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Fast Determination of Adenosine 5′-Triphosphate (ATP) and Its Catabolites in Royal Jelly Using Ultraperformance Liquid Chromatography

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ABSTRACT: To obtain insight into the metabolic regulation of adenosine 5′-triphosphate (ATP) in royal jelly and to determine whether ATP and its catabolites can be used as objective parameters to evaluate the freshness and quality of royal jelly (RJ), a rapid ultraperformance liquid chromatography (UPLC) method has been developed for feasible separation and quantitation of ATP and its catabolites in RJ, namely, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR), and hypoxanthine (Hx). The analytes in the sample were extracted using 5% precooled perchloric acid. Chromatographic separation was performed on a Waters Acquity UPLC system with a Waters BEH Shield RP18 column and gradient elution based on a mixture of two solvents: solvent A, 50 mM phosphate buffer (pH 6.5); and solvent B, acetonitrile. The recoveries were in the range of 86.0−102.3% with RSD of no more than 3.6%. The correlation coefficients of six analytes were high ($r^2 \geq 0.9988$) and within the test ranges. The limits of detection and quantification for the investigated compounds were lower, at 0.36−0.68 and 1.22−2.30 mg/kg, respectively. The overall intra- and interday RSDs were no more than 1.8%. The developed method was successfully applied to the analysis of the analytes in samples. The results showed that ATP in RJ sequentially degrades to ADP, AMP, IMP, HxR, and Hx during storage.

KEYWORDS: *ATP, catabolites, ultraperformance liquid chromatography, royal jelly*

INTRODUCTION

Royal jelly (RJ) is a milky white, gelatinous substance secreted by worker bees that constitutes the essential food for the larvae of the queen bee[1,2] and is believed to play an important role in the development of the queen bee.[3,4] In modern human nutrition the use of RJ has significantly increased because of the trend toward the use of healthy, organic, functional foods and dietary supplements. [5] RJ contains many important, biologically active compounds, such as free amino acids, proteins, sugars, fatty acids, minerals, and vitamins.[6−8] Among them, proteins account for 12−15% of fresh RJ and up to 50% of dried RJ; it is evident that proteins contribute significantly to honeybee biology[9,10] and the health promotion properties for mankind.[11] Other compounds identified in RJ include several nucleotides as free bases (adenosine, uridine, guanosine, iridin, and cytidine) and the phosphates adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), and adenosine 5'-triphosphate (ATP).[12,13]

Among the compounds in RJ, the pharmacological use of ATP has received increasing attention following reports of its benefit in the management of pain and the prevention of vascular disease and cancer.[14] The pathway of ATP catabolism has been extensively documented as a degradative sequence to ADP, AMP, inosine 5′-monophosphate (IMP), inosine (HxR), and hypoxanthine (Hx).[15] The *K* value, which is defined as the ratio of the sum of HxR and Hx to the sum of the ATP and its catabolites expressed as a percentage,[16] has been widely used as one of the freshness indices to evaluate the change in quality of raw fish after catch[17−19] and also might be a freshness index to evaluate the change in quality of RJ during storage. Given the interest in the metabolism of ATP and whether ATP-related compound contents can be used as objective parameters to evaluate the freshness and quality of RJ employed, it is necessary to establish a quick and reliable method for the simultaneous measurement of ATP and its catabolites in RJ, namely, ADP, AMP, IMP, HxR, and Hx.

To date, many different methods have been described for determination of ATP or its related compounds in biological fluids, herbal materials, and foodstuffs, including HPLC.[19−21] However, these methods, such as HPLC, suffer from a long analysis time. Ultraperformance liquid chromatography (UPLC) makes it possible to perform very high-resolution separations in short periods of time with minimal consumption of organic solvents,[22] which has attracted the attention of pharmaceutical and biochemical analysts.[23,24]

In this paper, a UPLC method for the fast simultaneous determination of ATP and its catabolites in RJ, namely, ATP, ADP, AMP, IMP, HxR, and Hx, was developed. The validated method was also successfully applied for the determination of ATP and its catabolites in RJ samples.

