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Short communication

# Short chain fatty acids analysis by capillary electrophoresis and indirect UV detection or laser-induced fluorescence

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**Abstract**

Short chain fatty acids are difficult to study using conventional techniques such as gas chromatography, because of their high volatility. Capillary electrophoresis (CE) and UV detection or conductimetry was used to study fatty acids of 6 carbon lengths or less. Difficulties in dissolving longer acids in aqueous buffer prevented analysis by CE. Recently indirect laser-induced fluorescence was used to study $C_6$ to $C_{18}$ fatty acids and the sensitivity of the detection was in the sub-micromolar range. In this paper we studied $C_5$ to $C_{18}$ branched, hydroxy or linear fatty acids using CE and UV indirect detection and we succeeded in obtaining good separations but with very poor sensitivity (limited to $10^{-5}$ *M*). In a second attempt we studied fatty acids after 5-bromomethylfluorescein derivation and analysis by CE and laser-induced fluorescence (LIF). The sensitivity was in the sub-nanomolar ($10^{-10}$ *M*) range but we could only study $C_8$ to $C_{11}$ fatty acids. Using CE–LIF, we quantitated these acids in normal and pathological serae. 

*Keywords:* Fatty acids

## 1. Introduction

Short chain fatty acids (SCFAs) are usually studied using gas chromatography (GC) of their butyl or methyl esters derivatives [1], or using high-performance liquid chromatography (HPLC) and UV detection of their 1-napthylamine or *p*-bromophenacylbromide derivatives [2,3]. The GC analysis of such volatile samples necessitates the need for time consuming sample preparation and a methylation or butylation step prior to analysis [4] and is not compatible with a great number of injectors (e.g., Ross injector). These molecules are quite difficult to analyze using capillary electrophoresis (CE), because of the poor solubility and the absence of a chromophore function. Most of studies on this topic used micellar electrokinetic chromatography (MEKC) [5] or isotachophoresis [6]. In most cases indirect UV detection [7] or conductimetry [8] were used. The literature suggests that very little work was conducted using CE to study fatty acids ranging from 6 to 15 carbon atoms in length. To our knowledge the work of Desbène and coworkers using CE and indirect laser-induced fluorescence was the only work on this topic [9,10]. They used sodium fluorescein salt as background signal generator, and a 5 m*M* borate, pH 9.2–ethanol (60:40, v/v) or 5 m*M* borate, pH 9.2 with 0.2 m*M* of hexamethyleneglycolmono-

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*n*-dodecyl ether buffers as electrolytes. The sensitivity of the detection in each case was around $5 \cdot 10^{-6}$ *M* for each acid. No work on small chain fatty acids or on more sensitive analysis of SCFA using CE–laser-induced fluorescence detection (LIF) has been reported.

SCFAs are associated to some pathologies as deficiencies of medium chain AcylcoA deshydrogenase, which are usually responsible of sudden infant death. The principal acids implied in this pathology are octanoic and decanoic acids. The symptoms are very different from patient to the patient and include nausea, hypoglycemia and vomiting. These symptoms could interfere with the diagnosis and therefore supports the analysis of SCFAs [11].

Because CE is well adapted to very small volume samples, it is well adapted to biological sample analysis. In this study we first looked for $C_5$ to $C_{18}$ hydroxylated, branched or linear fatty acids using CE and UV–indirect detection and we succeeded in obtaining good separations but with very poor sensitivity. In a second attempt we were looking for fatty acids after 5-bromomethylfluorescein derivation and analysis by CE and LIF. This dye was previously well studied for the analysis of palmitic acid in HPLC–LIF [12] and for the detection of cefuroxine and its carboxyl-containing analyte using LIF [13]. We developed an analysis using MEKC and LIF to detect some linear or branched SCFAs ranging from $C_8$ to $C_{11}$ carbon atoms to analyse microliter volumes of serae from patients.

