

**Overview of the LC×LC method optimisation program  
MDMO**

## Introduction

A brief overview of the LC×LC method optimisation program is provided. The program was written in-house in a MATLAB 2019b (The Mathworks, Natick, USA) environment, and works best using this version of MATLAB. Further details on the development, application and capabilities of the program can be found in [1-4].

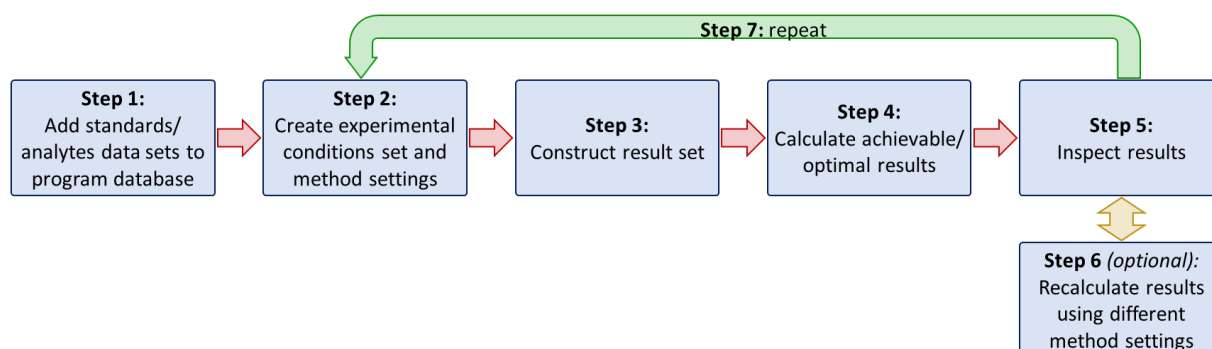
## Definitions

To simplify the program description, some concepts used by the program are first be defined:

Standards:	Compounds for which both the plate height data (reduced Van Deemter parameters and diffusion coefficient/molecular volume) and retention parameters are entered as input.
Analytes:	Sample specific compounds for which only the retention parameters are entered.
Standards set:	A group of standards.
Analytes set:	A group of analytes.
Experimental conditions set:	Contains the values (or ranges of values) of all the fixed and optimisable experimental parameters, as well as system information required to predict performance.
Permutation:	One combination of experimental parameter values.
Method settings set:	Contains settings specifying how certain values should be calculated, user defined restrictions to fine-tune the desirable results, and the optimisation objectives.
Results set:	Represents one optimisation attempt. Contains standard sets ( <sup>1</sup> D and <sup>2</sup> D), analyte sets (optional), experimental conditions set, method settings and results (if the results have been calculated already).
Results:	All the points (experimental conditions) remaining in the result set after performance was calculated. This can either be achievable results (if optimisation has not been performed yet), or optimal results.
Points:	Each point refers to one permutation with its corresponding performance values. Represents one set of experimental conditions (one analysis).
Calculate:	Process of calculating the performance for each permutation in the result set.
Optimise:	Removing points that are not optimal in terms of the optimisation objectives from the results set.
Simulate:	Process of predicting elution profiles of compounds using the algorithm designed by Stoll and co-workers [5,6].

## Program overview

The main steps of the LC×LC method optimisation program are provided in **Fig 1**. Each step will be further discussed in the following sections.



**Figure 1.** Main steps in the LC×LC method optimisation program.

### Step 1: Add standards and analytes data sets to program database

#### Standards set

Standards are used to predict kinetic performance. In the absence of measured plate height and retention values, generic values can also be entered for the standards, although this will influence the accuracy of the predictions. An example of a sample set is provided in **Fig 2**, with further details provided on the relevant parts indicated. Once created, standards sets are saved to the database of the program for future use or editing (**Fig 3**).

The screenshot shows the 'Create standard set' window with the following fields and options:

- Name of standards Set:** Phenolic RP 3
- Name of Column:** C18 Eclipse plus
- Retention model:**  Linear solvent strength,  Adsorption,  Neue-Kuss
- Temperature retention relationship:**  None (use one temperature only),  B value (van't Hoff equation),  Parameters at 2nd temperature (interpolate)
- For diffusion coefficients:**  Calculate using molecular volume,  Use diffusion coefficient value
- Buttons:** Load standards set from excel, Save standards set to excel

Identifier	Molecular Vo...	Reduced van Deemter			Retention parameters (at Temp.1)			Retention parameters (at Temp.2)			to delete				
		a	b	c	k0	S1	Temp.1 (C)	k0 (Te...	S1 (Te...	Temp.2 (C)					
1	Catachin	291.8000	1.0904	1.7642	0.1668	53.1964	24.8364	0	30	19.0402	23.1585	0	60	0	<input type="checkbox"/>
2	Vanillic Acid	174.3000	0.8544	2.6863	0.1414	41.8065	19.3678	0	30	18.8250	17.0910	0	60	0	<input type="checkbox"/>
3	Aspalathin	454.6000	0.9770	1.9613	0.1239	451.7...	28.9407	0	30	175.8...	27.8532	0	60	0	<input type="checkbox"/>

