EZ:faast™
USER'S MANUAL

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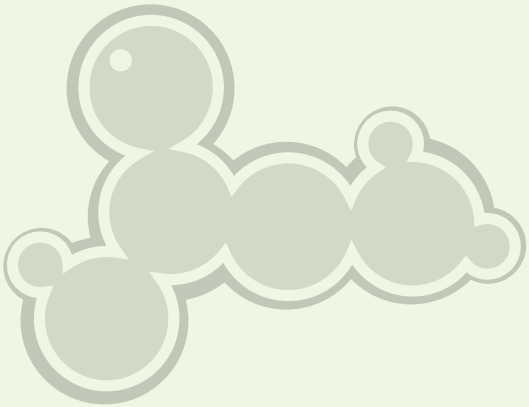
EZ faast™



EZ:faast™

Patent Pending

Amino Acid Analysis of Protein Hydrolysates by LC-MS

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EZ:faast™ For Amino Acid Analysis of Protein Hydrolysate by LC-MS

2570_L_6

Table of Contents

1.0 Kit Components

1.1 Reagents

Reagent 1	Internal Standard Solution	50mL
Reagent 2	Sodium Carbonate Solution	90mL
Reagent 3A	Eluting Medium Component I	60mL
Reagent 3B	Eluting Medium Component II	40mL
Reagent 4	Organic Solution I	4 vials, 6mL each
Reagent 5	Organic Solution II	50mL
SD	Protein Amino Acid, Standard Mixtures	2 vials, 2mL each

1.2 Supplies

Sorbent tips in racks 4x96
Sample preparation vials 4x100
Vial rack 1
Microdispenser, 20-100µL 1
Syringe, 0.6mL 10
Syringe, 1.5mL 10
EZ:faast AAA HPLC Column 1
Autosampler vials with inserts 4x100

User Manual 1
EZ:faast Demo Video and Reference CD 1

1.3 Materials Required but Not Supplied In Kit

- 100µL-1mL pipette (SoftGrip™ pipette [Phenomenex P/N AH0-5968] or equivalent)
- 30-300µL pipette (SoftGrip™ pipette [Phenomenex P/N AH0-5967] or equivalent)
- Pipette tips (Phenex™ [Phenomenex P/N AH0-5917 (200µL) and AH0-5920 (1mL)] or equivalent)
- Vortex
- Vials of an appropriate volume, with caps (see section 3.2)
- Pasteur pipettes for sample transfer (see section 3.4 step 15)
- Container for proper waste disposal
- Reagents and supplies for Protein Hydrolysis

2.0 Overview

2.1 Overview

The EZ:faast amino acid analysis procedure consists of a solid phase extraction step followed by a derivatization and a liquid/liquid extraction; derivatized samples are quickly analyzed by liquid chromatography-mass spectrometry. The solid phase extraction is performed via a sorbent packed tip that binds amino acids while allowing interfering compounds to flow through. Amino acids on sorbent are then extruded into the sample vial and quickly derivatized with reagent at room temperature in aqueous solution. Derivatized amino acids concomitantly migrate to the organic layer for additional separation from interfering compounds. Organic layer is then removed, evaporated, and re-dissolved in aqueous mobile phase and analyzed on a LC/MS system. Total sample preparation time takes around 8 minutes and analysis is performed in around 12 minutes for a total start to finish time of around 20 minutes.

A video included with this kit demonstrates the simplicity of the procedure. Please be aware that some sample preparation steps described in the video may be different than what is described in this users manual. Please use the video as a general guide, but follow the exact steps and sequence described in this manual.

2.2 Amino Acids in Physiological and Protein Hydrolysate Samples

The EZ:faast method has been developed for the analysis of amino acids from protein hydrolysates (Table 1). In total, over 60 aliphatic and aromatic amino acids or related compounds can be analyzed with this kit (Table 2). Additional amino acids and related compounds may be analyzed with this kit. A brief adjustment of chromatographic and MS conditions may be required. Please contact your Phenomenex technical consultant for method modifications and other LC and GC amino acid kits.

Table 1 **-** Protein Amino Acids analyzed by the EZ:faast Hydrolysate Amino Acid Analysis Kit by LC/MS

Abbreviation	Alternate Abbreviations	Chemical Name
ALA	A	Alanine
ARG	R	Arginine
ASN**	N	Asparagine
ASP	D	Aspartic Acid
C-C	$(Cys)_2$	Cystine
GLN**	Q	Glutamine
GLU	E	Glutamic Acid
HIS	H	Histidine
HLY	HLYS; OHLys	Hydroxylysine (2 isomers)
HYP	OHPro	4-Hydroxyproline
ILE	I	Isoleucine
LEU	L	Leucine
LYS	K	Lysine
MET	M	Methionine
PHE	F	Phenylalanine
PRO	P	Proline
SER	S	Serine
THR	T	Threonine
TYR	Y	Tyrosine
TRP*	W	Tryptophan
VAL	V	Valine

**TRP is completely lost during acid hydrolysis; use alternative hydrolysis procedure to analyze for TRP*
*** ASN and GLN are quantitatively converted to ASP and GLU during acid hydrolysis*

Table 2 **-** The comprehensive list of amino acids and related compounds prepared by EZ:faast for LC/MS analysis **(internal standards listed in bold)**

