Introduction

Novel drug leads are urgently sought after from natural products to combat currently incurable diseases or to deal with revolutionary viruses. To explore new drugable molecules, screening natural extracts and optimizing chromatographic separation conditions are fundamental for further studies. In recent years, the application of liquid chromatography–mass spectrometry (LC-MS) in the analysis of natural products has been continuously increasing, due to the greatly improved separation and detection capabilities. Nonetheless, the performance of LC-MS in the comprehensive analysis of chemical entities in natural products is still mediocre, because of a great number of compounds existing and large differences in concentrations of individual compounds. It also helps eliminate unexpected baseline disturbances and improve the resolution of LC-MS chromatograms. Unlike conventional deconvolution strategies, this method distinguishes the chemical properties of precursor ions through their dynamic retention behaviors. The algorithm is demonstrated with LC-MS datasets of control samples. In the application of such algorithms on a more complicated natural extract from *Lycium ruthenicum Murr.*, 206 precursor ions were facilely determined.

Keywords Deconvolution, untargeted molecular analysis, natural products, LC-MS, isotope ratio, retention behavior

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advantageous than TOF due to its greater sensitivity.

The objective of our work was to develop a LC-MS data preprocessing algorithm for detecting and clustering ions from natural products. An implementation of the algorithm was demonstrated by an open source MATLAB program. Since the molecular identification was based on the chromatographic retention, the program was named chromatographic retention assisted deconvolution (CRAD). The performance of CRAD was demonstrated with LC-qMS analysis datasets of control samples and a natural extract from *Lycium ruthenicum* Murr.

**Experimental**

The natural products extracted from wild *Lycium ruthenicum* Murr. growing on the Qinghai-Tibetan plateau were included in this study. Fresh fruits of *Lycium ruthenicum* Murr. were harvested and pulped. The juice was filtered through a ceramic membrane (0.1 μm pore) and the filtrate was collected. The 95% ethanol maceration of seeds and peels were collected by centrifugation. All the extraction solutions of *Lycium ruthenicum* Murr. were mixed. The extracts were lyophilized and stored at room temperature. The standard compounds of gallic acid and flavonoid derivatives such as apigenin, baicalein, hesperetin, naringenin, catechin, and quercetin were derived from China’s National Institutes for Food and Drug Control. A series of serine lactones were purchased from Sigma-Aldrich, including \(N\)-decanoyl-DL-homoserine lactone (DL), \(N\)-(3-oxodecanoyl)-DL-homoserine lactone (ODL), \(N\)-dodecanoyl-DL-homoserine lactone (DDL), \(N\)-(3-hydroxy-dodecanoyl)-DL-homoserine lactone (HDL), \(N\)-(3-oxododecanoyl)-L-homoserine lactone (ODDL), \(N\)-(3-hydroxy-dodecanoyl)-L-homoserine lactone (HDDL), \(N\)-(3-tetradecanoyl)-L-homoserine lactone (TDL). Detailed structures and hydrophobic properties of the selected chemical standards are summarized in Supporting Information (Table S1).

The analytes for LC-MS analysis were prepared in the mixed solvent of MeOH and \(H_2O\) (v:v = 50:50) at a concentration of 0.5 mg/mL. A mixture of flavonoid derivatives and gallic acids (M1), and a mixture of serine lactones (M2) were prepared by mixing the solutions of pure compounds at equal volumes. The analytes were stored in the dark at 4°C before analysis.

