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ON-LINE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–POST-COLUMN REACTION–CAPILLARY GAS CHROMATOGRAPHY ANALYSIS OF LIPIDS IN BIOLOGICAL SAMPLES

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SUMMARY

The versatility of on-line liquid chromatography and gas chromatography is further expanded by the addition of on-line derivatization. The on-line fractionation, derivatization and separation system is applied to the characterization of lipids in biological samples. Separation of the triglycerides from the phospholipids was accomplished on a narrowbore (2.0 mm I.D.) 5 μm silica column. The entire triglyceride fraction was transferred to a heated fixed bed reactor for esterification of the fatty acid constituents. Transfer of the derivatized zones to the gas chromatograph was accomplished by the use of a retention gap. Application of the system to the separation and characterization of *Staphylococcus aureus* is presented.

INTRODUCTION

The combination of liquid and gas chromatographic (LC and GC) separations as two modes in a multidimensional separation is hindered by the need for chemical derivatization of many polar compounds prior to their introduction into a gas chromatograph. Therefore the next logical progression in the development of on-line LC–GC interfacing[1,2] is the addition of post-column derivatization prior to GC analysis. Such a system would be ideally suited for biological separations where often the sample complexity requires both prefractionation to reduce interferences as well as derivatization to increase volatility and/or thermal stability.

One such area of application would be the analysis of biomass and community structure of bacteria. In studying the biomass community the determination of lipid constituents and their relative abundance is important. Researchers in this field have relied on a variety of techniques including agar films[3], colorimetric methods[4], and chitin assays[5] however each of these methods has its own shortcomings, which can lead to erroneous results. White *et al.*[6] developed a method, based on chromatography, requiring multiple separation steps, for determining community structure of

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TABLE I

COMPARISON OF THE OFF-LINE AND ON-LINE METHODS OF SEPARATION, ESTERIFICATION AND IDENTIFICATION OF TRIGLYCERIDES

Step	*Manual method*	*Time*	*On-line method*	*Time*
1	Develop preparative TLC plate	1 h*	Separate by LC	2 min
2	Scrape plate and extract lipids from plate media	20 min	Trap on fixed bed reactor	30 s
3	Esterification with methanolic hydrochloric acid	1 h	React on fixed-bed reactor	2 h
4	Extract into hexane add internal standard and blow down	20 min	Flush into GC injector	30 s
5	Reconstitute in hexane and vortex	15 min	Evaporate solvent in retention gap	25 min
6	GC analysis	30 min	GC analysis	30 min
	Total:	3 h 25 min		2 h 58 min
Automate:	No		Yes	

* 1–4 Samples/plate.

estuarine sediments. By purifying and measuring individual components, it is possible to begin to define some of the key components necessary to resolve the fungal population structure as a part of the total microbial assembly. The method was very labor intensive and time consuming, requiring many transfer steps which compromised sample intergity (Table I).

The method, described by White *et al.*[6], is carried out in a number of steps. First, the phospholipids and lipids are separated on a preparative thin-layer chromatography (TLC) plate. The plate is then scraped and the lipids extracted from the plate media. The triglycerides are then converted to the methyl esters with a methanolic hydrochloric acid solution and extracted into hexane. Internal standards are added and the solvent evaporated. After reconstitution the samples are injected on to a GC system for separation and identification. The major problem with such an approach is that the sample is subjected to several transfer steps leaving the sample liable to contamination and loss. Also the method requires a substantial amount of operator involvement thus limiting the sample throughput due to the inability to automate.

The use of on-line LC–GC with post-column derivatization would eliminate the manual transfer steps and thus the risk of loss or contamination, while increasing sample throughput through automation. In developing an on-line derivatization scheme for LC–GC several factors must be considered: (1) solvent miscibility (if solvents are immiscible reaction kinetics are severely impeded), (2) high-performance liquid chromatography (HPLC) and post-column reaction (PCR) solvent compatibility with GC column, (3) reaction time, and (4) side products. Preliminary work based on the method of White *et al.*[6], utilizing acidic esterification solvents proved unsatisfactory due to the susceptibility of GC stationary phases to acid hydrolysis. Thus, alternative reaction schemes were investigated.

