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Original Paper

Determination of Organic Acids in Orange Fruit by HPLC

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Abstract

Objective: To establish a method for rapid determination of 6 organic acids by HPLC. Methods: Ultimate C18 chromatographic column (4.6×250mm, 5μm) was used, the mobile phase was phosphate buffer solution (pH2.0), the flow rate was 0.5mL/min, and the detection wavelength was 210nm. Results: An HPLC analysis method for tartaric acid, malic acid, ascorbic acid, lactic acid, acetic acid, and citric acid was established, and the relative standard deviation was 1.38%~4.43%, the detection limit was 0.012~2.291 mg/L, and the recovery rate of organic acids was 89.7%~105.8%. Conclusion: This method has fast analysis speed, high reproducibility, and sensitivity, and is suitable for the determination of organic acids in oranges.

Keywords

High performance liquid chromatography, organic acid, orange

1. Introduction

Organic acids are important flavor nutrients in fruits and are widely found in various plant fruits. Some organic acids are also important metabolic intermediates in organisms (Guan, Cai, & Wang, 2009). The main organic acids in orange fruits are tartaric acid, malic acid and citric acid. Their composition and content are one of the important factors that determine the flavor and quality of orange fruits. Tartaric acid is the characteristic acid of orange fruits. It is not accumulated in other fruits. Its acidity is very strong and it is the main contributor to the acidity of oranges (Deblot, Cook, & Ford, 2006). The taste of fruits depends largely on the balance between sweetness, sourness and bitterness, and the taste quality depends on the harmony between these tastes. The type and concentration of organic acids regulate the "acid-base balance" and will inevitably affect the sourness of oranges. Therefore, accurate quantitative determination of organic acids plays an important role in fruit quality identification (Wen, & Zhang, 2009). Organic acids play a vital role in the environment. Taking oranges as an example, their multi-faceted impact can be clearly demonstrated. In the material cycle of the ecosystem, whether it is carbon cycle or nutrient cycle, organic acids play a key role. In the carbon cycle, organic acids

secreted by roots and produced by fruit decay during the growth of oranges participate in it, affecting the global carbon balance (Li, 2005). In terms of nutrient cycling, it can activate soil nutrients and facilitate orange absorption. For soil microorganisms, organic acids are carbon sources and energy sources, and can also regulate the structure of microbial communities and improve soil properties. In terms of water quality, organic acids contained in runoff from planting areas and processing wastewater affect the ecology of water bodies, either as carbon sources or as pollution. In the atmospheric environment, it indirectly regulates the climate by affecting the opening and closing of orange stomata.

Currently, the common analytical methods for organic acids include acid-base titration (Liu, 2004), thin layer chromatography (Feng, Du, & Wen, 2011), gas chromatography (Yu, 2010), ion chromatography (Wang, Li, & Chen, 2010), liquid chromatography, etc. (Yi, Yin, Lian et al., 2006; Cui, Duan, & Pan, 2010; Gratzfeld - Husgrn, 1996).

Acid-base titration is only applicable to macro-analysis and can only determine the total acid content; thin layer chromatography has low accuracy and sensitivity; gas chromatography requires complex derivatization, which is cumbersome and has large errors; ion chromatography has high requirements for sample processing, and the pre-treatment is very complex and time-consuming. The pretreatment of HPLC is relatively simple, which is suitable for the determination of non-volatile organic acids with high boiling points. It has the advantages of simple operation, high accuracy and good reproducibility. It can simultaneously determine multiple organic acids in samples and is currently widely used. This paper mainly establishes a method for the determination of organic acids by reversed-phase HPLC with fast analysis speed, high sensitivity and accurate results.

2. Experimental Part

2.1 Reagents and Equipment

Tartaric acid, malic acid, ascorbic acid, lactic acid, acetic acid, and citric acid are all analytical grade; methanol is analytical grade;

Ultrapure water. 100ml plastic volumetric flask, 100ml glass volumetric flask.

The oranges used in the experiment are sugar oranges.