**Figure 2.** Graphical interface window used to add a standards set.

	Name	Column name	Num. of compounds	Retention model	Temperature response	Diffusion coefficient	Delete	Edit/View
1	Example Phenolic HILIC	Amide	3	Add	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
2	Example Phenolic RP LSS	C18	3	LSS	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
3	Example Phenolic RP NK	C18	3	NK	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
4	Phenolics HILIC	Amide	10	Add	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
5	Phenolic RP	C18 Eclipse plus	10	LSS	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
6	Example HILIC DF	Amide	3	Add	Temp2	DiffusionCoefficient	<input type="checkbox"/>	<input type="checkbox"/>
7	Example RP DF	C18	3	LSS	Temp2	DiffusionCoefficient	<input type="checkbox"/>	<input type="checkbox"/>
8	HILIC Phenolic 5ds	Amide	10	Add	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
9	RP Phenolic 5ds	C18	10	LSS	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
10	HILIC Phenolic 5ds Ches	Amide	5	Add	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
11	RP Phenolic 5ds Ches	C18	5	LSS	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
12	HILIC phenolic min Nar	Amide	9	Add	Temp2	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>
13	RP phenolic min Nar	C18	9	LSS	None	MolecularVolume	<input type="checkbox"/>	<input type="checkbox"/>

**Figure 3.** Example of standards sets saved in the database.

### *Analytes*

Analytes are used to optimise resolution and orthogonality for a specific sample. Analyte retention parameters must be measured, generalised values cannot be used. This is generally done using scouting gradients. In the absence of analyte sets to use, the standards will be regarded as the sample analytes. An example of an analyte set is provided in **Fig 4**. Analyte sets are saved to the database of the program for future use or editing, in the same way as standard sets.

