Published in : Analytical and Bioanalytical Chemistry (2018), vol. 410, pp. 7987-7996

DOI: 10.1007/s00216-018-1421-z

Status : Postprint (Author's version)

INVESTIGATION OF MYCOBACTERIA FATTY ACID PROFILE USING DIFFERENT IONIZATION ENERGIES IN GC–MS

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ABSTRACT

Gas chromatography (GC) coupled with electron ionization (EI) mass spectrometry (MS) is a well-established technique for the analysis of volatile and semi-volatile compounds. The main advantage is the highly repeatable fragmentation of the compounds into the

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ion source, generating intense and diagnostic fragmentation when the ionization is performed at 70 eV; this is considered the standard ionization condition and has been used for creating many established databases, which are of great support in the analyte identification process. However, such an intense fragmentation often causes the loss of the molecular ion or more diagnostic ions, which can be detrimental for the identification of homologous series or isomers, as for instance fatty acids. To obtain this information chemical or soft ionization can be used, but dedicated ion sources and conditions are required. In this work, we explored different ionization voltages in GC–EI–MS to preserve the intensity of the molecular ion using a conventional quadrupole MS. Twenty, 30, 50, and 70 eV were tested using a mixture of fatty acid methyl esters standards. Intensity and repeatability ofthe most informative ions were compared. Twenty and 70 eV were then used to analyze the fatty acid composition of six different strains of mycobacteria. Two approaches were used for elaborating the data: (1) a single average spectrum of the entire chromatogram was derived, which can be considered (in terms of concept) as a direct EI–MS analysis; (2) the actual chromatographic separation of the compounds was considered after automatic alignment. The results obtained are discussed herein.

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KEYWORDS : ELECTRON IMPACT IONIZATION -- GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC–MS) -- FATTY ACID METHYL ESTERS (FAMES) -- Mass spectrometry -- Bacteria

Introduction

Electron impact (EI) ionization mass spectrometry (MS) is a well-established detection method for the analysis of volatile and semi-volatile compounds. Traditionally, EI ionization uses electrons of 70 eV kinetic energy. Although every molecular species has an ionization efficiency curve that is dependent on the ionization cross-section of the specific molecule, the use of 70 eVof kinetic energy usually exceeds the ionization energy required for most volatile and semivolatile molecules (generally below 15 eV) [1]. Under the 70 eVionization condition, intense and diagnostic fragmentation of the molecules occurs in a highly repeatable pattern, which is the basis of identification and quantitation. Therefore, the use of 70 eV has been considered the standard and many reference databases (e.g., NIST MS database) have been built using this condition. However, the intense fragmentation occurring at 70 eV can cause the loss of the molecular ion and can reduce the intensity of highly informative diagnostic ions. This fact can be detrimental to

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the identification of unknown analytes, homologous series, or isomers, as in the case of fatty acids analysis. The use of a gas chromatography (GC) step prior to MS detection supports the identification of many compounds based on their retention time or linear retention index (LRI), even if this is not always enough to avoid an ambiguous identification. These shortcomings have led to continual demand for lower-energy or Bsoft^ ionization techniques in MS, as well as the development of alternative detectors able to support compound identification, such as the vacuum ultraviolet detector which is discussed elsewhere [2, 3].

Regarding the MS detection, several soft ionization techniques have been developed over the years, such as chemical ionization [4, 5], field ionization [6, 7], and photoionization [8, 9]. A detailed discussion (advantages and limitations) of these techniques beyond the scope of this work and the reader is directed to the cited references for further details. An interesting alternative, rigorously studied in the 1980s by Maccoll [10, 11], is the use of lower electron kinetic energy and lower ion source temperature (< 100 °C) in the conventional EI ionization. The lower collision energy can possibly minimally exceed the ionization energy leading to less fragmentation and thus a higher signal of the molecular ion. However, this approach has never been routinely

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applied, since lower fragmentation repeatability and a general loss of sensitivity due to lower ionization efficiency are generally reported in the literature [1, 11]. However, few studies have been carried out since the 1980s to verify the performance of modern instruments under different ionization conditions [12–15].

In this work, we explored different ionization voltages using a conventional quadrupole MS, coupled to a prior gas chromatographic separation. Twenty, 30, 50, and 70 eV were evaluated via the use of two different mixtures of fatty acids methyl ester (FAME) standards. Furthermore, the advantages of a « softer » ionization energy were investigated for classification and identification support of the FAME profiles of six different species of mycobacteria.

Materials and methods

CHEMICAL AND REAGENTS

Methanolic trimethylsulfonium hydroxide solution (0.25 M), the 37-component FAME mixture (Supelco® 37 Component FAME Mix), and bacterial fatty acid methyl ester

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(Bacterial Acid Methyl Ester (BAME) Mix, Supelco) mixture were kindly provided by Supelco (Bellefonte, PA, USA). Methanol and tert-butyl methyl ether were of GC-grade and were purchased from Merck (Bellefonte, PA, USA).

SAMPLE PREPARATION

BACTERIAL STRAINS, CULTURE CONDITIONS, AND SAMPLE PREPARATION

Six mycobacteria species [*M. abscessus* (ABS), *M. bolletii* (BOL), *M. massiliense* (MAS), *M. avium* (AVI), *M. chimaera* (CHIM), and *M. bovis* BCG (BCG))] were used for all experiments. The considered species belong to three different mycobacterium complexes: (1) *Mycobacterium tuberculosis complex* (MTB), which includes BCG; (2) *M. avium complex* (MAC) which includes AVI and CHIM; (3) *M. abscessus complex* (MAB), which includes ABS, MAS, and BOL. All species were cultured aerobically (30 mL, 37 °C, 200 rpm shaking) in Difco Middlebrook 7H9 Broth (Becton Dickinson, Franklin Lakes, NJ) containing Tween 80, glycerol, and 10% Difco Middlebrook ADC enrichment (BD, Franklin Lakes, NJ). Bacterial growth was evaluated by measuring the optical density of the culture at 600 nm (OD_{600}). After an OD_{600} of 2.0–2.5 was reached, cultures were transferred to 50-mL conical flasks, placed on ice to arrest metabolism, and centrifuged (8000 rpm, 4 °C, 10 min). Five

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milliliters of culture supernatant was transferred to a 20-mL airtight glass headspace vial and used for other experiments, while the pellet was collected for the FAME analysis. Both samples were immediately stored at –20 °C until analysis. Three biological replicates were prepared per each strain (starting from the culture of the bacterium), for a total of 18 samples.

