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Journal of Chromatography A, 973 (2002) 203–210

JOURNAL OF CHROMATOGRAPHY A

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Analysis of aminofluorescein–fatty acid derivatives by capillary electrophoresis with laser-induced fluorescence detection at the attomole level: application to mycobacterial fatty acids

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Received 17 May 2002; received in revised form 30 July 2002; accepted 7 August 2002

Abstract

A method based on capillary electrophoresis coupled to laser-induced fluorescence detection was developed for the characterization of fatty acids including palmitic, stearic, oleic and tuberculostearic acids. The fatty acids were tagged by 4-aminofluorescein (AF) via a carboxylic acid-amine condensation promoted by *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) in non-aqueous solution. Using the optimized derivatization conditions, the fluorophore labeling of the fatty acids was achieved at the nanomolar level. The separation of palmitic, stearic, oleic and tuberculostearic–AF derivatives was achieved in less than 10 min, using 25 m*M* sodium borate buffer containing 30% of acetonitrile as running electrolyte. The concentration detection limit was found to be 5 n*M* while the minimum mass limit detection is around 30 attomol. This method was successfully applied to identification of mycobacteria via the characterization of tuberculostearic acid and found to be suitable for the detection of a minimum of 10^6 mycobacteria.
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Keywords: Derivatization, CE; Mycobacteria; Fatty acids, mycobacterial; Aminofluorescein

1. Introduction

Long chain fatty acids are usually characterized by GC–MS of their methyl or pentafluorobenzyl esters [1,2]. Reverse phase LC has been also successfully used for the separation of fatty acids labeled by UV dyes [3,4].

Capillary electrophoresis (CE) generally provides high efficiencies and fast analyses and therefore could be considered as a potent alternative method. Previous analyses of fatty acids by CE have focused on micellar electrokinetic chromatography [5] with indirect UV or fluorescence detection [6,7].

Laser-induced fluorescence detection (LIF) has been shown to be one of the most sensitive methods available for detection in capillary electrophoresis. As fatty acids lack any suitable chromophore or fluorophore, detection by LIF requires a derivatization. In the recent literature, there have been a

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PII: S0021-9673(02)01216-5

number of reports of flurophoric reagents designed for covalent labeling of fatty acids [8,9]. Many are based on the alkylation of carboxylate anions. Zuriguel et al. [10] have reported the CE separation of fatty acids derivatized using aqueous electrolyte with 5-bromomethylfluorescein but only up to the C_{11} fatty acid. The use of nonaqueous electrolyte was successfully applied for the separation of fatty acids either derivatized with near-infrared fluorophore [11] or free [12].

Other labeling reaction types have been developed which include reaction of carboxylic acids with fluorescent hydrazides, diazoalkanes and aromatic amines [8,9]. Amines can be covalently coupled to carboxylic acids via an amide bond. This could be done by initial conversion of the carboxylic acid to an acid chloride which then reacts with the amine, or by the direct reaction of the carboxylic acid with the amine, mediated by dicyclohexylcarbodiimide (DCC) or other related diimide reagents.

Recently, this method was successfully used for labeling various short-chain carboxylic acids (C_2–C_9) with 4-aminofluorescein and further analysis with CE coupled with laser induced fluorescence [13]. Even if the biphasic medium and huge excesses of reagents used by the authors are serious drawbacks of their method, this fluorophore seems to us suitable for the derivatization of long chain fatty acids. It is soluble in organic solvents, it possesses an amino function that can form stable carboxylic amides and a negative charge can be generated by opening the spirolactone in alkaline condition after the labeling reaction. Moreover, the dye fluorescence spectral properties (maximum excitation at 491 nm, maximum fluorescence emission at 520 nm) are fully compatible with the argon laser coupled to our CE instrument [14].

In this report, we explored the labeling of fatty acids by 4-aminofluorescein in non-aqueous medium and their separation by CE with LIF detection. The limitations of this approach including the reaction derivatization, detection and quantification are discussed. We also describe the suitability of this method for fatty acid analysis of crude extracts of *Mycobacterium tuberculosis* and its potential utility for identification of this pathogen in biological samples. Indeed, tuberculostearic acid is specific of the mycobacteria genus.

