ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 2, pp. 153-161. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © A. V. Lisitsa, N. A. Petushkova, I. P. Nikitin, V. G. Zgoda, I. I. Karuzina, S. A. Moshkovskii, O. V. Larina, O. G. Skipenko, L. O. Polyschuk, H. Thiele, A. I. Archakov, 2009, published in Biokhimiya, 2009, Vol. 74, No. 2, pp. 190-200.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-186, December 28, 2008.

# One-Dimensional Proteomic Mapping of Human Liver Cytochromes P450

**A. V. Lisitsa[1], N. A. Petushkova[1]*, I. P. Nikitin[1], V. G. Zgoda[1], I. I. Karuzina[1], S. A. Moshkovskii[1], O. V. Larina[1], O. G. Skipenko[2], L. O. Polyschuk[2], H. Thiele[3], and A. I. Archakov[1]**

*[1]Orekhovich Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, ul. Pogodinskaya 10, 119121 Moscow, Russia; fax: (495) 245-0857; E-mail: Natalia.Petushkova@ibmc.msk.ru*
*[2]National Research Center of Surgery, Russian Academy of Medical Sciences, Abrikosovsky Pereulok 2, 119992 Moscow, Russia*
*[3]Bruker Daltonik GmbH, Fahrenheitstrasse 4, Bremen, 28359 Germany; E-mail: ht@bdal.de*

Received May 29, 2008
Revision received June 18, 2008

**Abstract**—A method for constructing one-dimensional proteomic maps (1D-PM) based on mass spectrometric identification of proteins from adjacent slices of one-dimensional electrophoregram has been developed. For the proteomic mapping, gel lanes were sectioned into slices less than 0.2 mm thick and each slice was subjected to enzymatic hydrolysis. The resultant mixture of peptide fragments was analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) and liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS). Proteins were identified by the mass spectra obtained. Data on peptide fragments and corresponding identified proteins were presented as a 1D-PM. Proteomic maps were constructed by assigning individual proteins to gel slices based on number of matching peptides in a corresponding MS-data. On 1D-PM of human liver microsomal fraction, 18 proteins were identified in the region of 40-65 kDa. These included 12 membrane proteins belonging to the superfamily of cytochromes P450. Pooling of mass spectrometric data, obtained from several adjacent gel slices (molecular zooming) increased sequence coverage of CYP2A (cytochrome P450 family 2A). The maximal coverage of 66% significantly exceeded the level of 48% that could be obtained using one (even the most informative) slice. This method can be applied to the proteomic profiling of membrane-bound proteins.

**DOI**: 10.1134/S0006297909020059

*Key words*: proteomics, one-dimensional gel electrophoresis, molecular zooming, mass spectrometry, membrane-bound protein identification

The major task of proteomics consists of qualitative and quantitative analysis of proteins in a biological sample [1]. The approach based on a combination of protein separation by means of two-dimensional gel electrophoresis (2DE) followed by subsequent identification of proteins by peptide mass fingerprint (PMF) has been developed in proteomic studies for more than 10 years. The peptide mass fingerprint characterizes the spectrum of products of tryptic hydrolysis of proteins in gels (protein spots are excised from the gel) obtained by means of matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS). The resulting peptide mass spectra are then used for protein identification using programs for search in databases of decoded genomes [2-6]. However, the procedure of 2DE is poorly applicable for membrane proteins, which are basically insoluble under conditions required for isoelectrofocusing [7, 8]. Recently, a new proteomic strategy has been developed. Membrane (e.g. microsomal) proteins are separated by one-dimensional gel electrophoresis (1DE). The latter has evident advantage because membrane proteins are

*Abbreviations*: 1DE, one-dimensional gel electrophoresis; 2DE, two-dimensional gel electrophoresis; 1D-PM, one-dimensional proteomic maps; HLMG, human liver microsomal ghosts; LC-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption time-of-flight mass spectrometry; MS, mass spectrometry; PMF, peptide mass fingerprint.

* To whom correspondence should be addressed.

soluble in a buffer with SDS and the degree of protein separation allows identifying up to 20 proteins per gel segment [8-10]. A gel lane is then sectioned into segments (16 × 3 mm), each of which is subjected to tryptic hydrolysis, and the resulting mixture of peptide fragments is then analyzed by mass spectrometry (MS).

A new generation of proteomic methods for analysis of biomolecules is based on mass spectrometry with electrospray ionization of an analyzed sample [11]. Inventory of proteomes, including those containing membrane proteins, employs combination of the methods of one-dimensional electrophoresis (1DE) and high performance liquid chromatography coupled to a mass detector based on an ion trap (LC-MS/MS) [8-10, 12].

Although using the method of 1DE it is possible to separate proteins simultaneously on several lanes of a 1DE gel, its resolution capacity is lower than that of 2DE because of complexity of a protein mixture used for analysis [13]. For treatment of large massifs of mass spectrometric information obtained during protein separation by 1DE, the following approach has been proposed: a gel lane is sectioned into overlapping slices and protein profiles refer to types of tissue, gel number, or slice position on the gel [13]. Using the two latter modes of data treatment, it is possible to take into consideration systemic errors and to isolate significant areas on the 1DE gel.

In this study, we have investigated the possibility of 1DE proteomic mapping (1DE-PM) based on mass spectrometry data obtained using various segments of one-dimensional electrophoregrams. Using this approach, we have separated a complex protein mixture and represented each components of this mixture as a separate protein profile [14]. The superfamily of cytochromes P450 (CYPs), particularly, enzymes of CYP2A subfamily of human liver microsomes, were chosen as the research object for several reasons. Enzyme immunoassay revealed that (using tissue samples sufficient for proteomic studies) more than 20 CYPs are expressed by human liver [15] and the following forms represent 70% of the total amount of this heme protein: 1A2, 2A6, 2B6, 2C8/9/18/19, 2D6, 2E1, and 3A4/5 [15]. Members of CYP2A superfamily are the most abundant among them (these CYPs represent 30% of the total amount of cytochromes P450). In a practical aspect, cytochromes P450 of these families are especially interesting because they are involved in metabolism of more than 60% of all drugs. Hepatic cytochromes P450 are hydrophobic membrane proteins that precipitate at the stage of isoelectrofocusing in attempts to separate them by 2DE [16]. During separation by means of 1DE, all forms of cytochrome P450 are positioned within a rather narrow gel region [16] because many forms share significant similarity in amino acid sequence [17] and have basically identical molecular masses.

