Simultaneous determination of four organic acids in beverages by capillary electrophoresis coupled with ultraviolet detector

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Abstract

A simple and rapid capillary electrophoresis method with direct ultraviolet (UV) detection was set up for the determination of four organic acids in beverages. The method included dilution and filtration as simple sample preparation steps. The electrophoretic separation and detection of oxalic, malic, citric and lactic acids in wines and beers were performed in 8 min. For the method validation, linearity, detection and quantification limits, repeatability and recovery in wine and beer matrices were studied. Good linearity was observed from 25 to 500 mg/L for all acids excluding lactic acid, for which it started from 50 mg/L. The limits of quantitation of oxalic, malonic and citric acid were set 9.5 to 28.5 mg/L. Repeatability of this method was from 3.2 to 7.3%, recoveries ranged from 90.1 to 110.1%. The validated method was applied to the analysis of different wines and beers and showed great variability in their composition.

***Keywords**: Capillary electrophoresis, ultraviolet detection, beverages, organic acids.*

1. INTRODUCTION

Alcoholic drinks are widely used around the world. According to data from World Health Organization (WHO), total consumption of alcohol was estimated as 13.5 g/day/person, mostly spirits (50.1%), beer (34.8%) and wine (8%). The fermentation process, which is an irreplaceable part of drinking production, is the origin of the composition and content of organic acids in beverages. The content of succinic acid, acetic acid and lactic acid in beer generally increased after fermentation [1]. There is a difference of acid content in ales and lagers because of different ways of fermentation [2].

Organic acids play important roles in the liquors. Firstly, organic acids are partly responsible for beverages' taste and flavour. For example, malic acid, which is normally present in apples, affords a sour taste and apple flavour. Hence, the taste of the drink is

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adjusted by the mixture of organic acids. Furthermore, the stability of liquor depends on pH value, which controls propagation of microorganisms and fermentation [3]. In human body, succinic acid, acetic acid, citric acid, lactic acid and malic acid bolster the absorption of iron [4-5]. Marunaka et al revealed that insulin resistance can be ameliorated corresponding to an intake of weak organic acids by elevating the interstitial fluid pH in diabetes mellitus [6].

Various approaches for quantitative determination of main organic acids in beverages have been developed. A number of these methods are based on the chromatographic separation including ion-exchange chromatography with UV and RI [7-8] or amperometric detection [9], reversed phase chromatography with UV [10], diode array detector (DAD) [11-12], amperometric [12] or mass-spectrometric detection [13]. In the reported methods, the sample preparation mostly consists of dilution and filtration. However, sample clean-up by solid phase extraction with strong anion exchange resin was recommended in some studies [7-10]. Gas chromatographic methods with mass-spectrometric detection were also used [14-15]. GC-MS methods require more sophisticated sample pre-treatment: drying followed by derivatization [14] or continuous solid-phase extraction [15].

While chromatographic separation is the most widespread approach to the analysis of organic acids in beverages, capillary electrophoresis (CE) is the second favoured way [4]. Compared with GC and HPLC, capillary electrophoresis (CE) has the characteristics of high separation efficiency, short analysis time, low sample and chemical consumption and simple sample processing, which has a broad application prospect in the separation and analysis of complex samples and is commonly used in biological and food analysis. For analysis of organic acids in beverages, separation is done in capillary zone electrophoretic mode (CZE). Analytes are detected using UV detector or DAD using either indirect [16-18] or direct [19-20] detection strategy. Sample preparation is simple and requires only dilution and filtration. The electroosmotic flow is reversed by addition of flow modifiers to the background electrolyte (BGE) to speed up the separation of analytes. Indirect detection is achieved by addition of a chromophore to BGE and detection above 220 nm. Different compounds were successfully used as chromophores, for example, 3,5-dinitrobenzoic acid (DNB) [16], 2, 4-Dihydroxybenzoic acid [18], benzoic, boric, sorbic, phthalic acids and phosphate [21]. Successful direct UV detection was performed using aqueous phosphate buffer and detection below 200 nm [19-20].

