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PRELIMINARY APPLICATIONS OF CROSS-AXIS SYNCHRONOUS FLOW-THROUGH COIL PLANET CENTRIFUGE FOR LARGE-SCALE PREPARATIVE COUNTER-CURRENT CHROMATOGRAPHY

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SUMMARY

The cross-axis synchronous flow-through coil planet centrifuge with a 20-cm revolutional radius and a total capacity of 1600 ml was successfully applied to preparative counter-current chromatography of various biological samples, which include sea buckthorn extract, steroid reaction mixture, indole plant hormones, and dinitrophenylamino acids. The present system offers advantages of stable balance of the centrifuge, a large column capacity, and high resolution.

INTRODUCTION

The advantages of counter-current chromatography (CCC) have been described and demonstrated using a variety of support-free chromatographic systems[1–3]. Recently, a cross-axis synchronous flow-through coil planet centrifuge (X-axis CPC) has been developed for performing large-scale CCC[4,5]. The axes of revolution and the planetary motion form a cross to each other. The centrifugal-force field generated by this planetary motion provides efficient three-dimensional mixing of two solvent phases in a coiled column, thus doubling the partition efficiency of this unique CCC system. The preparative capability of this method has been demonstrated on gram-quantity separation of test samples with a pair of multilayer coiled columns connected in series[5].

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The present paper describes the preliminary applications of the X-axis CPC to the separations of various samples, which include crude sea buckthorn extract, steroid reaction products, indole auxins, and dinitrophenyl (DNP)-amino acid mixtures with suitable two-phase solvent systems.

EXPERIMENTAL

Apparatus

A second prototype of the X-axis CPC with a 20-cm revolutional radius was employed in the present experiments. Fig. 1 shows a photograph of the apparatus, which is equipped with a pair of identical multilayer coiled columns symmetrically mounted on each side of the centrifuge frame to provide perfect balance of the centrifuge system. The apparatus can be rotated up to 500 rpm which produces about 56 *g* on the axis of the column holder. Each separation column was prepared from 2.6-mm I.D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) by winding it onto the holder hub of 15-cm O.D. making multiple coiled layers. The β values (ratio of the radius of rotation to the radius of revolution) of the multilayer coil ranged from 0.375 at the internal terminal to 0.625 at the external terminal. Two multilayer coils were connected in series with a 0.85-mm I.D. PTFE tubing (Zeus Industrial Products) to provide a capacity of about 1600 ml. Each column consisted of 14 coiled layers between the flanges spaced 5 cm apart. These columns were laterally positioned at a distance of 10 cm left from the center of the holder shafts to improve the retention of the stationary phase[4].

