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Sequential depletion coupled to C18 sequential extraction as a rapid tool for human serum multiple profiling

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ABSTRACT

Sequential chemical depletion of serum coupled to C18 sequential extraction of peptides as a rapid tool for human serum multiple profiling is herein presented. The methodology comprises depletion with DTT and then with ACN; the extract thus obtained is then summited to fast protein digestion using ultrasonic energy. The pool of peptides is subsequently concentrated using C18-based Zip-tips and the peptides are sequentially extracted using different concentrations of ACN. Each extract is mass-spectrometry profiled with MALDI. The different spectra thus obtained are then successfully used for classification purposes. A total of 40 people, comprising 20 healthy and 20 non-healthy donors, were successfully classified using this method, with an excellent q-value < 0.05. The proposed method is cheap as it entails few chemicals, DTT and ACN, simple in terms of handling, and fast. In addition, the methodology is of broad application as it can be used for any study applied to serum samples or other complex biological fluids.

1. Introduction

Avoiding Serum's major protein components should be done by linking the protocol for depletion of such proteins to the proteomic profile of the disease under study [1,2]. Following this reasoning, diverse chemical depletion methods have been developed and employed in proteomics. Depletion with ACN has showed to render a serum reach in apolipoproteins, adequate for studies dealing with cardiovascular-related diseases [1,3], whereas, chemical depletion with DTT leads to a serum reach in immunoglobulin-like proteins, adequate to studies dealing with myelomas or lymphomas. In addition, DTT was found to be an excellent way to deplete major proteins whilst preserving an important number of them in solution [1,4]. Very recently, the combination of a sequential chemical extraction using ACN and then DTT has been successfully applied to human serum for the searching of osteoarthritis biomarkers [5].

Also as a step prior to mass spectrometry analyses in proteomics, Zip-tips (or similar devices) are regularly used as a fast way to desalt samples as well as to pre-concentrate the peptides present herein [6–9]. A step forward in the handling of Zip-tips for sample treatment in proteomics consists in the sequential elution of the peptides that are bound to the resin by using solutions of different composition [10]. Each solution extracts a different fraction of the peptides retained by the resin, which may be then employed for further research. This fractionation aids to reduce sample complexity, which ultimately leads to an increase in the number of peptides and proteins identified.

Finally, peptide profiling based on mass spectrometry has grown over the past 10 years to become the method of preference for the fast analysis of changes in protein expression patterns on biological systems, helping to achieve early disease detection, disease staging, therapeutic monitoring and prognosis of malignant diseases [11–13]. In the present work, the three steps mentioned above have been coupled for the first time (precipitations with DTT, with ACN and Zip-tips sequential elution), to the

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best of our knowledge, in order to develop an easy, fast and inexpensive method for the screening of human serum samples in proteomic studies aimed at biomarker discovery. We propose to deplete the samples with (i) chemical depletion promoted by DTT or (ii) by chemical sequential depletion. Then, to digest the remnant proteins, concentrate the correspondent peptides using Zip-tips and subsequently fractionate them by sequential elution with solutions of ACN of different concentrations. Finally, acquire MALDI-TOF mass spectrometry-based profiles of each eluate that can be analyzed with bioinformatics tools to find out differences between samples for classification purposes. The reproducibility and usefulness of this method are exemplified with the use of serum samples from a cohort of patients suffering from rheumatic diseases (RD) and healthy individuals.

2. Materials and methods

2.1. Reagents

DL-dithiothreitol (DTT, ≥99%), iodoacetamide (IAA, ≥99%), trifluoroacetic acid (TFA, 99% FOR LC-MS), acetonitrile (ACN, LC-MS CHROMASOLV), water (LC-MS CHROMASOLV) and ammonium bicarbonate (≥99%) were purchased from Sigma (Steinheim, Germany) and were used for protein precipitation, reduction and alkylation. Trypsin (sequencing grade) from Roche (Mannheim, Germany) was used for protein digestion. NuTips large 10–200 μl and C-18 for peptide separation were purchased from Glygen (Columbia, USA). α-Cyano-4-hydroxycinnamic acid puriss for MALDI-MS (Fluka, Germany) was used as MALDI matrix. Calibration 1, 4700 Proteomics Analyzer Calibration Mixture from ABSciex (Framingham, MA, USA) was used as a mass calibration standard for MALDI-TOF/TOF-MS measurements. Eppendorf DNA LoBind tubes were used for the sample store.

