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# Field-amplified sample injection brings surprise: an efficient method to determine the content of organic acids in chinese honey using high-performance capillary electrophoresis

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**Abstract.** Organic acids are responsible for the antimicrobial capacity of honey. Herein, field-amplified sample injection (FASI) was developed to improve the sensitivity of capillary electrophoresis in the determination of 7 organic acids in 8 honey samples from different geographical origins in China. Parameters that affect FASI were systematically investigated including pH, injection time, water plug length, buffer concentration, etc. The detection sensitivity was obviously enhanced at optimal conditions with an increased concentration factor of 8-to 72-fold. The detection limits of tartaric, protocatechuic, chlorogenic, oxalic, gallic, citric, and vanillic acids were 0.56, 0.26, 0.85, 0.90, 0.65, 0.52, and 0.55mg $L^{-1}$, respectively. The current developed method was successfully applied in the detection of organic acids in real honey samples.

**Key words.** Field-amplified sample injection, capillary electrophoresis, organic acids, honey.

## 1. Introduction

Honey is the natural sweet product made by honeybees from nectar of flowers or from the secretion of living parts of plants, which is combined with specific substances in honeybees to ripen.[1-3] Honey primarily contains water and highly concentrated sugar. The water content is approximately 17%. Sugar accounts for 95-99% of honey

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dry weight. The sugars include simple sugars such as fructose (about 38.2%), glucose (about 31.3%), and sucrose (about 1%). Other components includes organic acids (about 0.57%), proteins (about 0.26%), amino acids (about 0.1%), nitrogen (about 0.41%), and mineral (about 0.17%).[4] Some of these are the metabolism product of honeybees, and some are derived from plants.[5]

The antimicrobial and antioxidant capacity of honey is the combined activity of phenolics, peptides, organic acids, enzymes, etc.[6] Its hyperosmolarity (Aw: 0.6) and acidity (pH=3.4-6.1)detrimental for most micro-organisms.[1]Organic acids are responsible for the acidity although the content is only about 0.57%.[7]Organic acids can be obtained directly from nectar or be derived from sugars by enzymes secreted by honeybees when they transfer the nectar into honey.[8]Many organic acids from different regions of the world are detected in honey: aspartic acid, butyric, citricand others.[8-10]To establish methods of ascertaining the content of organic acid in honey is always a hot topic for analytical chemists.A rapid capillary electrophoresis (CE) method with direct ultraviolet (UV) detection was set up and developed to determine the most important nonaromatic organic acids in honey with a really simple treatment of the sample.[9]The use of two ion-exclusion columns coupled in series allowed the liquid chromatographic determination of the oxalic acid content in honey, which avoided interference from other components of the matrix.[10]Recently, organic acids in honey were determined repeatability by a rapid HPLC method.The analytical methods for the determination of organic acids in honey were reviewed by Mato's group.

As far as we know, as one of the most important analytical method, CE was less frequently used in the determination of organic acids in honey compared to other analytical methods, such as HPLC. Short-chain organic acids from three types of medicinal plants were analyzed with diode array-CE method, which can be applied on other natural products such as honey. The sensitivity of capillary-ultraviolet detection is strictly limited by its sample injection volume (nL grade) and narrow optical path length (id of the capillary). As one of the on-column concentration techniques, the field-amplified sample stacking injection (FASI) can greatly improve the detection sensitivity of CE-UV, which is based on the discrepancy of electrical resistivity between sample solution and background electrolyte(as shown in Figure 1). A new micellar electrokinetic CE method combined with FASI has been developed for the analysis of isonicotin amide and nicotinamide in whitening cosmetics and supplemented foodstuffs. It was found that components in honey are commonly different because of the seasonal climatic variations or a different geographical origin, even for honey of the same floral source.[2] Herein, we want to develop a method to determine7 typical organic acids (tartaric, protocatechuic, chlorogenic, oxalic, gallic, citric, and vanillic acids) in Chinese honey with CE. The molecular structures of these organic acids were shown in Scheme 1. The honey samples were collected from different geographical origins in Henan province of China during April, May, June, and July in 2016, as shown in Table 1(details of the location and species of sample). Influences of parameters such as pH, concentrations of buffer and hexadecyl trimethyl ammonium bromide (CTAB) solution, water plug length, injection voltage and time on the analysis were studied and the applicability of FASI in honey sample

was evaluated.

