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Development of a method for the analysis of nucleotides from the mantle tissue of the mussel *Mytilus galloprovincialis*☆

Sonia Lucía Blanco López[a,*], Jeanne Moal[b], Fuencisla San Juan Serrano[a]

[a]*University of Vigo, Genetics and Immunology, Department of Biochemistry, 36200 Vigo, Spain*
[b]*Laboratoire des Invertebrés Marins, IFREMER, Centre de Brest, B.P. 70, 29263 Plouzane, France*

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Abstract

Reversed-phase HPLC was applied to obtain a sensitive and efficient means for quantitating nucleotides in the mussel *Mytilus galloprovincialis*. We obtained a good separation of adenylic, guanylic, uridylic and cytidylic nucleotides. Adenine nucleotides play a critical role in the regulation and integration of cellular metabolism; particularly in the mantle tissue in the mussel, they are involved in the regulation of the enzyme glycogen phosphorylase, a key enzyme in the transfer of bioenergetic reserves (glycogen) to gametogenic development; it is of great importance to have a measure of the concentrations in vivo during the reproductive cycle of the organism. Different elution conditions were tested: isocratic versus step gradient elution, different mobile phase pH and the type and proportion of ion-pairing agent added to the mobile phase. The best method was selected and the separation and accurate determination of adenine, citidine, guanine and uridine nucleotides was accomplished within a 20-min run, with UV–Vis detection (254 nm).

Keywords: *Mytilus galloprovincialis*; Ion-pairing reagents; Mobile phase composition; Nucleotides

1. Introduction

Separation and measure of nucleotides, particularly ATP, ADP, AMP, has been achieved thanks to the development of HPLC, which seems to offer advantages compared to the widespread firefly luciferine–luciferase method for ATP assays [1–4], which is time consuming and technically difficult to apply. Several methods have been applied for measuring nucleotides in tissues from marine organisms [4–16].

Reversed-phase high-performance liquid chromatography was applied in order to obtain a sensitive and efficient means for quantitating nucleotides in the mussel *Mytilus galloprovincialis*. Our initial scope were adenylic nucleotides, but with the method we obtained a good separation of guanylic, uridylic and cytidylic nucleotides as well. Adenine nucleotides play a critical role in the regulation and integration of cellular metabolism; particularly in the mantle tissue in the mussel, they are involved in the regulation of the enzyme glycogen phosphorylase, a key enzyme in the transfer of bioenergetic reserves

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*Corresponding author. Tel.: +34-986-812-574; fax: +34-986-812-556.

E-mail address: slblanco@uvigo.es (S.L. Blanco López).

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(glycogen) to gametogenic development; it is of great importance to have a measure of the concentrations in vivo during the reproductive cycle of the organism, which provides data on the energetic status, and on the regulation of the enzyme, which seems to be different from that found in mammals [17].

Nucleotides may be separated and quantitated by reversed-phase chromatography, which is based upon the differences in polarity and solvophobic properties from the substances, since nucleotides have two different regions: a negatively charged phosphate group, depending on the pH, and the ribose-basic group, less polar. The stationary phase consists of octadecylsilane (ODS) groups covalently bonded to silica. Molecules are retained on these chains depending on their hydrophobic-solvophobic properties. The polar portion of the molecules tends to attach to the stationary phase, while the non-polar portion is readily eluted; depending on the polarity, analytes are separated in the column, eluting first the most polar substances. Retention times on the column may be reduced by means of addition of a polar solvent, such as methanol or acetonitrile, which act as organic modifiers. Principal factors implicated in these separations are: proportion of organic modifiers, temperature, salt concentration in mobile phase, pH and length of the hydrocarbon chain bonded to silica in the stationary phase. Studies on the development of new stationary phases (synthesis of new silanes and new silica-based stationary phase materials from commercially available silanes) for the HPLC separation of nucleosides and cyclic nucleotides have been carried out [18].

Reversed phase ion-pair chromatography (RP-IPC) has the same basis, with the addition of a molecule acting as an ion-pairing agent or counter-ion to the mobile phase. The advantage of this technique is the broad range of parameters which may be conveniently adjusted to optimize the separation method, including the concentration of organic modifier in the mobile phase, the type and concentration of buffer in the mobile phase and the type and concentration of the counter-ion. The choice of counter-ions depends on the solutes to be separated, but generally for the separation of acids a hydrophobic organic base is added to the mobile phase, such as a tertiary or quaternary amine.

