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REVIEW ARTICLE

SWATH-MS for metabolomics and lipidomics: critical aspects of qualitative and quantitative analysis

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Abstract

Introduction While liquid chromatography coupled to mass spectrometric detection in the selected reaction monitoring detection mode offers the best quantification sensitivity for omics, the number of target analytes is limited, must be predefined and specific methods developed. Data independent acquisition (DIA), including SWATH using quadrupole time of flight or orbitrap mass spectrometers and generic acquisition methods, has emerged as a powerful alternative technique for quantitative and qualitative analyses since it can cover a wide range of analytes without predefinition.

Objectives Here we review the current state of DIA, SWATH-MS and highlight novel acquisition strategies for metabolomics and lipidomics and opportunities for data analysis tools.

Method Different databases were searched for papers that report developments and applications of DIA and in particular SWATH-MS in metabolomics and lipidomics.

Results DIA methods generate digital sample records that can be mined retrospectively as further knowledge is gained and, with standardized acquisition schemes, used in multiple studies. The different chemical spaces of metabolites and lipids require different specificities, hence different acquisition and data processing approaches must be considered for their analysis.

Conclusions Although the hardware and acquisition modes are well defined for SWATH-MS, a major challenge for routine use remains the lack of appropriate software tools capable of handling large datasets and large numbers of analytes.

Keywords LC–MS · Data independent acquisition · SWATH · Metabolites · Lipids · Review

1 Introduction

Reliably identifying and accurately quantifying endogenous and exogenous compounds is the basis for understanding biological mechanisms including onset of disease, response to drug therapy, and general aspects of lifestyle and health. Medical conditions, therapeutic efficacy, and life style assessment are often associated with changes in the lipidome (Hájek et al. 2018) (Lanznaster et al. 2018) and/or metabolome (Casey and Yinfa 2019). The challenges in both metabolomics and lipidomics are the identification and quantification of many analytes with highly diverse chemical properties in various biological matrices. To successfully achieve these objectives, accurate analytical approaches in conjunction with comprehensive data processing workflows are needed. The Human Metabolome DataBase (HMDB) (version 4.0) (Wishart et al. 2018) currently lists 114,100 metabolites including lipids and other small molecules. While the metabolome is more diverse than the lipidome regarding functional groups, polarity range and pKa, the number of isomers is significantly higher in the lipidome, due to size and biochemistry (i.e. double bonds, total carbon number, and modifications). In terms of sensitivity and selectivity, mass spectrometry (MS) combined with separation techniques, such as gas chromatography (GC), liquid chromatography (LC), supercritical fluid chromatography (SFC) or capillary electrophoresis (CE) (Wishart 2016) offers the best options for analysis. Liquid chromatography coupled to tandem mass spectrometry has become widely accepted to investigate the metabolome and lipidome on an unbiased global scale or in a targeted approach focusing on specific metabolic pathways. Mass spectrometry-based metabolomics and lipidomics studies are generally

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conducted on triple quadrupole instruments (QqQ) using selected reaction monitoring (SRM) for quantitation or on high resolution mass spectrometers (HR-MS) using data dependent acquisition (DDA) for qualitative and quantitative analysis. While DDA provides valuable information on both the MS1 and MS2 levels, it suffers from two major drawbacks: (i) selection of the precursor ion can be challenging and relevant analytes can be missed, (ii) accurate and sensitive chromatogram based quantification can only be performed at the MS1 level since only single MS2 spectra are collected. In both scenarios, either hypothesis generating or hypothesis driven, metabolites need to be identified and quantified based on absolute or relative measures (Xiao et al. 2012). A major limitation in common workflows is the extraction of biologically relevant information based solely on retention time and accurate mass measurements (HR-MS) or on quantification only by SRM. In contrast, simultaneous comprehensive acquisition of precursor and product ions increases the reliability of the identification of unknowns and the quantification of known metabolites. The simplest way to obtain this information is to perform data independent acquisition (DIA) such as collision energy switching MS of everything (MS^E) (Wrona et al. 2005)) or all ion fragmentation (AIF (Naz et al. 2017), where precursor ions are not selected in Q1 and the total precursor ion population is submitted to fragmentation and subsequent mass analysis. Metabolomics studies have been performed using both techniques which rely strongly on the chromatographic performance, including retention time stability and narrow peak shapes, to be able to accurately align precursors and fragments within the run and over the analytical sequence.

Proteomics requirements drove the development of improved data independent workflows including SWATH (Gillet et al. 2012), PAcIFIC (Panchaud et al. 2011), FT-ARM (Weisbrod et al. 2012) and XDIA (Carvalho et al. 2010) all of which were reported between 2010 and 2012. Of the four, SWATH has become well established in proteomics and is widely used for the quantitative analysis of thousands of peptides/proteins from a single chromatographic run. (Anjo et al. 2017; Ludwig et al. 2018). While DDA selects the precursor ion for fragmentation in real time and with unit mass resolution, SWATH-MS involves the consecutive selection of pre-defined wide overlapping precursor ion windows with the first quadrupole (Q1) followed by fragmentation and simultaneous monitoring of precursors and fragments in the time-of-flight (TOF) or Orbitrap mass analyzers. Q1 window widths are typically of 20–25 Da but can be adjusted to optimize specificity and cycle time, and even changed during an analysis (Raetz et al. 2019; Zhang et al. 2015). In complex samples, such as tryptic digests, co-eluting peptides acquired within the same Q1 isolation window co-fragment and are recorded in the same MS2 spectrum, which may cause selectivity losses. Specific peptides can be quantified by extracting the fragment information from previously generated spectral libraries (Gillet et al. 2012) and searching spectral databases has also proved to be practical in other applications such as bioanalysis, metabolite identification, forensics, metabolomics and lipidomics (Bruderer et al. 2018; Tsugawa et al. 2015). However, the acceptance of SWATH-MS has been slower in these areas, even though first applications were already reported in 2012 (Hopfgartner et al. 2012). The use and application of SWATH-MS for low molecular weight compounds has recently been reviewed (Bonner and Hopfgartner 2018; Wang et al. 2019).

Even though both metabolomics and lipidomics aim to analyze endogenous metabolites, the challenges faced in quantification and data processing are unique due to the different chemical space, the lack of analyte-free matrices and the type of MS instruments used. A brief overview of the various techniques with their strengths and weaknesses are summarized in Table 1. The aim of this review is to critically discuss current SWATH-MS acquisition modes and software solutions for analyte identification and selective quantification, and to illustrate these with selected applications.