Ten milligrams of the pellet containing the bacteria cells was harvested and transferred to an Eppendorf tube. Bacteria cells were suspended in 10 μL of distilled water, and then 30 μL of methanolic trimethylsulfonium hydroxide solution (0.25 M) was added to simultaneously lyse the cell and transesterify the lipid fraction, according to the procedure reported by Müller et al. [16]. The reaction mixture was dried under a nitrogen stream and then dissolved in 200 μL of tertbutyl methyl ether/methanol (MeOH) mixture (10:1 v/v) and directly injected into the GC–MS system.

ANALYTICAL INSTRUMENTATION

All GC–MS applications were carried out on a Shimadzu GC2010 and a TQ8050 triple quadrupole mass spectrometer (Shimadzu, USA) equipped with an AOC-6000 autosampler. The single quadrupole acquisition mode was exploited on the TQ8050 MS.

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Injection volume was 1 μL in split mode (ratio 1:50). Data were acquired by using the GCMS solution software ver. 4.45 (Shimadzu).

The column employed was an SLB-5ms [(silphenylene polymer, practically equivalent in polarity to poly(5% diphenyl/95% methylsiloxane)], with the following dimensions: 30 m × 0.25 mm ID × 0.25 μm d_f (Supelco, Bellefonte, USA). The following GC temperature program was used: 50 °C (hold 1 min) to 300 °C at 5 °C/min, then to 350 °C at 20 °C/min. Helium head pressure (constant linear velocity mode 35 cm/s) was 48 kPa. Injection temperature, mode, and volume were 280 °C, split ratio 1:50, and 1 μL, respectively. A faster GC temperature program was run for analyzing the standards mixture starting at 130 °C; all other conditions were the same. MS conditions are reported in Table 1 and the electron ionization was 20, 30, 50, and 70 eV; filament emission current was manually adjusted for each electron ionization energy in accordance with the instrument operational manual, to 45, 50, 55, and 60 μA, respectively. Detector voltage was set according to the automatic tuning results, as with the other MS parameters (Table 1). The MS system was run in full-scan conditions: scan speed 2000 amu/ s; mass range 45–400 m/z. Interface and ion source temperatures were 200 and 250 °C, respectively.

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STATISTICAL ANALYSIS

Raw GC/MS data sets were post-processed using R-based package XCMS [17] (http://metlin.scripps.edu/download/) to create a compounds and intensity table, using 10 S/N threshold and extracting the most abundant mass ion for each peak. All statistical analyses were performed using R v3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

The data matrix obtained after alignment was first reduced on the basis of a frequency of observation cutoffs of 50 % (features present in at least two out of three samples within one class were retained for further statistical evaluation). Prior to statistical analyses, the relative abundance of compounds across chromatograms was normalized using probabilistic quotient normalization (PQN) [18] and log-transformed. To test for statistical significance, the Kruskal–Wallis (KW) test [19], with post hoc Dunn test and Benjamini–Hochberg (BH) correction [20], was used. A significance level of $p < 0.05$ was selected.

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Results and discussion

Since the systematic works carried out by Maccoll in the 1980s [10, 11], only sporadic papers explored the possibility to use low electron kinetic energy in EI ionization [12–15]. This can be explained, as suggested by Tranchida et al. [15], by the inherent advantages of the conventional ionization energy EI that « have inevitably put GC–MS users into a cozy routine ».

The goal of the present work is to verify whether the common drawbacks reported for the use of lower electron kinetic energy, namely low fragmentation repeatability and lower sensitivity [1], are still relevant on a modern instrument. Moreover, the advantages of less intense fragmentation are investigated herein for classificationand identification support of the FAME profiles of real-world samples. In fact, univocal identification of FAMEs may, in some case, be cumbersome. Although many ofthem can bechromatographically separated and assigned with the aid of the LRI filter, a confirmation of their identity through careful evaluation of the mass spectrum can be of great support, especially in real-world samples. Therefore, we wish to stress that this

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work will focus on the effect of different ionization energies on the MS profile and will not emphasize the role and utility of chromatographic separation.

COMPARISON OF DIFFERENT IONIZATION ENERGIES

The sensitivity, which was reported to be sacrificed using a lower ionization energy [1], was evaluated by analyzing a FAME standard mixture at 20 eV and 70 eV using the same detector voltage value (equal to 1 kV) (see Electronic Supplementary Material (ESM) Fig. S1). As reported in the literature [1], the signal intensity (TIC area) and the signal-tonoise ratio at 20 eV were lower compared to those at 70 eV (on average about 30% lower). However, when all the MS parameters, including the detector voltage were set on the basis of the auto-tuning conditions (adjustment based on the fragmentation of perfluorotributylamine, PFTBA), the loss in sensitivity was compensated, especially at 20 eV. ESM Fig. S2 reports the absolute TIC areas of the BAME mixture obtained using the different auto-tuning conditions reported in Table 1.

