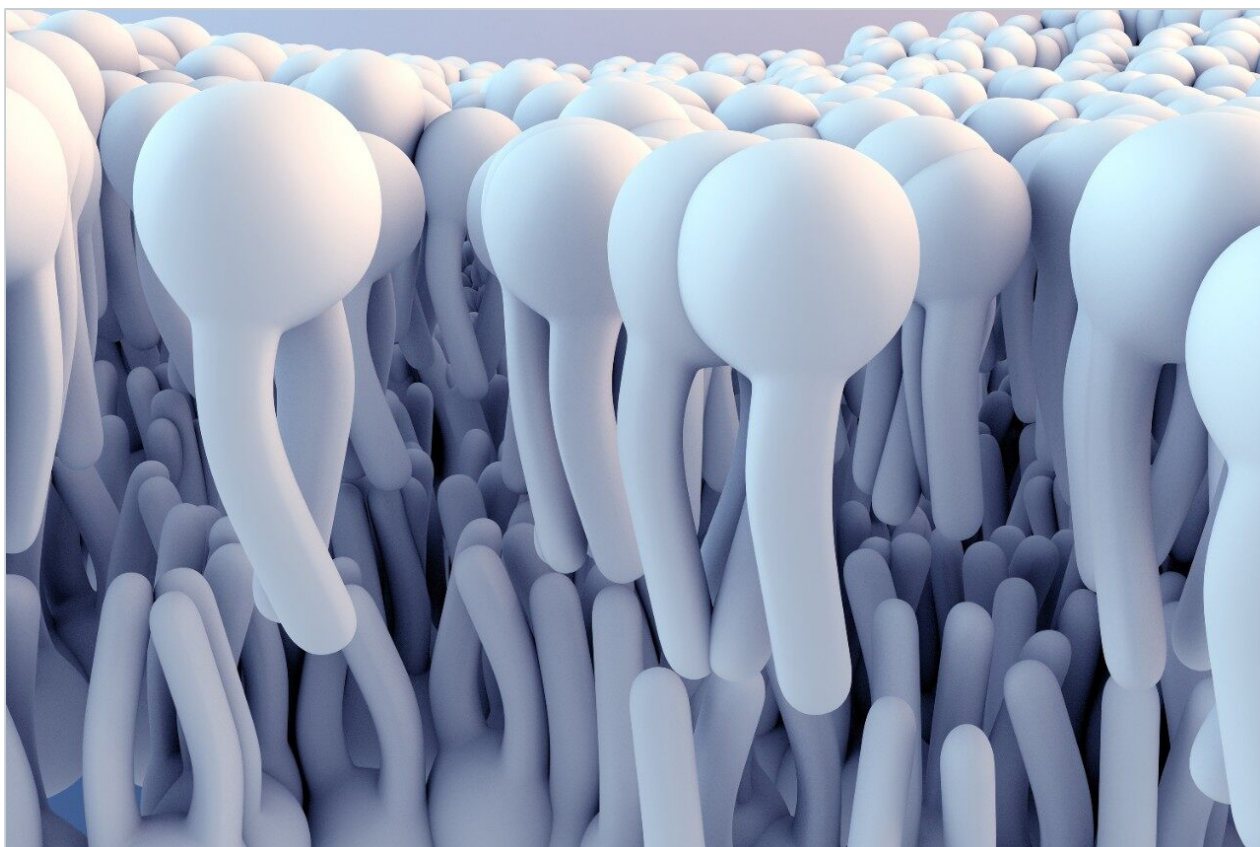


Nota applicativa

Lipid Analysis Using the ACQUITY RDa Detector with SmartMS Technology and UNIFI Application

Robert S. Plumb

Waters Corporation



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Abstract

The ACQUITY RDa Detector combined with ACQUITY UPLC I-Class PLUS, the waters_connect Software platform, and UNIFI Application facilitates detection and confirmation of lipids in large cohort studies. The mass accuracy, stability, and reproducibility of the ACQUITY RDa Detector along with intelligent library screening simplifies routine operation with clearly displayed results thus enabling operation by a range of users and facilitating rapid decision making and lipid confirmation.

