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Research Article

Transient isotachophoresis-capillary zone electrophoresis with capacitively coupled contactless conductivity detection for the analysis of essential amino acids in complex feed matrices

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Abstract

This study presents an innovative CE method that is combined with capacitively coupled contactless conductivity detection to analyse essential amino acids in animal feed. Conventional chromatographic methods for essential amino acids determination often involve time-consuming derivatization and complex procedures. Here, a transient isotachophoresis strategy is integrated into CE-capacitively coupled contactless conductivity detection to enhance analyte concentration and reduce matrix effects. Sodium ions were employed as the leading ion, while the slowest migrating essential amino acids, i.e., tryptophan (Trp), served as the pseudo-terminating ion. Acetic acid (pH 2.01) was used as the background electrolyte. Under optimized conditions, nine essential amino acids were adequately separated (resolution > 1.5) within 20 mins. The limits of detection ranged from 2.56 to 25.68 µM, representing a 19- to 25-fold improvement, as compared to conventional capillary zone electrophoresis. Intra- and inter-day precision (relative standard deviation, n = 3) for migration time and peak height were 0.28–6.20% and 0.51–8.55%, respectively, indicating good repeatability and reproducibility. Method accuracy was assessed via spike recovery experiments (5–15 µg/mL) in *Trichanthera gigantea* and *Pennisetum purpureum* feed samples, yielding recoveries between 80.67% and 116.5%. The developed method offers a rapid, sensitive, and minimal-solvent platform for essential amino acids screening in complex feed matrices. Its applicability to real samples and good analytical performance supports its potential for use in agricultural research, feed quality control, and regulatory compliance. This method promotes greener analytical workflows and provides a valuable tool for advancing sustainable livestock nutrition practices.

Keywords: amino acid, animal feed, capillary electrophoresis, online preconcentration, transient isotachophoresis

Introduction

Amino acids (AAs) are the building blocks of proteins, the fundamental unit of living organism and essential components of a well-balanced diet. They can be found in food and feed either in their free form or as the proteins' building blocks [1]. AAs have been linked to livestock growth, maturation, lactation, reproduction and intestinal wellness as well as being directly engaged in AA metabolism [2, 3]. The diet of poultry and livestock must include most of essential amino acids (EAAs) such as cysteine (Cys), leucine (Leu), lysine (Lys), histidine (His), methionine (Met), phenylalanine (Phe), isoleucine (Ile), threonine (Thr), tyrosine (Tyr), valine (Val) and tryptophan (Trp). AAs

composition is a key factor in the formulation of animal feeds, which is why the industry invests enormous resources and takes great care to achieve the best formulations. These aspirations resulting in introduction of synthetic supplementation with a precise blend of limiting AAs in diets to boost productivity, generate healthy animals and diminish enteric ailments [4]. Therefore, AAs profiling of animal feed is a beneficial tool that enables assessment of protein content while providing insight into feed quality through the monitoring of nutritionally indispensable AAs. Most ruminant and non-ruminant feeds, either from plant-based or animal-based source, contained a substantial number of AAs to meet animals' nutritional requirements. Chromatography has a long history of being utilized for determining amino acids in food and feed, and it is described in a number of methods [5-9] including ISO 13903:2005 and AOAC Official Method 994.12. AOAC 994.12 method [6] requires oxidation with performic acid followed by ion-exchange separation, but this destroys tyrosine. Meanwhile, ISO 13903:2005 method [9] employs HPLC with post-column derivatization for amino acid separation, necessitating high reagent use and longer run times. In such workflows, correction factors and recovery experiments are routinely applied to compensate for matrix effects and other method limitations. In numerous industries, feed samples are frequently complicated mixes with a variety of pigments, inorganic salts, organic acids, carbohydrates and various degradation products [10]. It is crucial to prepare the sample by eliminating or isolating the target ions of interest before performing chromatography analysis. A number of analytical strategies that can accurately separate AAs have been developed including LC-MS/MS [11], UHPLC-MS/MS [12] and CE [13, 14]. LC and CE coupled with light-absorption detector usually need to opt for wide variety of chromophore or fluorophore, derivatized typically post-or pre-column (pre- or post-capillary for CE). This is due to the vast number of EAAs such as Lys, Arg, Ala, Val, Leu, Ile, and Thr that lack of significant light absorption properties. Consequently, the analysis typically involved high demand on derivatization procedures, signifying drawback.

By contrast, several benefits come with capacitively coupled contactless conductivity detection (C^4D) [15] in CE. It is applicable to all ionic compounds without the need for derivatization or indirect approaches [16]. One of its features is stable baseline derived from the isolation of the high-voltage separation field from the low-voltage detection circuit, which suppresses unwanted interference [17]. Due to the cost-effective, simplicity of instrumentation and efficient analytical technique with low consumption of reagent and sample, rapid separation times, and a relatively high plate number, CE-C^4D together with online preconcentration was used in AAs monitoring in clinical, food and environmental sample [16-18]. Acid hydrolysis used strong HCl was typically used to cleave the proteins' peptide bond into free AAs. Nonetheless, the presence of strong HCl in the samples contributed to high conductivity of samples. As CE-C^4D operates by detecting the difference in conductivity of samples and BGE, higher conductivity of samples could reduce the sensitivity of instrument in general. This condition was proved by previous reported work where LODs were around one order of magnitude greater when samples were dissolved in 0.01 M HCl [19]. Therefore, it is important to introduce online preconcentration technique in analysis of AAs. Online preconcentration is one of the key advancements in CE that specifically addresses the sensitivity issues. The field-enhanced sample injection (FESI), ACN stacking, large-volume sample stacking (LVSS) and transient-ITP (tITP) are among reported preconcentration techniques for AAs analysis. According to Hodek et al., [19], ACN stacking successfully improved the LOD of CE in standard AAs sample yet failed in tobacco extract sample. The reasons behind ACN stacking failure towards tobacco leave were due to pH differences between samples and BGE and influence of ACN on pKa values of Aas. High ACN content alters the dielectric constant and shifts the pKa and ionisation state of AAs, disrupting the conductivity conditions required for efficient stacking. Therefore, an online preconcentration technique that is not affected by the changing of sample pH, needs to be introduced for analysis of EAAs in feed sample. Transient isotachophoresis (tITP) method was seen as an alternative, as it utilized multiple discontinuous solution medium, i.e., leading electrolytes (LE) and terminating electrolytes (TE) within the same capillary to create ITP effect and eventually concentrate EAAs. This online preconcentration technique was deemed suitable in determining EAAs in feed analysis due to the need for feed samples to undergo acid hydrolysis. This approach is in line with recent developments emphasizing the renewed application of ITP in modern CE workflows for real-world sample matrices [20]. Thus, this study investigated CE-C^4D to analyse EAAs with transient ITP (tITP) utilizing sodium ion as LE for preconcentration. The strength of this study lies in the integration of tITP with CZE–C^4D for analyzing hydrolyzed feed matrices, a challenging sample type due to its high salt content and low pH. The subsequent neutralization together with the optimized LE, TE and BGE conditions yielded a sample conductivity that remains compatible with tITP-based online preconcentration, as demonstrated by efficient stacking and separation of the hydrolyzed feed

samples. Additionally, the observed pseudo-terminating role of Trp within the matrix enabled stable and efficient stacking, which contributed to enhanced resolution and sensitivity. The method was applied to feed sample such as *Trichanthera gigantea* (Mandre de Agua) and *Pennisetum purpureum* (Napier grass) usually given to poultry and livestock, respectively. To the best of our knowledge, this tITP-CZE-C^4D technique for analysis of diverse EAAs in feed sample had not been reported yet. The outcome of this method was assessed in terms of its ability to produce precise and accurate qualitative and quantitative data.