Abbreviation	Alternate Abbreviation	Chemical Name	Retention Time (min)	Amino Acid MW	Derivative MW+1	Major MS^2 Frag. MW
EAM	—	Ethanolamine	2.6	61.08	148	106, 130
pGLU	—	Pyroglutamic acid	2.6	129.1	172	130
ARG	R	Arginine	2.7	174.2	303	286, 200
HARG	**—**	**Homoarginine**	**2.8**	**188.2**	**317**	**170, 257, 300**
GLN	Q	Glutamine	3.3	146.2	275	258, 215, 172
CIT	—	Citrulline	3.4	175.2	304	287, 244
ANS	—	Anserine	3.5	240.1	369	309, 212
MET-SO	—	Methionine Sulfoxide	3.5	165.2	294	234, 142
SER	S	Serine	3.7	105.1	234	174, 146, 216
ASN	N	Asparagine	3.8	132.1	243	201, 157
PHP	P-OHP	Proline-hydroxyproline (dipeptide)	3.8	228.2	357	297, 156
THE	—	Theanine	4.0	174.2	303	243, 215
HYP	—	4-Hydroxyproline	4.0	131.1	260	172, 200
3MHIS	—	3-Methyl-histidine	4.1	169.2	298	210, 238
1MHIS	—	1-Methyl-histidine	4.1	169.2	298	256, 238
GLY	G	Glycine	4.3	75.1	204	144, 162, 118
GPR	G-P	Glycine-proline (dipeptide)	4.3	172.2	301	158, 241
THR	T	Threonine	4.4	119.1	248	188, 230, 160
MET-SO_2	—	Methionine Sulfone	4.4	181.2	310	250, 222, 142
β-ALA	—	β-Alanine	5.2	89.1	218	158, 116
ALA	A	Alanine	5.2	89.1	218	158, 130
HLY	—	δ-Hydroxylysine	5.7	162.1	377	317
GABA	—	γ-Amino-n-butyric acid	5.7	103.1	232	172, 130
SAR	—	Sarcosine	5.7	89.1	218	158, 116
HTA	—	Histamine	5.8	111.1	284	198, 138
β-AIB	—	β-Aminoisobutyric acid	6.2	103.1	232	172, 130
ABA	—	α-Aminobutyric acid	6.6	103.1	232	172, 144
DABA	—	2,4-Diaminobutyric acid	6.7	118.1	333	273
ORN	O	Ornithine	6.9	132.1	347	287, 227, 201
CAR	—	Carnosine	6.9	226.2	441	381, 284, 353
LYS-ALA	K-A	Lysine-alanine (dipeptide)	6.9	217.2	432	317, 301, 170
MET	M	Methionine	7.1	149.2	278	218, 190
MET-*d3*	**—**	**Methionine-d3**	**7.1**	**152.2**	**281**	**193**
PRO	P	Proline	7.1	115.1	244	156, 184
Pe-Cys	—	Pyridyl Ethyl Cysteine	7.6	226.3	355	147, 106
LYS	K	Lysine	7.8	146.1	361	301, 170
ASP	D	Aspartic acid	7.9	133.1	304	216, 244
HIS	H	Histidine	7.9	155.1	370	284, 196, 224
TPR	—	Thiaproline	7.9	133.2	262	174, 202
HSER	—	Homoserine	8.1	119.1	230	188
VAL	V	Valine	8.3	117.1	246	158, 186

Table 2 - (continued)

Abbreviation	Alternate Abbreviation	Chemical Name	Retention Time (min)	Amino Acid MW	Derivative MW+1	Major MS^2 Frag. MW
—	—	Aspartame	8.3	294.3	423	—
GLU	E	Glutamic acid	8.4	147.1	318	258, 230, 172
TRP	W	Tryptophan	8.7	204.2	333	245, 273, 230
ETH	—	Ethionine	8.8	163.2	292	204
GLU-LYS	E-K	γ-Glutamyl-ε-lysine (dipeptide)	8.8	275.3	532	472, 412
Cm-Cys	—	Carboxymethyl Cysteine	8.8	179.2	350	290, 262
AAA	—	α-Aminoadipic acid	9.5	161.2	332	272, 244
LEU	L	Leucine	9.9	131.2	260	172
PHE	F	Phenylalanine	9.9	165.2	294	206
alLE	—	allo-Isoleucine	10.0	131.2	260	172, 200
ILE	I	Isoleucine	10.4	131.2	260	172, 130
ADN	—	Adrenaline	10.4	183.2	424	338, 382, 252
NLEU	—	Norleucine	10.5	131.2	260	172, 200
CYS	C	Cysteine	10.5	121.2	336	190, 248, 276
APA	—	Aminopimelic acid	11.0	175.2	346	286, 156, 198
DAM	—	Dopamine	11.0	153.1	412	266, 326
LAL	—	Lysinoalanine	11.1	233.2	430	370, 342
—	—	Pipecolic Acid	11.1	129.1	258	170,198,128
CTH	—	Cystathionine	11.3	222.3	479	419, 230
PABA	—	4-Aminobenzoic acid	11.5	137.1	266	224
HCYS	—	Homocysteine	11.6	135.2	350	204, 290
HPHE	**—**	**Homophenylalanine**	**11.6**	**179.2**	**308**	**220, 117, 308**
C-C	$(Cys)_2$	Cystine	11.7	240.3	497	248, 437, 306
TYR	Y	Tyrosine	12.4	181.2	396	308, 336, 294
DOPA	—	Dopamine	12.8	197.2	498	352, 412, 438
HC-CH	—	Homocystine	12.8	268.3	525	262, 465
Se-C-C	—	Selenocystine	12.9	334.1	593	296, 371

2.3 Storage and Stability

Store Reagents 1, 3B and 4 at 4°C. Store amino acid standard solutions in the freezer. All other components may be stored at room temperature. For your convenience, the bottom of the reagent box has been designed as a tray that can be easily lifted from the workstation and placed in the refrigerator when the kit is not in use for an extended period of time.