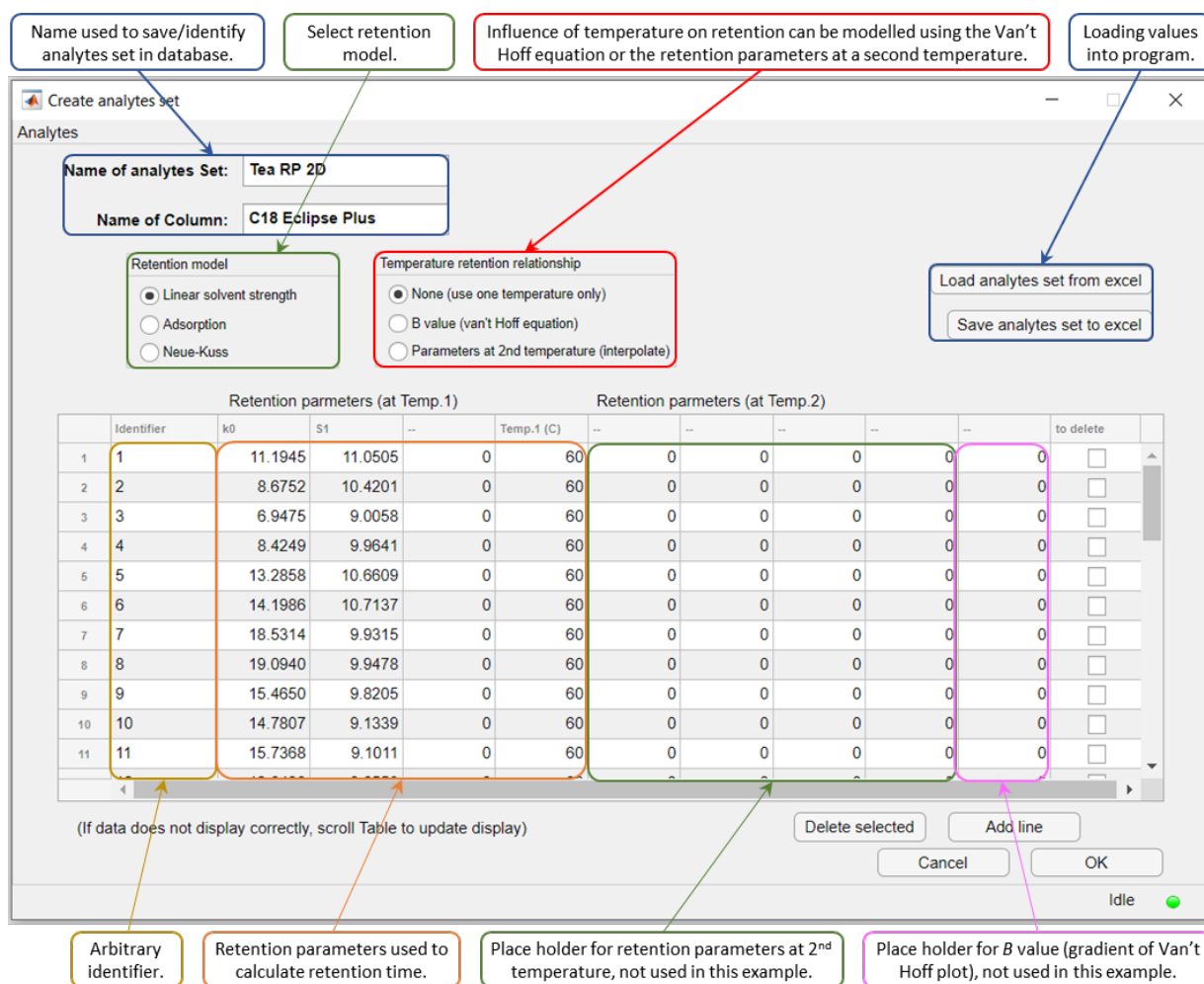


Figure 4. Graphical interface window used to add an analyte set.

## Step 2: Create experimental conditions set and method settings set

### Experimental conditions

In the experimental conditions set the values of various optimisable and fixed parameters are specified, as well as some restrictions. Apart from the standards/analytes' properties, these are all the values required to calculate performance. Conditions required include column dimensions, analysis and sampling times, flow rates, modulation parameters and gradients. Most optimisable chromatographic parameters, as well as gradient and column parameters can be varied between minimum and maximum values using user-defined step values. An example of an experimental conditions set is provided in Fig 5.

### Method settings set

In the method settings set the user can specify how certain values must be calculated and add specific restrictions to control the desired results. The optimisation objectives are also selected in the method settings set. An example of a method settings set is provided in Fig 6, along with a short description of the different features.

**MDMO app**

Main Database Optimization Modeling

Experimental conditions Method settings Generate results Display results

Name of conditions set: **Ches large test HILICxRP**  
 Description: limited diameter 1 and 3 Save to database

Select to load: RPxHILICset 1 Load

**1D 2D**

Solvent A Acetonitrile Water  
 Solvent B Water Acetonitrile  
 Gradient Gradient: 1 s... Shift: 1 step

Max 2D loop volume (uL): Set 180  
 Max inj% 2Vo: None  
 Max flow to mass sensitive detector (mL/min): None

1D Column	Min	Max	Step size	Additional discrete values	Num. of Values
Length (cm):	10	15	5		2
Diameter (mm):	1	1	0		1
Particle size (um):	1.7	1.7	0		1
Porosity:	0.626				
Resistance:	1000				
Maximum pressure:	400				

2D Column	Min	Max	Step size	Additional discrete values	Num. of Values
Length (cm):	5	5	2		1
Diameter (mm):	3	3	0		1
Particle size (um):	1.8	1.8	0	3.5	2
Porosity:	0.611				
Resistance:	1000				
Maximum pressure:	1200				

Column volumes required for re-equilibration: 1.5

**Total permutations: 8.709e+05**

Analysis time (min): 30 60 15 10 4  
 Sampling time (min): 0.2 1 0.1  
 1D eluent dilution (1: num): 4 19 5 4  
 1D eluent split (1: num): 0 1 1 2  
 1D Temperature: 30 30 0 1  
 2D Temperature: 60 60 0 1  
 1D Flow rate (uL/min): 5 35 5 7  
 2D Flow rate (mL/min): 2 3 1 2  
 2D %loopfill: 80 80 0 1  
 1D ini. volume (%1V0): 0.3 0.3 0 1  
 1D sample comp. (% B): 50  
 Concentration sample (%): 100  
 1D Dwell volume (uL): 32  
 2D Dwell volume (uL): 55  
 1D Extra column variance (uL2): 0  
 2D Extra column variance (uL2): 3.8

For Off-line and Stop-flow applications:  
 Cycle time (min): 1 1 1 1

1D gradient		2D gradient	
% time	% ss	% ss initial	% ss final
0	10	0	30:10:50
90	30:10:50	100	1:5:10 40:10:60
100	80		

Negative gradients will not be evaluated: 3 For full-in-fraction the time will be 100%: 18

