Thermo Scientific Liquid Chromatography Reagents

Introduction to HPLC Ion Pair Reagents

High-purity reagents with the selectivity needed for good separation.

In the past, reverse-phase HPLC analysis of highly charged acidic and basic compounds was frustrating and resulted in poor resolution. Important biomolecules such as amino acids, peptides, organic acids, polyamines and catecholamines had to be separated by ion exchange or by suppression techniques.

Thermo Scientific Ion Pair Reagents enable you to quickly and efficiently analyze charged compounds using reverse-phase techniques. Our ion pair reagents are simply dissolved in the HPLC solvent system, resulting in the formation of stable chromatographic complexes that can be separated using reverse-phase columns.

By using the correct ion pair reagents, you achieve:

- Increased or decreased retention, permitting controlled selectivity
- Resolution of complex ionic mixtures without using ion exchange columns
- Improved peak symmetry

Reverse-phase ion pair chromatography theories

Two principal theories have been proposed to explain reverse-phase ion pair chromatography. In the first theory, small polar ion pair reagents react with the ionized solute, forming neutral ion pairs. The second theorizes that an active ion exchange surface is produced in which long chain, nonpolar anions and cations are absorbed by the hydrophobic stationary phase.

To optimize chromatographic separations in ion pair elution systems, high-purity reagents of exceptional optical transparency are needed. Ion Pair Reagents are specially purified for ion pair chromatography and provide the selectivity needed for good separations.

Derivatization and Visualization Reagents for HPLC

Designed to provide selectivity and improve sensitivity.

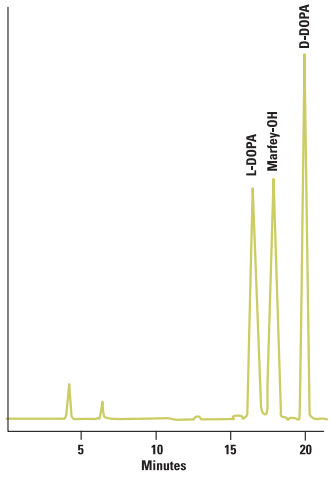
The lack of a universal HPLC detector that provides high sensitivity (as well as some degree of selectivity) established the need for suitable derivatization procedures. Derivatization is the chemical modification of an existing compound, producing a new compound that has properties more suitable for a specific analytical procedure. It is an analytical tool that can be used to provide both selectivity and improved sensitivity.

There are several requirements for derivatization protocol:

1. At least one acidic, polar functional group must be available for reaction on the parent compound.
2. A single derivative should be formed per parent compound.
3. The reaction should be reproducible under the given time and reaction conditions.
4. The reaction should proceed quickly and easily under mild conditions.
5. The reaction byproducts (if any) should not interfere with the chromatography, or with detection of the sample.

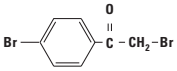
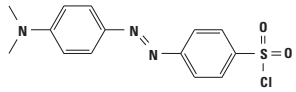
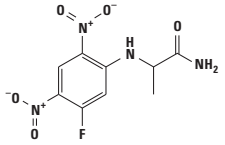
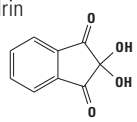
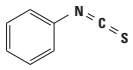
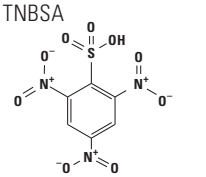
Pre- and post-chromatographic techniques are both used in HPLC derivatization. In addition, off-line and on-line reactions have been employed with both techniques. Pre-chromatographic (or pre-column techniques) offer more than greater selectivity and sensitivity in detection. Pre-column techniques can be used to enhance stability, improve resolution, improve peak symmetry and increase or decrease retention of solutes. FDAA (Marfey's Reagent) allows separation and quantification of optical isomers of amino acids (Figure 2). Post-chromatographic (or post-column) techniques are used primarily to provide selectivity and improve sensitivity.

We offer a variety of HPLC detection reagents for pre- and post-chromatographic techniques. All compounds and formulations are purified for chromatography, minimizing artifact formation.



