CIMac- αHSA Monoliths Increase Throughput and Sensitivity of Proteomic Studies of IVF Media by Rapid Albumin Depletion

by Tanja Panić-Janković[1], Urh Černigoj[3], Blaž Nemec[3], Urška Vidic[3], Jana Vidič[3], Rok Sekirnik[3], Nika Lendero Krajnc[3], Wei-Qiang Chen[1], Barbara Mahlberg[1], Goran Mitulović[1,2]

[1]*Clinical Institute of Laboratory Medicine, Medical University of Vienna, Vienna, Austria*

[2]*Proteomics Core Facility, Medical University of Vienna, Vienna, Austria*

[3]*BIA Separations, Ajdovščina, Slovenia*

Biomarker peptides and proteins are generally present at low concentrations and their analysis can be severely impeded by high concentrations of highly abundant proteins (HAP), such as human serum albumin (HSA). HAP removal is thus critical to increase sensitivity of detection of low-abundance proteins. Various techniques have been applied for albumin depletion, such as ultrafiltration, size-exclusion chromatography and affinity-based removal by immobilised antibodies. Despite their high specificity, affinity methods usually suffer from long processing times due to low flow rates that have to be employed when using chromatographic resins. Here an affinity-based method for improving sensitivity of biomarker detection in IVF culturing medium by using an albumin-specific antibody immobilised onto CIM® (Convective Interactive Monolith) monolith support is described. Due to their unique chromatographic properties, the use of monolithic columns enables very fast depletion (approx. 2 minutes per depletion cycle) and column reconditioning, thus significantly increasing sample preparation throughput.

During an IVF procedure, fertilised oocytes are cultivated in a medium, which is composed of human serum albumin, vitamins, amino acids, and other ingredients required for cellular development. The culturing medium is exchanged daily until the embryonic implantation into a uterus. A developing embryo secretes proteins that are diagnostic of the developmental conditions of the embryo, and serve as biomarkers for the ability for a successful implantation, and hence for a successful pregnancy. Depending on the medium manufacturer, amounts of HSA present in the IVF medium can vary from 5 mg/mL to over 10 mg/mL and has to be removed prior to proteomic studies. Previous studies reported ultracentrifugation, immunoaffinity depletion, depletion using peptide libraries, and ion-exchange chromatography as potentially useful tools for HSA depletion [1-8]. Although a certain degree of HSA depletion was demonstrated for each method, the depletion was not sufficient for increasing the sensitivity of detection required for proteomic analyses of IVF media.

Alternative mechanisms for HSA removal from IVF media were considered, which would combine a high affinity for HSA with speed of processing. The structure of a typical monolithic support used in chromatography comprises of relatively large flow through channels where convective flow is dominant. With particulate material the bulk of the surface area is contained within pores inside the spherical bead structure. The flow dynamics within the pores are dominated by diffusional processes which results in a flow rate dependent binding capacity. Polymethacrylate monoliths afford flow-rate independent binding capacity and resolution due to the convective nature of the flow, which allow for relatively short analysis times compared to traditional chromatographic supports. Large (>1.5 µm) flow-through channels modified with appropriate ligands offer binding sites that are easily accessible for large biomolecules. A relatively short path length lead to a low pressure drop over the matrix bed, rendering them particularly useful for fast separation/enrichment and purification of large macromolecules, including IgG, IgM, pDNA and various viruses [9]. Convective flow properties make polymethacrylate monoliths a promising material also in proteomic applications due to the possibility of increasing the speed and resolution of analyses [10]. The absence of diffusional limitations was shown for immobilised enzyme reactors utilising monoliths as a base support [11], which are a promising substitutes to classical off-line digestion, since an on-line, flow-through mode shortens processing times and reduces material costs by reusing protease enzymes [12].

