Application Note: 369

A High Throughput Approach for Metabolite Profiling and Characterization Using the LXQ Linear Ion Trap Mass Spectrometer

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Key Words

- LXQ™
- Surveyor Plus™
- Glyburide Incubation
- Mass Frontier™
- Metabolite Profiling
- MetWorks™

Introduction

Within the drug discovery environment, high sample throughput that provides comprehensive drug metabolite identification and profiling is highly desirable. Traditionally, triple quadrupole instruments running selected reaction monitoring (SRM or MRM) have been employed; however, SRM and MRM studies do not necessarily detect all of the drug metabolites being investigated. Additional detection methods including precursor ion and neutral loss scanning are often required, resulting in multiple LC-MS/MS injections. This experimental multiplicity is both time and sample consuming. A better approach is to use a mass spectrometer that delivers fast cycle time, high sensitivity and high quality MS^n spectra such as the LXQ so that all the structural information about potential metabolites can be collected in one run.

Glyburide is a potent sulfonylurea drug and has been used widely in the treatment of non-insulin-dependent diabetes mellitus for more than 25 years.[1-2] The metabolite analysis of glyburide has been carried out using a number of LC-MS/MS approaches, and hydroxylation has been found to be the major metabolic pathway in most cases.[3-6] In addition, other metabolites have also been identified. A previous report using a quadrupole/trap hybrid mass spectrometer claimed to be able to identify 14 metabolites of glyburide incubated with human liver microsomes. Multiple separate LC-MS/MS runs of 45 minutes each were required, which is not an ideal approach for high throughput metabolic profiling, particularly if sample and time are limited. In this report, a rapid LC-MS/MS method was developed for analyzing glyburide and its metabolites in a single run of approximately three minutes using the LXQ.

The rapid LC-MS/MS method made use of Data-Dependent™ acquisitions to study known metabolites and to uncover unexpected metabolites. In addition, data analysis was facilitated by MetWorks and Mass Frontier software that enhanced the screening and characterization of metabolites in complex matrices.

Experimental Conditions

Sample preparation

Glyburide was incubated using human hepatic microsomes at a concentration of 10 µM for 40 minutes and quenched with ACN. The sample was then centrifuged, and the supernatant was collected and re-constituted for further analysis. A 10 µL sample was injected into LC/MS system. A glyburide sample prepared at t=0 minute was used as a control.

HPLC:

LC System: Surveyor Plus HPLC System
Column: Hypersil GOLD™
(20×2.1 mm, 1.9 µm particle size)
Mobile phase: (A) water with 0.1% formic acid
(B) acetonitrile with 0.1% formic acid
Flow rate: 300 µL/min
Injection volume: 10 µL
Gradient:

t (min)	A%	B%
0.00	70	30
0.20	70	30
3.00	15	85
3.1	70	30
5.0	70	30

Mass Spectrometer

The LXQ linear ion trap mass spectrometer was operated in positive electrospray mode. The electrospray voltage was 5 kV. The capillary temperature was 275 °C, and the sheath gas flow was 25 units. An isolation width of 2 Da was used with a 30 ms activation time for MS/MS experiments. All scan events were acquired with one micro scan. Full scan MS spectra and Data Dependent MS/MS spectra were acquired with a 50 ms and 200 ms maximum ionization time respectively.

Results and Discussions

The MS method workflow is demonstrated in Figure 1. The *m/z* values of the parent drug and the predicted metabolites were put into the parent mass list so that the MS/MS analysis was preferentially performed on these ions. This list ensured that specific metabolites were examined in a similar fashion to SRM or MRM experiments. In addition, if none of the ions in the mass list were observed, the LXQ automatically performed MS/MS analysis on the most abundant ion(s) in the MS survey scan, thus ensuring the analysis of unpredicted species that could also be metabolites.

