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Review

From Grapevine Tissues to Proteome Coverage: Analytical Workflows for Protein Extraction, Fractionation and Mass Spectrometry

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Abstract

Grapevine (*Vitis* spp.) proteomics is an important tool for investigating berry development, stress responses, dormancy, root adaptation and extracellular defense processes. However, many grapevine tissues and extracellular matrices remain analytically challenging because of relatively low recoverable protein abundance and high levels of interfering compounds, including polyphenols, polysaccharides, sugars, pigments, lipids and structural polymers. This review provides a workflow-oriented overview of analytical strategies used in grapevine proteomics, covering protein extraction and clean-up, fractionation, digestion, mass spectrometry and bioinformatic processing. Matrix-adapted approaches based on TCA/acetone precipitation, phenol extraction and SDS-, urea- or guanidine-containing buffers are considered across major grapevine tissues and extracellular matrices. Gel-based, GeLC and gel-free LC–MS/MS workflows, peptide fractionation, label-free and isobaric quantification, data-independent acquisition (DIA) and post-translational modification (PTM)-oriented strategies are critically compared, with emphasis on their effects on protein recovery, proteome coverage, reproducibility and cross-study comparability. Despite advances in high-resolution LC–MS/MS, fully gel-free bottom-up workflows remain less frequently represented than gel-assisted or protein-level-prefractionated approaches. Future priorities include improved matrix-specific clean-up, workflow standardization, reference materials and quality-control procedures, and more transparent reporting of analytical and bioinformatic parameters.

Keywords: grapevine proteomics; protein extraction; gel-free bottom-up proteomics; mass spectrometry; matrix-specific workflows

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1. Introduction

Grapevine (*Vitis* spp.) is one of the most economically important perennial crops worldwide, underpinning table grape, raisin, juice and wine-related industries [1–3]. Beyond its economic relevance, grapevine represents a complex biological system characterized by diverse tissues, long developmental cycles and strong interactions between genotype, environment and management practices. These features, together with the chemical complexity of grape berries and other organs, make grapevine an important model for

studying the molecular basis of fruit development, stress responses and plant–environment interactions [4,5].

Proteins and peptides play central roles in these processes by regulating metabolism, cellular signaling, defense responses, structural organization and developmental transitions. Consequently, proteomics has become an important approach for investigating grapevine biology at the molecular level, enabling the characterization of protein networks associated with berry development, dormancy, root adaptation, stress responses and extracellular defense processes [6–8]. Recent integrative studies have shown that the Vitis proteome is highly dynamic and multifunctional, with coordinated protein-level changes contributing to developmental regulation and environmental adaptation. However, most of these studies have focused primarily on biological interpretation, while the analytical workflows that determine which proteins are extracted, detected, quantified and interpreted have received less systematic attention.

Numerous reviews have addressed proteomics methodologies in plant systems, providing broad overviews of protein extraction, separation, mass spectrometry and data analysis workflows [8–10]. More recent contributions have emphasized high-throughput proteomics, systems biology and integration with transcriptomics and metabolomics [11–14]. Although these reviews provide essential conceptual and technical frameworks, they do not fully capture the specific analytical constraints associated with grapevine tissues. Grapevine matrices differ markedly in composition: leaves and shoots are rich in pigments, waxes and phenolics; wood and roots contain lignin, suberin and structural polysaccharides; dormant buds contain starch, lipids and protective scales; berries contain sugars, organic acids, pigments and polyphenols; and apoplastic fluid or xylem sap contain very low protein concentrations. These matrix-specific features require tailored workflows that go beyond generic plant proteomics protocols.

Despite advances in mass spectrometry and bioinformatics, grapevine proteomics remains strongly influenced by matrix composition. Polyphenols, polysaccharides, sugars, organic acids, pigments, lipids, salts and other matrix-derived compounds can interfere with protein extraction, digestion, chromatographic separation and MS detection, thereby affecting proteome coverage and quantitative reliability [15–18]. Consequently, gel-assisted or protein-level-prefractionated workflows remain common, whereas fully gel-free bottom-up analysis of unfractionated whole-proteome extracts is less frequently represented. In this review, fully gel-free bottom-up proteomics refers to workflows in which whole-tissue or whole-matrix protein extracts are processed directly to peptides without electrophoretic protein-level separation or targeted subproteome enrichment; peptide-level clean-up or fractionation after digestion is considered compatible with this definition. Only a limited number of reviews have specifically addressed analytical methodologies for grapevine protein analysis. Earlier overviews, including Le Bourse et al. [19], mainly covered classical protein isolation, fractionation and characterization approaches and largely predate the widespread use of high-resolution LC–MS/MS, DIA and contemporary bioinformatic workflows. Thus, a systematic, matrix-specific evaluation of contemporary grapevine proteomics workflows remains lacking. Figure 1 illustrates how the biochemical characteristics of individual grapevine matrices determine the choice of extraction and separation strategies and subsequently influence peptide preparation, MS acquisition and data interpretation. It also emphasizes that matrix-dependent decisions made at the earliest stages of the workflow propagate through the analytical pipeline and ultimately affect proteome coverage, quantitative reliability and cross-study comparability.

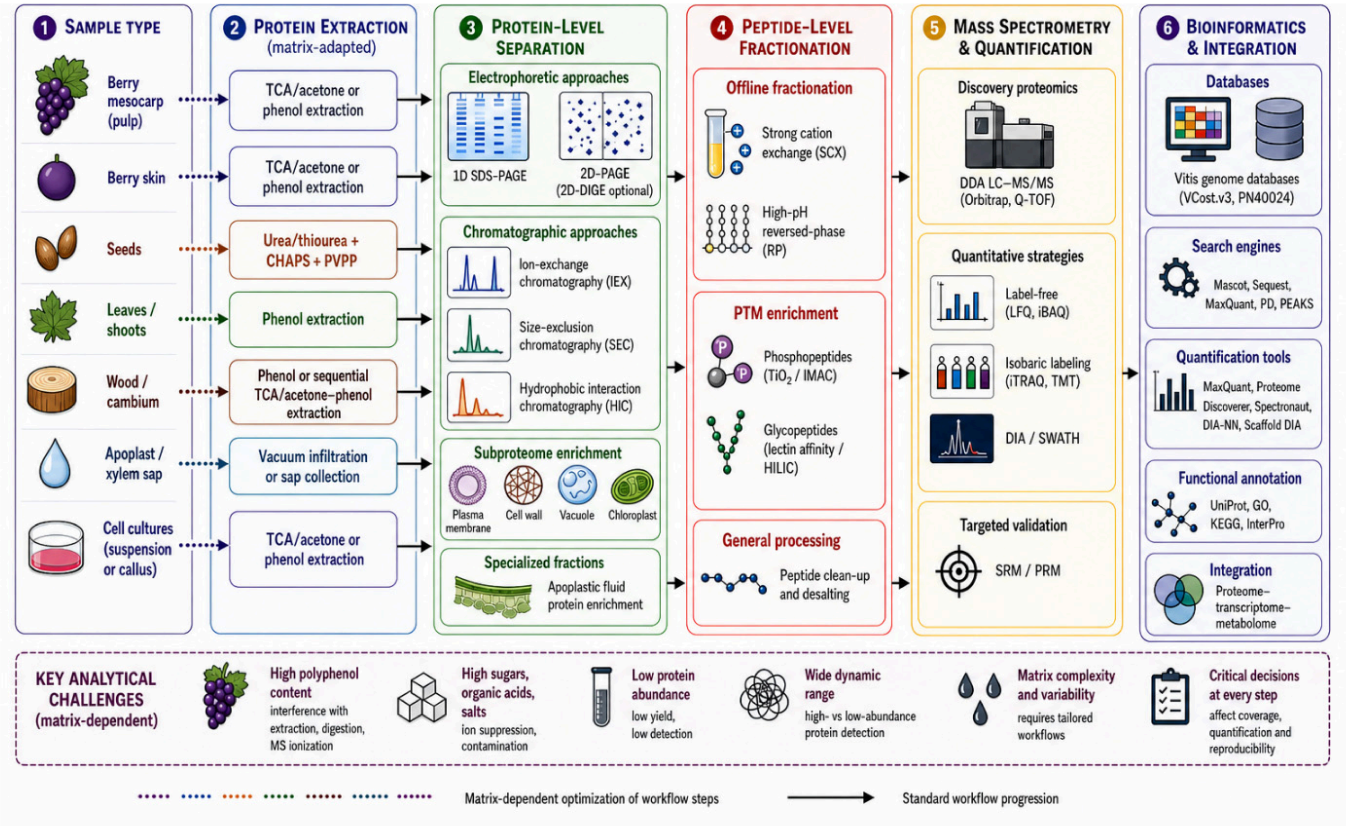


Figure 1. Matrix-dependent analytical workflow in grapevine proteomics, from tissue-specific sample preparation to protein extraction, fractionation, digestion, MS analysis and bioinformatic interpretation.

Accordingly, this review provides a workflow-oriented evaluation of grapevine proteomics, with emphasis on how matrix-adapted methodological choices influence protein recovery, peptide generation, proteome coverage, quantitative reproducibility and comparability among studies. The aim is to support the rational selection of matrix-appropriate workflows and to identify methodological limitations and priorities for greater standardization and reproducibility. This review is based on a comprehensive and structured survey of the scientific literature addressing analytical workflows in grapevine proteomics. Relevant publications were retrieved from major scientific databases, including Google Scholar, Web of Science, Scopus and PubMed, covering the period from the 1950s through September 2026. The search strategy combined general proteomics-related terms, such as "Vitis proteomics", "grapevine proteomics", "grape proteome" and "Vitis protein analysis", with methodological descriptors including "protein extraction", "protein precipitation", "protein fractionation", "gel electrophoresis", "gel-free proteomics", "protein digestion", "peptide fractionation", "LC–MS/MS", "mass spectrometry", "DDA", "DIA", "label-free quantification", "iTRAQ", "TMT", "phosphoproteomics" and "bioinformatics". Searches were further refined using matrix- and tissue-specific terms, including leaves, shoots, wood, buds, roots, berries, cell cultures, apoplastic fluid and xylem sap. The retrieved literature was qualitatively synthesized and organized according to the major stages of the proteomics workflow—protein extraction and clean-up, protein- and peptide-level fractionation, digestion and peptide preparation, mass spectrometric analysis, and bioinformatic processing—with particular emphasis on matrix-specific methodological requirements, analytical trade-offs and their potential impact on proteome coverage and quantitative reproducibility.

2. Evolution of Analytical Approaches in Grapevine Proteomics

Early research on grapevine proteins was primarily rooted in classical biochemistry and focused on the purification and characterization of individual proteins and enzymes from grapevine tissues. These studies were largely motivated by the need to understand varietal differences, metabolic processes and physiological functions in grapevine organs. Early work described soluble protein fractions and specific enzymes, including alcohol dehydrogenase, peroxidases, polyphenol oxidases and pathogenesis-related proteins, and established links between particular protein classes and stress responses or developmental processes [20–26]. Although these studies provided important information on individual grapevine proteins, they were limited to selected proteins or protein classes and did not provide a comprehensive view of the proteome.

In parallel with these biochemical approaches, one-dimensional and subsequently two-dimensional electrophoretic techniques were introduced to analyze proteins from seeds, berries and other grapevine tissues. These methods enabled the generation of protein fingerprints for cultivar discrimination and provided early insights into developmental and physiological variation at the protein level [27–31]. Together, these studies marked the transition from targeted protein characterization to broader protein profiling. However, despite representing an important step toward proteome-wide analysis, these approaches remained constrained by limited proteome representation, poor representation of low-abundance and hydrophobic proteins, and limited protein-identification capacity.

The emergence of plant proteomics in the late 1990s and early 2000s established standardized conceptual and technical frameworks for large-scale protein separation, identification and quantification in plants. Key developments included optimized two-dimensional polyacrylamide gel electrophoresis protocols, improved staining methods and the systematic integration of mass spectrometry into protein-mapping workflows [6,8,15,16,32–35]. Grapevine research rapidly adopted these approaches, applying 2D-PAGE in combination with MALDI-TOF/TOF or LC–MS/MS to investigate proteome changes in berry mesocarp and skins, leaves exposed to biotic and abiotic stresses, somatic embryogenesis and elicitor-treated cell cultures [36–42]. These gel-based workflows provided the first true proteomic views of grapevine tissues, but they remained biased toward abundant, soluble and well-resolved proteins.

Subsequent improvements in mass spectrometry gradually shifted grapevine proteomics toward peptide-centric LC–MS/MS workflows. Label-free quantification and isobaric labeling approaches, including isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tags (TMTs), enabled larger-scale protein identification and quantification, improved sensitivity and more flexible experimental designs. These strategies have been applied to berries, leaves, buds, roots and cell cultures to investigate developmental regulation and responses to environmental stimuli, often in combination with transcriptomic or metabolomic analyses [43–54]. This transition changed the analytical paradigm of grapevine proteomics from descriptive protein profiling toward high-resolution and quantitative proteome analysis.

Nevertheless, this transition has been incomplete. In contrast to some model plant systems, truly gel-free bottom-up proteomics of unfractionated whole-proteome extracts remains less frequently represented than gel-assisted or protein-level-prefractionated workflows in the literature surveyed here. Many workflows still include 2-DE, DIGE, 1D SDS-PAGE or GeLC-type steps, either for protein-level separation or as a pragmatic clean-up strategy before LC–MS/MS. This reflects the recalcitrant nature of grapevine matrices, where phenolics, pigments, polysaccharides, sugars, lipids, lignified cell-wall material and salts can interfere with digestion, chromatography and ionization. Therefore, the evolution of

grapevine proteomics should not be viewed simply as a replacement of gel-based methods by gel-free LC–MS/MS, but rather as a gradual diversification of matrix-adapted workflows.

More recently, data-independent acquisition (DIA) and related high-throughput quantitative strategies have begun to appear in grapevine proteomics. Compared with traditional data-dependent acquisition (DDA), DIA offers improved reproducibility, reduced missing values and more consistent quantification across complex sample series. These features are attractive for large-scale studies, although DIA requires highly reproducible chromatography and advanced computational processing [13,55–58]. DIA remains less widely used than DDA-based label-free or isobaric-label workflows but represents an important direction for future grapevine proteomics.

Alongside advances in global proteome profiling, methodological development has increasingly focused on defined subproteomes and post-translational modifications (PTMs). Dedicated workflows have been developed for cell wall and apoplastic proteomes, plasma membrane fractions, chloroplast-enriched fractions, xylem sap, wood, roots and cell cultures, requiring matrix-specific extraction, enrichment and fractionation [18,51,59–69]. In parallel, phosphoproteomic, glycoproteomic and acetylproteomic approaches access regulatory layers not captured by total protein abundance [10,70–73]. These approaches increase methodological demands on enrichment efficiency, site localization and quantitative normalization. Integrated proteomic and phosphoproteomic analysis of Vitis amurensis further illustrates the expanding scale of phosphorylation-focused workflows [73].

Within plant proteomics and systems biology, grapevine has evolved from a relatively under-studied crop to a well-established model for integrative omics research in perennial fruit species. High-quality reference genome resources, improved annotations and advanced bioinformatic tools have enabled increasingly comprehensive proteomic studies and integration with transcriptomic and metabolomic datasets [8,11,12,74–78]. Overall, grapevine proteomics has progressed from targeted biochemical characterization and electrophoretic protein profiling toward high-resolution, quantitative and integrative workflows, while retaining a strong dependence on matrix-adapted sample preparation and, in many cases, gel-assisted processing. This historical development is summarized in Figure 2.

