

A Guide to Finding Targets that are Expected but not Observed Within a UNIFI Screening Analysis

David Eatough and Jeff Goshawk
Waters Corporation, Milford, MA, USA

INTRODUCTION

Accurate mass screening in UNIFI® Scientific Information System is performed by converting the acquired data into a list of candidate components and then matching the components to a list of targets.¹ In some instances, a target that is known to be in a sample is not observed in the processed data. Additionally large, unidentified peaks may not have an associated candidate component. These issues usually occur as a result of parameter settings in the UNIFI analysis method. This document explains the circumstances under which targets appear to be missing, or peaks appear to be undetected, and provides a series of steps that aim to resolve these issues.

FINDING A TARGET THAT HAS NOT BEEN OBSERVED IN A SAMPLE

There are a number of reasons why an expected target may not be observed in a sample:

- There is another target, an alternative assignment, that is considered to be a better match to the candidate component.
- There is a candidate component just outside m/z or retention time tolerance.
- An ion has been detected but is considered to be an isotope of another component.
- An ion has been detected and subsequently discarded.
- There is a peak in the raw data, but the ion has not been detected.

Alternative assignment – multiple targets assigned to the same component

If multiple targets are assigned to the same candidate component then either all assigned targets or just the target considered to be the best match will be displayed as Identified in the Component Summary table of a UNIFI analysis, depending on the “Alternative Assignments” setting in the analysis method. The setting for the alternative assignments is in the “Target by Mass” section of the accurate mass screening analysis method as shown in Figure 1.

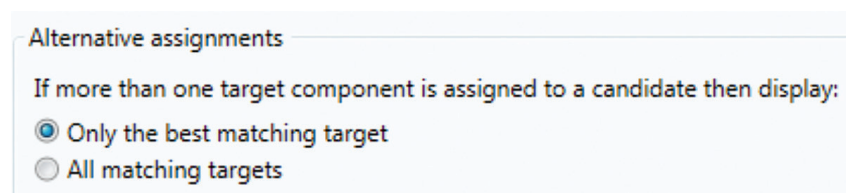


Figure 1. The Alternative Assignments options in the UNIFI Accurate Mass Screening Analysis Method.


When the “best matching target” option is selected then the other targets assigned to a component are not listed in the component summary table and may appear to be missing from the analysis.

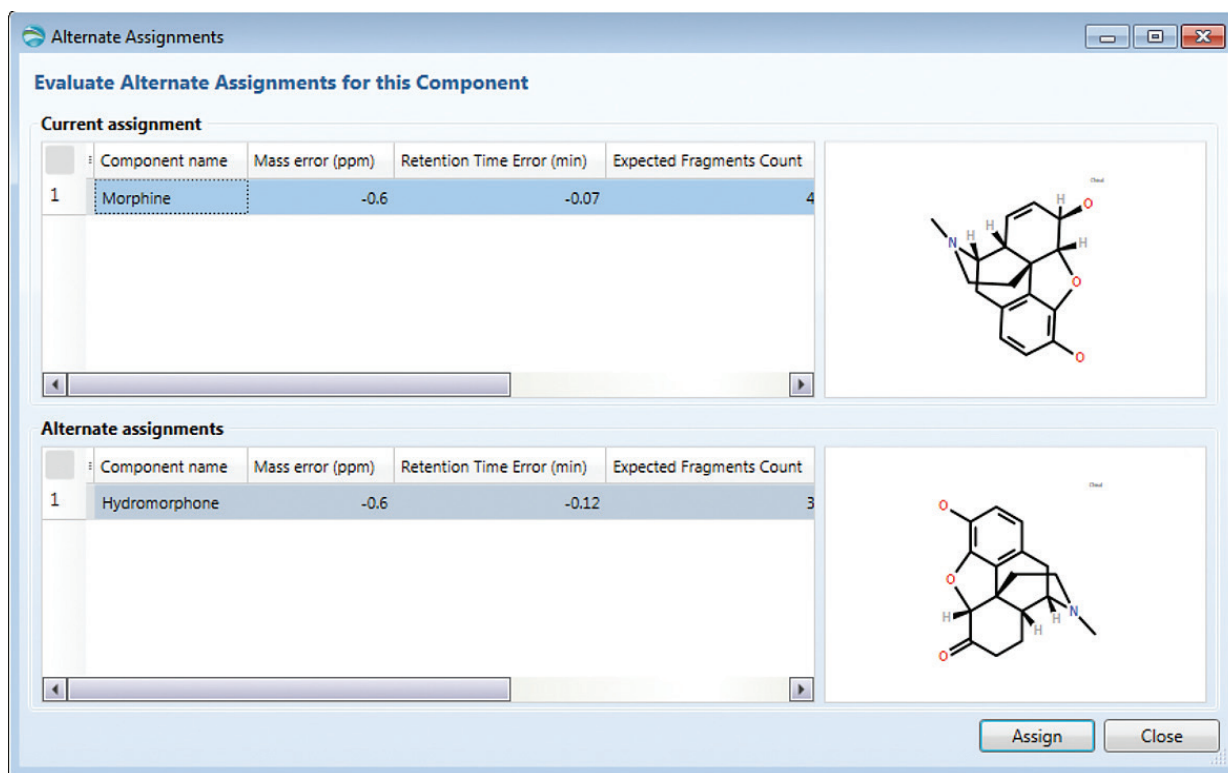
A straightforward way to determine whether an expected target has been deemed to be an alternative assignment is to apply the “Targets” filter. If the expected target is not in the list, as either Identified or Not Observed, then it is an alternative assignment to an identified component.

