

[54] **DIFFERENTIAL CHROMATOGRAPHIC METHOD**

3,399,972 9/1968 Skeggs et al. 23/253 R

[75] Inventors: **Charles D. Scott; W. Wilson Pitt, Jr.**, both of Oak Ridge, Tenn.

Primary Examiner—R. E. Serwin
Attorney, Agent, or Firm—John A. Horan; David S. Zachry; John B. Hardaway

[73] Assignee: **The United States of America as represented by the United States Atomic Energy Commission**

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[51] Int. Cl. **G01n 33/16, G01n 33/18, G01n 31/08**

[58] Field of Search **23/230 B, 232 C, 253 R, 23/230 R; 210/31 C, 198 C; 73/61.1 C**

[57]

ABSTRACT

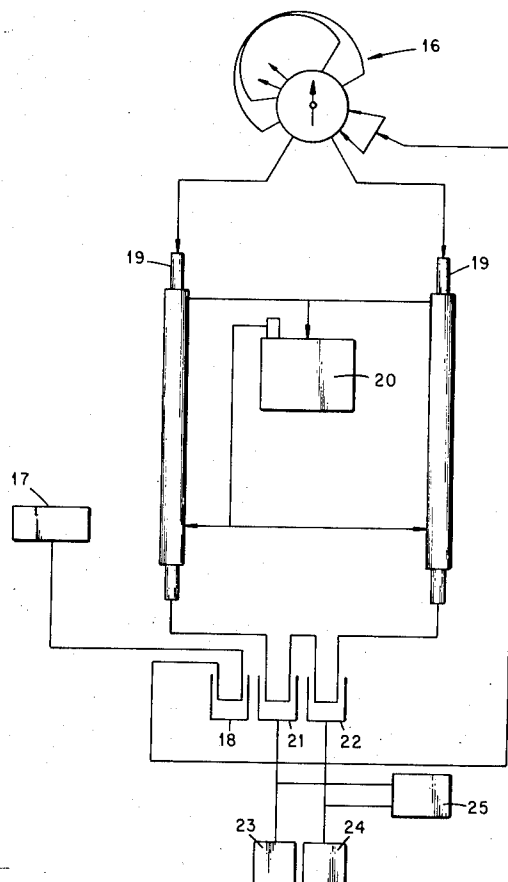
A method for carrying out differential chromatography by simultaneously introducing samples and eluent into at least two parallel chromatographic columns, photometrically monitoring the outflow from the columns, electronically subtracting the output of one photometer from that of another and plotting the difference as a function of time.

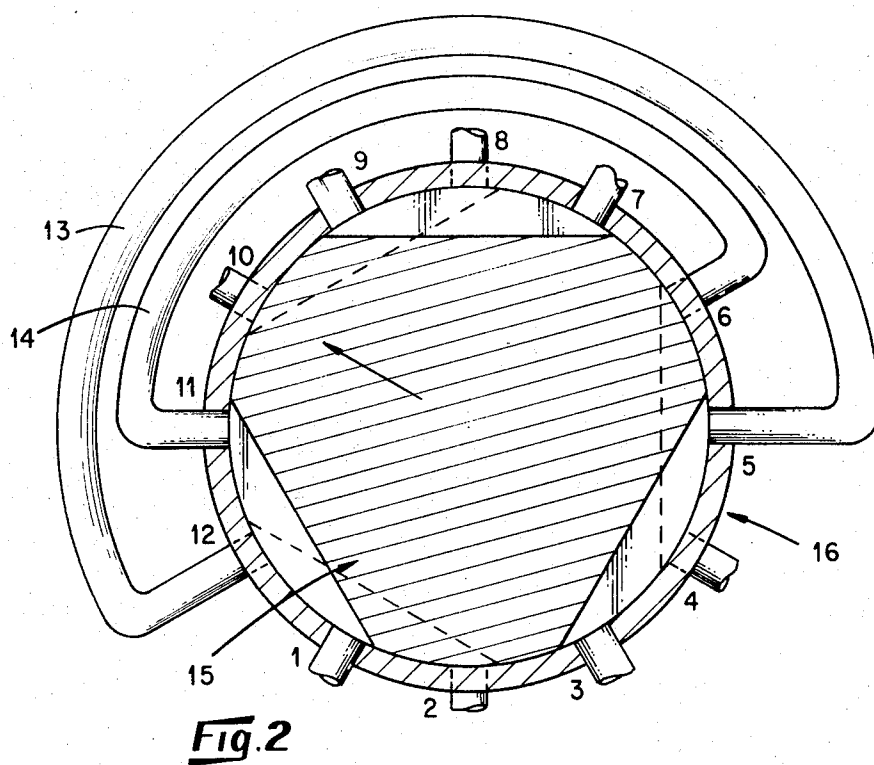
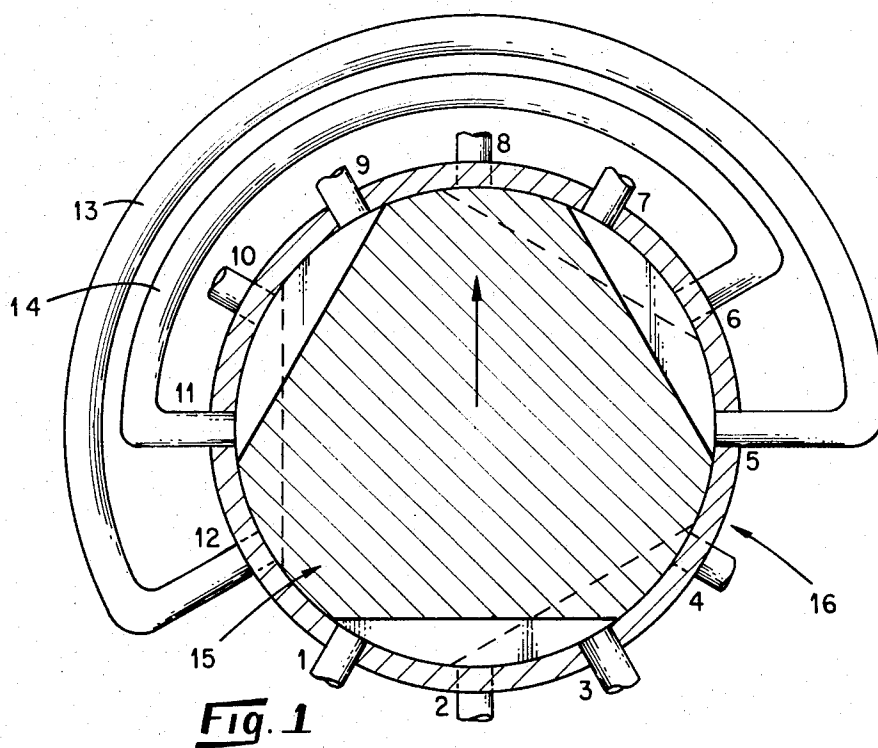
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9 Claims, 7 Drawing Figures