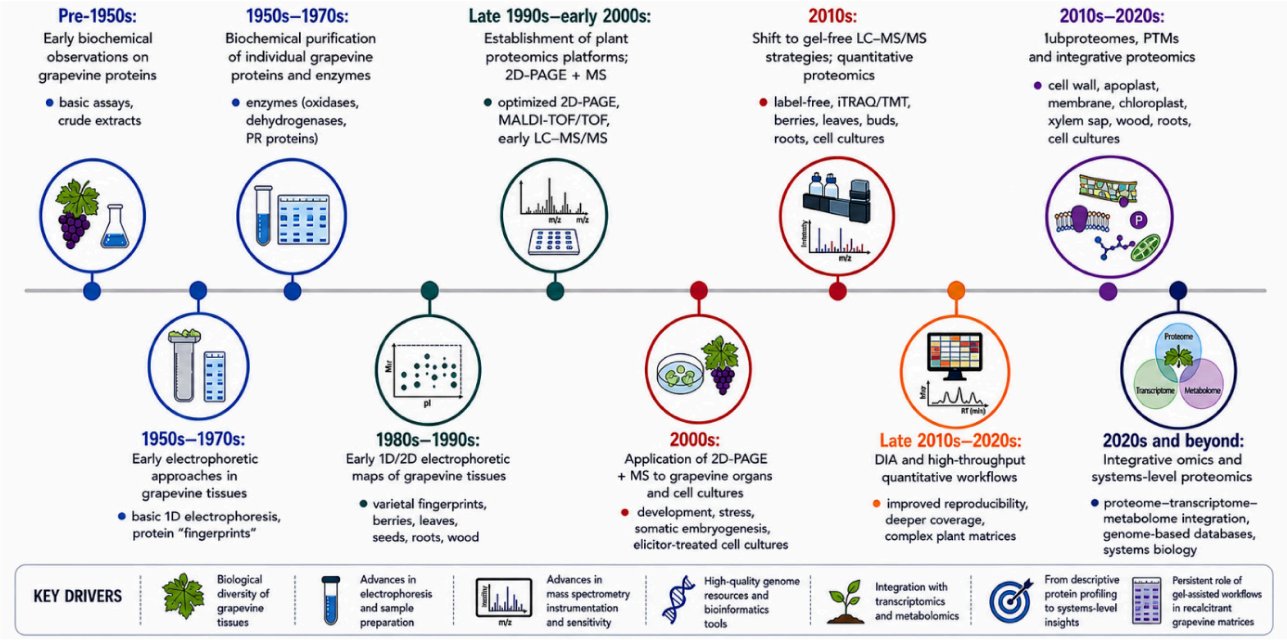


Figure 2. Evolution of analytical strategies in grapevine proteomics, from gel-based protein profiling to high-resolution quantitative and integrative MS-based workflows.

3. Protein Extraction from Grapevine Tissues and Cell-Based Matrices

Protein extraction is one of the most critical steps in grapevine proteomics because the biochemical composition of each tissue strongly determines protein recovery, extract purity, digestion efficiency and compatibility with downstream MS analysis. Grapevine organs typically combine relatively low protein abundance with high concentrations of interfering compounds, including polyphenols, polysaccharides, sugars, organic acids, pigments, lipids, lignin, suberin and salts. If not efficiently removed, these compounds can cause protein oxidation, aggregation, poor resolubilization, streaking in gel-based separations, impaired enzymatic digestion and ion suppression in LC–MS/MS workflows [15–18].

Because grapevine proteomics encompasses diverse matrices—including leaves, shoots, wood, buds, roots, berries, cell cultures, apoplastic fluid and xylem sap—no single extraction protocol is universally applicable. Instead, TCA/acetone precipitation, phenol extraction and SDS-, urea-, thiourea- or guanidine-based solubilization are adapted to specific tissues and analytical objectives, often combined with PVPP, reducing agents and additional clean-up procedures [37,40,51,79,80]. Figure 3 summarizes this matrix-dependent logic, from rapid freezing and grinding to extraction chemistry and downstream compatibility.

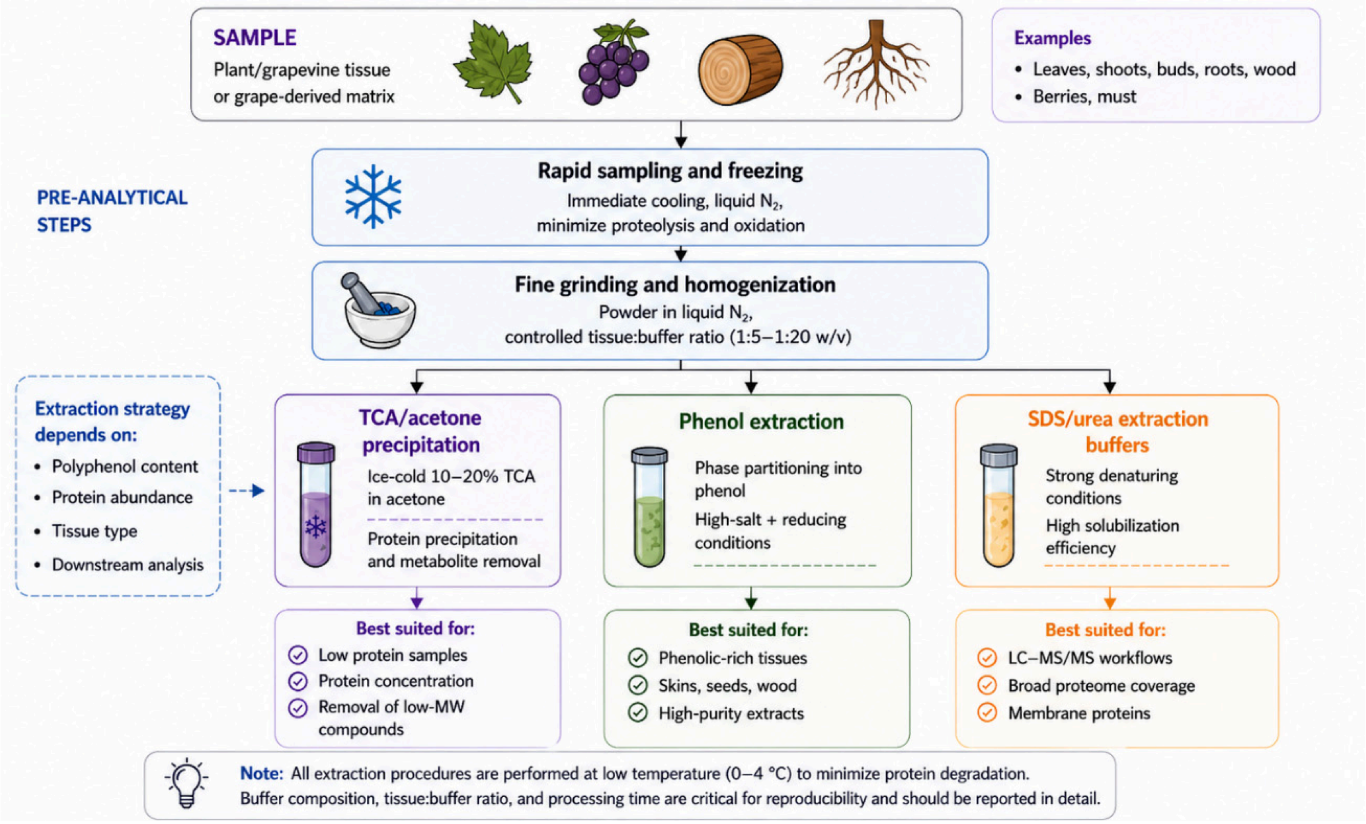


Figure 3. Matrix-dependent protein extraction strategies in grapevine proteomics and their compatibility with downstream analytical workflows.

3.1. General Principles of Plant Protein Extraction

Protein extraction strategies in plant proteomics were initially developed for herbaceous model systems and subsequently adapted to more recalcitrant plant tissues, including woody organs and phenolic-rich fruits. These workflows typically combine intensive mechanical disruption at low temperature with strong denaturing conditions and selective precipitation steps to separate proteins from interfering metabolites. In grapevine, however, extraction must be designed according to matrix composition, because the relative

abundance of phenolics, pigments, polysaccharides, sugars, lipids and structural polymers differs strongly between tissues [15,17,33].

Sample handling is therefore a critical determinant of extraction quality. Rapid freezing, fine grinding in liquid nitrogen and immediate exposure to ice-cold extraction or precipitation solutions are essential to limit proteolysis, oxidation and other chemical modifications, particularly in stress-related studies where protease activity and redox changes may be enhanced [8,16,35]. Three extraction strategies dominate grapevine proteomics. TCA/acetone precipitation, usually performed with 10–20% TCA in cold acetone supplemented with reducing agents and often PVPP, efficiently concentrates proteins and removes many low-molecular-weight metabolites. However, it may cause protein losses and produce pellets that are difficult to resolubilize, especially in phenolic-rich, lignified or lipid-rich matrices [15,17,80].

Phenol extraction partitions proteins into a buffered phenolic phase under high-salt and reducing conditions, followed by precipitation with ammonium acetate in cold methanol. This strategy generally produces cleaner extracts and is particularly suitable for phenolic-rich tissues such as leaves, berry skins, roots, buds and wood, although it is more labor-intensive and can cause losses during phase partitioning and precipitation [8,17,18].

SDS-, urea-, thiourea- and guanidine-based buffers provide strong protein solubilization and are especially useful in gel-free LC–MS/MS-oriented workflows. Their main limitation is incompatibility with digestion and MS analysis unless detergents, chaotropes, salts and reducing agents are efficiently removed by precipitation, ultrafiltration, filter-aided sample preparation (FASP), S-Trap-type workflows or other clean-up strategies [8,40,48,79].

Advanced clean-up strategies remain underrepresented in grapevine studies. FASP has been applied in a limited number of workflows, particularly in cell cultures and buds, whereas SP3-type bead-based approaches remain largely unexplored. Broader evaluation of such strategies could improve reproducibility, sample recovery and compatibility with high-throughput LC–MS/MS [8,10,48].

Across all extraction strategies, performance depends on tissue mass, tissue-to-buffer ratio, PVPP concentration, reducing agents, precipitation conditions, centrifugation parameters, number of washes, drying conditions and resolubilization buffers. Consistent reporting of these parameters is essential for comparing extraction efficiency and downstream analytical performance across studies.

3.2. Protein Extraction from Leaves, Shoots, Wood, Apoplastic Fluid and Xylem Sap

Leaves and shoots are among the most frequently analyzed grapevine tissues in proteomics, but they are chemically complex matrices rich in polyphenols, organic acids, waxes, pigments and cuticular components. Wood and other lignified organs are even more recalcitrant because they combine very low protein abundance with lignin, suberin and structural polysaccharides. Co-extracted phenolics and oxidative enzymes can promote protein oxidation, aggregation, degradation and charge heterogeneity during sample preparation. Therefore, extraction from these matrices requires rapid freezing, fine grinding in liquid nitrogen and low-temperature processing in the presence of reducing agents and polyphenol-binding compounds [18,80,81]. Representative workflows for leaves, shoots, wood, apoplastic fluid and xylem sap are summarized in Table 1.

Table 1. Representative protein extraction workflows for grapevine leaves, shoots, wood, apoplastic fluid and xylem sap.

Tissue/Matrix	Extraction/Sample Preparation Strategy	Fractionation/Separation	MS/Identification/Quantification	Ref.
Leaf apoplastic fluid	Vacuum infiltration–centrifugation followed by TCA/acetone–phenol clean-up	2-DE	MALDI-TOF/TOF	[62]
Leaves	Comparative ethanol/acetone, TCA/acetone and phenol-based extraction	2-DE	MALDI-TOF/TOF	[18]
Leaves	Comparative TCA/acetone, phenol and commercial-kit extraction	2-DE	Not specified	[80]
Leaf apoplastic fluid	Vacuum infiltration–centrifugation followed by ammonium acetate/methanol precipitation	Gel-free nanoLC workflow	nanoLC–MS/MS	[67]
Leaves	TCA/acetone precipitation followed by urea/thiourea/CHAPS solubilization	2-DE	MALDI-TOF	[82]
Leaves	TCA/acetone precipitation followed by SDS-compatible solubilization	Gel-free peptide-labeling workflow	iTRAQ/LC–MS/MS	[83]
Leaves	Phenol extraction	2-DE	MALDI-TOF/TOF	[84]
Leaves	Sequential TCA/acetone–phenol extraction	Gel-free in-solution digestion	LC–MS/MS	[85]
Leaves	Lysis-buffer extraction followed by TCA precipitation	Gel-free tryptic digestion	TMT/LC–MS/MS	[86]
Leaves	TCA/acetone precipitation with PVPP	Gel-free peptide-labeling workflow	iTRAQ/LC–MS/MS	[87]
Leaves	Comparative phenol extraction and TCA/acetone precipitation	2-DE	Not specified	[88]
Leaves	Phenol extraction followed by 2D clean-up	Gel-free workflow	LC–MS/MS/label-free	[89]
Leaves	TCA/acetone precipitation followed by guanidine denaturation and FASP-compatible clean-up	FASP/peptide fractionation	iTRAQ/nanoLC–MS/MS	[90]
Green stems	Sequential TCA/acetone–phenol extraction	2-DE	nanoLC–MS/MS	[81]
Leaves	TCA/acetone precipitation followed by phosphopeptide enrichment	Gel-free phosphopeptide workflow	iTRAQ/LC–MS/MS	[72]

Table 1. *Cont.*

Tissue/Matrix	Extraction/Sample Preparation Strategy	Fractionation/Separation	MS/Identification/Quantification	Ref.
Leaves	TCA/acetone precipitation	2-DE with phosphoprotein staining	MALDI-TOF/TOF	[91]
Leaves	Phenol extraction	2-DE	LC–MS/MS	[92]
Leaves	Phenol extraction	2-DE	MALDI-TOF-MS	[37]
Leaves	TCA/acetone precipitation followed by urea/thiourea/CHAPS solubilization	2-DE	MALDI-TOF/TOF	[93]
Xylem tissue/xylem sap	Urea/thiourea/Triton X-100 extraction followed by TCA precipitation	2-DE	MALDI-TOF/TOF	[94]
Leaves	Phenol extraction	2-DE	nanoHPLC-ESI-MS/MS	[95]
Leaves	Phenol extraction followed by FASP-compatible clean-up	FASP/gas-phase fractionation	LC–MS/MS/label-free	[44]
Leaves	TCA/acetone precipitation followed by SDS-compatible solubilization and FASP clean-up	FASP/SCX peptide fractionation	iTRAQ/LC–MS/MS	[96]
Shoots	TCA/acetone precipitation	2-DE	MALDI-TOF/TOF	[97]
Leaves	Guanidine-HCl extraction followed by FASP-based clean-up	FASP/gel-free workflow	LC–MS/MS/label-free	[48]
Leaves	Phenol extraction	2-DE	MALDI-TOF/TOF	[98]
Leaves/shoots	Urea/thiourea/CHAPS/SDS extraction followed by TCA/acetone precipitation	2-DE	LC-QTOF	[99]
Leaves	TCA/acetone precipitation	OFFGEL/gel-free workflow	LC–MS/MS/label-free	[100]
Stems	SDS-buffer extraction followed by phenol extraction	2-DE	nanoLC–MS/MS	[101]
Leaves	Phenol extraction	DIGE	MALDI-TOF/TOF	[102]
Shoot tips	Phenol extraction	2-DE	MALDI-TOF/TOF	[103]
Leaves	Urea/thiourea-based extraction followed by acetone/methanol precipitation	2-DE	MALDI-TOF/MALDI-TOF/TOF	[104]

Table 1. *Cont.*

Tissue/Matrix	Extraction/Sample Preparation Strategy	Fractionation/Separation	MS/Identification/Quantification	Ref.
Leaves/stems	Phenol extraction	2-DE	Edman sequencing	[105]
Leaf apoplastic fluid	Vacuum infiltration–centrifugation	Gel-free nanoLC workflow	LC–MS/MS	[106]
Leaves/apoplastic fluid	Apoplastic fluid extraction followed by ammonium sulfate precipitation	Chromatographic/affinity fractionation	LC–MS/MS	[107]
Xylem sap	Sap collection followed by ultrafiltration concentration	2-DE	MALDI-TOF/MS/MS	[61]
Xylem sap	Sap collection followed by protein precipitation	2-DE	LC–MS/MS	[108]
Xylem sap	Sap collection followed by TCA/acetone precipitation	Gel-free and/or 2-DE workflow	LC–MS/MS	[109]
Vascular sap/xylem sap	Pressure-chamber sap collection followed by freeze-drying and S-Trap digestion	DIA-compatible gel-free workflow	LC–MS/MS	[110]
Leaves	Phenol extraction followed by clean-up	2-DE/DIGE	MALDI-TOF/TOF	[111]
Leaves	TRIzol extraction followed by acetone precipitation	2-DE	LC–MS/MS	[112]
Leaves	TCA/acetone precipitation	2-DE	MALDI-TOF/TOF	[113]
Leaves	Phenol extraction	2-DE	MALDI-TOF/TOF	[105]
Wood	Sequential TCA/acetone–phenol extraction	2-DE	nanoLC–MS/MS	[63]
Leaves	Phenol extraction	2-DE	LC–MS/MS	[104]

In leaf and young shoot proteomics, TCA/acetone precipitation is widely used. Finely ground frozen tissue is typically treated with 10–20% TCA in cold acetone containing DTT and often PVPP, incubated at −20 °C, centrifuged, washed repeatedly with cold acetone and resolubilized in chaotropic buffers [80,97,114,115]. In gel-free variants, TCA/acetone precipitation is followed by resolubilization in SDS-, urea- or guanidine-containing buffers and subsequent clean-up [72,83,87,90,100,116].

