The Role of Chemistry Consumables in Biopharmaceutical Product Development

a report by
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Within the last decade, life science investigations have rapidly evolved from research and development (R&D) initiatives to the isolation and characterisation of commercialised biopharmaceutical products. In fact, of all new medicines in active clinical trials, some 27% are biopharmaceuticals.[1] This represents approximately 500 new biopharmaceutical candidates in clinical evaluation.[2] Intensified investigations in the areas of proteomics and biomarker discovery hope to accelerate the rate of drug discovery. These R&D initiatives continue at a wide variety of institutions including academic, small biotech start-ups and international pharmaceutical conglomerates. The realisation of robust, sensitive and accurate liquid chromatography (LC) and mass spectrometry (MS) technologies has been integral to the successful development of biopharmaceuticals.

Highly evolved instrumentation and methodologies have revolutionised the way biopolymers are analysed. For example, a prominent use of new separation and detection technologies is centred on peptide characterisation. In a well-characterised biopharmaceutical application, this might involve the simple determination of peptide purity using reversed-phase high performance liquid chromatography (HPLC). At the other extreme, the use of sophisticated hybrid LC/MS systems for the qualitative and quantitative characterisation of hundreds of peptides contained in a complex protein digest might be required.

The pace at which new LC and MS technologies evolve closely mimics the rapid advances seen with consumer electronic devices and software. Scientific instruments that push the limit of resolution and detection have become readily available to the scientific masses. For example, Waters' Corporation introduced the ACQUITY UPLC™ System in early 2004, which promises to revolutionise the quality and quantity of information obtained by LC. The ACQUITY UPLC System was specifically designed to take advantage of improvements in chromatographic resolution, sensitivity and speed afforded by using sub-2µm chromatographic media in traditional length columns. This integrated and optimised system solution was extended during the summer with the introduction of Waters' nanoACQUITY™ system designed as a 'next-generation' inlet for advanced MS analyses of biocompounds using electrospray ionisation methods at nano-flowrates.

Nano-flow LC interfaces coupled with high performance MS systems are necessary to obtain the high sensitivity required for the analysis of sample-limited or low abundance biopolymers such as peptides. Nano chromatography is performed using ≤150µm internal diameter HPLC columns operated at ≤1,000nL/min flows. Initially, many scientists studying proteomics became skilled in packing their own columns. This was done out of necessity since nano columns packed with high-performance sorbents were not commercially or readily available. In fact, separation sorbents (e.g. C_{18} reversed-phase materials) were generally obtained by unpacking a purchased analytical column and repacking the material into a nano column format.

Today, laboratory directors, professors, companies and investors are interested in results; that is, the application of advanced LC/MS techniques towards finding answers to challenging biological problems. Savings from self-packed nano columns will clearly be offset when factors such as the time required to pack, test and document column performance are calculated into the total cost of operation. Thus, self-packed columns may be a risky venture when precious samples are analysed.

Self-packed columns raise additional concerns in today's regulated environment with extensive validation and compliance requirements. Fortunately, several companies now offer sub-1mm internal diameter columns for LC/MS applications. For example, Waters' NanoEase™ capillary, trapping and nano columns containing high performance reversed-phase and ion-exchange sorbents are

1. *Bibby K, Davis J and Jones C, "Biopharmaceuticals-Moving to Centre Stage" IMS Global Consulting Report, (2003).*
2. *Walsh G,* Nature Biotechnology *(2003), 23, pp. 865–870.*

Figure 1: Total Ion Count (TIC) Chromatogram of an Enolase Protein Digest on a Waters' NanoEase™ Nano Column

