The Application of Porous Graphitic Carbon to Proteomic Studies

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An investigation of Porous Graphitic Carbon (PGC) as a stationary phase for the analysis of proteomic samples in a two-dimensional liquid chromatography (2D-LC) workflow is presented. A selectivity study performed on a standard mixture containing 15 peptides shows that the elution order obtained using PGC deviates significantly from conventional reversed phase selectivity. Furthermore PGC has been shown to retain and resolve small polar peptides otherwise unretained on standard reversed phase C18 stationary phases. The separation mechanism orthogonality compared to C18 combined with greater retentivity for polar analytes makes the use of PGC in 2 dimensional separations ideal. An analysis of a whole cell lysate by PGC prefractionation has been shown to result in an increase of nearly 6000 unique peptides compared with SCX as the first dimension separation of a 2D-LC-MS/MS experiment.

Introduction

Sample complexity is one of the biggest challenges in proteomics, as the original sample may contain many thousands of different proteins which after digestion will be converted to tens of thousands of peptides. The accurate separation, detection and identification of the peptides present in the sample are features of paramount importance to optimise in any proteomic workflow. Researchers often resort to multi-dimensional workflows (Figure 1) where LC analysis on two or more orthogonal LC phases is carried out to achieve greater resolution and therefore higher peptide identification numbers. The separation methods traditionally employed include Strong Cation Exchange (SCX), Hydrophilic Interaction Liquid Chromatography (HILIC) and high pH reversed phase HPLC [1-6] in the first dimension. Almost all approaches employ a reversed phase column in the second dimension. Each of these methods has advantages and disadvantages, which are summarised in Table 1.

The most popular combination of 2D-LC in proteomics is that of SCX-RP due to the high orthogonality that this combination of separation modes offers: separation in the first dimension is by charge and in the second dimension by reversed phase. While this has been used with much success its major disadvantage is the elution of the analytes from the first dimension in a high salt buffer which is incompatible with MS analysis, commonly performed online from the second dimension. This issue is overcome through trapping the peptides onto a C18 trap column, flowing through a suitable wash solvent through the column prior to transferring the peptides onto the analytical column. In a workflow which is already highly time consuming this additional step for each fraction adds significant time which although necessary is not value-added.

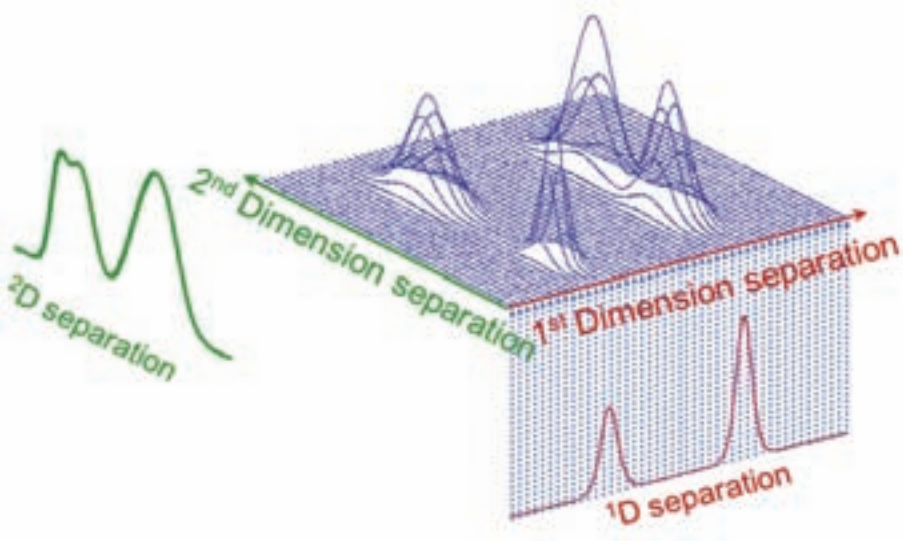

Figure 1: Illustration of the increase in resolution achievable through an offline two dimensional orthogonal separation. Fractions are collected at regular time intervals during the first separation (red chromatogram) and then each of these are analysed in a second orthogonal separation (blue chromatograms). If the sample was not separated on the first dimension but only on the second dimension phase the chromatography would be as shown in green. Complete resolution of four components is only possible through the two dimensional approach in this example.

