A rapid and simple method for the determination of organic acids in proteolytic enzymes by capillary electrophoresis with indirect ultraviolet detection

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Abstract

The use of organic acids (e.g. acetic acid and gluconic acid) as additives during protease production is regarded as one of the simplest alternatives to increase enzyme stability and activity in many industrial processes. However, no methods have been described for the determination of organic acids in proteases and their contents have not been established yet. In this work, a novel, rapid and simple method for the determination of organic acids in proteolytic enzymes by capillary electrophoresis (CE) with indirect ultraviolet (UV) detection has been developed. Under the optimized conditions, the method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ) and intra-day and inter-day repeatability. Later, a sample pretreatment based on a hydroalcoholic microextraction was carefully optimized to obtain good recovery and repeatability and determine acetic and gluconic acids in a commercial protease sample. The complete procedure was validated using the standard-addition calibration method, finding matrix effects on the studied compounds. Finally, acetic acid and gluconic acid were quantified at 80 mg/Kg (0.0080% (m/m)) and 69 mg/Kg (0.0069% (m/m)) in the protease sample, respectively.

Keywords: capillary electrophoresis; indirect UV detection; organic acids; protease; quality control; validation

1. Introduction

Protein hydrolysis can be used in a wide range of applications, from proteomic studies to cleaning and food biotechnology processes, and can be carried out by chemical or enzymatic processes [1–4]. While chemical processes (e.g. alkaline and acid hydrolysis) tend to be less eco-friendly, difficult to control and yield products with modified amino acids [5,6], enzymatic hydrolysis can be performed under milder conditions, hence avoiding the extreme environments required for chemical treatments [6,7]. Additionally, enzymes present substrate specificity, which allows the development of protein hydrolysates with better defined chemical and nutritional characteristics [7].

Proteases (also termed peptidases) are one of the most important groups of industrial enzymes and their designs range from small and simple catalytic units (around 20 kDa) to sophisticated protein-processing and degradation machines (0.7-6 MDa) [6,8]. Novel protease applications in industrial processes are constantly being introduced, but autolysis of proteases still remains the major limitation in enzyme production, which may lead to chain instability, low reaction rate and low substrate susceptibility [9,10]. Several methods have been described in the literature to increase enzyme stability, which include enzyme immobilization, cross-linking with chemicals or chemical modification of the amino acid side-chains [11–13]. Among them, the introduction of organic acids (e.g. acetic acid, gluconic acid and their salts, etc.) into the production medium is one of the simplest alternatives to increase enzyme stability and activity. These molecules are believed to provide additional points of hydrogen bonding with the enzyme surface, decrease dehydration and/or provide thermodynamic barriers to unfolding [14,15]. Despite its use in enzyme production is well-known, no dedicated

methods have been described for the determination of organic acids in proteases and their contents have not been established yet. Novel analytical methods in this field should provide further information about the content of organic acids during protease production, which could be used in quality control to ensure maximum enzyme stability and activity, as well as to track enzyme production.

Different techniques have been employed for organic acid determination, traditionally gas chromatography (GC) [16,17] and, more recently, liquid chromatography (LC) [18–21]. However, these techniques present some deficiencies. When using GC, organic acids need to be derivatized to make them volatile. LC, besides needing in general organic solvents, is time-consuming and limited by the narrow linear dynamic range and the susceptibility to matrix interferences. Capillary electrophoresis (CE) with direct [22–25] or indirect [26–34] ultraviolet (UV) detection has also been demonstrated, but to a lesser extent, despite the well-known benefits of this high-performance microscale electroseparation technique [35]. CE provides complementary and, very often, better separations than hydrophobicity-driven reversed-phase LC. Additionally, analyses can be performed using smaller amounts of sample and reagents, no organic solvents are necessary, separation times are considerably low and it offers good repeatabilites [35]. CE has proven to be a good choice for the determination of organic acids in several beverages [22–25,28–31], microbial fermentation process samples [32], engine coolants [33] and ionic liquids from biomass hydrolysates [34], where no more than a simple dilution is needed. In addition, CE and capillary isotachophoresis have also been reported for determination of anions of organic acids as counterions of basic drugs [36–38]. However, to the best of our knowledge, the use of CE for the analysis of organic

acids in more complex matrices containing a high protein content (such as enzymes, or specifically proteases) has never been reported.

