iTRAQ as a method for optimization: Enhancing peptide recovery after gel fractionation

Pieter Glibert, Katleen Van Steendam, Maarten Dhaenens and Dieter Deforce

Laboratory of Pharmaceutical Biotechnology, Ghent University, Belgium

At the dawn of a new era in label-free quantitation on high-resolution MS instruments, classical methods such as iTRAQ continue to provide very useful insights in comparative proteomics. The potential to multiplex samples makes this reporter-based labeling technique highly suited for method optimization as demonstrated here by a set of standard series. Instead of studying ratios of annotated proteins, we propose an alternative method, based on the analysis of the average reporter ratios of all the spectra from a sample or a large distinct subset herein. This strategy circumvents the bias, associated with the annotation and iTRAQ quantitation, leading to increased adequacy in measuring yield differences between workflows. As gel electrophoresis prior to MS analysis is highly beneficial, for example, as a fractionation step, the approach was applied to evaluate the influence of several parameters of the established in-gel digestion protocol. We quantified the negative effect of SYPRO Ruby staining and the positive effect of gel fixation prior to digestion on peptide yield. Finally, we emphasize the benefits of adding CaCl₂ and ACN to a tryptic in-gel digest, resulting in an up to tenfold enhanced peptide recovery and fewer trypsin missed cleavages.

Keywords:
Gel fractionation / In-gel digestion / iTRAQ / Method optimization / Quantification / Technology

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Over the years, isobaric labeling techniques have been applied on a large scale in different proteome studies where relative quantitation is required. Although label-free methods, such as SWATH (AB SCIEX) and HDMS® (Waters Corporation) are gaining popularity, label-based strategies remain important. The reporter-based labeling methods still have the ability to give complementary insights, especially in terms of minimizing technical variation by parallel quantitation of multiple samples [1, 2].

One of the main challenges in interpreting iTRAQ data is the underestimation of the fold change, partially caused by interfering masses in the silent region of the reporters and mixed MS/MS [3, 4]. Here, we present a new workflow for method optimization by means of isobaric tags (such as iTRAQ) to overcome these challenges without the need of complex data analysis tools. In-depth protocol knowledge can be achieved by dividing a (standard) peptide mixture into equal parts and differentially labeling them for each experimental condition under investigation. In the event of a digestion optimization, a protein sample can be split into equal parts, which are labeled and pooled after digestion [5]. The relative yield of each condition is then defined by all the associated reporter ions in a run instead of only focusing on the identified proteins. Additionally, the effect of different experimental conditions on a specific peptide set with similar physicochemical classes can be evaluated after annotation.

We first analyzed a standard series of known ratios to test the preciseness of this approach and more specifically validate the influence of contaminating peaks in the reporter.

Correspondence: Dr. Dieter Deforce, Laboratory of Pharmaceutical Biotechnology, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium
E-mail: Dieter.Deforce@UGent.be
Fax: +32-09-220-66-88

Abbreviations: Q, quadrupole; SR, SYPRO Ruby

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Validation of the iTRAQ method

