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Nanoparticle-assisted removal of protein in human serum for metabolomics studies

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Abstract

Among human body fluids, serum plays a key role for diagnostic tests and, increasingly, for metabolomics analysis. However, the high protein content of serum poses significant challenges for NMR-based metabolomics studies as it can strongly interfere with metabolite signal detection and quantitation. Although several methods for protein removal have been proposed, including ultrafiltration and organic solvent induced protein precipitation, there is currently no standard operating procedure for the elimination of protein from human serum samples. Here, we introduce novel procedures for the removal of protein from serum by the addition of nanoparticles. It is demonstrated how serum protein can be efficiently, cost-effectively, and environmentally friendly removed at physiological pH (pH 7.4) through attractive interactions with silica nanoparticles. It is further shown how serum can be processed with nanoparticles prior to ultrafiltration or organic solvent induced protein precipitation for optimal protein removal. After examining all the procedures, the combination of nanoparticle treatment and ultrafiltration is found to have minimal effect on the metabolite content leading to remarkably clean homo- and heteronuclear NMR spectra of the serum metabolome that compare favorably with other methods for protein removal.

TOC image

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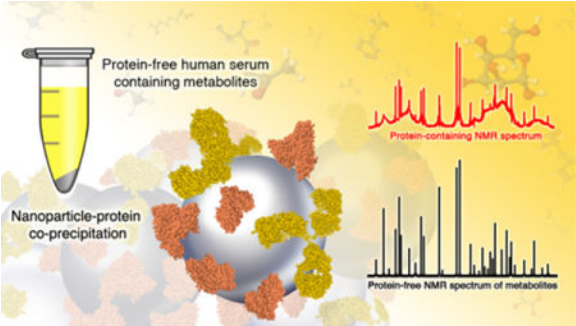
ASSOCIATED CONTENT

Additional information showing NMR spectra demonstrating reproducibility and controls, and ζ-potential determination. This information is available free of charge via the Internet at http://pubs.acs.org/.

Notes

The authors declare no competing financial interest.



Keywords

Human serum; NMR-based metabolomics; nanoparticle-assisted protein removal; ultrafiltration; organic solvent induced protein precipitation

INTRODUCTION

Metabolomics is a rapidly growing bioanalytical field for the comprehensive analysis of complex biological molecular systems with powerful applications in areas such as disease diagnosis and treatment,[1] toxicity assessment,[2] drug discovery,[3] and food science.[4] Since metabolism is the product of a multitude of biochemical pathways and the metabolome is a mirror of the phenotype, metabolic profiling of various biofluids and tissues has been demonstrated to provide powerful information about the state of a biological system.[5] The success of metabolomics studies critically relies on the collection, detection, and quantitation of metabolites, which can be perturbed by the presence of proteins and other biomacromolecules.[6–8] Because of the diversity of samples, a preparation protocol that works well for one type of biofluid or tissue is not necessarily transferable to other types of samples.[6,9]

Due to its richness in molecular components, human serum is routinely used for clinical blood tests and diagnostics, such as dissolved proteins, glucose, lipids, hormones, antibody/antigens, and metabolites, and it has become an attractive target for biomarker discovery and metabolomics.[10,11] Serum has a high protein content of 60–80 mg/mL consisting predominantly of albumins and globulins.[12] Unfortunately, this poses significant challenges for NMR-based metabolomics studies as it severely affects the spectral quality. Proteins possess a large number of mostly broad NMR peaks, which overlap with the resonances stemming from the metabolites, introducing a distorted baseline that makes identification and quantification of metabolites notably hard or even impossible. Certain proteins are known to interact with some of the metabolites, which can lead to the broadening or disappearance of metabolite peaks.[13,14] There is also the possibility of enzymatic metabolite degradation, which can have a negative impact on sample storage, quantitation, and reproducibility.

The one dimensional (1D) ^{1}H spectrum of raw pooled human serum (Figures 1a, S1a) illustrates these challenges: it shows sharp metabolite peaks with line widths of the order of a Hz that strongly overlap with the mostly broad protein peaks. Even in the two-dimensional

(2D) ^{13}C-^{1}H heteronuclear single quantum correlation (HSQC) spectrum of the same sample, which overall has substantially increased resolution as compared to the 1D spectrum (Figure 2a,c,e), many of the cross-peaks are very broad causing significant cross-peak overlap thereby reducing the number of identifiable metabolites by database query[15–17] and other means. Moreover, the presence of cross-peaks stemming from proteins can lead to cross-peak misassignments and false-positive metabolite identification.