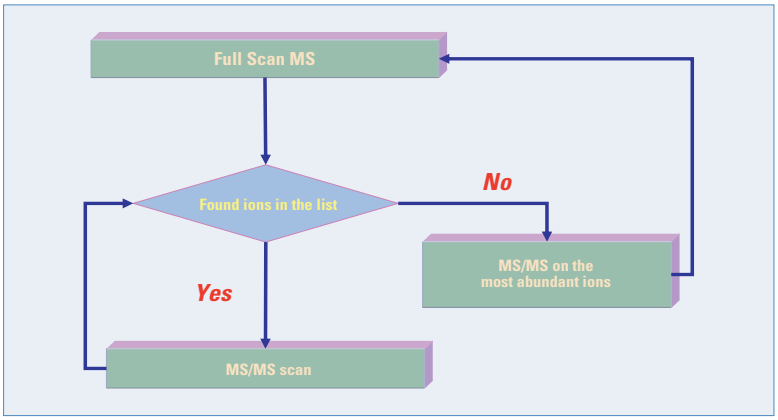
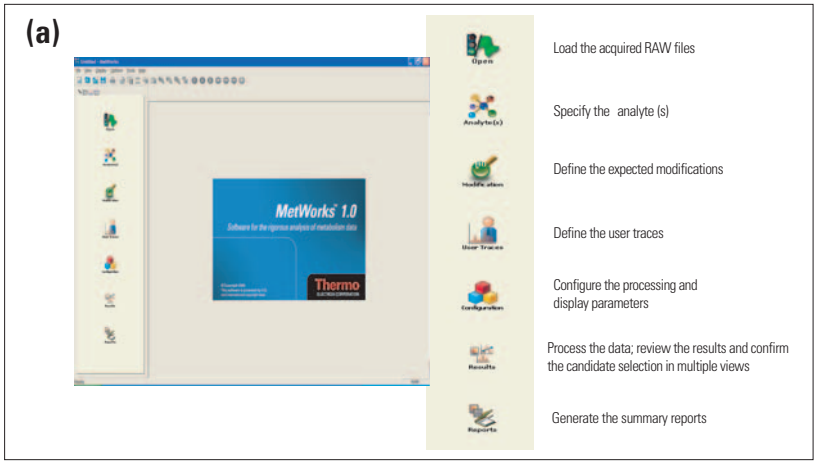


Figure 1: Schematic of MS method setup using precursor ion inclusion list

Glyburide LC-MS/MS data was processed with MetWorks software, which has an intuitive, user-friendly workflow as shown in Figure 2a. The embedded algorithm automatically searches all the possible metabolites based on the modifications specified by the user and generates the final report showing what metabolites have been detected. For glyburide, component detection results in a range of metabolites that are shown in Figure 2b. The components found in both sample and control are listed in the columns on the left with their chromatograms shown on the right. The top panel showed all the components found in the sample, while the middle panel showed those found in the control. The differential component detection results are shown in the bottom panel where all the identified components are marked with a green triangle. By clicking on the triangles, the corresponding MS or MS^n spectra would appear in the two spectrum placeholders at the bottom of the page. The component detection software automatically searched all biotransformations and screened the real metabolites from false positives.



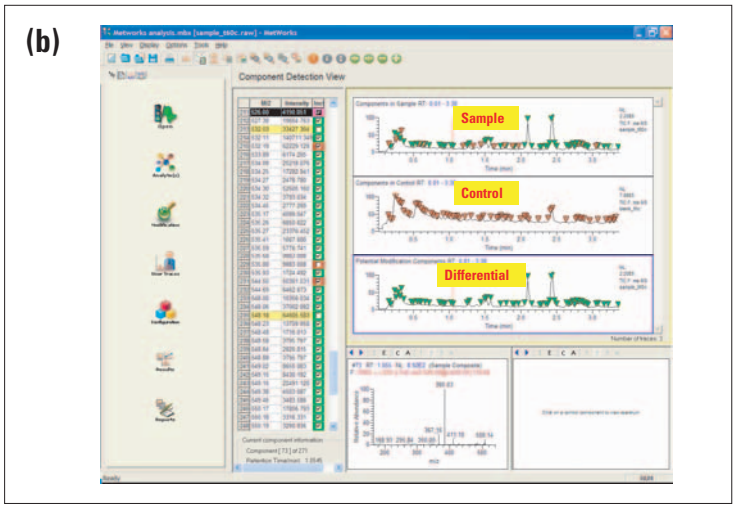


Figure 2: Using MetWorks for metabolic profiling data analysis
(a) Seven-step procedure of MetWorks;
(b) Component detection results of glyburide data using MetWorks