For many of the common 2D-LC combinations, the orthogonality of these separation methods have been shown [3], however Porous Graphitic Carbon (PGC) has so far been largely unexplored in this workflow, although it is known that there are selectivity differences between PGC and conventional C18-selectivity stationary phases [7-9]. PGC offers an alternative mechanism of interaction with the analytes, driven predominantly by induced dipole formed on the graphite surface [7-9]; this feature is known to result in increased retention of polar species. Furthermore, PGC has been shown to result in shape selectivity and separation of positional isomers [7-9].

These characteristics suggest potential orthogonal selectivity for the analysis of complex peptide mixtures.

In this study the orthogonality of PGC to C18 selectivity is compared for peptide analysis, including variation in C18 selectivity between different C18 phases. Using a whole cell lysate the performance of SCX-RP and PGC-RP were compared in a 2D-LC proteomics workflow to study the relative performance of the two combinations and quantify which provides the greatest number of unique peptides under similar conditions for this complex sample [10].

	SCX + RP	HILIC + RP	RP (High pH) + RP (Low pH)	RP + RP	PGC + RP
PROS	Established technique High orthogonality between phases	High orthogonality between phases More hydrophilic fragments retained	Higher resolution than SCX + RP	Established technique	High orthogonality between phases More hydrophilic fragments retained
CONS	Extensive sample preparation between analyses Peptide clustering according to charge	Long equilibration of HILIC phases Some peptide solubility is limited in high organic mobile phases	Limited availability of high-pH stable silica phases	Poor orthogonality	Strong retention of hydrophobic fragments Sensitive to ion pairing and modifier changes

Table 1: Advantages and disadvantages of different 2D-LC combinations for proteomics analyses

Selectivity Study

Evaluation of C18 selectivity variation between different C18-selectivity phases (EASY column 10cm x 75µm, 3µm particle; Hypersil GOLD KAPPA 10cm x 75µm, 5µm particle; Acclaim PepMap C18 15cm x 75µm, 3µm particle) and on PGC (Hypercarb KAPPA 10cm x 75µm, 5µm particle) was measured using the Pierce 15 peptide retention time calibration standard. The sample was loaded without further treatment in 1µL aliquots on a C18 trapping column. Peptides were separated using a linear gradient of water/acetonitrile + 0.1% formic acid at a flow rate of 350nLmin-1 (20 minute gradient on the C18 phases and 50 minute gradient on PGC).

Evaluation of variation between C18 selectivity and PGC on a protein digest containing phosphopeptides was carried out using a sample of digested enolase. Peptides were separated over a linear gradient of water/acetonitrile + 0.1% formic acid at a flow rate of 350nLmin-1 (30 minute gradient on the C18 phases and 60 minute gradient on PGC).

SCX vs PGC Study

The two-dimensional separation of a complex peptide mixture is represented schematically in Figure 2.

1st Dimension: Six 100 µg aliquots of enzymatically digested (trypsin) whole cell lysate from human SD1 cells was used to assess the performance of PGC and SCX columns (Each in triplicate, PGC column: Hypercarb 50 x 2.1mm, 5µm particle, SCX column: PolySULFOETHYL A 50 x 2.1mm, 5µm particle). Peptides were separated over a suitable linear gradient of water/acetonitrile + 0.1% TFA at a flow rate of 400µLmin-1. Fractions were collected at 30 s intervals, evaporated to dryness and re-suspended in 50µL Buffer A (0.1% TFA in water).