2 SWATH and data independent acquisition modes

In the simplest form of DIA acquisition with liquid chromatography two spectra are collected using alternating low and high collision energies; this is known as MS^E or All Ion Fragmentation (AIF)(Fig. 1a) (Wrona et al. 2005) (Naz et al. 2017). In fact, these approaches are not strictly MS/MS techniques as there is no precursor selection, but with only two experiments the cycle time can be short enabling coupling with ultra-high performance liquid chromatography (UHPLC). Selectivity is strongly dependent on the mass analyzer resolving power and establishing the precursor-fragment link relies on the performance of the LC and the signal–noise ratio of the mass spectral data.

Unlike DDA, DIA analysis collects MS2 (fragment ion) spectra from wide (> 1 Da) pre-defined precursor selection windows. An MS1 (full scan) spectrum is often collected for each cycle to help link the precursors and fragments.

The general advantage of SWATH (Fig. 1b-e) is the reproducible acquisition of MS2 data of any ionized precursor covered by the defined mass windows. In principle, in combination with high acquisition speed (100 Hz), this enables the quantification of any precursor and/or fragment by extracting ion chromatograms post-acquisition. Additionally, simultaneous monitoring of both MS1 and MS2 traces results in a more comprehensive data set from which additional links can be made (e.g. adducts and in-source fragments can be identified). Recording full scan

Table 1 Brief overview of the various mass spectrometric acquisition techniques with their strengths and weaknesses

Technique	DDA	SRM	MSE, AIF	SWATH	Scanning SWATH
Instruments	QqQ, QqTOF, Orbitrap	QqQ	QqTOF, orbitrap	QqTOF, orbitrap	QqTOF
Precursor selection mode	Real-time based on MS1 survey scan	Pre-defined	None	Pre-defined	Pre-defined
Precursor selection rules	Top N (most intense)	N/A	N/A	N/A	N/A
Precursor selection width	1 Da	1 Da	Full mass range	Wide (e.g. 10–50 Da),	Wide (50–100 Da)
Precursor selection range	Defined by survey scan	Defined by analyte list	Full mass range	Selected	Scanned
Product ion mass range	Full mass range	Selected *m/z* pairs	Full mass range	Full mass range	Full mass range
Product ion generation	CID of selected precursor	CID of selected precursor	CID of entire range; alternates with low energy (precursor) scan	All precursors in selected window	All precursors in window
Precursor-product determination	Known from selected precursor	Pre-defined	Match LC profiles	Match LC profiles	Match LC profiles
Compound identification	Library search	N/A	Library search	Library search	Library search
Qualitative performance	Very good	N/A	Fair	Good	Good
Quantitation modes	Precursor ion chromatograms Mass peak ratio to labelled standard	Fragment chromatograms	Fragment chromatograms Precursor chromatograms	Fragment chromatograms Precursor chromatograms	Fragment chromatograms
Quantitation performance	Acceptable	Excellent	Moderate	Very good	Very good
Strengths	Efficient use of time I Da precursor window (good selectivity) Widely accepted	Best quantitation (Gold Standard method)	Fast, HPLC compatibility No method development	Reproducible, consistent data acquisition Permanent record of all fragments Post-acquisition data analysis	Reproducible, consistent data acquisition Permanent record of all fragments Post-acquisition data analysis
Acquisition method development	Minimal	Extensive	No	Minimal	No
Weaknesses	Stochastic precursor selection Quantitation only from MS1 chromatograms or ion ratios	Limited compound coverage Method development can be time consuming	No MS precursor selectivity Deconvolution is essential and difficult for complex samples	Data is large, complex and requires special processing tools	Data is large, complex and requires special processing tools

Orbitrap includes various hybrid configuration and QqQ includes also the triple quadrupole linear ion trap QqQ_{LIT}

product ion spectra from all the Q1 isolation windows also allows flexibility in the post-acquisition choice of quantifier ions, for example $[M+H]^+$ or $[M\text{-}H]^-$ or any adducts or fragments that depend on the matrix, sample preparation or chromatographic system. This freedom of ion choice reduces the need for data reacquisition due to unexpected ion forms or previously unrecognized compounds, and increases flexibility in the data processing workflow.

While most SWATH approaches cover a defined mass range with several consecutive Q1 isolation windows, they vary in the number, size, and distribution of these windows.

Gillet et al. originally chose 25 Da to match their peptides library requirements (Gillet et al. 2012), but smaller or larger windows are possible or necessary depending on the absolute Q1 mass range, the acquisition speed of the mass spectrometer, the LC peak width and the required selectivity.

