HPLC method for the simultaneous quantification of the major organic acids in Angeleno plum fruit

Yanwei Wang¹, Jing Wang¹, Wei Cheng¹, Zhilei Zhao*¹ and Jiankang Cao²

¹Hebei University, College of Quality and Technical Supervision, Baoding, China
²China Agricultural University, College of Food Science and Nutritional Engineering, Beijing, China

zhaozhilei-3208@163.com

Abstract. A method was developed to profile major organic acids in Angeleno fruit by high performance liquid chromatography. Organic acids in plum were extracted by water with ultrasonication at 50°C for 30 min. The extracts were chromatographed on Waters Atlantis T³ C₁₈ column (4.6 mm×250 mm, 5 μm) with 0.01mol/L sulfuric acid and water as mobile phase, and flow rate was 0.5 ml/min. The column temperature was 40°C, and chromatography was monitored by a diode array detector at 210 nm. The result showed that malic acid, citric acid, tartaric acid, oxalic acid, pyruvic acid, acetic acid, succinic acid in Angeleno plum, and the malic acid was the major organic acids. The coefficient of determination of the standard calibration curve is R² > 0.999. The organic acids recovery ranged from 99.11% for Malic acid to 106.70% for Oxalic acid, and CV (n=6) ranged from 0.95% for Malic acid to 6.23% for Oxalic acid, respectively. The method was accurate, sensitive and feasible in analyzing the organic acids in Angeleno plum.

1. Introduction

Plum (Prunus salicina L) was flavorful and contains good nutrition, which has been one of the most commonly consumed stone fruits by domestic and foreign consumers [1]. Plum was rich in sugars, amino acids, vitamins, mineral elements [2-3], as well as organic acids. Organic acid is a function of both taste and aroma components, and the content of organic acids dictating the eating quality of the fruit. The sweetness of fruit mainly depends on the acids content [4-5]. The determination of organic acids commonly used titration, spectrophotometry, gas chromatography, fluorescence method, thin layer chromatography, enzymatic, etc. [6-9] Currently, high performance liquid chromatography method has been used in pineapple [10], oranges [11], peaches [12], apricots [13] and other fruit acids composition and content determination, however, the type and the content of organic acids in Angeleno fruit has not been reported at present. In this study, a rapid and effective method of quantitative analysis of organic acids in Angeleno fruit by high performance liquid chromatography was developed, and the content and the type of organic acids in Angeleno plum was analyzed.
2. Materials and methods

2.1. Reagents and instruments

Standards of oxalic acid, malic acid, tartaric acid, pyruvic acid, acetic acid, citric acid, succinic acid were obtained from Amresco company with purity of 99.0%. Sulfuric acid (GR). Deionized water.

Waters series HPLC system (American Waters companies) equipped with an evaporative light scattering detector (DAD, Waters 600E quaternary pump, 2998 photodiode array detector, Empower chromatography workstation), KQ-500E ultrasonic cleaner with numerical control (the Kunshan Ultrasonic Instrument Co., Ltd). Human ultra-pure water (Ultra Pure Water System). Electronic balance (AR1140, Ohaus Corporation). Flying pigeon centrifuge (TGL-10C, Shanghai An-ting scientific instrument factory). 5 mL disposable syringes, 0.45μm filter needle head aqueous, mortar, 100mL volumetric flask.

2.2. Methods

2.2.1. Chromatographic conditions. The extracts were chromatographed on Waters Atlantis T3 C18 column (4.6 mm x 250 mm, 5 μm) with 0.01mol/L sulfuric acid as mobile phase, and flow rate was 0.5 ml/min. The column temperature was 40℃, and chromatography was monitored by the photodiode array detector at 210 nm.

2.2.2. Sample preparation. The extraction of organic acids from plum samples was analyzed according to the method of Wang [14] and Zhang [15] with some modifications. For determining organic acids contents of Angeleno plum, sample was treated as follows, 5 g of fresh sample was ground into homogenized, followed by transferring to 50 mL volumetric flask and the ultrapure water was added to the scale, organic acids were extracted with ultra-sonication at 50℃ for 30 min. Then 8 mL extracts was transferred to the centrifuge tube, the samples were centrifuged at 10000 r/min for 15 min. The supernatant solution was filtered through a 0.45 μm nylon membrane filter.

