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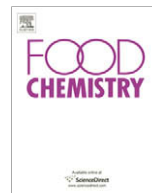

Analytical Methods

HPLC determination of adenosine in royal jelly

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ABSTRACT

A simple method is described for the determination of adenosine in royal jelly. The adenosine in the sample was extracted using 80% ethanol and analysed by reversed-phase high-performance liquid chromatography (HPLC). Chromatographic separation was performed using a Dionex HPLC system with a Waters Symmetry C18 column and gradient elution with a mixture of two solvents: solvent A, 0.4% phosphoric acid and solvent B, methanol. The effluent was monitored using a UV detector set at 257 nm. The average recoveries were 91.6–98.3% ($n = 6$) with standard deviation below 5.3%. The limits of detection and quantification were 0.017 and 0.048 μg/ml, respectively. The method has been successfully applied to the analysis of royal jelly samples. For 45 royal jelly products, the adenosine content varied from 5.9 to 2057.4 mg/kg.

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1. Introduction

Royal jelly (RJ) is a secretion from the hypopharyngeal and mandibular glands of worker bees (*Apis mellifera*) and is involved in the sexual determination of the queen bee (Nagai & Inoue, 2004). Some studies have reported the composition of RJ. The main components are water, proteins, sugars, lipids and other substances (Crane, 1990; Palma, 1992; Piana, 1996a). As a result of the increasing interest in RJ with respect to human health, the authentication of new active ingredients in RJ is becoming the subject of an increasing number of studies. In a recent paper, adenosine monophosphate (AMP) and adenosine monophosphate N1-oxide were found and identified in RJ (Noriko et al., 2006).

Adenosine is a naturally occurring purine nucleoside and is formed by the breakdown of adenosine triphosphate (ATP). ATP is the primary energy source in cells for transport systems and the action of many enzymes. Most of the ATP is hydrolysed to adenosine diphosphate (ADP), which can be further dephosphorylated to AMP. If large concentrations of ATP are hydrolysed, then some of the AMP can be further dephosphorylated to adenosine by the cell membrane associated with enzyme 5′-nucleotidase (Enzo, Maria, & Luciano, 2001). The pathway to adenosine is shown in Fig. 1.

Adenosine acts as a building block for nucleic acids and energy storage molecules, a substrate for multiple enzymes and an extracellular modulator of cellular activity (Alam, Szymusiak, Gong, King, & McGinty, 1999). The endogenous release of adenosine exerts powerful effects in a wide range of organ systems (Olah & Stiles, 1992, 1995). For example, adenosine has a predominantly hyperpolarising effect on the membrane potential of excitable cells, producing inhibition in vascular smooth muscle cells of coronary arteries and neurons in the brain (Basheer, Strecker, Thakkar, & McCarley, 2004). As an endogenous nucleoside, adenosine has been widely investigated in different products. For instance, adenosine is an important index for quality assessment of Lingzhi (*Ganoderma lucidum*) and Cordyceps (Gao et al., 2007; Gong, Li, Lia, Liu, & Wang, 2004). However, up to date, few studies have been reported on the adenosine content of RJ (Piana, 1996b).

The most frequently used procedures for the extraction of adenosine from samples are based on conventional liquid shaking or blending for a few minutes with a mixture of organic solvents or perchloric acid followed by clean-up of the extract and enrichment with SPE (solid-phase extraction). A different approach, using PLE (pressure liquid extraction) and SPE to extract adenosine from natural and cultured Cordyceps has recently been reported (Fan et al., 2006). However, these methods were found to be unsuitable for the extraction of adenosine from RJ due to the fact that it is more complex than other matrixes. Moreover, these extraction methods have certain drawbacks such as the consumption of large amounts of time and noxious solvent, and the necessity for one or more clean-up steps involving liquid–liquid partition or solid-phase extraction. An ideal leaching procedure should be exhaustive with respect to the constituents to be analysed, rapid, simple, inexpensive, environmentally friendly and amenable to automation for routine analysis (Sanchez, Priego, & Luque, 2007). Ultrasound is probably the simplest and most versatile method for sample extraction because the energy imparted facilitates and accelerates some of the steps, such as dissolution, fusion, and leaching amongst others; this method also resolves the problems of solvent and time consumption (Lavillaa, Vilasa, & Bendicho, 2008). In this study, a mixture of ethanol and water was utilised to extract

