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High-performance liquid chromatography and identification of mycobacteria

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Identification of mycobacteria by means of high-performance liquid chromatography (HPLC) of cell-wall mycolic acids was introduced at the end of the last decade but, despite its high reliability, it has not yet reached widespread use in clinical microbiology laboratories. The various combinations of long-chain mycolic acids, which are present in the cell wall of mycobacteria, have been shown by different analytical methods to be species-specific. When suitably processed mycolic acids are subjected to HPLC analysis a chromatogram is produced. Simple comparison of the profile with ones of known mycobacterial species allows identification to species level, without the need to recognize the individual compounds. Representative HPLC profiles of the more common mycobacterial species, which are presently scattered in various articles, are presented here with the aim of encouraging greater use of this identification technique, which combines rapidity and ease of performance with reliability, and high specificity with low cost.

Keywords: Mycobacteria; identification; HPLC; high-performance liquid chromatography.

INTRODUCTION

Three different approaches can be used, at present, for the speciation of mycobacteria. First, the widely, used conventional methods [1], based on the investigation of biochemical and cultural features of the strain, give reliable results provided that a sufficiently high number of tests is performed. The procedure is time-consuming and is limited in scope to the species for which a great amount of information is available, which has been based on a large number of strains studied.

An alterative method is represented by genetic investigations, aimed at the recognition, within the mycobacterial genome, of species-specific nucleotide sequences. DNA probe hybridization and genetic sequencing are the most important techniques based on this principle. Nucleic acid hybridization [2], which is user-friendly, rapid and specific, has attained a great acceptance in the last few years. However, it is available for only a few of the more common species. Genetic sequencing [3], which provides both a correct identification and phylogenetic information, can be considered the ultimate taxonomic procedure. Unfortunately, its use is restricted, at present, to a few research laboratories, and its acceptance in diagnostic mycobacteriology does not seem impending.

The third identification approach is represented by the analysis of mycobacterial fatty acids, which have been shown to be suitable for this purpose because of their unique and specific features. To this purpose several chromatographic techniques have been investigated. Thin-layer chromatography is suitable for resolving single mycolate types which are seen as spots on the chromatographic

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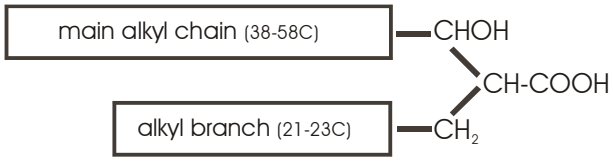

Fig. 1. Mycolic acid structure.

plate [4]. Gas-liquid chromatography (GLC) is used for the detection of characteristic cleavage products of mycobacterial alcohols and long-chain fatty acid [5, 6] or, in combination with mass spectrometry, for determination of the size, without pyrolysis, of mycolic acids [7]. Elution products are represented as peaks arranged according to the number of their carbon atoms. High-performance liquid chromatography (HPLC) - also called high-pressure liquid chromatography - is a separation technique which appears particularly suited to the study of high-molecular-weight compounds such as mycolic acids; it does not require the volatilization of the analytes, which is a major issue with high-boiling point compounds in GLC.

MYCOLIC ACIDS

Mycolic acids are high-molecular-weight β-hydroxy fatty acids, with a long alkyl chain branching at the α position (Fig. 1). They are present in the cell wall of a restricted number of bacterial genera. The number of carbon atoms of mycolic acids is at a minimum (22 to 38) in the genus *Corynebacterium* and gradually increases in other taxa characterized by the presence of such compounds in the cell wall: *Rhodococcus* (34 to 52), *Nocardia* (44 to 60), *Gordona* (48 to 66) *Tsukamurella* (64 to 78); the maximum value (60-90) occurs in the genus *Mycobacterium* [8]. Alcohol-acid fastness is related to mycolic acids, and it increases with their size and complexity, reaching its full expression in mycobacteria.

Additional oxygenated substituents may be present in the main chain and their presence differentiates seven main types of mycolic acids within the genus *Mycobacterium;* in many instances their combinations are species-specific.

HPLC

Chromatographic techniques are used to separate the compounds of a mixture by means of their different solubilities between two immiscible phases: the mobile and the stationary one. HPLC [9] is a liquid-solid chromatography in which, while the mobile phase is a liquid, the stationary one is made up of packed particulate material housed within a metal column. The liquid is forced at high pressure by highly efficient pumps and conveys the sample, introduced into the stream through an in-line injection port, into the column over the stationary phase. According to the physical partitioning between the two phases, single components of the mixture are eluted by the column at different times and are quantitatively tracked down by a detector and plotted (chromatogram) by a recorder (Fig. 2). Two different types of HPLC can be used, according to the polarity of the mobile and stationary phases. In the 'normal phase' a non-polar mobile phase and a polar stationary phase are used; the converse is true for 'reverse phase'. The use of an elution gradient, i.e. a continuous change in polarity, achievable by modifying the proportion of two solvents blended in the mobile phase, strongly improves the separation efficiency.

Various detection technologies are available; the most commonly used is based on the absorption by the analytes of the 254 nm wave-length light produced by an ultraviolet lamp, and allows an approximate sensitivity of 10^{-10} g/ml. A chemical modification of the compound (derivatization) is often necessary to allow the detectability of otherwise non-UV-absorbing separated components.

THE CHROMATOGRAM

The HPLC chromatogram is a graphic output (UV absorption versus elution time) in which separated analytes arriving at the detector are represented in the form of peaks (Fig. 3). The huge peak placed immediately after the injection point (the solvent front, with the non-mycolic fatty acids and the derivatization reagents) is disregarded and each subsequent peak corresponds to a single compound, with area under the peak related to its amount. Each fraction is identified by the corresponding retention time (RT) read on the abscissa, i.e. by the time taken by the analyte to travel from the injection port to the detector.