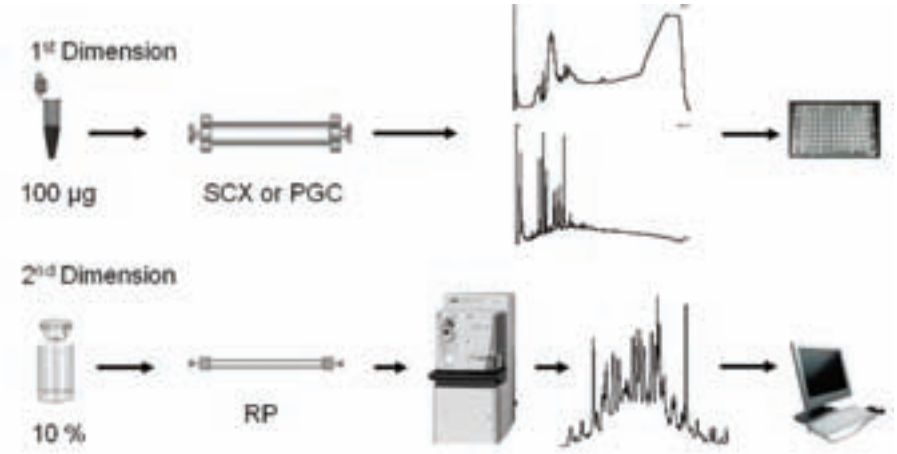

Figure 2: Schematic representation of the two dimensional separation workflow [10]

2nd Dimension: A 5µL aliquot (10% of starting extracted mass) of fraction numbers 10-80 (approx. 10µg total) was then injected onto a C18 column and separated online (30 min separation space) to an ion trap mass spectrometer with orbitrap technology (Thermo Scientific LTQ OrbitrapXL) operating in a data-dependent analysis mode. Data files were processed using ProteinPilot™ software and the numbers of unique peptides in each fraction were calculated (estimated FDR of 1%). Note: SCX fractions required a longer load time for the effective removal of non-volatile salts.

Results and discussion:

Selectivity study 1: 15 peptide standard

A simple data set of 15 peptides was used to initially study the differences in selectivity behaviour between PGC and standard reverse-phase columns. The peptide retention time calibration mixture contains 15 peptides of various lengths and hydrophobicities (Table 2). The spread of peptide variety provides a tool for initial assessment of the selectivity differences between PGC and three different C18-selectivity stationary phases. Due to the retentive behaviour of PGC, the gradient used for separations on PGC was extended to 80% acetonitrile whilst retaining the same rate of increase. Due to its polarity, peptide 1 (SSAAPPPPPR) was not retained on the trapping column.

In order to visualise the retention behaviour differences between PGC and standard reverse phase columns, normalised retention times (Rt) were calculated for each column. This allows compensation of any differences in column length. Normalised retention times were calculated as follows [3].

Equation 1

$$\text{Normalised } R_t = \frac{[R_t \text{ (peptide)} - R_t \text{ (first eluted peptide)}]}{[R_t \text{ (last eluted peptide)} - R_t \text{ (first eluted peptide)}]}$$

The calculated values were then used to generate plots to compare selectivities across the different stationary phases

#	Peptide Sequence	Average Mass	Hydrophobicity Factor (HF)
1	SSAAPPPPPR	985.522	7.56
2	GISNEGQNASIK	1224.6189	15.50
3	HVLTSIGEK	990.5589	15.52
4	DIPVPKPK	900.5524	17.65
5	IGDYAGIK	843.4582	19.15
6	TASEFDSAIAQDK	1389.6503	25.88
7	SAAGAFGPELSR	1171.5861	25.24
8	ELGQSGVDTYLQTK	1545.7766	28.37
9	GLILVGGYGTR	1114.6374	32.18
10	GILFVGSGVSGGEEGAR	1600.8084	34.50
11	SFANQPLEVVYSK	1488.7704	34.96
12	LTILEELR	995.589	37.30
13	NGFILDGFPR	1144.5905	40.42
14	ELASGLSFPVGFK	1358.7326	41.18
15	LSSEAPALFQFDLK	1572.8279	46.66

Table 2: List of peptides in the 15 peptide mix, sequence, mass and HF [11]

studied. When the normalised retention time plots of selected C18-selectivity columns (Hypersil GOLD, Acclaim PepMap and EASY-Column) were compared the relationship between their normalised retention times was found to be linear (R2 values of >0.998 obtained).