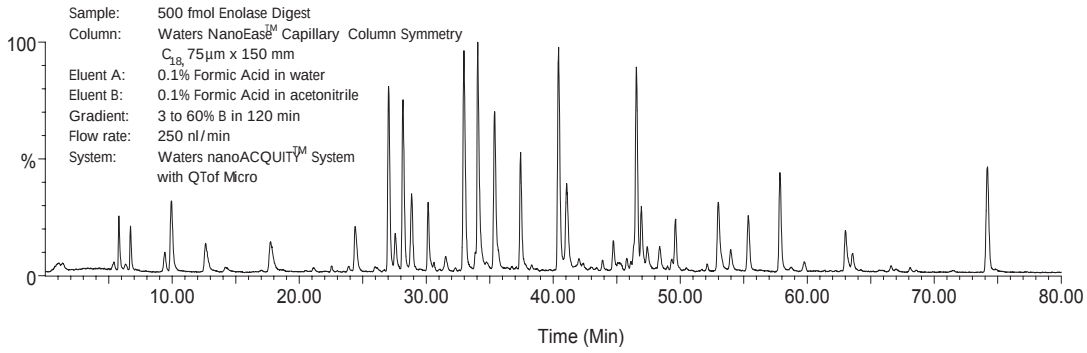
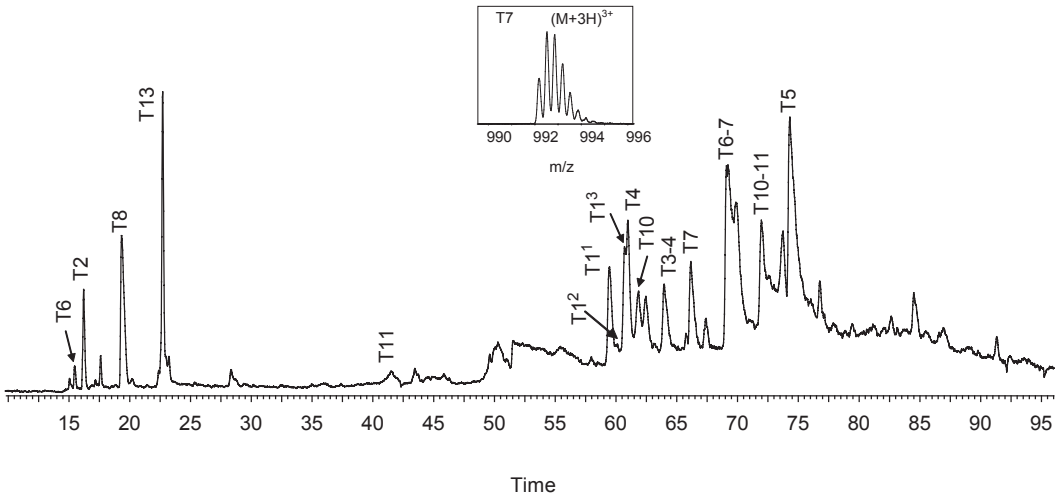


Figure 2: TIC from LC/MS of Trypsin Digested Bacteriorhodopsin in the Presence of Waters' RapiGest™ SF Reagent

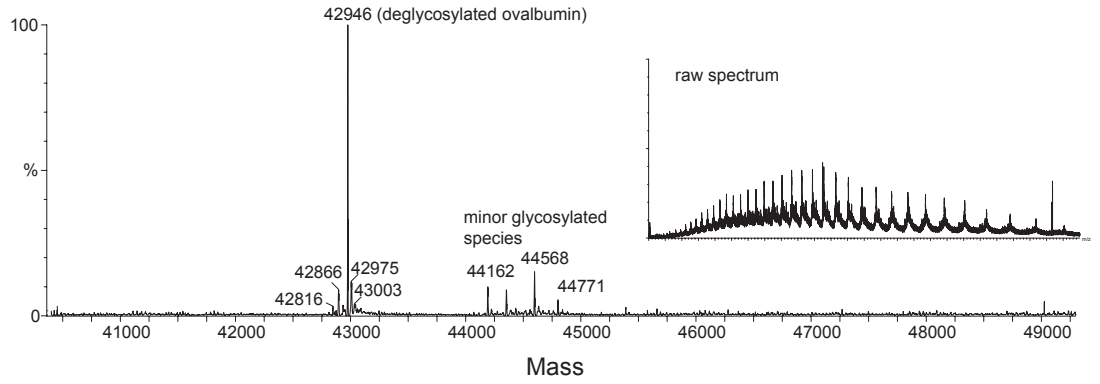


The identified tryptic peptides are labelled which represents about 97% of the amino acid sequence of the protein. Peaks labelled T11 through T13 are N-terminal peptides with different modifications. No digestion is observed when digestion is attempted with trypsin without RapiGest™ SF.

