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Dataset Brief

Proteomics of mouse liver microsomes: Performance of different protein separation workflows for LC-MS/MS

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The mouse liver microsome proteome was investigated using ion trap MS combined with three separation workflows including SDS-PAGE followed by reverse-phase LC of in-gel protein digestions (519 proteins identified); 2-D-LC of protein digestion (1410 proteins); whole protein separation on mRP heat-stable column followed by 2-D-LC of protein digestions from each fraction (3-D-LC; 3703 proteins). The higher number of proteins identified in the workflow corresponded to the lesser percentage of run-to-run reproducibility. Gel-based method yielded a number of predicted membrane proteins similar to LC-based workflows.

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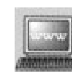

Keywords:
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Proteomics in its state-of-the-art already collected a lot of data sets derived from different sources of biomaterial. After a potency of different proteomics methodologies was established by inventory studies, the next task arises, which includes a selection of the best method for quantitative comparison between series of biological samples. As it is illustrated by several studies [1, 2], the best choice for mammalian liver microsome (LM) proteomics is a set of LC-MS-based pipelines. In order to make a background for comparative experiments on mouse LMs that are a traditional subject of our expertize [3], herein we compared three LC-MS-based proteomic techniques in terms of a number of identified proteins, run-to-run reproducibility and ability to detect membrane proteins. The latter, such as cytochromes P450 were often in a focus of clinical and functional mammalian liver studies [4]. Namely, we studied a performance of separation procedures since all MS measurements were conducted by ESI ion trap mass-spectrometer (MSD Trap, Agilent, USA). Probed separation techniques included (i) SDS-PAGE followed by slice trypsinolysis with reverse-phase HPLC-MS of resultant digests; (ii) 2-D-HPLC-MS of microsome proteome digest; and (iii) 3-D-LC-MS of the same where at first stage of procedure whole proteins were separated by heat-stable mRP HPLC (Agilent). A data set of proteomes derived by above procedures illustrates characteristic features of each of them and may help the investigator to select the best approach for both label-free and label-based quantitative proteomics. Special attention was paid to the ability of each separation scheme to find proteins with predicted transmembrane domains.

Male albino mice housed under specific-pathogen-free conditions were studied at the age of 3 months. All experimental protocols were approved by the local Animal Research Committee of the Institute of Biomedical Chemistry, Russian Academy of Medical Science. Nine mice were injected intra-peritonially with sodium phenobarbital in saline (3.2 mg/mL, 80 mg/kg) for cytochrome P450 induction. Twenty-four hours after the last injection, mice were sacrificed and livers were perfused with ice-cold isotonic KCl. Each three murine livers were pooled to obtain three triplicate microsomal samples for three runs of each separation method. Liver homogenates were prepared in potassium phosphate buffer (0.1 M, pH 7.4) and subfractionated by differential ultra-centrifugation at $105\,000 \times g$ and 4°C essentially as described previously [3] to obtain the

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Abbreviation: LM, liver microsomes

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pellet consisting of microsome ghosts. In all these studies, protein concentration was determined by the bicinchoninic acid method [5].

Electrophoretic separation (SDS-PAGE-based method) of microsomal proteins was carried out on a 1 mm thick 8×10 cm gel prepared at 10% acrylamide. A 20 μg portion of total protein in Laemmli sample buffer was loaded into a 7 mm well of the gel and separated at 20 mA. After protein separation, the entire sample lane was cut into 24 sequential slices of about 1.5 mm thickness. Proteins in each gel slice were destained and trypsin-digested using the protocol described previously [6].

Tryptic peptides were extracted by addition of 15 μL extraction solution (5% ACN, 0.5% formic acid) for 30 min and analyzed by LC-MS/MS. Reverse-phase nano-LC-MS/MS was performed using an Agilent 1100 nanoflow HPLC-Chip Cube system coupled to Agilent 1100 XCT Ultra Series MSD Trap (Agilent, USA). Tryptic peptides were separated on HPLC Chip (40 nL trap column, 75 μm $\times$ 43 mm analytical column, 5 μm C-18 SB-ZX, Agilent) using a linear gradient of 5–80% ACN in 0.1% formic acid over 60 min at flow rate 300 nL/min and detected by the ion trap in 300–1800 m/z range following the supplier's recommendations. Mass spectra were acquired in positive-ion mode with automated data-dependent MS/MS on the five most intense ions from precursor MS scans.

