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Methods for the Separation and Quantitative Determination of Nucleotides, Nucleosides, and Bases

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The results from the methods for the separation and quantitative determination of nucleotides, nucleosides, and bases† have been classified and discussed. Special consideration has been given to those methods used the most in the last decade—thin-layer chromatography and different types of high-performance liquid chromatography such as ion-exchange, reversed-phase and ion-pair methods, as well as spectrophotometric and titrimetric methods.
The bibliography includes 180 references.

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I. INTRODUCTION

At the present time the separation of the components of nucleic acids is mainly carried out using liquid chromatographic methods such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC); and the number of published studies using these techniques accounts for more than 80% of all the methods used for this purpose.[1,2]

The separation and quantitative determination of components of nucleic acids are very important in different fields of biochemical research and medicine. Components of nucleic acids are to be found in physiological liquids, cells, and tissues.

The presence of these components, their metabolites, as well as modified bases, nucleosides, and nucleotides is used to assess the catabolism of nucleic acids, their fermentative breakdown, and different functional and oncological diseases. No less important is the determination of trace components in commercial preparations of nucleosides and nucleotides. Their use in molecular biology for the synthesis of genetic structures as well as in enzymology as substrates imposes strict requirements for the chromatographic homogeneity of the preparations. Nucleotide analysis is necessary for confirmation of the structure and determination of the quality of chemically synthesised oligonucleotides. A new area in which the analysis of components of nucleic acids is being used is the food industry. Recently, a method for the analysis (using HPLC) of purines and pyrimidines in milk to determine its quality has been posed.[3]

All this indicates that the choice of method and conditions for separation and quantitative determination of the components of nucleic acids are of vital importance at the present time.

In this review we have classified those studies we consider the most interesting during the last ten years on methods for separating bases, nucleosides, and nucleotides such as TLC and HPLC, have indicated the conditions that permit their separation in complex mixtures, focussed attention on their quantitative determination, especially using TLC, and have briefly discussed other analytical methods. The length of this review does not permit a discussion on the problems connected with the preparation of samples for analysis, but they have been reported in detail in two reviews[4,5] and in the literature cited.

II. THIN-LAYER CHROMATOGRAPHY

During the last ten years TLC has become a highly effective analytical method that has been successfully used to solve many problems in different branches of biochemistry, bioorganic chemistry, and molecular biology. The use of modern instruments for quantitative detection and elution, batching systems together with an improved plate quality and development of high-performance thin-layer chromatography (HPTLC) and continuous thin-layer chromatography (CTLC) methods have converted TLC from a qualitative to a quantitative method and have placed it on the same level as HPLC.[6-13]

At the present time TLC has gained universal recognition as a result of the speed of conducting analyses, the relative simplicity of the equipment, its economy and versatility; and with the development of HPTLC, which has considerable advantages over conventional TLC, it has become highly efficient, rapid, sensitive, and reproducible.[14-16] These advantages are achieved with the use of a thin layer of sorbent (silica gel) with uniform particle diameter of about 5 μm, and with new methods for feeding the eluent, the use of special U-chambers for chromatography, an improved method of applying samples, and the use of efficient systems for processing data. The standard deviation of the results in the determination of nanogram quantities of a compound using this method does not exceed 1.5–2.3%. With all these advantages of HPTLC it is possible to separate up to 20 compounds simultaneously over a period of a few minutes and to detect femtogram quantities (10^{-15} g) of compounds.[17]

During the last 5–7 years a new method, CTLC, has been developed, combining the analytical possibilities of TLC and column liquid chromatography.[18] In CTLC the solvent is fed continuously to a thin layer of sorbent with sample previously applied. During separation of the compounds the solvent and compound being

† Referred to as components of nucleic acids in the text.

analysed are continuously removed from the chromatographic system, and at the same time a continuous detection method is carried out. With repeated use of the same plate, constant conditions for separation, complete saturation in the CTLC separation chambers, and a uniform soaking of the sorbent layer it is possible to obtain results with a reproducibility that is better than traditional TLC almost by a factor of 10. Using the continuous method it is possible to separate complex mixtures of compounds (which are separated with difficulty or not separated at all by other methods), and it is a versatile liquid chromatographic method with a very extensive potential for analysis.

A new stage has appeared in the development of TLC—the extensive use of instrumental methods for the quantitative determination of results of separation on a thin layer of adsorbent as well as automation of the whole chromatographic process have started.[17-21] Quantitative analysis in TLC is composed of several stages: introduction of the sample, separation of the components on a thin layer of sorbent, and quantitative determination of the results of analysis. After separation of the compounds on the plate they can be extracted by one of the following methods: removal of the compounds together with the adsorbent layer followed by their extraction; direct extraction from the adsorbent layer bonded to a support with elution by the pumping of an appropriate solvent system through it; extraction of the compounds by addition to the adsorbent layer of a porous material into which they pass together with the solvent; and direct transfer of the compounds being analysed into the detection systems. The methods for quantitative determination of separated compounds can be subdivided into direct and indirect ones. The first group contains all methods that involve the extraction of compounds prior to their quantitative determination. The different methods for analysing extracts and the ranges of detectable quantities of the compounds are shown in Table 1.[22]

