

Mass Spectrometry & Spectroscopy

Unlocking the complexity of metabolomics: Pushing the frontiers of targeted and untargeted methodologies

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Metabolomics is vital for understanding disease and enabling precision medicine by identifying biomarkers for diagnosis, prognosis, and predicting patient-specific treatment responses [1,2]. It also aids drug discovery by elucidating metabolic pathways, reducing toxicology costs, and improving trial design and patient selection [2].

Metabolites are numerous, highly diverse, and span a wide range of concentrations [1,3], creating analytical challenges. Capturing only abundant metabolites risks missing subtle but critical disease signatures. This complexity is key to understanding health and disease [3]. For instance, bladder cancer shows altered TCA cycle and fatty acid metabolism [1]; liver cancer exhibits changes in amino acid, bile acid, choline, fatty acid metabolism, and glycolysis [1]; obesity involves disruptions in glycolysis, TCA cycle, urea cycle, and glutathione metabolism [1]; and Alzheimer's disease shows alterations in amino acid, fatty acid, linoleic acid, cholesterol, glycine, serine, aspartate, glycerophospholipid, and polyamine metabolism [1].

Mass spectrometry powers metabolomics

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is indispensable for quantifying and characterising metabolites, but challenges remain in fragmenting and capturing enough MS2 ions for confident annotation [3]. The choice between data-dependent acquisition (DDA) or data-independent acquisition (DIA) can also be limited by scarce samples.

Collision-induced dissociation (CID) remains the backbone of metabolomics and lipidomics [4,5,6,7,8]. In CID, precursor ions collide with neutral gas, breaking weaker bonds (e.g., C–O) [6], yielding spectra useful for identifying functional groups like ethanolamine, phosphatidic, or glucuronic conjugates and for classifying metabolites such as lipids [6]. CID can provide limited structural data, such as fatty acyl composition [7,6,9], its strengths are speed, sensitivity and versatility [6].

However, CID cannot usually cleave stronger C–C bonds, leaving positional isomers, stereochemistry, and double bond locations unresolved [7,6,9]. This restricts its ability to distinguish regiospecific fatty acyls or structural subtleties, and in complex mixtures it biases toward intense ions, masking low-abundance but important metabolites [5,6,9,10].

Electron-activated dissociation (EAD) overcomes these limits, revealing stereochemistry and double bond positions critical to metabolomics and lipidomics [1,5,6,9,10,11]. By using tuneable energy electrons instead of collisions, EAD generates complementary cleavages [6,9], producing spectra rich in structural information [6,9]. It can identify subtle features in minor metabolites that CID misses, offering significant advances for structural characterisation [3,11,10].

Historically, the drawbacks to electron fragmentation have been speed and sensitivity: scan times of up to hundreds of milliseconds were required, limiting high-throughput use [3]. Multiple double bonds needed longer acquisition or greater sensitivity before decay [3]. Even so, EAD provides an orthogonal, high-resolution complement to CID, enabling the structural detail necessary to connect chemistry and biology [3,11].

Data acquisition for a complete picture of metabolomics

But fragmentation is only part of the story: equally important is how ions are selected—via DDA or DIA. In DDA, the instrument fragments the most intense MS1 ions without prior hypotheses, producing high-quality spectra and excelling for abundant metabolites or targeted workflows [3,8,12]. Yet with advances in DDA algorithms, it typically misses lower-intensity ions, suffers from stochastic selection and run-to-run variability, and produces incomplete datasets biased toward higher-abundant species [3,8,12].

DIA instead fragments all detectable ions across sequential m/z windows, producing reproducible, comprehensive datasets that include low-abundance metabolites [3,13]. However, DIA data are voluminous and noisy in complex samples, which limits the dynamic range [14]. To address this, SWATH DIA introduced overlapping Q1 windows, correlating fragments to precursors at each time point [14,15]. Still, conventional DIA suffered from low duty cycles and broad Q1 windows, with only 5–25% ion utilisation [16]. The Zeno trap when enabled raised this above 90%, boosting sensitivity four- to twenty-fold while preserving precision [16,17].

