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Routine analysis of short-chain fatty acids for anaerobic bacteria identification using capillary electrophoresis and indirect ultraviolet detection

Michel Arellano[a,b], Pascal Jomard[c], Said El Kaddouri[c], Christine Roques[c], Françoise Nepveu[a], François Couderc[d,*]

[a]*Laboratoire de Synthèse, Physico-Chimie, Radiobiologie, Service de Chimie Analytique, Oenologie et Bromatologie, Faculté des Sciences Pharmaceutiques, 35 Chemin des Maraîchers, 31062 Toulouse cedex 04, France*
[b]*Picometrics, 10 Avenue de l'Europe, 31520 Ramonville, France*
[c]*Laboratoire de Bactériologie, Virologie et Microbiologie Industrielle, Faculté des Sciences Pharmaceutiques, 35 Chemin des Maraîchers, 31062 Toulouse cedex 04, France*
[d]*Université Paul Sabatier, IPBS 205 Route de Narbonne, 31077 Toulouse cedex, France*

Abstract

The diagnosis of anaerobes can be difficult to perform, using classical biochemical tests. Characterization of metabolic end-products such as short-chain fatty acids (SCFA) was often used because of their reproducible biosynthesis. Despite this, SCFA are difficult to study using gas chromatography, due to their high volatility. Furthermore, the treatment of the samples are long and fastidious. Capillary electrophoresis and indirect UV detection (CE–indirect UV) is a well-known analytical method to study inorganic or organic anions. In this work, we validate the analysis of SCFA using CE–indirect UV detection. To do this, we studied the culture media of 98 anaerobic strains for the detection and quantitation of the following acids: succinic, pyruvic, acetic, lactic, propionic, 2-hydroxybutyric, butyric, 2-hydroxyvaleric, isovaleric, isocaproic, and 3-phenylpropionic. We verified that the CE–indirect UV detection analysis of SCFA for taxonomical data can be used as a mean for rapid identification for the study of anaerobes.

Keywords: Anaerobic bacteria; Fatty acids; Short-chain

1. Introduction

The diagnosis of anaerobic bacteria is sometimes difficult to do using classical biochemical tests, especially for poorly- and non-toxogenic species [1]. In the early 1960s, Prévot [2] recommended to complete these tests by studying end-products of metabolism. Among those molecules he considered, were the volatile and non-volatile organic acids which are synthesized along with the fermentation of the culture media glucides. These substances produced by anaerobes are of significant taxonomical interest. A wide number of publications described the use of different analytical means to obtain information on the nature and the quantity of different short-chain fatty acids (SCFA) which are found in the culture media. Most of the work on this topic was done using gas chromatography (GC). The following acids are quantified: succinic, pyruvic, acetic, lactic, propionic, 2-hydroxybutyric, butyric, 2-hydroxyvaleric, isovaleric, isocaproic and 3-

*Corresponding author.
E-mail address: couderc@ipbs.fr (F. Couderc)

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phenylpropionic [1,3]. Several methodologies using GC have been used. Sutter et al. [4] proposed to directly inject the acidificated culture medium in the column without prior treatment of the sample. Some authors used this analytical method with limited success to characterize anaerobic bacteria [5–7]. This method allowed very quick determination of volatile acids but did not succeed to identify non-volatile acids. In addition, ghosting and tailing peaks appear after a few injections and which promotes loss of sensitivity [5]. Lambert and Moss [8] analyzed the acids after obtaining butyl esters, and analyzed non-volatile and volatile SFCA. This method is relatively long because of the esterification step (overnight). In the same way, Holdeman et al. [9] preferred to extract the acids and derivitize to methyl esters, which are easily and quickly synthetised using diazomethane. Some of the very volatile esters (i.e. methyl acetate, methyl propionate) are difficult to quantify using GC and the reproducibility is greatly affected [10]. Carlier and Sellier [11] preferred to study methylated and butylated SCFA. Using gas chromatography/mass spectrometry, they succeeded to identify some SCFAs which are determinant for the identification of some particular strains of *Fusobacterium spp.* and *Clostridium spp.* Some authors used high-performance liquid chromatography (HPLC) to study SCFA in biological media [12].

Capillary isotachophoresis was also used to identify organic acids from selected microorganisms [13], but this application is uncommon as compared to the use of GC.

Some years ago, Huang et al. [14] presented the use of capillary electrophoresis and conductimetry for the analysis of organic acids in wines. They demonstrated that they could analyze aliphatic acids from C1 to C6 diacids, and hydroxy acids in a single run and that they could easily and rapidly quantify them. Several authors used CE and indirect UV detection as a rapid and sensitive analytical mean, to study rain water [15], wine [16], tap water [17] and river water [18]. Recently, in our laboratory we developed the analysis of different SCFAs. It was used for the analysis of SCFA in culture media of anaerobes [19]. In this publication we validate the methodology of the analysis of the SFCAs in culture media where bacteria are grown for 24 h. The CE–indirect UV analysis of the filtrated culture media has only a 10 mn duration for both volatile and non-volatile SCFA quantitation. We verified that the proposed methodology is new, rapid, easy, reproducible and can be a useful taxonomical means for the study of anaerobic bacteria.

