RESEARCH ARTICLE

# Enrichment of low molecular weight fraction of serum for MS analysis of peptides associated with hepatocellular carcinoma

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A challenging aspect of biomarker discovery in serum is the interference of abundant proteins with identification of disease-related proteins and peptides. This study describes enrichment of serum by denaturing ultrafiltration, which enables an efficient profiling and identification of peptides up to 5 kDa. We consistently detect several hundred peptide-peaks in MALDI-TOF and SELDI-TOF spectra of enriched serum. The sample preparation is fast and reproducible with an average CV for all 276 peaks in the MALDI-TOF spectrum of 11%. Compared to unenriched serum, the number of peaks in enriched spectra is 4 times higher at an S/N ratio of 5 and 20 times higher at an S/N ratio of 10. To demonstrate utility of the methods, we compared 20 enriched sera of patients with hepatocellular carcinoma (HCC) and 20 age-matched controls using MALDI-TOF. The comparison of 332 peaks at $p < 0.001$ identified 45 differentially abundant peaks that classified HCC with 90% accuracy in this small pilot study. Direct TOF/TOF sequencing of the most abundant peptide matches with high probability des-Ala-fibrinopeptide A. This study shows that enrichment of the low molecular weight fraction of serum facilitates an efficient discovery of peptides that could serve as biomarkers for detection of HCC as well as other diseases.

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**Abbreviations: HCC**, hepatocellular carcinoma; **HMW**, high molecular weight; **LMW**, low molecular weight; **SVM**, support vector machine

## 1 Introduction

Discovery of biomarkers for clinical use typically requires comparison of a large number of samples, which limits the applicability of many elegant proteomic methods [1, 2]. SELDI-TOF and SELDI-QqTOF analyses of serum were optimized for high-throughput comparison of biological samples as small as a few microliters [3]. Advanced statistical

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and computational methods were designed to compare the crude mixtures of proteins present in unfractionated serum. The results were surprisingly encouraging given the complexity of the problem and the performance of currently used markers [4–8]. Alpha-fetoprotein, the only serum marker for diagnosis of hepatocellular carcinoma (HCC), has reported sensitivity of 39–64% and specificity of 76–91% [9]. SELDI-based studies of HCC reported sensitivities of 61–90% and specificities of 76–95% [10–13]. Recent analyses raised the question of possible biases in profiling studies, which underscores the need for verification of biomarker identities [14–18]. The identification requires challenging complementary methods in SELDI-TOF experiments [12]. Even SELDI-QqTOF experiments did not sequence the identified biomarker candidates [3]. Here we report an improved protocol that allows an efficient comparison of samples by TOF MS, and identification of many peaks of interest in the low molecular weight (LMW) region by direct TOF/TOF sequencing. The utility of the method was tested in a pilot study of HCC, one of many diseases that could benefit from identification of molecular markers in serum.

## 2 Materials and methods

### 2.1 Materials

C8 magnetic bead desalting kits, CHCA, and MALDI 600-μm AnchorChip were purchased from Bruker Daltonics (Billerica, MA, USA). SELDI protein arrays were obtained from Ciphergen (Fremont, CA, USA). Microcon ultrafiltration membranes with 10–50-kDa cut-off were purchased from Millipore (Bedford, MA, USA). Red top vacutainer blood collection tubes (BD 366430) were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Tricine 10–20% gradient gels for SDS-PAGE and SYPRO Ruby stain were obtained from Invitrogen (Carlsbad, CA, USA). BCA protein assay was purchased from Pierce Biotechnology (Rockford, IL, USA). Other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA); solvents were of HPLC grade.

### 2.2 Blood samples

A single batch of serum aliquots (standard serum sample) was frozen at −80°C and was used throughout the study to perform method optimization and quality control. Blood samples of cancer cases and controls were obtained between October 2002–April 2003 in collaboration with the National Cancer Institute of Cairo University, Egypt. Controls were recruited among patients from the orthopedic fracture clinic at the Kasr El-Aini Hospital (Cairo, Egypt) and were frequency-matched by gender, rural versus urban birthplace, and age to cancer cases as described previously [19]. Blood samples for all participants were collected around 10 am, were processed within several hours of collection and then immediately frozen at −80°C in 1-mL aliquots. At first thaw, sub-aliquots of 50 μL were generated and stored at −80°C until mass spectrometric analysis. All measurements were performed on aliquots of twice-thawed sera.

### 2.3 Sample preparation

Serum (15 μL) was desalted on C8 magnetic beads according to the manufacturer's protocol (Bruker Daltonics) and eluted with 50% ACN. Microcon membranes were washed four times with 0.15 mL $dH_2O$. Samples were diluted with $dH_2O$ to a final concentration of 25% ACN (60 μL total volume) and ultrafiltered at $12\,000 \times g$ for 5 min using a 50-kDa Microcon membrane. Ultrafiltrate was dried in a centrifugal vacuum evaporator, reconstituted in 2 μL 5% ACN with 0.1% TFA and mixed 1:1 with CHCA matrix (3.3 mg/mL in 50% ACN). The sample (1 μL) was deposited on SELDI gold array or MALDI anchor chip and allowed to crystallize at room temperature.

