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TITLE:

Capillary Electrophoresis Mass Spectrometry Approaches for Characterization of the Protein and Metabolite Corona Acquired by Nanomaterials

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SUMMARY:

Here we present a protocol to characterize the complete biomolecular corona, proteins, and metabolites, acquired by nanomaterials from biofluids using a capillary electrophoresis – mass spectrometry approach.

ABSTRACT:

The adsorption of biomolecules from surrounding biological matrices to the surface of nanomaterials (NMs) to form the corona has been of interest for the past decade. Interest in the bio-nano interface arises from the fact that the biomolecular corona confers a biological identity to NMs and thus causes the body to identify them as “self”. For example, previous studies have demonstrated that the proteins in the corona are capable of interacting with membrane receptors to influence cellular uptake and established that the corona is responsible for cellular trafficking of NMs and their eventual toxicity. To date, most research has focused upon the

protein corona and overlooked the possible impacts of the metabolites included in the corona or synergistic effects between components in the complete biomolecular corona. As such, this work demonstrates methodologies to characterize both the protein and metabolite components of the biomolecular corona using bottom-up proteomics and metabolomics approaches in parallel. This includes an on-particle digest of the protein corona with a surfactant used to increase protein recovery, and a passive characterization of the metabolite corona by analyzing metabolite matrices before and after NM exposures. This work introduces capillary electrophoresis – mass spectrometry (CESI-MS) as a new technique for NM corona characterization. The protocols outlined here demonstrate how CESI-MS can be used for the reliable characterization of both the protein and metabolite corona acquired by NMs. The move to CESI-MS greatly decreases the volume of sample required (compared to traditional liquid chromatography – mass spectrometry (LC-MS) approaches) with multiple injections possible from as little as 5 µL of sample, making it ideal for volume limited samples. Furthermore, the environmental consequences of analysis are reduced with respect to LC-MS due to the low flow rates (<20 nL/min) in CESI-MS, and the use of aqueous electrolytes which eliminates the need for organic solvents.

INTRODUCTION:

Bio-nano interactions, at the interface between nanomaterial (NM) surfaces and biological matrices from which biomolecules adsorb to the NM, is an intense area of research underpinning nanosafety and nanomedicine[1]. This layer of adsorbed biomolecules confers a biological identity to NMs, thus cells identify them as "self", subsequently influencing cellular uptake, distribution and biological endpoints[2–4]. The vast majority of bio-nano studies have focused upon the proteins within the corona, and have demonstrated that proteins vary quantitatively and qualitatively between NMs of different compositions[5]. This variation is dependent upon both the properties of the NM such as size, functionalization, and material in addition to the properties of the biological matrix including composition and salt content[5]. A rising area of interest in the bio-nano interface are the metabolites that adsorb to NMs[6]. Several studies have demonstrated that, as with the proteins, NM properties are a key factor in determining the composition of the metabolites in the corona and that they can influence biological outcomes of NM exposure[6–8].

In studies of the biomolecular corona there are four distinct phases to its characterization, corona formation, the isolation of the biomolecules within the corona, their detection via qualitative or quantitative mass spectrometry and identification[9]. To date, a range of techniques have been adopted to isolate the protein corona including boiling in SDS-PAGE running buffer, solvent and salt extractions or direct digestion of proteins in situ on the surface of NMs. In terms of the metabolites in the corona, the isolation is more complex with various methods using solvents or even the dissolution of the NMs proposed as solutions[8, 10, 11]. However, unlike the protein corona a "one size fits all" approach is unlikely to apply to the isolation of metabolites from NMs, due to the wide chemical space occupied by the metabolome and the diversity of possible NMs. An alternative approach is to characterize the biological matrix before and after NM exposure with the difference in metabolite concentrations attributed to the adsorption of metabolites to NMs.[12]

This alternative approach would be applicable to all biofluids and NMs and although it does

require twice as much analysis, it consequently offers a much more precise measure of the metabolite corona and has no risk of low metabolite recoveries from the NM surface being mistaken for low binding of the specific metabolite.

The second step to the biomolecular corona analysis is the detection of the biomolecules. Traditionally for the proteins in the corona, this has been the remit of nano-LC-MS, the workhorse of proteomics; other approaches such as NMR[13], 1D and 2D SDS-PAGE have also been applied. In terms of the metabolite corona LC-MS[7], GC-MS[8] and direct infusion mass spectrometry have been utilized[14]. However, a new approach has recently started to gain traction, namely capillary electrophoresis-mass spectrometry (CE-MS)[11, 15] and has been present in NM labs as a standalone technique to characterize various physical and chemical properties of NMs[16]. CE-MS is an orthogonal separation technique to nanoLC-MS and GC-MS and is capable of enabling the detection of highly polar and charged metabolites[17, 18]. Furthermore, CE-MS is well suited for the analysis of proteins and their posttranslational modifications such as phosphorylation and glycosylation[19–22]. The final step, identification and data analysis can be performed in several ways depending upon if a quantitative / qualitative or targeted / untargeted approach is being used, this, however, falls outside the remit of this protocol.

CESI-MS, a combination of CE and a nanoESI interface, is a recent advance in CE technology utilizing a sheathless interface. This enables direct connection of ultra-low flow CE (<20 nL/min) to a high resolution mass spectrometer with no dilution, resulting in significantly improved detection sensitivity[23–25]. CESI-MS enables volume limited samples (< 10 µL) to be analyzed while maintaining a highly sensitive analysis[26]. CESI-MS also compares favorably to traditional low flow rate nanoLC-MS approaches used in proteomics and metabolomics in terms of reproducibility[27,28], throughput, and carry over making it an exciting prospect for the complete biomolecular corona characterization.

To highlight the potential of CESI-MS for the analyses of bio-nano interactions this work describes the sample preparation required to isolate the NM biomolecular corona from a single human plasma sample in such a way as to maximize data from both the protein and metabolite aspects of the complete biomolecular NM corona. While we report on the use of human plasma, this protocol would be equally appropriate for other biofluids such as blood serum, complete cell culture medium, cell lysate, cerebrospinal fluid or urine. The analyses of these two components (proteins and metabolites) will then be described using neutral coated CESI capillaries for the protein corona and bare fused silica capillaries for both cationic and anionic metabolites.

PROTOCOL:

Use of human biofluid was approved as per the IRB protocol guidelines from University of Leiden and Innsbruck Medical University. When human or animal biofluids are being investigated, ethical approval from the research institution is required, and has been obtained in the case of the results shown in this protocol. Furthermore adequate reporting should also be performed to ensure transparency and reusability of data in future work[9,29,30].

1. Preparation of background electrolytes (BGE)

NOTE: All solvents should be prepared in a suitable fume hood and adequate personal protective equipment must be used for all steps (labcoat, gloves and goggles). In each step, low binding plastic vials are required to minimize analyte loss and contamination of sample with salts leached from glassware.

1.1. Prepare 10% acetic acid BGE (v/v), pH 2.2 on the daily basis for metabolomics.

1.1.1. Into a 10 mL volumetric flask add 1 mL of acetic acid, followed by the addition of deionized (DI) water to the marked line and thorough mixing.

1.2. Prepare 100 mM acetic acid, pH 2.9 on the daily basis for proteomics.

1.2.1. Into a 10 mL volumetric flask add 57 µL of acetic acid, followed by the addition of DI water to the marked line and thorough mixing.

