Comparison of fractionation strategies to improve proteomic analysis using Gas-Phase Fractionation (GPF) and Data-Independent-Acquisition mass spectrometry (DIA-MS)

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INTRODUCTION

Mass spectrometry based quantitative proteomics aims to identify and quantify as many proteins as possible from complex biological samples. However, such projects often involve samples that differ in nature, quantity, and dynamic range, requiring careful adaptation of analytical strategies. In this context, **depth of analysis** is not the only criterion to consider, data **reproducibility** and **quantitative robustness** are equally critical. To meet these criteria, it is essential to optimize acquisition methods such as Data-Independent Acquisition (DIA) and carefully design experimental workflows, while also considering **instrument time constraints**, which are a limiting factor in high-throughput or large-scale studies. Strategies like Gas-Phase Fractionation (GPF) can help improve proteome coverage and sensitivity without compromising reproducibility. This study compares different GPF strategies to enhance DIA-MS workflows, aiming to find the best balance between depth, quantitative reliability, and analysis time.

OBJECTIVE

This study aims to evaluate and compare different fractionation strategies including classical approaches such as **cellular fractionation** and **SDS-PAGE**, as well as **Gas-Phase Fractionation (GPF)** to enhance the performance of DIA-MS workflows. The comparison was conducted on two distinct biological models: a **human cell lysate**, representing a conventional sample type, and **sturgeon eggs**, a more challenging sample with a high dynamic range. This approach allows us to assess the benefits and limitations of each strategy in terms of **protein identification depth**, **quantitative accuracy**, and **suitability for complex samples**.

MATERIAL AND METHODS

LC-MS & Instrumentation Settings:

- **LC System**: Thermo Ultimate 3000 RSLCnano
    - **Flow rate**: 200 nL/min
    - **Gradient**: 146 min
- **Instrument**: Thermo Scientific **Orbitrap Fusion Lumos Tribrid** mass spectrometer
    - Equipped with EASY-Spray ion source
    - DIA acquisition mode

Sample Preparation :

- **Human standard sample**:
    - Two fractionation strategies tested:
        - Gel-based separation
        - Gas-Phase Fractionation (GPF)
- **Sturgeon egg samples**:
    - Proteins extracted from 6 sturgeon eggs
    - Three fractionation methods tested:
        - Cellular fractionation
        - SDS-PAGE
        - GPF

