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ARTICLE

Online Cleanup of Accelerated Solvent Extractions for Determination of Adenosine 5′-Triphosphate (ATP), Adenosine 5′-Diphosphate (ADP), and Adenosine 5′-Monophosphate (AMP) in Royal Jelly Using High-Performance Liquid Chromatography

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Determination of the levels of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), and adenosine 5′-monophosphate (AMP) in royal jelly is important for the study of its pharmacological activities, health benefits, and adenosine phosphate degradation. In this study was developed a novel method to determine ATP, ADP, and AMP levels in royal jelly using accelerated solvent extraction (ASE) followed by online cleanup and high-performance liquid chromatography (HPLC) with diode array detection (DAD). The optimum extraction conditions were obtained using an 11 mL ASE cell, ethanol/water (5:5 v/v) as the extraction solvent, 1500 psi, 80 °C, a 5 min static time, and a 60% flush volume. Optimum separation of the three compounds was achieved in <25 min using a Waters XBridge Shield RP18 column with 0.05 mol L^{-1} $NH_4H_2PO_4$ (pH 5.70) and acetonitrile as the mobile phase. Detection was performed at 257 nm. The method was sensitive (LOD $\leq$ 0.32 mg L^{-1}), repeatable (RSD $\leq$ 4.5%), accurate (recovery rates of 87.6−94.2% with RSD $\leq$ 5.4), and precise (intraday RSD $\leq$ 8.5%, interday RSD $\leq$ 3.4%). The ASE extraction procedures developed here were compared with the classical adenosine phosphate extraction procedures (perchloric acid). The results indicate that the two techniques are similar in terms of recovery and reproducibility, but when other factors such as extraction time, environmental protection, and worker's health are considered, ASE is preferable to the classical extraction method. With this ASE-HPLC method, a minisurvey of ATP, ADP, and AMP levels in 15 samples of royal jelly of different origins was performed. Sample results indicated that the AMP concentration was 24.2−2214.4 mg kg^{-1}, whereas ATP and ADP were not detectable or present only at low levels.

KEYWORDS: ATP; ADP; AMP; royal jelly; accelerated solvent extraction; high-performance liquid chromatography

INTRODUCTION

Royal jelly (RJ) is secreted by the hypopharyngeal gland to feed young larvae and the adult queen bee. RJ is an interesting healthy and functional compound because it has several health-promoting and pharmacological properties (*1*); for example, RJ ameliorates physical fatigue after exercise, has immunomodulatory properties, and inhibits the development of atopic dermatitis such as skin lesions (*2*).

Chemical composition analysis has shown that RJ is mainly composed of proteins, sugars, lipids, vitamins, free amino acids, and a large number of bioactive substances (*3*). Numerous minor compounds, belonging to diverse chemical categories, and several nucleotides such as adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), and adenosine 5′-monophosphate (AMP) were separated from RJ by paper chromatography in 1964 (*4*). However, since then, no further study about adenosine phosphate in RJ has been reported.

For several years, the study of adenosine phosphate in foodstuffs and animal tissue has received special attention owing to its crucial roles in many biochemical pathways, its role as the universal currency of energy in biological systems, its function as a coenzyme in biochemical reactions, and its role as a precursor in RNA synthesis (*5*). Moreover, nucleotides are present in food, mainly as nucleoproteins, from which they are released and efficiently absorbed (*6*). Furthermore, in medicine, the pharmacological use of ATP has received increasing attention following reports of its benefits in treating pain, vascular disease, and cancer (*7*).

