Electronic Supplementary Material (ESI) for Analyst.

Table S1 Summary of boronic acid enrichment experimental conditions and results including the number of identified N-glycopeptides and the intensity ratio of glycopeptides versus nonglycopeptides.

Experimental conditions		ASF		HRP		IgG		RNB	
		Number of N-Glycopeptides	Relative Intensity (gly/nongly)	Number of N-Glycopeptides	Relative Intensity (gly/nongly)	Number of N-Glycopeptides	Relative Intensity (gly/nongly)	Number of N-Glycopeptides	Relative Intensity (gly/nongly)
Buffer composition (pH 10, 50mM)	$HCOONH_4$	11	31.29	16	208.39	3	420.07	5	34.61
	CH_3COONH_4	11	66.86	17	150.15	3	879.81	5	17.12
	NH_4HCO_3	12	72.00	17	236.08	3	516.65	5	20.20
Buffer concentration (pH 10, NH_4HCO_3)	10mM	9	33.82	14	113.53	3	158.07	5	42.93
	20mM	9	42.04	16	118.50	3	207.78	5	29.30
	50mM	12	53.36	17	123.77	3	288.56	5	117.67
	100mM	10	55.35	8	82.89	3	309.79	5	921.41
	200mM	0	31.53	1	98.70	0	63.05	5	773.65
pH of Buffer (50mM NH_4HCO_3)	7.00	5	33.74	7	52.44	3	163.77	0	3.26
	8.00	6	28.66	7	74.08	3	177.43	0	3.36
	9.00	7	30.43	11	47.07	3	241.63	2	7.82
	10.00	12	47.30	17	77.34	3	189.80	5	124.45
	11.00	8	35.70	14	81.59	3	139.80	5	151.45

Table S2 Summary of ZIC-HILIC enrichment experimental conditions and the number of enriched glycopeptides. (Intensity represented the total intensity of three selected N-glycopeptides.)

			ASF				HRP				IgG				RNB			
			Without Added Salt		With 20mM ABC		Without Added Salt		With 20mM ABC		Without Added Salt		With 20mM ABC		Without Added Salt		With 20mM ABC	
			NO. of Glycopeptides	Intensity	NO. of Glycopeptides	Intensity	NO. of Glycopeptides	Intensity	NO. of Glycopeptides	Intensity	NO. of Glycopeptides	Intensity	NO. of Glycopeptides	Intensity	NO. of Glycopeptides	Intensity	NO. of Glycopeptides	Intensity
Formic Acid System	FA Concentration of Loading Buffer (80% ACN with different concentrations of FA in aqueous solution)	0.50%	1	163.8	7	821.4	1	16.1	9	296.2	0	27.0	8	132.1	0	27.3	2	86.5
		1%	2	97.2	7	742.4	1	16.3	9	271.4	1	28.1	8	135.4	0	33.2	4	183.7
		3%	4	331.9	7	363.0	1	20.5	8	68.7	2	579.1	10	203.1	2	48.7	5	143.4
		5%	3	335.6	7	829.0	1	17.8	6	49.7	3	28.2	8	279.3	3	60.0	4	114.1
		8%	2	95.4	6	1081.4	3	89.5	4	165.9	1	30.3	8	300.4	2	30.1	3	59.5
		10%	1	31.3	6	793.5	1	9.5	1	46.7	0	25.0	7	173.2	0	27.5	2	32.9
Trifluoroacetic System	TFA Concentration of Loading Buffer (80% ACN with different concentrations of TFA in aqueous solution)	0.05%	6	4827.0	9	3840.4	2	268.4	16	1732.9	5	470.7	19	4394.9	1	640.2	5	1539.8
		0.10%	6	3647.3	14	3570.6	2	265.6	18	1532.4	6	353.3	21	3971.6	1	515.2	5	1904.2
		0.50%	6	2736.1	1	289.8	2	285.4	3	483.3	8	742.9	1	351.8	2	453.9	2	391.6
		1.00%	10	2956.8	1	273.3	6	296.5	3	239.4	10	312.9	0	288.5	5	1471.0	2	448.0
		3.00%	10	4906.8	1	389	9	2119.9	4	243.2	17	1414.7	0	250.9	5	1220.2	2	440.2
		5.00%	14	3059.8	3	579.2	14	2807.3	8	477.9	27	7853.8	13	1055.7	5	5843.4	3	422.9
ACN Concentration of Loading Buffer (TFA with different concentrations of ACN in aqueous solution)		30%	0	213.8	0	259.4	0	227.1	0	273.0	0	322.5	0	333.8	0	393.2	0	383.0
		50%	0	207.3	0	271.2	0	223.5	0	276.0	0	209.0	1	306.3	0	335.3	0	329.9
		60%	0	204.3	0	275.1	0	229.4	1	263.1	0	194.7	4	388.3	0	334.8	0	339.5
		70%	2	345.5	2	410.9	3	225.5	11	473.2	0	233.6	4	486.7	0	400.4	5	861.2
		80%	14	2675.4	14	2701.2	14	865.6	17	1928.6	27	2294.7	19	3131.2	3	1835.3	5	4383.9
		90%	14	4531.9	14	2225.3	16	1868.3	17	1330.6	19	3621.0	13	1203.4	5	3669.2	5	912.2

Notes:Intensity represents the total intensity of three selected N-glycopeptides.
The intensity values of the same series are comparable, and the intensity values of the different series are not comparable.

Table S3 Summary of PGC different eluents and the number of enriched glycopeptides.

