Integration of Chemistry and Chromatography: From Bioanalysis of Protein 3-Nitrotyrosine to Extreme Ultra-Pressure Liquid Chromatography (XUPLC)

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Background - Chromatography, Detection & Derivatisation

The identification and quantification of naturally occurring and exogenous substances in various biological fluids and tissues has been of high interest to a wide range of investigators in numerous disciplines for many decades. While numerous approaches have been described and proven valuable, since the 1970s growth in chromatographic-based methods, when used in conjunction with various detection technologies (UV, electrochemical (EC), fluorescence (F), and mass spectrometry (MS) and other techniques), have undergone maturation and acceptance to the extent that they represent the mainstay approach for determination of small molecules and peptides. During this period there has been a continual advance of HPLC instrumentation, to point where the conduct of separations using higher pressures is relatively common, an operational technique frequently referred to as ultrahigh-pressure liquid chromatography (UHPLC).

In conjunction with the advancement of chromatographic instrumentation, roughly during the same timeframe, there was a substantial increase in the quality and performance of chromatographic supports. During the early portion of this period, HPLC was largely conducted with columns packed with 10 µm particles packed into 4.6 mm x 250 mm columns; however, over time with improving technologies the transition to 5 µm, then 3 µm was observed and now sub-2µm supports (displaying a variety of bonding chemistries thus enabling the various retention processes commonly employed at present). In parallel to the use of smaller sized particles there has been a steady trend towards smaller internal diameter columns, from the conventional bore columns (4.6 mm) to nano-columns (75 µm), which are typically used in conjunction with UHPLC systems and nano-spray MS for detection.

During the early decades of the noted timeframe, the bioanalyst frequently utilised various derivatisation reactions as an essential component of method development. While the chemical modification of polar functional groups in GC-based methods was widely utilised to enhance analyte volatility, in liquid phase separations the use of derivatisation reactions was predominantly driven by the need for improved product detectability. From the period 1979-1999, various books on chemical derivatisation [1-6] were published that served to organise and provide numerous examples that were useful to the analytical chemistry community. Despite these substantial efforts to provide reliable reagents and develop established procedures for their application, especially in LC-based applications, there appears to be diminished utilisation of analytical derivatisation reactions in the past two decades. Obviously, one may question why this trend has emerged. For the determination of drug substances and associated metabolites over this time period, the development and integration of several key technologies; electrospray ionisation (ESI), MS as a quantitative detection technique and the increasing ease of interfacing LC with MS instrumentation, has lead to the widespread adoption of LC-MS as a preferred approach in drug bioanalysis. While LC-MS provides structural information there is all too often the perception that LC-MS based methods require minimal sample preparation and attention to chromatography. This position can lead to disregard of incorporation of derivatisation chemistry as a component of a method due to the additional required operational steps. Such attributes and perceptions have lead to the trend of adopting LC-MS based methodology as the preferred approach for the determination of drug substances (small molecules) in samples of biological origin.

Recent Trends - Derivatisation & Mass Spectrometry

Despite the trends previously noted, within the past decade there is a clear trend of the reemergence of derivatisation chemistry, but these chemical reactions can now be found integrated as an operation within MS based methods. As recently discussed by Bush [7], a quick web search of the terms 'mass spectrometry and derivatisation' results in numerous hits where chemical reactions have been used to enhance the MS aspect for various substances. As noted, many of the traditional derivatisation reagents, or updated analogues possessing structural features enabling ease of product ionisation, are becoming used to create a charged product thus enhancing detection efficiency in various MS formats. This enhanced interest in derivatisation chemistries appears to have motivated the publication of a recent book 'A Handbook of Derivatives for Mass Spectrometry' [8] and a series of reviews that recently appeared in the 'European Journal of Mass Spectrometry' [9-16]. Further evidence of increasing use of derivatisation reactions in MS is revealed by the web searches where various reagents and applications described by numerous commercial vendors can be found.

Complex Sample Analysis - Proteomics

While not exclusively, the previously noted considerations are largely confined to the

determination of a limited number of analytes in biological samples. Since the completion of the Human Genome Project (HGP) there has been a high degree of interest in the identification and quantification of proteins, the products of gene expression, in various cell types, an activity frequently referred to as global proteomics. Further investigations have lead to the realisation that gene expression does not always correlate with protein expression [17]; however, there appears to be approximately 10,000 or more proteins present in any particular cell type [18]. A further complication is the fact that proteins exhibit a wide dynamic concentration range, estimated to be about 10^7-10^8 in human cells and 10^{12} in plasma [19]. This situation substantially increases the difficulty in the determination of low abundance proteins, frequently thought to be of high importance with regard to cellular function.

Approaches to proteomics investigations have been based on top-down, determination of intact proteins, and bottom-up strategies, the so-called 'shotgun' approach used in the HGP. The bottom-up approach is especially appealing from the standpoint of chromatography and MS detection. However, as in genomic sequencing, the shotgun approach takes an already complex mixture, and through protein digestion creates a mixture of peptides, which by sheer number of analytes, has simply made the problem even more complex. While the bottom-up approach increases sample complexity, substantially increased chromatographic efficiency is obtained for peptides. Yates et al. [20] provides an overview of chromatography and MS issues associated with proteomics investigations in a recent review.

Global Proteomics - Sample Simplification

Given the aforementioned sample complexity situation associated with shotgun proteomics it is not surprising that researchers have sought approaches to simplify the task. In order to achieve this result and allow for relative quantification, lead Gygi et al. [17] to develop a derivatisation approach for proteomics investigations. These researchers developed a cysteine selective reagent, termed ICAT, which additionally featured a biotin-moiety and the ability to incorporate multiple deuterium atoms as a stable isotope, thus the availability of 'heavy' and 'light' versions of the reagent. The combination of selective

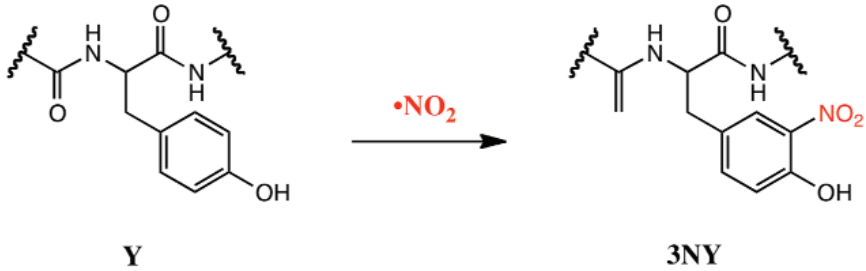

Figure 1: Post-translational nitro-oxidative modification of protein tyrosine

derivatisation, affinity chromatography (avidin-column), and mass shift simultaneously allowed for sample simplification and relative quantification by MS. Subsequently, Liu et al. described a similar overall approach, termed QCET, which utilised trypsin to enhance ^{16}O-^{18}O exchanges and a covalent reaction with a thiol specific resin [21].

