Rapid, Reliable Nucleotides Analyses, Using SUPELCOSIL LC-18-T HPLC Columns

SUPELCOSIL LC-18-T columns provide the high efficiency and selectivity needed for reversed phase HPLC analyses of nucleotides and nucleotide metabolites. Special treatment of the silica surface reduces the potential for interactions with these sensitive compounds. Nucleotides can be analyzed on a SUPELCOSIL LC-18-T column through conventional RP-HPLC or ion pairing RP-HPLC. In conventional RP-HPLC separations, nucleotides elute in order of decreasing number of phosphate groups, because of the corresponding increase in the hydrophobicity of the molecule. In ion pairing RP-HPLC, the ion pair reagent associates with the phosphate groups, creating a more hydrophobic complex, and increased retention, as the number of phosphate groups increases.

Key Words:
● nucleotides ● purines ● pyrimidines ● ATP

Nucleotides are the monomeric units of which deoxyribonucleic acids (DNA) and ribonucleic acids (RNA) are composed. Free nucleotides (Table 1) are found widely in cells, where they provide chemical energy for many biochemical reactions. Each nucleotide consists of a purine or pyrimidine base, a pentose sugar (ribose or deoxyribose), and one, two, or three phosphate groups. Figure A shows structures of the nucleotide adenosine triphosphate (ATP, a widely used intracellular energy source with a purine [adenine] base), the other common purine base (guanine), and the common pyrimidine bases (cytosine, thymine, uracil).

Knowledge of the type, concentration, and distribution of nucleotides in a cell gives insight into the cell's metabolic condition. Pool levels of nucleotides determine the rate and extent of DNA synthesis during mitosis, and of messenger RNA synthesis during protein synthesis.

Reversed phase HPLC (RP-HPLC) is an excellent tool for nucleotide analysis. It provides the high efficiency and selectivity needed to detect the often low levels of minor nucleotides or monitor the changes in levels of all nucleotides during cellular metabolism. RP-HPLC analyses are more stable than conventional ion-exchange methods of nucleotide analysis, and do not have the mobile phase limitations that plague the latter methods.

SUPELCOSIL™ LC-18-T columns are designed specifically for efficient and reliable RP-HPLC analysis of nucleotides and nucleotide metabolites. Special treatment of the silica surface prior to bonding the octadecyl (C18) groups to the particles reduces the potential for silanol or metal ion (chelation) interactions with these sensitive analytes. Both types of undesired interactions are stronger than the van der Waals interactions between the analytes and the bonded phase, and cause the analyte peaks to tail, reducing resolution and decreasing the sensitivity of the analysis.

Table 1. Common Nucleotides

Acronym	Nucleotide
A	Adenine
ADP	Adenosine diphosphate
ADP-Rib	Adenosine diphosphate-ribose
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CDP	Cytidine diphosphate
CMP	Cytidine monophosphate
CTP	Cytidine triphosphate
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HYP	Hypoxanthine
I	Inosine
IMP	Inosine monophosphate
NAD	Nicotinamide adenine dinucleotide
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
UDP	Uridine diphosphate
UMP	Uridine monophosphate
UTP	Uridine triphosphate
XAN	Xanthine

Nucleotides can be analyzed on a SUPELCOSIL LC-18-T column through conventional or ion pairing RP-HPLC methods. In conventional reversed phase separations the nucleotides elute in order of decreasing number of phosphate groups, because of the corresponding increase in the hydrophobicity of the molecule. Figure B shows a mixture of five adenine-derived nucleotides on a 25cm x 4.6mm SUPELCOSIL LC-18-T column (5µm particles). Analysis time is significantly reduced, with minor sacrifice in resolution, by using a 15cm x 4.6mm column containing 3µm particles (Figure C). The peak shape is excellent for either column.