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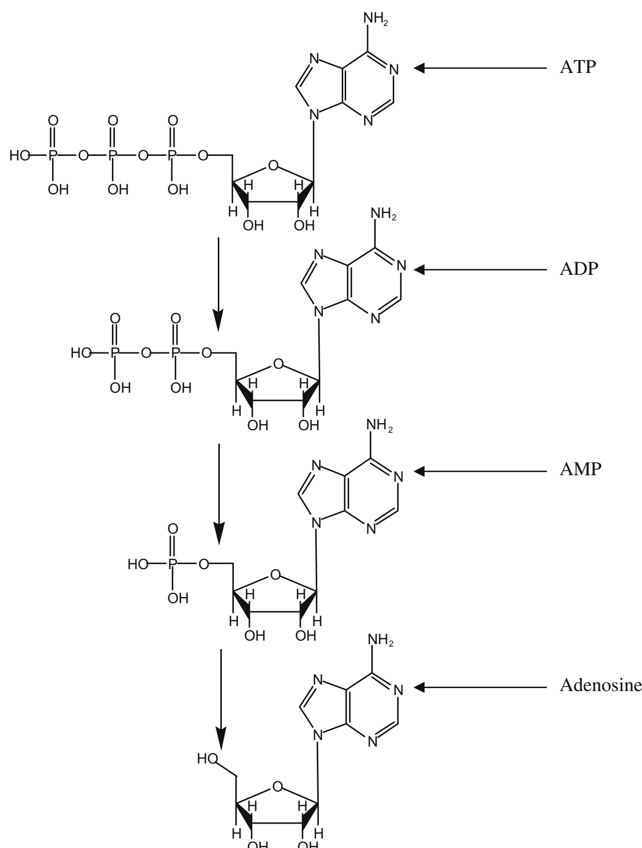

Fig. 1. Pathway to adenosine.

adenosine from RJ using ultrasonic-assisted extraction. The different factors affecting the efficiency of the extraction such as extraction solvent and extraction time were carefully optimised.

Capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) are currently the most commonly used separation techniques for adenosine in combination with detection by ultraviolet (Gong et al., 2004; Kieszling et al., 2004; Tzeng, Hung, Wang, Chou, & Hung, 2006), diode array and evaporative light-scattering (Yan, Luo, Wang, & Cheng, 2006), and mass spectrometry (Brink, Lutz, Volkel, & Lutz, 2006; Cahours, Dessans, Morin, Dreux, & Agrofoglio, 2000; Fan et al., 2006; Gao et al., 2007). In particular, as a conventional method, HPLC can be carried out using standard equipment in many laboratories and is also simple, sensitive and very suitable for monitoring adenosine. However, there are no detailed studies available concerning the distribution of adenosine 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 in RJ is therefore necessary and would be valuable for further study.

The purpose of this study was to develop a simple method for the quantitative analyses of adenosine in RJ. Adenosine was extracted with 80% ethanol and analysed by HPLC–UV. The validated method has been successfully utilised for determining adenosine content and investigating the range of adenosine content in RJ samples.

2. Materials and methods

2.1. Materials and reagents

Twenty-five commercial samples were purchased from supermarkets in Beijing. Twenty commercial samples of known origin were obtained from Pinghu apiary in China's Zhejiang province, a major RJ producing zone. All the samples were kept refrigerated at −18 °C before analysis. Adenosine standard was purchased from Sigma (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Phosphoric acid and absolute ethanol (analytical grade) were purchased from Beifen (Changping, Beijing). De-ionised water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA).

2.2. HPLC

HPLC analysis was carried out using a Dionex HPLC system (Dionex, USA), which included a P680 pump, an ASI-100 auto injector, a TCC-100 column oven and a 170UV UV detector, connected to Chromeleon software. A Symmetry C18 column (250 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 0.4% phosphoric acid (A) and methanol (B). The linear gradient conditions were: 0–25 min, 90% A; 25–35 min, 20% A; 35–40 min, 90% A and 40–65 min, 90% A. The flow rate was set at 0.9 ml/min and the injection volume was 20 μl. The detection wavelength was set at 257 nm. Identification of adenosine was based on retention time when co-injected with standards.