The use of a fixed bed resin was found to be both compatible with the GC stationary phase and a good catalyst for esterification. Key to the success of the system described was the use of a strong cation-exchange resin, of high capacity

(mequiv./g) for the catalysis of the esterification. The on-line system incorporated the use of a retention gap for the transfer of the large derivatized zones. The triglycerides tripalmitin and tristearitin, which are commonly found in biological systems, were used for evaluating the on-line system. Application of the system to the analysis of *Staphylococcus aureus* is presented.

EXPERIMENTAL

Liquid chromatography equipment

All LC separations were performed with a Waters M6000 pump (Waters-Millipore, Milford, MA, U.S.A.) and a Rheodyne injector (Rheodyne, Cotati, CA, U.S.A.) equipped with a 50-μl loop. The column used was 250 × 2.0 mm I.D. packed with LiChrosorb 60 A, 5 μm (EM Labs., Elmsford, NY, U.S.A.). The flow-rate was 0.3 ml/min and the mobile phase was heptane–isopropanol (7:3). The isopropanol was added to aid the misciblity of the LC mobile phase and the PCR solvent. Verification of triglyceride retention times was accomplished by connecting a refractive index detector (Waters Chromatography) or a UV detector (Kratos, Applied Biosystems, Foster City, CA, U.S.A.) set at 220 nm to the end of the column. The detector was then removed to prevent flow cell damage due to the back pressure of the PCR system. All solvents were HPLC grade and purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.).

TLC

All TLC experiments were preformed on pre-coated silica gel 60 A plates (EM Labs.). The development solvent was heptane–isopropanol (7:3). Triglyceride standards, tripalmitin and tristearitin, and phospholipid standards, dimyristoyl, dipalmitoyl and distearoyl, were purchased from Sigma (St. Louis, MO, U.S.A.) and solutions prepared in heptane at 1.0 mg/ml. Plates were spotted with 50 μl of solution. Upon development and drying, the plates were sprayed with 20% sulfuric acid in methanol and charred to determine migration distances.

Post-column reaction

All post-column reactions were performed using a Varian (Sunnyvale, CA, U.S.A.) 8500 syringe pump. The initial acidic esterification PCR system, consisted of a mixing/reaction loop of PTFE tubing, 0.010 in. I.D. and 1.0 ml total volume (Kratos), which had been tightly woven to minimize laminar dilution. Reaction solvents were methanol–isopropanol–acid (100:100:1) at 0.3 ml/min. The acids investigated were hydrochloric and trifluoroacetic acid.

The fixed-bed reactor system consisted of a column, 150 × 4.6 mm packed with a strong cation exchanger HC-15 (Hamilton, Reno, NV, U.S.A.). The fixed-bed reactor was packed by slurrying and packing in 5% glycerine under constant flow conditions. The PCR solvent was methanol–isopropanol (4:6). The isopropanol was added to the PCR solvent to aid in the miscibility of the mobile phase. The fixed-bed reactor was placed in an oven (Carle Instruments, Anaheim, CA, U.S.A.) and kept at 85–90°C. Elution times of the methyl esters were confirmed by placing a refractive index detector at the column outlet, the detector was then removed to prevent damage to the flow cell due to the system back pressure.