2.2. Serum samples

The human serum samples were obtained from anonymous donors in the Complejo Hospitalario Universitario de A Coruña, Spain. Patients with rheumatic disease (RD) and healthy controls were characterized radiographically. All patients signed the informed consent. The study was approved by the local ethics committee of Galicia (Spain). Serum samples were stored at −80 °C until further processing.

2.3. Protein depletion with DTT

Protein depletion with DTT was performed according to the protocol described by Warder et al. [4]. 2.2 μl of 500 mM DTT was added to 20 μl of pooled serum and vortexed briefly. The sample was incubated for 1 h at room temperature until a viscous white precipitate persisted, which is pelleted by centrifugation at 14,000g for 2 × 20 min. The supernatant was transferred into a clean LoBind tube and evaporated to dryness in a vacuum concentrator centrifuge Savant SPD121P SpeedVac (Thermo, Waltham, USA).

2.4. Sequential protein depletion

Sequential protein depletion was performed according to the protocol previously developed in our laboratory [5]. The sera were subjected to a sequential depletion protocol involving two precipitation steps: protein depletion was first performed with DTT as above described, whereas the second depletion was performed with ACN according to the protocol described by Kay et al. [3] with minor modifications. 20 μl of pooled serum was depleted with DTT and the supernatant was depleted with 57% (v/v) ACN, vortexed and sonicated for 10 min in an ultrasonic bath (Sonorex, Bandelin). The sample was then vortexed briefly and sonicated further for 10 min. The protein precipitate was pelleted by centrifugation at 14,000g for 10 min, and the supernatant was transferred into a clean LoBind tube and evaporated to dryness in a vacuum concentrator centrifuge.

2.5. In-solution digestion

Ultrasonic in-solution digestion was performed according to the ultrafast proteolytic digestion protocol previously developed in our laboratory [14]. The evaporated sample was resuspended in 20 μl of ammonium bicarbonate (AmBi) 25 mM and 10 μl of ACN were added, the sample was vortexed and sonicated for 1 min (50% amplitude) in the sonicator (SONOPULS HD 2200 with cup horn BB6 accessory, Bandelin). Protein cysteine residues were reduced with 2 μl 110 mM DTT, vortexed and sonicated again for 1 min (50% amplitude) in the sonicator. The resulting cysteines were then blocked with 2 μl IAA 600 mM and vortexed and sonicated again for 1 min (50% amplitude) in the sonicator. 10 μl of 110 mM DTT was added for IAA inactivation. The sample was diluted to a final volume of 200 μl with AmBi 12.5 mM. Afterwards, trypsin was added according to the ratio 1:20 (w/w) trypsin/protein and the digestion was performed in the sonicator operating at 50% amplitude for 5 min. Finally, 2 μl of formic acid 50% (v/v) was added to stop the enzyme activity. The digested serum was evaporated to dryness.

2.6. NuTip sequential elution (peptides separation)

The digested samples were reconstituted in TFA 0.1% (v/v) and different amounts of protein (30 μg, 20 μg and 18 μg) were loaded onto a NuTip C18 large. The peptides separation was performed according to the following protocol:

Conditioning procedure: aspirate and expel 5 times the same volume as the sample of 60–80% (v/v) ACN+0.1% (v/v) TFA, and wash 3 times with 0.1% TFA.

Sample binding: aspirate and expel the sample 50 times to allow the peptides to adsorb to the reverse phase material.

Sequential elution: aspirate and expel the same volume of different percentages of ACN 20 times for each one, from low to high ACN concentration. Collect the eluates in a clean LoBind tube and evaporate to dryness until MS/MS analysis. Following this procedure, different sets of experiments were performed:

(i) *First set*
Pooled serum from 5 RD patients was depleted with the DTT method and digested as described above. 30 μg of the digested sample was loaded by quintuplicate in a NuTip C18 large, following the previous protocol. The elution was performed with 0%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 55%; 80% (v/v) of ACN. Each eluate was collected in a clean tube and evaporated to dryness for MS/MS analysis. The experiment was performed again by triplicate with 18 μg of digested sample.

(ii) *Second set*
Pooled serum from 5 RD patients was depleted with the DTT method and digested. 20 μg of the digested sample was loaded by triplicate in a NuTip C18 large, following the previous protocol. The elution was performed with a new gradient of ACN: 0%; 4%; 7%; 10%; 14%; 17%; 20%; 25%; 35%; 60% (v/v), and each eluate was collected in a clean tube. All the eluates were evaporated to dryness for MS/MS analysis.

