# Acetonitrile adduct analysis of underivatised amino acids offers improved sensitivity for hydrophilic interaction liquid chromatography tandem mass-spectrometry

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## Abstract

LC-MS/MS method development for native amino acid detection can be problematic due to low ionisation efficiencies, in source fragmentation, potential for cluster ion formation and incorrect application of chromatography techniques. This has led to the majority of the scientific community derivatising amino acids for more sensitive analysis. Derivatisation has several benefits including reduced signal-to-noise ratios, more efficient ionisation, and a change in polarity, allowing the use of reverse phase chromatography. However, derivatisation of amino acids can be expensive, requires additional sample preparation steps, is more time consuming and increases sample instability, due to the most derivatised amino acids only be stable for finite amount of time. While showing initial promise, development of reliable hydrophilic interaction liquid chromatography (HILIC) separation methods has presented difficulties for the analyst including irreproducible separation and poor sensitivity. This study aimed to find a means to improve the detection sensitivity of the 20 protein amino acids by HILIC-MS/MS. We describe the use of previously undescribed amino acid-acetonitrile (ACN) adducts to improve detection of 16 out of the 20 amino acids. While all amino acids examined did form an ACN adduct, 4 had low intensity adduct formation compared to their protonated state, 3 of which are classified as basic amino acids. For 15 of the 20 amino acids tested, we used the ACN adduct for both quantification and qualification ions and demonstrated a significant enhancement in signal-to-noise ratio, ranging from 23% to 1762% improvement. Lower LODs, LOQs and lower ranges of linearity were also achieved for these amino acids. The optimised method was applied to a human neuroblastoma cell line (SH-SY5Y) with the potential to be applied to other complex sample types. The improved sensitivity this method offers simplifies sample preparation and reduces the costs of amino acid analysis compared to those methods that rely on derivatisation for sensitivity.

**Keywords**: Amino Acid; HILIC; Adduct; HILIC-MS/MS; LC-MS/MS; Metabolites;

## 1.1 Introduction

Amino acids are a class of small molecules present in many sample matrices. Amino acids are most commonly known for their role as the substrates for ribosomal protein synthesis, however only 22 protein amino acids (21 in eukaryotes and 22 in prokaryotes) are used in this process[1]. These 22 protein amino acids are not limited to being the constituents for protein synthesis but also have several metabolic roles including as neurotransmitters[2], modulating homeostasis[3], and mitochondrial functions[4]. Protein amino acid levels may give insight into many different factors including patient health from analysis of clinical samples[5] or nutritional quality in food samples[6]. Thus, analysis of amino acids is becoming increasingly important, as is the need for sensitive and robust analytical methods to identify and quantify them. In addition, toxic non-protein amino acids in the environment are attracting attention due to their putative links to neurological disorders[7-10].

Amino acids have been analysed by a variety of different analytical techniques. Multiple amino acids are usually present in samples, therefore separation is required before detection. Liquid chromatography (LC) and gas chromatography (GC) are the most commonly used forms of separation employed for amino acid analysis. Alternatively, non-chromatographic separation has also been used, the most common being capillary electrophoresis (CE)[11]. Detectors used for amino acids are typically either spectrophotometric (ultra-violet (UV), visible light (Vis), fluorescence detection (FLD))[12-13], or mass spectrometry (MS)[14-16]. As most amino acids don't have a chromophore, derivatisation is required to analyse all protein amino acids by spectrophotometry, however CE can perform indirect detection using specific electrolytes[17]. The three most commonly used techniques for the analysis of amino acids are amino acid analysers (ion chromatography with post column derivatisation detected with spectrophotometry), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Amino acid analysers typically fail to reach the sensitivity of the mass spectrometric analysis methods[18], and while GC-MS results are mostly comparable to LC-MS, this method requires additional sample clean up to obtain a clean spectra[12, 18]. Thus, LC-MS is the preferred method of analysis. Amino acids can be analysed either in their native form, or chemically modified (derivatised), the latter having several benefits including an increase in mass moving their *m/z* outside the noise seen in lower mass regions, thus increasing the signal-to-noise ratio (S/N). In addition, derivatisation improves limits of detection and alters polarity allowing the use of reverse phase chromatography[19]. Currently the most sensitive methods for amino acid analysis involve derivatisation, separation by reverse phase liquid chromatography (RPLC), and detection by multiple reaction monitoring (MRM) using a triple quadrupole mass spectrometer (TQMS). It would be beneficial however to analyse amino acids in their native form, reducing the analytical cost and sample preparation time, and avoiding instability issues in the derivatised analytes as all forms of derivatised-amino acids are only stable for a limited period of a time[19].

