Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 862 (2008) 86–92
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of 33 amino acids and dipeptides in spent cell culture media by gas chromatography-flame ionization detection following liquid and solid phase extraction

Tariq Mohabbat, Barry Drew *

SAFC Biosciences, Cell Sciences and Development, 13804 W. 107th Street, Lenexa, KS 66215, USA

Received 8 May 2007; received in revised form 25 September 2007; accepted 5 November 2007
Available online 13 November 2007

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 and dipeptides in spent cell culture media in under seven minutes. The method involves the use of the EZ:faast™ (Phenomenex) amino acid sample testing kit. Instrumental and assay precision, percent recovery, linear range, limit of detection and peak identity in highly complex cell culture media containing either soy hydrolysate or fetal bovine serum were validated using gas chromatography-flame ionization detector (GC-FID).

Keywords: Spent cell culture media; Amino acids derivatives; Dipeptide derivatives; EZ:faast kit; Liquid phase extraction; Solid phase extraction; Gas chromatography-flame ionization detector

1. Introduction

The development and optimization of cell culture media for the production of therapeutic proteins [1,2] and viral growth [3,4] is fundamental to the expanding biopharmaceutical industry. The optimization and utility of cell culture media requires a precise blend of nutrients such as essential and non-essential amino acids, carbohydrates, vitamins, lipids, growth factors, trace elements, minerals and many other compounds. 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. However, product syntheses and metabolic shifts lead to ever changing external cellular conditions in batch culture which can inhibit cellular growth, metabolism and overall product synthesis. Hence, there is a vital need to optimize cell culture media formulations and control such dynamic environmental changes so as to efficiently maximize cell growth, viability and specific productivity.

Production of biopharmaceuticals is directly related to understanding the biochemical mechanisms of cells in cell culture media. One of the major challenges in the analysis of multicomponent media samples is the difficulty in completely separating individual or a group of constituents in a single chromatographic analysis without very involved sample preparation. The analysis of cell culture media and supernatants, as well as non-standard plant- or animal-derived protein hydrolysates such as fetuin, yeastolate, soy or collagens, has created the need for analytical techniques that are capable of accurately quantifying free amino acids as well as amino acids not normally found in virgin cell culture media. However, analysis of the amino acid and dipeptide constituents of cell culture media has been problematic due to the presence of many other interfering components. Moreover, the significant differences in the chemical structure of the functional groups, ranging from nonpolar to highly polar and acidic to alkaline side chains may interfere with the derivatization or detection of the amino acids and dipeptides derivatives.

Previously, physiologically free amino acid analysis has been performed by integrated pulsed amperometric detection (IPAD) [5,6], capillary electrophoresis (CE) [7], high-performance liquid chromatography (HPLC) [8], liquid chromatography–mass spectrometry (LC–MS) [9], gas chro-

* Corresponding author. Tel.: +1 913 253 3536; fax: +1 913 253 3850.
E-mail address: barry.drew@sial.com (B. Drew).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.11.003

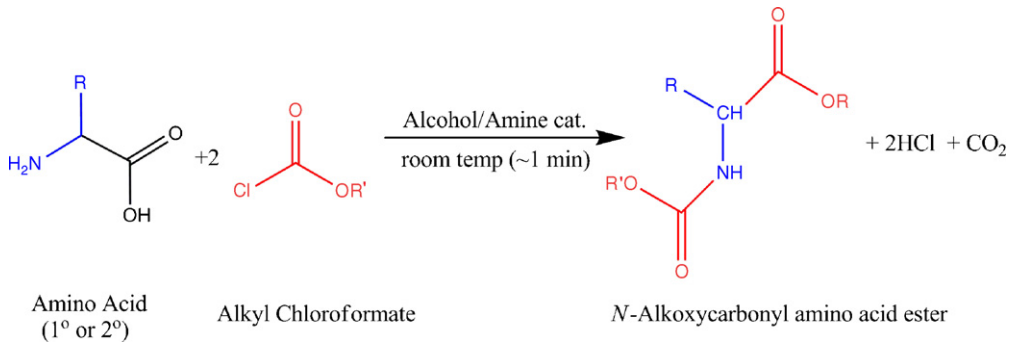

Fig. 1. Derivatization scheme of the alkylchloroformate esterification of amino acids.

matography (GC) [10–17] and gas chromatography–mass spectrometry (GC–MS) [16,18–20]. However, many of these methods have been impeded by labor intensive sample preparation, long analysis times, decreased resolution due to ion suppression, poor separation, low absolute sensitivity or dedicated and expensive instruments. Chromatographic methods of analysis have generally required a derivatization step to increase sensitivity and to improve selectivity of the analyte of interest. Both pre- and post-column derivatization have been used and their relative strengths and weaknesses discussed in detail [10,15,16,19–22]. Several liquid chromatographic methods have been developed for the determination of amino acid analysis. The most popular HPLC methods to date are *O*-phthalaldehyde (OPA) and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatized amino acids. These have several disadvantages. Most of these methods employ ultraviolet (UV) and fluorescent detection which either does not provide adequate sensitivity for the detection of amino acids or are limited to primary amines, especially, in cell culture media. The reaction of OPA with a primary amine and nucleophile, to produce a highly *N*-substituted isoindole has become a standard method for the trace analysis of amino acids. However, there are several

