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FOAM COUNTER-CURRENT CHROMATOGRAPHY OF BACITRACIN

I. BATCH SEPARATION WITH NITROGEN AND WATER FREE OF ADDITIVES

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SUMMARY

A foam counter-current chromatographic method utilizing a true counter-current movement between nitrogen and distilled water through a long narrow coiled tube is described. Samples introduced into the coil are separated according to their foaming capability: foam-active materials generate foam and quickly move with nitrogen toward one end of the coil while the remainder are carried with the liquid stream in the opposite direction and eluted through the other end of the coil. The utility of the method was demonstrated in the fractionation of commercial bacitracin (BC). Hydrophobic components including BC-A, BC-F and several minor components were enriched with foam and collected in decreasing order of hydrophobicity, whereas hydrophilic components were eluted with the liquid in decreasing order of polarity. The results indicate that foam-active components can be effectively separated by foam counter-current chromatography using nitrogen and distilled water without a surfactant or other additives.

INTRODUCTION

Recently, a great improvement in foam separation technology has been achieved by the development of foam counter-current chromatography (CCC)[1],

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which uses a long coiled column in a centrifugal force field. Introduction of a sample mixture into the coiled column, either batchwise or continuously, results in the separation of the sample components: molecules with a foam-producing capacity or foam affinity quickly move with the foaming stream and are collected through the foam outlet, whereas the remainder of the molecules are carried with the liquid stream in the opposite direction and eluted through the liquid outlet. This method has been successfully applied to various test samples: ionic compounds were collected with suitable surfactants, and surface-active proteins were separated in phosphate buffer solution to prevent denaturation of the molecules[1–3]. However, this original foam CCC method requires the removal of surfactants or other additives after fractionation.

As many natural products exhibit foaming capacity in an aqueous solution, foam CCC may be possible without surfactants or other additives for the isolation and enrichment of these natural products. In a previous paper[4], we briefly introduced a foam CCC method for bacitracin (BC) components using nitrogen gas and distilled water entirely free from surfactants and other additives. This paper describes in detail the successful application of foam CCC to the separation of BC components without any surfactants and other additives.

EXPERIMENTAL

Apparatus for foam CCC

Fig. 1 shows a cross-sectional view of the foam counter-current chromatograph. The apparatus holds a pair of holders symmetrically 20 cm from the central axis. The gear-driven holder (upper) is equipped with a coiled column, and the pulley-driven holder (lower) is used for mounting a counterweight for balancing the centrifuge system. The desired planetary motion of the gear-driven holder is produced by the use of a countershaft equipped with a gear and a toothed pulley. The stationary gear mounted on the central pipe of the centrifuge is coupled to the identical planetary gear affixed on the countershaft to rotate the countershaft on the rotary frame. This motion is further conveyed to the column holder by coupling the toothed pulley on the countershaft to an identical pulley on the coil holder shaft with a toothed belt. Consequently, as the rotary frame is driven by the motor, the coil holder undergoes a synchronous planetary motion in such a way that it revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction. As described elsewhere[1], this planetary motion permits the bundle of flow tubes to rotate with the rotary frame without twisting, thus allowing both gas and liquid to flow in and out through the rotating coil without the use of a conventional rotary seal device, which would become a potential source of leakage and cross-contamination. The revolutional speed of the apparatus can be regulated up to 1000 rpm with a speed control unit (Bodine Electric, Chicago, IL, U.S.A.).

The column design is illustrated schematically in Fig. 2. The coil, consisting of a 10 m × 2.6 mm I.D. PTFE tube with a capacity of 50 ml, is equipped with five flow channels: liquid feed line and foam collection line at the tail and gas feed line and liquid collection line at the head, while the sample feed line opens at the middle portion of the coil. In the actual column design, the gas and liquid feed lines each enter into the coil through a Kel-F three-way adaptor at the respective terminus