Phenol-based extraction has become a preferred strategy for phenolic-rich leaves, green stems and partially lignified tissues. Ground tissue is typically extracted in dense aqueous buffer containing 0.7 M sucrose, 0.1 M KCl, 50–100 mM Tris-HCl pH 7.5–8.0, 50 mM EDTA, 1–2% PVPP and 1–2% DTT or another reducing agent, usually at tissue-to-buffer ratios close to 1:1 (w/v). An equal volume of buffered or Tris-saturated phenol is added, followed by vigorous mixing, incubation on ice and phase separation by centrifugation at 10,000–12,000× g for 10–20 min at 4 °C. Proteins are precipitated from the phenolic phase with four volumes of 0.1–0.2 M ammonium acetate in methanol at −20 °C, often overnight, then washed repeatedly with cold methanol and/or acetone and resolubilized in chaotropic or denaturing buffers [37,44,71,84,89,95,98,103,105,111]. Comparative studies generally support the superior performance of phenol-based protocols over TCA/acetone alone in phenolic-rich grapevine matrices, with improved yield, spot resolution and reduced streaking [18,117].

For green stems, lignified shoots and wood, sequential strategies are often required. These commonly combine an initial organic wash or TCA/acetone precipitation with phenol partitioning, ammonium acetate/methanol precipitation and repeated methanol or acetone washes. Lignified tissues may require increased PVPP concentrations of 2–4%, longer precipitation times and centrifugation up to 20,000× g for 30 min to recover poorly soluble proteins [63,80,81]. Jogaiah et al. [80] specifically supported a modified TCA/acetone–phenol workflow for recalcitrant grapevine tissues.

A third group of workflows relies on strongly denaturing urea-, thiourea-, SDS- or guanidine-based buffers, particularly when the aim is to maximize solubilization of hydrophobic or membrane-associated proteins. Frozen tissue powder is extracted in 50–100 mM Tris-HCl or HEPES, pH 7.5–8.0, supplemented with 2–4% SDS or 7–8 M urea and 2 M thiourea, often with 2–4% CHAPS, 10–50 mM DTT or 5–10 mM TCEP, 1–2 mM EDTA, 1–2% PVPP and protease inhibitors, at tissue-to-buffer ratios of 1:5–1:10 (w/v). Extracts are incubated for 15–30 min on ice or at 4 °C and clarified at 15,000–20,000 g for 20–30 min, and proteins are precipitated with cold acetone or by methanol/chloroform partitioning to remove detergents, salts, chaotropes and residual phenolics [71,90,94,99,104,113,116]. These protocols improve solubilization but shift the main technical burden to downstream clean-up.

TRIzol-based extraction, followed by acetone precipitation and washing, has also been used in grapevine leaf proteomics when protein extraction is integrated with nucleic acid recovery or parallel molecular analyses, but it remains a less common alternative [112].

Specialized extracellular matrices require distinct workflows. Apoplastic proteins from leaves are typically recovered by vacuum infiltration–centrifugation. One optimized protocol uses approximately 30 g of leaves, vacuum infiltration with 0.1 M Tris-HCl pH 8.0, 0.5 M KCl, 0.006 M CHAPS and 2% Na_2SO_3 at 4 °C, followed by short vacuum cycles and centrifugation at approximately 5000× g for 15 min at 4 °C [62,67,106]. Because apoplastic fluid contains low protein concentrations and is prone to intracellular contamination, concentration and purification are essential. Direct TCA/acetone precipitation may co-precipitate sugars and produce viscous extracts, whereas combined TCA/acetone–phenol clean-up improves sample quality [62,106,107].

Xylem sap protein recovery is similarly challenging because of extremely low protein abundance. Exudates are collected from cut stems or canes, often after brief bleeding, kept on ice, filtered, and concentrated using centrifugal ultrafiltration devices with 3–10 kDa cut-offs. In a Chardonnay xylem sap study, exudate was collected after approximately 20 min bleeding, filtered through 0.2 µm filters, stored at −80 °C and concentrated about 40-fold using Amicon Ultra-15 and Centricon YM-10 filters (Millipore Corporation, Billerica, MA, USA) [61]. Proteins are then commonly precipitated with four to five volumes of cold acetone or 10–20% TCA/acetone at −20 °C for 1–2 h or overnight, washed with cold acetone and resolubilized [61,108–110].

Overall, leaves, shoots, wood, apoplastic fluid and xylem sap illustrate the central trade-off in grapevine protein extraction: TCA/acetone is simple and effective for soluble contaminants but may reduce solubility; phenol extraction provides cleaner extracts for phenolic-rich tissues but is labor-intensive and may cause losses; SDS/urea or guanidine buffers improve solubilization but require stringent clean-up. For recalcitrant wood and extracellular fluids, pre-analytical variation in sampling, concentration and purification may dominate over biological variation.

3.3. Protein Extraction from Buds, Dormant Tissues and Flower Clusters

Grapevine buds and other dormant organs are challenging matrices because they combine low protein abundance with starch, lipids, phenolics and lignified protective scales. Extraction protocols therefore emphasize strong denaturation, efficient contaminant removal, strict control of tissue-to-buffer ratios, low-temperature precipitation and repeated washing [49,118–120]. Representative workflows for buds, dormant tissues and flower clusters are summarized in Table 2. Although grapevine tendrils are relevant developmental organs, no dedicated proteomic workflow for tendril tissue was identified in the literature surveyed here.

Table 2. Representative protein extraction workflows for grapevine buds, dormant tissues and flower clusters.

Tissue/Matrix	Extraction/Sample Preparation Strategy	Fractionation/Separation	MS/Identification/Quantification	Ref.
Developing buds	Optimized TCA/acetone-, phenol- or urea-based extraction followed by chaotropic resolubilization	2-DE; immunoblotting	Immunodetection of tubulin isoforms; no MS-based identification	[119]
Buds	Borate-buffer extraction followed by ammonium acetate/methanol precipitation	1D SDS-PAGE	Protein-band profiling; no MS-based identification	[118]
Buds	Phenol extraction followed by methanol/chloroform precipitation and FASP digestion	Gel-free workflow; gas-phase fractionation	LC–MS/MS/label-free quantification	[49]
Buds	Urea/Triton-based extraction followed by TCA precipitation and acetone washing	iTRAQ workflow; high-pH RP peptide fractionation	LC–MS/MS/iTRAQ	[120]
Flower buds (from flower clusters)	Urea-based extraction followed by chloroform/methanol precipitation	iTRAQ workflow; SCX peptide fractionation	nanoLC–MS/MS/iTRAQ	[54]

Table 2. *Cont.*

Tissue/Matrix	Extraction/Sample Preparation Strategy	Fractionation/Separation	MS/Identification/Quantification	Ref.
Dormant buds	Phenol extraction followed by methanolic ammonium acetate precipitation	TMT workflow; high-pH RP peptide fractionation	LC–MS/MS/TMT	[121]
Dormant buds	Urea-based lysis followed by TCA precipitation and acetone washing	TMT workflow; high-pH RP peptide fractionation	LC–MS/MS/TMT	[122]

Phenol-based protocols are widely used for dormant buds because they efficiently separate proteins from phenolics, polysaccharides and other hydrophilic contaminants. Bud powder is typically homogenized in Tris–sucrose buffer containing KCl, EDTA, reducing agents and often PVPP, followed by phenol partitioning and ammonium acetate/methanol precipitation at −20 °C. Pellets are washed with methanol and/or acetone and resolubilized in urea/thiourea- or SDS-based buffers [49,79,119]. Wake and Fennell [118] used a simpler borate-buffer extraction followed by ammonium acetate/methanol precipitation, organic-solvent washing and resuspension in Laemmli buffer.

SDS/urea-based protocols are common in shotgun and quantitative bud proteomics. Buds are ground in liquid nitrogen and extracted with strongly denaturing buffers containing 8 M urea, detergents such as 1% Triton X-100 or 2–4% SDS, DTT and protease inhibitors, usually at tissue-to-buffer ratios of 1:4–1:5 (w/v). Sonication on ice is often used to improve solubilization, debris is removed by high-speed centrifugation, and proteins are precipitated with cold TCA/acetone, commonly at −20 °C for about 2 h. Pellets are washed three or more times with cold acetone, briefly dried and resolubilized in urea-based buffers [120–122]. Some workflows include additional precipitation or filter-based clean-up after reduction and alkylation to remove residual urea, detergents and salts [49,120].

Despite differences in chemistry, bud protocols share several principles: strong denaturation, controlled tissue-to-buffer ratios, low-temperature precipitation, repeated organic washes and inclusion of reductants, protease inhibitors and, where appropriate, PVPP. Phenol extraction provides a good compromise between purity and recovery in phenolic-rich dormant buds, whereas urea/SDS extraction improves solubilization but requires more stringent clean-up. Comparability between studies remains limited by differences in buffer composition, precipitation time, washing steps, drying conditions and resolubilization efficiency.

3.4. Protein Extraction from Roots and Rhizosphere-Associated Matrices

Grapevine roots and rootstocks represent particularly recalcitrant matrices because they contain structural polysaccharides, suberin, phenolics, starch and mucilage and, when collected from soil-grown plants, may retain humic substances, salts, mineral particles and microbial material. Root protein extraction therefore requires careful removal of adhering soil, rapid freezing and fine grinding in liquid nitrogen, together with efficient removal of phenolic and polysaccharide contaminants. Depending on the analytical objective, phenol-based extraction, sequential TCA/acetone–phenol protocols and precipitation-based workflows coupled with downstream clean-up have been applied to grapevine roots, whereas rhizosphere metaproteomics requires dedicated procedures for recovery of proteins from soil matrices [51,64–66,80,123]. Representative root and rhizosphere workflows are summarized in Table 3.

Table 3. Representative protein extraction workflows for grapevine roots, rootstocks and rhizosphere-associated matrices.

Extraction/Sample Preparation Strategy	Fractionation/Separation	MS/Identification/Quantification	Ref.
Phenol-based/PVPP total-protein extraction followed by SDS-buffer resolubilization	GeLC–MS/MS/in-gel digestion	LC-ESI-MS/MS/label-free quantification	[51]
Phenol-based/PVPP total-protein extraction followed by SDS-buffer resolubilization	GeLC–MS/MS	LC–MS/MS/label-free quantification	[64]
Phenol extraction + MeOH/acetone precipitation followed by SDS-buffer resolubilization	GeLC–MS/MS	LC–MS/MS/label-free quantification	[65]
NoviPure Soil Protein Extraction Kit	In-solution digestion + C18 SPE	Micro-LC–TripleTOF DDA	[66]
Modified TCA/acetone precipitation followed by FASP digestion	iTRAQ workflow with SCX peptide fractionation	LC–MS/MS/iTRAQ quantification	[123]
Comparative phenol vs. TCA/acetone extraction	2-DE	None	[88]

A direct comparison of extraction methods for *Vitis vinifera* roots was performed by Jellouli et al. [88], who evaluated TCA/acetone precipitation and phenol-based extraction. In the phenol protocol, frozen root powder was extracted in a sucrose-containing buffer supplemented with KCl, Tris-HCl, EDTA, PVPP, PMSF and β-mercaptoethanol, followed by partitioning with water-saturated phenol and precipitation of proteins from the phenolic phase with ammonium acetate in methanol. The resulting pellets were washed with methanol and acetone and resolubilized in a urea/thiourea/CHAPS-containing buffer. Compared with TCA/acetone precipitation, phenol extraction provided higher protein recovery, a greater number of resolved protein spots and reduced streaking in 2-DE, demonstrating its suitability for chemically complex grapevine root tissue [88].

Sequential TCA/acetone–phenol extraction provides an alternative when more extensive contaminant removal is required. Jogaiah et al. [80] found that a modified TCA/acetone–phenol protocol produced high-quality protein preparations from roots and other recalcitrant grapevine matrices. The workflow combined initial TCA/acetone precipitation and organic-solvent washing with phenol–SDS extraction and ammonium acetate/methanol precipitation. This extensive clean-up improves the removal of matrix-derived contaminants, although the additional handling may increase processing time and potential protein losses [80].

More recent quantitative root proteomics studies have coupled optimized total-protein extraction with SDS-based resolubilization and GeLC–MS/MS. In drought- and salt-stress studies of grapevine rootstocks, root material was thoroughly washed, frozen and ground in liquid nitrogen before protein extraction, and the recovered protein fractions were subsequently dissolved in SDS-containing buffer and processed by 1D SDS-PAGE followed by in-gel digestion and LC–MS/MS [51,64]. Thus, in these workflows, SDS primarily served as a strong resolubilization reagent after protein extraction rather than representing the principal extraction chemistry itself. Electrophoretic processing additionally provided an effective interface between chemically complex root extracts and downstream LC–MS/MS analysis.

A phenol-based workflow was used for proteomic analysis of nitrate responses in M4 grapevine rootstock [65]. Frozen root powder supplemented with PVPP was extracted using phenol and an aqueous buffer containing sucrose, EDTA, ascorbic acid, Triton X-100,

protease inhibitors and β-mercaptoethanol. Proteins recovered from the phenolic phase were subsequently purified by methanol- and acetone-based precipitation, and the final pellet was dissolved in an SDS-containing buffer before GeLC–MS/MS analysis [65]. This workflow illustrates the complementary functions of phenol extraction and subsequent SDS resolubilization: phenol partitioning facilitates the removal of phenolics and other matrix components, whereas SDS improves solubilization of the purified protein fraction before electrophoretic clean-up.

Precipitation-based extraction has also been successfully coupled to gel-free quantitative proteomics. In a comparative study of root cold resistance, Chen et al. [123] homogenized frozen root material in phosphate buffer and subsequently added pre-chilled TCA/acetone. Proteins were precipitated at low temperature, recovered by centrifugation, repeatedly washed with cold acetone and resolubilized in triethylammonium bicarbonate. The samples were then processed by filter-aided sample preparation, followed by iTRAQ labeling, SCX peptide fractionation and LC–MS/MS [123]. This approach demonstrates that TCA/acetone precipitation can be integrated into quantitative gel-free workflows when subsequent resolubilization and peptide-level clean-up are carefully controlled.

Rhizosphere metaproteomics represents a distinct analytical problem because the recovered proteome originates from a heterogeneous mixture of plant, microbial and soil-associated extracellular proteins. Bona et al. [66] therefore employed a dedicated soil-protein extraction procedure rather than a conventional grapevine tissue extraction buffer. Following protein recovery, the protein fraction was processed by reduction, alkylation and tryptic digestion, and the resulting peptides were purified before LC–MS/MS analysis [66]. Such workflows must simultaneously maximize recovery of proteins from different biological sources while minimizing interference from humic substances and other soil-derived components.