		Number of N-Glycopeptides							
		ASF		HRP		IgG		RNB	
		NO. of N-Glycopeptides	NO. of NonGlycopeptides	NO. of N-Glycopeptides	NO. of NonGlycopeptides	NO. of N-Glycopeptides	NO. of NonGlycopeptides	NO. of N-Glycopeptides	NO. of NonGlycopeptides
ACN Concentration of Washing Buffer (0.1% TFA with different concentration of ACN in aqueous solution)	10%	0	0	0	0	0	0	0	0
	20%	0	1	1	2	0	1	5	0
	30%	7	10	2	6	17	4	0	5
	40%	7	11	13	4	7	4	0	5
	50%	3	9	14	1	6	5	0	5
	80%	1	6	10	4	2	2	0	4

Table S4 Detailed information of the observed glycopeptides in ASF tryptic digest with boronic acid, ZIC-HILIC and PGC.

NO.	m/z	Glycan composition	Amino acid sequence	Boronic acid	ZIC-HILIC	PGC
A1	3018		VVHAVEVALATFNAESN#GSY LQLVEISR	Y	Y	
A2	3219	[HexNAc]	VVHAVEVALATFNAESN#GSY LQLVEISR		Y	
A3	3249	[Hex]5[HexNAc]4	LCPDCPLLAPLN#DSR	Y	Y	Y
A4	3377	[Hex]5[HexNAc]4	KLCPDCPLLAPLN#DSR		Y	Y
A5	3402	[Hex]5[HexNAc]4	NAESN#GSYLQLVEISR		Y	
A6	3452	[Hex]5[HexNAc]5	LCPDCPLLAPLN#DSR		Y	Y
A7	3580	[Hex]5[HexNAc]5	KLCPDCPLLAPLN#DSR	Y	Y	Y
A8	3605	[Hex]5[HexNAc]5	NAESN#GSYLQLVEISR	Y		Y
A9	3614	[Hex]6[HexNAc]5	LCPDCPLLAPLN#DSR	Y	Y	Y
A10	3742	[Hex]6[HexNAc]5	KLCPDCPLLAPLN#DSR	Y	Y	Y
A11	3767	[Hex]6[HexNAc]5	NAESN#GSYLQLVEISR	Y	Y	Y
A12	4016	[Hex]6[HexNAc]5	TFNAESN#GSYLQLVEISR	Y	Y	Y
A13	4639	[Hex]5[HexNAc]4	VVHAVEVALATFNAES#NGSY LQLVEISR	Y	Y	
A14	4842	[Hex]5[HexNAc]5	VVHAVEVALATFNAES#NGSY LQLVEISR	Y		
A15	5004	[Hex]6[HexNAc]5	VVHAVEVALATFNAESN#GSY LQLVEISR	Y	Y	
A16	5180	[Hex]5[HexNAc]4	RPTGEVYDIEIDTLETTCHV LDPTPLAN#CSVR		Y	Y
A17	5545	[Hex]6[HexNAc]5	RPTGEVYDIEIDTLETTCHV LDPTPLAN#CSVR	Y	Y	Y

Table S5 Detailed information of the observed glycopeptides in HRP tryptic digest with boronic acid, ZIC-HILIC-N and PGC.

NO.	m/z	Glycan composition	Amino acid sequence	Boronic acid	ZIC-HILIC	PGC
H1	1842	[Xyl][Hex]3[Fuc][HexNAc]2	NVGLN#R	Y	Y	Y
H2	2073	[Xyl][Hex]3[Fuc][HexNAc]2	PN#VSNIVR	Y		Y
H3	2276	[Xyl][Hex]2[Fuc][HexNAc]2	SILLDN#TTSFR			Y
H4	2321	[Hex]2[HexNAc]2	MGN#ITPLTGTQGQIR	Y		Y
H5	2532	[Fuc][HexNAc]	SFAN#STQTFFNAFVEAMDR		Y	
H6	2591	[Xyl][Hex]3[Fuc][HexNAc]2	PTLN#TTYLQTLR	Y	Y	Y
H7	2611	[Xyl][Hex]3[HexNAc]2	MGN#ITPLTGTQGQIR	Y	Y	Y
H8	2850	[Fuc][HexNAc]	GLIQSDQELFSSPN#ATDTIPLVR			Y
H9	3048	[Xyl][Hex]2[HexNAc]2	SFAN#STQTFFNAFVEAMDR	Y	Y	Y
H10	3087	[Xyl][Hex]3[Fuc][HexNAc]2	GLCPLNGN#LSALVDFDLR	Y	Y	Y
H11	3206	[Xyl][Hex]3[HexNAc]2	SFAN#STQTFFNAFVEAMDR		Y	Y
H12	3222	[Hex]3[Fuc][HexNAc]2	SFAN#STQTFFNAFVEAMDR			Y
H13	3323	[Xyl][Hex]3[Fuc][HexNAc]2	QLTPTFYDNSCPN#VSNIVR	Y	Y	Y
H14	3353	[Xyl][Hex]3[Fuc][HexNAc]2	SFAN#STQTFFNAFVEAMDR	Y	Y	Y
H15	3369	[Xyl][Hex]3[Fuc][HexNAc]2	SFAN#STQTFFNAFVEAM*DR	Y	Y	Y
H16	3508	[Xyl][Hex]2[Fuc][HexNAc]2	GLIQSDQELFSSPN#ATDTIPLVR			Y
H17	3525	[Xyl][Hex]3[HexNAc]2	GLIQSDQELFSSPN#ATDTIPLVR			Y
H18	3539	[Hex]3[Fuc][HexNAc]2	GLIQSDQELFSSPN#ATDTIPLVR			Y
H19	3606	[Xyl][Hex]3[Fuc][HexNAc]2	NQCRGLCPLNGN#LSALVDFDLR	Y	Y	Y
H20	3671	[Xyl][Hex]3[Fuc][HexNAc]2	GLIQSDQELFSSPN#ATDTIPLVR	Y	Y	Y
H21	3749	[Xyl][Hex]3[HexNAc]2	LHFHDCFVNGCDASILLDN#TTSFR		Y	Y
H22	3895	[Xyl][Hex]3[Fuc][HexNAc]2	LHFHDCFVNGCDASILLDN#TTSFR	Y	Y	Y
H23	4058	[Xyl][Hex]3[HexNAc]2	QLTPTFYDNSC(AAVESACPR)PN#VSNIVR-H2O		Y	Y
H24	4223	[Xyl][Hex]3[Fuc][HexNAc]2	QLTPTFYDNSC(AAVESACPR)PN#VSNIVR	Y	Y	Y
H25	4720	[Hex]3[Fuc][HexNAc]2 [Hex]3[Fuc][HexNAc]2	LYN#FSNTGLPDPTLN#TTYLQTLR	Y	Y	
H26	4837	[Xyl][Hex]3[Fuc][HexNAc]2 [Xyl][Hex]3[HexNAc]2	LYN#FSNTGLPDPTLN#TTYLQTLR	Y	Y	
H27	4983	[Xyl][Hex]3[Fuc][HexNAc]2 [Xyl][Hex]3[Fuc][HexNAc]2	LYN#FSNTGLPDPTLN#TTYLQTLR	Y	Y	Y