LC-MS detection was performed on a Shimadzu UHPLC-AB Sciex Triple Quad 5500 MS system. LC separation through hydrophilic interaction liquid chromatography (HILIC) was performed using XAmide HILIC column (10 μm particle size, \(\phi 4.6 \times 250 \text{ mm}, \text{Acchrom, Beijing, China}\)). The mobile phase was a mixture of \(H_2O\) and acetonitrile. According to the retention mechanism, the strong eluent in HILIC separations was water. Five stepwise elution gradients were designed, and the time spots to change the composition of the mobile phase were fixed (Table S2, Supporting Information). Reversed-phase liquid chromatography (RPLC) was conducted on a Capcell Core C18 column (2.7 μm particle size, \(\phi 4.6 \times 150 \text{ mm}, \text{Shiseido, Japan}\)). The mobile phase was a mixture of \(H_2O\) and MeOH. Isocratic flows were adopted for the analysis of M2 (Table S3, Supporting Information). The flow rates of both elution conditions were consistent at 1.0 mL/min, while half of the effluent was bypassed into waste. Mass spectrometric data were collected from 100 to 1200 u in either positive or negative ion modes. The electrospray ionization (ESI) condition included the ionspray voltage at 5.5 kV in positive mode and –4.5 kV in negative mode. Interface heater temperature was 550°C. Declustering potential (DP) was 130 V and the entrance potential (EP) was 10 V for LC-MS analysis. A collision energy of 48 eV and a collision gas pressure of 8 psi were adopted for LC-MS/MS analysis, while DP was raised to 200 V.

**Theory**

The preprocessing strategy described here is built on previous ideas of chemometric analysis.\(^6\) It also includes novel methods for extracting chromatographic peaks of possible real ions across liquid chromatograms by matching their retention behaviors with the Snyder–Soczewinski model. A general overview of the data preprocessing strategy is shown in Fig. 1. We will describe the three steps in detail, namely, PX-RelArea denoising, retention time prediction, and in-depth clustering. Their performances in denoising and identifying real ions are discussed in Results and Discussion.

**PX-RelArea denoising**

In this investigation, the mass scanning range was set as 100 and 1200 u, and the LC-MS dataset was sliced with an interval of 0.1 u in the \(m/z\) dimension. An extracted ion chromatogram (XIC) is constructed based on ion traces within a \(m/z\) window of 1 Da width, corresponding to the full width at half-maximum peak value (FWHM) of \(\pm 0.5\) Da of a normal qMS apparatus. Therefore, there are a total of 11001 XICs. Accordingly, an XIC is built by ion traces of the same compound or isobaric compounds. In general, the number of data points regarded as noise is significantly larger than that of real-ion signals in the LC-MS datasets. In another word, noises tend to generate numerous featureless peaks, while the number of peaks of real ions in an XIC is limited.\(^16\) Two simple parameters were controlled to filter the XICs containing noises only: the thresholds for peak numbers (PX) and relative peak area (RelArea). Peaks in an XIC with relative area higher than RelArea are counted. An XIC is considered containing real-ion peaks.
peaks and named as real-ion XIC (RIXIC), when the number of counted peaks inside the XIC is less than PX. Because the data of noises overweights that of the signals, the featured RIXICs should be distinguished from featureless noises. Discrete values of PX and RelArea are tested so that the number of RIXIC (NRIXIC) becomes a function of PX and RelArea. MATLAB helps to interpolate and smooth the function NRIXIC(PX, RelArea) automatically. Since we have a large number of XICs to analyze, NRIXIC becomes differentiable with respect to PX and RelArea. Taking the second order partial derivative of NRIXIC with respect to PX and RelArea leads to the Hessian matrix:

\[
H = \left[ \frac{\partial^2 N_{RIXIC}}{\partial PX \partial RelArea} \right] \tag{1}
\]

Investigating the positive/negative definite (PD or ND) properties of \( H \) helps distinguish the inflection points, where the Hessian matrix switches from PD(ND) to ND(PD). According to the matrix, \( H_{11} \), the first element of the \( 2 \times 2 \) matrix \( H \), is not equal to 0 and its determinant (\( \text{det}(H) \)) is positive.3 In the context of this research, inflection points to be selected would imply that \( H \) gradually changes from ND to PD. The noise data filters are set up using the PX-RelArea values of such inflection points.