Table 1. Methods for the quantitative determination of compounds after their extraction from thin layers of adsorbent.[22]

Quantities determined	1–100 mg	10–100 mg	10–100 ng
Methods of determination	gravimetry, titration, polarimetry	spectrometry, (UV, IR, NMR), mass spectrometry, isotopic methods, GLC refractometry	fluorescence, phosphorescence, biological methods

Extraction of the compounds being analysed together with removal of the adsorbent is carried out manually or by means of automated systems. The adsorbent zone is removed by suction into a suitable tank, from which the compound to be analysed is extracted. For the determination of microquantities of compounds, the adsorbent that is withdrawn into a microtube with solvent is separated by centrifuging or filtration through suitable filters, after which the eluate can be used directly for detection. The volume of eluate usually amounts to 50 – 100 μl and the degree of extraction to more than 90%.[23]

Direct extraction of the compounds being analysed from the adsorbent layer is carried out by preserving the integrity of the layer in the zone itself. The simplest version of this method is one which involves cutting of chromatograms on a flexible support into strips, which are placed in vessels with a solvent to extract the compounds being analysed.[24] A more complete solution to this method consists of the construction of an airtight apparatus and pumping suitable solvent systems through the strips around the zone containing the compound to be analysed. The eluate is then passed to a detection system. The total quantity of solvent passed through varies from 1 to 2 ml and the standard deviation of the results of analysis vary from 0.2 to 1.3% depending on the quantity of compound being analysed.[25] The use of this method is limited by the need for efficient separation of the compounds being analysed.

Simple methods for the direct elution of the separated compounds into microvolumes of solvent (40 – 100 μl) involve the pumping of a solvent through the adsorbent layer containing compound, injecting it on to strips with adsorbent bonded to filter paper, and preliminary concentration followed by elution.[26,27]

When the latter version is used the standard deviation of the results amounts to 0.005 – 0.01 μg.[27] The compounds extracted are detected using spectrophotometric, fluorometric, or radiochemical methods.

The direct quantitative determination of compounds on a chromatogram can be carried out by measuring the area of a spot or its colour intensity. However, this type of determination is associated with significant errors and is only suitable for reference measurements. More accurate methods require the use of special instruments. All the methods of this group are based on the principle of photometry, densitometry being used most frequently followed by spectrophotometric, fluorometric, and radiochemical methods, which have high sensitivity and made identification and quantitative determination possible for a large number of different compounds.[28-32] A comparison of optical methods for the quantitative determination of results from the separation of mixtures is presented in Table 2.[22] Other instrumental methods for the quantitative determination of the results have similar features to the optical methods.

Table 2. Comparison of optical methods for the quantitative determination of results.[22]

Method	Standard deviation, %	Required quantity of compound, μg	Time of analysis, min
Visual comparison	10—30	1—10	0.1
Extraction from layer followed by optical determination	2—5	10—100	20
Optical scanning based on measurements of:			
absorption of radiation	1.5—2.5	0.1—10	2
fluorescence	1—2	10^{-1}—10^{-1}	2

At the present time special equipment used for optical scanning in TLC is still not easy to obtain, as a result of which its widespread application in the laboratory has been somewhat restricted. The most widely used methods of optical scanning have certain limitations—they have insufficiently high chemical selectivity and a restricted area of application. There is a linear dependence of the signal on the content of compound in the zone over a narrow region

of measurement. For this reason the use of these methods for determining impurities at levels of less than 1–2% in preparations of nucleic acid components is virtually impossible.

In optical scanning the nature of sample application, the volume applied, separation, the shape of the spot, and the flow length are decisive factors in obtaining quantitative results. In order to reduce the detection limits, measurements should be made in a transmission system and not a reflection system. With strict adherence to specific conditions the standard deviation of the results of analysis is 1%.

In this connection it is demonstrated that TLC under pressure is a promising method for separating multicomponent mixtures of compounds with similar properties by quantitative scanning.

Radiochemical methods of detection in TLC are characterised by high sensitivity and good reproducibility. Radiochromatograms are evaluated by different methods—directly on the adsorbent layer as well as after extraction of the compound from the layer or by preliminary removal of sections of adsorbent together with the compound being separated. The removal of adsorbent for the analysis of radioactive compounds is carried out using mechanised and automated equipment. The equipment is based on the mechanical scraping off of sorbent and its transfer to the extraction chamber, where desorption of the compounds being determined takes place. The eluate passes through a filter system to collectors and for measurement. The equipment is suitable for the simultaneous analysis of several samples. This method can be used for chromatograms on which the zones of compounds are separated from each other by 2–6 mm.

