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MET-XAlign: A Metabolite Cross-Alignment Tool for LC/MS-Based Comparative Metabolomics

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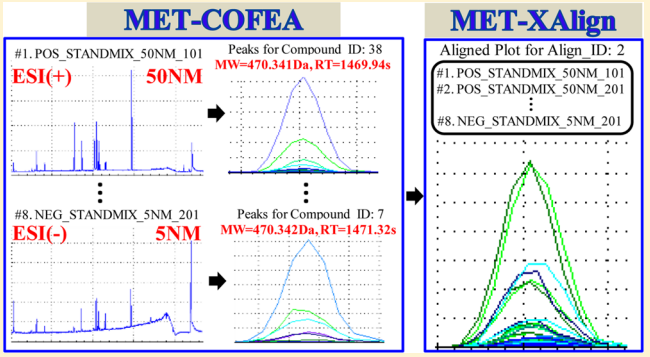
Supporting Information

ABSTRACT: Liquid chromatography/mass spectrometry (LC/MS) metabolite profiling has been widely used in comparative metabolomics studies; however, LC/MS-based comparative metabolomics currently faces several critical challenges. One of the greatest challenges is how to effectively align metabolites across different LC/MS profiles; a single metabolite can give rise to multiple peak features, and the grouped peak features that can be used to construct a spectrum pattern of single metabolite can vary greatly between biochemical experiments and even between instrument runs. Another major challenge is that the observed retention time for a single metabolite can also be significantly affected by experimental conditions. To overcome these two key challenges, we present a novel metabolite-based alignment approach entitled MET-XAlign to align metabolites across LC/MS metabolomics profiles. MET-XAlign takes the deduced molecular mass and estimated compound retention time information that can be extracted by our previously published tool, MET-COFEA, and aligns metabolites based on this information. We demonstrate that MET-XAlign is able to cross-align metabolite compounds, either known or unknown, in LC/MS profiles not only across different samples but also across different biological experiments and different electrospray ionization modes. Therefore, our proposed metabolite-based cross-alignment approach is a great step forward and its implementation, MET-XAlign, is a very useful tool in LC/MS-based comparative metabolomics. MET-XAlign has been successfully implemented with core algorithm coding in C++, making it very efficient, and visualization interface coding in the Microsoft.NET Framework. The MET-XAlign software along with demonstrative data is freely available at http://bioinfo.noble.org/manuscript-support/met-xalign/.



Liquid chromatography coupled with mass spectrometry (LC/MS) is an important analytical technology in metabolomics with several advantages over gas chromatography coupled with mass spectrometry (GC/MS) approaches.[1] For example, LC/MS does not require derivatization for polar compounds, is capable of analyzing a wider range of compounds, commonly uses low-energy electrospray ionization (ESI), and provides greater sensitivity. Untargeted metabolite profiling, especially LC/MS-based comparative metabolomics, has therefore been widely used to identify biomarkers by comparing the shared differences among the detected biologically meaningful features in hundreds to thousands of LC/MS samples that are produced from multiple well-designed comparative experiments.[2] However, until now, there were no universal LC/MS libraries that could be used to elucidate the chemical structures and identities of the metabolites.[3] The difficulty associated with creating such a resource arose because the extracted spectrum patterns for a single metabolite can vary greatly due to different instrument types and configurations as well as different experiment settings, such as solvent type, voltage, and ionization polarity.[4] Additionally, retention time shifts can be greatly affected by analytical conditions such as temperature, pressure, and humidity.[5] The varying spectrum patterns and retention time shifts together make it a difficult task to identify meaningful biomarkers from hundreds to thousands of LC/MS samples acquired with different experimental configurations, which has greatly impeded LC/MS-based comparative metabolomics studies. An efficient tool that can align identical metabolites across not only different samples but also different biological experiments would thus be highly advantageous.