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A suitable method for the extraction and analysis of adenosine phosphate is essential to the study of its activity in foodstuffs and tissues. The classical adenosine phosphate extraction method involves conventional liquid shaking for a few minutes with cold perchloric acid, neutralization with an alkaline solution, followed by centrifugation (*7*, *8*). Although this extraction method is suitable for the extraction of adenosine phosphate from some complex matrix materials, in practice it can cause environmental or health damage owing to the use of perchloric acid. Perchloric acid is extremely hazardous. It is corrosive to skin and eyes and should be handled with the utmost care. In addition, when perchloric acid is neutralized with alkaline solution, perchlorates are produced; these tend to be less reactive and more stable in the environment and are becoming a serious threat to human health and water resources (*9*). An ideal leaching procedure should be exhaustive with respect to the constituents to be analyzed, rapid, simple, inexpensive, environmentally friendly, and suitable for automation for routine analysis (*10*). One of the most promising sample preparation techniques is the accelerated solvent extraction (ASE) technique, which has the advantages of reducing solvent use and time involvement and allowing for automated sample handling (*11*). Although ASE has started to replace conventional extraction procedures in environmental (*12*) and food analysis (*13*), its application in the nucleotide field has been limited to adenosine analysis (*14*). In this study, a mixture of ethanol and water was used to extract adenosine phosphate from RJ using ASE. The different factors affecting the efficiency of the extraction, such as solvent composition, temperature, pressure, and static time, were carefully optimized.

High-performance liquid chromatography (HPLC) is currently the most commonly used separation technique for adenosine phosphate, used in combination with detection by ultraviolet light (*7*, *15*−*17*) and mass spectrometry (*5*, *18*). In particular, as a conventional method, HPLC can be performed using standard equipment in many laboratories and is simple, sensitive, and suitable for monitoring adenosine phosphate. However, no detailed studies have investigated the distribution of adenosine phosphate in RJ or the analytical methods used for its determination in RJ samples. Development of a simple and rapid HPLC method for the determination of adenosine phosphate in RJ is therefore necessary.

The aim of this study was to develop a simple method for the determination of ATP, ADP, and AMP levels in RJ using ASE and HPLC equipped with a diode array detector without requiring an additional cleanup step. Several extraction parameters, such as solvent composition, temperature, pressure, extraction cycles, and extraction time, have been optimized using spiked RJ samples to select the best conditions for analysis of three adenosine phosphates. The optimized procedure has been validated by comparison with the results provided by the classical method for the determination of ATP, ADP, and AMP levels in spiked samples. With the method developed here, a limited survey of ATP, ADP, and AMP levels in RJ was performed in some samples of different origins.

MATERIALS AND METHODS

Ten randomly selected commercial samples were purchased from supermarkets in Beijing. Five samples of known origin and bee species (*Apis mellifera*) were obtained from Pinghu Apiary in China's Zhejiang province, a major RJ producing zone. All of the samples were kept refrigerated at −18 °C before analysis. Adenosine 5′-triphosphate disodium salt (ATP), adenosine5′-diphosphate disodium salt, (ADP), and adenosine 5′-monophosphate sodium salt (AMP) were purchased from Sigma (St. Louis, MO). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Absolute ethanol, monoammonium phosphate (NH_4H_2PO), potassium hydroxide (KOH), potassium carbonate (K_2CO_3), and perchloric acid (PCA, $HCLO_4$) (analytical grade) were obtained from Beifen (Changping, Beijing). Sorbent (C18 resin, 20−45 μm, 60 Å) was obtained from Beijing Alltech. Hydromatrix was obtained from Varian (mesh 60). Deionized water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA).

Table 1. Selected ASE Operating Conditions for Extraction of ATP, ADP, and AMP from RJ Samples

extraction solvent	ethanol/water (5:5 v/v)	purge time (min)	1
pressure (psi)	1500	no. of cycles	1
temperature (°C)	80	cell volume (mL)	11
heat-up time (min)	5	total extraction time (min)	14−15[a]
static time (min)	5	total solvent used (mL)	15[a]
flush volume (%)	60		

[a] Per sample.

HPLC Analysis. HPLC analysis was performed using a Dionex HPLC system (Dionex, Sunnyvale, CA), which included a P680 pump, an ASI-100 autoinjector, a TCC-100 column oven, and a DAD 100 detector, connected to Chromeleon software. An XBridge Shield RP18 column (150 mm × 4.6 mm i.d., 5 μm) from Waters was used. The column temperature was maintained at 30 °C. The standards and samples were separated using a gradient mobile phase consisting of 0.05 mol L^{-1} $NH_4H_2PO_4$ (pH 5.70, A) and acetonitrile/water (60:40, v/v, B). The linear gradient conditions were as follows: 0−6.5 min, 100% A; 6.5−12.5 min, 100% B; 12.5−25 min, 100% A. The flow rate was set at 0.8 mL min^{-1}, and the injection volume was 20 μL. The detection wavelength was set at 257 nm.