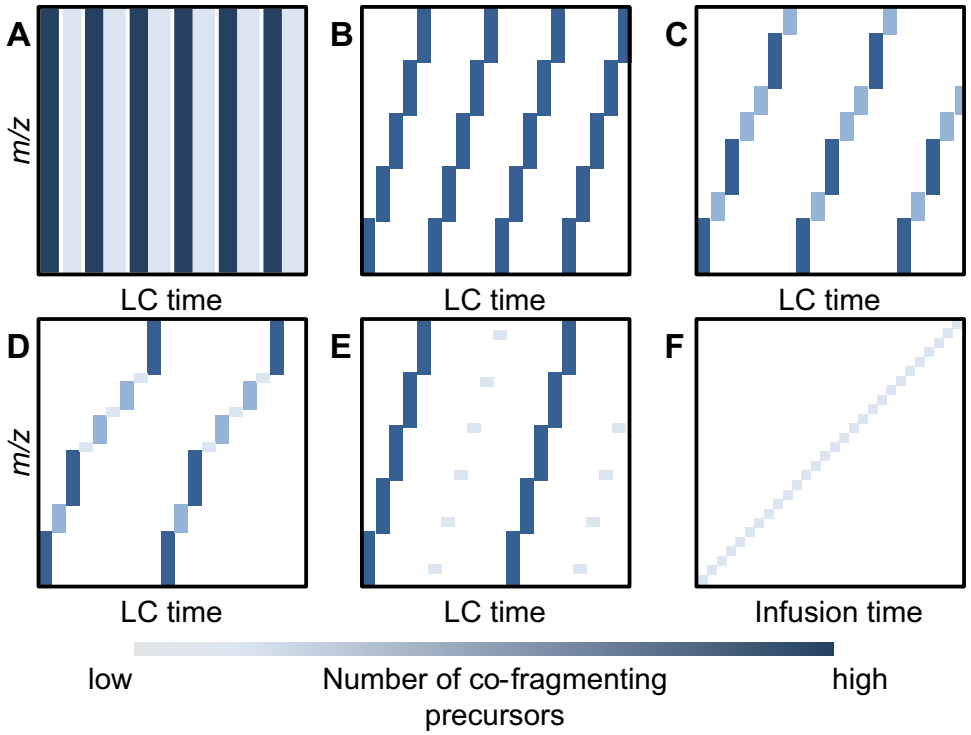

Fig. 1 DIA experiments in terms of Q1 selection size and the resulting cycle times. The color of the windows indicates the density of the precursor ion population that is covered by the Q1 isolation (dark blue = high, light blue = low). The width of each box indicates the same time increment except for 1F (infusion experiment). **a** MS^E or MS^{ALL}; transmission of the whole mass range of interest. Alternating collision energies are applied resulting in precursor (low energy, dark blue) and fragment (high energy, light blue) spectra. **b** Fixed window SWATH; Q1 isolation windows of the same width are equally distributed over the mass range of interest. A collision energy spread is generally applied for each window (e.g. 10–70 eV) to ensure some residual precursor ions are present with the fragment data. **c** variable SWATH; in contrast to fixed SWATH the mass range is separated into different width Q1 windows. **d** variable SWATH with targeted unit mass windows; similar to variable SWATH but additional discontinuous unit mass HR-SRM windows are applied. **e** Fixed SWATH with targeted unit windows; similar to fixed SWATH but additional discontinuous unit mass HR-SRM windows are used. **f** MS/MS^{ALL}; the mass range is covered with several hundred consecutive unit mass windows. Adapted with permission from (Bonner and Hopfgartner 2018)

The latter depends on the window size and distribution as analytes that share fragments, but have different precursor masses, are preferably separated in Q1. However, when studying metabolomics and lipidomics samples using SWATH-MS with fixed Q1 sizes, losses in selectivity are likely to occur due to the constant window sizes. Zhang et al. (Zhang et al. 2015). evaluated the concept of variable SWATH windows (Fig. 1c) for a specific matrix using swathTUNER and investigated three strategies to improve specificity: (i) equal precursor ion population (PIP) for each window (i.e. same number of ions per window); (ii) equal total ion current (TIC) within each window (i.e. same total signal per window) and (iii) scheduled SWATH where the precursor windows are changed at specific times during the LC analysis (Zhang et al. 2015). In terms of identification and quantification performance both methods of variable window SWATH generation were superior to fixed windows. They demonstrated that depending on the type of sample and the analytes of interest (i.e. which omics field) either PIP or TIC is the better choice. Furthermore, specific biologic pathways or analytes of interest can be targeted with increased spectral purity and MS2 selectivity in the variable SWATH Q1 windows (Fig. 1d) by narrowing the Q1 isolation (down to 1 amu), whilst still acquiring MS2 information for other precursors using larger isolation windows (Raetz et al. 2019; Schlotterbeck et al. 2019). Incorporation of consecutive targeted windows can compromise cycle times but, if a large number of unit mass windows have to be incorporated with the larger Q1 windows, additional discontinuous unit mass windows can be set (Fig. 1e). An important consideration for SWATH in metabolomics or lipidomics is cycle time limitations due to the chromatographic peak width. With one MS1 TOF scan and several MS2 scans cycle times are typically in the region of 0.6–1 s which match UHPLC peak widths and usually result in ca. 12 points across the LC peak for gradients between 15 and 30 min. However, it must be noted that narrowing the Q1 window size results in longer cycle times for a given precursor mass range and the accumulation times must be adjusted. For example, with a chromatographic peak of 12 s width, 50 SWATH windows with

an accumulation time of 20 ms can be acquired on a QqTOF instrument resulting in 12 points per LC peak.

A related technique, known as MSMS^{ALL}, also generates fragment spectra for all precursors in a given mass range, but uses a multitude of windows at unit resolution (Fig. 1f). Consequently, a very comprehensive data set is obtained and, as the window size is narrow, the assignment of precursors to the resulting fragment ions is quite straightforward. However, a downfall of the approach is the very long cycle time (several tens of seconds) that limits the method to infusion or flow injection acquisition. Thus, selectivity issues and potential matrix effects can arise due to the large ion population present and the lack of a prior separation. MSMS^{ALL} was first described as an acquisition mode for shotgun lipidomics (Simons et al. 2012) and has so far not established itself in any other omics field as a standard approach.

For quantification the size of the Q1 selection window can be critical in terms of selectivity (Fig. 2). As the Q1 window width increases, crosstalk at the MS2 level, depending on the analyzer resolving power, becomes more likely and limits the fragments used for quantification. Conversely, smaller windows are more selective and 1 amu windows correspond to traditional product ion scans.

