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Article

Determination of ATP and its metabolites in dietary energy supplements by capillary electrophoresis

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This study aimed to develop a simple and fast capillary electrophoresis (CE) method for the simultaneous determination of adenosine triphosphate (ATP) and its metabolites in dietary energy supplements. Reverse polarity separation mode for faster separation of the three strong negatively charged analytes and capillaries with a 25 µm inner diameter was employed. At –433 V/cm field strength at background electrolyte (BGE) consist with 0.1 M tris-HCl, 0.5 mM tetradecyltrimethylammonium chloride (TTAC) as positively charged surfactant and 0.3 mg/mL hydroxypropylmethylcellulose (HPMC) to reduce the electroosmotic flow (EOF), a complete separation of the three species were achieved in less than 15 minutes. The data acquisition was conducted at a wavelength of 254 nm. Three different commercialised dietary energy supplements were analysed.

Keywords: Determination of ATP. Capillary electrophoresis. Dietary energy supplement.

INTRODUCTION

Adenine nucleotides serve a significant role in biological systems since they function as units for DNA and RNA synthesis and as allosteric regulators of enzymes (Zhu *et al*., 2017). In addition, adenine nucleotides are correlated with bioprocesses in proliferation and cellular development (Agteresch, Rooijen, Berg, 2003), metabolism (Moreno-Sánchez *et al*., 2014), energy transfer (Stefan *et al*., 2012), and immune responses (Li *et al*., 2014).

Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (Figure 1), and other such compounds in the adenosine family are known to be involved in energy regulation, alteration of blood flow, and cellular signalling in the mammalian brain (Lusardi, 2009). ATP is decomposed into ADP, AMP, and inorganic phosphate. In this case, energy is released. Muscles use a significant amount of this energy to do mechanical work and to synthesise protein, urea, and metabolic intermediates. Thus, the main function of ATP in the body is to provide energy for many biochemical reactions (Khlyntseva *et al*., 2009).

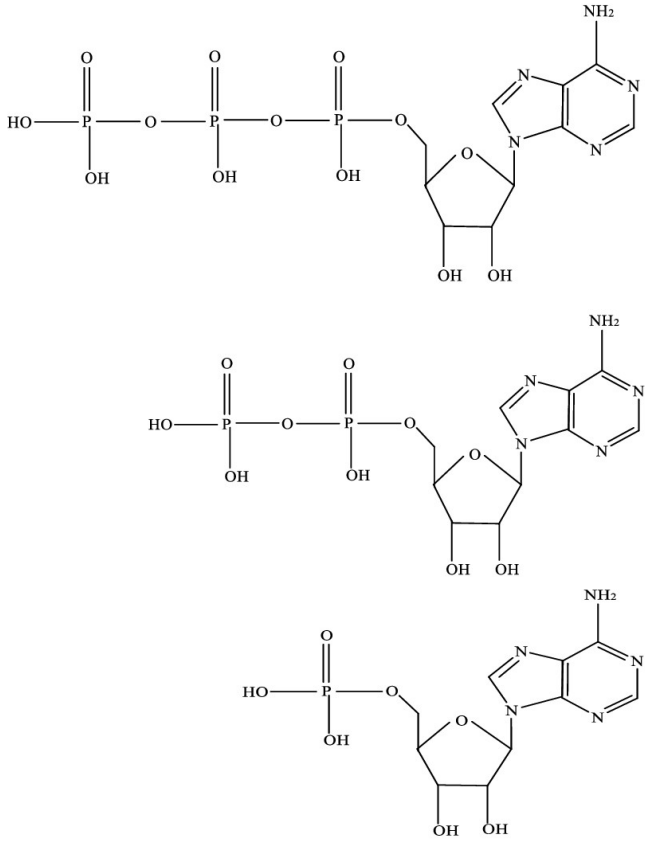

FIGURE 1 - Chemical structures of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP).

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ATP is a food ingredient. Meat, fish, and nuts provide a good source of ATP for our body. ATP has also been marketed as a dietary supplement. The World Anti-Doping Agency's prohibited list does not include ATP.