Hydrophilic interaction liquid chromatography (HILIC) is a form of chromatography that allows the separation of native amino acids. Analysis of native amino acids by HILIC-MS however struggles to reach the same level of sensitivity as the aforementioned derivatised RPLC-MS methods[20]. HILIC, similar to that of normal phase chromatography, employs a polar stationary phase for the retention of polar analytes. HILIC has a mobile phase comprised of a non-polar solvent with a small amount of water[21], where a partition is formed between these two components with a water layer being formed on the stationary phase. The analytes interact with this water layer via hydrophilic and electrostatic interactions allowing for chromatographic retention and separation[21-22]. HILIC is known for its poor peak shapes when implemented incorrectly[21-23], leading to the poorer sensitivity observed for native LC-MS/MS amino acid methods. HILIC requires careful optimisation to avoid poor peak shape, poor separation efficiency and poor reproducibility when compared to RPLC[21-23]. Additionally, HILIC often requires high buffer concentrations leading to ion suppression when coupled to MS. These issues coupled with amino acids having low ionisation efficiency, being prone to in-source fragmentation, and their low parent and fragment masses being present in regions of high noise, results in HILIC-MS/MS

methods having long development times while still being less sensitive compared to the more common derivatisation-based LC-MS/MS methods[19].

Currently, despite these short comings, HILIC is the best chromatography method for native amino acid separation. Here we examine improvements in the sensitivity of HILIC-TQMS achievable through investigating adduct formation. Compounds including alcohols, proteins and lipids form adducts in electrospray ionisation (ESI) which can result in increased sensitivity[24-26]. These adducts typically form with the LC mobile phase components, with some methods adding specific components to the mobile phase or post-column to allow adduct formation, increasing the detection sensitivity for these compounds[27]. Underivatised amino acids are known to from adducts and cluster ions comprising of multiple amino acids[28], heavy metals[29-30], alkali metals[31-32], and acids[33]. There is however limited information on the formation of amino acid adducts in HILIC-ESI-MS. Erngren and colleagues[31] describe the formation of amino acids adduct consisting of sodium $[M+2Na-H]^+$ and potassium $[M+2K-H]^+$, but this study was focused on their removal from plasma to improve overall metabolite sensitivity rather than using these adducts for increased sensitivity. Thus, the aims of the present study were to investigate the formation of amino acid adducts in ESI that may improve their sensitivity in order to develop a fast, sensitive non-derivatised protein amino acid method via HILIC-TQMS. We used signal-to-noise ratio (S/N), limits of detection (LOD) and quantification (LOQ) to demonstrate improvements, and we applied the optimised method to analyse the amino acids in a human neuroblastoma cell line.

## 2. Methods and materials

### *2.1 Standards*

A mixed standard of the 20 protein amino acids examined in this study was made from individual standards (L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, Sigma-Aldrich, Castle Hill, NSW, Australia). Individual standards were made up to 1000 µg/mL in 20 mM hydrochloric acid (HCl) to ensure solubilisation of all amino acids and 10 mM dithiothreitol (DTT) to prevent the formation of cystine. These individual standards were diluted and combined to a final concentration of 10 µg/mL. To limit freeze-thawing, the combined standard was aliquoted and stored at -20 °C until required for analysis. The standard was used to construct a standard curve for method development and validation (See section 2.6). Norvaline (L-norvaline, Sigma-Aldrich, Castle Hill, NSW, Sydney, Australia), a non-protein amino acid, was added to each standard used in the standard curve as an internal standard (ISTD) at 50 ng/mL to allow inter-run normalisation. The ISTD was added at the start of extraction (section 2.3) to account for any loss that may occur during this process.

