A method of calculating the second dimension retention index in comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry

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\textbf{A B S T R A C T}

A method was developed to calculate the second dimension retention index of comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC × GC/TOF-MS) data using \textit{n}-alkanes as reference compounds. The retention times of the \textit{C}_{n}−\textit{C}_{31} \textit{n}-alkanes acquired during 24 isothermal experiments cover the 0–6 s retention time area in the second dimension retention time space, which makes it possible to calculate the retention indices of target compounds from the corresponding retention time values without the extension of the retention space of the reference compounds. An empirical function was proposed to show the relationship among the second dimension retention time, the temperature of the second dimension column, and the carbon number of the \textit{n}-alkanes. The proposed function is able to extend the second dimension retention time beyond the reference \textit{n}-alkanes by increasing the carbon number. The extension of carbon numbers in reference \textit{n}-alkanes up to two more carbon atoms introduces <10 retention index units (iu) of deviation. The effectiveness of using the proposed method was demonstrated by analyzing a mixture of compound standards in temperature programmed experiments using 6 different initial column temperatures. The standard deviation of the calculated retention index values of the compound standards fluctuated from 1 to 12 iu with a mean standard deviation of 5 iu.

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1. Introduction

Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC × GC/TOF-MS) has increasingly been applied to analyze the chemical compositions of complex organic mixtures such as drug, food, fat, oil, and biological samples [1–6]. The two-dimensional gas chromatography separates compounds using two distinctly different capillary columns of different polarities. The interface between the two columns is called a modulator [7,8]. Compared to the 15–30 m length of the first dimension column, the length of the second dimension column is very short, in general only 0.5–2 m [7,9,10]. This means that the second dimension column’s separation is a very fast process and can be considered to be under pseudo-isothermal conditions before the next modulator pulse [11].

Spectrum library searching is widely used for compound identification in analyzing experiment data generated on GC × GC/TOF-MS instruments by finding the best mass spectrum matched compound recorded in the mass spectrum library [12]. However, the mass spectrum represents only partial information of the molecular structure of a compound. Identifying compounds based on spectrum matching only, therefore, may introduce a high rate of false-positive identifications, especially for the analysis of highly complex mixtures. For instance, it is likely that structurally similar molecular homologous compounds may have similar mass spectra and therefore, increase the chance of misidentifications. Additional molecular information may be employed to increase the identification confidence of a spectrum library match. One approach is to combine compound separation information with the mass spectrum matching. The \textit{n}-alkane-based retention index (\(I_{n}\)) has been playing an important role in one-dimensional gas chromatography to identify complex compounds [13,14], and several retention index libraries have been developed [12,15–18]. It is possible that the retention index of the second dimension column in a GC × GC/TOF-MS system can also enhance the confidence of compound identification.

The retention index values of compounds in the second dimension column of a GC × GC/TOF-MS system can be calculated from a second dimension retention map, which comprises a set of curves.
generated by fitting the retention time values of individual reference compound using an empirical column temperature–second dimension retention time function ($t_R^2 - 2T_e$ function). The second dimension retention index of any target compound, whose second dimension retention time falls between the second dimension retention times of two consecutive reference compounds, can be deduced from the retention index values of the two reference compounds via linear interpolation. Beens and co-workers first constructed a second dimension retention map from a continuous injection of n-dodecane [19], and then introduced five alkanes, i.e., C₉–C₁₃ [20]. Western and Marriott developed different second dimension retention map with repetitive, timed, and sequential injections and with injections at different temperatures and different temperature ramp rates using mixtures of reference compounds such as n-alkanes, 2-methyl ketone, fatty acid methyl ester, and alcohol standards [11,21]. Bieri and Marriott proposed solid-phase microextraction (SPME) [22] and a dual-injection system to extend the range of the second dimension map from C₁₀ to C₂₁ [23]. Meanwhile, some researchers applied the second dimension retention map to identify the unknown compounds in tobacco leaves [24] and cigarette essential oil [25].

