Journal of Chromatography B, 879 (2011) 1353–1360

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Characterization of amino acid profiles of culture media via pre-column 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization and ultra performance liquid chromatography☆

G. Fiechter, H.K. Mayer*

Department of Food Science and Technology, Food Chemistry Laboratory, BOKU - University of Natural Resources and Life Sciences Vienna, Muthgasse 11, A-1190 Vienna, Austria

ARTICLE INFO

Article history:
Received 16 July 2010
Accepted 2 February 2011
Available online 1 March 2011

Keywords:
UPLC™
Amino acid derivatization
6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate
Culture medium

ABSTRACT

The AccQ.Tag™ method, as a well-established protocol for amino acid analysis combining derivatization procedure, dedicated HPLC separation and fluorescence detection, was properly transferred and accordingly optimized for the application on ultra performance liquid chromatography (UPLC™) and UV detection. Capitalizing on *sub*-2 μm particles, this newly established UV-UPLC™ technique facilitated efficient chromatographic separation of 21 amino acid derivatives within 12 min. In addition, UPLC™ demonstrated significant improvements due to superior performance and reduced run times compared with the former 35 min of the original HPLC protocol. Using UV instead of fluorescence detection, amino acid quantification after pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) yielded appropriate sensitivities within the low *pmol* range with corresponding detection limits varying from 0.11 to 0.57 pmol per injection. Moreover, the UPLC™ method was applied to characterize changes in the free (*FAA*) as well as total amino acid (*TAA*) profiles specific to culture media at three distinctive stages of fermentation: fresh medium, fermentation broth after cell mass production prior to induction and after product expression at the end of fermentation. Amino acid profiles intrinsic to the fresh, sterilized medium indicated free, hence more bioavailable, amino acids at a *FAA*/*TAA* ratio of 40%, whereas ongoing fermentation implied a rather specific, successive decline in selective *FAA* concentrations. Thereby, the most distinctive variations in *FAAs* were highlighted by aspartic acid, serine and threonine, each exhibiting an almost complete uptake from the culture media (−96% to −99%), with remaining *FAA*/*TAA* ratios of 1%, 8%, and 1%, respectively. This indeed may indicate limitations and shortages within the nutrient broth. Thus, amino acid monitoring utilizing *high-throughput* chromatography, such as UPLC™, can be considered as a valuable tool to facilitate rapid adjustments of fermentation broths and to optimize culture media to specific requirements.

1. Introduction

Culture or growth media represent an indispensible tool of nowadays microbiology, designated to facilitate bacterial growth by supplementation of the required main nutrients with reference to a carbon supply and a nitrogen or amino acid source. However, depending on the desired application, their overall composition may vary significantly. *Defined* culture media constitute a well characterized chemical composition adjusted to a specific requirement only, whereas *complex undefined* or *basal* media originate mainly from hydrolysis of a typical protein source like casein, soy, beef or yeast with the exact composition being unknown. Due to their intrinsic complexity in nutrient structure and with optional fortification of specific compounds, such growth media may serve well for the non-selective cultivation of the majority of laboratory cultures. For such partial protein hydrolyzates, both the overall ratio as well as the specific composition of free, hence more bioavailable, amino acids is generally unclear. However, this might pose a certain interest, due to potentially required further amino acid supplementation in order to match particular requirements (e.g., adjustment to the amino acid uptake in course of fermentation).

With respect to amino acid characterization of hydrolyzates, the official AOAC reference method recommends ion-exchange chromatography in combination with post-column ninhydrin derivatization [1], although the variety of nowadays *state-of-the-art* pre-column derivatization techniques and the enhanced performance of *high-throughput* RP-chromatography [2–4] might emphasize an adequate alternative.

Most commonly applied protocols for amino acid quantification comprise pre-column derivatization with

☆ This paper is part of the special issue "Enhancement of Analysis by Analytical Derivatization", Jack Rosenfeld (Guest Editor).

* Corresponding author. Tel.: +43 1 47654 6170; fax: +43 1 47654 6196.
E-mail address: helmut.mayer@boku.ac.at (H.K. Mayer).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.02.003

either phenylisothiocyanate (PITC), *o*-phthalaldehyde (OPA), 9-fluorenylmethylchloroformate (FMOC), or 5-dimethylaminonaphthalene-1-sulfonyl chloride (DANSYL-Cl). However, no commercial "all-purpose" reagent has been established so far, as for all of the above mentioned techniques one or more key attributes still exhibited some inherent disadvantages. For instance, the necessity to remove any excess reagent (PITC), excess reagent that might interfere the separation (FMOC), the incapability to derivatize secondary amino groups (OPA) or even, long derivatization protocols at elevated temperatures (DANSYL-Cl) [5]. Pre-column derivatization utilizing 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), might constitute a feasible alternative, as to method characteristics that overcome some of these major drawbacks: AQC performs rapid ($t_{1/2}$ < 1 s) and quantitative derivatizations of primary and secondary amino groups in alkaline solution (pH 8.0–10.0) yielding stable (storable up to 1 month at 4 °C) derivatives. Besides, AQC derivatives allow fluorescence (excitation/emission at 248/395 nm) or UV detection (254 nm) with sensitivities at either *fmol* or low *pmol* level, respectively. Moreover, excess reagent hydrolyzes with water at a much slower rate forming 6-aminoquinoline (AMQ) as a fluorogenic side compound, thus despite its excessive concentration fortunately does not compromise separations due to the emission maxima found at 520 nm. For UV detection at 254 nm, however, AMQ implies equal absorption as the targeted derivatives and subsequently eluates as a massive peak dependent on eluent pH either prior or within the polar amino acids. The commonly applied, fairly facile, AQC derivatization protocols primarily include the buffering of sample solutions to match optimal (alkaline) derivatization pH, directly followed by the admixture of derivatization reagent (solution in aprotic solvents due to the reactivity towards water to form AMQ). However, to finalize derivatization, an additional heating step (55 °C for ~10 min) is needed to convert di-derivatized tyrosine (unstable phenolic AQC adduct) to its mono-form. Consequently, due to these advantageous characteristics, AQC has yet been successfully applied to various applications [5–17].

