Electronic Supplementary Information

Adapting aqueous biphasic systems to solid-phase extraction for bioanalytical applications: high-abundance protein depletion and prostate-specific antigen extraction from human serum

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ESI Materials and Methods

Materials

PEG with a molecular weight of 2000 g·mol^{-1} (PEG 2000) was purchased from Alfa Aesar. Citrate buffer at pH ≈ 7 was prepared using potassium citrate tribasic monohydrate ($C_6H_5K_3O_7.H_2O$, purity 99 wt%) from Acros Organics and citric acid 1-hydrate ($C_6H_8O_7.H_2O$, purity 99.5 wt%) from PanReac at 50 wt% $C_6H_5K_3O_7/C_6H_8O_7$ at a mass ratio of ca. 25. Throughout this work, when the weight percentage composition of citrate buffer is mentioned, it corresponds to the amount of ($C_6H_5K_3O_7/C_6H_8O_7$) without considering complexed water molecules in $C_6H_5K_3O_7.H_2O$ or the water contained in the buffer solution. Aqueous biphasic extraction chromatography (ABEC) resin with a monomethylated polyethylene glycol (Me-PEG) molecular weight of 2000 g·mol^{-1} (ABEC-2000 resin, 125-250 µm, 2% cross-linked bulk) was acquired from Eichrom. The human serum used was purchased from Sigma-Aldrich (H4522- Lot # SLCJ3824) and stored at -20 °C until use.

SE-HPLC mobile phase was prepared using sodium chloride extra pure (NaCl, purity ≥99.8 wt%) acquired from Labkem, sodium dihydrogen phosphate 1-hydrate ($NaH_2PO_4.H_2O$, purity 99 wt%) and sodium hydrogen phosphate 7-hydrate ($Na_2HPO_4.7H_2O$, purity 99 wt%), both obtained from PanReac.

Purified human immunoglobulin G (IgG) with a concentration of 29.4 mg·mL^{-1} was from Innovative Researched, Inc., and stored at -80 °C. Lyophilized human serum albumin (HSA) powder with purity ≥97wt% was from Sigma-Aldrich and stored at 4 °C. The commercial human prostate-specific antigen (PSA)-total ELISA kit (RAB0331) from Sigma-Aldrich was stored at 4 °C and used according to the manufacturer's instructions.

Depletion of immunoglobulin G and human serum albumin

To evaluate protein depletion efficiency, three sample pretreatment configurations were developed and investigated: aqueous biphasic systems (ABS), ABEC-2000-based solid-phase extraction (SPE), and protein precipitation (PP) using PEG 2000 or $C_6H_5K_3O_7/C_6H_8O_7$ aqueous solutions. The compositions of each component used in the preparation of these systems are detailed in Table S1 and were based on the ABS method.[1] To facilitate direct comparison and accelerate development, experimental conditions and compositions were aimed to be consistent across all three serum pretreatment configurations. However, due to experimental specificities of each configuration, slight adjustments in the separation protocols and component compositions were performed whenever required.

In the PP approach, the individual components of ABS configurations, namely PEG 2000 and $C_6H_5K_3O_7/C_6H_8O_7$, were separately evaluated. The mixtures were prepared by adding 10 wt% serum to various aqueous solutions of PEG 2000 or $C_6H_5K_3O_7/C_6H_8O_7$ at distinct weight percentages (within ± 10^{-4} g). For PEG-2000-induced PP, mixtures were stirred and centrifuged at 3500 rpm for 10 min, while for $C_6H_5K_3O_7/C_6H_8O_7$-induced PP, mixtures were left under agitation for 10 min and centrifuged at 13000 rpm for 20 min. Systems in which a protein-rich precipitate is formed upon adding $C_6H_5K_3O_7/C_6H_8O_7$ or PEG-rich aqueous solutions at certain concentrations were obtained. For ABS, compositions were based on the available ternary phase diagram for systems composed of PEG 2000, $C_6H_5K_3O_7/C_6H_8O_7$ and water.[2] The mixtures were

