Sensitive Analysis of Underivatized Amino Acids Using UHPLC with Charged Aerosol Detection

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Overview

Purpose: To develop fast and sensitive approaches for the analysis of underivatized free amino acids and peptides using the latest UHPLC column technology and charged aerosol detection.

Methods: Presented here are two distinct methods using the same UHPLC analytical column. The first method uses large amounts of ion-pairing agents to resolve 18 free amino acids. The second method uses less ion-pairing agent and an inverse gradient approach that enables a direct comparison of charged aerosol detection to traditional UV techniques. Protein digestion is monitored on the autosampler tray.

Results: The first method enabled the analysis of 18 amino acids in under 9 minutes with low nanogram sensitivity for all analytes. The tryptic digest method not only allowed the separation of amino acid residues and peptides, but the time course of digestion could be followed using this automated system. The results showed that charged aerosol detection is a complementary technique to the well established low wavelength UV with added sensitivity for small peptides and many amino acids.

Introduction

The need to determine free amino acid levels is common to many markets and industries. Unfortunately, separation and detection of underivatized amino acids is difficult as many of them are structurally similar, and few possess adequate chromophores. The combination of UV or fluorescence detection with derivatization, either pre- or post-column, may overcome some of these issues, but often adds time and complexity to the method. Presented in the first part of this poster is a simple UHPLC gradient method using a Thermo Scientific Acclaim RSLC Polar Advantage II 2.2 µm 2.1 × 250 mm column with charged aerosol detection for the measurement of 18 underivatized amino acids. Retention of hydrophilic amino acids was achieved by the addition of volatile ion-pairing agents (heptafluorobutyric acid HFBA, and trifluoroacetic Acid TFA) to the mobile phase. Although analyte response across a wide mass range measured by charged aerosol detection is non-linear, a new feature in the Thermo Scientific Dionex Corona ultra RS Charged Aerosol Detector (CAD™)–the internal power transformation algorithm–can be used to linearize the detector's output. This user-defined power function value (PFV) not only improves linearity but also positively impacts resolution and signal-to-noise at levels above the limit of quantification.[1,2] Several PFVs were used to illustrate the benefits of this new feature.

The second part of the poster examines the use of the Corona™ ultra RS™ Charged Aerosol Detector for universal quantification of amino acid and peptide residues in protein digest samples. Charged aerosol detection is a highly sensitive universal technique that can deliver near uniform response for non-volatile analytes.[3] One issue found with all nebulization-based techniques is that detector response will change during gradient elution due to changes in nebulization efficiency. This can easily be overcome by applying solvent compensation using an inverse gradient after the analytical column but before the detector.[4,5] The combination of the Corona ultra RS with the Thermo Scientific Dionex UltiMate 3000 RSLC system equipped with a dual gradient pump (DGP) forms a single analytical platform capable of delivering highly sensitive and near uniform results for non-volatile compounds. CAD and UV detection for monitoring digestion of bovine serum albumin by trypsin were compared. This technique offers a complementary approach to the traditional UV at 214 nm, provides more uniform response for both large and small peptides, and the ability to measure all free amino acids even those that are undetected by UV because of weak chromophores.

Methods

Sample Preparation

Standards were prepared as mixes at 2 mg/mL in deionized water. They were then serially diluted 10 different times to give 11 working standards for each mix.

A sample of liquid protein standard 2 mg/mL bovine serum albumin (Sigma Aldrich, St. Louis, MI.) was diluted 1/4 in 100 mM Tris HCl pH 8.5. Lyophilized trypsin from porcine pancreas 20 µg (Sigma) was reconstituted in 20 µL of 1 mM HCl. 1 mL of the diluted protein was added to the 20 µL of Trypsin solution and vortexed. This sample was then transferred to an autosampler vial and put on the tray set to 36 ^{0}C.The sample was injected at 9 time points over the next 21 hours with tray shaking prior to injection.