## 2. Experimental

### 2.1. Materials

A Dual Impact capillary electrophoresis integrated instrument (Europhor Instruments, now Zeta Technology, Toulouse Ramonville, France) was used for the CE–indirect UV application. A fused-silica capillary of 50 μm I.D.×320 μm O.D. (Polymicro Technology, Phoenix, AZ, USA) and of 42 cm effective length with 49 cm of total length was fitted on the cassette.

A modular injector and a high voltage power supply Prime Vision 1 (Europhor Instruments, now Zeta Technology), equipped with a modular LIF detector for capillary (Zeta Technology) and a 488 nm wavelength laser (532 MBS, 25 mW, Omnichrome, CA, USA) were used for CE–LIF studies. The capillary was a 75 cm×50 μm I.D.×375 μm O.D. fused-silica capillary (Polymicro Technology) and has an effective length of 42 cm.

For CE–indirect UV or CE–LIF experiments a 0.5 cm length detection window was made by burning the polyimide coating.

All chemicals were supplied by Aldrich (St. Quentin Fallavier, France). 12-Hydroxystearic acid was kindly provided by Professor J. Asselineau (CNRS, Toulouse, France).

### 2.2. SCFAs and CE–indirect UV detection

$C_5$ to $C_{18}$ acids were dissolved in 2 ml of a mixture of water–ethanol (8:2, v/v) and 2 ml of 0.1 *M* Tris pH 9.0. The migration buffer was 80 m*M* Tris, 10 m*M* benzoic acid pH 8.0, the voltage was +28 kV and the temperature 50°C. We used a hydrodynamic injection of 0.8 s (1.5 nl). Prior to analysis the capillary was rinsed with 0.2 *M* NaOH for 3 min, with water for 2 min, and then with separation buffer for 3 min. Samples were injected by hydrodynamic injection for 1 s (15 nl). Water was used as electroosmotic flow (EOF) marker.

### 2.3. Methylfluorescein esters SCFAs or long chain fatty acids

Derivation of standards was realised as described in [12]: 250 μl of a 125 μg/ml solution of 5-bromomethylfluorescein (5-BMF, Molecular Probe, Eugene, OR, USA) in acetonitrile were added to a mixture of 188 μl of a 100 μg/ml of SCFA standard in acetone and 48 μl of a 700 μg/ml 18-crown-6 with 20 mg of sodium carbonate, to react in an Eppendorf vial.

### 2.4. Calibrations of SCFAs and serae derivations

Standard solutions of the $C_8$, $C_9$, $C_{10}$ of concentrations ranging from $10^{-7}$ to $5\ 10^{-9}$ *M* were treated as the serum. 250 μl of serum were treated with 250 μl of a 1 *M* sodium hydroxide solution (water–methanol 30:70, v/v) for 3 h at 50°C. 250 μl