Traditional immunoaffinity chromatography enables enrichment of a target molecule from complex biological mixtures with high specificity, though the purification is limited by low flow rates permitted by traditional chromatographic supports (porous particles). Affinity CIM monoliths (BIA Separations, Ajdovščina, Slovenia) allow for a reduction in the sample preparation time, since the binding capacity and selectivity of affinity monolith resins are flow-rate independent [13]. Pseudo affinity monoliths with immobilised Mimetic Blue SA A6XL have been developed and tested for the depletion of HSA [14], but specificity for the antigen was low. Therefore immobilised antibodies are a preferred ligand in affinity purifications [15].

Oriented antibody immobilisation onto CIM monoliths, whereby immobilisation occurs via the antibody's carbohydrate moiety on a hydrazide or hydrazine-based supports, have been developed to enhance specificity of interaction with the antigen [16, 17]. It was reasoned that such high specificity immunoaffinity monoliths would offer significant advantages for the depletion of highly-abundant proteins. Here it is shown that polymethacrylate CIMac monoliths with oriented immobilisation of anti-Human Serum Albumin antibody (CIMac-αHSA) offers levels of albumin depletion from spent IVF medium that is compatible with proteomic analyses.

Experimental

CIMac monolith columns (pore size radius 700 nm, disc dimensions: 5.2 mm (I.D.) x 5 mm length, volume 0.106 mL) were provided by BIA Separations d.o.o. (Ajdovščina, Slovenia).

Polyclonal anti-Human Serum Albumin antibody (Sigma Aldrich) was immobilised by dissolving the antibody stock in 10 mM phosphate buffer, pH 7.0 (2 mg/mL final antibody concentration), then treated with phosphate buffer solution of $NaIO_4$ (10 mM in the same buffer) at room temperature to achieve oxidation of antibody glycosylated moieties to aldehyde functional groups. The reaction was quenched by addition of ethylene glycol (25 μL of ethylene glycol per mL of the antibody solution). Trace $NaIO_4$ and ethylene glycol were removed by buffer exchange into 50 mM MES, pH 5.2 by centrifugation (Amicon Ultra-4, MWCO10000, Merck Millipore) and the sample was pumped through CIMac-hydrazide columns at 0.5 mL/min.

Dynamic binding capacity at 50% breakthrough (DBC_{50}) studies of CIMac-αHSA were performed using purified human serum albumin (Sigma Aldrich) at 0.25 mg/mL, dissolved in 1x PBS buffer and loaded on the column generating a breakthrough curve. Different flow rates were tested, ranging from 0.1 to 1.0 mL/min, with The DBC capacity being calculated from R_t at which UV_{280} was half-maximal.

The IVF media used were Sydney IVF Cleavage/Blastocyst Medium (Cook, Australia) and VitroLife G1 PLUS /G2 PLUS medium (Vitrolife AB, Sweden).

Albumin depletion was performed on an ICS-5000 HPLC system at a flow rate of 100 μL/min for the sample loading and elution of unbound fraction (0-5 minutes) and 1000 μL/min for albumin elution. CIMac-αHSA was used at constant temperature of 40°C.

Two types of mobile phases were applied: commercially available solvents from Agilent (Buffer A, and Buffer B), as well as mobile phases prepared in house, where mobile phase A: 100 mM Phosphate buffer, pH 7.2 for loading, and mobile phase B: 2.1 M Phosphate buffer, pH 3.0 for elution.

Samples were diluted 1:4 (sample/loading buffer) in loading buffer prior to injecting 50 μL of the resulting IVF medium. Fractions were collected using Probot μ-sample collector (Dionex, Amsterdam, NL). UV absorption was recorded at 280 nm.

The mobile phase composition throughout the depletion of the albumin from the IVF media are given in Table 1.

The protein concentration of each medium was measured before depletion by Quick Start Bradford assay kit (Bio-Rad, US).

As a control method for HSA depletion, a depletion kit (ProteoPrep® Immunoaffinity Albumin & IgG Depletion Kit, SigmaAldrich, Wien, Austria) was used. This kit was used according to the manufacturer's manual.

Table 1: Composition of the separation gradient used for depletion of albumin from IVF media.