2.3. Sample extraction procedure

Cold samples were equilibrated at room temperature for 1 h and then homogenised before analysis. A total of 2.0 g of RJ was accurately weighed into a 50 ml volumetric flask. Then 5 ml of ultra-purified water and 40 ml of absolute ethanol were added and the mixture was shaken for 15 min at room temperature using an ultrasonic processor. The volume was made up to the mark with ultra-purified water. A sample from the solution was drawn into a pipette and filtered through a filtering cartridge containing a 0.45 μm nylon membrane using a disposable syringe set before HPLC analysis.

2.4. Preparation of the standard curve

Quantification was based on the external standard method. A stock solution of adenosine standard (1 mg/ml) was prepared by dissolving adenosine in 80% ethanol. The working standard solutions for linear calibration were prepared by diluting the stock solution to produce a concentration sequence of 0.1, 1, 5, 10, 100

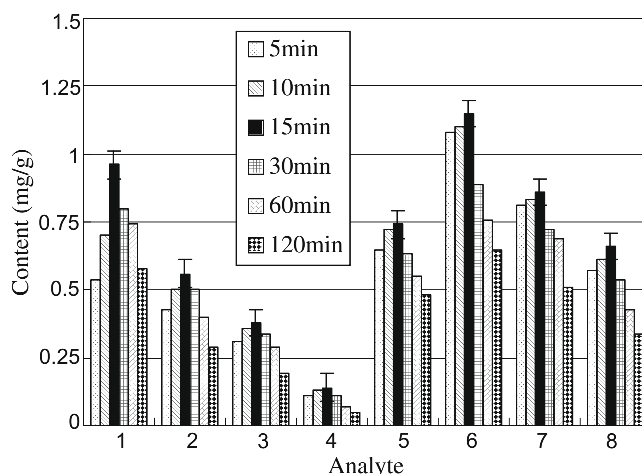

Fig. 2. Effect of time on the extraction of adenosine from RJ.

and 200 μg/ml. Both the stock and working solutions were kept at 4 °C for 1 month.

3. Results and discussion

3.1. Optimisation of sample extraction procedure

The water-soluble component of adenosine was extracted according to the previously developed procedures. However, these published extraction procedures were not suitable for RJ samples. There are many proteins (Crane, 1990; Palma, 1992; Piana, 1996a, 1996b) which should be removed from RJ samples before analysis. In a recently reported method, absolute ethanol was used to precipitate the protein effectively from the RJ samples (Zhou et al., 2007). In this study, different proportions of ethanol were tested to assess their extraction efficiency. The results indicated that 80% ethanol not only precipitated the protein effectively but also gave satisfactory recovery.

Extraction with an ultrasonic processor is common practice for the exhaustive extraction of adenosine from various products for quantitative analysis. This extraction method was therefore used in this work. The effect of time on the extraction efficiency was evaluated by using 80% ethanol for 5, 15, 30, 60 and 120 min. The results are shown in Fig. 2: increasing the extraction time from 5 to 15 min resulted in an improved yield of adenosine; the extraction time was greater than 15 min (30, 60 and 120 min), the loss of adenosine was observed. In accordance with the above observations, the extraction procedure was carried out for duration of 15 min using 80% ethanol. At the same time, it is worth noting that a longer extraction step led to a lower extraction yield of adenosine from RJ samples. Adenine, the degradation product of adenosine, can be formed at high temperatures by prolonged ultrasonic extraction (Kieszling et al., 2004).