MATERIALS AND METHODS

Reagents and Standards. Standards of the six individual compounds, adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-diphosphate disodium salt (ADP), adenosine 5′-monophosphate sodium salt (AMP), IMP, HxR, and Hx were purchased

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from Sigma. Acetonitrile was purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA). Perchloric acid ($HClO_4$), potassium dihydrogen phosphate (KH_2PO_4), potassium carbonate (K_2CO_3), dipotassium hydrogen phosphate trihydrate ($K_2HPO_4{\cdot}3H_2O$), potassium hydroxide (KOH), and magnesium sulfate ($MgSO_4$), all of analytical grade, were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd., China. All aqueous solutions were prepared with deionized water.

Stock solutions of ATP, ADP, AMP, IMP, HxR, and Hx were prepared in deionized water at concentrations of 1000 mg/kg and stored at −20 °C until use. Working standard solutions including all analytes were prepared by mixing stock standard solutions at the desired concentrations before use.

Sample Preparation. Fresh RJ samples were harvested at five different apiaries in different regions of Zhejiang province during the flowering period of *Brassica napus* with the high-RJ-producing Pinghu Italian-breed bees. Commercial samples marked as *B. napus* RJ were purchased from supermarkets in Beijing. All samples were stored at −20 °C until use. Three commonly used extraction methods for ATP and its catabolites were used and compared in this study.

Perchloric Acid Procedure for Extraction of ATP and Its Catabolites in RJ. A total of 0.5 g of RJ (0.15 g for RJ freeze-dried powder) was accurately weighed and transferred into a 10 mL centrifuge tube. Next, 5 mL of precooled perchloric acid (5%, precooled at −5 °C) was added, and the resultant solution was homogenized for 3 min with a polytron (VORTEX-5, Haimen Qilinbeier Instruments Co., Ltd., China). The homogeneous solution was then centrifuged at 12000*g* for 10 min (at 0 °C) and the supernatant decanted. Potassium hydroxide (400 μL, 6 mol/L) was added to precipitate the perchloric ion, and the pH of the perchloric extract was adjusted to 5.5−6.0 using 2 mol/L potassium carbonate. The extract was subsequently centrifuged for 3 min at 12000*g* (at 0 °C), and the supernatant was decanted and brought to a final volume of 5 mL with deionized water. The final extract was filtered through a 0.22 μm syringe filter (Millipore) prior to injection into the UPLC system.

Boiling Water Procedure for Extraction of ATP and ATP Catabolites in RJ. A total of 0.5 g of RJ (0.15 g for RJ freeze-dried powder) was accurately weighed and transferred into a 10 mL centrifuge tube. Next, 5 mL of boiling deionized water was added, and the resultant solution was homogenized for 1 min with a polytron (VORTEX-5, Haimen Qilinbeier Instruments Co., Ltd.). The centrifuge tube was incubated in a boiling water bath for 6 min. After cooling in ice for 30 s, the mixture was centrifuged for 10 min at 12000*g* (at 20 °C), and the supernatant was decanted and brought to a final volume of 5 mL with deionized water. The final extract was filtered through a 0.22 μm syringe filter (Millipore) prior to injection into the UPLC system.

Boiling Magnesium Sulfate Procedure for Extraction of ATP-Related Compounds in RJ. A total of 0.5 g of RJ (0.15 g for RJ freeze-dried powder) was accurately weighed and transferred into a 10 mL centrifuge tube. After 5 mL of boiling magnesium sulfate (0.2 mmol/L) was added, the subsequent sample preparation procedure followed that described for the boiling water procedure for extraction of ATP and ATP catabolites in RJ.

UPLC Analysis. All analyses were performed on a Waters Acquity UPLC system (Waters, Milford, MA, USA), including a binary solvent manager, a sample manager fitted with a 2 μL loop, and a tunable UV (TUV) detector, connected to Waters Empower 2 software. A Waters BEH Shield RP18 column (100 mm × 2.1 mm i.d., particle size = 1.7 μm) was used.