In order to overcome the aforementioned challenges, we have developed a novel alignment tool named MET-XAlign that effectively utilizes our previously published tool MET-COFEA.[6] MET-COFEA is a LC/MS data processing platform for metabolite compound feature extraction and annotation that can detect and cluster metabolite-related peak features into groups. For each metabolite compound feature consisting of a group of associated feature peaks, the neutral molecular mass can be deduced and the representative retention time of the compound can be estimated. The molecular mass and retention

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time are then used as the basis for cross-alignment of this metabolite across different samples and experimental configurations, even when the corresponding spectrum patterns vary between samples and experiments. On the basis of the deduced molecular mass and estimated retention time, MET-XAlign aligns the annotated metabolites together from all samples, if their corresponding molecular masses and retention times fall within a user-specified tolerance window.

In general, LC/MS-based comparative metabolomics requires analyzing different biological experiments and may use different experimental configurations, which in turn generate different metabolite-related spectrum patterns. For example, the extracted spectrum patterns for the same metabolite in the ESI(+) and ESI(−) ionization modes are totally different; therefore, the users must specify the correct ESI mode and allow MET-COFEA to select the corresponding configurable ESI ion species including possible fragments, adducts, and isotopes. Additionally, considering the detection sensitivity of the instrument, the detectable signal intensity for the same metabolite also varies with metabolite concentration level, collision voltage, etc. Thus, configuring the optimal parameters for the entire data set is problematic: if MET-COFEA is configured with more stringent parameters, the analysis of some data samples will miss some potentially important metabolites present at low concentrations or some of the associated peaks; on the other side, if MET-COFEA is configured with more relaxed parameters, the analysis procedure will be very time-consuming and will also generate many false-positive results, leading to less effective biomarker identification.

However, by combining MET-XAlign and MET-COFEA, we can overcome the potential pitfalls of MET-COFEA alone. In this report, by analyzing the mixed standard data sets and the untargeted biological data sets, we demonstrate that MET-XAlign in combination with MET-COFEA can effectively align the potential same metabolites across different samples and different experimental conditions. Additionally, we also find that the complementary information extracted from different samples, different experimental conditions may help to narrow down even specifically find the potential metabolite biomarker.

EXPERIMENTAL SECTION

Sample Preparation. A standard mixture sample was prepared containing umbelliferone (10 mmol/μL), 6-hydroxyflavone (10 pmol/μL), 3-hydroxyflavone (10 pmol/μL), chrysin (10 pmol/μL), naringenin (10 pmol/μL), quercetin (10 pmol/μL), 18β-glycyrrhetinic acid (10 pmol/μL), pygenic acid B (10 pmol/μL), rutin (10 pmol/μL), and soy B mixture containing Glc-Gal-GlcUA-soyasaponin B (0.5 pmol/μL), Rha-Gal-GlcUA-soyasaponin B (9.1 pmol/μL), Rha-Ara-GlcUA-soyasaponin B (4.3 pmol/μL), Gal-GlcUA-soyasaponin B (2.4 pmol/μL), and Ara-GlcUA-soyasaponin B (0.5 pmol/μL). All standards were purchased from Sigma with the exception of the soyasaponin B purified mixture, which was obtained from Mark Berhow of the USDA. A 1:10 dilution of the above mixture was also generated. A series of biological samples were extracted from leave and roots of four different individual *Medicago truncatula* ecotypes, respectively.

UPLC−MS Data Acquisition. The above two mixed standards samples for two different concentrations levels were analyzed in an Acquity UPLC system (Waters) interfaced with a Q-TOF Premier mass spectrometer (Waters). Both ESI(+) and ESI(−) ionization modes were adopted. The details for LC−TOF-MS analysis are described in the Supporting Information. Finally, four different experiment conditions were created and each of them contains two replicas marked as "_101" and "_201". Table S-1 (Supporting Information) gives the summary information about the 8 LC/MS metabolomics files. Additionally, the above real biological samples were also analyzed in the same UPLC−Q-TOF system and ionized in ESI(−); finally, 24 LC/MS data files were generated for a comparative metabolomics study through metabolite profiling.