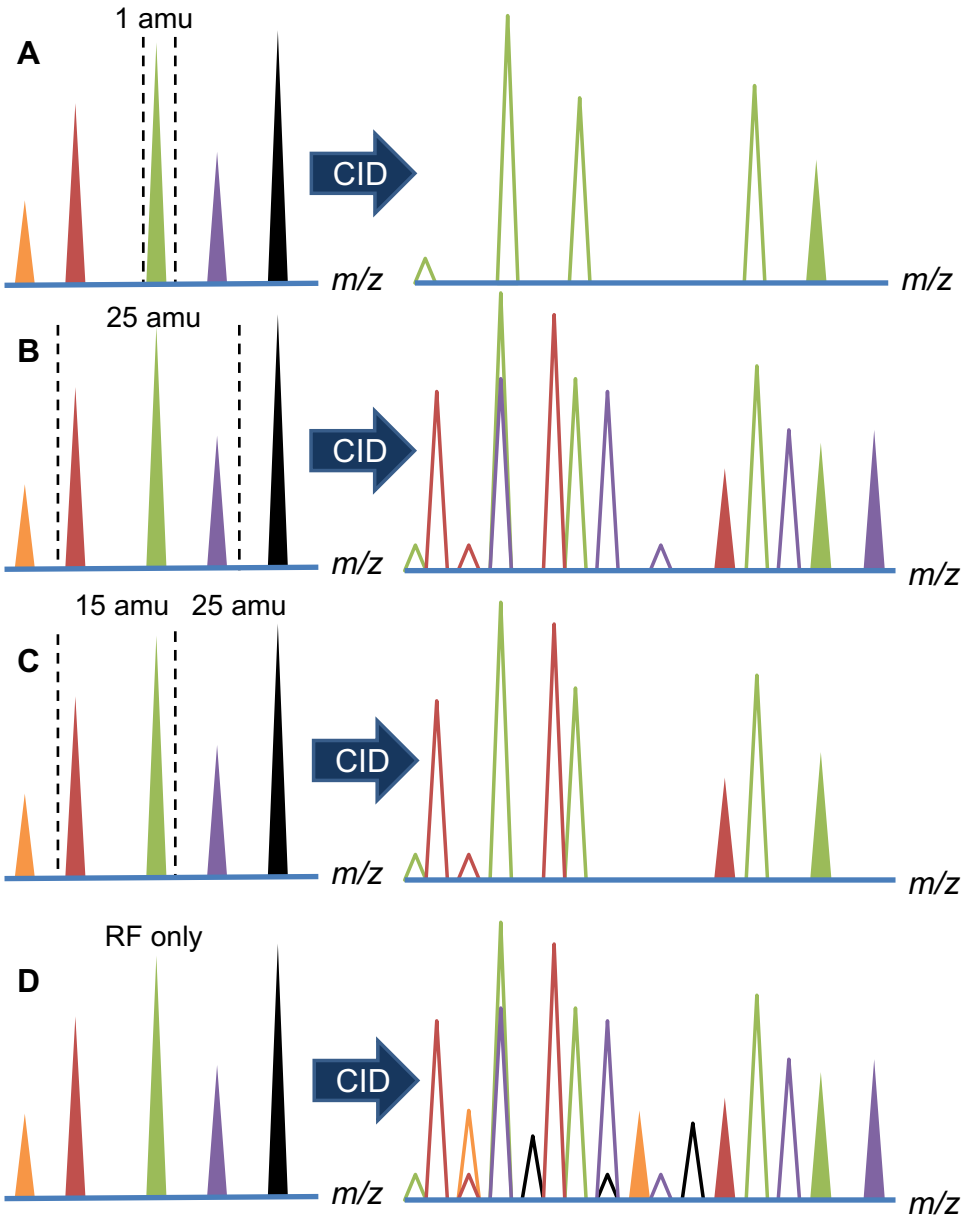

Fig. 2 General impact of the Q1 selection width (left) on the MS2 selectivity (right). Increasing the selection window width results in additional fragment ions but in many cases a compound-specific quantifier ion can be found, albeit with potentially reduced response (sensitivity). **a** for unit mass isolation, **b** for 25 unit isolation, fixed SWATH), **c** for 15 unit isolation (C, variable SWATH), and **d** Q1 open for MS^{E}. Solid peak in the fragment spectra correspond to residual precursor

The recent launch of SONAR (Moseley et al. 2018) and scanningSWATH (Messner et al. 2019) has opened new perspectives for increased Q1 selectivity. While for traditional SWATH Q1 fully transmits the selected mass range for each discrete Q1 window, these scanning techniques use a larger Q1 window that is scanned over the *m/z* range transmitting and fragmenting each precursor while it is within the window. Therefore, alignment of precursors and fragments is easier allowing improved MS2 spectral quality since fragment ions can only appear when the precursor is being transmitted. Consequently, faster chromatographic runs can be realized, and the throughput of omics measurements increased. SONAR has been described for lipidomics (Gethings et al. 2017a; King et al. 2019), proteomics (Juvvadi et al. 2018), and metabolomics (Gethings et al. 2017b). In contrast to proteomics (Messner et al. 2019), the benefits of scanningSWATH for metabolomics and lipidomics has not yet been demonstrated. Due to the limited number of publications for both scanning acquisition modes, they are not further discussed within this review.

The type of mass analyzer is an additional critical factor. Compared to QqTOF, trapping instruments typically require slower acquisition for high resolution and may require smaller Q1 selection widths to reduce the probability of including highly abundant species with very low abundant analytes of interest. In this scenario, the trap might fill before the low abundant analyte is detected with sufficient quality and might therefore be missed (Hopfgartner 2011). Ideally, such precursors would be separated in the Q1 dimension, allowing for better coverage of low abundant analytes, but this increases the number of windows and hence the cycle time. In TOF mass analyzers discrimination of lower abundant species is less likely, however the increased mass resolution of trap-based instruments could be beneficial for MS2 spectra and allow separation of MS1 or MS2 signals that are not separated on TOF instruments.

3 Software tools for processing DIA data

DIA data are highly complex containing thousands of features (*m/z* and retention time pairs) at both the MS1 and MS2 level for every sample, hence efficient data processing software is essential for qualitative and quantitative analysis. The main goals of the software are (i) the alignment of MS1 and MS2 data to determine precursor-fragment links; (ii) compound identification based on accurate mass and fragmentation pattern; (iii) confirmation of elemental composition using isotope ratios; (iv) retention time matching (if standards are available or using software prediction tools); (v) relative and/or absolute quantification of analytes. We note that the first goal can be easier if acquisition includes a collision energy spread so that residual precursor ions are also detected in the MS2 data. Overall the data processing routines should enable reliable extraction of biologically relevant, quantitative data in a manner.

The typical DIA qualitative data processing approach is matching to libraries (Bruderer et al. 2018) which contain MS2 spectra of compounds that have previously been recorded or reliably predicted, e.g. peptides (Tyanova et al. 2016) and lipids (Kind et al. 2013). In contrast to proteomics, false positive identifications for metabolomics and lipidomics cannot be excluded automatically and spectral matching needs to be manually confirmed. Retention time prediction can be useful for additional confirmation of analyte identity (Bruderer et al. 2017) and LC calibration mixes help avoid false positive identifications and can be especially important for isomer quantification.

SWATH enables building quantitative MS2 methods from a single LC–MS run post-acquisition as all the information of any precursor and the corresponding fragment ions are recorded simultaneously. This feature has been exploited in an approach referred to as SWATHtoMRM (Fig. 3), where based on MS1 and MS2 consensus spectra (Fig. 3a) and extracted ion chromatogram (XIC) alignment, SRM transitions were generated (Fig. 3b). Targeted methods were then developed that covered $1000-2000$ known and unknown metabolites from a single injection (Zha et al. 2018) and resulted in the discovery of potential biomarkers for colorectal cancer diagnosis. Relative to SWATH, the benefits of SRM reacquisition on QqQ instruments are higher sensitivity, increased linear dynamic range and the potential to resolve isobaric fragments that are not differentiated with larger Q1 isolation windows.

The NOFI (non outlier fragment interference) strategy described by Bilbao et al. (Bilbao et al. 2015) originally designed for peptide analysis by SWATH could also be applied for low molecular weight compounds. Automated inclusion or exclusion of selective fragment XICs after alignment with the MS1 XICs based on peak width and retention time, can reliably identify selective quantifier fragments and discard non-selective features. By selecting an appropriate set of MS2 signals outlier detection resulted in more accurate and precise quantification. The procedure has so far not been exploited for metabolomics and lipidomics but could easily be implemented in the processing routine of such data sets.