To show which identified components have alternative assignments, display the “Alternate assignments” column in the Component Summary table as shown in Figure 2.

Component name	Identification status	Observed m/z	Mass error (ppm)	Expected RT (min)	Observed RT (min)	Alternate assignments	Expected Fragments Found	Expected Fragments Count	F v E (%)	Response	Adducts
1 Methadone	Identified	310.2167	0.53	8.44	8.40	No	4	4	100.00	1650008	+H
2 Buprenorphine	Identified	468.3111	0.48	6.97	6.92	Yes	1	4	25.00	1255064	+H
3 Dihydrocodeine	Identified	302.1748	-0.86	1.70	1.61	No	4	4	100.00	1183109	+H
4 EDDP	Identified	278.1901	-0.66	7.29	7.29	No	3	3	100.00	1034520	+H
5 Codeine	Identified	300.1597	1.00	1.70	1.67	No	2	2	100.00	1020383	+H
6 Norbuprenorphine	Identified	414.2645	1.57	5.08	5.01	Yes	2	2	100.00	799941	+H
7 Morphine, 6 Monoacetyl	Identified	328.1545	0.50	2.10	2.04	No	4	4	100.00	719355	+H
8 Codeine, Nor	Identified	286.1436	-0.46	1.60	1.53	No	2	2	100.00	628601	+H
9 Morphine	Identified	286.1436	-0.62	1.10	1.03	Yes	4	4	100.00	427657	+H
10 Cotinine	Identified	177.1022	0.03	1.07	1.03	No	4	4	100.00	390839	+H
11 Amphetamine	Identified	136.1124	2.15	2.25	2.18	No	2	2	100.00	18898	+H

Figure 2. The Alternate Assignments column in the UNIFI component summary table.

The alternative assignments attributed to an individual component can be displayed by selecting the component in the Component Summary table and then clicking the “View alternate assignments” icon  which is located in the upper right-hand side of the frame of the Component Summary view.* This will launch the “Alternate Assignments” dialog which displays the target assigned to the component together with any other targets that satisfy the criteria to be assigned to the component (see Figure 3). Any of the targets listed in the “Alternate Assignments” dialog can be assigned to the component.



Alternate Assignments

Evaluate Alternate Assignments for this Component

Current assignment

Component name	Mass error (ppm)	Retention Time Error (min)	Expected Fragments Count
1 Morphine	-0.6	-0.07	4

Alternate assignments

Component name	Mass error (ppm)	Retention Time Error (min)	Expected Fragments Count
1 Hydromorphone	-0.6	-0.12	3

Assign Close

Figure 3. The Alternate Assignments dialog displays the target assigned to the component together with any alternative assignments.

* Prior to UNIFI v1.8.1 the tool tip was “Perform manual assignment”.

Alternative assignment – derived target assigned as an in-source fragment

In UNIFI the option to look for in-source fragments can be selected in the Target by Mass section of the analysis method, as shown in Figure 4.

If a target can be assigned to a component then that component cannot be considered as an in-source fragment of another target. However, if the target is derived, e.g. a metabolite of a target, then, if both an in-source fragment and a derived target can be assigned to the same component, the in-source fragment may take precedence. Note that at the time of writing, derived targets are only present in the Metabolite Identification analysis type in UNIFI. In future versions derived targets will be available in all screening analyses.

To determine whether an ion designated as an in-source fragment could also have been assigned as a derived target, display the “In Source Fragments” table in the Spectra window in the Review section of UNIFI and see whether the in-source fragment has an alternative assignment, as shown in Figure 5.

