Published in final edited form as:
Anal Chem. 2011 March 15; 83(6): 2187–2193. doi:10.1021/ac103023q.

Parallel Detection of Intrinsic Fluorescence from Peptides and Proteins for Quantification During Mass Spectrometric Analysis

Jason D. Russell[†,§], **Ryan T. Hilger**[†], **Daniel T. Ladror**[†], **Mark A. Tervo**[†,§], **Mark Scalf**[†], **Michael R. Shortreed**[†], **Joshua J. Coon**[†,‡,§], and **Lloyd M. Smith**[†,§]

[†] Department of Chemistry, University of Wisconsin-Madison, Madison, WI 53706

[‡] Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI 53706

[§] Genome Center of Wisconsin, University of Wisconsin-Madison, Madison, WI 53706

Abstract

Direct mass spectrometric quantification of peptides and proteins is compromised by the wide variabilities in ionization efficiency which are hallmarks of both the MALDI and ESI ionization techniques. We describe here the implementation of a fluorescence detection system for measurement of the UV-excited intrinsic fluorescence (UV-IF) from peptides and proteins just prior to their exit and electrospray ionization from an ESI capillary. The fluorescence signal provides a quantifiable measure of the amount of the protein or peptide present, while direct or tandem mass spectrometric analysis (MS/MS) on the ESI-generated ions provides information on identity. We fabricated an inexpensive, modular, fluorescence excitation and detection device utilizing an ultraviolet light-emitting diode for excitation in a ~300 nL fluorescence detection cell integrated into the fused-silica separation column. The fluorescence signal was linear over 3 orders of magnitude with on-column limits of detection in the low femtomole range. Chromatographically separated intact proteins analyzed using UV-IF prior to top-down mass spectrometry demonstrated sensitive detection of proteins as large as 77 kDa.

INTRODUCTION

Global and targeted protein analysis can provide powerful insights into the complexities of biological systems. Mass spectrometry (MS) has become a principal tool for comprehensive, high-throughput identification of proteins and their post-translational modifications (PTMs), permitting routine identification of thousands of proteins and PTMs from shotgun experiments. [1–3] Quantification of proteins and PTMs from these experiments, however, is required to further our understanding of the dynamics and subtleties of biological systems. MS-based quantification techniques can be broadly classified as label and label free.[4–7] Both approaches determine analyte concentration using gas-phase ions most commonly generated by electrospray ionization (ESI) and measured by MS. Yet highly variable peptide and protein ionization efficiencies often complicate quantification using gas-phase measurements.[8–13] In addition to matrix effects, instrument platform-specific mass spectrometer biases may be introduced, making cross-platform quantification comparisons difficult. A recent analysis of simple protein mixtures using MS-based quantification showed a high degree of variability across multiple labs using a variety of quantification techniques.[14] Although this variability may stem from multiple factors, clearly, technique robustness, dynamic range, and ease of implementation can affect quantification accuracy, precision, and reproducibility.

Correspondence to: Lloyd M. Smith.

Because of these difficulties in achieving quantification by gas-phase measurements of peptide or protein ions, we investigated the alternative of employing solution-phase measurements prior to ionization. The amino acids phenylalanine, tyrosine, and tryptophan exhibit ultraviolet light-induced intrinsic fluorescence (UV-IF). Tryptophan-containing analytes have higher molar absorptivities relative to species containing phenylalanine or tyrosine, and emit UV-IF at longer wavelengths. These chemical properties favor the measurement of UV-IF from tryptophan for the most sensitive native fluorescence assays. Capillary electrophoresis (CE) with laser-induced fluorescence (LIF) has been widely used for the separation and detection of compound mixtures exhibiting UV-IF.[15–20] For example, using CE-LIF, Lee and coworkers demonstrated a linear dynamic range of 5 orders of magnitude for tryptophan and 4 orders of magnitude for bovine serum albumin with low picomolar detection limits.[21] Separation by liquid chromatography (LC) followed by UV absorbance and UV-IF detection demonstrated that the ratio of intrinsic fluorescence to UV absorbance could discriminate tyrosine- and tryptophan-containing peptides within a protein digest.[22] Heath and Giordani expanded on this technique by performing ESI-MS after UV and UV-IF detection. The combination of techniques aid in peptide sequence assignment.[23] While UV laser sources are most commonly employed for experiments measuring UV-IF in capillaries,[15–19,21,24] advances in UV-Light-Emitting Diode (LED) technology have facilitated production of small, inexpensive LEDs with increased lifetimes, providing an attractive alternative to UV lasers. Sluszny *et al.* demonstrated nanomolar native fluorescence limits of detection for intact proteins using capillary zone electrophoresis with UV-LED excitation.[25]