prepared by weighing the appropriate amounts of each component in the presence of 10 wt% serum (within ± 10^{-4} g), stirred and centrifuged at 3500 rpm for 10 min. Systems in which the top phase is PEG-rich, the bottom phase is $C_6H_5K_3O_7$/$C_6H_8O_7$-rich and the solid interphase is enriched with depleted serum proteins were formed. In turn, ABEC-based SPE compositions were consistent with those used for ABS whenever possible. Lower $C_6H_5K_3O_7$/$C_6H_8O_7$ concentrations were also evaluated for technology development purposes. After weighing the adequate amounts of each component and serum (within ± 10^{-4} g), these mixtures were left under agitation for 10 min and centrifuged at 13000 rpm for 20 min. Systems in which the resin is placed above a $C_6H_5K_3O_7$/$C_6H_8O_7$-rich liquid phase were obtained.

For each method, systems were prepared in triplicate to obtain average values and the corresponding standard deviations. The resulting liquid or solid phases were carefully separated, and the concentrations of IgG and HSA were quantified in the liquid phases by SE-HPLC using established calibration curves. The weight of proteins in the solid phases was determined through mass balance considering the initial weight of IgG and HSA in commercial human serum samples.

A Chromaster HPLC system obtained from VWR Hitachi equipped with a Protein KW-802.5 analytical column (8 mm x 300 mm) coupled to a Protein KW-G 6B guard column, from Shodex was used. The mobile phase was prepared at pH ≈ 7, consisting of 50 mM sodium phosphate buffer and 0.3 M NaCl solution, and run isocratically at 0.5 mL·min^{-1} for 40 min. The temperature of the column oven and autosampler were 25 °C and 10 °C, respectively, whereas the injection volume was set at 25 µL and the wavelength at 280 nm.

Depletion efficiencies for IgG and HSA, DE_{IgG}% and DE_{HSA}%, were calculated using the following equations:

$$DE_{IgG}\% = \frac{w_{IgG}^{Depleted}}{w_{IgG}^{Initial}} \times 100 \quad (1)$$

$$DE_{HSA}\% = \frac{w_{HSA}^{Depleted}}{w_{HSA}^{Initial}} \times 100 \quad (2)$$

where $w_{IgG}^{Depleted}$ and $w_{HSA}^{Depleted}$ represent the weight of IgG and HSA in the interphase (for ABS), adsorbed onto the resin (for ABEC-based SPE), or present in the precipitate (for PP). $w_{IgG}^{Initial}$ and $w_{HSA}^{Initial}$ correspond to the initial weight of IgG and HSA in each system, respectively.

Table S1 Component compositions used in IgG and HSA depletion studies involving PP, ABS, and ABEC-based SPE. Compositions marked in grey and highlighted in bold refer to compositions selected to advance with PSA extraction studies.

Technique	Compositions (wt%)				
	PEG 2000	ABEC-2000	$C_6H_5K_3O_7/C_6H_8O_7$	Human serum	Water
PP	10	-	-	10	80
	15	-	-		75
	20	**-**	**-**		**70**
	25	-	-		65
	30	**-**	**-**		**60**
	-	-	0.1		99.1
	-	-	0.5		99.5
	-	-	1		99
	-	-	5		95
	-	-	10		80
	-	-	15		75
	-	-	20		70
	-	-	25		65
	-	-	**30**		**60**
ABS	10	-	20		60
	10	-	25		55
	10	**-**	**30**		**50**
	20	-	15		55
	20	-	20		50
	20	-	25		45
	20	-	30		40
	30	**-**	**10**		**50**
	30	**-**	**15**		**45**
	30	-	20		40
	30	-	25		35
	30	**-**	**30**		**30**
ABEC-based SPE	-	30	0		60
	-	30	0.1		59.9
	-	30	0.5		59.5
	-	30	1		59
	-	30	5		55
	-	30	10		50
	-	30	15		45
	-	30	20		40
	-	30	25		35
	-	10	30		50
	-	20	30		40
	-	**30**	**30**		**30**

