Analysis of low-molecular-mass organic acids using capillary zone electrophoresis–electrospray ionization mass spectrometry

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Abstract

A capillary zone electrophoresis–electrospray ionization-mass spectrometry (CZE–ESI-MS) method was developed to facilitate identification and determination of eleven low-molecular-mass (LMW) organic acids (i.e. oxalic, lactic, malonic, maleic, citric, tartaric, adipic, glutaric, gluconic, isosaccharinic and succinic acid) in different sample matrices. This CZE method was adapted to suit MS conditions. Sheath liquid, sheath flow and MS parameters were optimized to achieve high mass spectrometric sensitivity. The CZE–ESI-MS procedure showed good sensitivity (limit of detection of <0.05–0.7 mg/l for all acids), linearity (r^2=0.9925–0.9998) and reproducibility (2.09–5.34% RSD). The applicability of the CZE–ESI-MS was demonstrated on LMW organic acids in an ale sample. In addition the (here presented) method also provided quantification of fumaric, galacturonic and 2-ketoglutaric acid with high sensitivity.
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Keywords: Mass spectrometry; Organic acids

1. Introduction

Low-molecular-mass (LMW) organic acids are ubiquitous in environmental samples and are found in the concentration range from low µg/l to mg/l. In addition, LMW organic acids play an important role in biochemistry and food science. To monitor LMW organic acids, robust and sensitive analytical techniques are required. Today, gas chromatography–mass spectrometry (GC–MS) is the most common and sensitive technique for identifying LMW organic acids. However, preparation of the samples can be difficult, time-consuming and the acids often need to be derivatized before injection on the GC column.

When using derivatization procedures, pure standards must be available since the recovery need to be measured. Any sample pretreatment may involve risk for sample contamination and alteration of the content.

Capillary zone electrophoresis (CZE) is a relatively new separation technique that can achieve high separation efficiency. A large number of CZE methods have been developed for the analysis of LMW organic acids using UV absorbance detection [1,2]. Most methods are based on adding compounds with UV absorbing groups to the running electrolyte, thus making indirect UV absorbance detection possible. Often the electroosmotic flow is directed towards the anode through addition of cationic surfactants to the electrolyte. However, identification is a problem in complex matrices. Identification is restricted to spik-

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ing when using indirect UV absorbance detection but the analytes of interest can comigrate with matrix compounds. The development of CZE with electrospray ionization-mass spectrometry (CZE–ESI-MS) procedures is expected to improve identification.

CZE–ESI-MS has rapidly developed into a powerful analytical tool for charged species including haloacetic acids [3], amino acids [4], metal species [5,6], peptides and proteins [7] and has been extensively reviewed in the recent literature [8,9]. While CZE offers fast analysis and efficient resolution, MS provides high selectivity and sensitivity. With respect to LMW acids, to my knowledge there is only one publication concerning CZE–ESI-MS applications [10]. In that study succinic, maleic, malonic and glutaric acids were separated in an electrolyte consisting of pyromellitic acid, naphthalenedisulfonate, methanol together with diethylenetriamine as osmotic flow modifier. In this paper a CZE–ESI-MS method for the determination of eleven LMW acids, i.e. oxalic, lactic, malonic, succinic, maleic, glutaric, adipic, citric, tartaric, gluconic and isosaccharinic acid (ISA), is presented. Good separation was accomplished through adapting an existing CZE method to MS conditions [11]. Sheath liquid and flow together with MS parameters were optimized to achieve the highest sensitivity. Finally, the analysis of LMW organic acids found in ale, is to demonstrate the applicability of CZE–ESI-MS on this group of analytes in complex matrices.

2. Experimental

2.1. Chemicals

Ultra pure water (18 MΩ cm^{-1}) has been used throughout all experiments. Sodium hydroxide (analytical reagent grade), tris(hydroxymethyl)-aminomethane (Tris; analytical reagent grade), disodiumoxalate (analytical grade), gluconic acid (50%), malonic acid (>99%), maleic acid (>99%), succinic acid (analytical reagent grade), glutaric acid (99%), adipic acid (>99%), citric acid (analytical reagent grade), methanol and acetonitrile were from Merck (Darmstadt, Germany). 1,2,4-Benzenetricarboxylic acid (trimellitic acid; TMA: 99%), hexadimethrine bromide, lactic acid (85%) and L-tartaric

acid (99.5%) were from Aldrich (Steinheim, Germany) and 2-propanol was purchased from Labscan (Dublin, Ireland). Sodium α-D-isosaccharinate was prepared according to Glaus et al. [12].

