Polyion Complex Library Possessing Naturally Occurring Differentiation for Pattern-based Protein Discrimination

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Materials and Methods

Materials

Enzymes: β-Galactosidase from *Aspergillus oryzae* (GALAO), β-galactosidase from *Escherichia coli* (GALEC), α-amylase from *Aspergillus oryzae* (AMYAO), and amyloglucosidase from *Aspergillus niger* (AMYAN) were obtained from Sigma Chemical Co. (St. Louis, MO). **Substrates:** *o*-Nitrophenyl-β-D-galactopyranoside (oNPG, for GALAO and GALEC) and *p*-nitrophenyl-α-D-glucopyranoside (pNPG, for AMYAN) were obtained from Sigma Chemical Co. *p*-Nitrophenyl-α-D-maltoside (pNPM, for AMYAO) was obtained from Calbiochem (La Jolla, CA). **Human plasma proteins:** Albumin from human serum (ALB), immunoglobulin G from human serum (IMM), α_1-antitrypsin from human plasma (ANT), fibrinogen from human plasma (FIB), and apo-transferrin from human (TRA) were obtained from Sigma Chemical Co. **Others:** Poly(ethylene glycol)-*block*-poly(*N,N*-dimethylaminoethyl methacrylate) (PEG-*b*-PAMA) with average Mw (PEG) 5000 and Mw (PAMA) 12000 was obtained from Polymer Source inc. (Dorval, QC, Canada). 3-(*N*-Morpholino)propanesulfonic acid (MOPS) was obtained from Sigma Chemical Co. All chemicals used were of high-quality analytical grade and were used as received.

Determination of enzyme and plasma protein concentrations

Concentrations of enzymes and plasma proteins were determined from the absorbance at 280 nm using a spectrophotometer (UV-2450; Shimadzu Corporation, Kyoto, Japan), with extinction coefficients of 192075 M^{-1} cm^{-1} (GALAO), 1046760 M^{-1} cm^{-1} (GALEC), 106160 M^{-1} cm^{-1} (AMYAO), 145230 M^{-1} cm^{-1} (AMYAN), 34445 M^{-1} cm^{-1} (ALB), 203585 M^{-1} cm^{-1} (IMM), 19940 M^{-1} cm^{-1} (ANT), 654160 M^{-1} cm^{-1} (FIB), and 85115 M^{-1} cm^{-1} (TRA).[1]

*Titration of PEG-*b*-PAMA to enzymes*

Various concentrations of PEG-*b*-PAMA were incubated with anionic enzymes (62.5 nM GALAO, 15 nM GALEC, 187.5 nM AMYAO, and 168.8 nM AMYAN) in 10 mM MOPS (pH 7.0). After incubation for 30 min at 25°C, 120 μL of each solution was loaded into each well of 96-well plates (Half Area 96-Well Clear Flat Bottom UV-Transparent Microplates; Corning Inc., Corning, NY). Subsequently, 30 μL of substrate solution (25 mM oNPG, 25 mM pNPM, and 45 mM pNPG) in 10 mM MOPS (pH 7.0) was added to each well containing the corresponding enzymes so that the final concentrations were 50 nM GALAO and 5 mM oNPG; 12 nM GALEC and 5 mM oNPG; 150 nM AMYAO and 5 mM pNPM; 135 nM AMYAN and 9 mM pNPG. The time course of the increase in absorbance at 400 nm was then recorded using a microplate reader (Viento® nano; DS Pharma Biomedical Co. Ltd., Osaka, Japan). The samples were measured in triplicate.

Titration of plasma proteins to enzyme/polyelectrolyte complexes (EPCs)

Enzymes and PEG-*b*-PAMA were mixed in the ratio obtained by PEG-*b*-PAMA titration to enzymes to prepare EPCs in 10 mM MOPS (pH 7.0) (75 nM GALAO and 132 nM PEG-*b*-PAMA; 9 nM GALEC and 104.3 nM PEG-*b*-PAMA; 225 nM AMYAO and 162 nM PEG-*b*-PAMA; 202.5 nM AMYAN and 308.3 nM PEG-*b*-PAMA). Aliquots of 100 μL of each solution were loaded into each well of 96-well plates. Subsequently, 20 μL of various concentrations of plasma proteins was added to each well. After incubation for 30 min at 25°C, 30 μL of substrate solution (25 mM oNPG, 25 mM pNPM, and 45 mM pNPG) in 10 mM MOPS (pH 7.0) was further added to each well containing the corresponding enzymes so that the final concentrations were 50 nM GALAO, 88 nM PEG-*b*-PAMA, and 5 mM oNPG; 6 nM GALEC, 69.5 nM PEG-*b*-PAMA, and 5 mM oNPG; 150 nM AMYAO, 108 nM PEG-*b*-PAMA, and 5 mM pNPM; 135 nM AMYAN, 205.5 nM PEG-*b*-PAMA, and 9 mM pNPG. The time course of the increase in absorbance at 400 nm was then recorded using a microplate reader. The samples were measured in triplicate.