Separation of D- and L-DOPA on 100mm x 4.6mm C18
Conditions: A – 0.05 M triethylamine phosphate, pH 3.0;
B – acetonitrile.
Linear gradient: 10 to 40% B in 45 minutes, 2.0mL/minute, 25˚C, 340nm

Derivatization Reagents for HPLC

Functional Group	Description	Detection*	Page	Comments
Carboxylic Acid 	*p*-Bromophenacylate 	UV	**4-201**	Formulation of 1.0mmol/ml p-bromophenacyl bromide and 0.005mmol/ml crown ether in acetonitrile; pre-column; nanomole detection levels: λ_{max} = 260nm[1-7]
Primary Amine 	Dabsyl Chloride 	Vis	**4-204**	4-N, N-dimethylaminoazobenzene-4'-sulfonyl chloride (dabsyl chloride); pre-column; nanomole detection levels: λ_{max} = 436nm[8-14]
	FDAA, Marfey's Reagent 	UV	**4-200**	1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA); pre-column; nanomole detection levels: λ_{max} = 340nm. For chiral separations of amino acids.[15, 28-29]
	Ninhydrin 	Vis	**4-203**	Post-column; nanomole detection levels: λ_{max} = 570nm[22]
	PITC 	UV	**4-204**	Phenylisothiocyanate (PITC); pre-column; picomole detection levels: λ_{max} = 254nm[23-24]
	TNBSA  TNBSA MW 293.17	EC, UV	**4-201**	2,4,6-Trinitrobenzene-sulfonic acid (TNBSA); pre- or post-column; nanomole detection levels with EC and UV, GC - 0.85V; λ_{max} = 250nm[25-26]
Secondary Amine	Ninhydrin (see structure above)	Vis	**4-203**	Post-column; nanomole detection levels: λ_{max} = 440nm[22]
	PITC (see structure above)	UV	**4-204**	Phenylisothiocyanate (PITC); pre-column; picomole detection levels: λ_{max} = 254nm[23-24]

*EC = electrochemical; F = fluorescence; UV = ultraviolet; Vis = visible.

References

1. Durst, H.D., *et al.* (1975). *Anal. Chem.* **47,** 1797.
2. Borch, R.F., *et al.* (1975). *Anal. Chem.* **47,** 2437.
3. Grushka, E. *et al.* (1975). *J. Chromatogr.* **112,** 673.
4. Fitzpatrick, F.A. (1976). *Anal. Chem.* **48,** 499.
5. Nagels, L., *et al.* (1980). *J. Chromatogr.* **190,** 411.
6. Ahmed, M.S., *et al.* (1980). *J. Chromatogr.* **192,** 387.
7. Pierce Technical Bulletin: Preparation of Phenacyl and *p*-Bromophenacyl Derivatives for HPLC.
8. Stocchi, V., *et al.* (1985). *J. Chromatogr.* **349,** 77-82.
9. Chang, J.Y., *et al.* (1981). *Biochem. J.* **199,** 547-555.
10. Chang, J.Y., *et al.* (1982). *Biochem. J.* **203,** 803-806.
11. Vendrell, J., *et al.* (1986). *J. Chromatogr.* **358,** 401-413.
12. Chang, J.Y., *et al.* (1984). *J. Chromatogr.* **295,** 193-200.
13. Lin J.K., *et al.* (1980). *Anal. Chem.* **52,** 630-635.
14. Chang, J.Y., *et al.* (1981). *FEBS Letters* **132,** 117-120.
15. Marfey, P. (1984). *Carlsberg Res. Comm.* **49,** 591-596.
16. Jones, B.N. and Gilligan, J.P. (1983). *Amer. Biotech. Lab.* **12,** 46-51.
17. Fiedler, H.P., *et al.* (1986). *J. Chromatogr.* **353,** 201-206.
18. Fried, V.A., *et al.* (1985). *Anal. Biochem.* **146,** 271-276.
19. Jones, B.N., *et al.* (1983). *J. Chromatogr.* **266,** 471-482.
20. Seaver, S.S., *et al.* (Sept./Oct. 1984). *Biotechniques* 254-260.
21. Böhlen, P., *et al.* (1979). *Anal. Biochem.* **94,** 313-321.
22. Stein, W.H. and Moore, S. (1949). *Cold Spring Harbor Symp. Quant. Bio.* **14,** 179.
23. Heinrickson, R.L. and Meredith, S.C. (1984). *Anal. Biochem.* 137, 65-74.
24. Scholze, H. (1985). *J. Chromatogr.* **350,** 453-460.
25. Caudill, W.L., *et al.* (1982). *J. Chromatogr.* **227,** 331.
26. Caudill, W.L., *et al.* (1982). *Bioanalytical System's Current Separations* **4(4),** 59.
27. Andrews, J.L., *et al.* (1982). *Arch. Biochem. Biophys.* **214,** 386-396.
28. Aberhart, D.J., *et al.* (1985). *Anal. Biochem.* **151,** 88-91.
29. Szokan, G., *et al.* (1988). *J. Chromatogr.* **444,** 115-122.