Overall, the available studies indicate that no single extraction strategy is optimal for all grapevine root-associated matrices. Phenol-based extraction has demonstrated superior performance over TCA/acetone precipitation in direct comparative evaluation of grapevine roots, particularly with respect to protein recovery and 2-DE quality [88]. Sequential TCA/acetone–phenol procedures provide more extensive clean-up for highly recalcitrant material [80], whereas TCA/acetone precipitation coupled to FASP-compatible processing represents an alternative for quantitative gel-free workflows [123]. In rootstock proteomics, optimized protein extraction followed by SDS resolubilization and GeLC–MS/MS remains useful for obtaining MS-compatible samples from complex root matrices [51,64,65]. Rhizosphere samples, in contrast, require dedicated soil-protein extraction strategies [66]. Comparability among studies remains limited by differences in root washing, tissue input, extraction-buffer composition, tissue-to-solvent ratio, PVPP concentration, precipitation and washing conditions, resolubilization efficiency and downstream clean-up.

3.5. Protein Extraction from Grapevine Cell and Tissue Cultures

Grapevine cell and tissue cultures, including callus, embryogenic callus, non-embryogenic callus and suspension cultures, are generally less recalcitrant than lignified organs because they lack extensive secondary cell walls and suberized tissues. Nevertheless, their high water content, friable structure, extracellular polysaccharides and, in elicited cultures, accumulation of phenolics and secreted metabolites can dilute intracellular proteins and interfere with protein precipitation and solubilization. Most protocols therefore combine rapid harvesting, liquid-nitrogen freezing, cold precipitation and resolubilization in chaotropic or detergent-containing buffers. Representative workflows for cell cultures, culture medium and related extracellular fractions are summarized in Table 4.

Table 4. Representative protein extraction workflows for grapevine cell cultures and extracellular protein fractions.

Tissue/Matrix	Extraction/Sample Preparation Strategy	Fractionation/Separation	MS/Identification/Quantification	Ref.
Embryogenic and non-embryogenic callus	Phenol extraction with PVPP and ammonium acetate/methanol precipitation	2-DE	LC–ESI–MS/MS	[124]
Embryogenic and non-embryogenic callus	TCA/acetone precipitation with DTT followed by chaotropic resolubilization	2-DE	MALDI-TOF-MS	[125]
Embryogenic callus	TCA/acetone-based extraction followed by chaotropic resolubilization	2-DE	MALDI-TOF-MS	[126]
Cell suspension culture	TCA/acetone-based extraction	Gel-based protein profiling	MALDI-TOF	[127]
Cell suspension culture	TCA/acetone-based extraction	2-DE	MALDI-TOF and Q-TOF MS/MS	[128]
Cell suspension culture	Acetone pretreatment followed by guanidine/N-lauroylsarcosine extraction and chloroform–methanol precipitation	FASP with gas-phase fractionation	LC–MS/MS/label-free	[129]
Berry-derived suspension cultures	TCA/acetone precipitation followed by urea/thiourea/CHAPS resolubilization	Liquid-phase IEF followed by SDS-PAGE	MS identification of gel bands	[130]
Culture medium/extracellular proteins	Cell removal by filtration; extracellular-medium clean-up with PVPP, TCA precipitation and organic-solvent washes	2-DE	MALDI-TOF and LC–MS/MS	[131]

For embryogenic and non-embryogenic callus, TCA/acetone precipitation is frequently used. Frozen material is typically extracted with cold acetone containing about 10% TCA and DTT, incubated at −20 °C, centrifuged, washed with cold acetone and resolubilized in urea/thiourea/CHAPS-based IEF buffer [125]. Similar TCA/acetone-based strategies have been used in suspension-culture and elicitor studies [127,128]. These protocols are suitable for relatively soft cell biomass, although pellet resolubilization can become problematic when elicitation increases phenolic or secondary-metabolite accumulation.

Phenol extraction has also been applied to callus and embryogenic tissues when phenolics and extracellular polysaccharides compromise gel quality. Frozen biomass is homogenized in sucrose-containing buffer with Tris-HCl, KCl, EDTA, PVPP and reducing agents, mixed with buffered phenol, and proteins are precipitated from the phenolic phase with ammonium acetate/methanol at −20 °C. Pellets are washed with methanol and/or acetone and resolubilized in urea/thiourea/CHAPS buffer. This approach is particularly useful for embryogenic callus or friable aggregates prone to browning [8,124].

For shotgun proteomics of suspension cultures, stronger denaturing extraction protocols have been introduced. George et al. [129] harvested Cabernet Sauvignon suspension cells, stored them at −80 °C, and extracted approximately 1 g fresh weight after treatment with ice-cold 80% acetone containing 10 mM $Na_2S_2O_5$ at −20 °C for 12 h. After centrifugation at 11,000× *g* for 10 min at −4 °C, proteins were extracted from pellets

using 6 M guanidine-HCl, 1% N-lauroylsarcosine, 10 mM EDTA, 0.1 M tricine and 5% β-mercaptoethanol, followed by 5 min sonication, incubation at 75 °C for 10 min, centrifugation and chloroform–methanol precipitation. This provides stronger solubilization than classical 2-DE protocols and is suitable for gel-free LC–MS/MS [129].

For berry-derived suspension cultures, Sharathchandra et al. [130] used TCA/acetone-based extraction and reported protein yields of approximately 0.57–0.83 mg/g fresh weight, depending on culture stage, showing that even relatively homogeneous cell suspensions can differ in extraction yield according to developmental origin.

Extracellular and secreted proteomes require a different extraction logic. In grapevine suspension cultures, spent medium is separated from cells by filtration and used as an apoplast-like extracellular protein source. Such workflows avoid tissue disruption for the extracellular fraction but require efficient concentration and precipitation because secreted proteins are diluted in large culture volumes [131,132].

Overall, TCA/acetone precipitation is simple and effective for callus and suspension cells, especially for gel-based workflows, but may produce pellets that are difficult to resolubilize. Phenol extraction improves removal of phenolics and polysaccharides and is preferable for browning or highly elicited cultures. Guanidine/SDS-compatible workflows provide stronger solubilization and better compatibility with shotgun proteomics but require rigorous precipitation and clean-up. For extracellular proteomes, the main limitation is low protein concentration rather than tissue recalcitrance.

3.6. Protein Extraction from Grape Berries and Berry-Derived Matrices

Grape berries are among the most intensively studied grapevine matrices in proteomics, but they present a difficult extraction problem because of low protein abundance and high concentrations of sugars, organic acids, polyphenols, pigments and, depending on tissue type, lipids or storage proteins. Workflows therefore differ between whole-berry extracts and tissue-specific preparations of mesocarp/flesh, skins/exocarp and seeds. Representative berry workflows are summarized in Table 5.

Table 5. Representative protein extraction workflows for grape berries and berry-derived tissue fractions.

Tissue/Matrix	Extraction Method	Fractionation/Separation	MS/Identification/Quantification	Ref.
Berry flesh/mesocarp	Phenol extraction	2D-DIGE	LC–MS/MS	[133]
Berry mesocarp/skin	TCA/acetone precipitation	2-DE	MS identification	[134]
Ripe berry skin chloroplast fraction	Plastid enrichment followed by phenol extraction	Gel-free LC separation	LC–MS/MS	[69]
Berry cell wall fraction	Sequential cell-wall protein extraction	2-DE	MS identification	[40]
Whole berry/berry tissues	TCA/acetone precipitation	2-DE	MALDI-TOF MS	[39]
Grape juice/berry-derived chitinase fraction	Chromatographic purification from grape juice	Chromatographic purification	MS peptide mapping	[135]
Berry skin	Ammonium sulfate extraction and chromatographic enrichment	Biochemical enrichment/chromatography	Mass mapping	[136]

Table 5. *Cont.*

Tissue/Matrix	Extraction Method	Fractionation/Separation	MS/Identification/Quantification	Ref.
Berry skin	Phenol extraction	2-DE	MS identification	[137]
Berry skin	TCA/acetone washing followed by phenol extraction	Gel-free in-solution digestion	nanoLC–Orbitrap MS/MS/label-free	[138]
Grape seeds	Defatting followed by aqueous/TFA extraction	Direct MALDI profiling	MALDI-MS	[139]
Berry skin	Modified phenol extraction	Gel-free LC separation	LC–MS/MS/multi-omics quantification	[140]
Berry exocarp/mesocarp	TCA/acetone washing followed by phenol extraction	iTRAQ workflow with peptide fractionation	LC–MS/MS/iTRAQ	[43]
Berry mesocarp	TCA/acetone precipitation	2-DE	MALDI-MS/peptide mass fingerprinting	[36]
Berry plasma membrane fraction	Plasma membrane enrichment followed by membrane protein extraction	Membrane fractionation/2-DE	MS identification	[60]
Grape seeds	Water-soluble seed protein extraction	Protein fractionation	MS identification	[141]
Whole berry	Methanol/chloroform/water extraction	Gel-free LC-based workflow	LC–MS/MS/integrated omics	[42]
Muscadine berry	Phenol extraction	Gel-free shotgun workflow	LC–MS/MS/label-free	[45]
Berry exocarp/skin	TCA/acetone washing followed by phenol extraction	iTRAQ workflow with peptide fractionation	LC–MS/MS/iTRAQ	[132]
Whole berry/berry tissue	TCA/acetone washing followed by phenol extraction	iTRAQ workflow with peptide-level fractionation	LC–MS/MS/iTRAQ	[142]
Berry pericarp	Phenol extraction	SCX peptide fractionation	LC–MS/MS/iTRAQ	[47]
Mesocarp and exocarp	Phenol extraction	PTM enrichment: phospho-, glyco- and acetyl-peptides	LC–MS/MS/PTM profiling	[70]
Berry skin	Phenol extraction with PVPP	2-DE/biomarker-oriented profiling	MS identification	[143]
Berry skin	Phenol extraction	2-DE	MALDI-MS/LC–MS/MS	[38]
Berry skin	Phenol extraction with PVPP	2-DE	LC–MS/MS	[59]
Grape berry	Phenol extraction	Gel-free shotgun workflow	LC–MS/MS/label-free	[46]
Skin, pulp and seed	TCA/acetone precipitation	1D/2D or gel-based separation	LC–MS/MS/relative quantification	[144]
Grape berries	Phenol extraction followed by in-solution digestion	Gel-free LC separation	LC–MS/MS	[145]

Table 5. *Cont.*

Tissue/Matrix	Extraction Method	Fractionation/Separation	MS/Identification/Quantification	Ref.
Whole berry/deseeded berry tissue	Phenol extraction with repeated TCA/acetone washing	Gel-free workflow	LC–MS/MS	[53]
Skin, pulp and seed	Phenol extraction	2-DE/gel-based profiling	MS identification	[146]
Whole berries	Phenol extraction	DIA-compatible LC separation	LC-MSE/DIA	[55]
Whole berry	Vacuole isolation followed by urea/thiourea/CHAPS extraction	Gel-free peptide-level workflow	LC–MS/MS/quantitative proteomics	[52]
Whole berry	TCA/acetone precipitation	iTRAQ peptide workflow	LC–MS/MS/iTRAQ	[13]
Berry	Methanol/chloroform/water extraction	Gel-free LC-based workflow	LC–MS/MS/network analysis	[56]
Seed endosperm	Osborne fractionation	Sequential protein fractionation	MS identification	[147]
Whole berry	TCA/acetone precipitation	2D-DIGE	MS identification	[41]
Grape berry	Methanol/chloroform precipitation	FASP/DIA-type workflow	nanoUPLC–Q-TOF ion-mobility HDMS^E/label-free	[58]

Early proteomic studies of berry mesocarp and flesh and whole berries mainly used TCA/acetone precipitation or phenol extraction. In TCA/acetone workflows, frozen tissue is typically ground in liquid nitrogen and precipitated with cold TCA/acetone containing a reducing agent and often PVPP. Repeated acetone washing removes soluble metabolites before proteins are resolubilized in chaotropic buffers [36,37,39,42,146]. This approach is effective for removing sugars, organic acids and salts but may generate compact protein pellets that are difficult to resolubilize.

Phenol extraction is widely used when sugar, pigment and phenolic contamination compromises protein recovery. Powdered tissue is extracted in sucrose-containing buffer, typically with 0.7 M sucrose, Tris-HCl, KCl, EDTA, PVPP and DTT or β-mercaptoethanol, followed by addition of an equal volume of buffered or Tris-saturated phenol. Proteins are precipitated from the phenolic phase with 0.1–0.2 M ammonium acetate in methanol at −20 °C, often overnight, washed with methanol and/or acetone and resolubilized in urea/thiourea/CHAPS or SDS/urea buffers. Compared with TCA/acetone alone, phenol extraction generally provides cleaner extracts from phenolic-rich berry tissues, although it requires more handling and may increase protein losses during phase separation and precipitation [17,18,40].

Berry skins and exocarp are particularly recalcitrant because of their high concentrations of polyphenols, anthocyanins, tannins and cuticular components. Skin proteomics therefore relies mainly on phenol extraction or optimized TCA/acetone protocols with PVPP, low-temperature processing and extensive organic-solvent washing. These measures are intended to reduce pigments and phenolic carryover, which otherwise affects protein solubility and downstream separation [38–40,50,133,137,148].

Grape seeds represent a distinct berry sub-matrix because they contain storage proteins, lipids, tannins and other phenolics. Seed workflows may include defatting, extraction in denaturing buffers, precipitation and repeated washing before protein resolubilization. These strategies have been used for seed protein fingerprinting and characterization of seed proteins [139,141,147,149,150].

Specialized berry subproteomes require additional enrichment before protein extraction. Cell wall workflows use extensive washing and sequential extraction of wall-associated proteins, plasma membrane studies require membrane enrichment before solubilization, and chloroplast proteomics requires organelle enrichment before extraction [40,60,69]. These datasets represent defined protein fractions and are therefore not directly comparable with total berry proteomes.

Quantitative berry proteomics further requires compatibility with downstream labeling or high-resolution LC–MS/MS. DIGE depends on reproducible extraction and efficient resolubilization. iTRAQ/TMT, label-free and DIA workflows commonly use phenol-, TCA/acetone- or SDS/urea-based extraction followed by stringent clean-up, precipitation or filter-based processing [13,41,45,46,52,53,55,56,58,132,138,140,142,145]. The extraction strategy must therefore be selected together with the intended quantitative workflow rather than as an independent step.

Overall, berry extraction is strongly matrix dependent. TCA/acetone efficiently removes soluble metabolites but may compromise pellet resolubilization, particularly in skins and seeds. Phenol extraction generally produces cleaner preparations but is more labor-intensive. SDS/urea-based workflows improve protein solubilization and are well suited to shotgun proteomics but require efficient downstream clean-up. Seed, cell wall, plasma membrane and chloroplast fractions require additional matrix-specific processing and should be interpreted separately from total berry extracts.

3.7. General Critical Assessment

To facilitate direct comparison across the major grapevine matrices discussed in the preceding sections, their main analytical challenges and representative proteomic workflows are summarized in Table 6.

Across grapevine tissues, protein extraction involves a trade-off between protein recovery, extract purity, resolubilization efficiency and compatibility with downstream analysis [17,79,80]. TCA/acetone precipitation is simple and effective for removing soluble metabolites, salts and sugars, but may produce pellets that are difficult to resolubilize, particularly from phenolic-rich, lignified or lipid-rich tissues [15,33,35]. Phenol-based extraction generally provides cleaner preparations from matrices rich in polyphenols, pigments and polysaccharides, although it is more labor-intensive and may introduce protein losses during phase partitioning and precipitation [17,18,33]. SDS-, urea-, thiourea- and guanidine-based protocols improve solubilization of hydrophobic and membrane-associated proteins and are well suited to gel-free workflows, but require efficient downstream removal of detergents, chaotropes and other MS-incompatible components [8,10,151].

Table 6. Comparative overview of representative proteomic workflows across major grapevine tissues and extracellular matrices.

