EZ:faast™
USER'S MANUAL

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Patent Pending

Amino Acid Analysis of Protein Hydrolysates by GC-FID or GC-NPD

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EZ:faast™ For Amino Acid Analysis of Protein Hydrolysates by GC-FID or GC-NPD

2570_L_4

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
Reagent 6	Acid Solution	50mL
SD	Protein Amino Acid, Standard Mixture	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
ZB-AAA 10m x 0.25mm ID Amino Acid Analysis GC Column 1
FocusLiners™ 5

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
- Reagent and supplies for Protein Hydrolysis
- Septa (Auto-Sep T™ 11mm [SGE P/N 041883: fits Agilent or Carlo Erba instruments] or equivalent)

2.0 Overview

2.1 Overview

The EZ:faast amino acid analysis procedure consists of a solid phase extraction step followed by derivatization and liquid/liquid extraction; derivatized samples are quickly analyzed by gas chromatography with FID or NPD detection. 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. An aliquot from the organic is analyzed on a GC system with either a FID or NPD detector. Total sample preparation time takes around 8 minutes and analysis is performed in around 7 minutes for a total start to finish time of around 15 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 more than 40 aliphatic and aromatic amino acids and related compounds. Further amino acids and related compounds may be analyzed with this kit. A brief adjustment of gas chromatographic conditions may be necessary. Please contact Phenomenex for method modifications and other LC and GC amino acid kits.

Table 1 - Protein Amino Acids analyzed by the EZ:faast Amino Acid Analysis Kit for Protein Hydrolysates by GC:

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

**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 - Comprehensive list of amino acids and related compounds analyzed by the EZ:faast method as described in this manual. Mass spectral information provided for confirmation, when needed. **(internal standard listed in bold)**

Abbreviation	Alternate Abbreviation	Chemical Name	GC/MS Major Ions Observed (SIM)	
			Instrument: Agilent 5973	Instrument: Varian Saturn 2000
ALA	A	Alanine	130, 88	130, 70
SAR	—	Sarcosine	130, 217	130, 88
GLY	G	Glycine	116, 207	116, 102
ABA	—	α-Aminobutyric acid	144, 102	144, 102
VAL	V	Valine	158, 116	116, 98, 158
ß-ALA	—	β-Alanine	129, 158, 98	—
ß-AIB	BAIBA	β-Aminoisobutyric acid	158, 116	130, 84, 144
ß-ABA	—	β-Amino-n-butyric acid	88, 70	—
NORV	**—**	**Norvaline**	**158, 72**	**158, 116**
LEU	L	Leucine	172, 86	172, 130
alLE	—	allo-Isoleucine	172, 130	130, 101
ILE	I	Isoleucine	172, 130	130, 101
HSER	—	Homoserine	102, 128, 143	—
NLE	—	Norleucine	172, 86	—
$Et(OH)NH_2$	—	Ethanolamine	116, 117	—
THR	T	Threonine	160, 101	101
SER	S	Serine	146, 203	101, 86
Pe-Cys	—	Pyridyl ethyl Cysteine	—	—
PRO	P	Proline	156, 243	156, 114
GABA	—	γ-Amino-n-butyric acid	130, 144, 172	—
ASN	N	Asparagine	155, 69	113
PA	—	Pipecolic acid	—	—
TPR	—	Thiaproline	174, 147	174, 147
ASP	D	Aspartic acid	216, 130	216, 130
MET	M	Methionine	203, 277	101, 203, 129
HYP	OHPro	4-Hydroxyproline	172, 86	172, 86
ETH	—	Ethionine	203, 291, 143	—
GLU	E	Glutamic acid	230, 170	84, 142
PHE	F	Phenylalanine	206, 190	147, 128, 91
AAA	—	α-Aminoadipic acid	244, 98	98, 125
CYS	C	Cysteine	248, 162, 206	—
PABA	—	4-Aminobenzoic acid	265, 206, 163	—
HCYS	—	Homocysteine	142, 203	—
APA	—	α-Aminopimelic acid	198, 258, 286	198, 138, 112
GLN	Q	Glutamine	84, 187	84, 112
Cm-Cys	—	Carboxymethyl Cysteine	262, 203, 144	—
HA	—	Histamine	180, 168, 223	—
THE	—	Theanine	112, 215	—
MET-SO	—	Methionine Sulfoxide	182, 138, 229	—
DABA	—	2,4-Diamino-n-butyric acid	203, 142, 245	—
GLY-GLY	G-G	Glycine-glycine (dipeptide)	117, 144, 201	—
$MET\text{-}SO_2$	—	Methionine Sulfone	—	—