Relative Proteomics - Post Translational Modifications & Protein 3-Nitrotyrosine

Post-translational modification (PTM) is a term used to describe covalent chemical modification of various protein amino acid residues subsequent to their formation via gene expression. Prabakaran et al. have recently provided an extensive overview of observed PTMs and their potential biological role [22] and Dalle-Donne have similarly reviewed PTMs focused on alteration of proteins due to oxidative/nitrosative stress in disease states [23]. We are particularly interested in the detection of an oxidative PTM, 3-nitrotyrosine (3NY), which is formed due to oxidative processes [23, 24] wherein one of the phenolic *ortho*-positions undergoes a substitution reaction with the elements of NO_2 (Figure 1). Current evidence indicates that 3NY is a low abundance modification, whose biological roles as simply a biomarker or an evolved functional alteration continues to be debated [24-26]. Regardless of biological situation, as a low abundance modification with a frequency of occurrence of ranging from one (1) 3NY residue per 100,000 tyrosine residues in normal patients to one (1) 3NY residue per 1200 tyrosine residues in extracted atherosclerotic plaques [25].

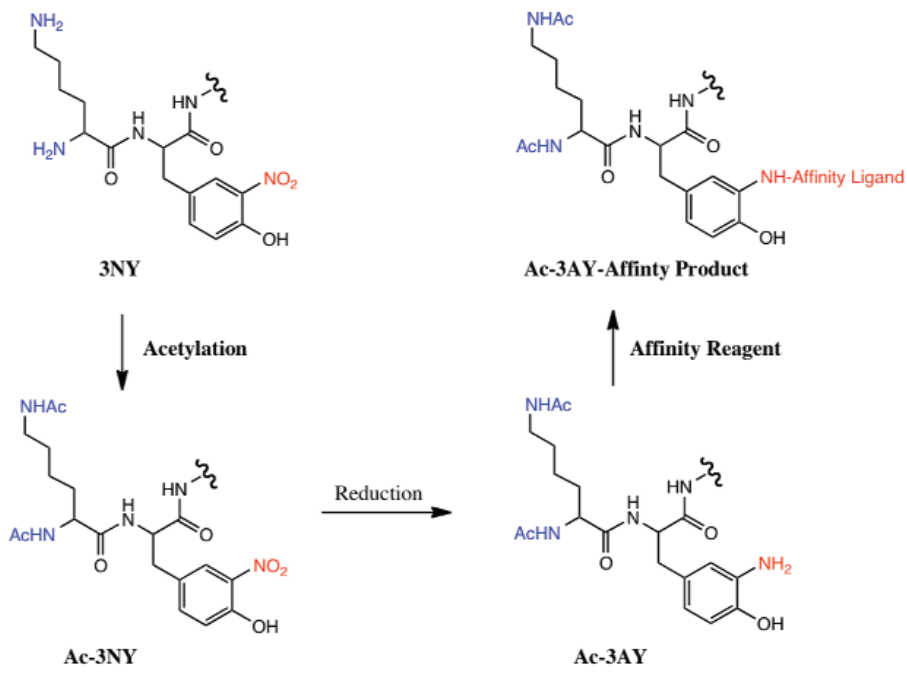

Figure 2: Required amine masking reactions used in methods employing affinity-based enrichment of 3NY-peptides based on ligand attachment on the 3AY moiety

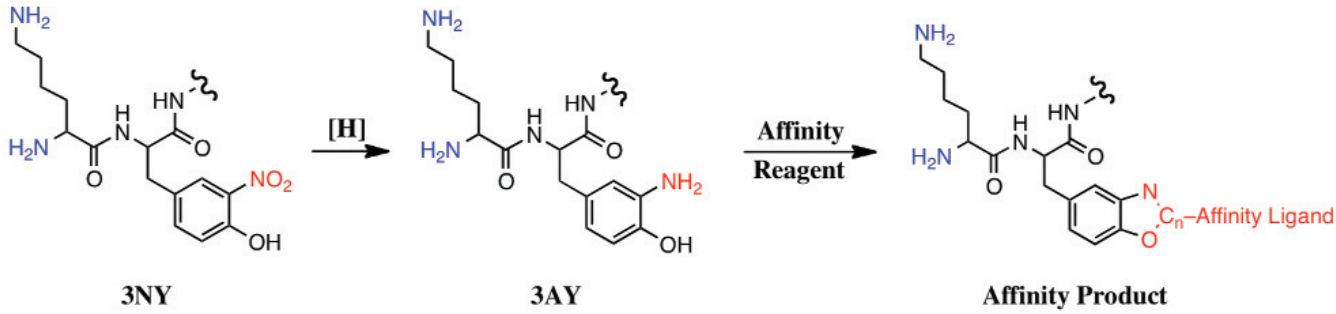

Figure 3: Derivatisation strategy envisioned to achieve extreme selectivity in derivatisation of 3AY-residues potentially allowed for multiple detection approaches

3NY Determination - Derivatisation of 3-AminoTyrosine for Enrichment

Past approaches for the determination of protein 3NY have been largely based on isolation of the protein mixture of interest, followed by enzymatic digestion and subsequent chromatographic fractionation (one or multiple dimensions), with detection by MS. Derivatisation reactions have been frequently employed in these various schemes as a way to achieve one or more of the following attributes: quantification or relative quantification, analyte enrichment, and formation of products amenable to fluorescence detection and/or MS detection. Two research groups have [27, 28] described approaches for the affinity enrichment of 3NY-peptides, the general approach being illustrated in Figure 2. In each of the methods, the free amines are first peracylated (3NY→Ac-3NY), followed by reduction of the nitro-moiety to the corresponding 3-aminotyrosine (Ac-3NY→Ac-3AY), which is utilised as the site of further chemical elaboration to attach an affinity ligand. One group [27] elaborated the Ac-3AY moiety to a free sulfhydryl (via reaction with N-succimidyl-S-acetylthioaceate followed by hydrolysis of the acetyl-group), which was captured, as previously noted [21], by covalent reaction with a thiol-specific resin to effect analyte enrichment. Release, followed by sulfhydryl-capping with iodoacetamide provided an analyte tool that was subsequently determined by MS. Alternatively, the Ac-3AY was reacted with N-hydroxysuccimidyl-biotin [28], thus enabling avidin affinity chromatography to accomplish a selective enrichment of the target peptides, which again were characterised by MS. While these two methods provide excellent examples of the utilisation of derivatisation, chromatography and MS for the determination of protein 3NY, there are numerous other approaches that have been described, which more or less follow the chemical strategy of nitro-group reduction followed utilisation of the resulting amine as the based of selectivity isolation steps. A recent review by Feeney et al. provides an overview of these methodologies [29].

