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Optimization of magnetic beads for MALDI-TOF MS analysis

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1. ABSTRACT

MALDI-TOF MS is being widely applied to analyze serum samples for the diagnosis of human diseases and for the identification of potential biomarkers. However, different types of magnetic beads afford different purification capability for low abundance proteins and peptides in body fluids. The aim of our study was to establish a proteome fractionation technique for choosing magnetic beads for proteomic analysis in breast cancer research. We used three different kinds of magnetic beads (MB-HIC C8, MB-IMAC-Cu and MB-WCX) to pre-analyze 28 breast cancer patients and 24 healthy control serum samples for proteomic profiling by MALDI-TOF MS analysis. The serum samples purified by MB-WCX group provided the best proteomic pattern .These samples had the most average peak numbers, the highest peak intensities, and the best capturing ability of low abundance proteins or peptides in serum samples. Therefore, we chose MB-WCX as our best magnetic beads to pre-analyze large-scale cancer and healthy control samples. The application of the standardized, pre-analytical serum samples with right type of magnetic bead-based fractionation will decrease variability of proteome patterns in human serum assessed by MALDI-TOF MS.

2. INTRODUCTION

Breast cancer is one of the most common malignant tumors in women. For the moment, it is estimated that there are about 1,200,000 women who suffer from breast cancer, and 500,000 have died from breast cancer worldwide (1). And, the incidence of breast cancer is rising. The morbidity and mortality varies in different countries and regions. In China, the morbidity increases more than 3% each year (2).

In the long, natural history of breast cancer, there seems early diagnosis appears to be the most appropriate tool to reduce disease-related mortality. At present, there is no early detection and diagnostic test with high sensitivity, specificity, which can be used as a clinical, routine screening tool. Therefore, the need for new biomarkers for breast cancer that can improve early diagnosis, monitoring of disease progression, therapeutic response, and detect disease recurrence is so important (3).

Despite the great effort in developing new therapies to improve the survival of breast cancer patients, the results are just modest with the exceptions being select early cancers (4-7). The majority of patients diagnosed with cancer are usually in the late stage of the disease. On

the other hand, if these cancers are diagnosed early, and the organs can be confined at the same time, the survival rate will exceed about 85% (4-5).Obviously, with the current therapies and treatments, only the improvements in early detection of cancer will lead to the improvements in cancer survival. So, it is crucial to develop high throughput tests to diagnose cancers earlier.

By using mass spectrometry (MS) to generate proteome profiles offers a wide range of possibilities for analyzing biological samples which contain complex mixtures of proteins or peptides. The aim of most profiling experiments is to identify changes in proteomic patterns that are related to a certain disease or clinical changes. Therefore, these patterns might be used to carry early diagnose, offer accurate prognosis, monitor the disease progression or response to treatment, and even to identify which patients are most likely to benefit from particular treatments (8-14).

The mass spectrometry instruments that are most commonly used to solve these clinical and biological problems use a matrix-assisted laser desorption, ionization (MALDI) ion source and a time-of-flight (TOF) detection system. In a word, the plate is inserted into a vacuum chamber, and the matrix crystals are struck with pulses from a nitrogen laser. The matrix molecules absorb energy from the laser, transfer it to the proteins causing them to desorb and ionize, and then produce a plume of ions in the gas phase.

Mass spectrometric analysis of proteins or peptides is most successful when the samples are analyzed in their purest state. The presence of salts and other small molecules can significantly interfere with the analysis, often with negative effects. Hence resulting suppression of the proteins or peptides of interest can severely reduce the amount of information that can be obtained from an experiment. Desalting and fractionation of the proteins or peptides mixtures are, therefore, required to separate out the compounds of interest. Alternatively, the specific enrichment of target molecules can be isolated based on affinity techniques. Therefore, molecules to be enriched will be absorbed reversibly by a complementary binding substance on an insoluble support. Magnetic beads are the way we do the fractionation. Magnetic beads are micro-particles available with a range of different surface chemistries providing a choice of functionality. These beads are used for sample preparation (for example, serum, plasma, urine, cerebrospinal and peritoneal fluid) before MALDI-TOF MS analysis (15).

We can use so many kinds of kits, such as MB-C3, C8 and C18, MB-WCX, WAX, MB-IMAC Fe Cu, MB-IAC Prot G, etc. These are available. The MB-HIC kits are based on super-paramagnetic micro-particles with a highly porous surface functionalized with hydrophobic coatings (C3, C8, C18). The MB-WCX kit is based on super-paramagnetic micro-particles with negatively charged functional groups at their surface enabling cation exchange chromatography. Binding of substances occurs due to reversible adsorption of charged molecules in the solution to immobilized ion exchange groups of opposite charge. The MB-IMAC Fe kit is designed for selective isolation and pre-concentration of phosphopeptides (e.g. protein digests) on the surface of super-paramagnetic micro-particles. Specific interaction between phosphorylated amino acid residues of peptides and magnetic beads is realized via the particle surface functionalized with immobilized polyvalent metal ions (e.g. Fe^{3+}). Using the MB-IMAC Cu Kit, proteins and peptides are separated on the basis of their affinity to Cu ions which have been immobilized by chelation. The immobilized complex has one coordination site available for interaction with amino acid side chains (e.g. –COO-) on the protein surface. The kit MB-IAC Prot G Kit was developed for specific capturing of antigens (e.g. peptides, proteins) from biological fluids based on their affinity to corresponding antibodies. And, we use AnchorChip targets instead of steel targets, because AnchorChip targets produce more homogenous matrix crystallization with better reproducibility of MS spectra than crystallization on steel targets (15).

But, choosing the right types of magnetic beads so that we can get as many as possible proteins and peptides for the further proteomics profiling analysis as possible is a major concern. The objective of this study was to assess the feasibility of choosing the correct magnetic bead based on MALDI-TOF MS analysis for breast cancer samples and healthy control individuals.