2. Experimental

2.1. Organisms and culture conditions

The following type strains (T) and referenced strains were obtained from the Collection of Institut Pasteur (CIP, Paris, France), from the ''Collection des anaérobies de l'Institut Pasteur'' (AIP, Paris, France) and from the ''National Collection of Type Cultures'' (NCTC, London, UK): *Fusobacterium gonidiaforme* AIP 3554 AT, *Fusobacterium navforme* AIP 10147 T, *Fusobacterium nucleatum subsp. fusiformis* NCTC 11326, *Fusobacterium nucleatum subsp. nucleatum* CIP 101130 T, *Fusobacterium nucleatum subsp. polymorphum* AIP 10098 AT, *Fusobacterium necrophorum* AIP 10145 T, *Fusobacterium russii* AIP 10146, *Porphyromonas gingivalis* CIP 103683 T, *Bacteroides fragilis* CIP 77.16 T, *Capnocytophaga ochracea* CIP 82.101 (*Capnocytophaga ochracea* includes facultative anaerobic strains with fermentative type metabolism. In this way its characterization frequently includes end-product formation [20]), *Propionibacterium acnes* CIP 53.117 T, *Peptostreptococcus anaerobius* AIP 101102 T, *Lactobacillus acidophilus* CIP 76.13 T, *Lactobacillus gallinarum* CIP 103611 T, *Actinomyces israelii* CIP 103259. *Fusobacterium nucleatum subsp. vincentii* ATCC 49256 was kindly provided by Pr Ch. Mouton (University of Laval, Québec). The other strains were human clinical isolates and were identified according to the described procedures [21,22]. *Fusobacterium nucleatum* strains were identified at the subspecies level according to 2-oxoglutarate reductase (OGR)/glutamate deshydrogenase (GDH) electrophoretic migration patterns [23,24]. Clinical isolates included 29 *F. nucleatum,* 6 *P. gingivalis*, 15 *Prevotella intermedia*, 3 *P. melaninogenica*, 17 *B. fragilis,* 18 *C. ochracea,* 7 *L. acidophilus,* 1 *Actinomyces odontolyticus*, 1 *Arachnia propionica* and 1 *Clostridium*

perfringens. Organisms were maintained under anaerobic conditions (Anaerobic Chamber, Bioblock Scientific, Illkirch, France) on Columbia blood agar (Biomérieux, Marcy l'Etoile, France). Before each experiment, bacteria were transfered in tryptone–glucose–yeast (TGY) broth (Diagnostics Pasteur, Paris, France) which was not supplemented with hemin. After 24 h of incubation at 37°C, the supernatants were collected for CE analysis.

2.2. Chemicals

Standard solutions were prepared from chromatographic or analytical reagent grade chemicals (Merck, Darmstadt, Germany; Sigma, St. Quentin Fallavier, France; Prolabo, Paris, France) by serial dilution with deionized 15 megaohms water (C2R, Montgiscard, France). Solutions of succinic (1.004 g/l), pyruvic (1.038 g/l), acetic (1.076 g/l), lactic (1.020 g/l), propionic (1.036 g/l), 2-hydroxybutyric (1.010 g/l), butyric (1.032 g/l), 2-hydroxyvaleric (1.000 g/l), isovaleric (1.122 g/l), isocaproic (1.008 g/l) and 3-phenylpropionic (1.036 g/l) acids (or sodium salts) were prepared. These solutions were mixed and diluted and the diluted mixture was used as the standard.

Benzoic acid (BA) (Sigma, St. Quentin Fallavier, France) and histidine (Aldrich, St. Quentin Fallavier, France) were the background electrolytes (BGE) and were prepared as a 10 m*M* stock solution; its pH value was adjusted to 6.0 by the addition of 1 *M* of Tris–Base buffer [pH 10.5, Tris[hydroxymethyl]aminomethane (Sigma, St. Quentin Fallavier, France)].

Tetradecyltrimethylammoniumbromid (TTAB) (Aldrich, St. Quentin Fallavier, France) was employed as an electroosmotic flow (EOF) modifier at a concentration of 1 m*M*.

2.3. Sample preparation

The sample dilution range was 0.76–502 mg/l for succinic acid, 9.16–259.50 mg/l for pyruvic acid, 0.08–519 mg/l for acetic acid, 2.55–255 mg/l for lactic acid, 1.23–259 mg/l for propionic acid, 2.05–252.50 mg/l for 2-hydroxybutyric acid, 1.52–258 mg/l for butyric acid, 2.88–250 mg/l for 2-hydroxyvaleric acid, 1.97–280.50 mg/l for isovaleric acid, 1.77–252 mg/l for isocaproic acid and 2.59–259 mg/l for 3-phenylpropionic acid.

TGY culture supernatants were filtered on a 0.2 mm membrane filter (Prolabo, Fontenay sous bois, France) and diluted 1:50 with deionised water.