Jordan *et al.* (2004) aimed to determine whether an enterically coated ATP composition was capable of enhancing physical performance after oral administration. They demonstrated that 225 mg of enteric-coated ATP supplementation per day for 15 days provided an increase in total bench-press lifting volume (Jordan *et al.*, 2004). Rathmacher *et al.* (2012) found that supplementation with 400 mg ATP/d for 15 days tended to reduce muscle fatigue while improving muscle low peak torque during repeated high-intensity exercise. These findings showed that dietary supplementation with ATP may have beneficial effects in both exercising and less active muscle tissue. Considering the functions of ATP and its metabolites, their determination in different matrices is important.

Firstly, the analysis of nucleotides was performed by HPLC. Conventionally, reversed-phase HPLC, anion-exchange HPLC, and ion-pair reversed-phase HPLC methods are time consuming and need large amounts of biological material. Another method for quantitative determination of ATP is based on the bioluminescence reaction catalysed by firefly luciferase with high sensitivity and specificity. However, the inhibitory effects of dehydroluciferyl adenylate (L-AMP) on the bioluminescence reaction, which is a side product of this reaction, result in unsatisfactory linearity and accuracy of ATP analysis (Wang *et al.*, 2019).

Capillary electrophoresis (CE) is exceptionally well-suited for examining adenine nucleotide levels due to its high separation efficiency and rapid analysis, inexpensive buffer salts, and smaller quantities of both buffer and sample. In general, in the literature HPLC methods have been used for the analysis of ATP and its metabolites.

The contents of ATP and its metabolites in yeast were determined using CE (Zhu *et al.*, 2017). The best separation was obtained in 60 mM borate (pH 9.50) with 1.0% (w/v) PEG, 50 µm i.d. × 44 cm (total length) fused silica capillary, 20 kV applied voltage, and 0.5 psi × 15 s injected volume at 210 nm. The separation time was approximately 40 min. A new method for CE laser-induced fluorescence (CE-LIF) was developed to quantify ATP in spermatozoa and oocytes (Zinellu *et al.*, 2010). ATP was separated in a buffer of 10 mmol/L tribasic sodium phosphate at pH 11.5, 40°C, normal polarity, and 22 kV with an uncoated fused silica capillary of 75 µm i.d. and 57 cm length in 5 min. Intracellular ATP, ADP, and AMP in *Mycobacterium smegmatis* was analysed by ion-pair reversed-phase high-performance liquid chromatography (Akhova, Tkachenko, 2019). They used a Luna C18 column (5 µm, 250 mm × 4.6 mm i.d.). They separated the compounds by (A) acetonitrile and (B) 50 mM monobasic potassium phosphate (pH 4.6) with 25 mM tetrabutylammonium hydrogen sulphate in a ratio of 0.5 (A):99.5 (B). The chromatographic separation completed in 28 min at 254 nm. The secretion of ATP and ADP for human and mouse platelets was determined by HPLC (Papen *et al.*, 2013). The analytes were separated with mobile phases A (90 mmol/L KH_2PO_4, 10 mmol/L K_2HPO_4, 4 mmol/L tetrabutylammonium sulphate, pH 6.0) and B (80 mmol/L KH_2PO_4, 20 mmol/L K_2HPO_4, 4 mmol/L tetrabutylammonium sulphate, 30% methanol, pH 7.2) using a linear gradient at 260 nm in 30 min. An ion-pairing HPLC method with fluorescence detection was developed for quantification of adenine nucleotides in primary astrocyte cell cultures (Bhatt *et al.*, 2012). They optimised the fluorescence derivatisation conditions and HPLC parameters to separate and quantitate the compounds. The nucleotides were converted to their respective 1,N6-etheno derivatives by incubating with chloroacetaldehyde at pH 4.5 and 60°C for 60 min. The separation was performed on an ODS column (5 µm, 250 mm × 4.6 mm) with fluorescence detection at Em=280 nm and Ex=410 nm in 20 min. In these studies, it is established that the analysis times are long.

The main objective of this study was to develop a fast and simple CE method for the simultaneous determination of ATP, ADP, and AMP in a dietary energy supplement.

