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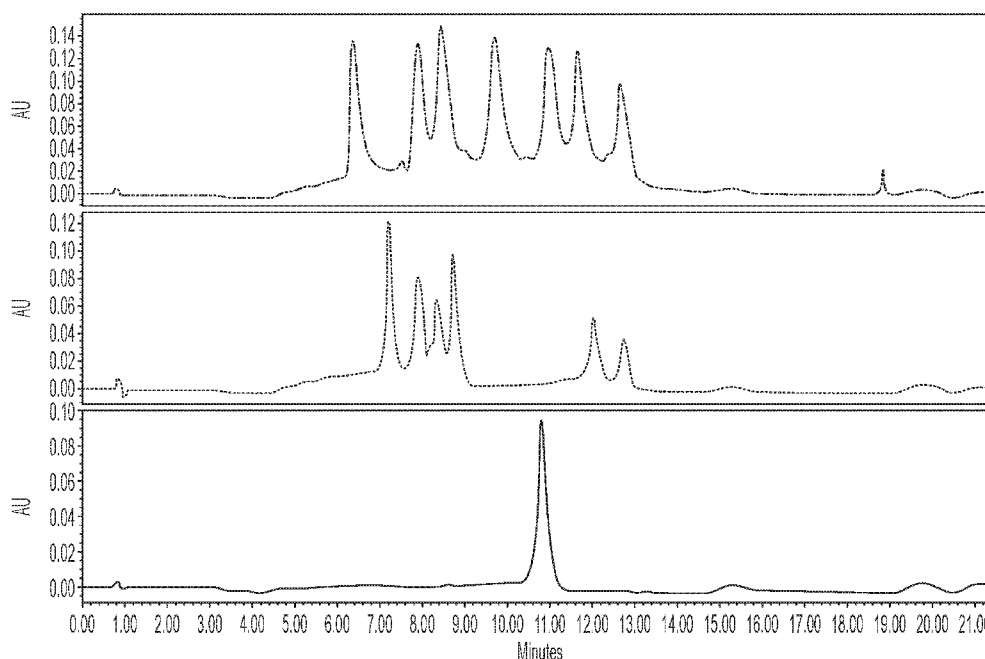


Figure 7

(57) **Abstract:** Aspects of the disclosure relate to liquid chromatography (e.g., HPLC) methods which enable high resolution separations of polynucleotides of various lengths, sequences, and/or base compositions in a highly tunable manner. In some embodiments, the disclosure describes liquid chromatographic methods for separating a nucleic acid (e.g., a polyadenylated nucleic acid, such as an mRNA) from a complex mixture by using multiple ion pairing agents in the same mobile phase system. Accordingly, in some embodiments methods described by the disclosure are useful for assessing the quality of pharmaceutical preparations comprising nucleic acids.

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ANALYTICAL HPLC METHODS

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of the filing date of U.S. Provisional Application Serial No. 62/547,462, filed August 18, 2017, entitled
5 “ANALYTICAL HPLC METHODS”, the entire contents of which are incorporated herein by reference.

BACKGROUND

Several methods of analyzing the purity of polynucleotides are known. For example, capillary electrophoresis (CE) is a methodology that is generally acceptable for RNAs <1500 nt
10 (more notably <1000 nt) in length; however, CE often demonstrates reduced resolution for larger polynucleotides and impurities must be present in high abundance to be detected. Additionally, methods in the literature describing RNA analysis on CE using custom gel matrices do not have the resolution seen in High Performance Liquid Chromatography (HPLC). Gel electrophoresis alone also exhibits low resolution for long polynucleotides.

15 HPLC methods utilized for large polynucleotides, such as mRNAs, may also have inferior resolution. For example, multiple polyadenylated RNAs in the same mixture (e.g. a mixture of multiple RNAs each having the same polyA tail size) may be challenging to resolve or coelute. Furthermore, temperature-dependent recovery often results in loss of resolution with long constructs. On-column sample degradation is also commonly observed when HPLC
20 methods are utilized.

SUMMARY

In some aspects, the disclosure relates to liquid chromatography (e.g., HPLC) methods which enable high resolution separations of polynucleotides of various lengths, sequences, and/or base compositions in a highly tunable manner. In some embodiments, methods described
25 by the disclosure are novel improvements of ion-pair reversed phase (IP-RP) HPLC methods. Without wishing to be bound by any particular theory, methods described by the disclosure are useful, in some embodiments, for assessing purity and monitoring integrity of long polynucleotide preparations (e.g., RNA generated by in vitro transcription), as well as analyzing compositions containing multiple polynucleotides of discreet sizes in varying ratios within a
30 single preparation.

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In some embodiments, methods described by the disclosure use multiple ion pairs in the same mobile phase system to tune selectivity of the chromatography and optimize resolution. The disclosure is based, in part, on the discovery that combining a size-selective ion pair with a composition-selective ion pair simultaneously drives nucleic acid selectivity toward a size-based separation with varying levels of resolution, while also shifting separation bias toward base composition to attain improved resolution over each as a single component. A size-selective ion pair can be characterized as a larger, bulkier, more hydrophobic ion pairing agent such as dibutylamine or a salt (e.g., an ammonium salt) thereof (for example, dibutylammonium acetate, (“DBAA”)) or hexylamine or a salt (e.g., an ammonium salt) thereof (for example hexylammonium acetate (“HAA”)). A composition-selective ion pair can be characterized as a smaller, less bulky ion pairing agent with shorter alkyl chains and less overall hydrophobicity, such as trimethylamine or a salt (e.g., an ammonium salt) thereof (for example, triethylammonium acetate, (“TEAA”)). Thus, in some embodiments, methods described by the disclosure are useful for high resolution analysis of long, polyadenylated polynucleotide preparations, for example pharmaceutical compositions comprising mRNA.

Accordingly, in some embodiments, the disclosure provides a method of separating a nucleic acid from a mixture comprising one or more additional nucleic acids or impurities, comprising the steps: contacting a stationary phase of a reverse phase chromatography column with the mixture; and eluting the nucleic acid with a mobile phase, wherein the mobile phase comprises one or more solvents and two or more ion pairs, such that the nucleic acid traverses the column with a retention time that is different than one or more other nucleic acids of the mixture.

In some embodiments, a column is an analytical column, or a preparative column. In some embodiments, a stationary phase comprises particles, such as porous resin particles. In some embodiments, particles are hydrophobic (e.g., comprise an intrinsically hydrophobic material such as polystyrene divinylbenzene) or comprise hydrophobic functional groups.

In some embodiments, particles have a diameter of about 2 μm to about 10 μm , about 2 μm to about 6 μm , or about 4 μm . In some embodiments, particles comprise pores having a diameter of about 100 $\text{\AA}$ to about 5000 $\text{\AA}$, about 100 $\text{\AA}$ to about 1000 $\text{\AA}$, or about 1000 $\text{\AA}$ to about 2000 $\text{\AA}$.

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In some embodiments, a mobile phase comprises one solvent. In some embodiments, a mobile phase is a mixture of a first solvent and a second solvent. In some embodiments, a mobile phase is a mixture of a first solvent solution and a second solvent solution.

In some embodiments, the volume percentage of a solvent solution (e.g., a first solvent solution or a second solvent solution) in a mobile phase is 0% to 100%. In some embodiments, a solvent solution (e.g., a first solvent solution or a second solvent solution) comprises one or more solvents and one or more ion pair (e.g., one or more ion pairing agent).

In some embodiments, the concentration of each of the one or more ion pair (e.g., each ion pairing agent) in a solvent solution (e.g., a first solvent solution or a second solvent solution) ranges from about 1 mM to about 2 M. In some embodiments, the concentration of each of the one or more ion pair (e.g., each ion pairing agent) in a solvent solution (e.g., a first solvent solution or a second solvent solution) ranges from about 1 mM-100 mM, 5 mM-100 mM, 5 mM-75 mM, 5 mM-50 mM, 5 mM-25 mM, 5 mM-10 mM, 10 mM-25 mM, 25 mM-50 mM, 25 mM-75 mM, 50 mM-100 mM or 75 mM-100 mM.

In some embodiments, one or more ion pair (e.g., ion pairing agent) is a cation selected from the group consisting of: trimethylamine or a salt thereof (e.g., triethylammonium salt), tetrabutylamine or a salt thereof (e.g., tetrabutylammonium salt), hexylamine or a salt thereof (e.g., hexylammonium salt), and dibutylamine or a salt thereof (e.g., dibutylammonium salt). In some embodiments, the triethylammonium salt is triethylammonium acetate (TEAA), the tetrabutylammonium salt is tetrabutylammonium phosphate (TBAP), the hexylammonium ion is hexylammonium acetate (HAA), or the dibutylammonium ion is dibutylammonium acetate (DBAA). In some embodiments, the one or more ion pairing agent is an inorganic molecule, for example unsubstituted ammonium salts (NH_4^+), lithium (Li), sodium (Na), potassium (K), etc., an organic cation (e.g., substituted ammonium ion), tris(hydroxymethyl)aminomethane (Tris), biological buffer (e.g., MOPS, HEPES, PIPES, etc.), or other charged or hydrophilic moiety.

In some embodiments, a solvent solution (e.g., a first solvent solution or a second solvent solution) comprises of one or more solvents selected from the group consisting of water, polar aprotic solvents (including, e.g., tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), acetonitrile, acetone, etc.), C_{1-4} alkanols, C_{1-6} alkandriols, and C_{2-4} alkanolic acids. In some embodiments, a solvent solution (e.g., a first solvent solution or a second solvent solution) comprises one or more solvents selected from the group consisting of water, acetonitrile, methanol, ethanol, isopropanol, and hexylene glycol.

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In some embodiments of methods described by the disclosure, the step of eluting the nucleic acid with a mobile phase comprises the step of passing a mobile phase through the column, wherein the mobile phase is a mixture of a first solvent solution and a second solvent solution, and wherein the first solvent solution and the second solvent solution independently
5 comprise one or more ion pair (e.g., one or more ion pairing agent) selected from the group consisting of: trimethylamine or a salt thereof (e.g., triethylammonium acetate), tetrabutylamine or a salt thereof (e.g., tetrabutylammonium acetate), hexylamine or salt thereof (e.g., hexylammonium acetate) and dibutylamine or a salt thereof (e.g., dibutylammonium acetate), such that a nucleic acid traverses the column with a retention time that is different than one or
10 more other nucleic acids of the mixture. In some embodiments, the trimethylamine salt is triethylammonium acetate (TEAA), the tetrabutylamine salt is tetrabutylammonium phosphate (TBAP), the hexylamine salt is hexylammonium acetate (HAA), or the dibutylamine salt is dibutylammonium acetate (DBAA). In some embodiments, the one or more ion pairing agent is a non-organic molecule, for example unsubstituted ammonium, lithium (Li), sodium (Na),
15 potassium (K), etc., an organic cation (e.g., substituted ammonium ion), tris(hydroxymethyl)aminomethane (Tris), biological buffer (e.g., MOPS, HEPES, PIPES, etc.), or other charged or hydrophilic moiety.

In some embodiments, the ratio of a first solvent solution to a second solvent solution is held constant during elution of a nucleic acid. In some embodiments, the ratio of a first solvent
20 solution to a second solvent solution is increased or decreased during elution of the nucleic acid.

In some embodiments, the concentration of each ion pair (e.g., ion pairing agent) in a mobile phase is held constant during elution of the nucleic acid. In some embodiments, the concentration of one or more ion pair (e.g., ion pairing agent) in the mobile phase is not held constant during elution of the nucleic acid.

25 In some embodiments, the retention time of a nucleic acid differs from the retention time of all other nucleic acids or impurities in a mixture by 0.1-10 minutes, 0.1-5 minutes, 0.1-1 minutes, 0.1-0.5 minutes, or 0.1-0.2 minutes.

In some embodiments, a nucleic acid comprises at least 100, at least 500, at least 1000, at least 2000, at least 3000, at least 4000, at least 5000, at least 6000, at least 7000, at least 8000, or
30 at least 9000 nucleotides.

In some embodiments, a nucleic acid is single stranded. In some embodiments, a nucleic acid is RNA or DNA. In some embodiments, a nucleic acid comprises 5' and 3' untranslated

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regions (UTRs). In some embodiments, a nucleic acid comprises a 5' 7-methylguanosine group (e.g., a 5' cap), or a 5' 7-methylguanosine group analog (e.g., a 5' cap analog). In some embodiments, a nucleic acid comprises a 3'-polyadenosine (polyA) tail.

In some embodiments, a mixture comprises a plurality of nucleic acids having tail length variants. In some embodiments, a mixture comprises degradation products (e.g., nucleic acid degradation products).

In some embodiments, methods described by the disclosure further comprise the step of detecting or isolating a nucleic acid, or detecting or isolating one or more additional nucleic acids or impurities from a mixture. In some embodiments, methods described by the disclosure are used to determine the potency of the mixture of nucleic acids.

In some embodiments, a nucleic acid is larger or smaller than one or more additional nucleic acids or impurities (e.g., additional nucleic acids or impurities present in a mixture), and a mobile phase comprises at least one size-selective ion pair (e.g., at least one size-selective ion pairing agent).

In some embodiments, a larger nucleic acid comprises 10-100%, 25-100%, 50-100%, 50-75%, 100-200%, 200-500% or 500-1000% more nucleotides than one or more additional nucleic acids or impurities (e.g., additional nucleic acids or impurities present in a mixture).

In some embodiments, a smaller nucleic acid comprises 10-100%, 25-100%, 50-100%, 50-75%, 100-200%, 200-500% or 500-1000% fewer nucleotides than one or more additional nucleic acids or impurities (e.g., additional nucleic acids or impurities present in a mixture).

In some embodiments, a size-selective ion pair is selected from HAA, DBAA, and TBAP.

In some embodiments, a nucleic acid has a similar size as one or more additional nucleic acids or impurities (e.g., additional nucleic acids or impurities present in a mixture), and a mobile phase comprises at least one composition-selective ion pair (e.g., at least one composition-selective ion pairing agent).

In some embodiments, a nucleic acid comprises 0-10%, 0-5%, or 0-1% more or fewer nucleotides than one or more additional nucleic acids or impurities (e.g., additional nucleic acids or impurities present in a mixture).

In some embodiments, a composition-selective ion pair is TEAA.

In some embodiments, a nucleic acid and one or more additional nucleic acids (e.g., additional nucleic acids in a mixture) have lengths of less than 2000 nucleotides, wherein two or

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more ion pairs are triethylammonium (e.g., TEAA) and dibutylammonium (e.g., DBAA), and wherein the ratio of triethylammonium (e.g., TEAA) to dibutylammonium (e.g., DBAA) ranges from about 5:1 to about 1:5, about 3:1 to about 1:3, about 2:1 to about 1:2, or about 1:1 to 1:3.

In some embodiments of methods described by the disclosure, the eluting step is isocratic with respect to mobile phase solvent composition. In some embodiments of methods described by the disclosure, the eluting step is isocratic or gradient with respect to the concentration of one or more ion pairs (e.g., concentration of one or more ion pairing agents).

In some embodiments, a mobile phase comprises two ion pairs, and the relative concentration of the ion pairs is varied during an eluting step. In some embodiments, the eluting step is gradient with respect to mobile phase solvent composition. In some embodiments, the eluting step is isocratic or gradient with respect to the concentration of one or more ion pairs.

In some aspects, the disclosure provides a pure mRNA sample comprising: a composition of an in vitro transcribed (IVT) RNA and a pharmaceutically acceptable carrier, wherein the composition comprises a nucleic acid separated by a method as described by the disclosure.

In some aspects, the disclosure provides a method of quality control of a nucleic acid (e.g., a polyadenylated nucleic acid) pharmaceutical composition, the method comprising: separating a nucleic acid from a mixture comprising one or more additional nucleic acids or impurities by a method as described by the disclosure; comparing the separated nucleic acid with a reference nucleic acid; and determining the quality of the nucleic acid based on a comparison of the separated nucleic acid with the reference nucleic acid.

In some embodiments, the nucleic acid (e.g., polyadenylated nucleic acid) pharmaceutical composition comprises RNA, such as mRNA or in vitro transcribed (IVT) RNA.

In some embodiments, the method of quality control of a nucleic acid comprises the step of comparing a HPLC chromatogram of the separated nucleic acid with a HPLC chromatogram of the reference nucleic acid. In some embodiments, the nucleic acid separated from the mixture is RNA (e.g., mRNA, dsRNA, ssRNA, etc.), or DNA (e.g., dsDNA, ssDNA, etc.). In some embodiments, the method further comprises comparing the nucleic acid separated from the mixture with a reference nucleic acid using an analytical method, for example polymerase chain reaction (e.g., qRT-PCR), nucleic acid sequencing, gel electrophoresis, restriction digest-based analysis, mass spectrometry, etc.

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Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF DRAWINGS

Figures 1A-1D show representative HPLC chromatograms comparing three alkylammonium ion pairing agents (HAA in Figure 1B; TBAP in Figure 1C; DBAA in Figure 1D) to the standard ion pairing agent for RNA and DNA separations, triethylammonium acetate (TEAA, Figure 1A). The samples tested were commercially available tailless ladders (50-1000nt and 500-9000nt) and three polyadenylated mRNAs (ORFan ~200nt, GFP is ~900nt, Luc is ~2000nt and comprises a mixture of Luc 60A and Luc 100A tail length variants). A PLRP-S 4000Å 8µm (Agilent Technologies) column was used for this experiment.

Figures 2A-2B show representative data for heterogeneous ion pairing agent tuning. Figure 2A shows representative chromatograms from mixed ion pairing systems (HAA/TEAA on top; DBAA/TEAA on bottom). Figure 2B shows representative data demonstrating introduction of low concentrations of a bulkier ion pair (e.g., HAA) to a 100 mM TEAA ion pairing system results in altered resolution of multiple species within a complex mixture. Increasing concentrations of HAA in 100 mM TEAA shift mRNAs of different sizes (e.g., T100 GFP and T60/100 Luc) apart, and resolution is lost between the tail variants of Luc.

Figures 3A-3F show use of mixed ion pairing agents for HPLC analysis of a mixture containing 7 different mRNAs. Each of the 7 mRNA molecules in the mixture had a different length, ranging from approximately 700 bp to about 3500 bp. Even at high concentrations, systems of DBAA alone failed to resolve the longest species sufficiently (Figure 3A), so mixed systems of DBAA and TEAA were investigated. Ratios of DBAA/TEAA tested were 5 mM DBAA/100 mM TEAA (Figure 3B); 15 mM DBAA/100 mM TEAA (Figure 3C); 75 mM DBAA/25 mM TEAA (Figure 3D); 75 mM DBAA/50 mM TEAA (Figure 3E); and 50 mM DBAA/50 mM TEAA (Figure 3F).

Figure 4 shows data indicating that retention of polyadenylated RNA molecules is strongly affected by the ratio of ion pairing agents used for HPLC.

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Figure 5 shows representative chromatograms for HPLC of nucleic acids using “pseudo-isocratic” conditions where acetonitrile was held constant and a gradient of TEAA concentrations was used. Retention was reduced by increasing the concentration of TEAA at constant DBAA and acetonitrile, resulting in high resolution separations by “ion pair gradient”.

5 Similar to an isocratic separation, the acetonitrile concentration was tuned to find the best separation at these TEAA/DBAA conditions.

Figures 6A-6D show representative HPLC chromatograms Figures 6A and 6B show a comparison of linear separation methods (Figure 6A) with constant organic phase (ACN):ion pair gradient elution (Figure 6B) for complex mixtures of mRNA. Figure 6C shows

10 representative chromatograms comparing HPLC solid phase (e.g., PLRP column vs. DNAPac column) performance in methods described by the disclosure Figure 6D shows representative chromatograms for quaternary ion pair (IP)/organic gradients as described by the disclosure.

Briefly, the concentrations of TEAA and DBAA are individually controlled, to tune the separation in each region of the elution gradient. Each chromatogram was produced using a
15 “reversed” gradient for reversed phase, with decreasing acetonitrile through the elution region to allow a wide range of compositions, and also includes the use of high DBAA concentration to separate the shorter species; increasing TEAA to is used to resolve mRNAs greater than 2kb.

Figure 7 shows representative chromatograms of a mRNA ladder (top), a mixture containing 6 different mRNAs (middle), and a single species mRNA composition (bottom) using
20 the ion pair gradient HPLC method described in Example 2.

Figure 8 shows HPLC chromatograms of pharmaceutical preparation samples from four development lots of a single-species mRNA pharmaceutical preparation. Representative chromatograms are shown for lot numbers 106004, 106017 and 106028 (left to right, respectively). TFF tangential flow filtration; hydroxyapatite purification; dT oligo dT
25 purification; IVT in vitro transcription; FFB final formulated bulk.

Figure 9 shows a comparison of chromatographic purity data obtained using mixed ion pair methods as described by the disclosure (bottom) with nucleic acid separation using thermally denaturing conditions (e.g., Transgenomic wave cartridge) or capillary electrophoresis (CE; fragment analyzer) methodologies.

30 Figure 10 shows representative HPLC chromatograms of a 65 °C heat-stressed mixture containing 7 different mRNAs. Degradation was observed in the regions before and between the peaks. The loss of area was more apparent in longer than shorter constructs.

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Figure 11 shows an HPLC chromatogram of a composition comprising two species of mRNA molecules which are of very similar size (~1900 bp) but differ in sequence. Data indicate HPLC methods as described by the disclosure are capable of resolving each molecule of the mixture.

5 Figure 12 shows a chromatogram of four mRNA constructs for investigation of the mass balance and carryover of HPLC methods described by the disclosure.

Figure 13 shows chromatograms demonstrating spike and recovery of the tailless mRNA molecules in a composition of standard full-length molecules having a polyA tail 100 nucleotides in length.

10 Figure 14 shows a schematic demonstrating RNase H was used to generate “5’ degradants”, or long polyadenylated species missing a short length of the 5’ end.

Figure 15 shows representative chromatograms of 5’ degradants separated using HPLC methods described by the disclosure.

15 Figure 16 shows RNaseH 5’ degradants of four lengths were purified by a tail-based method to isolate the 5’ truncated polyadenylated fragment as an analytical standard. Spike and recovery into full length, tailed mRNA at 50%, 25%, and 10% showed improved resolution and sensitivity for HPLC methods as described by the disclosure compared to FA.

Figure 17 shows a chromatogram demonstrating HPLC methods described by the disclosure reduce in-run degradation of samples.

20 Figure 18 shows a representative chromatogram of an mRNA ladder demonstrating that temperature robustness of methods described by the disclosure (e.g., resolution and peak area (recovery)) is preserved from 65 °C to 90 °C.

25 Figure 19 shows a representative chromatogram of a single species mRNA composition demonstrating that temperature robustness of methods described by the disclosure (e.g., resolution and peak area (recovery)) is preserved from 65 °C to 90 °C.

30 Figure 20 shows a representative chromatogram demonstrating that temperature robustness of methods described by the disclosure (e.g., resolution and peak area (recovery)) is preserved from 55 °C to 65 °C. The chromatogram on top represents a mRNA ladder containing 7 different mRNAs, and the chromatogram on the bottom represents a single drug product comprising six different species of mRNA.

Figure 21 shows a histogram demonstrating near 100% peak area of HPLC methods as described by the disclosure.

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Figure 22 shows analysis of three linearized plasmid templates (e.g., the same vector) comprising genes of different lengths using HPLC methods described by the disclosure at 65 °C and 50 °C. At the former temperature, both the polyT and polyA gene strands, as well as the two vector strands, were separated (vector doublet eluting at ~14 minutes). At 50 °C, the two of the double-stranded genes were separated from the double-stranded vector (top and bottom); the third gene is very close to the length of the vector and nearly coelutes (middle).

DETAILED DESCRIPTION

In some aspects, the disclosure relates to high performance liquid chromatography methods (e.g., HPLC methods) for analyzing polynucleotides (e.g., mixtures containing polynucleotides, such as RNA molecules). Typically, an HPLC apparatus comprises a reservoir containing a mobile phase, a sample input port, a chromatography column containing the stationary phase, and a detection apparatus. HPLC apparatus and methods for HPLC detection of RNA molecules are generally described, for example in U.S. Patent No. 8,383,340, the entire contents of which are incorporated herein by reference.

In some aspects, the disclosure relates to reversed phase ion pairing HPLC (RP-IP HPLC). Generally, RP-IP HPLC refers to a liquid chromatographic methodology in which retention of analytes on an HPLC column is modulated by addition of an ion pairing agent that alters electrostatic interactions between analytes in a sample (e.g., nucleic acids) and the stationary phase of the chromatography column. Without wishing to be bound by any particular theory, a single ion pairing agent is typically used. Larger, bulkier, more hydrophobic ion pairing agents with higher degree of alkylation are believed to drive selectivity toward a size-based separation, with varying levels of resolution. Smaller, less bulky ion pairing agents with shorter alkyl chains and less overall hydrophobicity are believed to shift bias toward base composition. In the context of HPLC analysis of certain polyadenylated molecules (e.g., mRNA), the polyA tail acts as a hydrophobic tag and biases selectivity, causing a loss of resolution between multiple RNAs of considerably different sizes depending on the ion pairing agent in use. Conversely, resolution between two RNAs of similar size may not be adequate using only a single highly alkylated bulky ion pair.

The disclosure is based, in part, on the discovery that inclusion of certain combinations of ion pairing agents (e.g., the combination of a size-selective ion pairing agent and a composition-selective ion pairing agent as described by the disclosure) during HPLC of samples

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containing polynucleotides results in improved resolution and detection of impurities in the samples. The addition of a less bulky ion pairing agent (e.g., a composition-selective ion pairing agent), in some embodiments, addresses the loss of resolution with increasing length, enhancing the size-based separation of polynucleotides greater than 2.5kb compared to single-ion pair systems. Resolution has been further improved through the novel elution strategy of an ion pair gradient, where the ion pairing agent ratio is manipulated at increasing, constant, or decreasing organic composition to provide high resolution separations. The combination of both classes of ion pairing agents in a single system enhances selectivity based on polynucleotide size and composition where the concentrations and ratios of the ion pairs to each other can be tuned to maximize resolution in specific applications. Therefore, due to the exceptional resolution that can be achieved, this is a preferred means of assessing purity of certain molecules (e.g., mRNAs, such as therapeutically relevant RNAs from 100-5000 nt and larger) compared to methods previously described in the art.

Accordingly, in some aspects, the disclosure provides a method of separating a nucleic acid from a mixture comprising one or more additional nucleic acids or impurities, comprising the steps: contacting a stationary phase of a reverse phase chromatography column with the mixture; and eluting the nucleic acid with a mobile phase, wherein the mobile phase comprises one or more solvents and two or more ion pairs, such that the nucleic acid traverses the column with a retention time that is different than one or more other nucleic acids of the mixture.

As used herein, an “ion pairing agent” or an “ion pair” refers to an agent (e.g., a small molecule) that functions as a counter ion to a charged (e.g., ionized or ionizable) functional group on an HPLC analyte (e.g., a nucleic acid) and thereby changes the retention time of the analyte as it moves through the stationary phase of an HPLC column. Generally, ion pairing agents are classified as cationic ion pairing agents (which interact with negatively charged functional groups) or anionic ion pairing agents (which interact with positively charged functional groups). The terms “ion pairing agent” and “ion pair” further encompass an associated counter-ion (e.g., acetate, phosphate, bicarbonate, chloride, citrate, nitrate, nitrite, oxide, sulfate and the like, for cationic ion pairing agents, and sodium, calcium, and the like, for anionic ion pairing agents). In some embodiments, one or more ion pairing agents utilized in the methods described by the disclosure is a cationic ion pairing agent. Examples of cationic ion pairing agents include but are not limited to certain protonated or quaternary amines (including e.g., primary, secondary and tertiary amines) and salts thereof, such as triethylammonium

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acetate (TEAA), tetrabutylammonium phosphate (TBAP), hexylammonium acetate (HAA) and dibutylammonium acetate (DBAA).

Protonated and quaternary amine ion pairing agents can be represented by the following formula:



wherein each R independently is hydrogen, optionally substituted aliphatic, optionally substituted heteroaliphatic, optionally substituted aryl or optionally substituted heteroaryl; provided that at least one instance of R is not hydrogen; and A is an anionic counter ion.

The term “aliphatic” refers to alkyl, alkenyl, alkynyl, and carbocyclic groups. Likewise, the term “heteroaliphatic” refers to heteroalkyl, heteroalkenyl, heteroalkynyl, and heterocyclic groups. The term “aryl” refers to a radical of a monocyclic or polycyclic (e.g., bicyclic or tricyclic) $4n+2$ aromatic ring system (e.g., having 6, 10, or 14 π electrons shared in a cyclic array) having 6–14 ring carbon atoms and zero heteroatoms provided in the aromatic ring system (“ C_{6-14} aryl”). The term “heteroaryl” refers to a radical of a 5–14 membered monocyclic or polycyclic (e.g., bicyclic, tricyclic) $4n+2$ aromatic ring system (e.g., having 6, 10, or 14 π electrons shared in a cyclic array) having ring carbon atoms and 1–4 ring heteroatoms provided in the aromatic ring system, wherein each heteroatom is independently selected from nitrogen, oxygen, and sulfur (“5–14 membered heteroaryl”). Suitable anionic counter ions include, but are not limited to, acetate, trifluoroacetate, phosphate, hexafluorophosphate, sulfate, methylsulfonate, trifluoromethylsulfonate, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP) and the like.

