

Targeted SCX Based Peptide Fractionation for Optimal Sequencing by Collision Induced, and Electron Transfer Dissociation

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Abstract

Electron transfer dissociation (ETD) of peptide ions has been introduced as a tool for mass spectrometry based peptide sequencing, complementary to the commonly used collision induced dissociation (CID). It has been proposed that ETD may have better performance than CID for more highly charged and/or larger peptides. Here, we compare the performance of ETD and CID on data generated in a large-scale proteomics experiment. First, tryptic proteolytic peptides of *Drosophila melanogaster* oocytes were off-line separated based on their in-solution net charge state using strong cation exchange chromatography (SCX), followed by an on-line reverse-phase (RP) liquid chromatography separation coupled to an ion trap mass spectrometer with ETD capabilities. The mass spectrometer selected MS peaks were subjected to both ETD and CID thus allowing a fair comparison. Around 2300 peptides were exclusively identified by CID and similarly more than 3000 by ETD with approximately 1400 by both ETD and CID. In total nearly 7,000 peptides were identified with a very conservative Mascot peptide cut-off score of 60 clearly verifying that ETD and CID are complementary techniques. In the early SCX fractions, which contain peptides with a 'low' net charge, more than 90% of the peptides could be successfully identified by CID whereas in the later SCX fractions more than 90% of the identified peptides could be successfully identified by using ETD only. The chosen strategy, with a combination of SCX and RP-LC-MS/MS, allows the user to make targeted decisions on whether to optimally use CID and/or ETD. Analysis of the sequence and amino acid contents of all identified peptides clearly revealed that the impressive performance of ETD for peptides possessing charge states above three do not require CID based sequencing which, at best, would be solely confirmatory.

Introduction

Several strategies are available for performing large-scale analyses of complex protein mixtures (Aebersold and Mann, 2003; Brunner et al., 2007; Kolkman et al., 2005; Krijgsveld et al., 2006; Shen et al., 2005; Witze et al., 2007). The 'shotgun' peptide-centric approach is popular for such analyses, involving the generation of in-solution tryptic digests of whole lysates. The complexity of the sample introduced into the mass spectrometer is reduced by using multidimensional separation techniques where, typically, the

first dimension consists of strong cation exchange (SCX) chromatography (Wu et al., 2003), hydrophilic interaction chromatography (HILIC) (Boersema et al., 2007) or peptide iso-electric focusing (IEF) (Cargile et al., 2004, Krijgsveld et al., 2006). In particular, the combination of SCX as a first dimension for separation of the peptides with nanoflow reversed phase (RP) chromatography has been shown to be extremely powerful (MacCoss et al., 2002). Unfortunately, when using such an approach there will be

an undersampling of the total peptide population. This is partly caused by the fact that the separation power of multidimensional chromatography is still insufficient and consequently too many peptides will co-elute and compete with each other for ionization and mass spectrometric sequencing. This drawback can be partly overcome by repeating the analysis for each sample several times since peptide sampling by the mass spectrometer is partly random (de Groot et al., 2007; Lipton et al., 2002; Liu et al., 2004; Shen et al., 2005). Another reason why not all peptides are successfully sequenced lies in the fact that most current electrospray based mass spectrometers have an optimal m/z range for analysis which lies between 300-1500 Th. In-solution digestion using trypsin may not allow a complete analysis due to certain proteolytic peptides falling outside this optimal window (MacCoss et al., 2002; Mohammed et al., 2008). Larger and highly charged tryptic peptides are often sequenced poorly by CID based tandem MS, partly due to insufficient mass resolution to assign the correct charge state for the precursor and product ions as well as poor fragmentation (Paizs and Suhai, 2005). All in all, new methods that will enable improved proteome coverage by using techniques complementary to CID, would be welcome.

Recently electron transfer dissociation (ETD) has been introduced as a new peptide sequencing method (Good et al., 2007; Syka et al., 2004) and through its mode of operation exhibits properties that are complementary to collision induced dissociation (CID). In ETD, an electron is transferred from a radical anion, usually fluoranthene, to the protonated peptide, inducing fragmentation and formation of c and z type ions. The exact mechanism of how ETD promotes fragmentation is however still under debate (Leymarie et al., 2003; Syka et al., 2004; Zubarev et al., 2000). It has been shown that ETD can be effective at fragmenting peptides with the higher charge state peptides that CID would often struggle to identify. However, earlier studies have shown that doubly charged peptides do not efficiently fragment in ETD experiments due to the fact that dissociation efficiency by ETD is related to the number of charges present on the precursor ion. To circumvent the difficulties of analyzing doubly protonated peptides by ETD techniques a limited amount of collisional activation is applied to precursor cations after electron transfer for more efficient fragmentation, so-called ETcaD (Pitteri et al., 2005; Swaney et al., 2007).