For a particular chromatographic method and column, the RT of a specific analyte is constant, provided that the column temperature and the flow

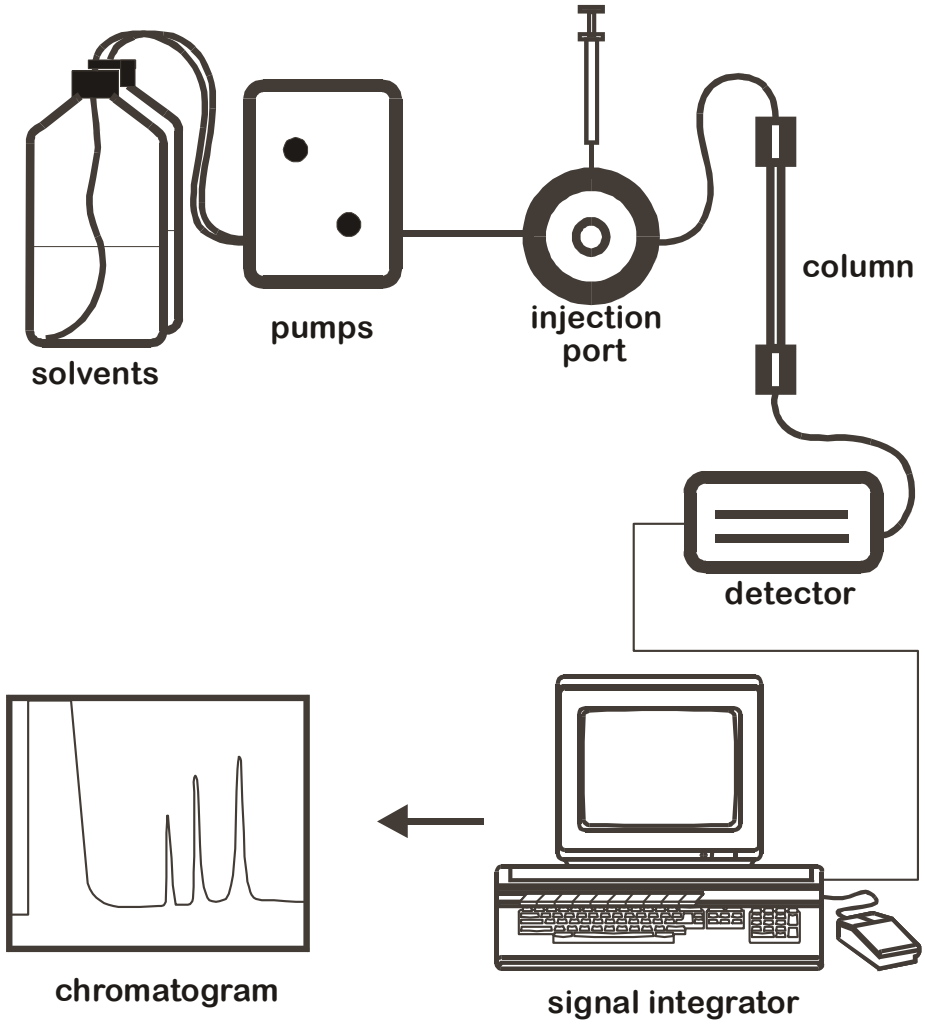

Fig 2. General outline of HPLC components.

rate of the mobile phase are also constant. The peaks of an unknown compound can be identified by comparison of their RTs with the ones present in a known mixture. A known substance can be added to the sample as an internal standard (IS), and the ratios of the RTs of single peaks to that of the IS can be used for the identification of peaks, thus overcoming the variations due to minor changes in the analytical process (e.g. column temperature or flow rate).

HPLC ANALYSIS OF MYCOLIC ACIDS

Bacterial identification by means of mycolic acid analysis was first used with the genera having the shorter of such compounds - such as *Corynebacterium, Rhodococcus* and *Nocardia* [10, 11] - but it is with mycobacteria that this technique has given its best results. Protocols include several steps: saponification, extraction, derivatization and chromatographic separation. Only minor changes have been made with time; the saponification period has been progressively shortened from 18 hours [10] to 1 hour [12] while the corresponding temperature has been raised from 85 to 121°C. The solvents mixed in the mobile phase have been changed from methanol-chloroform [10] to methanol-methylene chloride [13], and the elution period has been shortened from 65 min [10] to 21 min [14], and more recently to 10 min [13], because of the use of a shorter column and increased flow rate. A curvilinear gradient has also been tested [15].

THE METHOD

Minor differences may differentiate the basic: procedure; but a presently widespread approach has been chosen here.