Calibration. For the preparation of standard solutions, 2.00 mg of each standard compound was placed in a 10 mL volumetric flask and dissolved in ethanol/water (5:5, v/v). Beginning with this stock solution, six additional calibration solutions (0.5, 1.0, 50.0, 100, and 200 mg L^{-1}) were prepared by serial dilution with ethanol/water (5:5 v/v). The solutions were analyzed, and calibration curves were established by linear regression analysis (peak area vs concentration).

ASE Extraction. All of the extractions were performed using an ASE 350 system (Dionex). An aliquot of 1.5 g of the previously homogeneous sample and 3 g of C18 resin were placed in a samll beaker and blended together using a glass pestle to obtain a porous mixture to enable the extraction solvent to flow through the sample during the extraction. The mixture was poured into an 11 mL stainless steel extraction cell containing a cellulose filter in the cell outlet, which was packed by adding a layer of hydromatrix (1.5 g) at the top according to the manufacturer's recommendations.

The ASE parameters studied for optimization were composition of the extraction solvent (ethanol/water mixtures 7:3, 6:4, 5:5, and 4:6, v/v), extraction temperature (40, 60, 80, 100, and 120 °C), extraction pressure (1000, 1500, and 2000 psi), and static time (5 and 10 min). The flush volume was maintained at 60% of the cell. All experiments were performed in triplicate. The selected extraction conditions are summarized in **Table 1**.

The extracts were made up to a final volume of 20 mL with ethanol/water (5:5, v/v). After thorough mixing, an aliquot was filtered into vials using a 0.45 μm PTFE membrane from Phenomenex that had previously been shown to not retain the target analytes. After filtration, the raw extract was injected directly into the HPLC-DAD system without any further cleanup step.

Method Validation. Validation of the method was performed by evaluation of linearity (see above), limit of detection (LOD) (S/N ratio of 3), and limit of quantification (LOQ) (S/N ratio of 10), peak purity, repeatability, accuracy, and precision.

LOQ and LOD were determined by serial dilution of standard solutions. Peak purity was confirmed using the "peak purity" option in the Chromeleon software. Repeatability was confirmed by evaluating consistency of retention times and relative standard deviation (RSD) values. Accuracy was determined by spiking the RJ samples with three concentrations of the standard compounds (20, 50, and 100 mg L^{-1}). The precision (intra- and interday) of the method was evaluated by

analysis of four replicates of a real sample using the developed method over a 3 day period.

Comparison with the Classical Method (Perchloric Acid Extraction). The comparison experiments were performed using a RJ sample spiked with standard solution (spiking level = 50 mg L^{-1}). The spiked samples were analyzed using the optimized ASE conditions summarized in **Table 1** and the ASE procedure described above.

Alternatively, the same spiked samples were analyzed using the classical method (perchloric acid extraction) described previously (*7*) with minor modifications. Briefly, prior to the extraction stage, samples were homogenized using a supermixer blender system (Moulinex, Lyon, France). RJ samples (0.3 g) were vortex mixed for 3 min with 0.5 mL of ice-cold 8% PCA (v/v) in a 1.5 mL Eppendorf tube. After precipitation of the protein fraction (at 12000*g*, 10 min, 4 °C), the supernatant was removed and neutralized to pH 6.5 with 0.65 mL of 6 mol L^{-1} KOH and 40 μL of 2 mol L^{-1} K_2CO_3. The mixture was vortex mixed and centrifuged for 5 min, and the supernatant was collected in a 5 mL test tube. The volume was made up to 5 mL with 0.05 mol L^{-1} $NH_4H_2PO_4$ (pH 5.70). A sample from the solution was drawn into a pipet and filtered through a filtering cartridge with a 0.45 μm nylon membrane using a disposable syringe set before HPLC analysis.

