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Chiral analysis of aliphatic short chain organic acids by capillary electrophoresis

Short chain organic acids play an important role in different areas such as biochemistry, clinical chemistry, or the food industry. The enantiomeric ratio of chiral metabolites is an important parameter for the understanding of metabolic processes and in many cases it can serve diagnostic purposes. On the other hand, the presence of racemates in food products could indicate the use of organic acids as additives; this is not always permitted and needs to be controlled. The short chain of these acids makes difficult the three point interaction generally accepted as necessary for chiral recognition. Relatively recent publications have demonstrated the feasibility of their direct chiral separation in capillary electrophoresis by various techniques utilizing exchange capillary electrophoresis, macrocyclic antibiotics, cyclodextrins, ion-pair method, and transition metal complexes. The present article describes existing methods and strategies proposed to advance these areas.

Key Words: Anions; Organic acids; Carboxylic acids; Cyclodextrins; Enantiomers; Capillary electrophoresis

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1 Introduction

The impact of chirality in almost any process is well recognized. Different authors begin their reviews on the subject with the use of phrases such as: The number of scientific contributions dealing with one or other aspects of enantioseparation is literally exploding [1]. Capillary electrophoresis (CE) is demonstrably a good choice for enantiomeric resolutions using chiral selectors in the separation buffer which can provide very simple and automated method development. With this technology it is simple to construct and modify a chiral environment, which is the key to resolving these complex isomers. Another advantage of CE, mainly in body fluids analysis, is the small sample volume needed for injection and the reduced sample pretreatment. This point minimizes costs, errors, and artifacts. Most of the chromatographic methods proposed for organic acid analysis require different derivatization conditions, depending on the organic acid analyzed, because some of them undergo racemization, cyclization, or other effects during the sample treatment [2–4].

Comprehensive information on chiral CE analysis can be found in the literature [5–18]. However, most of the compounds described in these reviews have aromatic rings. Applications of the chiral separation of short chain organic acids in different matrices are very limited, mainly because the steric factors providing the basis for chiral recognition are very limited in these compounds.

Most biochemical reactions show enantiomeric selectivity and different enantiomers of the same compound can activate different metabolic pathways [19]. This is the reason for the interest in chiral analysis in biochemistry and clinical chemistry. On the other hand, the enantiomeric ratio of short chain organic acids can also be employed for detecting additives and different processes in food.

By way of example, some of the more characteristic acids and the differences between their enantiomeric forms will be summarized below.

Lactic acid is the optically active organic acid with the shortest chain and has two enantiomeric forms. It can be present in various organisms as a product of carbohydrate metabolism. L-Lactic acid originates by anaerobic glycolysis from its precursor pyruvic acid. This reaction is catalyzed by the enzyme lactate dehydrogenase having NADH + H^+ as a co-factor. Lactic acid may be elevated in urine of patients with primary lactic acidosis, hypo perfusion, shock, hypoxia, and other conditions. D-Lactic acid

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Abbreviations: ANSDA, 7-amino-1,3-naphthalenedisulfonic acid; ADAM, 9-anthryldiazomethane; BMF, 5-bromomethylfluorescein; AF, 4-aminofluorescein; BMMC, 4-bromomethyl-7-methoxycoumarin; PMC, polymethine cyanine; AAF, 5-(aminoacetamido)fluorescein; PDAM, 1-phenyldiazomethane; SA, sulfanilic acid; 2-HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; DM-β-CD, dimethyl-β-cyclodextrin; TM-β-CD, trimethyl-β-cyclodextrin.

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is not a human metabolite. It can be produced by the bacterial flora of the gut in pathological conditions, absorbed into the blood, and transported to different fluids such as urine. D-Lactic acid cannot be metabolized by mammals. In children with small short bowel and acidosis, the common intestinal flora consisting mainly of lactobacilli abundantly produces D-lactic acid from easily fermentable carbohydrates. Therefore, these bacteria directly cause shifts of bicarbonate, pH, and base excess and indirectly cause shifts of the anion gap, as well as hyperventilation [20]. Early recognition and appropriate management is essential to avoid morbidity secondary to this complication of short bowel syndrome. The lactate assays routinely used only measure L-lactate.