3. MATERIALS AND METHODS

3.1. Chemicals and calibrators

Gradient-grade alcohol and acetone were obtained from J.T. Baker (USA). p.a.trifluoroacetic acid and Ammonium acetate were purchased from Sigma-Aldrich(USA). The peptide calibrator containing Angiotensin II, the protein calibrator, and the matrix α-Cyano-4-hydroxycinnamic acid (CHCA) were purchased from Bruker Daltonics GmbH (Germany). For magnetic bead preparations, we used 0.2 mL of polypropylene tubes (8-tube strips) from Axygen Company (USA). Multiple needle sets and polypropylene vacuette without anticoagulants (EDTA, heparin and citrate) were obtained from BD Company (USA).

3.2. Blood samples

The blood samples were processed according to a standardized protocol.

1. After sample collection, the vacuette serum (collected in a red-top glass tube containing no preservatives or anticoagulant) were allowed to clot or to sediment at room temperature (about 25^0C) for at least half an hour and then centrifuged at 1500g for 15 min.

2. Samples were divided in aliquots of 100µl immediately and frozen at -80^0C refrigerator until use.

3. For proteomic fractionation, samples were thawed at room temperature (about 25^0C) for 15 min and processed immediately.

Table 1. Instrument parameter settings

ion source 1	20 kV
ion source 2	18.40kV
lens	7.50 kV
pulsed ion extraction	120 ns
nitrogen pressure	1700-2000 mbar

3.3. Patients

Serum samples were obtained from a total of 28 breast cancer patients the day before surgery. Breast cancer was histologically confirmed by surgical specimens and pathological validation. Breast cancer patients from our hospital were included from November 2006 till April 2007. The healthy control group consisted of 24 individuals, all from our hospital healthy check center.

3.4. Proteomic fractionation (Isolation of peptides)

Serums were subjected to fractionation using functionalized magnetic bead-bases kits (ClinProt Kits, Bruker Daltonics Inc., Fremont, CA). Magnetic beads (particle size, 1 mm) with hydrophobic interaction chromatography C8 (MB-HIC-C8), weak cation exchange chromatography (MB-WCX) were designed for capturing of proteins and peptides from biological samples to obtain specific profiles based on cationic exchange chromatography and immobilized metal-ion affinity chromatography (MB-IMAC-Cu). These were both used according to the manufacturer's protocols.

1. As recommended in the manufacturer's protocol, we added 5 µL magnetic beads to 10µL of a binding solution in a 0.2mL polypropylene PCR tube.

2. Then, we added 5 µL of sample and mixed it thoroughly by pipetting up and down several times.

3. We incubated the tube for 5 minutes.

4. Then, we separated the unbound solution using a magnetic bead separator.

5. After the magnetic bead separation and three times washings, the bound proteins/peptides were eluted from the magnetic beads according to the manufacturer's instructions (Profiling Kit Magnetic beads and Profiling, Bruker Daltonics GmbH, Leipzig, Germany).

6. A portion of the eluted sample was diluted (1:10) in a solution containing CHCA (0.6 g/L in 2:1 ethanol:acetone).

7. Then, 0.5 mL of the resulting mixture was spotted onto the AnchorChip target (Bruker Daltonics Inc., CA) and allowed to air dry for approximately 5 min at room temperature.

Generally, each sample for each conditioned experiment was conducted three times. AnchorChip targets produce more homogenous matrix crystallization with better reproducibility of MS spectra than crystallization on steel targets.

3.5. MS analysis (protein profiling)

For the proteomic analysis, we used a linear MALDI-TOF mass spectrometer (Microflex; Bruker Daltonics) with the following settings (Table 1):

Ionization was achieved by irradiation with a nitrogen laser (λ=337 nm) operating at 25 Hz.

Mass spectra were detected in linear, positive mode.

Mass calibration was performed with the calibration mixture of peptides and proteins in a mass range of 1000–10000 Da.

We measured 3 MALDI preparations (MALDI spots) from each magnetic bead fraction. For each MALDI spot, 400 spectra were acquired (50 laser shots at 8 different spot positions).

To increase the detection sensitivity, we usually use higher energy to shoot the spot positions first, and then shoot lower energy to save the spectra. This removes excess matrix with higher laser power before the data acquisition.

All signals with a signal-to-noise (S/N) ratio >5 in a mass range of 1000–10000 Da were recorded with the use of the flexControl tool acquisition software (Ver. 2.0; Bruker Daltonics).

We used the ClinProTools bioinformatics software (Ver. 1.0; Bruker Daltonics) for proteomic pattern recognition.

3.6. Data processing

The MS spectra for peaks of 1,000-10,000 m/z were generated by summarizing 400 laser shots (50 shots at 8 different spot positions). To increase detection sensitivity, excess matrix was removed with 10shots at a laser power of 90% before acquisition of spectra with 50 shots at a fixed laser power of 50%.

We exported all unprocessed spectra from the microflex MS in standard format .They covered a domain of 1,000-10,000 Da. To increase the accuracy, the average of eight spots was used to represent one serum sample. To normalize the spectra, we calculated the median intensity of each spectrum and subtracted it from the original spectra.

We analyzed the spectra obtained from all the samples by using ClinProTool 1.0 software, did the spectrum processing and generated proteomic fingerprints. Only the spectra obtained from magnetic beads were used in the analysis of this study.

Basically, we normalized all spectra to their own total ion count by summation of peak areas and recalibrated using the most prominent peaks, followed by baseline subtraction, peak defining, and calculation of peak intensities, peak numbers and areas. In order to evaluate the degree of variation among different proteome spectra, we first defined the peaks (in general, S/N>5 and minimal peak