MATERIAL AND METHODS

Chemicals and reagents

ATP, AMP, ADP, tris(hydroxymethyl) aminomethane HCl (tris HCl), tetradecyltrimethylammonium chloride

(TTAC), and hydroxypropylmethylcellulose (HPMC) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All solutions were prepared in ultrapure water (18.2 MΩ cm, Milli-Q Synthesis A10, Millipore, Burlington, MA, USA). A 0.2 µm nylon membrane filter (Supelco, Bellefonte, PA, USA) was used for filtration of the separation buffer, stock standard solutions injected to the capillary. The commercialised dietary energy supplement products used were Douglas labs' ATP 20, Bigjoy ATP Mito Energy, and Primeval Labs PRIM-ATP.

Apparatus

All experiments were conducted using an Agilent CE 7100 (Agilent Technologies) instrument equipped with an air-cooling system and a diode-array UV detector. The software provided with the HP ChemStation version B.02.01 was used to collect data. Uncoated fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were used to carry out the separation. Fused silica capillaries with various diameters, including 25 µm and 50 µm, were tested. The data acquisition was conducted at a wavelength of 254 nm. An Accumet AB15 pH meter was used for all pH measurements. Prior to electrophoresis, all the capillaries were rinsed with methanol, 0.1 M HCl, and 0.1 M sodium hydroxide, followed by the appropriate background electrolyte (BGE) for 5 min each. The capillary was cleaned by flushing water through for 5 min in between each rinsing step. Prior to reuse of any used capillaries, they were reconditioned by thoroughly flushing with water and appropriate running buffer for at least 10 min. The separation of adenosine nucleotides was studied in reverse polarity separation mode. TTAC was employed as the positively charged surfactant to reverse the electroosmotic flow (EOF).

Preparation of standards and samples

Standards of the analytes were prepared using nanopure water then stored in a freezer (–20°C) prior to use within 5 days. The standards were allowed to warm to room temperature before any solution was pipetted out of the container. Ten tablets of each commercial sample were chosen at random and weighed. They were then ground using a mortar and pestle and dissolved in nanopure water. After being filtered through Whatman filter paper, the solution was then diluted in water to 500 mL. Prior to CE analysis, this solution was filtered again using a 0.2 µm syringe filter.

RESULTS AND DISCUSSION

Separation optimisation

The three analytes of interest ATP, ADP and AMP become negatively charged in solvents with pH values above 6.6 (Table I) (Faizullin *et al*., 2013). Therefore, CE is an ideal technique for the analysis of the three compounds. However, ATP is unstable and degrades into its subsequent metabolite, ADP, in both acidic and highly basic media. The tris buffer at pH 7 was used as the BGE for the separation in order to minimise such decomposition during the CE analysis. Reverse polarity separation mode was employed for faster separation of the three strong negatively charged analytes. TTAC is a positively charged surfactant, and it was added to the running buffer to obtain a positively charged capillary wall, thereby reversing the EOF. Initial attempts to separate the three analytes using the BGE consisting of only 100 mM Tris buffer at pH 7.0 and 0.5 mM TTAC yielded only two peaks: one peak for comigrating ATP and ADP and a well-separated peak for AMP. To obtain better resolution between peaks and less analysis time, various analytical approaches, including mixing EOF modifiers with running buffer and varying the electric filed strength, were studied over the course of method optimisation.

TABLE I - pKa values of ATP, ADP and AMP

Compound	pKa value
ATP	6.51
ADP	6.36
AMP	6.21

Effect of buffer additives and electric field strength on separation

Several advantages are awarded to organic solvents in CE, e.g., higher separation efficiency, better separation selectivity, shortened analysis times, and higher solubility of some sample compounds (Porras, Kenndler, 2005; Kenndler, 2009). In this study, we analysed the effect of several options as EOF modifiers for improving the separation. To obtain a better separation, HPMC was mixed with the tris BGE. Because of the high viscosity, HPMC has the ability to reduce the EOF and, therefore, analytes spend more time in the capillary. As shown in Figure 2, the three analytes were fully separated when the tris-HCl BGE was mixed with 0.3 mg/mL HPMC. However, it took about 30 min for complete separation in the 50 µm I.D. fused silica capillary at a separation field strength of –236 V/cm.