3NY Determination - Chemistry for Extreme Derivatisation Selectivity

Examination of the reaction pathway of Figure 2 reveals an interesting fact; addition of the affinity moiety requires exclusive reaction with amine moiety of the 3AY residue. However, in the approaches noted above [27, 28] and others [29], in order to achieve reaction selectivity, it is necessary to block various other amine groups (Figure 2) or the affinity-bearing reagent will undergo reaction with various unwanted amine nucleophiles, which negatively effects analyte enrichment and reduction in sample complexity. A potentially high selectivity derivatisation approach would exploit the unique structural feature of peptide 3NY residues, the presence of ortho-functional groups. A conceptual reaction sequence is show in Figure 3, where the first step, similar to other methods, requires nitro-moiety reduction, but the second step invokes the use of each of the *ortho* functional groups to form a bicyclic product, thus inherently imparting a high degree of selectivity and, if properly designed, does not require any prior masking of other amines. Upon review

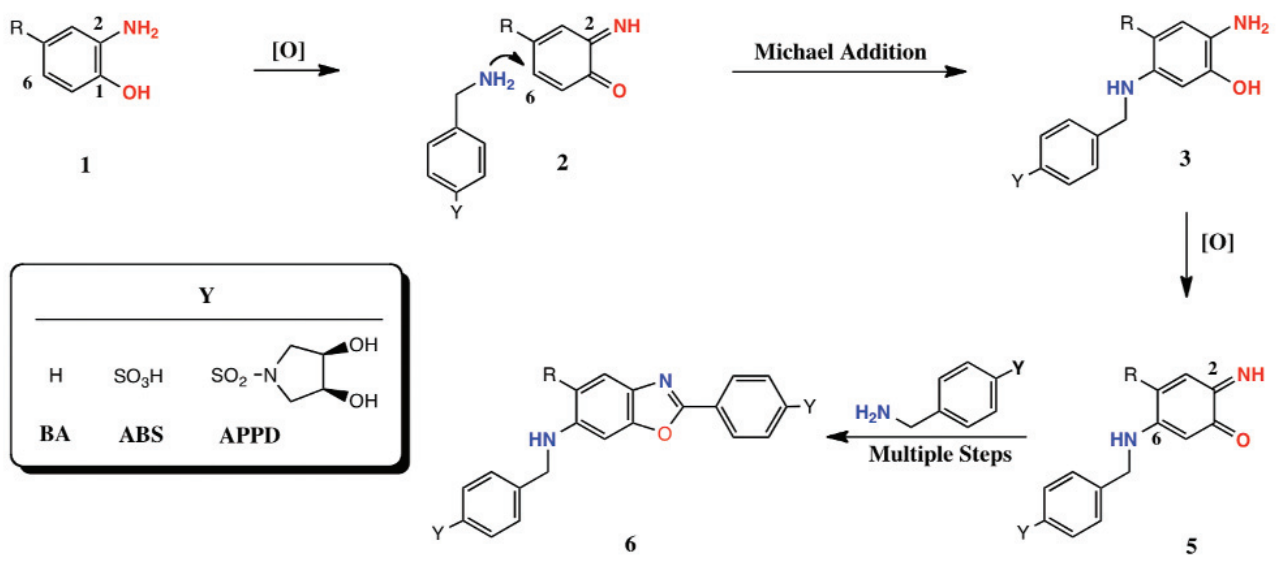

Figure 4: Proposed reaction pathway for the general transformation of 3AY residues to 6-substituted PBO products illustrating key transformation steps

of various reactions, benzylamine (BA) and/or diphenylethylenediamine (DPE), reagents previously used for the fluorogenic derivatisation of catechols, emerged as a candidates [30-32]. The prior work characterised the reaction as a conversion of the catechols-moiety to an efficiently fluorescent 2-phenybenzoxazole (BPO), upon exposure to excess BA or DPA at basic pH in the presence of potassium hexacyanoferrate ($K_3Fe(CN)_6$) and speculated that a key intermediate was the corresponding ortho-quinone. The known ease of oxidation 3AY types substances to the corresponding ortho-iminoquinones lead to the postulated reaction sequence shown in Figure 4. The reaction concept was confirmed in our laboratories [33], with an important structure difference noted in contrast to the original disclosure [31]. As illustrated in Figure 4, the reagent first undergoes addition to an intermediate iminoquinone (2), forming a 6-substituted-2-aminophenol (3), which undergoes an oxidatively mediated series of reactions to ultimately result in the formation of a 6-substituted 2-phenylbenzoxazole (6) (PBO). While most other 3NY derivatisation based methodologies required the masking of analyte amine nucleophiles prior to reduction of the 3NY moiety and utilisation of the nucleophile character of the resulting 3AY, this particular sequence is extremely selectively precisely due to the non-requirement of reacting electrophilic reagents with nucleophilic analyte functional groups and the utilisation of both functional groups, which are transformed to electrophilic intermediates (2 and 5) along the reaction pathway leading to fluorescent PBO formation (6). The presence of excess reagent does not result in the formation of a pool of matrix related interference products from reaction of the sample matrix. When the derivatisation reaction is conducted with BA, a relatively hydrophobic transformation of the target peptides occurs, which can result in inconvenient chromatographic retention. As a result, two reagent analogues were designed and synthesised, with the driving force being increased product polarity or extreme chromatographic selectivity. These substances (Figure 4) are referred to as **ABS** (Y = sulphonic acid) and **APPD** (Y = (3R,4S)-1- sulphonyl-pyrrolidine-3,4-diol). A noteworthy feature of entire regent family is the ease of incorporation of stable isotopes in each case, thus enabling relative quantification in proteomic investigations. Figure 5 illustrates the reaction scheme, experimental conditions, and fluorescence emission spectra (excitation 360 nm) obtained by APPD derivatisation of

