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Quantitative Analysis of Cytochrome P450 Isozymes by Means of Unique Isozyme-Specific Tryptic Peptides: A Proteomic Approach.

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Running title: MALDI TOF-based quantitation of CYPs

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Abbreviations: CYP – cytochrome(s) P450; PB – phenobarbital; MALDI – matrix-assisted laser desorption/ionization, TOF – time of flight; MS – mass spectrometry; PMF – peptide mass fingerprinting, IS – internal standard, ICAT - isotope-coded affinity tagging.

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Abstract

A novel MALDI-TOF MS method has been developed to quantitate cytochrome P450 isozymes based on their unique isozyme-specific tryptic peptides. It was shown that the molar ratio of CYP isozyme-specific peptides is linearly proportional to the mass peak area ratio of corresponding peptides not only in simple two peptide mixtures, but also in complex digest mixtures. This approach is applicable both to in-gel (as shown for CYP2B1 and CYP2B2) and in-solution digests (as shown for CYP1A2, CYP2E1, and CYP2C19) and does not require introduction of stable isotopes or labeling with ICAT. The relative and absolute quantitation can be performed after developing corresponding calibration curves with synthesized CYP isozyme-specific peptide standards. The absolute quantitation of human CYP isozymes was performed by using CYP2B2 isozyme-specific peptide (1306.7 Da) as the universal internal standard. The utility of this approach was demonstrated for two highly homologous (>97%) rat liver CYP2B1 and CYP2B2 and three human CYP isozymes belonging to two different families and three different subfamilies: CYP1A2, CYP2E1 and CYP2C19. In summary, we have demonstrated that MALDI TOF-based peptide mass fingerprinting of different cytochrome P450 isozymes can provide not only qualitative but quantitative data, too.

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Introduction

The superfamily of cytochrome(s) P450 (CYP) plays a key role in hepatic and extra-hepatic drug metabolism and qualitative and quantitative analysis of the CYP isozymes expression in a particular organ is critical in predicting a metabolic fate of a particular drug or in examination of the potential drug-drug interaction. The number of sequenced and named different P450 isozymes surpassed 3100 (dnelson.utm.edu/CytochromeP450.html) and the degree of sequence homology, particularly among CYPs belonging to the same subfamily is high (Nelson et al., 1996). None of the existing research approaches to the analysis of individual P450 forms that include: specific P450 inhibitors (Halpert et al., 1994; Kobayashi et al., 2003) or substrates (Kobayashi et al., 2002; Stresser et al., 2002), antibody-based identification (Gelboin et al., 1999; Shou et al., 2000) and mRNA-based analysis (Chow et al., 1999; Zhang et al., 1999) is in a position to provide reliable quantitative and qualitative information on the individual CYP composition in a given type of microsomes. First, only a minority of known P450 isozymes is fully characterized by substrate specificity and since they exhibit a broad, often overlapping substrate specificity there is no known substrate or inhibitor that is absolutely specific for an individual P450 isozyme. Second, the high degree of sequence homology among members of P450 superfamily confounds high specificity of antibody-based analysis, particularly among members of the same subfamily. Third, the application of a quantitative mRNA analysis for the evaluation of CYP isozymes expression, that once looked very promising, is questionable, too. It was shown that in many cases correlation between protein abundances and mRNA levels for numerous hepatic and extra-hepatic proteins is poor (Anderson and Seilhamer, 1997; Luss et al., 1997; Pradet-Balade et al., 2001; Chen et al., 2002; Huber et al., 2004). And most importantly, if

an unknown or an unexpected CYP isozyme is expressed in the microsomes under investigation none of these approaches will reveal it.

Over the last 10 years mass spectrometry based approach to the identification of proteins has become the method of choice for protein analysis in biological systems (Mann and Pandey, 2001). However, identification of different CYP isozymes represents quite a challenge even for proteomics. Main reason for this is that there is still no analytical method that can sufficiently separate different P450 isozymes either by electrophoretic or by chromatographic means. Consequently, one has to deal with mixtures containing more than one CYP and this superfamily is the largest group of enzymes that share a high degree of similarity in protein sequence. Our laboratory has previously shown that peptide mass fingerprinting (PMF) can be successfully applied to the identification and differential analysis of closely related cytochrome P450 isozymes in liver and brain microsomes from rat, mouse and rabbit (Galeva and Altermann, 2002; Duzhak et al., 2003; Galeva et al., 2003). In this study, on the examples of rat and human CYPs, we demonstrate that members of CYP superfamily possess unique isozyme-specific tryptic peptide(s) that could be utilized for differential qualitative and quantitative analysis by MALDI TOF mass spectrometry. Under “unique” we mean peptide sequences generated by trypsinolysis that differ from any other tryptic peptide derived from any other identified to-date protein.

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Experimental Procedures

Chemicals. Acetonitrile, urea and ammonium bicarbonate were purchased from Fisher (Pittsburgh, PA), α -cyano-4-hydroxycinnamic acid was purchased from Aldrich Chem. Co. (Milwaukee, WI) and additionally purified by re-crystallization, dithiothreitol was purchased from BIO-RAD, sodium iodoacetate (98% purity), β -lactoglobulin A from bovine milk and bovine serum albumin were purchased from SIGMA (St. Louis, MO) and sequencing grade modified trypsin purchased from Promega (Madison, WI). Human recombinant cytochromes P450 were purchased from Panvera/Invitrogen (Carlsbad, USA): CYP2C19 - lot number 26395B, CYP2E1 – lot number 12019A, and CYP1A2 – lot number 7166A.

Preparation of microsomes. Hepatic microsomes from adult male Sprague-Dawley rats were prepared by differential centrifugation as previously described (Alterman et al., 1993).

Peptide synthesis. Peptides, representing partial sequences of cytochromes CYP2B1 (F₃₅₉SDLVPIGVPHR₃₇₀, 1335.730 Da), CYP2B2 (F₃₅₉ADLAPIGLPHR₃₇₀, 1305.719 Da), CYP1A2 (Y₂₄₄LPNPALQR₂₅₂, 1070.587 Da), CYP2C19 (G₃₄₃HMPYTDVAVVHEVQR₃₅₇, 1737.826 Da), and CYP2E1 (F₃₆₀ITLVPSNLPHEATR₃₇₄, 1693.915 Da) were synthesized on an ACT 90 (Advanced ChemTech, Louisville, KY) by means of solid phase technique using Fmoc-protected amino acids. Peptides were purified by semi-preparative HPLC performed on a Summit HPLC system (Dionex, CA). The final peptide preparations were analyzed by MALDI-TOF MS and analytical reverse-phase HPLC, and were >99% pure. Stock solutions of each peptide at 10 mM were prepared in 50% acetonitrile/ water. All further dilutions were made with deionized water. To ensure reproducibility of the analytical approach peptide mixtures for

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standard curves were processed through ZipTips C₁₈ before MALDI TOF MS in the same way as the experimental tryptic digests.