To obtain a more in-depth sense for the performance of ETD as compared to CID, we performed a medium-scale proteome analysis of early *Drosophila melanogaster* embryos in which all peptides were subjected to CID and

ETD. We generated a dataset of approximately 7000 peptides that were sequenced by CID and ETD when applying a conservative Mascot cut-off score of 60. Annotated spectra were extracted from Mascot result files (.dat) that fulfil the score requirements using an in-house developed software tool, while systematic statistical analysis on the dataset were performed with simple Perl scripts. Strikingly, the result was the discovery for the overlap between the ETD and CID data-sets being less than 25%. Looking into the specifics on each peptide we conclude from the data that CID favours smaller and less basic peptides, whereas ETD favours longer and more highly charged and therefore more basic peptides. As SCX largely separate peptides on charge (Beausoleil et al., 2004), which is also clearly revealed by the current dataset, an optimal strategy can be proposed whereby the early fractions are predominantly analyzed by CID-MS, whereas the late fractions would solely require ETD.

Experimental

Fly stock and embryo collection and sample preparation

Wild-type OregonR flies were maintained by standard methods at 25 °C. Wild-type embryos were collected on agarose-agar plates, washed in water and dechorionated by incubation in 2.5% sodium hypochlorite for 90 s followed by another wash and kept at -20 °C. About 5 mg of embryos were lysed in 8 M urea and 50 mM ammonium bicarbonate. Cellular debris was pelleted by centrifugation at 20,000 g for 20 minutes. Prior to digestion, proteins were reduced with 1 mM DTT and alkylated with 2 mM iodoacetamide. The mixture was diluted 4-fold to 2 M urea using 250 μ L of 50 mM ammonium bicarbonate and 50 μ L of trypsin solution, 0.1 mg/mL, and incubated overnight at 37 °C.

Strong cation exchange

Strong cation exchange was performed using a Zorbax BioSCX-Series II column (0.8 mm i.d. \times 50 mm length, 3.5 μ m), a FAMOS autosampler (LC-packing, Amsterdam, The Netherlands), a Shimadzu LC-9A binary pump and a SPD-6A UV-detector (Shimadzu, Tokyo, Japan). Prior to SCX chromatography, protein digests were desalted using a small plug of C18 material (3 M Empore C18 extraction disk) packed into a GELoader tip (Eppendorf) similar to what has been previously described (Rappsilber et al., 2003), onto which \sim 10 μ L of Aqua C18 (5 μ m, 200 Å) material was placed. The eluate was dried completely and subsequently reconstituted in 20% acetonitrile and 0.05% formic acid. After injection, a linear gradient of 1% min^{-1} solvent B (500

mM KCl in 20% acetonitrile and 0.05% formic acid, pH 3.0) was performed. A total of 45 SCX fractions (1 min each, i.e., 50 μ L elution volume) were manually collected and dried in a vacuum centrifuge, of which 23, that contained most peptides, were subjected to our mass spectrometric analysis by RP-LC MS/MS.

Nanoflow-HPLC-MS

Dried residues were reconstituted in 50 μ L of 0.1 M acetic acid and were analyzed by nanoflow liquid chromatography using an Agilent 1100 HPLC system (Agilent Technologies) coupled on-line to a LTQ-XL mass spectrometer (Thermo-Fisher Scientific). The liquid chromatography part of the system was operated in a setup essentially as described previously (Licklider et al., 2002; Meiring et al., 2002). Aqua C18 (Phenomenex), 5 μ m resin was used for the trap column, and ReproSil-Pur C18-AQ, 3 μ m, (Dr. Maisch GmbH) resin was used for the analytical column. Peptides were trapped at 5 μ L/min in 100% solvent A (0.1 M acetic acid in water) on a 2 cm trap column (100 μ m i.d., packed in-house) and eluted to a 20 cm analytical column (50 μ m i.d., packed in-house) at ~100 nL/min in a 150-min gradient from 10 to 40% solvent B (0.1 M acetic acid in 8/2 (v/v) acetonitrile/water). The eluent was sprayed via standard coated emitter tips (New Objective), butt connected to the analytical column. The mass spectrometer was operated in the data dependent mode to automatically switch between MS and MS/MS ETD and MS/MS CID. Survey MS spectra were acquired from m/z 350 to m/z 1500 in the LTQ after accumulation to a target value of 30,000 in the linear ion trap. The two most intense ions were fragmented in the linear ion trap at a target value of 10,000. To prevent repetitive analysis of the same ion, dynamic exclusion technology (Thermo Fischer Scientific) was used.

