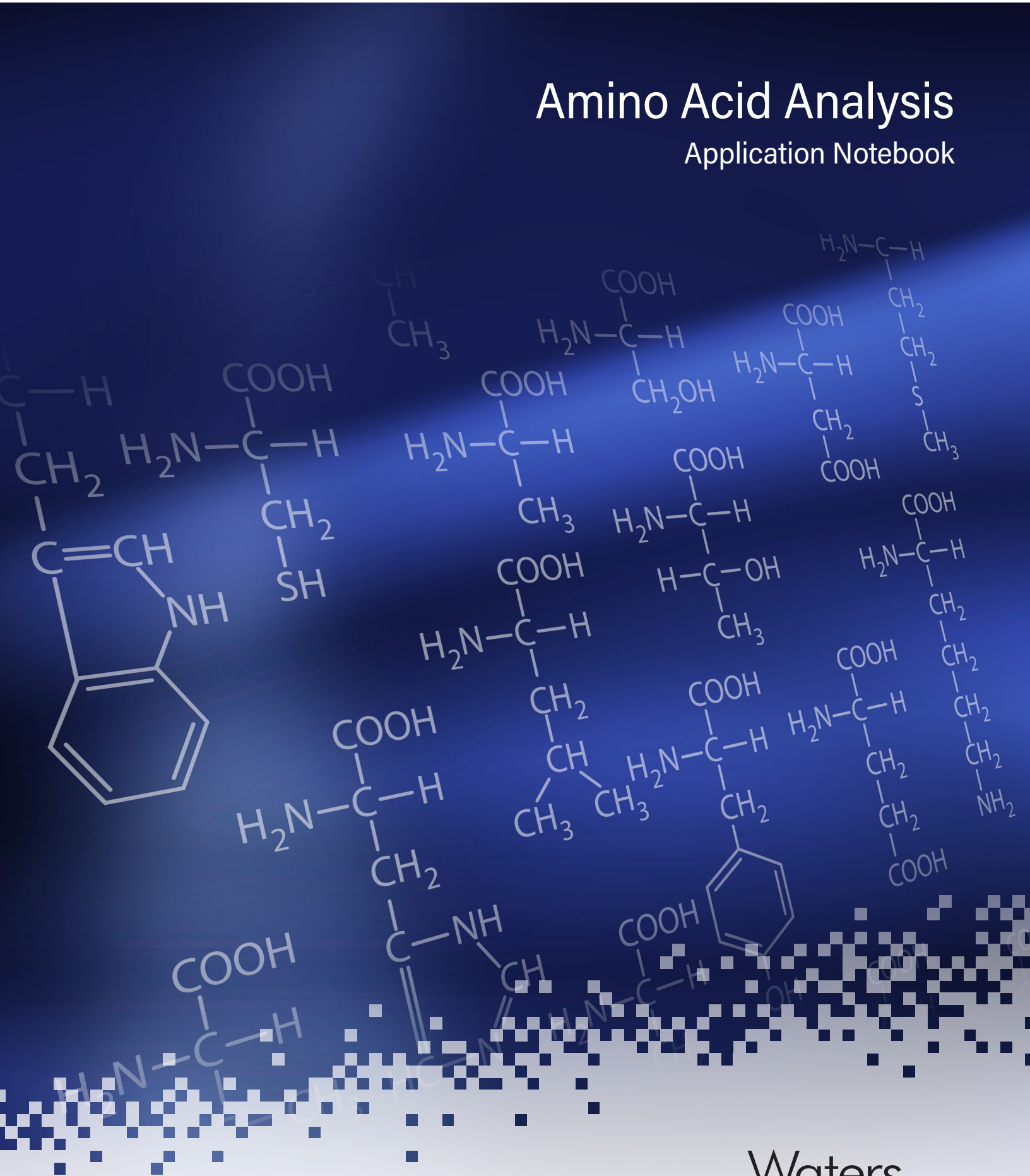
Amino Acid Analysis

Application Notebook



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[INTRODUCTION]

[HYDROLYSATES]

[CELL CULTURE]

[FOODS AND FEEDS]

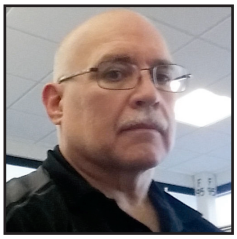
[OTHER AND REFERENCES]

NANCY ASTEPHEN

Nancy joined Waters Corporation as a Customer Support Specialist in 1986. Starting out working in the Life Science Marketing Lab focus on protein purification, the analysis of catecholamine's, amino acids, and peptides, supporting the Pico•Tag® and AccQ•Tag Ultra® methodologies the. Currently, Nancy is a Principal Project Manager for Waters Global Services team where she continues to drive world renowned service through improved customer satisfaction with successful installations, increased efficiency for the field engineers, and reduced costs.

Nancy received her B.S. / B.S. in Medical Technology, and Biology with a minor in Chemistry, from the Salve Regina University. Prior to Waters Nancy was employed at University of Massachusetts Medical Center in the Core Research Facility. She maintained both the spinning cup (Beckman) and gas phase sequencers (ABI), along with conducting amino acid analysis, peptide purification and gel electrophoresis.

ANDREW AUBIN



Andrew Aubin is the Manager of the Purification / SFx Systems Marketing Laboratory of Waters Corporation (Milford, MA). He has been actively involved in the development of new LC, SFC, and SFE product solutions for the pharmaceutical, fine chemical and life science markets more than 19 years. Currently, he is responsible for managing a team that provides application support for all of Waters Purification Products.

WEIBIN CHEN

Weibin Chen is a director in the scientific operations (SciOps) of Waters Corporation (Milford, MA). He has been actively involved in the development of new LC-MS-based solutions for biopharmaceutical analysis for more than 16 years. Currently, he is responsible for managing scientific projects that are designed to deliver novel analytical solutions to address the challenges and unmet analytical needs in the biopharmaceutical industry by LC-MS technologies. Weibin has a Ph.D. degree in Analytical Chemistry from Purdue University and published more than 40 peer-reviewed scientific papers/book chapters on biomolecule analysis.

RICHARD DAW

Richard Daw is a field technical support specialist at Waters Corporation. He performs demonstrations and provides technical and application support for all separation, MS, Informatics, and consumable product areas, to a wide range of analytical chemistry customers in the South East USA. Richard received an MChem in Chemistry from the University of Exeter in the UK and has a experience in a wide range of lab environments (Petroleum, Earth Science, Toxicology, Pharmaceuticals, Material Science, Tobacco, and Drugs of Abuse) prior to working at Waters.

DIANE M. DIEHL, Ph.D.

Diane joined Waters Corporation as a Senior Applications Chemist in 2001 and spent the next 10 years in various scientific manager and marketing roles within the Consumables Group, including Director of Applications. Her group developed LC, LC-MS, and GC-MS applications for new and existing LC columns and SPE products, covering all markets. In 2011, Diane was named the Director of New Product Portfolio Management. In 2013, Diane moved into the Pharmaceutical Vertical Marketing team as Director of Small Molecule Pharmaceutical Business Development, with focus on DMPK, Natural Products, and QC. In 2016, Diane became Senior Director of Pharmaceutical Market Development, covering Small Molecule Core Pharma, Biopharmaceuticals, DMPK, and Natural Products.

Prior to joining Waters, Diane received her B.S. (Chemistry), with a concentration in Business, from the University of Notre Dame. She was later awarded a Ph.D. (Analytical Chemistry) from Indiana University, under the direction of Professor Ronald Hites.

CATALIN DONEANU

A native of Romania, Catalin is a principal chemist at Waters. He develops bioanalysis applications for LCMS quantification of amino acids, peptides and proteins, as well as biopharmaceutical applications for mass spectrometry based characterization of protein therapeutics. Catalin's biggest career influence was his 7th grade chemistry school teacher. He received a Bachelor degree in chemistry from University of Bucharest and a doctorate in analytical chemistry from Oregon State University. Away from work Catalin enjoys biking, hiking, and ice skating with his daughter.

JOHN GEBLER

John Gebler has been with Waters for over 16 years focusing on Biopharmaceutical Strategic Business Development. He is responsible to develop Waters biopharmaceutical LC-MS business and strategy worldwide. John works with scientists from the pharmaceutical and biotech industry developing roadmaps for future Waters biopharma products, applications, and methods, while managing high-level technical collaborations with industry and academics. John's current focus is the characterization and bio-analysis of antibody drug conjugates (ADC's) and biosimilars. He received his PhD in Chemistry from The University of Utah.

ERIC GRUMBACH

Eric Grumbach is the Director, Product Marketing for Chromatography Systems within the Marketing Operations Group at Waters Corporation, Milford, MA. Eric and his team are responsible for the development and implementation of strategic and tactical plans to drive new business for Waters liquid and supercritical chromatography portfolio.

Eric has been at Waters for over 18 years, in various technical and business roles. He began the first 8 years of his career at Waters as an Applications Scientist in the Consumables Group. In 2008, he transitioned into a Global Product Marketing Manger role within Consumables Business Unit, taking on responsibility for a number of technologies including our industry leading ACQUITY® UPLC Columns, as well as leading the commercialization efforts for the 2.5 µm ***XP*** Columns. During this time, he authored two technology primers: Beginners Guide to UPLC [UltraPerformance Liquid Chromatography] and Comprehensive Guide to HILIC [Hydrophilic Interaction Chromatography].

In 2012, Eric transitioned into the Separation Technologies division, taking on product management responsibility for the ACQUITY UPLC H-Class System and led the development and commercialization efforts of the ACQUITY Arc® UHPLC System.

HILLARY HEWITSON

Hillary Hewitson is a Business Development Manager within the Pharmaceutical market segment at Waters Corporation, based in Milford, MA. Her focus is Pharmaceutical QC and manufacturing, including process analytical technologies. Previous to this role, she worked in development and application labs, performing a variety of pharmaceutical, protein, peptide, and amino acid separations. She participated in the development and commercialization of analytical standards for benchmarking mass spectrometry and was involved in the evaluations leading to advances with nano-scale column technology. Her first role with Waters was working in the QC lab at the chemistry manufacturing site.

Hillary obtained her Bachelor of Science in Biology from Bridgewater State University (MA) in 1995.

PAULA HONG, Ph.D.

Paula Hong, Ph.D. is a Principal Scientist at Waters Corporation. She received her B.S. in Chemistry from Bucknell University (Lewisburg, PA) and her doctorate in Inorganic Chemistry from The University of Pennsylvania (Philadelphia, PA). She began her career as an applications chemist at Waters Corporation focusing on UHPLC and sub-2-µm technology for the application of biomoelcules. More recently, she also has studied in the impact of instrumentation on numerous applications for both LC and SFC. She has authored journal articles, review articles, as well as white papers.

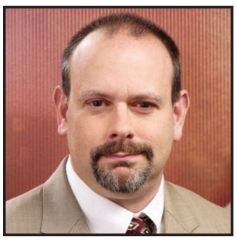
JO-ANN JABLONSKI

Jo-Ann Jablonski is a Principal Scientist in Separations Technology Division at Waters Corporation. Prior to joining Waters, Jo-Ann was a peptide chemist at two biotechnology firms where she synthesized, analyzed, isolated, and characterized peptides for research, process development, and manufacturing. Since joining Waters in 1998, Jo-Ann has provided technical support and training on Waters purification systems, in addition to isolating compounds from pharmaceuticals, natural product extracts, and other chemically diverse mixtures.

IGGY KASS

Iggy received his Ph.D. from The State University of New York in 1997, working under the direction of Professor Nicole S. Sampson, where he studied the relationship between protein structure and function. Iggy then joined Micromass, Inc. supporting various software applications in mass spectrometry. After three years, Iggy moved into the applications laboratory as a Product Specialist supporting high resolution, accurate mass time of flight mass spectrometry for Waters. This role focused on the analysis of peptides, oligomers, proteins, protein complexes and polymers as well as proteomic and metabolomic studies. In 2007, Iggy then took on a new role as Field Marketing Manager in support of Waters' marketing in the drug metabolism and pharmacokinetics area for the Americas. In 2014, Iggy moved into his current role in mass spectrometry sales.

JEFFREY R. MAZZEO, Ph.D.



Jeff received his Ph.D. from Northeastern University in 1992, working under the direction of Professor Ira Krull. Jeff joined Waters after graduation, focusing on chiral separations and ion analysis. After a short stint in the pharmaceutical industry, Jeff rejoined Waters in 2000 as Manager of the Applied Technology group in the Chemistry Operations division of Waters. Jeff was named Biopharmaceutical Business Director in 2007, a role in which he developed and implemented a strategy to grow Waters' business to biopharmaceutical firms. In 2012 Jeff took on a new role managing Waters' business in the chemical materials area. This area of Waters' business is focused on petroleum, polymers, chemicals, personal care products, and electronics. In 2014, Jeff took over the newly formed Health Sciences business, a critical growth area for Waters that is focused on the biomarker discovery to diagnostic continuum. In 2016, Jeff was named Vice President of the Consumables Group. In June of 2017, he was appointed to the role of Vice President, Global Marketing.

Dr. ROBERT PLUMB

Dr Robert Plumb is the Director of Metabolic Phenotyping and Stratified Medicine in the Waters Health Sciences Business Operations Division, based in Milford, MA.

Dr Plumb has published over 100 papers on the subject of HPLC-MS and NMR for bioanalysis, metabolomics and metabolite identification. He is a recognized expert in the use of liquid chromatography with mass spectrometry, capillary scale LC, purifications scale LC, and metabonomics, giving many invited papers at international meetings around the world.

After obtaining an honors degree in Chemistry from the University of Hertfordshire in 1992, he started work in at Glaxo Research and Development Drug Metabolism Department. During his time at Glaxo and later GlaxoWellcome he continued his research in liquid chromatography combined with NMR and mass spectrometry for metabolite identification and bioanalysis obtaining his Ph.D. in 1999. Dr Plumb continued his work for GlaxoWellcome with the responsibility of metabolite identification using HPLC/MS/NMR and new analytical technology development. In 2001 he moved to Waters Corporation in Milford, MA, USA where he was responsible for the Life Science Chromatography group and latterly LC-MS applications in the Pharmaceutical Market Development Group before becoming the Director of Metabolic Phenotyping. He is currently a visiting Professor in Analytical Chemistry at Kings College London, visiting Professor at Imperial College in the Dept Surgery and Cancer, and a Fellow of the Royal Society of Chemistry. In 2014 he was awarded Highly Cited Researcher by Thompson Reuters.

JOSEPH ROMANO

Mr. Romano is a Senior Manager for Waters Corporation. His current responsibilities include business development for global Environment and Food & Beverages companies. He has been with Waters for thirty-three years. At Waters he has held positions in customer education, applications laboratory, and marketing manager for environmental, food and beverages and natural products. Prior to Waters, Romano worked for DuPont as a methods development chemist in the analytical department. He has a master's degree in chemistry from Bridgewater State University, Bridgewater, Massachusetts.

THOMAS WHEAT, Ph.D.

Thomas E. Wheat earned his Ph.D. from the University of Illinois and held research positions at Northwestern University before joining Waters Corporation. At Waters, his work has focused on both bioseparations and small molecule separations, particularly with MS detection. He has developed techniques for purification, notably At-column Dilution, as well as tools for bioseparations, including Auto•Blend™ Plus. He has published and presented papers on analytical biochemistry, capillary electrophoresis, mass spectrometry, and preparative chromatography. Recently, he has focused on synergies in separation systems and novel approaches to multi-dimensional chromatography.

WATERS UPLC AMINO ACID ANALYSIS SOLUTION

Easy method transfer and significant run capacity gains in a high-throughput services lab

Client: A global biopharmaceutical firm headquartered in the U.S.

BACKGROUND

An analytical services laboratory supporting the efforts of its process development group performs LC-based analyses of amino acids from cell culture supernatants on an as-needed basis. The information gleaned from the analyses is used by the process development group to make decisions about how to best scale up the production of biopharmaceuticals.

To make the best informed decisions, the process development group must determine which amino acids are critical for producing the highest quality proteins at the fastest rate. Once these have been identified, the drug manufacturing process is modified so that cell cycle regulation results in optimal drug production.

The support lab employs five chemists, one of whom is dedicated to this project. HPLC has been the industry standard for years for performing the relevant amino acid analysis work. An HPLC separation typically takes slightly more than one hour to resolve the important compounds contained in the sample.

Results reported by the support lab are carefully checked for accuracy before being released by the laboratory. Turnaround time is also key given the number of experiments required to finetune the production process for a given protein.

"I was totally amazed that we were able to separate this set of amino acids in nine minutes when, not long ago, it took several hours."

Vice President,
Process Development

CHALLENGE

Process development submits samples to the support lab at the rate of 100 per month, or roughly five samples per eight-hour day. The support lab must derivatize each sample and then analyze it on one of the lab's HPLC instruments. Each HPLC separation takes 60 minutes from injection to injection.

With other demands on the lab as well, the lab director has had to outsource between 30% to 40% of the analyses and wait anywhere from two weeks to one month for results, depending on the number of samples sent out at one time.

THE SOLUTION

In an effort to keep the work in-house without hiring additional resources, reduce turnaround times, and cut costs, the lab director installed a Waters® ACQUITY UltraPerformance LC® (UPLC®) System.

In early 2005, the support lab introduced UPLC into the lab and, with only minor modifications, transferred its reversed-phase HPLC amino acid analysis method to an ACQUITY UPLC® System equipped with UV detection and 1.7-µm ACQUITY UPLC Column chemistries.

Because UPLC is based on the same fundamental principles as HPLC, important aspects of the HPLC separation, such as the elution order of the amino acids and resolution, were the same in both methods.

BUSINESS BENEFIT

The support lab was able to readily transfer an HPLC method to the UPLC system and obtain a 7X increase in speed without any perceptible loss of resolution. The significant improvement in sample throughput has increased capacity to the extent that requisite outsourcing of amino acid analyses has been eliminated.

The laboratory now has the ability to run all the analyses in-house – with capacity to spare – saving $56,000 in eight months while allowing the lab to exercise a greater degree of control over the process of generating test data.

The lab also reduced consumption of the solvent acetonitrile by 10X, which has resulted in a savings of $5,000 since installation of the ACQUITY UPLC System. In less than eight months, the lab has recouped its initial capital investment.

The adoption of UPLC has had other added benefits for this laboratory. Previously, it was not uncommon for an analyst to start an HPLC run just before leaving the lab for the day, only to arrive the next morning to find out that the results were unacceptable due to a needed modification or adjustment to the operating conditions. Now, due to the speed of UPLC, much more can be accomplished in an eight-hour day, minimizing the need to conduct overnight runs.

WATERS AND UPLC

The Waters UPLC Amino Acid Analysis Solution synergistically combines unique instrumentation, column chemistries, and software for data acquisition, processing, and support services. This system creates a singular solution with superior sensitivity, resolution, efficiency, and sample throughput.

When coupled with Waters mass spectrometers, UPLC provides a level of separation, quantification, and characterization previously unattainable with traditional HPLC methods.

UPLC is currently being employed by companies that rely heavily on HPLC, thus bringing their laboratories measurable improvements in analytical sensitivity, resolution, and speed. Ultimately, these firms are looking for meaningful ways to increase laboratory productivity, decrease operational costs, facilitate product development, and increase revenue generation.

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UPLC Amino Acid Analysis Solution

Thomas E. Wheat, Eric S. Grumbach, and Jeffrey R. Mazzeo
Waters Corporation, Milford, MA, USA

CLASSIC UPLC

INTRODUCTION

Analysis of amino acids is required in several different areas of research and is also a fundamental tool in various product analysis activities. These applications impose different requirements on the analytical method because the amino acids play different roles.

Amino acids are the basic constituents of proteins. For that reason, qualitative and quantitative analysis of the amino acid composition of hydrolyzed samples of pure proteins or peptides is used to identify the material and to directly measure its concentration.

Amino acids are also intermediates in a myriad of metabolic pathways, often not directly involving proteins. The amino acids are, therefore, measured as elements of physiological and nutritional studies. This has proven particularly important in monitoring the growth of cells in cultures, as used in the production of biopharmaceuticals.

Similar considerations lead to the analysis of foods and feeds to ensure that nutritional requirements are met. These diverse sample applications will all benefit from improved amino acid methods.

A comprehensive system-based solution for the analysis of amino acids has been recently developed. This solution provides better resolution and sensitivity, all achieved in a shorter analysis time than with previous methodologies. Its enhanced separation ensures that the analysis yields accurate and precise qualitative and quantitative results and that the method is exceptionally rugged.

This application solution is based on the well-understood and widely-used Waters® AccQ•Tag™ pre-column derivatization chemistry The derivatives are separated using the Waters ACQUITY UltraPerformance LC® (UPLC®) System for optimum resolution and sensitivity. System control, data acquisition, processing, and flexible reporting are provided within Empower® Software. The integrated total application solution ensures successful analyses.

UPLC Amino Acid Analysis Solution.

METHODS AND DISCUSSION

Ultimately, a new amino acid method must provide the right answer. Increased ruggedness, preferably with reduced labor and run times, are also desired characteristics of a successful laboratory system. These needs are met by combining AccQ•Tag Ultra amino acid analysis chemistries with the proven separation technology of the ACQUITY UPLC System; together they comprise the turnkey application solution called the Waters UPLC Amino Acid Analysis Solution.

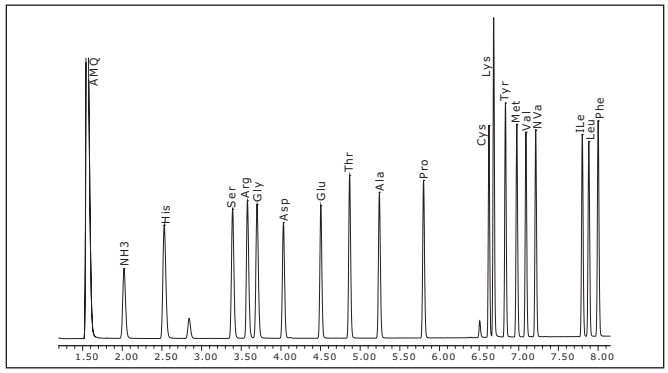

Figure 1. Separation of 50 pmoles of the amino acid hydrolysate standard with the UPLC Amino Acid Analysis Solution.

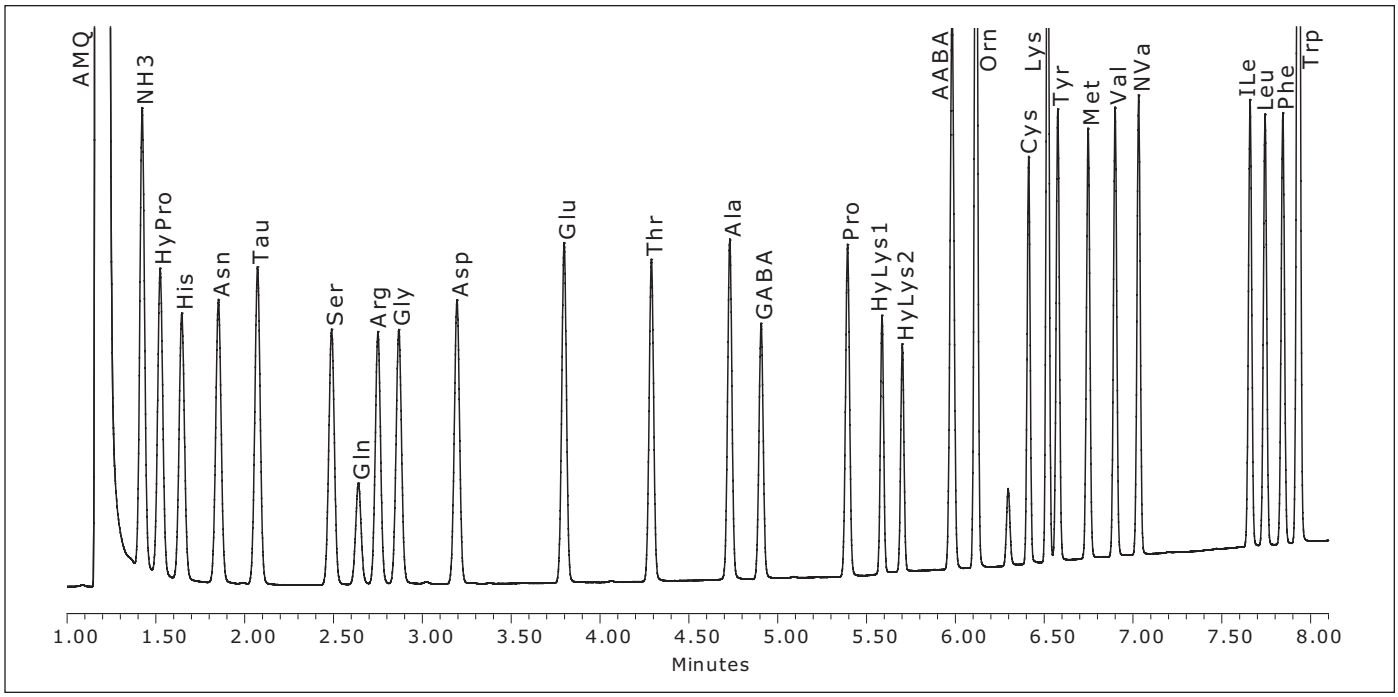

Figure 2. Separation of 10 pmoles of the amino acids commonly found in cell culture media. The UPLC Amino Acid Analysis Solution includes this modified separation method.

Analysis of a hydrolysate standard is shown in Figure 1. The amino acids are derivatized using AccQ•Fluor™ Ultra Reagent (Part Number: 186003836) (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Both primary and secondary amino acids react in a simple batch-wise derivatization, and samples are stable for several days. No special sample preparation is required, and the reaction occurs in a largely aqueous solution so it is very tolerant of buffer salts and other sample components. The excess reagent naturally hydrolyzes, and the by-product is chromatographically resolved from the derivatives. No special handling or extraction is required.

The derivatives are separated on an AccQ•Tag Ultra Column, 2.1 x 100 mm (Part Number: 186003837), a bridged ethyl hybrid (BEH) C18 1.7 µm particle specifically tested for separation of the amino acids. Packaged eluents are quality control (QC) tested with amino acid separations. They are provided as concentrates requiring only dilution with water before use. The instrument is the ACQUITY UPLC System with UV detection at 260 nm.

The resolution of the amino acids is 1.6 or greater to ensure accurate quantitation. Retention time reproducibility is on the order of hundredths of minutes, much less than a peak width, to ensure unambiguous identification of the amino acids. The detection is linear, over more than three orders of magnitude, to permit quantitative analysis of samples with disparate ratios of amino acids with an ample margin for samples of different concentration. The sensitivity of the method gives adequate signal-to-noise to quantitate at the level of 50 femtomoles on-column.

The method can be successfully used for a range of applications. The standard method shown in Figure 1 can also resolve the derivatives of cysteine commonly used in protein structure analysis. The products of performic acid oxidation that are part of assessing the nutritional quality of foods and feeds are also well-separated.

For monitoring the composition of media during the growth of cells in culture, additional amino acids must be resolved. This requires a different dilution of the AccQ•Tag Ultra Eluent A Concentrate and a higher separation temperature. The chromatogram used for monitoring cell culture media is shown in Figure 2.

CONCLUSION

These results describe the new Waters UPLC Amino Acid Analysis Solution, an Assured Performance Solution (APS), that come complete with application-focused chemistries, innovative UltraPerformance LC and MS technologies, methodology, documentation, and support to deliver the answers you need about amino acids, every time. Successful results are assured through the use of pre-tested derivatization and separation chemistry and the high resolution provided with the ACQUITY UPLC System.

This integrated analytical approach will give accurate and precise qualitative and quantitative results for a wide range of applications including protein and peptide hydrolysates, monitoring cell culture media, and measuring the nutritional value of food and feeds.

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Enhancement of the UPLC Amino Acid Analysis Solution with Flexible Detector Options

CLASSIC UPLC

Hillary B. Hewitson, Thomas E. Wheat, Diane M. Diehl

INTRODUCTION

The measurement of amino acids is important in many applications. Protein structure laboratories use it to confirm the identification and modification of proteins and peptides. Also, the sum of the amounts of amino acids gives the total concentration of the samples. Biopharmaceutical manufacturing facilities can optimize drug yield through careful monitoring and adjustments to the nutrient levels in cell cultures used in its production. In the animal feed industry, amino acid levels are measured as part of determining nutritional content. In each of these applications, it is essential to be able to quickly and accurately identify and quantitate amino acid levels. Incorrect results could result in poor batch yields, delay of product to market, or loss of product.

Waters provides a complete turnkey solution to meet the needs for each of these applications. The Waters UPLC® Amino Acid Analysis Solution was initially offered in 2006 as a total system solution that was available to users with a tunable UV (TUV) detector. Through the use of the application-specific quality tested columns, eluents, and derivatization chemistry, users can count on accurate results. Inclusion of pre-defined Empower® software methods provides users with powerful data generation and handling capabilities and allows rapid analysis and reporting of sample results. Recently, photodiode array and fluorescence detection have been added as options in the defined system, providing the users with equipment flexibility to satisfy the requirements of their laboratories, while maintaining the same quality results regardless of which detection option is chosen.

In this experiment, hydrolyzed samples of pure protein and of animal feed were analyzed using the Waters UPLC Amino Acid Analysis Solution with a TUV detector, photodiode array detector (PDA), and with fluorescence detection (FLR). Absolute amounts of amino acids as well as molar ratios were compared between TUV and FLR detection options for reproducibility, consistency, and accuracy as compared to expected values.

Figure 1. Waters UPLC Amino Acid Analysis Solution.

EXPERIMENTAL

LC conditions (Header 2)

LC system:	Waters ACQUITY UPLC® System
Column:	AccQ•Tag Ultra, 2.1 x 100 mm, 1.7 µm
Column temp:	55 °C
Sample temp:	20 °C
Flow rate:	700 µL/min
Mobile phase A:	1:20 Dilution of AccQ•Tag Ultra Eluent A Concentrate (prepared fresh daily)
Mobile phase B:	AccQ•Tag Ultra Eluent B
Needle washes:	Weak – 95:5 Water: Acetonitrile Strong – 5:95 Water: Acetonitrile
Gradient:	AccQ•Tag Ultra Hydrolysate Method (provided in the UPLC Amino Acid Analysis Solution)
Total run time:	9.5 min
Injection volume:	1 µL, Partial Loop with Needle Overfill (2 µL loop installed)
Detection:	UV (TUV), 260nm UV (PDA), 260nm, using 2D mode Fluorescence (FLR), λEx 266 nm, λEm 473 nm

Samples

Acid-hydrolyzed bovine serum albumin (BSA) and soybean meal samples were prepared in an independent laboratory as part of a collaborative study. The samples were supplied at an estimated concentration of 1.0 mg/mL in 0.1 M HCl sealed under argon in ampoules. Samples were stored at -80 °C until analysis.

Sample Dilution and Derivatization

The supplied samples were diluted with 0.1 M HCl prior to derivatization, as necessary, to assure accurate pipetting and complete derivatization. The samples were derivatized in batches, and were stable for up to one week at room temperature when tightly capped. Conditions, including suggested neutralization, for pre-column derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Application System Guide. The following sequential modified derivatization conditions were used for these samples.

1. 60 µL AccQ•Tag™ Ultra Borate Buffer
2. 10 µL diluted sample
3. 10 µL 0.1 N NaOH
4. 20 µL reconstituted AccQ•Tag Ultra Reagent

Acquisition and Processing Methods

The Waters UPLC Amino Acid Analysis Solution is provided with a CD that contains all the Empower methods necessary for acquisition and processing of the samples, as well as reporting of results. Details of the methods can be found in the Waters UPLC Amino Acid Analysis System Guide.

RESULTS AND DISCUSSION

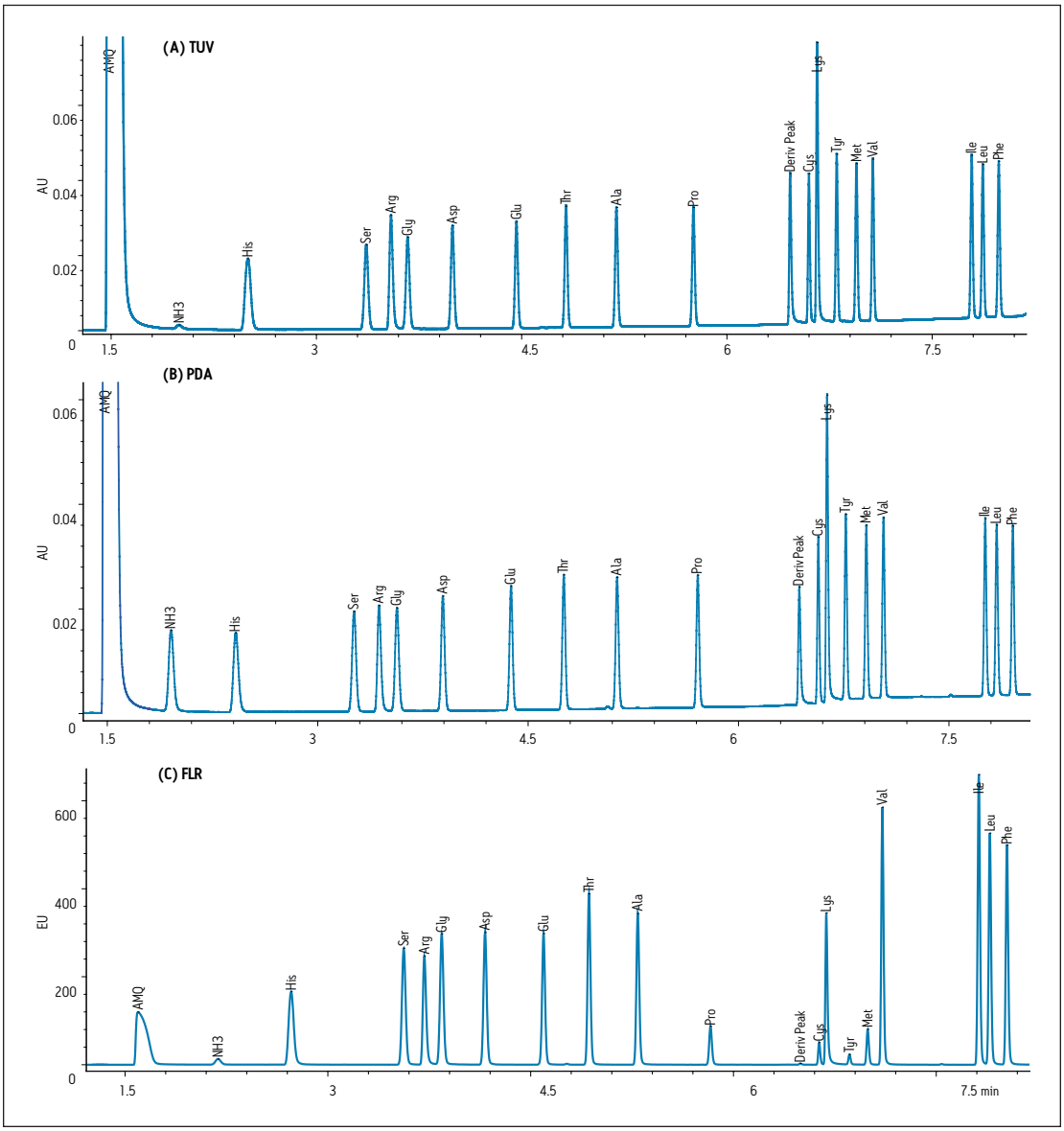

Figure 2. Analysis of Hydrolysate Standard, 10 pmoles of each amino acid on column (except Cystine at 5 pmoles), with (A) TUV detector, (B) PDA detector, and (C) FLR detector.