Mass Frontier software was used for structural identification of the glyburide metabolites. Its database searching capabilities can be coupled to a fragment prediction module for accurate structure characterization from MS^n data. This predictive fragmentation is crucial for identifying drugs and their metabolites. Another degree of confirmation in Mass Frontier software is the ability to do easy searches of a target component. For the glyburide parent drug, a search was performed against an in-house library using Mass Frontier, and the top hit was the correct compound (Figure 3a). More interestingly, the spectra comparison feature embedded in Mass Frontier provides a handy tool to determine the possible sites of biotransformations. For example, the MS/MS spectrum of one metabolite $[M+16+H]^+$ was compared to that of the parent drug $[M+H]^+$ (Figure 3b), and the comparison of fragmentation patterns readily pointed out that hydroxylation occurred on the ethyl chain.

Enhanced confidence in the structure identification of unknowns was achieved by combining a library search with the chromatographic elution time of the unknown and the mass of the precursor ion. As an example, the identities of four unpredicted metabolites were confirmed by using Mass Frontier to generate possible fragments and match against the MS/MS spectra (Figure 4). The biotransformation pathways that produced these previously unidentified metabolites include O-dealkylation (480, M-14), loss of cyclohexyl moiety by N-dealkylation (412, M-82), amide hydroxylation (369, M-125) and amide hydroxylation plus ethyl hydroxylation (385, M-109). Note that the ions 480, 369 and 385 are not reported in commonly cited literature on the metabolism of glyburide. With the aid of Mass Frontier, the major fragments for all four metabolites were assigned with structures.

The differentiation of isobaric metabolites was accomplished with the MS/MS spectra. For example, the extracted ion chromatogram of *m/z* 492 indicated that there were four dehydrogenation metabolites, and the challenge was to determine the location of the biotransformations (Figure 5a). The MS/MS spectra of the four dehydrogenation metabolites are depicted in Figure 5(b) to (e). The metabolites eluting at 1.33 min and 2.10 min have similar MS/MS spectra as shown in Figure 5(b) and (c). The major fragments, *m/z* 393 and 367 indicated that