Given the superior chromatographic performance of the *sub*-2 μm particle technology, yet being realized in ultra performance liquid chromatography (UPLC™) [18], the objective of this present study was to properly adapt the AccQ.Tag™ method (as a well-established protocol for amino acid analysis dedicated to HPLC separation, AQC pre-column derivatization and fluorescence detection) onto UPLC™ conditions. Since UV detection already proved excellent suitability in UPLC™ analyses of AQC derivatives of biogenic amines [19], method transfer was performed using UV instead of fluorescence detection. Moreover, capitalizing on this newly established fast-LC application, nutrient broths at different stages of fermentation were to be characterized quantitatively on their free (*FAA*) as well as total amino acid (*TAA*) contents.

2. Experimental

2.1. Chemicals and reagents

Commercial mixed amino acid hydrolyzate standard *type H* comprising 17 amino acids already dissolved in 0.1 M hydrochloric acid (HCl) (L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-leucine, L-isoleucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine each at 2.5 mM; with the exception of L-cystine which was 1.25 mM) was purchased from Pierce (Rockford, IL, USA), additional amino acids as solids (DL-alpha-n-amino butyric acid, L-tryptophan, L-citrulline, L-ornithine hydrochloride each at ≥99% purity) were obtained from Fluka (Buchs, Switzerland) or Sigma (St. Louis, MO, USA). Both AccQ.Tag™ Eluent A concentrate and AccQ.Fluor™ Reagent Kit (including derivatization reagent and borate buffer) were from Waters (Milford, MA, USA). Saturated phenol solution at biotechnology grade was received from Amresco (Solon, OH, USA), while all other reagents, chemicals and solvents exhibited either analytical or HPLC grade and were obtained from Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany). Ultrapure water prepared utilizing an Elga ultra-high quality water purification system (High Wycombe, Buckinghamshire, UK) was used in all experiments.

2.2. Amino acid standards and quantification

For the preparation of mixed amino acid standards, at first, 50 mM stock solutions of the respective crystalline amino acids (tryptophan, citrulline, and ornithine) were prepared in 0.1 M HCl. These stock solutions were then merged and diluted (1/20) to yield a 2.5 mM intermediate composite standard. Additionally, a 2.5 mM solution for alpha-amino butyric acid (AABA) as internal standard was prepared. Merging the composite standard (3 amino acids), the internal standard, and the commercial Pierce standard (17 amino acids at 2.5 mM already dissolved in 0.1 M HCl) and further diluting in ultrapure water yielded mixed amino acid standards ranging from 5 to 160 μM for each of the 20 analytes and constant 40 μM for AABA as internal standard.

Five microliters of these respective standard solutions were directly derivatized (equal to an additional 1/10 dilution) utilizing Waters AccQ.Fluor™ Reagent Kit and further used for system calibration. Seven calibration solutions ranging from 0.5 to 16 μM (0.5, 1, 2, 4, 6, 8, 16 μM) were submitted to UPLC™ analysis at an injection volume of 4 μL, respectively, indicating final calibration concentrations of 2–64 pmol per injection for each amino acid and 16 pmol/4 μL for the internal standard. The cystine concentrations (1–32 pmol/4 μL) were half that of the other amino acids, due to the reduced cystine amounts present in the commercial Pierce standard (only 1.25 mM for cystine). Using Waters Empower 2 chromatography software, non-weighted linear calibration functions in terms of the obtained peak response versus concentration of the 20 investigated amino acids, with AABA as internal standard were recorded.

For amino acid quantification in culture media, internal standard solution was admixed to processed samples prior to derivatization procedure, yielding constant amounts of 16 pmol AABA per 4 μL injection volume. Individual amounts for amino acids (free; unbound or after acid hydrolysis), recorded as pmol per injection, were subsequently converted to practical concentrations calculated as mg per L culture media.

2.3. Culture media samples

Sample material comprised 200 mL aliquots of colloidal suspended, *ready-for-use* nutrient broths at different stages of fermentation divided in: (i) fresh culture medium after sterilization, (ii) inoculated fermentation broth after cell mass production prior to induction, as well as (iii) after product expression at the end of fermentation. For the latter two, bacterial cell mass was separated via centrifugation, hence only the cleared media were submitted to analysis. Major nitrogenous constituents primarily originated from casein pepton and yeast hydrolyzate with auxiliary enhancement of selective amino acids. Since the analyzed samples were categorized as R&D and due to attended commercial interests, neither the exact media composition nor the used fermentation bacteria and the expressed recombinant product were revealed, however these factors were not mandatory objectives of this study.

2.4. Extraction of free amino acids (FAAs) from culture media

It was possible that the culture media could contain undigested proteins which were removed by precipitation as follows. Freeze-dried sample aliquots (8 mL) were re-hydrated in 4 mL 2.5% (w/v) 5-sulfosalicylic acid, incubated for 30 min at room temperature and passed through a fluted filter [20]. To provide matching derivatization conditions (alkaline pH), the obtained acidic eluates were neutralized with 0.5 M boric acid buffer, pH 9.0. An overall sample dilution of 1/15–1/35 brought the intrinsic amino acid concentrations into the calibration range. Centrifugation (16,000 × *g* at 10 °C for 15 min) further cleared the supernatants which were then used for derivatization utilizing the AccQ.Fluor™ Reagent Kit. All sample preparations were prepared as triplicate.

2.5. Total amino acids (TAAs) after acid hydrolysis

Degradation of oligomeric peptides/proteins towards their basic amino acids composition was accomplished, via micro-scale acid hydrolysis at elevated temperature utilizing Pico Tag™ Workstation (Waters, Milford, MA, USA). As to facilitate optimal hydrolytic yields (0.5–10 μg protein/peptide per reaction vial) as well as to match calibration range, culture media was stepwise diluted (up to 1/2000) with 20 mM HCl. Subsequently, 100 μL were then transferred into Pyrex glass reaction vials, that were placed into a hydrolysis container (simultaneous hydrolysis of 10 vials within one hydrolysis batch), and evaporated to dryness using the Pico Tag™ Workstation.

