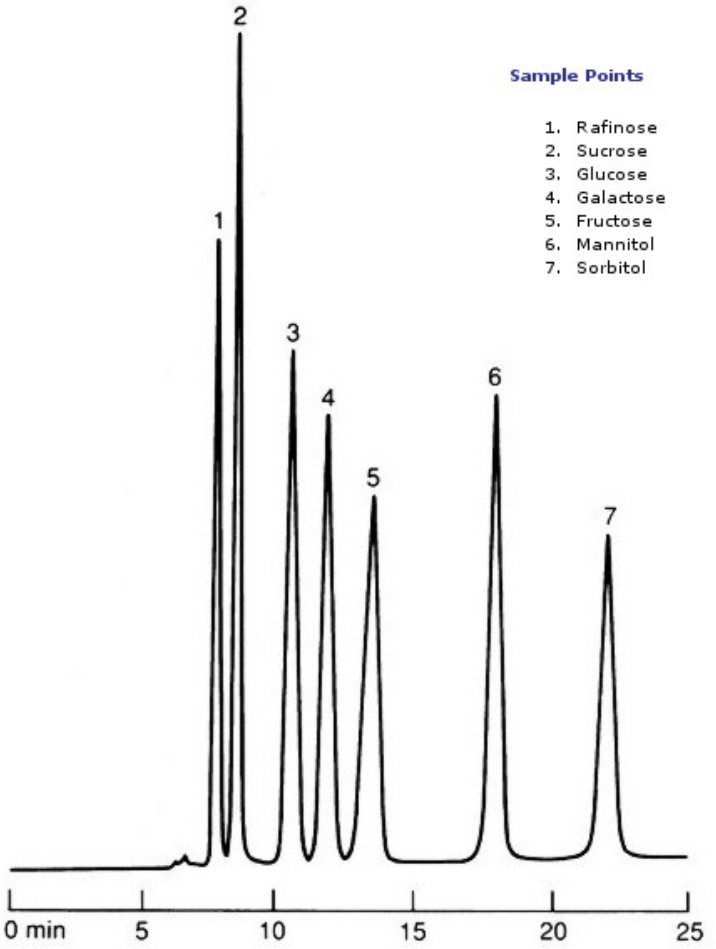
University of Lethbridge

Chromatography (More)

Voet & Voet: Pages 133-144



University of Lethbridge

Chromatography

Most powerful separation technique in Biochemistry

- Mixture is dissolved in 'mobile phase' and percolated through a 'stationary phase'
- Continuous process that subjects sample to repeated, identical separations

Chromatographic methods are classified according to:

1) type of mobile and stationary phase

eg. liquid-liquid, gas-liquid, ...

2) type of retarding force

eg. ion exchange, size exclusion, hydrophobic interaction, ...

University of Lethbridge

Ion Exchange Chromatography

Stationary phase is an insoluble, inert bead uniformly coated with charged functional groups

- **Anion exchange**: beads have positive charges and bind anions
- **Cation exchange**: beads have negative charges and bind cations

Ions (including proteins) in the mobile phase reversible bind stationary phase

- Ions compete for available binding sites based upon their concentration and strength of binding
- Typically, the mobile phase is chosen to maximize the binding of the sample of interest

Note: The net charge (and strength of binding) of a protein is highly dependent on the pH

University of Lethbridge

Stepwise vs. Gradient Elution

For IEC, bound proteins are removed or eluted by

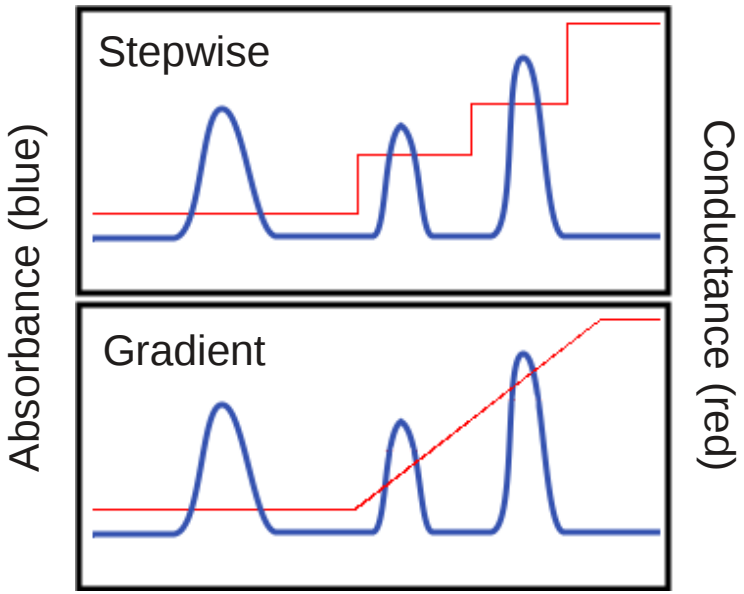
1) changing the concentration of a competing ion (ie. High salt concentration)

