User manual (v1.0)

Multivariate Optimization and Refinement Program for Efficient Analysis of Key Separations (MOREPEAKS) is an optimization program for one- and two-dimensional liquid chromatography. Otherwise visualization and analysis of gas chromatography and mass spectral data is also possible.

MOREPEAKS is released with the aim of improving the valorization of academic research towards society. MOREPEAKS is a freely available tool created by the enthusiasts of the Chemometrics and Advanced Separations Team (CAST, https://cast-amsterdam.org/), who wish to allow others to benefit from chemometric tools available in literature without requiring the computational skills. We aim to incorporate all tools developed at the University of Amsterdam, but a number of relevant tools published in literature.

When any bugs are encountered, please let us know at bug@cast-amsterdam.org.

For patch notes, please refer to https://cast-amsterdam.org/patchnotes/

If you would like to cite the MOREPEAKS software, please do so as the following:

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1. Installing MOREPEAKS

MOREPEAKS is downloadable from Zenodo: https://doi.org/10.5281/zenodo.5710442. Download either the "*Full_Installer*" or the "*RunTime_WebDownload*" version. The full installer includes MATLAB Runtime (The Mathworks, Inc.), which is necessary to run MOREPEAKS. The *RunTime_WebDownload* will download MATLAB Runtime when required. If you only update MOREPEAKS to a newer version, the *RunTime_WebDownload* is sufficient, as MATLAB Runtime is already installed.

Open the file. When prompted to choose an installation folder, choose any folder, except Program Files. MOREPEAKS might need to write files to its directory, and Program Files doesn't allow such rights. The default folder (AppData) is sufficient (Figure 1). Add a shortcut to the desktop if preferred.

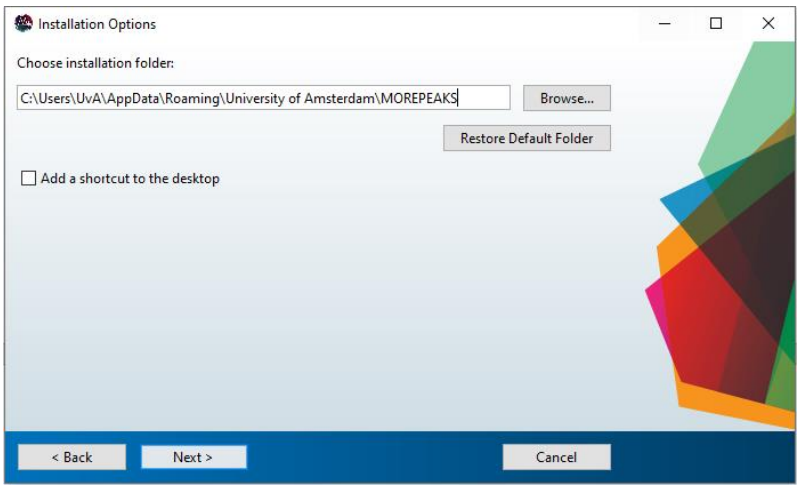

Figure 1. *Folder selection when installing MOREPEAKS.*

MOREPEAKS is a MATLAB-based user interface, therefore MATLAB Runtime is required. In contrary to MATLAB itself, Runtime is free of charge. MATLAB RunTime can be installed in the default Program Files folder (Figure 2).

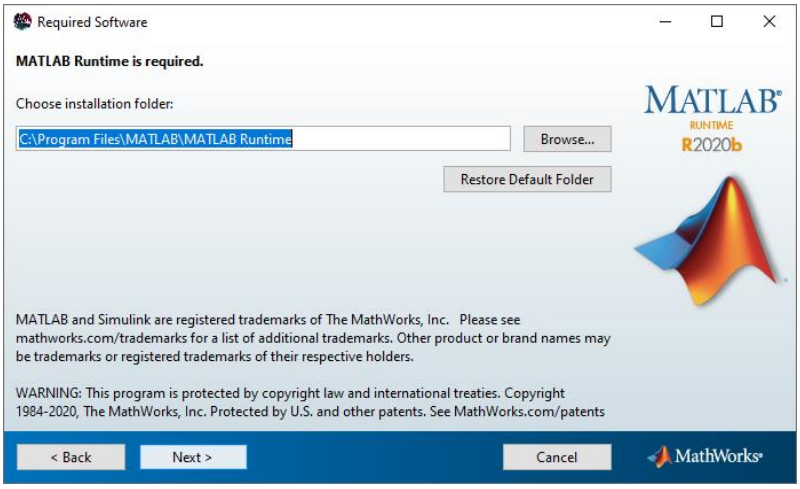

Figure 2. Installing MATLAB Runtime.

2. First time opening MOREPEAKS

2.1. Selecting a default MOREPEAKS folder

If it is the first time MOREPEAKS is opened, a welcome message is displayed (Figure 3). After pressing "*OK*" a folder selection tool will be opened. Select a preferred folder and MOREPEAKS will create a folder structure into which MOREPEAKS will store results. This folder is also where MOREPEAKS will store its created databases and standard settings (Figure 4).

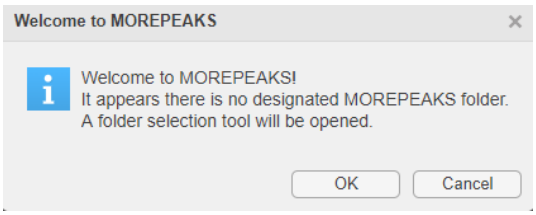

Figure 3. *Welcome to MOREPEAKS.*

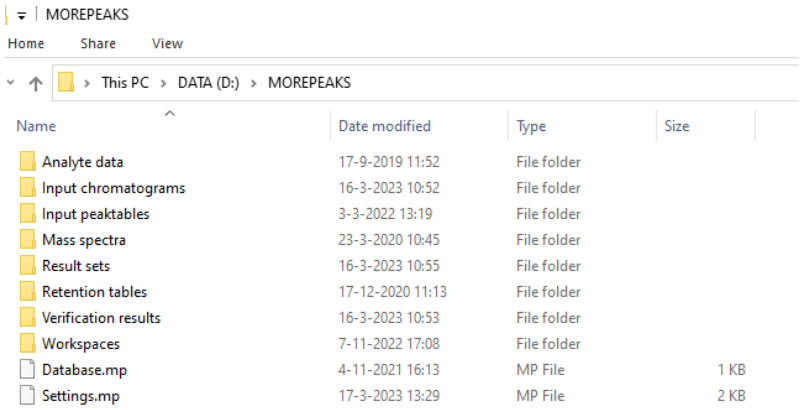

Figure 4. *MOREPEAKS folder structure.*

When a default MOREPEAKS folder is selected, the main menu is shown (Figure 5). The main menu contains shortcuts to various parts of the software and the option to load a previous workspace or save the current workspace. Saving the workspace allows you to continue with all current datasets, settings and results at a later time. If CompassXport is installed (more on CompassXport at Section 6), ".D" files containing mass spectrometry data can be converted to the ".mzXML" format, so that these can later be imported into MOREPEAKS.

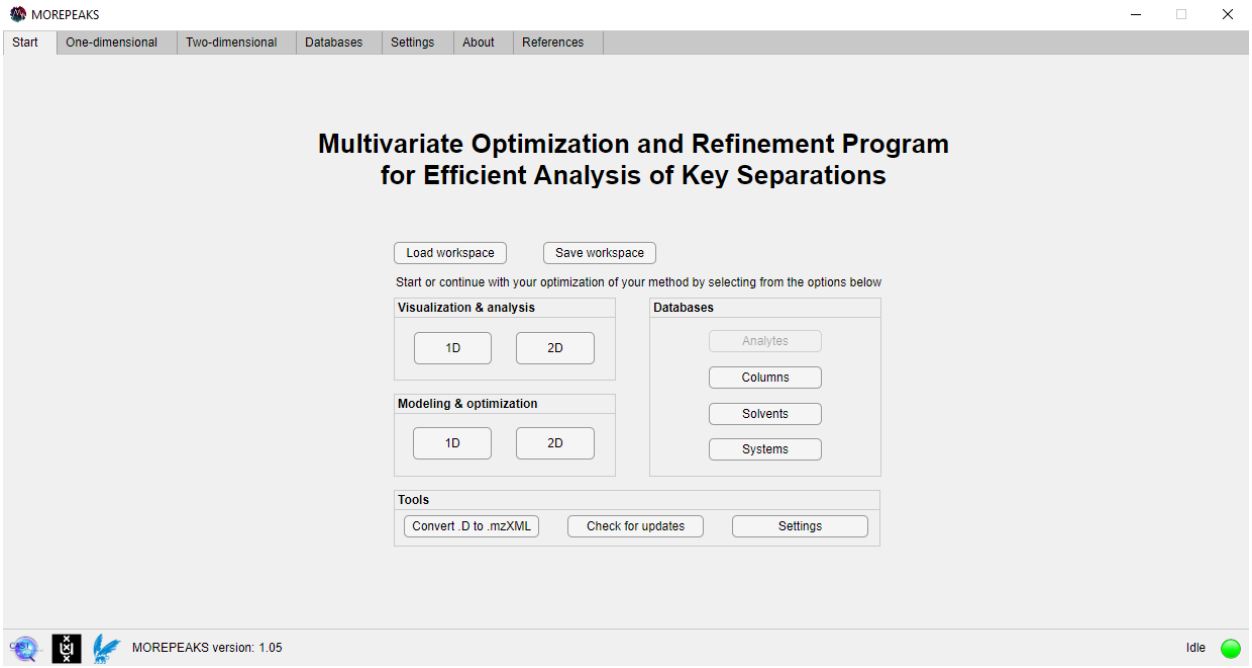

Figure 5. *MOREPEAKS main menu.*

2.2. Settings up databases

To optimize methods by retention modeling, some crucial information about the liquid chromatography (LC) system and used columns is needed. Via the shortcut on the main menu, one can quickly navigate to the required databases. The databases are also accessible via the tabs at the top of the user interface. Note that all required names are reference-labels for the software to know which variables are used. However, they are also reminders for the user about the used experimental conditions. MOREPEAKS will not use any names, for example for solvents, to look up information. Each individual database has a button in the bottom-right corner, to "*Save the database as standard*". When this button is pressed, MOREPEAKS will load in the default database whenever the software is in use.

2.2.1. System database

In the system database (Figure 6) one can add required one-dimensional (1D) LC-systems or systems for comprehensive two-dimensional (2D) LC (LC×LC). The dwell volume of an LC system delays the programmed gradient and is therefore needed for accurate retention modeling. The software requires a system name for a reference-label. The manufacturer however, is mainly used for self-reference for the user. if one is working with 1DLC, only the first dimension (^{1}D) dwell volume is required. If a 2DLC system is required, make sure the second dimension (^{2}D) dwell volume is also included. When all values are filled in in the right-most panel, one can add the system to the database by pressing "*Add system*". Any typos (or constants such as the dwell volume) can be adjusted in the table to the left if required.

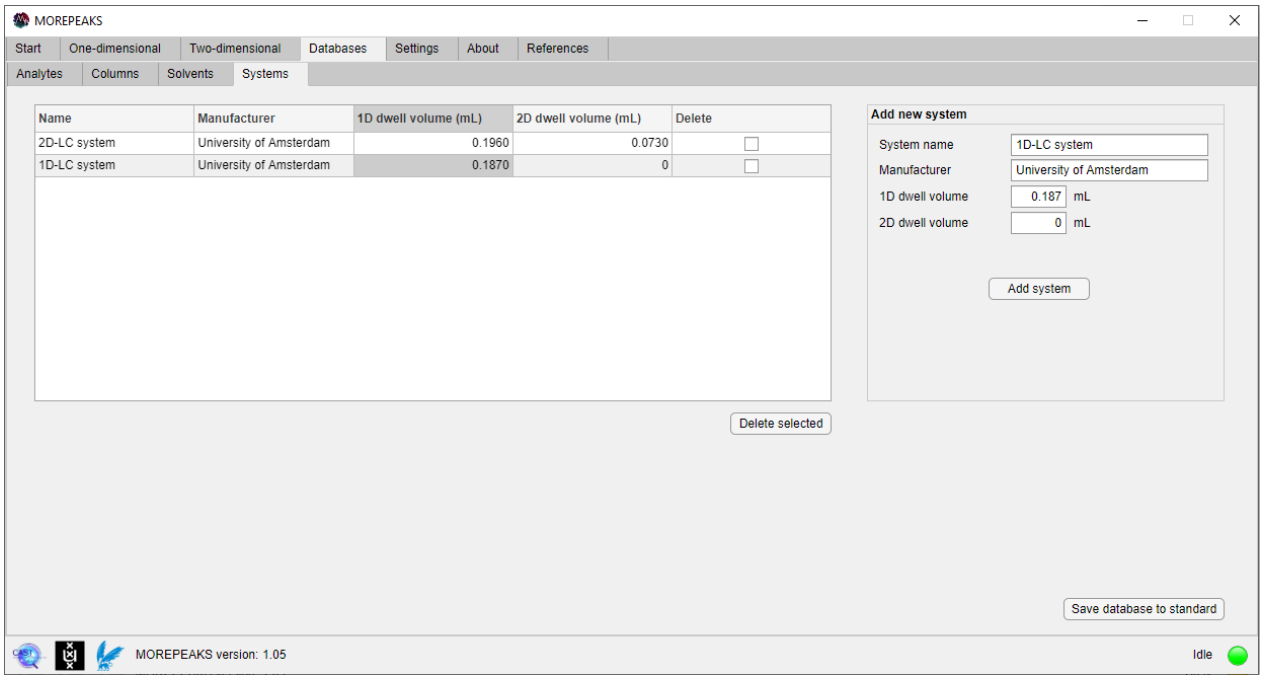

Figure 6. *System database.*

2.2.2. Solvent database

In the solvent database (Figure 7), used mobile phases can be stored. MOREPEAKS will not look up any information about the stored mobile-phases. All solvents are used for self-reference for the user. Before starting an optimization however, the system required at least one solvent to be present in the database. Add a solvent name and an optional manufacturer and then press "*Add solvent*" to add a solvent to the database.

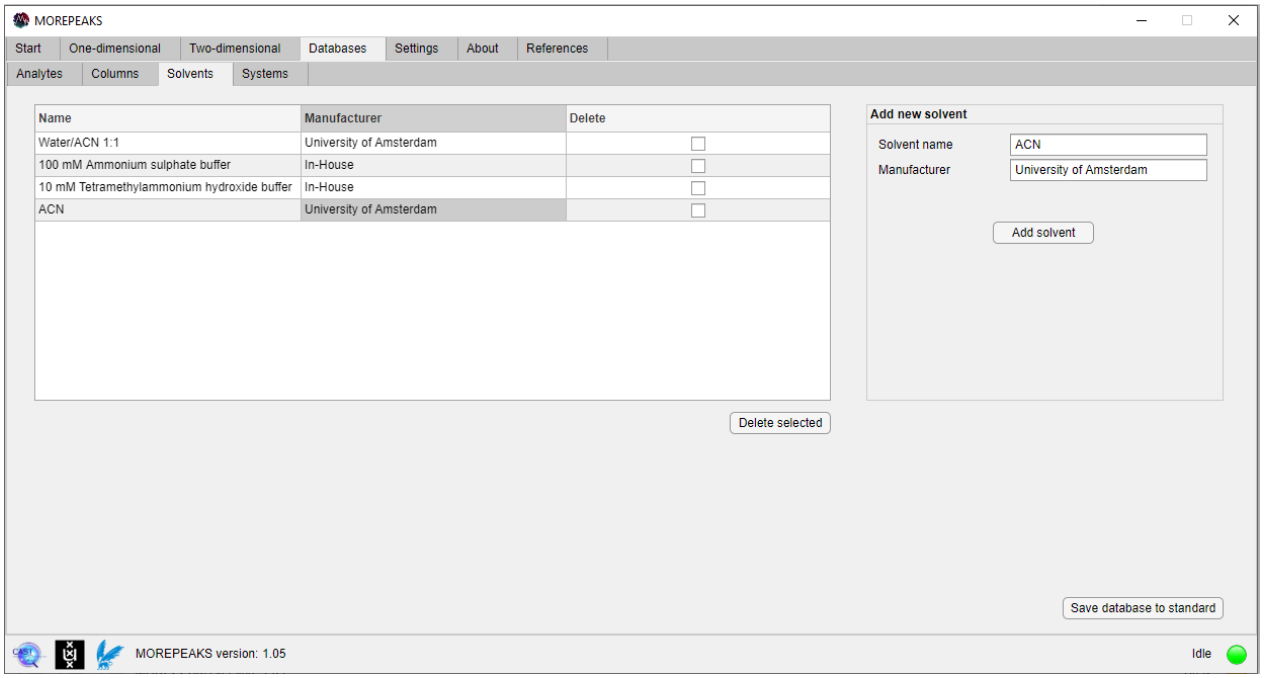

Figure 7. *Solvent database.*

2.2.3. Column database

In the column database (Figure 8), column information can be stored. Many aspects of the column dimensions are properties are used when performing retention modeling. Most importantly the column dead-volume. Other column dimensions are used to estimate peak widths and must therefore not be ignored. Also add a reference name and an optional manufacturer and press "*Add column*" to add it to the database. Column information can be adjusted in the table to the left.