2.4. Apparatus

An Europhor capillary electrophoresis system (Dual Impact, Europhor Instruments now Picometrics Ramonville, Toulouse, France) was used throughout the investigation. The ultraviolet detector was built into the CE instrument. The separation was performed on a 75 cm×75 mm I.D. fused-silica capillary column (Picometrics). The detection window was 7 cm from the capillary outlet. An ultraviolet wavelength of 220 nm was chosen to monitor the absorbance of the buffer solution. The capillary was rinsed with 1 *M* NaOH (1 min), with 0.1 *M* NaOH (1 min), with water (1 min) and then with separation buffer for 2 min. The separation voltage was −14 kV, resulting in an electrophoretic current of 4.8 mA, at a constant temperature of 20°C. The samples were injected by hydrodynamic injection (fixed vacuum: 1.5 p.s.i. relative to ambient pressure) for 2 s. Injection volumes measure 4 nL/s for a 75 mm I.D. capillary. The injection end of the capillary dips 1 cm into a 1.5 ml vial (Picometrics) filled with 1 ml sample. Migration time of electroosmotic flow was determined using a water injection. Electrophoregrams were recorded and processed with a PC 1000 data acquisition system (Thermo Separation Products, Freemont, CA, USA).

3. Results

3.1. CZE separation of SFCA

Fig. 1 shows a typical electropherogram of a test mixture containing the 11 compounds of interest in 10 m*M* BA–10 m*M* histidine–1 m*M* TTAB at pH 6.0. Peaks were identified by comparing migration times and spiking samples with known quantities of standard solutions of SFCA. The migration times of the analytes increase in the following order: (1) succinic acid, (2) pyruvic acid, (3) acetic acid, (4) lactic acid, (5) propionic acid, (6) 2-hydroxybutyric

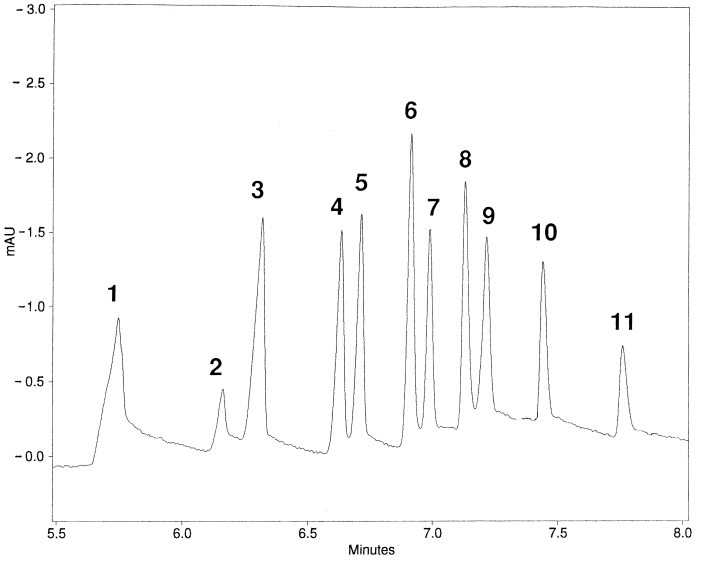

Fig. 1. Electropherogram of a mixture of the short-chain fatty acids in 10 m*M* BA–10 m*M* histidine–1 m*M* TTAB at pH 6.0. Migration order (peaks): (1) succinic acid, (2) pyruvic acid, (3) acetic acid, (4) lactic acid, (5) propionic acid, (6) 2-hydroxybutyric acid, (7) butyric acid, (8) 2-hydroxyvaleric acid, (9) isovaleric acid, (10) isocaproic acid, (11) 3-phenylpropionic acid. The migration time of the BOF was 11.44 mm.

acid, (7) butyric acid, (8) 2-hydroxyvaleric acid, (9) isovaleric acid, (10) isocaproic acid, (11) 3-phenylpropionic acid. The migration time of the ion is strongly dependent on the electric charge that it carries and its mass.

3.2. Precision and linearity

The precision [expressed in terms of relative standard deviation (RSD)] of the present method for the various analytes is summarized in Table 1. A mixture of the 11 anions in water was injected using the method settings given in the Experimental section. Six injections of standards were done sequentially. This operation was repeated over 3 days. The RSDs for the migration times are typically less than 0.58% for all compounds. The RSDs for the peak areas are typically in the range of 0.48–2.66%.

The linearity of the present method was investigated by analyzing standard solutions containing a mixture of 11 analytes with known concentrations ranging from 0.08 to 519 mg/l. From the electrophoregrams, the peak area is plotted against the concentration of the analyte to obtain the calibration graph for each analyte. The data points from the calibration graph were subjected to least-square regression analysis and the slope *a*, intercept *b* and correlation coefficients *R* for the various analytes were calculated and are given in Table 2. The linearity of the present method for the analytes is

Table 1
Elution times and standard deviation of elution time and area for the various SCFA ($n=18$)

SCFA	RSD (%) ($n=18$)		
	t.m.[a] (mn)	t.m.	Area
Succinic	5.73	0.43	0.92
Pyruvic	6.14	0.45	2.52
Acetic	6.29	0.46	2.50
Lactic	6.64	0.49	0.93
Propionic	6.71	0.52	1.12
2-Hydroxybutyric	6.92	0.52	1.17
Butyric	6.99	0.56	2.66
2-Hydroxyvaleric	7.13	0.56	0.48
Isovaleric	7.21	0.57	0.76
Isocaproic	7.44	0.58	1.31
3-Phenylpropionic	7.76	0.56	2.20

[a] t.m.=migration time.