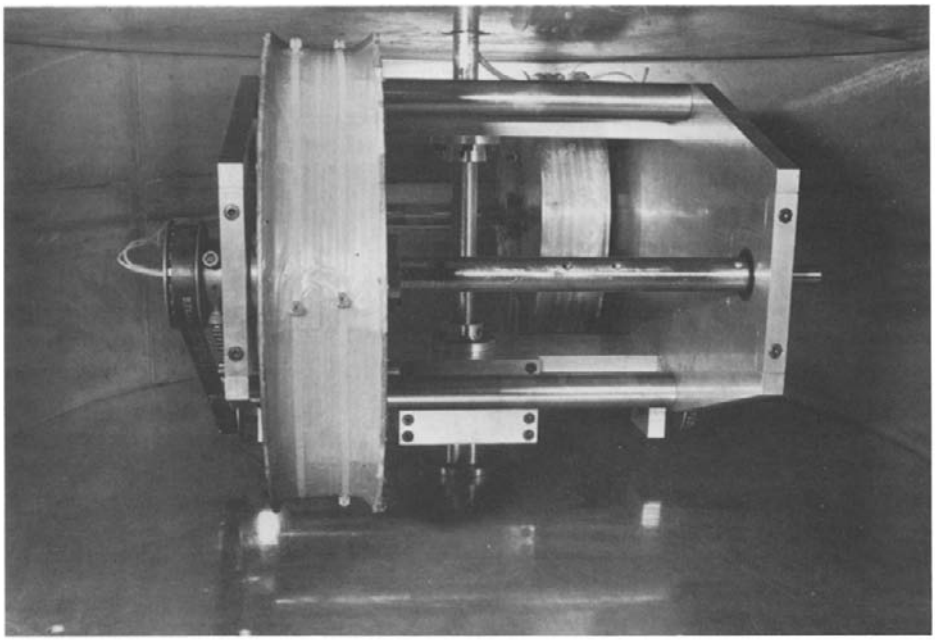


Fig. 1. Photograph of the second prototype of the cross-axis synchronous flow-through coil planet centrifuge with a 20-cm revolutional radius. A pair of large multilayer coils is symmetrically mounted, one on each side of the centrifuge frame, in the lateral position.

Sample and two-phase solvent system

Separations were performed on four sets of samples, *i.e.*, crude ethanol-extracted dried powder from sea buckthorn (*Hippophae rhamnoides* obtained from China), crude steroid reaction products, indole auxins (Sigma, St. Louis, MO, U.S.A.) and DNP-amino acids (Sigma). A suitable two-phase solvent system was selected for each set of these samples on the basis of solubility and partition coefficient values of the sample components. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and two phases separated shortly before application to the separation column.

Crude sea buckthorn extract. This sample is a dried powder from a crude ethanol extract of a medicinal herb called sea buckthorn (*H. rhamnoides*) and known to contain five major flavonoid compounds[6]. A two-phase solvent system composed of chloroform–methanol–water at a volume ratio of 4:3:2 was used for the separation. The sample solution was prepared by dissolving 100 mg of the crude powder in 50 ml of the above solvent mixture consisting of equal volumes of the upper and lower phases.

Steroid mixture. This crude synthetic steroid product was a complex mixture which contained more than ten different components detected by thin-layer chromatographic (TLC) analysis[7]. The separation was performed with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 6:5:4:2. The sample solution was prepared by dissolving 2.3 g of the steroid mixture in 20 ml of the above solvent system consisting of equal volumes of the upper and the lower phases.

Indole auxins. A synthetic mixture of indole-3-acetamide, indole-3-acetic acid, and indole-3-butyric acid was separated with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 3:7:5:5. The sample solution was prepared by dissolving 1 g of each compound, the total weight of 3 g, in 140 ml of the above solvent mixture consisting of equal volumes of the upper and the lower phases.

DNP-Amino acids. Two sets of sample mixtures were prepared, one used for the lower phase elution and the other for the upper phase elution, both with the same solvent system of chloroform–acetic acid–0.1 *N* hydrochloric acid at a volume ratio of 2:2:1. The first sample set for the lower phase elution consisted of 400 mg DNP-L-leucine (DNP-leu), 800 mg DNP-L-proline (DNP-pro), 800 mg DNP-β-alanine (DNP-β-ala), 400 mg diDNP-L-cystine [diDNP-$(cys)_2$], and 1600 mg DNP-DL-glutamic acid (DNP-glu). A total of 4 g of the above sample mixture was dissolved in 100 ml of the above solvent mixture consisting of equal volumes of the two phases. The second sample set for the upper phase elution consisted of 400 mg δ-N-DNP-L-ornithine (DNP-orn), 800 mg DNP-L-aspartic acid (DNP-asp), 800 mg DNP-DL-glutamic acid (DNP-glu), 400 mg diDNP-L-cystine [diDNP-$(cys)_2$], and 1600 mg DNP-L-alanine (DNP-ala). A total of 4 g of the above sample mixture was similarly dissolved in 100 ml of the solvent mixture.

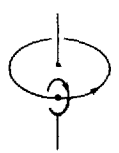
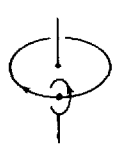
Experimental procedure

Each separation was performed as follows: a pair of multilayer coils was first entirely filled with the stationary phase. This was followed by injection of the sample solution through the sample port. Then the centrifuge was rotated at the optimum