The removal of protein in human serum and plasma has been a topic of research for several decades. Despite significant advances made, there is still no standard operating procedure for minimizing the adverse effects of proteins on serum analysis in NMR-based metabolomics for several reasons.[18–20] Currently, organic solvent-induced protein precipitation and ultrafiltration are two of the most popular methods, but they are not without drawbacks as is discussed below. A general procedure that can remove most if not all proteins and at the same time is efficient, reproducible, cost-effective, and environmentally friendly is still missing. Nanoparticles have been shown previously to interact with a variety of proteins.[21–23] In the present work, we introduce new protocols, which meet the above requirements by removing proteins from serum with the help of silica nanoparticles (SNPs) that co-aggregate with the serum proteins.

EXPERIMENTAL SECTION

A pooled serum sample from healthy humans was obtained from Innovative Research, Inc., Novi, MI). Bindzil 2040 silica nanoparticles (SNPs) were obtained from AkzoNobel and Eka Chemicals (Figure S7) with an average diameter of 20 nm (characterized previously[24]) and a negative surface charge at pH 7.4 (ζ-potential = −40.7 ± 5.9 mV, see Figure S8). The anionic SNPs were extensively dialyzed in 50 mM sodium phosphate buffer before they were added to serum. A 1D ^{1}H NMR spectrum of pure SNPs in the phosphate buffer, i.e. in the absence of serum (Figure S9), shows that the SNPs are essentially free of background signals.

Sample preparation protocols

Three different nanoparticle-assisted protein removal protocols have been developed in this work, with their workflows depicted in Figure 3. The first procedure is solely based on the use of SNPs without subsequent ultrafiltration or organic solvent induced protein precipitation (see left column of Figure 3). For 1 mL human serum sample it involves the following steps: (1) addition of 200 μL of 40% (w/w) aqueous suspension of SNPs to serum sample; (2) thorough mixing by vortexing for 1 min; (3) centrifugation at 14,500 rpm for 30 min at 4°C; (4) collection of supernatant; (5) addition of 300 μL 40% (w/w) aqueous suspension of SNPs and thorough mixing by vortexing for 1 min; (6) lyophilization of the sample; (7) addition of 650 μL D_2O and thorough mixing by vortexing for 1 min; (8) centrifugation at 14,500 rpm for 30 min at 4°C; (8) collection of 600 μL of supernatant for NMR measurements.

Nanoparticle-assisted protein removal can also be combined with ultrafiltration

For this purpose, an Omega Membrane filter (molecular weight cut-off of 1 kD) was used together with a Microsep Advance Centrifugal Device purchased from Pall Life Sciences (Port Washington, NY). For 1 mL human serum sample involves the following steps (see middle column of Figure 3): (1) addition of 100 μL of 40% (w/w) aqueous suspension of SNPs to serum sample; (2) thorough mixing by vortexing for 1 min; (3) centrifugation at 14,500 rpm for 30 min at 4°C; (4) collection of supernatant and ultrafiltration at 4,300 rpm for 30 min; (5) washing with 1 mL Milli-Q water at 4,300 rpm for 30 min; (6) pooling of filtrate from both the supernatant and the wash. In this way, over 95% of the original metabolite content of the serum sample is recovered. To obtain the final NMR sample, the pooled filtrate was then lyophilized and resuspended in 600 μL of 50 mM sodium phosphate buffer (pH 7.4) in D_2O for NMR measurements. For comparison, the ultrafiltration method without SNP pretreatment was also applied to serum, but with four additional wash cycles to achieve a comparable recovery rate.

Nanoparticle-assisted protein removal can also be combined with organic solvent induced precipitation

In this method (see right column of Figure 3), the SNP pretreated serum samples were subjected to either the addition of methanol or a combination of methanol/chloroform so that final ratios of methanol:H_2O = 2:1 (v/v) or chloroform:methanol:H_2O = 1:1:1 (v/v/v), respectively, were reached. In both cases, the resulting mixture was vortexed, incubated at −20°C for 30 min and centrifuged to remove the protein precipitate. The mixture was further diluted ten times with H_2O and frozen followed by lyophilization. The resulting powder was then resuspended in 50 mM phosphate buffer (pH 7.4) in D_2O for NMR measurements. For comparison, the organic solvent induced precipitation method was also applied to serum alone.

