Advances in the Determination of Nonprotein Amino Acids in Foods and Biological Samples by Capillary Electrophoresis

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ABSTRACT
There are hundreds of nonprotein amino acids whose importance in food and biological matrices is still unknown. Many of these compounds can be found in food as products formed during the processing, as metabolic intermediates or because they are added to increase functional and nutritional properties of food. Moreover, this kind of amino acids have also demonstrated to play relevant roles in the pharmaceutical and clinical fields since they may be used therapeutically in the treatment of some pathologies and their levels may be related with some diseases. These facts imply that the analysis of nonprotein amino acids can be useful to obtain relevant information in the food and biological fields. This article reviews the most recent advances in the development of analytical methodologies employing capillary electrophoresis for the achiral and chiral analysis of nonprotein amino acids in food and biological samples. With this aim, the most relevant information concerning the separation and detection of these compounds by capillary electrophoresis is discussed and detailed experimental conditions under which their determination was achieved in food and biological samples are given covering the period of time from 2015 to 2018.

KEYWORDS
Biological samples; capillary electrophoresis; food; nonprotein amino acids

Introduction
Hundreds of amino acids are known, but only 20 of them are part of proteins. These 20 proteinogenic amino acids have been widely studied; however, there are others that are not found in protein main chain either for lack of a specific transfer RNA and codon triplet or because they do not arise from protein amino acids by post-translational modifications. Many of these nonprotein amino acids (NPAAs) present an unknown origin and function, so it is difficult to attribute them a direct effect in the organism. Others have demonstrated to play relevant roles in the pharmaceutical and clinical fields since they may be used therapeutically for the treatment of some pathologies or have been related with some diseases. For instance, dihydroxyphenylalanine is used in Parkinson’s disease treatment, norleucine is related with the oxidative stress associated with Alzheimer’s disease (AD), and others such as γ-aminobutyric acid (GABA) and taurine have demonstrated to act as neurotransmitters to regulate synaptic transmission and memory. Moreover, NPAAs can provide information related to food quality and safety since some of them are present in food as products formed during processing or as additives to increase their nutritional value. Therefore, the determination of NPAAs constitutes an interesting tool to obtain information useful in the food, pharmaceutical and clinical fields. Consequently, it is necessary to develop analytical methods capable to accurately determine NPAAs in real samples.

Numerous works employing different techniques have been published reporting the determination of NPAAs. The most employed analytical techniques to face this challenge are High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and Capillary Electrophoresis (CE). Among these techniques, CE has emerged in the last decades as a potent separation technique thanks to its versatility, high efficiency and the low reagent and sample consumption required, among other advantages. In addition, its potential in the analysis of NPAAs has already demonstrated. The most employed CE modes to analyze NPAAs are Capillary Zone Electrophoresis (CZE) (based on the different mobility of the analyte in a conductive solution under the application of an electric field) and Micellar Electrokinetic Chromatography (MEKC) (whose separation is based on the different mobility of the analytes in a conductive solution that contains a micelle). Moreover, Electrogenic Chromatography (EKC) and Capillary Electrochromatography (CEC) are the most employed modes to carry out the enantoiseparation of chiral NPAAs using a chiral selector dissolved in the background electrolyte or a chiral stationary phase, respectively. Recently, the use of microchip electrophoresis (MCE) in the analysis of NPAAs has also become attractive. It presents some advantages over conventional systems such as the automatization, the lower sample and reagent consumption and its high efficiency.

The most common detection approach used in CE is the UV-Vis detector, although it requires a derivatization step.
because a high number of amino acids do not have sufficient UV absorption to be detected. Fluorescence detection has also been widely employed for the analysis of NPAAs due to its high sensitivity; however, a derivatization procedure is also needed due to the lack of fluorescence of most amino acids. Many derivatization reagents such as 2,3-naphthalenedicarboxaldehyde (NDA), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), fluorescent isocyanate (FITC), 9-fluorenylmethyl chloroformate (FMOCl), benzoyl chloride, 6-aminquinolyl-N-hydroxysuccinimidyl carbamate (AQC), dansyl chloride (DNS-Cl) and o-phthalaldehyd (OPA) have been employed. An interesting alternative to these detection approaches is mass spectrometry (MS) that presents higher sensitivity and selectivity than other systems among others. Citrulline, a precursor of arginine, participates in urea and NO cycles. As it can be seen in Table 1, CZE method with indirect UV detection using 3.0 mM SDS and 6% methanol as BGE allowed the simultaneous determination of 18 protein amino acids, hydroxyproline and hydroxylysine, and their by-products formed during derivatization. Also, by principal component analysis (PCA), hydroxyproline and hydroxylysine were selected as markers to discriminate between the authentic plastron and the adulterated one since these amino acids were not present in the other low-priced materials as it can be seen in Figure 1. Moreover, the combination of electrophoretic approach with PCA made possible the characterization of different kinds of juices which can be used as a tool to detect adulterations on industrial juice samples.

### Determination of nonprotein amino acids in foods by CE

The determination of NPAAs in food provides relevant information about food quality and safety. Different works have demonstrated the importance of analyzing NPAAs in food to detect adulterations, to evaluate nutritional quality of foods or to detect toxic effects, among others. When the NPAAs of interest are chiral, their enantiomeric determination also enables to obtain valuable information about the effects of food processing or storage or on the presence of adulterations. Table 1 shows that a wide variety of food matrices were analyzed including beverages (juice, milk, beer, water or functional drinks), vegetables, fermented products or shellfish. The analysis of NPAAs was mainly achieved using CZE and MEKC, although CEC and MCE were also employed. The detection systems most frequently used were UV and LIF detectors, despite of being necessary the use of a derivatization step. Other detectors less employed were mass spectrometry (MS) and capacitively coupled contactless conductivity (C4D). Li et al. developed a MEKC methodology enabling the simultaneous determination of hydroxyproline and hydroxylysine in different food samples (see Table 1). Both NPAAs are relevant components of protein collagen and they may be present in numerous food products. The developed MEKC methodology was applied to obtain amino acid profiles for authentic and fake plastron-derived functional product as a tool to detect adulterations with less expensive materials. The MEKC-UV method based on the use of Dns-CI as labeling reagent and 20 mM sodium tetraborate (pH 8.7) containing 0.1 M SDS and 6% methanol as BGE allowed the simultaneous separation of 18 protein amino acids, hydroxyproline and hydroxylysine, and their by-products formed during derivatization. Also, by principal component analysis (PCA), hydroxyproline and hydroxylysine were selected as markers to discriminate between the authentic plastron and the adulterated one since these amino acids were not present in the other low-priced materials as it can be seen in Figure 1. Moreover, the combination of hydroxyproline along with the 20 protein amino acids and cysteine in passion fruit juices was also performed by Passos et al. In this case, they proposed a MEKC approach with UV detection, using a 60 mM sodium tetraborate buffer (pH 10.1) containing 30 mM SDS and 5% methanol, and FMOCl as labeling reagent. The combination of the electrophoretic approach with PCA made possible the characterization of different kinds of juices which can be used as a tool to detect adulterations on industrial juice samples.

