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Automated Lipid Identification Using UPLC/HDMS^E in Combination with SimLipid

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APPLICATION BENEFITS

The workflow detailed in this application note employs UPLC®/ion mobility/time-of-flight (TOF) MS^E (HDMS^E) in combination with SimLipid Software for the high throughput and automated identification of lipids in complex biological matrices.

WATERS SOLUTIONS

ACQUITY UPLC® System

SYNAPT® G2 HDMS™

ACQUITY UPLC CSH™ C_{18} Column

T-Wave™

KEY WORDS

Lipid identification, lipids, biofuel, lipidomic, SimLipid, ion mobility, TOF, UPLC/HDMS^E

Lipids constitute one of the largest classes of macromolecules, together with nucleic acids, proteins, and carbohydrates. Lipids play key biological roles in all organisms and therefore their analysis is of major interest in nutritional, pharmaceutical, and biological research. More recently, the ability of lipids to store energy have also attracted the interest of the biofuel community. Therefore, there is a need to develop comprehensive analytical approaches that allow for the automatic analysis and identification of lipids in complex biological mixtures.

Chemically, lipids are hydrophobic or amphipathic small molecules (<1,500 Da) of biosynthetic origin, which can be counted in the order of tens of thousands. Because their chemical structures vary widely, lipids are classified in eight main categories, each with its own sub-classification hierarchy according to the LIPID MAPS lipid classification system.

To date, mass spectrometry (MS) is the technique of choice for a large scale lipid analysis (lipidomic analysis). MS-based lipidomic techniques are diverse in both method of sample introduction and detection. We have previously shown UPLC-based methods for lipid separation with a variety of unique chemistries.[1-4] UPLC separation is needed to fully address the complexity of lipid analysis in biological samples, both for reducing ion suppression from highly abundant lipids, and for improving separation of isomeric lipid species. Detection and quantification of lipid species becomes possible over a large dynamic range with greater full scan sensitivity than traditional methods.

However, UPLC/MS-based lipidomic analyses produce a large amount of data and data processing is the slowest step in any lipidomic workflow. Currently there are several online resources that can be used to facilitate lipid identification. The limitations associated with these resources include limited automation and reporting. It is indeed extremely time-consuming and laborious to manually interpret lipidomics datasets and identify lipids.

In this application note we introduce a combination of novel analytical (UPLC/HDMS^E) and informatic (SimLipid) tools for higher throughput and automated identification of lipid species from biological tissues.

EXPERIMENTAL

Sample Preparation

Total lipid extract from bovine liver was purchased from Avanti Polar Lipids and re-suspended in isopropanol/acetonitrile/water (2/1/1, v/v/v) at a final concentration of 0.1 mg/mL. 5 µL were injected into the system.

LC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC CSH C_{18} 2.1 x 100 mm, 1.7 µm
Column temp.:	55 °C
Flow rate:	400 µL/min
Mobile phase A:	Acetonitrile/water (60:40) with 10 mM ammonium formate and 0.1% formic acid
Mobile phase B:	2-Propanol/acetonitrile (90:10) with 10 mM ammonium formate and 0.1% formic acid
Injection volume:	5 µL
Weak wash:	Acetonitrile/water (60:40) in 0.1% formic acid
Strong wash:	2-Propanol/acetonitrile (90:10) in 0.1% formic acid

Gradient

Time (min)	% A	% B	Curve
Initial	60	40	Initial
2.0	57	43	6
2.1	50	50	1
12.0	46	54	6
12.1	30	70	1
18.0	1	99	6
18.1	60	40	6
20.0	60	40	1

MS conditions

MS system:	SYNAPT G2 HDMS
Acquisition mode:	MS^E and $HDMS^E$
Ionization mode:	ESI positive/negative
Capillary voltage:	2.0 KV (for positive) 1.0 KV (for negative)
Cone voltage:	30 V
Desolvation temp.:	550 °C
Desolvation gas:	900 L/Hr (nitrogen)
Source temp.:	120 °C
Acquisition range:	100 to 1200 *m/z*

RESULTS AND DISCUSSION

UPLC/HDMS^E analysis of lipids in real biological samples

To identify the complex variety of lipid species present in biological samples, a lipid analysis workflow was developed using the Waters® UPLC/ion mobility/TOF MS^E (HDMS^E) SYNAPT G2 HDMS System.