In this article, a CE-UV method has been developed and validated for simultaneous determination of four organic acids in alcoholic beverages. This method has been successfully applied to samples on the market for comparison of different beverages.

2. MATERIALS AND METHODS

2.1. Reagents and Solutions

All chemicals used in the experiments were of analytical reagent grade. Citric acid monohydrate (> 99%) and oxalic acid dihydrate (> 99%) were from MERCK (E. Merck, Darmstadt). Lactic acid (> 90.0%) was from VWR. Malonic acid (> 99%) was from SIGMA-ALDRICH. Cetyltrimethylammonium bromide (CTAB, > 99%) was from Fluka. Disodium hydrogen phosphate monohydrate was from MERCK. Sodium hydroxide solution (1 mol/L) was from Agilent technologies. Methanol (HPLC grade) was from Supelco. Phosphoric acid (85%) was from EMSURE.

Standard organic acid stock solutions (about 1,000 mg/L) were prepared in Milli-Q (MQ) water and were stored at 4°C. Working standard solutions were prepared weekly by diluting the stock solutions with MQ water. The stock solution of CTAB at 8 mM was also prepared. The final background electrolyte (BGE) solution (1 mM CTAB, 1% methanol and 180 mM Disodium hydrogen phosphate) was prepared weekly, was adjusted with phosphoric acid to pH 7.2, then degassed and filtered through a 0.2 µm cellulose membrane.

2.2. Sample preparation

12 beverages of different types (five wines and seven beers) were purchased from a liquor store in Sweden. Samples were stored at 4°C before analysis. Beers were degassed for about 30 min in an ultrasonic bath and then diluted with MQ water at an appropriate rate, filtered through a 0.2 µm cellulose membrane and analyzed immediately.

2.3. CE instrumentation and Electrophoresis Conditions

Experiments were carried out with an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany), equipped with a UV detector (UV). The detector was set at 192 nm for the direct detection of analytes. Analysis was performed using bare fused silica capillary (Agilent Technologies, Waldbronn, Germany) with 1mm diameter bubble cell, 50 µm i.d. and 56 cm effective length. The temperature was controlled and maintained at 25°C. For data acquisition, Agilent Lab Advisor software was utilized.

The electrolyte used for the separation of analytes consisted of 180 mM Na_2HPO_4, 1 mM CTAB, 15% (v/v) methanol, having the pH 7.2 adjusted with H_3PO_4.

The capillary was conditioned daily by flushing with 1 M NaOH (5 min), 0.01 M NaOH (5 min), Milli-Q water (5 min) and electrolyte solution (5 min) before batch analysis. After each run, the capillary was washed with the electrolyte for re-conditioning (2 min). After each 20th run, the capillary was re-conditioned with 0.01 M NaOH (2 min) and BGE (2 min).

The calibration solutions and samples were hydrodynamically injected in three seconds at 50 mbar pressure. The electrophoretic separation was conducted at inverted

polarity and constant voltage of -20 kV. Both calibration standards and samples were analyzed in triplicates.

2.4. Method validation

The optimized method was validated according to EC/657/2002 with selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy. Selectivity was determined by running blank samples, standard solutions, and spiked samples. The linearity of this method was checked by running a range of standard solutions from 5 to 500 mg/L. The calibration solutions were prepared each day before analysis. The limit of detection (LOD) and limit of quantification (LOQ) were determined from calibration curves. The LOD and LOQ have been estimated by the following equations:

$$LOD = \frac{3.3 \cdot \sigma}{S} \quad (1)$$

$$LOQ = \frac{10 \cdot \sigma}{S} \quad (2)$$

where σ is the residual standard deviation; S is the slope of the calibration curve.

Repeatability and recovery were evaluated by running six spiked samples at two different levels in two matrices (beer and wine). The repeatability was presented through relative standard deviation. Recovery was assessed by comparison between calculated concentration and expected one in percentage using the following equation:

$$R = \frac{C_1 - C_0}{\Delta C} \cdot 100\% \quad (3)$$

Where:

C_1 is a concentration of analyte in the sample before spiking calculated from calibration curve;

C_o is a concentration of analyte in the sample after spiking calculated from calibration curve;

ΔC is the expected increase in analyte concentration in the sample due to spiking.