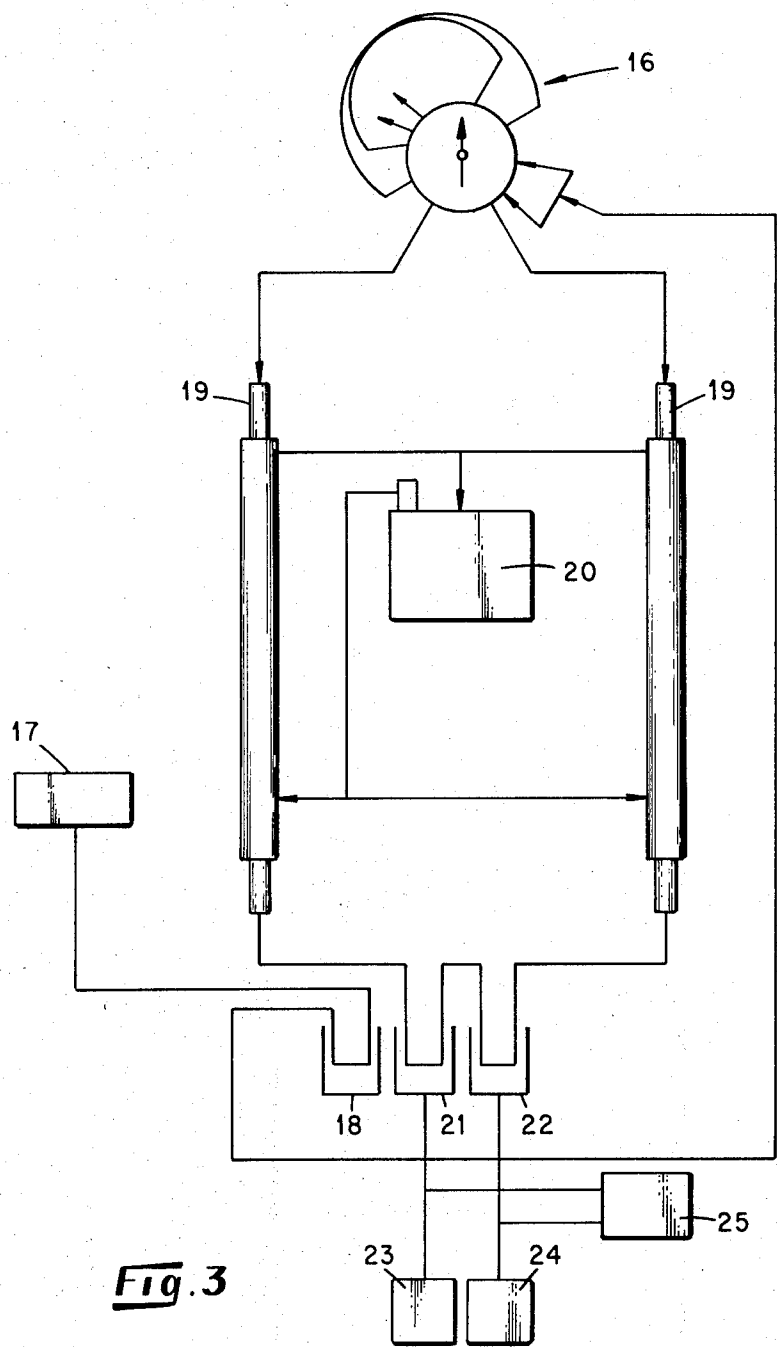


Fig. 3

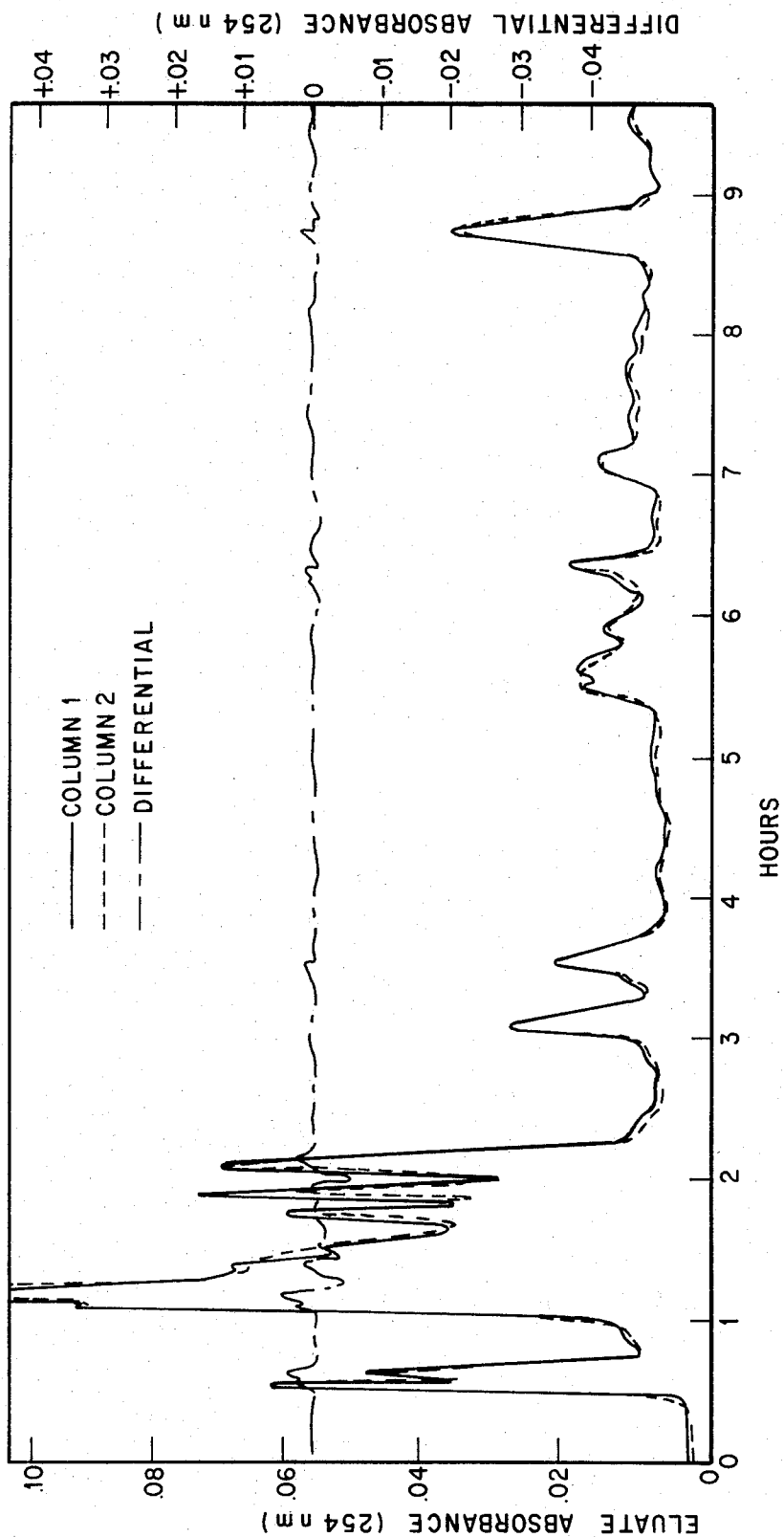


Fig. 4

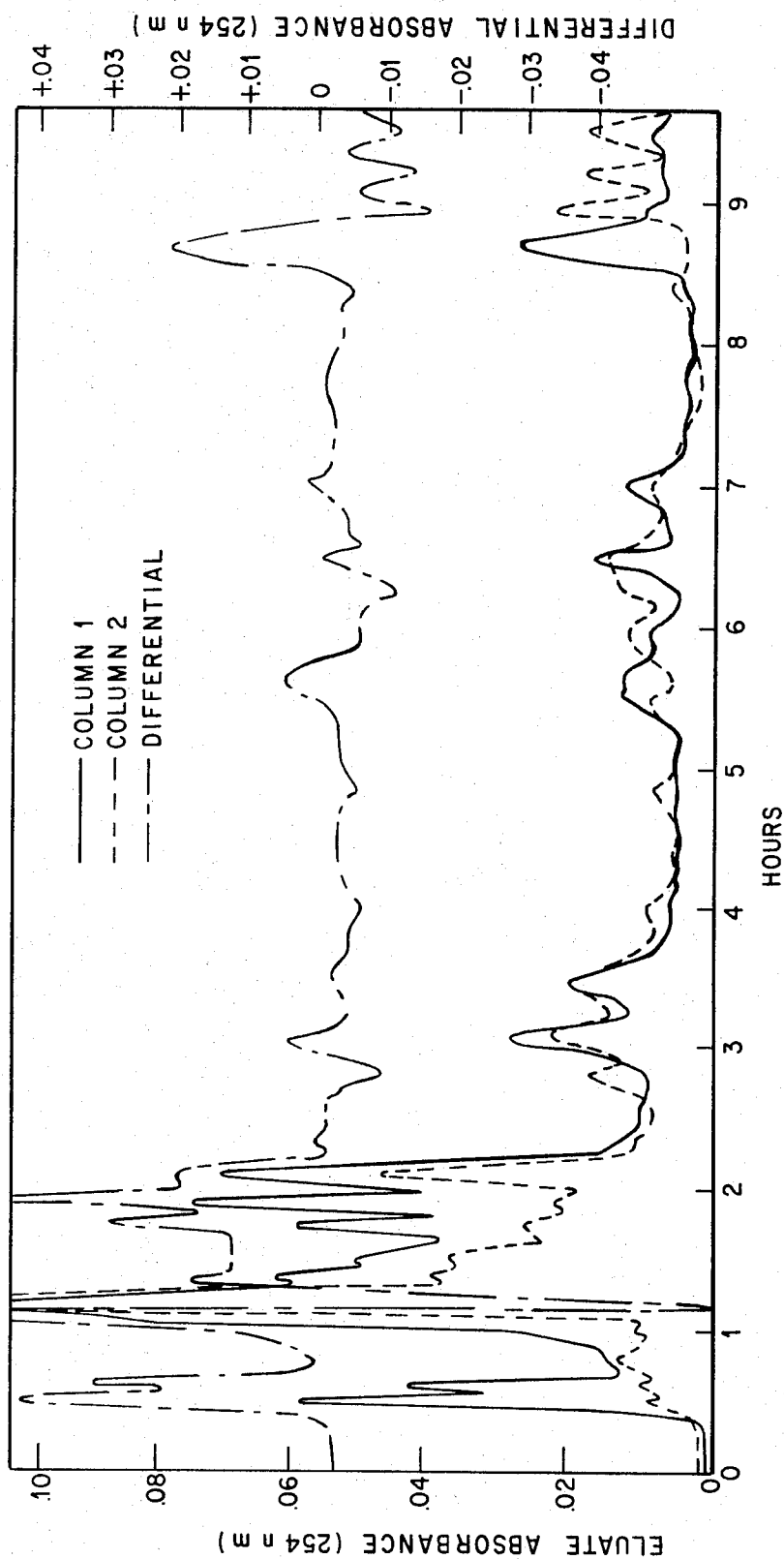


Fig. 5

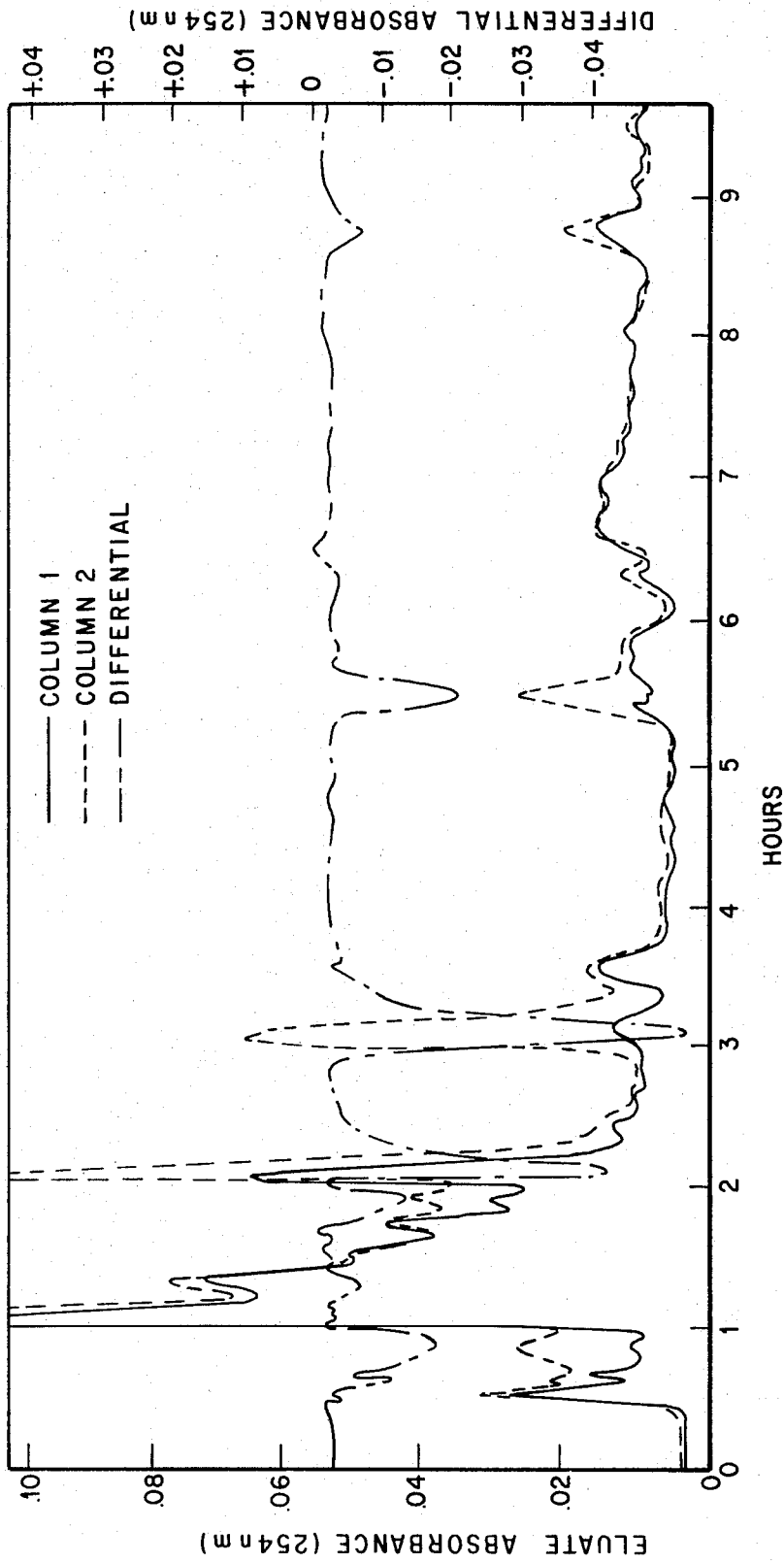


Fig. 6

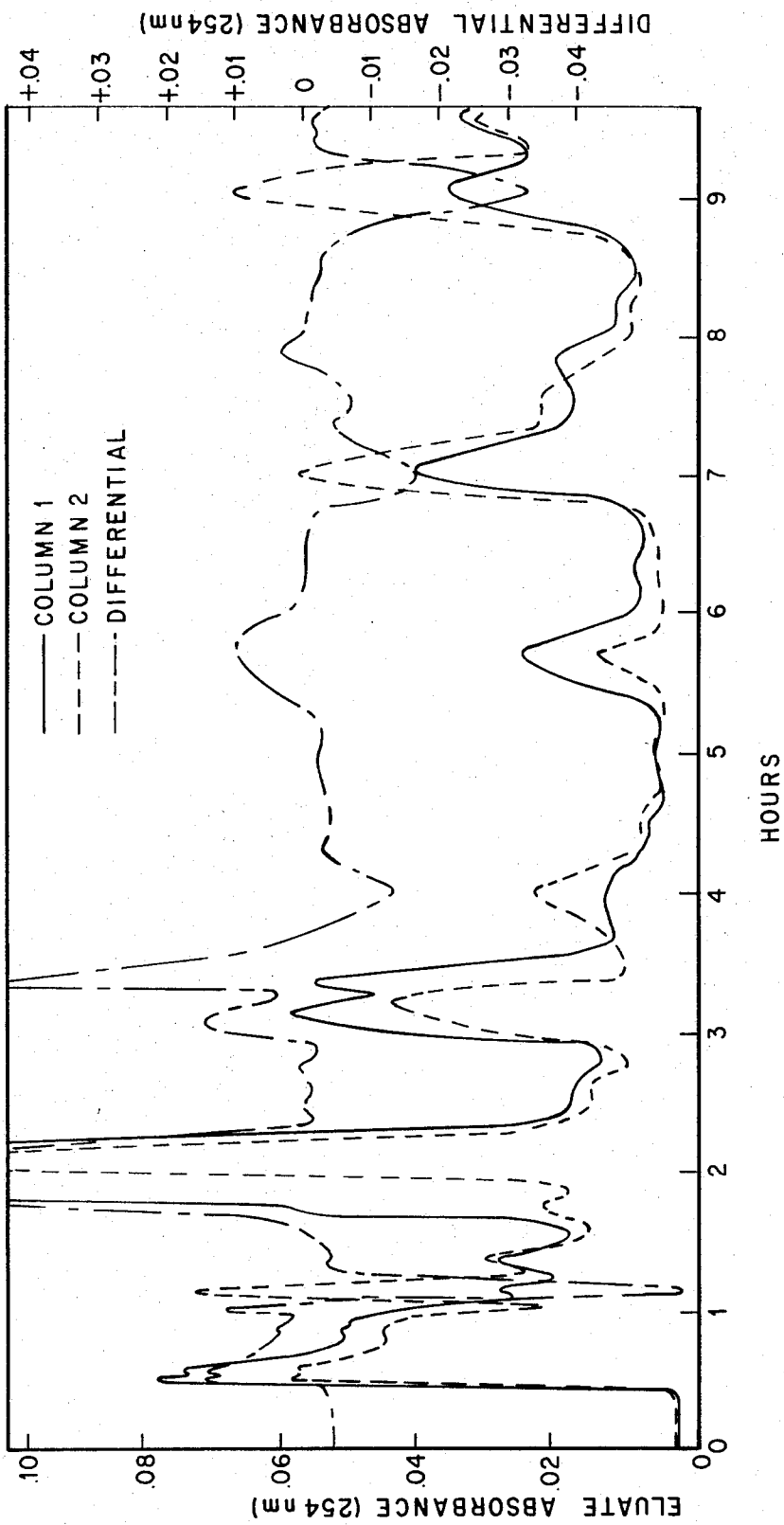


Fig. 7

DIFFERENTIAL CHROMATOGRAPHIC METHOD**BACKGROUND OF THE INVENTION**

Ion exchange chromatography has proven to be an extremely useful tool in many areas of technology. It is useful both as a means for separating molecular constituents, as in the separation of the rare earths, and as a means of chemical analysis. In the field of chemical analysis, ion exchange chromatography has proven to be invaluable in the analysis of physiologic fluid and in the analysis of water pollutants.

In analyzing physiologic fluids, such as urine, abnormalities of a particular sample can be detected by comparing the chromatogram of