(iii) *Third set*
Pooled serum from 5 RD patients was depleted following the protocol of sequential depletion as previously described. 20 μg of digested protein was loaded by triplicate in a NuTip

C18 large and the elution was performed with the new gradient of ACN: 0%; 4%; 7%; 10%; 14%; 17%; 20%; 25%; 35%; 60% (v/v). All the eluates were collected in a clean tube and evaporated to dryness until MS/MS analysis.

2.7. Comparison between control and RD pooled sera

Equal volumes of serum from 10 RD patients were grouped into two pools of five samples each to reduce the individual and biological variability, and the same grouping was done for 10 serum samples from control patients. The pools were depleted with the sequential depletion protocol and in-solution digested as described above. 20 μg of the digested pools was loaded onto a NuTip C18 large. The sequential elution was performed with 0%; 4%; 7%; 10%; 14%; 35% (v/v) of ACN. The eluates were evaporated to dryness until MS/MS analysis.

2.8. Comparison between control and RD individual sera

Twenty serum samples from each condition, RD and control conditions, were depleted individually with the sequential depletion protocol, and in-solution digested as described above. Table 4 of Supplementary material summarizes data from the patients included in this study and the correspondent protein concentrations of the sera. 20 μg of the digested samples was loaded onto a NuTip C18 large. The sequential elution was performed with 4%; 7%; 10%; 14% (v/v) of ACN. The eluates were evaporated and analyzed in quintuplicate (in order to increase reproducibility) by MALDI mass spectrometry, as described in the next section.

2.9. Mass spectrometry analysis

The evaporated samples were resuspended in TFA 0.1% (v/v). 1 μl of sample was hand-spotted onto a MALDI target plate (384-spot Teflon®-coated plate) and allowed to air dry at room temperature. Subsequently, 1 μl of a 3 mg/ml solution of α-cyano-4-hydroxycinnamic acid matrix in 0.1% (v/v) TFA and 50% (v/v) ACN was added to the dried peptide digest spots and again allowed to air dry. The samples were analyzed using a MALDI-TOF/TOF mass spectrometer model 4800 Proteomics Analyzer from ABSciex (Framingham, MA, USA) equipped with a Nd:YAG laser ($\lambda = 355$ nm) and 4000 Series Explorer™ Software (ABSciex). Internal calibration, data acquisition, processing and interpretation were carried out as recommended by the manufacturer. All mass spectra were externally calibrated using a standard peptide mixture (ABSciex).

Mass spectral analysis for each sample was based on an average of 1500 laser shots. Fragmentation spectra were acquired by selecting the 20 most abundant ions of each MALDI-TOF peptide mass map and averaging 2000 laser shots per fragmentation spectrum with medium energy CID. For MS/MS all peaks with an S/N > 10 were included in the database search. The analysis of MS/MS spectra was performed by applying the MASCOT (Matrix Science) search engine with the following parameters: (i) Swiss-Prot, 2011 Database (532,792 sequences; 188,961,396 residues); (ii) taxonomy, Homo sapiens (20,257 sequences); (iii) enzyme, trypsin; (iv) one missed cleavage; (v) fixed modifications, carbamidomethyl (C); (vi) variable modifications, oxidation (M); (vii) peptide mass tolerance up to 50 ppm; (viii) fragment mass tolerance up to 0.3 Da. The criteria used to accept the identification was significant homology scores achieved in MASCOT ($p < 0.05$).

2.10. PCA and statistical analysis

A Principal Component Analysis (PCA) was applied to the MS spectra of RD and control individual sera. Each spectrum was pre-processed with the 4000 Series Explorer Software using the following parameters: mass range of 500–4000 Da, peak density maximum 50 peaks per 200 Da, minimum signal to noise ratio (S/N) of 10, minimum area of 100, and maximum peak to spot ratio 500. Peaks were aligned with a peptide mass tolerance of 150 ppm and, finally, a representative spectrum was created for each sample with those peaks present in at least 4 of the 5 samples' spectra. Resulting peaks were filtered using a Fisher Test corrected with Benjamini Hochberg FDR, selecting those with a q-value < 0.05. Before applying the PCA, a single dataset was created for each elution percentage (i.e. 4%, 7%, 10%, and 14%) with the representative spectrum of every sample. Spectra on each dataset were converted into a 1 s and 0 s vector, where 1 means peak presence and 0 means peak absence. The PCA analysis and visualization were done using the RapidMiner v5.3 software (http://rapid-i.com/content/view/181/190/), configuring the PCA algorithm to reduce the dataset dimensionality down to 3 principal components.