Liquid Chromatography

System: UltiMate™ 3000 RSLC system
equipped with: HPG-3400RS, WPS-3000TRS, TCC-3000RS, DAD-3000RS, Corona ultra RS

Column: Acclaim™ RSLC Polar Advantage II
2.2 µm 2.1 × 250 mm

Free Amino Acid Method:

Mobile Phase A: 0.4% Heptafluorobutyric acid + 0.02% Trifluoroacetic acid in deionized water

Mobile Phase B: 0.1% Trifluoroacetic acid in acetonitrile

Column Temperature: 20 °C

Flow Rate: See Gradient Table

Gradient: See Table

Injection Volume: 2 µL

CAD settings: Filter: 3
Nebulizer Temp On at 10 °C
Power Function Value 1.0

Time (min)	Flow (mL/min)	% mobile phase B
0	0.4	99
1	0.4	99
3	0.7	95
10	0.7	40
12	0.7	40
13	0.7	95
17	0.4	99

Tryptic Digest Method:

System and Column: as above but now with a DGP-3600RS pump

Mobile Phase A: 0.1% Trifluoroacetic acid in deionized water

Mobile Phase B: 0.1% Trifluoroacetic acid in acetonitrile

Tray Temperature: 40 °C

Column Temperature: 35 ºC

Flow Rate: 0.75 mL/min

Gradient: See Gradient Tables

Injection Volume: 20 µL

Diode Array Settings: slit-width: narrow
reference: off
UV 1 at 254 nm
UV 2 at 214 nm

Analytical Gradient		
Time (min)	% mobile phase A	% mobile phase B
0	98	2
10	50	50
15	50	50
18	98	2
23	98	2

CAD settings: Filter: 3
Nebulizer Temp On at 10 ºC
Power Function Value 1.0
Flow Diverted 0.7 – 1.1 minutes

Compensation Gradient		
Time (min)	% mobile phase A	% mobile phase B
0	2	98
0.95	2	98
10.95	50	50
15.95	50	50
18.95	2	98
23	2	98

Data Analysis

Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) was used for all data collection and processing.

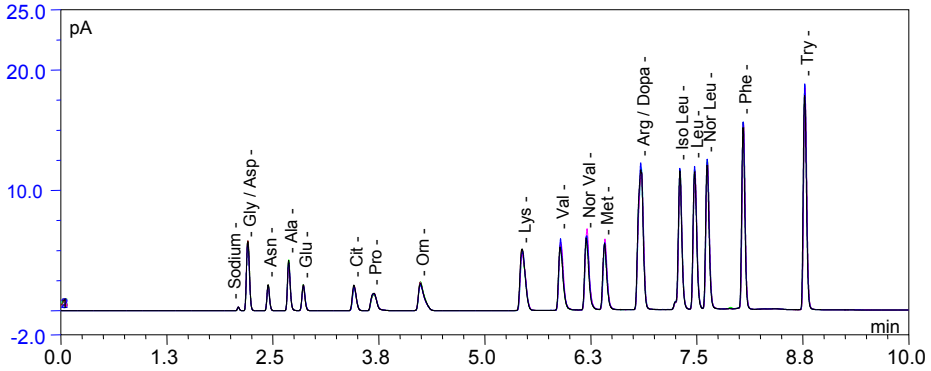
Results

Amino Acid Analysis

Adequate retention and resolution of the small highly polar amino acids (including glycine, serine, alanine, aspartic and glutamic acids) is one of the most difficult challenges when working with underivatized amino acids. While other HILIC techniques offer some potential solutions, they lack the resolution power for the full range of naturally occurring amino acids. The initial development of this method began at 100% aqueous with only the one ion pairing agent[5]. While this approach showed excellent resolution for these initial analytes, the overall retention time reproducibility was not adequate. This problem was overcome by the addition of a small amount of TFA into mobile phase A, the addition of 0.1% TFA to mobile phase B and the use of 1% organic phase B at the start of the analysis. Figure 1 shows an overlay of six injections of standard containing 19 amino acids at ~ 0.5 mg/mL. The retention time reproducibility was ≤ 0.1% for all peaks.