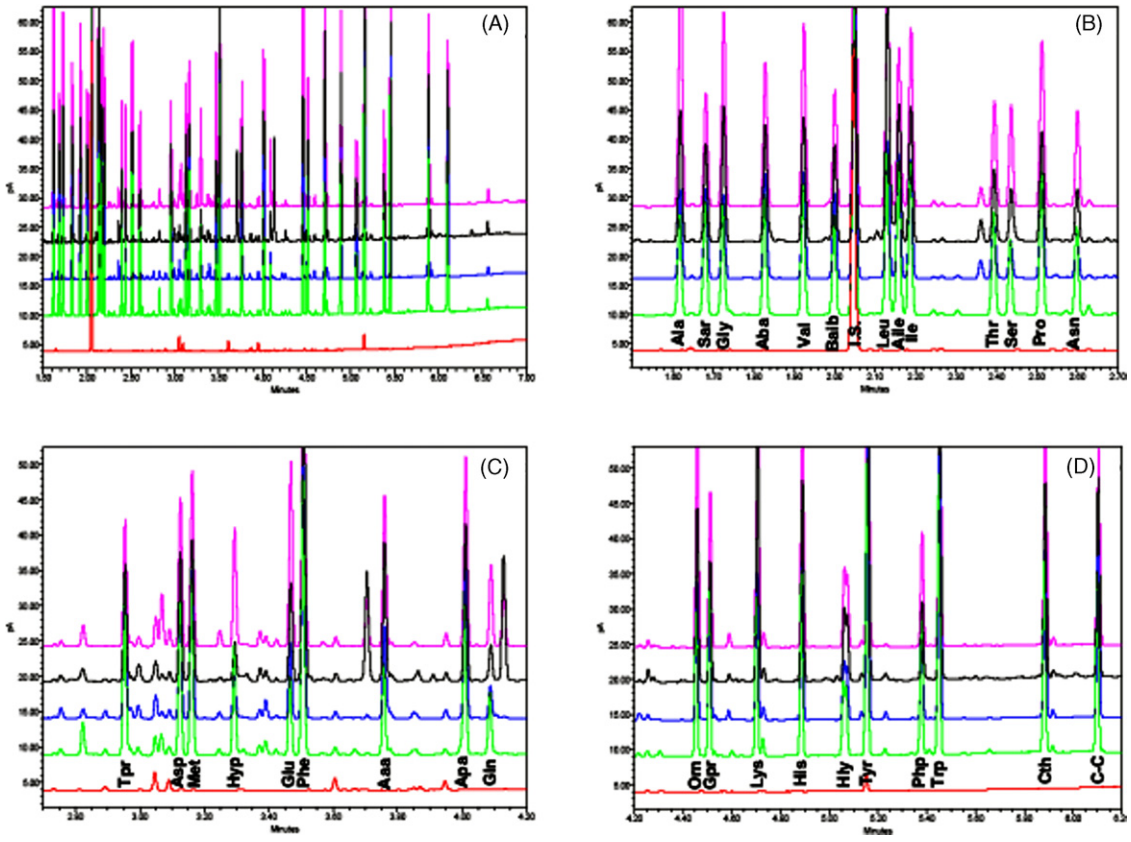

Fig. 2. Complete (A) and expanded ((B) 1.5–2.7 min, (C) 2.7–4.2 min and (D) 4.2–6.2 min) overlay chromatograms of a WFI blank (red trace) spiked with norvaline (I.S.), WFI blank with 10 nmol AA standard (green trace), blank media spiked with 10 nmol AA standard (blue trace), blank media spiked with 1% w/v hydrolysate and 10 nmol AA standard (black trace) and blank media spiked with 10% w/w FBS and 10 nmol AA standard (pink trace).

drawbacks to this method: the isoindole products are relatively unstable and decompose to nonfluorescent products; peptides derivatized with OPA fail to yield fluorescent products; and compounds containing more than one primary amine site, such as lysine and hydroxylysine exhibit quenching and have very low fluorescent yields. Although there have been other derivatizing agents developed for detection of amino acids by LC, none of these have focused on multi-site derivatization.