Other group of NPAAs analyzed by CE in the last years are betaines. Betaines are a group of amino acids derivatives whose structure presents a quaternary ammonium group (positively charged) and a carboxylic group. These compounds present osmoregulating properties in many plants to protect them from the environmental stress. L-carnitine and its main ester, acetyl-L-carnitine were the betaines analyzed in the period covered by this review. They are found in different mammalian tissues, plants and microorganisms and they play a key role in fatty acid metabolism. Carnitine is produced in low levels in humans, so it may be supplied from diet. Therefore, the development of analytical strategies capable of determine the content of these compounds in foods is required. Kong et al. developed a new CZE method with indirect UV detection using 3.0 mM melamine and 10% MeOH (pH 2.1) as BGE to quantify both L-carnitine and acetyl-L-carnitine in milk samples. An orthogonal experimental design (5) was employed to optimize the BGE pH and composition (melamine concentration and percentage of methanol). The LODs achieved for carnitine and acetyl-l-carnitine were 3.0 and 5.0 μM, respectively. As Figure 2 shows, the methodology was applied to the analysis of milk using the indirect UV detection since under normal CZE conditions with direct UV detection, carnitine cannot be detected. Thus, 14 kinds of milks were analyzed showing carnitine contents from 43.6 to 121.5 μM and acetyl-L-carnitine contents from 17.5 to 68.5 μM.

In the years covered by this review, the determination of GABA and citrulline by CE in food samples was reported. Besides being an important neurotransmitter in mammals, GABA has also demonstrated to present other physiological functions as regulator of cells, hormones and blood pressure, among others. Citrulline, a precursor of arginine, participates in urea and NO cycles. As it can be seen in Table 1, a MEKC methodology with LIF detection was developed by Zhu et al. using a flow-gated CE coupled with alternate injections (electrokinetic injection –5 kV for 0.3 s) in a microfabricated switch to perform simultaneously the determination of 17 protein amino acids, GABA, citrulline, phosphoryl...
Table 1. Characteristics of the analytical methodologies developed for the determination of NPAAs in foods by CE.

| NPA As                        | CE-mode/detection | Separation conditions* | Separation from: | Sample treatment Application | LOD † | References |
|------------------------------|-------------------|------------------------|------------------|------------------------------|-------|------------|
| Hydroxyproline               | MEKC-UV (214 nm)  | 20 mM sodium tetraborate and sodium phosphate +0.1M SDS +6% MeOH (pH 8.7); capillary, 50 μm × 50 cm; 25 kV, 25°C | 18 protein amino acids | Plastron, fish skin, pig skin, chicken tendon, calf tendon, pork, chicken and fish: dried, pulverized, dilution, alkali digestion, filtration and neutralization with HCl before derivatization with Dns-Cl | Potential of amino acids as markers of adulterations of plastron-derived functional foods | Hydroxyproline: 1.57 × 10⁻⁴ mg/mL, Hydroxylysine: 6.65 × 10⁻⁴ mg/mL | [21] |
| Hydroxylysine                |                   |                        |                  |                              |       |            |
| Carnitine and Acetyl-L-carnitine | CZE-indirect UV (200 nm) | 3.0 mM melamine +10% MeOH (pH 2.1); capillary, 75 μm × 20 cm; 10 kV, 20°C | 20 protein amino acids and Cystine | Extraction with ACN:MeOH (4:1 v/v), centrifugation, evaporation and dilution in water | Characterization and quantification of amino acid profile in passion fruit juices | L-carnitine: 3.0 μM, Acetyl-L-carnitine: 5.0 μM | [23] |
| γ-Aminobutyric acid          | MEKC-LIF (λex 442 nm; λem 485 nm) | 40 mM sodium tetraborate +60 mM SDS +2 mM HP-β-CD (pH 9.2); capillary, 10 μm × 10 cm; −25 kV, 25°C | 17 protein amino acids, phosphoryl ethanolamine and ethanolamine | 50-Fold dilution with water (previously degassed in ultrasonic bath) followed by NDA derivatization in presence of cyanide | Quantitation of 19 amino acids, phosphoryl ethanolamine and ethanolamine in beers | γ-Aminobutyric acid and Citrulline: 2.0-5.0 nM | [9] |
| Citrulline                   |                   |                        |                  |                              |       |            |
| Homoarginine β-N-Oxalyl-γ-,β-diaminopropionic acid | CZE-UV (195 nm) | 25 mM sodium borate +5 mM sodium sulfate (pH 9.2); capillary, 75 μm × 50 cm; 21 kV, 22°C | − | Extraction with MeOH-water (60:40 v/v) (under Ultra-Turrax), centrifugation, evaporation and dilution in sample buffer (10 mM sodium borate +5 mM sodium sulfate +12.84 mM hippuric acid), filtration prior to the CE analysis. Extraction with ethanol-water (60:40 v/v) (under rotating shaking), centrifugation, evaporation and dilution in the sample buffer (10 mM sodium borate +5 mM sodium sulfate +12.84 mM hippuric acid), filtration before CE analysis. | Simultaneous analysis of β-N-Oxalyl-γ-,β-diaminopropionic acid and Homoarginine in Lathyrus species | − | [24] |