SDS-PAGE. Electrophoresis was performed as described previously (Galeva and Altermann, 2002).

Enzyme assay. Cytochrome P450 isozymes concentrations in individual stock solutions were measured spectrophotometrically using the following extinction coefficients $\epsilon_{418} = 110 \text{ mM}^{-1} \text{ cm}^{-1}$ (for CYP2E1 and CYP2C19) and $\epsilon_{393} = 104 \text{ mM}^{-1} \text{ cm}^{-1}$ (for CYP1A2) (White and Coon, 1982). Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

Tryptic digestion and sample preparation. In-solution digest was performed according to (Stone and Williams, 1996), electrophoretically separated protein bands were excised by hand and in-gel digested as previously described (Rosenfeld et al., 1992). Corresponding aliquots (depending on the purpose of the experiment) of the synthesized isozyme-specific peptides were added after the tryptic digest both in case of in-gel and in-solution digests but before ZipTip step. In case of experiments involving absolute quantitation, 2.0 μl aliquot of 10 μM solution of internal standard (IS) peptide was added to 20 μl aliquot of either mixture of synthetic peptides (for calibration curves), or 20 μl aliquot of a tryptic digest. Six aliquots spiked with IS were prepared for each sample, and then each of this replicates was extracted separately with ZipTip C₁₈ according with the manufacturers instructions. Tryptic peptides bound to ZipTips C₁₈ were eluted onto the MALDI target plate with 0.5 μl of matrix solution (10 mg/ml of α -cyano-4 hydroxycinnamic acid in 50% acetonitrile/0.1% TFA). The deposited samples crystallized within 30 min upon evaporation of the solvent at ambient temperature. For experiments

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performed on Voyager DE STR we used 400-well Teflon coated MALDI target plates and in experiments executed on 4700 Proteomics Analyzer we used 192 well (192-6-AB) target plates.

MALDI-TOF mass spectrometry. Mass spectra were obtained on a Voyager DE STR (in case of CYP2B1 and CYP2B2 experiments) and 4700 Proteomics Analyzer (in case of CYP1A2, CYP2C19, and CYP2E1) MALDI-TOF mass spectrometers (Applied Biosystems). Voyager DE STR was operated in a positive reflector mode with the following parameters: acquisition mass range 700-3000 Da, accelerating voltage 20000 V, grid voltage 75%, mirror voltage ratio 1.12, guide wire 0.01%, low mass gate set at 700, extraction delay time 150 nsec and the laser power attenuator set at 2200. To compensate for the heterogeneity of the analyte crystallization and to cover as much target area as possible, a spiral search pattern was used when laser beam was moved from crystal area to crystal area manually with 5-6 laser shots at each firing position (total 100 shots/spectrum). 4700 Proteomics Analyzer was operated with a standard positive ion reflector acquisition program in the mass range 700-3000 Da (the focus mass was 1200 Da) and the laser power set at 4400. An automated spiral laser-firing pattern was used with 4 laser shots at each of 250 firing positions. The peak area values were determined by utilizing Applied Biosystems Data Explorer Version 4.5 for 4700 Proteomics Analyzer and Data Explorer Version 4.0 for Voyager DE STR. The mass peak list was exported and processed in Microsoft Excel. For both instruments the high voltage was turned on at least 40 min before start of a data collection and all samples were analyzed at the same laser power adjusted so that it would not produce saturated signals of analytes while producing analyte peaks with signal-to-noise ratio >5.

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Results and Discussion

Application of MALDI-TOF MS for qualitative analysis is well documented and is the basis for many current developments in proteomics (Gevaert et al., 2001). At the same time, the number of examples of the direct use of MALDI-TOF MS without introduction of stable isotopes for quantitative analysis of biomolecules is limited (Walker et al., 2000; Hlongwane et al., 2001; Bucknall et al., 2002; Helmke et al., 2004). Working with minute amounts of biological sample obtained from liver, brain, kidney or any other organ, would benefit from a simpler quantitative approach. Application of a stable isotope-based internal standard is one of such approaches (Mirgorodskaya et al., 2000; Mirgorodskaya et al., 2004). Another approach is to measure relative quantitation by comparing the mass peak areas or intensities of structurally similar compounds like tryptic peptides from different proteins without using an internal standard. Several recent studies explored the applicability of such approach to LC/MS electrospray ionization technique (Bondarenko et al., 2002; Chelius and Bondarenko, 2002).

With no existing general analytical approach capable of a reliable relative or absolute quantitation of a particular CYP isozyme in a complex biological sample, we decided to explore if tryptic peptide mass fingerprinting (PMF) based on MALDI TOF MS could be used as the basis for the development of a global analytical approach for quantitative analysis of CYP isozymes. Two very closely related cytochrome P450 isozymes, CYP2B1 and 2B2, were chosen for proof-of-concept experiments.

Proteomic analysis of CYP2B1 and CYP2B2. CYP2B1 is the major form of cytochrome P450 induced in liver of adult rats after exposure to phenobarbital (PB). PB also induces CYP2B2; although, it still is unclear to what extent. These two proteins are highly homologous

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(>97%) differing in only 14 amino acids out of 491. Their theoretical tryptic digests differ in 5 pairs of peptides, and 4 pairs of those peptides fall within the optimal MALDI TOF working range 800-2500 amu (Table 1). The first pair of peptides originates from the N-terminus (positions 1-21) and was very rarely found in experimental digests of purified CYPs or microsomal fractions (Fig. 1). One of the peptides in the second pair (positions 317-323) has MW that differs in 1 amu with one of the self-digest fragments of trypsin (842.439 vs. 841.502) and cannot be a reliable indicator because of the overlap of resolved isotopomers. The third pair of peptides presents an interesting case. The CYP2B2 sequence contains Arg followed by Pro, and as a result there is no cleavage in this position. In CYP2B1, Arg is followed by Leu and then by Pro, creating a more accessible cleavage site. However, in many experiments we found 1964.01 peak corresponding to missed cleavage (data not shown). Finally, a fourth pair of tryptic peptides appears to match the requirements for isozyme-specific tryptic peptides and was selected for further experiments. Since selected peptides originate from the same part of the molecule, position 359-370 (Fig. 1, inset) there should not be any doubt regarding their “equal accessibility” to tryptic digest and we do not see any peptides with missed cleavages in position 370 neither in CYP2B1 nor in CYP2B2 digests. It should be emphasized that both of those peptides are unique in a sense that there are no similar tryptic peptides in any of the proteins listed to date either in SwissProt or NCBI databases (over 140 000 entries). The mass peak intensities of these peptides were always among the strongest in more than a hundred of rat liver microsomal digests we have performed to date and their identity was confirmed by MS/MS (data not shown). The selected peptides were synthesized, mixed in different ratios and analyzed by

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MALDI-TOF MS. Figure 2 shows that the molar ratio of isozyme-specific tryptic peptides is linearly proportional to the mass peak area ratio of corresponding peptides.

