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Impact of sample preparation in peptide/protein profiling in human serum by MALDI-TOF mass spectrometry

Antonella Aresta [a], Cosima D. Calvano [a], Francesco Palmisano [a], Carlo G. Zambonin [a,*], Alessandro Monaco [b], Stefania Tommasi [b], Brunella Pilato [b], Angelo Paradiso [b]

[a] *Università degli Studi di Bari, Centro Interdipartimentale di Ricerca S.M.A.R.T., Dipartimento di Chimica, Via Orabona 4, 70126-Bari, Italy*
[b] *Istituto Tumori "Giovanni Paolo II", Via Hanheman 10, 70126-Bari, Italy*

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Abstract

The low molecular weight (LMW) serum proteome (<15 kDa) is the most generally informative from a medical point of view. Different sample pre-treatment approaches and devices for serum depletion in high-abundant proteins were tested in order to analyze, by MALDI-TOF-MS (both in "linear" and "reflectron" acquisition mode), the serum low molecular weight proteins/peptides. The best results in terms of detected ions number and abundance were obtained by using ultrafiltration of serum on 30 kDa molecular weight cut off membranes followed by miniaturized reverse-phase solid-phase extraction (μ-SPE) as sample pre-treatment; this procedure yielded also satisfactory within-sample and sample-to-sample repeatability (on both *m/z* values and peak intensity of the main observable ions). The procedure was finally applied to serum samples of breast cancer patients, and the relevant results compared to "normal" samples seem to be promising for the individuation of different profiles ("linear" and "reflectron" mode) and/or peptides capable of differentiating for malignancies ("reflectron" mode).

Keywords: MALDI-TOF-MS; Serum; Proteins; Biomarkers; Breast cancer

1. Introduction

The development of a malignant cancer depends on genetic mutations that lead to the activation of cellular oncogenes that take over the processes in the cell transforming it into an entity that continues to survive and proliferate. This transformed cell may undergo further mutations and develop into a fully malignant tumor cell. Although these changes occur at the DNA level, they must ultimately be expressed in terms of the protein content. Thus, changes in the cell that result from malignant transformation could be best followed by monitoring changes in the resulting protein expression [1,2].

The human serum proteome [3–5] is likely to contain, at variable concentration levels, most, if not all, human proteins. Serum proteome is then the most generally informative from a medical point of view; almost all body cells communicate with blood and many of them release at least part of their contents into blood upon damage or death.

Typical serum biomarkers associable with a cancer can be proteins over- or under-expressed, abnormally secreted, processed or modified as a consequence of the disease process, degraded due to abnormal activation of the proteolytic degradation pathways [6,7]. Unfortunately, the proteins that can be potential biomarkers are present in a very low concentration in serum. In fact, albumin, transferrins, immunoglobulins, and complement factors represent almost the 99% of serum protein content, while the remaining 1% is represented by lower abundance circulatory proteins as well as proteins that are excreted by not only live cells, but also apoptotic and necrotic cells. It has been reported [4,8] that a part of this fraction is represented by low molecular weight range (<15 kDa) proteins. The LMW serum proteome promises to contain a rich source of previously undiscovered biomark-

* Corresponding author. Tel.: + 39 080 5442506; fax: +39 080 5442026.
E-mail address: zambonin@chimica.uniba.it (C.G. Zambonin).

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ers [4,8], as biological processes give rise to cascades of enzymatically generated and proteolytically clipped biomarker fragments.

These low molecular weight molecules have been until very recently not completely characterized, since many problems exist for their detection by conventional techniques. For instance, the two-dimensional gel electrophoresis cannot be efficiently used for their separation because of many reasons: the mobility of peptides is high, making difficult their focusing on gel; the ability to bind different stains is small; their stability tends to reduce their irreversible denaturation [4,9].

As a result, investigators have turned to mass spectrometry (MS) techniques. Surface-enhanced laser desorption/ionization (SELDI) has been widely [2,10] used to acquire a "peptide/protein profile" for a high number of serum samples belonging to different populations (e.g. cancer *vs*. normal); the MS data set is then treated with appropriate statistical techniques in order to identify possible biomarkers, i.e. mass/charge (*m*/*z*) values able to represent/differentiate the two populations. SELDI is based on the use of functionalized probes (similar to a protein chip) possessing peculiar chromatographic or affinity-binding properties. Fast on-chip clean-up of the whole (untreated) sample is the main advantage of SELDI that is counterbalanced by the low resolution and mass accuracy of the dedicated analyzers (compared to MALDI) and by the fact that "profiles" are limited to bound peptides/proteins.

