tion of metabolic end-products derived from anaerobic bacteria. A brief survey of the HPLC monitoring of bacterial fermentations for the food industry is also given. The analysis of the structural components of the bacterial cell will be dealt with in the second part of the review.

1.1. Bacterial identification

An important area of microbiological studies is the taxonomic arrangement of the various bacterial species considered pathogenic for humans. Their classification is a preliminary step, which is strictly related to the problem of microorganism identification. In clinical specimens, the correct identification of the bacterial species responsible for infective processes in various areas of the human body is necessary to formulate correctly the aetiological diagnosis of infection. Therefore, identification is the central step of a triad (classification, identification and diagnosis) whose mutual relationships must be kept in mind when dealing with clinical microbiological problems.

1.2. Chemotaxonomy

Chemotaxonomy has been defined as the study of chemical variations in living organisms and the use of chemical characters in classification and identification[1].

As the morphological and physiological characteristics appeared to be insufficient to classify correctly some bacterial species, particularly anaerobes, attention has increasingly been directed to chemical methods capable of providing reliable characters in bacterial classification.

The application of chemotaxonomic techniques may bring more objectivity into the field of bacterial identification. The discovery of specific chemical markers can make bacterial identification more practical. Demonstration of specific markers may also reduce the variability of the results obtained from different laboratories performing physiological and biochemical identification tests. The chemotaxonomic approach can provide a better classification of bacteria, facilitating the identification of clinically important microorganisms[2–10].

Knowledge of the taxonomic characters of anaerobic bacteria has included the main structural components of the bacterial cell and the metabolites produced or utilized by these microorganisms. In fact, the chemotaxonomic approach has involved the elucidation of the cell wall structure, the determination of cellular fatty acids and DNA base composition and, more frequently, the qualitative and quantitative profile of exocellular short-chain acids[11,12]. Major applications include the analysis of some important macromolecules (peptidoglycan, lipopolysaccharide, DNA)[13] and the determination of relatively simple molecules (carboxylic acids, monosaccharides, amino acids, amines).

2. DETERMINATION OF EXOCELLULAR METABOLITES

The application of instrumental methods to the identification of anaerobic bacteria has involved mainly gas chromatography (GC)[14], a very sensitive and rapid technique which has proved useful for the diagnosis of infectious diseases[15–17]. The term GC–chemotaxonomy[18] has been used to describe the application of GC methods to the determination of metabolic products for the identification of a microorganism on the basis of chromatographic profiles of carboxylic acids, alcohols and amines.

In recent years, the use of HPLC to determine bacterial metabolites, especially short-chain acids including keto acids, hydroxy acids and phenolic acids, has become increasingly popular[19,20].

2.1. Short-chain acids

As the metabolism of anaerobic bacteria is characterized by a profile of acid end-products which is typical for each species, the determination of short-chain acids in culture media after growth of anaerobic bacteria is useful for the identification of these microorganisms. Also, the GC determination of short-chain acids in clinical specimens has been used for the presumptive identification of anaerobic infections[21]. GC procedures require separate treatment of samples for different classes of acids: volatile fatty acids can be chromatographed directly from the acidified cultural medium or after extraction with organic solvents, whereas non-volatile fatty acids require simple derivatization to methyl esters prior to GC analysis. On the other hand, GC often appears to have greater resolution, specificity and sensitivity than HPLC. Moreover, the continuous advances made in GC technology are not usually utilized in routine anaerobic analyses.

Technological improvements in HPLC instrumentation, especially of column selectivity and detector sensitivity, have shown that the HPLC determination of acidic metabolites is a suitable alternative to the GC method[19,20]. The flexibility of HPLC techniques in acidic metabolite analysis is evidenced by the following examples. In particular, the analytical possibilities deriving from the selection of a suitable column and especially from modification of the physico-chemical characteristics of the mobile phase are illustrated. Different modes of separation for the HPLC determination of carboxylic acids, including ion-pair chromatography, solvophobic chromatography and ion-exchange chromatography, were reviewed by Schwarzenbach[22]. Short-chain aliphatic acids are separated as such (without prior derivatization)[23,24] or as their phenacyl derivatives[25] on reversed phases. In the ion-pair chromatographic separation of dihydroxybenzoic acids on LiChrosorb RP-18 with 0.05 *M* phosphoric acid–methanol (7:3) + 0.005 *M* hexylamine, the pH is a very important parameter as it determines the concentration of the ionic form of the solutes. Quaternary ammonium salts dissolved in buffered water–methanol (as mobile phase) have also been used[26]. In solvophobic chromatography, the addition of acids or acidic buffers to the mobile phase lowers the pH and suppresses the ionization of the acidic functional groups of the solutes. Retention is the result of hydrophobic interactions between the hydrocarbon moiety of the solute and the octadecyl chains of the stationary phase. Separations are based on the hydrophobic properties of the solutes. The capacity factor of a solute is determined by the concentration of the organic modifier in the mobile phase. The separation selectivity can be affected by either the pH of the mobile phase or the nature of the organic modifier. To suppress the ionization of dicarboxylic acids, the addition of acidic buffers, such as sodium or potassium phosphate, sodium hydrogensulphate or sodium chlorate, has also been successfully used[27,28].

Ion-exchange materials have been used in many applications as stationary phases for the liquid chromatography of carboxylic acids. Owing to the development of new ion-exchange materials of small particle size and narrow size range, which are stable at higher pressures, improvements in the column efficiency and reductions in retention times were possible. Therefore, prepacked columns filled with efficient

ion-exchange materials became commercially available and columns of the HPX series (Bio-Rad Labs.) were used for organic acid separations[29,30]. When subjected to ion-moderated partition chromatography[31], the acids of the citric acid cycle elute according to their increasing pK_avalues. Partition chromatographic separations on ion-exchange resins of aromatic carboxylic acids have also been described[32]. Separations of carboxylic acids on cation-exchange resin columns adopted ion-exclusion chromatography coupled with ultraviolet monitoring of the effluent[33,34] or with conductimetric detection[35]. Dilute mineral acids[36] or dilute mineral acids modified with acetonitrile[37] were used as mobile phases for ion-exclusion chromatography.

A comparison between the GC and HPLC determination of acidic metabolism indicates that both techniques present some advantages and disadvantages, whose relative importance depends on the specific application. When simple analysis of volatile acids is required, the superior sensitivity of capillary GC with a flame ionization detector indicates that the use of this technique is preferable. When, on the other hand, the analysis of ionic, non-volatile or thermally labile compounds is necessary, HPLC has the following advantages: no need for methylation of non-volatile acids, the possibility of determining both volatile and non-volatile acids in a single chromatographic run and high sensitivity for aromatic acid determination.

2.1.1. Applications. One of the first applications of HPLC to bacterial metabolites concerned the fermentation products of several *Clostridium* species[36]. Sample preparation for HPLC analysis required only membrane filtration. Separation was performed on an Aminex column with $6.5 \cdot 10^{-3}$ *M* sulphuric acid. The effects of eluent concentration and column temperature on the retention times of various organic compounds were described. Effluents were monitored with refractive index (RI) and ultraviolet (UV) detectors. Alcohols absorb very little UV light above 200 nm. As pyruvic and fumaric acids absorb strongly at 210 nm, UV absorbance is suitable for detecting these compounds. The other organic acids yielded moderate UV and RI responses. HPLC traces of peptone–yeast extract–glucose cultures of *Clostridium* species were shown.

