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Journal of Chromatography A, 712 (1995) 31–43

JOURNAL OF CHROMATOGRAPHY A

Comparison of softwares used for the detection of analytes present at low levels in liquid chromatographic–mass spectrometric experiments

J. Visentini[a], E.C. Kwong[a], A. Carrier[b], D. Zidarov[b], M.J. Bertrand[b,*]

[a]*Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire-Dorval, Quebec H9R 4P8, Canada*

[b]*Regional Center for Mass Spectrometry, Department of Chemistry, University of Montreal, P.O. Box 6128, Montreal H3C 3J7, Canada*

Abstract

A comparison has been made between different approaches for detecting low-level analytes in the TIC traces of sample mixtures analysed by different liquid chromatographic–mass spectrometric (LC–MS) techniques. The approaches studied were contour mapping or "eagle's view" and a background treatment software, TICFilt, recently developed. Typical pharmaceutical samples including standards and plasma containing common drugs such as propranolol, phenothiazine, acetaminophen have been analysed in LC–MS experiments using ionspray, atmospheric-pressure chemical ionization and direct liquid introduction interfaces. The data obtained were examined by contour mapping and treated by TICFilt in order to detect low level elution peaks. Contour mapping can be efficient at higher masses ($> M_r$ 250) where the background is generally weaker but cannot always detect elution peaks at lower masses where background contribution is important. Furthermore, it cannot distinguish actual peaks from spikes which are often present in these experiments. Background treatment algorithms such as TICFilt, however, can not only eliminate spikes from the TIC trace but also offer a peak detection efficiency for unknown compounds which is constant throughout the mass range and independent of the mobile phase composition and the ionization technique used. Furthermore, background treatment algorithms also provide mass spectra with enhanced spectral information which is important in the identification of unknown drug-related species.

1. Introduction

The development of interfaces allowing the direct introduction of liquid samples into the mass spectrometer has considerably increased the range of compounds amenable to mass spectral analysis. For example, direct liquid introduction (DLI) [1], thermospray (TSI) [2,3], continuous-flow fast atom bombardment (CF-FAB) [4] and more recently atmospheric-pressure chemical ionization (APCI) [5–7], electrospray (ESI) [8,9], and IonSpray (ISP) [10–12] have all been used to identify eluents from an HPLC column. Since these techniques allow the eluent to enter partially or entirely into the ionization source, the solvents and buffers present in the mobile phase are also ionized, thereby generally producing high levels of chemical noise in the total-ion current (TIC) traces. The contribution of this background signal can lead to

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00234-0

problems in the analysis of unknown samples. For example, in pharmaceutical samples, metabolites or degradation products present at levels as low as 0.1% of the parent drug must often be identified. In screening for these substances in the full-scan mode, the degradation product peaks are often lost in background signal. This masking of low-level eluent peaks by background signal is worst in the low mass range ($< M_r$ 250–300).

Several approaches for the post-process background treatment of mass spectral data have appeared in the literature [13–22]. For LC–MS data, in particular, the TIC traces do not generally provide easily observed eluent peaks due to the abundance of mobile phase ions. Although peaks of interest can usually be detected by thorough screening of reconstructed mass chromatograms, which is necessary for unknowns, examination of all reconstructed mass chromatograms in a typical full-scan analysis is tedious and impractical. Furthermore, the traditional background subtraction routines involving the subtraction of a background spectrum from the spectrum of the analyte of interest is often of little use for low-level degradation products masked by chemical noise. This method is further complicated with gradient HPLC methods where the background spectra change with time.

Polynomial smoothing techniques [13] which can enhance the signal-to-noise ratios of low-level analyte peaks, improve the appearance of the profile but are of limited use since they do not extract significant ions hidden in the chemical noise. One of the most common approaches for the extraction of significant peaks from LC–MS data is contour mapping or "eagle's view" [14]. By plotting three axes (mass, time, and intensity) in a two-dimensional display (mass–time), the elution peaks appear over a few scans depending on the width of the chromatographic peak. The background noise in this type of plot will appear as a continuous band over a wide time-span, often occurring throughout the entire analysis time. This technique is most effective at higher mass ranges ($> M_r$ 400) where background noise is less abundant for most LC–MS interfaces. Pharmaceutical compounds, however, are often small molecules which fall in the lower mass range where background interferences are high.

An ideal background filter for LC–MS data would eliminate the contribution of solvent and buffer ions to the acquired mass spectra, recognize and remove noise spikes caused by experimental variations, and extract weak eluent peaks containing significant ions from the overall TIC trace. This type of approach would provide a background-corrected TIC trace indicating low-level eluent peaks as well as other more visible components. It would also provide treated mass spectra with enhanced intensities of significant analyte ions. A background treatment algorithm, TICFilt, has been developed with the above criteria in mind. The filtering of LC–MS data by TICFilt is based on the premise that the occurrence of background ions is more frequent than that of ions due to analytes. Its performance will be compared to the commonly used contour mapping approach.

2. Experimental

Acetaminophen (M_r 151) and propranolol (M_r 259) used in these experiments are USP reference standards obtained from American Chemicals (Rockville, MD, USA), phenothiazine (M_r 199) was purchased from Fluka Chemika (Ron Kon Koma, NY, USA). Compound X (M_r 454) was synthesized in-house. All compounds were used without further purification. The mobile phases used in these experiments were composed of given ratios of HPLC grade acetonitrile and distilled deionized water and contained 0.1% formic acid (approx. 99%, Sigma, St. Louis, MO, USA). All sample solutions were prepared in acetonitrile.