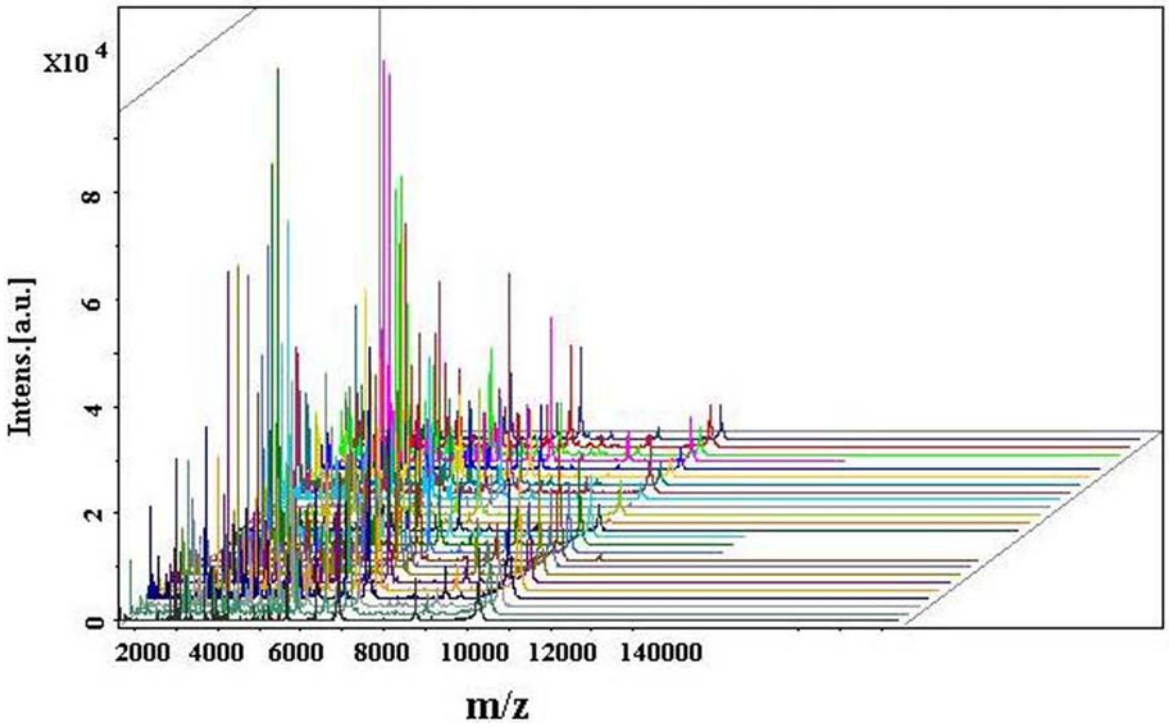

Figure 1. The Whole Quality of Mass Spectra by MB-WCX in Healthy Control Group.

intensity >1.6 arbitrary units) and carefully checked each corresponding peak throughout all of the spectra.

Then, we calculated the mean value of peak intensity, SD and CV (%) for each corresponding peak in our study. The degree of variation on the basis of the whole spectrum was thus determined by calculating the CV values for all of the peaks of the tested spectra. We defined CV >30% or $p<0.01$ as a significant difference.

4. RESULTS

4.1. Whole quality of mass spectra with three different types of magnetic beads fractionation

We compared all the origin mass spectra, including 28 breast cancer patients and 24 healthy control individuals, and drew the conclusion that with the pre-analysis of magnetic bead WCX, it is the best mass spectra. We can see the results from the following pictures (Figure 1**)**.

4.2. The coherence in the two groups- breast cancer group and the normal group

With the help of flexAnalysis software (Bruker Daltonics) and related statistical software, we compared the coherence in the two groups, breast cancer group and the control group. The conclusion we reached was almost the same with the whole quality of mass spectra, by using MB-WCX to pre-extract serum samples. The coherence in MB-WCX group was the best in these three types of magnetic beads.

4.3. The capability of seeking proteins and peptides in three types of magnetic beads

4.3.1. Analysis of peak numbers in the mass spectra

4.3.1.1. Breast cancer group

As we know, by using different types of magnetic beads to pre-extract our samples, we will get different kinds of proteins or peptides, especially low abundance proteins and peptides. The aim of proteomic pattern analysis of the cancer group and healthy control group is to find the differences in human diseases and seek for new potential biomarkers for early detection and early diagnosis of cancer. So, we hope to get more peaks in the MS analysis, which means we will have more detailed information from serum samples between cancer and healthy control group. At first, we did the comparison of peak numbers in the mass spectra in the breast cancer group after the pre-extraction by three types of magnetic beads, and got the results as follows (Table 2):

With the help of paired samples statistics data, we got the correlations in these three paired groups (Table 3).

Then, based on the results of paired samples statistics and correlations, we got paired samples test results (Table 4).

From the results in paired samples test, we drew the conclusions that the peak numbers in MB-WCX group are fewer than those in MB-Cu group and MB-HIC C8 group.

Table 2. Paired samples statistics in breast cancer group

Number	Group	Average peak numbers	Sample numbers	Std. Deviation	Std. Error Mean
Pair 1	WCX	121.2143	28	27.71596	5.23782
Pair 1	Cu	82.7143	28	27.37511	5.17341
Pair 2	WCX	121.2143	28	27.71596	5.23782
Pair 2	C8	70.1786	28	19.91473	3.76353
Pair 3	Cu	82.7143	28	27.37511	5.17341
Pair 3	C8	70.1786	28	19.91473	3.76353

Table 3. Paired samples correlations

Number	Group	Sample numbers	Correlation
Pair 1	WCX &Cu	28	0.213
Pair 2	WCX & C8	28	-0.189
Pair 3	Cu& C8	28	-0.045