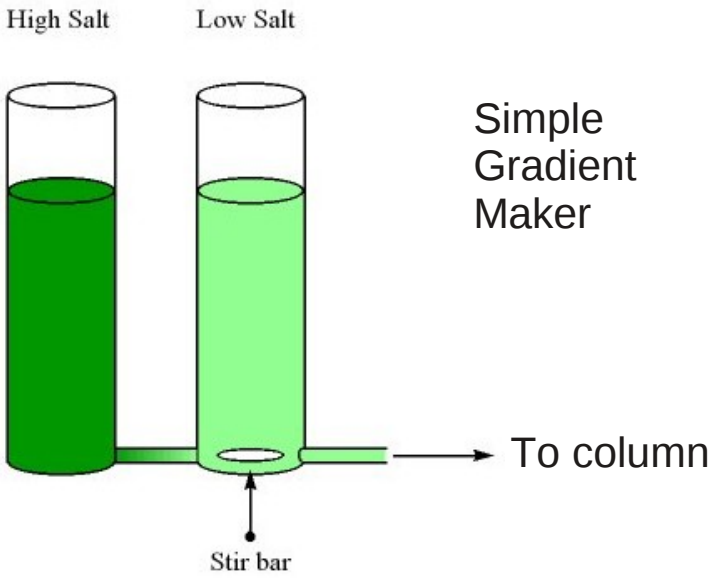
2) changing the pH of the mobile phase (ie. Changing the net charge of the protein)

The pH or salt concentration change can be abrupt or continuous

Stepwise elution – abruptly change mobile phase pH or salt concentration

Gradient elution – continuously change mobile phase pH or salt concentration using a gradient maker





University of Lethbridge

Matrix and Detection

Common ion exchange stationary phases

Strongly Basic	eg. Trimethylammonium (Q column)
Strongly Acidic	eg. Sulfonates (S column)
Weakly Basic	eg. Ethyleneamine (DEAE column)
Weakly Acidic	eg. Carboxylates (CM column)

Wide range of available detection methods:

UV absorbance	proteins have intrinsic UV absorbance
Fluorescence	requires labeling of sample prior to separation
Activity	requires assay (eg. Enzyme activity, ligand binding, ...)
Radioactivity	requires labeling of sample prior to separation
...	...