Protein identification was performed using Spectrum Mill MS Proteomics Workbench Rev A.03.03.078 (Agilent). Protein identifications were obtained by processing experimental data *versus* Swiss Prot mouse subset database, Release 56.6 of 16-December-2008. Search parameters were as follows: trypsin was used as the cutting enzyme, mass tolerance for the monoisotopic peptide window was set to ± 200 ppm, the MS/MS tolerance window was set to ± 0.5 Da and two missed cleavages were allowed. Cysteine acrylamide modification and oxidized methionine were chosen as variable modifications. The criteria of positive identification were set as following: 60% minimum scored peak intensity, δ-forward-reverse score >2; at least two peptides identifications with a confident score >7 and summarized protein score >14.

2-D-LC as the second method of the separation was performed as follows. Proteins were extracted using chloroform-methanol mixture, modified by reduction with 0.02 M dithiothreitol in 6M guanidine hydrochloride, pH 6.8 (1 h, 37°C), and subsequent carboxymethylation of SH groups with 0.17 M iodoacetamide (1 h, 37°C) [7]. After the same extraction procedure, the protein sediment was dissolved in 50 mM ammonium hydrocarbonate and sonicated in the sonication bath for 15 min at 4°C. After trypsinolysis (1 μg of enzyme/100 μg of sample protein, 37°C, 1 h; then 2/100 μg of sample protein, 37°C, overnight) formic acid was added to the preparations up to 0.1%. Preparations were then evaporated using Vacuum Concentrator 5301 (Eppendorf, Germany), dissolved in 0.1% formic acid and analyzed by LC-MS/MS. Two microliters of peptide mixtures, obtained from the digestion of the protein samples, were analyzed by 2-D nano-LC coupled with an ion trap mass spectrometer, using an Agilent 1100 nanoflow HPLC-Chip Cube system coupled to Agilent 1100 XCT Ultra Series MSD Trap (Agilent). Peptide mixtures were first separated by means of ion-exchange chromatography (Bio-SCXII column, 3.5 μm, 0.3 id $\times$ 50 mm, Zorbax, Agilent) sequentially using 10 mM, 25 mM, 50 mM, 0.2 M, 0.5 M, and 1 M ammonium formiate. Each salt step was on-line loaded onto the reversed phase 40 nL trapping column at a flow rate of 2 μL/min and then analyzed by LC-MS/MS which was performed as described above, as well as protein identification.

The 3-D-LC procedure was made using a specific heat-stable mRP-column as the first LC step. Reversed-phase separations of whole microsomal proteins were performed on Agilent 1100 LC system using an mRP-C18 column (4.6 mm $\times$ 50 mm, Agilent). The separations were archived by combined multisegmented and linear elution gradient exactly as described in [8] at column heating at 80°C. The gradient flow rate was 0.75 mL/min and detection was monitored at 280 nm. HPLC fractions were automatically collected by time into 1.5 mL plastic tubes, combined based on UV absorbance at 280 nm in four fractions and dried in a Vacuum Concentrator 5301 (Eppendorf). Each fraction was trypsin digested as described for 2-D-LC method and subjected to subsequent 2-D-LC-MS/MS analysis as mentioned above. As a result, protein identifications obtained for each separation workflow in triplicates were