Table 1. Validation of the iTRAQ method

| Sample | Label distribution | 114/115 log ratio | 116/117 log ratio | 114/117 log ratio | 116/115 log ratio | SD 114/115 | SD 116/117 | SD 114/117 | SD 116/115 |
|--------|-------------------|-------------------|-------------------|-------------------|-------------------|------------|------------|------------|------------|
| S1     | 1 2 3 4 5 6 7 8 9| 0.930             | -0.930            | 0.930             | -0.930            | 0.065      | 0.065      | 0.065      | 0.065      |
| S2     | 2 3 4 5 6 7 8 9 1 | -0.930            | 0.930             | -0.930            | 0.930             | -0.065     | -0.065     | -0.065     | -0.065     |
| S3     | 3 4 5 6 7 8 9 1 2 | 0.930             | -0.930            | 0.930             | -0.930            | 0.065      | 0.065      | 0.065      | 0.065      |
| S4     | 4 5 6 7 8 9 1 2 3 | -0.930            | 0.930             | -0.930            | 0.930             | -0.065     | -0.065     | -0.065     | -0.065     |
| S5     | 5 6 7 8 9 1 2 3 4 | 0.930             | -0.930            | 0.930             | -0.930            | 0.065      | 0.065      | 0.065      | 0.065      |
| S6     | 6 7 8 9 1 2 3 4 5 | -0.930            | 0.930             | -0.930            | 0.930             | -0.065     | -0.065     | -0.065     | -0.065     |
| S7     | 7 8 9 1 2 3 4 5 6 | 0.930             | -0.930            | 0.930             | -0.930            | 0.065      | 0.065      | 0.065      | 0.065      |
| S8     | 8 9 1 2 3 4 5 6 7 | -0.930            | 0.930             | -0.930            | 0.930             | -0.065     | -0.065     | -0.065     | -0.065     |
| S9     | 9 1 2 3 4 5 6 7 8 | 0.930             | -0.930            | 0.930             | -0.930            | 0.065      | 0.065      | 0.065      | 0.065      |

Linear regression

| Sample | Theoretical ratio | Experimental ratio |
|--------|-------------------|-------------------|
| S1     | 114              | 115               |
| S2     | 116              | 117               |
| S3     | 117              | 118               |

Identical parts of a peptide mixture were labeled with a different reporter label of the iTRAQ 4plex Kit (AB SCIEX, Framingham, MA, USA). Each sample (S1-S9) consists of 4 different combinations of the four labels resulting in known (theoretical) ratios. After MS analysis, the average 114/115, 116/117, 114/117, and 116/115 ratios are calculated from the spectra with reporter areas above the 0.3 threshold for each sample. For the linear regression analysis, both the slope and r values are presented for each ratio.

However, all the labels are suited for optimization studies shown in Fig. 1, probably caused by label-specific effects [7].
Next, we used this iTRAQ approach to optimize our in-gel digestion protocol, the technique that bridges the gap between two keystones methodologies in proteomics: gel electrophoresis and MS. Despite the gain in popularity of the gel-free methods, particularly due to highly sensitive mass spectrometers, gel electrophoresis still has an added value as a molecular weight fractionator, a purification step and in the study of more hydrophobic (membrane) proteins [8]. One very interesting application lies in PTM research where isobaric labels are used to measure stoichiometry: iTRAQ after gel fractionation is capable of reducing interfering precursors and thus enhancing quantitation accuracy [7, 9]. Most protocols separate the proteins by SDS-PAGE and visualize the fixed proteins by CBB, silver stain, or popular SYPRO Ruby (SR) stain [10]. After imaging, proteins in the gel bands are classically destained, reduced, and alkylated before digestion [11]. Numerous in-gel digestion strategies and optimized protocols are available: some focus on the reduction of the digestion time, while others implement alternative reagents and multiple proteases to augment peptide recovery [12]. However, the consequence of several well-established steps in many protocols on the robustness of the technique is not well documented. Yet, a large (interrun) variation in the yield of peptide extraction is a well-known downside of many in-gel digestion protocols and challenges optimization [11].

Here, we examine the impact of protein fixation and SR staining and the possible benefits of ACN and CaCl₂ on digest efficiency when gel electrophoresis is implemented as a sample preparation step prior to MS analysis. A HepG2 cell lysate was equally divided over 16 wells of two gels for the electrophoresis. For each condition, the extracted peptides were iTRAQ labeled and pooled immediately after the digest (Fig. 2A). Every experiment was carried out four times in parallel to allow swapping iTRAQ labels to compensate for possible variation in label efficiency or label accuracy due to label-specific contaminating peaks.

The gels were cut around the 50 kDa marker to create two fractions and test the possible impact of the protein molecular weight on the digest conditions. Next, the individual lanes were excised to be digested under the different conditions. For the standard condition 1, proteins were fixed within the gel with a 7% acetic acid, 10% MeOH solution twice for 10 min. After a short wash with Milli-Q, the gel bands were incubated overnight with the SR gel stain in the dark at room temperature. For alternative conditions 2 and 3, the staining (2) or fixation and staining step (3) were skipped. For condition 4, 1 mM CaCl₂ and 5% ACN were added to the trypsin buffer on a fixed and stained gel (Fig. 2A, right). After digestion, peptides were extracted with ACN in three steps, labeled, and pooled according to Fig. 2A. Data analysis was performed as described above.