2. Experimental

2.1. Instrumentation

Capillary electrophoresis (CE) separations were performed on a P/ACE 5000 capillary electrophoresis system (Beckman Instruments). The separations were monitored with a Beckman laser-induced fluorescence (LIF) detection system using a 4-mW argon-ion laser with the excitation wavelength of 488 nm and emission wavelength filter of 520 nm. The temperature of the capillary in the P/ACE instrument was controlled at 25 ± 0.1 °C. The electropherograms were acquired and stored on microcomputer using the PACE Station software (Beckman Instruments).

Absorption spectra were recorded on a UVIKON spectrophotometer. Spectrofluorometry was performed on a FLX instrument (Safa, Monaco).

GC–MS spectra were recorded on a Hewlett-Packard 5889 mass spectrometer working in EI mode and coupled with a Hewlett-Packard 5890 gas chromatograph equipped with an OV1 capillary column (8 m$\times$200 μm). The injector and interface temperature were seated at 290 °C.

MALDI-TOF MS analysis was carried out on a Voyager DE-STR (Perseptive Biosystems, Framingham, MA, USA) using linear and reflectron modes. Ionization was effected by irradiation with pulsed UV light (337 nm) from a N_2 laser. Instrument was operated in positive mode. Matrix solution consisted in saturated solution of 2,5-dihydroxybenzoic acid in water–acetonitrile (1:1, v/v).

2.2. Capillary electrophoresis analysis

All capillary electrophoresis analysis were performed on a 47 cm$\times$50 μm (I.D.) uncoated fused-silica capillary column. The samples were injected by applying 0.5 p.s.i. pressure for 5 s.

The running electrolyte consisted of 25 m*M* aqueous sodium borate–acetonitrile (70:30, v/v) pH 9. All fatty acid–AF derivatives were dissolved in the running electrolyte.

2.3. Derivatization of fatty acids

2.3.1. Materials and reagents

Standards fatty acids including myristic acid

(C_{14}), palmitic acid (C_{16}), stearic acid (C_{18}), tuberculostearic acid (C_{19}) (99–100% of purity) were purchased from Sigma–Aldrich as well as the 4-aminofluorescein (AF), 1-hydroxybenzotriazole (HOBT), *N′*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) and *N*,*N′*-dicyclohexylcarbodiimide (DCC). Acetonitrile (HPLC grade) and dimethylformamide (DMF) were supplied by Solvants Documentation Synthèse (Peypin, France). DMF was stored on activated 4-Å molecular sieves.

2.3.2. Derivatization protocol

To 500 nmol of fatty acids in 15 μl of DMF were added 1 μmol of EDC in 30 μl of DMF. The reaction mixture was stirred during 30 min at room temperature. Then, the AF (2 μmol in 40 μl of DMF) was added. Total volume reaction was adjusted to 100 μl. The mixture was stirred at room temperature during 2 h. The solvent was removed in vacuo and the residue was solubilized in the CE running buffer and analyzed. A similar derivatization protocol was used in experiments using HOBT which was added with EDC, in the same molar ratio.

2.3.3. Preparation of standards

Derivatization was carried out as above on a 5-mg scale of fatty acid. Purification was done on preparative thin layer chromatography (silica gel, elution with petroleum ether–ethyl acetate 2:1, v/v). After recovery of the products from the plate with ethyl acetate, they were analyzed and identified by MALDI-TOF; their purity was checked by CE.

2.4. Fatty acids from mycobacterium tuberculosis

A *M. tuberculosis* fraction of cell wall containing a mixture of phosphatidyl-*myo*-inositol mannosides [16] (PIMs, 50 μg) was submitted to alkaline hydrolysis (1 *M* NaOH for 2 h at 110 °C). After completion of the reaction and acidification, the fatty acids were extracted with hexane and divided in two fractions. The first fraction was derivatized as methyl esters with methanolic boron trifluoride while the other one was transformed in AF–fatty acids derivatives according to the protocol described above.

