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A Simple and Rapid Determination of ATP, ADP and AMP Concentrations in Pericarp Tissue of Litchi Fruit by High Performance Liquid Chromatography

Hai Liu[1,3], *Yueming Jiang*[1]*, *Yunbo Luo*[2] *and Weibo Jiang*[2]

[1]South China Botanic Garden, The Chinese Academy of Science, 510650 Guangzhou Leyiju, PR China

[2]College of Food Science and Nutritional Engineering, China Agricultural University, Qinghuadonglu, 100083 Beijing, PR China

[3]Graduate School of the Chinese Academy of Science, 100039 Beijing, PR China

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Summary

A simple and rapid method using high performance liquid chromatography (HPLC) was developed to determine levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) in litchi fruit pericarp tissue. This HPLC method used acetonitrile gradient elution and shortened the time required for determinations of adenosine phosphates. This analysis exhibited good repeatability (coefficients of variation 1.28–1.80 %) and recovery rate (94.7–97.1 %). The correlation coefficients of ATP, ADP and AMP with their peak areas at a range of 0–80 ng were 0.9946, 0.9994 and 0.9974, respectively. This method was applied to determine levels of adenosine phosphates in pericarp tissue of litchi fruit at harvest. There were 27.4 μg/g of ATP, 35.4 μg/g of ADP and 7.9 μg/g of AMP on a fresh mass basis.

Key words: HPLC, ATP, ADP, AMP, determination, litchi fruit, pericarp tissue

Introduction

Energy metabolism is an important metabolic pathway which maintains biochemical and physiological activities in plant tissues (*1*). Energy charge can be used to show energy status in plant tissues (*1,2*), while synthesis, degradation and restoration of membrane lipids depend on energy supply (*3–5*). The energy charge of cells can be calculated as ([ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP]), as described by Pradet and Raymond (*1*). Some of key enzymes concerning glycolysis, the Krebs' cycle, the electron transport system and oxidative phosphorylation are also regulated by the energy level of cells (*6,7*). Lipids are the essential components of plant cell membranes, and involvement of adenylate nucleotides in fatty acid biosynthesis in membrane lipid is well established (*5,8*). Thus, energy status of tissues of fruits and vegetables may play an important role in maintaining the integrity of cell membranes, which is related to postharvest life (*7,9*).

The luciferase system was mainly used to determine ATP concentration in organisms, while the analysis of ATP and ADP was performed by enzyme-linked immunosorbent assay (*10–12*). A high performance liquid chromatography (HPLC) analysis of ATP and its degradation products was also developed on reverse-phase columns using phosphate buffer as a mobile phase (*13,14*). Organic solvents, such as methanol (*15,16*) or acetonitrile (*14*), can be used to reduce the running time. In practice, these methods need a long running time and it is necessary to develop a simple and rapid method to analyze a large number of fruit samples (*14,17,18*).

*Corresponding author; Phone: ++86 20 37 252 905; Fax: ++86 20 37 252 831; E-mail: ymjiang@scib.ac.cn

There are no reports on the determinations of ATP, ADP and AMP contents by HPLC in plant tissue. The objective of this study was to develop a simple and rapid HPLC method for measurements of ATP, ADP and AMP content in pericarp tissue of litchi fruit.

Materials and Methods

Reagents

The high purity ATP, ADP and AMP standards, perchloric acid, potassium dihydrogen phosphate, potassium hydrogen phosphate and acetonitrile (HPLC grade) were purchased from Sigma Chemical Co. All reagents used were dissolved in deionized water, and then filtered with the Sybron/Barnstead Millipore system and a 0.45-μm filter paper.

Plant materials

Fruit of litchi (*Litchi chinensis* Sonn. cv. Huaizhi) at the commercially mature stage was harvested from an orchard in Guangzhou. Litchi pericarp was collected for extraction and determinations of ATP, ADP and AMP.

Preparations of standard stock solutions

ATP, ADP and AMP standards (1 mg) were each dissolved in 10 mL of deionized water to obtain ATP, ADP and AMP standard stock solutions at 100 μg/mL. Aliquots of the stock standard solutions were made by diluting them in deionized water at 0, 0.2, 0.5, 1, 2 and 4 μg/mL. A volume of 20 μL of each sample was taken for HPLC analysis.

