Extracellular polymeric substances (EPS) have a presumed determinant role in the structure, architecture, strength, filterability, and settling behaviour of microbial solids in biological wastewater treatment processes. Consequently, numerous EPS extraction protocols have recently been published that aim to optimize the trade off between high EPS recovery and low cell lysis. Despite extensive efforts, the obtained results are often contradictory, even when analysing similar biomass samples and using similar experimental conditions, which greatly complicates the selection of an extraction protocol. This study presents a rigorous and critical assessment of existing physical and chemical EPS extraction methods applied to mixed-culture biomass samples (nitrifying, nitritation-anammox, and activated sludge biomass). A novel fluorescence-based method was developed and calibrated to quantify the lysis potential of different EPS extraction protocols. We concluded that commonly used methods to assess cell lysis (DNA concentrations or G6PDH activities in EPS extracts) do not correlate with cell viability. Furthermore, we discovered that the presence of certain chemicals in EPS extracts results in severe underestimation of protein and carbohydrate concentrations by using standard analytical methods. Keeping both maximum EPS extraction yields and minimal biomass lysis as criteria, it was identified a sonication-based extraction method as the best to determine and compare tightly-bound EPS fractions in different biomass samples. Protein was consistently the main EPS component in all analysed samples. However, EPS from nitrifying enrichments was richer in DNA, the activated sludge EPS had a higher content in humic acids and carbohydrates, and the nitritation-anammox EPS, while similar in composition to the nitrifier EPS, had a lower fraction of hydrophobic biopolymers. In general, the easily-extractable EPS fraction was more abundant in carbohydrates and humic substances, while DNA could only be found in tightly bound EPS fractions. In conclusion, the methodology presented herein supports the rational selection of analytical tools and EPS extraction protocols in further EPS characterization studies.