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Determination of D-saccharic acid-1,4-lactone from brewed kombucha broth by high-performance capillary electrophoresis

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ABSTRACT

Kombucha is a health tonic. D-Saccharic acid-1,4-lactone (DSL), a component of kombucha, inhibits the activity of glucuronidase, an enzyme indirectly related with cancers. To date, there is no efficient method to determine the content of DSL in kombucha samples. In this paper, we report a rapid and simple method for the separation and determination of DSL in kombucha samples, using the high-performance capillary electrophoresis (HPCE) method with diode array detection (DAD). With optimized conditions, DSL can be separated in a 50 cm length capillary at a separation voltage of 20 kV in 40 mmol/L borax buffer (pH 6.5) containing 30 mmol/L SDS and 15% methanol (v/v). Quantitative evaluation of DSL was determined by ultraviolet absorption at λ = 190 nm. The relationship between the peak areas and the DSL concentrations, in a specified working range with linear response, was determined by first-order polynomial regression over the range 50–1500 μg/mL with a detection limit of 17.5 μg/mL. Our method demonstrated excellent reproducibility and accuracy with relative standard deviations (RSD) of less than 5% DSL content (n = 5). This is the first report to determine DSL by HPCE. We have successfully applied this method to determine DSL in kombucha samples in various fermented conditions.

1. Introduction

Kombucha, a fermentation tea beverage produced from a symbiosis of yeast species and acetic acid bacteria, has been consumed in Asia for over two millennia. It is becoming increasingly popular around the world today [1]. The beverage has been claimed to be a prophylactic agent and to be beneficial to human health [2–4]. Some of the claimed effects have been proven [5–9]. Studies have detected the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins and antibiotics in the brewed solution [10–15].

β-Glucuronidase is an enzyme responsible for hydrolyzing glucuronides in the lumen of the gut. This reaction generates toxic and carcinogenic substances. β-Glucuronidase promotes cancerous formations by hydrolysing conjugated glucuronides to their carcinogenic aglyconic compounds (normally, these carcinogenic compounds could be detoxified by glucuronide formation in the liver and then enter the bowel via bile). Thus, toxic aglycones are regenerated *in situ* in the bowel by bacterial β-glucuronidase. In humans, fecal β-glucuronidase activity was shown to be higher in colorectal cancer patients as compared to healthy controls, suggesting a role of this enzyme in carcinogenesis [15–16]. D-Saccharic acid-1,4-lactone (DSL) is the competitive inhibitor of β-glucuronidase [17]. The concentration of inhibiting effect of DSL differs from the substrates. For example, addition of the specific inhibitor DSL (1 mmol) could completely block the cleavage of the enzyme substrate, so nonspecific hydrolysis of the enzyme could be excluded [18]. β-Glucuronidase of the feces of a healthy human and of a human with colon cancer were markedly inhibited by DSL at the concentration from 0.03 to 0.15 mg/mL [19]. These studies have shown that only a small quantity of DSL can inhibit the activity of β-glucuronidase. DSL can also diminish greatly the loss of heparin, hyaluronic acid, sulphated glycosaminoglycans and glucuronic acid [14,15]. DSL has been discovered in kombucha with varied concentrations and are considered to be the most healthful component in kombucha [15,22–25]. Effective detection and identification of DSL in kombucha sample is of significance in explaining the beneficial effects of kombucha, and in the quantitative analysis in the secondary metabolism of DSL.