The term “optionally substituted” refers to being substituted or unsubstituted. In general, the term “substituted” means that at least one hydrogen present on a group is replaced with a permissible substituent, e.g., a substituent which upon substitution results in a stable compound, e.g., a compound which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, or other reaction.

In some embodiments, ion pairing agents are classified as a “size-selective” ion pairing agent or a “composition-selective” ion pairing agent. Without wishing to be bound by any particular theory, in a particular embodiment, a “size-selective” ion pairing agent is capable of promoting separation of an individual analyte (e.g., RNA) from impurities or from a mixture of analytes on the basis of size. Similarly, in a particular embodiment, a “composition-selective”

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ion pairing agent is capable of promoting separation of an individual analyte (e.g., RNA) from impurities or from a mixture of analytes on the basis of composition (e.g., base composition). In one embodiment, a size-selective ion pairing agent comprises four or more (e.g., 4, 5, 6, 7, 8, 9, 10, or more) carbon molecules per alkyl chain. In another particular embodiment, a
5 “composition-selective” ion pairing agent typically comprises three or less (e.g., 3, 2, or 1) carbon molecules per alkyl chain. In some embodiments, a size-selective ion pairing agent is selected from the group consisting of tetrabutylamine salts, for example tetrabutylammonium phosphate (TBAP), hexylamine salts, for example hexylammonium acetate (HAA) and dibutylamine salts, for example dibutylammonium acetate (DBAA). In some embodiments, a
10 composition-selective ion pairing agent is triethylammonium acetate (TEAA).

Ion pairing agents are generally dispersed within a mobile phase. As used herein, a “mobile phase” is an aqueous solution comprising water and/or one or more organic solvents used to carry an HPLC analyte (or analytes), such as a nucleic acid or mixture of nucleic acids through an HPLC column. Generally, a IP-RP HPLC mobile phase comprises a polar organic
15 solvent. Examples of polar organic solvents suitable for inclusion in a mobile phase include but are not limited to alcohols, ketones, nitrates, esters, amides and alkylsulfoxides. In some embodiments, a mobile phase comprises one or more organic solvents selected from the group consisting of acetonitrile, methanol, ethanol, propanol, isopropanol, dimethylformamide, methyl acetate, acetone, and dimethylsulfoxide (DMSO), hexylene glycol, polar aprotic solvents, C₁₋₄
20 alkanols, C₁₋₆ alkandriols, and C₂₋₄ alkanolic acids. In some embodiments, a mobile phase comprises acetonitrile. In some embodiments, a mobile phase comprises additional components, for example as described in U.S. Patent Publication US 2005/0011836, the entire contents of which are incorporated herein by reference.

The concentration of organic solvent in a mobile phase can vary. For example, in some
25 embodiments, the volume percentage (v/v) of an organic solvent in a mobile phase varies from 0% (absent) to about 100% of a mobile phase. In some embodiments, the volume percentage of organic solvent in a mobile phase is between about 5% and about 75% v/v. In some embodiments, the volume percentage of organic solvent in a mobile phase is between about 25% and about 60% v/v. In some embodiments, the concentration of organic solvent in a mobile
30 phase is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, or about 90% v/v.

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The pH of the mobile phase (e.g., the pH of each solvent solution of the mobile phase) can vary. In some embodiments, the pH of the mobile phase is between about pH 5.0 and pH 9.5 (e.g., about 5.0, about 5.5, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, or about 9.5). In some embodiments, the pH of the mobile phase is between about pH 6.8 and pH 8.5 (e.g., about 6.8, about 7.0, about 7.2, about 7.4, about 7.6, about 7.8, about 8.0, about 8.3, or about 8.5). In some embodiments, the pH of the mobile phase is about 7.0.

In some embodiments, the pH of the first solvent solution is between about pH 5.0 and pH 9.5 (e.g., about 5.0, about 5.5, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, or about 9.5). In some embodiments, the pH of the first solvent solution is between about pH 6.8 and pH 8.5 (e.g., about 6.8, about 7.0, about 7.2, about 7.4, about 7.6, about 7.8, about 8.0, about 8.3, or about 8.5). In some embodiments, the pH of the first solvent solution is about 7.0.

In some embodiments, the pH of the second solvent solution is between about pH 5.0 and pH 9.5 (e.g., about 5.0, about 5.5, about 6.0, about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, about 9.0, or about 9.5). In some embodiments, the pH of the second solvent solution is between about pH 6.8 and pH 8.5 (e.g., about 6.8, about 7.0, about 7.2, about 7.4, about 7.6, about 7.8, about 8.0, about 8.3, or about 8.5). In some embodiments, the pH of the second solvent solution is about 7.0.

In some embodiments, a mobile phase for use in HPLC methods as described by the disclosure is comprised of multiple (e.g., 2, 3, 4, 5, or more) solvent solutions. In some embodiments of HPLC methods described by the disclosure, the mobile phase comprises two solvent solutions (e.g., Mobile Phase A, and Mobile Phase B). In some embodiments, a solvent solution comprises one or more organic solvent (e.g., polar solvent, such as acetonitrile) and one or more ion pairing agents.

The concentration of two or more solvent solutions in a mobile phase can vary. For example, in a mobile phase comprising two solvent solutions (e.g., a first solvent solution and a second solvent solution), the volume percentage of the first solvent solution may range from about 0% (absent) to about 100%. In some embodiments, the volume percentage of the first solvent solution may range from about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, or about 90% v/v.

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Conversely, in some embodiments, the volume percentage of the second solvent solution of a mobile phase may range from about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, or about 90% v/v.

5 The concentration of one or more ion pairing agents in a mobile phase (e.g., a solvent solution) can vary. For example, in some embodiments, the concentration of each of the one or more ion pair (e.g., each ion pairing agent) in a solvent solution (e.g., a first solvent solution or a second solvent solution) ranges from about 1 mM to about 2 M (e.g., about 1 mM, about 2 mM, about 5 mM, about 10 mM, about 50 mM, about 100 mM, about 200 mM, about 500 mM, about 1 M, about 1.2 M, about 1.5 M, or about 2M), inclusive. In some embodiments, the
10 concentration of an ion pairing agent in a mobile phase (e.g., solvent solution) ranges from about 1mM-100mM, 5mM-100mM, 5mM-75mM, 5mM-50mM, 5mM-25mM, 5mM-10mM, 10mM-25mM, 25mM-50mM, 25mM-75mM, 50mM-100mM or 75mM-100nM. Examples of ion pairing agent concentrations include but are not limited to 5 mM DBAA/100 mM TEAA, 15
15 mM DBAA/100 mM TEAA, 75 mM DBAA/25 mM TEAA, 75 mM DBAA/50 mM TEAA, 50 mM DBAA/50 mM TEAA, and 50 mM DBAA/100 mM TEAA.

In some aspects, the disclosure relates to the discovery that ion pair gradients, where the ratio of certain combinations of ion pairing agents (e.g., a size-selective ion pairing agent and a composition-selective ion pairing agent) is manipulated at increasing, constant, or decreasing
20 organic composition, allow for high resolution separations of polyadenylated nucleic acids (e.g., mRNAs, such as IVT mRNAs). Accordingly, some embodiments of HPLC methods described by the disclosure comprise the step of passing a mobile phase through the column, wherein the mobile phase is a mixture of a first solvent solution and a second solvent solution, and wherein the first solvent solution and the second solvent solution independently comprise one or more
25 ion pair selected from the group consisting of: trimethylamine or a salt thereof (e.g., triethylammonium salt), tetrabutylamine or a salt thereof (e.g., tetrabutylammonium salt), hexylamine or a salt thereof (e.g., hexylammonium salt) and dibutylamine or a salt thereof (e.g., dibutylammonium salt), optionally wherein the triethylammonium salt is triethylammonium acetate (TEAA), the tetrabutylammonium salt is tetrabutylammonium phosphate (TBAP), the
30 hexylammonium salt is hexylammonium acetate (HAA), or the dibutylammonium salt is dibutylammonium acetate (DBAA), such that the nucleic acid traverses the column with a retention time that is different than one or more other nucleic acids of the mixture.

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In some embodiments, the ratio of the first solvent solution to the second solvent solution is held constant (e.g., isocratic) during elution of the nucleic acid. However, the skilled artisan will appreciate that in other embodiments, the relative ratio of the first solvent solution to the second solvent solution can vary throughout the elution step. For example, in some
5 embodiments, the ratio of the first solvent solution is increased relative to the second solvent solution during the elution step. In some embodiments, the ratio of the first solvent solution is decreased relative to the second solvent solution during the elution step.

The relative ratios of the one or more ion pairing agent in a mobile phase (or solvent solution) may vary or be held constant (e.g., isocratic) during the eluting step. In some
10 embodiments, the ratio of a first ion pairing agent is increased relative to a second ion pairing agent during the elution step. In some embodiments, the ratio of a first ion pairing agent is increased relative to a second ion pairing agent during the elution step. For example, in some embodiments, the ratio of triethylammonium (e.g., TEAA) to dibutylammonium (e.g., DBAA) ranges from about 1:100 to about 100:1, about 1:75 to about 75:1, about 1:50 to about 50:1,
15 about 1:25 to about 25:1, 5:1 to about 1:5, about 3:1 to about 1:3, about 2:1 to about 1:2, or about 1:1 to 1:3.

Any suitable HPLC column (e.g., stationary phase) may be used in the methods described by the disclosure. Generally, a "HPLC column" is a solid structure or support that contains a medium (e.g. a stationary phase) through which the mobile phase and HPLC sample
20 (e.g., a sample containing HPLC analytes, such as nucleic acids) is eluted. Without wishing to be bound by any particular theory, the composition and chemical properties of the stationary phase determine the retention time of HPLC analytes. In some embodiments of HPLC methods described by the disclosure, the stationary phase is non-polar. Examples of non-polar stationary phases include but are not limited to resin, silica (e.g., alkylated and non-alkylated silica),
25 polystyrenes (e.g., alkylated and non-alkylated polystyrenes), polystyrene divinylbenzenes, etc. In some embodiments, a stationary phase comprises particles, for example porous particles. In some embodiments, a stationary phase (e.g., particles of a stationary phase) is hydrophobic (e.g., made of an intrinsically hydrophobic material, such as polystyrene divinylbenzene), or comprise hydrophobic functional groups. In some embodiments, a stationary phase is a membrane or
30 monolithic stationary phase.

The particle size (e.g., as measured by the diameter of the particle) of an HPLC stationary phase can vary. In some embodiments, the particle size of a HPLC stationary phase

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ranges from about 1 μm to about 100 μm (e.g., any value between 1 and 100, inclusive) in diameter. In some embodiments, the particle size of a HPLC stationary phase ranges from about 2 μm to about 10 μm , about 2 μm to about 6 μm , or about 4 μm in diameter. The pore size of particles (e.g., as measured by the diameter of the pore) can also vary. In some embodiments, the particles comprise pores having a diameter of about 100 Å to about 10,000 Å. In some
5 embodiments, the particles comprise pores having a diameter of about 100 Å to about 5000 Å, about 100 Å to about 1000 Å, or about 1000 Å to about 2000 Å. In some embodiments, the stationary phase comprises polystyrene divinylbenzene, for example as used in PLRP-S 4000 columns or DNAPac-RP columns.

10 The temperature of the column (e.g., the stationary phase within the column) can vary. In some embodiments, the column has a temperature from about 20 °C to about 99 °C (e.g., any temperature between 20 °C and 99 °C. In some embodiments, the column has a temperature from about 40 °C to about 99 °C (e.g., any temperature between 40 °C and 99 °C, for example about 40 °C, about 50 °C, about 60 °C, about 70 °C, about 80 °C, about 90 °C, about 95 °C, or
15 about 99 °C). In some embodiments, the column has a temperature from about 60 °C to about 90 °C (e.g., any temperature between 60 °C and 90 °C). In some embodiments, the column has a temperature of about 65 °C.

In some embodiments, HPLC methods as described by the disclosure comprise the step of detecting or isolating a nucleic acid. Any detection apparatus or modality suitable for HPLC
20 may be used. Examples of HPLC detectors include but are not limited to absorbance detectors (e.g., UV/VIS detectors), fluorescence detectors, electrochemical detectors, and mass spectrometric detectors.

In some aspects, the disclosure relates to improved HPLC methods for detection of nucleic acids. As used herein, a “polynucleotide” or “nucleic acid” is at least two nucleotides
25 covalently linked together, and in some instances, may contain phosphodiester bonds (e.g., a phosphodiester “backbone”) or modified bonds, such as phosphorothioate bonds. An “engineered nucleic acid” is a nucleic acid that does not occur in nature. In some instances the nucleic acid is an engineered nucleic acid. It should be understood, however, that while an engineered nucleic acid as a whole is not naturally-occurring, it may include nucleotide
30 sequences that occur in nature. Thus, a “polynucleotide” or “nucleic acid” sequence is a series of nucleotide bases (also called “nucleotides”), generally in DNA and RNA, and means any chain of two or more nucleotides. The terms include genomic DNA, cDNA, RNA, any synthetic and

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genetically manipulated polynucleotides. This includes single- and double-stranded molecules; i.e., DNA-DNA, DNA-RNA, and RNA-RNA hybrids as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone.

The methods of the invention involve the analysis of samples (e.g., mixtures) comprising one or more nucleic acids, for example DNA or RNA. An RNA typically is composed of repeating ribonucleosides. It is possible that the RNA includes one or more deoxyribonucleosides. In preferred embodiments the RNA is comprised of greater than 60%, 70%, 80% or 90% of ribonucleosides. In other embodiments the RNA is 100% comprised of ribonucleosides. The RNA in a mixture is preferably an mRNA.

As used herein, the term "messenger RNA (mRNA)" refers to a ribonucleic acid that has been transcribed from a DNA sequence by an RNA polymerase enzyme, and interacts with a ribosome to synthesize protein encoded by DNA. Generally, mRNA are classified into two sub-classes: pre-mRNA and mature mRNA. Precursor mRNA (pre-mRNA) is mRNA that has been transcribed by RNA polymerase but has not undergone any post-transcriptional processing (e.g., 5' capping, splicing, editing, and polyadenylation). Mature mRNA has been modified via post-transcriptional processing (e.g., spliced to remove introns and polyadenylated region) and is capable of interacting with ribosomes to perform protein synthesis.

mRNA can be isolated from tissues or cells by a variety of methods. For example, a total RNA extraction can be performed on cells or a cell lysate and the resulting extracted total RNA can be purified (e.g., on a column comprising oligo-dT beads) to obtain extracted mRNA.

Alternatively, mRNA can be synthesized in a cell-free environment, for example by in vitro transcription (IVT). IVT is a process that permits template-directed synthesis of ribonucleic acid (RNA) (e.g., messenger RNA (mRNA)). It is based, generally, on the engineering of a template that includes a bacteriophage promoter sequence upstream of the sequence of interest, followed by transcription using a corresponding RNA polymerase. In vitro mRNA transcripts, for example, may be used as therapeutics in vivo to direct ribosomes to express protein therapeutics within targeted tissues.

Traditionally, the basic components of an mRNA molecule include at least a coding region, a 5'UTR, a 3'UTR, a 5' cap and a poly-A tail. IVT mRNA may function as mRNA but are distinguished from wild-type mRNA in their functional and/or structural design features which serve to overcome existing problems of effective polypeptide production using nucleic-acid based therapeutics. For example, IVT mRNA may be structurally modified or chemically

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modified. As used herein, a “structural” modification is one in which two or more linked nucleosides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide without significant chemical modification to the nucleotides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide “ATCG” may be chemically modified to “AT-5meC-G”. The same polynucleotide may be structurally modified from “ATCG” to “ATCCCG”. Here, the dinucleotide “CC” has been inserted, resulting in a structural modification to the polynucleotide.

A nucleic acid molecule (e.g., DNA or RNA) may comprise naturally occurring nucleotides and/or non-naturally occurring nucleotides such as modified nucleotides. In some embodiments, one or more nucleotides of a polynucleotide includes at least one chemical modification. In some embodiments, the chemical modification is selected from the group consisting of pseudouridine, N1-methylpseudouridine, 2-thiouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methoxyuridine, and 2'-O-methyluridine. Other exemplary chemical modifications useful in the mRNA described herein include those listed in US Published patent application 2015/0064235.

An “in vitro transcription template (IVT),” as used herein, refers to deoxyribonucleic acid (DNA) suitable for use in an IVT reaction for the production of messenger RNA (mRNA). In some embodiments, an IVT template encodes a 5' untranslated region, contains an open reading frame, and encodes a 3' untranslated region and a polyA tail. The particular nucleotide sequence composition and length of an IVT template will depend on the mRNA of interest encoded by the template.

A “5' untranslated region (UTR)” refers to a region of an mRNA that is directly upstream (i.e., 5') from the start codon (i.e., the first codon of an mRNA transcript translated by a ribosome) that does not encode a protein or peptide. In some embodiments, a 5' UTR encodes a 7-methylguanosine cap or a 7-methylguanosine group analog (e.g., a cap analog for example as described by Kowalska et al. RNA. 2008 Jun; 14(6): 1119–1131).

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A “3' untranslated region (UTR)” refers to a region of an mRNA that is directly downstream (i.e., 3') from the stop codon (i.e., the codon of an mRNA transcript that signals a termination of translation) that does not encode a protein or peptide.

5 An “open reading frame” is a continuous stretch of DNA beginning with a start codon (e.g., methionine (ATG)), and ending with a stop codon (e.g., TAA, TAG or TGA) and encodes a protein or peptide.

10 A “polyA tail” is a region of mRNA that is downstream, e.g., directly downstream (i.e., 3'), from the 3' UTR that contains multiple, consecutive adenosine monophosphates. A polyA tail may contain 10 to 300 adenosine monophosphates. For example, a polyA tail may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290 or 300 adenosine monophosphates. In some embodiments, a polyA tail contains 50 to 250 adenosine monophosphates. In some embodiments, a polyA tail contains up to 1000 adenosine monophosphates. In a relevant biological setting (e.g., in cells, in vivo, etc.) the poly(A) tail functions to protect mRNA from enzymatic degradation, e.g., in the cytoplasm, and aids in transcription termination, export of the mRNA from the nucleus, and translation.

15 In some embodiments, a mixture of nucleic acids comprises polyA tail length variants. As used herein, a “tail length variant” refers to a polynucleotide having an identical protein coding sequence to a full-length polynucleotide but having a polyA tail of a different length. 20 For example, if a full length polynucleotide encoding GFP comprises a polyA tail that is 100 nucleotides in length, a GFP tail length variant may have an identical coding sequence but comprise a polyA tail that is 60 nucleotides in length. Generally, tail length variants comprise a polyA tail that is shorter than a full length polynucleotide. In some embodiments, a polyA tail variant has a polyA tail that is between about 1 and about 200 nucleotides (e.g., any integer 25 between 1 and 200) shorter than a wild-type polynucleotide. In some embodiments, a polyA tail variant has a polyA tail that is more than 200 nucleotides shorter than a wild-type polynucleotide.

30 In some aspects, the disclosure relates to the discovery that HPLC methods using combinations of ion pairing agents enhance identification and separation of large polynucleotides. Thus, in some embodiments of methods described by the disclosure, a nucleic acid (e.g., a nucleic acid separated from a mixture) is larger than 100 nucleotides in length. In some embodiments, a nucleic acid (e.g., a nucleic acid separated from a mixture) is between

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about 100 and about 10000 nucleotides in length, about 200 and about 7500 nucleotides in length, or about 500 and about 5000 nucleotides in length.

In some embodiments, an mRNA (e.g., IVT mRNA) is a therapeutic and/or prophylactic mRNA. As used herein, the term “therapeutic mRNA” refers to an mRNA molecule (e.g., an IVT mRNA) that encodes a therapeutic protein. Therapeutic proteins mediate a variety of effects in a host cell or a subject in order to treat a disease or ameliorate the signs and symptoms of a disease. For example, a therapeutic protein can replace a protein that is deficient or abnormal, augment the function of an endogenous protein, provide a novel function to a cell (e.g., inhibit or activate an endogenous cellular activity, or act as a delivery agent for another therapeutic compound (e.g., an antibody-drug conjugate). As used herein, the term “prophylactic mRNA” refers to an mRNA molecule (e.g., an IVT mRNA) that encodes a prophylactic protein such as a vaccine antigen. Prophylactic proteins mediate a variety of effects in a host cell or a subject in order to prevent disease. Therapeutic and/or prophylactic mRNA may be useful for the treatment of the following diseases and conditions: bacterial infections, viral infections, parasitic infections, cell proliferation disorders, genetic disorders, and autoimmune disorders.

In some aspects, the disclosure provides HPLC methods for separating a nucleic acid from a mixture comprising one or more additional nucleic acids or impurities. A mixture may comprise between about 1 and about 100 nucleic acids. As used herein, the term “impurity” refers to a small molecule, protein, virus, bacterium, etc., that contaminates a composition comprising a desired nucleic acid (e.g., the nucleic acid sought to be separated from the mixture).

A nucleic acid may be larger or smaller than the one or more other nucleic acids or impurities in a mixture. For example, a larger nucleic acid may comprise about 10-100%, 25-100%, 50-100%, 50-75%, 100-200%, 200-500% or 500-1000% more nucleotides than the one or more additional nucleic acids or impurities in a mixture. Alternatively, a smaller nucleic acid may comprise about 10-100%, 25-100%, 50-100%, 50-75%, 100-200%, 200-500% or 500-1000% fewer nucleotides than the one or more additional nucleic acids or impurities in a mixture. In some embodiments, an impurity is a degradation product, for example a fragment (e.g., polynucleotide) that has been cleaved from a nucleic acid (e.g., an mRNA).

In some embodiments, HPLC methods as described by the disclosure are capable of separating nucleic acids that are closely related in size. For example, in some embodiments,

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HPLC methods as described by the disclosure are capable of separating nucleic acids that differ in size by about 1-2 nucleotides for a 100-mer polynucleotide, about 20 nucleotides for a 1000-mer polynucleotide, about 40 nucleotides for a 2000-mer polynucleotide, or about 100 nucleotides for a 5000-mer polynucleotide. in some embodiments, HPLC methods as described
5 by the disclosure are capable of separating nucleic acids that differ in size by about 1-2 nucleotides for a 10,000-mer polynucleotide.

In some embodiments, HPLC methods as described by the disclosure are capable of separating nucleic acids that are closely related in molecular weight (e.g., as expressed in Daltons (Da)). For example, in some embodiments, HPLC methods as described by the
10 disclosure are capable of separating nucleic acids that differ in molecular weight by about 680 Da to about 34,000 Da, about 1000 Da to about 20,000 Da, or about 5000 Da to about 10,000 Da.

Delivery of mRNA molecules to a subject in a therapeutic context is promising because it enables intracellular translation of the mRNA and production of at least one encoded peptide
15 or polypeptide of interest without the need for nucleic acid-based delivery systems (e.g., viral vectors and DNA-based plasmids). Therapeutic mRNA molecules are generally synthesized in a laboratory (e.g., by in vitro transcription). However, there is a potential risk of carrying over impurities or contaminants, such as incorrectly synthesized mRNA and/or undesirable synthesis reagents, into the final therapeutic preparation during the production process. In order to prevent
20 the administration of impure or contaminated mRNA, the mRNA molecules can be subject to a quality control (QC) procedure (e.g., validated or identified) prior to use. Validation confirms that the correct mRNA molecule has been synthesized and is pure.

Certain aspects of the disclosure relate to the discovery that HPLC methods described herein are useful, in some embodiments, for quality control of certain nucleic acid molecules
25 (e.g., polyadenylated nucleic acids, such as mRNA).

Accordingly, in some aspects, the disclosure provides a method of quality control of a nucleic acid (e.g., a polyadenylated nucleic acid) pharmaceutical composition, the method comprising: separating a nucleic acid (e.g., a polyadenylated nucleic acid) from a mixture comprising one or more additional nucleic acids or impurities by a method as described by the
30 disclosure; comparing the separated nucleic acid with a reference nucleic acid (e.g., a reference polyadenylated nucleic acid); and determining the quality of the nucleic acid based on a comparison of the separated nucleic acid with the reference nucleic acid.

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In some instances, the methods of the disclosure are used to determine the purity of an RNA sample. The term “pure” as used herein refers to material that has only the target nucleic acid active agents such that the presence of unrelated nucleic acids is reduced or eliminated, i.e., impurities or contaminants, including RNA fragments. For example, a purified RNA sample includes one or more target or test nucleic acids but is preferably substantially free of other nucleic acids. As used herein, the term “substantially free” is used operationally, in the context of analytical testing of the material. Preferably, purified material substantially free of impurities or contaminants is at least 95% pure; more preferably, at least 98% pure, and more preferably still at least 99% pure. In some embodiments a pure RNA sample is comprised of 100% of the target or test RNAs and includes no other RNA. In some embodiments it only includes a single type of target or test RNA.

A “reference nucleic acid” as used herein refers to a control nucleic acid (e.g. a polyadenylated nucleic acid, such as mRNA) or chromatogram generated from a control nucleic acid that uniquely identifies the nucleic acid separated from the mixture. The reference nucleic acid may be generated based on digestion of a pure sample and compared to data generated by HPLC of a mixture comprising the nucleic acid of interest. Alternatively it may be a known chromatogram, stored in a electronic or non-electronic data medium. For example, a control chromatogram may be a chromatogram based on predicted HPLC retention times of a particular RNA (e.g., a test mRNA). In some embodiments quality control methods described by the disclosure further comprise the step of comparing the nucleic acid separated from the mixture to the reference nucleic acid using an orthogonal analytical technique, for example polymerase chain reaction (e.g., RT-qPCR), nucleic acid sequencing, gel electrophoresis, mass spectrometry, etc.

For the purposes of the invention, ranges may be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

Moreover, for the purposes of the present invention, the term “a” or “an” entity refers to one or more of that entity; for example, “a protein” or “a nucleic acid molecule” refers to one or

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more of those compounds or at least one compound. As such, the terms “a” (or “an”), “one or more” and “at least one” can be used interchangeably herein. It is also to be noted that the terms “comprising”, “including”, and “having” can be used interchangeably. Furthermore, a compound “selected from the group consisting of” refers to one or more of the compounds in the list that follows, including mixtures (i.e., combinations) of two or more of the compounds.

EXAMPLES

Example 1

HPLC-based separation of mRNA was compared between three alkylammonium ion pairing agents (Hexylammonium acetate (HAA), Tetrabutylammonium phosphate (TBAP) and Dibutylammonium acetate (DBAA)) and triethylammonium acetate (TEAA, top left). The samples tested were commercially available tailless ladders (50-1000nt and 500-9000nt) and three polyadenylated mRNAs (ORFan is ~200nt, GFP is ~900nt, Luc is ~2000nt and comprises a mixture of A60 and A100 tail length variants). Representative chromatograms are shown in Figures 1A-1D.

In the standard TEAA method, an adequate separation is obtained for the tailless commercial ladder, but the polyadenylated mRNA species coelute regardless of length (Figure 1A). It was observed that polyadenylated mRNA also elute well past the elution point of the longest tailless species due to the hydrophobicity of the polyA tail; the shortened tail in the Luc sample has an exaggerated effect.

Each of the larger ion pairs screened gave a size-based separation, with the polyadenylated mRNAs eluting around the correct length according to the tailless commercial ladder (chemically unmodified) (Figures 1B-1D). Dibutylammonium acetate (DBAA) was selected for further investigation due to its high resolution and good mobile phase reproducibility.

The use of heterogeneous ion pairing agents for 1) tuning selectivity between ion pair character; and 2) resolving multiple nucleic acid species with homogenous polyA tails using a standard preparation grade polystyrene and divinylbenzene column (e.g., a PLRP column or a DNAPac-RP column) was investigated. Figure 2A shows representative chromatograms from mixed ion pairing systems (HAA/TEAA on top; DBAA/TEAA on bottom) for separation of four distinct mRNA species (ORFan, GFP, Chemically-modified Luc, MPLuc). Figure 2B shows

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representative data demonstrating introduction of low concentrations of a bulkier ion pair (e.g., HAA) to a 100 mM TEAA ion pairing system results in altered resolution of multiple species within a complex mixture. Increasing concentrations of HAA in 100 mM TEAA shift mRNAs of different sizes (e.g., T100 GFP and T60/100 Luc) apart, and resolution is lost between the tail variants of Luc.

Data indicate that mixed ion pairing systems preserved high resolution of the commercial ladders, and gave some separation of the polyadenylated mRNAs. Some tail bias is preserved, as the mRNAs, while separated, eluted with much larger species in the tailless ladder than their actual size.