In this work, we have developed a CE method with indirect UV detection for the determination of acetic acid and gluconic acid in proteases. Since most organic acids lack of a strong UV chromophore, the use of indirect UV detection offers an excellent alternative [26–34], without the need for any derivatization step or the use of a contactless conductivity detector [39–41]. After validating the method with standards, we optimized an appropriate sample pretreatment method for the extraction of organic acids from a commercial protease sample before CE-UV. The complete procedure was then validated using the standard-addition calibration method, before the accurate quantitation of the detected organic acids.

2. Materials and methods

2.1. Chemicals and samples

All the chemicals used in the preparation of solutions and buffers were of analytical reagent grade or better. Acetonitrile (ACN, HPLC grade), methanol (MeOH, HPLC grade), ethanol (EtOH, 96% (v/v)), ammonium hydroxide (NH_4OH, 25% (v/v)), acetic acid (glacial), gluconic acid, calcium chloride anhydrous, magnesium chloride anhydrous, sodium hydroxide, 2,6-pyridine dicarboxylic acid (2,6-PDC), cetyltrimethylammonium bromide (CTAB) and bovine serum albumin (BSA, molecular mass, M~ 66 kDa) were supplied by Merck (Darmstadt, Germany). The analyzed protease was commercially available as a solid powder mixture of combined proteases

123 derived from *Aspergillus oryzae*. Water with conductivity lower than 0.05 μS/cm was
124 obtained using a Milli-Q water purification system (Millipore, Molsheim, France).
125
126 **2.2. Electrolyte solutions**
127
128 The background electrolyte (BGE) was prepared with 20 mM 2,6-PDC, 0.3 mM CTAB,
129 30 mg/L Ca^{2+} and 30 mg/L Mg^{2+} in MeOH:water (10:90, v/v). The pH of the solution
130 was adjusted to 9.0 with NH_4OH. Before the analyses, the BGE was degassed by
131 sonication and filtered through a 0.20 μm nylon filter (Macherey-Nagel, Düren,
132 Germany).
133
134 **2.3. Apparatus and procedures**
135
136 pH measurements were made with a Crison 2002 potentiometer and a Crison electrode
137 52-03 (Crison Instruments, Barcelona, Spain). Centrifugal filtration at 25ºC was carried
138 out in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany).
139 Agitation during sample extraction was performed with a Vortex Genius 3 (Ika®,
140 Staufen, Germany).
141
142 **2.3.1. Standards and sample preparation**
143
144 An aqueous standard solution (1,000 mg/L) of each organic acid (acetic acid and
145 gluconic acid) was prepared and stored in a freezer at -20°C when not in use. Working
146 standard solutions were obtained by diluting the stock solutions with water. Standard
147 solutions were also used to spike the protease sample.

Under the optimized conditions, 20 mg of protease (solid powder) were mixed with 100 µL of 80% (v/v) EtOH (sample:solvent 1:5 m/v) and were incubated for 30 min with constant shaking (medium speed) in a vortex at room temperature. The mixture was then centrifuged at 10,000 x g for 10 min at 25 ºC. The extraction with EtOH 80% (v/v) was repeated twice and the supernatants were combined. The pooled supernatants were evaporated to dryness in a SpeedVac™ (Thermo Scientific, Waltham, MA, USA) and the solid residue was reconstituted with 100 µL of water or standard mixture at an appropriate concentration (spiked protease samples). Before the analysis, samples were filtered through a 0.20 µm nylon filter.