As an alternative to SELDI a number of groups have explored the possibility of proteins profiling by conventional MALDI coupled to an off-line sample pre-processing of biological samples [11]. This approach requires much more efforts but has the advantages of allowing an independent optimization of the sample enrichment/fractionation protocol. In order to increase sensitivity and confidence in protein identification several sample pre-processing steps have been devised including depletion columns for removal of albumin and other abundant proteins [8,12], molecular-weight cut-off cartridges [13], acetone precipitation [14], gel filtration [15], ion-exchange chromatography [8,16], isoelectric focusing [17] and electrophoresis [18]. However, the low accordance between data reported in the existing literature [4,19], suggests that the peptides eventually observed are strongly dependent on the sample pre-treatment method adopted, indicating that it has to be selected and optimized for the specific disease under investigation.

The objective of the present work was to compare different serum sample pre-treatment approaches for MALDI-TOF-MS analysis of low mass range proteins/peptides in terms of number and abundance of ions observed in the mass spectra recorded both in the *m*/*z* ranges of 3000–30,000 (linear mode) and, contrary to most existing studies on this topic, 1000–3000 (reflectron mode). The procedure that gave the best results, i.e. ultrafiltration of serum on 30 kDa molecular weight cut off membranes followed by micro-reversed-phase extraction (μ-SPE) was finally applied to the analysis of breast cancer women serum.

2. Experimental

2.1. Chemicals

All chemicals and solvents used were purchased from Sigma–Aldrich (St. Louis, MO, USA). Solvents were HPLC grade and were used without further purification.

Alpha-cyanohydroxycinnamic acid (CHCA) was used as MALDI matrix. Five milligrams of CHCA were dissolved in 1 ml of acetonitrile/0.1% trifluoroacetic acid (TFA) (9:1, v/v).

2.2. MALDI-TOF-MS

MS experiments were performed using a Micromass M@LDI^{TM}-LR (Waters MS Technologies, Manchester, UK) time-of-flight mass spectrometer equipped with a nitrogen UV laser (337 nm wavelength), a precision flat target plate sample introduction system bearing a micro-titer target plate (96 well, 3 mm diameter, 4.5 mm pitch, rectangular 12 by A-H with additional 24 near-point calibration wells), reflectron optics with effective path length of 2.3 m, a fast dual micro-channel plate (MCP) detector, and a high magnification (70×) camera system.

Positive ion spectra were acquired in reflectron mode. The following voltages were applied: pulse, 2500 V; source, 15000 V; reflectron, 2000 V; MCP 1850 V. The laser power densities were in the range of 10^7–10^8 W cm^{-2} with a laser pulse duration of 4 ns. The laser firing rate was 5 Hz, and 80 laser shots were used for each well. The 80 resulting spectra were averaged, background subtraction was applied, and the peaks were smoothed with a Savitzky–Golay algorithm.

A time lag focusing (TLF) delay of 500 ns was used between the time of the laser pulse and the application of the accelerating voltage. All samples were analyzed both in linear (in the *m*/*z* range 3000–30,000) and reflectron (in the range *m*/*z* 1000–3000) acquisition modes. Calibration was performed using a peptide mixture composed by the fragment 18–39 (2465.199 Da) of the adrenocorticotropic hormone, renin (1758.933 Da) and angiotensin (1296.687 Da) (reflectron mode) and a protein mixture composed by insulin beta chain (3497.0 Da), insulin (5735.0 Da), cytochrome *C* (12361.1 Da) (linear mode).

2.3. Serum samples collection and storage

Serum samples from patients with breast cancer were collected at the Istituto Tumori "Giovanni Paolo II" (Bari, Italy). The control group included five healthy women, whose mammography provided negative results for at least four consecutive years. The cancer group consisted of five serum samples from breast cancer patients at clinical category 3–5 as indicated from the BI-RADS classification of the American College of Radiology. All serum specimens collected were from women with similar age, body weight, diet, anamnesis and pharmacological therapies. Blood samples were collected with minimal stasis in evacuated tubes (Vacutainers) and allowed to clot by standing tubes at room temperature for 1 h. Then, the tubes were placed on ice for 2 h before to be centrifuged at 4 °C for 30 min at

1500 × *g*. Finally, aliquots of 0.1 ml were collected and stored at −20 °C. The aliquots were thawed only once.