A lipid mixture from bovine liver was separated using Charged Surface Hybrid (CSH) C_{18} UPLC (for more details on the chromatographic method, please see Reference 1). CSH C_{18} was made by applying controlled low-level positive charges to Ethylene Bridged Hybrid (BEH) particle surface, which was then bonded with C_{18}.[5] These low-level charges enhanced the lipid separations based on their acidic, basic, and neutral properties, providing excellent separation, peak shape, and chromatographic reproducibility, as shown in Figure 1.

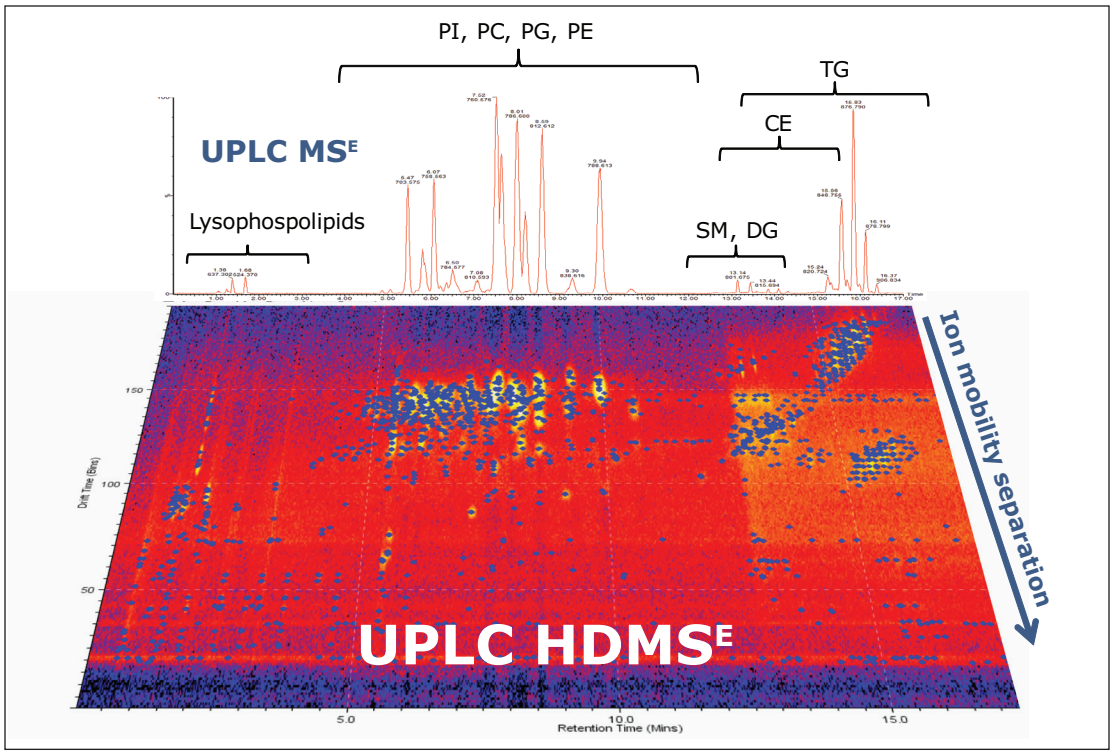

Figure 1. Representative ULPC/HDMS^E chromatogram. UPLC/HDMS^E increases peak capacity through the added dimension of ion mobility separation, which increases the specificity of lipid identification.