2.3.2. CE-UV

All CE-UV experiments were performed in a 7100 CE (Agilent Technologies, Waldbronn, Germany) with a diode-array detector (indirect UV detection at 254 nm). The method was adapted from the work of H. Turkia et al. [32], but substituting 2,3-PDC by 2,6-PDC and myristyltrimethylammonium hydroxide (MTAH) by CTAB as described by M. Navarro-Pascual-Ahuir et al. [29,30]. Separations were performed at 25°C in 58 cm total length (L_T) × 50 µm internal diameter (i.d.) × 365 µm outer diameter (o.d.) fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). All capillary rinses were performed at 930 mbar. New fused silica capillaries were flushed with 1 M NaOH (20 min), water (20 min) and BGE (15 min). The capillary was finally equilibrated by applying -20 kV (reversed polarity, cathode in the inlet) for 20 min. Samples (organic acid standards and protease samples) were injected at 50 mbar for 15 s. Between runs, capillaries were conditioned by rinsing with 1 M NaOH (1 min), water (1 min) and BGE (3 min). At the beginning and at the end of a sequence of

analysis of protease samples, a 250 mg/L standard mixture containing acetic and gluconic acid was analyzed as a quality control. Data acquisition was performed with ChemStation software (version C.01.06, Agilent Technologies). A specific macro provided with the software needed to be installed to transform the raw electropherograms with negative peaks into positive peaks.

The CE instrument was also used to estimate the total amount of protein in the commercial protease from absorbance measurements at 280 nm. A calibration curve was established by analyzing BSA standard solutions at concentrations between 50 and 1000 µg/mL. BSA standards and protease sample were injected in triplicate for 10 s at 50 mbar. Infusion experiments were performed without voltage and applying 50 mbar of pressure after the injection. Absorbance was measured from the height of the detected protein peaks.

2.3.3. Method validation

Quality parameters were calculated by measuring peak areas and migration times (t_m) from the electropherograms obtained for the organic acids. Studies of intra-day (n=6 with one capillary) and inter-day repeatability (n=9 over three alternate days and with a new capillary each day) were performed by analyzing a standard mixture of acetic acid and gluconic acid at 250 mg/L each or a spiked protease sample at 50 mg/L each. These values were calculated as a percentage of relative standard deviation (%RSD) of peak areas and t_m. LOD for each organic acid was experimentally established by injecting the standard mixtures at decreasing concentrations until the analytes could not be detected (S/N=3). LOQ was given as the lower concentration limit of the linear ranges. External

and standard-addition calibration methods were used for the quantification of acetic acid and gluconic acid in the protease sample. Calibration was performed at nine (from 1 to 500 mg/L) and six (from 10 to 500 mg/L) levels of concentration, for external and standard-addition calibration methods, respectively, in triplicate at each level. Concentrations for acetic acid and gluconic acid were determined in triplicate extrapolating from three independent standard-addition calibration curves.

3. Results and discussion

3.1. Analysis of standard solutions

Determination of organic acids by CE with indirect UV detection can be achieved adding a UV absorbing compound in the BGE, but separation conditions must be carefully selected to avoid comigration of the target compounds and sample matrix components [26–34]. From the variety of additives described for indirect UV dectection, 2,6-PDC was selected because it has been widely applied before with excellent performance [26–30]. CTAB, a surfactant that is commonly used in the analysis of organic acids, was added as a modifier to change the direction of the electroosmotic flow (EOF) (anodic EOF) [29,30]. As pH was adjusted to 9.0 [32], reversed polarity (i.e. negative voltage or anode in the outlet) was applied to ensure migration of the anionic organic acids towards the detector in the outlet end. Ca^{2+} and Mg^{2+} ions and MeOH were also added to the BGE to enhance separation efficiency and selectivity. On the one hand, cations of the alkaline earth group have strong tendencies to form partially dissociated complexes in solution with the anions of organic acids. The most important parameters affecting complexation equilibrium are the type and concentration of the cation and the pH of the BGE [42]. On the other hand, the addition