In most published studies, multiple injections of the reference compounds were accomplished in one chromatographic run, which would limit the reference compound identification and therefore the second dimension retention map would only cover a small portion of the two-dimensional retention time space. For example, using the n-alkanes from C₂ to C₉, the second dimension retention map would not cover the retention time space in which the more polar target compounds elute off the GC columns [21]. Covering a limited retention time space by the retention times of the reference compounds makes the accurate calculation of the retention index values of all target compounds a significant challenge in applying the GC × GC/TOF-MS system to analyze mixtures, like for example in metabolomics, where the target compounds in samples can range from being polar to non-polar.

The objectives of the current research was to employ n-alkanes as the reference compounds to create a second dimension retention map to cover most of the second dimension retention time space at various temperatures of the second dimension column in a GC × GC/TOF-MS system. An empirical $t_R^2 - 2T_e$ function was developed using the experimental retention times of the reference n-alkanes. The capability of the developed $t_R^2 - 2T_e$ function was explored for the prediction of retention index of target compounds whose retention times fall outside of the second dimension retention map, i.e., the retention time space that was not covered by the reference n-alkanes. In addition, a mixture of compound standards was used to demonstrate the accuracy of converting the second dimension retention time values to the retention index values in different experimental conditions.

2. Experimental

2.1. Materials

Two standard solutions were used in this study: (A) a mixture containing the C₂–C₄₀ n-alkanes (Sigma–Aldrich Corp., St. Louis, MO); (B) a mixture containing 76 compounds (8270 MegaMix, Restek Corp., Bellefonte, PA). All of the compounds were present at a purity of between 95% and 99% and prepared in dichloromethane at a concentration of 2.5 μg/mL per compound.

2.2. GC × GC/TOF-MS experiments

A LECO Pegasus® 4D GC × GC/TOF-MS instrument was equipped with an Agilent 6890 gas chromatography, featuring a LECO two stage cryogenic modulator (a cold pulse of liquid N₂-cooled N₂ gas and a hot pulse of heated air) and a secondary oven, and a Gerstel MPS2 autosampler. The first dimension column was a non-polar DB-5ms (phenyl arylene polymer virtually equivalent to a (5%-phenyl)-methylpolysiloxane) 30 m × 0.25 mm i.d. × 0.25 μm film thickness, and the second dimension column was a medium-polar BD-17 ((50%-phenyl)-methylpolysiloxane) 1.79 m × 0.1 mm i.d. × 0.1 μm film thickness. Both columns were obtained from Agilent Technologies (Agilent Technologies, Santa Clara, CA), and were connected by means of a press-fit connector before the modulator. The flow rate of ultra-high purity helium carrier gas (99.999%) was set to 1.0 mL/min in a constant flow with an initial head pressure of 65 psi.

A 2 μL liquid sample was injected into the liner using the splitless mode with the injection port temperature set at 260 °C. The ion source chamber was set to 230 °C, the detector voltage was 1700 V, and the electron energy was 70 eV. The thermal modulator was set to +20 °C relative to the primary oven. The second oven was set to +5 °C with respect to the primary oven. The mass spectra were acquired at a rate of 200 spectra per second with mass range set to m/z = 45–800. The modulation period for the temperature programmed experiments was set as 6 s, while it was set at 9 s for the isothermal experiments. Using the standard solution A, the first dimension column was set to isothermal at 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 265, 270 and 280 °C, respectively, with a 60 min analysis time. For the analysis of a mixture of standard solutions A and B, the first dimension column was programmed from 60, 80, 100, 120, 140, and 160 °C ramped to 280 °C at 5 °C/min with a hold time of 0.5 min at the initial temperature and 20 min at the end temperature, respectively.