2.2.3. Preparation of standard curve. A serial dilution of organic acids (oxalic acid malic acid, succinic acid, tartaric acid, citric acid, pyruvic acid and acetic acid) was made by dissolving the required amount of authentic standard in deionised water (dH2O). All standards were filtered through a 0.22 μm nylon filter before HPLC analysis Each standard was analysed in duplicate for calibration and their concentrations ranged from 1 to 800 mg/L for organic acids.

2.2.4. Determination of sample. Qualitative analysis of extracts was carried out according to the external standard method. The injection volume was 20μL.

3. Results and analysis

3.1. Mobile phase selectivity

3.1.1. The selection of mobile phase component. 0.01 mol/L Phosphoric acid, hydrochloric acid, nitric acid, P-toluenesulfonic acid, sulfuric acid was used as the mobile phase, respectively, the flow rate was at 0.5mL/min, and the column was thermostatic at 40℃. The standard mixture of organic acids and extract were analyzed by HPLC. The result shows that the Oxalic acid, malic acid, succinic acid and acetic acid can be well separated in the case of phosphoric acid and hydrochloric acid as mobile phase, but poor separation of citric acid and tartaric acid. In terms of nitric acid as the mobile phase, the two peaks of citric acid and tartaric acid overlap with poor separation. Replaced by P-toluenesulfonic acid, the result showed no peaks within 30 min and obtained the worst separation. When taking sulfuric acid as the mobile phase, seven kinds of organic acids had been separated effectively with completed peaks in 20 minutes, was tested to obtain the optimal chromatographic separation.

3.1.2. The selection of sulfuric acid concentration. 0.002, 0.005, 0.01, 0.05, 0.07 mol/L sulfuric acid was used as mobile phase with condition described in section 2.1.1, respectively. Results showed that
retention time was declined with the decreased of sulfuric acid, and the separation was poor. Retention time became longer when sulfuric acid increased, and the separation of oxalic acid and citric acid were poor, however, other organic acids were found to give a better separation. When the content of sulfuric acid was changed to 0.07 mol/L, oxalic acid and citric acid can not be separated. Results showed that 0.01 mol/L sulfuric acid as mobile phase could provide a good separation of seven kinds of organic acids. The results were showed in Figure 1, 2.

![Figure 1. Chromatogram of 7 organic acids standards](image)

1. Oxalic acid 2. tartaric acid 3. malic acid 4. pyruvic acid 5. acetic acid 6. citric acid 7. succinic acid

![Figure 2. Chromatogram of organic acids in plum fruit](image)

1. Oxalic acid 2. tartaric acid 3. malic acid 4. pyruvic acid 5. acetic acid 6. citric acid 7. succinic acid

3.2. Flow rate selectivity

The analysis were based on 0.01mol/L sulfuric acid as mobile phase, and column temperature was set at 40 °C with different flow rate, ranged from 0.3 to 0.6 mL/min, which were tested to obtain the optimal separation. The results showed that the flow rate was decreased, and the analytical time of separation was not affected markedly. When the flow rate increased, the column pressure increased at the same time and the analytical time was decreased. As a result, the flow rate was set at 0.5 mL/min as the optimal condition.

3.3. Column temperature selection

In order to obtain the optimal separation, we prepared column with 25 °C, 30 °C, 40 °C, 45 °C, 50 °C, respectively. The results showed that the column temperature was increased, and the analytical time of the organic acids was decreased, but the separation was satisfactory. However, the peak width of acetic acid was broadening when the column temperature was 50 °C. The column temperature at 40 °C was the optimal condition.

3.4. Linear range investigation

Under the chromatographic conditions described above, the calibration graphs were constructed by plotting peak area vs. concentration. Good linearity was achieved in the range from 0.001 to 1.0mg/ml
of the standard mixture studied for each organic acid in water with significant correlations. The coefficients of determination was $R^2 > 0.999$ (Tab. 1).