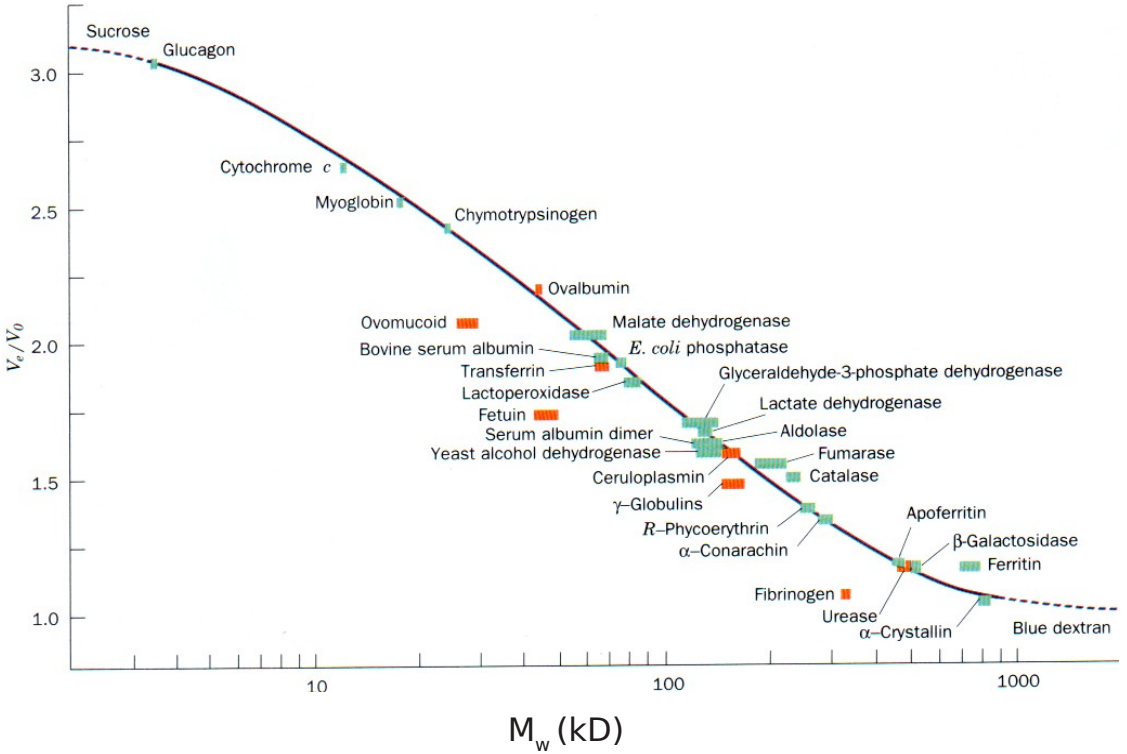
University of Lethbridge

Size Exclusion Chromatography

Separation based upon molecular size and shape

Stationary phase contains small pores that preferentially retard small samples

Behaviour of molecules can be quantitatively characterized (ie. M_w)



$V_t = V_x + V_o$, where V_t is the total column volume, V_x is volume occupied by the stationary phase and V_o is void (remaining) volume

The ratio of the elution and void volumes (V_e/V_o) varies approximately linearly with log M_w over a wide range

University of Lethbridge

SEC Matrices

Name	Type	Fractionation Range (kD)
Sephadex G-10	Dextran	0.05 – 0.7
Sephadex G-25	Dextran	1 – 5
Sephadex G-50	Dextran	1 – 30
Sephadex G-100	Dextran	4 – 150
Sephadex G-200	Dextran	5 – 600
Sepharose 6B	Agarose	10 – 4000
Sepharose 4B	Agarose	60 – 20000
Sepharose 2B	Agarose	70 – 40000
Bio-Gel P-2	Polyacrylamide	0.1 – 1.8
Bio-Gel P-6	Polyacrylamide	1 – 6
Bio-Gel P-10	Polyacrylamide	1.5 – 20
Bio-Gel P-30	Polyacrylamide	2.4 – 40
Bio-Gel P-100	Polyacrylamide	5 – 100
Bio-Gel P-300	Polyacrylamide	60 – 400

University of Lethbridge

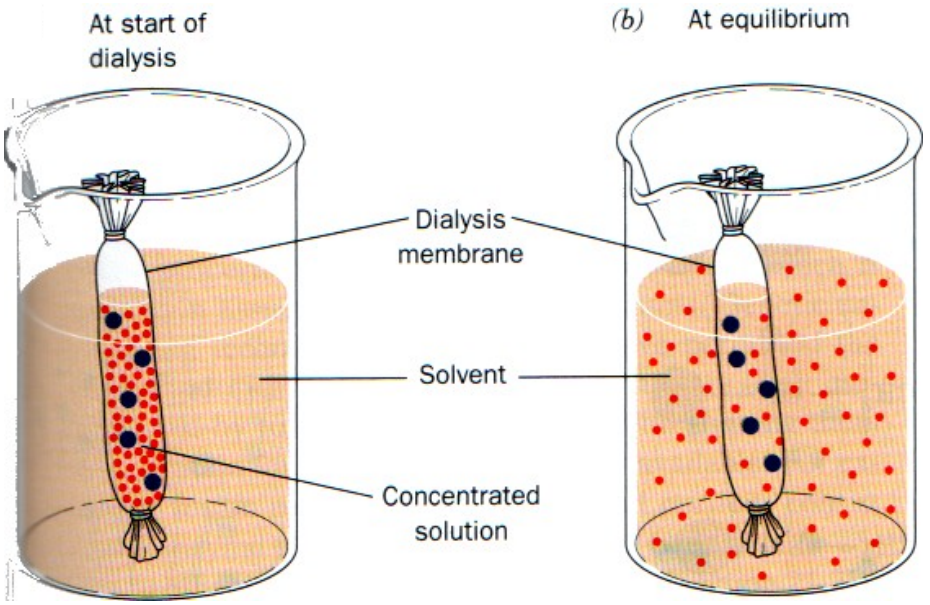
Dialysis

Dialysis is a commonly used, non-chromatographic filtration method

- **Preferred method for changing non-protein solutes (buffers, salts, ligands, etc.)**