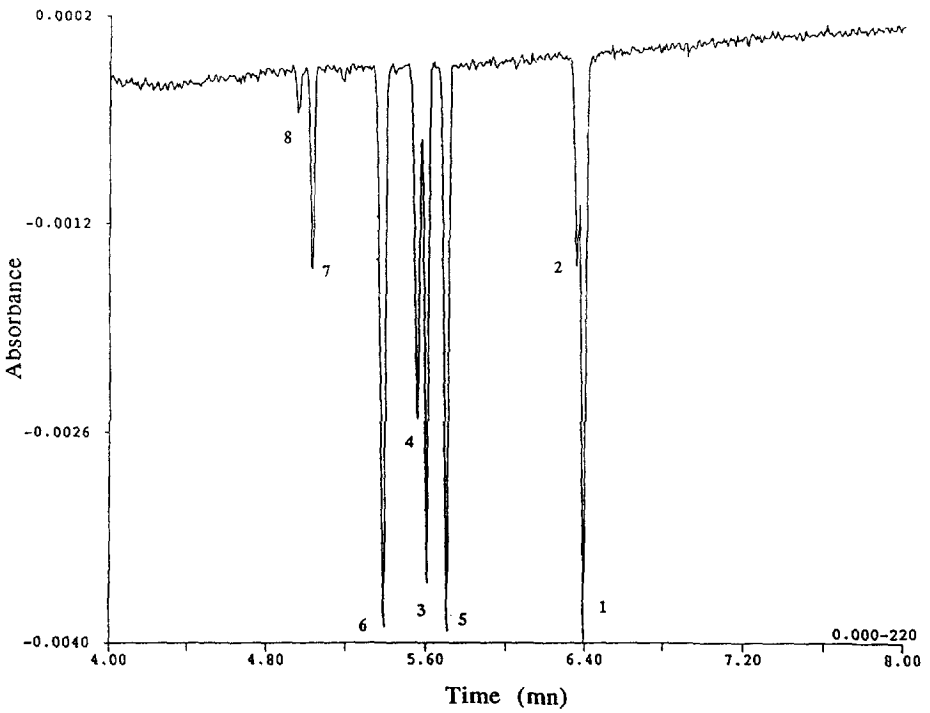


Fig. 1. Separation of some free short chain fatty acids and long chain fatty acids using CE–indirect UV detection. 1=*n*-Pentanoic acid (50 mg/l), 2=trimethylacetic (15 mg/l), 3=2-ethylhexanoic acid (15 mg/l), 4=3,5,5-trimethylhexanoic acid (25 mg/l), 5=*n*-octanoic acid (50 mg/l), 6=*n*-undecanoic acid (50 mg/l), 7=*n*-tetradecanoic acid (15 mg/l), 8=*n*-pentadecanoic acid (5 mg/l). mn=minutes.

of a 1 *M* sulfuric acid solution was then added and fatty acids were extracted three times with 500 μl of dimethyl ether. The organic phase was washed with water three times and the dimethyl ether was slowly evaporated to dryness. The obtained sample is diluted in 100 μl acetone. 5-BMF labelling conditions are as described in Section 2.3. Samples were diluted in a 10 m*M* borate pH 10.0 solution.

Table 1
Effective mobilities and minimum concentrations detected (*S/N*)=3 for branched, hydroxy or linear fatty acids using CE–indirect UV detection

| Fatty Acid | Effective mobility ($10^{-9}$ $m^2/V$ s) | Minimum concentration detected (μ*M*) | Carbon atom number |
|---|---|---|---|
| *n*-Pentanoic acid | −9.98 | 27 | 5 |
| Trimethylacetic acid | −4.18 | 55 | 5 |
| 2-Ethylhexanoic acid | −2.00 | 19 | 8 |
| 3,5,5-Trimethylhexanoic acid | −0.98 | 9 | 9 |
| *n*-Octanoic acid | −0.62 | 28 | 8 |
| *n*-Undecanoic acid | −0.41 | 15 | 11 |
| *n*-Tetradecanoic acid | −0.33 | 26 | 14 |
| *n*-Pentanoic acid | −0.27 | 16 | 15 |
| *n*-Octadecanoic acid | −0.23 | 17 | 18 |
| 12-Hydroxy-octadecanoic acid | −0.11 | 200 | 18 |

The effective modility $\mu_{eff}$ is calculated following the formula $\mu_{eff}=lLV^{-1}(t_e^{-1}-t_{eo}^{-1})$, where $l$ is the effective length and $L$ is the total length of the capillary. $V$ is the operating voltage, $t_e$ is the migration time of the analyte and $t_{eo}$ is the migration time of the EOF marker.

### 2.5. 5-Methylfluorescein esters of SCFAs and CE–LIF studies

Separations were carried out using a separation buffer containing 100 m*M* Borate pH 10 and various amounts of sodium dodecyl sulfate (SDS) and urea and by applying a separation voltage of 27 kV. Prior to analysis the capillary was rinsed with 0.1 *M* NaOH for 3 min, with water for 2 min, and then with separation buffer for 3 min. Samples were injected by hydrodynamic injection for 1s (15 nl). Water was used as the EOF marker.