Experimental

Materials and methods

L-amino acids, specifically essential amino acids (His, Phe, Thr, Arg, Met, Leu, Val, Lys, Ile, Trp), were purchased from Sigma-Aldrich. Glacial acetic acid (99%) were also from Sigma-Aldrich. Hydrochloric acid (37%), sodium acetate trihydrate, and MES were obtained from Merck, while sodium hydroxide (NaOH) pellets were supplied by QRec (Selangor, Malaysia). All chemicals were of analytical or reagent grade and used without further purification. Ultrapure water from a Milli-Q system (Millipore, Bedford, MA, USA) was used for all solution preparations. Stock solutions (1 mg/mL) of each EAAs was prepared by dissolving 10 mg of each compound in 10 mL of ultrapure water. Working standard mixtures were freshly prepared by diluting the stock solutions in 0.01 M HCl to obtain 10 µg/mL of each amino acid. Each amino acid had different concentrations when used in screening experiments (refer concentrations in figure or table captions). Stock solutions were stored at 4 °C, and working solutions were prepared daily. The background electrolyte (BGE) was prepared by diluting glacial acetic acid in ultrapure water to the required concentration. In BGE pH optimization experiments, acetic acid was first prepared as a 5 M solution at pH 2.01 and then diluted with water to obtain lower-concentration BGEs at pH 2.11, 2.19 and 2.29, as verified by pH measurement. Sodium acetate trihydrate, NaOH, and HCl for pH adjustment or capillary conditioning were prepared using appropriate amounts of each chemical dissolved in ultrapure water.

CE instrumental conditions

All experiments were conducted using an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) with a Tracedec® C^4D detector (Innovative Sensor Technologies, Austria). The separation cassette was configured with the C^4D signal filter activated (–12 dB, gain 150%) to reduce baseline drift. The capillary used was bare fused silica (Polymicro Technologies, USA) with 50 µm i.d., 375 µm o.d., 64.5 cm total length, and 56 cm effective length. The separation was performed at 30 kV and 25 °C. Sample injection was hydrodynamic at 50 mbar for 5 s unless stated otherwise. Data were processed using OpenLAB CDS ChemStation (Agilent), and electropherograms were visualised using OriginPro. All separations were performed using acetic acid at 5 M pH 2.01 as the BGE unless otherwise stated. For BGE pH optimization experiments, acetic acid at pH 2.11, 2.19 and 2.29 were prepared by diluting it with water, followed by pH-meter verification. All solutions were filtered through 0.45 µm membranes prior to use. New capillaries were conditioned by flushing with 1.0 M NaOH and 0.1 M NaOH (10 min each), distilled water (5 min), and running buffer (5 min) at 930 mbar. Daily preconditioning before runs included 0.1 M NaOH (3 min), water (4 min), and BGE (3 min). During continuous separation with the 5 M acetic acid (pH 2.01), the current remained stable at 11–13 µA, with no detectable baseline drift or loss of efficiency over repeated runs. Bare fused silica capillaries were recognized as chemically stable when operated in the pH range of approximately 2 to 9 [21], enabling the use of strongly acidic BGEs without deteriorating performance or causing significant wall degradation. Nevertheless, as 50 µm i.d. capillaries were more prone to Joule heating than narrower alternatives, regular rinsing and conditioning between runs were implemented to preserve long-term performance. Capillaries were flushed with 0.1 M NaOH (4 min) and distilled water (5 min). Acetic acid also provided a more stable C^4D baseline and better peak shape than alternative acids such as lactic acid, which showed higher conductivity and a reduced detector dynamic range.

Transient isotachophoresis-capillary zone electrophoresis-capacitively coupled contactless conductivity detection

The tITP-CZE-C^4D method was developed by introducing a short plug of sodium leading electrolyte (LE) in front of standard/sample. Sodium acetate trihydrate (250 mM) was hydrodynamically injected into the capillary system to allow sodium to act as leading ion (LE). During tITP, initially BGE had filled the capillary, then a short LE plug containing sodium was added into the capillary by HDI (between 10 s to 60 s during optimization). The next step was to inject the sample containing the analytes of interest hydrodynamically (HDI) at 50 mbar for 100 s. Trp was identified as the terminating ion in the tITP-CZE-C^4D method, based on its low effective mobility under acidic conditions. The analytes were separated using a voltage of 30 kV unless other stated.

Samples pretreatment

Two feed samples, *Trichanthera gigantea* and *Pennisetum purpureum* (Napier grass), were obtained from local farmers in Johor and Kedah, Malaysia, for

method development and performance evaluation. Crude samples were finely ground to pass a 0.25 mm sieve and homogenised prior to analysis. Approximately 50 mg of homogenised feed was transferred into a screw-cap vial and mixed with 10 mL of 6 M HCl. The tightly sealed vials were hydrolysed in an oven at 110 °C for 16 h. The acid hydrolysis procedure was adapted with minor modifications from a previously published method [14]. After filtration through an ash-free filter, the hydrolysate was aliquoted (500 µL) and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 500 µL of water, then diluted with 10 parts ACN to 1-part MeOH/water (1:1, v/v) at 4–8 -fold dilution. The appropriately diluted solution was used for tITP-CE-C^4D analysis.

Method validation

Evaluation of quality, consistency and reliability of th e analytical results were evaluated by using method v alidation's results. All samples were done in triplicate to measure precision, reproducibility and repeatabilit y of the developed method. RSD of three repeated me asurement were measured intra-day and inter-day to a ssess analytical precision. The concentrations corresp onding to the peak heights for S/N of 3 and S/N of 10 was used to determine LODs and LOQ respectively. S ensitivity enhancement factor (SEF) was monitored b y comparing the LODs between tITP-CZE method wi th conventional CZE method. For precision and accur acy studies, the concentration range evaluated for the mixed standard solution was 48.96–85.36 µM, and re covery experiments were performed by spiking hydro lysed feed samples at three levels (5, 10 and 15 µg/m L; low, medium and high, respectively).