2.5. Uncertainty estimation

The uncertainty of this method u_c was estimated through the Nordtest approach [31]. Uncertainty sources were considered in two big groups. Uncertainty due to random effects is assessed by repeatability u_{sr}. Uncertainty due to systematic effects u_{bias} was considered through the root mean square of bias error found from spiking experiments. All other uncertainty sources from the purity of standard substances, volumetric operations, etc were expected to be negligible. The combined uncertainty was calculated through the following equation:

$$\boldsymbol{u_c = \sqrt{u_{sr}^2 + u_{bias}^2}}$$

3. RESULTS AND DISCUSSIONS

3.1. Electrolyte composition

3.1.1. Indirect detection

An attempt to create a system for indirect detection of organic acids was made. To perform an indirect detection of analytes, the BGE must contain a chromophore. In studies [16, 22], 3,5-dinitrobenzoic acid was used as a chromophore at a concentration of 10 mM. According to the work [16], the following background electrolyte should be used: 10 mM DNB, 0.2 mM CTAB as EOF modifier, pH 3.6 adjusted with HCl. Result showed that it was impossible to dissolve DNB in water to prepare a 10 mM solution practically. Furthermore, it was reported that the solubility of DNB in water at 25°C is only around 1.3 g/L which is equal to 6 mM. A small amount of ethanol was added for aiding dissolution of DNB, however, DNB precipitated back when the solution was diluted with water. Heating and ultrasonication approaches were unsuccessful, so analysis of organic acids was decided to try direct detection mode.

3.1.2. Direct detection

For direct detection, two buffer compositions were tested. Both buffers were phosphate-based, which had a selectivity modifier and CTAB as EOF modifier.

Buffer A containing 7.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 2.5 mM CTAB and 0.24 mM $CaCl_2$ (as selectivity modifier) at pH 6.4 was prepared and the expected pH value was adjusted by HCl. A similar system was successfully used in the study [21] for analysis of citric, malic, lactic, tartaric, succinic and acetic acids in wines. Separation was made at a constant voltage of -25 kV. This buffer was then applied to analyze a mixed standard solution containing all four acids (around 250 mg/L each), a noisy background was seen, and analytes were not separated. Since there were many positive and negative peaks in the background, it was impossible to refer any peak to an analyte. An electropherogram obtained from a mixture of acids using buffer A was presented in Figure 1.

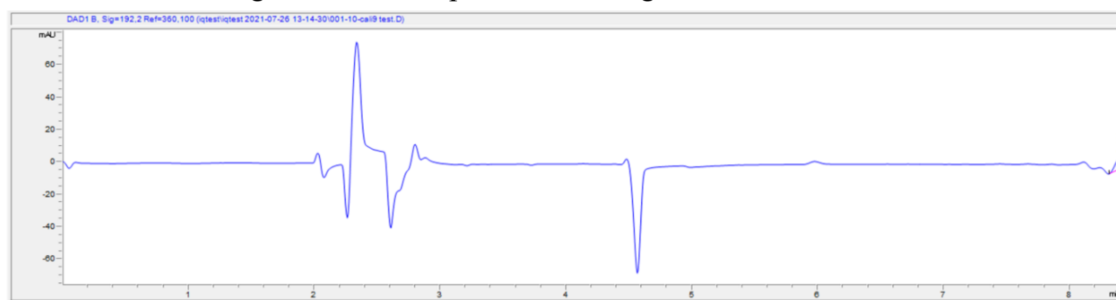

Figure 1. *Electropherogram of four organic acids when buffer A is used as electrolyte*

The similar buffer used by the authors contained tetradecyltrimethylammonium hydroxide (TTAOH) as an EOF modifier. TTAOH has a shorter alkane chain than CTAB and a different counterion. CTAB has a critical micelle concentration (CMC) of 1.3 mM

[23], while that of TTAOH is higher since its chain is shorter - 1.8 mM [24]. It is known that if the EOF modifier is present at concentrations lower than its CMC, its monomers adhere to the capillary walls, which results in the reverse of EOF [25]. So, it is possible that at such a high concentration, CTAB forms micelle and the concentration of monomer CTABs which should be adhered to capillary wall is not enough and flow is not stable for the separation of organic acid by buffer A.