When the same retention time normalisation was applied to data obtained from elution on PGC, significantly more deviation from linearity was observed with PGC (shown in Figure 3).

Increased retention can be observed generally; this is more pronounced for smaller, less hydrophobic peptides (3-5). Interestingly, the elution order of larger, hydrophobic peptides is altered on PGC compared to C18. For example peptide 15, the most hydrophobic of the mixture, elutes last on C18. On PGC however, it elutes third from last, showing that on PGC the selectivity is not driven by hydrophobic interactions alone.

Clear selectivity differences between PGC and all three C18-selectivity columns were observed. In the case of C18 phases, peptides 2 and 3 were poorly resolved, with a retention time difference of 0.6 minutes; this is unsurprising when considering the very small difference in hydrophobicity between them (HF of 15.50 and 15.52 respectively). When this is transferred to PGC, the 2 peptides are resolved by over 4 minutes, despite the very small difference in hydrophobicity. This is mirrored for peptides 6 and 7 (HF 25.88 and 25.24 respectively). Peptides 10 and 11 co-elute on all three C18 phases studied (HF 34.50 and 34.96 respectively); however in the case of PGC, these were separated by at least 0.7 minutes. Interestingly, peptides 11 and 12, which are fully resolved on all C18 phases, were found to co-elute on PGC.

Selectivity study 2: increasing sample complexity with digested enolase

The 15 peptide retention time calibration standard is designed to provide a wide scale of hydrophobicities in the mixture. The variation from pure hydrophobic selectivity with PGC should be transferable to a sample mixture that would better re-create the conditions of a real proteomic sample mixture. To this end, digested enolase spiked with four synthetic phosphospeptides was used for further tests (Table 3). This sample set contains 22 tryptic peptides and 4 synthetic phosphopeptides of various chain lengths and hydrophobicities. Since PGC is known to feature increased retention of polar analytes, direct on-column loading without trapping was applied in order to assess the retention differences for small, polar

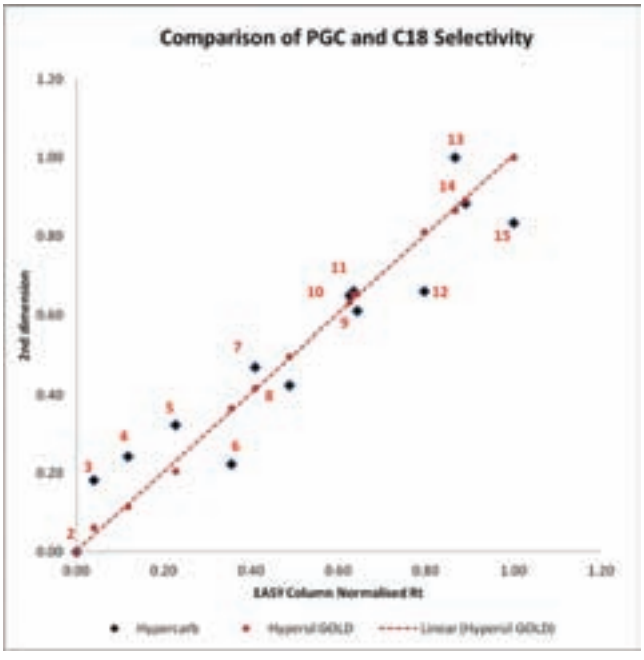

Figure 3: Normalised retention plot of C18-selectivity column 2 (Hypersil GOLD, red circles) and PGC (black diamonds) vs. C18-selectivity column 1 (EASY). Normalised retention times were calculated according to Equation 1 [3]. Comparison of PGC with C18-selectivity media shows deviation from linearity and therefore altered selectivity.

