Uniting targeted (Zeno MRM-HR) and untargeted (SWATH) LC-MS in a single run for sensitive high-resolution exposomics.

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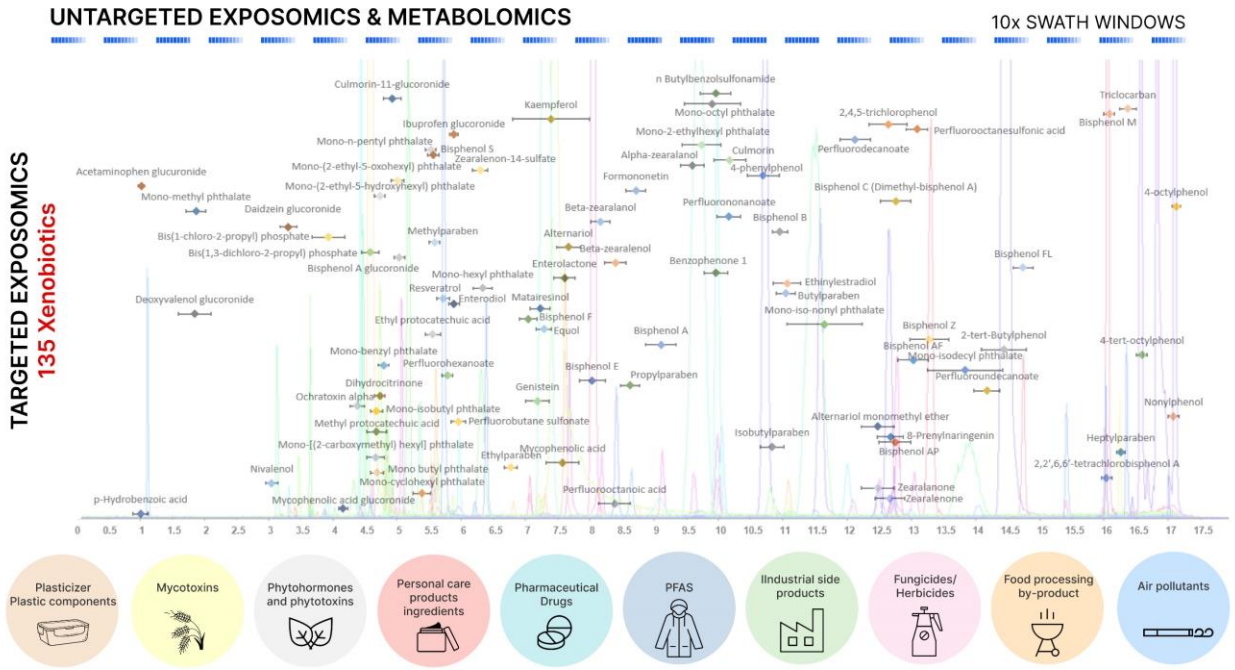
ABSTRACT: Traditionally, chemical exposure has been assessed by low-resolution mass spectrometry via targeted approaches due to the typically extremely low concentration of such compounds in biological samples. Nevertheless, untargeted approaches are now becoming a promising tool for a broader investigation of the exposome, covering additional compounds, their biotransformation products and possible metabolic alterations (metabolomics). However, despite broad compound coverage, untargeted metabolomics still underperforms in ultra-trace biomonitoring analysis. To overcome these analytical limitations, we present the development of the first combined targeted/untargeted LC-MS method, merging MRM-HR and SWATH experiments in one analytical run, making use of the Zeno technology for improved sensitivity. MRM transitions were optimized for 135 highly diverse toxicants including mycotoxins, plasticizers, PFAS, personal care products ingredients and industrial side products as well as potentially beneficial xenobiotics such as phytohormones. As a proof of concept, standard reference materials of human plasma (SRM 1950) and serum (SRM 1958) were analyzed with both, Zeno MRM-HR + SWATH and SWATH-only methodologies. Results demonstrated a significant increase in sensitivity represented by the detection of lower concentration levels in spiked SRM materials (mean value: 2.2x and 3x more sensitive for SRMs 1950 and 1958, respectively). Overall, detection frequency was increased by 45% (from 22 to 40 positive detections) in MRM-HR+SWATH mode compared to the SWATH-only. This work presents a promising avenue for addressing the outstanding challenge in the small-molecule omics field: finding balance between high sensitivity and broad chemical coverage. It was demonstrated for exposomic applications but might be transferred to lipidomics and metabolomics workflows.



Introduction

Humans are exposed to a myriad of chemicals throughout their lifespan, including industrial pollutants, food nutrients and contaminants, synthetic products in cosmetics among many others. With that, an increasing interest in the measurement of known and novel chemicals as well as their possible effect in human health is now taking place specially under the recently coined idea of exposomics. First conceptualized by Wild[1], the exposome has been defined by Miller and Jones[2] as "*the cumulative measure of environmental influences and associated biological responses throughout the lifespan, including exposures from the environment, diet, behavior, and endogenous processes"*.