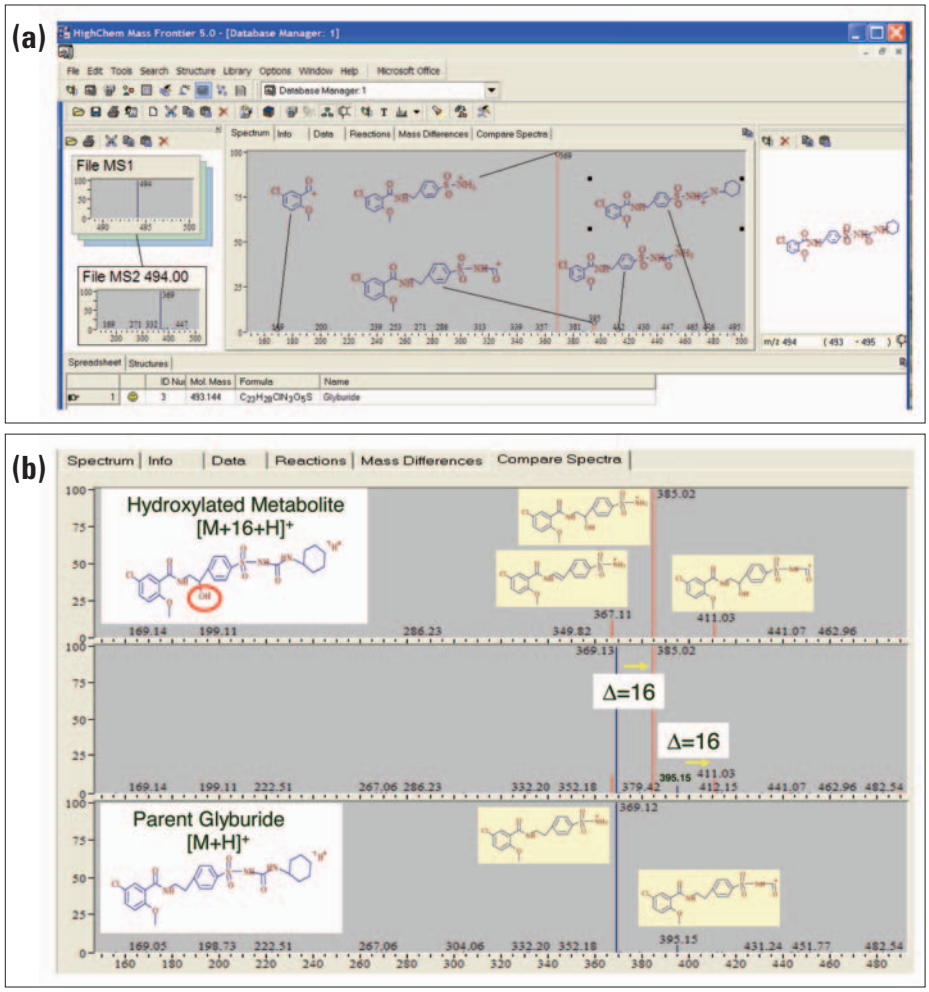


Figure 3: Application of Mass Frontier for metabolism study of glyburide
(a) Library search results for glyburide; **(b)** Spectra comparison for identification of hydroxylated metabolites

dehydrogenation occurred at the left side of cyclohexyl moiety, and *m/z* 169 indicated that the dehydrogenation occurred at the right side of benzyl ring. Therefore, the biotransformation very likely occurred on the ethyl group.

Figure 5(d) and (e) depicted the MS/MS spectra for the metabolites eluting at 1.58 min and 2.32 min. The major fragments ions at *m/z* 395 and 369 indicated that the transformation occurred on the cyclohexyl ring.

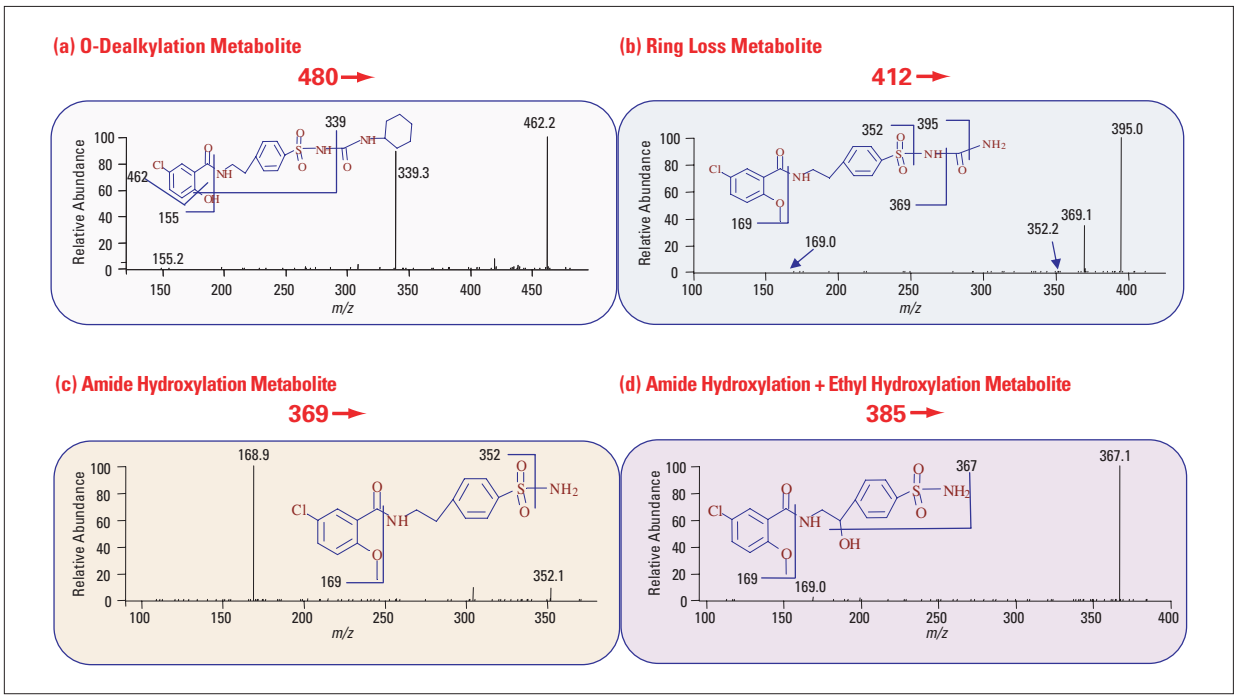


Figure 4: MS/MS Spectra of four unpredicted metabolites. **(a)** MS/MS spectrum of O-dealkylation glyburide (*m/z* 480); **(b)** MS/MS spectrum of ring loss metabolite (*m/z* 412); **(c)** MS/MS spectrum of metabolite by amide hydroxylation (*m/z* 369); **(d)** MS/MS spectrum of metabolite by amide hydroxylation + ethyl hydroxylation (*m/z* 385).