### 2.4 SELDI-TOF MS

PBS II Protein Chip Array reader (Ciphergen) was externally calibrated using the $[M+H]^+$ ions of five peptides in the range 1084–7033 $m/z$. All mass spectra were recorded in positive-ion mode at 20 Hz with an 80 ns delay. Saturated solution of CHCA matrix (1 μL) in 50% aqueous ACN containing 0.1% TFA was added to each sample for SELDI-TOF MS analysis.

### 2.5 MALDI-TOF MS

Samples were analyzed using an Ultraflex MALDI TOF/TOF mass spectrometer (Bruker Daltonics) equipped with pulsed ion extraction ion source. Ionization was achieved by irradiation with a nitrogen laser ($\lambda$ = 337 nm) operating at 20 Hz. Ions were accelerated at +19 kV with 80 ns of pulsed ion extraction delay. Each spectrum was detected in linear positive mode and was externally calibrated using a mixture of peptide/protein standards between 1 and 4 kDa. Mass spectra were analyzed using the Flex Analysis and ClinProTools softwares (Bruker Daltonics). Raw data were exported as text files for further analysis.

### 2.6 Gel electrophoresis

Proteins were analyzed by SDS-PAGE using a 10–20% gradient tricine gel and visualized by staining with SYPRO Ruby (Invitrogen) according to manufacturer's protocol.

### 2.7 Analysis of TOF MS spectra

To reduce the noise and dimensionality of the raw spectra, we used a binning procedure (100-ppm step) that divides the $m/z$ axis into 23 846 bins in the 0.9–10 kDa region. The maximum intensity within each interval was used as the

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protein expression variable for each spectrum. The baseline of each spectrum was estimated by obtaining the minimum intensity within a shifting widow size of 50 bins. Spline approximation was used to regress the varying baseline and the regressed baseline was subtracted from the spectrum. Each spectrum was normalized by dividing by its total ion current and the spectra were scaled to have an overall maximum intensity of 100. We used two methods for peak detection. One method was used to define S/N ratios and number of peaks [5]. The second method [5] was modified for our study to calculate peak intensities for biomarker discovery. In this latter method, a bin was identified as a peak if the sign of the intensity's slope changed from positive to negative. Peaks with intensity below a pre-defined threshold line were considered as noise and were discarded. To account for drift in *m/z* location in different spectra, two peaks were coalesced into a window if they differed in location by at most 7 bins or 0.03% relative mass. The maximum intensity in each window was defined as the peak-intensity variable. To distinguish cancer patients and healthy individuals, the processed spectra were split into training and testing (independent) datasets. The training dataset was used to select *m/z* windows, to compare their intensities using a random variance *t*-test, and to build a support vector machine (SVM) classifier. Prediction accuracy of the classifier was evaluated using the independent dataset. These functions were performed in BRB-ArrayTools 3.1 software (NCI, Bethesda, MD, USA) [20, 21]. Other analyses were carried out in MATLAB (MathWorks, Natick, MA, USA), Clin ProTools (Bruker Daltonics) and Splus (MathSoft Inc., Cambridge, MA, USA) analytical software packages.

## 3 Results

Denaturing ultrafiltration enriches the LMW fraction of serum and plasma (Fig. 1). Removal of proteins greater than 50 kDa including albumin appears efficient. SYPRO Ruby staining detected at most traces of albumin in the ultrafiltered serum. Some proteins smaller than 50 kDa are also removed as shown by the SDS-PAGE in Fig. 1. This is consistent with the manufacturer's definition of the 50-kDa cutoff based on retention of analytes. It is expected that partial removal of analytes occurs below the specified cutoff. Our studies focus on peptides <5 kDa because of the biological importance of this fraction of serum and optimal performance of the MALDI-TOF/TOF instrument in this LMW region. Minimal losses are expected in the <5-kDa region provided that protein-protein interactions are disrupted. In this study, ultrafiltration was carried out in the presence of 25% ACN, which allowed removal of high molecular weight (HMW) proteins including albumin with concurrent enrichment of the LMW fraction sufficient for detection of several hundred peptides. Quantification of proteins in the desalted ultrafiltrate by BCA assay (Pierce) showed a 15% increase in the protein content with the addition of