### *2.2 SH-SY5Y Cell culture*

SH-SY5Y (human neuroblastoma) cells (passage 20) were cultured in a T75 flask in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% glutamax and 10% fetal bovine serum (FBS). Once fully confluent, cells were seeded into 4 wells of a 12-well plate at 480,000 cells per well and left for 24 hrs. After 24 hrs these adherent cells were washed three times with phosphate buffered saline (PBS) then the plate was snap frozen with liquid nitrogen and stored at – 80 °C prior to analysis.

### *2.3 Amino acid extraction*

A 500 ng/mL solution of the ISTD was prepared in ultrapure water and added to the 4 wells of the well plate that was to be extracted. Cells were scrubbed off the well-plate using a cell scraper, and the suspended cell solution was transferred to a 2 mL tube. Trichloroacetic acid (TCA) was added to the sample to give a final concentration of 10% (w/v) TCA. The sample was then subjected to probe sonication (Qsonica Q125 Sonicator) for 30 seconds at 50% power twice to ensure complete lysis, with samples left on ice for 1min between repeats. Samples were then centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was collected and transferred to a new 2 mL tube. The pellet was then washed with 200 µL of cold 10% (w/v) TCA and the samples centrifuged again with the supernatant collected and transferred to the same 2 mL tube, this was then repeated to allow for triplicate washes of the pellet resulting in a final volume for the supernatant containing tube being 1.4 mL. The remaining pellets were resuspended in 200 µL of 0.1% (w/v) triton X -100 for later protein concentration determination. The free amino acid containing samples were then sublimated by freeze-drying (Martin Christ, alpha 2-4 LD plus) at 0.1 mbar and - 80 °C for 16 hrs. The freeze-dried samples were then reconstituted in 500 µl of 20 mM HCl with 10 mM DTT and spun through a 0.22 µm membrane filter (Ultrafree-MC LG Centrifugal 0.2 µm pore size PTFE Membrane Filter (UFC30LG25)) for 10 min at 5000 g. Samples were then stored at -80 °C until analysis. Prior to LC-MS/MS analysis, the samples were diluted 1:10 with acetonitrile (ACN) to match the initial chromatographic mobile phase conditions (90:10 ACN:$H_2O$).

### *2.4 Bicinchoninic acid assay for protein quantification*

4% (w/v) copper(II) sulphate ($CuSO_4$) was diluted 1 in 50 with bicinchoninic acid (BCA) solution (Sigma-Aldrich, Castle Hill, NSW, Australia). 10 µl of each sample was added to a well in a 96 well-

plate, this was done in triplicate for each sample. A 7-point calibration curve was constructed (25 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL and 2000 µg/mL) of bovine serum albumin (BSA) in 0.1% (w/v) triton X -100, with 10 µL of each point also being loaded into a well of the same 96 well-plate, this was also performed in triplicate. 100 µL of the $CuSO_4$ BCA solution was added to each well of the 96 well plate. Colour was left to develop for 2 hours at room temperature. The 96 well-plate was then read in a Tecan Infinite M1000 PRO monochromator microplate reader. The absorbance of each well was read at a wavelength of 562 nm with the number of flashes being set to 25.

*2.5* Hydrophilic interaction liquid chromatography (*HILIC)triple quadrupole mass spectrometry (TQMS)*

Chromatographic separation of the 20 amino acids (Figure 1) was performed on a Shimadzu Nexera X2 UHPLC coupled to a Shimadzu 8060 triple quadrupole mass spectrometer (TQMS) for detection, with a Waters BEH Amide column (2.1×100 mm, 1.7 µm particle size), a flow rate of 0.8 mL/min and a column oven temperature of 30°C. Solvent A consisted of 80 mM ammonium formate in ultrapure water + 0.6% Formic acid (FA), Solvent B consisted of acetonitrile (ACN) + 0.6% FA. The amino acids were eluted using the following stepped gradient of Solvent B (separation shown in Figure 1): 0.00 min 90%, 3.50 min 90%, 5.50 min 80%, 9.25 min 80%, 9.30 min 70%, 11.20 min 70%, 11.20 min 90%, 14.00 min 90%. Injection volume was set to 5 µl and no carry over was observed following an injection of the highest concentration of standard used (1000 ng/mL), with each sample and standard being injected in triplicate. The TQMS was run with an ESI source in positive mode with the following source parameters: 0.1 kV interface voltage, 400 °C interface temperature, 225 °C desolation line (DL) temperature and 400 °C heat block, 3 L/min nebulising gas flow, 17 L/min heating gas flow, and 3 L/min drying gas flow. Nitrogen was used for drying, heating and nebulising gas, while argon was used for the collision gas. Prior to analysis, multiple reaction monitoring (MRM) ion transitions were established for the protonated amino acids and the amino acid adducts (Table 1). MRMs were segmented based on the elution window of the corresponding amino acid (Figure 1), with the dwell time for each transition set to 20 msec, allowing for the total cycle time to never exceed 300 msec.