Fractionation strategies

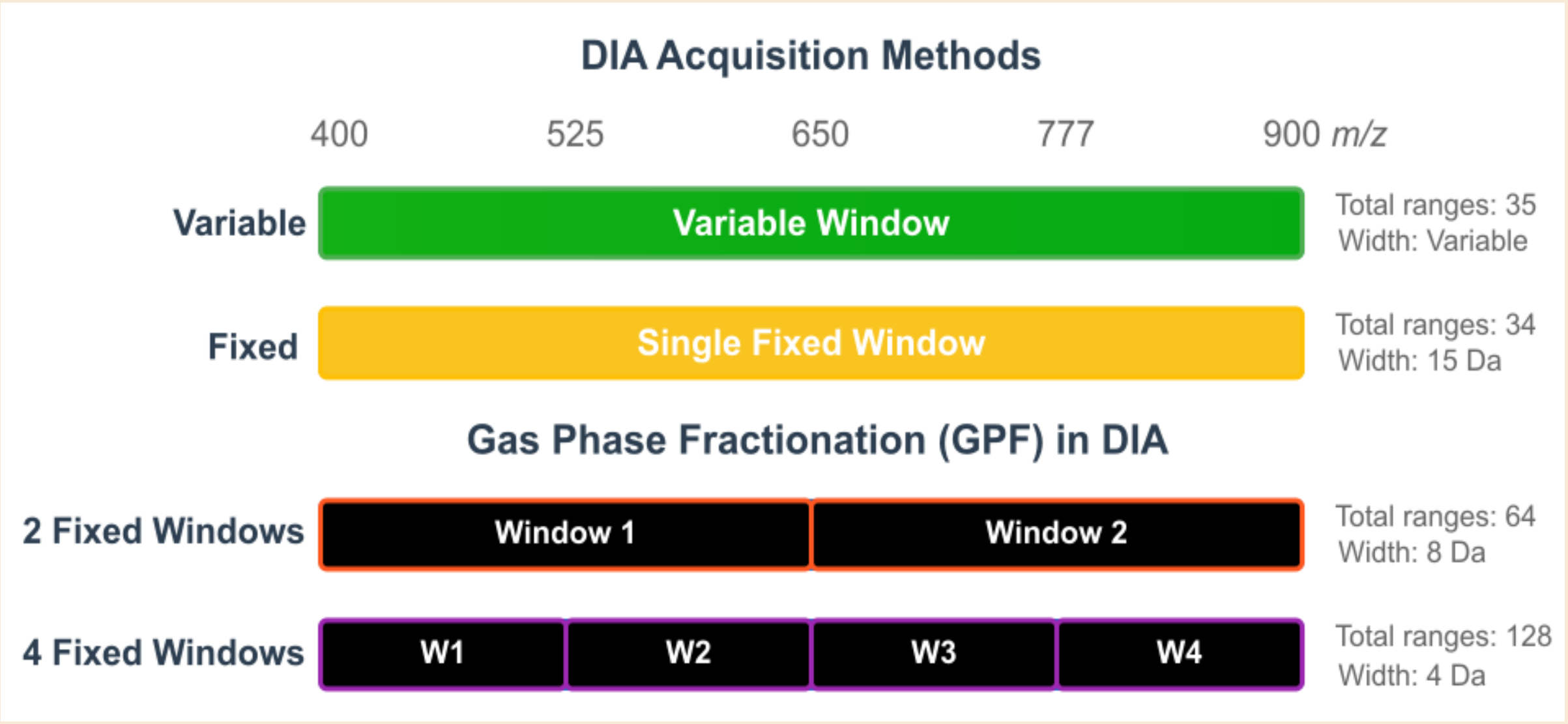

Figure 1. **Data-Independent Acquisition (DIA) and Gas-Phase Fractionation (GPF) strategies.** DIA fragments precursor ions regardless of intensity across wide isolation windows, co-fragmenting all co-eluted peptides simultaneously. The variable window approach optimizes precursor selection across the full mass range, while fixed windows provide uniform coverage. Gas-Phase Fractionation (GPF) enhances MS/MS coverage by dividing the *m/z* range into smaller, sequential acquisition windows through multiple sample injections. GPF requires as many sample injections as the number of windows (e.g., GPF2 requires 2 injections, GPF4 requires 4 injections).