Another buffer (buffer B) was tested for the analysis of organic acids. The composition of the buffer B was the following: 180 mM Na_2HPO_4, 1 mM CTAB, 15% (v/v) methanol, having the pH 7.2 adjusted with H_3PO_4 [23]. Methanol acted as a selectivity modifier. The use of methanol and high concentration of phosphate buffer were used to obtain the electrolyte with high viscosity and, therefore, reduced its mobility, which retained analytes longer in the capillary and subsequently improved resolution. Vorarat et al [23] used the voltage of -15 kV for the separation. In this work, the separation of analytes at -20 kV and -15 kV were compared. Results showed that all peaks were separated from each other in both cases. The total run time was 12 min for the voltage of -15 kV and 10 min for the voltage of -20 kV. As a shorter time for analysis, the voltage of -20 kV was chosen for further work. The electropherogram of the optimum condition was present in Figure 2.

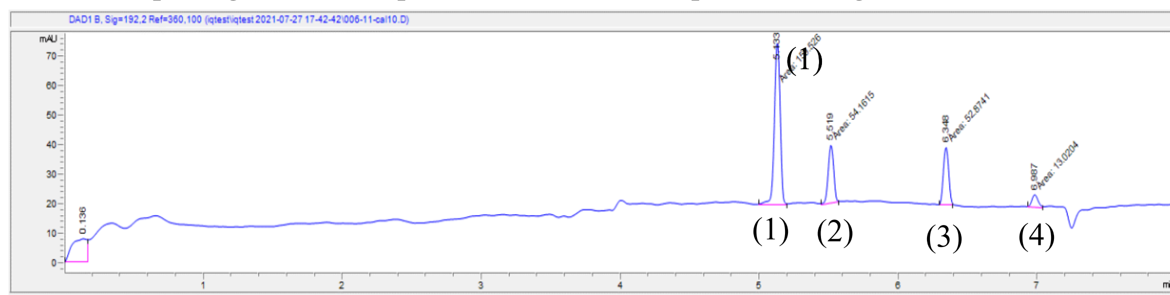

__Figure 2__. Electropherogram of 4 organic acids when solution B is used as electrolyte , the peaks illustrated (1) oxalic acid, (2) malonic acid, (3) citric acid, (4) lactic acid

In Figure 2, all the peaks of four organic acids are separated from each other. All the peaks are sharp, the total run time was 10 min including 2 min flushing with BGE before injection. This optimum method has been chosen for method validation.

3.2 Validation

3.2.1 Selectivity

The electropherograms of a wine sample, wine spiked sample were presented in Figure 3. Selectivity was ensured by the comparison of migration times corresponding to peaks of a sample to migration times of analytes in standard solutions. It can be seen from Figure 3 that there were several additional peaks in a spiked sample (B) compared to non-spiked sample, (A) whose migration times were corresponding to those in the standard solution.