2.4. Sample preparation

2.4.1. Miniaturized reversed-phase solid-phase extraction (μ-SPE)

Vivapure C18 Micro-spin columns (Sartorius, Goettingen, Germany) were washed (twice) with 200 μl of 80% Methanol in TFA 0.1% and centrifuged at 400 × *g* for 1 min. Columns were equilibrated (twice) with 200 μl of 5% Methanol in TFA 0.1% and centrifuged at 400 × *g* for 2 min. Fifty microliters of biological sample (serum diluted 1:5 with a guanidine HCl 1 M in 0.5% TFA aqueous solution or serum treated with ultrafiltration or dealbuminization devices) were loaded onto the column and centrifuged at 400 × *g* for 2 min. The column was washed (twice) with 200 μl of 20% methanol in 0.1% TFA and centrifuged at 400 × *g* for 2 min. A further centrifugation step at 13,000 × *g* for 30 s was performed to discard all the residual TFA. 4 μl of 75% of Acetonitrile in TFA 0.1% were loaded onto the column and centrifuged at 400 × *g* for 30 s and 13,000 × *g* for 30 s in order to completely elute the sample.

2.4.2. Selective albumin removal followed by μ-SPE

Four hundred microliters of the anti-HAS affinity resin suspension were loaded into the spin column (Vivapure Anti-HSA kit, Sartorius) and 180 μl of human serum, added with 20 μl ammonium bicarbonate, were loaded to the same spin column. The sample was incubated on a rotary shaker for 15 min at room temperature and then centrifuged at 400 × *g* for 1 min. Two hundred microliters of binding buffer (10 mM Tris–HCl, pH 7.4) were added to the spin column, incubated for further 2 min on a rotary shaker and centrifuged at 400 × *g* for 1 min. The effluent was mixed with 200 μl of binding buffer, loaded again onto the spin column and centrifuged at 400 × *g* for 1 min. The effluent (albumin-depleted serum) was collected and subjected to μ-SPE as described above.

2.4.3. Ultrafiltration followed by μ-SPE

Columns with polyethersulfone membranes (Vivaspin 500, 30 kDa MWCO) were activated loading 50 μl of 0.1% TFA followed by centrifugation at 15,000 × *g* for 10 min; effluent was discarded. 400 μl of human serum (diluted 1:5 with TFA 0.5%, in order to denaturate serum proteins and consequently disrupt protein-protein interactions) were loaded and centrifuged at 5000 × *g* for 30 min. The effluent was collected and subjected to μ-SPE as described above.

3. Results and discussion

Most existing studies dealing with the search for different serum proteomic profiles by MALDI-TOF privileged the linear acquisition mode, to increase the investigated *m/z* range and

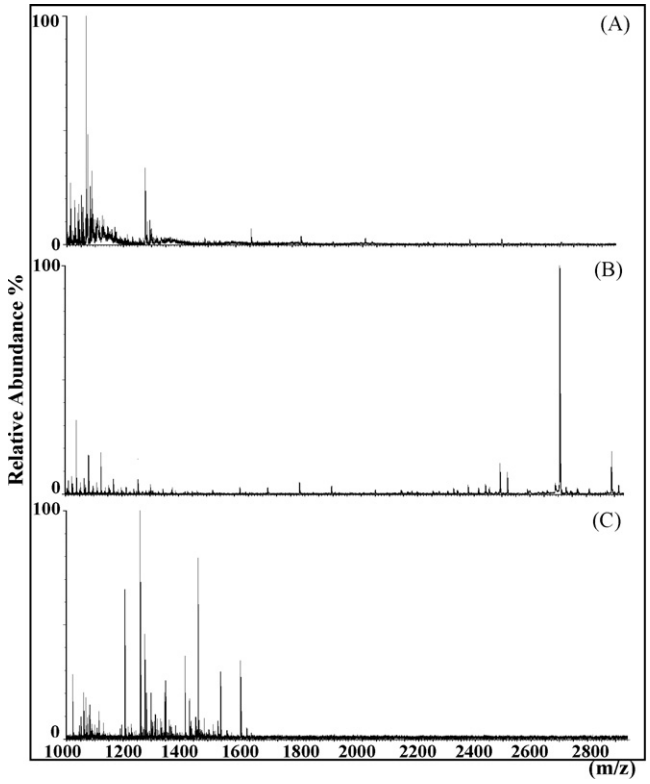

Fig. 1. MALDI-TOF-MS spectra (reflectron acquisition mode, 1000–3000 Da) obtained in the analysis of a healthy donor serum samples. Clean-up method: (A) reverse-phase μ-SPE, (B) dealbuminization followed by reverse-phase μ-SPE and (C) ultrafiltration (30 kDa molecular weight cut off membranes) followed by reverse-phase μ-SPE.