the sample with that of a normal or standard sample. Also the effects of drugs or other medication on the abnormal chemical makeup may be visually observed by comparing before and after chromatograms. Thus, in the field of physiologic fluid analysis, ion exchange chromatography is valuable as a diagnostic aid as well as an aid in determining treatment efficacy.

In water pollution control, it has become necessary to identify and quantify refractory organic constituents that can result in chlorinated residuals. The efficiency of a sewage treatment process can be determined by comparing chromatogram samples taken before and after treatment. Also the purity of the treated product may be ascertained by comparing a chromatogram of the treated product with that of an acceptable standard. The usefulness of ion exchange chromatography as an analytical tool in water pollution control is thus evident.

However, many of the fluids which can be used to produce chromatograms are composed of a great number of constituents. Urine, for example, has over 700 molecular constituents and over 50 molecular species have been identified in secondary sewage effluent. On top of the number of different chromatogram peaks which must be compared in analyzing samples, there is a problem in that chromatograms from different ion exchange columns or even the same column with a different eluent history will not produce peaks at the same point in time. Therefore, conventionally produced chromatograms are not superimposable for purposes of comparison. Thus, the job of comparing chromatograms is tremendously tedious and time consuming.

SUMMARY OF THE INVENTION

It is thus an object of this invention to provide a process whereby chromatograms may be quickly and easily compared.

This object as well as other objects is accomplished by simultaneously introducing eluent into identically packed ion exchange columns, flowing the eluent through the columns at substantially the same flow rate, introducing equal volumes of comparative samples into the eluent flow streams at substantially the same flow rate, photometrically analyzing the eluent outflow from the columns and electronically producing a differential chromatogram from the output of the photometers.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of a twelve-port valve used in the sample loading process of this invention.

FIG. 2 is similar to FIG. 1 but the valve is in the operational condition.

FIG. 3 schematically illustrates the apparatus used in the practice of this invention.

FIGS. 4-7 are chromatograms produced according to the process of this invention.

DETAILED DESCRIPTION

According to this invention, it has been found that the combination of several critical steps can be used to produce a differential chromatogram. As used within this disclosure the term "differential chromatography" or "differential chromatogram" means the practice of, or the charts produced by the practice of electronically comparing the differences between chromatograms of similar samples, which differ in the quantities of molecular constituents which are present, and charting this difference as a function of time of outflow from ion exchange columns.

The process of this invention may be carried out using simple ion exchange chromatography or it may be preferably carried out using the more sophisticated techniques of high pressure and eluent gradient ion exchange chromatography, which techniques are well known in the art.