When designing any small molecules "omics" such as exposomics or metabolomics, one of the first decisions is typically the choice between targeted and untargeted approaches. The first normally relies on the use of low-resolution instruments and aims at providing quantitative data with increased sensitivity when compared to high resolution instruments. These methods normally include tens to hundreds of compounds and are commonly used for confirming previous hypothesis. In exposomics, for example, these methods are used for large biomonitoring studies due to their capability of providing quantitative information for trace-level chemicals[3], ultimately providing policy markers with information for the science-to-policy interface regarding chemical exposure[4]. Untargeted approaches, on the other hand, have the advantage of detecting, in principle, any compound at a given concentration that is able to be ionized and detected. For that reason, untargeted methodologies have gained momentum in the last years specially under the strategies termed suspect screening analysis (SSA) and non-targeted analysis (NTA), which enables for the screening of hundreds to thousands of (potentially novel and relevant) chemicals[4]. In addition, untargeted data acquisition approaches can be used for the assessment of the "effects" part of the exposure by detecting the normally more abundant endogenous metabolites, i.e., via metabolomics studies.[5]

When acquiring such type of data using an LC-MS or LC-HRMS set ups, different acquisition modes can be selected. For targeted analysis, multiple reaction monitoring (MRM) is the common choice, and it is based on the detection of usually two fragments for each analyte allowing for high sensitivity and specificity. For untargeted data, on the other hand, data dependent acquisition (DDA) and data independent acquisition (DIA) are the most commonly used approaches. The first relies on the selection of the n most intense *m/z* detected at MS1 level to further selection and fragmentation (MS^2 acquisition), normally leading to good quality MS^2 spectra of a few of the detected features.[6] DIA, on the other hand, has the capability of fragment virtually all detected signals and later performing spectra deconvolution afterwards in order to assign each fragment to the most likely precursor. As a result, the number of MS^2 spectra acquired is higher, leading to better possibilities of identification specially for features with lower intensity that would not be selected by DDA experiments. As a downside, deconvolution algorithms are not 100% accurate and MS^2 spectra become either more polluted (with miss assigned fragments) or less informative (with missing fragments assigned to a different precursor), which ultimately leads to worsened spectra matching. SWATH (sequential window acquisition of all theoretical fragments) is one type of DIA acquisition mode in which sequential ranges of precursor isolation windows are selected and fragmented, allowing for the acquisition of MS^2 data for virtually all ions detected at MS^1 level.[7,8]

Fiehn and Cajka (2016)[9] reviewed targeted and untargeted metabolomics data in order to evaluate the possibility of having one method with good performance for both using high resolution mass spectrometry (HRMS). As future directions, the authors highlight the efforts needed towards the combination of targeted and untargeted metabolomics approaches via untargeted MS1 data acquisition and high-resolution MRM transitions for targeting multiple metabolites. More recently, Bird et al (2023)[10] have introduced a new workflow based on orbitrap and/or TOF systems (SQUAD, Simultaneous Quantitation and Discovery Analysis) aiming at combining both targeted and untargeted with the main objective of finding the equilibrium between untargeted and targeted approaches in a single experiment.

This paper describes, to the best of our knowledge, the first targeted/untargeted, single-injection LC-MS method for integrated exposomics and metabolomics, combining the advantages of both approaches, i.e., ultra-high sensitivity (targeted) and broad coverage and retrospective data analysis (untargeted). The use of the recently introduced Zeno technology allowed for significant increase in sensitivity. In addition to presenting the results obtained for different xenobiotics in targeted mode (135 compounds) as well as the results for the analysis of standard references materials (SRM 1950 and SRM 1958), we also describe the method development process that is expected to support other researchers working in the field of small molecule omics.

Experimental Section

Chemicals and Sample preparation

Multi-component standard solutions containing 135 compounds (**Table S1**) were prepared at six different concentrations levels. Concentrations were compound-dependent and are described in details in **Table S5.**

Standard reference materials (1950 and 1958) were purchased from NIST and stored at -80°C. In total, 9 replicates of each SRM were prepared following the protocol previously described by Jamnik et al (2022)[11]. For extraction, 30 µL of SRM materials (1950 and 1958 – NIST) were mixed with 120 µL of extraction solution (ACN/MeOH 50:50, v/v) in Eppendorf tubes and sonicated for 10min on ice. The extraction solution contained labeled IS (**Table S2**) for accounting for the total variation (sample preparation + analytical). A 2 h protein precipitation step was performed at -20°C afterwards, followed by centrifugation for 10 min at 18,000 x g at 4°C. The supernatant (120 µL) was transferred to a new Eppendorf tube and dried under vacuum at 4°C overnight. From three of the nine replicates, three were reconstituted using 120µL of ACN/H2O 10:90,

v/v). The additional 6 replicates were used to build a matrix-matched calibration curve spiked at the same levels as the standard solutions in solvent (Cal01 to Cal100, **Table S4**). Samples were vortexed for 10min after reconstitution and centrifuged at 18,000g and 4°C. Finally, 100uL of the supernatant was transferred to an amber glass vial for data acquisition. Sample preparation protocol was chosen to maximize chemical coverage[12].