All components are guaranteed for 12 months from the date of purchase when stored at recommended temperatures and used as described in this manual. Please review the Instruction Manual included with the Drummond® Dialamatic Microdispenser for recommended usage and warranty information. Please observe recommendations for solvent bottle handling and syringe cleaning in Section 6.0 of this manual.

2.4 Safety

Although the concentration of all toxic components in any of the reagent bottles is low, for safety reasons the sample preparation station should be placed in an exhaust hood and protective gloves and goggles should be worn. When working with biological fluids, please take any necessary precautions to prevent infection with blood borne pathogens. Appropriate bio-safety precautions and disposal of bio-hazardous wastes should be followed.

3.0 Sample Preparation Procedure

3.1 Setup

The EZ:faast kit packaging has been designed as an efficient workstation. It holds a reagent tray, a vial rack, a pipette rack and a section for sorbent tips and vials. To speed up sample preparation it is recommended that the workstation be arranged as shown in figure 1a. When the kit is not in use for several days, the reagent tray (figure 1b) may be conveniently removed and placed in the refrigerator.

Workstation Arrangement - (Figure 1)

To speed up sample preparation it is recommended that the workstation be arranged as shown below.

Figure 1a

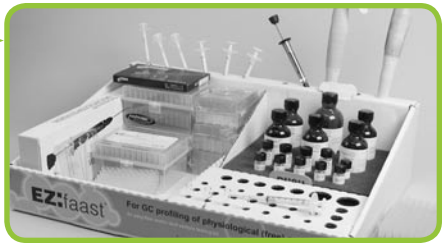


Figure 1b

3.2 Preparing the Eluting Medium

The volume of prepared Eluting Medium depends upon the number of samples to be analyzed during the day (200µL/sample). The eluting medium should be prepared fresh each day:

1. Use capped vials of appropriate size (not included) for preparation of the Eluting Medium
2. Combine 3 parts Reagent 3A (Eluting Medium Component I) with 2 parts Reagent 3B (Eluting Medium Component II) in an appropriate sized vial (see Table 3, page 6, for reagent volumes based on number of samples). Mix briefly.
3. Store prepared eluting medium during the day at room temperature. Discard any unused mixture at the end of the day.

Table 3 - For your convenience check the table below to determine the volume of Eluting Medium components needed depending on your number of samples:

Number of Samples	Reagent 3A Eluting Medium Component I	Reagent 3B Eluting Medium Component II
2	300µL	200µL
4	600µL	400µL
7	900µL	600µL
12	1.5mL	1.0mL
14	1.8mL	1.2mL
19	2.4mL	1.6mL
24	3.0mL	2.0mL
29	3.6mL	2.4mL
34	4.2mL	2.8mL
39	4.8mL	3.2mL
44	5.4mL	3.6mL
49	6.0mL	4.0mL

3.3 Protein Sample Hydrolysis

3.3.1 Background

There are numerous published methods for protein hydrolysis; all are compatible with analysis by the EZ:faast procedure with minor modifications to the described method. The most common methods use acid hydrolysis with 6M HCl in either liquid or vapor phase. (Stein and Moore, Methods in Enzymology 6, 819-831, 1963; Tarr *et. al.* In Microcharaterization of Proteins {J.E. Shively, ed.}, Humana Press {1986}). While these methods give good results for a majority of amino acids, there are several amino acids that are either partially or completely destroyed by such methods and alternate hydrolysis methods must be used. For convenience, a common liquid and vapor phase method are described; hydrolysis reagents and supplies are not included with the EZ:faast kit.

3.3.2 Vapor Phase Hydrolysis

The following is a sample method for vapor phase hydrolysis as a reference; other methods may work better for your application:

1. Transfer 1-20 nanomoles of protein into an autosampler vial insert.
2. Lyophilize sample in a vacuum concentrator.
3. In a hydrolysis vessel add: 989µL 6N constant boiling HCl, 10µL 5% Phenol, and 1µL betamercaptoethanol.
4. Add vial inserts into hydrolysis vessel and cap with minaret valve.
5. Place vessel in an ice bucket, and purge with nitrogen and vacuum several times and seal vessel under vacuum.
6. Hydrolyze in oven at 110ºC for 24 hours.
7. Cool vessel and remove vial inserts.
8. Dry down any remaining acid in sample vial using a speed vac evaporator. Pipette 100µL of Reagent 1 into sample vial to re-dissolve amino acids. (Section 3.4.2)
9. Perform EZ:faast procedure as per manual.

3.3.3 Liquid Phase Hydrolysis

The following is a sample method for liquid phase hydrolysis as a reference; other methods may work better for your application.

1. Transfer 5-25 nanomoles of protein into a glass test tube (10x150mm).
2. Lyophilize sample in a vacuum concentrator.
3. Add 100 µL of 6N HCl containing 4% Thioglycolic acid to tube.
4. Purge air from tube with vacuum and flame seal tube.
5. Hydrolyze in oven at 110ºC for 22 hours.
6. Cool tube, break seal, and perform EZ:faast procedure as per manual.