GC equipment

All GC experiments were preformed on a Varian 3400 equipped with a flame ionization detector. A "retention gap", consisting of an uncoated 50 m × 0.32 mm fused-silica capillary (Polymicro Technologies, Phoenix, AZ, U.S.A.), was connected to the head of the column with a zero dead volume union. The column used was a 25 × 0.32 mm I.D. 5% phenylmethyl silicone (Hewlett-Packard, Palo Alto, CA, U.S.A.). Deactivation of the retention gap was achieved[7] by filling with a solution of hexamethyldisilazane–pentane (1:1) (Petrarch Systems, Bristol, PA, U.S.A.) capping the ends, and placing in an oven set at 100°C for 17 h. After heating the retention gap was then connected to an LC pump and washed with toluene and methanol prior to use. The retention gap was connected to a Rheodyne 7040 switching valve mounted directly above the injection port. A short length of 0.020 in. I.D. stainless-steel tubing was epoxied to the end of the fused-silica retention gap to both eliminate dead volume and make a gas tight seal to the switching valve. Injections were on-column with a sample volume of 50 μl. The carrier gas was helium at a linear velocity of 52 cm/min. The GC oven was maintained at 100°C for 25 min during the injection/evaporation procedure and then ramped at the rate of 7°C/min to 250°C.

Sample preparation

Dried *Staphylococcus aureus* cells were purchased from Sigma. The lipids were extracted from the *Staphylococcus aureus* using the Bligh-Dyer[8] technique. Approximately 100 mg of dried cells were weighed and transferred to a 250-ml separatory funnel containing water (15 ml), methanol (37.5 ml) and chloroform (18.8 ml). After allowing the sample to react overnight, 18.8 ml each of water and chloroform were added to the solution breaking the system into two phases. The lipid containing layer (chloroform) was collected and the chloroform evaporated by placing under a stream of air. The sample was then redissolved in heptane and analyzed with the LC–PCR–GC system. A portion of the heptane solution was blown down and reacted without any preseparation. A solution of methanol–chloroform–hydrochloric acid (10:1:1) was added to the residue and allowed to reacted for 1 h at 100°C. The methyl esters were then extracted into heptane and analyzed directly by GC.

RESULTS AND DISCUSSION

Phospholipid detection

In developing the LC separation it was necessary to find an alternative method of detection for the phospholipids, due to insensitivity of the refractive index detector. At the concentrations used in this study the phospholipids could not be distinguished from the baseline with the refractive index detector. The phospholipids were believed to be retained on the head of the silica column due to the highly polar phosphate groups. Since the phospholipids lack a good chromophore, UV and fluorescence detection could not be used, and an alternative method was needed for determining the retention time of the phospholipids. Thus, silica gel plates were obtained which were manufactured from the same gel as the HPLC column, and the triglyceride and phospholipid standards were chromatographed under the same mobile phase conditions as the HPLC column. Development of the plates confirmed that the phospholipids were contained at the head of the LC column, thus preventing their

fatty acid constituents from interfering with the GC analysis. The LC column was periodically backflushed to remove the phospholipids from the head of the column.

Typical off-line separation and derivatization schemes

Analysis of the triglycerides is usually carried out off-line by the formation of the fatty acid methyl ester in the presence of a strong acid, with subsequent extraction of the methyl ester into an organic solvent and separation by GC[9–11]. Table I lists the steps involved in an off-line separation and derivatization scheme. Initially an acidic PCR solvent was used with the on-line LC–PCR–GC system. To increase the reaction rate of the lipid (organic layer) and the reaction solvent (aqueous layer) isopropanol was added forming a single-phase system. To verify that the addition of the isopropanol did not cause the formation of the isopropyl esters of the fatty acids, the isopropyl esters by palmitic and stearic acid were synthesized and chromatographed by GC. No response was ever detected for the isopropyl esters of either fatty acid.