The main parameters for comparison of the two methods were recovery, reproducibility, and information such as solvent consumption, extraction time, and environment and health effects.

Analysis of Real Samples. In total, 15 RJ samples of different origins were analyzed using the developed ASE-HPLC method to assess concentrations and distributions of ATP, ADP, and AMP.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions. Adenosine phosphates are often not retained on columns under conventional reversed-phase chromatographic conditions due to their extremely high polarity (*8*). To limit ionization and obtain good resolution, a gradient elution with phosphate buffer as the mobile phase is often used in reversed-phase liquid chromatography (*7*). In this study, the concentration and pH of phosphate buffer were optimized: 0.02, 0.04, 0.05, 0.06, and 0.10 mol L^{-1} concentrations of $NH_4H_2PO_4$ were used to separate three targets. The results show that 0.05 mol L^{-1} $NH_4H_2PO_4$, 0.06 mol L^{-1} $NH_4H_2PO_4$, and 0.1 mol L^{-1} $NH_4H_2PO_4$ can limit the ionization and eliminate peak tailing of target compounds. However, high salt concentrations may lead to column damage, so 0.05 mol L^{-1} $NH_4H_2PO_4$ was selected as the mobile phase. Different pH values can also affect separation. To acquire better separation and selectivity and higher resolution, different pH values of 0.05 mol L^{-1} $NH_4H_2PO_4$ were investigated. The results show that pH 5.70 can provide sufficient resolution and the best peak shapes (see **Figure 3a**). Therefore, the pH of the mobile phase was fixed to 5.70.

In the ultraviolet spectra of the HPLC-DAD chromatograms of reference, maximum absorbance values of ATP, ADP, and AMP were about 203 and 257 nm. A stable baseline was found at about 257 nm, so 257 nm was selected as the detection wavelength. A representative chromatogram of the separation of the standard mix and real RJ samples is shown in **Figure 3**.

Optimization of the ASE Method for ATP, ADP, and AMP Extractions in RJ. First, ethanol was selected as the extraction solvent because it can effectively extract adenosine phosphate from a complex matrix (*19*) and precipitate the protein from the RJ samples (*20*). In addition, the mixture of ethanol and water as the extraction solvent is environmentally acceptable and does not affect the health of the experimenter.

To identify the most suitable ASE conditions for ATP, ADP, and AMP extraction, an experimental design was conducted using RJ samples spiked with standard solution (spiking level = 100 mg L^{-1}). The main investigated parameters were temperature (40, 60, 80, 100, nd 120 °C), pressure (1000, 1500, and 2000 psi), static time (5 and 10 min), and extraction solvent (ethanol/water 7:3, 6:4, 5:5, and 4:6 v/v).

First, the extraction solvent and extraction temperature were optimized while maintaining constant pressure (1500 psi) and static time (5 min). The effects of temperature and solvent proportion on the extraction recoveries of ATP, ADP, and AMP in RJ are shown in **Figure 1**. The results show that extraction solvent and extraction temperature can markedly affect the extraction yield. After comparison, ethanol/water 5:5, v/v, and extraction temperature 80 °C were selected because they provided satisfactory extraction yields (≥90%) for all analytes. In addition, it should be noted that extraction recoveries of AMP were >100% (102−120%), whereas those of ATP and ADP were low (75%), when the extraction temperature was set at 100 °C; this is likely due to degradation of ATP and ADP, which has not been reported previously.

Sequentially, the extraction pressure and static time were optimized while maintaining constant extraction solvent (ethanol/water 5:5, v/v) and extraction temperature (80 °C). As shown in **Figure 2**, the results show that the extraction recoveries of the three analytes slightly increased with increasing pressure and static time. As the ex-length (10 min) of the static cycle or more than one cycle of extraction (data not shown) did not obviously affect the extraction efficiency, the extraction time was set to 5 min to ensure a rapid extraction. An increase in the applied pressure to 2000 psi resulted in darker extracts, with broad peaks at the beginning of the chromatogram due to the coextraction of other matrix components, which can damage the chromatographic column; therefore, it was decided to use 1500 psi as the extraction pressure in this study.