A 50 mM phosphate buffer (pH 6.5) (mobile phase A) and 100% acetonitrile (mobile phase B) were used to separate the compounds of interest. Buffers were prepared by diluting 50 mL of a stock solution of 0.6 M K_2HPO_4/0.4 M KH_2PO_4 with 1 L of deionized water. The pH was adjusted to 6.5 with concentrated phosphoric acid. The two solvents were filtered and degassed before use. Gradient elution was carried out as follows: 0−2.5 min, 100% A; 2.5−3.0 min, linear gradient to 30% B; 3.0−5.5 min, 30% B; 5.5−6.0 min, linear gradient back to 100% A (initial conditions); 2 min equilibration wash with 100% A. The flow rate was set at 0.50 mL/min, and the injection volumes for all samples and standards were 1.0 μL. The column temperature was set at 30 °C. The peaks were detected at 257 nm.

RESULTS AND DISCUSSION

Optimization of Extraction Method. There are three commonly used methods for the extraction of ATP and its catabolites from biological tissues and foodstuffs, namely, (a) perchloric acid followed by neutralization;[19,25] (b) boiling water coupled with a heating process (to inactivate ATPase);[26] and (c) boiling saline solution coupled with a heating process (to inactivate ATPase).[27] Perchloric acid is a widely used agent to extract ATP-related compounds from intact cells due to its strong acidic nature;[19] it can not only extract ATP and inactivate ATPase simultaneously but also precipitate protein effectively. Boiling water or saline solution coupled with a heating process to inactivate ATPase has also been used for extraction of ATP-related compounds from biological tissues. Unlike the perchloric acid method, the boiling water or saline solution/heating method does not require neutralization. In this study, 5% precooled perchloric acid, boiling 0.2 mmol/L magnesium sulfate solution, and boiling water were used to extract ATP and its catabolites in RJ, respectively. The comparison experiments were performed using a RJ sample spiked with standard solution containing the six analytes (spiking level = 50 mg/mg per analyte), and three replications were used. The extraction efficiencies of the three methods are listed in Table 1.

Table 1. Comparison of Extraction Efficiencies of Three Methods for ATP and Its Catabolites in Royal Jelly

	recoveries[a] (%)		
analyte	method 1[b]	method 2[c]	method 3[d]
ATP	88.3 ± 5.4 d	60.9 ± 7.3 e	61.4 ± 8.9 e
ADP	94.2 ± 4.8 d	73.8 ± 6.0 e	72.5 ± 7.5 e
AMP	95.8 ± 3.9 d	123.4 ± 8.9 e	127.8 ± 7.1 e
IMP	96.7 ± 4.1 d	117.9 ± 6.7 e	119.2 ± 5.8 e
HxR	98.3 ± 2.9 d	97.5 ± 2.6 d	98.1 ± 3.1 d
Hx	93.1 ± 4.6 d	92.7 ± 3.0 d	94.1 ± 1.9 d

[a]Data are expressed as the mean ± SD%; $n = 3$. Values within rows with different letters were significant at p value <0.05 based on Duncan's multiple-range test. [b]Method 1: 5% precooled perchloric acid followed by neutralization. [c]Method 2: boiling water coupled with a heating process. [d]Method 3: boiling 0.2 mmol/L magnesium sulfate solution coupled with a heating process.

The results indicated that the recoveries of six analytes in RJ ranged from 88.3 to 98.3% when the 5% precooled perchloric acid method was used. However, when the boiling water method was used, the recoveries of ATP and ADP in RJ were 60.9 and 73.8%, respectively, significantly lower than those of the perchloric acid method ($p < 0.05$), but the recoveries of AMP and IMP in RJ were 123.4 and 117.9%, respectively, obviously higher than those using the perchloric acid method ($p < 0.05$). Similar results were achieved when boiling 0.2 mmol/L magnesium sulfate solution was used; the recoveries of ATP, ADP, AMP, and IMP were 61.4, 72.5, 127.8, and 119.2%, respectively.