With conventional fluorescence detection (AQC), many of the problems with sensitivity have been improved. However, fluorescent detection is limited in the sensitivity of analysis due to the variation of light intensity with excitation sources such as mercury and xenon arc. Moreover, the major hydrolysis product and unreacted derivatizing reagents elute around 38 minutes thus swamping the detector and dwarfing some analyte peaks. Lastly, some unidentified peaks, produced from side reaction with the excess derivatizing reagent also appear on the chromatogram and can negatively affect accurate quantitation and sensitivity.

Gas chromatography has been in use for the past few decades; however, as most biologically active compounds are nonvolatile, thermally labile, ionic or of high molecular weight, derivatization was usually necessary. A major advancement in the derivatization of amino acids came with the use of alkylchloroformates [12–15] as a very rapid, efficient class of derivatizing reagent, as well as its utility in analyzing amino acids in biological matrices [14,16,21,23]. The method described here, using a commercially available reagents kit, EZ:faast^{TM} (Phenomenex), shows excellent accuracy and precision as well as very short analysis time (<7 min) for the baseline resolution of 33 amino acid and dipeptide peaks present in spent cell culture media. Additionally, the method is shown to be free of interferences from other matrix components present in serum and hydrolysates that are added to cell culture media and can be applied to other physiological fluids and food samples as well. This GC-FID method has been critical in our efforts to optimize cell culture media for higher growth and productivity for different cell lines.

2. Experimental

2.1. Chemicals

All reagents including standards (200 μM each), GC column (10 m × 0.25 mm ZB-AAA) and liner were provided in

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

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.[a]	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
Aile	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

[a] I.S. is norvaline and is spiked at 20 nmols in accordance with SOP.

the EZ:faast™ amino acid analysis sample testing kit by Phenomenex Inc. (Torrance, CA, USA).

2.2. Cell culture media

Amino acid- and protein-free NS0 media (labeled "Blank Media") were prepared and spiked with amino acid standards, insulin, ultrafiltered hydrolysate (SAFC Biosciences, Lenexa, KS, USA), and/or fetal bovine serum (FBS) (SAFC Biosciences, Lenexa, KS, USA). Minimum essential media (alpha modification) (SAFC Biosciences, Lenexa, KS, USA) was used without any modification.

2.3. Equipment

GC-FID was performed using an Agilent Technologies Inc. (Palo Alto, CA, USA) 6890N series gas chromatography system with dual flame ionization detectors and a 7683 series dual-tower and autosampler, all controlled by the Waters Corporation (Milford, MA, USA) Empower™ 2 Software package.

2.4. 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 1 min and then increased at 32 °C/min 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 was set at 320 °C and had a H_2 (ultrapure 5.0) flow of 35 mL/min and an oxidizer flow (hydrocarbon free air) of 350 mL/min. All gases were supplied by Praxair Inc. (Danbury, CT, USA).

2.5. Sample treatment

Amino acid- and protein-free NS0 cell culture media were prepared in house. The blank media was aliquoted to three containers, and insulin was added to a final concentration of 1.0 mg/L for each of the last two aliquots. Ultrafiltered hydrolysate was added to a final concentration of 1% w/v to the second aliquot and FBS added to a final concentration of 10% v/v to the final aliquot. Any unused portion of the media was kept at 4 °C during the course of the experiment.

One hundred microliters of cell culture media, 100 μL of internal standard (norvaline at 200 μM) and 50 μL of amino acid standards (each standard at 200 μM) were combined in a glass vial and mixed by two short bursts on a vortex. An ion exchange resin solid phase extraction (SPE) tip was attached to a 1.5 mL syringe and the solution was pulled slowly through to completion. Three hundred microliters of wash solution was added to the glass vial and also pulled slowly through the SPE tip to completion. The 1.5 mL syringe was removed while leaving the SPE tip inside the glass vial. Two hundred microliters of a premixed elution buffer was then added to the vial. The piston of a 0.6 mL syringe was pulled halfway up the barrel and attached to the SPE tip. Elution buffer was drawn into the SPE resin inside the tip to just before the filter plug and the sorbent material was quickly expelled into the glass vial. This step was repeated until all of the material had been expelled. Fifty microliters of derivatizing reagent was added to the glass vial and the mixture was vortexed vigorously for 8 sec. The solution was allowed to react for 1 min and the vortexing step repeated. One hundred microliters of organic reagent was then added to the emulsion and vortexed vigorously for 5 sec. The mixture was allowed to stand for 1 min for phase separation. One hundred microliters of weak acidic solution was added to stop the reaction and the mixture vortexed vigorously for 5 sec. After 1 min of the phase separation, an aliquot of the upper organic layer was transferred to a spring-loaded microinsert within an autosampler vial. Two microliters of the extract are injected into the GC-FID for analysis.