The effect of the different ionization energies (20, 30, 50, and 70 eV) on the fragmentation pattern was evaluated by applying the respective auto-tuning setting and by considering the relative intensity of the ions in the mass spectrum, which is generally not affected by

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the absolute intensity. Two standard mixtures of FAMEs (see ʙMaterials and methods^) were analyzed to cover a wider range of different fatty acids. Characteristic fragments from different classes of fatty acids were selected to assess whether the fragmentation patterns were consistent and repeatable and to measure the profile changes. The main ions that generally characterized the fragmentation of carboxylic acid derivatives resulted from αcleavage and the McLafferty rearrangement. The former may occur at either side of the carbonyl group to produce $[M - CH_3O]^+$ and $[COOCH_3]^+$. While the McLafferty rearrangement strictly refers to loss of an alkene from molecular ions of saturated carboxylic acids, it can be extended to any alkene loss that can be described as a transfer of a γ-H to a double-bonded atom through a six-membered transition state [1]. ESM Tables S1–S3 report the relative intensity of the diagnostic ions for both standard mixtures divided into saturated, unsaturated, hydroxylated, and other (iso-, anteiso-, and cyclopropyl-) FAMEs. The overall repeatability of the fragmentation was evaluated by considering the coefficient of variation (CV%) of the relative intensity over three replicates.

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Table 1. *MS tuning energy conditions*

Electron ionization energy (eV)	Filament emission current (μA)	Detector voltage (V)	Lens 1	Lens 2	Lens 3	Lens 4	Q pre-Rod
20	45	1.22	2.3	– 14.1	– 1.2	– 21.2	– 3.5
30	50	1.17	– 1.0	– 16.1	– 1.5	– 17.4	– 16.0
50	55	1.08	– 0.3	– 19.0	– 0.7	– 10.2	– 3.5
70	60	1.04	– 0.2	– 19.5	– 0.5	– 10.4	– 3.5

The averaged CV% values among the different classes were consistent at the different ionization energies, being about 2.0% for saturated (2.4% at 20 eV and 1.7% at 70 eV) and about 3% for the unsaturated, hydroxyl, and other FAMEs at all the ionization energies tested. Figures 1 and 2 and ESM Fig. S3 report the comparison of the relative intensity of the molecular ion using the different ionization energies for each compound. In all cases, the theoretically expected trend was confirmed with an increment of the intensity of molecular ion by decreasing the ionization energy. Moreover, the intensity of the molecular ion generally increased with the length of the carbon chain, as a result of the stabilization of the charge within the molecule, although the presence and position of double bonds, hydroxyl, or methylene groups can influence this pattern [21]. This general trend is clearly depicted in Fig. 1, which illustrates the series of saturated FAMEs.

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The intensity of the molecular ion at 20 eV was about 4.5 times higher than at 70 eV for Me.C6:0 and Me.C8:0 (low chain FAs), while about 2-fold higher for all the others (higher MW). For the unsaturated FAMEs up to a carbon chain of C18, the same trend of an increase of carbon chain length was observed with an average increment of about 3-fold between 20 eVand 70 eV. Longer unsaturated FAMEs (> C18) do not follow this trend, although the molecular ion [M^+] remained significantly more intense at 20 eV compared to 70 eV. In general, molecular ion intensity decreases with the increment of the double bond number in the FA backbone, but in all cases the molecular ion [M^+] remained significantly more intense at 20 eV compared to 70 eV. For instance, an increment of 3.6-fold and 2.4-fold was observed for the molecular ion [M^+] at 20 eV compared to 70 eV for Me.C20:5n3 and Me.C22:6n3, respectively (Fig. 2), facilitating its detection.

Finally, the relative intensity bar plot depicting the molecular ion intensity of other FAMEs (hydroxyl and methyleneFAMEs) is reported in ESM Fig. S3. The same trend related to the length of the carbon chain and the influence of the substituent group was observed.

The less intense fragmentation, along with the higher intensity of the most diagnostic fragments (when lower ionization energy is applied), is particularly important when real-world samples are analyzed. In fact, small changes in instrumental parameters,

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imperfect chromatographic resolution, and background noise can mask some informative fragments. Some examples are discussed herein to highlight the benefits that can be derived from using lower ionization energy.