NMR measurements

All NMR spectra were collected on a Bruker AVANCE 600 MHz spectrometer equipped with a cryogenically cooled probe at 298 K. 1D ^{1}H NMR spectra of the model mixture were collected with 32,768 complex points, 16 ppm spectral width with 64 scans. 2D ^{13}C-^{1}H HSQC spectra of the model mixture were collected with 512×2048 (N_1×N_2) complex points with 16 scans per increment. All NMR data were zero-filled, Fourier transformed, and phase-corrected. 1D ^{1}H NMR spectra were processed using the ACD/1D NMR manager version 12.0 (Advanced Chemistry Development, Inc.). 2D NMR spectra were processed using NMRPipe.[25] Identification of metabolites from the 1D and 2D NMR spectra was done using the online COLMAR database query (http://spin.ccic.ohio-state.edu/index.php/colmar).[26]

RESULTS

The effectiveness of protein removal of the three different SNP-assisted methods depicted in Figure 3 was directly assessed from their corresponding NMR spectra (Figures 1, 2, 4). The results for protein removal using previously established methods, namely ultrafiltration or organic solvent induced precipitation without the use SNPs are shown for comparison.[13]

SNP-assisted protein removal without additional treatments

Figure 1b shows that that the treatment by nanoparticles alone is quite effective as the spectral baseline is substantially improved over the original serum spectrum (Figure 1a). This approach consists of two steps: after the initial addition of SNPs causing protein-nanoparticle co-aggregates they are removed by centrifugation. Further addition of SNPs followed by lyophilization and resuspension leads to the further improvement, i.e. flattening, of the baseline as shown in Figure S6. Still, the spectral quality does not fully match the one of the ultrafiltration method alone (Figure 1c). Hence, while the SNP approach alone is effective, it can be complemented by additional treatment depending on the application and the spectral quality required.

SNP addition with ultrafiltration

Ultrafiltration is an alternative approach, which separates biomacromolecules from small molecules purely based on size. Since metabolites are much smaller than proteins, ultrafiltration can in principle remove proteins and other biomacromolecules when using an appropriate filter pore size cutoff. It has been found previously that ultrafiltration is an effective method for protein removal in metabolomics studies of serum in terms of sensitivity, protein removal efficiency, and reproducibility.[14] This is confirmed by Figure 1c and Figure S1, which show the excellent baseline property and details of low-concentration metabolite peaks, e.g. in the 2.6 – 3.0 ppm range, of human serum treated by ultrafiltration using an ultrafiltration device (1 kD molecular weight cutoff). However, in practice the limited capacity of ultrafiltration devices causes severe restrictions making it extremely difficult to filter a sample that is rich in protein such as serum, especially when the sample has a high protein content forming a sticky suspension that resists passage through the filter. In such cases, multiple dilutions and wash cycles are required, which involve each time the time-consuming ultrafiltration by centrifugation step, causing a significant dilution (or loss) of sample that may result in the detection of fewer metabolites.

As an alternative, the combination of nanoparticle treatment with subsequent ultrafiltration represents an attractive approach for the removal of protein signals from the NMR spectra. Figures 1d, S1d demonstrate the excellent performance of this method producing the highest quality NMR spectrum among the methods described here (Figures 1, 2) in terms of the flatness of the baseline, the sharpness of the peaks, as well as the signal-to-noise ratio. The spectral quality of the 2D ^{13}C-^{1}H HSQC spectrum improves considerably making it amenable to automated peak-picking and database query, e.g. using the COLMAR HSQC web server.[17,26,27] Moreover, the reproducibility of this method for three independent batches of SNPs and serum samples is very high as is demonstrated in Figure S3. The SNP addition to human serum reduces the amount of protein in the sample to a level where the ultrafiltration method still works efficiently. For the successful implementation of the nanoparticle addition method the addition of an optimal amount of SNPs to the serum sample is important, which in this work corresponded to 10 – 20% (v/v) of 40% (w/w) SNP. It is generally recommended to first perform a test run in order to estimate the optimal amount of SNPs for which ultrafiltration works without significant blockage.