2.3. Raw data reduction

The LECO ChromaTOF software (version 4.21), equipped with the National Institute of Standards and Technology (NIST) MS database (NIST MS Search 2.0, NIST/EPA/NIH Mass Spectral Library; NIST 2002), was used for instrument control, spectrum deconvolution, and compound identification. By manually visualizing the raw data, we estimated the peak widths on the first dimension column ranges from 2 to 3 modulation periods, while the peak width on the second dimension column ranges from 0.2–0.5 s. Manufacturer recommended parameters for ChromaTOF were used to reduce the raw instrument data into a compound peak list. These parameters are: baseline offset = 0.5 s; smoothing = auto; peak width in first dimension = 6s; peak width in second dimension = 0.1 s; signal-to-noise ratio = 100; match required to combine peaks = 500; R.T. shift = 0.08 s; minimum forward similarity match = 600. The true peak spectrum was also exported as part of the information for each peak in absolute format of intensity values.

2.4. Hold-up time of the second dimension column

The hold-up time in the second dimension column $2t_M$ varies with different column temperatures and head pressure. The head pressure was set constant as 65 psi for all experiments in this study. To a fixed temperature of the second dimension column $2T_e$, $2t_M$ can be calculated from the retention times of three homologues with equidistant carbon number as follows [26]:

$$2t_M = \frac{t_{R_1}t_{R_3} - t_{R_2}^2}{(t_{R_3} - t_{R_2}) - (t_{R_2} - t_{R_1})}$$

where $t_{R_1}$, $t_{R_2}$ and $t_{R_3}$ are the continuous retention times of three homologues such as C9–C13-alkanes. In the current study, the isothermal experiments were carried out at 24 different temperatures of the second dimension column. Multiple n-alkanes
were analyzed at the same column temperature of the second dimension column. Therefore, we can obtain more than one hold-up times in one temperature using Eq. (1). Using the mean hold-up time values at different temperatures, the hold-up time at different column temperatures of the second dimension column was fitted as follows:

\[ t_{M} = 0.0000004(2T_{R})^2 - 0.0025(2T_{R}) + 1.6184 \]  
(2)

where \( t_{M} \) is the fitted hold-up time in the second dimension column and \( 2T_{R} \) is the second dimension column temperature in \( ^{\circ} \)C. The fitting accuracy \( R^{2} \) was 0.9773.

2.5. Selection of the \( 2t_{R} - 2T_{R} \) function

The sample of the \( n \)-alkanes was analyzed multiple times on the \( GC \times GC/TOF-MS \) system by setting both the first and the second dimension columns in isothermal mode. After correcting the hold-up time of the second dimension column using Eq. (2), a second dimension retention map of all \( n \)-alkanes could be constructed with the relationship of column temperature and retention time.

In order to make it possible to extend the second dimension retention map beyond the reference compounds, it is better to incorporate the carbon number of the \( n \)-alkanes reference compounds into the \( 2t_{R} - 2T_{R} \) function. The carbon number in each \( n \)-alkane (\( n \)) and temperature of the second column (\( 2T_{R} \)) were first transformed by a logarithmic function. Then a multiple dimension polynomial fitting method was used to get a comprehensive \( 2t_{R} - 2T_{R} \) function as follows:

\[ 2t_{R} = exp[p_1n + p_2n^2 + p_3n^3 + p_4n^4 + p_5n^5 + p_6n^6 + p_7n^7 + p_8n^8 + p_9n^9 + p_{10n^{10}}] \]  
(3)

where \( n \) is \( \log(n) \), \( T \) is \( \log(2T_{R}) \), and each \( p_i \) is the parameter of the \( 2t_{R} - 2T_{R} \) function. The numeric values of \( p_i \) can be generated by fitting the retention time values of all \( n \)-alkanes reference compounds.