Use of mixed ion pairing agents for HPLC analysis of a mixture containing 7 different mRNAs was investigated next. Each of the 7 mRNA molecules in the mixture had a different length, ranging from approximately 700 bp to about 3500 bp. Even at high concentrations, systems of DBAA alone failed to resolve the longest species sufficiently (Figure 3A), so mixed systems of DBAA and TEAA were investigated. Ratios of DBAA/TEAA tested were 5 mM DBAA/100 mM TEAA (Figure 3B); 15 mM DBAA/100 mM TEAA (Figure 3C); 75 mM DBAA/25 mM TEAA (Figure 3D); 75 mM DBAA/50 mM TEAA (Figure 3E); and 50 mM DBAA/50 mM TEAA (Figure 3F).

Data indicated that the retention time of the mRNA molecules was strongly affected by the ratio of ion pairing agents, as shown in Figure 4 and Table 1 below:

Table 1

Ratio DBAA/TEAA	% acetonitrile (starting gradient)
5/100	15.5-17
15/100	19-20.5
50/100	25-26
50/50	30-32
75/25	35-38
100/0	40-42.5

Furthermore, data indicated that higher resolution of mRNA species was obtained at higher ion pair concentrations. However, each ratio of ion pairing agents elutes at a very narrow range (<2%). It was also observed that shorter length nucleic acids (e.g., <1-2kb) were better resolved

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by higher DBAA concentration (and also exhibited higher retention) and that TEAA was necessary for high resolution with longer nucleic acid constructs (lower retention).

Given the narrow elution range of each of the size-based systems described above, as well as a need for less retentive conditions (e.g., higher TEAA:DBAA ratios) to better resolve late-eluting, longer nucleic acid species, “pseudo-isocratic” conditions where acetonitrile was held constant were investigated (Figure 5). Retention was reduced by increasing the concentration of TEAA at constant DBAA and acetonitrile, resulting in high resolution separations by “ion pair gradient”. Similar to an isocratic separation, the acetonitrile concentration was tuned to find the best separation at these TEAA/DBAA conditions.

Figures 6A-6D show representative HPLC chromatograms. Figures 6A and 6B show a comparison of linear separation methods (Figure 6A) with constant organic phase (ACN):ion pair gradient elution (Figure 6B) for complex mixtures of mRNA. Figure 6C shows representative chromatograms comparing HPLC solid phase (e.g., PLRP column vs. DNAPac column) performance in methods described by the disclosure. Figure 6D shows representative chromatograms for quaternary ion pair (IP)/organic gradients as described by the disclosure. Briefly, the concentrations of TEAA and DBAA are individually controlled, to tune the separation in each region of the elution gradient. Each chromatogram was produced using a “reversed” gradient for reversed phase, with decreasing acetonitrile through the elution region to allow a wide range of compositions, and also includes the use of high DBAA concentration to separate the shorter species; increasing TEAA to is used to resolve mRNAs greater than 2kb.

Example 2

One embodiment of a method for chromatographic separation of polynucleotides is described below:

Mobile phase A: 100 mM triethylammonium acetate (TEAA)/50 mM dibutylammonium acetate (DBAA) in water

Mobile phase B: 100 mM triethylammonium acetate (TEAA)/50 mM dibutylammonium acetate (DBAA), 50% v/v acetonitrile in water

Column: DNAPac RP 2.1mm x 100mm length (Thermo Fisher part # 0889239)

Run time: 24 min

Run temperature: 65 °C

Autosampler Temperature: 4 °C

RNA concentration prior to injection: 0.1- 0.5 mg/mL

Injection Volume: 20 µL

Detection: UV absorbance @ 260 nm

System: Waters H-Class UPLC or equivalent

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Gradient: shown below in Table 1

Table 1

Time (min)	% MPA	%MPB	Flow (mL/min)
0	75%	25%	0.35
1.5	75%	25%	0.35
4.5	50%	50%	0.35
19.0	44%	56%	0.35
19.5	0%	100%	0.35
20.0	0%	100%	0.35
20.1	75%	25%	0.35
24.0	75%	25%	0.35

- 5 Figure 7 shows representative HPLC chromatograms for a mRNA ladder (top), mixture containing 6 different mRNAs, and single mRNA species composition that were produced using the method described above.

The method described above was then used to assess in-process samples, process variants, and lot-to-lot purity of a single-species mRNA pharmaceutical preparation. Figure 8 shows HPLC chromatograms of pharmaceutical preparation samples from four development lots; in the first (16031), process variants (e.g., hydroxyapatite (HA) purified, oligo dT purified, tangential flow filtration (TFF) purified, reverse phase purified, and combinations thereof) were compared to the crude TFF purified reaction. A gain in purity was observed with additional dT or RP purification apparent (Figure 8, 16030). The next three lots (16004, 17, and 28) compare available process retains across a similar process. The impact of dT purification on the crude IVT RNA is clear. It was observed that both lots 16017 and 16028 show a drop in purity of the final material after HPLC purification, likely due to in-process degradation not cleared by the tail-based purifications. The three lots of final material (final formulated bulk, FFB) show similar final purity, indicating good process reproducibility.

20 The method described above was then used to separate the RNA molecules present in a mixture containing 7 different mRNAs. Figure 9 shows a comparison of separations obtained using mixed ion pair methods as described by the disclosure (bottom) with nucleic acid separation using a non-porous HPLC reverse phase column (e.g., Transgenomic wave cartridge) (top) or capillary electrophoresis (CE; fragment analyzer) (middle) methodologies.

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Example 3

The mixture containing 7 different mRNAs described in the Examples above was heat stressed to generate time points from 10 minutes to 2 hours. HPLC was performed according to the method described in Example 2. Figure 10 shows the resulting chromatograms. Degradation products were observed in the regions before and after each mRNA peak; loss of area was more apparent in longer molecules as opposed to shorter molecules, due to the greater chance of hydrolysis of longer molecules.

Whether the methods described by the disclosure are capable of separating two mRNA molecules having a similar size but difference base composition (e.g., sequence) was investigated. Figure 11 shows an HPLC chromatogram of a composition comprising two species of mRNA molecules which are of very similar size (~1900 bp) but differ in sequence. Data indicate HPLC methods as described by the disclosure are capable of resolving each molecule of the mixture.

Example 4

Mass balance (Table 2) and carryover (Table 3) of HPLC methods described by the disclosure were investigated for mRNA constructs. Briefly, mass balance was derived by running RNA samples through an analytical HPLC column, followed by injection of the same amount of RNA samples into the HPLC without a column in-line. Peak area of column mRNA samples was compared to theoretical area (e.g., peak area of RNA sample with no column in line) using the following formula: $(\text{Area (post column recovery)})/(\text{Area(theoretical)})/100$. Carryover was derived by running RNA samples through an analytical HPLC column, followed by elution of a blank sample (no RNA). Residual RNA (carryover) was calculated by the following formula: $(\text{Area (blank)})/(\text{Area(method)})/100$. Figure 12 shows a chromatogram for each construct tested for mass balance.

Table 2: Mass Balance

Construct	Area(theoretical)	Area(post-column recovery)	Mass balance
mRNA 1	2958531	3111960	105%
mRNA 2	2840752	2730302	96%
mRNA 3	2599871	2417156	93%
mRNA 4	2763281	2634380	95%

Table 3: Carryover

Sample	Method Area	Blank Area	% carryover
Ladder	120627688	161196	0.13%
mRNA 1	11955430	24025	0.20%
mRNA 2	14515379	12758	0.09%
mRNA 3	12593173	51242	0.41%
mRNA 4	11251251	56737	0.50%
mRNA 5	12898349	28182	0.22%

Example 5

Figure 13 shows chromatograms demonstrating spike and recovery of the tailless mRNA molecules in a composition of standard full-length molecules having a polyA tail 100 nucleotides in length. Data indicates that HPLC methods described by the disclosure separate tailless and tailed molecules having the same sequence.

Figure 14 shows a schematic demonstrating RNase H was used to generate “5’ degradants”, or long polyadenylated species missing a short length of the 5’ end. RNase H nicks the RNA side of an RNA/DNA duplex, so by introducing a DNA-containing guide oligo of a specific sequence to an mRNA sample, strand scission can be targeted to a single site on the RNA. The 3’ end, or 5’-degraded side, was of interest, in part, because the 5’ degradant is a critical product that is not detected by other HPLC methods. By artificially targeting degradation to discrete lengths, the resolution of methods described by the disclosure was probed.

Figure 15 shows representative chromatograms of 5’ degradants separated using HPLC methods described by the disclosure. Full length mRNA is overlaid in black with each crude RNase H reaction. It was observed that separation was achieved down to the -41 reaction, which went to approximately 50% completion as evidenced by the close doublet. On the left side of each chromatogram, the short 5’ fragment was detected early in the elution. This two-sided look at the two fragments of the molecule is indicative of the size range observed in random degradation. In addition, a comparison of the -90 or -114 to a tailless standard (Figure

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13), which is essentially -105 on the 3' end, shows the tail bias; much less separation is achieved by removing ~100 bases on the 5' end vs. removing the 100 hydrophobic A's from the 3' end.

Resolution of degraded products between fragment analyzer (FA) methodology and HPLC methods described by the disclosure were compared. Figure 16 shows RNaseH 5'

5 degradants of four lengths were purified by a tail-based method to isolate the 5' truncated polyadenylated fragment as an analytical standard. Spike and recovery into full length, tailed mRNA at 50%, 25%, and 10% showed improved resolution and sensitivity for HPLC methods as described by the disclosure compared to FA.

10 Example 6

Reproducibility of HPLC methods as described by the disclosure was investigated. A mRNA ladder, which was a mixture of seven mRNAs of lengths between 450 nt and 3400 nt, was run according to a method as described by the disclosure. The purified fraction was collected and then reinjected to investigate in-run degradation. Figure 17 is a chromatogram
15 demonstrating that the integrity of the purified fraction is largely preserved, with only small changes to the peak valleys and slight loss of the longest mRNA, which will degrade the fastest compared to the shortest mRNA. The composition of the mRNA ladder before and after reinjection are shown in Table 4.

20 Table 4

LADDER COMPOSITION	
INITIAL	REINJECTION
21%	20%
12%	13%
15%	15%
17%	18%
13%	14%
11%	11%
10%	8%

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Figures 18 and 19 show representative chromatograms demonstrating that temperature robustness of methods described by the disclosure (e.g., resolution and peak area (recovery)) is largely preserved from 55 °C to 90 °C. Figure 18 depicts a chromatogram of the mRNA ladder described above, while Figure 19 depicts a single-species mRNA composition. The significance of temperature robustness is that the temperature of analysis can be reduced to minimize in-run degradation, without adding analytical artifacts or losing recovery. Temperature robustness was also preserved at lower temperatures (Figure 20). Figure 21 shows a histogram demonstrating near 100% peak area of HPLC methods as described by the disclosure.

Example 7

Methods described by the disclosure were applied for analysis of a DNA template. It was observed that the DNA template was slightly more retained than RNA of the same size, due to increased hydrophobicity, but elutes within the same acetonitrile range (25-28%) under the conditions specified above. Temperature may also be varied to either stabilize or denature the DNA duplex. Figure 22 shows analysis of three linearized plasmid templates (e.g., the same vector) comprising genes of different lengths using HPLC methods described by the disclosure at 65 °C and 50 °C. At the former temperature, both the polyT and polyA gene strands, as well as the two vector strands, were separated (vector doublet eluting at ~14 minutes). At 50 °C, the two of the double-stranded genes were separated from the double-stranded vector (top and bottom); the third gene is very close to the length of the vector and nearly coelutes (middle).

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CLAIMS

What is claimed is:

1. A method of separating a nucleic acid from a mixture comprising one or more additional nucleic acids or impurities, comprising the steps:
 - 5 i. contacting a stationary phase of a reverse phase chromatography column with the mixture; and
 - ii. eluting the nucleic acid with a mobile phase, wherein the mobile phase comprises one or more solvents and two or more ion pairs, such that the nucleic acid traverses the column with a retention time that is different than one or more other
10 nucleic acids of the mixture.
2. The method of claim 1, wherein the column is an analytical column, or a preparative column.
- 15 3. The method of claim 1, wherein the stationary phase comprises particles.
4. The method of claim 3, wherein the particles are porous resin particles.
5. The method of claim 3, wherein the particles are hydrophobic or comprise hydrophobic
20 functional groups.
6. The method of claim 3, wherein the particles have a diameter of about $2\mu\text{m}$ – about $10\mu\text{m}$, about $2\mu\text{m}$ – about $6\mu\text{m}$, or about $4\mu\text{m}$.
- 25 7. The method of claim 3, wherein the particles comprise pores having a diameter of about $100\text{\AA}$ to about $5000\text{\AA}$, about $100\text{\AA}$ to about $1000\text{\AA}$, or about $1000\text{\AA}$ to about $2000\text{\AA}$.
8. The method of claim 1, wherein the mobile phase is one solvent.
- 30 9. The method of claim 1, wherein the mobile phase is a mixture of a first solvent and a second solvent.

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10. The method of claim 1, wherein the mobile phase is a mixture of a first solvent solution and a second solvent solution.

11. The method of claim 10, wherein the volume percentage of the first solvent solution in the mobile phase is 0% to 100%.

12. The method of claim 10, wherein the first solvent solution comprises one or more solvents and one or more ion pair.

13. The method of claim 12, wherein the concentration of each of the one or more ion pair in the first solvent solution is 1mM-100mM, 5mM-100mM, 5mM-75mM, 5mM-50mM, 5mM-25mM, 5mM-10mM, 10mM-25mM, 25mM-50mM, 25mM-75mM, 50mM-100mM or 75mM-100mM.

14. The method of claim 12, wherein the one or more ion pair is selected from the group consisting of: triethylammonium salt, tetrabutylammonium salt, hexylammonium salt and dibutylammonium salt, optionally wherein the triethylammonium salt is triethylammonium acetate (TEAA), the tetrabutylammonium is tetrabutylammonium phosphate (TBAP), the hexylammonium is hexylammonium acetate (HAA), or the dibutylammonium is dibutylammonium acetate (DBAA).

15. The method of claim 12 or 13, wherein the first solvent solution comprises of one or more solvents selected from the group consisting of water, polar aprotic solvents (including, e.g., THF, DMSO, DMF, acetonitrile, acetone, etc.), C₁₋₄ alkanols, C₁₋₆ alkandriols, and C₂₋₄ alkanoic acids.

16. The method of claim 15, wherein the first solvent solution comprises one or more solvents selected from the group consisting of water, acetonitrile, methanol, ethanol, isopropanol, and hexylene glycol.

17. The method of claim 10, wherein the volume percentage of the second solvent solution in the mobile phase is 0% to 100%.

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18. The method of claim 10, wherein the second solvent solution comprises one or more solvents and one or more ion pair.

19. The method of claim 18, wherein the concentration of each of the one or more ion pair in the second solvent solution is 1mM-100mM, 5mM-100mM, 5mM-75mM, 5mM-50mM, 5mM-25mM, 5mM-10mM, 10mM-25mM, 25mM-50mM, 25mM-75mM, 50mM-100mM or 75mM-100mM.

20. The method of claim 18, wherein the one or more ion pair is selected from the group consisting of: triethylammonium, tetrabutylammonium, hexylammonium and dibutylammonium, optionally wherein the triethylammonium salt is triethylammonium acetate (TEAA), the tetrabutylammonium salt is tetrabutylammonium phosphate (TBAP), the hexylammonium is hexylammonium acetate (HAA), or the dibutylammonium salt is dibutylammonium acetate (DBAA).

21. The method of claim 18, wherein the second solvent solution comprises one or more solvents selected from the group consisting of water, polar aprotic solvents (including, e.g., THF, DMSO, DMF, acetonitrile, acetone, etc.), C₁₋₄ alkanols, C₁₋₆ alkandriols, and C₂₋₄ alkanolic acids.

22. The method of claim 21, wherein the second solvent solution comprises one or more solvents selected from the group consisting of water, acetonitrile, methanol, ethanol, isopropanol, and hexylene glycol.

23. The method of claim 1, wherein step (ii) comprises:
passing a mobile phase through the column, wherein the mobile phase is a mixture of a first solvent solution and a second solvent solution, and wherein the first solvent solution and the second solvent solution independently comprise one or more ion pair selected from the group consisting of: triethylammonium, tetrabutylammonium, hexylammonium and dibutylammonium, optionally wherein the triethylammonium is triethylammonium acetate (TEAA), the tetrabutylammonium is tetrabutylammonium phosphate (TBAP), the

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hexylammonium is hexylammonium acetate (HAA), or the dibutylammonium is dibutylammonium acetate (DBAA)

such that the nucleic acid traverses the column with a retention time that is different than one or more other nucleic acids of the mixture.

5

24. The method of claim 23, wherein the ratio of the first solvent solution to the second solvent solution is held constant during elution of the nucleic acid.

25. The method of claim 23, wherein the ratio of the first solvent solution to the second solvent solution is increased or decreased during elution of the nucleic acid.

10

26. The method of claim 23, wherein the concentration of each ion pair in the mobile phase is held constant during elution of the nucleic acid.

27. The method of claim 23, wherein the concentration of one or more ion pair in the mobile phase is not held constant during elution of the nucleic acid.

15

28. The method of any one of the preceding claims, wherein the retention time of the nucleic acid differs from the retention time of all other nucleic acids or impurities in the mixture by 0.1-10 minutes, 0.1-5 minutes, 0.1-1 minutes, 0.1-0.5 minutes, or 0.1-0.2 minutes.

20

29. The method of any one of the preceding claims, wherein the nucleic acid comprises at least 100, at least 500, at least 1000, at least 2000, at least 3000, at least 4000, at least 5000, at least 6000, at least 7000, at least 8000, or at least 9000 nucleotides.

25

30. The method of claim 29, wherein the nucleic acid is single stranded.

31. The method of claim 29, wherein the nucleic acid is RNA or DNA.

32. The method of claim 29, wherein the nucleic acid comprises 5' and 3' UTRs.

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33. The method of claim 29, wherein the nucleic acid comprises a 5' 7-methylguanosine group or a 5' 7-methylguanosine cap analog.

34. The method of claim 29, wherein the nucleic acid comprises a 3'-polyadenosine tail.

35. The method of claim 28, wherein the mixture comprises tail length variants.

36. The method of claim 1, wherein the mixture comprises degradation products.

37. The method of any one of claims 1 to 36, further comprising the step:

iii. detecting or isolating the nucleic acid.

38. The method of claim 37, further comprising the step:

iv. detecting or isolating the one or more additional nucleic acids or impurities.

39. The method of claim 37 or 38, wherein the method is used to determine the potency of the mixture of nucleic acids.

40. The method of any one of the preceding claims, wherein the nucleic acid is larger or smaller than the one or more additional nucleic acids or impurities, and the mobile phase comprises at least one size-selective ion pair.

41. The method of claim 40, wherein the larger nucleic acid comprises 10-100%, 25-100%, 50-100%, 50-75%, 100-200%, 200-500% or 500-1000% more nucleotides than the one or more additional nucleic acids or impurities.

42. The method of claim 40, wherein the smaller nucleic acid comprises 10-100%, 25-100%, 50-100%, 50-75%, 100-200%, 200-500% or 500-1000% fewer nucleotides than the one or more additional nucleic acids or impurities.

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43. The method of claim 40, wherein the size-selective ion pair is selected from tetrabutylammonium, hexylammonium, or the dibutylammonium, salt optionally wherein the ion pair is selected from HAA, DBAA, and TBAP.

5 44. The method of any one of claims 1-39, wherein the nucleic acid has a similar size as the one or more additional nucleic acids or impurities, and the mobile phase comprises at least one composition-selective ion pair.

45. The method of claim 44, wherein the nucleic acid comprises 0-10%, 0-5%, or 0-1% more
10 or fewer nucleotides than the one or more additional nucleic acids or impurities.

46. The method of claim 44, wherein the composition-selective ion pair is a triethylammonium salt, optionally wherein the triethylammonium is TEAA.

15 47. The method of claim 1, wherein the nucleic acid and the one or more additional nucleic acids have lengths of less than 2000 nucleotides, wherein the two or more ion pairs are TEAA and DBAA, and wherein the ratio of TEAA to DBAA is ranges from about 1:20 to about 20:1, from about 5:1 to about 1:5, about 3:1 to about 1:3, about 2:1 to about 1:2, or about 1:1 to 1:3.

20 48. The method of claim 1, wherein said eluting is isocratic with respect to mobile phase solvent composition.

49. The method of claim 48, wherein said eluting is isocratic or gradient with respect to the concentration of one or more ion pairs.

25 50. The method of claim 49, wherein mobile phase comprises two ion pairs, and the relative concentration of the ion pairs is varied during the eluting.

51. The method of claim 1, wherein said eluting is gradient with respect to mobile phase
30 solvent composition.

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52. The method of claim 51, wherein said eluting is isocratic or gradient with respect to the concentration of one or more ion pairs.

53. A pure mRNA sample comprising: a composition of an in vitro transcribed (IVT) RNA
5 and a pharmaceutically acceptable carrier, wherein the composition comprises the nucleic acid separated by the method of any one of claims 1 to 52.

54. A method of quality control of a nucleic acid (e.g., a polyadenylated nucleic acid) pharmaceutical composition, the method comprising:

- 10 i. separating a nucleic acid (e.g., a polyadenylated nucleic acid) from a mixture comprising one or more additional nucleic acids or impurities by the method of any one of claims 1 to 52;
- ii. comparing the separated nucleic acid with a reference nucleic acid; and
- iii. determining the quality of the nucleic acid based on a comparison of the
15 separated nucleic acid with the reference nucleic acid.

55. The method of claim 54, wherein the comparing step comprises comparing a HPLC chromatogram of the separated nucleic acid with a HPLC chromatogram of the reference nucleic acid.

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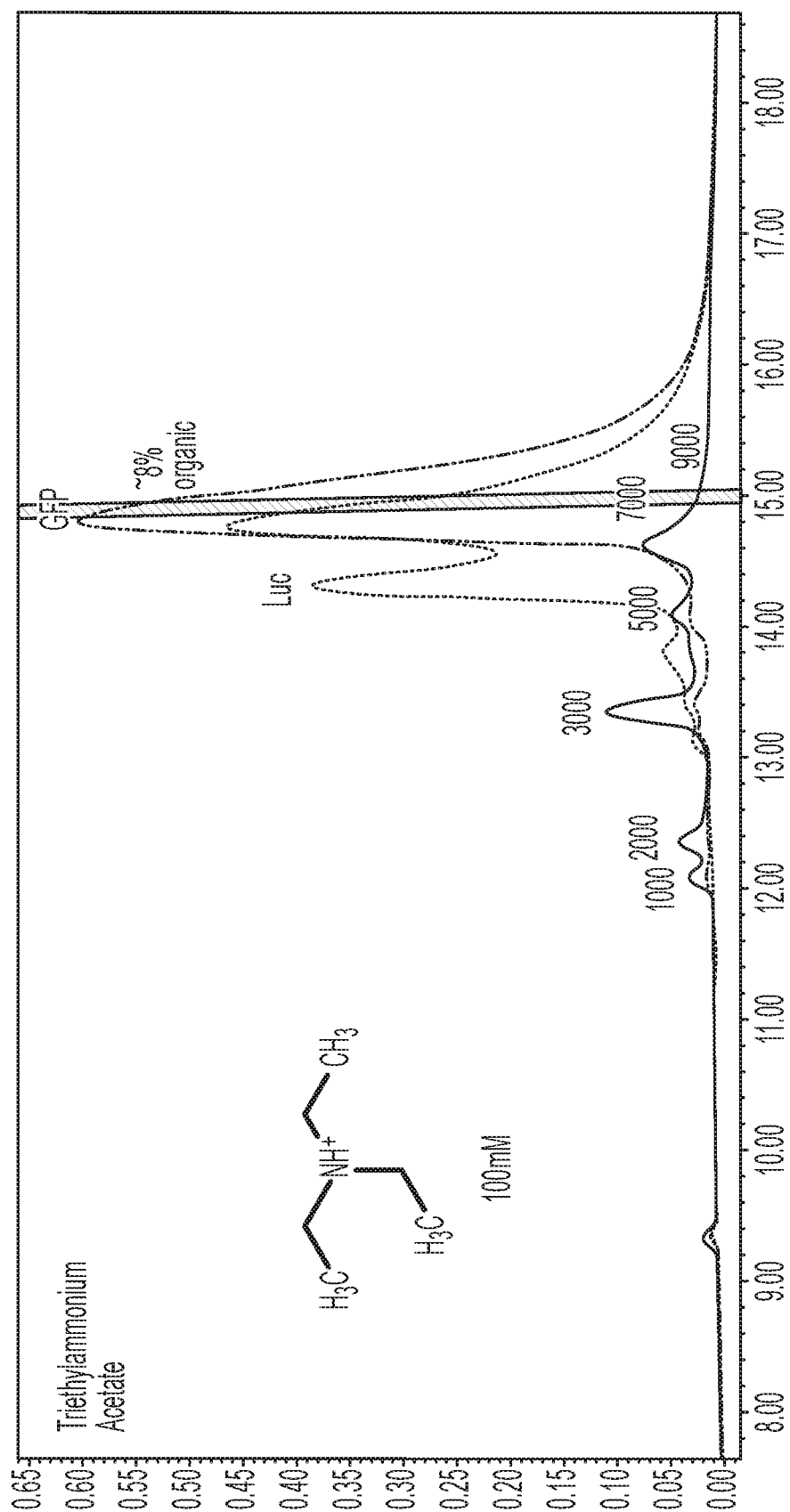