In previous studies, interrogation of biomolecules by UV-IF and LC-MS utilized LC flow-splitting, microliter flow rates, microfluidic devices, or separate analyses.[23,26,27] However, LC-MS analyses are routinely performed in capillary columns in the nanoflow regime for enhanced ESI-MS sensitivity.[13] The fluorescence excitation and detection system and associated techniques described here provide simultaneous UV-IF and MS detection compatible with common nanoLC-ESI-MS platforms. Our strategy eliminates the need to perform separate UV-IF and MS analyses and minimizes LC dead volume; a critical parameter affecting chromatographic performance, analysis time, and reproducibility at nanoliter flow rates. We designed and constructed a modular fluorescence excitation and detection system employing a UV-LED excitation source, simple optics, and a sensitive photomultiplier. The design permits facile interfacing with MS inlets and allows the quick exchange of inexpensive source and optical components to accommodate fluorescence studies using a variety of excitation and emission wavelengths. To allow simultaneous collection of fluorescence and MS data in the nanoflow regime, we integrated a fluorescence detection cell (~ 300 nL) with electrospray emitter into a single fused-silica capillary separation column employing splitless nanoflow gradient elution. Here, we characterize the excitation and detection device and evaluate its utility as a tool for label-free protein analysis.

EXPERIMENTAL SECTION

Chemicals and Reagents

Tryptophan-containing peptides LPRSAYWHHITG, WWGKKYRASKLGLAR, FLWGPRALV, and YGGFLRRIRPKLKWDNQ were purchased from AnaSpec (Fremont, CA) in > 95% purity and were reconstituted in 0.2% formic acid for immediate use. AQUA QuantPro™ peptides were purchased from Thermo Scientific (Bremen, Germany) in solution form with manufacturer validated concentrations within +/− 25% of the stated amount. High-purity formic acid and acetonitrile were purchased from Fisher Scientific (Waltham, MA). A NANOpure® Diamond™ deionization system (Barnstead International; Dubuque, IA) was used to generate 18.2-MΩ water used in LC analyses. All additional

standard proteins and reagents were obtained from Sigma Aldrich (Milwaukee, WI) unless otherwise specified.

Fluorescence Excitation and Detection Device

The design of the fluorescence excitation and detection system was modeled after that of Sluszny and co-workers[25] and adapted for use with nanoLC (Figure 1 and S.I. Figure 1). A capillary LC column with detection cell and integrated electrospray tip (*vide infra*) was mounted to a breadboard by sliding it through a PEEK tubing sleeve secured into a locating groove on a fixed metal rod. The last 5 to 10 mm of the capillary containing the detection cell extended out over an AlInGaN UV-LED (UVTOP-280-TO39BL, Sensor Electronic Technology, Columbia, SC). The capillary detection cell was placed 11 mm above the center of the LED's integrated ball lens. The LED was placed inside an aluminum sleeve supporting a bandpass interference filter (Semrock, Rochester, NY) with a center wavelength of 280 nm and a bandwidth of 20 nm. A second fused silica ball lens with a diameter of 4 mm (ISP Optics, Irvington, NY) was placed between the LED and the capillary. This lens was mounted on a 3-D translation stage and positioned such that the capillary was nearly resting upon the lens. UV-IF was collected at 90° relative to excitation light using a fused silica plano-convex lens with diameter of 0.5 inch and focal length of 19 mm (Newport, Irvine, CA). This lens was also mounted on a 3-D translation stage. UV-IF was passed through an aperture with a diameter of 0.5 inch positioned 95 mm beyond the collection lens. This aperture was at the entrance of a black delrin tube (1 inch i.d., 75 mm long) that housed a longpass colored-glass filter with a cut-on wavelength of 324 nm (Newport) followed by a bandpass interference filter with a center wavelength of 357 nm and a bandwidth of 44 nm (Semrock). The LED was powered by a LED power supply (LED PS, SandHouse Design, FL) operated at 5–20 mA. Light detection was performed by a R928 photomultiplier tube (Hamamatsu Photonics, Hamamatsu, Japan). The breadboard supporting the entire system was mounted on a 3-D translation stage to allow fine positioning of the electrospray tip at the MS inlet. Signal from the photomultiplier was passed through a home-built current to voltage converter. The voltage was sampled using a PCI-6035E card (National Instruments, Austin, TX) within a personal computer running Windows XP. Signal processing was done using a program written in LabVIEW 8.5 (National Instruments, Austin, TX). Signal was sampled at a rate of 100 kHz and 10^4 samples were averaged to produce one recorded data point resulting in the generation of ten data points per second.

