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Determination of total ribonucleotide pool in plant materials by high-pH anion-exchange high-performance liquid chromatography following extraction with potassium hydroxide

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Abstract

A new, improved method that only requires a potassium hydroxide extraction procedure is presented for the analysis of a full nucleotide pool in plant materials. Quantification was performed by high-pH anion-exchange chromatography (HPAEC) with UV detection after a potassium hydroxide extraction, and allowed the quantification of 13 linear ribonucleotides in a single run. The method has been validated by comparison of six extraction methods and also by measurement of the intracellular nucleotide levels of three plant species (cell cultures and leaves). The evolution of the nucleotide pool of *Nicotiana tabacum* cell culture during growth has also been measured, and showed an increase in the pool until the fifth day, where the growth rate reaches a maximum, after which a decrease was observed.

Keywords: Ribonucleotide; HPAEC; Plant; Cell culture; Leaves

1. Introduction

The quantitative determination of nucleotides is important in many areas of biochemical research, because of their central role in the metabolism of living organisms. Measurement of that pool is generally achieved by HPLC, and it is a well-established procedure in the cases of animal tissues, prokaryotes and lower eukaryotes such as yeasts [1–4], but less established in the case of plants, especially when the pool should be as complete as possible [5,6]. The reason for this lack of interest arises from severe interferences by low-molecular-weight materials, such as phenolic compounds and chlorophyll precursors or degradation products, which are extracted together with the nucleotides [5]. Meyer and Wagner [5] have developed a procedure for sample preparation. However, it has the inherent problems of purification, which are time consuming and above all, it leads to the loss of compounds. To circumvent the obstacle of purification, we have concentrated our work on ion-exchange separation (already used for partial pool nucleotide measurement in acid condition [7,8]), but at alkaline pH. This method has never been used before for nucleotide separation of plant extracts. In fact, recent progress in ion-exchange columns with the pellicular resin initially developed for the separation of sugars in alkaline medium [9] allowed a good separation of nucleotides, as well as a good life time of the stationary phase [10].

The nucleotide pool is composed of linear nucleotides employed as precursors for nucleic acids and in bio-energetic processes involved in the synthesis of macromolecules, including polysaccharides, phospholipids and glycolipids (for reviews see [11,12]), and also of cyclic nucleotides, which are key regulatory molecules. The cyclic nucleotides in higher plants have been a topic of contention for a long time, but now it is clear that these compounds perform important roles [13].

From these considerations, and taking into account the importance of nucleotide assays in plant samples, it appeared

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useful to us to develop an analytical technique for the complete nucleotide pool determination that is as simple and reliable as possible. We tried to set up an ion-exchange HPLC method that, without any preparation of samples except for potassium hydroxide extraction of cell suspensions, allowed the direct simultaneous separation of the complete pool of nucleotides present in a plant cell. The present study reports the analytical conditions for the separation of 16 (thirteen linear and three 3′–5′ cyclic) nucleotides, and validates the present HPLC method by the quantification of the 13 linear nucleotides in several plant species (*Nicotiana tabacum*, *Arabidopsis thaliana* and *Lycopersicum esculentum*) and tissues (cell suspension and leaves).

2. Experimental

2.1. Chemicals and chromatographic standards

All HPLC eluents and standards were prepared with 18 MΩ cm deionized water. Sodium acetate was obtained from Acros Organics (Noisy le Grand, France) and HPLC-grade sodium hydroxide solution was supplied by Fisher Scientific Labosi (Elancourt, France). The NaOH solution was stored under neutral gas and kept for no more than 3 months after opening, to avoid problems of carbonate formation. Ultrapure standards of the individual nucleotides, purine and pyrimidine bases were purchased from Sigma–Aldrich (St. Quentin Fallavier, France), except for the cyclic GMP and CMP purchased from Biolog Life Science Institute (Bremen, Germany). Individual standard solutions were stored at −30 °C in water and remade every 6 months. A solution of 16 standards was freshly prepared in 1 M KOH for daily calibration.

2.2. HPLC systems

HPLC was performed with a liquid chromatography system consisting of a GP-40 quaternary gradient pump and an AS-50 autosampler (Dionex, Sunnyvale, CA, USA), a Spectrafocus optical scanning detector (Thermo Separation Products, Darmstadt, Germany), an SPD-6AV UV detector (Shimadzu, Croissy Beaubourg, France) and an CTO-10AS oven (Shimadzu, Croissy Beaubourg, France). Data acquisition and analysis were performed by Chromeleon software (Dionex).

The HPLC column used in this work was a Dionex CarboPac PA-100 (250 mm × 4.0 mm) equipped with a guard column. The stationary phase is composed of 10 μm diameter microporous substrate agglomerated with 285 nm microbeads quaternary ammonium functionalized latex, with a high pH range stability from 0 to 14.