After chromatographic separation, lipids were ionized and entered in the mass spectrometer, where they passed through the Ion Mobility Separation (IMS) cell. A T-Wave mobility separator uses a repeating train of DC pulses to propel ions through the gas-filled cell in a mobility dependent manner. Lipids migrate with characteristic mobility times (drift times) according to their size and shape. For example, differences in the acyl chain length or number of double bonds affect the shape and size of lipid molecules, resulting in characteristic drift times.

Therefore, IMS provides an additional degree of separation besides chromatography, improving peak capacity over conventional UPLC, as shown in Figure 1. This leads to a better separation of lipid species and increased selectivity.

Finally, the lipid ions exiting the IMS cell were fragmented in the transfer T-Wave cell in MS^E mode, which utilizes parallel low and elevated collision energy to acquire both precursor and product information for virtually every detectable ion in a single analytical run.[4] The transfer T-Wave delivers the mobility separated ions to the time-of-flight mass analyzer, which records ion arrival times (or drift times).

The combination of IMS and MS^E – known as HDMS^E – provides increased specificity and hence confidence for lipid identification in complex biological mixtures, reducing false-positive identifications. HDMS^E has several advantages over previous methods traditionally used to obtain the product ion data, such as multiple reaction monitoring or data dependent acquisition. Indeed, no prior knowledge of the lipid ions of interest is required and there is no loss of data due to poor duty cycle. Therefore, HDMS^E acquisition is ideal for the rapid analysis of unknown lipid mixtures in biological samples.

The data generated by UPLC/HDMS^E was extracted using Waters® MS^E Data Viewer, software developed for visualization, processing, and interpretation of multi-dimensional MS or HDMS data, as shown in Figure 2. MS^E Data Viewer uses a Waters proprietary algorithm, Apex 4D, to assign a unique retention time, drift time, *m/z*, and intensity to each individual lipid ion in the mixture. Precursor and product spectra are then aligned according to retention and drift times and linked together. An example of this application is shown in Figure 2, in which a lipid extract from bovine liver was analyzed by UPLC/HDMS^E and MS^E Data Viewer was used to process the datasets, which were then used for lipid identification through SimLipid Software.