Independent of the MS acquisition mode, multivariate data analysis (MVA) on MS1 or MS 2 level applied to a sample-feature table can be an additional powerful tool for the evaluation of large data sets typically faced in the omics fields (Bonner and Hopfgartner 2016). Although, SRM and DDA data can can be used for MVA but both approaches have limitations. For example, with SRM only the predefined analytes can be evaluated and potential changes of other analytes will be ignored. For DDA, the lack of LC data points

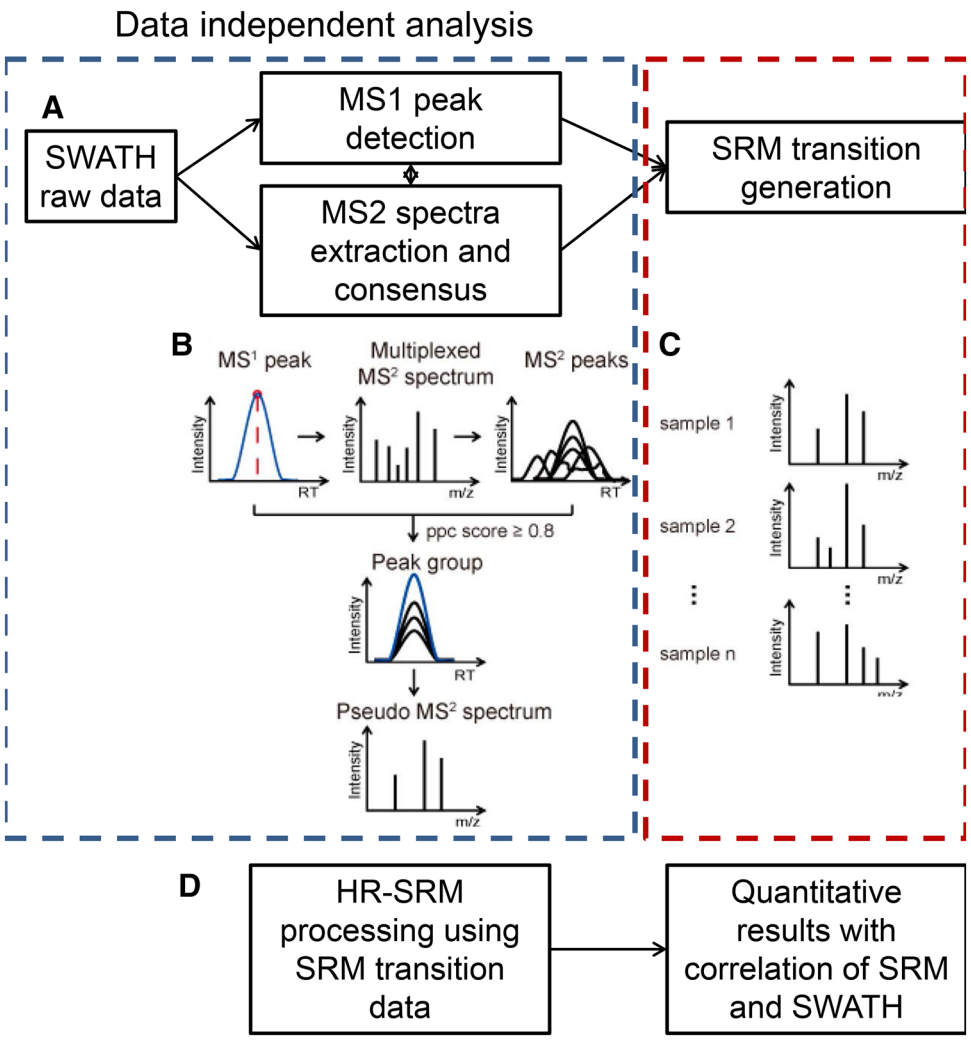

Fig. 3 SWATHtoMRM workflow (adapted from (Zha et al. 2018)). **a** SWATH raw data interpretation with consensus of the MS1 and MS2 XICs. **b** Alignment of the MS1 and MS2 XICs for peak group and SRM transition generation. **c** SRM analysis based on previous SWATH data. **d** Consensus of SWATH HR-SRM quantification with the SRM quantitative data. Adapted with permission from (Zha et al. 2018)

in MS2 limits the MVA to the precursor level and important MS2 feature changes might be missed. Furthermore, the stochastic nature of DDA precursor ion selection compromises reproducibility. Classical metabolomics workflows include several experiments but on cost of analysis time and sample consumption: (i) MS1 which gives sufficient data points for the peak-picking and detection and (ii) MS2 acquisitions for compound identification. In contrast, the simultaneous, consistent acquisition of MS1 and MS2 spectra by SWATH allows MVA to be used on any of the experimental data, separately or in combinations. In one approach, MVA is first performed on the MS1 data to identify precursor features of interest and subsequently the corresponding MS2 SWATH windows are assessed to aid elucidation of compound identity (Klont et al. 2020).

While many software tools, both open-source and commercial, are available for visualization of spectral and chromatographic data, automated solutions for data interpretation and especially quantification are still needed. Two approaches, the freely available MS-Dial (Tsugawa et al. 2015) and propriety MasterView (Sciex), are able to identify compounds using either library searching and retention time or suggest lipid sum compositions. Users can create MS1 or MS2 quantitation methods in separate software, such as MultiQuant (proprietary, Sciex) or the free MRMPROBS (Tsugawa et al. 2013). Another issue facing open-source or free solutions can be the conversion of data from the vendor's to an open format. Although free programs such as MSconvert (Adusumilli and Mallick 2017) or the Analysis Base File converter (ABF [Tsugawa, 2015 #125]) can perform the conversion, the result can be problematic as metadata are not included in the final files and data quality can suffer during conversion. Further, the conversion process converts the already large SWATH raw files, e.g. ~ 50–200 MB, to much larger files, e.g. converted data ~ 200–500 MB and processed file ~ 400–800 MB.

Another approach to dealing with complex SWATH data in lipidomics was demonstrated by Raetz et al. (Raetz et al. 2019) where unit isolation windows for phospholipids were used. But, as several hundred MS2 experiments

were performed per injection, common data processing approaches were impractical. However predicting precursor-fragment combination the method exporter module of LipidView (Sciex), allowed the specific generation of lipid species quantification methods. In this way library searching was unnecessary and XICs of the possible fragments were directly extracted from each unit mass isolation window for identification and quantification. A similar strategy for the automated generation of metabolomics methods seems conceivable and would facilitate the workflow for metabolomics, but metabolite fragments are difficult to predict and appropriate spectral libraries would be needed. In fact, given the variety of collision cell designs and fragmentation processes, a number of databases would be required. In addition, differences in collision energies and their range, might result in different spectra and requires extensive processing.

As reported by Siegel et al. (Siegel et al. 2014 and Sun et al. 2018a), homologues of compounds and classes can be easily detected from specific MS2 fragments (e.g. functional group) or by selective neutral losses (e.g. from a derivatization agent). With a collision energy spread precursor candidates are available in the MS2 data which helps to identify precursors of interest and gives hints about structural moieties. However it does not always support quantification in MS2 as the same fragment can be generated from different precursors. Generally, analytes of interest that have structural similarities and are close in *m/z* compared to the Q1 window size are more susceptible to cross-talk and care must be taken when choosing the quantifier ion. Further, adduct or neutral loss related interferences might occur even if no obvious MS1 cross-talk is observable at the $[M+H]^+$/$[M\text{-}H]^-$ level.