2.2. Samples and background electrolyte

During optimization a sample consisting of 30 mg/l of each acid was prepared fresh each morning from 1000 mg/l stock solutions. Stock solutions were kept dark and cool (4 °C). Before evaluation of linearity, repeatability and limit of detection all stock solutions were newly prepared.

An earlier developed CZE–UV procedure for the analysis of LMW organic acids was adapted to CZE–ESI-MS conditions. The background electrolyte (BGE) from the CZE–UV procedure consisted of 2 m*M* TMA, 5 m*M* Tris, 0.6 m*M* Ca(OH)$_2$ and 0.6 m*M* of tetradecyltrimethylammonium bromide (TTAB), at pH 8.5 [11]. The mass-flow-rate of disturbing background components was kept at a minimum to enhance the sensitivity for the target analytes [13]. Accordingly, sodium hydroxide was replaced by ammonium hydroxide and calcium hydroxide was removed to avoid the presence of adduct forming ions, such as sodium and calcium, in the ion source [14]. Calcium hydroxide had earlier been used to enhance separation, but the use of a longer CZE–MS capillary circumvented this problem. However, the migration order of the LMW organic acids was altered. The surfactant TTAB was removed to avoid a decrease in sensitivity, which can be seen over time due to adsorption of surfactant to the counter electrode [15]. Instead hexadimethrine bromide (polybren) was used, which main advantage is that it can be flushed through the capillary before the BGE [16]. This yields a semipermanent wall coating and the possibility to use a surfactant-free electrolyte, which diminishes interference of the surfactant on the MS measurements. The resulting BGE consisted of 2 m*M* TMA and 5 m*M* TRIS. The pH was set to 8.5 by adding 1 *M* ammonium hydroxide. The pH of the electrolyte was measured with a 744 pH meter coupled to a combination glass electrode, both from Metrohm (Herisau, Switzerland). The pH meter was calibrated daily between pH 7.0–10.0 with fresh pH buffers from Scharlau (Barcelona, Spain).

2.3. CZE–ESI-MS instrumentation

A ^{3D}CE system with built-in diode-array detector, a 1100 series LC–MSD mass spectrometer, a G1603A CE–MS adapter kit, and a G1607 CE–ESI-MS kit (all Agilent Technologies, Waldbronn, Germany) were used for all CZE–ESI-MS measurements. Separations were carried out in a fused-silica capillary (Agilent Technologies) of total length of 103 cm×50 μm I.D. Before daily use and mounting in the nebulizer needle, the capillary was conditioned by flushing with water (5 min), 0.1 *M* HCl (3 min), water (5 min), 1 *M* NaOH (4 min), water (5 min), BGE (2 min), polybren (5 min) and BGE (10 min). Between injections the capillary was preconditioned for 3 min by flushing with BGE. Injection was made hydrostatically at 30 mbar for 10 s. The applied separation voltage was −17 kV (anode at the detection side) and the current decreased from −2.5 to −1.9 μA during one CZE run. The temperature of the capillary was 25 °C. An Agilent 1100 series pump equipped with a 1:100 splitter delivered 4 μl/min sheath liquid consisting of 0.25% (v/v) formic acid in 2-propanol–water (50:50, v/v). Sheath liquid was pumped outside of the CZE capillary to establish a stable electrical connection between the tip of the capillary and the ground. All analyses were made in the negative ion electrospray ionization mode and with selected ion monitoring (SIM). All ions were detected as their molecular ion ($[M-H]^-$). The analytes were divided into two SIM groups in order to enhance sensitivity for lactate, gluconate and ISA. These ions have a longer separation time, which affect the peak shape negatively and thereby the detection sensitivity. Table 1 summarizes the instrumental operating parameters of the

ESI-MS and the SIM parameters. Evaluation was based on three evaluation parameters, i.e. peak area, peak height and signal-to-noise ratio (S/N). Only peak area is discussed in the Results section when these three evaluation parameters coincide.

3. Results and discussion

3.1. Sheath liquid

To find the appropriate sheath liquid composition three organic solvents, methanol, acetonitrile and 2-propanol, and three volatile organic acids, formic, acetic and TMA, were evaluated. Preliminary methanol was used as the organic solvent, with which the three acids were tested. TMA performed poorly as a conductor, which resulted in fluctuations in current and an irregular baseline. Using acetic acid resulted in more stable conditions in the ion source but poor ionization for the majority of the target compounds. The use of formic acid resulted in both stable ion source conditions and adequate ionization. Formic acid was evaluated at three different concentrations; 0.25, 0.5 and 0.75% (v/v) and 0.25% (v/v) gave best results with regard to peak area, peak height and S/N. The selection of volatile organic acid and the optimization of the concentration was preliminary made with a methanol-based sheath liquid, however, the results were verified using a 2-propanol-based sheath liquid.