LC Column Fabrication

Micro-capillary columns containing an integrated detection cell and ESI emitter (Figure 1) were fabricated in-house. Fused silica tubing (360 μm o.d. × 200 μm i.d.) was used to prepare analytical columns slurry-packed to 15 cm in length with 3.5 μm, 130 Å particles of XBridge reversed-phase material (Waters Corporation, Milford, MA) for peptide separations and Magic C18 AQ, 5 μm, 300 Å particles (Michrom Bioresources Inc., Auburn, CA) for intact protein separations. Chemical frits were cast by heat-induced polymerization of a silicate solution using a 125 °C soldering iron.[13] Integrated electrospray tips were fabricated using a model P-2000 laser puller (Sutter Instrument Co., Novarto, CA) incorporating a 7–10 mm detection window between the chemical frit and electrospray tip. Precolumns were constructed from 360 μm o.d. × 200 μm i.d. fused silica with a cast chemical frit and slurry-packed with 8–10 cm of the same reversed-phase material used for either peptide or protein separations.

LC-MS/MS

Gradient chromatographic elution was performed using a nanoACQUITY Ultra Performance LC® system (Waters Corporation) at 500–1000 nL min^{-1} analytical flow rates.

An LTQ Orbitrap Velos enabled for ETD (Thermo Scientific, Bremen, Germany) was used for all MS and tandem MS (MS/MS) analyses. All MS selected ion chromatograms were generated from Orbitrap MS^1 scans.

RESULTS AND DISCUSSION

General considerations

Three of the standard 20 amino acids found in proteins are fluorescent - phenylalanine, tyrosine, and tryptophan.[28] Tryptophan is the most sensitive of the three by a substantial factor, due to both a larger molar absorptivity and a greater quantum yield for fluorescence.[29] Its fluorescence emission occurs at a longer wavelength than either phenylalanine or tyrosine, permitting optical discrimination.[30] Tryptophan is also a rare amino acid occurring infrequently in the proteomes of commonly studied organisms.[31] Tryptophan represents 1.1% of the amino acids in the yeast (*Saccharomyces cerevisiae*) proteome (S.I. Figure 2). This scarcity is an advantage in the present study, as it means that fewer peptides or proteins will exhibit a fluorescence signal, reducing the requirements for their separation prior to fluorescence determinations (effectively reducing sample complexity). Yet, greater than 86% of yeast proteins contain tryptophan and 77% will produce a tryptophan-containing tryptic peptide permitting the majority of proteins in this model organism to be interrogated (S.I. table 1). Based on these considerations we chose tryptophan as our target for on-line determination of peptide or protein concentration.

Fluorescence detector design and characterization

We utilized a combination of emission filters that maximized the UV-IF signal-to-noise of tryptophan while minimizing signal collected from tyrosine and phenylalanine (S.I. Figure 3). We selected a 280 nm UV-LED over a UV laser for its ease of implementation, low cost, and compact size. Initial experiments used a 12-V marine battery and home-built circuit to operate the UV-LED between 15–30 mA. Subsequent experiments used a power supply designed to operate the UV-LED at constant current. The power supply provided more consistent LED output and increased LED lifetimes by ~ 25%. The average LED lifetime was 250 h while interfaced with the mass spectrometer. NanoLC separations are commonly performed in packed fused silica capillaries with 360 μm outer diameters and 50–200 μm inner diameters. To reduce chromatographic band broadening, we integrated the detection cell into the fused silica separation column, which was terminated by the electrospray emitter. This design eliminated the need for bulky unions at the column, detection cell, and emitter junctions and allowed for routine LC backpressures exceeding 4000 psi. The choice of capillary inner and outer diameters had a significant impact on fluorescence signal (S.I. Figure 4). We ultimately selected the 360 μm o.d. × 200 μm i.d. capillary as a compromise between signal intensity, capillary strength, and ability to use standard 360 μm o.d. nanoLC fittings.