The liquid chromatographic system used for DLI analyses consisted of a Carlo Erba Phoenix-20 high-pressure syringe pump (Carlo Erba Str. Milano, Italy) connected to a Valco C14W 60 nL valve (Valco Instrument Co., Houston, TX, USA) in order to minimize dead volume between the pump and capillary tubing or liquid chromatographic columns. The columns used in

the DLI-LC–MS experiments consisted of laboratory made capillary reversed-phase columns (Spherisorb ODS-2, particle diameter 5 μm, 200 mm × 250 μm I.D.). Mass spectral analyses were performed on a VG Trio-1 mass spectrometer equipped with differential pumping (analyzer 50 l/s, source 240 l/s) and using an in-source μ-thermospray interface [23]. Data handling and control of the mass spectrometer were provided by the LAB BASE (version 2.01) data system. All spectra were recorded in the filament-on mode. The TICFilt program version for this instrument was written in Borland C^{++} on an IBM compatible computer PC (80386 or above with Windows software) and execution time for moderate data files requires only a few seconds.

All APCI and ESI LC–MS experiments were performed on a HP 1090 liquid chromatograph equipped with an Intersil (Metachem, Torrance, CA, USA) 5 μm ODS-2 (100 × 3 mm I.D.) column and coupled to a PE/Sciex API III triple quadrupole mass spectrometer via an IonSpray or an APCI interface operating at atmospheric pressure. Full-scan mass spectra over the mass range m/z 120–600 were acquired and examined using PE/Sciex Tune 2.4 and MacSpec 3.22 softwares respectively. The contour mapping feature of the API III MacSpec software was used as a typical example of this type of approach for treating LC–MS data. The background treatment algorithm, TICFilt, was written in C language and runs on Macintosh or IBM compatible computers with Windows. Execution time of this version of TICFilt is in the order of 1 min. Treated data can be accessed and viewed through MacSpec software.

3. Results and discussion

Although direct introduction interfaces have made the routine use of LC–MS possible, the problem of dealing with background interferences remains an important challenge. Current background treatment softwares associated with LC–MS systems often represent mass spectral data through contour mapping which plots mass vs. scan number vs. intensity as shown below by the typical contour plot in Fig. 1a. This contour mapping approach can be used to distinguish peaks from the constant chemical background signal due to mobile phase ions. For example, a chromatographic peak will appear as a discrete band such as that observed at about m/z 150 and 2.1 min in Fig. 1a while the background ions are observed as constant bands throughout the analysis time.

Once a peak is found on the contour plot, spectra immediately before and after the elution peak are subtracted from the spectrum or average spectrum due to the species of interest in order to improve the signal-to-background ratio. Other methods simply subtract baseline noise from the TIC chromatogram. These methods, however, do not filter data and are therefore vulnerable in cases where background is intense. It is very difficult to find elution peaks at low levels amidst heavy noise levels due to mobile phase ions unless background ions are distinguished from elution peak ions. Furthermore, the presence of spikes in the TIC chromatogram may complicate the analysis of data and the contour plot. The whole process may become rather time-consuming.

A background treatment algorithm which filters data will enhance the quality of the TIC chromatogram and of the mass spectra simultaneously, thereby saving data treatment time. Furthermore, if data are filtered, the number of cases where elution peaks are lost in the mass of background peaks will be minimized. An efficient computer algorithm for LC–MS data treatment of sample mixtures containing unknowns must, therefore, be able to remove background signal without eliminating low-level analyte peaks. TICFilt, a background treatment algorithm developed by our group [15], is based on the hypothesis that background ion signals in a mass spectrum will occur with a higher frequency than ion peaks due to analyte species which should have intensities distributed in a random fashion. The algorithm which basically includes three steps–background treatment, noise and spikes treatment and elution peak recognition–will from the raw data generate a treated TIC