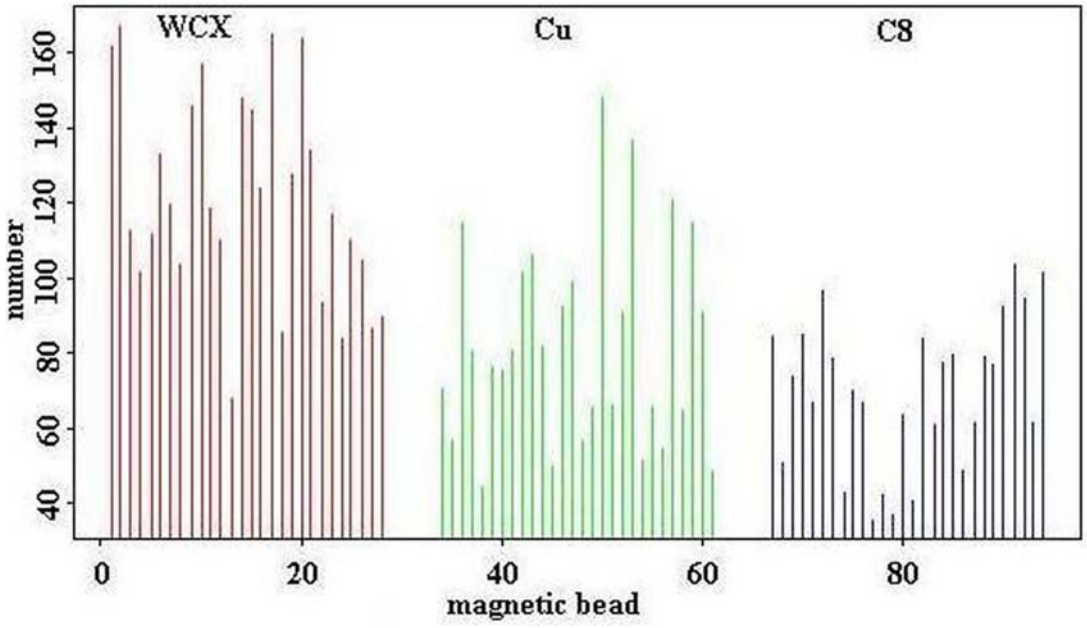

Figure 2. Peak Numbers Statistics of Breast Cancer Samples in MB-WCX, MB-Cu and MB-HIC C8 groups. (Red. MB-WCX, green- MB-Cu, blue- MB-HIC C8).

Then, we divided the samples into three paired groups to compare the result. In the paired WCX-Cu group and paired WCX- C8 group, $p<0.01$, was of great significance. But, little difference was seen in the paired Cu-C8 group.

Based on all above data, we drew the following conclusion of these three types of magnetic beads. Peak numbers obtained from each sample are shown along the vertical axis and individual tests are shown on the horizontal axis. (In the picture red designates the MB-WCX group, green designates the MB-Cu group and blue designates the MB-C8 group.) Figure 2 shows the results of the mass spectra information of the breast cancer group. We can easily see that the most peaks we got were from the MB-WCX pre-extraction.

By using 28 single samples mass spectra, we did a same sample –different group comparison picture. The results also showed us the same samples in the peak numbers after the pre-extraction of MB-WCX, MB-Cu and MB-HIC C8, as shown in figure 3 below. Each spot delegates one single serum sample in the breast cancer group, and we joined the same sample with three groups' results to see which one was the best choice. The horizontal axis designates the three different groups, and the vertical axis designates the peak numbers we got with each serum sample.

4.3.1.2. Healthy control group

Just as we did in the breast cancer group, we conducted the same peak statistics in the healthy control group with 24 serum samples to compare the pre-extraction ability. The results are shown as follows in Table 5, 6 and 7:

Using the results shown in Table 5, Table 6 and Table 7, we conducted the paired samples statistics for the healthy control group, and analyzed the correlations and paired sample test. We found that the results were consistent with those in the breast cancer group. We got more peaks using MB-WCX, than MB-Cu, and the least effective was MB-HIC C8. The correlations and the test of two paired were almost the same as the results of the peak

Table 4. Paired samples test

Number	Group	Mean	Std.Deviation	Std.Error Mean	Sig.(2-tailed)
Pair1	WCX-Cu	8.50000	34.55698	6.53066	0.000
Pair2	WCX-C8	1.0371	37.0599	7.00330	0.000
Pair3	Cu-C8	2.53571	34.56554	6.53227	0.066

Table 5. Paired samples statistics in healthy control group

Number	Peak numbers	Peak numbers	Sample numbers	Std. Deviation	Std. Error Mean
Pair 1	WCX	136.3750	24	29.24233	2.83269
Pair 1	Cu	104.3333	24	13.87731	2.83269
Pair 2	WCX	136.3750	24	29.24233	5.96907
Pair 2	C8	75.0000	24	28.20577	5.75748
Pair 3	Cu	104.3333	24	13.87731	2.83269
Pair 3	C8	75.0000	24	28.20577	5.75748

Table 6. Paired samples correlations

Number	Group	Sample numbers	Correlation
Pair 1	WCX& Cu	24	-0.015
Pair 2	WCX & C8	24	0.072
Pair 3	Cu & C8	24	0.252

Table 7. Paired samples test

Number	Group	Mean	Std.Deviation	Std.Error Mean	Sig.(2-tailed)
Pair1	WCX-Cu	32.04167	32.55294	6.64484	0.000
Pair2	WCX-C8	61.37500	39.14834	7.99112	0.000
Pair3	Cu-C8	29.33333	28.12189	5.74036	0.000