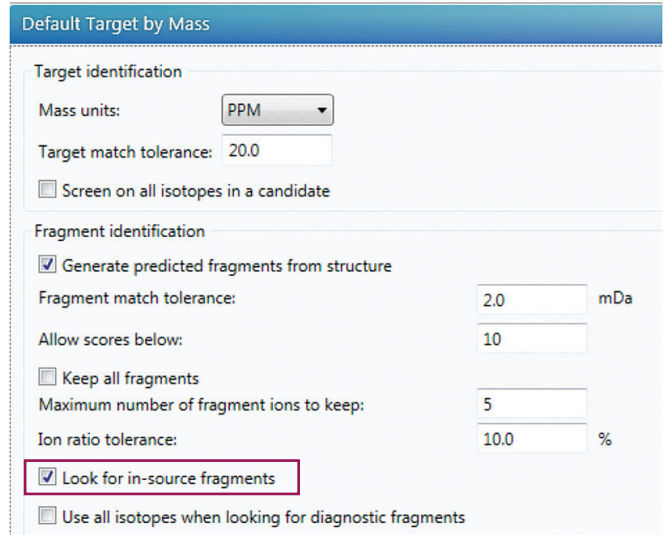


Figure 4. Option to look for in-source fragments.

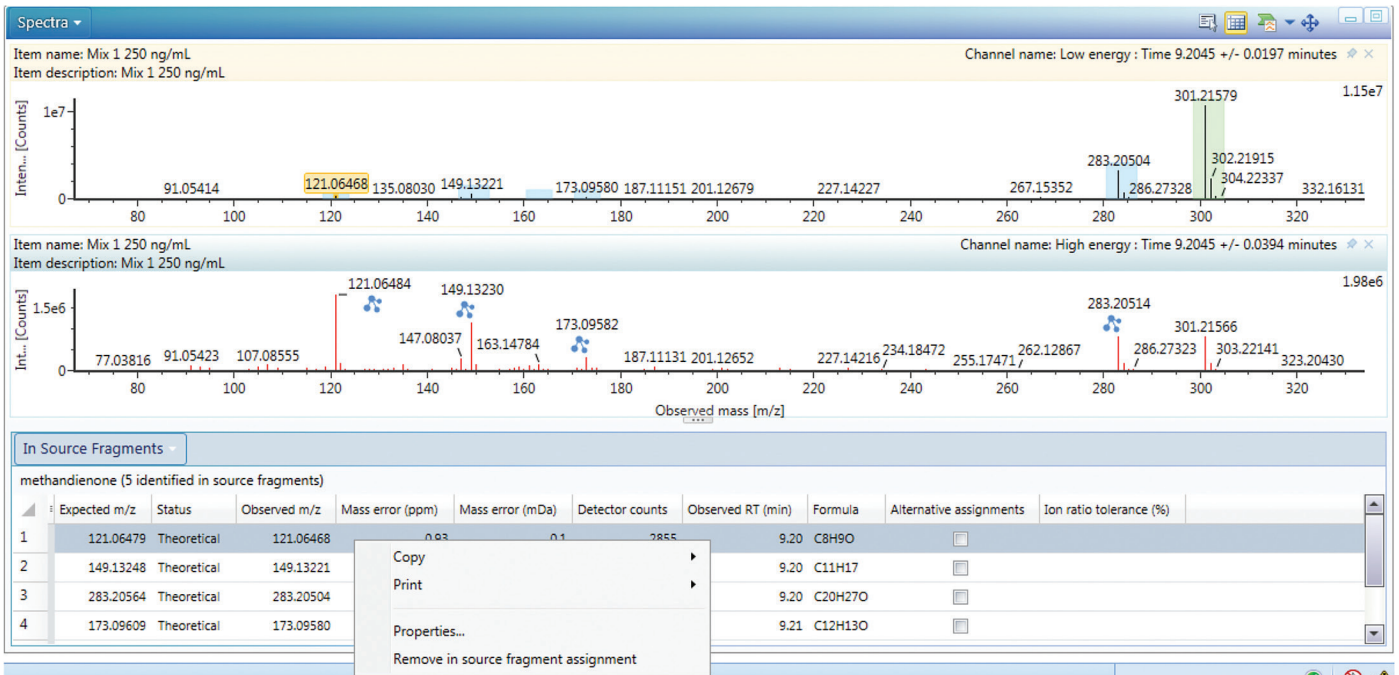


Figure 5. The “In Source Fragments” table in the Spectra window.

The in-source fragment assignment can be removed by positioning the cursor over the fragment entry in the table, clicking the right-hand mouse button and selecting “Remove in source fragment assignment” from the resulting context menu. Upon removing the in-source fragment assignment, the component will be automatically reassigned as a derived target.* If more than one derived target can be assigned to the component then the alternatives can be viewed through the Alternate Assignments dialog as discussed in the previous section.

If it is felt that in-source fragments are being assigned to components instead of derived targets too frequently, consider deselecting the option to look for in-source targets in the analysis method.

CANDIDATE COMPONENT JUST OUTSIDE TOLERANCE FOR TARGET ASSIGNMENT

To determine whether there is a candidate component just outside the m/z and/or retention time tolerance of a target, the expected retention time and m/z values of all expected adducts for the target can be manually compared to observed retention times and m/z values of the candidate components.