2.2 Instruments and Equipment

See Table 1 for the main instruments and equipment

Table 1. Main Instruments and Equipment

Serial number	Instrument	Model	Manufacturer	Place of production	Remarks
1	High Performance Liquid Chromatograph	FL2200II	Fuli Analytical Instruments Co.	Beijing	
2	Ultrasonic cleaner	SK7200H	Shanghai Ke-guide Ultrasonic Instrument	Shanghai	

			Co.	
			Shanghai Precision Scientific Instrument	
3	pH meter	PHS-3E	Co.	Shanghai

2.3 Preparation of Sample Solution

Accurately weigh 100g of fresh oranges, crush them, add 100mL of distilled water, extract with high-frequency ultrasound for 40min, and dilute the filtered solution to a 250mL volumetric flask for use. Filter through a 0.45μm filter membrane before injection, and calculate the content of each organic acid.

2.4 Preparation of Standard Solution

Accurately weigh 50mg of ascorbic acid, 250mg of tartaric acid, 500mg of malic acid and citric acid, 1000mg of lactic acid and acetic acid, dissolve them in mobile phase and dilute to 50mL as standard stock solution. Dilute the standard stock solution with mobile phase to different concentrations for injection analysis, and establish a linear regression equation with organic acid concentration (mg/mL) as the horizontal axis and peak area (mAU) as the vertical axis.

2.5 Instrument Parameters

The chromatographic column is Ultimate XB→C18 column (4.6x250(mm), 5μm); the mobile phase is methanol

-0.01 mol/L; K2HP04(3:97) solution, and the pH value is adjusted to 2.0 with phosphoric acid. The flow rate is

0.5 mL/min, the injection volume is 20μm, the detection wavelength is 210 nm, the column temperature is 25℃, and it is filtered with a 0.45μm

filter membrane before use and ultrasonically degassed.

2.6 Precision Experiment

The same concentration of organic acid standard was injected 6 times, the peak area and retention time of each organic acid were measured, and the RSD value was calculated.

2.7 Recovery Rate Experiment

Accurately weigh 6 portions of 100g orange samples with known organic acid content, add each organic acid component, prepare sample solution according to the method of "2.3", and measure the recovery rate.

3. Results and Discussion

3.1 Chromatogram

The mixed organic acid standard solution was injected and analysed according to the best test conditions, and the baseline separation of six organic acids was achieved within 20 min, and the chromatograms of the organic acid standards were obtained, and the results are shown in Figure 1.

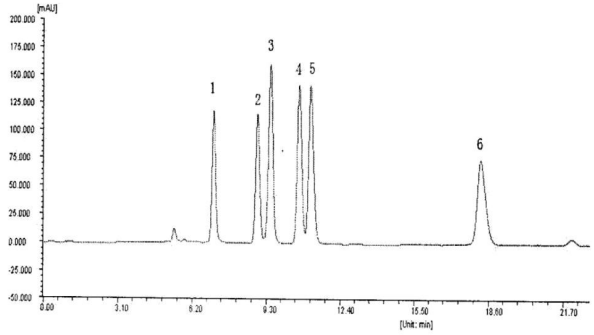

Figure 1. Chromatograms of 6 Organic Acids

Note. 1 Tartaric acid; 2.L-malic acid; 3 Ascorbic acid; 4.Lactic acid; 5.Acetic acid; 6.Citric acid

3.2 Standard Curve, Detection Limit and Precision of each Component

Weigh 50mg of ascorbic acid, 250mg of tartaric acid, 500mg of malic acid, 500mg of citric acid, 1000mg of lactic acid and 1000mg of acetic acid accurately, and then dissolve them with mobile phase to 50mL as the standard reserve solution. The standard stock solution was diluted into different concentrations with the mobile phase, and the linear regression equation was established with the concentration of organic acids (mg/mL) as the horizontal coordinate and the peak area (mAU) as the vertical coordinate. The results are shown in Table 3-1.Repeat the injection of the same concentration of organic acid standard 6 times, determine the peak area and retention time of each organic acid, and calculate the RSD value. The results are shown in Table 2.