**Retention time prediction**

The chromatographic behavior of a chemical could be theoretically predicted according to the Snyder–Soczewinski model (Eqs. (2) and (3)). Specifically, the capacity factors of analytes in liquid chromatography is mathematically correlated with the composition of the mobile phase. When the chromatographic retention of an analyte is based on a partitioning mechanism, such as the separation in RPLC, the logarithm capacity factor (\( \log k \)) is linearly correlated with the ratio of water (Eq. (2)). Or else, when the retention is based on the surface adsorption mechanism, such as the separation in HILIC, the linear plots in the log-log form match with the experimental results more closely (Eq. (3)).4 In both separation modes, \( k_w \) represents the extrapolated retention of the solute in pure solvent of less elution strength (i.e., water of RPLC or organic modifier of HILIC). Additionally, \( k_w \) is related to the oil-water distribution ratio (\( k_w \)) based on the Collander equation, wherein "m" and "n" are the fitting constants (Eq. (4)).5 Therefore, the capacity factor (\( k \)) or retention time (\( t_R \)) of a compound is correlated to its chemical property.

\[
\log k = \log k_w - SC_B \tag{2}
\]

\[
\log k = \log k_w - S \log C_B \tag{3}
\]

\[
\log k_w = -n \log k_o + n \tag{4}
\]

Given an elution gradient, the accumulative ratio of strong eluent \( C_B (\mathcal{T}_B) \) can be calculated by integrating the instantaneous ratio of strong eluent in the course of retention time (Eq. (5)). Hence, logarithm retention time (\( \log t_R \)) of the chromatographic peak of a real ion should fit with \( \mathcal{T}_B \) or \( \log(\mathcal{T}_B) \) linearly. The retention behavior of a compound in RPLC or HILIC is thus defined by the slope and intercept of the fitting line (i.e., "\( S \)" and "\( P \)" (Eq. (6) or (7))). We make use of a minimum threshold for linear fit goodness (\( R^2 \)) at 0.9 to identify the peaks of possible real ions.

\[
\mathcal{T}_B = \int_0^t C_B(t)dt/t_R \tag{5}
\]

\[
\log t_R = SC_B + P \tag{6}
\]

\[
\log t_R = S \log \mathcal{T}_B + P \tag{7}
\]

**In-depth clustering**

Contrastive from other deconvolution algorithms, CRAD clusters the detected ions into bins not only by their retention times but also based on their dynamic retention behaviors, which is shown by analyzing with a series of different mobile phases. Different real-ion peaks are clustered into the same bin if they have similar retention behaviors. Specifically, the difference between the slopes ("\( S \)" in Eqs. (6) and (7)) is less than 0.005, and the difference between intercepts ("\( P \)" in Eqs. (6) and (7)) is less than 0.05. Such threshold values are set after iterative inspections of different real-ion peaks belonging to the same chemical standard. Consequently, the chromatographic peaks clustered into the same bin unequivocally indicate ions coming from the same molecule or molecules with similar physicochemical properties. The in-depth clustering progress further divides the ions into separated bins according to their monoisotopic ratios. On account of the complexity of ionization progress, the same molecule is often ionized in various statuses, such as molecular ions, protonated or deprotonated ions, ion adducts, ion clusters, and fragments. As such, a bin of clustered ions was assigned as noise, if the number of ions inside was less than two. Subsequently, the ions in the same bin were further clustered into a smaller group if the differences between their measured isotopic ratios were less than 0.2.