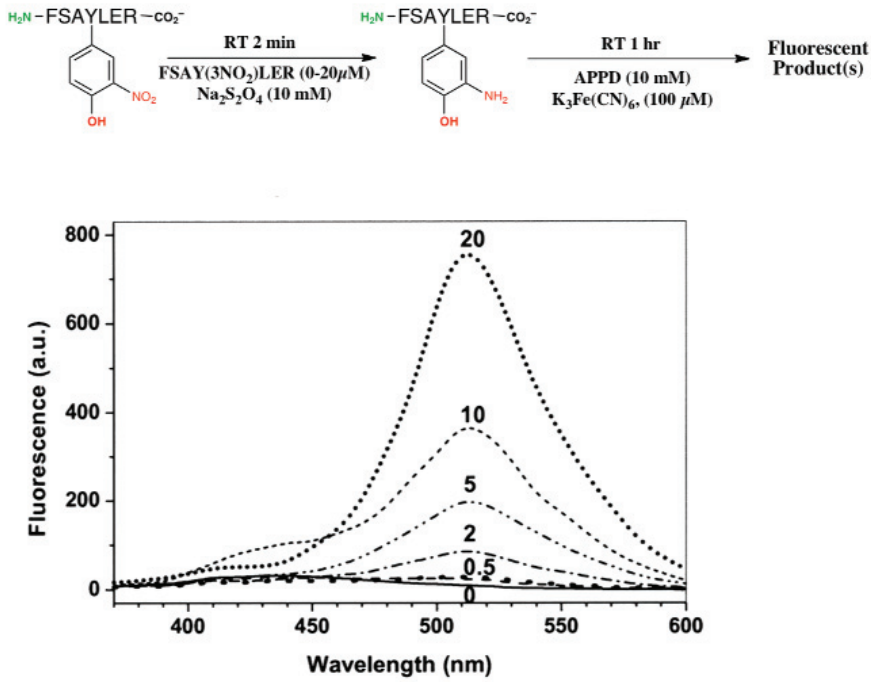

Figure 5: Reaction steps and conditions used in the fluorogenic derivatisation of a synthetic peptide present on mouse phosphorylase b protein. Fluorescence spectra reprinted by permission from ref. 35

the nitrated tyrosine residue of $^{545}FSAY(NO_2)LER^{551}$, a peptide sequence found in mouse muscle phosphorylase b (Ph-b). Examination of the emission spectra reveal a maximum at 510 nm and a slight emission at approximately 430 nm that appeared to be related to degradation of the reagent in the presence of the oxidant as this minor intensity band was observed in a control sample (peptide absent).

Characterisation of the peptide reaction mixture by MALDI-TOF MS revealed the fluorescence band (Figure 5) to be composed of three APPD related PBO products as shown in Figure 6. In each case the expected selective modification of 3AY was realised. Based on relative signal intensity, the products were found to be presence in the following amounts: Type I, 73%; Type II, 20% and Type III, 6%. The first two types are apparently related to the reagent trapping intermediate 2 (Figure 4), with Type II likely formed as a result of subsequent oxidative steps resulting in a dealkylation reaction to leave the 6-amino-substitued product (Type II), while the Type III product results from an intramolecular attached of intermediate 2 (Figure 4) by the peptide N-terminal amine. Similar results have been observed for the related reagent ABS (Figure 4, inset) [34]. For fluorometric measurements, this result has no impart as all

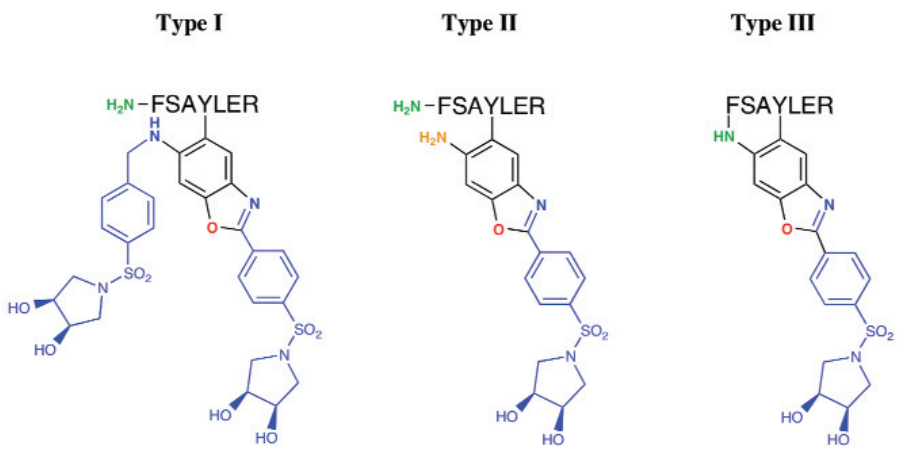

Figure 6: Various 6-substituted PBO type products obtained from derivatisation of the model peptide with APPD.

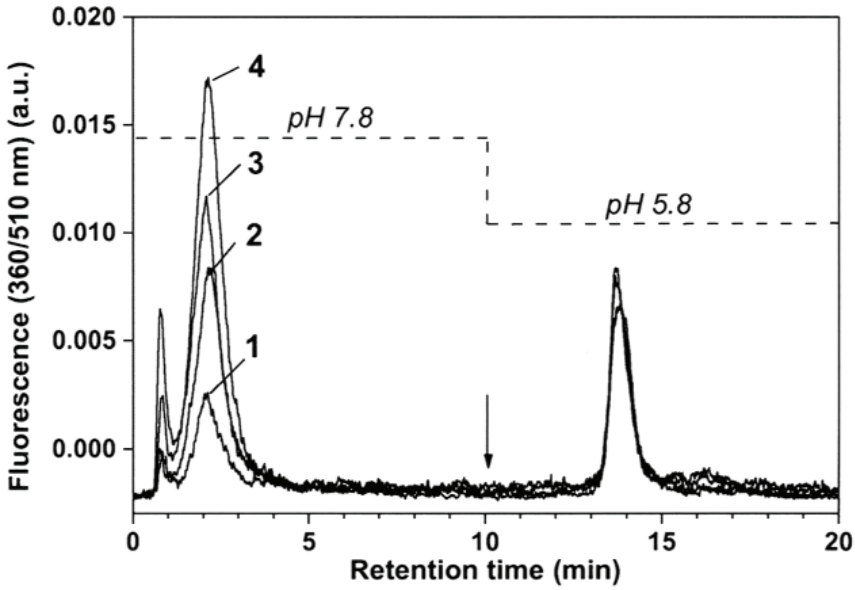

Figure 7: Boronate affinity chromatography of nitrated phosphorylase b protein (1 µg), which had been subject to tryptic digestion and 1 spiked into varying amounts lysate from C2C12 cultured cells (trace 1, 0 mg; trace 2, 0.2 mg; trace 3 0.5 mg; trace 4 1.0 mg) and subjected to the derivatisation protocol with APPD as show in figure 5. The time period from 0-10 minutes represented flow-through unbound material, while the period from 13-18 minutes represents bound 3NY-peptides that were converted to AAPD-PBO derivatives. Refer to the text for further description and ref. 35 for experimental details. The figure is reproduced by permission from ref. 35.

product types display the same fluorescence emission spectra. When these products were subjected to LC-MS/MS characterisation, excellent identifying y-type and b-type ions were formed, and unwanted derivative fragmentation was not observed [35].