SNP addition with organic solvent induced protein removal

Organic solvent induced protein precipitation is the most commonly used sample preparation method of serum. In this procedure, organic solvent(s) are added to the serum, which denature and precipitate proteins so that they can be subsequently removed by centrifugation, while the organic solvent(s) are removed via a rotary evaporator or lyophilization.[13,28] In Figures 4a,b & S2a,b, ^{1}H 1D NMR spectra of human serum samples are depicted after they have been treated by the addition of methanol or methanol/chloroform. The figures show that the broad protein peaks have been largely removed by both methods, although after treatment with methanol alone, residual protein signals cause a well-visible curved baseline under the crowded peak regions as well as a broad lipid feature around 1.2 ppm. Chloroform treatment results in a very similar spectrum with a baseline that is slightly less curved. Obviously, considering the toxic nature of chloroform for humans and the environment, it is desirable to limit its usage for high-volume routine applications. Also the removal of methanol from methanol-water mixtures has to be done with appropriate care.

In our experience, the use of SNPs as a pretreatment step considerably reduces the use of organic solvent. As shown in Figure S2a,c, the pretreatment of SNPs improved the flatness of the baseline as well as the peak details as is visible, for example, in the spectral regions around 1.9 and 2.3 ppm. Thus the addition of SNPs is useful to eliminate signals stemming from proteins as well as lipids, whereby the latter are more difficult to be removed by methanol alone. The combined usage of SNPs with the methanol-based organic solvent method (MeOH:water = 2:1 (v/v)) achieves equally good results as the chloroform-based method.

DISCUSSION

The ability to acquire clean NMR spectra of human serum is a prerequisite for high-quality metabolomics studies. The presence of high concentration of protein in serum is manifested in the form of broad groups of resonances, which lead to a curved baseline that adversely affects spectral analysis in terms of peak identification and quantitation. This applies to 1D and 2D NMR-based analysis alike (Figures 1, 2). Existing methods for protein removal can overcome these challenges, but their use is not without drawbacks: ultrafiltration devices get easily clogged and organic solvent-based removal is time-consuming and potentially impacts the environment.

The ability of nanoparticles to interact with proteins has been reported previously.[21,29] Silica-based nanomaterials are generally non-toxic, colloidal, chemically stable, and they have low cost.[24] Here, we demonstrate that SNPs provide an effective means for protein removal from solution as can be seen for serum in Figure 1b. The best results are obtained, however, when the SNP addition method is combined with either ultrafiltration or an organic solvent based approach. For the combined SNP-ultrafiltration method, it shortens the time until the lyophilization step by at least a factor of two to below 50 minutes.

We have recently shown that electrostatic nanoparticle-metabolite interactions can be utilized to discriminate between metabolites with different charge properties.[24] These

studies were applied to samples, such as urine, where essentially no protein is present. Importantly, in the presence of protein, as is the case for serum, the combination of SNP-addition with ultrafiltration does not cause any loss of metabolite signals, including the ones from charged metabolites, as can be seen when comparing Figures 1c and 1d. The reason for this is that SNP-protein interactions are much stronger than SNP-metabolite interactions and, therefore, the surfaces of the SNPs are first covered with protein. Once all protein has been removed and SNPs are added, SNP-metabolite interactions lead to the disappearance of NMR signals of specific metabolites facilitating their identification as reported previously.[24] Ultrafiltration of these samples leads to the recovery of essentially all metabolites by "washing" them off at the filter. This is demonstrated for a metabolite model mixture in Figure S4. For protein-free serum, two metabolites that interact more strongly with the SNPs may not come off the SNPs during ultrafiltration (see Figure S5). It should be emphasized that this effect is only relevant for samples that are already free of protein, whereas for protein-rich serum with adequate SNP content unwanted metabolite-SNP interactions do not impose restrictions, as the final metabolite content is not affected by the protein removal treatment.