Tissue/Matrix	Main Analytical Challenge	Representative Extraction/ Sample Preparation	Fractionation/ Digestion	MS/Identification/ Quantification	Ref.
Leaves	Polyphenols, pigments, waxes and polysaccharides	Phenol or TCA/acetone extraction; PVPP-assisted clean-up; guanidine- or SDS-compatible extraction followed by FASP where required	2-DE/DIGE; FASP; in-solution digestion; peptide fractionation	MALDI-TOF/TOF; LC–MS/MS; label-free; iTRAQ/TMT	[18,44,48,83,90,102]
Wood/lignified tissues	Very low protein abundance; lignin, suberin and structural polysaccharides	Sequential TCA/acetone–phenol or phenol-based extraction with extensive contaminant removal	2-DE; gel-based prefractionation	nanoLC–MS/MS; MS-based spot identification	[63,80,81]
Buds/dormant tissues	Low protein abundance; starch, lipids, phenolics and protective scales	Phenol extraction; urea/Triton-based extraction with TCA/acetone clean-up; FASP-compatible preparation	2-DE; FASP; high-pH RP peptide fractionation	MALDI-TOF/TOF; LC–MS/MS; label-free; iTRAQ/TMT	[49,120–122]
Roots/rootstocks	Phenolics, suberin and structural polysaccharides; soil- and humic-derived contaminants	Phenol/PVPP extraction; sequential TCA/acetone–phenol extraction; TCA/acetone followed by FASP	2-DE; GeLC/in-gel digestion; FASP with peptide fractionation	LC–MS/MS; label-free; iTRAQ	[51,64,65,80,123]
Berries/berry tissues	Sugars, organic acids, polyphenols and pigments; lipids and storage proteins in seeds	Phenol or TCA/acetone extraction; tissue-specific clean-up or subproteome enrichment	2-DE/DIGE; gel-free in-solution digestion; FASP; peptide fractionation	MALDI-MS; LC–MS/MS; label-free; iTRAQ; DIA	[36,43,45,55,58,138]
Apoplastic fluid	Very low protein abundance and risk of intracellular contamination	Vacuum infiltration–centrifugation followed by precipitation, concentration and/or clean-up	2-DE or gel-free digestion	MALDI-TOF/TOF; nanoLC–MS/MS	[62,67,106]
Xylem sap	Extremely low protein abundance and residual salts	Sap collection followed by filtration, ultrafiltration/concentration, precipitation or S-Trap processing	2-DE or gel-free/S-Trap digestion	MALDI-TOF/MS/MS; LC–MS/MS; DIA	[61,108–110]

Quantitative information on total protein recovery is surprisingly scarce in the grapevine proteomics literature. Only a limited number of methodological studies report protein yield normalized to tissue mass, and even fewer compare extraction methods directly using the same biological material. In a direct comparison of phenol and TCA/acetone extraction, Jellouli et al. [88] reported protein yields of 2.23 and 1.45 mg g^{-1} fresh weight (FW), respectively, from grapevine leaves and 1.06 and 0.045 mg g^{-1} FW from roots. The corresponding 2-DE analyses resolved 613 versus 428 spots in leaves and 612 versus 250 spots in roots, demonstrating that the phenol protocol improved both measured protein recovery and electrophoretic resolution. Jogaiah et al. [80] reported quantitative protein yields across several grapevine matrices using their optimized TCA/acetone–phenol procedure. Reported values were approximately 1.98 mg g^{-1} FW in leaves, 4.83 mg g^{-1} FW in buds, 1.75 mg g^{-1} FW in flower clusters, 1.14 mg g^{-1} FW in berries and 1.02 mg g^{-1} FW in roots. These values illustrate substantial matrix-dependent differences in extractable protein, but they should not be interpreted as universal tissue-specific reference ranges because they were obtained using a single extraction workflow and defined experimental material.

Importantly, a higher measured protein yield does not necessarily correspond to a better proteomics-compatible extract. Sebastiana et al. [18] directly compared phenol, ethanol/acetone and TCA/acetone extraction of grapevine leaves and obtained apparent protein yields of 3.78, 20.55 and 16.57 mg g^{-1} FW, respectively. Despite its substantially lower measured yield, phenol extraction produced the highest-quality 2-DE separation and 532 resolved spots, compared with 166 after ethanol/acetone and 362 after TCA/acetone extraction. The authors attributed the discrepancy partly to interference from co-extracted non-protein compounds in colorimetric protein quantification. This direct comparison therefore shows that total protein yield, extract purity and analytical performance should be considered together rather than treating protein concentration alone as an indicator of extraction efficiency [18].

The quantitative values reported in these methodological studies must also be distinguished from proteome coverage. Numbers of resolved 2-DE spots reflect electrophoretic complexity and extraction quality, but individual spots may contain more than one protein, while multiple spots may correspond to different isoforms or modified forms of the same protein. Consequently, spot counts cannot be interpreted as the number or proportion of proteins represented in the grapevine proteome. Likewise, numbers of proteins identified in independent LC–MS/MS studies cannot be directly compared as measures of workflow performance when tissue type, extraction chemistry, fractionation depth, protein input, LC configuration, MS platform, sequence database and identification criteria differ. Most of the studies surveyed here were designed to investigate biological responses rather than to benchmark the fraction of the proteome recovered by different analytical workflows.

Thus, despite the large number of grapevine proteomics studies, systematic quantitative benchmarking remains a major data gap. For most matrices, including berries, wood, apoplastic fluid and xylem sap, comparable data linking tissue input, total protein recovery, extract purity, peptide recovery and the number of proteins subsequently identified by modern LC–MS/MS are either absent or inconsistently reported. This limitation is particularly relevant to the further development of fully gel-free bottom-up workflows, for which sufficient protein recovery alone is not adequate; the extract must also support efficient digestion, peptide recovery, chromatographic separation and MS detection. Matrix-specific studies that directly compare established phenol- or TCA/acetone-based protocols with FASP, S-Trap, SP3 and related gel-free processing strategies using common quantitative endpoints would therefore be especially valuable.

More generally, inconsistent methodological reporting continues to limit comparison across studies [9,34,152]. Tissue mass, fresh or dry weight basis, buffer volume, tissue-to-buffer ratio, PVPP concentration, precipitation conditions, centrifugation parameters, number of washes, drying conditions, resolubilization efficiency and the method used for protein quantification are not always reported in sufficient detail [16,153,154]. Future grapevine proteomics studies should therefore report protein yield relative to a clearly defined tissue input, together with basic extract-quality and downstream-performance metrics. Where appropriate, these should include protein recovery, peptide recovery, digestion efficiency and the number of reproducibly identified proteins, while clearly distinguishing direct workflow comparisons from cross-study observations [35,155,156].

4. Fractionation and Separation

Proteome complexity in grapevine tissues often requires fractionation to reduce sample complexity, remove residual contaminants, enrich defined protein pools and improve detection of low-abundance proteins [10,12,15,16,34]. Grapevine studies have used 1D SDS-PAGE, 2-DE, 2D-DIGE, GeLC-type workflows, liquid-phase isoelectric focusing, chromatographic prefractionation and compartment-specific enrichment across leaves, shoots, roots, wood, buds, cell cultures, berries, apoplastic fluid, xylem sap and seed fractions [9,32,37,40,61,62,67,149,157].

Over time, the field has shifted from protein-map generation toward protein-level clean-up, targeted enrichment and compartment-resolved profiling [28,55,79,146,158–160]. The choice of strategy depends on matrix composition, extraction chemistry, extract quality and the required analytical depth [74,153]. The main approaches are summarized in Figure 4.

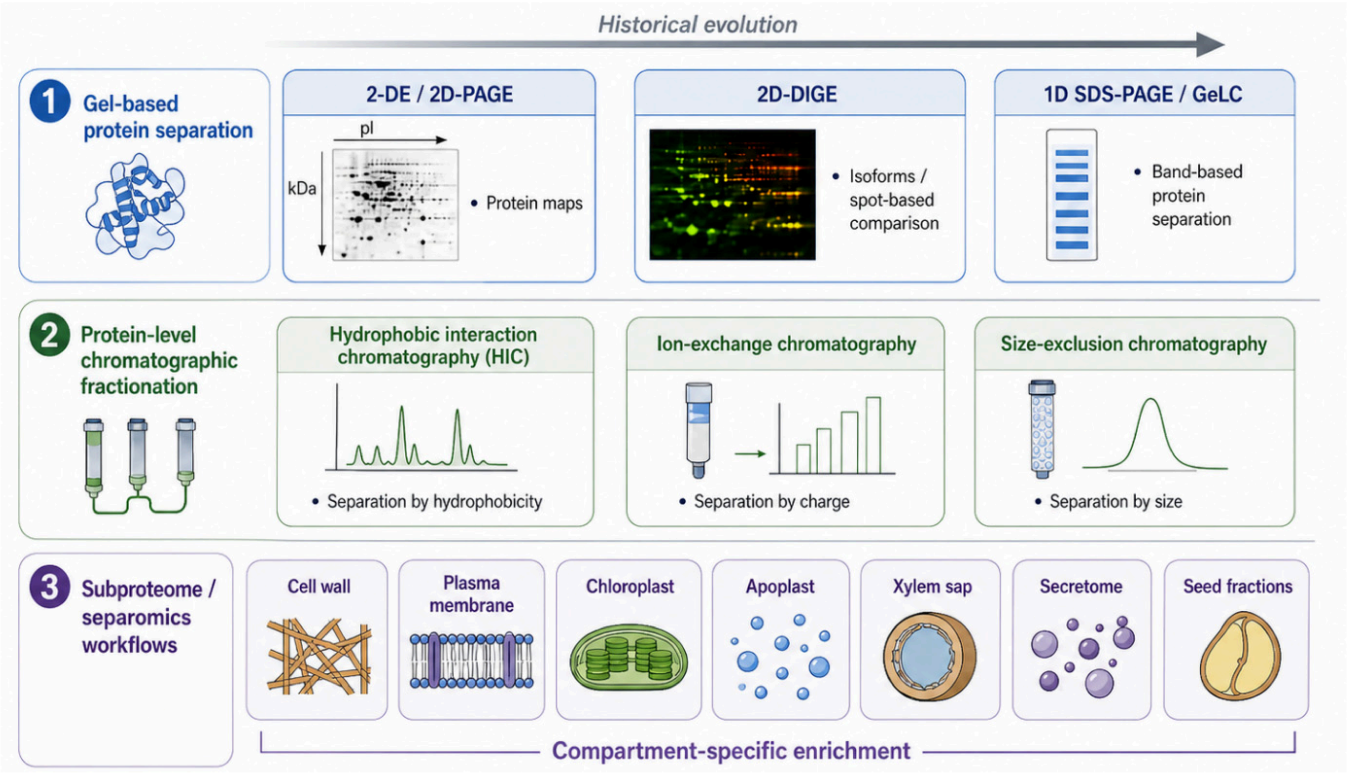


Figure 4. Major protein fractionation and separation strategies used in grapevine proteomics, including gel-based, chromatographic and subproteome-enrichment approaches.

4.1. Gel-Based Protein Separation

Gel-based electrophoresis plays a foundational role in grapevine proteomics. In 2-DE workflows, extracted proteins are typically resolubilized in buffers containing urea, thiourea, CHAPS or related detergents and reducing agents, separated by isoelectric focusing on immobilized pH gradients, and then resolved by SDS-PAGE. Broad pH 3–10 gradients and narrower ranges such as pH 4–7 have been used, depending on the analytical objective. This approach has been applied to leaves, roots, shoot tips, green stems, wood, apoplastic fluid, callus, cell suspensions and berry tissues [18,36–39,63,79–81,103,105,146].

Although gel conditions differ among studies, the analytical purpose is similar. Delaunois et al. [62] separated whole-leaf and apoplastic-fluid proteins on 18 cm pH 3–10 non-linear IPG strips followed by 12% SDS-PAGE, whereas Jogaiah et al. [80] used 13 cm pH 3–10 strips for recalcitrant grapevine tissues. Comparable workflows were used for callus, suspension cultures, roots and berries to generate protein maps and select spots for MS identification [36,40,124–126,143].

In method-comparison studies, 2-DE also served as a practical quality-control readout for extraction efficiency. Spot number, streaking, sharpness, background staining and distribution across pI and molecular-mass ranges provided direct evidence of extract cleanliness and protein resolubilization. This was particularly relevant for phenolic-rich leaves, roots, berry skins and woody tissues, where residual phenolics, pigments, polysaccharides and salts impaired isoelectric focusing and spot resolution [18,79,80,117]. This role is especially important when extraction protocols are compared, because differences in streaking and spot recovery provide an immediate indication of residual matrix effects before downstream MS analysis.

The main limitation of 2-DE is selective proteome representation. Abundant and soluble proteins are favored, whereas hydrophobic, extreme-pI, very large, very small and low-abundance proteins are underrepresented. Abundant photosynthetic proteins may dominate leaf gels, while residual matrix components reduce reproducibility in roots, wood and berry skins. Thus, 2-DE remains useful for protein-map generation, isoform visualization and extraction-quality assessment, but not for comprehensive proteome representation.

4.2. DIGE as a Quantitative Gel-Based Format

Two-dimensional difference gel electrophoresis improves relative spot quantification by labeling protein samples with spectrally distinct cyanine dyes and resolving them on the same 2D gel together with an internal standard [9,35,37,124]. This reduces gel-to-gel variability compared with conventional 2-DE.

In grapevine, DIGE has been applied to leaves, berry flesh and exocarp, postharvest or withered berries and cell cultures [41,102,111,132,161]. DIGE is useful when protein-spot resolution and relative quantification are both required, but it retains the principal limitations of 2-DE. Poorly soluble, hydrophobic, low-abundance and extreme-pI proteins remain underrepresented, and performance still depends strongly on extraction purity and efficient resolubilization.

4.3. One-Dimensional SDS-PAGE, GeLC and Liquid-Phase IEF

Several grapevine workflows use 1D SDS-PAGE, short-gel separation or GeLC-type processing primarily as clean-up and prefractionation before MS [37,48,55]. Proteins are separated or concentrated into gel bands and subsequently subjected to in-gel digestion [10]. Compared with 2-DE, these approaches provide less intact-protein information but improve downstream compatibility by reducing residual detergents, salts, phenolics and other contaminants [8,15].

GeLC-type workflows are particularly useful for roots, berry skins, seeds and SDS-containing extracts. In grapevine rootstock studies, proteins solubilized in SDS-containing buffers were subjected to partial 1D SDS-PAGE before in-gel digestion and LC–MS/MS [51,64,65]. In these workflows, the gel serves mainly as a clean-up interface between extraction and MS rather than as a high-resolution protein map.

Liquid-phase isoelectric focusing provides an alternative form of protein-level fractionation. Sharathchandra et al. [130] separated cytosolic proteins from berry-derived suspension cultures using a MicroRotofor cell before SDS-PAGE. Recovery of proteins in liquid fractions can facilitate handling of complex cytosolic extracts and reduce some practical limitations of classical 2-DE.

4.4. Protein-Level Chromatographic Prefractionation

Protein-level chromatographic prefractionation is less common than gel-based separation in grapevine proteomics but provides complementary selectivity [10,15,33]. Hydrophobic interaction, ion-exchange and size-exclusion chromatography separate intact proteins according to hydrophobicity, charge or hydrodynamic size before digestion and MS analysis [8,10,19]. These approaches can reduce sample complexity and enrich defined physicochemical protein classes [34,153].

In grapevine tissues, however, chromatographic prefractionation has been used less systematically than 2-DE, DIGE or GeLC-type approaches [158–160]. This likely reflects the need for clean, concentrated protein extracts and the additional procedural complexity associated with protein-level chromatography [37,59]. Nevertheless, it may be useful for membrane-associated, extracellular and seed protein fractions, particularly when coupled with modern LC–MS/MS workflows [10,67,147].

Accordingly, chromatographic protein prefractionation is best considered a complementary rather than dominant strategy in grapevine tissue proteomics. Broader application will require matrix-compatible extraction, standardized loading conditions and clear reporting of fraction pooling, protein recovery and downstream compatibility [16,35].

4.5. Subproteome-Specific Enrichment and Separomics Workflows

Subcellular and compartment-specific enrichment is used to access protein pools that may be masked in total extracts, including cell wall, plasma membrane, chloroplast, apoplast, xylem sap, seed and secreted proteins [8,34,61,62,67,147,157,160]. Because enrichment determines which proteins enter the analytical workflow, these procedures represent fractionation strategies in their own right.