Small, charged, very hydrophilic compounds, like nucleotides, often are analyzed by ion pair chromatography. In ion pairing analyses of nucleotides, the ion pair reagent associates with the phosphate groups. Retention increases as the number of phosphate groups increases, because the number of ion pair molecules that can associate with the compound also increases, creating a more hydrophobic complex. This is demonstrated in Figure D, where the adenine-derived nucleotides elute in the

Figure A. Structures of Nucleotides and Bases

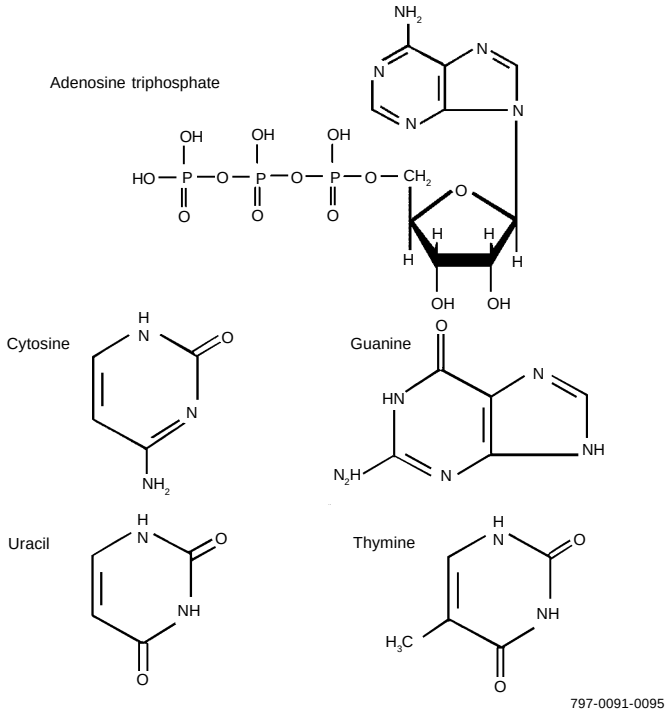

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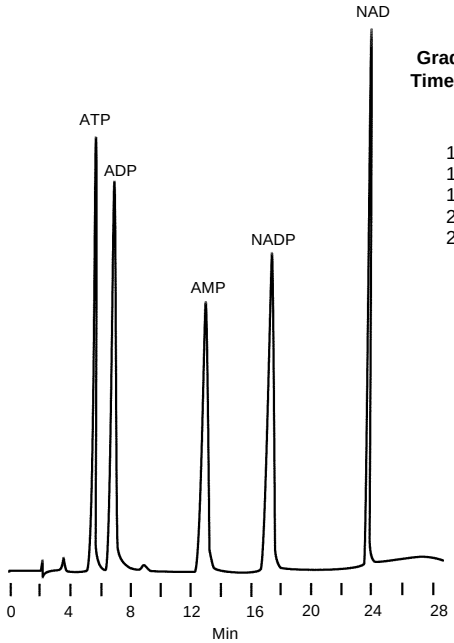
Figure B. Nucleotides on a SUPELCOSIL LC-18-T Column
(25cm x 4.6mm ID, 5µm particles)

Column: **SUPELCOSIL LC-18-T, 25cm x 4.6mm ID (5µm particles)** with Supelguard LC-18-T guard column
Cat. No. **58971**
Mobile Phase: A = 0.1M KH_2PO_4, pH 6
B = A:methanol, 90:10
gradient program shown on figure
Flow Rate: 1.3mL/min
Det: UV, 254nm



Gradient Program

Time (min)	% B
0	0
9	0
15	25
17.5	90
19	100
23	100
24	0

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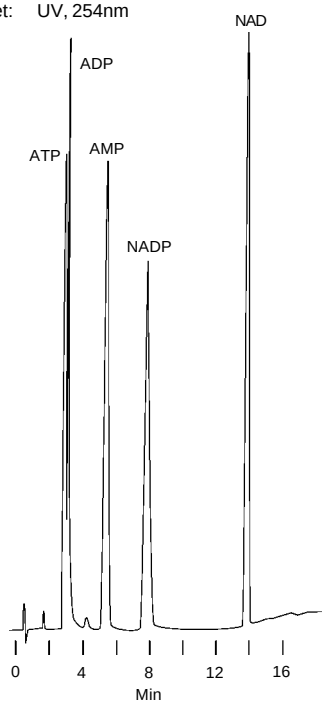
Figure C. Nucleotides on a SUPELCOSIL LC-18-T Column
(15cm x 4.6mm ID, 3µm particles)

Column: **SUPELCOSIL LC-18-T, 15cm x 4.6mm ID (3µm particles)** with Supelguard LC-18-T guard column
Cat. No. **58970**
Mobile Phase: A = 0.1M KH_2PO_4, pH 6
B = A:methanol, 90:10
gradient program shown on figure
Flow Rate: 2.0mL/min
Det: UV, 254nm



