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Review

Extracellular vesicle isolation as an analytical determinant in mass spectrometry-based omics: A review

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Abstract: Extracellular vesicle (EV) isolation critically influences data quality in mass spectrometry (MS)-based omics. Common descriptors such as recovery and purity are insufficient to predict analytical performance, particularly in biofluid-derived samples where co-isolated non-vesicular background can dominate molecular readouts. Thus, the key issue is not only EV recovery but also the extent to which background components are carried into the final fraction. This review treats EV isolation as part of the analytical system rather than a neutral preparative step. Precipitation-, size exclusion chromatography-, and centrifugation-based workflows are evaluated based on EV enrichment, background carryover, reproducibility, and measurable molecular coverage. Notably, high recovery does not necessarily translate into improved analytical depth if dominant background remains unresolved. Precipitation-based methods illustrate this trade-off clearly: while advantageous for scalability and low-input samples, they often co-enrich substantial background, limiting EV-specific interpretation. Accordingly, their use is best considered in combination with additional cleanup steps or with appropriate analytical caution. Overall, workflow selection should be guided by analytical suitability rather than yield alone. For MS-based EV omics, the most effective approach is the one that produces a reproducible and interpretable fraction with sufficient access to EV-associated molecular features.

Keywords: extracellular vesicles, mass spectrometry, proteomics, metabolomics, lipidomics

1. Introduction

Extracellular vesicles (EVs) have attracted considerable attention because they carry proteins, lipids, glycans, and nucleic acids that reflect the physiological and pathological states of their cells of origin. Consequently, EV-associated cargo has become an important target in biomarker research across diverse biofluids, particularly plasma and serum. Mass spectrometry (MS)-based omics has further advanced this field by enabling comprehensive molecular profiling beyond a limited set of predefined markers.[1-3] However, in EV omics, the molecular information obtained by MS is not determined solely by biological origin. It is also strongly influenced by how EVs are isolated prior to analysis. Plasma and serum exemplify

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this challenge: although they contain EVs, they are also dominated by abundant soluble proteins, lipoprotein-associated particles, and other non-vesicular components. As a result, isolation procedures do not simply enrich EVs but also co-determine the extent and composition of background material that is introduced into the analytical workflow.[4-6]

Fig. 1 schematically illustrates the relationship between EV enrichment and co-isolated background, depicting EV isolation as a process that simultaneously shapes both components and ultimately determines the measurable molecular space in MS-based analyses. When non-vesicular components remain abundant, they can dominate MS readouts and obscure lower-abundance EV-associated signals. Accordingly, higher particle recovery does not necessarily lead to increased EV-specific molecular information. Likewise, fractions that appear acceptable based on single metrics such as particle count or total protein may still perform poorly in downstream proteomic, metabolomic, lipidomic, or glycomic analyses if analytically dominant background is not adequately controlled. Considering these factors, EV isolation directly governs what becomes detectable, what remains masked, and how confidently detected molecules can be attributed to EV-enriched material.[7-9]

On this basis, this review considers EV isolation not as a neutral preparative step but as an integral component of the analytical system. Rather than comparing workflows solely in terms of recovery, we examine how precipitation-, size exclusion chromatography-, and centrifugation-based approaches shape the composition and analytical usability of the resulting fractions. Emphasis is placed on their impact on