Evaluation of organic solvents showed that acetonitrile worked very badly because it caused a high background signal. Methanol on the other hand gave satisfying ionization, but 2-propanol gave outstanding results for both electrical connection and ioniza-

Table 1
ESI-MS operating parameters (after optimization) and SIM parameters

ESI-MS parameters		SIM parameters					
		Analyte	$[M-H]^-$	Dwell time (ms)	Analyte	$[M-H]^-$	Dwell time (ms)
Nebulizer gas pressure	12 p.s.i.	Oxalate	89	86	Malate	115	86
Nebulizer gas flow-rate	5 l/min	Lactate	89	232	Citrate	191	86
Sheath liquid flow-rate	4 μl/min	Malonate	103	86	Tartrate	149	86
Nebulizer gas temp.	190 °C	Succinate	117	86	Gluconate	195	232
Capillary voltage	3400 V	Glutarate	131	86	ISA	179	232
Fragmentation voltage	55 V	Adipate	145	86			

tion of the acids. This agrees well with findings of other authors, reporting the suitability of 2-propanol as organic solvent in the sheath liquid [3,14]. Three different concentrations of 2-propanol were evaluated, i.e. 30, 50 and 70% (v/v). Evaluating the peak area, height and S/N there seems to be no unity between the evaluated parameters. The peak area favored 70% of 2-propanol, but for peak height a 50% concentration was favored, while the S/N showed best results of 30%. A compromise using 50% (v/v) of 2-propanol was made.

Sheath liquid flow-rate was varied from 3 to 6 μl/min. At 3 μl/min, the small flow could not establish the electrical contact necessary for CZE separation. By increasing the flow-rate from 4 to 6 μl/min the signal intensity decreased, probably due to dilution of the sample. A sheath liquid consisting of 2-propanol–water (50:50, v/v) and 0.25% formic acid (v/v) at a flow of 4 μl/min was used throughout the experiments.

3.2. Fragmentation voltage

Fragmentation voltage was optimized within the range 25–65 V. The fragmentation voltage is applied on the exit end of the glass capillary that leads ions from the ion source (at atmospheric pressure) to the high vacuum regions in the MS. The applied voltage affects both the fragmentation of ions and the transport of these. The increase in signal intensity when the voltage was increased from 25 to 55 V was probably caused by a more efficient ion transport. A distinct drop in signal intensity at 65 V likely indicates an extensive fragmentation. The best results were seen for the fragmentation voltage of 55 V for all acids except the polyhydroxy acids (i.e. gluconic and ISA) which showed best response at the highest fragmentation voltage. For further analysis a fragmentation voltage of 55 V was chosen.

3.3. Nebulizer gas pressure

Nebulizer gas pressure was varied from 8 to 16 p.s.i. (1 p.s.i.=6894.76 Pa). The signal response increased with increasing gas pressure from 8 to 12 p.s.i.. All acids showed almost an equally good response to all nebulizer pressures between 12 and 15 p.s.i., but at 16 p.s.i. the signal decreased sig-

nificantly. An increase in nebulizer pressure induces an increase in the electroosmotic flow (EOF), due to a suction effect reported by several authors [17,18]. This results in a shorter time for the acids to separate on which gives changes in migration time and causes co-migration and different migration order. This was seen between 13 and 16 p.s.i., therefore 12 p.s.i. was chosen since it gave a good separation of ten of the eleven acids. To shorten the time for analysis a higher nebulizer gas pressure can be chosen.

3.4. Nebulizer gas flow-rate

The nebulizer gas flow-rate was varied from 4 to 11 l/min. A flow-rate of 4 l/min induces memory effects between the injections possibly caused by insufficient evaporation of sample from the capillary tip. The use of 4 l/min was therefore not suitable, even though it gave highest signal intensity. An increase in nebulizer gas flow-rate from 4 to 5 l/min leads to a decrease in signal intensity of 5–8%, probably due to a larger dispersion of the sample aerosol. A flow-rate of 5 l/min was chosen since it gave the best result for all acids.

3.5. Nebulizer gas temperature

The nebulizer gas temperature was varied from 110 to 200 °C. The signal intensity increased for almost all acids as the nebulizer gas temperature was increased. A distinct drop in intensity at 200 °C for most of the acids and the fact that citric acid had a maximum intensity at 170 °C, was the reason that 190 °C was used in the continuation of the optimization.