As a critical part of this invention two columns must be identically packed with ion exchange resin particles having a size of about 10 to 40 microns but preferably within the range of about 10 to 15 microns. The particles may be either spherical or irregular as long as they are within the operable size range. The condition of identical packing cannot be achieved by gravity packing of the columns but must be achieved by dynamically packing the columns. As is explained in *J. Chromatog.* 42 (1969) 263-265, dynamic packing is achieved by forcing a slurry of about 25 to 50 volume percent resin particles suspended in the intended eluent column at a velocity which is greater than the natural settling velocity of the resin. The packing efficiency is further improved by filling the column with a liquid, such as water, to be displaced during the process of dynamic packing. This prevents any air pockets from forming during the packing process.

After the columns are packed, the individual flow rates of each are determined by flowing eluent through each column with means which are to be described below. If the flow rates of the two columns are not within 2 percent of each other based on the faster flowing column, a resistance is added to the eluent delivery line of the faster flowing column. The resistance is in the form of a porous metal disc held in a compression fitting through which the eluent must flow.

Another critical part of the process of this invention comprises maintaining both of the columns at the same temperature during the chromatography process. This is best accomplished by using a common constant temperature bath for both columns. However, other means for maintaining both columns at the same temperature may also be used.

As was previously mentioned, simultaneity of process steps on the two columns is extremely critical for carrying out the process of this invention. If the samples and eluents are not simultaneously introduced into the columns, the chromatogram peaks will be "out of phase," thus producing a completely unintelligible differential chromatogram. For this purpose it has been found that a double-ganged six-port valve as is shown in *Clin.*

Chem. 18 (1972) 767-770 is useful for producing simultaneous introduction of samples. This results in a 12-port valve, which is schematically shown in FIGS. 1 and 2. FIG. 1 shows the valve in the sample introduction position. Samples are introduced into ports 7 and 8, thus filling loops 13 and 14 with equal volumes of samples. Eluent is introduced into ports 3 and 4 and flows out of ports 1 and 2 through two ion exchange columns connected to ports 1 and 2. Central shaft 15 can be rotated 60°, as shown in FIG. 2, thus placing filled loops 13 and 14 into communication with eluent flowing through ports 3 and 4 thus simultaneously introducing both samples into their respective columns through ports 1 and 2. It should be noted that this can be done without interrupting the eluent flow through ports 3 and 4. The sample introduced through port 8 ends up being ejected through port 2 into the ion exchange column, and in a like manner, the sample introduced through port 7 is ejected through port 1 into the other ion exchange column.

FIG. 3 shows an apparatus arrangement for carrying out the process of this invention. Reference numeral 17 represents eluent introduction means. Such means may include means for introduction of an eluent whose concentration varies with time, such as is used in eluent gradient chromatography. For reference purposes the eluent is fed through photometer reference cell 18 prior to being split and introduced into the 12-port valve 16 described above. This section of the line, prior to the split, may be provided with high pressure means and valve relief means if desired. Eluent flows through ports 1 and 2 into parallel ion exchange columns 19. The two identically packed ion exchange columns 19 are maintained at the same temperature by common bath 20.

The outflow from columns 19 contains separated constituents which flow out in their respective order. The different constituents are detected by photometers 21 and 22. Photometers 21 and 22 are conventional dual-beam, dual-wave-length (254 and 280 nm) photometers used in liquid column chromatography. A more complete discussion of the photometers is found in *J. of Chromatog.* 51 (1970) 175-181. The output from photometers 21 and 22 is recorded by recording potentiometers 23 and 24. The output from photometers 21 and 22 is also fed to recording potentiometer 25 which provides a differential chromatogram. For the sake of simplicity, three individual recording potentiometers 23, 24 and 25 are illustrated. However, in actuality it has been found convenient to use a single multipoint recording potentiometer. A six point potentiometer (Minneapolis Honeywell Brown Electronik, Model Y) with a span of 10 millivolts has been found to be useful in the process of this invention.

The process of this invention is, of course, not limited to use with columns containing ion exchange resin; but may be used with any chemical separation particles such as those disclosed in application Ser. No. 306,062(70) commonly assigned herewith. Such particles include not only ion exchange resins but also selective sorbents such as silica, zirconia, hydroxylapatite and alumina. The common ion exchange resins which constitute the preferred embodiments of this invention include those comprised of styrene cross-linked with divinyl benzene, as well as those containing epoxy-polyamine, phenolic and acrylic lattices. Various functional groups are attached to the above resins for use

in the ion exchange process. Such resins can be in either the cationic or anionic state.

Having generally described the invention, the following example is given as a further illustration of the process of this invention.