The candidate components can be displayed in the Component Summary table by selecting the “Candidates” filter. The candidate components can be sorted by Observed m/z or Observed RT, as appropriate, to enable the comparison to the expected retention time and m/z values of the target. If a candidate component is found that is just outside the m/z and/or retention time tolerance, it is possible the tolerance(s) are not generous enough or that there is an interference causing inaccuracy in the measurement of m/z or retention time for the target. If this is the case, then specific tolerances can be set for the target if so desired as shown in Figure 6.

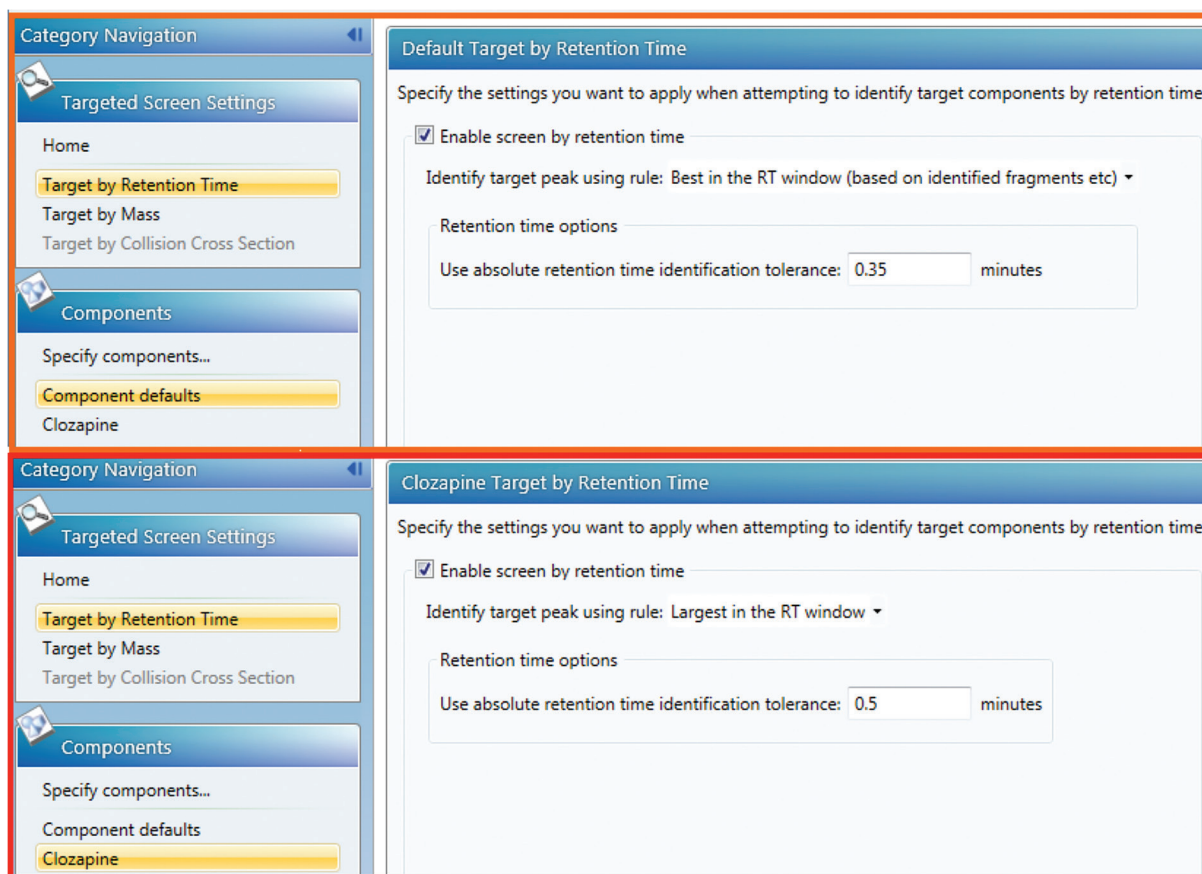


Figure 6. An example of default and target specific settings for retention time in the analysis method.

* Note that in some instances more than one in-source fragment may be assigned to a component. All in-source fragments have to be removed for the derived target(s) to be assigned to the component.

Note that in some cases it might be helpful to limit the candidates displayed to those in the vicinity of the target. This can be achieved by the construction of an appropriate filter. For example, if the adduct of an expected target has a m/z value of 301.2159 and a retention time of 9.2 minutes, then a filter such as that shown in Figure 7 would limit the candidates displayed to those close to the target.

Figure 7. A filter designed to display candidate components within 0.1 Da and 1 minute of a target with expected m/z and retention time values of 301.2159 and 9.2 respectively.

The target ion is an isotope of another component

It is possible that a target is not observed because the ion equivalent to the monoisotopic mass of the target is considered to be an isotope of a component. The parameters which control the isotope clustering in UNIFI are shown in Figure 8.