TABLE I

SUMMARY OF EXPERIMENTAL CONDITIONS

Expt. No.	*Sample*	*Solvent system*	*Volume ratio*	*Mobile phase*	*Flow-rate*	*Planetary* motion*	*Elution mode*	*Revolution speed*
1	Sea buckthorn extract (100 mg)	Chloroform Methanol Water	4 3 2	Lower phase	120 ml/h	P_I	Head-Tail	450 rpm
2	Steroid reaction mixture (2.4 g)	*n*-Hexane Ethyl acetate Methanol Water	6 5 4 2	Lower phase	240 ml/h	P_I	Head-Tail	450 rpm
3	Indole auxins Indole-3-acetamide (1 g) Indole-3-acetic acid (1 g) Indole-3-butyric acid (1 g)	*n*-Hexane Ethyl acetate Methanol Water	3 7 5 5	Lower phase	240 ml/h	P_I	Head-Tail	450 rpm
4	DNP-amino acid mixture DNP-L-leucine (400 mg) DNP-L-proline (800 mg) DNP-β-alanine (800 mg) diDNP-L-cystine (400 mg) DNP-DL-glutamic acid (1600 mg)	Chloroform Acetic acid 0.1 *N* Hydrochloric acid	2 2 1	Lower phase	120 ml/h	P_{II}	Tail-Head	500 rpm
5	DNP-amino acid mixture DNP-L-ornithine (400 mg) DNP-L-aspartic acid (800 mg) DNP-DL-glutamic acid (800 mg) diDNP-L-cystine (400 mg) DNP-L-alanine (1600 mg)	Chloroform Acetic acid 0.1 *N* Hydrochloric acid	2 2 1	Upper phase	120 ml/h	P_{II}	Head-Tail	500 rpm

* Planetary motion P_I:  ; P_{II}: 

Large circles indicate revolution around the central axis of the centrifuge and small circles, rotation around the holder axis.

revolutional speed while the mobile phase was pumped into the column at a selected flow-rate in the proper elution mode predetermined by the preliminary experiments (see Table I). Effluent from the outlet of the coiled column was continuously monitored with a UV monitor (LKB Uvicord S) at 278 nm and collected in test tubes with a fraction collector (LKB Ultrorac). Aliquot of each fraction was diluted with a known volume of methanol and the absorbance was determined at a suitable wavelength with a spectrophotometer (Zeiss Model PM 6).

Determination of proper elution mode

The CCC method utilizing a CPC requires a knowledge of hydrodynamic motion and distribution of two solvent phases in the rotating coil. In the high-speed CCC system, two solvent phases are usually distributed unilaterally in the coil in such a way that one phase (head phase) occupies the head and the other phase (tail phase) the tail of the coil. (Here, head–tail relationship refers to the Archimedean screw force which tends to drive all objects of different density toward the head of the coil.) Consequently, satisfactory retention of the stationary phase is attained by eluting either the head phase from the tail toward the head or the tail phase from the head toward the tail.

In the present X-axis CPC method, the above hydrodynamic distribution is further modified by the mode of planetary motions P_I and P_{II} illustrated in Table I (bottom) when the coil is mounted in the lateral position on the holder[4]. Although various mechanisms involved in the above hydrodynamic phenomena are extremely complex and difficult to explain on the basis of our present knowledge, the proper elution mode can be easily determined by a series of simple preliminary runs using a