Table 2 - (continued)

Abbreviation	Alternate Abbreviation	Chemical Name	GC/MS Major Ions Observed (SIM)	
			Instrument: Agilent 5973	Instrument: Varian Saturn 2000
ORN	O	Ornithine	156, 70	156, 139, 114
GPR	G-P	Glycine-proline (dipeptide)	70, 300	153, 114
LYS	K	Lysine	170, 128	153, 170, 128
THR-ASP	T-D	Threonine-aspartic acid (dipeptide)	218, 360, 130	—
HIS	H	Histidine	282, 168	267, 222, 136
Se-C-C	—	Selenocystine	—	—
HLY	OHLys	Hydroxylysine (2 isomers)	129, 169	87, 129
TYR	Y	Tyrosine	206, 107	164, 107
DAP	—	Diaminopimelic acid	256, 168	—
PHP	P-OHP	Proline-hydroxyproline (dipeptide)	156, 186	156, 114
TRP	W	Tryptophan	130	130
NTYR	—	3-Nitrotyrosine	152, 209	—
LYS-ALA	K-A	Lysine-alanine (dipeptide)	170, 224, 153	—
DA	—	Dopamine	179, 136, 123	—
CTH	—	Cystathionine	203, 272	146, 114
DOPA	—	3,4-Dihydroxyphenylalanine	222, 123	—
C-C	$(Cys)_2$	Cystine	248, 216	114, 173
HC-HC	$(Hcys)_2$	Homocystine	230, 188, 128	—
ARG-SUC	—	Arginino-succinic acid	441, 326	—

2.3 Storage and Stability

Store Reagents 1, 3B and 4 at 4°C (do not freeze!). 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, which can be easily lifted from the work station 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 7.0 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

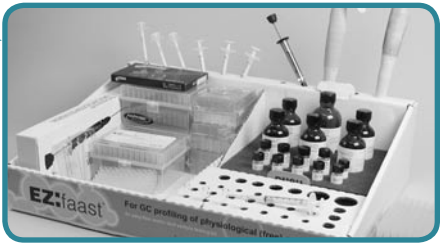


Figure 1b

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 5, 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 a 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 is shown; 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 Sample Preparation by SPE and Derivatization

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 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. Transfer sample (if necessary) to EZ:faast sample vial and proceed.

   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 greater than 1.5 (but less than pH 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 both cases, calculate the multiplication factor for quantitative analysis by taking into account the amount of sample, and the volumes of HCl, Reagent 2, or water used.*

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 VERY small steps.

Caution: Do not quickly pull back the piston. Try to take at least one minute to pass the sample through the sorbent tip. Watch as the liquid accumulates inside the syringe barrel and move the piston only as the accumulation slows down. The syringe should be capable of drawing all sample and subsequent wash into the barrel. 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°, push the tip into the vial gently 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 drawing air through the 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.

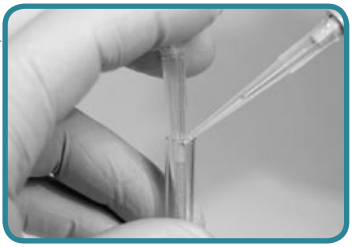
Note: *save the syringe, as it can be reused with many other samples. For convenience place it into the pipette rack.*

7. Pipette 200µL Eluting Medium (prepared fresh each day, section 3.2) into the same sample preparation vial (see Figure 3).
8. Pull back the piston of a 0.6mL 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 Reagent 4 and 5 as they will contaminate the sample! Use the included Microdispenser for 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 reaction to proceed 1 minute or more. The emulsion will gradually separate into two layers.