223 of MeOH to the BGE reduce the EOF, hence increasing t_m and resolution. Furthermore,
224 the relative permittivity of MeOH is lower than that of water. This provides additional
225 possibilities for improving separation, because complexation reactions and formation of
226 high degree complexes are promoted in a low relative permittivity medium [32].
227
228 Figures 1 A-B show the electropherograms obtained by CE-UV (at 254 nm) for a blank
229 (i.e. pure water) and a 50 mg/L standard mixture of acetic acid and gluconic acid. The
230 raw electropherogram was transformed to show acetic acid (t_m ~ 5.5 min) and gluconic
231 acid (t_m ~ 7.3 min) as positive peaks. Both organic acids were originally detected as
232 negative peaks due their low absorption and the use of a BGE that contained a high UV
233 absorbing compound (2,6-PDC). Peak identification was performed by comparing t_m in
234 mixtures at different concentrations with those obtained in individual standard solutions.
235
236 In order to establish the external calibration curves, standard mixtures of both organic
237 acids were analyzed at concentrations between 1 and 500 mg/L. As can be observed in
238 Table 1-A, the method was linear between 2.5 and 500 mg/L for acetic acid and
239 between 5 and 500 mg/L for gluconic acid (coefficients of determination (R^2) > 0.998,
240 in both cases). LODs were 1 and 2.5 mg/L and LOQs were 2.5 and 5 mg/L for acetic
241 acid and gluconic acid, respectively. Intra-day repeatability of peak areas and t_m were
242 good, and %RSD values were 1.5 and 2.0% for peak areas and 0.8 and 1.5% for t_m (for
243 acetic acid and gluconic acid, respectively), which are the typical values for CE-UV
244 [35]. Inter-day repeatability was also satisfactory, and %RSD values were 2.0 and 2.5%
245 for peak areas and 2.7 and 3.5% for t_m (for acetic acid and gluconic acid, respectively).
246
247 **3.2. Sample pretreatment optimization**

For the analysis of the organic acids in the protease sample, a sample pretreatment was necessary because the sample matrix contained a high protein content (i.e. 50% m/m of protein in the studied protease sample, from the UV absorbance at 280 nm). Different procedures for the extraction of acetic acid and gluconic acid were tested, which were originally developed for the analysis of organic acids in food [43] or for the precipitation of the most abundant proteins from blood plasma or serum [44,45]. The procedures consisted of extracting with 80% (v/v) EtOH (sample:solvent 1:2 m/v), 80% (v/v) EtOH with 1 M NaOH (sample:solvent 1:2 m/v) and ACN (sample:solvent 1:6 m/v). Peak identification was performed by spiking the protease sample with a standard mixture at a concentration of 50 mg/L of acetic acid and gluconic acid and comparing t_m with those obtained for standard mixtures. Figures 2 A-C show the electropherograms obtained by CE-UV for the spiked protease samples after extracting in the different conditions. As it is shown in Figure 2, only the use of 80% (v/v) EtOH (sample:solvent 1:2 m/v) (Figure 2 A) allowed a reliable identification of the extracted acetic acid and gluconic acid (t_m ~ 5.5 and 7.3, respectively, as in the standards, see Figure 1). For this reason, 80% (v/v) EtOH was chosen for further studies about organic acid recoveries.

Different ratios between the protease sample and the hydroalcoholic solvent were tested to optimize recoveries: 1:2 m/v (50 mg sample, 100 µL solvent), 1:5 m/v (20 mg sample, 100 µL solvent) and 1:10 m/v (20 mg sample, 200 µL solvent). The organic acid recoveries were estimated from a comparison of the peak areas obtained for the protease sample spiked at 50 mg/L with the standard mixture and the unspiked protease sample. For the calculations, a 100% recovery was considered for the 50 mg/L standard mixture of acetic acid and gluconic acid (Figure 1 B). As an example, Figures 3 A-B

show the electropherograms obtained by CE-UV after extraction with 80% (v/v) EtOH (sample:solvent ratio of 1:5 m/v) for the protease sample spiked at 50 mg/L with acetic acid and gluconic acid and the unspiked protease sample, respectively. These results can be compared with Figure 1 B that shows the electropherogram obtained by CE-UV for a 50 mg/L standard mixture of the organic acids. Recoveries for acetic acid and gluconic acid with the 1:2 m/v ratio were 40% and 59%, respectively, 50% and 81% with the 1:5 m/v ratio, and 51% and 70% with the 1:10 m/v ratio. As can be observed, recoveries for acetic acid did not change significantly, especially between ratios 1:5 and 1:10 (around 50% in both cases). However, recoveries for gluconic acid were maximum (81%) with the 1:5 ratio and decreased (70%) when the solvent proportion was increased. For this reason, extraction with EtOH 80% (v/v) (sample:solvent 1:5 m/v) was selected for further method validation.