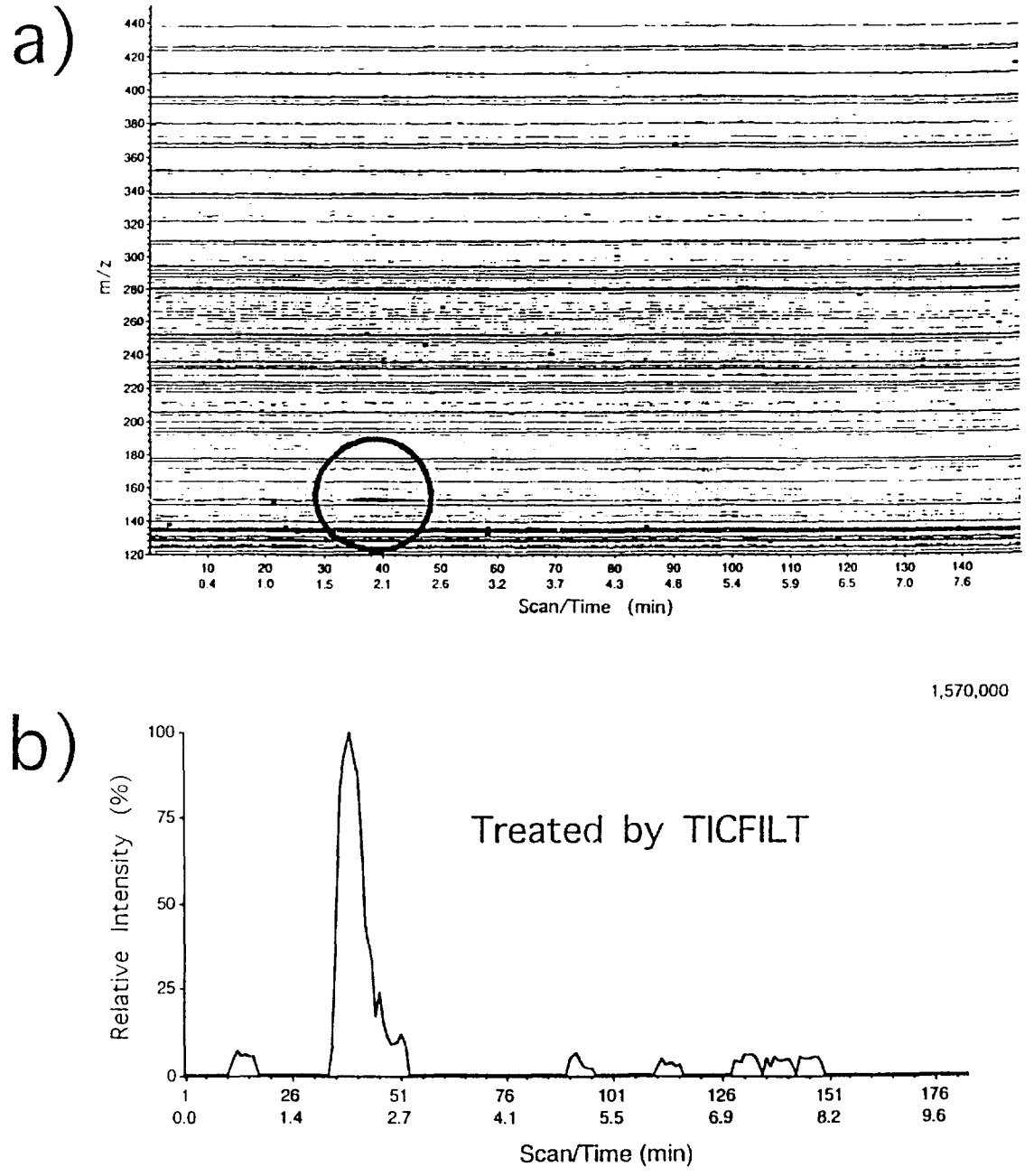

Fig. 1. (a) Contour plot and (b) TICFilt treated data for a 0.025 mg/ml solution of acetaminophen in acetonitrile obtained by LC–APCI (heated nebulizer)-MS.

and treated mass spectra from which most interferences are eliminated.

Upon entering the TICFilt program, the user must select the files to be treated and may define parameters to be used, such as mass range or scan interval of interest, if they are different from the default conditions (entire acquisition ranges). Once these values are established, TIC-

Filt uses the mass/intensity lists of the raw data file and calculates the frequency of occurrence of each mass in order to perform its background treatment [15]. Noise and spike treatment is then performed by defining appropriate parameters which are related to experimental conditions. Signals which adhere to the given definition of spikes are then eliminated from the data file. Finally, the elution peak recognition portion of TICFilt determines the total number of elution peaks and filters each mass with respect to the elution peaks found. The treated mass/intensity list is then plotted by the commercial software from which raw data was taken. The data output from this program consists of a treated TIC trace with enhancement of elution peaks and its corresponding mass spectra which have been background treated.

The TICFilt data filtering method was compared to the contour plot approach for LC–MS data obtained using IonSpray, APCI and DLI interfaces. Fig. 1a, for example, illustrates the contour plot of a 0.025 mg/ml solution of acetaminophen analyzed by LC–APCI-MS. An elution peak can be observed as a distinct band (highlighted) at about 2.1 min in this contour plot. When this data was treated by TICFilt, a peak at this retention time is isolated from the total TIC trace as shown in Fig. 1b. The results given in Fig. 2 indicated that when LC–MS analyses are performed at a lower concentration (0.5 μg/ml), the peak due to acetaminophen (highlighted) at about 2.1 min and m/z 150 is not easily observed on the contour plot (Fig. 2a) but that TICFilt is still able to extract significant ions from the overall acquired data (Fig. 2b). This efficiency of TICFilt in extracting elution peaks from the TIC trace is particularly more important at lower masses ($< M_r$ 250) where the constant and often intense background signal masks the elution peak bands.

Fig. 3a presents the TIC obtained for compound X in rat plasma, which contributes a substantial background signal, obtained by LC–IonSpray-MS. Although a signal is not easily observed in the original TIC trace (Fig. 3a), TICFilt indicates that an elution peak occurs at approximately 10 min (Fig. 3b). The mass spectrum at the apex of this peak contains a base peak at m/z 455 that corresponds to the parent ion of compound X. TICFilt also allows a narrow scan range to be selected which is useful when the elution peak is quite small in the normalized TIC. The effect of this option is demonstrated in Fig. 3c where the elution peak of compound X now appears as prominent. This option also enhances the elution profile of another peak which was revealed in Fig. 3b. This compound shows a base peak at m/z 416 in its mass spectrum and although it may be a by-product of compound X its origin is still uncertain.

Similarly, results obtained from experiments conducted by LC–APCI-MS for a phenothiazine sample in rat plasma (Fig. 4a) also showed that TICFilt is efficient at extracting elution peaks from the overall signal acquired (Fig. 4b). The elution peak for phenothiazine was found to be clearly distinguished from background signal (Fig. 4b). Again the narrow scan option can be advantageous in enhancing low-level elution peaks as shown in Fig. 4c where the scan range to be treated was limited to scans 50–183. Another small elution peak is also revealed in the TIC around 9 min with a base peak at m/z 417. Similar results were also obtained for an acetaminophen standard analyzed by DLI (Fig. 5). From this figure it can be seen that the noise-filtering ability of TICFilt is very efficient in eliminating the heavy background fluctuations in the TIC that could otherwise be mistaken for analyte signals. The results from these experiments indicate that regardless of the ionization technique employed for the LC–MS analysis, TICFilt can successfully isolate significant ions from the mass of ions produced in the source.