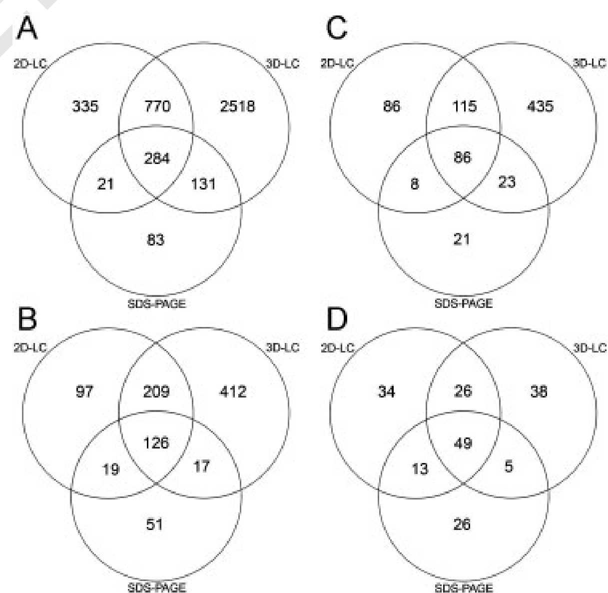

Figure 1. Venn diagrams comparing protein identification lists delivered by SDS-PAGE, 2-D-LC and 3-D-LC separation procedures. (A) all proteins identified; (B) only proteins identified in three replicates; (C) identified proteins with at least one transmembrane helix predicted [9]; (D) proteins with at least one transmembrane helix predicted and which were identified in three replicates.

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compared with the spectra being recorded using the same conditions of MS and database search being done using the same Spectrum Mill parameters, as described above.

Protein and peptide identification data on project are submitted to PRIDE, the publicly available repository (http://www.ebi.ac.uk/pride/). Accession numbers are 8848–8850, 8851–8853 and 8854–8856 for 2-D-LC-MS data; 3-D-LC-MS data and SDS-PAGE followed by reverse-phase LC data, respectively. The PRIDE Converter application (v1.11 and v1.14; URL http://code.google.com/p/pride-converter/) was used to convert MS data into valid PRIDE xml files.

Total number of proteins identified with selected search conditions in all experiments was as high as 4142. Comparison of data sets generated by three separation procedures illustrated that a majority of proteins (3703) from whole list was identified from mRP-based 3-D-LC-MS (Fig. 1A, Table 1). 2-D-LC-MS and SDS-PAGE-LC-MS yielded 1410 and 519 proteins, respectively. Then we evaluated how reproducible identifications were from one technical run of the separation method to another, because only those proteins that appear in most and, preferably, in all replicates may be used for comparative study. From this point of view, SDS-PAGE-based method performed somewhat better (41% proteins identified in all three runs), than 2-D-LC and 3-D-LC (see Table 1) supporting the evidence that the addition of chromatography steps decreases overall reproducibility. At the same time, an absolute number of proteins identified by the gel-based technique remained notably smaller (Table 1).

Another feature of the methods in the study was their ability to identify proteins with transmembrane domains. Since an annotation of proteins in available public resources usually contains their intracellular location without specific indication that this protein is actually embedded to the membrane, we used one of the known methods of transmembrane helix prediction [9](http://www.cbs.dtu.dk/services/TMHMM-2.0/). According to the prediction, a ratio of membrane proteins among identified lists tended to be increased in SDS-PAGE method (Table 1). This fact is especially observed in the sets of proteins reproducibly

Table 1. Performance of SDS-PAGE, 2D-LC and 3D(mRP)-LC separation workflows coupled with ESI Ion Trap MS

Separation method	Number of proteins identified	Number of proteins identified in three runs of each method	Reproducibility[a)]	Number of proteins with predicted membrane helices[b)] (Ratio)	Number of proteins with predicted membrane helices in three runs (Ratio)
SDS-PAGE	519	213	41%	138 (27%)	93 (44%)
2-D-LC	1410	451	32%	295 (21%)	122 (27%)
3-D-LC	3703	764	21%	659 (18%)	118 (15%)

a) Ratio between proteins identified in three technical runs and total number of proteins.
b) Transmembrane helices were predicted at http://www.cbs.dtu.dk/services/TMHMM-2.0/[9]. The protein was accounted if it contained at least one predicted transmembrane helix.
Run-to-run reproducibility of protein sets and a proportion of proteins with predicted membrane domain.