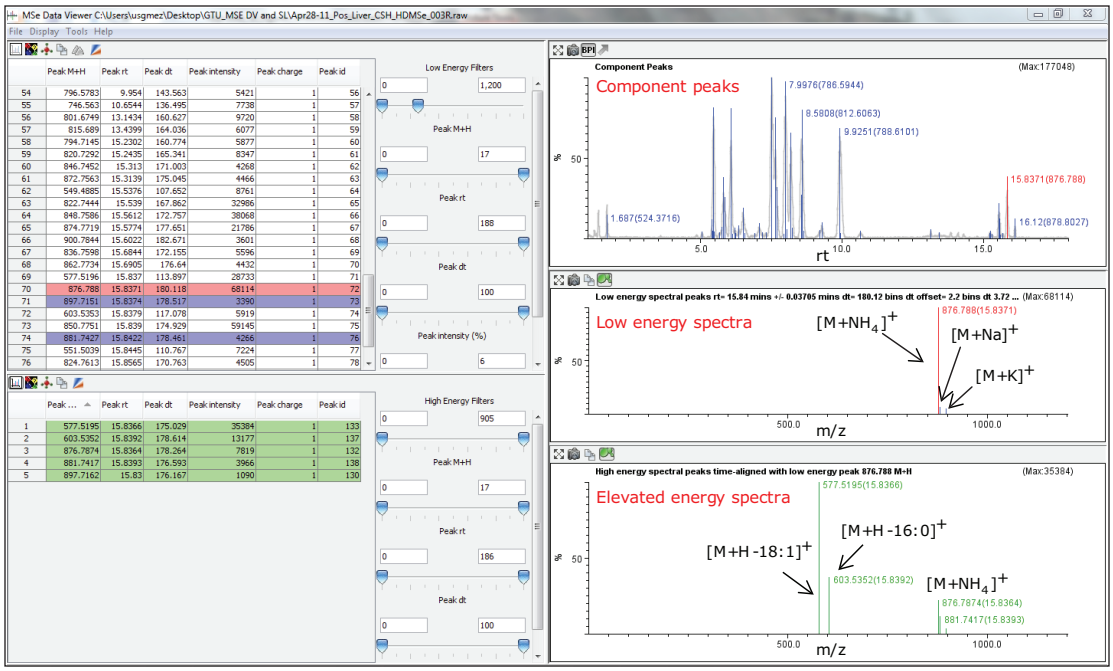

Figure 2. MS^E Data Viewer aligns precursor and corresponding product ion spectra for each lipid species sorting them by retention and drift times. The red highlighted m/z 876.788 shows the selected lipid species (ammonium adduct of 16:0/18:1/18:1 TG). The blue highlighted m/z 881.7427 (sodium adduct of 16:0/18:1/18:1 TG) and 897.7151 (potassium adduct of 16:0/18:1/18:1 TG) show co-eluting lipid species within the retention- and drift-time tolerance window. The elevated energy product ions at m/z 577.5195 and 603.5352 are due to neutral loss of the 18:1 and 16:0 respectively.

Lipid identification using SimLipid Software

The dataset processed in MS^E Data Viewer can be imported into SimLipid, a powerful lipid identification software from Premier Biosoft, shown Figure 3. SimLipid accepts the experimental UPLC/MS^E and UPLC/HDMS^E data (retention time, *m/z*, drift time, and intensity values) in their native file format. Then the software matches the exact masses of the precursor and product ions of unknown lipids with those on an *in silico* database containing over 22,000 lipid species belonging to the major lipid classes (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, prenols, saccharolipids, and polyketides). Table 1 shows the major features of SimLipid. Users can perform MS and MS/MS lipid search for high resolution data with an error tolerance of 1 to 20 ppm or 0.1 to 2000 mDa. SimLipid supports $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, $[M+C_5H_{12}N]^+$, $[M+Li]^+$ ions in the positive ion mode and $[M-H]^-$, $[M+AcO]^-$, $[M+Cl]^-$, $[M-CH_3]^-$, $[M+OAcO]^-$, and $[M+HCOO]^-$ in the negative ion mode, as shown in Table 1.

Feature	SimLipid
Lipid categories	Glycerophospholipids, Sphingolipids, Fatty Acyls, Glycerolipids, Sterols, Prenols, Saccharolipids, Polyketides
Input file format	Text, Excel, mzData, mzXML, and Waters MS^E and HDMS^E files
Manual entry of peak list	Yes
Peak List Sorting (on the basis of precursor ion *m/z*, charge state, retention time, intensity, drift time, and Scan number)	Yes
Search parameters template	Yes
Isotopic peak correction	Yes
Lipid profiling using MS data	Yes
Lipid structural elucidation using MS/MS data	Yes
Search lipids in database	Yes, search by lipid abbreviation/mass/ chemical composition/lipid ID
Ion mode	Positive ion mode: $[M+H]$, $[M+NH_4]$, $[M+Na]$, $[M+C_5H_{12}N]$, $[M+Li]$, and $[M+K]$ Negative ion mode: $[M-H]$, $[M+AcO]$, $[M+Cl]$, $[M-CH_3]$, $[M+OAcO]$, and $[M+HCOO]$
Report generation and export of results	Excel/CSV/ HTML/JPEG/PNG formats
Lipid 2D structure display or export	Yes
Lipid fragment and their 2D structure display	Yes
Trigger product ion data analysis from an MS lipid profile	Yes
High throughput MS and MS/MS data analysis	Yes
Comprehensive MS and complementary MS/MS or MS^E data in excel/CSV/HTML formats	Yes
Comparative report to validate lipids identified with different Adducts/Ion modes in Excel/CSV/ HTML formats	Yes

Table 1. Major features of SimLipid Software.