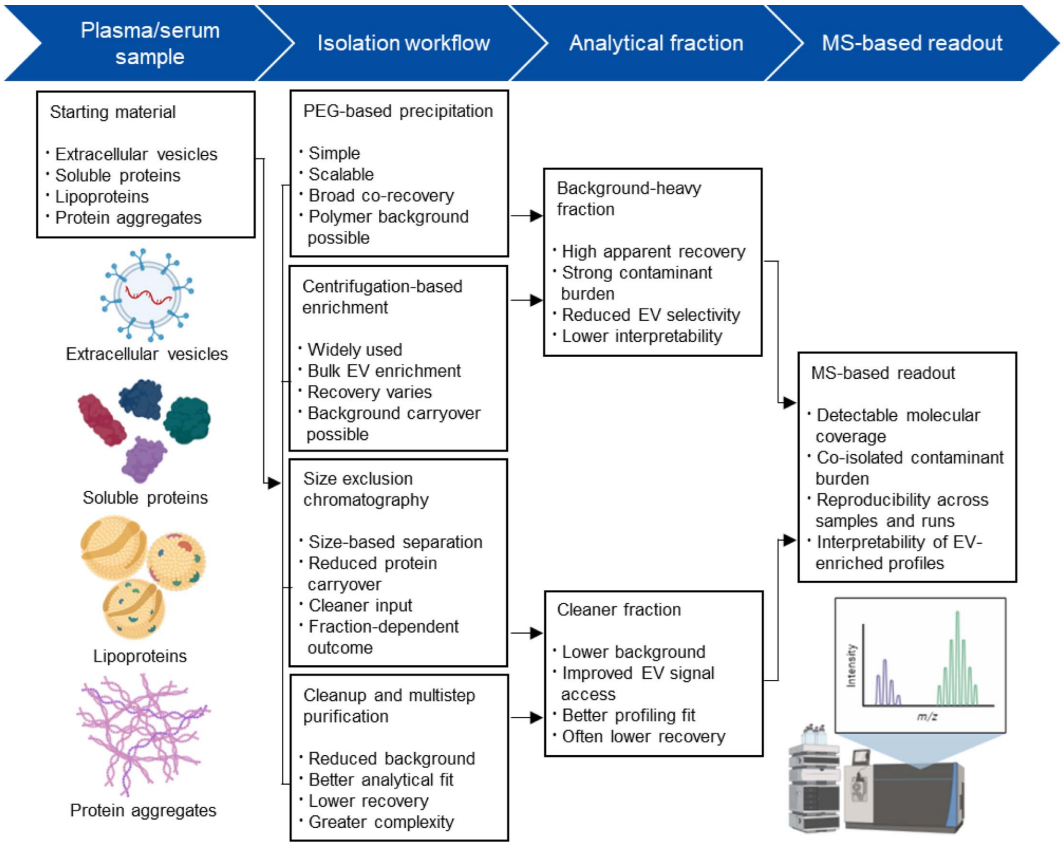

Fig. 1. EV isolation workflows define the analytical fraction reaching MS-based omics. Different workflows alter the burden of co-isolated background and therefore influence downstream molecular coverage, contaminant burden, and interpretability.

background carryover, measurable molecular space, and downstream MS performance.[10-12]

2. EV Isolation and Its Analytical Consequences

In mass spectrometry-based EV workflows, isolation not only enriches vesicles but also defines the composition of the fraction available for molecular analysis. This is particularly important in complex biofluids, where EVs are co-recovered with varying amounts and classes of non-vesicular material depending on the enrichment strategy employed.[4,13,14] Consequently, isolation workflows differ not only in recovery efficiency but also in the composition of the resulting fractions, which in turn influences their analytical behavior. Some methods co-isolate substantial non-vesicular components, whereas others remove a significant portion of this background. As a result, a fraction may contain a high number of particles yet remain analytically dominated by background, while another may yield less total material but offer greater analytical utility due to improved signal specificity.[13-17]

These differences have direct implications for proteomic, metabolomic, lipidomic, and increasingly, glycomic analyses. In MS-based EV glycomics, for instance, background carryover presents a particularly severe challenge. Abundant non-vesicular plasma proteins, such as immunoglobulins and transferrin, are heavily glycosylated. If these components are co-isolated, their overwhelming glycan signals can easily mask the intrinsic, biologically relevant EV surface glycome. The critical factor is not only whether EVs are recovered, but which molecular components are retained and how they are represented in the final fraction. Preparations that retain abundant plasma-derived background may produce MS readouts dominated by non-vesicular signals despite favorable recovery metrics. In contrast, workflows that reduce background carryover can yield more interpretable molecular profiles, even when overall recovery is lower. From this perspective, EV isolation functions as an integral component of the analytical system, as it directly shapes the measurable molecular space and influences data interpretation. This distinction becomes particularly important in biomarker-oriented studies, where conclusions often rely on subtle yet biologically meaningful molecular differences.[8,9,18]