1D Isocratic hold (min): 0 2D Isocratic hold (s): 0

Idle

**Annotations:**

- Name and description used to save/identify experimental conditions set in database.
- Solvent selection (used to calculate viscosity).
- Select gradient type. Options are linear or multi-step in 1D; full-in-fraction or (multi-step) shifting in 2D.
- Maximum sample loop volume (instrumental restriction).
- Maximum injection volume unto the 2D (expressed as % of void volume, used to limit injection band broadening).
- Maximum flow to detector, used to calculate split required (and effect on dilution).
- 1D and 2D column parameters. Optimisable parameters are length, diameter and particle size. Porosity is a column characteristic. Maximum pressure of column or system (whichever is lowest) is an instrumental restriction.
- Column volumes required for re-equilibration of 2D column.
- System characteristics, used in calculation of retention time and peak variance.
- Optimisable chromatographic parameters.
- Minimum value of range.
- Maximum value of range.
- Step size between values.
- Additional values (values not part of range).
- Total number of values considered in optimisation for each parameter.
- As an example: the values that will be considered for analysis time is 10, 30, 45 and 60 min.
- Dilution of 1D eluent with weak 2D solvent before the modulation valve. Expressed as: 1 part 1D eluent: x parts weak solvent.
- Splitting of 1D eluent before the modulation valve. Expressed as: 1 part 1D eluent to 2D: x parts 1D eluent to waste.
- Temperature in 1D and 2D.
- Flow rate in 1D and 2D.
- Filling percentage of sample loops.
- Injection volume in 1D. Expressed as % of 1D column void volume.
- Sample solvent composition (expressed as % strong solvent). Used to calculate injection band broadening in 1D.
- Relative sample concentration. Used to compare dilution factor between different sets of optimisation results with varying sample concentrations.
- Only applicable for off-line and stop-flow applications where the cycle time is different from the sampling time.
- Possible 1D and 2D gradients. Time is expressed as a percentage of the total gradient time.
- Number of different gradients (calculated from gradient table).
- Total number of permutations is the number of different combinations of experimental parameters for which performance must be calculated (product of the number of values of each optimisable parameter). Computational time will increase with a larger number of permutations.
- Isocratic hold time before start of gradient in 1D and 2D. Input using format: minimum value:step size:maximum value

Figure 5. Graphical interface window used to create an experimental conditions set.

**MDMO app**

Main Database Optimization Modeling

Experimental conditions Method settings Generate results Display results

Name of method settings: **Basic - example**

Description: Inj eq with no separation space restr. Save to database

Select to load: Same MS1 min 10min Load

**Method settings**

Mode: On-line

Analysis time: Retention time of last 1D peak

1D compounds elute in time: Yes

2D compounds elute in time: Yes

1D peak gradient compression: No

2D peak gradient compression: No

Correct 1D peak capacity: Last eluting peak

Correct 2D peak capacity: Last eluting peak

1D injection band-broadening: None

2D injection band-broadening: 1. Volume effect x 2ke/2kss

To calculate 2kss: 1D solvents are inverse of 2D solvents

Orthogonality: Asterisk method

Required resolution: 1.5

Variance injection profile (delta inj): 8

**Optimization objectives**

Objective 1: Analysis time

Objective 2: Peak capacity

Objective 3: Dilution factor

Objective 4: Resolution

Objective 5: None

**Advanced settings**

Minimum peak capacity: 50 Minimum analysis time: 0

Maximum dilution factor: 100 Maximum analysis time: 60

1D: First peak elute between 0 % and 100 % of analysis time window

1D: Last peak elute between 0 % and 100 % of analysis time window

2D: First peak elute between 0 % and 100 % of analysis time window

2D: Last peak elute between 0 % and 100 % of analysis time window

Maximum undersampling per peak: None

Maximum average undersampling: None

Maximum injection band broadening (% peak width): None

Reduce predicted injection bandbroadening for excessive increase: Yes

If peak width increase more than 500 % set increase percentage to 500 %

1D: Calculate analyte kinetic performance using: Ave. peak variance of st...

2D: Calculate analyte kinetic performance using: Ave. peak variance of st...

Idle

**Callouts:**

- Specify whether conditions where compounds elute after the end of analysis time (1D) and cycle time (2D) should be included in the results.
- Name and description used to save/identify method settings in database.
- Choose between on-line, off-line and stop-flow.
- Analysis time can either be total analysis time (set in experimental parameters), or elution time of last compound in 1D.
- Objectives of the method optimisation. Maximum of four objectives is recommended (only four can be visually displayed on the Pareto front). More objectives increase the number of points in the optimised results. Options are: 1) Analysis time (minimise) 2) Peak capacity (maximise) 3) Dilution factor (minimise) 4) Resolution score (maximise) 5) Orthogonality (maximise)
- Restrictions used to limit the final Pareto-optimised results to the area of interest.
- Specify separation window where first and last analytes should elute. This function is used when the user suspects the sample contains less or more retained compounds than in the analyte set. Effectively ensures separation space is available for compounds eluting before or after those used in the optimisation.
- Restriction parameters used to avoid conditions where excessive undersampling occurs. Maximum undersampling factor ( $\beta$ ) can be specified per peak, or for the average of all peaks.
- Restriction parameter used to avoid conditions where excessive injection band broadening in the 2D occur.
- Limit predicted injection band broadening to % of peak variance. Used when the injection equation overestimates injection band broadening for weakly retained peaks.
- Peak variance of analyte compounds is estimated from the standards using one of three approaches: 1) Use average peak variance of the standards compounds after inj. band broadening is calculated; 2) Use average peak variance of standards before inj. band broadening is calculated, and calculate injection band broadening for each analyte compound; 3) Use average plate count of standards and calculate peak variance for each analyte
- Value dependent on injection profile. For square injection profile, a value of  $\delta^2_{inj}=12$  is used (variance of rectangular plug), but generally a value between 4 and 8 is accepted. Used to calculate injection band broadening.
- Value is used as the desired resolution when calculating the overall resolution score using the Derringer desirability function.
- Select method to calculate orthogonality. Options are: \*1) Asterisk method \*2) Minimum convex hull
- Describes relationship between 1D and 2D mobile phases to calculate the retention factor in the sample solvent on the 2D column. Options are: 1) inverse of 2D solvents; 2) same as 2D solvents; 3) strong solvents in 2D; 4) weak solvents in 2D
- Corrects 1D and 2D injection band broadening. Options are: 1) none; 2) simulation; 3) volume effect  $\sigma^2_{inj} = (V_{inj}/(F \times \delta_{inj}))^2$ ; 4) volume effect x  $2k_e/2k_{ss}$ ;  $\sigma^2_{inj} = (V_{inj}/(F \times \delta_{inj}))^2 \times (k_e/k_{ss})^2$ ; 5) zone and step-gradient compression  $\sigma^2_{inj} = (V_{inj}/(F \times \delta_{inj}))^2 \times ((1+k_e)/(1+k_{ss}))^2 \times (1+(k_e - k_{ss})/(k_{ss} + k_{s0}))^2$  simplifies to  $\sigma^2_{inj} = (V_{inj}/(F \times \delta_{inj}))^2 \times ((1+k_e)/(1+k_{ss}))^2$  if the initial 2D mobile phase is weaker than the sample solvent
- Choose to correct peak capacity for separation space usage. Options are: 1) no correction; 2) separation space (first and last eluting peaks); 3) first eluting peak; 4) last eluting peak
- Peak variance can be calculated with or without <sup>b</sup>Poppe's gradient compression factor.