The operating conditions were optimized for each of the three detectors to give the highest signal-to-noise ratio. The results were compared, and a representative chromatogram of an amino acid hydrolysate standard is shown for each detector in Figure 2.

It has been widely accepted that a TUV is more sensitive than a PDA, and that a FLR is much more sensitive than UV detection. In addition, it is also believed in general that a FLR detector will give more selectivity, while a PDA can give UV spectral information to confirm peak identity and purity. The data was analyzed with these assumptions in mind, to see if they were true in this application solution.

The response for 10 pmoles on column is almost identical for the TUV and PDA detectors, while the FLR gives quite a different response. The TUV has lower noise than the PDA detector by approximately a factor of two, so the sensitivity as signal-to-noise is higher for the TUV by about the same factor.

With the FLR detector, we observe that the derivatives of the different amino acids have different fluorescence yields, and thus different sized peaks. The excitation and emission spectra are identical for all the amino acids. The differences do not seem to be related to spectral shifts. Tyrosine is the smallest peak in the fluorescence chromatogram, and, therefore, dictates the limit of quantitation. The usable range for both the TUV and FLR detectors in the application is 50 fmoles to 50 pmoles on column.

Peak identity and purity are often assessed based on spectral properties using a PDA detector. Figure 3 shows the UV spectra for five examples of AccQ•Tag derivatized amino acids, including acids, bases, neutrals, and doubly-derivatized molecules. The chemical distinctions between amino acids do not yield any useful spectral differences that could be used for peak identification. Therefore, the major value of using a PDA detector in the UPLC Amino Acid Analysis Solution is in the instrument flexibility created for other applications that require its use.

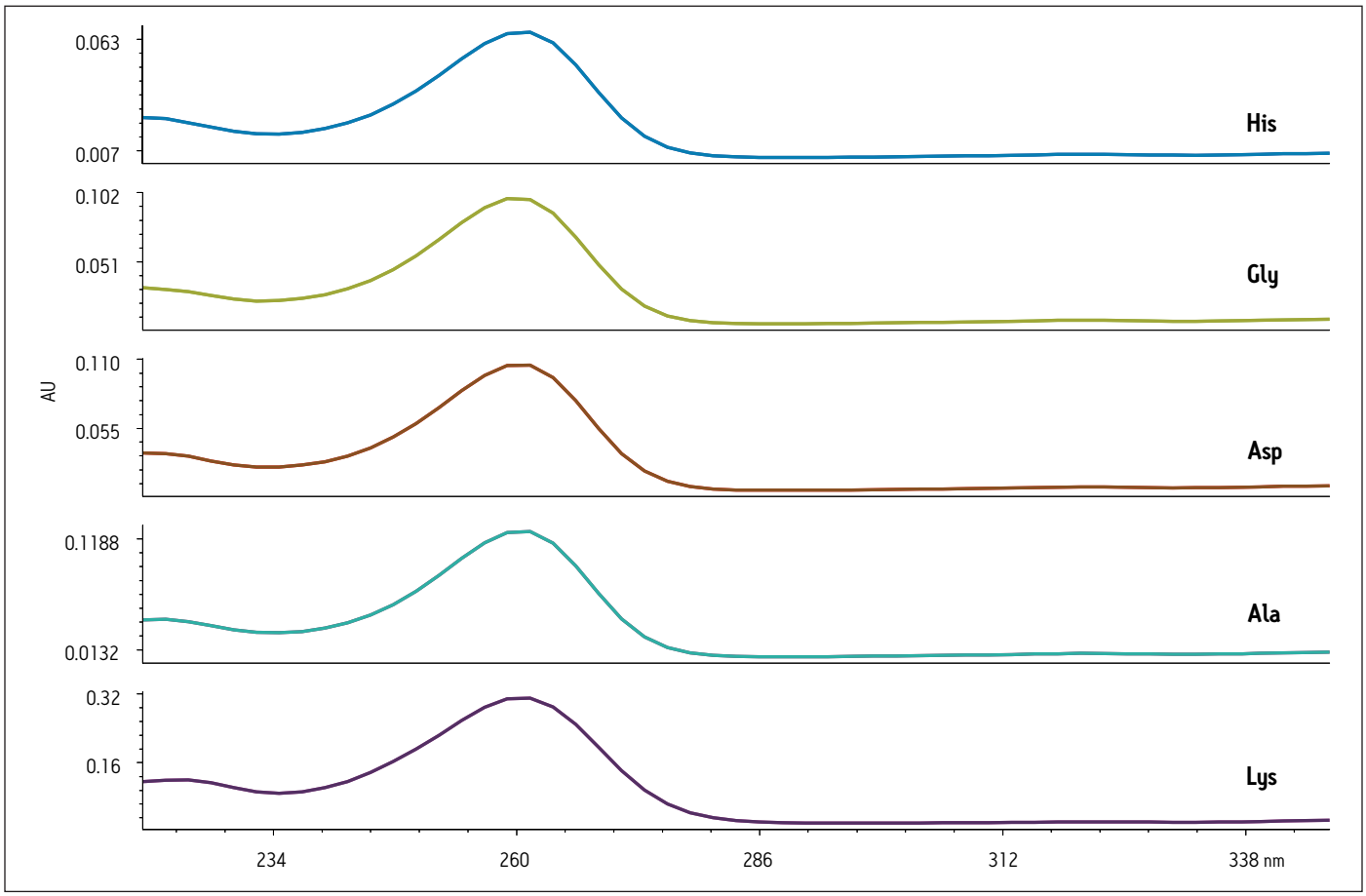

Figure 3. UV Spectra for various AccQ•Tag derivatized amino acids.

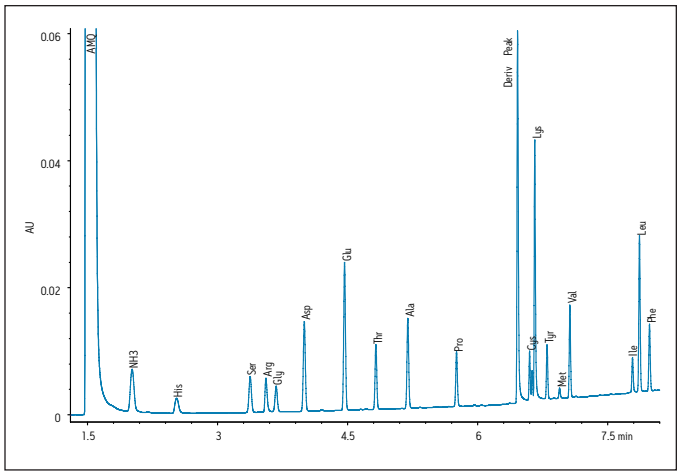

Figure 4. Analysis of BSA hydrolysate sample with UV detection, approximately 9 ng on column.

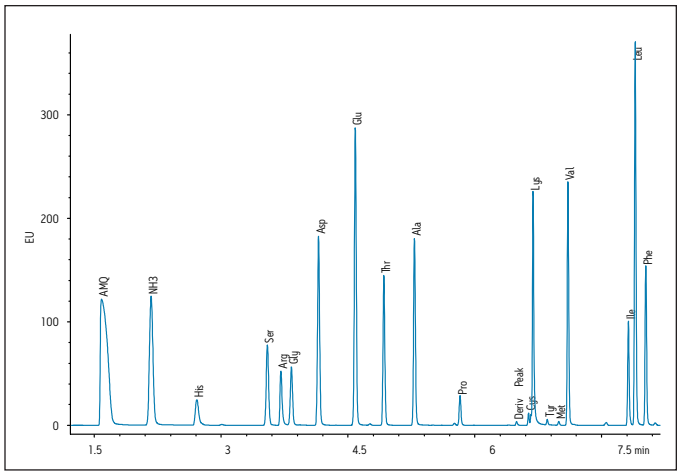

Figure 5. Analysis of BSA hydrolysate sample with fluorescence detection, approximately 9 ng on column.

Figures 4 and 5 show chromatograms with the same load of BSA hydrolysate on the column. Again, there is a difference in response for the amino acid peaks between the UV and fluorescence detectors. However, since the sample analysis is calibrated against a standard analyzed under the same conditions, no differences in the final result should be expected.

The accuracy of the results for both detectors is demonstrated by the quantitative results seen in Table 1. For all sample types, the 75 data points represent five days of analysis, each with independent sample dilutions, fresh mobile phase preparation, and each diluted sample derivatized five separate times, and injected in triplicate. The amino acid composition is expressed as residues per mole of BSA. Tryptophan and cysteine/cystine are excluded from the calculations because they are destroyed by the acid hydrolysis. The measured results for each detector match each other very well in addition to agreeing with the expected composition values.

			*Observed Residues
Amino Acid	**Expected Residues**	**TUV**	**FLR**
His	17	15.36 ± 0.19	15.73 ± 0.16
Ser	28	26.00 ± 0.08	25.90 ± 0.41
Arg	23	22.37 ± 0.08	22.39 ± 0.20
Gly	16	22.37 ± 0.08	16.65 ± 0.42
Asp	54	55.47 ± 0.21	55.18 ± 0.32
Glu	79	80.68 ± 0.20	80.27 ± 0.44
Thr	33	31.92 ± 0.06	32.01 ± 0.07
Ala	47	47.51 ± 0.15	47.40 ± 0.16
Pro	28	28.35 ± 0.14	28.92 ± 0.13
Lys	59	57.78 ± 0.38	57.83 ± 0.99
Tyr	20	20.19 ± 0.08	20.67 ± 0.34
Met	4	4.16 ± 0.15	4.04 ± 0.05
Val	36	35.67 ± 0.13	35.38 ± 0.13
Ile	14	13.15 ± 0.16	13.44 ± 0.16
Leu	61	63.13 ± 0.28	63.18 ± 0.28
Phe	27	26.57 ± 0.13	27.00 ± 0.33

Table 1. Comparison of observed with expected composition derived from known sequence of BSA for both UV and fluorescence detection.

**Average of 75 data points (25 derivatizations, each injected in triplicate).*

The analysis of complex animal feed hydrolysate samples with both UV and fluorescence detection is shown in Figures 6 and 7. As with the analysis of the BSA hydrolysate, the difference in response for amino acids between the detectors does not mean that one detector is more suitable for quantitation than the other. This fact is further supported by the comparison of measured absolute amounts of the same samples with both detectors. Table 2 shows the mean weight % values for both TUV and FLR for the 75 data points. The ratio of amount of each amino acid to amount of feed hydrolysate was expressed using the residue molecular weights of the amino acids. Since each analysis was calibrated relative to a standard with the same detector, the quantitative results are the same.

The reliability of the method is demonstrated with the reproducibility of the results over a large number of determinations that intentionally includes the variability that would be possible in routine analysis. These variations include multiple columns, eluents, and derivatizations. The largest contribution to variability in the method is due to the pipetting steps in the sample preparation. The addition of an internal standard to the sample to be hydrolyzed will correct for pipetting variability. Norvaline is the preferred internal standard for this purpose.

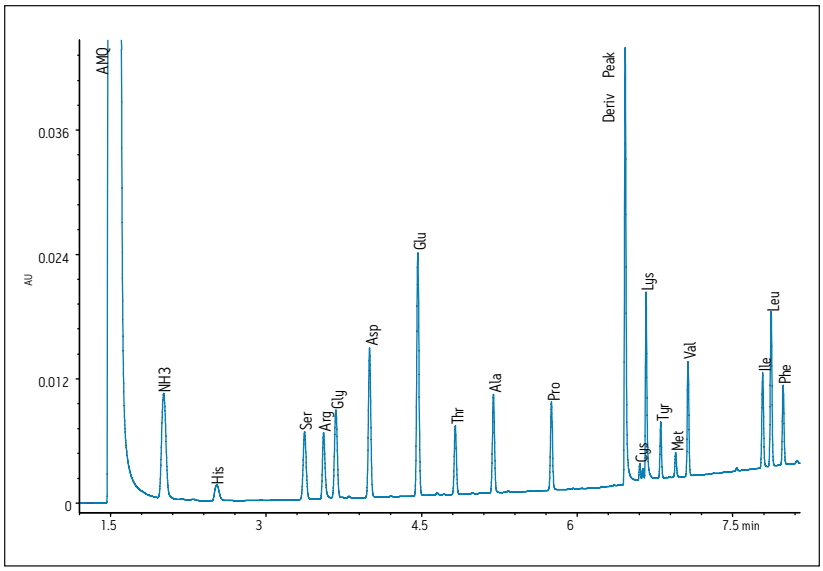

Figure 5. Analysis of BSA hydrolysate sample with fluorescence detection, approximately 9 ng on column.

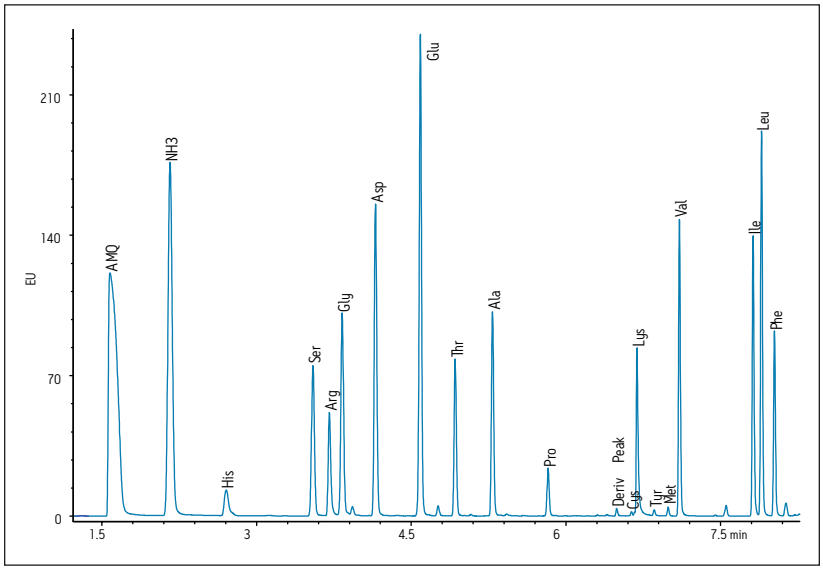

Figure 7. Analysis of soybean meal hydrolysate sample with fluorescence detection, approximately 6 ng on column.

	*Combined Mean	
Amino Acid	TUV	FLR
His	1.87 ± 0.06	1.98 ± 0.13
Ser	3.65 ± 0.07	3.58 ± 0.21
Arg	5.82 ± 0.13	5.74 ± 0.35
Gly	2.98 ± 0.07	2.87 ± 0.16
Asp	9.06 ± 0.17	8.92 ± 0.54
Glu	14.49 ± 0.28	14.36 ± 0.87
Thr	2.92 ± 0.06	2.90 ± 0.17
Ala	3.17 ± 0.08	3.15 ± 0.18
Pro	3.86 ± 0.08	3.87 ± 0.23
Lys	4.76 ± 0.11	4.80 ± 0.32
Tyr	2.90 ± 0.07	3.06 ± 0.21
Met	1.08 ± 0.03	1.08 ± 0.06
Val	3.66 ± 0.07	1.08 ± 0.06
Ile	3.45 ± 0.07	3.48 ± 0.20
Leu	6.12 ± 0.13	6.10 ± 0.35
Phe	3.92 ± 0.09	3.94 ± 0.22

Table 2. Weight/Weight % Comparison of TUV and FLR results for soybean meal hydrolysate; approximately 6 ng hydrolysate injected on column.

**Average of 75 data points (25 derivatizations, each injected in triplicate) ± Standard Deviation.*

CONCLUSION

The Waters UPLC Amino Acid Analysis is extended to three detector choices: TUV, PDA, and FLR. All three detectors give the same qualitative and quantitative result.

Historically, fluorescence detection has often been desired in amino acid analysis to provide enhanced sensitivity and to give specificity in the analysis of complex samples. The low variable fluorescence yield for the amino acids means that sensitivity is limited to the least responsive amino acid, specifically tyrosine. The analyses of pure protein and complex animal feed hydrolysates in this experiment shows that fluorescence and UV detectors both give accurate and consistent results with the Waters UPLC Amino Acid Analysis Solution.

It is generally true that cleanliness limits the usable sensitivity in any amino acid analysis method. Both the UV and fluorescence detectors give good analytical results well below the typical background limits. The Waters UPLC Amino Acid Analysis Solution provides a complete turnkey analytical method for the analysis of hydrolysate samples that allows the selection of a detector that not only meets the needs of the application, but also that of other assays in the laboratory as well. Regardless of the detector option chosen for the application, the ruggedness of the total system solution ensures highly reliable and rapid identification and quantitation of amino acids, with no interference or ambiguity.

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Amino Acid Analysis of Proteins, Cell Culture Media, Foods, and Feeds

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Thomas Wheat[1]; Mark Benvenuti[1]; Greg LeBlanc[2]; Jennifer Burgess[1]; Patricia McConville[1]
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H-CLASS UPLC

INTRODUCTION

The analysis of amino acids is essential in a variety of investigational projects as well as in routine QC and process monitoring. Successful analysis requires consideration of sample handling, separation and detection of the amino acids. A system has been developed that is intended to provide accurate and precise analysis over a wide range of sample types. The amino acids are derivatized by reaction with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and separated using reversed phase UPLC. The suitability of this method has been validated across several applications. The amino acid composition of acid hydrolyzed pure proteins was compared to expected values from the known sequence. Amino acids as metabolic indicators were measured in growing cell culture media and various foods and beverages. The nutritional content of feeds was also determined after hydrolysis. Optimization of handling for each sample type provided more reliable results. The extreme sensitivity of the derivatization and separation techniques simplified some aspects of sample preparation while facilitating optimization of others. Particularly for feed samples, the use of microwave hydrolysis techniques provided a practical addition to the overall analytical process. Feed samples were subjected to acid hydrolysis both with and without performic acid oxidation as well alkaline hydrolysis for a full profile of amino acid and, therefore, protein content. Sample handling throughput was much higher with this approach and was compatible with the analytical throughput of the system. These experiments demonstrate the suitability of the newly developed sample handling procedures and amino acid analysis system.

METHODS

Conditions :
System: ACQUITY UPLC H-Class System with TUV, or PDA, or FLR Detector
Column: AccQ•Tag™ Ultra, 2.1 X 100mm
Eluent A: 100% AccQ•Tag Ultra Eluent A
Eluent B: 90:10 Water, AccQ•Tag Ultra Eluent B
Eluent C: Water
Eluent D: 100% AccQ•Tag Ultra Eluent B
Injection Volume: 1.0 µl
Detection: UV@ 260 nm
Internal Standard: Norvaline (NVa)
Flow Rate: 0.7 ml/min
Run Time: 10.2 minutes
Note: The parameters listed above are common to all sample methods. Each method, however, uses a different quaternary gradient and column temperature. Complete details can be found in the ACQUITY UPLC H-Class Amino Acid Analysis System Guide (Waters).
Method 1: Used for Hydrolysates and Cell Culture Analyses
Method 2: Used for hydrolyzed Foods and Feeds

Standard Mixture used for Cell Culture or Free Amino Acids:

To 400 µl of 0.1N HCl are added 100 µl of Protein Hydrolysate Standard and 50 µl stock 5 mM aliquots of HypPro, Asn, Tau, Gln, GABA, HyLys, AABA, Orn, Nva, (internal standard) and Trp. This results in a Cell Culture Standard Mix at 250 pmol/µl and is stable for one month when stored at -20°C.

Standard Mixture used for Foods and Feeds:

To 650 µl of 0.1N HCl are added 100 µl of Protein Hydrolysate Standard and 50 µl stock 5 mM aliquots of Cya, Tau, $MetSO_2$, AABA, and Nva, (internal standard). This results in a Foods and Feeds Standard Mix at 250 pmol/µl and is stable for one month when stored -20°C.

Derivatization of Standard Mixtures:

To a total recovery vial (186000384C) is added 70 µl AccQ•Tag Ultra Borate Buffer and 10 µl standards mix. The vial is vortexed to mix these components. 20 µl of reconstituted AccQ•Tag Ultra reagent powder is added, the solutions vortexed and heated @ 55°C for ten minutes. The concentration of each analyte is now 25 pmol/µl.

Derivatization Chemistry

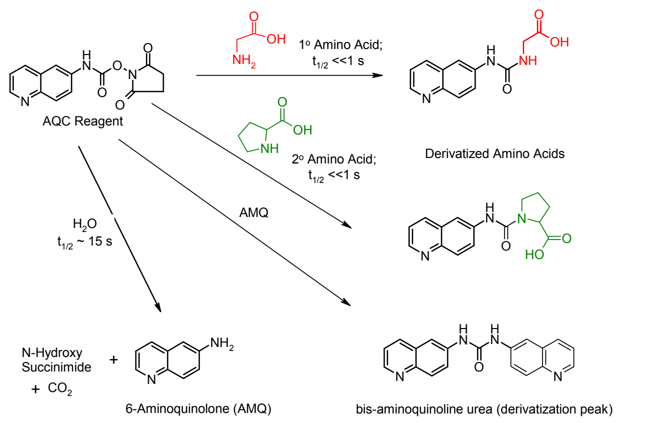

Figure 1. Reaction of AQC reagent with amino acids. The 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent reacts with both primary and secondary amines. Excess reagent reacts with water to form 6-aminoquinoline (AMQ). Subsequently, AMQ can react with excess AQC reagent to form a bis urea. Both of these side products do not interfere with the identification of any of the amino acids. The derivatives are stable for days, permitting batch-wise processing. Derivatization chemistry is used in both the UPLC® AAA Solution and in the ACQUITY UPLC H-Class Amino Acid Analysis System.

RESULTS

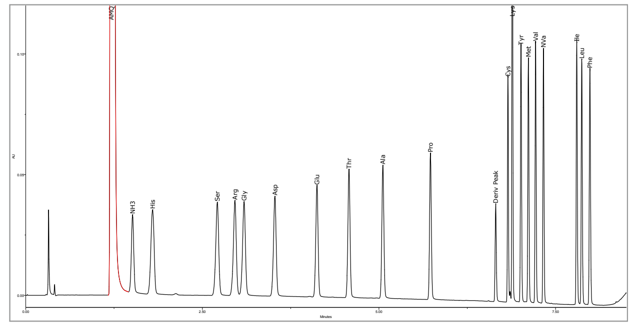

Figure 2. Protein Hydrolysate Standard with Norvaline Internal Standard. 25 pmoles on column and norvaline added as internal standard

Component Summary For Area

	Injection	His (µV*sec)	Ala (µV*sec)	Phe (µV*sec)
1	1	122445	114286	120924
2	2	122315	115531	122037
3	3	122742	115758	122485
4	4	123547	116499	123257
5	5	123113	116031	122684
6	6	122883	115944	122654
Min		122315	114286	120924
Max		123547	116499	123257
Mean		122841	115675	122340
Std. Dev.		451	753	797
% RSD		0.367	0.651	0.651

Component Summary For Retention Time

	Injection	His (min)	Ala (min)	Phe (min)
1	1	1.791	5.047	7.989
2	2	1.791	5.049	7.988
3	3	1.791	5.051	7.987
4	4	1.794	5.051	7.987
5	5	1.791	5.052	7.987
6	6	1.793	5.050	7.984
Min		1.791	5.047	7.984
Max		1.794	5.052	7.989
Mean		1.792	5.050	7.987
Std. Dev.		0.001	0.002	0.002
% RSD		0.075	0.033	0.022

Table 1 and 2. Area and Retention Time Reproducibility. Reliability is summarized for three indicator peaks that elute early, late, and in the middle.

Peak Name	Conc (pmol/uL), Ave Peak Areas 0.05	0.075	1.0	2.5	25	50
AMQ	6033932	6020972	6101437	6056268	5069175	3648294
NH3	4064	4810	9094	14396	86830	155693
His	167	304	4064	9618	104287	204119
Ser	334	330	4052	9459	102749	199653
Arg	230	320	3994	9516	102770	201123
Gly	591	682	4260	9608	100540	196412
Asp	211	272	4053	9704	101645	190396
Glu	238	303	3988	9330	100969	190784
Thr	238	329	4115	9617	104228	201799
CM Cys	197	310	4110	9726	105289	211792
Ala	269	336	4029	9447	102180	195455
PE Cys	240	339	4281	10014	109782	224055
Pro	219	308	3807	8916	96538	187201
D-Peak	94086	84767	94312	89565	62635	46575
Cys	172	262	3566	8315	91527	179095
Lys	334	499	6810	15852	173792	329877
Tyr	217	320	4177	9874	106770	209182
Met	176	268	4049	9354	104998	204492
Val	274	374	4127	9765	105725	204246
NVa	219	331	3893	8911	99315	198500
Ile	373	435	4323	9899	106378	205866
Leu	225	300	4084	9521	103758	201006
Phe	237	348	4312	9970	106180	208031

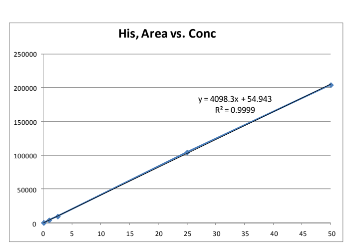

Table 3. Linearity of peak area with respect to amount derivatized. Reliable quantitation is possible over the range of 50 fmoles to 50 pmoles.

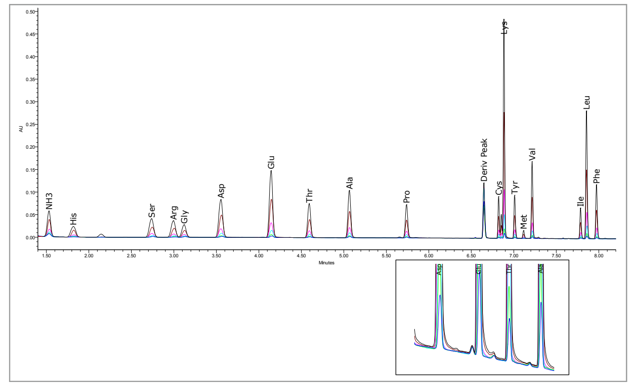

Figure 3. Hydrolysate of Bovine Serum Albumin over a 100-fold concentration range. Hydrolyzed BSA was diluted to extend over 1-100pmoles derivatized. The retention times and peak shapes are unaffected by concentration. The proportions of the amino acids are constant over the range derivatized.

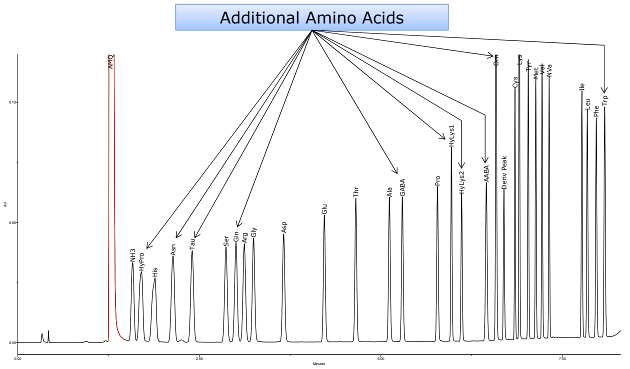

Figure 4. Analysis of Cell Culture Media. For monitoring the nutritional status of a growing cell culture, a larger panel of amino acids must be analyzed. The most important metabolic indicators are well-resolved with the standard chromatographic conditions.

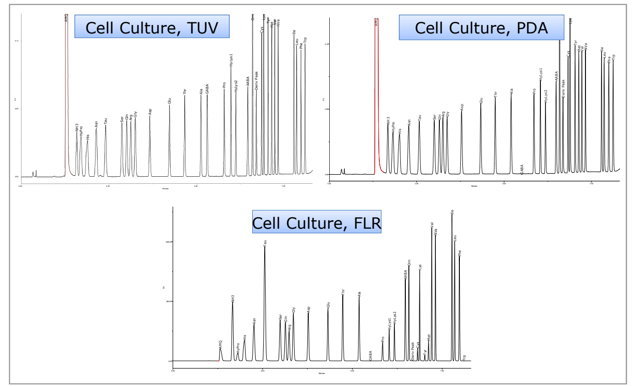

Figure 5. Alternative detectors for AAA. The system can be based on any of three detectors. The tunable UV and PDA, operating in single wavelength mode give essentially identical results and sensitivity. The spectral utility of the PDA is limited because all the derivatives have the same spectrum. Normally, a fluorescence detetctor is expected to yield extra sensitivity, but here the amino acids give different responses. The least fluorescent amino acid, tyrosine, limits sensitivity to the same range as a TUV. Tryptophan is completely undetectable.

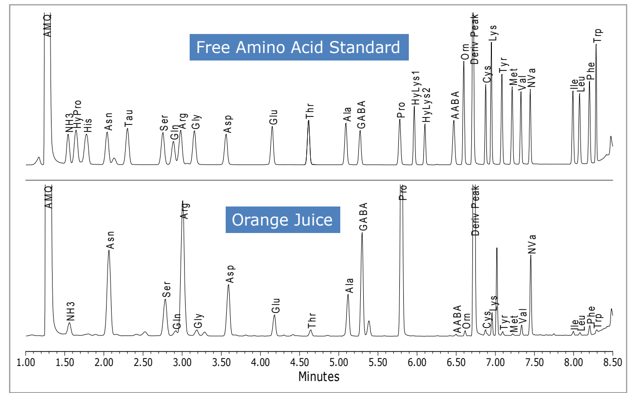

Figure 6. Free Amino Acids in Orange Juice. Non-protein amino acids serve as indicators of metabolic pathways reflecting the genetic origin and history of the "pant extract".

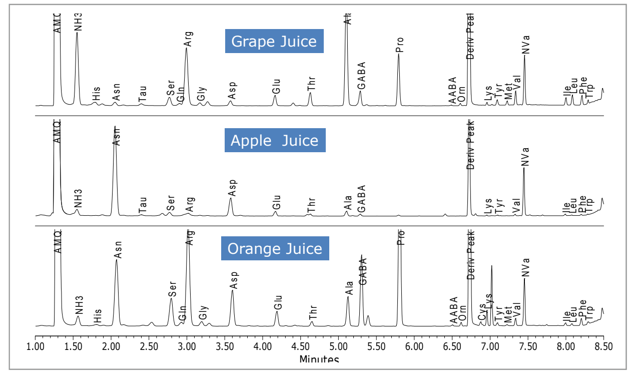

Figure 7. Free Amino Acids in Fruit Juices. The different genetic origins and history of different beverage products are consistent with the free amino acid profiles. For the analysis, norvaline is added as the internal standard.

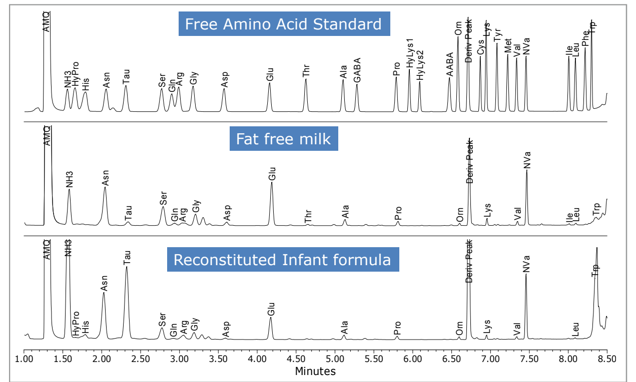

Figure 8. Free Amino Acids in Milk. Milk, like any biological sample, has a characteristic profile of non-protein amino acids. This profile is useful for identification and quality assessment. The profile can be used for adding nutrients to meet particular needs. It is known that cow's milk is inadequate for nutrition of human infants. As analyzed here, taurine is added when preparing infant formula to meet this requirement. For the analysis, norvaline is added as the internal standard.

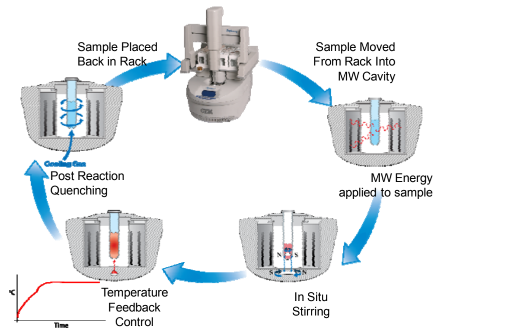

Figure 9. Microwave Hydrolysis of Feeds. The use of microwave hydrolysis (CEM Discover SP-D) accelerates the processing of feed samples from 24 hours to about 20 minutes while improving control of hydrolysis variables.

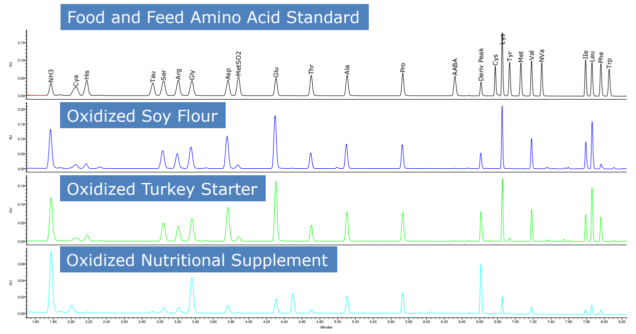

Figure 10. Determining Nutritional Value of Different Feeds. The proportions of amino acids, particularly including the sulfur amino acids can indicate how well a feed meets health needs.