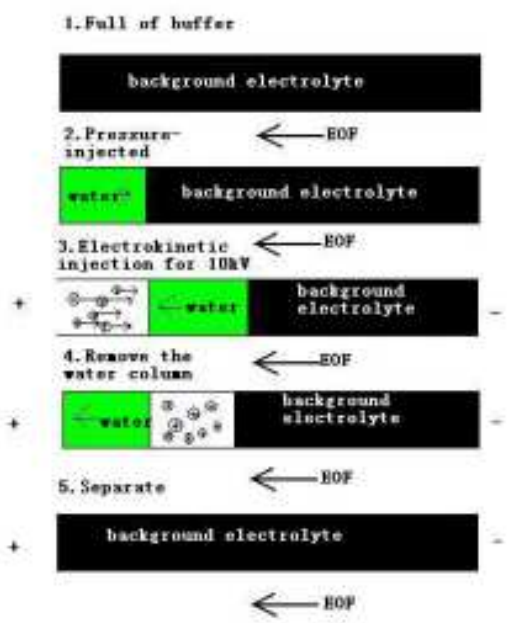


Fig. 1

## The mechanism and process of FASI-CE

Table 1. The mechanism and process of FASI-CE

| **Table 1** Geographical distribution of honey samples. The units of longitude and latitude are degree. | | | | |
|---|---|---|---|---|
| Sample | Area | Longitude | Latitude | Species |
| $S_1$ | Huanggou Cun | 119.52 | 36.85 | Tong nectar |
| $S_2$ | Dongjiang Cun | 114.32 | 34.67 | Tong nectar |
| $S_3$ | Lianglou Cun | 115.25 | 34.05 | Acacia honey |
| $S_4$ | Yuanping Cun | 114.32 | 34.67 | Acacia honey |
| $S_5$ | Chenling Cun | 110.35 | 32.08 | Acacia honey |
| $S_6$ | Xiaoyouhe Cun | 112.45 | 35.04 | Acacia honey |
| $S_7$ | Anping Cun | 112.68 | 41.89 | Rape nectar |
| $S_8$ | Dongshui Cun | 114.82 | 58.62 | Vitex honey |

## Materials and Methods

### Reagents and Materials

1 mg $ml^{-1}$stock standard solutions of tartaric, protocatechuic, chlorogenic, oxalic, gallic, citric, and vanillic acids (Analytical grade, Aladdin Reagent, Shanghai, China) were individually prepared with ultrapure water (UPW) which was prepared with Ultra-Pure-Water purification system (Z14030617, Youpu, Chengdu, China) and stored at 4 °C away from direct light. Then these solutions were diluted with water to desired concentration prior to use. Honey samples, including chaste, False Acacia, and rape flower honeys, were collected from Jiyuan, Anyang, Jiaozuoin Henan provinces of China. All other chemicals, including sodium hydroxide, hydrochloric acid, sodium tetraborate, phosphoric acid, CTAB, hydrochloric acid, are of analytical grade. Background electrolyte (BGE)(pH=8.5-9.0) were prepared with 20 mol $L^{-1}$sodium tetraborate and phosphoric acid. 0.7 mmol $L^{-1}$CTAB was used as electro-osmotic flow modifier, and water was used as selective modifier. All other solutions were prepared with water and stored at 4 °C in the refrigerator. Before use, these solutions were filtered through degassed by ultra-sonication for 10 min.