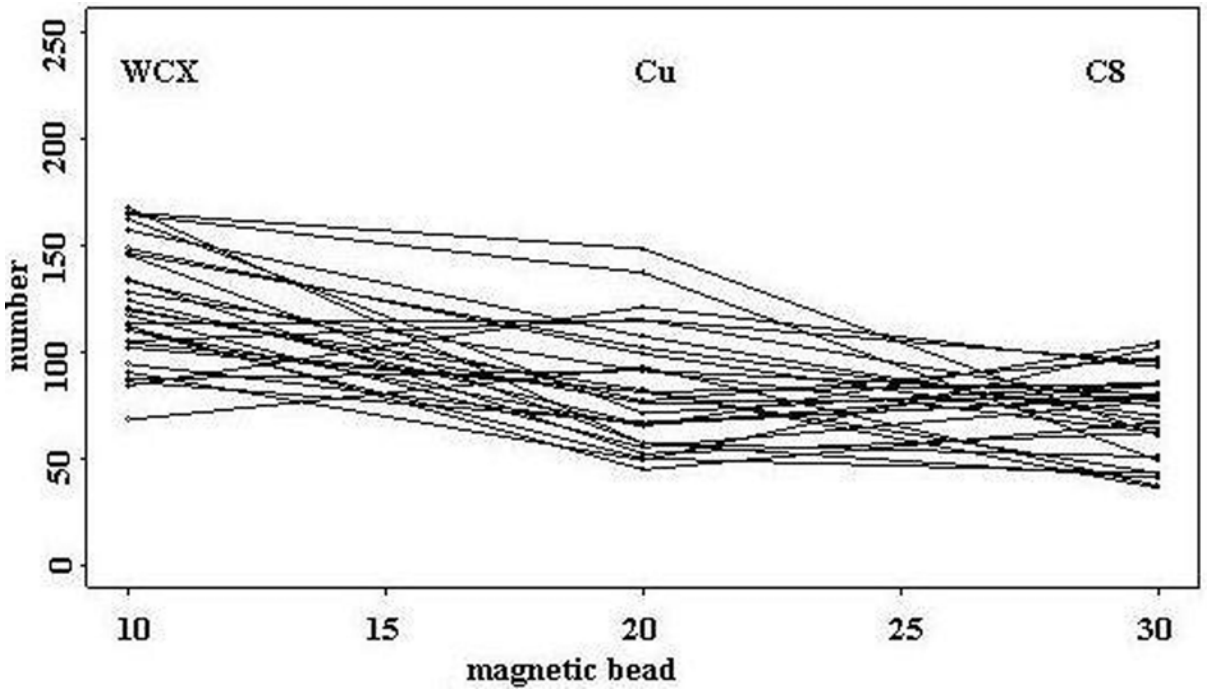

Figure 3. Same Sample –Different Group Comparison of MB-WCX, MB-Cu and MB-HIC C8 group.

statistics. The only difference was that in all the three paired groups, $p<0.01$, held great significance.

With all the data, we performed the peak numbers analysis of healthy control samples in MB-WCX, MB-Cu and MB-HIC C8 groups. Figure 4 shows the obvious results. The result was consistent with those in breast cancer group. The only deviation from breast cancer group was the p value in the paired group MB-Cu and MB-C8, <0.01. This was of great significance.

We also did the same sample-three MB group analysis. The result is shown below in Figure 5:

4.3.2. Analysis of peak intensities in the mass spectra

4.3.2.1. Breast cancer group

By using the flexAnalysis software and relative statistic software, we analyzed the total peak intensities in the 28 breast cancer samples, and we got the peak intensities statistics shown in see Table 8.

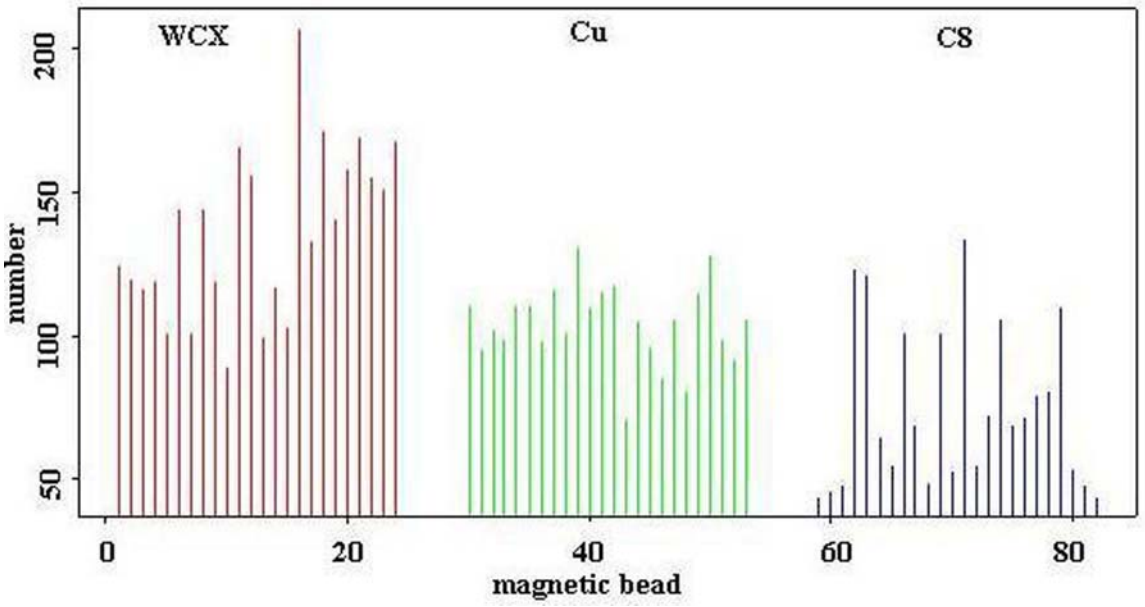

Figure 4. Peak Numbers Statistics of Healthy Control Samples in MB-WCX, MB-Cu and MB-HIC C8 groups. (Red. MB-WCX, green- MB-Cu, blue- MB-HIC C8).

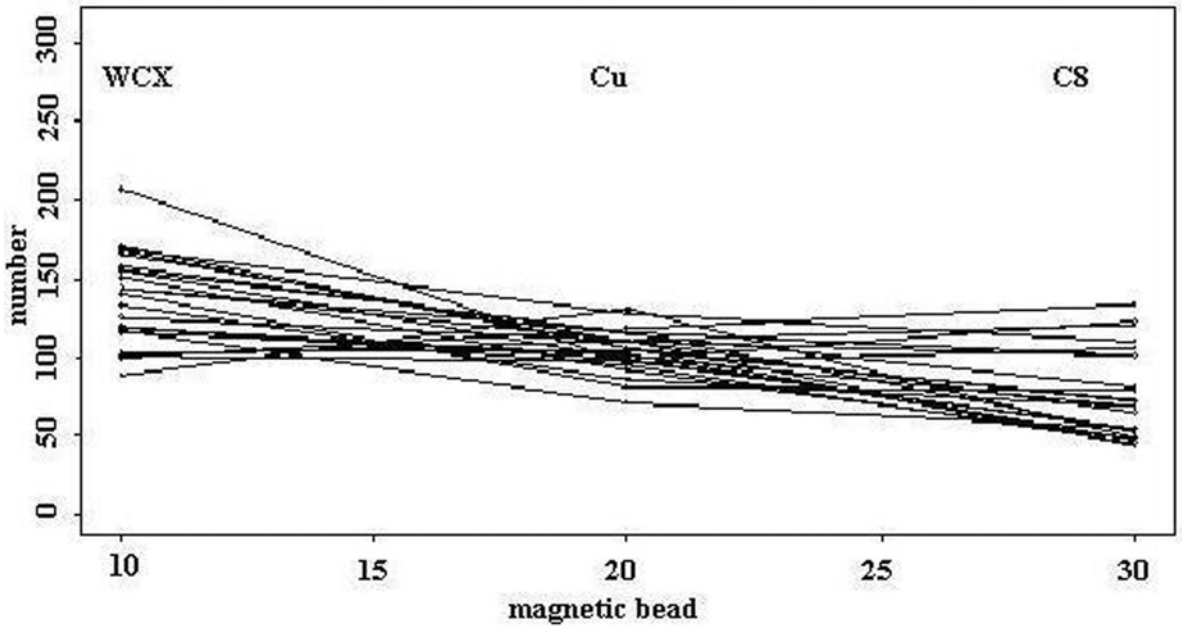

Figure 5. Same Sample –Different Group Comparison of MB-WCX, MB-Cu and MB-HIC C8 group.