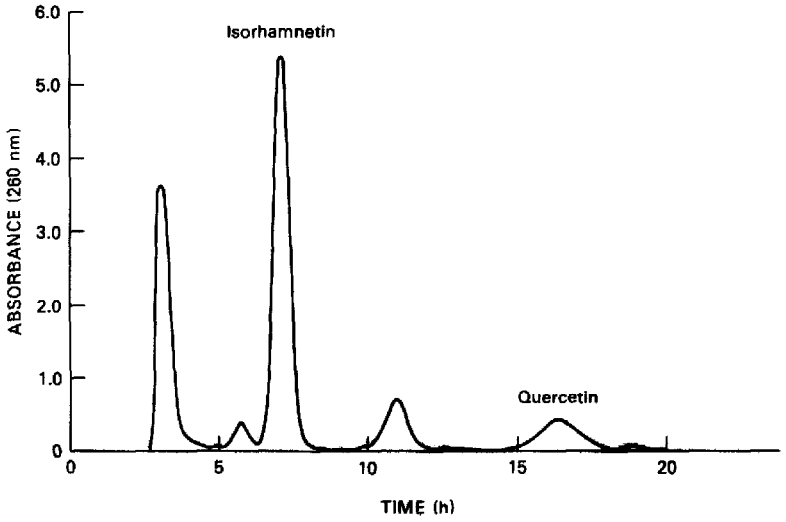


Fig. 2. Chromatogram of crude sea buckthorn extract (100 mg) with chloroform–methanol–water (4:3:2). Experimental conditions: column: multilayer coil, 2.6 mm I.D., 1600 ml capacity; mobile phase: lower non-aqueous phase; elution mode: head to tail; flow-rate: 120 ml/h; revolution: 450 rpm (P_I); retention: 85.5%.

short coil. In addition, a set of phase distribution diagrams previously reported[4] will predict the hydrodynamic behavior of the two phases in a variety of commonly used two-phase solvent systems. The experimental conditions employed in the present studies are summarized in Table I.

RESULTS

Separation of crude sea buckthorn extract

Fig. 2 shows a chromatogram of the sea buckthorn extract obtained from a set of experimental conditions indicated in the diagram. A 100-mg quantity of the crude sample was completely separated into five major flavonoid components. Partition efficiency can be calculated from the chromatogram according to the conventional gas chromatographic formula, $N = (4\ t_R/W)^2$, where N is a partition efficiency expressed in terms of theoretical plates, t_R, retention time of the peak maximum, and W,

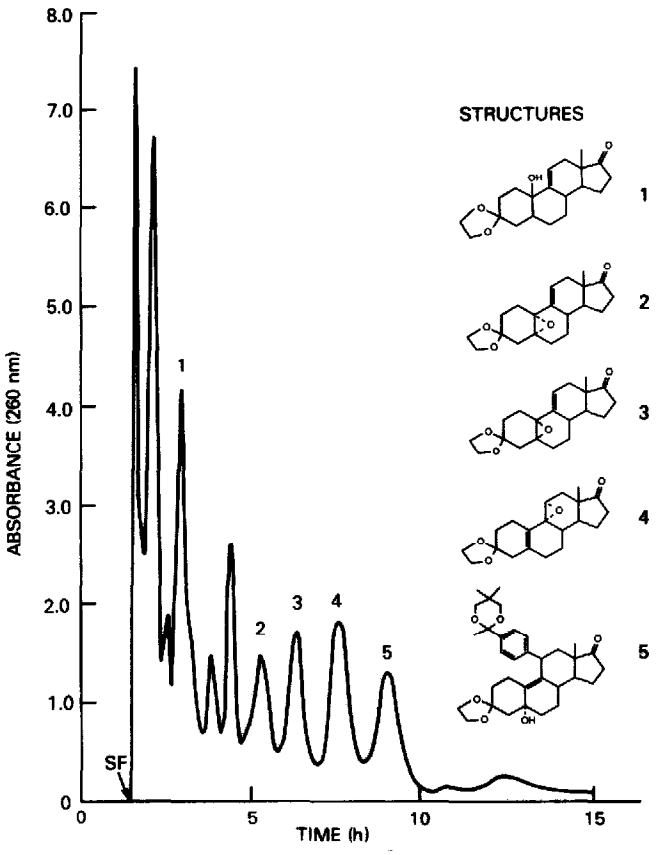


Fig. 3. Chromatogram of steroid reaction mixture (2.4 g) with *n*-hexane–ethyl acetate–methanol–water (6:5:4:2). Experimental conditions: column: multilayer coil, 2.6 mm I.D., 1600 ml capacity; mobile phase: lower aqueous phase; elution mode: head to tail; flow-rate: 240 ml/h; revolution: 450 rpm (P_1); retention: 71.3%.

the peak width expressed in the same unit as t_R. Partition efficiencies of peaks 3, 4, and 5 in Fig. 2 were calculated by the above formula as 720, 900, and 720, respectively. In this experiment, the head to tail elution mode was applied and planetary motion P_I was used at a revolutional speed of 450 rpm. The retention of the stationary phase was 85.5% of the total column capacity.