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| NPAA                  | CE-mode/detection | Separation conditions \(^a\)                          | Separation from:                                                                 | Sample treatment                                                                 | Application                                                                                   | LOD \(^b\) | References |
|----------------------|------------------|------------------------------------------------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|-----------|------------|
| Pyroglutamic acid    | MEKC-UV (200 nm) | 25 mM sodium tetraborate +50 mM SDS (pH 9.1); capillary, 50 \(\mu\) m x 56 cm; 30 kV, 35°C | Organic acids, 13 protein amino acids, Cystine and Tryptamine                      | Water solution, basic hydrolysis (with \(\alpha\)-amylase), thermal sterilization, microfiltration and fermentation (tapioca starch and yeast) before CE analysis | Simultaneous determination of lactic acid and its organic impurities in fermentatively products | –         | [23]       |
| Taurine              | CZE- C\(^4\)D    | Commercial equipment: 20 mM CHES +10 mM NaOH (pH 9.5); capillary, 25 \(\mu\) m x 11.5 cm; 10 kV, temperature, not indicated | Citrate and Carbonate                                                              | Sonication (to remove dissolved gases) and 40-fold dilution for energy drink           | Determination of Taurine in energy drink                                                   | 14.4 mg/L | [24]       |
|                      | MCE-LIF (\(\lambda_{ex}\) 635 nm; \(\lambda_{em}\) 495 nm) | 100 mM sodium borate (pH 9.9); glass microchip with a simple cross channel design; separation channel, (60 mm x 25 \(\mu\) m x 70 \(\mu\) m (length x depth x width)), 45 mm from injection to the detector; applied potential and temperature, not indicated | Lysine and Vitamin B\(_3\)                                                         | Twofold dilution with 40 mM sodium borate, pH adjustment (8.60), derivatization with Cy5 and dilution with 10 mM sodium borate (pH 9.88) | Analysis of amino acids (Lysine and Taurine) and vitamin B\(_3\) in functional drinks        | 8.2 mg/L  | [26]       |
| \(\beta\)-N-Methylamino-\(L\)-alanine | CZE-UV (192 nm) | 250 mM sodium phosphate (pH 3.0); capillary, 50 \(\mu\) m x 46 cm; 25 kV, 17°C | Four \(\beta\)-N-Methylamino-\(L\)-alanine isomers                                  | Acid hydrolysis, drying and dilution with HCl, clean-up, drying and re-dilution with HCl prior to CE analysis | Separation of five \(\beta\)-N-Methylamino-\(L\)-alanine isomers and quantification of \(\beta\)-N-Methylamino-\(L\)-alanine in cycad, mussel and lobster samples | 0.50 nM   | [27]       |
|                      | CZE-(QqQ)MS\(^2\) ESI+: (4.0 kV); sheath liquid: MeOH:water (50:50 v/v) containing 0.1% formic acid at 1.0 \(\mu\) L/min, Flow and temperature of dry gas, and nebulizer gas pressure not indicated | 5 M formic acid =10% (v/v) ACN (pH 1.55), capillary, 50 \(\mu\) m x 100 cm; 20 kV, 17°C |                                    |                                                                                         |                                                                                              | 0.25 \(\mu\)g/mL and 20 mg/g | [28]       |
| Domoic acid          | MCE-LIF (\(\lambda_{ex}\) 475 nm; \(\lambda_{em}\) 535 nm) | 5 mM sodium tetraborate (pH 9.2); glass microchip-cross-channel design; separation channel, (49 mm x 30 \(\mu\) m x 80 \(\mu\) m (length x depth x width)), 27 mm from injection to the detector; 400 V/cm, temperature, not indicated | –                                  | Extraction with MeOH:water (1:1 v/v), centrifugation, filtration and derivatization with FITC | Determination of Domoic acid in shellfish tissues                                           | 0.28 nM   | [19]       |

\(^a\) Conditions

\(^b\) Limits of detection
ethanolamine (PEA) and ethanolamine (ETA) in beers. Using NDA as labeling reagent and 40 mM sodium tetraborate containing 60 mM SDS and 2 mM HP-β-CD as running buffer (pH 9.2) allowed a high separation efficiency for all these compounds within 90 s using a capillary length of 10 cm. The LODs obtained for amino acids, PEA and ETA with the proposed methodology were from 2.0 to 5.0 nM. The quantitative results obtained in eight different brands of beer showed that GABA, alanine and valine were the most abundant in all samples whereas citrulline, glutamine and methionine were the less abundant (indeed, the content of citrulline was lower than 40 μM in all beer brands analyzed). These differences in amino acids composition were in agreement with the differences among the characteristics of the samples (i.e. differences in flavor, raw materials, processing or enzyme activity).[19]

Sacristán et al.[24] developed a CZE methodology to analyze homoarginine and β-N-Oxalyl-L-α,β-diaminopropionic acid, that are the main NPAA in grass pea seeds (Lathyrus species). Lathyrus species are a rich source of proteins and are cultivated for human consumption. However, a high consumption of these species may produce a disease known as “lathyrism” responsible for humans and animal’s paralysis. The scientific committee of the Spanish Agency for Food safety and Nutrition recommends an occasional consumption of Lathyrus being the safe consumption lower than 1.5 mg/g for humans, but further research needs to be performed to ensure these safety values. [46] The developed CZE methodology employed a BGE (pH 9.2) containing 25 mM sodium borate and 5 mM sodium sulfate, and UV detection, and enabled the simultaneous determination and quantification of homoarginine and β-N-Oxalyl-L-α,β-diaminopropionic acid in L. sativus (grass pea) and L. cicera (red pea). Sample preparation was carried out by two different extraction protocols based on the use of a rotating shaker (24 h) and an Ultra-Turrax (1 min) with ethanol-water (60:40 v/v) as extraction solvent. Despite of the fact that no significant differences were found between the two extraction protocols, the Ultra-Turrax method, which is simpler and faster, and demonstrated to provide higher yield results than the rotating shaking method, was selected to analyze all samples. Different Lathyrus cicera and Lathyrus sativus species were analyzed to evaluate the levels of homoarginine and β-N-Oxalyl-L-α,β-diaminopropionic acid showing that homoarginine contents (from 8.08 mg/g to 12.44 mg/g) were higher than the contents of β-N-Oxalyl-L-α,β-diaminopropionic acid (from 0.79 to 5.05 mg/g) in all samples. Moreover, the results obtained showed that β-N-Oxalyl-L-α,β-diaminopropionic acid levels for L. cicera species were lower than the recommended ones but this was not the case for L. sativus species, whose values exceeded those recommended.[24]