The 10^7 heat-killed *M. tuberculosis* cells were treated for 2 h with 200 μl of 1 *M* NaOH at 110 °C. The fatty acids were then extracted, divided in two pools, and derivatized as above to their methyl esters and AF derivatives.

3. Results and discussion

3.1. Optimization of precolumn derivatization

The derivatization of fatty acids was performed with 4-aminofluorescein (AF) through a condensation reaction between the amino group of the derivatizing agent and the carboxylic group of the fatty acid to form an amide bond. Carbodiimides, such as dicyclohexylcarbodiimide (DCC) are required to promote the bond formation, they activate the carboxyl group of the carboxylic acid towards nucleophilic attack by the amine group of the fluorophore. DCC is the most common reagent and has been used extensively in peptide synthesis as a fast and efficient coupling reagent. This strategy was also used for short-chain fatty acid labeling with AF in aqueous solution [13].

We took advantage of both fatty acids and AF solubility in organic solvents to conduct this two-step reaction in dimethyformamide (DMF). EDC was selected instead of DCC since higher solubility in DMF was obtained. The derivatization reaction proceeds according to the following scheme (Fig. 1).

The excitation and fluorescence spectra for AF and C_{14}–AF had been recorded. It was found that the wavelengths for maximum absorbance of AF and C_{14}–AF are very similar (492 and 495 nm, respectively) Their maximum emission for these two compounds were found identical at 518 nm. Thus the AF tagging reaction conferred to the solute excitation and emission wavelengths that perfectly match the spectral line of the argon laser (488 nm) and the filter of the detector (520 nm). The molar absorptivity is higher for AF (75 400 mol/cm) than that of C_{14}–AF derivative (37 300 mol/cm) but C_{14}–AF signal fluorescence at 518 nm is more intense than AF revealing that the quantum efficiency of C_{14}–AF is twenty times higher than that of the dye, in agreement with the literature data found [13,14].

The labeling reaction was optimized for myristic acid (C_{14}, see Fig. 1) in terms of molar ratio of EDC (2 equiv.), reaction time (120 min) and temperature

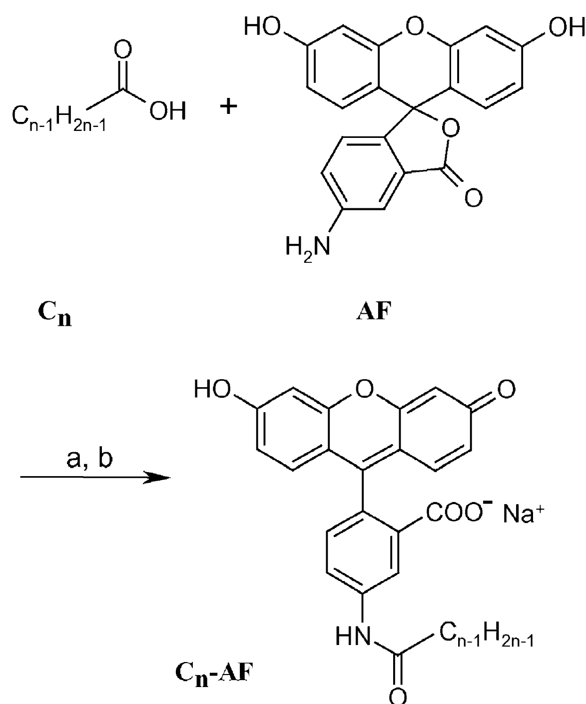

Fig. 1. Derivatization of fatty acids with aminofluorescein. (a) EDC, DMF; (b) 25 m*M* sodium borate–acetonitrile.

of reaction (20 °C). The detailed optimum conditions are described in the experimental section. An authentic standard of the reaction product (C_{14}–AF) was prepared and was controlled by MALDI-TOF analysis. The mass spectrum shows an intense peak at *m/z* 558 assigned to the protonated molecular ion of the myristic acid derivative.