Results and Discussion

Optimization of basic CZE–C^4D

Several conditions were optimized for CZE-C^4D separation before introducing an effective online preconcentration method for EAAs. The migration time and the separation between analytes were both directly influenced by the BGE. The primary factor in selecting a suitable BGE was the ability of the analytes to ionize [22]. EAAs have pKa values ranging from 1.85 to 2.83, which means they carry a net positive charge in an aqueous BGE when the pH is close to 2. Furthermore, it is preferable to employ acidic conditions in order to ensure that these compounds become completely positively charged and to suppress or reduce the full term? (EOF) [23]. Consequently, this study employed a positive polarity separation voltage. Several electrolytes were tested as buffer solutions in the previous study, including citric acid, acetic acid, formic acid, and lactic acid [23]. We found that acetic acid provided more stable baselines and significantly better His peak resolution as compared to lactic acid (peaks were broader with lactic acid). This difference in performance might be attributed to the varying conductivity between these two buffer solutions. Lactic acid might have higher conductivity than acetic acid, potentially limiting the dynamic range of the C^4D detector due to excessively high background conductivity. Under these conditions the detector operates closer to its upper response limit, so small conductivity differences between neighbouring zones are less efficiently translated into peak height, leading to reduced sensitivity and poorer peak resolution. In contrast, the lower-conductivity acetic acid BGE produced a lower baseline signal, allowing the C^4D detector to resolve the conductivity gradients associated with each EAA more clearly and thus improving Rs and overall separation performance.

Since EAAs ionization and migration behaviour were pH-dependent, maintaining BGE's pH was essential for ensuring stable and repeatable baselines. This explained why acetic acid, at various concentrations between 0.1 and 10 M (pH 1.16–3.3) [17] was commonly used during the separation of EAAs when using a C^4D detector. When acetic acid was used as BGE, EAAs appeared as negative peaks because EAAs zones had lower conductivities as compared to the BGE. An adequate separation of EAAs could be readily achieved in acetic acid solutions at pH ~ 2. This pH range was near the acidic dissociation constant (pKa) of most EAAs. BGE with varying pH values (acetic acid diluted with water), including pH 2.01, 2.11, 2.19, and 2.29, were evaluated. As depicted in **Figure 1**, a pH of 2.29 provided the highest sensitivity as compared to other pH levels.

However, it was observed that not all EAAs were well separated under these conditions. When the pH was lowered, there was a concurrent increase in buffer concentration resulting in longer migration times. As the EAAs migrated more slowly, this allowed for better separation between the EAAs before reaching the detector due to the reduced EOF. It was observed that acetic acid at pH 2.01 enabled the separation of Phe and Trp. While this could be improved slightly by reducing the pH further, further reduction in pH was not attempted to maintain capillary effectiveness and prevent damage. Thus, the optimum performance in terms of separation resolution was achieved using acetic acid at pH 2.01. This condition allowed for the separation of all ten EAAs and Ala as IS, with resolution of Rs > 1.5, which was considered acceptable, even though it led to increased migration time (still under 30 mins). The migration order was Lys < Arg < His < Ala (IS) < Val < Ile < Leu < Thr < Met < Phe < Trp which is similar with the expected effective mobilities of protonated EAAs at pH ≈ 2.01, where Trp moved the slowest among the EAAs due to

its comparatively larger hydrophobic side chain and weaker interaction with the acidic BGE. In addition to its comparatively larger hydrophobic side chain, the slower migration of Trp reflects its lower effective electrophoretic mobility at the working pH, where its protonation state and molecular size lead to a slightly reduced velocity as compared with the other protonated EAAs.

It should be noted that by increasing the buffer concentration will lead to a decrease in peak height and analytical sensitivity. This decrease might be attributed to a reduction in the relative permittivity (εr) of the medium, making it more challenging for the signal to flow between the C^4D electrodes, especially when water was no longer the primary solvent [24]. Thus, to achieve both good resolution and sensitivity, the next step is to introduce online preconcentration techniques while maintaining acetic acid at pH 2.01 as the BGE. The optimization of C^4D settings involved adjusting both the output voltage of the frequency synthesizer and the gain of the input signal. Firstly, the output voltage was fine-tuned with three settings: 0 dB, -12 dB, and -18 dB. Trials demonstrated that -12 dB was the optimal output voltage, offering better peak sensitivity as compared to -18 dB. However, when applying 0 dB, it led to instrument errors due to exceeding the current limit. Next, the gain of the input signal was optimized with four settings: 75%, 100%, 150%, and 200%. Interestingly, 75%, 100%, and 150% gains showed no significant differences in terms of sensitivity, migration time, and resolution. However, when applying a 200% gain, instrument errors occurred because the separation current exceeded the instrument's internal limit and these conditions were therefore excluded from further work. Additionally, combining a voltage setting of -18 dB with a gain below 150% also resulted in instrument errors linked to the current limit, so these combinations were likewise discarded. Consequently, the best combination of C^4D settings identified was -12 dB for the output voltage and 150% for the gain, and these settings were consistently used throughout the study. Additionally, the offset value was consistently set between 18500 and 18600 units to maintain a consistent baseline throughout the study. This was achieved by adjusting the maximum offset value to maximum at 150.

One of the initial steps was to determine the most suitable type of injection for EAAs, as it was crucial in selecting the appropriate online preconcentration method for this study. Two injection methods were explored: hydrodynamic injection (HDI) and electrokinetic injection (EKI). The major difference between tITP and EKS was that HDI was utilised to inject samples during (tITP). Preliminary experiments were conducted using HDI (pressure at 50 mbar) and EKI (applied voltage of 5 kV for 10 s, which is a common injection duration) to introduce the EAAs into the capillary.

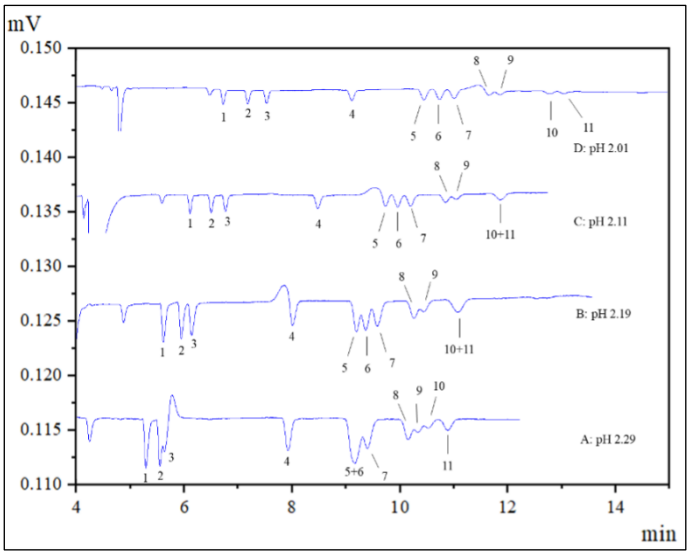


Figure 1. Effect of pH buffer. A: pH 2.29, B: pH 2.19, C: pH 2.11, and D: pH 2.01. BGE: 5 M acetic acid. 10 µg/ml of essential amino acids mixed standard solution was dissolved in 0.01 M HCl with 5 µg/ml of internal standard. CE condition: Analytes introduced via HDI 50 mbar for 10 s. Separation voltage at 30 kV and 25°C. Conductivity detector condition: High sensor, voltage -12 dB, gain 150%, offset value in range 18500-18600 unit. Peak identification: (1) Lysine, (2) Arginine, (3) Histidine, (4) Alanine (Internal standard), (5) Valine, (6) Isoleucine, (7) Leucine, (8) Threonine, (9) Methionine, (10) Phenylalanine, (11) Tryptophan