Yet DIA in metabolomics faces redundancy: many small molecules fragment to the same ions. For instance, choline-containing lipids, such as phosphatidylcholine and sphingomyelin, generate the highly efficient diagnostic phosphocholine product ion at m/z 184 (i.e., the protonated phosphocholine headgroup) in positive-ion MS/MS. In DDA, pairing this fragment with an isolated precursor m/z cleanly confirms the choline-lipid class for that specific precursor. But in DIA, multiple co-isolated precursors within a window can simultaneously contribute to the same m/z 184 signal, obscuring which phosphatidylcholine or sphingomyelin species—and which fatty-acyl composition or isomer—produced it [3,18,19]. This redundancy complicates unambiguous identification and quantitation, underscoring the need for narrow Q1 windows and high scan speed in metabolomics DIA [3]. DIA thus requires both high speed and narrow Q1 windows [3]. Ultimately, DDA and DIA are complementary: DDA provides clean spectra for abundant metabolites, while DIA ensures broad coverage of scarce but significant molecules, though running both remains time-consuming and sample-intensive [3].

A transformative new platform for high-throughput metabolomics

To address many challenges in metabolomics and other omics analyses, a new MS system has been introduced with radically improved capabilities that promise to transform the study of metabolomics, lipidomics (and proteomics). Prof. Dr. Nicola Zamboni's group at ETH Zürich studies metabolic regulation, cellular decisions, and pathological states, using cutting-edge MS/MS to reveal metabolite complexity and support personalised health research [4,3,4,20]. Zamboni emphasises that biologically critical metabolites are often of low-abundance, requiring advanced acquisition strategies and instrumentation. In general, the ZenoTOF 8600 system, with innovations like the OptiFlow Pro ion source, DJet ion guide and QJet ion guide, and the Zeno trap, improves ion generation, transmission, and detection, yielding a tenfold sensitivity increase over the ZenoTOF 7600+ system [21]. This enhances peak intensity tenfold and S/N ratio 12.9-fold, detecting more features in complex samples [21]. Indeed, metabolomics experiments showing up to 30-fold sensitivity gains and lipidomics revealing 2–3 times more features (see Figure 1) [3,11]. Spectra are exceptionally clean, enabling precise denoising, deisotoping, and peak detection [3,11].

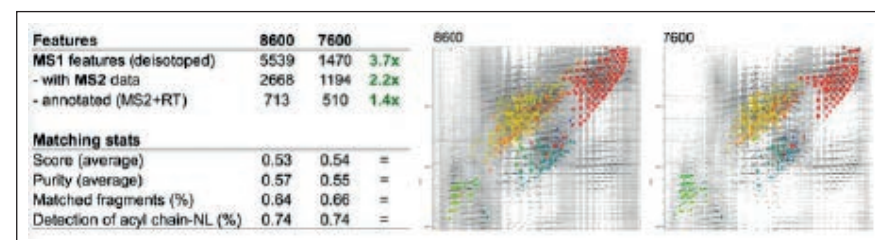


Figure 1: To verify the impact on detection and identification of complex samples, Zamboni and his team analysed a lipid extract by DDA (positive-ion mode, 15-minute reverse phase LC, top-10, 5 ms accumulation per MS²). They observed a two- to three-fold increase in the number of detected features, and a 50% increase in putatively MS2-annotated lipids ion by matching against a library of theoretical MS²-fragments (LipidOracle), without any apparent loss in match quality [11].

The system maintains high sensitivity across wide dynamic ranges, capturing both abundant and scarce metabolites, which is critical as metabolite concentrations span orders of magnitude [3]. Zamboni observed a six-order-of-magnitude intra-scan dynamic range (10–10 million), enabling improved MS1/MS2 feature detection using DDA and DIA workflows, and facilitating aggressive peak-picking to detect and characterise more metabolites than previously possible [3,11].

Ultrafast data-independent acquisition unlocks the complexity of metabolomics

With DDA, they found the number of features detected increased two- to three-fold in a lipidomics experiment compared with the ZenoTOF 7600 system [3,11]. Double the number of precursors were fragmented, and by matching the data against a library of theoretical MS2-fragments, there was approximately a 50% increase in putatively MS2-annotated lipids, without any loss in match quality observed [3,11]. Even in a challenging dilution series, they were able to detect many more features without any tangible loss in matching statistics [3,11]. For instance, at a 1000x dilution, they detected five times more features (see Figure 2) [11]. In a similar metabolomics experiment with DDA, the ZenoTOF 8600 system provided 80% more identifications [11]. This translates to being able to detect and identify more of the metabolites, especially the less abundant ones, and with the same confidence that they had with the more abundant ones [3]. Moreover, the improved sensitivity also meant that their DDA experiments with EAD could be sped up by a factor of three to five [3].