A: Flow-through fraction (0-10 minutes)

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01 MSRPLSDQEKRKQISVRGLAGVENVTELKKNFNRHLHFTLVKDRNVATPRDYYFALAHTV
02 RDHLVGRWIRTQQHYYEKDPKRIYYLSLEFYMGRTLQNTMVNLALENACDEATYQLGLDM
03 EELEEIEEDAGLGNGGLGRLAACFLDSMATLGLAAYGYGIRYEFGIFNQKICGGWQMEEA
04 DDWLRYGNPWEKARPEFTLPVHFYGRVEHTSQGAKWVDTGVVLAMPYDTPVPGYRNNVVN
05 TMRLWSAKAPNDFNLKDFNVGGYIQAVLDRNLAENISRVLYPNDNFFEGKELRLKQEYFV
06 VAATLQDIIRRFKSSKFGCRDPVRTNFDAFPDKVAIQLNDTHPSLAIPELMRVLVDLERL
07 DWDKAWEVTVKTCAYTNHTVLPEALERWPVHLLETLLPRHLQIIYEINQRFLNRVAAAFP
08 GDVDRLRRMSLVEEGAVKRINMAHLCIAGSHAVNGVARIHSEILKKTIFKDFYELEPHKF
09 QNKTNGITPRRWLVLCNPGLAEIIAERIGEEYISDLDQLRKLLSYVDDEAFIRDVAKVKQ
10 ENKLKFAAYLEREYKVHINPNSLFDVQVKRIHEYKRQLLNCLHVITLYNRIKKEPNKFVV
11 PRTVMIGGKAAPGYHMAKMIIKLITAIGDVVNHDPVVGDRLRVIFLENYRVSLAEKVIPA
12 ADLSEQISTAGTEASGTGNMKFMLNGALTIGTMDGANVEMAEEAGEENFFIFGMRVEDVD
13 RLDQRGYNAQEYYDRIPELRQIIEQLSSGFFSPKQPDLFKDIVNMLMHHDRFKVFADYEE
14 YVKCQERVSALYKNPREWTRMVIRNIATSGKFSSDRTIAQYAREIWGVEPSRQRLPAPDE
15 KIP
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B: Capture release fraction (13-18 minutes)

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01 MSRPLSDQEKRKQISVRGLAGVENVTELKKNFNRHLHFTLVKDRNVATPRDYYFALAHTV
02 RDHLVGRWIRTQQHYYEKDPKRIYYLSLEFYMGRTLQNTMVNLALENACDEATYQLGLDM
03 EELEEIEEDAGLGNGGLGRLAACFLDSMATLGLAAYGYGIRYEFGIFNQKICGGWQMEEA
04 DDWLRYGNPWEKARPEFTLPVHFYGRVEHTSQGAKWVDTGVVLAMPYDTPVPGYRNNVVN
05 TMRLWSAKAPNDFNLKDFNVGGYIQAVLDRNLAENISRVLYPNDNFFEGKELRLKQEYFV
06 VAATLQDIIRRFKSSKFGCRDPVRTNFDAFPDKVAIQLNDTHPSLAIPELMRVLVDLERL
07 DWDKAWEVTVKTCAYTNHTVLPEALERWPVHLLETLLPRHLQIIYEINQRFLNRVAAAFP
08 GDVDRLRRMSLVEEGAVKRINMAHLCIAGSHAVNGVARIHSEILKKTIFKDFYELEPHKF
09 QNKTNGITPRRWLVLCNPGLAEIIAERIGEEYISDLDQLRKLLSYVDDEAFIRDVAKVKQ
10 ENKLKFAAYLEREYKVHINPNSLFDVQVKRIHEYKRQLLNCLHVITLYNRIKKEPNKFVV
11 PRTVMIGGKAAPGYHMAKMIIKLITAIGDVVNHDPVVGDRLRVIFLENYRVSLAEKVIPA
12 ADLSEQISTAGTEASGTGNMKFMLNGALTIGTMDGANVEMAEEAGEENFFIFGMRVEDVD
13 RLDQRGYNAQEYYDRIPELRQIIEQLSSGFFSPKQPDLFKDIVNMLMHHDRFKVFADYEE
14 YVKCQERVSALYKNPREWTRMVIRNIATSGKFSSDRTIAQYAREIWGVEPSRQRLPAPDE
15 KIP
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Figure 8: Proteomic determination of peptides via capillary LC with FTICR MS detection and data analysis using a Seaquest search against mouse and rabbit databases. Details are described in ref. 35.

3NY Determination - Extreme Chromatographic Selectivity

The APPD reagent features a *cis*-diol moiety, which allows derivatisation products to be isolated with extremely high selectivity via the use of boronate affinity chromatography. In a 'mock' real sample, tryptic digests of the lysate obtained from C2C12 cultured cells were combined in varying amounts (Figure 7: trace 1, 0 mg protein; trace 2, 0.2 mg protein; trace 3, 0.5 mg protein; and trace 4, 1.0 mg protein) with nitrated Ph-b (1 µg contained 100 pmol 3NY residues) and the complete derivatisation scheme conducted (reduction from 3NY to 3AY, followed by reaction with APPD). These reaction mixtures were subjected to boronate affinity chromatography/fluorescence detection (Figure 6), at pH 7.8, with eluent from the first 10 minutes being collected as a single fraction, followed by a shift to pH 5.8, with the 13-18 minute eluent range being collected. The non-APPD related substances were not retained, while the AAPD derivatives were first selectively retained (pH 7.8), then subsequently released in a mobile phase of increased acidity (pH 5.8) [35]. In each case, the recovery of APPD derivatives appears to be similar, despite the increasing quantities of other proteins, which at the extreme is a situation where only 1 of 200 proteins contains a 3NY residue if one assumes a 50 kDa average molecular weight for each protein. In a separate experiment, when non-retained and retained fractions were subjected to proteomic analysis, with Figure 8A showing the sequence coverage found in the flow-through fraction and Figure 8B indicating the sequence coverage for the fraction retaining by the boronate affinity interaction. Upon critical examination of these data, it is noted that the flow-through fraction exhibits a larger sequence coverage as compared to the affinity captured fraction, with several instances of Y-residues in the former that were not observed in the latter. This is not a failure of the methodology, but rather to be expected as Ph-b was not exhaustively nitrated and nitration of Y-residues is known to be highly variable [24, 25]. Further all peptides noted in the affinity-captured fraction did contain nitrated Y (3NY), thus validating the extreme selectivity of the approach.