Table S6 Detailed information of the observed glycopeptides in IgG tryptic digest with boronic acid, ZIC-HILIC-N and PGC.

NO.	m/z	Glycan composition	Amino acid sequence	Boronic acid	ZIC-HILIC	PGC
I1	2210	[Hex]4[HexNAc]2	EEQFNSTFR	Y		Y
I2	2226	[Hex]3[HexNAc]2[Fuc]1	EEQYNSTYR	Y		Y
I3	2252	[Hex]3[HexNAc]3	EEQFNSTFR			Y
I4	2242	[Hex]4[HexNAc]2	EEQYNSTYR	Y		Y
I5	2397	[Hex]3[HexNAc]3[Fuc]1	EEQFNSTFR		Y	
I6	2430	[Hex]3[HexNAc]3[Fuc]1	EEQYNSTYR		Y	Y
I7	2455	[Hex]3[HexNAc]4	EEQFNSTFR		Y	
I8	2487	[Hex]3[HexNAc]4	EEQYNSTYR		Y	Y
I9	2560	[Hex]4[HexNAc]3[Fuc]1	EEQFNSTFR		Y	
I10	2567	[Hex]6[HexNAc]2	EEQYNSTYR			Y
I11	2592	[Hex]4[HexNAc]3[Fuc]1	EEQYNSTYR		Y	
I12	2602	[Hex]3[HexNAc]4[Fuc]1	EEQFNSTFR		Y	Y
I13	2608	[Hex]5[HexNAc]3	EEQFNSTFR		Y	
I14	2617	[Hex]4[HexNAc]4	EEQFNSTFR		Y	
I15	2633	[Hex]3[HexNAc]4[Fuc]1	EEQYNSTYR		Y	Y
I16	2754	[Hex]5[HexNAc]3[Fuc]1	EEQYNSTYR			Y
I17	2763	[Hex]4[HexNAc]4[Fuc]1	EEQFNSTFR		Y	Y
I18	2779	[Hex]5[HexNAc]4	EEQFNSTFR		Y	
I19	2795	[Hex]4[HexNAc]4[Fuc]1	EEQYNSTYR		Y	Y
I20	2804	[Hex]3[HexNAc]5[Fuc]1	EEQFNSTFR		Y	Y
I21	2811	[Hex]5[HexNAc]4	EEQYNSTYR		Y	
I22	2820	[Hex]4[HexNAc]5	EEQFNSTFR			Y
I23	2836	[Hex]3[HexNAc]5[Fuc]1	EEQYNSTYR		Y	Y
I24	2883	[Hex]4[HexNAc]3[Fuc]1[NeuAc]1	EEQYNSTYR		Y	
I25	2926	[Hex]5[HexNAc]4[Fuc]1	EEQFNSTFR		Y	Y
I26	2940	[Hex]4[HexNAc]4[NeuAc]1	EEQYNSTYR			Y
I27	2958	[Hex]5[HexNAc]4[Fuc]1	EEQYNSTYR		Y	Y
I28	2966	[Hex]4[HexNAc]5[Fuc]1	EEQFNSTFR		Y	Y
I29	2999	[Hex]4[HexNAc]5[Fuc]1	EEQYNSTYR		Y	Y
I30	3086	[Hex]4[HexNAc]4[Fuc]1[NeuAc]1	EEQYNSTYR		Y	Y
I31	3128	[Hex]5[HexNAc]5[Fuc]1	EEQFNSTFR		Y	
I32	3160	[Hex]5[HexNAc]5[Fuc]1	EEQYNSTYR		Y	
I33	3248	[Hex]5[HexNAc]4[NeuAc]1[Fuc]1	EEQYNSTYR		Y	Y
I34	3257	[Hex]4[HexNAc]5[Fuc]1[NeuAc]1	EEQFNSTFR			Y
I35	3451	[Hex]5[HexNAc]5[NeuAc]1[Fuc]1	EEQYNSTYR		Y	
I36	3613	[Hex]6[HexNAc]5[NeuAc]1[Fuc]1	EEQYNSTYR		Y	
I37	3726	[Hex]6[HexNAc]5[NeuAc]2	EEQFNSTFR			Y

Table S7 Detailed information of the observed glycopeptides in RNB digest with boronic acid, ZIC-HILIC-N and PGC.