3. Results and discussion

3.1. Multiple profiling with DTT depletion

The sample treatment illustrated in Fig. 1 was followed in this set of experiments. The enzymatic digestion of a complex proteome generates a pool of peptides. Such peptides can be selectively separated through the interactions that they can establish with an immobilized solid phase, such as the one included in the C18-based NuTips©. C18 contains large hydrocarbon chains, which interact preferentially with hydrophobic peptides. As depicted in Fig. 1, in this optimization study we used a C18 solid phase resin. Thus, after protein digestion, the hydrophobic peptides are retained using the C18-based NuTip and eluted with different

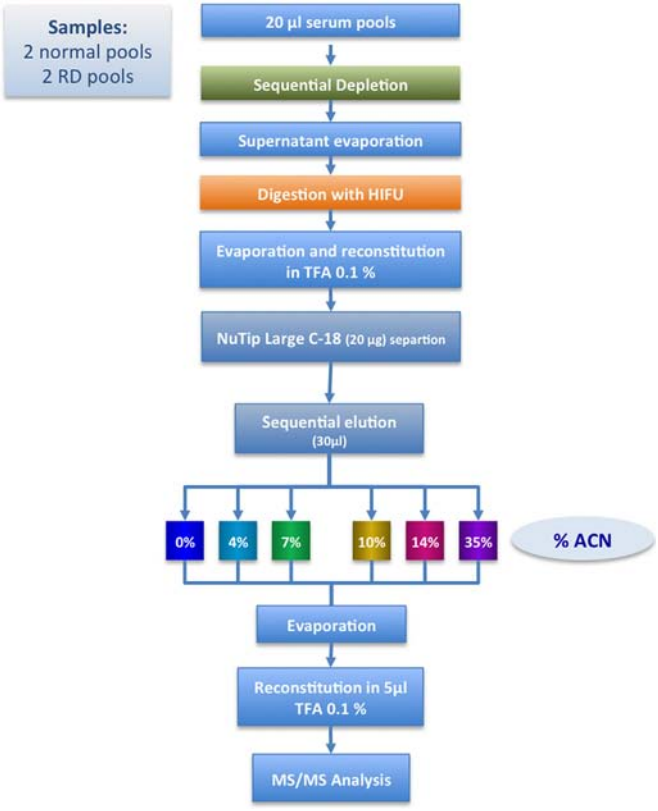

Fig. 1. Sample treatment corresponding to the first set of conditions assayed for DTT depletion and ACN sequential elution.

amounts of ACN. A number of variables affecting this procedure were identified and optimized to maximize the number of peptides extracted, as explained below.

3.1.1. Influence of the total protein mass loaded

Care must be taken to avoid overloading the NuTips with peptides, as the amount of resin contained in the NuTips has been designed to retain peptides from a digestion of up to 25 μg of protein. Therefore, it can be saturated easily. In addition, some peptides are retained preferentially in the resin. The aforementioned drawbacks hamper the study if MALDI is used as an ionization source, because the presence of some few peptides at high concentration will lead to signal suppression of the less abundant ones. This means that the spectrum obtained in such situation is going to have few intense peaks dominating it. This is not desirable if the objective is to find differences by comparing the *m*/*z* peaks obtained from a series of spectra from different samples. To study the influence of the mass of protein treated, in the first set of NuTip sequential elution assays, two amounts were assessed, 30 and 18 μg. For the highest amount a total of 14 non-redundant proteins were identified as shown in