DATA ANALYSIS METHODS

One of important applications of LC/MS-based comparative metabolomics is to identify candidate biomarkers by comparing the shared differences across a large number of metabolomics data files from different experiments. On the basis of our proposed compound-based alignment strategy, MET-XAlign is dedicatedly developed, which can identify the potential same metabolites by incorporating with our previously published tool, MET-COFEA. The typical application procedures using MET-COFEA and MET-XAlign for LC/MS-based comparative metabolomics include (1) using MET-COFEA to analyze each data file and output all possible metabolites mainly characterized by the deduced neutral molecular mass and estimated retention time and (2) using MET-XAlign to align the potential same metabolites across experiments and finally identify the biological meaningful candidate biomarkers. In the following section, MET-COFEA is briefly described, then the compound-based alignment strategy and the related MET-XAlign are described in detail, finally the LC/MS-based comparative metabolomics using MET-COFEA and MET-XAlign is comprehensively illustrated.

MET-COFEA. MET-COFEA[6] is a pipeline and visualization tool that has been implemented with core code in C++ and visualization interfaces in.NET that can be downloaded from http://bioinfo.noble.org/manuscript-support/met-cofea/. MET-COFEA mainly includes the compound feature extraction module and the compound feature annotation module. The compound feature extraction module aims to extract individual peaks that correspond to one of a compound's feature from each LC/MS raw data input. The compound feature annotation module aims to cluster/group peaks based on peak shapes and retention time, annotate the detected clusters/groups, and identify compound-associated peak clusters/groups.

Compound-Based Alignment. Shifts in retention time for given compounds occur commonly between LC/MS experiments due to change in the specific analytical conditions, such as temperature, pressure, and humidity.[5] The chromatographic retention time should be aligned prior to any statistical or multivariate analysis;[7] therefore, alignment, which aims to correct the shift of chromatographic retention time among different runs is a necessary procedure. There are many algorithms related to chromatogram alignment, including correlation-optimized warping (COW),[5] dynamic time warping (DTW),[8] COW-TIC,[9] COW-CODA,[10] and DTW-CODA,[11] all of which aim to align the whole chromatogram. XCMS,[12] MAVEN,[13] and MZmine RANSAC[14] adopt an iteration strategy of peak grouping/matching and peak retention time correction across samples, which consider the detected peaks as one-dimensional peak features and align the detected chromatographic peaks rather than the whole chromatogram. Another alignment method, utilized by LCMSWARP[15] and LC/MS image-based alignment,[16] can be used to correct mass

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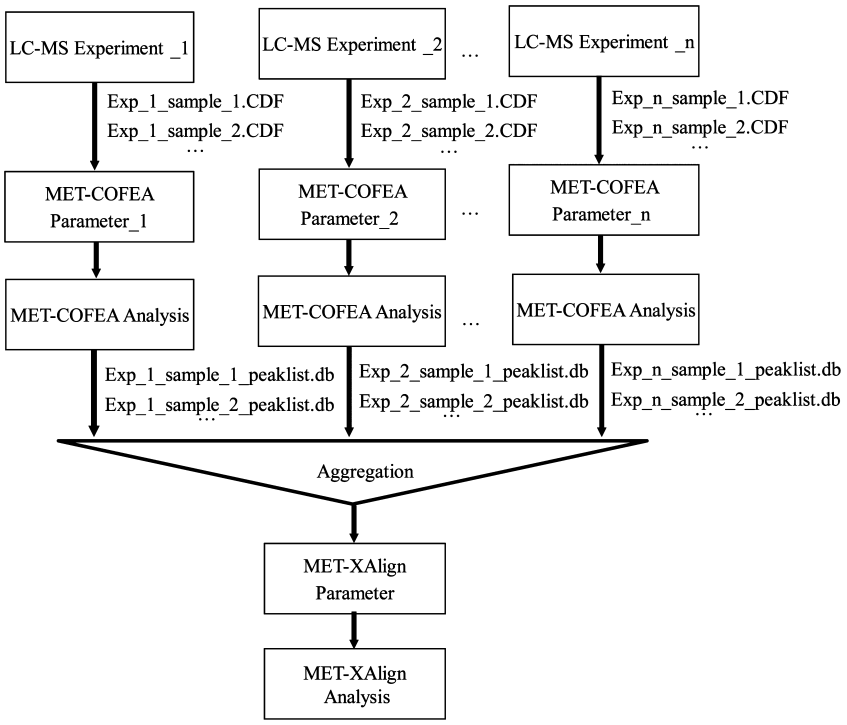

Figure 1. Proposed flowchart of LC/MS-based comparative metabolomics analyses using both MET-COFEA and MET-XAlign.

drift and elution time variations based on two-dimensional LC/MS features.