When employing background filters to treat LC–MS data, it is also important to enhance the quality of the mass spectra. For example, in Fig. 6a, the parent ion of acetaminophen at m/z 152 is lost in the legion of background peaks of the raw mass spectrum. When the spectra immediately before and after the peak of interest identified using the contour plot are subtracted from the mass spectra of the acetaminophen (Fig. 6b), the parent ion at m/z 152 is now the base peak but there are also several other

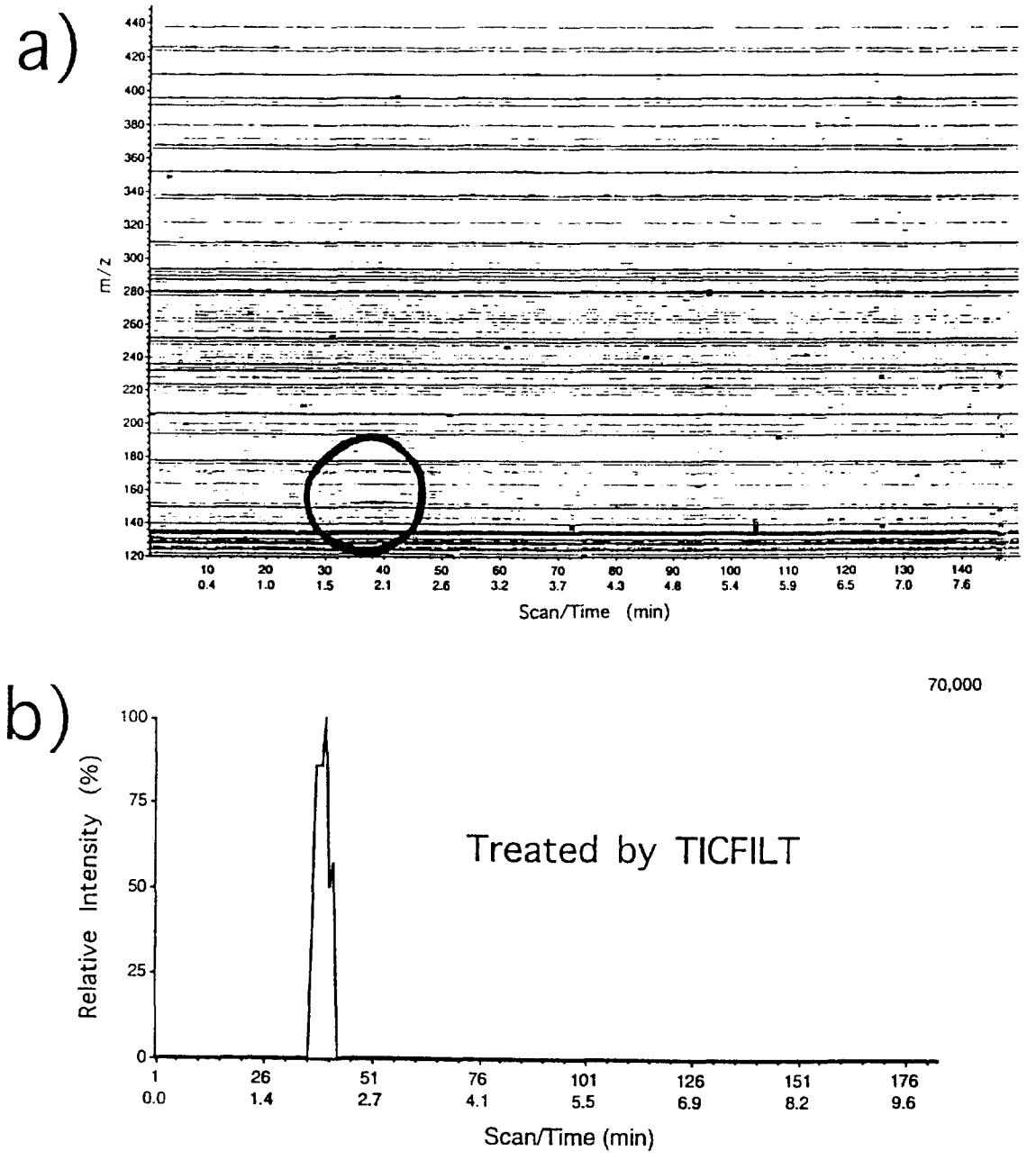

Fig. 2. (a) Contour plot and (b) TICFilt treated data for a 0.5 μg/ml solution of acetaminophen in acetonitrile obtained by LC–APCI (heated nebulizer)-MS.

background peaks which can be seen in this background-corrected spectrum. The corresponding TICFilt treated spectrum (Fig. 6c) however contains only a few peaks with the base peak at m/z 152. This improvement in signal-to-noise ratio due to background treatment can be