On the basis of the results above obtained, the optimum ASE conditions were identified and are shown in **Table 1**.

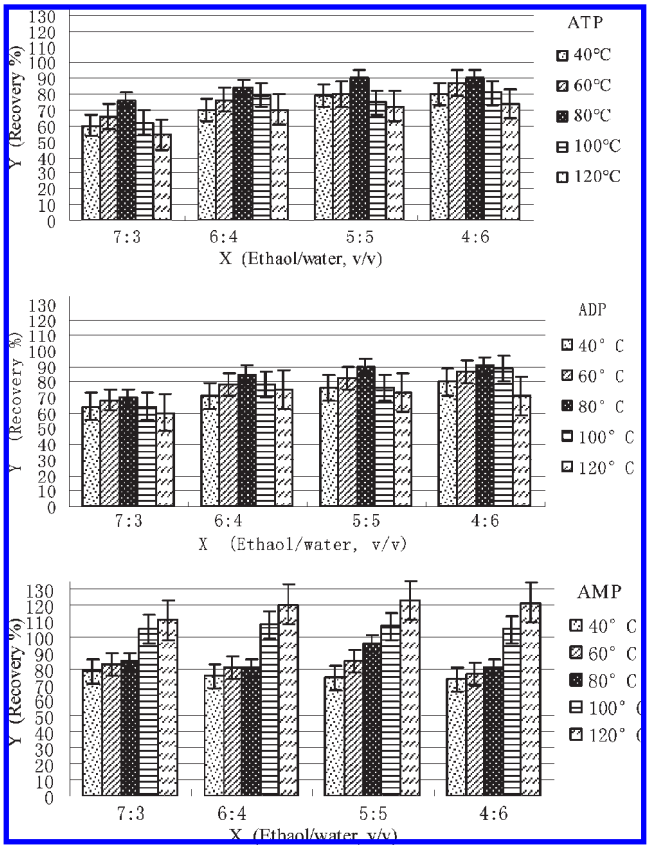

Figure 1. Effects of solvent proportion and temperature on recovery [constant pressure (1500 psi) and static time (5 min) were maintained].

Selection of ASE Online Cleanup. In any extraction process, it is common for the extract to require additional cleanup steps prior to analysis. ASE can easily achieve online cleanup by adding various sorbent materials directly to the ASE cell, producing a clean extract ready for analysis. Different sorbents are used for removing different interferences. ATP, ADP, and AMP are not retained by C18 resins due to their high polarity (*16*). Moreover, C18 resins can efficiently absorb lipids and pigments in RJ, as outlined in the manufacturer's recommendations (*21*). Therefore, in this study, C18 resins were selected as cleanup sorbents. After optimization, the rate of sample and the C18 resin are selected at a 1:2 (mass ratio). From real sample chromatography (**Figure 3b**), the chromatographic interference was shown to be low after an ASE online cleanup. Furthermore, the cleaner extracts can extend the life of the HPLC column. Under the conditions used in the present study, 150 injections could be performed without loss of resolution. There is only a small increase in column pressure with column aging.