Ultraviolet detection has been usually employed since it permits the measurement of the majority of unmodified nucleotides, nucleosides and bases with good sensitivity, adequate for the quantitation of nucleotides in most tissue extracts [19]; recent methods are referenced [20–23], although fluorescence detection has been applied obtaining a best sensitivity, especially for many nucleosides and bases in tissue extracts and cyclic nucleotides in physiological fluids [19]; recent methods employing fluorescence detection are also referenced [24–26].

2. Experimental

2.1. Chemicals

All the chemicals were of analytical grade. Standards of 5′-nucleotides and nucleosides, tetrabutylammonium (TBA) hydroxide, trichloracetic acid (TCA) and tri-*n*-octylamine were supplied by Sigma (Madrid, Spain), 1,1,2-trichlorotrifluoroethane was from Sigma–Aldrich, TBA hydrogensulphate from Supelco, Spain, $NaH_2PO_4 \cdot H_2O$ and methanol LiChrosolv grade by Merck (Barcelona, Spain). Buffers were prepared using ultrapure water (MilliQ gradient A10 — Millipore).

2.1.1. Standard solutions

As stock solution a mixture of 20–120 μ*M* precise of nucleotides from the kit Sigma K100-25A of 5′-nucleotides and nucleosides was used. Guanosine was excluded from the stock solution because of its low solubility in water. It was made up in ultrapure water, divided in several aliquots and stored at −20°C; it was stable for at least 6 months.

2.2. Equipment

Nucleotides were separated by HPLC on a reversed-phase ODS column (125×4 mm, 5 μm particle size, 120 Å pore diameter) Hypersil-ODS from Hewlett-Packard (Madrid, Spain), with a guard-column (4×4 mm, 5 μm) from the same firm. The apparatus consisted of a G1329A thermostated injector, a G1322A degasser modulus, a G1311A quaternary pump and a G1315A diode array detection (DAD) system, from HP Series 1100.

Table 1
Calibration curves for nucleosides and nucleotides. HPLC conditions: gradient time 8–12 min 5% methanol, time 12–20 min 20% methanol; buffer 0.15 *M* NaH_2PO_4 pH 6.0; TBA hydroxide 3.7 m*M*

	Slope	Intercept	Residual SD	Correlation coefficient
AMP	7302.06617	−4.33235	4.68701	0.99991
ADP	6963.18683	−19.92704	2.39949	1.00000
ATP	7338.29646	15.73772	1.38410	1.00000
A	7833.89128	−2.16413	0.93163	0.99999
GMP	8692.26740	19.28373	5.35802	0.99990
GDP	7419.86205	−3.11960	2.16916	0.99996
GTP	7468.85123	−6.62073	2.41944	0.99998
CMP	3632.51185	1.73720	0.41685	1.00000
CDP	3696.26890	−8.53045	4.10468	0.99972
CTP	3399.60353	2.31369	0.20165	1.00000
C	3716.30867	1.90342	0.27162	1.00000
UMP	4072.50141	0.27846	0.60935	1.00000
UDP	4384.60299	−0.73670	1.12426	0.99998
UTP	4753.17968	−5.99219	1.51528	0.99994
U	4512.28373	−1.85096	1.86506	0.99994

2.3. Sample preparation

The biological material used was mantle tissue from *Mytilus galloprovincialis.* Animals were collected and quickly dissected, the mantle tissue was deep-frozen in liquid nitrogen (−196°C) and crushed in a precooled mortar (Retsch MM2000) to a fine powder.

2.3.1. Nucleotide extraction

The method employed was from Moal et al. [13].

A subsample (200 mg) of the frozen powder was extracted by cold trichloroacetic acid 0.5 *M* in a proportion 1/10 (w/v), mixed in a vortex for 15 s and allowed to stand for 10 min (4°C). It was then centrifuged (3379 *g*, 10 min). The supernatant was neutralized with fresh amine freon solution 0.5 *M* (1:4, v/v) [27]. The neutralized sample was immediately analyzed by HPLC.