Historically, several methods have been used in analyzing components in brewed kombucha, and these include high-performance liquid chromatography (HPLC) [10], thin-layer chromatography (TLC) [13], and mass spectrometry (MS) [15]. The majority of the existing literature used gas chromatography (GC) methods to determine DSL [20–24]. The main drawback of these methods is the complexity in sample pretreatment procedures and in the intricate chemical reactions required [20]. These methods involve many reactive substances and intermediates. Another worrisome point is

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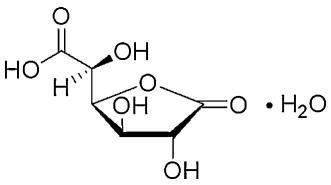

Fig. 1. Molecular structures of DSL.

the lack of technical parameters reported in the GC works. In our lab, even after repeated efforts we were never able to achieve sufficient resolution of the standard DSL following the published protocols. Up to today, there is no report on the determination and separation of DSL in brewed kombucha samples using HPCE. In this paper, a simple and selective HPCE method with DAD is established for the separation and quantitative determination of DSL in different kombucha samples.

2. Experimental

2.1. Materials

D-Saccharic acid-1,4-lactone monohydrate ($C_6H_8O_7 \cdot H_2O$, analytical grade, >99.7%) (DSL), of which the molecular structure was shown in Fig. 1, was purchased from Sigma (Germany). Methanol (HPLC grade, >99.9% purity) was purchased from TEDIA (Fairfield, OH, USA). Sodium dodecyl sulphonate (SDS) was purchased from Merck, Darmstadt, Germany (analytical grade, >99.7%). Distilled, deionized water (Milli-Q Water Systems, Millipore Corporation, Bedford, MA, USA) was used to prepare all aqueous solutions. All other chemicals used were of analytical reagent grade. There were five kombucha tea samples: two were from our laboratory (Lab of Microbiology, Anhui Agriculture University, Hefei, China); others were collected from three individual households in Anhui.

2.2. Instrumentation

All CE separations were conducted on a Beckman P/ACETM MDQ Capillary Electrophoresis system (Beckman Coulter, USA) equipped with an autosampler and a DAD detector. System control and data capture were processed with 32 Karat 5.0 software. The electrophoresis separation was performed on a fused-silica capillary of 75 μm I.D. × 50 cm (40 cm effective length).

2.3. Sample preparation

The DSL standard was prepared by quantitatively diluting the stock solution with pure water, and the stock solution was diluted to the desired concentrations just prior to use. Two milliliter of each kombucha sample was taken to lyophilize at −80 °C for more than 12 h. Afterwards the lyophilized material was extracted by sonication for 20 min with 4 mL pure water. Finally, the samples were centrifuged at 8000 r/min. Each supernatant was filtered through a syringe cellulose acetate filter (0.22 μm) prior to HPCE analysis. All samples were properly diluted, and duplicates were made.

2.4. Chromatography

Running buffer was made up with 40 mmol/L borax buffer ($Na_2B_4O_7$–KH_2PO_4), 30 mmol/L SDS, 15% methanol (v/v), with pH of 6.5. Sample solutions, standard solutions and the running buffer were all filtered through a syringe cellulose acetate filter (0.22 μm) prior to use. CE was performed at separation voltage of 20 kV with the running buffer. Sample injection was performed hydrodynamically at 3.45 kPa for 5 s. Capillary temperature was controlled at 25 °C and detection wavelength was set at 190 nm. The capillary was washed with 0.1 mol/L NaOH and pure water for 2 min, respectively, and then rinsed with running buffer for 5 min at 137.9 kPa before the next run.

3. Results and discussions

3.1. Selectivity

The method of addition of standard DSL sample to kombucha was used to prove qualitatively the effective separation of DSL and to determine the exact retention time of the eluted DSL peak. The chromatograms of the standard DSL sample and the separation of DSL from other components in a representative kombucha sample are showed in Fig. 2. "a" points to the peak presenting DSL levels. Excellent baseline separation was obtained between DSL and other components. Migration time for DSL was 20.6 min.