EXAMPLE

Two chromatographic columns, 0.33 cm I.D. × 150 cm, were fabricated from nominal ¼ inch O.D. type 316 seamless, stainless steel tubing and the stationary phase consisted of 12- to 15-μ-diameter, anion exchange resin (Aminex A-27, Bio Rad Laboratories). This resin is a polystyrene — divinyl benzene copolymer with quaternary ammonium active sites. The resin was first dynamically preppacked into a cartridge and then extruded as a packed bed into the chromatographic columns using an eluent pump. The columns were then operated through one complete chromatographic sequence, after which the individual eluate flow rates were determined and adjusted so that they were within 2 percent of each other. This was done by adding flow resistance, in the form of a porous metal disc in the eluent delivery line, to the column system with the higher flow rate.

An automated dual-chamber gradient generation system was used to generate the eluent buffer concentration gradient; and a single, positive-displacement pump (MilRoyal D pump manufactured by the Milton Roy Co.) was used to force the eluent through the columns in parallel. The eluent was an ammonium acetate — acetic acid buffer (pH 4.4). The acetate concentration was 0.015 molar for the first two hours. The acetate concentration was then linearly increased from 0.015 molar to 6 molar over the next 28 hours. The final 2 hours of the 32-hour run was at 6 molar. Operating pressures of up to 3,000 psi were used.

The two columns were enclosed in stainless steel jackets containing a heating fluid that was temperature-controlled and circulated.

The twelve-port sample injection valve of FIG. 1 was used to allow direct introduction of two samples simultaneously into the separate columns. The external sample loops 13 and 14 were filled at ambient pressure; then after the internal connecting ports had been reoriented by turning the valve handle, the two samples were simultaneously injected into the column eluent streams without terminating the eluent flow.

The two column monitors were dual-beam, dual-wave-length, flow photometers previously described. One flow cell in each monitor was used as a reference cell in which the eluent passed prior to entering the high pressure pump and the other cells were used for the column eluate stream.

In each test, the column eluate flow rates were adjusted to 10.5 ml/hr., and the column temperature was varied from ambient (25°C ± 1°C) during the initial 6 hours to 60°C thereafter during the remaining part of the 32 hours of a typical run.

The variation in column eluent buffer concentration during the run (i.e., the concentration gradient) consisted of approximately the first 21 ml of eluent that was 0.015 M in acetate, followed by a linear increase to 6 M for the next 294 ml and then a final 21 ml of 6 M in acetate.

The reproducibility of the two columns was determined by separating two identical urine samples on the two columns. In this case (FIG. 4) the graphical repre-

sentations of the absorbances of the column eluates at 254 nm are almost superimposable, with the differential signal giving only minor indications of difference. Only the first 9 hours of a 32-hour run are shown in FIGS. 4-7. In the other three chromatograms (FIGS. 5-7) different samples are compared. Differences between the urine of a normal subject (column No. 1) versus that of a subject with a cancer (column No. 2) are notable (FIG. 5), while the effects of drug therapy can be studied by comparing the urine of a subject before (column No. 1) and after (column No. 2) the ingestion of the drug (FIG. 6). Similarly, the effects of a processing step can be evaluated by comparing a process stream before and after the processing step. As an example, chlorination greatly affects the molecular contaminants in the effluent from the primary stage of a sanitary sewage plant (FIG. 7) as indicated by both positive and negative changes in the differential signal. Column No. 1 contained primary sewage effluent before chlorination and column No. 2 after chlorination.

It is thus seen that the process of this invention provides a method by which chromatograms may be readily compared and differences quickly ascertained. By merely visually scanning the differential signal, one is able to readily ascertain any difference which exists between samples. These differences may then be used to diagnose diseases or to determine the efficacy of a treatment. While the invention has been described in terms of two columns in parallel, it is apparent that more than two columns may be operated in parallel so that more than one sample may be compared with a standard.

What is claimed is:

1. A method for carrying out differential chromatography comprising the steps of:
flowing eluent through at least two substantially iden-

tically packed chromatographic columns at substantially the same flow rates;
simultaneously introducing substantially equal volumes of samples into the eluent flow streams;
maintaining said columns at substantially the same temperature;
photometrically monitoring the outflow from each column by photometer means;
differentially comparing the output of one photometer with that of another photometer; and
plotting the difference in said outputs so as to produce a differential chromatogram.

2. The method according to claim 1 wherein said columns are identically packed by forcing a slurry of chemical separation particles into said columns at a velocity which is greater than the natural settling velocity of said particles.

3. The method according to claim 1 wherein said columns are packed with chemical separation particles selected from the group consisting of ion exchange resins and selective sorbents.

4. The method according to claim 3 wherein said particles are within a size range of 10 to 40 microns.

5. The method according to claim 4 wherein said particles are within a size range of 10 to 15 microns.

6. The method according to claim 1 including the further step of plotting individual chromatograms for each of said columns.

7. The method according to claim 1 wherein said eluent increases in concentration over time.

8. The method according to claim 1 wherein said samples are two different urine specimens.

9. The method according to claim 1 wherein said columns and flowing eluent are maintained at a pressure greater than atmospheric.

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