Figure 1A

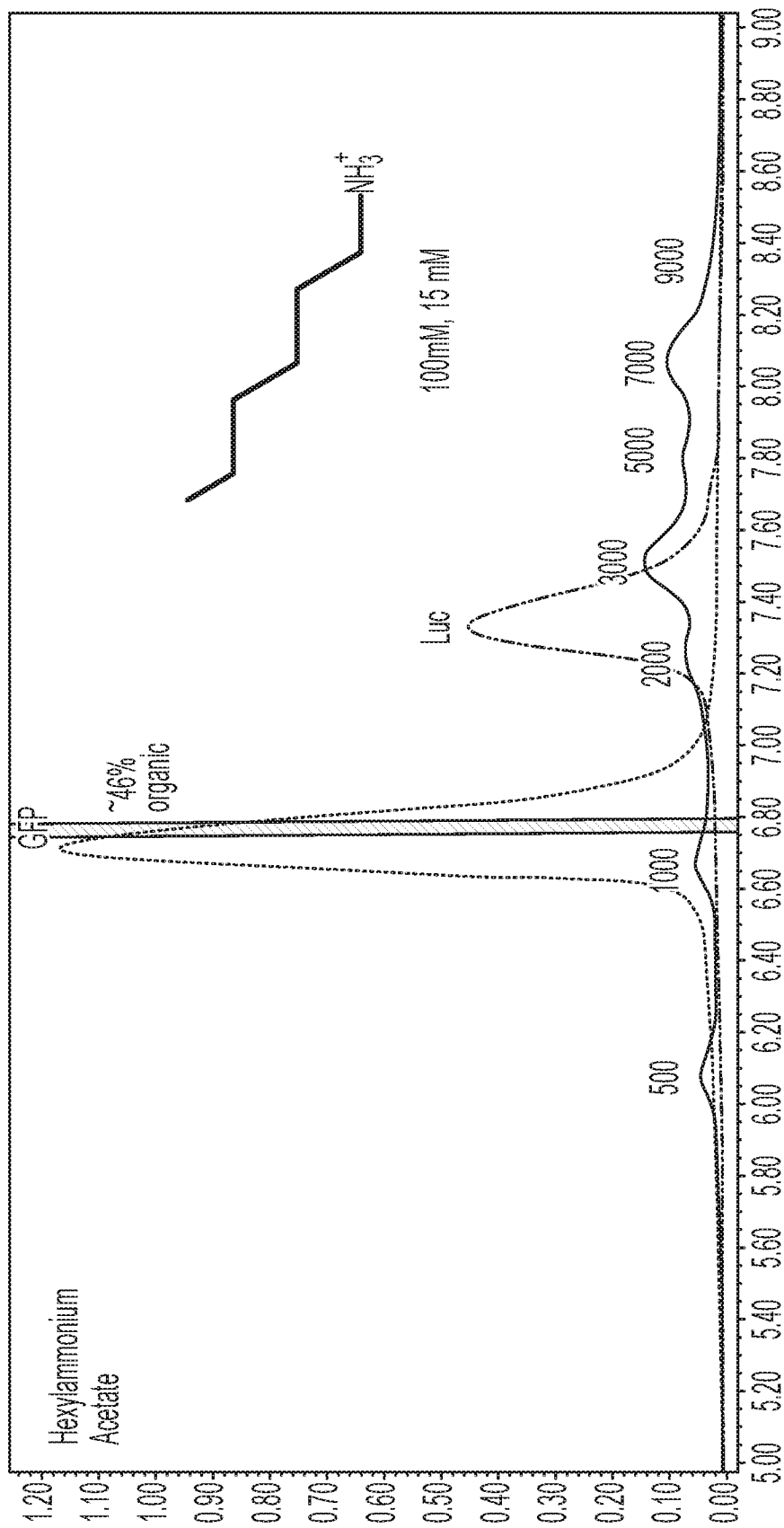


Figure 1B

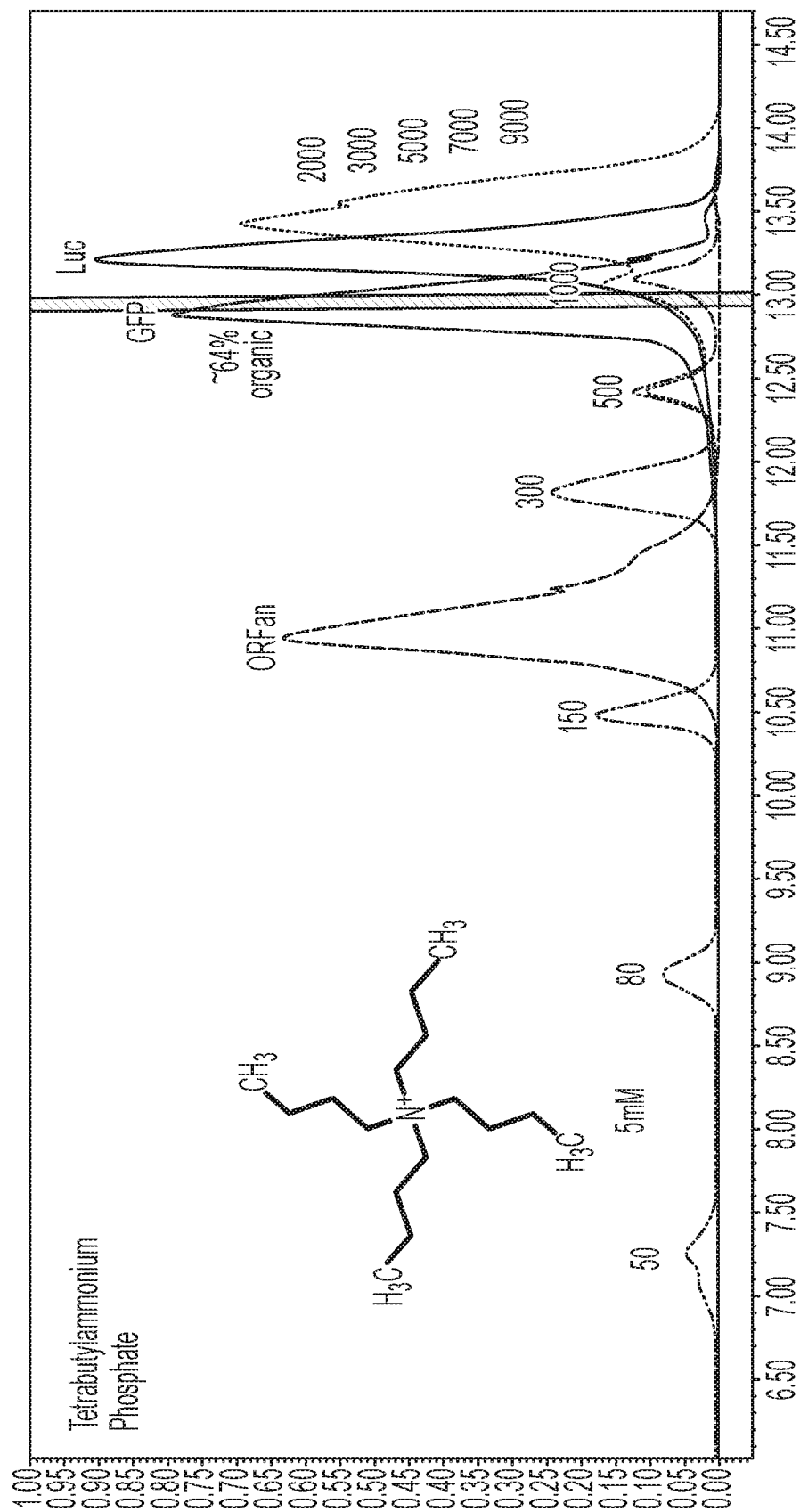


Figure 1C

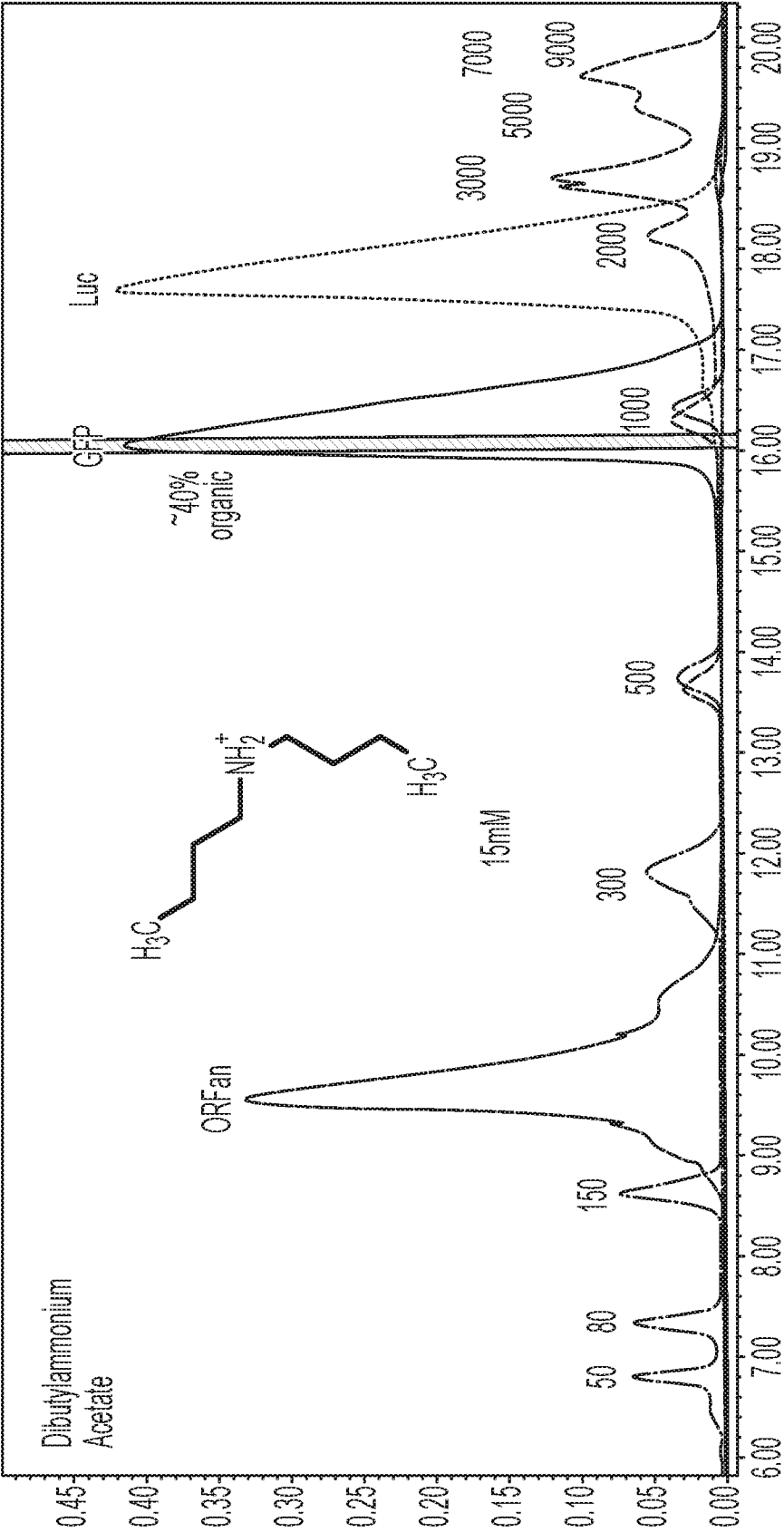


Figure 1D

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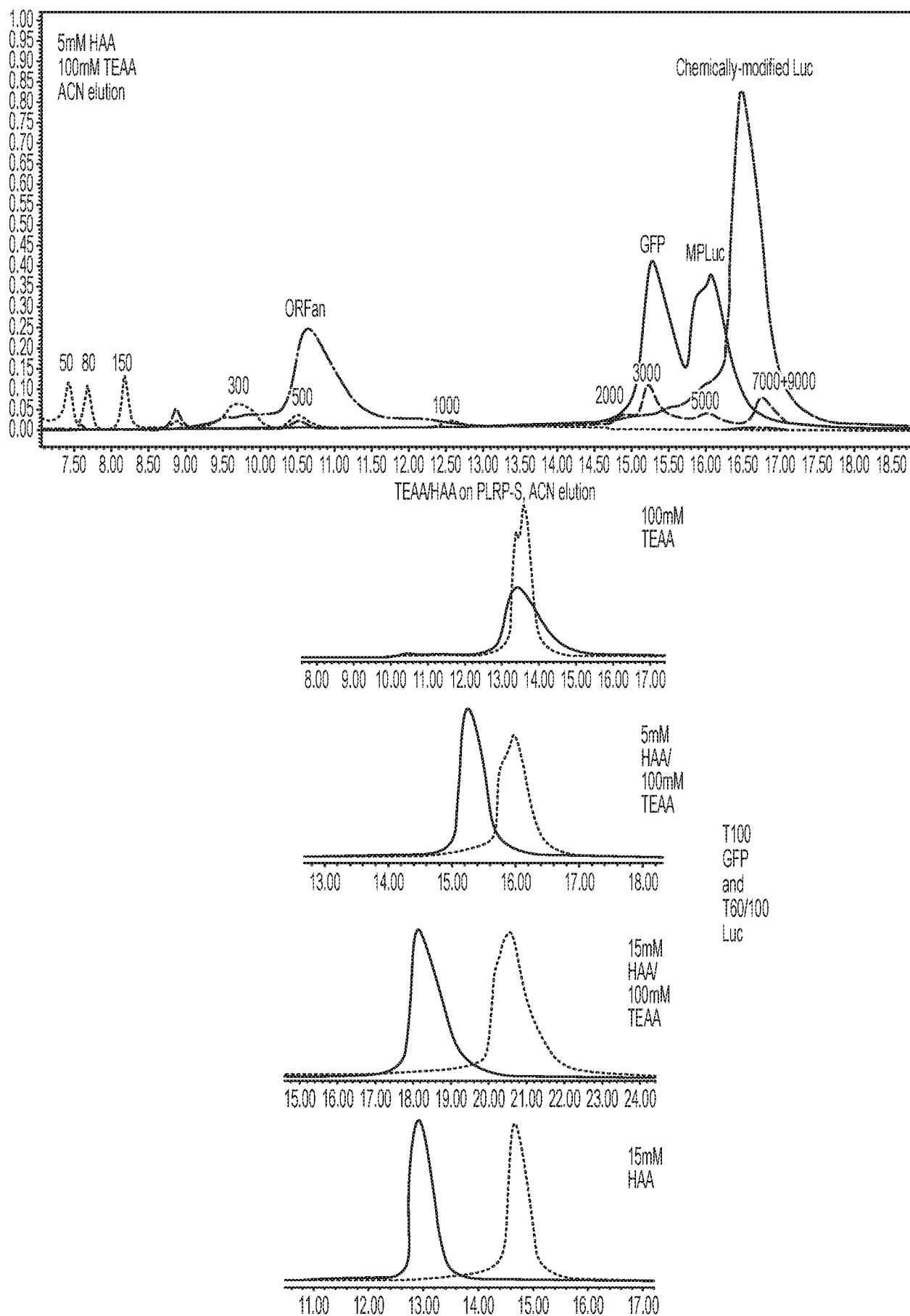


Figure 2A

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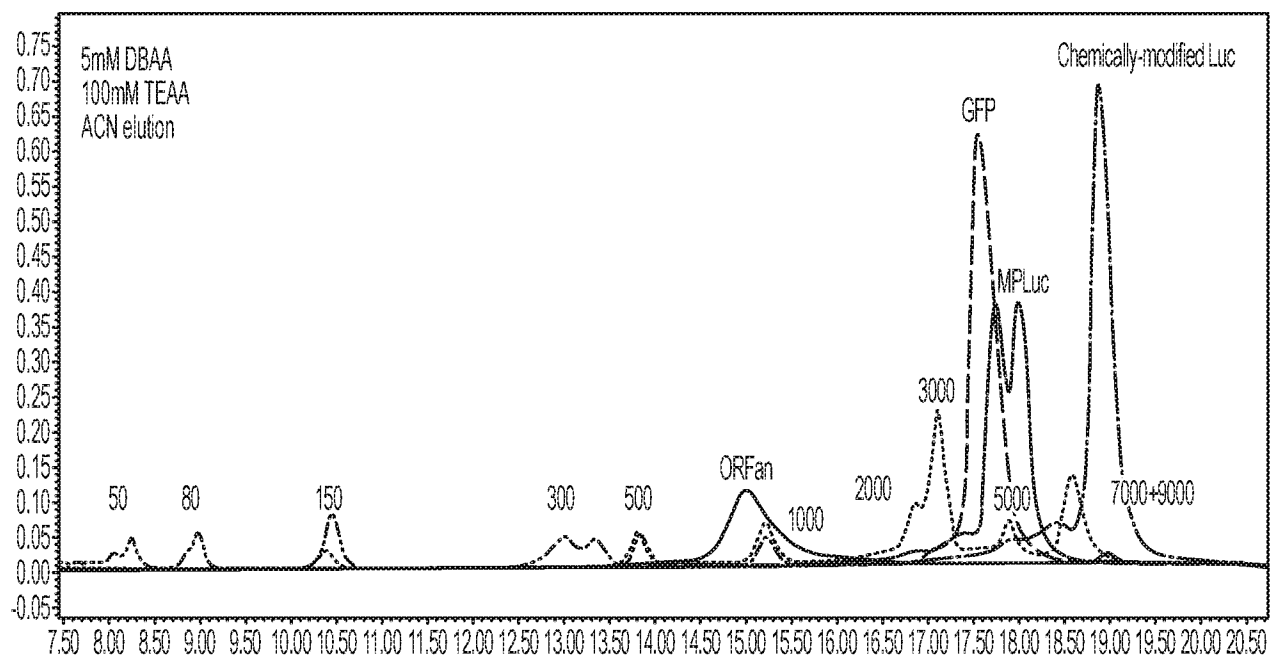


Figure 2B

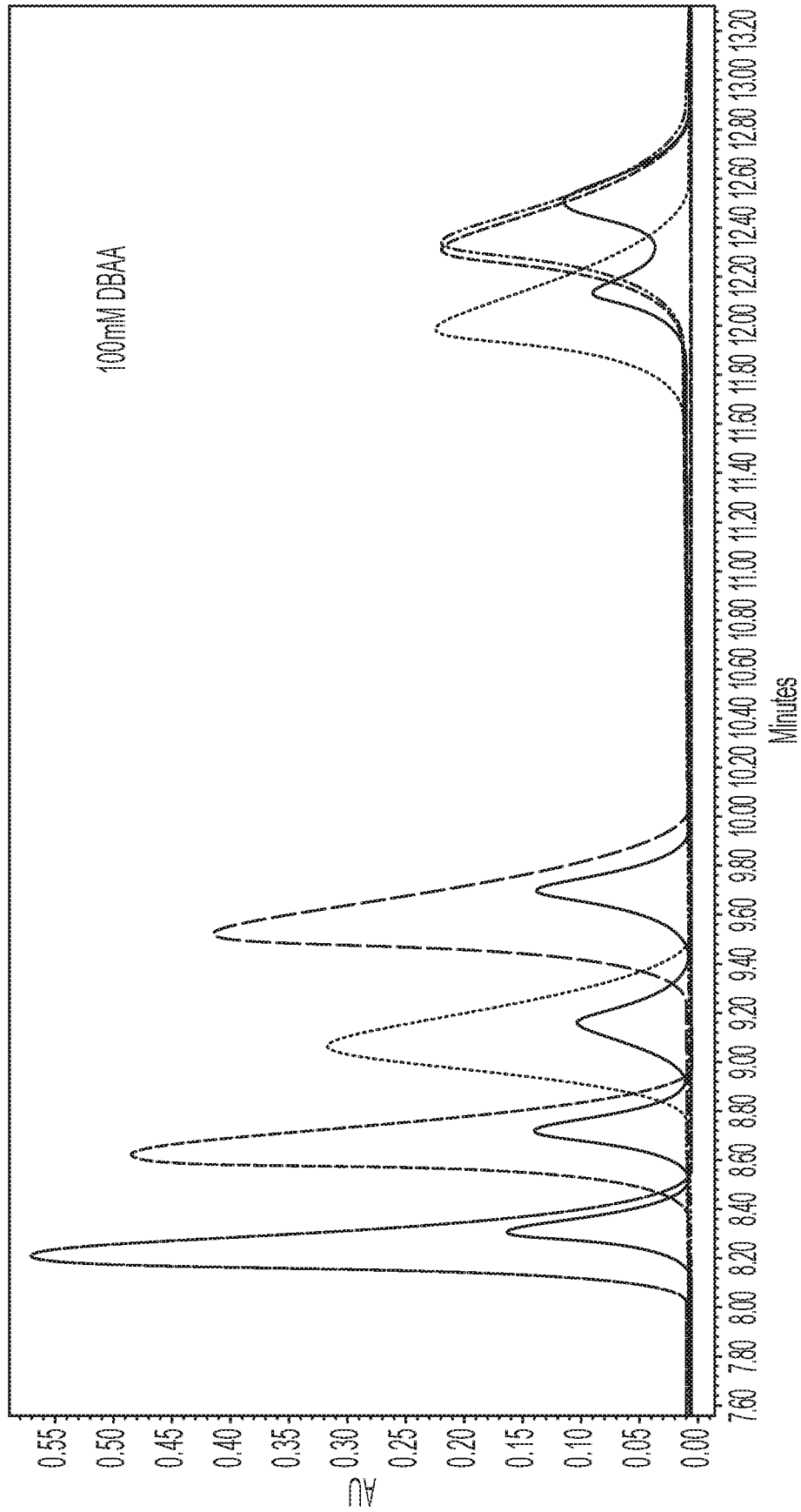


Figure 3A

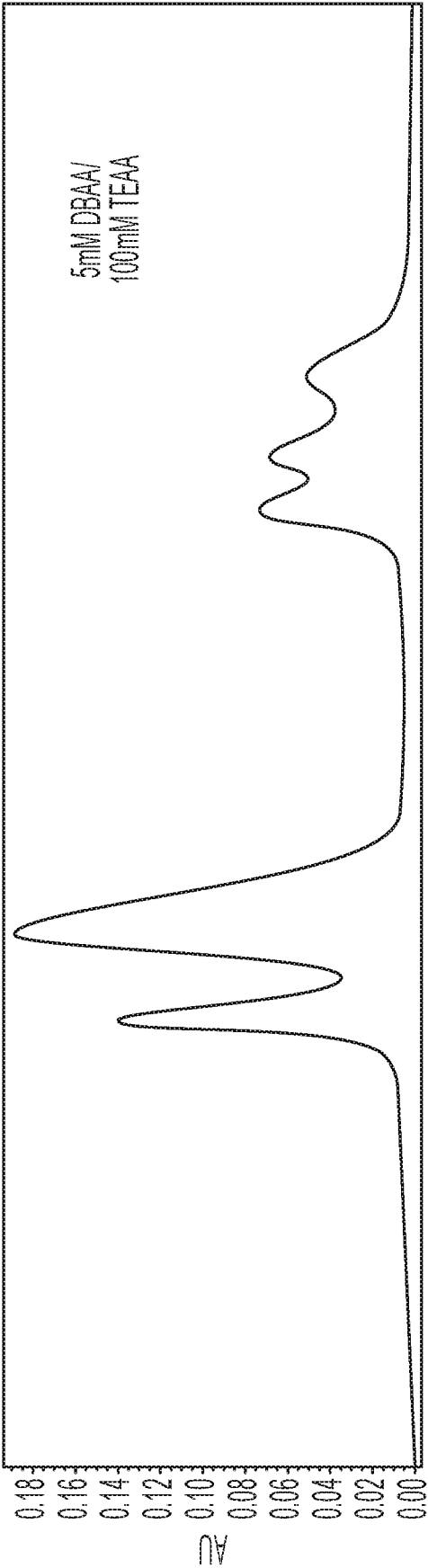


Figure 3B

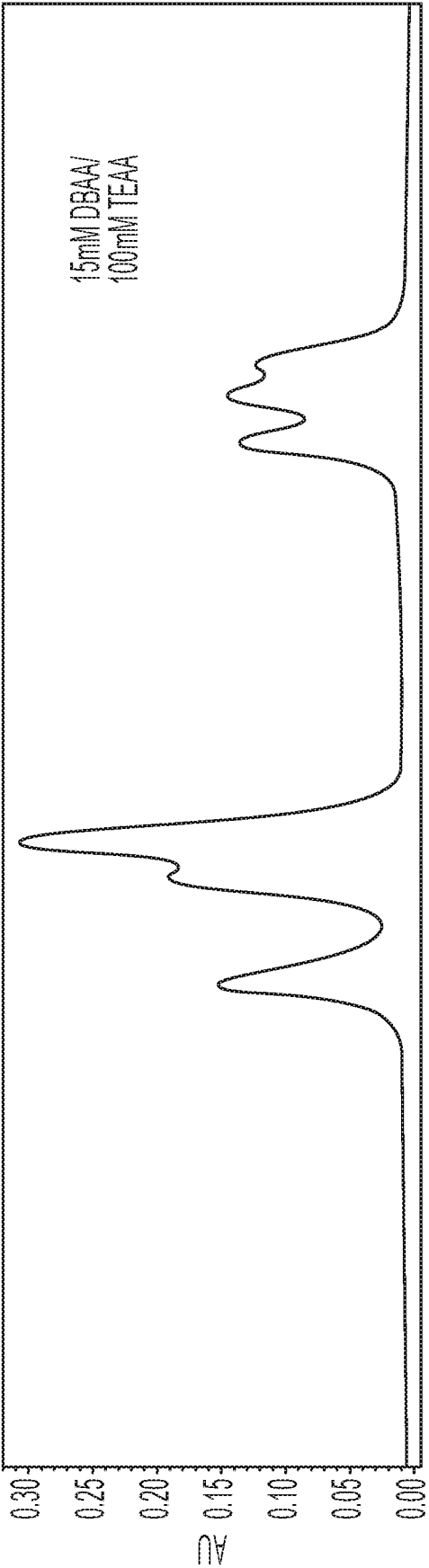


Figure 3C

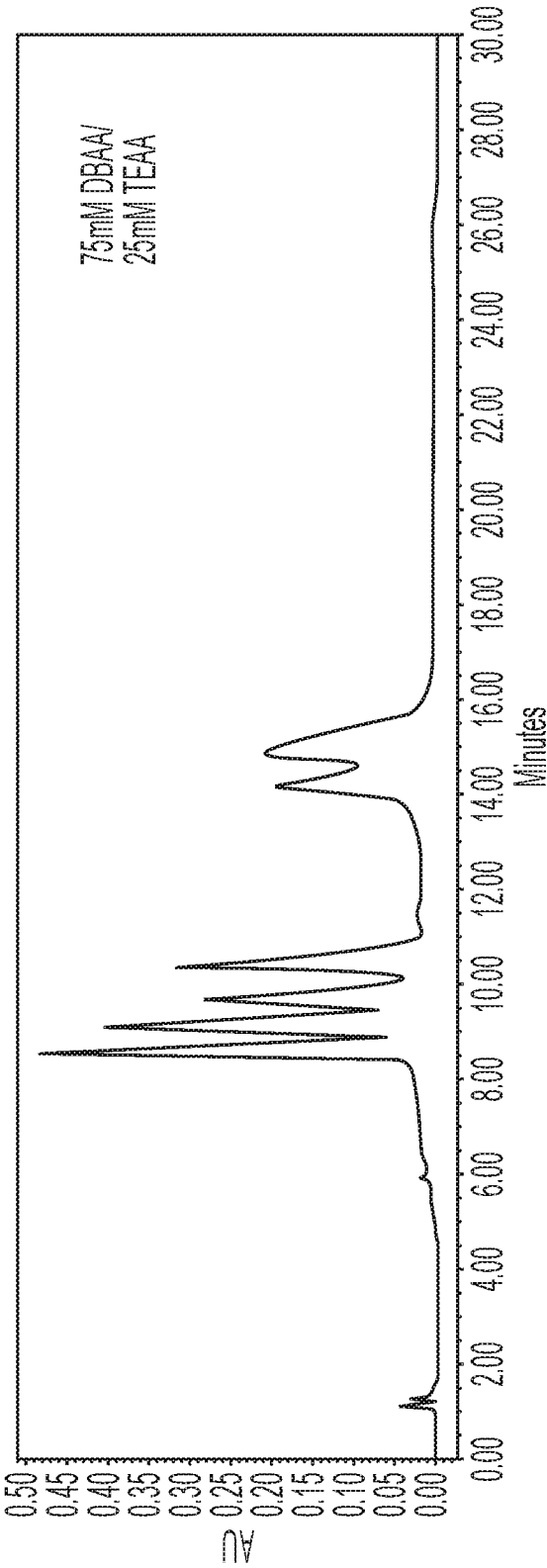


Figure 3D

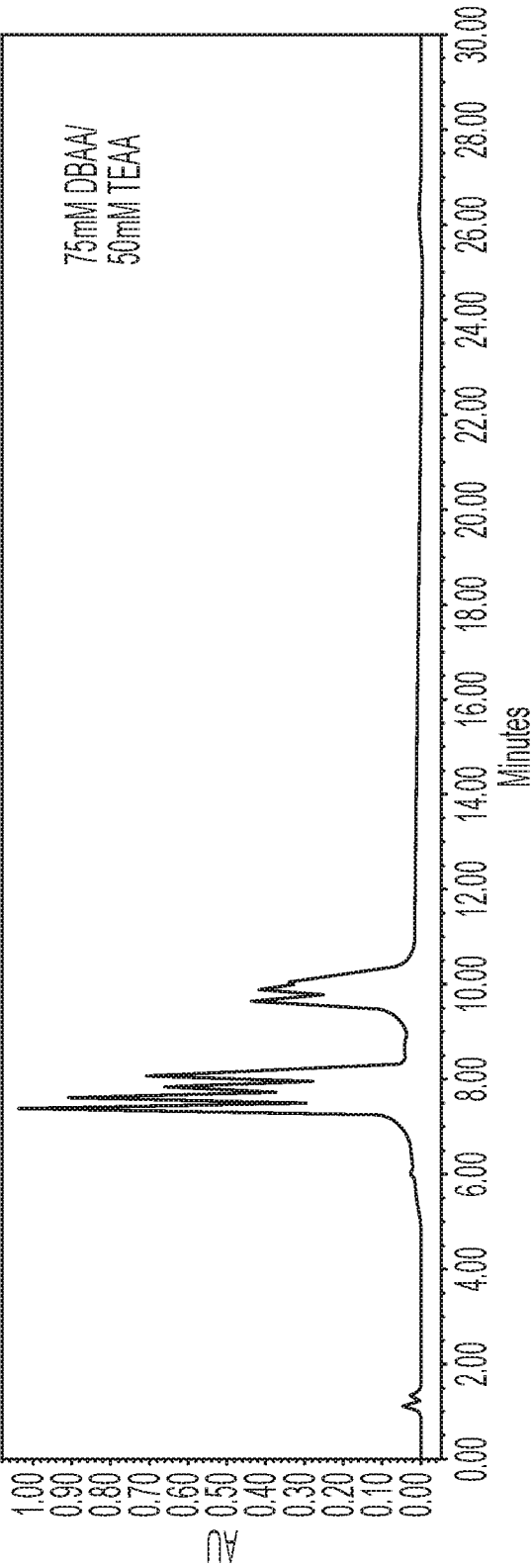


Figure 3E

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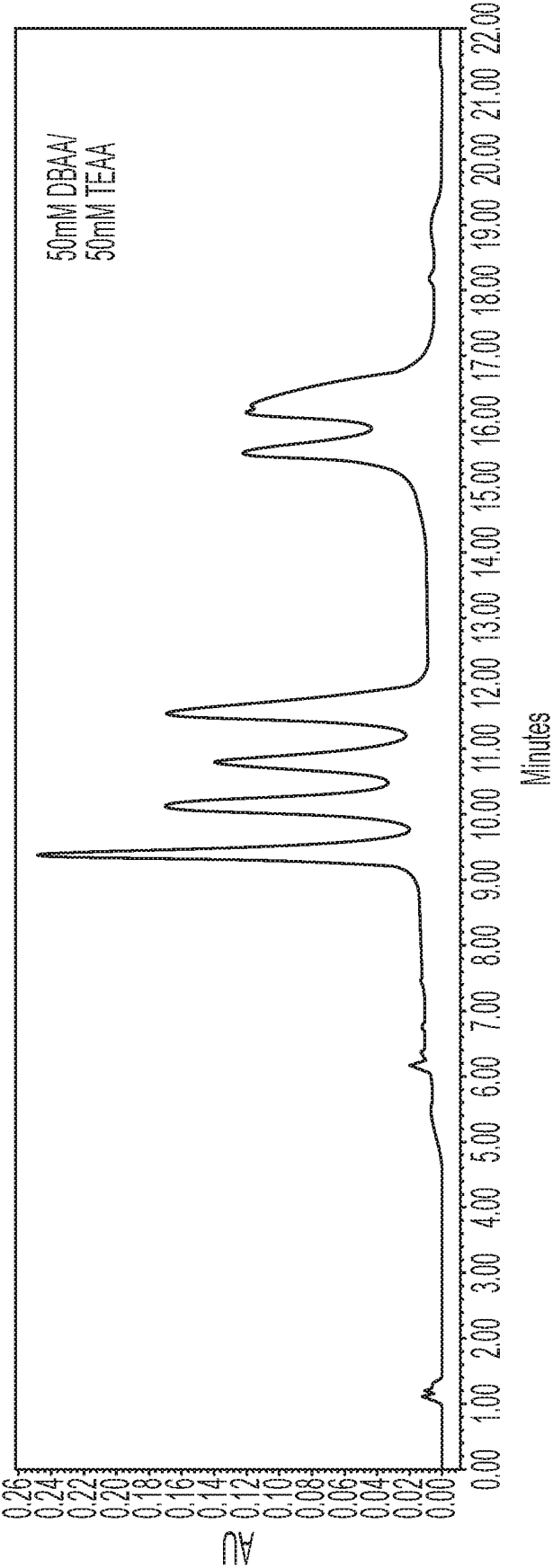