Sharp peaks of analytes and stable baseline separation were achieved as compared to EKI . Additionally, another factor to consider was that EKI could create a bias wherein ions with higher mobilities enter the capillary more quickly than ions with lower mobilities, resulting in a stronger detector response. While this was advantageous for analyzing high-mobility ions, it could be detrimental to low-mobility ions. Crucially, this study focused on analyzing analytes with lower effective mobility as compared to most matrix ions found in feed samples. Therefore, HDI injection was chosen as the preferred method for this study. A 10 s HDI time provided the best results, offering a sharp peak, better peak height, and improved resolution. However, when the injection time was extended to 20 seconds, broad peaks were observed. This broadening of peaks might have occurred because the sample volume became overloaded, preventing the formation of sharp zones when the separation voltage was applied. While HDI alone was a straightforward method, there was a need to enhance the instrument's sensitivity to achieve better LOD. Consequently, tITP was developed to improve sensitivity. In tITP, both LE and TE were used to concentrate the wide sample zones. It was anticipated that when analytes were injected by HDI during tITP-CZE-C^4D, sharp peaks could once again be achieved, even with a high sample volume injection.

Optimization of tITP-CZE-C^4D

Improving sensitivity became crucial once the separation selectivity was optimized to enhance the sensitivity for EAAs. This was necessary because many peptides, including AAs, could not be detected at low concentrations using basic CZE. By introducing tITP into CZE separation, the target analytes were concentrated into distinct zones within the tITP system, located between an LE (which had higher electrophoretic mobility, μ_{ep} than the analytes) and TE (which had lower μ_{ep} than the analytes). After destacking, the targeted analytes were separated by CZE. Na^+ as LE established the fast-migrating front for cationic EAAs stacking. The choice of Na^+ defines which analytes were able to be stacked in sequence: EAAs with effective mobilities lower than Na^+ will be focused between the LE (Na^+zone) and the terminating zone. Under these conditions, EAAs could be arranged and concentration profiles optimized according to their mobilities relative to Na^+. Upon injection, this Na^+ zone migrated quickly and acted as the migration boundary for faster-moving analytes. Trp was employed as the pseudo-terminating ion, being the slowest migrating EAA under the selected conditions. In other words, Trp did not form a classical tITP with a defined TE, but its low effective mobility created a trailing conductivity boundary that confined the stacked EAA zones and stabilised the tITP–CZE transition. Therefore, we refer to Trp as a ‘pseudo-terminator’ operationally, acknowledging that the system corresponds to a sample-induced tITP mode rather than a full ITP system with a dedicated TE reservoir.

In this study, we focused on optimizing four factors: LE concentration (mM), LE injection time (s), sample injection time (s), and TE injection time (s) using BBD. We also investigated the interactions among these factors. The principal parameters for tITP-CZE-C^4D were optimized under conditions minimizing potential matrix effects, ensuring clear interpretation of each factor’s impact. It was noteworthy that the BBD was applied to the aqueous BGE to establish a robust baseline method. In this study, the center points were established at a value of 5. Accordingly, based on the equation for determining the number of experimental points (N), we needed to complete 29 runs. The collected data and the relationship between the independent variables and the responses were analyzed using regression model analysis. The design employed in this study permitted the representation of the response through a first-order polynomial fit. In terms of statistical analysis of the model, the model and its individual terms were considered statistically significant when the P-values < 0.05. In this context, the models for the responses, namely peak height, resolution, and migration time, were found to be significant. Specifically, the D model terms (related to TE injection time) were not significant for both migration time and peak height, whereas the B model terms (related to sample injection) were significant for resolution. These observations underscored the significant impacts towards the responses when the significant terms were varied throughout the study. According to this statistical analysis, the predicted R^2 were in reasonable agreement with adjusted R^2 values, with the differences being less than 0.2 for all responses. Additionally, to confirm that the model accurately represents the experimental results with a confidence level of 95%, the p-values for lack of fit were commonly used. The fact that a non-significant lack of fit was achieved for all responses indicated that the model fitted well with the experimental data, which was desirable. In this study, LE concentration and sample injection time were found to have a dominant effect on peak height, resolution, and migration time, respectively. For the peak height response in this model, LE concentration and LE injection time were positively related, while sample injection time and TE injection time were inversely related. This suggested that peak height increased with higher salt concentration and LE injection time but decreased with longer sample injection time and TE injection time.

To create the 3D contour plots shown in **Figure 2**, it was necessary to maintain the values of the variables C and D. These plots demonstrated that increasing LE concentration while minimizing sample injection could lead to improved peak height, resolution, and migration time. To further enhance the preliminary results, additional screening analysis such as fractional factorial design (FFD) can be introduced. FFD helps in developing a more comprehensive model that allows us to explore the actual minimum and maximum factor values for each response, leading to the best performance in the tITP system. While the BBD determined optimal factor values for each EAA, only the optimal factor value for Thr was considered in this study. This decision was made because Thr occupied a relatively central position among the AAs (peak #8) and had the lowest resolution relative to the other EAAs in CZE. This was crucial when selecting the optimal tITP condition to ensure efficient resolution of Thr. Selecting the analyte with the most difficult separation as the target response for optimization could streamlined data interpretation and saves time and effort. Based on the overall optimization results, the optimized values for this tITP system were; LE concentration 250 mM, sample injection time 100 s, LE injection time 10 s and TE injection 50 s. LE concentration was found to have a dominant impact, particularly on peak height and migration time. To investigate the relationship between this variable and these two responses, we varied the concentration of LE at four different levels, ranging from 100 to 250 mM. These experiments were conducted at the optimum voltage of 30 kV, with constant sample, LE, and TE injection times.

The results are summarized in **Figure 3**, which illustrates (a) peak height and (b) migration time as functions of LE concentration. In **Figure 3a**, it was evident that as the Na^+ concentration increased, the peak height of each analyte also increased significantly, except for the last analyte (11), which was Trp. The Na^+ peak consistently surged as the concentration rose, indicating higher Na^+ conductivity. This was because higher Na^+ conductivity required more time to establish the conductivity difference between the LE and BGE, resulting in a very high peak from sodium ions before it returned to the baseline for analyte separation. Additionally, it was observed that the slower migration associated with higher LE concentration led to a steep increase in migration time, as shown in **Figure 3b**. This phenomenon provided the AAs with ample time to concentrate into distinct zones within the sample before destacking and migrating in the typical CZE manner after the electromigration dispersion of LE.