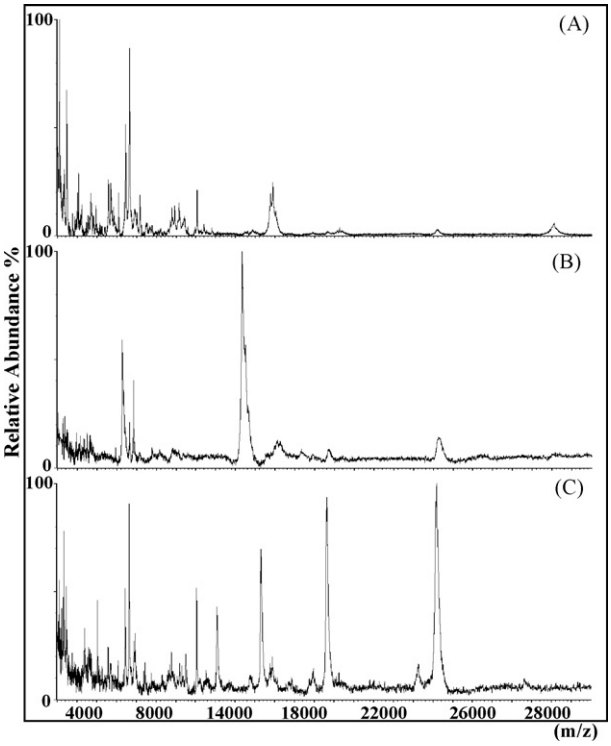

Fig. 2. MALDI-TOF-MS spectra (linear acquisition mode, 3000–30000 Da) obtained in the analysis of a healthy donor serum samples. Clean-up methods: (A) reverse-phase μ-SPE, (B) dealbuminization followed by reverse-phase μ-SPE and (C) ultrafiltration (30 kDa molecular weight cut off membranes) followed by reverse-phase μ-SPE.

the sensitivity of the method. However, this has brought to the common practice to use serum proteins to distinguish between healthy and pathological patients that were not identified [19], raising questions about their real usefulness. Thus, contrary to this trend, in the present work also the reflectron acquisition mode was employed to improve the mass resolution of very LMW peptides (i.e., <3500 Da); this could potentially allow the attribution after database search of peaks suspected to be of a certain usefulness as biomarkers. It is true that the identity of these molecules is not absolutely necessary for their use as biomarkers, but without this knowledge, the method will remain empirical and probably difficult to validate, reproduce and standardize [20]. Of course, an apparent drawback is that reflectron mode has a low *m/z* upper limit; however, very LMW peptides have been recently correlated to tumor pathologies [2].

Thus, the aim of the present work was the comparison of different sample preparation approaches for MALDI-TOF-MS analysis of low mass range proteins/peptides in serum, in order to achieve satisfactory repeatability (on both *m/z* values and peak intensity) and the higher number and abundance of ions observed in MALDI spectra recorded in the *m/z* ranges of 1.000–3.000 (reflectron mode) and 3.000–30.000 (linear mode), respectively.

To detect really meaningful signatures of the patient status on serum peptides/proteins profiles, pre-analytical and analytical variability must be within acceptable limits such that they do not obscure variations induced by the pathological status. This aspect can be very problematic, particularly when samples are collected at different times, by different operators (even at the same place) and then stored (under non-standardized conditions), and finally sent to a mass laboratory for analysis.

Thus, several pre-analytical variable parameters (namely: serum-clot contact time, clotting temperature, number of freeze-thaw cycles) were carefully considered, adopting a standardized collection/storage protocol (see Section 2).