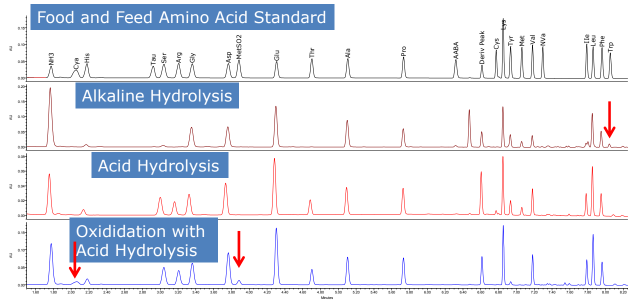

Figure 11. Complete AAA of feeds. Complete characterization of feed requires different sample handling for accurate determination of all the important amino acids. Oxidation gives the best values for the sulfur-amino acids while alkaline hydrolysis is required for tryptophan measurement.

CONCLUSIONS

- **A new system for amino acid analysis has been developed and tested**
- **Protein hydrolysates, free amino acids in cell culture and foods, and nutrional value can be measured**
- **Microwave hydrolysis reduces sample preparation time**
- **The ACQUITY UPLC H-Class Amino Acid Analysis System yields reliable, accurate, and precise determinations**

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Amino Acid Analysis of Proteins, Cell Culture Media, Foods, and Feeds

AUTOMATING AMINO ACID ANALYSIS USING ROBOTIC SAMPLE PREPARATION INSTRUMENTATION

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CLASSIC UPLC AND AUTOMATION

INTRODUCTION

Amino acids play an important role in many biological and physiological processes. Their importance has led to the use of amino acid analysis across a wide number of applications and research endeavors. These include protein structure characterization, determining the nutritional value of foods and feeds, monitoring cell cultures, and clinical research applications involving metabolic pathways. In all cases, the measurements must be accurate and precise. The analytical process must have the highest throughput consistent with reliable results. We have developed two robust protocols that include both the derivatization of amino acids and chromatographic analysis. The Waters UPLC® Amino Acid Analysis Solution has been applied across the range of applications, including protein characterization and the analysis of cell culture media. For physiological amino acids, the MassTrak™ Amino Acid Solution provides the same characterization for research use only. Both methods provide faster and more robust analyses than traditional methods. The limiting step in the analysis process is now in sample handling. A robotic sample preparation instrument has been optimized for the amino acid derivatization. Results are compared to the established manual process for reproducibility, accuracy and precision. The specific variables requiring optimization are described in detail. This automation can reduce the possibility of human error when preparing large number of samples. These results will demonstrate how a robotic sample derivatization combined with a robust analytical solution can lead to higher throughput with accurate and precise amino acid analysis.

Figure 1. Tecan Freedom EVO system, as configured for amino acid analysis.

METHODS

DERIVATIZATION CHEMISTRY

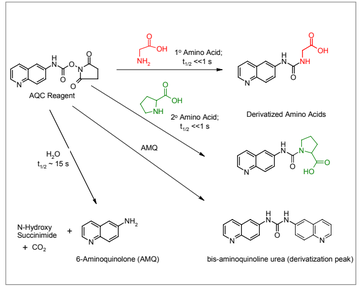

Figure 1. Reaction of AQC reagent with amino acids. The 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent reacts with both primary and secondary amines. Excess reagent reacts with water to form 6-aminoquinoline (AMQ). Subsequently, AMQ can react with excess AQC reagent to form a bis urea. Both of these side products do not interfere with the identification of any of the amino acids. The derivatives are stable for days, permitting batch-wise processing. Derivatization chemistry is used in both the UPLC® AAA Solution and MassTrak AAA Solution.

CHROMATOGRAPHIC CONDITIONS

UPLC® Amino Acid Analysis Solution

Column: AccQ•Tag™ Ultra 2.1 x 100mm, 1.7µm
Mobile Phases: AccQ•Tag Ultra Eluent A and Eluent B
Flow Rate: 0.7 mL/min
Injection Volume: 1.0 µL
Gradient: AccQ•Tag Ultra Standard Gradient
Column Temp: 55 °C
Detection: UV @ 260 nm
Instrument: ACQUITY UPLC® System with TUV

MassTrak Amino Acid Analysis Solution

Column: MassTrak™ AAA 2.1 x 150 mm, 1.7µm
Mobile Phases: MassTrak™ AAA Eluent A and Eluent B
Flow Rate: 0.4 mL/min
Injection Volume: 1.0 µL
Gradient: MassTrak AAA Standard Gradient
Column Temp: 43 °C
Detection: UV @ 260 nm
Instrument: ACQUITY UPLC System with TUV

SAMPLE PREPARATION

Depending on the application, sample preparation utilizes either the UPLC Amino Acid Analysis Solution Kit or the MassTrak Amino Acid Analysis Solution Kit. Each kit includes: amino acid standards, Total Recovery Vials, and the respective Derivatization Kit consisting of Reagent, Borate Buffer, and Reagent Diluent.

For derivatization of standards, 10µL of standard, 70 µL of borate buffer, and 20 µL of reagent are mixed. For derivatization of plasma, 20 µL of supernatant, 60 µL of borate buffer, and 20 µL of reagent are mixed.

AUTOMATION

Instrument: Tecan Freedom EVO®
Arms: Liquid Handling Arm (LiHa)
Tube Robotic Arm (PnP)
Deck Positions: Tip Wash Station
Disposable Tip Stations (3) with Tip Disposal Waste Chute
Plate Carrier with 3 - 48 well plates
Capping Station
Te-Shake/ Heater

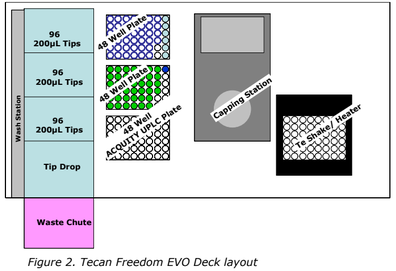

Figure 2. Tecan Freedom EVO Deck layout

The automated pre-column derivatization is performed using the Tecan Freedom EVO system. The derivatization steps are identical to those performed in a manual derivatization. The configuration of the deck layout is shown above. Up to 40 individual samples may be set to run in a batch. Each sample is derivatized in the capping station. Steps 1—8 are performed in sequence for each sample. These steps are repeated for each sample before proceeding to Step 8.

The exact sequence of steps is as follows:

1. Robotic arm grabs capped vial.
2. Robotic arm transports vial to capping station.
3. Robotic arm removes vial cap.
4. LiHa selects fresh pipette tip and aspirates Borate Buffer from plate 1. LiHa dispenses 70 µl of borate buffer into vial on capping station.
5. LiHa selects fresh pipette tip and aspirates sample from plate 2. LiHa dispenses 10 µl of sample into vial on capping station. LiHa mixes sample and borate buffer.
6. LiHa selects fresh pipette tip and aspirates reagent from plate 2. LiHa dispenses 20 µl of reagent into vial on capping station. LiHa mixes reagent and sample.
7. Robotic arm caps vial.
8. Robotic arm transports vial to original position in plate 1.
9. Steps 1-8 are repeated for each remaining sample.
10. Robotic arm transports all sample vials to heating block.
11. After 10 min at 55 °C, robotic arm moves all capped vials to ACQUITY UPLC plate.

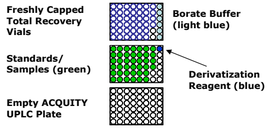

Figure 3. Tecan Freedom EVO sample/reagent layout

RESULTS AND DISCUSSION

The automated derivatization for the UPLC AAA Solution was evaluated for accuracy, precision and stability and compared to the manual derivatization for accuracy. Matching sets of six samples were prepared both manually and by an automated derivatization completed by the Tecan Freedom EVO. An overlay of the 25 pmole on column standard is shown below.

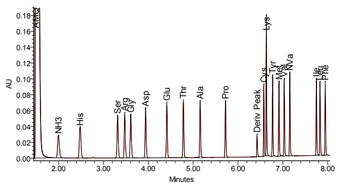

Figure 4. Overlay Chromatograms of UPLC AAA solution Hydrolysate standard. Comparison of manual preparation (black) and Tecan Freedom EVO preparation (red).

Amino Acid	Manual Mean Area Count	Manual % RSD	Tecan Mean Area Count	Tecan % RSD
His	96027	3.31	97885	1.70
Ser	95560	3.39	97790	1.67
Arg	89309	3.12	91685	1.64
Gly	93673	3.21	95527	1.67
Asp	94927	3.30	99203	1.76
Glu	94072	3.39	97742	1.74
Thr	98060	3.50	99341	1.63
Ala	98428	3.43	97581	1.69
Pro	96331	3.43	91682	1.70
Cys	80926	2.96	82216	2.00
Lys	158198	3.27	161957	1.63
Tyr	99040	3.31	100481	1.64
Met	97426	3.37	99191	1.57
Val	98756	3.42	99942	1.60
Ile	99261	3.43	101213	1.61
Leu	96887	3.47	98510	1.59
Phe	98534	3.30	99904	1.56

Table 1. Comparison of Tecan Freedom EVO and manually derivatized UPLC AAA Solution standards. Mean data is for matching sets of six. The precision for the Tecan derivatized samples is better than those prepared manually.

UPLC AAA Standard		
Amino Acid	Mean Area Count	% RSD Area
His	98906	1.72
Ser	98475	1.73
Arg	92533	1.71
Gly	96233	1.73
Asp	99360	1.83
Glu	96920	1.83
Thr	100105	1.73
Ala	98414	1.7
Pro	92493	1.73
Cys	82470	1.82
Lys	162572	1.64
Tyr	100776	1.64
Met	100413	1.66
Val	101002	1.64
NVa	89062	1.67
Ile	102209	1.65
Leu	99379	1.64
Phe	101365	1.59

Table 2. Precision of Tecan Freedom EVO derivatization for UPLC AAA Solution standards. The derivatization of 40 samples was completed 90 minutes. Precision over the complete set at better than 2% RSD meets the requirements of amino acid analysis. The software permits user selection of 1 - 40 samples.

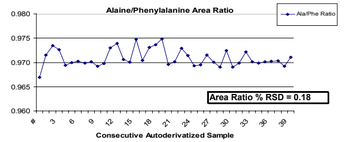

Figure 5. Measurement of Alanine/Phenylalanine Ratio for UPLC AAA Hydrolysate standard prepared using Tecan Freedom EVO. Each amino acids is derivatized at a different rate. Therefore, the ratio of alanine to phenylalanine is a measure of reagent stability and completeness of reaction. The constant ratio over 40 samples in 90 minutes indicates that the process is consistent over the entire set.

MassTrak AAA Standard, 40 Replicates, 25 pmole on column			
Amino Acid	Area %RSD	Amino Acid	Area %RSD
PSer	2.11	Ala	1.97
HyPro	1.97	GABA	2.00
PEA	1.99	AADA	1.98
His	2.15	Pro	1.98
Asn	1.96	BAIB	2.00
3MH	1.96	Hyl1	1.94
Tau	1.99	Hyl2	1.93
1MH	1.96	AABA	1.97
Ser	1.98	Cyst	1.97
Gln	1.98	Orn	2.33
Carn	1.97	Cys	2.03
Arg	1.98	Lys	1.95
Gly	1.94	Tyr	2.15
Ans	2.02	Met	1.96
EA	2.01	Val	1.98
Asp	1.99	Nva	1.98
Sar	2.16	Ile	1.97
Glu	1.96	aIle	1.98
Cit	1.95	Leu	1.97
B-Ala	1.99	HCys	1.93
Thr	1.98	Phe	1.97
		Trp	2.00

Table 3. Precision of Tecan Freedom EVO derivatized MassTrak AAA Solution standards. Area precision for all amino acids in the 40 replicate derivatized standards is better than achieved manually.

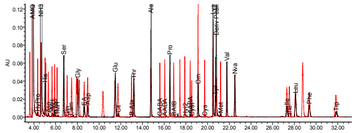

Figure 6. Overlay of Tecan Freedom EVO derivatized MassTrak AAA Solution standard (red) and deproteinized plasma sample (black). The same automation procedure is successfully applied to both standards and complex samples.

CONCLUSION

- **Automation of the pre-column derivatization steps of UPLC AAA and MassTrak AAA Solutions is successfully demonstrated using the Tecan Freedom EVO system.**
- **Comparison between the Tecan and manual process shows less variability and the reduction of human error with the automated solution.**
- **Precision in both the UPLC AAA Solution and the MassTrak AAA Solution meets the requirements of routine amino acid analysis.**
- **Automation allows for 1 - 40 samples to prepared in a batch within 90 minutes.**
- **The Waters® AAA Solutions combined with the Tecan automated system allows for a reliable, time savings solution for the high throughput laboratory.**

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Automating Amino Acid Analysis using Robotic Sample Preparation Instrumentation

Improving Sample Handling for Amino Acid Analysis

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[1]Waters Corporation; Milford, MA 01757; [2]CEM Corporation; Matthews NC 28106; [3]Tecan US; Research Triangle Park, NC 28106

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CLASSIC UPLC WITH MICROWAVE HYDROLYSIS

INTRODUCTION

Amino acid analysis provides fundamental information for many applications and research endeavors. These include protein structure characterization, determining the nutritional value of foods and feeds, monitoring cell cultures and other processes, and clinical research applications involving metabolic pathways. In all cases, the measurements must be accurate and precise with the highest throughput consistent with reliable results. We have previously described development of a robust protocol for derivatization of amino acid samples followed by chromatographic analysis. This UPLC® Amino Acid Analysis Solution has been applied across the range of applications with increased analytical throughput. With faster and more robust analysis, however, the limiting steps in the analysis process are now in sample handling. Two opportunities exist for improving throughput. One is preparation of sample hydrolysates, and the other is the derivatization. Microwave hydrolysis has been suggested to reduce the hydrolysis process from many hours to several minutes. We have optimized that technique for samples of pure proteins and for complex samples represented by biopharmaceutical formulations. The comparison of these tests with conventional oven-based hydrolyses will be reported. The factors in optimization will be described in detail. Secondly, a robotic sample preparation instrument has been optimized for the amino acid derivatization. Results will be compared to the established manual process. The specific variables requiring optimization will be described in detail. The total workflow of optimum rapid hydrolysis, robotic sample derivatization, and a robust analytical solution leads to higher throughput for accurate and precise amino acid analysis.

DERIVATIZATION CHEMISTRY

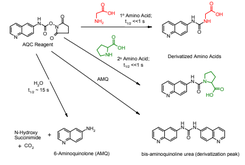

Figure 1. Reaction of AQC reagent with amino acids in UPLC® AAA Solution and MassTrak AAA Solution. The 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent reacts with both primary and secondary amines. Excess reagent reacts with water to form 6-aminoquinoline (AMQ). Subsequently, AMQ can react with excess AQC reagent to form a bis urea. These side products do not interfere with the identification of any of the amino acids. The derivatives are stable for days, permitting batch-wise processing.

METHODS

SAMPLE PREPARATION

Depending on the application, sample preparation utilizes either the UPLC Amino Acid Analysis Solution Kit or the MassTrak Amino Acid Analysis Solution Kit. Each kit includes: amino acid standards, Total Recovery Vials, and the respective Derivatization Kit consisting of Reagent, Borate Buffer, and Reagent Diluent.

For derivatization of standards and samples, 10µL of amino acid solution, 70 µL of borate buffer, and 20 µL of reagent are mixed.

HYDROLYSIS

Instrument: CEM Discover Protein Hydrolysis
Conditions: As described in figure legends
Protein Solutions for hydrolysis:
A: Horse Heart Myoglobin, 1mg/mL in Water.
B: Horse Heart Myoglobin, 1mg/ml in formulation
Bovine Serum Albumin, 1mg/ml in formulation
Biopharmaceutical formulation:
Sucrose, 25mg/mL
NaCl, 0.15M
NaH_2PO_4, 0.025M
Na_2HPO_4, 0.025M
Tween 80, 0.10M

AUTOMATED DERIVATIZATION

Instrument: Tecan Freedom EVO®
Arms: Liquid Handling Arm (LiHa)
Tube Robotic Arm (PnP)
Deck Positions: Tip Wash Station
Disposable Tip Stations (3) with Tip Disposal
Waste Chute
Plate Carrier with 3 - 48 well plates
Capping Station
Te-Shake/ Heater

CHROMATOGRAPHY

Instrument: ACQUITY UPLC System with PDA

CHROMATOGRAPHIC CONDITIONS

UPLC® Amino Acid Analysis Solution
Column: AccQ•Tag™ Ultra 2.1 x 100mm, 1.7µm
Mobile Phases: AccQ•Tag Ultra Eluent A and Eluent B
Flow Rate: 0.7 mL/min
Injection Volume: 1.0 µL
Gradient: UPLC AAA Solution Standard Hydrolysate Gradient
Column Temp: 55 ºC
Detection: UV at 260 nm

MassTrak Amino Acid Analysis Solution
Column: MassTrak™ AAA 2.1 x 150 mm, 1.7µm
Mobile Phases: MassTrak™ AAA Eluent A and Eluent B
Flow Rate: 0.4 mL/min
Injection Volume: 1.0 µL
Gradient: MassTrak AAA Standard Gradient
Column Temp: 43 ºC
Detection: UV at 260 nm

RESULTS AND DISCUSSION

MICROWAVE HYDROLYSIS

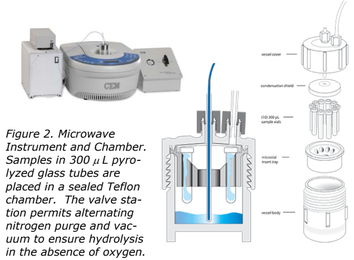

Figure 2. Microwave Instrument and Chamber. Samples in 300 µL pyrolyzed glass tubes are placed in a sealed Teflon chamber. The valve station permits alternating nitrogen purge and vacuum to ensure hydrolysis in the absence of oxygen.

The chamber contains a pool of 6N HCl/0.1% phenol. Samples are placed in the chamber dry for vapor phase hydrolysis, or 40 µL of the same acid is added directly to each tube for liquid phase hydrolysis. The instrument was operated at constant temperature for the times as described in the figure legends. The optimum time and temperature combination for vapor phase hydrolysis was determined based on accuracy of amino acid composition for pure myoglobin.

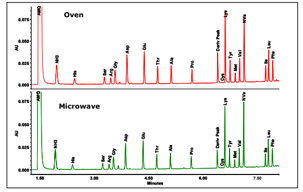

Figure 2. Comparison of microwave and conventional vapor phase hydrolysis of pure Horse Heart Myoglobin.

Amino Acid	Known Composition	150W, 150ºC, 10min	150W, 150ºC, 20min	150W, 165ºC, 10min	150W, 165ºC, 20min
His	11	6.47	9.73	9.72	10.06
Ser	5	6.86	4.87	4.65	4.23
Arg	2	1.61	1.97	1.98	1.96
Gly	15	29.78	18.10	18.25	16.96
Asp	10	25.71	11.83	11.77	11.55
Glu	19	14.99	20.81	20.62	21.32
Thr	7	5.02	5.54	5.28	5.51
Ala	15	21.29	16.68	16.74	16.28
Pro	4	2.07	4.00	4.02	4.28
Lys	19	9.47	17.86	17.49	18.44
Tyr	2	0.69	1.81	1.75	1.78
Val	7	3.63	4.81	4.91	5.50
Ile	9	5.14	7.13	7.37	7.34
Leu	17	10.50	16.98	17.60	17.05
Phe	7	5.77	6.88	6.84	6.75

Table 1 . Time and temperature optimization of vapor phase microwave hydrolysis, shown by the accuracy of Myoglobin composition. The higher temperature and longer time was chosen for further experiments, although loss of labile amino acids is seen.

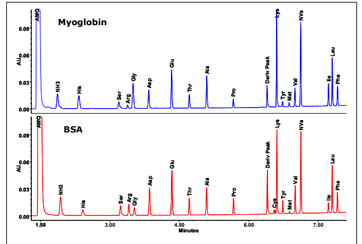

Figure 3. Liquid phase hydrolysis of two different proteins in a typical formulation, using the previously determined optimal microwave parameters (165ºC, 20min).

Amino Acid	Known Composition	Vapor Phase Average	Vapor Phase Std Dev	Liquid Phase Average	Liquid Phase Std Dev
His	11	12.59	0.60	10.73	0.12
Ser	5	1.59	0.43	4.06	0.51
Arg	2	1.78	0.19	2.11	0.05
Gly	15	18.28	1.17	15.99	0.26
Asp	10	11.16	0.45	10.54	0.17
Glu	19	26.47	1.50	19.54	0.35
Thr	7	2.89	0.48	6.19	0.47
Ala	15	21.90	1.53	15.36	0.52
Pro	4	5.95	0.33	4.29	0.07
Lys	19	7.78	3.17	19.23	0.20
Tyr	2	0.18	0.08	1.71	0.08
Val	7	7.77	0.36	6.52	0.24
Ile	9	8.51	0.49	8.20	0.26
Leu	17	17.71	1.04	17.45	0.09
Phe	7	4.46	0.79	7.09	0.10

Amino Acid	Known Composition	Vapor Phase Average	Vapor Phase Std Dev	Liquid Phase Average	Liquid Phase Std Dev
His	17	20.18	0.75	16.86	0.21
Ser	28	8.49	1.40	23.07	3.09
Arg	23	20.04	1.28	24.41	0.37
Gly	16	24.15	1.18	18.24	0.35
Asp	54	61.97	2.38	56.11	0.79
Glu	79	112.63	3.28	81.99	1.17
Thr	33	13.94	1.69	29.33	1.61
Ala	47	71.39	4.13	47.23	1.39
Pro	28	40.21	1.35	29.40	0.35
Lys	59	25.26	6.46	59.41	1.12
Tyr	20	1.35	0.22	16.17	0.45
Val	36	45.23	1.19	35.57	0.55
Ile	14	13.78	0.94	13.76	0.22
Leu	61	64.67	4.25	63.15	0.53
Phe	27	16.70	3.28	27.29	0.38

Table 2a . Comparison of vapor and liquid phase microwave hydrolysis (165ºC, 20min) for Myoglobin (top) and BSA (bottom) prepared as a typical biopharmaceutical formulation. The known proteins are prepared in a solution of buffer and stabilizers, typical of biopharmaceuticals, that may interfere with hydrolysis. Liquid phase hydrolysis gives better fidelity to expected composition. The results are the averages of 4 tubes in each of 3 independently hydrolyzed vessels, indicating good compositional precision.

Amino Acid	Vapor Phase Average	Vapor Phase % RSD	Liquid Phase Average	Liquid Phase % RSD
His	0.019	2.874	0.034	4.029
Ser	0.006	15.262	0.029	1.083
Arg	0.022	2.454	0.055	4.246
Gly	0.010	1.369	0.015	4.093
Asp	0.053	3.990	0.092	4.859
Glu	0.104	2.235	0.150	4.824
Thr	0.010	11.058	0.044	2.772
Ala	0.038	2.314	0.047	4.153
Pro	0.028	2.380	0.041	3.440
Cys	0.001	3.122	0.004	5.954
Lys	0.022	15.070	0.108	5.540
Tyr	0.001	5.678	0.039	5.366
Met	0.000	14.159	0.006	4.715
Val	0.031	2.159	0.050	5.370
Ile	0.010	3.900	0.022	5.828
Leu	0.049	4.942	0.103	4.054
Phe	0.016	7.193	0.058	4.615
Total	0.422		0.898	

Table 2b . Comparison of vapor and liquid phase microwave hydrolysis (165ºC, 20min) of BSA prepared as a biopharmaceutical formulation. The reported results are given in mg/mL in starting solution. The recovery of mass is much higher with liquid phase hydrolysis and the reproducibility is better and more consistent. The results are the averages of 4 tubes in each of 3 independently hydrolyzed vessels. The precision indicates good control of the hydrolysis process. Liquid phase hydrolysis is preferred for complex samples.

AUTOMATED DERIVATIZATION

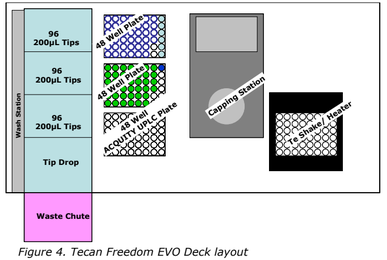

Figure 4. Tecan Freedom EVO Deck layout

The automated derivatization for the UPLC AAA Solution was evaluated for accuracy, precision and stability and compared to the manual derivatization for accuracy. Matching sets of six samples were prepared both manually and by an automated derivatization completed by the Tecan Freedom EVO. An overlay of the 25 pmole on column standard is shown below.

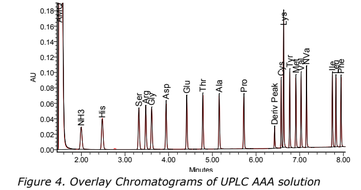

Figure 4. Overlay Chromatograms of UPLC AAA solution Hydrolysate standard. Comparison of manual preparation (black) and Tecan Freedom EVO preparation (red).

Amino Acid	Manual Mean Area Count	Manual % RSD	Tecan Mean Area Count	Tecan % RSD
His	96027	3.31	97885	1.70
Ser	95560	3.39	97790	1.67
Arg	89309	3.12	91685	1.64
Gly	93673	3.21	95527	1.67
Asp	94927	3.30	99203	1.76
Glu	94072	3.39	97742	1.74
Thr	98060	3.50	99341	1.63
Ala	98428	3.43	97581	1.69
Pro	96331	3.43	91662	1.70
Cys	80926	2.96	82216	2.00
Lys	158198	3.27	161957	1.63
Tyr	99040	3.31	100481	1.64
Met	97426	3.37	99191	1.57
Val	98756	3.42	99942	1.60
Ile	99261	3.43	101213	1.61
Leu	96887	3.47	98510	1.59
Phe	98534	3.30	99904	1.56

Table 3. Comparison of Tecan Freedom EVO and manually derivatized UPLC AAA Solution standards. Mean data is for matching sets of six. The precision for the Tecan derivatized samples is better than those prepared manually.

UPLC AAA Standard		
Amino Acid	Mean Area Count	% RSD Area
His	98906	1.72
Ser	98475	1.73
Arg	92533	1.71
Gly	96233	1.73
Asp	99360	1.83
Glu	96920	1.83
Thr	100105	1.73
Ala	98414	1.7
Pro	92493	1.73
Cys	82470	1.82
Lys	162572	1.64
Tyr	100776	1.64
Met	100413	1.66
Val	101002	1.64
NVa	99062	1.67
Ile	102209	1.65
Leu	99379	1.64
Phe	101365	1.59

Table 4. Precision of Tecan Freedom EVO derivatization for UPLC AAA Solution standards. The derivatization of 40 samples was completed 90 minutes. Precision over the complete set at better than 2% RSD meets the requirements of amino acid analysis. The software permits selection of 1 - 40 samples.

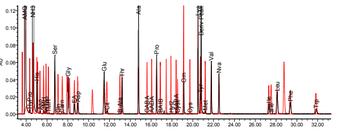

Figure 5. Overlay of Tecan Freedom EVO derivatized MassTrak AAA Solution standard (red) and deproteinized plasma sample (black). The same automation procedure is successfully applied to both standards and complex samples.

CONCLUSIONS

- **Waters UPLC Amino Acid Analysis Solution and MassTrak Amino Acid Analysis Solution provide robust total solutions for many applications.**
- **Microwave hydrolysis can be used to reduce the hydrolysis time from about 20 hours to 20 minutes.**
- **Microwave hydrolysis gives good compositional accuracy for both simple and complex samples.**
- **Complex samples, such as biopharmaceutical formulations, are best hydrolyzed in the liquid phase**
- **Automation of the pre-column derivatization steps of UPLC AAA and MassTrak AAA Solutions is successfully demonstrated using the Tecan Freedom EVO system.**
- **Comparison between the Tecan and manual process shows the reduction of human error with the automated solution.**
- **Precision of automated derivatization meets the requirements of routine amino acid analysis.**
- **The Waters® AAA Solutions combine with the CEM microwave hydrolysis and Tecan automation for a reliable, time saving solution for the high throughput laboratory.**

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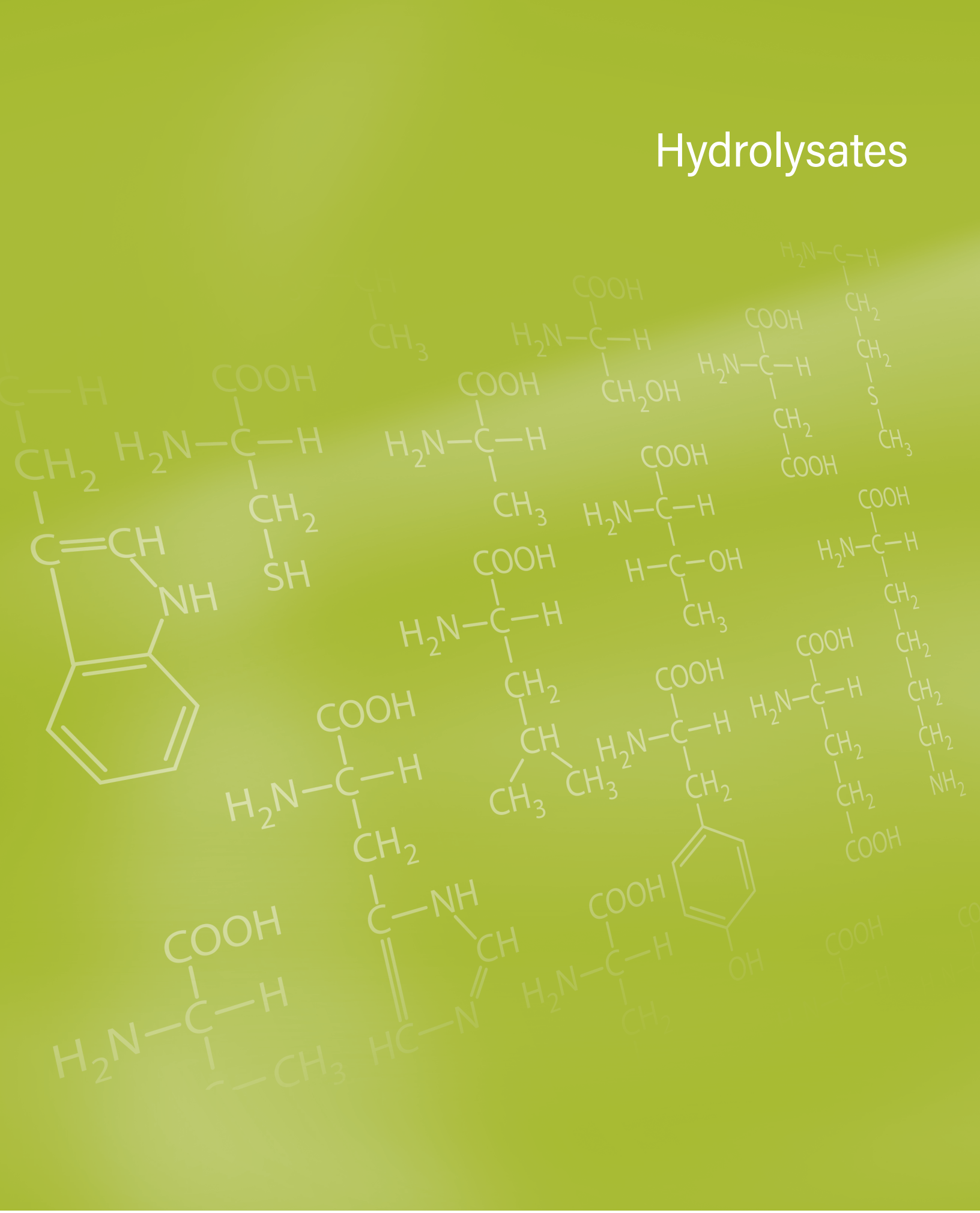
Improving Sample Handling for Amino Acid Analysis

Hydrolysates



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Waters AccQ•Tag Method for Hydrolysate Amino Acid Analysis

HPLC

Prof Ian D Wilson[1] and Robert S Plumb[2]
[1]Dept. Surgery & Cancer, Imperial College London, UK, [2]Waters Corporation, Milford, MA, USA

EXPERIMENTAL

LC conditions

Column:	Waters AccQ•Tag, 3.9 x 150 mm
Column temp.:	37 °C
Mobile phase:	A ternary gradient elution profile using:
Eluent A:	AccQ•Tag Eluent A
Eluent B:	Acetonitrile
Eluent C:	MilliQ® water
Flow rate:	1.0 mL/min
Detection:	470 Scanning Fluorescence Detector (5µL flow cell) EX: 250 nm EM: 395 nm Filter: 0.5 Gain: 100
Injection volume:	5 µL
Sample:	AQC-Derivatized Amino Acid Standard (50 pMoles)

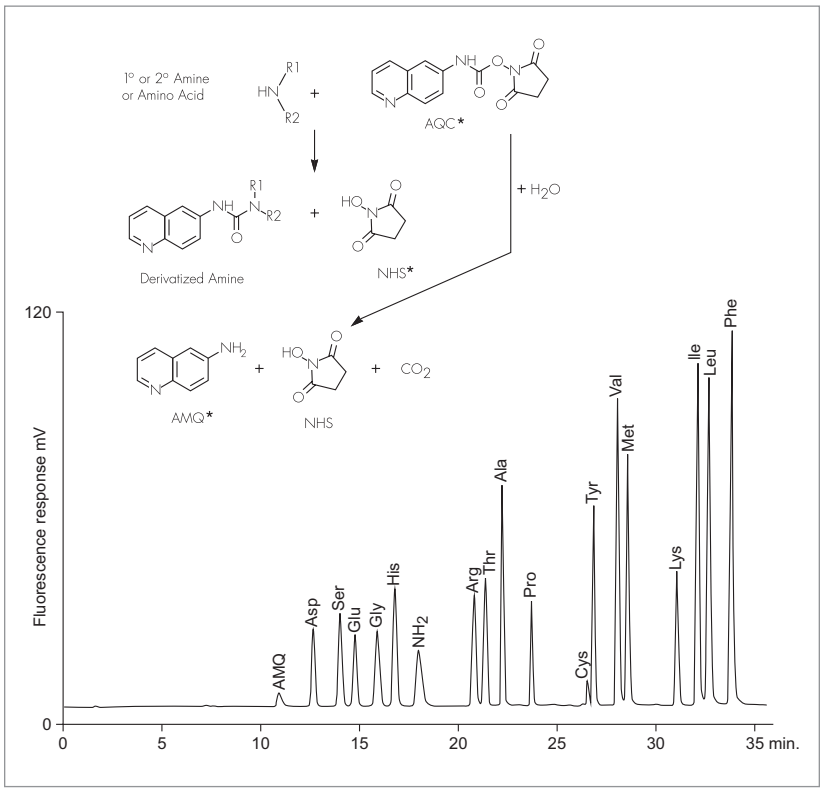

Figure 1. Both primary and secondary amines rapidly react with AQC to produce highly stable, fluorescent derivatives. Optimized chromtaographic conditions provide baseline or near-baseline separation of the hydrolysate amino acids.