NO.	m/z	Glycan composition	Amino acid sequence	Boronic acid	ZIC-HILIC	PGC
R1	1935	[Hex]5[HexNAc]2	SRN#LTK	Y	Y	Y
R2	2097	[Hex]6[HexNAc]2	SRN#LTK	Y	Y	Y
R3	2259	[Hex]7[HexNAc]2	SRN#LTK	Y	Y	Y
R4	2421	[Hex]8[HexNAc]2	SRN#LTK	Y	Y	Y
R5	2583	[Hex]9[HexNAc]2	SRN#LTK	Y	Y	Y

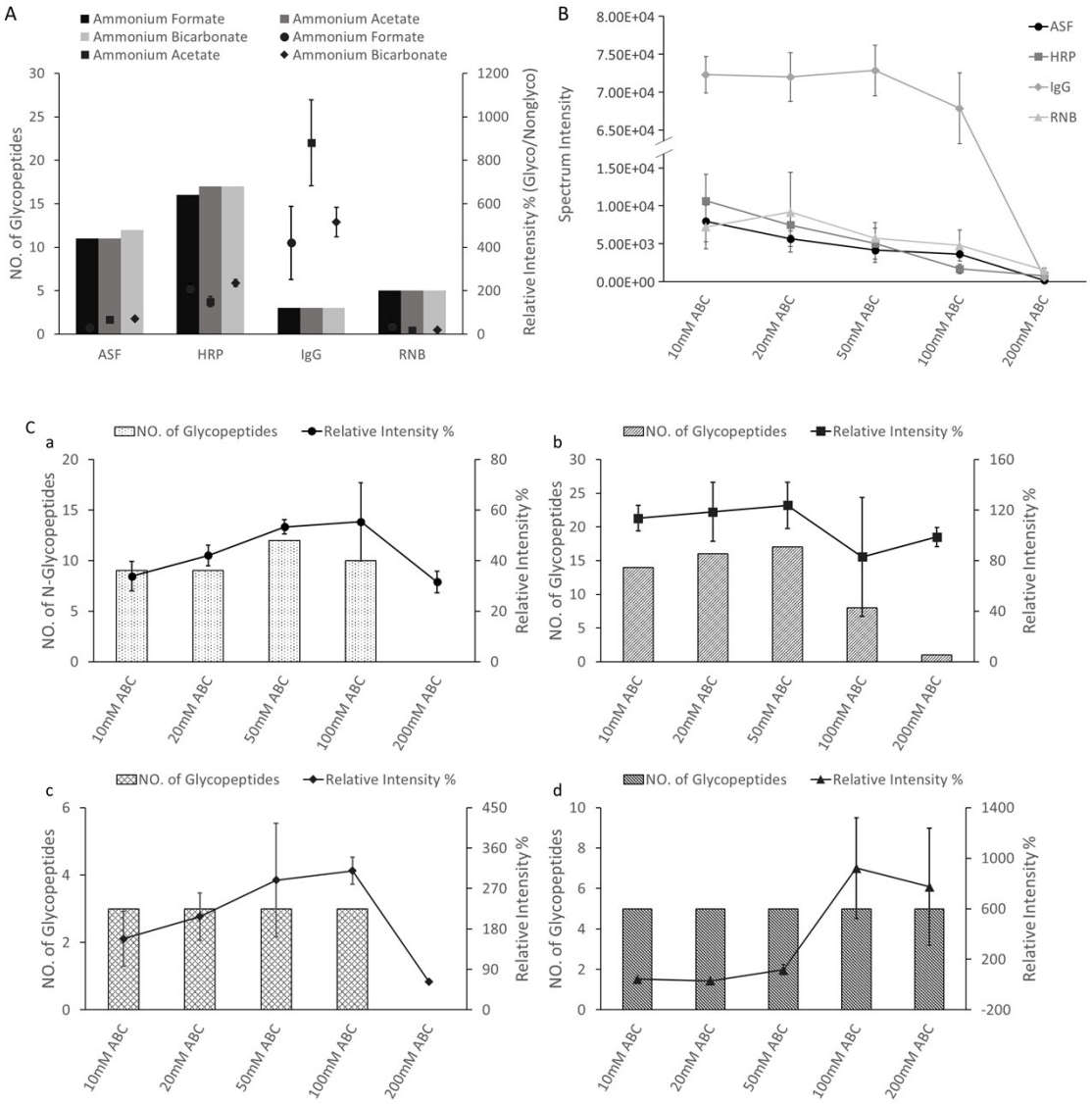

Fig. S1 The effects of buffer types and buffer concentration on boronic acid enrichment method. (A) The number of identified N-glycopeptides and the relative intensity when $HCOONH_4$, CH_3COONH_4 and NH_4HCO_3 were used as binding buffer. (B) The trend of spectrum total intensity when the binding buffer concentration was varied. (C) The number of identified N-glycopeptides and the relative intensity when changing the binding buffer concentration. (a) glycopeptides drived from ASF, (b) glycopeptides drived from HRP, (c) glycopeptides drived from IgG, (d) glycopeptides drived from RNB. (Relative intensity = sum of glycopeptides intensity / sum of nonglycopeptides intensity)