Acidic PCR solvent

The preliminary results obtained with an acidic post column reactor solvent and a mixer/reaction loop proved to be quite unsatisfactory. The first acidic solution

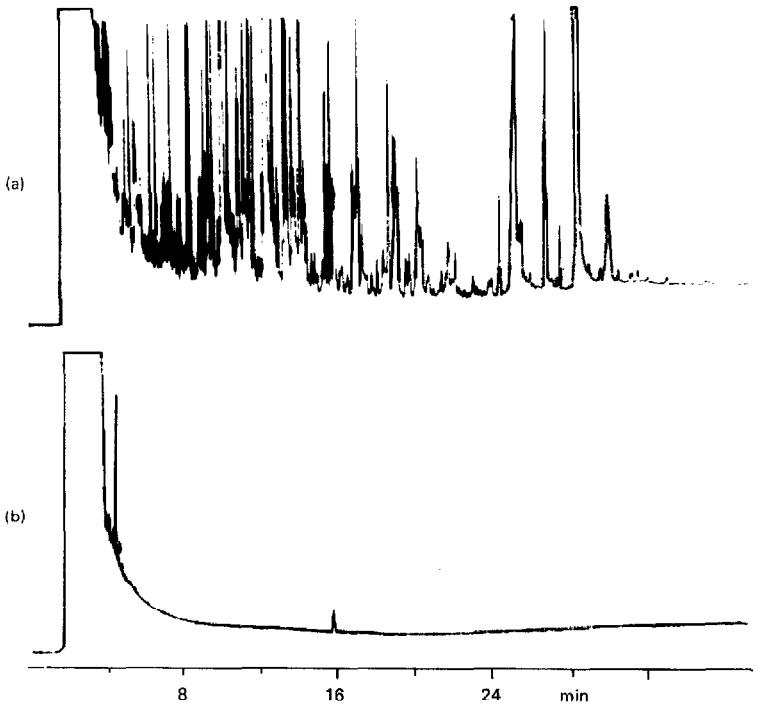

Fig. 1. GC traces of (a) the on-line separation and derivatization of a standard solution of tripalmitin using an acidic post-column derivatization solution of methanol–isopropanol–trifluoroacetic acid (100:100:1) (Notice the excessive column bleed due to the injection of an acidic solution); (b) off-line derivatization prior to GC with methanol–chloroform–hydrochloric acid (10:1:1), with extraction into hexane. (Notice lack of sensitivity, 10 μl injection.)

employed, methanol–isopropanol–hydrochloric acid (100:100:1), produced several major peaks in the GC separation, which interfered with the resulting methyl ester separation. Dilution of the acid was examined as a means of eliminating the spurious peaks. Though the intensity of these peaks were greatly reduced they were not eliminated. Furthermore, the reaction rate of the methanolysis was severely decreased. An organic acid, trifluoroacetic acid, was examined as a catalyst. However, once again the separation showed several interfering peaks (Fig. 1). Reduction of the acid content did not eliminate this problem and therefore other reaction schemes were investigated. The spurious peaks in the acid catalyzed reactions were throught to be due to column phase hydrolysis caused by the injection of the acidic solutions.

Alternative derivatization schemes

Many off-line derivatization schemes for the formation of methyl esters of fatty acids are commercially available[10,11] however, the chemicals employed were not readily compatible with the post-column reactor pump. Thus, it was felt that the acid catalyzed reaction scheme used previously had to be modified in one of two ways: (1) addition of an anion-exchange column after the reaction loop to remove the acid prior to GC injection, or (2) removal of the acid from flow stream completely with the substitution of a strong cation-exchange column for the reaction loop.

The first of these choices was not investigated for several reasons including: (1) removal of the acid would deplete the anions thus requiring either continuous replacement of the anion-exchange column or additional pumping capability for regenerating the ion-exchange capability of the column, and (2) the column would add to the variance of the transfer zone thus increasing the volume of the zone.

Fixed-bed reactor

The second alternative, the addition of a strong cation-exchange column, was chosen since complete removal of the acid would eliminate the acidic hydrolysis of the GC stationary phase. Since the acid groups on the cation-exchange column are used as a catalyst, they will not be consumed by the reaction, thus eliminating the need for column regeneration. Also, by carrying out the reaction in a packed bed of very small particles (10 μm) the zone should maintain its narrow width, whereas the original mixer/reaction loop, though modified to minimize the differences in the laminar flow, adds a significant amount of variance to the zone. The width of the zone transferred is an important criteria for successful LC–GC interfacing[12,13] due to the minute injection volumes tolerated by GC.