Gradient Program

Time (min)	% B
0	0
6	0
10	25
11.5	90
12.5	100
17	100
18	0

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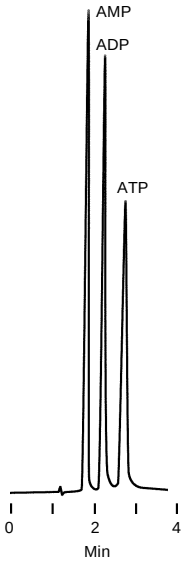
Figure D. Isocratic Ion Pair Separation of Nucleotides

Column: **SUPELCOSIL LC-18-T, 15cm x 4.6mm ID (3µm particles)** with Supelguard LC-18-T guard column
Cat. No. **58970**
Mobile Phase: A = 0.1M KH_2PO_4 + 4mM tetrabutylammonium hydrogen sulfate, pH 6
B = A:methanol, 70:30, pH 7.2
isocratic elution, A:B = 15:85
Flow Rate: 1.5mL/min
Det: UV, 254nm



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reverse order of that in Figure C. Note the analysis time is less than three minutes, which demonstrates the value of the 3µm particles for rapid analysis. We recommend a 15cm x 4.6mm, 3µm SUPELCOSIL LC-18-T column for gradient ion pair methods, because it will allow shorter equilibration time.

Figures E and F show gradient ion pair analyses of various nucleotide standards on 15cm x 4.6mm SUPELCOSIL LC-18-T columns containing 3µm particles. The elution pattern difference between Figures E and F, particularly for the ADP/GTP pair, is accounted for by the difference in the mobile phase. This shows the power of RP-HPLC in enabling you to fine tune a separation by using slight mobile phase adjustments. A real sample of nucleotides, obtained by alkaline extraction of whole blood, is shown in Figure G.

For maximum reproducibility and minimum equilibration time when using gradient ion pairing, keep the concentration of the ion pair reagent constant throughout the gradient. We recommend adding it in equal amounts to both mobile phase components. When working with biological extracts, or any sample that may contain insoluble matter, we strongly recommend using a Supelguard™ LC-18-T guard column to protect the analytical column.

Figure E. Gradient Ion Pair Separation of Nucleotides

Column: **SUPELCOSIL LC-18-T, 15cm x 4.6mm ID (3µm particles)** with Supelguard LC-18-T guard column
Cat. No. **58970**
Mobile Phase: A = 0.1M KH_2PO_4 + 4mM tetrabutylammonium hydrogen sulfate, pH 6
B = A:methanol, 70:30, pH 7.2
gradient program shown on figure
Flow Rate: 1.5mL/min
Det: UV, 254nm

Gradient Program

Time (min)	% B
0	0
2.5	0
5	30
10	60
13	0
17	0
18	100

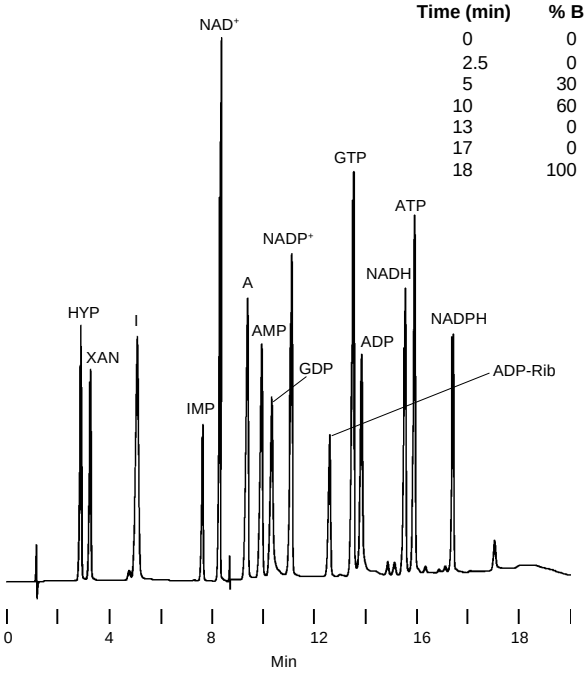

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Figure F. Gradient Ion Pair Separation of Nucleotides