Extraction of prostate-specific antigen

The different approaches previously employed in the depletion of IgG and HSA were then applied to address the extraction of PSA in the liquid phase for further analysis. The selected mixture compositions to advance with these studies are marked in grey and highlighted in bold in Table S1. 10 wt% human serum spiked with clinically relevant concentrations of PSA (4 ng·mL^{-1}) was added to each system.[3] To assess possible interferences from the human serum matrix, systems with non-spiked human serum were used as controls. The systems were prepared in triplicate to obtain mean values and respective standard deviations, and the phases were separated by employing the same procedure previously described. The concentration of PSA in the liquid phases was quantified using PSA ELISA kits following the protocol provided by the manufacturer, while the weight of PSA in the solid phases was obtained through a mass balance. The recovery yields of PSA, $RY_{PSA}\%$, were calculated according to the following equation:

$$RY_{PSA}\% = \frac{w_{PSA}^{Recovered}}{w_{PSA}^{Initial}} \times 100 \quad (3)$$

where $w_{PSA}^{Recovered}$ represents the weight of PSA determined in each system's phases (top/inter/bottom phases for ABS, resin/liquid phase for ABEC-based SPE, precipitate/liquid phase for PP, and $w_{PSA}^{Initial}$ corresponds to the initial weight of PSA added to each system.

ESI Results

Depletion of immunoglobulin G and human serum albumin

Table S2 Depletion efficiencies of IgG and HSA using PEG-2000-induced and $C_6H_5K_3O_7/C_6H_8O_7$-induced PP in the presence of 10 wt% human serum.

Depletion efficiencies (%)		
System	**IgG**	**HSA**
	Mean ± SD	**Mean ± SD**
10 wt% PEG 2000	42 ± 2	0
15 wt% PEG 2000	67 ± 3	2 ± 2
20 wt% PEG 2000	80 ± 3	5 ± 2
25 wt% PEG 2000	56 ± 5	0
30 wt% PEG 2000	24 ± 3	0
0.1 wt% $C_6H_5K_3O_7/C_6H_8O_7$	0	0
0.5 wt% $C_6H_5K_3O_7/C_6H_8O_7$	0	2 ± 1
1 wt% $C_6H_5K_3O_7/C_6H_8O_7$	0	0.1 ± 0.2
5 wt% $C_6H_5K_3O_7/C_6H_8O_7$	0	0.7 ± 1.0
10 wt% $C_6H_5K_3O_7/C_6H_8O_7$	0	0
15 wt% $C_6H_5K_3O_7/C_6H_8O_7$	0	0
20 wt% $C_6H_5K_3O_7/C_6H_8O_7$	3.1 ± 0.5	0
25 wt% $C_6H_5K_3O_7/C_6H_8O_7$	63 ± 1	0
30 wt% $C_6H_5K_3O_7/C_6H_8O_7$	95.1 ± 0.4	6.5 ± 0.3

Table S3 Depletion efficiencies of IgG and HSA obtained using ABS composed of PEG 2000 and $C_6H_5K_3O_7/C_6H_8O_7$ in the presence of 10 wt% human serum.