Then with the statistics of peak intensities, we got the samples correlation data, and found the results shown in Table 9.

Finally, we did the paired samples test and drew the conclusion shown Table 10 and we performed the peak intensities analysis pictured in Figure 6 and Figure 7.

In Figure 6, the horizontal axis shows three different MB groups and the vertical axis designates the peak intensities in each serum sample. Red designates the MB-WCX group, green designates the MB-Cu group, and blue designates the MB-C8 group.

From the results in Figure 6 and Figure 7, we learned that the peak intensities in MB-WCX group were the highest of these three groups. The paired results in MB WCX-Cu group and MBWCX-C8 group, $p<0.01$, was of great significance. However, there was no significant difference between MB Cu and theC8 group.

Table 8. Paired statistics of peak intensities

Number	Group	Peak intensities	Sample number	Std. Deviation	Std. Error Mean
Pair 1	WCX	390504.8252	28	139978.40833	17170.28059
Pair 1	Cu	227212.7501	28	90856.58477	17170.28059
Pair 2	WCX	390504.8252	28	139978.40833	26453.43267
Pair 2	C8	249289.7375	28	100580.36678	19007.90266
Pair 3	Cu	227212.7501	28	90856.58477	17170.28059
Pair 3	C8	249289.7375	28	100580.36678	19007.90266

Table 9. Paired samples correlations of peak intensities

Number	Group	Sample number	Correlation
Pair 1	WCX& Cu	28	-0.030
Pair 2	WCX &C8	28	0.148
Pair 3	Cu & C8	28	-0.217

Table 10. Paired samples test of peak intensities in breast cancer group

Number	Group	Mean	Std.Deviation	Std.Error Mean	Sig.(2-tailed)
Pair1	WCX-Cu	163292.1	169119.21976	31960.53	0.000
Pair2	WCX-C8	141215.1	159813.83398	30201.98	0.000
Pair3	Cu-C8	-22077.0	149450.08141	28243.41	0.441

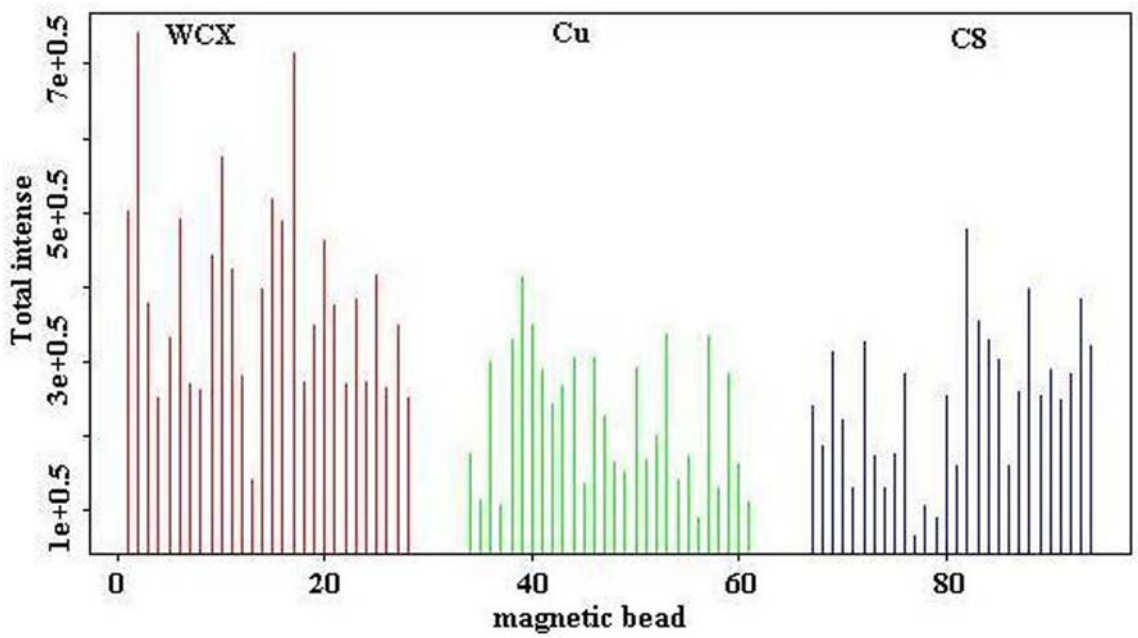

Figure 6. Peak Intensities Analysis in MB-WCX, MB-Cu and MB-HIC C8 groups. (Red. MB-WCX, green- MB-Cu, blue- MB-HIC C8).

4.3.3.2. Healthy control group

We did the same analysis of 24 healthy control serum samples by using the flexAnalysis software and relative statistic software, and we found that the peak intensity results in Table 11.

From the result shown in Table 11, we found that of the compared results from three paired groups, the highest peak intensities were from the MB-WCX group, then the MB-Cu and MB-HIC C8 groups.

We did the paired samples correlation of these 24 serum samples, and got the results shown in Table 12.

Finally, we did the paired samples test of the healthy control group. The result is shown below in Table 13. We found that there was no significant different in paired group MB WCX-Cu, but there was great significant difference in two paired groups, MB WCX-C8 and paired group MB Cu-C8. The p values were both <0.01.

Then, we performed the peak intensities mass spectra analysis shown in Figure 8 and Figure 9. From this data, we extracted know the information of these three kinds of magnetic beads.

