Name	Mr (Da)	Sequence
PP1	2093.861	TASDTDSS**Y**#AIPTAGMSPSR
PP2	1758.794	SVENLPEAGI**T**#HEQR
PP3	1137.529	N**S**#VEQGRRL
PP4	1343.587	APPDNLPSPGG**S**#R
PP5	1302.549	LIEDNE**Y**#TAR
NP6	1263.621	APPDNLPSPGGSR
PP7	1313.599	R**S**#DGGHTVLHR
PP8	1931.83	ENIMR**S**#ENSESQLTSK
PP9	2229.981	QLGEPEK**S**#QDSSPVL**S**#ELK
PP10	2309.947	QLGEPEK**S**#QD**S**#SPVL**S**#ELK
PP11	963.3587	SV**S**#DYEGK
GluFib	1569.67	EGVNDNEEGFFSAR

Table 1.

Action		Description
ReagentAVial=	R1	Definition of vials containing transport (0.1% TFA) liquid or TFE as wash liquid. Here, R1 = 0.1%TFA; R2 = TFE.
ReagentBVial=	R2	
ReagentCVial=	R3	
ReagentDVial=	R4	
PrepVial=	R1	
UdpInjectValve	Position=Inject	WPS Inject valve is switched into inject position.
UdpSyringeValve	Position=Needle	The needle valve switches injection needle online with the syringe.
UdpDraw	From=ReagentAVial, Volume=16.000, SyringeSpeed=400, SampleHeight=4.000	Injection needle is filled with transport liquid (0.1% TFA).
UdpMixWait	Duration=2	The injection procedure is halted for stabilization of the fluidic in the injection needle.
UdpDraw	From=ReagentAVial, Volume=0.000, SyringeSpeed=GlobalSpeed, SampleHeight=4.000	Injection needle remains in the vial containing transport liquid.
UdpInjectValve	Position=Load	Injection valve switches: sample is transferred into the sample loop
UdpDraw	From=SampleVial, Volume=Volume, SyringeSpeed=400, SampleHeight=7.000	Sample is aspired into the injection needle and sample loop.
UdpMixWait	Duration=2	The injection procedure is halted for stabilization of the fluidic in

		the injection needle.
UdpDraw	From=SampleVial, Volume=0.000, SyringeSpeed=GlobalSpeed, SampleHeight=7.000	Injection needle remains in the sample vial.
UdpDraw	From=ReagentAVial, Volume=20.000, SyringeSpeed=400, SampleHeight=4.000	Sample in injection needle is transferred into the sample loop
UdpMixWait	Duration=2	The injection procedure is halted for stabilization of the fluidic in the injection needle.
UdpDraw	From=ReagentAVial, Volume=0.000, SyringeSpeed=GlobalSpeed, SampleHeight=4.000	Injection needle remains in the sample vial.
UdpInjectValve	Position=Inject	Sample Injection.
UdpInjectMarker		Data system obtains start signal from autosampler.
UdpMixNeedleWash	Volume=300.000	Injection needle is washed with default wash solvent (20% iso-propanol).
UdpMixWait	Duration=1200	Autosampler waits for 20 minutes until the TFE wash procedure starts.
UdpInjectValve	Position=Load	Injection Valve is switched to load position..

UdpDraw	From=ReagentBVial, Volume=100.000, SyringeSpeed=6000, SampleHeight=4.000	100 µl TFE is aspired into the sample loop.
UdpInjectValve	Position=Inject	TFE from the sample loop is injected onto the trap column (either TC 1 or TC2).
UdpMixWait	Duration=60	Autosampler waits for 1 minute to start the injection needle wash.
UdpMixNeedleWash	Volume=300.000	Injection needle is washed with default wash solvent (20% iso-propanol).

Table 2.