3.3.4 Limitations of Hydrolysis Methods

While 6N HCl acid hydrolysis is the most common procedure, there are several limitations to this method. ASN and GLN are deamidated to ASP and GLU, and thus are quantitated as a mixture. Peptide bonds of hydrophobic amino acids (VAL, ILE, LEU) may be difficult to break and require additional hydrolysis time (up to 72 hours). Residual oxygen in the hydrolysis vessel can increase the thermal breakdown of hydroxyl and sulfur containing amino acids (typical recoveries for SER, THR, HYP, and TYR range between 50-90%, MET ranges from 25-75%). Reducing hydrolysis time improves recoveries but reduces other amino acid yields (see above). GLY yields tend to exceed 100% (especially for low level samples) due to background protein contamination. Finally, both TRP and CYS are completely destroyed by acid hydrolysis and must be analyzed by alternate methods (see below). The above listed limitations are based on hydrolysis chemistry and are not related at all to the EZ:faast process.

3.3.5 Alternate Methods and References

For TRP analysis the use of either 4N Methane Sulfonic Acid, Dodecanethiol/HCl, or Thioglycolic acid has been shown to generate some useful results for TRP, however yields tend to be low for all of these methods.

For CYS analysis reduction and alkylation to generate either carboxymethyl cysteine or pyridylethyl cysteine are the preferred methods for detection for EZ:faast procedure (cysteic acid cannot be detected by EZ:faast). Procedures for useful hydrolysis methods can by found in the following references:

- Stein and Moore, Methods in Enzymology 6, pp 819-831 (1963)
- Tarr in: *Microcharacterization of Proteins* (Shively, ed.) Humana Press, (1986)
- Miedel *et. al.* J. Biochem. Biophys. Methods 18, pp 37-52 (1989);
- Strydom *et. al.* in *Techniques in Protein Chemistry IV* (Angeletti, ed., 1993)
- Jones *et. al.* J. Liquid Chromatography 4, pp 454-486 (1981)

For additional information contact your Phenomenex Technical Representative.

3.4 Preparation of Protein Hydrolysates for Liquid-Chromatographic Analysis

Please first refer to section 3.2 if you have not prepared fresh Elution Medium yet. The freshly prepared Eluting Medium vial may be placed in one of the empty slots in the reagent tray.

1. For each sample, line up one sorbent tip and one glass sample preparation vial in the vial rack (Figure 2). Be aware of some variability in vial opening and sorbent tip dimensions, which may prevent the tip from reaching to the bottom of the sample preparation vial. Match vials and sorbent tips before dispensing samples and reagents.
2. Add the sample as follows:
   For vapor phase hydrolysates: Dry down any remaining acid in sample vial using a speed vac evaporator. Pipette 100µL of Reagent 1 into sample vial to re-dissolve amino acids. (Section 3.4.2)

   For liquid phase hydrolysates: pipette 100µL (or less) of the hydrolysate sample and 200µL of Reagent 2 into a vial (keep the ratio of hydrolysate: reagent 2 = 1:2) and mix briefly. The mixture should have a pH>1.5 (but, <5.0). Check the pH of one sample with pH paper; all other samples prepared by the same procedure should have a similar pH. Pipette 25µL of mix and 100µL Reagent 1 into each sample preparation vial.

Note: *In either case, for quantitative analysis calculate the multiplication factor by taking into account the amount of sample, and volumes of HCl, Reagent 2, or water used prior to SPE sample cleanup.*

Note: *Amino acid standard mixtures come with the correct pH. No pH adjustment is needed as described above. Just add 100µL Reagent 1 to the amino acid standard mixture, and proceed with the SPE as described at step 3.*

Glass Vial Line Up - (Figure 2)

For each sample, line up one sorbent tip and one glass sample preparation vial in the vial rack.

Figure 2

3. Attach a sorbent tip to a 1.5mL syringe and loosen the syringe piston; immerse the tip and pass the solution in the sample preparation vial through the sorbent tip by SLOWLY pulling back the syringe piston, in steps.
   Caution: Do not quickly pull back the piston. The syringe should be capable of drawing all sample and subsequent wash into the barrel. Watch as the liquid accumulates inside the syringe barrel and move the piston only as the accumulation slows down. Try to take at least 1 minute to pass hydrolysate or standard solution samples through the sorbent tip. If you run out of piston range, detach the sorbent tip, expel the solution from syringe barrel, then reattach the sorbent tip and proceed with sample preparation.

Note: *the sorbent tip should stay in the sample preparation vial through steps 3-10 (see Figure 3) even when dispensing reagents. In case the sorbent tip cannot reach to the bottom of the vial tilt the vial to 45° and proceed with the SPE step.*

4. Pipette 200µL HPLC grade water into the same sample preparation vial.
5. Pass the water through the sorbent tip and into the syringe barrel SLOWLY. Drain the liquid from the sorbent bed by pulling air through the sorbent tip.
6. Detach the syringe from the sorbent tip while keeping the tip inside the sample preparation vial. Discard the liquid accumulated in the syringe.

Note: *save the syringe, as it can be reused with many other samples. For convenience place it into the pipette rack.*

7. Pipette 200µL Eluting Medium (prepared fresh each day, section 3.2) into the same sample preparation vial.
8. Pull back the piston of a 0.6 mL syringe halfway up the barrel and attach the sorbent tip used in steps 3-6.

Keep the Sorbent Tip in the Vial - (Figure 3)

Keep the sorbent tip in the sample preparation vial through steps 3-10, even while dispensing HPLC grade water at step 4 and eluting medium at step 7.