LC-HRMS instrumentation and parameters

A LC Inifintiy II 1290 (Agilent) coupled to a Zeno TOF 7600 with a Turbo V source (SCIEX) was used. Chromatographic separation was based on the same method described by Jamnik[11] *et al* (2022). In summary, a 20 min reversed-phase gradient was employed on an HSS T3 column (Waters), with a flow rate of 0.4 mL/min and an injection volume of five microliters.

LC-MS data acquisition was performed for both a SWATH-only method and the newly developed Zeno MRM-HR + SWATH methods. Ion source parameters were standardized across both methodologies, as follow: GS1 – 50 psi; GS2 – 50 psi; curtain gas – 35 psi; CAD gas – 9; temperature – 550 °C. In both cases, MS^1 data was acquired from *m/z* 100 to 1000 Da, with declustering potential at 80V (-80 V for negative mode) and collision energy at 10V (-10 V for negative mode). Spray voltage was set at 5500 V for positive ioniozation mode (ESI+) and -4500 V for negative ionization mode (ESI-).

For the developed Zeno MRM-HR + SWATH data, accumulation time was set at 0.05 for individuals MRM transitions and at 0.02 for each SWATH window (n= 10). SWATH windows were optimized individually for each SRM using the SWATH windows optimizer provided by SCIEX. Both SWATH-only and MRM-HR+SWATH methods had the exact same parameter for SWATH acquisition, including windows range, declustering potential (80 or -80V) and CE, as described in details in **Table S3**. For MRM experiment, Start/Stop option was selected, with TOF Start Mass at *m/z* 50 and TOF Stop Mass set as compound-dependent by adding 2 *m/z* units to precursor *m/z*. Retention time windows, declustering potentials and collision energies were adjusted for each compound (see Results section) and Zeno Pulsing was activated at a threshold of 8000 cps.

To allow for a point of comparison, the cycle time for the SWATH-only method was adjusted to a similar value as the maximum scan time for the MRM-HR + SWATH method. To evaluate possible differences in increased accumulation time for MS^1 or SWATH windows, two different approaches were taken. For the Zeno MRM-HR+SWATH in negative mode, accumulation time for MS^1 was increased from 0.05 s to 0.15 s (for both SRM 1958 and SRM 1950). For positive mode, on the other hand, MS^1 accumulation time was kept the same as for MRM-HR + SWATH method (0.05 s) and, instead, accumulation times for SWATH-only windows were increased such as total cycle time would compare to maximum cycle time for MRM-HR + SWATH, as previously explained. For instance, maximum scan time for Zeno MRM-HR +SWATH method was 0.435 s while SWATH-only total cycle time was 0.25 s (0.05 s for MS1 and 10 x 0.02 s for each SWATH window). Therefore, at the maximum number of overlapping MRM windows, the total cycle time would be 0.685 s. Based on that, the SWATH-only method for positive mode was set as total scan time of 0.643 by keeping MS1 acquisition at 0.05 s and increasing SWATH windows accumulation time from 0.02 to 0.05 s. For direct comparison, key method parameters are also summarized in **Table 1.**

Table 1. Parameters employed across the different MS methods. For SWATH-only, cycle time represents the total scan time (constant across the whole chromatographic run). For MRM-HR+SWATH, cycle time represents the maximum scan time, since cycle time changes along the chromatographic run due to its dependency on the number of overlapping MRM transitions.

	NEG		POS	
	Swath Only	MRM-HR+ SWATH	Swath Only	MRM-HR+ SWATH
MS^1 accumulation time (s)	0.15	0.05	0.05	0.05
Total number of SWATH Windows	10	10	10	10
SWATH Window accumulation time (s)	0.06	0.02	0.05	0.02
MRM Transitions	-	83	-	52
Accumulation time MRM transitions (s)	-	0.05	-	0.05
Cycle time (s)	0.843	0.763	0.643	0.685

Finally, the data acquisition sequence was set such as data for MRM-HR + SWATH and SWATH-only methods would be acquired sequentially for the same sample. That approach was taken in order to minimize batch effects when comparing the results for the same sample between the two different methodologies.

