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(54) Title: IMPROVED LIQUID CHROMATOGRAPHIC MEDIA FOR POLYNUCLEOTIDE SEPARATION

(57) Abstract

Nonporous beads having an average diameter of about 0.5–100 microns are suitable for chromatographic separation of mixtures of polynucleotides when the beads comprise a nonporous particle which are coated with a polymer or which have substantially all surface substrate groups endcapped with a non-polar hydrocarbon or substituted hydrocarbon group. The beads provide efficient separation of polynucleotides using Matched Ion Polynucleotide Chromatography.

TITLE OF THE INVENTION

IMPROVED LIQUID CHROMATOGRAPHIC MEDIA FOR POLYNUCLEOTIDE SEPARATION

FIELD OF THE INVENTION

5 The present invention is directed to the separation of polynucleotides using nonporous beads. More specifically, the invention is directed to the chromatographic separation of both single stranded and double stranded polynucleotides by chromatography using chromatographic columns containing nonporous beads, where the beads comprise either organic or 10 inorganic particles which are coated with a polymer, or non-polar substituted polymer, and/or which have substantially all surface substrate groups substituted with a non-polar hydrocarbon or non-ionic substituted hydrocarbon.

BACKGROUND OF THE INVENTION

15 Separations of polynucleotides such as DNA have been traditionally performed using slab gel electrophoresis or capillary electrophoresis. However, liquid chromatographic separations of polynucleotides are becoming more important because of the ability to automate the analysis and to collect fractions after they have been separated. Therefore, columns for 20 polynucleotide separation by liquid chromatography (LC) are becoming more important.

Silica-based columns are by far the most common LC columns. Of these, reverse phase silica-based columns are preferred because they have high separation efficiencies, are mechanically stable, and a variety of 25 functional groups can be easily attached for a variety of column selectivities.

Although silica-based reverse phase column materials have performed adequately for separating single stranded DNA, these materials have not performed well for separating double stranded DNA. The peaks from double stranded DNA separations using silica-based materials are badly shaped or broad, or the double stranded DNA may not even elute. Separations can take up to several hours, or the resolution, peak symmetry, and sensitivity of the separation are poor.

High quality materials for DNA separations have been based on polymeric substrates, as disclosed in U.S. Patent No. 5,585,236. There exists a need for silica-based column packing material and other materials that are suitable for separation of double stranded DNA.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide a chromatographic method for separating polynucleotides with improved separation and efficiency.

This and other objects of the invention, which will become apparent from reading the following specification, have been achieved by the method of the present invention in which polynucleotides are chromatographically separated using a column containing nonporous beads, where the beads comprise either organic or inorganic particles which are coated with a polymer, or non-polar substituted polymer, and/or which have substantially all surface substrate groups substituted with a non-polar hydrocarbon or non-ionic substituted hydrocarbon.

Disclosed herein is a method for separating a mixture of polynucleotides. The method comprises flowing a mixture of polynucleotides

having up to 1500 base pairs through a separation column containing beads having an average diameter of 0.5 to 100 microns, and separating the mixture of polynucleotides. The beads comprise nonporous particles coated with a hydrocarbon or non-polar substituted polymer or having substantially all surface substrate groups reacted with a non-polar hydrocarbon or substituted hydrocarbon group. Precautions are taken during the production of the beads so that they are substantially free of multivalent cation contaminants and the beads are treated, for example by an acid wash treatment, to remove any residual surface metal contaminants. In one embodiment, the beads are characterized by having a DNA Separation Factor (as defined hereinbelow) of at least 0.05. In a preferred embodiment, the beads are characterized by having a DNA Separation Factor of at least 0.5.

The separation is preferably by Matched Ion Polynucleotide Chromatography (MIPC) as defined hereinbelow. The beads preferably have an average diameter of about 1 - 5 microns. The nonporous particle is preferably selected from silica, silica carbide, silica nitrite, titanium oxide, aluminum oxide, zirconium oxide, carbon, insoluble polysaccharides such as cellulose, and diatomaceous earth, or any of these materials that have been modified to be nonporous. The nonporous particle is most preferably silica, which preferably is substantially free from unreacted silanol groups. The particles can be prepared by non-covalently bonded coatings, covalently bonded coatings, or reaction of the silanol groups with hydrocarbon groups.

The nonporous particle can be coated with a polymer. The polymer is preferably selected from polystyrenes, polymethacrylates, polyethylenes, polyurethanes, polypropylenes, polyamides, cellulose, polydimethyl siloxane,

and polydialkyl siloxane. The polymer is optionally unsubstituted or substituted with hydrocarbon groups or other groups having nonionic substituents. The polymer can be optionally substituted with hydrocarbon groups having from 1 to 1,000,000 carbons, the hydrocarbon groups optionally being alkyl groups with from 1 to 100 carbons and preferably from 1 to 24 carbons. Hydrocarbon groups from 24 to 1,000,000 are described herein as hydrocarbon polymers and have the constituency of hydrocarbon groups as defined herein.

The reaction of organosilanols (e.g. $HO\text{-}Si\text{-}R_3$) or alkoxy- (e.g., $RO\text{-}Si\text{-}R_3$) silanes with silica supports without polymerization can also produce good packings. The method produces a dense monolayer of functional groups of alkyl or alkylsubstituted, ester, cyano, and other nonionic groups. The use of monofunctional dimethyl silanes ($X\text{-}Si(CH_3)_2\text{-}R$) provides a homogeneous organic coating with a minimum of residual Si-OH groups. Monochlorosilane reagents are preferred, if the required organic functionality can be prepared. These reactions are reproducible and provide high quality packing materials. Unreacted, accessible silanols can be left after the initial reaction. The nonporous particle is preferably endcapped with a tri(lower alkyl)chlorosilane (preferably a trimethylchlorosilane) to block residual reactive silanol sites following the coating or hydrocarbon substitution. Alternatively, all of the silanol sites can be reacted with an excess of the endcapping reagent to extinguish all reactive silanol groups. Endcapping of the nonporous particle can be effected by reaction of the nonporous particle with the corresponding hydrocarbon substituted silane, such as trialkyl chlorosilane (eg. trimethyl chlorosilane) or by reaction with the corresponding hydrocarbon substituted

disilazane, such as dichloro-tetraalkyl-disilazane (eg. dichloro-tetramethyl-disilazane).

The method of the present invention can be used to separate double stranded polynucleotides having up to about 1500 to 2000 base pairs. In
5 many cases, the method is used to separate polynucleotides having up to 600 bases or base pairs, or which have up to 5 to 80 bases or base pairs.

The method is performed at a temperature within the range of 20°C to 90°C to yield a back-pressure not greater than 10,000 psi. The method also preferably employs an organic solvent that is water soluble. The solvent is
10 preferably selected from the group consisting of alcohols, nitriles, dimethylformamide, esters, and ethers. The method also preferably employs a counter ion agent selected from trialkylamine acetate, trialkylamine carbonate, and trialkylamine phosphate. The most preferred counter ion agent is triethylammonium acetate or triethylammonium hexafluoroisopropyl
15 alcohol.

The method also preferably comprises supplying and feeding solutions entering the separation column with components having process solution-contacting surfaces which contact process solutions held therein or flowing therethrough. The process solution-contacting surfaces are material which
20 does not release multivalent cations into aqueous solutions held therein or flowing therethrough, so that the column and its contents are protected from multivalent cation contamination The process solution-contacting surfaces are preferably material selected from the group consisting of titanium, coated stainless steel, passivated stainless steel, and organic polymer. Multivalent
25 cations in eluent solutions and sample solutions entering the column are also

preferably removed by contacting the solutions with multivalent cation capture resin before the solutions enter the column so as to protect the resin bed from multivalent cation contamination. The multivalent capture resin is selected from cation exchange resin and chelating resin. The column and process solutions held therein or flowing therethrough are preferably substantially free of multivalent cation contaminants. The polynucleotides are separated by Matched Ion Polynucleotide Chromatography.

Also disclosed herein is a method for separating a mixture of polynucleotides, comprising flowing a mixture of polynucleotides having up to 1500 base pairs through a separation column containing beads having an average diameter of 0.5 to 100 microns, and separating the mixture of polynucleotides by Matched Ion Polynucleotide Chromatography. The beads comprise nonporous particles coated with a polymer or having substantially all surface substrate groups reacted and/or endcapped with a non-polar hydrocarbon or substituted hydrocarbon group. The beads are characterized by having a DNA Separation Factor of at least 0.05. The column and process solutions held therein or flowing therethrough are substantially free of multivalent cation contaminants. The method is performed at a temperature within the range of 20°C to 90°C to yield a back-pressure not greater than 10,000 psi. An organic solvent that is water soluble is used in the performance of the method.

Also disclosed herein is a bead comprising a nonporous particle coated with a polymer. The bead has an average diameter of 0.5 to 100 microns and is characterized by having a DNA Separation Factor of at least 0.05. In a preferred embodiment, the bead is characterized by having a DNA

Separation Factor of at least 0.5. The bead preferably has a diameter of about 1 - 5 microns. The nonporous particle is preferably selected from silica, silica carbide, silica nitrite, titanium oxide, aluminum oxide, zirconium oxide, carbon, insoluble polysaccharides such as cellulose, and diatomaceous earth, 5 or any of these materials that have been modified to be nonporous. The nonporous particle is most preferably silica, which preferably has minimum silanol groups. The polymer is preferably selected from polystyrene, polymethacrylate, polyethylene, polyurethane, polypropylene, polyamide, cellulose, polydimethyl siloxane, and polydialkyl siloxane, and is preferably 10 unsubstituted, alkylated, or alkyl or aryl substituted, or alkylated with a substituted alkyl group methyl-substituted, or ethyl-substituted. The polymer can be alkylated with alkyl groups having 1 - 22 carbon atoms, preferably, 8 - 18 carbon atoms.

Also disclosed herein is a bead comprising a nonporous particle having 15 substantially all surface substrate groups reacted with a hydrocarbon group and then endcapped with a non-polar hydrocarbon or substituted hydrocarbon group, preferably a tri(lower alkyl)chlorosilane or tetra(lower alkyl)dichlorodisilazane. The bead has an average diameter of 0.5 to 100 microns and is characterized by having a DNA Separation Factor of at least 20 0.05. The bead preferably has a diameter of about 1 - 5 microns.

The nonporous particle is preferably selected from silica, silica carbide, silica nitrite, titanium oxide, aluminum oxide, zirconium oxide, carbon, insoluble polysaccharides such as cellulose, and diatomaceous earth, or any of these materials that have been modified to be nonporous. The nonporous 25 particle is most preferably silica, which preferably has minimum silanol

groups. Endcapping of the nonporous particle can be effected by reaction of the nonporous particle with trimethyl chlorosilane or dichloro-tetraisopropyl-disilazane.

BRIEF DESCRIPTION OF THE DRAWINGS

5 FIG. 1 is a schematic representation of how the DNA Separation Factor is applied to a separation.

FIG. 2 is a schematic drawing of a cross-section of a representation of a reverse phase bead with a silica core and endcapping shielding.

FIG. 3 is a schematic drawing of a cross-section of a representation of 10 a reverse phase bead with a silica core and polymer shielding.

FIG. 4 is a MIPC separation of pUC18 DNA-*Hae*III digestion fragments on a column containing alkylated poly(styrene-divinylbenzene) beads. Peaks are labeled with the number of base pairs of the eluted fragment.

FIG. 5 is a MIPC separation of pUC18 DNA-*Hae*III digestion fragments 15 on a column containing nonporous 2.1 micron beads of underivatized poly(styrene-divinylbenzene).