Figure 3

9. Wet the sorbent with Eluting Medium; watch as the liquid rises through the sorbent particles and stop when the liquid reaches the filter plug in the sorbent tip.
10. Eject the liquid and sorbent particles out of the tip and into the sample preparation vial. Repeat step 9 and 10 until the sorbent particles in the tip are expelled into the sample preparation vial. Only the filter disk should remain in the empty tip; see Figure 4. Discard the empty tip. Keep the syringe as it can be reused with many other samples.
11. Using the adjustable Drummond Dialamatic Microdispenser (included), transfer 50µL Reagent 4 into the sample preparation vial.

    Caution: Avoid cross-contamination by not touching the inner wall of the sample vial with the tip of the Microdispenser. The piston will ensure proper transfer of liquids into the vial without the need of touching the vial wall. Use the same Microdispenser with both Reagents 4 and 5. There is no need to change Microdispenser tips between uses, or to wash the dispenser between uses of Reagent 4 and 5.

Warning: Do not use regular pipettes and tips with Reagents 4 and 5 as they will contaminate the sample! Dedicate the Microdispenser to Reagents 4 and 5 ONLY!

Note: *for all subsequent sample preparation steps use a vortex mixer set in the touch (pulse) mode (to about 80% of max speed) for any mixing operations.*

12. Emulsify the liquid in the vial by repeatedly vortexing for about 5-8 seconds. During vortexing hold the sample vial firmly between fingers, and keep it straight as you push it onto the vortex plate. Do not let the vial wobble, otherwise liquid may come out of the vial. Allow reactions to proceed 1 minute or more.

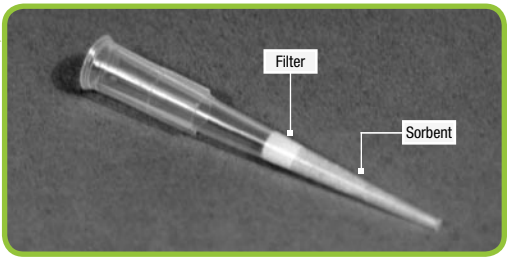
Note: *a longer reaction time than 1 minute at step 12 and 13, or later at step 14, does not affect results.*

13. Re-emulsify the liquids in the vial by vortexing again for about 5 seconds. Allow the reaction to proceed for one additional minute or more.
14. Transfer with the Microdispenser 100µL Reagent 5 (2 x 50µL for convenience), and mix for about 5 seconds. Let the reaction proceed for one more minute.

Sorbent Tip - (Figure 4)

Wet the sorbent with Eluting Medium and stop before it gets to the filter then eject the liquid and sorbent particles out of the tip.

Figure 4



15. Transfer part of the (upper) organic layer (about 50µL) into an autosampler vial (included) using a Pasteur pipette. Avoid the transfer of aqueous layer along with the organic layer! Evaporate to (almost) dryness in a gentle stream of nitrogen. Do not leave samples in the nitrogen stream for more than 3 minutes! Re-dissolve in 70-100µL mixture of mobile phase components: 10mM ammonium formate in water: 10mM ammonium formate in methanol 1:2, v/v. Transfer the reconstituted sample into an insert (included), and place the insert into the same autosampler vial. The sample is ready for LC/MS analysis.

3.5 Optimizing Sample Preparation Time

For experienced users, sample preparation and derivatization proceeds in 7-8 minutes per sample. This process can be further improved if samples are prepared in multiples of three. For example, dispense Reagent 1 in three vials successively, with the same pipette tip. At step 11, add Reagent 4 and perform subsequent steps with three samples in succession. Vortex all three vials simultaneously. During the two-minute wait in steps 12-13, prepare the next three samples. After preparing the third sample group, return to the first group of samples and proceed with step 14.

4.0 LC/MS Analysis

4.1 Column For EZ:faast LC/MS Analysis:

The HPLC column included in the kit should be based on the flow rate most compatible with your LC/MS system:

- Flow 0.5 mL/min: EZ:faast AAA-MS column 250 x 3.0 mm
- Flow 0.25mL/min: EZ:faast AAA-MS column 250 x 2.0 mm

Column should be equilibrated by running a blank gradient. Column can be stored in mobile phase when not in use.

Note: *because of column length, and the use of a sorbent with small particle size and a mobile phase of high viscosity (methanol/water), the expected column backpressure is 200-220 bar (2,900-3,200 psi). The column supplied with the kit will tolerate this backpressure very well.*

4.2 Instrument Settings:

LC

Mobile phase:	A: 10mM Ammonium formate in water B: 10mM Ammonium formate in methanol	
Gradient:	00.00min 11.00 11.01 13.00 Re-equilibrate column for 4 to 6 min before next injection depending on HPLC system used.	68%B 83%B 68%B 68%B
Flow rate:	0.50mL/min. for 3.0mm ID column 0.25mL/min. for 2.0mm ID column	
Column temperature:	35°C	
Injection volume:	5µL	

MS

Either ESI or APCI may be used

Mode:	Positive Ion
Scan range:	100-600 m/z
ESI ion source temperature:	365°C
APCI ionization chamber temperature:	450°C

4.3 Tuning the Mass Spectrometer

Some mass spectrometers require a concentrated calibration solution for tuning the instrument (if not then calibration solution III {see section 4.5} can be used). To prepare the concentrated solution, dispense 200µL aliquots of standard and Reagent 1 into each of two sample vials. Perform the SPE and derivatization steps to each vial as described by the EZ: faast procedure (section 3.4). Transfer the organic layers from the two sample vials into one autosampler vial and evaporate to near dryness with a nitrogen stream. Reconstitute in 200µL of 1:2 mobile phase A:B mixture and use for tuning the mass spectrometer.