Taurine is the only sulfur-containing amino acid analyzed by CE in food in the reviewed period. This NPAA can be found in mammalian tissues in high concentration levels and it presents important physiological and therapeutic functions such as bile acid conjugation, maintenance of calcium homeostasis,[47] liver protection and treatment of low blood pressure.[48] Taurine is the most employed component
| NPAA                  | CE-mode/detection | Separation conditions | Separation from: | Sample treatment                                                                 | Application                                                                 | LODb | Ref |
|----------------------|-------------------|-----------------------|------------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------|------|-----|
| Homocysteine         | MEEK-LIF (λex 488 nm; λem 520 nm) | 50 mM borate + 30 mM SDS + 30 % MeOH (pH 9.5); capillary, 50 μm × 50 cm; 21 kV, 21 °C | Arginine, asymmetric dimethyl L-arginine and Monomethyl-L-arginine | Protein precipitation with 5-sulfosalicylic acid, evaporation to dryness under vacuum, redissolution in BGE and derivatization with CFSE before CE analysis. | Determination of Homoarginine, Homocysteine and Ornithine metabolic derivatives in fluids from Type 2 diabetics with peptic ulcer bleeding | 0.12 nM | [30] |
| Ornithine            | CZE-LEDIF (λex 405 nm; λem 486 nm) | 1% PVP + 10 mM HEPES (pH 7.0); PVP coated capillary, 75 μm × 28 cm; ~20 kV, temperature, not indicated | Arginine, Glutamic acid, Aspartic acid and Histamine | Protein precipitation by heating, derivatization with NDA before CE injection. | Measurement of branched chain amino acid uptake in 3T3-L1 cells | -- | -- |
| Citrulline           | CZE-LIF (λex 488 nm; λem 502 nm) | 15 mM borate + 1.4 mM SBEC + 10 % DMSO (pH 9.2); 10 kV, capillary and temperature, not indicated | Arginine, Glutamic acid, Aspartic acid and Histamine | Fluorogenic derivatization with NDA. | Analysis of amino acid neurotransmitters in brain dialysis samples | Citrulline: 0.36 μM Taurine: 0.42 μM | [32] |
| Taurine              | CZE-(IT)MS² and ESI+ (4.5 kV); sheath liquid: MeOH/water (60:40 v/v) containing 0.5% formic acid at 5.0 μL/min; dry gas: 5 L/min at 200 °C; nebulizer gas pressure: 8 psi | 0.8 M formic acid + 15 % MeOH (pH 1.96); capillary, 50 μm × 85 cm; 30 kV, 20 °C | Arginine, Glutamic acid, Aspartic acid and Histamine | Centrifugation and dilution prior to CE analysis | Determination of amino acids in urine samples | γ-aminobutyric acid: 4 μM and Ornithine: 4.8 μM Citrulline: 7.7 μM Hydroxyproline: 3.7 μM Alloisoleucine: 2.5 μM | [31] |
| Taurine              | MCE-LIF (λex 445 nm; λem 480 nm) | 20 mM carbonate (pH 10.0); 27 kV, capillary and temperature, not indicated | Arginine, Glutamic acid, Aspartic acid and Histamine | Protein precipitation with ACN, derivatization with FITC and dilution before CE analysis. | Determination of plasma levels of Arginine and Citrulline in preterm and full-term neonates | -- | [32] |
| Taurine              | MEEK-SDED | 20 mM phosphate + 20 mM SDS (pH 10.0); capillary, 25 μm × 40 cm; 12 kV, temperature, not indicated | Arginine, Glutamic acid, Aspartic acid and Histamine | Fluorogenic derivatization with NDA. | -- | -- | [33] |
| Taurine              | EKC-LIF (λex 488 nm; λem 543.5 nm) | 90 mM borate + 35 mM α-CD (pH 10.0); capillary, 5 μm × 6.2 cm; ~23 kV, temperature, not indicated | Arginine, Glutamic acid, Aspartic acid and Histamine | On-line derivatization with 20 mM NBD-F/250 μM HCl in 50 % MeOH. | Monitoring the in vivo dynamics of amino acids biomarkers of metabolism in adipose tissue | 2.7 μM | [37] |
| Taurine              | CZE-(TOF)MS and ESI+ (4.0 kV); sheath liquid: MeOH/water (50:50 v/v) containing 0.5 μM reserpine at 10 μL/min; dry gas: flow not indicated at 300 °C; nebulizer gas pressure: 10 psi | Commercial electrophoresis buffer for anion and cation analysis; capillary, 50 μm × 80 cm; applied potential and temperature, not indicated | Arginine, Glutamic acid, Aspartic acid and Histamine | Extraction with water, protein precipitation with ACN, evaporation to dryness under nitrogen stream, redissolution in BGE. | Determination of Taurine in human tear fluid | 0.18 μM | [33] |

**Table 2.** Characteristics of the analytical methodologies developed for the determination of NPAAs in biological samples by CE.
| Homocysteine | MEKC-UV (λabs 285 nm) | CZE-LIF (λex 473 nm; λem 510 nm) | CZE-(QqQ)MS2 and ESI+ (4.5 kV); sheath liquid: MeOH:water (50:50 v/v) containing 5 mM acetic acid at 6.0 µL/min; dry gas: 61 L/min at 160°C; nebulizer gas pressure: 34.5 kPa | 0.02 µM | [31] |
|---|---|---|---|---|---|
| 0.8 µM | 0.3 M TEA + 0.1 M formic acid + 50 µL CTAB (pH 3.9); capillary, 50 µm × 21.5 cm; −12 kV, 25°C | Cysteine, Cysteine–Glycine | Reduction with DTT before TCDI derivatization. | Rapid detection of total Homocysteine and Cysteine in human plasma | [35] |
| 0.2 µM | 0.1 M phosphate + 30 mM TEA + 25 M CTAB + 2.5 M SDS + 2.5% PGE-600 (pH 2); capillary, 50 µm × 23.5 cm; −17 kV, 30°C | Cysteine | Derivatized with TCDI, extraction with chloroform-ACN. | Determination of Homocysteine and Cysteine levels in human plasma and urine | [36] |
| 13 pM | 180 mM sodium citrate (pH 7.5); capillary, 50 µm × 50 cm; 15 kV, room temperature | Glutation, Cysteine, γ-glutamylcysteine | Cells lines were resuspended in PBS and incubated with NEM. Single cells were incubated with TMPAB-o-M and mixed with the running buffer. | Chemical cytometry of thiols in human colon cancer and breast cells | [37] |
| 35 nM | 5 M acetic acid; capillary, 50 µm × 60 cm; 25 kV, 20°C | Glutamic acid, Cysteine, Methionine | Blood samples + EDTA, centrifugation. For aminothiols analysis: Mix samples with DTT, IAA and ACN, centrifugation and analysis of the supernatant. | Determination of Homocysteine as potential biomarkers of amyotrophic lateral sclerosis | [38] |
| NPA | CE-mode/detection | Separation conditions | Separation from: | Sample treatment | Application | Ref |
|-----|-------------------|-----------------------|-------------------|-----------------|-------------|-----|
| γ-Aminobutyric acid | MEKC-LIF (\( \lambda_{ex} \), 450 nm; \( \lambda_{em} \), 480 nm) | 25 mM phosphate +30 mM SDS (pH 8.3); capillary, 25 μm × 50 cm; 29 kV, 25 °C | 17 protein amino acids | Derivatization with NDA cyanide and internal standard (\( \gamma \) norvaline) (9:1:1:1, sample:NDA/cyanide: \( \gamma \) norvaline) | Measuring amino acid secretions from islets of Langerhans | [15] |
| Hydroxyproline | MEKC-LIF (\( \lambda_{ex} \), 492 nm; \( \lambda_{em} \), 520 nm) | 40 mM cholate + 40 mM deoxycholate + 40 mM tetraborate (pH 9.2); capillary, 10 μm × 10 cm; −25 kV, room temperature | Proline, 4 propyl dipeptides | Acid Hydrolysis, 100-fold dilution, addition of EDTA, OPA and derivatization with NBD-F prior to CE analysis. | Rapid determination of free propyl dipeptides and Hydroxyproline in urine | 70 nM [18] |
| Pyroglutamic acid | CZE-UV (\( \lambda_{abs} \), 200 nm) | 40 mM CHES/NaOH (pH 10.2); capillary, 25 μm × 15 cm; 30 kV, 25 °C | Paracetamol | Protein precipitation with ACN and NH4OH, centrifugation and analysis of the supernatant. | Quantification of Paracetamol and Pyroglutamic acid in serum | 1.3 μg/mL [39] |
| Betaine | CZE-(QqQ)MS2 and “ESI+” (4.0 kV); sheath liquid: MeOH:H2O (50:50 v/v); containing 5 mM ammonium acetate at 60 μL/min; dry gas: 6 L/min at 300 °C nebulizer gas pressure: 6 psi | 10 % MeOH v/v + 5 % formic acid v/v; capillary, 75 μm × 120 cm; 28 kV, 20 °C, pressure assistance: 40 mbar | Choline, dimethylglycine | Protein precipitation with ACN, evaporation to dryness under vacuum, redisolution in water. | Simultaneous quantification of Choline, Betaine and Dimethylglycine in human plasma | 0.62 μM [40] |
| 3,4-Dihydroxyphenylalanine | EKC-(IT)MS2 and “ESI+” (−4.0 kV); sheath liquid: MeOH:water (50:50 v/v) containing 0.1 % formic acid at 3.3 μL/min; dry gas: 5 L/min at 200 °C nebulizer gas pressure: 3 psi | 180 mM M-β-CD +40 mM HP-β-CD + 2 M formic acid (pH 1.2); capillary, 50 μm × 120 cm; 30 kV, 15 °C | Phenylalanine, Tyrosine, Dopamine, Norepinephrine and Epinephrine | Precipitation of proteins with ACN (plasma/ACN, 1:2), centrifugation, dilution of the supernatant with formic acid, sonication and filtration. | Simultaneous enantioseparation of all the chiral constituents of the Phenylalanine-Tyrosine metabolic pathway | l-3,4-Dihydroxyphenylalanine: 54 nM [41] |

