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Journal of Chromatography A, 781 (1997) 497–501

JOURNAL OF CHROMATOGRAPHY A

Short communication

Capillary electrophoresis and indirect UV detection as a fast and simple analytical tool for bacterial taxonomy

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Abstract

Short-chain methylated fatty acids are difficult to study using conventional techniques such as gas chromatography, because of their high volatility. Moreover, the treatment of the samples is long and fastidious. Fatty acids are very interesting substances for the characterization of anaerobic bacteria. In this work we develop a new fast and simple method using capillary electrophoresis and indirect UV detection for the analysis of different short-chain fatty acids. We analyzed culture media of different anaerobic bacteria and found some concentration patterns which are discriminant at the species level. Prior to analysis the culture media were simply filtrated and diluted. © 1997 Elsevier Science B.V.

Keywords: Fatty acids

1. Introduction

The bacterial taxonomy needs many various investigation procedures based on contents characterization (sugars, quinones, DNA, etc.) and enzymatic functions [1]. It is also well known that fatty acids have an important taxonomic part [2]. In this way, the detection and the quantitation of short-chain fatty acids (SCFA) produced during fermentation in culture media is routinely used by many laboratories to confirm anaerobic bacteria identification [3,4].

Usually, short-chain hydroxylated or non-hydroxylated fatty acids are studied using gas chromatography (GC) or GC–mass spectrometry (GC–MS). Therefore, the very important volatility of the non-hydroxylated SCFA esters makes GC quantitation of these compounds not easy. A GC study of volatile SCFA (free acids) and another GC study of methyl or butyl esters are needed, inducing time-consuming sample preparations and several GC runs [3,4].

Capillary electrophoresis (CE) is now a well known analytical technique [5,6]. It allows without specific sample preparation to detect and to quantify concentration levels down to 1 μmol l^{-1} [6].

Numerous studies presented organic acids characterization by capillary electrophoresis and indirect UV detection or conductimetry [7,8]. However, to our knowledge, very few applications of electromigrative analysis have been performed to quantify organic acids produced by microorganisms. Microcapillary isotachophoresis and conductimetry were solely used in such a way [9]. They concerned SCFA bacterial production on vegetal substrates

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0021-9673/97/$17.00
PII S0021-9673(97)00310-5

without being extended to the identification field of clinical isolates.

In this work we propose a new bacterial taxonomic analytical method to identify SFCA in culture media using CE and indirect UV detection.

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) and from the 'Collection des Anaérobies de l'Institut Pasteur' (AIP, Paris, France): *Fusobacterium nucleatum* subsp. *nucleatum* CIP 101130 T, *Fusobacterium necrophorum* AIP 10145 T, *Porphyromonas gingivalis* CIP 103683 T, *Bacteroides fragilis* CIP 77.16 T, *Capnocytophaga ochracea* CIP 82.101, *Propionibacterium acnes* CIP 53.117 T, *Peptostreptococcus anaerobius* AIP 101102 T. *Prevotella melaninogenica* and *Clostridium perfringens* were clinical isolates and were identified according to the described procedures [1,5]. Organisms were maintained anaerobically (Anaerobic chamber, Bioblock Scientific) on Columbia blood agar (Biomérieux). Before each experiment, bacteria were transferred on TGY broth (Diagnostics Pasteur, Paris, France). 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 MΩ water (C2R, Montgiscard, France). Solutions of succinic (1.004 g l^{-1}), pyruvic (1.038 g l^{-1}), acetic (1.076 g l^{-1}), lactic (1.020 g l^{-1}), propionic (1.036 g l^{-1}), 2-hydroxybutyric (1.010 g l^{-1}), butyric (1.032 g l^{-1}), 2-hydroxyvaleric (1.000 g l^{-1}), isovaleric (1.122 g l^{-1}), isocaproic (1.008 g l^{-1}) and 3-phenylpropionic (1.036 g l^{-1}) 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) and histidine (Aldrich, St. Quentin Fallavier, France) which were the background electrolytes (BGE) and were prepared as 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)]. Tetradecyltrimethylammoniumbromide (TTAB) (Aldrich) was employed as electroosmotic flow (EOF) modifier and its concentration was 1 m*M*.

2.3. Apparatus

An Europhor CE system (Dual Impact, Europhor Instruments is now Zeta Technology 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 μm I.D. fused-silica capillary column (Thermo Separation Products, Freemont, CA, USA). 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 μA, 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; 1 p.s.i.=6894.76 Pa) for 2 s. Injection volumes measure 4 nl s^{-1} for a 75 μm I.D. capillary. The injection end of the capillary dips for 1 cm in a 1.5 ml vials (Thermo Separation Products) filled with 1 ml sample.