2. NM preparation

2.1. Vigorously disperse NMs in water using published guidelines[31–33].

NOTE: In the development of this protocol, 7 NMs were used as follows: 100 and 1,000 nm carboxylated polystyrene were used for protein and metabolite corona, respectively. 100 nm unmodified polystyrene NMs, 13 nm anatase TiO_2 NMs which were unmodified or coated with either polyacrylate or PVP polymers and 22 nm unmodified SiO_2 NMs were used for both protein and metabolite coronas. While the method was developed and demonstrated using these NMs, it should be noted that this approach would be applicable for a wide array of NM compositions, sizes, shapes, and morphologies.

2.2. Characterize particle size using either dynamic light scattering[34,35], single particle inductively coupled plasma mass spectrometry[36], nanoparticle tracking analysis[37], or transmission electron microscopy and surface charge as zeta potential using a zeta sizer[34].

3. Biomolecular corona formation

3.1. Split 2 mL of human plasma (or biofluid of choice) into 1 mL aliquots, one for the metabolite and protein corona and the second for plasma metabolome characterization.

3.2. Incubate 1 mg/mL of NMs in the human plasma for 1 h at 37 ˚C while gently mixing at 500 rpm in a thermomixer. Following incubation centrifuge the sample at 4,000 x *g* for 15 min at 4 ˚C to pellet the NMs.

3.3. Collect the plasma supernatant for metabolite corona analysis. Retain the NM-protein corona complex for protein corona characterization.

4. Protein corona isolation

4.1. Resuspend the NM-protein complex (the pellet) in 1 mL of 10x PBS Buffer and vortex vigorously for 2 min to remove unbound proteins. Centrifuge the PBS solution at 4,000 x *g* for 15 min at 4 ˚C to pellet the NMs, and carefully remove the supernatant without disturbing the pellet.

4.2. Resuspend the pellet in 1 mL of ammonium bicarbonate buffer (ABC) (100 mM, pH 8.0) and vortex vigorously for 2 min to remove unbound proteins and undesired salts. Centrifuge at 4,000 x *g* for 15 min at 4 ˚C to pellet the NMs; remove and discard the supernatant. Repeat this step.

4.3. Reduce protein disulfide bonds by dissolving the NM-protein pellet in 20 µL of ABC buffer (100 mM pH 8) containing 10 mM dithiotheritol. Incubate the reduction solution for 30 min at 56 ˚C.

4.4. To digest proteins, add 2 µg of sequencing grade Trypsin to 20 µL of ABC containing 0.1% surfactant. Incubate the digest solution for 16 h at 37 ˚C.

4.5. Alkylate the sample by the addition of 20 µL of iodoacetamide at 55 mM in 100 mM ABC and incubate at room temperature for 20 min.

4.6. Add 20 µL of 0.1 M HCl to cleave the surfactant and leave for 10 min at room temperature.

4.7. Enrich and desalt peptides using a C18 packed desalting pipette tip.

4.7.1. Wet the tip with 80 µL of acetonitrile 5 times, clean with 80 µL of DI water 5 times, draw up and elute the sample 10 times, desalt the sample by flushing 80 µL of 0.5 % formic acid 5 times and elute the sample with 80 µL of 1% formic acid 50% acetonitrile into a clean low binding PCR tube.

4.8. Lyophilize the samples and store at -20 ˚C until analysis.

5. Metabolite corona isolation

5.1. Take the control plasma and the supernatant from the NM plasma incubation from Step 3.3 and keep on ice during sample preparation.

5.2. Take 50 µL from each into separate vials and dilute 1 in 10 with DI water. Take 50 µL of each diluted sample and move to new vials for step 5.3.

5.3. Add 200 µL chloroform, 250 µL methanol and 350 µL DI water and vigorously vortex mixture for 2 min. Centrifuge for 10 min at 20,800 x *g* at 4 ˚C.

5.4. Take 500 µL of supernatant and filter through a 3 kDa centrifugal filter for 2 h at 10,000

x *g* at 4 ˚C. Take 430 µL of ultrafiltrate and dry in a speed vac. Samples can now be frozen prior to analysis.

6. Preparation of CESI-MS system[38]

NOTE: Prior to the installation of the capillaries, check that the inlet and outlet end of the capillaries are intact and that there are no visible breakages in the capillary.

6.1. Installation of a neutral coated capillary for protein corona analysis[39].

6.1.1. Place a new neutral coated capillary (90 cm length with 30 µm internal diameter) into the CESI instrument following the manufacturer guidelines.

6.2. Preparation of CESI capillaries for proteomic analysis.

6.2.1. Flush the separation capillary in the forward direction using 100 mM of HCl for 5 min at 100 psi and check for droplet formation at the outlet of the capillary. Flush separation capillary with BGE at 100 psi for 10 min and then DI water at 100 psi for 30 min (this is only required for new capillaries).

6.2.2. Flush both the separation capillary and the conductive capillary with BGE at 90 psi for 5 min and ensure droplets form at the outlet end of both capillaries.

6.2.3. Flush the separation capillary with DI water at 90 psi for 10 min, then 50 psi for 10 min with 0.1 M HCl, followed again by 90 psi for 10 min with DI water and finally BGE at 90 psi for 10 min. Couple CESI to the MS using a nanospray source and adapter as previously described[38].

6.3. Installation of bare fused silica capillary for metabolite corona characterization[38].

6.3.1. Place a new bare fused silica capillary (90 cm length with a 30 µm internal diameter) into the CESI instrument following manufacturer guidelines.

6.4. Preparation of CESI capillaries for metabolomics analysis.

6.4.1. Flush the separation capillary in the forward direction using 100% MeOH at 50 psi for 15 min and check for droplet formation at the outlet of the capillary. Flush both the separation capillary and the conductive capillary with BGE at 90 psi for 5 min and ensure droplets form at the outlet of both capillaries.

6.4.2. Flush the separation capillary with DI water at 90 psi for 10 min, then 50 psi for 10 min with 0.1 M NaOH, followed again by 90 psi for 10 min with DI water and finally BGE at 90 psi for 10 min. Couple CESI to the ESI-MS using a nanospray source and adapter as previously described.[38]

7. CE-MS for protein corona analysis

7.1. Resuspend samples from step 4.8 in 20 µL of 50 mM ammonium acetate pH 4.0.

7.2. Perform 5 psi 10 s hydrodynamic injection of sample. Separate using a 30 kV separation voltage with the following pressure gradient: 0-10 min at 1 psi, 10-35 min at 1.5 psi and 35-45 min at 5 psi using 100 mM, pH 2.9 acetic acid as BGE.

7.3. Collect mass spectra between 250-2000 *m/z* with top-10 data dependent fragmentation acquisition.

7.4. Between samples, rinse capillary with 0.1 M HCl for 3 min at 100 psi and 10 min 100 psi of BGE for separation capillary and for 3 min at 100 psi for the conductive liquid capillary.

8. CESI-MS for metabolite corona analysis

NOTE: All samples must be analyzed twice, once in positive mode for the detection of cations and once in negative mode for the detection of anions. The control plasma samples need to be analyzed at least 5 times.

8.1. Resuspend NM exposed and unexposed plasma samples from step 5.4 in 430 µL of DI water and vigorously vortex for 2 min. Filter through a 0.1 µm membrane filter.

8.2. Take 95 µL of filtrate and add 5 µL of internal standards at 200 µM for cations (L-methionine sulfone) and 400 µM for anions (2,2,4,4-D4-citric acid) and vortex vigorously. Centrifuge at 4,000 x *g* at 4 ˚C for 10 min prior to CESI-MS analysis.

8.3. Inject sample hydrodynamically for 30 s (cations) and 40 s (anions) at 2 psi.

8.4. Separate metabolites in 10% acetic acid with a separation voltage of 30 kV in forward (cations) and reverse (anions) mode. Reverse separation mode is assisted with 0.5 psi forward pressure on the separation capillary.