4 SWATH quantification on MS1 and MS2 level

SWATH allows quantification via post-acquisition data processing which reduces the need for sample reacquisition (Hopfgartner et al. 2012). While SWATH-MS has been evaluated for its ability for label-free relative quantification (Huang et al. 2015), absolute quantification using liquid chromatography is still difficult due to several factors: (i) the large number of analytes to be measured (up to several hundreds); (ii) the lack of suitable internal standards for many of the analytes of interest; (iii) the cost of labeled standards even if they are available. Nevertheless, several approaches to partially cope with these limitations have been described recently. Metabolomics often relies on relative quantification, but it should be recognized that both relative changes in metabolite levels and their absolute abundance can be relevant to the evaluation of biological data as suggested by Liebisch et al. (Liebisch et al. 2017) for lipidomics. Due to the organization of lipids into structural classes, quantitation is generally easier in lipidomics than in metabolomics. Class separation strategies, which allow the use of a single or small number of internal standards per lipid class, can be realized by using hydrophilic interaction chromatography (HILIC) (Cífková et al. 2012; Hines et al. 2017; Schwalbe-Herrmann et al. 2010), SFC (Lísa and Holčapek 2015; Takeda et al. 2018) or normal phase chromatography(NP) (Donot et al. 2013). This approach is possible as the species from a given class co-elute based on class specific characteristics and therefore experience the same ionization conditions, and is widely accepted by the lipidomics community (Burla et al. 2018; Liebisch et al. 2017). HILIC chromatographic class separation approaches are valid for accurate absolute quantification at the MS1 level but may show limitations for low abundant species due to isotope related cross talk (Raetz et al. 2019). As all the species within a class co-elute, the M + 2 isotope of a lipid with a higher number of double bonds overlaps with the monoisotopic signal of the following lipid (e.g. PC 34:2 M + 2, *m/z* 760.5767 vs. PC 34:1 M, *m/z* 760.5851). In contrast, reverse phase liquid chromatography (RPLC) allows the separation of lipids based on their fatty acyl length and unsaturation and therefore isotopic crosstalk is not a factor. But while isobaric interferences are reduced, the species from each class do not elute in the same analytical environment so the possibility of single internal standard quantification is limited and data processing more labor-intensive. Rampler et al. (Rampler et al. 2018) have demonstrated a promising lipidomics workflow using fully 13C-labeled yeast strains as an internal standard that supports accurate quantification and is compatible with RPLC.

Compared to the lipidome, the chemical space of the metabolome is more diverse and classification is based on metabolic pathways and functional groups which complicates absolute quantification as class-based approaches cannot be realized. Ruskic et al. (Ruskic et al. 2018) implemented a parallel derivatization setup where the sample was derivatized with a non-labeled agent while in parallel a standard mix was matched with a labeled reagent. While this procedure allows for absolute quantification with a single point calibration, it discriminates the metabolome in favour of the reactive functional groups. Due to the large Q1 isolation, this heavy/light approach is susceptible to MS2 crosstalk mostly where the heavy/light precursor co-elute and generate the same fragments.

Generally, when designing SWATH experiments and selecting the internal standards it is important to remember that large Q1 isolation windows can reduce selectivity resulting in isobaric fragments and increased risk of inaccurate quantification. Figure 4 illustrates the crosstalk between product ion spectra of tryptophan and its d5-analog for individual MS2 spectra (Fig. 4a, b) and the combination of both (Fig. 4c). Ideally, the IS and corresponding analyte

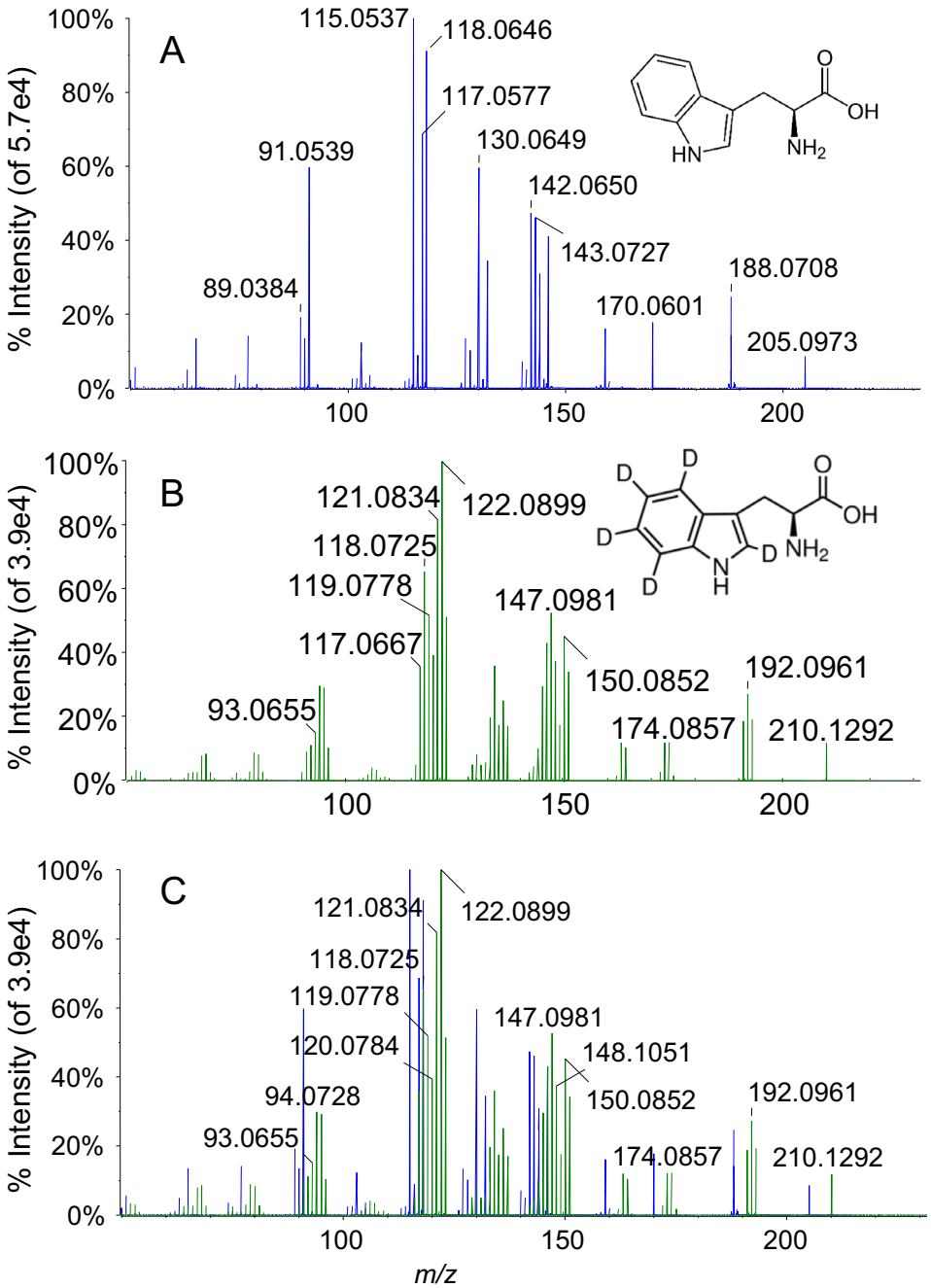

Fig. 4 Individual product ion spectra of tryptophan (**a**) and tryptophan-d5 (**b**), SWATH MS2 spectrum of a mix of the analyte and the deuterated standard (**c**). Both B and C show considerable overlap of the labelled and unlabeled fragments, complicating interpretation and quantitation. This situation could be solved with scanning SWATH