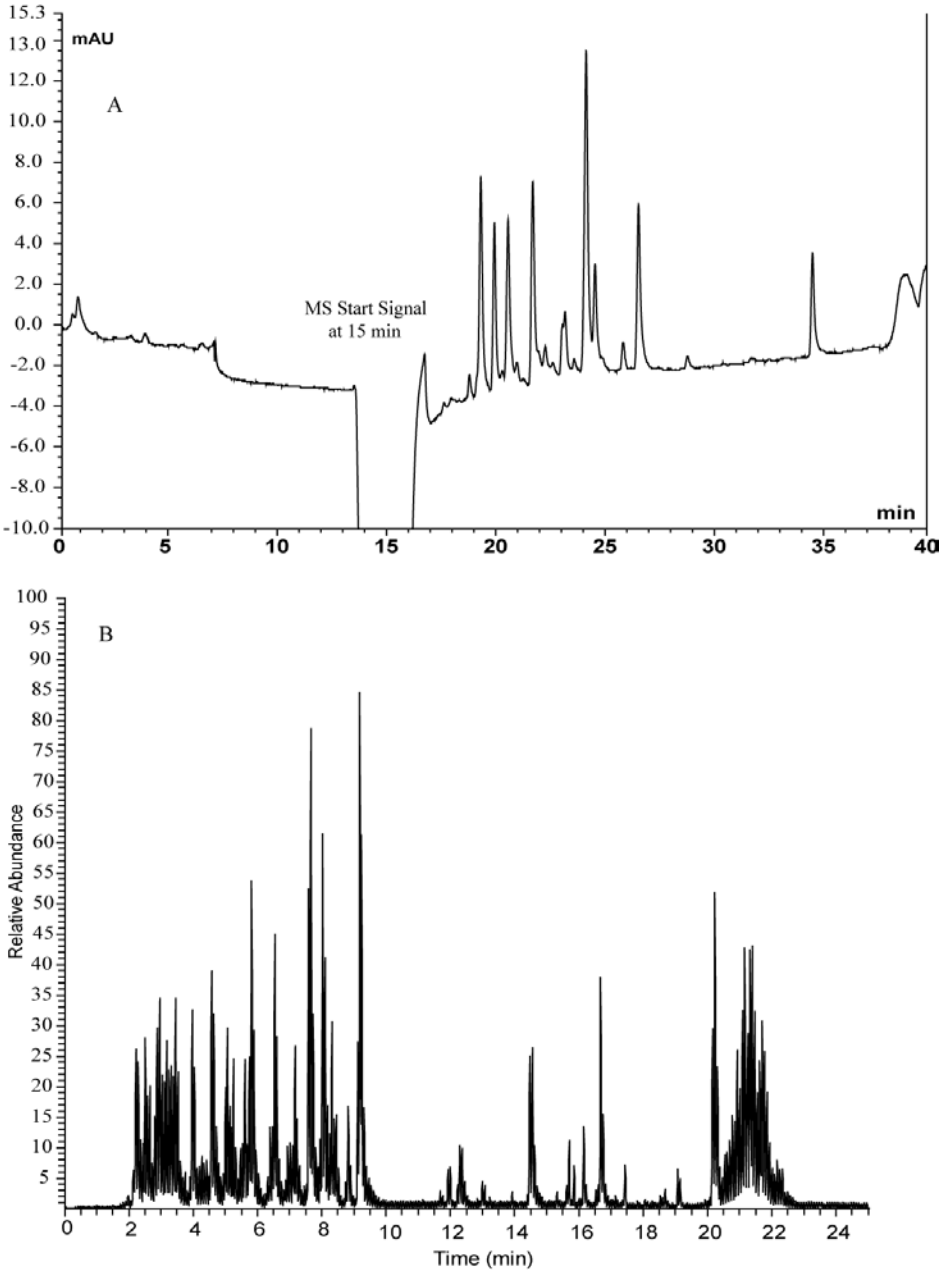
mAU
A
MS Start Signal
at 15 min
min
B
Relative Abundance
Time (min)

Figure 1.