2.3. Chromatographic conditions

Eluent A consisted of 30 mM NaOH (pH 12.5) and eluent B of 1 M sodium acetate (pH 9.4). The elution profile applied consisted of an initial isocratic phase of 100% A (5 min). From 5 to 15 min the gradient increased linearly from 0 to 25% of B. The gradient was increased with a slightly convex curve (curve number 8 in Chromeleon software) from 25 to 50% of B from 15 to 35 min. From 35 to 47.5 min, the gradient was increased linearly from 50 to 75% of B, and then the gradient was increased to 100% to 48 min. An isocratic phase was held at these conditions to 60 min. The gradient was decreased to 0% of B in 0.1 min and maintained at these conditions to 75 min. All eluents were sparged with purified helium gas (Helium C, Air Liquide, Paris, France) before and during use. The flow rate was set at 1 ml/min and the column thermostated at 30.0 ± 0.1 °C.

2.4. Identification and quantification of metabolites

Calibration curves of the different standards, as well as runs of cell extracts, were monitored by a diode array detector set up for data acquisition of absorbance between 250 and 300 nm, and quantitative analysis was performed at 254 nm (chosen as a mean value between the maximum of absorption of purine and pyrimidine nucleotides). Assignment of peaks in the samples was achieved by comparing both retention times and absorption spectra of the standards. When comparison of absorption spectra was not applicable (due to the inadequate concentration of that particular compound), the assignment was performed by co-chromatogram with the appropriate standard, and added to the sample solution in such a concentration as to approximately double the area of the uncertain peak. Metabolite concentrations were measured by comparison with calibration curves prepared with known quantities of standards. Calibration curves, prepared for each metabolite, were constructed by plotting peak area versus amount for the following amounts: 15, 31, 156, 312 and 625 pmol. The concentrations of metabolites were determined automatically (Dionex Chromeleon software) by interpolation of the integrated peak areas against the calibration curve.

2.5. Cell lines and plants: growth conditions and preparation of cell extracts

Cell suspensions of *N. tabacum* cv Xanthi, *A. thaliana* and *L. esculentum* were grown in Murashige and Skoog modified medium, pH 5.6, containing MS salt [14], 5 mg l^{-1} nicotinic acid, 3 mg l^{-1} calcium pantothenate, 2 mg l^{-1} glycine, 0.5 mg l^{-1} thiamine–HCl, 0.5 mg l^{-1} folic acid, 0.5 mg l^{-1} pyridoxine–HCl, 0.05 mg l^{-1} biotine, 100 mg l^{-1} myo-inositol, 200 mg l^{-1} glutamine, 0.11 mg l^{-1} kinetine, 0.165 mg l^{-1} 2,4-dichlorophenylacetic acid and 30 g l^{-1} sucrose, at a constant temperature of 25 °C and a continuous illumination (100–105 μmol m^{-2} s^{-1} light radiance obtained with neon tubes: Osram L36W/77 Fluora, Appro5, Dijon, France), on a rotary shaker (140 rpm). Cell suspensions were subcultured every 7 days and used for assay 4 days after subculturing.

An aliquot of the cell suspension (900 μl) was immediately mixed with 100 μl of 10 M KOH and 400 mg of glass beads (diameter 0.25–0.5 mm, Bioblock, Illkirch, France) that had been weighed beforehand. The biomass concentration of samples was determined on the cell culture remainder after filtration and washing.

Plants of *N. tabacum* var. Petit Havana, *A. thaliana* var. Columbia and *L. esculentum* were cultured in a greenhouse, at 60–65 $\mu mol\ m^{-2}\ s^{-1}$ light radiance obtained with neon tubes (a combination of Mazdafluor Prestiflux Brilliant 840 and Mazdafluor Prestiflux Incandia 830, Appro5, Dijon, France), with a 12/12 h, 25/20 °C light/dark cycle and 60% relative humidity. Young leaves were detached, a small piece was cut between two secondary leaf veins, weighed and immediately immersed in 1 ml of 1 M KOH and 400 mg of glass beads. Samples could be stored at that stage in liquid nitrogen. Samples were then disrupted on a bead beater (Fast-prep 101, Q-biogene, Illkirch, France) for 30 s at a speed of 6.5 (two disruptions cycles for the leaf samples), and centrifuged at 4 °C and 14 000 × *g* for 5 min. The supernatant was then immediately injected.