Dialysis membranes contain small pores that allows molecules smaller than the pore size to freely diffuse (eg. Non-protein solutes)

- **Molecules larger than the pore size are retained (eg. Proteins)**



University of Lethbridge

Dialysis (example calc.)

Assume you have 10.0 mL of a protein solution in a solution containing 1.00 M NaCl. If you dialyze the protein sample (until equilibrated) against a 0.500 L solution containing 0.100 M NaCl what is the concentration of NaCl in the protein solution?

$$(C_{NaCl,\ protein})(V_{protein}) + (C_{NaCl,\ dialysate})(V_{dialysate}) = (C_{NaCl,\ total})(V_{total})$$

$$(1.00\ M)(0.0100\ L) + (0.100\ M)(0.500\ L) = (C_{NaCl,\ total})(0.510\ L)$$

$$[\ (0.0100\ moles) + (0.0500\ moles)\ ] / (0.510\ L) = (C_{NaCl,\ total})$$

$$0.118\ M = (C_{NaCl,\ total})$$

Process can be repeated by replacing the dialysate until desired solute concentrations are obtained

University of Lethbridge

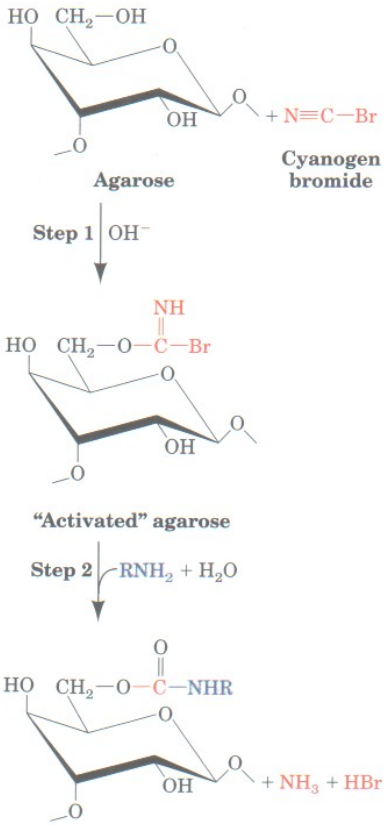
Affinity Chromatography

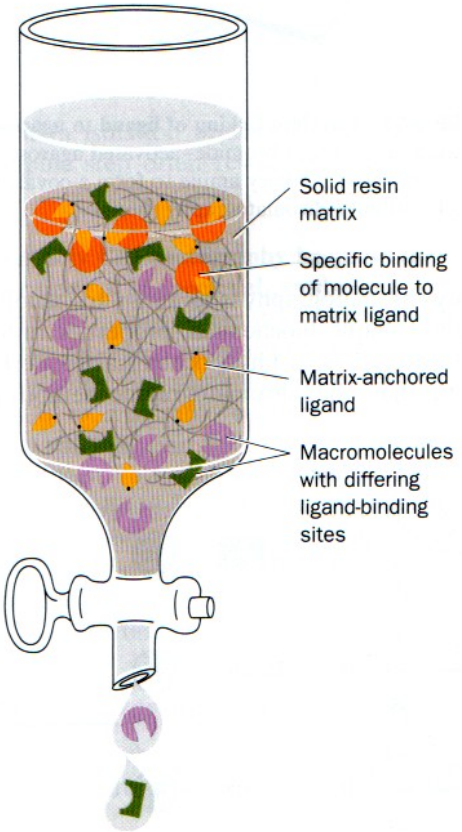
Exploits characteristic or engineered ligand binding properties of proteins

- **Recombinant DNA technology can be used to add a specific tag (binding activity) to a recombinant protein (fusion protein)**
- **Elution is typically accomplished by adding excess ligand**