Compound optimization

Two key parameters namely collision energy (CE) and declustering potential (DP, a voltage applied to the orifice to minimize solvent clusters and help on desolvation of ions) were optimized by either direct infusion (DP) or using a LC-MS method (CE). For DP, sub-mixes of all compounds at either 10 or 100 ng/mL (depending on ionization efficiency) of all standards were constantly injected in the source using a syringe pump set at 5µL/min. Automatic DP optimization was performed in SCIEX OS in the range from 10 to 300 V. For CE optimization, MS^2 spectra was first acquired using DDA with inclusion list for each of the sub-mixes at in the range of 100 to 1000 ng/mL. MS^2 spectra were acquired using the collision energy spread (CES) at 35±15 V. This allows for a combined (and representative) spectra of 3 collision energies: 20, 35 and 50 V. Next, the 5 most intense fragments (or fragments known to work best in our targeted approach using low-resolution instruments[11]) were selected for further optimization. Finally, an MRM-HR method was created with all selected

transitions, and sub-mixes were injected multiple times using the above described LC-MS method with one specific CE for each injection, in the range from 15 to 60 V, with steps of 5 V (total of 9 injections per submix). Peak areas were used to select the best fragment and CE.

Data analysis and quality check

Targeted data analysis (MRM-HR) was performed using SCIEX OS version 3.0.0.3339. Regardless of the MS mode of acquisition, extracted ion chromatograms for SWATH-only (MS^1 level) and MRM+SWATH (MS^2 level) were extracted using the default window of 0.02 Da as recommended by SCIEX. Chromatograms were integrated by the AutoPeak method and smoothed using noise filter algorithm with the Low option. Concentrations were calculated for all compounds detected in non-spiked SRMs by the use of standard addition method.

For quality control, peak areas of the internal standards in ESI- mode were inspected in MS^1 mode for both SWATH-only and MRM-HR+SWATH. From the labeled IS employed, none was found to be ionized in ESI+ so, relative standard deviation values (RSD) were only estimated to be similar to ESI-. Peak areas for MRM+SWATH mode have presented higher RSD **(Table S4),** which may be explained by the lower values for peak areas. These may additionally be related to the fact that accumulation time at MS^1 for SWATH-only in ESI- mode was set three times higher than MRM-HR + SWATH, therefore providing better sensitivity. Nevertheless, it is also important to highlight that this variation is likely to be considerably smaller for the MS^2 data in MRM-HR + SWATH due to the increased peak areas provided by the Zeno Pulsing technique, which is, in fact, the data presented and evaluated along this work. In addition, labeled IS were added to the extraction solution and, therefore, account for the sum of the variances of both extraction process and analytical run. For PFOS for example, recovery at very low concentration was reported[11] as 56 ± 25%, representing 50% of variance only related to sample preparation. As the average RSD was significantly below this value, we may infer that analytical variance was minimal. Total variance for recovery was also reported[11] for genistein and estradiol at 30% and 14%, respectively, while RSD in our analysis were at 24% and 23% for MRM-HR+SWATH data.

Finally, MS^2 matching for both exogenous (xenobiotics) and endogenous (metabolites) compounds was performed in MS-DIAL (version 5.2.240218.2) against Mass Bank of North America (MoNA) and MS-DIAL spectral libraries. For ESI+, *LC-MS/MS Positive Mode* (MoNA - 99,260 spectra) and *ESI(+)-MS/MS from authentic standards* (MS-DIAL, 324,191 spectra) were used. For ESI-, *LC-MS/MS Negative Mode* (MoNA - 47,058 spectra) and *ESI(-)-MS/MS from authentic standards* (MS-DIAL - 44,669 spectra) were employed. A spectral match of 70% was used as a threshold with no limitation at the lower end for dot and reverse dot score products (since data was manually curated afterwards).

Balancing accumulation time, RT window and total cycle time

Considering the significant number of compounds included in each method, i.e., 83 in ESI- mode and 52 in ESI+, it is essential to optimize the total cycle time needed to acquire 1xMS^1 + 10xSWATH windows + 1xMS^1 + 83xMRM transitions. This is related to the fact that, as a rule of thumb, a chromatographic peak should have at least between six and ten data points[13] in order to achieve satisfactory gaussian shape and reproducibility. Consequently, the above-mentioned acquisition cycle has to be repeated at least six times along the width of every chromatographic peak for proper quantification. Therefore, accumulation time for each of the mentioned experiments must be fine-tuned. The parameters found to be the most important are the accumulation time and the retention time window of each MRM transition, both due to the high number of overlapping MRM transitions. To optimize RT windows, MS^1 level data was acquired for the mixture of analytical standards at the highest concentration level (individual concentrations for each compound can be found in **Table S5)**. From this data, we retrieved the peak width for each compound, and opted for a RT window that would be defined by ± 0.75 x peak width. For example, methylparaben (RT = 5.55 min) was detected with a baseline peak width of ~6.6 s, and, therefore, RT window was set at ±5 s. Manual fine-adjustment was also performed after, especially for compounds which are prone to suffer from relatively larger RT shifts (such as phthalates and glucuronide conjugates). Final RT windows are described for each compound in **Table S1. Figure 1** presents the final and optimized methods with extracted ion chromatograms, RT and RT windows for each compound of the target panel.