2.6. Testing of the extraction procedure

Six different extraction procedures of nucleotides were tested on leaves of *N. tabacum* var. Petit Havana, chosen for their mechanical and chemical resistance: boiling water for 5 min [1], ice-cold 75% ethanol [10], 60% acetonitrile [15], potassium hydroxide at 30 mM [16], perchloric acid at 0.5 M [3,5,10,17] and potassium hydroxide at 1 M (our method). In each case, 100 mg of leaves/ml were used with 400 mg of glass beads and followed by two sample disruptions in a bead beater, centrifugation and immediate injection. For the perchloric acid extraction procedure, a potassium hydroxide neutralization at 0 °C followed by a centrifugation was realized before injection. That procedure allows to eliminate perchlorate ions into potassium perchlorate, practically insoluble at cold temperature. This neutralization did not affect nucleotides content in perchloric acid extract and allows the use of this extract with anion-exchange column without perturbation of retention time.

2.7. Measurement of nucleotide pool according to the cell culture age

A cell suspension of *N. tabacum* cv Xanthi was grown in Murashige and Skoog modified medium (see above) at a constant temperature of 25 °C and under continuous illumination (100–105 $\mu mol\ m^{-2}\ s^{-1}$), on a rotary shaker (140 rpm). At 1, 2, 3, 4, 5, 6 and 7 days after subculturing, samples were treated as previously described and analysed for their nucleotide pool. For each sample, an aliquot allowed quantification of the biomass in fresh weight after filtration and dry weight after drying at 100 °C to constant weight.

3. Results

3.1. Development of the HPLC method

Until now, separation of partial nucleotide pools (adenosine and/or uridine nucleotides) of plant extract has been achieved with anion exchange for the most recent studies [7,8] and for the almost total pool with an ion-pairing liquid chromatography method preceded by fastidious steps of sample purification to eliminate various compounds of plant metabolism [5].

High-pH anion-exchange chromatography was chosen for this work because of the powerful separation capacity of this type of stationary phase and also for advantages brought by the lack of interference from low-molecular-weight compounds with the plant cell material.

A pulsed amperometric detector system was chosen initially because of its excellent sensitivity for compounds containing multiple hydroxyl groups. However, it was discovered early in this study that nucleotides detection was not very sensitive to electrochemical detection compared with UV detection. We have found that a good nucleotide pool separation requires an inverted NaOH gradient from 30 to 0 mM, which did not allow good ionization of hydroxyl groups and thus good detection of nucleotides. Therefore, UV detection was chosen for further development.

The gradient profile employed in this work may seem long, but with ion exchange, the column state should be identical to ensure valid retention times.

3.2. Separation of metabolite standard

The separation of a 16-metabolite standard in a single run has been achieved, along with baseline separation of virtually all of the standards (Fig. 1). All the data and statistics are presented in Table 1. In all cases, variation in retention time for compounds was less than 0.4 min. The limits of detection of the individual metabolites ranged from 6 to 15 pmol injected. All the calibration curves were linear in the range of amount tested from 15 to 625 pmol injected, with correlation coefficients or r^2 values between 0.980 and 0.998. The relative standard deviation of the standard areas determined on each standard did not exceed 8.1%. The peak width did not exceed 0.62 min, except for cGMP with 1.17 min, due to its very high affinity for the column. Finally, the number of theoretical plates for the column has been calculated for three amounts of standard, and varied less than 10% between the higher and lower amount injected.

3.3. Separation of compounds into plant samples

In our extraction method, all the soluble compounds in alkali solution were collected, and many undetermined peaks were present on chromatograms. In order to identify the peaks, all the different plant samples (species and cellular

