

A Rapid, Simultaneous Determination of 33 Amino Acids and Dipeptides in Spent Cell Culture Media by Gas Chromatography-Flame Ionization Detection Following Solid and Liquid Phase Extraction

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Abstract

A rapid, sensitive and reproducible gas chromatographic method with flame ionization detection is described for the simultaneous identification and quantification of 33 amino acids (AA) and dipeptides in spent cell culture media in under 7 minutes. The method involves the use of the EZ:fast™ Amino Acid Analysis sample testing kit. Instrumental and assay precision, % recovery, linear range, limit of detection and peak identity in highly complex cell culture media containing either soy hydrolysate or Fetal Bovine Serum (FBS) were validated using a gas chromatography system with dual flame ionization detectors (GC-FID).

Introduction

The development and optimization of cell culture media for the production of therapeutic proteins and viral growth is fundamental to the emerging biotechnological and pharmaceutical research into biomolecular mechanisms, genetic engineering and drug discovery. The choice of culture medium and stoichiometric balance of complementing nutrients can have a significant and irremediable impact on the growth, function and relative phenotype of cells and metabolic flux of the biochemical pathways. However, product syntheses and metabolic shifts lead to ever-changing external cellular conditions in batch culture which can inhibit cellular growth, metabolism and overall production. There is a vital need to optimize cell culture media formulations and control such dynamic environmental changes to efficiently maximize cell growth, viability and specific productivity.

The analysis of the amino acid and dipeptide constituents of cell culture media has been problematic due to the presence of many other components. Moreover, the significant differences in the chemical structure of the functional groups, ranging from nonpolar to highly polar, may interfere with the derivatization or detection of the amino acid and dipeptide derivatives. Previously, physiologically free amino acid analysis has been performed by Capillary Electrophoresis (CE), High Performance Liquid Chromatography (HPLC), Liquid Chromatography - Mass Spectrometry (LC-MS), Gas Chromatography (GC) and Gas Chromatography - Mass Spectrometry (GC-MS). However, many of these methods have been impeded by long sample preparation or analysis times, decreased resolution due to ion suppression, poor separation, low absolute sensitivity and dedicated or expensive instruments that are difficult to maintain and operate.

The method described here, following the derivatization scheme in Figure 1, shows excellent accuracy and precision as well as very short analysis time (< 7 minutes) for the baseline resolution of 33 amino acid and dipeptide peaks present in spent cell culture media. Using the Agilent 6890N series GC-FID with a 7683 series dual-tower and autosampler, more than 250 samples can be derivatized and run in a 24 hour period. Additionally, the method is shown to be free of interferences from amino acids and dipeptides present in serum and hydrolysates that are added to cell culture media and can be applied to other complex physiological fluids and food samples as well.

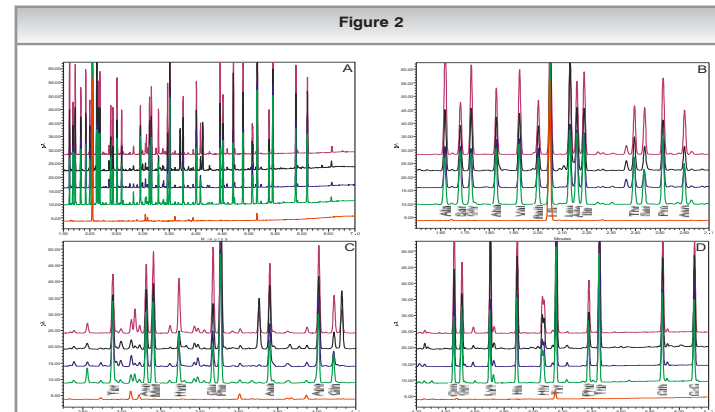
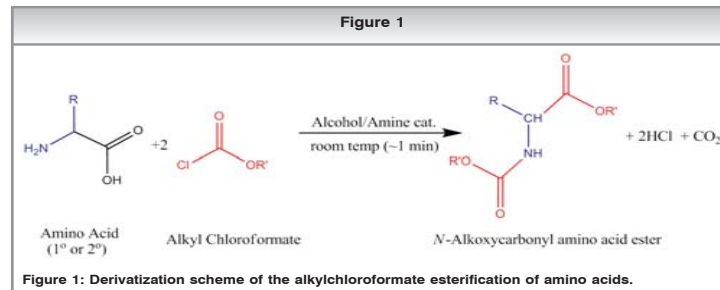