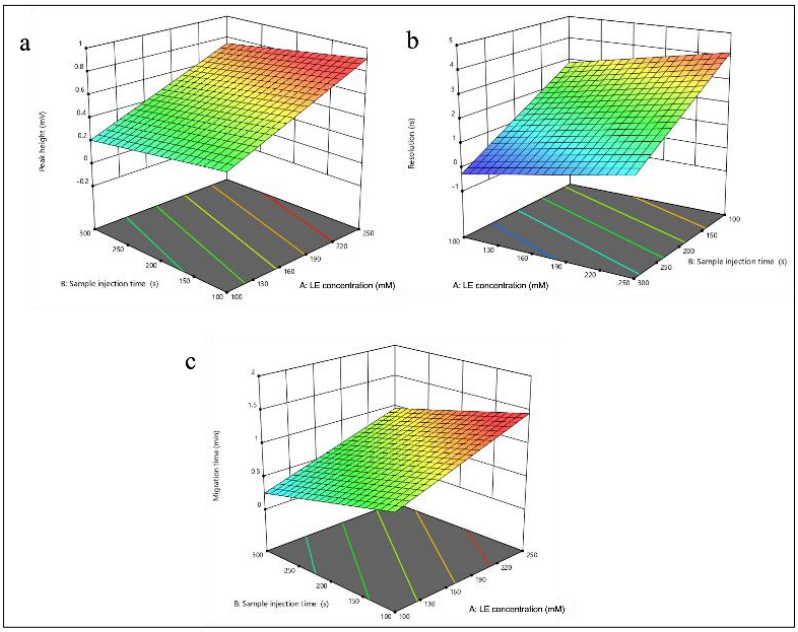


Figure 2. Box–Behnken Design Response Surface Methodology 3D contour plots for (a) peak height (b) resolut ion and (c) migration time

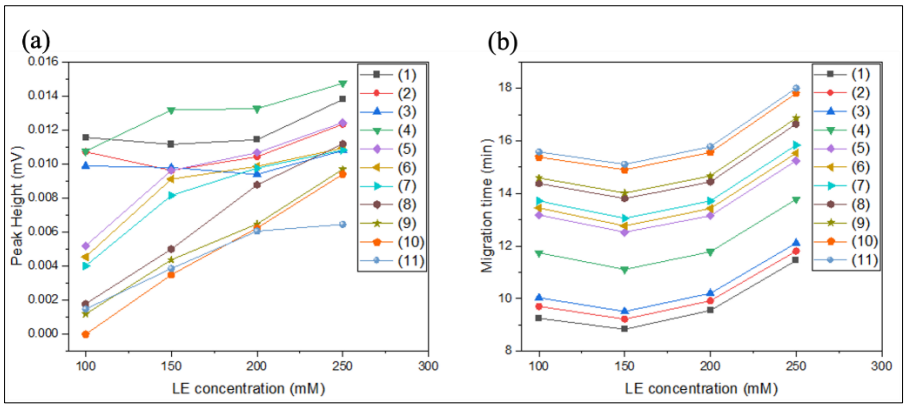


Figure 3. Effects of leading electrolyte concentration (mM) on (a) peak height and (b) migration time of 10 µg/ml EAAs and 5 µg/ml alanine (internal standard) mixture were dissolved in 0.01 M HCl. LE: sodium acetate trihydrate 250 mM (60 s), and sample (102 s). Peak identification: (1) Lysine, (2) Arginine, (3) Histidine, (4) Alanine (Internal standard), (5) Valine, (6) Isoleucine, (7) Leucine, (8) Threonine, (9) Methionine, (10) Phenylalanine, (11) Tryptophan

A similar effect was observed when increasing the LE injection time, resulting in a noticeable surge in migration time and peak height. This surge might be attributed to a longer LE plug that allowed analytes to concentrate more efficiently in the ITP zone before exiting. Since ITP occurred near the capillary entrance, it provided more time for AAs to concentrate into a self-sharpening zone before transitioning to typical CZE separation, resulting in sharp and narrow peak heights. On the other hand, increasing the sample injection time led to broader peaks, especially for slower AAs, and unsatisfactory resolution. This was likely due to sample overloading, which prevented the analytes from concentrating into discrete zones and led to diffusion between them. This diffusion occurred when the analytes did not have sufficient time to concentrate into the self-sharpening zone and were forced to migrate away from the ITP zone towards the detector. **Figure 4** supports this explanation, as increasing sample injection time correlates with a decrease in migration time, giving them less time to preconcentrate. The decrease in migration time with increasing sample injection time might be due to the lower conductivity of the sample zone thus dominating the capillary, causing a faster electrophoretic velocity. In conclusion, LE concentration, LE injection time, and sample injection time all played crucial roles in preconcentrating the analytes during the tITP system.

The concentration of the sample matrix played a significant role in the effectiveness of enrichment, making it essential to examine its effect in this study. As widely known, strong HCl was used to hydrolyze proteins into free amino acids by cleaving the peptide bonds. Therefore, it is important to investigate the impact of HCl presence in this tITP-CZE-C^4D method. The EAAs, along with Al (IS), were prepared in water and in two different concentrations of HCl, namely 0.01 M and 0.6 M. The concentration of the running buffer was as optimized previously, which was 5 M acetic pH 2.01. As depicted in **Figure 5**, higher sensitivity was attained when EAAs were dissolved in 0.01 M HCl, followed by water and 0.6 M HCl. Peak height was improved by dissolving EAAs in 0.01 M HCl as compared to water, while a minor shift in migration time was observed. Conversely, the peaks for EAAs dissolved in 0.6 M HCl were almost invisible, indicating that stacking did not favour in this condition. In the tITP–CZE–C^4D configuration used here, this behaviour indicated that efficient tITP stacking only occurs when the sample conductivity remains sufficiently lower than that of the 5 M AcOH BGE. This might be explained by the fact that different concentrations of HCl in the sample matrix could result in differential electric conductivities between the sample and running buffer. The strength of the electric field was greater in the sample plug when the conductivity of the sample region was lower than that of the running buffer. Consequently, analytes migrated quickly to the sample/buffer border, where the rate of electrophoretic migration slowed down, causing analytes to stack. This qualitative picture was consistent with the field-amplified stacking and Kohlrausch regulating function concepts that describe how conductivity gradients control the local electric field and focusing efficiency in tITP systems. In contrast, when the sample region had a higher conductivity as compared to the BGE, stacking was not preferred in this condition. This phenomenon explained why the EAAs dissolved in 0.6 M HCl produced almost invisible peaks, as the high conductivity and ionic strength of 0.6 M HCl (22.085 S/m and 597.966 mM,

respectively, according to PeakMaster version 5.4) lowered the electric field of the sample region as compared to the BGE. This made it impossible for stacking to occur, even at higher concentrations of acetic acid in BGE, which would produce higher conductivity and ionic strength (0.558 S/m and 14.883 mM for 10 M acetic acid , respectively, according to PeakMaster version 5.4), while stacking remained effective at sample conductivity below ~0.4 S/m, such as in 0.01 M HCl (0.4122 S/m).