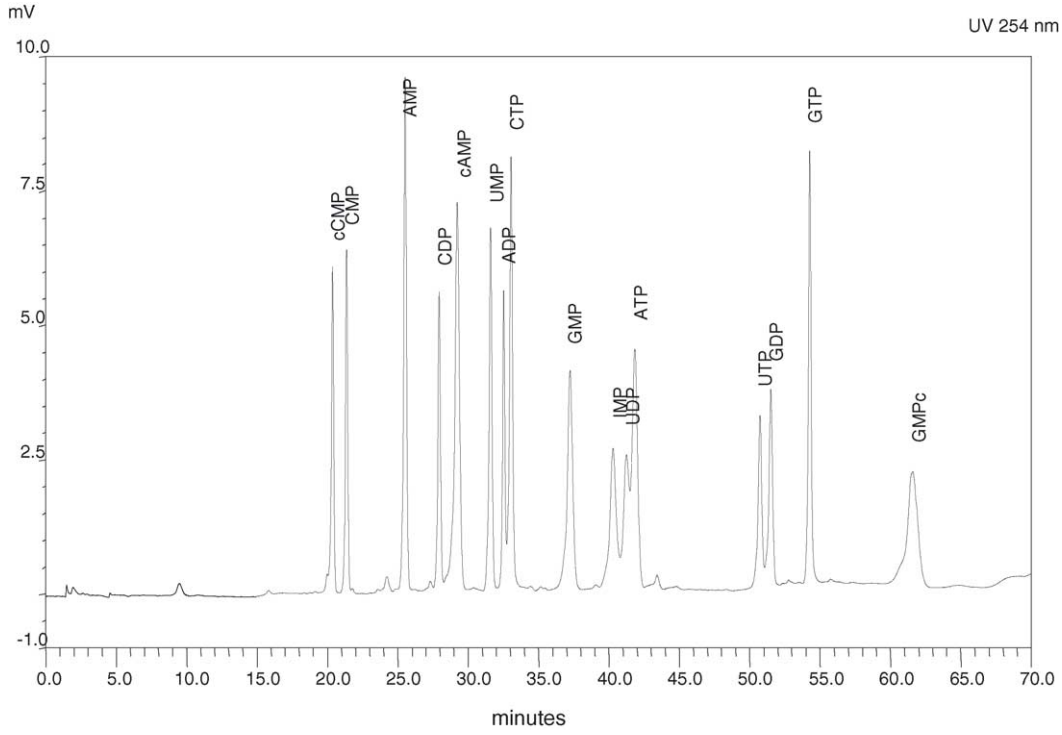

Fig. 1. Simultaneous separation of the complete series (mono-, di- and triphosphate) of the four nucleosides (adenosine, guanosine, cytidine and uridine) added to IMP and of the three 3′–5′ cyclic nucleotides (cAMP, cGMP, cCMP).

Table 1
Statistical analysis of data for metabolite standards

Component	Retention time (min) ± SD	Limit of detection (pmol injected)	Standard calibration curve (regression coefficient)[a]	Peak width[b] (min)	Area detection repeatability[c]	Theoretical plates[d]		
						15 pmol	312 pmol	625 pmol
cAMP	28.6 ± 0.1	6	99.7	0.45	2.7	65 028	68 647	67 340
AMP	24.4 ± 0.0	6	99.7	0.27	7.5	133 327	139 255	137 800
ADP	32.2 ± 0.1	12	98.3	0.26	4.4	223 452	245 999	234 079
ATP	42.8 ± 0.3	12	99.4	0.61	4.6	94 710	81 984	80 723
cCMP	17.3 ± 0.1	6	99.4	0.32	6.3	39 365	49 500	49 228
CMP	19.5 ± 0.0	6	99.7	0.22	3.4	103 594	122 424	117 786
CDP	27.2 ± 0.1	6	99.7	0.24	3.8	203 280	201 758	202 413
CTP	32.9 ± 0.2	6	99.6	0.32	2.8	137 738	171 225	167 647
cGMP	64.3 ± 0.2	12	99.7	1.17	6	49 215	52 072	51 895
GMP	37.3 ± 0.4	6	99.6	0.52	4.4	88 566	86 925	85 989
GDP	52.0 ± 0.1	12	99.8	0.28	3.6	544 063	560 213	534 980
GTP	54.7 ± 0.0	6	99.1	0.33	5.1	493 219	457 399	457 025
IMP	40.7 ± 0.4	12	99.8	0.62	4.2	60 548	74 193	73 842
UMP	31.0 ± 0.1	12	99.7	0.28	3.1	170 534	198 351	198 277
UDP	41.6 ± 0.4	15	98.9	0.59	4.7	104 867	85 529	86 217
UTP	51.2 ± 0.1	15	98.0	0.31	8.1	962 610	441 260	451 288

Statistics were determined for 10 injections. Theoretical plates measure for 15, 312 and 625 pmol injected.

[a] Linear regression coefficient for calibration curve used to quantify metabolite standards (from 15 to 625 pmol).

[b] Peak width measure for 312 pmol injected.

[c] Accuracy of concentration measurements for metabolite standards (relative percentage of standard deviation), 312 pmol injected.

[d] European standard: number of theoretical plates = $5.54 \times (T_r/Wh)^2$ with T_r the retention time and Wh the peak width at 50% height (half width).