Most mass spectrometers will not allow for concomitant tuning for a large number of ions as required for amino acid profiling. This impediment can be easily overcome by creating time segments (periods) in the run file where a selected group of ions are analyzed within each segment. This use of segments allows for optimal tuning for a large number of desired amino acids.

A suggested breakdown of the MS analysis into three segments looks as follows:

Time	Suggested Tune AA	AA in Range	AA at End of Range
0-4.8 min	ASN and THR	R,S,G,T	THR
4.8-9.3 min	MET and ASP	A,M,P,K,D,H,V,E (W)	GLU (or TRP)
9.3-13 min	LEU and C-C	L,F,I,C-C,Y	TYR

A suggested breakdown of the MS analysis into six segments looks as follows:

Time	Suggested Tune AA	AA in Range	AA at End of Range
0-4.8 min	ASN and THR	R,S,G,T	THR
4.8-6.5 min	ALA	A	ALA
6.5-7.7 min	MET and PRO	M,P	PRO
7.7-9.3 min	VAL and ASP	K,D,H,V,E (W)	GLU (or TRP)
9.3-10.7 min	LEU and PHE	L,F,I	ILE
10.7-13 min	C-C and TYR	C-C,Y	TYR

The segment time limits and amino acids to tune for may be different depending on instrument and application. HPLC pumps having larger gradient delay times will produce longer retention times and segments must be adjusted accordingly.

4.4 Calibration Standards

For quantitation purposes, aliquots of the amino acid standard mixture should be prepared following the Sample Preparation by SPE and Derivatization procedure described in this manual in Section 3.4. With standard samples no pH adjustment is necessary. Just add 100µL of Reagent 1 (Internal Standards Solution) and proceed with sample preparation.

The protein hydrolysate amino acid standard mixture (SD) is composed of the following amino acids (200nmoles/mL each):

ALA	GLU	HYP	MET	TRP*
ARG	GLY	ILE	PHE	TYR
ASP	HIS	LEU	PRO	VAL
C-C	HYL	LYS	SER	

**TRP can be analyzed only in hydrolysates prepared under alkaline conditions.*

***Note:** the amino acids included in SD are the most widely analyzed for protein hydrolysates. For assistance with additional amino acids in your hydrolysate sample, please contact Phenomenex.*

4.5 Calibration Procedure

Use the following standard amino acid mixtures and make duplicate injections of each to generate the desired calibration:

Calibration Solution

I. 10µL of SD solution, plus 100µL Internal Standard solution (2 nmoles of each amino acid, and 20 nmoles of each IS)

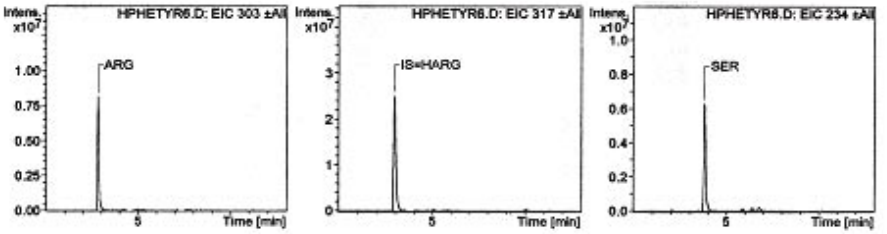
II. 50µL SD solution, plus 100µL IS (10 nmoles of each amino acid, and 20 nmoles of each IS)

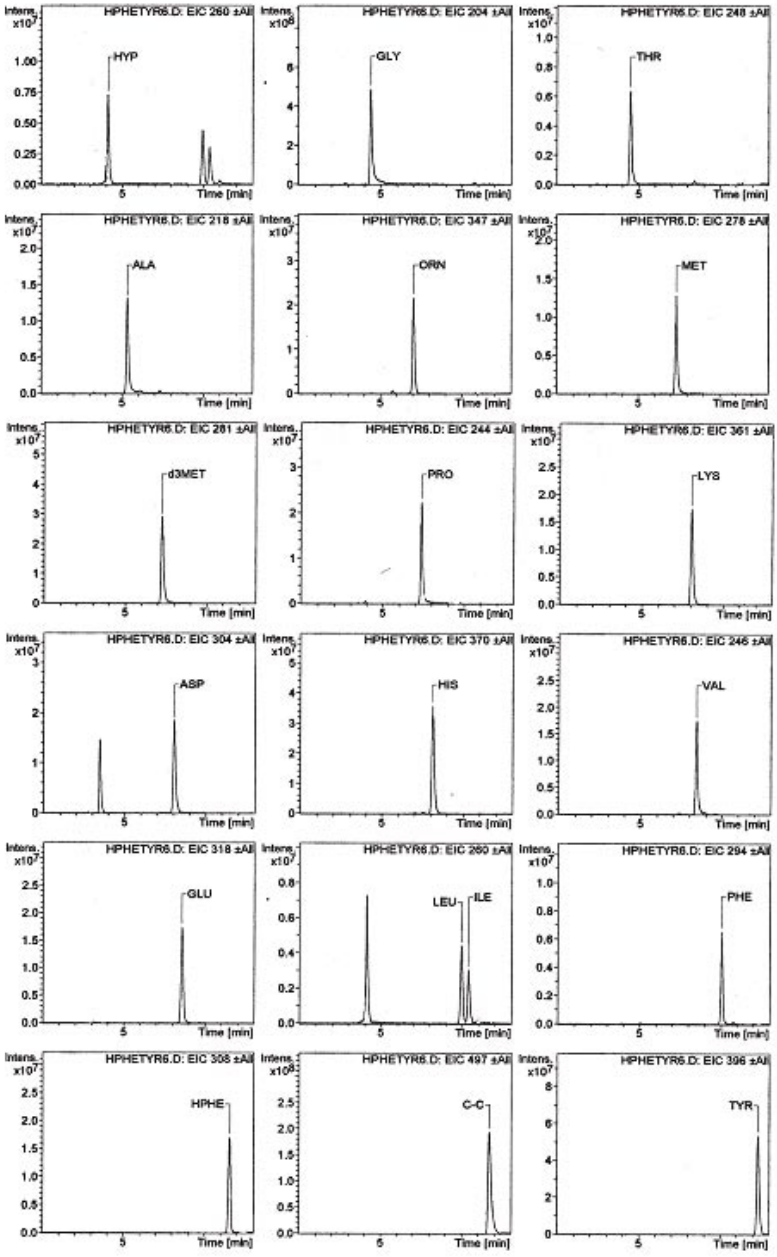
III. 100µL SD solution, plus 100µL IS (20 nmoles of each amino acid, and 20 nmoles of each IS)