Table 2
Calibration datas of the SCFA ($n=6$), regression equations of the calibrations, where (x) is the concentration, (y) the area, and R the linear regression coefficient

Short-chain fatty acids (SCFA)	Regression equation	r
Succinic	$y=234.78x-797.28$	0.9998
Pyruvic	$y=19.50x-34.01$	0.9998
Acetic	$y=178.67x-250.65$	0.9996
Lactic	$y=50.76x-108.85$	0.9997
Propionic	$y=91.58x-28.67$	0.9999
2-Hydroxybutyric	$y=69.20x-170.01$	0.9996
Butyric	$y=71.26x-8.65$	0.9999
2-Hydroxyvaleric	$y=35.67x+61.53$	0.9998
Isovaleric	$y=76.47x-49.39$	0.9998
Isocaproic	$y=69.73x-35.08$	0.9999
3-Phenylpropionic	$y=34.71x+39.27$	0.9999

excellent, with correlation coefficients better than 0.9990.

3.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was evaluated from eight independent blanks, which were spiked to produce a peak height, close to three times the baseline noise for each of the analyte anions, which was determined as described by Romano and Krol [18]. The LOD was estimated by taking three times the standard deviation of the peak areas obtained from these solutions and calculating the corresponding concentrations from the calibration lines (Table 3). Each acid was analyzed separately.

The LOQ was defined as the minimal level at which the measurement precision is satisfactory for quantitative analysis. It was estimated by taking ten times the standard deviation of the peak areas obtained from the eight blanks and subsequently calculating the corresponding concentrations from the calibration lines (Table 3).

The LOD were from 0.02 to 2.75 mg/l and the LOQ were from 0.08 to 9.16 mg/l with respect to the 11 different anions. LOD and LOQ could probably be improved by the injection of larger volumes or more concentrated samples.

The LOQ/LOD ratios for all the ions are quite constant and equal to about 3.

Table 3
Limits of detection (LOD) and quantification (LOQ) of the SFCA

Short-chain fatty acids (SCFA)	LOD (mg/l)	LOQ (mg/l)
Succinic	0.23	0.76
Pyruvic	2.75	9.16
Acetic	0.02	0.08
Lactic	0.77	2.55
Propionic	0.37	1.23
2-Hydroxybutyric	0.61	2.05
Butyric	0.46	1.52
2-Hydroxyvaleric	0.87	2.88
Isovaleric	0.59	1.97
Isocaproic	0.53	1.77
3-Phenylpropionic	0.79	2.59

3.4. Analysis of TGY culture media of anaerobes

As an application of CE–indirect UV to SCFA studies, concerning taxonomic interest, we have examined TYG culture media obtained from different anaerobic strains.

As a first step, we have determined the composition in SCFAs of TGY culture media of type and reference strains corresponding to 17 defined anaerobic species (and subspecies), including particularly 10 Bacteroidaceae. Table 4 presents these reference taxonomical data (expressed in mM) obtained using CE–indirect UV detection. Fig. 2 is an electropherogram of *Fusobacterium nucleatum subsp. nucleatum* CIP 101130 T. Among Gram negative anaerobic bacilli, fusobacterial strains are characterized by their high production of butyric acid and absence or low production of succinic, pyruvic, isovaleric, isocaproic and 3-phenylpropionic acids. Some differences are observed in SCFA production by the four *Fusobacterium nucleatum* type strains, but their use for identification at the subspecies level can not be concluded. *P. gingivalis* CIP 103683T electrophoregram is characterized by the detection of high level of isovaleric acid. *B. fragilis* CIP 77.16T and *C. ochracea* CIP 82101 produce a mixture of succinic, pyruvic, acetic and lactic acids with a particular high level of succinic acid (with respect to *C. ochracea* CIP 82101) and no or poor levels of propionic and butyric acids.

Gram positive reference bacilli present specific electrophoregrams. Despite this, among the lac-

Table 4
Concentration of SCFA (m*M*) for the tested references anaerobes

SCFA (m*M*) / Species	Succinic	Pyruvic	Acetic	Lactic	Propionic	2-Hydroxybutyric	Butyric	2-Hydroxyvaleric	Isovaleric	Isocaproic	3-Phenylpropionic
A. israelii	1.56	0	15.09	58.21	0	0	0	0	0	0	0
A. odontalis	1.66	9.07	2.04	36.68	0	0	0	0	0	0	0
A. propionica	1.91	0	15.97	44.38	0	0	0	0	0	0	0
B. fragilis	2.23	4.63	4.76	12.99	1.43	0	1.11	0	0	0	0
C. ochracea	35.95	5.26	20.57	2.51	0	0	0	0	0	0	0
C. perfringens	1.85	0	23.99	6.54	2.84	2.13	26.85	10.39	0	0	0
F. russii	0.10	0	10.72	16.81	0	0	25.68	7.59	0	0	0
F. gonidioformans	0.13	0	1.98	26.96	0.38	4.27	54.44	2.15	0	0	0
F. naviforme	0.55	0	5.26	36.63	0	0	34.2	0	0	0	0
F. necrophorum	0.21	0	10.09	1.05	3.34	2.3	52.38	3.59	0	0	0
F. nucleatum subsp. vincentii	0.05	0	8.95	0	4.96	0	42.8	21.3	0	0	0
F. nucleatum subsp. fusiforme	0	0	6.74	16.89	0	0	55.37	0	0	0	0
F. nucleatum subsp. polymorphum	0	0	9.6	27.73	0	0	49.58	0	0	0	0
F. nucleatum subsp. nucleatum	0	0	10.98	11.6	3.62	0	60.11	0	0	0	0
L. acidophilus	0.15	0	0.02	28.63	3.34	5.35	0	0	0	0	0
L. gallinorum	0.16	0	0	25.26	2.48	5.01	0	0	0	0	0
L. johnsonii	0.18	0	0	24.95	3.67	3.96	0	0	0	0	0
P. gingivalis	0	0	16.20	2.28	3.02	0	38.54	0	11.22	0	0
P. melaninogenica	10.04	0	9.16	19.22	0	0	0	0	5.58	0	0
P. acnes	2.28	8.57	4.59	44.31	7.18	0	0	0	0	0	0
P. anaerobius	0	0	21.85	5.84	0	0	10.60	0	6.08	20.29	7.19
Medium control	1.93±0.06	0	3.02±0.06	2.83±0.30	0.77±0.07	0	6.14±0.15	0	0	0	0