2.4. Calibration

The HPLC system was calibrated by injecting three standard mixtures: the stock solution (23–117 μ*M*), and two solutions of higher concentrations (30–160 and 60–320 μ*M*). Linearity was obtained in the range assayed (relationship amount/area). Table 1 shows regression data; the regressions obtained for each of the compounds were optimal (correlation coefficient of at least 0.9997). Detection limits of these compounds at 253.7 nm were approx. 50 pmol injected [19]; in our method we obtained a signal-to-noise ratio from 1.2 to 3.3, which would represent an adequate sensitivity for the quantification of nucleotides in most tissue extracts. Table 2 shows data for replicate analysis ($n=12$).

Table 2
Mean and standard deviations of replicate analyses ($n=12$) of the standard. Results are indicated for peaks of interest for our purposes: A, AMP, ADP and ATP

Component	Quantity (mean±SD) (nmol)
A	0.2734±0.01215
AMP	0.5458±0.0966
ADP	0.9158±0.0793
ATP	1.2042±0.04867

3. Results

In order to develop the method of separation of adenine nucleotides, as initial condition, elution buffer 0.15 *M* NaH_2PO_4 was used, an ion-pairing reagent was added, and methanol was used as organic modifier. Separation conditions were adjusted experimentally to obtain a good resolution of the different peaks.

A previous study of the retention times for the different nucleotides and nucleosides in our column, and the effects of several parameters on retention times and resolution was made.

Firstly, retention times for each of the groups of the nucleosides adenosine, uridine, guanosine and citidine and its nucleotides was determined. Four solutions were prepared, each of them containing the nucleoside and nucleotides related: solution A containing adenosine (A), AMP, ADP and ATP; solution U containing uridine (U), UMP, UDP and UTP; solution G containing, GMP, GDP and GTP (guanosine was not included because of its low solubility in water) and solution C containing citidine (C), CMP, CDP and CTP. Each one of these solutions was injected and eluted with the isocratic method described by Moal et al. [13]: 0.15 *M* NaH_2PO_4 buffer containing 3.29 m*M* tetrabutylammonium, 5% methanol, pH 6. The order in the elution within each group was determined.

A solution containing all of the nucleotides and nucleosides was injected and eluted under these conditions, in order to determine the resolution in a single run. The elution profile is shown in Fig. 1. Separation was achieved within 15 min, but peaks A, UTP and GTP overlap, and the same for CTP and GDP.

Under these conditions of isocratic elution, lower proportions of organic modifier were assayed, in order to increase retention times and to achieve the separation of peak A from UTP and GTP. Totally satisfactory results were not obtained, and elution time was too long, having an effect in increasing of peak broadening (ATP) as shown in Fig. 2, so we started to apply different methanol gradient elutions.

In order to achieve a better separation of the peaks eluting first, by means of obtaining longer retention times at the start of the elution, the conditions assayed were gradient elutions; a reference gradient taken from Pijnenburg et al. [14] was initially used, and further modifications were done. At the beginning of the elution we employed buffer 100% to increase the differences between polarities of mobile and stationary phases, and started a gradient of methanol at minute 5 to achieve the elution of the more strongly retained peaks in a shorter time; the gradient achieved 10% methanol at minute 20. Separation of peak A is obtained, but UTP and GTP, and U and UMP overlap, as seen in Fig. 3.