available and produced in a fashion similar to that used for analytical columns (i.e. ≥1mm internal diameter). State-of-the-art techniques have resulted in commercially available nano columns that have performance characteristics equal to those of analytical columns (see *Figure 1*). These analytical tools have evolved to such a point that laboratory scientists no longer need to be expert chromatographers in order to realise the synergies from LC/MS methods. Efforts can now be appropriately focused on research activities directed towards the development of new drugs, improved treatment regimes or enhanced diagnostics capabilities.

Recently, attention has focused on how sample preparation affects the quality of collected data and interpreted results for well-characterised proteins. Biopharmaceutical corporations have invested heavily in acquiring sophisticated scientific instrumentation and informatic technologies. However, the collected and software-reduced data obtained from these systems are only as good as the quality of the analysed samples. For example, peptide mapping is frequently performed during the investigations directed towards the development of a new, protein-based biopharmaceutical. A variety of well-documented chemical and enzymatic methods are used for sample preparation prior to LC, MS or LC/MS analyses of the generated peptides. The use of high-quality trypsin is commonly employed for protein digestions since this enzyme normally creates predictable peptide fragments of moderate length based on the amino acid sequence of the target compound. With this simple and inexpensive reagent, techniques have been developed that take advantage of sophisticated LC/MS methods such as peptide mass fingerprinting. While many readily soluble proteins are effectively digested with trypsin, many proteins of biological importance (e.g. membrane proteins) are resistant to digestion due to their hydrophobic nature. Even though hydrophobic proteins can be solubilised prior to digestion, most surfactants significantly inhibit endoprotease activity, which can yield incomplete digestion and poor peptide mass fingerprinting results. Furthermore, it is often necessary to remove the

Figure 3: Deconvoluted Spectrum of Ovalbumin Deglycosylated in the Presence of PNGase F and Waters' RapiGest™ SF (insert, raw spectrum)



No deglycosylation was observed when RapiGest was omitted from the PNGase F containing solution.

denaturants (e.g. sodium dodecyl sulphate (SDS)) post-cleavage since surfactants frequently interfere (e.g. ion suppression) with the mass analysis of biopolymers. Recent investigations have resulted in the introduction of novel surfactants that are well suited for this demanding application.

Waters' RapiGest™ SuperFect (SF) reagent is an acid-labile detergent that effectively denatures proteins and does not interfere with trypsin activity. In addition, the generated peptides are free from adduct or other modifications that are frequently encountered using other protein solubilisation reagents (e.g. carbamylation via the use of urea). Since the RapiGest™ SF reagent is acid-labile (i.e. degraded in the presence of 0.1% trifluoroacetic acid (TFA)), it can easily be removed prior to LC/MS or matrix-assisted laser desorption time-of-flight (MALDI TOF) analysis. Proteolytic-resistant proteins such as the membrane-bound bacteriorhodopsin is completely digested overnight in the presence of RapiGest™ SF (see *Figure 2*). Over the past year, several manuscripts featuring this reagent for simple in-solution digestion, in-gel digestion, membrane protein digestion and whole cell lysate digestion have been published.[3–7] On-going investigations support the use of this reagent to solubilise glycoproteins prior to the enzymatic cleavage of N-linked carbohydrates (see *Figure 3*).