3. Recovery, Purity, and Analytical Value

Metrics such as particle count, total protein, and particle-to-protein ratio are widely used to evaluate EV isolation performance. While these parameters provide useful descriptive information, they do not, on their own, resolve whether the recovered material is genuinely enriched in EV-associated molecules or dominated by co-isolated background. For MS-based proteomic, metabolomic, lipidomic, and glycomic applications, analytical value depends less on total yield and more on the proportion of EV-associated molecular content within the measurable fraction.[1,2,9]

The limited ability of these metrics to distinguish EV-associated material from co-isolated background becomes evident in comparative studies of serum-derived EVs. For example, precipitation-based methods have been reported to yield particle counts substantially higher than those obtained by ultracentrifugation, in some cases by approximately 80- to 300-fold.[19] Although such increases may initially suggest improved EV recovery, these preparations often exhibit lower particle-to-protein ratios and a higher burden of co-isolated proteins compared to more selective approaches, particularly size exclusion chromatography (SEC)-based workflows.[13,15-17,20] These observations indicate that elevated particle counts can arise from the co-recovery of non-vesicular material rather than increased EV selectivity.[19,20] For instance, comparative quantitative studies have demonstrated that while precipitation methods may co-isolate over 90 % of total plasma proteins (e.g., albumin and abundant apolipoproteins), SEC workflows can eliminate up to 95 % of this soluble protein background. Consequently, despite exhibiting significantly lower total particle recovery than PEG-based methods, SEC-enriched fractions often yield a 2- to 3-fold higher number of EV-specific protein identifications in LC-MS/MS analyses.[13,20]

Table 1. Representative physicochemical overlap between small EVs and major serum non-EV components

Component	Diameter (nm)	Density (g/mL)	Markers	Potential impact on MS-based EV omics
Small EVs	~30–200	1.130–1.190	CD9, CD63, CD81	Primary analytical target
HDL	5–12	1.063–1.210	ApoA1, ApoA2	Strong interference in lipidomic analysis; density overlap with EV-associated fractions
LDL	18–25	1.019–1.063	ApoB	Potential co-isolation in density-based workflows
VLDL	30–80	0.930–1.006	ApoB, ApoE	Size overlap with larger EVs; possible co-isolation
Chylomicrons	75–1200	<0.930	ApoB-48	Marked size overlap with large EVs; strong matrix effect in some samples
Albumin	~7	1.350	ALB	Major source of soluble protein carryover and signal masking

This stark contrast provides a clear empirical basis for the argument that high material yield does not intrinsically equate to high analytical value.

The observed increase in particle counts accompanied by elevated co-isolated protein content is consistent with the compositional complexity of plasma and serum, which contain abundant non-vesicular constituents, including albumin, immunoglobulins, fibrinogen, apolipoproteins, and lipoprotein particles.[4-6,21] When such components constitute a substantial fraction of the isolate, increases in particle count or total protein no longer correspond to broader EV-associated molecular coverage, but instead reflect increased background carryover. Under these conditions, conventional yield-based metrics lose their interpretive value for omics applications.

From an analytical perspective, a workflow may therefore appear efficient based on recovery metrics while remaining suboptimal for downstream analysis if dominant background components continue to occupy most of the measurable molecular space. Conversely, lower total recovery can yield a more informative and interpretable fraction when background interference is effectively reduced. This distinction is particularly relevant in plasma-derived samples, where extensive overlap between EVs and lipoproteins in size and density complicates the interpretation of recovery-based metrics. Accordingly, recovery and analytical value do not necessarily correlate: increased material yield does not guarantee improved access to EV-associated molecular features.[8,9,22,23] As summarized in *Table* 1, the physicochemical overlap between EVs and major non-vesicular components in plasma and serum further limits the reliability of recovery-based evaluation.[4,5,21] For MS-based omics workflows, analytical suitability—defined by the balance between EV enrichment and background reduction—therefore provides a more meaningful basis for assessing isolation performance than recovery alone.