Developments in Amino Acid Analysis

Improvements in amino acid analysis by ion exchange chromatography have involved the analytical system, as well as the instrumentation. Systems have been developed (by varying buffer pH or ionic strength) that work to displace the amino acids into discrete bands. The buffer systems are compatible with single- or two-column analysis of amino acids found in protein hydrolyzates or physiological fluids. Buffer systems are determined by the counter ion used (sodium or lithium) and by the method of buffer changes introduced to the resin (step changes or gradient elution).[9-15] The most commonly used buffering component, citrate, is suitable for solutions below pH7.[16] Buffers are prepared either with citric acid or an alkali salt.

Unfortunately, for high-sensitivity work, citric acid is a significant contributor to amino acid contamination. Therefore, to achieve consistent analyses, it is essential to use high-purity reagents for buffer preparation.

Alternatives to ion exchange are available for the separation of amino acids. Because amino acid analysis is one of the basic protein chemistry tools available, more rapid and sensitive methods for separation and quantitation are desirable.[17] Several pre-column derivatization methods using reverse-phase HPLC have been developed.

Two of the most widely used of these methods involve the formation of dansyl[18-19] or o-phthalaldehyde (OPA)[20-24] derivatives of amino acids prior to HPLC analysis. Both methods offer greater sensitivity and shorter analysis time than post-column derivatization techniques. Other methods include the quantitative derivatization of amino acids with phenylisothiocyanate (PITC) and the separation and quantitation of the resulting phenylthiocarbonyl derivatives via HPLC. These derivatives are stable enough to eliminate in-line derivatization.

Note: Please refer to page **3-122** for details of references

Sample Preparation and Hydrolysis

The extraction and purification of proteins play an important role in determining amino acid content. These methods are based on one or more of their physical characteristics (e.g., solubility, molecular size, charge, polarity and specific covalent or noncovalent interactions).

The techniques commonly used to separate proteins and peptides include:

- Reverse-phase HPLC
- Polyacrylamide gel electrophoresis
- Gel filtration
- Ion exchange chromatography
- Affinity chromatography
- The table below provides a more detailed list of methods for fractionating peptide mixtures.[25]

Hydrolysis

Most protein samples require some form of chemical treatment before their component amino acids are suitable for analysis. Protein and peptide samples must be hydrolyzed to free amino acids from peptide linkages. Acids (usually HCl) are the most widely used agents for hydrolyzing proteins.

A simplified hydrolysis procedure involves refluxing the protein with excess HCl, then removing the excess acid in vacuum.[26] The lyophilized protein then is suspended in constant boiling 6 N HCl and introduced into the hydrolysis tube. The sample is frozen by immersing the tube in dry ice and acetone. Before sealing, the tube is evacuated to avoid formation of cysteic acid, methionine sulfoxide and chlorotyrosine.[27] This procedure minimizes decomposition of reduced S-carboxymethylcysteine and analyzes S-carboxymethylated proteins. Hydrolysis is conducted at 110°C (with the temperature accurately controlled) for 20-70 hours by Moore and Stein's method.[28] After hydrolysis, residual HCl is removed in a rotary evaporator. The residue is dissolved in water and brought to the appropriate pH for addition to the analyzer column.[28]

Methods for the fractionation of peptide mixtures.