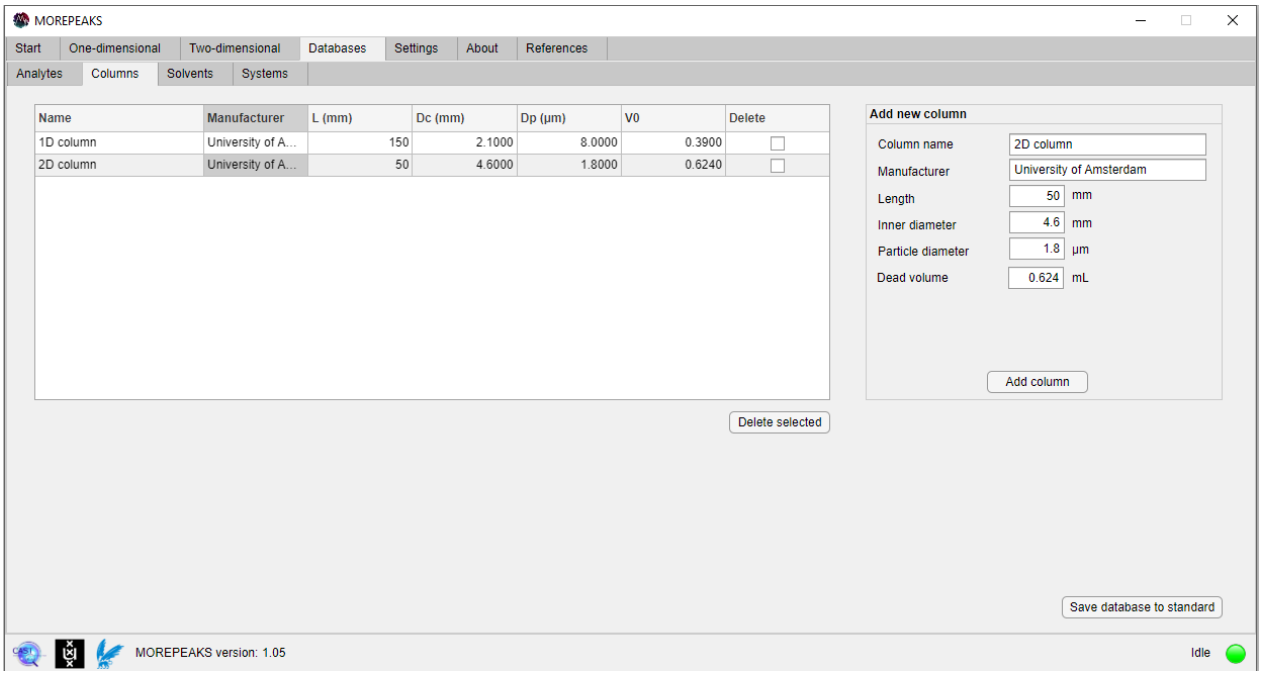

Figure 8. *Column database.*

2.2.4. Analyte database

The analyte database (Figure 9) is the location where MOREPEAKS will save retention parameters for analytes that are used in the current optimization and can not be filled in by the user. When retention parameters are known, one can see them here. Also an Akaike Information Criterion (AIC) is provided and is a measure of the quality of the fit [1]. By using the dropdown menu on the right side, one can adjust which parameters are shown. Parameters differ per system, retention mechanism, column, mobile phase and used modifier. If a one suspects the retention parameters for certain analytes are wrong, due to a different retention behavior, the Active-state per analyte can be turned off. If an analyte is not active, it won't be taken into account during retention modeling and thus the optimization protocol.

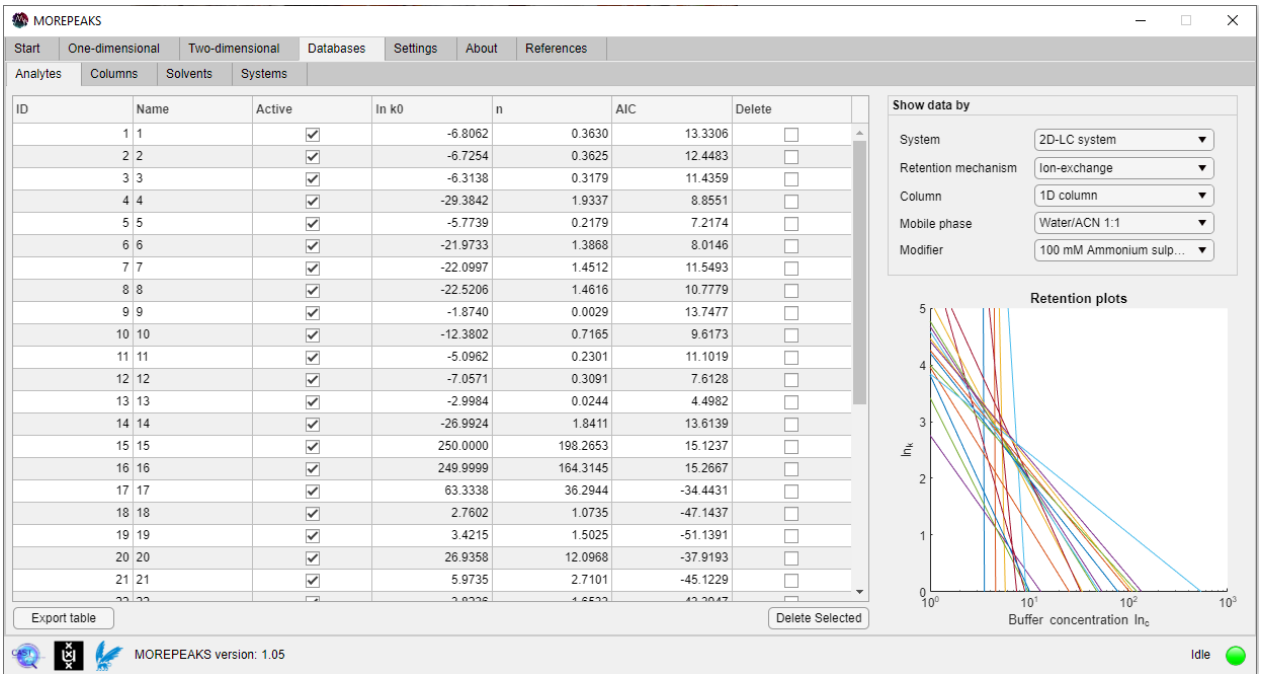

Figure 9. *Analyte database.*

3. Starting an optimization

3.1. Measuring scanning gradients and retention model selection

To start optimizing a separation, retention models need to be established. Commonly, at least two or three scanning gradients are performed to be able to make an estimation of the retention parameters. Scanning gradients typically make use of simple linear modifier-gradients that vary in time. Then retention models can be established based on different elution times. More on measuring scanning gradients can be found in work by Den Uijl *et al.* [2] The minimum number depends on the retention mode, and more importantly the used retention model. MOREPEAKS has five different retention models built in. The linear solvent (LSS) model [3,4], the adsorption model [5,6], the mixed-mode model [7,8], the quadratic model [4], and the Neue-Kuss model [9,10]. Typically used retention models for some elution modes are given in Table 1. For more information about retention modeling, please refer to an excellent review by Den Uijl *et al.* [11]

Retention mode	Retention models	Literature	Parameters
Reversed-phase LC (RPLC)	LSS model	[4,12]	2
	Quadratic model	[4]	3
	Neue-Kuss model	[9,10]	3
Normal-phase LC (NPLC)	Adsorption model	[5]	2
Hydrophilic interaction chromatography (HILIC)	Adsorption model	[13]	2
	Mixed-mode model	[7]	3
	Neue-Kuss model	[14]	3
Ion-exchange chromatography (IEX)	Adsorption model	[6]	2

Table 1. *Typically used retention models for RPLC, NPLC, HILIC and IEX.*

3.2. Importing scanning gradient data

When scanning gradients are performed, retention times for each analyte need to be determined on all scanning gradients. If the separation is monitored via mass spectrometry (MS), automated peak-pairing can be performed in the "*Peak-pairing tool*". The "*Peak-pairing tool"* can also be used to manually create a peak table. More on this tool in Section 4. Otherwise, a retention table has to be made by hand in for example Windows Excel (Figure 10). Make sure analytes follow each other in a row-wise fashion. Thus analyte 1 first, then the second analyte, etc. First, all ^{1}D retention times in minutes should be given. The first column should represent the retention time in the ^{1}D from the first scanning gradient. The second column the retention time in the ^{1}D in the second scanning gradient, etc.

If an LC×LC measurement has taken place, provide all the ^{2}D retention times in seconds in a column-wise fashion immediately after all ^{1}D retention times are given. First, the retention time in the ^{2}D of the first scanning gradient, followed by the retention time in the ^{2}D of the second scanning gradient, etc.

		min	min	sec	sec
		$^{1}D\ t_{r,1}$	$^{1}D\ t_{r,2}$	$^{2}D\ t_{r,1}$	$^{2}D\ t_{r,2}$
		A	B	C	D
Analyte 1 →	1	43,01282	48	93,51234	240,937
	2	44	48	87,15	207,562
	3	43,15518	46,26071	83,91151	193,4747
Analyte 4 →	4	40,88914	42,51817	83,16114	187,0176
	5	40,91828	42	76,77058	169,787
	6	40,6797	42	75,11427	166,987
	7	42,42047	45,61528	73,02368	162,6072
	8	41,48551	44,12002	68,24055	148,149
	9	42,46483	48	67,04419	147,412
Analyte 10 →	10	41,27592	43,24692	58,84175	122,2221
	11	43,30559	46,16231	56,65352	116,7767
	12	40,80588	42	54,70992	109,962
	13	41,45186	42	52,32135	103,012
	14	44,55134	49,90445	49,99394	96,96488
	15	69,94989	90	64,6109	138,387
	16	71,75868	96	65,91484	143,212
	17	80,3694	102,8833	69,28656	148,8738
	18	111,2925	144	64,02806	136,087
	19	103,4343	135,9711	61,53014	130,1654
	20	90,26532	128,3663	59,30554	124,3976
	21	97,97765	134,1449	55,23697	113,4
	22	103,6156	138,0327	47,89592	93,11512
	23	129,4055	180	47,6	92,587
	24	142,3321	210	64,23745	133,962
	25	160	234	51,2	105,537
	26	155,515	228	42,27432	85,637
	27	156,751	228	36,44377	69,287
	28	142	214,4233	38,925	96,87496
	29	172	246	44,45	94,637
	30	184	264	37,35	68,062
	31	190	276	43,45	89,212
	32	198,9656	288	39,43793	65,162
	33	187,5801	264,6687	74,63247	164,8259
	34	246,276	338,1166	76,34595	169,1998
	35	88	120	63,475	138,062
Analyte 36 →	36	40	43,19969	72,425	159,802
	37				

Figure 10. *Example retention table.*

Depending on the number of dimensions, navigate either to "*One-dimensional – Modeling & optimization – Import data*" or to "*Two-dimensional – Modeling & optimization – Import data*" via the navigation panels at the top of the user interface or via the shortcuts in the main menu ("*Start"*).

When importing a retention table, flip the switch in the "*Control panel*" on the top-right side to "*Peaktable*" (Figure 11). If raw data is imported, make sure the switch is set at "*Raw data*". More on importing raw data in Section [X]. When the switch is set to "*Peaktable*", the data file can be imported by pressing the "*Load*" button in the "*Peaktable panel*". MOREPEAKS automatically navigates to its own "*Input peaktables*" folder. Select the required peak table.

After this, MOREPEAKS requires information about the acquisition of the data. First of all, on the top-left side, select the system parameters: The used LC(×LC) system, required retention mechanism, and the used columns, mobile phases and modifiers. Figure 11 shows the "*Two-dimensional*" tab. But, of course, the "*One-dimensional*" tab doesn't require information about any second dimension.

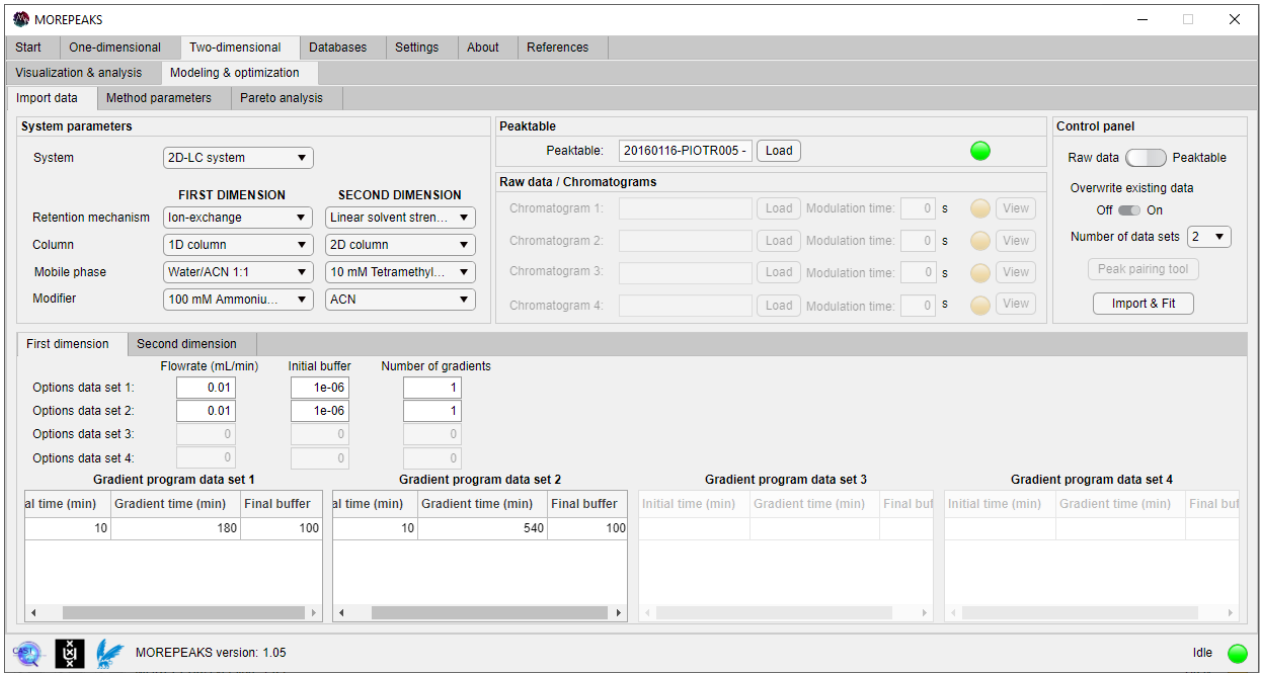

Figure 11. *Importing data.*

At the bottom, provide the used method parameters, such as the used flowrate, initial buffer concentration (input for IEX) or modifier fraction and the gradient program. The number of data sets can be selected in the "*Control panel*" on the top-right side. When performing 2DLC, apply the ^{2}D method parameters by navigating to the "*Second dimension*" tab. The gradient program for the ^{2}D should be provided in seconds (Figure 12). When all system and method parameters are correct and the peak table is loaded. Press "*Import & Fit*" in the "*Control panel*". MOREPEAKS should now indicate that it is calculating retention parameters (Figure 13). When MOREPEAKS is finished, you will be

navigated to the "*Method parameters*" tab. The "*Analyte database*" should have analyte information available now (Section 2.2.4).

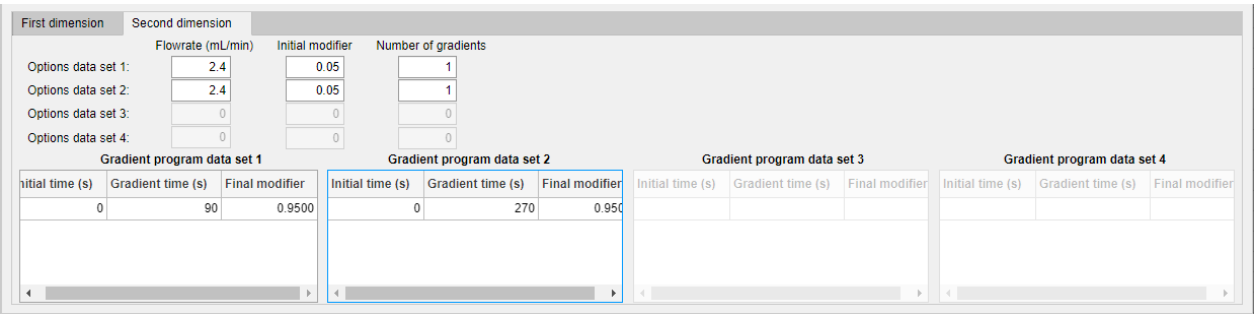

Figure 12. *Second dimension gradient program.*

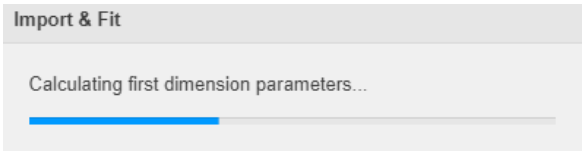

Figure 13. *MOREPEAKS indicates that retention parameters are being estimated.*

3.3. Selecting optimization ranges in the "method parameters" tab

3.3.1. "Simple" gradient parameters

3.3.1.1. Selecting method parameters

When retention parameters are estimated, it is possible to predict retention times for other gradient profiles. To the left, the system parameters used when importing data are automatically provided (Figure 14). And the method parameters of one of the scanning gradient will automatically be provided in the method tables. The method parameters in the tables can be adjusted to allow the software to calculate retention times and quality descriptors for different conditions.

Each method parameter has four adjustable values. The minimum and maximum value, its step size and the total number of steps that being taken. The step size and number of steps will automatically be adjusted if one of them is changed. To illustrate, if the final buffer concentration has the following four values: Min: 80, Max: 100 and step size: 5. The software will automatically adjust the number of steps to 4, since 80+5*4=100. And when allowed to compute results will calculate different combination of 80, 85, 90, 95, 100. Note that this is one additional value as to the number of steps. The graphs show the minimum and maximum values and thus provides an indication of the range of method parameters that are being considered. When values in the method parameter table are set, MOREPEAKS will calculate each possible combination of values. This means that the number of methods that needs to be evaluated grows exponential with the number of steps. The total number of combination will be shows in the top-right of the user interface in the "Control Panel", next to the "*Compute*" button. Depending on your computer specification, it is recommended limit the number of methods to 1000-3000. A good practice is to first calculate method parameters using larger steps, and then "zoom-in" (*i.e.*

reducing the step size and search in that particular area) on areas that show the highest values for the used quality descriptors.

