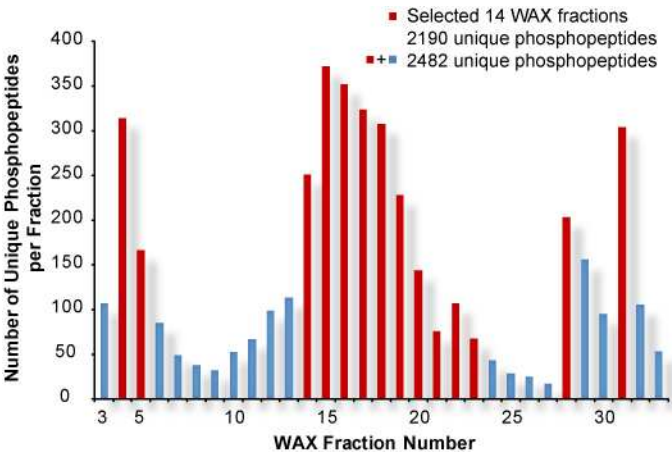fraction number. The blue bars represent the results by the use of a 30 minute gradient and the
red bars represent the results by the use of a 140 minute gradient.

Supplementary Material

**Supplementary Figure S1. Number of unique phosphopeptides identified in WAX fractions
resulting from the separation of a SCX phosphofraction**. The number of unique
phosphopeptides identified per fraction is plotted versus the fraction number. The red bars
represent 14 WAX fractions which contain over 85% of all identified phosphopeptides.

21
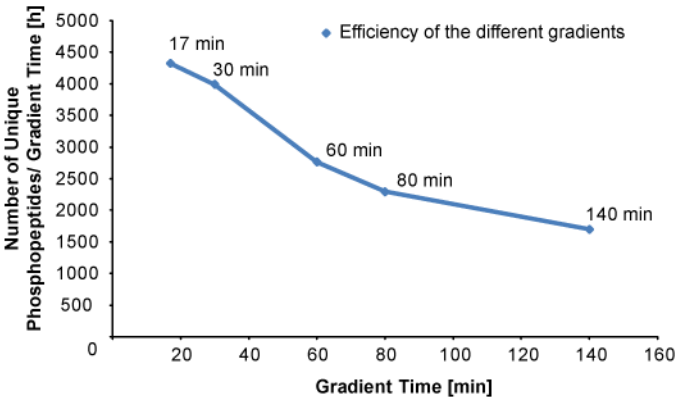
Supplementary Figure S2. Number of unique phosphopeptides identified per hour gradient time plotted against the gradient time for each of the RP-LC-MS gradients revealing that shorter gradients (i.e. 30 min.) are more efficient.
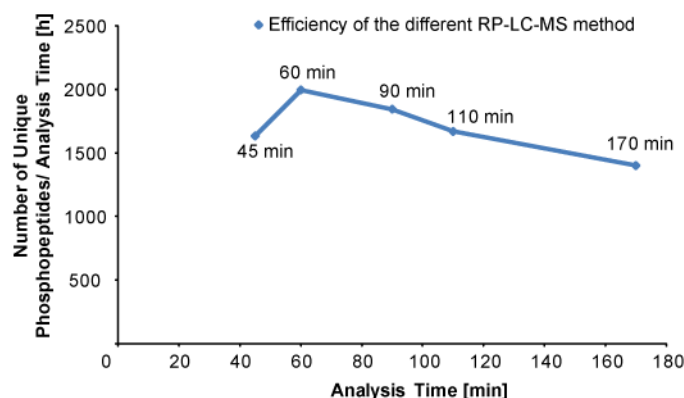
Supplementary Figure S3. Efficiency expressed as the number of unique phosphopeptides identified per hour analysis time plotted against the analysis time for each of the LC MS analyses.

total gradient time [min]	length gradient step 1 [min]	end concentration gradient step 1 [%B]	length gradient step 2 [min]	end concentration gradient step 2 [%B]	percentage of SCX fraction analyzed	number of unique phosphopeptides
17	17	40	-	-	0.33%	1223
30	30	40	-	-	0.67%	1993*
60	60	32	-	-	1.00%	2762
80	57	26	23	50	1.20%	3054
140	107	25	33	50	2.00%	3960*
90 (3x30)	30	40	-	-	3x0.67%	2669** (1944; 1925; 2110)
420 (3x140)	107	25	33	50	3x2.00%	5090** (3849; 4045; 3986)

Supplementary Table 1. Overview of experimental parameters. Indicated are the different used LC-MS gradients, the % of the SCX fraction used for the analysis and the number of unique phosphopeptides identified by the use of the described gradient. *The number of unique phosphopeptides for the 30 min and 140 min gradient are the average of the three measurements. **The number identified with the 90 (3x30) min and 420 (3x140) min represent the result of the combined dataset of the three individual analysis and the individual results are reported in brackets.

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Supplementary Table 2. An excel table containing the unique phosphopeptides identified by each RP-LC gradient and combination of WAX and each RP-LC gradient.