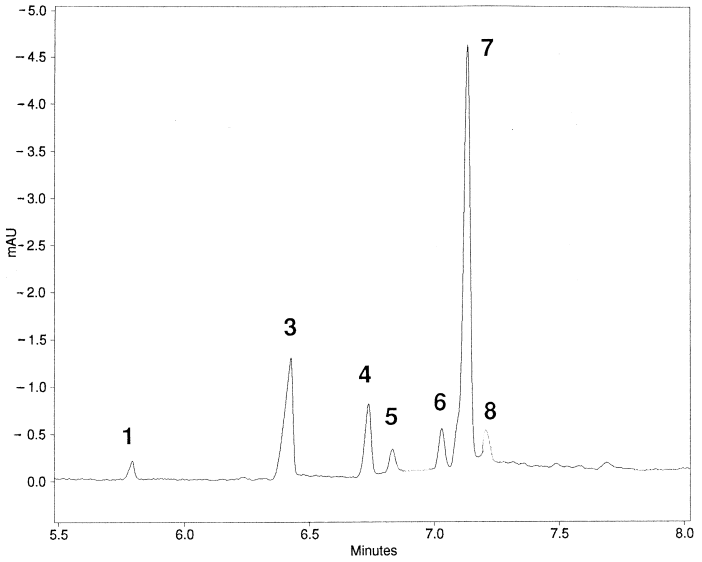

Fig. 2. Electropherogram of the short-chain fatty acids of *Fusobacterium nucleatum subsp.* nucleatum CIP 101130 T. Migration order (peaks): (1) succinic acid, (3) acetic acid, (4) lactic acid, (5) propionic acid, (6) 2-hydroxybutyric acid, (7) butyric acid, (8) 2-hydroxyvaleric acid.

tobacilli reference strains, it appears that differentiation at the species level cannot be performed using CE analysis alone.

In a second step, electrophoregrams (or the obtained quantitative data) of anaerobes from human clinical isolates were analyzed. Table 5 shows the analytical data of the 98 tested strains corresponding to ten different anaerobic species preliminary identified on the basis of classical biochemical tests [4,22]. We present the means and the standard deviations of the molar concentrations of the different SCFAs produced by the tested anaerobes. Fig. 3 presents the graphic results of Table 5. We see that the histograms of the seven genus are very different and that the concentrations of the acids may be of taxonomic interest in the identification of the genus and/or the species which were studied.

Among the different species, we confirm that *C. ochracea* has very large amount of succinic, acetic and lactic acids, while the other acids are at very low concentrations or not detected. *F. nucleatum* produces a very high concentration of butyric acid, with concentrations below 10 m*M* for acetic, propionic and 2-hydroxyvaleric acids and very low quantities of the other acids. It differs strongly from *P. gingivalis*, because of the presence of isovaleric acid in the culture media of this bacteria. *B. fragilis* and *L. acidophilus* have quite similar histograms, but *L. acidophilus* displays the presence of 2-hydroxybutyric acid (5 m*M*), which is absent in the other bacteria. *P. intermedia* and *P. melaninogenica* have similar SCFA profiles with different concentrations. They are very low for *P. intermedia* and approach the concentrations of these acids seen in the media, while *P. melaninogenica* has succinic, acetic and lactic acids in concentrations higher than 10 m*M*. Moreover, 2-hydroxyvaleric acid is not detected in the culture media of *P. melaninogenica*.

The quantities of the different acids in a genus could be used to identify a species as it has been done before using GC and mass spectrometry [3,25].

4. Discussion

Considering our results, some differences with the values obtained by GC are to be noted. Three reasons can be given to such differences: (i) it is well known that culture conditions influence greatly the quantitative results (culture medium, growth duration) [1]. Our culture conditions may be different from the other authors (they do not describe precisely their culture media). We did not supplement the culture medium with hemin, which is known to modify the concentrations of the different SCFA in the culture media [26–28]; (ii) in our method we do not use any extraction or derivation steps which can modify the quantities detected; (iii) no loss of volatile acids takes place in CE as compared to GC where the volatility of some acids is comparable to the volatility of the solvent which may induce a partial loss of the sample in the injector. We wanted to compare the obtained SCFAs with our culture media (without hemin) and the culture media which are used elsewhere. We quantitated succinic acid in culture media of ten strains of *Bacteroides fragilis* supplemented with hemin or not. We observed a ratio of 14.6±10.7 between the medium containing hemin and the same one without hemin. It confirms the results of Macy et at. [27]. Fig. 4 presents some differences recorded between the GC and CE analytical methodologies. A lack of succinic acid de-