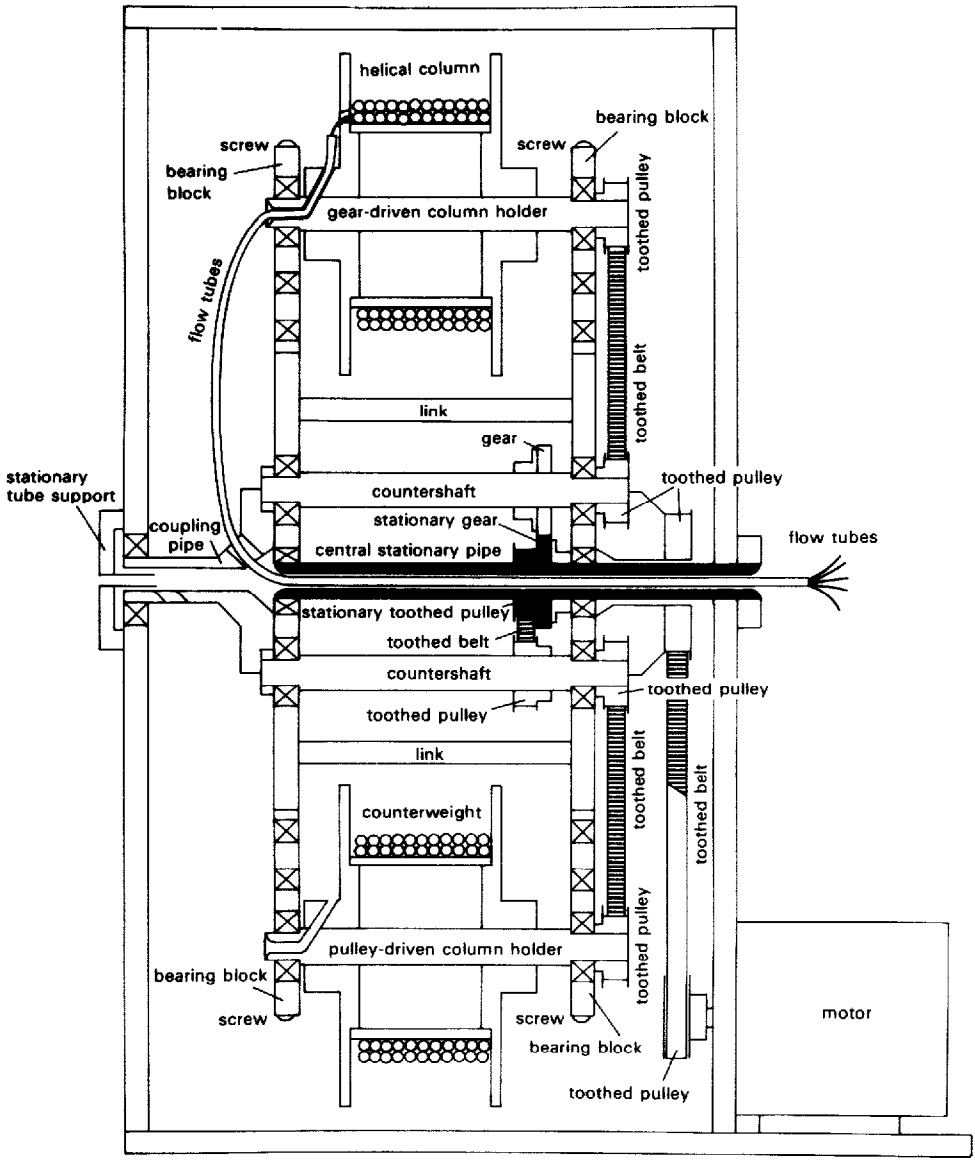

Fig. 1. Illustration of foam counter-current chromatograph.

where the tubing extends into the coil for about one turn (50 cm) to prevent the fed fluid from flowing back through the nearby outlet at the same terminus. The liquid is pumped with a Milton-Roy Minipump through the tail and collected from the head while nitrogen is introduced through the head directly from a gas cylinder at 80 p.s.i., and the generated foam is collected from the tail as indicated in Fig. 2 (the head tail relationship of the rotating coil is conventionally defined by an Archimedean screw

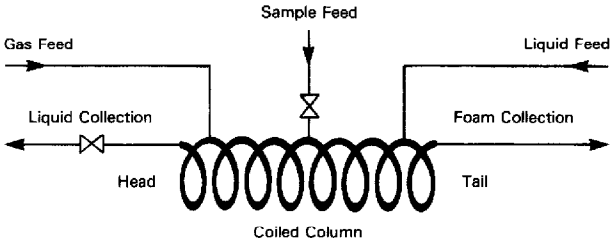

Fig. 2. Column design for foam CCC.

force where all objects of different densities are driven toward the head of the coil). The liquid flow through the liquid collection line is regulated with a needle valve (Washington Valve, Rockville, MD, U.S.A.) while the foam collection line is left open to the air.