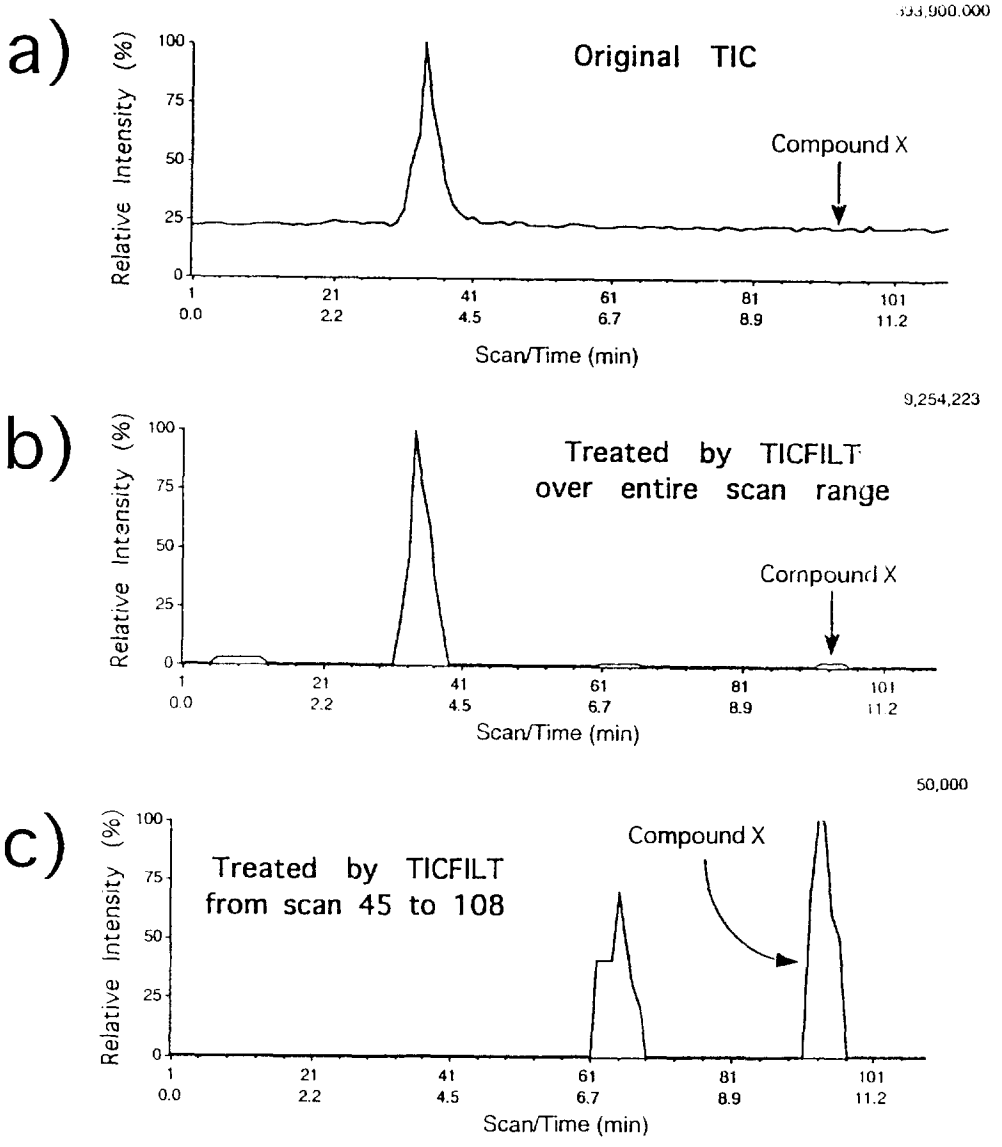

Fig. 3. Total-ion chromatograms of compound X (0.0025 mg ml) in rat plasma obtained by LC–IonSpray-MS. (a) Original TIC, (b) TICFilt treated TIC and (c) TICFilt treated TIC over the limited scan range 45–108.

particularly useful for the analysis of unknown samples where the analyst must identify parent ions of compounds in a sample mixture.

Fig. 7 compares the quality of spectra obtained for a propranolol sample by examining the raw mass spectrum, the contour mapping background-subtracted spectrum, and the TICFilt treated spectrum. In the original mass spectrum (Fig. 7a), again the parent ion at m/z 260 is lost in the legion of background ions. When the contour plot was used to guide in background subtraction (Fig. 7b), the parent ion became the base peak with several other background ion peaks present in the treated spectrum. The TICFilt treated spectrum (Fig. 7c) illustrates that the extraction of significant ions from the overall raw spectrum,

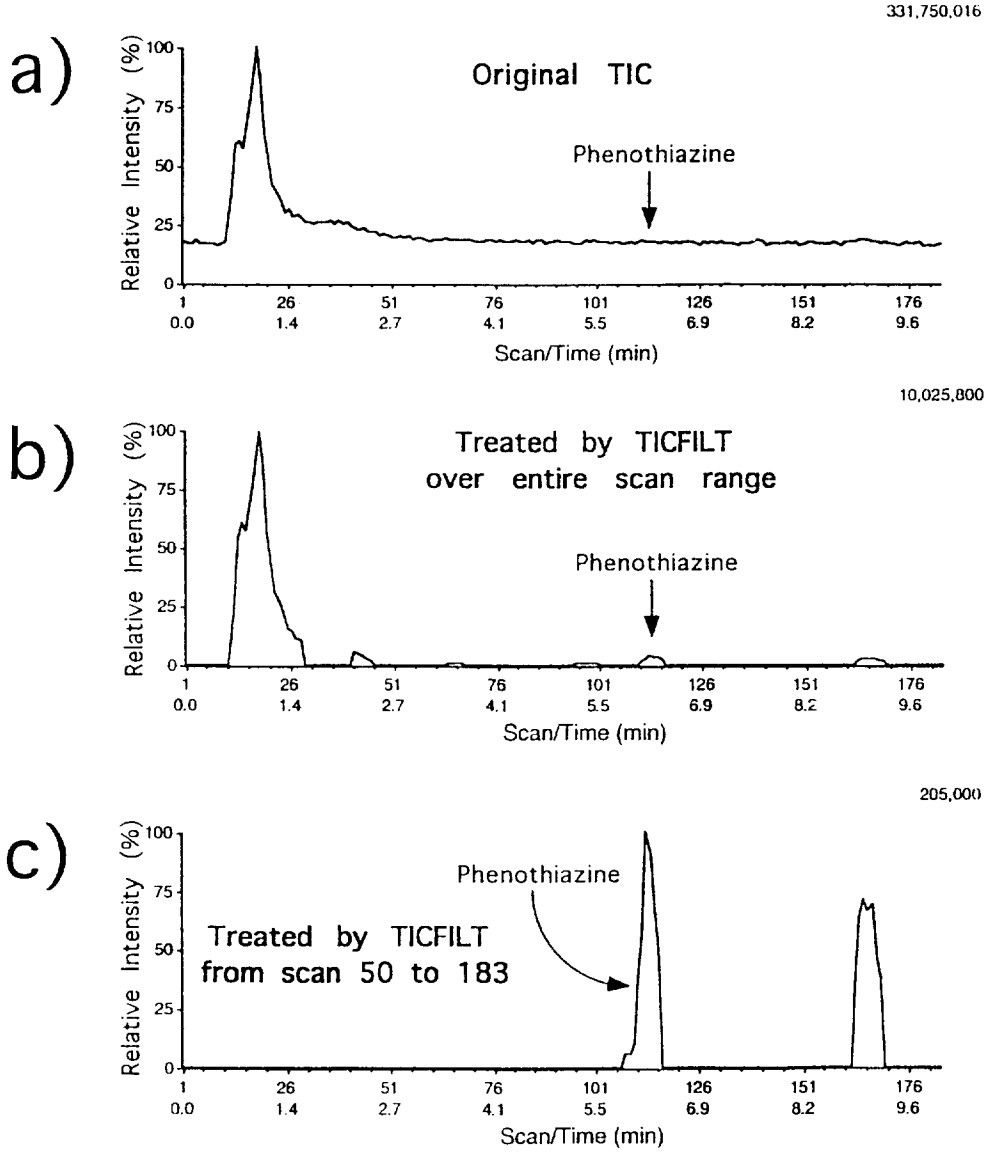

Fig. 4. Total-ion chromatograms of phenothiazine (0.0025 mg/ml) in rat plasma obtained by LC–APCI (heated nebulizer)-MS. (a) Original TIC, (b) TICFilt treated TIC and (c) TICFilt treated TIC over the limited scan range 50–183.