Note: *a longer reaction time than 1 minute each at steps 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 (50µL twice for convenience) and mix for about 5 seconds. Let the reaction proceed for one more minute.
15. Pipette (DO NOT use the Microdispenser for this purpose!) 100µL Reagent 6, and vortex for about 5 seconds. The emulsion will separate into two layers. The upper, organic layer contains amino acid derivatives to be analyzed by gas-chromatography (see GC set up and calibration in section 4). Sample this layer directly from the sample prep vial or use a Pasteur pipette to transfer part of it into an autosampler vial.

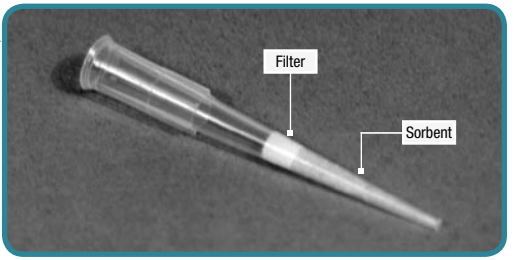
3.5 Optimizing Sample Preparation Time

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 at steps 12-14, prepare the next three samples.

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



4.0 Gas Chromatographic Analysis

4.1 Column For EZ:faast Amino Acid Analysis in Protein Hydrolysates by GC (included)

The Zebron ZB-AAA GC column for Protein Hydrolysate comes without a cage. Connect the ends of the column in the usual manner; rest the column coil on the oven bracket. Keep the pieces of thermal thread spaced evenly around the column coil. The maximum column temperature is 320/340°C.

4.2 Instrument Conditions:

Constant Flow Mode (recommended) - GC-FID/NPD Settings

Injection	Split 1:15@250°C, 1.5-2µL (with hot needle, see section 4.6)
Carrier Gas	Helium 1.5mL/min constant flow
Oven program	32°C/min from 110° to 320°C
Detector	320°C

Constant Pressure Mode - GC-FID/NPD Settings

Injection	Split 1:15 @ 250°C, 2.0µL
Carrier Gas	Helium, 8 psi (60 kPa) or Hydrogen 30 kPa
Oven Program	35°C/min from 110° to 320°C
Detector	320°C

For your convenience we have included the GC methods for both the Varian Saturn™ 2000 and Agilent 6890 GC systems on the reference CD included with the kit. To use these included methods: copy the method folder into the appropriate method folder in your software and load.

4.3 Mode of operation

For best resolution, a rate of 35°C/min is preferred with instruments operating in constant pressure mode only. Electronic Pressure Control (EPC) or Advanced Flow Controller (AFC) equipped instruments should be operated preferably in constant flow or constant velocity mode. With these instruments a temperature gradient of 30-32°C/min is fast enough to elute the least volatile derivatives (e.g. that of cystine) with similar retention times to constant pressure mode. If the instrument is not equipped with the EPC option, you may use a pressure raise of 3kPa/min.

4.4 Liners

Use the best deactivated liners supplied by the instrument manufacturer. Good results were obtained with FocusLiners™ (Phenomenex P/N AG0-4680; fits Agilent and Varian injectors). In general, the liner should carry a plug of silanized quartz or pesticide grade glass wool.

4.5 Injection

- Split injection at a ratio of 1:10 to 1:20 is recommended
- Injection volumes of 1.5-2µL are optimal

Quasi-splitless injection mode, will produce a 5 to 10 fold increase in sensitivity with some instruments. In this mode, the split valve should be closed for an initial 5 to 7 seconds. Before selecting this injection mode it should be checked experimentally that no significant discrimination of late eluting amino acid derivatives takes place in comparison with common split injection. Alternatively, instruments equipped with EPC/AFC can be operated at double initial head pressure for 6-10 seconds.