**Figure 6.** Graphical interface window used to create a method settings set.

<sup>a</sup>Symbols:  $\sigma^2_{inj}$ : peak variance caused by the injection process;  $V_{inj}$ : injection volume;  $F$ : flow rate;  $\delta_{inj}$ : standard deviation of injection plug profile;  $k_e$ : retention factor at moment of elution;  $k_{ss}$ : retention factor in sample solvent;  $k_0$ : retention factor in initial mobile phase.; <sup>b</sup>[7,8]; <sup>c</sup>Simulated according to [5,6]; <sup>d</sup>[9]; <sup>e</sup>[10].



### Step 3: Construct result set

A result set represents one optimisation attempt. A result set is constructed by selecting the relevant standards sets and analytes sets (optional), along with an experimental conditions set and method settings set (**Fig 7**). A result set can be created, calculated and optimised.

**Create result set**

Result set name: Prot HILICxRP cal inj

**Kinetic optimization**

1D standard set: Phenolics HILIC  
2D standard set: Phenolic RP

**Resolution optimization**

1D analyte set: Prot HILIC 1D  
2D analyte set: Prot RP 2D

If none is selected the standard sets will be used

**Experimental conditions and method settings:**

Conditions set: Prot large test HILICxRP  
Method settings: Basic - example

**Recalculate existing resultset using different method settings**

New result set name: Prot HILICxRP sim inj

Existing result set: Prot RPxHILIC cal

New method settings: Same MS1 sim

Select to load: Prot RPxHILIC sim

**Apply to selected to result sets in table:**

Calculate, Optimize, Calculate & optimize, Display, Delete, Save

**Current loaded result sets**

Name	Permutations	NumberResults	Calculated	Optimized	Mode	Conditions	Method	Standards1D	Standards2D	Analytes1D	Analytes2D
1 Prot RPxHILIC cal	115200	407	✓	✓	On-line	Prot large test RPxHILIC	Same MS1 res 2	RP Phenolic 5ds	HILIC Phenolic 5ds	Prot RP 1D	Prot RP 2D
2 Prot RPxHILIC sim	115200	385	✓		On-line	Prot large test RPxHILIC	Same MS1 res 2 sim 40-60	RP Phenolic 5ds	HILIC Phenolic 5ds	Prot RP 1D	Prot RP 2D