Using one-dimensional proteomic mapping, it was possible to refer mass spectrometric data to protein localization of the lane of a 1D gel. The principle of 1D-PM is based on scaling of a gel lane by molecular masses or peptide sequences of the separated proteins. The region of the electrophoregram lane (40-65 kDa corresponding to molecular masses of CYPs) was sequentially sectioned into 40 slices, and each slice was then subjected to trypsinolysis and MS analysis. The use of matrix-assisted and electrospray methods of peptide ionization provided additional advantages to the one-dimensional mapping.

## MATERIALS AND METHODS

**Materials.** The following reagents were used in this study: phenylmethylsulfonyl fluoride, 2,5-dihydrobenzoic acid, Tris, EDTA, NADPH, dithionite, trypsin, and sodium deoxycholate from Sigma-Aldrich (USA); acetonitrile and trifluoroacetic acid from ICN (USA); Coomassie Brilliant Blue G-250 from Fluka (Germany); mercaptoethanol, dithiothreitol, SDS, glycerol, Bromophenol Blue, and ammonium bicarbonate from Acros Organics (USA). Other reagents of chemically pure and pure for analysis grades were produced by domestic suppliers.

Morphologically unchanged pieces of human liver obtained by resection during surgical treatment of liver were used as the material for this study. Four samples (of 8-10 g) were obtained from the Department of Pathological Anatomy, Russian Research Center for Surgery, Russian Academy of Medical Sciences. Human liver microsomes were isolated from the surgical material within 30 min after excision by differential centrifugation. Additionally purified preparations of microsomal membranes (ghosts) were obtained as described previously [18]. Protein was determined by the method of Bradford [19] using bovine serum albumin as a standard.

**Electrophoresis of proteins of human liver microsomal ghosts (HLMG).** For separation of proteins by 1DE, HLMG samples (20 μg of protein) were diluted with buffer containing 0.06 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, mercaptoethanol, and Bromophenol Blue used at a ratio 1 : 10 (v/v). Proteins were separated using a Mini-Protean III Cell (Bio-Rad, USA). After protein separation by mass, the gels were stained with Coomassie Brilliant Blue as described earlier [20].

**Tryptic hydrolysis of proteins in polyacrylamide gel** stained with Coomassie Brilliant Blue G-250 was carried out as described earlier [20]. The region of the gel lane of 40-65 kDa (molecular masses of cytochromes P450) was initially frozen and then sectioned into thin (about 0.2 mm) slices using a manual microtome. Each slice (about 40 in total for the selected range of molecular masses; Fig. 1, a and b) was then treated as an independent sample. These slices were washed with water three times and then were destained by incubating in a mixture

containing 50% acetonitrile (v/v) in 100 mM ammonium bicarbonate, pH 8.9, at 56°C for 20 min and then in 100% acetonitrile for 20 min. After acetonitrile removal, gels were dried and treated with 5-8 μl of trypsin solution (25 ng/μl of modified trypsin in 50 mM ammonium bicarbonate) (depending on initial size of the gel slice) at 37°C for 12 h. After trypsinolysis, a 15-μl aliquot of mixture containing 5% acetonitrile in water and 0.5% formic acid was added and the solution of peptides layered over the gel was taken for mass spectrometric analysis.

**Time-of-flight mass spectrometry.** A mixture of proteolytic peptides extracted from gel (0.3 μl) was mixed with an equal volume of α-cyano-4-hydroxycinnamic acid (a saturated solution prepared using 0.5% trifluoroacetic in 50% aqueous acetonitrile, which was then diluted 2-fold). The resulting mixture was applied on five positions of a mass spectrometry AnchorChip target (Bruker Daltonics, Germany) and dried in air.

Mass spectra were recorded in the reflex mode using an accelerating voltage of 25 kV on a Bruker Ultraflex spectrometer (Bruker Daltonics) equipped with the delayed extraction system Bruker PAN$^{TM}$. Resulting spectra were processed using the Bruker FlexAnalysis 2.2 software and the SNAP option as the peak detection algorithm. The mass spectra were treated in the data warehousing and bioinformatics information system ProteinScape v.1.2 (Bruker Daltonics) using the 200 most intense peaks. Proteins were identified by peptide mass fingerprint (PMF) databases search using the Mascot (Matrix Science, USA) and ProFound (Proteometrix, USA) programs. The accuracy of mass detection $MH^+$ was 0.2 Da assuming the possibility of methionine oxidation and modification of cysteine residues by acrylamide. The range of allowable protein masses was 40-100 kDa (for ProFound). The search employed the NCBI database (http://www.ncbi.nlm.nih.gov/sites/gquery).

**Liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS)** was carried out using a nanoflow high performance liquid chromatography coupled with an Agilent 1100 SL Series MSD Trap ion-trap (Agilent Technologies, USA). Chromatographic separation of peptides was achieved by means of a linear gradient (5-80%) of acetonitrile in 0.1% formic acid (for 40-60 min) at the flow rate of 2 nl/min using a 180 μm capillary column. Detection employed an ion-trap in the range of m/z 200-1800. Proteins were identified by database search using Mascot and the following search parameters: accuracy of determination of mass peptide ions ±1.5 Da, possibility of methionine oxidation and modification of cysteine residues by acrylamide.