3.6. Capillary voltage

The capillary voltage was varied between 3300 and 3600 V. A slight increase in signal intensity at 3400 V was seen, but at 3500 V the conditions in the ion source seemed to be less stable (i.e. capillary current fluctuated). Even 3600 V was tested but discharges at the capillary end were observed as a blue glow. A capillary current of 3400 V was chosen.

3.7. Linearity, limit of detection and reproducibility

The quality of the developed procedure was evaluated by determining linearity, limit of detection (LOD) and reproducibility of area, migration time and corrected peak area (i.e. peak area/migration time) (Table 2). LOD was determined by an infinite dilution strategy. The concentration, at which the peak height of an acid coincides with the $3\times$ noise level, was considered to represent the LOD. During the determination of LODs it was evident that all acids had a different detector response. The procedure was more sensitive in the SIM mode than other CZE–ESI-MS and CZE–UV methods using the hydrodynamic injection mode [10,11,19]. Especially oxalic and maleic acid shows a good response to the optimized settings. Linearity of the method was evaluated in three sample matrices to study how the procedure responds to the presence of interfering ions. The different sample matrices were ultra-pure water where little or no disturbing ions are expected to be present, surface water from a forest pond and tap water where several inorganic and organic ions are present. Known aliquots of standard solutions were directly added to the different water types (that had been filtered). The concentration range in the ultra pure water was 0.25–80 mg/l, while it was 1–20 mg/l in the pond water and tap water. The linearity was overall good in all three matrices. The response was similar between ultra-pure water and tap water, but there was a larger concentration interval in the ultra-pure water determination. The response in surface water was slightly lower. This depends likely on a larger variation of organic ions and to a higher concentration of these in the pond water. When comparing reproducibility for peak area, migration time and corrected peak area it is obvious that most of the variation in the developed procedure arises from instabilities in the ion source. The low variation in the migration time, in comparison to the higher variation in peak area confirms this. In CZE–UV, where the detection is on-column, the reproducibility is considered good when it lies between 1 and 2% RSD [20]. However, in CZE–ESI-MS the detection is postcolumn and the difficulties in coupling CZE and MS because of the low electro-osmotic flow, give rise to a larger variation in signal response. In addition, the quantitative reproducibility of ESI-MS is itself poor, no better than several percent.

3.8. Quality of the capillary end cutting

With the CZE–ESI-MS ionization needle used in this study it was necessary to mount the capillary

Table 2

Quality parameters, i.e. limit of detection (LOD; 3×noise), linearity (0.25–80 mg/l in ultrapure water and 1–20 mg/l in surface and tap water) and reproducibility for peak area, migration time and corrected peak area (30 mg/l, $n=9$)

Analyte	LOD (mg/l)	Linearity			Reproduction		
		r^2, $n=9$ Ultra-pure water	r^2, $n=6$ Surface water	r^2, $n=6$ Tap water	Area RSD (%)	Mig. time (RSD, %)	Corr. peak area (RSD, %)
Oxalate	0.05[a]	0.9973	0.9853	0.9947	5.37	0.21	5.34
Lactate	0.09	0.9994	0.9991	0.9994	3.60	0.33	3.69
Malonate	0.05	0.9925	0.9851	0.9997	3.22	0.24	3.24
Succinate	0.1	0.9990	0.9992	1.000	2.07	0.35	2.09
Glutarate	0.06	0.9988	0.9997	0.9999	4.51	0.62	4.21
Adipate	0.06	0.9994	0.9996	0.9998	3.28	0.38	3.13
Malate	0.05[b]	0.9955	0.9953	0.9999	4.59	0.24	4.52
Citrate	0.7	0.9941	0.9858	0.9951	3.18	0.26	3.18
Tartrate	0.09	0.9956	1.000	0.9996	3.00	0.31	2.86
Gluconate	0.05	0.9998	0.9991	0.9989	2.50	0.83	2.13
ISA	0.07	0.9991	0.9988	0.9997	3.72	0.84	3.39

[a] $S/N=20$.
[b] $S/N=50$.

every day, to avoid introduction of sodium hydroxide in the ion source (from the conditioning procedure). An everyday variation of the exact positioning of the capillary gave rise to differences in signal intensity and migration time in day-to-day analyses. This is caused by the difficulty of making a clean cut of the capillary end that leads into the ion source. Since the positioning of the capillary is slightly different each day the small variation in the capillary end cutting affects the ion spray direction as well as the conditions for the electrical connection. During one experiment where a new capillary was taken in use, sensitivity dropped significantly. Several attempts (six) to make a clean cut were made but none of these cuts gave as high signal intensity as the original capillary that was used during optimization, illustrating the importance of the quality of the capillary cutting.