\( x \)-CD: \( x \)-cyclodextrin; ACN: acetonitrile; BIS-TRIS propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CFSE: 5-carboxyfluorescein succinimidyl ester; CHES: 2-(N-cyclohexylamino)ethane sulfonic acid; CTAB: hexadecyltrimethylammonium bromide; CZE: capillary zone electrophoresis; DMSO: dimethyl sulfoxide; DTT: dithiothreitol; EDTA: tetrasodium salt of ethylenediaminetetraacetic acid; EMMA: electrophoretically mediated micro-analysis; FITC: fluorescein isothiocyanate; HP-β-CD: 2-hydroxypropyl- \( \beta \)-cyclodextrin; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IAA: iodoacetic acid; IPA: 2-propanol; (IT)MS: ion trap; LEDIF: light emitting diode induced fluorescence; LIF: laser-induced fluorescence; M-J/CD: methyl- \( \beta \)-cyclodextrin; MEKC: micellar electrokinetic chromatography; MeOH: methanol; M5: tandem mass spectrometry; NBD-F: 4-fluoro-7-nitro-2,1,3-benzoxadiazole; NDA: 2,3-naphthalenedicarboxaldehyde; NEM: N-ethylmaleimide; ODS: octadecyl silica; OPA: o-phthalaldehyde; PBS: phosphate buffered saline; PGE-600: polyethylene glycol 600; PVP: polyvinylpyrrolidone; (QqQ)MS2: triple quadrupole mass spectrometry; SBE: sulfobutylether- \( \beta \)-cyclodextrin; SDED: serial dual-electrode detection; SDMA: symmetric dimethyl-L-arginine; SDS: sodium dodecyl sulfate; TCDI: 1,1-thiocarbonyldiimidazole; TDLFP: transverse diffusion of laminar flow profiles; TEA: triethanolamine; TMPAB-o-M: 1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiaza-s-indacene; (TOF)MS: time-of-flight mass spectrometry.

\( a \)-Capillary dimensions expressed as internal diameter × effective length (cm to the detector).

\( b \)LODs units expressed as in the original work.
in the formulation of energy and sport drinks that have gained popularity among athletes as a consequence of their energetic properties. Nevertheless, attention should be paid to the consumption of these products (especially for patients with heart disease or hypertension) since high levels of taurine intake may produce undesirable effects even in healthy people. As it can be seen in Table 1, taurine was determined using different modes of CE such as CZE, MEKC and MCE. A CZE method with C18D was developed for the determination of taurine in energy drinks. The results obtained with an instrument with a coaxial flow-gating interface (FGI) were compared with those obtained with an Agilent commercial equipment showing similar LODs (14.4 mg/mL and 8.2 mg/mL, respectively). Both methods were able to determine lower percentages of taurine than the declared value (4000 mg/L). The coaxial FGI presents some characteristics comparable with common commercial CE instrument such as repeatable sample injection and improved total analysis time (73 s and 225 s, respectively). As Table 1 shows, the separation was achieved using 20 mM CHES and 10 mM NaOH (pH 9.5) as separation buffer and only a 40-fold dilution step of samples was needed to analyze them by CE. Wu et al. developed a methodology using MCE with LIF detection to determine taurine, lysine and vitamin B3 in functional drinks. The use of field-amplified sample stacking (FASS) combined with reverse-field stacking as on-line preconcentration strategy allowed improving the sensitivity and the separation efficiency in comparison with conventional MCE-LIF method. After optimizing different electrophoretic and derivatization variables, the use of 100 mM sodium borate (pH 9.88) as running buffer and sulfoindocyanine succinimidyl ester (Cy5) as derivatization reagent enabled the quantification of lysine, taurine and vitamin B3 (within 4 min) in eight functional drinks showing a lower concentration of taurine in one of them than the values declared in the label.

Pyroglutamic acid is an interesting cyclical NPAA that may be produced in protein biosynthesis or as an intermediate in metabolic and transport pathways and it can be found as a free acid or bound at the N terminal group of proteins and peptides. This NPAA is usually found in urine, plasma, bones and other tissues, and it can also be present naturally in food or can be employed in beauty or dietary formulations. The only article published in the reviewed period reporting the separation of pyroglutamic acid by CE was aimed to determine lactic acid and its organic impurities in fermented products. Among these impurities, taurine and pyroglutamic acid were the NPPAs identified. The MEKC methodology developed consisted of using a 25 mM sodium tetraborate buffer containing 50 mM SDS (pH 9.1) and UV detection (200 nm). Sample
treatment including an enzyme-assisted extraction procedure and a fermentation process was accomplished. Thus, ten organic acids, thirteen protein amino acids, cysteine, tryptamine, taurine and pyroglutamic acid were identified and separated from lactic acid in fermentation broth of different renewable resources. It was observed a major unknown component before the lactic acid peak in some samples and it was identified as pyroglutamic acid using MS spectra followed by the standard confirmation. The methodology enabled to detect 0.3 ppm of pyroglutamic acid in presence of 718, 400 ppm of lactic acid.