The detection limit using a radiochemical method is $10^{-7}-10^{-4}$ Ci and depends on the energy of the elementary particles formed by decay of nuclei, the conditions of chromatographic analysis, and the features of the detection system. The standard deviation of the results is 1%. When the samples are analysed there is a linear relationship between the quantity of compound being analysed and the signal of the detection system over a wide range of measurements.[17]

The choice of stationary phase in TLC is decisive in achieving the required selectivity in the separation of mixtures to be analysed. The necessity for highly efficient chromatographic separation presents specific requirements for the choice of adsorbent. Cellulose modified with polyethyleneimine and silica gel have been widely used for the separation of complex mixtures of nucleic acid components. The use of plates with a sorbent layer applied in advance leads to a considerable simplification in the TLC method. Commercial plates provide a better reproducibility of results than with plates prepared in the laboratory because of the high degree of uniformity in composition and thickness of the sorbent. Several firms produce silica gel and polyethyleneimine (PEI)-cellulose plates that are suitable for separation of the components of nucleic acids.[33] The most convenient material as a support is aluminium foil, which can be bent, cut, and made into plates of the required shape. The efficiency of separation depends on the grain size, specific surface area, pore size, and moisture content.[12]

Good separation of compounds occurs on PEI-cellulose when the content of PEI is 0.5–1% and with low pH values of the eluent.[34] The mobility of each product is determined by the magnitude of negative charge, which depends on the pH of the eluent. Boric acid systems are used to separate deoxyribo- and ribo- derivatives. Borate complexes of ribonucleotides have an additional negative charge and have lower mobility than deoxyribo- derivatives. Multistep or gradient elution with salt solutions of varying concentration is used to separate compounds according to the number of their phosphate groups.[35–39] The separation of uncharged compounds—purine and pyrimidine bases and nucleosides—is carried out on unmodified cellulose or silica gel.

Silica gel is used to separate different mixtures of components of nucleic acids. The selection of suitable solvent systems permits separation with respect to both the type of nitrogenous base and the type of carbon residue.[40–44] Good separation of complex mixtures containing 5′- and 5′-poly-phosphates of purine nucleosides occurs with the use of systems containing ammonia as a strong base, which prevents the formation of hydrogen bonds between the amino groups of the compounds being analysed and silica gel. For the separation of nucleoside 5′-polyphosphates, acidic systems containing isobutyric acid are the most suitable; for nucleosides the most suitable are "reversed-phase" systems having a high concentration of salts such as $(NH_4)_2SO_4$ and NaCl.

For the separation of components of nucleic acids silica gel has been successfully used in recent years in HPTLC, which is characterised by highly efficient separation and a low detection limit for compounds. With the use of adsorbents such as aminopropylated silica gel, which has anion-exchange properties, and silica gel modified with hydrocarbon radicals, a number of studies have been carried out on the separation of components of nucleic acids.[45–53]

The best results on the separation of complex mixtures of nucleic acid components that differ according to the type of nitrogenous base, hydrocarbon residue, and number of phosphate groups have been obtained using two-dimensional chromatography and mainly on PEI-cellulose.[54–57] Some data on the separation of different mixtures of components of nucleic acids are presented in Table 3.

III. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The progress made using HPLC is due to its high resolving power, mild conditions for separation, and efficient design of apparatus. The use of high-pressure chromatographs with high-sensitivity detectors, new types of sorbent, and also the design of spectrophotometers that permit ultramicroanalysis to be carried out have contributed towards providing HPLC with a lead over all other methods in its use for the separation of components of nucleic acids and has made it possible to separate effectively microgram and nanogram quantities of compounds in a short time.[61–68]

A general tendency in HPLC is the change-over to the use of chemically modified silica gels as sorbents with particle diameter of 5–10 μm and even 3μm. Detection is carried out using fluorescence, UV-photometric, electrochemical, or atomic emission detectors,[68–77] while identification of the components can be carried out using a combination of HPLC and mass spectrometry.[78–80]

For the separation of components of nucleic acids, those forms of HPLC such as ion-exchange chromatography, reversed-phase chromatography and ion-pair reversed-phase chromatography are used almost exclusively. Adsorption ("direct-phase") HPLC is used at the present time only in isolated cases.[81]

1. Ion-exchange High-performance Liquid Chromatography

Ion-exchange chromatography (IEC) is a traditional method for separating the components of nucleic acids.[34,65,82] In ion-exchange chromatography, separation of the compounds is based on the difference in charges on their molecules; hydrophobic interactions also play a definite part. At the present time a large number of different ion exchangers for HPLC are produced. Generally, these

Table 3. Separation of the components of nucleic acids by thin layer chromatography.

