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SIMPLIFIED METHODS FOR PREPARATION OF MICROBIAL FATTY ACIDS FOR ANALYSIS BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

Analysis of bacterial metabolites and constituents by gas chromatography (GC) with frequency-pulsed electron-capture detection (FPECD) has been suggested as an approach to rapid identification of infection in man. In such methods conversion of analytes to electrophoric derivatives is obligatory. Present methods for analysis of microbial carboxylic acids by GC—FPECD use trichloroethylation. In order to simplify and possibly automate such sample preparation procedures, we investigated pentafluorobenzylation via extractive alkylation and via solid-supported reagents. The sensitivity in terms of minimum amounts of bacteria detectable were determined for the trichloroethyl and pentafluorobenzyl derivatives and results from solid-supported reagents were compared to extractive alkylation.

INTRODUCTION

Gas chromatography (GC) is widely used in clinical microbiology to assist in the identification of anaerobic bacteria [1]. Identification of such bacteria is based upon the presence of abundant short-chain volatile carboxylic acids in spent media. As such, the instrumentation required and sample preparation are not complex. Profiling of long-chain carboxylic acids has also been applied to the identification of bacteria propogated in vitro [2] utilizing simple sample preparation for GC with flame-ionization detection.

Recently, a number of investigators have proposed that monitoring of the longer-chain carboxylic acids and/or other compounds directly from body fluids of infected patients can be used to detect infection and assist in identifying bacteria [3—7]. Brooks and co-workers [3—5] have reported on the

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use of GC with frequency-pulsed electron-capture detection (FPECD) for the detection of infection in man by profiling carboxylic acids, amines and sugars. This technique was used in order to improve speed sensitivity and specificity of analyses. However, since few compounds of biological origin are electrophoric, derivatization with halogen-containing agents is obligatory. Brooks and co-workers [3—5] have converted carboxylic acids to the trichloroethyl esters and both amines and hydroxyl groups to the trifluoroacyl derivatives but these methods have been criticized as being difficult to perform [8].

Nevertheless, GC for the detecton of infections and identification of bacteria is potentially promising [3—7]. It is important therefore that this approach be further studied with the aim of its development into a more practical research and clinical tool. We therefore investigated approaches to simplifying and automating sample preparation procedures.

EXPERIMENTAL

Materials

Pentafluorobenzyl bromide (PFBBr) was purchased from Caledon Labs. (Georgetown, Canada); trichloroethanol (TCE) and heptafluorobutyric anhydride (HFBA) were obtained from Supelco (Mississauga, Canada). Tetrapentylammonium iodide was obtained from Kodak and was converted into tetrapentylammonium hydroxide (TPeAOH) at a concentration of 0.1 *M* in methanol by standard methods published elsewhere [9, 10]. The macroreticular resin XAD-2 and preparative thick-layer plates were purchased from BDH (Toronto, Canada). Solvents were obtained from Canlab Labs. (Toronto, Canada). Catlin's Medium for bacterial incubations was obtained from Gibco (Burlington, Canada).

Synthesis of standards

The pentafluorobenzyl (PFB) derivatives of the carboxylic acids were prepared by a modification of previously described method [9—11]. Briefly, the acids were reacted with a 10% molar excess of PFBBr in acetone (rather than dimethylformamide used in earlier methods) with potassium carbonate as a catalyst. This reaction mixture was stirred for 1 h and at that time methylene chloride and water were added and the mixture was shaken. The aqueous phase was removed by aspiration and the methylene chloride phase further washed with water, dried with sodium sulphate, decanted and evaporated to dryness. Preparative-scale thick-layer chromatography on 2-mm thick silica gel 60 F254 was used to purify these PFB esters. The plates were developed in hexane and the compounds visualized on the fluorescent plates by shortwave ultraviolet light. Purified derivatives were weighed and served as standards. All PFB esters of ten to twenty carbon atoms straight-chain carboxylic acids were so prepared.

Gas chromatography with frequency-pulsed electron-capture detection

GC analyses were carried out using a Hewlett-Packard 5790 gas chromatograph equipped with a Grob injector for splitless injection onto capillary columns

and a frequency-pulsed electron-capture detector. The capillary column was 15 m × 0.25 mm I.D. The phase was DB-I with a thickness of 0.25 μm. The output of the electron-capture detector was recorded on a Hewlett-Packard 3390 recording integrator.