Figure 2: Complete (A) and expanded (B, C and D) overlay chromatograms of a WFI blank (red trace), 10 nmol AA standard (green trace), blank media spiked with 10 nmol AA standard (blue trace), blank media spiked with 1% w/w hydrolysate and 10 nmol AA standard (black trace) and blank media spiked with 10% FBS and 10 nmol AA standard (pink trace).

Materials and Methods

Cell Culture Media

Amino acid and protein-free NS0 media (labeled "blank media") were prepared and spiked with amino acid standards, insulin, ultrafiltered soy hydrolysate (SAFC Biosciences Catalog No. 58903) and/or FBS (SAFC Biosciences Catalog No. 12103).

Instrument

Agilent 6890N series gas chromatography system with dual flame ionization detectors with a 7683 series dual-tower and autosampler, all controlled by the Waters® Empower™ software package (Milford, Massachusetts, USA).

Reagents

All reagents, including standards, GC column (10 m x 0.25 mm ZB-AAA) and liner were provided by the Phenomenex, Inc., EZ:fast™ Amino Acid Analysis sample testing kit.

GC Conditions

Carrier gas (ultrapure 6.0 He) flow-rate was kept constant at 1.5 mL/min. The oven temperature was held at the initial temperature of 110 C for one minute and then ramped at 32 C/min up to a final temperature of 320 C with no final hold. The temperature of the injection port was 250 C, split at 1:20. The inlet was set at 250 C, the detector at 320 C, with a H₂ (ultrapure 5.0) flow of 35 mL/min and an oxidizer flow (hydrocarbon free air) of 350 mL/min.