8.5. Set mass spectrometer to collect data in positive (cations) or negative (anions) mode over the mass range 65-1000 *m/z.* Between samples, rinse capillary with 0.1 M HCl, 0.1 M NaOH, DI water and BGE at 50 psi for 2 min.

REPRESENTATIVE RESULTS:

The described method is the first to characterize both the proteins and metabolites in the NM biomolecular corona using the same human plasma sample. This method is capable of detecting >200 proteins and >150 metabolites, thus enabling the most comprehensive overview of the complete biomolecular corona to be determined, enabling enhanced understanding of NMs cellular attachment, uptake and impacts.

The use of CESI-MS for both proteomics (**Figure 1**) and metabolomics (**Figure 2**) separation and detection shows good separation windows on both capillaries for each approach.

This method is capable of distinguishing between the proteins in the corona across a wide range of NMs compositions, thus demonstrating the methods' applicability to NMs across a broad range of physical and chemical characteristics (**Table 1**).

The unique fingerprints of the metabolite corona can also be distinguished using the metabolomic branch of this methodology (**Figure 3**). Although this approach utilizes a passive approach to characterize the NM metabolite corona it is still capable of uncovering interesting insights into the role of metabolites in the biomolecular corona such as the differential adsorption of isomers (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Exemplar electropherogram of a SiO_2 protein corona separation using CESI-MS following an on-particle digest.

Figure 2: Example of extracted ion electropherograms obtained from endogenous compounds in plasma by CE-MS. The numbered compounds are as follows: 1. Ornithine; 2. Lysine; 3. Arginine; 4. Histidine; 5. Creatine; 6. Glycine; 7. Alanine; 8. Valine; 9. Isoleucine; 10. Leucine; 11. Serine; 12. Threonine; 13. Asparagine; 14. Methionine; 15. Glutamine; 16. Glutamic acid; 17. Phenyl-D5-alanine; 18. Phenylalanine; 19. Tyrosine; 20. Proline; 21. Methionine sulfone (internal standard). Published under an open access Creative Commons CC BY license and reproduced from Zhang et al[17].

Table 1: Analysis of protein corona across an array of 6 NMs. Heat map of protein classes identified on silica (SiO_2), titania (TiO_2), PVP capped titania (TiO_2-PVP), dispex capped titania (TiO_2-Dispex), polystyrene and carboxylated polystyrene NMs. Percentages refer to the proteins' abundance relative to total protein content based upon top 3 peptides abundance for each protein. Given the differences observed in the relative abundances of proteins in the different NM coronas, it is clear that this proposed protocol is sufficiently sensitive to differentiate between the protein corona of different NMs. Figure reproduced from a CESI-MS analysis of the protein corona, published under an open access Creative Commons CC BY license[11].

Figure 3: Targeted analysis of the cationic metabolites in the NM corona. Adsorption of cations to SiO_2 and TiO_2 NMs. Red refers to the initial concentration of metabolites whereas blue refers to the concentration of metabolites following a 1 h incubation with the NMs at 37 ˚C. The reduction in metabolite concentration in the solution is a result of metabolite adsorption to the NM surface. No significant adsorption of metabolites was observed for the polystyrene NMs. Box plots represent the minimum and maximum values, median and interquartile ranges. Figure reproduced from a CESI-MS targeted analysis of the metabolite corona published under an open access Creative Commons CC BY license[15].

Figure 4: Targeted analysis of the anionic metabolites in the NM corona with a focus on isomer adsorption. Adsorption of anions to a range of titania NMs shows clear differences between various isomers such as the sugar phosphates. Red refers to the initial concentration of metabolites whereas blue refers to the concentration of metabolites following a 1 h incubation with the NM at 37 ˚C. The reduction in metabolite concentration in solution is a result of metabolite adsorption to the NM surface. No significant adsorption of metabolites was observed for the SiO_2 and the polystyrene NMs. Box plots represent the minimum and maximum values, median and interquartile ranges. Figure reproduced from a CESI-MS targeted analysis of the metabolite corona published under an open access Creative Common CC BY license[15].

Table 2: Reproducibility of CESI-MS technical replicates for peptides and cationic metabolites. RSD: relative standard deviation, as a measure of reproducibility. The reproducibility of CESI-MS in-terms of peak areas and migration times is very good, with all analytes having a mean RSD <15% and migration times <2.2%. These results demonstrate the high degree of confidence that can be attributed to the data collected using this technique and confirm that variations detected are due to genuine differences in sample composition (enrichment on NMs) rather than analytical variation. Table 2 is generated from results presented previously[11,15].

DISCUSSION:

This is the first method proposed to characterize the complete biomolecular corona incorporating proteins and metabolites, from the same sample, using CESI-MS. The ability to characterize both the proteins and metabolites from the same sample will significantly increase the amount of data collected from a single experimental exposure enabling new insights into the process of corona formation and its impacts for NMs toxicity and efficacy as nanomedicines. Furthermore, CESI-MS is an ideal platform to characterize both classes of biomolecules with just a change of capillary required. Moving forward, new methods developed for non-aqueous CE will enable lipidomics to be performed using CE-MS[40] thus it is now possible to analyze proteins, metabolites and lipids using a single platform. The current conventional approaches would require two dedicated LC-MS platforms, one for the protein corona and one for the metabolite corona. Thus, this approach can collect twice as much data using a single analytical platform.

There are some caveats to this approach particularly with the metabolite corona as this protocol proposes an indirect measurement of the corona. As such, problem may arise when the metabolite levels are present at high concentrations relative to the NMs and the subsequent drop in metabolite concentration in the matrix is small. However, unlike with the protein corona, it is unlikely that a “one size fits all” approach to isolating the metabolite corona will be developed due to each NM having unique surface chemistries. Going forward it is more likely that NM specific approaches to isolate the metabolite corona will be developed and validated however; this will be applicable to only that specific NM. Despite this, the CESI-MS will still be a highly suitable platform for the characterization of the polar charged metabolites regardless of how they are isolated. Also noteworthy is that if a pellet does not form during the centrifugation steps designed to pellet the NMs, a higher centrifugal force may be required; this is most likely to occur with less dense NMs. It is also critical that all filtering steps are adhered to within the protocol due to the narrow bore of the capillaries these can easily become blocked if any particulate

matter has not been adequately eliminated from the sample.

While the protein corona has been an intense area of research for a number of years the ability to combine that with the metabolite corona is a new field of interest for scientists investigating the bio-nano interface[6,14]. To date, the majority of these studies have focused upon the non-polar, lipid fraction of the metabolite corona[9,28]. This current method enables the characterization of the highly polar and charged metabolites in the corona which includes important metabolites involved in energy metabolism, glycolysis, and DNA synthesis. As a result, when combined with the proteomics workflow, a more holistic characterization of the biomolecular corona is possible. This enables a more in-depth analysis of bio-nano interactions moving forward. This method enables enhanced mechanistic insights into receptor-mediated endocytosis via protein and metabolite interactions with membrane receptors. Furthermore, inter and intra corona interactions between proteins and small molecules may be explored; for example, it has been recently shown that the proteins in the corona play a key role in the recruitment of metabolites[15]. It is worth noting that the representative results in **Figure 3** and **Figure 4** are absolute quantification of metabolites, however, an untargeted approach using multivariate data analysis to compare all CE-MS features in controls compared to exposed plasma would also elucidate metabolite binding. Thus, there is a significant scope to investigate how, and which, metabolites and proteins influence their respective recruitment into NM coronas. These additional studies may help design coronas to optimize delivery of nanomedicines or uncover further hurdles to their development such as the suppression of pharmaceutical action because of the biomolecular corona blocking or masking targeting functionalities.