Callout Statement

Demonstrating the use of the ACQUITY RDa Detector for the robust reproducible analysis of target lipids in biological fluids.

Introduction

The “Omics” analysis of large-scale population studies such as epidemiological, biobanking, translational medicine etc, has increased over recent years with the advent of techniques such as high throughput gene sequencing and metabolomics.^{1,2} These sample sets can range from several hundred to several thousand in size and require high throughput, reproducible data to aid understanding of such diverse areas as; disease progression, population health, age, gender, environmental, and dietary effects.³ More recently, lipidomics has shown great promise in the understanding of diseases such as cancer and diabetes and is being employed as a front-line omics tool in these large studies.^{4,5}

The detection, identification, and categorization of lipid(s) of biological interest is often a time consuming and challenging process requiring LC separation, HRMS-MS/MS data, and statistical analysis to fully characterize the sample – especially if complex isomer analysis is required. Once a potential biological marker of disease has been hypothesized and identified a focused analysis for specific lipids of interest or class, changes can be performed to test the validity of the biomedical hypothesis. This lipidomic analysis of blood and tissue samples from large cohort studies requires a robust, rapid, and simple LC-MS platform for the detection and confirmation these investigative lipids in the samples.

The ACQUITY RDa Detector is a high-resolution time-of-flight (ToF) mass analyzer, combining mass accuracy with robustness, reproducibility, and simple operation. When combined with the ACQUITY UPLC,

the ACQUITY RDa Detector simplifies the analysis of lipidomics samples. Here we highlight the capability of the system with UNIFI Application for the analysis of lipids in large biological data sets.

Experimental

Sample Preparation

The Avanti Lipidomics SPLASH mix was diluted 1:50 in IPA and vortex mixed prior to injection. The LC-MS analysis was performed on the commercially available Avanti Lipids SPLASH mix and NIST Plasma. Plasma preparation was by a simple protein precipitation using ice cold IPA (20 μ L of plasma diluted with 600 μ L IPA and 200 μ L diluted SPLASH mix), vortex mixed, placed at 2–8 °C for 2 hours, and centrifuged to remove particulates. No additional clean up was performed.

Method Conditions

The LC-MS was performed on an ACQUITY UPLC I-Class PLUS connected to an ACQUITY RDa Detector, the data analysis was performed using the software platform waters_connect with UNIFI Application.

LC Conditions

LC system(s):	ACQUITY UPLC I-Class PLUS
Column(s):	ACQUITY UPLC CSH C ₁₈ 1.7 μ m (2.1 x 100 mm)
Column temp.:	55 °C
Sample temp.:	8 °C
Flow rate:	0.4 mL/min
Mobile phase A:	60:40 ACN:Water, 0.1% formic acid 10mM ammonium formate
Mobile phase B:	90:10 IPA:ACN, 0.1% formic acid

Injection volume:

1 μ L

Gradient Profile for both 12-minute and 20-minute Methods

Time (min)	Buffer A (%)	Buffer B (%)
Initial	50	50
0.5	47	53
4.0	45	55
7.0	35	65
7.5	20	80
10.0–11.0	1	99
11.1–12.0	50	50

Time (min)	Buffer A (%)	Buffer B (%)
Initial	60	40
2.0	57	43
2.1	50	50
12.0	46	54
12.1	30	70
18.0	1	99
18.1–20	60	40

MS Conditions

MS system:	ACQUITY RDa Detector
Ionization mode:	Positive ion electrospray and negative ion electrospray
Acquisition range:	100–800 <i>m/z</i>
Capillary voltage:	3 kV

The ACQUITY RDa Detector settings were default, with fragmentation energy set to a custom value of 150–170 v.