**Results and Discussion**

**Denoising effect based on the PX-RelArea assessments**

In the PX-RelArea denoising progress, all the 11001 XICs were assigned as RIXICs, when PX and RelArea were set as 10 and 0.8. Thus, no denoising effect was observed. However, \( N_{RIXIC} \) decreased at lowered RelArea and PX. When the Hessian matrix determinant (\( H \)) of \( N_{RIXIC} \) against the values of PX and RelArea was plotted in a two-dimensional surface with PX and RelArea as the coordinates, a clear boundary line could be obtained by curve fitting using a tenth order polynomial, and the inflection points which outline the boundary could be marked. Starting from the boundary line, \( N_{RIXIC} \) grew rapidly at raised RelArea and PX. Nonetheless, the false positive rate was significantly increased. As a result, the performance of denoising gradually deteriorated in a vertical direction of the boundary line. On the contrary, selecting the parameters on the boundary line afforded consistent denoising performances (see Figs. S1 and S2, Supporting Information). RIXICs contained a limited number of peaks, which were probably peaks of real ions. When PX was set at a high value, RIXICs containing more minor peaks were picked, since RelArea monotonously decreased with increasing PX on the boundary line. For example, parameters set on the boundary line at PX 1 and RelArea 0.5 only permitted picking lofty major peaks. On the contrary, the inflection point at PX 10 and RelArea 0.2 on the boundary line should be better because most elution peaks were attained while noises were reduced effectively. After PX-RelArea denoising, a proportion of 91.3 - 99.5% XICs were found featureless and eliminated (see Table S5, Supporting Information). Of note, the former algorithms such as the algorithm for various forms of chromatography hyphenated mass spectrometry (XCMS) and the algorithm of matched
filtration with experimental noise detection (MEND) defined RIXIC according to a rule of no more than 5 peaks with $S/N$ ratios beyond 10 in one XIC.\textsuperscript{5,6} Herein, for our algorithm, we have chosen RelArea 0.2 as the threshold, thus isobaric ion peaks of low $S/N$ ratios could be picked. Meanwhile, the threshold of maximum peak numbers per XIC (PX) is set as 10, which is twice the value set in the former algorithms. Thus, our algorithm allows the detection of more isobaric ions in the analysis of complex sample matrices.

Identifying real-ion peaks based on chromatographic retention behaviors

According to the Snyder-Soczewinski model, the logarithm retention time of real-ion peaks should be linearly correlated with the volumetric ratio of water in RPLC (Eq. (6)) or the logarithm volumetric ratio of organic modifier in HILIC (Eq. (7)). If the variance ($R^2$) of the fitting line is lower than 0.9, the LC-MS peaks are considered as noises and removed. Such a denoising effect allowed the precise annotation of RIXICs. Because the chromatographic retention behavior of an ion was defined by the fitting line according to the Snyder–Soczewinski model, a minimum of three chromatographic conditions should be screened. Moreover, the column and mobile phase should be properly selected to afford moderate retention of the examined analytes, which requires: (i) all the compounds in the sample matrices are eluted out of the column in more than three different elution conditions; and (ii) the retention time of a compound is varied in two altered elution conditions. The influences of pH and salt additives in mobile phases on the retention behaviors of ionic compounds (e.g., peptides) obey the theory proposed by Hováth.\textsuperscript{19} Nonetheless, pH and salt additives of mobile phases have marginal influences on the retention behaviors of less polar analytes, because of their very low dissociation constants. Thus, the retention behaviors of less polar analytes should also obey the Snyder–Soczewinski model, in the cases when pH or ionic strength of mobile phases are adjusted. On the other hand, salts can easily contaminate MS apparatus and the addition of concentrated salt additives into a mobile phase is generally unfavorable in LC-MS analysis. Considering compounds of low polarity may account for a large portion of natural products, no acid or salt additives were added into the mobile phase when developing the method. Here, the retention behaviors of ions in M1 were investigated in HILIC separation mode. Compounds in M1 could be eluted out of the column within 30 min by the designed gradient flows. However, under the elution conditions from gradient 3 to gradient 5, the analytes of apigenin and baicalein in M1 could not be retained on the column properly and their retention times were close to the dead time ($\sim$3.6 min). In addition to their retention times of 4.18 and 3.99 min in gradient 1 and 2, three points were obtained for plotting the fitting line of the Snyder–Soczewinski model to investigate their retention behaviors. Accordingly, the retention behaviors of detected ions were shown in the diagrams, when more than three points in each line were attainable (Fig. 2(a)). The real-ion peaks were distinguished from background noises if their retention behaviors fitted with the Snyder–Soczewinski model well and their retention behaviors were outlined in thick lines. In order to show the effectiveness of CRAD in identifying real-ion peaks, the retention behaviors of noise peaks were also plotted but using thinner lines. After eliminating noise peaks with irregular retention behaviors, it was found that the chromatographic peaks of all the chemical standards were successfully assigned as real-ion peaks (as listed in Table S6, Supporting Information). Based on the XICs of identified real ions, the total ion current (TICs) of M1 was reconstructed (Fig. 2(b) black line). Meanwhile, the diluted analyte showed minor peaks, which were invisible in the LC-UV chromatogram (Fig. 2(b) blue line), but the steep gradients at 8 and 18 min caused two severe disturbances on the baseline, because of large variance between the physicochemical properties of different eluting solvents. LC-MS exhibited higher sensitivity and greater tolerance towards the baseline disturbances. However, it was also difficult to identify the time region when the analytes were eluted