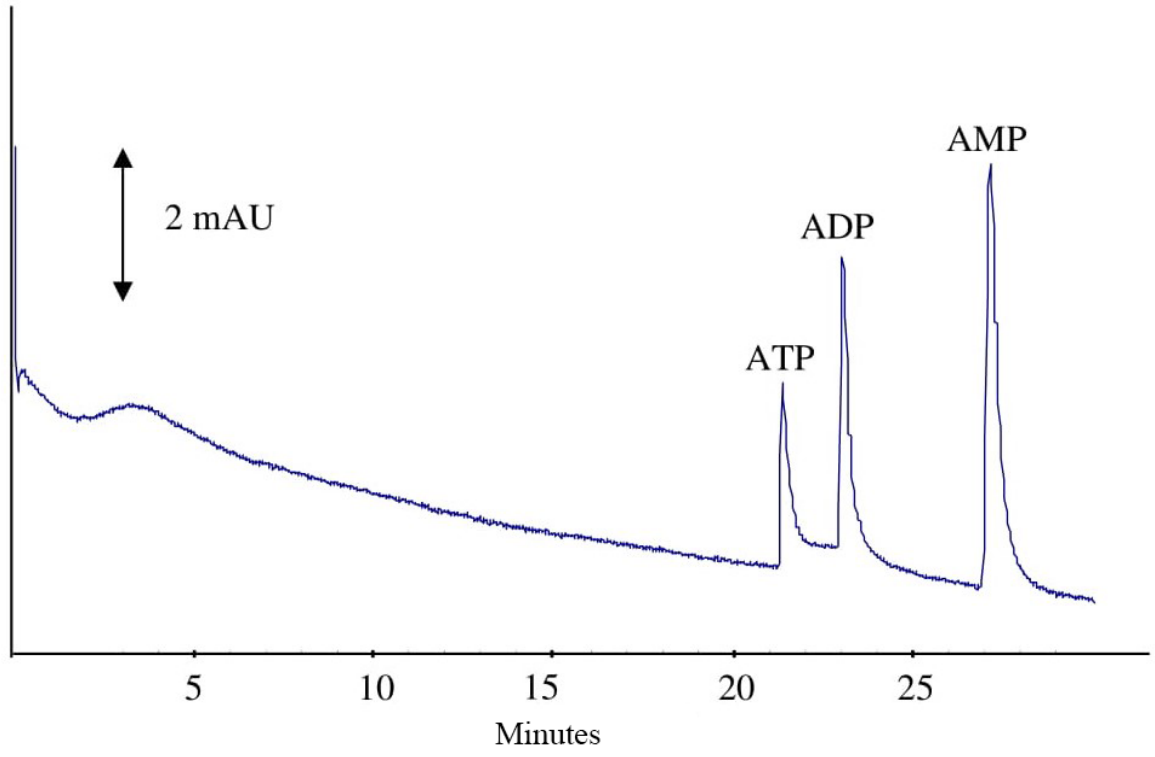

FIGURE 2 - Capillary electrophoresis (CE) separation of a 0.5 mM mixture of ATP, ADP, and AMP standards: 0.1 M tris buffer with 0.5 mM tetradecyltrimethylammonium chloride (TTAC) and 0.30 mg mL^{-1} hydroxypropylmethylcellulose (HPMC) with 50 µm i.d.x 63.5 (53.5) cm in length capillary at field strength of –236 V/cm and UV detection at 254 nm.

Therefore, to further reduce the analysis time, the separation field strength was increased. Increasing the field strength has number of benefits. While it increases the electrophoretic mobility of the analytes and the EOF, as well as shortening the analysis time, it also increases the sharpness of the peaks and improves the resolution. The migration time of the analytes also decreases. However, at higher field strengths the Joule heating in the capillary increases and, as a result, the reproducibility of the migration times decreases. To address the Joule heating issue, capillaries with a 25 µm inner diameter were employed to effectively dissipate the heat generated in the capillary. At –433 V/cm filed strength at BGE consist with 0.1 M tris-HCl and 0.3 mg/mL HPMC, a complete separation of the three species were achieved in less than 15 minutes (Figure 3).