3.3. Quantification of organic acids

Once the sample pretreatment was optimized, validation of the complete procedure was performed with the spiked samples by means of investigating intra-day and inter-day repeatability, as well as the standard-addition calibration method to find possible matrix effects. Intra-day repeatability of peak areas and t_m were good, taking into account the complexity of the extracts, and %RSD values were 4.5 and 7.1% for peak areas and 0.3 and 0.5% for t_m (for acetic acid and gluconic acid, respectively). Inter-day repeatability was also satisfactory, and %RSD values were 7.4 and 9.9% for peak areas and 3.3 and 4.4% for t_m (for acetic acid and gluconic acid, respectively). As can be observed in Table 1-B, the linearity ranges were slightly shorter with the standard-addition calibration method compared to the values obtained with the external calibration

method (2.5-500 mg/L vs. 25-500 mg/L for acetic acid and 5-500 mg/L vs. 50-500 mg/L for gluconic acid, see Table 1 A-B), and LODs and LOQs were ten times higher. Moreover, the sensitivity given by the linear regression slopes significantly decreased (Table 1 A-B), indicating a matrix effect, which needed to be taken into account for an accurate quantification of the organic acids in the protease sample. The standard-addition calibration method allowed determining that the total concentration of acetic acid and gluconic acid in the protease sample was 80 mg/Kg (0.0080% (m/m)) and 69 mg/Kg (0.0069% (m/m)), respectively.

4. Conclusions

In this work, a rapid and simple CE method with indirect UV detection for the separation of two organic acids in less than 8 min was successfully developed and validated. The method was applied to the determination of acetic acid and gluconic acid in a commercial protease sample, after optimizing an appropriate sample pretreatment based on extraction with 80% v/v of EtOH. Intra-day and inter-day repeatability were good, but the standard-addition calibration method was necessary for a reliable quantification due to the presence of matrix effects. LODs were low enough for an accurate quantification of both acids in the protease sample at 80 mg/Kg (0.0080% (m/m)) and 69 mg/Kg (0.0069% (m/m)), respectively. The developed method could be easily applied for monitoring acetic acid and gluconic acid in the production of proteases or other enzymes, but also could be explored to analyze other organic acids as part of the quality control process. More broadly, the proposed method could be also investigated for the analysis of organic acids in other complex matrices with high protein content, as quality indicators in a wide range of applications, dealing with food,

biological and environmental samples. In the applications dealing with other organic acids and/or complex samples, the method would probably need to be slightly reoptimized to ensure appropriate results.

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The authors declare no conflicts of interest.

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Figure legends

Figure 1. CE-UV electropherograms (254 nm) obtained for (A) a blank (i.e. pure water) and (B) a 50 mg/L standard mixture of acetic acid and gluconic acid.

Figure 2. CE-UV electropherograms (254 nm) obtained for a spiked protease sample at 50 mg/L with acetic acid and gluconic acid after extracting the organic acids with (A) 80% (v/v) EtOH (sample:solvent 1:2 m/v), (B) 80% (v/v) EtOH with 1 M NaOH (sample:solvent 1:2 m/v) and (C) ACN (sample:solvent 1:6 m/v).

Figure 3. CE-UV electropherograms (254 nm) obtained after extracting the organic acids with 80% (v/v) EtOH (sample:solvent 1:5 m/v) for (A) a spiked protease sample at 50 mg/L with acetic acid and gluconic acid and (B) an unspiked protease sample.