Table 1. The calibration linear and coefficients of determination of organic acids.

| Compounds    | Linear equations                   | coefficients($R^2$) |
|--------------|------------------------------------|---------------------|
| Oxalic acid  | $y = 0.00000006 x + 0.00492105$    | 0.9990              |
| Tartaric acid| $y = 0.00000029 x + 0.00462126$    | 0.9995              |
| Malic acid   | $y = 0.00000051 x + 0.00169815$    | 0.9999              |
| Pyruvic acid | $y = 0.0000009 x + 0.00788941$     | 0.9998              |
| Acetic acid  | $y = 0.00000009 x + 0.00404933$    | 0.9992              |
| Citric acid  | $y = 0.00000041 x + 0.00437906$    | 0.9997              |
| Succinic acid| $y = 0.00000105 x + 0.00503900$    | 0.9995              |

3.5. Precision inspection

As shown in Table 2, the precision of the method was determined by calculating the coefficient of variation (%CV) of analyte concentration of six repeat mixture standard analysed with the HPLC method. The % CV of the concentration values of organic acids ranged between 0.95% and 6.23%.

Table 2. Precision of the method (n = 6).

| Compounds    | Concentration/(mg/ml) | Average value | RSD/% |
|--------------|-----------------------|---------------|-------|
| Oxalic acid  | 0.0134                | 0.0138        | 2.96  |
| Tartaric acid| 0.0118                | 0.0120        | 0.81  |
| Malic acid   | 0.5246                | 0.5222        | 0.95  |
| Pyruvic acid | 0.0081                | 0.0082        | 0.78  |
| Acetic acid  | 0.0052                | 0.0053        | 2.64  |
| Citric acid  | 0.0231                | 0.0218        | 4.76  |
| Succinic acid| 0.0425                | 0.0423        | 7.21  |

3.6. Repetitive inspection

Approximately 5 groups of the same mixed sample of plums was evaluated by the method has been mentioned in 2.2.2. The results shown that the average content of oxalic acid, tartaric acid, malic acid, pyruvic acid, acetic acid, citric acid and succinic acid was 0.085, 0.102, 6.328, 0.081, 0.051, 0.243, 0.255, 0.220 mg/g•Fw, and the CV was 1.83%, 1.79%, 2.31%, 4.26%, 3.79%, 2.38%, 1.97%, respectively. The results show that the repeatability of the method was satisfactory.

3.7. Recovery

The recovery of analytes after extraction from plum spiked with a mixture of standards was analysed in triplicate in order to assess the effectiveness and accuracy of the extraction step. The results confirmed that the separation and analysis conditions were accurate for all compounds (Table 3). The mean of the percentage recovery were 106.70%, 104.44%, 99.11%, 104.61%, 101.18%, 101.55%, 105.15%, for oxalic acid, tartaric acid, malic acid, pyruvic acid, acetic acid, citric acid, succinic acid, respectively. For the recovery experiment, plum samples were spiked with all compounds at the same time prior to extraction and HPLC analysis.

Table 3. The recovery of the method.

| Compounds    | Initial value /(mg/ml) | Added value /(mg/ml) | measured value /(mg/ml) | Average recovery % |
|--------------|------------------------|----------------------|-------------------------|-------------------|
| Oxalic acid  | 0.02                   | 0.03                 | 0.05                    | 106.70            |
The quantitative and qualitative determination of organic acids in plum fruit samples was achieved by the method described in section 2.2.2 and the chromatographic conditions as described in section 2.2.1. The composition and content of organic acids in Angeleno plum were determined. As showed in Table 4, malic acid content was 6.328 mg/g, which showed the highest content in Angeleno plum, citric acid and succinic acid were much lower than malic acid, the contents was 0.243 mg/g and 0.221 mg/g, respectively. Acetic acid content was 0.051 mg/g, which content was the lowest.