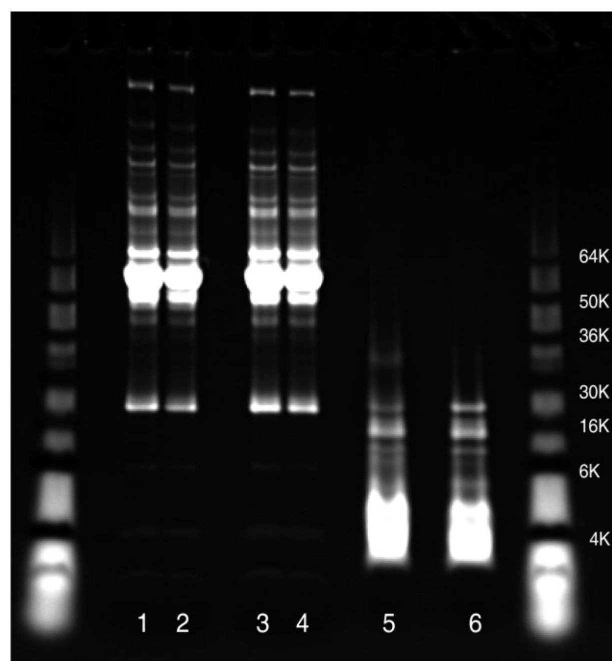


**Figure 1.** SDS-PAGE analysis of human plasma and serum. Lanes 1 and 2, unfiltered plasma, lanes 3 and 4, unfiltered serum, lane 5, enriched LMW plasma and lane 6, enriched LMW serum; 2 μg total protein was applied per lane and visualized by SYPRO Ruby staining.

ACN (0.30 ± 0.029 μg with 25% ACN; 0.26 ± 0.027 μg without ACN during ultrafiltration). Further experiments are needed to compare various denaturing ultrafiltration conditions [22–24].

The enrichment procedure begins with desalting of serum on C8 magnetic beads, followed by ultrafiltration on a 50-kDa cut-off membrane as described in Materials and methods. The desalted ultrafiltrate can be analyzed on any MS platform of choice. Here we show SELDI-TOF (Fig. 2) and MALDI-TOF (Fig. 3) mass analysis of the LMW fraction spotted with CHCA matrix. The figures align three spectra of independently processed aliquots of a serum standard (out of 9 SELDI spectra and 15 MALDI spectra used to estimate CV). We optimized the method for MALDI-TOF analysis of the 1–5-kDa region using an Ultraflex mass spectrometer (Bruker Daltonics). This instrument allows direct identification of peptides in this mass range by TOF/TOF sequencing.

The method is reproducible as shown by the calculated CV. The mean CV across all peaks ($n = 194$) in the 9 SELDI-TOF replicate spectra is 17%. The mean CV across all peaks ($n = 276$) in the 15 MALDI-TOF replicate spectra is 11%. Reproducibility was determined for smoothed, baseline-corrected, and normalized spectra (Fig. 4). Signal processing decreases the CVs approximately two times, which is mostly accounted for by the normalization step. Table 1 compares CVs of small (1–3% maximum peak intensity), medium (5–15% maximum peak intensity), and large (>25% maximum

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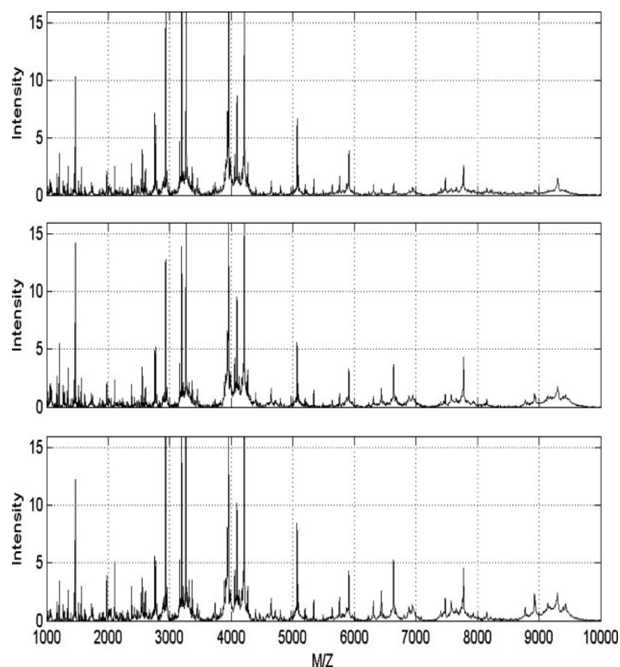


**Figure 2.** SELDI-TOF spectra, three independently prepared samples of enriched LMW fraction of serum.

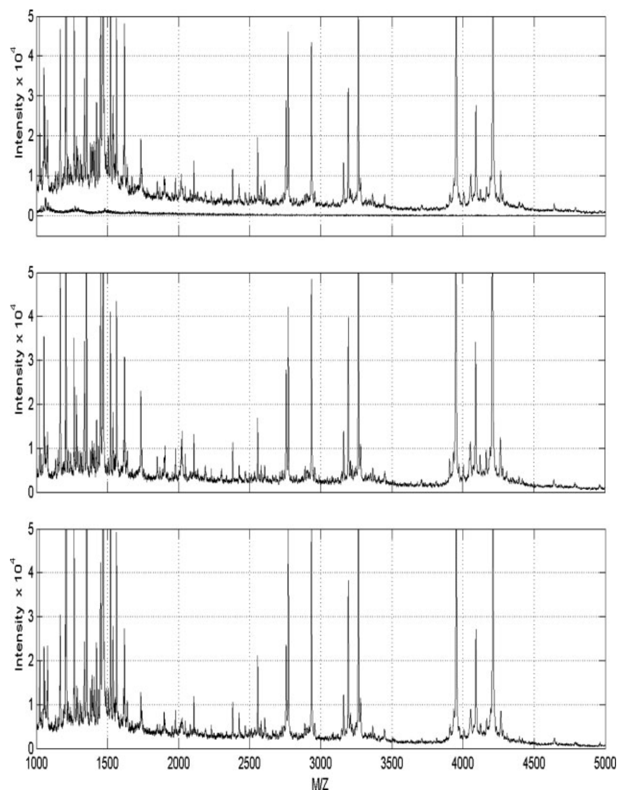


**Figure 3.** MALDI-TOF spectra, three independently prepared samples of enriched LMW fraction of serum. A spectrum of CHCA matrix without sample is presented for comparison in the top panel.