**Proteome mapping.** One-dimensional proteome maps of HLMG proteins were analyzed using the 1D-ZOOMER software (http://projects.ibmh.msk.su/oldzoomer/projects/hlm2004/guest.pl) by treating more than 200 time-of-flight and 60 chromato-mass spectra. The proteome map contained proteins reliably identified by the search systems. The 1D-PM is presented as a table that consists of horizontal rows (each of which corresponds to one protein or a group of proteins) and columns representing the sequential number of the excised slice of the gel (Table 1). Values in the cells reflected either peak number in the fingerprint of peptide masses coinciding with the list of masses for theoretical proteolysis of a particular protein (the PMF-index in the

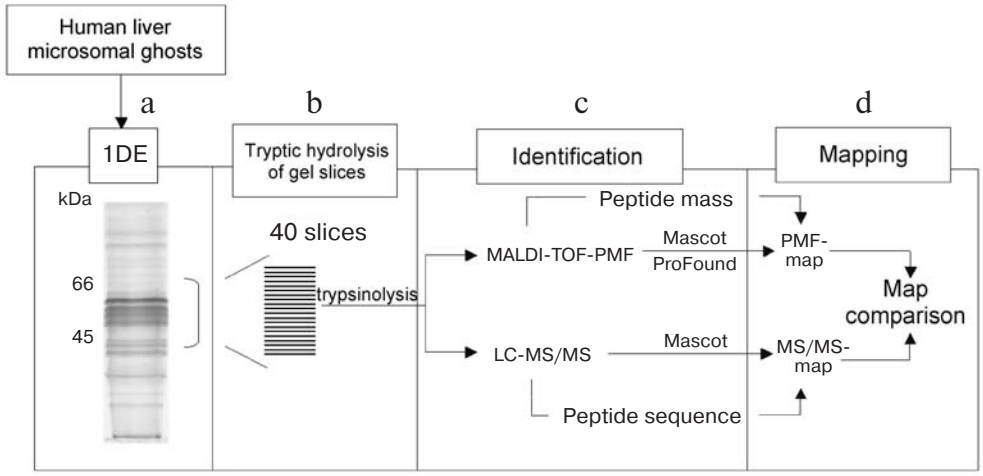


**Fig. 1.** Scheme of one-dimensional proteome mapping of cytochromes P450. a) Separation of liver microsomal ghost proteins by one dimensional electrophoresis; b) sectioning of the region (40-65 kDa) of the 1D-electrophoregram into 40 slices using a manual microtome and tryptic hydrolysis of each gel slice; c) protein identification by liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) and by peptide mass fingerprint (PMF) using matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) data; d) map generation by combining data obtained by the PMF method (PMF-map) or LC-MS/MS (MS/MS-map) with identified proteins and slice numbers.

**Table 1.** One-dimensional proteomic map of human liver microsomal ghost (sample No. 3) generated by MALDI-TOF PMF (a) and LC-MS/MS data (b). Proteins were sorted by decrease in their molecular masses. Cytochromes P450 (CYP) exhibiting amino acid sequence similarity exceeding 80% were pooled into groups by families and defined as CYP3A4/5/7, CYP2A6/7/13, and CYP2C9/10/19. Black background shows values of the index exceeding the mean for all the values placed onto the map by more than one standard deviation. The frame marks cells in which the value of the PMF-index does not reach the level of statistical significance, but MS/MS-data confirm protein identification. Data on 20 (18) of 40 slices are shown in this table. These slices are related to the region of localization of proteins belonging to the cytochrome P450 superfamily