Figure 3F

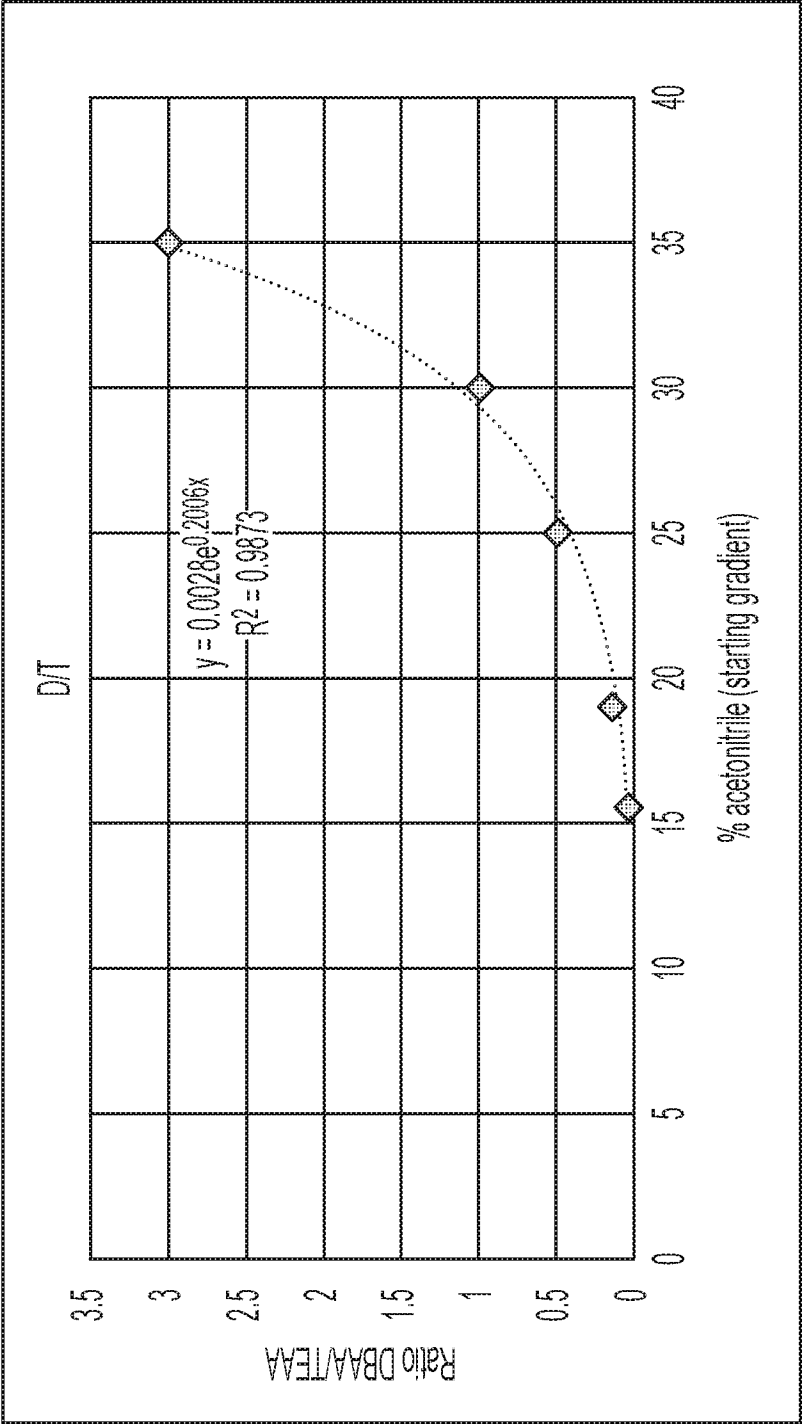


Figure 4

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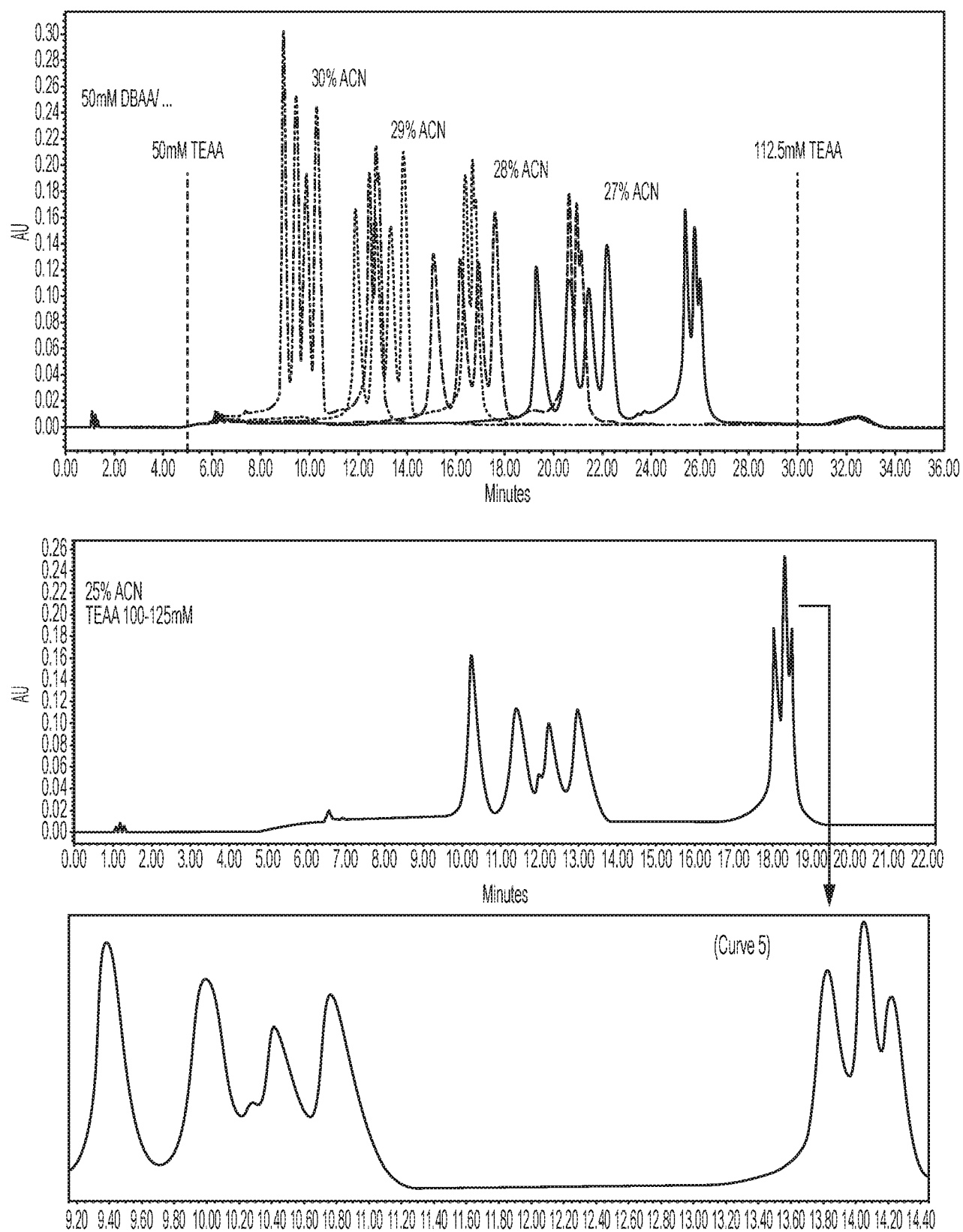