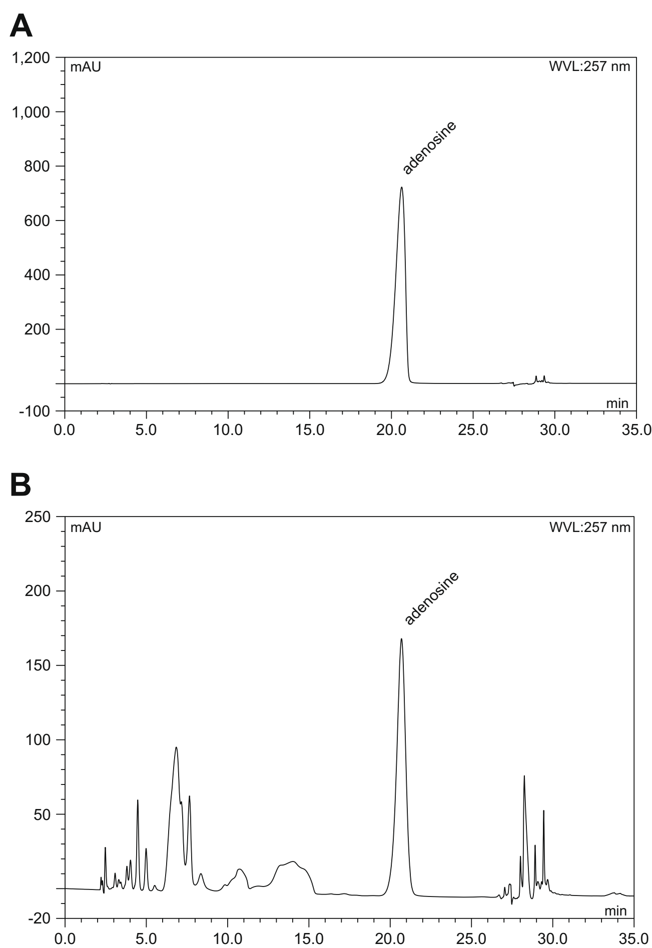

Fig. 3. Typical HPLC–UV chromatograms of (A) adenosine standard and (B) actual samples.

3.2. Optimisation of chromatographic conditions

Since adenosine contains a polar ribose group, ionisation needs to be limited without compromising resolution; to achieve this gradient elution with a mobile phase containing acid is often used in reversed-phase liquid chromatography. In this study, the composition of the mobile phase was optimised by adding different acids (formic, acetic and phosphoric acids) to the aqueous phase to determine their effects on the enhancement of resolution, inhibition of adenosine ionisation and elimination of peak tailing of the target compounds. As a result, a mobile phase containing phosphoric acid was selected together with methanol which gave satisfactory resolution and a stable baseline. To improve separation selectivity and increase efficiency, different percentages of phosphoric acid were investigated. Finally, a mobile phase consisting of methanol and 0.4% phosphoric acid was chosen for the determination of adenosine in RJ. Typical HPLC–UV chromatograms of adenosine standard and actual samples are presented in Fig. 3.

The ultraviolet spectrum of the adenosine reference showed maximum absorbance values at around 204 and 257 nm. A stable baseline was found at around 257 nm. Thus, in this study, 257 nm was selected as the detection wavelength.

3.3. Stability test

Since adenosine can degrade to adenine (Kieszling et al., 2004) a stability test should be carried out. In this study we examined the

Table 2
Recovery data (mean ± SD), intra- and inter-day accuracy and precision values of the method.

Spiked (mg/kg)		20	50	100	200
Recovery	Intra-day[a]	93.6 ± 4.4	95.3 ± 2.3	98.3 ± 3.3	97.9 ± 2.4
	Inter-day[b]	91.6 ± 5.3	96.4 ± 3.5	97.2 ± 2.9	96.7 ± 3.1

[a] Within-day precision (repeatability) six replicates.
[b] Day-to-day precision (repeatability) six replicates.

Table 1
Stability of adenosine standard solution and actual samples stored at room temperature for 2, 5, 10 and 24 h.

Solution type	Actual adenosine concentration (μg/ml)	Observed concentration(μg/ml)				Mean (μg/ml)	RSD%
		2 h	5 h	10 h	24 h		
Standard solution	10.00	9.89	9.92	9.91	9.94	9.93	0.42
Sample 1	0.38	0.36	0.36	0.35	0.35	0.36	3.40
Sample 2	1.69	1.62	1.63	1.64	1.61	1.64	1.90
Sample 3	0.61	0.58	0.60	0.58	0.57	0.59	2.79
Sample 4	3.92	3.92	3.86	3.81	3.79	3.86	1.57

Table 3
Content (mg/kg) of adenosine in actual RJ samples ($n = 3$).