### Instrumentation

The real samples were firstly oscillated for 30 min in Multi-functional Oscillation Extractor (Hua-ou company, Jintan, China). The honey solutions were evaporated with a rotary evaporator (Ya-rong Biochemistry Instrument Company, Shanghai, China). CE experiments were performed on an Agilent G1600AX Capillary Electrophoresis (Agilent, Santa Clara, USA) instrument equipped with a UV detector. Uncoated fused-silica capillary tube of 40 cm (31.5 cm to detector, 50 $\mu$m id $\times$ (355 $\pm$ 10) $\mu$m od) with a polyimide outer coating was bought from Yongnian Optical Fiber (Hebei, China). Ultrasonic cleaning instrument (KQ5200DE, Ultrasonic Instrumental Company, Kunshan, China) and a centrifuge (Xiangyi H1650, Changsha, China) were used for real sample pretreatment. All solid reagents were balanced with a precision electronic balance (Beijing Sartorius Limited Company, Beijing, China). The Oasis HLB solid phase extraction (SPE, 6cc LP Extraction Cartridge) column was bought from Waters Company in USA.

### Real Sample Preparation

10 g of honey samples (from Jiyuan, Anyang, and Jiaozuo in Henan province) were solved in 30 mL hydrochloric acid (pH=2) and oscillated by 10 min.The extracted solution was eluted for 3 times with 3 mL hydrochloric acid (pH=2). The eluted solution was centrifuged for 10 min at 7000 r $min^{-1}$. The supernatant was eluted with 10 mL water in aSPE column, followed by 5 mL methanol and 10 mL hydrochloric acid. Then the column was eluted with methanol. 20 mL eluent was collected and the solvent was evaporated with rotary evaporation. The final extract was solved with 1 mL methanol and filtered through a 0.22 $\mu$m membrane prior to the analysis by FASI-CE procedure.

### Experiment Procedures

### Procedure of CE

New capillaries were rinsed sequentially with 0.1 mol $L^{-1}$ NaOH for 10 min, water for 10 min, 0.1 mol $L^{-1}$ HCl for 10 min, water for 10 min, and buffer solution for 8 min. Then these capillaries were rinsed with 0.1 mol $L^{-1}$ NaOH for 3 min, water

for 1 min, buffer solution for 5 min in sequence for daily use, and were rinsed with water for 10 min when all experiment finished every day. Hydrodynamic injection was carried out at 0.8 psi. All CE procedure was controlled by an Agilent Capillary Electrophoresis Online System.

**Procedure for FASI-CE**

A water plug was introduced into the capillary with hydrodynamic injection at 0.8 psi. A high negative voltage (-8kV) was then applied to electro-kinetically when introduce the sample into the capillary for a time of 30 s. When both electrode reservoirs were filled with BGE, the separation of tartaric, protocatechuic, chlorogenic, oxalic, gallic, citric, and vanillic acids was performed at a voltage of -25 kV across the capillary.

**Results and Discussion**

**Optimization of FASI Conditions**

An online pre-concentration method was adopted to improve the detection sensitivity of FASI-CE. Analytes are not always suitable for FASI-CE when it can be separated by direct CE. So, in the current study, the feasibility of FASI-CE in the detection of organic acids was evaluated firstly. Namely, the stacking factors, including pH, concentration of buffer solution, concentration of CTAB, water plug length, injection voltage and time were investigated one by one to get the best FASI conditions.

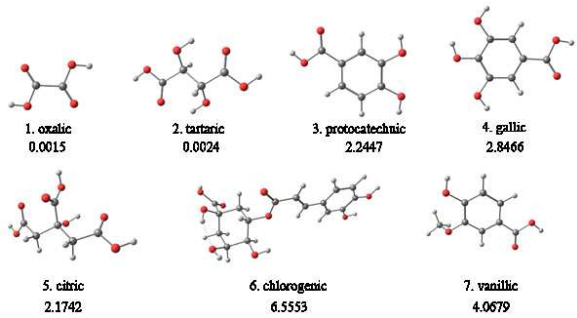


Fig. 2. The optimized geometry and dipole moment of 7 organic acids, which was calculated at the M06-2X/6-31G (d,p) theoretical level. The dark gray balls are carbon atoms, the shallow gray balls are hydrogen balls, while the red balls are oxygen atoms.