4. Co-Isolated Background and Reduced Analytical Depth

In many EV omics datasets, the primary limitation is not insufficient material recovery but the persistence of analytically dominant background, which restricts access to lower-abundance EV-associated signals. When plasma-derived proteins or lipoprotein-associated particles are retained in the isolate, EV-derived features may remain present yet become difficult to detect due to their lower relative abundance.[8,9,22] This phenomenon can be explicitly defined as spectral masking—an analytical condition in mass spectrometry where highly abundant, strongly ionizing background molecules (e.g., plasma proteins or lipoproteins) suppress the ionization or outcompete the precursor ion selection of lower-abundance EV-associated molecules, rendering them analytically undetectable. As used here as a practical descriptor, spectral masking occurs because co-isolated background components limit access to EV-associated signals through multiple contributing factors, including higher relative abundance, dynamic range compression, and biased MS/MS sampling. Co-isolated background components can limit access to EV-associated molecular signals through multiple contributing factors, including higher relative

abundance, more efficient ionization, and preferential selection during MS/MS acquisition. In addition, effects such as dynamic range compression and precursor ion competition can further bias detection toward dominant species. As a result, weaker EV-associated signals may be underrepresented or absent in the final dataset, even when present in the original sample, leading to a measurable molecular space that is narrower than the actual composition of the isolate.[24,25] Lipoprotein-associated particles provide a representative example of this effect. High-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL) overlap with EVs in size and/or density, making their separation challenging in plasma-based workflows. When these particles are co-isolated, proteins such as ApoB or ApoE can dominate MS/MS acquisition events. Under such conditions, the limited detection of EV-associated molecules reflects not their absence, but the preferential sampling of analytically dominant non-vesicular species.[4,5,21] Similarly, in EV metabolomics, the presence of abundant free metabolites in the biofluid matrix can lead to a comparable spectral masking effect. High-concentration small molecules from the surrounding plasma or serum may overshadow the intrinsic metabolic cargo of EVs, effectively narrowing the measurable metabolic space and complicating the attribution of detected signals to the vesicular interior.

In polyethylene glycol (PEG)-based precipitation, residual polymer contamination introduces an additional but related constraint. PEG and PEG-based detergents produce characteristic ion series with repeating 44 Da mass differences corresponding to ethylene oxide units, and may also appear as multiply charged ions.[26] When present at sufficient levels, these species can interfere with signal detection, suppress peptide ionization, and bias MS/MS acquisition toward contaminant-derived ions. In complex samples, such interference may be difficult to recognize because polymer species of varying chain lengths do not necessarily coelute and may partially overlap with peptide-rich regions of the chromatographic profile.[24-26]

The preferential detection of abundant background components, together with ion suppression and biased MS/MS sampling, can also be interpreted in terms of diminishing analytical return. Beyond a certain point, increased material recovery yields limited gains in EV-associated molecular coverage, as additional material becomes progressively enriched in already dominant background components, including plasma proteins, lipoproteins, and other non-vesicular species. Under these conditions, the analytical return per unit of recovered material plateaus: apparent yield increases, while EV-specific informational content does not expand proportionally.[8,18,22,23] Accordingly, contamination in EV omics should not be considered solely in terms of nominal purity. Instead, it directly shapes what remains measurable and how the resulting data can be interpreted by constraining the observable signal space. These analytical limitations are particularly evident in precipitation-based enrichment workflows, where substantial co-isolation of background components is often unavoidable.[8,9,22]