Preparation of resin

The resin (XAD-2) was prepared from the commercially available product by methods described elsewhere [11]. The inorganic preservatives and the fines were removed by suspension in water, aspiration of the supernatant and repeating this procedure until the supernatant was clear and at neutral pH. The water was removed by washing with ethanol, methanol and then the organic contaminants were removed by successive washing in a Soxhlet extractor with methanol and then with diethyl ether. For this experiment the resin was stored at room temperature in a sealed bottle until used.

Impregnation of resin

The resin XAD-2 (100 mg) was suspended in 4 ml of trichlorofluoromethane (Freon 11). PFBBr (50 μl) was added to this mixture and the Freon 11 (b.p. 24°C) was evaporated by shaking in a waterbath at 30°C for 1 h. As the volatile Freon 11 evaporated the PFBBr was deposited homogeneously on the surface of the beads.

In vitro quantitative bacteriology

Clinically derived strains of *Escherichia coli* were incubated in Catlin's medium at 37°C for 6 h. This incubate was diluted 10-, 100- and 1000-fold in phosphate-buffered saline. Aliquots of 0.1 ml from each dilution were sub-cultured in triplicate on the solid medium. The culture was incubated overnight at 37°C and the mean number of colonies found on the plate from each dilution was calculated. The total number of bacteria in the initial incubate was determined and this permitted calculation of the concentration of bacteria in Catlin's medium. Aliquots of this medium of sufficient volume to give $2.2 \cdot 10^{10}$, $2.2 \cdot 10^{9}$, $2.2 \cdot 10^{8}$ and $2.2 \cdot 10^{6}$ bacteria were removed and centrifuged to prepare the pellet. This permitted calculation of the minimum amount bacteria detectable.

Hydrolysis

The pellets were hydrolyzed by heating in 0.1 *M* potassium phosphate for 1 h at 100°C. The alkaline hydrolysate was subjected to one of three reaction conditions: formation of trichloroethyl esters; pentafluorobenzylation with extractive alkylation; and pentafluorobenzylation with solid-supported reagent.

Derivatization

Trichloroethyl ester formation. Trichloroethyl esters were synthesized by methods previously described [3—5]. Tetracosanoic acid was added to the hydrolysate to serve as an internal standard. The aqueous hydrolysate was acidified and extracted with chloroform. The organic phase was isolated by aspiration of the water layer and was then dried with sodium sulphate and concentrated to 100 μl. Trichloroethanol and HFBA were added and the

reaction mixture was warmed at 37°C for exactly 32 min. The chloroform solution was then washed once with 100 μl of 0.1 *M* hydrochloric acid and subsequently 100 μl of 0.1 *M* sodium hydroxide. The reagents and solvents were carefully evaporated and the residue was taken up in 100 μl of xylene—ethanol containing 1 μg of PFB-nonanoate which served as an external standard. This solution was analyzed by GC—FPECD.

Pentafluorobenzylation by extractive alkylation. The hydrolysate containing the internal standard was diluted to 4 ml with phosphate buffer at pH 7.4. Methylene chloride (2 ml) was added followed by 10 μl of PFBBr and 100 μl of 0.1 *M* TPeAOH in methanol. The reaction mixture was shaken for 5 min and centrifuged to separate the organic and aqueous phases. The aqueous phase was aspirated and the methylene chloride was decanted onto a short column of florisil and the PFB esters were then eluted with 20 ml of diethyl ether. This was evaporated to dryness and taken up in 500 μl of toluene containing 1 μg of external standard. The solution was analyzed as described above.

Derivatization using impregnated reagent. The hydrolysate containing internal standard was diluted to 4 ml with 0.1 *M* phosphate buffer, pH 7.4. This was transferred to a tube containing the impregnated reagent and shaken for 1 h. The resin was isolated by filtration and the PFB derivatives eluted with diethyl ether. This was evaporated to dryness, reconstituted in toluene and analyzed as described above.

Qualitative studies

Fatty acid profiles of *Staphylococcus aureus* were generated by two methods of pentafluorobenzylation. Compounds generated from hydrolysate were compared with standards consisting of the PFB esters of carboxylic acids of known molecular structure. Compounds for which standards were not available were tentatively identified by comparison to literature values of relative retention times [12].

RESULTS AND DISCUSSION

The sample preparation procedures employed in the original work of Brooks and co-workers [3—5] were basically a two-step process involving extraction and, subsequently, derivatization but complicated by intermediate washings and the requirement of exacting reaction times. In addition, because HFBA is labile to water, anhydrous conditions were essential during the derivatization step. In our hands, the procedure was effective but technically complex, requiring considerable skill and attention to detail. Similar observations have been reported by others [8].