Other sources of variability are certainly represented by all the steps of the analytical method (namely: clean-up procedure, denaturing or not-denaturing conditions [13], choice of MALDI matrix, sample:matrix ratio, instrumental conditions [21]); in fact, analysis of plasma protein constituents by means of mass spectrometry has so far yielded a bewildering number of protein identifications with little accordance between studies [4]. Thus, unique standardized protocols for both preparation and analysis of serum sample have not been suggested until now.

Table 1
Within sample ($N=5$) and sample to sample ($N=5$) precision obtained for serum samples from healthy donors on both *m/z* values and peak intensity of the main observable ions ($S/N \geq 3$) in the relevant MALDI-MS spectra

Mean *m/z* (monoisotopic)	*m/z*				Peak intensity			
	Within sample		Sample to Sample		Within sample		Sample to Sample	
	S.D.	CV (%)	S.D.	CV (%)	Mean relative (%)	S.D.	Mean relative (%)	S.D.
(A) Reverse-phase μ-SPE								
1025.739	0.042	0.004	0.054	0.005	16.31	2.17	17.81	2.26
1036.748	0.052	0.005	0.065	0.006	12.63	4.26	13.30	4.99
1066.113	0.049	0.005	0.044	0.004	100,00	0	100,00	0
1168.852	0.047	0.004	0.093	0.008	7.09	2.19	7.23	2.34
1296.757	0.052	0.004	0.065	0.005	12.18	3.54	12.70	4.65
1759.072	0.089	0.005	0.093	0.005	3.61	2.22	3.89	2.60
1830.158	0.092	0.005	0.113	0.006	3.61	0.49	4.07	0.53
2058.267	0.107	0.005	0.120	0.005	3.19	0.33	3.33	0.45
(B) Dealbuminization followed by reverse-phase μ-SPE								
1020.656	0.042	0.004	0.069	0.007	6.31	2.37	6.42	2.41
1465.591	0.072	0.005	0.093	0.006	3.63	0.26	4.30	0.37
1716.880	0.072	0.004	0.114	0.007	8.18	3.54	8.48	3.67
1829.951	0.097	0.005	0.122	0.007	12.19	4.22	12.31	4.26
1943.018	0.097	0.005	0.129	0.007	4.61	0.99	6.10	1.31
2541.056	0.134	0.005	0.177	0,007	16.64	2.79	16.88	2.83
2753.228	0.146	0.005	0.191	0.007	100,00	0	100,00	0
2937.312	0.150	0.005	0.194	0.007	17.56	1.11	18.68	1.18
(C) Ultrafiltration followed by reverse-phase μ-SPE								
1020.534	0.039	0.004	0.057	0.055	28.34	7.88	25.34	13.40
1206.618	0.055	0.005	0.068	0.056	65.83	12.90	70.83	15.00
1260.566	0.056	0.004	0.070	0.056	100.00	0.00	100.00	0.00
1350.691	0.057	0.004	0.069	0.051	28.16	9.17	29.66	12.60
1418.614	0.071	0.005	0.089	0.063	38.75	8.58	35.63	12.36
1465.716	0.072	0.005	0.090	0.061	81.69	12.96	70.69	15.30
1545.688	0.074	0.005	0.090	0,058	30.98	14.98	50.18	20.40
1616.725	0.076	0.004	0.099	0.061	35.21	10.12	45.21	12.86

Sample pre-treatment: (A) reverse-phase μ-SPE, (B) dealbuminization followed by reverse-phase μ-SPE and (C) ultrafiltration (30 kDa molecular weight cut off membranes) followed by reverse-phase μ-SPE. Acquisition mode: reflectron.

First of all, different sample pre-treatment methods were tested in order to detect the higher ions number and abundance. It has been recently demonstrated [11] that serum sample fractionation, desalting and pre-concentration, before MALDI-MS analysis (linear mode), can be easily achieved using "home-made" miniaturized reverse-phase solid-phase extraction. Thus, the micro-SPE approach was initially tested on carefully selected healthy donors serum samples, directly applying samples onto μ-SPE columns. Fig. 1A shows the relevant MALDI-TOF-MS spectrum acquired in reflectron mode (1000–3000 Da). Furthermore, in order to deplete the high molecular weight proteins, such as albumin, and to enrich the LMW fraction, healthy donors serum samples were previously subjected to a dealbuminization step or to an ultrafiltration step on 30 kDa molecular weight cut off membranes, and finally loaded onto the μ-SPE columns. Fig. 1B and C, respectively, reports the relevant MALDI-TOF-MS spectra acquired in reflectron mode (1000–3000 Da).