CESI-MS with its nano scale flow rates of around 20 nL/min and minimal use of organic solvents offers an improvement in green and economic credentials over standard LC-MS and nanoLC-MS in terms of solvent use, the main challenges for metabolomics and proteomics studies, respectively. CESI-MS offers highly reproducible results for both migration time and peak area which compare favorably with LC-MS based methods[11,15]. Furthermore, compared to nanoLC-MS, carry-over is not an issue in CESI-MS. Therefore, extensive system clean-up between sample analyses is not required, which greatly improves sample throughput[11].

In summary, the proposed workflow is the first to detail an approach to characterize both the protein and metabolite components of the complete biomolecular corona of NMs. This unlocks a new aspect of bio-nano interactions, the metabolite corona, thus enabling the collection of twice as much data from the same sample, enabling a more complete understanding of interactions at the bio-nano interface.

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DISCLOSURES:

J.A.T is an employee of AB Sciex UK Ltd whom are participating as an industry partner on the ACEnano project, J.A.T and all other authors have no conflict of interest in this work.

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Figure 1

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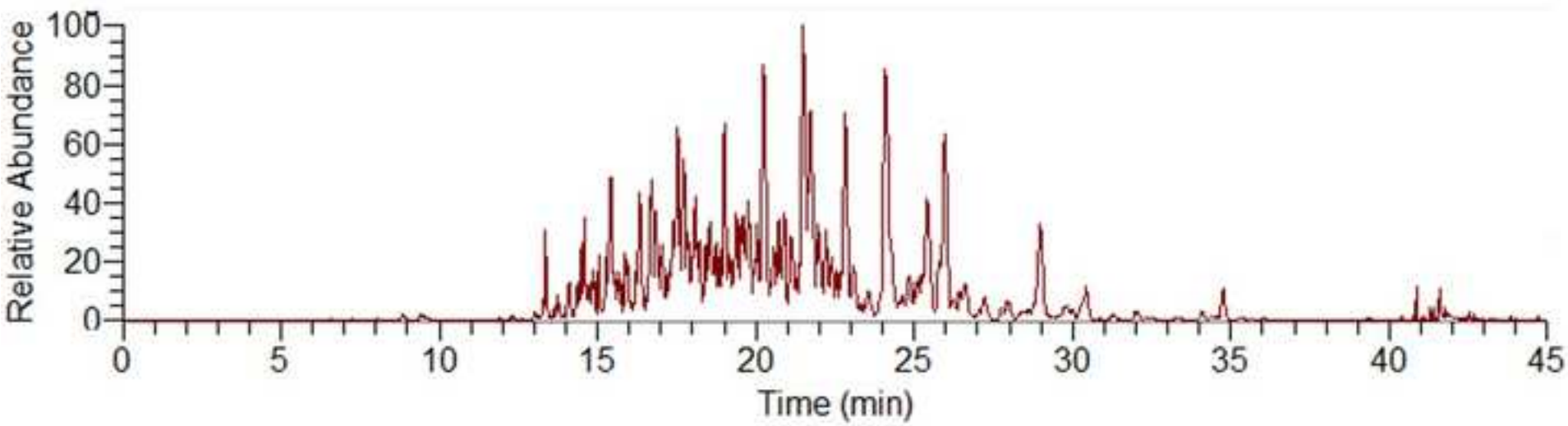

Figure 2

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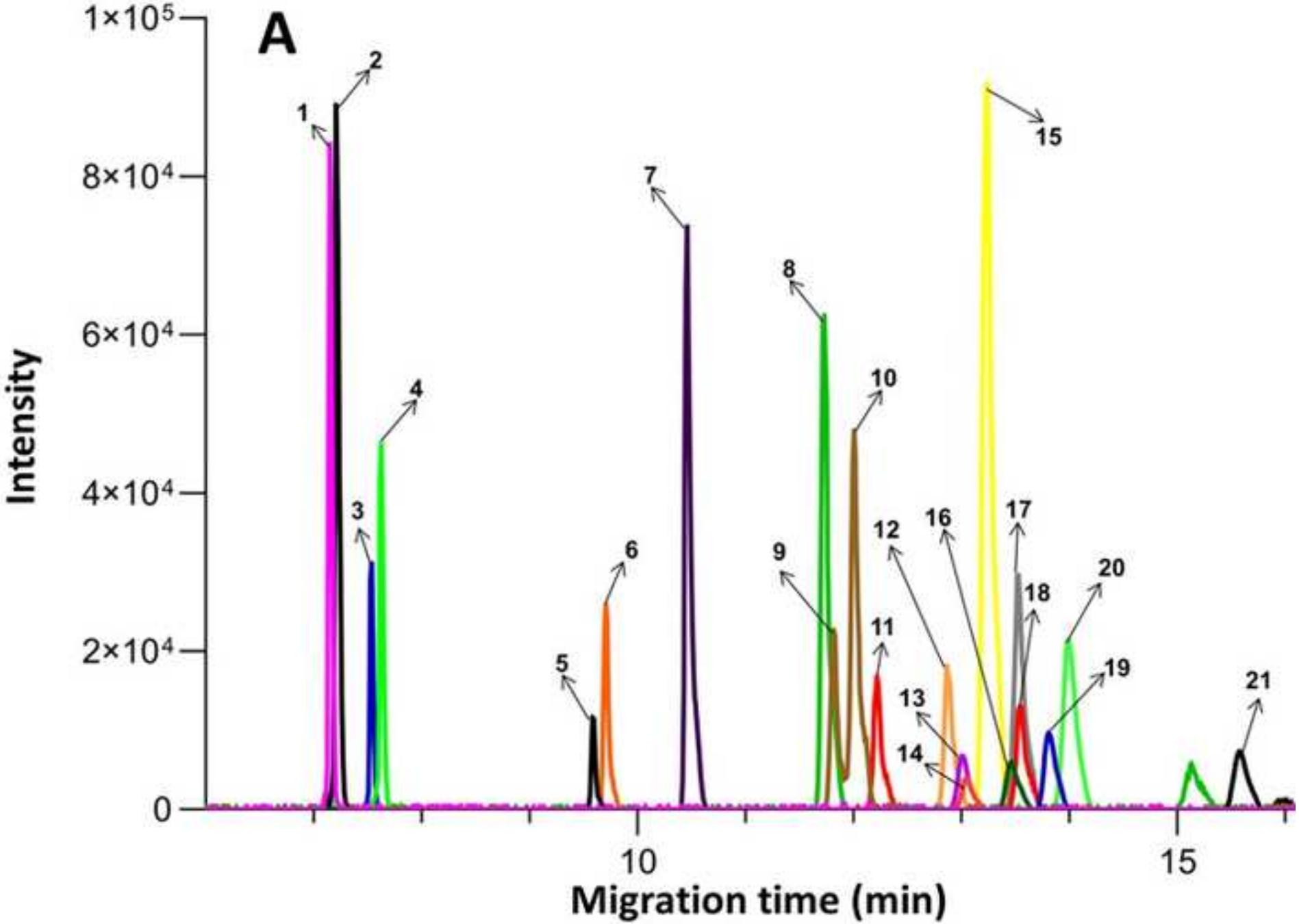