FIG. 6 is a Van't Hoff plot of the retention factor $1/T\ [^{\circ}K^{-1}]$ with alkylated poly(styrene-divinylbenzene) beads showing positive enthalpy using acetonitrile as the solvent.

20 FIG. 7 is a Van't Hoff plot of the retention factor $1/T\ [^{\circ}K^{-1}]$ with underivatized poly(styrene-divinylbenzene) beads showing positive enthalpy using acetonitrile as the solvent.

FIG. 8 is a Van't Hoff plot of the retention factor $1/T\ [^{\circ}K^{-1}]$ with alkylated poly(styrene-divinylbenzene) beads showing negative enthalpy using 25 methanol as the solvent.

FIG. 9 is a separation using alkylated beads and acetonitrile as solvent.

FIG. 10 is a separation using alkylated beads and 50.0% methanol as the solvent.

5 FIG. 11 is a separation using alkylated beads and 25.0% ethanol as the solvent.

FIG. 12 is a separation using alkylated beads and 25.0% vodka (Stolichnaya, 100 proof) as the solvent.

FIG. 13 is a separation using alkylated beads and 25.0% 1-propanol as

10 the solvent.

FIG. 14 is a separation using alkylated beads and 25.0% 1-propanol as the solvent.

FIG. 15 is a separation using alkylated beads and 10.0% 2-propanol as the solvent.

15 FIG. 16 is a separation using alkylated beads and 10.0% 2-propanol as the solvent.

FIG. 17 is a separation using alkylated beads and 25.0% THF as the solvent.

FIG. 18 is an isocratic/gradient separation on non-alkylated

20 poly(styrene-divinylbenzene) beads.

DETAILED DESCRIPTION OF THE INVENTION

In its most general form, the subject matter of the present invention is the separation of polynucleotides by Matched Ion Polynucleotide Chromatography utilizing columns filled with nonporous beads having an

25 average diameter of about 0.5 -100 microns; preferably, 1 - 10 microns; more

preferably, 1 - 5 microns. Beads having an average diameter of 1.0 - 3.0 microns are most preferred.

In U.S. Patent No. 5,585,236, Bonn et al. had characterized the polynucleotide separation process as reverse phase ion pairing chromatography (RPIPC). However, since RPIPC does not incorporate certain essential characteristics described in the present invention, another term, Matched Ion Polynucleotide Chromatography (MIPC), has been selected. MIPC as used herein, is defined as a process for separating single and double stranded polynucleotides using non-polar beads, wherein the process uses a counter ion agent, and an organic solvent to desorb the polynucleotide from the beads, and wherein the beads are characterized as having a DNA Separation Factor of at least 0.05. In a preferred embodiment, the beads are characterized as having a DNA Separation Factor of at least 0.5.

The performance of the beads of the present invention is demonstrated by high efficiency separation by MIPC of double stranded and single stranded DNA. We have found that the best criterion for measuring performance of the beads is a DNA Separation Factor. This is measured as the resolution of 257- and 267-base pair double stranded DNA fragments of a pUC18 DNA-*Hae*III restriction digest wherein the distance from the valley between the peaks to the top of one of the peaks, over the distance from the baseline to the valley. Referring to the schematic representation of FIG. 1, the DNA Separation Factor is determined by measuring the distance "**a**" from the baseline to the valley "**e**" between the peaks "**b**" and "**c**" and the distance "**d**" from the valley "**e**" to the top of one of the peaks "**b**" or "**c**". If the peak heights

are unequal, the highest peak is used to obtain "**d**." The DNA Separation Factor is the ratio of **d/(a+d)**. The peaks of 257- and 267-base pairs in this schematic representation are similar in height. Operational beads of the present invention have a DNA Separation Factor of at least 0.05. Preferred
5 beads have a DNA Separation Factor of at least 0.5. In an optimal embodiment, the beads have a DNA Separation Factor of at least 0.95.

Without wishing to be bound by theory, we believe that the beads which conform to the DNA Separation Factor as specified herein have a pore size which essentially excludes the polynucleotides being separated from
10 entering the bead. As used herein, the term "nonporous" is defined to denote a bead which has surface pores having a diameter that is less than the size and shape of the smallest DNA fragment in the separation in the solvent medium used therein. Included in this definition are beads having these specified maximum size restrictions in their natural state or which have been
15 treated to reduce their pore size to meet the maximum effective pore size required. Preferably, all beads which provide a DNA Separation Factor of at least 0.05 are intended to be included within the definition of "nonporous" beads.

The surface conformations of nonporous beads of the present
20 invention can include depressions and shallow pit-like structures which do not interfere with the separation process. A pretreatment of a porous bead to render it nonporous can be effected with any material which will fill the pores in the bead structure and which does not significantly interfere with the MIPC process.

Pores are open structures through which eluent and other materials can enter the bead structure. Pores are often interconnected so that fluid entering one pore can exit from another pore. We believe that pores having dimensions that allow movement of the polynucleotide into the interconnected pore structure and into the bead impair the resolution of separations or result separations that have very long retention times. In MIPC, however, the beads are "nonporous" and the polynucleotides do not enter the bead structure.

The term polynucleotide is defined as a linear polymer containing an indefinite number of nucleotides, linked from one ribose (or deoxyribose) to another via phosphoric residues. The present invention can be used in the separation of RNA or of double- or single-stranded DNA. For purposes of simplifying the description of the invention, and not by way of limitation, the separation of double-stranded DNA will be described in the examples herein, it being understood that all polynucleotides are intended to be included within the scope of this invention.

Chromatographic efficiency of the column beads is predominantly influenced by the properties of surface and near-surface areas. For this reason, the following descriptions are related specifically to the close-to-the-surface region of the polymeric beads. The main body and/or the center of such beads can exhibit entirely different chemistries and sets of physical properties from those observed at or near the surface of the polymeric beads of the present invention.

The beads of the invention comprise a nonporous particle which has non-polar molecules or a non-polar polymer attached to or coated on its surface. In general, the beads comprise nonporous particles which have

been coated with a polymer or which have substantially all surface substrate groups reacted with a non-polar hydrocarbon or substituted hydrocarbon group, and any remaining surface substrate groups endcapped with a tri(lower alkyl)chlorosilane or tetra(lower alkyl)dichlorodisilazane as described
5 above.

The nonporous particle is preferably an inorganic particle, but can be a nonporous organic particle. The nonporous particle can be, for example, silica, silica carbide, silica nitrite, titanium oxide, aluminum oxide, zirconium oxide, carbon, insoluble polysaccharides such as cellulose, or diatomaceous
10 earth, or any of these materials which have been modified to be nonporous. Examples of carbon particles include diamond and graphite which have been treated to remove any interfering contaminants. The preferred particles are essentially non-deformable and can withstand high pressures. The nonporous particle is prepared by known procedures. The preferred particle size is
15 about 0.5 -100 microns; preferably, 1 - 10 microns; more preferably, 1 - 5 microns. Beads having an average diameter of 1.0 - 3.0 microns are most preferred.

Because the chemistry of preparing conventional silica-based reverse phase HPLC materials is well-known, most of the description of the beads of
20 the invention herein is presented in reference to silica. It is to be understood, however, that other nonporous particles, such as those listed above, can be modified in the same manner and substituted for silica in the process of the invention. For a description of the general chemistry of silica, see Poole, Colin F. and Salwa K. Poole, *Chromatography Today*, Elsevier:New York
25 (1991), pp. 313-342 and Snyder, R. L. and J. J. Kirkland, *Introduction to*

Modern Liquid Chromatography, 2nd ed., John Wiley & Sons, Inc.:New York (1979), pp. 272-278, the disclosures of which are hereby incorporated herein by reference in their entireties.

The nonporous beads of the invention are characterized by having
5 minimum exposed silanol groups after reaction with the coating or alkylation reagents. Minimum silanol groups are needed to reduce the interaction of the DNA with the substrate and also to improve the stability of the material in a high pH and aqueous environment. Silanol groups can be harmful because they can repel the negative charge of the DNA molecule, preventing or
10 limiting the interaction of the DNA with the stationary phase of the column. Another possible mechanism of interaction is that the silanol can act as ion exchange sites, taking up metals such as iron (III) or chromium (III). Iron (III) or other metals which are trapped on the column can distort the DNA peaks or even prevent DNA from being eluted from the column.

15 Silanol groups can be hydrolyzed by the aqueous-based eluent. Hydrolysis will increase the polarity and reactivity of the stationary phase by exposing more silanol sites, or by exposing metals that can be present in the silica core. Hydrolysis will be more prevalent with increased silanol groups. The effect of silanol groups on the DNA separation depends on which
20 mechanism of interference is most prevalent. For example, iron (III) can become attached to the exposed silanol sites, depending on whether the iron (III) is present in the eluent, instrument or sample.

The effect of metals can only occur if metals are already present within the system or reagents. Metals present within the system or reagents can get
25 trapped by ion exchange sites on the silica. However, if no metals are

present within the system or reagents, then the silanol groups themselves can cause interference with DNA separations. Hydrolysis of the exposed silanol sites by the aqueous environment can expose metals that might be present in the silica core.

5 Fully hydrolyzed silica contains a concentration of about 8 μmoles of silanol groups per square meter of surface. At best, because of steric considerations, a maximum of about 4.5 μmoles of silanol groups per square meter can be reacted, the remainder of the silanol being sterically shielded by the reacted groups. Minimum silanol groups is defined as reaching the

10 theoretical limit of or having sufficient shield to prevent silanol groups from interfering with the separation.

Numerous methods exist for forming nonporous silica core particles. For example, sodium silicate solution poured into methanol will produce a suspension of finely divided spherical particles of sodium silicate. These

15 particles are neutralized by reaction with acid. In this way, globular particles of silica gel are obtained having a diameter of about 1 - 2 microns. Silica can be precipitated from organic liquids or from a vapor. At high temperature (about 2000°C), silica is vaporized, and the vapors can be condensed to form finely divided silica either by a reduction in temperature or by using an

20 oxidizing gas. The synthesis and properties of silica are described by R. K. Iler in *The Chemistry of Silica, Solubility, Polymerization, Colloid and Surface Properties, and Biochemistry*, John Wiley & Sons:New York (1979).

W. Stöber et al. described controlled growth of monodisperse silica spheres in the micron size range in *J. Colloid and Interface Sci.*, 26:62-69

25 (1968). Stöber et al. describe a system of chemical reactions which permit

the controlled growth of spherical silica particles of uniform size by means of hydrolysis of alkyl silicates and subsequent condensation of silicic acid in alcoholic solutions. Ammonia is used as a morphological catalyst. Particle sizes obtained in suspension range from less than 0.05 μm to 2 μm in
5 diameter.

Nonporous silica core beads can be obtained from Micra Scientific (Northbrook, IL) and from Chemie Uetikkon (Lausanne, Switzerland).

To prepare the nonporous beads of the invention, the nonporous particle is coated with a polymer or reacted and endcapped so that
10 substantially all surface substrate groups of the nonporous particle are blocked with a non-polar hydrocarbon or substituted hydrocarbon group. This can be accomplished by several methods.

The organic bonded-phase siloxane coating can be made as a monomolecular layer or as a polymerized multilayer coating. Packings with
15 so-called monomolecular organic layers are normally prepared by reacting the surface silanol groups of siliceous-base particles with mono-, di-, or trifunctional chloro-, dimethyl-, amino-, siloxy-, or alkoxy-silanes. Typical monofunctional reactants used in these reactions include X-Si-R, where X = Cl, OH, OCH_3, or OC_2H_5, and R is an organic radical. FIG. 2 is a schematic
20 representation of a bead 20 having a silica core 22 and a monomolecular organic layer. (The figure does not necessarily reflect the morphology or pore structure of the beads of the invention and is meant for illustrative purposes only.)