Fig. 1 *Molecular ion relative intensity of saturated FAME series*

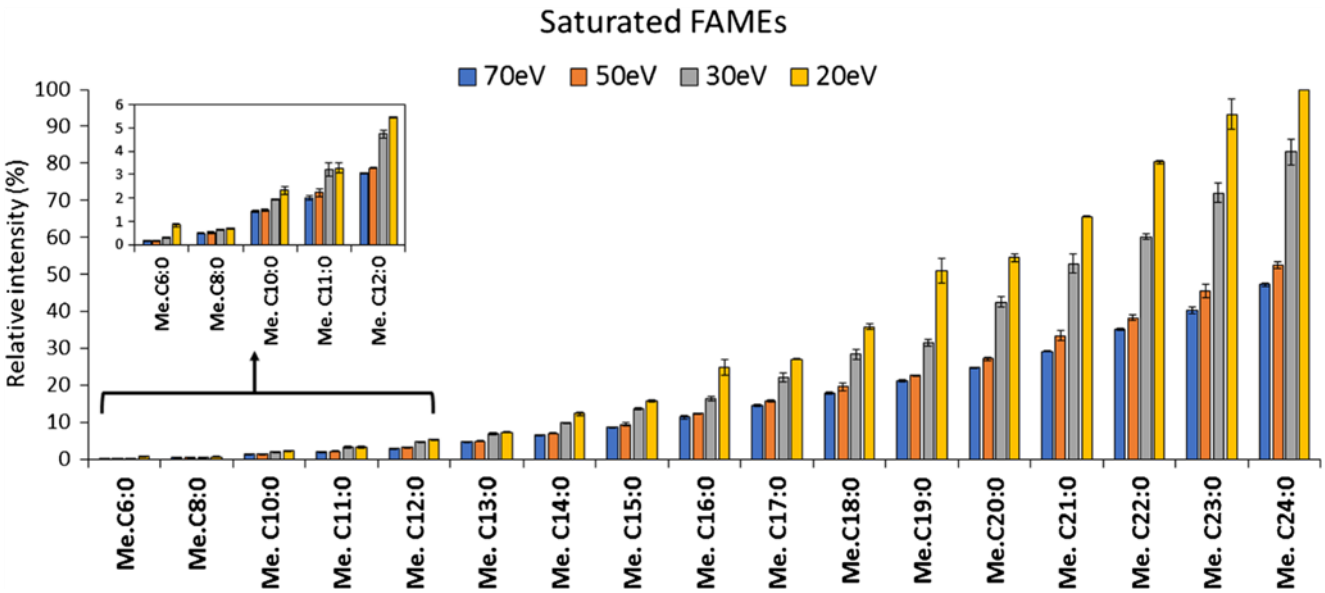

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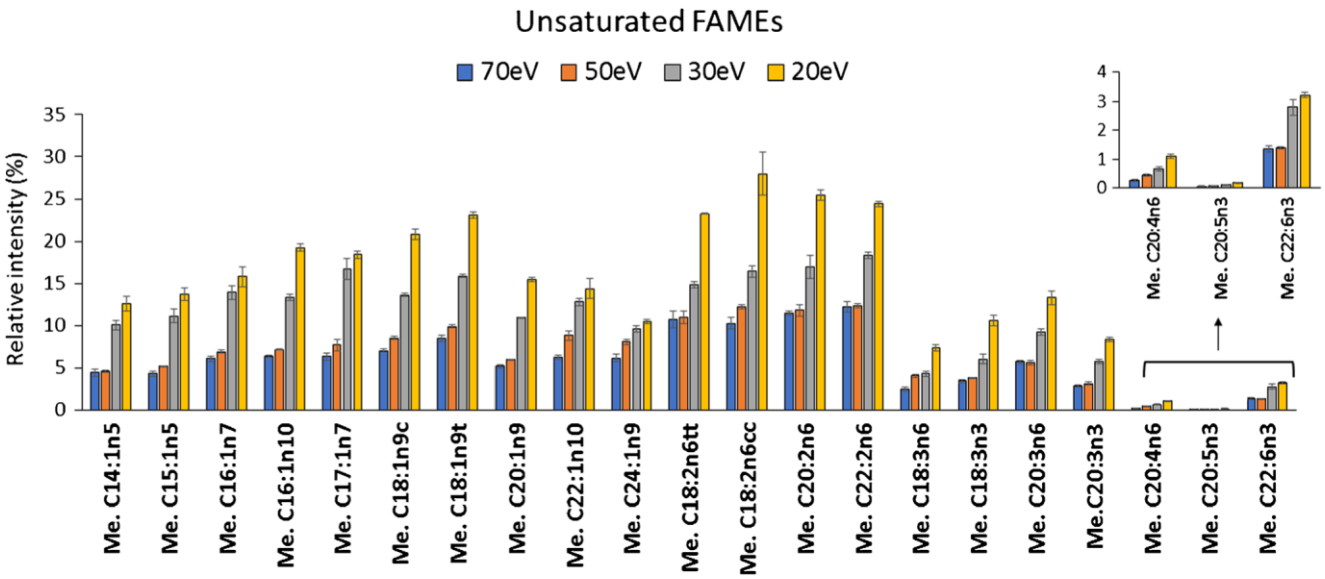
***Fig. 2** Molecular ion relative intensity of unsaturated FAME series*



Generally, there are no fragments that permit location or stereochemistry determination of the double bond in positional isomers, because the double bond can migrate to any position when the alkyl chain is ionized in the MS [1, 21]. This is particularly true for mono- and dienoic fatty acids. However, the position of non-conjugated double bonds in polyenoic fatty acids (≥ 3 double bonds) can be elucidated by the presence of some diagnostic ions [21]. Figure 3 reports a comparison of the mass spectra obtained at different ionization energies for Me.C18:3n3 and Me.C18:3n6 (all-cis). An increase of intensity of the diagnostic fragments deriving from the α-fragmentation, at 236 m/z and 194 m/z, respectively, and the ω-fragmentation, 108 m/z and 150m/z, respectively, can be observed by reducing the ionization energy, thus simplifying the assignment of the

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FAME isomer. The relative intensity of the molecular ion and the α- and ωfragments was not significantly different ($p > 0.05$) between 70 eV and 50 eV, but did increase significantly ($p < 0.05$) at 30 eV and 20 eV. They increased about 2.5–3 times in the spectra obtained at 20 eV; in particular, the ω-fragment of Me.C18:3n3 was almost the base peak at 20 eV (94.8%) compared to 70 eV (37.3%), where it was 2.5 times lower. The two C18:3 FAME standards, indeed, were not distinguishable on the basis of the mass spectrum at 70 eV (both library match over 90%), while the mass spectra acquired at 20 eV enhanced the different intensities of the diagnostic ions.

Another example is the comparison of the cyclopropyl FAME and the unsaturated analogue with an alkyl chain one carbon longer, which presented a very close library match (both matches were > 90% for both peaks), since the cyclopropyl ring appeared to rearrange giving a fragmentation similar to a double bond. Figure 4 shows the mass spectra of Me.9,10-methylene-C16:0 and Me.C17:1n7. The only ion that may be considered diagnostic was the molecular ion (282 m/z), which was higher in the unsaturated fatty acid rather than in the cyclic, isobaric analogue. With both high and low voltage, the molecular ion was 7-fold higher in the unsaturated FAME, although using 20 eV the molecular ion was about three times higher than using 70 eV in both cases

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([Me.C17:1n7]$^+$ = 18.4% versus 6.3% and [Me.9,10-methyleneC16:0]$^+$ = 2.9% versus 0.9% at 20 and 70 eV, respectively).

The mass spectra of hydroxyl FAMEs give highly specific patterns that help locate the position of the –OH group [21]. In fact, the McLafferty ion differs according to the –OH position, being 74 m/z in 3-OH moieties and 90 m/z for 2-OH.

Furthermore, the 2-OH moieties are characterized by an intense fragment corresponding to [M – $COOCH_3$]$^+$, which increased by about 1.5 times at 20 eV compared to 70 eV. The most informative fragment in the 3-OH moieties fragmentation pattern was associated with the α-cleavage (103 m/z) of the carbon carrying the oxygen atom: this was observed in the two standards contained in the mix (Me.3-OH C12:0 and Me.3-OH C14:0), in which it remained the base peak regardless of the ionization energy used. Rarely, the molecular ion was of significant intensity at 70 eV, but using 20 eV we had an average intensity increase of about 7-fold (ESM Table S3).