Zone transfer

Because of the large transfer volumes (column diameters of 2.0 mm generate transfer volumes of approximately 0.2 ml) and the low concentration of triglycerides (note Fig. 1b, 10 μl injection of methylpalmitate standard) the retention gap[14–18] technique was employed. The retention gap technique is ideally suited for trace work since it allows the transfer of several hundred microliters of sample to the GC system, and as shown by Grob[19]. The application of Grob's method to the LC–GC interface is an obvious and effective extension of the technique. Injections of up to 2 ml have been reported in the literature[14] when using concurrent solvent evaporation. Initially short retention gaps were examined (10 and 21 m) in this work. However, as noted by

Grob[19] polar molecules such as methanol and isopropanol require longer retention gaps due to their higher heats of vaporization. Grob also noted that heptane floods a longer section of capillary than hexane or pentane, therefore requiring a longer retention gap. The 10- and 21-m retention gaps were not sufficiently long enough to allow complete solvent evaporation, because the sample loop was injected by flushing with carrier gas (fast injection). The alternative injection technique, concurrent solvent evaporation, was not used because the high PCR temperatures did not allow accurate flow-rates, a requirement for this technique. Therefore, a long, 50 m, retention gap was used to accommodate the longer evaporation times required by the polar LC and PCR solvents.

On-line LC–PCR–GC

Fig. 2 shows a block diagram of the apparatus used for this study. The system was simplified by the removal of the LC detector prior to the post column reactor, since the triglycerides were completely excluded from the separation column. The LC detector at the exit of the fixed-bed reactor column was also removed, since the (non-polar) methyl esters did not exhibit any retention on the (polar) reactor column.

The first switching valve (Fig. 2) allows the trapping of the triglyceride zone on the reactor column (to prevent evaporation of the solvent, the inlet and outlet ports in the trapped position of the valve were plugged). A multicapillary eluent splitter[20] was initially used to skim the derivatized zone, as it eluted from the fixed-bed reactor column, and transfer it to the second valve. However, the splitter was removed due to the excessive back pressure (> 2000 p.s.i.g.) caused by the high viscosity of the eluent (33% isopropanol). This was necessary because the reactor column was a medium pressure resin (< 1500 p.s.i.g.). The second switching valve is used for trapping the derivatized lipid zones for on column injection.

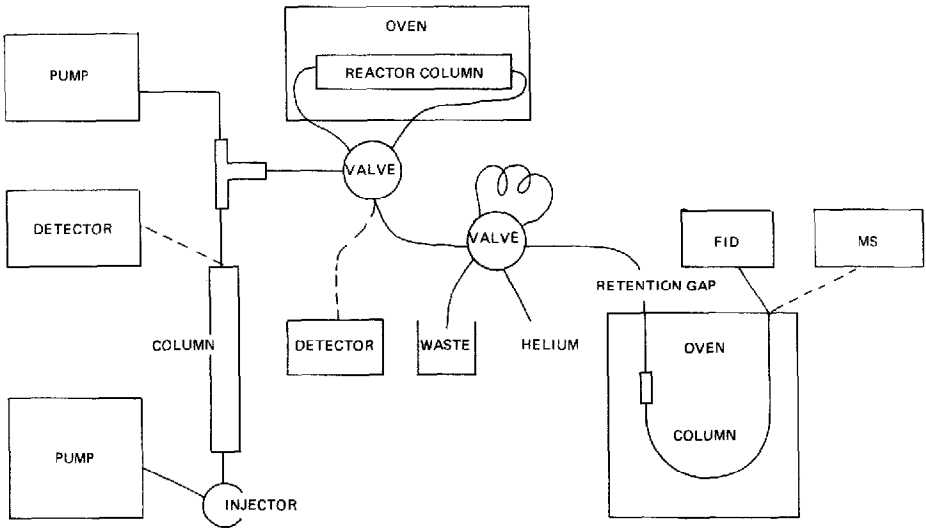

Fig. 2. Block diagram of the on-line LC–PCR–GC system. The detectors were removed after determining the elution times of the triglycerides and methyl esters respectively. The use of a retention gap allowed the transfer of the large derivatized zone. FID = Flame ionization detector; MS = mass spectrometer.