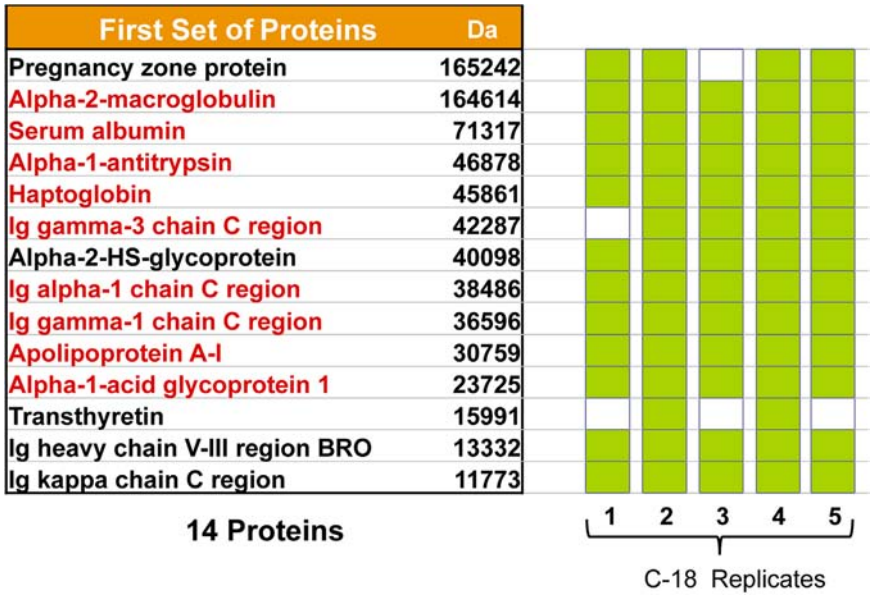

Fig. 2. Proteins identified by MALDI-MS/MS. Replicates were done using one NuTip for each line. Replicates were carried out on the same sample (reproducibility of extraction).

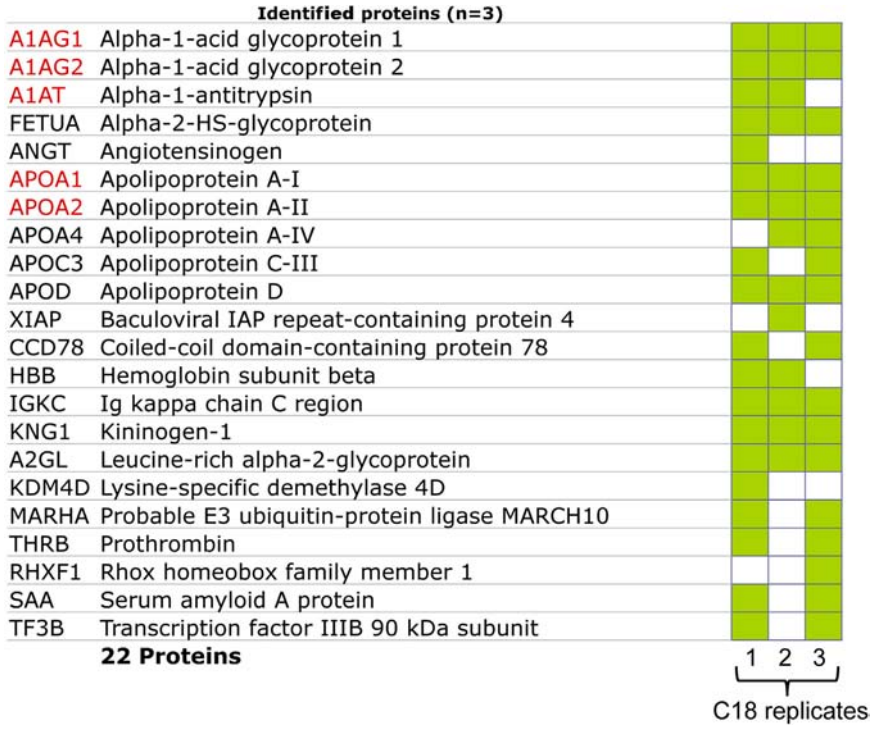

Fig. 3. Proteins identified by MALDI-MS/MS. Technical replicates. Three different samples corresponding to the same serum were treated as described in the experimental section.

Fig. 2 (13 ± 1, range 12–14, and $n=5$). The number of major proteins (20 most abundant proteins in serum, top 20) found was 9. When the total mass of protein loaded in the NuTips was set to 18 μg, the number of non-redundat proteins identified rose to 21 (19 ± 2, range 16–20, $n=3$), from which 12 belong to the top 20 group. Another interesting finding from this set of experiments is that the number of proteins identified increases until the ACN concentration reaches 35% v/v. Then, growing rates of ACN do not affect significantly the number of proteins identified. It was concluded that it is important to avoid loading above the upper limit of the NuTips, because the number of proteins identified was higher with 18 μg than with 30 μg of total protein.

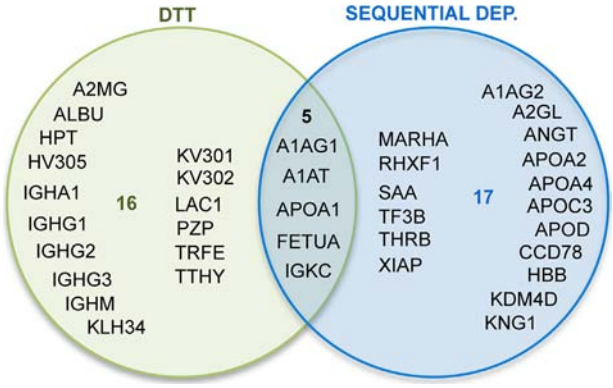

Fig. 4. Venn diagram showing the proteins identified and common proteins for the sample treatments performed using (i) depletion with DTT and (ii) sequential depletion.