3. Results and discussion

The analytical method described is suitable for rapid and highly sensitive determination of primary and secondary amino acids, physiological amines and dipeptides present in cell culture media, as well as other complex biological matrices such as fermentation broths, urine, cerebral spinal fluid, wine or grain samples. This procedure, which consists of solid phase

Table 2
Relative lower limit of detection and linearity range by GC-FID

Name	Limit of detection (nmol)	Range of linearity (nmol)	Correlation coefficient
Ala	0.039	0.2–50	0.998
Sar	0.064	0.2–50	0.995
Gly	0.090	0.2–50	0.998
Aba	0.021	0.2–50	0.999
Val	0.031	0.2–50	0.999
Baib	0.054	0.2–50	1.000
Leu	0.034	0.2–50	0.999
Aile	0.028	0.2–50	1.000
Ile	0.026	0.2–50	0.999
Thr	0.021	0.2–50	0.988
Ser	0.025	0.2–50	0.996
Pro	0.042	0.2–50	0.998
Asn	0.060	0.2–30	0.994
Tpr	0.024	0.2–30	0.990
Asp	0.027	0.2–50	0.991
Met	0.026	0.2–50	1.000
Hyp	0.043	0.2–50	0.984
Glu	0.027	0.2–50	0.999
Phe	0.030	0.2–50	0.998
Aaa	0.095	0.2–50	0.998
Apa	0.028	0.2–30	0.999
Gln	0.025	0.2–30	0.996
Orn	0.012	0.2–50	0.990
Gpr	0.036	0.2–30	0.999
Lys	0.034	0.2–50	0.989
His	0.037	0.2–50	0.997
Hly	0.039	0.2–30	0.998
Tyr	0.060	0.2–50	0.999
Php	0.044	0.2–30	0.998
Trp	0.016	0.2–80	0.996
Cth	0.022	0.2–30	0.998
C–C	0.034	0.2–50	0.997

extraction followed by a single-step, rapid derivatization and liquid/liquid phase extraction step, can be completed in under 3 min per sample when multiple samples are prepared. This procedure ensures the selective removal of interfering compounds from cell culture media additives such as serum and hydrolysates which can lead to poor chromatographic results while analyzing for amino acids. The use of GC with FID as a detector is highly sensitive and can rapidly detect 33 amino acid and dipeptide peaks in less than 7 min at very low concentrations of the analytes present in the cell culture broth. The simple derivatization procedure (Fig. 1) and the ease with which the derivatives can be separated make this method feasible for routine analysis with high throughput. Moreover, the *N*-alkoxycarbonyl ester derivatives have been observed by us to be stable for at least 5 days at room temperature (%R.S.D. less than 2%; data not shown).

As shown in Fig. 2, all amino acids (10 nmol of each standard) were baseline resolved and detected in water for injection (WFI), amino acid- and protein-free NS0 blank media, blank media with 1% w/v ultrafiltered soy hydrolysate and blank media with 10% v/v FBS. In WFI, a small peak eluted near the same retention time as tyrosine. This peak was present in the blank even after changing both the column and liner and is considered to be an artifact of the assay. However, the total area of the peak was negligible to overall concentration of tyrosine typically found in cell culture media and hence had no effect on the overall quantitation of this analyte. Hydroxylysine (RT = 5.06 min) appears as two slightly resoloved, distinct peaks but merges into one single peak at higher concentrations (not shown). From Fig. 2, the complexity of cell culture media and its components can be seen; however, the separation and resolution of each peak is high enough to allow for the identification and quantitation of the amino acids and dipeptides of interest. The addition of nutrient rich hydrolysate and serum introduces the presence of dipeptides which are not within the range of standards used and cannot be identified by the current methodology. These include the peaks at both 3.72 and 4.13 min.

Table 1 shows the specificity of repeated injections ($n = 6$) on the overall retention time. In each matrix, the standard deviation of the retention time of any of the amino acid standards never exceeded 0.1 s. Moreover, if all injections are considered together (approximately 800 injections on the same column), the retention time standard deviation for any amino acid analyte peak never increased more than 0.3 s, demonstrating the high reproducibility for identifying peaks using retention time.

Table 3
Intra- and inter-assay precision (%R.S.D., $n = 6$ injections)