Procedure for foam CCC

Separation was initiated by the simultaneous introduction of distilled water at the desired flow-rate from the tail and nitrogen at 80 p.s.i. from the head into the rotating coil at 500 rpm while the needle valve on the liquid collection line was fully open (13.5 turns). After a steady-state hydrodynamic equilibrium had been reached, the pump was stopped and 0.5 ml of a sample solution containing bacitracin (Sigma, St. Louis, MO, U.S.A.) at 1% (w/v) in distilled water was injected through the sample port. After the desired standing time, opening of the needle valve was adjusted to the desired level and the pumping resumed. Effluents through the foam and liquid outlets were each manually fractionated at 15-s intervals. Elution curves of BC from the foam and liquid outlets were obtained by spectrophotometric analysis of each fraction at a measurement wavelength of 234 nm, and an aliquot of each fraction was also analysed by reversed-phase high-performance liquid chromatography (HPLC).

HPLC conditions

A chromatograph equipped with a constant-flow pump (LC-6A; Shimadzu, Kyoto, Japan) was used with a variable-wavelength UV detector (SPD-6A; Shimadzu) operated at 234 nm. The separations were performed on Capcell Pak C_{18} (150 mm × 4.6 mm I.D.) (Shiseido, Tokyo, Japan) with methanol–0.04 *M* disodium hydrogenphosphate solution (62:38) as the mobile phase at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Bacitracin (BC) is a basic cyclic peptide antibiotic commonly used as a feed additive for livestock. It consists of more than 20 components, but the chemical structures of these components are still unknown except the major active component BC-A and its oxidation product BC-F. Under the present HPLC conditions, BC was separated into more than 15 components, as shown in Fig. 3. Generally, hydrophilic compounds show shorter retention times than hydrophobic compounds under reversed-phase HPLC conditions. We consider that BC-A is more hydrophilic than BC-F, which elutes earlier in HPLC. In this study, we used this definition to establish

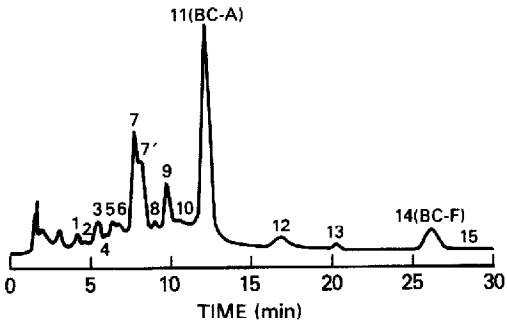

Fig. 3. High-performance liquid chromatogram of the original bacitracin. For HPLC conditions, see Experimental.

the polarity of BC components; special attention was also paid to peaks 3, 7, 11 and 14 to evaluate the separation of bacitracin components by foam CCC.

Optimization of foam CCC conditions

Using a set of fixed conditions for nitrogen flow-rate, sample size, fractionation rate and the column rotation speed, as described under Experimental, we investigated the effects of the liquid flow-rate, opening of the needle valve at the liquid outlet and standing time after sample injection on the separation efficiency. The results are summarized in Table I. Liquid flow-rates lower than 3.2 ml/min failed to elute foam, whereas flow-rates higher than 3.2 ml/min gave a less efficient separation between peaks 11 and 14. Consequently, 3.2 ml/min was selected as the liquid flow-rate. The degree of opening of the needle valve at the liquid outlet was found to be an important parameter. When the valve was opened less than 0.5 turn (fully open: 13.5 turns), all peaks showed less efficient separation. On opening the valve between 0.5 and 0.8 turn, peak 7 eluted from the foam outlet, whereas the same peak eluted from the liquid outlet with the valve opening between 0.8 and 1.2 turn. Valve opening more than 1.2 turn gave no foam fraction. Accordingly, we adjusted the needle valve opening to 0.8 turn in the subsequent work. The length of the standing time after sample injection also affects the foam separation. Standing times less than 5 min gave an inefficient separation between peaks 11 and 14, and no foam eluted at standing times over 5 min. Therefore, 5 min was selected as the optimum standing time. The standing time permits the injected sample components to distribute along the length of the coil according to their foam-producing capacity.