The first observation one can made on the spectra shown in Fig. 1 is that the profiles obtained for the three purification procedures are different, clearly indicating how different can be the information that can be drawn by the same sample just adding one step in the pre-treatment procedure. In particular, after the μ-SPE procedure (see Fig. 1A) few peptides were detected in the explored range, likely due to a suppression effect [22,23] on the ionization of LMW peptides that could be ascribed to the high molecular weight proteins that have not been removed from the sample.

Better results were obtained after the selective albumin removal (Fig. 1B), even if the number of peptides detected still remained low. This evidence was not completely unexpected, since it was already reported [24–26] that albumin is a serum transport protein that binds a large number of LMW compounds; then, its removal may result in the probable loss of low abundance proteins, such as cytokines, lipoproteins and peptides.

As can be seen in Fig. 1C, the most satisfactory results were obtained in the case of ultrafiltration followed by μ-SPE, since a number of LMW compounds were clearly observable in the spectrum. As already reported [13], the improvement associated to this procedure, compared to the dealbuminization step, mainly arises from the possibility to work in denaturing conditions that, consequently, disrupt protein–protein interactions. This was not possible in the case of selective albumin removal due to intrinsic limitation of the technique. It is worth noting that better results were obtained working at a low centrifugation speed and by diluting the sample (see Section 2). According to previously reported data [13,27], the most abundant species were found in the *m/z* range below 2000. It is likely that the increased presence of peptides with *m/z* values lower than 2000, could interfere with the ionization of peptides with higher molecular weight, also considering that, working in reflectron mode, sensitivity decreases with mass increase [28].

Fig. 2A, B and C reports the MALDI-TOF-MS spectra relevant to the same samples of Fig. 1A, B and C, respectively, acquired in "linear mode" in the *m/z* range 3000–30000. As

Table 2
Within sample ($N=5$) and sample to sample ($N=5$) precision obtained for serum samples from healthy donors on both *m/z* values and peak intensity of the main observable ions ($S/N \geq 3$) in the relevant MALDI-MS spectra

Mean *m/z* (average)	*m/z*				Peak intensity			
	Within sample		Sample to Sample		Within sample		Sample to Sample	
	S.D.	CV (%)	S.D.	CV (%)	Mean relative (%)	S.D.	Mean relative (%)	S.D.
(A) Reverse-phase μ-SPE								
3096.82	1.04	0.033	1.44	0.046	100,00	0	100	0
3457.61	1.35	0.039	1.86	0.054	74.46	15.41	77.25	16.88
6433.93	2.43	0.038	3.02	0.047	51.74	14.40	55.32	17.20
6635.04	1.99	0.030	3.21	0.048	87.82	28.29	86.52	30.19
10050.11	3.25	0.032	4.12	0.041	23.62	11.77	27.16	13.58
13878.93	3.33	0.024	4.46	0.032	24.74	4.40	22.35	5.06
28103.54	6.40	0.023	7.89	0.028	11.81	4.88	12.08	5.51
(B) Dealbuminization followed by reverse-phase μ-SPE								
6060.81	1.04	0.017	1.84	0.030	60.00	17.29	64.42	24.41
6352.24	1.03	0.016	1.93	0.030	22.71	16.32	25.32	18.37
6632.63	1.35	0.020	2.35	0.035	44.46	15.40	46.23	17.22
12123.92	2.43	0.020	4.24	0.035	100,00	0	100	0
22265.51	4.59	0.021	7.78	0.035	13.82	4.45	12.31	5.26
(C) Ultrafiltration followed by reverse-phase μ-SPE								
3322.84	1.30	0.04	1.89	0.06	78.98	17.29	77.05	20.00
5030.19	1.43	0.03	1.74	0.04	44.87	9.17	42.36	12.36
6633.61	1.61	0.05	2.67	0.04	90.45	8.58	87.56	10.66
10046.77	2.43	0.02	3.89	0.04	53.25	22.96	50.22	22.00
13298.75	2.89	0.02	3.78	0.03	69.35	19.98	70.00	20.36
16630.38	3.04	0.02	4.89	0.04	90.60	10.12	88.23	14.56
22179.69	4.09	0.02	7.98	0.04	100.00	0.00	96.00	5.23

Sample pre-treatment: (A) reverse-phase μ-SPE, (B) dealbuminization followed by reverse-phase μ-SPE and (C) ultrafiltration (30 kDa molecular weight cut off membranes) followed by reverse-phase μ-SPE. Acquisition mode: linear.

can be observed in Fig. 2C, ultrafiltration followed by μ-SPE confirmed to be the most promising approach, clearly showing the best results in terms of number and abundance of ions observed in the spectrum, compared to the other clean-up procedures (see Fig. 2A and B), confirming the strong dependence of the detectable proteins/peptides on the clean-up procedure.