Among factors known to contribute to difficulties associated with quantitative application of MALDI TOF MS, most significant are the heterogeneity of analyte crystallization (Cohen and Chait, 1996; Garden and Sweedler, 2000) and the ion suppression effect (Kratzer et al., 1998; Knochenmuss et al., 1999). To control reproducibility and to compensate for heterogeneity of analyte-matrix co-crystallization we standardized and to some degree optimized the conditions for data acquisition (see Experimental Procedures). The evaluation of the ion suppression effect was performed by spiking digests of bovine serum albumin (BSA) and β -lactoglobulin A (β -LGA) with synthesized CYP2B1 and CYP2B2 isozyme-specific peptides in various ratios. Figure 3 shows a representative MALDI-TOF mass spectrum of 2 pmol of the BSA digest containing CYP2B1 and CYP2B2 isozyme-specific tryptic peptides in 1:1 ratio. In both cases a linear response between the molar ratio and the corresponding mass peak areas was observed. Figure 4 illustrates such dependence for digests of BSA (Panel A) and β -LGA (Panel B) spiked with synthesized CYP2B1 and CYP2B2 specific peptides.

Next, we applied the developed method to the microsomal sample separated on SDS-PAGE gel. Rat liver microsomes were obtained from untreated male rats. Previously it was shown that such microsomes do not contain CYP2B1 and CYP2B2 (Galeva and Altermann, 2002; Galeva et al., 2003; Nisar et al., 2004). Twenty μ g of total microsomal protein were electrophoresed on 10% SDS PAGE. Several bands with an apparent molecular mass of 50-60 KDa were excised and subjected to tryptic digest. The band containing CYP2D2 (sequence identity to CYP2B1 and CYP2B2 41%) was chosen for further experiments. The tryptic digest of CYP2D2 was

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spiked with synthesized CYP2B1 and CYP2B2 specific tryptic peptides in different ratios to simulate digests of CYP2B1 and CYP2B2 and then analyzed by MALDI TOF MS. To determine the relative amounts of CYP2B1 and CYP2B2 a calibration curve was developed using corresponding synthetic peptides (Fig. 5). As is seen from Fig. 5 there was a good correlation between the experimental points (open circles) and the calibration curve.

Proteomic analysis of human CYP1A2, CYP2E1 and CYP2C19. Based on the results obtained from experiments with CYP2B1 and CYP2B2 we decided to explore if PMF MALDI TOF-based quantitative approach is applicable to other CYP isozymes and particularly to human CYPs. The human genome encodes 57 cytochrome P450 genes. Thirty-five of these genes encode P450s belonging to families 1 to 4 (Danielson, 2002). CYPs associated with families 1 to 3 are the key enzymes of Phase I in human drug metabolism, while members of CYP4 family are mainly involved in fatty acid and arachidonic acid metabolism. The remaining 14 CYP families for the most part are implicated in steroid metabolism.

First of all, considering large number of human CYPs and high degree of homology between members of CYP subfamilies, we needed to establish if all of human CYPs possess unique isozyme-specific tryptic peptides. To this end we undertook a data base search for unique isozyme-specific tryptic peptides of human P450s. The following set of requirements was considered in this search. Suitable tryptic peptide candidates should not have any similar counterparts (homologues) preferably in any organism, or, at least, in humans; have a mass between 900 and 1900 Da, to achieve best possible accuracy and resolution in MALDI TOF spectrum; ideally have an Arg at the C-terminus, since Arg-ending peptides produce much stronger MS signals in MALDI MS than Lys-ending; and, do not contain any missed cleavages.

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A list of isozyme-specific tryptic peptides was developed using PAWS software (Genomic Solutions) to generate simulated tryptic digests and ScanProsite search engine (<http://au.expasy.org/tools/scanprosite>) to scan protein sequences from Swiss-Prot, TrEMBL and PDB with a user-entered pattern (in our case candidate tryptic peptides). As it turns out, all human CYPs have from 2 to 14 unique isozyme-specific tryptic peptides and the complete list encompasses several hundred peptides. Table 2 shows predicted unique isozyme-specific tryptic peptides for three human CYPs (CYP1A2, CYP2E1, and CYP2C19) that were used in further experiments. As can be seen from Table 2 all of them have 8-9 unique isozyme-specific tryptic peptides. Clearly, not all of these peptides will show up in a tryptic digest and/or produce strong signal in the MALDI TOF mass spectrum. To perform reliable quantitation we needed to establish which one of the candidate peptides is consistently formed during trypsinolysis and generates strongest MS signal. To this end all three purified isozymes were subjected to tryptic digest (Fig. 6). It should be noted that we performed multiple digests differing in conditions (in-solution vs. in-gel, with or w/o reduction and alkylation, 37°C overnight vs. 58°C 45 min) with each of these isozymes and the results were consistent. For each CYP there was at least one isozyme-specific tryptic peptide that produced a dominant mass peak in the corresponding PMF MALDI TOF mass spectrum (Fig.6 and marked in bold in Table 2). Sequences of these peptides were confirmed by MS/MS (data not shown). It should be emphasized that these major isozyme-specific tryptic peptides were conserved even in simplified digests performed without destaining of gel bands, reduction and alkylation (cf. panels A and B, Fig. 6). Some other predicted unique isozyme-specific peptides were seen in the digest mass spectra too, but their peaks were not as strong. The identified major isozyme-specific tryptic peptides were synthesized and used for