3.2. Calibration and working range

Standard curves were established from serial dilution of the stock DSL solutions. The relationship between peak area and the amount of substance applied, in a specified working range with linear response, was determined by first-order polynomial regres-

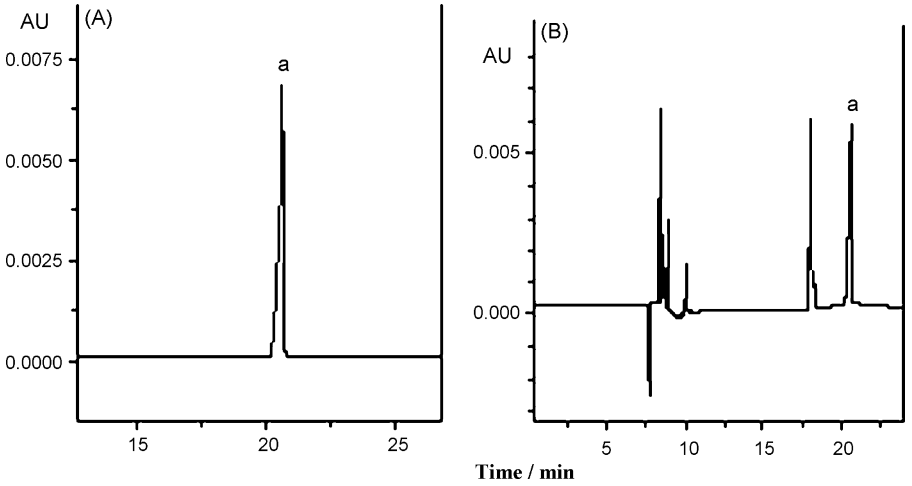

Fig. 2. (A) HPCE electropherogram of standard sample of DSL(a) and (B) HPCE electropherogram showing the separation of DSL(a) from other components in a representative kombucha sample; working conditions as in experimental.

Table 1
Determination of the recovery from this method[a].

Known content of DSL (μg/mL)	Added content (μg/mL)	Found content[b] (μg/mL)	Recovery (%)	RSD (%)
78.9	50	125.2	97.1	3.26
78.9	200	271.5	97.4	3.05
78.9	500	543.07	94.5	4.89

[a] Working conditions as in experimental.
[b] Average from five experiments.

sion over the range 50–1500 μg/mL for DSL. The limit of detection (LOD) is at 17.5 μg/mL with a signal-to-noise ratio of 5 ($S/N = 5$). The regression equation was $y = 65.43x + 304.79$, where y is the peak area and x the concentration of standard DSL (μg/mL) ($n = 8$, $R^2 = 0.9941$).

3.3. Repeatability

Repeatability was determined by repeated analysis of a homogeneous sample using the same analytical procedure and the same equipment in the same laboratory. A representative sample was determined by HPCE analysis. The proposed method demonstrated excellent repeatability with relative standard deviations (RSD) of less than 5% for DSL ($n = 10$).

3.4. Accuracy

The accuracy of the method, which gives information about the recovery of the analyte from the sample and about matrix effects, was examined by spiking experiments, using in-system calibration of sample solutions of known (previously determined) DSL content. The results were given in Table 1.

3.5. Separation and quantitative determination of DSL

According to the procedure of experiment, sample solutions were prepared for HPCE analysis. DSL separation and quantitative determination of DSL in different kombucha samples were investigated. The results are shown in Table 2.

Great differences of DSL content were shown in different kombucha samples. This difference may be due to the different origins of kombucha strains and different nutrition conditions [22,25]. Different strains may have dissimilar pathways of metabolism in fermented kombucha. Thus, DSL contents vary in kombucha samples.

3.6. Optimization of buffer parameters

We designed five different buffer systems to separate the standard sample.

When borax–phosphate buffer was at 100 mmol/L (pH 9.2), the sample could not be detected. The electrosmotic flow (EOF) fluctuates severely, most likely because of the high concentration of the buffer. When the buffer was reduced to 50 mmol/L (pH 9.2), it was still impossible to detect the DSL sample because the sample peak was too close to the EOF peak. Three voltage values, 18, 20 and 25 kV, were tried, respectively, and the sample peak could not be separated from the high EOF peak. As the voltage increased, the migration time of the sample peak decreased. There were several small peaks following the high EOF peak with one nearly overlapping the sample peak when voltage was at 20 kV.