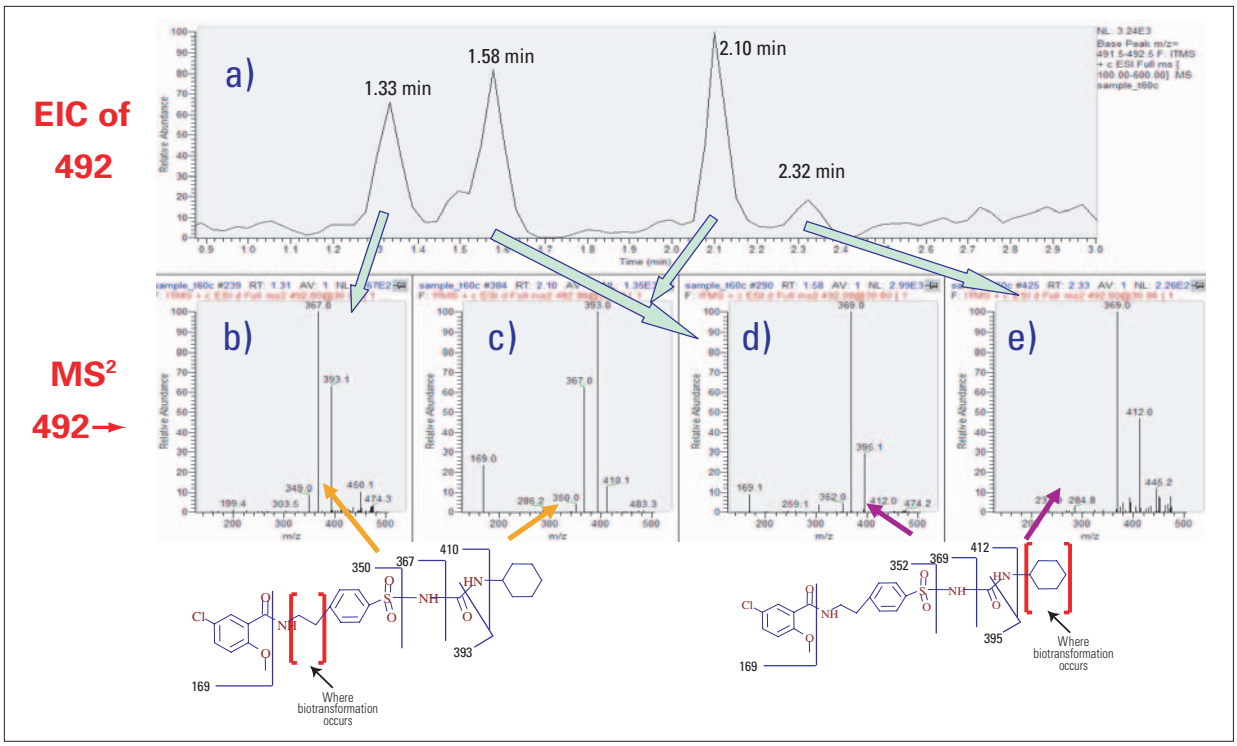
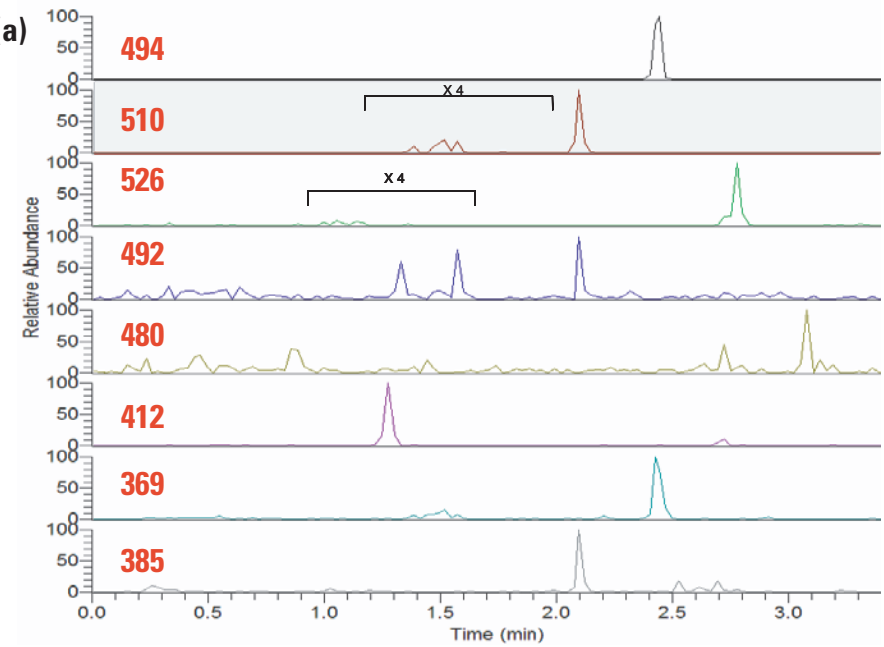