Table 4. Organic acids content in Angeleno plum.

| Varieties | Oxalic acid | Tartaric acid | Malic acid | Pyruvic acid | Acetic acid | Citric acid | Succinic acid |
|-----------|-------------|---------------|------------|--------------|-------------|-------------|---------------|
| Angeleno  | 0.085 ± 0.012 | 0.102 ± 0.004 | 6.328 ± 0.078 | 0.081 ± 0.0001 | 0.051 ± 0.0004 | 0.243 ± 0.001 | 0.221 ± 0.006 |

4. Conclusions

The solvent system chosen for the analysis of organic acids in Angeleno plum exhibited good baseline stability and low background noise. The organic acids profile in Angeleno plum fruit with HPLC system is a difficult task because of several interferences. The proposed method allows the quantification of malic acid, citric acid, tartaric acid, oxalic acid, pyruvic acid, acetic acid, succinic acid in Angeleno plum fruit. The analysis is simple, rapid and does not require any complicated sample preparation, and only one reversed-phase HPLC column is used for the separation in less than 25 min. This HPLC-DAD method is a versatile method that can be used with a simple UV-vis detector. Therefore, the proposed method is set up to analyze up to 7 organic acids in order to control the product quality. The proposed HPLC method is accurate, sensitive and feasible to determine organic acids in Angeleno plum with direct injection after sample extraction.

Acknowledgments

This work was financially supported by Natural Science Foundation of China (31201430); Natural Science Foundation of of HeBei (C2013201113); Special Fund for Agro-scientific Research in the Public Interest (201303075). Correspondence author: Zhao Zhilei.

References

[1] Faragher J D and Chalmers D J 1977 Regulation of an-thocyanin synthesis in apple skin. III. involvement of phenylalanine ammonia-lyase J. Func. Plan. Bio. 4 133-41
[2] Zhang L, Yao S L, and Wang J H 2008 Detection and analysis of nutrients in the fresh fruit of Jiu Qian plum J. South West Agr. Sci. 21 1664-66
[3] Li J, Xu Z, and Zhou L 2011 Determination of nutrients in Wild Cherry Plum in Xinjiang J. Xinjiang Agr. Sci. 47 2145-49
[4] Zhao Y H, and Li X L 2009 Progress of research on the accumulation of sugar and acid in fruit J. Agr. Sci. and Tech. 8 110-12
[5] BERUTER J 2004 Carbohydrate metabolism in two apple genotypes that differ in malate accumulation J.
Plan. Phys. 161 1011-29

[6] Zhang L X, Zhang T F, and Li L Y 1981 Technical biochemical experiments (Beijing: Higher Education Press) p 115-118

[7] Zhu X L, Gao Y, and Su Q D 2004 Determination of non-volatile organic, higher fatty acids and low molecular weight carbohydrates in tomatoes by capillary gas chromatography J. Food Che. 25 152-57

[8] Huang Z X, and Dai R Z 1985 Determination of malic acid in wine by fluorescent spectrophotometry J. Food and Fer. Ind 26

[9] Lei D F 2006 Modern biochemistry and molecular biology instruments and equipment (Beijing: Science Press) p 222

[10] Zhang X M, Du L Q, and Xie J H 2007 HPLC determination of sugars in Pineapple fruit J. Food Chem. 28 450-52

[11] Li Y K, and Pan S Y 2006 Evaluation of orange juice sugar composition by HPLC J. Food Che. 27 190-92

[12] Wu B H, Li S H 2003 The fur and fruit flesh color for the effects and correlation research of sugar and acid content in peach J. Agr. Sci. of China 36 1540-44

[13] Zhang L L, Liu W S 2010 Determination of sugars and acids of five varieties of apricots by HPLC J. Fruit Sci. 27 119-23

[14] Wang Y Y, Hu W Z, and Pang K 2008 HPLC-ELSD determination of soluble sugars content in apples J. Ana. and Detect. 34 129-31

[15] Zhang Y Z, Li P M, and Cheng L L 2010 Developmental changes of carbohydrates, organic acids, amino acids, and phenolic compounds in Honey crisp, apple flesh J. Food Chem. 123 1013-18