Sensing of plasma protein analytes

Enzymes and PEG-*b*-PAMA were mixed at the optimal ratio determined taking into account both differences in activity changes between plasma proteins and signal-to-noise ratio in the absorbance increase (7.5 nM GALAO and 13.2 nM PEG-*b*-PAMA; 1.5 nM GALEC and 17.3 nM PEG-*b*-PAMA; 75 nM AMYAO and 54 nM PEG-*b*-PAMA; 202.5 nM AMYAN

and 308.3 nM PEG-*b*-PAMA) in 10 mM MOPS (pH 7.0). Aliquots of 100 μL of each solution were loaded into each well of 96-well plates. Subsequently, 20 μL of plasma protein analytes (shown in Table S1) was added. After incubation for 30 min at 25°C, 30 μL of substrate solution (25 mM oNPG, 25 mM pNPM, and 45 mM pNPG) in 10 mM MOPS (pH 7.0) was further added to each well containing the corresponding enzymes so that the final concentrations were 5 nM GALAO, 8.8 nM PEG-*b*-PAMA, and 5 mM oNPG; 1 nM GALEC, 11.5 nM PEG-*b*-PAMA, and 5 mM oNPG; 50 nM AMYAO, 36 nM PEG-*b*-PAMA, and 5 mM pNPM; 135 nM AMYAN, 205.5 nM PEG-*b*-PAMA, and 9 mM pNPG. The time course of the increase in absorbance at 400 nm was then recorded using a microplate reader for 40 min (GALAO), 80 min (GALEC), and 120 min (AMYAO and AMYAN). This process was repeated for seven plasma protein analytes with three EPC in six replicates each, providing a data set matrix of 3 EPC × 7 analytes × 6 replicates. This data set matrix was subjected to classical linear discriminant analysis using SYSTAT 13 (Systat Inc., Evanston, IL). Similar procedures were also performed to discriminate 21 unknown plasma protein analytes.