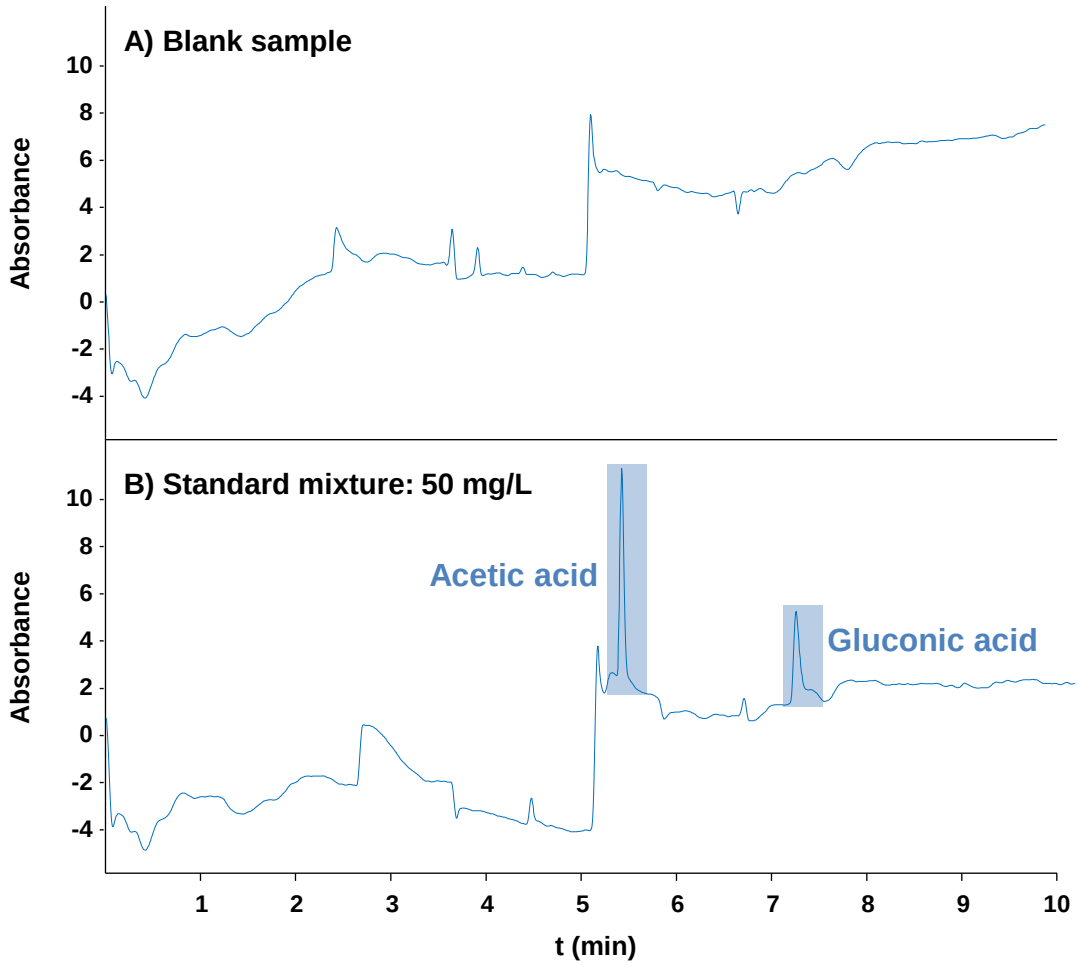
A) Blank sample
B) Standard mixture: 50 mg/L
Acetic acid
Gluconic acid
Absorbance
Absorbance
t (min)
10
8
6
4
2
0
-2
-4
1
2
3
4
5
6
7
8
9
10