are separated in the Q1 dimension (*m/z* 205 and *m/z* 210) and are preferably not selected for fragmentation in the same Q1 selection window. Due to the vast number and diversity of analytes this is only possible for a few analytes and cross-talk is likely to occur. In the example, the precursor ions are separated by only 5 Da and many of the intense fragments are common to both forms. Consequently, more attention needs to be given to the data processing, and the MS2 spectra need to be carefully evaluated. Applying the procedure described by Schwaiger-Haber et al. (Schwaiger-Haber et al. 2019) metabolites where a fully ^{13}C-labeled yeast extract is spiked into the sample, might allow for increased MS2 selectivity with reduced risk of isobaric overlap as labeling is present on all carbon atoms.

5 SWATH metabolomics applications

With the improvement in QqTOF instrument performance, particularly the acquisition speed (up to 100 Hz), the use of multiple Q1 windows has become acceptable for metabolomics. Zhu et *al.* (Zhu et al. 2014) performed a comparative

study between DDA, SWATH, and MS^{All} for metabolite identification and concluded that DDA generates the best MS2 spectra, but that SWATH outperforms MS^{All} spectral quality and maximizes compound coverage. HR-MS in general has also become an attractive alternative to SRM as there is no need to tune the transitions or to predefine the analytes of interest. A key advantage of MS2 quantitation is the large improvement in signal-to-noise, due to the increased selectivity compared to MS1, and despite the loss in absolute signal intensity the limit of quantification is improved (Tonoli et al. 2012). Bonner et al. have applied an LC-SWATH-MS approach for simultaneous monitoring of drug metabolism and metabolome changes in rat urine samples (Bonner and Hopfgartner 2016). MS1 and MS2 data allowed for numerous data analysis approaches, including: (i) detection of metabolites by prediction; (ii) metabolite detection by mass defect filtering; (iii) quantification from HR-MS precursor or fragment chromatograms; (iv) use of spectral libraries with in-house or commercial databases. Multivariate analysis can be performed based on the data from the full scan TOF or from any or all of the MS2 experiments. The authors characterized 28 vinpocetine metabolites, mostly mono- and di-hydroxylated forms, and detected time dependent changes in the levels of several endogenous metabolites (e.g. dihydroxyindole, phenylacetylglycine) after the administration of a single dose of the model drug vinpocetine to rats.

Sun et al. applied SWATH to investigate the relationship between the levels of progesterone in follicular fluid and oocyte quality of women with higher progesterone level (> 1.5 ng/mL) compared to a control group (< 1.5 ng/mL) (Sun et al. 2018b). SWATH spectra were acquired from *m/z* 100 to 1200 in positive and negative mode. Using principal component analysis several features were detected at different levels between the two groups. In addition to accurate mass and isotopic pattern, MS2 fragments could be used to identify compounds in the metabolome with significant changes (e.g. 8-hydroxyguanosine, 4-hydroxynonenal, ATP, estradiol, and L-carnitine) which lead to the conclusion that progesterone has an adverse effect on oocyte quality.

DDA and SWATH data from positive and negative ion modes were mined by Sun et al. to identify compounds contained in the traditional Chinese medicine called Ziwan and tested for their potential as anti-depressants using mouse brain slices (Sun et al. 2018a). Characteristic product ions were used to extract precursor candidates of specific classes (e.g. triterpenes, m/z 191.1789) in addition to matching the MS2 data to an in-house library. In total, 131 compounds (organic acids, peptides, terpenes, flavonoids and others) could be identified by their data-mining approach. The SWATH data set had a higher precursor coverage than DDA (131 vs. 120), but DDA was able to obtain MS2 spectra of certain compounds (peptides and glycosides) that were not recorded by SWATH due to matrix effects.

6 SWATH lipidomics applications

Quantitative lipidomics measurements are commonly performed using shotgun-MS or via a class separation approach (i.e. NP, HILIC, SFC). Both strategies are preferred over species separation (typically RPLC), as all the species from a class co-elute and therefore undergo the same matrix effects. Applications with only a single internal standard for quantification were described either for the whole sample or for each lipid class of interest (Cífková et al. 2012). Generally, the lipidomics community considers methods using a single or small set of IS per class to be the reference standard and while the MS acquisition has to meet the general validation criteria it is not further specified (Liebisch et al. 2017).

Shotgun lipidomics are usually conducted on QqQ instruments in SRM where the sample is introduced by direct infusion or flow injection. The main advantages of this approach are ease of data processing and the speed and sensitivity of the analysis. The main advantages of HR-MS for shotgun approaches are the reduced risk of isobaric interferences and the availability of full scan MS2 spectra (Almeida et al. 2015; Simons et al. 2012).

As mentioned earlier, the main difference between polar metabolites and lipids lays in their chemical space. While the use of SWATH for metabolomics is increasing, lipidomics approaches using LC-SWATH-MS are scarce. This is mainly due to the vast number of lipid isomers and levels of unsaturation and the resulting difficulty of using larger Q1 isolation windows – especially for class separations (Fig. 4). As all potential fragments are mainly due to fatty acids/alcohols/sphingoid bases and headgroups, differentiation of lipid species becomes more difficult the as the precursor selection window gets larger. Therefore, the majority of the current LC-SWATH approaches are limited to RPLC where chromatographic resolution of isobaric features and the corresponding fragments is critical. Class separation approaches such as HILIC or SFC are generally limited to DDA-like workflows with unit mass isolation of the precursor where qualitative assessments of the class identity are possible but quantification at the MS2 level is lacking. Consequently, the traditional LC-SWATH methodology in combination with accepted quantification strategies (class separation) is not able to profit from the benefits of sequential large isolation windows and is limited to MS1 quantification with reduced qualitative assessments on MS2 (Fig. 4b). In contrast, RPLC separation of lipids by fatty acyl profile complicates the quantification routine but allows for larger SWATH Q1 windows as the LC resolution reduces the MS2 overlap significantly.