The pH of the buffer solution is a dominant factor for the separation speed and efficiency. The electro-osmotic flow may be changed by higher pH. The solubility of organic acid is scarcely influenced by pH of the buffer solution, but pH is a significant factor that affects the separation degree. The CE diagrams of 7 organic acids in buffer solutions with different pH values were shown in **Figure 3**. The theoretically optimized geometry and calculated dipole moment was listed in **Figure 2**. Generally speaking, the polarity of a molecule is higher when it has large dipole moment value. Peaks of vanillic and chlorogenic acids were detected because of higher polarity and the resulting higher force acquired from the electronic field. Peaks of oxalic and tartaric appear at the final because of its nearly zero dipole moment. It can be

found that the migration time slightly increases when pH rises from 8.0 to 10.0. The resolution is the highest when pH lies between 8.5 and 9.0. The order of detected peak of the 7 organic acids can be understood by their dipole moment values.

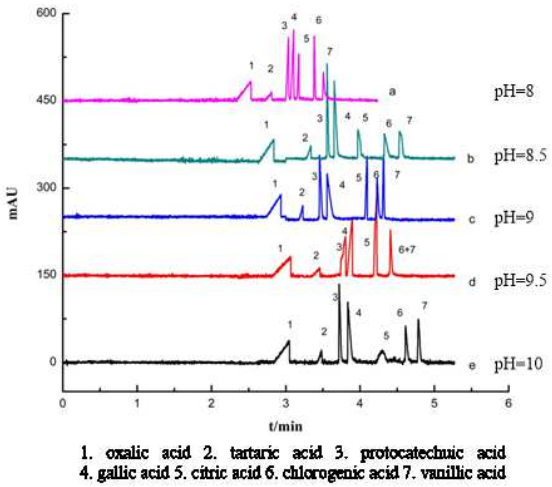


Fig. 3. The effects of pH on separation of 7 organic acids. The concentrations of organic acids are all 10 mgL$^{-1}$. The conditions are as in Table 1

**Effects of Concentration of Buffer Solution**

The electro-osmotic flow slows down when the concentration of buffer solution increases. The CE diagrams with different buffer concentration(10 to 50 mmol L$^{-1}$) were shown in **Figure 4**. The resolution is the best when the buffer concentration is 20 mmol L$^{-1}$. When the concentration is higher than 20 mmol L$^{-1}$, resolution decreases largely. Moreover, it has been known that the migration speed was highly influenced in high viscosity solution.Considering the compromise between resolution and migration time, 20 mmol L$^{-1}$was used for the next experiments.

**Effect of Concentration of CTAB**

As shown in **Figure 5**, it was found that forward detection took longer time (over half an hour) than reverse detection in the current study, so CTAB was used to change the direction of electro-osmotic flow. Electric double layer forms when cationic surfactant such as CTAB is absorbed on the wall of capillary. The electric double layer makes organic acids migrate to positive pole, and electro-osmotic flow speed decreases, which is favorable for the separation.Resolution increases with a CTAB concentration in the range of 0.5 and 0.9 mmol L$^{-1}$, and decreases when the concentration of CTAB is more than 0.7 mmol L$^{-1}$. So the 0.7 mmol L$^{-1}$CTAB was finally used for the following experiments.

**Effect of water plug length**

Higher stacking efficiency was obtained when a short water plug was pre-injected before sample injection in FASI.Along with the increasing length of water plug, the total amount of analyte introduced into capillary by FASI decreased. Therefore, the length of water plug was kept as short as possible to get the best efficiency. Herein,