| a | | | | | | | | | | | | | | | | | | | | | | Protein name | kDa | b | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Slice number | | | | | | | | | | | | | | | | | | | | | | | | Slice number | | | | | | | | | | | | | | | | | | | |
| 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | | | | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 |
| 16 | 22 | 20 | 25 | 14 | 1 | 1 | 1 | 1 | 3 | 0 | 1 | 1 | 3 | 1 | 2 | 2 | 3 | 4 | 1 | 1 | | carboxylesterase | 62.5 | 12 | 21 | 25 | 25 | 26 | 3 | 3 | 6 | 5 | 2 | 2 | 1 | 3 | 4 | 2 | 4 | 7 | 5 | 8 |
| 5 | 8 | 3 | 3 | 3 | 1 | 1 | 1 | 0 | 2 | 2 | 3 | 1 | 2 | 1 | 1 | 2 | 1 | 3 | 3 | 0 | | cyp1B1hum | 60.8 | 8 | 5 | 7 | 3 | 8 | 4 | 7 | 9 | 5 | 5 | 4 | 7 | 6 | 8 | 9 | 9 | 7 | 9 | 8 |
| 0 | 0 | 0 | 0 | 2 | 1 | 6 | 7 | 13 | 15 | 7 | 7 | 5 | 4 | 4 | 3 | 3 | 3 | 2 | 2 | 2 | | UDP glycosyltransferase | 60.7 | 7 | 4 | 7 | 6 | 8 | 8 | 13 | 20 | 20 | 23 | 22 | 18 | 9 | 10 | 11 | 9 | 5 | 7 | 10 |
| 2 | 2 | 1 | 3 | 7 | 7 | 15 | 10 | 3 | 4 | 3 | 5 | 4 | 4 | 3 | 2 | 1 | 3 | 4 | 1 | 1 | | flavin monooxygenase | 60.1 | 7 | 6 | 5 | 4 | 14 | 25 | 28 | 19 | 5 | 3 | 6 | 5 | 5 | 5 | 6 | 7 | 7 | 4 | 6 |
| 3 | 4 | 4 | 3 | 2 | 0 | 3 | 4 | 5 | 10 | 6 | 3 | 1 | 3 | 5 | 4 | 4 | 3 | 4 | 0 | 2 | | cyp4F2hum | 59.9 | 5 | 5 | 5 | 8 | 5 | 7 | 4 | 12 | 14 | 14 | 9 | 7 | 6 | 5 | 7 | 10 | 6 | 8 | 10 |
| 2 | 2 | 2 | 4 | 2 | 1 | 3 | 3 | 2 | 4 | 4 | 7 | 6 | 8 | 4 | 3 | 3 | 3 | 2 | 1 | 1 | | cyp4A11hum | 59.6 | 8 | 12 | 9 | 11 | 11 | 10 | 8 | 11 | 10 | 13 | 10 | 11 | 16 | 12 | 9 | 15 | 12 | 11 | 13 |
| 0 | 0 | 0 | 0 | 2 | 3 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 2 | 2 | 2 | 2 | 3 | 2 | 1 | 1 | | cyp1A2hum | 58.3 | 7 | 3 | 8 | 3 | 9 | 7 | 12 | 9 | 8 | 2 | 6 | 8 | 5 | 9 | 5 | 11 | 9 | 6 | 12 |
| 3 | 4 | 5 | 5 | 4 | 1 | 1 | 0 | 2 | 2 | 1 | 3 | 3 | 3 | 3 | 4 | 3 | 4 | 3 | 2 | 5 | | cyp1A1hum | 58.2 | 9 | 5 | 8 | 3 | 9 | 10 | 9 | 10 | 6 | 6 | 5 | 8 | 7 | 5 | 6 | 10 | 8 | 8 | 11 |
| 4 | 1 | 0 | 2 | 2 | 1 | 1 | 1 | 3 | 2 | 2 | 3 | 3 | 4 | 3 | 6 | 3 | 5 | 4 | 1 | 2 | | cyp3A43hum | 57.9 | 2 | 5 | 3 | 4 | 3 | 4 | 0 | 5 | 2 | 6 | 4 | 2 | 6 | 6 | 3 | 5 | 4 | 4 | 7 |
| 9 | 7 | 2 | 4 | 3 | 3 | 4 | 4 | 4 | 8 | 10 | 16 | 19 | 20 | 9 | 8 | 8 | 9 | 9 | 4 | 6 | | cyp3A4/5/7 | 57.3 | 5 | 6 | 11 | 5 | 8 | 9 | 7 | 10 | 8 | 8 | 11 | 13 | 16 | 22 | 8 | 11 | 9 | 10 | 11 |
| 2 | 2 | 2 | 2 | 2 | 1 | 1 | 3 | 3 | 13 | 18 | 16 | 7 | 6 | 4 | 2 | 2 | 3 | 5 | 3 | 4 | | cyp2E1hum | 56.8 | 4 | 4 | 6 | 5 | 6 | 6 | 5 | 8 | 9 | 23 | 29 | 22 | 5 | 6 | 6 | 7 | 4 | 5 | 8 |
| 2 | 3 | 2 | 2 | 1 | 0 | 1 | 1 | 1 | 5 | 3 | 11 | 13 | 12 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | | ATP syntase | 56.6 | 5 | 5 | 4 | 4 | 4 | 5 | 6 | 6 | 5 | 4 | 4 | 13 | 19 | 14 | 5 | 7 | 6 | 7 | 8 |
| 3 | 2 | 3 | 3 | 2 | 2 | 5 | 5 | 5 | 7 | 7 | 8 | 6 | 11 | 15 | 19 | 16 | 3 | 4 | 4 | 6 | | cyp2A6/7/13 | 56.6 | 4 | 7 | 6 | 7 | 9 | 6 | 7 | 6 | 8 | 5 | 8 | 6 | 8 | 19 | 25 | 25 | 22 | 10 | 11 |
| 2 | 3 | 2 | 4 | 2 | 4 | 3 | 2 | 3 | 2 | 4 | 3 | 3 | 8 | 3 | 2 | 0 | 0 | 1 | 3 | 4 | | cyp2D6hum | 55.9 | 6 | 6 | 4 | 6 | 7 | 9 | 9 | 10 | 8 | 6 | 11 | 7 | 8 | 14 | 8 | 10 | 9 | 10 | 10 |
| 5 | 6 | 4 | 7 | 4 | 1 | 2 | 2 | 1 | 8 | 7 | 8 | 6 | 7 | 3 | 2 | 2 | 0 | 2 | 1 | 2 | | cyp2C8hum | 55.8 | 4 | 4 | 4 | 4 | 9 | 4 | 4 | 9 | 11 | 9 | 13 | 7 | 14 | 20 | 5 | 7 | 6 | 8 | 8 |
| 9 | 9 | 9 | 12 | 6 | 0 | 7 | 7 | 11 | 13 | 8 | 6 | 5 | 6 | 7 | 7 | 7 | 7 | 6 | 4 | 6 | | cyp2C9/10/19 | 55.5 | 9 | 7 | 9 | 7 | 10 | 9 | 9 | 23 | 27 | 22 | 20 | 6 | 4 | 9 | 7 | 12 | 8 | 7 | 11 |
| 4 | 4 | 1 | 4 | 3 | 1 | 1 | 1 | 2 | 0 | 1 | 2 | 1 | 3 | 13 | 18 | 22 | 20 | 16 | 4 | 2 | | epoxide hydrolase | 52.9 | 3 | 3 | 5 | 4 | 6 | 5 | 6 | 8 | 5 | 4 | 3 | 3 | 5 | 4 | 25 | 35 | 31 | 30 | 26 |
| 4 | 3 | 4 | 3 | 1 | 0 | 0 | 1 | 1 | 2 | 2 | 3 | 0 | 2 | 2 | 3 | 2 | 3 | 6 | 10 | 10 | | actin | 41.0 | 6 | 4 | 3 | 2 | 4 | 4 | 6 | 4 | 4 | 3 | 5 | 5 | 5 | 4 | 4 | 5 | 5 | 6 | 3 |
| | | | | 60 kDa > | | | | | | | | | | | 50 kDa > | | | | | | | | | | | | | 60 kDa > | | | | | | | | | | | 50 kDa > | | | |

case of MALDI-TOF mass spectrometry) or the number of peptide sequences related to these proteins (the MS/MS-index in the case of tandem spectrometry). Thus, the index values in a cell reflected degree of protein concordance with the mass spectrum obtained from a particular gel slice.

## RESULTS

**Proteomic mapping of human liver microsomal ghosts.** One-dimensional proteomic maps were generated for four samples of HLMG using protein separation in polyacrylamide gel. Depending on sample and electrophoretic conditions, 13-19 separate protein bands were recognized after gel staining (Fig. 1a). The region of 40-65 kDa demonstrated the most intensive staining, where 2-3 main wide bands and several minor bands were recognized. Positioning of each protein on the polyacrylamide gel was determined by sectioning of this region of the gel (Fig. 1b) into thin slices (0.2 mm) and concordance MS-data for this protein with the slice number. Each gel slice was analyzed by MALDI-TOF and LC-MS/MS (Fig. 1c). Searching a sequence database using mass spectrometry data resulted in identification of 14 various forms of cytochrome P450. In addition, we identified flavin monooxygenase, ATP-synthase, epoxide hydrolase, actin, carboxylesterase, and UDP-glycosyltransferase. The four latter proteins were detected in all HLMG samples, and in subsequent experiments they served as markers for detection of cytochrome P450 localization on 1D-PM.