No single method is optimal for all conceivable metabolomics sample conditions. In practice, the choice of protein removal is dictated by the number and size of serum samples. If a sample volume is of the order of a few milliliters, the combined SNP-addition and ultrafiltration method is very powerful because it overcomes the limited capacity of the filtration device. If a sample has a smaller volume in the hundreds of microliters, SNP addition in combination with the methanol-based precipitation method works well, since in this case the solvent can be removed by lyophilization in a short period of time. The nanoparticle-assisted protein removal method has a good potential for applications also to other types of protein-rich metabolomics samples, such as plasma and tissue samples.

CONCLUSIONS

In summary, novel SNP-based procedures for the removal of protein have been presented here. SNPs can either be used alone or as an effective complement to ultrafiltration or organic solvent induced precipitation. The use of silica nanoparticles during the preparation of serum samples provides an efficient, cost-effective, and environmentally sustainable means to remove a substantial amount of protein in protein-rich serum allowing the collection of high-quality 1D and higher dimensional NMR data suitable for accurate and quantitative metabolomics studies. These properties should make SNP addition attractive for the routine screening of serum samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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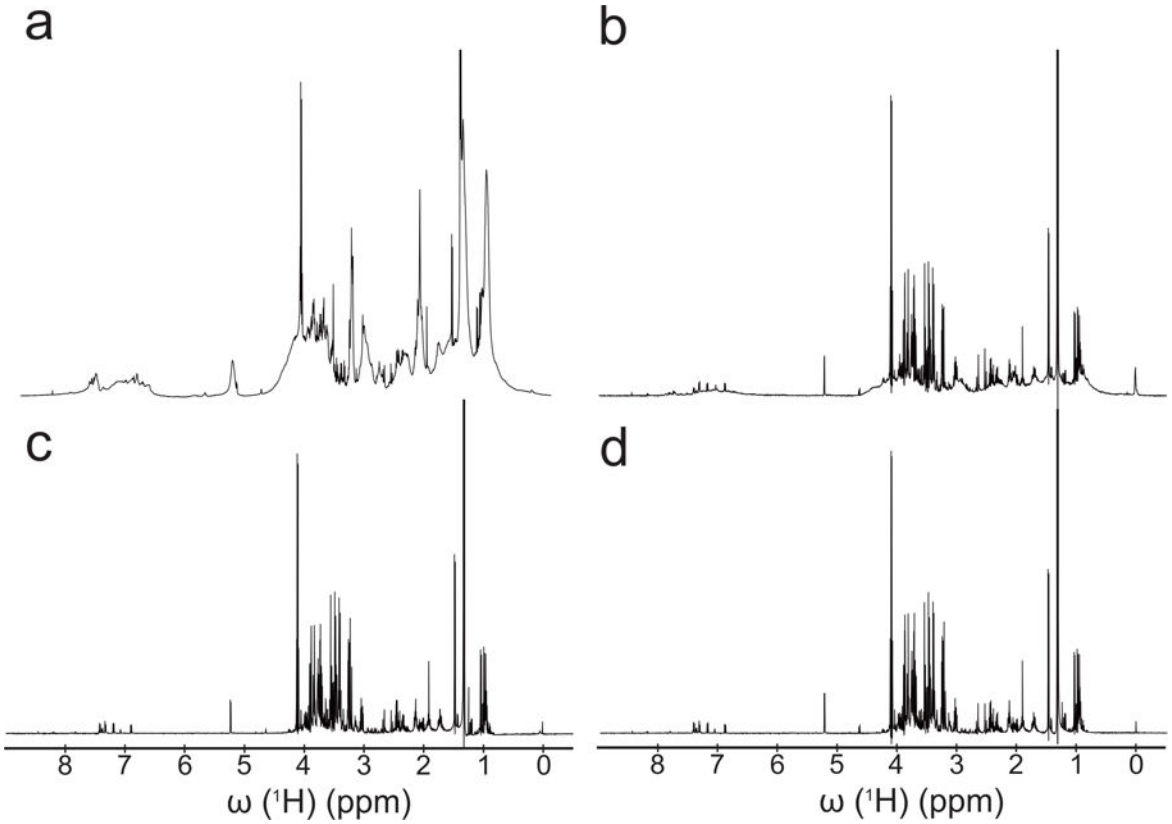

Figure 1.