Figure 1

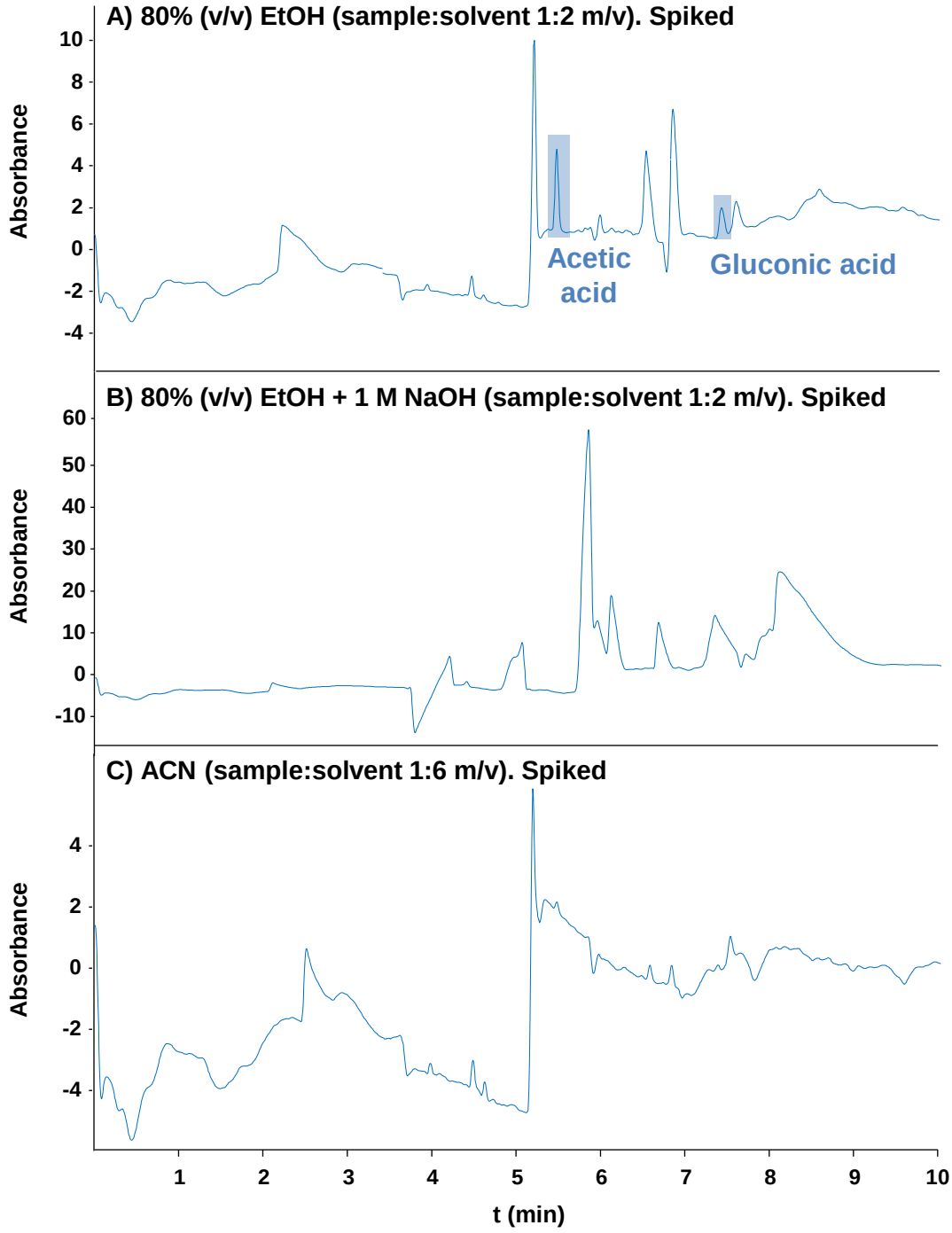
A) 80% (v/v) EtOH (sample:solvent 1:2 m/v). Spiked
Absorbance
10
8
6
4
2
0
-2
-4
Acetic acid
Gluconic acid
B) 80% (v/v) EtOH + 1 M NaOH (sample:solvent 1:2 m/v). Spiked
Absorbance
60
50
40
30
20
10
0
-10
C) ACN (sample:solvent 1:6 m/v). Spiked
Absorbance
4
2
0
-2
-4
1
2
3
4
5
6
7
8
9
10
t (min)