5. PEG-Based Precipitation in MS-Based EV Omics

PEG-based precipitation provides a useful framework for illustrating the divergence between material recovery and analytical interpretability in EV omics. Its continued use is largely driven by practical considerations, including procedural simplicity, compatibility with limited sample input, and suitability for high-throughput processing.[27, 28] However, these operational advantages do not inherently confer analytical selectivity on the resulting fractions.[12] PEG precipitation typically produces a heterogeneous fraction rather than a selectively enriched EV isolate. Although EVs are present, the recovered material frequently includes substantial amounts of soluble proteins, lipoprotein-associated particles, protein aggregates, and other non-vesicular components. In plasma and serum, where such background constituents are already abundant, this lack of selectivity becomes particularly consequential.[5,16,19] As a result, the limitation of PEG-based precipitation lies not only in the co-recovery of background material, but in the

Table 2. Analytical comparison of major EV isolation workflows for MS-based omics

Workflow	Principle	Strengths	Background	MS impact	Limitations
PEG-based precipitation	Polymer-mediated bulk precipitation	Simple; scalable; low-input compatible	Soluble proteins; lipoproteins; polymer residue	High apparent recovery; low selectivity	Broad co-recovery; cleanup often needed
SEC	Size-based separation	Lower protein carryover; cleaner input	Residual lipoproteins; fraction-dependent carryover	Favorable for discovery profiling	Recovery may decrease; fraction selection matters
Centrifugation-based enrichment	Sedimentation by centrifugal force	Widely used; bulk EV enrichment	Aggregates; lipoproteins; sedimentable background	Recovery acceptable; background highly protocol-dependent	Variable selectivity; limited class resolution
Cleanup and multistep purification	Sequential orthogonal cleanup	Lower background; better analytical fit	Reduced but residual carryover possible	Better clarity; better access to low-abundance EV signals	More complex; slower; cumulative sample loss

expansion of analytically non-informative components relative to EV-associated molecular content. Under these conditions, increases in total recovered material do not necessarily translate into improved analytical value for EV-focused proteomic, metabolomic, lipidomic, or glycomic analyses. Preparations may therefore exhibit high particle counts while remaining limited in EV-associated molecular coverage.[8,12,22]

Residual PEG-derived species introduce an additional layer of analytical complexity. As described above, these species generate characteristic ion series with 44 Da spacing and can interfere with ionization efficiency as well as MS/MS acquisition.[26] In parallel, precipitation-derived fractions often retain substantial lipoprotein-associated background, including ApoB- and ApoE-containing particles. When both polymer-derived and lipoprotein-associated components remain abundant, MS/MS sampling can become disproportionately allocated to these analytically dominant species, thereby limiting the detection of lower-abundance EV-associated molecules. Under such conditions, apparent gains in recovery yield limited analytical benefit.[4,5,16,26] Accordingly, increases in particle yield observed in precipitation-based workflows require careful interpretation. When elevated particle counts are accompanied by low particle-to-protein ratios or substantial lipoprotein carryover, the outcome is more consistent with broad co-recovery than with enhanced EV selectivity. Misinterpretation is particularly likely when PEG-derived fractions are treated as analytically equivalent to more selectively purified isolates, as observed differences may reflect co-isolated background as much as intrinsic EV composition. Post-isolation cleanup strategies—including washing, repeated precipitation, ultracentrifugation, and size exclusion chromatography—can partially reduce background carryover while retaining some practical advantages of precipitation-based workflows. Although these approaches do not eliminate the underlying trade-off, they demonstrate that the analytical performance of PEG-based methods depends strongly on subsequent processing steps.[15,29-31] When applied with these considerations in mind, PEG-based precipitation can remain a practical option in specific contexts, particularly for low-input or high-throughput applications. However, without appropriate analytical control—especially in discovery-oriented proteomic, metabolomic, lipidomic, or glycomic studies—it may generate fractions in which apparent recovery overestimates EV-associated analytical value. This comparison is summarized in *Table* 2, where major EV isolation strategies are evaluated in terms of analytical suitability for MS-based omics rather than recovery alone.[12,31,32]