The concentration of each internal standard (IS)--HARG, MET-d3 and HPHE-- in calibrators and samples prepared for chromatographic analysis is 200 nmoles/mL. While the use of the ideal internal standard will vary based on instrument and application, we recommend using HARG as the internal standard for ARG and other early eluting amino acids; MET-d3 as the internal standard for SER through GLU (TRP), (middle eluting amino acids); and HPHE for LEU through TYR (late amino acids).

Remember: the SD vial should be placed in the freezer after use! Allow standards to reach room temperature before use.

Extracted Ion Chromatograms for protein amino acids, and for the internal standards included in Reagent 1 are shown below.




HPHETYR6.D: EIC 260 ±All
HYP
HPHETYR6.D: EIC 204 ±All
GLY
HPHETYR6.D: EIC 248 ±All
THR
HPHETYR6.D: EIC 218 ±All
ALA
HPHETYR6.D: EIC 347 ±All
ORN
HPHETYR6.D: EIC 278 ±All
MET
HPHETYR6.D: EIC 261 ±All
d3MET
HPHETYR6.D: EIC 244 ±All
PRO
HPHETYR6.D: EIC 361 ±All
LYS
HPHETYR6.D: EIC 304 ±All
ASP
HPHETYR6.D: EIC 370 ±All
HIS
HPHETYR6.D: EIC 246 ±All
VAL
HPHETYR6.D: EIC 318 ±All
GLU
HPHETYR6.D: EIC 260 ±All
LEU
ILE
HPHETYR6.D: EIC 294 ±All
PHE
HPHETYR6.D: EIC 308 ±All
HPHE
HPHETYR6.D: EIC 497 ±All
C-C
HPHETYR6.D: EIC 306 ±All
TYR
Time [min]

4.6 Amino Acid and Protein Quantitation Calculations

For additional information regarding protein quantitation calculations, as well as example calculation spreadsheets please refer to reference CD included in kit.

5.0 Sample Storage and Stability

Some amino acids are chemically unstable in physiological fluids (e.g., progressive decline of plasma glutamine and cystine in time), and also in standard mixtures. Keep samples and standard mixtures in the freezer. Old amino acid standard mixtures and mixtures which have not been stored properly should not be used for instrument calibration. Order fresh mixtures (see ordering info on page 18 of this manual).

Samples prepared for LC-MS analysis following the procedure outlined in this manual may be stored for several days in a freezer before analysis. Storing dry samples is preferable to storing reconstituted samples. Dry down the organic solvent as described in section 3.4 step 15, cap vials, and place them in the freezer. For longer storage we recommend that the organic layer be desiccated with anhydrous sodium sulfate before solvent removal, vials be capped and placed in the freezer. Since sample preparation is expeditious with this procedure we recommend analyzing samples prepared freshly.

6.0 Cleaning and Care of Supplies

The Drummond® Dialamatic Microdispenser should be flushed with isopropanol:acetone (approx. 1:1) at the end of the day. Please review the Drummond Microdispenser users manual for further care and use notes. The same organic mix is recommended as wash for both manual syringes and autosamplers.

Always tightly cap the reagent bottles when not in use in order to avoid solvent evaporation and alteration of reagent composition. Cover the racks holding sorbent tips when not in use to prevent contamination.

7.0 Quality Assurance

All components of the EZ:faast Amino Acid Analysis kit are subjected to rigorous quality control testing. These measures help to ensure the best results. If poor results occur, please contact your Phenomenex technical consultant or distributor.

8.0 Product Limitations

Phenomenex Analyte Specific Reagent products are not intended for clinical use. Because they are not intended for clinical use, no claim or representation is made or intended for their clinical use (including, but not limited to diagnostic, prognostic, therapeutic or blood banking). It is the user's responsibility to validate the performance of Phenomenex products for any particular use, since the performance characteristics are not established. Phenomenex products may be used in clinical diagnostic laboratory systems after the laboratory has validated their complete system as required by the Clinical Laboratory Improvements Amendments of 1988 (CLIA '88) regulation in the U.S. or equivalent in other countries

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ordering information

EZ:faast™ Kit

Each kit includes: a EZ:faast AAA LC column (or ZB-AAA GC column and liners), sample prep and derivatization reagents, sample prep vials, AA standard mixtures, SPE sorbent tips, vial rack, autosampler vials with inserts come with MS kits, Microdispenser for Reagents 4 and 5, and demo video.