Data extraction and analysis

The MS2-data was extracted from the raw data file with a beta-release of Bioworks 3.4 (Thermo-Fisher Scientific) into separate spectrum (.dta) files using the Sequest preprocessor, without additional filtering. The standard method of charge state assignment is to use the Charger program (Thermo-Fisher Scientific, (Sadygov et al., 2008)) on every ETD tandem mass spectrum. We let the Charger program analyze our experimental data to assess its performance. All other analyses were performed without the dependency on the Charger program (Sadygov et al., 2008) via concatenating the spectra in Mascot Generic Files (.mgf), where the CHARGE field for each peak list corresponding to an individual spectrum would contain values

from 2+ to 7+. Tandem MS ion searches were performed with Mascot 2.2 (Matrix Science inc.) on a concatenated database of *Drosophila melanogaster* sequences in the Swissprot and the TrEMBL databases, with a peptide tolerance of 3.0 Da and a MS/MS tolerance of 0.9 Da. Subsequently, the Mascot result files (.dat) were retrieved from the server and each underwent an extraction procedure. For all SCX fractions, the highest scoring peptide hit for each spectrum was retrieved. Sequence, ion score, charge state and precursor mass were stored in a text file. Similarly, for protein identifications the best protein hit for each peptide identification was retrieved. Protein identification required a minimum of two peptides to be identified. All algebraic operations regarding peptides and protein identifications were performed with Perl (Activestate Perl 5.8.8) scripts and visualized with Microsoft Excel 2007. All the Perl scripts, the original input text files and the Excel workbook (in Excel 2007 format) are made available in the supplementary material (https://bioinformatics.chem.uu.nl/supplementary/vdtoorn_etdclid/). All raw data with identifications has been submitted to the PRIDE repository at the EBI (<http://www.ebi.ac.uk/pride>, in a project with the name "Targeted SCX based peptide fractionation for optimal sequencing by collision induced, and electron transfer dissociation", accession numbers 8697-8742 inclusive).

Results and Discussion

Drosophila melanogaster embryos were lysed and the peptide mixture generated by trypsin proteolysis was subjected to SCX fractionation (Figure 1). Twenty three 1 minute fractions were subjected to analysis by RP-LC-MS/MS. The linear ion trap mass spectrometer was operated in the data dependent mode and switched automatically between MS, ETD and CID.

Precursor ion charge state determination

The linear ion trap mass spectrometer has limited mass resolution when performing a standard scan and therefore correct determination of the precursor ion charge state requires additional time consuming scans. A specific method of charge state determination is available for ETD MS spectra, which exploits the knowledge that the transferred electron(s) might not necessarily induce dissociation, but lead to intact peptides ions with a reduced charge state. The Charger program, which is part of the Bioworks package (Thermo-Fisher Scientific (Sadygov et al., 2008)) tries to determine charge states using these charge reduced species that are present within the ETD spectra. An overview of Charger program output for the mass spectrometric data