Figure 5: Identification and characterization of dehydrogenated metabolites (*m/z* 492)
(a) Extracted ion chromatogram (EIC) of 492 in MS; **(b)–(e)** MS/MS spectra of 492

Glyburide, and 19 metabolites, were identified and characterized in approximately three minutes. The extracted ion chromatograms of the parent drug and metabolites are depicted in Figure 6a, and a summary table is shown in Figure 6b. Six mono-oxidation metabolites (*m/z* 510, M+16), five di-oxidation metabolites (*m/z* 526, M+32), and four dehydrogenation metabolites (*m/z* 492, M-2) were identified. In addition, four unpredicted metabolites were also found. These metabolites were all identified from their retention time, precursor ion *m/z* and MS/MS spectra.

In addition to a more comprehensive identification of the metabolites, the study with the LXQ enabled elimination of false positives. For example, two ions, *m/z* 412 and *m/z* 414 were detected at the same retention time with an approximate 3:1 intensity ratio. The LXQ acquired MS/MS spectra of both of them, and they share the same fragmentation patterns with a 2 amu shift. Considering the fact that there is one chlorine in glyburide, it is safe to conclude that *m/z* 414 is an isotopic peak of *m/z* 412 (Figure 7a). However, this ion was mistakenly identified as a unique metabolite using a quadrupole/trap hybrid mass spectrometer where two different scan methods were required to detect these two ions.[6] Furthermore, the application of MetWorks readily eliminates degradation products from the metabolite candidates. As an example, two species, formed from: (a) dehalogenation and oxidation (476, M-18) and (b) cyclohexyl loss followed by oxidation (427, M-67) were previously reported[6] as metabolites. However, both species were observed in the control with similar abundance as in the incubation sample. In addition, their MS/MS spectra are identical. Therefore MetWorks automatically eliminates them from the possible metabolite list (Figure 7b).

(a)



(b)

Drug and Metabolites	#
Parent Drug (494)	1
Hydroxylation (510)	6
Di-Hydroxylation (526)	5
Dehydrogenation (492)	4
O-Dealkylation (480)	1
Ring Loss (412)	1
Amide hydroxylation (369)	1
Amide hydroxylation + Ethyl hydroxylation (385)	1
Total Number of Metabolites	**19**

Figure 6: Summary of identification and characterization of glyburide with its 19 metabolites
(a) Extracted ion chromatograms of parent drug and metabolites;
(b) Summary of metabolic profiling results