Min	%A	%B
0.00	100.00	0.00
9.00	100.00	0.00
9.01	0.00	100.00
12.00	0.00	100.00
12.10	100.00	0.00
16.00	100.00	0.00

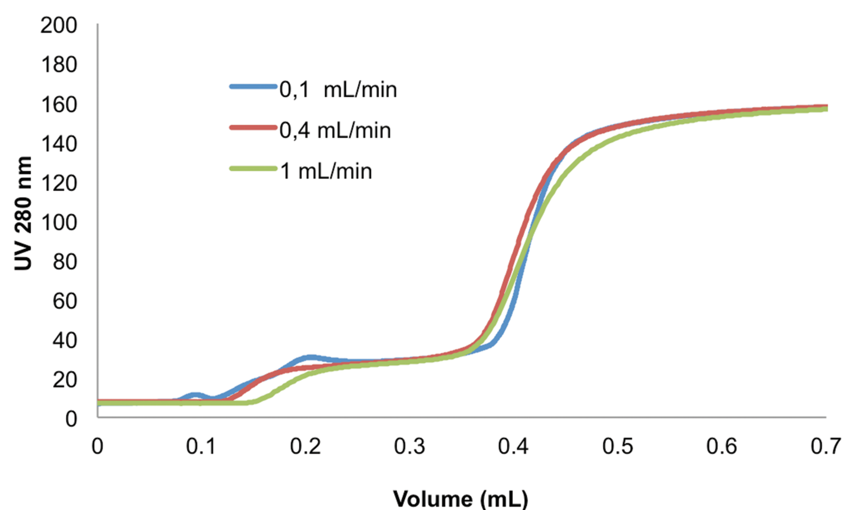


Figure 1: Dynamic binding capacity (DBC) CIMac-αHSA for HSA is flow rate-independent. HSA (0.25 mg/mL) was loaded onto CIMac-αHSA at 0.1, 0.4 and 1 mL/min and break-through was determined by measuring absorption at 280 nm. Note that the initial increase in UV absorbance at 0.1 mL corresponds to the impurities present in HSA sample. A major increase in UV absorbance at 0.4 mL corresponds to HSA breakthrough.