To the bottom of the hydrolysis container, 200 μL of 6 M HCl containing 1% (v/v) phenol were added, followed by a purging step with nitrogen. Thus, hydrolysis was performed in oxygen-free, hydrochloric vapor-phase for 20 h, at which the Pico Tag™ oven temperature was set to 112 °C.

The obtained dry residues after hydrolysis were then directly submitted to AQC derivatization using AccQ.Fluor™ Reagent Kit. To monitor possible carry-overs during hydrolysis, one blank sample (ultrapure water), undergoing an identical sample treatment, was integrated in each hydrolysis batch. Each culture media was hydrolyzed as triplicate.

2.6. Pre-column AQC derivatization of FAAs and TAAs

Amino acid derivatization with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using AccQ.Fluor™ reagent was conducted according to the Waters AccQ.Tag™ pre-column derivatization procedure [21]. Briefly, for standard solutions, 5 μL were mixed with 35 μL AccQ.Fluor™ borate buffer; while for *FAAs*, 5 μL sample extract, 5 μL internal standard (AABA at 40 μM) and 30 μL borate buffer were mixed. Thus, 40 μL derivatization mixtures were obtained. The dry residues (*TAAs*) after hydrolysis were incorporated in 20 μL internal standard solution (20 μM AABA in 10 mM HCl) and combined with 60 μL borate buffer.

Ten (for *FAAs*) or 20 μL (for *TAAs*), respectively, of reconstituted derivatization reagent (~10 mM AccQ.Fluor™ reagent in acetonitrile) were then admixed to the buffered samples (1/10 dilution for *FAAs*; no dilution for *TAAs*), immediately vortexed and left at room temperature for 1 min. Samples were then transferred into a heating block and derivatization finalized for 10 min at 55 °C. Prior to injection, samples were further filtered through a 0.20 μm regenerated cellulosic membrane (Sartorius, Goettingen, Germany).

2.7. Method transfer and UPLC™ conditions

Chromatographic separation of amino acid derivatives was performed on an Acquity™ Ultra Performance LC (UPLC™) system (Waters, Milford, MA, USA) equipped with an Acquity™ tunable UV detector (TUV; 190–700 nm) incorporating a 500 nL flow cell. Data acquisition was achieved via the Waters Empower 2 chromatography software package.

Column setup comprised a Waters Acquity UPLC™ BEH C_{18} column (1.7 μm, 2.1 mm × 50 mm) with pre-connected 0.20 μm guard frit that was kept constant at 37 °C during elution. Mobile phase A consisted of Waters AccQ.Tag™ Eluent A (concentrate diluted 1/11 with ultrapure water), and mobile phase B was 60% (v/v) acetonitrile in ultrapure water.

A preexisting HPLC protocol designed for separations of AQC amino acid derivatives utilizing Waters Nova-Pak™ C_{18} columns (4 μm; 3.9 mm × 150 mm) and fluorescence detection (AccQ.Tag™ method) [21], was transferred to the UV-UPLC™ system, and consequently optimized, as to the known interferences (hydrolyzed AQC excess reagent!), that are associated when changes in detection strategy are performed (transfer fluorescence to UV detection).

Four microliters derivatized amino acid standard or sample solution (*FAAs* or *TAAs*) were injected onto the column, and eluted at a flow rate of 0.4 mL min^{-1} according to the established gradient profile: initial – 0.4 min/0–2% B; 0.4–5.0 min/2–6% B; 5.0–6.3 min/6–10% B; 6.3–11.0 min/10–33% B and finally holding at 33% B until 11.5 min, thus implying a completed elution of all amino acid derivatives. Subsequent an interconnected cleaning purge (11.5–12.0 min/33–100% B; 12.0–13.0 min/100–100% B), the column was returned to 0% B within 1 min and further re-equilibrated for 4 min at initial conditions resulting in a total cycle time of 18 min before the next injection. UV detection of AQC derivatives was set to 254 nm.

3. Results and discussion

3.1. Method transfer from HPLC to UPLC™

Since chemistry of Nova-Pak™ C_{18} columns, originally used in the corresponding HPLC protocol, matches that of Acquity UPLC™ BEH C_{18} columns, a proper method transfer to UPLC™ ought to result in an identical elution pattern as obtained by the former HPLC technique. Moreover, to facilitate a reliable authentication, peak identities were further verified by comparing retention times with those of pure standard solutions, as well as by co-chromatography of spiked samples.

Based on established methodologies [22–24], assay transfer to UPLC™ was performed due to scaling by means of dimensional parameters. However, changing from fluorescence to UV detection implied utmost significance throughout the method transfer, since the excess of derivatization reagent hydrolyzes to 6-aminoquinoline (AMQ) that exhibits hardly any fluorescence response indeed, but shows a major peak when using UV detection at 254 nm. As AMQ elutes prior to the polar amino acids at early retention times, gradient profile needed further optimization in order to increase the resolution between AMQ and aspartic acid.

Emphasizing the enhanced performance of *sub*-2 μm LC particles utilized in UPLC™, the hereby newly established method facilitated an excellent separation of all 21 amino acid derivatives (AABA included) within 12 min only (Fig. 1a). The transferred AccQ.Tag™ method reported here, reduces the run time from 35 min for classical HPLC techniques [16,21,25] to 12 min with UPLC™ while retaining the elution pattern. In addition, while the former techniques could not completely resolve the arginine/threonine, cystine/tyrosine, valine/methionine and isoleucine/leucine pairings, the higher plate count of UPLC™ separated these compounds ($Rs \geq 1.5$, Table 1) as well as allowing the addition of citrulline, ornithine and tryptophan to the profile.