Components separated	Adsorbent	Solvent systems	Note	Refs.
Bases, deoxyribonucleotides deoxyribonucleosides	silufol (Kavalier, Czechoslovakia)	dioxan–isopropanol–2 M ammonia–1 M ammonium acetate (3:2:4.7:0.3); dioxan–isopropanol–5 M ammonia–1 M ammonium acetate (3:1.8:4.9:0.3)	–	[44]
Bases, deoxyribonucleosides	silufol (Kavalier, Czechoslovakia)	dioxan–ammonia (7:3); isopropanol–ammonia (8:2); dioxan–isopropanol–ammonia (4:4:2)	radiochemical detection	[44]
	RP-silica gel C_{12} (Kodak, USA)	1.5 mM TBA + 5% methanol; 0.4 M ammonium phosphate, pH 3.5; 0.1 M ammonium acetate, pH 6.9 + 5% acetonitrile		[51]
Ribonucleotides	NH_2-silufol	1 M acetic acid–ethanol (10:1), pH 3.2	–	[52]
Bases, ribo- and deoxyribo-nucleosides and nucleotides	silica gel GF-254 (HPTLC), (Merck, FRG); PEI-cellulose (Merck, FRG)	Chloroform–methanol–acetic acid (3:2:1); chloroform–methanol–water (81:15:4)	nucleotides remain at start	[58]
		Chloroform–methanol–acetic acid (3:2:1) in 0.1 M boric acid		[58]
Ribo- and deoxyribo-nucleotides	PEI-cellulose (Merck, FRG)	0.25 M acetic acid (9 cm), 0.8 M formic acid (4 cm)–direction 1; 0.22 M Tris-HCl, pH 8.0, in methanol (10 min), methanol (10 min)–direction 2	two-dimensional chromatography	[59]
Ribonucleotides	PEI-cellulose (Merck, FRG)	direction 1–0.2 M LiCl (2 min), 1 M LiCl (6 min) 1.6 M LiCl (75 min); 0.5 M sodium formate, pH 3.4 (0.5 min) 2 M sodium formate, pH 3.4 (2 min), 4 M sodium formate, pH 3.4 (60 min)–direction 2	two-dimensional chromatography	[59]
Ribonucleoside 5′-mono-, 5′-di-, and 5′-tri-phosphates	silufol (Kavalier, Czechoslovakia)	dioxan–isopropanol–water–ammonia (4:2:4:1); dioxan–water–ammonia (6:4:1)	radiochemical detector	[43]
		dioxan–isopropanol–ammonia–1 M ammonium acetate (3:2:4.7:0.3)		[44]
	NH_2-silica gel (HPTLC), (Merck, FRG)	0.2 M NaCl in a mixture of: methanol–water (3:7); 0.2 M NaCl mixed with methanol, ethanol, and chloroform		[47]
Deoxynucleoside 5′-mono-5′-di- and 5′-tri-phosphates	NH_2-silufol	1 M acetic acid–ethanol (10:1), pH 6.5	radiochemical detector; two-dimensional chromatography	[52]
	NH_2-silufol	1 M acetic acid–ethanol (10:1), pH 6.5		
Ribo- and deoxyribo-nucleoside 5′-triphosphates	PEI-cellulose (Merck, FRG)	direction 1–1 M LiCl with 5% boric acid, pH 7.0, washing with methanol, 2 M sodium formate (pH 3.4)–1.6 M LiCl (1:1) at 4 °C; 0.85 M potassium phosphate, pH 3.4–direction 2	"	[60]
Ribo- and deoxyribo-nucleosides	cellulose (Merck, FRG)	0.2 M; 2 M; saturated solution of $(NH_4)_2SO_4$	"	[35]

are either modified copolymers of styrene with divinylbenzene containing a relatively high proportion of the latter or supports based on microporous silica gels.[83] Anion exchangers usually contain quaternary ammonium groups (Partisil-10 SAX, Ultrasil-10 AX, Lichrosorb AN, etc.), less often they contain tertiary amino groups (Nucleogen DEAE). Quite often aminopropylated silica gel (APS-Hypersil, Lichrosorb-NH_2) is used as an anion exchanger. The advantage of ion exchangers based on silica gel is the highly effective separation and short analysis time achieved by high rates of mass exchange, although the stability of these sorbents is not high and the operating pH range for the mobile phase is from 2 to 7.5.[59–63,83–85] Cation exchangers most frequently contain alkylsulphonate groups (Partisil-10 SCX, Ultrasil-10 CX). In recent years ion-exchange derivatives of rigid hydrophilic resins such as Spherone, TSK-Gel-PW,[84] and others have been used quite frequently.

Anion-exchange chromatography is widely used for the analysis of nucleotides, in particular, nucleoside polyphosphates.[86–97] Gradient elution is more frequently used. Although this permits separation of more than 20 compounds over a short period of time, much time is spent in regenerating the column after analysis. Moreover, the salt concentration gradient leads to a drift in the base line of the UV detector. The drift can be compensated by the addition of a calculated quantity of acetone to the initial buffer or by partial replacement of phosphate by chloride, inclusion of which in the mobile phase can, however, lead to corrosion of the chromatographic equipment.[98–100]

An isocratic system of separation does not impose such high requirements of purity for the reagents being used and gives better reproducibility of results than with gradient elution. A disadvantage of isocratic chromatography is the appreciable width of peaks with large K' values for retention of the chromatographic zone, which reduces the sensitivity and accuracy of analysis.[101,102]

The order of elution of nucleotides during anion-exchange HPLC is not always easy to interpret. Perhaps the only rule that has no exceptions is the order of elution of nucleoside polyphosphates: NMP, NDP, NTP.‡ It is a more complex problem with isomeric nucleoside monophosphates and with the separation of ribo- and deoxyribo-nucleotides. Thus, Nguyen and co-authors[102] observed the following orders of elution (column with Partisil-10 SAX sorbent, 3% MeOH in 0.08 M phosphate buffer as eluent, pH 4.15):

1) CMP, UMP, AMP, GMP
2) dCMP, TMP, dAMP, dGMP
3) 2′-UMP, 2′-AMP, 2′-CMP, 2′-GMP,
4) 3′-UMP, 3′-CMP, 3′-AMP, 3′-GMP
5) dCMP, CMP, 3′-CMP, 2′-CMP
6) UMP, 2′-UMP, 3′-UMP
7) AMP, dAMP, 3′-dAMP, 2′-AMP, 3′-AMP
8) 3′-dGMP, GMP, dGMP, 3′-GMP, 2′GMP.