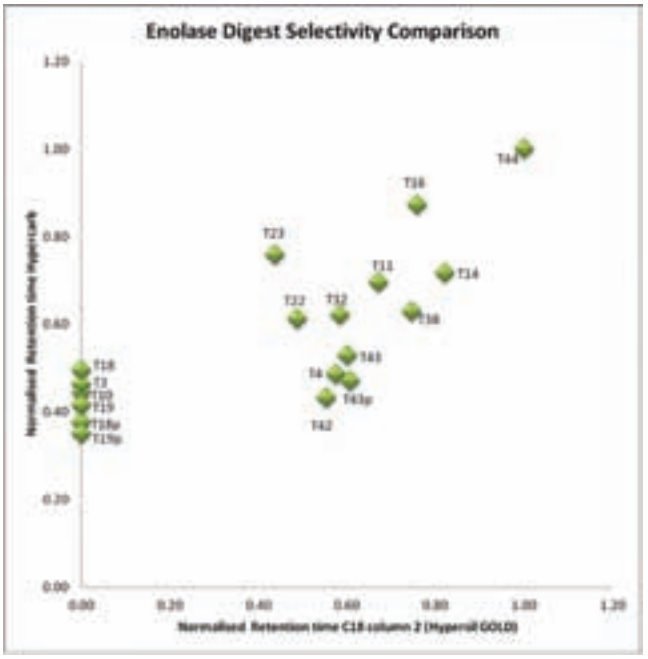

Figure 4: Normalised retention time plot of detected peptides present in digested enolase and eluted on PGC and C18 column 2 (Hypersil GOLD). An arbitrary retention time of zero minutes is assigned to those peptides unretained on C18 column 2 and the retention time window started at time zero. Peptides fully retained on PGC are not considered. On the y axis are 6 polar peptides unretained on C18, but retained and separated on PGC. The large degree of scattering observed for the remaining peptides shows the difference in selectivity between PGC and traditional reversed-phase phases.

peptides usually lost in the loading on traditional reversed-phases. Relative hydrophobicities were calculated for all peptides according to prediction algorithms widely available [11].

Due to the mass range limits set in the method (Method 2), peptides T28-T19 were not detected.

Retention differences

Compared to reversed-phase, PGC was found to retain and resolve 6 peptides, including 2 phosphorylated peptides, otherwise lost on loading in reverse phase (T19p, T18p, T19, T10, T3 and T18). However, 3 large peptides in the mid-hydrophobicity range were not eluted on PGC (T51, T6 and T44). The strong retention is not solely due to hydrophobic interactions, since peptides at the higher end of the hydrophobicity scale were successfully eluted.

Firstly, peptides are overall more strongly retained on PGC than on C18-selectivity column. Each fragment features a greater retention time than its counterpart in reversed phase. Secondly, in classic reversed-phase the retention time of peptides analysed increases linearly with increasing hydrophobicity. This behaviour is not observed in PGC, where hydrophobicity appears to have less effect on selectivity. This also applies to the polar peptides unretained on direct-loading on standard reversed phase.

Plotting normalised retention times for PGC against C18-column 2 shows that the two phases demonstrate alternative selectivities to each other (Figure 4). On the y axis are six small, hydrophilic peptides unretained on C18 column 2 and therefore have a normalised retention time value of zero. On PGC retention and separation of the six peptides is achieved. A clear lack of linearity in the plot is observed for the remaining peptides retained and eluted on both C18 column 2 and PGC. This denotes a difference in selectivity behaviour between the two phases. Interestingly, deviation from linearity is greater in the digested enolase samples than the 15 peptide standard, further showing the suitability of PGC as an alternative stationary phase in proteomic analyses.