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quantitative analysis of human CYPs. In all further experiments CYP2B2 isozyme-specific peptide (1306.7 Da) was used as an internal standard (IS). The calibration curves for the absolute quantitation of CYP isozymes were generated using mixtures of four peptides (IS peptide plus three synthetic isozyme-specific peptides). Each MALDI target spot contained 20 pmol of IS peptide and from 500 fmol to 70 pmol of the synthetic CYP1A2 and CYP2E1 specific peptides and from 500 fmol to 50 pmol of the synthetic CYP2C19 specific peptide. Linear regression analysis data presented on Fig. 7 indicate that for all three isozymes the peak area ratios are linear with the amount of the synthesized isozyme-specific peptides. Subsequently, we prepared two mixtures of purified CYPs with different molar ratios based on their concentrations determined spectrophotometrically by UV-Vis spectra and then spiked them with IS peptide and performed in-solution tryptic digest. A representative MALDI TOF mass spectrum of a combined digest of all three CYP isozymes is shown in Fig. 8. The peak area ratios of isozyme-specific peptides to IS peptide was measured from MS spectra and the concentrations of all three CYPs in a given mixture were determined simultaneously using the developed calibration curves. The CYP isozymes concentrations measured by MALDI TOF MS were generally higher than the concentrations measured spectrophotometrically in individual CYP stock solutions (Table 3). Somewhat elevated values of CYP concentrations measured by MALDI TOF MS compared to UV-Vis measurement reflect the fact that the mass spectrometric method measures the apoprotein amount, while UV-Vis measures holoenzyme (CYP molecules containing heme moiety). Since in cytochrome(s) P450 the prosthetic heme group is not covalently bound to the apoprotein (except CYP4As), part of the P450 molecules lose it relatively easily. All three CYP isozymes used in this study were recombinant proteins produced

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from over-expressed plasmid in *E. coli*. According to the manufacturer's certification (Panvera/Invitrogen) their specific content varied from 10 nmol of spectral P450 per milligram of protein in case of CYP2C19 to 12 nmol/mg protein for CYP2E1, and 16 nmol/mg protein for CYP1A2. These values indicate presence of heme-depleted CYPs and/or existence of some protein impurity. And, indeed, the proteomic analysis identified presence of β -galactosidase from *E. coli* in all preparations of these isozymes (data not shown). In line with these findings, we established that CYP concentrations calculated based on protein measurements in CYP isozymes stock solutions were consistently higher than MALDI TOF MS measured values (Table 3). Noteworthy also is the fact that the higher the P450 specific content (i.e. CYP purity) is, the better is the correlation between MALDI TOF MS and UV-Vis measured values (Table 3, CYP1A2 vs. CYP2E1 vs. CYP2C19).

Due to the low sequence similarity among the predicted isozyme-specific tryptic peptides, we did not design the IS peptide, but rather decided to use CYP2B2 specific tryptic peptide (1305.7 Da) as the universal internal standard. However, it should be emphasized, that isozyme-specific peptides for CYP2C19 and CYP2E1 originate from the same part of the CYP molecule as CYP2B1 and CYP2B2 specific peptides, while CYP1A2 specific peptide comes from a different part of the molecule. If this trend can be confirmed in further studies then a single internal standard per CYP family/subfamily could be designed what in turn might increase accuracy of this approach.

In summary, a novel MALDI-TOF MS method has been developed to quantitate cytochrome P450 isozymes based on their unique isozyme-specific tryptic peptides. This approach is applicable both to in-gel (as shown for CYP2B1 and CYP2B2) and in-solution digests (as shown

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for CYP1A2, CYP2E1, and CYP2C19) and does not require introduction of stable isotopes or labeling with ICAT. The relative and absolute quantitation can be performed after developing corresponding calibration curves with synthesized isozyme-specific peptide standards. The absolute quantitation of human CYP isozymes was performed by using CYP2B2 isozyme-specific peptide (1306.7 Da) as the universal internal standard. The utility of this approach was demonstrated for two highly homologous (>97%) rat liver CYP2B1 and CYP2B2 and three human CYP isozymes belonging to two different families and three different subfamilies: CYP1A2, CYP2E1 and CYP2C19. Furthermore, the data obtained point to the fact that sample preparation and data acquisition conditions need to be carefully controlled in order to obtain optimal results. In more general context, our data along with some other recent publications (Hlongwane et al., 2001; Bucknall et al., 2002; Mims and Hercules, 2003; Alterman et al., 2004; Helmke et al., 2004) suggest that application of MALDI-TOF mass spectrometry for relative or direct quantitation is a valid alternative to stable isotope approach. Particularly advantageous in direct quantitation by MALDI TOF is the simplicity of the sample processing. The data presented along with new findings involving CYP2A6 and CYP2A13 (manuscript in preparation) strongly suggest that this technique could become a universal method for the quantitative analysis of CYP isozyme expression. Studies involving relative and absolute quantitation of human CYP isozyme expression in liver and other organs are currently in progress in our laboratory.

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Footnotes

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Figure legends.

Figure 1. MALDI mass spectrum of a tryptic peptide mass fingerprint of SDS-PAGE band containing CYP2B1/CYP2B2. Filled circles indicate mass peaks corresponding to common CYP2B1/2B2 tryptic peptides. Open circles correspond to CYP2B1-specific tryptic peptides and triangles to CYP2B2-specific tryptic peptides. Inset: expanded view showing resolution attained.

Figure 2. A linear dependence between molar ratio of CYP2B1/CYP2B2 specific tryptic peptides and corresponding monoisotopic peak areas. Each data point represents the average \pm S.D. of data collected in six experiments.

Figure 3. A representative MALDI TOF mass spectrum of BSA tryptic digest spiked with synthesized CYP2B1 and CYP2B2-specific tryptic peptides (peak 1306.735 is CYP2B2-specific peak and 1336.739 is CYP2B1-specific).

Figure 4. A linearity plot of monoisotopic peak areas of CYP2B1 and CYP2B2 isozyme specific tryptic peptides spiked into BSA (Panel A) and b-LGA (Panel B) tryptic digests. Each data point represents the average \pm S.D. of data collected in six experiments.

Figure 5. Relative quantitation of CYP2B1/2B2 isozymes. Triangles represent monoisotopic peak areas of synthesized CYP2B1/CYP2B2 isozyme specific tryptic peptides mixtures used to build calibration curve. Open circles represent monoisotopic peak areas of synthesized CYP2B1/CYP2B2 isozyme specific tryptic peptides spiked into a tryptic digest of a band excised from SDS-PAGE and containing CYP2D2. Calibration curve and experimental samples were

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extracted with ZipTip C₁₈ and then eluted with MALDI matrix on target. Each data point represents the average \pm S.D. of data collected in six experiments.