5. DISCUSSION

Proteomic pattern analysis is one of the most promising new approaches for seeking for potential biomarkers and classifying cancer versus non-cancer samples. But, before MALDI-TOF MS analysis, we must

Table 11. Paired samples statistics

Number	Group	Peak intensities	Sample number	Std. Deviation	Std. Error Mean
Pair 1	WCX	504661.0000	24	168280.07409	34350.02628
Pair 1	Cu	381614.0375	24	181043.25197	36955.29906
Pair 2	WCX	504661.0000	24	168280.07409	34350.02628
Pair 2	C8	254950.5250	24	84071.13265	17160.94809
Pair 3	Cu	381614.0375	24	181043.25197	36955.29906
Pair 3	C8	254950.5250	24	84071.13265	17160.94809

Table 12. Paired samples correlations

Number	Group	Sample numbers	Correlation
Pair 1	WCX & Cu	24	-0.413
Pair 2	WCX& C8	24	-0.019
Pair 3	Cu & C8	24	-0.113

Table 13. Paired samples test of peak intensities in healthy control group

Number	Group	Mean	Std.Deviation	Std.Error Mean	Sig.(2-tailed)
Pair 1	WCX-Cu	123047.0	293709.19857	59953.14	0.052
Pair 2	WCX-C8	249710.5	189510.30231	38683.63	0.000
Pair 3	Cu-C8	126663.5	208016.17054	42461.12	0.007

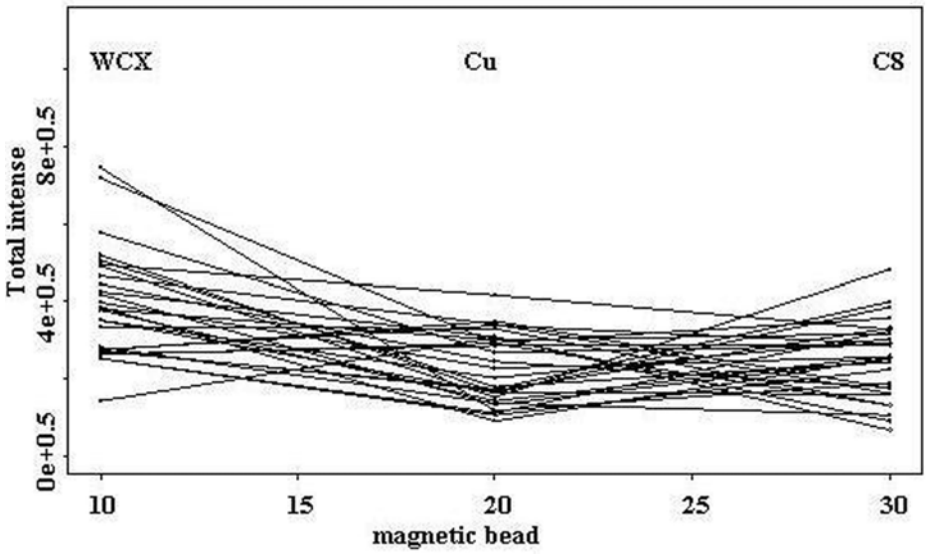

Figure 7. Same Sample-Different Group Peak Intensities Analysis in MB-WCX, MB-Cu and MB-HIC C8 Groups.

pre-analyze all these kind of complex mixture samples-serum. Our aim of is to extract as many proteins or peptides from the serum as possible especially low abundance ones. The process of fractionation of proteins or peptides from samples is a difficult and complex process, as we know little about the real components in the mixture of serum samples. According to the characteristics of samples, we should select different methods to extract proteins or peptides for good quality and high reproducibility mass spectra. Only by doing these can we lay a good foundation for the finding of differential proteins or peptides and seeking for potential biomarkers.

Proteome fractionation of serum samples is one of the most important and basic steps in proteomic analysis. If we use the wrong method to fractionate proteins and peptides from mixtures of body fluid, we could get little or no sample to analyze. In order to solve these problems, in the MALDI-TOF MS analysis, we used magnetic particles, which we called magnetic bead, a mean pore size of 40 nm, and a specific surface area of 100 cm^2 /g, to extract the mixture in the serum samples, especially the low abundance proteins or peptides. This high sensitivity and reproducibility of the novel magnetic-bead-based platform gives us a good support for proteomic analysis. Some reported that use of porous particles for sample pretreatment is more sensitive than surface capture on chips, because spherical particles have larger combined surface areas than small-diameter spots. Using the magnetic bead technology, we can find more different proteome signals in the MALDI-TOF MS analysis. The application of magnetic bead fractionation in combination with MALDI-TOF is appropriate for the detection of low concentrations of proteins and peptides in serum samples.

Though using a magnetic-bead-based platform is more convenient for proteomic analysis, we still have to pay special attention to the details such as what kind of magnetic bead we can choose, the real mass range we want to explore, etc. In order to solve these problems, we did our