3.1.2. *Influence of the gradient of ACN used in the sequential extraction process*

As explained above and shown in Fig. 1 of supplementary content (Supplementary Fig. 1), the group of non-redundant proteins identified remains almost constant after the seventh extraction (35% ACN). This suggests that further extractions and higher concentrations of ACN are not necessary. Therefore, the gradient was changed to a range with lower amounts of ACN, comprising between 0% and 60% v/v (0%; 4%; 7%; 10%; 14%; 17%; 20%; 25%; 35%; 60%). According to the results explained above, for these sets of experiments the total mass of protein loaded in the NuTips was 20 μg. Now, the results showed a total of 21 proteins identified (19 ± 1, range 18–20, and $n=3$). It was also verified that 12 of the 21 proteins identified (57%) were in the list of the top 20 most abundant proteins in human serum.

3.1.3. *Reproducibility of the method*

Fig. 2 presents the reproducibility for the sequential elution method using NuTips. An excellent absolute reproducibility, higher than 80%, was obtained for the five technical replicates performed with a total of 30 μg of protein mass digested and loaded (first set).

It can be also verified that 9 of the 14 proteins identified are included in the top 20 of the most abundant proteins in serum.

3.2. *Multiple profiling with chemical sequential depletion*

As for previous research done in our laboratory [1,5], sequential chemical depletion was expected to result in a serum with lower content in high abundant proteins, thus rendering a pool of peptides with a compressed range of concentrations and increased number of peaks in the MALDI spectra. The mass of protein used and the ACN elution gradient chosen were those found in Section 1. Fig. 3 shows the results of 3 technical replicates. The number of proteins identified was 22. All proteins were identified within the extracts

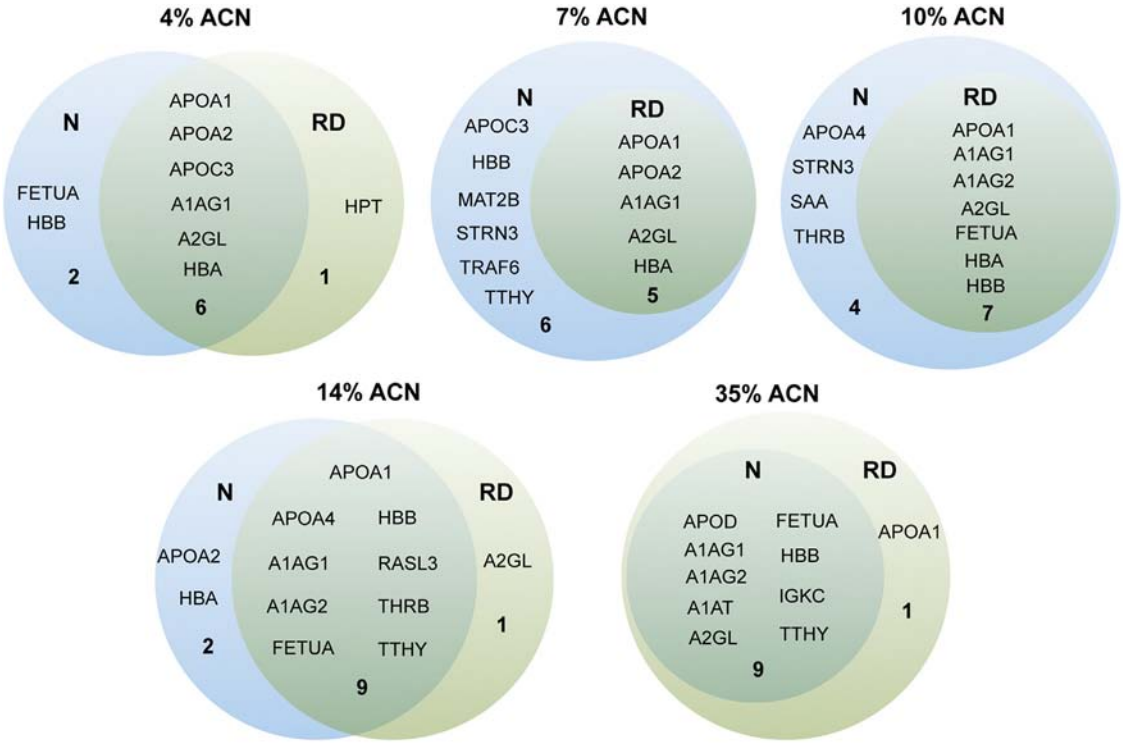

Fig. 5. Venn diagrams showing the proteins identified and common proteins for sera pools belonging to healthy (N) and non-healthy (RD) donors. The sample treatment was done using sequential depletion coupled to sequential extraction. % refers to the ACN concentration used in the sequential extraction procedure (% v/v).

obtained with an ACN concentration range comprised between 0% and 35% v/v. Proteins identified in a minimum of two replicates constituted 82% of the total ($n=3$). For this case only 5 of 22 were high abundant proteins.