‡ Abbreviations for the components of nucleic acids are given in accordance with the IUPAC–IUB recommendations (Molekulyar. Biolog., 1972, **6**(1), 167–171).

As can be seen, the pattern is very complex. It is clear that hydrophobic interactions do not play a conclusive role. Although adenine is protonated to a considerable degree at pH 4.15, adenine nucleotides are eluted after UMP and TMP. At lower pH values, however, AMP "overtakes" UMP, but the order of UMP and GMP is retained.

Bases and nucleosides are separated on anion exchangers as a result of hydrophobic interactions. Non-ionic interactions with the support are partly suppressed by an increase in temperature and the addition of organic solvents to the mobile phase.[103,104] With an isocratic system it is possible to separate bases, nucleosides, and nucleotides simultaneously.

Recently, the need to speed up and simplify analyses and also the necessity for analysing very complex samples containing bases, nucleosides, and their mono- and poly-phosphates as well as UDPG, NAD, NADP, and related compounds have led to a certain decline in interest in anion-exchange chromatography and have stimulated research in the field of ion-pair RPC (see below). Combined chromatographic systems that consist of two anion-exchange and reversed-phase columns are also capable of separating such mixtures effectively, but the method of analysis in this case is considerably more complex.[105,106]

As regards cation-exchange chromatography, this method has been used traditionally for the analysis of bases. Taking into account the fact that at low pH values the charge of bases differs by no more than one, separation can readily be carried out in an isocratic system. Examples of the separation of components of nucleic acids are given in Table 4.

2. Reversed-Phase Chromatography

Chromatography in which the eluent is more polar than the stationary phase is known as reversed-phase. Usually the stationary phase is in the form of a silylalkyl ether, while aqueous buffer solutions and/or their mixtures with polar organic solvents are used as the mobile phase.[108] The widespread use of RPC is due to a whole number of advantages:[108,109] a) compounds of very diverse classes can be separated, and compounds that have marked differences in polarity and structure can be separated in one chromatographic cycle (for example, a mixture of nucleosides and their phosphates); b) the method has high efficiency and selectivity; c) the columns are rapidly regenerated; d) the method can be used to analyse very dilute samples with a large volume; e) the supports are commercially available.

The following disadvantages of the RPC method should be mentioned:[108] a) the stationary phases are only stable over the pH range 2–7.5; b) interaction of the molecules being separated with the stationary phase has a composite mechanism because of the presence of residual silanol groups (Si–OH) on the surface of the stationary phase; c) the retention mechanism of compounds in RPC has been insufficiently studied. Evidently these shortcomings are not fundamental problems and it is nearly always possible to select the optimum combination of stationary and mobile phases for the resolution of any given task.

Microporous silica gels with chemically bonded hydrocarbon groups, most frequently n-octadecyl (C_{18} or ODS) or n-octyl groups (C_8), are used in RPC as stationary phases.[109] In order to obtain them, silica gels are usually treated with alkylsilyl halides. However, because of steric hindrance, up to 30% of the silanol groups present on the surface of the silica gel remain unmodified; a limited portion of them can be removed by repeated treatment, with trimethylsilylating agents, and by "capping".[109–111] The properties of the stationary phases depend on the type of grafted ligand, the concentration of alkyl and available silanol groups on the surface, the characteristics of the initial silica gel, the specific surface area, the specific pore volume, etc.[110–120]

In one review[108] there is a list of more than 70 supports, which were being produced by over 50 firms. It may be assumed that by now the number of supports being produced has increased even further.

Table 4. Examples of the separation of components of nucleic acids by ion-exchange HPLC.

Sample No.	Type of sample	Support*; column dimensions, mm	Composition of mobile phase and type of elution	Order of elution of sample components	Complete analysis time, min	Refs.
1	synthetic mixture	TSK gel K-anion-BW (9 μm); 0.19×450	30 mM $(NH_4)HPO_4$, pH 7.15	Cyd < Urd < Cyt < Ura < Guo < Ado < Gua < Ade < CMP < UMP < 2′-CMP < 3-CMP < 2′UMP < 3′-UMP < GMP < AMP < 2′-GMP < 2-AMP < 3′-GMP < 3′-AMP	60	[87]
2	synthetic mixture	Partisil 10-SAX (10 μm); 4.6×250	Gradient up to 0.3 M KH_2PO_4 and 0.3 M KCl, pH 3.3 → 4.9	CMP < AMP < UMP < IMP < GMP < UDP < CDP < ITP < CTP < ATP < GTP < NADH < NADPH < UDPG	~40	[94]
3	cellular extracts	APS-Hypersil (5 μm); 5.0×100	Gradient up to 0.5 M KH_2PO_4 and 0.8 M KCl, pH 2.9	XMP < CMP < AMP < NAD^+ < TMP < IMP < GMP < CDP < UDPG < ADP < dADP < TDP < GDP < CTP < ATP < dATP < GTP	~15	[95]
4	rat liver extract	Lichrosorb-NH_2 (5 μm); 4.6×150	0.1 M K_2HPO_4, 0.1 M NH_4Cl, 20% MeCN, pH 6.5	AMP < GMP < $NADP^+$ < ADP NADPH < GDP < ATP < GTP	20–25	[106]
5	DNA hydrolysates from fibroblast cultures	Ultrasil-10CX (10 μm); 4.6×250	0.02 M $(NH_4)H_2PO_4$, pH 2.3	Thy < Gua < Cyt < Ade < m^5Cyt	~15	[107]

* Particle diameter given in brackets.