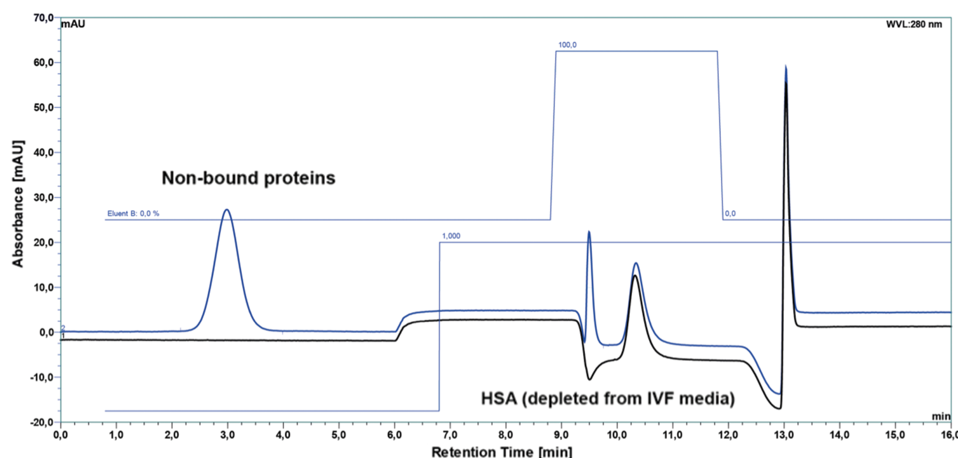
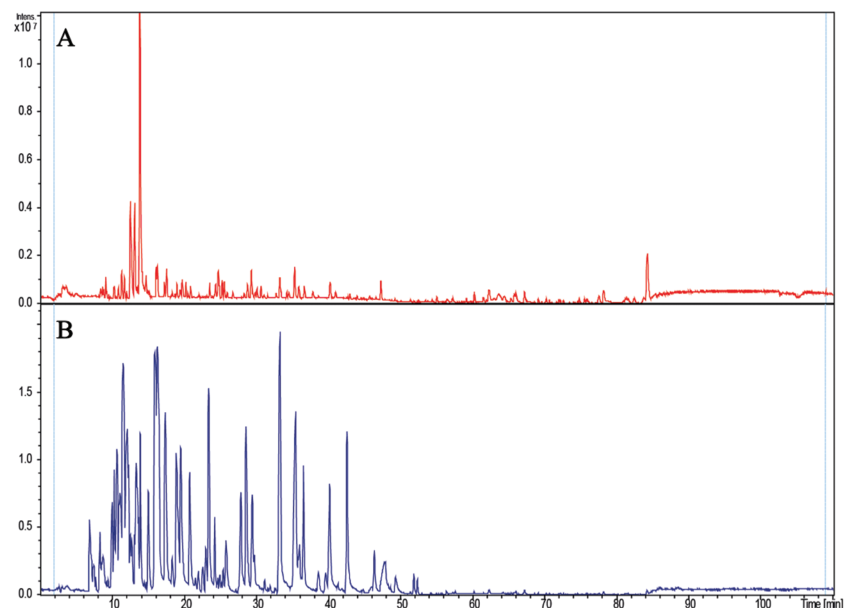
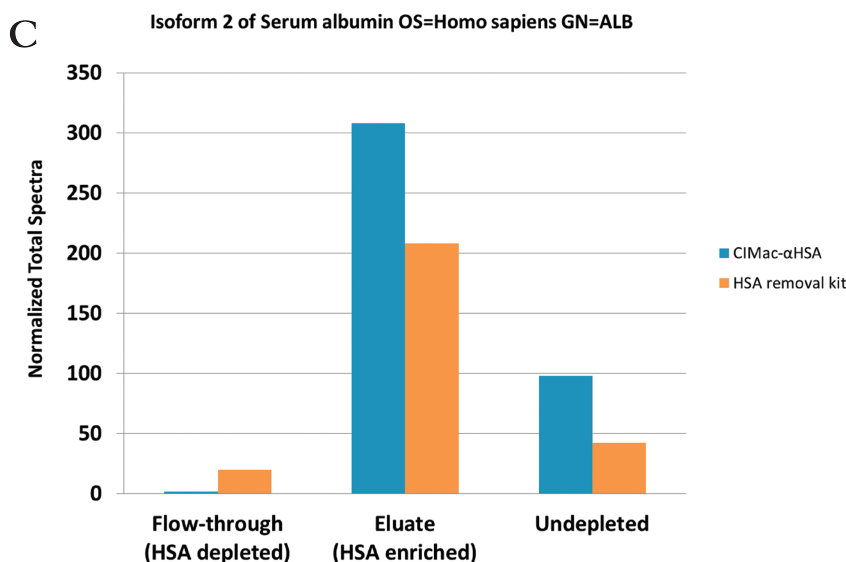


Figure 2: Albumin depletion of spent IVF media upon embryo cultivation using CIMac-αHSA monolithic column (blue = IVF media, black = blank injection of buffer A). Flow rate for sample loading and albumin binding was 0.1 ml/min. The flow rate was increased to 1.0 mL/min for elution of albumin, using the conditions specified in Table 1. The UV detection was performed at 280 nm.