Data Management

MS software: waters_connect

Informatics: waters_connect

Results and Discussion

The most common modes of chromatographic analysis of lipids in blood products, such as serum and plasma, are reversed-phase LC or HILIC. With reversed-phase LC more polar lipid elute at the beginning of the chromatogram and the least polar at the end, while with HILIC analysis the lipids are eluted from the column by class with the least polar lipids eluting first. The extra chromatographic resolution provided by reversed-phase LC makes it the ideal platform profiling of lipid in biofluids. Two different reversed-phase methods were employed in this study, a 12-minute and 20-minute separation, the first being a faster screening method and the second being a higher resolution LC method for characterization. The data displayed in Figure 1 shows the profile observed from an injection of the NIST commercially available plasma using the 12-minute method, the top LC-MS chromatogram shows positive ion mode acquisition, and the bottom BPI shows negative ion mode acquisition. The LPC, LPE, and FFA lipids eluted between 0.75 and 1.7-minutes with the FFA showing a greater response in negative ion mode, the PI, PE, PG, PS, PC, and Cer lipids eluted between 2.5 and 5.5-minutes with a stronger response being observed in positive ion mode, the SM and DG's eluted between 6.5 and 8-minutes with the DG's again showing a greater response in positive ion mode, and finally the TAG and CE, being the most lipophilic eluted at the end of the analysis between 8.5 and 9.5-minutes were exclusively detected in positive ion mode.

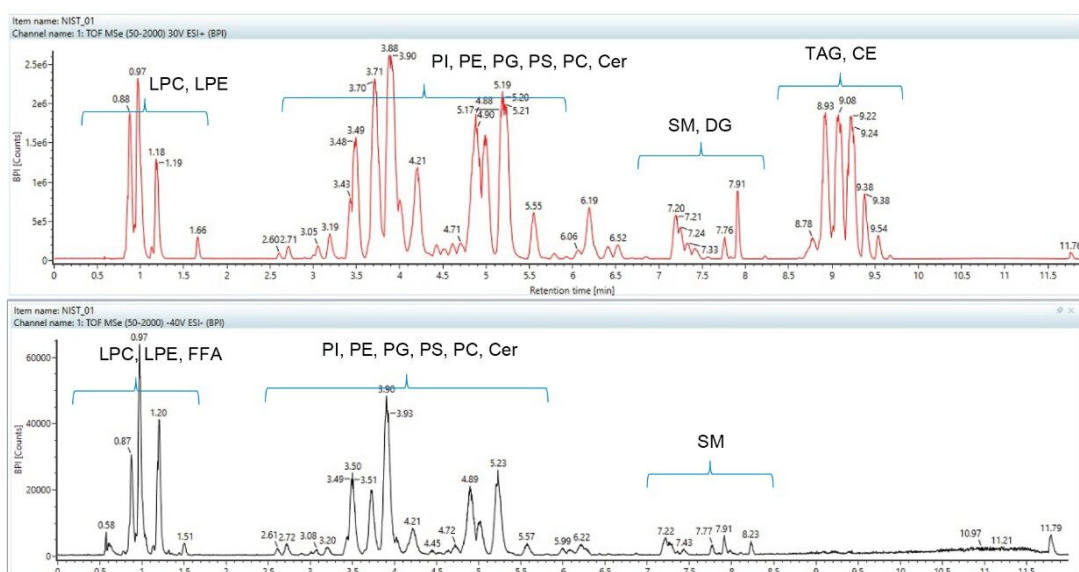


Figure 1. A representative positive ionization mode BPI (top/red), and negative ionisation mode BPI (bottom/black) for the NIST plasma sample injected on the ACQUITY RDa Mass Detector.