Table 2. Linear Regression Equations for the Six Organic Acids

organic acid	Regression equation	Linear range (mg/mL)	Correlation coefficient	Minimum Detection Limit(mg/L)	RSD（%）
Tartaric acid	Y=2.9031-0.06X	0.05~5	0.9961	0.680	1.62
L-Malic acid	Y=5.9031-0.06X	0.1~10	0.9773	1.001	2.97
Ascorbic acid	Y=1.9635-0.07X	0.05~1	0.9289	0.012	4.43
Lactic acid	Y=8.9457-0.06X	0.2~20	0.9778	0.417	3.01
Acetic acid	Y=7.7901-0.06X	0.2~20	0.9895	2.291	2.89
Citric acid	Y=4.0282-0.06X	0.1~10	0.0760	0.059	1.38

The linear correlation coefficients of the regression equations of the organic acids were 0.9289-0.9973, which indicated that the linear relationship was good. The detection limit of each organic acid was obtained when the sex-to-noise ratio (S/N) was 3:1. The RSD values of the organic acids were less than 5%, indicating that the results were sensitive and reproducible Table 3.

Table 3. Repeatability of Organic Acid Retention Time Data Sheet

Organic acid	Average retention time (min)	Relative standard deviation (%)
Tartaric acid	7.0770	1.732
L-Malic acid	8.7913	0.089
Ascorbic acid	9.3723	0.752
Lactic acid	10.4790	0.076
Acetic acid	10.8177	0.145
Citric acid	18.0473	0.251

The relative standard deviations (RSD) of the retention times of the six organic acids were in the range of 0.089%-1.732%, indicating that the retention times were basically constant under the determined chromatographic conditions, and the method has good reproducibility.

3.3 Test Results of Organic Acid Content and Recovery Rate in Orange Samples

A total of 6 samples of 100g of orange with known organic acid content were accurately weighed, each organic acid component was added, and the sample solution was prepared according to the method of "2.3", and the recovery rate was determined, and the results are shown in Table 4.

Table 4. Data Sheet for Recovery Experiments

Organic acids	Content of organic acid（mg/g）	Recovery rate（%）
Tartaric acid	3.645	105.8
L-Malic acid	1.393	101.2
Ascorbic acid	0.145	89.7
Lactic acid	0.056	92.5
Acetic acid	0.051	94.3
Citric acid	0.338	96.1

The results of orange samples and the recovery rate of spikes showed that the recoveries of the six organic acids ranged from 89.7% to 105.8%, indicating that the sample treatment method could meet the requirements for the determination and analysis of organic acids in oranges.

3.4 Safety and Operational Precautions

After the food sample was extracted by homogenate and centrifuged, the sample solution was filtered through a 0.3μm filter membrane to $(NH_4)\ 2HPO_4$

A H_3PO_4 buffer solution (pH=2.7) was a mobile phase, separated by high performance liquid chromatography on a C18 column, detected by a UV detector at 210nm, and the organic acid content was determined by a peak height or peak area standard curve.

This method was extracted from GB/T 5009.157-2003, and the detection limits were: tartaric acid 0.1 μg/mL, malic acid 0.3 μg/mL, citric acid 0.5 μg/mL, and succinic acid 0.2 μg/mL.

3.5 Precautions

3.5.1 Quality of Reagents and Water

The reagents utilized in this method are of analytical purity grade. This high-grade purity ensures the reliability and accuracy of the experimental results. The test water holds significant importance as well. It is either - distilled water or water with an equivalent level of purity. To further enhance the quality of the water used in the process, it undergoes vacuum - filtration through a 0.45μm filter membrane. This meticulous filtration process removes any potential impurities or particulate matter that could interfere with the analysis, thereby safeguarding the integrity of the entire experimental procedure.

3.5.2 Calibration During Sample Solution Determination

When engaging in the determination of the sample solution, a crucial step is the calibration of the standard solution. This calibration needs to be performed at regular intervals. Specifically, the standard solution should be calibrated every three samples. This frequent calibration is essential because it helps in maintaining the accuracy of the measurement results. During the calibration process, the correction coefficient is recalculated. This recalculation accounts for any potential variations or errors that might have occurred during the experiment, ensuring that the final measurement results are as precise as possible.