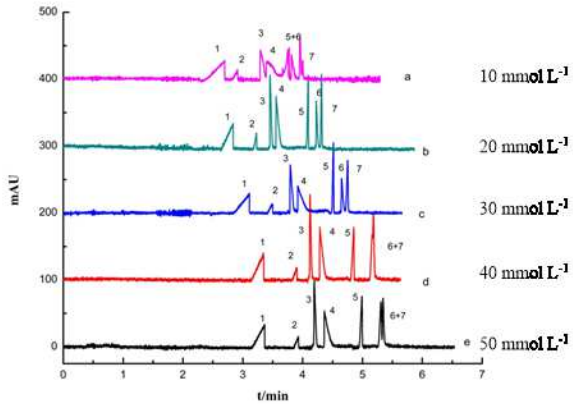


Fig. 4. The effects of concentration of buffer solution on separation of 7 organic acids. The peak numbers are the same as in Figure 2. The concentrations of organic acids are all 10 $mgL^{-1}$. The conditions are as in Table 1

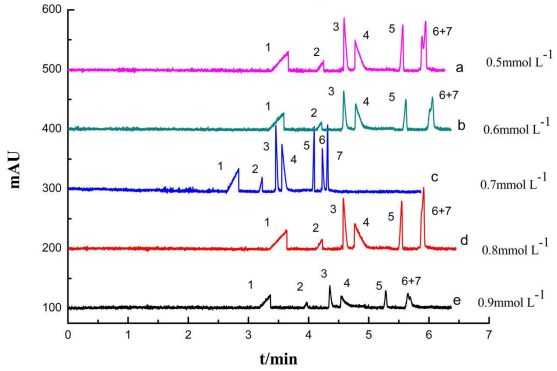


Fig. 5. The effects of concentration of CTAB on separation of 7 organic acids.The peak numbers are the same as in Figure 2

different water plug length (1, 2, 5, 8 and 10 s) was evaluated. As shown in **Figure 6**, the results indicated that 2s was the optimal water injection time.

**Effect of voltage and injection time**

Effects of voltage on stacking efficiency were investigated. When the injection voltage increased from -5 to -25 kV at the step of -5 kV, the migration time became shorter and shorter, and heating and background noise became intense. The migration time was too long at -5 kV. Moreover, the analytical sensitivity was no so good at low voltage. Considering the maximum voltage that CE instrument can endure is -30 kV, -25 kV was chosen as the optimal condition. At the same time, the injection time was further optimized. The results were shown in **Figure 7**. The peak areas of all organic acids were the largest when the injection time was 30 s. When the injection time was longer than 30 s, the overloaded organic acids led to a decrease of peak area. So 30 s was selected as the optimal injection time.

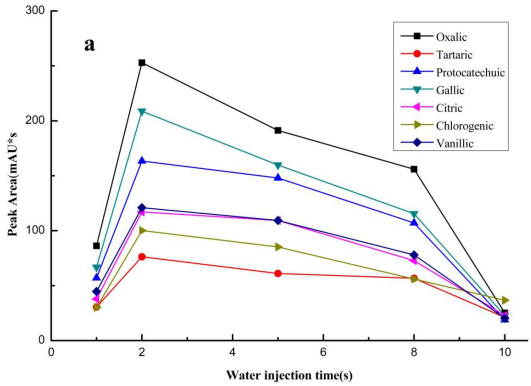


Fig. 6. Effect of water plug length on peak area

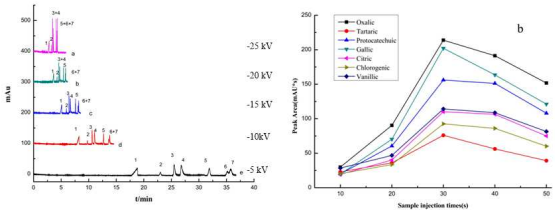


Fig. 7. The effects of voltage and injection time on separation of 7 organic acids. The peak numbers in the left one are the same as in Figure 2.

**Comparison between FASI-CE and direct CE**

The separation performance using the optimal conditions was evaluated by comparing the separation efficiency of FASI-CE and direct CE.FASI behaved a large pre-concentration ability, which could be found from the obvious enhancement in detection sensitivity as shown in **Figure 8**.The enhancement factors for oxalic, tartaric, protocatechuic, gallic, citric, chlorogenic, and vanillic acids were 13-, 8-, 36-, 15-, 72-, 26-, and 55-fold, respectively. The enhancement factor was defined as $(A/C)/(A_0/C_0)$, where A and $A_0$ are the corrected peak areas of the analyte under stacking and normal conditions; $C$ and $C_0$ are analyte concentrations under stacking and normal conditions, respectively. Analytical characteristic data of the devised FASI-CE method were summarized in **Table 2**.