The acid metabolites present in cultures of a group of clinically significant anaerobic bacteria, including *Peptostreptococcus anaerobius, Bacteroides fragilis* and *Clostridium difficile*, have been successfully determined[37]. This method involved organic acid extraction with diethyl ether–sodium hydroxide from bacterial culture supernatants. The double extraction improved the reliability of the procedure by selectively removing the acids from potentially interfering compounds. Separation was performed on an Aminex HPX-87H column using $3.5 \cdot 10^{-3}$ *M* sulphuric acid–10.8% acetonitrile as the mobile phase, and effluents were monitored at 210 nm. *B. fragilis* and *C. difficile* cultures contained acids which had not been detected by commonly employed GC procedures. In fact, α-ketoglutaric, *p*-hydroxyphenylacetic and phenylacetic acids were determined in the extracts of *B. fragilis*, and *p*-hydroxyphenyl acetic, phenylacetic, 3-phenylpropionic and indoleacetic acids together with *p*-cresol in those of *C. difficile*.

Adams *et al.*[38] presented the results of the application of the HPLC procedure of Guerrant *et al.*[37] to the analysis of acid products from cultures of *Clostridia, Salmonellae* and *Lactobacilli.* Chromatograms of culture extracts of *Clostridium perfringens, Clostridium difficile* grown in cooked-meat medium, *Salmonella sofia,*

Salmonella infantis, *Salmonella typhimurium*, *Lactobacillus casei* and *Lactobacillus fermentum* grown anaerobically in peptone–yeast extract–glucose medium were shown. The acid profiles of bacterial cultures showed that considerable differences existed among the genera and among the species tested. The procedure has the greatest value when used qualitatively, as in the development of profiles of microbial metabolites for quality control purposes, and when used semi-quantitatively as an aid to bacterial identification.

Another example of the applicability of HPLC to the determination of exocellular acid metabolites has involved black-pigmented *Bacteroides*. The organic acids produced by eight oral *Bacteroides* species were determined by HPLC, after extraction with diethyl ether–sodium hydroxide[39]. The chromatographic profiles were typical of the individual species examined: *Bacteroides gingivalis* produced phenylacetic and *p*-hydroxyphenylacetic acids, *Bacteroides intermedius* produced major amounts of succinic and formic acids. These results indicate that the application of HPLC to the determination of the organic acids produced by oral *Bacteroides* is very useful for classifying these bacteria. The capacity factors of the carboxylic acids of a standard mixture under the same chromatographic conditions as described in ref. 39 are reported in Table 1. The chromatographic profiles of acidic end-products from two *Bacteroides* species are shown in Fig. 1.

The production of volatile and non-volatile fatty acids has been revealed with an Aminex column, for the rapid identification of *Clostridium, Bacteroides, Fusobacterium, Peptostreptococcus, Peptococcus, Veillonella* and *Propionibacterium* species[40]. The results illustrated the usefulness of HPLC in determining acidic end-products as an additional means for rapid differentiation between closely related anaerobic bacterial species. Pyruvic and malonic acids were found in some species of all the genera with the exception of *Fusobacterium*. Butyric acid was produced by all the species of *Clostridium, Fusobacterium, Peptococcus, Peptostreptococcus* and *Veillonella*. Butyric acid was most often detected when *Clostridium* or *Fusobacterium*

TABLE 1

CAPACITY FACTORS OF CARBOXYLIC ACIDS IN THE HPLC ANALYSIS OF A STANDARD MIXTURE

Chromatographic conditions: column, Aminex HPX-87H (30 cm × 7.8 mm I.D.); mobile phase, $3.5 \cdot 10^{-3}$ *M* sulphuric acid–10.8% acetonitrile; flow-rate, 0.4 ml/min; detector, spectrophotometer at 210 nm and 0.08 a.u.f.s.

Carboxylic acid	*k'*	*Carboxylic acid*	*k'*
Oxalic	0.122	Propionic	1.677
α-Ketoglutaric	0.433	Isobutyric	1.933
Pyruvic	0.555	Butyric	2.111
Malonic	0.622	Isovaleric	2.411
Methylmalonic	0.755	Valeric	3.000
Succinic	0.788	*p*-Hydroxyphenylacetic	3.222
Lactic	1.000	Isocaproic	3.455
Fumaric	1.233	Caproic	4.422
Formic	1.288	Phenylacetic	5.011
Acetic	1.366		

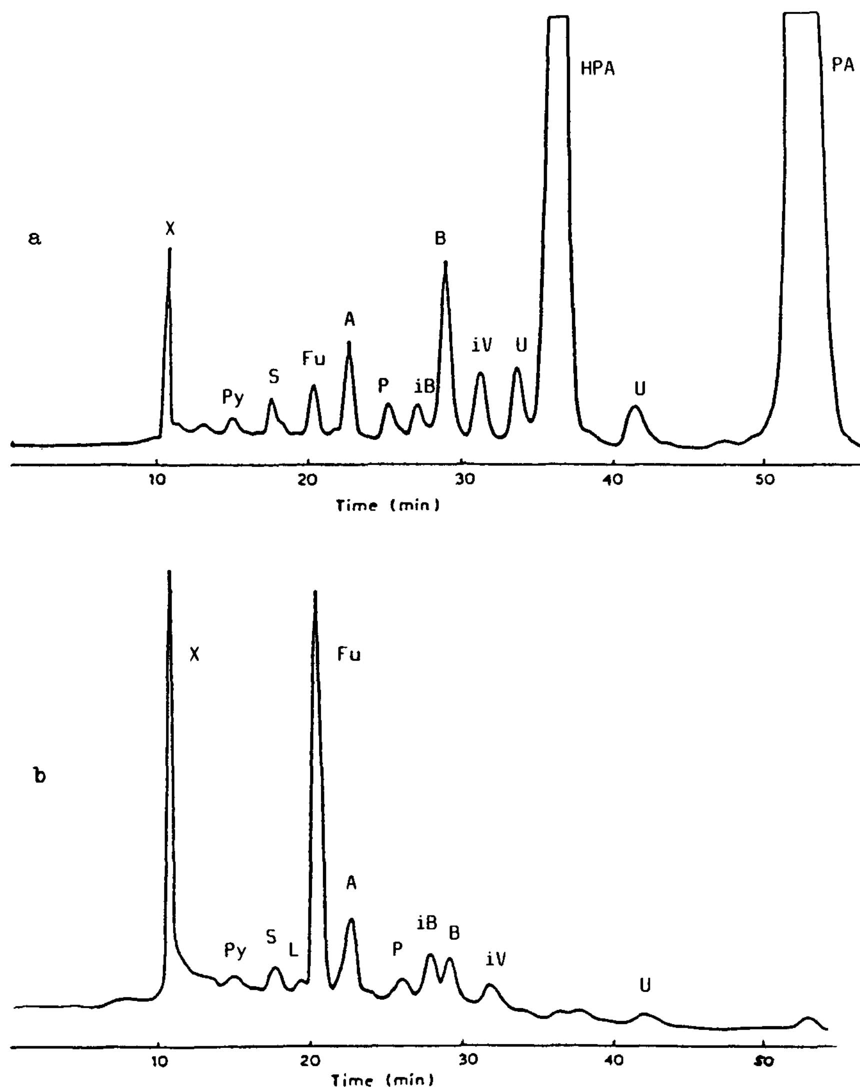

Fig. 1. HPLC of short-chain acids produced by (a) *Bacteroides gingivalis* and (b) *Bacteroides endodontalis*. X = solvent and non-adsorbed components. Acids: Py = pyruvic; S = succinic; L = lactic; Fu = fumaric; A = acetic; P = propionic; iB = isobutyric; B = butyric; iV = isovaleric; HPA = *p*-hydroxyphenylacetic; PA = phenylacetic. U = unidentified components.

species were present in blood cultures[41]. Large amounts of phenylacetic acid were produced by 75% of *Bacteroides* species; phenylacetic acid was produced also by a few species of *Clostridium* and *Fusobacterium*. Large amounts of caprylic acid were detected only in *Clostridium tetani* cultures. The detection of 4-methylvaleric acid in stool specimens was proposed as a screening test to reveal *Clostridium difficile* involvement in diarrhoeal disease[42,43]. Although the presence of isocaproic acid is, in principle, not sufficient to distinguish *in vitro C. difficile* from other *Clostridia* species

which also produced this acid, the detection of isocaproic acid *in vivo* in patients suffering from diarrhoeal disease can be considered a specific marker of the presence of *C.difficile*.