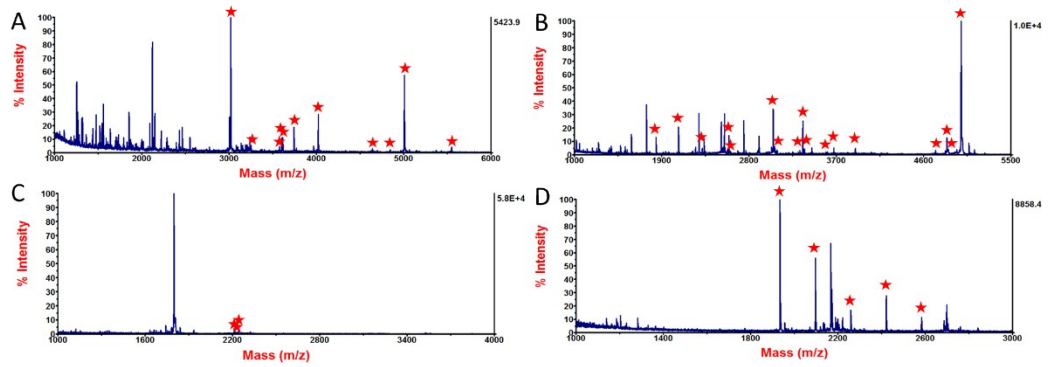

Fig. S2 MALDI-TOF-MS spectra of the ASF, HRP, IgG and RNB digests under the optimal experimental conditions: (a) glycopeptides drived from ASF, (b) glycopeptides drived from HRP, (c) glycopeptides drived from IgG, (d) glycopeptides drived from RNB. Glycopeptides peaks are marked with the red star.

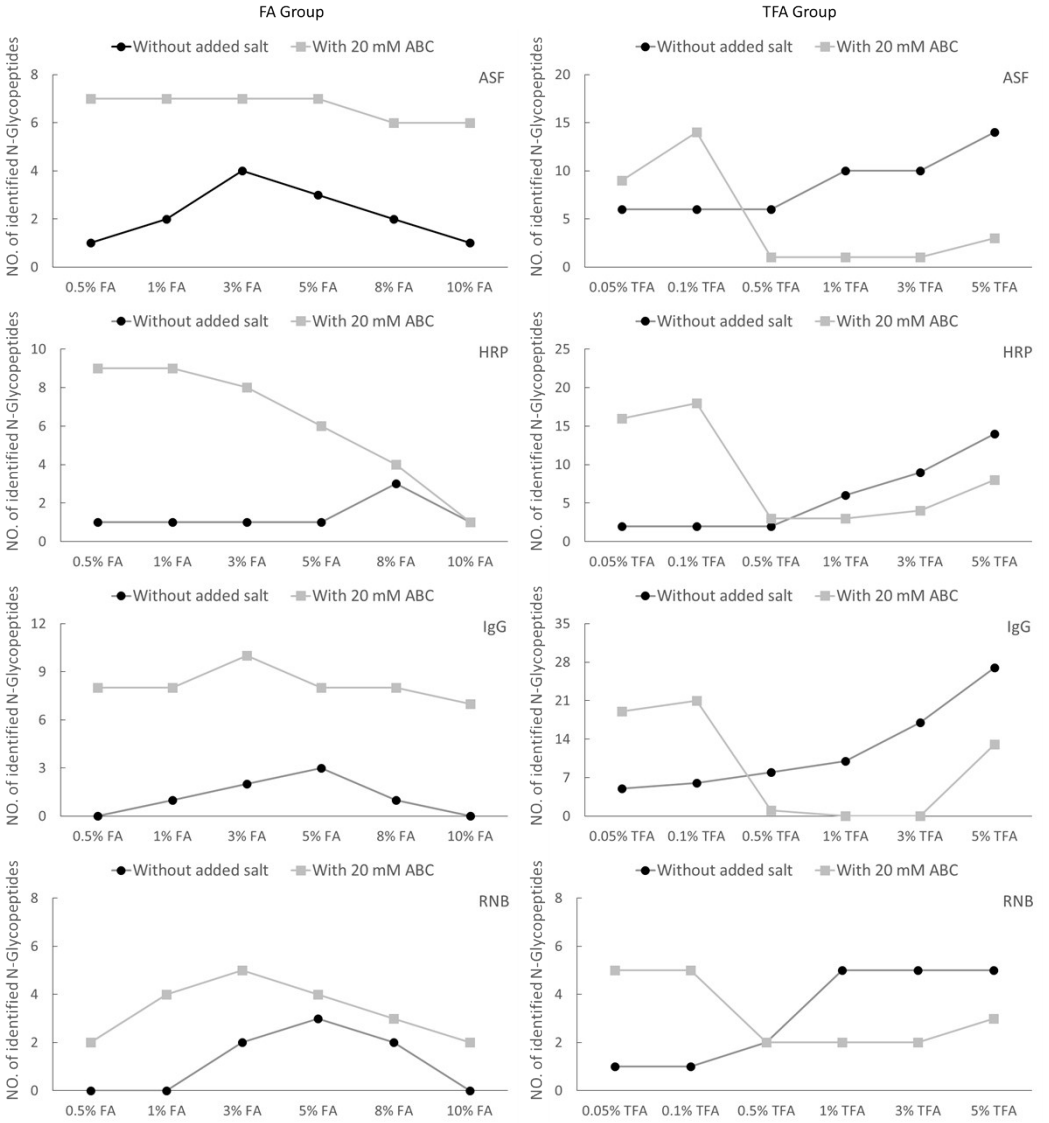

Fig. S3 The effects of the ion-pairing reagent (TFA) using FA as acid control and the presence of salt in loading buffer on ZIC-HILIC enrichment method. The numbers of identified N-glycopeptides by

ZIC-HILIC with FA group and TFA Group were counted. The dark dot represented the solution without added salt, the light square represented the solution with 20mM NH_4HCO_3.