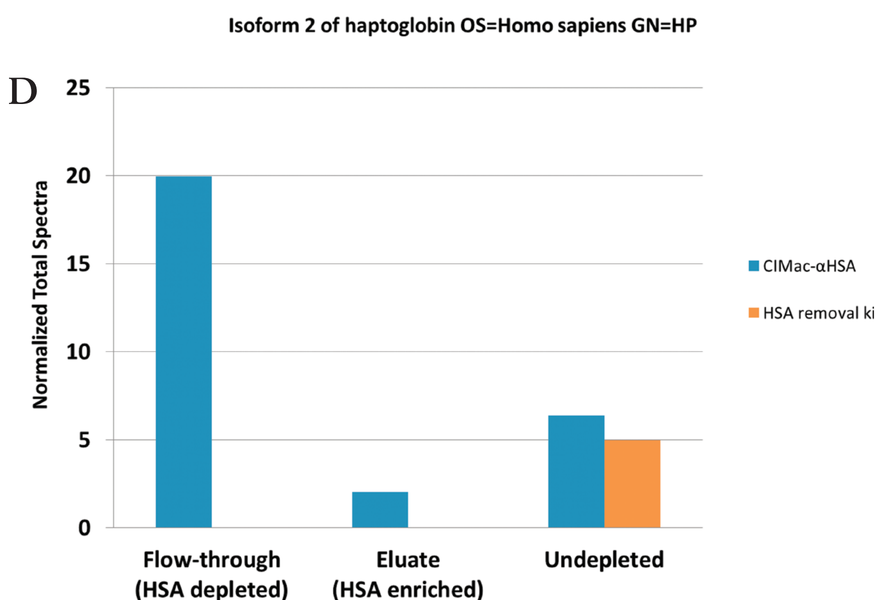


Figure 3: LC-MS/MS chromatograms and normalised spectral counts for proteomic analysis of naïve and CIMac-αHSA depleted IVF samples. A) LC-MS/MS chromatogram of CIMac-αHSA-depleted IVF sample B) MS base peak chromatogram of native trypsin-digested IVF sample; C) normalised spectral counts for HSA from flow-through (HSA depleted) and elution (HSA enriched) fractions and undepleted IVF media; D) normalised spectral counts for haptoglobine from flow-through (HSA depleted) and elution (HSA enriched) fractionsand undepleted IVF media.

The control group was followed with standard in-solution digestion procedure and the HPLC/MS analysis was performed as described earlier [18]. Collected fractions were digested using Trypsin Gold (Promega GmbH, Wien, Austria) and the resulting peptides were separated on an Acclaim PepMap C18 column (3 µm particles, 100 Å pore size, 75 µm ID, 250 mm length). The column was operated using an UltiMate 3000 RSLC nano HPLC system (ThermoFisher Scientific, Germering, Germany) as described in [18]. Mass spectrometric detection was performed on a maXis Impact q-ToF mass spectrometer (Bruker, Bremen, Germany) in positive electrospray mode and using collision-induced dissociation. Singly charged ions were excluded from dissociation, and fragmented ions were excluded from further fragmentation for 60 seconds. Resulting raw mass spectra were transformed into Mascot Generic Format (MGF) files for database search by a script in Data Analysis 4.2 (Bruker, Bremen, Germany), and database search was performed using Protein Scape 3.1 (Bruker, Bremen, Germany) and Mascot Version 2.5.1.1 (Matrix Science, London, UK). Swissprot at its most current version (April 2015) was searched for trypsin-digested proteins (Homo sapiens) with up to two miscleavages allowed; mass precision for MS was set at 50 ppm and at 0.05 Da for MS/MS spectra. Peptides with a Mascot score >15 were accepted as valid hits.

Results and Discussion

Achieving a high loading capacity for the target analyte is a crucial parameter for depletion column performance, therefore the DBC of CIMac-αHSA depletion columns for human serum albumin was first established. It was estimated that IVF media contain 5-10 mg/mL of human serum albumin (50 µL injected per proteomic analysis), therefore the target DBC was ~1.0 mg HSA per mL of support due to the HSA amount in spent IVF-media. Oriented antibody immobilisation procedure for CIMac-αHSA columns resulted in 1.3 mg/mL DBC50 (Figure 1). Loading of HSA at increasing flow rates (0.1 – 1.0 mL/min) led to no decrease of capacity, demonstrating flow-independent HSA binding onto CIMac-αHSA (Figure 1).