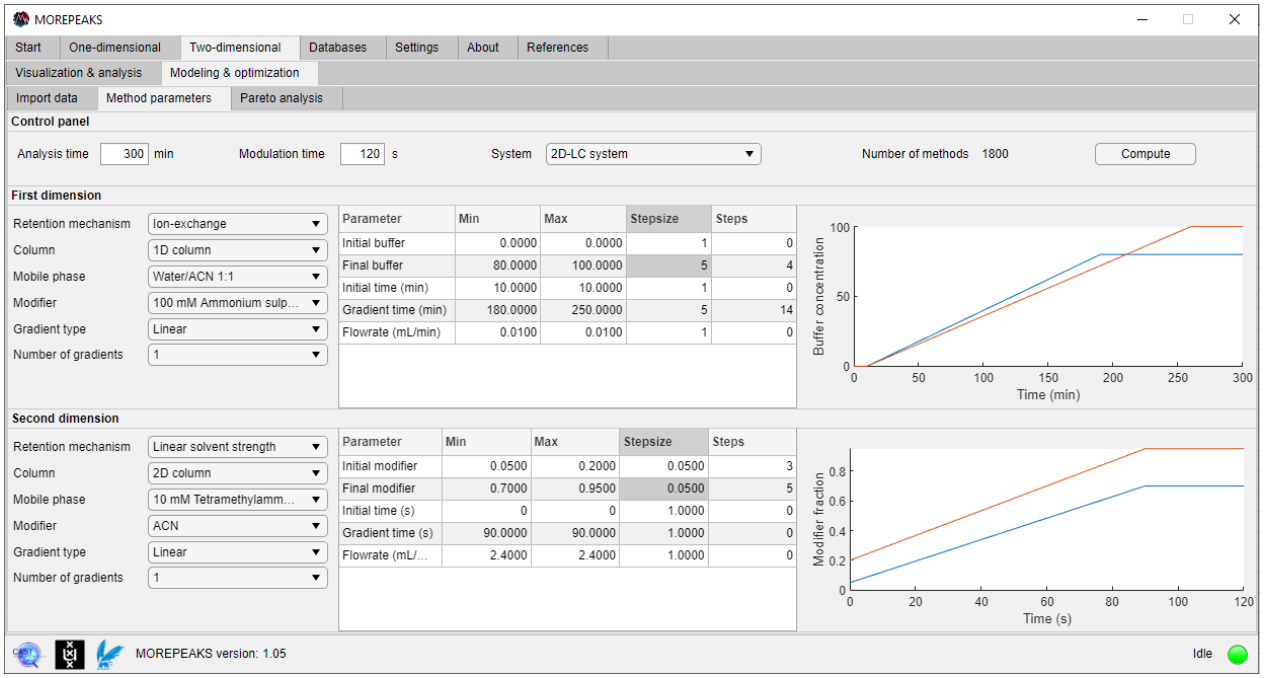

Figure 14. *Selection method parameters.*

In the "*Control panel*" at the top, the maximum allowed analysis time can also be provided. Each separation that has peaks eluting later than this value will be flagged and deemed inappropriate. Furthermore, if LC×LC is being predicted, the modulation time needs to be provided here. If one is happy with the currently selected ranges, press "*Compute*". MOREPEAKS will now calculate each different combination of methods (Figure 15). If too many methods were selected and calculations take too long, the "*Cancel*" button can be pressed. MOREPEAKS will finish its current calculation and will return to the "*Method parameter*" tab shortly.

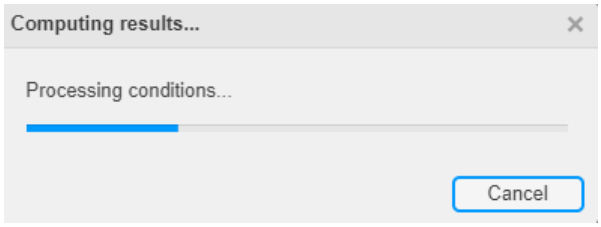

Figure 15. *Computing results.*

3.3.1.2. Pareto analysis of results

When MOREPEAKS is done calculating, it automatically navigates to the "*Pareto analysis*" tab. On the left side, each calculation is shown based on two quality descriptors. In Figure 16 the last eluted peak and the product of resolution scores are shown. If datapoints are shown as an asterix (*) instead of a filled circles, the calculation was flagged because something went wrong. More on this later in this section. On the right side, the predicted chromatogram is shown. Depending if a 1D or 2D separation is calculated, the chromatogram is either shown as a surface (below in Figure 16) or a 2D representation of a 1D separation (*i.e.* time versus signal). If the background or chromatogram shows red (see Figure 17), the current chromatogram is flagged.

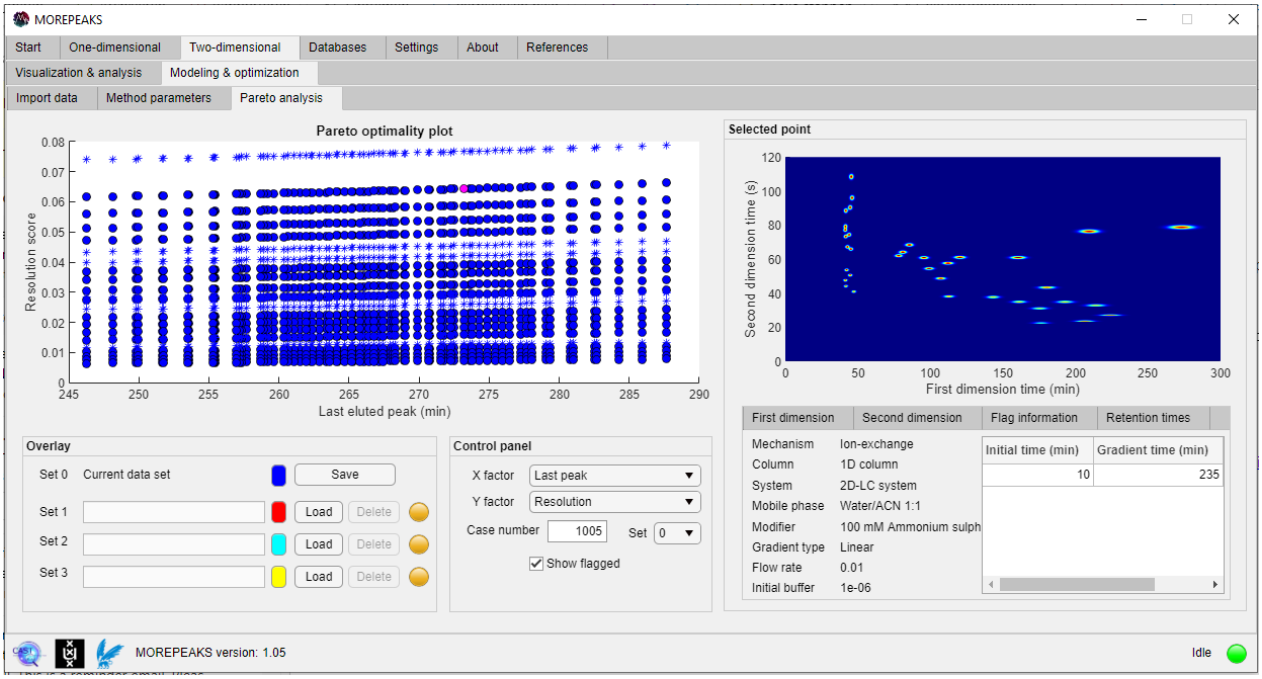

Figure 16. *Pareto analysis tab.*

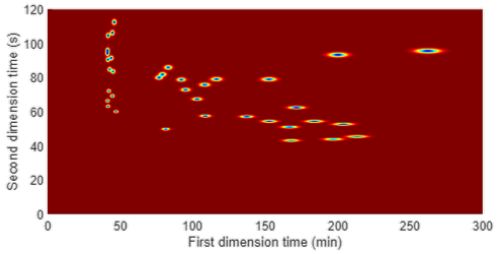

Figure 17. *Example of a flagged chromatogram.*

By clicking on a point in the "*Pareto optimality plot*" one can navigate through each calculation. Each calculation is also provided with a case number. In the "*Control panel*" in the bottom-middle section of the user interface, one can also navigate by case number if necessary. Here, the "*Pareto optimality plot*" can also be forced to not show flagged calculations (Figure 18). However, navigating by case number still guides a user to this

calculation. In the "*Control panel*" one can also change the quality descriptors of the Pareto optimality plot.

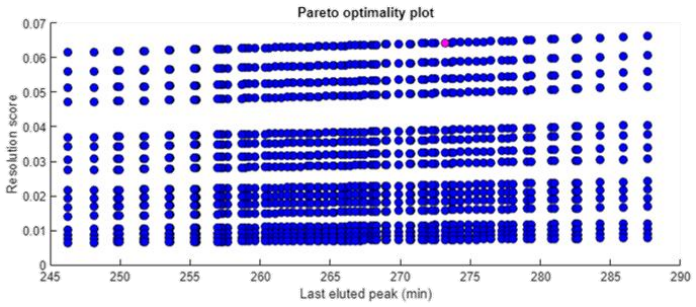

Figure 18. *Pareto optimality plot that doesn't show flagged calculations.*

On the bottom-left of the screen in the tab panel, all ^{1}D (and if available, ^{2}D (Figure 19)) experimental conditions can be navigated through. Furthermore, flag information can be seen (Figure 20). Chromatograms can be flagged due to a last eluted peak that exceeds the maximum allowed analysis time, not eluted peaks (*i.e.* modifier or buffer concentration remained too low and a peak is retained in the column) or in the case of LCLC, wrap-around (*i.e.* an analyte is retained too long and elutes in the next modulation) can occur

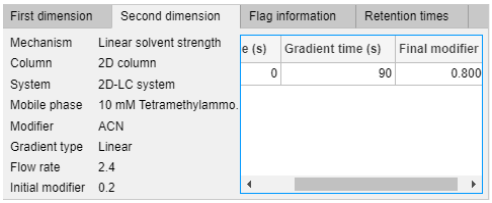

Figure 19. *^{2}D method parameters.*

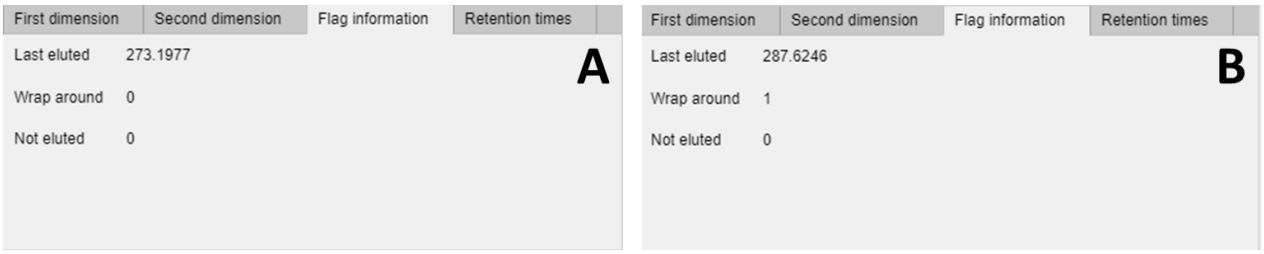

Figure 20. *Flag information.* ***A****) Successful separation.* ***B****) Unsuccessful separation.*

Lastly, the predicted retention times are provided in a table (Figure 21). From this menu, one can also export all retention times and method parameters to an Excel file by clicking "*Export*" (Figures 22 and 23).

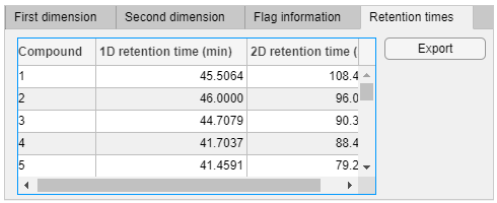

Figure 21. *Retention-time table and "Export" button.*

	A	B	C	D
1	Names	1D retention time (min)	2D retention time (s)	
2	1	45,50641145	108,4528793	
3	2	45,99999997	96,00002851	
4	3	44,7079448	90,342875	
5	4	41,70365727	88,46354867	
6	5	41,45914013	79,24683475	
7	6	41,33984945	77,14994862	
8	7	44,01787193	74,35614249	
9	8	42,80276603	67,1861919	
10	9	45,23241664	65,89346155	
11	10	42,26142104	53,56925227	
12	11	44,73395197	50,50001118	
13	12	41,40294214	47,49960895	
14	13	41,72593207	44,04751101	
15	14	47,22789288	40,79242283	
16	15	78,13075306	61,96188844	
17	16	81,01986113	64,06309069	
18	17	85,50347603	68,33960293	
19	18	120,3597842	61,00258261	
20	19	112,1516433	57,54199964	
21	20	99,14800886	54,38041483	
22	21	107,1501685	48,53587056	
23	22	112,7497357	38,02355816	
24	23	143,2405724	37,63434166	
25	24	160,5200128	60,87780159	
26	25	180,3289568	43,20551951	
27	26	175,3283366	31,03923969	
28	27	176,3297131	22,55695038	
29	28	161,2068044	34,77741177	
30	29	192,7364681	34,75128348	
31	30	206,527161	23,67951483	
32	31	214,1370462	32,80693985	
33	32	224,0931895	26,94626829	
34	33	209,5054829	76,30400794	
35	34	273,1976636	78,7318789	
36	35	95,58261395	60,79299837	
37	36	41,59984612	73,28277096	
38				

Retention times | Method parameters

Figure 22. *Predicted retention times in Excel.*

	A	B	C	D	E	F	G	H	I
1	System	2D-LC system							
2	Analysis time	300	min			Modulation time	120	s	
3	1D retention mechanism	Ion-exchange				2D retention mechanism	Linear solvent strength		
4	1D column	1D column				2D column	2D column		
5	1D mobile phase	Water/ACN 1:1				2D mobile phase	10 mM Tetramethylammonium hydroxide buffer		
6	1D modifier	100 mM Ammonium sulphate buffer				2D modifier	ACN		
7	1D flow rate	0,01	mL/min			2D flow rate	2,4	mL/min	
8	1D gradient type	Linear				2D gradient type	Linear		
9	1D time frame	10	245	min		2D time frame	0	90	s
10	1D buffer concentration	0,000001	90	mmol/L		2D modifier content	0,2	0,8	
11									

Figure 23. *Experimental conditions in Excel.*

In the bottom-left corner, the current results can be save to be able to compare them to other results later on. By clicking "*Save*", MOREPEAKS will navigate to its "*Result sets*" folder and allows one to save it here.

3.3.2. "Complex" gradient parameters

3.3.2.1. Selecting method parameters

The method parameter tables allow for multiple gradients (Figure 24). If an additional gradient is added, the number of different parameter is the table is adjusted. For each gradient, select a gradient time (*i.e.* if the first gradient time equals 50 min and the second equals 70 minutes, the total gradient time will sum this to 120 min) and maximum buffer concentration or modifier fraction.

If a separation is predicted in 2D, the gradient type of the ^{2}D might be adjusted from linear, to shifted, two-step shifted (as shown in Figure 24) or continuous. A shifted gradient adjusts the ^{2}D gradient starting from the calculated column time until the analysis time (as shown in the "*Control panel*" at the top of the screen by varying multiple constants. The graphs will only show the shifted conditions for the minimum values. Two-step shifted gradients work similar, but additional values can be selected to further optimize the method. Continuous gradients consist of one slow gradient throughout the entire ^{2}D. The increase in parameters however, results in an large growth of number of methods if one is not careful. In the following example, 7290 methods are calculated while most variables do not differ or only have 2-8 different values.

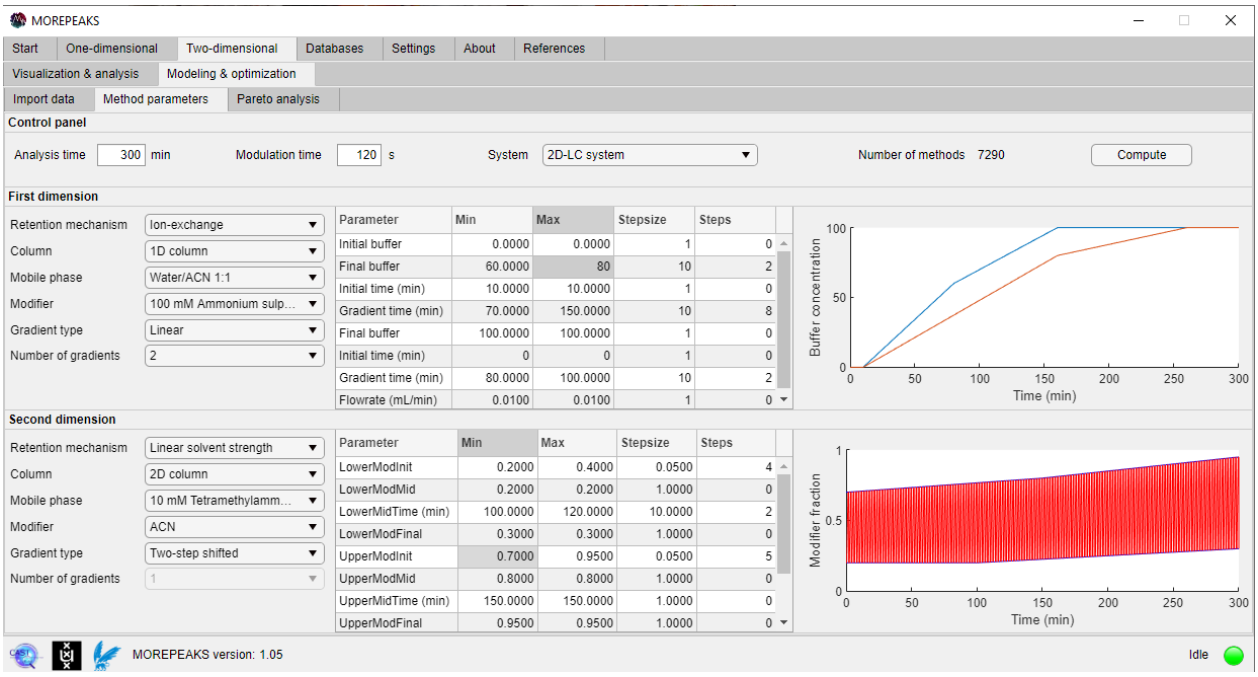

Figure 24. *Method parameters for two [1]D gradients and a two-step shifted [2]D gradient profile.*

3.3.2.2. Pareto analysis of results

The “Pareto analysis” tab will look similar to Section 3.3.1.2, but with a slightly less predictable Pareto plot (Figure 25).