Effect of silica nanoparticle addition and ultrafiltration on 1D ^{1}H spectra of 1 mL pooled human serum. **a)** Without any protein removal, **b)** with protein removal using nanoparticle addition followed by lyophilization and resuspension, **c)** after ultrafiltration only, and **d)** after nanoparticle addition, centrifugation, and supernatant extraction followed by ultrafiltration. 10 μM DSS was added as internal standard for all the samples (signal at 0 ppm).

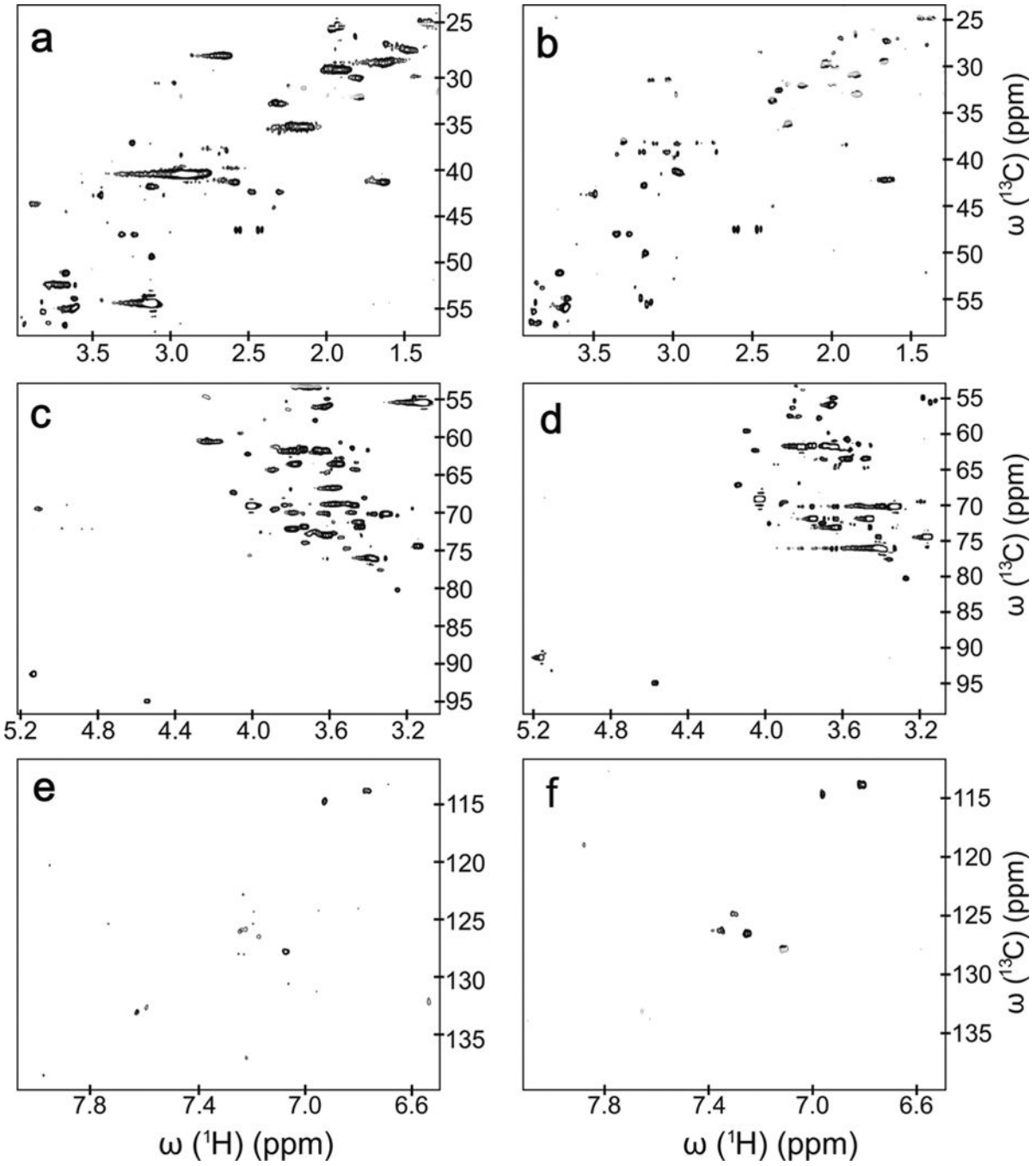

Figure 2.