Robustness and Reproducibility

The reproducibility of the ACQUITY RDa Detector UPLC-MS system was determined by the 400 repeat injections of the extracted NIST plasma spiked with SPLASH lipidomix was analysed, this test was performed using the 20-minute method giving a total continuous analysis time of 6 days. The data displayed in Figure 2 shows the reproducibility of the system for three representative endogenous lipids along with their respective internal standards, one eluting at the beginning (LPC 16:0 tR 1.1-minutes), one mid (PC 34:1 tR 5.8-minutes), and one eluting at the end of the gradient (TAG 52:4 tR 15.4 minutes). The retention time reproducibility RSD of the three lipids and internal standards was 0.77%, 0.81%, 0.52%, and 0.56%, 0.07%, and 0.09% for the LPC, LPC Istd d7 LPC, PC, IStd d7 PC, TAG, and IStd d7 TAG respectively. Over the 400 injections the LPC retention time varied by 0.1-minutes over the entire analysis, the PC retention varied by only 0.15-minutes over the entire analysis and the TAG retention varied by less than 0.1-minutes over the entire analysis. The MS signal response over the 400 injection analysis showed signal variance (RSD) of 3.2%, 4.1%, 4.1%, 4.7%, 5.5%, and 9.8% for LPC and IStd d7 LPC, PC, IStd d7 PC TAG, and IStd d7 TAG respectively.

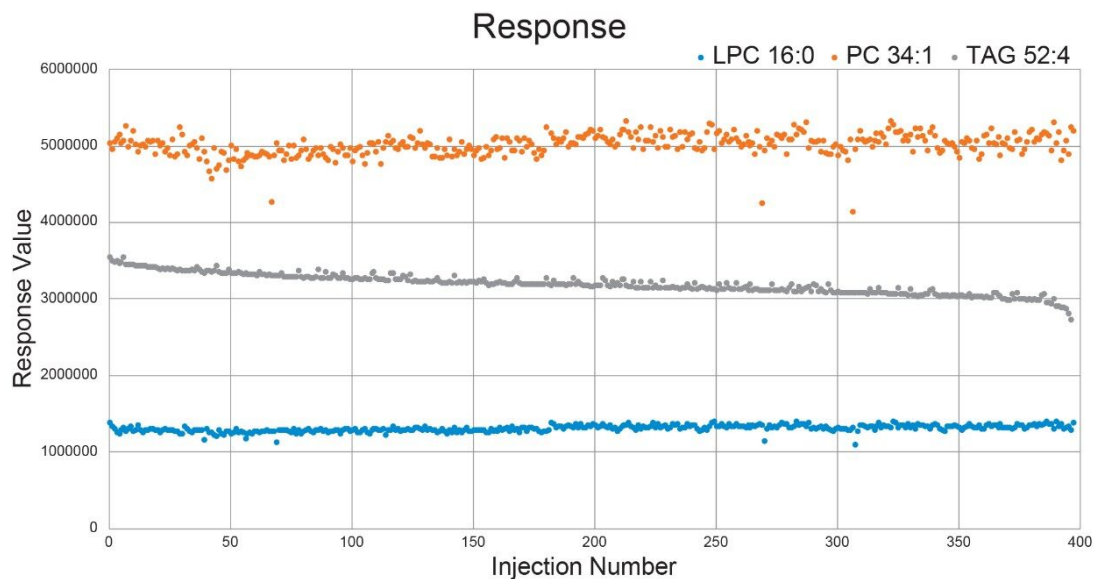


Figure 2. The response stability (non-normalized) over a 400 injection analysis for the three endogenous lipids investigated in this application.