Figure 3

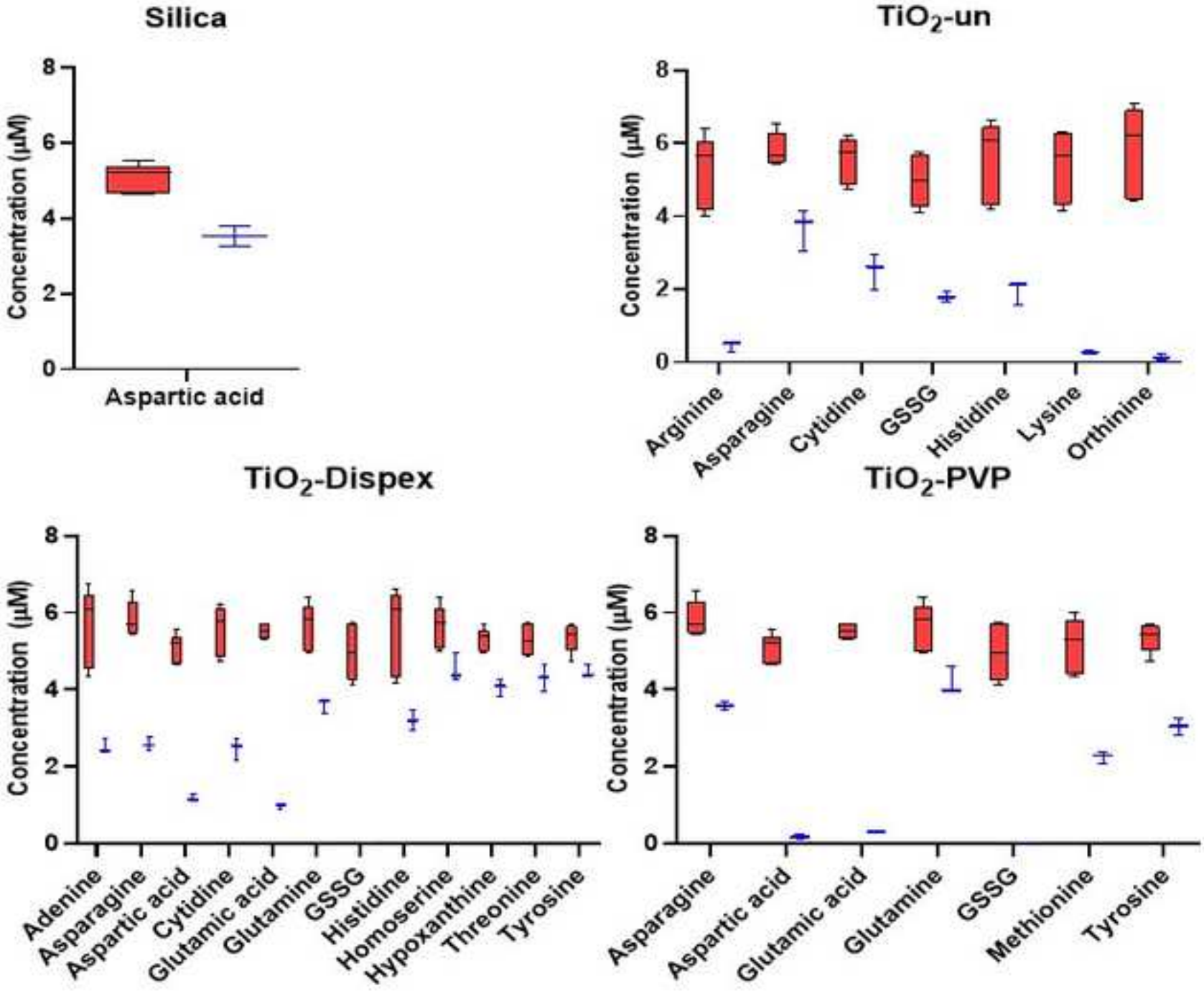

Figure 4

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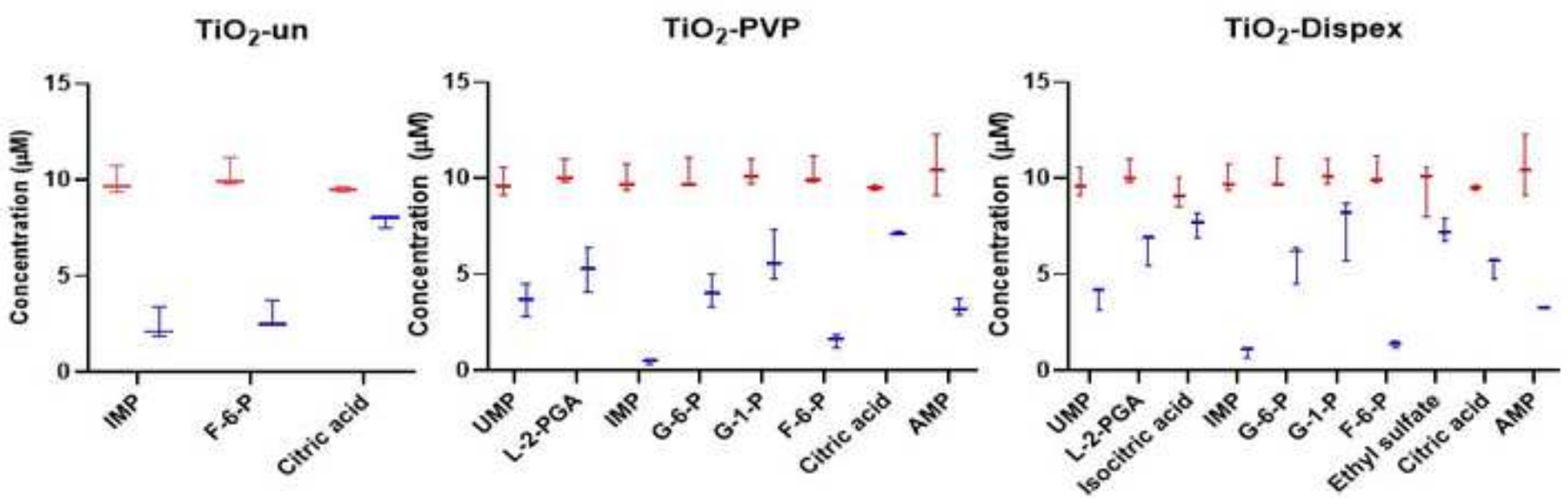

Table 1

Click here to access/download;Table;Table 1.xlsx

	PS-Carb		PS		TI		TI-PVP	
Protein	**ppm**	stdev	**ppm**	stdev	**ppm**	stdev	**ppm**	stdev
Fibrinogens	**13%**	0.20%	**6%**	1.20%	**43%**	1.10%	**41%**	0.80%
Apolipoproteins	**5%**	1.10%	**3%**	0.50%	**0%**	0.00%	**1%**	0.10%
Complement components	**7%**	1.50%	**1%**	0.60%	**8%**	0.70%	**6%**	0.30%
Immunoglobulin	**1%**	0.20%	**2%**	0.60%	**22%**	2.50%	**20%**	2.80%
Serum albumin	**15%**	0.60%	**61%**	1.70%	**1%**	0.00%	**0%**	0.00%
Vitronectin	**32%**	1.20%	**10%**	0.40%	**5%**	0.40%	**8%**	1.20%
Clusterin	**1%**	0.20%	**10%**	0.50%	**0%**	0.00%	**0%**	0.00%
Inter-alpha-trypsin inhibitor heavy chains	**3%**	0.30%	**1%**	0.10%	**1%**	0.50%	**1%**	0.00%
Kininogen-1	**3%**	0.00%	**0%**	0.00%	**1%**	0.10%	**3%**	0.60%
Histidine-rich glycoprotein	**7%**	2.50%	**0%**	0.00%	**1%**	0.20%	**1%**	0.10%
Alpha-2-HS-glycoprotein	**0%**	0.10%	**0%**	0.10%	**2%**	0.60%	**4%**	0.70%
Prothrombin	**0%**	0.20%	**0%**	0.00%	**5%**	0.40%	**7%**	0.60%
Serotransferrin	**0%**	0.00%	**0%**	0.10%	**0%**	0.00%	**0%**	0.00%
Plasminogen	**0%**	0.10%	**0%**	0.10%	**2%**	0.00%	**2%**	0.20%
Gelsolin	**0%**	0.20%	**0%**	0.00%	**1%**	0.30%	**2%**	0.10%
Beta-2-glycoprotein 1	**1%**	0.30%	**0%**	0.10%	**1%**	0.00%	**0%**	0.00%
Vitamin D-binding protein	**0%**	0.10%	**2%**	1.60%	**0%**	0.00%	**0%**	0.00%
Vitamin K-dependent protein S	**0%**	0.00%	**0%**	0.00%	**1%**	0.10%	**1%**	0.10%
Plasma kallikrein	**1%**	0.10%	**0%**	0.00%	**1%**	0.10%	**1%**	0.10%
Hemopexin	**0%**	0.00%	**0%**	0.00%	**0%**	0.00%	**0%**	0.00%
others	**9%**	0.30%	**3%**	0.50%	**6%**	1.00%	**5%**	0.00%