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Separation of Low Levels of Isoleucine from Leucine Using the ACQUITY UPLC H-Class Amino Acid System

H-CLASS

Richard C. Daw
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Resolution of leucine and isoleucine at levels as low as 0.05% Ile/Leu using the UPLC® Amino Acid Analysis Solution.

WATERS SOLUTIONS

UPLC Amino Acid Analysis Solution

ACQUITY UPLC® H-Class System

AccQ•Tag™ Ultra Chemistry

Empower® 3 Software

KEY WORDS

Amino acid analysis, leucine, isoleucine, European Pharmacopoeia, USP

INTRODUCTION

The European Pharmacacopoeia (Ph. Eur.) defines requirements for the qualitative and quantitative composition of amino acids and mixtures of amino acids. The requirements for allowed impurities are also defined. Manufacturers of amino acids are legally bound to prove that their amino acids meet these specifications before they can distribute their products in Europe.

Leucine (Leu) is a branched-chain α-amino acid and is produced by the fermentation process. During this process, isoleucine can be produced as a by-product. The European Pharmacopoeia states that leucine and isoleucine should have a resolution of 1.5 to levels as low as 0.05%. This application note is intended to demonstrate that the Waters ACQUITY UPLC H-Class Amino Acid System can be used to suitably resolve isoleucine from leucine at these low levels.

The Waters ACQUITY UPLC H-Class Amino Acid System combines UPLC separation technology with AccQ•Tag Ultra derivatization chemistry, providing improved resolution and sensitivity, leading to improved sample characterization, all achieved within a shorter analysis time than conventional methodologies.

The amino acids are derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) under largely aqueous conditions. The derivatives are then separated utilizing the ACQUITY UPLC H-Class System, enabling analysts to achieve accurate, precise, and robust amino acid analysis utilizing a reversed-phase separation, and quantification with either UV or fluorescence detection.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV at 260 nm
Column:	AccQ•Tag Ultra C_{18}, 2.1 x 100 mm, 1.7 µm
Sample temp.:	20 °C
Column temp.:	43 °C
Injection vol.:	0.8 µL
Flow rate:	0.7 mL/min
Mobile phase A:	AccQ•Tag Eluent A
Mobile phase B:	90/10 Water/Eluent B
Mobile phase C:	Water
Mobile phase D:	AccQ•Tag Eluent B

Gradient:

Time	%A	%B	%C	%D	Curve
0.00	10.0	0.0	90.0	0.0	N/A
0.29	9.9	0.0	90.1	0.0	11
5.49	9.0	80.0	11.0	0.0	7
7.10	8.0	15.6	57.9	18.5	6
7.30	8.0	15.6	57.9	18.5	6
7.69	7.8	0.0	70.9	21.3	6
7.99	4.0	0.0	36.3	59.7	6
8.59	4.0	0.0	36.3	59.7	6
8.68	10.0	0.0	90.0	0.0	6
10.20	10.0	0.0	90.0	0.0	6

Data Management

Empower 3 Software, SR2

Standards, reagents, separation column, and turnkey methodologies within Empower Software projects, are sold as a system solution.

Sample preparation

To a leucine solution, different amounts of isoleucine were spiked to prepare isoleucine/leucine mixtures at 0.0, 0.05, 0.1, and 0.2%. A calibration standard and samples were prepared by transferring 70 µL Borate buffer and 10 µL of the standard/sample to a Waters total recovery vial, vortexing to mix. The derivatization reagent was dissolved in 1 mL of acetonitrile and then 20 µL of the solution was transferred to each vial. Each vial was capped, vortexed, and then heated to 55 °C for 10 minutes prior to analysis.

RESULTS AND DISCUSSION

A calibration standard of 17 amino acids was prepared. The standard consisted of a single point calibration curve with each standard at a concentration of 50 pmoles/µL (except cystine at 25 pmoles/µL). As can be seen from the figure below, isoleucine and leucine were resolved at around 7.8 minutes.

The 0.0% Ile/Leu (unspiked) was analyzed and the leucine sample was found to be free of interferences at the retention time of isoleucine. The spiked Ile/Leu samples at 0% (Black line) 0.05% (Blue line), 0.1% (Brown line), and 0.2% (Green line) were analyzed and the peaks were found to have a USP resolution of 2.0 for the 0.05%, 0.1%, and 0.2% levels.

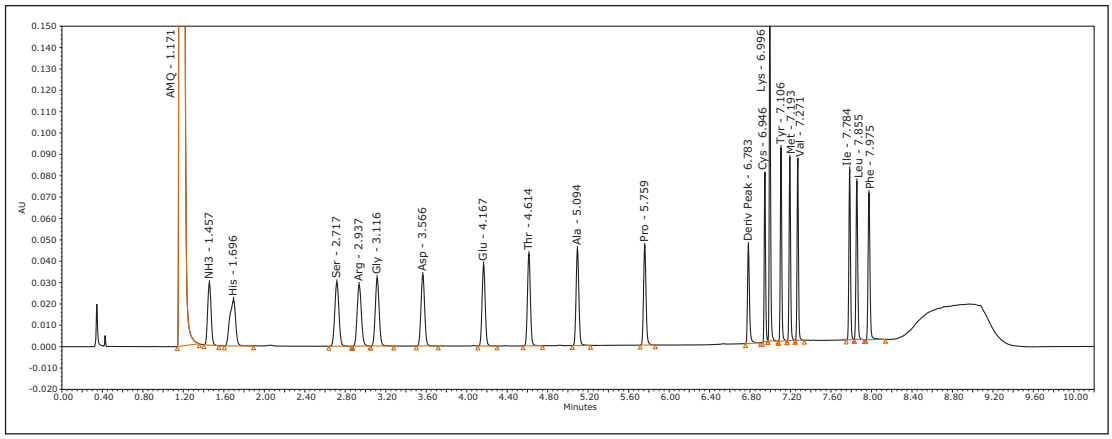

Figure 1. Standard chromatogram.

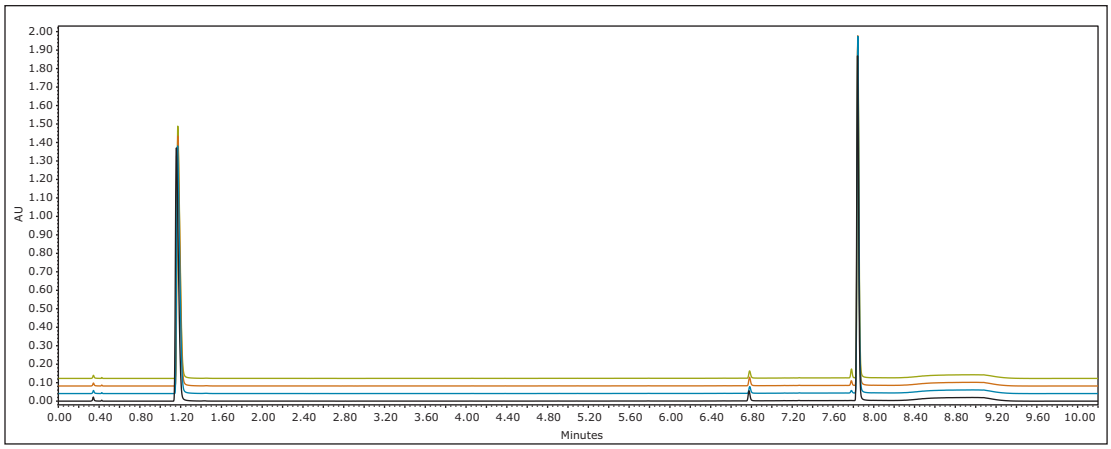

Figure 2. 0%, 0.05%, 0.1%, and 0.2% Ile/Leu chromatogram overlay.

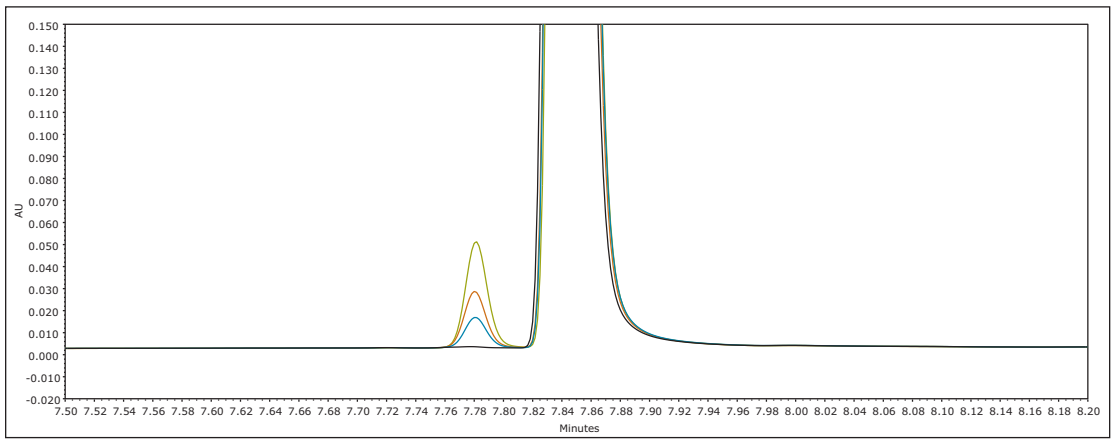

Figure 3. 0%, 0.05%, 0.1%, and 0.2% Ile/Leu chromatogram overlay (zoomed).

The Ile area was found to be linear when compared to the %Ile/Leu, with an R^2 of 0.9999.

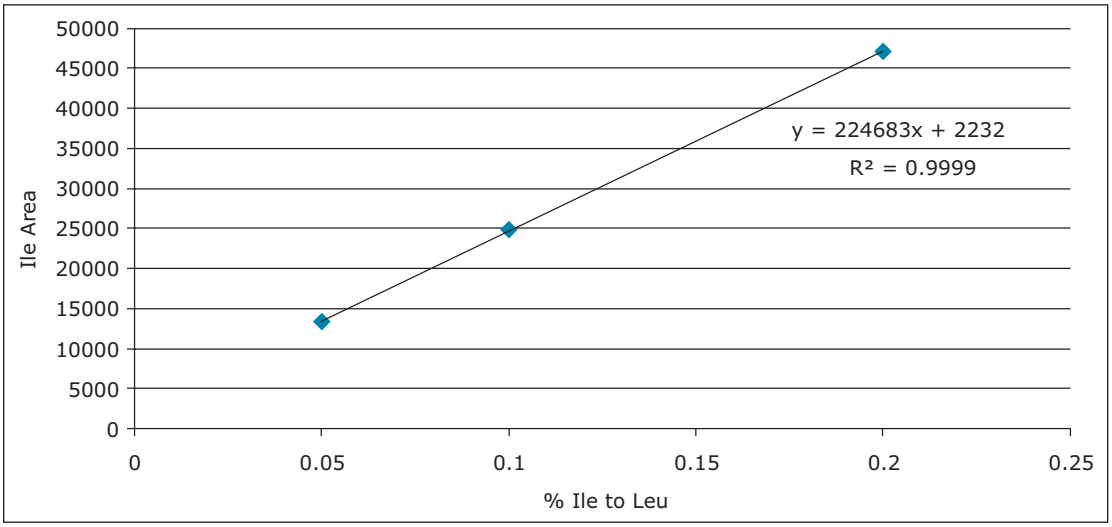

Figure 4. Linearity of Ile area vs. %Ile/Leu.

CONCLUSIONS

The Waters ACQUITY UPLC H-Class System provided chromatographic separation of all 17 amino acids in a commercially available amino acid mix within a very short run time. Baseline resolution of isoleucine and leucine was confirmed at levels as low as 0.05% Ile/Leu, meeting the regulatory requirements for these components.

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Amino Acid Analysis of Pure Protein Hydrolysate with Waters UPLC Amino Acid Analysis Solution

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CLASSIC UPLC AND ACID HYDROLYSIS

INTRODUCTION

Amino acid analysis is used in the protein structure laboratory to provide two kinds of information. First, the total quantity of amino acids is a direct measure of the amount of protein in a sample. Second, the measurement of the proportions of amino acids provides information to confirm the identity of the protein and to detect modifications. Both applications require robust, accurate, and sensitive measurements that both identify and quantitate the amino acids. There is increasing need for these labs to provide these correct results faster and more economically.

The Waters UPLC® Amino Acid Analysis Application is a turnkey solution to address these needs. This total system solution includes a well established and understood sample derivatization kit, eluents, chromatographic column, and a separation system based on the ACQUITY UPLC® using UV detection under Empower® Software control.

In this experiment, this system solution is used to measure the composition and concentration of a known protein. The accuracy of the determination is compared to the known correct results.

Figure 1. Waters UPLC Amino Acid Analysis Solution

EXPERIMENTAL

Sample

Acid-hydrolyzed bovine serum albumin (BSA) samples were prepared in an independent laboratory as part of a collaborative study. The samples were supplied at an estimated concentration of 1.0 mg/mL in 0.1 M HCl sealed under argon in sealed ampoules. Samples were stored at -80 °C until analysis.

Sample derivatization

The sample was diluted 1:10 with 0.1 M HCl prior to derivatization. The standard derivatization protocol was modified to include neutralization of excess acid with 0.1 M NaOH. Conditions for derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Application System Guide (P/N 71500129702).

- 10 µL of samples diluted 1:10 with 0.1 M HCl
- 10 µL 0.1 N NaOH
- 60 µL AccQ•Tag™ Ultra Borate Buffer
- 20 µL AccQ•Tag Ultra Reagent

LC conditions

LC system:	ACQUITY UPLC System with TUV detection at 260
Column:	AccQ•Tag Ultra 2.1 x 100 mm, 1.7 µm Part Number: 186003837
Column temp.:	55 °C
Flow rate:	700 µL/min
Mobile phase A:	1:20 Dilution of AccQ •Tag Ultra Eluent A concentrate Part Number: 186003838
Mobile phase B:	AccQ•Tag Ultra Eluent B Part Number: 186003839
Gradient:	AccQ•Tag Ultra Hydrolysate Method
Injection volume:	1 µL

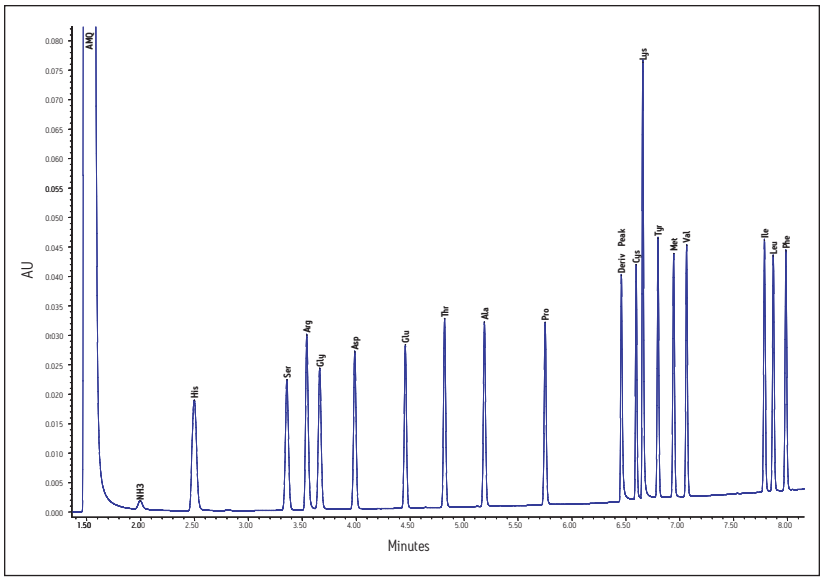

Figure 2. Analysis of Amino Acid Hydrolysate Standard, 10 pmoles each on column.

RESULTS

Figure 2 shows the chromatogram of a standard that contains amino acids typically found in protein hydrolysate samples. Each amino acid is 10 pmoles on column. For quantitative analyses, a three point calibration at 0.5, 10, and 25 pmoles on column was applied.

The analysis of a typical BSA hydrolysate is shown in Figure 3. The estimated starting concentration is consistent with this chromatogram representing a total of 9 ng of protein on column. This analysis was repeated a total of 75 times, over five separate days, with two columns and a total of five mobile phase preparations. The 75 injections represent five independent sample dilutions, each dilution derivatized five separate times. Each derivatized sample was injected in triplicate.

The amino acid composition, expressed as residues/mole of protein, was compared to the value expected from the known sequence. Table 1 shows the mean and standard deviation for each amino acid over all 75 analytical injections. Tryptophan and cysteine/cystine are excluded from the calculation because they are destroyed by acid hydrolysis. The measured molar composition agrees well with the expected values from the sequence. The reliability of the UPLC Amino Acid Analysis Application is confirmed by the reproducibility of results over the large number of determinations that intentionally includes the variability that could arise from multiple columns, eluents, and derivatizations.

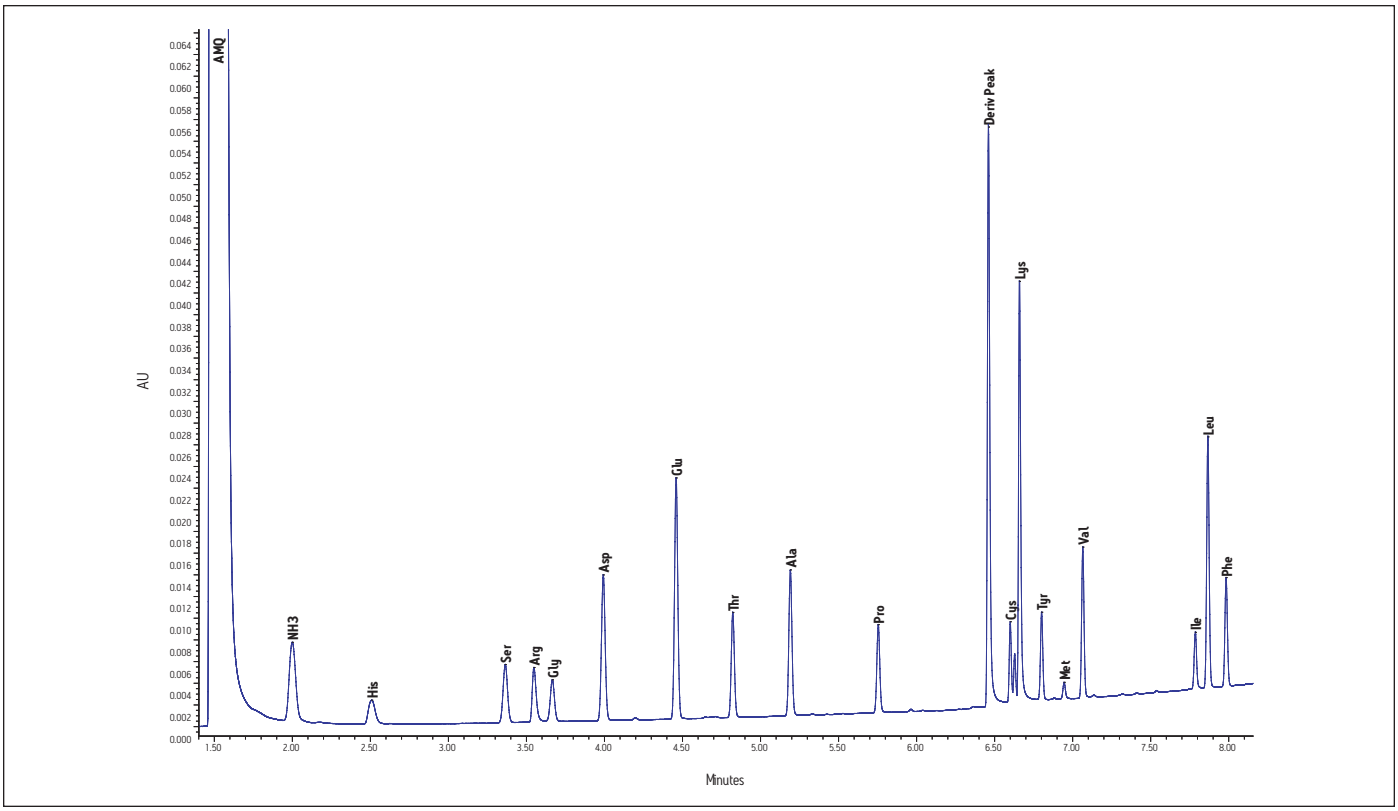

Figure 3. Analysis of BSA Hydrolysate Sample, approximately 9 ng on column.

Amino acid	Expected residues	*Observed residues
His	17	15.36 ± 0.19
Ser	28	26.00 ± 0.08
Arg	23	22.37 ± 0.08
Gly	16	17.68 ± 0.20
Asp	54	55.47 ± 0.21
Glu	79	80.68 ± 0.20
Thr	33	31.92 ± 0.06
Ala	47	47.51 ± 0.15
Pro	28	28.35 ± 0.14
Lys	59	57.78 ± 0.38
Tyr	20	20.19 ± 0.08
Met	4	4.16 ± 0.15
Val	36	35.67 ± 0.16
lle	14	13.15 ±0.15
Leu	61	63.13 ± 0.19
Phe	27	26.57 ± 0.13

Table 1. Comparison of Observed with Expected Composition Derived from Known Sequence of BSA.
*Average of 75 data points (25 derivatizations, each injected in triplicate; mean value ± standard deviation)

Sample I.D.	Total µg AA/ mL hydrolysate
BAS 1-1	699.64
BAS 1-2	694.09
BSA 1-3	697.88
BSA 1-4	698.21
BSA 1-5	695.43
BSA 2-1	716.66
BSA 2-2	717.87
BSA 2-3	715.65
BSA 2-4	709.75
BSA 2-5	707.93
BSA 3-1	714.93
BSA 3-2	711.50
BSA 3-3	708.46
BSA 3-4	708.14
BSA 3-5	708.10
BSA 4-1	674.43
BSA 4-2	678.43
BSA 4-3	683.07
BSA 4-4	678.62
BSA 4-5	678.94
BSA 5-1	591.40
BSA 5-2	604.30
BSA 5-3	571.78
BSA 5-4	571.57
BSA 5-5	599.18
Mean	677.43
Standard Deviation	47.98
% RSD	7.08

Table 2. Reproducibility of method for amount of BSA protein in sample.

The analytical data was used to calculate absolute amount of protein in the sample. The amount of each amino acid were expressed as the residue molecular weight. The sum of weights of the amino acids is equal to the weight of the protein. Table 2 summarizes the result of the 75 determinations with the mean for the triplicate injections shown for each derivatization. The measured amount corresponds to 0.7 mg/mL in the starting material. The estimated amount used to prepare the hydrolysate has not been independently verified. It should be noted in addition that this measurement of protein amount does not include the contribution of cysteine/cystine and tryptophan, as they are mostly destroyed by the hydrolysis of the protein.

The reproducibility of determination of the 75 analyses gives a RSD of 7%. Detailed examination shows that much of this variance is due to the difference of experiment 5 from the other four analyses. Since all of the replicates in experiment 5 are lower than the others, this variance is consistent with a difference in pipetting in the initial sample preparation. The addition of an internal standard to the sample to be hydrolyzed will improve the reliability of the final analytical result. Norvaline is the preferred internal standard for this purpose

CONCLUSION

Protein structure and biopharmaceutical laboratories rely on accurate quantitation of amino acids to confirm the identity and amount of protein in their samples. The analyses shown here demonstrate that Waters UPLC Amino Acid Analysis Application can provide assured results for these laboratories.

The molar ratios of amino acids are reproducible over multiple derivatizations and replicate injections. The measured composition agrees with that expected from the sequence of BSA.

Determination of the absolute amount of protein in the samples is determined by summing the residue weights of amino acids. The reproducibility of this measurement is on the order of ±7%. The largest contribution to this variance is the initial sample dilution. Incorporation of an internal standard in hydrolysis will improve the precision of the determination. Norvaline is recommended as an internal standard for the method.

The Waters UPLC Amino Acid Analysis Application provides a complete turnkey analytical method for analyzing protein hydrolysate samples. The ACQUITY UPLC System gives very high resolution for certain peak identification and ease of integration. With standard UV detection, all the derivatized amino acids have similar extinction coefficients to facilitate quantitative analysis. Sensitivity levels corresponding to nanograms of protein can be achieved routinely. The ruggedness of this turnkey system solution ensures rapid and unequivocal identification of proteins, with no interference or ambiguity. The pre-tested column, eluents and reagents ensure that the user will not spend time adjusting the method. The small amount of sample required for good analyses contributes to long column life and minimizes the chance of failure during a series of runs. The high resolution ensures reliable peak identification and quantitation so that runs need not be repeated. The pre-defined methods and reports simplify reporting of results. These analytical benefits are obtained with a short analysis time for the high throughput required for the routine determination of protein composition and concentration.

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AMINO ACID ANALYSIS OF PROTEIN HYDROLYSATES USING UPLC® AMINO ACID ANALYSIS SOLUTION

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Hillary B. Hewitson, Thomas E. Wheat, Diane M. Diehl
Waters Corporation, Milford, MA

CLASSIC UPLC AND ACID HYDROLYSIS

INTRODUCTION

Amino acid analysis is used in the protein structure laboratory to provide two kinds of information. The total quantity of amino acids is a direct measure of the amount of protein in a sample. Additionally, the measurement of the proportions of amino acids provides information to confirm the identity and to detect modifications. Both applications require robust, accurate, and sensitive measurements that identify and quantitate the amino acids.

An amino acid analysis system solution has been developed for these applications. It includes a defined combination of instrument, chemistry, and methods. The standard configuration for this solution includes a tunable UV detector, but additional detector options provide flexibility to meet the needs for other assays in the laboratory.

The quantitative properties of the method were evaluated with the tunable UV (TUV), photo diode array (PDA), and fluorescence (FLR) detectors. Sensitivity, linearity and reproducibility were compared. Factors influencing quantitative derivatization were systematically optimized. Acid hydrolysates of bovine serum albumin were used as the model protein to test the accuracy of the analysis for measurement of composition and total protein concentration.

METHODS

Chromatographic Conditions

Standard UPLC® Amino Acid Analysis Solution instrument and chemistry configurations were used for all experiments. No changes were made to mobile phases, column or derivatization chemistry, or operating conditions.

Sample Preparation and Evaluation

Linearity and Reproducibility—A 2.5µmol/mL Hydrolysate standard was serially diluted to give concentrations of 10, 50, 100, 250, and 500 pmol/µL. The amount injected corresponded to 1, 5, 10, 25, and 50 pmol on column. Six replicate 50 pmol injections were made to test reproducibility.

Protein Hydrolysate—Acid-hydrolyzed bovine serum albumin was prepared in an independent laboratory as part of a collaborative study and supplied at an estimated concentration of 1.0 mg/mL in 0.1 M HCl, sealed under argon in glass ampoules. Samples were diluted 1:11 with 0.1 M HCl.

- 10 µL diluted sample
- 10 µL 0.1 N NaOH
- 60 µL AccQ-Tag™ Ultra Borate Buffer
- 20 µL AccQ-Tag™ Ultra Reagent

The protein hydrolysate analysis was repeated over five separate days, with two columns and a total of five mobile phase preparations. Independent sample dilutions were prepared on five days. On each day, the sample was derivatized five separate times. Each derivatized sample was then injected in triplicate. These 75 analyses are described below.

Detector Options

Figure 1A 50 pmole Standard with TUV Detection

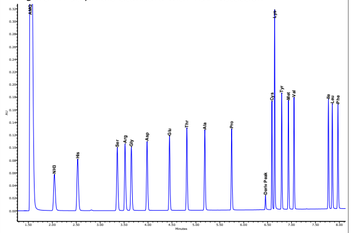


Figure 1B 50 pmole Standard with PDA Detection

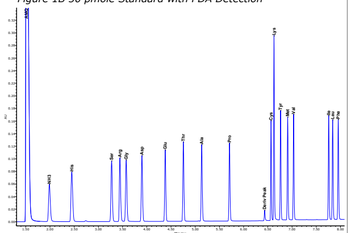


Figure 1C 50 pmole Standard with FLR Detection

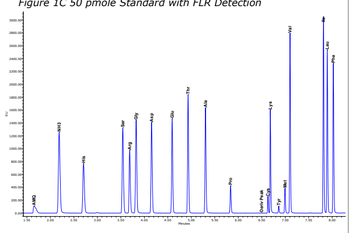
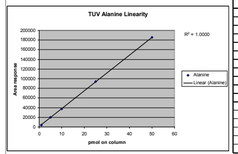


Figure 1. Chromatographic comparison of detector options with 50 pmole Hydrolysate Standard. Figure 1A (TUV) and 1B (PDA) show similar chromatographic profiles, while Figure 1C (FLR) is characterized by different peak sizes because of differences in fluorescence yields among the amino acids.

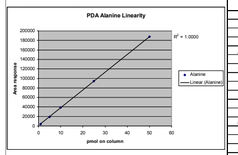
Quantitative Properties

Figure 2A Quantitation with TUV



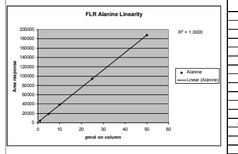
TUV	50 pmol RT Std Dev (min)	50 pmol Area % RSD	Correlation Coefficient 1 - 50 pmol
His	0.013	0.16	1.000
Ser	0.006	0.17	0.999
Arg	0.004	0.19	1.000
Gly	0.004	0.19	1.000
Asp	0.003	0.19	1.000
Glu	0.002	0.18	1.000
Thr	0.001	0.21	1.000
Ala	0.001	0.17	1.000
Pro	0.001	0.16	1.000
Cys	0.001	0.19	1.000
Lys	0.001	0.25	1.000
Tyr	0.001	0.23	1.000
Met	0.001	0.21	1.000
Val	0.001	0.21	1.000
Ile	0.001	0.22	1.000
Leu	0.001	0.19	1.000
Phe	0.001	0.25	1.000

Figure 2B Quantitation with PDA



PDA	50 pmol RT Std Dev (min)	50 pmol Area % RSD	Correlation Coefficient 1 - 50 pmol
His	0.023	0.31	1.000
Ser	0.010	0.26	1.000
Arg	0.008	0.27	1.000
Gly	0.007	0.32	1.000
Asp	0.005	0.33	1.000
Glu	0.003	0.32	1.000
Thr	0.002	0.26	1.000
Ala	0.001	0.24	1.000
Pro	0.001	0.27	1.000
Cys	0.001	0.24	1.000
Lys	0.001	0.13	1.000
Tyr	0.001	0.25	1.000
Met	0.001	0.25	1.000
Val	0.001	0.25	1.000
Ile	0.001	0.22	1.000
Leu	0.001	0.20	1.000
Phe	0.001	0.15	1.000

Figure 2C Quantitation with FLR



FLR	50 pmol RT Std Dev (min)	50 pmol Area % RSD	Correlation Coefficient 1 - 50 pmol
His	0.013	0.33	1.000
Ser	0.008	0.33	1.000
Arg	0.006	0.32	1.000
Gly	0.006	0.35	1.000
Asp	0.005	0.34	0.999
Glu	0.004	0.34	0.999
Thr	0.003	0.38	1.000
Ala	0.003	0.27	1.000
Pro	0.002	0.37	1.000
Cys	0.001	0.33	0.999
Lys	0.001	0.34	0.998
Tyr	0.001	0.37	0.999
Met	0.001	0.38	0.999
Val	0.001	0.36	0.999
Ile	0.001	0.40	0.999
Leu	0.001	0.33	0.999
Phe	0.001	0.35	0.999

Figure 2. Reproducibility and linearity data for TUV (2A), PDA (2B), and FLR (2C) detector options, with Alanine plotted for each. Each plot is accompanied by a table for all amino acids that show the standard deviation of retention time and % RSD of area for replicate injections of 50 pmoles.

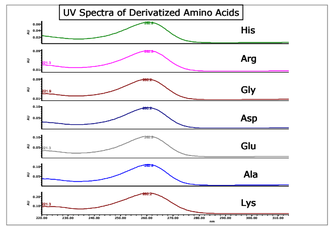


Figure 3. UV spectra for selected amino acid peaks at spectral resolution setting of 1.2 nm width on PDA detector. All the amino acids have the same absorbance maximum at 260 nm and same spectral shape. The PDA does not provide peak identification or peak purity assessment in this application.

Controlling Derivatization

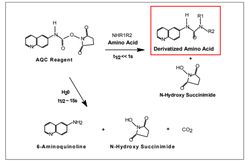


Figure 4. Reaction Mechanism for the derivatization of amino acids and subsequent hydrolysis of excess AQC Reagent

Requirements for optimal derivatization

- Optimal reaction is between pH 8 and pH 10
  - Standard derivatization cocktail includes 70 µL of borate buffer at pH 8 to neutralize sample acidity
  - If sample is equivalent to 0.1 M HCl, no neutralization is required
  - For higher concentrations of acid, replace part of the borate buffer volume with a volume of NaOH equal to the sample volume. The concentration of NaOH should match the sample acid concentration
- Ensure a 5x reagent excess
  - Derivatization cocktail contains ~250 nmoles of reagent
  - A standard derivatization cocktail is used for 1 µL injection from 100 µL total volume
  - Sample should not exceed 50 nmoles of amino acids
  - An injection could contain at most 500 pmoles divided among the many peaks
  - Useful guidelines
    - 1 pmole on column or 100 pmoles total of least abundant amino acid
    - 1—3 µg total protein in derivatization cocktail

Experimental approach to unknown samples

- Test two level of neutralization with sodium hydroxide
- Test two concentration levels
- Evaluate
  - Total peak area
  - Ratio of Ala/Phe
  - The amount of mono-derivatized Lys can also indicate the level of excess reagent in sample

Table 1. Evaluating quality of derivatization of BSA Hydrolysate. Asp, Glu, Ala, and Lys are most sensitive to incomplete derivatization, while Phe is least affected. The areas shown are corrected for dilution. Peak areas are not significantly larger with additional neutralization. Dilution of the sample to increase reagent excess does not increase yield. The Ala/Phe ratio is constant across all conditions. These observations confirm complete derivatization of the sample.