Benchmarking with SRM

Evaluation of detection levels (sensitivity)

One of the main parameters that allows for a sensitivity comparison between two methods is signal-to-noise ratio (S/N). Nevertheless, comparing S/N between different levels of MS data, i.e., MS^1 for SWATH-only and MS^2 for MRM-HR+SWATH would not provide a meaningful comparison. One of the main reasons for this is the expected increase in specificity when dealing with MS^2 data, resulting, frequently in a flat (non-existent) baseline which, consequently, makes noise estimation impractical. In addition, S/N estimation algorithms included into SCIEX OS (namely Standard Deviation and Peak to Peak) require a noise region to be defined. Nevertheless, due to the use of very narrow RT windows for MRM experiments, selecting this noise region becomes unrealistic and, as a consequence, estimated S/N values turn out to be not representative. For these reasons, we have opted to report the lowest detected concentration level detected for each analyte in the spiked matrix as a comparative basis for each method's sensitivity. This approach was taken for all compounds that were not detected in non-spiked matrix (for compounds detected in non-spiked matrix, see next section). For SWATH-only

Results and Discussion

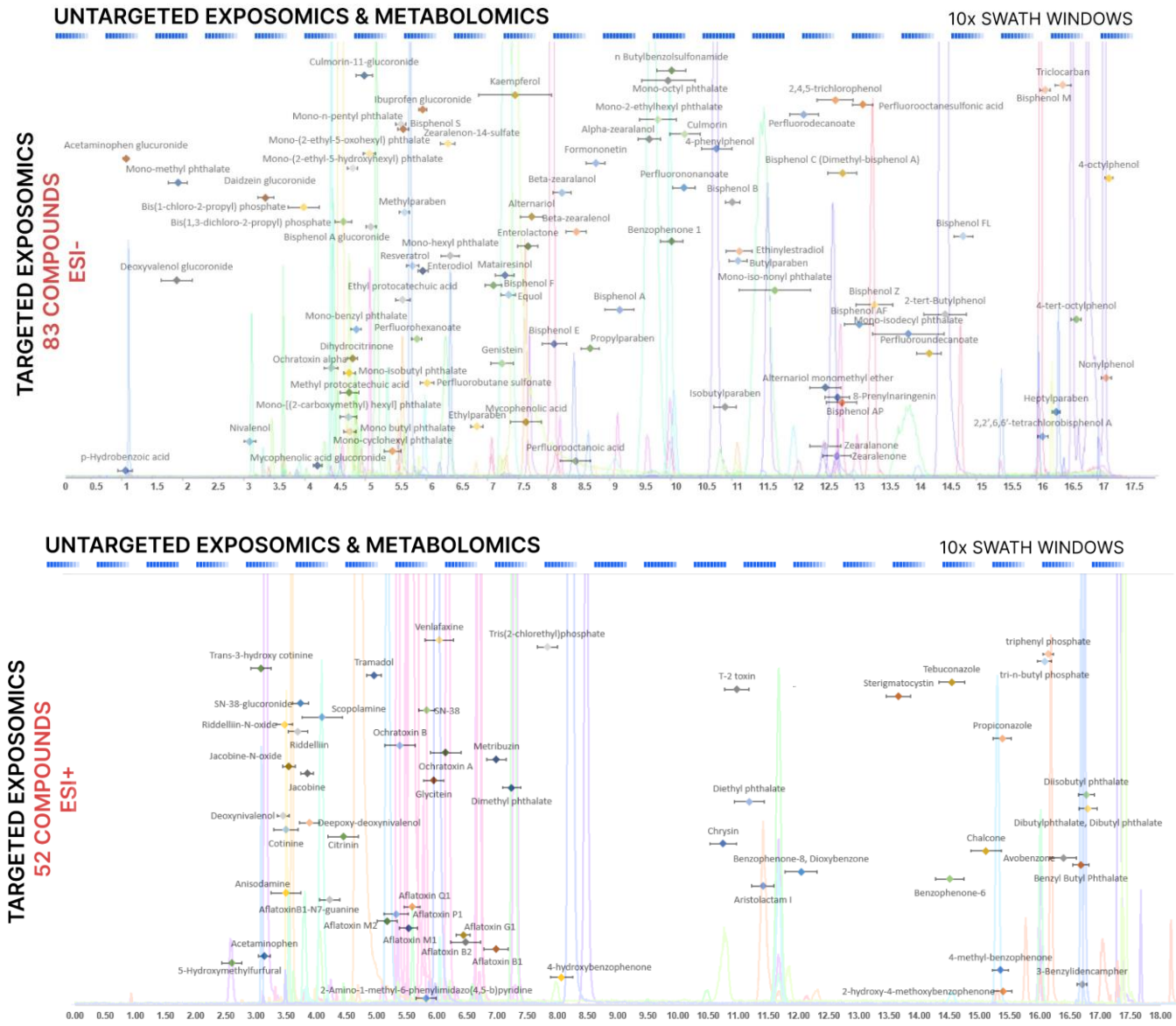


Figure 1. Overview of extracted ion chromatograms, retention times and detection windows in both ESI- and ESI+ mode. A total of 135 compounds from a variety of chemical classes were included in the MRM-HR experiments, with 83 compounds in ESI- mode and 52 in ESI+ mode. In addition to the targeted quantification of the toxicant panel, the acquisition of SWATH data allows for the untargeted screening of additional xenobiotics, their biotransformation products as well as endogenous metabolites (no peaks depicted).