	Matrix																	
	Blank media						Hydrolysate						10% FBS					
	d_1	d_2	d_3	d_4	d_5	d_x	d_1	d_2	d_3	d_4	d_5	d_x	d_1	d_2	d_3	d_4	d_5	d_x
Ala	0.2	0.4	0.6	0.3	0.6	0.5	0.2	0.1	0.4	0.3	0.7	0.3	0.4	0.6	0.4	0.4	0.6	0.5
Sar	0.2	0.5	0.6	0.4	0.6	0.5	0.3	0.1	0.5	0.3	0.8	0.4	0.5	0.5	0.6	0.5	0.8	0.6
Gly	0.3	0.5	0.6	0.4	0.6	0.5	0.2	0.2	0.6	0.5	1.0	0.5	0.6	1.0	1.3	0.7	1.4	1.0
Aba	0.2	0.5	1.1	0.3	1.1	0.6	0.5	0.4	2.6	1.3	1.2	1.2	0.5	0.7	0.6	0.5	0.8	0.6
Val	0.1	0.4	0.5	0.3	0.5	0.4	0.2	0.1	0.5	0.2	0.7	0.3	0.4	0.5	0.5	0.5	0.6	0.5
Baib	0.2	0.5	0.6	0.4	0.6	0.5	0.2	0.1	1.4	0.3	0.7	0.5	0.4	0.5	0.6	0.4	0.7	0.5
Leu	0.2	0.5	0.5	0.3	0.5	0.5	0.6	0.2	4.7	0.6	0.8	1.4	0.5	0.9	1.1	0.6	1.1	0.8
Aile	0.1	0.4	0.5	0.3	0.5	0.4	0.6	0.3	0.4	0.6	0.8	0.5	0.4	0.4	0.6	0.4	0.6	0.5
Ile	0.2	0.4	0.5	0.3	0.5	0.5	0.3	0.1	0.3	0.3	0.6	0.3	0.4	0.6	0.6	0.4	0.7	0.5
Thr	0.3	0.5	1.1	0.4	1.1	0.6	0.4	0.2	2.6	0.3	0.9	0.9	0.5	0.9	2.0	0.5	1.1	1.0
Ser	0.5	1.0	1.4	0.6	1.4	0.9	0.6	0.6	3.6	0.7	1.1	1.3	0.5	0.8	2.7	0.6	1.3	1.2
Pro	0.2	0.4	0.6	0.3	0.6	0.5	0.3	0.1	0.6	0.3	0.7	0.4	0.4	0.6	1.0	0.5	0.7	0.6
Asn	0.3	0.5	0.7	0.4	0.7	0.6	0.4	0.2	1.3	0.4	0.9	0.6	0.5	0.6	2.5	0.5	1.1	1.0
Tpr	0.7	0.6	0.6	0.6	0.6	0.7	0.4	0.2	1.1	0.3	0.8	0.5	0.6	0.6	1.7	0.5	1.2	0.9
Asp	0.3	0.5	0.7	0.5	0.7	0.6	0.5	0.2	1.4	0.3	0.9	0.7	0.5	0.7	2.6	0.5	1.1	1.1
Met	0.3	0.5	0.6	0.5	0.6	0.5	0.6	0.2	1.9	0.4	0.9	0.8	0.5	0.6	2.3	0.5	1.0	1.0
Hyp	0.6	0.8	1.2	0.9	1.2	0.9	1.0	0.9	2.6	1.3	2.5	1.7	0.8	1.2	4.4	1.0	2.0	1.9
Glu	0.3	0.5	0.7	0.5	0.7	0.6	0.6	0.2	1.6	0.4	0.9	0.7	0.5	0.6	3.1	0.5	1.1	1.2
Phe	0.2	0.4	0.6	0.4	0.6	0.5	0.9	0.3	1.6	0.4	0.8	0.8	0.4	0.5	2.8	0.5	1.1	1.1
Aaa	0.8	2.0	1.8	0.7	1.8	1.4	1.7	1.7	2.9	1.1	2.2	1.9	0.8	1.2	4.1	1.1	2.0	1.8
Apa	0.3	0.5	0.7	0.6	0.7	0.6	0.7	0.3	2.1	0.6	1.1	0.9	0.4	0.8	4.1	0.6	1.3	1.5
Gln	0.6	0.9	0.7	1.1	0.7	1.0	1.5	0.7	3.1	1.0	1.5	1.5	0.5	1.4	5.8	1.1	1.6	2.1
Orn	0.3	0.5	0.7	0.8	0.7	0.7	1.0	0.3	2.6	0.8	1.4	1.2	0.5	0.9	5.1	0.9	1.5	1.8
Gpr	0.5	0.5	0.7	0.8	0.7	0.7	1.0	0.3	2.8	0.8	1.4	1.3	0.5	1.0	5.2	0.8	1.4	1.8
Lys	0.4	0.5	0.6	0.9	0.6	0.7	1.1	0.3	2.8	1.0	1.5	1.3	0.6	1.0	5.2	0.9	1.5	1.8
His	0.4	0.5	0.7	1.0	0.7	0.7	1.1	0.3	2.9	0.9	1.5	1.3	0.5	1.0	5.5	0.9	1.7	1.9
Hly	0.5	0.5	0.7	1.4	0.7	0.9	1.3	0.4	2.5	1.2	1.8	3.7	0.7	1.4	6.0	1.3	1.9	2.3
Tyr	0.5	0.6	0.9	1.1	0.9	0.9	1.5	0.6	3.3	1.0	1.7	1.6	0.7	1.3	5.9	1.1	1.6	2.1
Php	1.1	0.6	0.7	1.2	0.7	1.0	1.2	0.4	3.0	1.2	2.0	1.6	0.6	1.3	5.6	1.2	1.6	2.0
Trp	0.4	0.5	0.7	1.3	0.7	0.9	1.1	0.2	2.9	1.1	1.9	1.4	0.6	1.2	5.4	1.2	1.5	2.0
Cth	0.4	0.5	0.6	1.4	0.6	1.0	1.0	0.3	2.7	1.0	1.9	1.4	0.5	1.2	5.2	1.2	1.4	5.0
C–C	0.5	0.5	0.7	1.3	0.7	1.0	1.0	0.2	2.7	1.0	2.0	1.4	0.4	1.5	5.0	1.2	1.5	1.9