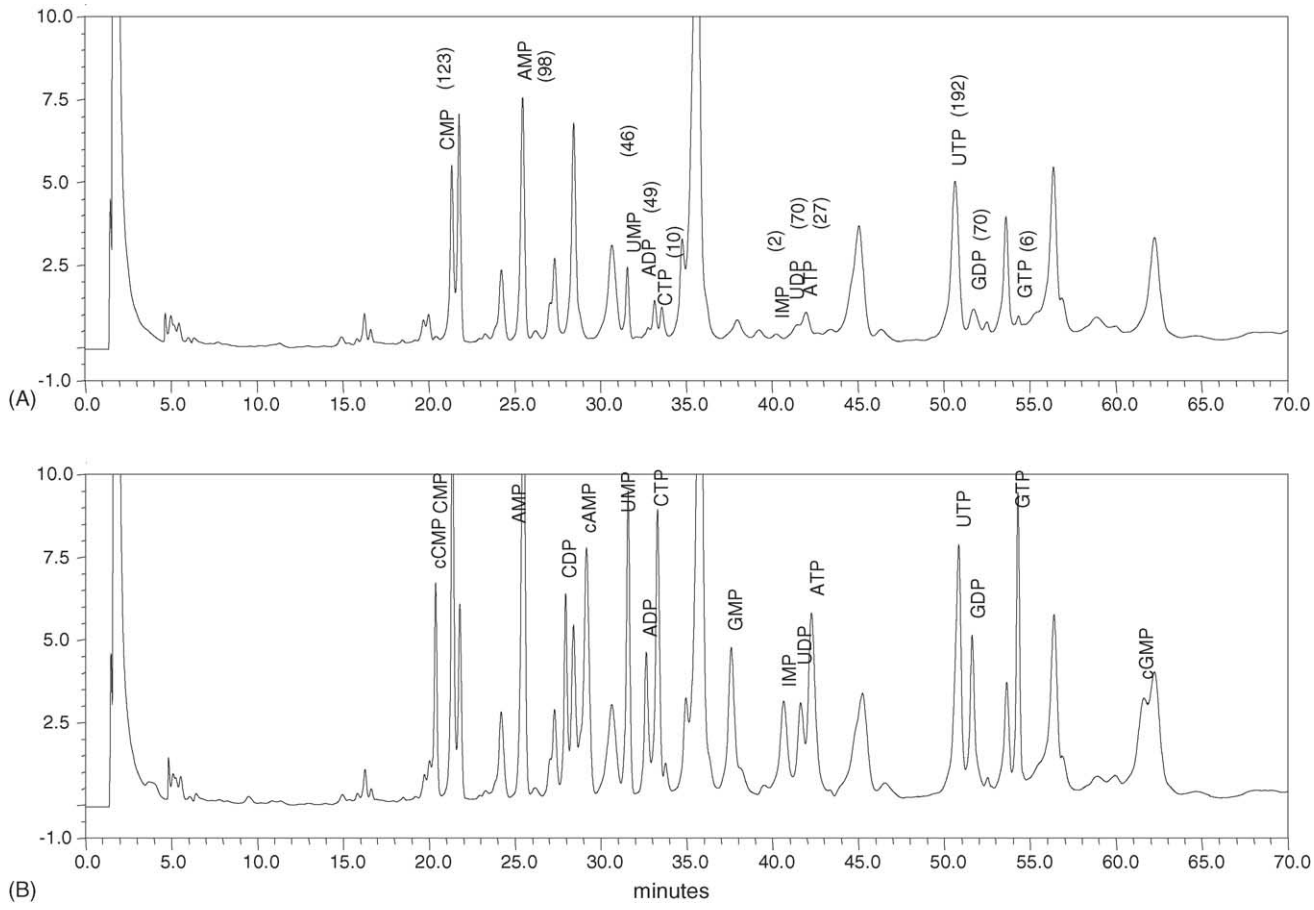

Fig. 2. Chromatograms of potassium hydroxide (1 M) extracts of an old tobacco leaf (100 mg of fresh weight in 1 ml of KOH) without (A) or with (B) addition of a standard mixture at 312 pmol injected. This sample is very rich in nucleotides and contaminant peaks, and therefore represents the hardest conditions of elution met. Nucleotides concentrations indicated between brackets are expressed in nmol g^{-1} fresh weight.

states) were analysed with or without addition of standard mixture. The results obtained with an old tobacco leaf are presented in Fig. 2. This example clearly shows that all 16 nucleotides can be separated. Furthermore, a higher diversity of nucleotides can be detected (Fig. 2B). In order to preserve the life time of the column, we recommend to change the pre-column as soon as the retention time variation is higher than 0.5 min. Under these conditions, the column can be used for up to 500 injections.

3.4. Stability and extraction of compounds in potassium hydroxide

The effect of alkali extraction (1 M KOH) on the recovery percentage of each standard compound was tested (Table 2), and only a small percentage was lost during extraction for most nucleotides; furthermore, the storage of the standard in KOH for up to 3 days at room temperature did not significantly alter the relative concentration of each compound (data not shown). On the basis of these results, we can, therefore, confirm that the compounds under evaluation are not modified by processing. In alkali extraction conditions, it is known [9,10] that UDP sugars are hydrolysed in UMP. We have verified this in our conditions and confirmed that UMP in the samples represents the sum of free UMP in the cell and all the UDP sugars hydrolysed during the extraction [10].