The mass accuracy of the ACQUITY RDa Detector was evaluated using the Avanti SPLASH mix both positive and negative ion mode. A total of 9 lipids were analysed in positive ion mode, these lipids were typically observed as $[M+H]$, $[M+NH_4]$, and as $[M+Na]$ species. The average system mass accuracy was determined to be ± 2.4 mDa/ ± 2.0 ppm with a maximum deviance observed of 1.9 mDa/4.8 ppm. In negative ion mode 6 lipids were evaluated and demonstrated an average system mass accuracy of ± 1.2 mDa/ ± 1.7 ppm, and maximum deviance observed of -1.8 mDa/-2.7 ppm. Lipids identified were typically seen as both M-H and as M+COOH⁻ species. The mass accuracy over the 400 sample analytical batch was determined to be ± 0.7 mDa (± 1.5 ppm) for LPC, ± 1.0 mDa (± 1.9 ppm) for IStd d7 LPC, ± 6.0 mDa (± 7.9 ppm) for PC, ± 4.7 mDa (± 6.2 ppm) for IStd d7 PC, ± 8.9 mDa (± 10.2 ppm) for TAG, and ± 9.5 mDa (± 11.3 ppm) for IStd d7 TAG. The data in Figure 3 gives an example of the analysis of the 15:0, 18:1 (d7) PC lipid using the ACQUITY RDa UPLC-MS UNIFI System.

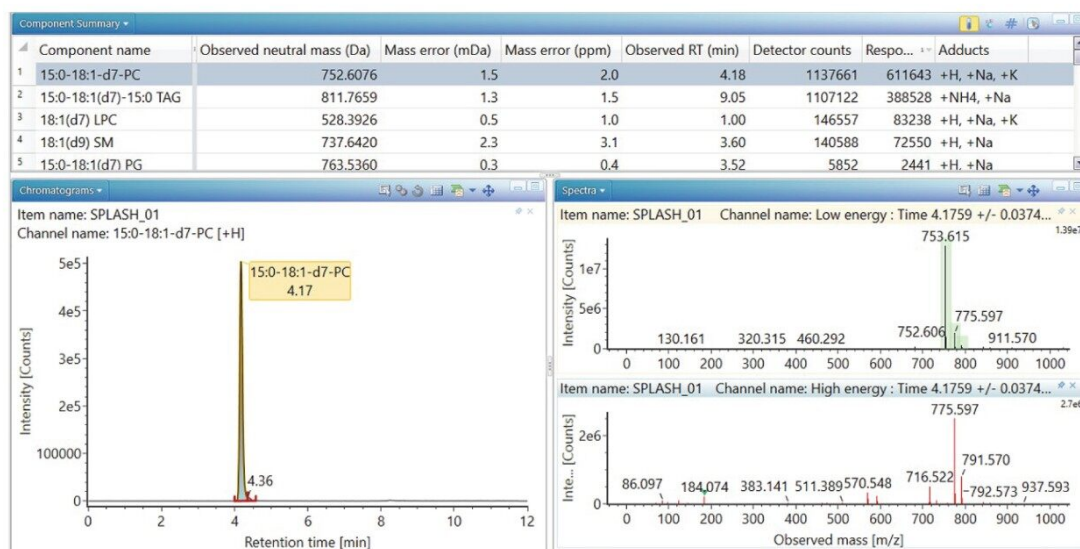


Figure 3. UNIFI display showing table of identifications in the SPLASH Lipidomics injection giving a summary for each compound, an XIC for the identified lipid:PC, is displayed along with its low (top/green) and high (bottom/red) cone voltage mass spectra.

These results demonstrate that the ACQUITY RDa Detector provided robust reproducible data over extensive acquisition times and sample set sizes; with excellent retention time reproducibility, consistent mass accuracy and a stable response. It is, therefore, ideally suited for the routine analysis of large lipid data sets where the lipids of interest have been previously characterized.

Targeted Library Search

The detection and confirmation of target lipid(s) requires not only highly reproducible MS accuracy, MS response, and chromatographic performance, but diagnostic fragment ion information and informatics to process these data sets. The lipid precursor and fragment ion data were acquired by employing a low and high cone voltage in the MS source. The data displayed in Figure 4 is an example of the data obtained for the endogenous lipid LPC 16:0 where the fragmentation information for the parent lipids was achieved using a high energy cone voltage ramp. The high cone voltage spectra has been annotated (blue markers) to indicate which ions within the spectra correspond to a matched theoretical fragment, this can be expanded to display details of the fragment ion including structure and mass accuracy match. The acquired LC-MS data were processed using the UNIFI application to confirmation lipid identity. This was achieved by comparing the lipid retention time, precursor, and fragment ion information against the UNIFI library. A bespoke library was generated from commercially available MS spectral libraries. Within the UNIFI application, libraries can be

also be generated from import of .mol files. Here the structure and chemical formula are generated and additional information such as retention times, specific to individual methods, can also be entered.