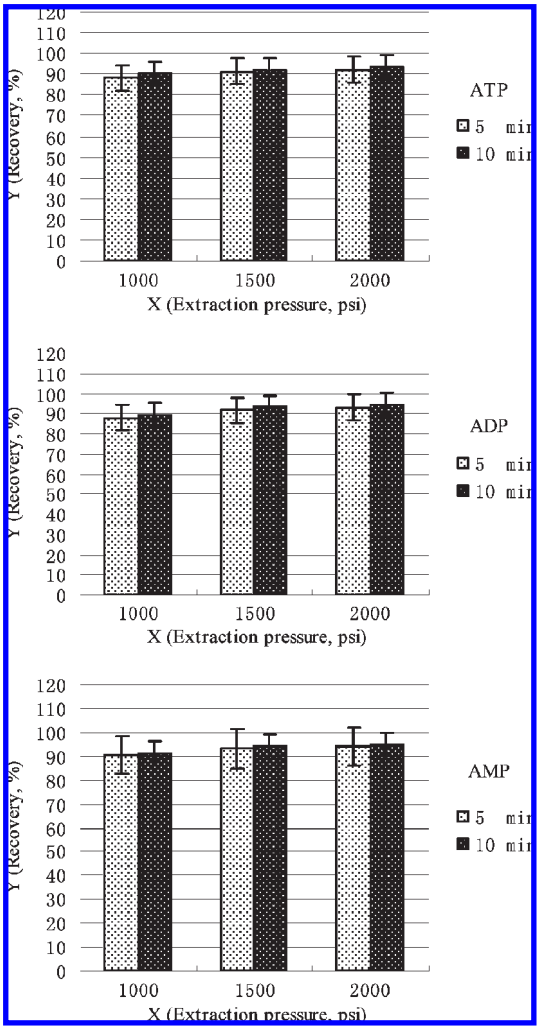

Figure 2. Effects of extraction pressure and static time on recovery [constant extraction solvent (ethanol/water 5:5, v/v) was maintained].

Method Validation. Validation of the method is the process by which it can be shown that an analytical method is suitable for its intended use. Calibration curves were calculated using peak areas at six standard concentrations, the range of which was proportional to the concentration of analyte in the prepared whole RJ samples. The concentration range was linear, from 0.5 to 200 mg L^{-1}, for all analytes. The regression coefficient for all calibration curves was >0.999 (**Table 2**).

On the basis of signal-to-noise ratios of 3 and 10, the LOD and LOQ were determined using standard solutions of three targets subjected to HPLC and analyzed using the methodology described above (HPLC Analysis). The details are summarized in **Table 2**.

The identity and purity of the peaks of interest were confirmed on the basis of retention times and spectral data available. Repeatability could be deduced by stable retention times, and relative standard deviations (RSDs) were $<4.5\%$ for five injections (**Table 2**).

Accuracy, determined at three concentrations (20, 50, and 100 mg L^{-1}), was found to be satisfactory (recovery rates between 87.6 and 94.2%, with RSD $<$ 5.4%, are shown in **Table 2**) for all compounds.

Intraday and interday precision values, assessed by analysis of a real sample over a 3 day period (days 1, 2, and 3) are shown in **Table 3**. The intraday precision was between 4.4 and 8.5% and the interday precision was between 0.9 and 3.4% for three analytes. The results show that the method has good reproducibility.

Comparison with Classical Method. To validate the optimized ASE method for determination of ATP, ADP, and AMP in RJ, the procedure was used to determine levels of three targets in spiked samples (50 mg L^{-1}) and the results were compared with those obtained using the classical method (perchloric acid extraction). As shown in **Table 4**, no significant differences at a 95% confidence limit were observed between the results obtained by the two methods. Moreover, application of the ASE procedure allows a significant reduction in the analysis time, allows automated and high-throughput analysis, and avoids the use of perchloric acid, with its associated human health and environmental pollution risks.

Analysis of Real Samples. The method was applied to real samples collected from beekeepers and supermarkets. In total, 15 samples were analyzed. The ATP content ranged from not detectable (ND) to 184.1 mg kg^{-1}, ADP content from ND to

Table 3. Intra- and Interday Precisions of the Method (Sample 1, Values in mg kg^{-1}, RSD % in parentheses)

	intraday precision (n = 4)			
analyte	day 1	day 2	day 3	interday precision(n = 3)
ATP	50.2 (4.4)	49.1 (4.9)	51.4 (5.2)	50.2 (2.3)
ADP	64.3 (4.9)	67.3 (5.3)	63.1 (6.4)	64.9 (3.4)
AMP	704.3 (6.8)	715.3 (8.5)	705.8 (7.7)	708.5 (0.9)