The NPAA \(\beta\)-N-methylamino-L-alanine is a toxin present in nature which is related to many neurodegenerative pathologies such as the amyotrophic lateral sclerosis, Alzheimer’s dementia or Parkinson’s disease.[53] This NPAA presents some relevant structural isomers. Three of them, namely 2,4-diaminobutyric acid (2,4-DAB), N-(2-aminoethyl)glycine (AEG) and \(\beta\)-amino-N-methyl-alanine have been found in food matrices (e.g. microalgae and mollusks). The major exposure pathway to \(\beta\)-N-methylamino-L-alanine is the dietary intake so the development of high selective methods able to separate the isomers of this NPAA is crucial.[54] However, \(\beta\)-N-methylamino-L-alanine analysis may be a hard task since all its isomers have the same monoisotopic mass and similar physicochemical properties making difficult their discrimination. Recently, two different methodologies based on the use of CZE with UV and MS detection were developed by Kerrin et al.[28] to carry out the separation of \(\beta\)-N-methylamino-L-alanine and four of its isomers in a mussel tissue reference material. A simple sample treatment based on protein hydrolysis in acid conditions followed by Oasis-MCX cartridge cleanup procedure without any derivatization step was employed. To develop the CZE-UV methodology, the effect of different separation variables, such as the running buffer composition, buffer concentration, organic modifiers and pH and instrumental parameters, such as temperature and voltage, were evaluated. Under the optimized conditions (see Table 1) \(\beta\)-N-methylamino-L-alanine and its isomers could be separated. However, the LOD (20 mg/g, dry mass) obtained for \(\beta\)-N-methylamino-L-alanine using this method was much higher than the reported content in cyanobacteria and mussels (300 \(\mu\)g/g and 10 \(\mu\)g/g, respectively). Afterwards, in order to improve the sensitivity, these authors developed a new methodology by CZE-MS. First, to select compatible CE-MS conditions, the phosphate BGE was replaced by 5 M formic acid containing 10% acetonitrile and a custom interface was built with a straight tube enclosing the CE capillary which eliminated the plugging problems previously obtained. A 50% aqueous MeOH containing 0.1% formic acid was used as sheath liquid. Before the analysis by CZE-MS, a strong cation low conductivity solvent. This approach allowed to achieve a LOD of 16 ng/g (dry mass) for \(\beta\)-N-methylamino-L-alanine enabling the quantification of this NPAA in real samples (cycad leaves, lobster tail meat and lobster tomalley).[29]

Domoic acid is other neurotoxic water soluble tricarbonylic acid which is present in numerous types of shellfish and seafoods usually consumed as part of the human diet. The consumption of high levels of this NPAA may be responsible for Amnesic Shellfish Poisoning (ASP), a disease whose symptoms are cardiac arrhythmias, abdominal cramps and neurological dysfunction, among others. Therefore, analytical methods are needed to assess a safe content of this compound in food.[55] A CEC method, based on the use of a packed capillary column with octadecyl silica (ODS) particles (using a supplementary pressure) and LIF detection, was employed to analyze traces of domoic acid in shellfish samples. As it can be seen in Table 1, this is the only work in which CEC was applied to the analysis of NPAA s in the period of time reviewed in this article. A solid–liquid extraction followed by a clean-up procedure and a derivatization step with NBD-F was achieved before CEC analysis that was carried out in positive and negative voltage using 5 mM phosphate buffer containing 60% acetonitrile (pH 2.5), enabling a LOD for domoic acid as low as 10 ng/mL.[15] The developed methodology was compared with a HPLC-MS/MS method showing similar LODs and RSD results, and better recoveries in the case of CEC. MCE was also employed to determine domoic acid in shellfish tissues. The use of a 5 mM sodium tetraborate buffer (pH 9.2), FITC as derivatization reagent and LIF detection, allowed the determination of domoic acid within 60 s with a LOD of \(2.8 \times 10^{-10}\) M which enabled to assess the accomplishment of the official regulatory limit of 20 \(\mu\)g domoic acid/g wet tissue. The method constitutes a potent alternative to carry out the detection of this toxin since it presents some advantages such as simplicity, sensitivity and high separation speed.[19]

Chiral analysis of NPAA s in food is of high interest to guarantee food quality, authenticity and safety. Even though the l-enantiomer is the natural form, d-enantiomers of NPAA s can be found in food due to a racemization during food processing, a microbiological processes or by the fraudulent addition of racemic mixtures in the particular case of supplemented foodstuffs,[56] for which regulations establish the use of the l-enantiomer. As a consequence of the different properties and biological activity that the enantiomers may have, their individual determination in foods present a high interest. In fact, the enantioselective determination of NPAA s has demonstrated to be relevant to detect food adulterations[42] or to evaluate manufacturing processes.[57] During the period of time covered by this review, only one work has been focused on the enantiomeric separation of NPAA s by CE in food.[29] New analytical methodologies were developed reaching the enantiomeric separation of eight NPAA s by EKC. After FMOC derivatization, the optimized separation conditions consisted of the use of a 100 mM formate buffer (pH 2.0) and an anionic cyclodextrin (sulfated-\(\alpha\)-CD or sulfated-\(\gamma\)-CD depending on the amino acid). Figure 3 shows the electropherograms corresponding to the enantiomeric separation of the NPAA s investigated under the optimized conditions. The figures of merit of the developed method were shown to be adequate for determining l-citrulline and its enantiomeric impurity in
food supplements. LODs of $2.1 \times 10^{-7}$ M and $1.8 \times 10^{-7}$ M were achieved for D- and L-citrulline, respectively. L-citrulline was quantified in six samples (three new and three submitted to a long storage time) where D-citrulline was not detected in any case showing that storage time did not originate racemization.

**Determination of nonprotein amino acids in biological samples by CE**

From a biological point of view, the determination of NPAAs has a special relevance since many of them are key compounds in metabolic pathways or are related with different pathologies. In fact, several diseases related with metabolic dysfunctions originate abnormal quantities of amino acids in body fluids. Thus, the determination of NPAAs in different biological fluids can be used for the early detection of different cancer types, as diagnostic tool to inspect vesicoureteral reflux samples, to detect an immature enzymatic system in preterm neonates, as indicator of ocular surface diseases, to assess the embryo viability in assisted reproduction, as indicator of pathologies such as coronary artery disease, diabetes renal insufficiency or Alzheimer’s disease, or even for clinical toxicology laboratory diagnostics. These examples show the relevance of the determination of NPAAs in biological fluids and the imperative need to develop high sensitive methodologies able to detect these compounds at the low levels at which they are present in biological samples. Table 2 summarizes the characteristics of the CE methodologies developed for the analysis of NPAAs in the period covered by this article. As it can be observed in this table, the preferred detection mode was LIF followed by MS$^2$ and UV. The CE approaches developed have been employed to analyze a broad range of samples: urine, plasma, serum, tear fluid, saliva, human embryos or human colon cancer and breast cells, among others (see Table 2).