SimLipid assigns a probability score to the unknown lipid structure according to the best fit of the experimental *m/z* values with the theoretical *m/z* values of both precursor and product ions of the SimLipid database, shown in Figure 3. By matching the exact masses of the characteristic product ions, in addition to precursor ions, SimLipid is able to identify isomers with similar *m/z*, reducing the misidentification of lipid structures.

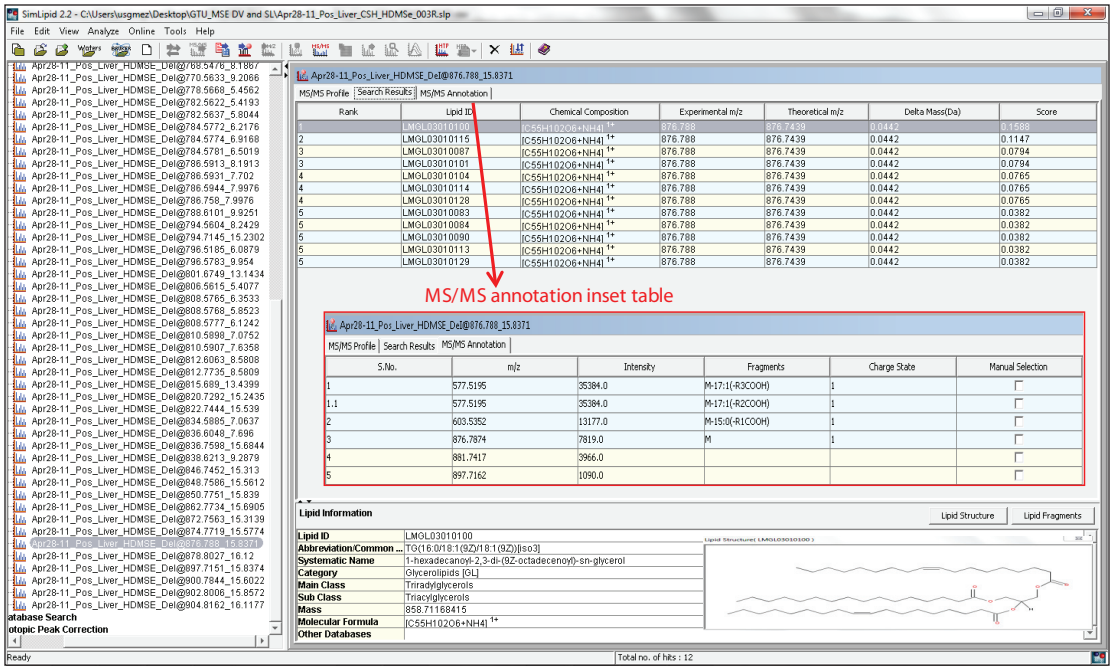

Figure 3. SimLipid Software identifies lipid species using accurate mass precursor and product ion information. The rows in the inset table highlighted in blue indicate experimental product ions matched to the in silico generated product ions.