Chromatography - Extreme Peak Capacity

Presently, numerous manufacturers offer UHPLC systems that typically are capable of operation in the 10-15K psi regime, thus

allowing sub-2µm particles to be taken advantage of. Such columns are generally available in lengths up to 25 cm before pressure becomes an operational limitation. Peak capacity for these 1D UHPLC systems is generally reported to be less than 300 and more typically observed to be under 200 in practice [36, 37]. For applications requiring additional peak capacity, multidimensional separations are often employed. One technique that has received a significant amount of attention is the multidimensional protein identification technology (MudPIT) approach, which was pioneered by Yates for the analysis of peptides. [38, 39]. This approach combines cation-exchange and reversed-phase particles into a single capillary column, allowing step-wise elution of peptides from the cation-exchange portion of the column followed by reversed-phase gradient elution of the peptides. Another commercially available approach from Waters Corporation implemented reversed-phase separations of peptides in both the first and second-dimensions by operating a high/low pH scheme. The selectivity is sufficiently altered by operating at pH 10 and subsequently at pH 2 to give orthogonal separations [40]. More recently, attention has been given to the pre-fractionation of intact proteins. In these methods, proteins are separated and fractions collected with each fraction then being subjected to enzymatic digestion prior to LC/MS/MS analysis for protein identification and quantification [41, 42]. While greater peak capacity is achieved for all three of these approaches, it comes at a cost of greater complexity in instrumentation and increased analysis time. When possible, it has generally been desired to utilise single-dimensional separations as opposed to the multidimensional approaches often needed for greater peak capacity. Operational simplicity is a significant reason for this. Data analysis tends to be more straightforward for single-dimensional approaches as well. However, the pressure limit of available instrumentation has presented a significant hurdle in generating greater peak capacities for single-dimensional approaches in a reasonable amount of time. In 2005, Yates showed a custom separation system capable of operating at an upper pressure limit of 20,000 psi, yielding peak capacities of ~1,000 in 400 minutes [43]. Alternatively, there have been efforts utilising methods on current instrumentation that have given peak capacities of ~750 in 600 minutes when employing custom-packed metre long columns [44]. Research by the Jorgenson group has long focused on implementing pressures far beyond what is commercially available, routinely operating above 45,000 psi and as high as 100,000 psi [45]. Recently, efforts have been made to combine columns greater than 1 meter in length packed with sub-2µm particles for operation at, or exceeding, 30,000 psi. Columns were fabricated via a slurry-packing methodology, with an example of such a packing apparatus shown in Figure 9 [46]. Separations utilising these columns were accomplished via a constant pressure approach with a Haskel pneumatic driven liquid pump serving as the driving force. This extreme ultra-performance liquid chromatography system (XUPLC) is based on the nanoAcquity UPLC platform from Waters Corporation with nearly all components being commercially available and software control accomplished via the existing interface (Figure 10). Briefly, gradients are loaded in reverse on to a gradient storage loop. The sample injection is then made and placed at the end of the storage loop [37]. Changing the state of the appropriate valves then allows the Haskel pump to drive the sample and gradient onto the column while protecting the nanoAcquity from the ultrahigh-pressures. This system is typically operated at 30,000 psi although much higher pressures are theoretically possible given the capability of the pump. An example of chromatograms typical of this system is observed in Figure 11. Peak capacities of approximately 770 in a total analysis time of 400 minutes are possible with this system [46].

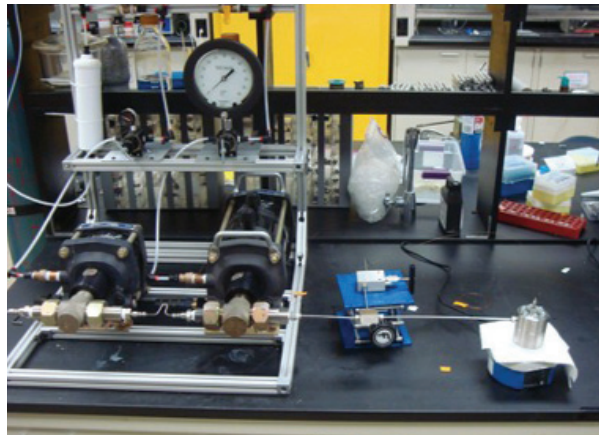

Figure 9: An apparatus used for packing capillaries (typically 75 µm id x 1-2 m) for used in XUPLC separations.

Summary and Conclusions

Review of trends in chromatographic based bioanalytical methods over the past several decades provides for interesting conclusions to be reached. Initially various derivatisation chemistries for the enhancement of detectability were frequently employed, particularly in the case of limited analytes. As chromatographic systems and chromatographic phase development significantly advanced, together with the advent of quantitative MS techniques, method development shifted towards these technologies, with less emphasis on derivatisation techniques. However, with increasing emphasis on the bioanalysis of complex mixtures there was a return towards the use of derivatisation reactions, but now

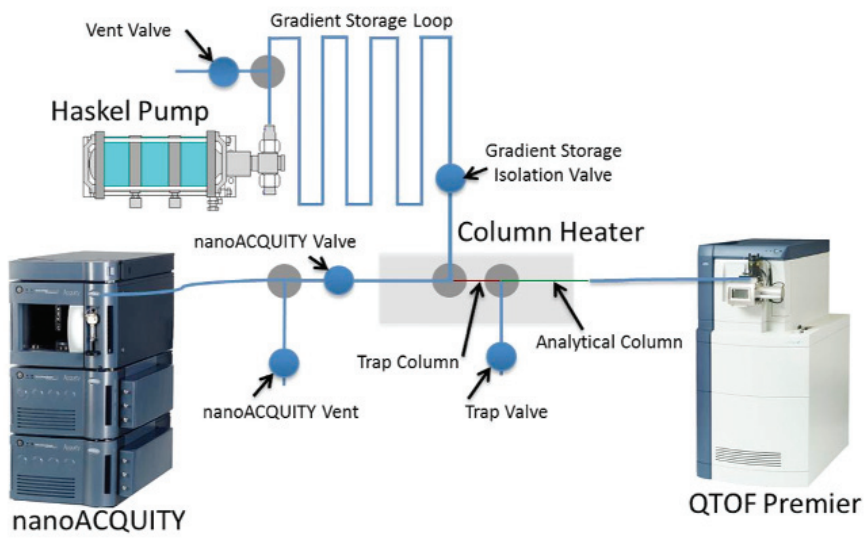

Figure 10: Schematic diagram for the XUPLC add-on to a commercially available UPLC system and associated MS.