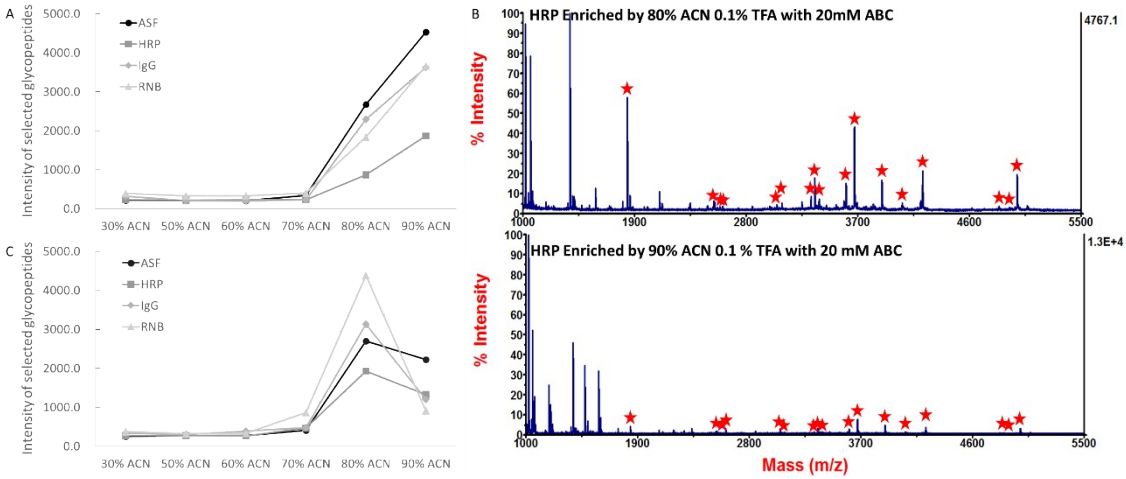

Fig. S4 The effect of different concentration ACN on ZIC-HILIC enrichment by using TFA solution. (A) without added salt group (B) the mass spectrums of HRP enriched by 0.1% TFA with 20mM ABC in 80% ACN solution and 90% ACN solution (C) with 20 mM ABC group.

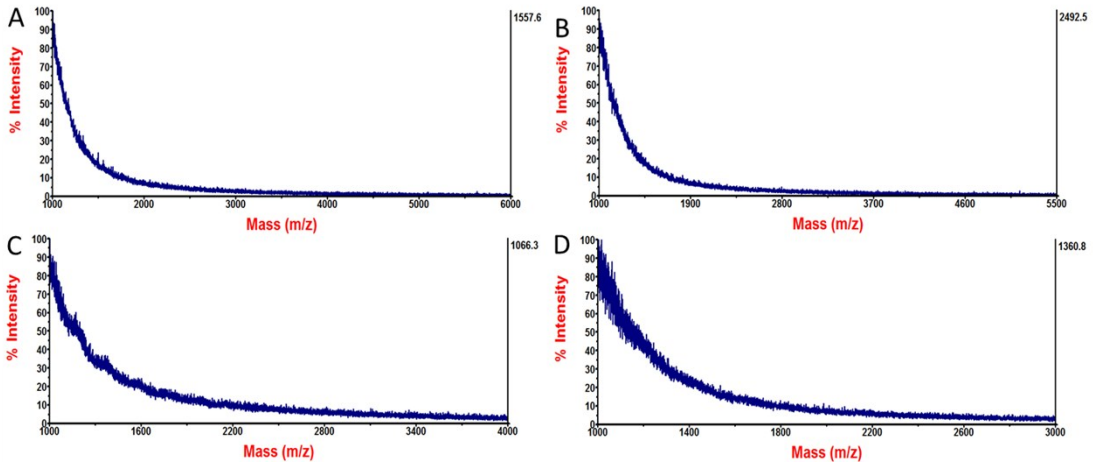

Fig. S5 MALDI-TOF-MS spectrums of loading buffer after loaded three times. (A) ASF, (B) HRP, (C) IgG, (D) RNB.