Colour code:	**0%**	5.00%	**10%**	30.00%	**60%**

TI-Dispex		Silica	
ppm	stdev	ppm	stdev
9%	0.60%	17%	2.90%
0%	0.00%	17%	5.70%
8%	0.30%	12%	1.90%
7%	1.30%	12%	0.20%
1%	0.10%	8%	1.40%
14%	0.40%	2%	0.30%
0%	0.00%	1%	0.00%
0%	0.10%	4%	0.50%
2%	0.40%	3%	0.30%
0%	0.00%	1%	0.10%
22%	2.00%	1%	0.30%
21%	0.30%	0%	0.00%
0%	0.00%	4%	1.70%
1%	0.10%	2%	0.50%
1%	0.00%	1%	0.20%
0%	0.10%	1%	0.00%
0%	0.00%	0%	0.00%
1%	0.10%	0%	0.00%
1%	0.20%	0%	0.00%
0%	0.00%	2%	0.20%
9%	0.50%	14%	1.30%

Table 2

Analyte	Mean RSD peak area	Mean RSD migration time
1928 peptides intraday (n=3)	15%	0.30%
27 cations intra-day (n=16)	5.80%	2.20%
27 cation inter-day (n=36)	8.60%	1.80%

Table of materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1,4-Dithiothreitol	Sigma	10197777001	
2,2,4,4-D4-citric	Simga	485438-1G	Internal standard anion
Ammonium Acetate >98%	Sigma	A1542	
Ammonium Bicarbonate >99.5%	Sigma	09830	
Capillary cartridge coolant	Sciex	359976	
CESI 8000 instrument	Sciex	A98089	
CESI Cap	Sciex	B24699	
CESI Vials	Sciex	B11648	
Chloroform	Sigma	650498	Toxic, use in a fume hood
Glacial Acetic acid	Sigma	A6283	
Hydrochloric acid 1mol/L	Sigma	43617	
Iosoacetamide	Sigma	I1149	
L-methionine sulfone	Sigma	M0876-1G	Internal standard cation
Methonal (LC-MS Ultra Chromasolve)	Sigma	14262	Use in a fume hood
Microvials	Sciex	144709	
nanoVials	Sciex	5043467	
Neutral Surface Cartridge 30 µM ID x 90 cm total length	Sciex	B07368	
OptiMS Adapter for Sciex Nanospray III source	Sciex	B07363	B07366 and B83386 for Thermo mass spectrometers, B85397 for Waters mass spectrometers, B86099 for Bruker mass spectrometers
OptiMS Fused-Silica Cartridge 30 µm ID x 90 cm total length	Sciex	B07367	
Phospahte buffered saline 10x	Sigma	P7059	
Pierce C18 Tips, 100 µL bed	Thermo Fisher Scientific	87784	

Polystyrene 100 nm	Polysciences Inc	876
Polystyrene carboxylated 100 nm	Polysciences Inc	16688
Polystyrene carboxylated 1000 nm	Polysciences Inc	08226
Rapigest SF (surfactant)	Waters	186001860
Sequencing Grade modified Trypsin	Promega	V5111
SiO2 Ludox TM-40	Sigma	420786
Sodium Hydroxide solution	Simga	72079
TiO2 Dispex coated	Promethion particles	Bespoke particles
TiO2 PVP coated	Promethion particles	Bespoke particles
TiO2 uncoated	Promethion particles	Bespoke particles

UNIVERSITY OF BIRMINGHAM

School of Geography, Earth & Environmental Sciences

Dr Andrew Chetwynd
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4th September 2020

Dear Dr. Bajaj,

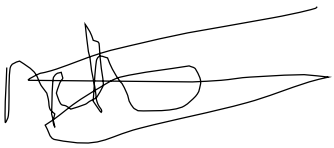
Please find attached our revised manuscript for consideration as a video article in the Journal of Visualized Experiments "Capillary Electrophoresis Mass Spectrometry approaches for characterization of the protein and metabolite corona acquired by nanomaterials" by Andrew J. Chetwynd, Wei Zhang, Klaus Faserl, James A. Thorn, Iseult Lynch, Rawi Ramautar, and Herbert Lindner. This is an original contribution and is not under consideration anywhere else. All co-authors have approved the submission.

We believe the amended manuscript and the point-by-point response to the editor and reviewers comments carefully and thoughtfully address the helpful questions and comments that arose during the review process.

We are confident that the manuscript will be of interest to JoVE readers/viewers, we look forward to receiving, and future comments in due course.

Thank you for your consideration of the manuscript.

Yours sincerely,



Dr. Andrew Chetwynd

School of Geography, Earth and Environmental Sciences,
University of Birmingham

Response to editor and reviewers comments.

We would like to thank the editor and the 3 reviewers for their time and consideration of our manuscript and welcome their questions and suggestions that have helped us to improve our manuscript. We are particularly grateful for the quick turnaround of our manuscript by the editorial board and reviewers given the current ongoing worldwide pandemic. All changes in the manuscript have been marked using red text and responses to individual comments can be found below.

Editorial Comments:

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the guidelines of your institutions human research ethics committee.

We have now added this statement as requested and added that future work using this protocol on human or animal samples would require similar ethical approval.

2) 3.1: What is the source of human plasma?

When we developed the method we used two sources of plasma, one was a non-profit blood bank and sources from the Sanquin blood bank (Leiden, The Netherlands), while the other was sourced from an in house donor who had provided informed consent in line with the ethical guidelines of the institution. For the protocol, we are just splitting 1 sample into 2 halves for the proteomics and metabolomics workflows, and any source of plasma would work but the source to use would depend on the research question being addressed in the particular study.

3) 4.13: unclear what is being done here.

We have now added more detail to this step and removed the term Zip Tip which is a trade name. With the re-organization of the protocol this section is now 4.7 and 4.7.1.

• **Protocol Numbering:**
1) Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary.
We have changed the protocol to reflect this layout.
2) All steps should be lined up at the left margin with no indentations.
We have changed the protocol to reflect this layout.
3) Please add a one-line space after each protocol step.

We have changed the protocol to reflect the requested layout.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) Merge shorter related steps if needed. There can be up to 4 related actions per step.
3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
5) Notes cannot be filmed and should be excluded from highlighting.

We have made the requested corrections and provided the relevant highlighting.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have made corrections to better suit these guidelines and in response to the reviewers comments and suggestions.

• **Figures:**
o Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

The requested corrections have been made.

o Fig 2, 3: Define error bars.