Accordingly we tested two alternative techniques for the derivatization of carboxylic acids to highly electrophoric pentafluorobenzyl esters using hydrolysate of bacteria propagated in vitro as a microbiological model. This represented the simplest matrix expected in microbiological problems and methods unsuccessful in this matrix would not be useful in more complex matrices such as cerebrospinal fluid or plasma. This would give some indication of the potential applicability of this technique at the clinical level.

Extractive alkylation is frequently used because extraction of organic acids

TABLE I

CARBOXYLIC ACIDS IDENTIFIED IN A *STAPHYLOCOCCUS AUREUS* HYDROLYSATE

I = Iso; AI = anteiso; O = straight chain, saturated.

Number of carbon atoms	Configuration	Relative response (%) (normalized to most intense peak)
14	I*	2.5
	O**	3.2
15	I*	100
	AI*	27
	O**	0.68
16	O**	4.5
17	I*	2.4
	AI*	5.6
	O**	0.47
18	I*	4.1
	AI*	1.5
19	O**	3.5
	O*	0.37
	AI*	0.67
20	I*	0.4
	O**	0.7

*Identified by comparison to known standards.
**Identified on the basis of data from the literature.

from aqueous medium and derivatization are simultaneous [13]. The fatty acid profiles of the bacterial hydrolysates of the bacteria determined via pentafluorobenzylation using extractive alkylation were similar to those reported by other workers using different techniques [6, 7, 12] (Table I). In particular, we also detected odd-numbered fatty acids which are not biosynthesized by mammals. If present in either plasma or cerebrospinal fluid of infected mammals, such compounds may eventually be used as indicators of infection [14].

Analysis utilizing pentafluorobenzylation by extractive alkylation is more sensitive than analysis based on trichloroethylation. As shown in Fig. 1 a recognizable profile can be obtained with approximately 10^8 bacteria using the PFB derivatives whereas 10^{10} bacteria are required in the case of trichloroethylation. The microbiological sensitivity, in terms of minimum number of detectable organisms, is not defined by the response factor of the pentafluorobenzyl esters. In the absence of any interfering compounds, the limit of detection for a straight-chain carboxylic acid is 10—20 ng. However, the microbiological sensitivity is limited by extra-microbial carboxylic acids. These compounds have been previously identified as palmitic and stearic acids and are universal contaminants of laboratory reagents and glassware [15]. The presence of these extra-microbial acids distorts the profile when less than 10^8 bacteria are hydrolyzed providing the pentafluorobenzylation is the derivatization reaction. Since this number constituted bacteria in log phase, perhaps fewer viable bacteria are required in either older in vitro broth cultures or in vivo due