An increased concentration of TBA hydrogensul-

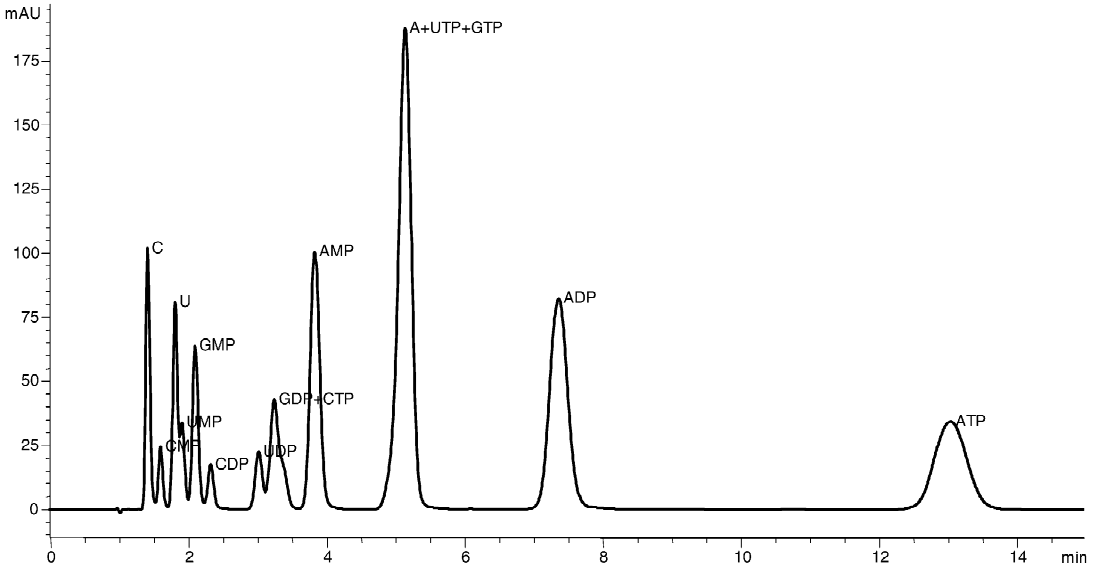

Fig. 1. Elution profile of nucleotides and nucleosides applying an isocratic elution. Conditions: buffer 0.15 *M* NaH_2PO_4 pH 6.0; TBA hydrogensulphate 3.29 mM; methanol 5%.

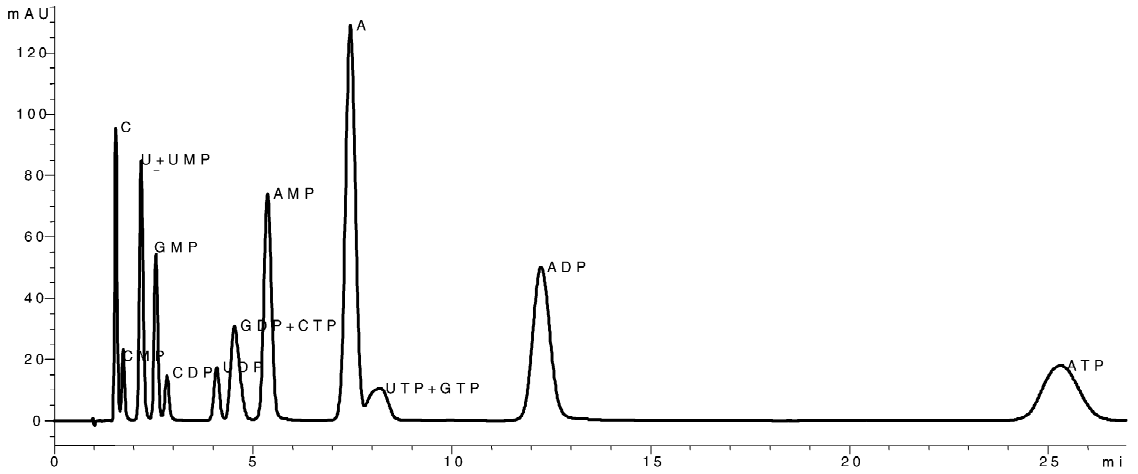

Fig. 2. Effect of a decrease in the proportion of organic modifier on the elution profile of nucleotides and nucleosides. Isocratic elution conditions: buffer 0.15 *M* NaH_2PO_4 pH 6.0; TBA hydrogensulphate 3.29 mM; methanol 2%.

phate up to 4 m*M* in the initial isocratic method resulted in a better separation of peaks U and UMP, but peak A is eluted with GTP and UTP. A gradient elution results in the resolution of peak A, but UTP and GTP coelute. The next step was to combine this increased concentration of ion-pairing agent and the methanol gradient.