Figure 5

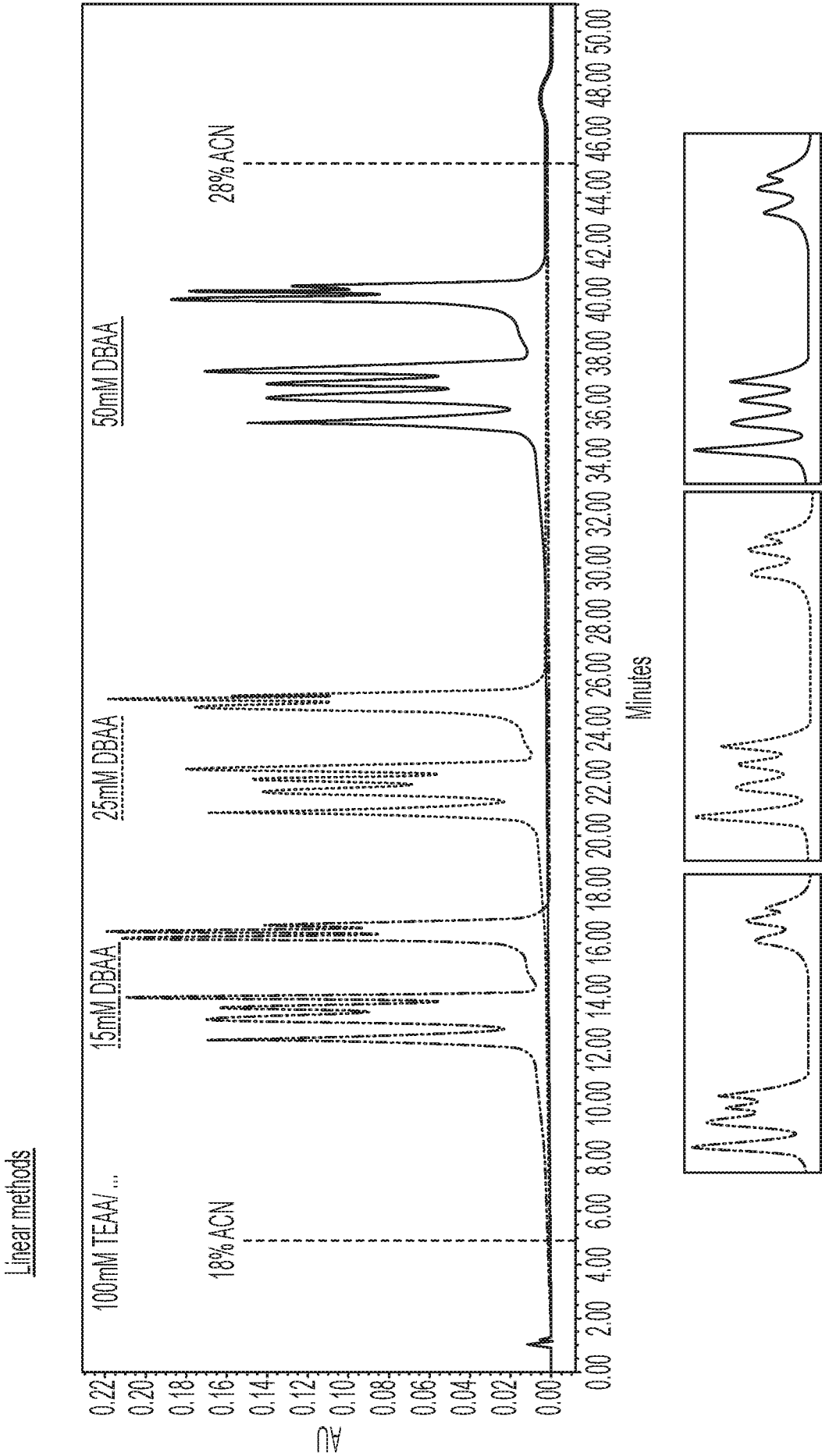


Figure 6A

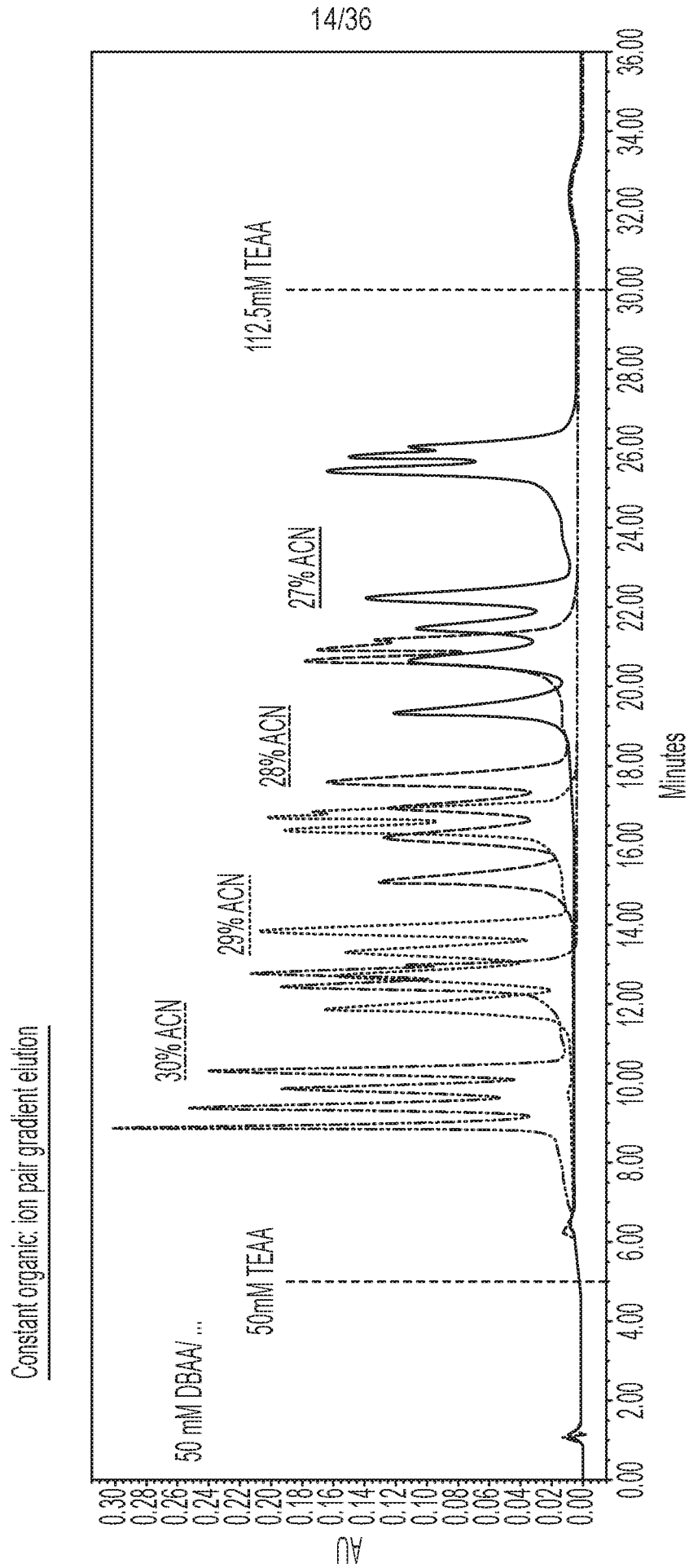


Figure 6B

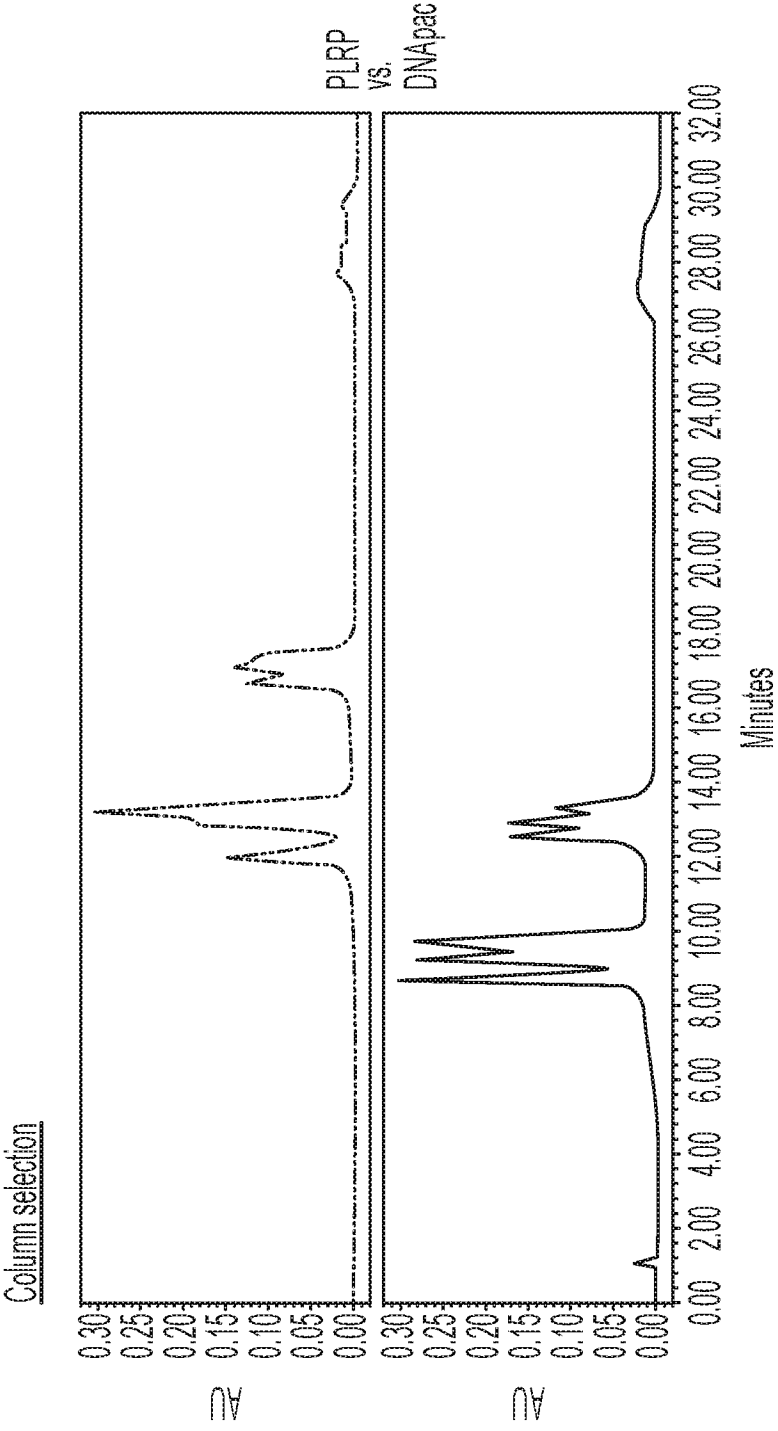


Figure 6C

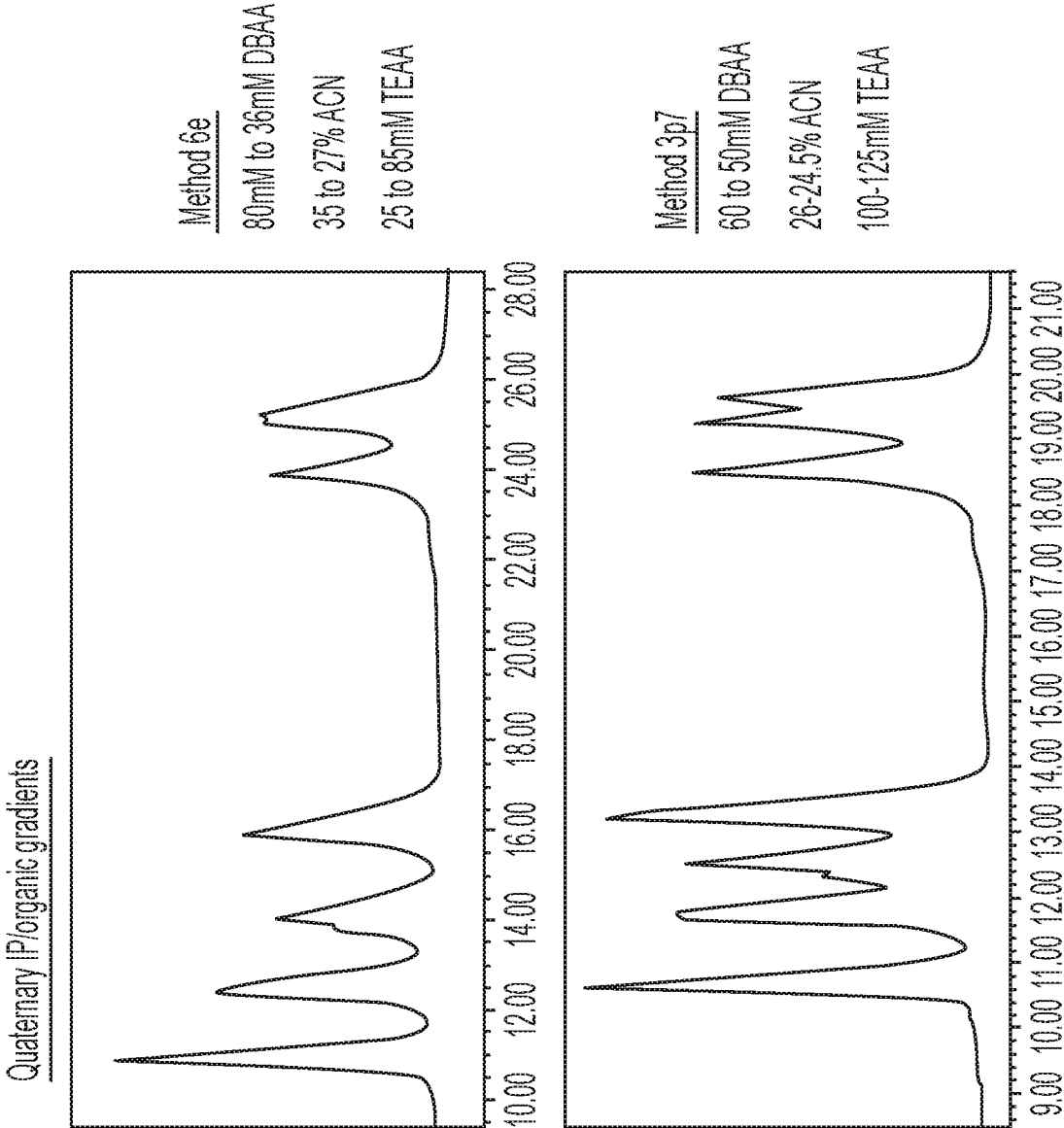


Figure 6D

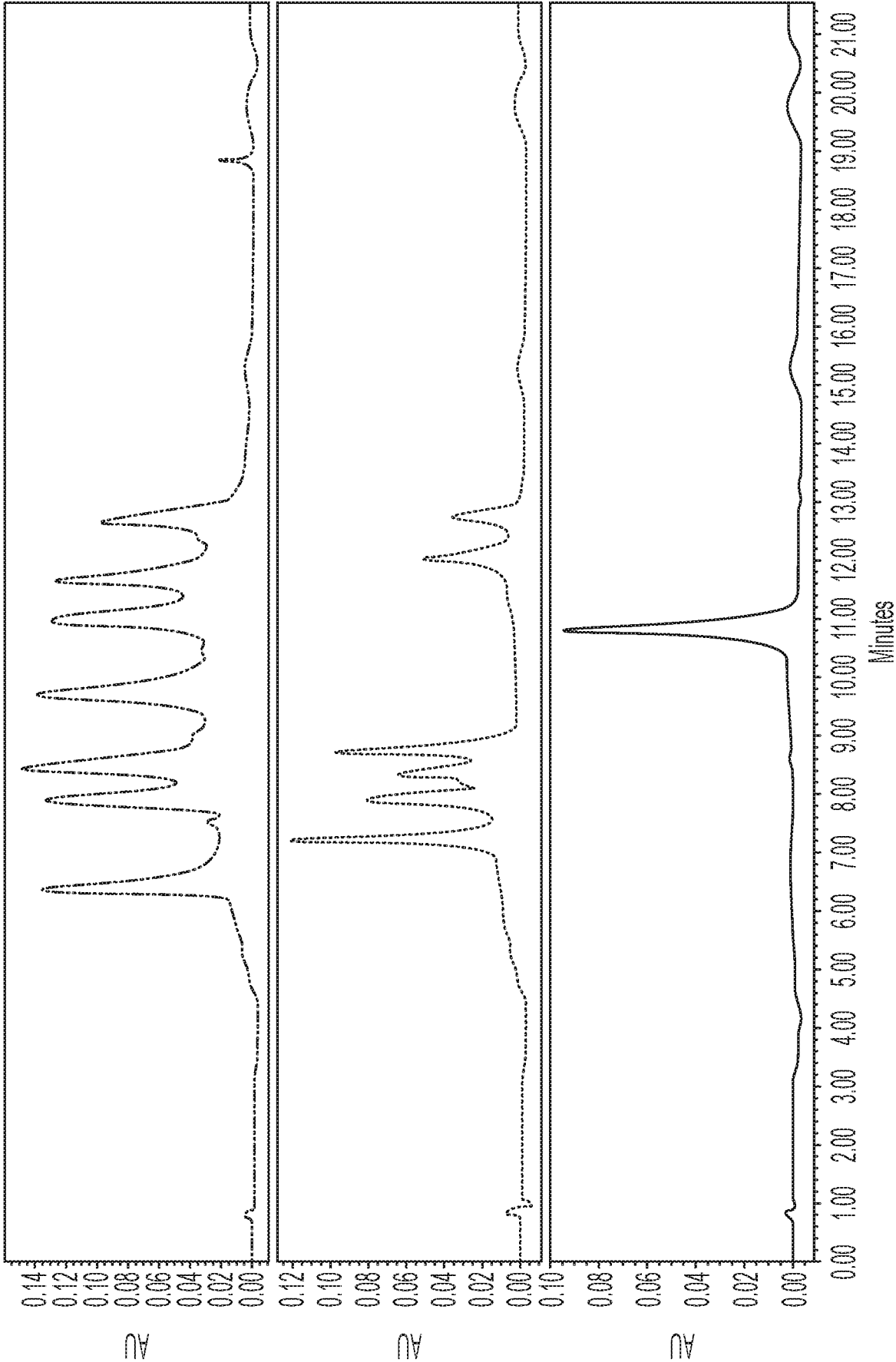


Figure 7

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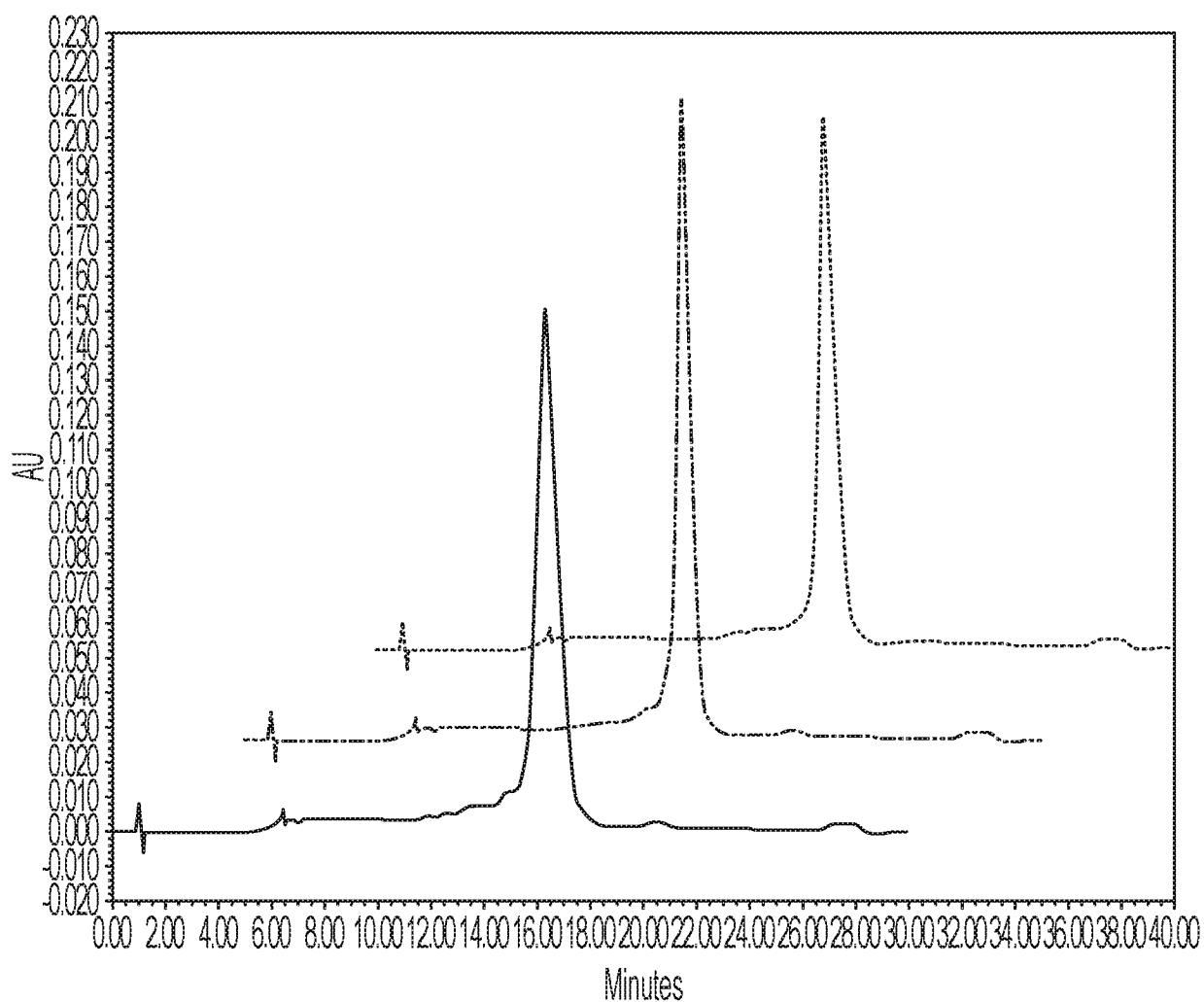
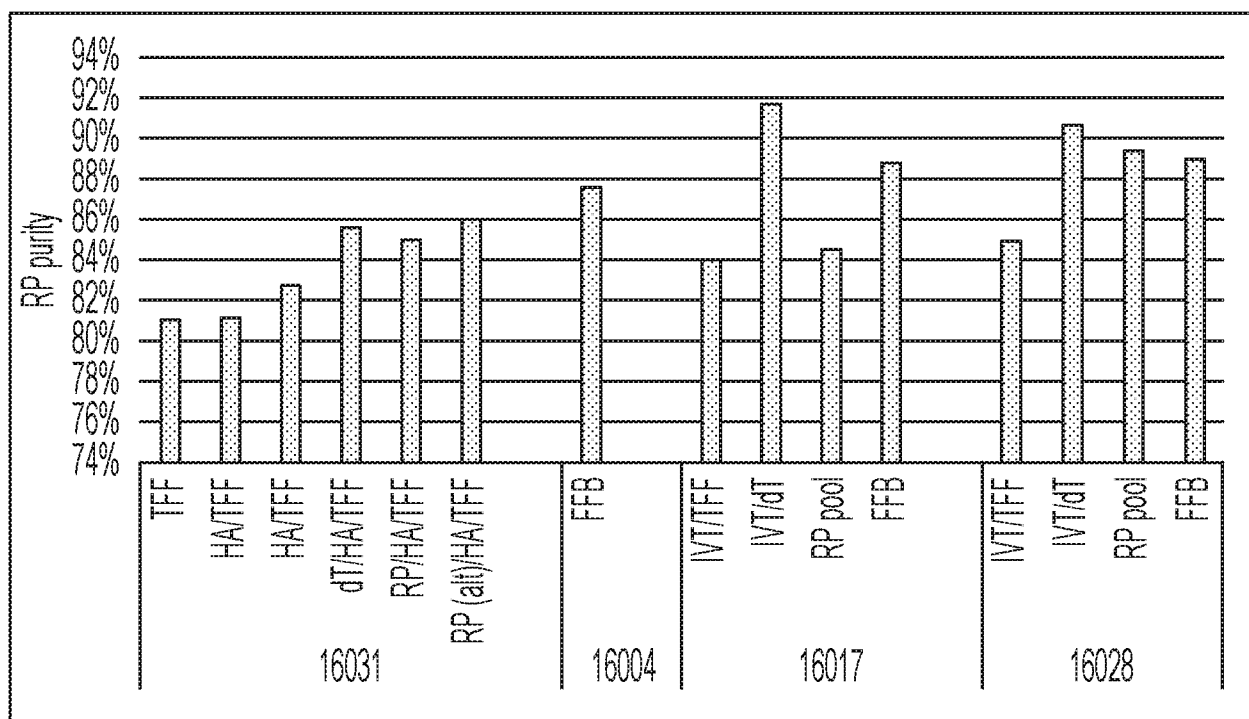


Figure 8

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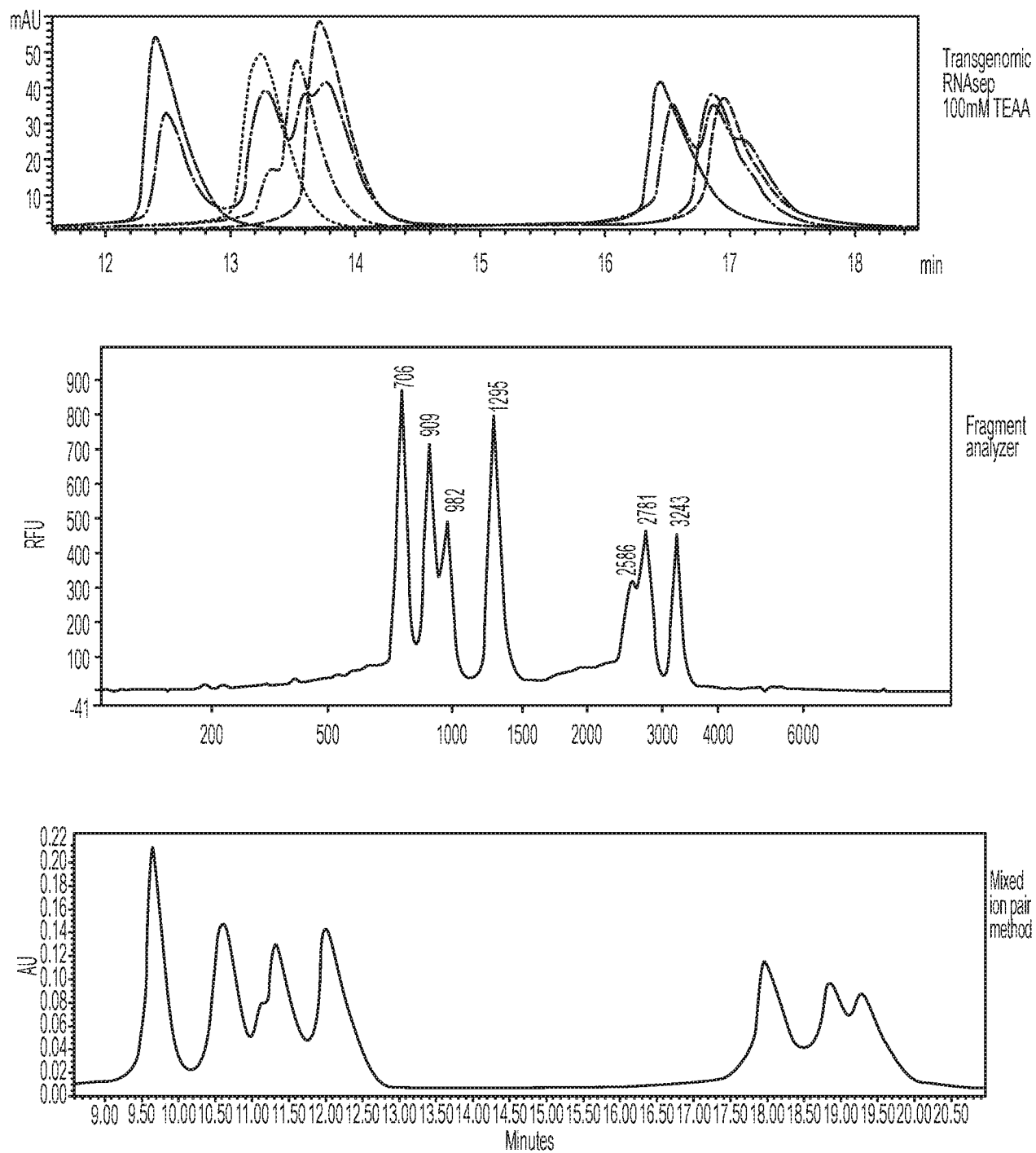


Figure 9

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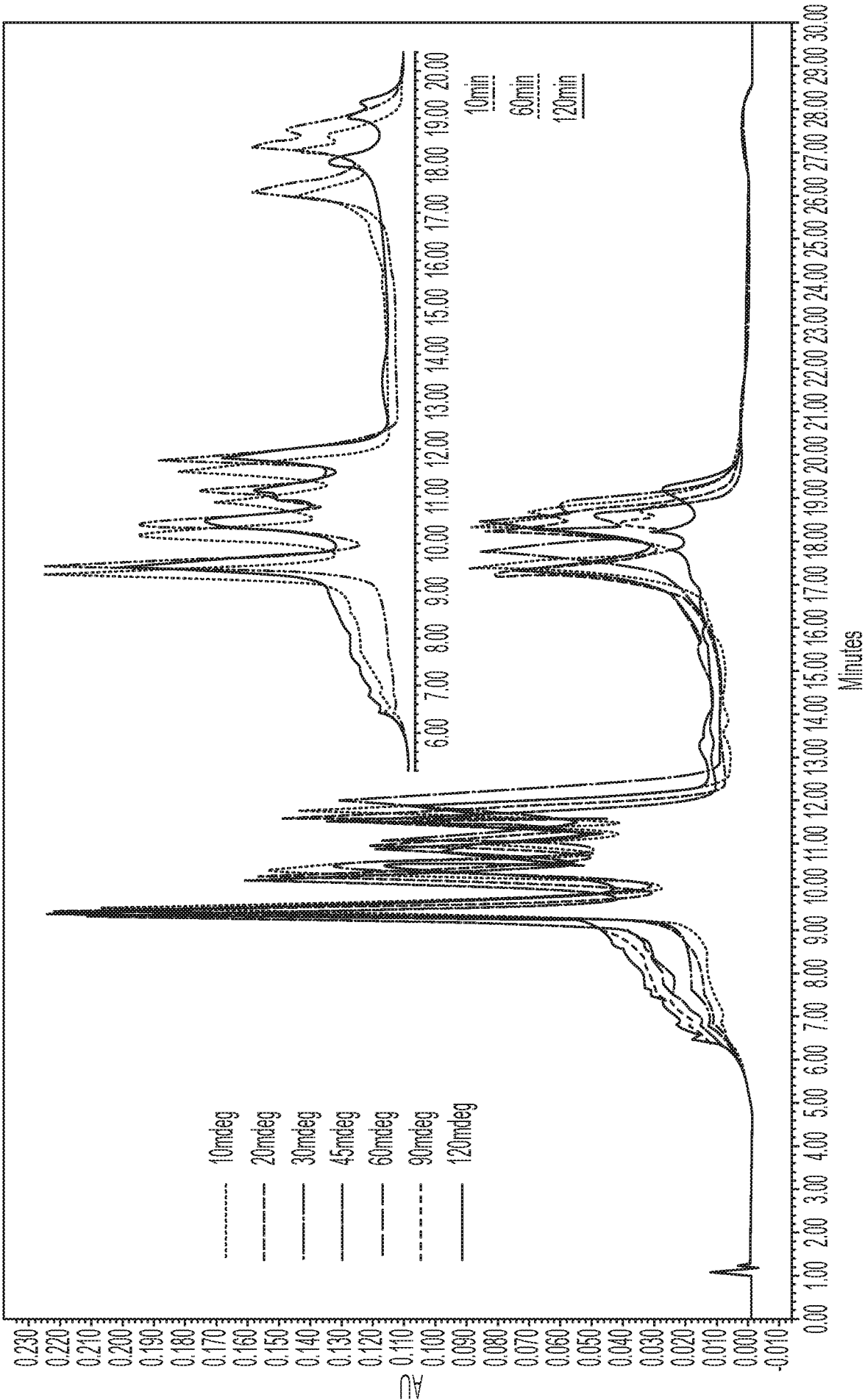


Figure 10

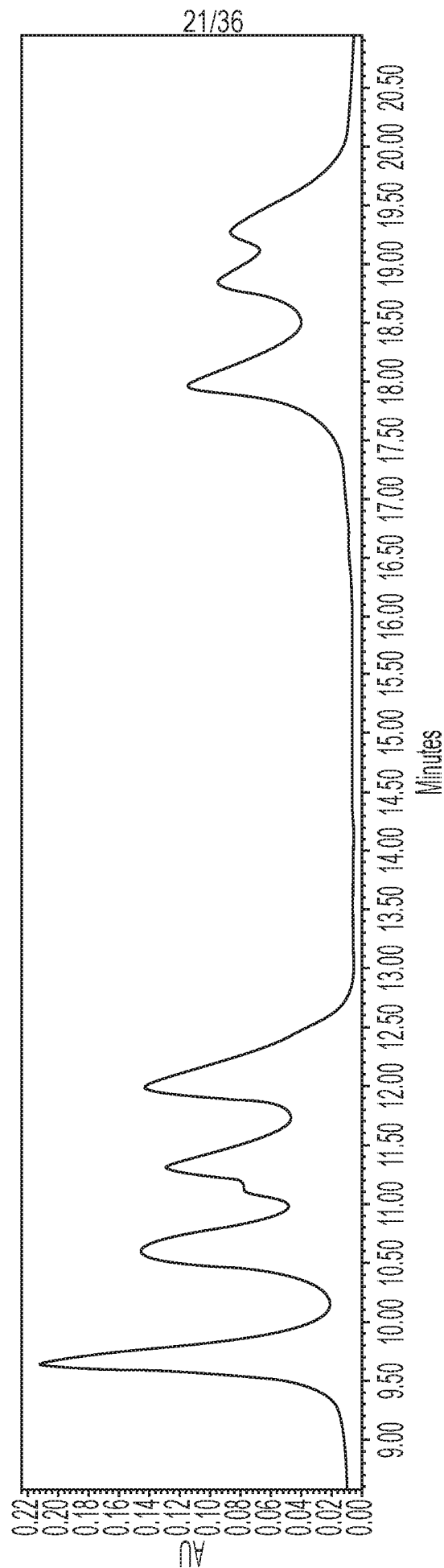


Figure 10 continued

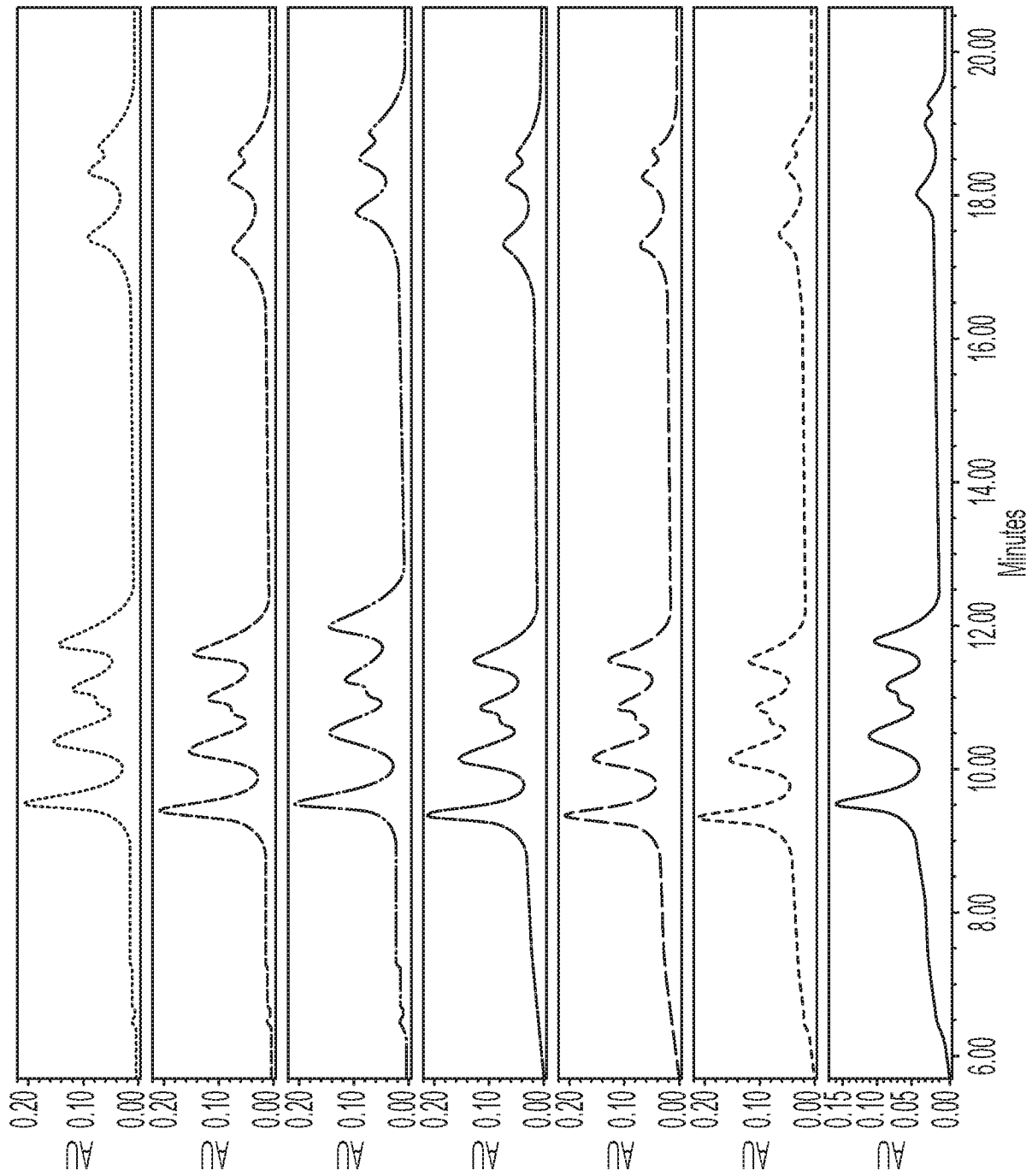


Figure 10 continued

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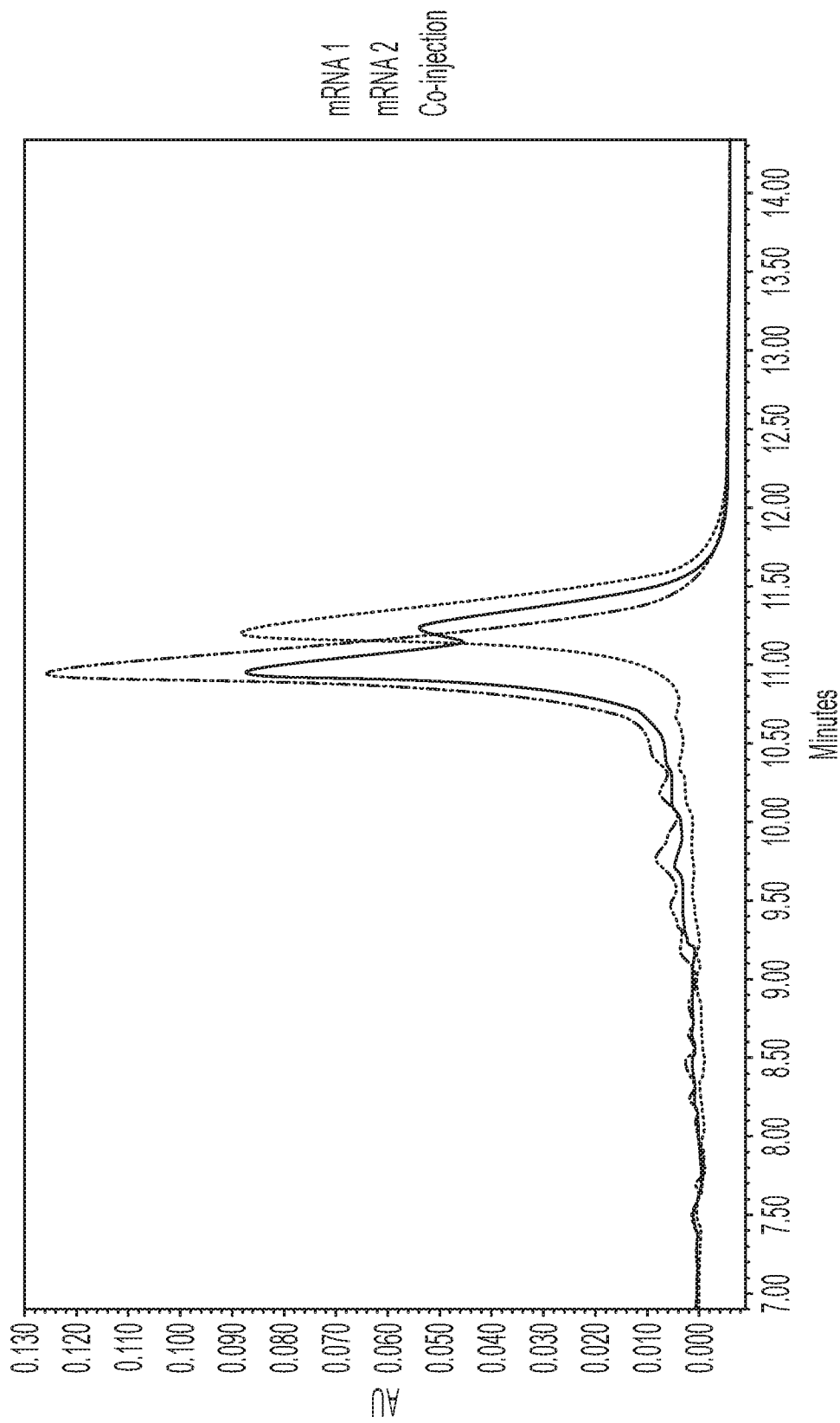


Figure 11

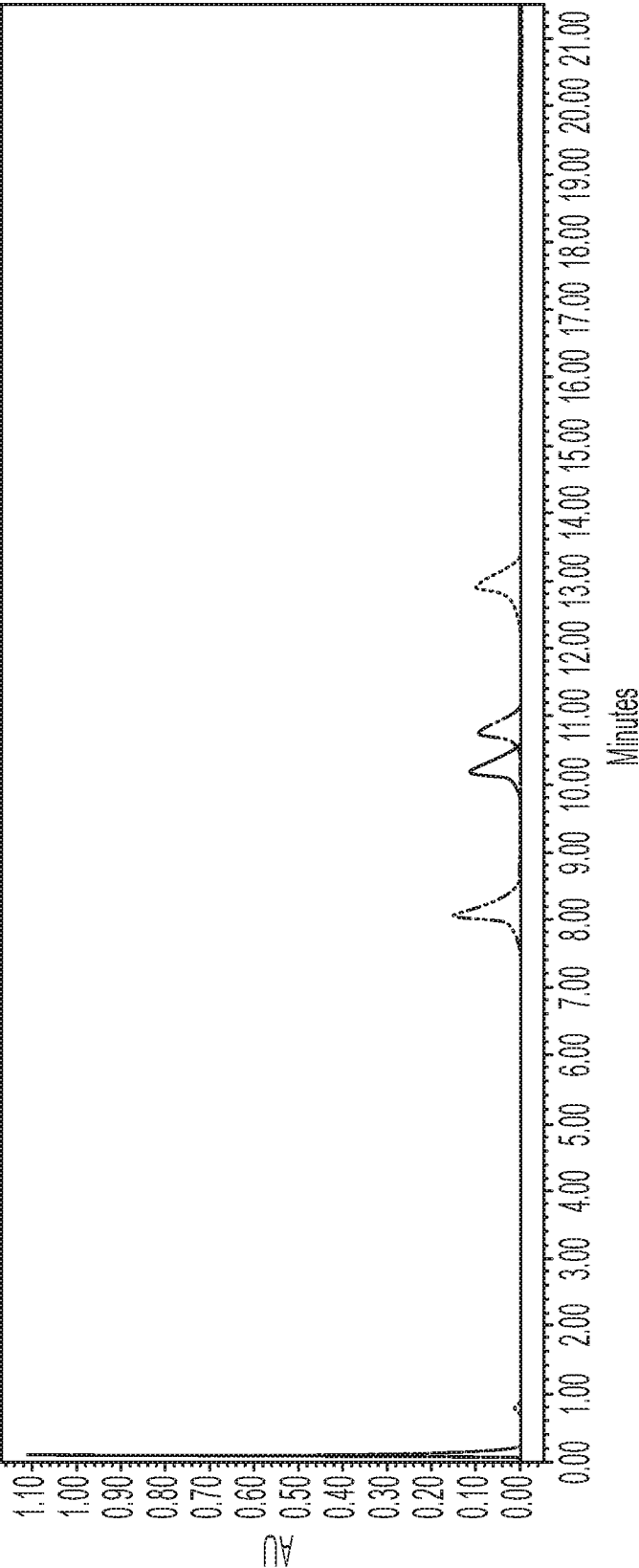


Figure 12

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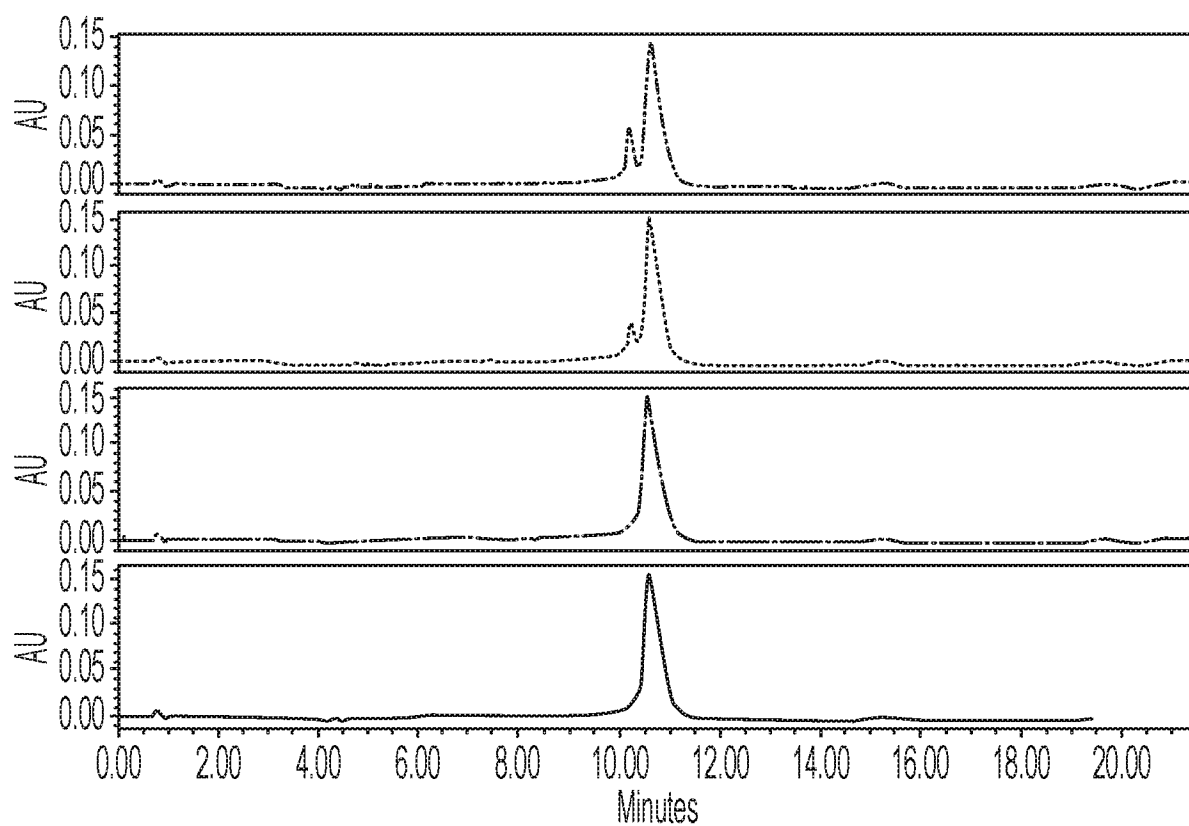
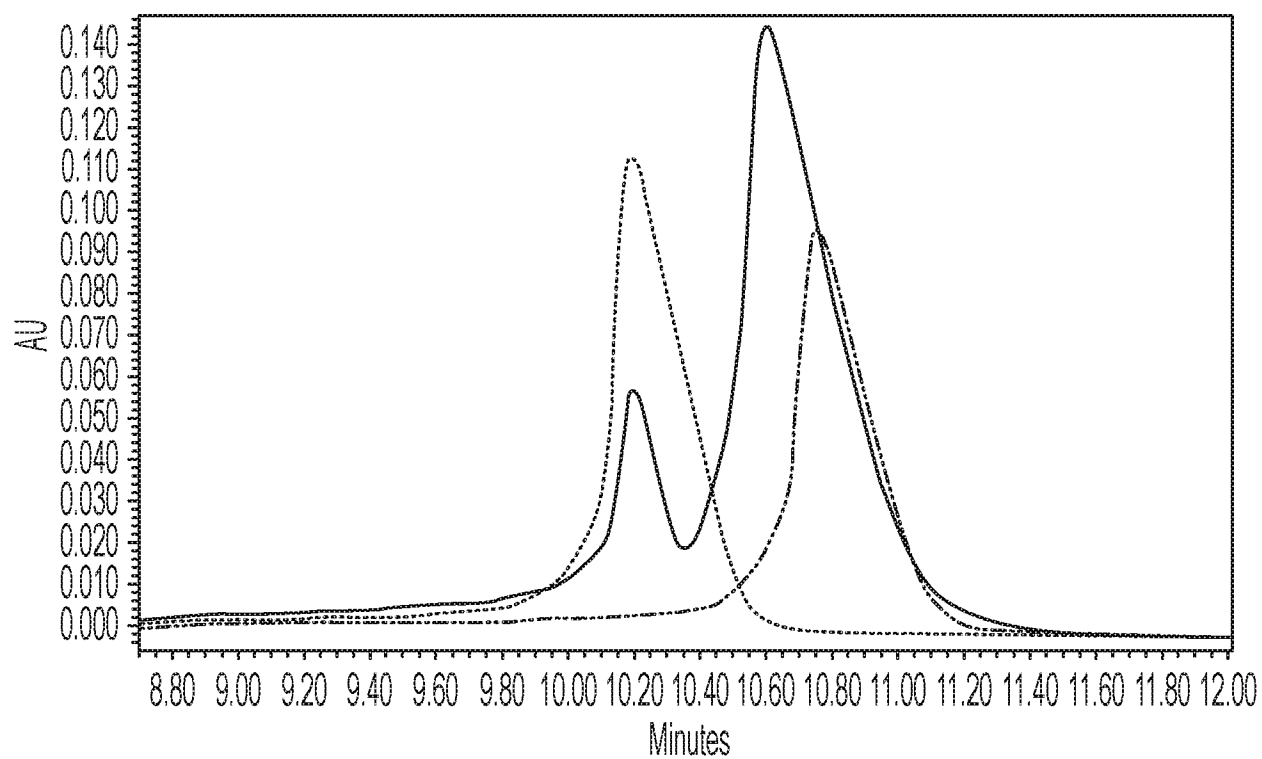