Mobile phases

Mobile phase A consisted of 0.06 mol/L dipotassium hydrogen phosphate and 0.04 mol/L potassium dihydrogen phosphate dissolved in deionized water and adjusted to pH=7.0 with 0.1 mol/L potassium hydroxide, while mobile phase B consisted of 100 % acetonitrile. Air bubbles in the two solutions were driven away using an ultrasonic instrument.

Extraction of ATP, ADP and AMP from litchi fruit pericarp tissue

Litchi fruit pericarp tissue (2 g) was rapidly frozen in liquid nitrogen and homogenized into powder. Adenosine phosphates were extracted from the powder with 10 mL of 0.6 mol/L perchloric acid in the ice bath for 1 min by the method of Yang *et al.* (*12*). The extraction mixture was centrifuged for 10 min at 6 000×*g* (Beckman J20-2) and 4 °C, and 6 mL of the supernatant was taken and quickly neutralized to pH=6.5–6.8 with 1 mol/L KOH solution. The neutralized supernatant was then allowed to stand for 30 min in an ice bath to precipitate most of the potassium perchlorate, which was removed by paper filtration. The filtrate solution was filtered again through a 0.45-μm filter. The final filtrate solution was made up to 8 mL and then stored at –30 °C prior to the analysis.

HPLC analysis

The HPLC (Gold 125 Solvent System, Beckman Instruments Inc., USA) conditions were as follows: an Ultrasphere ODS EC 250×4.60 mm column (Beckman Instruments Inc., USA) was equipped with a Beckman 125 pump system. Peaks were detected and analyzed at 254 nm by a Gold 168 diode array detector. HPLC separation was achieved using continuous gradient elution. The elution program was as follows: 0 min 100 % A, 0 % B; 2 min 95 % A, 5 % B; 4 min 80 % A, 20 % B; 5.3 min 75 % A, 25 % B and 6 min 100 % A, 0 % B. Finally, the program took a further 1 min to return to the initial conditions and stabilize. Flow rate of the mobile phase was 1.2 mL/min, while the injection volume was 20 μL. The total retention time was about 5 min and the gradient was run for 6 min to ensure full separation. ATP, ADP and AMP in the samples were identified by comparison with retention time of standards, while the concentrations of ATP, ADP and AMP were determined using the external standard method. Data were expressed as means of six replicate determinations.

Recovery trial

Three standards (ATP, ADP and AMP) were added into litchi fruit pericarp tissue. The extraction was carried out according to the above-mentioned method. The concentration of each standard added for the trial was 2 μg/mL. The whole experiment was repeated six times.

Statistical analysis

Quantitative data from the HPLC analysis were compared using either the coefficients of variation (CV) or analysis of variance (ANOVA). Least significant difference was used to compare the means.

Results and Discussion

HPLC analysis and calibration curves of ATP, ADP and AMP

ATP and its breakdown products exhibited a great absorbance at 254 nm (*13*). As shown in Fig. 1, ATP, ADP and AMP were separated well and detected at 254 nm in this study. Acetonitrile instead of methanol used as mobile phase gave a rapid and better separation of ATP, ADP and AMP (data not shown) because it has higher polarity than methanol. Ryder (*13*) obtained a

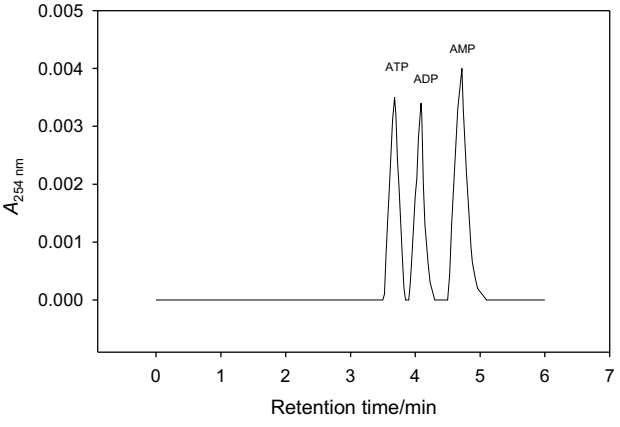

Fig. 1. HPLC chromatogram of a standard mixture of ATP, ADP and AMP

sufficient resolution of adenosine phosphates within 16 min using an isocratic system. Veciana-Nogues *et al.* (*16*) reported a good separation of adenosine phosphates using 30 % methanol gradient conditions. In this study, the separation and determination of ATP, ADP and AMP needed only 6 min and, thus, it is very convenient to analyze a large number of samples.