Ligand is attached to inert, porous matrix by one of several chemical methods

Note: A portion of the ligand is sterically blocked by the attachment to the matrix





Paper Chromatography

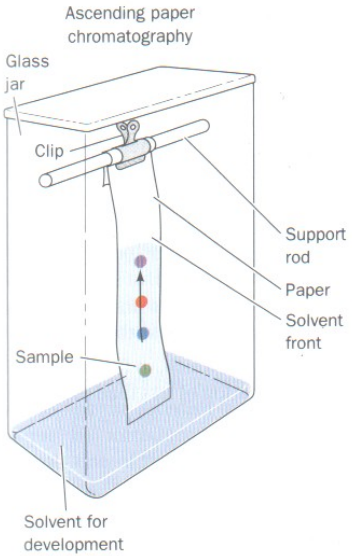
Historically important technique used to separate amino acids, nucleotides and metabolites

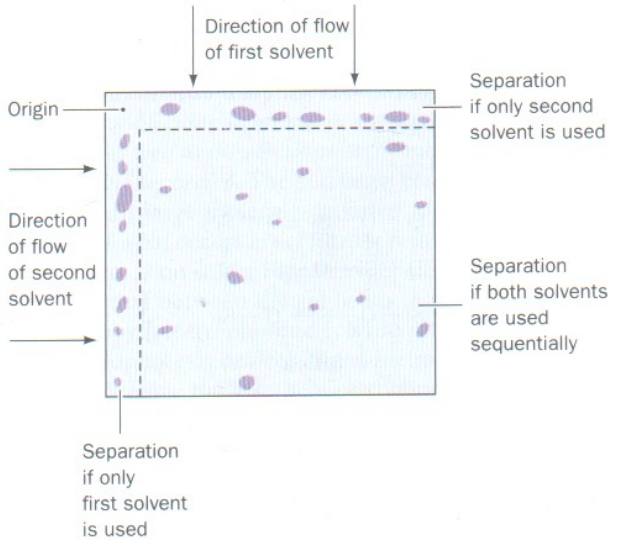
Stationary phase is cellulose-based paper

Mobile phase can be more polar or less polar than the stationary phase

- **water, acetic acid, formic acid are more polar than stationary phase**
- **organic solvents (hexane, ketones, ethers) are less polar**

Separates small, relatively polar samples due to polar stationary phase (typically non-recoverable)





Resolving power can be readily improved by adding a second dimension using a second solvent system

University of Lethbridge

Thin Layer Chromatography

Improvement over paper chromatography

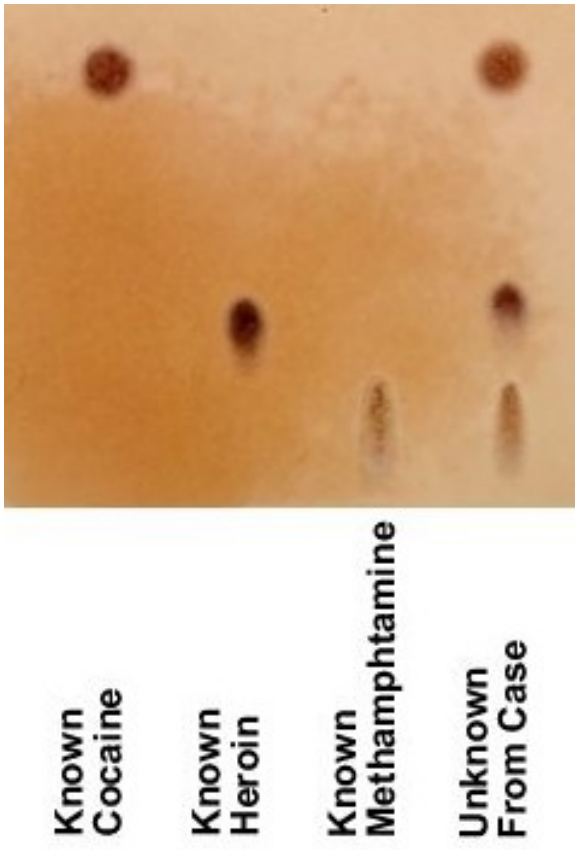
Stationary phase can be any of a variety of materials (ie. Silica gel – polar, C18 – non polar) attached to a glass or plastic plate