Figures and Tables

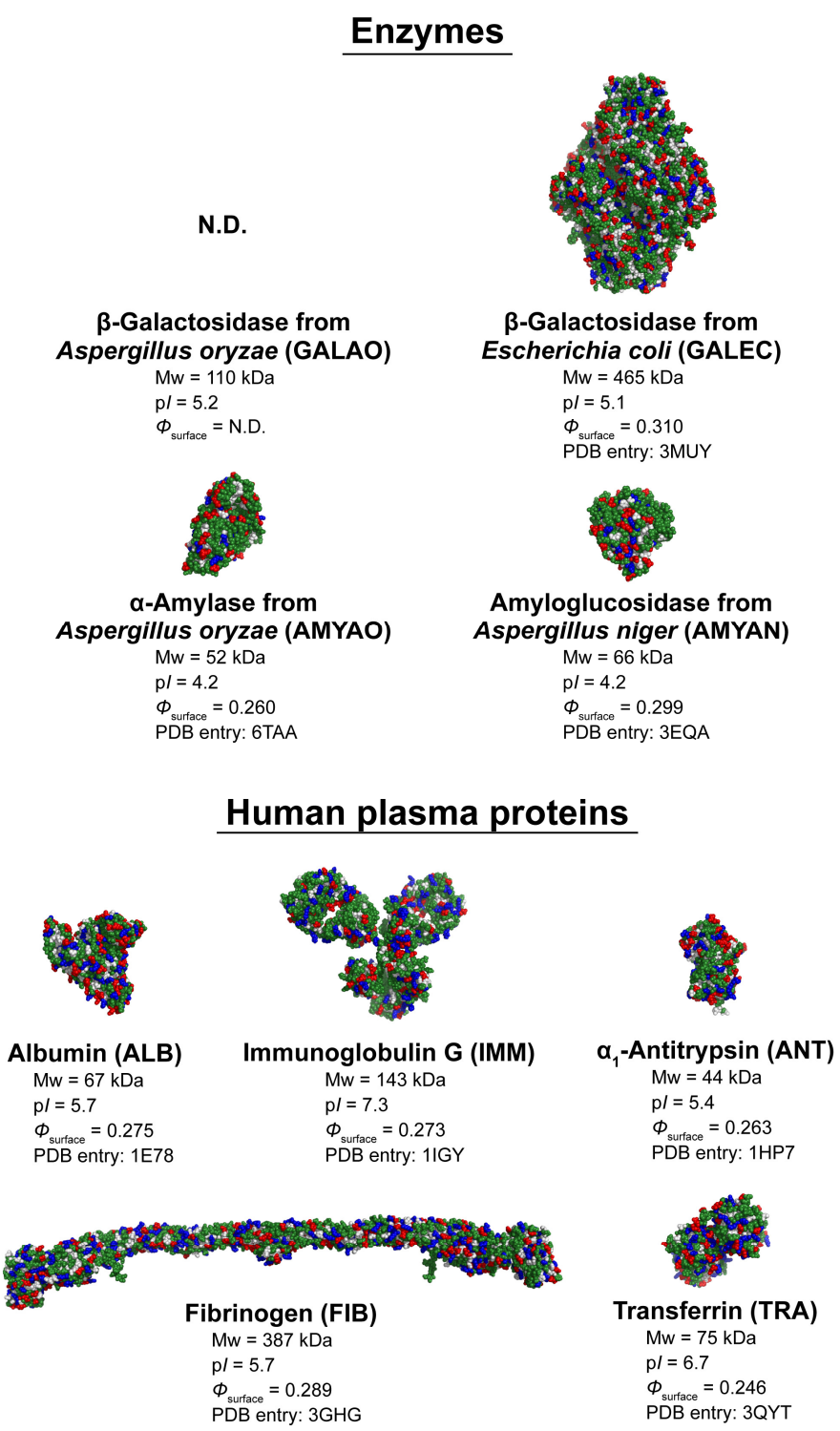


Figure S1. Properties of anionic enzymes and human plasma proteins. Color scheme for enzymes and proteins: anionic residues, red; cationic residues, blue; hydrophobic residues, gray; hydrophilic residues, green. The surface hydrophobicity ($\Phi_{surface}$) of enzymes and proteins based on the Miyazawa-Jernigan hydrophobicity scale was estimated using the previously described method[2] with the accessible surface areas of enzymes and proteins calculated by the program GETAREA.[3] The crystal structure of GALAO is not available in the Protein Data Bank (PDB).