To determine whether the monoisotopic mass of the target has been considered to be an isotope of a component, examine the m/z values of the components within tolerance of the expected retention time of the target. With the settings shown in Figure 8, if there is a component within retention time tolerance and with an m/z value 0.5, 1, 1.5, 2, 3 or 4 Da lower than the expected m/z value of the target, then it is possible that the ion corresponding to the monoisotopic m/z of target has been clustered as an isotope for the component. As a result of this, a component would not have been created for the ion corresponding to the monoisotopic m/z of target. To verify whether this has happened examine the low energy spectrum of the component to determine whether it contains an ion at the correct m/z for the target.

Figure 8. 3D isotope clustering parameters.

Upon looking at the low energy spectrum, decide whether the isotope clustering has been performed correctly and if not adjust the isotope clustering parameters accordingly. One way to ensure that every ion has a corresponding component is to set the "Maximum number of isotopes per cluster" value to 1.

The component was discarded

In the "Target by Mass" section of the analysis method there are two parameters that control the number of candidate components used during certain stages of the processing, as shown in Figure 9.

Maximum candidates per sample to use during screening and discovery : 20000

Maximum candidates per sample to keep after screening and discovery : 10000

Figure 9. Parameters controlling the number of candidate components to use during screening and discovery and to retain following screening and discovery.

The "Maximum candidates per sample to use during screening and discovery" parameter controls the number of candidate components used during the screening and discovery process. The setting of 20000 shown in Figure 9 indicates that if more than 20000 candidates have been found in the sample then only the 20000 most intense will be used for screening and discovery, the lower intensity candidates are discarded. To determine whether this has occurred for any of the samples processed in an analysis, select Tools > Show processing results within the Review section of UNIFI and look for entries similar to that shown in Figure 10.

19	Warning	Oct 14, 2016 10:38:23 GDT	There were 24151 candidates to screen which is more than the maximum number 20000 specified in the method. The 4151 with the lowest response have been removed.
----	---------	---------------------------	---

Figure 10. A warning indicating a number of candidate components have been removed prior to screening and discovery.

The second parameter shown in Figure 9 controls the number of candidates to retain following screening and discovery. Using the figure of 10000 as an example, if 321 components had been identified as targets during screening they will be automatically retained together with up to 10000 candidate components making a maximum of 10321 components. Those candidate components that pass the discovery filters set in the analysis method (common fragment ions, common neutral losses, etc.) take precedence over those that do not pass the discovery filters. If more than 10000 candidates pass the discovery filters then the 10000 most intense are retained. If less than 10000 candidates pass the discovery filters then they are all retained together with the most intense candidates that have not passed a discovery filter, up to a maximum of 10000 candidate components. Any other candidate components are discarded. To determine whether this has occurred for any samples in the analysis, once again select Tools > Show processing results within the Review section of UNIFI and look for entries similar to that shown in Figure 11.


23	Info	Oct 14, 2016 10:38:24 GDT	Identified 1 target components.
24	Info	Oct 14, 2016 10:38:28 GDT	There were 20000 unidentified candidates which is more than the maximum number 10000 specified in the method. These were sorted with those past list have been removed.

Figure 11. Information indicating the number of candidate components removed following screening and discovery.

If information similar to that shown in Figures 10 and 11 is present in the processing results it may be that the candidate equivalent to an expected target was detected but subsequently discarded. Using the information in the processing results the settings shown in Figure 9 can be updated so that more, or all, candidates are retained.

The ion was discarded or not detected

To determine whether an ion was detected at the m/z value and retention time of the target, display the ions detected during 3D-peak processing in the 3D Viewer in the Investigate section of UNIFI. To select the relevant low energy data, display the 3D Viewer, click the "Select 3D data" button (highlighted in Figure 12), select the appropriate option and click "Replace". (Note, for ion mobility (IMS) data pick the low energy IMS channel).

Next, click the grid button, , to show the list of detected ions, as shown in Figure 13.

The full list of ions can be interrogated to determine the presence or absence of an ion corresponding to that of the expected target. Alternatively, a filter can be set up by clicking the down arrow to the right of the filter icon on the right-hand side of the peak list, as shown in Figure 14.

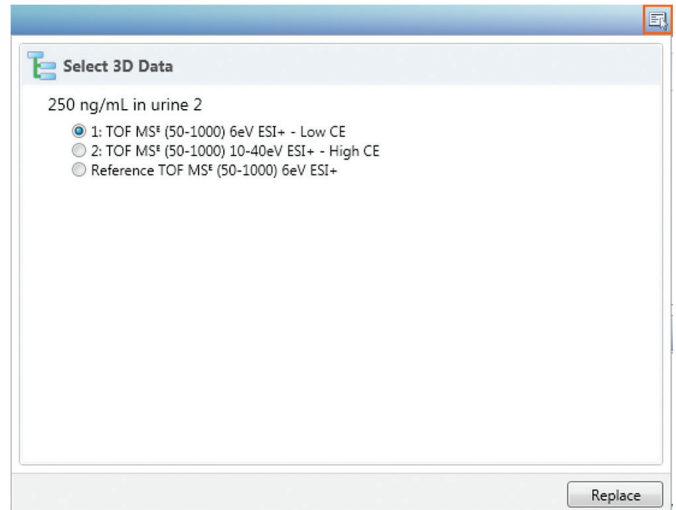


Figure 12. Selecting 3D data for the 3D viewer.