Amino Acid	22x Dilution	22x Dilution w/1x Neutralization	22x Dilution w/2x Neutralization	10x Dilution	50x Dilution
Asp	285890	275352	285142	279360	292750
Glu	419716	403876	419364	408600	436900
Ala	244024	234278	241010	237960	249950
Lys	469590	440264	449416	459790	471400
Phe	148126	141438	147246	143930	153250
Ala/Phe ratio	1.647	1.656	1.637	1.653	1.631

Sample Analysis

Figure 5A Analysis of Hydrolyzed BSA with TUV Detection

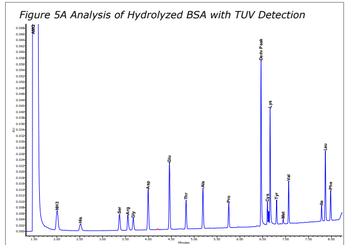


Figure 5B Analysis of Hydrolyzed BSA with FLR Detection

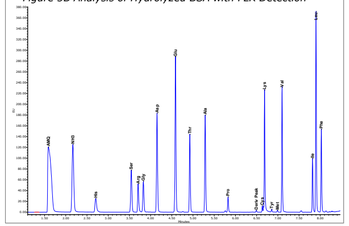


Figure 5. Analysis of Bovine Serum Albumin (BSA) Hydrolysate with TUV (5A) and FLR (5B) detection, approximately 9 ng on column. While the responses vary for the amino acids between the detectors, quantitation is consistent, as shown in Tables 2 and 3.

Table 2. Comparison of calculated and expected composition derived from known sequence of BSA for both UV and fluorescence detection. Results are the mean ± standard deviation for 75 replicate determinations.

Amino Acid	Expected Residues	*Observed Residues TUV	FLR
His	17	15.36 ± 0.19	15.73 ± 0.16
Ser	28	26.00 ± 0.08	25.90 ± 0.41
Arg	23	22.37 ± 0.08	22.39 ± 0.20
Gly	16	17.68 ± 0.20	16.65 ± 0.42
Asp	54	55.47 ± 0.21	55.18 ± 0.32
Glu	79	80.68 ± 0.20	80.27 ± 0.44
Thr	33	31.92 ± 0.06	32.01 ± 0.07
Ala	47	47.51 ± 0.15	47.40 ± 0.16
Pro	28	28.35 ± 0.14	28.92 ± 0.13
Lys	59	57.78 ± 0.38	57.83 ± 0.99
Tyr	20	20.19 ± 0.08	20.67 ± 0.34
Met	4	4.16 ± 0.15	4.04 ± 0.05
Val	36	35.67 ± 0.13	35.38 ± 0.13
Ile	14	13.15 ± 0.16	13.44 ± 0.16
Leu	61	63.13 ± 0.28	63.18 ± 0.28
Phe	27	26.57 ± 0.13	27.00 ± 0.33

*** Average of 75 data points (25 derivatizations, each injected in triplicate)**

Table 3. Determination of the protein concentration of a sample. The amino acid amounts are expressed as the residue molecular weights, and the sum of these amounts is equal to the weight of the protein. The BSA hydrolysate was derivatized five times on each of five separate days, and each derivatization injected in triplicate. The values reported in Table 3 are the means of the triplicate injections. The apparent variability in the concentration measurement is approximately 7%. Detailed examination shows that much of the variance of the data can be attributed to pipetting errors. The TUV replicates from Day 5 are much lower for all derivatization preparations as compared to the other four days. This is consistent with a volumetric error in the initial dilution on that day. With the FLR detector, the first derivatization on Day 5 is much higher than any other derivatization on any day. This is consistent with a pipetting error in that derivatization cocktail. Addition of an internal standard to the sample to be hydrolyzed will improve the reliability of the final analytical result.

Sample ID	Total µg AA/mL Hydrolysate TUV	FLR
BSA 1-1	699.64	664.27
BSA 1-2	694.09	656.91
BSA 1-3	697.88	665.76
BSA 1-4	698.21	655.55
BSA 1-5	695.43	655.40
BSA 2-1	716.66	747.45
BSA 2-2	717.87	741.41
BSA 2-3	715.65	746.38
BSA 2-4	709.75	748.39
BSA 2-5	707.93	733.88
BSA 3-1	714.93	721.30
BSA 3-2	711.50	721.43
BSA 3-3	708.46	710.33
BSA 3-4	708.14	725.01
BSA 3-5	708.10	681.68
BSA 4-1	674.16	730.93
BSA 4-2	678.43	729.37
BSA 4-3	683.07	721.29
BSA 4-4	678.62	732.51
BSA 4-5	678.94	740.78
BSA 5-1	591.40	927.97
BSA 5-2	604.30	743.11
BSA 5-3	571.78	740.98
BSA 5-4	571.57	742.96
BSA 5-5	599.18	726.54
Mean	677.43	724.46
Std Dev	47.98	53.21
% RSD	7.08	7.34

CONCLUSION AND SUMMARY

- **The UPLC® Amino Acid Analysis Solution can be used to accurately measure amino acid composition and to derive the protein concentration of a sample.**
- **The system can include different detectors and the same final result is obtained with all detectors.**
- **Optimal sample derivatization is critical for accurate quantitation of amino acids.**
- **Consistent results over a large number of replicates confirms robustness of the analytical solution.**

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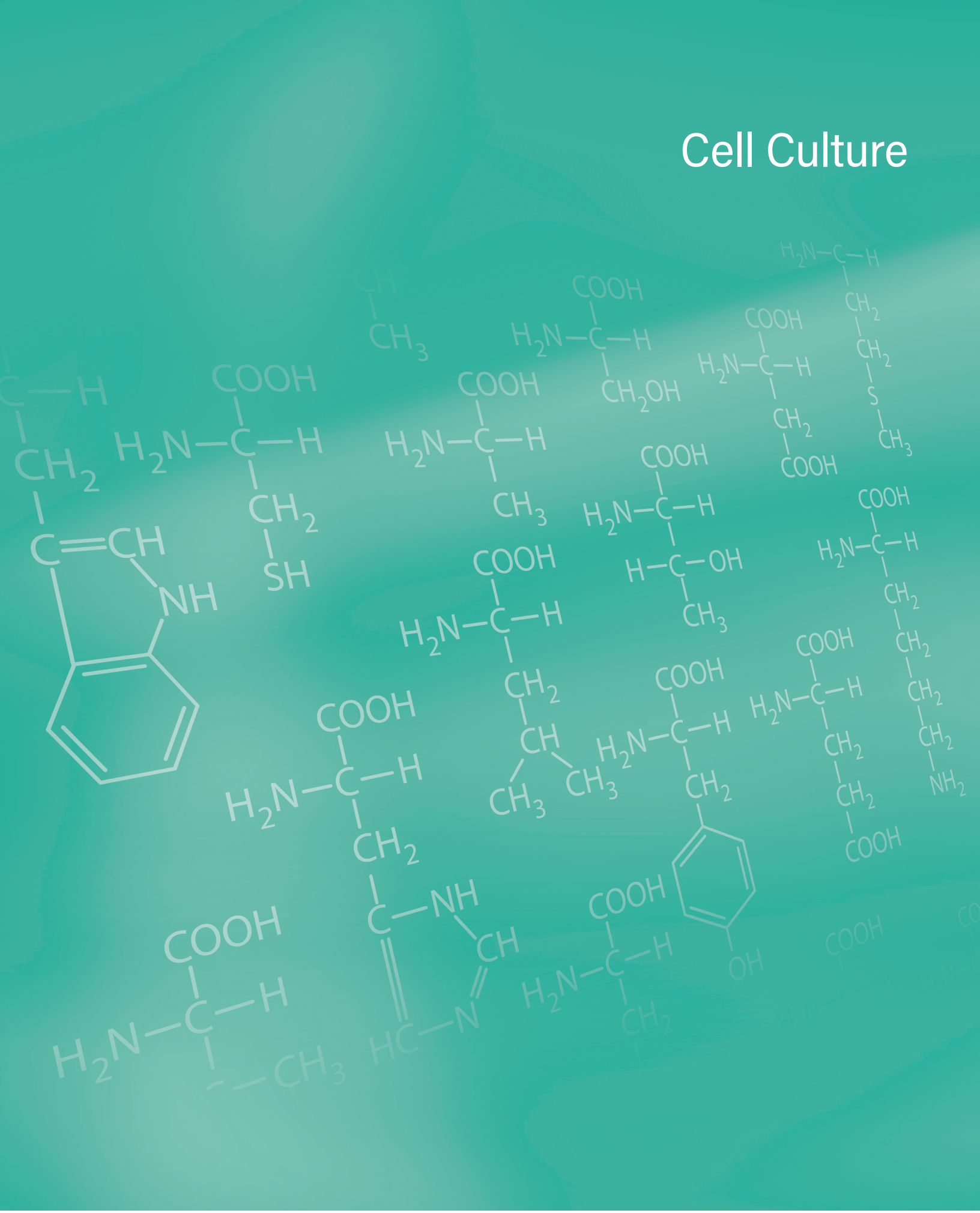
Amino Acid Analysis oif Protein Hydrolysates Using UPLC® Amino Acid Analysis Solution

Cell Culture



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Monitoring Cell Culture Media with the Waters Amino Acid Analysis Solution

CLASSIC UPLC

Paula Hong, Thomas E. Wheat, Jeffrey R. Mazzeo, and Diane M. Diehl
Waters Corporation, Milford, MA, USA

INTRODUCTION

Cell culture techniques are routinely used to produce proteins intended for use as biopharmaceuticals. The culture conditions must be optimized to ensure that the protein is produced without structural modification and in the highest possible yield. These preferred conditions will often be different for each clone investigated, so a large number of optimization experiments may be required. This assessment of growth conditions must also consider the changes in the media that occur as a consequence of cell growth, that is, the consumption of nutrients and the release of waste products. The monitoring and optimization are complex because of the large number of physical and chemical parameters that have an effect. The experiments described here are focused on one particular class of components, the free amino acids.

Amino acids are important as the constituents of proteins, but they also serve as intermediates in many metabolic pathways. They are provided as individual amino acids in the growth media to satisfy both types of nutritional requirements. The concentration of amino acids in the media changes both from consumption of some amino acids and release of others by the growing cells. Monitoring these dynamic conditions is part of the optimization process, and the observed changes in concentration can be used to schedule a "feeding" of the culture or replacement of the medium. The Waters UPLC® Amino Acid Analysis Solution (Figure 1) provides a suitable way to monitor these changing nutrient levels.

The Waters UPLC Amino Acid Analysis Solution is a turnkey offering that encompasses instrumentation, derivatization chemistry, separation chemistry, software, and support. The solution includes defined conditions suitable for the assay of the amino acids commonly found in mammalian cell culture media. We show here the use of this defined method in monitoring a growing culture.

Figure 1. Waters UPLC Amino Acid Analysis Solution.

EXPERIMENTAL

Conditions for derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Solution System Guide.

Samples of serum-free cell culture medium were obtained at daily time intervals from a bioreactor that was actively producing a biopharmaceutical protein. The medium was diluted 1:4 with 0.1 M HCl. A 10 µL aliquot of the dilution, with no additional sample preparation, was derivatized using the standard AccQ•Tag™ Ultra protocol.

LC conditions

LC System:	WATERS ACQUITY UPLC® System with TUV detection at 260nm
Column:	AccQ•Tag Ultra, 2.1 x 100 mm, 1.7 µm PN: 186003837
Column temp:	60 °C
Flow rate:	700 µL/min
Mobile phase A:	1:10 Dilution of AccQ•Tag Ultra A concentrate
Mobile phase B:	AccQ•Tag Ultra B PN: 186003838
Gradient:	AccQ•Tag Ultra Cell Culture Method PN: 186003839
Injection volume:	1 µL

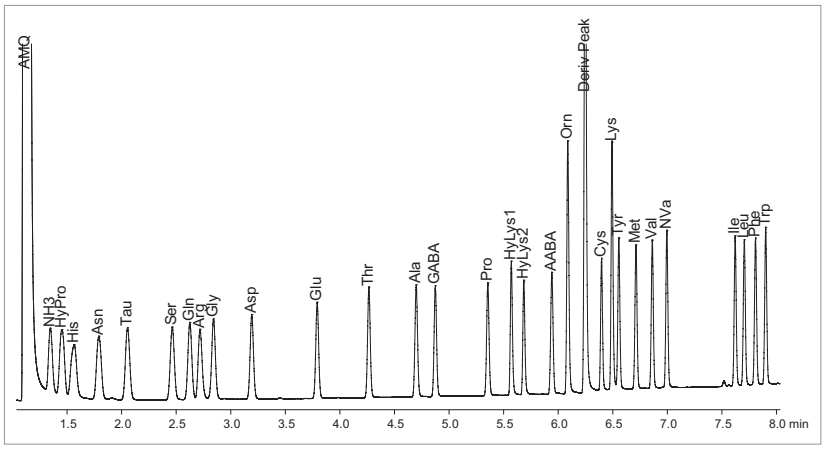

Figure 2. Analysis of standards of amino acids commonly found in cell culture media.

RESULTS

An analysis of amino acid standards representing the compounds commonly found in cell culture media is shown in Figure 2. This separation is obtained using the mobile phases and separation conditions that are part of the standard UPLC Amino Acid Analysis Solution. No adjustment of mobile phase pH or changes in composition are required. The resolution and reproducibility are sufficient for unambiguous peak identification and for reliable quantitation.

This method was applied to samples taken from an active bioreactor at daily intervals. These results are overlaid in Figure 3, and a second overlay in Figure 4 magnifies the region of the chromatogram which includes the amino acids that change most significantly during this growth experiment. The chromatographic characteristics observed with the standards are preserved with the authentic samples. The significant amino acids are readily identified and are sufficiently well-resolved for quantitation. There are a few small unidentified peaks that do not interfere with the amino acids.

The comparison of the 1, 3, and 6 day samples clearly shows the decline in concentration for some amino acids, notably glutamine, and the increase in others, such as alanine. These changes can be expressed quantitatively as plotted in Figure 5. All the amino acids can be quantitated, but only a few are shown as examples, including glutamine which increases in concentration with feeding.

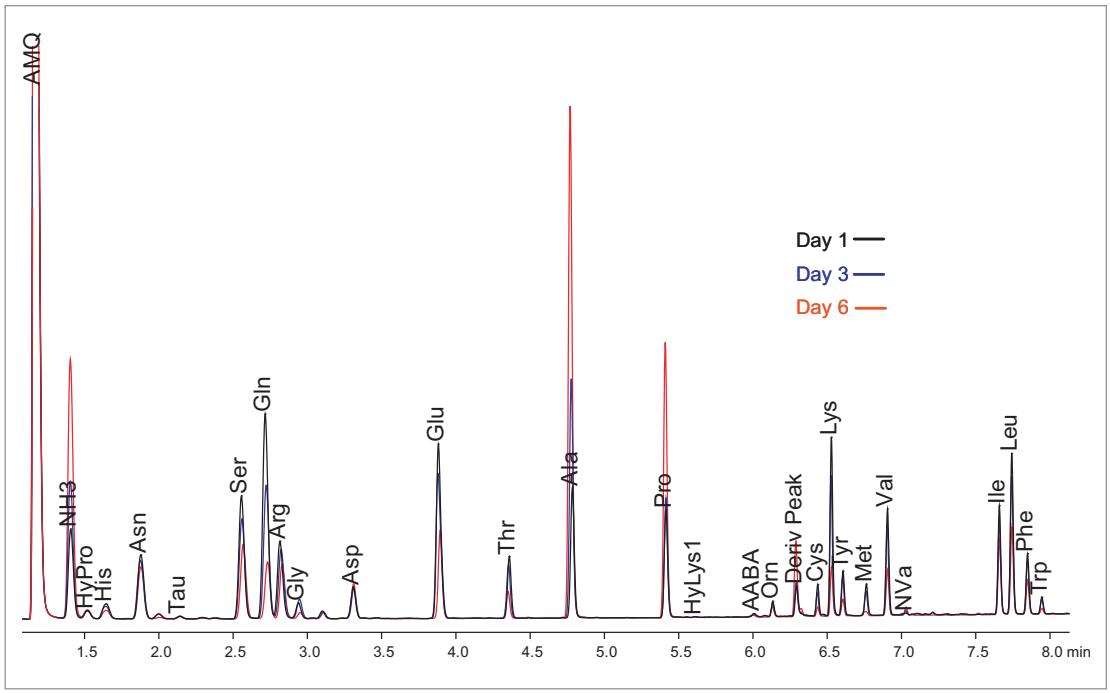

Figure 3. Analysis of amino acids in cell culture media after 1, 3, and 6 days of culture.

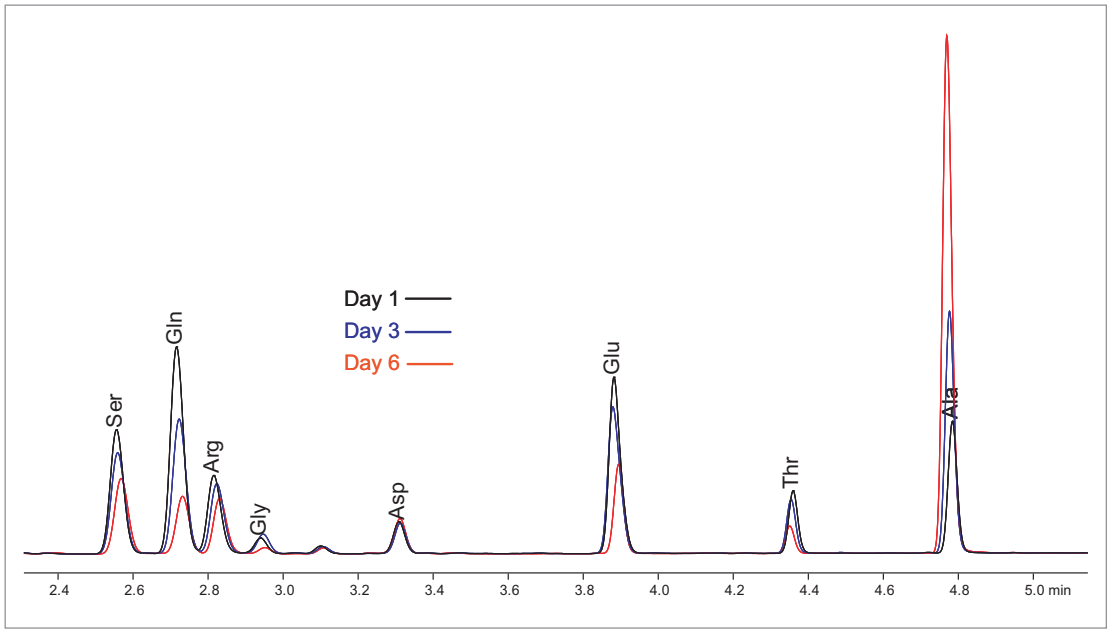

Figure 4. Analysis of critical amino acids in cell culture media after 1, 3, and 6 days of culture.

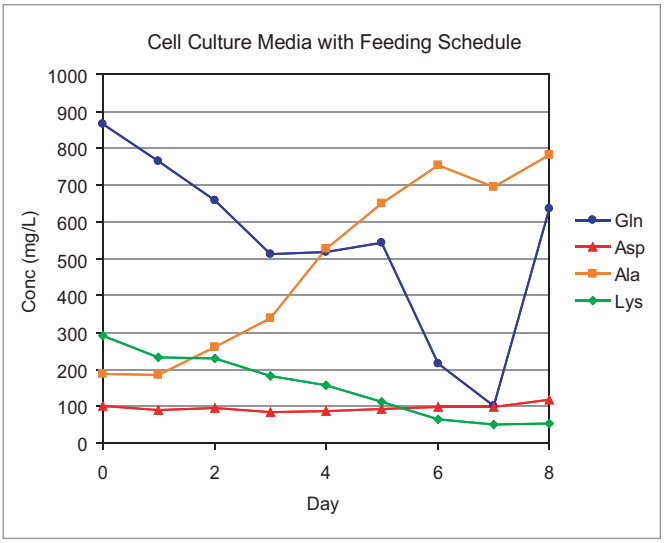

Figure 5. Quantitative trends in amino acid concentration during cell culture.

CONCLUSION

The Waters UPLC Amino Acid Analysis Solution has been used for the analysis of mammalian cell culture media. The standard method provides the chromatographic resolution required for peak identification. No sample preparation beyond simple dilution is required.

The analysis proves rugged and reproducible over a series of samples. No interferences are observed. The quantitative analysis is suitable for monitoring changes in concentration over time and for recognizing the proper time for a scheduled feeding. These analytical results are obtained with a short run time compatible with the high throughput requirements for optimizing growth conditions.

The Waters UPLC Amino Acid Analysis Solution provides a complete turnkey analytical method for monitoring amino acids in mammalian cell culture media. The pre-tested column, eluents and reagents ensure that the user will not spend time adjusting the method. The small amount of sample required for good analyses contributes to long column life and minimizes the chance of failure during a series of runs. The high resolution ensures reliable peak identification and quantitation so that runs need not be repeated. These analytical benefits are obtained with a short analysis time for the high throughput required for the optimization of cell culture conditions.

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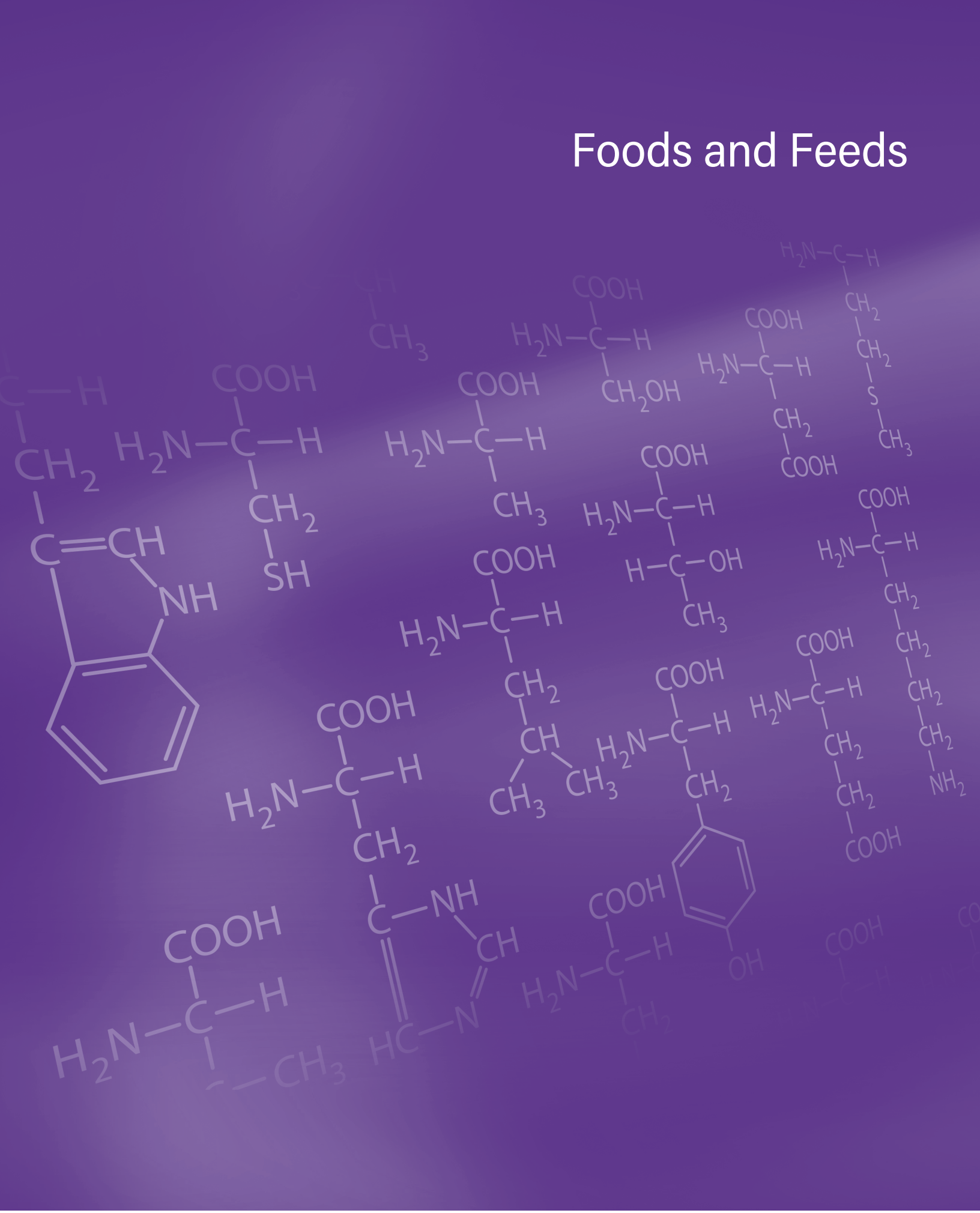

IMPROVING FOOD PROCESS CONTROL USING THE UPLC AMINO ACID ANALYSIS SOLUTION

Hillary B. Hewitson, Thomas E. Wheat, Diane M. Diehl

CLASSIC UPLC

INTRODUCTION

Food products have different desirability and value based in part on palatability and flavor profile. Controlling the manufacture of these food products to consistently meet the expectations and requirements of the consumer can be a difficult challenge. The materials and process that are combined to achieve that finished product can introduce many variables that can ultimately alter the product characteristics.

To ensure product quality, consistency in starting materials is required. Food constituents can have different characteristics based on origin and seasonal variation. The natural products may also have been processed by another manufacturer. All of these factors become further complicated when applying them to a complex process, such as fermentation.

It is, therefore, necessary to verify the quality of the starting materials. Chemical changes in those materials during processing can be monitored to ensure maximum yield of good product. The analysis of the final product assures that consumers always receive quality product. One useful class of chemical markers is amino acids.

Free amino acids can exist as nutrients, metabolic intermediates, or as waste products to a biological process and can be used to identify the genotype and origin of a plant. In a similar fashion, amino acids can be used to relate a profile to valuable characteristics in high value foods. By monitoring the free amino acids during and after a process, it is possible to identify and control physiology that affects the product quality.

Waters turnkey application, UPLC Amino Acid Analysis Solution, has been used to monitor free amino acids in raw materials, processes, and final product quality of food products. As an example of this application, we have followed a yeast fermentation in the production of a beer. Characterization of raw materials is demonstrated with the free amino analysis of three different starting fermentation barley malts. Each of these malt varieties were then carried through a fermentation process, and the changes in free amino acid levels were observed at different stages of the fermentation. Finally, free amino acid analysis was performed on multiple lots of two commercially-produced pale ales to differentiate the brands and determine the manufacturing lot consistency.

Figure 1: Waters UPLC® Amino Acid Analysis Solution

EXPERIMENTAL

Sample Handling

Starting Barley Malts

- 2-row Pale Malt; 6-row Pale Malt; 2-row Pilsener Malt
- Samples were collected from early suspension of malts and stored at -80 °C prior to analysis.
- Each thawed sample was centrifuged for one minute at 16110 RCF x g, then the supernatant was diluted 1:10 with 0.1 M HCl.
- Derivatization volumes: 70 µL Borate buffer, 10 µL diluted sample, 20 µL AccQ•Tag™ Ultra reagent

Brewing Fermentation

- Samples: Beginning, 24 hour, day 4, and end of primary fermentation
- Samples were drawn from fermentor at intervals during primary fermentation and stored at -80 °C prior to analysis.
- Each thawed sample was centrifuged for one minute at 16110 RCF x g, then the supernatant was diluted 1:10 with 0.1 M HCl.
- Derivatization volumes: 70 µL Borate buffer, 10 µL diluted sample, 20 µL AccQ•Tag Ultra reagent

Commercial Pale Ales

- Two lots each of two brands of pale ale
- Samples were collected from 12 oz. bottles, and stored at -80 °C prior to analysis.
- Each thawed sample was centrifuged for one minute at 16110 RCF x g, then the supernatant was diluted 1:10 with 0.1 M HCl.
- Derivatization volumes: 50 µL Borate buffer, 10 µL diluted sample, 20 µL 0.1 M NaOH (to neutralize excess acid), 20 µL AccQ•Tag Ultra reagent

Sample Derivatization

The derivatization reagent reacts with both primary and secondary amines at an optimal pH of 8.5. The batch-derivatized samples are stable at room temperature for up to one week when tightly capped. Conditions for pre-column derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Solution System Guide (P/N 71500129702).

ACQUISITION AND PROCESSING METHODS

The Waters UPLC Amino Acid Analysis Application Solution is provided with all the Empower methods necessary for acquisition and processing of the samples, as well as reporting of the results. Details of the methods can be found in the Waters UPLC Amino Acid Analysis Solution System Guide.

Chromatographic Conditions

LC System:	Waters ACQUITY UPLC® System
Column:	AccQ•Tag Ultra, 2.1 x 100 mm, 1.7 µm
Column Temp:	60 °C
Sample Temp:	20 °C
Flow Rate:	700 µL/min.
Mobile Phase A:	1:10 Dilution of AccQ•Tag Ultra Eluent A with Milli-Q® water
Mobile Phase B:	AccQ•Tag Ultra Eluent B
Weak Needle Wash:	95:5 Water:Acetonitirile
Strong Needle Wash:	5:95 Water:Acetonitrile
Gradient:	AccQ•Tag Ultra Cell Culture Method (provided in the UPLC Amino Acid Analysis Solution)
Total Run Time:	9.5 min
Injection Volume:	1 µL, Partial loop with needle overfill
Detection:	UV (TUV), 260 nm

RESULTS AND DISCUSSION

Three batches of the same recipe of beer were produced, each using different starting malts (barley grain). These malts were chosen to represent some of the varietal, regional, seasonal, and malting process differences. This selection reflects the genetic varieties (2-row Pale Malt and 6-row Pale Malt) and malting processes (2-row Pale Malt and 2-row Pilsener Malt). The segment of the amino acid analysis chromatogram of Figure 2 shows the differences in free amino acid levels for the various starting malts. Some amino acids are constant across the different grain varieties, but asparagine (Asn) is higher for the 2-row Pilsener malt. Glutamine (Gln) levels are different for each of the starting malts.

These same three batches used to compare the starting malts were monitored throughout the entire process. Figure 3 shows time-dependent changes throughout primary fermentation. Some amino acids, such as valine (Val), were slowly depleted throughout fermentation. Others, like lysine (Lys), decreased dramatically in the first day of fermentation, and then maintained a constant level. Ornithine (Orn), on the other hand, increased during the first four days of fermentation, then dropped once the yeast activity ceased.

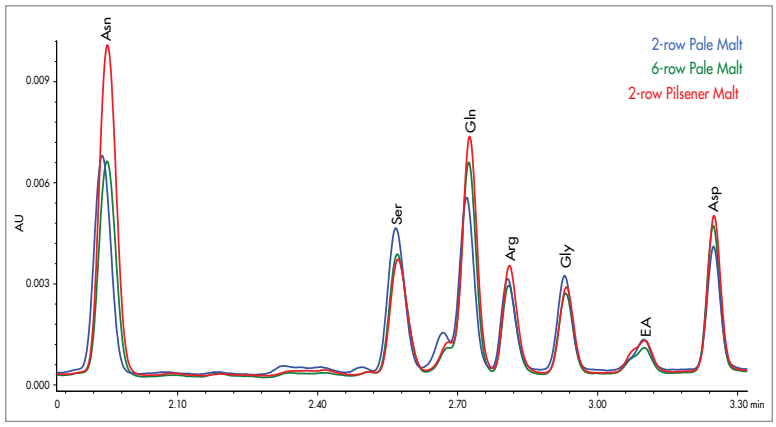

Figure 2: Amino acid differences between the different starting barley malts.

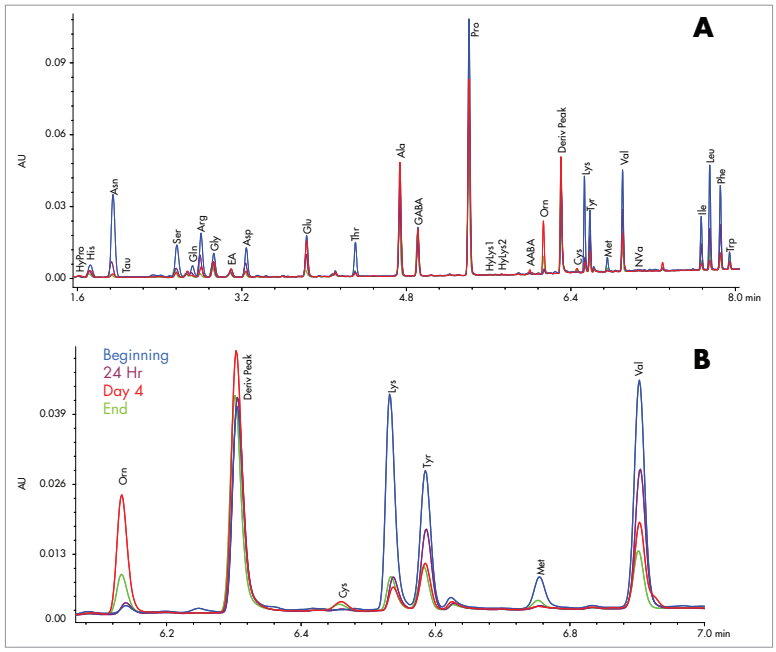

Figure 3: (A) Amino acid levels at various stages of a primary beer fermentation process, using the UPLC Amino Acid Analysis Solution. (B) Magnified region showing time dependent changes throughout the fermentation process.