3.5.3 Selection of Extraction Agent

The choice of extraction agent for the organic acids in the extraction sample is a critical decision. In this case, 80% ethanol is employed for this purpose. This particular concentration of ethanol offers several distinct advantages. Firstly, it can ensure the complete extraction of organic acids from the sample. This means that a higher yield of the target organic acids can be achieved, maximizing the information obtained from the sample. Secondly, and equally importantly, using 80% ethanol helps in avoiding the dissolution of proteins present in the sample. Protein dissolution can have a detrimental impact on the lifetime of the column. By preventing protein dissolution, the column's performance and longevity are maintained, allowing for more consistent and reliable analyses over an extended period of time.

4. Summary

In this study, high-performance liquid chromatography (HPLC) analysis methods for tartaric acid, malic acid, ascorbic acid, lactic acid, acetic acid, and citric acid were established, and the relative standard deviation was 1.38%-4.43%, the detection limit was 0.012-2.291 mg/L, and the recovery rate of organic acids was 89.7%-105.8%. This method has a fast analytical speed, high reproducibility, and sensitivity, and is suitable for the determination of organic acids in oranges.

The experiment used 210-280nm ultraviolet light to scan various calculations and found that 6 kinds of organic acids had strong absorption at about 210nm, so 210nm was selected as the detection wavelength for the test. The experiments investigated sulfuric acid, metaphosphoric acid, phosphate, and other marine liquids as mobile phases, and found that under the conditions of the chromatographic column used in the test, the dipotassium phosphate solution, as a weak acid ionization inhibitor, had almost no absorption in the ultraviolet region, so the separation effect was the best. Since the higher concentration of the buffer salt solution has an effect on both the pump and the column, and the lower concentration is not conducive to separation, a buffer salt concentration of 0.01mol/L was chosen. The addition of a small amount of methanol to the mobile phase significantly improved the peak pattern (Zhang, Zhou, Ji et al., 2008), and a 5% methanol addition was experimentally determined. The mobile phase pH, which has a greater influence on the separation of organic acids, was investigated, and it was found that the separation of organic acids was good in the lower pH range, but the pH value that was too low was more damaging to the column. The Ultimate Column was used for the test with a low pH range, so the mobile phase pH 2.0 was selected for the test. The column temperature has a certain effect on the retention of organic acids, and the test shows that the organic acids are better retained at lower column temperatures. Different columns have different retention properties for organic acids, and the column separates organic acids well at lower mobile phase flow rates (0.5 ml/min). The flow rate was too high, and some of the organic acid chromatographic peaks overlapped.

Most of the organic acids in tangerines are water-soluble acids, which have good solubility in water (Li, 2005). In this experiment, an appropriate amount of pure water and high-frequency ultrasonic assisted extraction were used to extract the organic acids in oranges, and good results were obtained. Through the experiment, it was determined that the ultrasonic extraction achieved the best efficiency at about 40 min.

High-performance liquid chromatography was used to determine a variety of organic acids in oranges at the same time, which saved time and achieved good results. Ascorbic acid is difficult to determine in routine analysis due to its poor stability. The accuracy and recovery of ascorbic acid in the test are somewhat different from those of other organic acids. Therefore, further research is still needed on the test conditions for the determination of ascorbic acid by high-performance liquid chromatography.

The high performance liquid chromatography method determined by the experiment was used to determine the organic acids of more than 100 different orange varieties in China, and good results were obtained, and the feasibility of the method was also illustrated.

5. Conclusion

Ultimate C18 (4.6×250mm, 5μm) as the chromatographic column and 0.01mol/L phosphate as the mobile phase, the tartaric acid, malic acid, ascorbic acid, lactic acid, acetic acid and citric acid in the orange samples were simultaneously determined by high performance liquid chromatography at a wavelength of 210nm, and the analysis time was not more than 20min. This method has the advantages of simple processing, high accuracy, good reproducibility and fast analysis speed, and is suitable for the rapid analysis of organic acid content in oranges and other fruits.

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