Despite the massive AMQ peak at the onset of the elution window (Fig. 1a), a slight modification at initiation of the transferred gradient profile yielded further segregation of AMQ, thus no inter-

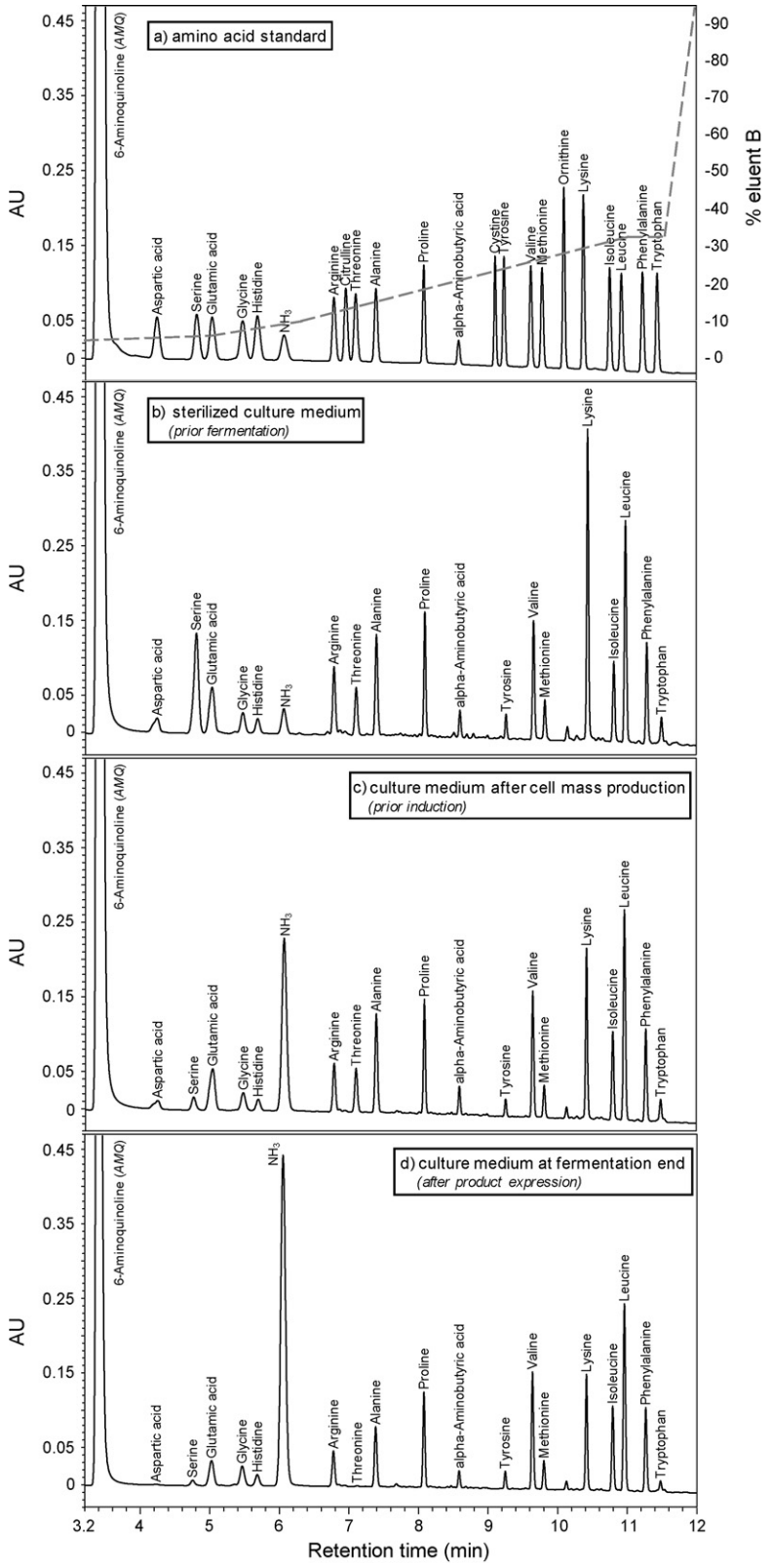

Fig. 1. UPLC™ chromatograms for (a) separation of amino acid standards (16 pmol/4 μL injection volume, respectively; alpha-amino butyric acid as internal standard; chromatographic conditions as in Section 2.7), (b) *FAA* profiles of fresh, sterilized culture medium, (c) inoculated fermentation broth prior to induction, and (d) after product expression at the end of fermentation.

ferences with aspartic acid were observed (Rs AMQ/aspartic acid 5.1). Using UPLC™, peak widths$_{50\%}$ ranged from somewhat over 5 s at early retention to around 2 s at the end of elution. Given the optimized peak shapes, the estimated peak capacity [26] implied 60–130 resolvable peaks per gradient time, depending on the referenced amino acid.

However, commercial UV-UPLC™ dedicated AQC amino acid solutions as recently realized by the Waters AccQ.Tag Ultra™

Table 1
Performance parameters of the applied UPLC™ method for the determination of *FAAs* and *TAAs* in culture media.

Amino acids	LOD[a]			LOQ[a]			Chromatographic performance[b]		
	On column (pmol)[c]	*FAA* (mg L^{-1})[d]	*TAA* (mg L^{-1})[d]	On column (pmol)[c]	*FAA* (mg L^{-1})[d]	*TAA* (mg L^{-1})[d]	$Width_{50\%}$	Resolution[e]	Peak capacity
Aspartic acid	0.57	6.6	18.8	2.04	23.7	67.7	5.6	5.1^{AMQ}	62
Serine	0.24	2.2	6.2	0.85	7.8	22.4	5.1	3.8	62
Glutamic acid	0.49	6.3	18.1	1.77	22.8	65.1	5.5	1.5	60
Glycine	0.25	1.6	4.6	0.89	5.8	16.6	5.6	2.8	59
Histidine	0.24	3.2	9.2	0.86	11.6	33.3	5.3	1.4/2.4^{NH3}	62
Arginine	0.27	4.1	11.6	0.96	14.7	42.0	3.5	5.4^{NH3}	84
Citrulline	0.28	4.3	12.4	1.02	15.7	44.8	3.3	1.8	87
Threonine	0.30	3.1	8.9	1.07	11.2	32.0	3.6	1.5	80
Alanine	0.46	3.6	10.2	1.65	12.9	36.9	3.2	3.1	90
Proline	0.24	2.4	6.9	0.87	8.8	25.1	2.5	8.6/7.1^{AABA}	113
Cystine	0.11	2.3	6.6	0.40	8.3	23.8	1.9	8.4^{AABA}	134
Tyrosine	0.23	3.6	10.3	0.83	13.1	37.4	2.3	2.2	116
Valine	0.33	3.3	9.6	1.18	12.1	34.5	2.5	5.7	109
Methionine	0.26	3.4	9.8	0.95	12.4	35.5	2.5	2.3	105
Ornithine	0.44	5.0	14.4	1.57	18.1	51.8	2.2	4.8	110
Lysine	0.54	6.9	19.8	1.95	25.0	71.3	2.2	4.5	105
Isoleucine	0.31	3.5	10.1	1.11	12.8	36.4	2.5	5.7	103
Leucine	0.31	3.5	10.1	1.11	12.7	36.4	2.5	2.3	103
Phenylalanine	0.23	3.3	9.5	0.83	12.0	34.2	2.6	4.2	106
Tryptophan	0.21	3.7	–[f]	0.75	13.3	–[f]	2.6	2.9	105