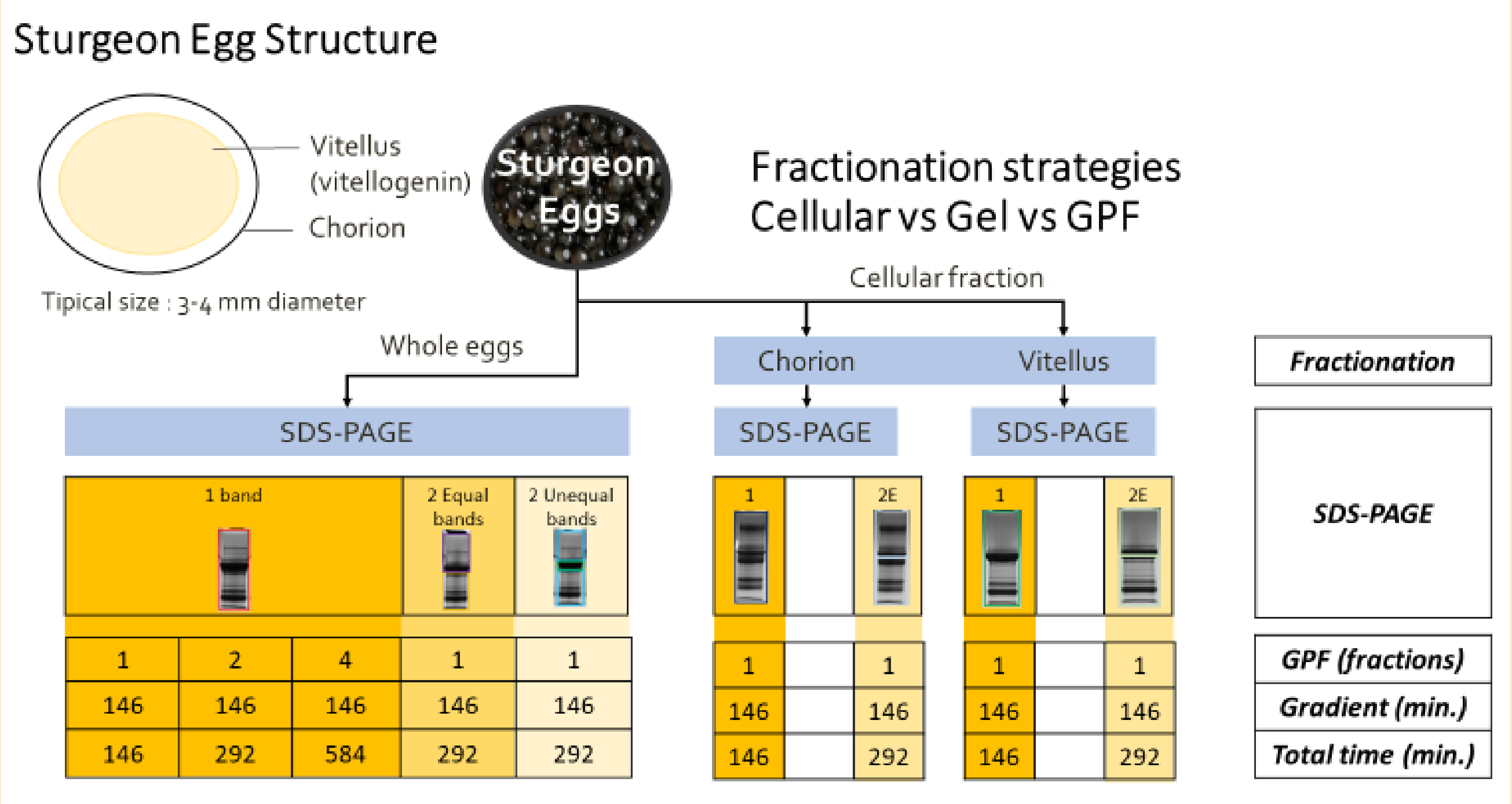

Figure 2. **Sturgeon egg samples fractionated using different experimental strategies.** Samples were first fractionated into **chorion** (outer layer) and **vitellus** (inner content), as vitellogenin is predominantly localized in the vitellus fraction, allowing for more targeted proteomic analysis of each egg compartment. Each cellular fraction was then further separated by gel electrophoresis into two bands. Additionally, whole egg proteins were separated by gel electrophoresis as a single band, into two equal bands, or into two unequal bands, with the unequal band approach strategically isolating the highly abundant **vitellogenin** protein in one fraction to enhance proteome depth analysis of the remaining proteins. These physical fractionation strategies were compared to gas-phase fractionation (GPF) approaches that achieve similar complexity reduction through multiple analytical injections.