2-Hydroxybutyric, 2-hydroxyglutaric, and glyceric acids play a similar role in the diagnosis of congenital disorders [21, 22]. Recent descriptions of diseases producing abnormal chiral metabolites have created the need for individual enantiomer analysis. Due to the lack of specific clinical symptoms in these diseases, enantiomeric analysis is vital for diagnosis enabling prompt therapeutic intervention [21].

In a very different area, lactic acid is a major organic acid in sake, as well as in wine, and is thought to have a great influence on the taste. Whereas naturally occurring lactic acid bacteria are used in the traditional brewing of sake, lactic acid addition has recently been predominant in order to simplify sake brewing [23]. Although rigorous sensory studies are still needed, authors claim that D-lactic acid in water has a different sour taste from L-lactic acid.

L-Tartaric acid is distributed in plants, especially grapes. D- and *meso*-tartaric acids have not been found to occur in nature, but they can be used in food products provided that they are declared on the label. Therefore, the enantiomeric determination of tartaric acid as a food additive is very important for the safety and quality control of food products [24].

As in the other cases, malic acid exists in nature in only one enantiomeric form, viz. L-malic acid. In some countries, the use of synthetic racemic mixtures of malic acid is legally allowed in food products with a declaration of the addition on the label. Therefore the enantiomeric determination of malic acid as a food additive is important in safety and quality control of food products [25].

This paper will be devoted mainly to the chiral separation of non-aromatic short chain organic acids. Amino acids, profusely studied, have not been included. However, they are mentioned when their separation can add some knowledge to the separation of organic acids, e.g. in the case of α-hydroxy acids.

2 Indirect separation by derivatization to diastereomers

The diastereomeric derivatizing method is described as one of the most effective procedures for small molecules, and for aliphatic and non-chromophoric compounds [26]. Nevertheless, derivatization of the carboxylic group is complicated, as it frequently requires several steps and racemization can take place during the derivatization process.

Although improved detectability is an important reason to perform a derivatization procedure, there can be other reasons as well in chiral separation. These goals include (i) transformation into diastereomers to obtain separation without chiral selectors, (ii) provision of more points for steric interactions with the chiral selector. That is the reason why pre-column derivatization is almost always employed in this case. Only labeling reactions, i.e. with a covalent bond formed between the analyte and the labeling reagent, will be considered under this point. The derivatizing agents used for the carboxylic group in CE methods have been collected in **Table 1**.

Fluorescence labeling of carboxylic acids with alkyl halides usually requires the use of organic solvents and phase transfer, because the reactivity in aqueous solution is very low [27]. Alkyl bromides, such as phenacyl bromide and 4-bromomethyl-7-methoxycoumarin, are commonly applied. A disadvantage of these reagents is their poor selectivity [28]. In indirect derivatization procedures, the carboxylic acid function is first activated by a carbodiimide, e.g. dicyclohexyl-carbodiimide, or the water soluble *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride, or 2-bromo-1-methylpyridinium iodide and is subsequently reacted with a fluorescent alcohol or amine [29].

Table 1. Main derivatization reagents for carboxylic acids in capillary electrophoresis.

Reagent	CE mode – detection	Compound	Ref.
AAF	MECC-LIF	Phenoxy herbicides	[56]
	CZE-UV	Acidic monosaccharides	[30]
PDAM	CZE-LIF	Dicarboxylic acids	[34]
SA	CZE-UV	Acidic monosaccharides	[30]
ADAM	CZE-LIF	Carnitines and acyl-carnitines	[57, 58]
AF	CZE-LIF	Carboxylic acids	[35]
ANDSA	CZE-UV/LIF	Phenoxy herbicides	[31, 59]
	CZE-LIF	Insecticides	[60]
BMF	CZE-LIF	Fatty acids	[61]
BMMC	CZE-LIF	Resin acids	[62]
PMC	CZE-NIR-LIF	Fatty acids	[63]

Table 2. Direct chiral separations of non-aromatic-short-chain organic acids by CE.