Cell wall proteomics relies on sequential washing and extraction of wall-associated proteins, often with salt- or detergent-containing buffers. Plasma membrane studies require membrane enrichment, typically by differential centrifugation and partitioning procedures, before protein solubilization [60]. Chloroplast-enriched skin fractions similarly require organelle enrichment before downstream analysis [69]. These workflows provide access to defined subproteomes but are not directly comparable with total-tissue extracts.

Apoplastic fluid and xylem sap present a different analytical problem because protein abundance is very low. Early grapevine studies used 2-DE after concentration, whereas more recent workflows employ gel-free nanoLC–MS/MS or DIA-type analysis after collection, filtration, concentration and desalting [61,62,67,106,108–110]. In these matrices, concentration and clean-up are integral to fractionation.

The same principle applies to secreted proteins from grapevine cell cultures, where the culture medium must be separated from cells, concentrated and purified before 2-DE or LC–MS/MS [131,133].

4.6. Seed and Targeted Protein Fractionation

Seed-focused studies include direct MALDI-MS protein fingerprinting and sequential extraction of water-soluble or endosperm protein fractions followed by electrophoretic or MS-based identification [139,141,147]. Targeted studies of enriched grape proteins, including chitinases, β-1,3-glucanases, β-glycosidases and miraculin-like proteins, generally use biochemical enrichment or chromatographic purification before MS [26,162–164].

These workflows are analytically useful but intentionally restrict the analyzed protein pool. They should therefore be interpreted as targeted protein-fraction studies rather than whole-proteome workflows and should not be directly compared with global tissue proteomics [158,165].

4.7. Critical Assessment

Fractionation strategies in grapevine proteomics have shifted from classical protein-map generation toward clean-up, targeted enrichment and compartment-resolved profiling [158–160]. The 2-DE approaches remain valuable for extraction assessment, isoform visualization and major abundance changes, while DIGE improves relative quantification but shares the same basic representation limitations [9,35,37,124,146].

GeLC and 1D SDS-PAGE provide effective clean-up for contaminated or SDS-containing extracts, whereas chromatographic prefractionation offers orthogonal selectivity but remains underused [10,19,37,55,129]. Subproteome enrichment improves access to defined protein pools but generates datasets that are not directly comparable with total-tissue proteomes [62,67,147,157].

Across all approaches, fractionation performance remains strongly dependent on extraction quality [15,33]. Residual phenolics, polysaccharides, salts, detergents and chaotropes can compromise electrophoretic and chromatographic separations [37,80]. Future studies should therefore report the key parameters needed for comparison, including protein load, IPG range and gel conditions, number of bands or fractions, chromatographic mode, fraction-pooling strategy, enrichment procedure and quality-control criteria [9,34,152]. Consistent reporting is essential for distinguishing biological variation from variation introduced during separation [16,35].

5. Digestion and Peptide Preparation

After protein extraction and, where applicable, fractionation, proteins are converted into MS-compatible peptides [15,33]. Digestion efficiency and peptide recovery influence identification depth and quantitative reproducibility [34,152]. Grapevine studies mainly use two approaches: in-gel digestion after 2-DE, DIGE or SDS-PAGE, and gel-free in-solution, filter-aided or trap-based digestion after extraction and clean-up [8,10]. Workflow selection depends on the preceding separation step, extract composition, and the need to remove detergents, chaotropes, salts, phenolics, pigments and other MS-incompatible compounds [158,165]. The main digestion and peptide-preparation workflows are summarized in Figure 5.

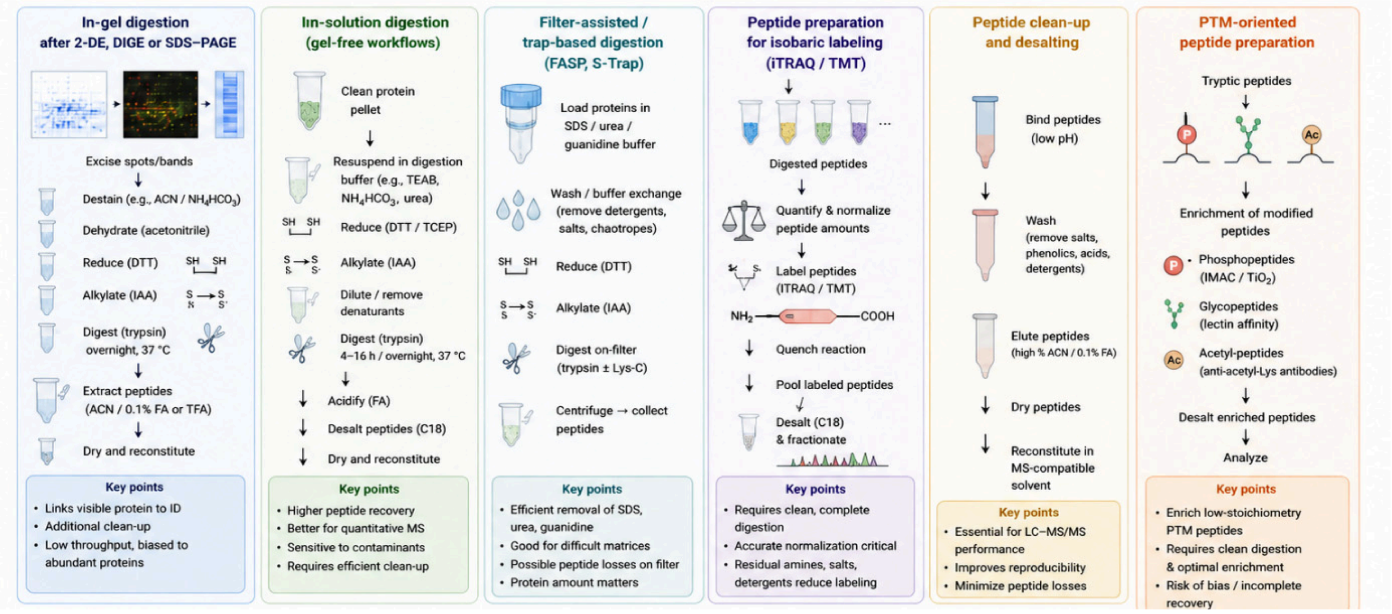


Figure 5. Principal digestion and peptide-preparation workflows in grapevine proteomics, including in-gel and gel-free strategies for MS-based analysis.

5.1. In-Gel Digestion After 2-DE, DIGE and SDS-PAGE

Early grapevine proteomics relied mainly on digestion after gel-based separation. Protein spots from 2-DE or DIGE and bands from 1D SDS-PAGE/GeLC workflows were excised and digested, most commonly with trypsin. This approach was applied to leaves, shoots, stems, roots, wood, apoplastic fluid, xylem sap, buds, callus, cell suspensions and berry tissues [36,37,39,41,62,79,80,99,105,111,115,125,146].

A typical workflow includes destaining, dehydration, reduction, alkylation and tryptic digestion, followed by peptide extraction from the gel [10,15]. Trypsin remains the dominant enzyme because it generates peptides well suited to MALDI-TOF/TOF, LC–MS/MS and database searching [33,152].

In-gel digestion links a visible spot or band directly to MS identification and provides additional clean-up during washing and destaining [34,35]. This is useful for grapevine matrices rich in phenolics, pigments, salts, detergents or polysaccharides [37,62,80]. However, the approach is labor-intensive, relatively low-throughput and biased toward abundant, well-resolved proteins [8,152]. Incomplete peptide recovery and poor representation of hydrophobic or low-abundance proteins further limit identification depth [15,158].

5.2. In-Solution Digestion in Gel-Free Workflows

Gel-free workflows increasingly use in-solution digestion after extraction, precipitation and clean-up. This strategy is common in label-free LC–MS/MS and isobaric-labeling workflows such as iTRAQ and TMT [44–46,53,71,72,83,86,87,89,90,100,116,123,145].

Cleaned proteins are typically reduced, alkylated and digested with trypsin [15,33,152]. Lys-C may be used before trypsin when proteins are solubilized in high urea concentrations because it tolerates stronger denaturing conditions [10,152]. After digestion, peptides are acidified, desalted and prepared for LC–MS/MS [8]. Alternative proteolysis has also been explored. Perutka and Šebela [166] demonstrated peptide and protein digestion using a grape-derived cysteine protease at pH 3, although this remains a specialized rather than routine bottom-up workflow.

In-solution digestion avoids gel-based protein selection and is better suited to quantitative shotgun proteomics, multiplex labeling and high-resolution LC–MS/MS [6,10,35,152].

It is, however, more sensitive to extraction-derived contaminants. Residual SDS, guanidine, high urea concentrations, salts, phenolics, pigments, lipids and reducing agents can impair digestion, peptide recovery and LC–MS/MS performance [15,165]. Efficient clean-up is therefore essential [80,158].

5.3. Filter-Aided and Trap-Based Digestion Workflows

Filter-aided sample preparation (FASP) links strong denaturing extraction with MS-compatible digestion. Proteins retained on molecular-weight cut-off filters undergo buffer exchange, reduction, alkylation and on-filter digestion, while detergents, salts and chaotropes are removed [44,48,90,116].

FASP is useful for grapevine tissues and cell cultures extracted under strongly denaturing conditions. George et al. [49], for example, applied a modified FASP workflow to bud proteins using ultrafiltration followed by sequential Lys-C and trypsin digestion. Related filter-based approaches have been used in grapevine cell-culture shotgun proteomics [48].

S-Trap-type workflows use protein immobilization followed by contaminant removal, on-support digestion and peptide recovery. They are suited to difficult or low-input matrices, including vascular sap, because clean-up and digestion are combined in a single workflow [110]. Performance depends on protein input, sample cleanliness, binding efficiency and peptide recovery.

5.4. Peptide Preparation for Isobaric Labeling

iTRAQ and TMT workflows require efficient digestion, accurate peptide normalization and removal of compounds that interfere with labeling or reporter-ion quantification [6,10,47,70,90,152]. Grapevine studies of leaves, buds, roots and berries generally combine protein clean-up, reduction, alkylation, tryptic digestion, peptide desalting, isobaric labeling and peptide fractionation before LC–MS/MS [13,43,46,52,72,83,87,90,116,120–123,142].

In bud proteomics, TCA/acetone-cleaned proteins were resolubilized in urea-containing buffer before TMT labeling [122]. Root cold-stress studies similarly used TCA/acetone precipitation followed by digestion and iTRAQ labeling [123]. Quantitative reliability depends on digestion completeness, peptide recovery, labeling efficiency and normalization [10,152]. Residual contaminants are particularly problematic in phenolic- and pigment-rich tissues such as berry skins, leaves and dormant buds [80,158,165].

5.5. Peptide Clean-Up and Desalting

Peptide clean-up is required in both gel-based and gel-free workflows [8,10,15]. Residual detergents, salts, phenolics, urea, ammonium bicarbonate or TEAB, reducing agents, alkylating reagents and labeling by-products must be removed before LC–MS/MS [6,165]. Reversed-phase C18 cartridges, StageTips and related solid-phase extraction formats are commonly used for this purpose [10,35].

Peptides recovered from gel pieces may require limited additional processing when already compatible with MALDI or LC–MS/MS [165]. Gel-free workflows, especially those involving iTRAQ, TMT, FASP, S-Trap or PTM enrichment, generally require more stringent desalting [10,47,70]. For apoplastic fluid, xylem sap and culture secretomes, clean-up must also minimize peptide losses because of the low protein input [61,62,67].

5.6. PTM-Oriented Peptide Preparation

PTM analysis requires selective enrichment of modified peptides after digestion. Grapevine phosphoproteomic, glycoproteomic and acetylproteomic workflows therefore include additional peptide-processing steps. Melo-Braga et al. [70] enriched phosphopeptides by TiO_2 chromatography, N-glycopeptides by ZIC-HILIC followed by PNGase A

treatment, and lysine-acetylated peptides by anti-acetyl-lysine immunoaffinity enrichment. IMAC and metal-oxide approaches are also used for phosphopeptide enrichment, whereas glycopeptide and acetyl-peptide strategies depend on the target modification.

Modified peptides are generally present at low stoichiometry and may be masked by abundant unmodified peptides. Incomplete digestion and residual detergents, salts or phenolics can reduce enrichment efficiency and bias PTM datasets. Digestion, desalting, enrichment and peptide recovery therefore need to be optimized as a single workflow.

5.7. Matrix-Specific Considerations

Although digestion chemistry is broadly similar across grapevine studies, matrix composition strongly affects peptide preparation.

In buds and dormant tissues, low protein abundance and co-extracted storage compounds increase losses during precipitation, washing and buffer exchange. Efficient digestion and, where applicable, validated iTRAQ/TMT labeling are therefore important [49,120–122].

Root and rhizosphere samples may contain residual phenolics, suberin-associated contaminants, salts, SDS and humic substances. In-gel digestion provides additional clean-up, whereas in-solution and isobaric-label workflows require more rigorous contaminant removal. Rhizosphere metaproteomics is additionally affected by differential recovery of plant and microbial proteins and by peptide losses during clean-up [51,64–66,117].

Cell cultures are compatible with both in-gel and gel-free digestion. FASP and in-solution workflows are particularly suitable for strongly denaturing shotgun protocols, whereas extracellular culture media are limited by low protein concentrations and peptide losses during precipitation, desalting and drying [129,131,133].

Berry digestion depends on efficient removal of sugars, organic acids, phenolics and pigments. Seeds additionally contain lipids and storage proteins. In-gel digestion provides useful clean-up but limits analysis to resolved proteins, whereas gel-free digestion avoids restricting analysis to gel-resolved proteins but is more sensitive to residual TCA, phenol, SDS, urea, sugars, salts and pigments [36,40,55,60,69]. Subcellular fractions may also contain detergents or enrichment reagents that interfere with digestion.

Apoplastic fluid and xylem sap are constrained mainly by low protein abundance. Early studies used in-gel digestion after protein concentration, whereas more recent workflows use gel-free digestion followed by nanoLC–MS/MS, avoiding gel-based selection of proteins [61,62,67,106,108–110]. Concentration, desalting and digestion must therefore be balanced to minimize sample loss.

5.8. Critical Assessment

Digestion and peptide preparation are important but often insufficiently reported stages in grapevine proteomics [15,35,152]. In-gel digestion provides effective clean-up and a direct link between protein spots or bands and MS identification, but it is low-throughput and biased toward gel-resolved proteins [37,158]. In-solution, FASP and trap-based workflows provide broader proteome access and are better suited to quantitative LC–MS/MS, but require tighter control of contaminants and digestion conditions [6,10,48].

Direct comparisons linking sample-preparation strategy to downstream MS identification depth are particularly rare. One notable exception is the study of George et al. [48], which compared SDS-PAGE/in-gel processing with FASP coupled to gas-phase fractionation using the same *Vitis riparia* leaf material. FASP-GPF produced a 24% increase in the number of reproducibly identified proteins relative to the SDS-PAGE workflow [48]. This result provides direct quantitative evidence that sample-preparation strategy can affect protein identification depth, but it should not be interpreted as a 24% increase in absolute proteome

coverage because the proportion of the complete grapevine proteome represented by either workflow was not determined.

A major challenge is the frequent use of extraction reagents that are incompatible with digestion or MS, including SDS, guanidine, high urea concentrations, salts, phenol residues, pigments, lipids and reducing agents [15,80,165]. Peptide preparation can therefore introduce substantial analytical bias. Incomplete digestion, insufficient contaminant removal, peptide loss during clean-up and inefficient labeling can alter the final set of identified and quantified proteins [6,10,152,160].

Future studies should report protein input, digestion strategy, enzyme type and enzyme-to-substrate ratio, reduction and alkylation conditions, digestion duration, denaturant removal, clean-up and desalting procedures, peptide recovery, labeling efficiency and missed-cleavage control. PTM studies should additionally report enrichment strategy and site-localization criteria [8,34,152]. Consistent reporting of these parameters is required for assessing reproducibility and separating biological variation from sample-preparation effects [35].