Table 2
Standard nucleotides stability in the KOH extraction solution (31 pmol)

	Recovery (%)		
	Mean	Mini	Maxi
cAMP	97.0	95.7	98.5
AMP	99.5	97.6	102.6
ADP	95.0	89.0	100.6
ATP	95.6	94.3	96.5
cCMP	97.5	95.4	98.6
CMP	96.0	94.0	98.4
CDP	96.0	93.1	98.4
CTP	99.0	97.3	101.9
cGMP	94.8	92.6	96.1
GMP	94.8	90.5	97.7
GDP	101.1	97.9	103.5
GTP	97.4	95.1	99.8
IMP	97.7	96.3	98.6
UMP	97.7	96.6	99.3
UDP	96.7	95.0	98.2
UTP	94.0	90.3	97.1

Table 3
Extraction yield of nucleotides family from tobacco leaves by various protocols (expressed in percentage compared to KOH 1 M extraction)

	Extraction procedure					
	Boiling water	Ethanol 75%	Acetonitrile 60%	KOH 30 mM	Perchloric acid 0.5 M	KOH 1 M
$\sum A$	63	36	11	28	88	100
$\sum C$	4	0	35	80	65	100
$\sum G$	51	55	62	3	58	100
$\sum U$	5	1	90	83	78	100
IMP	72	110	109	76	113	100
Total	46	58	81	66	77	100

For each data, the standard deviation does not exceed 20%. $\sum X$: sum of series, the extraction yield of nucleotide X is expressed in percentage of the total nucleotide content.

Concerning the plant samples, we have verified that the nucleotide pool was stable at 4 °C for 20 min. After that time, an increase in the monophosphate nucleotides pool was observed, in spite of the presence of 1 M KOH in the sample and of the good stability of standard into KOH. Therefore, samples were injected immediately after extraction or defrosting from liquid nitrogen, where we have verified their stability for at least 15 days (data not shown).

Concerning the extraction procedure, we have tested six different protocols already used for nucleotide extraction [1,5,10,15,16] and ours, namely 1 M KOH. The perchloric acid extraction which is the most frequently used technique has been tested with a potassium hydroxide neutralization before injection. The results (each extraction procedure records the mean values of three determinations) show that extraction with 1 M KOH was identical or better than those tested for all four families of nucleotides, and are identical with the ethanol and acetonitrile extraction for the IMP (Table 3). The alkaline extraction was the most efficient technique because of cell wall solubilization at high pH. Intracellular component was more accessible after cell wall pectic and hemicellulosic polysaccharides were dissolved in potassium hydroxide [18].

3.5. Measurement of nucleotide pool in different plant species and tissues

The nucleotide concentrations were determined in three different plant species in cell suspension culture and in leaves (Table 4). These are mean values of three determinations. For the three species tested, many compounds are present in the leaves but absent or in lower concentration in the cell culture. This is the case for CMP, AMP, ADP and IMP, while the three compounds ATP, UTP and GTP are present in higher concentration in the cell culture than in leaves.

The adenylate charge, defined as ((ATP + 1/2ADP)/(ATP+ ADP + AMP)), is close to 0.9 for the three cell culture species, but decreased to approximately 0.5 in the leaves of the three species.

Table 4
Concentration of nucleotides in different plant tissues and species (nmol g^{-1} fresh weight)

	Nicotiana tabacum		*Arabidopsis thaliana*		*Lycopersicon esculentum*	
	Cell	Leave	Cell	Leave	Cell	Leave
AMP	8.5 ± 3.1	32.9 ± 7.3	17.0 ± 8.7	58.6 ± 4.9	–	54.8 ± 8.7
ADP	8.3 ± 2.6	23.4 ± 13.0	19.0 ± 2.8	76.5 ± 10.1	37.7 ± 13.5	30.2 ± 3.9
ATP	123.6 ± 14.1	47.5 ± 11.0	145.1 ± 17.4	40.3 ± 7.5	190.4 ± 8.4	50.2 ± 7.4
CMP	–	TR	–	TR	–	TR
CDP	–	8.9 ± 2.4	TR	–	–	–
CTP	3.6 ± 1.0	11.6 ± 2.5	9.3 ± 1.5	TR	–	–
GMP	TR	–	TR	8.9 ± 5.5	10.2 ± 3.5	–
GDP	–	–	–	–	–	–
GTP	23.4 ± 3.1	TR	25.9 ± 4.0	TR	24.3 ± 33.7	TR
IMP	–	20.3 ± 7.0	–	8.2 ± 2.1	–	20.3 ± 7.4
UMP	187.6 ± 14.3	73.5 ± 1.6	178.0 ± 31.2	80.9 ± 4.7	174.2 ± 70.9	50.2 ± 8.5
UDP	–	23.2 ± 6.9	TR	17.7 ± 3.8	58.8 ± 17.2	15.8 ± 3.2
UTP	48.7 ± 15.4	6.9 ± 2.2	18.0 ± 8.8	20.1 ± 4.3	27.4 ± 11.8	5.8 ± 1.7
$\sum A$	34.8	41.8	43.9	56.4	43.6	59.5
$\sum C$	0.9	8.3	2.3	0.0	0.0	0.0
$\sum G$	5.8	0.0	6.3	2.9	6.6	0.0
$\sum U$	58.5	41.7	47.5	38.1	49.8	31.6
Ratio ATP/ADP	14.9 ± 0.3	2.0 ± 0.6	7.6 ± 0.2	0.5 ± 0.2	5.1 ± 0.4	1.7 ± 0.2
Adenylate energy charge[a]	0.91	0.57	0.85	0.45	0.92	0.48