These figures (now 3 and 4) are all box plots as such the middle line is the mean, the boxes the interquartile range and the "error bars" are the minimum and maximum values. Due to the reproducibility of the data for these results the typical box shape has been lost. We have added a note to the figure capture to reflect this.

• **Tables:** Please remove the embedded Table from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.

We have now uploaded these as excel sheets

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial

sounding language in your manuscript are Rapigest SF™, MilliQ, ZipTip, nanospray, etc
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

This has now been accounted for in this manuscript, we do however have one remaining query for this. The Rapigest used to solubilize the peptides in step 4.4 is a tradename (Waters Ltd.) however other surfactants proved to be inadequate. Should we say surfactant here and then state in the materials tables that it's Rapigest SF which is what we have changed it to for now?

The term nanospray is not a commercial name but a technical name for the type of source being used.

• **Table of Materials:**
1) Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
3) Please sort in alphabetical order.

We have now amended our Table of Materials to reflect this request.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript is very interesting and describes the experimental procedures to prepare a plasma sample to evaluate proteins and metabolites adsorption on nanomaterial particles, in order to evaluate the formed corona. It requires English revision in terms of orthography, text composition, punctuation, and others. The results are interesting to be published in this journal and are an innovation in terms of concomitant proteome and metabolome evaluation on corona nanomaterials using CESI-MS - a technique that has not been previously been used for this purpose.

The authors thank the reviewer for their positive critique of our work, we believe that we have now addressed all of their comments and queries in our response below.

Major Concerns:
Line 134: where was this sample obtained? Were ethics issues considered? What was the criteria for selection of this single sample?

This is a protocol as such we didn't obtain samples for this manuscript, the representative results come from previous studies we have carried out and they had relevant ethical approval which can be found in the cited research. We do however appreciate the concern over ethical approval and have now added a statement on the relevant ethical approval from the research institution. This information has been added now (see lines 119-120). The choice of human plasma was taken due to its prevalence in biomolecular corona studies and, therefore, used in this protocol for the characterization of the biomolecular corona. However, this protocol would also be applicable to serum or other biofluids such as CSF or urine which we now state in the introduction (see lines 111-112).

Line 140: but if the aim of this part of the experiment was the corona NM metabolome characterization, why was the supernatant analyzed? I suppose the non-adsorbed metabolites would be analyzed in the other plasma aliquot (which was not incubated with the NM), and a comparison between total metabolome with metabolome adsorbed on NM would be performed)

The reviewer is correct, unlike the proteins in the corona there is no "one method suits all" approach to characterize the metabolites adsorbed to the nanoparticles due to their very different chemistries. As such, we characterize the matrix before and after NM exposure. This is described in the introduction lines 81-85. We have also added to the discussion the relative drawbacks of characterizing the metabolite corona using this approach in response to a similar comment from Reviewer 2.

Lines 141 and 142: I understand that metabolome absorbed on NM should also be analyzed in this sample

In this work, we have examined which metabolites have been depleted from the matrix rather than eluting adsorbed metabolites from the NM surface. This approach also provides information on the extent of metabolite adsorption to the NMs when employing proper controls and suffers from less uncertainty regarding recovery efficiency for each individual metabolite and is less costly than analyzing the amounts recovered from the NMs and the amounts remaining in solution and performing mass balance to understand

if some of each metabolite is still attached to the NM surface.

Figure 1: a discussion on the difference of different proteins classes on the surface of different NMs should have been performed

This is a representative result of the protocol rather than a research result per se; Our aim here is just to demonstrate its applicability, while the primary research on which this protocol is based thoroughly discusses the implications of these proteins in the corona. We have however added a sentence to the legend to state that this protocol allows for easy differentiation between the corona composition of different nanomaterials.

Figure 2: why proteome NM corona was evaluated on 6 NMs while metabolome was evaluated with 4 NMs?

As with Figure 1 these are representative results of the protocol, in the primary research these representative results come from all 6 materials were indeed studied but the PSNPs did not adsorb the metabolites being investigated. We have added a note to this effect to the figure legends to avoid confusion for the readers.

Minor Concerns:
1) It seems that the submitted version was not the final one, since some English mistakes have been found, and texts highlights are observed (as well some marked corrections)

We have carried out another proof read and corrected English language issues. The highlighted text in this version is at the request of JoVE for the segment to be filmed.

2) What was the equipment configuration (describe equipment details)? What was the nanomaterial composition?

The CE set up is described through steps 6 and 7 of the protocol, the nanosource being used is compatible with a wide range of mass spectrometer manufacturers and mass spectrometry types (i.e. time of flight, Orbitraps, FT-ICR, triple quads). Therefore, the technical aspects of the mass spectrometry side are not included as most are manufacturer and mass analyzer dependent. However, where these details are universal such as ionization mode, fragmentation and m/z range they have been included in steps 6-8.

We have added the details of the NMs we applied this too in the Note at the start of section of the protocol lines 120-126. We also make it clear that these can be substituted for any other NMs that may be of interest, we do not wish to limit the readers choice of material inadvertently by implying that only these materials are acceptable for this method as we do not require any NM specific steps. The NMs used to develop these methods have been detailed in the Materials Tables, in the case of the TiO_2 these were bespoke particles though we provide details of the manufacturer,

Lines 193 - 195: why is it necessary to flush a neutral coated capillary with NaOH on the conditioning step?

The reviewer is correct - this is an error from when the manuscript was restructured prior to original submission and has now been deleted.
Figure 1: insert the color legend according to proteins abundance

We have added a color legend to the latest version of the figure.

Line 318: include "not" between "is" and "significant"

We are confused as to why the reviewer suggests this as this would mean that we are saying that there is no scope to investigate the protein and metabolite coronas. The aim of this protocol is to enable this.

Reference number 14 has already been cited (10); I mean, it has been cited twice.

We thank the reviewer for their observation; we have now corrected this in the manuscript.

Reviewer #2:
Manuscript Summary:
Andrew et al. present a protocol for the joint characterization of protein and metabolite coronas on nanomaterials (NMs). The authors briefly introduce state-of-the-art research and protocols to determine the protein and metabolite corona on NMs after incubation in biological fluids. The need for optimized protocols and various approaches for characterization of NM coronas are mentioned and discussed. The protocol is divided in eight sections, comprising steps of sample and buffer preparation, preconditioning of the CE instrument and capillary and data acquisition. The NMs are incubated in human blood plasma and protein and metabolite corona isolation are performed subsequently from the same sample. Preconditioning protocols to set up the CE-MS system for analysis are described in detail for both methods. Relevant instrumental parameters for data acquisition are provided. The authors demonstrate the capabilities of their protocol with representative results for protein and metabolite coronas on NMs of various materials. Intra- and inter-day reproducibility for migration times and peak areas are provided. The authors discuss the impact and possible application of their protocol in the context of nanomedicine. The advantage of the joint characterization of proteins and metabolites on the NMs corona are mentioned and discussed. The results are compared with relevant publications in the field. The advantages of CE-MS over nanoLC-MS are briefly mentioned and discussed.

We thank the reviewer for their thoughtful and insightful comments on our manuscript. We have adjusted our work to address their comments and concerns. These changes are detailed below alongside their comments.

Major Concerns:
The authors should consider to briefly point out important steps in data analysis. This also includes strategies for quantification (I see that you have determined absolute concentrations for metabolites in plasma. What type of calibration are you using?)

We have not included data analysis, as the focus of this protocol is the sample preparation to isolate the corona and preparation of these samples for CE-MS analysis. Given the broad range of vendor specific and open access software describing a single approach would limit the applicability of this protocol. However, we have added to the discussion lines 340-343 that an absolute quantification for metabolites can be performed as well as an untargeted approach (though we do not have representative data to show for this with this particular application yet) whereas the protein corona aspect was developed using a bottom up approach. We have added a line in the introduction 101-103 to clarify this and explain why we took this approach.