Technique	Properties of Peptide Molecules Exploited
Centrifugation	Solubility
Size exclusion chromatography	Size
Ion exchange chromatography	Charge, with some influence of polarity
Paper electrophoresis	Charge and size
Paper chromatography	Polarity
Thin layer electrophoresis	Charge and size
Thin layer chromatography	Polarity
Polyacrylamide gel electrophoresis	Charge and size
High-performance liquid chromatography (HPLC)	Polarity
Gas chromatography	Volatility of derivatives
Counter-current extraction	Polarity; sometimes specific interactions
Affinity chromatography	Specific interactions
Covalent chromatography or irreversible binding	Disulfide bond formation; reactivity of homoserine lactone

References

1. Braconnot, H. (1820). *Ann. Chem. Phys.* **13**, 113.
2. Mulder, G.J. (1848). *J. Prakt. Chem.* **38**, 294.
3. Kjeldahl, J.G.C.T. (1883). *Z. Anal. Chem.* **22**, 366.
4. Rattenbury, J.M. (1981). *Amino Acid Analysis,* Ellow Horwood, publisher, Chicester, England, Chapter 1.
5. Martin, A.J.P. and Synge, R.L.M. (1941). *Biochem. J.* **35**, 1358.
6. Elsden, S.R. and Synge, R.L.M. (1944). *Proc. Biochem. Soc. IX.*
7. Synge, R.L.M. (1944). *Biochem. J.* **38**, 285.
8. Stein, W.H. and Moore, S. (1949). *Cold Spring Harbor Symposia Quant. Biol.* **14**, 179.
9. Liao, T.H., *et al.* (1973). *Anal. Chem.* **45**, 2286.
10. Hare, P.E. (1972). *Space Life Sciences* **3**, 354.
11. Dus, K., *et al.* (1966). *Anal. Biochem.* **14**, 41.
12. Thomson, A.R. and Miles, B.J. (1964). *Nature* **203**, 483.
13. Robinson, G.W. (1975). *J. Chromatogr.* **3**, 416.
14. Benson, J.V. (1972). *Anal. Biochem.* **50**, 477.
15. Benson, J.V. and Patterson, J.A. (1965). *Anal. Chem.* **37**, 1108.
16. Bates, R.G. and Pinching, G.D. (1949). *J. Amer. Chem. Soc.* **71**, 1274.
17. Jones, B.N. and Gilligan, J.P. (1983). *American Biotech. Lab.* December, 46-51.
18. Weidmeier, V.T., *et al.* (1982). *J. Chromatogr.* **231**, 410-417
19. DeJong, C., *et al.* (1982). *J. Chromatogr.* **241**, 345-359.
20. Lindroth, P. and Mopper, K. (1979). *Anal. Chem.* **51**, 1667-1674.
21. Jones, B.N., *et al.* (1981). *J. Liq. Chromatogr.* **4**, 565-586.
22. Turnell, D.C. and Cooper, J.D.H. (1982). *Clin. Chem.* **28**, 527-531.
23. Umagat, H., *et al.* (1982). *J. Chromatogr.* **241**, 324-334.
24. Jones, B.N. and Gilligan, J.P. (1983). *J. Chromatogr.* **266**, 471-482.
25. Allen, G. (1981). Laboratory Techniques in Biochemistry and Molecular Biology, T.S. Work and R.H. Burdon, Eds., Elsevier/North-Holland, Biomedical Press.
26. Moore, S. and Stein, W.H. (1963). *Meth. Enzymol.* **6**, 819.
27. Eveleigh, J.W. and Winter, G.D. (1970). Protein Sequence Determination, S.B. Needleman, Ed., Springer-Verlag, pp. 92-95.
28. Moore and Stein, *op. cit.*