	0.078	0.069	0.11	0.029	0.047
B0 23e3	0.294	0.091	0.23	0.04	0.25	0.251	0.016	0.243	0.186	0.161	0.051
B0 24e3	0.007	0.033	0.339	0.252	0.102	0.372	0.143	0.183	0.086	0.059	0.029
C 463	0.104	0.113	0.023	0.03	0.213	0.006	0.526	0.263	0.021	0.155	0.455
C 887	0.358	0.028	0.673	0.609	0.244	0.676	0.044	0.578	0.002	0.31	0.029
	Leu	**MOx**	**His**	**Lys**	**Arg**	**Ile**	**Asn**	**Gln**	**Trp**	**HydPro**	**Total Amino acids**
B0 08e3	0.954	0.221	0.893	0.043	0.766	0.574	0.279	0.134	0.935	0.787	0.858
C 890	0.432	0.037	0.985	0.003	0.058	0.028	0.016	0.003	0.925	0.153	0.079
B0 14e3	0.06	0.002	0.188	0.127	0.154	0.226	0.271	0.035	0.294	0.103	0.053
B0 23e3	0.11	0.059	0.266	0.13	0.214	0.208	0.053	0.004	0.256	0.12	0.132
B0 24e3	0.306	0.096	0.44	0.001	0.067	0.008	0.032	0.016	0.184	0.165	0.072
C 463	0.096	0.732	0.177	0.118	0.902	0.229	0.184	0.202	0.072	0.573	0.346
C 887	0.576	0.054	0.552	0.016	0.127	0.259	0.111	0.013	0.712	0.203	0.147