Bacterial growth conditions, treatment of culture supernatants and operating conditions employed by several workers[36–40,44,45] in the HPLC determination of carboxylic acids from different bacteria are summarized in Table 2. Very important factors are composition of the medium and incubation time. As these factors significantly affect short-chain acid production by microorganisms[46–48], it is difficult to compare the results obtained by different workers. There is no universally accepted ideal growth medium for microorganisms, which differ widely in their individual growth requirements. For qualitative analysis the choice of a growth medium may be important. As the proportions of individual products vary considerably during growth, standard incubation times should be adhered to. Krausse and Ullmann[45] used a 48–72-h incubation time for anaerobes in Schaedler broth, while other workers used 24–48-h incubation in peptone–yeast extract–glucose broth[36,37]. The use of a defined medium can often solve the problem of media variability, but this is almost a "mirage" in the case of fastidious microorganisms[15]. Unfortunately, a synthetic medium with a defined chemical composition for cultivating most anaerobic bacteria has not yet been formulated by microbiologists. This drawback constitutes a limit to the whole subsequent analytical procedure, including treatment of culture supernatant and HPLC analysis. In our experience, the utilization of a synthetic medium originally developed by Socransky *et al.*[49] to cultivate *Bacteroides gingivalis* appears to be very promising: chromatograms of spent cultures obtained on a yearly basis proved to be virtually identical, as the relative standard deviation of individual acids ranged from 2 to 5% (unpublished results). Preliminary experiments with this type of medium indicate that it can be successfully utilized to analyse other species of anaerobic bacteria. From these considerations it appears that most of the efforts to standardize growth conditions for HPLC experiments should be directed to the development of a new synthetic medium capable of allowing the satisfactory growth of all anaerobic species of clinical interest. Treatments of the culture supernatants prior to the HPLC analysis differ from one another: single or double extraction procedures provide different recoveries of the carboxylic acids and affect the final quantitative data. The recoveries of selected acids in water extracted with diethyl ether were determined by Adams *et al.*[38]. The values ranged from 22% for pyruvic acid to 72% for isovaleric acid. The recoveries of known concentrations of acids after double extraction with diethyl ether–sodium hydroxide from water were determined by Corio *et al.*[50]: the values for volatile fatty acids decreased with increasing carbon chain length (from 40% for acetic acid to 12% for isovaleric acid). This behaviour was opposite to that mentioned above involving extraction with diethyl ether alone. These data indicate that different extraction procedures can give rise to different results. The values for non-volatile acids were in the range 32–36%, with the exception of succinic acid (15%). As the yields for some acids were too low, it would be useful to examine alternative methods, *e.g.*, solid-phase extraction, for the sample treatment prior to HPLC analysis.

The addition of acetonitrile to the mobile phase as an organic modifier differentiates some HPLC procedures[37–39] from the other methods listed. Changes in acetonitrile concentration cause a shift in the retention time of some compounds. For

TABLE 2

CHROMATOGRAPHIC CONDITIONS IN THE HPLC DETERMINATION OF CARBOXYLIC ACIDS PRODUCED BY SEVERAL BACTERIAL SPECIES

Bacteria	*Growth*[a]	*Treatment*	*Mobile phase*	*Temperature (°C)*	*Flow-rate (ml/min)*	*Detection*	*Ref.*
Clostridium	PYG 24–48 h	Filtration	6.5 m*M* H_2SO_4	30	0.7	RI/UV	36
Peptostreptococcus, Bacteroides, Clostridium	PYG 24–48 h	Extraction with diethyl ether–NaOH	3.5 m*M* H_2SO_4–10.8% CH_3CN	20	0.5	UV, 210 nm	37
Clostridium, Salmonella, Lactobacillus	CMM PYG 4 d PYG 4 d	Extraction with diethyl ether–NaOH	3 m*M* H_2SO_4–CH_3CN (90:10)	50	0.6	UV, 210 nm	38
Bacteroides	BM 6 d	Extraction with diethyl ether–NaOH	3.5 m*M* H_2SO_4–10.8% CH_3CN	20	0.4	UV, 210 nm	39
Azospirillum, Desulfovibrio	Salts	–	2.25 m*M* H_2SO_4	85	0.8	UV, 210 nm	44
Campylobacter	Schaedler 72 h	Extraction with diethyl ether	6.5 m*M* H_2SO_4		0.8	UV, 210 nm	45
Clostridium, Bacteroides, Fusobacterium, Peptostreptococcus, Peptococcus, Veillonella, Propionibacterium	Schaedler 48–72 h	Extraction with diethyl ether	6.5 m*M* H_2SO_4	22	0.8	UV, 210 nm	40

[a] PYG = Peptone-yeast extract-glucose; CMM = cooked meat medium; BM = bacteroides medium.

example as the concentration of acetonitrile was decreased from 10.8 to 5%, as described by Guerrant *et al.*[37], the retention times of fumaric and *p*-hydroxyphenyl acetic acids increased more than those of other acids, facilitating the identification of the former acids. Separation of the acids was optimized by adjusting the concentrations of sulphuric acid and acetonitrile in the eluent. The separation of some acids which elute close together with $6.5 \cdot 10^{-3}$ *M* sulphuric acid alone (succinic–lactic, fumaric–formic–acetic, *p*-hydroxyphenylacetic–2-methylvaleric, caprylic–phenylacetic)[40] could be optimized by manipulating the eluent pH, by adjusting the organic modifier concentration and by changing the column temperature. The values of column temperature and flow-rate employed by several workers cover wide ranges. It is important to point out that it will be useful in the future to select optimum conditions for each chromatographic variable. Standardization of the chromatographic conditions will make it possible to compare the qualitative and quantitative data obtained by different laboratories.

2.2. Substrates utilized by bacteria

A new approach that has proved useful for taxonomic and identification purposes in clinical laboratories is based on establishing the utilization of a complex mixture of defined substrates by bacteria.

2.2.1. Carbohydrates. An improved procedure for determining carbohydrates, alcohols and organic acids in fermentation mixtures metabolized by intestinal microflora has been described[51]. A fermentation mixture containing four carbohydrates was incubated at 37°C for 24 h. The compounds were separated on an Aminex HPX-87H column with 0.028 *M* sulphuric acid. Effluents were monitored with UV and RI detectors in series. The metabolic profiles for the fermentation of glucose, fructose, lactose and sucrose by several intestinal microorganisms (*Escherichia coli, Klebsiella pneumoniae* and *Streptococcus faecalis*) were characterized and compared. The fermentation patterns indicated that all three microorganisms produced acetic acid from the four sugars studied. Additionally, levulinic acid was formed by *E. coli* and formic acid by *S. faecalis*. The sucrose fermentation pattern of *E. coli* was different from those of the other three sugars. The concentrations of both the sugars metabolized and the analytes produced were calculated. All three microorganisms metabolized glucose to a higher degree than did the other three sugars.