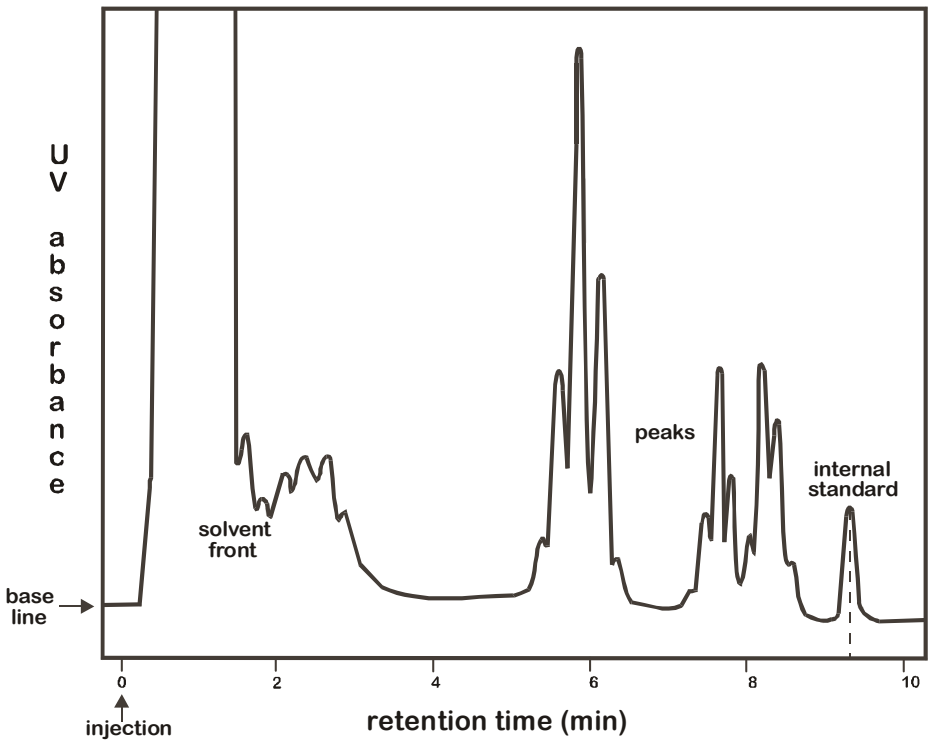

Fig. 3. Elements of a chromatogram. Retention times of single peaks are defined by the intersection with the abscissa axis (e.g. the retention time of the internal standard reads about 9.2 min).

Sample preparation

One loopful of bacteria grown on sold medium (either Lowenstein Jensen or Middlebrook 7H11) is suspended in 2 ml of ethanolic potassium hydroxide (25% of KOH in 50% ethanol) in a glass tube with a Teflon-lined screw cap. After vortexing for 30 s it is saponified for 1 h al 121°C in an autoclave.

Fatty acids are extracted by vortexing for 30 s, after addition of 2 ml of chloroform and acidification with 1.5 ml of 18.5% hydrochloric acid.

The bottom chloroform layer is transferred to a new screw-capped tube and dried under a stream of air at 85°C. After the addition of 0.2 ml of 1% potassium bicarbonate the sample is further evaporated.

The derivatization [16] of mycolic acids is performed for 30 min at 85°C by adding 1 ml of chloroform containing 0.5% of 0.1 mM bromophenacyl bromide plus 0.005 mm dicyclohexyl-18-crown-6 ether per ml of acetonitrile.

Once cooled to room temperature, 0.5 ml of 18.5% hydrochloric acid and 0.5 ml of methanol are added and the tube is vortexed for 30 s. The chloroform layer is finally removed, evaporated and stored al 4°C until use.

Before injection of 5 µ1 volumes, each sample is suspended in 100 µl of chloroform, spiked with 5 µl of a high-molecular-weight standard and filtered through a 0.45 µm pore membrane.

HPLC conditions

For the chromatographic separation, an HPLC instrument equipped with a reverse phase C_{18} Ultrasphere XL analytical cartridge column and with a detector set at 254-260 nm can be used. The gradient of solvents, methanol (M) and methylene chloride (MC), is changed from the initial conditions (98% M:2% MC) to 80% M:20% MC in 1 min and, linearly, to 35% M:65% MC in 9 min. At the end of the run the column is re-equilibrated to the initial conditions (98% M:2% MC) in 0.5 min and held there for 1.5 min. A flow rate of 2.5 ml min^{-1} is used throughout.

Patten identification

According to usual conventions only peaks greater than or equal to 2% of total peak heights are considered while the fractions eluted within the first 4 min of the run are ignored. Every valid peak is identified on the basis of its relative retention time (RRT) determined with the internal standard

and labelled according to the scheme proposed by the Centers for Disease Control (Table 1).

REPRESENTATIVE PATTERNS

Individual HPLC peaks may represent mixtures of mycolic acids whose exact composition has not been determined. This, however, does not affect the discriminating power of the technique: the identification of mycobacteria is in fact feasible on the basis of the number, the position (RRT) and the relative height of individual peaks present in a chromatogram. They are, in a good quality chromatographic separation, consistent parameters and allow the superimposition of profiles drawn from members of the same species.

Table 1. Peaks identification (modified from [38]). Single peaks are labeled with the name corresponding to the range in which their relative retention times (RRT) fall (the RRT of each peak is calculated by dividing its retention time by the one of the internal standard)

Peack name	RRT (range)
F1	0.438-0.459
F2	0.475-0.496
F3	0.495-0.515
F4	0.512-0.532
A1	0.532-0.551
A4	0.566-0.585
A5	0.594-0.612
M1	0.620-0.638
M2	0.640-0.657
M3	0.674-0.691
M4	0.694-0.710
M5	0.702-0.718
M6	0.724-0.740
B1	0.740-0.755
B2	0.760-0.775
B3	0.783-0.797
B4	0.801-0.815
B5a	0.816-0.830
B5b	0.832-0.845
B6	0.844-0.857
B7	0.862-0.875
B8	0.878-0.891
B9	0.895-0.907
E1	0.912-0.924
E2	0.927-0.939

Different mycobacterial species yield profiles with a great variety of peak arrangements, the two most common patterns being characterized by peaks crowded in one or two major clusters. Triple clusters and scattered peak sequences are also found less frequently.

The number of profiles published so far is mainly limited to the more common species; their gross features, along with respective references are summarized in Table 2.

Within the *M. tuberculosis* complex two different profiles are present [13, 14, 17]: that shared by *M. tuberculosis, M. bovis, M. africanum* and *M. microti* and that which characterizes *M. bovis* BCG (Fig. 4).