data, MS^1 level is reported since compounds could be detected at lower levels when compared to MS^2 level 1 (which may be explained for the non-specific CE chosen for each compound). For MRM-HR +SWATH data, on the other hand, MS^2 level data showed larger values for peak areas when compared to MS^1 data, as also expected due to the optimized CE and the use of the Zeno. In summary, detected concentrations in MRM-HR+SWATH had a 2.1x and 2.3x times higher mean concentration for SRM 1950 in ESI- and ESI+ mode, respectively. For SRM 1958, a four times higher mean detected concentration was found for ESI-, while ESI+ presented a two-fold increase. Even for compounds in which the lowest detected level was the same between MRM-HR + SWATH and SWATH-only, there were prevalent larger peak areas for the first, as depicted in **Figure 2** for four exemplary compounds. The complete description of all detected levels for both methods is reported in **Table S4**.

Detection and quantification of xenobiotics

Different xenobiotics were detected in both SRM 1958 and SRM 1950 and are summarized in **Table 2**. Concentrations were calculated based on the calibration curves built for MRM-HR + SWATH and SWATH-only methods (**Tables S6 and S7**). Concentration values were very similar for most of the compounds (with the exception of nonylphenol and dibutylphatalate for SRM 1958 and PFOA and nonylphenol for SRM 1950). The results showcase the higher capability for compound detection. From the total of 21 compounds detected in SRM 1958, roughly 43% (9) were detected in MRM-HR+SWATH mode only, covering different classes (bisphenols, PFAS, phthalates, and herbicides/pesticides such as metribuzin). Similarly, 47% of the compounds detected in SRM 1950 (9 out of 19) were found only with the use of the combined method.

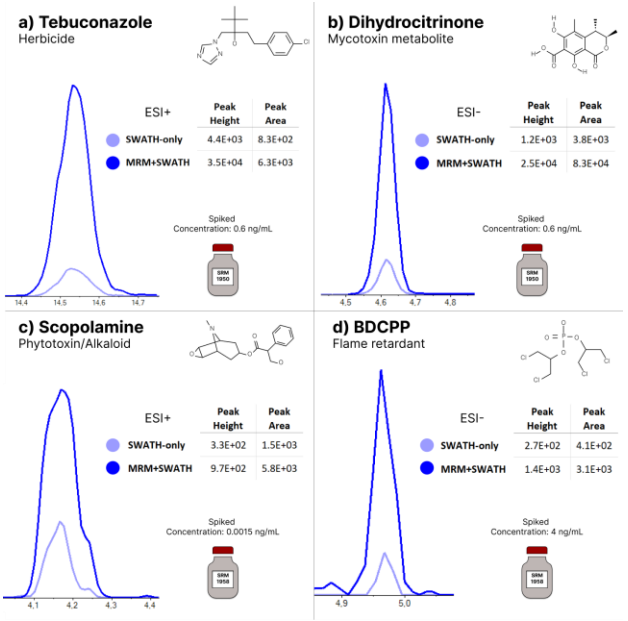


Figure 2. Extracted ion chromatograms for the lowest detection levels of four illustrative compounds in a) SRM 1950 – ESI+; b) SRM 1950 – ESI-; c) SRM 1958 – ESI+ and d) SRM 1958 ESI-. Despite not being able to detect each compound at a lower concentration level, MRM-HR+SWATH approach is able to provide larger peak areas and, consequently, better sensitivity.

Table 2. Detected compounds with calculated concentration for MRM-HR+SWATH x SWATH-only.