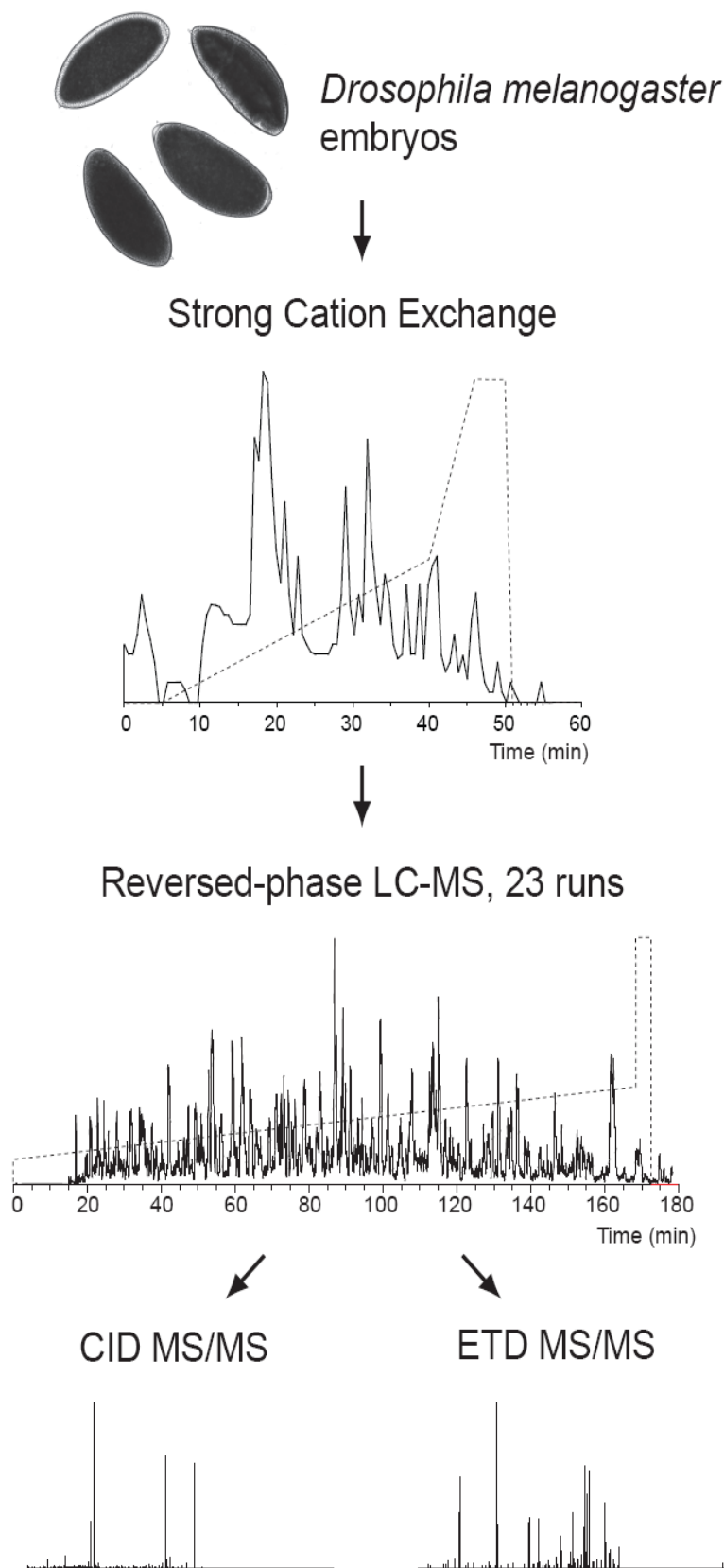


Figure 1: Scheme of the experimental setup. Tryptic peptide digests of *Drosophila melanogaster* embryo lysates were first separated by off-line strong cation exchange (SCX), where each fraction was analysed by reversed-phase liquid chromatography on-line coupled to a mass spectrometer. Individual peptide ions were selected by the mass spectrometer and fragmented using sequentially both collision induced dissociation (CID) and electron transfer dissociation (ETD).