The lack of information on their method of synthesis and properties produces a certain difficulty in operating with commercial supports. It is therefore not surprising that a comparative study of the chromatographic properties of commercial supports has been the subject of a large number of studies, the results of which can be reduced to the following principles:[121-129]

1. In the RPC of nonpolar compounds the selectivity of separation does not depend very much on the type of support and the efficiency also does not vary significantly.

2. The separation of compounds containing polar groups is affected not only by the type of support but also by the difference in batches of supports of a single type. In this case asymmetry and/or broadening of the chromatographic peaks often occur, which is usually due to the presence of available silanols on the surface and its contamination with heavy metals.

3. Supports with a high content of residual silanols (Nucleosil-C_8, Partisil-ODS2, Partisil-ODS3) have inferior stability in aqueous salt mobile phases compared to capped supports (Nucleosil-C_{18}, Partisil-CDS_3, CCS-C_8, Ultrasil-ODS, and others) because of the more rapid dissolving of the silica gel matrix and the possible irreversible adsorption of different compounds.

Reversed-phase chromatography is a most important and widely used method for separating the components of nucleic acids that is based on the high selectivity of nonpolar supports with respect to heterocyclic bases.[130-140] In studies on the effect of RPC parameters on the separation of components of nucleic acids it was found that the content of the organic solvent in the mobile phase does not alter the selectivity of separation but only affects the retention; the pH and ionic strength can affect the selectivity of separation.[129-131]

Bases and nucleosides can be separated using RPC with low concentrations of organic solvents. In order to suppress nonspecific interactions a buffer (usually phosphate, pH 4–6) is added in low concentration (10–50 mM) to the eluent. As can be seen from Table 5, bases are eluted first, then the corresponding nucleosides, and finally deoxynucleosides, with purines usually being retained to a greater extent than pyrimidines. In solving frequent problems the analytical method can be simplified by varying the composition of the mobile phase. Thus, Fraser and Pearson[141] carried out the separation of a mixture at pH 2.5. In this case dU and T were effectively separated, while dA, dG, and dC, which were not of interest to the authors, were hardly retained at all on a reversed-phase column.

Clinical samples and also hydrolysates of tRNA can contain up to 20 minor nucleosides, so analysis of such complex samples requires the use of gradient elution (Nos.1–3, Table 5). The problems associated with the analysis of minor nucleosides in the clinic have been examined in detail in two reviews[4,5] and in one study[133] where the authors succeeded in separating and determining quantitatively more than 20 nucleosides in samples of physiological origin. Elution was carried out in a multistage MeOH gradient and the analysis time amounted to over 100 min. For the analysis of a less complex sample the same authors used isocratic elution; the analysis time was reduced by a factor of nearly 15. Examples of the separation of bases and nucleosides are given in Table 5.

The presence of a negatively charged phosphate group makes nucleotide molecules more hydrophilic than molecules of nucleosides and bases, so that nucleotides are retained to a lesser degree on reversed-phase columns. Mobile phases without organic solvents are used for the separation of nucleotides; the pH is most frequently about 4–5, which suppresses the ionisation of a second phosphate group, and the concentration of buffer is increased considerably in comparison to the RPC of nucleosides.

An increase in the ionic strength of the mobile phase leads to an increase in retention time of nucleotides to acceptable values during RPC (this effect has been studied in detail[97,129,130]), while at the same time because of the increase in inherent UV absorption of the mobile phase the sensitivity of the detector is reduced.

Nucleoside polyphosphate molecules are even more hydrophilic. The order of elution for polyphosphates (NTP < NDP < NMP) in RPC is the reverse of that in anion-exchange chromatography.

When the simultaneous separation of nucleotides and nucleosides in the mobile phase is required, methanol must be added and gradient elution carried out.[147-150]

Examples of the separation of nucleotides are given in Table 6.

Table 5. Examples of the separation of bases and nucleosides by reversed-phase HPLC.