The chromatograms for the fermentation of lactose by *E. coli* using RI or UV detection were shown. When the 0- and 24-h samples were compared, a decrease in the amount of lactose and an increase in the amounts of lactic, acetic, levulinic, isobutyric, butyric and isovaleric acids were found.

The applicability of HPLC to the rapid identification of *Bacteroides* species has been evaluated by analysing the effects of their glycosidase patterns on the composition of a defined chemical medium after aerobic incubation[52]. The defined chemical medium contained six carbohydrates and lyxose was added as an internal standard. Each *Bacteroides* isolate was inoculated into the medium and incubated at 37°C for 1 h. After centrifugation, the supernatants were injected into an Aminex HPX-87H column to determine carbohydrates and acid metabolic products. *Bacteroides fragilis* metabolized some carbohydrates (raffinose, lactose, glucose) and produced seven new peaks (B–H). Peaks B and C were identified as succinic and lactic acid, respectively. The production of peak H was a metabolic feature of *B. fragilis*,

TABLE 3

CAPACITY FACTORS OF CARBOHYDRATES SELECTED FOR THE PREPARATION OF CONTROL MEDIUM

Chromatographic conditions: column, Aminex HPX-87H (30 cm × 7.8 mm I.D.); mobile phase, $3.5 \cdot 10^{-3}$ *M* sulphuric acid; flow-rate, 0.4 ml/min; detector, spectrophotometer at 196 nm and 0.04 a.u.f.s.

Carbohydrate	*k'*	*Carbohydrate*	*k'*
Raffinose	0.088	Galactose	0.544
Cellobiose	0.188	Xylose	0.555
Trehalose	0.188	Rhamnose	0.588
Maltose	0.200	Lyxose	0.622
Lactose	0.222	Arabinose	0.733
Glucose	0.422	Salicin	1.655
Mannose	0.522		

which could be distinguished from the other *Bacteroides* species. The capacity factors of carbohydrates to be selected for the preparation of control medium under the same chromatographic conditions as described in ref. 52 are reported in Table 3. The HPLC profiles of both the defined chemical medium and *Bacteroides thetaiotaomicron* supernatant after aerobic incubation are shown in Fig. 2.

Polystyrene–divinylbenzene cation-exchange resins with a metallic counter ion and water as the eluent proved useful for the separation of carbohydrates, whereas the hydrogen form with an acidic eluent was employed for carbohydrate[53–56] and organic acid analysis[57,58]. HPLC separation of carbohydrates, alcohols, aldehydes, ketones and carboxylic acids on a cation-exchange resin in the hydrogen form was described by Pecina *et al.*[59]. The chromatographic behaviour of 63 substances of the above classes on an HPX-87H column was investigated. The effects of column temperature on the capacity factors of the examined compounds were described. The column temperature appeared to be a very important parameter for optimizing the separation of these substances. Optimized separations of carbohydrates, acids, aldehydes, ketones and alcohols were described. Operating conditions for the HPLC separation of monosaccharides and disaccharides were reviewed by Robards and Whitelaw[60].

2.2.2. Amino acids. In a study of numerous species of *Clostridium*[61], the isolated strains were inoculated into a chemically defined medium, containing primarily ten amino acids, and incubated aerobically at 37°C for 1 h. After centrifugation, the supernatants were derivatized with *o*-phthalaldehyde for 1 min in the presence of saturated boric acid. Samples were injected into an Ultrasphere-ODS column and effluents were monitored at 340 nm by spectrophotometry. Elution was performed with 30 m*M* sodium phosphate (pH 6.5), programming a 25–70% methanol gradient. The total time required for chromatographic analysis was *ca.* 50 min. A chromatogram of the medium after incubation with *Clostridium sordelii* showed that this micro-organism metabolized many of the components of the control medium and produced seven new peaks (A–G). The chromatographic profile showed both the utilization of defined substrates (as demonstrated by the elimination of the peak corresponding to an amino acid) and the appearance of new peaks corresponding to compounds produced by the metabolism of the amino acids contained in the medium. Multiple

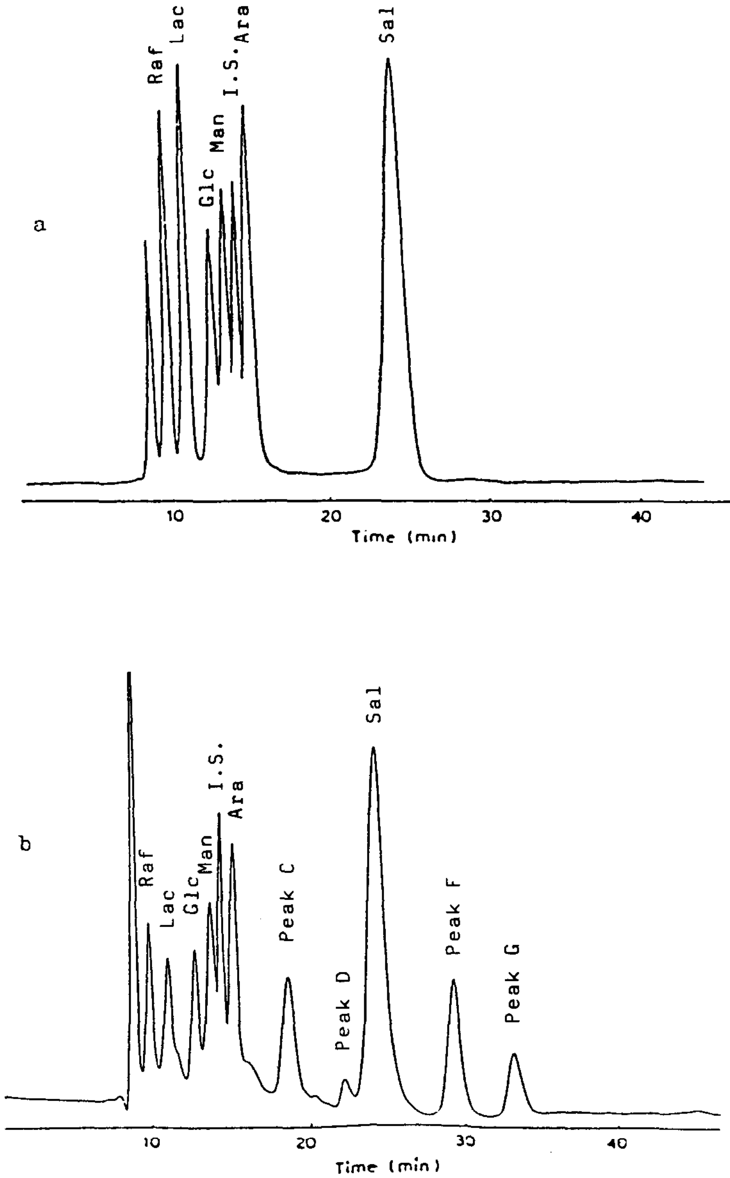

Fig. 2. HPLC profile of (a) the defined chemical medium and (b) *Bacteroides thetaiotaomicron* supernatant after aerobic incubation. Raf = Raffinose; Lac = lactose; Glc = glucose; Man = mannose; Ara = arabinose; Sal = salicin; internal standard (I.S.) = lyxose; peak C = latic; D = acetic; F = isobutyric; G = butyric.

isolates of various *Clostridium* species gave consistent patterns of medium utilization that could be used for identification. *Clostridium sordelii* was the only indole-positive t*Clostridium* species that produced peak D and also a very large peak E. These features can be used for distinguishing *Clostridium sordelii* from the other clostridial species.