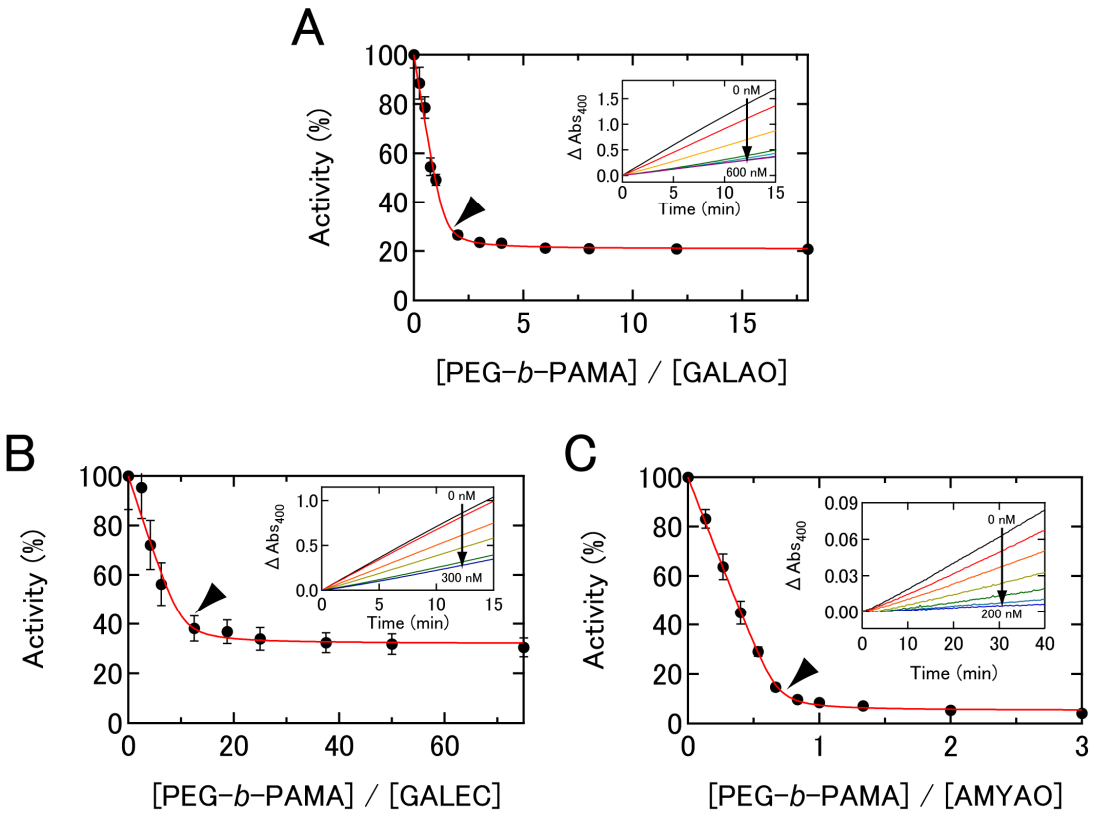


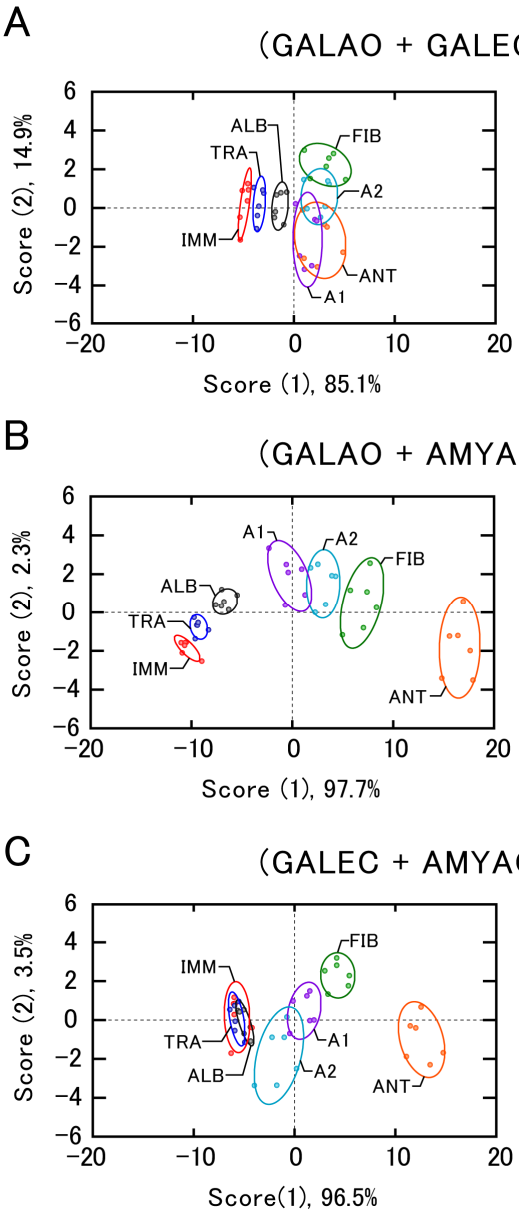
Figure S2. Changes in activity of GALAO, GALEC, or AMYAO. Titration of PEG-*b*-PAMA to 50 nM GALAO (A), 12 nM GALEC (B), and 150 nM AMYAO (C) in 10 mM MOPS (pH 7.0). The arrows indicate the mixing ratios used in Figure 2. The insets show the time course of increase in absorbance at 400 nm in the presence of various concentrations of PEG-*b*-PAMA.

Table S1. A matrix of final concentrations of analytes for discrimination study

	ALB	IMM	ANT	FIB	TRA
Analyte 1 (ALB)	100 nM				
Analyte 2 (IMM)		100 nM			
Analyte 3 (ANT)			100 nM		
Analyte 4 (FIB)				100 nM	
Analyte 5 (TRA)					100 nM
Analyte 6 (A1)	25 nM	25 nM	25 nM	25 nM	
Analyte 7 (A2)	25 nM	25 nM	25 nM		25 nM

Table S2. Data set matrix of v/v_0 for individual plasma protein analytes generated from the EPC sensor array (GALAO, GALEC, AMYLA, and AMYAN) against seven analytes