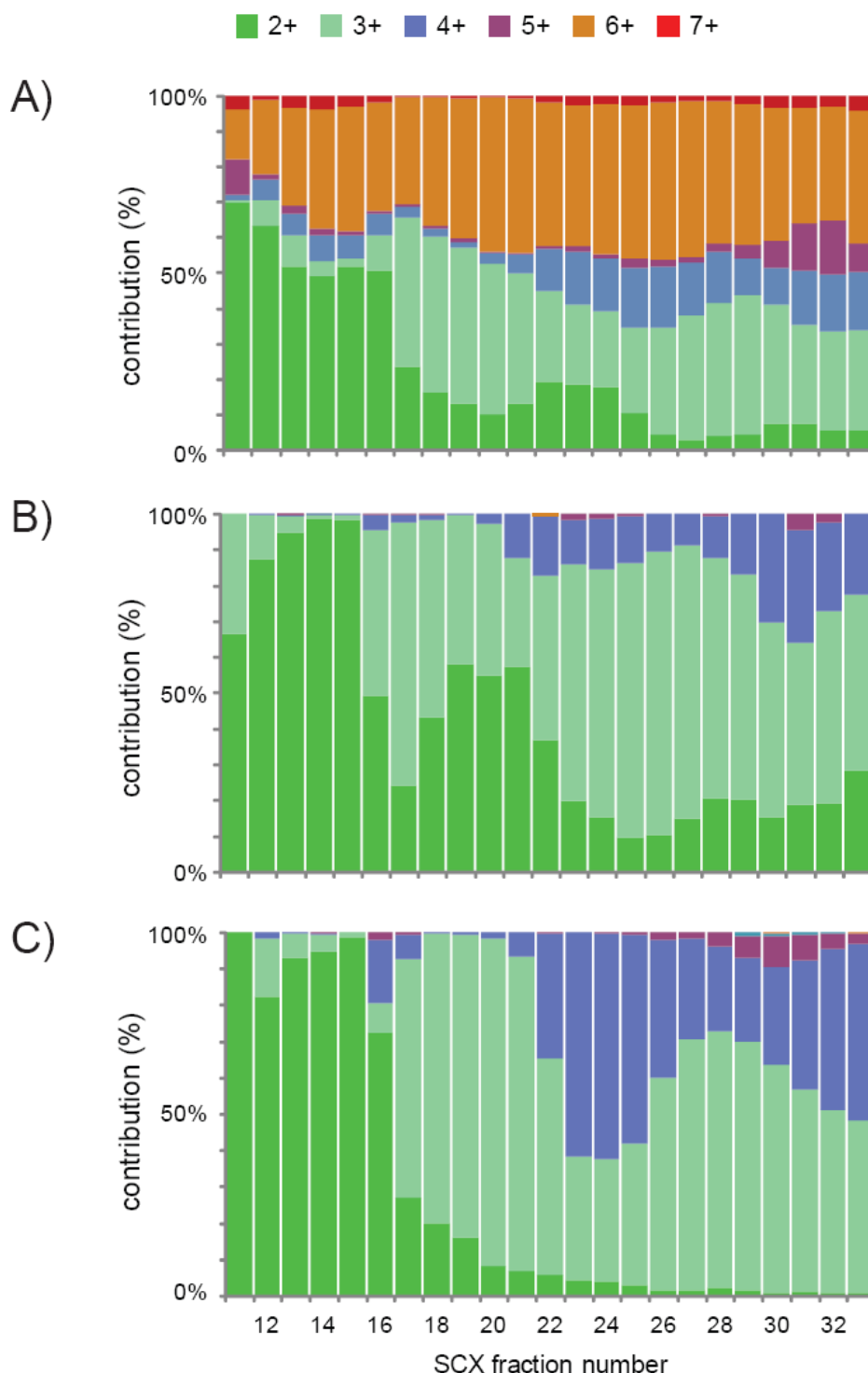


Figure 2: Correct determination of precursor ion charge states are vital for optimal peptide sequencing. In (A) the charge states were determined by the Bioworks 'charger' preprocessor program. For each SCX fraction the relative contribution of ions of each charge state is shown. The charge assigned in (B) and (C) were determined by the charge of the highest scoring peptide (minimum peptide score of 60) in a Mascot database search. Mascot was instructed to search with the charge state being between 2+ and 7+ for each MS-spectrum. The charge state contributions for both CID data (B) and ETD data (C) are shown. The color legend at the top provides correlation with the assigned charges. The absolute number of identified peptides in each SCX fraction is listed in supplementary Figure 4.

acquired is shown in Figure 2A. We started our comparison at fraction 11 which was the first to contain a reasonable number of peptides, and stopped at fraction 33. As expected, there is a trend of increasing peptide charge states with increasing fraction number for the SCX run. Notably, in every SCX fraction analysis many spectra are, quite unrealistically, assigned as 6+ charge states, especially compared to the number of peptides with 4+ and 5+ charges, suggesting there is a weakness in identifying 3+ peptides confidently possibly caused by poor peak detection. In order to check the confidence in charge state assignment we instructed the Mascot search engine to consider all charge states between 2+ and 7+ for each submitted ETD spectrum. The peptide sequence that was assigned with the highest Mascot score was assumed to indicate the correct charge state. For the identifications we set an ion score cut-off of 60 to solidify our assumption, which allowed a False Discovery Rate (FDR) of below 0.3 % for all peptide rich fractions as determined by the use of a decoy database search (Supplementary figure 1). Figure 2B and C summarize charge state trends detected by Mascot based discrimination. Figure 2B indicates the charge states for all peptides sequenced by CID, whereas figure 2C contains the analogous data obtained from the ETD analysis. From the data presented in figure 2C it is apparent that, indeed, the higher number of 6+ charge states assigned by the Bioworks Charger program were erroneous. When the 6+ and 7+ charged peptides are removed from the Charger results (supplementary figure 2), the results show improved, though not perfect, agreement with the data presented in Figure 2C. Our analysis reveals that for all peptides successfully identified charges of up to 4+ are detected frequently but higher charge states are much less common. The higher number of identified peptides in lower fractions for CID as compared to the higher number of identified peptides in higher SCX fractions (Figure 2B and 2C) indicate that CID has a preference for 2+ peptide precursor ions, whereas ETD is relatively more successful in sequencing higher charge state precursor peptide ions.