Harpold and Wasilauskas[62] evaluated HPLC for the rapid identification of obligately anaerobic Gram-positive cocci by using the same method as described above. Chromatograms of a derivatized *P. anaerobius* supernatant and of a derivatized *G. anaerobia* supernatant after 1 h of incubation were shown. Medium utilization indices for *Peptostreptococcus* species (*micros, magnus, anaerobius asaccharolyticus, prevotii*) and *Staphylococcus saccharolyticus* were calculated. *P. magnus* utilized only serylleucine. *P. anaerobius* utilized serine, alanine, serylleucine, phenylalanine and leucine. A scheme for identifying Gram-positive anaerobic cocci was proposed on the basis of the differential utilization of some amino acids (phenylalanine, histidine, serine).

These positive experiments prompted Radin *et al.* [63] to apply this method to *Bacteroides* species. Some profiles representative of the most significant species, belonging to the *B. fragilis* group were shown. Even if the metabolic activities involved in the amino acid utilization appear to be much more reduced for *Bacteroides* than *Clostridium*, it was possible to propose a scheme which affords the correct identification of the examined microorganism within about 2 h. It is important to emphasize that, for the species belonging to the *B.fragilis* group, incubation can occur in aerobiosis and the time of incubation can be reduced to 30 min. In conclusion, one can say that this identification approach is rapid and reliable. The capacity factors of amino acids under the same chromatographic conditions as described in ref. 63 are reported in Table 4.

The operating conditions employed in the HPLC determination of different substrates (carbohydrates and amino acids) and their metabolic end-products by several bacterial species are summarized in Table 5.

2.3. *Perspectives of HPLC in diagnostic bacteriology*

The application of instrumental chromatographic techniques for diagnostic purposes has extensively involved the GC analysis of biological fluids from different areas of the human body. A chromatographic profile might be valuable if it demonstrated that the disease process was originally infectious or non-infectious. Brooks[15] pointed out that the direct analysis of biological fluids is the ultimate goal in

TABLE 4

CAPACITY FACTORS OF AMINO ACIDS IN THE HPLC ANALYSIS OF DERIVATIZED UNINOCULATED CONTROL MEDIUM

Chromatographic conditions: column, Ultrasphere-ODS (15 cm × 4.6 mm I.D.); mobile phase, gradient from 25 to 70% methanol in 30 m*M* sodium phosphate (pH 6.5); flow-rate, 1 ml/min; detector, spectrophotometer at 340 nm.

Amino acid	*k'*	*Amino acid*	*k'*
Asparagine	3.391	Alanine	11.43
Serine	4.913	Tryptophan	14.73
Glutamine	6.043	Valine	15.30
Histidine	6.304	Phenylalanine	15.73
Glycine	8.695	Leucine	17.39
Arginine	9.130	Lysine	19.26

the GC identification of a specific infectious disease. Many pathogenic micro-organisms that grow slowly or with difficulty might be identified so that a more appropriate therapy could be started much earlier.

The use of HPLC for the analysis of clinical specimens presents some technical drawbacks in comparison with GC. There is a pitfall in the direct injection of biological samples, owing to the possibility of clogging the capillary which connects the injector to the analytical column. In contrast, samples subjected to filtration, extraction with organic solvents or concentration by solid-phase extraction are suitable for HPLC analysis.

The identification of components present in biological fluids is a much more demanding task than identifying metabolites produced by microorganisms in spent culture media. In fact, clinical specimens, in most instances, are difficult to obtain, whereas the amount of spent culture media is usually unlimited. The types of components found in pathological body fluids are often less predictable than metabolites produced by bacteria in spent culture media. The components detected in biological fluids may derive not only from the metabolism of the infecting micro-organism but also from the host response to infection. Determining reproducible HPLC profiles from various sources will provide specific information that will help in identifying a particular disease.

While many reports have dealt with the use of GC for diagnostic purposes, application of HPLC to the analysis of clinical specimens are limited to only a few specific cases.

HPLC has been used for the determination of histamine and other biological amines in sputum samples from patients with asthma, pneumonia, chronic bronchitis and cystic fibrosis[64,65]. Separation was performed on a Spherisorb 3 ODS column with 0.2 *M* sodium acetate and tetrahydrofuran (75:25), adjusted to pH 5.1 with hydrochloric acid. Detection of the *o*-phthalaldehyde-derivatized amines was achieved using a fluorescence spectrometer (emission at 430 nm and excitation at 350 nm). The results of HPLC analysis strongly supported the hypothesis that bacteria might contribute to the presence of histamine in sputum from patients with infective lung disease. Sputum bacteriology for both chronic bronchitis and cystic fibrosis samples revealed the presence of *Pseudomonas aeruginosa, Haemophilus influenzae* and *Staphylococcus aureus*, together with anaerobic species in the former disease and *H. influenzae* in the latter[65].

Another very interesting application of HPLC in clinical bacteriology is in the analysis of gingival crevicular fluid, which is considered a promising medium for the detection of chemical markers of periodontal disease activity[66]. Gingival crevicular fluid contains a large number of components derived not only from the host tissues but also from the sub-gingival bacterial plaque; hence a wide range of molecules may be evaluated as potential markers. The metabolism of sugars and amino acids by the microbial community in periodontal pockets produces an array of both acidic and basic end-products. The concentration of acidic metabolites in sub-gingival plaque has been evaluated to be in the millimolar range. The aim of this approach was to provide the clinician with a rapid and sensitive means of diagnosis of active periodontal breakdown. The detection of a characteristic end-product profile and/or of a specific marker in clinical samples can be utilized to indicate the presence of both a quiescent or active microflora in the infected site. The limitation of this approach is that only

TABLE 5

CHROMATOGRAPHIC CONDITIONS IN THE HPLC DETERMINATION OF SUBSTRATES FOR SEVERAL BACTERIAL SPECIES

Bacteria	*Substrate*	*Column*	*Mobile phase*	*Temperature (°C)*	*Flow-rate (ml/min)*	*Detection*	*Ref.*
–	–	Aminex HPX-87H	0.005*M* H_2SO_4	70	0.7	RI	59
E. coli, K. pneumoniae, S. faecalis	Carbohydrates	Aminex HPX-87H	0.028 *M* H_2SO_4	40		UV + RI	51
B. fragilis	Carbohydrates	Aminex HPX-87H	$3.5 \cdot 10^{-3}$ *M* H_2SO_4	20	0.3	UV, 196 nm	52
Clostridium	Amino acids	Ultrasphere-ODS	25–70% methanol	–	1	UV, 340 nm	61
Peptostreptococcus	Amino acids	Ultrasphere-ODS	25–70% methanol	–	1	UV, 340 nm	62

(sub)microlitre amounts of gingival fluid can be retrieved; this means that analytical techniques requiring both minimum sample handling and detection of these metabolites in a single run, must be used. Detection methods have been developed, using HPLC[67], luciferase-linked enzyme assays[68], GC[69] and isotachophoresis[70]. The use of HPLC is attractive as different classes of acids, including phenolic acids, can be determined in a single analysis. However, the detection methods for bacterial end-products, such as hydroxy acids, dicarboxylic acids and amines, at concentrations present in gingival crevicular fluid, require considerable modification in order to be useful in diagnostic laboratories. We are currently evaluating the potential of HPLC in this area where detection of millimolar concentrations in sub-microlitre volumes is necessary. Further improvements in HPLC technology will be necessary to determine picomole amounts of most of these molecules. At present, electron-capture GC seems capable of offering some advantages over the available HPLC procedures.