Another ion-pairing agent was assayed: TBA hydroxide (40% solution in water), because of its easier handling and availability. The conditions assayed were the same already optimized for the previous ion-pairing agent (TBA hydrogensulphate): 4 m*M* TBA hydroxide, pH 6.0, and the initial gradient. The result is the same obtained with TBA hydrogensulphate, as seen in Fig. 4; under these conditions, AMP and CTP still coelute. To achieve a

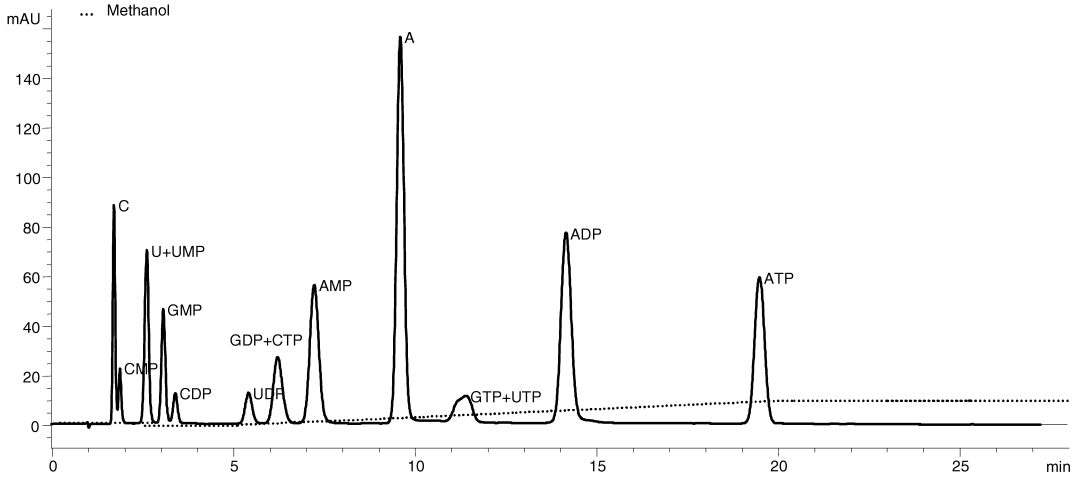

Fig. 3. Effect of a methanol gradient on the elution profile of nucleotides and nucleosides. Gradient: time 0–5 min: 100% buffer 0.15 *M* NaH_2PO_4 and 0% methanol; time 5–20 min: 90% buffer and 10% methanol; TBA hydrogensulphate 3.29 m*M*.

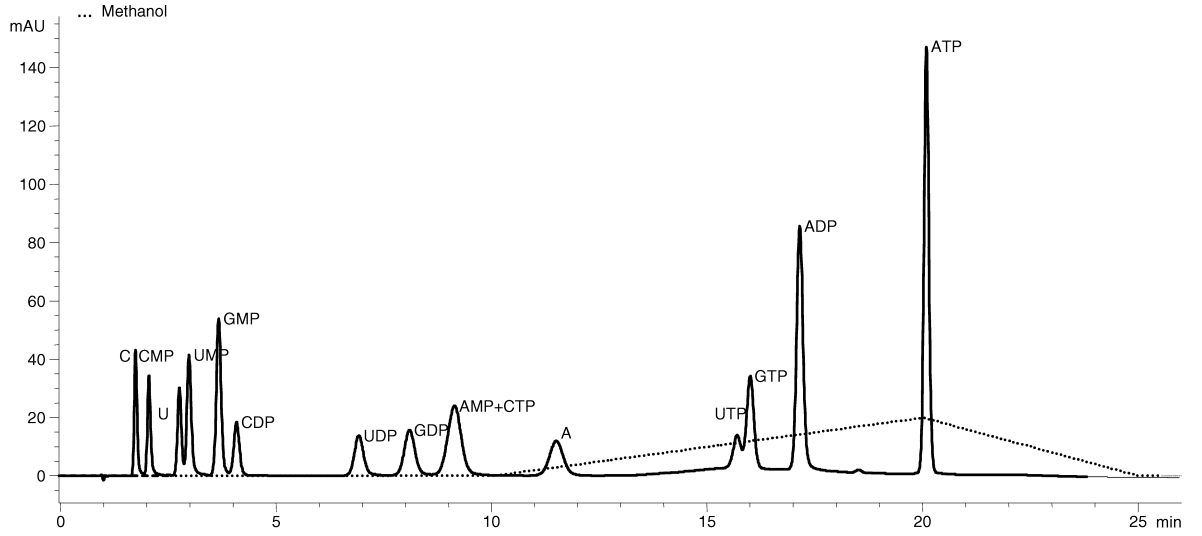

Fig. 4. Elution profile of nucleotides and nucleosides with gradient time 10–20 min 0–20% methanol, buffer 0.15 *M* NaH_2PO_4 pH 6.0; TBA hydroxide 4 m*M*.