Table 5
Concentrations of SCFA (m*M*) for the tested clinical anaerobes

SCFA (m*M*) Species	Strains	Succinic	Pyruvic	Acetic	Lactic	Propionic	2-Hydroxybutyric	Butyric	2-Hydroxyvaleric	Isovaleric	Isocaproic	3-Phenylpropionic
B. fragilis	17	1.88±1.11	1.62±2.30	3.98±2.23	15.96±20.20	1.97±2.37	0	0.59±0.77	0	0	0	0
C. ochracea	18	42.28±14.79	4.68±4.09	27.68±7.80	27.36±72.21	0	0	0	0	0	0	0
F. nucleatum	29	0.06±0.07	0	8.45±2.39	0.45±1.35	7.65±5.64	0.52±.07	47.97±7.94	3.08±3.58	0	0	0
L. acidophilus	7	0.09±0.10	0	0	30.39±9.09	2.90±0.27	5.11±0.30	0	0	0	0	0
P. gingivalis	6	0.24±0.28	0	8.74±2.54	2.38±1.12	2.35±2.64	0	24.35±7.50	1.72±0.75	8.06±3.41	0	0
P. melaninogenica	3	14.95±2.92	1.77±3.07	15.60±6.71	10.02±2.82	4.37±4.64	0	1.38±2.05	0	1.59±0.50	0	0
P. intermedia	15	4.78±4.95	0	3.76±3.11	0.92±1.73	4.54±3.49	0	2.73±6.42	1.37±3.73	1.48±2.97	0	0

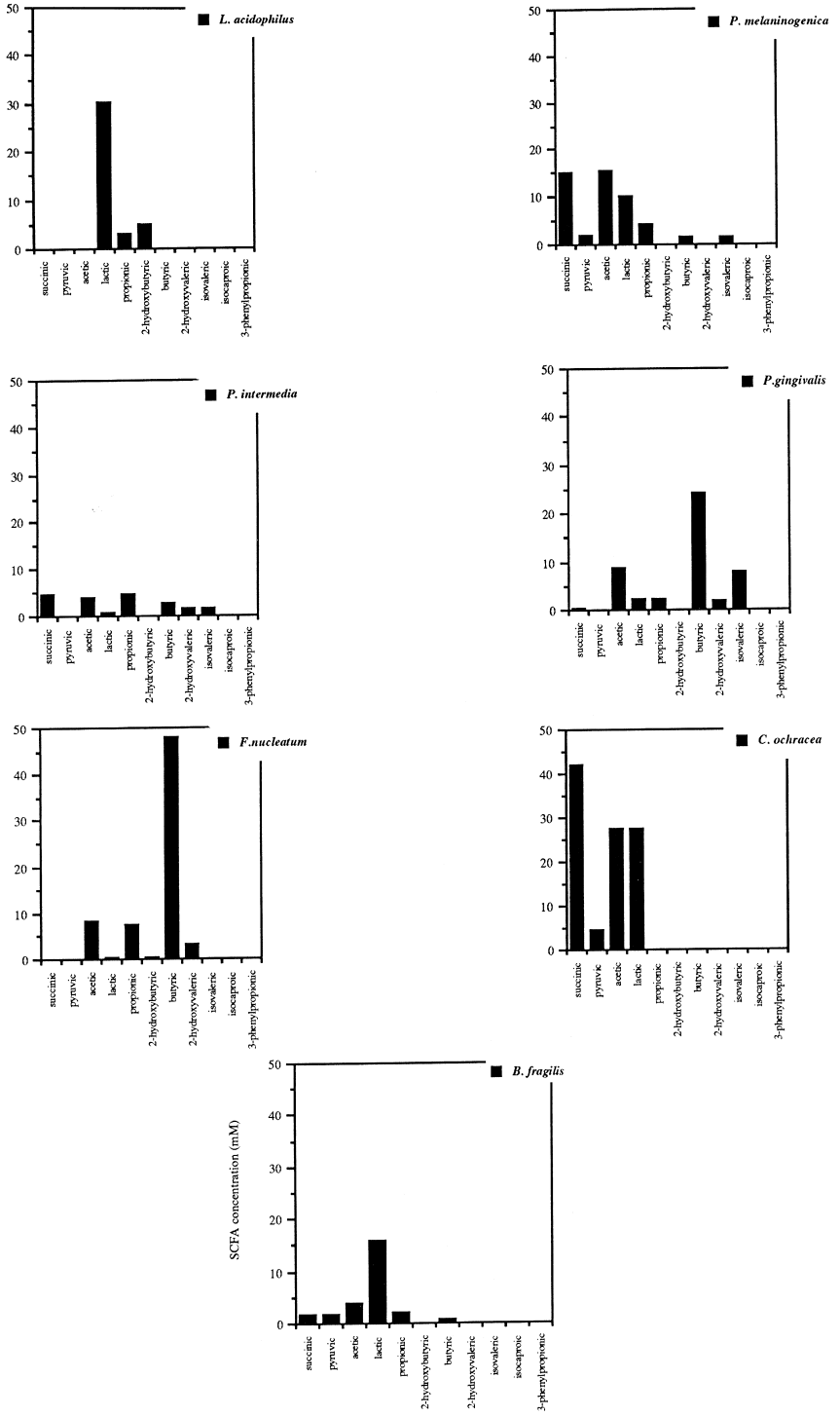

Fig. 3. Histograms presenting the concentrations of the different SCFAs for the tested references anaerobes.