CIMac-αHSA was then tested for albumin depletion from IVF medium (Figure 2).

LC-MS/MS analysis of tryptic-digested proteins from eluted fractions and subsequent proteomic database search (conducted as described elsewhere

[18]) revealed that flow-through fraction contained unbound proteins, whereas albumin was only detected in eluate upon increasing the concentration of mobile phase B to 100% (Figure 2). No difference was observed when two different mobile phase compositions (Agilent and own mobile phases) were used, suggesting that the method is robust.

Based on normalised spectral counting, the amount of serum albumin was significantly reduced in the CIMac-αHSA treated sample compared to native sample (300 counts versus 2.5 counts, respectively). Detection limits for proteins that could not be detected in native samples was increased. Spectral count for haptoglobine, considered to be an important indicator for pregnancy and embryo development [19-22], increased from 2 to 20 counts, a ten-fold improvement.

Although 31 proteins have been identified in both fractions, the depleted fraction contained approximately twice as many unique proteins compared to non-depleted fraction and in the sample, which was depleted using the depletion kit from Sigma as a quality control and comparative method (Table 2).

Importantly, despite the relatively high concentration of albumin in analysed samples (5 - 10 mg/mL depending on batch and manufacturer), no proteins or peptides were identified in blank samples, This is of great importance since carry-over can pose a significant problem to subsequent analysis; a chromatographic challenge that was intensively investigated earlier [23-25]

Conclusions

Depletion of highly abundant proteins, such as albumin is an important step for achieving a higher coverage of low abundant proteins in proteomic analyses of biological samples. For analysis of spent IVF media and identification of putative biomarkers, albumin depletion has to be performed prior to analysis of secreted proteins. CIMac-αHSA facilitates identification of low abundant proteins through albumin depletion, and generally increases the number of proteins identified.

The use of monolithic depletion columns enables very fast depletion (Rt for HSA-depleted fraction ~ 2.0 min), allowing faster elution of albumin and fast column reconditioning (total cycle 16 min), thus significantly increasing the throughput of proteomic analyses as compared to other approaches, e.g. using spin columns.

This work was financially supported through FP7 project Prot-HiSPRA,www.prot-hispra.eu

Table 2: Number of proteins identified in CIMac-αHSA-treated, commercial HSA depletion kit, and untreated samples of IVF media.

CIMac-@HSA	Non-Depleted Medium	Depleted Medium
Proteins ID	74	122
Unique Proteins	43	91
Common	31	
HSA Depletion Kit		
Proteins ID	82	59
Unique Proteins	36	23
Common	18	

References

1. Baussant, T., et al., Effective depletion of albumin using a new peptide-based affinity medium. Proteomics, 2005. **5**(4): p. 973-977.

2. Echan, L.A., et al., Depletion of multiple high-abundance proteins improves protein profiling capacities of human serum and plasma. Proteomics, 2005. **5**: p. 3292-3303.

3. Mahn, A., et al., Depletion of highly abundant proteins in blood plasma by hydrophobic interaction chromatography for proteomic analysis. Journal of Chromatography B, 2010. **878**(15-16): p. 1038-1044.

4. Mortezai, N., et al., Tandem Affinity Depletion: A Combination of Affinity Fractionation and Immunoaffinity Depletion Allows the Detection of Low-Abundance Components in the Complex Proteomes of Body Fluids. Journal of Proteome Research, 2010. **9**(12): p. 6126-6134.

5. Afkarian, M., et al., Optimizing a Proteomics Platform for Urine Biomarker Discovery. Molecular & Cellular Proteomics, 2010. **9**(10): p. 2195-2204.

6. Shi, T., et al., Targeted Quantification of Low ng/mL Level Proteins in Human Serum without Immunoaffinity Depletion. Journal of Proteome Research, 2013. **12**(7): p. 3353-3361.