Using bi- and trifunctional reactants, such as R_2SiX_2 and $RSiX_3$, for the
25 surface modifications, up to two Si-X groups per bonded functional group

remain unreacted. After treatment with water, hydrolysis of these unreacted groups takes place, and additional silanol groups are formed (sometimes in a polymer matrix) in about the same concentration as the bonded organic functional groups present in the packing. These acidic organo-silanol groups can significantly affect the retention behavior of solutes and adversely influence the stability of the packing in aqueous solutions at pH > 7.

Thus, incomplete reaction of the surface with the silane reagent, or the formation of new Si-OH groups from using bi- or trifunctional modifiers, can result in a population of residual acidic Si-OH groups that are readily accessible to molecules of the mobile phase or sample. Therefore, the recent trend is toward (a) a dense monolayer of functional groups instead of partial coverage and (b) the use of monofunctional dimethylsilanes [X-Si$(CH_3)_2$-R] to provide a homogeneous organic coating with a minimum possibility of residual Si-OH groups. Monochlorosilane reagents are preferred, if the required organic functionality can be prepared. If two of the R groups in the monofunctional modifier are methyl, surface coverage can be as high as about 4 μmoles per square meter of organic (based on carbon analysis). In the latter case, residual Si-OH groups on the silica surface are unavailable for chromatographic interactions with most solutes because of steric shielding.

The reaction of organosilanols (*e.g.*, HO-Si-R_3) or organoalkoxy- (*e.g.*, RO-Si-R_3) silanes with silica supports without polymerization can also produce good packings. These reactions are relatively reproducible, provided that traces of water or other reactive species are absent. Unreacted, accessible silanols can be left after the initial reaction, but these can be removed by

capping of the packing with chlorotrimethylsilane (providing the *R* groups do not react with the latter silane).

According to one method, the nonporous particle is coated with a polymer coating. Suitable polymers for use in coating the particle include chain reaction polymers and step reaction polymers, for example, polystyrene, polymethacrylate, polyethylene, polyurethane, polypropylene, polyamide, insoluble polysaccharides such as cellulose, polydimethyl siloxane, polydialkyl siloxane, and related materials. The polymer coating can be attached to the nonporous particle by means of a multi-coating process so that complete shielding of the surface is achieved.

In the last few years, new bonded phase packings, known as polymer-coated or polymer-encapsulated packings, have been introduced based on techniques used to prepare immobilized stationary phases for open tubular column gas chromatography. In this case, the phases are prepared by mechanically coating either bare silica or presilanized silica microparticles with a poly(siloxane) or poly(butadiene) prepolymer, which is then immobilized by peroxide, azo-tert-butane, or gamma radiation-induced chemical crosslinking reactions. FIG. 3 is a schematic illustration of a coated bead 30 having a silica core 32 and polymer coating 34. (The figure does not necessarily reflect the morphology or pore structure of the beads of the invention and is meant for illustrative purposes only.)

An alternative method comprises a combination of covalent bonding with a vinyl-containing silane molecule and then polymerizing a coating on the surface of the particles. A second coating can be applied if residual silanol groups or metal groups are present.

In a variation of this method, the silica surface is first modified by reaction with vinyltrichlorosilane, followed by polymerizing acrylic acid derivatives to and over the derivatized silica surface. The availability of a large number of useful monomers and prepolymers has enabled a wide variety of reverse phase, polar, and ion exchange packings to be prepared using the same general reaction. Also, since the general approach does not depend on the chemistry of the underlying substrate, materials other than silica, for example, alumina and zirconia, can be modified and used under conditions for which silica is unsuitable, for example, with mobile phases outside the pH range 2 - 7.5. Returning to silica, presilanization decreases the number of active silanol groups, which are then further shielded by the polymeric film anchored over the surface. In reverse phase liquid chromatography, these packings have shown improved chromatographic properties compared to monomeric, chemically bonded phases for the separation of basic solutes. Polymer-encapsulated packings have a film thickness of about 1 nm to maintain reasonable mass transfer characteristics. A description of the this procedure has been published by H. Engelhart et al. (*Chromatographia*, 27:535 (1989)).

The polymer-coated beads prepared according to either of the above methods can be used in their unmodified state or can be modified by substitution with a hydrocarbon group. Any hydrocarbon group is suitable. The term "hydrocarbon" as used herein is defined to include alkyl and alkyl substituted aryl groups, having from 1 to 1,000,000 carbons, the alkyl groups including straight chained, branch chained, cyclic, saturated, unsaturated nonionic functional groups of various types including, aldehyde, ketone, ester,

ether, alkyl groups, and the like, and the aryl groups including as monocyclic, bicyclic, and tricyclic aromatic hydrocarbon groups including phenyl, naphthyl, and the like. Methods for hydrocarbon substitution are conventional and well-known in the art and are not an aspect of this invention. The hydrocarbon can
5 also contain hydroxy, cyano, nitro groups, or the like which are considered to be non-polar, reverse phase functional groups. The preferred hydrocarbon groups are alkyl groups, and the description of suitable substitution processes hereinbelow are presented as alkylation for purposes of simplification and not by way of limitation, it being understood that aryl substitution by conventional
10 procedures are also intended to be included within the scope of this invention.

The polymer-coated beads can be alkylated by reaction with the corresponding alkyl halide such as the alkyl iodide. Alkylation is achieved by mixing the polymer-coated beads with an alkyl halide in the presence of a Friedel-Crafts catalyst to effect electrophilic aromatic substitution on the
15 aromatic rings at the surface of the polymer blend. Suitable Friedel-Crafts catalysts are well-known in the art and include Lewis acids such as aluminum chloride, boron trifluoride, tin tetrachloride, etc. Substitution with hydrocarbon groups having from 1 to 1,000,000 and preferably from 1 to 22 carbons can be effected by these processes. Hydrocarbon groups having from 23 to
20 1,000,000 carbons are referenced herein as hydrocarbon polymers.

Alkylation can be accomplished by a number of known synthesis procedures. These include Friedel-Crafts alkylation with an alkyl halide, attachment of an alkyl alcohol to a chloromethylated bead to form an ether, etc. Although the preferred method for alkylating the polymer-coated beads
25 of the present invention is alkylation after the polymer coating has been

formed on the nonporous particle, an alternative method of alkylation is to polymerize alkylated monomers to form an alkylated polymer coating on the nonporous particle. In this embodiment, the monomers will be substituted with alkyl groups having any number of carbon atoms, for example, from 1 to 100, 1 to 50 or 1 to 24, for example, depending upon the requirements of the separation variables.

As an alternative to polymer coating, the nonporous particle can be functionalized with an alkyl group or other non-polar functional group including cyano, ester, and other non-ionic groups, followed by a complete endcapping process to reduce silanol and metal interaction. Endcapping of the nonporous particle can be achieved by reacting the particle with trialkyl chlorosilane or tetraalkyl dichlorodisilazane, such as, for example, trimethyl chlorosilane or dichloro-tetraisopropyl-disilazane.

A large number of factors influence the success of the bonding reactions and the quality of the final bonded-phase product. The rate and extent of the bonding reaction depends on the reactivity of the silane, choice of solvent and catalyst, time, temperature, and the ratio of reagents to substrate. Reactive organosilanes with Cl, OH, O*R*, $N(CH_3)_2$, $OCOCF_3$, and enolates as leaving groups have been widely used. The dimethylamine, trifluoroacetate, and enol ethers of pentane-2,4-dione are the most reactive leaving groups, although economy, availability, and familiarity result in the chlorosilanes and alkoxysilanes being the most widely used, particularly among commercial manufacturers. Initially, reactions can be almost stoichiometric but, as the surface coverage approaches a maximum value, the reaction becomes very slow. For this reason, reaction times tend to be

long (12 - 72 hours), reaction temperatures moderately high (in most cases, around 100°C) and, in the case of chlorosilanes, an acid acceptor catalyst (*e.g.*, pyridine) is used. Some reagents, such as the alkylsilyl enolates and alkylsilyldimethylamines, do not require additional catalyst, or even solvent, to carry out the reaction. The most common solvents employed are toluene and xylene, although other solvents, such as carbon tetrachloride, trichloroethane, and dimethylformamide (DMF), have been recommended as being superior. Since the bonding reactions are carried out by refluxing in an inert atmosphere, solvents are often selected based on their capacity to be a good solvent for the organosilanes and to attain the desired reaction temperature at reflux. Except for 3-cyanopropylsiloxane bonded phases, the high reactivity of chlorosilanes towards certain polar functional groups (*e.g.*, OH, etc.) precludes the use of these groups for the preparation of polar, reverse phase bonded phases. Alkoxysilanes containing acidic or basic functional groups are autocatalytic and the bonded phases are usually prepared by refluxing the silane in an inert solvent at a temperature high enough to distill off the alcohol formed by the condensation reaction with the surface silanol groups. Bonding of neutral, polar ligands generally requires the addition of a catalyst, such as toluene-4-sulfonic acid or triethylamine, in the presence of sufficient water to generate monolayer coverage of the silica. The presence of water speeds up the hydrolysis of the alkoxy groups of the adsorbed organosilane, which tends to react with surface silanol groups rather than polymerize in solution. It seems to be a general problem in the preparation of polar bonded phases that surface silanol groups are blocked by physically adsorbed organosilanes, giving rise to a lower bonded phase density after workup than

the maximum theoretically predicted. The bonded phase density can be increased by repeating the reaction a second time or exposed silanol groups minimized by endcapping.

Although most bonded phases are prepared from organosilanes containing a single functionalized ligand bonded to silicon, with the remaining groups being leaving groups and/or methyl groups, more highly substituted organosilanes can also be used. Bifunctional organosilanes, such as 1,3-dichlorotetraisopropyldisilazane, are able to react with surface silanol groups at both ends of the chain, forming a bonded phase that is more hydrolytically stable than bonded phases formed from conventional organosilanes. The bidentate organosilanes have reactive sites that more closely match the spacing of the silanol groups on the silica surface and provide a higher bonded phase coverage than is achieved with dichlorosilanes with both leaving groups attached to the same silicon atom. For alkyldimethylsilanes, increasing the length of the alkyl group increases the hydrolytic stability of the bonded phase relative to that of the trimethylsilyl bonded ligands. Increasing the chain length of the methyl groups increases the hydrolytic stability of the bonded phase, but reduces the phase coverage due to steric effects. The use of monofunctional organosilanes containing one or two bulky groups, for example, isopropyl or t-butyl, on the silicon atom of the silane can become more important in the preparation of bonded phases for use at low pH. The bulky alkyl groups provide better steric protection to the hydrolytically sensitive siloxane groups on the packing surface than does the methyl group.

The general process of coating and endcapping of a silica substrate is well-known technology. However, the general understanding of those who

have used these materials is they are not suitable for high performance double stranded DNA separations. However, the beads of this invention are formed by a more careful application of the coating and end-capping procedures to effect a thorough shielding of the silica core, the resulting beads having the ability to perform rapid separations of both single stranded and double stranded DNA which are equal to or better than those achieved using the alkylated nonporous polymer beads disclosed in U.S. Patent No. 5,585,236, for example.