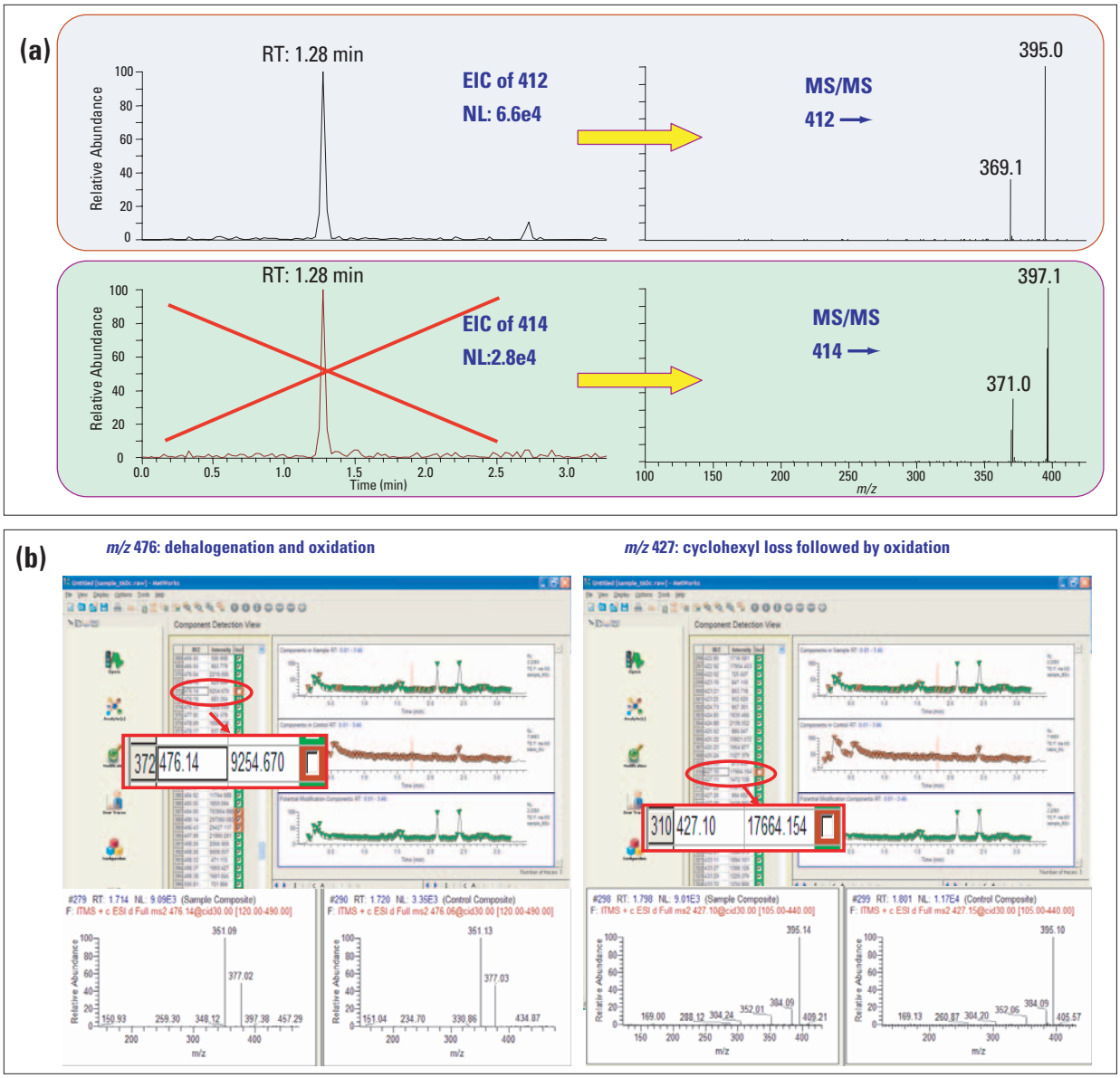


Figure 7. Elimination of false positive **(a)** Identification of isotopic peak (*m/z* 414); **(b)** Identification of degradation products *m/z* 476 and *m/z* 427 using MetWorks

Conclusions

A high throughput LC/MS/MS approach has been developed for metabolic profiling using the LXQ linear ion trap mass spectrometer. The identification and characterization of glyburide along with 19 metabolites was accomplished in a single LC run of approximately three minutes using the fast cycle time, high sensitivity, and excellent spectral quality of this instrument. Compared to previous literature reports, this approach provided a more comprehensive identification of metabolites and eliminated false positives. The use of MetWorks and Mass Frontier software enabled rapid and confident analysis of complicated metabolic profiling data.

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