For protein identification in gel slices, we used two mass spectrometric methods. This results in two types of mass spectrometric data (MS-data). In the case of MALDI-TOF, results represented peptide fingerprints, which we defined as PMF-data. In the case of LC-MS/MS, the MS/MS data included a set of peptide sequences identified by mass spectra of secondary peptide fragmentation. For 1D peptide mapping, we have used protein abundance index [22]; this is a quantitative characteristics reflecting concordance between MS-data on each slice and an identified protein. For example, in Table 1 (part a) black background marks the value of PMF-index in the line corresponding to CYP4F2. The value indicates that the time-of-flight mass spectrum of slice No. 29 contained 10 peaks matched with masses of peptides of theoretically calculated products, which would be obtained during CYP4F2 amino acid cleavage by trypsin. On average, the PMF-index included 130 $\pm$ 27 m/z peaks, whereas MS/MS-index contained from 4 to 17 peptide amino acid sequences.

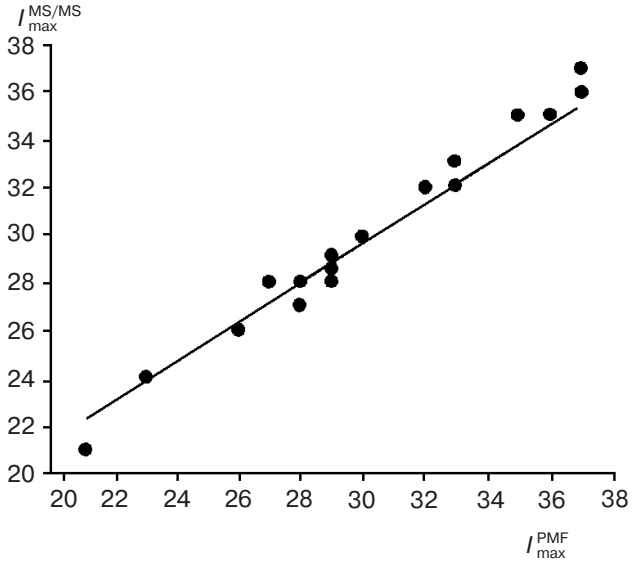


**Fig. 2.** Concordance between one-dimensional proteomic maps generated by PMF- and MS/MS-data. $I_{max}^{PMF}$ and $I_{max}^{MS/MS}$ are section numbers in which the indexes for particular proteins were maximal.

During one-dimensional protein mapping, names of identified proteins were placed in the lines of the middle part of the table depending on the decrease in their molecular masses (Table 1). In the table cells (intersects of gel slice number and protein name), the index value determining concordance between MS-data and a particular protein is indicated. In accordance with the two types of mass spectrometry methods (Fig. 1, c and d), two 1D-PMs were generated for each HLMG sample: the PMF-map (Table 1a) and MS/MS-map (Table 1b). Both types of 1D-PM were characterized by unequivocal distribution of the protein index in gel slices. For example, in the case of epoxide hydrolase (see Table 1a) the first, significantly differing from the background level, PMF-index appeared in slice No. 34, then this index gradually increased, reached the maximal value for this protein in slices No. 36 and 37, and then gradually decreased in subsequent gel slices. Similar dependence on the gel slice number was also observed in the case of the MS/MS-index (Table 1b): the latter reached high values for epoxide hydrolase in the slices No. 33-38. Table 1 also shows that high values of the index marked with black background are located in diagonal cells of the 1D-PM.

**Comparison of 1D-PMs and analysis of their properties.** For estimation of reliability of the results obtained, we compared 1D-PMs based on two types of mass spectrometry data (Table 1, a and b). Figure 2 shows results of this comparative analysis. The gel slice number corresponding to the maximal value of the index is shown for each protein. The abscissa shows the numbers of gel slices in which the PMF-index reaches maximal value, and the ordinate shows the numbers of gel slices with maximal values of the MS/MS-index. Each point on the plot corresponds to one of the identified proteins. For example, in the case of CYP4F2 the PMF-index and the MS/MS-index reached maximal value in the gel slice No. 29 and No. 28, respectively (Table 1). The data points of Fig. 2 approximated a straight line with correlation coefficient 0.87. This suggests that 1D-PMs obtained using PMF- and MS/MS-data were basically consistent. Since one of the correlation parameters is a slice coordinate, one may suggest that 1D-PM contains information about protein localization in the gel lane.

## DISCUSSION

Proteome mapping includes separation of a complex protein mixture for subsequent mass spectrometric identification of proteins. Usually protein separation employs 2DE, which is not applicable for separation of hydrophobic proteins [10]. An alternative method is 1DE, avoiding protein aggregation during isoelectrofocusing. Its evident advantages in separation of hydrophobic proteins are accompanied by loss in resolution, because proteins with similar molecular mass values exhibit similar migration versus the molecular size. This complicates protein identification and quantitative determination by optical density (because it is impossible to assign a particular protein with particular band of the gel).

Insufficient resolution of 1DE is a significant limitation in the case of cytochromes P450, a group of membrane proteins characterized by similarity in amino acid sequences and therefore similar values of their molecular masses. In addition, similarity in amino acid sequences results in identical or similar mass spectra, which cannot discriminate MS-data for structurally related forms of this enzyme.

However, in spite of these difficulties the development of a proteome catalog for members of the cytochrome P450 superfamily is an important task [14, 23, 24]. Cytochromes P450 are responsible for biotransformation of more than 60% of drugs [25]. They are involved in numerous metabolic processes, such as biosynthesis of steroid hormones, bile acids, as well as in formation of unsaturated fatty acids. However, the main physiological role of these enzymes consists in detoxification of xenobiotics [26].