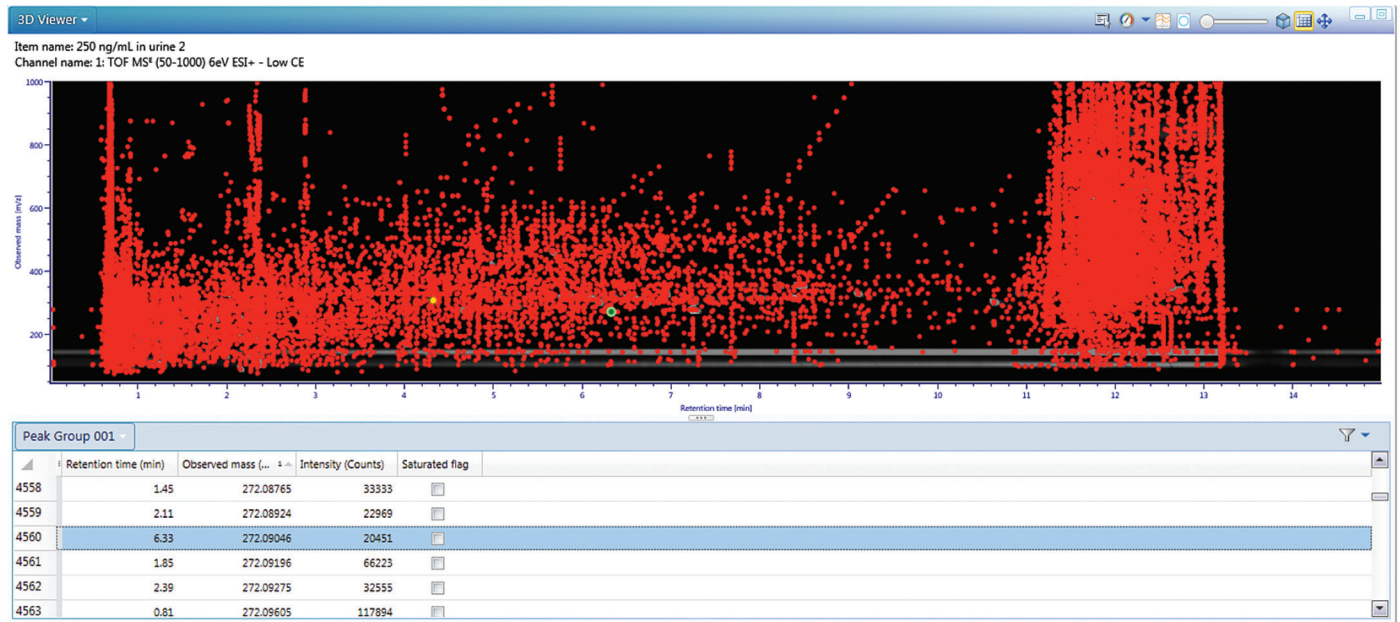


Figure 13. The detected ions as shown in the 3D viewer.

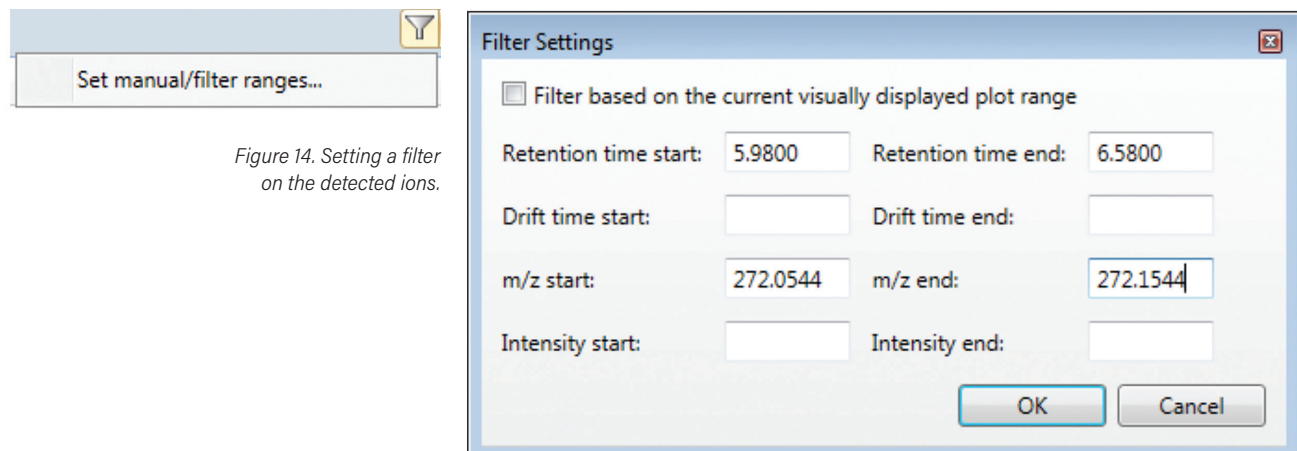


Figure 14. Setting a filter on the detected ions.