Figure 13

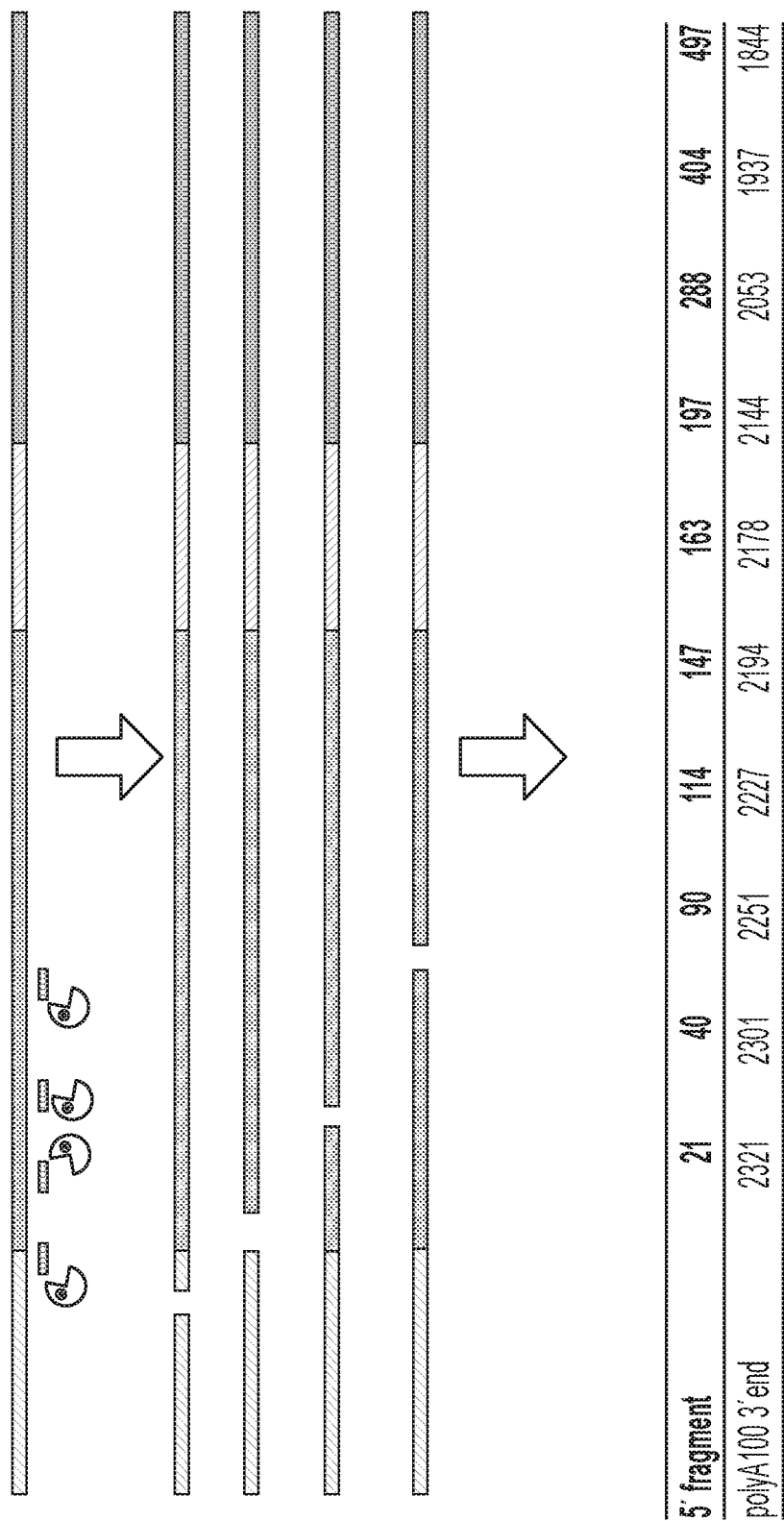


Figure 14

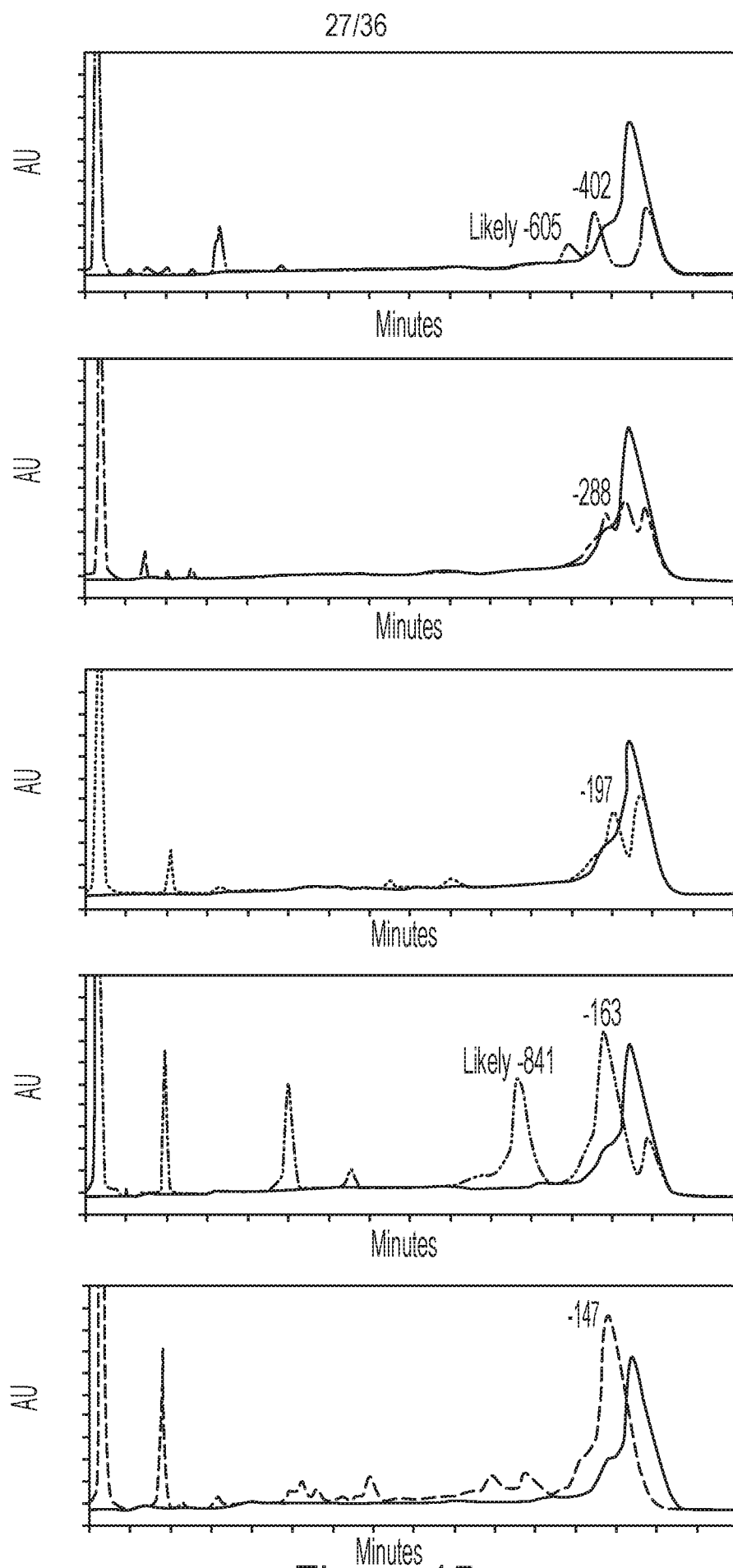


Figure 15

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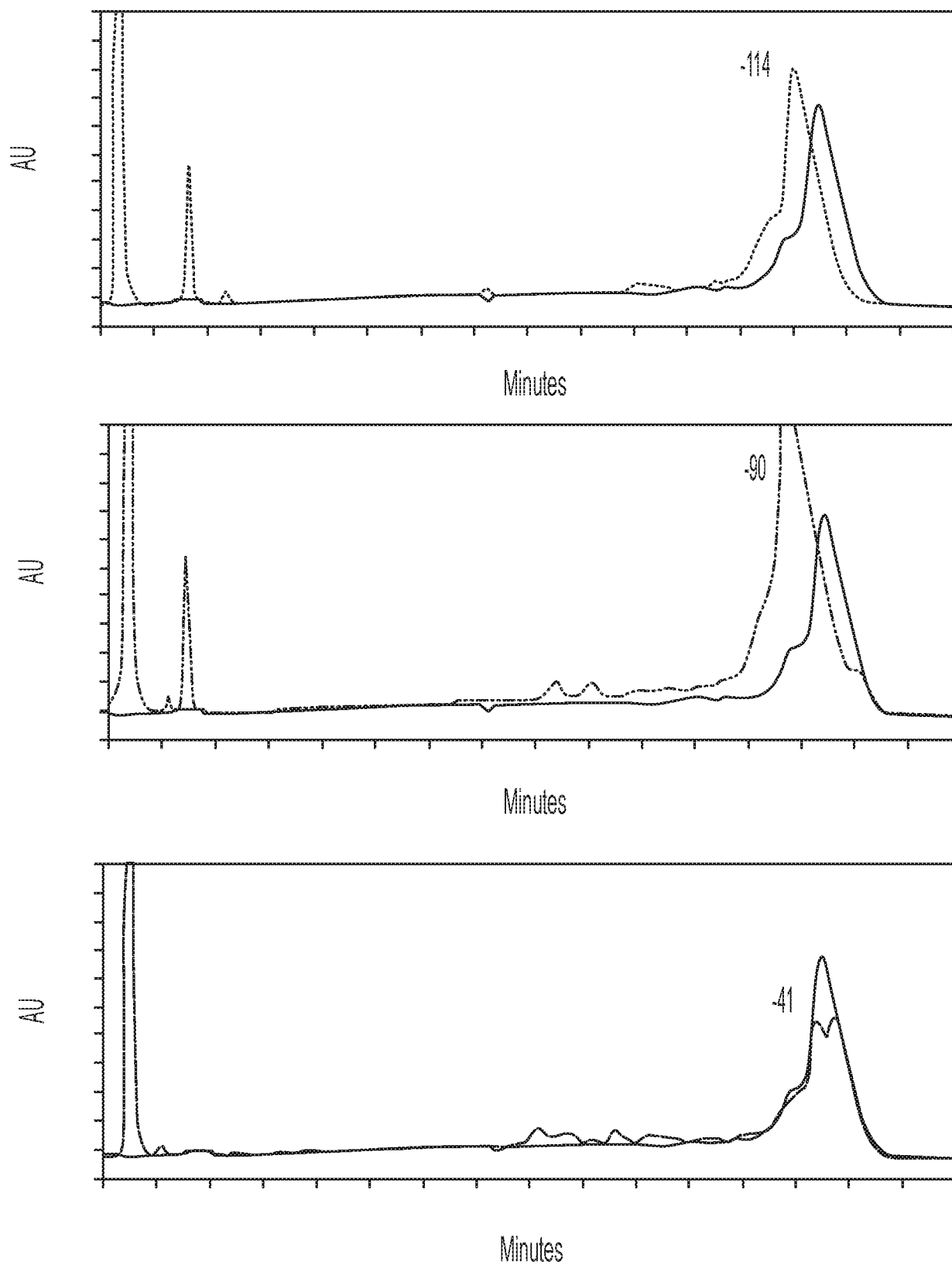
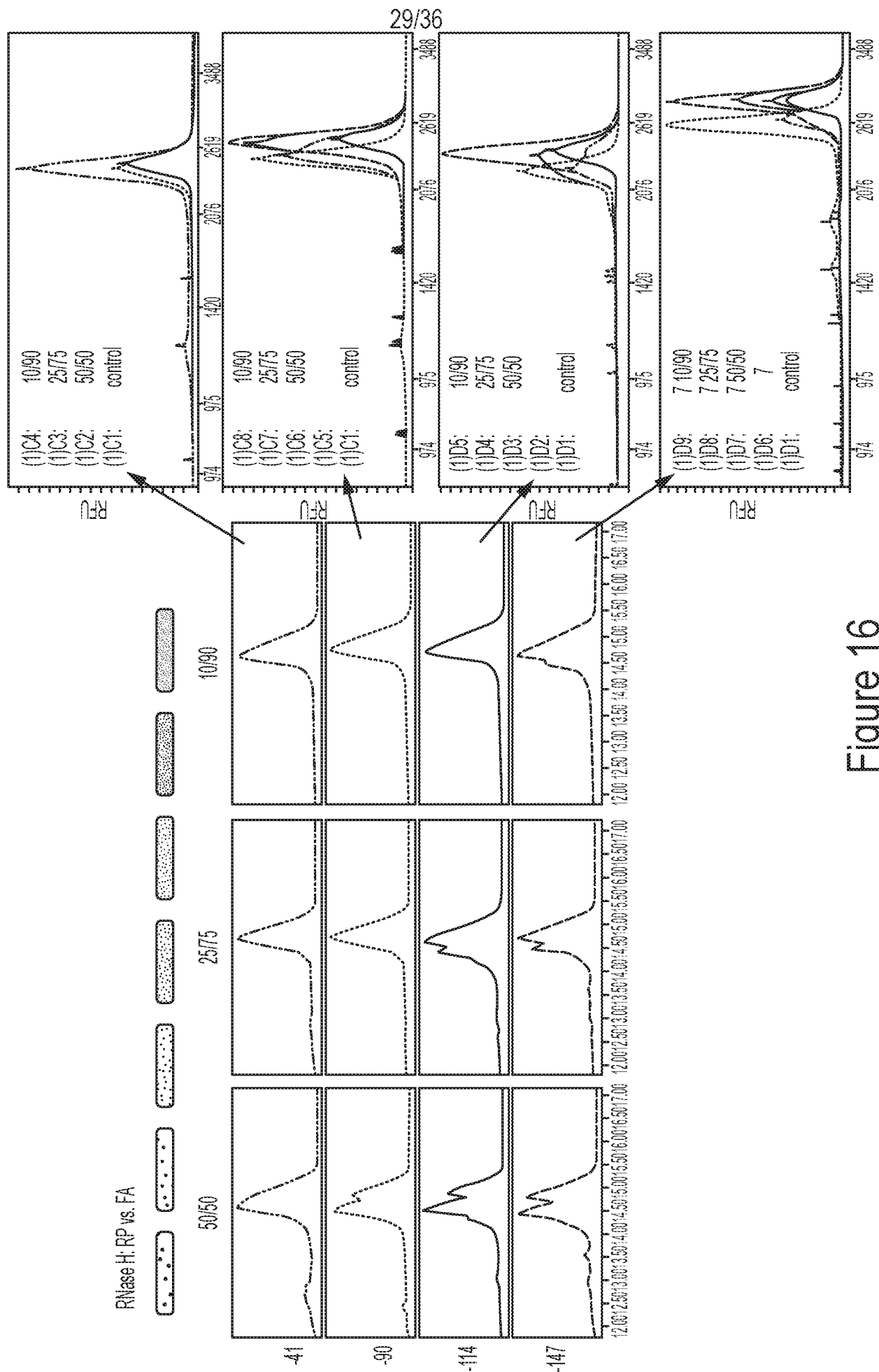


Figure 15 continued



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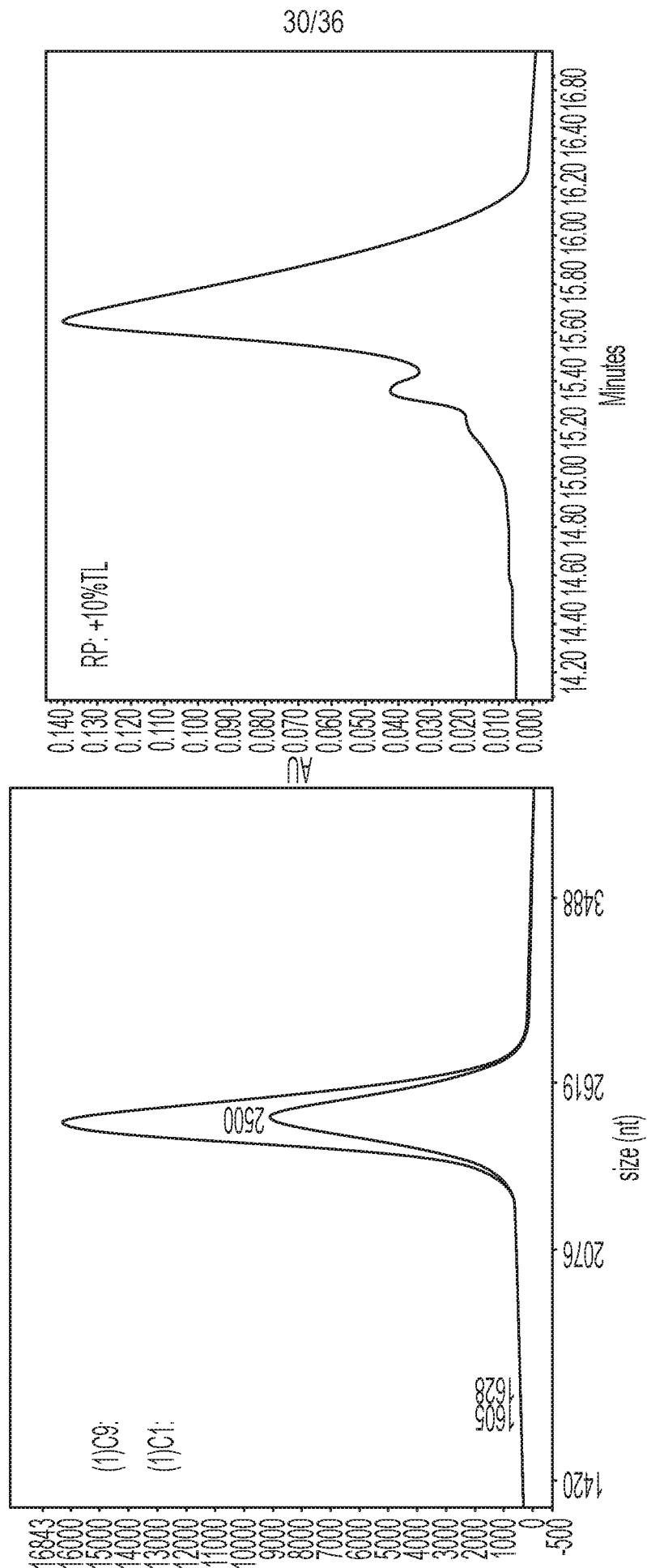


Figure 16 continued

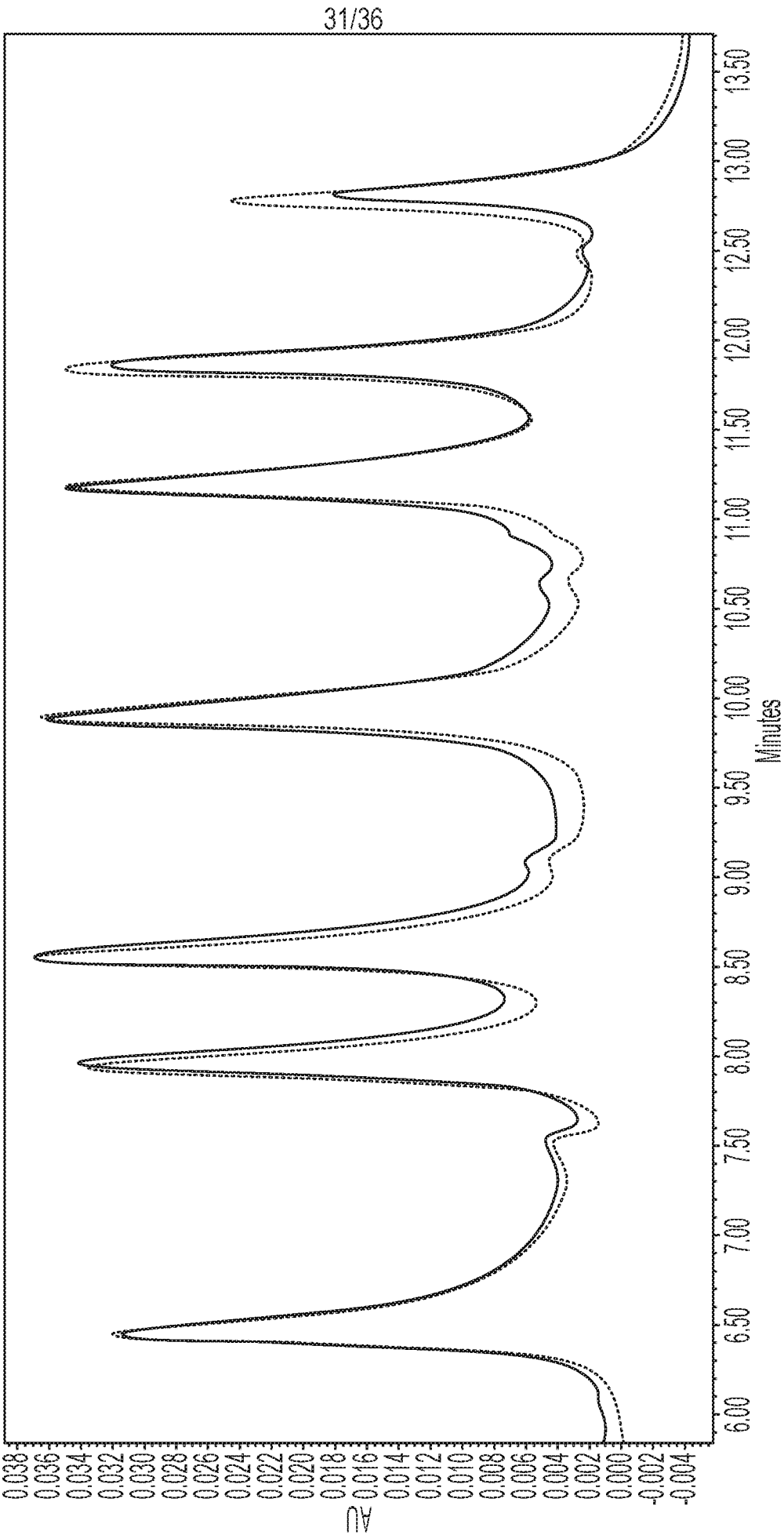


Figure 17

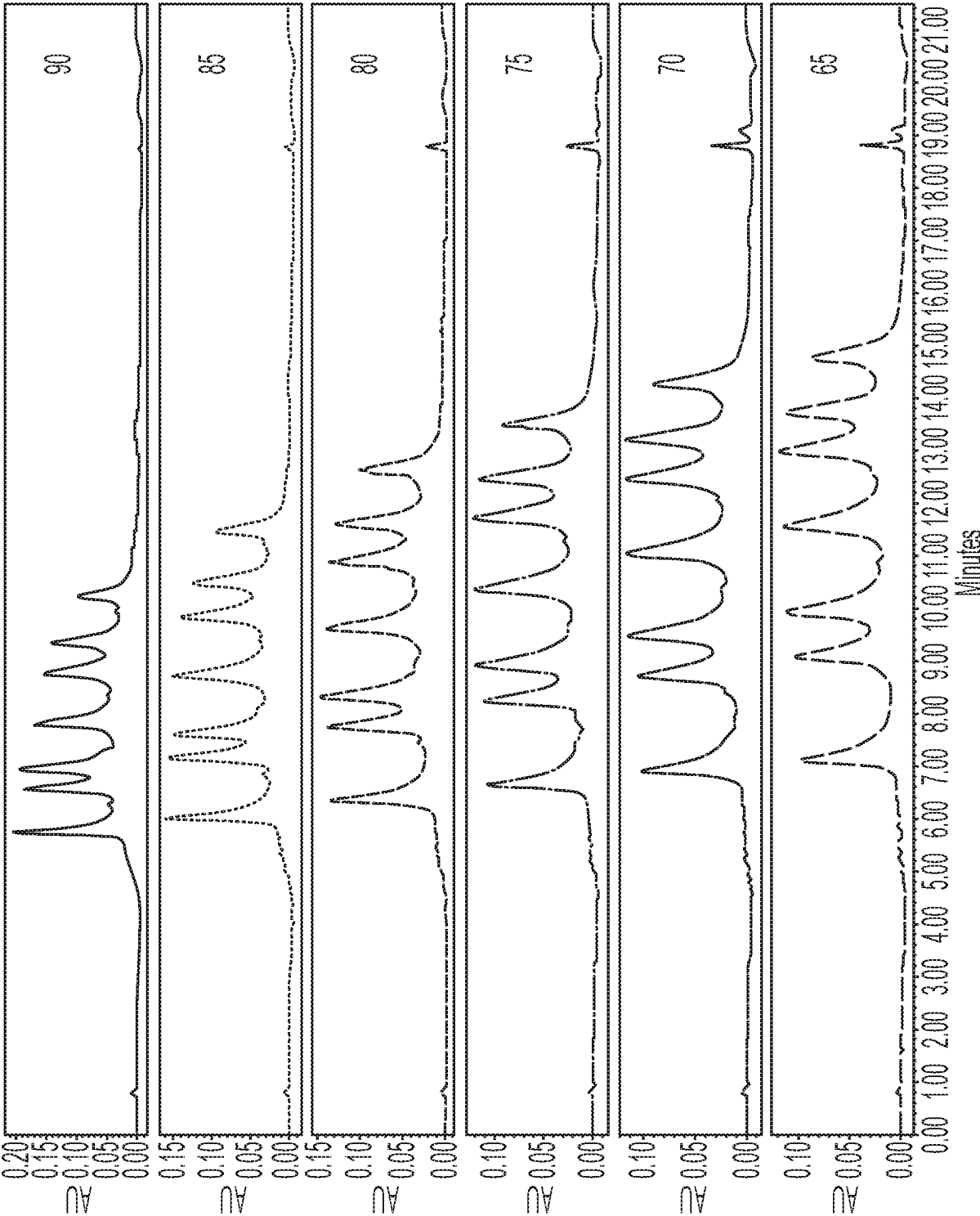


Figure 18

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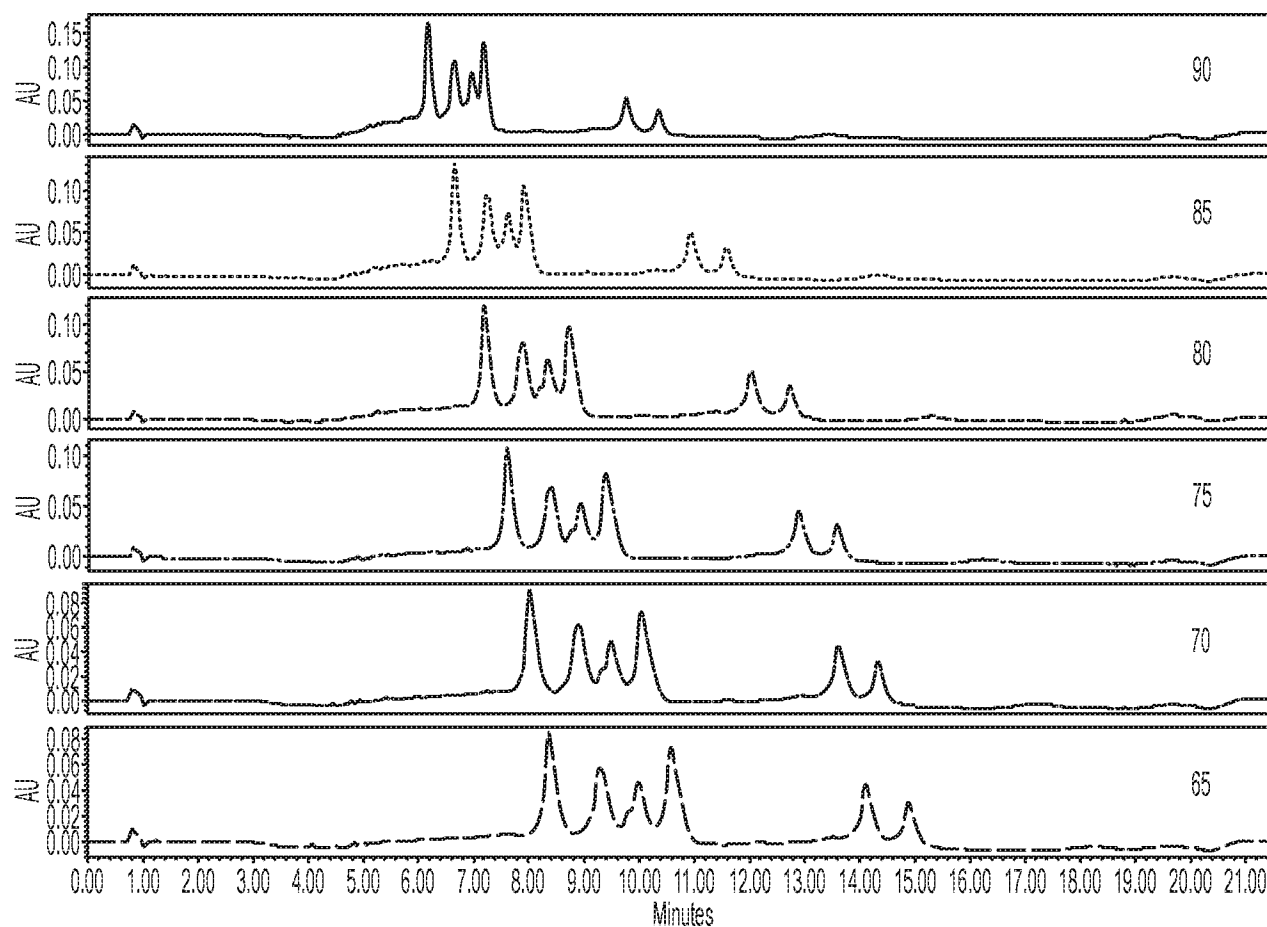