*2.7 Method comparison and validation*

A calibration curve of 12 points was developed for the protonated and ACN adduct analytes (0.01 ng/mL, 0.1 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, 750 ng/mL and 1000 ng/mL). Repeatability was determined from calculation of %RSD from 7 repeat injections from one point in the standard curve. LOD and LOQ were determined with a S/N ratio of 3.3 and 10 respectively. All data analysis and method validation was conducted on Shimadzu Labsolutions.

## 3. Results & Discussion

### *3.1 Chromatography and source conditions optimisation*

Chromatography was performed on a Waters BEH Amide column. This column has a pH range of 2 – 11, thus a large range of buffers/additives (including formic acid, ammonium formate, ammonium acetate, acetic acid, and ammonium hydroxide) could be tested at different pH values. The best results regarding intensities in the MS and peak shape were obtained with 0.1% formic acid, however the addition of ammonium formate was required to separate isomers such as leucine and isoleucine. At higher pH values (>3.2) poor peak shape was observed for glutamic acid and aspartic acid, requiring the addition of a large concentration of formic acid (0.8%) to both solvents resulting in the pH of the aqueous solvent (solvent A) being 3.2, which is close to the isoelectric point of aspartic acid (2.98). However, as the pH was dropped, chromatographic resolution between these compounds and their non-acidic derivatives, glutamine and asparagine was lost. Separation of these compounds is required as carbon 13 (a naturally occurring isotope of carbon) ($^{13}C$) containing glutamine and asparagine become isobars of glutamic acid and aspartic acid as their mass is increased by ~ 1 Da. Thus, to ensure separation of these compounds, ammonium formate concentration was increased as formic acid's concentration was increased, leaving the final concentration of ammonium formate at 80 mM in the water with + 0.6% formic acid added to both ACN and water to cause the lower pH. The high ammonium formate concentration (80 mM) would typically result in ion suppression and a decrease in the column's longevity however the percentage of water never exceeds 30%. Thus, there is never more than 24 mM on the column. Besides glutamic acid and a $^{13}C$ glutamine, and aspartic acid and a $^{13}C$ asparagine, other compounds of the same *m/z* in this method included isoleucine and leucine, norvaline and valine, and glutamine and lysine. A stepped gradient was developed to allow for a relatively short method (< 20 min between injections) for HILIC while also allowing for the separation of all these isomers and isobars. Column temperature did not assist with separation of isomers so was maintained at 30 °C for consistency, with the flow rate being optimised to 0.8 ml/min. These combined parameters allowed for all compounds to be eluted within 9 min, with the remaining time required to wash and re-equilibrate the column for the next run.

Positive and negative ionisation were examined with better S/N and intensities for the protonated masses and ACN adducts obtained in positive mode. Parameter were optimised for both protonated and adduct methods and were found to be identical with the biggest S/N and intensity improvement found by lowering the voltage to 0.1 kV, the recommended lowest setting of the instrument, and increasing the interface temperature to the maximum setting (400°C). This extremely low voltage is similar to a study conducted by Sørensen *et al.,*[34] who demonstrated that lowering the needle voltage to 0 in an ESI increased the S/N ratio 40-50 times for polar amino acids when compared their ionisation at 4 kV. Despite optimisation, sensitivity of some of the protonated amino acids was still poor in the TQMS. Glycine had the highest LOD and LOQ of all protonated amino acids with 100 ng/mL and 320 ng/mL respectively. Other amino acids including alanine, aspartic acid, cysteine, and histidine also had higher LODs and LOQs (>10 ng/mL) than the other amino acids. Interestingly, the low S/N and poor intensity was not observed in selected ion monitoring (SIM) scans (Using the first quadrupole (Q1)) for these amino acids, indicating these high LODs are most likely due to poor fragmentation. Collision gas pressure and collision voltage were investigated to see if any settings or combination of settings could improve fragmentation, however none were observed.