Separation of steroid mixture

The chromatogram of a crude reaction mixture of synthetic steroids showed fairly well resolved multiple peaks (Fig. 3). A total of 2.4 g of the crude steroid mixture was efficiently separated in 15 h. In this separation, planetary motion P_I was applied at a revolutional speed of 450 rpm. Retention of the stationary phase was 72%. Five steroids corresponding to peaks 1–5 were determined by NMR analysis as indicated on the right side of the chromatogram (Fig. 3). The desired product was found at peak 5 and yielded 310 mg of the crystalline material at high purity.

Separation of indole auxins

Fig. 4 shows a chromatogram of the synthetic mixture of indole plant hormones. A 3-g quantity of the sample was well separated. Planetary motion P_I was applied at the revolutional speed of 450 rpm. Retention of the stationary phase was 33.5%. Partition efficiencies of indole-3-acetamide, indole-3-acetic acid, and indole-3-butyric acid were calculated as 580, 580, and 590, respectively.

Separation of DNP-amino acids

In the first experiment, the lower non-aqueous phase was used as the mobile phase and the separation was performed under planetary motion P_{II} at a revolutional

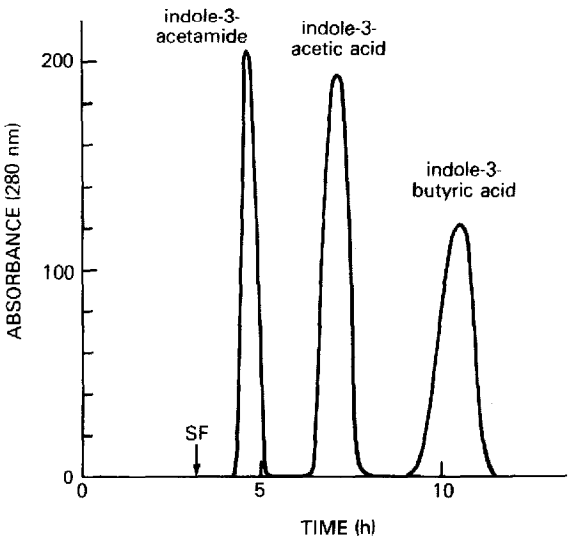


Fig. 4. Chromatogram of indole auxins (3 g) with *n*-hexane–ethyl acetate–methanol–water (3:7:5:5). Experimental conditions: column: multilayer coil, 2.6 mm I.D., 1600 ml capacity; mobile phase: lower aqueous phase; elution mode: head to tail; flow-rate: 240 ml/h; revolution: 450 rpm (P_I); retention: 34%.

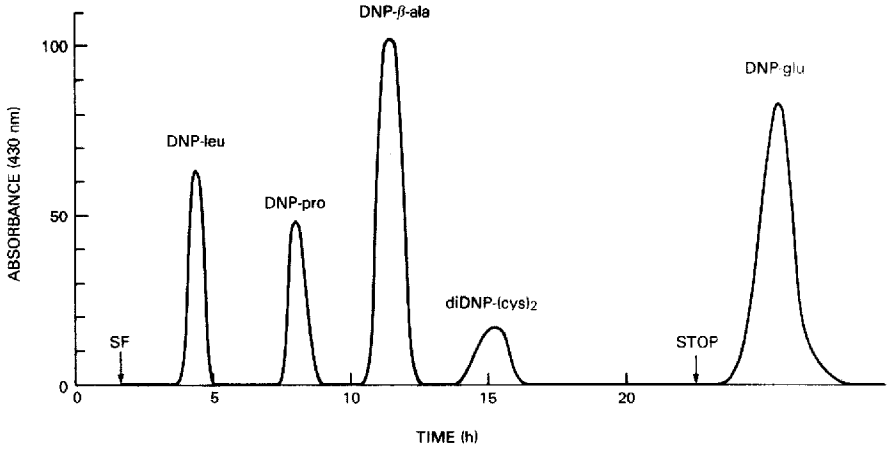


Fig. 5. Chromatogram of DNP-amino acid mixture (4 g) with chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1). Experimental conditions: column: multilayer coil, 2.6 mm I.D., 1600 ml capacity; mobile phase: lower non-aqueous phase; elution mode: tail to head; flow-rate: 120 ml/h; revolution: 500 rpm (P_{II}); retention: 83%.

speed of 500 rpm. Fig. 5 shows the chromatogram in which 4 g of the sample mixture were efficiently separated in 23 h. Retention of the stationary phase was 83%.