Figure 6. Representative tryptic peptide mass fingerprinting MALDI TOF mass spectra of isolated human CYPs. Panel A – CYP1A2 digest (asterisks denote 1A2 tryptic peptides); panel B – CYP1A2 simplified digest without destaining, alkylation and reduction; panel C – CYP2C19 digest (asterisks denote 2C19 tryptic peptides); panel D – CYP2E1 digest (asterisks denote 2E1 tryptic peptides).

Figure 7. Absolute quantitation standard curves: panel A – CYP1A2 standard curve; panel B – CYP2E1 standard curve; panel C – CYP2C19 standard curve. Each data point represents the average \pm S.D. of data collected in six experiments.

Figure 8. Representative tryptic peptide mass fingerprint MALDI TOF mass spectrum of a combined tryptic digest of CYP1A2, CYP2C19 and CYP2E1.

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Table 1. Comparison of tryptic peptides that differ between CYP2B1 and CYP2B2.

CYP isozyme	Start - End	Peptide Sequence	MH⁺ (calculated)
CYP2B1	1-21	MEPT <u>I</u> LLLLALLVG <u>F</u> LLLLVR	2350.484
CYP2B2	1-21	MEPS <u>I</u> LLLLALLVG <u>F</u> LLLLVR	2336.468
CYP2B1	317-323	YPHV <u>A</u> EK	843.429
CYP2B2	317-323	YPHV <u>T</u> EK	873.439
CYP2B1	327-336	EIDQVIGSHR(<u>L</u> PT <u>L</u> DDR)	1153.589
CYP2B2	327-343	EIDQVIGSHR <u>P</u> PS <u>L</u> DDR	1933.965
CYP2B1	359-370	F <u>S</u> DL <u>V</u> PIG <u>V</u> PHR	1336.738
CYP2B2	359-370	F <u>A</u> DL <u>A</u> PIG <u>L</u> PHR	1306.719

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Table 2. List of predicted unique tryptic peptides for CYP1A2, CYP2E1 and CYP2C19.

CYP	Start - End	Sequence	Monoisotopic Mass	Length, aa
1A2	80-90	IGSTPVLVLSR	1140.687	11
	244-252	YLPNPALQR	1070.587	9
	267-277	TVQEHYQDFDK	1408.626	11
	297-306	ASGNLIPQEK	1055.561	10
	378-392	HSSFLPFTIPHSTTR	1726.879	15
	393-403	DTTLNGFYIPK	1267.645	11
	432-447	FLTADGTAINKPLSEK	1703.909	16
	489-500	VDLTPIYGLTMK	1349.726	12
2C19	60-73	IYGPVFTLYFGLER	1673.882	14
	74-84	MVVLHGYEVVK	1272.690	11
	236-247	NLAFMESDILEK	1408.691	12
	250-261	EHQESMDINNPR	1468.636	12
	343-357	GHMPYTDAVVHEVQR	1737.826	15
	384-399	GTTILTSLSVLHDNK	1698.915	16
	400-410	EFPNPEMFDPR	1377.602	11
	422-432	SNYFMPFSAGK	1247.564	11
2E1	64-76	FGPVFTLYVGSQR	1469.767	13
	101-110	GDLPAFHHR	1119.557	10
	113-123	GIIFNNGPTWK	1245.651	11
	150-159	EAHFLLEALR	1197.651	10
	188-195	HFDYNDEK	1066.436	8
	345-359	QEMPYMDAVVHEIQR	1844.855	15
	360-374	FITLVPSNLPHEATR	1693.915	15
	409-420	FKPEHFLNENGGK	1458.725	12
423-434	YSDYFKPFSTGK	1438.677	12	

Table 3. Comparison of concentrations of three CYP isozymes measured by different techniques^a.

CYP isozyme	Concentration of CYP isozymes in mixture 1, pmol/μl			Concentration of CYP isozymes in mixture 2, pmol/μl			CYP isozyme specific content, nmol P450/mg protein
	MALDI TOF MS	UV-VIS	BCA	MALDI TOF MS	UV-VIS	BCA	
CYP 1A2	7.6 ± 1.6	6.7	8.4	1.8 ± 0.5	2.0	2.6	16.3
CYP 2E1	7.8 ± 1.1	5.7	8.7	11.5 ± 1.5	8.1	12.3	12.3
CYP 2C19	5.3 ± 1.0	3.2	6.6	8.5 ± 0.3	4.6	10.5	10.0

^a CYP isozymes mixtures were prepared from stock solutions of purified proteins. UV-Vis and protein (BCA) concentrations were measured in corresponding stock solutions. The MALDI TOF MS data reported represent the average ± SD from six measurements.