[a] Limits of detection (LOD) and quantification (LOQ) calculated according to DIN 32645.
[b] Based on standard solutions comprising 20 amino acids (+internal standard AABA) at 16 pmol per injection, respectively.
[c] Amount per 4 μL injection volume.
[d] For a complete sample work-up including derivatization procedure.
[e] Unless otherwise noted, stated resolution relates to the preceding amino acid.
[f] Complete degradation of tryptophan throughout acid hydrolysis.

method, may demonstrate even further enhanced performance yielding separations within 8 min [27–29].

3.2. Amino acid quantification using AQC derivatives and UV detection

UV detection of AQC derivatives at 254 nm indicated linearity within the applied calibration range of 2–64 pmol (per 4 μL injection volume) with correlation coefficients of >0.999 for all analytes, respectively. Moreover, due to the two NH_2 groups accessible for AQC derivatization, cystine, lysine as well as ornithine implied a nearly doubled UV response, thus providing approximately 1.5–2.0-fold steeper slope coefficients.

Limits of detection (LOD) and quantification (LOQ) for each amino acid, were calculated on the basis of calibration functions according to DIN 32645 [30], applying an uncertainty of 33% ($k = 3$) and 95% significance. Hereof resulting instrumentation sensitivities were stated as pmol per injection, hence relate to the actual on-

Table 2
FAA and *TAA* contents of the analyzed nutrient broths respective to (i) fresh, sterilized culture medium before fermentation, (ii) fermentation broth after cell mass production prior to induction and, (iii) after product expression at the end of fermentation.

Amino acids	Milligram amino acids per liter culture media						Percentage ratio		
	FAA			*TAA*			*FAA*/*TAA*		
	Before fermentation	Prior induction	After fermentation	Before fermentation	Prior induction	After fermentation	Before fermentation	Prior induction	After fermentation
Aspartic acid	291	175	13[a]	2240	1689	1301	13	10	1
Serine	1286	144	51	2002	737	670	64	20	8
Glutamic acid	914	835	409	5446	4731	3993	17	18	10
Glycine	186	162	161	850	722	624	22	22	26
Histidine	256	190	166	638	503	468	40	38	36
Arginine	849	604	404	1167	782	632	73	77	64
Threonine	386	357	4[a]	1149	852	474	34	42	1
Alanine	570	550	314	1076	912	678	53	60	46
Proline	690	642	491	2698	2400	2073	26	27	24
Cystine	–[b]	–[b]	–[b]	18[a]	21[a]	23[a]	–	–	–
Tyrosine	192	137	127	513	385	356	38	36	36
Valine	650	681	590	1678	1390	1281	39	49	46
Methionine	291	233	191	536	359	291	54	65	66
Lysine	1261	664	430	2007	1252	1008	63	53	43
Isoleucine	496	533	478	1344	1069	999	37	50	48
Leucine	1403	1321	1101	2152	1721	1595	65	77	69
Phenylalanine	784	728	613	1253	1012	918	63	72	67
Tryptophan	274	236	107	–[c]	–[c]	–[c]	–	–	–
Total	10,778	8190	5649	26,766	20,535	17,383	40	40	33

[a] Below LOQ; regarding the corresponding amino acids and the respective sample preparation.
[b] Not detectable; below LOD.
[c] Not detectable; due to degradation of tryptophan throughout acid hydrolysis.

column amounts of ACQ amino acids derivatives. In addition, these analytical amounts were further converted to practical amino acid concentrations presented as mg per L culture media, in due consideration of the complete sample work-up (including dilutions and derivatization, Table 1). Consequently, obtained LODs/LOQs (on-column) varied from 0.11/0.40 pmol (cystine) to 0.57/2.04 pmol (aspartic acid), thus implying an overall accordance with values (low μM) stated by other authors also applying UV detection [9,27,28]. In contrast, due to the less sensitive response when working with UV, fluorescence detection (LOD reaching nM/fmol) was reported to be of superior sensitivity [16,31]. Including also the sample work-up, the accordingly resulting amino acid concentrations in terms of LOQs ranged from 6 to 25 mg L^{-1} for *FAAs* and 17 to 71 mg L^{-1} for *TAAs*.

Intra-day precision of peak areas (amino acid standard 16 pmol/4 μL injected 6-times within one day) varied <1%, whereas inter-day variability for multiple derivatizations of amino acid standards (derivatized 6-times within one month) ranged between 0.9% (ornithine) and 2.9% (aspartic acid).

3.3. Characterization of amino acid composition of nutrient broths during fermentation

3.3.1. FAAs

The analyzed sample set comprised three progressive fractions of nutrient broths each designated to a different fermentation step throughout the production of a biotechnological compound, differentiated in: (i) fresh culture medium after sterilization, (ii) inoculated fermentation broth prior to induction (after cell mass production), and (iii) culture medium at the end of fermentation (after product expression). UPLC™ analysis of both *FAAs* (Fig. 1b–d) and *TAAs* (Fig. 2b–d) enabled superior separations throughout all analyzed fractions, hence interference-free quantification of all targeted amino acids. The specific *FAA* and *TAA* concentrations, the total amounts for each fraction, as well as the corresponding ratios for *FAA*/*TAA* are summarized in Table 2.