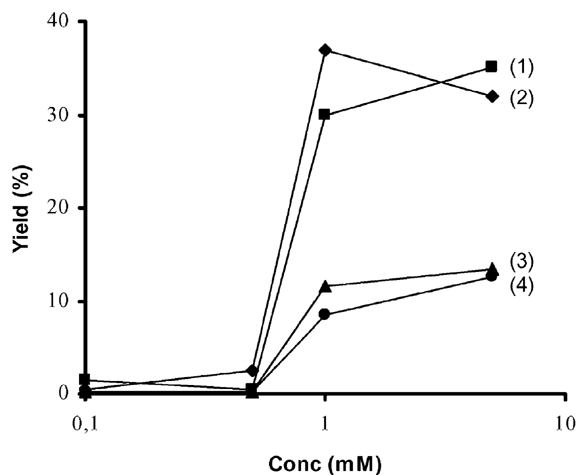

Fig. 2. Derivatization yields for reactions of C_{14} and C_{19} with aminofluorescein. Lines 1 and 2: C_{14} with (■) and without (♦) HOBT. Lines 3 and 4: C_{19} with (●) and without (▲) HOBT.

According to the Fig. 2, we observed successful derivatization of 500 nmol of myristic acid at concentrations as low as 5 10^{-3} *M*, unfortunately no coupling product could be detected at micromolar or submicromolar concentrations of acid. At 500 μ*M* concentration, the yield of the reaction is very low compared with the observed yield at 1 m*M*. All yields were calculated from observed peak area of C_{14}–AF and remaining AF in the CE analysis, corrected for their relative quantum efficiencies.

To improve the condensation of amino groups with acids, *N*-hydroxybenzotriazole (HOBT) has been extensively and successfully used in peptidic synthesis. HOBT was tried as an additive in our reactions, but, no improvement of the yield was observed, a small decrease of the yield was even obtained in some cases (Fig. 2) and finally the electropherogram shows many peaks assigned to reaction sub-products. Consequently, HOBT was not selected for the derivatization reaction.

At the lowest fatty acid concentration, we investigated whether elevated temperatures and extended reaction times might increase the yield of the derivatization reaction. We found that overnight reaction at 37 °C lead only to a very small increase in the yields of derivatized product but this improvement was not found significant enough.

3.2. Capillary electrophoresis analysis of fatty acid–AF derivatives

The precolumn derivatization introduced the uncharged 4-aminofluorescein (Fig. 1). The fatty acid derivatives were then treated at high pH in order to open the AF spirolactone to a carboxylate group which introduced a negative charge. Separation was then carried out in a basic buffer at pH 9, using 25 m*M* sodium borate containing 30% of acetonitrile (v/v) as the running electrolyte.

The C_{14}–AF was hydrodynamically injected at the anode (injected volume of approximately 6 nl). The applied field strength for the separation was +638 V/cm with a current of 42 μA.

Due to the borate electrolyte system the electro-

osmotic flow (EOF) is very strong and its direction is towards the cathode which is at the ground potential. This flow is able to sweep the negatively charged fatty acid derivatives toward the cathode past the detection point. The electropherogram of the derivatization reaction of C_{14} is given in Fig. 3. Peak 3 was assigned to C_{14}–AF by coinjection with a purified sample (see Section 2). Peak 4 was assigned to unreacted AF, while peak 5 was assigned to a minor contaminant from the commercial AF reagent.