Catabolism of ATP normally results in a fast and temporary accumulation of AMP and IMP[28] under hot and acid conditions, and the degradation of IMP to HxR and finally

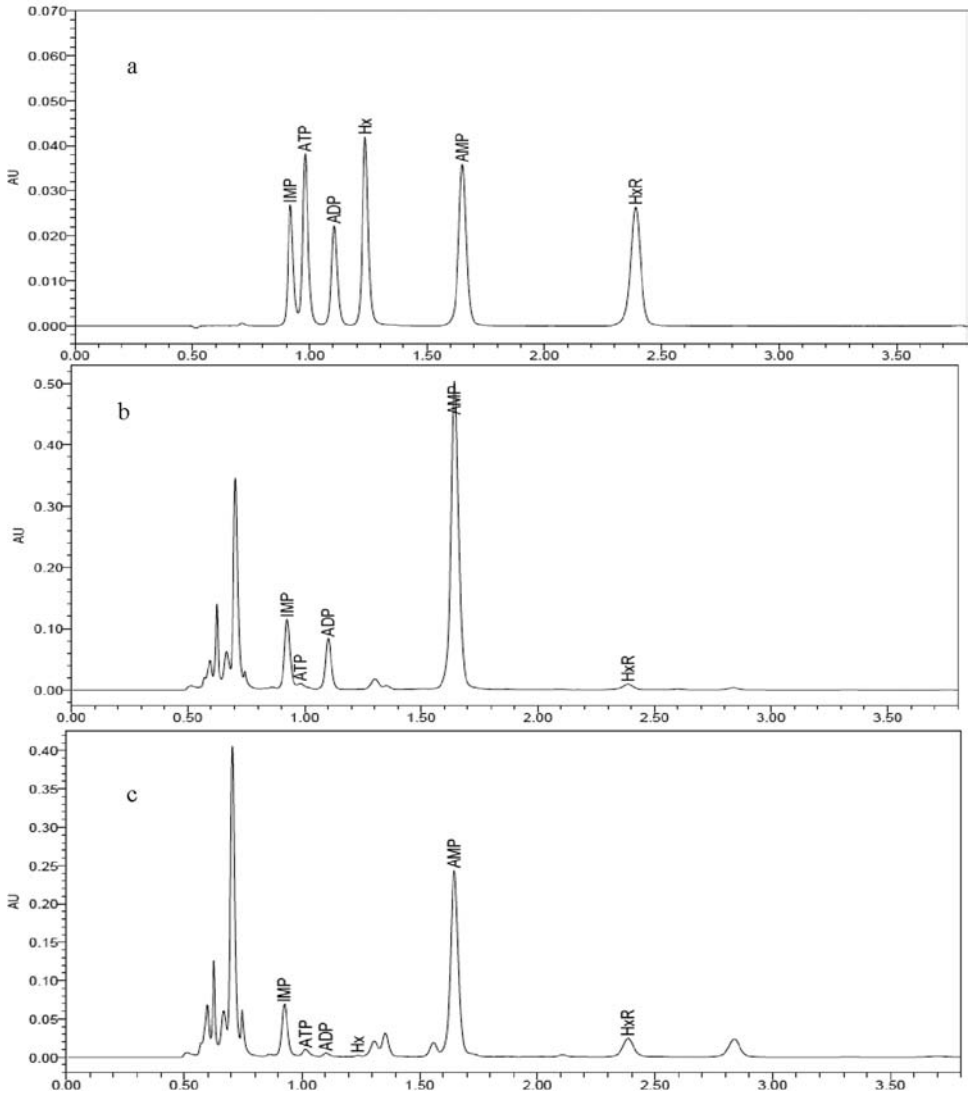

Figure 1. UPLC chromatograms of standard solutions of ATP-related compounds and the samples: (a) standard solutions; (b) fresh RJ sample harvested of known origin; (c) commercial RJ sample.

Hx occurs at a slower rate.[29] Because RJ is an acidic matrix (pH 3.5−4.5) and a heating process is necessary to inactivate ATPase when water or saline solution is used for extraction of ATP-related compounds, ATP in RJ can degrade to AMP and IMP during the extraction process. That is why when the methods with a heating process are used, the recoveries of ATP and ADP are lower, and the recoveries of AMP and IMP are higher than those using the perchloric acid method. Thus, the perchloric acid method was finally selected as the optimal method for the extraction of ATP and its catabolites in RJ.