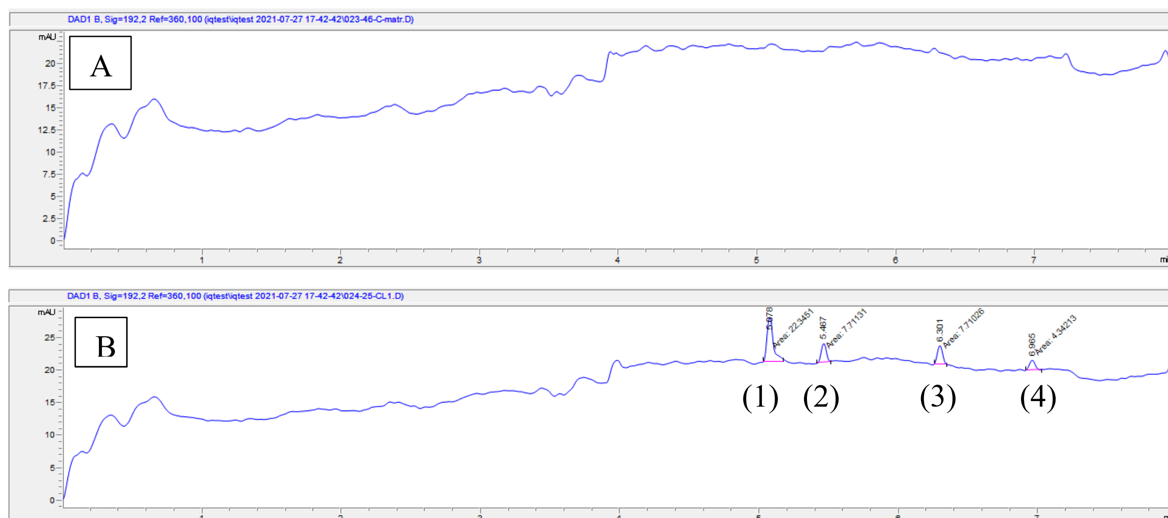

Figure 3. *Electropherograms of wine sample (A) and wine spiked sample (B), the peaks illustrated (1) oxalic acid, (2) malonic acid, (3) citric acid, (4) lactic acid*

3.2.2 Calibration curves (Linearity)

The relationship of analytes' peak areas with respective standard deviation and their respective concentrations is plotted in Figure 4.

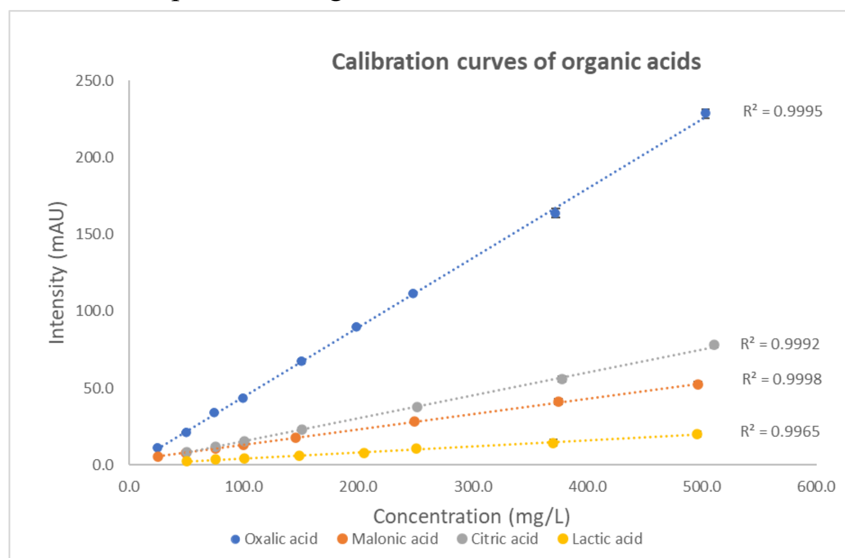

Figure 4. *Calibration curves of four organic acids*

The linearity range of oxalic acid and malonic acid was from 25 to 500 mg/L, while those of the others was from 50 to 500 mg/L. As it can be seen in the Figure 4, all four calibration curves yielded an R^2 value higher than 0.995. Furthermore, the residual plots, which are presented in Figure 5, showed the random distribution of residuals. Therefore, the calibration curves can be used for further calculation.