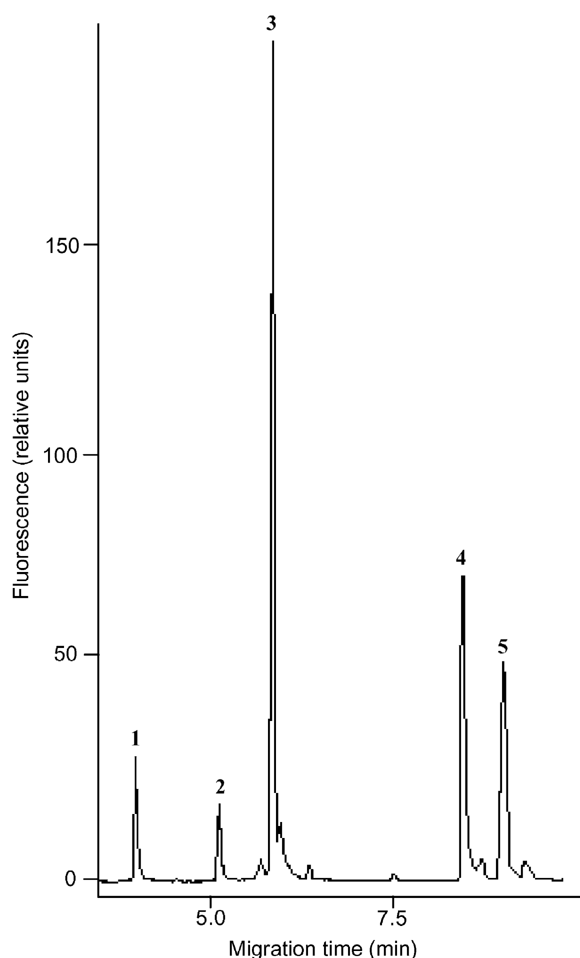

Fig. 3. Electropherogram of C_{14}–AF (100 μ*M*). Conditions: fused-silica capillary 47 cm×50 μm I.D. 25 m*M* aqueous sodium borate containing 30% acetonitrile (v/v) as running electrolyte; separation 30 kV (42 μA); 25 °C, LIF detection (488/520 nm). For identification of peaks, see text.

Peaks 1 and 2, of lower intensities, are by-products of the derivatization reaction.

A calibration curve of C_{14}–AF showed excellent linearity ($r^2 = 0.997$) over a concentration range from 1.7 μ*M* down to 5 n*M* (each point done in triplicate in three independent experiments).The concentration detection limit was found to be 5 n*M* ($S/N = 2$) and the minimum mass detection limit was 30 attomol using a 50-μm I.D. capillary. The concentration detection limit was determined using 5-s injection time corresponding to a volume of 6 nl. The previous study on AF–short chain fatty acids (C_5 to C_9), reported a concentration detection limit of 50 n*M* and a mass detection limit of 9 nmol [13].

The capacity of CE to separate fatty acid–AF derivatives was then investigated. The fatty acids were chosen to cover the molecular mass range of the fatty acids found in the mycobacterial cell wall, these are palmitic (C_{16}), stearic (C_{18}) and tuberculostearic (C_{19}), the latter one is specific of the mycobacteria genus and could be used as a marker of mycobacterial infection, including tuberculosis.

The CE conditions described above were applied to a mixture of C_{14}, C_{16}, C_{18} and C_{19}–AF derivatives. Since the EOF is in the opposite direction to the electrophoretic mobility of the negatively charged analytes, the AF–fatty acid derivatives migrated at the cathode in the order of decreasing mass-to-charge ratio, i.e. C_{19}–AF, which has the highest mass-to-charge ratio, migrated first followed by C_{18}–AF, C_{16}–AF and C_{14}–AF.

An electropherogram of this fatty acid–AF mixture is shown in Fig. 4. Peak identities were confirmed by the coinjection of individual standards, prepared and purified independently. As expected labeled fatty acids migrated in order of decreasing molecular mass. A complete separation of all these AF–fatty acid derivatives was obtained, except for the C_{18} and C_{19} which are resolved approximately at 30% of the valley. The electropherogram shown in Fig. 4 also revealed a decrease of peak intensities from C_{14}–AF to C_{19}–AF in agreement with the observed reaction yields for C_{14} and C_{19} reported on Fig. 2. This phenomenon is not yet well understood. Indeed, it was advanced by Gallaher et al. [15] that the difference of labeling between propionic acid (C_3, 13%) and pelargonic acid (C_9, 63%) arises from the increased solubility of propionic acid which