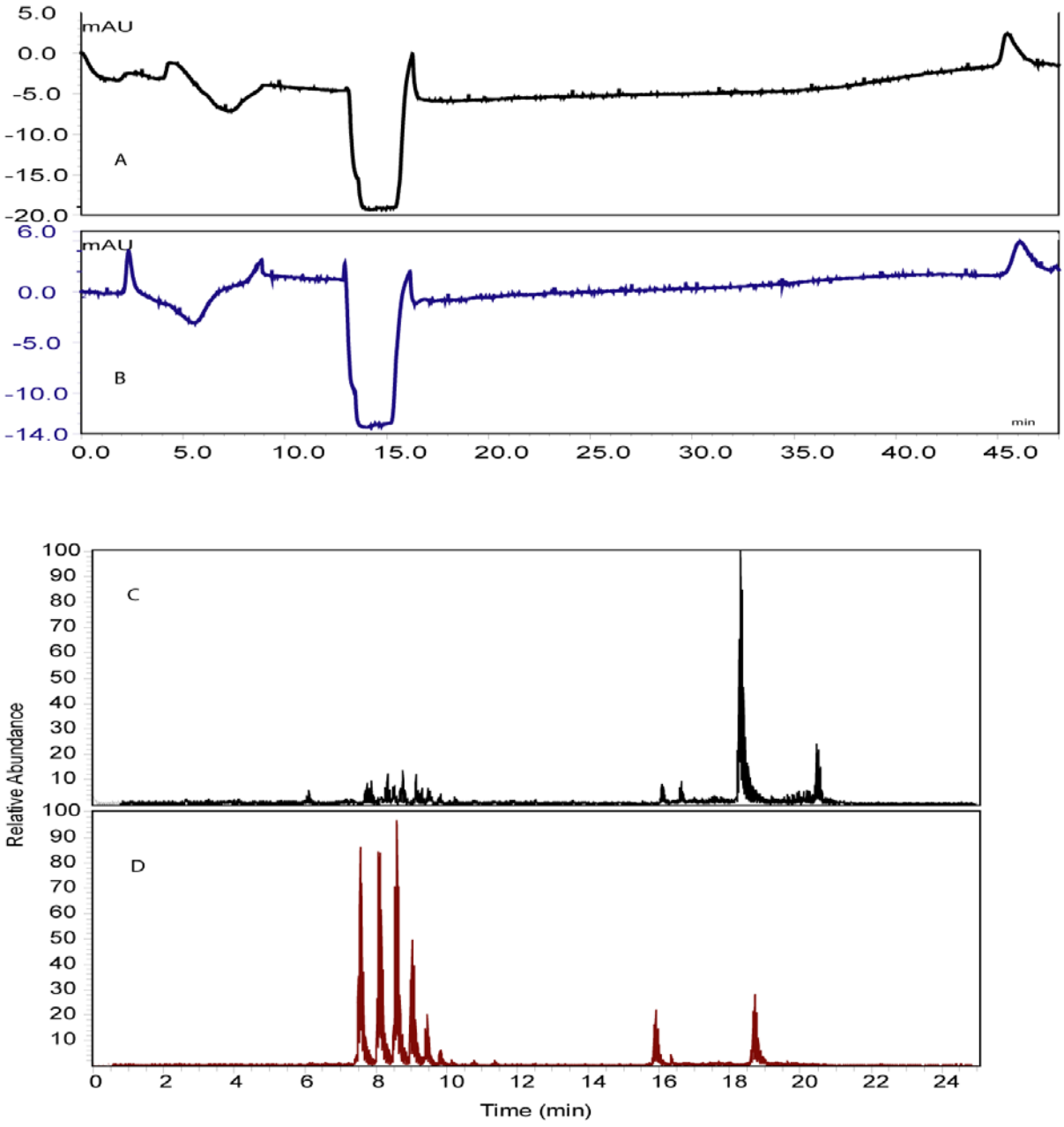
5.0
mAU
0.0
-5.0
-10.0
-15.0
-20.0
A
6.0
mAU
0.0
-5.0
-10.0
-14.0
B
min
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
100
90
80
70
60
50
40
30
20
10
C
Relative Abundance
D
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (min)

