Complete Amino Acid 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 sulfur 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 derivatized 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

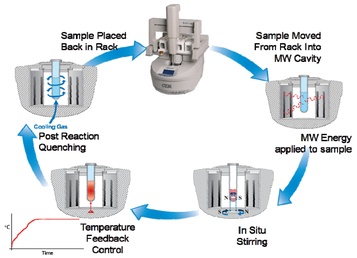

Figure 3. Microwave Hydrolysis Procedure

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.

Derivatization 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 neutralization step was carried out prior to derivatization.

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 ad 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 ad 3

RESULTS

Derivatization Chemistry

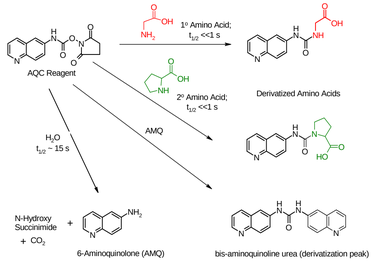

Figure 4. Reaction of AQC reagent with amino acids. The 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent reacts with both primary and secondary amines. Excess reagent reacts with water to form 6-aminoquinoline (AMQ). Subsequently, AMQ can react with excess AQC reagent to form a bis urea. 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. Derivatization 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.

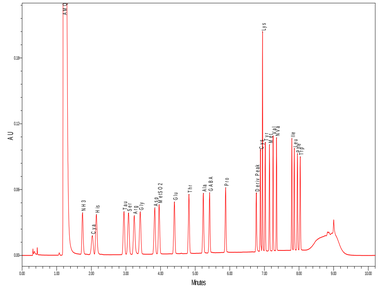

Figure 5. Food and Feed Standard with Nva added as internal standard. 25pmoles on column.

Optimization 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 hydrolyzed 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.

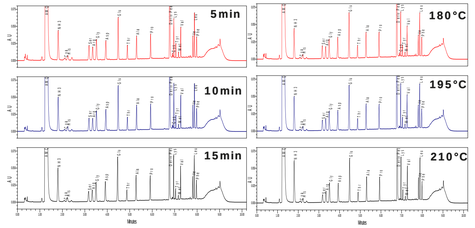

Figure 6.a. Effect of varying time of hydrolysis while keeping temperature constant at 195°C ; 6.b. Effect of varying temperature while keeping time constant at 15min.

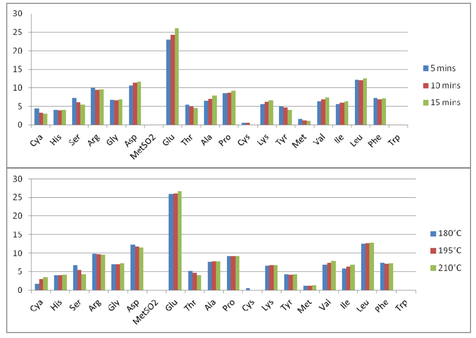

Figure 7. Total Amino acids in mg/g of solid feed: 7.a. when the time is varied keeping temperature constant at 195°C ; 7.b. when the temperature is varied keeping time constant at 15min

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.

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

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

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.

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

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

Complete Nutritional Analysis

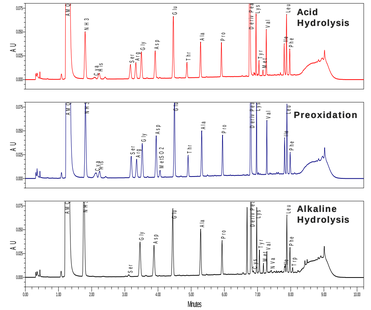

Figure 8. Three different methods of hydrolysis required to fully characterize the amino acid composition in food and feed samples.

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

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

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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