Characterizing the intrinsic *FAA* composition, the sterilized medium (step 1) represented a heterogenic amino acid profile featuring 10,800 mg *FAAs* per L in total, and individual amounts varying from 180 mg L^{-1} to 1400 mg L^{-1}, hence implying leucine (13%), serine (12%), and lysine (12%) at the most abundant concentrations.

Moreover, cell mass production (step 2) and subsequent protein expression (step 3) yielded a successive decline in total *FAA* concentrations (−24% and −48%, respectively), pointing out their preferable uptake during fermentation as to their higher bioavailability.

The found variations strongly depended on the individual amino acids (Fig. 3a), indicating either a predominate uptake during cell mass production (step 1 → step 2) (e.g., serine $-1142\ mg\ L^{-1}_{(step\ 1\rightarrow 2)}$ *vs.* $-93\ mg\ L^{-1}_{(step\ 2\rightarrow 3)}$; lysine −596 *vs.* −234 mg L^{-1}); or more often the reverse, an intense decline during expression (step 2 → step 3) (e.g., glutamic acid $-79\ mg\ L^{-1}_{(step\ 1\rightarrow 2)}$ *vs.* $-426\ mg\ L^{-1}_{(step\ 2\rightarrow 3)}$; threonine −29 *vs.* −353 mg L^{-1}; alanine −20 *vs.* −236 mg L^{-1}).

However, the most distinctive changes in *FAAs* were highlighted by aspartic acid, serine and threonine, each exhibiting an almost complete uptake from the nutrient broth (−96% to −99%) along the entire fermentation process (step 1 → step 3). This in fact may strongly indicate arising limitations of selective amino acids, thus showing either an insufficient supplementation or a well designed fermentation process with an efficiently calculated amino acid uptake.

3.3.2. TAAs

To monitor possible carry-overs during vapor-phase hydrolysis (especially serine and glycine), the therefore integrated blank sample (ultrapure water), implied clean chromatograms and no traces of amino acids (Fig. 2a), thus emphasizing the Pico Tag™ Workstation as a reliable tool for the simultaneous hydrolysis of multiple samples.

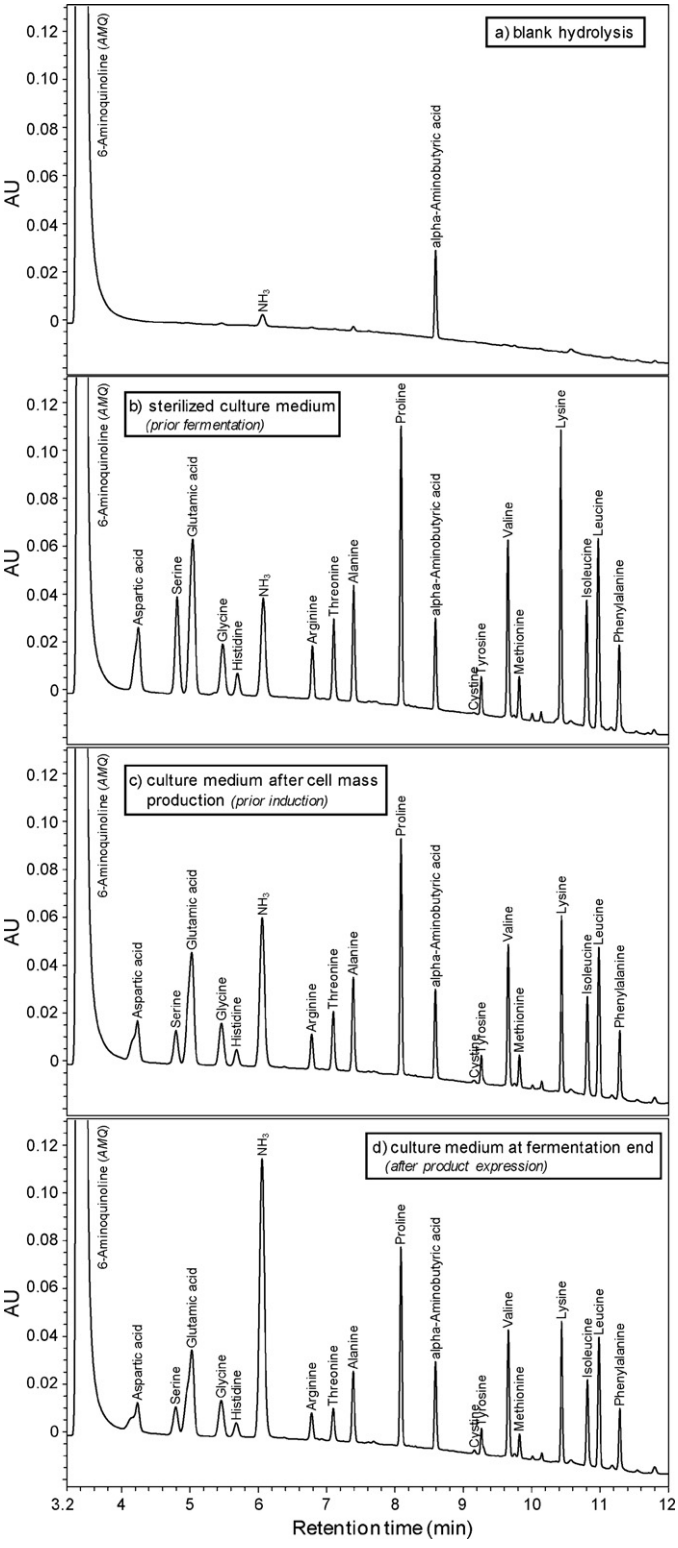

Fig. 2. UPLC™ of *TAA* profiles for (a) blank hydrolysis (ultrapure water), (b) fresh, sterilized nutrient broth, (c) fermentation broth after cell mass production prior to induction, and (d) culture medium after product expression at the end of fermentation.