Depletion efficiencies (%)		
System composition	**IgG**	**HSA**
	Mean ± SD	**Mean ± SD**
10 wt% PEG 2000 + 20 wt% $C_6H_5K_3O_7/C_6H_8O_7$	63 ± 3	18 ± 1
10 wt% PEG 2000 + 25 wt% $C_6H_5K_3O_7/C_6H_8O_7$	96.7 ± 0.1	27 ± 3
10 wt% PEG 2000 + 30 wt% $C_6H_5K_3O_7/C_6H_8O_7$	97 ± 3	84 ± 7
20 wt% PEG 2000 + 15 wt% $C_6H_5K_3O_7/C_6H_8O_7$	77 ± 1	20 ± 2
20 wt% PEG 2000 + 20 wt% $C_6H_5K_3O_7/C_6H_8O_7$	98.6 ± 0.2	49 ± 2
20 wt% PEG 2000 + 25 wt% $C_6H_5K_3O_7/C_6H_8O_7$	97 ± 2	94 ± 2
20 wt% PEG 2000 + 30 wt% $C_6H_5K_3O_7/C_6H_8O_7$	97 ± 5	97 ± 2
30 wt% PEG 2000 + 10 wt% $C_6H_5K_3O_7/C_6H_8O_7$	85 ± 3	40 ± 7
30 wt% PEG 2000 + 15 wt% $C_6H_5K_3O_7/C_6H_8O_7$	99 ± 1	91.3 ± 0.5
30 wt% PEG 2000 + 20 wt% $C_6H_5K_3O_7/C_6H_8O_7$	100	98 ± 2
30 wt% PEG 2000 + 25 wt% $C_6H_5K_3O_7/C_6H_8O_7$	99.5 ± 0.8	96 ± 2
30 wt% PEG 2000 + 30 wt% $C_6H_5K_3O_7/C_6H_8O_7$	99.1 ± 0.1	96.8 ± 0.7

Table S4 Depletion efficiencies of IgG and HSA using ABEC-based SPE in the presence of 10 wt% human serum.

Depletion efficiencies (%)		
System composition	**IgG**	**HSA**
	Mean ± SD	**Mean ± SD**
0 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	0	0
0.1 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	46 ± 3	57 ± 1
0.5 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	44 ± 3	56 ± 1
1 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	18 ± 2	38 ± 2
5 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	25 ± 3	15 ± 2
10 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	19 ± 12	13 ± 6
15 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	6 ± 1	2 ± 1
20 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	47 ± 3	0
25 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	93.8 ± 0.1	18 ± 1
30 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ + 30 wt% ABEC-2000	97.7 ± 0.4	93.2 ± 0.1
0 wt% ABEC-2000 + 30 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$	95.1 ± 0.4	6.5 ± 0.3
10 wt% ABEC-2000 + 30 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$	91.9 ± 0.9	2 ± 1
20 wt% ABEC-2000 + 30 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$	98.1 ± 0.2	54.8 ± 0.3

Extraction of prostate-specific antigen

Table S5 Recovery yield of PSA in the top phase, interphase, and bottom phase of ABS formed in the presence of 10 wt% human serum spiked with 4 ng·mL^{-1} of PSA.

Recovery yields (%)			
System	**Top phase**	**Interphase**	**Bottom phase**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**
10 wt% PEG 2000 + 30 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$	19 ± 3	79 ± 6	2 ± 3
30 wt% PEG 2000 + 10 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$	103 ± 8	1 ± 2	0
30 wt% PEG 2000 + 15 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$	43 ± 2	55 ± 1	2 ± 1
30 wt% PEG 2000 + 30 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$	1 ± 1	97.7 ± 0.6	1.3 ± 0.8

Table S6 Recovery yields of PSA in the liquid phase and resin/precipitate of ABEC-based SPE, PEG-2000-induced and $C_6H_5K_3O_7$/$C_6H_8O_7$-induced PP in the presence of 10 wt% human serum spiked with 4 ng·mL^{-1} of PSA.

Recovery yields (%)		
System	**Liquid phase**	**Resin/Precipitate**
	Mean ± SD	**Mean ± SD**
30 wt% ABEC-2000 + 30 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$	9 ± 2	91 ± 2
20 wt% PEG 2000	113 ± 11	0
30 wt% PEG 2000	25 ± 0	75 ± 0
30 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$	0	100

ESI References

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