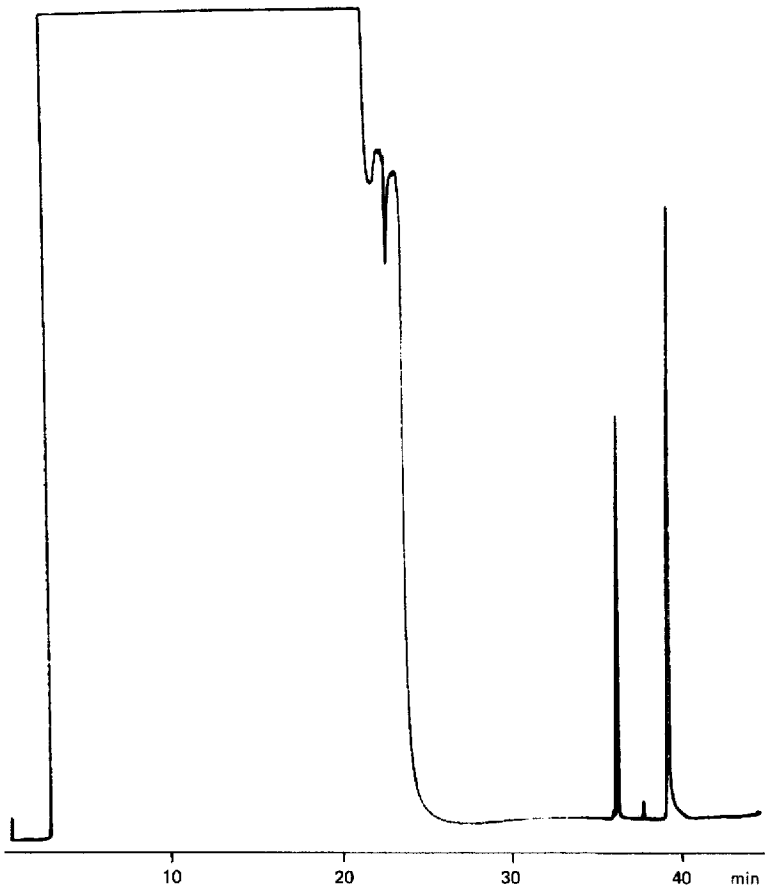

Fig. 3. Separation and derivatization of standards, tripalmitin and tristearitin, on the on-line LC–PCR–GC system using the fixed bed reactor catalysis column. LC conditions: mobile phase, heptane–isopropanol (7:3); flow-rate, 0.3 ml/min; column, LiChrosorb Si 60 A 250 mm × 2.0 mm. PCR conditions: solvent, isopropanol–methanol (4:6); flow-rate, 0.3 ml/min. GC conditions: on-column injection, 50 μl; retention gap, 50 m × 0.32 mm deactivated; separation column, 25 m × 0.32 mm 5% phenylmethylsilicone; hold 100°C for 25 min; ramp at 7°C/min to 280°C.