It is worth noting that other approaches for the removal of interference compounds were tried without success (lower sensitivity and number of detectable ions), i.e. protein precipitation, two-steps ultrafiltration, addition of surfactants, zip-tip (data not shown).

The subsequent step of the work was to achieve satisfactory within-sample and sample-to-sample repeatability (on both *m/z* values and peak intensity of the main observable ions ($S/N \geq 3$)) for the three procedures under investigation, by performing replicate measurements ($N = 5$) on samples from healthy donors ($N = 5$). The relevant results are shown in Tables 1A, B and C ("reflectron mode") and 2A, B and C ("linear mode"), respectively.

Even if all the above mentioned procedures gave good precision results, further experiments were performed using ultrafiltration followed by μ-SPE, since it was the procedure that gave the better results in terms of peaks number and intensities. It is worth noting that the good repeatability was likely to be ascribed also to the really homogeneous selection of the serum samples donors.

Once the pre-analytical and analytical conditions were optimized, the procedure was finally applied to serum samples of breast cancer patients ($N = 5$); the relevant MALDI-TOF-MS spectra ("reflectron mode") are shown in Fig. 3. Even if, at the first sight, the spectra could seem identical to the one reported in Fig. 1C (healthy subject), a more careful inspection of the less abundant peaks revealed the presence of *m/z* ions not observable in the case of healthy donors. Table 3 reports the *m/z* values relevant to the peaks observable (with the exception of those arising from the matrix) in the spectra obtained from both healthy and pathological samples, and the relevant presence (+) or absence (−). As can be seen, the majority of the peaks were present in the spectra relevant to both healthy and pathological samples; however, four peaks were observed only in the case of pathological samples while others were found only in healthy donors samples.

Due to the fairly good repeatability of the present approach, attribution attempts of the peaks present in the spectra could be undertaken. The idea is then to use the list of the masses obtained for a database search using ExPASy FindPept [29]. This database has been conceived to identify peptides that result from unspecific cleavage of proteins from their experimental masses (since

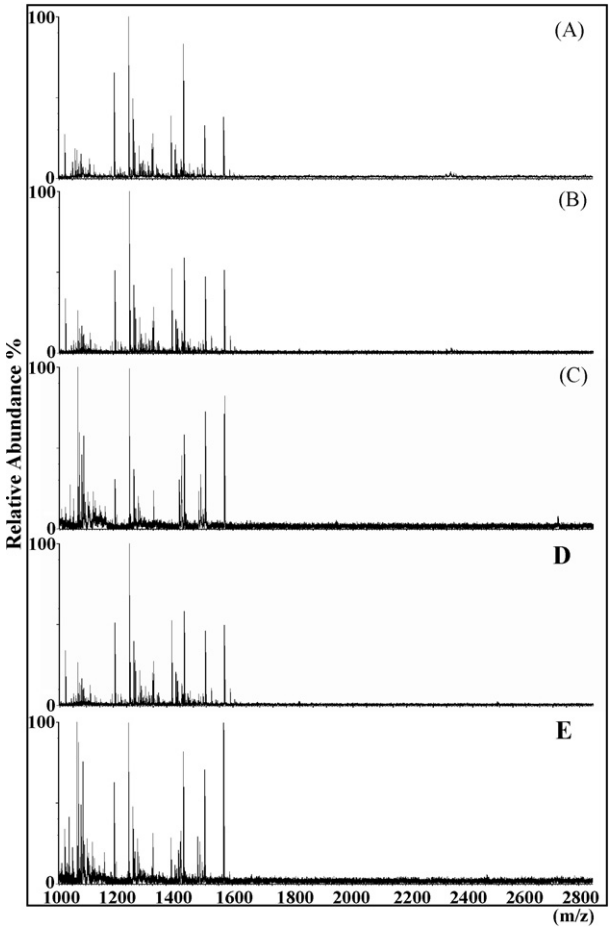

Fig. 3. MALDI-TOF-MS spectra (reflectron acquisition mode, 1000–3000 Da) obtained in the analysis of serum samples of breast cancer patients ($N = 5$). Clean-up method: ultrafiltration (30 kDa molecular weight cut off membranes) followed by reverse-phase μ-SPE.