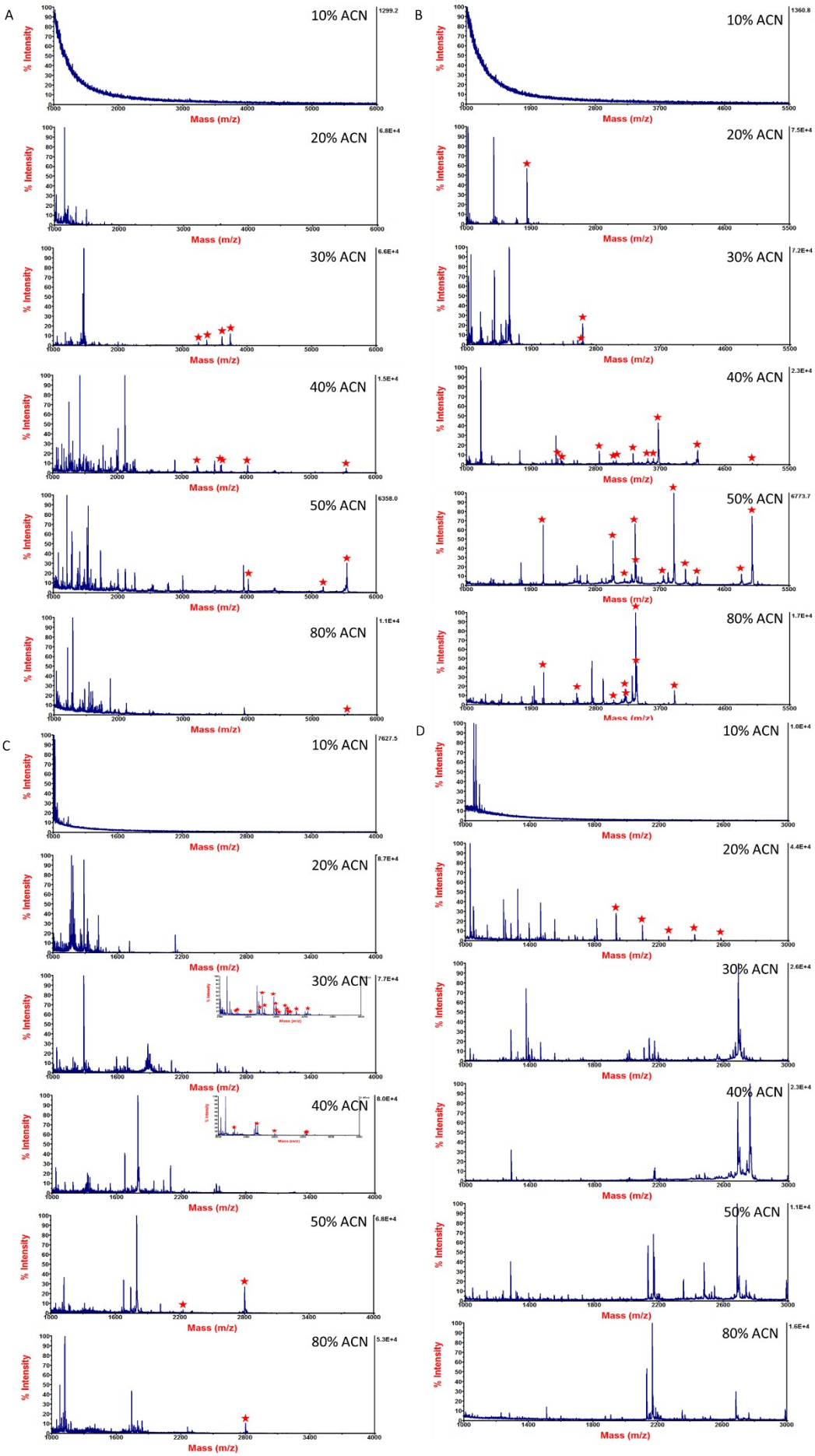
A
10% ACN
20% ACN
30% ACN
40% ACN
50% ACN
80% ACN
B
10% ACN
20% ACN
30% ACN
40% ACN
50% ACN
80% ACN
C
10% ACN
20% ACN
30% ACN
40% ACN
50% ACN
80% ACN
D
10% ACN
20% ACN
30% ACN
40% ACN
50% ACN
80% ACN
% Intensity
Mass (m/z)

Fig. S6 MALDI-TOF-MS spectrums obtained by using PGC to separate tryptic products from four standard proteins. The content of ACN in the eluent was varied to obtain the MS spectrums. (A) glycopeptides drived from ASF, (B) glycopeptides drived from HRP, (C) glycopeptides drived from IgG, (D) glycopeptides drived from RNB. ('★' indicated the N-glycopeptides.)

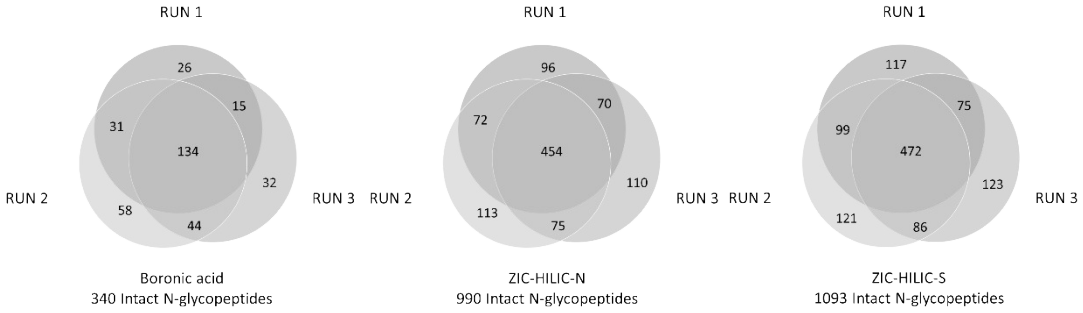

Fig. S7 Three technical replicates of boronic acid, ZIC-HILIC-N and ZIC-HILIC-S.