As a result, true analytical peaks can be easily distinguished from superfluous or unknown peaks by visual or computational constraints (*i.e.,* retention window) relative to known standards.

The limit of detection and range of linearity are shown in Table 2 for each of the standard analytes. The limit of detection (LOD) was based on the least squares regression analysis and determined using the following equation:

$$\mathrm{LOD} = \frac{3.3\sigma}{S} \quad (1)$$

where σ is the residual standard deviation of the regression line and S is the slope of the calibration curve.

According to the ICH guidelines [24,25], the standard deviation of the response used to determine LOD can be determined using the residual standard deviation of a regression line in the range of the detection limit. For this experiment, the chromatographic peak area ratio of each standard analyte to the internal standard (norvaline) over the range of concentrations from 0.02 to 5.0 nmol was plotted and the residual standard deviation of the linear regression of each determined. The slope over the regression lines for each concentration tested to its corresponding peak area ratio was also determined and used in Eq. (1) to determine the LOD for each analyte.

The range of linearity is limited to the overall amount of total amino acids that can be effectively bound to the cation exchange resin used in the solid phase extraction (SPE) of the amino acids from the sample matrix. The binding limit value is given as 1.2 μmol of total amino acids [26]. For matrices with higher salinity, the overall total binding efficiency of amino acids can be reduced by cations (calcium, sodium, potassium or magnesium) from salts competitively binding to the cation exchange sorbent resin and consequently reducing the availabity of binding sites for amino acids. For salinities consistent with cell culture media and lower, we have observed no competive influence. For instance, we have observed that the percent recovery between amino acid standards in WFI, amino acid-free media or 150 mM phosphate buffered saline (PBS) to be identical, while the use of saturated sodium chloride solution caused a significantly decreased efficiency in the binding on amino acid standards resulting in a percent recovery of about 5% of the expected concentrations (data not shown).

In Table 3, the intra- and inter-assay precision are shown. One of the advantages to repeat injections is that a low %R.S.D. indicates a homogenous liquid extraction during the final step of the methodology. Specifically, a higher value would indicate some of the sorbent resin or aqueous phase was pulled up during the liquid extraction and aliquoted to the sample vial. Based on prior experience, a cumulative (*i.e.*, the summation of all analytes) %R.S.D. of ≤1.0 would indicate a clean liquid extraction. Generally a cumulative %R.S.D. below 3% is acceptable. In Table 3, it is evident that day 3 for the blank NS0 media containing 10% FBS is slightly higher than the cumulative %R.S.D. deemed acceptable for quantitative accuracy which indicates a poor extraction on that day. When protein-containing raw materials, such as hydrolysate or FBS, are added to cell culture media, the final liquid extract using the aforementioned methodology can be slightly opaque. A 15–30 sec low speed centrifugation will separate this material out of the organic layer yielding a very homogeneous, organic extract.

Table 4
Percent recovery of amino acids/dipeptides in various matrices spiked at 10 nmol with each AA ($n = 6$, mean ± S.D.)