Amino Acid Abbreviation Key							
glycine	Gly	proline	Pro	methionine	Met	phenylalanine	Phe
aspartic acid	Asp	ornithine	Orn	3,4-dihydroxy-phenylalanine	DOPA	tryptophan	Try
asparagine	Asn	lysine	Lys	isoleucine	Iso Leu	serine	Ser
glutamic acid	Glu	valine	Val	leucine	Leu	tyrosine	Tyr
citrulline	Cit	nor-valine	Nor Val	norleucine	Nor Leu	histidine	His

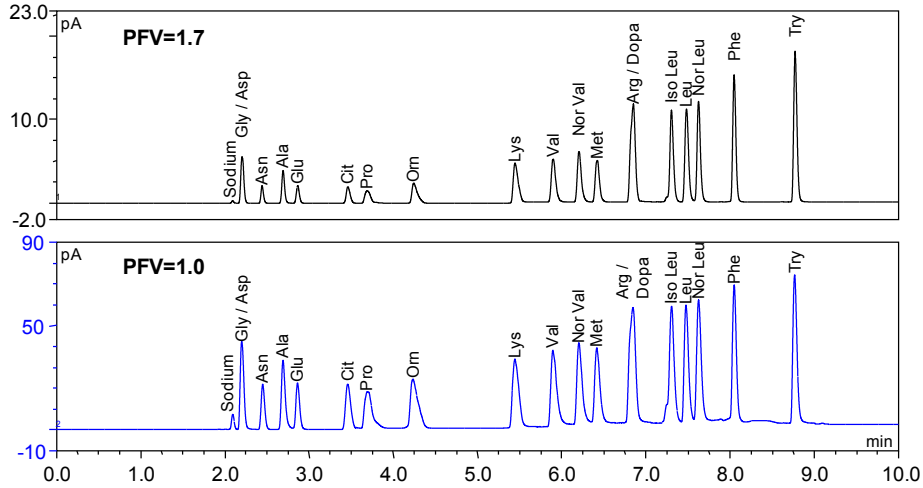
FIGURE 1: Repetitive analysis (n=6) of 19 amino acid standards.



In order to obtain good reproducibility with the method, the resolution between several analytes had to be sacrificed. Glycine and aspartic acid coeluted as seen in Figure 1. Serine and threonine also showed similar retention times as glycine but were not included in the standard mixture. Arginine and 3,4-dihydroxyphenylalanine (DOPA) also coeluted, but as DOPA is not typically found in proteins, this should not be an issue. Tyrosine was not included in this mixture due to solubility issues but it has a retention time similar to isoleucine.

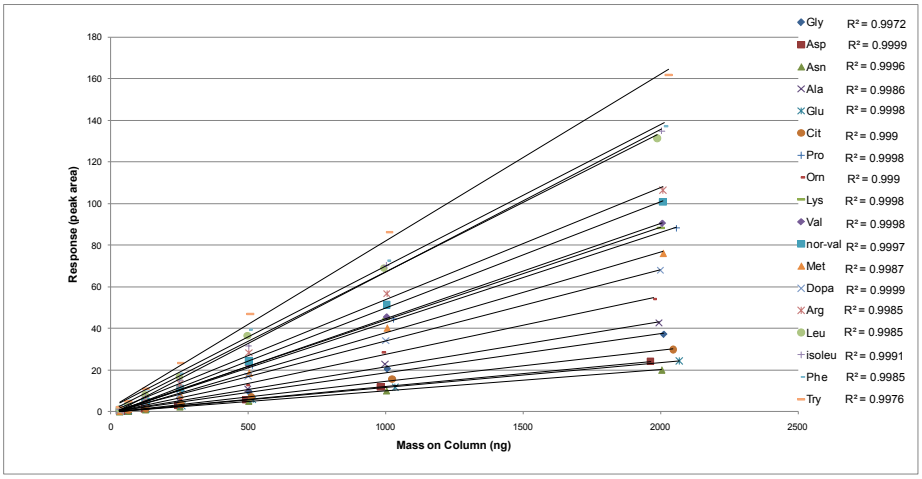
The CAD is a non-linear detector. As discussed previously, the use of a power transformation can correct for the curvature in the line and result in linearity over several orders of magnitude. The power transformation can also help to improve other observed chromatographic results.[1] Figure 2 shows the effect of changes in the PFV on the chromatographic separation of a 0.5 mg/mL mix when analyzed with a PFVs of 1.7 and 1.0 (no power transformation). The PFV of 1.7 typically results in an ~20% increase in calculated resolution, a 4 fold increase in the signal-to-noise ratio (at the level shown) and improved peak asymmetry for all analytes. However the use of the power function value has no impact on the absolute limit of detection for the method.