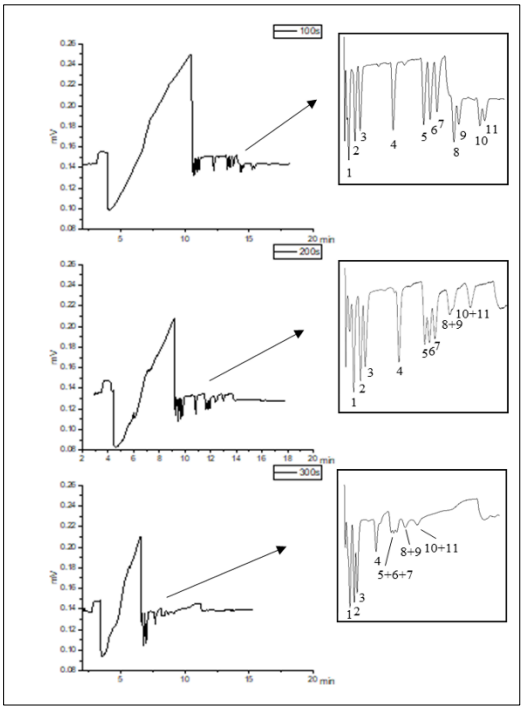


Figure 4. Effect of sample injection time (s). 10 µg/ml EAAs and 5 µg/ml alanine (internal standard) mixture were dissolved in 0.01 M HCl. CE condition: HDI 50 mbar. Separation voltage at 30 kV and 25°C. tITP-CZE-C^4D method: LE: sodium acetate trihydrate 250 mM (60 s), TE: Trp. Other condition and peak identification as depicted in Figure 3

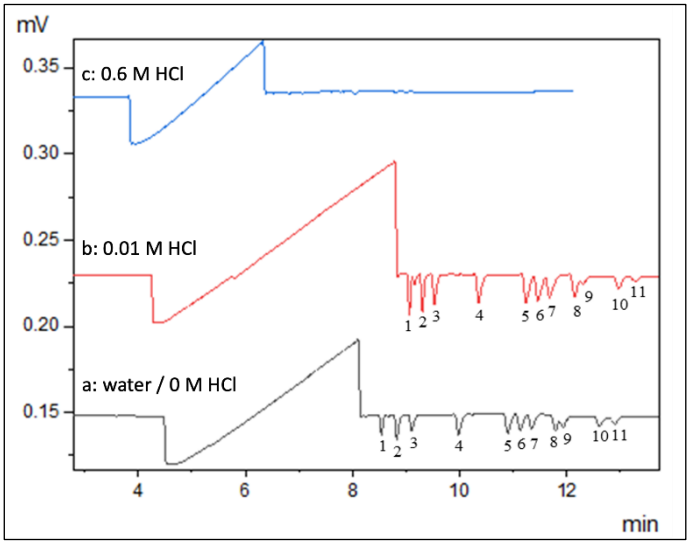


Figure 5. Effect of sample matrix concentration. A: Essential amino acids was dissolved in water, B: Essential amino acids was dissolved in 0.01 M HCl and C: Essential amino acids was dissolved in 0.6 M HCl. Other conditions and peak identification were as optimized before in Figure 3

Additionally, the difference in conductivity between 0.01 M HCl and 0.6 M HCl was further confirmed by the fact that there was a surge in current observed when increasing the sample matrix concentration from 0.01 M HCl to 0.6 M HCl (12 to 14 µA). This was because the high ionic strength of 0.6 M HCl induced high conductivity in the solution, making it easier for electric current to flow and resulting in higher electric current production during the separation. Consequently, the electrophoretic velocity of analytes also increased, as observed in Fig.5, where the migration time shortened from 8.6 to 6.5 mins for Lys as the concentration of HCl increased from 0.01 to 0.6 M, respectively. This condition made it unfavorable for EAAs to be dissolved in 0.6 M HCl for this analysis. Furthermore, EAAs dissolved in 0.01 M HCl exhibited a higher number of theoretical plates as compared to water, with values of 210,460 and 165,092 for Lys dissolved in 0.01 M HCl and water, respectively, indicating sharper peaks were produced in the 0.01 M HCl sample matrix. However, 10 out of 11 AAs dissolved in water had several theoretical plates higher than 60,000, as compared to only 6 AAs when dissolved in 0.01 M HCl. Therefore, water was chosen as the best sample matrix for the analysis of EAAs, including Ala, in the tITP-CZE-C^4D method. From the above statements, it could be concluded that it was crucial to eliminate or minimize the presence of HCl during sample preparation to conduct the analysis successfully and effectively.

Based on previous studies, the addition of organic solvents such as ACN to sample successfully improved separation efficiency and LOD [19, 24, 25]. This was because it reduced the conductivity of the sample, amplifying the electric field and allowing sample stacking. The velocity of analytes in the sample zone was influenced by the conductivity and dielectric constants of the sample medium [22]. Therefore, by dissolving the sample in a low-conductivity solvent like water with a percentage of organic solvent, sample stacking could be achieved more easily. This improvement in sample stacking was crucial since the samples in this study were prepared in highly conductive 6 M HCl, and the residual HCl content after dryness in the sample was unknown. As mentioned in previous sections, the presence of a high concentration of HCl in the sample reduced the analytical sensitivity of tITP-CZE-C^4D. Thus, the addition of an organic solvent to the sample might help enhance separation efficiency and LOD when applied to feed samples. Although organic solvents were introduced after completion of the BBD optimization, the percentages of organic modifiers were carefully adjusted so that the current, viscosity and migration times remained within the operational window as defined by the initial design. Mixtures of 10 µg/mL EAAs in a mixed standard solution in 0.01 M HCl with water or 20%, 50%, and 60% v/v of each organic solvent (MeOH or ACN) were hydrodynamically injected at a pressure of 50 mbar for 100 s to assess the impact of organic solvent addition on separation efficiency. The subsequent introduction of organic solvents into the sample matrix is a recognized as a strategy to adapt the method for more complex samples. Addition of organic solvents after main optimization ensured that potential matrix effects could be assessed and tuned, thus preserving interpretability of the BBD experiment and ensuring robust final method performance. These results showed that the addition of organic solvent in the sample led to an increase in migration time and, simultaneously, a noticeable improvement in resolution for both MeOH and ACN. This could be attributed to the decrease in current value for the separation as the percentage of organic solvent increased (from 11 µA for water to 8 µA for 60% ACN). However, the peak height and separation efficiency decreased significantly in the case of the organic solvent MeOH as the percentage v/v increased. In contrast, insignificant changes in peak height and peak efficiency were observed for the organic solvent ACN. Therefore, mixtures of 10 µg/mL EAAs mixed standard in 0.01 M HCl with mixture of 10 ACN:1 MeOH/water 1:1 v/v ratio was conducted to assess the peak height, migration time and separation efficiency. Surprisingly, increased peak height, separation efficiency, and good separation of EAAs were achieved. For example, the fastest-moving EAA, Lys, exhibited separation efficiencies of 127,998 and 180,551 plates for samples diluted with water and the ACN:MeOH mixture, respectively. For Trp, the EAA with the slowest migration, the efficiencies were 70,981 and 92,710 plates for samples diluted in water and the ACN:MeOH mixture, respectively. Therefore, the samples diluted with the organic solvent mixture of 10 ACN:1 MeOH/water 1:1 v/v exhibited increased separation efficiency and reasonable separation resolution for all EAAs ($Rs > 1.2$). Consequently, this organic solvent mixture was selected as the dilution solution for both the mixed standard solution and real samples.