Three different regions (rows) of two 2D ^{13}C-^{1}H HSQC spectra (columns) of 1 mL pooled human serum. Panels **a,c,e**: selected regions of same 2D spectrum *without* any protein removal; Panels **b,d,f**: selected regions of same 2D spectrum *with* protein removal using nanoparticle addition followed by ultrafiltration.

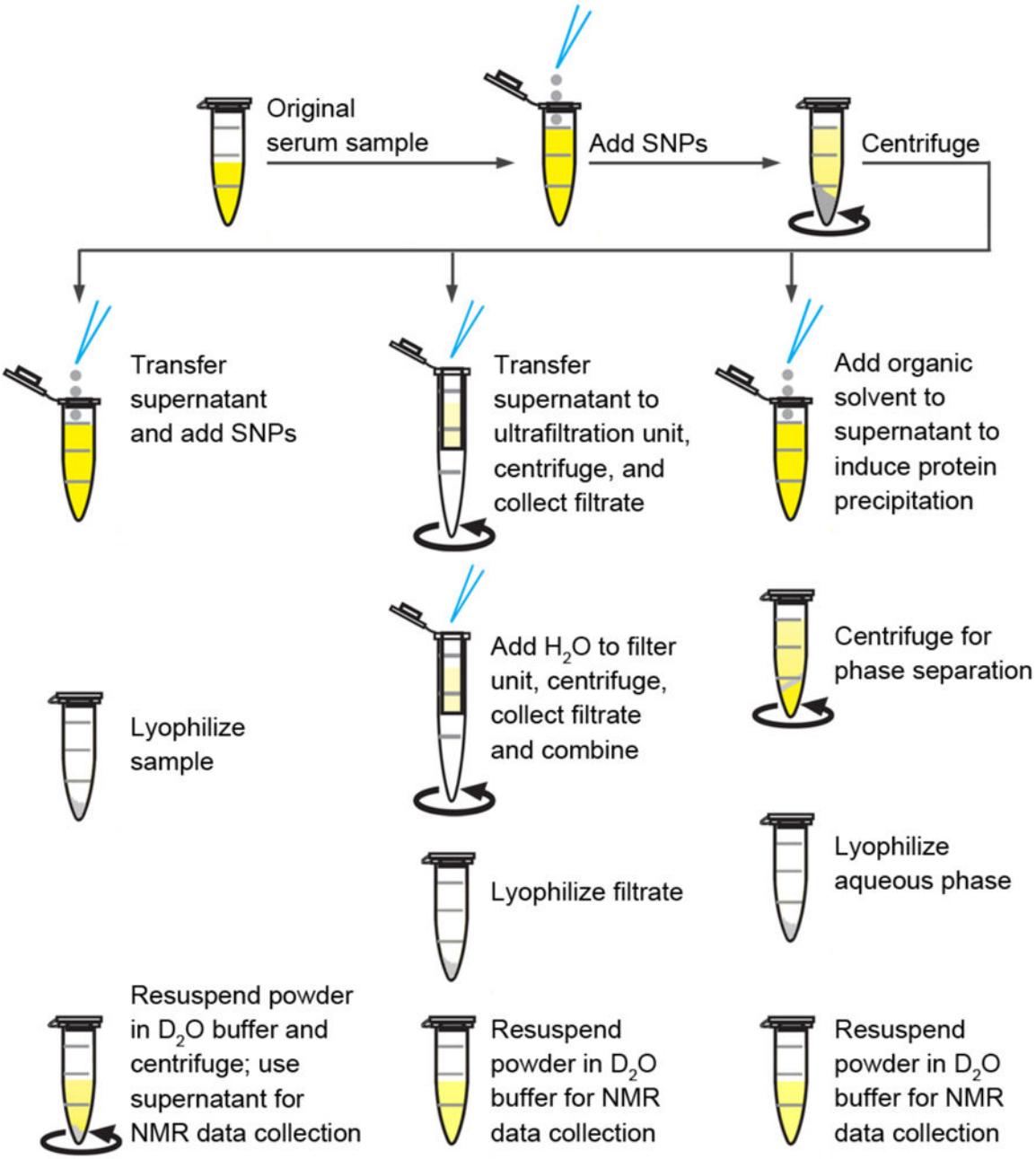

Figure 3.