During development of a proteome catalog, it is hard to show preference to a particular method of protein identification. Now the method of LC-MS/MS is widely used for these purposes (including qualitative [27] and quantitative [23] determination of cytochromes P450 in microsomes of animal liver cells). In this study, the mass spectrometric methods based on matrix and electrospray ionization were considered as mutually complementary ones. Using these methods, we have

analyzed the proteome profile of cytochrome P450 in human liver microsomes. Profiling employed the method of 1D-PM [14], which can be considered as a variant of molecular zooming of a gel fragment. Using one-dimensional mapping, we proposed to obtain information on protein localization in a gel and to determine qualitative and quantitative composition of the analyzed sample. On a 1D-PM (Table 1) we put proteins identified by mass spectral search in the database of known sequences by means of standard programs Mascot (www.matrixscience.com) and Profound (prowl.rockefeller.edu) for MALDI-TOF spectra or only Mascot for LC-MS/MS spectra (Fig. 1). Sequential processing of slices resulted in detection and identification of hydrophobic HLMG proteins. In the analyzed gel region corresponding to molecular masses of 40-66 kDa, we identified 18 microsomal membrane proteins. The identified proteins were positioned in the 1D-PM by the decrease in the molecular mass as shown in Table 1 for one of the samples (similar results were obtained for the other three HLMG samples).

In addition to cytochromes P450 (forms 1A1, 1A2, 1B1, 2A6/7/13, 2E1, 2C8, 2C9/10/19, 2D6, 3A4/5/7, 3A43, 4A11, 4F2), this region of the gel lane contained other proteins: epoxide hydrolase, actin, carboxylesterase, and UDP-glycosyltransferase. These proteins were detected in all samples of human liver and were used as "markers" for determination of molecular masses of proteins on the proteomic map. Table 1 shows that the gel region containing cytochromes P450 on the upper side is limited by carboxylesterase (62.5 kDa) and UDP-glycosyltransferase (60.7 kDa), and on the lower side it is limited by epoxide hydrolase (52.9 kDa) and actin (41.0 kDa). The protein identification was statistically significant (by both peptide mass fingerprint and MS/MS-spectra). The numbers of gel slices in which the index of these protein markers reached the highest values allowed assigning the 1D-PM region of molecular masses from 60 to 50 kDa. On a 1D-PM within these limits, there were 11 slices in which all identified forms of cytochrome P450 were positioned (Table 1). Table 1 shows that the highest values of the MS-index were also high (versus other cells in the row) in some adjacent gel slices. For example, in the row of Table 1 defined as CYP2E1 high index values were observed in slices No. 29-31 (Table 1b).

At the initial stage of 1D-PM analysis the highly homologous forms of cytochromes P450 were pooled into three groups: CYP2A6/7/13, CYP2C9/10/19, and CYP3A4/5/7. Amino acid sequence identity exceeded 80% in these groups (http://projects.ibmh.msk.su/cpk), and so the maximal index values corresponding to these proteins were found in one gel slice.

Integration of MS-data obtained using several adjacent slices increased amino acid coverage of a protein localized in these slices. Table 2 (a and b) shows an example of such integration for cytochromes P450 family CYP2A. The rows of Table 2a contain information on mass peptide fragments, and the rows of Table 2b show indentified peptide sequences. Table columns correspond to numbers of gel slices. Background indicates a number of gel slice in which a particular peptide (or its molecular mass) was detected. Table 2 shows that the largest number of peptides was found in gel slice No. 35. Data of peptide mass fingerprints (Table 2a) were not specific for the CYP2A family. For example, the mass of 1356.84 (No. 24, Table 2) marked with an asterisk is typical not only for proteolytic products of sequences of the CYP2A family, but also for cytochrome CYP1B1. In the columns "Accompanying proteins" of Table 2, such "degenerate" masses are marked with the note that they do not belong to the family of CYP2A proteins. In contrast with the molecular mass values, peptide sequences are more specific with respect to the CYP2A family. Table 2b shows that LC-MS/MS-identified peptides longer than seven amino acid residues were detected only in proteins of the CYP2A family. Peptides specific for CYP2A were detected in gel slices No. 33-36, whereas shorter nonspecific peptides (No. 76-89, Table 2b) were basically detected in all analyzed slices.

Data of Table 2 show that integration of the MS-data from adjacent slices increases sequence coverage of particular proteins. The column of Table 2a corresponding to slice No. 35 contained the most peptide masses assigned to proteins of the CYP2A family; however, four mass/charge values (2169.10, 1685.94, 1221.81, and 1956.00 Da) were not found. They were found in adjacent slices numbered 33, 34, and 36. Integration of information obtained from slices 33-36 resulted in 50% coverage, whereas in the most informative slice No. 35 the coverage level was 38%.

A similar situation was also observed for the MS/MS data (Table 2b). For example, slice No. 35 did not contain seven peptides included in Table 2b under the numbers 55, 62, 65, 70, 72, 73, and 74. Inclusion of these peptides from the adjacent slices numbered 33, 34, and 36 resulted in the increase of sequence coverage from 30% (for slice No. 35) to 35%. In general, integration of PMF- and MS/MS-data from the slices numbered 33-36 increased the sequence coverage up to 66%; this significantly exceeds the level of 48% obtained using the single most informative slice No. 35. Similar results were also obtained by Lim et al. [28]; combining two mass spectrometric methods they obtained 70% level of sequence coverage. For other analyzed liver microsomal samples integration of the MS-data from adjacent slices also increased sequence coverage of CYP2A (average value $12 \pm 5\%$).