Figure 2

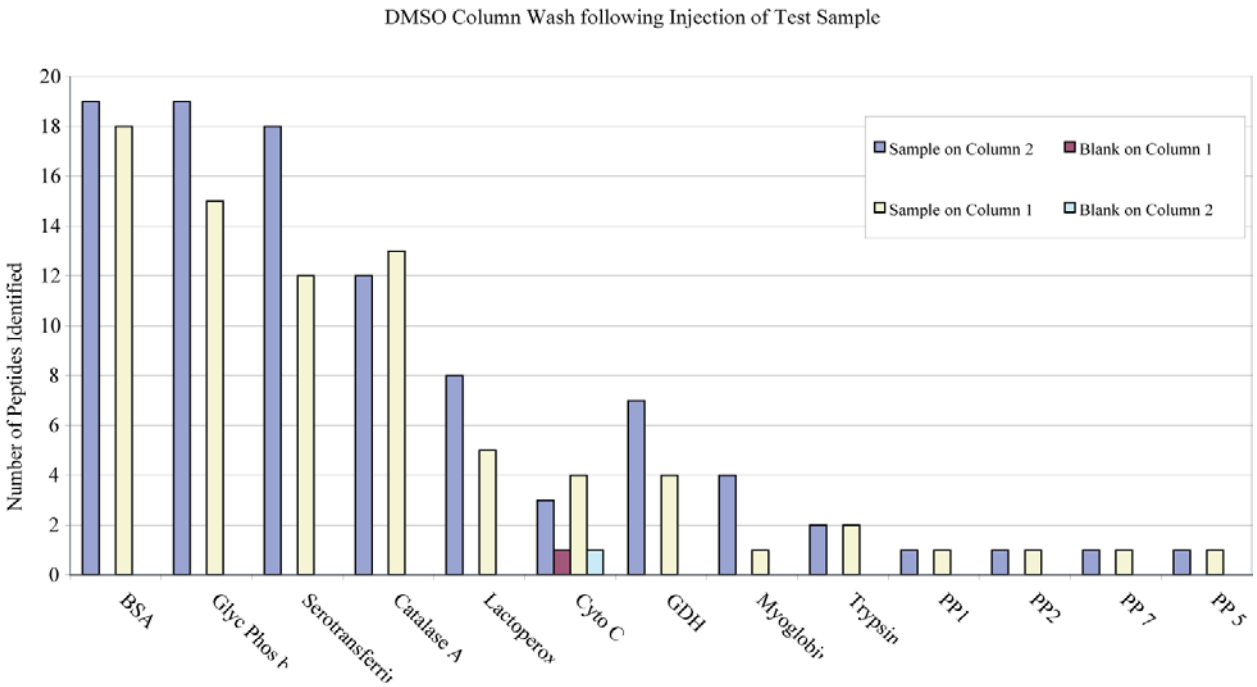

Figure 3

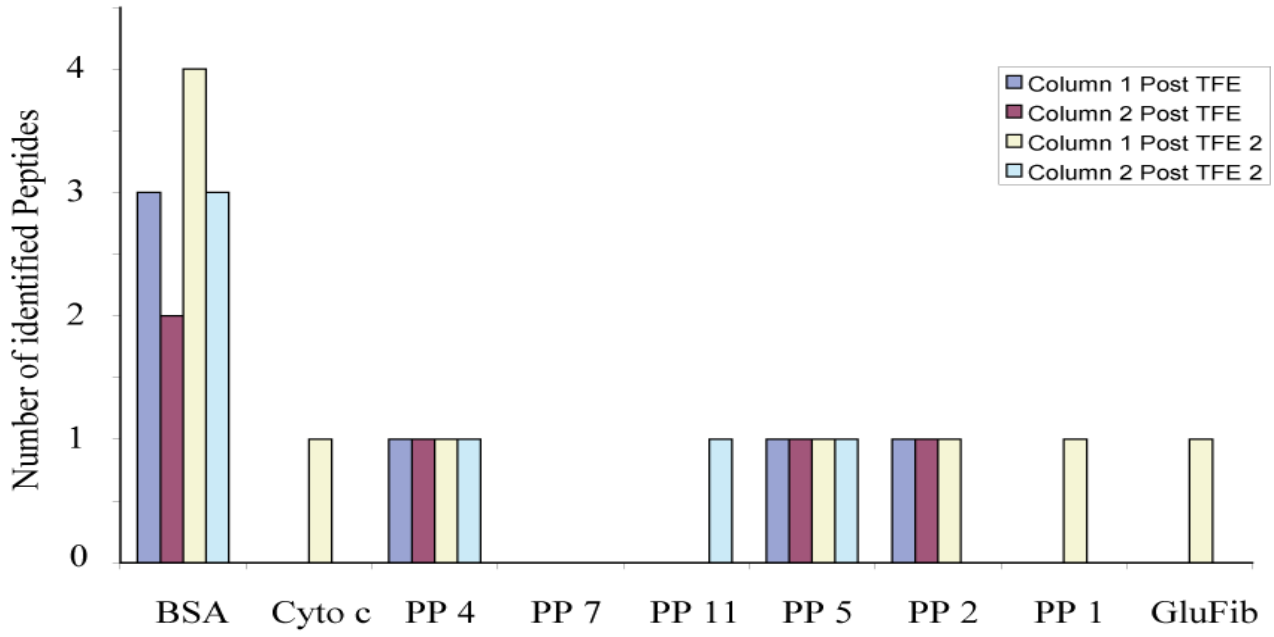

Figure 4

Legends to Supplemental Tables and Figures

Table 1. Sequences and molecular masses of the ten synthetic phosphopeptides and two non-phosphorylated peptides used for the performance test of the separation system. Phosphorylated residues are followed by a # symbol.

Figure 1. HPLC separation and analysis of the test sample. (A) the UV chromatogram, and (B) the BPC obtained after injecting 500 fmol (10 µl) of the test sample in the HPLC system. Salts, detergents and other impurities are flushed off from the trap column before it is switched online with the separation column. Since no separation on the column is performed during the first 10 min, the mass spectrometric data acquisition was started at 15 min after switching the trap column online with the separation column.

Figure 2. Carryover can be missed by UV chromatography. Blank (0.1% TFA) injections following injection of test sample and flushing of the injection path with 20% i-PrOH resulted in blank UV chromatograms at 214 nm on both column 1 (A) and column 2 (B). However, BPCs obtained on the MS for column 1 (C) and column 2 (D) clearly show that a significant amount of carryover is present in the separation system.

Figure 3. Effects of flushing the HPLC system with DMSO. The graph shows the number of peptides per protein identified during a sample run and after a single flushing of the injection path and the transfer tubing between the sample loop and the trapping column with 100 µl DMSO. This procedure yielded a clean chromatographic system, with only one peptide from cytochrome c identified in blank runs on both columns. However, the use of DMSO induced serious damage to tubing and injection valves and its use was abandoned.

Figure 4. Importance for peptide detection on column re-equilibration after flushing with TFE. The graph shows that the number of peptides identified by MS dropped significantly between identical injections of sample when the column was flushed with TFE but not properly equilibrated thereafter. Here, the column performance recovered only after 4 consecutive injections. In order to avoid this, and completely remove TFE from the separation system after flushing, an acetonitrile gradient is applied before conditioning with aqueous phase.