A unique HPLC pattern (Fig. 4) is produced by *M. kansasii* [13, 14, 18] although at least two different genotypes may be found within this species, only one of which reacts with commercially available DNA probes [19].

M. avium and *M. intracellulare*, confused in the *M. avium* complex (MAC), cannot be differentiated using conventional tests, while they may be differentiated by means of hybridization with commercial genetic probes. Their HPLC profiles (Fig. 5) are characterized, in most cases, by differences in relative heights of selected peaks which correlate with genetically identified single species [14, 18, 20]. The majority of the so-called X strains, i.e. isolates hybridizing with MAC-comprehensive DNA probe but not with the species-specific ones for *M. avium* and *M. intracellulare* [21], show the representative mycolic acid pattern of *M. intracellulare*. The pattern of *M. scrofulaceum* (Fig. 5), is not very different from the aforesaid patterns but is, however, usually recognizable [14, 22].

M. gordonae [13, 14, 20, 22, 23] is the only mycobacterial species of which two different HPLC patterns are known; the more frequent type is characterized by a single cluster of peaks (Fig. 4), while the two-cluster pattern (Fig. 5) is present in about 20% of isolates.

The detection of a group of mycobacteria with an HPLC profile (Fig. 5) resembling (but clearly not identical to) that of *M. xenopi* [20, 24], and thus called *M. xenopi*-like [20], was the first clue to the description of the new species *M. celatum* [25].

Table 2. Rough visual features of HPLC profiles of more frequently encountered mycobacterial species

Taxon	Peak arrangement	References
M. abscessus	Double late cluster	12, 31
M. asiaticum	Single late cluster	13
M. aurum	Major early cluster	40
M. avium complex	Double cluster	14, 18, 20
M. bovis BCG	Single late cluster	13
M. celatum	Double cluster	24, 27
M. chelonae	Double late cluster	12
M. conspicuum	Major early cluster	29
M. *fortuitum*	Double late cluster	12, 18, 31
M. gastri	Single late cluster	13
M. genavense	Triple late cluster	26, 27
M. *gilvum*	Triple early cluster	41
M. gordonae	Single or double cluster	13, 14, 20, 22, 23
M. interjectum	Double cluster	22
M. kansasii	Single late cluster	13, 14, 18
M. malmoense	Continuous late sequence	27
M. marinum	Single late cluster	13
M mucogenicum	Major early cluster	31
M. nonchromogenicum	Major early cluster	18, 28
M. obuense	Quadruple early cluster	41
M. parafortuitum	Double early cluster	40
M. peregrinum	Double late cluster	18
M. phlei	Major early cluster	17
M. poriferae	Major early cluster	40
M. pulveris	Double early cluster	41
M. scrofulaceum	Double cluster	14, 22
M. simiae	Triple late cluster	26, 27
M. szulgai	Single late cluster	13, 18
M. terrae	Major early cluster	28, 29
M. tuberculosis complex	Single late cluster	13, 14, 17
M. xenopi	Double cluster	20, 24

Three late clusters of peaks are the signature of *M. simiae* and *M. genavense* (Fig. 6). However, despite the similarity, an accurate identification of peaks allows their recognition [26, 27].

HPLC greatly simplifies the identification of species included in the *M. terrae* complex (Fig 7). While the similar profiles of M. *terrae* and *M. nonchromogenicum* are characterized by distinct RTs of peaks in the first cluster [18, 28, 29], a completely different pattern is presented by *M. triviale.*

Different patterns (Fig. 8) have been reported for the two subspecies of *M. chelonae* for which recently the elevation to species status has been acknowledged [30]. Although similar, they allow the differentiation of *M. chelonae* [12] from *M. abscessus* [12, 31]. The differentiation of *M. chelonae-like o*rganisms (Fig. 8) is straightforward and they have recently been included in the novel species *M. mucogenicum* [32] because of their evidently '*M. chelonae*-unlike' HPLC pattern [31]. So far undecided is the HPLC status of the species related to *M. fortuitum* (Fig. 8). The majority of authors [12, 33] assert the inability of the technique to distinguish between the new species into which the previous taxon *M. fortuitum* has been split [30, 34] *(M. fortuitum, M. peregrinum* and the two unnamed taxa of the *M. fortuitum* third-biovariant complex). However, the pattern of *M. peregrinum* [18] and two evidently different chromatograms of *M. fortuitum* [18, 31] are reported in the literature. Moreover, in a third paper [12], a unique profile, fortunately identical to one of the two just cited about *M. fortuitum* is assigned to the three species,