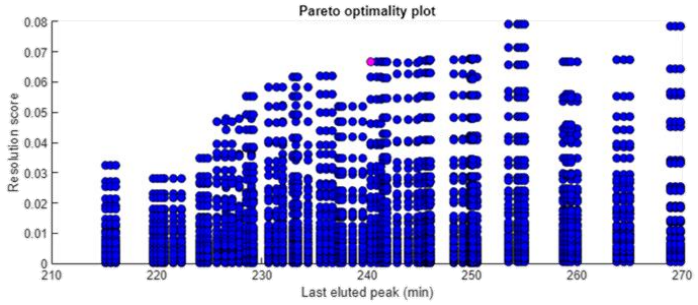

Figure 25. *Pareto optimality plot of the two-step shifted gradient.*

If one wants to compare the obtained Pareto optimality plot with the previous obtained results from Section 3.3.1.2, click “*Load*” in the “*Overlay*” panel in the bottom-left corner and select the previously saved results. The “Pareto analysis” tab can now look similar to Figure 26. Colors for the Pareto plot can be chosen by clicking the color in the “*Overlay*“ panel. If one is not interested in comparing the results, one can click “*Delete*” to remove the results from the plot.

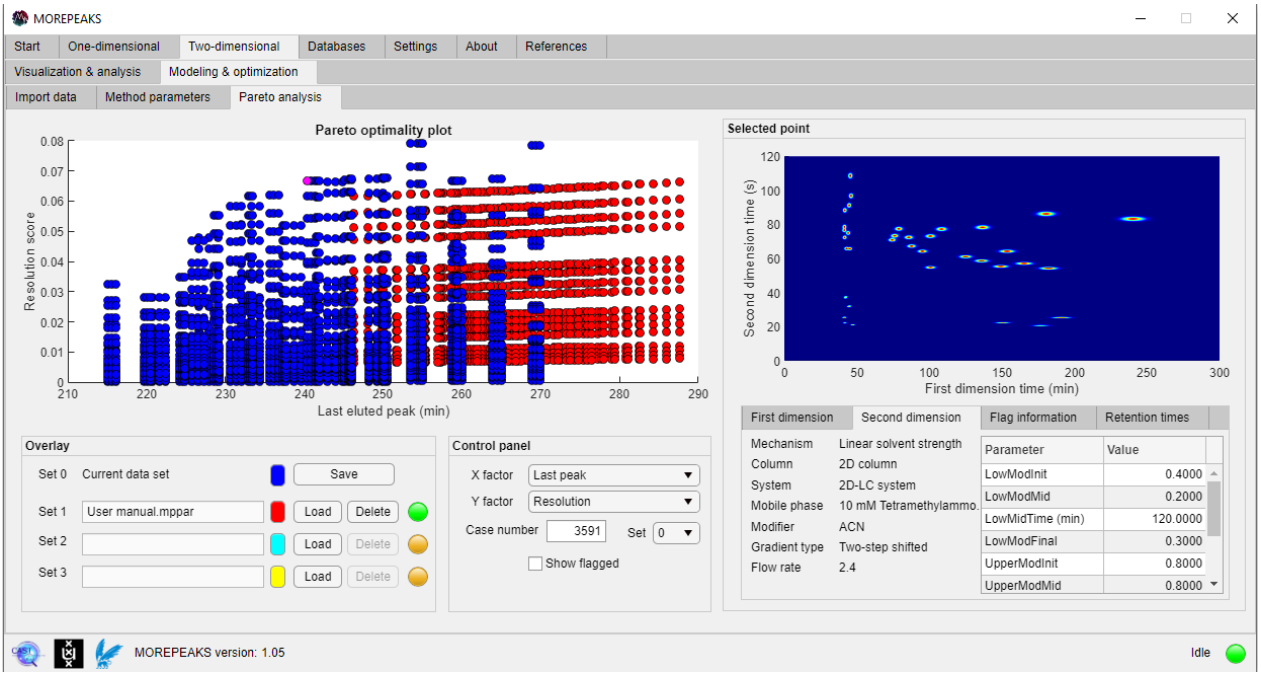

Figure 26. *Comparing result sets in the "Pareto analysis" tab.*

4. Peak-pairing tools

Retention modeling requires you to relate retention times to experimental conditions. MOREPEAKS has the option to create these lists of retention times built-in. In the "*Import data*" tab, in the "*Control panel*" on the top-right side of the user interface in both "*One-dimensional*" – "*Modeling & optimization*" and "*Two-dimensional*" – "*Modeling & Optimization*" the option to open the "*Peak pairing tool*" will be enabled when enough chromatograms are loaded into the software. To load in chromatograms into MOREPEAKS, flip the switch in the "*Control panel*" to "*Raw data*". MOREPEAKS allows extensions: .xlsx, .csv, .arw, .mzXML and .cdf.

4.1. One-dimensional peak pairing

Click the "*Browse & Load*" buttons to select a file. The chromatograms can be viewed by clicking the "*View*" button. When all files are imported into MOREPEAKS, click "*Peak pairing tool*" in the "*Control panel*" to open it. If there are two chromatograms that have MS data present (.mzXML and .cdf files), the option for "*Automated peak pairing*" will be available (Section 4.1.2). Otherwise, only manual peak-tracking is allowed (Section 4.1.1). Below an example of the peak-pairing tool is provided with two chromatograms loaded in (Figure 27).

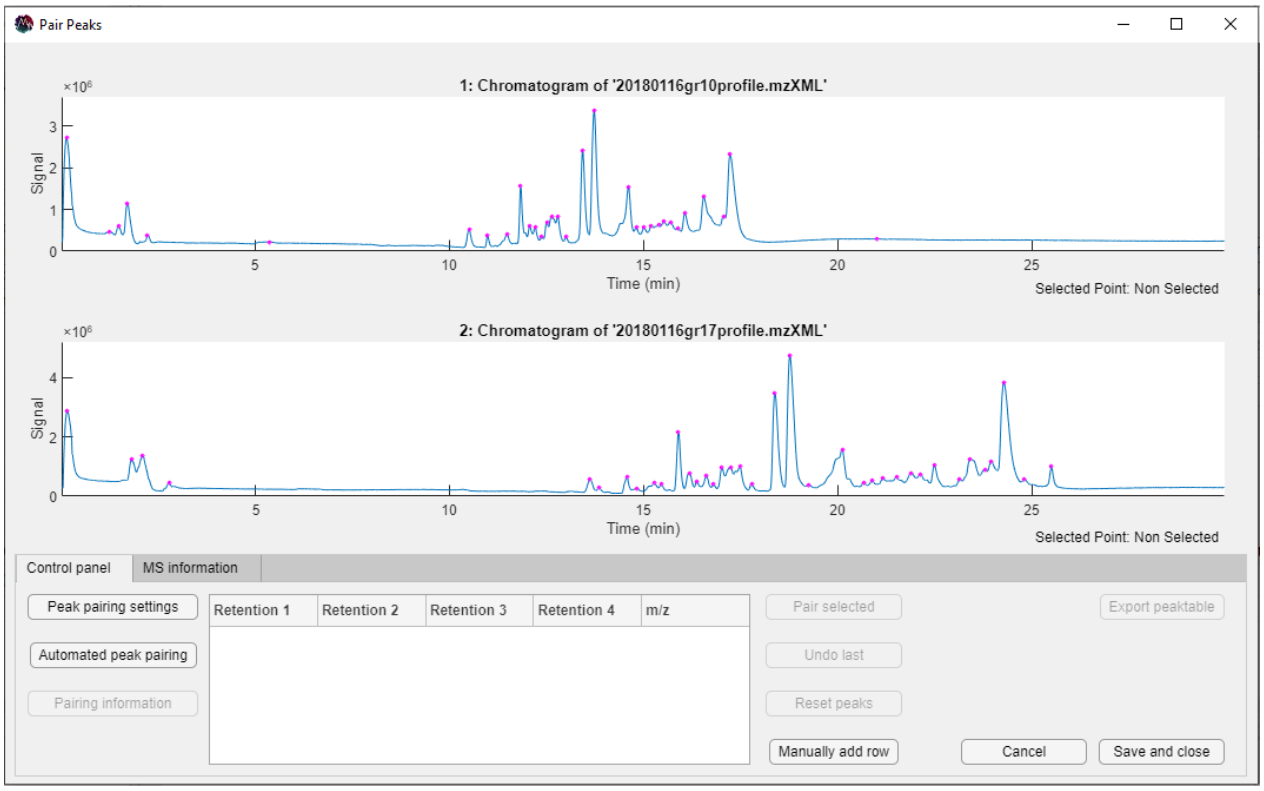

Figure 27. *One-dimensional peak-pairing tool.*

4.1.1. Manual peak-tracking

To manually pair peaks, find a peak on both chromatograms that is similar and select it by clicking on it. Underneath the chromatogram, the selected time point is shown and on the chromatogram a circle is placed to indicate which peak is selected. Then click "*Pair selected*" in the "*Control panel*". The peak pairing tool adds the peak to the table as shown in Figure 28 and will show on the chromatogram that the peak is paired by provided it with a number. If more than two chromatograms are present, peaks need to be selected on all chromatograms before the selected can be paired. Values in the table can be adjusted if the peak detection is slightly off. Furthermore, row can be added by clicking "*Manually add row*" to provide information about peaks that were not detected by the peak-detection algorithm.

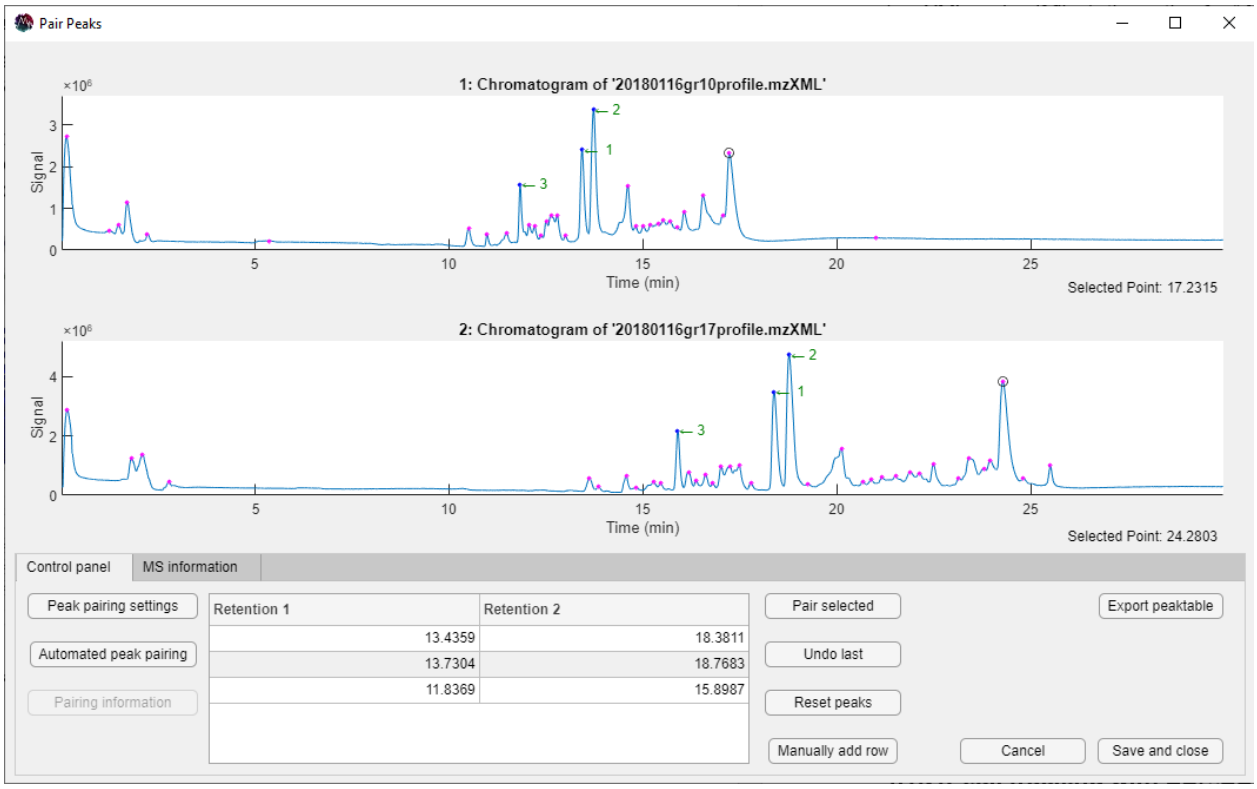

Figure 28. *Manual peak pairing in progress. Three peaks have already been paired, a fourth peak-pair is selected.*

If MS data is provided, one can navigate to the "*MS information*" tab. If "*Select MS*" is turned on and you click on any point in the chromatogram, the software will provide the mass spectra of that point. Indicated by a cross on its location (Figure 29). When in doubt if two peaks are equal to each other, use this feature to compare the spectra. Furthermore, one can show extracted ion-currents (XIC) from specific mass-to-charge (*m/z*) values on the right hand side to determine if peaks are equal (Figure 30). Back in the "*Control panel*" tab, by pressing "*Export peaktable*" the retention table will be exported for later use. When peak-tracking is done, click "*Save and close*". When clicking "*Cancel*", all information is forgotten.

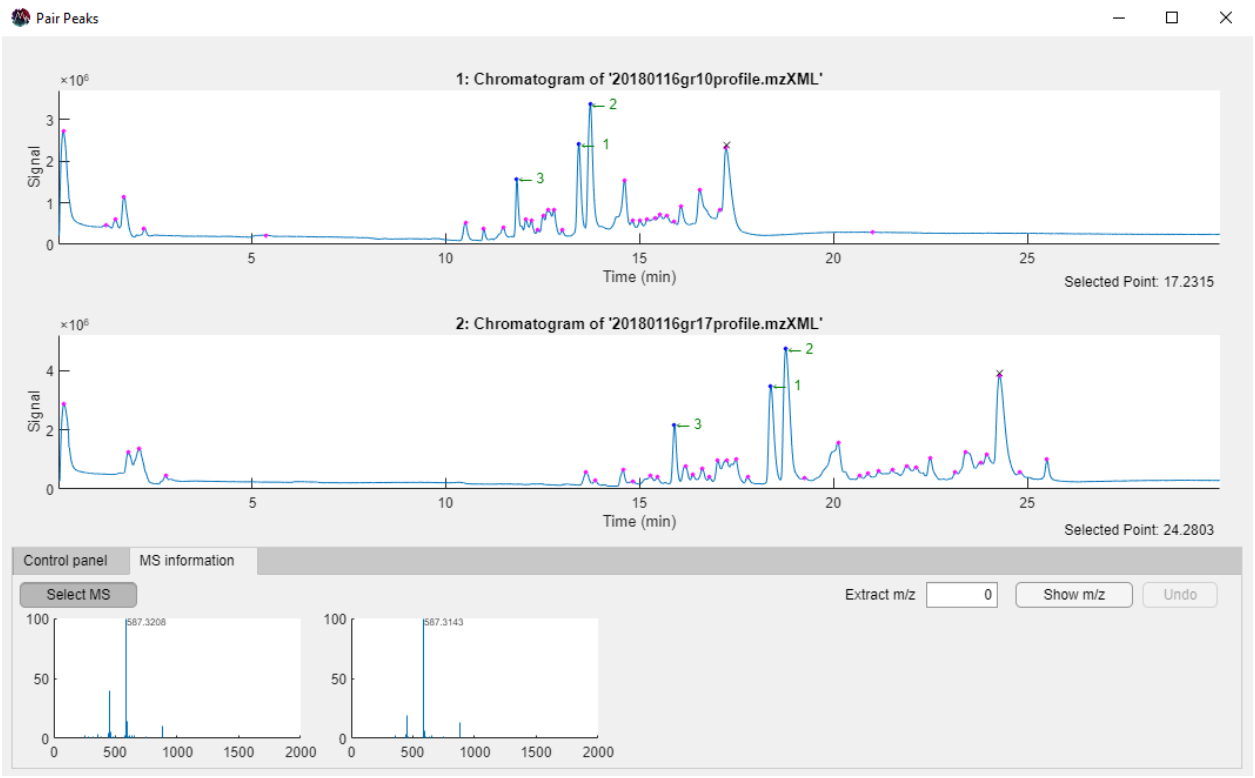

Figure 29. *Mass spectra of time points from each chromatogram.*

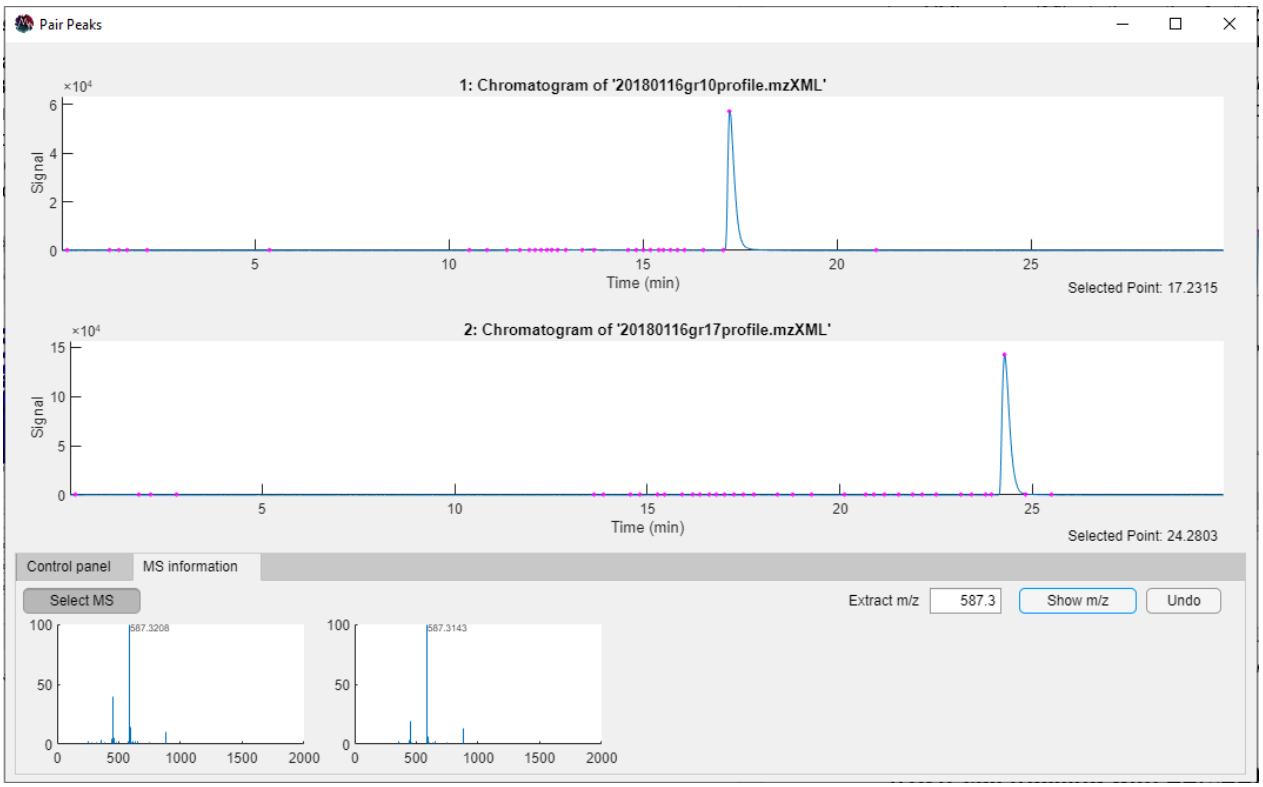

Figure 30. *XIC of a particular m/z value.*

4.1.2. Automated peak tracking with LC-MS data

If two LC-MS files are loaded in, the option to automatically track peaks is available. Simply press "*Automated peak pairing*" and the software will show that it is in progress (Figure 31).