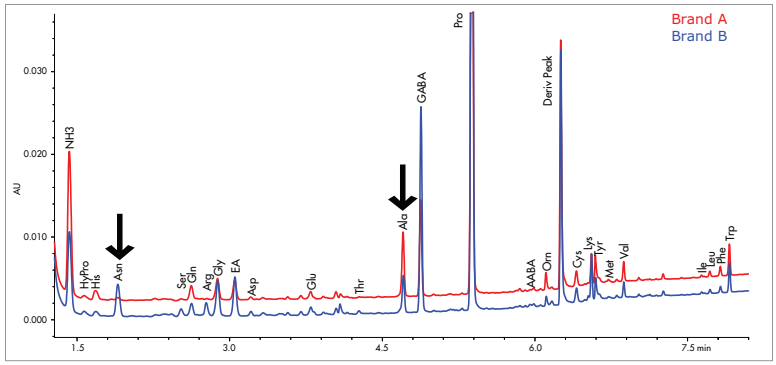

Figure 4: Different free amino acid profile of two commercially-produced pale ales. Arrows indicate significant differences between brands.

Table 1: Lot-to-lot variability for one brand of pale ale. The values (expressed as pmoles/µL sample) include triplicate injections of two derivatizations.

	Lot 1		Lot 2	
Amino Acid	Mean	Std Dev	Mean	Std Dev
His	81.25	0.85	91.40	1.35
Asn	18.83	0.69	19.18	0.46
Ser	12.45	0.91	11.55	0.62
Arg	5.94	0.46	4.49	0.26
Gly	156.58	3.02	169.21	1.43
EA	146.13	2.40	156.45	4.50
Asp	16.42	1.27	14.11	0.22
Glu	49.06	1.47	49.22	0.40
Thr	5.58	1.61	3.54	0.12
Ala	357.08	6.09	342.39	1.89
GABA	475.44	8.68	487.51	2.22
Pro	3479.25	42.23	4091.81	14.83
Orn	36.70	1.26	45.19	0.34
Cys	41.75	0.52	46.05	0.39
Tyr	100.27	1.99	99.40	0.39
Met	13.12	1.64	12.91	2.13
Val	101.21	1.73	73.73	0.76
Ile	12.87	0.39	9.49	0.34
Leu	26.03	0.41	19.58	0.31
Phe	41.36	0.55	34.08	0.29
Trp	104.12	1.88	107.50	1.17

To demonstrate the analysis of the method for finished products, two commercially-produced pale ales were analyzed for their free amino acid content. The chromatogram in Figure 4 shows the differences between the two brands. Brand B has higher amounts of free amino acids than Brand A. In particular, there is a significant amount of asparagine in Brand B, while it is virtually absent from the Brand A. Alanine, on the other hand, is higher in Brand A.

Multiple lots were tested for each brand of the pale ales. Table 1 shows the average amounts for two separate lots of Brand B of pale ale. The small standard deviations for the six determinations allows for recognition of differences between the lots. While the asparagine and glutamic acid levels are the same in each lot, within the variability of the data set, significant differences can be observed for proline, valine, and alanine. These differences could indicate a change to the materials or process.

CONCLUSION

The full characterization of food materials, processes, and products is essential to assuring consistent product quality. Monitoring the free amino acid profile can be a useful tool in assessing the stages of production as well as a final product.

Waters UPLC Amino Acid Analysis Solution was successfully applied to all stages of the process. Neither modifications to the method nor any special sample preparation was required.

Differences observed in the levels of amino acids for the starting malts show how raw materials can be verified for consistency prior to use in a manufacturing process.

Quantitative changes during the fermentation process were observed. Both increasing and decreasing concentrations of amino acids were readily measured.

Analysis of finished product showed differences both between brands as well as between lots of a single brand. These reliable measurements of chemical markers can reflect palatability or consumer acceptance of the product.

Waters UPLC Amino Acid Analysis Solution is a total system solution that is well suited to provide meaningful results for these applications. The small amounts of sample required make additional sample handling unnecessary in many cases. The rapid and reproducible results provide confidence to the user in making quality decisions about the material and processes that go into making a food product.

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APPLICATION OF THE UPLC AMINO ACID ANALYSIS SOLUTION TO THE ANALYSIS OF ANIMAL FEED HYDROLYSATES

Hillary B. Hewitson, Thomas E. Wheat, Diane M. Diehl

CLASSIC UPLC AND ACID HYDROLYSIS

INTRODUCTION

There are many factors that contribute to the health and growth of agricultural animals. Monitoring those factors is an important part to optimizing that growth, and therefore increasing profitability. Determination of the total amount of amino acids in the feeds provides critical information about the nutritional content. Based on this information, feeds can then be blended in an economical way while still assuring that the nutritional requirements of the animals are met.

The analysis of amino acids in animal feed samples is difficult due to the complex nature of the samples. The presence of a high concentration of non-protein constituents, including minerals, can interfere with the derivatization of amino acids and their subsequent analyses. These interferences can result in misidentification of the amino acids due to changes in retention over a series of analyses. Derivatization and chromatographic errors stemming from interferences will also result in incorrect quantitation of the amino acids. Unreliable amino acid analysis can lead to the costly and time consuming need to reanalyze samples. In the worst case, production may become uneconomical, and the animals may even become endangered.

Waters has developed the UPLC® Amino Acid Analysis Solution as a turnkey solution for the analysis of amino acids in a variety of applications. By combining the well-established pre-column derivatization chemistry of AccQ•Tag™ (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) with the sensitivity and resolution of UltraPerformance LC® (UPLC), this method consistently gives the right answer, even for these complex feed samples. Because the eluents, derivatization chemistry, and columns are application-tested, there is no need for method adjustments. A CD containing software projects for the management software (Empower™) chromatography data system is supplied as part of the system. Results are quickly generated and reported.

Figure 1. Waters UPLC Amino Acid Analysis Solution.

The reliability of the application solution was tested in a collaborative study across multiple laboratories (the inter-laboratory results will be reported elsewhere). Four animal feed hydrolysates, two soybean samples and two complex animal feed blends, were analyzed. The robustness and reproducibility of the method were evaluated in multiple experiments that included the typical sources of variability.

The complete sample set was analyzed on five separate days. Each daily experiment included fresh eluent and sample preparations, as well as five replicate derivatizations, injected in triplicate for each sample. A total of 75 data points were generated for each sample type. The ruggedness of the method was further tested by the inclusion of multiple columns throughout the five days of analysis.

EXPERIMENTAL

Samples

Swine diet, poultry diet, whole soybean, and soybean meal samples were acid-hydrolyzed in an independent laboratory as part of a collaborative study. The samples were supplied at an estimated concentration of 1.0 mg/mL in 0.1 M HCl and sealed under argon in ampoules. Samples were stored at -80 °C until analysis. The standard was NIST 2389 Amino Acids in 0.1 mol/L HCl Reference Material, and it was diluted to 5, 100, and 250 pmol/µL.

Sample Derivatization

The samples were diluted 1:16 with 0.1 M HCl prior to derivatization. The standard derivatization protocol was modified to include neutralization of excess acid with 0.1 M NaOH. The derivatization reagent reacts with both primary and secondary amines. The samples were derivatized in batches, and are stable at room temperature for up to one week when tightly capped. Conditions for pre-column derivatization and analysis are described in detail in the Waters UPLC Amino Acid Analysis Solution System Guide (P/N 71500129702). These derivatization conditions were modified to include additional base.

1. 60 µL AccQ•Tag Ultra Borate Buffer
2. 10 µL diluted sample
3. 10 µL 0.1 N NaOH
4. 20 µL reconstituted AccQ•Tag Ultra Reagent

ACQUISITION AND PROCESSING METHODS

The Waters UPLC Amino Acid Analysis Application Solution is provided with a CD that contains all the Empower methods necessary for acquisition and processing of the samples, as well as reporting of the results. Details of the methods can be found in the Waters UPLC Amino Acid Analysis Solution System Guide.

Chromatographic Conditions

LC System:	Waters ACQUITY UPLC® System
Column:	AccQ•Tag Ultra, 2.1 x 100 mm, 1.7 µm (two columns were alternated) Part Number: 186003837
Column Temp:	55 °C
Sample Temp:	20 °C
Flow Rate:	700 µL/min.
Mobile Phase A:	1:20 Dilution of AccQ•Tag Ultra Eluent A with MilliQ® water (prepared fresh daily) Part Number: 186003838
Mobile Phase B:	AccQ•Tag Ultra Eluent B Part Number: 186003839
Weak Needle Wash:	95:5 Water:Acetonitirile
Strong Needle Wash:	5:95 Water:Acetonitrile
Gradient:	AccQ•Tag Ultra Hydrolysate Method (provided in the UPLC Amino Acid Analysis Solution)
Total run time:	9.5 min
Injection volume:	1 µL, Partial Loop with Needle Overfill
Detection:	UV (TUV), 260nm

RESULTS AND DISCUSSION

The contributions of all method factors to the variability of retention time of the NIST standard is summarized in Table 1. The reported value is the mean of fifteen injections, three injections on each of five days. The preparation of fresh mobile phases and the use of different columns throughout the five days did not contribute any instability to the retention time. The most variable amino acid, Histidine, shows a range of four seconds between the shortest and longest retention times.

Figure 2 shows the separation of each of the different animal feed samples. The increased complexity of the diet samples does not result in any additional extraneous peaks or shifts in retention time that would result in the incorrect identification or quantitation of the amino acids.

The retention times of the amino acids in the four different feed samples are shown in Table 2. The reported retention time is the mean for the fifteen analyses of that sample on a single day. The amino acids have the same retention time, independent of sample type. The greatest deviation, again for Histidine, is approximately two seconds.

Amino Acid	Mean	Std Dev	% RSD
His	2.536	0.026	1.03
Ser	3.377	0.014	0.43
Arg	3.565	0.021	0.60
Gly	3.680	0.015	0.39
Asp	3.999	0.010	0.24
Glu	4.465	0.010	0.23
Thr	4.830	0.012	0.26
Ala	5.200	0.014	0.27
Pro	5.765	0.017	0.30
Cys	6.604	0.012	0.18
Lys	6.668	0.015	0.23
Tyr	6.807	0.012	0.17
Met	6.954	0.014	0.21
Val	7.074	0.015	0.22
Ile	7.796	0.018	0.23
Leu	7.878	0.019	0.24
Phe	7.995	0.018	0.23

Table 1. Summary of retention times, in minutes, for the hydrolysate standard throughout the five days of analyses. The standard deviations obtained assures no misidentification of amino acids.

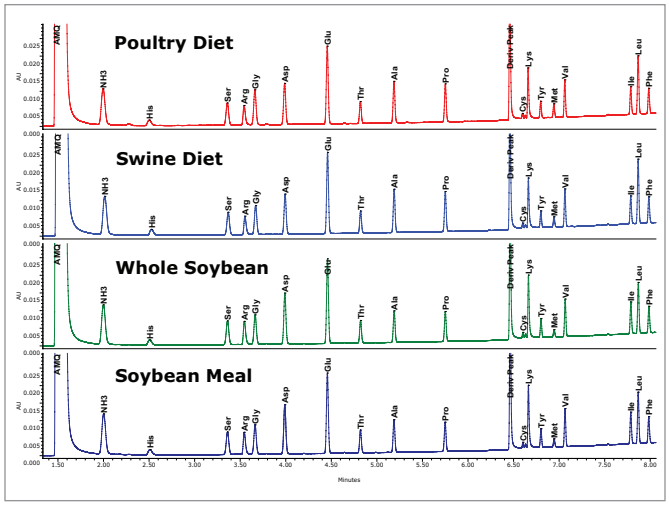

Figure 2. Representative chromatogram for each of the animal feed hydrolysates, 6 ng on column, using the UPLC Amino Acid Analysis Solution.

Amino Acid	Poultry Diet	Swine Diet	Whole Soybean	Soybean Meal	Mean	Std. Dev.
His	2.513	2.504	2.526	2.534	2.521	0.016
Ser	3.363	3.358	3.370	3.373	3.367	0.008
Arg	3.546	3.544	3.553	3.555	3.551	0.006
Gly	3.665	3.661	3.671	3.673	3.668	0.006
Asp	3.990	3.987	3.995	3.997	3.993	0.005
Glu	4.457	4.455	4.461	4.462	4.459	0.004
Thr	4.820	4.819	4.823	4.823	4.822	0.002
Ala	5.189	5.187	5.190	5.191	5.189	0.002
Pro	5.752	5.751	5.750	5.751	5.751	0.001
Cys	6.599	6.601	6.602	6.603	6.602	0.001
Lys	6.661	6.663	6.662	6.663	6.663	0.001
Tyr	6.799	6.800	6.801	6.801	6.801	0.001
Met	6.944	6.945	6.945	6.945	6.945	0.000
Val	7.064	7.065	7.064	7.066	7.065	0.001
Ile	7.787	7.788	7.787	7.787	7.787	0.001
Leu	7.868	7.869	7.868	7.869	7.869	0.001
Phe	7.985	7.985	7.985	7.986	7.985	0.001

Table 2. Retention time summary, in minutes, for the different sample types from one day of analyses. Each reported value represents the mean value of fifteen injections.

Poultry Diet	Day 1	Day 2	Day 3	Day 4	Day 5	Average	% RSD
His	2.08	2.10	2.13	2.08	2.12	2.10	1.09
Ser	6.12	6.11	6.26	6.11	6.11	6.14	1.08
Arg	4.89	4.91	4.91	4.91	4.94	4.91	0.36
Gly	9.14	9.15	9.16	9.16	9.16	9.15	0.09
Asp	9.84	9.85	9.81	9.87	9.79	9.83	0.33
Glu	16.21	16.21	16.21	16.26	16.20	16.22	0.15
Thr	4.13	4.13	4.14	4.13	4.13	4.13	0.11
Ala	8.51	8.49	8.48	8.50	8.45	8.49	0.29
Pro	7.68	7.69	7.68	7.70	7.70	7.69	0.16
Cys	0.34	0.34	0.35	0.35	0.34	0.35	1.47
Lys	4.49	4.55	4.64	4.58	4.58	4.57	1.16
Tyr	2.37	2.35	2.34	2.32	2.35	2.35	0.80
Met	2.08	2.08	2.00	2.06	2.07	2.06	1.66
Val	5.45	5.43	5.42	5.42	5.42	5.43	0.24
Ile	3.96	3.95	3.94	3.94	3.96	3.95	0.20
Leu	9.22	9.21	9.14	9.17	9.21	9.19	0.36
Phe	3.81	3.80	3.75	3.76	3.79	3.78	0.66

Whole Soybean	Day 1	Day 2	Day 3	Day 4	Day 5	Average	% RSD
His	1.96	1.99	2.03	1.95	2.02	1.99	1.84
Ser	6.61	6.59	6.61	6.62	6.61	6.61	0.16
Arg	5.69	5.83	5.84	5.76	5.87	5.80	1.22
Gly	7.92	7.75	7.78	7.87	7.78	7.82	0.91
Asp	12.09	12.12	12.01	12.12	12.04	12.08	0.39
Glu	17.63	17.59	17.55	17.64	17.53	17.59	0.28
Thr	4.27	4.25	4.25	4.25	4.25	4.25	0.19
Ala	6.44	6.39	6.38	6.41	6.37	6.40	0.44
Pro	6.06	6.03	6.05	6.06	6.06	6.05	0.24
Cys	0.37	0.39	0.39	0.39	0.39	0.39	1.99
Lys	5.32	5.57	5.62	5.47	5.56	5.51	2.17
Tyr	2.67	2.65	2.66	2.63	2.66	2.66	0.54
Met	1.15	1.14	1.09	1.15	1.13	1.13	2.04
Val	5.47	5.45	5.47	5.47	5.44	5.46	0.30
Ile	4.59	4.57	4.58	4.56	4.57	4.57	0.21
Leu	8.09	8.05	8.03	8.05	8.05	8.05	0.24
Phe	4.04	4.03	4.04	4.00	4.05	4.03	0.46

Swine Diet	Day 1	Day 2	Day 3	Day 4	Day 5	Average	% RSD
His	2.15	2.19	2.22	2.21	2.23	2.20	1.47
Ser	6.20	6.19	6.20	6.19	6.20	6.20	0.08
Arg	4.54	4.63	4.61	4.63	4.66	4.62	0.98
Gly	7.66	7.55	7.56	7.60	7.58	7.59	0.56
Asp	9.46	9.50	9.48	9.52	9.44	9.48	0.32
Glu	16.87	16.86	16.86	16.85	16.81	16.85	0.15
Thr	4.16	4.16	4.15	4.16	4.15	4.16	0.06
Ala	8.82	8.77	8.77	8.79	8.75	8.78	0.33
Pro	8.08	8.07	8.11	8.10	8.11	8.09	0.23
Cys	0.34	0.35	0.36	0.36	0.35	0.35	2.25
Lys	4.41	4.56	4.59	4.56	4.55	4.53	1.62
Tyr	2.35	2.34	2.35	2.32	2.36	2.34	0.64
Met	1.61	1.58	1.52	1.53	1.56	1.56	2.49
Val	5.50	5.49	5.50	5.50	5.48	5.49	0.16
Ile	4.04	4.00	4.00	3.99	4.00	4.00	0.48
Leu	10.19	10.16	10.14	10.13	10.17	10.16	0.23
Phe	3.95	3.95	3.93	3.93	3.95	3.94	0.32

Soybean Meal	Day 1	Day 2	Day 3	Day 4	Day 5	Average	% RSD
His	2.04	2.06	2.06	2.05	2.09	2.06	0.91
Ser	6.34	6.36	6.34	6.34	6.35	6.35	0.10
Arg	5.64	5.63	5.63	5.63	5.68	5.64	0.36
Gly	7.97	7.83	7.98	7.85	7.86	7.90	0.90
Asp	11.89	11.96	11.90	11.98	11.88	11.92	0.36
Glu	16.97	16.99	17.01	17.03	16.95	16.99	0.19
Thr	4.38	4.37	4.36	4.36	4.38	4.37	0.18
Ala	6.78	6.75	6.73	6.77	6.74	6.76	0.33
Pro	6.02	6.01	6.02	6.02	6.03	6.02	0.11
Cys	0.39	0.39	0.40	0.40	0.40	0.40	1.54
Lys	5.55	5.64	5.66	5.64	5.64	5.63	0.76
Tyr	2.71	2.70	2.69	2.66	2.69	2.69	0.61
Met	1.22	1.28	1.22	1.26	1.26	1.25	1.86
Val	5.59	5.59	5.60	5.60	5.59	5.59	0.13
Ile	4.61	4.61	4.61	4.60	4.62	4.61	0.16
Leu	8.22	8.19	8.16	8.17	8.21	8.19	0.28
Phe	4.06	4.03	4.02	4.01	4.04	4.03	0.46

Table 4. Mole % amounts for each of the four animal feed sample types. The reported values for each day are an average of fifteen data points, the overall average representing 75 independent analyses.

The quantitative reproducibility, expressed as mole %, is shown in Table 4. For all four sample types tested across the five days of analysis, the mole % is typically better than 1% RSD. The sulfur-containing amino acids were not protected during hydrolysis, and are higher.

The composition of the four feed samples is, in general, similar. There are, however, clear distinctions. For example, Aspartate is lower in the blended feeds than in the soybean samples. Valine, on the other hand, is almost identical.

These 75 determinations reflect many sources of variability, including pipetting errors, incomplete derivatization, injector error, integration error, standard and sample preparation error, sample hydrolysis, and coelution of unidentified sample components with the amino acids. Complete examination of the raw data for each of the individual injections shows that the largest contribution to the variability is pipetting. The addition of an internal standard to the sample to be hydrolyzed will improve reliability. Norvaline is the preferred internal standard for this purpose.

CONCLUSION

The measurement of the total amount of amino acids plays a key role in the efforts to assess the quality and appropriateness of animal feeds for the best growth and production. While there are many methods available to obtain that information, the complexity of these kinds of samples can compromise the reliability of the results. In that case, samples might require reanalysis, or, in the worst case, the most economical growth conditions can be misjudged.

The summary of this study of animal feed hydrolysates demonstrates that the Waters UPLC Amino Acid Analysis Solution can provide accurate, reliable, and timely information. The robustness of the chromatography is shown by retention time stability of the standard across multiple columns, eluents, and days of analysis. The complexity of the samples does not distort retention time or other chromatographic features.

Quantitation is reliable and reproducible. The sensitivity of the analytical method requires only small aliquots of these complex feed samples.

These experiments show that the Waters UPLC Amino Acid Analysis Solution can be used to determine the nutritional content of animal feeds. The turnkey solution provides the derivatization chemistry, the chromatographic column and eluents, and the software for data acquisition and reduction so that the end user can be assured accurate and precise results.

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September 2008 720002804EN KK-PDF

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ACQUITY UPLC FOR THE RAPID ANALYSIS OF AMINO ACIDS IN WINE

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CLASSIC UPLC

INTRODUCTION

Amino acids serve as the nitrogen source for yeasts during the fermentation of wine. Hernández-Orte et al[1] have reported that amino acids are also a mark of the quality of wine because they contribute to the wine's taste, aroma and color. Besides scientifically determining the quality of wine, analysis of amino acids can also classify a wine based on its amino acid concentrations rather than relying solely on the human palate.

Previously, methods for analyzing amino acids in wine have taken anywhere from 46.5 minutes on a Luna C_{18} bonded silica column (4.6 x 250 mm, 5 µm) protected by a 4.6 x 20 mm sentry guard column[1] to 25 minutes on a narrow bore C_{18} HP Amino Acid Analysis column (2.1 x 200 mm) protected by a 2.1 x 15 mm guard column[2].

The Waters® UPLC® Amino Acid Analysis Solution performs this separation in only 8.5 minutes on an AccQ•Tag™ Ultra Column (2.1 x 100 mm) with an in-line filter, which is 4.8 and 2.6 times faster, respectively, than previous HPLC methods, while still maintaining baseline resolution of a 17 standard amino acid mixture (Figure 1).

The Waters UPLC Amino Acid Analysis Solution.

EXPERIMENTAL

Materials

Twenty wines, four of each variety (shiraz, chardonnay, cabernet, sauvignon blanc and merlot) were purchased from a local liquor store. All samples were stored at room temperature until analysis. Amino acid standard solution, AccQ•Tag Ultra borate buffer, and AccQ•Tag Ultra reagent were all included in the UPLC Amino Acid Analysis Application Solution. Water was purified with a Milli-Q system (Millipore, Billerica, MA).

UPLC conditions

The Waters ACQUITY UPLC® System consisted of a Binary Solvent Manager (BSM), a Sample Manager fitted with a 2 µL loop, and a Tunable UV (TUV) detector. The system was controlled and data collected using Empower™ 2 Software. Separations were performed on a 2.1 x 100 mm ACQUITY UPLC AccQ•Tag Ultra Column with an in-line filter at a flow rate of 0.70 mL/min.

Column temperature was set at 55 °C and injection volumes for all samples and standards were 1.0 µL. Water/acetonitrile (95:5) was used as the weak needle wash solvent and water/acetonitrile (5:95) was used as the strong needle wash solvent. Mobile phase components and gradient conditions are outlined in Table 1. Detection was set at 260 nm using a sampling rate of 20 points per second and a time constant of 0.4 seconds.

Sample preparation

An 80 µL aliquot of AccQ•Tag Ultra borate buffer was added to a total recovery vial. Wine (20 µL, previously diluted 1:8 with water) was added along with 20 µL of reconstituted AccQ•Tag Ultra reagent into the vial. The vial was placed on a vortex mixer for 5 seconds and then allowed to stand at room temperature for 1 minute before being placed in a heating block at 55 °C for 10 minutes. After 10 minutes, vials were removed and analyzed using the ACQUITY UPLC System. All wine samples were prepared and analyzed in triplicate.

Calibration

A standard solution of 17 amino acids, included as part of the AccQ•Tag Ultra Chemistry Package, was prepared. The standard consisted of a one point calibration curve with standard concentrations of 50 pmoles/µL for all amino acids except cystine, which had a concentration of 25 pmoles/µL (Figure 1).

Time (min)	% A	% B	Curve
0.0	99.9	0.1	-
0.54	99.9	0.1	6
5.74	90.9	9.1	7
7.74	78.8	21.2	6
8.04	40.4	59.6	6
8.64	40.4	59.6	6
8.73	99.9	0.1	6
9.5	99.9	0.1	6

Table 1. The Waters UPLC Amino Acid Analysis Solution gradient separation conditions.

Solvent A = AccQ•Tag Ultra Eluent A:Water (5:95)

Solvent B = AccQ•Tag Ultra Eluent B

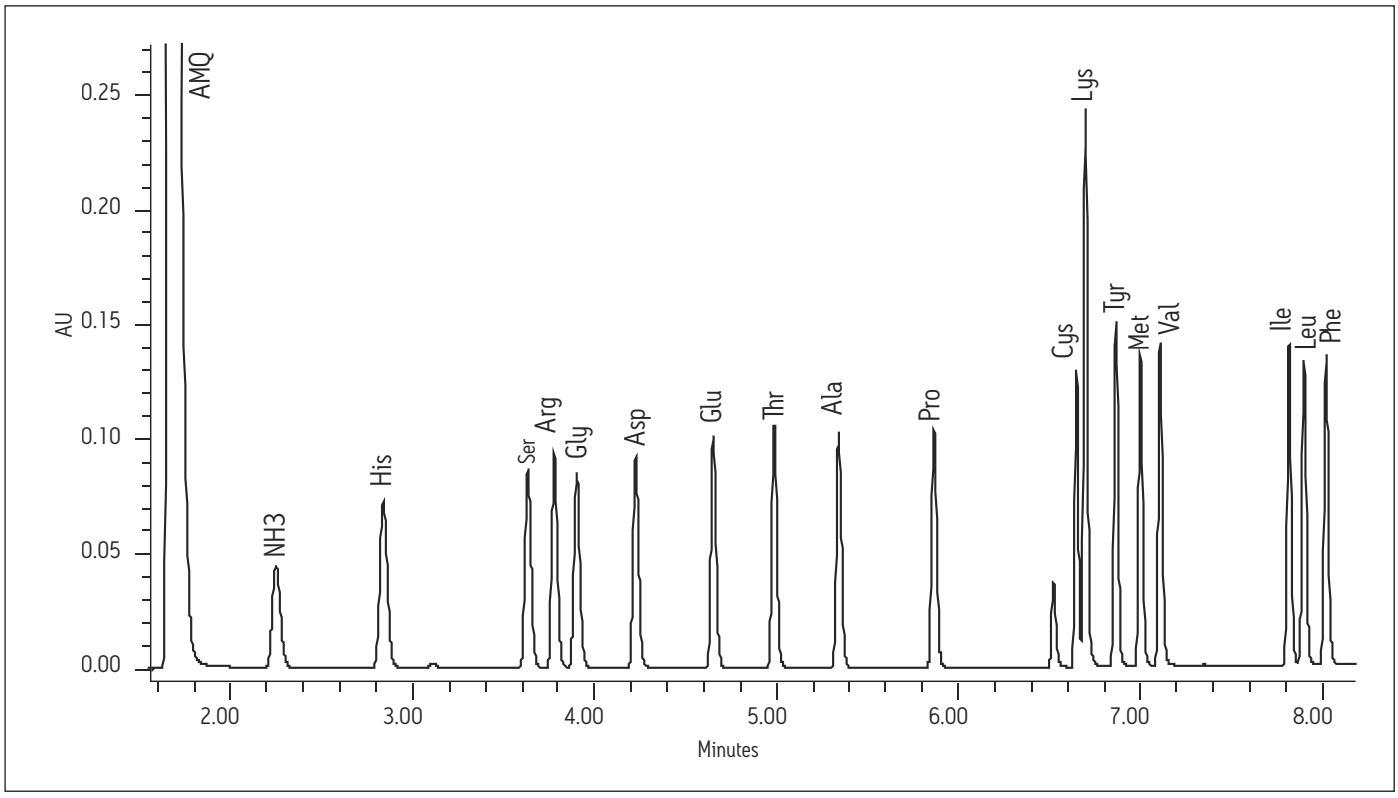


Figure 1. Separation of 50 pmoles of amino acid hydrolysate standard (25 pmoles cystine) with the UPLC Amino Acid Analysis Solution.

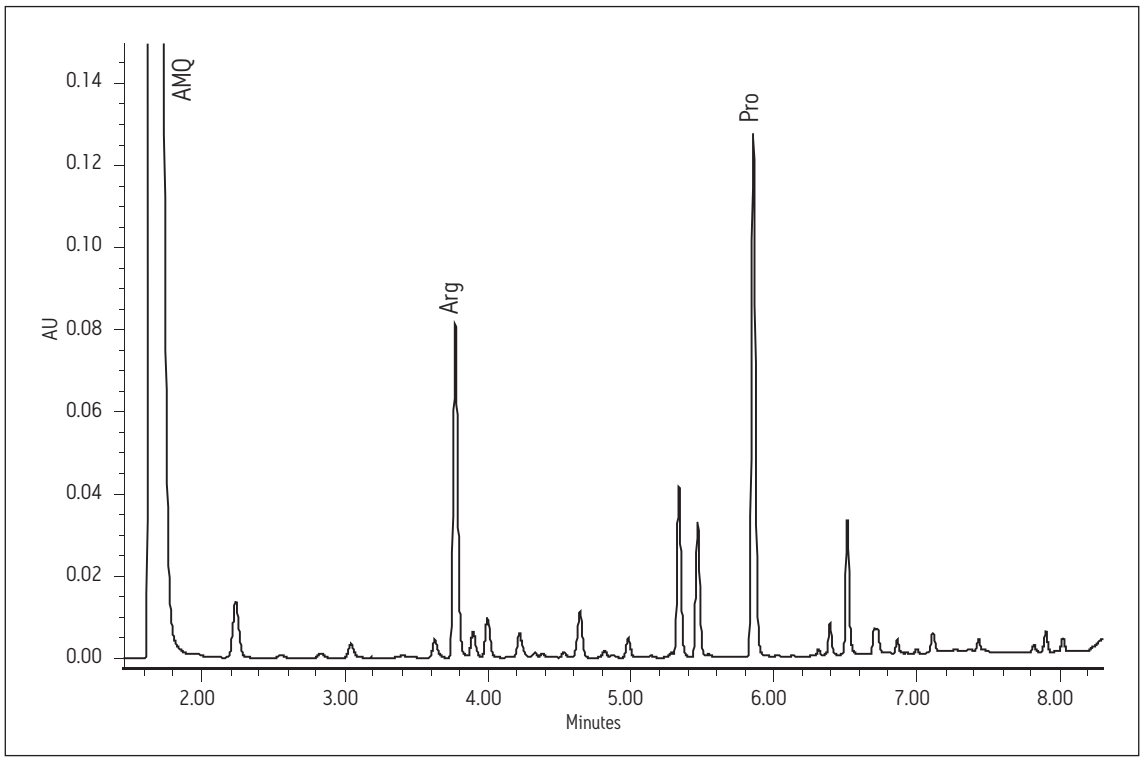


Figure 2. Separation of sauvignon blanc with the UPLC Amino Acid Analysis Solution.

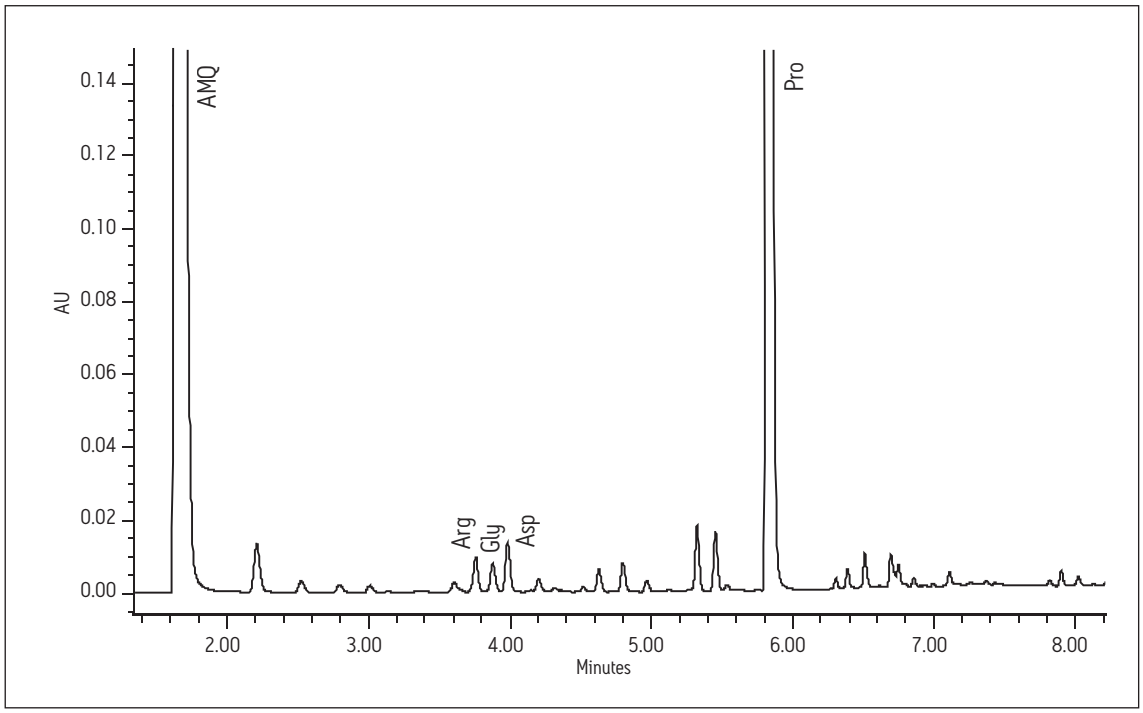


Figure 3. Separation of cabernet with the UPLC Amino Acid Analysis Solution.

RESULTS

Using the Waters UPLC Amino Acid Analysis Solution, each of the 17 amino acids was baseline resolved in less than 10 minutes. The analysis time of 8.5 minutes was significantly faster than the previous reported times of 45 minutes and 26 minutes. Figures 2 and 3 show the chromatograms of sauvignon blanc 2 and cabernet 4, respectively.

Area counts from injections of derivatized amino acids in the wine samples were compared to the response from the derivatized amino acid standard solution for each amino acid. Concentrations (in µg amino acid per mL) were calculated using Empower 2 Software. All 17 of the amino acids present in the standard solution were found in all of the wine samples, with the exception of cystine, which was not found in one of the chardonnay samples. The total amino acid content (sum of the 17 amino acids in the standard) for each wine can be seen in Figure 4.