3.9. Analysis of LMW organic acids with CZE–ESI-MS

A separation of ten of the eleven acids was accomplished by the optimization (Fig. 1). The baseline shift, seen after 16 min in Fig. 1, is a result of the settings of the SIM descriptor (i.e. it is divided
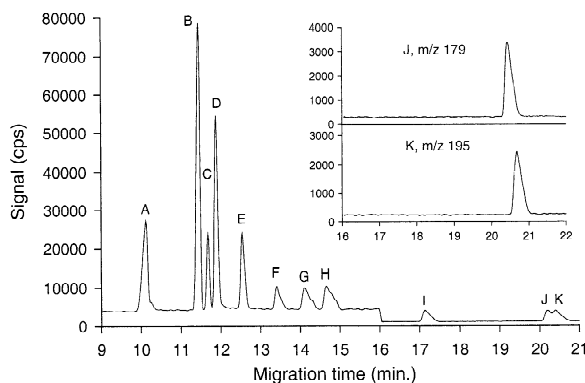
into two parts in order to increase the dwell-times for lactic, gluconic and isosaccharinic acid). Gluconic and isosaccharinic acid co-migrate, but the SIM detection compensates for this, and both can be detected at different mass. Since the capillary must have a certain length to reach the MS detector the analysis time is prolonged which causes tailing of the last six peaks. To compensate for this an attempt to use a higher separation voltage (<-17 kV) was made which induced such high electrical field strength over the UV window when the unresolved sample plug passed that the capillary cracked at that position, as well as the resolution decreased for some analytes. Consequently, the separation voltage was kept at -17 kV. Besides the acids that were used during optimization the methodology works well for fumaric, D-galacturonic and 2-ketoglutaric acid.

The developed method was applied on a sample of ale (Fig. 2). The sample was only filtered before analysis. Peak identification was performed by matching the unknown peak migration time with that of the standard solution in combination with mass identification. Calibration curves were made by plotting peak area vs. concentration for known solutions. The large concentration of citric and lactic acid caused unsymmetrical peak shapes and a dilution of the sample was made. The high concentration of lactic and citric acid probably originates from the ale character of the beer.

Fig. 1. Separation of LMW organic acids (20 mg/l) by CZE–ESI-MS with optimized parameters, see Table 1. Conditions: capillary: 103 cm×50 µm I.D.; electrolyte composition: 2 m*M* TMA and 5 m*M* Tris, pH 8.5; voltage: −17 kV. Large diagram: total ion electropherogram, small diagrams: single ion electropherograms. (A) Oxalate, (B) malate, (C) malonate, (D) citrate, (E) tartrate, (F) succinate, (G) glutarate, (H) adipate, (I) lactate, (J) isosaccharinate, (K) gluconate.
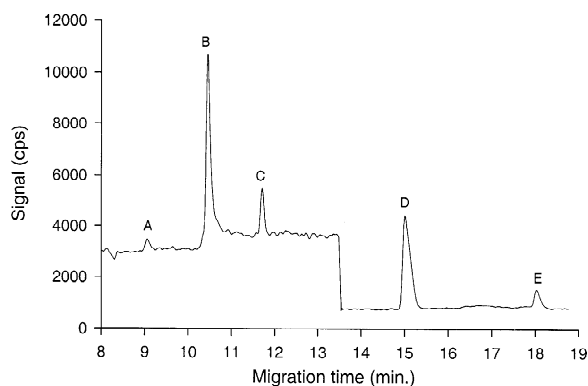

Fig. 2. Ale sample (diluted 1:10). Instrumental settings as in Fig. 1. (A) Oxalic acid (6.4 mg/l), (B) citric acid (170 mg/l), (C) succinic acid (39 mg/l), (D) lactic acid (260 mg/l), (E) gluconic acid (79 mg/l).

4. Conclusions

The optimized settings gave stable separation conditions for ten of the eleven acids in 21 min (gluconic and isosaccharinic acid co-migrate). The developed methodology showed adequate limit of detection that lies in the range of <0.05 to 0.1 mg/l for all acids except citric acid, using the hydrodynamic injection mode. The sensitivity is due to SIM detection, which also makes identification of co-migrating ions possible. With care concerning the cutting of the capillary and its position in the ion source the procedure also showed excellent linear response in different matrices as well as repeatability. The developed CZE–ESI-MS procedure is more sensitive and provides a better selectivity and identification compared to UV detection, which makes the analysis of LMW carboxylic acids in complex matrices more reliable.

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