Of all the above alignment algorithms, none is based on the final potential structural feature of the compound. Chae et al.[17] proposed an approach called "peak block" to perform alignment, which can contribute to preserve the shape and area of the peak. MET-COFEA clusters/groups the detected peak features into compound groups and for each compound group annotates the relationship of each peak feature with the deduced neutral molecular mass. Here, we utilize the annotation information together with the deduced molecular mass, the common base of a compound group that is independent of experimental conditions, for metabolite compound-based cross-alignment. The principle of the compound-based alignment can be briefly described as that the compounds will be aligned together across different samples if the deduced molecular mass and estimated retention time fall within an acceptable tolerance window. Similar to the traditional peak alignment procedure, there are two important criteria for compound-based alignment: reference determination and alignment matching strategy.[5] Alignment is aiming to correctly find all the potential same objects in global and therefore usually is a time-consuming procedure. To consider the practical alignment requirements in speed and precision, the current MET-XAlign provide the user two options: simple alignment but with a fast speed and deep alignment with more consideration. The algorithm details of the proposed compound-based alignment are described in the Supporting Information.

MET-XAlign. The above compound-based alignment strategy is based on the MET-COFEA compound feature extraction and annotation results. MET-XAlign, as an alignment tool, has been developed independently. It mainly consists of an algorithm core and user interfaces. The algorithm core, i.e., compound-based alignment, is implemented in standard C++ using Standard Template Libraries (STL). The user interfaces mainly include interactive parameter configuration interfaces and data visualization components, which are implemented using the Microsoft.NET platform and combined with an open source graph library, named ZedGraph (http://sourceforge.net/projects/zedgraph/), for creating 2D line and bar graphs. The interactive parameter configuration interfaces allow users to load the analysis results for each sample exported from MET-COFEA as a.db database file in SQLite (http://www.sqlite.org/) and configure the necessary parameters for alignment. The visualization components allow users to load and view the alignment results that are exported as an.aligndb database file in SQLite.

LC/MS-Based Comparative Metabolomics Analysis Using MET-COFEA and MET-XAlign. LC/MS is capable of analyzing a wider range of compounds than GC/MS. Therefore, LC/MS has been widely used in comparative metabolomics. However, the lack of universal LC/MS libraries and the varying spectrum patterns for the same metabolite across different samples and experiments make LC/MS-based comparative metabolomics analysis a challenging task. We successfully developed MET-COFEA, which enables each possible metabolite compound and the associated chromatographic peak features to be extracted and annotated. The annotated peak features, deduced neutral molecular mass, and the estimated compound retention time can be used together to align the same metabolite across samples. MET-COFEA alone has several potential pitfalls: (1) it needs to configure and optimize parameters according to experimental conditions and (2) the measured concentration of the metabolite must be comparable, and the instrument configurations must be the same in the case of one set parameter to process a group of data. However, in comparative metabolomics, the concentrations of the metabolites can vary broadly, and the data can be generated using different instrument configurations in different laboratories. In summary, MET-COFEA with one fixed set of parameters cannot balance the different analysis requirements