FIGURE 2: Comparison of the effect of different PFVs on chromatographic performance.



As already mentioned, the major reason for using the power transformation is to improve the linear dynamic range of the detector. The level of response depends on chromatographic conditions and is different for analytes that are eluting early (using nearly 100% aqueous conditions) compared to those eluting later (when the solvent composition is greater than 50% organic). This phenomenon prevents an optimal PFV choice for all analytes. Figure 3 displays the response curves for all 19 amino acids analyzed with a PFV set to 1.5. While some analytes such as phenylalanine and tryptophan require PFVs of 1.7 the value of 1.5 is optimal for the majority of the amino acids. The correlation coefficients for levels ranging from 30 to 2000 ng on column are ≥ 0.997 for the 7 point curves shown in Figure 3. The difference in the slopes of the lines is mostly the effect of the organic content on nebulization efficiency at the elution point for the given compound. This phenomenon can be corrected with the application of an inverse gradient as discussed in the next section.

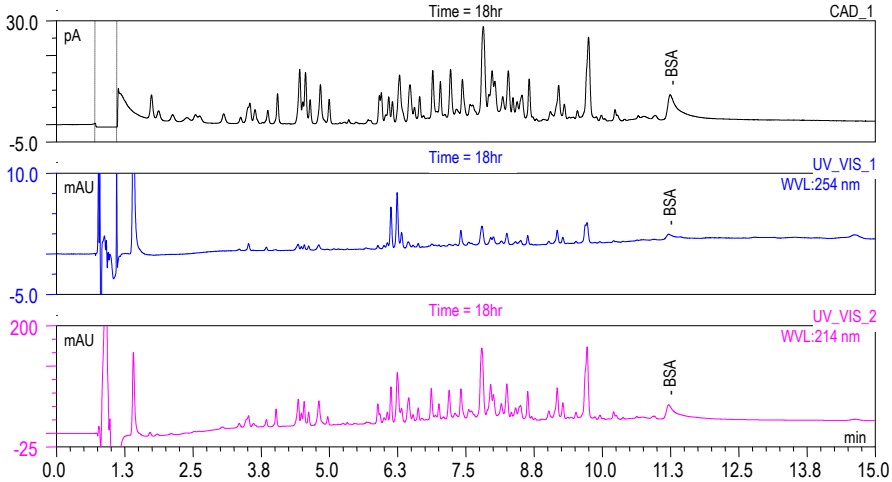
FIGURE 3: Linear calibration curves for all amino acids with a PFV of 1.5.



Analysis of Tryptic Digests

Selective cleavage of proteins by the enzyme trypsin is often used to help characterize larger proteins. While this typically requires an MS to obtain structural information, quantitative analysis relies on detection by UV at 214 nm. This is a well established technique and detects the amide bonds of the peptide that are UV active. However, depending on the specific protein, the type of protease used, and the time of digest, each can result in the production of varying amounts of small peptides and free amino acids, which are not always detected. Figure 4 compares the results from the CAD with those using UV at 254 and 214 nm. The UV at 254 nm is more selective and fewer peaks are observed relative to the other two detection channels. The low wavelength UV and the CAD appeared to show similar sensitivity and peak abundances for the majority of the peaks. However, the major difference can be observed in the region between 1 and 4 minutes of the chromatogram. Using this reversed phase chromatography system, peaks that elute earlier include free amino acids and small peptides, and these compounds exhibit much lower UV absorbance. As an inverse or compensation gradient is being used with the CAD, a more uniform response for free amino acids, small and large peptides is obtained. Their relative abundance is estimated more accurately without the need for individual standards.