### *3.2 ACN adduct formation*

All amino acids tested were found to form an adduct which added an additional 41 Da to the protonated amino acid (Table 1) which was attributed to ACN ($[M+H+ACN]^+$). During the use of the third quadrupole (Q3) in SIM or MS1 scan mode, the intensity of the ACN adduct was low and

undetectable for 5 amino acids at 1000 ng/mL; however Q1 scans resulted in an increase in the intensity for all but 3 amino acids (arginine, lysine and histidine) (see Supplementary data Figure S1-20). Interestingly, these amino acids include 3 of the 4 amino acids that did not improve S/N or intensities when the ACN adduct was used for MRM transitions (see section 3.3). For 7 of the amino acids (tryptophan, threonine, tyrosine, phenylalanine, proline and glutamine) the protonated mass intensities and S/N did not differ significantly between the different quadrupole SIM scans (Q1 vs Q3), however 10 of the amino acids an increase in the intensity of protonated masses was seen for when analysed with a Q3 SIM or with a Q3 scan (see Supplementary data), suggesting that the ACN adduct dissociates in the first quadrupole or collision cell without collision energy. All ACN adducts produced fragments of the protonated amino acid mass (Table 1) at low collision energies supporting the ease of dissociation differences observed in the Q1 and Q3 scans mentioned above. This fragmentation is similar to many derivatisation methods that produce the protonated mass as major fragments[19]. This transition was used as a qualifier ion for the majority of amino acids, apart from tyrosine and glycine where it was used as their quantifier ion. Serine was the only amino acid that did not use this transition as, while it formed (See supplementary Table S1), it occurred in a region of high noise, leaving its two best transitions being the ACN adduct to fragment transitions (147.15 *m/z to* 60.15 *m/z* and 42.15 *m/z*). The Serine-ACN adduct *m/z* was 147.15, similar to the protonated masses of glutamine and lysine (147.10 *m/z* and 147.20 *m/z* respectively). While a high-resolution mass spectrometer can differentiate between these masses, they cannot be differentiated with the low resolution of a TQMS, and glutamine and serine were not baseline separated in the chromatography employed. Fragments produced by the serine-ACN adduct were unique with no overlap with glutamine or lysine and were therefore selected for the analysis. The formation of the ACN adduct for four of these amino acids, arginine, histidine, lysine, and glutamine, was low and the S/N did not improve upon the protonated form.

### *3.3 Basic amino acids, glutamine, and glutamic acid*

Two ACN adduct transitions were established for glutamic acid (see Supplementary data) however neither improved on the protonated quantifier transitions in terms of S/N or intensity. However, the ACN adduct to fragment transition was an improvement over the qualifying protonated transition. The final MRM transitions selected for glutamic acid included 1 protonated transition and 1 ACN adduct transition. As previously mentioned, most HILIC amino acid methods choose to use only 1 transition per amino acid[15, 35-36], thus the inclusion of this adduct transition, while not improving quantification, still greatly improves the specificity for analysing glutamic acid. The detection of the 16/20 protein amino acids (and the NPAA ITSD norvaline) were improved by the implementation of targeting the ACN amino acid adduct. Four amino acids, histidine, lysine, arginine, and glutamine were not improved with the implementation of using the ACN adduct for analysis. For these amino acids, the ACN adduct was observed but at low intensities and a reduction in S/N. While glutamine followed the trend of the other 16 amino acids which had the ACN adduct intensities drop when analysed using a Q3 SIM opposed to a Q1 SIM, arginine and histidine significantly increased in S/N when analysed in a Q3 SIM, with lysine-ACN not being observed at 1000 ng/mL in either a Q1 or Q3 SIM (see Supplementary data). This may suggest that these ACN adducts have a stronger interaction and do not readily fragment like the other amino acids, however this may also be due to the first quadrupole filtering out more noise prior to its analysis from the third, thus improving the S/N. Further optimisation of both source and collision cell parameters did not yield improved results (see Supplementary data for MRM information). Excluding glutamine, the remaining 3 amino acids are classified as the basic amino acids, with isoelectric points up to 10.76 (arginine). Thus, to determine if the ACN adduct formation was dependant on the charge state of the amino acid, a series of direct infusions (no chromatography employed) were performed under basic conditions. Mobile phase was composed of 90:10 ACN: water, buffered with 10 mM ammonium hydroxide (~ pH 10). Basic conditions did not improve the S/N or the intensity for the