(–) Not detectable; TR: trace; $\sum X$: sum of series, the extraction yield of nucleotide X is expressed in percentage of the total nucleotide content.
[a] Adenylate energy charge is defined as ((ATP + 1/2ADP)/(ATP + ADP + AMP)).

Table 5
Concentration of the nucleotides in nmol g^{-1} fresh weight during growth of the suspension culture of tobacco cells

	Day						
	1	2	3	4	5	6	7
ADP	6.1 ± 3.6	11.9 ± 3.5	10.8 ± 3.4	18.2 ± 6.8	16.4 ± 9.0	7.7 ± 1.6	7.8 ± 1.4
ATP	91.1 ± 14.4	175.7 ± 20.3	161.4 ± 18.4	264.9 ± 23.6	130.6 ± 77.0	37.8 ± 3.2	21.9 ± 2.2
GDP	–	–	–	–	–	–	9.6 ± 0.5
GTP	22.9 ± 6.4	34.6 ± 5.9	30.6 ± 4.1	56.3 ± 3.2	19.4 ± 13.1	6.5 ± 1.2	1.5 ± 1.5
UMP	130.6 ± 18.2	239.6 ± 88.5	245.1 ± 18.7	420.6 ± 18.7	171.6 ± 12.3	38.7 ± 4.1	16.8 ± 4.5
UTP	25.8 ± 13.3	70.7 ± 37.4	63.6 ± 20.1	122.3 ± 19.2	52.4 ± 13.9	11.2 ± 2.1	1.8 ± 0.0
Adenylate energy charge[a]	0.97 ± 0.22	0.98 ± 0.16	0.97 ± 0.16	0.97 ± 0.12	0.94 ± 0.77	0.92 ± 0.11	0.87 ± 0.12
Ratio ATP/ADP	14.9 ± 0.6	14.8 ± 0.3	14.9 ± 0.3	14.5 ± 0.4	8.0 ± 0.8	4.9 ± 0.2	2.8 ± 0.2
Dry biomass	44.5 ± 5.8	48.9 ± 2.9	60.5 ± 4.2	96.1 ± 1.7	196.1 ± 65.1	322.9 ± 66.1	439.1 ± 14.6
Growth rate[b] (j^{-1})	0.02	0.10	0.24	0.59	1.04	0.65	0.36

Nucleotides are expressed in pmol mg^{-1} of fresh weight. Values are the mean of three independent replicates. (–) Not detectable.
[a] Adenylate energy charge is defined as ((ATP + 1/2ADP)/(ATP + ADP + AMP)).
[b] Growth rate is defined as ((biomass at day x − biomass at day $x-1$)/(biomass at day $x-1$))/(day x − day $x-1$).

The quantification of cyclic nucleotides was not possible due to their very low concentrations. We have tried to use lyophilized cell cultures to increase the concentration during the extraction procedure, but even with a cellular concentration 10 or 20 times higher, no cyclic nucleotides could be detected. In fact, a previous study had measured the cyclic nucleotides in tobacco cell culture and shown that concentrations were approximately a thousand times lower than for linear nucleotides [17].

3.6. Effect of cell culture age on the nucleotide pool

For the nucleotide pool (Table 5), the concentrations were constant over the first 3 days of growth after subculturing, a maximum was reached on the fourth day with an average increase of a factor of two, then the nucleotide concentration gradually decreased until the seventh day. That fall in concentration was also accompanied by a fall in the ratio ATP/ADP from the fifth day from 14.5 to 2.8 at the seventh, and by a fall in the adenylate energy charge from 0.97 to 0.87. Concerning the growth rate, the maximum is reached between the fourth and fifth days, agreeing with the maximum of the triphosphate charge.

4. Discussion

Determination of the nucleotide pool in cell extract is mainly performed either by ion-pairing or by ion-exchange HPLC methods. However, until now, no method to our knowledge has analysed 16 ribonucleotides (13 linear and 3 cyclic) in a single injection. Moreover, techniques applied to plant extracts are not frequent and generally require a fastidious purification step of the sample before injection.