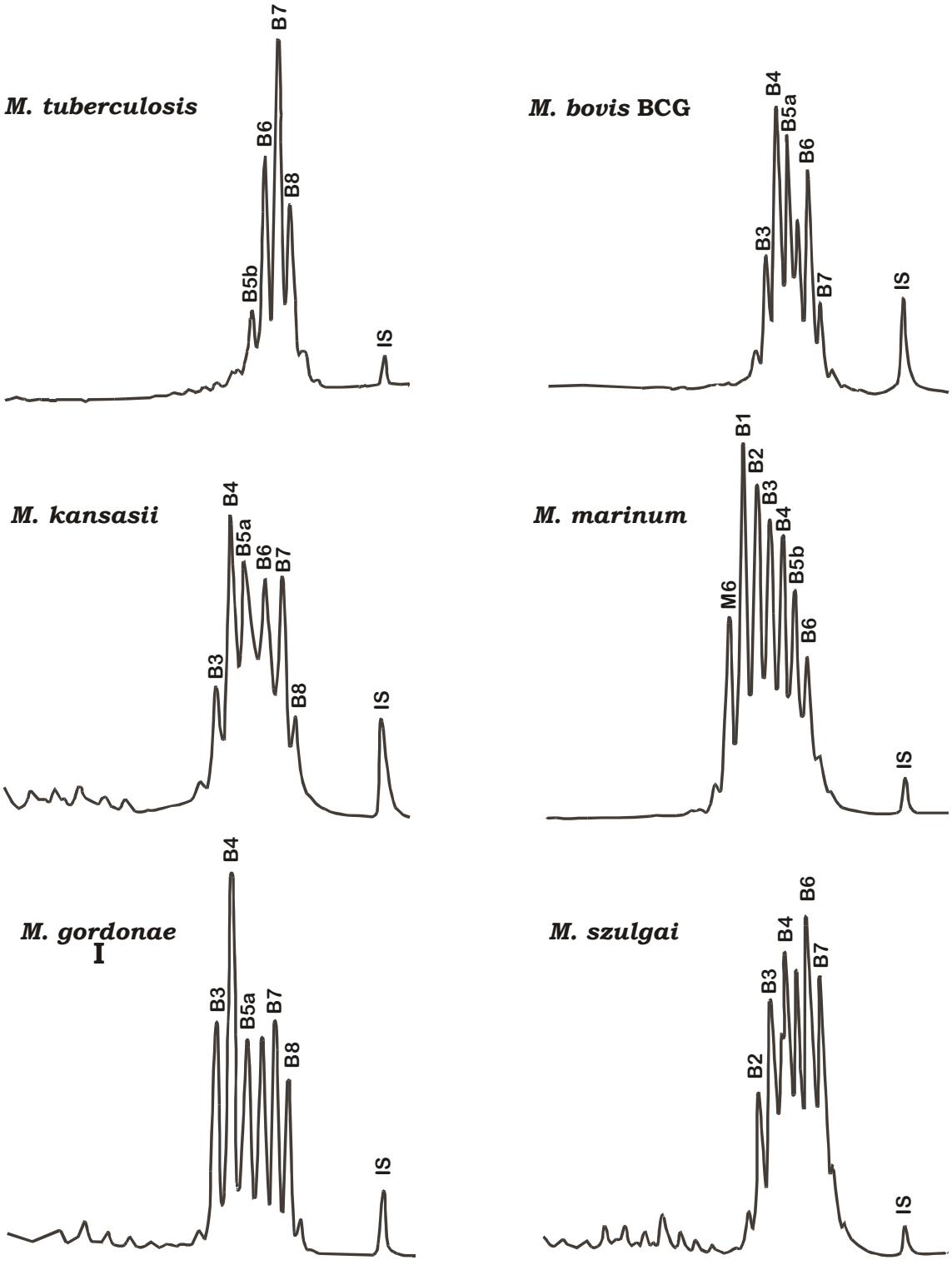

Fig. 4. Representative chromatograms of several mycobacterial species characterized by single clusters of peaks.

or rather, to the three biovars included in the species *M. fortuitum* which, at that time, was undivided. In repeated analyses which we performed at 1 week intervals on the same *M. fortuitum* culture, both profiles were obtained. Furthermore, the presence of *M. fortuitum* isolates characterized by atypical HPLC profiles has been reported [33, 35].

FUTURE PROSPECTS

Although HPLC of mycolic acids is a rapid technique, it requires bacterial colonies grown in culture and this step can take from one to several weeks according to the mycobacterial species. However, efforts have recently been devoted to the use of HPLC analysis on early cultures or even