in this case, is more efficient and less time-consuming when TICFilt is used for data treatment. Another example of the efficiency of TICFilt to produce significant treated spectra is given in Fig. 8. This figure presents the raw and treated spectra of acetaminophen (150 ng) obtained by LC–DLI-MS. Since this technique uses the mobile phase as a reagent gas for chemical ionization, the intensities of the mobile phase ions are often very important which causes problems in identifying the signal due to the analyte in the normalized spectrum. As can be seen from Fig. 8 (lower spectrum) the spectrum of the analyte is clearly enhanced after data treatment by the algorithm clearly showing the protonated molecular ion at m/z 152 and frag-

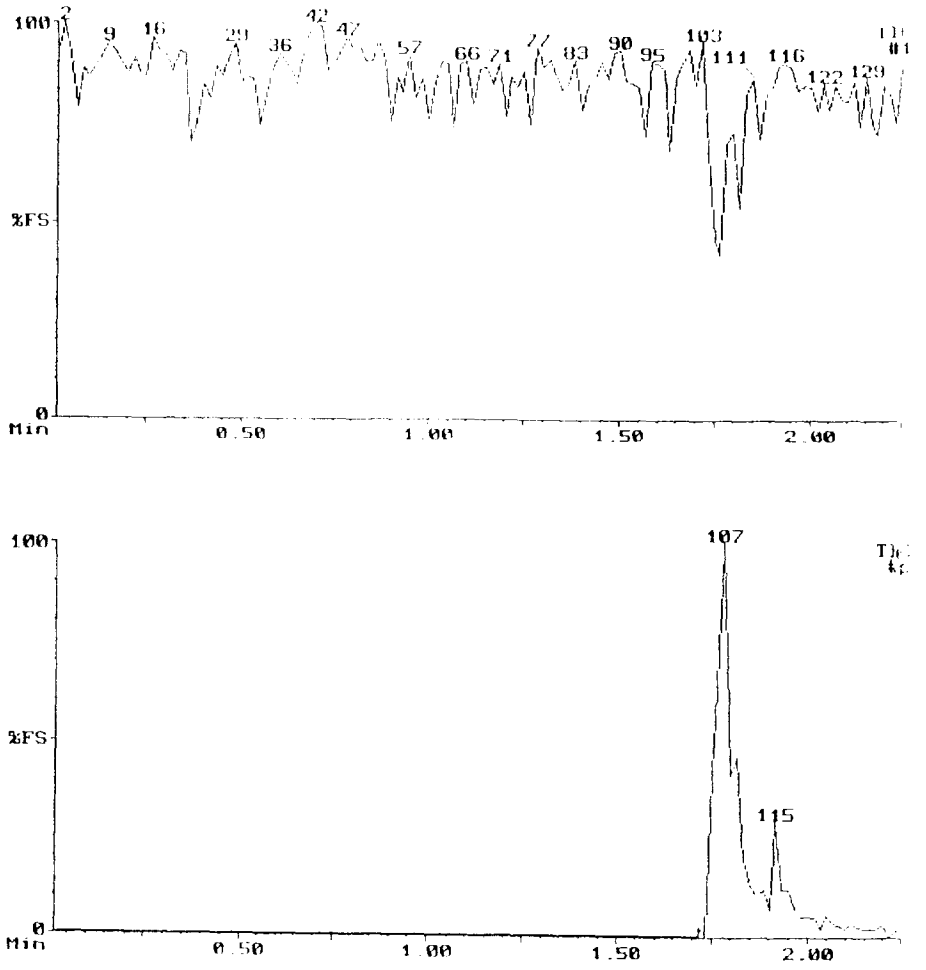

Fig. 5. Total-ion chromatograms obtained for a 150-ng injection of acetaminophen by LC–DLI-MS.

ment at m/z 134. In spite of their relatively high intensities, plasma ions at m/z 42 and 83 due to the protonated monomer and dimer of acetonitrile are absent from the treated spectrum (lower spectrum).

4. Conclusion

The data presented in this comparative study indicate that peak detection approaches can be extremely useful in identifying elution peaks of low-level components in TIC chromatograms obtained by current direct introduction LC–MS techniques. Contour mapping types of approaches can be efficient at higher masses because background is generally lower but they are limited at lower masses ($< M_r$ 250) where the background generated by the mobile phase is usually more intense. Background treatment algorithms such as TICFilt, however, tend to be efficient throughout the mass range. Furthermore, background treatment algorithms are independent of the ionization technique and the composition of the mobile phase used. When elution peaks or spikes are observed in the TIC, contour mapping will detect both as elution peaks varying only in their width. Background treatment algorithms such as TICFilt can, how-