![Image](image-url)
because the interference of isobaric ions could be attenuated. When the elution condition when the compounds were well resolved, the isotope ratio calculated was found to be more precise in a ±0.2 range of the measured value. In addition, the true monoisotopic ratio of a detected ion should be within a ±0.2 (see Table S4, Supporting Information). In another mode, the monoisotopic ratios on the qMS apparatus could be no more than 0.2 (see Table S4, Supporting Information). Nonetheless, it was found that the difference between measured and theoretical values of different ions in the same cluster may not always be easily understood. Without a reasonable explanation, the identified real-ion peaks could be false positive results, or real-ion peaks belonging to different analytes. Therefore, during the untargeted molecular screening of natural extracts, the identified real ions require further confirmation with other experimental techniques, such as the tandem mass spectrometry analysis (MSn).

**Clustering identified real ions based on monoisotopic ratios**

The picked ions were clustered into the same bin if their retention behaviors were the same. Quite often, it was observed that a pair of ions with a \( m/z \) value difference of 1 u exhibited the same retention behaviors. However, such a pair of ions can be either isotopomers or protonated/deprotonated ion (\([M+H]^+\) or \([M-H]^-\)) and molecular status ion (\([M]^+\) or \([M]^+\)) (Figs. S4 and S5, Supporting Information). Nonetheless, it was found that the ratio between the intensities of protonated/deprotonated ion and its molecular ion was often much higher than the normal ratio between the intensities of isotopomers. In many cases, the measured isotope ratios were often higher than the actual values because isotopomer pairs were merged with protonated ion pairs. In order to exclude the mutual influences between protonated ion pairs and isotopomer pairs and to identify the molecular status ions, the ions in the same bin were further divided into smaller groups based on isotope ratios. It was found that the difference between measured and theoretical monoisotopic ratios on the qMS apparatus could be no more than 0.2 (see Table S4, Supporting Information). In another word, the true monoisotopic ratio of a detected ion should be within a ±0.2 range of the measured value. In addition, the isotope ratio calculated was found to be more precise in an elution condition when the compounds were well resolved, because the interference of isobaric ions could be attenuated. Ultimately, the ions clustered in the same group have both the similar retention behaviors and monoisotopic ratios. Therefore, their chemical structures should be quite similar. It is noteworthy that the molecular status ions of serine lactones and gallic acid were unable to be detected. Instead, gallic acid presented its fragment as the major ion \([M-COOH]^+\) (see Table S6, entry C4 and C5, Supporting Information). On the other hand, CRAD successfully identified real ions with \( m/z \) values which were generally ~56 u higher than the molecular weight of serine lactones. Such detected ions were probably their ion adducts \([M+Na+CH_3OH]^+\) (see Table S6, entry L1–L6, Supporting Information). By further inspecting \( m/z \) values of identified real ions in each clustered bin, we can conclude that the ion with the strongest MS response is not necessarily the molecular status ion of the targeted analyte. Meanwhile, the correlation between \( m/z \) values of different ions in the same cluster may not always be easily understood. Without a reasonable explanation, the identified real-ion peaks could be false positive results, or real-ion peaks belonging to different analytes. Therefore, during the untargeted molecular screening of natural extracts, the identified real ions require further confirmation with other experimental techniques, such as the tandem mass spectrometry analysis (MSn).