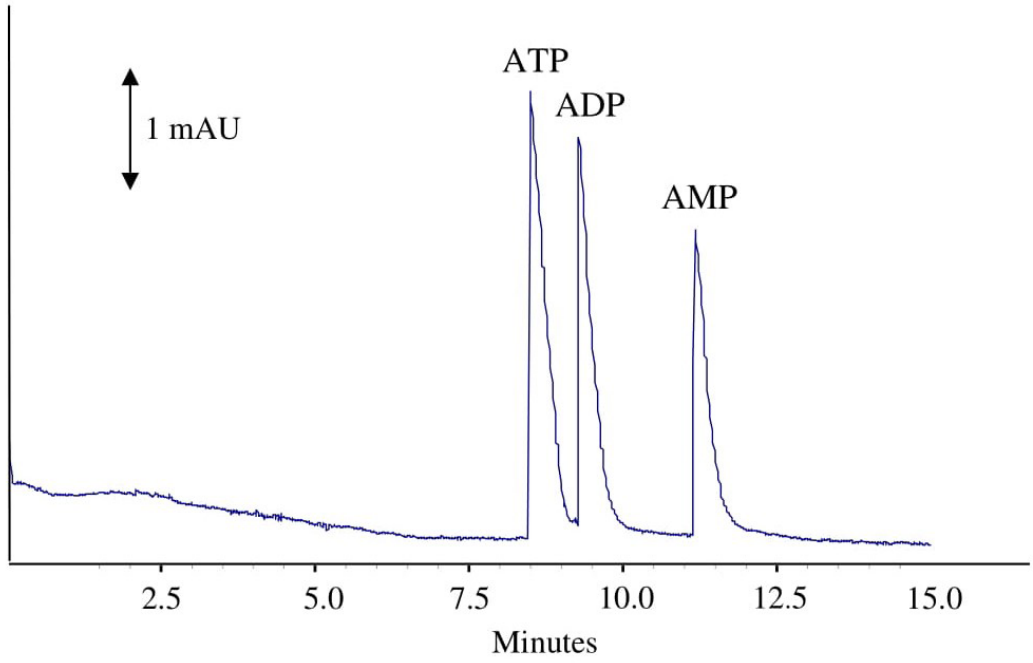

FIGURE 3 - CE separation of a 0.5 mM mixture of ATP, ADP, and AMP standards: 0.1 M tris buffer with 0.5 mM TTAC and 0.30 mg mL^{-1} HPMC with 25 µm i.d.x 63.5 (53.5) cm in length capillary at field strength of –433 V/cm and UV detection at 254 nm.

With the current separation conditions, nanomolar limits of detection could be achieved for all three analytes (Table II). The complete separation was achieved in less than 13 min from sample injection.

The resolution for the separation of ATP and ADP was 1.2, while the resolution for the separation of ADP and AMP was 3.2. The calculated tailing factors were obtained in the acceptable range of $0.5 \leq T \leq 2$, and the theoretical plate numbers of all compounds were higher than 2500. The retention times, theoretical plate numbers, limit of detection (LOD), and limit of quantification (LOQ) of the compounds are given in Table II. The lowest concentration that can be quantified (LOQ) with acceptable accuracy and precision was evaluated at a signal-to-noise ratio of 10. The LOD was evaluated at a signal-to-noise ratio of 3.

TABLE II - Important parameters of compounds

Compound	Retention time	Theoretical plates numbers	LOQ	LOD
ATP	8.75±0.53	2500	500 nM	200 nM
ADP	9.60±0.45	2621	600 nM	250 nM
AMP	11.55±0.62	3335	800 nM	350 nM

Sample preparation for ATP dietary energy supplements

Three different samples spiked with 10, 50, and 100 µM of ATP (Table III) were used for determining the precision and recovery. Precision was expressed as the relative standard deviation of the control sample concentrations. To demonstrate the accuracy of the method, a sample was analysed before and after the addition of known amounts of ATP.