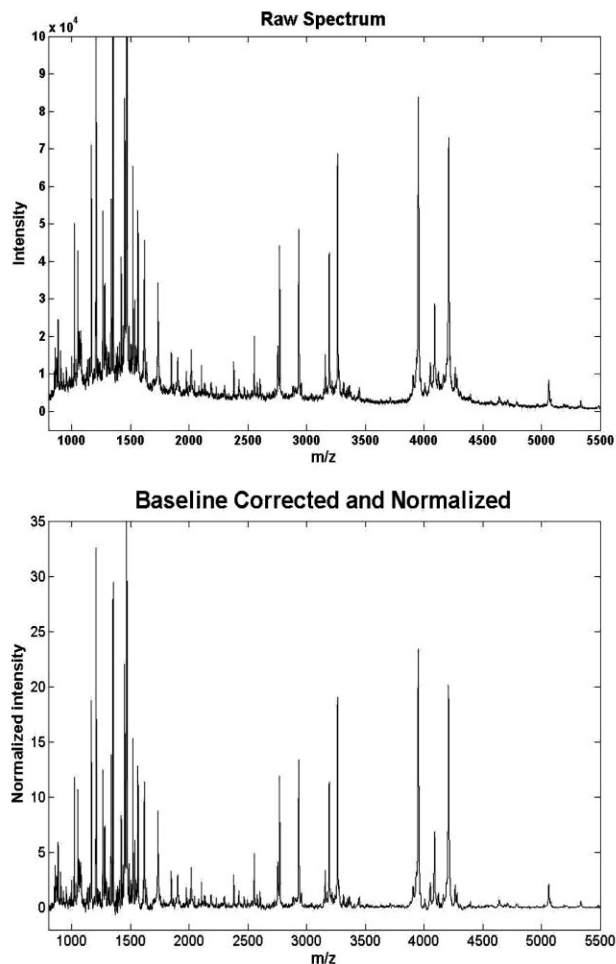


**Figure 4.** MALDI-TOF spectrum of a standard serum sample processed by smoothing, baseline correction, and normalization. Top panel: raw spectrum; bottom panel: processed spectrum.

**Table 1.** CV for SELDI-TOF ($n = 9$) and MALDI-TOF ($n = 15$) spectra of independently processed aliquots of standard serum; five peaks were randomLy selected for each group

| | | CV1 | CV2 | CV3 | CV4 | CV5 | Mean CV |
|---|---|---|---|---|---|---|---|
| SELDI | High | 9.4 | 9 | 16.1 | 20.6 | 12.6 | 13.6 |
| | Medium | 17.4 | 23 | 29 | 8.8 | 11.3 | 17.9 |
| | Low | 18.1 | 13.2 | 14.6 | 14.1 | 19.3 | 15.9 |
| MALDI | High | 9.3 | 7.8 | 7.2 | 8.9 | 8.3 | 8.3 |
| | Medium | 12.4 | 9.3 | 10.1 | 13.9 | 6.8 | 10.5 |
| | Low | 8.8 | 10.4 | 9.2 | 16.8 | 5.3 | 10.1 |

peak intensity) peaks. Five peaks were randomLy selected in each category for this comparison. The mean CV ranges from 8% to 11% for MALDI-TOF and from 14% to 18% for SELDI-TOF spectra in the three intensity groups. The CVs do not vary substantially with signal intensity.

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MALDI-TOF spectra of enriched serum prepared as described above have, in our hands, substantially better quality than spectra of C8 desalted serum without ultrafiltration (Fig. 5). The increase in signal quality is demonstrated by a 4-fold increase in the number of peaks at an S/N of 5 and a 25-fold increase at an S/N of 10. It is expected that removal of highly abundant proteins by ultrafiltration under denaturing conditions will improve detection of LMW peptides. We have optimized the procedure such that the eluate of the C8 desalting step is diluted with $dH_2O$ to a final concentration of 25% ACN for the ultrafiltration step. Under these conditions the recovery of peptides from ultrafiltration on the 50-kDa membranes is adequate and reproducible.