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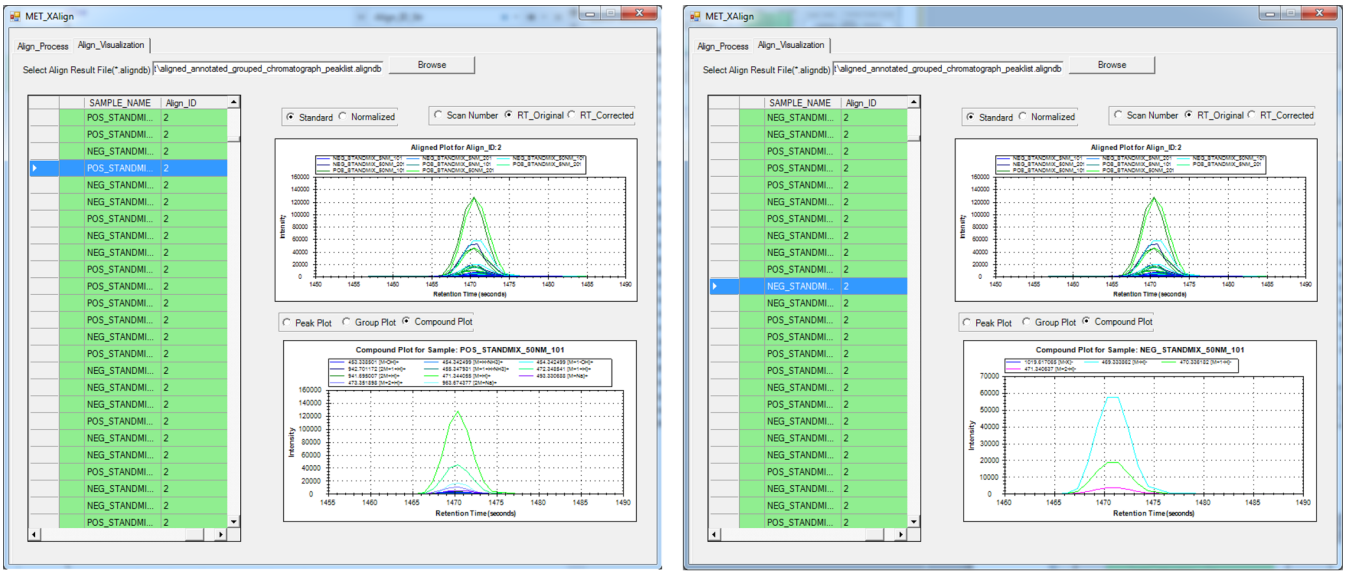

Figure 2. Visualization of the MET-XAlign alignment result for the metabolite named "18β-glycyrrhetinic acid", marked by unique Align_ID = 2. (A) The annotated metabolite fragment peaks from sample file "POS_STANDMIX_50NM" are plotted in the right bottom panel. (B) The annotated metabolite fragment peaks from sample file "NEG_STANDMIX_5NM" are plotted in the right bottom panel.

Table 1. Outline Performance of MET-COFEA and MET-XAlign by Analyzing LC/MS-Based Mixed Standard Data Acquired at Different Concentration Levels and Different ESI Modes

MET-COFEA analysis results			
file name	no. Peak_ID	no. Group_ID	no. of Compound_ID
POS_STANDMIX_50NM_101.CDF	2012	580	1243
POS_STANDMIX_50NM_201.CDF	1945	542	1179
POS_STANDMIX_5NM_101.CDF	1593	766	907
POS_STANDMIX_5NM_201.CDF	1880	863	1038
NEG_STANDMIX_50NM_101.CDF	269	96	140
NEG_STANDMIX_50NM_201.CDF	186	60	101
NEG_STANDMIX_5NM_101.CDF	227	109	121
NEG_STANDMIX_5NM_201.CDF	242	116	140
MET-XAlign alignment results			
no. of *Align_ID*		2551	
no. of *Valid Align_ID*[a]		13	

[a] *"Valid Align_ID"* represented one potential metabolite that has been found in all of the LC/MS data files.

among comparative metabolomics experiments. However, the analysis, including metabolite feature extraction and annotation for each sample and alignment across all samples, can be separated. On the basis of this, our dedicated alignment tool named MET-XAlign and its combination with MET-COFEA can overcome these difficulties in LC/MS-based comparative metabolomics.

Figure 1 shows the flowchart of LC/MS-based comparative metabolomics analyses using both MET-COFEA and MET-XAlign. By combining MET-COFEA and MET-XAlign, users can configure the optimal parameters according to the settings of each biological experiment and individually run MET-COFEA to analyze the corresponding samples, which will extract and annotate all the associated features of the detectable metabolites and output them as database files. The exported database files from MET-COFEA can be aggregated and analyzed in MET-XAlign according to the user-configured optimal alignment parameters. The combination of MET-COFEA with MET-XAlign makes it practical to align the potential same metabolite compounds (known or unknown) in LC/MS-based comparative metabolomics data.