FIGURE 4: The analysis of amino acid residues and peptides formed during the hydrolysis of BSA by trypsin. A comparison of relative peak abundance obtained by UV and charged aerosol detection.



The consistency of the digest depends on experimental conditions such as incubation time, temperature and other factors. In Figure 5, overlays are shown for CAD traces with reaction times of 2 hrs, 21 hrs and control, where no trypsin was added to the BSA sample. This time course was conveniently accomplished by heating the autosampler tray to a specific temperature and adding delay times prior to injections. This automation permitted the constant monitoring of the reaction without significant labor. The three chromatograms are displayed at the same scale and the relative abundance of the original BSA protein peak is clearly seen to diminish over time.

Table 1 shows the three detector channels raw area values for the two time points, with and without the addition of Trypsin. The percent area recovery for the CAD for all eight time points from 0 to 21 hours was within 12% of the expected value. The data from the 21 hour point was the closest to expected indicating a 100.2% recovery or within 0.5% of the expected value. The results using UV detection for the same analysis varied from 59 to 74% recovery at 254 nm and 47 to 78% at 214 nm. These results are expected since the production of small peptides and free amino acid increases over time and these compounds typically have poor chromophores. This is not a significant issue for the CAD since the total mass of analytes does not change and analyte response remains consistent when inverse gradient is used.

FIGURE 5: Time course for trypsin digestion of BSA monitored by the CAD.

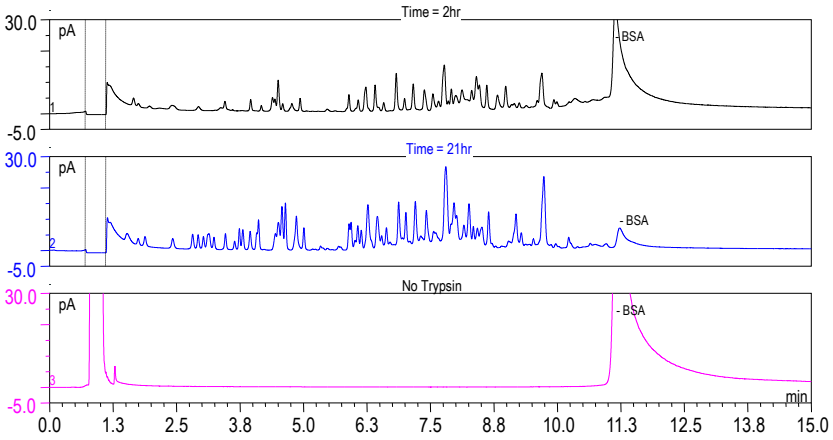


Table 1: Comparison of charged aerosol detection and UV data at 2 and 21 hours.

Table 1	2 hours			21 hours		
	CAD	UV 254	UV 214	CAD	UV 254	UV 214
Area BSA no Trypsin	26.73	3.10	180.26	26.73	3.10	180.26
Total Raw Area	23.69	2.30	141.69	26.79	1.90	91.74
% Area Recovery	88.7%	74.1%	78.6%	100.2%	61.4%	50.9%

Conclusion

- The fast and reproducible analysis of underivatized amino acids can be accomplished with the new RSLC Polar Advantage II column and Corona ultra RS detector.
- More than 18 amino acids were resolved in under 9 minutes. The CAD provides sensitivity at low nanogram on-column levels for all analytes.
- The power transformation feature available in the Corona ultra RS is effective at linearizing the data while also providing improvements in peak symmetry and resolution.
- The use of charged aerosol detection with gradient compensation in amino acid and peptide analysis can provide better quantitative results than traditional UV techniques.

References

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