Analytes	GALAO	GALEC	AMYLO	AMYAN
ALB	1.4700	1.3639	1.1164	1.1080
ALB	1.4475	1.0677	0.8660	0.9674
ALB	1.3241	1.2873	0.9877	0.7534
ALB	1.3259	1.2350	1.1133	1.0194
ALB	1.3662	1.0667	0.9896	0.9853
ALB	1.3093	1.0815	0.9027	1.0068
ANT	2.3620	1.6894	4.1830	1.4528
ANT	2.0487	1.4131	4.2640	1.2193
ANT	2.0282	1.7930	4.6502	1.0013
ANT	1.8238	1.6983	4.9104	1.2331
ANT	1.7091	1.2517	4.4794	1.1634
ANT	2.0877	1.4378	4.3724	1.1515
FIB	2.2567	1.0244	2.6467	2.0255
FIB	1.9766	0.8062	2.5891	1.4463
FIB	1.9558	0.8794	3.0712	1.2857
FIB	1.7528	0.9715	3.0513	1.4096
FIB	1.6132	0.7026	2.8247	1.3339
FIB	2.0339	0.7697	2.8245	1.2353
IMM	0.8354	1.2424	1.1459	1.0838
IMM	0.8852	1.4516	0.7296	1.0147
IMM	0.9085	1.1011	0.7960	1.1349
IMM	0.9009	1.0013	0.7833	1.0759
IMM	0.8151	0.9994	0.8205	0.9102
IMM	0.8798	0.9427	0.7971	0.9578
TRA	1.0928	1.3676	0.9983	0.9892
TRA	1.1246	1.0494	0.7108	0.9837
TRA	1.0797	1.2485	0.8062	1.0409
TRA	1.0808	1.1643	0.8149	1.0961
TRA	0.9727	0.9857	0.8883	0.9182
TRA	1.1032	1.0194	0.8045	1.0264
A1	1.9761	1.3401	1.8887	0.9179
A1	2.0324	1.0459	1.9587	1.1144
A1	2.0177	1.2457	2.2866	1.0081
A1	1.7680	1.2450	2.3677	1.1811
A1	1.6653	0.9739	2.2893	1.0344
A1	2.0064	1.0150	2.2317	1.0433
A2	1.9463	1.7756	1.1841	1.0705
A2	1.8993	1.3541	1.5385	0.9936
A2	1.8494	1.8000	1.6240	0.9838
A2	1.7543	1.6705	2.0183	1.1071
A2	1.5952	1.1857	1.8339	1.0281
A2	1.9291	1.3688	1.7789	1.1038



Analytes	%correct
ALB	100
ANT	67
FIB	83
IMM	100
TRA	100
A1	33
A2	33
Total	74

Analytes	%correct
ALB	100
ANT	100
FIB	83
IMM	83
TRA	100
A1	100
A2	83
Total	93

Analytes	%correct
ALB	67
ANT	100
FIB	100
IMM	0
TRA	17
A1	83
A2	67
Total	62

Figure S3. Classification accuracy of sensor arrays consisting of two EPCs for discrimination of seven analytes: (A) GALAO and GALEC, (B) GALAO and AMYAO, (C) GALEC and AMYAO. Two discriminant functions of enzyme activity patterns in discriminant score plots were obtained by linear discriminant analysis. The 95% confidence ellipses for the individual plasma protein analytes are shown. The values are taken from the jackknifed classification matrix based on the linear discriminant analysis model, i.e., Mahalanobis distances to the centroid of each group.