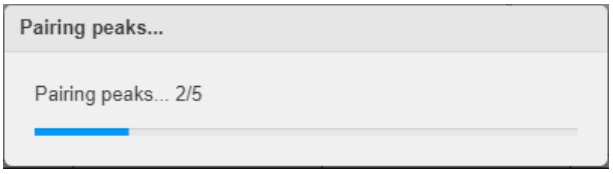

Figure 31. *Automated peak pairing in progress.*

When MOREPEAKS is done tracking peaks, it will show all tracked peaks on the chromatogram. The provided color indicates how sure the algorithm is about the quality of the match. Colors go from green-orange-red to indicate this, green is sure, red is unsure (Figure 32).

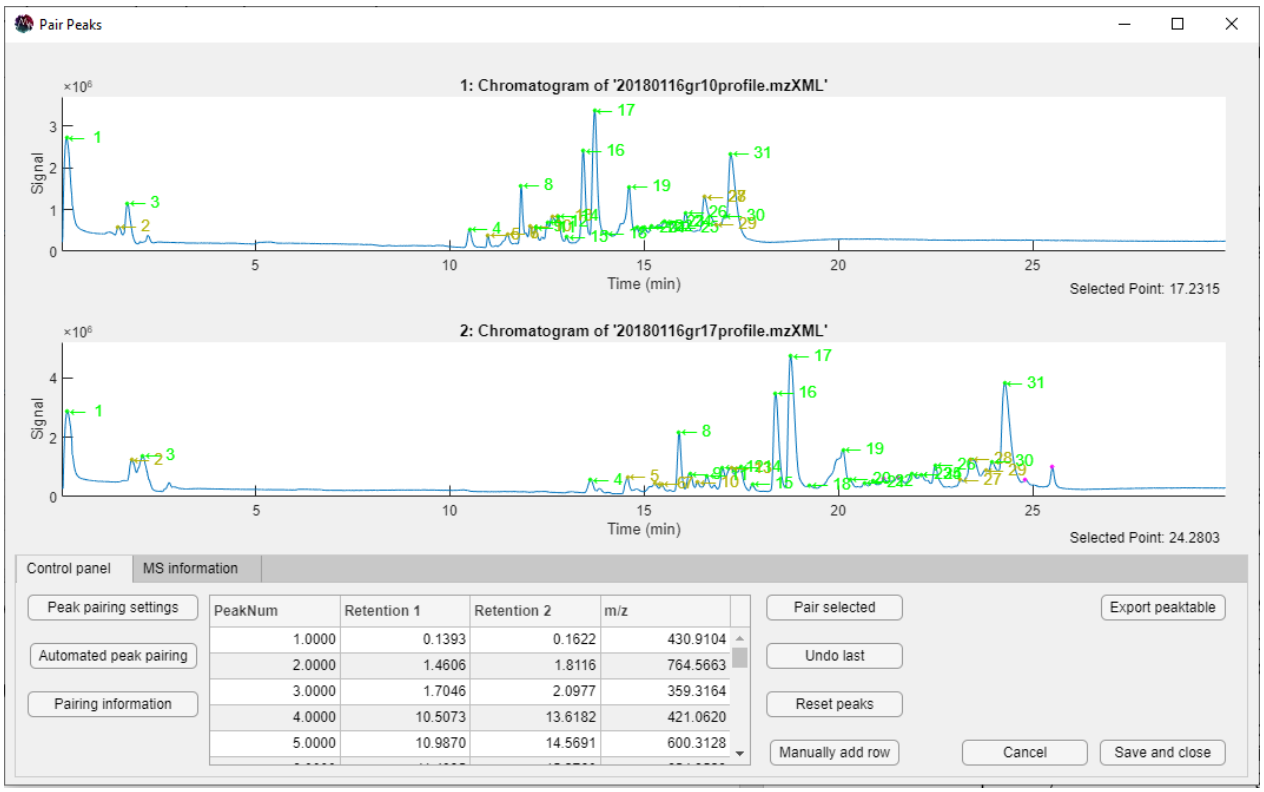

Figure 32. *One-dimensional peak tracking is done.*

Information about the quality of the peak tracking can be view by clicking "*Pairing information*". This will open a new window. The window will show a summary of the scores and the cases that were paired with a score less than 40% (Figure 33), and can be navigated through to show the average relative retention as a single line and all values that deviate from this line as dots (Figure 34). Also histograms can of all scores can be

seen by going to the “*Scores*” tab (Figure 35). Furthermore, all individual scores can be seen by clicking “*Data table*”.

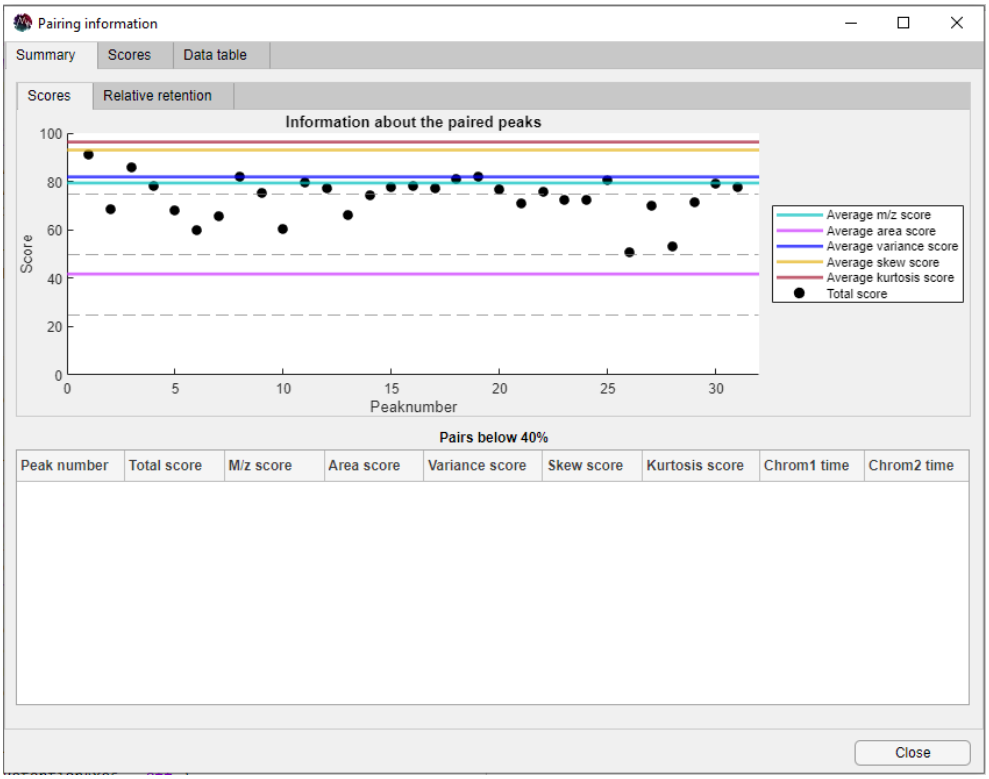

Figure 33. *Pairing information, quick overview.*

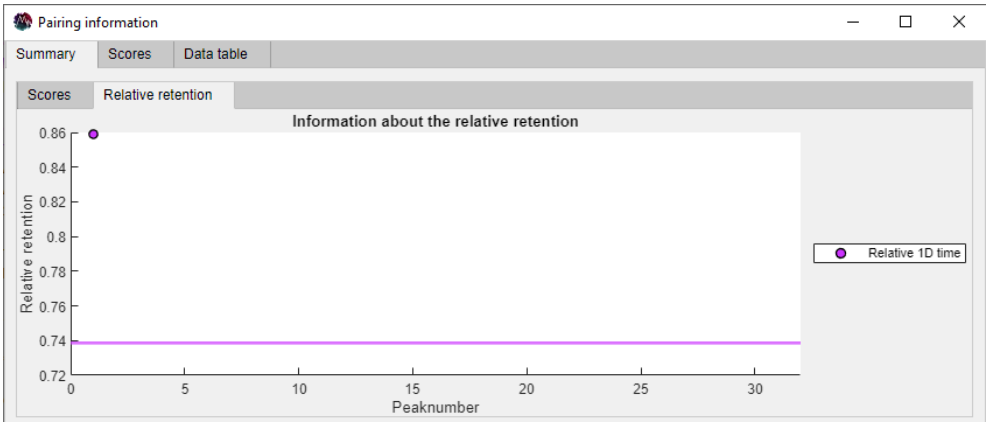

Figure 34. *Pairing information, relative retention.*

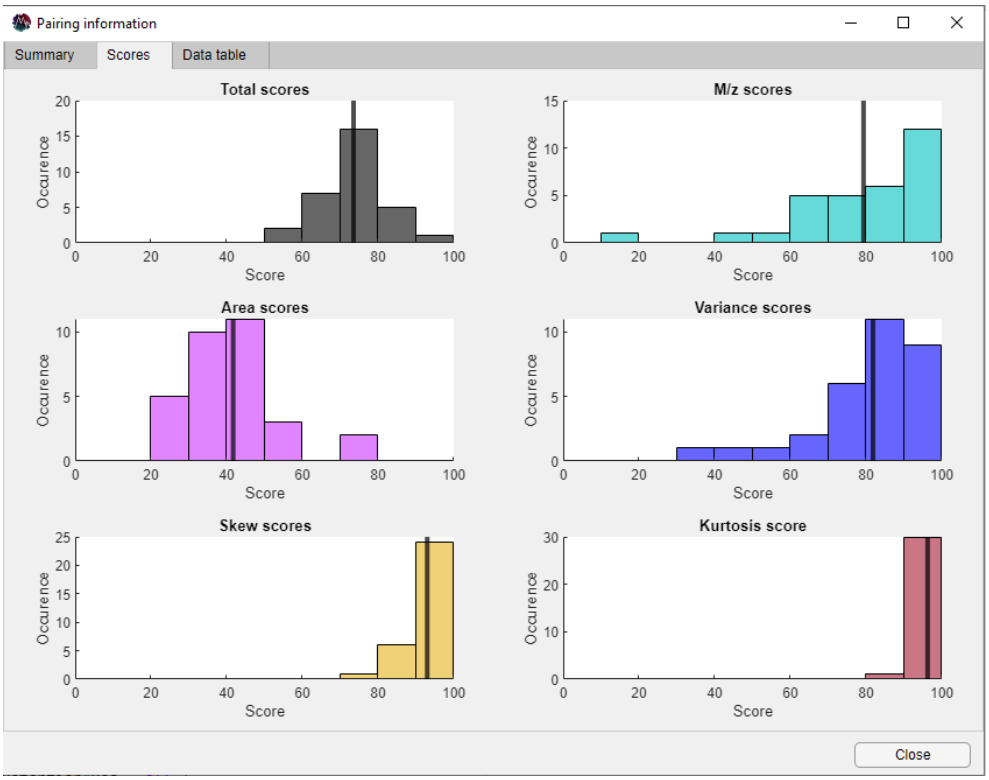

Figure 35. *Pairing information, histograms of scores.*

Peak-tracking settings can be adjusted by clicking the "*Peak pairing settings*" button. More information about these settings and how the peaks are paired can be found in the manuscript by Pirok *et al.* [15] By pressing "*Export peaktable*" the retention table will be exported for later use. When peak-tracking is done, click "*Save and close*". When clicking "*Cancel*", all information is forgotten.

4.2. Two-dimensional peak pairing

To load in raw LC×LC data, turn the switch in the "*Control panel*" to "*Raw data*". Then fill in the modulation times in seconds into the edit field next to the "*Load*" buttons in the "*Raw data / Chromatograms*" panel. Then click "*Load*" and select the chromatograms. Below an example of the peak-pairing tool is provided with two chromatograms loaded in. Press "*View*" to see the chromatogram. When all files are imported into MOREPEAKS, click "*Peak pairing tool*" in the "*Control panel*" to open it. If there are two chromatograms that have MS data present (.mzXML and .cdf files), the option for "*Automated peak pairing*" will be available (Section 4.1.2). Otherwise, only manual peak-tracking is allowed (Section 4.1.1). Below an example of the peak-pairing tool is provided with two chromatograms loaded in (Figure 36). The slider to the right of each chromatogram can be used to adjust the color intensity, this can render the visualization of low-abundant analytes more clearly, as done in the chromatogram to the left.

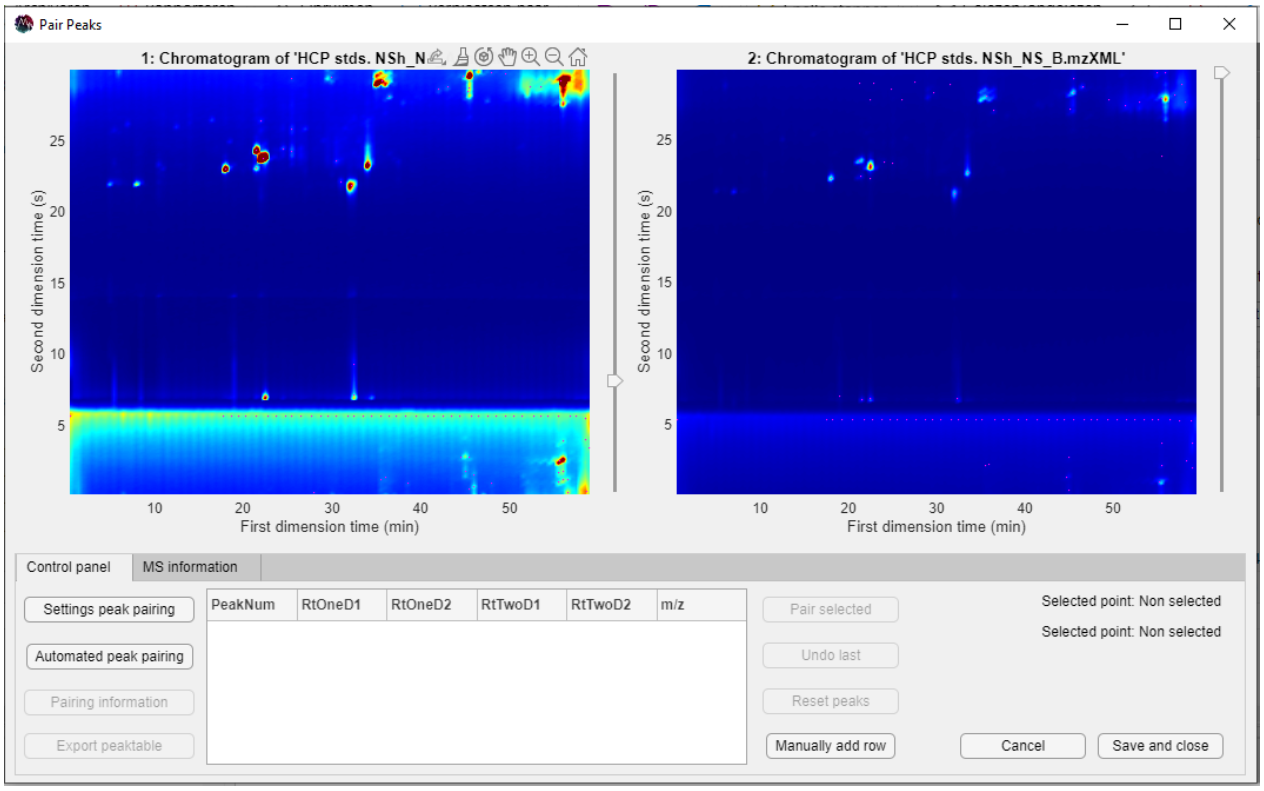

Figure 36. *Two-dimensional peak-pairing tool*

4.2.1. Manual peak-tracking

To manually pair peaks, find a peak on both chromatograms that is similar and select it by clicking on it. To the right in the "*Control panel*", the selected time points are shown and on the chromatogram a circle is placed to indicate which peak is selected. Then click "*Pair selected*" in the "*Control panel*". The peak pairing tool adds the peak to the table as shown in Figure 37 and will show on the chromatogram that the peak is paired by provided it with a number. If more than two chromatograms are present, peaks need to be selected on all chromatograms before the selected can be paired. Values in the table can be adjusted if the peak detection is slightly off. Furthermore, row can be added by clicking "*Manually add row*" to provide information about peaks that were not detected by the peak-detection algorithm.