The migration time of electroosmotic flow was determined using water injection. Electrophoregrams were recorded and processed with a PC 1000 data acquisition system (Thermo Separation Products).

2.4. Sample preparation

The sample dilution range was 0.76–502 mg l^{-1} for succinic acid, 9.16–259.50 mg l^{-1} for pyruvic acid, 0.08–519 mg l^{-1} for acetic acid, 2.55–255 mg l^{-1} for lactic acid, 1.23–259 mg l^{-1} for propion-

ic acid, 2.05–252.50 mg l^{-1} for 2-hydroxybutyric acid, 1.52–258 mg l^{-1} for butyric acid, 2.88–250 mg l^{-1} for 2-hydroxyvaleric acid, 1.97–280.50 mg l^{-1} for isovaleric acid, 1.77–252 mg l^{-1} for isocaproic acid and 2.59–259 mg l^{-1} for 3-phenylpropionic acid.

3. Results and discussion

The SCFA of taxonomic interest to be detected in culture media are presented in Table 1. They can be distributed in three categories, non-hydroxylated and non-keto mono acids, hydroxylated and keto mono acids, diacid.

The electrophoretic mobility μ can be written as $\mu \equiv Z/\sqrt{M}$ [10]. It means that the mobilities of the acids are dependent on the number of carboxylic groups, on the pK_a values which are influenced by hydroxy and/or keto groups and on the molecular mass. For the non-hydroxylated and non-keto mono acids, the mobilities decrease when the molecular mass increase. Hydroxylated and keto mono acids are eluted following their pK_a values and molecular mass. Succinic acid has two charges and migrates first.

A typical electropherogram of a tested bacteria is given in Fig. 1. Identification of short-chain fatty acids was performed according to the electopherogram of a mixture of the SCFA (in broth medium) (Fig. 2). The products were identified by comparison

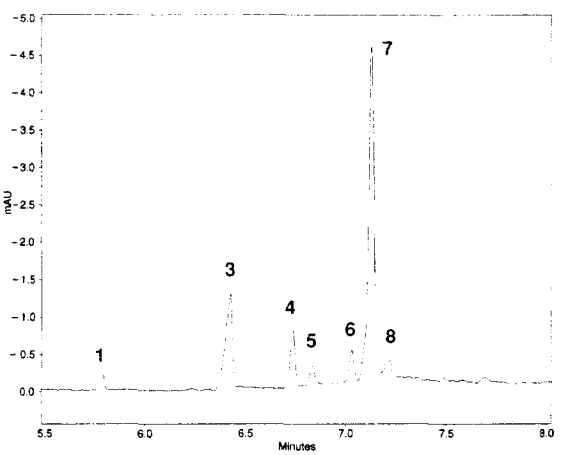

Fig. 1. Electropherogram of the short-chain fatty acids of *Fusobacterium nucleatum* subsp. *nucleatum* CIP 101130 T. Migration order (peaks): (1) succinic acid, (3) acetic acid, (4) lactic acid, (5) propionic acid, (6) 2-hydroxybutyric acid, (7) butyric acid, (8) 2-hydroxyvaleric acid.

with the reference samples and by measurement of their retention time. The detection (absence or presence) of SCFA is of primary importance in the identification scheme of anaerobic bacteria. Table 2 presents the mean value in quantity (mM l^{-1})±standard error calculated for each tested strain, on three experiments. Values of medium control are also given. Considering these, some SCFA productions are to be considered as non-significant, especially production of succinic acid in

Table 1
Formula, elution times, mobilities and pK_a values of SCFA found in culture media using CE and indirect UV detection

	Name of SCFA	Formula	Elution time (min)	Mobility (cm^2 s^{-1} V^{-1})	pK_{a1}	pK_{a2}
Nonhydroxylated and non-keto mono-acids	Acetic	CH_3–COOH	6.32	57.64	4.75	
	Propionic	CH_3–CH_2–COOH	6.71	54.29	4.87	
	Butyric	CH_3–$(CH_2)_2$–COOH	6.99	52.12	4.81	
	Isovaleric	$(CH_3)_2$–CH–CH_2–COOH	7.21	50.53	4.77	
	Isocaproic	$(CH_3)_2$–CH–$(CH_2)_2$–COOH	7.44	48.96	4.84	
	3-Phenylpropionic	C_6H_5–$(CH_2)_2$–COOH	7.76	46.94	4.37	
Hydroxylated and keto mono-acids	Pyruvic	CH_3–C(O)–COOH	6.16	59.14	2.49	
	Lactic	CH_3–CH(OH)–COOH	6.64	54.86	3.08	
	2-Hydroxybutyric	CH_3–CH_2–CH(OH)–COOH	6.92	52.64	4.70	
	2-Hydroxyvaleric	CH_3–$(CH_2)_2$–CH(OH)–COOH	7.13	51.09	4.75	
Diacid	Succinic	HOOC–$(CH_2)_2$–COOH	5.73	63.58	4.21	5.63