One of the features of HPLC is that, whereas selectivity is achieved in the separation step on the analytical column, sensitivity and improved selectivity can be achieved in the detection step. When comparing HPLC and GC analysis, the inherent differences between the two methods affect the selectivity of the separation and of the detection system. As far as the separation in GC is concerned, it is the volatility of the compounds that determines the selectivity of the chromatographic procedure; on the other hand, solubility of chemical compounds in the mobile phase is the major determinant in HPLC. It should be noted that in HPLC the mobile phase can be selected in such a way as to improve the solubility of the substances under study and therefore to increase selectivity. A similar flexibility is achieved in GC by selecting a polar or non-polar stationary phase from a wider array of commercially available types. In terms of detectors, both GC and HPLC present the possibility of using different detection systems. Mass spectrometric, photodiode-array, fluorescence, electrochemical and electron-capture detectors all improve selectivity, facilitating peak identification; when applying these techniques, peak identification is not based solely on retention times.

The type of detector to be used should be selected according to the amounts of the substances to be analysed and the nature of the compounds to be determined. In general, UV and fluorescence methods are suitable for the more sensitive detection of substances, *e.g.*, from nanomoles to 10 pmol. As not all substances show UV or fluorescence properties, chemical derivatization can be used to obtain these properties to increase the sensitivity[71–77]. An example is most aliphatic acids, which are normally detected at the millimole level only, whereas their detection limit can reach the nanomole level when they are derivatized. Labelling of substances with reagents that afford structures with UV bands is the most popular means of derivatization. Phenyl, *p*-bromophenacyl bromide, 2-naphthacyl bromide and other types of reagents for precolumn labelling of carboxylic acids have been reported[78,79].

The application of labelling procedures to bacterial metabolites could improve the HPLC analysis of biological fluids from different sources, as suggested by the results of Tsuchiya *et al.*[80]. They determined the fatty acid composition of phospholipids from several oral *Streptococci*, following derivatization with a fluorescent reagent (4-bromomethyl-7-acetoxycoumarin). These results showed that the HPLC method was at least 500 times more sensitive than GC with flame ionization detection but less sensitive (50%) than GC with electron-capture detection. By

analogy, the application of similar labelling procedures to the HPLC analysis of bacterial metabolites in biological fluids could provide sensitivity comparable to GC with frequency pulse-modulated electron-capture detection as proposed by Edman and Brooks[4] for the diagnosis of infectious diseases. All these considerations suggest that HPLC is potentially useful in bacteriological laboratories, especially for the diagnosis of anaerobic infections.

3. BACTERIAL FERMENTATIONS

An important application of HPLC procedures is the monitoring of bacterial fermentations during the industrial preparation of various food products (beer, wine, cheese, yogurt, sauces). Qualitative data are often sufficient for these applications; however, HPLC analysis carried out under controlled conditions can provide excellent quantitative data.

The chromatographic profile of a sample of soya sauce obtained from a mixed fermentation, including *Lactobacilli*, yeasts and fungi, was considered a useful indicator of product integrity and stability. A peak present in the final part of the chromatogram was assigned to benzoic acid, an additive commonly employed for the storage of some food products[38].

4. METABOLITES ASSOCIATED WITH THE PURINE AND PYRIMIDINE PATHWAYS

The biological importance of purine compounds is emphasized by their occurrence in nucleic acids and their participation in biosynthetic reactions involving nucleotides. A number of methods for the HPLC analysis of free bases, nucleosides and nucleotides has been published. Isocratic separations on ion-exchange columns[81] have proved to be more time consuming than analyses performed by reversed-phase HPLC[82,83]. Mechanisms of RP-HPLC retention, structure–retention relationhips of purines and pyrimidines in reversed-phase systems, effects of mobile phase on purine and pyrimidine retention characteristics and ion-pairing techniques were reviewed by Scoble and Brown[84]. The roles of the mobile phase parameters pH, organic modifier and ionic strength were examined in reversed-phase chromatographic systems[85].

As the retention times of purines are highly affected by changes in pH using reversed-phase systems[86], various pH values of potassium phosphate buffers were tested in an HPLC separation of purines (adenine, 6,8-dihydroxypurine, 2-hydroxypurine, hypoxanthine, purine, uric acid, xanthine) and their anaerobic and aerobic degradation products (4-ureido-5-imidazolecarboxylic acid, 4-amino-5-imidazolecarboxylic acid, 4-aminoimidazole, formiminoglycine, allantoin, ureidoglycolate) using UV detection at 205 nm[87]. The best resolution of purines and their anaerobic degradation products was achieved at pH 3.7, whereas the separation of uric acid and its aerobic degradation products was achieved at pH 3.1.

Interesting applications have involved the determination of the pathway of purine degradation by *Clostridium purinolyticum*[87,88], the study of the metabolism of a xanthine drug by microorganisms[89] and the determination of ribonucleoside triphosphates in *Escherichia coli*[90]. The degradation of purines by aerobic microorganisms starts from uric acid by a pathway yielding allantoin, allantoic acid, ureidoglycolate and finally urea and glyoxylate[91]. HPLC was used to determine the

pathway of adenine degradation by *Clostridium purinolyticum*, a species of obligate purine-fermenting bacteria[92]. The decomposition of adenine by cell-free extracts of *C. purinolyticum*, was found to proceed via hypoxanthine, xanthine and imidazole derivatives. The strict dependance on selenium compounds for the growth of this anaerobic species was thought to be due to the presence of some selenoenzymes which have key functions in the catabolic breakdown of purines and energy generation[93]. The possibility of a purine ring cleavage in the imidazole moiety yielding pyrimidine derivatives has been investigated[88]. *C. purinolyticum* decomposed uric acid via pyrimidine derivatives under selenium starvation conditions, producing acetate, formate, glycine, ammonia and carbon dioxide. The results of the HPLC determination of uric acid, 4,5-diaminouracil and 6,7-dimethyllumazine[88] showed that under selenium starvation conditions an alternative pathway is used for uric acid decomposition. The discovery of the new pathway led to the speculation that some sort of diabetes, induced by the pyrimidine alloxan[94], might be due to a microbiological origin of this compound.

An HPLC method has been developed for the determination of pentoxifylline and its major metabolites in microbial extracts[89]. Pentoxifylline is widely used in the treatment of patients with cerebrovascular and peripheral vascular diseases[95]. Seven metabolites of pentoxifylline have been identified in mammals, including man[96]; the major metabolites are a secondary alcohol and two carboxylic acids[97]. The strategy of microbial models of mammalian metabolism[98] was planned to seek microorganisms that metabolize pentoxifylline in a manner similar to mammals. The developed procedure was used for investigating the metabolism of pentoxifylline by *Nocardia corallina*. Initial extraction of acidified media with dichloromethane-2-propanol (4:1) was required. HPLC with a methanol–0.02 *M* phosphoric acid (pH 5.0) (3:7) mobile phase and UV detection at 275 nm permitted detection of μg/ml concentrations of the xanthine compounds. This technique was previously used to determine pentoxifylline and secondary alcohol metabolite in plasma[99] and carboxylic acid metabolites in urine[100].