		Detected Compound	Calculated Concentrations (ng/mL)	
			MRM-HR + SWATH	SWATH-only
SRM 1958	ESI (-)	TCBPA	1.8	**ND**
		Bisphenol S (BPS)	0.023	0.028
		Ethylparaben	0.0018	**ND**
		Methylparaben	0.49	0.66
		5-OH-MEHP	0.8	1.25
		Mono-benzyl phthalate (MBeP)	0.5	0.7
		n- butylbenzolsulfonamide	32	24
		Nonylphenol	14	30
		Perfluorononanoate	1.8	**ND**
		Perfluorooctanoic acid (PFOA)	5.7	5
		Perfluorooctanesulfonic acid (PFOS)	4.8	5.8
		Propylparaben	0.06	**ND**
	ESI (+)	2-hydroxy-4-methoxybenzo-phenone (BP-3)	2	**ND**
		Acetaminophen	56	53
		Benzyl Butyl Phthalate	4.3	**ND**
		Avobenzone	57	**ND**
		Cotinine	4.9	4.7
		Dibutylphthalate	100	22
		Diethylphthalate	13	**ND**
		Metribuzin	1	**ND**
		Trans-3-hydroxy-cotinine	1.6	2
SRM 1950	ESI (-)	Bisphenol A (BPA)	3.7	4.8
		Ethylparaben	0.21	**ND**
		Methylparaben	4.6	4.2
		n- butylbenzolsulfonamide	54	42
		Nonylphenol	3.5	14.5
		Perfluorononanoate	1.4	**ND**
		Perfluorooctanoic acid (PFOA)	6.3	3.9
		Perfluorooctanesulfonic acid (PFOS)	5.1	6
		Propylparaben	0.29	**ND**
	ESI (+)	2-hydroxy-4-methoxybenzo-phenone (BP-3)	4.8	**ND**
		4-methyl-benzophenone	2.9	**ND**
		Acetaminophen	820	670
		Benzyl Butyl Phthalate	2.3	**ND**
		Avobenzone	87	**ND**
		Cotinine	123	124
		Dibutylphthalate	80	241
		Diethylphthalate	57	**ND**
		Metribuzin	2.4	**ND**
		Trans-3-hydroxy-cotinine	50	45

Annotation of endogenous metabolites for integrated metabolome analysis

For evaluating the performance of each method for compound annotation, the non-spiked samples of SRM 1950 and SRM 1958 were investigated, independently, for both MRM-HR+SWATH and SWATH-only using MS-DIAL (two sets of triplicate non-spiked samples see Material and Methods, Data analysis and quality control section for detailed information). As an initial assessment, the number of features obtained using the same processing method was evaluated. As observed in **Table 3**, MRM-HR+SWATH data retrieved a larger number of features for all experiments. When comparing the total number of MS^2 matching retrieved from the libraries, SWATH-only data initially seemed to outperform the newly developed MRM-HR+SWATH method.

Table 3. Performance variables for MS2 matching for endogenous compounds against open-source MS2 libraries. Results indicate a superior matching frequency for SWATH-only data. Nevertheless, manual data curation evidences a similar matching rate/score between both methods, confirming the capability of the newly developed methodology to assess different parts of the metabolome.

		Method	Number of features	Number of MS^2 matches
SRM 1958	ESI(+)	SWATH-Only	2806	237 (8%)
		MRM-HR +SWATH	3602	178 (5%)
	ESI(-)	SWATH-Only	2236	113 (5%)
		MRM-HR +SWATH	2610	62 (2%)

SRM 1950	ESI(+)	SWATH-Only	2990	179 (6%)
		MRM-HR +SWATH	3832	184 (5%)
	ESI(-)	SWATH-Only	2477	145 (6%)
		MRM-HR +SWATH	2915	91 (3%)

After manual curation of the data and removal of false positives, it was observed that annotation of compounds was, in fact, of high similarity between both methods. More specifically, all compounds that could be reasonably annotated (minimum of 2 matching ions and mass error below 5 ppm for parent compound) in SWATH-only mode had the same suggested compound in MRM-HR+SWATH, with highly comparable matching scores (dot product, reverse dot product and total score). Mirror plots of annotated compounds for SRM 1950 in both ESI+ and ESI- including matching scores is described in details in **Tables S7 and S8, along with all mirror plots for both methodologies (Figures S1-S45).** Since a large overlap was observed for the compounds annotated between SRM 1958 and SRM 1950 in both ionization modes, we opted to not include mirror plots for the first as a way to avoid repetitive description of the data. Despite the clear similarity in MS^2 matching performance, it is important to highlight that SWATH-only method indeed outperformed MRM-HR+SWATH data in the quality of a few spectral matches, more prominently for low abundance compounds (peak height <1000 counts). **Figure 4** presents two illustrative cases of spectral matches. The first (phenylalanine, **Figure 4a**) showcases an optimal MS^2 match with the library for both SWATH-only and MRM-HR+SWATH, outlining the high MS^2 spectral similarity between both methods. **Figure 4b,** on the other hand, intends to exemplify a case for which, despite the good matching score for both methods, spectra similarity is lower across both methods, with missing fragments for the deconvoluted MS^2 spectrum from MRM-HR+SWATH. This may be explained by the lower accumulation time for both MS^1 and MS^2 data, possibly hindering the deconvolution process (i.e., matching fragments with precursor ions). The same trend can be observed, for instance, for citric acid **(Figure S1)** and tyrosine **(Figure S5)** and as highly similar spectral matches between both methods (with peak heights all above 1500) as well as for 4-nitrophenol **(Figure S21)** and glychocolic acid **(Figure S22)** as less reliable matches in MRM-HR+SWATH due to missing fragments in the deconvoluted spectra and with peak heights for both compounds at around 300 counts