Description	Order No.	Unit
GC-FID Free (Physiological) Amino Acid Analysis Kit	KG0-7165	ea
GC-MS Free (Physiological) Amino Acid Analysis Kit	KG0-7166	ea
GC-FID Protein Hydrolysate Kit	KG0-7167	ea
GC-MS Protein Hydrolysate Kit	KG0-7168	ea
LC/MS Free (Physiological) Amino Acid Analysis Kit with 250 x 2.0mm column	KH0-7337	ea
LC/MS Free (Physiological) Amino Acid Analysis Kit with 250 x 3.0mm column	KH0-7338	ea
LC/MS Protein Hydrolysate Kit with 250 x 2.0mm column	KH0-7339	ea
LC/MS Protein Hydrolysate Kit with 250 x 3.0mm column	KH0-7340	ea
GC Free (Physiological) Amino Acid Standards (SD1, SD2 & SD3) 2mL/vial x 2	AG0-7184	ea
GC Protein Hydrolysate Standard (SD) 2mL/vial x 2	AG0-7263	ea
LC/MS Free (Physiological) Amino Acid Standards for LC (SD1, SD2, & SD3) 2mL/vial x 2	AL0-7500	ea
LC/MS Protein Hydrolysate Standard (SD) 2mL/vial x 2	AL0-7501	ea

ordering information

Phenex™ Vials

This universal vial can be used in any autosampler that utilizes a 12 x 32mm vial. It may be used in place of crimp top and snap ring top vials. Eliminates the need of stocking many different style vials. The top screws down in 1/3 turn and eliminates the chore of crimping, de-crimping and snapping caps on. Cap comes with a bonded-in septa that eliminates septa slipping into vials. Vials and caps with bonded-in septa come in one convenient kit pack.

Description	Order No.	Unit
Clear wide mouth vial, cap and septa kit pack with:		
Rubber/PTFE septa	AH0-4610	1000/pk
Silicone/PTFE septa	AH0-4613	1000/pk
PTFE/Silicone/PTFE septa	AH0-4616	1000/pk
Amber wide mouth vial, cap and septa kit pack with:		
Rubber/PTFE septa	AH0-4619	1000/pk
Silicone/PTFE septa	AH0-4622	1000/pk
Clear wide mouth vial, cap with pre-slit septa:		
Silicone/PTFE septa	AH0-7507	1000/pk

EZ:faast - Amino Acid Analysis of Protein Hydrolysates by LC-MS

Quick Reference Guide

Summary of Procedure:

1. For each sample line up one sorbent tip and one glass sample preparation vial in the vial rack.
2. **Vapor phase hydrolysate:** Dry down any remaining acid in sample vial using a speed vac evaporator. Pipette 100µL of Reagent 1 into sample vial to re-dissolve amino acids. (Section 3.4.2)

   Liquid phase hydrolysate: Pipette 100µL sample hydrolysate, and 200µL Reagent 2 into a glass vial and mix briefly. If pH>1.5, pipette 25µL of mix and 100µL Reagent 1 into each sample preparation vial. (Section 3.4.2)
3. Attach a sorbent tip to a 1.5mL syringe; pass the solution in the sample preparation vial through the sorbent tip by **slowly** pulling back the syringe piston.
4. Pipette 200µL water into the sample preparation vial.
5. **Slowly** pass the solution through the sorbent tip and into the syringe barrel.
6. Detach the sorbent tip, and discard the liquid accumulated in the syringe.
7. Pipette 200µL Eluting Medium (prepared fresh each day, section 3.2) into the sample preparation vial.
8. Pull back the piston of a **0.6mL syringe** halfway up the barrel and attach the sorbent tip.
9. Wet the sorbent with Eluting Medium;stop when the liquid reaches the filter plug in the sorbent tip.
10. Eject the liquid and sorbent out of the tip and into the sample preparation vial. Repeat, until all sorbent particles in the tip are expelled into the sample preparation vial. Discard the empty tip.
11. Using the Drummond Dialamatic Microdispenser, transfer 50µL Reagent 4.
12. **Emulsify** by repeatedly vortexing the solution for about 5 seconds. Allow reaction to proceed for about 1 minute.
13. Vortex the solution again for a few seconds to re-emulsify the content of the vial. Allow the reaction to proceed for at least one additional minute.
14. Using the Microdispenser, transfer 100µL Reagent 5, and re-emulsify by vortexing for about 5 seconds. Let the reaction proceed for 1 minute.
15. Transfer part of the (upper) organic layer (50-100µL) using a Pasteur pipette into an autosampler vial. Avoid transferring aqueous layer along with the organic layer. Evaporate the solvent SLOWLY to almost dry under a gentle stream of nitrogen (3 min). Stop when sample almost dry. Re-dissolve amino acid derivatives in 100µL (or less) of a mixture of LC mobile phase components A:B 1:2 (v/v). Transfer the reconstituted sample into an insert, and place the insert in the same autosampler vial. The reconstituted sample is ready for LC/MS analysis.

LC-MS Analysis

LC

Mobile phase:	A: 10mM Ammonium formate in water B: 10mM Ammonium formate in methanol
Gradient:	0.00min 68%B 11.00 83%B 11.01 68%B 13.00 68%B
	Re-equilibrate column for 4 to 6 min before next injection depending on HPLC system used.
Flow rate:	0.50mL/min. for 3.0mm ID column 0.25mL/min. for 2.0mm ID column
Column temperature:	35°C
Injection volume:	5µL

MS

Either ESI or APCI may be used

Mode:	Positive Ion
Scan range:	100-600 m/z
ESI ion source temperature:	365°C
APCI ionization chamber temperature:	450°C

Detach Here

ordering information

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