A cursory examination of the data set (Table 2) showed proline to be the most abundant amino acid throughout all 20 different wine samples with the exception of sauvignon blanc 1, which contained slightly more arginine than proline (367 and 339 µg/mL respectively). Concentrations of proline ranged from 158 µg/mL in sauvignon blanc 2 (42% of the total amino acid content for that wine) to 3,178 µg/mL in merlot 1 (94% of the total amino acid content for that wine). Results can be seen in Figures 5 and 6.

After proline, analysis of Table 2 becomes rather complicated. Further analysis of the data is aided by using multivariate statistical methods (SIMCA-P+ version 11.5 from Umetrics[3]), i.e. Partial Least Squares Discriminate Analysis (PLS-DA) and Orthogonal Partial Least Squares (OPLS). As shown in Figure 7, PLS-DA clearly shows the variance between the wines, differentiating not just red from white but also the individual varietals. Figure 8 indicates that high proline is the distinguishing feature in red wines while higher arginine, aspartate and glutamate concentrations are indicative of a white wine.

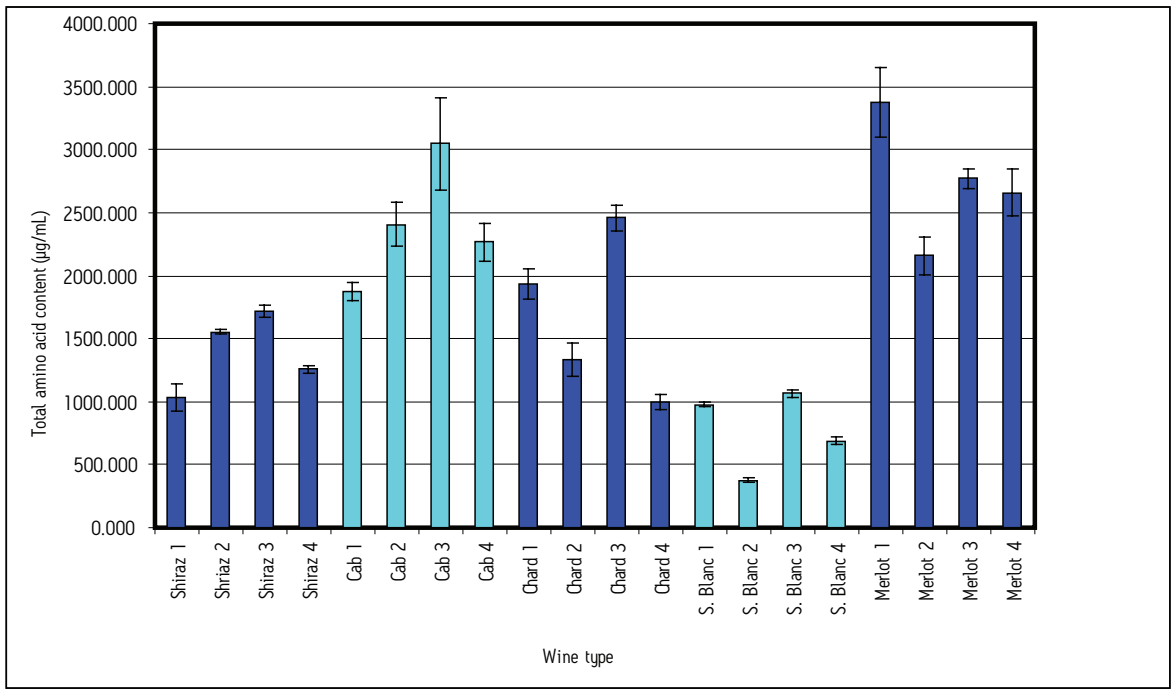
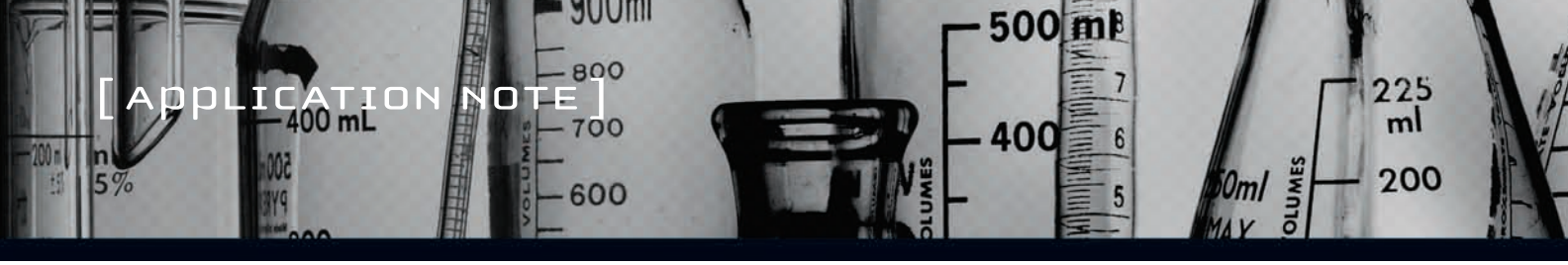


Figure 4. Total amino acid content in wine samples (n=3).



	Amino acids in wine, µg/mL							
	His	Ser	Arg	Gly	Asp	Glu	Thr	Ala
Shiraz 1	6.2	6.1	22.9	11.3	9.1	16.3	5.4	19.5
Shriaz 2	11.1	11.2	46.2	21.7	19.5	34.6	10.5	38.8
Shiraz 3	3.5	14.2	76.2	21.9	17.0	36.0	11.9	47.7
Shiraz 4	4.9	9.1	8.7	18.1	17.1	27.5	9.9	35.3
Cab 1	3.9	7.4	6.3	13.2	10.5	17.3	6.5	32.8
Cab 2	2.4	6.2	5.5	11.6	10.1	14.2	5.4	20.5
Cab 3	11.0	8.4	44.9	16.1	13.5	19.4	7.8	32.5
Cab 4	16.7	18.4	130.1	30.6	48.1	46.2	20.6	59.5
Chard 1	13.9	20.1	94.0	24.4	38.2	50.9	17.5	63.3
Chard 2	8.1	13.4	48.8	15.8	30.8	23.3	10.0	30.6
Chard 3	18.3	30.4	258.8	20.6	26.1	76.3	23.3	148.1
Chard 4	16.7	16.2	91.6	15.6	38.9	42.2	12.2	58.6
S. Blanc 1	6.8	14.8	367.6	14.3	27.3	45.6	13.2	87.6
S. Blanc 2	5.4	8.7	48.4	7.4	20.2	36.4	6.2	36.0
S. Blanc 3	12.5	12.3	297.1	15.7	18.2	54.0	9.0	76.4
S. Blanc 4	8.0	8.2	95.4	12.5	19.1	31.5	7.1	38.2
Merlot 1	9.9	8.6	49.6	18.4	10.8	24.9	7.4	29.3
Merlot 2	5.9	6.9	25.7	14.8	10.0	18.2	6.2	21.4
Merlot 3	4.6	10.1	28.1	17.1	15.7	25.6	8.7	30.6
Merlot 4	8.3	10.2	35.6	18.8	15.5	25.0	9.6	37.5

	Amino acids in wine, µg/mL								
	Pro	Cys	Lys	Tyr	Met	Val	Ile	Leu	Phe
Shiraz 1	903.8	3.7	6.2	3.3	1.1	5.7	2.1	5.3	4.8
Shriaz 2	1272.2	2.2	25.7	8.8	3.8	11.5	4.9	15.7	12.2
Shiraz 3	1395.6	1.5	25.9	6.7	4.4	14.3	6.2	18.6	13.9
Shiraz 4	1064.7	1.3	18.6	3.7	2.4	10.0	3.7	11.3	11.4
Cab 1	1736.4	1.2	7.9	4.1	1.5	8.2	3.0	6.7	6.8
Cab 2	2294.4	2.6	9.8	2.8	1.5	6.3	2.4	6.4	6.1
Cab 3	2833.1	5.6	15.6	7.5	2.2	7.9	3.5	9.2	7.9
Cab 4	1760.7	3.0	35.5	9.5	6.5	21.9	9.8	24.9	22.3
Chard 1	1456.8	3.2	35.5	20.9	9.2	21.5	11.6	35.4	22.7
Chard 2	1062.6	0.0	27.5	8.3	5.5	10.1	5.8	20.7	12.4
Chard 3	1766.3	1.7	16.3	13.2	4.4	16.7	5.8	17.7	13.6
Chard 4	578.7	0.6	26.4	20.0	6.3	18.5	9.0	24.3	19.9
S. Blanc 1	339.2	0.7	8.9	9.7	2.9	12.4	4.2	12.2	11.1
S. Blanc 2	158.1	0.8	9.6	6.8	2.3	8.0	2.6	8.9	8.7
S. Blanc 3	500.0	2.6	11.1	12.0	2.8	13.8	4.9	11.5	12.9
S. Blanc 4	388.1	1.9	18.2	10.1	4.0	10.6	5.3	16.4	11.7
Merlot 1	3178.4	3.3	7.8	5.7	1.5	5.7	2.0	5.9	6.7
Merlot 2	2009.0	1.1	8.6	3.8	1.5	7.0	2.8	6.8	7.3
Merlot 3	2569.2	7.8	14.5	4.3	2.3	9.9	4.1	10.4	9.9
Merlot 4	2440.9	1.3	12.6	4.7	2.5	9.4	4.0	12.0	11.1

Table 2. Individual amino acid content in wines (µg/mL, mean of three replicates).

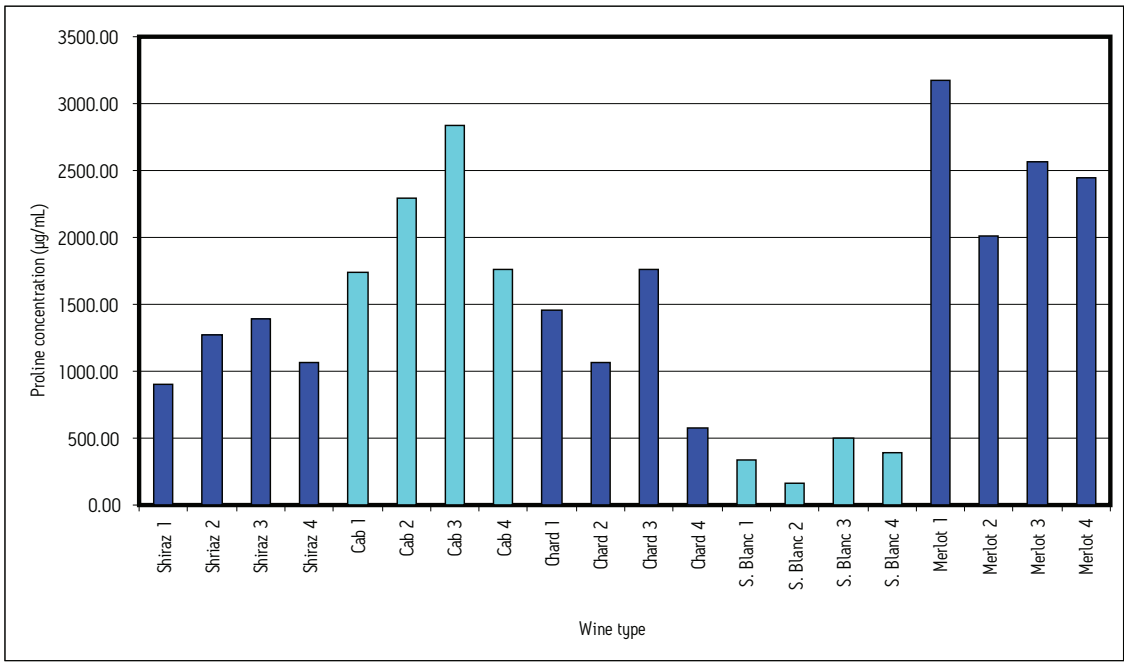


Figure 5. Proline concentrations in wine samples.

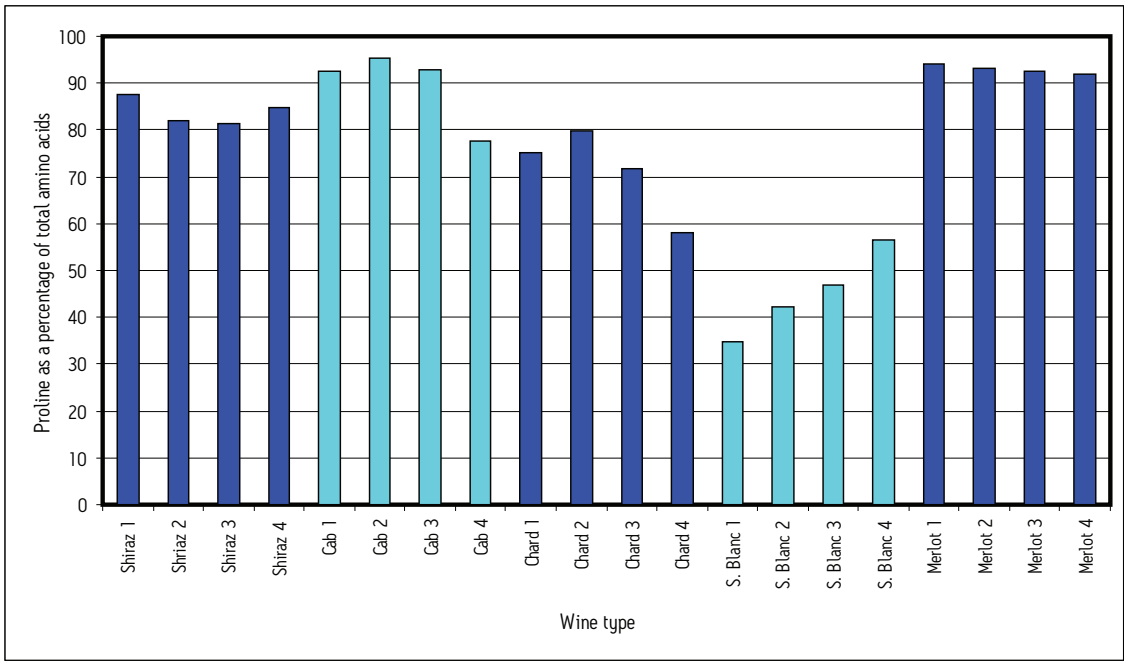


Figure 6. Proline amount in each wine sample relative to total amino acids in sample.

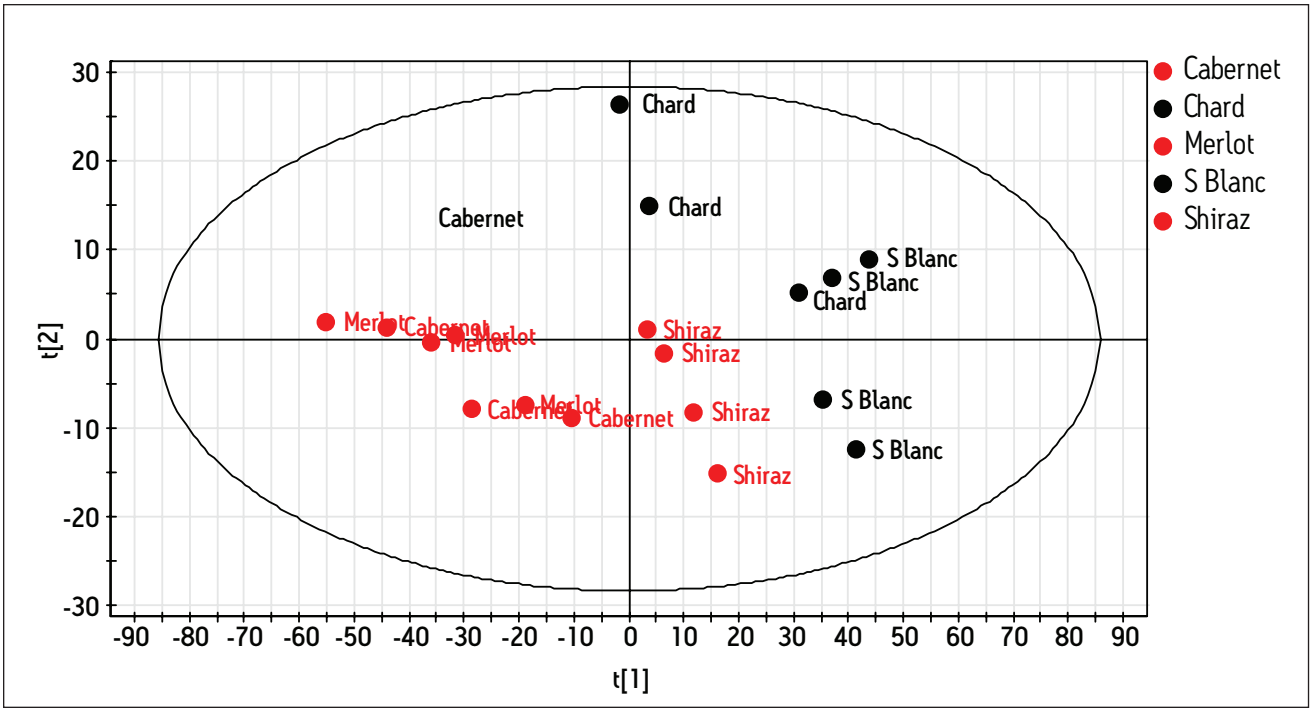


Figure 7. Amino acids in red and white wines: PLS-DA scores plot.

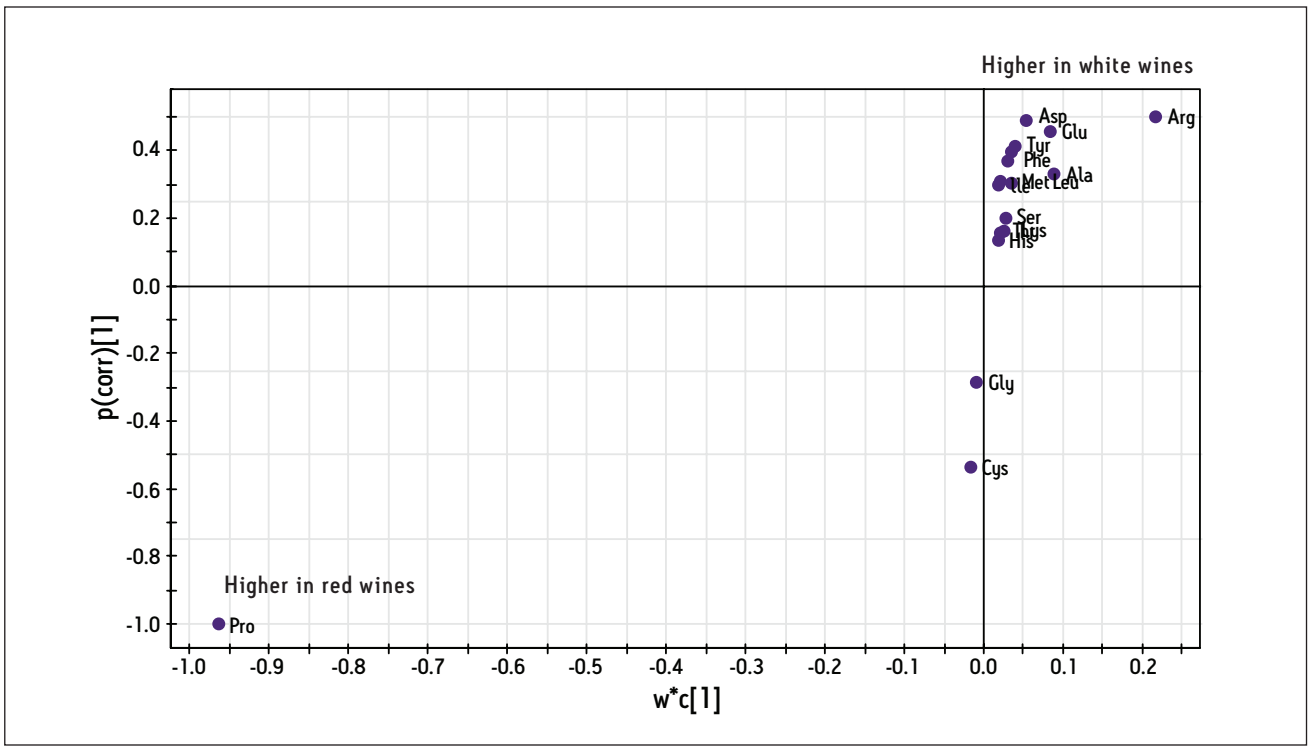


Figure 8. Amino acids in red and white wines: OPLS s-plot.

CONCLUSION

The Waters UPLC Amino Acid Analysis Solution provides significantly reduced analysis time of amino acids and baseline resolution of 17 different amino acids found in wine. The ability to decrease analysis time while also providing baseline resolution results in more productive and efficient laboratories. Applying the Waters UPLC Amino Acid Analysis Solution will greatly benefit any laboratory interested in analyzing amino acids.

Further data analysis using more advanced tools, such as Principal Component Analysis (PCA) and Orthogonal Partial Least Squares (OPLS), provides scientists with additional information from the large, complex data sets in a simplified format.

According to Herbert et al[2], "In order to have routine analysis for wine certification" an amino acid method for wines must be able to accurately detect and quantitate the most prominent amino acids in wine, must have low detection limits for other amino acids and must have a short analysis time. The Waters UPLC Amino Acid Analysis Solution meets these demanding requirements and could be used for wine certification, quality control or identification of adulteration.

Waters UPLC Amino Acid Analysis Solution.

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2. Herbert P., Barros. P., Ratola, N., Alves,A., Journal of Food Science, Vol. 65, No. 7 2000.

3. SimcaP+ Version 1.5 is available form Umetrics (www.umetrics.com), Wilmington, MA.

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DETERMINATION OF AMINO ACIDS IN BEERS USING THE UPLC AMINO ACID ANALYSIS SOLUTION

CLASSIC UPLC

Mark E. Benvenuti and Paula Hong
Waters Corporation, Milford, MA USA

INTRODUCTION:

Beer is a complex matrix consisting of over 100 components. Water, ethanol and carbohydrates are the major constituents of beers and ales. However, there are many minor compounds, some of which are critical for proper taste and quality. One class of compounds, amino acids, is metabolized by yeast during fermentation, leading to the formation of critical flavor components. Therefore, the monitoring of amino acids is essential to demonstrate product consistency and ensure customer satisfaction.

Current HPLC methods for amino acids require run times that exceed 30 minutes, with poor resolution between many amino acids. Here we shall demonstrate the efficacy of the Waters® UPLC® Amino Acid Analysis Solution to resolve 27 amino acids and an internal standard in less than 10 minutes and apply this capability to amino acid analysis of several beer and ale samples.

EXPERIMENTAL:

Conditions:

System: ACQUITY UPLC® with Tunable UV Detector
Method: Cell culture
Column: AccQ•Tag™ Ultra, 2.1 x 100 mm
Temperature: 60 °C
Injection volume: 1.0 µL
Detection: UV @ 260 nm
Data: Empower™ software

Standard preparation:

A stock 1000 pmol/µL stock mixed amino acid standard was prepared per the cell culture method 1,2,3. An intermediate 100 pmol/µL mixture was prepared by mixing 100 µL of stock with 900 µL water. The working derivatized standard was prepared by adding 10 µL of the 100 pmol/µL mixture to 70 µL borate buffer followed by 20 µL AQC derivatization reagent in a total recovery vial and mixing well. The mixture was heated for 10 minutes at 55 °C, cooled to room temperature then injected. The concentration is 10 pmol/µL for the analytes of interest except Cystein (Cys) which is 5 pmol/µL.

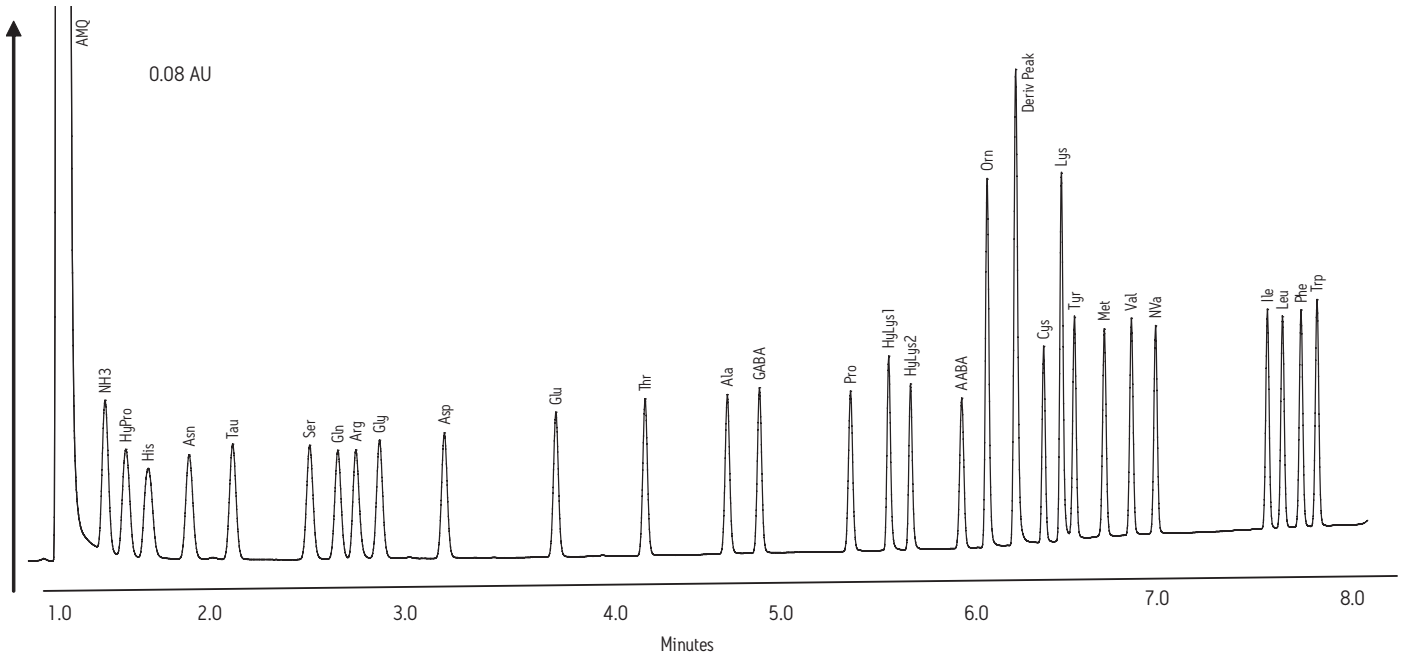

Figure 1. Chromatogram of 10 pmol/µL amino acid standard.

Analyte	RT	Area	Analyte	RT	Area
Hypro	0.522	0.736	HyLys1	0.013	0.874
His	0.581	0.743	HyLys2	0.014	0.86
Asn	0.417	0.853	AABA	0.017	0.856
Tau	0.329	0.798	Orn	0.017	0.846
Ser	0.236	0.708	Cys	0.014	0.885
Gln	0.219	0.725	Lys	0.014	0.841
Arg	0.217	0.762	Tyr	0.013	0.846
Gly	0.167	0.754	Met	0.012	0.809
Asp	0.126	0.817	Val	0.012	0.822
Glu	0.074	0.413	Nva	0.012	0.413
Thr	0.05	1.029	Ile	0.01	0.964
Ala	0.035	0.903	Leu	0.01	0.983
GABA	0.03	0.816	Phe	0.01	0.857
Pro	0.019	0.845	Trp	0.01	0.9

Table 1. Reproducibility data for amino acid standard, RT and Area (RSD), 5 injections.

Sample preparation:

14 samples of beer and ale were purchased commercially. These included domestic regular, light, non-alcoholic, dark beers and an imported Belgian ale. Approximately 100 mL of each beer was sonnicated to remove carbonation. If the sample appeared excessively cloudy or turbid, it was filtered through a 0.45 micron hydrophilic filter. 200 µL of each beer and ale was mixed thoroughly with 160 µL water and 40 µL of a 1000 pmol/µL Norvaline (Nva - internal standard) solution. The preparation of the internal standard is described in the Cell Culture Method 1,2,3. This resulted in a 1:2 dilution (400 µL total volume) of the beer made 100 pmol /µL in internal standard.

10 µL of this mixture was then mixed with 70 µL of borate buffer and 20 µL of AQC derivitazation reagent and heated as described in the standard preparation section. This working sample mixture, now a 20 fold dilution of the beer, made 10 pmol /µL in internal standard (similar to the working standard) was injected.

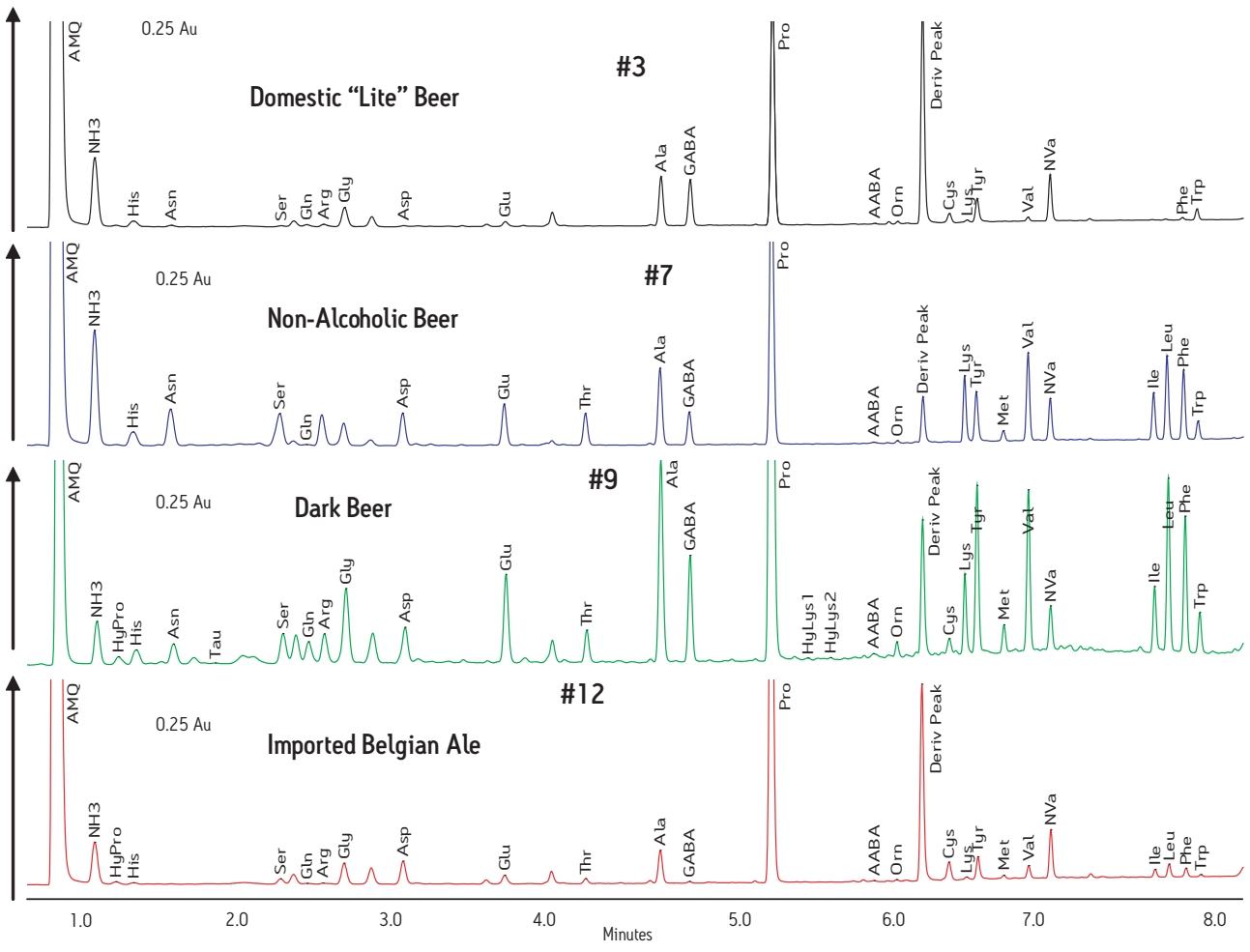

Figure 2. Chromatographic profiles of amino acid content for various beer types.