The beads of the invention are also characterized by having low amounts of metal contaminants or other contaminants that can bind DNA. The preferred beads of the present invention are characterized by having been subjected to precautions during production, including a decontamination treatment, such as an acid wash treatment, designed to substantially eliminate any multivalent cation contaminants (e.g. Fe(III), Cr(III), or colloidal metal contaminants). Only very pure, non-metal containing materials should be used in the production of the beads in order that the resulting beads will have minimum metal content.

Care must be taken during the preparation of the beads to ensure that the surface of the beads has minimum silanol or metal oxide exposure and that the surface remains nonporous.

To achieve high resolution chromatographic separations of polynucleotides, it is generally necessary to tightly pack the chromatographic column with the solid phase nonporous beads. Any known method of packing the column with a column packing material can be used in the present invention to obtain adequate high resolution separations. Typically, a slurry of

the beads is prepared using a solvent having a density equal to or less than the density of the beads. The column is then filled with the bead slurry and vibrated or agitated to improve the packing density of the beads in the column. Mechanical vibration or sonification are typically used to improve packing density.

For example, to pack a 50 x 4.6 mm i.d. column, 2.0 grams of beads can be suspended in 10 mL of methanol with the aid of sonification. The suspension is then packed into the column using 50 mL of methanol at 8,000 psi of pressure. This improves the density of the packed bed.

The separation method of the invention is generally applicable to the chromatographic separation of single stranded and double stranded polynucleotides of DNA and RNA. Samples containing mixtures of polynucleotides can result from total synthesis of polynucleotides, cleavage of DNA or RNA with restriction endonucleases or with other enzymes or chemicals, as well as polynucleotide samples which have been multiplied and amplified using polymerase chain reaction techniques.

The method of the present invention can be used to separate double stranded polynucleotides having up to about 1500 to 2000 base pairs. In many cases, the method is used to separate polynucleotides having up to 600 bases or base pairs, or which have up to 5 to 80 bases or base pairs.

In a preferred embodiment, the separation is by MIPC. The nonporous beads of the invention are used as a reverse phase material that will function with counter ion agents and a solvent gradient to produce the DNA separations. In MIPC, the DNA fragments are matched with a counter ion agent and then subjected to reverse phase chromatography using the

nonporous beads of the present invention. Counter ion agents that are volatile, such as trialkylamine acetate, trialkylamine carbonate, trialkylamine phosphate, etc., are preferred for use in the method of the invention, with triethylammonium acetate (TEAA) and triethylammonium hexafluoroisopropyl
5 alcohol being most preferred.

To achieve optimum peak resolution during the separation of DNA by MIPC using the beads of the invention, the method is performed at a temperature within the range of 20°C to 90°C to yield a back-pressure not greater than 10,000 psi. In general, separation of single-stranded fragments
10 should be performed at higher temperatures.

We have found that the temperature at which the separation is performed affects the choice of solvents used in the separation. When the separation is performed at a temperature within the above range, an organic solvent that is water soluble is preferably used, for example, alcohols, nitriles,
15 dimethylformamide (DMF), esters, and ethers. Water soluble solvents are defined as those which exist as a single phase with aqueous systems under all conditions of operation of the present invention. Solvents which are particularly preferred for use in the method of this invention include methanol, ethanol, 1-propanol, 2-propanol, tetrahydrofuran (THF), and acetonitrile, with
20 acetonitrile being most preferred.

Two compounds (in this case, DNA fragments of different size) can only be separated if they have different partition coefficients (K). The Nernst partition coefficient is defined as the concentration of an analyte (A) in the stationary phase divided by its concentration in the mobile phase:

$$K = \frac{[A]_s}{[A]_m}$$

The partition coefficient (K) and the retention factor (k) are related through the following equations:

5

$$K = \frac{n(A)_s V_m}{n(A)_m V_s} \quad \text{and} \quad k = \frac{n(A)_s}{n(A)_m}$$

the quotient V_m/V_s is also called phase volume ratio (Φ). Therefore:

$$k = K\Phi$$

To calculate the sorption enthalpies, the following fundamental

10 thermodynamic equations are necessary:

$$\ln K = -\frac{\Delta G}{RT}, \quad \ln k = -\frac{\Delta G_{sorp}}{RT} + \ln \Phi \quad \text{and } \Delta G_{sorp} = \Delta H_{sorp} - T\Delta S_{sorp}$$

By transforming the last two equations, we obtain the Van't Hoff equation:

$$\ln k = -\frac{\Delta H_{sorp}}{RT} + \frac{\Delta S_{sorp}}{R} + \ln \Phi$$

15

From a plot ln k versus $1/T$, the sorption enthalpy ΔH_{sorp} can be obtained from the slope of the graph (if a straight line is obtained). ΔS_{sorp} can be calculated if the phase volume ratio (Φ) is known.

20 Experiments on silica beads coated with poly(styrene-divinylbenzene) also give a negative slope for a plot of ln k versus $1/T$, although the plot is slightly curved.

If the acetonitrile is replaced with methanol, the retention factor k decreases with increasing temperature, indicating the retention mechanism is

25 an exothermic process ($\Delta H_{sorp} < 0$).

The thermodynamic data (as shown in the Examples hereinbelow) reflect the relative affinity of the DNA-counter ion agent complex for the beads of the invention and the elution solvent. An endothermic plot indicates a

preference of the DNA complex for the bead. An exothermic plot indicates a preference of the DNA complex for the solvent over the bead. The plots shown herein are for alkylated and non-alkylated surfaces as described in the Examples. Most liquid chromatographic separations show exothermic plots.

5 In addition to the beads themselves being substantially metal-free, we have also found that, to achieve optimum peak separation during RPIPC, the separation column and all process solutions held within the column or flowing through the column should be substantially free of multivalent cation contaminants (e.g. Fe(III), Cr(III), and colloidal metal contaminants). As 10 described in commonly owned, copending U.S. application Ser. No. 08/748,376, (filed November 13, 1996), this can be achieved by supplying and feeding solutions entering the separation column with components which have process solution-contacting surfaces made of material which does not release multivalent cations into the process solutions held within or flowing 15 through the column, in order to protect the column from multivalent cation contamination. The process solution-contacting surfaces of the system components are preferably material selected from the group consisting of titanium, coated stainless steel, passivated stainless steel, and organic polymer. Metals found in stainless steel, for example, do not harm the 20 separation, unless they are in an oxidized or colloidal partially oxidized state. For example, 316 stainless steel frits are acceptable in column hardware, but surface oxidized stainless steel frits harm the DNA separation.

For additional protection, multivalent cations in eluent solutions and sample solutions entering the column can be removed by contacting these 25 solutions with multivalent cation capture resin before the solutions enter the

column to protect the resin bed from multivalent cation contamination. The multivalent capture resin is preferably cation exchange resin and/or chelating resin.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

All references cited herein are hereby incorporated by reference in their entirety.

Procedures described in the past tense in the examples below have been carried out in the laboratory. Procedures described in the present tense have not been carried out in the laboratory, and are constructively reduced to practice with the filing of this application.

EXAMPLE 1

C-18 Bonded Phase Standard Phase

To a 1000-mL round bottomed flask, add 200 g of nonporous, 2 µm silica and one small stirring egg. Transfer flask with silica to an oven and heat at 125°C overnight (*i.e.*, at least 8 hours). Have heating mantle and condenser set up.

The C-18 bonding reagent, n-octadecyldimethylsilane, is a waxy white solid to semi-solid at room temperature. To transfer, open the bottle in a hood and gently warm with a heat gun (note: pressure can build up in stored chlorosilane bottles, and they should be handled as if they were HCl, as upon contact with moisture, HCl is the side product).

To a second flask, transfer 125 g of the n-octadecylmethylchlorosilane reagent, 10 mL of chloroform, 400 mL of toluene, and 65 mL of pyridine. Mix

the liquid reagents by swirling, and then add to the dried silica and swirl until all of the silica is suspended. Attach the refluxing condenser and bring the mixture to reflux for 15 hours. Let the mixture cool, such that refluxing has stopped. Add the capping reagent package of 20 mL of trimethylchlorosilane, 6 mL of hexamethylsilane in 20 mL of toluene. Resuspend the mixture and bring the system back to reflux for 6 hours. Let the mixture cool to room temperature.

Transfer to a Buchner funnel and wash with three 200-mL aliquots of methanol, followed by three 200-mL aliquots of acetone. Air dry for at least 0.5 hour, and then dry in the oven at 100°C overnight.

Submit sample for elemental analysis, and percent carbon.

Dried bonded phase is now ready for column packing.

EXAMPLE 2

CN Bonded Phase, Cyano Phase

To a 1000-mL round-bottomed flask, add 200 g of nonporous, 2 μm silica, one stirring egg, and place in an oven at 125°C overnight (*i.e.*, at least 8 hours) to dry. To the dried silica, add 100 mL of the 3-cyanopropylmethyldichlorosilane, 10 mL of chloroform, 450 mL of toluene, and 50 mL of pyridine. Suspend the mixture and bring to reflux for 15 hours. Cool filter and wash in a Buchner funnel with one 200-mL aliquot of toluene, followed by two 200-mL aliquots of methanol. Transfer to a beaker and add 300 mL of 50:50 methanol:water, pH 5.5 with HCl. Suspend and let sit at room temperature for 1 hour. Filter onto Buchner funnel and wash phase with methanol and acetone. Transfer to the 1000-mL round-bottomed flask and dry in oven overnight.

Next, endcap by adding 20 mL of trimethylchlorosilane, 6 mL of hexamethyl-disilane, 350 mL of toluene, 10 mL of chloroform, and 25 mL of pyridine to the dried bonded phase, and bring to reflux for 6 hours. Cool the resulting mixture, transfer to a Buchner funnel, and wash with three 200-mL aliquots of methanol, followed by three 200-mL aliquots of acetone. Air dry for at least 0.5 hour, and then dry in the oven at 100°C overnight.

Submit a sample for elemental analysis.

The bonded phase is now ready for column packing.

EXAMPLE 3

Dioctyl Silyl Phase - C-8X2

Repeat all of the steps for CN bonded phase, but replace 3-cyanopropylmethyldichlorosilane with 100 mL of dioctyl dichlorosilane.

EXAMPLE 4

Acid Wash Treatment

The procedures of Example 1 are repeated but the silica is washed with 500 mL of 100 mM HCl and then water prior to drying. The product is washed with 500 mL of 100 mM HCl after cooling and prior to the methanol wash.

EXAMPLE 5

The product of Example 1 is coated with 100 mL of dichloromethane containing 1 gram of divinylbenzene and 10 mg of benzoylperoxide. The dichloromethane is removed by rotary evaporation until the monomer is coated onto the beads. While rotating very slowly, the temperature is increased to 70°C for 8 hours. The product is washed with methanol.

This procedure is repeated with the product of Example 4.

EXAMPLE 6

The procedure of Example 5 is repeated with stearyldivinyl benzene in place of divinylbenzene. This procedure is repeated with the product of Example 4.

5 EXAMPLE 7

Fifteen (15) grams of the nonporous silica particles, 50 mL of 2,2,4-trimethylpentane, and 25 mL of vinyltrichlorosilane are refluxed for 2 hours. The modified silica is then washed several times with both 2,2,4-trimethylpentane and acetone and dried at 80°C.

10 Five (5) grams of the vinyl-coated silica particles prepared as described above are placed in a round bottom flask. Twenty-five mL of acetonitrile containing 2 g of a vinyl monomer (divinylbenzene, styrene, acrylonitrile, acrylic acid, butyl methacrylate, or 2-hydroxy methacrylate) are added and the mixture well dispersed. Twenty-five mL of acetonitrile 15 containing 0.2 g of dibenzoyl peroxide is added, and the mixture is refluxed for 2 hours.