Table 3
List of the *m/z* values of the peaks observable (with the exception of those arising from the matrix) in the spectra (acquired in "reflectron" mode) obtained from both healthy and pathological samples, and the relevant presence (+) or absence (−)

m/z (monoisotopic)	Healthy	Pathological
1020.534	+	+
1036.721	+	+
1081.234	+	+
1096.801	+	−
1113.403	+	+
1125.370	−	+
1139.350	−	+
1140.803	+	−
1167.930	−	+
1185.321	+	−
1206.618	+	+
1228.234	+	+
1260.566	+	+
1276.514	+	+
1282.513	+	+
1347.471	−	+
1350.691	+	+
1418.614	+	+
1433.210	+	+
1435.001	+	−
1455.070	+	+
1465.716	+	+
1487.712	+	+
1536.801	+	+
1545.688	+	+
1567.708	+	+
1616.725	+	+

Table 4
Within sample ($N=5$) and sample to sample ($N=5$) precision obtained for serum samples from cancer patients on both *m/z* values and peak intensity of the four peaks found only in pathological samples in the relevant MALDI-MS spectra

Mean *m/z* (monoisotopic)	*m/z*				Peak intensity			
	Within sample		Sample to Sample		Within sample		Sample to Sample	
	S.D.	CV (%)	S.D.	CV (%)	Mean relative (%)	S.D.	Mean relative (%)	S.D.
1125.370	0.046	0.004	0.063	0.006	3.45	1.17	3.72	1.50
1139.350	0.057	0.005	0.065	0.006	3.63	1.26	3.93	1.49
1167.930	0.059	0.005	0.074	0.006	3.52	1.31	3.81	1.51
1347.471	0.071	0.005	0.081	0.006	12.18	6.14	12.56	6.30

in serum are active different proteases) [30], taking into account artefactual chemical modifications and post-translational modifications (PTM). The experimentally measured peptide masses are compared with the theoretical peptides calculated from a specified Swiss-Prot/TrEMBL entry; this means that only one entry at time can be considered for the comparison. Thus, due to this intrinsic limitation of the search engine, 107 entries were selected for the match on the basis of an existing database of circulating peptide/proteins in human blood [30] from literature data. After database search, all the *m/z* ions shown in Table 3 could be attributed to peptides arising from proteins normally present in serum, such as circulatory proteins, coagulation and complementary factors, cytokines, transport proteins, growth factors and hormones, with the only exception of four peaks observable only in the pathological samples. Table 4 reports their standard deviation and coefficients of variation on the mass values and intensities. The peaks at *m/z* 1125.370, 1139.350, 1347.471 were matched only with osteopontin precursor while the *m/z* 1167.930 could arise from three possible proteins, i.e. vitronectin, uteroglobin-related protein 2 precursor and ubiquitin-activating enzyme E1 (a1s9 protein); all these proteins are known to be abnormally expressed in many cancer diseases [31–33] (of course, these results could not be significant from a clinical point of view, due to the low number of analyzed samples).

Serum samples of breast cancer patients were also analyzed in "linear mode", even if no significant differences were observed between healthy and pathological individuals (data not shown).

4. Conclusions

Different clean-up procedures for the MALDI-TOF-MS analysis (in both "linear" and "reflectron" acquisition mode) of low molecular weight proteins/peptides in serum were tested; the higher number and abundance of ions were obtained using ultrafiltration of serum on 30 kDa molecular weight cut off membranes followed by micro-reversed-phase extraction (μ-SPE). The procedure achieved also satisfactory repeatability. The method was finally tested on serum from breast cancer women to look for the existence of molecular masses capable of differentiating for malignancies. The findings obtained using the "reflectron" mode (rarely adopted in this field) were quite encouraging, even if few samples have been analyzed due to the difficult to find "similar" patients. Furthermore, a more complete and reliable investigation will be possible with the development of more powerful databases able to identify serum peptides originated by a-specific cleavages by unknown proteases.

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