2.6. Evaluating the performance of the proposed \( 2t_{R} - 2T_{R} \) function

The retention index of a \( C_n \)-alkane, an alkane with \( n \) carbons, is defined as \( 100 \times n \). Since the second dimension column can be considered under pseudo-isothermal conditions [11], the Kovats retention index for isothermal experimental conditions can be calculated to estimate the retention index value of a target compound in the second dimension column as follows [27]:

\[ I = 100n + 100 \left( \frac{\log(t_1) - \log(t_2)}{\log(t_{n+1}) - \log(t_1)} \right) \]  
(4)

where \( I \) and \( t_i \) are the retention index and adjusted retention time of the target compound in the second dimension column, respectively, and \( t_1 \) and \( t_{n+1} \) are the adjusted retention times of two \( n \)-alkanes between which the target compound eluted off the second dimension column.

Two methods were employed to evaluate the performance of the proposed \( 2t_{R} - 2T_{R} \) function. In the first approach, the fitted \( 2t_{R} - 2T_{R} \) curves of the \( C_{n-1} \)-alkane and \( C_{n+1} \)-alkane were used to predict the retention index of the \( C_n \)-alkane at different temperatures of the second dimension column by linear interpolation using Eq. (4). The distribution of the absolute retention index deviations (\( d_{abs} \)) between the predicted values and the true values, and the root mean square error (RMSE) were then used as evaluation criteria:

\[ d_{abs} = |t'_p - I| \]  
(5)

\[ RMSE = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (t'_p - I)^2} \]  
(6)

where \( t'_p \) is the predicted value of retention index for the \( i \)th data point, \( I \) is true retention index value of a given \( n \)-alkane by definition, \( d_{abs} \) is the absolute deviation of the \( i \)th data point, and \( N \) is the total number of the data points used in calculation.

For the temperature programmed data of MegaMix compounds, the mean standard deviation of all compounds (\( STD \)) was used to evaluate the performance of \( 2t_{R} - 2T_{R} \) function. A larger value of \( STD \) indicates the instability of the testing \( 2t_{R} - 2T_{R} \) function for converting the retention time values of all compounds into the retention index values. The \( STD \) of all compounds is defined as follows:

\[ T_j = \frac{1}{M_j} \sum_{i=1}^{M_j} I_i \]  
(9)

\[ STD_j = \sqrt{\frac{1}{M_j} \sum_{i=1}^{M_j} (I_i - I)^2} \]  
(10)

\[ STD = \sqrt{\frac{1}{N} \sum_{j=1}^{N} STD_j} \]  
(11)

where \( M_j \) is the number of retention index values for the \( j \)th compound, \( T_j \) is the mean of calculated retention index values of the \( j \)th compound, \( STD_j \) is the standard deviation of the \( j \)th compound, and \( N \) is the number of identified compounds in the MegaMix.

3. Results and discussion

In the current study, \( n \)-alkanes (\( C_7-C_{40} \)) were chosen as the reference compounds to create a second dimension retention map to cover most of the retention time space defined by the two dimension chromatogram of a \( GC \times GC/TOF-MS \) system. An empirical \( 2t_{R} - 2T_{R} \) function was further developed to construct the second dimension retention map from the isothermal data of the \( n \)-alkanes. The constructed second dimension retention map was extended into the retention time area that was not covered by the reference \( n \)-alkanes for the calculation of the compound retention index in the second dimension column. A mixture of \( n \)-alkanes and MegaMix was analyzed on \( GC \times GC/TOF-MS \), from which \( C_7-C_{31} \) alkanes and 47 MegaMix compounds were identified.

3.1. Experiment repeatability

A mixture of solution A (\( n \)-alkanes) and solution B (MegaMix) were analyzed with 6 replicate injections every day for five continuous days. For the experiments performed within one day, the standard deviation of the second dimension retention time of each identified compound ranges from 0 to 0.0225 s. Fig. 1 displays the cumulative probability curves of the day-to-day experiments, i.e., a total of 30 replicate analyses in five days. The standard deviation of the second dimension retention time of the \( n \)-alkanes fluctuated from 0.0015 to 0.0046 s and of compounds detected from the MegaMix it fluctuated from 0.0037 to 0.0059 s, with mean standard deviation values of 0.0022 and 0.0043 s, respectively. The standard deviation of 90% of the second dimension retention time values in all 30 experiments were <0.005 and 0.01 s for the \( n \)-alkanes and the compounds in the MegaMix, respectively. Such a small standard deviation of the second dimension retention time indicates that the instrument has a very good repeatability in measuring the retention time of compounds separated on the second dimension column during the experiment period.