The products are extracted with acetonitrile and then acetone to remove unreacted monomers and oligomers from the particle.

In the case of the acrylic acid-modified silica, extractions with water are 20 also carried out.

The packing materials are dried at 80°C prior to packing.

EXAMPLE 8

Standard Procedure for Testing the Performance of Separation Media

Separation particles are packed in an HPLC column and tested for 25 their ability to separate a standard DNA mixture. The standard mixture is a

pUC18 DNA-*Hae*III digest (Sigma-Aldrich, D6293) which contains 11 fragments having 11, 18, 80, 102, 174, 257, 267, 298, 434, 458, and 587 base pairs, respectively. The standard is diluted with water and five µL, containing a total mass of DNA of 0.25 µg, is injected.

5 Depending on the packing volume and packing polarity, the procedure requires selection of the driving solvent concentration, pH, and temperature. The separation conditions are adjusted so that the retention time of the 257, 267 peaks is about 6 to 10 minutes. Any one of the following solvents can be used: methanol, ethanol, 2-propanol, 1-propanol, tetrahydrofuran (THF), or

10 acetonitrile. A counter ion agent is selected from trialkylamine acetate, trialkylamine carbonate, trialkylamine phosphate, or any other type of cation that can form a matched ion with the polynucleotide anion.

As an example of this procedure, FIG. 4 shows the high resolution of the standard DNA mixture using octadecyl modified, nonporous

15 poly(ethylvinylbenzene-divinylbenzene) beads. The separation was conducted under the following conditions: Eluent A: 0.1 M TEAA, pH 7.0; Eluent B: 0.1 M TEAA, 25% acetonitrile; Gradient:

Time (min)	%A	%B
0.0	65	35
3.0	45	55
10.0	35	65
13.0	35	65
14.0	0	100
15.5	0	100
16.5	65	35

The flow rate was 0.75 mL/min, detection UV at 260 nm, column temp. 50°C. The pH was 7.0.

As another example of this procedure using the same separation conditions as in FIG. 4, FIG. 5 is a high resolution separation of the standard
5 DNA mixture on a column containing nonporous 2.1 micron beads of underivatized poly(styrene-divinylbenzene).

EXAMPLE 9

This example demonstrates the high resolution separation of DNA restriction fragments using octadecyl modified, nonporous silica reverse
10 phase material, as described in Example 1. The experiment is conducted under the following conditions: Column: 50x4.6 mm i.d. Mobile phase: 0.1 M TEAA, pH 7.0. Gradient: 8.75 - 11.25% acetonitrile in 2 minutes, followed by 11.25 - 14.25% acetonitrile in 10 minutes, 14.5 - 15.25% acetonitrile in 4 minutes, and by 15.25 - 16.25% acetonitrile in 4 minutes. Flow rate 1
15 mL/min. Column temperature: 50°C. Detection: UV at 254 nm. Sample: Mixture of 0.75 µg pBR322 DNA-*Hae*III restriction digest and 0.65 µg Φx174 DNA-*Hinc* II restriction digest.

A high resolution separation is obtained by optimizing the concentration of triethylammonium acetate (TEAA), shape of the gradient curve, column temperature, and flow rate. The resolution of peaks is continuously enhanced in going from 25 mM to at least 125 mM of TEAA.
5 The gradient is optimized by decreasing the steepness of the gradient curve with increasing fragment lengths of DNA molecules. The best separations of double-stranded DNA molecules are accomplished at about 30°C to 50°C. Denaturation of DNA at higher than about 50°C prevents utilization of higher column temperatures for double-stranded DNA fragments, although single-
10 stranded DNA separations can be performed at temperatures up to 80°C and higher.

EXAMPLE 10

If the gradient delay volume is minimized, the separation of PCR products and hybrid DNA derived from various sources of DNA, including
15 living and dead organisms (animal and plant), as well as parts of such organisms (*e.g.*, blood cells, biopsies, sperm, etc.) on octadecyl modified, nonporous poly-(ethylvinylbenzene-divinylbenzene) coated beads can be achieved with run times under 2 minutes.

The analysis of PCR products and hybrid DNA usually requires only
20 separation and detection of one or two species of known length. Because of this, the resolution requirements are considerably less severe than for separations of DNA restriction fragments. Such less stringent resolution requirements allow the utilization of steep gradients and, consequently, lead to still shorter run times. The recovery rate for a DNA fragment containing
25 404 base pairs is about 97.5%.

Unlike capillary electrophoresis (CE), PCR samples do not have to be desalted prior to analysis by MIPC. This represents a decisive advantage of MIPC over CE. With MIPC, it is thus possible to achieve a fully automated analysis of PCR samples if an automatic autosampler is utilized. Moreover, 5 since the volume of sample injection is known, in contrast to CE, quantitation over several orders of magnitude can be achieved without the need for an internal standard, hence allowing the quantitation of gene expression, as well as the determination of virus titers in tissues and body fluids. A fully automated version of the method of the invention can be used to discriminate 10 (distinguish) normal from mutated genes, as well as to detect oncogenes, bacterial and viral genome polynucleotides (hepatitis C virus, HIV, tuberculosis) for diagnostic purposes. Moreover, adjustment of column temperature allows one to moderate the stringency of hybridization reactions or to separate heteroduplex from homoduplex DNA species.

15 The suitability of the polymer-coated beads of the invention for clinical use is described under the following conditions: Column: 50x4.6 mm i.d. Mobile phase: 0.1 M TEAA, pH 7.0. Gradient: 11.25 - 13.75% acetonitrile in 1 minute, followed by 22.5% acetonitrile for 6 seconds, and 11.25% acetonitrile for 54 seconds. Flow rate: 3 mL/min. Column temperature: 20 50°C. Detection: UV at 256 nm. Sample: 20 µl of a PCR sample. In the separation, the following elution order is obtained: 1=unspecific PCR product, 2=PCR product having 120 base pairs, 3=PCR product having 132 base pairs, and 4=PCR product having 167 base pairs.

PCR methods and processes are described by R. K. Saiki et al. in 25 *Science*, 23):1350-1354 (1985) and K. B. Mullis in U.S. Patent No. 4,863,202.

These references are incorporated herein by reference for a more complete description of methods and processes for obtaining PCR samples which can be separated using the method of the present invention.

The repetitive analysis of PCR products using the method of the
5 invention is highly reproducible under the described analytical conditions. The results are not in any way influenced by the preceding injection. The present method is highly suitable for routine use under real conditions in clinical laboratories.

EXAMPLE 11

10 The following describes a separation of single-stranded DNA. A silica-C18 column, as described in Example 1, 1.5 micron, 30x4.6 mm i.d., is used with a linear gradient of 2.5 - 12.5% acetonitrile in 0.1 M triethylammonium acetate in 40 minutes at 1 mL/min and 40°C. A mixture of p(dC)12-18 and p(dT)12-18 oligonucleotides is separated, with the first mixture eluting
15 between 5 and 15 minutes, and the second mixture eluting between 15 and 30 minutes.

EXAMPLE 12

Sorption Enthalpy Measurements

Four fragments (174 base pair, 257 base pair, 267 base pair, and 298
20 base pair, found in 5 µl pUC18 DNA-*Hae*III digest, 0.04 µg DNA/µl) of a DNA digest are separated under isocratical conditions at different temperatures using C-18 alkylated poly(styrene-divinylbenzene) polymer beads. Conditions used for the separation are: Eluent: 0.1 M triethylammonium acetate, 14.25% (v/v) acetonitrile at 0.75 mL/min, detection at 250 nm UV,
25 temperatures at 35, 40, 45, 50, 55, and 60°C, respectively. A plot of ln k

versus *1/T* (FIG. 6) shows that the retention factor *k* is increasing with increasing temperature. This indicates that the retention mechanism is based on an endothermic process ($\Delta H_{sorp} > 0$).

The same experiments on non-alkylated poly(styrene-divinylbenzene)
5 beads (FIG. 7) give a negative slope for a plot of ln *k* versus *1/T*, although the plot is slightly curved.

The same experiments on alkylated poly(styrene-divinylbenzene) beads but the acetonitrile solvent is substituted with methanol (FIG. 8) gives a plot ln *k* versus *1/T* shows the retention factor k is decreasing with increasing
10 temperature. This indicates the retention mechanism is based on an exothermic process ($\Delta H_{sorp} < 0$). Replacing the alkylated and non-alkylated polymer beads with silica beads having a coating of alkylated poly(styrene-divinylbenzene) and non-alkylated alkylated poly(styrene-divinylbenzene) will give the same results.

15 EXAMPLE 13

Separations with alkylated poly(styrene-divinylbenzene) beads

Eluents are chosen to match the desorption ability of the elution solvent to the attraction properties of the bead to the DNA-counter ion complex. As the polarity of the bead decreases, a stronger (more organic) or
20 higher concentration of solvent will be required. Weaker organic solvents such as methanol are generally required at higher concentrations than stronger organic solvents such as acetonitrile.

FIG. 9 shows the high resolution separation of DNA restriction fragments using octadecyl modified, nonporous poly(ethylvinylbenzene-
25 divinylbenzene) beads. The experiment was conducted under the following

conditions. Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M tetraethylacetic acid (TEAA), pH 7.2; gradient: 33-55% acetonitrile in 3 min, 55-66% acetonitrile in 7 min, 65% acetonitrile for 2.5 min; 65-100% acetonitrile in 1 min; and 100-35% acetonitrile in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, column temp. 51°C. The sample was 5 µl (= 0.2 µg pUC18 Hea III digest).

Repeating the above procedure replacing the acetonitrile with 50.0% methanol in 0.1 M TEAA gives the separation shown in FIG. 10.

Repeating the above procedure replacing the acetonitrile with 25.0% ethanol in 0.1 M TEAA gives the separation shown in FIG. 11.

Repeating the above procedure replacing the acetonitrile with 25% vodka (Stolichnaya, 100 proof) in 0.1 M TEAA gives the separation shown in FIG. 12.

The separation shown in FIG. 13 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M TEAA, pH 7.3; gradient: 12-18% 0.1 M TEAA and 25.0% 1-propanol (Eluent B) in 3 min, 18-22% B in 7 min, 22% B for 2.5 min; 22-100% B in 1 min; and 100-12% B in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µl (= 0.2 µg pUC18 DNA-*Hae*III digest).

The separation shown in FIG. 14 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M TEAA, pH 7.3; gradient: 15-18% 0.1 M TEAA and 25.0% 1-propanol (Eluent B) in 2 min, 18-21% B in 8 min, 21% B for 2.5 min; 21-100% B in 1 min; and 100-15% B in

1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µl (= 0.2 µg pUC18 DNA-*Hae*III digest).

The separation shown in FIG. 15 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M TEAA, pH 7.3; gradient: 35-55% 0.1 M TEAA and 10.0% 2-propanol (Eluent B) in 3 min, 55-65 % B in 10 min, 65% B for 2.5 min; 65-100% B in 1 min; and 100-35% B in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µl (= 0.2 µg pUC18 DNA-*Hae*III digest).

The separation shown in FIG. 16 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.05 M TEA_2HPO_4, pH 7.3; gradient: 35-55% 0.05 M TEA_2HPO_4 and 10.0% 2-propanol (Eluent B) in 3 min, 55-65% B in 7 min, 65% B for 2.5 min; 65-100% B in 1 min; and 100-65% B in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µl (= 0.2 µg pUC18 DNA-*Hae*III digest).