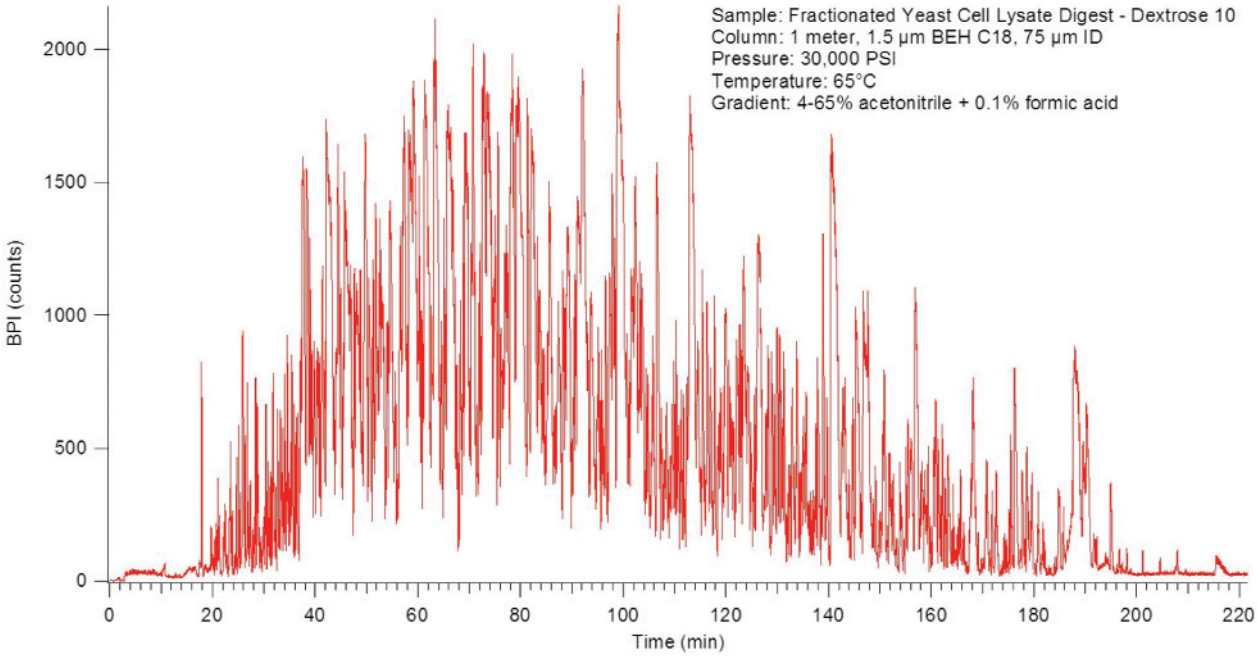

Figure 11: Chromatogram generated from the determination of yeast cell lysate digest using the XUPLC system shown in Figure 10.

in combination with advanced LC-MS platforms. Examples of the extremes of chromatographic selectivity and peak capacity were provided. Overall, it can be concluded that there are still ample opportunities for the integration of chemistry and chromatography, which is only limited by the resourcefulness of the modern bioanalytical chemist.

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References

1. D.R. Knapp, "Handbook of Analytical Derivatisation Reactions", John Wiley & Sons, New York, 1979.

2. R.W. Frei and J.F. Lawrence, eds, "Chemical Derivatisation in Analytical Chemistry", Volume 1: Chromatography, Elsevier, Amsterdam, 1981.

3. R.WQ. Frei and J.F. Lawrence, eds, "Chemical Derivatisation in Analytical Chemistry", Volumn 2: Separation and Continuous Flow Techniques, Elsevier, Amsterdam, 1982.

4. H. Lingeman and W.J.M. Underberg, eds, "Detection-Oriented Derivatisation Techniques in Liquid Chromatography", Chromatographic Science Series Volume 48, Marcel Dekker, New York, 1990.

5. K. Blau and J. Halket, eds, "Handbook of Derivatives for Chromatography", 2nd ed., John Wiley & Sons, Chichester, England, 1993.

6. T. Toyo'oka, ed., "Modern Derivatisation Methods for Separation Sciences", John Wiley & Sons, Chichester, England, 1999.

7. K.L Bush, "Derivatisation in Mass Spectrometry", Spectroscopy, November 1, 1990.

8. V. Zaikin and J.M. Halket, "A Handbook of Derivatives for Mass Spectrometry", IM Publications LLP, Chichester, UK, 2009.

9. J.M. Halket and V.G. Zaikin, "Derivatisation in Mass Spectrometry - 1. Silylation", Eur. J. Mass Spectrom, 9(1), 1-21 (2003).

10.V.G. Zaikin and J.M. Halket, "Derivatisation in Mass Spectrometry - 2. Acylation", Eur. J. Mass Spectrom, 9(5), 421-434 (2003).

11.J.M. Halket and V.G. Zaikin, "Derivatisation in Mass Spectrometry - 3. Alkylation", Eur. J. Mass Spectrom, 10(1), 1-19 (2004).

12.V.G. Zaikin and J.M. Halket, "Derivatisation in Mass Spectrometry - 4. Formation of Cyclic Derivatives", Eur. J. Mass Spectrom, 10(4), 555-568 (2003).

13.J.M. Halket and V.G. Zaikin, "Derivatisation in Mass Spectrometry - 5. Specific Derivatisation of Monofunctional Compounds", Eur. J. Mass Spectrom, 11(1), 127-160 (2005).

14.V.G. Zaikin and J.M. Halket, "Derivatisation in Mass Spectrometry - 6. Formation of Mixed Derivatives of Polyfunctional Compounds ", Eur. J. Mass Spectrom, 11(6), 611-636 (2003).

15.J.M. Halket and V.G. Zaikin, "Derivatisation in Mass Spectrometry - 7. On-line Derivatisation/Degradation", Eur. J. Mass Spectrom, 12(1), 1-13 (2005).

16.V.G. Zaikin and J.M. Halket, "Derivatisation in Mass Spectrometry - 8. Soft Ionization Mass Spectrometry of Small Molecules", Eur. J. Mass Spectrom, 12(2), 79-115 (2003).

17.S.P. Gygi, Y. Rochon, B.R. Franza and R. Abersold, "Correlation Between Protein and mRNA Abundance in Yeast", Mol. Cell. Biol. 19, 1720-1730 (1999).

18.S. Julka and F. Regnier, "Quantification in Proteomics through Stable Isotope Coding: A Review", J. Proteome Res., 3, 350-363 (2004).

19.L.V. Schneider and M.P. Hall, "Stable

Isotope Methods for High-Precision Proteomics", Drug Discovery Today, 10(5), 353-363 (2005).

20.J.R. Yates, C.I. Ruse and A. Nakorchevsky, "Protreomics by Mass Spectrometry: Approaches, Advances and Applications", Annu. Rev. Biomed. Eng., 11, 49-79 (2009).

21.T. Liu, W.-J. Qian, E.F. Strittmatter, D.G. Camp, G.A. Anderson, B.D. Thrall and R.D. Smith, "High-Throughput Comparative Proteome Analysis Using a Quantitative Cysteinyl-peptide Enrichment Technology", Anal. Chem., 76, 5345-5353 (2004).

22.S. Prakakarn, G. Lippens, H. Steen and J. Gunawardene, "Post-Translational Modification: Nature's Escape from Genetic Imprisonment and the Basis for Dynamic Information Encoding", WIREs Syst Biol Med (2012), doi: 10.1002/wsbm.1185.