There was a good linear relationship among ATP, ADP and AMP concentrations at a range of 0–80 ng against their peak areas, with correlation coefficients being 0.9946, 0.9994 and 0.9974, respectively, at the 5 % level. Using 2 μg/mL standards, the CV of the levels of ATP, ADP and AMP were 1.80, 1.28 and 1.30 %, respectively, all below 5.00 % (Table 1), which indicated excellent repeatability for the analysis of the three compounds.

Table 1. Comparative HPLC analyses of ATP, ADP and AMP

Compound	Means[a]	CV/%
ATP	0.6111	1.80
ADP	0.7667	1.28
AMP	0.8663	1.30

[a]Data were expressed as peak areas (mm^2) (N=6)

Recovery trial

High precision was important to determine the concentrations of ATP, ADP and AMP (*14,19*). The results indicated that the mean recovery rates of ATP, ADP and AMP were 94.9, 94.7 and 97.1 %, respectively. These were very high recovery rates bearing in mind the complexity of the analyses. The CV of ATP, ADP and AMP were 4.3, 3.5 and 1.4 %, respectively, all below 5.0 %, exhibiting good precision.

Analyses of ATP, ADP and AMP of litchi fruit pericarp tissue

This HPLC method was applied to determine the concentrations of ATP, ADP and AMP of pericarp tissue in litchi fruit at harvest. Fig. 2 shows the chromatograms of ATP, ADP and AMP extracted from the litchi pericarp tissue. There were 27.4 μg/g of ATP, 35.4 μg/g of ADP and 7.9 μg/g of AMP on a fresh mass (FM) basis. In this study, 2 g of fresh litchi fruit pericarp tissue was enough to determine the concentrations of ATP, ADP and AMP. Furthermore, the authors used this method to analyze the concentrations of ATP, ADP and AMP of banana and longan fruits, and found 10.9 μg/g FM of ATP, 12.2 μg/g FM of ADP and 12.8 μg/g FM of AMP in banana fruit peel and 6.6 μg/g FM of ATP, 8.1 μg/g FM of ADP and 7.1 μg/g FM of AMP in longan fruit skin.

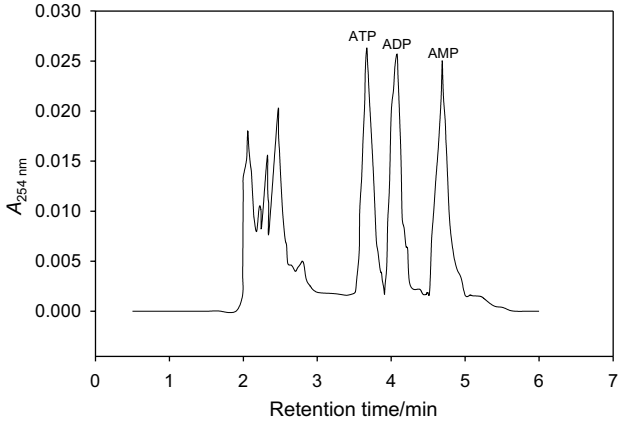

Fig. 2. HPLC chromatogram of ATP, ADP and AMP from litchi fruit pericarp tissue

In this study, the HPLC method could not separate the peaks eluting between 2 and 3 min, whose ultraviolet absorption spectrum was at 254 nm (Fig. 2). Özogul *et al.* (*14*) reported that the unknown compounds in herring tissue could be the degradation products of ATP, such as inosine monophospate and hypoxanthine. However, their identification in litchi fruit pericarp tissue needs further investigation.

Conclusion

In conclusion, an HPLC analysis of ATP, ADP and AMP concentrations in litchi fruit pericarp tissue was simple and rapid to use. We suggest that the improved method for identification and quantification of ATP, ADP and AMP should be a valuable tool in analyzing a large number of fruit samples.

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