Comparison of performance between ETD and CID

In order to compare the performance between the ETD and CID methods we calculated the number of unique peptides identified in each SCX fraction. We separated the identified peptides (Mascot peptide score > 60) into three categories (Figure 3); peptides exclusively identified by CID (blue), peptides exclusively identified by ETD (red), and peptides mutually identified by both CID and ETD (green). We also analyzed data using a cut-off of 40, which is presented in Supplementary Figure 1B. From Figure 3A it

can be observed there are approximately three broad maxima in the total peptide numbers observed over the full SCX run around fractions 13/14, around fractions 18/19, and around fractions 25 to 29. These maxima correspond most likely to the elution profiles of differentially charged peptides in the SCX separation i.e. the 2+, 3+ and >4+ peptides, conforming to the results shown in Figures 2B and 2C. Around the first maximum (i.e. 2+ net charge) many more unique peptides are found with CID compared to ETD. Moreover, ETD adds little to the overall number of peptide identifications for these SCX fractions (note: supplemental activation was applied for the 2+ peptides analyzed by ETD). SCX fractions, containing 3+ peptides, 17 onwards, show a significant increase in peptides identified with ETD and from fraction 25 ETD outperforms CID where by using only ETD data, 90% of the total number of peptides identifications can be attained. It should be noted that ETD peptide fragment ions originating from 3+ peptides will have a maximum charge of 2+ while CID fragments from a similar precursor will have a maximum charge of 3+ thus making CID spectra potentially more difficult to interpret using an LTQ which has a limited resolving power. Figure 3B shows the cumulative identification of peptides over all SCX fractions revealing that the total number of peptides identified is dominated by CID in the early fractions and by ETD in the later SCX fractions.

The overall result is 6710 spectral annotations (peptide identifications), with a MASCOT score cut-off of 60. CID uniquely identified 2309, while ETD obtained 3040 unique identifications and 1361 were identified by both CID and ETD (see also supplementary Figure 4). Interestingly, in a recent comparative study of CID and ETD Molina et al. (2008) (Molina et al., 2008) reported that the use of ETD hardly added to the number of peptide identifications already made through CID, i.e. CID outperformed ETD as far as number of peptide identification were concerned. This apparent discrepancy between these results and ours, wherein ETD identifies more peptides than CID, can be largely explained by the differences in experimental design. In the present work, by using SCX as peptide pre-fractionation technique, a more targeted analysis was performed towards sequencing of highly charged, larger peptides, which we show are more successfully sequenced by ETD. However, observed differences in the outcome of these two experiments may also originate from the different search engines used for peptide identifications (i.e. Spectrum Mill versus Mascot) and different mass spectrometers. Our findings are actually more consistent with the results reported by Good et al. (2007); (Good et al., 2007; Molina et al., 2008).

When switching to the context of unique protein identifications which we based upon the identification of a minimum of two unique peptides, the numbers for ETD, CID and the overlapping group are 327, 277 and 247 respectively (Figure 3C). It is clear that the trend observed

for peptides identifications is reflected in protein identifications.

We delved further into the data and determined the basic residue (Arginine, Lysine and Histidine) occurrence in the