Sample number	Content, mg/kg (mean ± SD)	Sample number	Content, mg/kg (mean ± SD)	Sample number	Content, mg/kg (mean ± SD)
1	9.6 ± 0.2	16	1104.3 ± 12.1	31	1413 ± 13. 8
2	42.2 ± 0.7	17	10.8 ± 0.1	32	18.3 ± 0.2
3	15.3 ± 0.1	18	100.4 ± 0.9	33	40.3 ± 0.5
4	98.1 ± 1.1	19	10.6 ± 0.1	34	8.3 ± 0.1
5	32.3 ± 0.7	20	79.4 ± 0.7	35	20.3 ± 0.2
6	10.2 ± 0.1	21	300.2 ± 4.5	36	51.7 ± 0.9
7	70.4 ± 0.9	22	60.2 ± 0.4	37	190.3 ± 3.9
8	41.3 ± 0.4	23	89.4 ± 1.9	38	70.2 ± 1.1
9	5.9 ± 0.2	24	709.3 ± 7.4	39	467.3 ± 6.1
10	120.3 ± 1.5	25	15.2 ± 0.3	40	21.2 ± 0.3
11	97.4 ± 0.9	26	100.6 ± 2.7	41	82.2 ± 1.4
12	43.6 ± 0.8	27	2057.4 ± 18.9	42	700.3 ± 9.6
13	798.9 ± 11.3	28	46.7 ± 1.3	43	1319.7 ± 16.1
14	2050.6 ± 25.9	29	500.2 ± 10.2	44	7.8 ± 0.1
15	49.4 ± 1.1	30	24.7 ± 0.7	45	92.1 ± 1.2

stability of adenosine in both the standard solutions and the actual RJ samples.

The stability of the adenosine standard solution maintained at room temperature was tested by repeated HPLC analysis of the same concentration (10 μg/ml) at different times during the day, and comparing the adenosine concentration with that in a freshly prepared standard solution. The results are shown in Table 1. No significant differences in adenosine concentration were found (RSD < 0.5%), indicating that the adenosine standard solutions were quite stable at room temperature.

The stability of adenosine in RJ samples was assessed by comparison of the results of repeated HPLC analysis of four samples stored for 2, 5, 10 and 24 h at room temperature containing known concentrations of adenosine. No significant decrease in adenosine concentration in the RJ samples was observed, thus indicating that the samples were stable for 24 h. The stability data are summarised in Table 1.

3.4. Linearity and calibration standards

The linearity of the method was calculated using various concentrations of adenosine (0.1, 1, 5, 10, 100 and 200 μg/ml) and repeating the experiments in triplicate. Calibration plots for adenosine yielded the linear relationship $y = (3.0303 \pm 0.0042)x - (0.8318 \pm 0.0039)$, where y and x are the peak area (mAU) and concentration of the standard solution (μg/ml), respectively. Linear regression showed good linearity in the range of 0.1–200 μg/ml with a correlation co-efficient of 0.9997. This allows the determination of adenosine over a wide range of concentrations.

3.5. Limit of detection and limit of quantification

Based on signal-to-noise ratios of 3 and 10, the limit of detection (LOD) and limit of quantification (LOQ) were determined using standard solutions of adenosine subjected to HPLC and analysed using the methodology described in Section 2.2. The LOD and LOQ were 0.017 and 0.048 μg/ml, respectively.

3.6. Recovery, accuracy and precision

Recovery was examined by adding a known amount of adenosine standard to RJ samples. The mixtures were extracted and analysed using the method described above. Table 2 shows the recoveries of adenosine from RJ samples.

The mean extraction recoveries of adenosine from RJ were found to range from 91.6% to 98.3%. The intra- and inter-day accuracy and precision values of the method are presented in Table 2. The co-efficient of variation of both inter- and intra-day analysis varied from 2.4% to 5.3%. These results show that the method is accurate and precise as evidenced by the high recovery and low CV values.

3.7. Application of the analysis to actual samples

The method was applied to actual samples collected from beekeepers and supermarkets. In total, 45 samples were analysed, and the adenosine content ranged from 5.9 to 2057.4 mg/kg. The details are summarised in Table 3. According to the results, some RJ samples were rich in adenosine, but there was a large variation in adenosine content amongst the samples. Time of harvest, storage conditions and the flower of origin likely account for the considerable variation of adenosine content in RJ samples.

4. Conclusions

In this study, a HPLC method has been established for the qualitative and quantitative analyses of adenosine in RJ. The method is simple, sensitive and reliable. From the results of analyses of a selection of actual RJ samples, adenosine was found to be relatively abundant in some products. Having in mind that adenosine plays a vital role in the activity of cells in living organisms, the method described here can be used to study the activity of adenosine in RJ. Comparing the results of the analyses, we found a substantial variation in the adenosine content of the RJ samples tested. It will be interesting in future research to investigate the factors led to the variation in the adenosine content of different RJ samples.

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