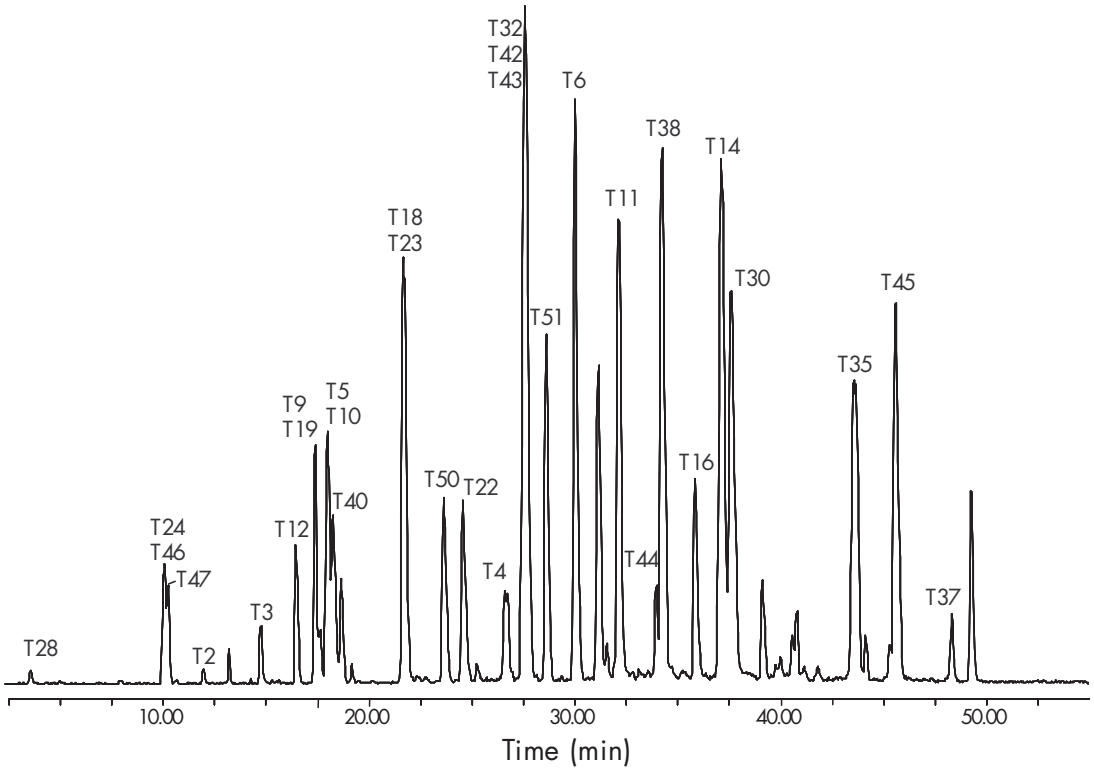
While peptide mapping is frequently used to analyse an isolated protein, applications exist where analytical data from the intact protein is desired. For example, results from peptide mapping experiments can be correlated with intact protein analysis to better define the protein of interest. An especially powerful tool for intact protein analysis is MS. In these experiments, the undigested protein is infused or injected into a mass spectrometer where the molecular weight is accurately measured. In addition to obtaining an accurate molecular weight for the analysed protein, mass analysis can also give information on protein modifications, such as glycosylation, oxidation, etc. This information can then be compared with the peptide mapping data to confirm the site(s) of modification.

Due to MS using high vacuum sources as part of the ionisation and detection technology, it is important to minimise the introduction of non-volatile salts into the mass analyser. Salts, if not removed, can suppress the ionisation of the analysed intact proteins and lead to poor detection sensitivity. A variety of published sample preparation procedures report the use of 'offline' devices (e.g. Ziptips® C4 from Millipore Corporation) that can effectively be used to remove salts from the proteins prior to analysis. In an attempt to increase sample throughput in automated LC/MS applications, Waters introduced its MassPREP™ Online Desalting Cartridge (2.1x10mm) for protein desalting prior to mass spectral analysis.

The MS-compatible, reversed-phase material contained in these online devices successfully 'traps'

3. *Yu Y-Q, Gilar M, Lee P J, Bouvier E S P and Gebler J C, "Enzyme-Friendly, Mass Spectrometry-Compatible Surfactant for In-Solution Enzymatic Digestion of Proteins",* Anal. Chem. *(2003), 75, pp. 6,023–6,028.*
4. *YYu Y-Q, Gilar M and Gebler J C, "A Complete Peptide Mapping of Membrane Proteins: A Novel Surfactant Aiding the Enzymatic Digestion of Bacteriorhodopsin",* Rapid Commun. Mass Spectrom. *(2004), 18, pp. 711–715.*
5. *Stover T, Amari J V, Mazsaroff I, Yu Y-Q, Gilar M and Gebler J C, "RapiGest™ SF Denaturant Tool for Improved Trypsin Digestion of Monoclonal Antibodies",* Genetic Engineering News *(2003), 23, pp. 48–49.*
6. *Arnold R J, Hrncirova P, Annaiah K and Novotny M V "Fast Proteolytic Digestion Coupled with Organelle Enrichment for Proteomic Analysis of Rat Liver",* Journal of Proteome Research *(2004), 3, pp. 653–657.*
7. *Nomura E, Katsuta K, Ueda T, Toriyama M, Mori T and Inagaki N "Acid-labile surfactant improves in-sodium dodecylsulfate polyacrylamide gel protein digestion for matrix-assisted laser desorption/ionization mass spectrometric peptide mapping",* J. Mass Spectrom. *(2004), 39, pp. 202–207.*