RESULTS AND DISCUSSION

For the mixed standard data files, we configure MET-COFEA's parameters for each experimental set according to the concentration levels and ESI modes and then ran MET-COFEA separately according to the configured parameters. The configured parameters for MET-COFEA were manually adjusted for multiple times to ensure that all of the molecular peaks of the standards, $[M - H]^-$ for ESI(−) and $[M + H]^+$ for ESI(+), could be detected. Note that the configured parameters for MET-COFEA will affect the performance of compound feature extraction and compound feature annotation, and the more relaxed parameters will result in more chromatographic peaks, more Group_IDs, and more Compound_IDs.

The MET-COFEA analysis results for each LC/MS data file were exported and stored as individual database files with the extension of ".db". All analysis results were loaded into MET-

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XAlign for alignment. Figure S-1 (Supporting Information) shows a screenshot of MET-XAlign for loading the analyzing result files outputted from MET-COFEA. After configuring the alignment parameters for MET-XAlign and running MET-XAlign, the alignment results across all samples were exported into a database file with the extension of ".alignndb", which can be specifically recognized and loaded back into MET-XAlign for visualization and verification. Figure 2 shows the alignment of one of our standard compounds, "18β-glycyrrhetinic acid" in MET-XAlign. There were 11 peak features in ESI(+) mode, while only 4 peak features in ESI(−) mode were detected. None of the extracted peak features is overlapped. On the basis of these observations, we concluded that the spectrum pattern of the metabolite "18β-glycyrrhetinic acid" in ESI(+) and ESI(−) profiles were totally different. However, the deduced neutral molecular mass and retention time were close; therefore, they could be aligned together and represented by the same Align_ID = 2. By inspecting the MET-XAlign results for the same Align_IDs, users can have a comprehensive understanding about the spectrum patterns of the metabolite with different experimental settings, which is especially useful in comparative metabolomics studies.

The analyzing results from MET-COFEA and the alignment results from MET-XAlign for the 8 mixed standard files are given in Table 1. In Table 1, the valid Align_ID means that the potential same metabolite has been found in all 8 LC/MS profiles. We observed that there were 2 551 Align_IDs in total, but only 13 valid Align_IDs were found across all of the 8 sample files, which corresponded to the 13 of the known standard metabolites. Of course, the user can adopt a more relaxed strategy to define a valid Align_ID by setting up a percentage ratio of the number of sample files that found the potential same metabolite over the total number of sample files. Figure S-2 (Supporting Information) shows the relationship of the number of Valid Align_ID varies with the percentage of the sample file number.

From Table S-1 (Supporting Information), we observed that the raw file acquired at ESI(+) were usually larger than the raw file acquired at ESI(−), when other experimental conditions were the same. Similarly, from Table 1, we also observed that more chromatographic peaks in ESI(+) than in ESI(−) were detected when both the experimental conditions and the MET-COFEA parameter configurations were comparable. We concluded that the same metabolite was more easily ionized in ESI(+) mode than in ESI(−) mode with otherwise the same experimental conditions; therefore, there were more related ion peaks that were detected by mass spectrometry. This conclusion was further verified by the example metabolite "18β-glycyrrhetinic acid" depicted in Figure 2.

This characterization difference in spectrum pattern is both beneficial and detrimental. More ion peaks mean more features, which can improve specificity in library searching and metabolite identification. However, a greater number of fragment peaks can also decrease the intensity of the fragment peaks and thereby reduce the sensitivity of detection. In order to extract some very low molecular peak $[M + H]^+$ for the metabolite at ESI(+), we need to configure MET-COFEA with very relaxed parameters for mass trace extraction and subsequent chromatographic peak detection. Additionally, LC/MS technique typically utilizes ESI as the ionization method, which can result in high levels of chemical background noise. The relaxed parameter configuration for chromatographic peak detection, which will unavoidably yield more false-positive peaks from noise and solvent, will at the same time make data processing with MET-COFEA more time-consuming.