Peptide	Sequence	Mass [M+H]+	Relative Hydrophobicity *[10]
T18p	NVPLpYK	813.3912	11.61
T19p	HLADLpSK	863.4028	9.35
T43p	VNQIGpTLSESIK	1368.6776	25.34
T43pp	VNQIGpTLSEpSIK	1448.6439	25.16
T28	AAGHDGK	655.3163	-3.61
T12	ANIDVK	659.3728	9.49
T33	NPNSDK	674.2109	-1.32
T10	GVLHAK	723.4517	4.65
T3	SVYDSR	726.3422	6.77
T18	GILFVGSGVSGGEEGAR	733.425	17.4
T40	NVPLYK	745.446	13.22
T19	HLADLSK	783.4367	13.92
T32	YDLDFK	800.383	24.57
T22	TFAEALR	807.4365	20.6
T42	AADALLLK	814.5038	24.85
T23	IGSEVYHNLK	1159.6111	18.17
T11	NVNDVIAPAFVK	1286.7011	32.81
T43	VNQIGTLSESIK	1288.7112	26.15
T16	LGANAILGVSLAASR	1412.8225	37.51
T4	GNPTVEVELTTEK	1416.7224	27.16
T14	AVDDFLISLDGTANK	1578.8015	41.21
T38	TAGIQIVADDLTVTNPK	1755.9492	40.14
T44	AAQDSFAAGWGVMVSHR	1789.8444	35.69
T45	SGETEDTIFADLVVGLR	1821.9234	48.31
T6	SIVPSGASTGVHEALEM	1840.9227	31.69
T51	IEEELGDNAVFAGENFHHGDK	2328.0533	30.08

Table 3: Tryptic peptides present in digested enolase

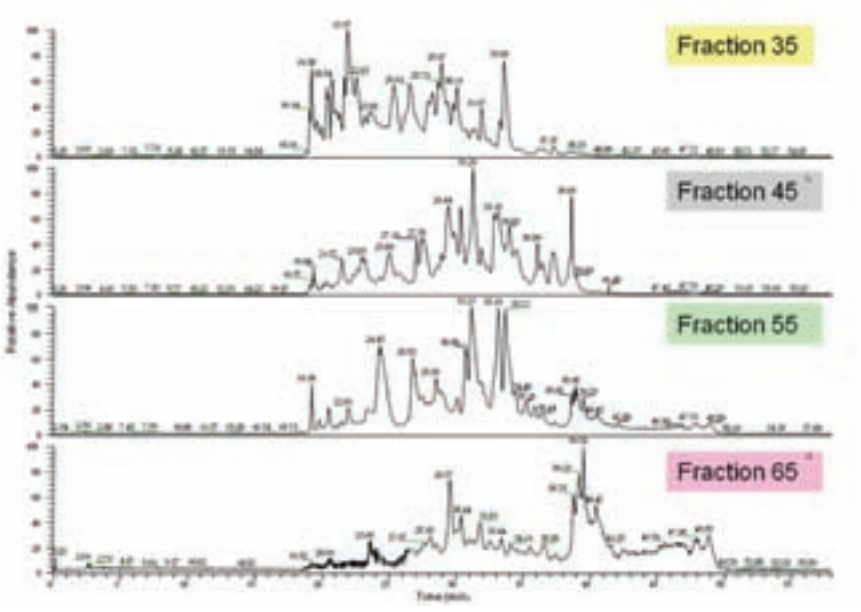

Figure 5: Second dimension reversed phase chromatograms for selected PGC fractions spanning the fractions collected

SCX vs PGC study

After establishing the orthogonality of PGC to C18 reversed phase selectivities it is useful to quantify the differences between the methods in a 2D-LC proteomics workflow. A direct comparison was therefore carried out using a split whole cell lysate sample with analysis in an SCX-RP and a PGC-RP 2D-LC proteomics workflow [10] (Figure 2). The chromatography for the first dimension separation is shown in the upper row of Figure 2 for the SCX and PGC separations. Fractions were collected every 30 seconds and selected reversed phase chromatograms for early, middle and late elution range PGC (1st dimension) fractions are shown in Figure 5. If the two phases were not orthogonal a