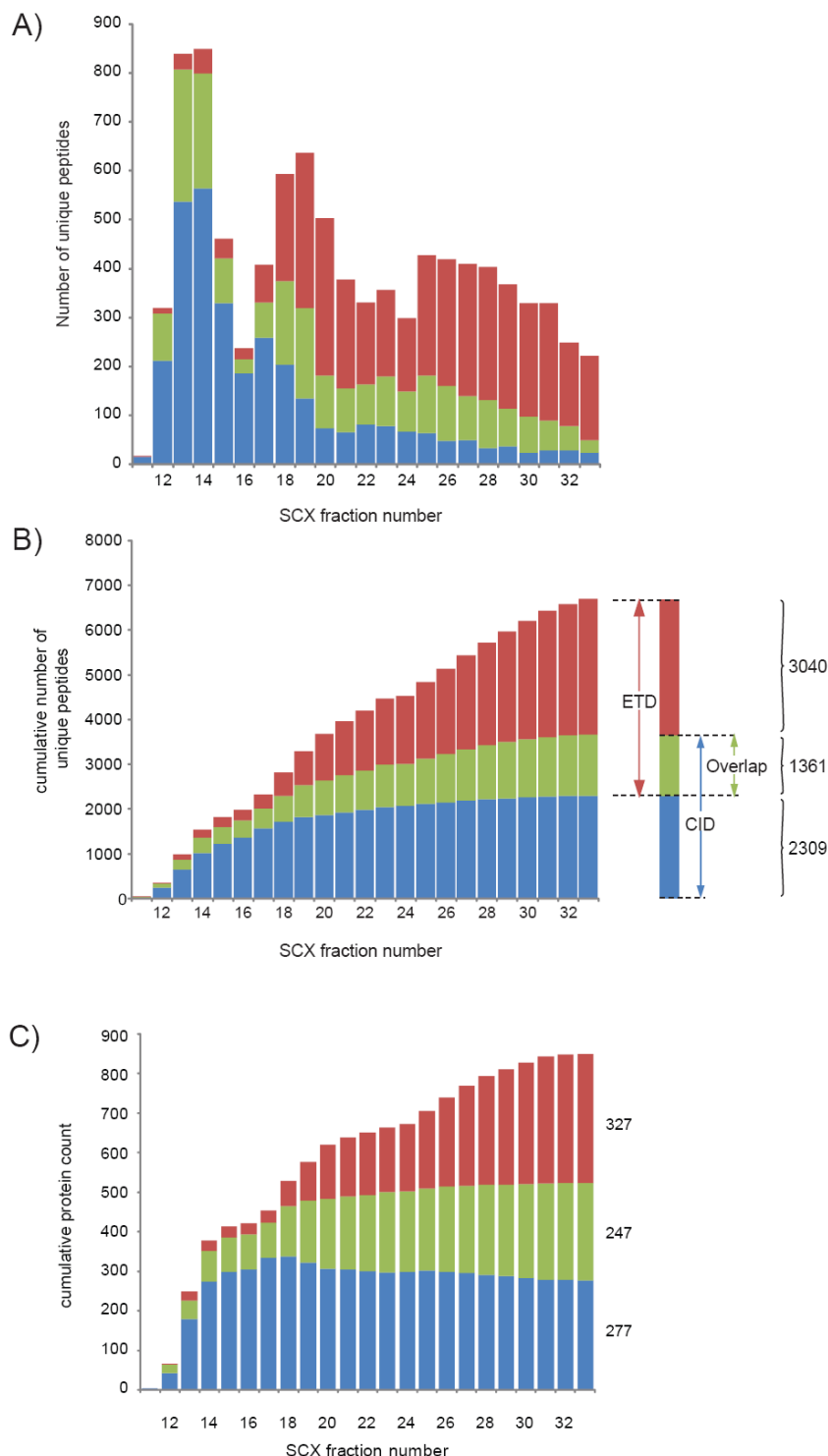


Figure 3: ETD and CID are complementary. (A) Number of unique peptide identifications per SCX fraction. The number of peptides exclusively identified with CID are in blue, the number of peptides exclusively identified with ETD are in red, and the number of peptides identified with both activation methods are in green. (B) The cumulative number of unique peptides identified based on all SCX fractions. On the right the accumulated peptide numbers are shown. The absolute number of identified peptides in each individual SCX fraction is listed in supplementary Figure 4. (C) The cumulative number of unique proteins over all SCX fractions.

sequenced peptides with respect to SCX fraction with the expectation to find an increasing number of basic residues in the later SCX fractions. In Figure 4A and 4B the results of this analysis is provided for the peptides identified by CID and ETD, respectively. Although the data for the CID

and ETD sets show some resemblance, there are also some significant differences observed. For instance, as expected, ETD fails to identify peptides with no basic residues, contrary to CID which is able to identify a few in SCX fraction 11. Moreover, in agreement with our charge state analysis (see