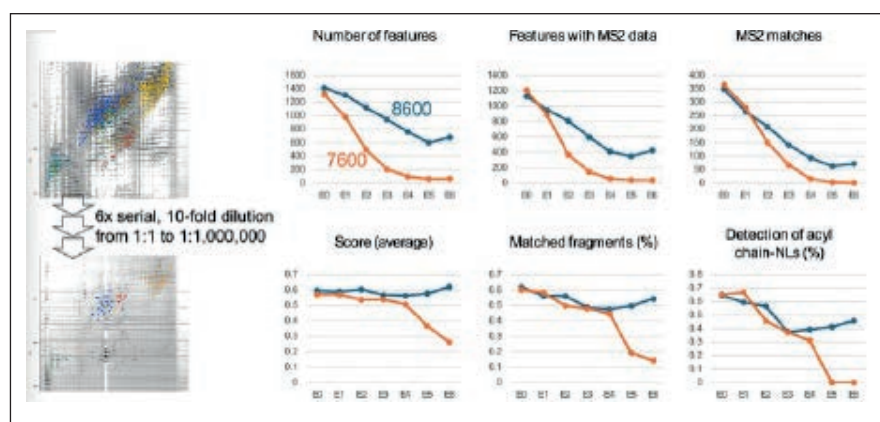


Figure 2: To test whether the increased sensitivity also applied to the most challenging analyses, Zamboni and his team analysed a dilution series on a short 2.2-minute reverse phase LC-MS method. At a 1000x dilution, the ZenoTOF 8600 system detected 5 times more features, with no tangible loss in matching stats [11].

For DIA, they used the new ZT Scan DIA 2.0 approach [11,21]. ZT Scan DIA 2.0 leverages the improved sensitivity and speed of the ZenoTOF 8600 system, to deliver DIA with a narrow isolation window over a wide m/z range in less than a second, without any compromise in spectral quality, LLOQ or dynamic range of detection [3,11]. The system can adjust the Q1 isolation size from 5 to 20 Da, depending on target m/z range and desired cycle time, and perform up to 858 MS2 scans per second [11]. The ZT Scan DIA 2.0 data can be transformed to DDA-like data automatically, with apparent precursor hops of 1 to 4 Da, that is, one-fifth of the Q1 isolation window [11]. This translates to the detection and identification of 50% more lipids based on MS2 data than DDA [11]. Zamboni and his team pushed the ZT Scan DIA 2.0 even further, using a very fast, 2.2-minute LC gradient and fragmenting all the precursors in a cycle time of 400 ms, including MS1. The result was the putative annotation of 1300 lipids (see Figure 3) [3,11].

ZT Scan DIA 2.0 was also tested successfully by Zamboni to analyse the metabolomics of other complex matrices, including milk, gut extract, and serum [3]. In plants, bitter-tasting alkaloids like those in *Swertia chirayita* (Roxb.) may be too scarce to appear in DDA-driven MS2 scans, leaving gaps in annotation [3,11]. Thus, while DDA provides clarity for abundant molecules, it cannot overcome the problem of complexity on its own [3]. Even so, a fast DDA analysis using the ZenoTOF 8600 system on *S. chirayita* plant extracts produced higher quality spectra and provided 80% more annotations (see figure 4)—across all of the classes, compared with the ZenoTOF 7600 system [3,11]. The breakthrough though came from ZT Scan DIA 2.0 with filtered spectra, which proved particularly powerful, putatively identifying 50% to 100% more compounds than DDA on the ZenoTOF 8600 system (see Figure 4) [3,11].

The technological advances

The OptiFlow Pro ion source incorporates the reliability and efficiency of the Turbo V ion source while providing flexibility for quickly switching flow rates [21]. It uses an orthogonal spray and V heater design with improved geometry to enhance electrospray ionisation (ESI) droplet desolvation and sensitivity [22,23]. Its pre-optimised probe/electrode positions and interchangeable towers allow easy switching between different ionisations (e.g., ESI and atmospheric pressure chemical ionisation [APCI]) and different flow rate regimes [22].

Ion transfer from the source to the mass spectrometer is critical for MS sensitivity [24]. Conventional orifice-and-skimmer designs scatter ions, reducing transmission, especially for low-abundance species [24]. The single-stage QJet ion guide overcomes this using ionic quadrupoles and RF fields: after the supersonic gas jets expand to form Mach discs, collisional and RF focusing refocus the ions into a narrow beam, enhancing

Lipid Mix	15 min DDA top-10 5 ms	15 min ZT Scan DIA 300-1000 m/z Q1 6 Da 898 Da/s	2 min ZT Scan DIA 100-1000 m/z Q1 20 Da 3000 Da/s
MS1 scans	2324	918	374
- scans per second (Hz)	2.6	1.0	2.6
MS2 scans	20285	597618	87142
- MS2 per second	23	651	660
Features	10290	4374	5019
- with MS2 data	4308	4276	4946
- annotated (MS2+RT)	633	975	1322
Quality			
Score (average)	0.51	0.49	0.48
Purity (average)	0.69	0.43	0.30
Matched fragments (%)	0.51	0.69	0.77
Detection of acyl chain-NL (%)	0.55	0.73	0.80