ACN adduct forms of these amino acids. These amino acids had adequate sensitivity and specificity and could therefore still be analysed in their protonated form.

*3.4 Comparison between protonated mass and ACN adduct mass*

As mentioned above the optimal source parameters for the protonated form where also found to be the optimal source conditions for the ACN adduct. By using the ACN adduct as the targeted parent mass for the quantifier and qualifier ions of the sixteen amino acids which showed ACN adduct formation, ranges of linearity increased, and lower LODs and LOQs were observed. S/N improvements ranged from a 24% increase (asparagine) to a 1879% increase (aspartic acid, see Figure 2). Alanine, glycine and aspartic acid had the highest LODs and LOQs using the protonated mass for the MRM transitions, and here showed some of the greatest improvements in S/N when compared to their respective ACN adduct. Aspartic acid-ACN had an improvement of 1879% as previously stated, alanine-ACN had the second highest improvement with 1762% and glycine-ACN had the fourth highest with 1011% (Figure 2). Similar trends of S/N improvements were also observed with the qualifier ions. The internal standard norvaline had an increase in S/N similar to its constitutional isomer, valine (Figure 2). The cause of the improvement in S/N using the ACN adduct formation is similar to that of derivatised methods as both methods increased mass of the amino acid, effectively shifting the mass of the fragment ions out of the higher noise regions associated with smaller masses[19], despite only being a small mass increase (41 Da). This is further demonstrated by the smaller amino acids such as glycine and alanine (<100 Da) having two of the highest increases in S/N.

Like the protonated form, the glycine-ACN adduct still had the poorest LODs and LOQs compared to all the other ACN adducts (See Table 2). However, when compared to the protonated form, there was an approximately 10 times improvement of the LOD and LOQ from 100 ng/mL and 320 ng/mL to 3.1 ng/mL and 9.5 ng/mL, and the lower point of the linear range decreased from 500 ng/mL to 10 ng/mL. Histidine contained the highest LOD and LOQ of the optimised method as it was still analysed using its protonated form (see section 3.3). Alanine-ACN and proline-ACN had the lowest LOD of 0.016 ng/mL, with tryptophan obtaining the lowest protonated amino acid LOD of 0.35 ng/mL. Linear ranges of up to 5 orders of magnitude were obtained using the ACN adducts, whereas only 3 orders of magnitude was obtained with the method focusing on protonated amino acids. Both methods had high repeatability with %RSD values below 10%.

When it comes to targeted methods for native amino acids, TQMS provides the most sensitive and specific methods, however many methods reduce the specificity and only use one MRM transition for each amino acid[15, 35-36]. This may be due to the large number of analytes being monitored in these protein amino acid methods, reducing the number of transitions to ensure adequate points are collected across the chromatographic peak, or due to most amino acids having poor fragmentation and only having one observable fragment ion. The use of amino acid-ACN adducts increased specificity with the identification of two fragments ions for each analyte.