Effects of pH and solvent on UV-IF

UV-IF of indole-containing compounds, tryptophan, and tryptophan-containing peptides and proteins is modulated by local environment factors, including pH, solvent polarity, temperature, and structure.[32–42] We examined the effects of solvent polarity and pH relevant to low-pH reversed-phase LC-MS separations. Tryptophan-containing peptides were dissolved in 0.2% formic acid with increasing percentages of acetonitrile, approximating a chromatographic gradient (S.I. Figure 5, top). Each peptide solution was infused through an unpacked fused-silica analytical column and UV-IF was measured on-line using the fluorescence detector described above. For each 10% increase in acetonitrile concentration up to 40%, we saw a 4–10% increase in peptide UV-IF signal. A majority of tryptic peptides will elute from a C18 column in this 10–40% acetonitrile range. The trend begins to

diminish at higher percentages of acetonitrile. We examined the effects of mobile-phase pH on UV-IF by infusing peptide solutions in 0.2% formic acid titrated with ammonium hydroxide to the desired pH. The UV-IF signal for three of the four peptides increased modestly with increasing pH (up to 4), at which point they began to level off (S.I. Figure 5, bottom). Interestingly, the UV-IF signal for the peptide containing tyrosine adjacent to tryptophan continued to increase across the tested range, although again the effect is small. The effects of both pH and solvent composition are relatively modest in magnitude, suggesting that these factors do not substantially compromise the utility of the UV-IF detection.

Peptide and protein digest LC analysis

To assess fluorescence detector performance during data-dependent LC-MS/MS analyses, we analyzed an equimolar (+/− 25%) mixture of 15 high-purity AQUA (Absolute QUAntification) peptides.[43] These particular AQUA peptides were chosen, in part, for their ability to yield a strong and consistent positive ion ESI response, and thus this set of fifteen is not likely to be representative of all peptides in a proteome. Normalized UV-IF and MS base-peak chromatographic traces are shown in Figure 2. Importantly, the signal intensity obtained from each of the single tryptophan peptides not containing tyrosine, were similar within a factor of roughly two. The peptide WPGYLNGGR, containing both a tryptophan and tyrosine residue, produced a lower intensity signal relative to the other single tryptophan-containing peptides. It has been reported that intrinsic fluorescence can be affected by both peptide/protein sequence and conformation; and particularly by the presence of aromatic and ionizable residues.[41,44–47] However, in this particular case, we suspect that a low concentration of the peptide is the main factor responsible for the lower signal of WPGYLNGGR, since its normalized UV-IF intensity was in good agreement with its normalized MS intensity. This interpretation is supported by the observation that the signal intensity from the peptide NSWGTDWGEK, containing two nonadjacent tryptophan residues that are sufficiently distanced to minimize intramolecular fluorescence quenching, was roughly two-fold greater that of the other single tryptophan-containing peptides. As expected, no UV-IF signal was detected for peptides lacking tryptophan or tyrosine residues. These results indicate the utility of using UV-IF for obtaining an orthogonal measure of peptide concentration during an LC-MS/MS analysis, independent of the vagaries of peptide ionization efficiency.