Limitations

Despite the clear method capability towards a more sensitive and comprehensive analysis combining metabolomics and exposomics as well as targeted and untargeted in a single run, the method is not out of its limitations. Depending on the number of compounds, optimizing chromatographic separation, collision energy and declustering potential and total cycle time is time-intensive, especially for a relatively large number of compounds as described herein. We also acknowledge the lack of labeled internal standards for absolute quantification. Despite adding such compounds would lead to a more accurate calculation of the concentrations, it should be considered that additional MRM transitions would be required, further elongating total cycle time. Regarding the untargeted data, there is still the need to evaluate the influence (if any) of different SWATH accumulation times in terms of signal intensity, quality of deconvoluted spectra and, ultimately, in the total number of annotated compounds. Moreover, total accumulation times (and consequently, cycle times) may still be fine-tuned. One example is the MRM accumulation time, which can be set as compound-dependent rather than as a unique value for all, depending on overlapping transitions and total cycle time.

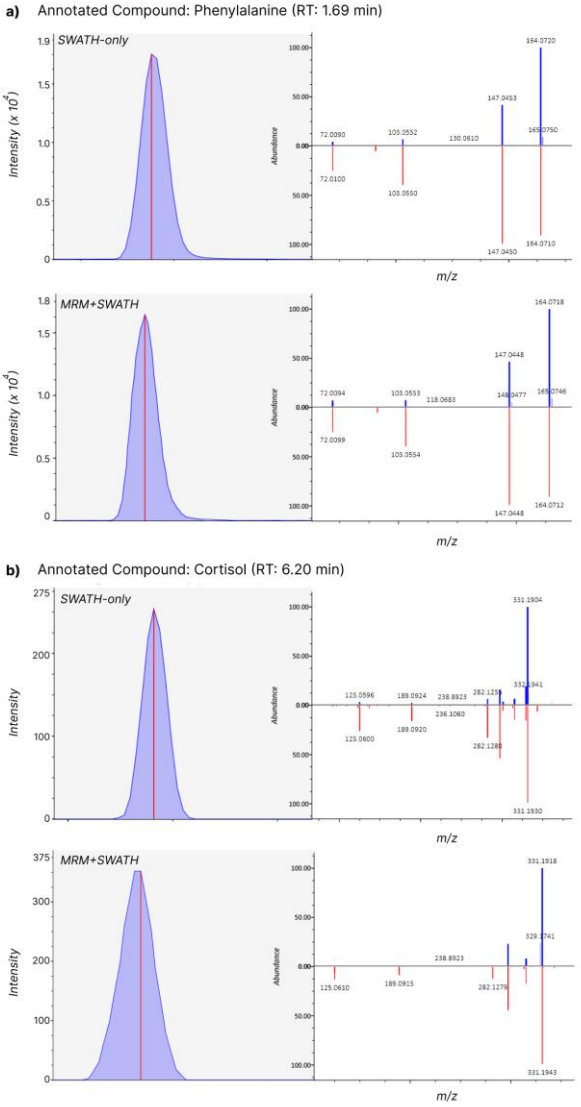


Figure 3. Exemplary MS^2 spectra matching for a) phenylalanine and b) cortisol as highly and low correlated spectra (respectively) for SWATH-only and MRM-HR+SWATH methods. For cortisol, three fragments are not present in the deconvoluted spectrum of MRM-HR+SWATH (*m/z* 282, 189 and 125).

Conclusion and Future Perspectives

We described, a combination of targeted and untargeted approaches for a single-injection LC-HRMS exposomics/metabolomics. The method is also the first of its type to make use of the Zeno trap in MRM-HR mode, recently developed with the aim to correct for duty cycle issues related to TOF instruments, allowing for increased sensitivity. The results highlight the method capabilities and its potential to detect xenobiotics present at low concentrations that otherwise would not be detected (or would be poorly detected) using a SWATH-only methodology. The method also allows for future customized developments, not only in terms of which compounds to include in the targeted list, but also regarding the choice of a semi or absolute quantitation. In the field of metabolomics, for instance, this approach could be employed to detect poorly ionized and/or low concentration compounds, e.g., endogenous hormones. Additionally, in applications in which limited amount of sample is available (such as for serum samples for premature infants) or sample amount is limited by the sample collection technique (such as dried blood spots or volumetric microsampling devices) may also benefit, producing targeted and untargeted data acquisition in a single injection. In conclusion, we demonstrated that this type of data acquisition shows great potential for future application in any field of small molecules "omics" and can be an interesting alternative for the long-time discussed compromise between sensitivity and coverage.

ASSOCIATED CONTENT

Supporting Information

Supplementary information provided in Excel (Supplementary tables S1 to S7) and in PDF Document (Supplementary tables S87 and S9, Figures S1 to S45) are included and provide with additional information regarding the method and the results

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Author Contributions

The manuscript was written through contributions of all authors.

DATA AVAILABILITY

The raw data files have been submitted to the Metabolights data repository.

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