Sample#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Type	**American Beer**	**American Beer**	**Lite Beer**	**Lager Beer**	**Mexican Beer**	**Dutch Beer**	**Non-Alcoholic**	**NY Micro Brew**	**Dark American Beer**	**Chinese Beer**	**Canadian Stout**	**Belgian Ale**	**California Micro Brew**	**American Brown Ale**
HyPro	17.4	15.1	ND	27	13.9	23.3	ND	39.8	66.6	22.9	24.4	17.7	28	29.6
His	152.3	96.9	53.8	120.8	78.4	156.3	153.7	117	136.1	76.8	180	12.4	122	140.8
Asn	272.3	69.7	12.6	87.6	14.1	16.2	346.4	55.3	172.3	ND	25.7	ND	55.7	19.5
Tau	ND	ND	ND	ND	ND	ND	ND	ND	4	ND	ND	ND	19.8	ND
Ser	113.2	25.4	9.3	40.5	19.3	17.3	333.4	52.1	240.3	13.4	29.9	37.5	64.7	24.7
Gln	129.2	51.4	19.5	24	29	36.5	9.4	22.4	196.9	11.9	97.9	3.3	24.1	14.5
Arg	137.6	29.9	21.8	96.5	175.2	186.6	ND	40.6	249.4	156.6	193.8	4.1	ND	50.4
Gly	326.3	258.8	147.7	256.3	249.7	263	ND	342.3	605.6	238.7	429.6	147.9	380.6	362.1
Asp	231.7	113.8	6.5	67.5	14.8	19.4	252.3	40.4	287.7	11.8	70.3	153.7	23.7	20.6
Glu	243.3	132.6	32.3	117.1	65.4	105.7	278.8	100.9	564.4	64.9	166	56.4	98.2	63.9
Thr	66.5	10.5		24.9	9.2	4.3	197.8	23.9	192.9	3.5	7.8	27.4	27.3	7
Ala	928.5	567.5	268.2	582	520.4	614	463.9	1219.6	1162.9	383.6	1114.3	168.2	807.5	531.8
GABA	431.9	368.1	244.8	700.1	522	457.7	190.8	911.8	586.1	494.5	484.2	7.8	930.9	903.5
Pro	2007.9	1733.4	1359.6	4473.7	2067.1	3421.5	1867.5	5797.5	6238.7	2858.7	3940.8	2685.2	4271.7	2806.2
HyLys1	ND	ND	ND	ND	ND	ND	ND	5.3	12.1	ND	ND	ND	ND	ND
HyLys2	ND	ND	ND	ND	ND	ND	ND	2.8	15.6	ND	ND	ND	ND	ND
AABA	12.2	ND	4.2	15.7	8.5	7.7	6.5	25.1	42.2	ND	13.9	5	20.5	11.1
Orn	12.3	9.1	5.4	7.6	11.2	7.5	6.7	53.9	34.6	7.3	15	3.4	1.1	27.2
Cys	26.4	33.3	26.9	38.6	27.6	26.4	ND	66.1	38	36.4	29.9	49	35.9	25.4
Lys	95.1	26.5	5.2	36.2	13.5	9.8	172.5	2.8	199.7	8.2	35.7	6.1	9.2	27
Tyr	379.4	251.3	99.5	491.8	320.5	264.5	235.3	527.9	686.7	336.9	616.8	86.7	533.5	587.1
Met	26.7	8.5	ND	17.4	12.6	7.3	49.9	14.9	114.2	ND	21	12.6	23.8	7.7
Val	406.6	181.2	14.9	378.8	294.4	244	388.6	885.9	670.9	179.3	666.8	48.7	446.7	430.5
Ile	134.9	38.3	ND	108.4	59.7	41.2	203.9	95.3	293.5	19.1	191.5	29.3	114.4	30.6
Leu	267.3	69.6	ND	184	98.5	54.8	375.2	115.5	765.5	29.1	416.7	51.6	189.9	158.9
Phe	311.3	140	8.2	260.6	236.6	174.2	302.8	333.2	586.5	148.5	487.2	33.2	241.7	339.2
Trp	99.8	65.6	40.8	119.8	85.5	149.1	76.1	157	166.4	107.6	123.5	7.6	125.3	151.9

Table 2. Amino acid content of beers sampled, units are pmol/µL, ND = not detected.

RESULTS AND DISCUSSION:

Figure 1 is a chromatogram of the cell culture standard. Table 1 is reproducibility data (RSD) for retention time and area for 5 injections of this standard. An overlay of the chromatograms of several of the beers analyzed is found in Figure 2. Table 2 lists the quantitated amounts for the amino acids in the 14 beer samples tested.

The differences in amino acid content, both qualitative and quantitative, for the samples tested are quite evident. Proline (Pro) was found in all samples tested and at a high level, not surprising given the fact that beer yeast cannot ferment proline. On the other hand, Taurine (Tau) and Hydroxy-L-lysine (HyLys) were absent or at a very low level. In general the darker beers had higher amino acid content than light beers.

Also note that in Figure 2 there are many unidentified peaks, possibly amino acids not included in the standard mixture or other compounds that contain an amino group that would react with the AccQ•Fluor™ reagent. Since the methodology is fully compatible with mass spectrometry detection, it is possible to positively identify these additional compounds, which may also be of critical importance to product consistency.

CONCLUSION:

We have successfully demonstrated application of the Waters UPLC amino acid analysis solution (AAA) to determine the amino acids found in several different beers and ales. This method demonstrated excellent resolution of all sample components with a 10 minute cycle time. Simple sample preparation and analysis times that are approximately three times faster than traditional HPLC methods make the UPLC AAA solution ideal for demonstrating consistency of beer production.

REFERENCES:

1. Wheat, E, Grumbach, E and Mazzeo et al. “A new Amino Acid Analysis Application Solution”. Waters Corporation, 2006: 720001683EN.
2. “UPLC Amino Acid Analysis Application Solution System Guide” Sections 4, 6, Waters Corporation, 2006: 720001565EN.
3. Paula Hong, Private Communication.

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COMPLETE AMINO ACIDS ANALYSIS OF FOODS AND FEEDS

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INTRODUCTION

Amino acid analysis is used for the quantification of one group of essential nutrients in foods and feeds. To provide a robust, reproducible and accurate method for the quantification of all the amino acids, both sample handling and chromatographic separation must be taken into consideration. In sample handling, the samples must be hydrolyzed so that the proportions of amino acids can be measured. Multiple hydrolysis approaches must be used because all amino acids are not equally stable. Each sample must be subjected to 3 different hydrolysis protocols namely acid hydrolysis used to determine the total protein composition; acid hydrolysis following performic acid oxidation required to measure sulphur containing amino acids and alkaline hydrolysis used to assess tryptophan recovery. Sample hydrolysis is a time consuming process requiring special equipment. Microwave hydrolysis implementation for all three protocols results in improved control of hydrolysis conditions with better accuracy, reproducibility, speed and robustness.
In this work we show the implementation of all three hydrolysis protocols for raw feeds, such as, soy bean meal, as well as complete mixed feeds. Chromatographic separation is used to identify and quantify the released amino acids. Reversed-phase UPLC® of amino acids derivatised with 6-aminoquinolyl-N-hydroxysuccinimidyl (AQC) provides high resolution and sensitivity.

Figure 1. *Microwave Hydrolysis CEM Discover SP-D*

Figure 2. *ACQUITY UPLC H-Class and H-Class Bio Amino Acid Analysis system*

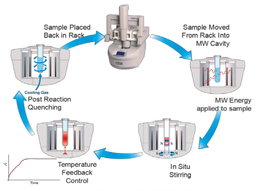

Figure 3. *Microwave Hydrolysis Procedure*

METHODS

Conditions :
System: ACQUITY UPLC H-Class System with TUV Detector
Column: AccQ•Tag™ Ultra, 2.1 X 100mm
Eluent A: 100 % AccQ•Tag Ultra Eluent A
Eluent B: 90:10 Water, AccQ•Tag Ultra Eluent B
Eluent C: Water
Eluent D: 100% AccQ•Tag Ultra Eluent B
Injection Volume: 1.0 µl
Detection: UV@ 260 nm
Internal Standard: Norvaline (NVa)
Flow Rate: 0.7 ml/min
Run Time: 10.2 minutes
Note: The same set of samples were subjected to three different hydrolysis protocols. The chromatography conditions detailed above were used to identify and quantify the released amino acids from the three different protocols.

Standard Mixture used for Foods and Feeds:
100 µl of Protein Hydrolysate Standard and 50 µl of stock 5 mM aliquots of Cya, Tau, $MetSO_2$, GABA, Trp and Nva, (internal standard) were added to 600 µl of 0.1N HCl. This standard mix of Foods and Feeds at 250 pmol/µl is stable for one month when stored at -20°C.

Derivatisation of Standard Mixtures:
70 µl of AccQ•Tag Ultra Borate Buffer and 10 µl of standards mix were added to a total recovery vial (186000384C). The vial was vortexed for 10seconds. 20 µl of reconstituted AccQ•Tag Ultra reagent powder was added to this mix, the solution was vortexed for 10 seconds and heated @ 55°C for ten minutes. Final concentration of each analyte is 25 pmol/µl.

For the raw and complete mixed feeds samples, a neutralisation step was carried out prior to derivatisation.

Sample Handling

A. Performic Acid Oxidation (Pre-oxidation):
a. 6ml of freshly prepared performic acid solution (9:1 formic acid: 30% hydrogen peroxide) was added to the sample weighed into a 35 ml reaction vessel with stir bar
b. The reaction mix was incubated in a water bath at 50°C for 1 hour.
c. Residual performic acid solution was evaporated in a N-Evaporator at 50°C to dryness
d. 5 ml of 6N HCl was added to the dry sample in the 35 ml vessel.
e. Sample was hydrolyzed at 195 °C for 15 minutes.

B. Acid Hydrolysis:
a. 5 ml of 6N HCl was added to the sample weighed into a test tube
b. For microwave conditions, see Table 2 and 3.

C. Alkaline Hydrolysis:
a. 5 ml of 4N NaOH was added to the sample weighed into a test tube
b. For microwave conditions, see Table 2 and 3.

RESULTS

Derivatisation Chemistry

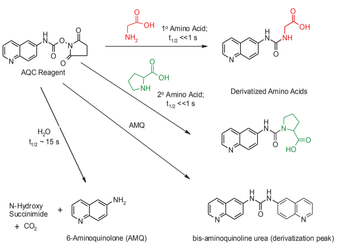

Figure 4. *Reaction of AQC reagent with amino acids. The 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent reacts with both primary and secondary amines. Excess reagent reacts with water to form 6-aminoquinoline (AMQ). Subsequently, AMQ can react with excess AQC reagent to form a bis urea. Both of these side products do not interfere with the identification of any of the amino acids. The derivatives are stable for days, permitting batch -wise processing. Derivatisation chemistry is used in both the UPLC® AAA Solution and in the ACQUITY UPLC H-Class Amino Acid Analysis System.*

The Foods and Feeds standard prepared using the *AccQ•Tag™ Ultra Amino Aid Analysis kit* is run on the *ACQUITY UPLC H-Class Bio system*, Figure 5.

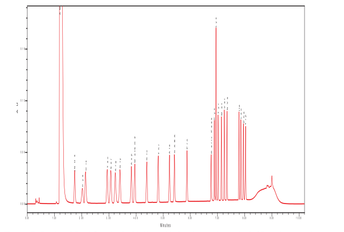

Figure 5. *Food and Feed Standard with Nva added as internal standard. 25pmoles on column.*

Optimisation Of Hydrolysis Protocols

Hydrolysis conditions must be optimized for microwave processing. The samples were subjected to varying hydrolysis time with constant temperature. The samples were also hydrolysed at different temperature points while holding the time of hydrolysis constant, (Figure 6.a. and 6.b.). The total amount of amino acid yield from the above mentioned optimization experiments are summarized in Figures 7a. and 7b.

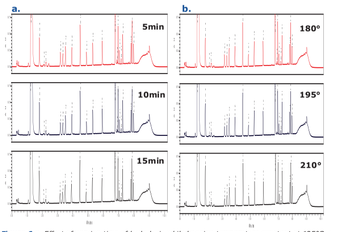

Figure 6.a. *Effect of varying time of hydrolysis while keeping temperature constant at 195°C ;* ***6.b.*** *Effect of varying temperature while keeping time constant at 15min.*

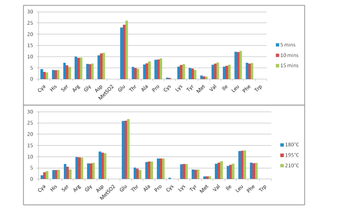

Figure 7. *Total Amino acids in mg/g of solid feed:* ***7.a.*** *when the time is varied keeping temperature constant at 195°C ;* ***7.b.*** *when the temperature is varied keeping time constant at 15min.*

Hydrolysis is sensitive to both time and temperature. Some amino acids are gradually destroyed during hydrolysis while others are released slowly. Optimization was tested by varying temperature (180 °C, 195 °C and 210 °C) and time (5min, 10min, 15min and 30min). Amino acid weights were normalized to Phenylalanine to detect trends.

The proportions of amino acids in Table 1 suggest that increasing the temperature and time during acid hydrolysis results in a decrease in the yield of serine, an unstable amino acid. Valine and isoleucine yields increase at the higher temperatures and times. The shortest hydrolysis time is associated with lower yields of many of the amino acids but most dramatically the hydrophobic ones.

Table 1. *Normalized weights of amino acids with respect to Phenylalanine during acid hydrolysis optimization experiments*

HCl	180°C	195°C				210°C
	15Min	5Min	10Min	15Min	30Min	15Min
Cya	0.225	0.609	0.469	0.421	0.636	0.479
His	0.558	0.553	0.571	0.563	0.538	0.569
Ser	0.919	0.992	0.898	0.768	0.686	0.585
Arg	1.343	1.360	1.365	1.347	1.318	1.309
Gly	0.951	0.933	0.955	0.969	0.982	0.998
Asp	1.676	1.440	1.654	1.643	1.440	1.594
$MetSO_2$	0.000	0.000	0.000	0.000	0.000	0.000
Glu	3.551	3.144	3.498	3.637	3.762	3.682
Thr	0.710	0.741	0.717	0.642	0.588	0.561
Ala	1.048	0.902	1.010	1.098	1.062	1.071
Pro	1.261	1.185	1.269	1.289	1.190	1.264
Cys	0.078	0.093	0.081	0.000	0.000	0.000
Lys	0.904	0.759	0.911	0.938	0.825	0.924
Tyr	0.587	0.681	0.681	0.573	0.610	0.581
Met	0.171	0.221	0.181	0.167	0.198	0.191
Val	0.933	0.871	0.998	1.034	1.115	1.099
Ile	0.808	0.765	0.859	0.897	0.947	0.947
Leu	1.713	1.661	1.735	1.758	1.735	1.753
Phe	1.000	1.000	1.000	1.000	1.000	1.000
Trp	0.000	0.000	0.000	0.000	0.000	0.000

The purpose of the Alkaline hydrolysis is to obtain the best value for tryptophan. The amount of tryptophan is low and more variable than observed for some other amino acids. The yield of tryptophan does not show any trends with increasing temperature or time. Valine increases with an increase in temperature and time. The yields of the polar amino acids are relatively low in alkaline hydrolysis compared to the acid hydrolysis. The observed proportions of amino acids in the alkaline hydrolysis are more variable than with acid hydrolysis suggesting that more aggressive hydrolysis conditions should be considered for more complete characterization, Table 2.

Table 2. *Normalized weights of amino acids with respect to Phenylalanine during alkaline hydrolysis optimization experiments.*

NaOH	180°C	195°C				210°C
	15Min	5Min	10Min	15Min	30Min	15Min
Cya	0.025	0.000	0.000	0.000	0.000	0.000
His	0.280	0.000	0.000	0.000	0.000	0.000
Ser	0.556	0.242	0.167	0.095	0.084	0.000
Arg	0.660	0.000	0.000	0.000	0.000	0.000
Gly	1.355	1.766	1.520	1.392	1.287	1.254
Asp	2.038	2.385	2.092	2.005	1.873	1.785
$MetSO_2$	0.000	0.000	0.000	0.000	0.000	0.000
Glu	4.101	4.568	4.394	4.229	4.123	4.106
Thr	0.346	0.000	0.000	0.000	0.000	0.000
Ala	1.371	1.721	1.578	1.508	1.472	1.465
Pro	1.347	1.467	1.420	1.334	1.322	1.295
Cys	0.089	0.097	0.090	0.098	0.069	0.094
Lys	0.977	1.085	1.027	0.991	0.999	0.987
Tyr	0.781	0.967	0.973	0.979	0.996	0.998
Met	0.247	0.391	0.368	0.331	0.317	0.322
Val	0.720	0.542	0.537	0.545	0.568	0.619
Ile	0.468	0.160	0.162	0.171	0.260	0.248
Leu	1.704	1.696	1.735	1.725	1.756	1.759
Phe	1.000	1.000	1.000	1.000	1.000	1.000
Trp	0.129	0.322	0.281	0.268	0.299	0.284

Complete Nutritional Analysis

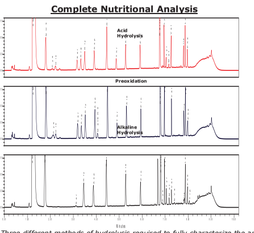

Figure 8. *Three different methods of hydrolysis required to fully characterize the amino acid composition in food and feed samples.*

Table 3.*Total Amino acids in mg/g of solid feed using three different protocols.*

AA (mg/solid g)	Acid Hydrolysis	Pre-oxidation	Alkaline Hydrolysis
Cya	3.012	9.394	0.000
His	4.029	3.490	0.000
Ser	5.495	6.807	0.563
Arg	9.647	9.700	0.000
Gly	6.936	7.786	8.258
Asp	11.761	13.891	11.895
MetSO2	0.000	1.445	0.000
Glu	26.038	29.075	25.094
Thr	4.595	5.495	0.000
Ala	7.864	8.284	8.945
Pro	9.229	9.686	7.918
Cys	0.000	0.000	0.579
Lys	6.712	7.139	5.878
Tyr	4.100	0.000	5.808
Met	1.199	0.000	1.965
Val	7.404	7.930	3.234
Ile	6.422	6.775	1.014
Leu	12.584	13.418	10.236
Phe	7.159	7.030	5.934
Trp	0.000	0.000	1.593
Total AA	**134.185**	**147.345**	**98.914**

CONCLUSIONS

- **Three protocols are required to describe completely the nutritional content of a sample.**
- **Microwave hydrolysis can be used with all three protocols, dramatically reducing sample preparation time from 24 hours to 15 mins.**
- **The amino acid analysis procedure is compatible with all three protocols.**
- **The combination of microwave hydrolysis and UPLC amino acid analysis improves throughput for nutritional analysis of feeds to approximately 50 samples in a 24 hour period.**

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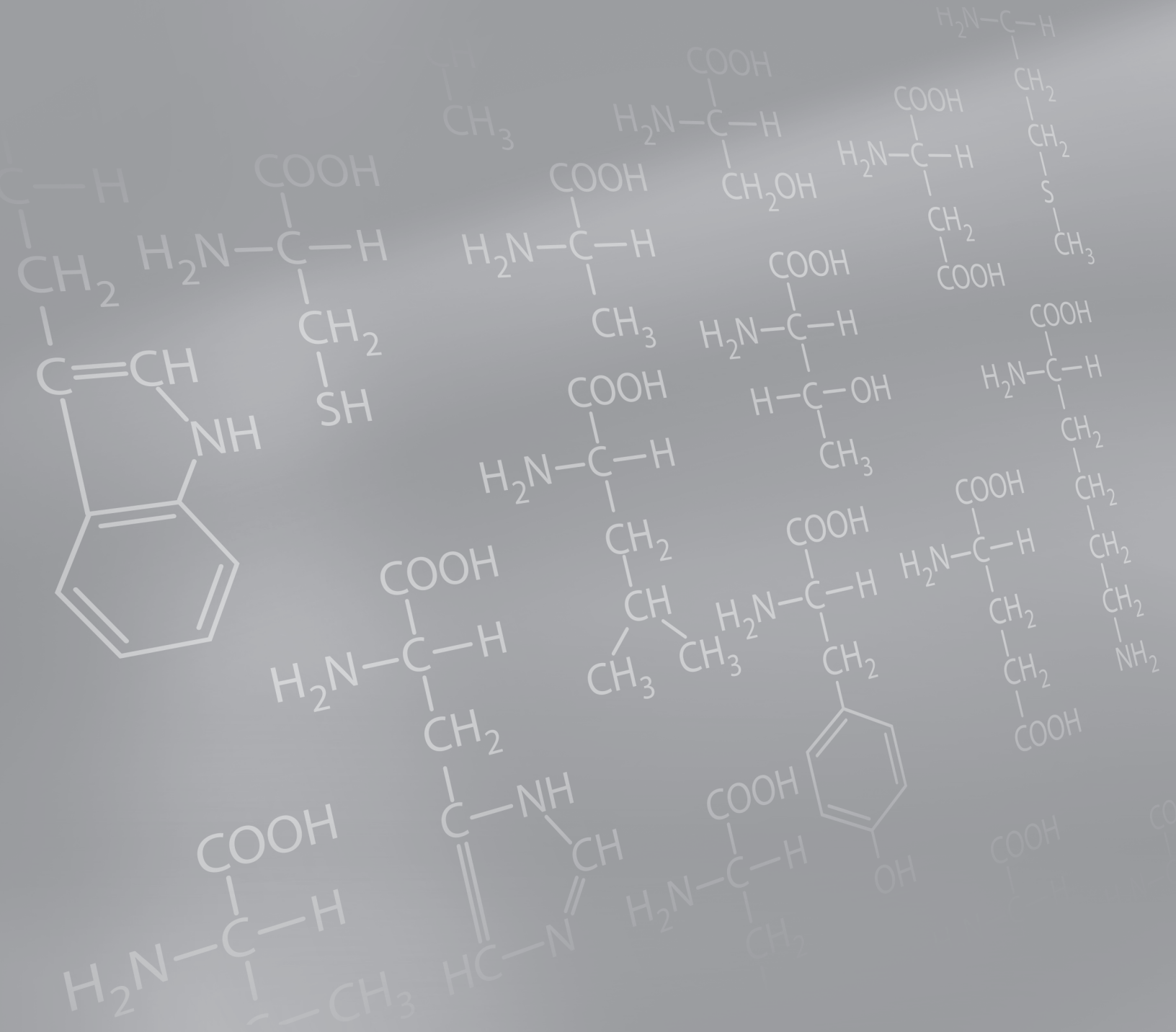
H-CLASS AND MICROWAVE HYDROLYSIS

Complete Amino Acid Analysis of Food and Feeds

Other and References



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A Validated Bioanalytical Method for the Quantification of Biogenic Amines Using UPLC-MS/MS

Prof Ian D Wilson[1] and Robert S Plumb[2]
[1]Dept. Surgery & Cancer, Imperial College London, UK, [2]Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

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KEYWORDS

Biogenic amines, UPLC-MS/MS, validated assay, amino acids, AccQ•Tag

INTRODUCTION

Blood concentrations of amino acids and other amino-containing compounds change in response to different conditions, including toxicity and metabolic diseases (inborn errors, metabolic syndrome, diabetes, obesity, cancer, etc). As such, there is a need for a rapid, targeted method for their quantification in biological fluids to support metabolic phenotyping studies. Currently, mass spectrometry- (MS) based methods for the quantification of amino compounds using MS for detection include procedures based on GC-MS, CE-MS/MS, and LC-MS/MS.[1-3] Reverse-phase liquid chromatography (RPLC)-based methods are complicated by the polar, amphoteric nature of these analytes, which can result in poor retention and make direct analysis impractical for all but a few analytes. While alternative modes of liquid chromatography (cation exchange, HILIC, ion-pairing) can be used, they are not without disadvantages. As a result, modifying the chromatographic properties of this class of compounds to form more retentive derivatives for RPLC remains popular. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ•Tag reagent) was originally used to analyze primary and secondary amines via fluorescence detection, but can also enable detection of these analytes via LC-MS. This application note describes the use of AccQ•Tag Ultra reagent combined with UPLC-ESI-MS/MS for the analysis of amino acids and biogenic amines in human plasma or serum via a high-throughput, sensitive, and specific method.

EXPERIMENTAL

Human plasma/serum (10 µL) is mixed with Optima™ grade water (10 µL), 5 µL of a 10 µg/mL internal standard (IS) mixture, and 40 µL of cold isopropanol–0.1% formic acid (v/v). Next, it is vortexed to precipitate proteins. After 20 minutes (-20 °C), samples are centrifuged (≥10,000 g, 10 minutes). For derivatization, 10 µL of supernatant mixed with 70 µL of borate buffer (pH 8.6) was treated with 20 µL of the AccQ▪Tag Ultra reagent solution (prepared according to Waters® instructions) with vortex mixing, heated at 55 °C (10 minutes), then diluted 1 in 100 with Optima grade water for UPLC-MS.

Method conditions

The method was validated, as far as practicable; using the approach outlined in the FDA "Guidance for Industry" Bioanalytical Methods – which covers aspects such as intra- and inter-assay precision, specificity, carryover, recovery, matrix effects, and stability.

LC conditions

LC system:	ACQUITY UPLC I-Class
Detection:	Xevo TQ-S
Column:	ACQUITY UPLC HSS T3, 1.8 µm, 2.1 mm x 150 mm (P/N 186003540)
Column temp.:	45 °C
Sample temp.:	4 °C
Injection volume:	2 µL
Flow rate:	0.6 mL/min
Mobile phase A:	0.1% formic acid in H_2O (v/v)
Mobile phase B:	0.1% formic acid in ACN (v/v)

Gradient

The starting composition was 4% B held for 0.5 minutes, before increasing to 10% over 2 minutes, then to 28% over 2.5 minutes, increasing to 95% for 1 minute to wash the column, and finally returning to 4% B for a 1.3 minute re-equilibration step.

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI+
Acquisition range:	MRM detection
Capillary voltage:	1 kV
Collision energy:	Analyte-dependent
Cone voltage:	50 V

Data management

MassLynx® Software with TargetLynx™ Application Manager

RESULTS AND DISCUSSION

The AccQ•Tag Ultra reagent reacts with primary and secondary amines, giving derivatives with good chromatographic and mass spectrometric properties. MS conditions for positive ESI were obtained via direct infusion of the individual derivatives for determination of the parent ion and best multiple reaction (MRM) transitions, dwell times, cone voltages, collision energies, etc.

The AccQ•Tag Ultra reagent gives rise to a common fragment ion at *m/z* 171, generated by a loss of the aminoquinoline (AMQ) moiety; in most – but not all – cases the combination of parent ion and this common fragment was selected for detection. Monoamines form a mono-ACQ derivative, giving the [M+AccQ+H]+ ion, while polyamines (e.g. cystine, lysine) can form derivatives containing more than one ACQ unit. The chromatographic method requires only 7.5 min/sample including column re-equilibration, allowing high-throughput analysis (Figure 1). A good separation is provided for a number of isomers/isobars such as 1- and 3-methylhistidine; sarcosine, β-alanine, and alanine; γ-amino-n-butyric acid, β-aminoisobutyric acid, and α-amino-n-butyric acid; isoleucine and leucine.

METHOD VALIDATION

Because obtaining true biological blanks for these analytes is not possible, standard curves and QC samples were prepared in 50:50 water–methanol (v/v). Investigated were the linearity; lower and upper limits of quantification (LLOQ, ULOQ); inter- and intra-day accuracy and precision; specificity; carry over; recovery; matrix; and analyte and derivative stability. Standard curves were linear over the range 1–400 µm/mL (effectively 2–800 µm/mL for plasma/serum due to sample dilution prior to derivatization) with correlation coefficients (R) of 0.993 or better determined over the three days of the validation. Stock solutions of the underivatized analytes were stable when either left for six hours at ambient temperature or frozen at -20 °C for 48 hours. The derivatized analytes were stable at either ambient temperature or in the autosampler at 4 °C for up to one week. Carryover was found to be acceptable at 11% or less for all of the analytes tested while carryover of the internal standards was less than 1%.

The intra- and inter-day performance of the assay was evaluated by the analysis of samples on three separate occasions on three separate days over the concentration range of 2–800 µm, and the method was found to be reproducible and accurate. For both intra- and inter-day analysis, a lower limit of quantification of (LLOQ) of 2 µm was obtained using isotopically labeled internal standards for 17 of the amino acids (inter-day data in Table 1). A further eight of the target gave acceptable results using a LLOQ of 6 µm, while the remaining seven compounds considered for absolute quantification had LLOQs of 20 µm (Table 1). The ULOQ for all analytes was 800 µm. The additional 20 amino compounds monitored here for relative quantification were not included in the validation study.

The intra- and inter-day precision of the assay ranged from 0.91–16.9% for the intra-day determinations and 2.12–15.9% for the inter-day comparison. The corresponding values for intra- and inter-day accuracy were 0.05–15.6% and 0.78–13.7% respectively. Specificity, in terms of matrix and other interferences, was assessed and found acceptable for matrix to analyte (at the LLOQ), matrix to internal standard, analyte to internal standard, and internal standard to analyte interference, etc.

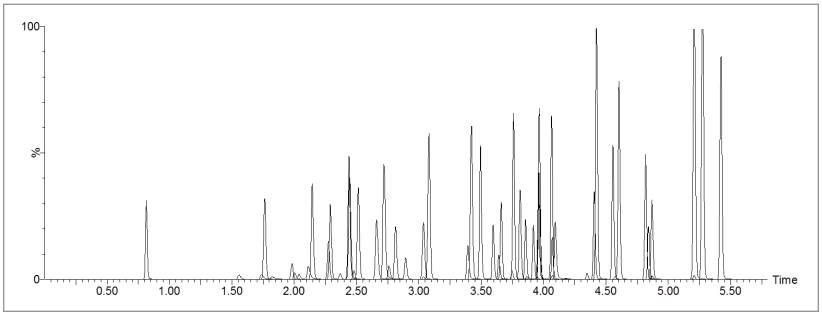

Figure 1. Chromatographic separation of all of the analyte standards monitored by the method at 400 µm (240 fmol on-column).

Amino acid	Mean Inter-day Concentrations (Coefficients of Variation (%))						
	1 µM	3 µM	10 µM	30 µM	150 µM	300 µM	400 µM
4-hydroxyproline	1.05 (10.7)	3.08 (5.03)	10.4 (5.17)	31.0 (4.75)	156 (4.12)	311 (4.88)	415 (4.21)
Alanine	1.2 (10.2)	3.08 (4.54)	10.03 (3.95)	31.7 (3.37)	157 (4.06)	314 (3.88)	417 (4.02)
Arginine	–	–	10.5 (8.49)	30.3 (8.75)	153 (7.92)	298 (8.18)	494 (6.87)
Aspartic acid	0.97 (16.9)	3.07 (6.20)	10.3 (2.82)	31.4 (3.86)	155 (3.73)	307 (3.59)	411 (4.90)
Asparagine	0.95 (8.06)	2.90 (3.29)	9.79 (3.06)	30.0 (1.80)	151 (3.69)	300 (5.97)	401 (2.12)
Carnosine	–	–	10.48 (5.31)	31.0 (6.54)	151 (3.31)	305 (5.45)	394 (6.41)
Cystine	1.07 (8.41)	3.24 (9.51)	11.3 (6.23)	33.2 (2.50)	166 (7.67)	3.23 (5.62)	434 (3.75)
Ethanolamine	1.03 (5.04)	3.12 (4.22)	10.3 (1.15)	31.2 (3.97)	155 (3.26)	303 (5.05)	404 (2.36)
Glutamic acid	1.0 (7.11)	3.04 (2.23)	10.3 (3.44)	31.9 (2.49)	158 (4.17)	312 (2.63)	416 (2.67)
Glutamine	4.36	3.38	9.66 (6.01)	29.4 (3.73)	147 (4.07)	289 (4.72)	383 (5.74)
Glycine	–	–	10.6 (4.59)	32.1 (3.30)	155 (2.61)	302 (4.48)	399 (4.48)
Histidine	–	3.14 (7.60)	10.4 (5.75)	32.3 (4.41)	156 (5.39)	313 (4.84)	417 (5.49)
Isoleucine	1.03 (3.13)	3.07 (2.25)	10.3 (1.37)	31.6 (3.16)	157 (3.66)	312 (4.49)	419 (3.02)
Leucine	1.00 (6.38)	3.02 (2.00)	10.2 (1.05)	31.4 (2.54)	156 (3.59)	315 (4.55)	426 (3.47)
Lysine	1.06 (4.66)	3.12 (3.62)	10.3 (4.57)	31.9 (3.38)	157 (4.83)	312 (3.90)	416 (3.37)
Methionine	1.06 (3.13)	3.10 (3.22)	10.3 (1.63)	31.7 (3.50)	157 (2.98)	309 (4.14)	413 (2.59)
Phenylalanine	1.05 (3.97)	3.07 (2.50)	10.3 (1.57)	31.6 (3.50)	156 (3.37)	309 (4.36)	410 (2.84)
Proline	–	3.00 (8.45)	10.0 (7.15)	31.4 (5.56)	157 (4.35)	314 (5.01)	421 (3.73)
Serine	–	3.12 (4.92)	10.2 (3.97)	31.6 (2.57)	158 (3.50)	309 (5.47)	417 (3.68)
Threonine	1.04 (3.72)	3.04 (2.39)	10.3 (1.74)	31.8 (2.96)	156 (2.95)	311 (3.60)	414 (1.68)
Tryptophan	1.12 (3.95)	3.21 (2.28)	10.8 (0.91)	33.2 (3.02)	166 (3.38)	324 (2.92)	434 (2.34)
Tyrosine	1.09 (8.80)	3.17 (7.44)	10.6 (3.47)	31.5 (5.83)	162 (3.76)	321 (7.47)	438 (4.01)
Valine	1.03 (3.56)	3.06 (3.61)	10.3 (3.50)	31.4 (2.19)	157 (3.31)	309 (2.44)	417 (2.27)
β-amino-iso-butyric acid	–	3.10 (3.58)	10.3 (1.87)	31.3 (3.74)	153 (6.46)	300 (4.22)	396 (4.42)
Citrulline	–	3.12 (9.83)	10.3 (4.42)	31.2 (4.63)	158 (3.60)	312 (5.54)	413 (3.31)
Cystathionine	1.06 (11.3)	3.16 (4.94)	10.6 (4.46)	32 (2.65)	160 (3.58)	323 (3.37)	431 (3.98)
3-methylhistidine	–	–	9.90 (4.38)	29.2b (5.39)	145 (5.25)	288 (3.88)	398 (3.15)
1-methylhistidine	–	–	10.8 (3.84)	30.6 (5.76)	153 (7.10)	305 (6.06)	413 (5.27)
Hydroxylysine	–	–	10.2 (10.7)	30.6 (5.29)	154 (4.26)	303 (2.71)	404 (3.89)
Ornithine	–	–	9.53 (12.7)	29.5 (3.30)	151 (6.92)	304 (4.84)	414 (4.51)
Aminoadipic acid	1.07 (4.10)	3.00 (3.59)	10.2 (1.79)	31.8 (3.87)	156 (6.51)	308 (3.59)	406 (2.71)
α-amino-n-butyric acid	–	3.12 (8.81)	10.5 (3.95)	31.4 (2.64)	154 (4.73)	295 (6.25)	393 (1.61)
Sarcosine	1.09 (5.18)	3.15 (4.43)	10.5 (5.14)	31.4 (4.14)	154 (3.67)	316 (4.14)	426 (3.01)
γ-amino-n-butyric acid	(16.9)	3.05 (7.15)	10.2 (4.15)	31.4 (3.58)	154 (5.57)	303 (3.15)	396 (4.62)

Table 1. Inter-day mean concentration data and coefficients of variation for the quantified analytes.

CONCLUSIONS

The use of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ•Tag reagent) as a derivatizing reagent for the targeted analysis of amino acids and other amino-containing compounds provides these analytes with good reverse-phase liquid chromotography and mass spectrometry properties, and allows the sensitive and specific analysis of this class of compounds in human serum and plasma in methods that are well suited to high-throughput analysis using UPLC.

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January 2018 720006130EN IH-SIG