Figure 19

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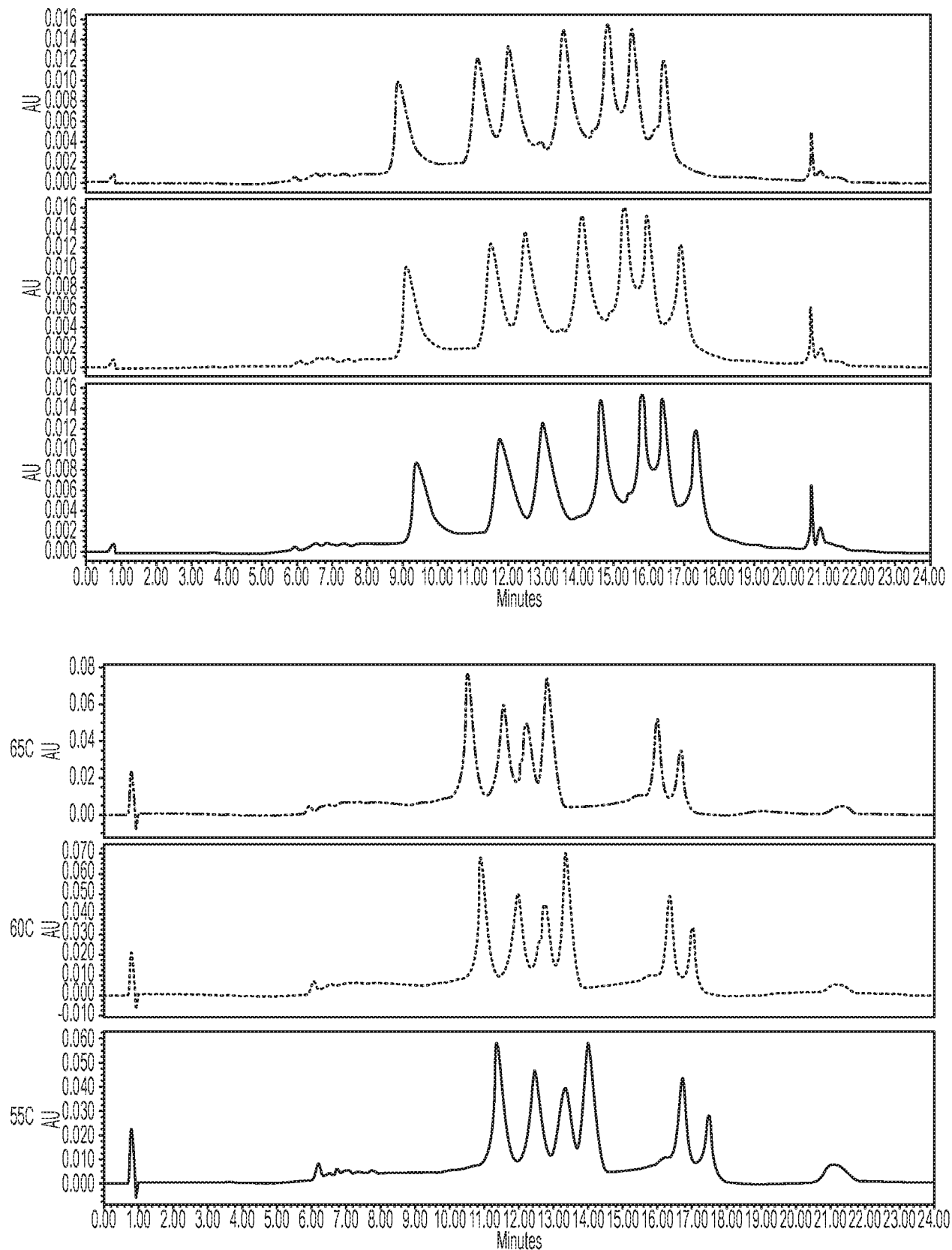


Figure 20

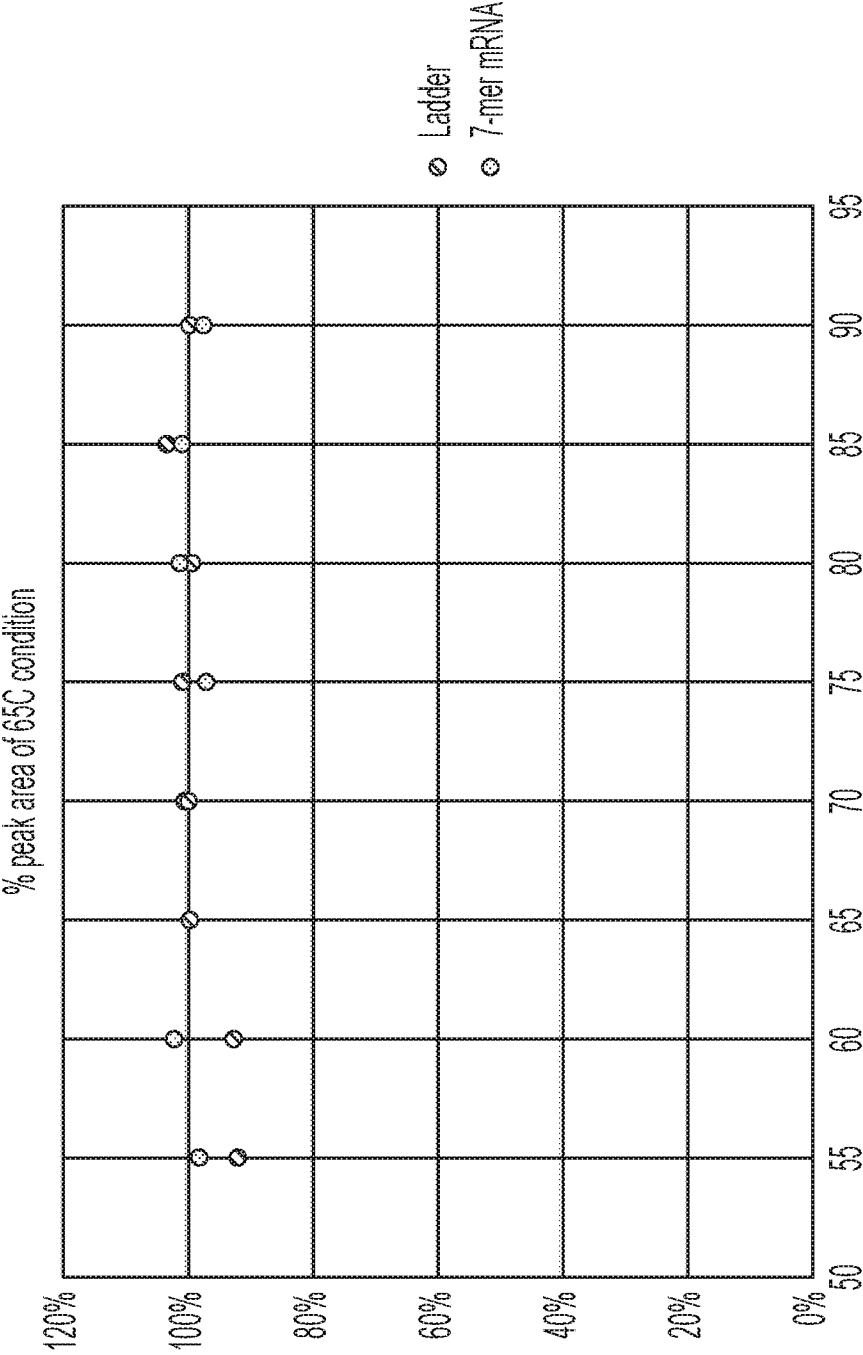


Figure 21

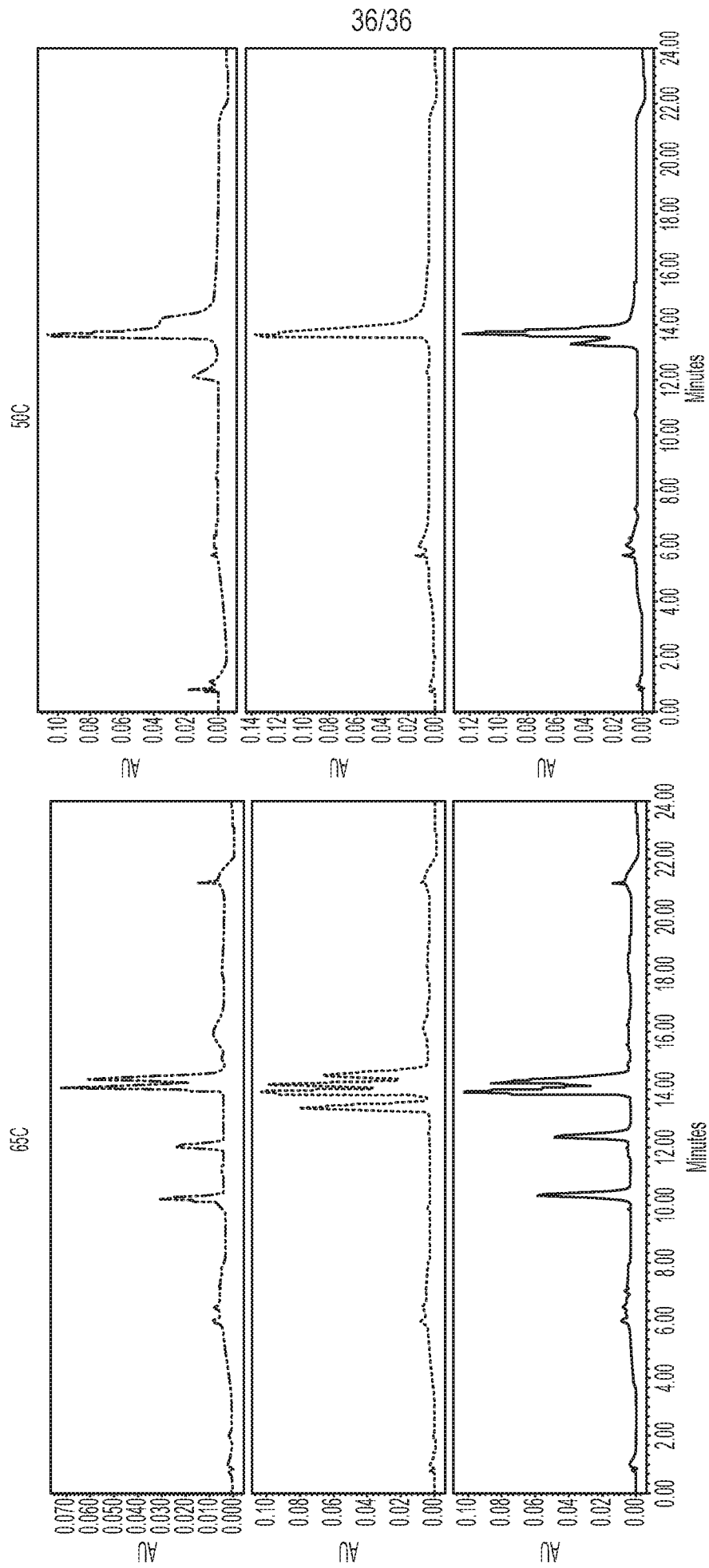


Figure 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/046990

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/10 (2006.01) G01N 30/02 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PATENW, CAPlus, BIOSIS, EMBASE, MEDLINE: ion pair, reverse phase, liquid chromatography, alkylammonium, and like terms.

IPC/CPC: C12N15/10, C12N15/101, G01N30/02, B01D15/08, B01J20/00, B01J39/00, B01J41/00, B01J43/00, B01J45/00, B01J47/00, B01J49/00

Esp@cenet, PubMed: Applicant/Inventor search.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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☒ Further documents are listed in the continuation of Box C

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"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
13 December 2018

Date of mailing of the international search report
13 December 2018

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/US2018/046990
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2003/031580 A2 (TRANSGENOMIC, INC.) 17 April 2003 Abstract; Examples 3 and 4; Pages 26, 41, 42, 48; Claim 57; Table 6; Figure 2	1-55
X	US 2016/0369243 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 22 December 2016 Paragraphs [0383], [0384]	53
X	WO 2016/193206 A1 (CUREVAC AG) 08 December 2016 Example 5; Pages 44-50, 73	53
A	US 2002/0003109 A1 (GJERDE et al.) 10 January 2002 Claims 1, 4, 7; Examples 1-19	1-55
A	US 8383340 B2 (KETTERER et al.) 26 February 2013 Columns 9, 10; Examples 1-7; Claim 13	1-55
A	CLOSE, E. D., et al., "Nucleic acid separations using superficially porous silica particles", Journal of Chromatography A, 2016, Vol. 1440, pp 135-144 Page 137	1-55
P,X	WO 2018/096179 A1 (CUREVAC AG) 31 May 2018 Claims 13, 16, 21, 27	1-55

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INTERNATIONAL SEARCH REPORT		International application No. PCT/US2018/046990	
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2003/031580 A2	17 April 2003	WO 03031580 A2	17 Apr 2003
		AU 2002334892 A1	22 Apr 2003
		US 2003225261 A1	04 Dec 2003
		US 7135289 B2	14 Nov 2006
US 2016/0369243 A1	22 December 2016	US 2016369243 A1	22 Dec 2016
		US 10006007 B2	26 Jun 2018
		AU 2010328310 A1	28 Jun 2012
		AU 2010328310 B2	21 May 2015
		AU 2012362113 A1	14 Aug 2014
		AU 2012362113 B2	03 Aug 2017
		AU 2015215938 A1	10 Sep 2015
		AU 2015215938 B2	18 Jan 2018
		AU 2017251767 A1	16 Nov 2017
		AU 2017251767 B2	16 Aug 2018
		AU 2018202479 A1	26 Apr 2018
		AU 2018264152 A1	06 Dec 2018
		BR 112012013875 A2	03 Nov 2015
		CA 2783032 A1	16 Jun 2011
		CA 2862377 A1	04 Jul 2013
		CN 102947450 A	27 Feb 2013
		CN 102947450 B	23 Nov 2016
		CN 107090436 A	25 Aug 2017
		EP 1979364 A2	15 Oct 2008
		EP 2510099 A2	17 Oct 2012
		EP 2510099 B1	09 Aug 2017
		EP 2578685 A2	10 Apr 2013
		EP 2798064 A1	05 Nov 2014
		EP 2798064 B1	31 Aug 2016
		EP 3112467 A1	04 Jan 2017
		EP 3112467 B1	14 Feb 2018
		EP 3144389 A1	22 Mar 2017
		EP 3144389 B1	09 May 2018
		EP 3287525 A1	28 Feb 2018
		HK 1203559 A1	30 Oct 2015
		JP 2013512690 A	18 Apr 2013
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
		JP 2016171804 A	29 Sep 2016
		KR 20130009944 A	24 Jan 2013
		KR 101878502 B1	13 Jul 2018
		KR 20180081836 A	17 Jul 2018
		SG 181564 A1	30 Jul 2012
		SG 10201408129X A	27 Feb 2015
		US 2009286852 A1	19 Nov 2009
		US 8278036 B2	02 Oct 2012
		US 2013111615 A1	02 May 2013
		US 8691966 B2	08 Apr 2014
		US 2013261172 A1	03 Oct 2013
		US 8748089 B2	10 Jun 2014
		US 2011143436 A1	16 Jun 2011
		US 8808982 B2	19 Aug 2014
		US 2013197068 A1	01 Aug 2013
		US 8835108 B2	16 Sep 2014
		US 2011143397 A1	16 Jun 2011
		US 9012219 B2	21 Apr 2015
		US 2015184123 A1	02 Jul 2015
		US 9163213 B2	20 Oct 2015
		US 2015315572 A1	05 Nov 2015
		US 9371511 B2	21 Jun 2016
		US 2014315988 A1	23 Oct 2014
		US 9371544 B2	21 Jun 2016
		US 2015038558 A1	05 Feb 2015
		US 9750824 B2	05 Sep 2017
		US 2013189741 A1	25 Jul 2013
		US 2014328825 A1	06 Nov 2014
		US 2016251629 A1	01 Sep 2016
		US 2017043037 A1	16 Feb 2017
		US 2018265848 A1	20 Sep 2018
		WO 2007024708 A2	01 Mar 2007
		WO 2011071931 A2	16 Jun 2011
		WO 2011071936 A2	16 Jun 2011
		WO 2013102203 A1	04 Jul 2013
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2016/193206 A1	08 December 2016	WO 2016193206 A1	08 Dec 2016
		CN 107873055 A	03 Apr 2018
		EP 3303583 A1	11 Apr 2018
		US 2018298372 A1	18 Oct 2018
US 2002/0003109 A1	10 January 2002	US 2002003109 A1	10 Jan 2002
		US 6503397 B2	07 Jan 2003
		AU 7157798 A	24 Nov 1998
		AU 725928 B2	26 Oct 2000
		AU 7577298 A	30 Dec 1998
		AU 731812 B2	05 Apr 2001
		AU 7162098 A	24 Nov 1998
		AU 740718 B2	15 Nov 2001
		AU 7579698 A	30 Dec 1998
		AU 743789 B2	07 Feb 2002
		AU 9021998 A	08 Mar 1999
		AU 744476 B2	28 Feb 2002
		AU 6461098 A	29 Sep 1998
		AU 746851 B2	02 May 2002
		AU 8769398 A	01 Mar 1999
		AU 750394 B2	18 Jul 2002
		AU 1294299 A	24 May 1999
		AU 751737 B2	29 Aug 2002
		AU 1077999 A	03 May 1999
		AU 752932 B2	03 Oct 2002
		AU 1230301 A	03 Jul 2001
		AU 784283 B2	02 Mar 2006
		AU 1297599 A	24 May 1999
		AU 2765301 A	17 Sep 2001
		AU 4250400 A	07 Nov 2001
		AU 4734701 A	17 Sep 2001
		AU 4800000 A	22 Jan 2001
		AU 5911301 A	07 Nov 2001
		AU 7384100 A	17 Apr 2001
		AU 8707801 A	27 May 2002
		AU 9695898 A	03 May 1999
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
		AU 2002309588 A1	05 Nov 2002
		CA 2284164 A1	17 Sep 1998
		CA 2285307 A1	05 Nov 1998
		CA 2286350 A1	05 Nov 1998
		CA 2288603 A1	17 Dec 1998
		CA 2288813 A1	17 Dec 1998
		CA 2298019 A1	18 Feb 1999
		CA 2298630 A1	25 Feb 1999
		CA 2308314 A1	14 May 1999
		CA 2309760 A1	22 Apr 1999
		CA 2375746 A1	22 Mar 2001
		CA 2393849 A1	28 Jun 2001
		CA 2406374 A1	01 Nov 2001
		EP 0984978 A1	15 Mar 2000
		EP 1002137 A1	24 May 2000
		EP 1017466 A1	12 Jul 2000
		EP 1017841 A1	12 Jul 2000
		EP 1023306 A1	02 Aug 2000
		EP 1023306 B1	13 Aug 2003
		EP 1023463 A1	02 Aug 2000
		EP 1025113 A1	09 Aug 2000
		EP 1027121 A1	16 Aug 2000
		EP 1030933 A1	30 Aug 2000
		EP 1042503 A1	11 Oct 2000
		EP 1056528 A1	06 Dec 2000
		EP 1231993 A1	21 Aug 2002
		EP 1274837 A2	15 Jan 2003
		EP 1278583 A1	29 Jan 2003
		EP 1331976 A2	06 Aug 2003
		JP 2001515217 A	18 Sep 2001
		JP 3345832 B2	18 Nov 2002
		JP 2000516638 A	12 Dec 2000
		JP 2001504235 A	27 Mar 2001
		JP 2001512702 A	28 Aug 2001
		JP 2001521729 A	13 Nov 2001
		JP 2001524133 A	27 Nov 2001
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
		JP 2002506425 A	26 Feb 2002
		JP 2002506426 A	26 Feb 2002
		JP 2003509662 A	11 Mar 2003
		JP 2003531389 A	21 Oct 2003
		JP 2004506873 A	04 Mar 2004
		US 5772889 A	30 Jun 1998
		US 5972222 A	26 Oct 1999
		US 5986085 A	16 Nov 1999
		US 5997742 A	07 Dec 1999
		US 6017457 A	25 Jan 2000
		US 6024878 A	15 Feb 2000
		US 6027898 A	22 Feb 2000
		US 6030527 A	29 Feb 2000
		US 6056877 A	02 May 2000
		US 6066258 A	23 May 2000
		US 6103112 A	15 Aug 2000
		US 6136195 A	24 Oct 2000
		US 6156206 A	05 Dec 2000
		US 6174441 B1	16 Jan 2001
		US 6177559 B1	23 Jan 2001
		US 6187539 B1	13 Feb 2001
		US 6210885 B1	03 Apr 2001
		US 6218153 B1	17 Apr 2001
		US 6251272 B1	26 Jun 2001
		US 6258264 B1	10 Jul 2001
		US 6265168 B1	24 Jul 2001
		US 6287822 B1	11 Sep 2001
		US 6309549 B1	30 Oct 2001
		US 6342161 B1	29 Jan 2002
		US 6355165 B1	12 Mar 2002
		US 2001008761 A1	19 Jul 2001
		US 6355417 B2	12 Mar 2002
		US 6355791 B1	12 Mar 2002
		US 6372130 B1	16 Apr 2002
		US 6372142 B1	16 Apr 2002
		US 6419824 B1	16 Jul 2002
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Publication Number	Publication Date	Publication Number	Publication Date
		US 6455692 B1	24 Sep 2002
		US 6461819 B1	08 Oct 2002
		US 6471866 B1	29 Oct 2002
		US 6475388 B1	05 Nov 2002
		US 2001030156 A1	18 Oct 2001
		US 6482317 B2	19 Nov 2002
		US 6485648 B1	26 Nov 2002
		US 2001023848 A1	27 Sep 2001
		US 6488855 B2	03 Dec 2002
		US 2001047961 A1	06 Dec 2001
		US 6491821 B2	10 Dec 2002
		US 2001032817 A1	25 Oct 2001
		US 6521123 B2	18 Feb 2003
		US 2002038786 A1	04 Apr 2002
		US 6524480 B2	25 Feb 2003
		US 2001042714 A1	22 Nov 2001
		US 6576133 B2	10 Jun 2003
		US 2002185441 A1	12 Dec 2002
		US 6579459 B2	17 Jun 2003
		US 2001023290 A1	20 Sep 2001
		US 6642374 B2	04 Nov 2003
		US 2002092811 A1	18 Jul 2002
		US 6652745 B2	25 Nov 2003
		US 2003064524 A1	03 Apr 2003
		US 6838242 B2	04 Jan 2005
		US 7138518 B1	21 Nov 2006
		US 2003165941 A1	04 Sep 2003
		US 7225079 B2	29 May 2007
		US 2001051715 A1	13 Dec 2001
		US 2002036169 A1	28 Mar 2002
		US 2002102563 A1	01 Aug 2002
		US 2002123063 A1	05 Sep 2002
		US 2002153312 A1	24 Oct 2002
		US 2002158017 A1	31 Oct 2002
		US 2002169309 A1	14 Nov 2002
		US 2002197629 A1	26 Dec 2002
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
		US 2002197638 A1	26 Dec 2002
		US 2003057154 A1	27 Mar 2003
		US 2003075503 A1	24 Apr 2003
		US 2003102260 A1	05 Jun 2003
		US 2003144500 A1	31 Jul 2003
		US 2003175723 A1	18 Sep 2003
		US 2004034211 A1	19 Feb 2004
		US 2007161010 A1	12 Jul 2007
		WO 0102418 A1	11 Jan 2001
		WO 0119485 A1	22 Mar 2001
		WO 0146687 A2	28 Jun 2001
		WO 0166216 A1	13 Sep 2001
		WO 0166218 A1	13 Sep 2001
		WO 0180964 A1	01 Nov 2001
		WO 0181566 A2	01 Nov 2001
		WO 0240130 A1	23 May 2002
		WO 9840395 A1	17 Sep 1998
		WO 9848913 A1	05 Nov 1998
		WO 9848914 A1	05 Nov 1998
		WO 9856797 A1	17 Dec 1998
		WO 9856798 A1	17 Dec 1998
		WO 9907899 A1	18 Feb 1999
		WO 9909203 A1	25 Feb 1999
		WO 9919514 A1	22 Apr 1999
		WO 9919517 A1	22 Apr 1999
		WO 9922839 A1	14 May 1999
		WO 9923257 A1	14 May 1999
		WO 02086444 A2	31 Oct 2002
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
US 8383340 B2	26 February 2013	US 2010048883 A1	25 Feb 2010
		US 8383340 B2	26 Feb 2013
		AU 2007338360 A1	03 Jul 2008
		AU 2007338360 B2	02 Jun 2011
		CA 2670727 A1	03 Jul 2008
		CN 101563457 A	21 Oct 2009
		CN 101563457 B	12 Mar 2014
		DE 102006061015 A1	26 Jun 2008
		EP 2092064 A1	26 Aug 2009
		EP 2092064 B1	15 Sep 2010
		JP 2010512767 A	30 Apr 2010
		JP 5307724 B2	02 Oct 2013
		KR 20090098968 A	18 Sep 2009
		KR 101183173 B1	14 Sep 2012
		WO 2008077592 A1	03 Jul 2008
WO 2018/096179 A1	31 May 2018	WO 2018096179 A1	31 May 2018
End of Annex			