To evaluate the approach on a more biologically relevant sample, we digested commercially available proteins containing varying numbers of tryptophan residues and subjected them to online LC-MS/MS and UV-IF detection. UV-IF and MS base-peak chromatograms for three single proteins are given in Figure 3a–c. Ribonuclease (0 Trp, Figure 3a) did not yield an appreciable peptide UV-IF response. Myoglobin (2 Trp, Figure 3b) produced two distinct UV-IF peaks. The major peak was produced by a peptide containing two non-adjacent tryptophan residues. The minor peak was produced by a peptide containing both tyrosine and phenylalanine. Transferrin (8 Trp, Figure 3c) produced 6 major peaks containing tryptophan and more than a dozen low-level peaks. Low-level UV-IF peaks corresponded to tryptophan-containing missed-cleavage peptides, peptides containing tryptophan and oxidized methionine, tyrosine-containing peptides, or peptides that could not be unambiguously identified.

The analysis of transferrin revealed complications when trying to correlate UV-IF, MS, and database outputs in an efficient manner. UV-IF peak correlation to identified peptides from database outputs based on the retention time or spectrum number was often insufficient to confidently match peptide sequences to UV-IF peaks. Several tryptophan-containing peptides and UV-IF peaks often found in the same retention-time window complicated peak-sequence assignment. We achieved the most confident assignment of UV-IF peaks to

peptide sequences by comparing selected ion chromatograms (SIC) of identified peptides to UV-IF peaks. The combination of SIC peak shape, especially peak width, and retention time was often helpful in the sequence assignment process when multiple tryptophan-containing peptides were identified over a small retention time window. To facilitate the process of comparing peptide sequences with SICs and UV-IF peaks, we developed the software FluorAid (S.I. Figure 6). FluorAid allows the user to select a peptide identified from database searching and automatically produces a SIC for the selected peptide. A UV-IF chromatogram is produced on the same graph centered on the retention time of the identified peptide with a user-defined window. The user can quickly scroll through the list of identified peptides to determine the best match. We have found this simple software tool to be of great help for analysis of UV-IF and MS results. The software is open source and freely available at http://www.chem.wisc.edu/~coon/software.php.

Peptide linear dynamic range and quantification

We evaluated the linear dynamic range of detection for UV-IF peptide analysis using peak area integration of three tryptophan-containing peptides identified from a tryptic digest of lysozyme (6 Trp). The UV-IF linear dynamic range was comparable to that of MS ion current in the range of 25 femtomoles to 8 picomoles. UV-IF r^2 values exceeded 0.99, with similar responses for all three peptides (S.I. Figure 7). The UV-IF response of the peptide WWCNDGR, containing two adjacent tryptophan residues, was similar to those containing a single tryptophan, suggesting internal fluorescence quenching. Similar linear dynamic ranges were generated from other single protein digests (data not shown). In nearly all cases, MS SICs provided lower limits of detection (~ 5 fmol) by a factor of 3–5. However, when peptides contained multiple non-adjacent tryptophan residues, UV-IF produced limits of detection approaching sub-fmol levels.

To further assess the use of UV-IF for peptide/protein quantification, an estimated equimolar mixture of conalbumin, transferrin, serum albumin, enolase, and aldolase containing 10, 8, 5, 4, and 3 tryptophan residues, respectively, was digested and analyzed in triplicate. As expected due to the relatively low frequency of tryptophan-containing peptides, the UV-IF chromatogram is considerably less complex than the MS base-peak chromatogram (Figure 4). We compared peak area estimates for resolved or partially resolved tryptophan-containing peptides filtered to a 1% False Discovery Rate (FDR) to peak areas generated from MS SICs (Figure 4) of the same peptides listed in the accompanying table.[48] Peptides identified as missed-cleavage products or containing oxidized methionine residues were grouped for quantification. Response was normalized to the highest responding peptide for UV-IF or MS, respectively. Conalbumin, transferrin, and enolase produced multiple tryptophan-containing peptides. Serum albumin and aldolase each produced one confidently identified peptide and were excluded for quantification. Upon summation and normalization of peak areas from the tryptophan-containing peptides, the UV-IF results yield relative abundances of the three proteins of 0.9:1.0:0.8, while the MS results yield a ratio of 0.7:1.0:0.5. Thus a somewhat improved measure of protein relative abundance was obtained from the UV-IF data than from the MS data, along with improved run-to-run reproducibility. As evident from the standard deviations shown in Figure 4, there is greater variability in the MS peak areas than in the UV-IF peak areas (the average standard errors of the mean for the two sets of data are 4.2% for the UV-IF, versus 7.8% for the MS). There is little correlation between the two responses ($r^2 = 0.36$) for the 31 tryptophan-containing peptides identified from this sample. Yet, both methods produced comparable quantification at the protein level. This suggests these solution- and gas-phase peptide measurements are highly orthogonal providing complementary information about vastly different peptide attributes, i.e., intrinsic fluorescence and ionization/transmission efficiencies. These data demonstrate

that UV-IF can be used to estimate protein abundance from simple protein digests using peptides identified from parallel MS/MS analyses.