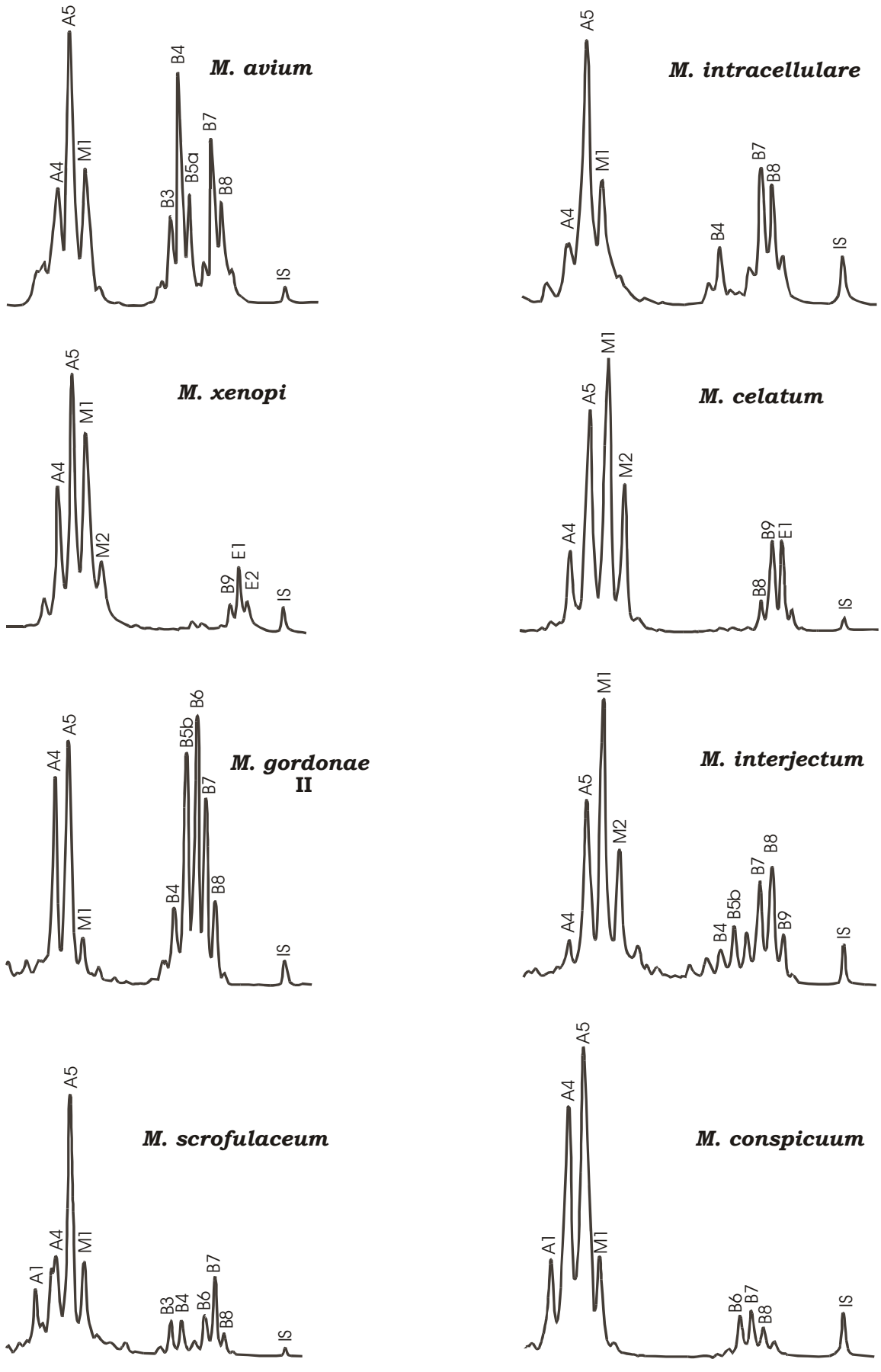

Fig. 5. Representative chromatograms of several mycobacterial species characterized by two clusters of peaks.

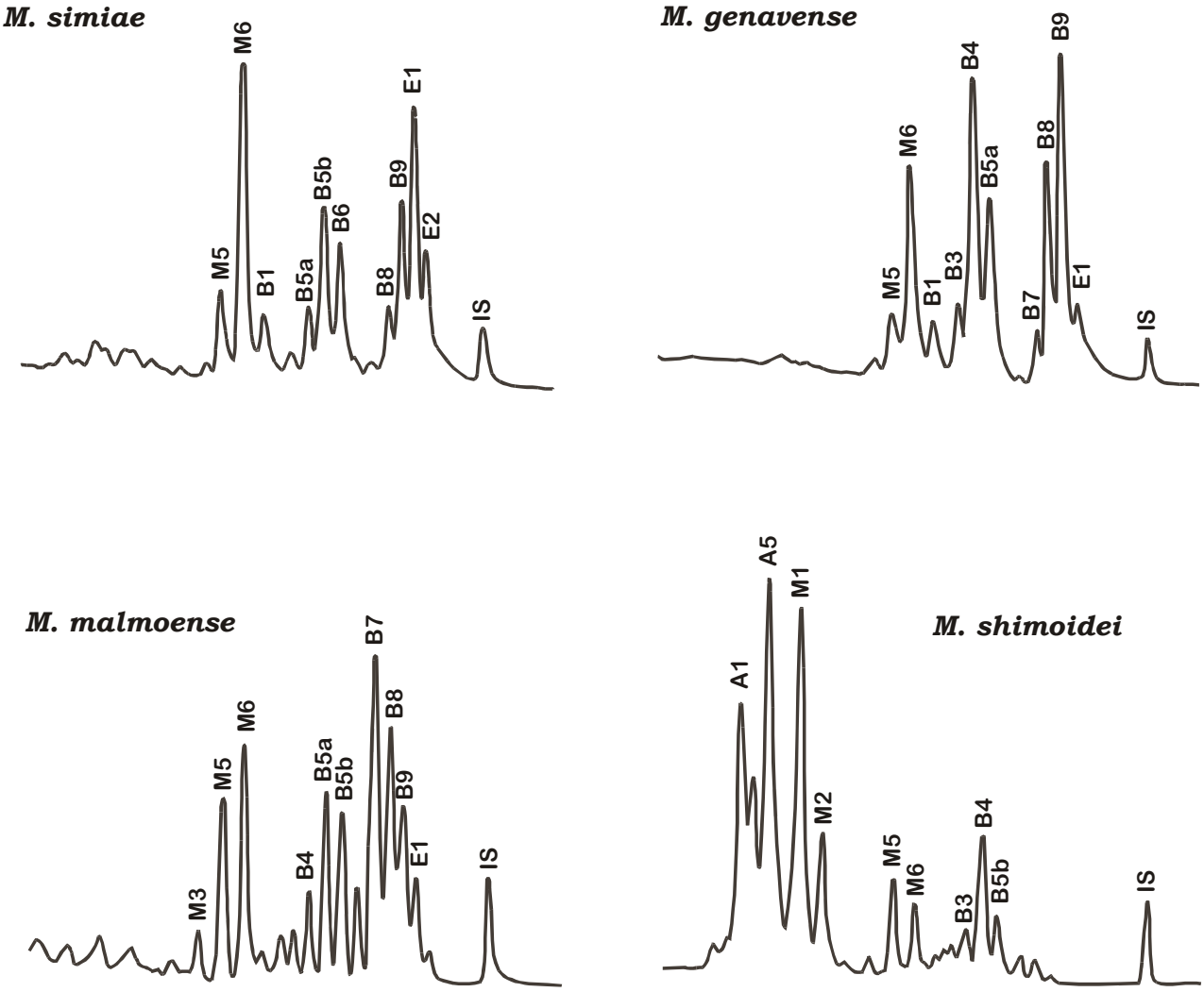

Fig. 6. Representative chromatograms of several mycobacterial species characterized by complex peaks arrangements.

directly on clinical samples.
Some results have been achieved with early radiometric cultures, still producing very low signals. A sufficiently recognizable profile can be obtained after an average of 20 days from the inoculation of the sample. The addition of oleic acid-albumin-dextrose-catalase enrichment to the radio-labelled vial, some days before the HPLC analysis, and the centrifugation of the whole broth culture, are, however, needed 136].