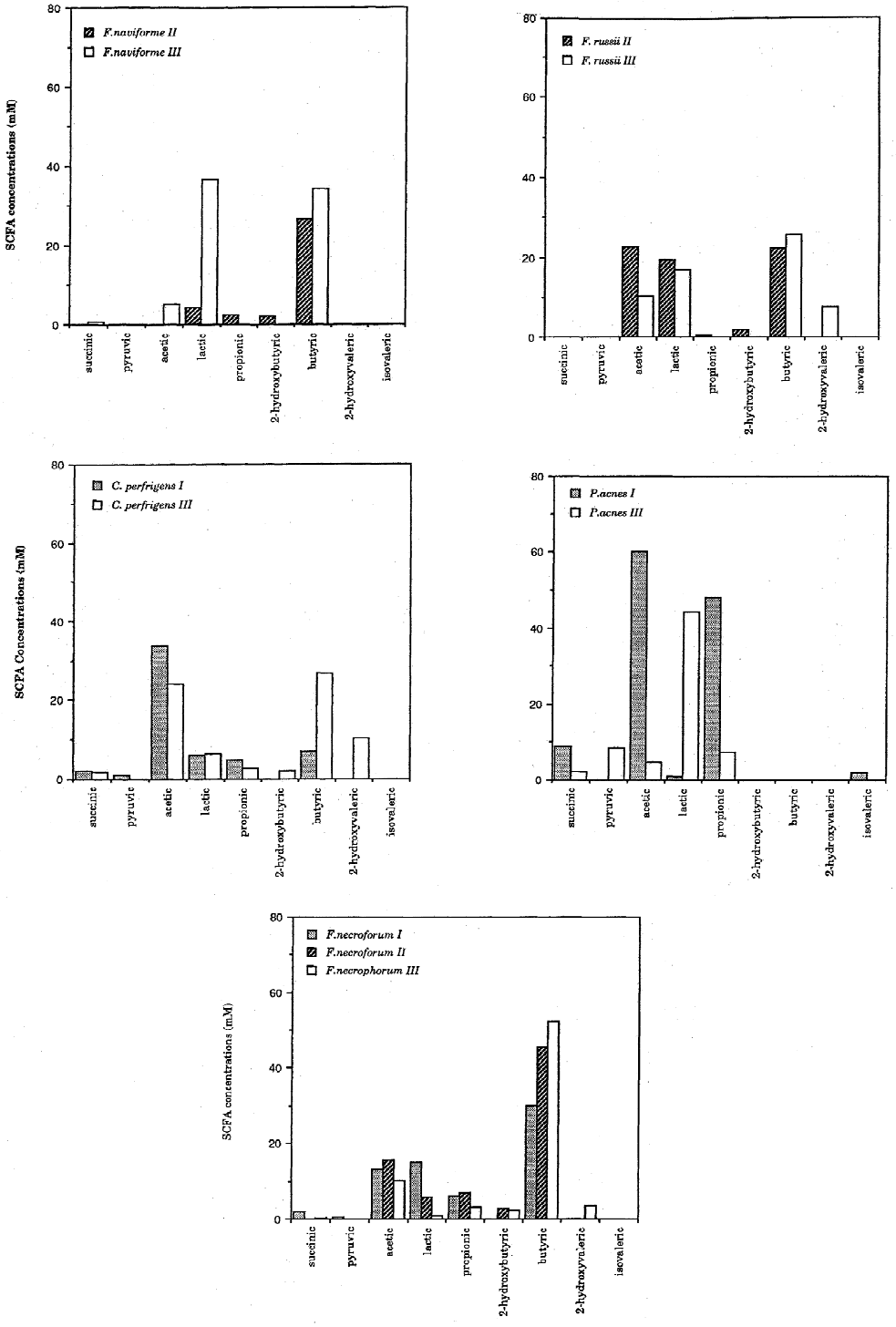

Fig. 4. Comparison of the SCFAs quantitated by different authors. I is Ref. [1], II is Ref. [25], III is our work.

tected by our new method is noteworthy, particularly for *B. fragilis*. This is probably due to the lack of the hemin in the culture media. Despite this, the majority of the strains studied were able to produce succinic and/or butyric acids, as reported earlier [29] which is considered characteristic of anaerobes. Acetic and lactic acids are present at very different concentrations between the studied genus and/or species and may have high values in the range of 58 m*M* for lactic acid (*L. acidophilus* and *A. propionica*) and 21 m*M* for acetic acid (*C. ochracea* and *P. anaerobius*). In some instances they cannot be detected. Pyruvic, propionic, 2-hydroxybutyric, succinic and isovaleric acids have lower values or are not present in the culture media and may be considered as characteristic of some genera. Isocaproic and 3-phenylpropionic acids which where described to be characteristic of *Clostridium* [30], were not identified in *C. perfringens*, culture medium but only for *P. anaerobius*.