Separation of bacitracin components

The separation of BC components was carried out using the optimum foam CCC conditions determined above. Elution curves for BC from the foam and liquid outlets were obtained manually by spectrophotometric analysis of each fraction, which were also analysed by reversed-phase HPLC.

Foam fraction. The elution curve for BC from the foam outlet shows three major peaks, as indicated by arrows in Fig. 4. The fractions corresponding to these three peaks were subjected to HPLC analysis. As shown in Fig. 5A, the most hydrophobic compounds with the longest retention time in HPLC analysis corresponding

TABLE I

OPTIMIZATION OF OPERATING CONDITIONS FOR FOAM COUNTER-CURRENT CHROMATOGRAPHY OF BACITRACIN

Sample size, 5 mg in 0.5 ml of distilled water; nitrogen gas pressure, 80 p.s.i.; revolution speed, 500 rpm; fractionation rate, 15 s per tube.

Parameter	*Conditions*	*Results*
Liquid flow-rate	<3.2 ml/min	Failure to elute foam
	>3.2 ml/min	Lower efficiency of separation (peaks 11 and 14)
Needle valve at liquid outlet	<0.5 turn open	Lower efficiency of separation (all peaks)
	0.5–0.8 turn open	Peak 7 elutes from foam outlet
	0.8–1.2 turn open	Peak 7 elutes from liquid outlet
	>1.2 turn open	No foam
Standing time after sample injection	<5 min	Lower efficiency of separation (peaks 11 and 14)
	>5 min	Intermittent foam elution

to peaks 14 and 15 were eluted in the first fraction with a small amount of less hydrophobic components corresponding to peaks 11 and 13. Each component showed substantial enrichment relative to that in the original sample solution. Peaks 14 and 15 were enriched 2.8 and 2.2 times, respectively. Peak 15 is hardly visible in the HPLC trace of the original sample owing to the low concentration, but the same peak

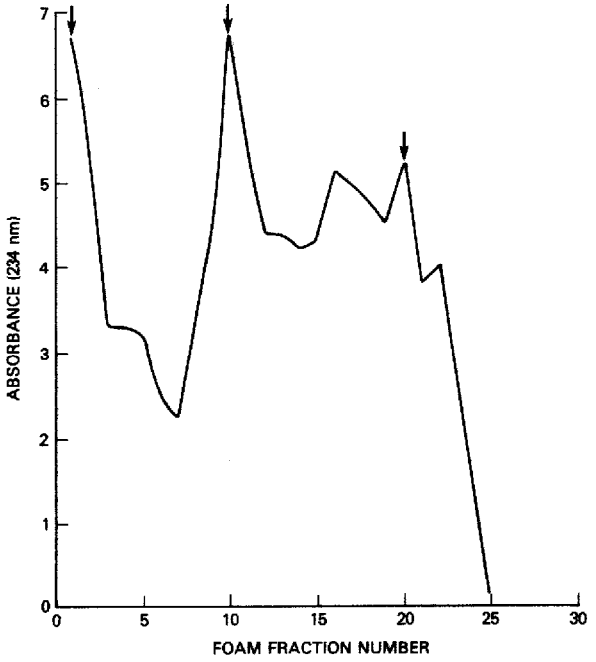

Fig. 4. Elution curve of bacitracin from foam outlet. Foam CCC conditions: liquid flow-rate, 3.2 ml/min; needle valve at liquid outlet, 0.8 turn open; standing time after sample injection, 5 min; nitrogen pressure, 80 p.s.i.; revolution speed, 500 rpm; sample size, 5 mg in 0.5 ml of distilled water; fractionation rate, 15 s per tube.