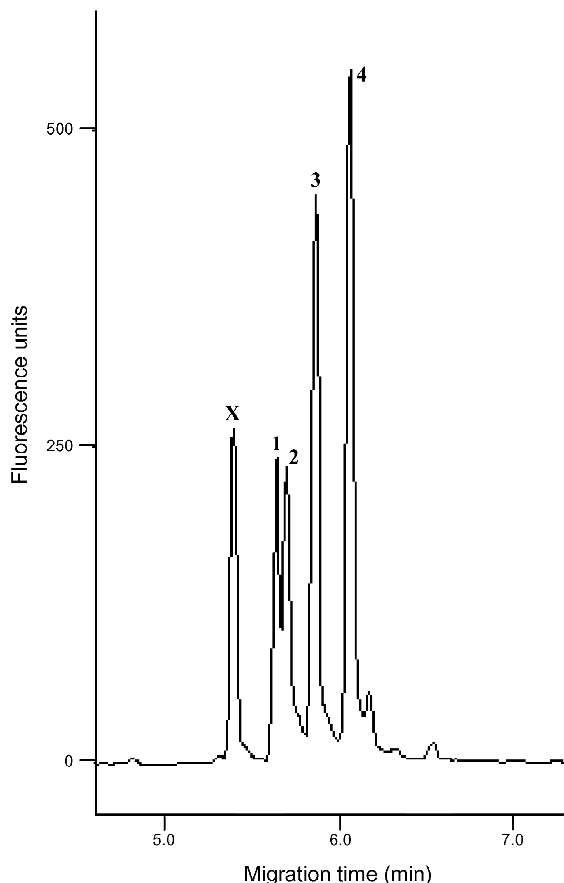

Fig. 4. Electropherogram of a mixture of AF-labelled fatty acids (200 μ*M*). Peak assignment: (1) C_{19}–AF, (2) C_{18}–AF, (3) C_{16}–AF, (4) C_{14}–AF, (X) artefact from derivatization. Separation conditions: see legend to Fig. 3.

could led to unfavorable reaction kinetics in the derivatization step.

The influence of organic solvents as modifiers of the running electrolyte and the sodium borate concentration were studied. Switching from acetonitrile to methanol as co-solvent in the electrolyte resulted in a drastic decrease of the resolution exemplified by the fact that C_{18} and C_{19} were no longer separated. Separation of C_{18}–AF and C_{19}–AF was not improved using electrolyte with lower borate concentration (12.5 m*M* instead of 25 m*M*) but decreased migration times were obtained. Faster analysis resulted from the increase of the EOF due to the decrease of the borate concentration. Finally, as it can be seen on Fig. 4, the best compromise in terms of analysis time and resolution was reached when the running electrolyte was 25 m*M* borate and 30% acetonitrile. Separation of C_{14}–AF to C_{19}–AF fatty acid derivatives was achieved in less than 10 min.

These electrophoretic conditions were applied with success to the separation of a mixture of C_{16}, oleic acid ($C_{18:1}$) and C_{19} as shown in Fig. 5.

3.3. Application to M. tuberculosis fatty acid analysis

The fatty acids recovered from *M. tuberculosis* PIMs were divided in two pools. The first pool was derivatized as methyl esters while the other one was transformed in AF–fatty acids derivatives according to our protocol. The methyl ester fatty acids were analyzed by GC–MS (Fig. 6 A) revealing the presence of two major fatty acids, C_{16} and C_{19}, and small amounts of C_{18} and $C_{18:1}$. The electropherogram of the second fraction which is shown on Fig. 6B is in very good agreement with the results from