Figure 4: TIC of Waters' MassPREP™ Enolase Digestion Standard Noting the Identified Tyrpsin Cleavage Peptides



proteins (e.g. hydrophobic monoclonal antibodies) allowing the salts to be washed to waste prior to elution of the desalted protein onto the mass spectrometer for analysis. With an optimised LC/MS method, cycle times as low as three minutes are achievable. Of equal importance, is the fact that Waters' MassPREP™ Online Desalting Cartridge, when used with an appropriate HPLC system injector and injector wash solution, can be used for the sequential analyses of different samples (e.g. 50 to 100 in an overnight method) due to the lack of protein carry-over from prior injections.

While inadequate sample preparation can seriously compromise the quality of collected data, deficiencies in system or column performance can also seriously affect the quality of results. In today's pharmaceutical quality control (QC) laboratories, a series of system and column performance qualification tests are routinely performed prior to the analysis of test compounds. The importance of performing these tests is significantly increased with the analysis of valuable volume and concentrated limited samples as found in proteomics and biomarker investigations. A recent article entitled "HPLC Standard Controls as a Requirement in Proteomics"[8] highlights the importance of using biological standards (i.e. peptides) in a performance qualification procedure for proteomics research.

Commercially available peptides (e.g. angiotensin peptide series) can be purchased to create a useful test sample. However, some investigations require more complex test mixtures (e.g. trypsin digests of specific proteins). The generation of reproducible and stable tryptic digests for system and column performance testing can be problematic. Frequently, the enzyme digested samples contain undigested protein(s), trypsin or large hydrophobic peptides. Ideally, a QC-certified standard contains only peptides.

To address this need, in 2003, Waters introduced the MassPREP™ Peptide and Protein Digestion Standards (see *Figure 4*). These samples are produced using highly reproducible digestion methods that yield high-quality standards free of artifact components. They are QC-tested prior to lyophilisation, packaged to maximise stability and shipped with certificates of analyses for documentation archives. In addition to high quality standards, a variety of MassPREP™ MALDI matrices are purified, QC-tested and packaged to provide reproducible MALDI TOF analysis of important, concentration limited samples. In combination, the availability and use of high quality peptide, protein digests and MALDI matrices can reduce the time and associated expense in preparing these important chemistry consumables prior to the analysis of extremely valuable samples.

Today's biopharmaceutical organisations have

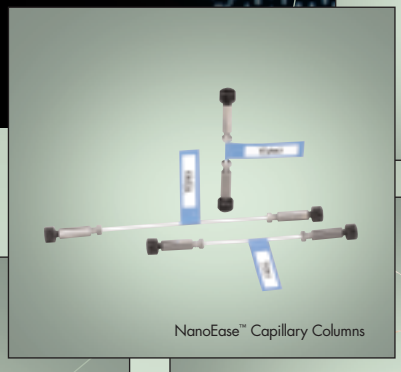
8. Wefr T, "HPLC Standard Controls as a Requirement in Proteomics", LCGC North America *(2004), 22, pp. 354–361.*

invested heavily in instrumentation (e.g. high performance LC (HPLC), MS and LC/MS) and have trained staff to accelerate the discovery and development of new therapeutic and diagnostic products. Obtaining chromatograph resolution of complex biological mixtures at MS-compatible flows, generating quality digests from isolated proteins, removing interfering substances prior to high-throughput MS analysis and assuring proper system, column and method performance prior to the analyses of valuable samples are some of the frequently overlooked yet important factors that affect the quality of generated data and interpreted results. Scientific instrument, software and chemistry manufacturers such as Waters Corporation are paying careful attention to the details responsible for the successful realisation of reproducible, sensitive and faster analytical methods. In particular, this article highlights the importance of using high-quality chemistry consumables as part of a 'total integrated system solution', which will maximise return on investment and help achieve biopharmaceutical research, development and commercial objectives. ■