6. Cleanup and Multistep Purification Strategies

Many EV workflows incorporate additional cleanup steps, as single EV enrichment methods often leave substantial unresolved background that limits downstream omics analyses. The rationale for these multistep designs is not increased procedural complexity per se, but the need to modify the composition of the fraction entering analysis. This is typically achieved

by combining orthogonal separation principles. For example, precipitation may be followed by washing or SEC, while centrifugation-based enrichment may be extended with density-based purification. In plasma-derived samples, additional steps such as depletion of abundant proteins or lipoprotein-associated particles may also be applied prior to mass spectrometry.[13,15,21,31,33] The value of such additional cleanup depends on the analytical objective. Discovery-oriented workflows generally benefit from more extensive background reduction, whereas targeted or higher-throughput approaches may tolerate greater carryover if the signals of interest remain stable and quantifiable.[9,22,33] Furthermore, asymmetric flow field-flow fractionation (AF4) has emerged as a powerful, high-resolution, and label-free alternative for EV separation.[34,35] By fractionating particles based on their hydrodynamic size within a gentle, cross-flow fluidic channel, AF4 not only preserves vesicle structural integrity but also successfully resolves EV subpopulations (such as exomeres and small EVs) from the soluble protein background. From an MS-based omics perspective, while AF4 yields exceptionally pure fractions with minimal spectral masking, the inherent sample dilution and lower absolute loading capacity often dictate the need for subsequent sample concentration steps prior to downstream molecular profiling.

For completeness, it is also essential to consider other highly specific or emerging isolation methods, such as density gradient ultracentrifugation (DGUC), immunoaffinity capture, and microfluidics. DGUC remains a benchmark for resolving EVs from lipoproteins based on buoyant density (typically 1.13–1.19 g/mL), drastically reducing protein and lipid background to yield high-confidence MS datasets, albeit at the cost of lower throughput and potential material loss. Immunoaffinity capture, utilizing antibodies against canonical tetraspanins (e.g., CD9, CD63, CD81), offers unparalleled specificity for EV subtypes; however, the resulting fractions are often low in total mass, necessitating highly sensitive nano-LC-MS/MS platforms. Furthermore, microfluidic technologies enable rapid, low-input EV isolation with integrated on-chip processing. While highly advantageous for translational diagnostics, scaling microfluidic yields to meet the sample volume requirements of comprehensive multi-omics profiling remains an ongoing analytical challenge.

The secondary purification steps can reduce background dominance, even at the cost of decreased total recovery. In some cases, this trade-off enhances analytical performance, as lower-abundance EV-associated features become more detectable once the most analytically dominant components are removed. As a result, a smaller yet more selective fraction may offer greater analytical value than a larger, less refined isolate.[14,15,17,18] Nevertheless, these benefits are not universal. Each additional processing step carries potential drawbacks, including sample loss, altered vesicle recovery, increased handling time, and reduced throughput. Such limitations are particularly relevant for low-input studies, large cohort analyses, or translational applications that require standardized and scalable workflows. Therefore, analytically optimal workflows may not always be practically feasible.[15,21,31,33]

The benefits of background reduction are particularly pronounced in discovery-oriented proteomics, metabolomics, lipidomics, and glycomics, where low-abundance EV-associated features can be masked or suppressed by highly abundant non-EV proteins or lipids present in the sample matrix. In such cases, additional purification improves the detectability of these features by reducing analytically dominant components that would otherwise interfere with downstream measurements. Conversely, targeted validation or high-throughput workflows may tolerate residual background if the signals of interest remain reproducible and quantitatively robust. Thus, the optimal degree of purification depends on the analytical goal, and no single level of background reduction is universally appropriate across all study designs.[9,22,33] For these reasons, multistep purification is best understood not as a universal solution to EV isolation, but as a strategy for tuning the analytical input. Its utility ultimately depends on whether the resulting change in the analytical fraction meaningfully improves the downstream readout.[8,9,21,33]