A correlation between the accumulation of pyrophosphate and the increased level of ribonucleoside triphosphates in *Escherichia coli* in the presence of some inhibitors (6-azauracil, 6-mercaptopurine, 5-fluorouracil and hydroxyurea) of nucleic acid synthesis was demonstrated by Kukko and Kallio[90]. Ribonucleoside triphosphate concentrations were determined by HPLC using isocratic elution with 0.2 *M* sodium phosphate (pH 6.0)–0.025 *M* tetrabutylammonium hydroxide–10% methanol and detection at 260 nm.

The results obtained in the applications mentioned above indicate that reversed-phase HPLC can be useful not only for monitoring the activities of purine-metabolizing enzymes but also for studying the metabolism of purine drugs.

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6. ABSTRACT

The chemotaxonomic approach to the identification of pathogenic bacteria for clinical purposes is surveyed. Primary interest is focused on the applications of HPLC to the determination of metabolic products from anaerobic bacteria. The use of HPLC is attractive as different classes of short-chain acids can be determined in a single analysis. Chromatographic conditions are extensively described, emphasizing the effects of changing variables on the HPLC profiles of analytes. The application of labelling procedures to bacterial metabolites can markedly increase the sensitivity of the analysis of pathological fluids. HPLC appears to be potentially useful in clinical bacteriology for the diagnosis of anaerobic infections.

REFERENCES

1 M. Goodfellow and D. E. Minnikin, in M. Goodfellow and D. E. Minnikin (Editors), *Chemical Methods in Bacterial Systematics*, Academic Press, London, 1985, pp. 1–15.
2 M. Goodfellow and R. G. Board (Editors), *Microbiological Classification and Identification*, Academic Press, London, 1980.
3 D. B. Drucker, *Microbiological Applications of Gas Chromatography*, Cambridge University Press, Cambridge, 1981.
4 D. C. Edman and J. B. Brooks, *J. Chromatogr.*, 274 (1983) 1.
5 N. R. Krieg (Editor), *Bergey's Manual of Systematic Bacteriology*, Vol. 1, Williams and Wilkins, Baltimore, 1984.
6 G. Gottschalk (Editor), *Methods in Microbiology*, Vol. 18, Academic Press, London, 1985.
7 I. Brondz, *Dr. Thesis*, University of Oslo, Oslo, 1985.
8 M. Goodfellow and D. E. Minnikin (Editors), *Chemical Methods in Bacterial Systematics*, Academic Press, London, 1985.
9 I. Brondz and I. Olsen, *J. Chromatogr.*, 380 (1986) 1.
10 I. Brondz and I. Olsen, *J. Chromatogr.*, 379 (1986) 367.
11 H. N. Shah and M. D. Collins, *J. Appl. Bacteriol.*, 55 (1983) 403.
12 H. N. Shah, R. A. Nash, J. M. Hardie, D. A. Wheetman, D. A. Geddes and T. W. McFarlane, in M. Goodfellow and D. E. Minnikin (Editors), *Chemical Methods in Bacterial Systematics*, Academic Press, London, 1985, pp. 317–340.
13 R. C. Seid, H. Schneider, S. Bondarew and R. A. Boykins, *Anal. Biochem.*,124 (1982) 320.
14 L. V. Holdeman, E. P. Cato and W. E. C. Moore, *Anaerobe Laboratory Manual*, Virginia Polytechnic Institute and State University, Blacksburg, VA, 4th ed., 1977.
15 J. B. Brooks, *Adv. Chromatogr.*, 15 (1977) 1.
16 D. C. Edman, R. B. Craven and J. B. Brooks, *CRC Crit. Rev. Clin. Lab. Sci.*, 14 (1981) 133.
17 C. W. Moss, *J. Chromatogr.*, 203 (1981) 337.
18 D. B. Drucker, in J. R. Norris (Editor), *Methods in Microbiology*, Vol. 9, Academic Press, New York, 1976, p. 52.
19 E. Postaire, F. Schnirer and J. C. Darbord, *Bull. Inst. Pasteur*, 86 (1988) 145.
20 D. B. Drucker, *Microbiological Applications of High-Performance Liquid Chromatography*, Cambridge University Press, Cambridge, 1987.
21 S. L. Gorbach, J. W. Mayhew, J. G. Bartlett, H. Thadepalli and A. B. Onderdonk, *J. Clin. Invest.*, 57 (1976) 478.
22 R. Schwarzenbach, *J. Chromatogr.*, 251 (1982) 339.
23 B. Libert, *J. Chromatogr.*, 210 (1981) 540.
24 C. Droz and H. Tanner, *Schweiz. Z. Obst. Weinbau*, 118 (1982) 434.
25 R. L. Patience and J. D. Thomas, *J. Chromatogr.*, 249 (1982) 183.
26 P. Jandere and H. Engelhardt, *Chromatographia*, 13 (1980) 18.
27 B. S. Buslig, C. W. Wilson and P. E. Shaw, *J. Agric. Food Chem.*, 30 (1982) 342.
28 K. G. Wahlund, *J. Chromatogr.*, 218 (1981) 671.
29 D. N. Buchanan and J. G. Thoene, *Anal. Biochem.*, 124 (1982) 108.
30 N. Kubadinow, *Zuckerindustrie*, 107 (1982) 1107.