Analyte	Sample	CE mode – detection	Chiral selector	Ref.
DL-Malic acid	Apple juice	CZE-UV	Cu^{2+}-L-tartaric acid complex	[25]
DL-Tartaric acid	Food products	CZE-UV	Cu^{2+}-D-quinic acid complex	[24]
2-Hydroxybutyric acid	Standards	CZE-UV	α-CD, 2-HP-β-CD	[45]
2-Hydroxyisocaproic acid	Standards	CZE-UV	α-CD, γ-CD, 2-HP-α-CD, 2-HP-β-CD, DM-β-CD, TM-β-CD	[45]
2-Hydroxy-3-methylbutyric acid	Standards	CZE-UV	α-CD, 2-HP-α-CD, 2-HP-β-CD, DM-β-CD	[45]
DL-Lactic acid	Serum, Amniotic fluid, Cerebrospinal fluid, Urine	CZE-UV	2-HP-β-CD	[47]
	Sake	CZE-UV	2-HP-β-CD	[23]
	Food products	CZE-UV	2-HP-β-CD	[46, 64]

This reaction principle has been used for bigger compounds than short chain organic acids, e.g. phenoxy acid herbicides, acidic monosaccharides, sialogangglosides with 7-aminonaphthalene-1,3-disulfonic acid (7-ANDA) [30–33]. The labeling of a dicarboxylic acid with 1-pyrenyldiazomethane (PDAM) can be performed without the addition of carbodiimide [34]. Due to steric hindrance only the 1-pyrenylmethyl monoester can be formed.

Kibler et al. derivatized short chain organic acids (C_5–C_9) in aqueous solution with 4-aminofluorescein as fluorophore and dicyclohexylcarbodiimide as activating agent and the procedure includes a phase-transfer and re-extraction process [35]. For all these compounds separation in CE has been reported. Although there are no chiral compounds among them, the resulting structures present aromatic rings and, if a chiral centre exists, they could be separated following the typical strategies.

3 Direct separation

Direct CE separations described have been summarized in **Table 2**. It is necessary to point out that, although much more elegant, these methodologies suffer from the problem of limited sensitivity in UV detection, because short chain organic acids do not contain a chromophore group, and the problem is acerbated by the UV-absorption of the added selector.

3.1 Ligand-exchange capillary electrophoresis

The separation mechanism of ligand-exchange CE is based on the formation of diastereomeric ternary mixed metal complexes between the chiral selector ligand and the analytes. Resolution is due to the difference in complex stability constants of the two mixed complexes with the analyte enantiomers [36, 37].

The method, first applied in HPLC, was shown to be also applicable to the chiral separation of aromatic hydroxy acids using copper(II) complexes of L-hydroxyproline or aspartame as chiral selectors [38]. It was then applied to capillary electrophoresis using an on-line combination of two capillaries filled with either chiral selective or achiral background electrolytes. Thus, they provided optimum chiral selectivity and optimum detection sensitivity. Yet again the analytes were aromatic compounds.

More recently, ligand-exchange capillary electrophoresis was also applied to 11 aromatic α-hydroxy acids and different degrees of resolution were obtained. Aliphatic α-hydroxy acids did not show resolution, according to the authors. Only at high selector concentration were some aliphatic α-hydroxy acids partially resolved, but serious detection problems occurred [36].

Later on, malic acid was enantioseparated by ligand exchange CE using copper(II) L-tartrate as a chiral selector. The running conditions for optimum separation of malic acid were found to be 1 mM copper(II) sulfate –1 mM L-tartrate (pH 5.1) with an effective voltage of –20 kV at 30°C, using direct detection at 280 nm. With this system D- and L-malic acid in apple juice were analyzed successfully. Resolution of the racemic was approximately 4, as can be seen in **Figure 1** [25].