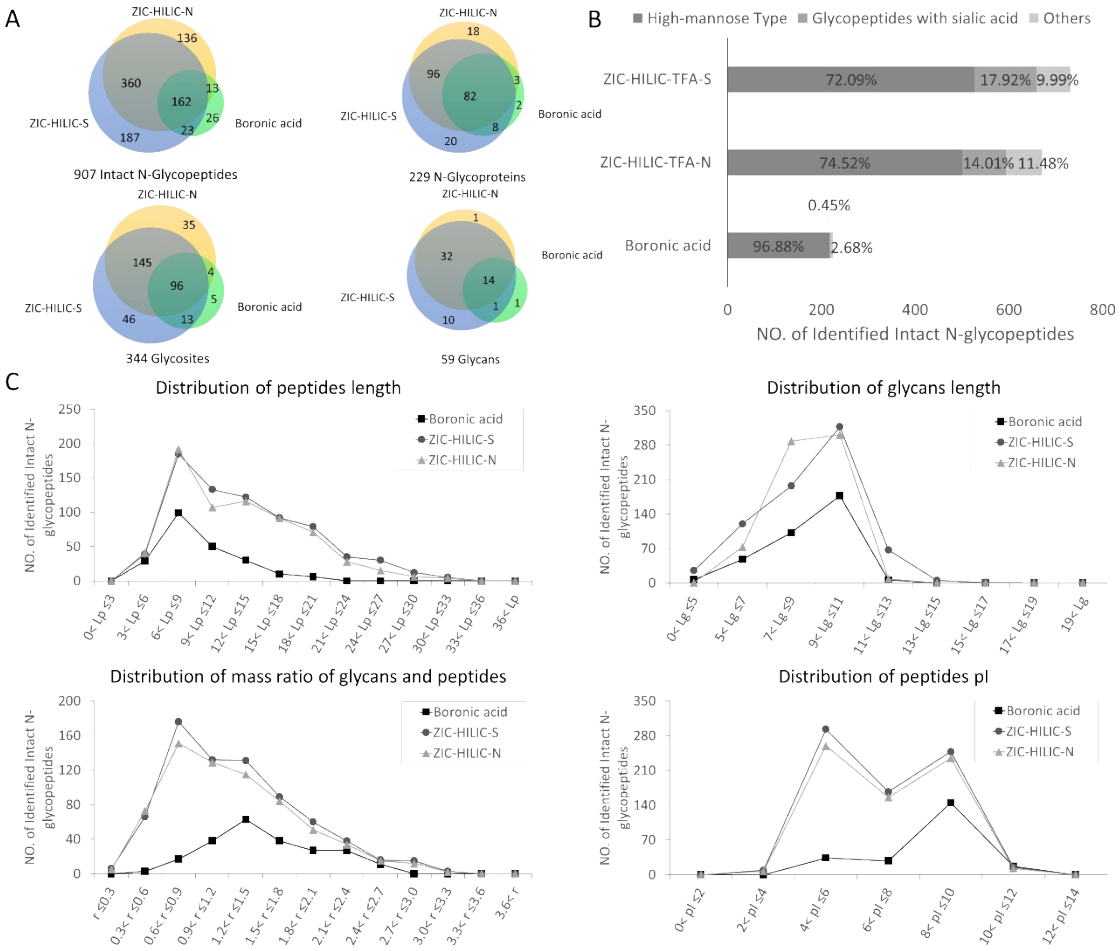

Fig. S8 Intact N-glycopeptides enrichment methods comparison with glycopeptides identified in more than 2 of the 3 replicates in each method. (A) Venn diagram of intact N-glycopeptides, N-glycoproteins, N- glycosites and glycans enriched with different methods were shown. (B) The proportion of different glycan types in intact glycopeptides identified by Boronic acid, ZIC-HILIC-N

and ZIC-HILIC-S. (C) The distributions of the peptides length (Lp), the length of glycans, the mass ratio of glycans versus peptides and the pI distribution of peptides enriched with Boronic acid, ZIC-HILIC-N and ZIC-HILIC-S.

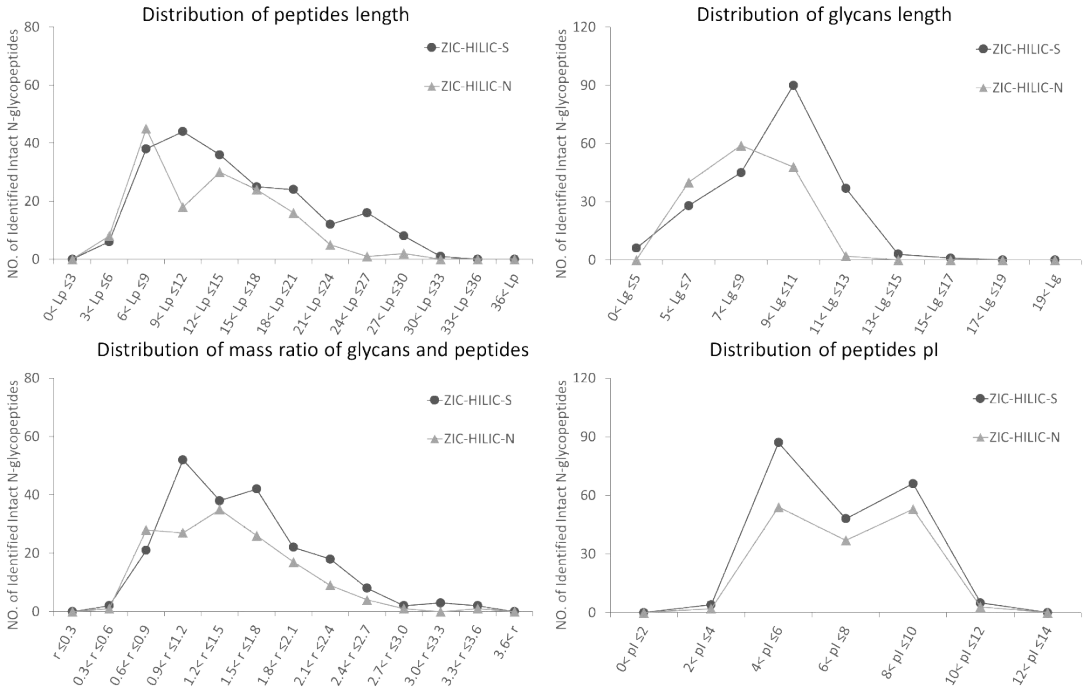

Fig. S9 The distributions of the peptides length (Lp), the length of glycans, the mass ratio of glycans versus peptides and the pI distribution of unique glycopeptides identified by ZIC-HILIC-N and ZIC-HILIC-S, respectively.