Fig. 2 shows the relation between the second dimension retention time and the first dimension retention time of all \( n \)-alkanes detected in the 6 temperature programmed experiments performed in one day. The retention times of all \( n \)-alkanes measured
in the same experiment are connected by a solid line. There is an inflection point in each curve, at which the temperature of the second dimension column was 285 °C and was held for 20 min.

3.2. Construction of the second dimension retention map

Fig. 3 shows the second dimension retention map constructed by applying the proposed $2t_R - 2T_e$ function using the isothermal experimental data of the n-alkanes (C7–C31). The dots are the experimental data and the solid lines represent the curves fitted by the $2t_R - 2T_e$ function. Even though the retention time of each n-alkane has limited coverage of the second dimension retention time, the retention time of all reference n-alkanes cover all of the retention time area less than 6 s in the second dimension retention time. This makes it possible to calculate the retention indices of target compounds from the corresponding retention time values without the extension of the retention space of the reference compounds. The proposed $2t_R - 2T_e$ function uses the retention time data and carbon number of all reference compounds for empirical parameter fitting. The variation introduced by each retention time data or the retention time data of an individual reference compound does not significantly affect the fitting, which results a set of evenly dis-

Fig. 1. Cumulative probability curves of standard deviation of the second dimension retention times of the n-alkanes (in blue) and compounds detected in MegaMix (in red). A mixture of n-alkanes and MegaMix was analyzed on a GC × GC/TOF-MS system operated in temperature programmed mode with 6 replicate injections every day for five continuous days (For interpretation of references to colour in this figure legend, the reader is referred to the web version of this article.).

Fig. 2. Retention time map of the n-alkanes analyzed in temperature programmed mode with different initial first oven temperatures of 60 (□), 80 (■), 100 (▲), 120 (+), 140 (▲) and 160 (○) °C, respectively. There is an inflection point in each curve, at which the temperature of the second dimension column was 285 °C and was held for 20 min.

Fig. 4. The second dimension retention map constructed from the isothermal data of the n-alkanes from C7 to C31 using the proposed empirical function. The $2t_R - 2T_e$ curves for C27-, C28-, C29-, and C30-alkane were predicted based on the retention time values of n-alkanes from C7 to C31 using the proposed $2t_R - 2T_e$ function.
Table 1

<table>
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<th>Compound name</th>
<th>Second dimension retention index (iu)</th>
<th>Mean</th>
<th>STD</th>
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</table>

The table shows the second dimension retention indices of 47 identified MegaMix compounds analyzed in 6 temperature programmed experiments with different initial temperatures of the first dimension column.

3.3. The predictive capability of the proposed function

In order to assess the predictive capability of the proposed $t_{R} - 2T_e$ function, the fitted curves of the C$_n$-alkane and the C$_{n+1}$-alkane were used to predict the retention index of the C$_n$-alkane at different temperatures of the second dimension column by applying linear interpolation. The mean absolute deviation of the reference compound C$_n$-alkane was then calculated by comparing the difference between the predicted retention index value and the true retention index value at different column temperatures. The RMSE for all n-alkanes reference compounds is 9 iu.

In order to quantitatively analyze the performance of the proposed $t_{R} - 2T_e$ function, the fitted curves of the C$_n$-1-alkane and the C$_{n+1}$-alkane were used to predict the retention index of the C$_n$-alkane at different temperatures of the second dimension column by applying linear interpolation. The mean absolute deviation of the reference compound C$_n$-alkane was then calculated by comparing the difference between the predicted retention index value and the true retention index value at different column temperatures. The RMSE for all n-alkanes reference compounds is 9 iu.