RESULTS

Human standard

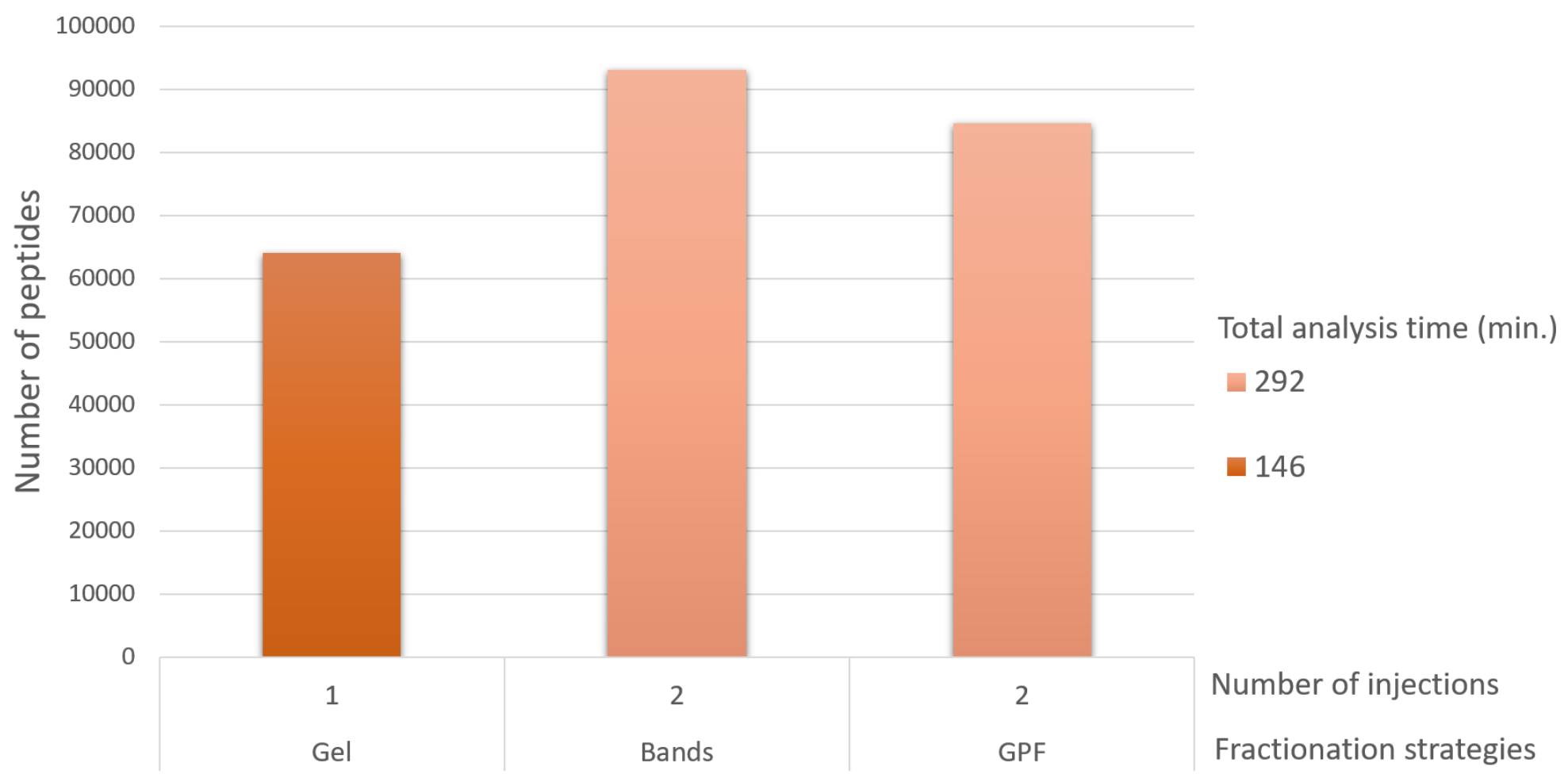

Figure 3. **Impact of Fractionation Strategies on Peptide Identification and Analysis Time**. Gel-based fractionation provides 10% more identifications than GPF. However, whether using gel-based or GPF fractionation, performing two separate analyses still provides better coverage than a single band, single injection classical gel approach but it effectively doubles the analysis time.