4.6 Sampling

Both autosampler and manual sampling can be performed. If manual sampling is preferred, hot needle injection is recommended to prevent discrimination of components with high boiling temperatures. With this technique the sample plug is completely drawn into the syringe barrel, leaving the needle empty. The needle is inserted and kept in the hot injector for about two seconds before injection.

4.7 Calibration Standards

For quantitation purposes, mixtures of amino acid standards should be prepared following the Sample Preparation by SPE and Derivatization procedure described in this manual in Section 3.4. Amino acid standard mixtures should be stored in the freezer! With standards, no pH adjustment is necessary. Simply add 100µL Reagent 1 (Internal Standard solution) and proceed with sample preparation.

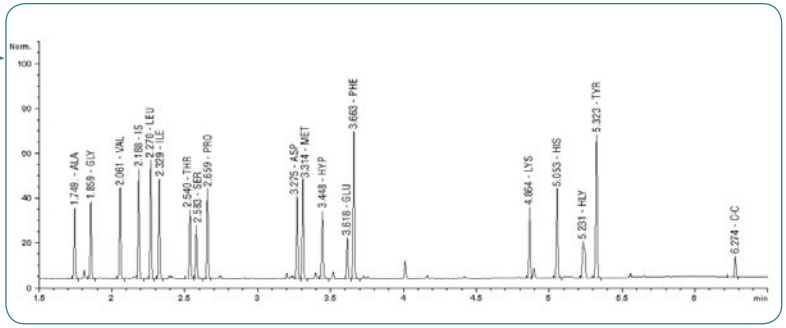
The amino acid standard mixture for protein hydrolysates (SD) is composed of the following amino acids (200 nmoles/mL each):

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

**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.*

Figure 5



A typical chromatogram of the amino acids standard solution included in this kit. Column and instrumental settings as specified in section 4.1-4.2.

4.8 Calibration Procedure

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

Calibration Solution:

I. 25µL of SD solution, plus 100µL Reagent 1(5 nmoles of each amino acid, and 20 nmoles IS)

II. 50µL SD solution, plus 100µL Reagent 1(10 nmoles of each amino acid, and 20 nmoles IS)

III. 100µL SD solution, plus 100µL Reagent 1(20 nmoles of each amino acid, and 20 nmoles IS)

The concentration of the internal standard (IS) in the sample prepared for chromatographic analysis is 200 nmoles/mL.

***Note:** Disregard the first 3-5 injections when performing method calibration. These injections act as primers and mask active sites inside the liner and chromatographic column. Use subsequent duplicate runs for calibration.*

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

4.9 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 Troubleshooting

Problem	Cause	Solution
Decrease in peak height for some amino acids (components of amino acid standard mixtures SD2 or SD3)	Possibly related to improper sample storage	See sample standard storage, section 6.0
Decrease in peak height for basic amino acids	Old eluting medium	Prepare eluting medium daily based on the number of samples to be analyzed on that day
Decrease in peak height or missing peaks for late eluting (C-C), or polar amino acids (SER, HYP)	Improper injection	Optimize your injection by comparing split mode with quasi-splitless, or alternatively, with an increased initial head pressure during injection. Follow the hot needle injection technique for manual injections, as indicated in section 4.6.
	Improper liner	Use deactivated liners, see section 4.4. Analyze samples only after making priming injections.
Decrease in peak height for early eluting amino acids	Sample too concentrated; the capacity of the SPE tip exceeded	Use less sample for preparation, see section 3.4, step 2. Constantly monitor the area for the IS peak.
	Volatile derivatives evaporated during removal of residual reagents with nitrogen gas	Adjust nitrogen flow to minimal; stop evaporation before sample gets completely dry
Low peak height for late eluting derivatives	Carrier gas leak	Check instrument for leaks (reinstall the column and check o-ring on liner)
Peak height for IS (Norvaline) lower compared to other early eluting amino acids in the standard mix	Pipetting error	Recalibrate pipette used for dispensing Reagent 1. Constantly monitor area for IS.
Ghost peaks	Pipette tips used for dispensing reagents or for transferring prepared samples may be a source of contaminant peaks	Use polypropylene pipette tips of appropriate quality (see ordering info on page 15)
	Interfering peaks may result from extracted contaminants in plastic sample preparation vials	Use the vials provided in the kit. For autosampler vials order Phenomenex P/N AH0-4610 and for inserts AH0-4604.
Early deterioration of column performance	Residual sample preparation reagents degrade column stationary phase	Make sure to sample only the upper, organic layer. Remove the first 20cm of column and re-install.
Interfering peaks, drug metabolites	Physiological sample anticoagulants, like citrate or citric acid may interfere in the amino acid profile	Samples collected with EDTA and heparin anticoagulants are recommended. Confirm peak identity based on mass- spectrum.