Minor Concerns:
81-88:
The authors should discuss problems regarding this indirect determination of the metabolite corona. The approach only works when NMs are concentrated enough to result in a significant change in metabolites concentration by adsorption. Consequently, this method might be difficult for biologically/medicinally relevant NM concentrations. The same problem arises when metabolites with high concentrations (compared to the NMs) are considered.

This is an excellent point the reviewer has made, we have added a discussion of these limitations to the manuscript on lines 297 to 306.

105-107:
I agree in terms of throughput and carry-over with the authors. CE-MS offers fast separation and equilibration and due to the lack of stationary phase, carry-over is reduced. However, reproducibility has always been an issue in CE-MS. Having a look at your RSD data on migration times of cationic and anionic metabolites I agree that RSD values of ~2% are good for CE, however they are not comparable to RSD in retention times usually observed in (nano)LC (0.1-0.5%). The authors should cite relevant literature underlining their claim or discuss this point more detailed.

We have changed the sentence slightly to remove reference to analytical flow LC-MS and now refer to only nanoflow LC-MS to make a fairer comparison concerning the flow rate. While the reviewer is correct that CE-MS has previously had issues with RT reproducibility, the same is also true of nanoLC-MS; however recent advances in technology for both separation approaches have improved RT/MT reproducibility. We include a reference to a recent review of nLC-MS which finds that reported RT RSDs range from 0.2 – 3.4 % across 25 studies covering peptides and metabolites.

122/125:
Is the pH value determined/measured after dilution of the acid?

If the quality of acid and DI water being used is as stated in this manuscript then adding the stated volumes will reproducibly generate the same pH in our experience.

130-132:
Please provide a reference for the determination of particle size (ideally the protocol used by the authors)

We have added references to relevant methods; we suggested a range of approaches due to the diverse array of NMs being studied and certain sizing techniques are not suitable for all NMs.

136/137:
Please provide information on how to add the NMs to the plasma (are they solved in a stock solution or are they added as solid?)

This is explained in what is now step 2.1: particles are to be added to the plasma as a solution dispersed in water. We have also stated in what is now step 3.2 what concentration

to use for the plasma incubation.

218/219:
Reading HCD" I suggest that you are using an Orbitrap instrument for data analysis. This should be mentioned here since the fragmentation parameters (28% normalized energy) cannot be directly transferred to instruments from other vendors.

The reviewer is correct that the proteomics portion of this approach was developed using an Orbitrap instrument. However, JoVE do not permit the use of brand names. As such we have changed this to state DDA/DIA fragmentation methods, as this CE set-up will work across a broad range of instrument manufacturers and the reviewer is correct that 28% normalisation energy does not translate to QToF instruments.

221:
Is HCl directly flushed towards the MS interface? Do you encounter any problems with contaminating the MS by flushing with non-volatile compounds?

Yes, the HCl is flushed towards the MS interface. However, a 90 cm capillary with internal diameter of 30 µm and just 100 PSI for 5 minutes pushes a little over 1 µL total volume of HCl through the capillary. At this time there is no ESI voltage being applied so the HCl does not reach the interface and evaporates at room temperature, thus it does not contaminate the MS.

232:
Please provide detailed information about internal standards used for the protocol.

These details have now been added to the protocol and to the materials table.

236-238:
I suppose that for cationic metabolite analysis you have residual EOF (despite the low pH value of the BGE) in your system since you do not need to support the separation with additional pressure. Can you comment on this?

Due to the use of a bare fused silica capillary for metabolomics we indeed retained a residual EOF at the employed BGE conditions and, therefore, an additional pressure step was not required. This was only needed with systems without an EOF, such as with the neutral coated capillary used for the proteomics.

Figure 1:
What does the percentages in the heat map represent? How were these values determined?

As suggested by the reviewer we have amended the caption for the table to state that the percentages reported refer to the percentage of overall protein abundance based upon the intensity of the 3 most intense peptides in the protein.

Table 1:
The numbers of (technical?) replicates used to calculated RSD should be provided with table 1.
We have revised as suggested and stated in the legend that these are technical replicates.

324-330:
I do not support the claim that CE-MS is a more green technology, especially compared to nanoLC. Solvent consumption in nanoLC-MS (commonly used in proteomics) is very low and in my view negligible compared to the high energy demands of the mass spectrometers and the high consumption of single-use plastic in laboratories.

We have tempered our statement over solvent usage only and stated the economic impact of this. Still when compared to nLC-MS for proteomics the "flow rate" is at least 10x lower in CESI and no organic solvents are used other than approximately 2 µL of methanol for the conditioning of the bare fused silica capillary. Compared to the flow rates used for metabolomics the CESI "flow rates" could be up to 100x lower and also use hardly any organic solvent. Reducing the environmental impact even in small ways can be regarded as an improvement even if power demands of the MS (which could be from reusable sources) or high use of single use plastic in labs have bigger environmental footprints overall.

Reviewer #3:
Manuscript Summary:
The authors described a detailed protocol for characterization of proteins and metabolites adsorbed on nanoparticles using capillary electrophoresis mass spectrometry. The protocol is well written and provides detailed information, which will be useful for other researchers in the field. I have some minor comments to help the authors improve the manuscript further.

We thank the review for their positive appraisal of our work and we hope the following changes to our manuscript adequately address their comments.

Minor Concerns:
1) In terms of figures, some example electropherograms of peptides and metabolites will be useful for the readers.

As suggested, we have now included representative electrophoretograms for the proteins and metabolites as figure 1 and 2 respectively.

2) Lines 98-99: Two nice review papers about CE-MS for proteomics could be cited here. Zhang et al. Trends in Analytical Chemistry. 2018, 108, 23-37. Shen et al. Trends in Analytical Chemistry,
2019, 120, 115644.

We have added both of the suggested references to the manuscript.

3) Lines 157-158: Why alkylate the sample at the peptide level not at the protein level as usual?

Alkylating after digestion removes the need to quench the alkylation step and thus reduces the number of steps required.

4) The authors used 30-cm-long capillaries for CE-MS with the CESI instrument. Based on our experience, it is hard to use that short of capillaries for CE-MS. Please double check.

We thank the reviewer for highlighting this, the lengths stated were an error, which have now corrected to 90 cm.

5) Lines 193-195: Is it necessary to flush the neutral capillary with NaOH before CE-MS?

The reviewer is correct this is an error from when the manuscript was restructured during drafting of the manuscript. To clarify NaOH on a neutral capillary would strip the dynamic neutral coating and is thus not used.

6) Figure 1, are the percentages calculated by mass? Some explanation will be helpful.

As suggested by the reviewer we have amended the caption for the table to state that the percentage reported refers to the percentage of overall protein abundance based upon the intensity of the 3 most intense peptides in the protein.

Assessing the suitability of capillary electrophoresis–mass spectrometry for biomarker discovery in plasma-based metabolomics

Author: Rawi Ramautar, Yvan Heyden, Thomas Hankemeier, et al

Publication: Electrophoresis

Publisher: John Wiley and Sons

Date: May 2, 2019

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The Nanomaterial Metabolite Corona Determined Using a Quantitative Metabolomics Approach: A Pilot Study

Author: Rawi Ramautar, Iseult Lynch, James A. Thorn, et al

Publication: Small

Publisher: John Wiley and Sons

Date: Apr 2, 2020

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Corona Isolation Method Matters: Capillary Electrophoresis Mass Spectrometry Based Comparison of Protein Corona Compositions Following On-Particle versus In-Solution or In-Gel Digestion

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