- allows for many less polar stationary phases when compared to 'paper chromatography'

Mobile phase can be more polar or less polar than the stationary phase
- water, acetic acid, formic acid are very polar
- organic solvents (hexane, ketones, ethers) are very non polar

Often used to separates non polar organic compounds (eg. Lipids)

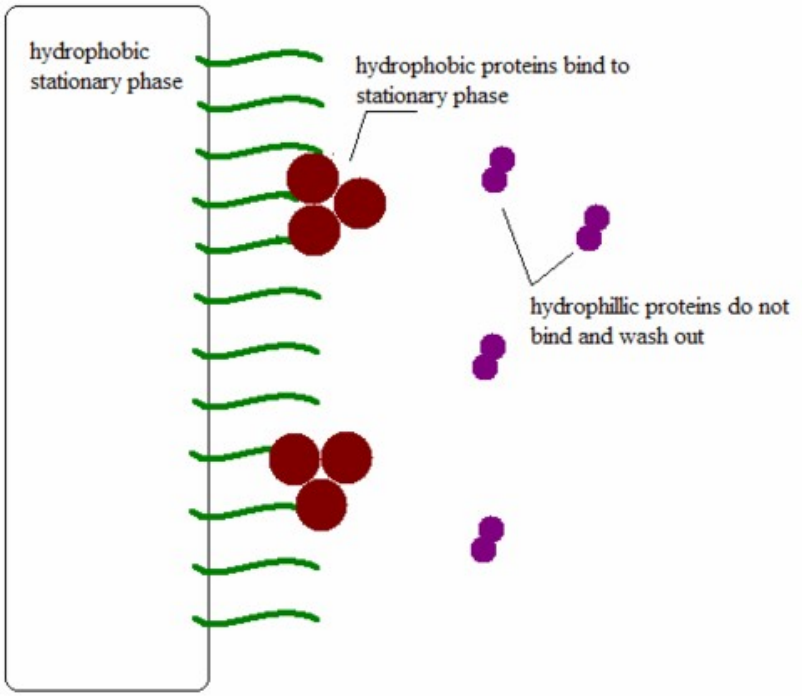
Has largely replaced paper chromatography due to the variety of stationary phases and their mechanical stability



Often requires comparison to standard samples

Hydrophobic Interaction Chromatography

- Stationary phase is lightly substituted hydrophobic matrix
  - Separation is based upon the hydrophobic surface of a native protein
  - Difficult to predict how a protein will behave
- Mobile phase is highly polar (high salt, aqueous solution)
  - Elute by decreasing ionic strength (lowering salt concentration)



- Particularily useful for separating:
  - A) membrane associated proteins
  - B) protein subunits

Reverse Phase Chromatography

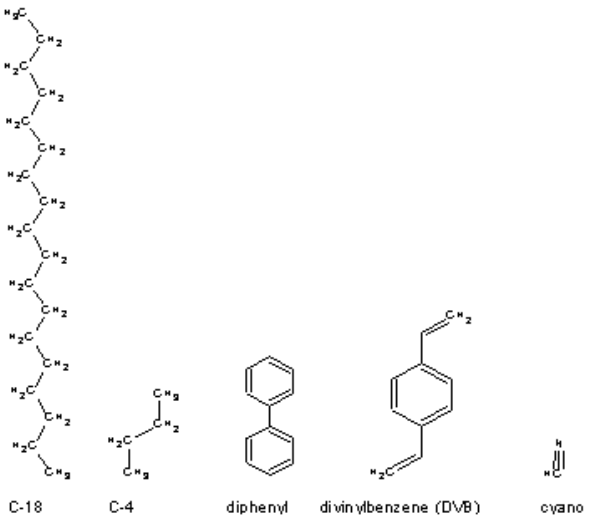
Conceptually similar to Hydrophobic Interaction Chromatography (HIC)

Stationary phase is far more hydrophobic in Reverse Phase

- Essentially an immobilized organic solvent layer

Mobile phase is the more polar solvent (hence reverse phase)

- Unlike HIC, proteins are denatured when bound or partitioned into stationary phase
- Elute samples with organic solvents



Wide variety of stationary phases allow separation of virtually any non-polar or weakly polar compound

Note: Require powerful pumps due to exceptionally large back pressures (up to 5000 psi)