The sample peak was also close to the high EOF peak when the concentration of the buffer was decreased to 25 and 40 mmol/L, respectively, and the limit was quite high.

In the borax–phosphate buffer of 40 mmol/L (pH 9.2) supplied with SDS at 50 mmol/L and 7.5% of acetonitrile (v/v) the limit was very high. There was a peak which was not changed with the concentration of sample and therefore it was not the sample peak. The specific sample peaks were neither in the buffer of borax–phosphate (pH 9.2) at 40 mmol/L with 0.05% of hydroxy propyl cellulose nor in the borax–phosphate buffer (pH 9.8) at 30 mmol/L after running for more than 25 min.

Since the sample is a weak acid, theoretically a basic buffer should be used to detect the sample. But from the data above, it is clear that strong basic buffer is not suitable for the task. It is most likely because the acid is too weak to be ionized for separation.

In order to optimize the buffer system, citrate–phosphate buffer with varied concentrations, pH values and pressure were designed to separate the sample. A buffer pH 2.5 was used and the pressure was adjusted to 0, 6.88, 13.75, 20.62 kPa, respectively. There were no sample peaks without pressure. Sample peaks appeared when pressure was applied. It was observed that the higher the pressure, the shorter the migration time, and the smaller and the more miscellaneous the peaks become. After a few tries, pressure was fixed at 6.88 kPa, and voltage adjusted to 20 and 25 kV, respectively. The results showed that the higher the voltage, the lower and flatter the peaks, and the longer the migration time. So 20 kV is a suitable value for electrophoresis. Under this condition there was a big peak for standard DSL sample. The peak position changed after the sample was diluted in methanol. Furthermore, the area of the peaks did not change accordingly with the dilution of the sample. A high peak appeared when the sample was dissolved in water. The peak was almost invisible at 190 nm, but was clearer at 276 nm.

The concentration of acid citrate–phosphate buffer (pH 2.5) was decreased to 60 and 80 mmol/L, respectively. Both the standard sample and the sample appeared at 4.2 min. Unfortunately, these peaks could not be totally separated because of the flatter bottom of the peaks. Observations were similar with citrate–phosphate buffer of 50 mmol/L both at pH 3.0 or pH 4.5.

The sample did not appear until 25 min in 50 mmol/L phosphate buffer (pH 4.0).

Though the sample could be separated in acid buffer either dissolved in methanol or in water, the sensitivity was too low with flatter peaks and high EOF. Pressure higher than 6.88 kPa did not benefit the separation of the sample.

Neutral phosphate buffers of 25 mmol/L were designed and the peak of standard DSL, with good shape and proportionally to the concentration of the sample, appeared at 12 min from pH 5.5 to pH 8.5. Yet the standard DSL added in the kombucha sample could not

Table 2
DSL content in different kombucha samples[a].

Sample[b]	DSL content (μg/mL)[c]
A	70.98 ± 2.39
B	ND
C	57.99 ± 1.98
D	132.72 ± 5.13
E	78.90 ± 2.85

ND: not detected.
[a] Working conditions as in experimental.
[b] Samples A, B, C were collected from families.
[c] Average from five experiments.

be separated from two front peaks of some other components of kombucha, even though 30 mmol/L SDS or 15% of acetonitrile (v/v) was added in buffers.

A neutral borax–phosphate buffer of 40 mmol/L was also designed with pH being adjusted to 5.5, 6.5, 7.5 and 8.5, respectively. Standard DSL could be separated well at pH 6.5 from the point of view of resolution and shorter migration time. Yet the standard DSL added in the kombucha sample could not be satisfactorily disjoined from other peaks, so addictives were considered to be used in the experiment.