The calibration curves were made with peak area versus concentration. The linear ranges of oxalic, tartaric, protocatechuic, gallic, citric, chlorogenic, and vanillic acids are 0.7-80, 0.5-50, 0.2-15, 0.6-45, 0.5-35, 0.8-60, and 0.5-40 mg $mL^{-1}$, respectively. The detection limits are 0.90, 0.56, 0.26, 0.65, 0.52, 0.85, and 0.55 mg $mL^{-1}$ for oxalic, tartaric, protocatechuic, gallic, citric, chlorogenic, and vanillic acids, respectively. The current FASI-CE-UV method is less time-consuming and less expensive than other techniques such as HPLC and GC-MS.[13]

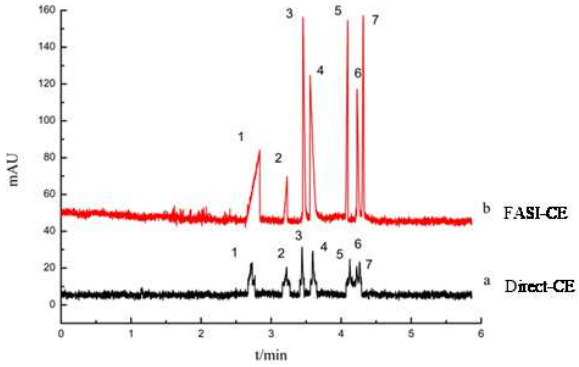


Fig. 8. Electropherogramsof 7 organic acids (10 mgL$^{-1}$) obtained by directed CE (B) and FASI-CE (A). The peak numbers are the same as in Figure 2

**Table 2.** Regression equation, correlation coefficient, linear range, enrichment factor (EF) and LOD.

| **Compound (Organic acids)** | Regression equation | Correlation coefficient | Linear Range (mg L-1) | RSD(%)a | EFb | Detection limits (mg L-1) |
|---|---|---|---|---|---|---|
| | | | | Peak Migration Area time | | |
| | | | FASI-CE | | | FASI-CE CE |
| **Oxalic** | y=1.10x+76.64 | 0.9912 | 0.7-80 | 4.2 2.6 | 13 | 0.90 45 |
| **Tartaric** | y=0.45x+46.37 | 0.9228 | 0.5-50 | 7.5 3.7 | 8 | 0.56 48 |
| **Protocatechuic** | y=6.30x+49.79 | 0.9769 | 0.2-15 | 6.3 2.4 | 36 | 0.26 5.2 |
| **Gallic** | y=5.16x+33.51 | 0.9981 | 0.6-45 | 8.2 1.8 | 15 | 0.65 8.3 |
| **Citric** | y=4.30x+24.60 | 0.9690 | 0.5-35 | 6.7 3.5 | 72 | 0.52 5.5 |
| **Chlorogenic** | y=1.95x+12.86 | 0.9683 | 0.8-60 | 5.8 2.7 | 26 | 0.85 53 |
| **Vanillic** | y=8.01x+88.54 | 0.9843 | 0.5-40 | 6.1 1.9 | 55 | 0.55 3 |
| **EF=(A/A$_0$)/(C/C$_0$)** | | | | | | |

**Real Sample Analyses**

The current devised method was applied under optimal conditions to determine 7 organic acids in 8 honey samples collected from different geographical places. The samples and their spiked recoveries were treated as in the section of Real Sample Preparation. The electropherogram of 7 organic acids was shown in **Figure 3**. The data were summarized in **Table 3**. The recoveries are all higher than 90%, which indicates that the method is suitable and acceptable for the current samples. Oxalic was detected in all samples. Tartaric and protocatechuic were only detected in $B_6$. Chlorogenic was only found in $B_1$. It can be concluded that the different contents of the organic acids were probably attributed to different geographical places, honey

species and other factors.