Reproducibility and kinetics of the esterification

Fig. 3 shows the derivatization and separation of the standards tripalmitin and tristearitin on the LC–PCR–GC system. The reaction rate of the esterification was determined by injecting a solution of tripalmitin (1.2 mg/ml) on the LC–PCR–GC system and varying the reaction time while recording the GC response. Fig. 4 is a plot of the data from this experiment. From the graph it appears that a reaction time of 2 h was sufficient to convert all of the triglyceride present to the methyl ester. Reproducibility of the system was determined by injecting a solution, containing tripalmitin and an internal standard, tristearitin, on the system using a reaction time of 2 h. The average relative standard deviation using the internal standard averaged 9%. Tristearitin was chosen as the internal standard to account for any changes in reac-

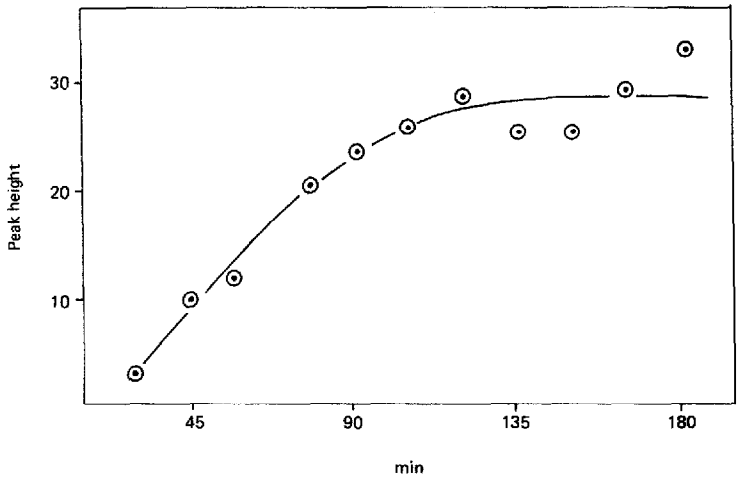

Fig. 4. Plot of the GC response *versus* reaction time for the fixed-bed reactor system. GC injection volume was 50 μl.

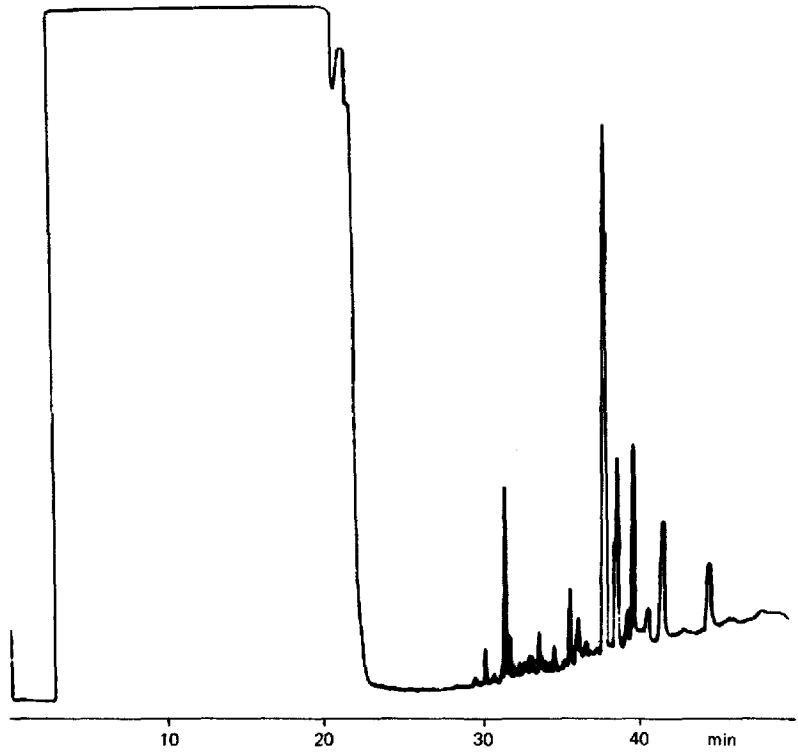

Fig. 5. Derivatization of the entire lipid fraction of *Staphylococcus aureus* separated by the Bligh-Dyer technique. GC conditions as in Fig. 3.