Name	Matrix		
	Blank media	Hydrolysate	10% FBS
Ala	96.4 ± 1.2	100.7 ± 2.4	100.5 ± 4.4
Sar	92.2 ± 1.1	92.5 ± 2.1	88.4 ± 0.7
Gly	95.1 ± 1.8	98.0 ± 5.8	96.3 ± 1.9
Aba	95.3 ± 1.4	98.5 ± 2.6	102.9 ± 1.1
Val	95.0 ± 0.8	96.8 ± 3.8	94.9 ± 2.6
Baib	99.8 ± 1.4	97.2 ± 1.4	103.5 ± 1.1
Leu	101.1 ± 0.4	104.1 ± 2.6	97.5 ± 1.6
Aile	97.8 ± 1.9	104.8 ± 1.4	94.7 ± 0.5
Ile	98.1 ± 1.7	99.0 ± 4.5	96.5 ± 3.3
Thr	77.6 ± 2.1	86.6 ± 7.6	94.8 ± 2.3
Ser	60.7 ± 8.3	65.3 ± 12.5	101.9 ± 6.5
Pro	92.6 ± 1.9	93.1 ± 4.0	88.3 ± 1.7
Asn	73.5 ± 7.6	76.3 ± 15.3	101.2 ± 3.2
Tpr	92.3 ± 3.2	90.4 ± 2.4	75.1 ± 2.3
Asp	86.1 ± 8.7	96.2 ± 12.1	98.1 ± 11.7
Met	95.7 ± 1.3	83.9 ± 2.8	89.7 ± 2.0
Hyp	51.4 ± 7.0	40.6 ± 8.0	85.3 ± 5.0
Glu	109.8 ± 9.1	103.7 ± 13.5	105.1 ± 20.0
Phe	110.1 ± 2.5	110.0 ± 2.2	96.5 ± 2.2
Aaa	107.9 ± 3.8	126.0 ± 12.0	97.2 ± 10.9
Apa	116.5 ± 4.2	115.0 ± 4.4	112.5 ± .4
Gln	72.6 ± 4.8	73.0 ± 12.0	86.7 ± 4.4
Orn	110.4 ± 4.7	110.3 ± 5.1	116.2 ± 4.4
Gpr	95.8 ± 3.4	96.2 ± 7.7	89.6 ± 2.9
Lys	104.4 ± 4.6	95.8 ± 9.6	120.7 ± 6.8
His	108.7 ± 2.3	106.6 ± 6.4	103.9 ± 5.1
Hly	96.6 ± 11.6	89.2 ± 14.3	100.5 ± 15.9
Tyr	109.3 ± 4.1	112.1 ± 5.3	98.4 ± 4.5
Php	85.5 ± 3.5	82.2 ± 9.7	82.9 ± 3.6
Trp	118.3 ± 5.6	109.1 ± 5.6	98.1 ± 5.7
Cth	106.3 ± 3.4	112.8 ± 5.2	115.7 ± 7.6
C–C	108.0 ± 2.1	119.9 ± 6.9	104.5 ± 6.3

The percent recoveries in each of the matrices are shown in Tables 4 and 5. In amino acid-free and protein-free NS0 media (Table 4), the percent recovery was within an 80–120% range for 27 of the 32 analytical standards (based on the ratio of the peak area of the analyte to the peak area of the internal standard), in accordance with the International Conference of Harmonization (ICH) guidelines for analytical validation [24,25]. The five which were below the 80% criteria were threonine, serine, asparagine, glutamine and hydroxyproline, which all have polar, neutral side chains. Polar functional groups reduce the volatility of the derivatized compound allowing degradation within the injection port. For example, threonine has one more methyl group than serine giving the threonine a slightly more nonpolar character. Consequently the less polar threonine has a higher percent recovery (77.6%) than the serine (60.7%). When 1% w/v ultrafiltered hydrolysate was added to the blank NS0 media described above, four of the five amino acids previously mentioned were below the 80% criteria, with only threonine improving (86.6%) in analyte recovery. When 10% v/v BSA was added to the blank NS0 media described above, the percent recovery for the five amino acids which were below the

Table 5
Percent recovery of amino acids/dipeptides in minimum essential medium alpha modification ($n = 6$, mean ± S.D.)

Name	Matrix		
	Experimental	Theoretical	% Recovery
Ala	29.6 ± 0.3	28.1	105.5
Gly	78.8 ± 3.1	66.6[a]	118.3
Val	43.2 ± 1.3	39.3	110.1
Leu	39.8 ± 0.4	39.9	99.6
Aile	2.6 ± 1.3	0.0	–
Ile	41.5 ± 1.7	40.0	103.6
Thr	44.6 ± 4.0	40.3	110.7
Ser	21.1 ± 2.1	23.8	88.7
Pro	32.5 ± 2.4	34.7	93.7
Asn	29.5 ± 3.3	33.3[a]	88.5
Asp	24.9 ± 1.3	22.5	110.7
Met	9.1 ± 0.2	10.1	90.4
Glu	57.2 ± 2.0	51.0[a]	112.2
Phe	18.0 ± 0.5	19.4	92.8
Gln	134.4 ± 15.2	199.8[a]	67.3
Lys	40.5 ± 3.3	39.7	102.0
His	19.4 ± 0.7	20.0	97.3
Tyr	19.4 ± 0.7	19.9	97.5
Trp	4.2 ± 0.2	4.9	85.7
C–C	8.3 ± 0.2	10.0	83.0

[a] Outside the range of linearity (see Table 2).

80% criteria improved dramatically. One amino acid, thioproline (75.1%), fell below the 80% criteria, while one amino acid, lysine (120.7%), was slightly outside the 120% criteria. While these amino acids are relevant to cell culture media and the percent recovery is not closer to complete recovery, the assay is highly reproducible which means differences between formulations, passages, cell lines and days in culture can be determined with high precision.