**Table 3.** Analytical results of 7 organic acids in real honey samples

| **Sample** | Oxalic (mg/kg) | Tartaric (mg/kg) | Protocatechuic (mg/kg) | Gallic (mg/kg) | Citric (mg/kg) | Chlorogenic (mg/kg) | Vanillic (mg/kg) |
|---|---|---|---|---|---|---|---|
| $\mathbf{S}_1$ | 41.35 | ND | ND | ND | 0.73 | ND | ND |
| $\mathbf{S}_2$ | 36.84 | ND | ND | ND | 5.31 | ND | ND |
| $\mathbf{S}_3$ | 44.64 | ND | ND | ND | ND | 9.61 | ND |
| $\mathbf{S}_4$ | 39.25 | ND | ND | ND | 1.21 | ND | ND |
| $\mathbf{S}_5$ | 43.35 | ND | ND | 6.87 | ND | ND | ND |
| $\mathbf{S}_6$ | 4.63 | 46.96 | 11.49 | 7.13 | ND | ND | ND |
| $\mathbf{S}_7$ | 9.87 | ND | ND | ND | ND | ND | 2.79 |
| $\mathbf{S}_8$ | 33.46 | ND | ND | ND | ND | ND | ND |

**ND* Not detected**

**The Recovery of Typical Sample**

The recovery of these organic acids in sample $S_6$was investigated based on results of real samples. Different concentration of standard solution was added into 1 g sample and mixed well with rotary oscillator. The mixture was diluted to 10 ml with water, filtered with ultrathin film, and degassed with ultrasonic before detection. Parallel experiments were performed for three times and take their average as the final results, which were archived in **Table 4**. The recoveries of organic acids ranged from 89.5% to 97.1% and the RSDs ranged from 0.82% to 3.49%.

**Table 4.** Recoveries of Organic acids in Real Sample ($S_6$).

| **Compound (Organic acids)** | **Measured (mg/kg)** | **Added (mg/kg)** | **Found (mg/kg)** | **Recoveries(%)** | **RSD(%) n=3** |
|---|---|---|---|---|---|
| | | 10.0 | 13.58 | 92.19 | |
| Oxalic | 4.63 | 10.0 | 13.77 | 93.48 | 1.73 |
| | | 10.0 | 13.46 | 91.38 | |
| | | 44.0 | 82.50 | 90.71 | |
| Tartaric | 46.96 | 44.0 | 83.40 | 91.68 | 1.12 |
| | | 44.0 | 83.60 | 91.93 | |
| | | 10.0 | 19.59 | 90.73 | |
| Protocatechuic | 11.49 | 10.0 | 19.87 | 92.03 | 2.02 |
| | | 10.0 | 20.11 | 93.14 | |
| | | 7.30 | 13.66 | 94.66 | |
| Gallic | 7.13 | 7.30 | 13.95 | 96.67 | 2.07 |
| | | 7.30 | 14.01 | 97.09 | |
| | | 1.20 | 1.09 | 90.70 | |
| Citric | ND | 1.20 | 1.11 | 92.50 | 3.13 |
| | | 1.10 | 0.98 | 89.51 | |
| | | 1.20 | 1.13 | 94.57 | |
| Chlorogenic | ND | 1.10 | 1.04 | 94.54 | 0.82 |
| | | 1.10 | 1.05 | 95.45 | |
| | | 1.25 | 1.14 | 91.20 | |
| Vanillic | ND | 1.25 | 1.13 | 90.40 | 3.49 |
| | | 1.25 | 1.18 | 94.48 | |

**ND* Not detected**

## Conclusion

A simple online pre-concentration method was developed for improving the sensitivity and efficiency of CE-UV in the determination of 7 types of organic acids in 8 honey samples from different geographical places of Henan province. As compared with direct CE, the sensitivity of FASI-CE was greatly enhanced. This method is simpler and cheaper than other analytical methods. The results suggested that this method might be potential used for the detection of different organic acids in different samples with a low concentration.

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