The applicability of the method was tested in a pilot study of HCC. We compared spectra of patients with HCC ($n = 20$) and matched controls ($n = 20$) to examine whether we can identify peptides associated with the disease. Overlay of the spectra in ClinProTools software (Bruker Daltonics) is shown in Fig. 6. The picture enlarges the 1200–1900-Da region to better visualize the differences. There are several peaks that strikingly differ between the two groups. This is highlighted in the overlay of average spectra ($n = 20$ for each group) presented in Fig. 7. To carry out a preliminary statistical comparison of this pilot dataset, we first identified peaks in a training set of 10 HCC cases and 10 controls. Our methods defined a total of 332 peaks in the MALDI-TOF spectra yielding a $332 \times 20$ matrix of peak intensities. This was used to compare the HCC cases and controls by random variance *t*-test (BRB Array Tools 3.1). The *t*-test identified 45 differentially abundant peaks ($p < 0.001$) that were used to build an SVM classifier. Compared to controls, 34 peaks are increased in HCC and 11 peaks are decreased. The classifier correctly predicted the presence of HCC in 18 of 20 spectra (90% sensitivity and specificity) in an independent test set (10 cases and 10 controls). The independent test spectra were not used for either peak finding or definition of the SVM classifier. Since the test set is small, the data should be viewed as preliminary. The identified peaks are not confirmed biomarkers of HCC and a larger set of samples will have to be used together with multivariate analyses to validate these encouraging pilot results.

The sequence of the most abundant discriminating peptide (*m/z* 1465.6 Da) was defined by TOF/TOF sequencing as DSGEGDFLAEGGGVR, which matches with high probability (MASCOT score 127) the sequence of des-Ala-fibrinopeptide A (Fig. 8). The possibility to directly sequence the peptides of interest is a powerful feature of this method.

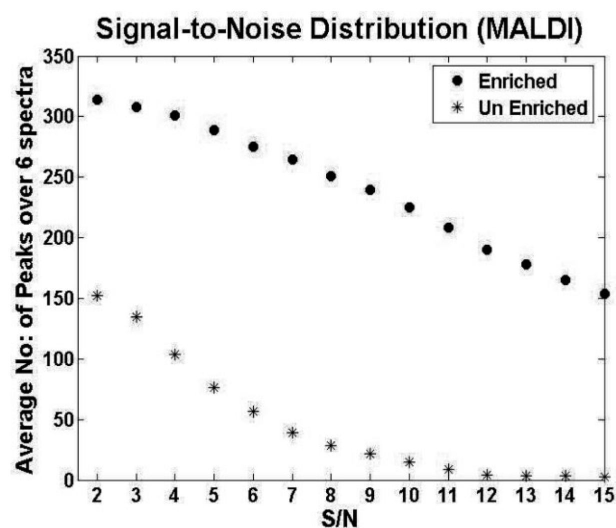


**Figure 5.** Enrichment of samples by denaturing ultrafiltration expressed as number of peaks at an S/N ratio of 2–15.

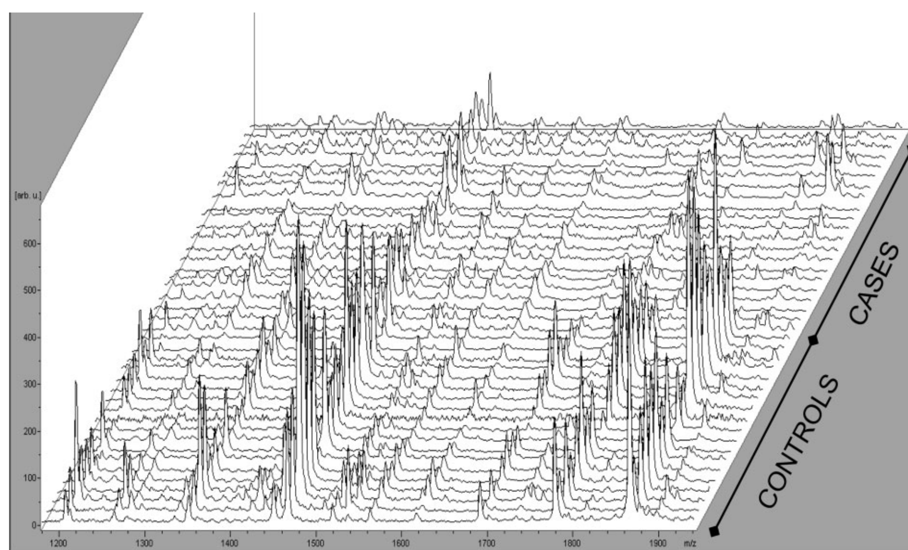


**Figure 6.** Alignment of 20 HCC cases and 20 controls, mass region of 1200–1900 Da, ClinProTools (Bruker Daltonics).

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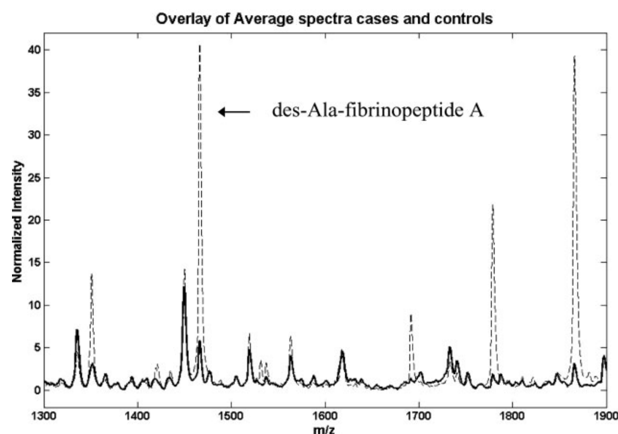


**Figure 7.** Average spectra of HCC cases and matched controls. Full line: Cases ($n$ = 20); broken line: controls ($n$ = 20).