7. Jones, K.A., et al., Immunodepletion Plasma Proteomics by TripleTOF 5600 and Orbitrap Elite/LTQ-Orbitrap Velos/Q Exactive Mass Spectrometers. Journal of Proteome Research, 2013. **12**(10): p. 4351-4365.

8. Hakimi, A., et al., Assessment of reproducibility in depletion and enrichment workflows for plasma proteomics using label-free quantitative data-independent LC-MS. Proteomics, 2014. **14**(1): p. 4-13.

9. Podgornik, A., et al., Fast separation of large biomolecules using short monolithic columns. Journal of Chromatography B, 2013. **927**: p. 80-89.

10. Josic, D. and J.G. Clifton, Use of monolithic supports in proteomics technology. Journal of Chromatography A, 2007. **1144**(1): p. 2-13.

11. Vodopivec, M., et al., Characterization of CIM monoliths as enzyme reactors. Journal of Chromatography B, 2003. **795**(1): p. 105-113.

12. Nicoli, R., et al., Trypsin immobilization on an ethylenediamine-based monolithic minidisk for rapid on-line peptide mass fingerprinting studies. J. Chromatogr., A, 2009. **1216**: p. 2695.

13. Gunasena, D.N. and Z. El Rassi, Organic monoliths for hydrophilic interaction electrochromatography/ chromatography and immunoaffinity chromatography. Electrophoresis, 2012. **33**: p. 251-261.

14. Urbas, L., et al., Depletion of high-abundance proteins from human plasma using a combination of an affinity and pseudo-affinity column. Journal of Chromatography A, 2009. **1216**(13): p. 2689-2694.

15. Jiang, T., R. Mallik, and D.S. Hage, Affinity Monoliths for Ultrafast Immunoextraction. Analytical Chemistry, 2005. **77**(8): p. 2362-2372.

16. Rajak, P., M.A. Vijayalakshmi, and N.S. Jayaprakash, Production and characterization of monoclonal antibodies (mAbs) against human serum albumin (HSA) for the development of an immunoaffinity system with oriented anti-HSA mAbs as immobilised ligand. Journal of Pharmaceutical and Biomedical Analysis, 2013. **78–79**: p. 154-160.

17. Brne, P., et al., Development and characterization of methacrylate-based hydrazide monoliths for oriented immobilization of antibodies. Journal of Chromatography A, 2009. **1216**(13): p. 2658-2663.

18. Mitulović, G., et al., An improved method for tracking and reducing the void volume in nano HPLC–MS with micro trapping columns. Analytical and Bioanalytical Chemistry, 2003. **376**(7): p. 946-951.

19. Berkova, N., et al., Haptoglobin is

present in human endometrium and shows elevated levels in the decidua during pregnancy. Molecular Human Reproduction, 2001. **7**(8): p. 747-754.

20. Gatzka, C., et al., Isolierter Haptoglobinabfall in der Schwangerschaft: Zufallsbefund oder pathologisch? Zentralbl Gynakol, 2002. **124**(02): p. 120-122.

21. Raijmakers, M.T.M., et al., Haptoglobin and its association with the HELLP syndrome. Journal of Medical Genetics, 2003. **40**(3): p. 214-216.

22. Montskó, G., et al., Noninvasive embryo viability assessment by quantitation of human haptoglobin alpha-1 fragment in the in vitro fertilization culture medium: an additional tool to increase success rate. Fertility and Sterility, 2015. **103**(3): p. 687-693.

23. Mitulović, G., et al., Preventing Carryover of Peptides and Proteins in Nano LC-MS Separations. Analytical Chemistry, 2009. **81**(14): p. 5955-5960.

24. Dolman, S., et al., Investigation of carryover of peptides in nano-liquid chromatography/ mass spectrometry using packed and monolithic capillary columns. Journal of Chromatography B, 2013. **912**(0): p. 56-63.

25. Maes, K., et al., Strategies to reduce aspecific adsorption of peptides and proteins in liquid chromatography–mass spectrometry based bioanalyses: An overview. Journal of Chromatography A, 2014. **1358**(0): p. 1-13.