Figure 1

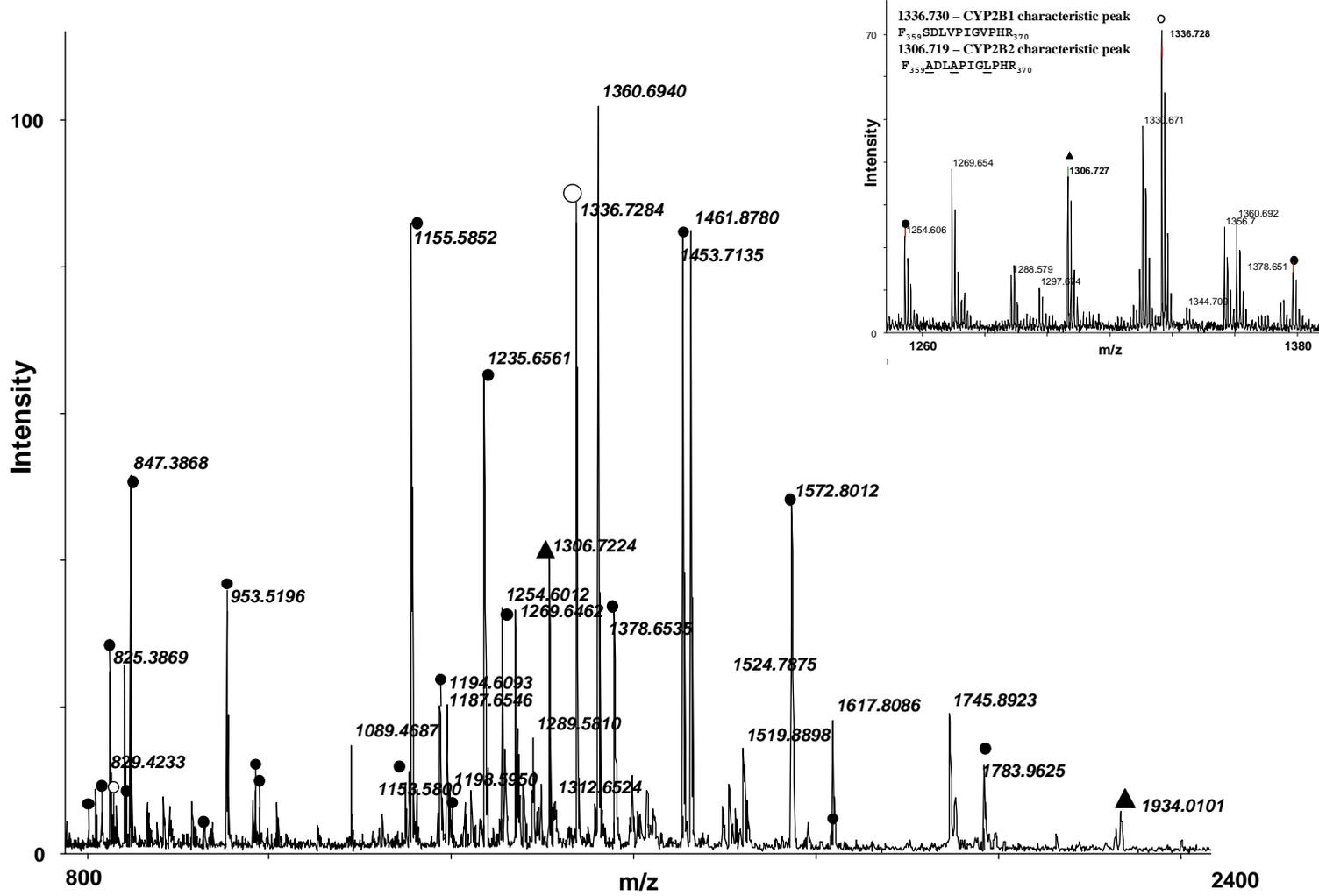


Figure 2

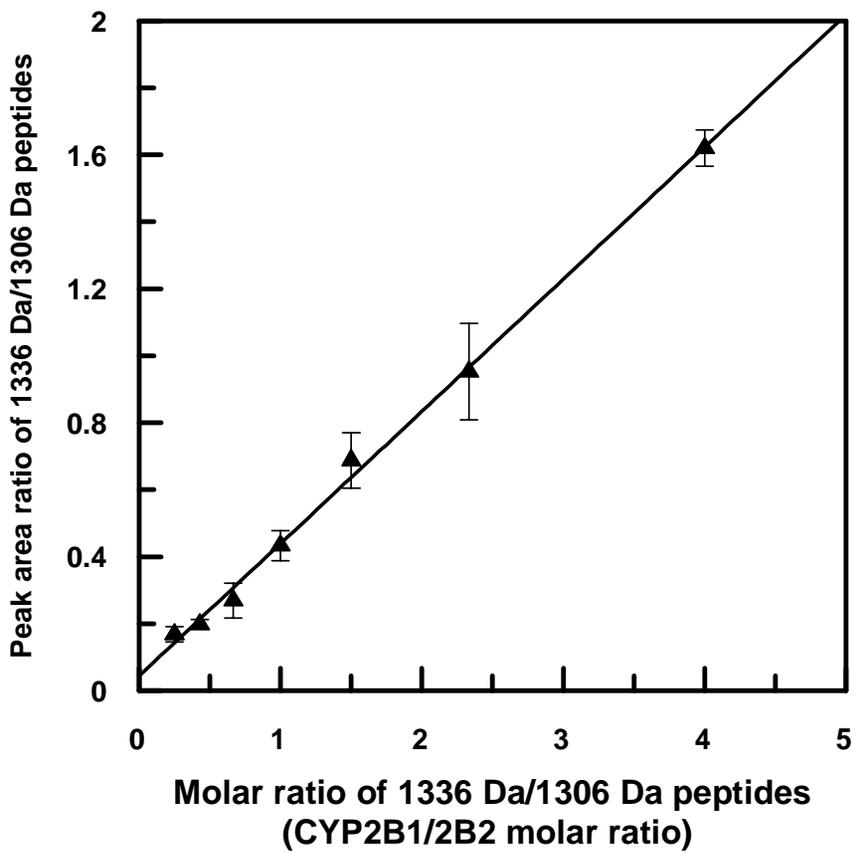
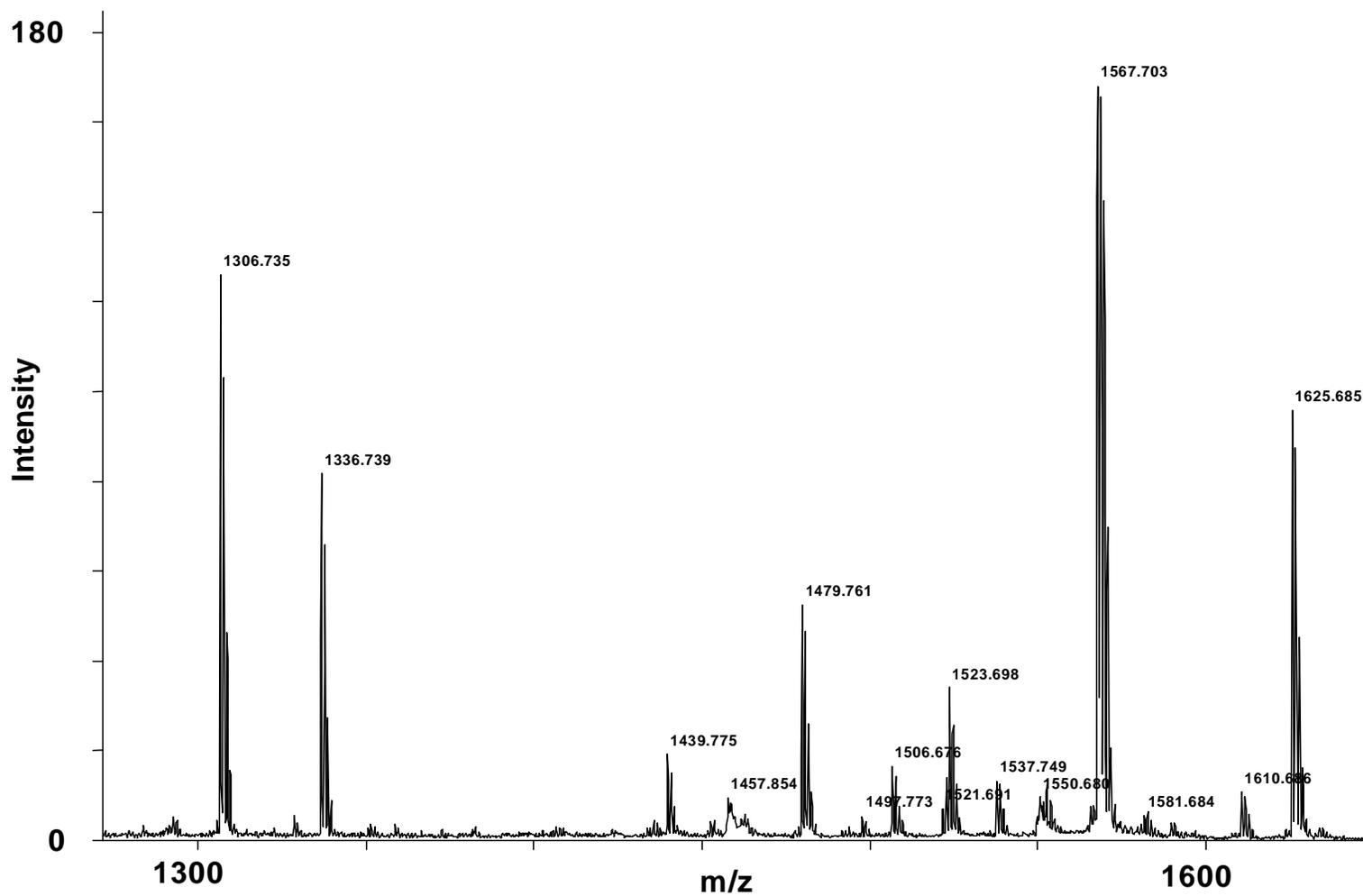