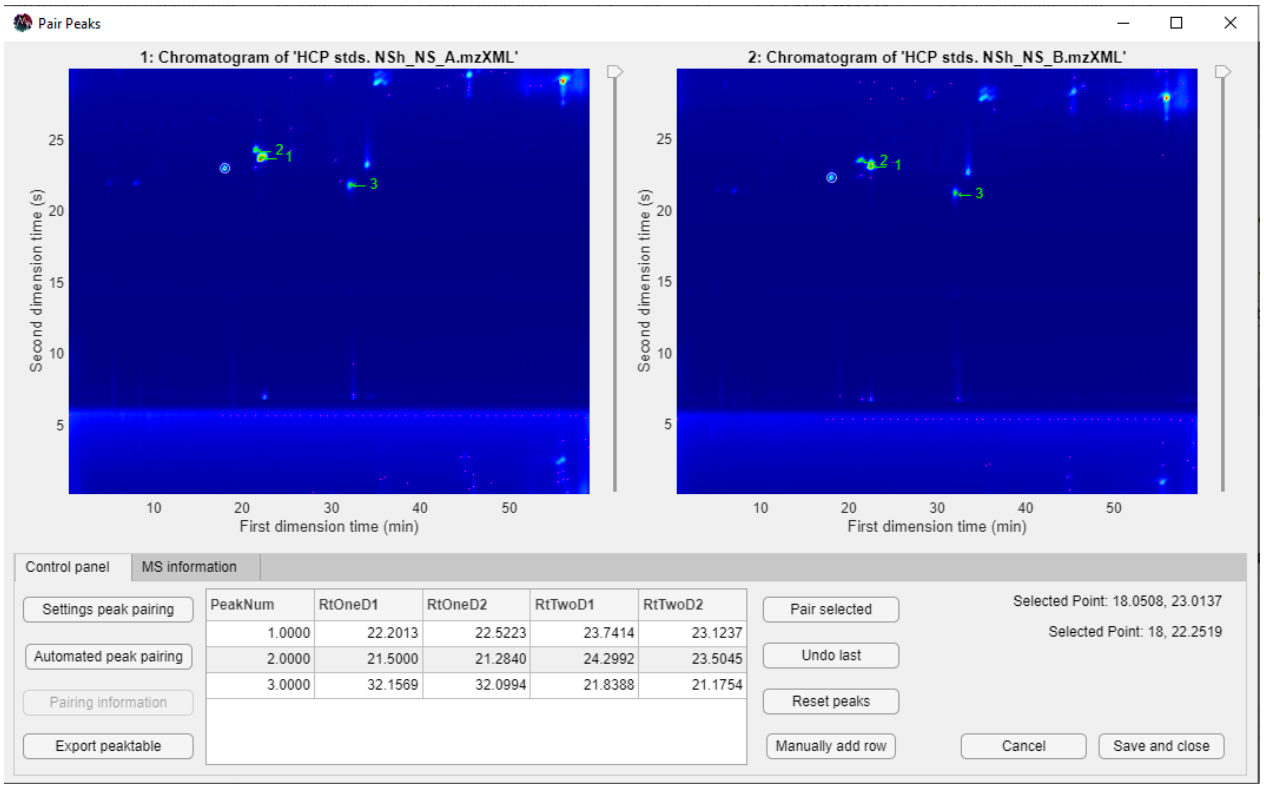

Figure 37. *Manual peak pairing. Three peaks are already paired, a fourth peak pair is already selected.*

If MS data is provided, one can navigate to the "*MS information*" tab. If "*Select MS*" is turned on and you click on any point in the chromatogram, the software will provide the mass spectra of that point. Indicated by a cross on its location (Figure 38). When in doubt if two peaks are equal to each other, use this feature to compare the spectra. Furthermore, one can show extracted ion-currents (XIC) from specific mass-to-charge (*m/z*) values on the right hand side to determine if peaks are equal (Figure 39).

Back in the "*Control panel*" tab, by pressing "*Export peaktable*" the retention table will be exported for later use. When peak-tracking is done, click "*Save and close*". When clicking "*Cancel*", all information is forgotten.

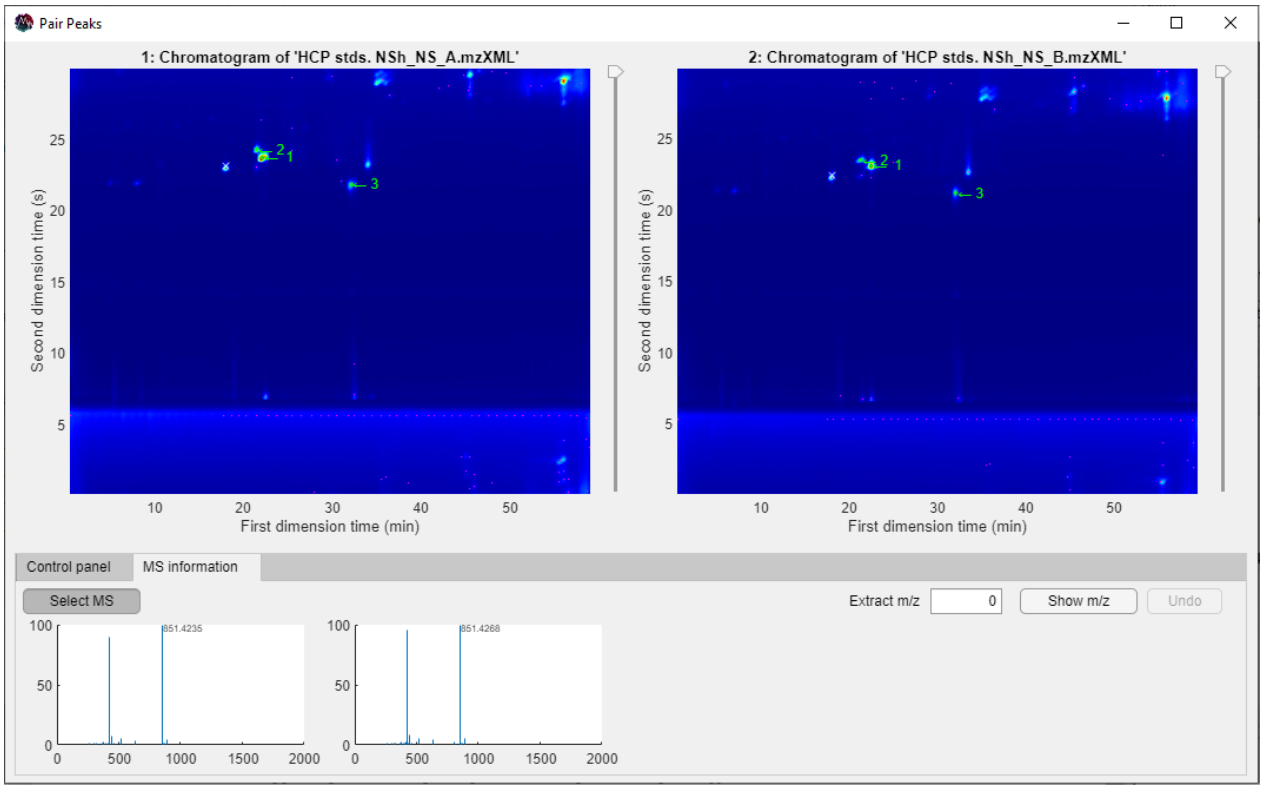

Figure 38. *Mass spectra of time points from each chromatogram.*

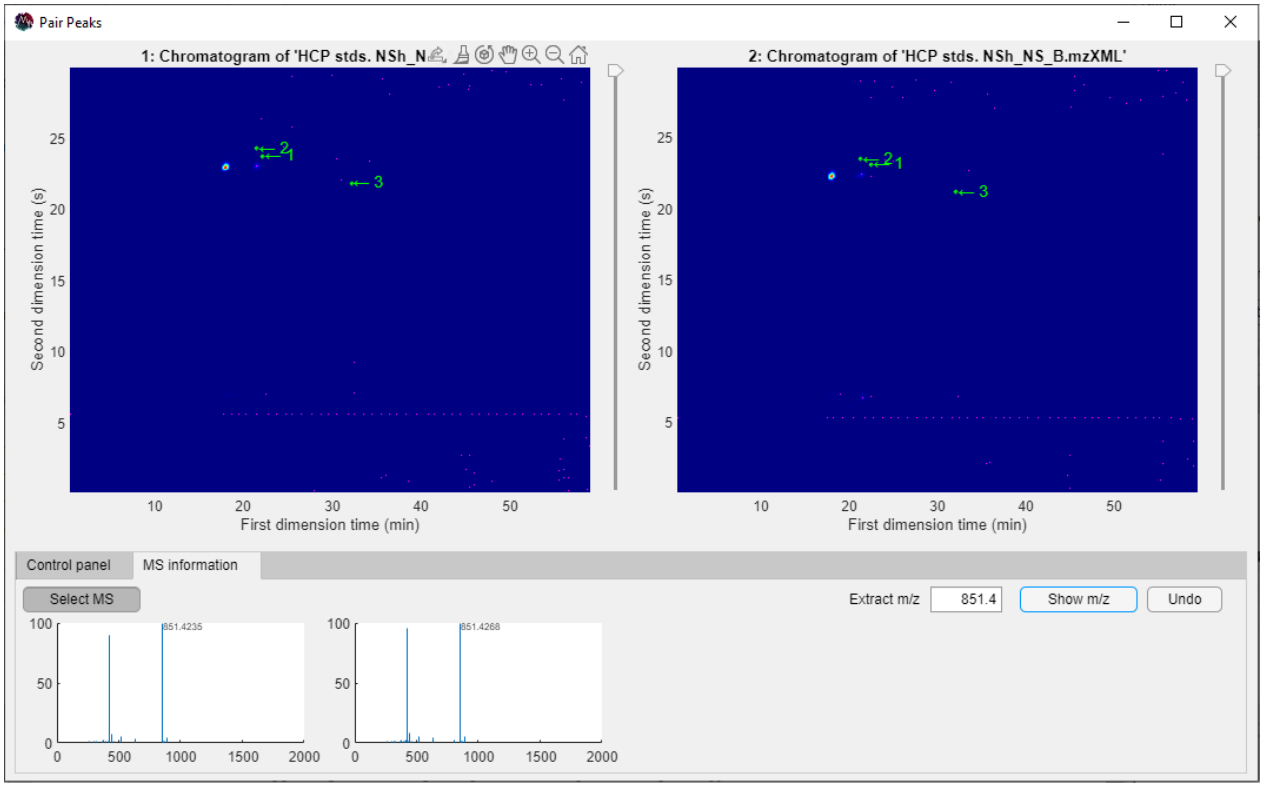

Figure 39. *XIC of a particular m/z value.*

4.2.2. Automated peak tracking with LC×LC-MS data

If two LC-MS files are loaded in, the option to automatically track peaks is available. Simply press "*Automated peak pairing*" and the software will show that it is in progress (Figure 40).

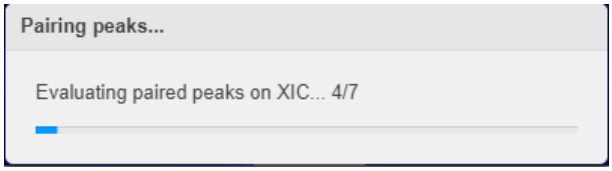

Figure 40. *Peak pairing is in progress.*

When MOREPEAKS is done tracking peaks, it will show all tracked peaks on the chromatogram. The provided color indicates how sure the algorithm is about the quality of the match. Colors go from green-orange-red to indicate this, green is sure, red is unsure (Figure 41).

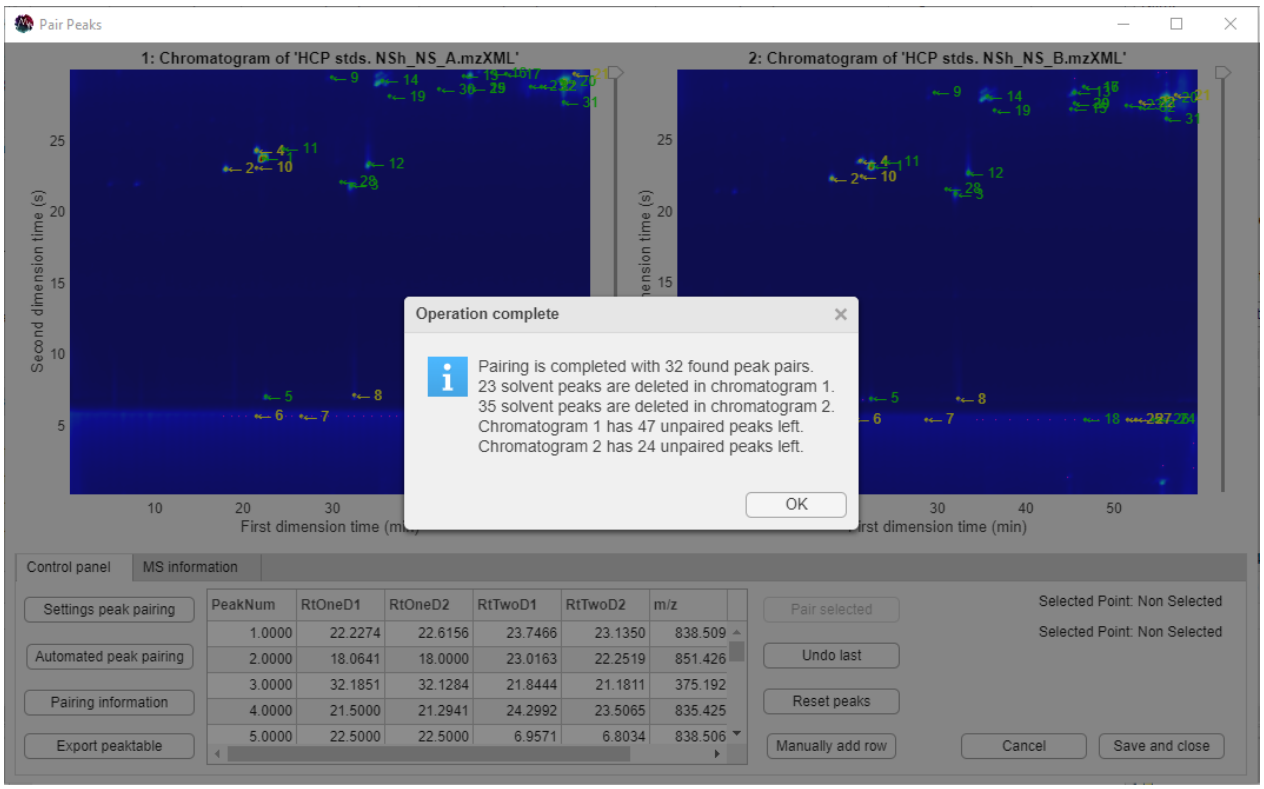

Figure 41. *Two-dimensional peak tracking is done.*

Information about the quality of the peak tracking can be view by clicking "*Pairing information*". This will open a new window. The window will show a summary of the scores and the cases that were paired with a score less than 40% (Figure 42), and can be navigated through to show the average relative retention as a single line and all values that deviate from this line as dots (Figure 43). Also histograms can of all scores can be

seen by going to the "*Scores*" tab (Figure 44). Furthermore, all individual scores can be seen by clicking "*Data table*".