Table 2
Concentrations of SCFA (mmol l^{-1}) for the tested anaerobes

Species	Succinic	Pyruvic	Acetic	Lactic	Propionic	2-Hydroxy-butyric	Butyric	2-Hydroxy-valeric	Iso-valeric	Isocap-roic	3-Phenyl-propionic
B. fragilis	4.13±0.18	0	13.34±0.73	29.56±1.87	3.65±0.23	0	0	0	4.32±0.33	0	0
C. ochracea	8.19±0.43	9.79±0.23	5.92±3.30	3.21±0.21	0	0	0	0	10.09±0.78	0	0
F. necrophorum	1.90±0.01	0	12.87±1.44	31.96±1.07	13.42±0.72	1.96±0.01	52.86±1.54	5.33±0.19	0	0	0
F. nucleatum	1.90±0.00	0	14.70±0.17	15.66±0.04	3.09±0.06	4.98±0.14	69.02±1.14	1.24±0.03	0	0	0
P. gingivalis	0	0	16.20±0.69	2.28±0.02	3.02±0.28	0	38.54±1.70	0	11.22±0.52	0	0
P. melaninogenica	10.04±0.16	0	9.16±0.77	19.22±0.53	0	0	0	0	5.58±0.02	0	0
P. acnes	2.28±0.06	8.57±0.04	4.59±0.05	44.311±3.40	7.18±0.59	0	0	0	0	0	0
P. anaerobius	0	0	21.85±0.47	5.84±0.10	0	0	10.60±0.35	0	6.08±0.27	20.29±1.11	7.19±0.22
C. perfringens	1.85±0.02	0	23.99±0.52	6.54±0.98	2.84±0.12	2.13±0.06	26.85±0.05	10.39±0.19	0	0	0
Medium control	1.86±0.02	0	3.90±0.17	3.50±0.07	0	0	7.73±0.45	5.84±0.64	0	14.49±0.83	0

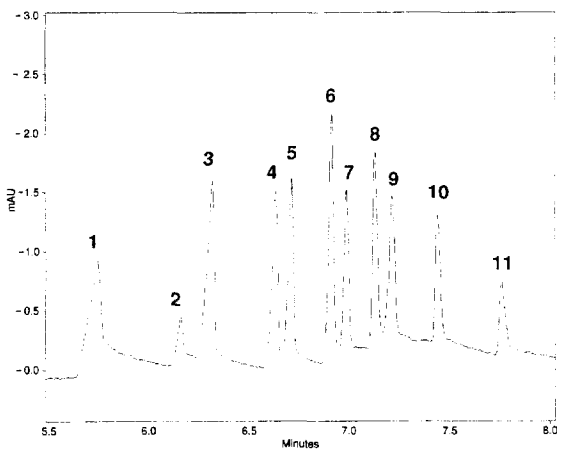

Fig. 2. Electropherogram of a mixture of the short-chain fatty acids in 10 m*M* BA–10 m*M* histidine–1 m*M* TTAB at pH 6.0. Migration order (peaks): (1) succinic acid, (2) pyruvic acid, (3) acetic acid, (4) lactic acid, (5) propionic acid, (6) 2-hydroxybutyric acid, (7) butyric acid, (8) 2-hydroxyvaleric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

culture media of *F. necrophorum*, *F. nucleatum* and *C. perfringens*. The registered concentrations are in the range of the one previously observed using conventional methods such as GC [11]. These bacteria expressed only small differences in redox potential between substrates and terminal products, resulting in accumulation of the latter in the medium. These end products are constant for a given species, and are designated as 'metabolic type'. The electropherograms of the Gram positive bacilli tested (i.e. *Clostridium perfringens* and *Propionibacterium acnes*) demonstrated very well the diversity in acid fermentation products, as well as the specificity of this production.

In the same way, the quantitation of SCFA in culture media was considered as discriminant at the species level for some genus or subgroups expressing poor biochemical characteristics (i.e. *Fusobacterium*) [3,12].

If conditions of sample preparation and analysis were optimized during this first study, further works have to be performed. Application of CE analysis in the field of anaerobic bacteriae identification has to be validated on a more extended number of strains for each of the tested strains.

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