The separation shown in FIG. 17 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M TEAA, pH 7.3; gradient: 6-9% 0.1 M TEAA and 25.0% THF (Eluent B) in 3 min, 9-11 % B in 7 min, 11% B for 2.5 min; 11-100% B in 1 min; and 100-6% B in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µl (= 0.2 µg pUC18 DNA-*Hae*III digest).

EXAMPLE 14

Isocratic/gradient separation of dsDNA

The following is an isocratic/gradient separation of dsDNA on a polystyrene coated silica base material. Isocratic separations have not been performed in DNA separations because of the large differences in the selectivity of DNA/alkylammonium ion pair for beads. However, by using a combination of gradient and isocratic elution conditions, the resolving power of a system can be enhanced for a particular size range of DNA. For example, the range of 250-300 base pairs can be targeted by using an eluent of 0.1 M TEAA, and 14.25% acetonitrile at 0.75 mL/min at 40°C on 50 x 4.6 mm crosslinked polystyrene coated silica reverse phase column, 2.0 micron. The pUC18 DNA-*Hae*III digest was injected under isocratic conditions and 257, 267 and 298 base pairs DNA eluted completely resolved. Then the column was cleaned from larger fragments with 0.1M TEAA/25% acetonitrile at 9 minutes. FIG. 18 shows a separation using the same elution conditions but performed on a poly(styrene-divinylbenzene) polymer based column. In other examples, there might be an initial isocratic step (to condition the column), then a gradient step (to remove or target the first group of DNA at a particular size), then an isocratic step (to separate the target material of a different size range) and finally a gradient step to clean the column.

While the foregoing has presented specific embodiments of the present invention, it is to be understood that these embodiments have been presented by way of example only. It is expected that others will perceive and practice variations which, though differing from the foregoing, do not

depart from the spirit and scope of the invention as described and claimed herein.

The claims defining the invention are as follows:

1. A method for separating a mixture of double-stranded polynucleotides, comprising:

a) flowing a mixture of said polynucleotides having up to 1500 base pairs through a separation column containing beads which are a member selected from the group consisting of silica, silica carbide, silica nitrite, titanium oxide, aluminium oxide, zirconium oxide, carbon, insoluble polysaccharide, and diatomaceous earth, the beads having an average diameter of 0.5 to 100μm, the beads comprising particles coated with a hydrocarbon polymer or a non-polar hydrocarbon substituted polymer, or particles having substantially all polar groups reacted with a non-polar hydrocarbon or substituted hydrocarbon group, wherein the particles are substantially free from multivalent cations which are free to interfere with polynucleotide separation, and the particles have a DNA Separation Factor of at least 0.05; and

b) separating said mixture of polynucleotides.

2. The method of claim 1, wherein the beads are silica.

3. The method of claim 1 or claim 2, wherein the mixture of polynucleotides is in an eluant solution containing a chelating agent.

4. The method of any one of claims 1 to 3, wherein the mixture of polynucleotides includes a counter ion selected from the group consisting of trialkylamine acetate, trialkylamine carbonate, a trialkylamine phosphate, and mixtures thereof.

5. The method of any one of claims 1 to 4, wherein the beads are non-porous.

6. The method of any one of claims 1 to 5, wherein the beads have been contacted with a solution containing a trialkylamine phosphate.

7. A method for separating a mixture of double-stranded polynucleotides, said method being substantially as hereinbefore described with reference to any one of the examples.

8. A separation column containing beads for use in a method for separating a mixture of double-stranded polynucleotides wherein the beads are selected from the group consisting of silica, silica carbide, silica nitrite, titanium oxide, aluminium oxide, zirconium oxide, carbon, insoluble polysaccharide, and diatomaceous earth, the beads comprising particles coated with a hydrocarbon polymer or a non-polar hydrocarbon substituted polymer, or particles having substantially all polar groups reacted with a non-polar hydrocarbon or substituted hydrocarbon group, wherein the particles are substantially free from multivalent cations which are free to interfere with polynucleotide separation and have a separation factor of 0.5.

9. The separation column of claim 8, wherein the beads are silica.

10. The separation column of claim 8 or claim 9, wherein the beads have had contact with a chelating agent.

11. The separation column of any one of claims 8 to 10, wherein the beads have had contact with a trialkylphosphate.

12. A separation column containing beads for use in a method for separating a mixture of double-stranded polynucleotides, said column being substantially as hereinbefore described with reference to any one of the examples.

13. Beads for use in a method for separating a mixture of double-stranded polynucleotides wherein the beads are selected from the group consisting of silica, silica carbide, silica nitrite, titanium oxide, aluminium oxide, zirconium oxide, carbon, insoluble polysaccharide, and diatomaceous earth, the beads comprising particles coated with a hydrocarbon polymer or a non-polar hydrocarbon
5 substituted polymer, or particles having substantially all polar groups reacted with a non-polar hydrocarbon or substituted hydrocarbon group, wherein the particles are substantially free from multivalent cations which are free to interfere with polynucleotide separation and have a separation factor of 0.5.

14. The beads of claim 13, wherein the beads are silica.

10 15. The beads of claim 13 or claim 14, wherein the beads have had contact with a chelating agent.

16. The silica beads of any one of claims 13 to 15, wherein the beads have had contact with a trialkylphosphate.

17. Beads for use in a method for separating a mixture of double-stranded polynucleotide,
15 said beads being substantially as hereinbefore described with reference to any one of the examples.

Dated 15 November 1999
TRANSGENOMIC, INC.