## 3. Results and discussion

### 3.1. Separation of linear, branched and hydroxy fatty acid using CE and indirect UV detection

Because of the paucity of work on the use of CE and indirect UV detection to study SCFAs and long chain fatty acids, we attempted a separation of different linear hydroxy and branched fatty acids, to determine if we could obtain an easy separation of these molecules. In addition we determined if we were able to separate carboxylic acids ranging from $C_5$ to $C_{18}$ with sufficient sensitivity. Fig. 1 shows an electropherogram of different linear and branched fatty acids. Table 1 presents the mobilities observed for these acids. $C_{16}$ and $C_{18}$ acids could not be separated. All these acids have similar $pK_a$ values and are totally ionized at pH 10.0. We see that the absolute values of effective mobilities decrease when the molecular masses increase. Moreover, the branched fatty acids have higher mobilities than the linear ones. This could be due to the fact that the molecular volume of branched species are smaller than the linear species thus minimizing the hydrophobic effect [14].

The calculated minimum concentrations detected are in the range $10^{-5}$ *M*. These concentrations are not well adapted to the analysis of SCFAs in sera, which are reported to be in the micromolar concentration range [15].

### 3.2. Separation of 5-methylfluorescein esters SCFAs

To achieve the best separation between the different SCFAs, we studied the influence of the concentration of SDS in the buffer on the resolution between the $C_8$ and the $C_9$ and $C_{10}$ and the $C_{11}$ fatty acids. The optimal concentration to separate these species was between 20 and 40 m*M*, with a much higher current in the latter case. To separate acids with a carbon atom number higher than 11 we attempted to use high concentrations of urea as preconised by Terabe et al. [16]. A concentration of 4 *M* of this chaotropic agent was necessary. Table 2 indicates the electrophoretic mobilities of the different SCFAs we have studied. As observed in the CE indirect UV studies, the branched species (2-ethylhexanoic acid) is eluted prior to the linear isomer. We were not able to study $C_5$ and $C_6$ SCFAs because they eluted with the blank peaks. Moreover the acids longer than $C_{11}$ coeluted and we could not observe any separation. The sensitivity limits were around $10^{-10}$ *M* for $C_8$, $C_9$, $C_{10}$, $C_{11}$ linear SCFAs.

Table 2
Effective mobilities and minimum concentrations detected ($S/N$)=3 for branched or linear fatty acids esters of 5-methylfluorescein using CE–LIF

| Fatty acid | Effective mobility ($10^{-9}$ $m^2/V$ s) | Minimum concentration detected ($10^{-10}$ *M*) |
|---|---|---|
| 2-Ethylhexanoic acid | −56.3 | Undetermined |
| *n*-Octanoic acid | −45.5 | 4.0 |
| Nonanoic acid | −38.5 | 4.1 |
| Decanoic acid | −33.8 | 5.4 |
| Undecanoic acid | −32.2 | 5.6 |

Effective mobilities are calculated as in Table 1.

Fig. 2 shows a separation of standard SCFAs. Fig. 3 indicates a non-pathological serum after a 3000 fold dilution in water. The linear $C_8$, $C_9$ and $C_{10}$ can be identified. We constructed a calibration curve after saponification and extraction of standard diluted in 250 μl of water. The calibration curve plotted as the log of the concentration of SCFA and the log of the fluorescence is linear between $5 \cdot 10^{-7}$ and $2 \cdot 10^{-9}$ *M*, with a slope of 0.818 and an intercept of 11.794 ($r$=1.000) for $C_8$ and a slope of 0.961 and an intercept of 12.855 ($r$=0.997) for $C_{10}$. The relative standard deviations are smaller than 9.7%.

The quantitations of $C_8$ and $C_{10}$ in pathological and non-pathological sera are shown in Table 3. In the two pathological sera the quantitation of the linear $C_8$ was not possible, because of coelution of other peaks. A GC–MS study on fatty acids methyl esters was unsuccessful in identifying the nature of the coeluted species. The concentrations of the $C_8$ and $C_{10}$ acids are in agreement with the ranges indicated in the literature [15].