In some cases peptides identified by peptide mass fingerprint did not overlap (partially or totally) with peptides ionized in the electrospray. The MALDI-TOF method revealed peptides that were not identified by MS/MS-spectra. Thus, combined use of the methods

**Table 2a.** Distribution of peptide masses of cytochromes P450 (family CYP2A) identified by peptide mass fingerprint (MALDI-TOF) in gel slices. The frame marks proteins found in the region of putative localization of proteins of the CYP2A family (i.e. in slices numbered from 33 to 36), CYP2A's masses absent in the slice No. 35 are underlined

| No. | Mass, Da | Slice number | Accompanying proteins, which do not belong to the CYP2A family but which contain peptides with the indicated mass |
|---|---|---|---|
| 1 | 877.31 | 20 | 3A43 |
| 2 | 1341.79 | 20 21 22 23 | |
| 3 | 1451.82 | 20 21 | |
| 4 | 2245.08 | 22 23 24 | |
| 5 | 2960.36 | 22 23 | 2C9/10/19 |
| 6 | 1417.85 | 24 26 27 28 29 30 31 32 33 34 42 | flavin monoxigenase.UDP |
| 7 | 1978.93 | 25 26 27 | flavin monoxigenase |
| 8 | 2181.03 | 25 26 | |
| 9 | 1435.81 | 26 27 28 29 30 32 42 | ATP syntase,2C9/10/19 |
| 10 | 1493.84 | 26 27 28 29 30 31 | UDP,4A11 |
| 11 | 918.59 | 27 | |
| 12 | 666.32 | 28 31 | 3A4/5/7, 3A43 |
| 13 | 2445.18 | 28 29 | UDP.4F2 |
| 14 | 1433.81 | 29 30 31 32 | |
| 15 | 1845.91 | 29 30 31 | 1B1.2C8.2E1 |
| 16 | 1246.81 | 29 30 31 | 2E1 |
| 17 | 1120.77 | 30 | 2E1 |
| 18 | 1815.94 | 31 32 33 34 35 36 | ATP syntase |
| 19 | 2169.10 | 31 32 33 | 1B1.4A11 |
| 20 | 1685.94 | 32 33 | 2D6 |
| 21 | 1125.79 | 33 34 35 36 | |
| 22 | 1128.74 | 33 34 35 36 | |
| 23 | 1184.75 | 33 34 35 36 | 1A2.2C8.2C9/10/19.4F2 |
| 24 | 1356.84* | 33 34 35 36 44 | 1B1 |
| 25 | 1450.80 | 33 34 35 36 37 38 | |
| 26 | 1474.88 | 33 34 35 36 41 | |
| 27 | 2047.06 | 33 34 35 36 | 3A43 |
| 28 | 1205.81 | 34 35 36 | 2C9/10/19 |
| 29 | 1221.81 | 34 | |
| 30 | 1323.85 | 34 35 | |
| 31 | 1327.78 | 34 35 | 2C9/10/19 |
| 32 | 1421.84 | 34 35 36 | 2C8 |
| 33 | 1899.90 | 34 35 36 | |
| 34 | 1051.73 | 35 36 37 | |
| 35 | 1144.75 | 35 | 2C9/10/19 |
| 36 | 1262.84 | 35 36 | 2E1, 3A4/5/7 |
| 37 | 1616.85 | 35 36 | |
| 38 | 2478.31 | 35 36 | |
| 39 | 2773.50 | 35 | 3A43 |
| 40 | 1956.00 | 36 37 | |
| 41 | 1790.92 | 38 39 40 41 42 | actin, 3A4/5/7 |
| 42 | 1861.98 | 38 | 2E1 |
| 43 | 2165.06 | 38 39 40 | |
| 44 | 1954.07 | 39 40 41 | actin |
| 45 | 2429.19 | 39 40 | |
| 46 | 1774.85 | 40 | 2C8, 3A4/5/7 |
| 47 | 2197.10 | 40 | |
| 48 | 919.49 | 43 44 45 | actin |
| 49 | 1139.55 | 43 | ATP syntase |
| 50 | 1916.00 | 43 44 45 | |
| 51 | 1803.89 | 44 | 2C8 |
| 52 | 1969.93 | 44 45 | |
| 53 | 2571.19 | 44 45 | |

**Table 2b.** Distribution of peptides of cytochromes P450 (family CYP2A) identified by secondary fragmentation spectra (MS/MS) in gel slices. The frame marks proteins found in the region of putative localization of proteins of the CYP2A family (i.e. in slices numbered from 33 to 36); peptides absent in slice No. 35 are underlined

| No. | Peptide sequence | Slice number | Accompanying proteins that do not belong to the CYP2A family but which contain peptides with the indicated amino acid sequence |
|---|---|---|---|
| 54 | MQEEEKNPNTEFYLK | 35 | |
| 55 | AKMPYMEAVIHEIQR | 33 34 | |
| 56 | MPYMEAVIHEIQR | 33 34 35 36 | |
| 57 | IQEEAGFLIDALR | 33 34 35 36 | |
| 58 | EALVDQAEEFSGR | 35 | |
| 59 | YGPVFTIHLGPR | 33 35 | |
| 60 | GEQATFDWVFK | 33 34 35 36 | |
| 61 | SDAFVPFSIGK | 35 | |
| 62 | GYGVVFSNGER | 33 34 | |
| 63 | FGDVIPMSLAR | 33 34 35 36 | |
| 64 | NYTMSFLPR | 34 35 36 | |
| 65 | DKEFLSLLR | 34 | |
| 66 | HVGFATIPR | 34 35 36 | |
| 67 | DFIDSFLIR | 34 35 36 | |
| 68 | RFSIATLR | 34 35 36 | |
| 69 | YGFLLLMK | 33 34 35 36 | |
| 70 | FRDFFLPK | 33 34 | |
| 71 | TLDPNSPR | 33 35 | |
| 72 | DIDVSPK | 34 36 | |
| 73 | DFGVGKR | 34 | |
| 74 | HPEVEAK | 36 | |
| 75 | VHEEIDR | 33 34 35 36 | |
| 76 | GSVLR | 24 30 | |
| 77 | GIEER | 34 36 | |
| 78 | MLLV | 21 26 28 35 36 | |
| 79 | FEDR | 34 | |
| 80 | VIGK | 21 31 34 | |
| 81 | LGPR | 24 25 31 32 33 35 36 37 38 | |
| 82 | DAVR | 28 32 38 | |
| 83 | EAIR | 21 24 37 | |
| 84 | GDR | 21 22 23 25 26 27 28 29 30 33 34 35 36 37 38 | CYP2D6 |
| 85 | YLK | 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 | CYP2A6/7/13; epoxide_hydrolase; prot_disulfide_isomerase_ER60_precursor |
| 86 | IPR | 23 24 25 26 28 30 31 32 34 35 36 38 | CYP2E1hum; CYP2A6/7/13 |
| 87 | DLR | 21 23 24 25 26 28 29 30 31 32 33 34 35 36 37 38 | CYP2A6/7/13; CYP4A11hum |
| 88 | LIR | 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 | CYP2A6/7/13; CYP3A4/5/7; UDP_glycosyltransferase_2B7 |
| 89 | KNP | 22 23 24 35 36 37 38 | CYP2A6/7/13; cyp1A1hum; cyp1A2hum |

MALDI-TOF and LC-MS/MS increases the quality of protein identification [28, 29].