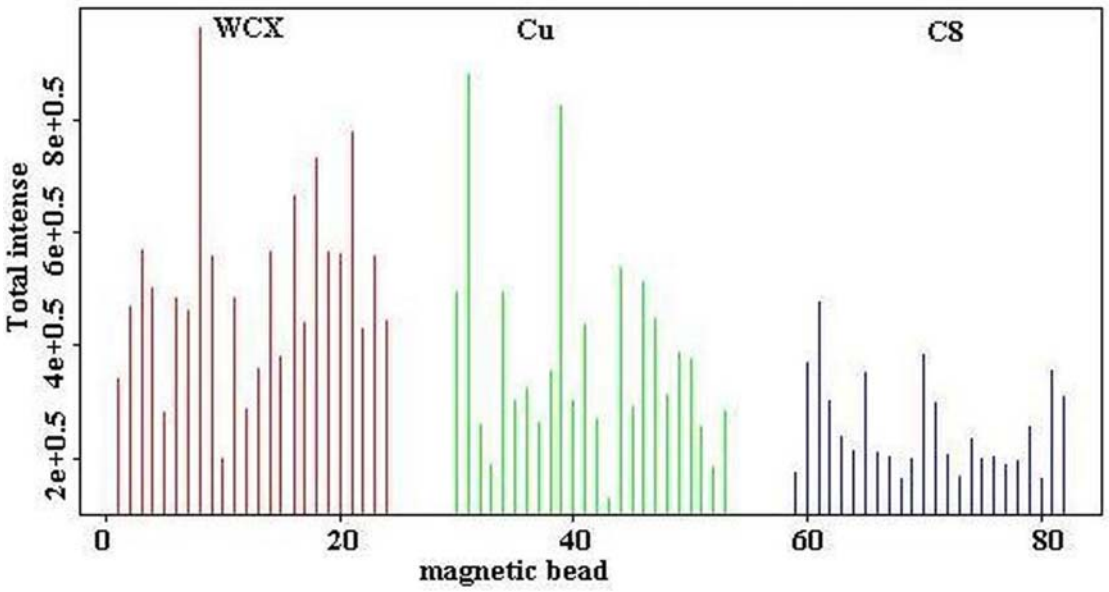

Figure 8. Peak Intensities Analysis in MB-WCX, MB-Cu and MB-HIC C8 groups. (Red. MB-WCX, green- MB-Cu, blue- MB-HIC C8).

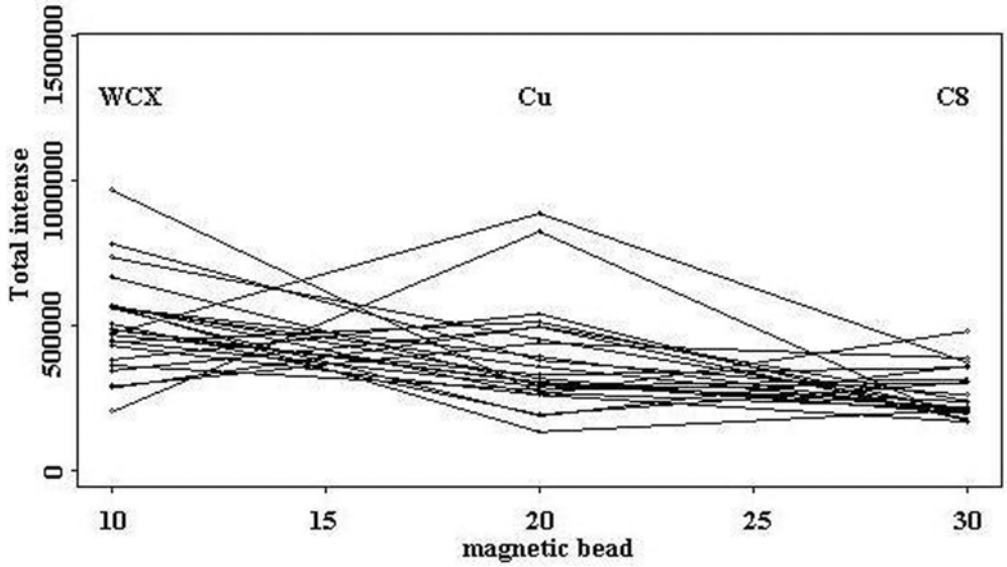

Figure 9. Same Sample-Different Group Peak Intensities Analysis in MB-WCX□MB-Cu and MB-HIC C8 groups.

research about how to choose the right magnetic bead for breast cancer, and got some experience that can be used for reference.

In our research, we chose 28 breast cancer patient serum samples as the cancer group and 24 healthy control serum samples as control group, made the pre-extraction with three kinds of magnetic beads (MB-WCX, HIC C8 and IMAC-Cu) and compared all the mass spectra results.

We compared the whole quality of cancer group and normal group, the coherence in the group, the peak numbers and peak intensities in both groups and the most peaks' mass range we could get in the MALDI-TOF analysis.

With the help of flexAnalysis software and statistical software, we did the data analysis and chose WCX magnetic beads as the best for our breast cancer research, since the compared quality and the coherence in both the cancer and control groups was the best of the three sets of magnetic beads. Then, we did the peak numbers and peak intensities analysis in the cancer group and control groups. In the cancer group, the average peak numbers in MB-WCX group was more than those of MB-Cu and MB-HIC C8, with great significant difference, $p<0.01$. But, little was found in MB-Cu and MB-HIC C8. In control

group, the results were similar to the cancer group, and there was still great difference in these three groups(MB-WCX ,MB-Cu and MB-HIC C8) as $p<0.01$.

The peak intensities' results were distinguished in the cancer group and control groups. In the cancer group, the result was similar to the average peak numbers, though the peak intensities were much higher than those of MB-Cu and MB-HIC C8. Almost none was found in MB-Cu and MB-HIC C8. The result from the control group was different from the results of average peak numbers, there was almost no difference between MB-WCX group and MB-Cu group, but significant difference was found in the two paired groups that were MB-WCX group with MB-HIC C8 and MB-Cu group with MB-HIC C8 , $p<0.01$.

At last, we made a comparison of the mass range distribution. Because different kinds of magnetic beads have different capability in capturing proteins and peptides in chromatography, this is of fundamental importance in the MALDI-TOF MS analysis. Human serum is a very complex biological sample. We know little of the mixture of so many proteins and peptides. So at the beginning of our research design, we had to take many factors into account, and hope to get as many proteins and peptides from serum samples as possible. We had no real target data of what is useful and what is not. For this reason, the objective of pre-extraction is developed for enrichment and purification proteins and peptides in serum samples, especially low abundance ones. If we know the mass range of the aim protein, we can choose the exact kind. For example, by pre-extraction of MB-Cu, the most peaks' mass range is about 2,000-5,000 Da. For MB-HIC C8, it is about 1000-4000 Da, but we can not get much larger ones. But for MB-WCX, we will get a wide range of the mass from 1,000-10,000 Da.

In our research we chose MB-WCX as the best magnetic bead for pre-extraction samples, because the research objective was to get as much as proteins and peptides as possible, so that we can obtain more information from serum samples, settle a good foundation for cancer diseases research and afford more candidate proteins and peptides for biomarker finding.

Based on the above information, we chose the right magnetic bead according to the aim of the research, which will afford more convenience, save more time and get better quality origin mass spectra, so that we can improve the sensitivity and reproducibility in MALDI-TOF MS analysis.

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