Application to top-down LC-MS

We investigated the use of intrinsic fluorescence for the interrogation of intact proteins using top-down LC-MS. Estimated equimolar quantities (~1 pmol ea.) of transferrin, a-casein, and ribonuclease were individually analyzed with composite UV-IF and MS SICs, as shown in Figure 5. Protein relative responses for each detector were determined by peak area integration. MS protein response scaled with protein molecular weight: ribonuclease (13.7 kDa) produced the greatest response, followed by α-casein (24.5 kDa) and transferrin (77 kDa). UV-IF response increased with the number of tryptophan residues. Ribonuclease (0 Trp) produced very little UV-IF, whereas α-casein (2 Trp) and transferrin (8 Trp) produced increasingly greater responses. The peak area of transferrin was ~4 times larger than α-casein and very closely scaled with the number of tryptophan residues in each protein in agreement with previously reported observations.[25] Monitoring UV-IF during top-down analysis may be a promising application for the detection, identification, and quantification of intact proteins. High-quality top-down spectra often require significant spectral averaging, leading to very long MS/MS duty cycles and few measured points across a peak. UV-IF does not suffer the same duty cycle penalties and may offer a label-free approach for intact protein quantification, offering substantially lower limits of detection for both large and tryptophan-rich proteins. Moreover, UV-IF may be particularly advantageous for detection and quantification of larger (> 30 kDa) or poorly ionizing proteins for which the correlation between MS signal and protein abundance is low.

CONCLUSIONS

An inexpensive, modular, nanoLC-compatible fluorescence detection system was constructed and interfaced to a high-resolution tandem mass spectrometer allowing parallel collection of solution-phase ultraviolet light-induced intrinsic fluorescence and gas-phase mass spectrometric data of proteins and peptides. We integrated a separation column, fluorescence cell, and electrospray emitter into a single fused-silica capillary accommodating nanoflow-rates with low dead volume. Device performance was characterized using peptide standards and simple protein digest mixtures demonstrating sensitive label-free UV-IF detection of peptides containing naturally-occurring fluorescent amino acids. UV-IF dynamic range was linear over three orders of magnitude with low femtomole limits of detection for tryptophan-containing peptides. Proof-of-principle studies showed the quantification of simple protein mixtures using peptides generated from tryptic digests. At the intact protein level, UV-IF-based quantification correlated well with that obtained from MS ion current. However, at the peptide level, there was little correlation between the UV-IF and MS responses. These results indicate that the UV-IF and MS responses of peptides are highly orthogonal and thus complementary in nature. We demonstrated sensitive analysis of intact proteins using UV-IF and suggest its utility as a label-free quantification method during top-down MS/MS interrogation. When UV-IF is measured in parallel with MS/MS analysis of either peptides (bottom-up) or intact proteins (top-down), quantitative information can be obtained along with information on protein identity and structure in a high-throughput fashion. Although the primary emphasis of this work has been on the detection, identification, and quantification of peptides and proteins by measurement of intrinsic fluorescence, the device design is readily adapted to any emission and excitation wavelengths and could thus be widely utilized for fluorescence detection in nanoLC-MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Gloria M. Kreitinger for preparation of AQUA peptide samples, Craig D. Wenger for advice with software programming, Alicia Williams for aid in manuscript preparation, and A.J. Buretta for contributions to figure illustrations. This work was supported by the NIH NGHRI grants P01 GM081629 and P50 HG004952 to LMS and JJC. RTH was supported by a NIH NHGRI training grant to the Genomic Sciences Training Program (5T32HG002760).