6.0 Sample Storage and Stability

Some amino acids are chemically unstable in physiological fluids (e.g., progressive decline of plasma 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 mixture from Phenomenex (see ordering info in Phenomenex catalog).

Samples prepared for gas-chromatographic analysis following the procedure outlined in this manual may be stored for several days in a freezer before analysis. For longer storage we recommend that samples be desiccated with anhydrous sodium sulfate, vials be capped and placed in the freezer. Since sample preparation is expeditious with this procedure we recommend analyzing samples prepared freshly.

7.0 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.

8.0 Quality Assurance

All components of the EZ:faast GC 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.

9.0 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.

ordering information

EZ:faast™ Kit

Each kit includes: a ZB-AAA GC column (or EZ:faast AAA LC column), GC liners with GC kits, 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

SGE FocusLiners™

Description	GC Model No.	Dimensions ID ID x L x OD(mm)	Material* (deactivated)	Quartz Wool (Y/N)	Mfr P/N	Order No.	Unit
ThermoQuest (Carlo Erba) Single Taper/ Gooseneck Liner	8000 series	5 x 105 x 8.0	B (y)	Y	092046	AG0-4679	5/pk
Agilent Technologies Single Taper/ Gooseneck Liner	5880/5890 /6890	4 x 78.5 x 6.3	B (y)	Y	092003	AG0-4680	5/pk
PerkinElmer Single Taper/ Gooseneck Liner	Autosystem	4 x 92 x 6.2	B (y)	Y	092095	AG0-4681	5/pk
Shimadzu Single Taper/ Gooseneck Liner	17B	3.4 x 95 x 5	B (y)	Y	092068	AG0-4683	5/pk
Varian Single Taper/ Gooseneck Liner	1075/77	4 x 72 x 6.3	B (y)	Y	092025	AG0-4684	5/pk
Varian Double Taper/ Gooseneck Liner	1078/79	3.4 x 54 x 5	B (y)	Y	092036	AG0-4685	5/pk

** B=Borosilicate; Deactivated=Yes (y) or No (n)*

EZ:faast - Amino Acid Analysis of Protein Hydrolysates by GC-FID, GC-NPD

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. Pipette (DO NOT use the Microdispenser for the purpose) 100µL Reagent 6, and re-emulsify by vortexing for about 3 seconds. The upper, organic layer contains amino acid derivatives to be analyzed by gas chromatography on a ZB-AAA column.

Gas Chromatographic Analysis

Constant Flow Mode (recommended) - GC-FID/NPD Settings

Injection	Split 1:15 @ 250°C, 2.0µL
Carrier Gas	Helium, 1.5mL/min
Pressure Increase	3 kPa/min (alternative setting to constant flow)
Oven Program	32°C/min from 110° to 320°C
Detector	320°C

Constant Pressure Mode - GC-FID/NPD Settings

Injection	Split 1:15 @ 250°C, 2.0µL
Carrier Gas	Helium, 8 psi (60 kPa) or Hydrogen 30 kPa
Oven Program	35°C/min from 110° to 320°C
Detector	320°C

Detach Here

ordering information

EZ:faast™ Kit

Each kit includes: a ZB-AAA GC column (or EZ:faast AAA LC column), GC liners with GC kits, 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.

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