Sturgeon eggs

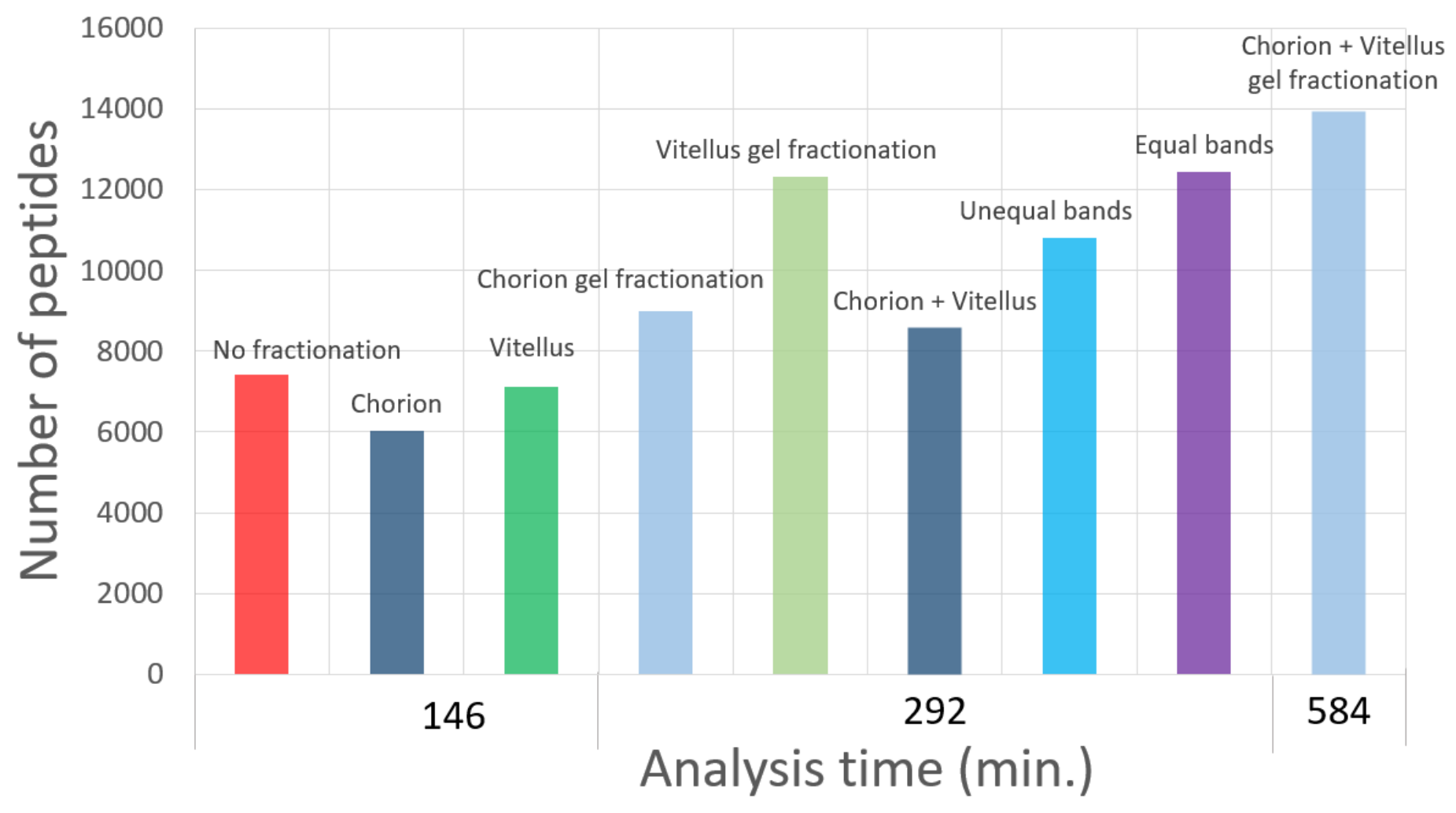

Figure 4. **Impact of sample fractionation strategies on peptide identification in sturgeon egg proteome analysis.** Sequential fractionation significantly enhances proteome coverage. Cellular fractionation (chorion/vitellus separation) increases peptide detection compared to whole egg analysis Gel-based fractionation consistently provides deeper proteome coverage than cellular fractionation alone, regardless of the banding strategy (equal or unequal bands). The combination of cellular and gel-based fractionation yields the highest peptide count (~14,000 peptides), highlighting the strong impact of pre-analytical strategies on proteome depth. However, such workflows can be time-consuming and may present challenges for large sample cohorts.