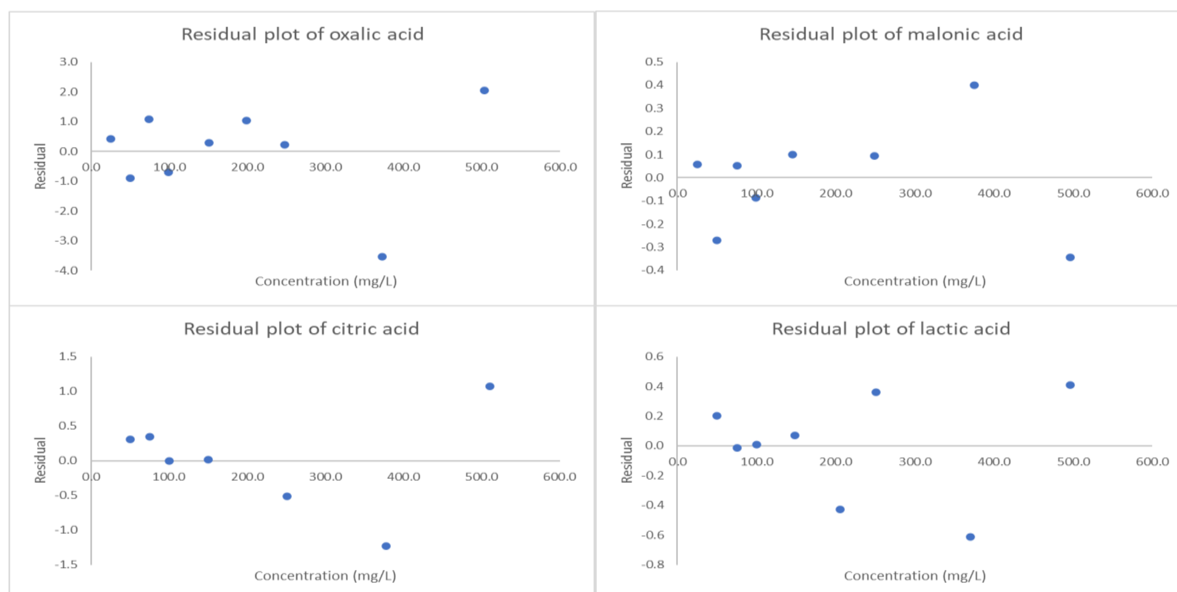

Figure 5. *Residual plots of four calibration curves for organic acids*

The repeatability of migration times is usually poor due to the fluctuation of electroosmotic flow, which is an inherent weakness of capillary electrophoresis [27]. Some other causes are unstable temperature, current, pH, ionic strength, presence of air bubbles and siphoning effect [28-30]. To ensure the migration time for batch analysis, each standard solution was run randomly in triplicate, the migration time for each peak was recorded and the repeatability of migration times was expressed by the relative standard deviation (RSD) value presented in Table 1.

Table 1. *Relative standard deviation of migration times of four organic acids*

Analyte	***Oxalic acid***	***Malonic acid***	***Citric acid***	***Lactic acid***
RSD (%)	0.65%	0.72%	0.82%	0.97%

As it was shown in Table 1, RSD values of migration time for all studied acids are below 1%, which can be considered as good repeatability. The good precision can be explained by the stable temperature (25°C) set by the instrument, and the BGE was periodically changed to a fresh portion during an analysis of the batch. CTAB, acting as a surfactant, can be another reason for the stable migration time of organic acids [27].

3.2.3 Limit of detection and limit of quantification

Using calibration curves consisting of four first points from the whole concentration range, the LOD and LOQ have been estimated from equation (1) and (2) and can be found in Table 2.

Table 2. *Estimated limit of detection and limit of quantification*

Analyte	***Oxalic acid***	***Malonic acid***	***Citric acid***	***Lactic acid***
LOD (mg/L)	8.3	6.2	3.1	9.4
LOQ (mg/L)	25.1	18.9	9.5	28.5

3.2.4. Repeatability

Six replicates of spiked samples in each matrix were analyzed by the optimized method. The peak area was recorded for each compound in electropherograms. The repeatability was assessed by the relative standard deviation of a peak area, which is presented in Table 3. To estimate acceptable repeatability s, the Horwitz function was used:

$$s = 2^{(1-0.5\cdot\log C)} \quad (5)$$

where C is a concentration of analyte in the spiked sample.

Table 3. *Relative standard deviation of peak area in 6 replicated measurements*

Matrix	***RSD of peak area***			
	Oxalic acid	***Malonic acid***	***Citric acid***	***Lactic acid***
Wine	3.4%	7.3%	4.0%	4.8%
Beer	3.2%	3.2%	5.8%	3.8%
Acceptance (s)	8.9%	8.9%	6.4%	8.0%

From Table 3, the RSD of peak area of each acid varied from 0.8% to 7.3%. RSD was lower than the maximum limit for repeatability s found from the Horwitz function for each acid at both spiking levels. That meant this method met the requirement for repeatability.