Table 1

Name	Matrix				Average
	Calibration Standard	Blank Media	Hydrolysate	10% FBS	
Ala	1.617±0.000	1.618±0.000	1.619±0.000	1.619±0.000	1.618±0.001
Sar	1.681±0.000	1.681±0.000	1.682±0.000	1.682±0.000	1.682±0.001
Gly	1.724±0.000	1.725±0.000	1.726±0.000	1.726±0.000	1.725±0.001
Aba	1.827±0.000	1.828±0.000	1.829±0.000	1.829±0.000	1.828±0.001
Val	1.923±0.000	1.923±0.000	1.924±0.000	1.924±0.000	1.924±0.001
Baib	2.001±0.000	2.000±0.000	2.001±0.000	2.001±0.001	2.001±0.000
I.S. ¹	2.049±0.000	2.049±0.000	2.050±0.000	2.050±0.000	2.050±0.001
Leu	2.129±0.000	2.130±0.000	2.131±0.000	2.131±0.001	2.130±0.001
Alie	2.158±0.000	2.158±0.000	2.159±0.000	2.159±0.000	2.159±0.001
Ile	2.187±0.000	2.188±0.000	2.189±0.000	2.189±0.001	2.188±0.001
Thr	2.393±0.000	2.394±0.000	2.395±0.000	2.396±0.001	2.395±0.001
Ser	2.435±0.001	2.435±0.000	2.437±0.000	2.437±0.000	2.436±0.001
Pro	2.511±0.000	2.512±0.000	2.513±0.000	2.513±0.000	2.512±0.001
Asn	2.599±0.001	2.599±0.000	2.600±0.000	2.601±0.000	2.600±0.001
Tpr	2.952±0.001	2.953±0.000	2.954±0.000	2.954±0.000	2.953±0.001
Asp	3.124±0.001	3.124±0.000	3.126±0.000	3.126±0.000	3.125±0.001
Met	3.160±0.001	3.161±0.000	3.162±0.000	3.163±0.000	3.162±0.001
Hyp	3.293±0.001	3.292±0.001	3.294±0.000	3.295±0.001	3.294±0.001
Glu	3.466±0.001	3.467±0.001	3.468±0.000	3.469±0.000	3.468±0.001
Phe	3.506±0.001	3.507±0.000	3.508±0.000	3.509±0.000	3.508±0.001
Aaa	3.757±0.001	3.759±0.001	3.759±0.000	3.760±0.000	3.759±0.001
Apa	4.008±0.001	4.010±0.000	4.011±0.000	4.012±0.000	4.010±0.002
Gln	4.087±0.001	4.087±0.000	4.089±0.000	4.090±0.000	4.088±0.002
Orn	4.454±0.001	4.459±0.000	4.458±0.000	4.459±0.001	4.458±0.002
Gpr	4.509±0.001	4.509±0.001	4.511±0.000	4.512±0.000	4.510±0.001
Lys	4.702±0.001	4.703±0.000	4.706±0.000	4.706±0.001	4.704±0.002
His	4.887±0.001	4.888±0.000	4.890±0.000	4.890±0.001	4.889±0.002
Hly	5.059±0.001	5.060±0.000	5.062±0.000	5.063±0.001	5.061±0.002
Tyr	5.155±0.000	5.156±0.001	5.158±0.000	5.158±0.001	5.157±0.002
Php	5.378±0.001	5.379±0.001	5.381±0.000	5.381±0.000	5.380±0.002
Trp	5.450±0.001	5.452±0.001	5.454±0.000	5.454±0.000	5.453±0.002
Cth	5.883±0.001	5.884±0.001	5.886±0.001	5.887±0.000	5.885±0.002
C-C	6.100±0.001	6.103±0.001	6.103±0.001	6.105±0.001	6.103±0.002

Table 1: Specificity and amino acid/dipeptide retention times (min±S.D., six injections per matrix) by GC-FID under various matrices spiked at 10 nmol with each AA.

¹I.S. is Norvaline and is spiked at 20 nmols in accordance with SOP.

Table 2

Name	Matrix		
	Blank Media	Hydrolysate	10% FBS
Ala	95.6±1.3	102.3±1.6	104.0±2.1
Sar	92.0±1.6	93.3±2.1	90.3±2.8
Gly	93.0±2.6	97.4±5.2	99.4±4.0
Aba	97.6±1.7	102.9±2.3	100.5±2.1
Val	95.6±1.1	104.0±2.3	96.0±3.2
Baib	101.9±1.1	100.1±1.1	109.0±3.4
Leu	105.5±1.7	105.9±1.0	102.9±2.5
Alie	98.8±2.4	103.9±5.0	96.5±2.5
Ile	99.1±1.8	102.6±4.2	101.6±3.5
Thr	85.0±2.9	96.6±4.3	98.5±3.5
Ser	74.1±7.1	83.2±8.0	116.9±3.3
Pro	93.9±2.6	97.0±3.9	92.8±2.6
Asn	78.4±5.5	88.7±7.2	101.1±2.0
Tpr	88.1±2.7	91.4±3.0	72.4±2.1
Asp	85.1±1.5	101.0±1.2	101.9±3.4
Met	99.7±1.4	89.1±3.0	88.6±3.3
Hyp	66.0±12.6	68.8±10.7	115.9±3.2
Glu	96.4±2.4	92.4±4.6	91.2±4.8
Phe	107.3±1.3	108.6±2.1	94.4±2.6
Aaa	101.1±5.1	117.8±7.8	95.1±6.9
Apa	105.4±3.0	105.3±3.5	93.1±3.1
Gln	82.9±6.0	79.5±8.0	96.9±2.3
Orn	107.9±2.3	103.0±6.0	108.5±3.5
Gpr	98.7±3.1	99.3±7.1	92.8±1.9
Lys	108.6±2.7	98.2±4.2	120.9±3.0
His	109.9±2.6	108.7±2.7	100.9±3.2
Hly	106.2±2.2	92.0±3.9	98.9±2.1
Tyr	108.3±1.3	108.2±2.2	92.1±2.4
Php	96.3±4.8	93.7±9.3	88.2±1.9
Trp	112.1±2.4	101.9±3.6	99.3±1.5
Cth	100.5±1.9	99.0±1.8	102.6±1.6
C-C	104.7±1.2	111.9±1.5	94.5±1.7