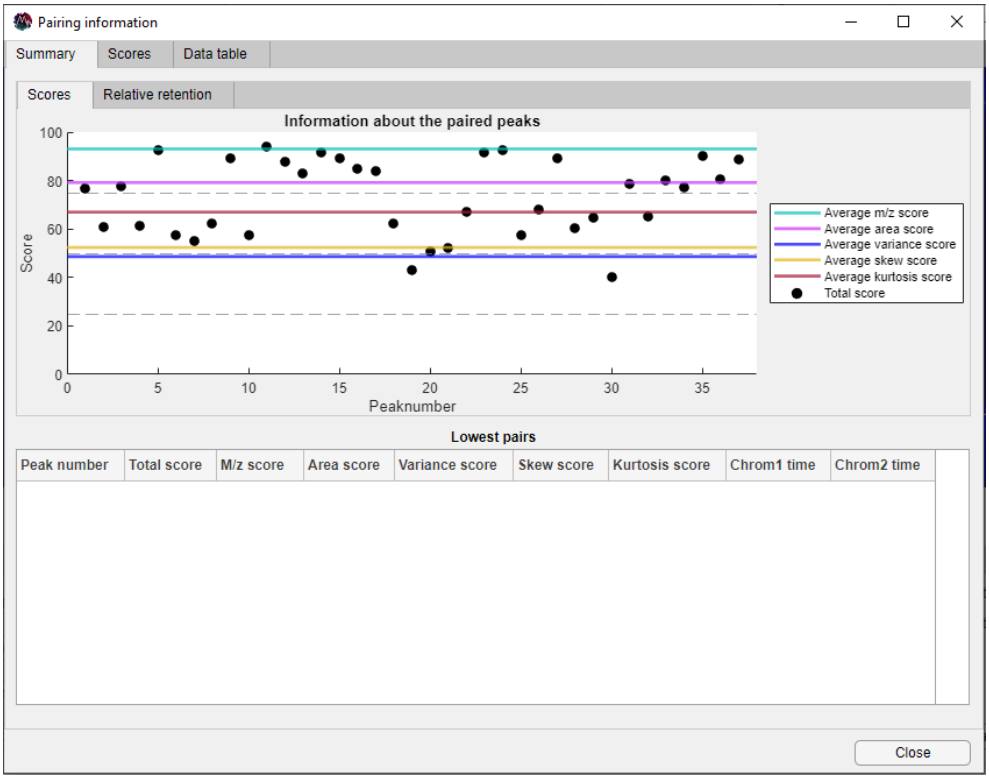

Figure 42. *Pairing information, quick overview.*

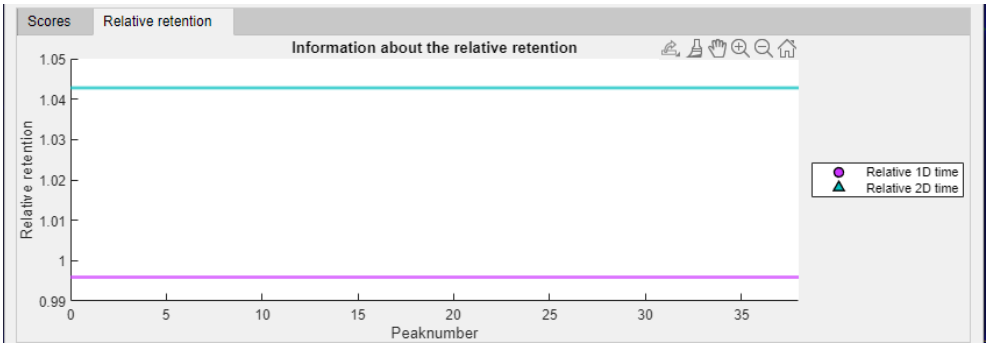

Figure 43. *Pairing information, relative retention.*

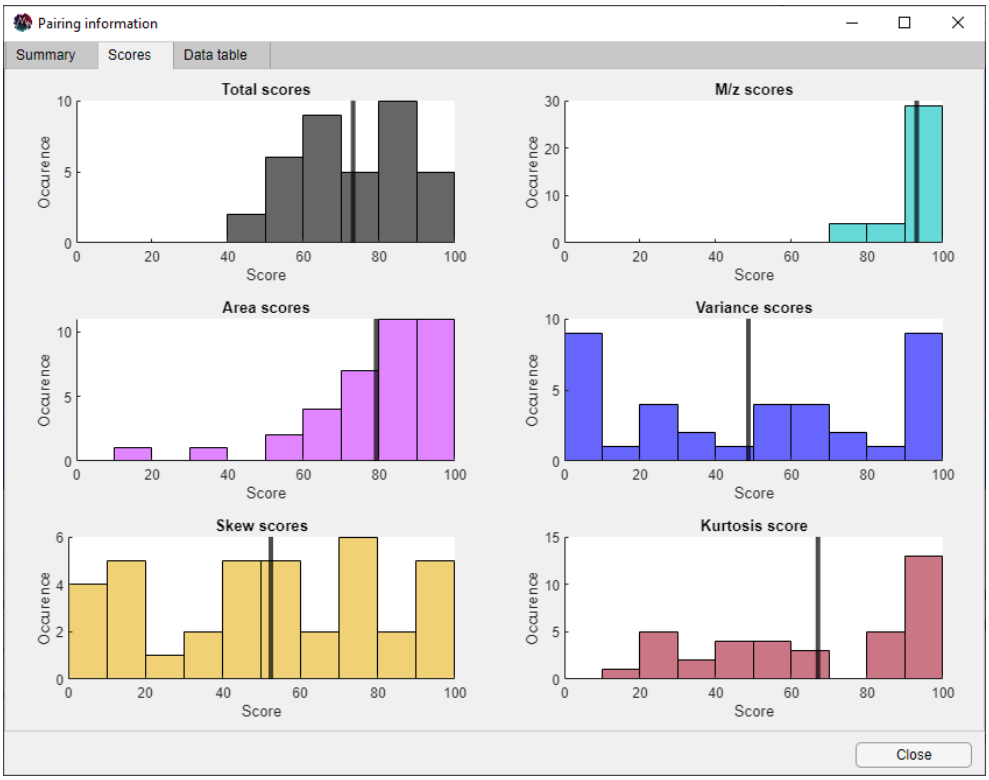

Figure 44. *Pairing information, histograms of scores.*

Peak-tracking settings can be adjusted by clicking the "*Peak pairing settings*" button. More information about these settings and how the peaks are paired can be found in the manuscript by Molenaar *et al.* [16] By pressing "*Export peaktable*" the retention table will be exported for later use. When peak-tracking is done, click "*Save and close*". When clicking "*Cancel*", all information is forgotten.

5. Visualization & analysis

The "*Visualization & analysis*" tab in both 1D and 2D mode can be used to visualize chromatograms. Typically, data formats don't change from LC to gas chromatography (GC). As typically it is time versus signal, or maybe MS data is present. Both formats however can be loaded into MOREPEAKS. This tab can therefore also be used to analyze GC(×GC) datafiles. When loading a 2D chromatogram, provide a modulation time in seconds in the "*Data*" tab in the bottom of the screen then press "*Load*" and select the correct file. For a 1D chromatogram, simply press the "*Load*" button. If mass data is present, on the right the "*Spectral information*" tab will be visible (Figure 45). Otherwise, the right side stays grayed out. Clicking on any point in the chromatogram will change the mass spectrum on the right side to the point where just has been clicked. When analyzing a 2D chromatogram, the intensity of the colors can be adjusted by the slider next to the chromatogram. Detected peaks will be shown in the bottom-left.

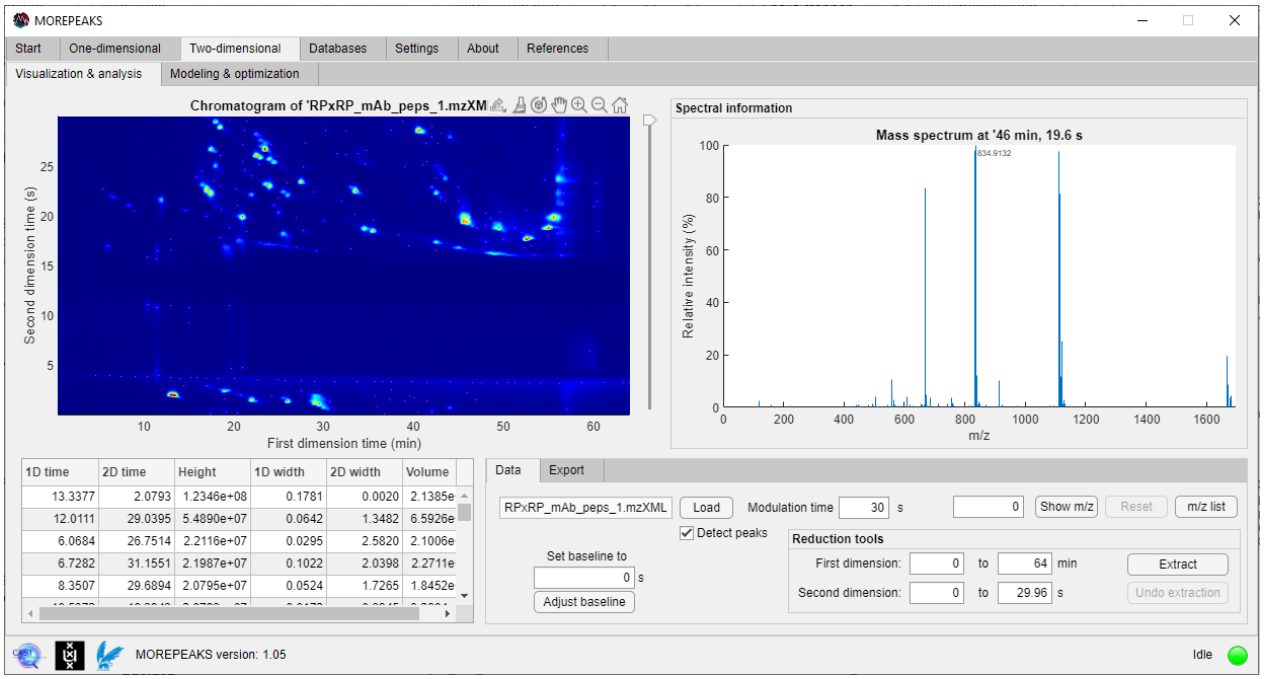

Figure 45. *Two-dimensional "Visualization & Analysis" tab.*

When loading in 2D datasets, the offset, or baseline, in the ^{2}D can be adjusted to shift the chromatogram up and down, as in Figure 46.

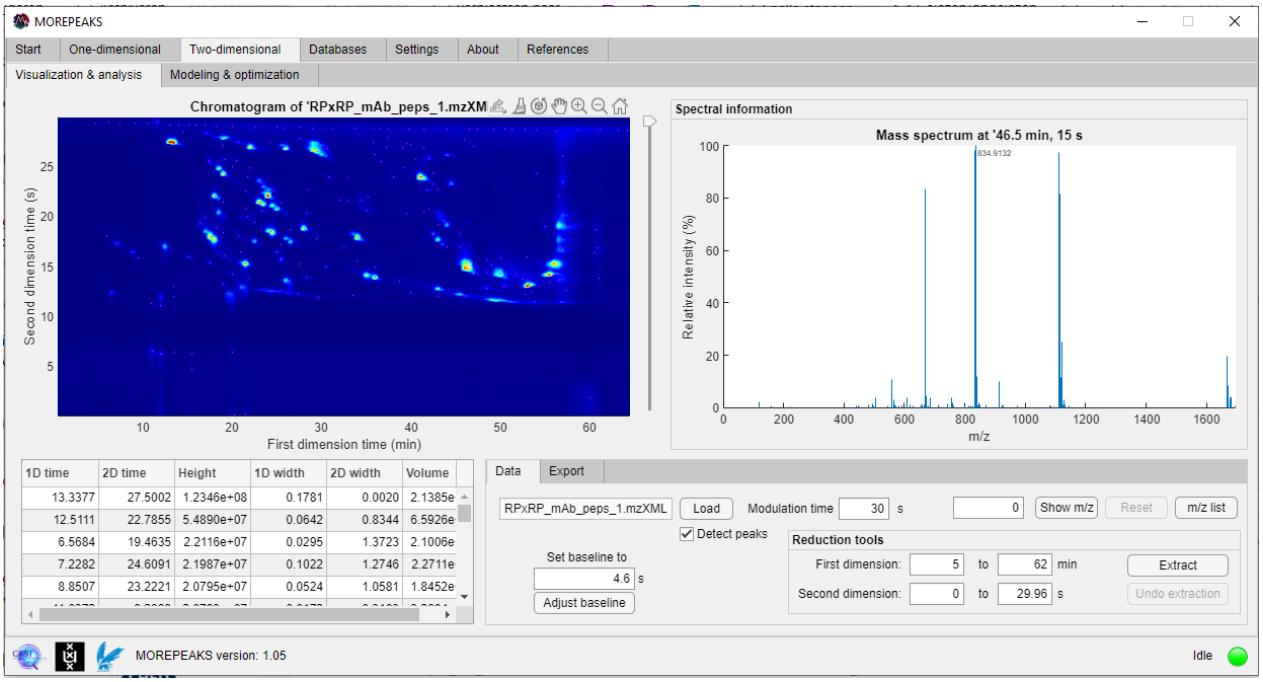

Figure 46. *Offset adjusted chromatogram.*

By filling in a *m/z* value in the right side of the screen and clicking "*Show m/z*", and XIC of the chromatogram will be shown as in Figure 47.

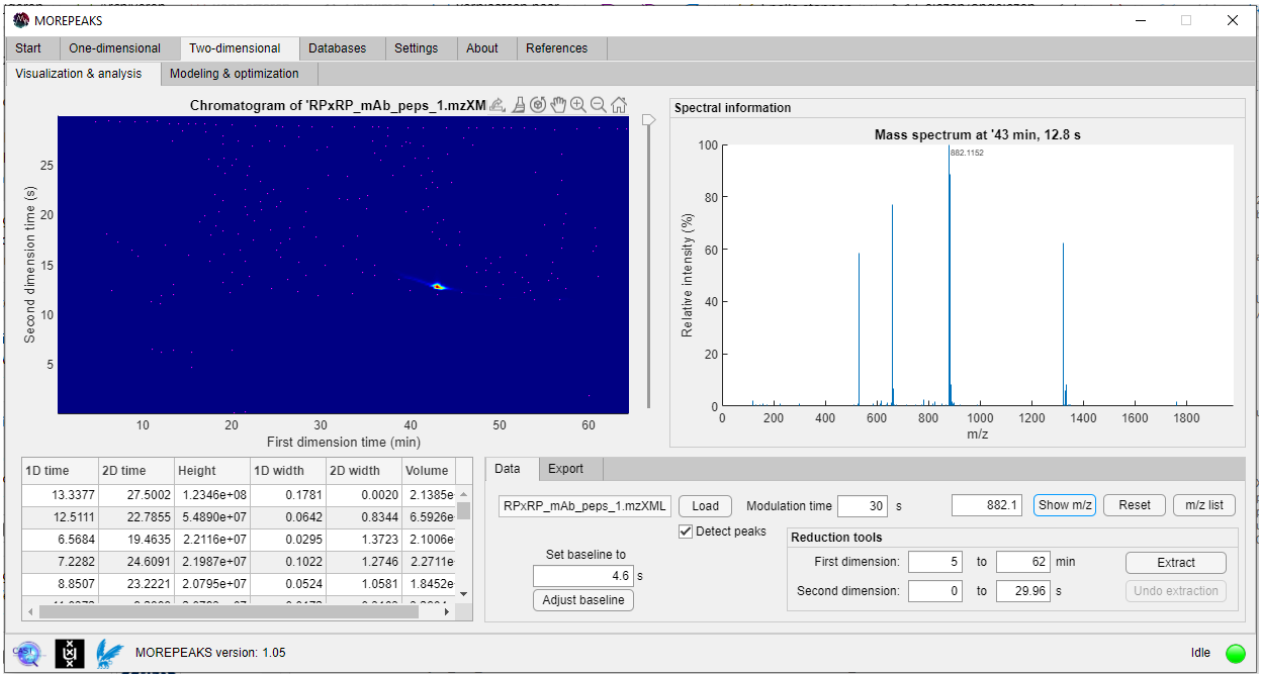

Figure 47. *XIC of the LC×LC-MS data.*

If one would like to see a sum of different *m/z* values, create a list in Excel as in Figure 48 and click "*m/z list*". Then select the Excel file with the *m/z* values. MOREPEAKS will now show a sum of all *m/z* values in the list as in Figure 49.

	A
1	882,1152
2	834,6614
3	714,0141
4	899,4582
5	
6	

Figure 48. *Example m/z list in Excel.*

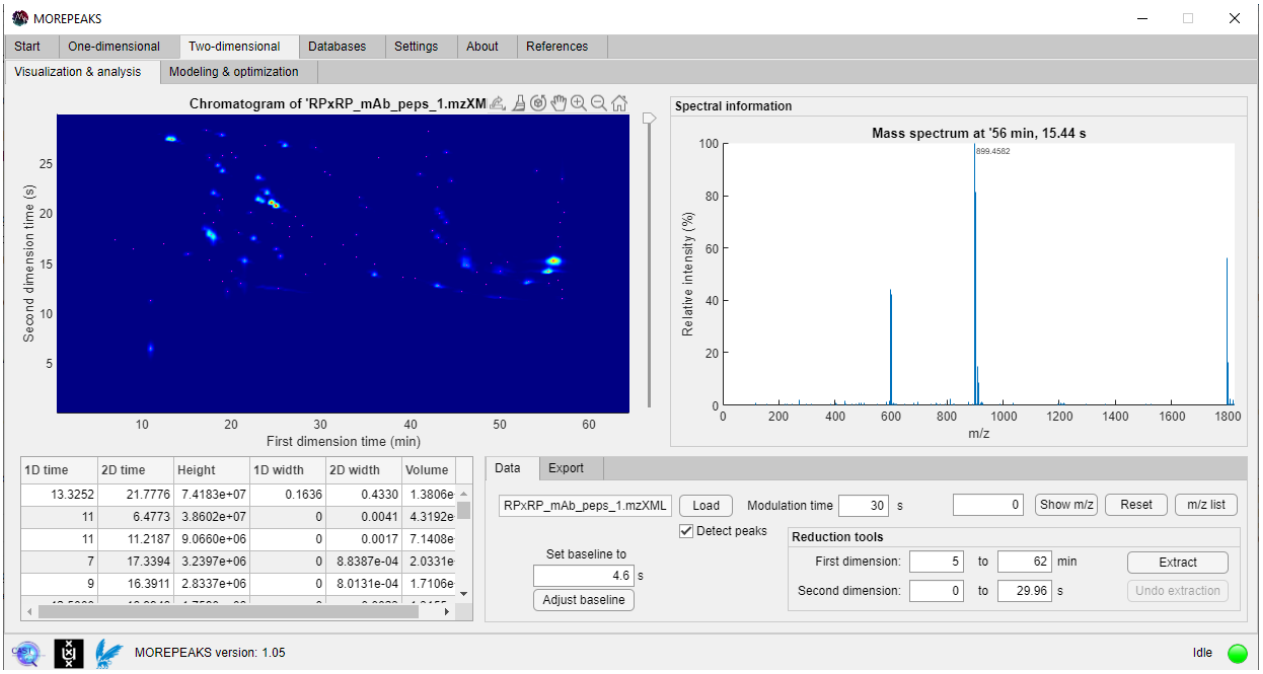

Figure 49. *MOREPEAKS showing the sum of m/z values from Figure 48.*

Part of the chromatogram can be extracted by using the "*Extraction tools*" panel. Zooming-in on the chromatogram can already be done by hovering the mouse above the chromatogram and selecting the "*Zoom*" function, but the extraction tools will also extract all peaks that are present in that part of the chromatogram and leaves all other peaks out of the peak list. The current peak list can be exported in the "*Export*" tab (Figure 50), from here the current mass spectra can be exported. Moreover, if the chromatogram is needed to create a peak list (Section 4), the chromatogram can be exported to the "*Import data*" menu from the "*Modeling & optimization*" tabs.

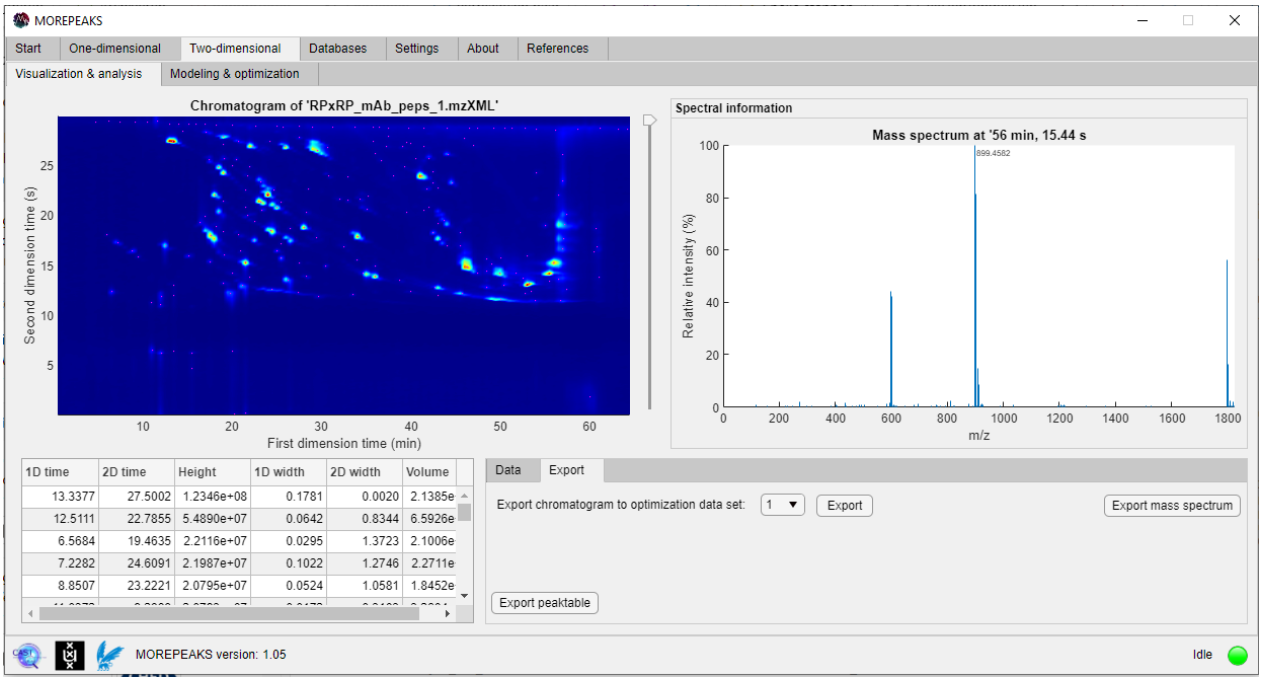

Figure 50. *Exporting in the "Visualization & Analysis" tab*

6. The settings menu

In the settings menu (Figure 51), many of the core settings of MOREPEAKS can be adjusted. All settings will be explained below.