Some of the developed CE methodologies have been applied to the simultaneous analysis of different NPAAs, being CE coupled to LIF the approach mainly used in this kind of analysis. For instance, Liang et al., developed a MEKC-LIF methodology to achieve the simultaneous determination of homocysteine, homoaarginine and five related metabolites (including ornithine and citrulline) after derivatization with 5-carboxylfluorescein succinimidyl ester (CESE). A baseline separation was possible in 10 min using as BGE a 50 mM borate buffer at pH 9.5 containing 30 mM SDS and 30% MeOH. LODs reached were between 0.12 and 1.70 nM which are much lower than other reported in previous works for some of the analyzed compounds by fluorescence (sensitivity was improved from 5 to 600 fold times). This methodology was fully validated using plasma and urine samples from type 2 diabetics with peptic ulcer bleeding. Also, an interesting CE-LIF method for the high-speed monitoring of branched chain amino acids uptake in 3T3-L1 cells was developed by Harstad and Bowser. The interest in the measurement of these amino acids and their downstream metabolites (where GABA, ornithine, citrulline and taurine can be included) is related to the fact that they play relevant roles in the tricarboxylic acid cycle and adipocyte lipogenesis. To carry out the analysis, analytes were sampled using microdialysis, on-line derivatized with a fluorescent reagent, separated by CE and detected by LIF using the device shown in Figure 4(a). Under optimal conditions, the separation was obtained in less than 30 s (see Figures 4(b,c)). Other CE-LIF methodology developed within the time covered in this review has been proposed to achieve the simultaneous analysis of different NPAAs (ornithine, citrulline, norvaline and norleucine) along with 17 protein amino acids in plasma. This was a polymer-based technology.
separation method in the presence of mixed micelles and the analytes were derivatized with naphthalene-2,3-dicarboxaldehyde (NDA). In spite of the fact that the analysis time was too high (180 min), the high level of resolution obtained using this methodology allowed the accurate quantitation of amino acids in plasma without the need for protein filtration. Taking in mind that the presence of free amino acids in plasma could be used for the early detection of different cancer types, this methodology will be useful for clinical diagnosis employing amino acids as biomarkers.

The simultaneous analysis of several NPAAs not only was performed by CE with LIF detection but also using the hyphenation of CE with MS. Thus, the targeted analysis of amino acids in urine, including 20 protein amino acids, β-alanine, the dipeptide carnosine and 5 NPAAs (GABA, ornithine, citrulline, hydroxyproline and alloisoleucine) was carried out by CE-MS2. After optimizing the parameters related to BGE, CE-MS interface and MS detection, the method, based on the use of 0.8 M formic acid at pH 1.96 containing 15% MeOH and a pH stacking procedure to increase the sensitivity (a plug of 12.5% ammonium solution was injected before the sample), enabled the separation of the 27 analyzed compounds in less than 30 min with LODs ranging from 0.63 to 29 μM. Once the method was validated according to FDA and ICH guidelines, its feasibility was demonstrated by analyzing urine samples from children with vesicoureteral reflux which proved that the CE-MS2 method could be considered as a possible auxiliary diagnostic tool to inspect vesicoureteral reflux samples.

Figure 4. (a) Schematic on the online MD-CE system. (b) Full electropherogram and (c) expanded one from an online analysis of CE analysis of 3T3-L1 cells supernatant after 30 min of incubation with Ringer solution, glucose, isoleucine, leucine and valine. Experimental conditions: BGE, 90 mM borate containing 35 mM α-CD (pH 9.8); capillary, 50 μm × 30 cm; voltage, 21 kV. Peaks: (1) lysine, (2) isoleucine, (3) leucine, (4) ornithine, (5) methionine, (6) phenylalanine, (7) valine, (8) ornithine, (9) β-Alanine, (10) glutamine, (11) alanine, (12) threonine, (13) β-alanine, (14) glycine, (15) NBDOH, (16) taurine, (17) internal standard, (18) glutamate and (19) aspartate. Reprinted from Ref. [18] copyright (2016) with permission from ACS publications.
and a serial dual-electrode to conduct the detection. In this indirect detection mode, bromide is oxidized to bromine which reacts quantitatively and rapidly with taurine, so that the decrease in the bromine current can provide taurine concentration. Once optimized the parameters affecting the analytical performance (bromine concentration, dual-electrode potentials and CE separation conditions), taurine was baseline separated from other interfering amino acids within 18 min. The LOD obtained for taurine by the developed methodology (0.18 μM) was compared with those obtained by other detection modes such as pulse and direct amperometric detection or LIF, being the LOD obtained by the indirect method lower than or similar to the LOD obtained by both amperometric methods and higher than the LOD obtained by LIF detection (but it requires a derivatization step which makes the process more labor-intense). The successful application of this methodology for determining taurine in tear fluids shows the potential of this device to be applied in clinical analysis. An interesting work recently published proposes an on-line microdialysis (MD)-CE method with LIF detection to measure the in vivo dynamics of amino acids (taurine, GABA and 10 proteinogenic amino acids) biomarkers of metabolism in adipose tissue. This method was applied to the monitoring of amino acids dynamics in mice adipose tissue in near real time (22 s). The LOD obtained for taurine (which represents taurine concentration out of the probe and before its derivatization with 4-fluoro-7-nitrobenzofurazan (NBD-F)) was 2.7 μM. To demonstrate the potential of the developed strategy, in vivo changes were assessed after administering an insulin stimulation. In this way, it could be observed that taurine, alanine and valine levels raised within the first 5 min after insulin delivering and reaching a second baseline corresponding to higher amounts of amino acids than those present initially.

In addition to the two aforementioned works, in which CE strategies were employed to measure the level of taurine in biological fluids, there are other three articles in which taurine, among other compounds, is analyzed by CE. On the one hand, the use of a CE-MS platform for the metabolomics analysis of saliva samples from patients with oral squamous cell carcinoma and healthy controls enabled to propose taurine along with other 24 metabolites as oral cancer specific markers, and, on the other hand, MEKC-LIF was employed to obtain the amino acids profiles in the culture media used in embryo cultivation after in vitro fertilization in order to assess the embryo viability in assisted reproduction. In a first attempt, Celá et al., used the transverse diffusion of laminar flow profiles (TDLFP) methodology to achieve the on-line derivatization of amino acids with NDA. Using a BGE composed of 35 mM borate, 55 mM SDS, 2.7 M urea, 1 mM BIS-TRIS propane and 23 mM NaOH, the derivatives of 18 protein amino acids, taurine, cysteine and the dipeptide Ala–Gln were baseline resolved in 50 min. However, due to the limitations of this methodology, the method was subsequently modified to provide better separation conditions in terms of analysis time. First, the BGE was changed to avoid urea and to decrease the pH of 9.8 since both effects contribute to the dissolution of carbon dioxide giving rise to a modification of the BGE ionic strength that originated increased values for migration times due to the alteration of the micelle-analyte distribution equilibrium. The optimum BGE, based on the use of 73 mM SDS, 6.7% 1-propanol (v/v) + 0.5 mM HP-β-CD +135 mM boric acid/NaOH (pH 9.0), enabled the baseline resolution of the analytes in 46 min. Regarding the on-line derivatization, it was accomplished using electrochemically mediated microanalysis (EMMA) which improved the LODs (a LOD of 12 nM was reached for taurine). Thus, the improved MEKC-LIF methodology was applied to the noninvasive analysis of human embryos to establish if there was a correlation between the potential of embryos to be developed and the variation in amino acids levels. Statistical analysis of the data showed that the discrimination between successfully and unsuccessfully implanted embryos was partial probably because of the small number of statistically significant samples.