Table 2. Calibration, Repeatability, and Accuracy for Three Standard Compounds, Including Regression Equation, Correlation Coefficient (r^2), LOD, LOQ, Retention Time (RT; RSD % in Parentheses, n = 5), and Recovery Rates (RSD % in Parentheses, n = 4)

						recovery		
analyte	linear eq	r^2	LOD (mg L^{-1})	LOQ (mg L^{-1})	RT (min)	20 mg L^{-1} spike	50 mg L^{-1} spike	100 mg L^{-1}spike
ATP	$y = 3.7629x + 1.1521$	0.9991	0.11	0.32	4.23 (3.95)	87.6 (5.2)	90.2 (4.8)	92.4 (3.6)
ADP	$y = 3.7595x + 1.2196$	0.9997	0.10	0.29	4.66 (4.23)	89.1 (5.4)	88.6 (5.3)	90.8 (4.1)
AMP	$y = 2.6652x + 0.5008$	0.9999	0.08	0.25	6.19 (4.50)	92.4 (4.4)	90.9 (4.9)	94.2 (3.9)

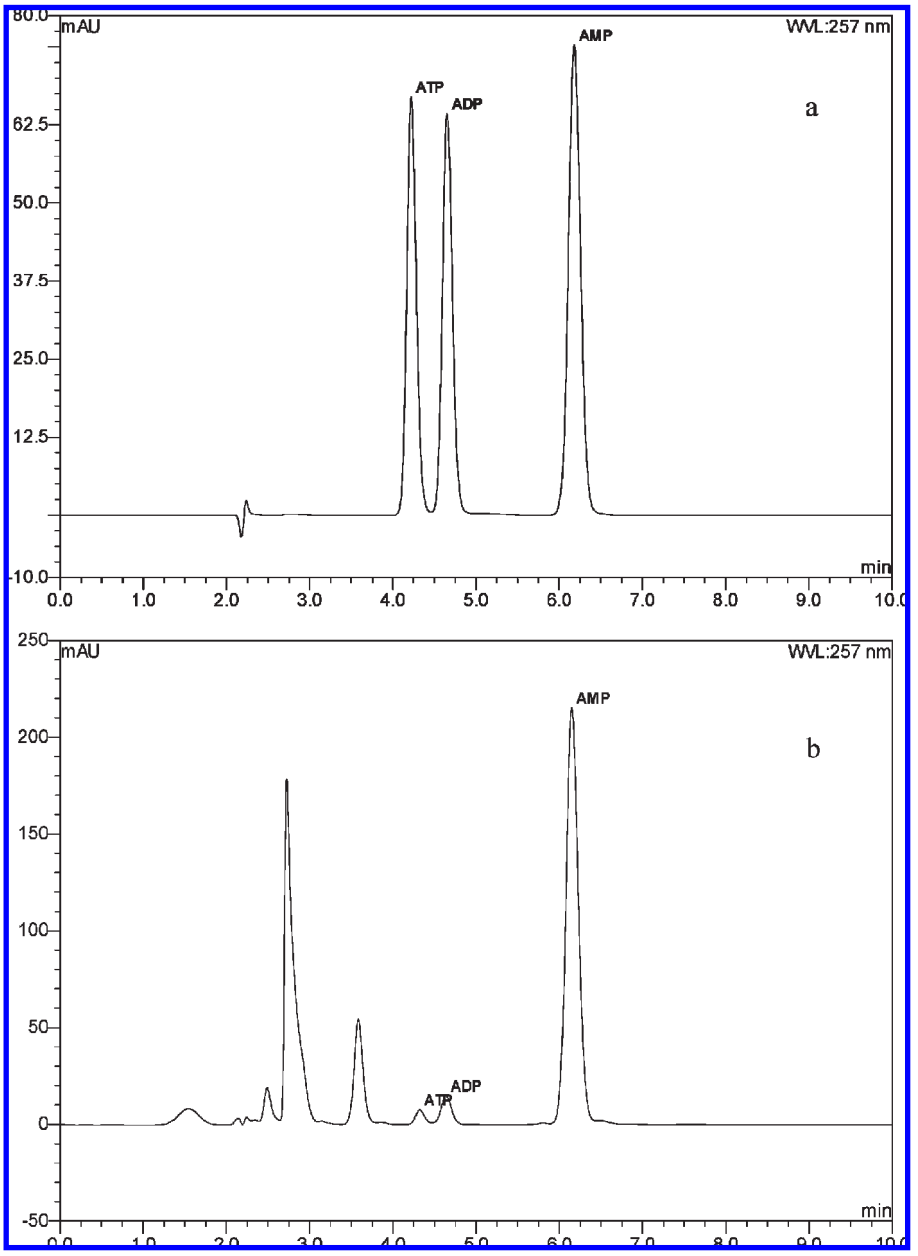

Figure 3. Typical HPLC chromatograms of (**a**) mix standards and (**b**) real samples.