Table 2: % recovery (±%RSD) of amino acids/dipeptides in various matrices spiked at 10 nmol with each AA (n=6) by GC-FID.

Table 3

Name	Limit of Detection	Range of Linearity	Correlation Coefficient
Ala	0.64	1 - 30	0.994
Sar	0.31	1 - 25	0.998
Gly	0.77	1 - 30	0.991
Aba	0.64	1 - 30	0.994
Val	0.76	1 - 30	0.991
Baib	0.60	1 - 30	0.995
Leu	0.60	1 - 30	0.995
Alie	0.60	1 - 30	0.995
Ile	0.84	1 - 30	0.990
Thr	0.61	1 - 20	0.991
Ser	0.63	1 - 20	0.990
Pro	0.84	1 - 30	0.990
Asn	0.43	1 - 20	0.996
Tpr	0.40	1 - 25	0.997
Asp	0.70	1 - 25	0.992
Met	0.59	1 - 30	0.995
Hyp	0.90	1 - 20	0.984
Glu	0.54	1 - 30	0.996
Phe	0.59	1 - 30	0.995
Aaa	0.92	1 - 30	0.990
Apa	0.52	1 - 25	0.995
Gln	0.53	1 - 20	0.993
Orn	0.67	1 - 30	0.993
Gpr	0.72	1 - 30	0.992
Lys	0.72	1 - 30	0.992
His	0.65	1 - 30	0.994
Hly	0.57	1 - 30	0.995
Tyr	0.64	1 - 30	0.994
Php	0.71	1 - 25	0.991
Trp	0.68	1 - 25	0.991
Cth	0.34	1 - 30	0.998
C-C	0.39	1 - 30	0.998

Table 3: Relative lower limit of detection and linearity range by GC-FID.

Conclusion

The analytical method described within is suitable for the rapid and highly sensitive determination of primary and secondary amino acids present in cell culture media, fermentation broths, determination of physiological amines and dipeptides. This procedure consists of solid phase extraction of amino acids from the matrix of interest, followed by a single, rapid derivatization (~1 min) and a liquid/liquid phase extraction step. The overall sample prep takes about 3 minutes/sample, with a total analysis time of 7 minutes per sample (Figure 2) or 3.5 minutes using the dual tower mode. The derivatives are stable for 48 hours at room temperature. This method is well suited for highly complex matrices such as blood, plasma, urine, cerebral spinal fluid, wine or grain samples containing interfering proteins, urea and other impurities that can lead to poor chromatographic results while analyzing for amino acids. This procedure ensures the selective removal of interfering compounds from serum and hydrolysates, that are usually added to cell culture media, thus yielding highly reliable (Table 1) and reproducible data (Table 2). The method offers excellent linear range and very low detection (Table 3) for the simultaneous identification and quantification of 33 baseline resolved amino acids and dipeptides in spent cell culture media using GC-FID.

The simple derivatization procedure and the ease with which the derivatives can be prepared and resolved make this method highly attractive for routine, high throughput analysis. It is of paramount importance that when apparent differences are observed in data from samples of different cell lines grown in the same or different cell culture media, the methodology of the analytical analysis is such that small but real differences can be discerned with relatively high degree of certainty. Additionally, the applicability of this method to other highly complex biological matrices makes it an ideal assay for other clinical or biological research.