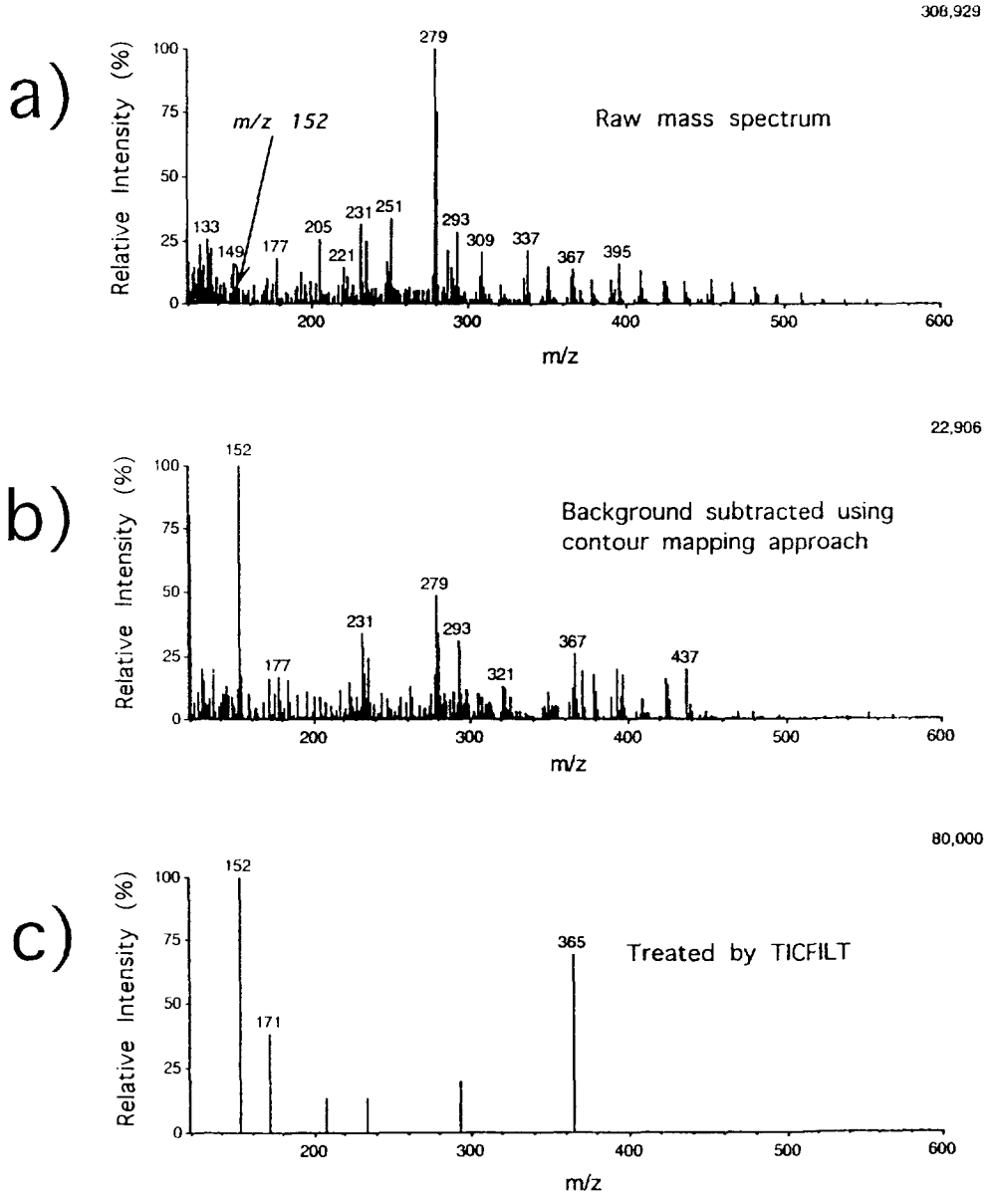

Fig. 6. Comparison of mass spectral quality for: (a) raw data, (b) data treated using the contour mapping approach and (c) data treated by TICFilt for a 0.5 μg/ml acetaminophen standard analyzed by LC–APCI (heated nebulizer)-MS.

ever, remove spikes present in the TIC and reveal only analyte components. A further advantage of background treatment algorithms resides in their ability to produce treated mass spectra in which most of the interfering signals have been eliminated. This feature is useful in revealing the masses due to the analyte especially at lower masses where analyte peaks are often not observed in the raw spectrum because of their low relative intensities. Thus, background