In the second experiment, the upper aqueous phase was used as the mobile phase in the head to tail elution mode while planetary motion P_{II} was applied at 500 rpm. Fig. 6 shows the chromatogram obtained from a 4-g quantity of the sample mixture. All components are well separated in 22 h. Retention of the stationary phase was 45%.

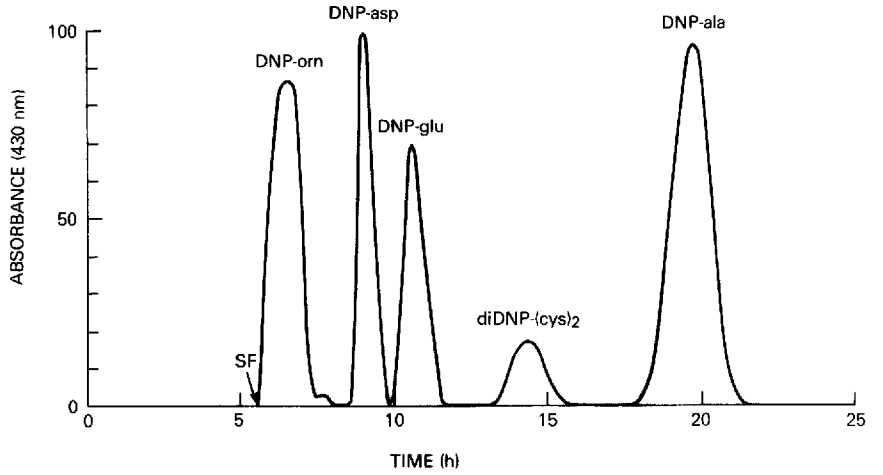


Fig. 6. Chromatogram of DNP-amino acid mixture (4 g) with chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1). Experimental conditions: column: multilayer coil, 2.6 mm I.D., 1600 ml capacity; mobile phase: upper aqueous phase; elution mode: head to tail; flow-rate: 120 ml/h; revolution: 500 rpm (P_{II}); retention: 45%.

DISCUSSION

In the present experiments, preparative capacity of the X-axis CPC was demonstrated in gram-quantity separations of various samples. Partition efficiencies of these separations are mostly over several hundred theoretical plates. These results clearly demonstrate that the present method is capable of separating several grams of samples at a high partition efficiency.

In the separation of the cruder sea buckthorn extract with the chloroform–methanol–water (4:3:2) system, an increase of the sample size to 1 g or more resulted in extensive carryover of the stationary phase from the column. This detrimental effect may be attributed to emulsification in the rotating column as suggested by a long settling time of the sample solution[8]. This clearly suggests that one must search for better conditions to improve the retention of the stationary phase mainly by trial and error experiments using the settling time as a parameter. In the present applications, both retention of the stationary phase and effective partition process were produced by the unique three-dimensional force field generated by the X-axis CPC. The method is suitable for gram-quantity separations of natural and synthetic products with various two-phase solvent systems with a broad spectrum in physical properties.

The operational conditions of the X-axis CPC can be properly adjusted in not only the revolutional speed, flow-rate of the mobile phase, and elution mode of the head to tail or tail to head, but also the mode of planetary motion P_I or P_{II}. Selection of multiple parameters and conditions will facilitate the choice of various kinds of solvent systems suitable for particular applications.

A pair of identical multilayer coils was connected in series and symmetrically mounted one on each side of the centrifuge frame. This doubles the capacity of the separation column to improve both partition efficiency and sample loading capacity. It further provides an important advantage of the care-free stable balance of the centrifuge system without the use of a counterweight which would require accurate weight adjustment according to the density of the applied solvent system. In order to perform large-scale preparative separations, the present centrifuge was designed to hold a pair of large columns at a 20-cm revolutional radius, and to operate at a revolutional speed not exceeding 500 rpm. The present X-axis CPC system will be extremely useful for large-scale separation and extraction in research laboratories and industrial plants.

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