The blank means the compound was not detected during the experiment.

tributed curves. In order to quantitatively analyze the performance of the proposed $t_{R} - 2T_e$ function, the fitted curves of the C$_n$-1-alkane and the C$_{n+1}$-alkane were used to predict the retention index of the C$_n$-alkane at different temperatures of the second dimension column by applying linear interpolation. The mean absolute deviation of the reference compound C$_n$-alkane was then calculated by comparing the difference between the predicted retention index value and the true retention index value at different column temperatures. The RMSE for all n-alkanes reference compounds is 9 iu.

3.3. The predictive capability of the proposed function

In order to assess the predictive capability of the proposed $t_{R} - 2T_e$ function, isothermal data of the n-alkanes only from C$_7$ to C$_{26}$ were used to get the fitted parameters for the function defined in Eq. (3). The fitted function was subsequently used to create the predicted $t_{R} - 2T_e$ curves for the n-alkanes from C$_{27}$ to C$_{31}$. Such an extension of the retention time space cannot be achieved using the literature-reported functions because the carbon number of the n-alkanes was not incorporated into these $t_{R} - 2T_e$ functions [11,21,23,24]. Fig. 4 displays the second dimension retention map of the n-alkanes from C$_7$ to C$_{31}$. The dots are the isothermal experimental data. The curves of C$_{27}$–C$_{31}$ n-alkanes were predicted by the proposed function fitted by the retention time values of the n-alkanes. Compared to the curves of the n-alkanes from C$_7$ to C$_{31}$ in Fig. 3 that were fitted using all the isothermal data of the n-alkanes from C$_7$–C$_{31}$, it is obvious that the deviation of the predicted curves increased with the increase of the carbon number in an n-alkane, especially in the upper area of the retention map.

Fig. 5 shows the mean absolute deviation of the retention index between the predicted values and the true values (defined by the number of carbon atoms in the n-alkanes). The predicted values of the retention index using either the entire isothermal data set, i.e., the isothermal retention times of the n-alkanes from C$_7$ to C$_{31}$, or the isothermal data of C$_7$–C$_{26}$ n-alkanes, are very close to the true values with a mean deviation of less than 8 iu. It can also be noted that the mean absolute deviation is independent in the retention
4. Conclusion

In this study, the homologue n-alkanes (C7–C31) were chosen as the reference compounds to build a second dimension retention map for GC x GC/TOF-MS. Compared to the existing literature reports, the new second dimension retention map covers all the 6 s retention time area in the second dimension retention time space, which makes it possible to calculate the retention index values of the majority of compounds directly from linear interpolation of two consecutive n-alkanes. An empirical $t_R^2 - 2T_e$ function was developed to incorporate the carbon number of the n-alkanes and the column temperature of the second dimension column. The proposed function is able to extend the second dimension retention time beyond the reference n-alkanes by increasing the carbon number. The predictive analysis demonstrated that the extension of the proposed function for two carbons introduces deviation less than 10 iu.

Forty-seven compounds were identified from the MegaMix containing 76 compound standards on GC x GC/TOF-MS. Using the proposed $t_R^2 - 2T_e$ function, the standard deviations of the converted retention index values of the 47 identified compounds fluctuated from 1 to 12 iu with a mean standard deviation of 5 iu, while the standard deviation of the second dimension retention time fluctuates from 0 to 0.5 s (data were not shown). Of the 47 identified compounds, only three compounds have the standard deviations of the second dimension retention index larger than 10 iu. These results show that the compound retention index values in the second dimension column of a GC x GC/TOF-MS system can be obtained by converting the retention time values of these compounds in the second dimension column using the proposed $t_R^2 - 2T_e$ function, i.e., the second dimension retention mapping.

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