Kodama et al. used various selectors other than copper(II) amino acid complex, such as copper (II)-D quinic acid, for α-hydroxy acids and described the chiral resolution of tartaric acid in food products [24]. Factors affecting chiral resolution, migration time, and peak area of tartaric acid were studied. The running conditions for optimum separation of tartaric acid were found to be 1 mM copper(II) sulfate–10 mM d-quinic acid (pH 5.0) with an effective voltage of –15 kV at 30°C, using direct detection at 250 nm, and resolution of racemic tartaric acid was approximately 1.3. With this system, chiral resolution of dl-tartaric acid in food products, such as grape juice, red wine, sake, and

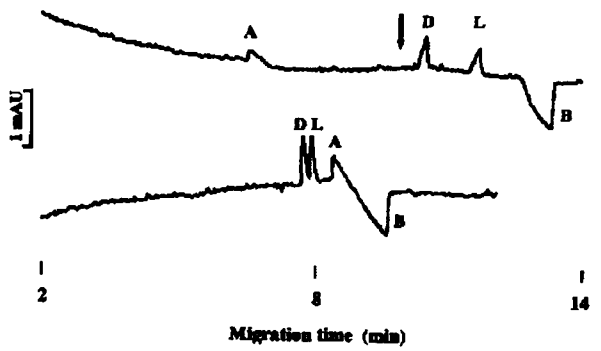

Figure 1. Electropherograms of racemic malic acid. The BGE is (pH 5.1) 1 mM copper(II) sulfate containing 1 mM (upper) or 2 mM (lower) L-tartaric acid. A and B are unknown peaks. Reproduced with permission [25].

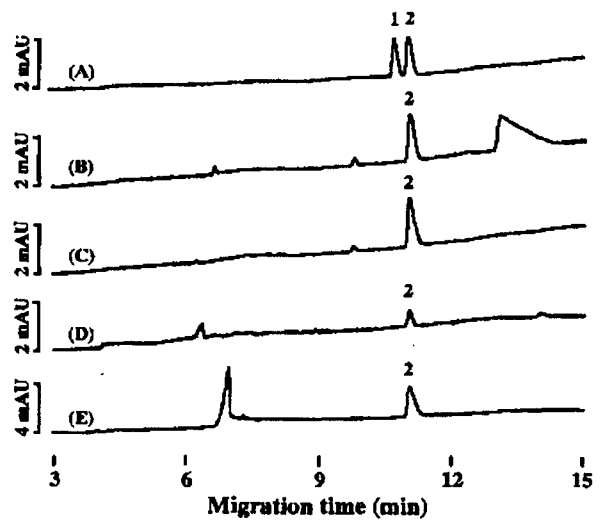

Figure 2. Electropherograms of grape juice, red wine, sake, and tablet candy. (A) standards solution (1 mM racemic tartaric acid); (B) grape juice diluted 10-fold; (C) red wine diluted 30-fold; (D) sake diluted threefold; (E) tablet candy diluted 50-fold; (1) D-tartaric acid; (2) L-tartaric acid. Reproduced with permission [24].

tablet candy, was conducted successfully, as can be seen in **Figure 2**.

A new chiral selector, *N*-(2-hydroxy-octyl)-L-4-hydroxyproline/Cu(II) complex, was used for the direct separation of underivatized α-amino acids [39]. The possible structure of the copper complex proposed by the authors might make it useful for α-hydroxy acid analysis, as was described above for other ligands.

3.2 Macrocyclic antibiotics

The macrocyclic antibiotics have been shown to exhibit powerful enantioselectivity towards numerous compounds [40]. There are a number of ways that one can alter enantioselectivity in the macrocyclic antibiotic-based separation schemes. They can be enantioselective for anionic compounds using the glycopeptides. Within a given class of antibiotics such as the glycopeptides, enantioselectivity may also be altered by the use of micelles, uncoated vs. coated capillaries, or manipulation of operating parameters such as pH or organic modifiers.

By use of dilute solutions of ristocetin A a number of carboxylic-acid-containing compounds not effectively separated by other glycopeptides have been separated. These compounds include 2-bromo-3-methylbutyric acid [41]. Nevertheless, they exhibit some drawbacks such as strong absorption in the UV wavelengths and adsorption on the capillary wall. The use of coated capillaries with either counter current or partial filling-counter current methods can be successfully applied [42].