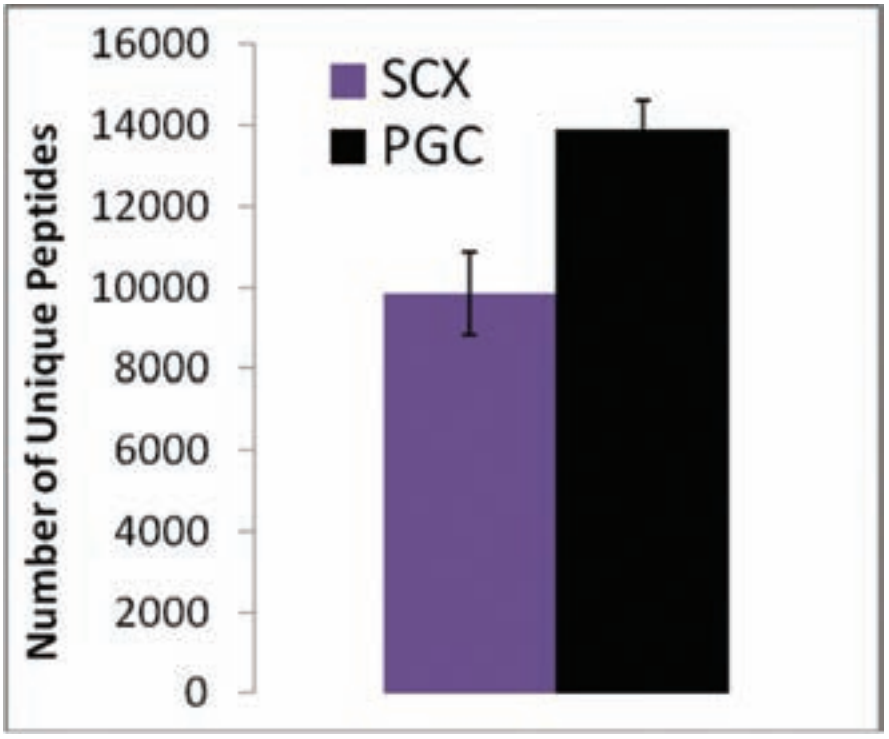

Figure 6: Number of unique peptides detected using a SCX-RP and PGC-RP 2D-LC proteomics workflow [10]

limited band of peptides would be expected in each chromatogram that moved through the time window with the fraction number. The spread of peaks observed in all of these fractions indicates high orthogonality between the two phases as predicted from the selectivity study.

The number of peptide identifications is directly related to the effectiveness of the separation and so to quantify the performance of PGC versus SCX as a first dimension in a 2D-LC proteomics workflow the number of unique peptides detected was quantified using each approach. This analysis was carried out in triplicate on the same whole cell lysate and the results are shown in Figure 6. Using the PGC-RP approach 14,000 unique peptides were detected on average, ~40 % increase relative to the SCX-RP workflow [10]. This significant increase in peptide identification combined with the elimination of the desalting step in the workflow makes PGC an attractive phase for routine 2D-LC analysis of proteomic samples.

Conclusion

From these experiments it has been shown that PGC is orthogonal to C18 stationary phases and results in deviation from an elution order based solely on peptide hydrophobicity both for a peptide calibration standard and an enolase digest. By applying direct on-column loading, PGC was shown to retain and resolve six polar peptides (including 2 phosphopeptides) which were otherwise lost on conventional C18 columns. The different selectivity of PGC compared to C18 suggests it is a good alternative to traditional stationary phases used routinely as a first dimension for proteomic sample pre-fractionation.

In a direct comparison PGC offers superior performance to SCX as an off-line first dimension stationary phase in a 2D-LC-MS/MS proteomic study. Realistic biological quantities of starting material (approximately 100µg digested whole cell lysate) result in the identification of around 14,000 peptides at an estimated FDR of 1%. Only 10% (approx 10µg) of this material was injected onto the second dimension. Replicate analyses show good reproducibility and an increase of approximately 40% in peptide identifications when compared to SCX and without requiring desalting. A comparison of the properties of the most popular 2D-LC combination of a SCX-RP with the PGC-RP approach presented here are summarised in Table 4.

Ideal characteristics	SCX (most commonly used)	PGC (new approach)
Orthogonal to reversed phase	✓	✓
Wide elution window	✓	✓
Peptides soluble in mobile phase	✓	✓
Simple sample handling	x Requires desalting	✓
Stable across pH range and at different temperatures	x (only some show this characteristic)	✓

Table 4: Comparison of the ideal characteristics of a first dimension chromatography phase for 2D-LC for SCX and PGC phases

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[11] LSS Slope Calculator© 2010 http://hs4.proteome.ca/SSRCalc/Slope/