3.2.5 Recovery

The range of recovery for each acid was depicted in Table 4.

Table 4*. Recovery of organic acids in two different levels in three matrices*

Recovery	***Oxalic acid***	***Malonic acid***	***Citric acid***	***Lactic acid***
Wine	94.0 - 105.9%	91.5 - 109.7%	90.4 - 110.1%	94.1 - 107.5%
Beer	90.1 - 104.1%	90.3 - 97.8%	90.4 - 106.7%	92.2 - 104.5%

From the table, the recovery of all organic acid in each matrix was in the range of 90 - 110%, which meant that the method was reliable to determine organic acid in wine and beer. The dataset for calculated concentration and recovery for each spiked sample was put in the supporting information.

3.5. Estimation of uncertainty

Total uncertainty was estimated by the Nordtest approach as described in Table 5. To calculate expanded uncertainty, coverage factor $k = 2$ was chosen for 95% confidence level.

***Table 5**. Uncertainty of analysis method estimated by Nordtest approach*

Matrix	***Expanded uncertainty, k = 2***			
	Oxalic acid	***Malonic acid***	***Citric acid***	***Lactic acid***
Wine	6.8%	16.5%	8.2%	10.0%
Beer	7.9%	6.4%	11.6%	9.2%

Compared to HPLC method of Park et al, whose quantification using low UV wavelength detection generally faced unstable baselines, this limitation can be overcome by CE-UV. Cheaper and less consuming chemicals and solvents were advantageous aspect for using CE-UV compared to HPLC method. Meanwhile, all the validated parameters can be compatible with this study [3]. In the work [23], the authors have applied the method on matrices orange and lime juices and having full validation of lactic acid and tartaric acid. In our research, two approaches for CE-UV including indirect and direct detection were compared for practical purposes. Different electrolytes were tested for optimum condition. The effects of pH value of buffer and the content of methanol have not been investigated. In the next project, it would be recommended if these values will be evaluated for the optimum method. Furthermore, there were several unknown peaks in electropherogram of samples, which may be assigned for other organic acids. Therefore, to assess total organic acids in beers and wines, the scope of this method can be promisingly widened by adding more analytes.

3.3. Sample analysis

All samples were diluted with water before analysis with appropriate dilution factor. For confirmation of a peak corresponding to an analyte, peaks were identified using the migration times. Within the run, migration times were mostly stable and differ within 1.2%. The content of organic acid in sample was calculated by the following equation:

$$C\left(\frac{mg}{L}\right) = k.\frac{S_{analyte} - intercept}{slope} \qquad (6)$$

Where k was dilution factor, $S_{analyte}$ was the area of peak corresponding to analyte, intercept and slope were taken from calibration curve. Results of the analysis of samples are presented in Figure 6.