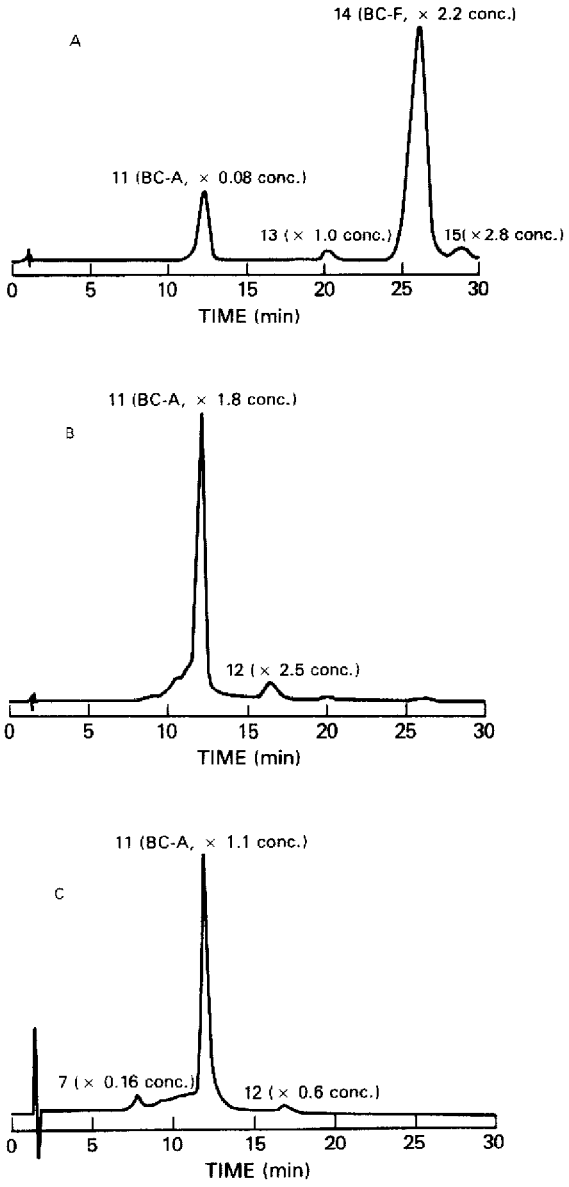

Fig. 5. High-performance liquid chromatograms of bacitracin in foam fractions. For HPLC conditions see Experimental. (A) First foam fraction; (B) tenth foam fractions; (C) twentieth foam fraction.

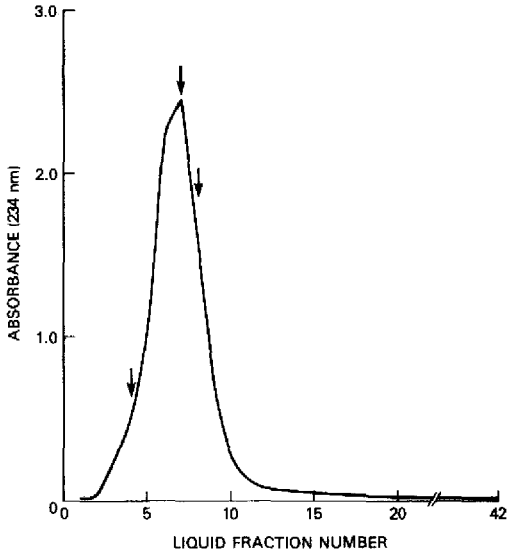

Fig. 6. Elution curve of bacitracin from liquid outlet. Foam CCC conditions are described in Fig. 4.