Figure 3



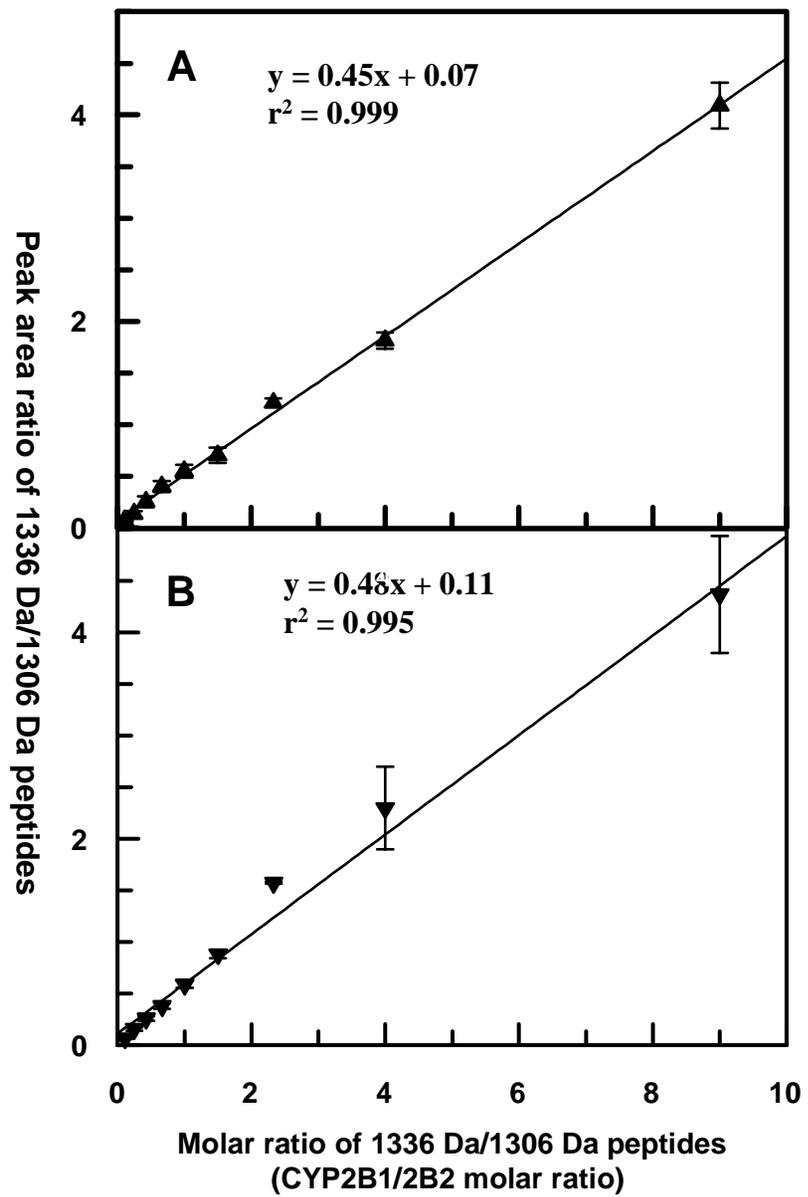


Figure 4

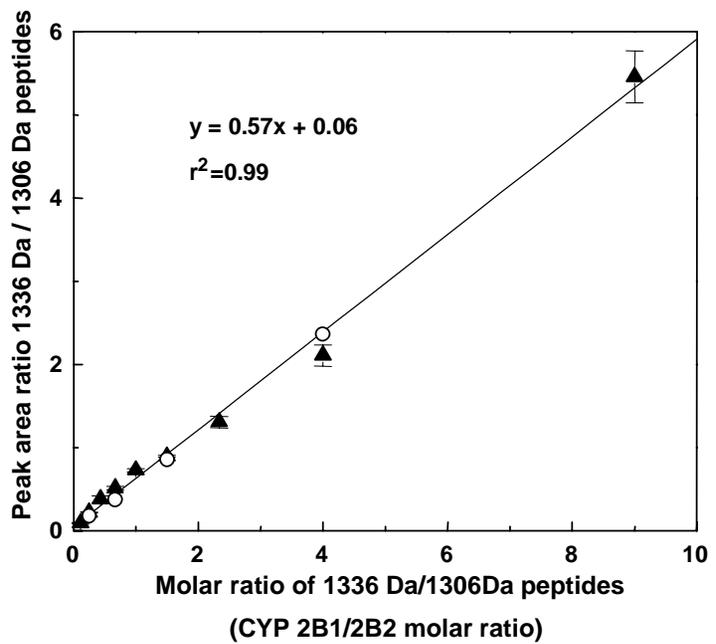


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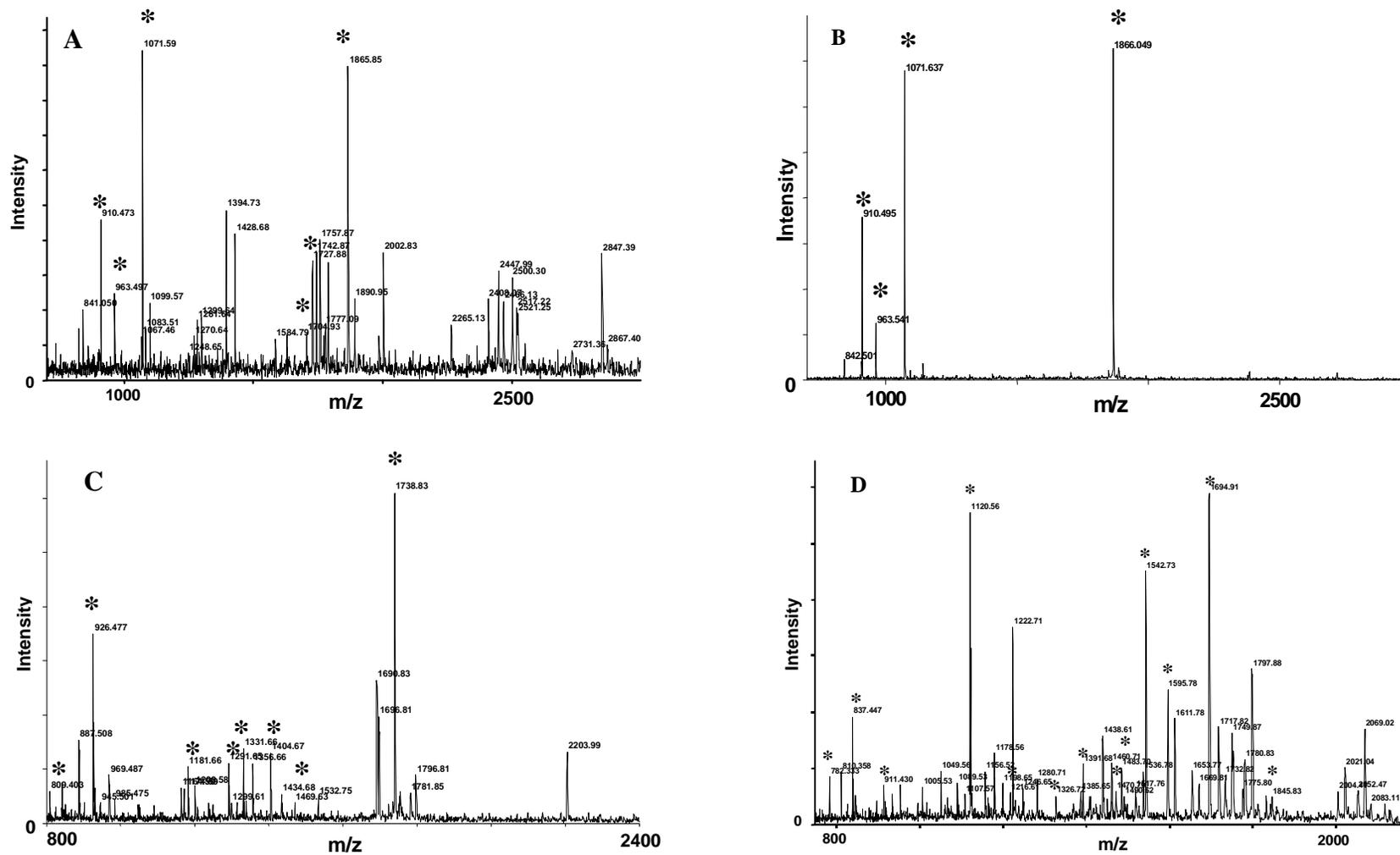