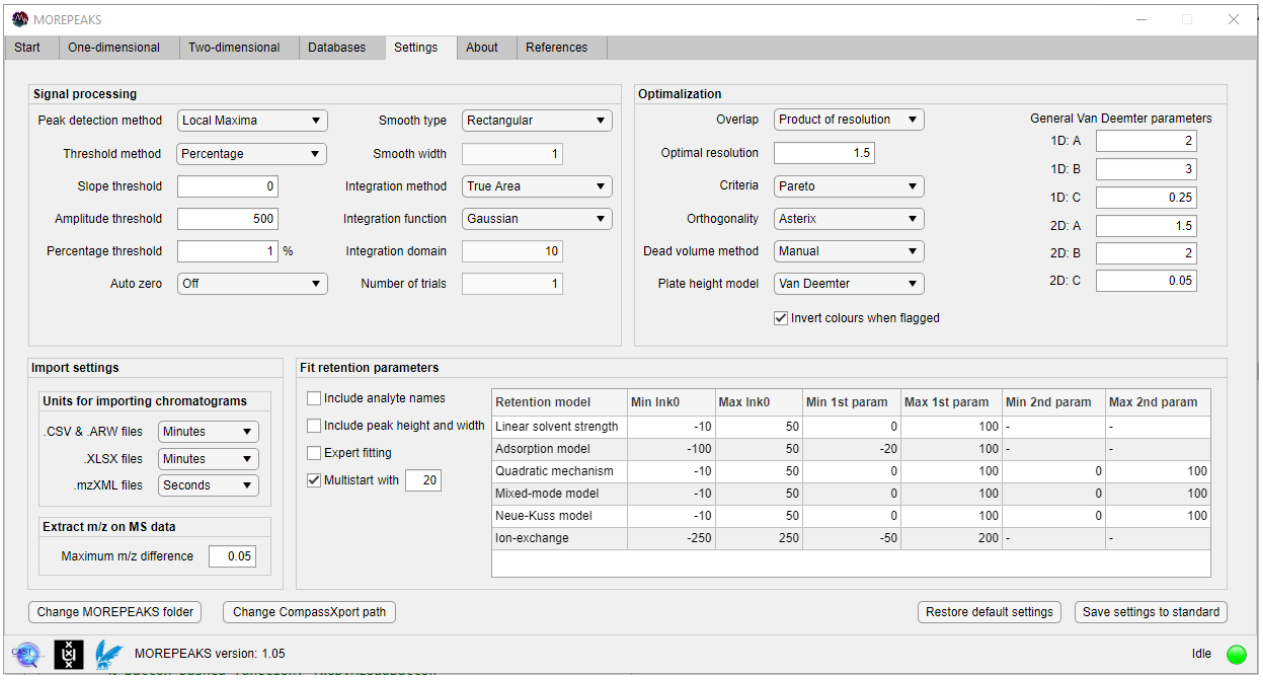

Figure 51. *The settings menu.*

6.1. Signal processing

Here many settings are given, however most of these settings will only be available in later version of MOREPEAKS, but they received a place holder here. Eventually MOREPEAKS should allow the user the choice between different peak-detection algorithms, smoothing types, integration methods etc. Currently, the only three adjustable settings are:

- **Threshold method:** Peak-detection algorithms need minimum settings, otherwise the peak-detection methods will eventually detect all noise that is present in the chromatogram. MOREPEAKS allows two different ways of setting the minimum signal-intensity threshold.
    - **Percentage:** MOREPEAKS will define the maximum signal-intensity in the chromatogram and will base the intensity threshold on a percentage from this maximum intensity.
    - **Amplitude:** When this option is selected, the intensity threshold will be set at a user-defined value.
- **Amplitude threshold:** The user-defined intensity threshold. This value will be used when "*Threshold method*" is set to "*Amplitude*".
- **Percentage threshold:** The user-defined percentage threshold. When the "*Threshold method*" is set to "*Percentage*", the intensity threshold will be determined by the given percentage of the maximum intensity.

6.2. Optimalization

Here settings about the calculation of quality descriptors are given.

- **Overlap:** This determines how the resolution score of a predicted chromatogram is given. 2D resolution is determined by the resolution metric of Schure *et al.* [17]
    - **Product of resolution:** When this mode is selected, the product of all individual resolutions between peaks is used as a quality descriptor.
    - **Sum of resolution:** When this mode is selected, the resolution score will be given by the sum of all resolutions.
    - **Average resolution:** When this mode is selected, the resolution score will be given by the sum of resolution scores divided by the number of peaks.
- **Optimal resolution:** When resolutions are calculated, all resolutions are normalized to a resolution score. This is a value between 0 and 1. All resolution are normalized to this optimal resolution value. Furthermore, all resolution values above this value will be set to this value. In principle, a resolution of 1.5 indicates baseline separation.
- **Criteria:** Determines how the optimum is being chosen. At the moment, only Pareto plots are allowed.
    - **Pareto:** Optimum is chosen by Pareto optimality plots.
- **Orthogonality:** Here the orthogonality metric for a 2D separation can be determined. More orthogonality metrics are planned for future versions.
    - **Asterix:** The resolution metric by Camenzuli & Schoenmakers [18].

- **Dead volume method:** This controls how the column dead volume is determined.
    - **Manual:** During retention modeling, it is assumed the user has provided an experimental value for the column dead volume in the column database.
- **Plate height model:** This controls how the peak width is determined.
    - **Plate number:** MOREPEAKS will estimate the plate number per analyte based on peak widths provided in the peak tables (see Section 6.4)
    - **Van Deemter:** MOREPEAKS will calculate peak widths by individual Van Deemter parameters. These parameters need to be provided per analyte in the "*Analyte database*". All peaks will receive the "*General Van Deemter parameters*" initially.
    - **M Van Deemter:** Peak widths will be determined, assuming all analytes make use of the "*General Van Deemter parameters*".
- **Invert colours when flagged:** When this option is turned on, MOREPEAKS will show flagged chromatograms in red during the "*Pareto analysis*" (Section 3.3.1.2). Otherwise, chromatograms will be shown normally.
- **General Van Deemter parameters:** These Van Deemter parameters will be given to each analyte for the determination of peak widths.

6.3. Import settings

Here the time units are given that MOREPEAKS expects when loading in certain file types and values for XIC are provided.

- **Units for importing chromatograms:** The time units that MOREPEAKS expects files to be formatting in.
- **Extract *m/z* on MS data:** This value is used as a maximum *m/z* difference when XICs are determined. If no *m/z* value can be found within this range, the value is set to 0.

6.4. Fit retention parameters

Here options can be adjusted when determining retention parameters during the import phase of an optimization.

- **Include analyte names:** When this box is checked, MOREPEAKS will assume the first column of peak tables (see Section 3.2) is the analyte name. Otherwise, the first column is assumed to the retention time of the first scanning gradient and analytes will receive a number as an identifier.
- **Include peak height and width:** This option is in the test phase. When this box is checked, MOREPEAKS will assume additional information about each peak is provided in the peak tables. First all the retention times as explained in Section 3.2, then all peak heights in the same format and than all peak widths in the same format. This option allows to have better estimates of the peak shapes when predicting. When using this option, make sure the "*Plate height model*" is set to "*Plate number*" (see Section 6.2)

- **Expert fitting:** When this box is turned on, the user is no longer limited to a maximum of four datasets when importing data from a peak table. Further information is provided in Section 7.1.
- **Multistart with [XX]:** Normally, retention parameters are estimated from one starting point. This may render the search algorithm to provide a local minimum instead of a global minimum. When this option is checked, MOREPEAKS will determine retention parameters from different starting point. The number of starting points need to be provided by the user. This will render the determination of retention models more computational heavy and therefore it will take longer to determine retention models.
- **Retention parameter table:** The table to the left provides the limits of the above explained *multistart* search.

6.5. Change path and restore/save settings buttons

These buttons will allow the user to change folder paths, save the current settings as standard and restore the default settings.

- **Change MOREPEAKS folder:** When this button is clicked, a user can change the default MOREPEAKS folder as explained in Section 2.1.
- **Change CompassXport path:** When clicking this button, the user is prompted to set the path to the CompassXport executable to be able to convert .D files to .mzXML from the MOREPEAKS main menu. More on CompassXport in Section 7.2.
- **Restore default settings:** This button will restore all default settings of the MOREPEAKS software.
- **Save settings to standard:** When this button is pressed, MOREPEAKS will save the current settings and will use these whenever the program is launched.

7. Advanced retention modeling & settings

MOREPEAKS allows for more accurate retention modeling and allows the use of CompassXport if available.

7.1. Expert fitting

If the option for "*Expert fitting*" is checked in the "*Settings*" menu (see Section 6.2), the software is no longer limited to four datasets when importing peak table (see Section 3.2). If the option is checked, and one would click "*Import & Fit*" in the "*Import data*" tabs for both 1D and 2D optimization, MOREPEAKS will prompt the user to input a number of datasets that will be loaded in (Figure 52).

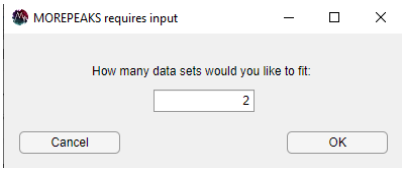

Figure 52. *MOREPEAKS requires input when "Expert fitting" is enabled.*

When a number of datasets is provided, the "*Expert fitting*" window will pop-up (Figure 53). The user is prompted to input all system settings, and the method parameters for the first dataset. In principle, all values provided here are the same as one would input in the regular "*Import data*" tab. When the user is happy about the current parameters, click "*Next data set*" in the upper-right corner.

Figure 53. *The "Expert fitting" window.*

MOREPEAKS will now lock all system parameters for the following datasets. Provide the method parameters for each (scanning) gradient separately and click "*Next data set*" when all parameters are provided. Make sure all method parameters match the acquisition parameters and the retention times for this dataset are provided in the column matching the dataset (*i.e.* the third method parameters should match the third column in the peak table). Finish providing method parameters, and after the final dataset click "Start import & fit" (Figure 54). MOREPEAKS will now determine retention parameters based on all input datasets that were provided.

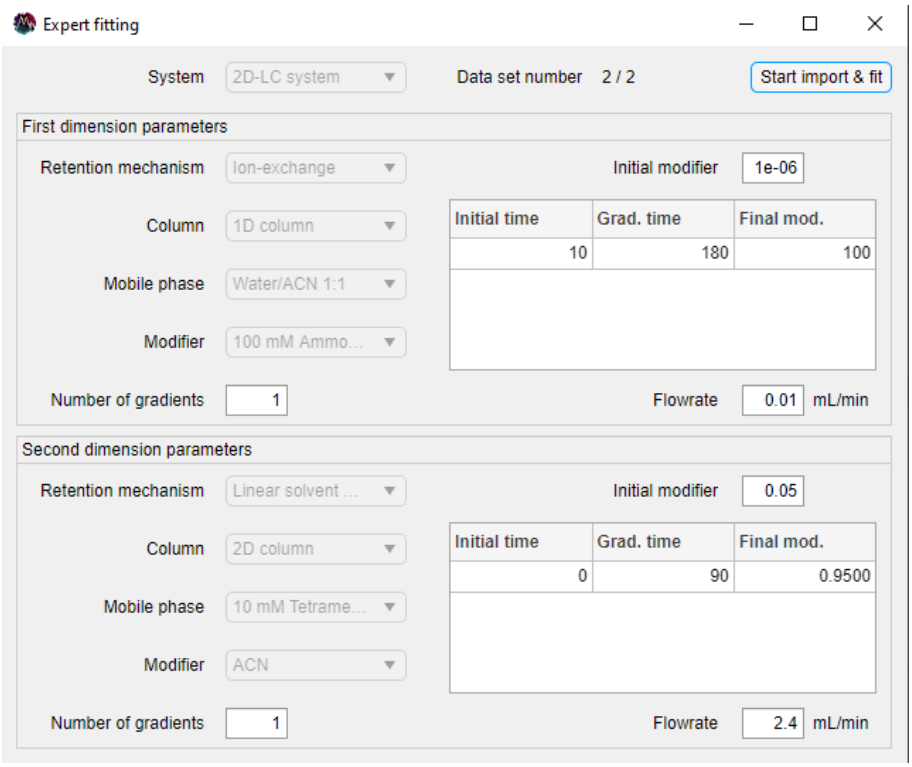

Figure 54. *Last input required during "Expert fitting". System parameters are locked.*

7.2. CompassXport

MOREPEAKS allows the use of CompassXport by Bruker*. The software is free, but needs registration. The software can be downloaded here. Using CompassXport, Bruker and Agilent mass data files can be converted to .mzXML. When installed, the path to CompassXport needs to be provided to MOREPEAKS via the "*Settings*" menu (Section 6.4). See Figure 55 for an example of the selection of the correct file. From now on the "*Convert .D files to .mzXML*" button in the "*Main menu*" can be used.

* The developers are in no way associated with Bruker and provide this as a service. We are in no way responsible for any misuse or loss/corruption of data.

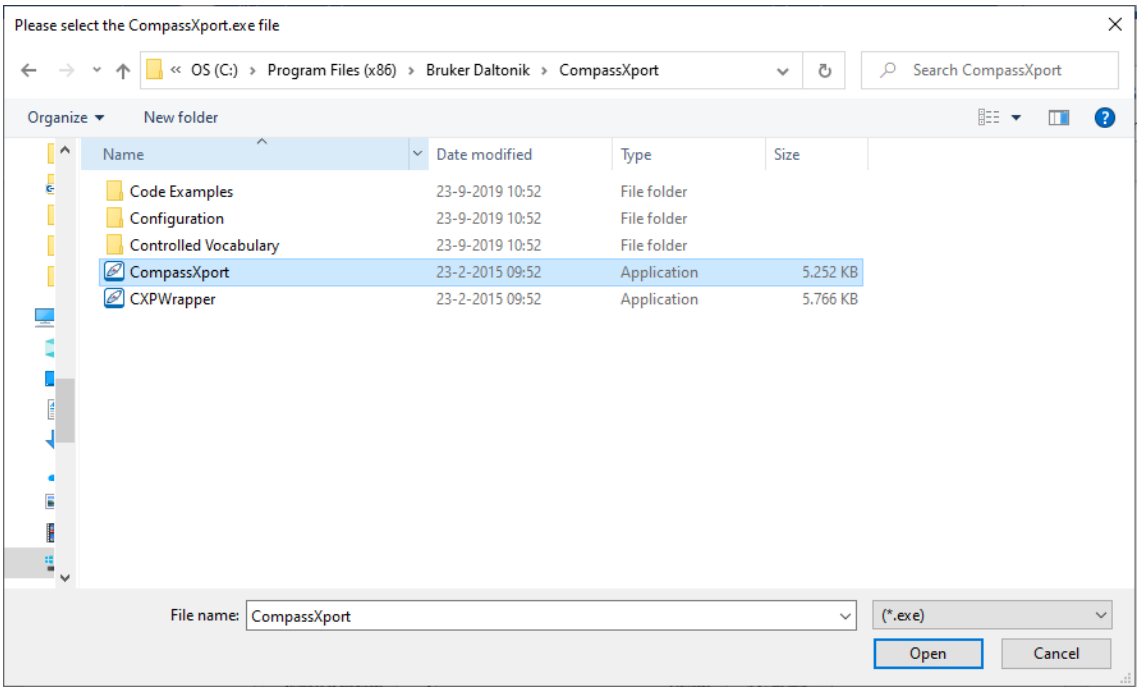

Figure 55. *Selecting the CompassXport.exe file.*

8. Recommendations from the developers

The developers have used the software themselves for the optimization of different samples by 1D [19,20] and 2D [21] LC separations and used some of the core code as example for fully automated method development [22,23].

We highly recommend to turn on "*Multistart*" (Section 6.4). This will make the determination of the retention models more accurate. And save these settings to standard.

Next to CompassXport, other free software exist to convert MS datafiles to .mzXML. One we use personally is MSConvert from ProteoWizard* [24]. MSConvert can be downloaded here. When converting .D files to .mzXML using ProteoWizard, might sure the zlib compression is turned off and Write index and TPP compatibility are turned on, else MOREPEAKS cannot read the files properly. Also, when files are becoming too large, one might consider a threshold count of about 2000-3000. This will reduce the file size, and for most purposes, more *m/z* values per time point are not needed.

Furthermore, save the database as a standard. Maybe not all systems and columns are used during all optimization protocols, but it is highly convenient to have them stored. Values can always be overwritten if needed and saved in a workspace. MOREPEAKS will always give preference to the values that are saved in the workspace over the database.

* We are in no way associated with ProteoWizard and we are in no way responsible for any misuse or loss/corruption of data.

Don’t forget to save results sets when during Pareto assessments. It is good to compare different results set to see if an optimum can be achieved in a shorter amount of time, or with a less complex gradient.

Don’t predict too many method parameters at once. The number of different combinations grows exponentially with the number of parameters and this might results in very long waiting time. Moreover, depending on the memory of the used PC, MOREPEAKS might overload it. And lastly, it is difficult to select Pareto points if the Pareto optimality plot is cluttered with thousands of different points and it might slow the software down.

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