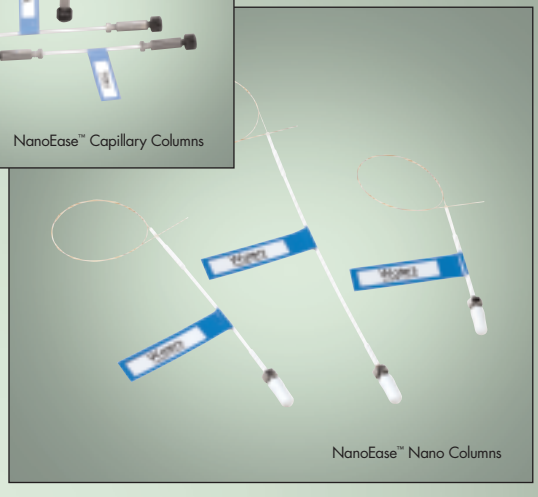
High Quality MS and LC/MS Results

Waters is an international leader in the development of reagents and chromatography-based technologies for the isolation and characterization of biomolecules. Whether you are using LC/MS or gel electrophoresis with MALDI techniques, Waters has mass spectrometry consumables that will improve the quality of your results.

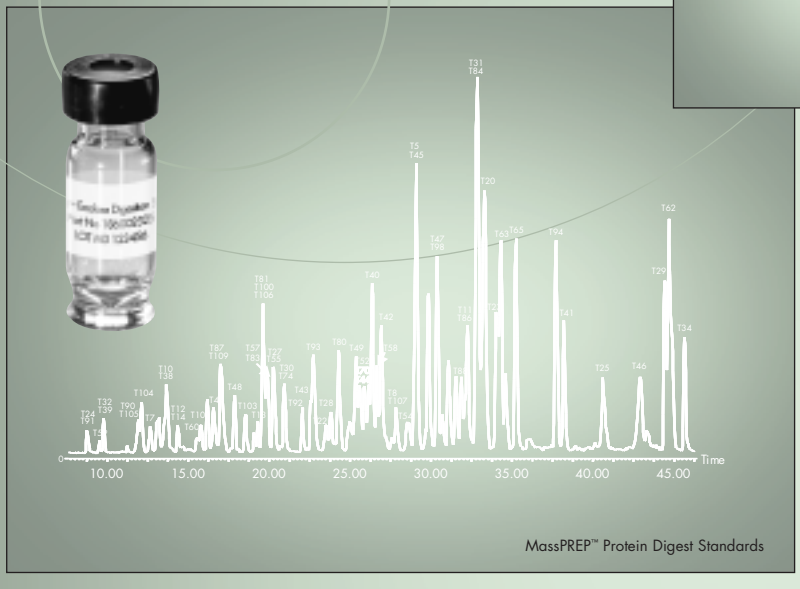
NanoEase™ Trapping Columns



NanoEase™ Capillary Columns



NanoEase™ Nano Columns



MassPREP™ Protein Digest Standards

The NanoEase™ column family consists of capillary, nano and trapping formats that utilize several of Waters' premier, mass spec compatible separations media. These columns help scientists separate, concentrate, fractionate or desalt sample-limited, complex samples in either 1-D or 2-D LC/MS applications. To ensure optimal system performance prior to the analyses of your valuable samples, we recommend the routine use of Waters' MassPREP™ Peptide and Protein Digestion Standards.

Visit us at **www.waters.com/lifesciences** to learn more about our complete portfolio of MS and LC/MS products, including:

- RapiGest™ SF Reagent
- MassPREP™ Standards and Matrices
- NanoEase™ Columns
- 1-D and 2-D Kits for LC/MS

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