Three different ways to use silica nanoparticles (SNPs) for serum sample preparation for NMR-based metabolomics. Left column: use of SNPs alone; middle column: use of SNPs for pretreatment followed by ultrafiltration; right column: use of SNPs for pretreatment followed by organic solvent-induced protein precipitation.

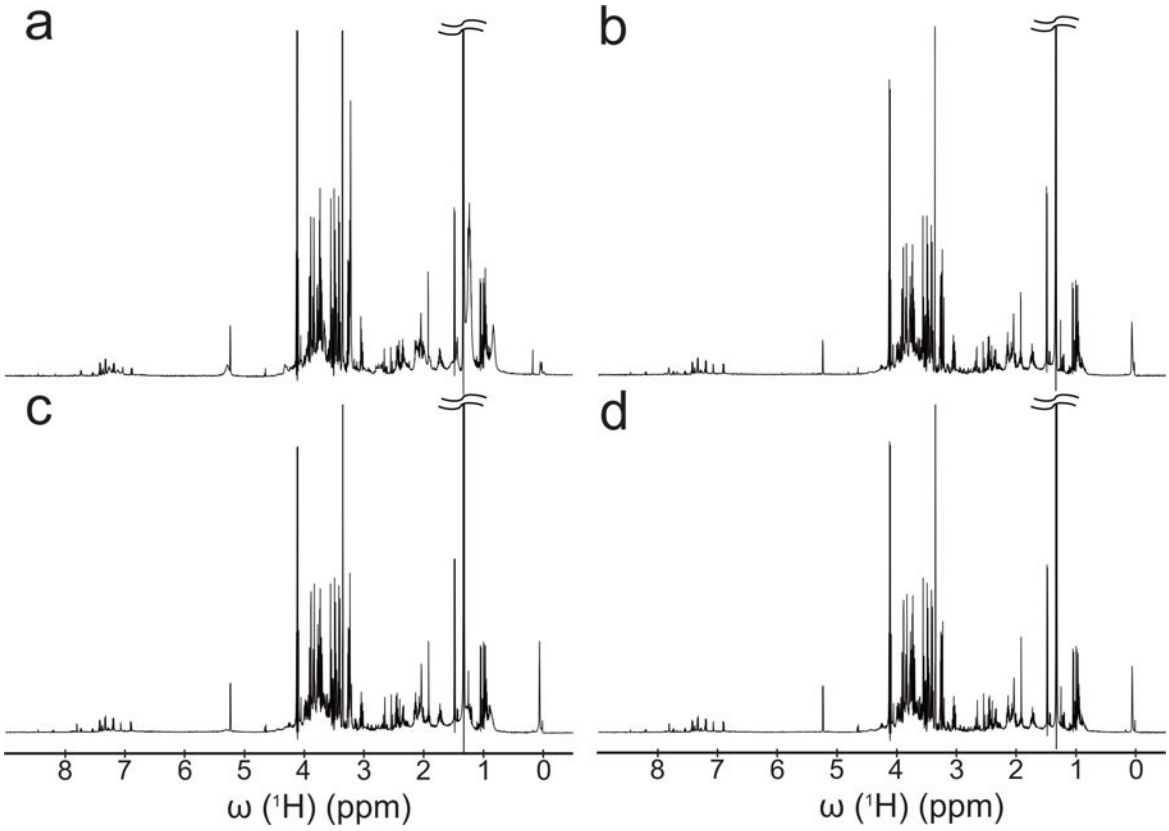

Figure 4.

Effect of silica nanoparticle addition and organic solvent induced protein precipitation on 1D ^{1}H spectra of 1 mL pooled human serum. **a)** Methanol and H_2O (2:1 (v/v)) method, **b)** chloroform, methanol, and H_2O (1:1:1 (v/v/v)) method, **c)** nanoparticle addition, centrifugation, and supernatant extraction followed by the extraction of methanol and H_2O (2:1 (v/v)), **d)** nanoparticle addition, centrifugation, and supernatant extraction followed by the extraction of chloroform, methanol, and H_2O (1:1:1 (v/v/v)). In Panels a,c, the strong, sharp peak at 3.4 ppm belongs to methanol, which stems from the organic solvent used for protein precipitation.