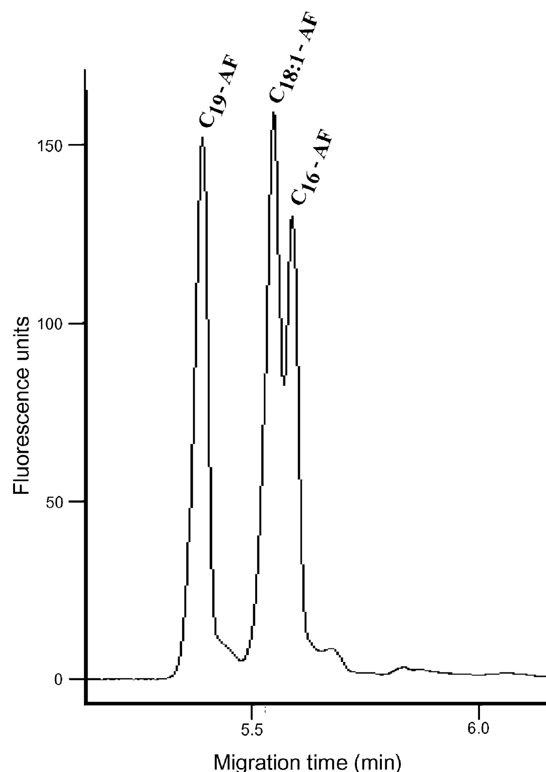

Fig. 5. Electropherogram of a mixture of C_{19}–AF, $C_{18:1}$–AF and C_{16}–AF. Separation conditions were as in Fig. 3.

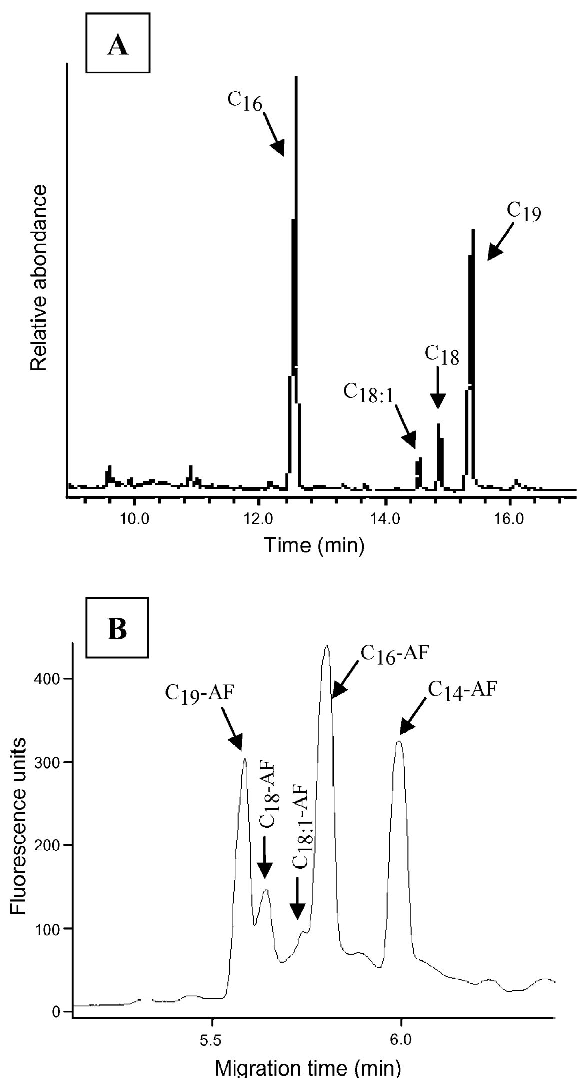

Fig. 6. Analysis of a mixture of fatty acids obtained from a PIM fraction from *M. tuberculosis* (50 μg of PIMs, see text). (A) GC–MS of the fatty acid methyl esters; (B) electropherogram of fatty acid–AFs, C_{14}–AF added as internal standard.

GC–MS analysis. AF fatty acid derivatization and CE analysis is a powerful tool for the separation and identification of mycobacterial fatty acids, including C_{19}.