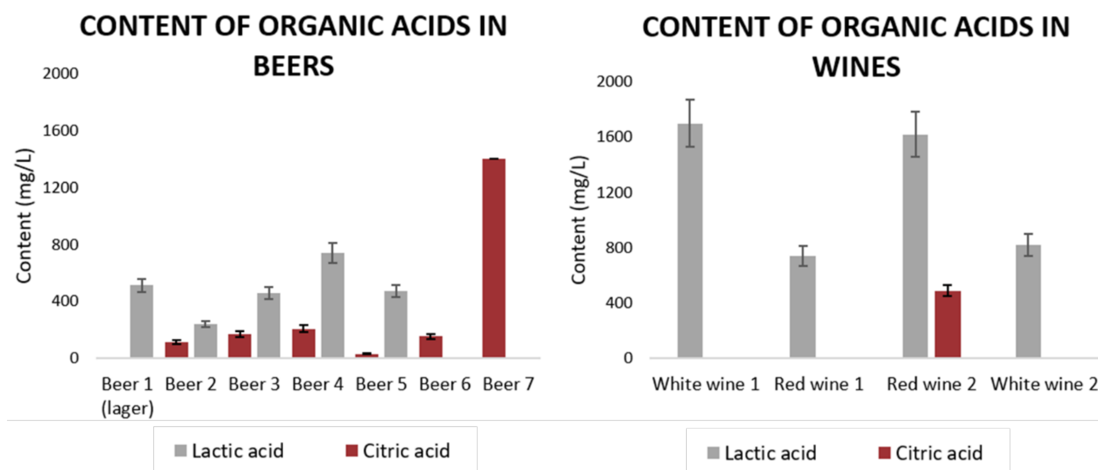

Figure 6. *Content of organic acids in beers and wines*

A high concentration of citric acid in beers which ranged from 32 to 1,400 mg/L can be due to the following facts. Firstly, citric acid is a popular preservative that is added to soft drinks. Secondly, citric acid naturally occurs in fruits, so it is present in fruits used to brew beverages. Similarly, citric acid naturally occurs in grapes which is the main material for wine production. Oxalic acid cannot be detected in any beers or wines with LOD (8.3 mg/L) reported above except for one beer sample with concentration lower than LOQ (25.1 mg/L). This result was similar to Liu's study in which there is no oxalic acid reported in red wines and beers with LOD of 7.5 mg/L [17]. No malonic acid was detected in beverages even though it is naturally present in fruits. The reason can be its low original concentration in fruits used for brewing or/and its loss during brewing.

Malic acid naturally occurring in different fruits and berries is converted into lactic acid during the fermentation process. Therefore, lactic acid may be found in all brewed beverages. The results for lactic acid in wines, which was from 740 to 1,700 mg/L, was corresponding to study of Park et al [3] with HPLC method for organic acid determination. Meanwhile, the content of lactic acid in beer is from 243 to 741 mg/L which was higher than reported value of this study, from 95.9 to 226.4. The differences can be explained by different sources of materials for making beer, different production chains, customer orientation features and other compositions.

4. CONCLUSION

The CE method with direct UV detection for quantitative and qualitative determination of four organic acids in beverages was developed and validated in this work. The samples were diluted, filtered, and then injected into the capillary for analysis. The validation result indicated good linearity, selectivity, recovery, and repeatability. This method was then successfully applied into real samples analysis. Malonic acid and oxalic acid were not detected in any samples except for 1 beer sample with concentration of oxalic acid lower

than LOQ. Citric acid and lactic acid were found in most beer samples in the range from 32 to 173 mg/L and from 243 to 741 mg/L respectively. In wine, lactic acid was found in the range of 740 to 1,700 mg/L, which is higher than beer.

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Xác định đồng thời các acid hữu cơ trong đồ uống bằng điện di mao quản sử dụng detector UV

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Tóm tắt

Phương pháp điện di mao quản kết hợp với detector UV để xác định và định lượng đồng thời 04 acid hữu cơ gồm malonic acid, citric acid, lactic acid và oxalic acid trong đồ uống đã được xây dựng và thẩm định trên nền mẫu bia và rượu vang. Mẫu được pha loãng với tỉ lệ phù hợp và phân tích với điều kiện đã tối ưu trên thiết bị. Các hợp chất được phân tách bằng mao quản với chất điện ly gồm Na_2HPO_4 180 mM, CTAB 1 mM và Methanol 15%, điều chỉnh pH tới 7,2. Tổng thời gian phân tích mẫu là 8 phút. Phương pháp đã được thẩm định trên các tiêu chí tính chọn lọc, độ tuyến tính, giới hạn phát hiện, giới hạn định lượng, độ lặp lại và độ thu hồi. Khoảng tuyến tính được xác định từ 25 đến 500 mg/L cho citric acid, malonic acid và oxalic acid, với lactic acid, đường chuẩn được xây dựng từ 50 đến 500 mg/L. Giới hạn phát hiện và giới hạn định lượng của các acid được xác định từ 3,1 đến 28,5 mg/L. Độ lặp lại thấp hơn giới hạn cho phép theo công thức Horwitz. Độ thu hồi của phương pháp đạt trong khoảng 90 - 110%. Phương pháp được ứng dụng thành công để phân tích 12 mẫu đồ uống gồm bia và rượu vang trên thị trường Thụy Điển.

Từ khóa: *điện di mao quản, UV, đồ uống, acid hữu cơ.*