## 4 Discussion

Analysis of unfractionated serum samples by SELDI-TOF allows comparison of a large number of samples, but the resolution of the method is limited. It has been proposed that fractionation may be needed to detect less abundant proteins and peptides [17, 25]. More than 95% of serum proteins are represented by 22 species; albumin alone represents about 50% of the serum proteome. Disease-associated proteins are typically found in the remaining fraction [22, 26]. This suggests that removal of HMW carriers without loss of the LMW fraction should improve MS-based biomarker discovery.

We adapted denaturing ultrafiltration, used previously for improved LC-MS/MS and FT-ICR-MS [22, 23, 27], for MALDI TOF/TOF analysis of serum. Our method combines desalting/concentration on C8-derivatized magnetic beads [24, 28] with denaturing ultrafiltration [22]. Desalting on magnetic beads has a higher capacity compared to SELDI surfaces and minimizes handling volumes (compared to small columns, *etc.*). The paramagnetic properties of the particles allow easy handling and automation of the procedure [24]. We selected the 50-kDa cut-off in the presence of ACN, because it efficiently eliminates albumin and other large abundant proteins and allows isolation of an enriched peptide fraction (Figs. 1–5). This method is more effective for isolation of the LMW fraction than immunodepletion, and facilitates comparison of large numbers of samples. It was shown that the abundant HMW proteins bind other peptides/proteins [29–31]. Immunodepletion of the HMW carriers, which can lead to loss of disease-associated peptides, has also been proposed as an enrichment strategy for recovery of the biomarker candidates [32]. Disruption of the interactions by various denaturing conditions including organic solvents is used to improve recovery of peptides [22, 33].

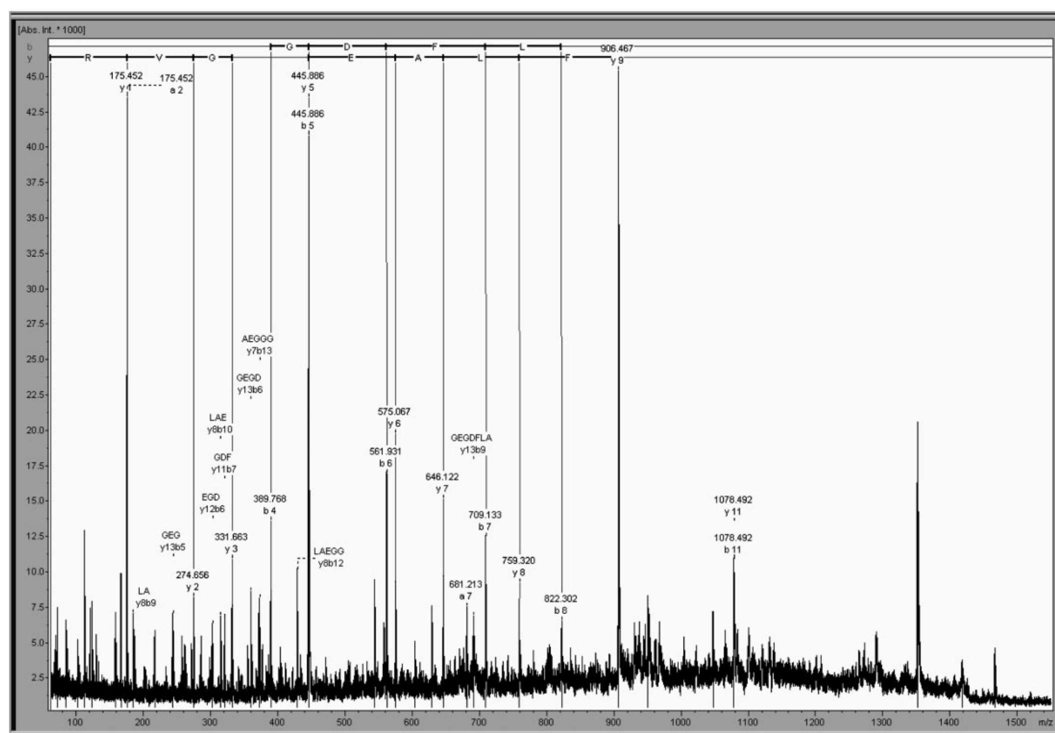


**Figure 8.** MALDI-TOF/TOF spectrum of peptide with mass 1465.6 Da. Sequence DSGEGDFLAEGGGVR matches with high probability des-Ala-fibrinopeptide A (MASCOT score 127).