7. How EV Isolation Should Be Evaluated for MS-Based Omics

As EV isolation workflows become increasingly complex, evaluating their performance becomes correspondingly more challenging. Traditional metrics —such as particle count, total protein, or the particle-to-protein ratio—are commonly used as proxies for EV enrichment, but none alone can determine whether the resulting fraction is suitable for downstream proteomic, metabolomic, lipidomic, or glycomic analysis. A workflow may appear effective based on a single metric, yet still yield data that are unreliable or difficult to interpret.[8,9,33] In practice, the key consideration is whether the isolated fraction is analytically fit for its intended purpose. This extends beyond EV enrichment to include background burden, compatibility with the analytical platform, reproducibility across replicates, and stability across runs or batches. A preparation that performs adequately in one context may not do so in another, even when nominally identical isolation procedures are applied.[22,23,33]

The limitations of commonly used proxy metrics become evident upon closer examination. Particle counts cannot distinguish EVs from similarly sized non-vesicular particles, and bulk protein measurements do not differentiate EV-associated cargo from persistent background. Even detection of EV markers provides only partial insight when contaminant markers are not considered. Consequently, a workflow may appear favorable under one proxy yet remain analytically compromised under another.[5,16,32] A more informative evaluation requires consideration of both EV enrichment and contaminant burden. Measures of enrichment—such as detection of EV-associated markers, reproducibility of molecular profiles, and recovery of EV-derived analytes—remain essential. However, assessment of contaminant burden is equally critical, particularly when abundant plasma proteins or lipoprotein-associated markers persist. Evaluating only one aspect risks overestimating overall workflow performance.[4,5,21,23]

This issue becomes especially important in comparative studies across biological groups, where differences in contaminant burden may be misinterpreted as biological variation. If contaminant levels vary with workflow or sample set, apparent biological differences may instead reflect differences in background carryover. Accordingly, evaluation must extend beyond single-sample characterization to include consistency across representative samples, batches, and processing runs.[8,22,33] In practical terms, workflow assessment requires criteria that extend beyond simple yield-based metrics. Key considerations include whether a method expands detectable molecular coverage or primarily increases bulk recovery, whether it improves access to low-abundance EV-associated features or leaves dominant background unresolved, and whether it demonstrates sufficient consistency to support robust comparisons across samples. Although such criteria are less straightforward than particle-based measures, they more accurately reflect the analytical demands of EV omics.[18,22,23,33] No single benchmark is universally applicable across all EV studies. Discovery proteomics, targeted validation, metabolomics, lipidomics, glycomics, and translational cohort analyses impose distinct requirements on the isolated fraction. Accordingly, evaluation of EV isolation workflows for MS-based omics is most informative when enrichment, contaminant burden, reproducibility, and measurable molecular coverage are considered in an integrated manner rather than in isolation.[1,2,9,22,33]

8. How EV Isolation Workflows Should Be Chosen in Practice

Selection of EV isolation workflows becomes more straightforward once the downstream analytical objective is clearly defined. Approaches that rely primarily on recovery metrics can lead to misleading comparisons. Fractions suitable for broad enrichment, targeted nucleic acid analysis, or certain functional assays may be suboptimal for discovery-oriented proteomic, metabolomic, or lipidomic applications. The primary consideration is therefore the nature of the signal to be recovered.[9,22,33] In discovery-oriented profiling, the practical objective is typically not

maximal particle recovery, but improved access to low-abundance EV-associated molecular features. Under such conditions, workflows that effectively reduce analytically dominant background are generally more valuable than those that maximize bulk recovery while leaving contaminant burden unresolved. Broad enrichment strategies may still be appropriate, provided that the resulting fraction remains compatible with the downstream analytical platform.[8,15,17,22]