Rao et al. (Rao et al. 2016) applied $MSMS^{All}$ based lipidomics to study early change in acute kidney injury (AKI) in male C57BL/6 mice. They covered a mass range of 200–1200 m/*z* with unit mass isolation in positive and negative ion mode to identify specific lipid sum compositions. They were able to identify a set of ether linked PE and PC that were significantly increased in AKI and subsequently monitored the most abundant ones in MSI to locate the changes in tissue sections.

Cahill et al. (Cahill et al. 2018) reported the application of SWATH-MS with laser microdissection-liquid vortex capture/electrospray ionization mass spectrometry (LMD-LVC/ESI-MS) for the online differentiation of mouse brain tissue regions under different analytical conditions (i.e. solvents, ionization polarity, spectral acquisition). They applied three SWATH Q1 windows of 75 Da (total m/z 700–925) to overcome observed isobaric interferences in MS2 data (e.g. PC(32:0)/PE(35:0)) based on selective head group ion fragments or neutral losses. Overall, they showed that SWATH-MS yielded higher accuracy for PCA-LDA applied to the mouse brain model and improved tissue differentiation compared to MS1 measurements.

Yan et al. (Yan et al. 2018) applied SWATH-MS to identify plasma lipids associated with schizophrenia (SCH) onset and its treatment to better understand the mechanisms of disease and therapeutic options. The authors used fixed 40 Da windows from 100 to 1160 m/z in positive and negative ion modes using RPLC. They were able to identify 445 lipids from 17 lipid classes, of which 47 lipid species from 9 classes (CE, AC, LPC, LPE, PC, p-PC, p-PE, SM, and TG) were deregulated in naïve SCH compared to the healthy control. After antipsychotic treatment of SCH, 50 lipid species out of 9 classes (CE, Cer, FA, GlcCer, LPC, PC, p-PC, SM, and TG) were altered.

Schlotterbeck et al. (Schlotterbeck et al. 2019) applied a variable window SWATH approach to a clinical study of the platelet lipidome of patients with cardio vascular diseases (stable angina pectoris and acute coronary syndrome) compared to healthy subjects. Twenty six Q1 windows (with variable size, varSWATH) were distributed over the mass range from 30 – 1000 Da in addition to targeted five Da windows. Variable SWATH Q1 window sizes were created using swathTUNER (Zhang et al. 2015) based on PIP from previously generated DDA data. As oxidized lipid species are of major interest in these patients, one Da Q1 windows specifically targeted four oxidized PC sum compositions and merged with the automatically generated varSWATH windows. 600 lipid species from more than 12 classes could be identified in positive and negative ion mode and the four targeted oxPCs showed significant increases in the cardiovascular disease group compared to the healthy control.

Raetz et al. have demonstrated a pseudo-scheduled hybrid approach that merges product ion scans and fixed window SWATH-MS (Raetz et al. 2019). Large Q1 windows of 100 unit mass and 50 unit mass Q1 windows were chosen to cover simultaneously selected species of interest in a given retention time window. As a HILIC-based class separation was used prior to the MS acquisition, each lipid class could be covered with a specific unit mass method. Overall the authors demonstrated that applying HR-SRM approaches using unit mass SWATH isolation to lipidomics can be beneficial in terms of detection limits and improved accuracy due to the reduced isotopic cross-talk and flexibility in the choice of the quantifier fragment.

King et al. (King et al. 2019) were the first to describe a SWATH approach using a scanning quadrupole for the analysis of the lipidome. MS2 data were acquired by scanning the mass range of 350–950 m/z using a Q1 selection width of 10 Da with alternating high and low collision energies for the analysis of liver extracts from sitaxentan-treated PXB Mice. They demonstrated that 16 triglycerides were elevated by the sitaxentan treatment. The authors concluded that SONAR allows for fast data acquisition while maintaining specificity and allowing for highly reliable identifications with robust relative quantification.

7 Conclusions

High quality quantitative data covering a variety of compounds over a wide dynamic range is a key aspect that has led to the wide acceptance of SWATH. While SRM still offers the best quantification performance, predefining analytes and the need to develop specific methods usually limits its applicability to smaller scale omics measurements and requires further method development if new analytes are added. SWATH can be used without major method development and can cover additional analytes and forms, such as adducts and fragments, without modification. Standardization of methods and workflows for metabolomics and lipidomics seems likely and, as in proteomics, implementation of reference compounds for retention time calibration or for performance comparisons will be highly beneficial. As a result, method transfer between laboratories will be straightforward and reproducible and, if acquisition is unchanged, the data from sample cohorts can be used in different studies by targeting different compounds (fragment ions).

One limitation for DIA approaches, however, is absolute quantification. While label free relative quantification has been shown to be easily realizable using SWATH, the ultimate goal for any omics application should be absolute quantification. Several approaches have demonstrated robust quantitative data for a selection of analytes, but the full potential of DIA and especially SWATH cannot currently be exploited due to the lack of suitable labeled reference material. Novel ideas and approaches are needed to support

absolute quantitative aspects of SWATH, for example, using 13C-labeled strains of microorganisms or differential isotope labeling (DIL) might be convenient and feasible.

While the potential of SWATH using scanning modes for Q1 has been demonstrated for lipidomics using RPLC (King et al. 2019), the performance for class separation based elucidation and absolute quantification of the lipidome has so far not been shown. From a technical point of view, the scanning approaches appear highly promising for routine metabolomics and lipidomics due to the potential of increased selectivity with a consequent decrease in LC–MS runtime and simpler data analysis.

Improvements in hardware and acquisition modes for SWATH have allowed researchers to acquire high quality DIA data simply and quickly, however a major challenge for routine use is the lack of software tools to deal with these large and comprehensive data sets. Two aspects are critical: (i) data processing tools supporting the identification of unknown analytes based on their MS1 and MS2 information and, (ii) processing software that provides a reliable and efficient way of automatically extracting information from complex data. From a user perspective, the integration of analyte identification and quantification on both MS1 and MS2 levels in a single platform is needed and would facilitate the migration from SRM and DDA approaches towards DIA.

User friendly software solutions and larger analyte coverage with truly quantitative standards could lead to a paradigm change in MS-based quantitative metabolomics and lipidomics, but the lack of options for rapid data processing and comprehensive quantification currently deters potential users from switching to DIA approaches. Further, if the emerging fields of ion mobility spectrometry and scanning SWATH are also considered, current software solutions will be inadequate for the larger and even more complex data. Despite these challenges, the additional, and sometimes redundant, information from complex DIA experiments will allow novel holistic processing approaches that provide deeper coverage with increased reliability and confidence.

Finally, DIA methods have attractive unique features, namely generation of a digital record of the sample that can be mined retrospectively as further knowledge is gained and, with standardized acquisition schemes, the ability to use samples in multiple studies.

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