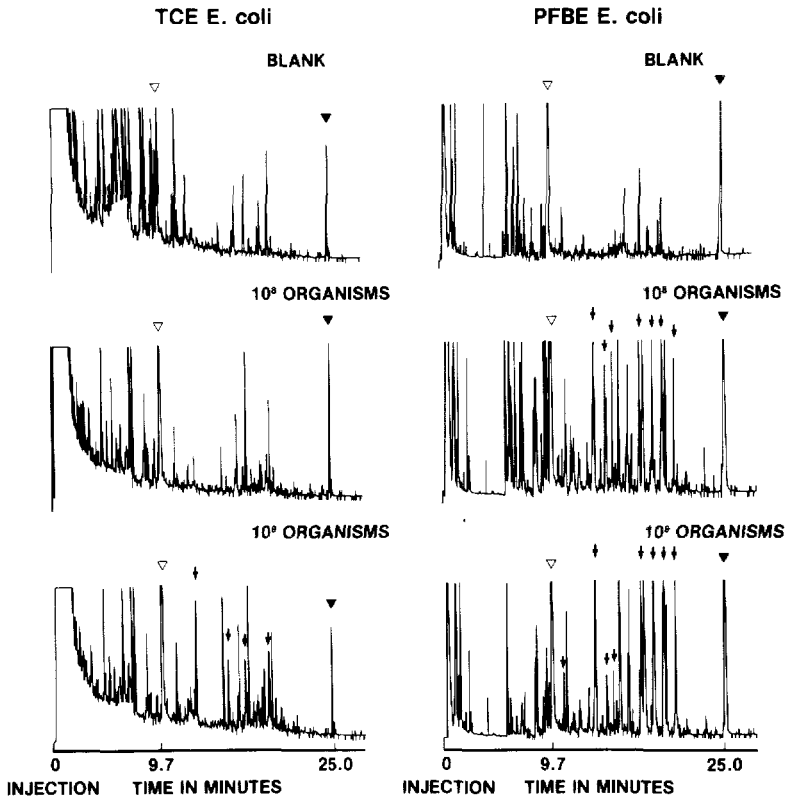

Fig. 1. Comparison of sensitivity for trichloroethyl esters and pentafluorobenzyl esters formed from *Escherichia coli* hydrolysate. Peaks: (▽) external standard, PFB-nonanoate; (▼) internal standard, tetracosanoic acid ester; (↓) peaks that are present in greater than twice the amounts observed in the background trace.

to the contribution of carboxylic acids by dead bacteria. This may account for the complex GC traces reported by Brooks and co-workers [3—5] and others [6, 7].

Extractive alkylation of carboxylic acids is predicated upon extraction of analyte into water-immiscible organic solvent. Such methods frequently, and certainly in this instance, require: (a) centrifugation to separate emulsions, (b) aspiration of the aqueous layer and (c) chromatographic separation of catalyst and analyte [9, 10, 13]. This last step is necessary because phase transfer catalyst co-extracts with the PFB derivatives and generates unacceptably large solvent fronts. For these reasons extractive alkylation is not readily amendable to automation. This would be an important goal in order to incorporate these techniques into the routine clinical or research laboratory [16].

Recently, we reported an approach to the automation of the simultaneous isolation and pentafluorobenzylation of carboxylic acids directly from aqueous medium that was based upon solid supported reagent [11]; a class of reagents currently under investigation to develop automated analytical procedures [17—19]. This study tested the applicability of solid supported reagent to the analysis of compounds present in hydrolysate of bacteria and have found that

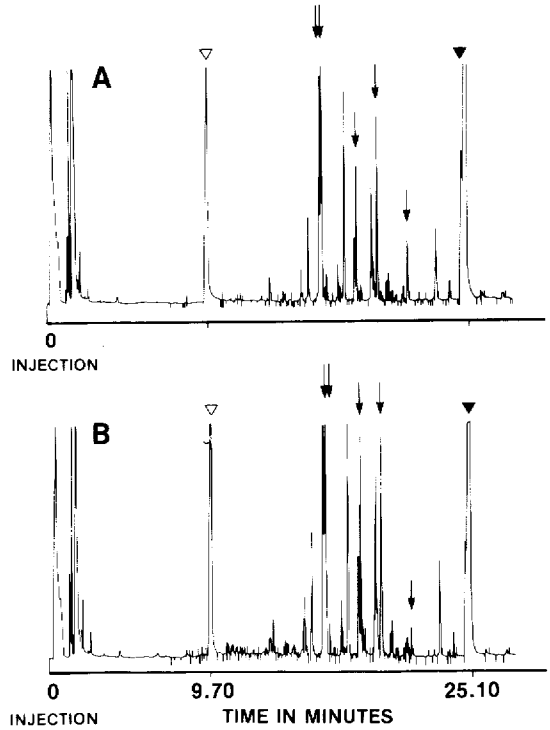

Fig. 2. Comparison of two derivatization techniques for carboxylic acids in hydrolysate of *Staphylococcus aureus*. (A) Pentafluorobenzylation with extractive alkylation ($2.2 \cdot 10^8$ organisms); (B) pentafluorobenzylation using impregnated reagent ($2.2 \cdot 10^8$ organisms). Peaks: (↓) peaks that are present in greater than twice the amounts observed in background trace; (▽) external standard, PFB-nonanoate; (▼) internal standard, PFB-tetracosanoate.

impregnated reagent and extractive alkylation give very similar traces (Fig. 2). Thus, the new solid-supported reagent is capable of derivatizing the same range of compounds and from the same matrix as extractive alkylation which is a more established method. However, the former method is more suitable to future developments in automation as the solid-supported reagent could be prepared commercially in bulk and since all sample preparation is based on filtration.

Despite the present problems from background contaminants, it is apparent that, owing to the technical simplicity, both extractive alkylation and the solid-supported reagents can be useful techniques in studies on the application of GC—FPECD to microbiology. That role could be improved by reducing the background noise through appropriate cleaning of glassware, purifying of reagents, etc. However, even more important, would be development of methods for separating bacteria and bacterial components from those derived mammalian sources.

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