The first attempts to detect mycolic acids of *M. tuberculosis* and MAC directly on smear-positive clinical specimens were made very recently. However, fluorescence detectors which are approximately 200-fold more sensitive than UV ones, seem to be required for the analysis of the very low amounts of mycolic acids which may be found in these kinds of sample [37]. Wider experimentation is required to ascertain whether this technique has the potential for competing with nucleic acid amplification systems for the early detection of mycobacteria in uncultured samples.

The recognition of profiles is a critical topic in the HPLC-based identification of mycobacteria. The first attempt to overcome this problem and to put the HPLC approach into the reach of the less experienced analysts has led to the development of decision trees which, once fed with the height ratios of selected peaks, direct the microbiologist to the correct identification of the strain [13, 20]. The more sophisticated evolution of this is represented by a computer-aided pattern recognition algorithm [18, 38]. Field validation of both approaches has given very promising results, with high rates of correct identifications [13, 20, 33, 38].

The computer-assisted approach will, before long, probably replace the visual recognition of chromatograms of frequently encountered mycobacterial species. Interpretation of the less

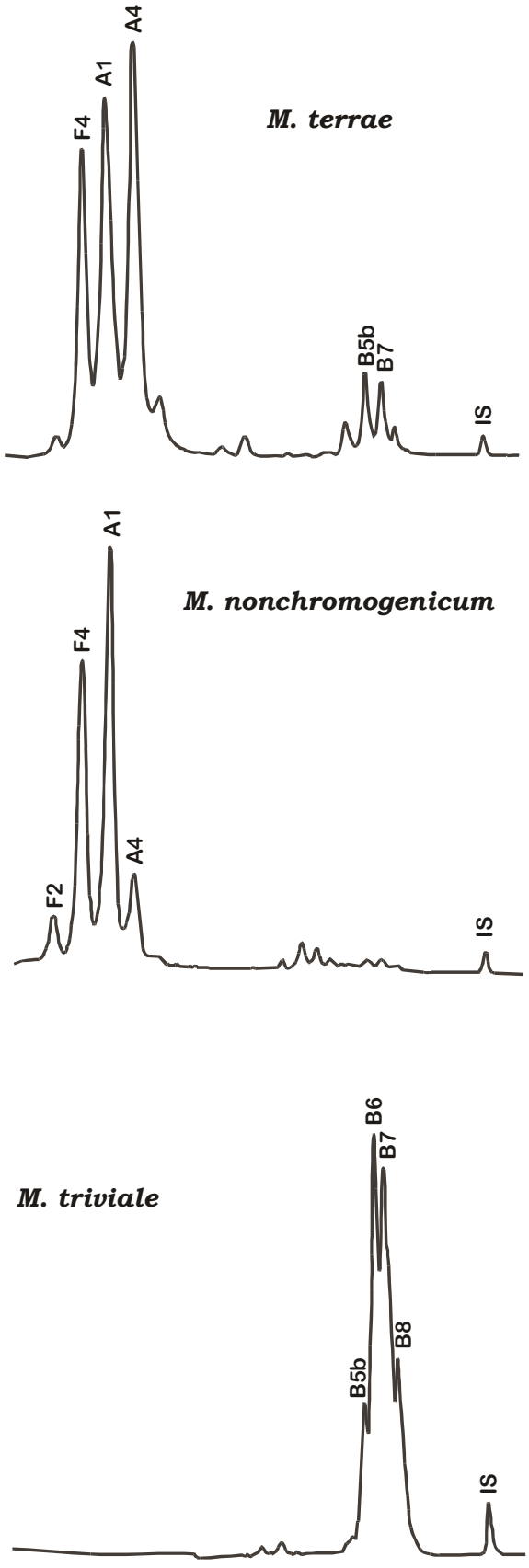

Fig.5. Representative chromatograms of species included in the *M. terrae* complex.

common profiles will, however, still benefit from visual inspection by a skilled mycobacteriologist.

CONCLUSIONS

The long-standing need for a reliable method for rapid identification of mycobacteria is becoming acute because of the steady increase in the number of species, possibly of different clinical significance and antimicrobial susceptibility, which are involved in infections, particularly in immunocompromised patients.

HPLC is indeed a rapid method, as the whole procedure may be performed in 3 hours or less, much less than the weeks needed by conventional procedures. In comparison with the equally rapid technique of genomic hybridization, HPLC has the advantage of being suitable for the identification of most mycobacteria. Nucleic acid probes are in fact commercially available for a limited number of species, which, though they are the ones encountered more frequently, are also the less problematic. Most importantly, in the presence of an unknown mycobacterium, HPLC analysis provides, instead of a negative result, an unusual profile, alerting the possibility of a previously unrecognized species. This adds an heuristic value to the technique, making it also suitable for taxonomic research.

In comparison with other strategies of lipid analysis, HPLC has a clearly better species-specificity and discriminatory power. Thin-layer chromatography and gas-liquid chromatography exhibit a limited number of patterns, many of which are shared by different and often unrelated species [39]. The consistency of mycolic acid patterns within the various species of mycobacteria is remarkable if compared with the variability of other phenotypic characters. Only minor variations, possibly related to the age of the cultures tested, have in fact been detected with a few rapidly growing isolates.