Optimum Chromatographic Conditions. Adenosine nucleotides are a class of compounds with extreme polarity due to the presence of multiple phosphate groups that may interfere with chemical determination. In particular, the nucleotide phosphates are not retained on a column under conventional reversed-phase chromatographic conditions due to their extremely high polarity.[30] However, the presence of an ion-pairing agent or phosphate buffer enhances their retention and separation on the LC column. In this study, phosphate buffer was selected as the mobile phase. Different mobile phase concentrations (40, 45, 50, 55, and 60 mmol/L) and pH values (5.5, 6.0, 6.5, and 7.0) were evaluated and compared to determine the best peak resolution conditions. As a result, a mobile phase containing 50 mmol/L phosphate buffer (pH 6.5) was selected, which gave satisfactory resolution and a stable baseline.

RJ is a complex matrix, which contains many proteins,[6,7] pigments, esters,[2] and other biologically active substances.[8−10] Although most proteins can be precipitated by perchloric acid, some liposoluble compounds, such as pigments or esters, cannot be precipitated simultaneously. Those compounds will remain on the column and are difficult to be washed off with isocratic elution by phosphate buffer and thus can damage the chromatography column. Because acetonitrile has strong eluting power in both reversed- and normal-phase chromatography, using it as a mobile phase might be an effective way to wash off compounds remaining on the column. In this study, gradient elution of 50 mM phosphate buffer (pH 6.5) (mobile phase A) and 100% acetonitrile (mobile phase B) was finally chosen for the separation. ATP and its catabolites in the samples were identified by comparison of retention times against those obtained from standard stock solutions. Typical chromatograms of standard and sample under the optimized chromatographic conditions are shown in Figure 1.

Stability Test. For high-throughput analysis, samples usually have to remain in the autosampler for a long time, which should be considered carefully for unstable analytes. In this study, the stabilities of six analytes were tested by injection of standard solutions (50 mg/kg per analyte) at 0, 1, 2, 4, 8, and 12 h. The results showed that the six analytes were stable during the test time period (Table 2). Thus, the sample manager was controlled at 20 °C, and the quantitation of investigated compounds was preformed within 12 h.

Table 2. Stability and Intra- and Interday Precisions of the Investigated Compounds

		intraday (n = 6)[a]		interday (n = 6)[a]	
analyte	stability RSD (%)	accuracy[b] (%)	RSD (%)	accuracy (%)	RSD (%)
ATP	1.5	96.8	1.2	97.8	1.6
ADP	1.3	96.5	0.5	96.1	1.8
AMP	0.9	100.9	1.4	98.4	0.9
IMP	1.1	97.3	1.0	100.2	1.1
HxR	0.8	99.5	0.6	99.7	1.2
Hx	0.6	100.3	0.7	96.7	0.9

[a]Data expressed as mean value. [b]Accuracy (%) = 100 × mean of measured concentration/nominal concentration.

Calibration Curves and Limits of Detection (LOD) and Quantification (LOQ). Water stock solutions containing six reference compounds were prepared and diluted to appropriate concentrations for the generation of calibration curves. Seven concentrations of the solution were analyzed in triplicate, and the calibration curves were generated from peak areas of standards to their concentration. The results are shown in Table 3. The high correlation coefficients ($r^2 \geq 0.9988$) indicated good correlations between concentrations of the investigated compounds and their peak areas within the test ranges.

The LOD and LOQ were defined as 3 and 10 times the signal/noise ratios, respectively. They were separately determined by serial dilution of the standard solution using the described UPLC conditions in triplicate. The data in Table 3 show that the LOD and LOQ for the investigated compounds were lowered to 0.36−0.68 and 1.22−2.30 mg/kg, respectively.

Precision and Recovery. Intra- and interday variations were chosen to determine the precision of the developed method. For the intraday variability test, the mixed standards solution (50 mg/kg per analyte) was analyzed for six replicates within 1 day, whereas for the interday variability test, the solution was examined in duplicates for consecutive 3 days. The overall intra- and interday variations of the six analytes were no more than 1.8% (the relative standard deviations). The results listed in Table 2 indicated satisfactory intraday and interday variabilities.