**Callout boxes:**

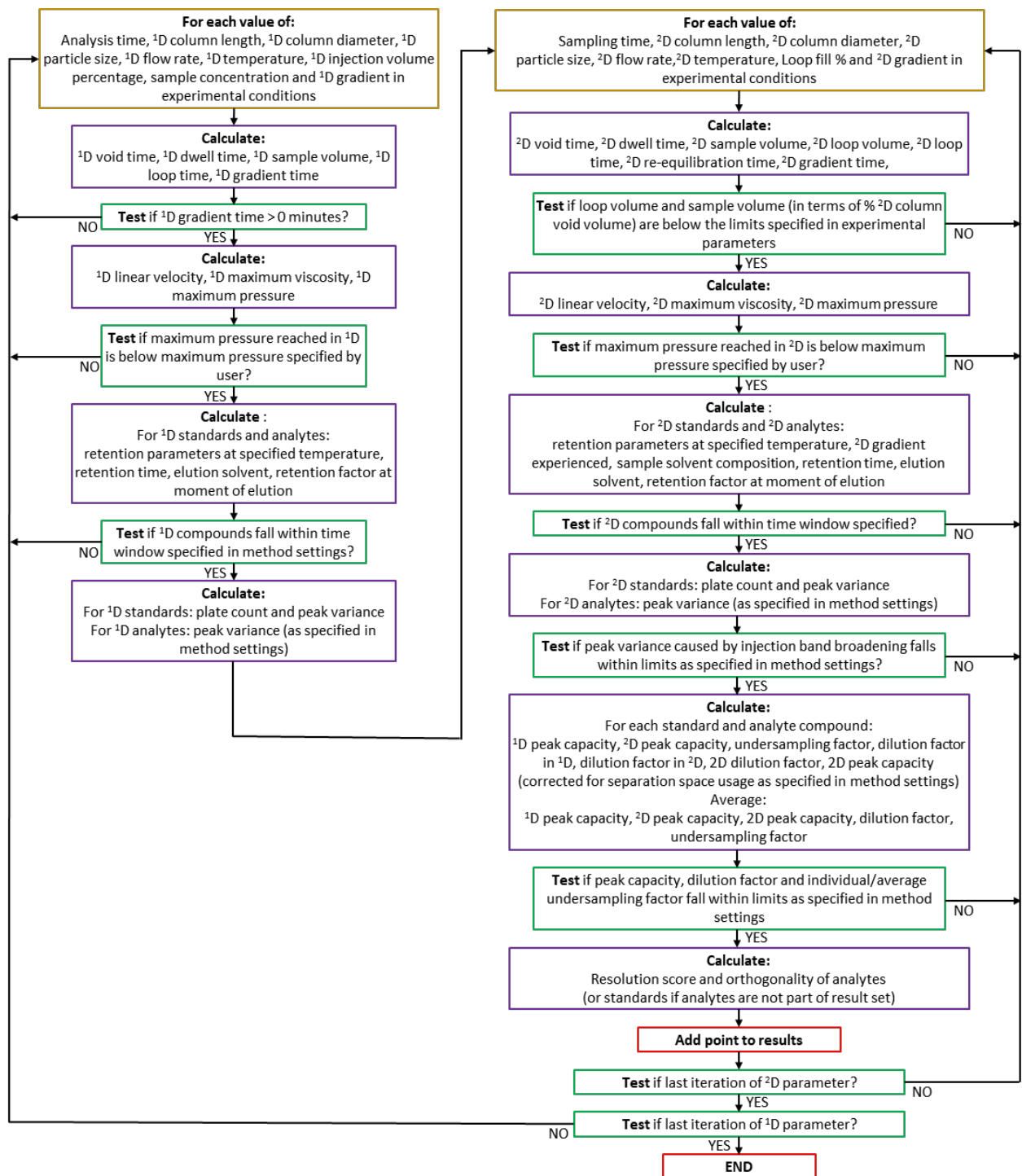
- Create new result set. Start new optimisation.
- Name used to save/identify result set in database.
- Select standards set for 1D and 2D. Should have same compounds in correct order.
- Select analytes set for 1D and 2D. Should have same compounds in correct order. Selecting analytes sets is optional. In absence of analytes sets, standards will be considered the analytes.
- Select experimental conditions set and method settings set which should be used to calculate results.
- When creating a result set there is the option of 1) just creating it (leaving calculation to a time which is more convenient); 2) creating and calculating it (end result will contain suboptimal conditions as well, can be optimised afterwards), 3) or creating, calculating and optimising it.
- Details and status of results sets currently loaded into memory.
- Total number of permutations considered in the optimisation (from experimental conditions set).
- Number of calculated results remaining in the result set. This can either be achievable results (if optimisation has not been performed yet), or optimal results.
- Indicates whether result set has been computed, or just created.
- Indicates whether result set has been optimised.
- Name of experimental conditions set, method settings set, standards sets and analytes sets used in constructing the result set.
- Step 6. The optimised results of an existing result set can be recalculated using new method settings set. Only the points remaining in the result set will be used, not the total number of permutations in the experimental conditions set.
- Name of new result set.
- Existing result set containing the points that must be recalculated.
- New method settings that must be used in recalculating the results.
- Functions that can be applied to all the selected results sets in the table. Allows user to select more than one result set for calculations and/or optimisation, which is useful for running calculation overnight.