**Application of CRAD on the analysis of real natural extracts from Lycium ruthenicum Marr.**

CRAD was further employed in preprocessing raw LC-MS datasets of natural extracts from Lycium ruthenicum Marr. It was reported that the hydrophilic ingredients of Lycium ruthenicum Marr. contained a substantial amount of anthocyanines, which were flavonoids. Thus, it was assumed the five chromatographic gradients successfully applied on the flavonoid mixture of M1 would be suitable for screening the hydrophilic ingredients in Lycium ruthenicum Marr. After preprocessing by CRAD, the retention behaviors of real-ion peaks could be recognized in the presence of a great amount of noises (see Fig. S6, Supporting Information). As a result, the searching range of natural products was significantly clarified by the peaks in deconvolved XICs. It was also noteworthy that CRAD enabled the accurate determination of minor compounds (Fig. 3).

A total of 206 ions were discovered, which were clustered in 18 bins in negative ion mode and 7 bins in positive ion mode.
CRAD efficiently picked the ion peaks without any prior knowledge of the analytes or reference databases. The existence of identified ions was confirmed by the LC-MS/MS analysis. Furthermore, the analytical results of major compounds and many minor compounds were corroborated by previous reports, such as the anthocyanins identified with molecular weights of 634.5, 515.1, 593.3, 949.3, 963.4, 609.2, 933.3, and 641.1.\textsuperscript{20,22} Meanwhile, CRAD also recognized a number of isobaric ions, such as ions 593.3 and 623.1 in N10, N12, and N14; ions 381.1 in N2, N3, and N4; and ions 485.2 in N7 and N9. These compounds could not be discriminated by their mass or retention time in one separation condition. Such isobaric ions with close isotope ratios but different retention behaviors were probably isomers, because their fragmental ions were quite similar (SI). Although cis-trans isomers of anthocyanins in \textit{Lycium ruthenicum Murr.} were reported before (e.g., ions 949.3), our comprehensive analysis revealed that more isomers existed in a relatively low mass range.\textsuperscript{23} The result also showed that the anthocyanins in \textit{Lycium ruthenicum Murr.} attained strong retention on the hydrophilic stationary phase (i.e., N11 - N18 and P7). Meanwhile, a variety of low molecular compounds with relatively shorter retention times in the region from N2 to N10 accounted for a substantial portion of the whole mixture. In addition, the fraction N1 was found to be poorly retained, and its retention behavior was greatly influenced by the elution conditions (i.e., slope –2.7697 and intersection 0.2149). Such phenomena suggested the compound was retained through weak ionic interactions, which was easily disrupted by water in the mobile phase.

Conclusions

CRAD is a new algorithm providing a unique perspective in distinguishing real-ion signals from noises in LC-MS datasets of natural products. The natural compounds are identified in complex sample matrices through their chemical properties, which are reflected by the chromatographic retention times and behaviors. Together with many other criteria, such as XIC features (i.e., the number of peaks and their relative peak areas), isotopic ratios, and fragmental ions, CRAD is capable of denoising and identifying the real ions of unknown compounds from natural products, without the need for reference compounds or databases. The program is freely available with an open source license, and can be downloaded from https://github.com/CRAD.

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Supporting Information

Details of the selected chemical standards and liquid chromatographic separation methods employed for their investigation; denoising effects of CRAD at different parameter settings; LC-ESI-MS/MS spectrum of precursor ions in \textit{Lycium ruthenicum Murr.} determined by CRAD. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci.

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