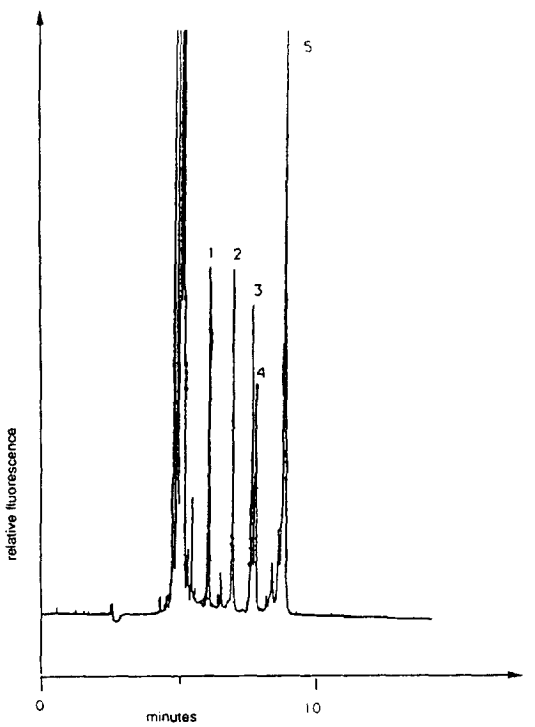


Fig. 2. Separation of SCFA 5-methylfluorescein esters by CE–LIF using a 100 m*M* borate pH 10, 20 m*M* SDS, 4 *M* urea buffer: 1=$C_8$, 2=$C_9$, 3=$C_{10}$, 4=$C_{11}$, 5=$C_{14}$ and $C_{16}$ standards fatty acids at a concentration of $10^{-8}$ *M*.

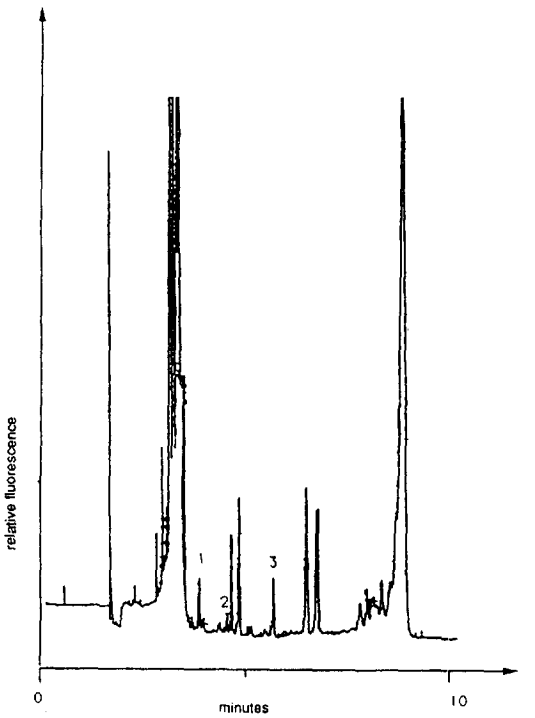


Fig. 3. Non pathological serum after a 3000 fold dilution in water. 1=$C_8$, 2=$C_9$ and 3=$C_{10}$ can be identified. $C_8$ and $C_{10}$ are quantitated. Same separation conditions as in Fig. 2.

## 4. Conclusion

In this study we attempted to study SCFAs using CE–indirect UV adsorption and CE–LIF. Although better separations for a large variety of acids is possible with CE–indirect UV detection, the sensitivity is mediocre. On the contrary, CE–LIF study gives a much better sensitivity, but due to the high mass of the fluorescent species and to the presence of unreacted bromofluorescein, only $C_8$ to $C_{11}$ acids can be separated and detected.

Table 3
Quantitation of $C_8$ and $C_{10}$ 5-methylfluorescein esters in serae

| Fatty acid | *n*-Octanoic acid ($\mu M$) | *n*-Decanoic ($\mu M$) |
|---|---|---|
| Serum I | 11.7 | 26.4 |
| Pathological serum II | Undetermined | 16.8 |
| Pathological serum III | Undetermined | 13.6 |
| Literature values [15] | 5–19 | 5–17 |

## Acknowledgments

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