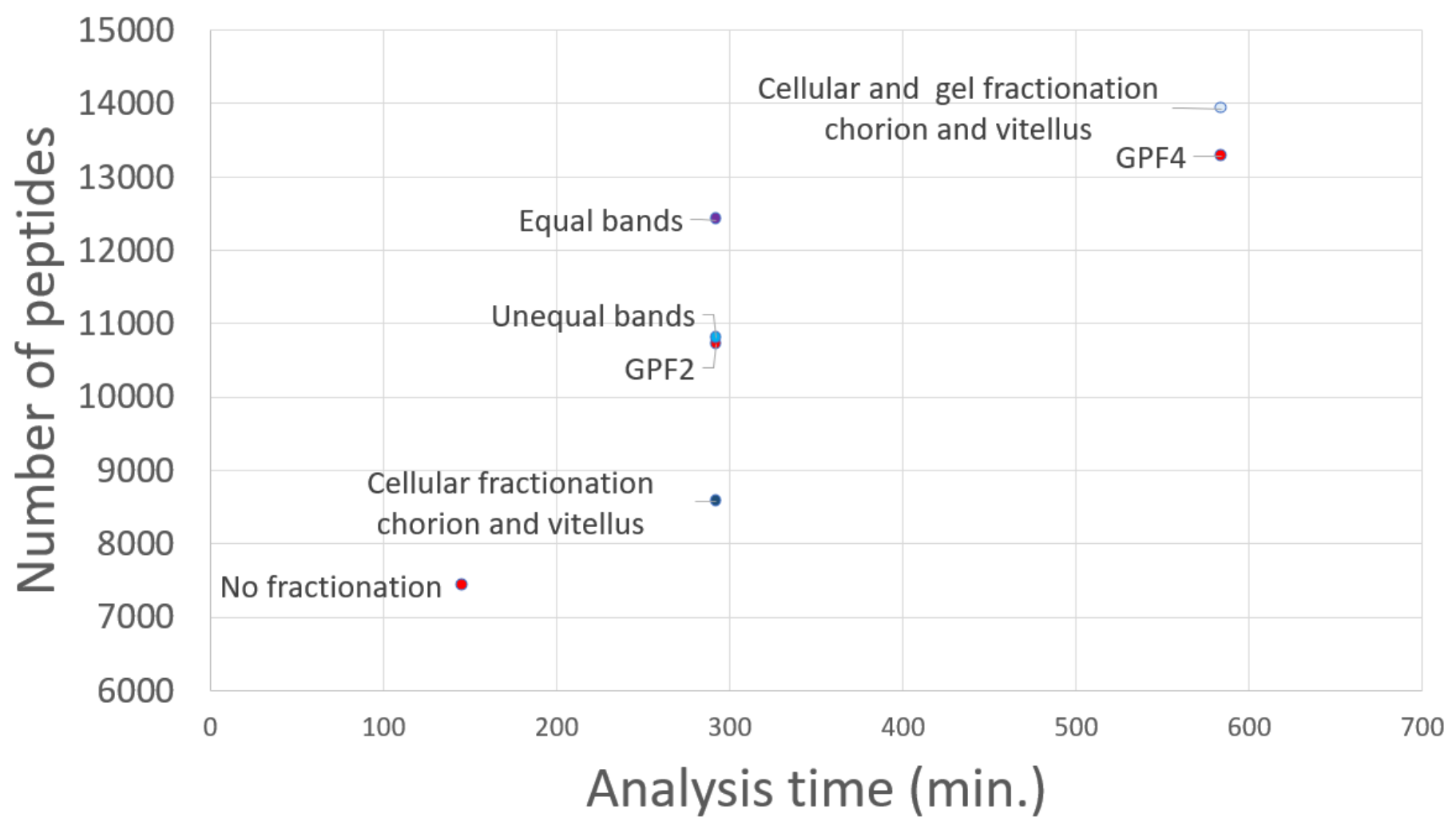

Figure 5. **Time-efficiency comparison of fractionation strategies for sturgeon egg proteome analysis.** This analysis evaluates the trade-off between peptide identification performance and required analysis time across different fractionation approaches. While combined cellular and gel fractionation achieves maximum proteome depth, it requires extensive preparation and long analysis times. GPF4 efficiently matches the performance of complex experimental designs with significantly improved reproducibility and reduced hands-on time. This demonstrates that gas-phase fractionation provides an optimal solution for routine proteomics, delivering comparable results to intensive physical fractionation methods while maintaining methodological consistency.

CONCLUSION

Pre-analytical sample fractionation significantly enhances proteome coverage, with combined cellular and gel fractionation achieving the highest number of peptide identifications. While physical fractionation offers greater depth than gas-phase fractionation, it also introduces added experimental complexity and potential reproducibility challenges Gas-phase fractionation (GPF) provides an optimal balance between improved coverage and methodological robustness, making it the preferred approach for our quantitative proteomics on sturgeon eggs. However, experimental design must be carefully adapted to the sample's complexity and dynamic range, the desired proteome coverage, available acquisition time, and the level of robustness required for accurate quantitative analysis with sufficient data points across chromatographic peaks, as well as the instrument's scan speed - with newer generation instruments potentially making GPF even more competitive compared to classical fractionation approaches.