Figure 6

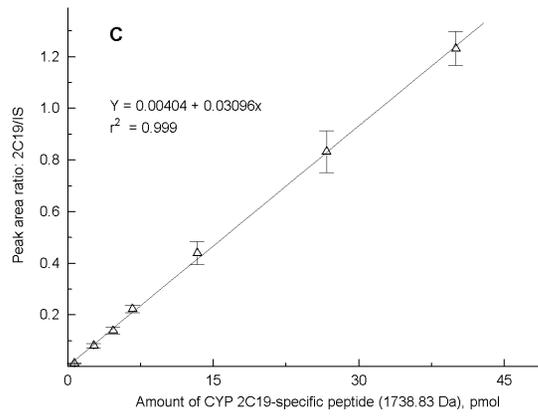
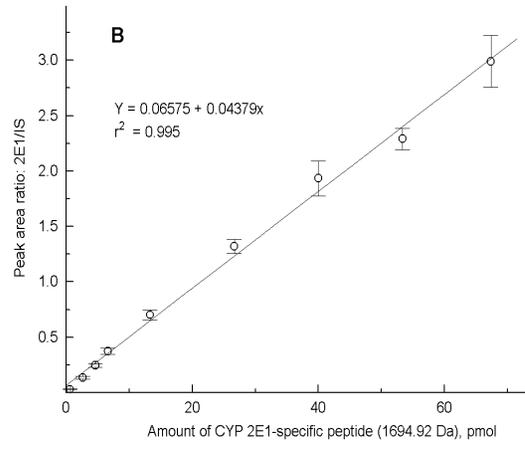
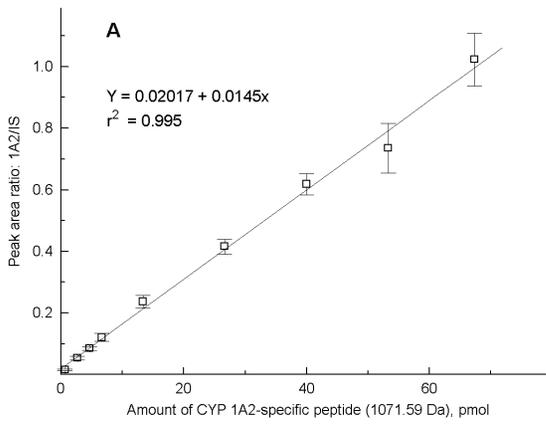


Figure 7

Figure 8