The following analysis was performed in order to demonstrate the lipid identification workflow. A bovine liver lipid extract was analyzed by UPLC/HDMS^E and processed using MS^E Data Viewer. Among the list of ions generated, the mixture contained an abundant lipid component at *m/z* 876.788 (low level collision energy mass corresponding to the precursor ion), 15.84 min (retention time, rt), and 180.12 ms (drift time, dt), shown in Figure 2. Such information could be associated with different lipid structures and does not provide the specificity needed to elucidate the chemical structure of the unknown lipid. However, HDMS^E provided additional information on characteristic product ions (*m/z* 577.5195 and 603.5352) derived from the parallel use of elevated collision energy in the same analytical run, shown in Figure 2. These datasets were incorporated in SimLipid, which was able to automatically confirm the identity of the unknown lipid as the triacylglyceride 16:0/18:1/18:1 [iso3], by matching the experimental masses of the precursor and product ions with the theoretical masses contained in its database, shown in Figure 3. In fact, using precursor MS search, only *m/z* 876.788 was identified by SimLipid as the ammonium adduct $[M+NH_4]^+$ of the triacylglycerol species with 52 carbons and two double bonds (52:2). However by adding the MS/MS search, SimLipid was able to automatically identify the characteristic fragments at *m/z* 577.5195 and 603.5352 as the molecular ion after neutral losses of the ammoniated fatty acyl groups 18:1 and 16:0 respectively, shown in Figure 3. Therefore, SimLipid allowed determination of the nature of the acyl chains substituent and discriminated among isomeric triacylglycerol species in a single step, shown in Figure 3. The high throughput MS and MS/MS data analysis feature in SimLipid allows automatic identification of product ions and hence structure for each precursor ions observed at different retention time points in batch mode. Each batch run is assigned a unique ID and a comprehensive report can be generated. Multiple batch runs can be launched simultaneously reducing the analysis time considerably.

In addition, SimLipid automatically displays the chemical structure of the identified precursor ion, and annotates the corresponding fragments, shown in Figures 3 and 4. Additional information on the identified lipid such as lipid abbreviation, systematic name, composition, and links to open-access database were also made available for easy reference. Furthermore, to facilitate accurate quantitation of lipids from biological mixtures, SimLipid provides a high-throughput module to calculate isotope percentages and correct the experimental peak intensities observed for their isotopic overlaps, shown in Table 1. Finally, SimLipid can annotate mass spectra with the lipids identified for MS^E data and generate reports in different formats (CSV, XLS, HTML, JPEG, and PNG) for information sharing and further processing of the data, shown in Figure 4 and Table 1.