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The process was streamlined for fast processing of small samples (15 µL serum). In our hands, the enrichment is substantial compared to C8-desalted serum without ultrafiltration (Fig. 5). This method is reproducible with about 10% mean CV across identified peaks (Fig. 5). Reproducibility is critical for biomarker discovery. The enrichment is higher than previously reported likely because of optimal denaturing conditions using ACN and use of different magnetic bead particles [24]. Analysis of unfractionated serum requires careful selection of a batch of magnetic beads with adequate performance [34]. We did observe efficient elimination of albumin and other large proteins [23]. Traces of albumin and presence of other HMW contaminants in our samples did not limit our ability to obtain well-resolved reproducible spectra (Fig. 2). However, we did see elimination of proteins smaller than 50 kDa (Fig. 1). This may be due to short filtration times, which limits contact of ACN with the membrane. Detailed analysis of filters with lower cut-off under various denaturing conditions is needed to further optimize the enrichment for specific applications.

Our method was applied to a pilot analysis of HCC, one of many diseases that could benefit from improved classification based on molecular markers in serum. HCC is a common cancer worldwide with as many as 500,000 new cases each year [35]. Egypt is a country with high rates of chronic HCV infection and associated HCC [36, 37]. The 40 samples analyzed in this study are a subset of a large study of HCC we conducted in Egypt [19]. We proposed that a set of peptides associated with HCC could be present in serum and serve as a biomarker. Cancer biomarkers in serum are not necessarily new antigens; they are often modified peptides and fragments of proteins [22, 26, 38]. Proteases and peptidases are reportedly deregulated in HCC [39–41], and it is reasonable to expect that cancer-specific fragments of proteins will be found in the LMW region [12].

Here we demonstrate that our method is efficient for discovery of peptides associated with HCC. We should emphasize that the described peptides are not validated biomarkers. Larger studies and additional analyses are needed to confirm that these biomarker candidates have utility. Nonetheless, the results are encouraging. We identified 45 peptides that are associated with HCC and classify the disease with 90% prediction accuracy in this small pilot. Importantly, the method allows direct identification of the peptides by TOF/TOF sequencing as demonstrated by the identification of the peak at $m/z$ 1465.6 as a fragment of fibrinopeptide A.

Recent studies noted that human serum contains fragments of relatively common proteins [42]. These fragments are expected to be present in the ultrafiltered serum under our experimental conditions. A fragment of fibrinopeptide A was in fact the most abundant peptide in our study (Fig. 7) and in a comparable study of ultrafiltered serum using FT-ICR [27]. It is not clear whether these fragments and their PTMs can be used for disease classification. The specificity of these fragments and modified peptides was not explored. The composition of the peptide mixture in our samples remains to be defined. The distinct pattern of peptides in HCC is most likely related to differential proteolytic activity in cancer patients. It will be important to define whether the peptides represent cancer-related antigens, fragments derived from the activity of tumor-related proteases, or host response. The use of combinations of peptides to define disease status was not studied extensively. Currently, it is unclear whether a combination of peptides related to tumor-related enzymatic activities or host response can provide an efficient biomarker.

In summary, we describe a sensitive high-throughput platform for discovery of biomarker candidates among peptides in the LMW fraction of serum. This method combines C8 desalting and denaturing ultrafiltration for simultaneous measurement of several hundred peptides by MALDI-TOF. Biomarkers can improve disease classification, early detection and intervention, assessment of disease progression, and possibly long-term outcomes. It will be important to use all available information (quantity, sequence, modifications, etc) to find optimal peptide biomarkers and their combinations. The presented method is expected to facilitate the discovery of biomarkers among peptides in the LMW fraction of serum.

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## 5 References

[1] Veenstra, T. D., Conrads, T. P., Hood, B. L., Avellino, A. M. *et al.*, *Mol. Cell. Proteomics* 2005, *4*, 409–418.

[2] Hanash, S., *Nature* 2003, *422*, 226–232.

[3] Conrads, T. P., Fusaro, V. A., Ross, S., Johann, D. *et al.*, *Endocr. Relat Cancer* 2004, *11*, 163–178.

[4] Petricoin, E. F., Zoon, K. C., Kohn, E. C., Barrett, J. C. *et al.*, *Nat. Rev. Drug Discov.* 2002, *1*, 683–695.

[5] Coombes, K. R., Fritsche, H. A., Jr., Clarke, C., Chen, J. N. *et al.*, *Clin. Chem.* 2003, *49*, 1615–1623.

[6] Qu, Y., Adam, B. L., Thornquist, M., Potter, J. D. *et al.*, *Biometrics* 2003, *59*, 143–151.

[7] Ressom, H. W., Varghese, R. S., Abbel-Hamid, M., Eissa, S. A. *et al.*, *Bioinformatics* 2005, *21*, 4039–4045.

[8] Yasui, Y., Pepe, M., Thompson, M. L., Adam, B. L. *et al.*, *Biostatistics* 2003, *4*, 449–463.

[9] Lopez, L. J., Marrero, J. A., *Curr. Opin. Gastroenterol.* 2004, *20*, 248–253.