3.3. Depletion with DTT versus sequential chemical depletion

The Venn diagram shown in Fig. 4 illustrates the differences between the methods of depletion assessed in this work. Only five unique proteins were common to both methods. In addition, 12 abundant proteins (from the top 20) were found with the DTT depletion, whilst 5 were the abundant proteins identified using the sequential depletion method. Both methods present similar reproducibility. Taking into consideration the optimization process, the method of choice to perform multiple sequential profiling was the sequential chemical depletion because the presence of high abundant proteins negatively influences the profile of each sequential extraction. Further to these considerations, the sequential extractions were reduced from 10 to 6, as some of the sequential extractions were found to provide the same information in terms of proteins identified (see Supplementary Table 1). Therefore, the following sequential ACN gradient v/v was finally selected: 0%, 4%, 7%, 10%, 14% and 35%.

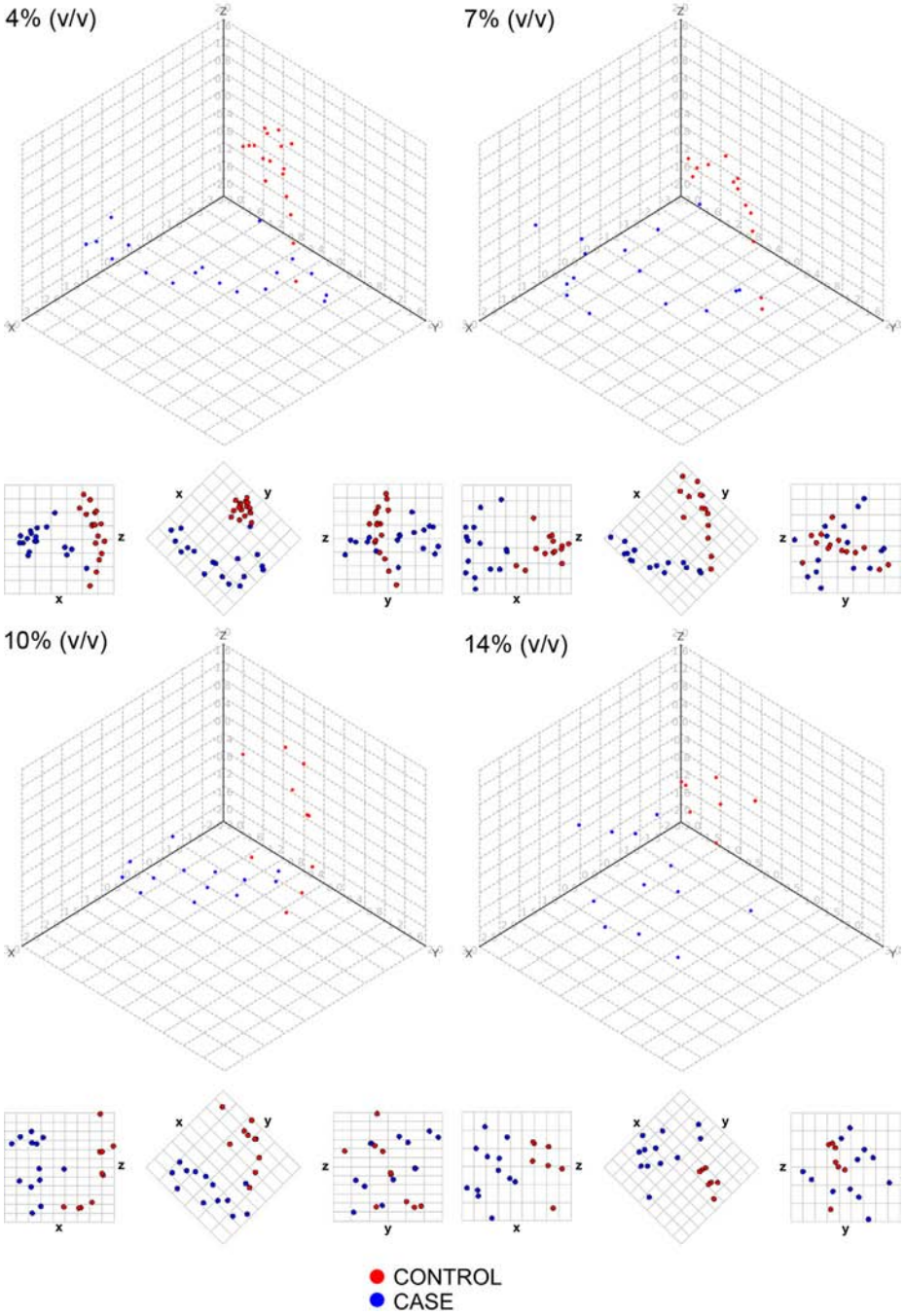

Fig. 6. PCA diagrams showing the classification obtained with the profiles from the MALDI analyses of 20 healthy subjects (Controls, red dots) and 20 patients with rheumatic disease (Case, blue dots), whose sera were individually submitted to the sequential depletion plus sequential extraction sample treatment as explained in the experimental section. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Differential protein profiling between human sera from rheumatic disease and control donors

Sera from 10 healthy individuals were pooled into two samples (five sera each) to reduce interindividual variability [5]. Sera from 10 donors with rheumatic disease (RD) were pooled in the same manner. Each pool was submitted to the sequential chemical depletion followed by the sequential elution process described above (see Supplementary Fig. 2). Fig. 5 shows the Venn diagrams illustrating those proteins identified in each of the analyzed fractions of the pools, as well as the comparison of the proteins found for healthy and non-healthy pools, which are listed in Supplementary Table 2. The peptide matches obtained by MALDI-TOF mass spectrometry and the identification score of each protein are explained in Supplementary Table 3. It may be seen that each group (healthy versus non healthy) has a characteristic and different pattern of proteins for each fraction of ACN.

3.5. Classification of serum samples by their peptide profiles