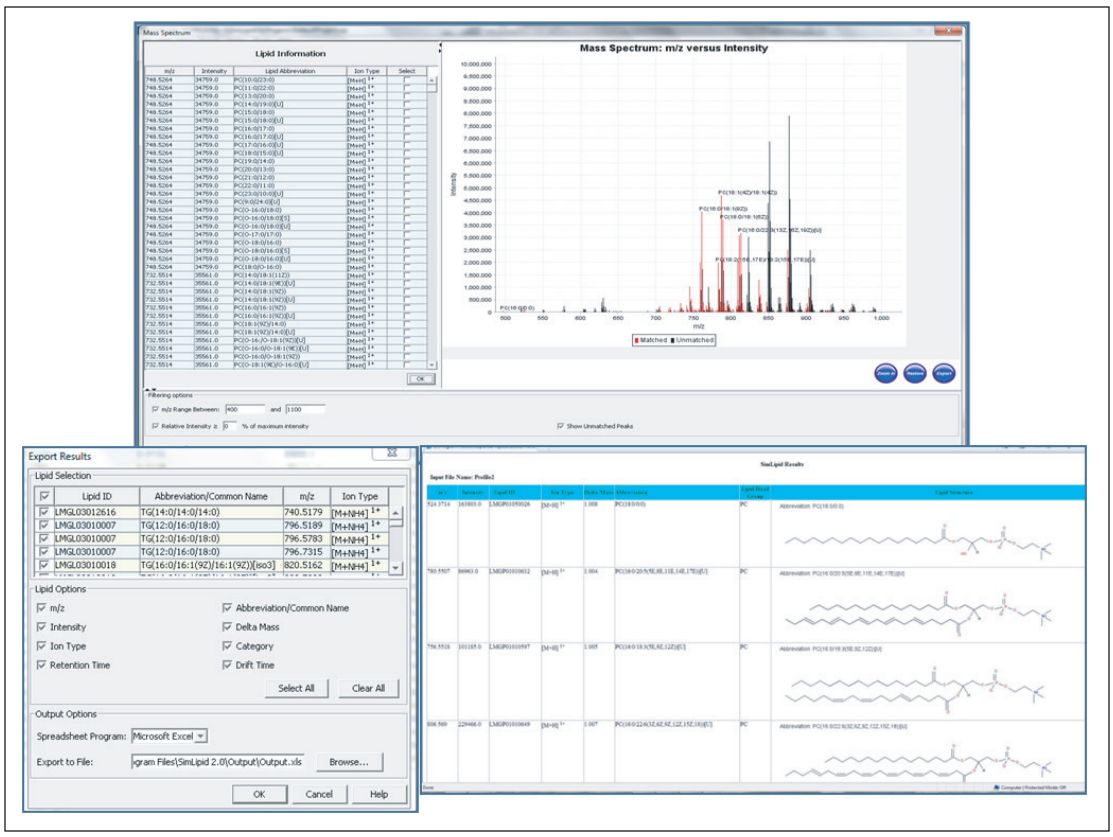

Figure 4. SimLipid Software generates report in different file format, which can be used for further statistical analysis, publication, or sharing information with colleagues.

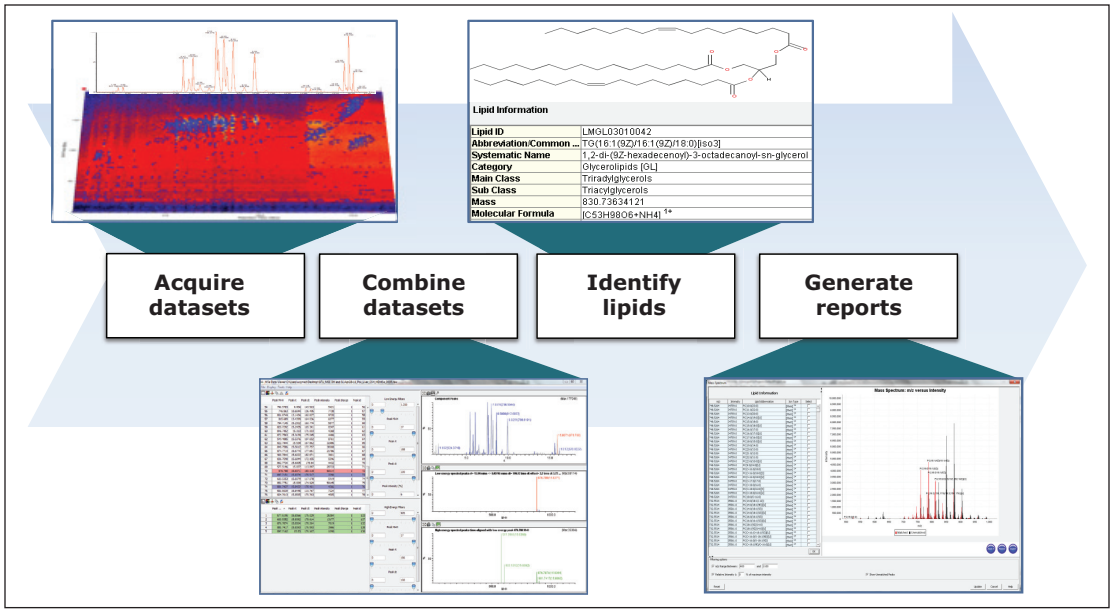

Figure 5. The Waters global lipid identification workflow encompasses a combination of analytical and informatic tools.

CONCLUSIONS

The workflow detailed in this application note provides a simple and robust solution for the high-throughput, automated identification of lipids using novel analytical and informatic tools, as shown in Figure 5.

The use of UPLC coupled to HDMS^E provides multiple degrees of orthogonal separation, delivering unprecedented peak capacity. The addition of the SimLipid Software to our workflow offers an integrated informatics solution for lipid identification, utilizing the datasets generated by Waters' instruments.

In conclusion, the combination of UPLC/HDMS^E and SimLipid allows for a confident identification of lipid species in a biological mixture.

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