TABLE III - Recovery and precision of the determination of ATP

Added (µM)	Found (µM)	Recovery (%)	RSD (%)
10	10.5	105	5.8
50	51.6	103	4.1
100	102.2	102	3.6

For the standard addition assay, sample solutions were spiked with aliquots of stock standard solutions of ATP, ADP, and AMP to obtain final concentrations within the specified ranges. Using the solutions of the tablets and the new standards of ATP (2.4 mM), ADP (2.5 mM), and AMP (2.6 mM), a series of standard solutions were made to obtain a standard addition curve (Table IV). Each compound was determined by three repeated analyses (n=3). The ATP concentration was in the range of 50 to 400 µM. The ADP and AMP concentration were in the range of 10 to 100 µM.

TABLE IV - Standard addition solutions.

Supplement (µL)	ATP (µL)	ADP (µL)	AMP (µL)	Water (µL)
4000	1000	240	231	529
4000	625	182	175	1018
4000	505	121	116	1258
4000	252	61	58	1629
4000	125	24	23	1828

By analysing the tablets, it made it clearer to see if the optimised method that was developed was applicable to other ATP or its metabolites and is able to monitor them (Figure 4). There was no interfering peak in the retention times corresponding to the analytes. Therefore, the proposed method was considered to be selective

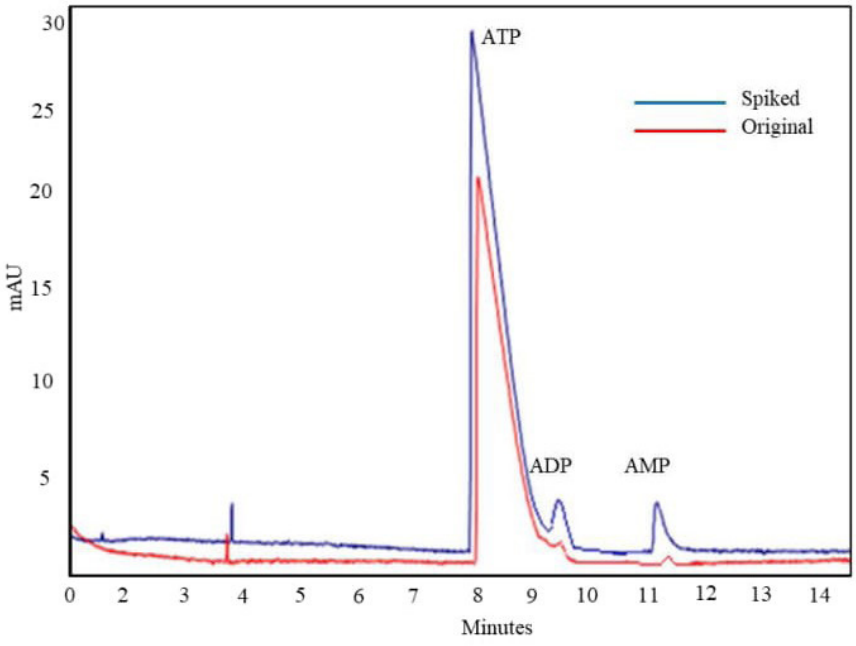

FIGURE 4 - Electropherogram of the analytes in the Douglas tablets (spiked vs. unspiked).

The tablets were also tested in order to determine the true concentration of ATP in the supplements. The results from the experiments show that the sample contained Douglas labs' AMP also, but it was below the LOD. The results from the ATP determination are presented in Table V.

TABLE V - Results of ATP determination in dietary energy supplement

Tablets	Labeled value (mg)	Experimental value (mg)
Douglas labs' ATP 20	20	21.30±1.25
BigJoy ATP Mito Energy	90	92.45±3.74
Primeral Labs Pim-ATP	150	151.86±4.32

The analytical utility of the presented method for determining ATP in tablets has been confirmed.