The advantage of our MET-XAlign over the peak-based alignment approaches is obvious as the alignment principle of MET-XAlign is fundamentally based on compound-related characteristics instead of each individual chromatographic peak characteristic. We chose MZmine RANSAC,[14] a peak-based alignment approach, for comparison. We specified 20 s as the retention time tolerance for both MZmine RANSAC and MET-XAlign and 0.1 Da for the m/z tolerance of MZmine RANSAC and for molecular mass tolerance of MET-XAlign. MZmine RANSAC could be applied to align the 4 ESI(+) files or 4 ESI(−) files separately and generated 204 and 100 aligned chromatographic peaks, respectively, but could not be applied to align the 8 mixed ESI files. However, the approach that combined MET-COFEA and MET-XAlign was successfully applied to analyze all of the three scenarios and generated 198, 21, and 13 valid Align_IDs, respectively. Herein, we can conclude that MET-XAlign has a wider application scope and suits for analyzing different LC/MS data sets.

For the 24 untargeted biological data files, the same analysis procedures were adopted, and our comparative metabolomics study included 3 scenarios: to comprehensively analyze (1) the 12 leaf data files, (2) 12 root data files, and (3) 24 leaf + root data files, respectively. Table S-2 (Supporting Information) gives the analysis result information for the untargeted LC/MS metabolomics data at the 3 different analysis scenarios. We found 36 and 71 the potentially same metabolites in the *Medicago truncatula*'s leave and roots, respectively, on which the different ecotypes can be clearly distinguished/clustered.[6] Additionally, the 16 potential same metabolites in scenario 3 are expected to provide a more meaningful biological insight for the *Medicago truncatula*'s leave and roots at different ecotypes. Supporting Information (Figure S-3−S-5) show the screenshots of MET-XAlign's user interfaces for loading the MET-COFEA's analyzing results and the comprehensive visualization of alignment results.

CONCLUSIONS

We have developed a novel alignment tool named MET-XAlign, which can be combined with our previously published tool named MET-COFEA for LC/MS-based comparative metabolomics analysis. Our proposed approach separates data analysis of individual samples by allowing users to configure different independent parameters for MET-COFEA according to experimental conditions and to optimize downstream metabolite alignment. The analysis results from each sample data across different experiments can be aggregated and aligned by MET-XAlign. Therefore, the same potential metabolite not only from different samples but also from different biological experiments can be aligned. Compared with the traditional peak-based alignment strategies, our alignment approach aim to cross-align metabolites and make the base on the representative retention time and neutral molecular mass of the metabolite deduced from its multiple associated peaks. Although the extracted spectrum patterns for the same metabolite can vary greatly due to differences in factors such as concentration, ionization mode, and voltage, the deduced neutral molecular mass and retention time can be used as a basis for alignment, which is independent of experimental conditions. The analysis results for the mixed standard data set and the untargeted biological data set demonstrate the promise of MET-XAlign,

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which in turn enables and empowers LC/MS-based comparative metabolomics studies. As a subsequent platform of MET-COFEA, MET-XAlign is also equipped with user-friendly interfaces for alignment analysis and also allows loading back the alignment results for further inspection and validation. In MET-XAlign, differences in the grouped peak features of the same metabolite due to different experimental conditions can be comprehensively visualized, compared, and analyzed.

In some specific application, the retention time can change dramatically, and many different isomers with the same molecular mass may occur. Herein, the adopted alignment strategy in MET-XAlign for LC/MS-based comparative metabolomics may face some potential pitfalls, such as how to set the optimal retention window and how to distinguish the isomers with the same molecular mass. Currently, we are in an effort to develop a more reliable metabolite cross deep alignment approach that can comprehensively combine the information including the retention index, molecular mass, and spectrum matching results (the extracted spectrum pattern against our to-be-built instrument independent ESI spectrum library). We expect that the integration can well solve the above-mentioned pitfalls and facilitate LC/MS-based comparative metabolomics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01324.

Additional experimental details and results (PDF)

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Notes

The authors declare no competing financial interest.

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