With other conditions being equal, the pH of borax-phosphate buffer was adjusted to 4.5, 6.5, 7.5 and 8.5, respectively. The migration time increased with increased pH values, and the resolution of DSL in kombucha samples was not satisfied at pH 4.5. Therefore, based on the criteria of high separation efficiency and shorter running time, pH 6.5 was considered as the optimal value.

The concentration of the buffer not only affects the Zeta electric potential of the interior face of capillary column, but also changes the viscosity coefficient of buffers and the diffusion coefficient of solutes to be analyzed. Thus it affects the resolution and migration time of the solute. The concentration of borax buffer of pH 6.5 was controlled to 20, 40 and 80 mmol/L, respectively, and all treatments were supplied with 30 mmol/L SDS and 15% methanol (v/v). The sample peak was disturbed by background noise and was badly shaped in the buffer of 20 mmol/L. The migration time was much longer in the buffer of 80 mmol/L, and the sample peak was separated very well with high resolution and shape in the buffer of 40 mmol/L. Therefore, we settle on a suitable concentration of 40 mmol/L.

Surfactants and organic modifiers in the buffer would affect the resolution and the selectivity of solutes to be analyzed. In order to reach a compromise between these two interactive factors, a simultaneous optimization strategy based on an iterative progression process was designed. Based on the electrophoresis conditions optimized above the effects of methanol and SDS were further probed. When methanol was added in the buffer to 6% (v/v) the low selectivity happened, and the standard DSL added in kombucha could not be separated satisfactorily from the miscellaneous peaks of other kombucha components, no matter which concentration values, 10, 30 or 50 mmol/Lof SDS, were used. With methanol being fixed to 15%, the peak of DSL would overlap the frontal one when SDS was at 10 mmol/L. Good separation with longer migration time (>25 min) happened when SDS was at 50 mmol/L. Finally we found that 15% of methanol plus 30 mmol/L SDS would bring acceptable separation with a sufferable migration time of 20 min.

The use of a surfactant (SDS) might increase the resolution between DSL and other similar molecules in sample and therefore the migration time because of the negative charge of sulfate group of the SDS. On the other hand, an increase of concentration of organic solvent could enhance the solubility of DSL in system here. Since organic solvents decrease the EOF and Joule's heat during electrophoresis, high voltage should be used to increase the resolution of electrophoresis.

For the borax buffer with parameters optimized above, voltage of 16 kV during electrophoresis would bring quite good separation of DSL with migration time longer than 25 min. 25 kV resulted in a low resolution because of the heat effect of increased current, whereas 20 kV leaded to satisfactory separation with migration time for 20.6 min.

4. Conclusions

The GC method [20,21] involves complicated pretreatments and chemical reactions for kombucha samples. It makes the determination of DSL in kombucha very difficult in common labs. The modified GC method [22], though with short migration time, could not be repeated with multiple attempts in our lab.

The HPCE method newly established in this study for the separation and determination of DSL in kombucha samples was highly selective, reproducible and simple to use. Meanwhile, this proposed method has adequate reproducibility and accuracy with RSD less than 5% ($n = 5$). It has been successfully used to determine the content of DSL in several kombucha samples. The shortcomings of HPCE method optimized here are (1) quite long migration time and (2) relatively high LOD in comparison to GC (which is a common shortcoming of HPCE).

The results of this paper showed that the main DSL constituents can be well separated in a 40 cm length capillary at a separation voltage of 20 kV in a 40 mmol/L borax buffer (pH 6.5) containing 30 mmol/L SDS and 15% methanol (v/v). Baseline separation can also be obtained.

The relationship between peak areas and concentrations of DSL, in a specified working range with linear response, was established; and quantitative evaluation of DSL in different kombucha samples was determined by HPCE method. The result also showed that the DSL content were different in various kombucha samples, consistent with previous reports [22,25].

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