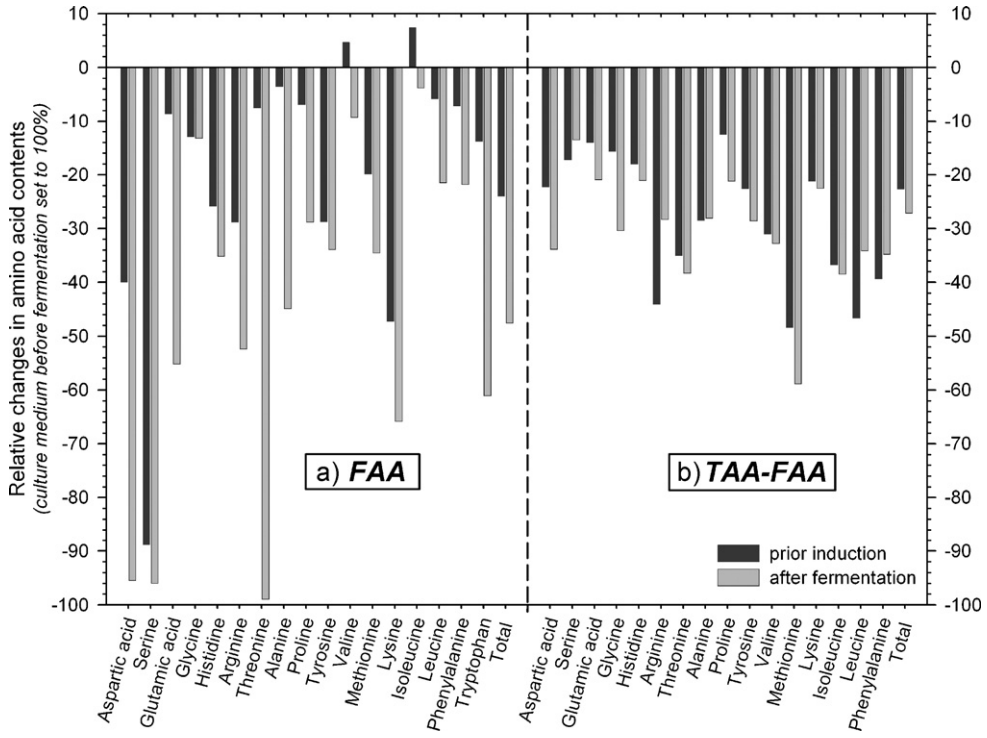

Fig. 3. Relative variations of (a) *FAAs* and (b) bound amino acids (*TAA–FAA*) during fermentation; fresh sterilized, culture medium was set as initial reference with 100%.

Starting from 26,800 mg L^{-1} intrinsically present in the sterilized medium, the overall *TAA* amounts declined over 20,500 mg L^{-1} ($-23\%^{\text{step } 1\rightarrow 2}$) after cell mass production to reach finally 17,400 mg L^{-1} ($-35\%^{\text{step } 1\rightarrow 3}$) at the end of fermentation.

Opposing the total *FAA* and *TAA* amounts, the obtained ratio suggested that the first two fractions primarily comprised protein/peptide bound amino acids at approximately ~60% (*FAA*/*TAA* 40%), respectively. Whereas at fermentation end, the ratio raises to 68%, hence highlighting the anticipated increased uptake of *FAAs* due to their higher bioavailability.

However, the individual *FAA*/*TAA* ratios at initial composition as well as the variations during fermentation may differ significantly (Fig. 4). For some amino acids, the fermentation resulted either in a moderately or drastically declining *FAA*/*TAA* ratio, most evident for serine (decline step 1 → 3 from 64% to 8%), threonine (34% to 1%) or aspartic acid (13% to 1%). Moreover, the reverse, a slightly increasing *FAA*/*TAA* ratio (e.g., isoleucine, valine, phenylalanine) or even retaining at almost constant ratios (e.g., tyrosine, proline) throughout all fermentation steps could be observed as well.

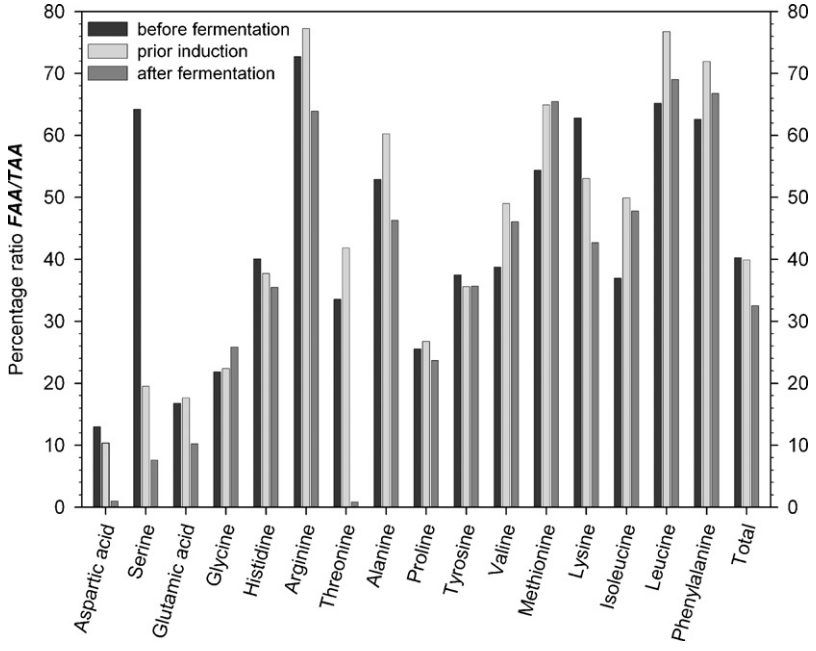

Fig. 4. Impact of consecutive fermentation steps on the *FAA*/*TAA* ratios.

3.3.3. Bound amino acids

However, as *FAAs* are inevitably included in the analyzed *TAA* amounts, their partly significant changes during fermentation (step 1 → 3: e.g., serine −1235 mg L^{-1}, arginine −445 mg L^{-1}, lysine −830 mg L^{-1}, glutamic acid −505 mg L^{-1}) will mask the actual variation within the peptide bound amino acids. Therefore, abstracting *FAA* amounts and only evaluating the remaining proportion (virtual estimation of peptide bound amino acids) seemed more appropriate to highlight proceeding modifications.

