Short Communication

Analysis of amino acids in cell culture supernatant using capillary electrophoresis with contactless conductivity detection

CE-C\textsuperscript{4}D methods for the analysis of amino acids (AAs) are presented. Combining the results from two methods with acetic acid and cyclodextrin-based BGEs, 20 proteinogenic AAs could be analyzed using CE. CE-C\textsuperscript{4}D was also, for the first time, applied to analyze free AAs in samples of mammalian cell culture supernatant. After dilution as only sample preparation, combining the results of the two CE methods allowed monitoring the concentration changes of 17 AAs in samples taken during the cultivation of CHO cells.

**Keywords:**
Amino acid / Capillary electrophoresis / Cell culture / Contactless conductivity

**Correspondence:** Professor Åsa Emmer, Division of Applied Physical Chemistry, Department of Chemistry, School of Engineering Sciences in Chemistry, Biotechnology and Health, KTH Royal Institute of Technology, Teknikringen 38, SE-100 44 Stockholm, Sweden
E-mail: aae@kth.se

**Abbreviations:** HAc, acetic acid; HEC, 2-hydroxyethyl cellulose

Biopharmaceuticals are being increasingly used to treat diseases including cancer, immunological, respiratory, and neurodegenerative diseases. A majority of the therapeutic proteins are produced using mammalian cells that are cultured and fed until protein expression occurs [1]. The cell culture is a complex mixture, including nutrients such as saccharides, amino acids (AAs) and vitamins, and high concentrations of salts and buffering components. The concentrations of free AAs provide information about the viability of the cells and can affect the quality of the protein product [2], and are often monitored using HPLC-UV-based methods. Electrophoretic methods can enable fast, high-resolution separations using small amounts of samples and other chemicals. As a result, a higher throughput and increased possibilities for at-line monitoring could be obtained. For example, CE-LIF has been applied to monitor the uptake of 14 AAs during beer fermentation [3], and in another contribution an online monitoring system including microfluidic components for cell counting and filtration followed by CE separation of the metabolic markers glucose, Gln, Leu/Ile and lactate was developed [4].

Since most AAs lack native UV absorbing or fluorescent groups, chemical derivatization, which can be both time consuming and difficult, must be performed to enable direct optical detection of AAs. Employing C\textsuperscript{4}D, derivatization can be avoided, and the use of CE- and ME-contactless conductivity detection for AA analysis was recently reviewed [5]. CE-C\textsuperscript{4}D methods have been applied to analyze AAs in diverse samples, including clinical samples like plasma, serum, and urine, and samples of food, beverages, and soil. When AAs are analyzed as cations with CE-C\textsuperscript{4}D, acetic acid (HAc) as BGE can provide a good buffering capacity and a stable C\textsuperscript{4}D baseline. Coufal et al. first used an HAc-based BGE to separate 20 native AAs in a capillary with 66.5 cm separation length, and applied it for AA analysis in different natural samples [6]. In other contributions, a BGE of 1.7 M HAc and 0.1% hydroxyethylcellulose was employed for CE-C\textsuperscript{4}D analysis of 18 of 20 proteinogenic AAs in human plasma within 60 min [7], and of 20 proteinogenic AAs, and 12 other biogenic compounds, in amniotic fluid in approximately 65 min [8].

The analysis time can be reduced by employing shorter separation channels. However, the separation of complex samples containing many AAs and/or other components typically becomes more challenging. Tüma et al. approached this by using three methods with different HAc concentration to separate 28 AAs in blood plasma, urine, saliva, and cerebrospinal fluid in an 18 cm effective length capillary followed by C\textsuperscript{4}D [9].