Examine the *m/z* values and retention times of the ions in the peak list and/or zoom into the relevant region in the 3D viewer in order to determine the presence or absence of a particular ion. If there is an ion present then the target is not being displayed as an identified component due to one of the reasons already explored above.

If there is not an ion corresponding to the target this could be due to one of several reasons.

1. The ion was detected but then discarded because the number of ions detected exceeded the number to retain as set in on the "Find 3D Peaks" section of the analysis method, see Figure 15.

To determine whether this is the case, select Tools > Show processing results from the Review section of UNIFI and determine whether there are any warnings similar to those displayed in Figure 16.

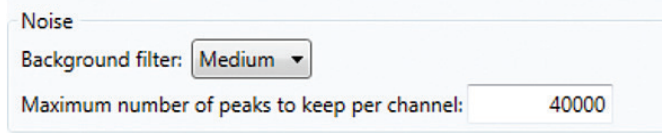


Figure 15. Setting in the Find 3D Peaks section of the analysis method that controls the number of ions retained following peak detection.

If such warnings are present it could be that the target ion was one of the smaller ions removed. To verify this, change the "Maximum number of peaks to keep per channel" to a value at least equal to the size of the largest number of low energy peaks detected so as not to discard any low energy ions. For example, looking at the processing results in Figure 16 a value of 271123 would ensure that no ions are discarded from the processed sample.

Processing Results			
	Level	Date/Time	Message
1			----- PLG Log Entries -----
2	Info	Oct 14, 2016 10:28:34 GDT	About to run the PLG on the 'Wash' sample.
3	Info	Oct 14, 2016 10:28:34 GDT	About to do lock mass processing on the 'Reference TOF MS* (50-1000) 6eV ESI+' channel.
4	Debug	Oct 14, 2016 10:28:35 GDT	Created a lock mass reference table with 14 entries.
5	Debug	Oct 14, 2016 10:28:59 GDT	Using a chromatographic peak width of 0.0420 minutes.
6	Debug	Oct 14, 2016 10:28:59 GDT	Using MS resolution of 26997.7.
7	Warning	Oct 14, 2016 10:30:13 GDT	Reduced the number of peaks from 220986 to the 40000 most intense peaks as specified in the method.
8	Debug	Oct 14, 2016 10:30:13 GDT	Detected 40000 peaks in channel 1: TOF MS* (50-1000) 6eV ESI+ - Low CE. Some of these may not pass the final quality filters.
9	Warning	Oct 14, 2016 10:32:24 GDT	Reduced the number of peaks from 271123 to the 40000 most intense peaks as specified in the method.
10	Debug	Oct 14, 2016 10:32:24 GDT	Detected 40000 peaks in channel 2: TOF MS* (50-1000) 10-40eV ESI+ - High CE. Some of these may not pass the final quality filters.
11	Debug	Oct 14, 2016 10:34:28 GDT	The lock mass correction will be applied.

Figure 16. Warnings indicating that some of the detected ions have been discarded.

- If no peaks are being discarded then it is possible that there is an interference that is causing the peak of interest to be missed by the 3D peak detection algorithm. If, upon looking at the data in the 3D Viewer, this appears to be the case, consider setting manual peak widths to about half the apparent value of the full width of the peak at half the peak height and reprocessing.
- If, after reprocessing (peak detection and full application processing), the target is still not found, then try changing the value of the "Background filter" (see Figure 15) to a less severe setting. The background filter attempts to find ions that have a response across the chromatographic run that does not vary significantly, and remove them so they are not mistaken for peaks. The amount of variation permitted for a background ion depends upon the severity of the background filter with the "high" setting removing the most ions.
- Finally, if the target is suspected to be present at a very low level and the low energy intensity threshold in the "Find 3D Peaks" section of the analysis method, as shown in Figure 17, is above the minimum of 5 counts, the value can be reduced to try and detect the ion of interest.

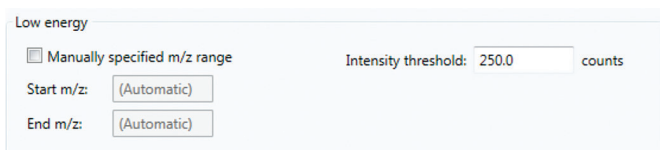


Figure 17. The intensity threshold setting for the low energy data.