The recoveries were preformed by adding a known amount of individual standards into a certain amount of RJ. Three replicates were performed for the test. The mixture was extracted and analyzed using the methods described above. The recoveries were in the range of 86.0−102.3%, with RSDs no more than 3.6% (Table 4). These results indicated that the present method can be used for quantitative analyses of ATP and its catabolites in RJ.

Table 4. Spiked Recoveries and RSDs of the Investigated Compounds

analyte	original (mg/kg)	spiked (mg/kg)	found (mg/kg)	recovery[a] (%)	RSD (%)
ATP	18.2	10.0	27.1	89.0	2.7
		40.0	56.2	95.0	3.0
		80.0	93.7	94.4	1.7
ADP	32.4	10.0	41.5	91.0	1.6
		40.0	71.3	97.3	2.2
		80.0	110.9	98.1	2.0
AMP	1121.6	10.0	1130.2	86.0	3.1
		40.0	1159.1	93.8	2.3
		80.0	1203.4	102.3	2.1
IMP	653.1	10.0	661.8	87.0	3.6
		40.0	690.4	93.2	2.9
		80.0	728.4	94.1	3.0
HxR	51.3	10.0	60.6	93.0	3.1
		20.0	70.8	97.5	1.7
		40.0	91.4	100.3	1.3
Hx	12.0	10.0	20.9	89.0	3.4
		20.0	30.9	94.5	2.9
		30.0	41.3	97.7	2.5

[a]Recovery (%) = 100 × (amount found − original amount)/amount spiked). The data represent the average of three determinations.

Application for Analysis of Actual RJ Samples. The validated UPLC method was applied for the determination of ATP and its catabolites in 15 RJ samples. Among them, 10 commercial samples marked as *B. napus* RJ were purchased from supermarkets in Beijing, and 5 fresh samples of known origin were obtained from five different apiaries in Zhejiang

Table 3. Linear Regression Data, LOD, and LOQ of the Investigated Compounds

	linear regression data[a]				
analyte[b]	regression eq[c]	linear range (mg/kg)	r^2	LOD (mg/kg)	LOQ (mg/kg)
ATP	$Y = (8604.3 \pm 23.1)X - (652.1 \pm 32.5)$	0.50−96.62	0.9995	0.43	1.41
ADP	$Y = (9812.6 \pm 35.2)X - (263.9 \pm 12.8)$	0.52−98.24	0.9997	0.68	2.30
AMP	$Y = (7895.7 \pm 11.9)X - (617.7 \pm 16.8)$	0.40−86.89	0.9988	0.42	1.40
IMP	$Y = (8516.4 \pm 26.7)X + (129.4 \pm 9.8)$	0.40−85.68	0.9998	0.46	1.52
HxR	$Y = (5987.7 \pm 19.6)X - (254.8 \pm 16.5)$	0.26−41.35	0.9993	0.52	1.69
Hx	$Y = (9628.5 \pm 36.4)X + (328.6 \pm 15.8)$	0.21−32.86	0.9997	0.36	1.22

[a]Resolutions of the peaks in the UPLC profile of reference compounds were calculated using Waters Empower TM^2 software. [b]All samples were prepared and analyzed in triplicate. [c]The data in the regression equations are presented as Y = (mean ± SD)X ± (mean ± SD).