Considering that the most relevant differences were observed at 20 eV, this ionization energy, along with the conventional 70 eV, were used for evaluation of real-world samples.

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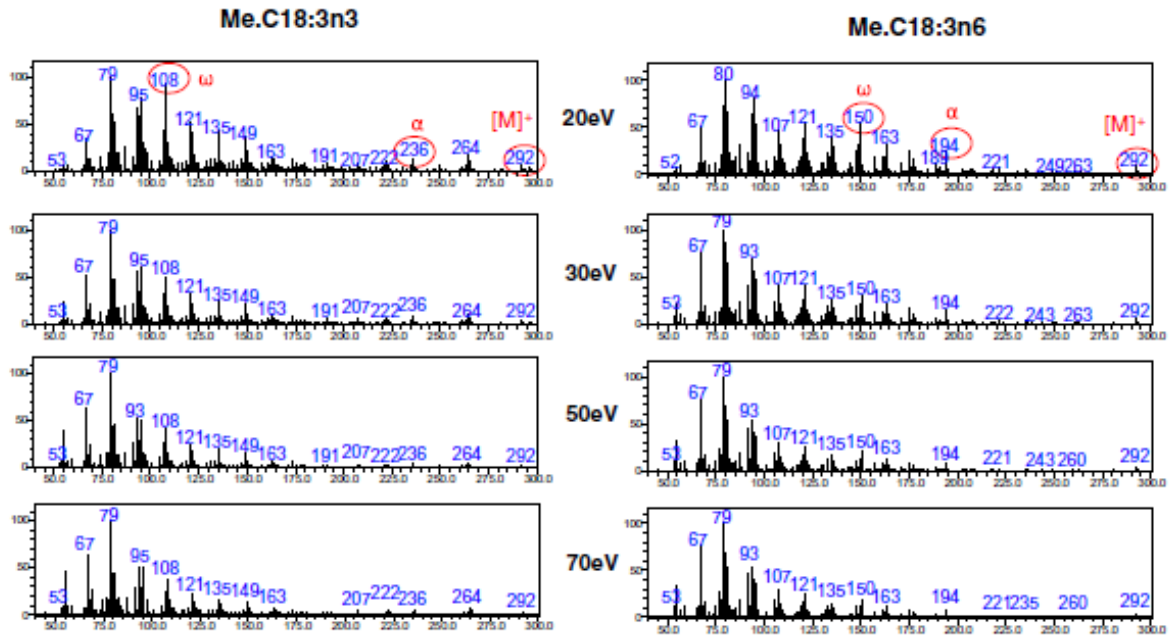
Fig 3



Mass spectra of Me.C18:3n3*ccc* and Me.C18:3n6*ccc* obtained at different electron ionization energy

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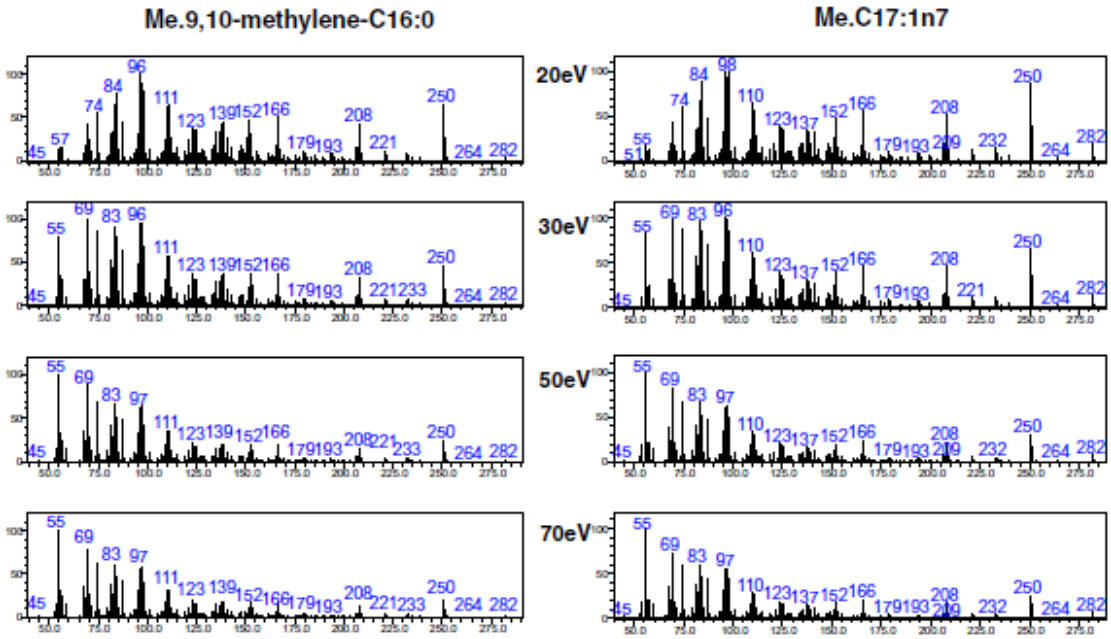
Fig 4



Mass spectra of Me.9,10-methylene-C16:0 and Me.C17:1n7 obtained at different electron ionization energy. Molecular ion 282 *m/z*

REAL-WORLD SAMPLE ANALYSES

The fatty acid profile has been extensively used for taxonomic classification of bacteria [22–26] and commercial databases are available to support their identification [27]. Bacterial species from three different mycobacterium complexes were included in this study: (1) the MTB complex (BCG), (2) the MAB complex (MAS, ABS, and BOL), and (3) the MAC complex (AVI and CHIM). All the samples were analyzed twice using the same GC

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conditions, but once with the MS set at 70 eVand one at 20 eV. The data obtained were elaborated with two different approaches: (1) using the mass spectrum averaged from the entire chromatogram (TIC) and considering ion fragments as the features for the data analysis, which can be conceptually seen as a direct EI–MS analysis; (2) considering the chromatographic separation and using peaks as the features for the data analysis. The results obtained are discussed separately herein.