The choice of workflow is influenced not only by analytical objectives, such as access to low-abundance EV-associated features, but also by practical constraints related to study design, sample availability, and throughput requirements. Large cohort studies and low-input experiments often necessitate simpler, more scalable workflows, which may favor precipitation-based approaches despite their analytical limitations. These fractions should not be considered analytically equivalent to more selectively purified isolates, and their limitations should be explicitly acknowledged and mitigated where possible through additional cleanup, depletion strategies, or targeted quality control measures.[12,31,32]

It is useful to distinguish among enrichment, cleanup, and evaluation as separate but interrelated functions within a workflow. Enrichment facilitates the recovery of EV-containing material, cleanup reduces analytically dominant background, and evaluation determines whether the resulting fraction is fit for the intended analytical application. This distinction helps ensure that workflow selection, even when driven by logistical constraints, supports subsequent steps necessary for analytical suitability.[9,22,33] For this reason, evaluation should be incorporated at an early stage.

Pilot comparisons in omics-based studies typically provide more reliable guidance than assumptions based solely on workflow classification. Even workflows described by the same methodological label may differ substantially in parameters such as rotor configuration, column characteristics, fraction collection strategy, washing intensity, and post-enrichment processing. Analytical suitability must therefore be established empirically rather than inferred from nomenclature.[15-17,32] Workflow selection also influences how results should be interpreted and reported. When substantial co-enriched background persists, detected proteins, lipids, or glycans should not be uncritically attributed to EV origin. A more appropriate formulation is that such molecules were identified within EV-enriched fractions generated under defined isolation conditions. This conservative wording more accurately reflects the underlying analytical context.[1-3]

Precipitation-based workflows may be advantageous when throughput and scalability are primary considerations, whereas size exclusion chromatography is often preferable when reduction of soluble background is critical. Density-based purification and multistep workflows may be justified when deeper molecular profiling is required and some loss of recovery is acceptable. In practice, workflow selection reflects a balance among sample constraints, tolerance for background, and the analytical demands of the intended readout.[11,12,21,31,32,36]

9. Conclusion

Discussion of EV isolation has traditionally emphasized recovery, purity, and operational convenience. While these descriptors remain informative, they are insufficient to explain what becomes analytically accessible once samples are subjected to mass spectrometry. In biofluid-derived workflows, including those based on plasma and serum, the central consideration extends beyond recovery of EV-containing material to the extent to which the resulting fraction remains influenced by non-vesicular background. This perspective reframes the role of isolation in EV omics. Isolation workflows differ not only in procedural design but also in their impact on background carryover, subtype representation, and compatibility with downstream analytical platforms. Increased recovery does not necessarily translate into greater EV-associated molecular insight when dominant background components persist. Conversely, reduced recovery may yield a more informative dataset when the measurable molecular space is sufficiently cleared to reveal lower-abundance EV-associated features.

PEG-based precipitation provides a clear illustration

of this trade-off. Its practical advantages—particularly in scalable and low-input contexts—are well established. However, its broad enrichment characteristics can preserve substantial levels of soluble proteins, lipoprotein-associated particles, and polymer-derived interference, all of which complicate EV-focused molecular interpretation. Accordingly, PEG-based approaches are most appropriately considered as components of multistep workflows rather than as standalone solutions for EV-specific analysis. Increasingly, the key question in EV omics is not the magnitude of recovery alone, but whether enrichment, cleanup, and evaluation are appropriately aligned with the analytical platform and the biological objective. From this standpoint, the most effective workflow is not the one that maximizes material yield, but the one that produces a fraction that is analytically interpretable, reproducible, and fit for purpose.

Future progress in EV proteomics, metabolomics, lipidomics, and glycomics will depend on more systematic evaluation of isolation strategies in terms of enrichment efficiency, background reduction, reproducibility, and measurable molecular coverage. Integrating these factors will improve the ability to distinguish workflow-dependent background from true biological signal and support more rational selection of isolation approaches based on analytical suitability rather than recovery alone.

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