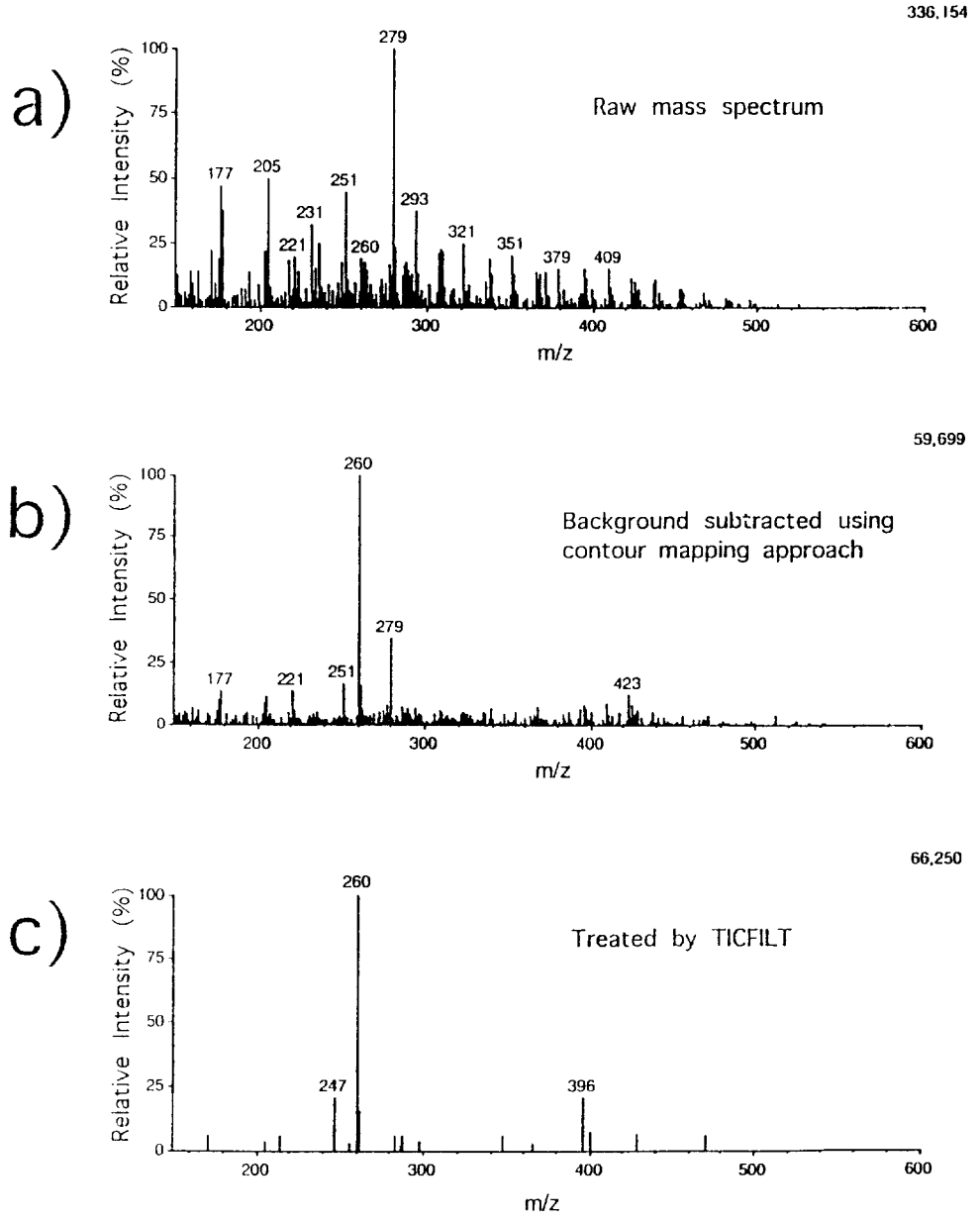

Fig. 7. Comparison of mass spectral quality for: (a) raw data, (b) data treated using the contour mapping approach and (c) data treated by TICFILT for a 0.0025 mg/ml propranolol standard analyzed by LC–IonSpray-MS.

treatment algorithms such as TICFilt, provide high quality TIC and mass spectral data simultaneously, thereby saving data processing time and facilitating data interpretation involving unknown species as in the case of degradation or metabolic studies. The algorithm is in all aspects equivalent to single-ion chromatograms traces, however, TICFilt is more advantageous because

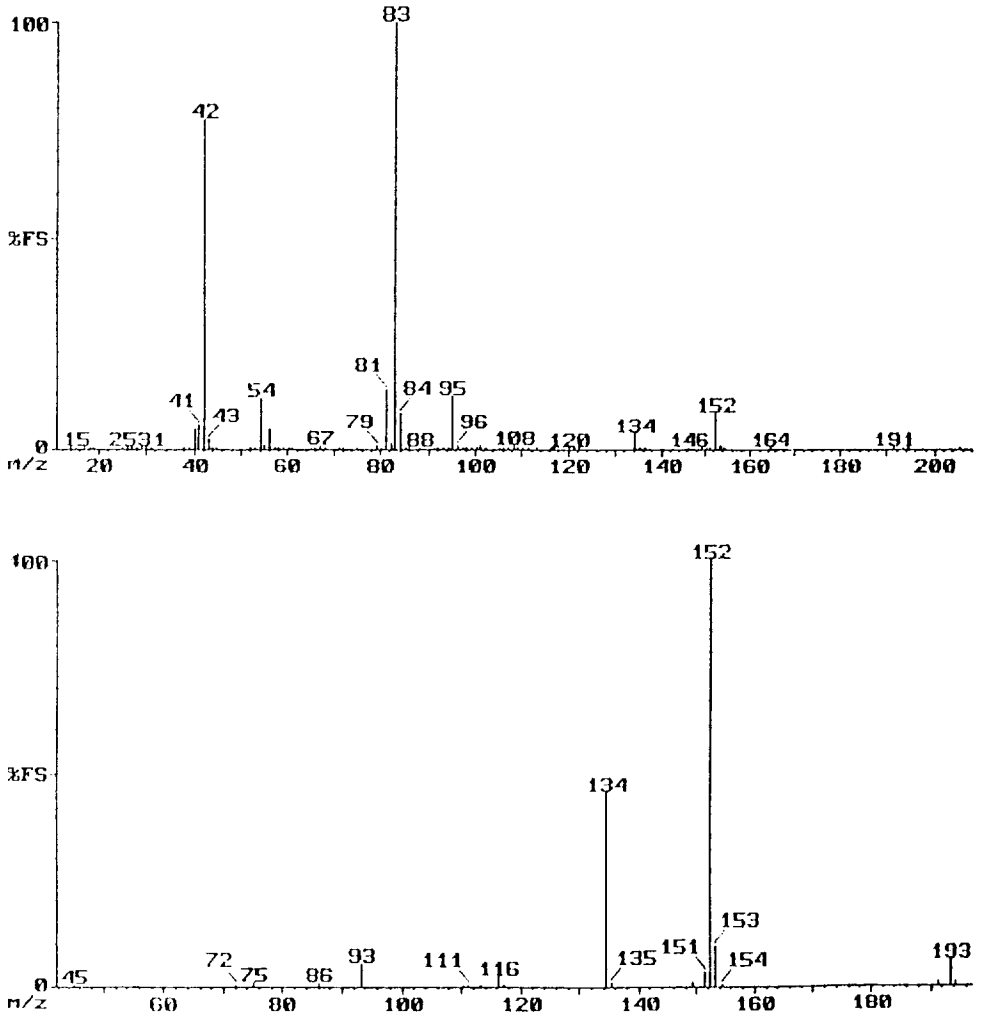

Fig. 8. Comparison of mass spectral quality for (top) raw data and (bottom) data treated by TICFilt for a 150-ng injection of acetaminophen analyzed by LC–DLI-MS.

it can detect the elution peaks without requiring any information on the nature of the eluting compound.

Acknowledgements

The authors wish to acknowledge the financial support of the Natural Sciences and Engineering Research Council of Canada (NSERC) and Hydro-Quebec that has permitted this work.

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