Furthermore, it was observed that a waiting time of a few minutes, should be given after diluting the samples with the organic solvent before injecting them into the instrument. This was because an additional reduction in current was observed when the diluted samples were directly injected into the instrument, resulting in a significant shift in migration time and a decrease in peak height. After allowing time for the equilibration between the analytes and organic solvent to occur, reproducibility of the current was achieved between runs. To maintain good repeatability, reproducibility, and reliable detection results for all EAAs, the surrounding conditions were controlled,

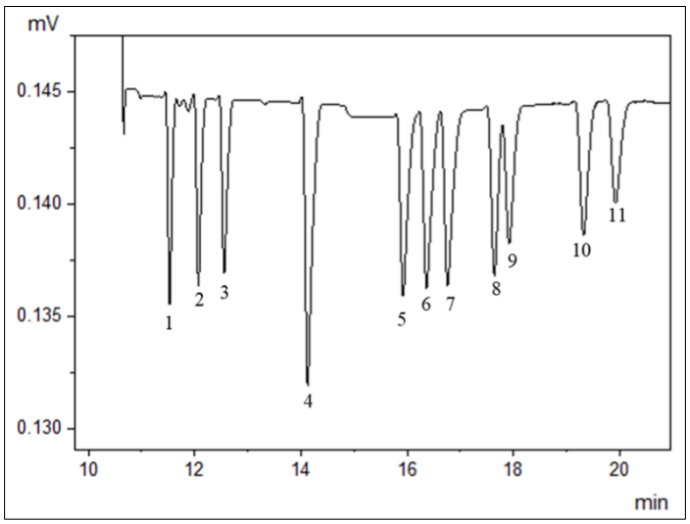


Figure 6. Electropherogram of a mixture containing 10 µg/ml EAAs and 5 µg/ml alanine (internal standard) with optimized tITP-CZE-C^4D method (LE: sodium acetate trihydrate 250 mM (50 mbar for 60 s), TE: Trp). Peak identification as depicted in Figure 3

and all working solutions were prepared prior to analysis to prevent degradation of standards and samples. Fig.6 illustrates electropherogram for optimized tITP-CZE-C^4D. The optimized conditions were 250 mM sodium acetate trihydrate as LE, injected for 60 s, and Trp as TE. Our tITP-CZE-C^4D method utilized constant voltage operation which was in aligned with standard operating mode for CE in amino acid and related to biomolecule analysis [26], nonetheless it was also acknowledged that constant current mode in CE instrument offered an alternative approach with potential benefits for migration time reproducibility. Incorporating constant current protocols could therefore be considered in future work, especially for analyses involving complex sample matrices or where enhanced repeatability was required

Method validation

The optimized tITP-CZE-C^4D method conditions were 250 mM sodium acetate trihydrate as LE, injected for 60 s, and Trp as TE. To comprehensively assess the effectiveness of tITP-CZE-C^4D for EAAs, method validation including evaluating intra-day and inter-day precision (RSD%), linearity, LOD, LOQ, and SEFs were conducted. Calibration curves using five concentrations of the mixed standard solution of EAAs along with the IS Ala were created. These EAAs exhibited excellent linearity values ($R^2 > 0.995$) within the investigated concentration range. For precision and accuracy studies, the concentration range evaluated for the mixed standard solution was 48.96–85.36 µM, and recovery experiments were performed by spiking hydrolysed feed samples at three levels (5, 10 and 15 µg/mL; low, medium and high, respectively). Inter-day precision analysis was conducted on three separate days. For intra-day precision, the RSD values for migration time ranged from 0.28 to 1.67%, and for peak height, they were all below 1.95%. In the case of inter-day precision, RSD values for migration time and peak height varied from 2.21 to 6.20 and 2.52 to 8.55, respectively. With RSDs (n = 3) consistently below 10.0% for both intra and inter-day precision, it could be concluded that the method exhibits good repeatability and reproducibility. To determine the LOD and LOQ, low concentrations of EAAs were used and calculated based on the observed baseline noise at signal-to-noise ratios of 3:1 and 10:1, respectively. The results indicated that LOD and LOQ ranged from 2.56 to 25.68 µM and 27.02 to 77.82 µM, respectively. In the context of real feed samples, where most EAAs are present at concentrations well above the µM range, the achieved LODs/LOQs were sufficient for quantitative profiling, as demonstrated by the recoveries and application results. The SEFs were within the range of 19 to 25. The SEFs observed for tITP relative to CZE were obtained by comparing peak heights under their respective optimized conditions. This demonstrated that the method provided significant sensitivity enhancements compared to typical injections in basic CZE (10 s). For Mandre de Agua and Napier grass, mean recoveries across the three spike levels fell within 80.67–116.47%, with n = 3 replicate analyses per level and per matrix, confirming acceptable accuracy of the method. Taken together, the linearity,

precision, LOD/LOQ and spike-recovery results indicate that the method satisfies commonly accepted criteria for quantitative amino-acid determination in feed matrices in line with the principles of ICH Q2(R1) for analytical procedure validation [27]. Our developed method achieved LODs in the low to mid-µM range, which was comparable to Wang et al. UHPLC-MS/MS method [28] for most amino acids, and reasonably close to Zhao et al. LC-MS method [29] for selected amino acids. This suggests our tITP-CZE-C^4D method was a viable alternative for amino acid quantification, especially when MS-based access was limited or green chemistry was prioritized.

Application of tITP-CZE-C^4D to real samples

The tITP-CZE-C^4D method was utilized to analyze EAAs in alternative and commercial feed samples: *Trichanthera gigantea* (Mandre de Agua) and *Pennisetum purpureum* (Napier grass), respectively. During the analysis of the real feed samples, significant shifts in the migration time of the analytes and poor separation of the analytes were observed.

Additionally, there was an increase in current (from 11 µA to 12 µA) when running the samples under the same voltage conditions (30 kV) as the standard. A higher current was observed during separation, leading to faster migration of analytes in the capillary. This shift might have occurred due to the presence of endogenous components (such as chloride from the sample preparation technique) in the samples, which contributed to the sample's conductivity. To ensure that the separation conditions matched those of the standard, the applied voltage was reduced to 25 kV. **Figure 7** illustrates the electropherograms for both samples. Table 1 presents the contents of EAAs in the two samples. It should be noted that the hydrolysis procedure obliged to be done to release free amino acids was known to affect Met and Trp, which explains the comparatively smaller Met and Trp peak in the electropherogram.