6. Mass Spectrometry Platforms and Acquisition Modes

Mass spectrometry enabled the transition of grapevine proteomics from identification of individual gel-resolved proteins to high-throughput, peptide-centric and quantitative analysis [6,158,165]. Early MALDI-TOF and MALDI-TOF/TOF analysis of 2-DE spots was progressively complemented and largely replaced by LC–ESI–MS/MS, nanoLC–MS/MS and high-resolution Q-TOF, TripleTOF and Orbitrap-type platforms [10,15,151]. More recently, DIA and specialized workflows for PTM analysis, vascular fluids, secretomes and defined subproteomes have further expanded analytical scope [7,70,160].

The principal configurations include MALDI-based identification of gel-resolved proteins, LC–ESI–MS/MS and nanoLC–MS/MS shotgun workflows, DDA, iTRAQ/TMT-based acquisition, label-free LC–MS/MS, DIA and specialized approaches for low-input, extracellular, subcellular and PTM-enriched samples [47,48,165].

6.1. MALDI-TOF and MALDI-TOF/TOF in Gel-Based Proteomics

Early grapevine proteomics relied extensively on MALDI-TOF and MALDI-TOF/TOF to identify proteins separated by 2-DE or DIGE. Protein spots were excised, digested with trypsin and analyzed by MALDI. Peptide mass fingerprints were searched against protein, EST or broad plant databases, while TOF/TOF analysis provided additional sequence confirmation. This strategy was widely applied to leaves, shoots, stems, roots, wood, apoplastic fluid, cell cultures and berry tissues [18,36–39,42,62,94,97,102,111].

MALDI was well suited to classical gel-based proteomics because individual spots could be linked directly to protein maps, extraction comparisons and differential abundance patterns [158,165]. MALDI-TOF/TOF increased identification confidence by providing fragment-ion spectra in addition to peptide mass fingerprints [15].

Its limitations largely reflected those of gel-based workflows. Analysis was restricted to visible and sufficiently abundant spots, co-migration could complicate identification, and hydrophobic, low-abundance and extreme-pI proteins were underrepresented [9,37]. Early studies were also constrained by incomplete grapevine sequence databases and reliance on EST or cross-species resources [43,77]. MALDI-TOF/TOF was therefore important for early proteome mapping but provided limited identification depth [158,165].

6.2. LC–ESI–MS/MS, Q-TOF and nanoLC–MS/MS Workflows

LC–ESI–MS/MS shifted grapevine proteomics from spot-based to peptide-centric analysis and increased sensitivity and dynamic range [158,165]. Initially used to complement

MALDI identification of gel-derived peptides, LC–MS/MS subsequently became the main platform for shotgun proteomics [47,48,146].

Q-TOF and ion-trap instruments were used in early and intermediate studies after 2-DE, 1D SDS-PAGE or direct peptide preparation [39,40,81,89,99,115]. Compared with peptide mass fingerprinting, LC–MS/MS provided greater peptide-separation capacity and more sequence information from complex mixtures and overlapping gel spots.

Modern workflows increasingly use nanoLC–MS/MS, with peptides separated on nanoscale reversed-phase C18 columns before tandem MS. The low flow rates improve sensitivity and reduce sample consumption, which is particularly useful for apoplastic fluid, xylem sap, secretomes, leaf extracts and berry subproteomes [44,63,67,90,106,110,116].

High-resolution Orbitrap, Q Exactive, Q-TOF and TripleTOF platforms have enabled analysis of thousands of peptides in complex samples. Their mass accuracy, resolving power and MS/MS performance support label-free, isobaric-label, PTM-oriented and DIA workflows.

6.3. Data-Dependent Acquisition (DDA)

Most shotgun grapevine proteomics studies have used DDA. An MS1 survey scan is followed by fragmentation of selected precursor ions, and the resulting MS/MS spectra are used for database searching and protein identification. DDA has been applied in label-free, iTRAQ/TMT, phosphoproteomic and nanoLC–MS/MS studies of leaves, buds, roots, cell cultures, berries, apoplastic fluid and xylem sap [44,67,71,72,89,90,116].

DDA provides high-quality MS/MS spectra but preferentially samples abundant precursors and can undersample low-abundance peptides in complex mixtures. This affects leaves dominated by abundant photosynthetic proteins, berries with broad dynamic ranges and low-protein matrices such as apoplastic fluid and xylem sap. Peptide prefractionation, longer LC gradients, dynamic exclusion, narrower isolation windows and high-resolution instrumentation reduce but do not eliminate stochastic precursor selection.

6.4. Isobaric-Label Acquisition: iTRAQ and TMT

iTRAQ and TMT have been widely used for quantitative grapevine proteomics. Peptides from different samples are labeled, pooled, fractionated and analyzed by LC–MS/MS, with quantification based on reporter-ion intensities. These workflows have been applied to leaves, buds, roots and berries in studies of stress responses, dormancy and berry development [13,43,46,52,71,72,83,86,87,90,116,120–123,142].

Their main advantage is multiplexing, which allows several treatments, genotypes or developmental stages to be compared within the same analytical design [6,47]. Quantitative accuracy depends on efficient digestion and labeling, peptide normalization, pooling and fractionation [10,152]. Co-isolation of unrelated peptides can cause ratio compression; narrow isolation windows, high-resolution MS/MS and peptide prefractionation can reduce this effect [47,152].

6.5. Label-Free LC–MS/MS

Label-free LC–MS/MS is increasingly used in grapevine proteomics. Protein abundance is estimated from spectral counts or MS1 precursor intensities across separate LC–MS/MS runs. Applications include leaves, shoot tips, buds, roots, cell cultures, apoplastic fluid, vascular sap, whole berries, berry skins and muscadine berry tissues [44–46,48,53,56,67,89,110,129,140,145].

Label-free quantification avoids chemical labeling and is flexible for large or complex experimental designs [6]. It is also suitable for low-input extracellular matrices where sample amount may limit multiplexed labeling [61,62,67]. Because samples are analyzed separately,

however, quantitative reliability depends on reproducible sample preparation, LC separation, instrument performance, computational alignment and normalization [7,10,15,48].

6.6. Data-Independent Acquisition (DIA) Workflows

DIA has more recently been introduced into grapevine proteomics, particularly for complex berry, leaf and vascular-fluid samples where missing values limit comparative analysis [110]. By collecting fragment-ion information more systematically than DDA, DIA can improve quantitative completeness and reproducibility.

Sagawa et al. [110] applied a DIA-compatible workflow to vascular sap after S-Trap processing. Riebel et al. [55] used an LC–MS^E/DIA-like approach for grape berry profiling, while Corrêa et al. [58] combined FASP with nanoUPLC–Q-TOF ion-mobility $HDMS^E$ and label-free quantification during berry ripening.

Compared with DDA, DIA reduces stochastic precursor sampling and improves quantitative consistency. It requires stable chromatography, advanced data processing and, depending on the workflow, spectral libraries or robust library-free identification. DIA remains less common than DDA-based label-free and iTRAQ/TMT workflows in grapevine proteomics but is well suited to large comparative datasets and low-protein matrices [110].

6.7. MS Analysis of Low-Input and Specialized Matrices

Apoplastic fluid, xylem sap, culture secretomes, berry subproteomes, seeds and rhizosphere-associated samples require adapted MS workflows because of low protein amounts, residual matrix components or mixed protein origins.

Early xylem sap studies used concentrated proteins and MALDI-based identification of gel-resolved spots [61,108]. Later studies adopted LC–MS/MS and DIA after sap collection, filtration, concentration and digestion, improving detection of lower-abundance vascular proteins [109,110]. Apoplastic-fluid proteomics followed a similar transition from 2-DE/MALDI-TOF/TOF [62] to gel-free nanoLC–MS/MS [67,106].

Culture secretomes require sensitive LC–MS/MS because proteins are diluted and coexist with salts, sugars, elicitors and secreted metabolites. Rhizosphere metaproteomics additionally requires high-resolution LC–MS/MS and stringent clean-up because peptide mixtures contain plant, microbial and extracellular proteins together with soil-derived contaminants [66].

Berry subproteomes also require matrix-adapted acquisition. Plasma membrane and chloroplast fractions are generally analyzed by LC–MS/MS after membrane or organelle enrichment [60,69], whereas cell-wall proteomics relies on MS/MS identification of proteins released from enriched wall fractions. Seed studies range from direct MALDI-MS fingerprinting to LC–MS/MS analysis of purified protein fractions [40,139,141,147].

6.8. PTM-Oriented MS Workflows

PTM-oriented proteomics requires enrichment of modified peptides followed by MS acquisition capable of reliable peptide identification and site localization [70,72,167]. Grapevine phosphoproteomic, glycoproteomic and acetylproteomic studies have used LC–MS/MS to characterize modification-specific subproteomes [70–72].

These workflows depend strongly on mass accuracy, fragmentation quality, peptide coverage and modification-aware database searching. Because site assignment relies on fragment-ion evidence, poor MS/MS quality or incomplete sequence coverage can reduce localization confidence.

6.9. Critical Assessment

Grapevine proteomics has progressed from gel-spot MALDI identification to high-resolution LC–MS/MS-based quantitative workflows [6,158,165]. MALDI-TOF and MALDI-TOF/TOF remain useful for spot-based analysis but provide limited identification depth [37,165]. High-resolution LC–ESI–MS/MS and nanoLC–MS/MS on Q-TOF, TripleTOF and Orbitrap-type platforms now form the basis of modern grapevine proteomics [10,15,151].

Acquisition mode shapes precursor sampling, identification completeness and quantitative behavior [6,168]. DDA provides confident identification but favors abundant peptides and generates missing values [10,15]. iTRAQ/TMT enables multiplexed quantification but requires control of labeling efficiency, fractionation and ratio compression [47,70]. Label-free LC–MS/MS is flexible but depends on chromatographic reproducibility and computational alignment [7,48]. DIA improves quantitative completeness and reproducibility but requires stable LC performance and more advanced data processing [110]. PTM-oriented workflows add further requirements for enrichment, fragmentation and site localization [70–72,167].

MS performance also remains dependent on upstream sample preparation [15,33]. Residual phenolics, pigments, salts, detergents, chaotropes, lipids, humic substances and incomplete digestion can impair chromatography, ionization and peptide identification regardless of instrument performance [80,165]. Studies should therefore report instrument type, LC configuration, acquisition mode, mass resolution, precursor isolation width, fragmentation method, dynamic exclusion, labeling and fractionation strategy, database version, FDR criteria and quantitative settings [8,34,152]. Consistent reporting is essential for comparison across tissues, platforms and acquisition modes [16,35].

7. Protein Identification, Quantification and Bioinformatic Processing

Protein identification and bioinformatic processing in grapevine proteomics have progressed from spot-based searches after gel electrophoresis to large-scale, genome-supported LC–MS/MS datasets with dedicated quantitative and functional annotation pipelines [15,158,165]. Early studies relied mainly on peptide mass fingerprints or MS/MS spectra from 2-DE spots searched against public protein, EST or broad plant databases [43,165]. Modern workflows use high-resolution LC–MS/MS combined with label-free, iTRAQ/TMT, DIA, PTM-oriented or proteogenomic analysis [6,7,70,151]. Data processing therefore depends on the upstream analytical strategy, including gel-based separation, shotgun LC–MS/MS, isobaric labeling, DIA, PTM enrichment, subproteome analysis and metaproteomics [8,160,168]. The main processing steps are summarized in Figure 6.

7.1. Sequence Databases and Search Spaces

Reliable protein identification depends strongly on database quality. Early grapevine studies used NCBI non-redundant databases, Viridiplantae collections, EST resources, partial cDNA libraries or laboratory-specific Vitis datasets. These resources enabled initial large-scale identifications but limited identification completeness and complicated discrimination among homologous proteins, isoforms and modified variants [36,43,158].

The release of Vitis vinifera genome assemblies and improved annotation increased identification confidence. Modern workflows increasingly use genome-derived Vitis protein databases, UniProt/Swiss-Prot, NCBI Vitis resources and cultivar-specific datasets. Resources such as GrapeIS and VitisNet, together with improved 12X and VCost annotations, also support functional annotation, pathway analysis and cross-omics integration [76,146].

Database selection is particularly important in host–pathogen, extracellular and rhizosphere studies. Apoplastic-fluid datasets involving Plasmopara viticola may contain both host and pathogen peptides and therefore require combined or sequential database

searches [106]. Rhizosphere metaproteomics requires broader search spaces covering plant, bacterial, fungal and extracellular proteins [66]. Subproteome datasets additionally require localization and contamination assessment.

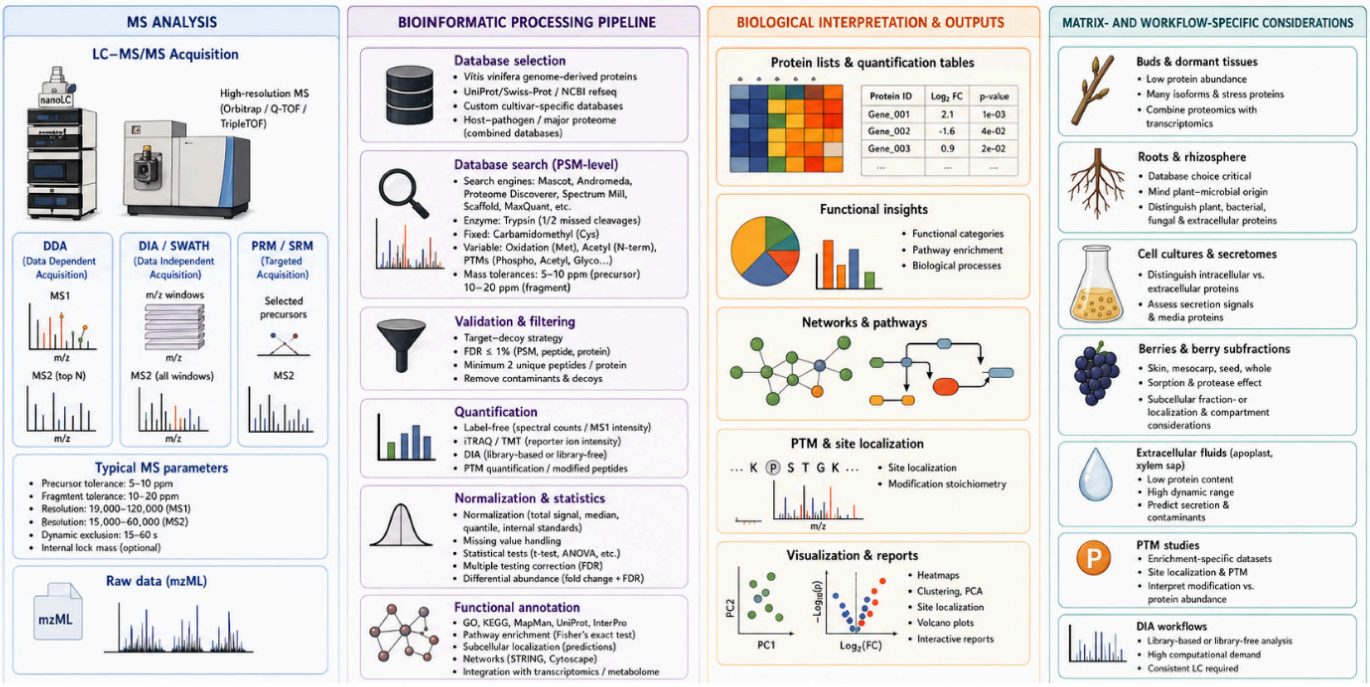


Figure 6. Overview of protein identification, quantification and bioinformatic processing in grapevine proteomics, from MS data acquisition to functional interpretation.

7.2. Database-Search Pipelines and Identification Criteria

Early 2-DE and DIGE workflows combined gel-image analysis with MALDI-TOF, MALDI-TOF/TOF or LC–MS/MS identification of selected spots. These approaches were widely used for leaves, shoots, stems, wood, roots, apoplastic fluid, callus, cell cultures and berry tissues [37,38,62,79,105,111,124,146]. Identification confidence was often limited by database incompleteness, spot co-migration and sparse MS/MS evidence.

Modern LC–MS/MS datasets are processed with search engines and software environments such as Mascot, MaxQuant/Andromeda, Proteome Discoverer, Spectrum Mill, Scaffold and Progenesis QI [6,15,152]. Search parameters typically include enzyme specificity, missed cleavages, fixed and variable modifications and precursor and fragment mass tolerances [10,15,151,152].