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[10] Schwegler, E. E., Cazares, L., Steel, L. F., Adam, B. L. *et al.*, *Hepatology* 2005, *41*, 634–642.

[11] Zhu, X. D., Zhang, W. H., Li, C. L., Xu, Y. *et al.*, *World J. Gastroenterol.* 2004, *10*, 2327–2329.

[12] Paradis, V., Degos, F., Dargere, D., Pham, N. *et al.*, *Hepatology* 2005, *41*, 40–47.

[13] Poon, T. C., Yip, T. T., Chan, A. T., Yip, C. *et al.*, *Clin. Chem.* 2003, *49*, 752–760.

[14] Liotta, L. A., Lowenthal, M., Mehta, A., Conrads, T. P. *et al.*, *J. Natl. Cancer Inst.* 2005, *97*, 310–314.

[15] Petricoin, E. F., Fishman, D. A., Conrads, T. P., Veenstra, T. D. *et al.*, *Proteomics* 2004, *4*, 2357–2360.

[16] Baggerly, K. A., Morris, J. S., Coombes, K. R., *Bioinformatics* 2004, *20*, 777–785.

[17] Diamandis, E. P., *Mol. Cell. Proteomics* 2004, *3*, 367–378.

[18] Ransohoff, D. F., *J. Natl. Cancer Inst.* 2005, *97*, 315–319.

[19] Ezzat, S., Abdel-Hamid, M., Eissa, S. A. L., Mokhtar, N. *et al.*, *Int. J. Hyg. Environ. Health* 2005, *208*, 329–339.

[20] Simon, R., Radmacher, M. D., Dobbin, K., McShane, L. M., *J. Natl. Cancer Inst.* 2003, *95*, 14–18.

[21] Simon, R., *Br. J. Cancer* 2003, *89*, 1599–1604.

[22] Tirumalai, R. S., Chan, K. C., Prieto, D. A., Issaq, H. J. *et al.*, *Mol. Cell. Proteomics* 2003, *2*, 1096–1103.

[23] Harper, R. G., Workman, S. R., Schuetzner, S., Timperman, A. T. *et al.*, *Electrophoresis* 2004, *25*, 1299–1306.

[24] Villanueva, J., Philip, J., Entenberg, D., Chaparro, C. A. *et al.*, *Anal. Chem.* 2004, *76*, 1560–1570.

[25] Ransohoff, D. F., *J. Natl. Cancer Inst.* 2005, *97*, 315–319.

[26] Pieper, R., Gatlin, C. L., Makusky, A. J., Russo, P. S. *et al.*, *Proteomics* 2003, *3*, 1345–1364.

[27] Bergen, H. R. III, Vasmatzis, G., Cliby, W. A., Johnson, K. L. *et al.*, *Dis. Markers* 2003, *19*, 239–249.

[28] Zhang, X., Leung, S. M., Morris, C. R., Shigenaga, M. K., *J. Biomol. Tech.* 2004, *15*, 167–175.

[29] Zhou, M., Lucas, D. A., Chan, K. C., Issaq, H. J. *et al.*, *Electrophoresis* 2004, *25*, 1289–1298.

[30] Liotta, L. A., Ferrari, M., Petricoin, E., *Nature* 2003, *425*, 905.

[31] Lowenthal, M. S., Mehta, A. I., Frogale, K., Bandle, R. W. *et al.*, *Clin. Chem.* 2005, *51*, 1933–1945.

[32] Mehta, A. I., Ross, S., Lowenthal, M. S., Fusaro, V. *et al.*, *Dis. Markers* 2003, *19*, 1–10.

[33] Fu, Q., Garnham, C. P., Elliott, S. T., Bovenkamp, D. E. *et al.*, *Proteomics* 2005, *5*, 2656–2664.

[34] Villanueva, J., Philip, J., Chaparro, C. A., Li, Y. *et al.*, *J. Proteome Res.* 2005, *4*, 1060–1072.

[35] Montalto, G., Cervello, M., Giannitrapani, L., Dantona, F. *et al.*, *Ann. N. Y. Acad. Sci.* 2003, *963*, 13–20.

[36] Kew, M. C., *Clin. Lab Med.* 1996, *16*, 395–406.

[37] Nada, O., Abdel-Hamid, M., Ismail, A., El Shabrawy, L. *et al.*, *J. Clin. Virol.* 2005, *34*, 140–146.

[38] Schulz-Knappe, P., Zucht, H. D., Heine, G., Jurgens, M. *et al.*, *Comb. Chem. High Throughput Screen.* 2001, *4*, 207–217.

[39] Zhou, X. D., *Hepatobiliary Pancreat. Dis. Int.* 2002, *1*, 35–41.

[40] Sun, Z., Yang, P., *Lancet Oncol.* 2004, *5*, 182–190.

[41] Feitelson, M. A., Pan, J., Lian, Z., *Surg. Clin. North Am.* 2004, *84*, 339–354.

[42] Koomen, J. M., Shih, L. N., Coombes, K. R., Li, D. *et al.*, *Clin. Cancer Res.* 2005, *11*, 1110–1118.

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