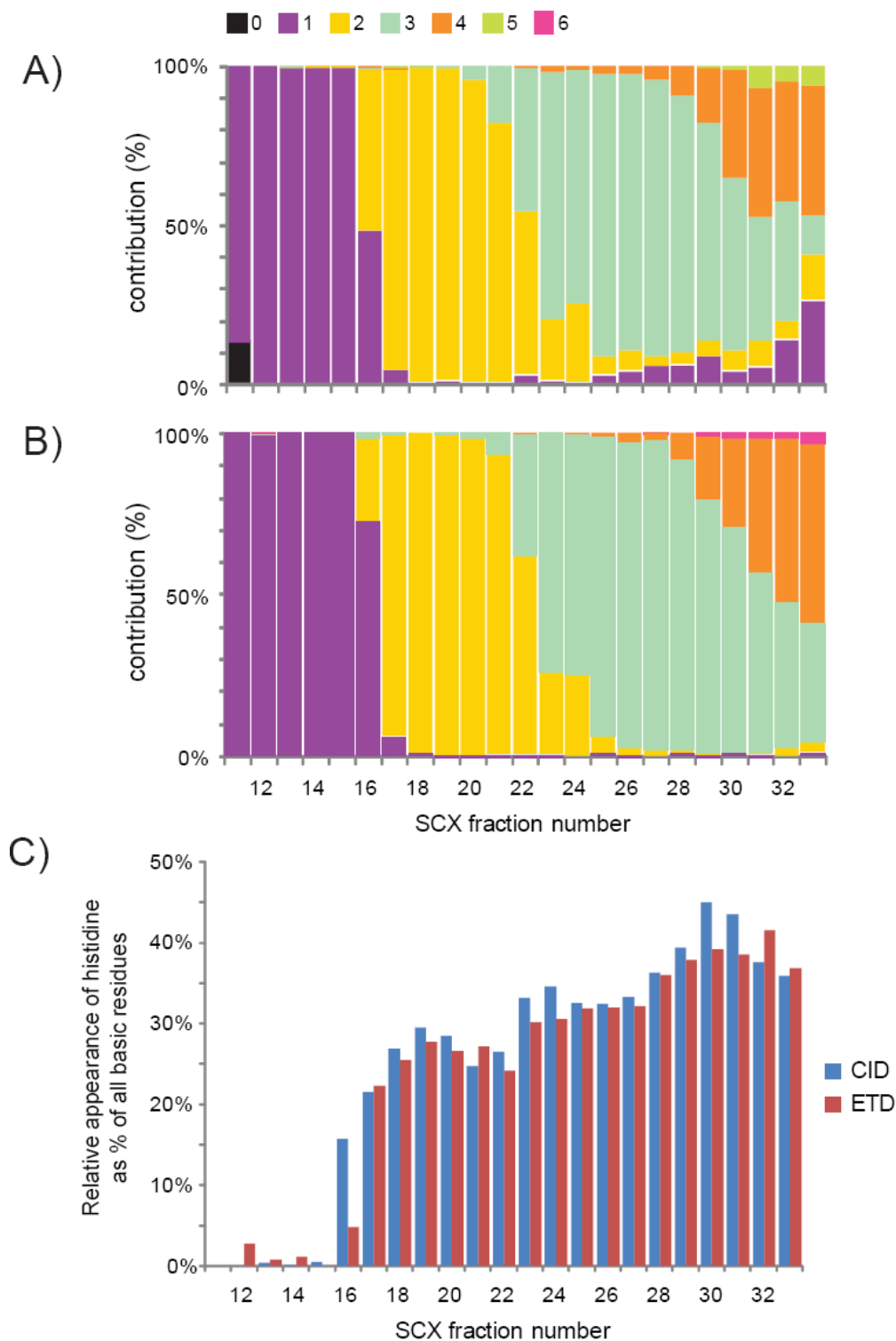


Figure 4: Abundance of basic residues in identified peptides. (A) Relative appearance of basic residues in peptides identified in each SCX fraction by CID. The colors code denotes the number of basic residues per peptide. (B) Relative appearance of basic residues in peptides identified in each SCX fraction by ETD. (C) Relative appearance of histidine basic residues as % of all basic residues in peptides identified in each SCX fraction by CID (blue) and ETD (red). The absolute number of identified peptides in each individual SCX fraction is listed in the supplementary Figure 4.

Figure 2), the contribution of peptides with an increasing number of basic residues gradually increases over the SCX run. We observed a marked increase in the appearance of a basic Histidine in the identified peptide sequences from SCX fraction 16/17 onwards (see figure 4C), which we believe is responsible, alongside Arginine and Lysine, for the distinctive shift in the peptide net charge and the observed peptide charge within the mass spectrometer found for these SCX fractions. The CID data (figure 4A) indicates initially a decrease in the number of peptides with 1 and 2 basic residues and then a steady increase in the number of peptides with 1 and 2 basic residues which is in contrast with the apparent clear separation and observed increase of peptide net charge for the SCX chromatography. These identifications might be false positives or artefacts of the SCX method. It should be noted that in these fractions the total number of identified peptides is rather low, making conclusive remarks about these fractions statistically less valid. Incidentally, results obtained with a lower Mascot score cut-off indicate such peptides are still present in the later fractions however, it can be seen that the clear patterns as found in figure 2A and 2B are gradually lost (Supplementary data figure 3) with decreasing score threshold, indicating that the number of false positives likely increases.

Conclusion

Although suggested previously, it is very apparent, from our data, that CID and ETD identify largely complementary peptide data sets. Whereas smaller, less basic tryptic peptides are ideally sequenced by CID, larger and more basic peptides with higher charges are more readily sequenced by ETD. As shown here, these general characteristics make SCX an ideal pre-fractionation method for the peptides, as this peptide separation is largely based on charge. Our data indicate that the most efficient and simple use of sequencing time can be achieved when a switch is made between CID and ETD from the SCX fractions wherein peptides with 3 or more charges become dominant. The complementarities of the two techniques in conjunction with SCX is also reflected when observed in the context of protein identifications. When instruments with an inherent higher MS resolution with both CID and ETD capabilities become more readily available (Meiring et al., 2002), decision making on the charge state should be made “on-the-fly”, making it easier to decide on the activation method to be used. However, when the peptide separation power in SCX is further improved, so that fractions of different charge states could be baseline resolved, such an on-the-fly assessment of the charge is not really required.

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