Target–decoy strategies are now widely used to control false discovery rates [8,168]. Modern studies generally apply peptide-spectrum match as well as peptide- and protein-level FDR thresholds of approximately 1% or lower [16,35]. Some workflows require at least two unique peptides per protein, whereas single-peptide identifications may be retained when supported by stringent FDR control and high-quality sequence-unique MS/MS evidence. This is particularly relevant in grapevine because many proteins belong to large homologous families, including PR proteins, chitinases, peroxidases, glycosidases, stilbene synthases, heat-shock proteins, tubulins and transporters [158,165]. Single-peptide identifications should therefore be reported explicitly and interpreted cautiously [8,152].

Proteogenomic approaches provide an additional layer of annotation. Genome-anchored peptide evidence can refine gene models, validate predicted coding sequences and support the identification of previously unannotated proteins. Integration of proteomic data with Vitis genomic resources therefore extends protein identification beyond conventional database searching [77].

7.3. Quantification Strategies

Quantification depends on the analytical platform. Conventional 2-DE uses normalized spot volume, whereas DIGE uses fluorescence-based relative quantification with internal standards [37,41,132]. Both approaches remain useful for protein isoforms and abundant spot-level changes but are limited by gel resolution, co-migration and selective spot picking [158].

Label-free LC–MS/MS estimates protein abundance from spectral counts or MS1 precursor intensities. NSAF-based spectral counting has been used in grapevine bud and cell-culture studies, while intensity-based workflows use peptide feature detection, retention-time alignment, peak integration and protein-level summarization [44,48,67,110,129]. Reliable label-free quantification requires reproducible chromatography, consistent peptide recovery, normalization and appropriate treatment of missing values.

iTRAQ and TMT workflows quantify peptides from reporter-ion intensities. Processing includes reporter-ion extraction, isotope correction, channel normalization and protein-level summarization. These approaches have been applied to leaves, buds, roots and berries [13,46,52,71,72,83,87,90,100,120–123,142]. Their main limitations include incomplete labeling, co-isolation, ratio compression and normalization errors.

DIA and LC–MS^{E} require distinct computational workflows based on systematic fragment-ion acquisition, retention-time alignment and spectral-library or library-free analysis. DIA generally improves quantitative reproducibility and reduces missing values but requires stable chromatography and more complex computational validation [55,110].

7.4. Statistical Processing and Differential Abundance Criteria

Statistical treatment varies considerably among grapevine proteomics studies [6–8]. Gel-based studies generally compare normalized spot intensities using fold-change thresholds and univariate tests [133,158]. LC–MS/MS workflows additionally require normalization, replicate assessment, missing-value handling, statistical testing and, increasingly, multiple-testing correction [11,48,140].

Rootstock studies illustrate this variation. Prinsi et al. [51] quantified proteins from precursor intensities normalized to total validated protein signal and defined differential abundance using a two-fold threshold combined with a Student's t-test. In a related study, Prinsi et al. [65] applied a peptide FDR $\leq 1\%$, required at least two unique peptides per protein, and again normalized protein intensities to the total validated signal before functional interpretation.

Quality control is essential because grapevine matrices differ markedly in extraction efficiency and chemical complexity [15,80,165]. Useful metrics include protein yield, numbers of identified peptides and proteins, missed-cleavage rate, labeling efficiency, reporter-ion balance, peptide-intensity distributions, replicate correlation, PCA separation, fraction consistency, FDR and missing-value percentage [6,7,129]. Inconsistent reporting of these parameters limits cross-study comparison and makes analytical variation difficult to separate from biological effects [8,16,35].

7.5. PTM-Oriented Bioinformatic Processing

PTM analysis requires identification of both modified peptides and modification sites. Grapevine phosphoproteomic, glycoproteomic and acetylproteomic workflows therefore use modification-specific database searches together with stringent FDR control and site-localization scoring [70–72].

PTM datasets are interpreted at the levels of modified peptide, localization site, protein and biological function [70,72]. Changes in modified-peptide abundance may reflect altered modification stoichiometry, total protein abundance or both. PTM data are therefore

most informative when interpreted together with total proteome measurements [70,151]. Functional analysis may include motif analysis, kinase–substrate prediction and pathway enrichment, although grapevine-specific PTM annotation remains less developed than in model species [72,160].

7.6. Functional Annotation and Pathway Analysis

After identification and quantification, proteins are assigned to functional categories, pathways and subcellular compartments [6,158,165]. Early studies relied mainly on broad functional classes, whereas recent LC–MS/MS studies use resources and tools such as Gene Ontology, KEGG, MapMan, Mercator, UniProt, InterPro, Blast2GO, STRING and Cytoscape [7,140,160].

Functional annotation remains challenging because many Vitis proteins belong to large, sequence-redundant families and remain incompletely characterized [158,165]. Mapping to Arabidopsis, potato or other plant orthologues can facilitate pathway interpretation but may obscure grapevine-specific functions [7,160,169]. Subcellular and extracellular datasets require additional filters. Apoplastic, xylem sap, secretome, plasma membrane, chloroplast and cell-wall proteomes should be evaluated using signal-peptide prediction, transmembrane-domain analysis, localization prediction and contamination assessment [60,62,67,69,106,109]. These steps are necessary because enriched fractions may contain cytoplasmic contaminants or proteins released during sample handling.

Proteomic data are also increasingly integrated with transcriptomic, metabolomic and network analyses, particularly in bud, berry and stress-response studies. Such integration requires consistent normalization and mapping among gene, transcript and protein identifiers because transcript and protein abundance are not necessarily correlated.

7.7. Matrix- and Workflow-Specific Considerations

Bioinformatic interpretation remains matrix-dependent.

In buds and dormant tissues, modern studies use label-free NSAF-based spectral counting or iTRAQ/TMT quantification, but low protein abundance, large gene families and integration with transcriptomic data complicate interpretation [49,120–122].

Root and rhizosphere studies depend strongly on database selection and protein-origin assignment. GeLC–MS/MS rootstock datasets are not directly comparable with iTRAQ-based studies unless search spaces, normalization and statistical thresholds are aligned. Rhizosphere metaproteomics further requires separation of plant, bacterial, fungal and extracellular protein assignments [51,64–66,123].

Secretome datasets require signal-peptide prediction and assessment of cell-lysis contamination because proteins detected in culture medium may be genuinely secreted, cell-wall-associated or released from damaged cells [48,131,132].

Berry datasets span 2-DE/DIGE, iTRAQ/TMT, label-free DDA, DIA, PTM analysis and targeted purification. Skin, mesocarp, seed, whole-berry and subcellular datasets should therefore not be considered directly equivalent. Enriched cell-wall, plasma membrane and chloroplast fractions require localization-based interpretation, whereas seed fingerprinting and targeted purification may rely on protein profiles or purified-protein confirmation rather than global database-driven quantification [55,59,60,69,139,141].

7.8. Critical Assessment

Protein identification and bioinformatic processing in grapevine proteomics have progressed from spot-based searches to genome-supported, quantitative and modification-aware workflows [6,15,158,165]. This has improved identification depth and identification

confidence but increased dependence on database selection, search parameters, protein inference, quantification algorithms, normalization and statistical criteria [8,160,168].

Incomplete sequence information was a major limitation of early studies [77,165]. Current challenges are reproducibility, missing-value control, quantitative accuracy, protein grouping and integration across platforms [6,7,129]. Label-free, iTRAQ/TMT, DIA and PTM datasets are not directly comparable unless identification criteria, normalization and statistical processing are transparent and consistent. Host–pathogen, extracellular and rhizosphere datasets require additional care because spectra may originate from multiple biological sources [66,70,110].

Future studies should report database version, search engine, enzyme specificity, missed cleavages, fixed and variable modifications, mass tolerances, FDR thresholds, minimum peptide criteria, protein inference rules, quantification and normalization methods, missing-value handling, statistical filters and the functional annotation pipeline [8,16,152]. PTM studies should additionally report site-localization criteria and modified-peptide normalization [70,72], while DIA studies should describe spectral-library or library-free processing parameters [110]. Extracellular, subcellular and metaproteomic datasets require localization, contamination and protein-origin assessment [66,111,160].

8. Methodological Challenges and Future Perspectives in Grapevine Proteomics

Despite substantial methodological progress, grapevine proteomics remains strongly constrained by matrix chemistry and heterogeneous, tissue-specific workflows. Across leaves, shoots, wood, buds, roots, cell cultures, berries, apoplastic fluid and xylem sap, protein extraction still relies largely on TCA/acetone precipitation, phenol extraction and SDS-, urea- or thiourea-based buffers. These approaches can generate analyzable samples, but their performance depends strongly on tissue composition: polyphenols, polysaccharides, pigments, lignin, suberin, starch, sugars, salts, lipids and soil-derived contaminants can affect protein solubility, digestion, chromatography and MS ionization. Consequently, variation introduced during sample preparation can contribute substantially to differences in the proteins ultimately identified and quantified [15,17,18,34,80].

Fully gel-free bottom-up workflows using unfractionated whole-proteome extracts remain less common than gel-assisted or protein-level-prefractionated approaches. The 2-DE, 2D-DIGE, 1D SDS-PAGE and GeLC approaches are still used as separation or clean-up steps, which can bias datasets toward abundant, soluble and gel-resolved proteins [17,18,37,40,41,79–81,111]. FASP has been applied in leaves, cell cultures and buds [48,49,129], S-Trap-type processing has appeared in vascular sap [110], whereas SP3-type bead-based preparation is essentially absent from the grapevine literature surveyed here. Systematic evaluation of these gel-free clean-up strategies against established phenol- and TCA/acetone-based procedures is therefore warranted, particularly for phenolic-rich, lignified and low-input matrices [8,10,12,17,78].

More fundamentally, controlled head-to-head benchmarking remains rare. As detailed in Section 3.7, early method-comparison studies mainly evaluated total protein yield and 2-DE quality [18,80,88], whereas direct benchmarking at the level of modern shotgun proteomics is exceptional. George et al. [48] provide one notable example: using the same *Vitis riparia* leaf material, FASP coupled with gas-phase fractionation produced 24% more reproducibly identified proteins than SDS-PAGE/in-gel processing. Beyond such isolated studies, the available evidence is insufficient to determine which extraction, clean-up and digestion strategy performs best for a given grapevine matrix.

This gap is methodologically important because protein yield and 2-DE spot counts cannot be used as surrogates for downstream MS performance, and protein-identification

counts from independent studies are confounded by differences in tissue, cultivar, protein input, fractionation, LC configuration, MS platform, database and identification criteria. Future studies should therefore use split-sample designs in which the same homogenized material is processed in parallel while downstream analytical conditions are kept as constant as possible. Evaluation should include protein recovery relative to tissue mass, extract purity, peptide recovery, digestion efficiency, missed-cleavage rate, chromatographic reproducibility, numbers of reproducibly identified peptides and proteins, quantitative coefficient of variation and missing-value frequency. Matrix-stratified benchmarking is especially needed for berries, lignified tissues, dormant buds, roots, apoplastic fluid and xylem sap.

Benchmarking should be accompanied by matrix-specific standard operating procedures rather than a single universal protocol. Critical parameters—including tissue mass, tissue-to-buffer ratio, PVPP concentration, reducing agent, precipitation chemistry, incubation time, centrifugation, washing, drying and resolubilization—are still reported inconsistently [37,40,51,64,79]. Consensus workflows should therefore be defined separately for leaves and shoots, lignified tissues, dormant buds, roots, berries, cell cultures, apoplastic fluid and xylem sap, and linked explicitly to intended downstream formats such as gel-based proteomics, GeLC–MS/MS, fully gel-free LC–MS/MS, DIA, PTM-oriented analysis or targeted subproteomes [6,8,35,75,160].

Quality control is another priority. Although high-resolution MS, genome-anchored databases and target–decoy-based FDR control are increasingly common, many studies still lack matrix-matched reference materials, process controls or inter-batch benchmarks. Future work should incorporate pooled reference samples, repeated QC injections and, where appropriate, internal peptide standards or protein spike-ins, and should report protein yield relative to a defined tissue input, peptide recovery, missed-cleavage rate, numbers of identified peptides and proteins, replicate correlation, chromatographic stability and missing-value percentage. Matrix-defined reference materials would facilitate interlaboratory comparability and future meta-analysis [7,35,74,76,77].

Bioinformatic and quantitative processing also require harmonization. Genome-anchored Vitis databases have improved protein assignment, but differences remain in database version, search engine, enzyme specificity, fixed and variable modifications, mass tolerances, FDR thresholds, minimum peptide criteria, protein inference, normalization and missing-value handling [6,8,75,160]. These differences are particularly important for homologous protein families and when comparing label-free, iTRAQ/TMT and DIA datasets. Studies should therefore report these settings transparently, apply stringent FDR control and describe batch correction, statistical testing and multiple-testing correction where relevant [6,35,44,52,110,142].

Subcellular, extracellular and PTM-oriented workflows require additional validation because enriched fractions are not directly comparable with whole-tissue proteomes. Apoplastic fluid, xylem sap, cell-wall, plasma-membrane, chloroplast, secretome and seed datasets should include contamination and localization assessment, while phosphoproteomic, glycoproteomic and acetylproteomic studies should report enrichment efficiency, peptide input, site-localization criteria and modified-peptide normalization [60–62,67,69–73,106,109,147]. Mechanistic studies of protein lysine acetylation during berry ripening [170] should likewise be distinguished from global MS-based acetylome profiling.

Overall, grapevine proteomics has matured into a technically diverse field, but future progress will depend on validated, matrix-aware and quantitatively benchmarked workflows rather than continued proliferation of unbenchmarked protocols. Priorities include direct comparison of FASP, S-Trap and SP3 with established phenol- and TCA/acetone-based procedures, development of matrix-specific SOPs and reference materials, implementation of robust QC and harmonization of protein identification and quantification criteria. No

single workflow is likely to be universally optimal; extraction, clean-up, digestion, fractionation, MS acquisition and bioinformatic processing must be co-optimized according to matrix composition and analytical objective. Moving toward experimentally benchmarked and transferable workflows should improve reproducibility and biological interpretability across grapevine proteomics studies.

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Abbreviations

The following abbreviations are used in this manuscript:

2-DE	Two-dimensional gel electrophoresis
2D-DIGE	Two-dimensional difference gel electrophoresis
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
DIGE	Difference gel electrophoresis
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
EST	Expressed sequence tag
FASP	Filter-aided sample preparation
FDR	False discovery rate
GeLC–MS/MS	Gel electrophoresis–liquid chromatography–tandem mass spectrometry
HCD	Higher-energy collisional dissociation
HILIC	Hydrophilic interaction liquid chromatography
IEF	Isoelectric focusing
IMAC	Immobilized metal affinity chromatography
IPG	Immobilized pH gradient
iTRAQ	Isobaric tags for relative and absolute quantitation
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LC–ESI–MS/MS	Liquid chromatography–electrospray ionization–tandem mass spectrometry
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MALDI-TOF/TOF	Matrix-assisted laser desorption/ionization tandem time-of-flight
MeOH	Methanol

MIAPE	Minimum Information About a Proteomics Experiment
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^E	Data-independent acquisition using alternating low- and elevated-energy scans
NCBI	National Center for Biotechnology Information
NSAF	Normalized spectral abundance factor
PCA	Principal component analysis
PMSF	Phenylmethylsulfonyl fluoride
PNGase A	Peptide-N-glycosidase A
PR	Pathogenesis-related
PTM	Post-translational modification
PVPP	Polyvinylpolypyrrolidone
Q-TOF	Quadrupole time-of-flight
QC	Quality control
RP	Reversed-phase
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPE	Solid-phase extraction
SP3	Single-pot solid-phase-enhanced sample preparation
SOP	Standard operating procedure
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine
TEAB	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TMT	Tandem mass tags
TOF	Time-of-flight
UPLC	Ultra-performance liquid chromatography
ZIC-HILIC	Zwitterionic hydrophilic interaction liquid chromatography

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