The occurrence of patterns common to more than one species is not unusual in conventional identification procedures; however, shared HPLC profiles are, if ever, very rare. In many cases a gross similarity can be resolved with peak identification by means of RRTs. Only when the recognition of two profiles relies on different heights of the same peaks may some difficulty exist. This ambiguity has been noted mainly between a few strains of *M. scrofulaceum* and MAC [35].

The need for repetitions of tests because of profiles that are not well defined, due to poor preparation or

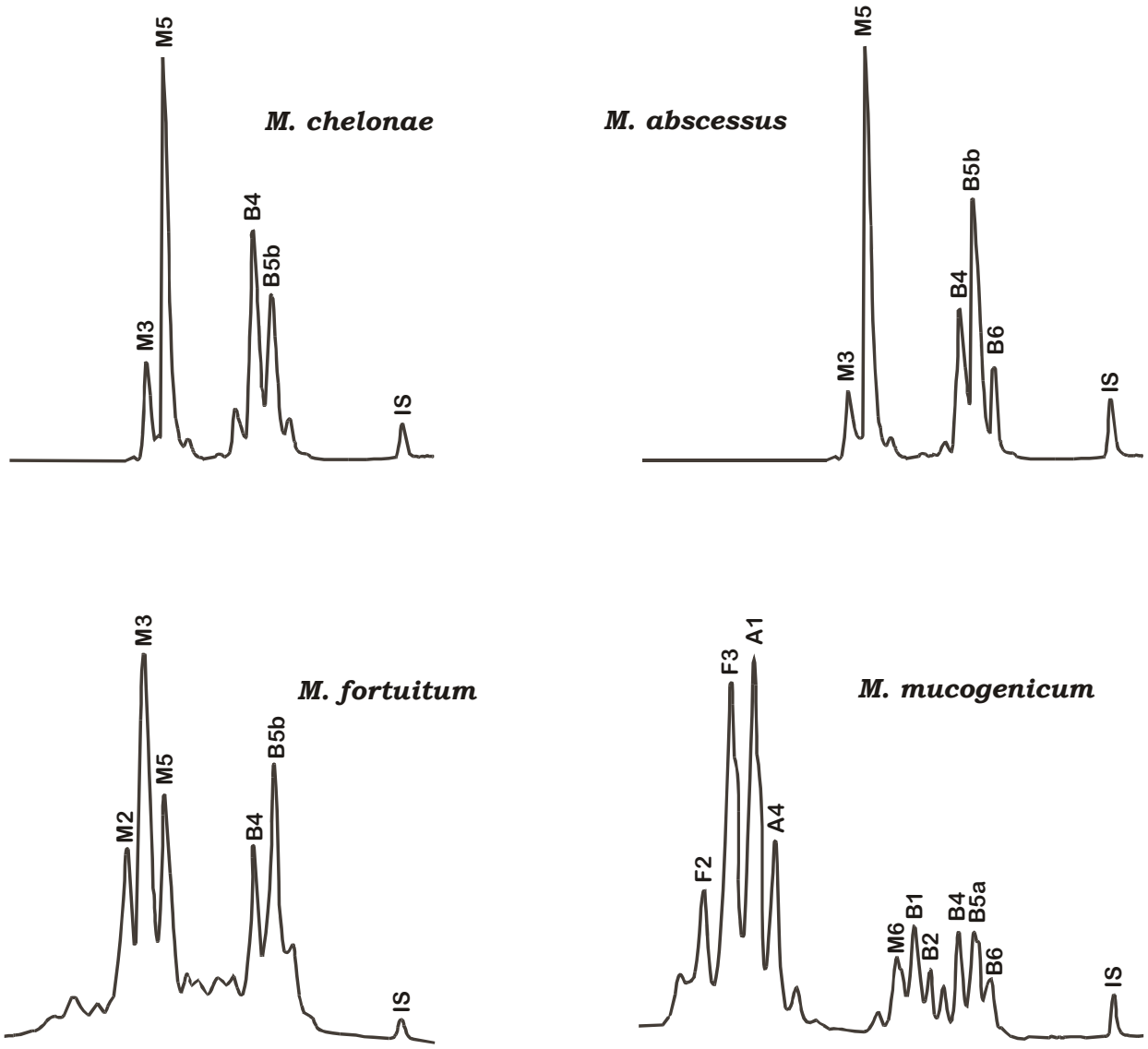

Fig. 8. Representative chromatograms of several rapidly growing mycobacterial species.

elution of a sample, are rare and do not exceed 5% of cases.

The presence of mixed mycobacterial cultures is a major problem with almost every identification procedure. Under these circumstances misleading chromatograms are plotted when HPLC analysis is used and the very unusual profile arising from the superimposition of patterns is, however, often an indication of the anomaly.

Apart from the HPLC equipment (the price of a basic model, inclusive of the controlling software, is about $30 000) the cost of each identification is quite low: materials do not exceed $5, and the personnel time has been estimated to be 45 min per run [33].

The recent adoption of a standardized labelling system has finally overcome the confusion characterizing many previously published profiles, the peaks of which were arbitrarily and differently identified. This will certainly help, allowing a more accurate comparison of profiles and acceptance of the technique. The reliability of HPLC used alone for the speciation of mycobacteria has been assessed on a very large number of strains [33, 35]. Rapidity, reproducibility, ease of execution, cost effectiveness, suitability and discriminating power for the largest number of species make this approach an excellent primary method for the identification of mycobacteria in a level III laboratory.

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