In this study, we have generated a proteomic map of HLMG membrane proteins. Using this map, we have determined differences between mass-spectra of cytochrome P450 forms closely related by molecular mass (and therefore by primary structure). Our approach of one-dimensional mapping can be used for revealing primary characteristics of marked changes in composition of liver microsomal fraction.

This work was supported by the Russian Federal Agency for Science and Innovations (contract 02.512.11.2105).

## REFERENCES


1. Halligan, B. D., Slyper, R. Y., Twigger, S. N., Hicks, W., Olivier, M., and Green, A. S. (2005) *J. Am. Soc. Mass Spectrom.*, **16**, 302-306.
2. Hufnagel, P., and Rabus, R. (2006) *J. Mol. Microbiol. Biotechnol.*, **11**, 53-81.
3. Lubec, G., Afjehi-Sadat, L., Yang, J.-W., and John, J. P. P. (2005) *Progr. Neurobiol.*, **77**, 90-127.
4. Afjehi-Sadat, L., Shin, J.-H., Felizardo, M., Lee, K., Slavc, I., and Lubec, G. (2005) *Biochim. Biophys. Acta*, **1747**, 67-80.
5. Damodaran, S., Dlugos, C. A., Wood, T. D., and Rabin, R. A. (2006) *Eur. J. Pharm.*, **547**, 75-82.
6. Chen, Y., Kwon, S. W., Kim, S. C., and Zhao, Y. (2005) *J. Proteome Res.*, **4**, 998-1005.
7. Zuo, X., Echan, L., Hembach, P., Tang, H. Y., Speicher, K. D., Santoli, D., and Speicher, D. W. (2001) *Electrophoresis*, **22**, 1603-1615.
8. Xiong, Y., Chalmers, M. J., Gao, F. P., Cross, T. A., and Marshall, A. G. (2005) *J. Proteome Res.*, **4**, 855-861.
9. Wang, Y., Al-Gaart, A., Seibert, C., Sharif, A., Lane, C., and Griffiths, W. J. (2006) *Biochem. Soc. Trans.*, **34**, 1246-1251.
10. Simpson, R. J., Connolly, L. M., Eddes, J. S., Pereira, J. J., Moritz, R. L., and Reid, G. E. (2000) *Electrophoresis*, **21**, 1707-1732.
11. Link, A. J., Eng, J., Schieltz, D., Carmack, E., Mize, G., Morris, G., Garvik, B., and Yates III, J. R. (1999) *Nature Biotechnol.*, **17**, 676-682.
12. Nisar, S., Lane, C. S., and Wilderspin, A. F. (2004) *Drug Metab. Dispos.*, **32**, 382-386.
13. Supek, F., Peharec, P., Krsnik-Rasol, M., and Smuc, T. (2008) *Proteomics*, **8**, 28-32.
14. Petushkova, N. A., Kanaeva, I. P., Lisitsa, A. V., Sheremetyeva, G. F., Zgoda, V. G., Samenkova, N. F., Karuzina, I. I., and Archakov, A. I. (2006) *Toxicology in vitro*, **20**, 966-974.
15. Anzenbacher, P., and Anzenbacherova, E. (2001) *Cell. Mol. Life Sci.*, **58**, 737-747.
16. Kanaeva, I. P., Petushkova, N. A., Lisitsa, A. V., Lokhov, P. G., Zgoda, V. G., Karuzina, I. I., and Archakov, A. I. (2005) *Toxicology in vitro*, **19**, 805-812.
17. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., and Estabrook, R. W. (1996) *Pharmacogenetics*, **6**, 1-42.
18. Archakov, A. I., Bachmanova, G. I., Blinder, L. V., Zhikhareva, V. O., Nanaeva, I. P., Karyakin, A. V., Karuzina, I. I., Khaitlina, S. Z., Borovyagin, V. L., and Galuschenko, I. V. (1977) *Biokhimiya*, **42**, 100-112.
19. Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248-254.
20. Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988) *Electrophoresis*, **9**, 255-262.
21. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.*, **68**, 850-858.
22. Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., and Mann, M. (2005) *Mol. Cell. Proteomics*, **4**, 1265-1272.
23. Lane, C. S., Nisar, S., and Griffiths, W. J. (2004) *Eur. J. Cancer*, **40**, 2127-2134.
24. Galeva, N., and Alterman, M. (2002) *Proteomics*, **2**, 713-722.
25. Ingelman-Sundberg, M., and Rodriguez-Antona, C. (2005) *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **360**, 1563-1570.
26. Archakov, A. I., and Bachmanova, G. I. (1990) in *Cytochrome P450 and Active Oxygen*, Taylor and Francis, London-New York, pp. 1-81.
27. Nisar, S., Lane, C. S., Wilderspin, A. F., Welham, K. J., Griffiths, W. J., and Patterson, L. H. (2004) *Drug. Metab. Dispos.*, **32**, 382-386.
28. Lim, H., Eng, J., and Yates, J. R. (2003) *J. Am. Soc. Mass Spectrom.*, **14**, 957-970.
29. Zhu, K., Kim, J., Yoo, C., Miller, F. R., and Lubman, D. M. (2003) *Anal. Biochem.*, **75**, 6209-6217.