31 F. G. R. Reyes, R. E. Wrolstad and C. J. Cornwell, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 126.
32 L. M. Jahangir and O. Samuelson, *Anal. Chim. Acta*, 92 (1977) 329.
33 V. T. Turkelson and M. Richards, *Anal. Chem.*, 50 (1978) 1420.
34 R. T. Marsili, *J. Chromatogr. Sci.*, 19 (1981) 451.
35 P. R. Monk and P. G. Iland, *Food Technol. Aust.*, 36 (1984) 16.
36 G. G. Ehrlich, D. F. Goerlitz, J. H. Bourell, G. V. Eisen and E. M. Godsy, *Appl. Environ. Microbiol.*, 42 (1981) 878.
37 G. O. Guerrant, M. A. Lambert and C. W. Moss, *J. Clin. Microbiol.*, 16 (1982) 355.
38 R. F. Adams, R. L. Jones and P. L. Conway, *J. Chromatogr.*, 336 (1984) 125.
39 L. Radin, A. Costa, M. van Steenbergen, J. De Graaff and G. A. Botta, *IV Convegno delle Sezioni Liguri, Genoa, 1984*, Associazone Microbiologi Clinici Italiani, Tecnostampa, Reggio Emilia, 1986, p. 267.
40 R. Krausse and U. Ullmann, *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg., Abt. 1 Orig., Reihe A*, 265 (1987) 340.
41 R. S. Edson, J. E. Rosenblatt, J. A. Washington and J. B. Stewart, *J. Clin. Microbiol.*, 6 (1982) 1059.
42 J. B. Brooks, O. L. Nunez-Montiel, B. J. Wycoff and C. W. Moss, *J. Clin. Microbiol.*, 20 (1984) 539.
43 C. Potvliege, M. Labbe' and E. Yourassowsky, *Lancet*, ii (1981) 1105.
44 W. H. Loh, N. Santoro, R. H. Miller and O. H. Tuovinen, *J. Gen. Microbiol.*, 130 (1984) 1051.
45 R. Krausse and U. Ullmann, *Zbl. Bakteriol. Parasitenkd. Infektionskr. Hyg., Abt. 1 Orig., Reihe A*, 260 (1985) 342.
46 J. W. Mayhew, A. B. Onderdonk and S. L. Gorbach, *Appl. Microbiol.*, 4 (1975) 472.
47 P. N. Levett and K. D. Phillips, *J. Clin. Pathol.*, 38 (1985) 82.
48 A. E. van den Bogaard, M. J. Hazen and C. P. van Boven, *J. Clin. Microbiol.*, 3 (1986) 523.
49 S. S. Socransky, J. L. Dzink and C. M. Smith, *J. Clin. Microbiol.*, 22 (1985) 303.
50 R. Corio, P. A. Dusi, L. Radin and C. Lucarelli, in Emmezeta (Editor), *20th National Congress, Italian Society of Clinical Biochemistry, Rome*, La Grafica Briantea, Como, 1988, p. 117.
51 L. F. Ross and D. C. Chapital, *J. Chromatogr. Sci.*, 25 (1987) 112.
52 L. Radin, A. Arzese, C. Lucarelli and G. A. Botta, *J. Chromatogr.*, 459 (1988) 331.
53 M. E. Cieslak and W. C. Herwig, *J. Am. Soc. Brew. Chem.*, 40 (1982) 43.
54 K. M. Brobst and H. D. Scobell, *Starke*, 34 (1982) 117.
55 F. E. Wentz, A. D. Marcy and M. G. Gray, *J. Chromatogr. Sci.*, 20 (1982) 349.
56 J. C. du Preez and J. P. van der Walt, *Biotechnol. Lett.*, 5 (1983) 357.
57 L. A. Th. Verhaar and B. F. M. Kuster, *J. Chromatogr.*, 210 (1981) 279.
58 K. Brunt, *J. Chromatogr.*, 246 (1982) 145.
59 R. Pecina, G. Bonn, E. Burtscher and O. Bobleter, *J. Chromatogr.*, 287 (1984) 245.
60 K. Robards and M. Whitelaw, *J. Chromatogr.*, 373 (1986) 81.
61 D. J. Harpold, B. L. Wasilauskas and M. L. O'Connor, *J. Clin. Microbiol.*, 22 (1985) 962.
62 D. J. Harpold and B. L. Wasilauskas, *J. Clin. Microbiol.*, 25 (1987) 996.
63 L. Radin, R. Corio and G. A. Botta, *Biochim. Clin.*, 13 (1989) 993.
64 J. L. Devalia, B. D. Sheinman and R. J. Davies, *J. Chromatogr.*, 343 (1985) 407.
65 B. D. Sheinman, J. L. Devalia, S. J. Crook ad R. J. Davies, *Agents Actions*, 17 (1985) 449.
66 M. A. Curtis, I. R. Gillett, G. S. Griffiths, M. F. J. Maiden, J. A. C. Sterne, D. T. Wilson, J. M. A. Wilton and N. W. Johnson, *J. Clin. Periodontol.*, 16 (1989) 1.
67 M. A. Curtis and C. W. Kemp, in B. Guggenheim (Editor), *Cariology Today*, Karger, Basle, 1983, p. 212.
68 S. Robrish, M. A. Curtis, S. A. Sharer and W. H. Bowen, *Anal. Biochem.*, 136 (1984) 503.
69 G. A. Botta, L. Radin, A. Costa and G. Blasi, *J. Periodont. Res.*, 20 (1985) 450.
70 W. M. Edgar, M. W. J. Dodds and S. M. Higham, *Biochem. Soc. Trans.*, 14 (1986) 977.
71 J. F. Lawrence, *Organic Trace Analysis by Liquid Chromatography*, Academic Press, New York, 1981, Ch. 7.
72 R. E. Majors, H. G. Barth and C. H. Lochmüller, *Anal. Chem.*, 54 (1982) 323R.
73 R. E. Majors, H. G. Barth and C. H. Lochmüller, *Anal. Chem.*, 56 (1984) 300R.
74 H. Lingeman, W. J. M. Underberg, A. Takadate and A. Hulshoff, *J. Liq. Chromatogr.*, 8 (1985) 789.
75 D. Perrett and S. R. Rudge, *J. Pharm. Biomed. Anal.*, 3 (1985)3.
76 I. S. Krull, C. M. Selavka, C. Duda and W. Jacobs, *J. Liq. Chromatogr.*, 8 (1985) 2845.
77 K. Imai and T. Toyo'oka, in R. W. Frei and K. Zech (Editors), *Selective Sample Handling and Detection in High-Performance Liquid Chromatography*, Part A, Elsevier, Amsterdam, 1988, p. 209.

78 R. L. Patience and J. D. Thomas, *J. Chromatogr.*, 249 (1982) 183.
79 P. C. Bossle, J. J. Martin, E. W. Sarver and H. Z. Sommer, *J. Chromatogr.*, 267 (1983) 209.
80 H. Tsuchiya, M. Sato, I. Namikawa, T. Hayashi, M. Tatsumi and N. Takagi, *J. Clin. Microbiol.*, 24 (1986) 81.
81 E. Nissinen, *Anal. Biochem.*, 106 (1980) 497.
82 N. D. Brown, J. A. Kintzios and S. E. Koetitz, *J. Chromatogr.*, 177 (1979) 170.
83 W. E. Wung and S. B. Howell, *Clin. Chem.*, 26 (1980) 1704.
84 H. A. Scoble and P. R. Brown, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 1.
85 M. Zakaria, P. R. Brown and E. Grushka, in A. Zlatkis, R. Segura and L. S. Ettre (Editors), *Advances in Chromatography 1981,* University of Houston, Houston, TX, 1981, pp. 451–471.
86 P. R. Brown, A. M. Krstulovic and R. A. Hartwick, *Adv. Exp. Med. Biol.*, 76A (1977) 610.
87 P. Duerre and J. R. Andreesen, *Anal. Biochem.*, 123 (1982) 32.
88 P. Duerre and J. R. Andreesen, *Arch. Microbiol.*, 131 (1982) 255.
89 R. V. Smith, S. K. Yang, P. J. Davis and M. T. Bauza, *J. Chromatogr.*, 281 (1983) 281.
90 E. Kukko and T. Kallio, *Biochem. Int.*, 8 (1984) 1.
91 G. D. Vogels and C. van der Drift, *Bacteriol. Rev.*, 40 (1976) 403.
92 P. Duerre, W. Andersch and J. R. Andreesen, *Int. J. Syst. Bacteriol.*, 31 (1981) 184.
93 P. Duerre and J. R. Andreesen, *J. Gen. Microbiol.*, 128 (1982) 1457.
94 M. Pojek and B. Rocic, *Experientia*, 36 (1980) 78.
95 H. Denck, *Arzneim.-Forsch.*, 23 (1973) 571.
96 H. J. Hinze, G. Bedessem and A. Soder, *Arzneim.-Forsch.*, 22 (1972) 1144.
97 H. J. Hinze, *Arzneim.-Forsch.*, 22 (1972) 1492.
98 R. V. Smith and J. P. Rosazza, *J. Nat. Prod.*, 46 (1983) 79.
99 D. A. Chivers, D. J. Birkett and J. O. Miners, *J. Chromatogr.*, 225 (1981) 261.
100 J. Chamberlain and K. W. Jolley, Hoechst U.K., personal communication, 1981.