For the fresh medium (before fermentation), peptide bound amino acids revealed glutamic acid by far as the predominate constituent (28%), succeeded by proline and aspartic acid (12% each), while for the corresponding *FAA* profile, these amino acids were of minor significance only (9%, 6%, 3%, respectively). Considerable differences, however, may indicate a fortification of selective amino acids, that are consequently present within fraction of *FAAs*.

The declining concentrations of bound amino acids (*TAA–FAA*) in course of fermentation (step 1 → 3) (Fig. 3b) implicated proceeding cleavages of peptide bonds, thus an supplementary amino acid uptake from this fraction. Similar to the *FAAs* (see Fig. 3a), most bound amino acids showed successive declines till fermentation ended (step 3) reaching up to −40% of initial concentrations (exp. methionine −59%). Moreover, a systematic evaluation of *FAAs* as well as *TAAs*, may imply a rough estimation on the metabolism of peptide bound amino acids in the course of fermentation, thus further facilitate optimization of nutrient broths.

4. Conclusion

The Waters AccQ.Tag™ method as being designed for HPLC and fluorescence detection was successfully transferred onto UPLC™ and further optimized for UV detection. Adapted from this well characterized amino acid analysis solution, the newly established UV-UPLC™ method, combining *high-throughput* chromatography with sensitive AQC derivatization, facilitated rapid and reliable separations of 21 amino acids within 12 min only. Thus, using UPLC™ offered superior performance compared to the former conventional HPLC protocol.

Moreover, the established method was successfully utilized to evaluate proceeding modifications of *FAA* as well as *TAA* profiles intrinsic to nutrient broths during a fermentation process. Distinctive variations as to the preferable uptake of specific amino acids during cell mass production or expression of recombinant products could be observed. By identifying possible amino acid limitations during fermentation and highlighting arising shortages in supplementation material, such rapid techniques may contribute a valuable tool to properly re-adjust culture media in applied fermentation processes.

References

[1] W. Horwitz (Ed.), Official Methods of Analysis of AOAC International, vol. I, 17th ed., AOAC International, Gaithersburg, MD, USA, 2002, p. 994.12 (Chapter 4, p. 6C).
[2] D.T.-T. Nguyen, D. Guillarme, S. Rudaz, J.-L. Veuthey, J. Sep. Sci. 29 (2006) 1836.
[3] D. Guillarme, D.T.-T. Nguyen, S. Rudaz, J.-L. Veuthey, J. Chromatogr. A 1149 (2007) 20.
[4] D.T.-T. Nguyen, D. Guillarme, S. Rudaz, J.-L. Veuthey, J. Chromatogr. A 1128 (2006) 105.
[5] I. Molnár-Perl (Ed.), Quantitation of Amino Acids and Amines by Chromatography, Journal of Chromatography Library, vol. 70, Elservier, Amsterdam, Netherlands, 2005, p. 120 (Chapter 1.2).
[6] S.A. Cohen, D.P. Michaud, Anal. Biochem. 211 (1993) 279.
[7] D.J. Strydom, S.A. Cohen, Anal. Biochem. 222 (1994) 19.
[8] C. Van Wandelen, S.A. Cohen, J. Chromatogr. A 763 (1997) 11.
[9] H.J. Liu, J. Chromatogr. A 670 (1994) 59.
[10] D.L. Crimmins, R. Cherian, Anal. Biochem. 244 (1997) 407.
[11] M. Reverter, T. Lundh, J.E. Lindberg, J. Chromatogr. B: Biomed. Appl. 696 (1997) 1.
[12] H. Liu, M.C. Sañuda-Peña, J.D. Harvey-White, S. Kalra, S.A. Cohen, J. Chromatogr. A 828 (1998) 383.
[13] J. Díaz, J.L. Lliberia, L. Comellas, F. Broto-Puig, J. Chromatogr. A 719 (1996) 171.
[14] S.A. Cohen, K.M. De Antonis, J. Chromatogr. A 661 (1994) 25.
[15] L. Bosch, A. Alegria, R. Farre, Food Sci. Technol. Int. 11 (2005) 33.
[16] L. Bosch, A. Alegria, R. Farre, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 831 (2006) 176.
[17] M. Murkovic, K. Derler, J. Biochem. Biophys. Methods 69 (2006) 25.
[18] M.E. Swartz, J. Liquid Chromatogr. Relat. Technol. 28 (2005) 1253.
[19] H.K. Mayer, G. Fiechter, E. Fischer, J. Chromatogr. A 1217 (2010) 3251.
[20] H.K. Mayer, C. Rockenbauer, H. Mlcak, Lait 78 (1998) 425.
[21] Waters AccQ, Tag Chemistry Package (Instruction Manual), Waters Corp., Milford, MA, USA, April, 1993.
[22] D. Guillarme, D.T.T. Nguyen, S. Rudaz, J.-L. Veuthey, Eur. J. Pharm. Biopharm. 68 (2008) 430.
[23] D. Guillarme, D.T.-T. Nguyen, S. Rudaz, J.-L. Veuthey, Eur. J. Pharm. Biopharm. 66 (2007) 475.
[24] Y. Yang, C.C. Hodges, LC–GC North Am. 23 (2005) 31.
[25] I. Kabelová, M. Dvořáková, H. Čížková, P. Dostálek, K. Melzoch, J. Food Compost. Anal. 21 (2008) 736.
[26] S.A.C. Wren, J. Pharm. Biomed. Anal. 38 (2005) 337.
[27] I. Boogers, W. Plugge, Y.Q. Stokkermans, A.L.L. Duchateau, J. Chromatogr. A 1189 (2008) 406.
[28] W. Liming, Z. Jinhui, X. Xiaofeng, L. Yi, Z. Jing, J. Food Compost. Anal. 22 (2009) 242.
[29] H.B. Hewitson, T.E. Wheat, D.M. Diehl, Am. Lab. 41 (2009) 22.
[30] DIN 32645:1994-05, Chemical Analysis; Decision Limit; Detection Limit And Determination Limit; Estimation in Case of Repeatability; Terms, Methods, Evaluation, Beuth Verlag, Berlin, Germany, 1994.
[31] P. Hernández-Orte, M.J. Ibarz, J. Cacho, V. Ferreira, Chromatographia 58 (2003) 29.