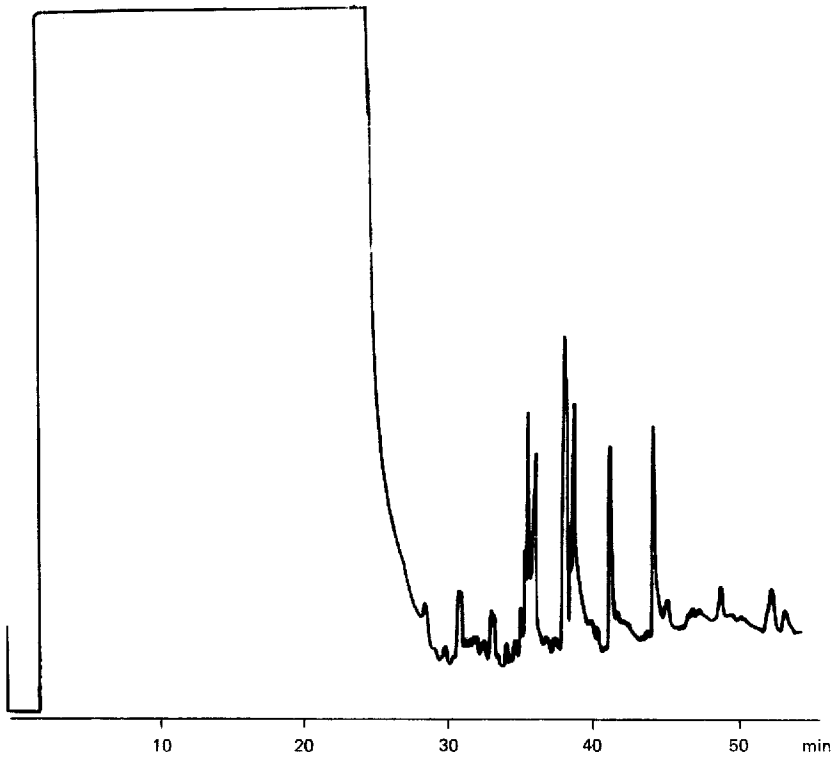

Fig. 6. Separation and derivatizaton of the *Staphylococcus aureus* lipid fraction on the on-line LC–PCR–GC system. LC and GC conditions as in Fig. 3. Note the elimination of the fatty acid constituents from the phospholipids, see Fig. 5.

tion rate caused by fluctuations in temperature and solvent composition. Linearity of the system was determined by injecting solutions of the lipids in the concentration range 0.09–0.5 mg/ml. The correlation coefficients for tripalmitin was 0.992. Examination of a plot of the residuals indicated good linearity over this range.

Application to samples of biological interest

The system was applied to the separation and identification of the triglycerides which are contained in *Staphylococcus aureus*. Fig. 5 is a GC trace of all of the lipid constituents that were extracted by the Bligh-Dyer technique from the *Staphylococcus aureus* cells (*i.e.* no LC fractionation prior to GC). The sample was then run on the LC–PCR–GC system (Fig. 6) with a noticeable difference in composition due to the exclusion of the phospholipids from the derivatization and GC separation steps. Confirmation of the lipid constituent was performed by GC–mass spectrometry off-line. Information gained by determining the fatty acids which compose the biomass community is important for determining whether communities have suffered genetic damage due to environmental pollution.

CONCLUSIONS

The addition of a post column reactor to the on-line LC–GC increases the range of analyses for the multidimensional LG–GC experiment dramatically. Although the system presented did not decrease analysis time significantly for the lipid separations as compared to existing TLC methods (Table I), it did show the feasibility of the technique. Unfortunately, the particular reaction chosen was limited by slow kinetics, while the use of faster reagents, such as boron trifluoride, would be prohibitively expensive unless pulsed into a reactor coil only while the peak of interest elutes. Nevertheless, the value of having a completely automated system is significant as compared to the manual procedures currently in use. Future work will focus on improving the kinetics of the derivatization reaction, and applying PCR to other biological samples.

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