Is there a peak in the raw data?

If, after pursuing the steps above, an expected target remains elusive, the raw data can be interrogated through extracting mass chromatograms and/or examining the 3D data, to establish whether or not there is a peak in the data that is consistent with the target ion.

Within the Investigate section of UNIFI, select the "Targets" filter followed by the expected target which should appear in the Components list with a "Not Observed", -, status. If the target of interest is not present in the list then it is an alternative assignment to a component (see the alternative assignments section in this document).

St...	Name
4	Clozapine
5	Milnacipran
6	Nicotine
7	Perphenazine
8	Scopolamine
9	Tianeptine
10	Triprolidine

Figure 18. Unobserved targets in the component list in the Investigate section of UNIFI.

Select the target for which a chromatogram is to be extracted and click the right-hand mouse button. From the context menu that appears, select the name of the target followed by the option to "Extract mass chromatograms for expected mass with adducts", as shown in Figure 19.

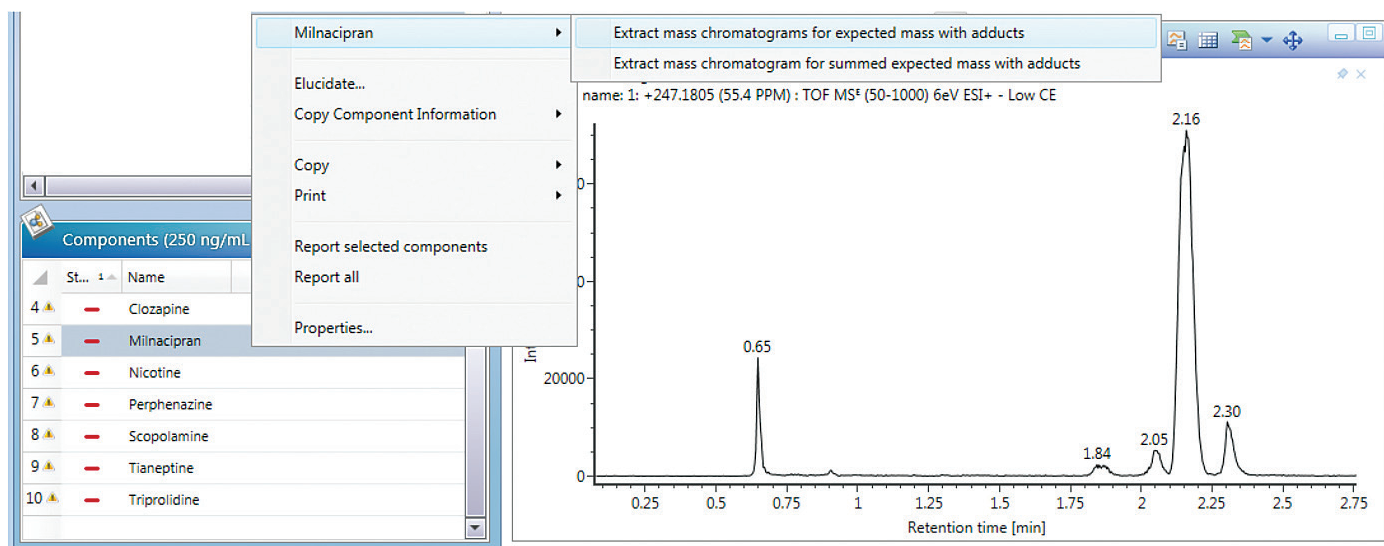


Figure 19. Extracting mass chromatograms for a target from the component list in the Investigate section of UNIFI.

If there is not a peak on the chromatogram at the expected retention time, then the instrument on which the data has been acquired is unable to measure enough ions to detect the target. If there is a peak on the chromatogram then the 3D peak detection algorithm has not detected the peak either due to an interfering ion or because the peak on the mass chromatogram does not correspond to the summit of the peak in 3D-dimensions.¹

To determine why the peak shown on the mass chromatogram has not been detected, display the low energy data in the 3D viewer in the Investigate section of UNIFI. Zoom into the appropriate region of the data and look at the data in both map and perspective mode to try and determine whether a peak should have been detected by the 3D-peak detection algorithm.

If it is concluded that a peak present in the data has not been detected by the software this information should be provided to your Waters representative together with the supporting data. This will enable either an explanation to be provided or improvements to the 3D-peak detection algorithm to be made.

References

1. Goshawk JA, Eatough D, and Wood M, Componentization Following 3D-Peak Detection in the UNIFI Scientific Information System. (p/n [720005480EN](#)).

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters, The Science of What's Possible, and UNIFI are registered trademarks of Waters Corporation. All other trademarks are the property of their respective owners.

©2016 Waters Corporation. Produced in the U.S.A. December 2016 720005818EN LM-PDF

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com