This work is a part of the EU-funded project iConsensus, integrated control and sensing platform for biopharmaceutical cultivation process high-throughput development and
production (https://www.iconsensus.eu), with the overall goal of developing new and improved means of bioprocess monitoring. Herein, CE-C^4D methods for the analysis of free AAs in cell culture supernatant are presented. HAc (Riedel de Haën/Honeywell Fine Chemicals, Seelze, Germany) with 0.1% 2-hydroxyethyl cellulose (HEC, average molecular weight ca 1 300 000) to suppress the EOF and protect the capillary wall from unwanted adsorption was selected as BGE. CE-C^4D analyses were performed using an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) equipped with a Tracedec® C^4D detector (Innovative Sensor Technologies, Strasshof, Austria) and a 35.4/49 cm effective/total length capillary (Polymicro Technologies, Phoenix, AZ, USA, 375/50 μm od/id). The C^4D detection parameters were as follows: frequency, “high”; voltage: 50%; gain: 50%. The capillary was rinsed with 1 M NaOH, 0.1 M NaOH, water, and BGE for 20 min each before its first use, with 0.1 M NaOH and water for 10 min each, and BGE for 15 min each new analysis day, and with BGE for 5 min in between runs. CE runs were performed at 25°C and 20 kV, and samples were injected hydrodynamically (50 mbar, 3 s). Data treatment was performed using OpenLAB CDS ChemStation (Agilent), and after exporting the data, electropherograms were plotted using SigmaPlot version 12.5 (Systat Software, San Jose, CA, USA).

Tüma et al. previously developed CE-C^4D methods employing HAc at different concentrations to tune the separation selectivity [9]. In the present work, a HAc concentration of 0.5 M was selected, and the separation selectivity was instead tuned using CD additives. α-CD (Sigma-Aldrich, St Louis, MO, USA) concentrations of 10, 20, and 40 mM were evaluated, whereas β-CD (Sigma-Aldrich) was added at 5 or 10 mM due to solubility limitations. The resulting electropherograms for a sample of 20 proteinogenic AAs, at 0.1 mM each, and some of the evaluated BGE compositions are shown in Fig. S1. The only AAs that could be completely separated in all the evaluated BGEs were Gly, Ala, Cys, and Asp, and baseline resolution separation of 20 AAs in the 35.4 cm capillary using a single BGE was not possible. Using a BGE with 0.5 M HAc, 0.1% HEC, and 40 mM α-CD, here referred to as method A, 16 AAs (Fig. 1A: Lys, His, Arg, Gly, Ala, Val, Ile, Thr, Asn, Gln, Met, Tyr, Phe, Pro, Cys, Asp) could be completely separated. With a BGE of 0.5 M HAc, 0.1% HEC, 20 mM α-CD, and 5 mM β-CD (method B), 14 AAs (Fig. 1B: Lys, His, Arg, Gly, Ala, Leu, Ser, Thr, Asn, Trp, Glu, Pro, Cys, Asp) were baseline resolved. Combining the results of the two methods allowed detecting all of the 20 AAs. The intraday average RSD of migration times, based on a series of 7 runs with a 20 AA mixture at 0.1 mM, were 1.1% (range 0.4–1.7%) for method A and 0.6% (range 0.3–1.1%) for
method B (see Table 1). The interday average RSD of migration times, based on 3 days, were 1.3% (range 0.5–2.0%) for method A and 0.8% (range 0.1–2.0%) for method B. The LOD values were in the range of 0.01–0.05 mM for both methods, which for most AAs is sufficient for the intended application. The method’s linearity was determined in the range of 0.025–1.5 mM (0.05–1.5 mM for Cys and Asp), resulting in average $R^2$ values of 0.9976 (range 0.9863–0.9999) for method A, and 0.9964 (range 0.9746–0.9996) for method B (Table 1). Data for the individual AAs are shown in Tables S1 and S2.