Vancomycin and teicoplanin were evaluated for the analysis of UV non-absorbing compounds such as aspartic and glutamic acid enantiomers. Vancomycin was found to be the most useful chiral selector. The optimized method (10 mM sorbic/histidine, pH 5, and 10 mM of vancomycin), with indirect detection at 254 nm, was used for the analysis of real samples such as tooth dentine and beer [43]. Although they are dicarboxylic aliphatic amino acids, the method could be tested for other carboxylic acids not having an amino group.

3.3 Cyclodextrins

The ability of cyclodextrins to discriminate on the basis of steric differences may be modified and enhanced by chemical derivatization. β-Cyclodextrin, the least soluble native cyclodextrin, is usually utilized for the derivatization. Uncharged groups, like methyl, hydroxyethyl, hydroxypropyl, carboxymethyl, and acetyl, introduce additional interaction points in the molecule and modify both the depth of the cavity and the free cross-section of its smaller opening [44].

Direct chiral resolution of the aliphatic α-hydroxy acids: 2-hydroxybutyric, 2-hydroxy-3-methylbutyric, and 2-hydroxyisocaproic acids was developed by Kodama et al. using 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD). The resolution for lactic acid enantiomers in the assayed conditions was only 1.02. The separation was performed in a poly(vinyl alcohol)-coated capillary with electroosmotic flow almost suppressed using 60 mM phosphate buffer (pH 6.0) and −30 kV applied voltage. They found that the resolution and migration time of all the α-hydroxy acids increased with increasing amount of 2HP-β-CD used. It was considered that the increased migration times resulted both from complexation and from the increased viscosity of the buffer due to the high cyclodextrin concentration. For those acids which are commercially available as two separate isomers (lactic and 2-hydroxyisocaproic),

the L-isomer was found to move faster than the D-isomer and, according to the authors, this means that the D-isomer formed a stronger complex with 2HP-β-CD. It was also found that an increase in the bulkiness of the alkyl group in these acids lowered the amount of 2HP-β-CD required for chiral separation [45].

Afterwards, chiral resolution of native DL-lactic acid in food products was performed by capillary electrophoresis using 2HP-β-CD as a chiral selector. Various factors affecting chiral resolution, migration time, and peak area of lactic acid were studied. The running conditions for optimum separation of lactic acid were found to be 90 mM phosphate buffer (pH 6.0) containing 240 mM 2HP-β-CD with an effective voltage of −30 kV at 16°C, using direct detection at 200 nm. In order to enhance the sensitivity, sample injection was performed at a pressure of 5 kPa for 200 s. On-line sample concentration was accomplished by sample stacking. Yoghurt and sake were analyzed successfully [46].

Optimization of the separation conditions of the two optical isomers of lactic acid in standards and body fluids by a factorial design has also been reported [47]. First different chiral selectors were systematically investigated and then three quantitative factors (cyclodextrin concentration, and background buffer pH and concentration) were evaluated by means of a mathematical experimental design. Optimal conditions for obtaining a resolution higher than 1.5 were: phosphate buffer 200 mM at pH = 6.00 with 413 mM 2HP-β-CD, −20 kV of applied potential and polyacrylamide coated capillary.

The method was applied to the identification of both isomers in body fluids such as, amniotic fluid, plasma, cerebrospinal fluid, and urine and it has been validated for quantification in amniotic fluid and plasma. The corresponding electropherograms can be seen in **Figure 3** and **Figure 4**, respectively. Samples were just centrifuged and diluted (1 : 4) prior to the analysis.

Introduction of ionizable groups, like carboxyl, phosphate, alkyl sulfate, or methylamino groups, into the cyclodextrin changes the dimensions of the cavity and adds groups capable of coulombic interactions. The charged cyclodextrins are highly efficient in the separation of enantiomers of the opposite charge.

Cationic and amphoteric β-cyclodextrins have been used as chiral selectors for the enantiomeric separation of acidic racemates [18], but all the compounds described in the paper present one aromatic ring.

During our work with lactic acid enantiomers, 6-monoamino-6-deoxy-β-CD was also tested [47]. It provided baseline resolution for both isomers, but 2HP-β-CD was chosen for further optimization and validation because it is more easily available.