SINGLE AVERAGED GC–MS SPECTRUM

The single average spectrum of the entire GC–MS chromatogram acquired at 20 eV was compared, in terms of diagnostic information gathered, to that acquired at 70 eV.

The MSaverage spectrum ofthe entireGC–MS(subtracted from the chemical noise at two points, at the beginning and at the end of the analysis) was obtained from all the samples and aligned. The two data matrices obtained (one from the samplesacquiredat 20eVand one from the samples acquiredat 70 eV) were treated separately, normalized, and log-transformed. Normalization was applied to minimize unwanted systematic variations between samples, and logarithmic transformation reduced the influence of larger values over trace compounds. Principal component analysis (PCA) was used to visualize the

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sample clustering after mean-centering and scaling. When the entire matrices were used, an overall explained variance of 68% and 57% was obtained for the analyses carried out at 20 and 70 eV, respectively (ESM Fig. S4).

A further data reduction step was carried out to minimize noise and redundancy, and to extract the most useful information. A multiple class test, the Kruskal–Wallis (KW) test [28], was performed to retain only features significantly different ($p < 0.05$) between groups after Benjamini–Hochberg (BH) correctiontominimize the false discovery rate [20]. Considering the 20 eV data matrix, a total of 256 featureswereretained. For samples acquired at 70 eV, no features resulted significantly different after the BH correction. These results can be explained by (1) an overall higher signal at 20 eV due to the higher detector voltage (1.22 V), which led to better distinction of the diagnostic fragments from the noise (less relative intensity of the bleeding fragments, such as 207, 281, and 355 m/z) ; (2) less abundance of low mass fragments such as 74 and 55 m/z in favor of higher intensity of more informative ions at 20 eV (Fig. 5).

As a result of the lack of statistical significance, no further elaboration was performed on the 70 eV matrix, while the data obtained from the 20 eV matrix was further visualized using the PCA on the 256 retained features giving a good discrimination between the six

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mycobacteria (Fig. 6). Species from the MAB complex were clustered as a group as well separately from each other. BCG, CHIM, and AVI were clearly separated, but the MAC complex was not clearly distinguishable as a single cluster.

CHROMATOGRAPHIC SEPARATION

The chromatographic FAME profile of every sample was then evaluated using the data acquired at 70 eV. Data acquired at 20 eV were used for identification support. The XCMS package in R was used to align the compounds across all the samples. The data matrix obtained was cleaned by removing siloxane and other contaminants. An additional filter was then applied by removing all the features that were not present in at least two samples within the same group, affording a final matrix of 29 peaks (the PCA score plot obtained using this refined matrix is reported in ESM Fig. S5). Then, as for the elaboration performed for the entire MS spectrum, a multiple class test (the Kruskal–Wallis test with BH correction) was performed to retain only features significantly different ($p < 0.05$) between groups. A total of 24 features were retained. The PCA score plot obtained using these significantly different features is reported in Fig. 7.

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The three main complexes (MTB, MAC, and MAB) are clearly separated. It is interesting to notice that Leao et al. [29] suggested that the mycobacterial species MAS and BOL cannot be clearly separated from ABS on the basis on DNA data and thus suggested to consider them as subspecies. Our results confirmed this difficulty, although a slight discrimination can be observed. Anyway, they were both separated from BOL.

Table 2 reports the putative identification of the entire matrix acquired at 70 eV. The table contains the experimental LRI and the LRI reported in the literature (mainly from [30] and in the NIST database), along with the MS similarity match with the library both at 70 and 20 eV for comparisonpurposes. The compounds that were significantly different ($p < 0.05$) after the KW test are marked with an asterisk.

Most of the compounds were putatively identified on the basis ofthe combination ofa dual filter: the MSsimilarity with the NIST17 library (≥ 80%) and the experimental LRI within a ±5 range compared to the literature. Compounds that did not match with the previous filters were tentatively assigned after a careful study of the mass spectrum, which was supported by the information from the 20 eV ionization analysis and from the LipidWeb website (http://www.lipidhome.co.uk/ms/ masspec.html) and relative references [31, 32].

Published in : Analytical and Bioanalytical Chemistry (2018), vol. 410, pp. 7987-7996

DOI: 10.1007/s00216-018-1421-z

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Compound 4 was assigned generically as Me.C14:1, as also reported by Tranchida et al. [30] at the same LRI. It gave a very good similarity match with Me.C14:1n5 but no correspondence with the LRI was found.

Compounds 18, 19, and 22 were not found in the library and they all had a very similar mass spectrum. The library returned for all of them a good similarity with hydroxylated FAMEs but with different carbon chains. Although it was not possible to assign the specific position of the double bond and the –OH group, diagnostic fragments to assign them as Me.OH-C18:1 were present, namely 294 m/z (the prominent peak in the 20 eV mass spectrum) corresponding to the loss of water $[M - 18]^+$, 280 m/z corresponding to the loss of methanol $[M - 32]^+$, and the molecular ion at 312 m/z, which was present at a very low intensity in the 20 eV spectrum (1.25%) and completely absent in the 70 eV spectrum (ESM Fig. S6 shows the mass spectrum of compounds 18 and 20 at 70 eV, as an example).

The chemical class information of compounds 21 and 24 was derived from the library (both were recognized as FAMEs) but a match of greater than 80% was not found. Compound 21 presented a relatively high intensity of the FAME characteristic fragment ion, i.e. $[M - CH_3O]^+$, and the related molecular ion (310 m/z), but no correspondence was

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DOI: 10.1007/s00216-018-1421-z

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found with either MS spectrum or LRI. Therefore, it was generically assigned with its chemical formula ($C_{20}H_{38}O_2$). Compound 24 was tentatively identified as an ethyl ester since the prominent ion was 88 m/z, which is the equivalent of 74m/z (McLaffertyion) for ethylesters (no informative differences were observed between 20 and 70 eV, ESM Fig. S7). However, its LRI does not correspond to Et.C21; thus we hypothesized from the molecular ion (i.e., 354 m/z) that it may be a branched ethyl ester corresponding to the general formula $C_{23}H_{46}O_2$.