Figure 2

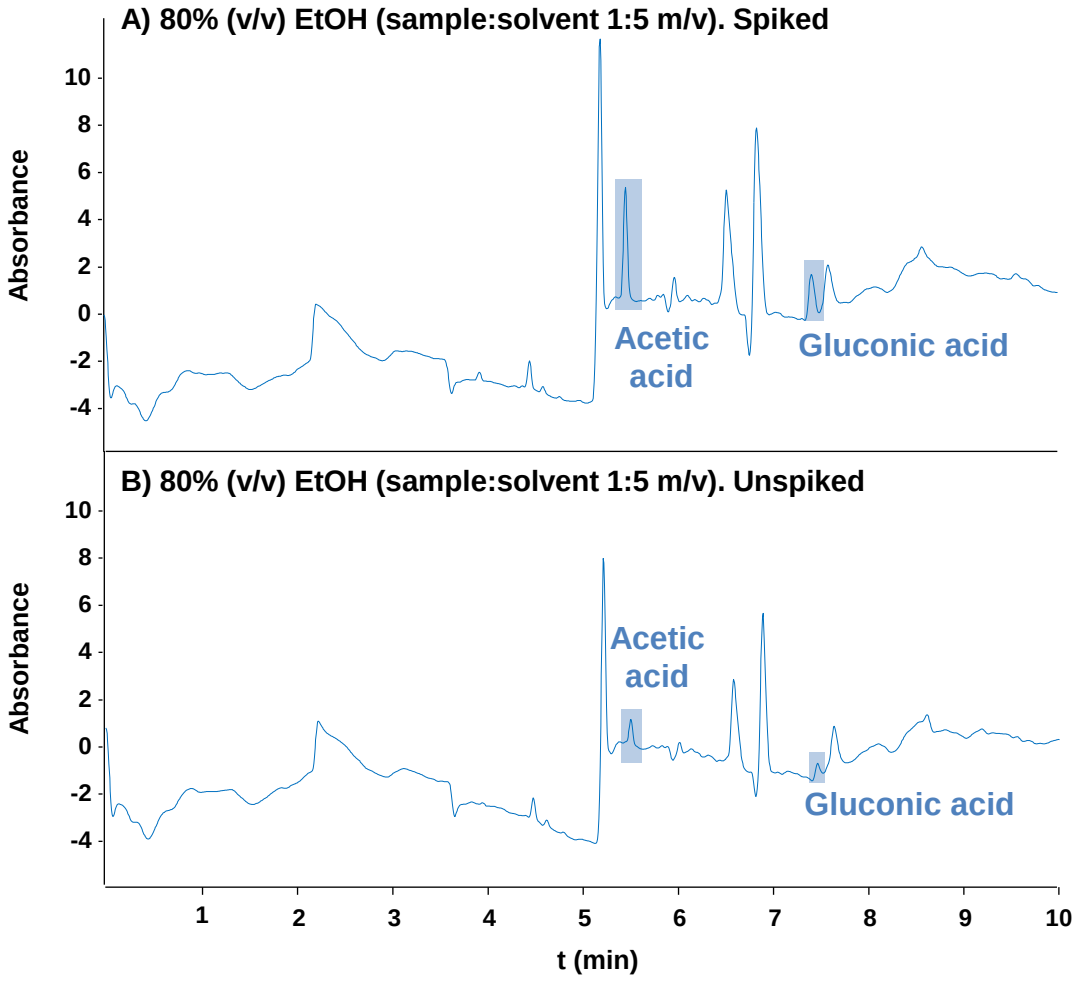
A) 80% (v/v) EtOH (sample:solvent 1:5 m/v). Spiked
Absorbance
10
8
6
4
2
0
-2
-4
Acetic acid
Gluconic acid
B) 80% (v/v) EtOH (sample:solvent 1:5 m/v). Unspiked
Absorbance
10
8
6
4
2
0
-2
-4
Acetic acid
Gluconic acid
1
2
3
4
5
6
7
8
9
10
t (min)

Figure3

Table 1. Calibration curves, regression coefficients (R^2), linear ranges, LOD and LOQ obtained by CE-UV for acetic acid and gluconic acid using (A) external calibration and (B) standard-addition calibration.

Quality parameters	(A) External calibration	
	Acetic acid	**Gluconic acid**
Calibration curve	y = 0.545x - 2.35	y = 0.290x - 0.707
R^2	0.998	0.999
Linear range (mg/L)	2.5-500	5-500
LOD (mg/L)	1	2.5
LOQ (mg/L)	2.5	5
	(B) Standard-addition calibration	
Calibration curve	y = 0.371x - 5.93	y = 0.187x - 2.57
R^2	0.998	0.995
Linear range (mg/L)	25-500	50-500
LOD (mg/L)	10	25
LOQ (mg/L)	25	50