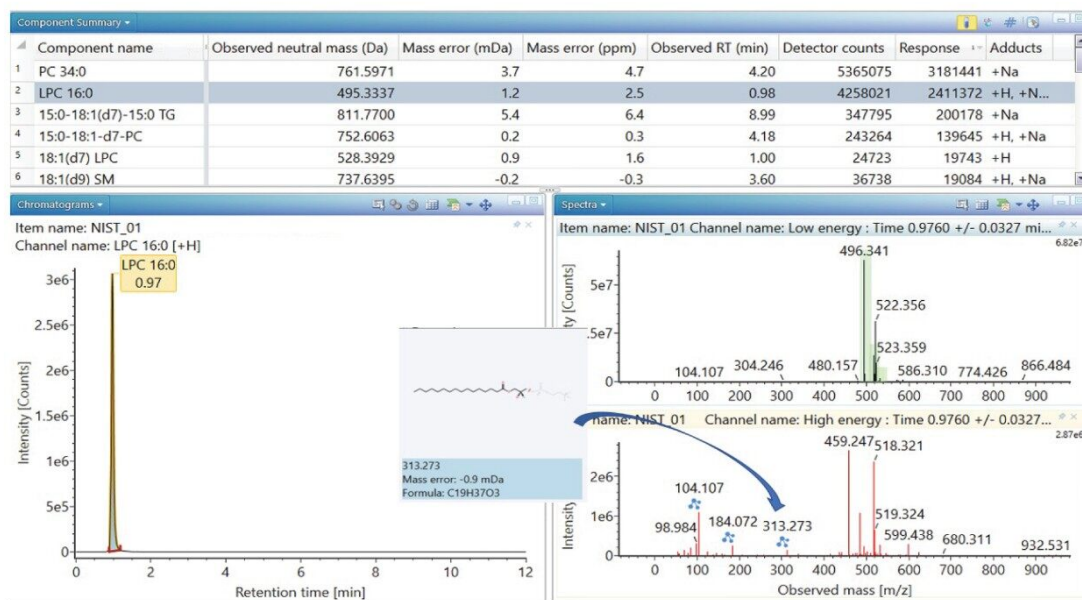


Figure 4. UNIFI display showing processed NIST plasma data, including the table of identifications, matched against the library search giving a summary for each compound. An XIC for the identified lipid:LPC 16:0 is displayed along with its low (top/green) and high (bottom/red) cone voltage mass spectra, within the high cone voltage spectra the blue marks indicate identified fragment ions corresponding to the identified lipid.

The results identified lipids present within the sample, with information such as mass accuracy, retention time, detector counts, response, and adducts used as identification criteria for lipid assignment. The UNIFI spectral view functionality allows individual lipid fragments to be identified and matched with theoretically derived fragmentation data produced from the .mol file.

Conclusion

The ACQUITY RDa Detector combined with ACQUITY UPLC I-Class PLUS and waters_connect Software platform with UNIFI Application provides a robust system for simple, routine analysis of lipids using a

retention time, accurate mass library database. The UNIFI application provides an easily tailored solution, with an integrated library searching tool, simplifying and fast-tracking analysis and processing times. This produced clearly displayed results that are easily converted to a report format to enable rapid decision making. The data provided by the ACQUITY RDa Detector shows excellent mass accuracy and coverage, combined with a powerful software platform ideally suited to target analysis applications in research laboratories.

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