Finally, compound 25 was tentatively identified as hexacosanal by combining both the chemical class information derivable from lighter characteristic fragments by the library search (from the 70 eV), and the higher m/z diagnostic fragments related to the length of the saturated aldehyde, namely the molecular ion (380 m/z, visible only from the 20 eV data at a relative intensity of 1%) and the characteristic fragments at $[M - 18]^+$ (loss of water) and $[M - 46]^+$ (deriving from the loss of $H_2O+CH_2=CH_2$) [32] (ESM Fig. S8).

Furthermore, the experimental LRI corresponded with the data reported in the online NIST database (https://webbook.nist.gov/cgi/cbook.cgi?ID=C26627850&Units=SI&Mask=2000#Gas-Chrom) for the same compound.

Published in : Analytical and Bioanalytical Chemistry (2018), vol. 410, pp. 7987-7996

DOI: 10.1007/s00216-018-1421-z

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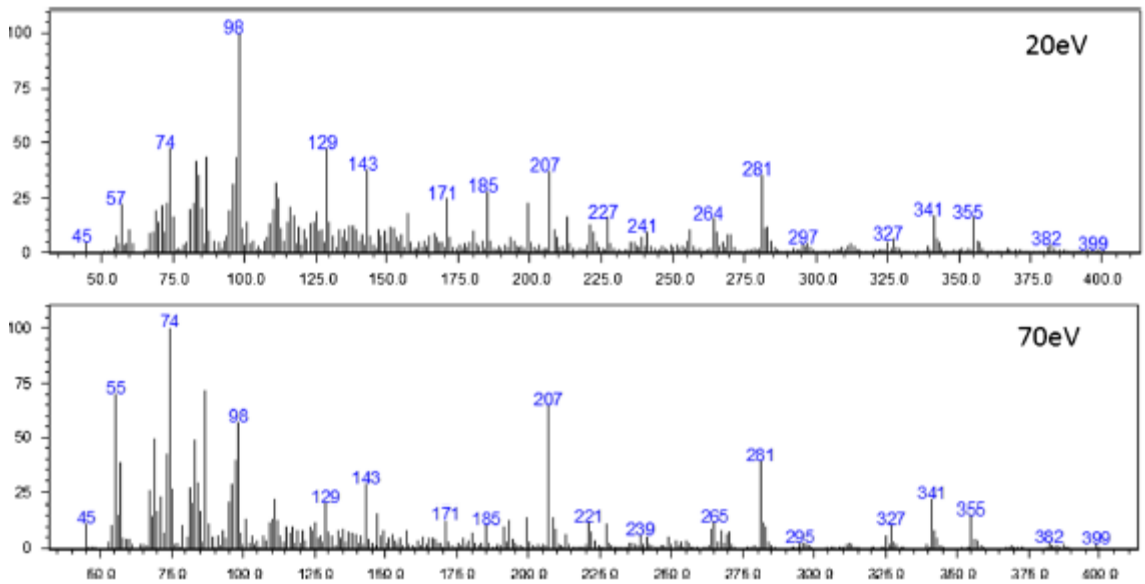
Fig 5



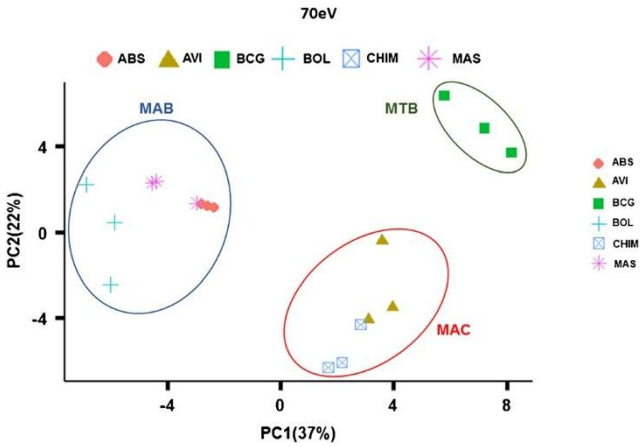
Comparison of the entire average chromatogram obtained from an ABS sample acquired at 20 eVand 70 eV

Published in : Analytical and Bioanalytical Chemistry (2018), vol. 410, pp. 7987-7996

DOI: 10.1007/s00216-018-1421-z

Status : Postprint (Author's version)

Fig. 6



Principal component score plot of the six mycobacteria strains (three biological replicates per group) obtained using the 256 features significantly different after Kruskal–Wallis analysis and Benjamini– Hochberg correction. MS Data matrix obtained from the entire chromatogram acquired at 20 eV. Ovals are only for illustrative purposes

Published in : Analytical and Bioanalytical Chemistry (2018), vol. 410, pp. 7987-7996

DOI: 10.1007/s00216-018-1421-z

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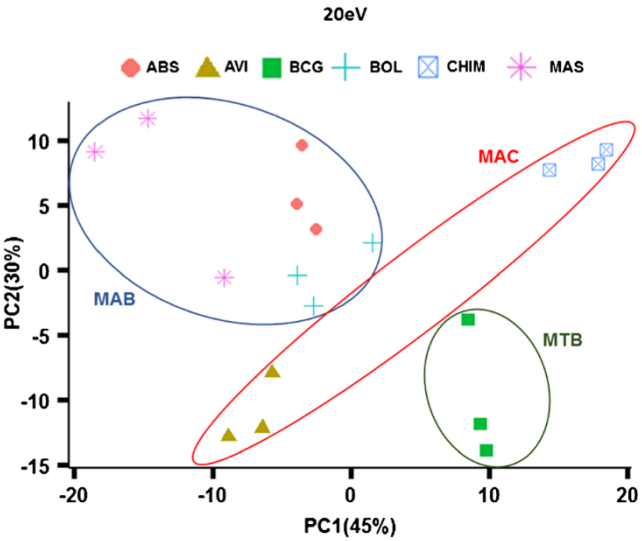
Fig. 7