good separation of AMP and CTP, slight modifications of the gradient and a range of concentrations of TBA hydroxide were assayed. The gradients were: Gradient 1: 5′–20′ 10% methanol; Gradient 2: 10′–20′ 20% methanol; and Gradient 3: 8′–12′ 5% methanol, 12′–20′ 20% methanol; it seemed to be optimal to apply a methanol gradient more gradual at the start and stronger after elution of AMP, to accelerate the elution of the resting compounds. The range of TBA hydroxide concentrations assayed was from 3.7 m*M* to 4.3 m*M*. The best results for our initial scope (quantification of adenine nucleotides) were obtained with the Gradient 3, detailed in Table 3, and a concentration of 4 m*M* of TBA hydroxide as can be seen in Fig. 5.

This separation achieved, the method was applied to samples from *Mytilus* mantle tissue, after extraction of nucleotides as described above. A sample amount of 10 μl was injected in the HPLC and separation of the nucleotides and nucleosides was obtained, resulting of application in further studies (Fig. 6).

Table 3
Modification of the gradient elution applied to obtain resolution of peaks U and UMP and UTP and GTP

Time	Methanol (%)	Buffer (%)
0	0	100
8	0	100
12	5	95
20	20	80
25	0	100

3.1. Peak purity analysis

To assess whether a peak comprises the component that was assigned to it on the bases of its retention time, spectral analysis was used; this assessment is based on the comparison of spectra recorded during the elution of the peak. In order to confirm the identity and purity of each peak in the sample, spectral data were stored and compared to the spectra of the original peak, corresponding to a standard (Fig. 7).

4. Discussion

As exposed above, the adaptation of a method for separation and quantification of nucleotides was based in modifications on the retention times given by changes in the polarity of the eluent, by modifications in the proportion of the organic modifier, and changes in the proportion of the ion-pairing

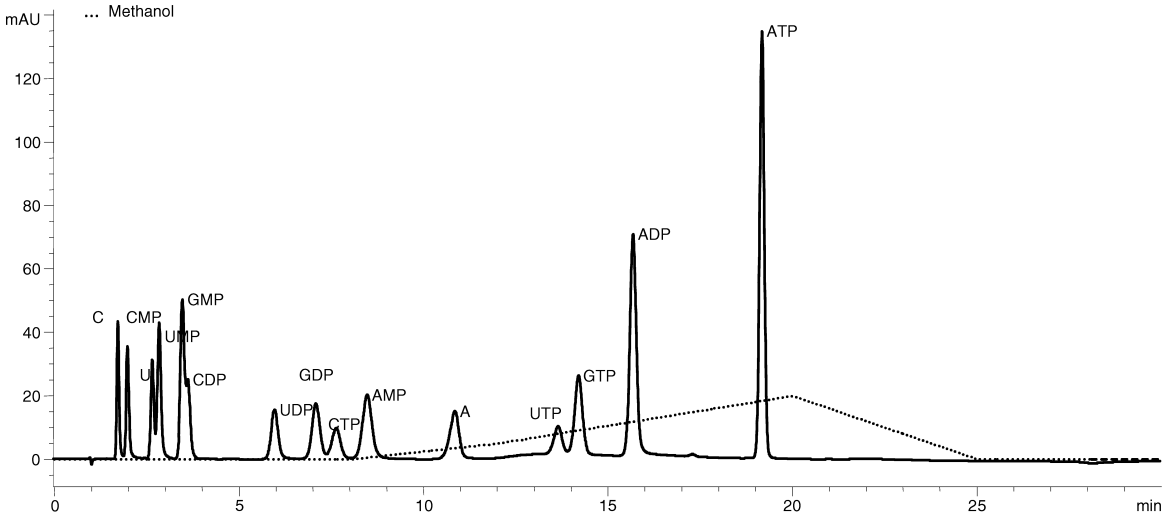

Fig. 5. Elution profile of nucleotides and nucleosides with gradient time 8–12 min 5% methanol, time 12–20 min 20% methanol; buffer 0.15 *M* NaH_2PO_4 pH 6.0; TBA hydroxide 4 m*M*.

agent, until a good separation of the peaks of interest for our studies (adenine nucleotides) was achieved in a reasonable analysis time (less than 30 min).