Overall, the results obtained clearly showed that each eluted fraction (i) presents a characteristic pattern of proteins within the same group of donors (healthy or non-healthy) and (ii) presents a characteristic pattern of proteins for different groups of donors (healthy versus non-healthy). These results were promising, and thus the next step was to compare the profiles of peptides obtained from each fraction. That is, we evaluated the possibility of using those MS spectra obtained by the presented method with classification purposes, in order to avoid the time-consuming MS/MS profiling. Interestingly, the pipette tips pre-packed with C18 resin are commonly used in the proteomic field for desalt and preconcentrate the sample prior to MS analysis with a single elution [6,7,15], but the present work reports for the first time a sequential chemical depletion coupled to peptide sequential elution using C18 tips as a way to reduce dynamic range of protein concentrations and fractionate high complex samples such as digested human serum. To this end, a differential study was done using the serum of 20 healthy individuals and 20 rheumatic disease (RD) patients.

From all the ACN solutions used to extract the peptides from the C18 resins, the best results in terms of classification using PCA at a level of q-value < 0.05 were obtained with the concentrations of 4%, 7%, 10% and 14% v/v. The corresponding PCAs are presented in Fig. 6 and show that for all the extracting conditions the classification is achieved in at least two of the planes. This demonstrates the usefulness of the herein proposed methodology of sample treatment to find out differences between groups and thus allowing classification in proteomic studies on serum samples.

4. Conclusions

Sequential chemical depletion of serum coupled to C18 sequential extraction of peptides as a rapid tool for human serum peptide profiling has been successfully proven. The methodology comprises depletion with DTT and then with ACN, and the extract thus obtained is then submitted to fast protein digestion using ultrasonic energy. Next, the pool of peptides obtained is concentrated using C18-based Zip-tips and the peptides are sequentially extracted using different concentrations of ACN. Each extract is mass-spectrometry profiled using MALDI-TOF technology. The different spectra consequently obtained can be then successfully used for classification purposes. To evaluate its usefulness a total of 40 people, comprising 20 healthy and 20 RD people, were classified using this protocol with an excellent q-value < 0.05 and we obtained a peptide profile to discriminate between these two conditions. The proposed method is cheap as it entails few chemicals and is easy in terms of handling and is not time consuming, thus enabling high throughput. In addition, the methodology is of broad application as it can be used for any proteomic study on human serum samples. Finally, although the power of this protocol for disease biomarker discovery using proteomics is clearly prompted in the present work, further experiments should be performed on larger cohorts of patients and controls to validate its utility in clinical routines.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.02.050.

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