**Figure 7.** Graphical interface window used to create and manage result sets.



#### Step 4. Calculate and optimise results

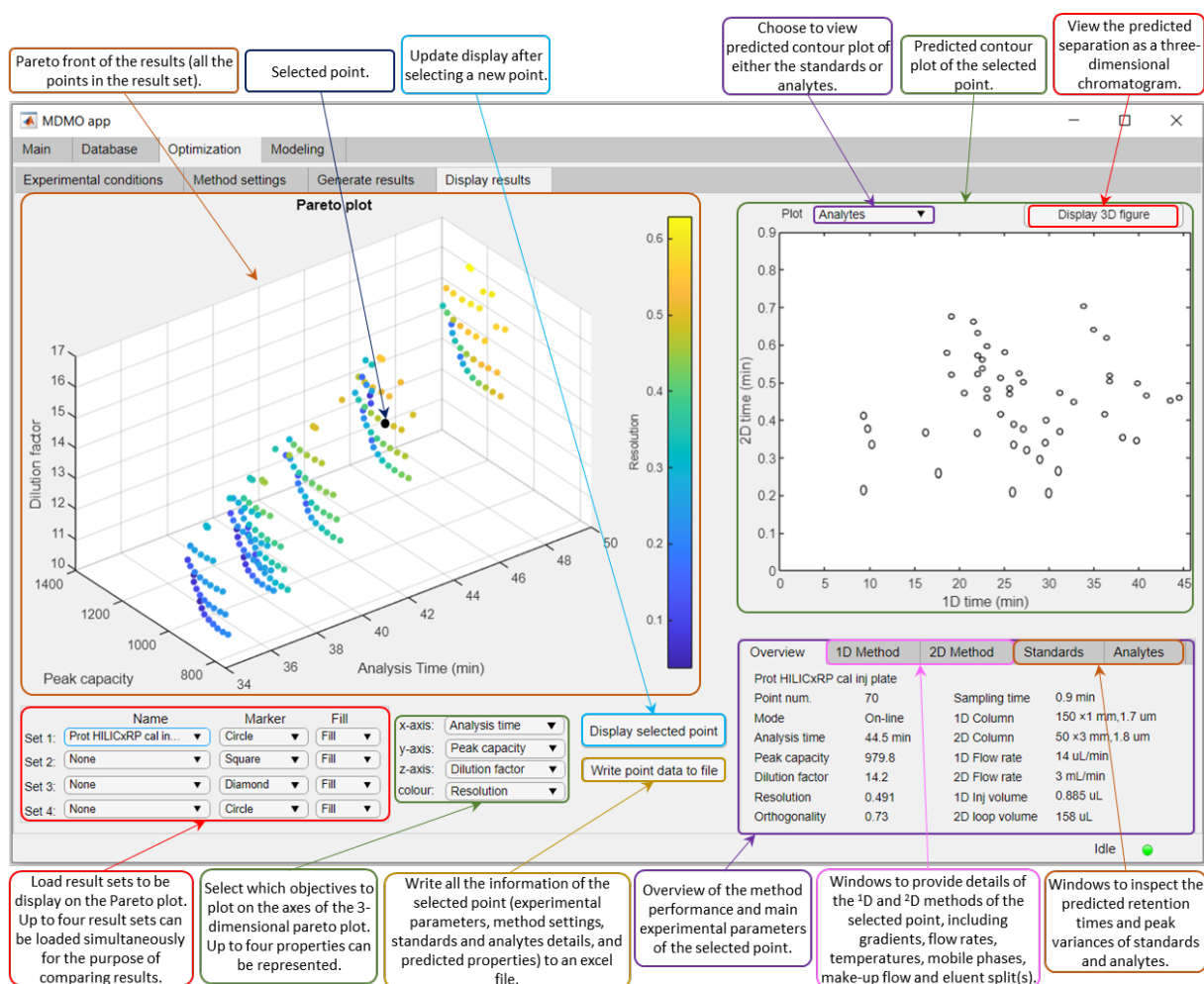
Calculation is performed using an iterative process where performance for each permutation in the experimental conditions set is calculated. The algorithm used to calculate the results is shown in **Fig 8**. After calculation is complete, multi-objective optimisation is performed by comparing the values of the optimisation objectives of each point in the results with each other point in the results. Only points that display the best performance in terms of the optimisation objectives are kept, while the points resulting in sub-optimal performance are removed from the results.