Methods A and B were applied to analyze AAs during the cultivation of CHO cells (TurboCell™ [10]; Rentschler Biopharma, Laupheim, Germany) in a 4-L reactor (Belach Bioteknik, Stockholm, Sweden). The samples were kindly provided by the division of Bioprocess Design, Department of Industrial Biotechnology, KTH Royal Institute of Technology, Stockholm, Sweden. After sampling from the reactor, centrifugation to collect the supernatant was performed, and before CE analysis, the supernatant was diluted 1:10 with water. Different dilution factors were evaluated, and 1:10 provided the best compromise concerning resolution and sensitivity. Samples taken 1 h after the reactor was inoculated with the cells (day 0, marking the start of the process), and after 4 and 8 days of the process, respectively, were analyzed using CE-C4D. Electropherograms for the samples from day 4 are shown in Fig. 1C and D, for methods A and B, respectively. As seen, few interfering peaks are observed, and most of the AAs shown in Fig. 1A and B can be detected. Peaks belonging to inorganic cations of salts included in the cell culture medium were detected at around 2–3 min, that is, before the first AAs at ~5 min. Due to the high concentrations of Arg, separation between Arg and His, which was complete for the set of standard AAs, was lost. The resolution between Arg and His could be increased by further diluting the sample, which would, however, affect the detectability of other AAs, or by increasing the HAc concentration in the BGE. At increased HAc concentrations, overlaps between the matrix peak at ~9 min in the electropherograms and several AA peaks were, however, observed. Cys was the only one of the 20 AAs that was not detected in the cell culture supernatant samples, which may be due to a low concentration and high detection limit of this late migrating AA, and/or its conversion to the oxidized dimer form cystine. Using a single capillary, a total of 90 injections of cell culture supernatant were performed without compromising the stability of the capillary (70 injections during method development with different HAc-based BGes and 20 injections using the final methods). The changes of the AA concentrations during the cultivation process are shown in Fig. 2. While some of the 20 AAs could be detected using both methods A and B, in

| Table 1. Average RSD of migration times (intray: $n = 7$, interday: $n = 3$) and peak area ($n = 3$), and linearity assessment for AAs analyzed with CE-C4D. The linearity data were determined with a 20 AA mixture at 0.025, 0.05, 0.1, 0.5, 1.0, and 1.5 mM ($n = 3$ for each concentration) |
|-----------------------------------------------|
|                                  | Intraday | Interday | Intraday | $R^2$ value | Slope | Intercept |
|-----------------------------------------------|
| Method A                                  | 1.1      | 1.3      | 5.5      | 0.9976       | 0.048  | 4.9E–04   |
| Method B                                  | 0.6      | 0.8      | 5.3      | 0.9964       | 0.054  | −7.4E–05  |

Figure 2. Quantification of AAs in cell culture medium taken during the cultivation of CHO cells. Sampling was performed at the beginning of the process (day 0, first bar) and after 4 (second bar) and 8 (third bar) days of cultivation, respectively. The error bars represent the standard deviation of concentration for the individual AAs and method (denoted as A: BGE: 0.5 M HAc, 0.1% HEC, and 40 mM α-CD, or B: 0.5 M HAc, 0.1% HEC, 20 mM α-CD, and 5 mM β-CD).
Fig. 2 the results for one method per AA are shown. The selection of method was based on the estimated error for the given AA. For most AAs, very similar results were, however, obtained when comparing the two methods (see Fig. S2). Figure 2 shows that most AA concentrations increased from day 0 through 8. This is particularly evident, for example, for Thr, Gly, Leu, Ser, and Asp. For Gln, the concentration was instead found to decrease comparing the day 0 data to the later measurements. Combining the results of the two CE-C4D methods, 17 AAs could be detected in the cell culture supernatant samples, with no other sample pre-treatment than dilution. Compared to current HPLC-based methodologies, the presented approach has several advantages. For HPLC-UV analysis, the sample preparation, including derivatization using commercial kits, takes approximately 1 h, followed by an up to 1 h HPLC run. Thus, despite the use of two CE methods, the combined time for sample preparation and CE is shortened by more than a factor of 2, and includes much less manual handling. Method A has a run time of less than 30 min. Method B could be terminated once the last AA that could not be separated in method A (i.e., Glu) has reached the detector, which would take an additional ~22 min. Furthermore, going from HPLC to CE, the consumption of chemicals and cost per analysis are significantly reduced.

In conclusion, CE-C4D was, to the best of our knowledge for the first time, applied to analyze AAs in mammalian cell culture supernatant. In these samples, 17 out of 20 available AAs could be detected using a combination of two methods. This makes CE-C4D a very promising technique for the selected application considering the complexity of such samples, containing high concentrations of salts, nutrients, metabolites, surfactants, and cell debris, and the simple and limited amount of sample preparation. Compared to HPLC-UV-based methods, the analysis time, sample and reagent consumption, and cost per analysis are significantly reduced.

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The authors have declared no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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