Table 4. Comparison of the Recovery and Precision (RSD % in Parentheses) Obtained for ATP, ADP, and AMP in Spiked Samples by ASE and Classical Methods (n = 4)

analyte	spike	ASE method	classical method
ATP	50 mg L^{-1}	90.2 (4.8)	88.5 (5.6)
ADP	50 mg L^{-1}	88.6 (5.3)	90.1 (5.9)
AMP	50 mg L^{-1}	90.9 (4.9)	91.4 (5.1)

195.2 mg kg^{-1}, and AMP content from 24.2 to 2214.4 mg kg^{-1} (**Table 5**). According to the results, there was a large variation in ATP, ADP, and AMP contents among the samples. Differences in time of harvest, storage conditions, and the flower of origin likely account for the considerable variation in ATP, ADP, and AMP contents in RJ samples.

The freshness index of the RJ is currently an exciting area of beekeeping research. Many researchers have attempted to identify new indices to assess the freshness of RJ. The ATP and its relative catabolites (such as ADP and AMP) have been used to evaluate the freshness of some products (*22*), so our method

Table 5. Contents of ATP, ADP, and AMP in Real RJ Samples (RSD % in Parentheses, n = 4)

	content (mg kg^{-1}) for analyte		
sample	ATP	ADP	AMP
1	50.2 (4.4)	64.3 (4.9)	704.3 (6.8)
2*[a]	119.3 (5.2)	134.2 (4.9)	1811.8 (7.6)
3	13.2 (4.9)	10.2 (5.1)	750.3 (6.3)
4*[a]	184.1 (4.8)	195.2 (5.3)	1980.2 (11.8)
5	nd[b]	38.8 (4.5)	50.2 (5.4)
6	61.2 (4.8)	64.9 (5.2)	650.2 (7.8)
7	91.4 (4.7)	137.2 (6.1)	740.2 (7.4)
8	20.4 (4.2)	nd	450.2 (3.6)
9	nd	nd	24.2 (5.1)
10	nd	nd	64.2 (6.3)
11	98.4 (5.2)	101.4 (6.4)	600.6 (8.4)
12	43.6 (6.2)	50.6 (5.1)	500.4 (7.8)
13	98.9 (5.7)	102.5 (4.3)	646.7 (5.6)
14*[a]	134.8 (5.9)	142.2 (5.1)	2214.4 (11.4)
15	nd	nd	109.2 (5.6)

[a] Asterisk indicates lyophilized RJ. [b] Not detectable.

could be used to study the catabolites of ATP in RJ and assess the freshness of RJ from the breakdown of ATP.

Here we demonstrate the use of an ASE online extraction and cleanup method in conjunction with HPLC-DAD for determination of ATP, ADP, and AMP in RJ. Compared with the classical method for RJ analysis, this procedure is faster and more environmentally friendly because it avoids the use of noxious solvents. Sample manipulation is high-throughput because the ASE procedure is automated, thereby allowing good precision and accuracy over a wide concentration range. The proposed ASE-HPLC method was successfully used to determine ATP, ADP, and AMP levels in 15 RJ samples. Comparing the results of the analyses, we found a substantial variation in the ATP, ADP, and AMP contents of the RJ samples tested. It will be interesting to investigate further the factors responsible for the variation in ATP, ADP, and AMP contents of different RJ samples and assess the RJ freshness from the ATP breakdown.

ABBREVIATIONS USED

ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; RJ, royal jelly; PLE, pressurized liquid extraction; ASE, accelerated solvent extraction; HPLC, high-performance liquid chromatography; DAD, diode array detector.

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