CONCLUSION

In conclusion, a rapid and basic CE method for the analysis of ATP in dietary energy supplements has been developed. The improved CE method for the identification and quantification of ATP, ADP, and AMP should be important in the analysis of different matrices.

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REFERENCES

Agteresch HJ, Rooijen MHC, Berg JWO, Minderman-Voortman GJ, Wilson JHP, Dagnelie P. Growth inhibition of lung cancer cells by adenosine 5'-triphosphate. Drug Dev Res. 2003;60(3):196–203.

Akhova AV, Tkachenko AG. HPLC–UV method for simultaneous determination of adenosine triphosphate and its metabolites in *Mycobacterium smegmatis*. Acta Chromatogr. 2019;31(1):45–8.

Bhatt DP, Chen X, Geiger JD, Rosenberger TA. A sensitive HPLC-based method to quantify adenine nucleotides in primary astrocyte cell cultures. J Chromatogr B. 2012;889:110–5.

Faizullin D, Zakharchenko N, Zuev Y, Puzenko A, Levy E, Feldman Y. Hydration of AMP and ATP molecules in aqueous solution and solid films. Int J Mol Sci. 2013;14(11):22876–90.

Jordan AN, Jurca R, Abraham EH, Salikhova A, Mann JK, Morss, GM, et al. Effects of oral ATP supplementation on anaerobic power and muscular strength. Med Sci Sports Exerc. 2004;36(6):983–90.

Kenndler, E. Organic solvents in CE. Electrophoresis. 2009;30(Suppl 1):S101–11.

Khlyntseva SV, Bazel YR, Vishnikin AB, Andruch V. Methods for the determination of adenosine triphosphate and other adenine nucleotides. J Anal Chem. 2009;64(7):677–93.

Li S, Li X, Chen X, Geng X, Sun J. ATP release channel pannexin1 is a novel immune response gene in Japanese flounder *Paralichthys olivaceus*. Fish Shellfish Immunol. 2014;40(1):164–73.

Lusardi TA. Adenosine neuromodulation and traumatic brain injury. Curr Neuropharmacol. 2009;7(3):228–37.

Moreno-Sánchez R, Marín-Hernández A, Saavedra E, Pardo JP, Ralph SJ, Rodríguez-Enríquez S. Who controls the ATP supply in cancer cells? Biochemistry lessons to understand cancer energy metabolism. Int J Biochem Cell Biol. 2014;50:10–23.

Papen M, Gambaryan S, Schütz C, Geiger J. Determination of ATP and ADP secretion from human and mouse platelets by an HPLC assay. Transfus Med Hemother. 2013;40(2):109–16.

Porras, SP, Kenndler E. Are the asserted advantages of organic solvents in capillary electrophoresis real? A critical discussion. Electrophoresis. 2005;26(17):3203–20.

Rathmacher JA, Fuller JC, Baier SM, Abumrad NN, Angus HF, Sharp RL. Adenosine-5'-triphosphate (ATP) supplementation improves low peak muscle torque and torque fatigue during repeated high intensity exercise. J Int Soc Sports Nutr. 2012;9(1):48–54.

Stefan E, Düser MG, Zarrabi N, Dunn SD, Börsch M. Elastic deformations of the rotary double motor of single F0F1-ATP

synthases detected in real time by Forster resonance energy transfer. Biochim Biophys Acta. 2012;1817(10):1722–31.

Wang L, Li Y, Guo R, Li S, Chang A, Zhu Z, Tu P. Optimized bioluminescence analysis of adenosine triphosphate (ATP) released by platelets and its application in the high throughput screening of platelet inhibitors. PLOS ONE. 2019;14(10):e0223096.

Zhu P, Wang S, Wang J, Zhoua L, Shi P. A capillary zone electrophoresis method for adenine nucleotides analysis in *Saccharomyces cerevisiae*. J Chromatogr B. 2017;1008:156–63.

Zinellu A, Pasciu V, Sotgia S, Scanu B, Berlinguer F, Leoni G, et al. Capillary electrophoresis with laser-induced fluorescence detection for ATP quantification in spermatozoa and oocytes. Anal Bioanal Chem. 2010;398(5):2109–16.

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