Increasing differences in polarity between the mobile and the stationary phase resulted in increased retention times of the different nucleotides, as expected. The ion-pairing agent has the effect of increasing retention times for most polar compounds, nucleotide triphosphates, by blocking the phosphate function by a lipophilic one. The elution time was

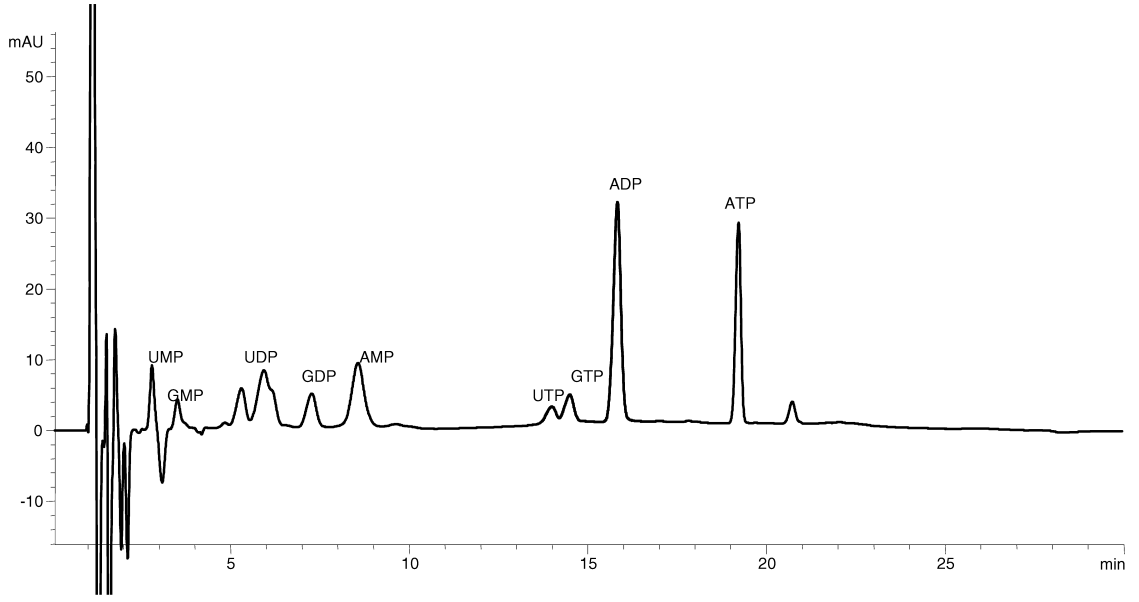

Fig. 6. An example of the separation of nucleotides in one of the samples from *Mytilus galloprovincialis* mantle tissue, extracted as described in the text.