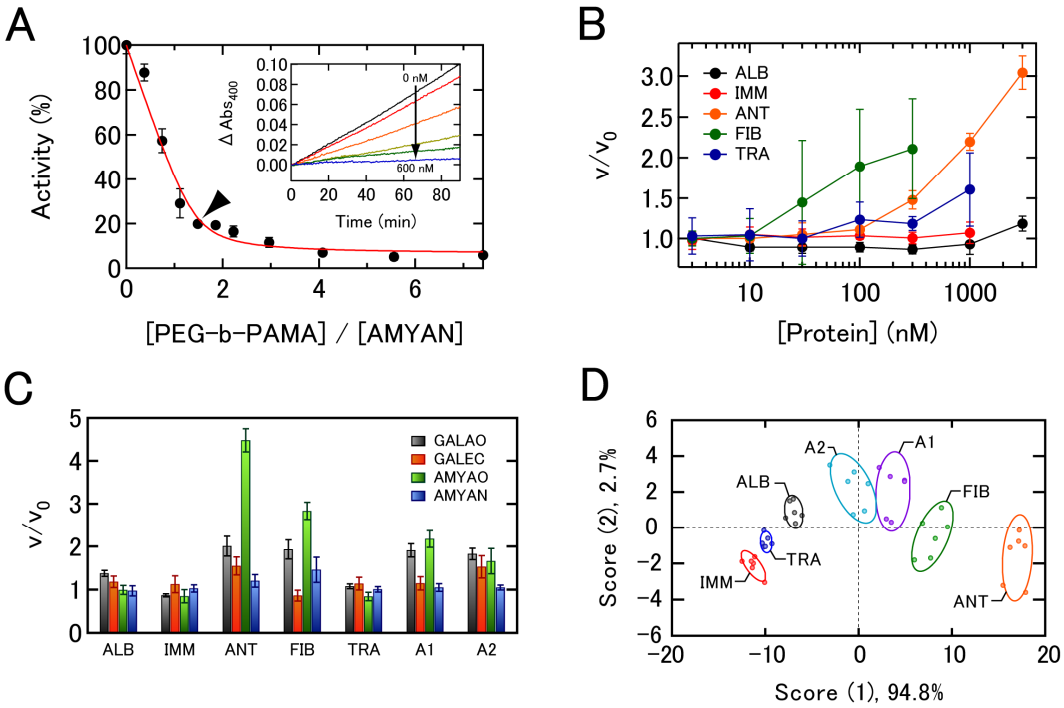


Figure S4. Pattern-based sensing of plasma protein analytes using four EPCs. (A) Titration of PEG-*b*-PAMA to 135 nM AMYAN in 10 mM MOPS (pH 7.0). The arrow indicates the mixing ratio used in (B). The inset shows the time course of increase in absorbance at 400 nm in the presence of various concentrations of PEG-*b*-PAMA. (B) Titration of five plasma proteins to the mixture of 135 nM AMYAN and 205.5 nM PEG-*b*-PAMA in 10 mM MOPS (pH 7.0). The y-axis indicates the ratio of velocity of absorbance increase before (v_0) and after (v) addition of plasma proteins. (C) Enzyme activity patterns for seven analytes obtained by four EPC (GALAO, GALEC, AMYAO, and AMYAN). Each value represents the average of six parallel measurements with standard deviation. (D) Discriminant score plot of the first two discriminant functions of enzyme activity patterns analyzed by linear discriminant analysis. The 95% confidence ellipses for the individual plasma protein analytes are shown.

Table S3. Discrimination of 21 unknown plasma protein analytes by the EPC sensor array

Identification	Enzyme activity pattern			Verification	Accuracy
	GALAO	GALEC	AMYAO		
ALB	1.3374	1.3441	0.9953	ALB	YES
ANT	1.7897	1.7398	4.7284	ANT	YES
FIB	1.6566	0.7690	3.0940	FIB	YES
IMM	0.9357	1.1016	0.8873	IMM	YES
TRA	1.0772	0.9996	1.0140	TRA	YES
A1	1.7535	0.9977	2.6385	A1	YES
A2	1.7275	1.4708	2.0824	A2	YES
ALB	1.2938	1.0955	0.9767	ALB	YES
ANT	1.8310	1.5736	5.2256	ANT	YES
FIB	1.7411	0.6435	3.2949	FIB	YES
IMM	0.8970	1.1034	0.7850	IMM	YES
TRA	1.0737	0.9465	0.9286	TRA	YES
A1	1.7866	0.9013	2.8737	FIB	NO
A2	1.7482	1.4772	1.9503	A2	YES
ALB	1.3077	1.1028	1.0279	ALB	YES
ANT	1.8336	1.5175	4.8239	ANT	YES
FIB	1.8206	1.0555	3.2787	FIB	YES
IMM	0.9101	1.0202	0.9183	IMM	YES
TRA	1.0583	1.1062	0.8873	TRA	YES
A1	1.7988	1.1880	2.4099	A1	YES
A2	1.7288	1.5054	1.6254	A2	YES

References

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[2] Lienqueo, M.E.; Mahn, A.; Asenjo, J.A., *J. Chromatogr. A*, **2002**, 978, 71.
[3] Fraczkiewicz, R.; Braun, W., *J. Comput. Chem.,* **1998**, 19, 319.