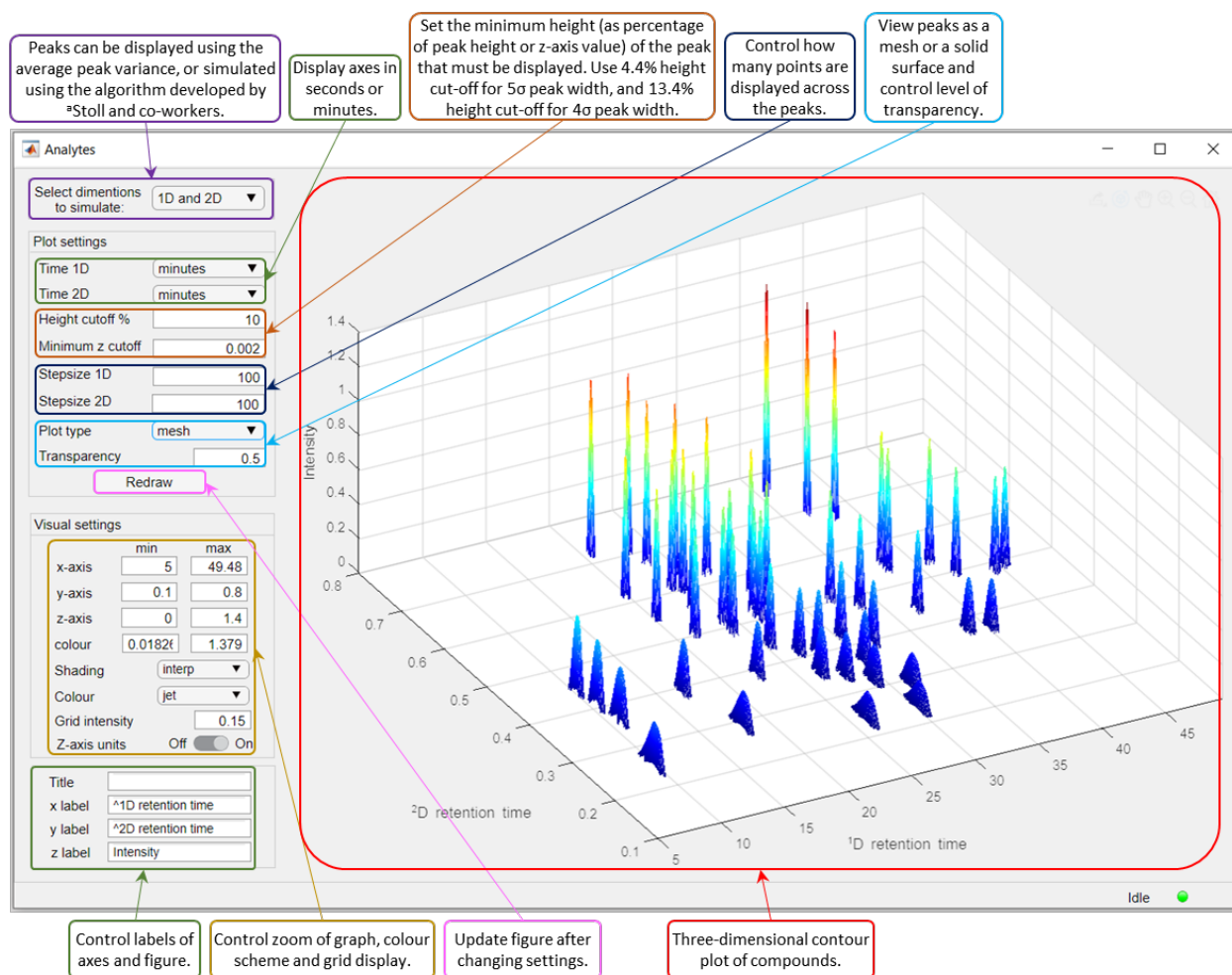
**Figure 8.** Algorithm used by program to calculate results.

## Step 5. Inspect results

After calculating and optimising the results, the points on the Pareto front can be inspected to find suitable conditions. The user can view the experimental parameters associated with each point, as well as the predicted contour plot for the standards and analytes (**Fig 9**). The predicted contour plot can also be viewed as a three-dimensional surface plot, using either the calculated peak variances, or by simulating each peak (for more accurate elution profiles). An example of such a three-dimensional surface plot is provided in **Fig 10**.



**Figure 9.** Graphical interface window used to view and inspect points on the Pareto front.



**Figure 10.** Graphical interface window to view and manage the predicted three-dimensional contour plot of the standards or analytes. <sup>a</sup>[5,6].

### **Step 6. Recalculate results using different method settings set**

After calculating and optimising the results of a result set, it is possible to recalculate the performance for each point in the result set using different method settings (**Fig 7**). Only the performance for the limited number of points remaining on the Pareto front are recalculated, not the complete set of permutations in the original experimental conditions set.

Recalculating a result set is primarily used to increase the accuracy of the injection band broadening predictions by using the simulation model (see [4]). Performing a full optimisation using the simulation model is not possible due to the high computational demand, but when recalculating a result set the number of points that must be simulated is reduced to a manageable number.

### **Step 7. Repeat**

Due to the large number of optimisable parameters, the total number of permutations in a result set can easily become excessively large. For this reason, it is advisable to start the initial optimisation attempt using broad ranges of values for the optimisable variables, but with large increments (step-

sizes) between values. This optimisation attempt will not provide the true optimum conditions, but rather an indication of the range of values which will likely result in the optimum conditions. In the next optimisation attempt, this narrower range of values with smaller increments between values can be used to get closer to the true optimal conditions. This process can be repeated until satisfactory results are obtained.

### **Summary**

The LC×LC method optimisation program is a powerful method development tool capable of simultaneously optimising most experimental parameters. The user can control how specific values are calculated and utilise a range of settings to refine the results to suit specific circumstances. The program is however currently still a research tool, and can still be improved significantly. For example, including a peak-detection and peak-tracking algorithm [11] to automatically obtain retention parameters from scouting gradients will be a great addition to the program.

## 5 References

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