Figure 3: In lipidomics, ZT Scan DIA 2.0 led to 50% more MS2-based identifications than DDA (15-minute method), with better matching, with the exception of spectral purity, pointing to an increase in chimeric MS2 spectra. ZT Scan DIA 2.0 was pushed even further, to fragment all precursors in a cycle time of 400 ms, including MS1. In a 2.2-minute LC-MS, Zamboni and his team putatively annotated 1300 lipids by MS2 [11].

Evidence	MS2 score	tima score	MS2 Filter	Zeno Trap	Unique structures
Low	>0.2	>0.5	DDA	On	4.1x
			ZTScan 20 Da	On	
			ZTScan 20 Da Q1	On	
Intermediate	>0.3	>0.5	DDA	On	3.5x
			ZTScan 20 Da	On	
			ZTScan 20 Da Q1	On	
High	>0.4	>0.6	DDA	On	2.3x
			ZTScan 20 Da	On	
			ZTScan 20 Da Q1	On	

Figure 4: Analysis of metabolome extracts of the plant *Swertia chirayita* (Roxb.) using a fast mixed mode 3.5-minute LC reversed-phase anion-exchange method by DDA and ZT Scan DIA 2.0 (Isolation 20 Da, Q1-filtered). The aggregation of scores and final ranking were done by tima. Three alternative scoring schemes were applied: One with stringent cutoffs, passing only high-confidence hits supported by a strong match to the MS2 library, one with a loose scheme suited for discovery of new natural products, and one with an intermediate scheme between the two. The low-evidence scheme, With ZT Scan DIA 2.0, the results varied depending on the stringency scheme. In the low-evidence scheme, ZT Scan DIA 2.0 with Q1 filtering identified more than four times the number of unique structures than DDA. Even without Q1 filtering, ZT Scan DIA 2.0 identified more unique structures than DDA. These increases observed with and without Q1 filtering, over DDA were also observed with the intermediate- and high-stringency schemes, though to a lesser extent, which may have been curtailed due to the increasing stringency [2].

sensitivity across m/z ranges [24]. The dual-stage DJet ion guide, utilising multipoles and tapered electrodes, enhances ion focusing and transmission at higher gas pressures, thereby boosting sensitivity in systems such as the ZenoTOF 8600 system [25].

Continuous development of SWATH DIA has led to ZT Scan DIA 2.0, which leverages the Zeno trap [14, 26]. The Zeno trap boosts sensitivity by increasing duty cycle to >90% [16]. Conventional TOF systems lose the majority of ions—75–95% duty cycle—due to orthogonal injection timing mismatches [16], but the Zeno trap temporarily holds ions and releases them in synchronised bursts with the TOF pulse [16]. This improves ion detection four- to twenty-fold without sacrificing resolution or scan speed, capturing more MS/MS events per LC peak [16]. Speed, or with DIA equated to the number of MS/MS per unit time, is achieved by the high-speed scanning of Q1 and TOF pulse rate. This enables over an order of magnitude more MS/MS to be acquired vs. Zeno SWATH DIA.

In ZT Scan DIA 2.0, these gains enhance metabolomics performance [16,17]. DIA fragments all ions within defined m/z windows, requiring high sensitivity and efficient duty cycles to detect both abundant and low-abundance metabolites [16,17]. By maximising detected ions, the Zeno trap enables capture of scarce metabolites and

lipids while maintaining throughput and reproducibility, producing deeper, richer, and more reproducible datasets [16,17].

The final technological component is the optical detector, which Zamboni credits as the “secret sauce” of the ZenoTOF 8600 system for its “fantastic optics” [3]. Combined with Mass Guard technology, it allows high ion current operation while minimising contamination [21]. The Mass Guard technology filters ions before mass separation using T Bar electrodes in the Q0 region, removing contaminants and producing a cleaner sample plume. This reduces matrix effects, even with residue on the source curtain plate, maintaining peak analyser sensitivity up to twice as long and optimising instrument uptime [27,28].

For researchers like Zamboni, ZT Scan DIA 2.0 - leveraging the OptiFlow Pro ion source, QJet ion guide, DJet ion guide, Zeno trap, and new optical detector plus Mass Guard technology - finally enables unbiased, high-throughput metabolomics workflows that can capture the complexity of biology [3,11].

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