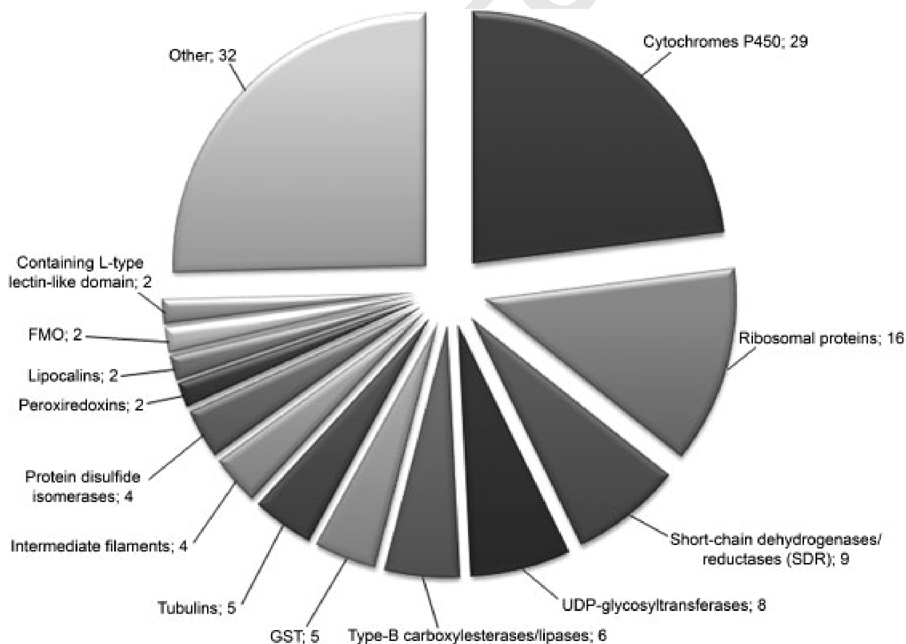

Figure 2. Protein superfamily and family distribution in the list of 126 proteins identified in all three runs of SDS-PAGE, 2-D-LC and 3-D-LC separation procedures. Proteins are classified according the system published in www.uniprot.org, except the ribosomal proteins that belong to different families and combined here for better appreciation. FMO, flavin-containing monooxygenases.

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found in all three runs of each method (Fig. 1D; Table 1). Gel-based procedure surprisingly could identify an absolute number of such proteins (93) closely comparable with such numbers for 2-D-LC (122) and 3-D-LC (118).

Having no reason to analyze differentially expressed proteins we paid some attention to identities of those proteins that formed a subset always identified by all methods in all runs (126 gene products). The proteins of this list are expected to be identified in any proteomics study of mouse LMs (see the distribution of proteins in Fig. 2). Of them, the most widely presented group was a cytochrome P450 superfamily, a fact, which would be explained by use of drug-induced liver for this study. This also supports the opinion that LC-MS-based proteomics is a good tool for simultaneous measurements of cytochromes P450 in different biosamples [10]. Remarkably, GSTs, tubulins, protein disulfide isomerases, peroxiredoxins, carbonic anhydrase, calreticulin and ATP synthase-β subunit registered in this core proteome subset (Fig. 2) are also found in rodent 'hit-parade' of most often identified differentially expressed proteins [11]. This observation again supports the opinion that differentially expressed proteins identified in many proteomics papers relate to most abundant and MS-compatible protein species.

Presented proteome results obtained from mouse LMs by three separation methods followed by ESI-Ion Trap MS can lead to conclusion that the SDS-PAGE separation has not lost importance despite its simple handling and a lesser potency in terms of an absolute number of identified proteins. The gel-based procedure is especially useful in context of membrane proteins and sometimes may be recommended as a method of choice for following LC-MS. Another advantage of this method is the fact that tryptic peptides united to proteins after algorithm-based calculations are a priori co-localized in the same slice [12]. Thus, a validity of identification according to the SDS-PAGE procedures is somewhat higher than that in methods without extensive separation of whole proteins.

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The authors have declared no conflict of interest.

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