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

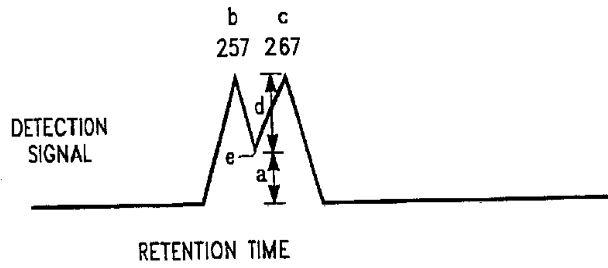

FIG.–1

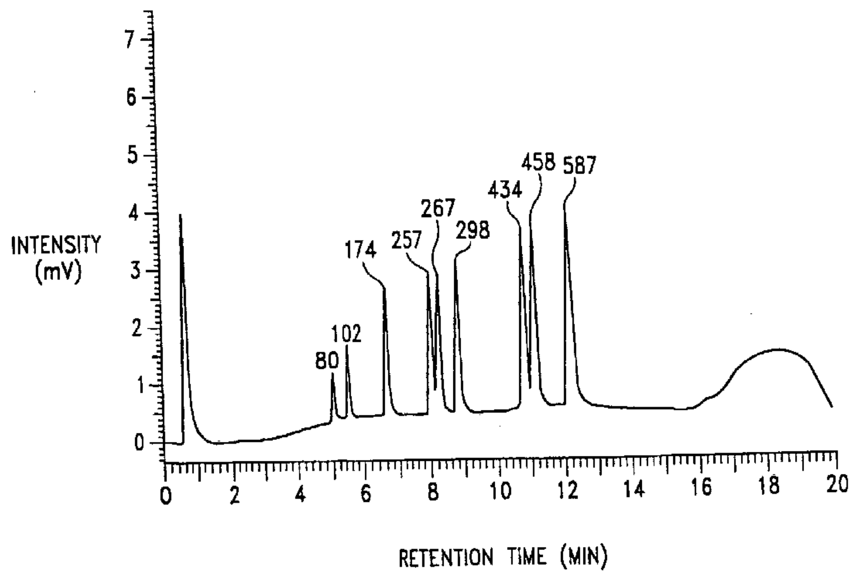

FIG.–4

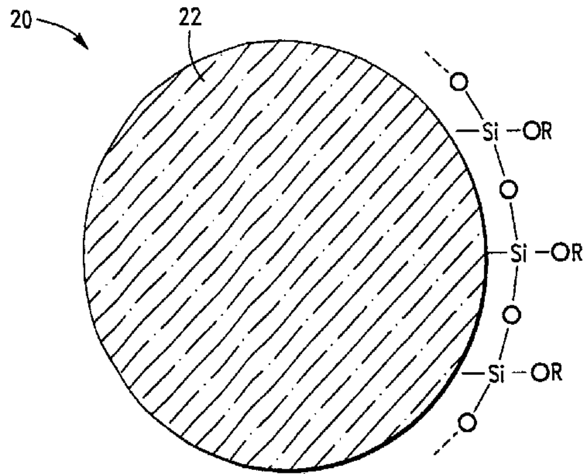

FIG.–2

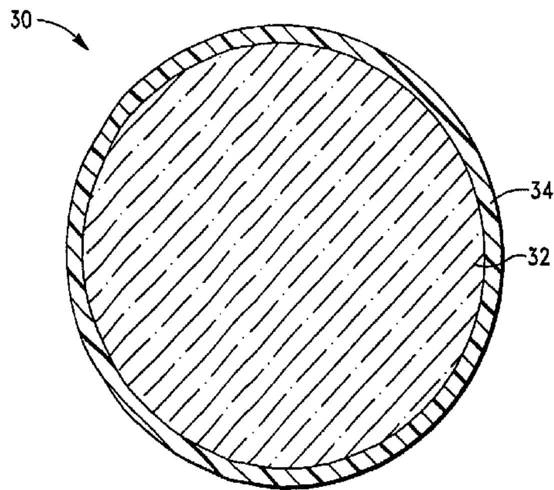

FIG.–3

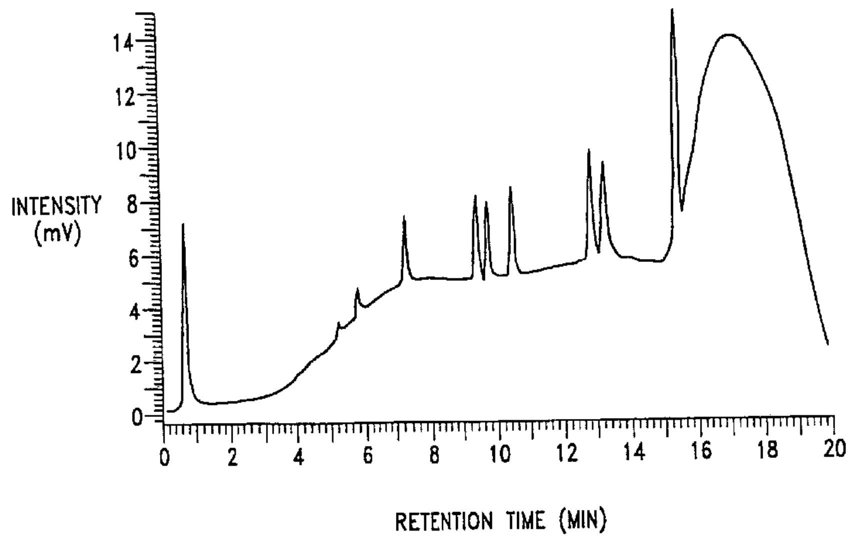

FIG.–5

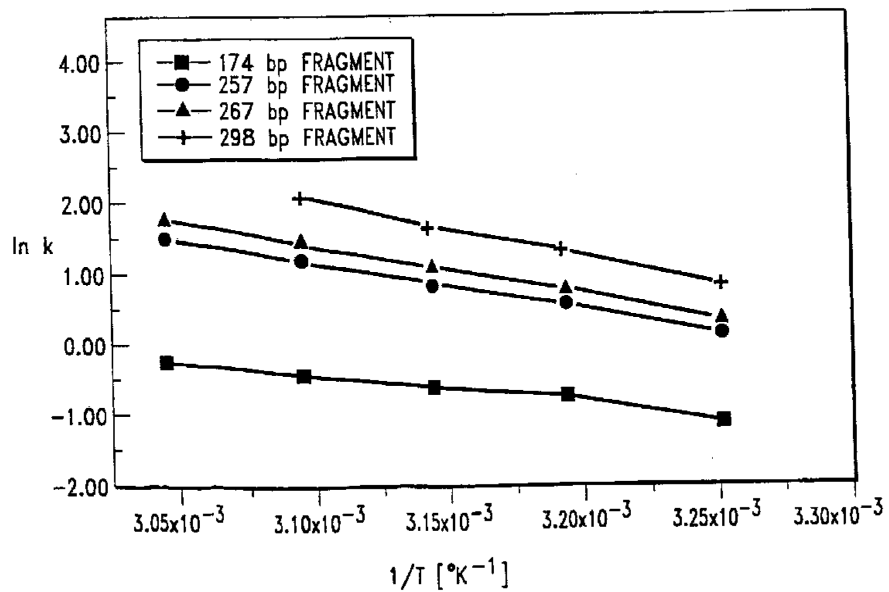

FIG.–6

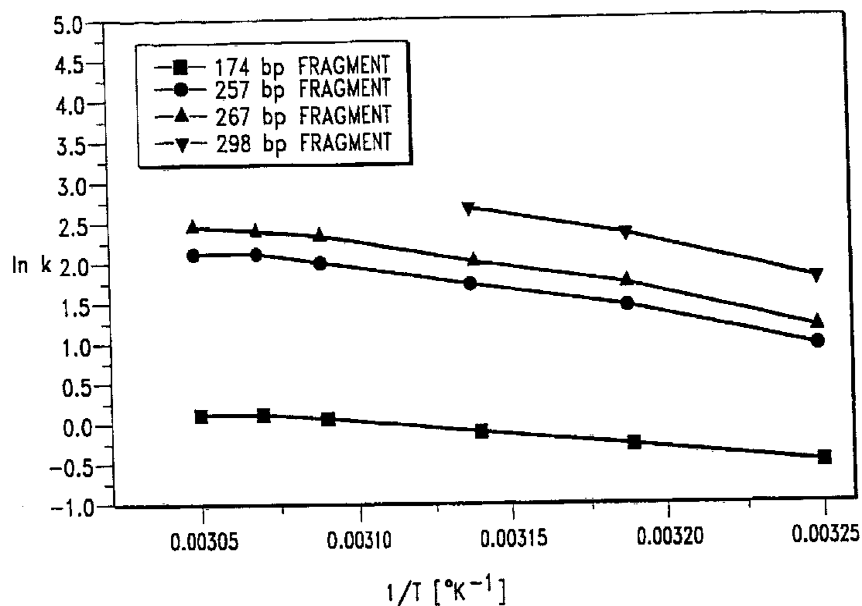

FIG.-7

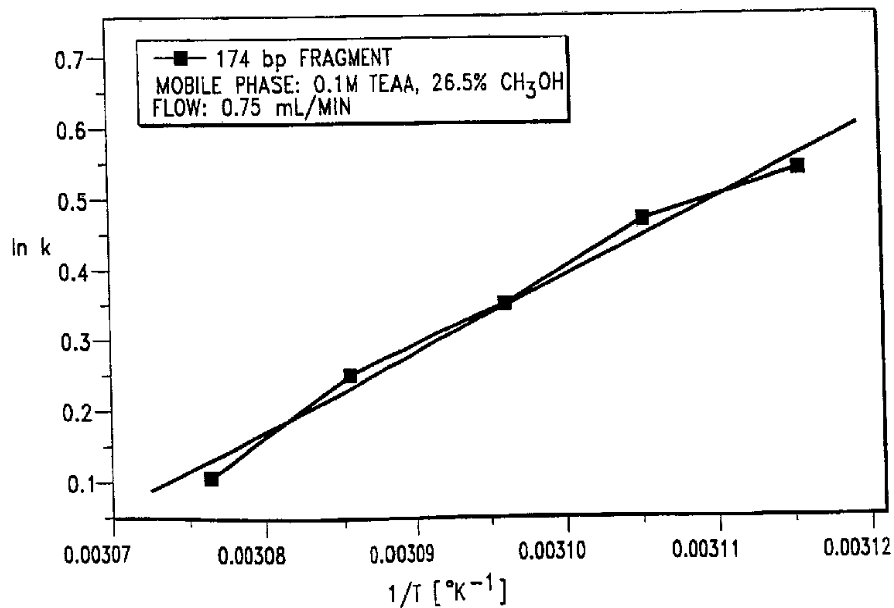

FIG.-8

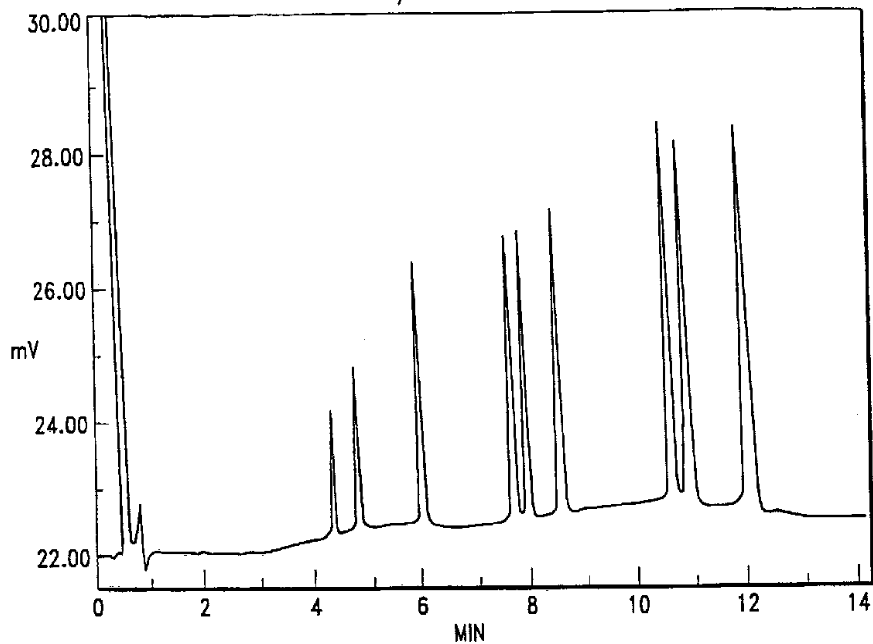