Homocysteine is a low molecular weight aminothiol of high relevance in biological processes since higher levels in plasma or serum have been related with different pathologies such as coronary artery disease, diabetes renal insufficiency or Alzheimer’s disease, among others. Along with homocysteine, it is also relevant to take into consideration the proteinogenic amino acid cysteine because the ratio cysteine/homocysteine reflects the bioavailability of homocysteine. Ivanov et al., established a CE-UV approach based on the use of 1,1’-thiocarbonyldiimidazole (TCDI) as derivatizing reagent for determining the levels of homocysteine and cysteine in plasma. In this work, an electrokinetic injection with pH mediated stacking was employed, and a LOD of 0.8 μM for homocysteine was achieved. Subsequently, the authors improved the methodology introducing several modifications which enabled to achieve a LOD of 0.2 μM. These modifications included the use of a liquid–liquid extraction with chloroform-ACN to purify the sample and determine homocysteine and cysteine levels in urine (the previous approach was not suitable for determining both analytes in matrices like urine in which salt levels vary considerable), a different composition of the running buffer (see Table 2), and an in-capillary preconcentration strategy based on the combination of field amplified sample stacking and pH mediated stacking. In this way, homocysteine and cysteine levels were determined in plasma and urine samples from healthy subjects and patients with kidney disorders (see Figure 5), observing a decrease in the homocysteine levels in urine from patients with kidney disorders. Other detection modes different from UV were also hyphenated with CE to perform the determination of homocysteine in biological samples. Thus, LIF was used as detection mode in a high-sensitive CE method developed for chemical cytometry of homocysteine and other thiol compounds (cysteine, glutathione and γ-glutamylcysteine) within human colon cancer (HCT-29) and breast (MCF-10A) single cells. Here, 1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiazaza-s-indacene (TMAPB-o-M) was employed as labeling reagent in a postcolumn sheath flow cuvette. Conversely, MS detection has demonstrated to be a powerful
Since the levels of these compounds are altered in plasma of amyotrophic lateral sclerosis (ALS) patients, their determination is relevant because they could be pointed out as potential biomarkers of this disease. Prior to analyze these compounds by CE-MS\(^2\), the protein depletion of plasma samples was performed using DTT and cold acetone, and IAA was added to the sample to avoid the oxidation of thiol compounds by CE-MS\(^2\), the protein depletion of plasma samples was performed using DTT and cold acetone, and IAA was added to the sample to avoid the oxidation of thiol groups. After validating the CE-MS\(^2\) methodology, it was possible to carry out the quantification of 4-hydroxyproline and prolyl dipeptides in urine samples. The developed methodology included the treatment of urine samples with OPA, which blocking primary amines, and the derivatization of the secondary amines with 4-fluoro-7-nitro-2,1,3 benzoxadiazole. Then, using a mixture of borate, cholate and deoxycholate at 40 mM each (pH 9.2) as running buffer, proline, 4-hydroxyproline and 4 propyl dipeptides were separated in just 30 s achieving LODs at the nM level. An interesting investigation accomplished by Hložek et al. demonstrated the suitability of CE for clinical toxicology laboratory diagnostic. High anion gap metabolic acidosis habitually complicates paracetamol poisoning and is normally attributed to lactic acidosis, renal failure or compromised hepatic function. But, it can also be due to the accumulation of pyroglutamic acid (or 5-oxoproline). Therefore, 5-oxoprolinemia could be considered to diagnose patients with acidosis after acute paracetamol overdose. Then, to determine paracetamol and pyroglutamic acid levels in serum samples from patients after ingestion of paracetamol, these authors developed a CE-UV method based on a simple sample treatment and the employ of 40 mM CHES/sodium hydroxide at pH 10.2 as BGE. By using this methodology, it was possible to carry out the quantification of pyroglutamic acid in case of paracetamol overdose. Finally, Forteschi et al., designed an isotope dilution CE-MS\(^2\) method to detect, for the first time, betaine, choline and dimethylglycine simultaneously in plasma samples, since they provide relevant information related to methyl groups flow in very relevant biological processes, particularly in folate deficiency stages. Under the conditions detailed in Table 2, the compounds were detected in 22 min achieving LODs of 0.43, 0.62 and 0.31 \(\mu\text{M}\) for choline, betaine and dimethylglycine, respectively. Based on the concentration of the three analytes measured by the application of the developed CE-MS\(^2\), it was possible to find differences between the plasma samples of healthy controls and patients with chronic kidney disease.

During the period of time covered in this review just a research work described the enantioselective determination of a chiral NPPA in a biological sample. Namely, Sánchez-López et al., developed a CE-MS\(^2\) methodology for the simultaneous enantioseparation of all the chiral constituents of the phenylalanine-tyrosine metabolic pathway; the protein amino acids phenylalanine and tyrosine, the catecholamines...
dopamine, norepinephrine and epinephrine and the NPPA 3,4-dihydroxyphenylalanine (DOPA)).[41] The method, consisting of the use of 180 mM methyl-β-cyclodextrin plus 40 mM 2-hydroxypropyl-β-CD in 2 M formic acid (pH 1.2) as BGE and a large volume sample stacking as in-capillary preconcentration step, enabled for the first time, the simultaneous enantiomeric separation of all the chiral compounds involved in this metabolic pathway, in 90 min with LODs from 40 to 150 nM. The usefulness of the developed method was demonstrated through the successful analysis of some of the compounds investigated in rat plasma samples (Figure 6).

Concluding remarks

This article reviews the works dealing with the determination of nonprotein amino acids in food and biological samples published from 2015 to 2018. Nonprotein amino acids are related with the quality and safety of food and they have also been considered as biomarkers of some pathologies. For this reason, the determination of these compounds in real samples is relevant. During the period of time covered by this review, the analysis of nonprotein amino acids by CE in food and biological samples was mainly achieved using MEKC and CZE modes. The most employed detection systems include direct and indirect UV absorption, LIF, capacitively coupled contactless conductivity, electrochemical detection and mass spectrometry. In general, the absence of chromophore and fluorophore groups in nonprotein amino acids required a derivatization step in order to enable their determination or to improve the sensitivity. Thus, many labeling reagents were employed such as NDA, NBD-F, FITC, FMOC-CI, AQC, DNS-CI or OPA. CE and MCE are attractive strategies providing good sensitivity and selectivity to perform the analysis of a broad range of complex food samples such as beverages, vegetables, fermented products or shellfish and biological samples such as urine, plasma, serum, tear fluid and saliva. Moreover, the chiral separation of nonprotein amino acids in food and biological samples has also demonstrated to provide relevant information of these samples, but the number of publications in the last years is scarce. In fact, the enantiomeric separation of nonprotein amino acids (through the addition of a chiral selector in the background electrolyte in EKC or the use of a chiral stationary phase in CEC) provides interesting information about food quality and safety or about the diagnosis or treatment of some pathologies. Since the function of numerous nonprotein amino acids has not been investigated yet and their presence in real samples is still unknown, the interest of the development of analytical methodologies capable to analyze these compounds in real samples is of high interest for scientists.

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