Sample No.	Type of sample	Support; column dimensions, mm	Composition of mobile phase and type of elution	Order of elution of sample components	Complete analysis time, min	Refs.
1	clinical analyses	Ultrasphera-ODS (5 μm); 4.6 × 150	MeOH gradient, 10 mM KH_2PO_4, pH 4.9	Hx < dCyd < Ado < Ino < dGuo < Thd < Ade < dAdo	25	[142]
2	clinical analyses	Hypersil-ODS (3 μm); 4.6 × 50 + 4.6 × 150	MeOH gradient, phosphate buffer, pH 6.0	Cyt < Ura < Cyd < Hx < Xan < Urd < Ade < Ino <	~20	[143]
3	tRNA hydrolysates or tissue extracts	μ-Bondapak C_{18} (10 μm); 4.0 × 300	MeOH gradient, phosphate buffer, pH 5.1 (36 °C)	Guo < dUrd < dCyd < Thd < Ado < dAdo Cyd < Urd < m^1Ado < m^5Cyd < m^7Guo—about 15 nucleosides in all	35-40	[144]
4	DNA and RNA hydrolysates	Supelcosil C_{18}-DB (5 μm); 4.6 × 150	8% MeOH in 0.05 M KH_2PO_4, pH 4.0	Cyd < dUrd < dUrd < m^5dCyd < Ino < Guo < dIno < dGuo < dThd < Ado < dAdo	7	[145]
5	human placental extract	Micropak CH-10 (10 μm); 4.0 × 300	MeOH gradient, phosphate buffer, pH 6.5	Xam < Hx < NAD^+ < Ino < Ado < Ade	~20	[146]

Table 6. Examples of the separation of nucleotides by reversed-phase HPLC.

Type of sample	Support, column dimensions, mm	Composition of mobile phase and type of elution	Order of elution of sample components	Complete analysis time, min	Refs.
Bacterial cellular extracts	μ-Bondapak C_{18} (10 μm); 4,0×300	0,2 M $(NH_4)_2HPO_4$, pH 5,1	GTP < GDP < ATP < ADP < AMP	<20	[119]
Extracts of erythrocytes, hepatocytes, and different tissues	LiChrospher 100 CH-18 (5 μm); 4,0×250	Phosphate buffer, pH 6.0; gradient up to 7.5% MeCN	GTP < GDP < GMP < IMP < ATP < ADP < Urd < AMP < $NADP^+$ < NAD^+ < Guo < Ado	30—40	[131]
Products of the chemical phosphorylation of nucleosides	Ultrasphere-ODS (5 μm); 4,6×250	10% MeOH, 0,05 M $(NH_4)H_2PO_4$	AMP < 2′-AMP < Ade < 2′, 3′-cAMP < 3′-AMP < 3,5′-cAMP < Ado	10—20	[140]
DNA and rat liver hydrolysates	Spherisorb-ODS (10 μm); 4,6×500	0.5 or 0.75 M Na_2HPO_4, pH 5,5	dCMP < m^5dCMP < TMP < dGMP < dAMP	~125	[147]
Human liver extracts	Spherisorb-ODS (10 μm); 4,6×500	25 mM NaH_2PO_4 pH 5,5	ATP < ADP < AMP < $NADP^+$ < NAD^+	120	[151]

3. Ion-Pair Reversed-Phase Chromatography

Ion-pair (IP) reversed-phase chromatography (RPC) is a form of RPC used to separate compounds that dissociate in aqueous organic solvents. Compounds containing lipophilic groups capable of forming ion pairs with ions of the opposite charge from the components of the sample being analysed are added to the mobile phase; the compounds are sodium dodecylsulphate, quaternary ammonium bases or zwitterions.[152–154]

As has already been stated, nucleotides, in particular nucleoside polyphosphates, are only weakly retained on reversed-phase sorbents because of their low hydrophobic capacity. There are two methods of increasing the retention of ionised molecules during RPC. Firstly, chromatography can be conducted with that pH of eluent at which the acid (or basic) dissociation of the molecules of compound being separated is insignificant. This method, often called "ion suppression",[155] in relation to nucleotides is to a certain extent used in those studies that were discussed in the previous section. Secondly, nucleotides are suitable candidates for IP RPC; in this case an increase in retention of the ions is achieved by introduction of hydrophobic ions of the opposite charge into the mobile phase.

A number of studies[155-157] have been devoted to studying the mechanism of IP RPC. We can list here the main conclusions of these investigations:

1. The presence of hydrophobic ions in the mobile phase strengthens the adsorption of ions of the opposite charge. The more hydrophobic the ion, the greater is the increase in K' for the counter-ion. Consequently, the effect occurs at lower concentrtions for a more hydrophobic ion. Thus, addition of 2.5 mM TBA to the mobile phase increases K' for the nucleotides to a greater extent than addition of 5 mM TEA or 20 mM TMA.[158]

2. The dependence of K' on the concentration of hydrophobic counter-ion for ionic components of a mixture most frequently follows a bell-shaped curve; nucleotides are no exception to this rule.[159] The position of the K' maxima varies somewhat for different compounds under the same conditions. As a rule, the concentration of ion-pair agent is selected in the region of K' maxima of the components of the mixture being separated.

3. The presence of hydrophobic ions in the mobile phase slightly reduces the retention of nonionic compounds; for example, addition of TBA to the eluent reduces the K' values of nucleosides.[159]

4. As in conventional RPC, an increase in the concentration of organic solvent decreases the retention time of both ionic and non-ionic components in IP RPC.