FIG.–9

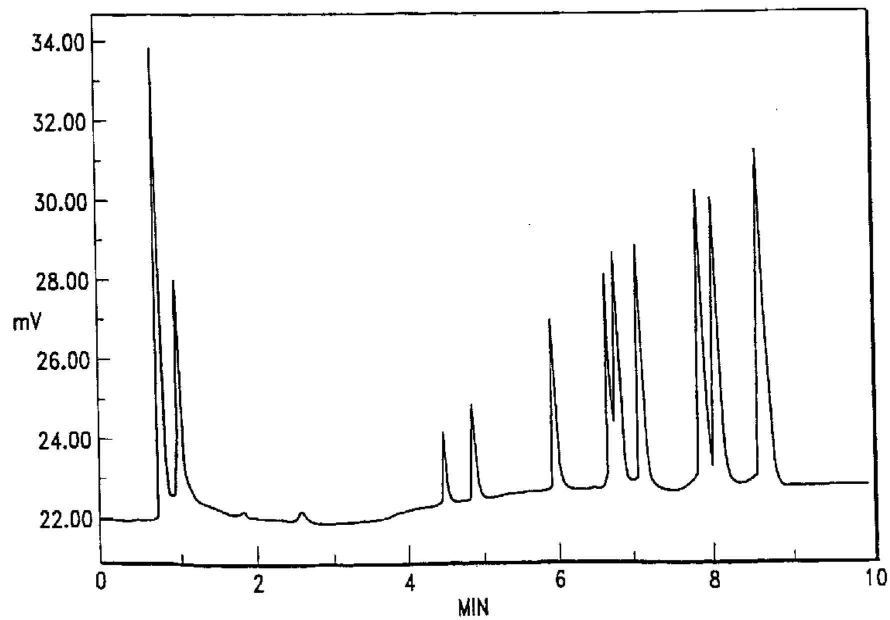

FIG.–10

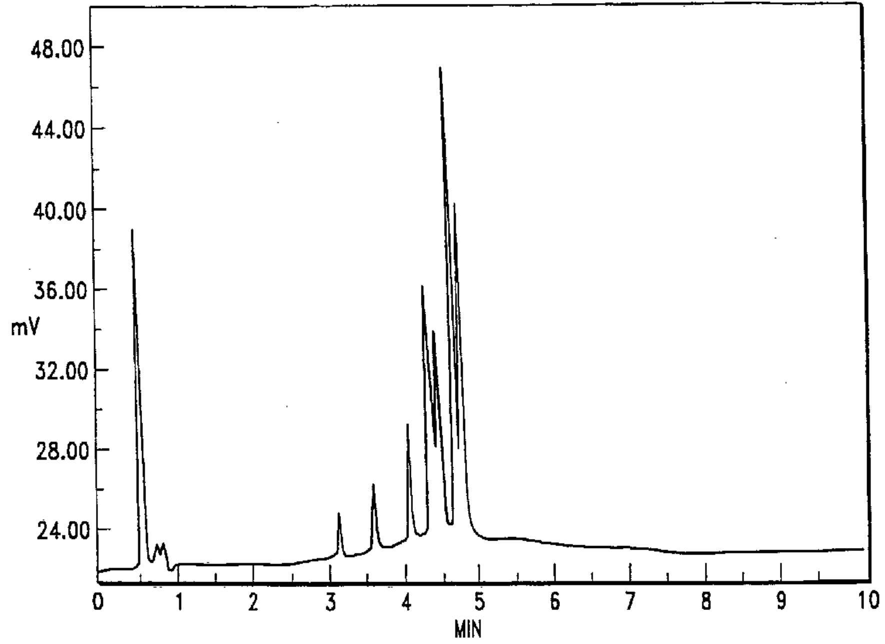

FIG.-11

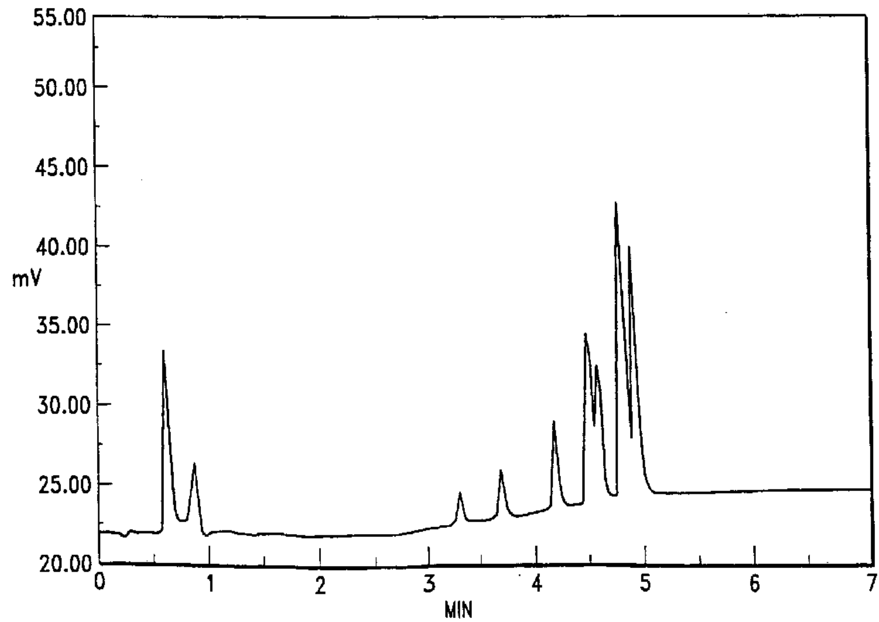

FIG.-12

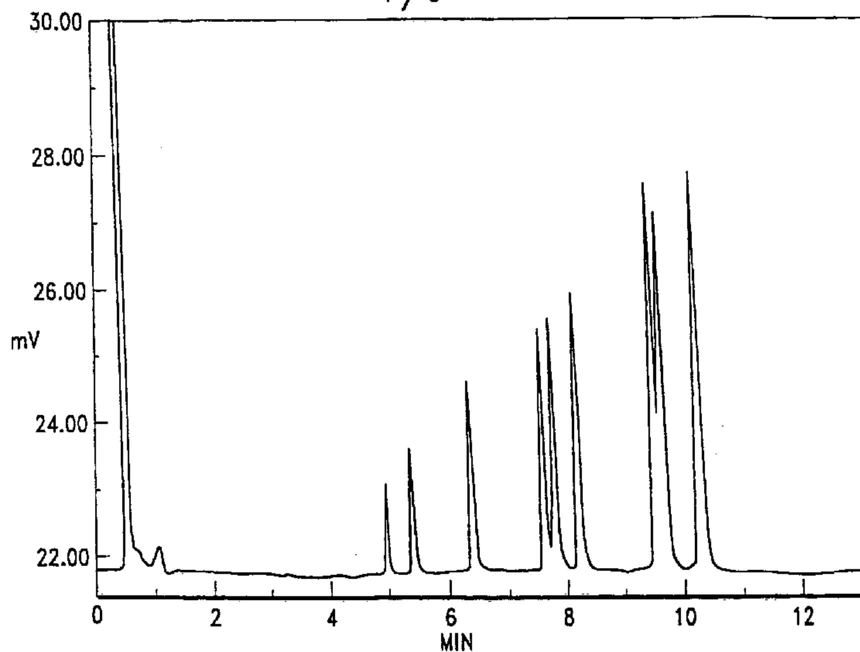

FIG.–13

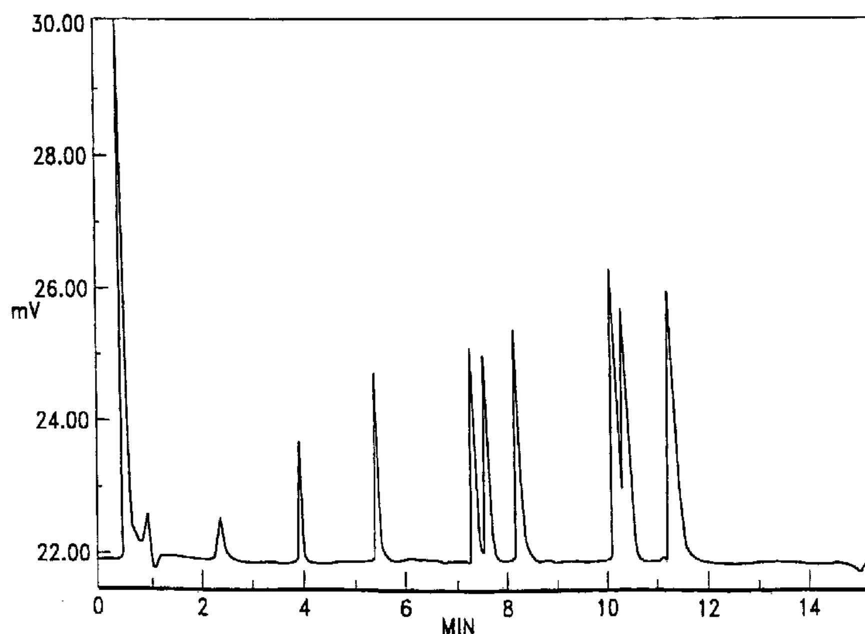

FIG.–14

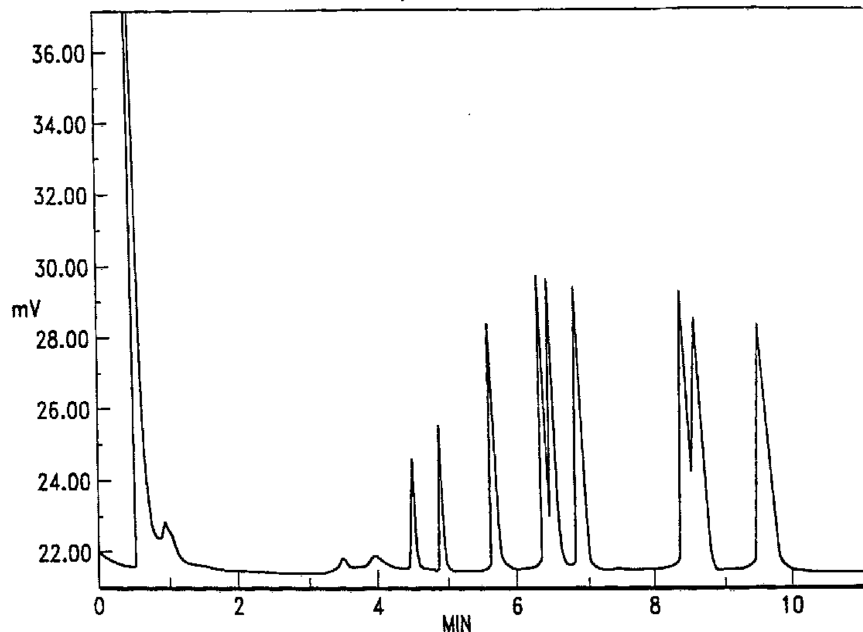

FIG.-15

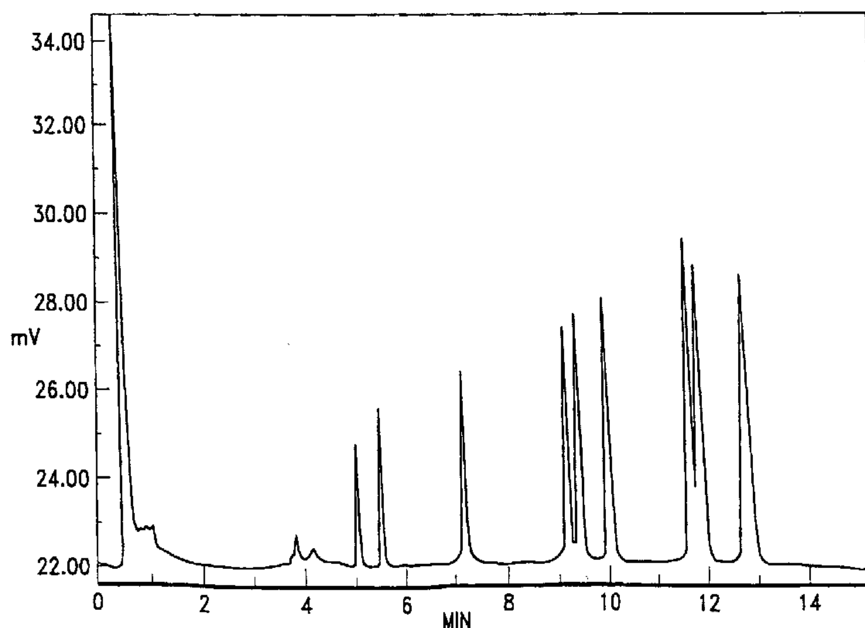

FIG.-16

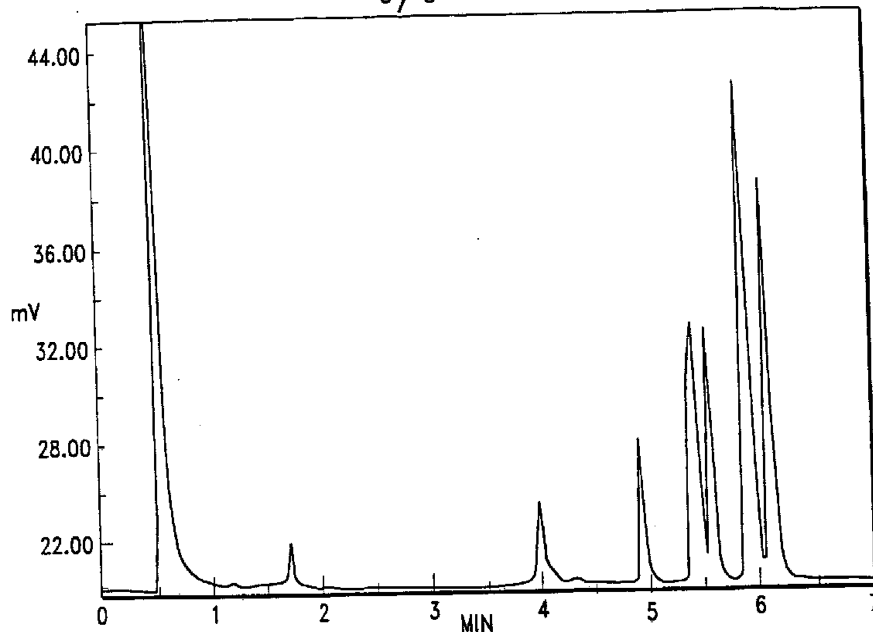

FIG.–17

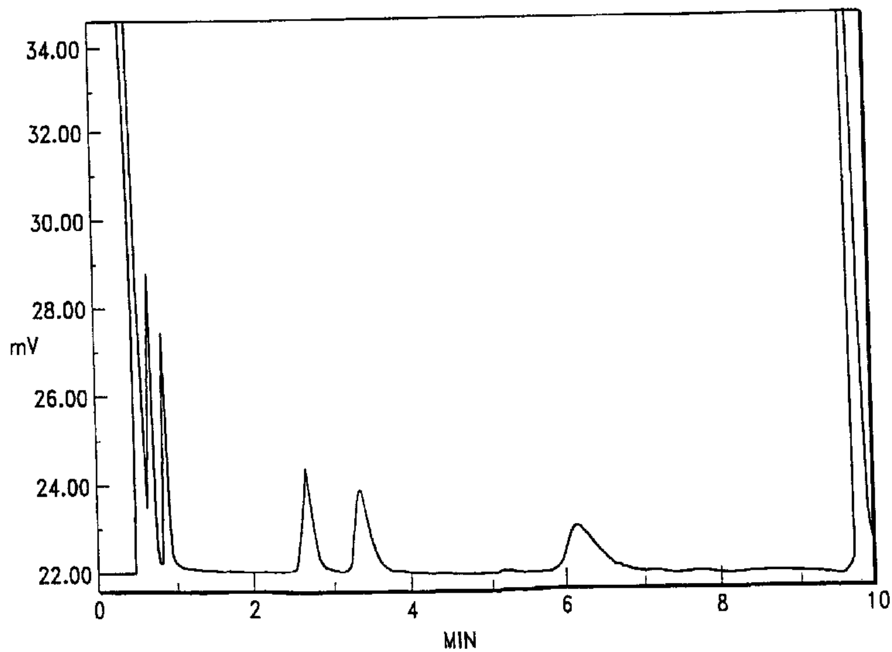

FIG.–18