Column: **SUPELCOSIL LC-18-T, 15cm x 4.6mm ID (3µm particles)** with Supelguard LC-18-T guard column
Cat. No. **58970**
Mobile Phase: A = 0.03M KH_2PO_4 + 10mM tetrabutylammonium hydrogen sulfate, pH 7.5
B = methanol
gradient program shown on figure
Flow Rate: 0.7mL/min
Temp.: 35°C
Det: UV, 254nm
Inj.: 10µL water containing 1mM each analyte

Gradient Program

Time (min)	% B
0	8.3
24	28.4

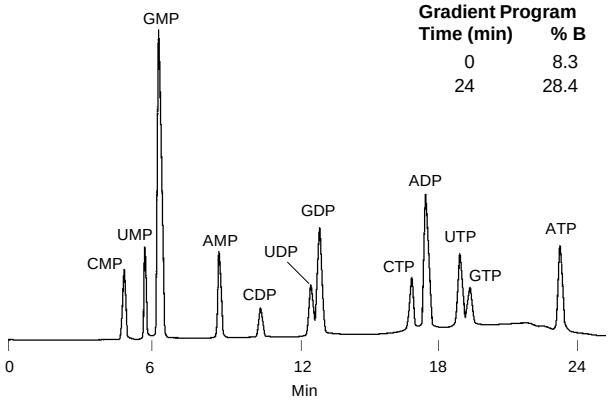

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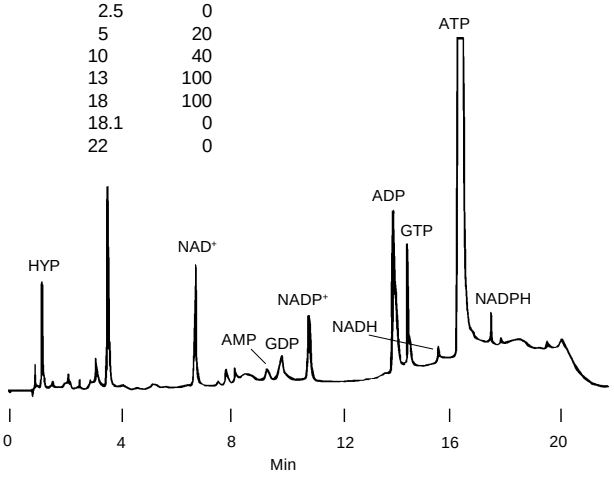
Figure G. Nucleotides from Whole Blood

Column: **SUPELCOSIL LC-18-T, 15cm x 4.6mm ID (3µm particles)** with Supelguard LC-18-T guard column
Cat. No. **58970**
Mobile Phase: A = 0.1M KH_2PO_4 + 4mM tetrabutylammonium hydrogen sulfate, pH 6
B = A:methanol, 70:30, pH 5.5
gradient program shown on figure
Flow Rate: 1.5mL/min
Det: UV, 254nm
Inj.: 50µL alkaline extract of whole blood

Gradient Program

Time (min)	% B
0	0
2.5	0
5	20
10	40
13	100
18	100
18.1	0
22	0



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Ordering Information:

Description	Cat. No.
SUPELCOSIL LC-18-T Modular HPLC Columns	
3µm particles	
15cm x 4.6mm ID	**58970**
15cm x 4.0mm ID	**58970-C40***
15cm x 3.0mm ID	**58970-C30***
5µm particles	
25cm x 4.6mm ID	**58971**
25cm x 4.0mm ID	**58971-C40***
25cm x 3.0mm ID	**58971-C30***
Supelguard LC-18-T Guard Columns	
2cm cartridge columns (5µm particles)	
For 4.6mm and 4.0mm ID analytical columns	
Kit (one cartridge, holder, connecting hardware)	**59620**
Replacement cartridges (pk. of 2)	**59621****
For 3.0mm ID analytical columns	
Replacement cartridges (pk. of 2)	**59621-C30****

*Requires reusable column endfittings, Cat. No. **5-5200**

Requires stand-alone (Cat. No. **5-9660) or integral (Cat. No. **5-5205**) guard column holder.

Other column dimensions available as custom products; please inquire.

For more information, or current prices, contact your nearest Supelco subsidiary listed below. To obtain further contact information, visit our website (www.sigma-aldrich.com), see the Supelco catalog, or contact Supelco, Bellefonte, PA 16823-0048 USA.

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