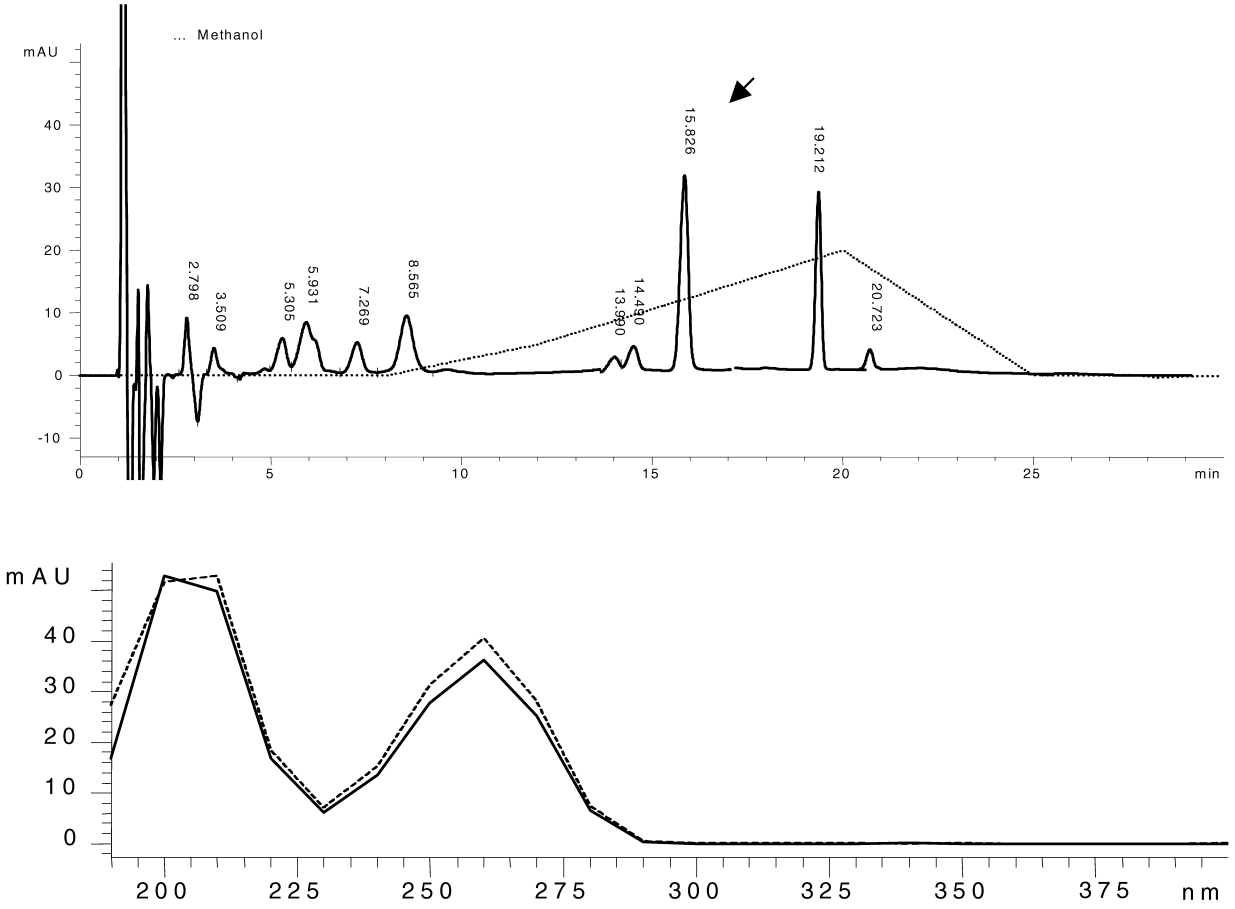

Fig. 7. Spectral comparison of peaks corresponding to a standard of ADP and a sample from *Mytilus* mantle tissue (peak eluting at 15.826 min corresponding to the elution time of ADP).

delayed as a function of the number of phosphate functions.

Elution of the nucleotides and nucleosides is dependent upon the amount of organic modifier present and can be easily manipulated with a methanol gradient. A decrease in the proportion of methanol used in isocratic elution, or a gradual methanol gradient has the effect of a longer retention of some of the retained compounds, allowing the resolution of peaks of interest, when coelution is present, which was the case of AMP and CTP. Then, larger amounts of organic modifier can be added to elute other highly retentive compounds rapidly.

The effect of pH was assayed too, as an added variable, to optimize the separation, and it was

Table 4
Separation conditions finally applied

Mobile phase	A: 0.15 *M* NaH_2PO_4 TBA hydroxide (4 m*M*) pH 6 B: methanol gradient:		
		A	B
	0′–8′	100%	0%
	8′–12′	95%	5%
	12′–20′	80%	20%
	25′–30′	100%	0%
Flow	1 ml/min		
Detection	254 nm		
Injection	10 μl		
Column	Hypersil-ODS (5 μm), Hewlett-Packard 125×4 mm		

observed that a decrease in pH in the range 5.7–6.6 has the effect of decreasing the retention times of the compounds retained in the column over 6 min.

In conclusion, the method finally applied for the separation of nucleotides from the *Mytilus* mantle tissue resumed in Table 4, enables an adequate separation in the biological samples of interest (Fig. 6).

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