This protocol was then applied to the analysis fatty acids from whole cells of *M. tuberculosis*; 10^7 heat-killed *M. tuberculosis* cells were submitted to alkaline hydrolysis. The fatty acids were then extracted, divided in two pools, and derivatized as above to their methyl esters and AF derivatives. Parallel GC–MS and CE analysis revealed the presence of mainly four types of fatty acids C_{16}, C_{18}, $C_{18:1}$ and C_{19}.

We investigated whether this method might be sensitive enough to be used for the detection of tuberculostearic acid in patient sputum which contains from 10^3 and 10^6 *M. tuberculosis* cells. The latter protocol was applied to samples containing a number of mycobacteria from 10^3 up to 10^6. Identification of tuberculostearic acid on the electropherogram was unambiguously obtained for 10^6 mycobacteria, but not for lower amounts of cells. This protocol must be improved in order to consider its application for the diagnosis of pulmonary tuberculosis from the patient's sputum.

4. Conclusion

In summary, we have optimized and evaluated the tagging of long chain fatty acids by AF to allow their sensitive detection by CE coupled to LIF detection. The derivatization reaction has several advantages including: (i) specific attachment of the tag in nonaqueous solution, and (ii) no sample clean up. These features allowed the high-resolution separation of the fatty acids which are found in mycobacteria, C_{16}, C_{18}, $C_{18:1}$ and C_{19} with sensitivities down to 30 attomol. The main limitation is the low yield of the derivatization reaction at low concentrations, this must be improved to take full advantage of the very high sensitivity of the LIF detection.

This method was successfully applied for the characterization of tuberculostaric acid in samples containing up to 10^6 mycobacteria, but the derivatization conditions must be optimized at submicromolar concentration in order to reach the characterization of tuberculostaric acid from 10^3 *M. tuberculosis* cells and to develop a tuberculosis diagnosis.

Acknowledgements

This work was supported by a grant from the Ministère de la Recherche et de la Technologie (Action Concertée Incitative "Molécules et Cibles Thérapeutiques"). We thank D. Bounery for GC–MS

analysis and Beckman (France) for technical assistance.

References

[1] J.P. Cartier, Bull. Inst. Pasteur 83 (1985) 57.
[2] L. Larsson, G. Odham, G. Wesredahl, B. Olsson, J. Clin. Microbiol. 25 (1987) 893.
[3] M. Ikeda, K. Shimada, T. Sukaguchi, Chem. Pharm. Bull. 30 (1982) 2258.
[4] G. Gucnikov, W. Beck, H. Engelhardt, J. Microcol. Sep. 6 (1994) 565.
[5] F.B. Erim, X. Xu, J.C. Kraak, J. Chromatogr. A 694 (1995) 471.
[6] E. Drange, E. Lundanes, J. Chromatogr. A 771 (1997) 301.
[7] A.M. Desbène, C.J. Morin, N.L. Mofaddel, R.S. Groult, J. Chromatogr. A 716 (1995) 1.
[8] P.S. Mukherjee, H.T. Karnes, Biomed. Chromatogr. 10 (1996) 193.
[9] Y. Yasaka, M. Tanaka, J. Chromatogr. B 659 (1994) 139.
[10] V. Zurigel, E. Caussé, J.D. Bounéry, G. Nouadje, N. Siméon, M. Nertz, R. Salvayre, F. Couderc, J. Chromatogr. A 781 (1997) 233.
[11] D.L. Gallaher, M.E. Johnson, Anal. Chem. 72 (2000) 2080.
[12] M. Jussila, S. Sundberg, A. Hopia, M. Mäkinen, M.L. Riekkola, Electrophoresis 20 (1999) 111.
[13] M. Kibler, K. Bächmann, J. Chromatogr. A 836 (1999) 325.
[14] R.P. Hangland, Handbook of Fluorescent Probes and Research Chemicals, 6th ed, 1996.
[15] D.L. Gallaher, M.E. Johnson, Analyst 124 (1999) 1541.
[16] K.-H. Khoo, A. Dell, H.R. Morris, P.J. Brennan, D. Chatterjee, Glycobiology 5 (1995) 117.