Table 5. ATP and Its Catabolites in Royal Jelly Samples

sample[b]	mg/kg[a]					
	ATP	ADP	AMP	IMP	HxR	Hx
FRJ1	50.8 ± 0.9	235.4 ± 9.8	1023.4 ± 32.5	612.7 ± 11.9	16.9 ± 0.6	ND[c]
FRJ2	52.1 ± 1.0	251.3 ± 11.2	1234.5 ± 23.5	552.5 ± 9.6	20.4 ± 0.1	ND
FRJ3	49.6 ± 1.2	264.2 ± 12.3	967.8 ± 19.5	564.3 ± 13.5	31.4 ± 0.6	ND
FRJ4	58.3 ± 1.3	253.7 ± 12.5	1356.4 ± 25.7	690.7 ± 16.2	26.7 ± 0.4	ND
FRJ5	46.2 ± 0.9	248.7 ± 11.9	1138.7 ± 31.2	712.5 ± 15.2	23.7 ± 0.9	ND
RJ6	5.2 ± 0.3	38.9 ± 0.9	967.5 ± 21.3	510.6 ± 10.7	41.3 ± 0.4	2.3 ± 0.2
RJ7	7.3 ± 0.1	36.7 ± 0.4	897.6 ± 25.4	602.8 ± 16.2	39.4 ± 0.6	3.8 ± 0.3
RJ8	6.8 ± 0.5	41.2 ± 0.6	1024.7 ± 30.7	569.4 ± 10.8	38.7 ± 0.4	2.5 ± 0.2
RJ9	4.2 ± 0.2	40.6 ± 1.0	697.6 ± 21.5	552.7 ± 12.5	41.6 ± 0.7	4.6 ± 0.4
RJ10	3.8 ± 0.2	68.9 ± 1.3	894.2 ± 19.7	625.4 ± 15.6	56.4 ± 0.9	6.1 ± 0.6
RJ11	6.5 ± 0.3	43.5 ± 0.7	1029.7 ± 16.4	580.2 ± 16.4	40.2 ± 1.1	4.7 ± 0.1
RJ12	7.5 ± 0.5	50.6 ± 1.6	964.2 ± 23.4	496.7 ± 12.2	39.6 ± 0.6	3.5 ± 0.2
RJ13	3.8 ± 0.4	110.7 ± 2.6	679.6 ± 19.5	761.4 ± 16.4	46.7 ± 0.9	7.6 ± 0.6
RJ14	4.9 ± 0.6	38.4 ± 1.4	1011.9 ± 26.8	569.3 ± 15.8	39.2 ± 1.2	2.1 ± 0.1
RJ15	3.8 ± 0.2	41.6 ± 0.9	698.7 ± 16.4	659.4 ± 12.3	56.7 ± 1.6	1.6 ± 0.3

[a]Data expressed as the mean ± SD, mg/kg RJ; $n = 3$. [b]FRJ1−FRJ5 were the five fresh RJ samples of known origin harvested from apiaries in Zhejiang province, China. RJ6−RJ15 were the 10 commercial samples purchased from supermarkets in Beijing. [c]ND, not detected.

province during the flowering period of *B. napus*. All of the samples were kept at −18 °C before analysis. The results of the analysis are summarized in Table 5.

The average ATP and ADP contents in fresh RJ harvested from apiaries were 51.4 ± 4.4 and 250.7 ± 10.4 mg/kg, respectively, significantly higher than those in RJ purchased from supermarkets, which were 5.4 ± 1.5 and 51.1 ± 23.0 mg/kg, respectively. It is also noteworthy that ATP contents in all samples from supermarkets were <10 mg/kg, obviously lower than those in fresh samples. In contrast, HxR and Hx contents in RJ purchased from supermarkets were 44.0 ± 7.0 and 3.9 ± 1.9 mg/kg, respectively, markedly higher than those in fresh RJ, which were 23.8 ± 5.6 mg/kg and lower than the detection limit, respectively.

The results showed that ATP in RJ could sequentially degrade to ADP, AMP, IMP, HxR, and Hx during storage, similar to byproducts of ATP catabolism in other biological tissues[20,25,30] and foodstuffs.[27,31]

The developed UPLC method is rapid, sensitive, and accurate for the simultaneous qualitative and quantitative determination of ATP and its catabolites in RJ, which suggests UPLC can be a powerful tool for the analysis of components in bee products. This method could also be applied to biochemical and nutritional research.

Because ATP in RJ could sequentially degrade to ADP, AMP, IMP, HxR, and Hx during storage, it will be interesting in future research to study if the *K* value, which is widely used as a freshness index in fish samples, can be used to predict the freshness and quality of RJ using the validated UPLC method.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; IMP, inosine monophosphate; HxR, inosine; Hx, hypoxanthine; RJ, royal jelly; UPLC, ultraperformance liquid chromatography.

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