GC analysis of metabolic end-products has been preliminary presented as of taxonomic interest for classification of the family bacteria [9] and in identification at the *Fusobacterium* species level [25], especially, when the analysis of volatile acids and non-volatile acids including 2-hydroxy acids [3,11] are concerned.

We particularly studied the SCFA production of Gram negative bacilli including different species of Fusobacteria and subspecies of *Fusobacterium nucleatum*. In the current taxonomic system, Fusobacteria are distinguished from Bacteroides, Prevotella Porphyromonas and Leptotrichia by their production of butyric acid from carbohydrate and peptone sources. In contrast to the few Bacteroidaceae, which also produce butyric acid, the metabolite end products of *Fusobacterium* do not include isobutyric or isovaleric acids. Among Gram negative bacilli, only some Fusobacteria were able to produce 2-hydroxybutyric acid. According to this last observation, the tested Bacteroides species (B. fragilis) and Prevotella and Porphyromonas (*P. gingivalis, P. melaninogenica* and *P. intermedia*) do not synthesize 2-hydroxybutyric acid.

The *Fusobacterium* genus comprises non-fermentative or weakly fermentative strains for which only a few reliable biochemical tests are available for their identification. Among those non-gas-producing, including *F. nucleatum, F. russii* and *F. naviforme*, the production of 2-hydroxybutyric and 2-hydroxyvaleric acids was noted by Carlier [25]. In spite of this, the production of 2-hydroxybutyric and 2-hydroxyvaleric acids cannot be used to identify strains to the species level because of the variable amounts detected, especially in the case of *F. nucleatum* (e.g. type strains and clinical isolates). Among 29 *F. nucleatum* clinical isolates, whose culture media were tested, 2-hydroxybutyric was not detected in 23 samples and 2-hydroxyvaleric acid was not found in 11 samples. Theses results indicates that the identification of hydroxy acid cannot be used as a discriminant test.

The tested Fusobacteria type strains able to produce gas (e.g. *F. gonidiaformans* and *F. necrophorum*) are characterized by the production of 2-hydroxybutyric acid. They can be differentiated by comparing the level of acetic acid production (respectively 1.98 and 10.09 mol/l). According to these observations, the complete characterization of some Fusobactenum species needs further classical biochemical tests such as indole and lipase detection.

If we consider the subspecies classification (OGR/GDH electrophoretic migration) of the 29 clinical isolates of *F. nucleatum*, SCFA analysis does not discern great differences. Despite this, we have to point out the possible correlation between the predominance of *F. nucleatum subsp. vinventii* and secondly of *F. nucleatum subsp. nucleatum* (20/29 strains) and the predominance of bacteria which do not produce lactic acid, among clinical isolates (22/29 strains). This observation needs further experimentation.

Hydroxylated acids are of great interest in identifying other species. In this way, 2-hydroxybutyric acid is also produced by Clostridia (i.e. *C. perfringens*) and Lactobacilli. In this latter case, the different CE profiles observed between *L. gallinarum, acidophilus* and *johnsoni* give rise to a specific electrophoregram of *L. acidophilus* complex strains without distinction of the three cited Lactobacilli. This observation was never mentioned before.

Considering variations observed on data from clinical isolates, some SCFA especially acetic, propionic, succinic, pyruvic acids are unique in the differentiation of some anaerobes at the genus level (e.g. Gram positive bacilli). They also allow one to confirm the identification of some strains at the

species level, e.g. differentiation of *P. melaninogenica* and *P. intermedia* among the saccharolytic black-pigmented Bacteroidacae.

Among the classical chromatographic methods used in the taxonomic area, gas-liquid chromatography of fatty acids methyl esters is widely used [10]. The introduction of the Microbial Identification System (MIS) has enable personnel in diagnostic laboratories to perform cellular fatty acid analyses on a variety of microorganisms [31–33]: A gas chromatographic method using a capillary column is used to perform the analysis of cellular fatty acids (derivatized as methyl esters) and the obtained chromatographic profiles are automatically searched against reference profiles stored in a computer-generated library. Some authors used the MIS for anaerobes identification ([34,35], for a chemotaxonomic review on anaerobes see [36]). Moreover, the GC study of hydroxylated (trimethylsilyl derivatives) and non-hydroxylated long chain fatty acid methyl esters was described by Calhoon [37]. This method led to differentiation of anaerobes on the basis of *n*-C16:O to 3-OH-C16:O ratio [38] and 3-hydroxy isoheptadecanoic acid [39]. This last method was not computerized.

Long chain fatty acids (hydroxylated and non-hydroxylated) were recently studied using CE [40,41] and we are developing the use of this methodology to achieve anaerobic identification as well as using SFCA and these acids.

Another interesting point regarding the use of capillary electrophoresis and indirect UV detection to quantitate SCFA or long chain fatty acids of anaerobes, is the cost of one analysis (0.04$ per analysis including usable and fused-silica capillary, the price of an instrument is around 35,000$). These analytical means can be used routinely in the clinical laboratories.

5. Abbreviations

BGE	background electrolytes
CE	capillary electrophoresis
EOF	electroosmotic flow
GDH	glutamate deshydrogenase
MIS	microbial identification system
OGR	2-oxoglutarate reductase
SCFA	short-chain fatty acids
TGY	tryptone–glucose–yeast
TTAB	tetradecyltrimethylammonium bromid

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