5. An increase in the concentration of salts reduces the retention time of ionic components in IP RPC.

6. The relationship between retention times and pH in IP RPC is the reverse of that with ion suppression; the retention time is longer if the eluate molecules are ionised and capable of forming an ion air. Thus, at pH 3.0 addition of TBA increases K' for TMP and UMP and has no effect on K' for CMP and AMP, which at pH 3.0 are zwitterions; at pH 5.7 TBA increases K' for all nucleotides.[160–162]

We should mention that the actual mechanism of IP RPC has not so far been established. Hypothetical "ion-pair", "dynamic ion-exchange", and "ionic-interaction" mechanisms have been discussed in one review.[155]

Some examples of the analysis of components of nucleic acids using IP RPC are given in Table 7. We have restricted ourselves here to five examples but the number of studies on the IP RPC of nucleotides is very considerable.[152,163–169]

The order of elution of nucleoside polyphosphates is the same as that in anion-exchange chromatography. It is interesting to note that in 2.5 mM TBA the order of elution of mononucleotides also corresponds to the order of elution in anion-exchange chromatography at low pH. In 20 mM TMA, UMP is eluted after CMP, as in normal RPC.

In all of the examples in Table 7 apart from No.4, an isocratic system of elution was used, although in some cases nucleoside polyphosphates with considerable different molecular charges were separated, in other cases nucleotides and nucleosides were separated. On the whole, gradient elution is used less often in IP RPC than in ion-exchange chromatography or in RPC for mixtures of nucleosides and nucleotides, which is a definite advantage.

As was mentioned above, for the analysis of very complex samples either quite elaborate systems combining IEC and RPC are used or IP RPC is used, and this is illustrated by example No.4 in Table 7.

Table 7. Examples of the separation of components of nucleic acids by IP RPC.

Sample No.	Type of sample	Support; column dimensions, mm	Composition of mobile phase and type of elution	Order of elution of sample components	Complete analysis time, min	Refs.
1	Various tissue extracts	Lichrosorb RP-8 (RP-18); 4,0×250	0.3% TBA; 0.65 KH_2PO_4, pH 5.8; 240 ml MeCN, water up to 1100 ml	AMP < ADP < GTP < UTP < ATP	~10	[155]
2	Various tissue extracts	Ultrasphere ODS (5 μm); 4,6×250	2,5 мМ TBA; 12% MeCN pH 2,5 (HCOOH)	CMP < AMP < GMP < UMP < IMP < XMP	~30	[158]
3	Hydrolysates of chemically synthesised oligonucleotides	Partisil-5C_8; 5 (μm) 4,6×250	2.0 мМ TBP; 0.005 М KH_2PO_4, pH 4,9; 7% MeOH	dCyd < dCMP < dGuo < Thd < dGMP < TMP < dAdo < dAMP <	~60	[159]
4	Various mixtures	Servachrom ODS-S: 100; 4,6×250	5.0 mM TBA; phosphate buffer, pH 6.0; gradient up to 10% MeCN	Cyd < Urd < CMP < Guo < UMP < GMP < Ado < CDP < AMP < UDP < GDP < CTP < ADP < UTP < GTP < ATP	~60	[170]
5	DNA hydrolysate	Ultrasphere-C_8 (5 μm); —	10 mM hexanesulphonate; 5% MeCN; 10 mM phosphate buffer, pH 2.9	Ura < Hx < Guo < Cyt < m^5Cyt < Ade	10	[171]

Although the separation in example No.4 is not so impressive as in two studies,[105,106] it was obtained in a considerably simpler system, and it may be assumed that separation of a 16-component mixture is within the limits of IP RPC, especially when gradient elution is used.

Finally, nucleic bases can also be separated at low pH values under conditions of IP RPC by introduction of suitable components to the mobile phase (see, examples No.5, Table 7 and No.5, Table 4).

Thus, ion-pair chromatography is the most promising method of HPLC for the analysis of nucleotides or their mixtures with nucleosides.

IV. OTHER METHODS FOR DETERMINING THE COMPONENTS OF NUCLEIC ACIDS

In the last 8–10 years there has been a development of methods for potentiometric titration of components of nucleic acids based on the acid-base properties of purine and pyrimidine bases, methods based on the selective oxidation of the *cis*-1,2-glycol group of the ribose part of ribonucleosides and their phosphates, and methods based on colour reactions of purine bases and the carbon part of the nucleosides.

For the separate analysis of multicomponent mixtures of nucleic acid components, differential alkalimetric or acidimetric titration in the medium of organic solvents is used without requiring preliminary separation of the different purine and pyrimidine derivatives.[172-174]

Potentiometric titration methods are specific and accurate. The disadvantages in using them relate to the large consumption of samples (20–200 mg) and the impossibility of determining impurities that constitute less than 10%.

In order to make a selective determination of ribonucleosides and their phosphates, methods for the oxidation of the *cis*-1,2-glycol group with periodate are used with subsequent titrimetric or colorimetric analysis of the iodate formed by oxidation of the compounds being analysed.[175-176]

The colour reactions used to determine the components of nucleic acids are based on the following properties: adenine is diazotised to give azo compounds with α-naphthylamine; guanine is reacted with Folin's reagent; purine nucleotides form coloured ribose compounds with orcein and deoxyribose compounds with diphenylamine.[177-180]

Methods for oxidation of the *cis*-1,2-glycol group in ribose and the colour reactions which are used to determine the components of nucleic acids are selective and sensitive and are used for the analysis of a specific class of compounds, but they are not suitable for the differentiated determination of nucleic acid components within this class.

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