The data reported in the present experimental study demonstrate, for the first time to the best of our knowledge, that by using an appropriate HPLC column and proper chromatographic conditions, it is possible to separate and quantify simultaneously the complete series (mono-, di- and triphosphate) of the four nucleosides (adenosine, guanosine, cytidine and uridine) added to IMP on various plant extracts without any preparation of the sample.

Rapid extraction of cell pools and inactivation of metabolic enzymes is essential to obtain true intracellular metabolite concentrations that reflect steady state metabolism because of the very short half-lives (<4 s) of metabolites in vivo [19]. In many cases, that has been achieved by extraction with water or solvent, but those methods are not suitable for plant cell extracts (especially with leaves) because of the cell wall resistance even with a bead beater treatment (Table 3), or by perchloric acid extraction also less performant into extraction yield of nucleotides especially those of cytidine and guanosine family. Therefore, the concentrated alkaline extraction method (1 M) in the case of plant cells is better for several reasons. Extraction yield was the highest, and extraction by pipetting of cells directly into concentrated potassium hydroxide solution is instantaneous, resulting in denaturation of metabolic enzymes and stabilization of different compounds. Furthermore, alkali extract of the sample did not modify the retention time of compounds because of a similar composition to the HPLC mobile phase. Finally, without any treatment of cell suspensions, such as filtration or centrifugation before metabolism inactivation, we are sure to be as near as possible to the in vivo conditions of the composition of the metabolic compounds. However, for a particular application, it is possible to increase the concentration (by a factor of 10) of the sample by using filtered cells directly extracted into 1 M KOH, but with the inherent problem of nucleotide pool modification linked to filtration.

The results obtained for the various plant species studied are consistent with available data on various plant species, and especially on tobacco [5,6,12,20,21], and validate the analytical technique.

Our results on cell cultures have shown a high concentration of triphosphate nucleotides (ATP, GTP and UTP) and a low concentration of di- and monophosphate nucleotides compared with those in leaves, where less triphosphate and more di- and monophosphate nucleotides were present (ex-

cept for the UMP, but this represents the sum of UMP and all the UDP sugars hydrolysed by the extraction procedure). The difference in triphosphate nucleotide rate between cell culture and leaves has already been shown [5] and demonstrates a higher metabolism activity in the cell culture due to their higher growth rate. Similarly young leaves contain a higher quantity of triphosphate nucleotides than old leaves.

The adenylate energy charge value is close to 0.9 in the three cell culture species tested, as reviewed previously on many plant species [5]. Concerning the adenylate energy charge in leaves, our values (0.5 on average, see Table 4) are lower than those described by Ashihara in 2003 [20], on average 0.8 for tobacco, tea and *Catharanthus roseus*, but closer to those found in the leaves of cereals [6].

The different components of the nucleotide pool for the three species in cell cultures are, a high uridine nucleotide pool between 47 and 58%, followed by an adenosine nucleotide pool between 35 and 44%, 6–7% for the guanosine nucleotide pool and a few percent for the cytidine pool, in agreement with previous studies (for a review see [12]). In the case of leaves, that distribution becomes 42–59% for the adenosine nucleotide pool, followed by 32–42% for the uridine nucleotide pool, and a few percent for the guanosine and cytidine nucleotide pool.

Concerning the cyclic nucleotides, UV detection combined with the very low concentration of those compounds did not allow their detection. However, that limitation in the case of plant extracts should not overshadow the interest in other biological extracts, where the cyclic nucleotides are as concentrated as the linear nucleotides [1]. Furthermore, in our technique, there are no contaminant compounds present at the same retention times as the three cyclic nucleotides, and that raises the possibility for detection with a more sensitive detector.

The study of the nucleotide pool according to the cell culture age has shown that at the fourth day, the nucleotides concentration is higher and in accordance with the maximum growth rate obtained between the fourth and the fifth days. Those observations are in agreement with a previous study [22] on the same subject (but with another cell cultivar and Linsmaier–Skoog medium), and confirm that the fourth day after subculturing is the richest in nucleotides. The main difference with the previous study is the evolution of the ATP/ADP ratio, which stays at a constant level between days 7 and 10 during all the growth, whereas it fell rapidly in our study after the optimum growth rate at the fifth day, from 14.8 to 2.8 at the seventh day. That could be explained by the fact that the biomass at the end of the culture is very different in the two cases, and in a higher concentration in our cultures.

5. Conclusion

In conclusion, in the present study we have demonstrated that, by using an original extraction technique for nucleotides in alkaline medium combined to an HPAEC method, it was possible to obtain simultaneous separation of 13 linear and 3 cyclic nucleotides from plant material without any purification step. Due to its simplicity and reproducibility, this HPLC method is of great interest for the study of the nucleotide pool in plant material under different physiological or pathological states, and offers the possibility for measurement of cyclic nucleotides on other biological samples or with other more sensitive detection techniques.

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