In these real-sample applications, the separation therefore reflects a combination of the tITP-optimized conditions and the properties of the hydrolysed matrix, and the quantitative evaluation was focused on those EAAs that remain stable under the hydrolysis conditions. By eliminating derivatization and using a short separation time of less than 20 min, together with low sample and reagent consumption in the CE-C4D system, the developed method provides a faster, simpler, and more environmentally friendly alternative for routine essential amino acid profiling. It is worth noting that unidentified peaks were observed in between and after the EAAs peaks. However, these unidentified peaks did not interfere with the separation of the target analytes. The additional peaks observed in the hydrolysed feed electropherograms did not match the migration times of any of the 10 EAAs or the internal standard and were therefore not assigned to the target analytes.

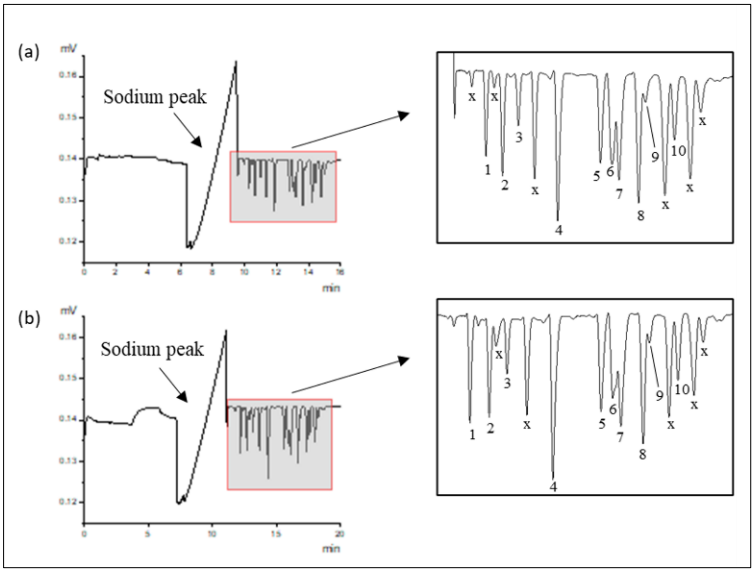


Figure 7. Electropherogram of essential amino acids during transient isotachophoresis–capillary zone electro phoresis–capacitively coupled contactless conductivity detection method in real feed samples. (a) Mandre de Agua and (b) Napier grass. The peak was under dilution factor of 8 and 4 for Mandre de Agua and Napier respectively. Other conditions were conducted under optimized condition as mentioned in experimental part with some modification in separation voltage (25 kV). Peak identification for 1-10 as depicted in Figure 3 and (x) was unknown peak

On this basis, they were treated as matrix components that did not affect integration of the EAA peaks used for quantification. To assess the accuracy of the method, recovery test was conducted by adding 5, 10, and 15 µg/mL of mixed standard EAAs to Mandre de Agua and Napier grass samples (n = 3). The suggested tITP-CZE-C^4D method demonstrated its success in determining EAAs in feed samples as evidenced by satisfactory spiking recoveries (80.67% and 116.47%). The performance of the developed tITP–CZE–C^4D method was evaluated in light of existing amino acid analysis protocols such as ISO 13903:2005 [9] and AOAC standard method 994.12 [6]. While all three methods relied on acid hydrolysis for protein digestion, substantial differences arise in the subsequent analytical workflows. AOAC standard method 994.12 for instance, required performic acid oxidation prior to hydrolysis to oxidize sulfur-containing amino acids. However, this approach led to the destruction of tyrosine, which limits its applicability for complete EAA profiling. Similarly, ISO 13903:2005 method relied on HPLC analysis with derivatization, which introduced additional reagents, increased chemical consumption, and lengthens analysis time. In contrast, our CE-based method did not require derivatization or oxidative pretreatment, thereby preserving the integrity of all EAAs. The integration of tITP stacking enhanced detection sensitivity without relying on external concentration.. With an aqueous BGE system, the method achieved high-resolution separation with reduced reagent consumption.

Table 1. The results of the AAs content and recovery in *Trichanthera gigantea* (Mandre de Agua) and *Pennisetum purpureum* (Napier grass)

EAA	Mandre de Agua				Napier grass			
	Content (g/kg)	Added (µg/mL)	Recovery	RSD (%)	Content (g/kg)	Added (µg/mL)	Recovery	RSD (%)
Lys	10.79	5	102.51	1.94	9.02	5	96.82	9.86
		10	110.88	1.42		10	108.58	8.30
		15	107.39	5.43		15	98.81	4.08
Arg	16.73	5	98.46	1.08	9.13	5	102.94	0.09
		10	101.54	4.42		10	89.97	4.96
		15	97.72	2.71		15	96.05	5.70
His	5.78	5	111.94	4.65	3.63	5	110.26	1.14
		10	114.48	0.98		10	111.99	0.57
		15	110.25	7.01		15	106.36	1.87
Val	13.41	5	103.62	4.12	9.31	5	98.83	4.39
		10	106.40	3.57		10	92.50	8.42
		15	101.39	3.88		15	102.29	2.74
Ile	20.31	5	91.04	6.39	10.16	5	88.14	3.07
		10	95.09	1.05		10	89.02	8.18
		15	87.95	6.56		15	93.80	4.69
Leu	19.55	5	102.08	3.74	13.63	5	101.68	5.77
		10	103.80	3.94		10	93.72	6.62
		15	100.16	3.91		15	100.68	9.81
Thr	28.59	5	102.39	4.43	18.10	5	93.32	2.72
		10	99.58	5.87		10	99.81	0.78
		15	99.83	2.47		15	88.29	3.97
Met	3.63	5	116.47	1.71	0.81	5	87.48	5.73
		10	105.46	5.95		10	80.67	2.56
		15	110.80	5.60		15	110.35	10.14
Phe	13.63	5	102.58	4.64	8.45	5	97.49	8.70
		10	101.47	4.93		10	97.90	10.01
		15	98.73	2.74		15	108.09	4.66
Trp	ND	5	-	-	ND	5	-	-
		10	-	-		10	-	-
		15	-	-		15	-	-

ND= not detected; Cys= cysteine; Leu= leucine; Lys= lysine; His= histidine; Met= methionine; Phe= phenylalanine; Ile= isoleucine; Thr= threonine; Tyr= tyrosine; Val= valine; Trp= tryptophan

Conclusion and future insights

This study presents a robust and sensitive tITP–CZE–C^4D method for determining EAAs in animal feed. Initial optimization of conventional CZE parameters (buffer, voltage, injection) improved separation and sensitivity. Introducing tITP by using sodium as leading ion and Trp as a pseudo-terminating electrolyte further enhanced stacking without organic solvents. The method achieved clear separation of 9 out of 10 EAAs in ~20 min, with low LODs and good validation (precision, accuracy). Its application to *Trichanthera gigantea* and *Pennisetum purpureum* feed confirmed practical utility and recovery performance. Unlike conventional stacking methods reliant on high salinity or organics, this approach suits a wider range of sample matrices. Its simplicity and fast analysis make it a promising screening tool in animal nutrition. With proper regulatory and industry support, this method holds strong potential for routine use in feed quality control labs.

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