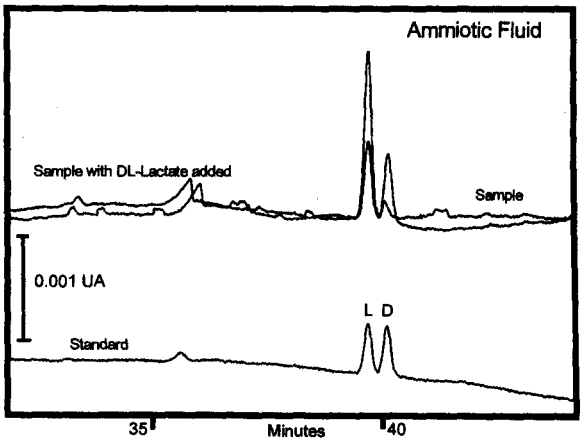

Figure 3. DL-Lactic acid enantioseparation in amniotic fluid. The figure shows three overlaid electropherograms corresponding to standards, sample and sample with standards coinjected. BGE is 200 mM phosphate buffer at pH 6.00 with 413 mM of 2-HP-β-CD as chiral selector added. Reproduced with permission [47].

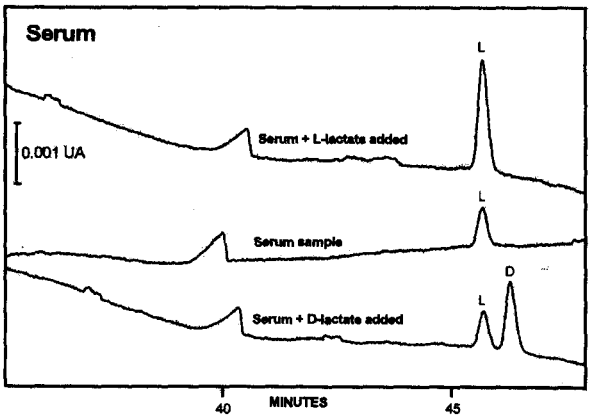

Figure 4. DL-Lactic acid enantioseparation in serum. The figure shows three overlaid electropherograms corresponding to standards, sample, and sample with standards coinjected. BGE is 200 mM phosphate buffer at pH 6.00 with 413 mM of 2-HP-β-CD as chiral selector added.

3.4 Ion-pair method

Following the concept of Schurig et al. of unified enantioselective chromatography [48], the methods used in HPLC could also be applied in CE. In this sense quinine and quinidine are effective counter-ions for the separation of enantiomers of carboxylic acids. The chiral counterions are generally used with organic mobile phases to promote a high degree of ion-pair formation and indirect UV detection [49]. A counter-current flow method with quinine-free buffer in the detection window has also been applied [50]. However, in all cases only organic acids with aromatic rings or derivatized amino acids with free carboxylic group have been tested.

Interactions of L-malic acid with open chain polyammonium cations have been studied [51] and they could serve as a basis for future developments in this area.

3.5 Transition metal complexes

A significant improvement of separation selectivity can be achieved in capillary electrophoresis by exploiting the formation of complexes which modify the analyte electrophoretic mobilities. Many organic compounds with electron-donor properties can form weak charge-transfer complexes with suitable metals. These types of complexing equilibria can be exploited in the separation of both species involved in complex formation: metals and organic compounds.

Unlike much larger resolving agents used for separations of chiral organic molecules, small, highly charged organic salts containing multiple asymmetric carbons and possessing high aqueous solubility would appear to act most effectively as chiral resolving agent for transition metal complexes [52–54]. Antimonyl-d-tartrate has been employed for the separation of a racemic mixture of $[Ru(phen)_3]^{2+}$ and for $[Ni(phen)_3]^{2+}$. The reaction could probably be employed in the opposite sense.

The selective complex formation of molybdate (MoO_4^{2-}) with the enantiomers of lactic, 2-methylbutyric, and some aromatic α-hydroxy acids has been studied [55] and it could serve for future developments in capillary electrophoresis separation of these compounds.

4 Conclusion

Direct chiral resolution of short chain organic acids has been shown to be possible by CE, not only for standards, but also for relatively complicated samples as food products or body fluids. As in many other areas of this subject, knowledge needs to be systematized and further investigations must be done to obtain more general methods.

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[JSS 1313]