Principal component score plot of the six mycobacteria species (three biological replicates per group) using the 24 significantly different features obtained at 70 eV. Ovals are only for illustrative purposes

Published in : Analytical and Bioanalytical Chemistry (2018), vol. 410, pp. 7987-7996

DOI: 10.1007/s00216-018-1421-z

Status : Postprint (Author's version)

Table 2 *Putative identification of the 29 compounds detected in the bacterial cultures, along with their CAS number, experimental (exp) linear retention index (LRI) and reported in the literature (lit), and the mass spectrum similarity (MS %) at 70 and 20 eV*

	Compound name	CAS	R_t	LRI exp	LRI lit	MS %	
						70 eV	20 eV
1*	2(3*H*)-Furanone, 5-ethyldihydro-5-methyl-	2865-82-9	9.78	1052	1051	80	–
2*	Et.C7:0	106-30-9	11.06	1097	1101	82	71
3	Me.C8:0	111-11-5	11.77	1122	1122	90	91
4*	Me.C14:1	56219-06-8	26.55	1694		83	–
5*	Me.C14:0	124-10-7	27.16	1722	1724	94	85
6	Hexadecanal	629-80-1	29.16	1816	1815	95	86
7*	Me.C15:0	7132-64-1	29.30	1823	1825	93	86
8*	Me.C16:1n9(*Z*)	56875-67-3	30.81	1896	1897	91	76
9*	Me.C16:1n7(*Z*)		30.99	1906	1903	90	80
10*	Me.C16:0	112-39-0	31.35	1923	1925	95	77

Published in : Analytical and Bioanalytical Chemistry (2018), vol. 410, pp. 7987-7996

DOI: 10.1007/s00216-018-1421-z

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	Compound name	CAS	R_t	LRI exp	LRI lit	MS % 70 eV	MS % 20 eV
11*	Me.10-Me-C16:0	2490-51-9	32.13	1964	1968	84	70
12	Me.C17iso	6929-04-0	32.58	1986	1988	88	79
13	Me.C17anteiso	2490-49-5	32.74	1996	2001	90	79
14*	Me.C17:0		33.30	2024	2026	93	80
15*	Me.C18:1n9(*Z*)	2777-58-4	34.67	2097	2098	92	73
16*	Me.C18:1n9(*E*)	1620-36-1	34.79	2104	2104	90	79
17*	Me.C18:0	112-61-8	35.16	2125	2126	95	75
18	Me.OH-C18:1		35.48	2142			
19*	Me.OH-C18:1		35.63	2151			
20*	Me.10-Me-C18:0	2490-19-9	35.86	2163	2165	91	76
21*	C20H38O2	112-79-8	36.09	2176			
22*	Me.OH-C18:1		36.25	2187			

Published in : Analytical and Bioanalytical Chemistry (2018), vol. 410, pp. 7987-7996

DOI: 10.1007/s00216-018-1421-z

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Compound name	CAS	R_t	LRI exp	LRI lit	MS %	
					70 eV	20 eV
23* Me.C20:0	1120-28-1	38.66	2326	2326	90	76
24* C23H46O2		39.51	2377			
25* Me.C22:0	929-77-1	41.90	2527	2528	84	76
26* Me.C24:1n9(*Z*)	2733-88-2	44.54	2704	2703	80	64
27* Me.C24:0	2442-49-1	44.89	2729	2729	91	75
28* Hexacosanal	26627-85-0	46.41	2837	2833		
29* Me.C26:0	5802-82-4	47.69	2930	2931	86	60

*Features significantly different ($p < 0.05$) after Kruskal–Wallis multiclass analysis, with BH correction

Conclusion

The aim of this paper was to explore the performance of a modern quadrupole EI–MS instrument when operated at lower (20, 30, 50 eV) than conventional (70 eV) ionization energy. The loss in sensitivity, expected using low ionization energies (caused by the

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lower ionization efficiency), was compensated by using the detector parameters as set by the autotuning. In contrast to what is reported in the literature, the fragmentation pattern in terms of relative intensity was very repeatable, with an average CV% of less than 5% (exceptions are present when the relative intensity of the ion under consideration is less than 0.1%) at all the ionization energies tested.

Moreover, the softer fragmentation obtained at 20 eV simplified the interpretation of the mass spectra by providing more information on diagnostic ions, whose signals were significantly higher in intensity compared to at 70 eV. Nevertheless, the lack of MS databases recorded at lower ionization energy requires users with knowledge of the fragmentation and rearrangement processes in the EI to understand which ions are relevant and which ones can be disregarded. That said, it is the authors' opinion that the additional and complementary information obtained using both 20 and 70 eV is worth the extra time spent running the samples. This extra information is also useful to support the assignment of compounds not recorded in common databases and/or for which a pure standard is not commercially available.

Moreover, in this specific application, because of the more informative nature of the mass spectrum profile, the 20 eV average MS spectrum from the entire TIC was

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DOI: 10.1007/s00216-018-1421-z

Status : Postprint (Author's version)

succesfully exploited as a screening tool for species differentiation (conceptually similar to a direct EI–MS analysis), and followed by peak-by-peak identification and sample profiling.

Although other studies on different chemical classes would be desirable to evaluate the performance of using a low ionization energy on a conventional EI–MS system, this work is an attempt to move the GC–MS users away from the Bcozy routine^ [15] and invite researchers to investigate and question some basic assumptions, which may benefit from the technological advancement of modern instruments.

ACKNOWLEDGEMENTS

The authors thank Shimadzu and Supelco for their support. NTM strains were kindly provided by Dr. Jerry Nick, National Jewish Health and the BCG was from Harvard Medical School.

Compliance with ethical standards

CONFLICTS OF INTEREST The authors declare that they have no conflict of interest.

Published in : Analytical and Bioanalytical Chemistry (2018), vol. 410, pp. 7987-7996

DOI: 10.1007/s00216-018-1421-z

Status : Postprint (Author's version)

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