23.I. Dalle-Donne, A. Scalont, D. Glustarini, E. Cavarra, G. Tell, G. Lungarella, R. Colombo, R. Rossi and A. Milzani, "Proteins as Biomarkers of Oxidative/Nitrosative Stress in Diseases: The Contribution of Redox Proteomics", Mass Spec. Rev., 24, 55-99 (2005).

24.R. Radi, "Protein Tyrosine Nitration: Biochemical Mechanisms and Structural Basis of Functional Effects", Acc. Chem. Res., 46(2), 550-559 (2013).

25.J.M. Souza, G. Peluffo and R. Radi, "Protein Tyrosine Nitration - Functional Alteration or Just a Biomarker?", Free Radical Biol. Med., 45, 357-366 (2008).

26.M.B. Feeney and C. Schöneich, "Tyrosine Modifications in Aging", Antioxid. Redox Signal., 17(11), 1571-79 (2012).

27.Q. Zhang, W.-J. Qian, T.V. Knyushko, T.R.W. Clauss, S.O. Purvine, R.J. Moore, C.A. Sacksteder, M.H. Chin, D.J. Smith, D.G. Camp II, D.J. Bigelow and R.D. Smith, "A Method for Selective Enrichment and Analysis of Nitrotyrosine-Containing Peptides in Complex Proteome Samples", J. Prot. Res., 6, 2257-2268 (2007).

28.N. Abello, B. Barroso, H.A.M. Kerstjens, D.S. Postma and R. Bischoff, "Chemical Labeling and Enrichment of Nitrotyrosine-Containing Peptides", Talanta, 80, 1503-1512 (2010).

29.M.B. Feeney and C. Schöneich, "Proteomic Approaches to Analyze Protein Tyrosine Nitration", Antioxid. Redox Signal., 19(11), 1247-56 (2013).

30.H. Nohta, A. Mitsui and U. Ohkura, "Spectrofluorimetric Determination of Catecholamines with 1,2-Diphenylethylenediamine", Anal. Chim. Acta, 165, 171-176 (1984).

31.H. Nohta, M.-K. Lee and Y. Ohkura, "Fluorescent Products of the Reaction for the Determination of Catecholamines with 1,2-Diphenylethylenediamine", Anal. Chim. Acta, 267, 137-139 (1992).

32.H. Nohta, T. Yukizawa, M. Yoshimura, J. Ishida and M. Yamaguchi, "Aromatic Glycinonitriles and Methylamines as Pre-Column Fluorescence Derivatisation Reagents for Catecholamines", Anal. Chim. Acta, 344, 233-240 (1997).

33.J.P. Pennington, C. Schöneich and J.F. Stobaugh, "Selective Fluorogenic Derivatisation with Isotopic Coding of Catechols and 2-Aminophenols with Benzylamine: A Chemical Basis for the Relative Determination of 3-Hydroxytyrosine and 3-Nitrotyrosine Peptides", Chromatographia, 66, 649-659 (2007).

34.V.S. Sharov, E.S. Dremina, N.A. Galeva, G.S. Derstenecker, X.-B. Li, R.T. Dobrowsky, J.F. Stobaugh and C. Schöneich, "Fluorogenic Tagging of Protein 3-Nitrotyrosine and 4-(aminomethyl)-benzenesulfonic acid for Quantitative Analysis of Protein Nitration", Chromatographia, 71, 37-53 (2010).

35.E.S. Dremina, X.-B. Li, N.A. Galeva, V.S. Sharov, J.F. Stobaugh and C. Schöneich, "A Methodology for Simultaneous Fluorogenic Derivatisation and Boronate Affinity Enrichment of 3-Nitrotyrosine-Containing Peptides", Anal. Biochem., 418, 184-196 (2011).

36. J.A. McLean, B.T. Ruotolo, K.J. Gillig, D.H. Russell, "Ion moibility-mass spectrometry: a new paradigm for proteomics", International Journal of Mass Spectrometry, 240, 301-315 (2005).

37.J.T. Stobaugh, "Strategies for Differential Proteomic Analysis by Liquid Chromatography-Mass Spectrometry", Ph.D. Thesis, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA (2012).

38.D.A. Wolters, M.P. Washburn, J.R. Yates, "An Automated Multidimensional Protein Identification Technology for Shotgun Proteomics", Anal. Chem., 73, 2183-2191 (2009).

39.M.P. Washburn, R. Ulaszek, C. Deciu, D.M. Schieltz, J.R. Yates, "Analysis of Quantitative Proteomic Data Generated via Multidimensional Protein Identification Technology", Anal. Chem., 74, 1650-1657 (2002).

40.M. Gilar, P. Olovova, A.E. Daly, J.C. Gebler, "Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in first and second dimensions" J. Sep. Sci., 28, 1694-1703 (2005).

41.J. Martosella, N. Zolotarjova, H. Liu, G. Nicol, B.E. Boyes, "Reversed-Phase High-Performance Liquid Chromatographic Prefractionation of Immunodepleted Human Serum Proteins to Enhance Mass Spectrometry Identification of Lower-Abundant Proteins", J. Prot. Res., 4, 1522-1537 (2005).

42.J.T. Stobaugh, K.M. Fague, J.W. Jorgenson, "Prefractionation of Intact Proteins by Reversed-Phase and Anion-Exchange Chromatography for the Differential Proteomic Analysis of Saccharomyces cerevisiae", J. Prot. Res., 12, 626-636 (2012).

43.Y. Shen, R. Zhang, R.J. Moore, J. Kim, T.O Metz, K.K. Hixson, R. Zhao, E.A. Livesay, H.R. Udseth, R.D. Smith, "Automated 20 kpsi RPLC-MS and MS/MS with Chromatographic Peak Capacities of 1000-1500 and Capabilities in Proteomics and Metabolomics", Anal. Chem., 77, 3090-3100 (2005).

44.F. Zhou, Y. Lu, S.B. Ficarro, J.T. Webber, J.A. Marto, "Nanoflow Low Pressure High Peak Capacity Single Dimension LC-MS/MS Platform for High-Throughput, In-Depth Analysis of Mammalian Proteomes", Anal. Chem., 84, 5133-5139 (2012).

45.K.D. Patel, A.D. Jerkovich, J.C. Link, J.W. Jorgenson, "In-Depth Characterization of Slurry Packed Capillary Columns with 1.0-µm Nonporous Particles Using Reversed-Phase Isocratic Ultrahigh-Pressure Liquid Chromatography", Anal. Chem., 76, 5777-5786 (2004).

46. E.G. Franklin, "Utilization of Long Columns Packed with Sub-2 µm Particles Operated at High Pressures and Elevated Temperatures for High-Efficiency One-Dimensional Liquid Chromatographic Separations", Ph.D. Thesis, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA (2012).