With no obvious differences in the calculated average ratios between high and low molecular weight fractions, these data files were merged for further analysis. In Fig. 2B, the conditions 2–4 are compared against condition 1, the standard protocol where proteins are extracted from a fixed and SR-stained gel. iTRAQ quantified large variations in peptide recovery between different gel digests. This coordinate emphasizes the importance of replicate analysis, especially during technical optimization. However, the main advantage of applying this strategy for method development is that the entire peptide ion yield is taken into account. Unlike most studies, we do not rely on the amount of identified proteins directly affected by
unexpected modifications and inherent to different steps under investigation such as gel staining and fixation. SR staining clearly has a negative influence on peptide recovery in each replicate as shown by the increased “fixation no SR/standard” ratios where no staining was applied. Peptide loss is a known downside of fluorescent methods, yet here we show that this is not due to unexpected modifications, but rather to a loss of on average ±40% of the extracted peptides. When implementing gel electrophoresis as a fractionation or purification step, staining should thus be avoided. Surprisingly, however, fixation of a gel with acetic acid and MeOH hinted toward a positive effect on peptide recovery as suggested by three of the four significantly positive “fixation no SR/no fixation no SR” ratios (one-sample t-test, p < 0.0001; Fig. 2B, asterisk). As mentioned earlier, when looking at annotated peptides one can select certain populations of peptides within the ratio distribution (e.g., left and right of the mean; Supporting Information Methods 2A for details) [6]. When these subsets of labeled peptides are compared based on certain properties such as the number of missed cleavages and grand average of hydrophobicity (GRAVY) scores, one can coordinately define the gain of a certain methodology for specific characteristic sequences. Since fixation yielded a higher amount of peptides, we hypothesize that skipping fixation possibly results in spontaneous migration of proteins and peptides out of the gel. On the other hand, gel fixation resulted in an increased number of missed cleavages, which suggests that fixation could restrict the number of accessible clipping sites. Finally, despite the use of a modified trypsin, addition of CaCl₂ and ACN at the time of digestion was found to be very
beneficial to the yield of the digestion resulting in an up to tenfold increase in peptide recovery. To our knowledge, this is the first time that the advantages of those additives are quantified specifically for in-gel digestion. The advantage of including Ca\(^{2+}\) was previously explained by the beneficial effect on trypsin autocleavage during in-solution digestion protocols but is often overlooked since modified trypsin which is less susceptible to autocleavage, became a golden standard [11]. This significant difference is probably induced by the stabilizing effect of Ca\(^{2+}\) ions on trypsin and improvement of the protein accessibility in the presence of ACN [13]. CaCl\(_2\) and ACN supplementation does not result in recovery of more hydrophobic peptides as shown by the similar GRAVY scores but does diminish the amount of trypsin-missed cleavages from 26 to 17% (Supporting Information Methods 2B).

Isobaric tags are mostly applied to study the proteome expression between different biological samples. The label-based bias or variation induced by the required annotation of the proteins is often overlooked or corrected for by using complex postacquisition tools. Our standard series emphasize the accuracy of the iTRAQ technique in quantifying small loading differences between samples when analyzing the average ratios of all the data in a run. iTRAQ allows for multiplexing and internal replicates, which makes the technique highly suitable for method optimization. Using all the data excludes the variation associated with protein identification and quantitation, caused by unexpected modifications or fragmentation. For gel electrophoresis, particularly when used as a fractionation and purification tool for subsequent MS analysis, we recommend fixation of the gel and skipping SR staining before digestion. Finally, we corroborate the positive influence of CaCl\(_2\) and ACN addition in tryptic in-gel digestion protocols to increase reproducibility, above all in automated workflows [7].

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