Table 5 shows the percent recovery of amino acids in minimum essential medium (alpha modification), a commercially available and nonproprietary media. Three of the amino acids (glycine, asparagine, glutamic acid and glutamine) were far outside the range of linearity of standards and will not be included in the discussion. A fourth amino acid, asparagine, was only slightly outside the range of linearity and the recovery was 88.5%. Allo-isoleucine, which is not present in the formulation, was detected at low concentrations which indicate the interconversions between its stereoisomers leucine and isoleucine. All of the other amino acids fell within the 80–120% range and showed the utility of this method for determining amino acid concentrations in commercially available cell culture media.

4. Conclusion

Improved production of biopharmaceuticals is directly related to better understanding how cells behave in cell culture media. This necessitates a very thorough analysis of the media constituents. However, multicomponent samples, such as serum cell culture media, and other physiological specimen are extremely difficult to separate completely in a single chromatographic analysis without laborious and time consuming sample preparation. For the trace analysis of amino acids and dipeptides, the chromatographic system must be capable of delivering results with a high sensitivity, selectivity and a high degree of precision. The described GC-FID method allows for the simultaneous separation, detection and baseline resolution of 33 amino acids and dipeptides within a very complex matrix with reliable quantitation in under 7 min. GC was chosen to meet these requirements by combining liquid–liquid and SPE extraction with FID detection. This method's detection is extremely sensitive, with detection limits comparable with or better than those of other chromatographic systems. Moreover, selectivity is enhanced, since very few endogenous compounds are derivatized. For added confirmation, selectivity was investigated by GC-FID for possible interference of media components such as serum, hydrolysates, and insulin.

Acknowledgments

We thank Drs. Michael Wathen and Tivadar Farkas for input and for critical reading of the manuscript.

References

[1] J.E. Dally, J. Gorniak, R. Bowie, C.M. Bentzley, Anal. Chem. 75 (2003) 5046.
[2] M. Schneider, I.W. Marison, S.U. von, J. Biotechnol. 46 (1996) 161.
[3] L. Maranga, C.F. Goochee, Biotechnol. Bioeng. 94 (2006) 139.
[4] S.C. Wu, C.C. Liu, W.C. Lian, Vaccine 22 (2004) 3858.
[5] P. Jandik, J. Cheng, N. Avdalovic, Methods Mol. Biol. 211 (2003) 155.
[6] P. Jandik, J. Cheng, N. Avdalovic, J. Biochem. Biophys. Methods 60 (2004) 191.
[7] C. Prata, P. Bonnafous, N. Fraysse, M. Treilhou, V. Poinsot, F. Couderc, Electrophoresis 22 (2001) 4129.
[8] Z. Deyl, J. Hyanek, M. Horakova, J. Chromatogr. 379 (1986) 177.
[9] J.Y. Kwon, M. Moini, J. Am. Soc. Mass Spectrom. 12 (2001) 117.
[10] Amino Acid Analysis by Gas Chromatography, CRC Press, Boca Raton, FL, 1987.
[11] P. Husek, J. Chromatogr. 91 (1974) 483.
[12] P. Husek, J. Chromatogr. 113 (1975) 139.
[13] P. Husek, FEBS Lett. 280 (1991) 354.
[14] P. Husek, J. Chromatogr. B Biomed. Appl. 669 (1995) 352.
[15] P. Husek, J. Chromatogr. B Biomed. Sci. Appl. 717 (1998) 57.
[16] M.J. Nozal, J.L. Bernal, M.L. Toribio, J.C. Diego, A. Ruiz, J. Chromatogr. A 1047 (2004) 137.
[17] R.W. Zumwalt, J. Desgres, K.C. Kuo, J.E. Pautz, C.W. Gehrke, J. Assoc. Off Anal. Chem. 70 (1987) 253.
[18] H. Bruckner, A. Schieber, Biomed. Chromatogr. 15 (2001) 166.
[19] K.R. Kim, J.H. Kim, E. Cheong, C. Jeong, J. Chromatogr. A 722 (1996) 303.
[20] P. Vesely, L. Lusk, G. Basarova, J. Seabrooks, D. Ryder, J. Agric. Food Chem. 51 (2003) 6941.
[21] A. Namera, M. Yashiki, M. Nishida, T. Kojima, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 776 (2002) 49.
[22] J.L. Bernal, M.J. Nozal, L. Toribio, J.C. Diego, A. Ruiz, J. Sep. Sci. 28 (2005) 1039.
[23] P. Husek, H.M. Liebich, J. Chromatogr. B Biomed. Appl. 656 (1994) 37.
[24] International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures, ICH-Q2A, Geneva 1995.
[25] International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: methodology, ICH-Q2B, Geneva 1996.
[26] EZ:faast™ For Free (Physiological) Amino Acid Analysis by GC-FID or GC-NPD User's Manual. 2006.