Prinsen et al[15] developed a HILIC-MS/MS method for the analysis of native amino acids using the same column used in this study and quantified 24 amino acids. LODs and LOQs were reported in µM (LOD ~ 8.9 ng/mL to 15 ng/mL) (LOQ ~ 7.5 ng/mL to 47 ng/mL), and apart from glycine, all were higher than the values reported here (excluding those listed as 0.0 µM). Du and Huang[37] used HILIC and parallel reaction monitoring (PRM) in an orbitrap-MS and also had higher LODs (2.7 ng/mL to 16 ng/mL) than those observed with the method using the ACN adduct formation presented here. Another HILIC-TQMS method using the same column as the present study developed by Yuan *et al.*[38], reported a lower limit of detection (LLOD) range of 0.25 to 11 ng/mL. This method avoided the use of ammonium formate to improve sensitivity. They were able to achieve this by having unique ion transitions for leucine and isoleucine and thus didn't resolve them chromatographically. Additionally,

while Yuan *et al.*[38] reported unique quantifier ion transitions these two isomers shared their qualifying ion transition. No unique ions for these isomers were observed in the present study thus resulting in the need for chromatographic separation. This allowed the selection of the highest intensity transition as the quantifier ion, as opposed to the less intense unique ions. One of these unique ion transitions was used for the protonated isoleucine as a qualifier ion (132.20 *m/z* to 69.10 *m/z*) however in the present study it was not a unique ion as leucine was also observed to have this transition, albeit at a lower intensity than isoleucine. All LLOD values reported for the amino acids that formed the ACN adduct in the study by Yuan *et al.*[38], were above the present studies LODs, however the protonated arginine, lysine and histidine all had lower LLODs compared to the LODs reported in the present study, most likely due to the absence of ammonium formate.

The HILIC-TQMS method developed here is still not as sensitive as the most sensitive derivatised RPLC-MS/MS methods. Salazar et al[16] and Wang et al[39] used the ACQ derivatisation and reported LODs in the low fmol range which are up to an order of magnitude lower than those reported here. Ziegler et al[40] utilised another derivatisation reagent, 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl), and once again the LODs reported where lower than that for the amino acid ACN adduct LODs reported here. Thus, while derivatised methods still show an increase in sensitivity relative to native amino acid analysis, the use of ACN adducts simplifies the sample preparation, reduces the costs of analysis and provides greatly increased sensitivity for the analysis of amino acids in cell lysates and potentially other complex samples.

### *3.6 Analysis of SH-SY5Y cell lysates*

19 out of the 20 protein amino acids were present in the cell lysate and could be detected with the HILIC-MS/MS method utilising ACN adduct analysis. Concentrations in cells were found to be between 4.3 ng/mg of protein (cysteine) and 644.5 ng/mg of protein (glutamine). Both glutamine and its acidic form glutamic acid had the highest levels detected in the cells with 644.5 ng/mg of protein and 562.8 ng/mg of protein respectively. These levels were much higher than the next amino acid which was alanine at 188.7 ng/mg of protein. These high levels are consistent with the current knowledge about amino acid levels in humans, as glutamine is known to be the most abundant amino acid, being readily converted to glutamic acid (glutamate)[41]. This could also be due to the growth medium, DMEM, being supplemented with L-alanyl-L-glutamine (glutamax), a dipeptide consisting of glutamine and alanine, which was the second highest amino acid detected. The only amino acid not detected was methionine. Methionine, an essential amino acid, could not be detected in the SH-SY5Y cell lysate, possibly due to its low abundance as other studies have reported it as one of the lower concentration amino acids in some sample types including skeletal muscle[42] and leukocytes[43]. This is of course dependent on sample type and currently there is no data on SH-SY5Y amino acid levels. Samples were concentrated an additional 4 times via freeze-drying and reconstitution in a smaller volume (100 µL), but this amino acid was still undetectable.

## 4. Conclusions

A sensitive HILIC method was developed for 20 of the protein amino acids. By targeting previously undescribed ACN adducts formed in ESI, improved sensitivity was achieved for 16/20 protein amino acids tested (and the ISTD). There were 4 amino acids that did no exhibit improvement from targeting the ACN adduct form however this ACN adduct was still observed in the ESI at a low abundance. This method is still limited by the ACN adducts of these 4 amino acids having higher LOD, and LOQs, nonetheless qualification and quantification was greatly improved for 16 of the protein amino acids.

## 5. Authorship contributions

All authors conceptualized the original project. J.P.V. conducted the laboratory work and wrote the original manuscript with D.P.B, M.P.P, M.T.W and K.J.R contributing to the final manuscript.

## 6. Conflicts of Interest

The authors declare no conflict of interest.

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