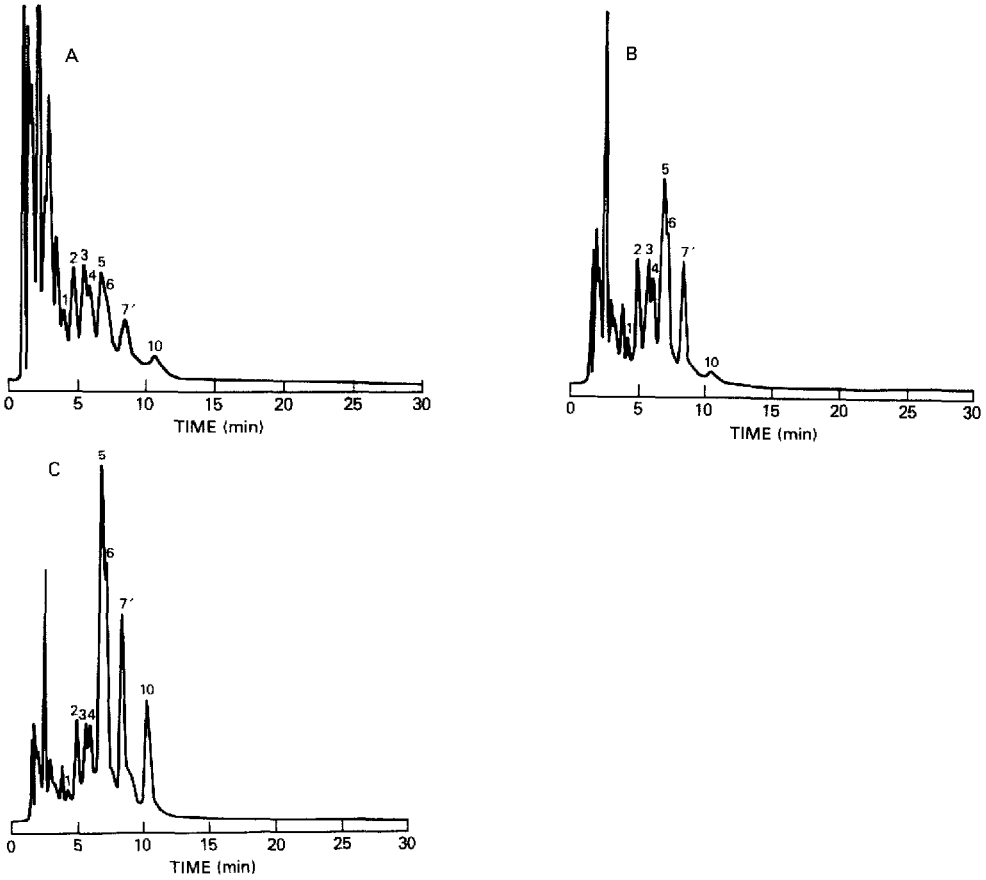

Fig. 7. High-performance liquid chromatograms of bacitracin in liquid fractions. For HPLC conditions, see Experimental. (A) Fourth liquid fraction; (B) sixth liquid fraction; (C) ninth liquid fraction.

is clearly observed in this chromatogram. In the tenth fraction (Fig. 5B), BC-A (peak 11) was almost isolated from other components while enriched 1.8 times. In the twentieth fraction (Fig. 5C), peak 7 appeared on the chromatogram while peak 11 still remained as the major peak. Components with higher polarity, including peaks 1–6, were undetected in the foam fraction. These results clearly indicate that the BC components are separated in the order of hydrophobicity of the molecules and enriched in the foam fractions.

Liquid fraction. As shown in Fig. 6, the elution curve from the liquid fractions is different from that obtained from the foam fractions. Although only a single peak is observed, HPLC analysis of fractions 4, 6 and 9, as indicated by arrows, yielded interesting results.

The HPLC trace of the fourth liquid fraction shows, in addition to peaks 1–10, a group of more hydrophilic components which are hardly visible in the chromatogram of the original sample owing to the low concentration (Fig. 7A). In the sixth fraction, these hydrophilic compounds tend to disappear and peaks 2–10 dominate (Fig. 7B). In the ninth fraction, peaks 5 and 7′ still remain as the main peaks whereas the intensity of peak 10 is considerably enhanced compared with that in the sixth fraction (Fig. 7C). Later fractions showed no additional peaks. These results clearly indicate that BC components elute in decreasing order of their polarity in the liquid fractions. As described above, the liquid fractions obtained from our prototype apparatus gave inefficient fractionation of hydrophilic components. Nevertheless, these results clearly demonstrate the potential capability of the present foam CCC method.

CONCLUSION

Using foam CCC, we were able to separate the components of BC in the order of their hydrophobicity without any surfactants or other additives. The method provides a number of advantages over other chromatographic methods: (1) enrichment and concentration of foam-active components; (2) minimum decomposition or deactivation of biological samples; (3) no adsorptive sample loss into the solid support matrix; (4) no risk of contamination; (5) easy recovery of the sample after fractionation; and (6) low cost in operation. Therefore, we believe that the method has great potential in the isolation and enrichment of various natural and synthetic products in research laboratories and industrial plants. The method also permits continuous operation by continuous sample feeding. These results will be reported elsewhere in the near future.

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