References

1. Mann M, Hendrickson RC, Pandey A. Annu Rev Biochem 2001;70:437–473. [PubMed: 11395414]
2. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Anal Bioanal Chem 2007;389:1017–1031. [PubMed: 17668192]
3. Aebersold R, Mann M. Nature 2003;422:198–207. [PubMed: 12634793]
4. Heck AJR, Krijgsveld J. Expert Rev Proteomics 2004;1:317–326. [PubMed: 15966828]
5. Gant-Branum RL, Kerr TJ, McLean JA. The Analyst 2009;134:1525–1530. [PubMed: 20448914]
6. Iliuk A, Galan J, Tao W. Anal Bioanal Chem 2009;393:503–513. [PubMed: 18843486]
7. Zhu W, Smith JW, Huang C-M. J Biomed Biotechnol 2010;2010:1–6.
8. Wilm M, Mann M. Anal Chem 1996;68:1–8. [PubMed: 8779426]
9. El-Faramawy A, Siu KWM, Thomson BA. J Am Soc Mass Spectrom 2005;16:1702–1707. [PubMed: 16095913]
10. Marginean I, Kelly RT, Prior DC, LaMarche BL, Tang K, Smith RD. Anal Chem 2008;80:6573–6579. [PubMed: 18661954]
11. Schmidt A, Karas M, Dülcks T. J Am Soc Mass Spectrom 2003;14:492–500. [PubMed: 12745218]
12. Chernushevich IV, Bahr U, Karas M. Rapid Commun Mass Spectrom 2004;18:2479–2485. [PubMed: 15384129]
13. Ficarro SB, Zhang Y, Lu Y, Moghimi AR, Askenazi M, Hyatt E, Smith ED, Boyer L, Schlaeger TM, Luckey CJ, Marto JA. Anal Chem 2009;81:3440–3447. [PubMed: 19331382]
14. Turck CW, Falick AM, Kowalak JA, Lane WS, Lilley KS, Phinney BS, Weintraub ST, Witkowska HE, Yates NA. Mol Cell Proteomics 2007;6:1291–1298. [PubMed: 17513294]
15. Swaile DF, Sepaniak MJ. J Liq Chromatogr 1991;14:869–893.
16. Chang HT, Yeung ES. Anal Chem 1993;65:2947–2951.
17. Timperman AT, Oldenburg KE, Sweedler JV. Anal Chem 1995;67:3421–3426. [PubMed: 8686891]
18. Okerberg E, Shear JB. Anal Chem 2001;73:1610–1613. [PubMed: 11321317]
19. Okerberg E, Shear JB. Anal Biochem 2001;292:311–313. [PubMed: 11355869]
20. García-Campaña AM, Taverna M, Fabre H. Electrophoresis 2007;28:208–232. [PubMed: 17136735]
21. Lee TT, Yeung ES. J Chromatogr A 1992;595:319–325.
22. Schlabach TD, Wehr TC. Anal Biochem 1982;127:222–233. [PubMed: 6819783]
23. Heath TG, Giordani AB. J Chromatogr A 1993;638:9–19.
24. Lacroix M, Poinsot V, Fournier C, Couderc F. Electrophoresis 2005;26:2608–2621. [PubMed: 15948219]
25. Sluszny C, He Y, Yeung ES. Electrophoresis 2005;26:4197–4203. [PubMed: 16252334]
26. Lee J, Tripathi A. Biotechnol Prog 2007;23:1506–1512. [PubMed: 17994758]
27. Wilkins JA, Xiang R, Horváth C. Anal Chem 2002;74:3933–3941. [PubMed: 12199558]
28. Permyakov, EA. Luminescent Spectroscopy of Proteins. CRC Press; Boca Raton: 1993.

29. Lakowicz, JR. Principles of Fluorescence Spectroscopy. 3. Springer Science+Business Media, LLC; New York City: 2006.
30. Fuller RR, Moroz LL, Gillette R, Sweedler JV. Neuron 1998;20:173–181. [PubMed: 9491979]
31. Cagney G, Amiri S, Premawaradena T, Lindo M, Emili A. Proteome Sci 2003;1:5. [PubMed: 12946274]
32. White A. Biochem J 1959;71:217–220. [PubMed: 13628557]
33. Van Duuren BL. J Org Chem 1961;26:2954–2960.
34. Kirby EP, Steiner RF. J Phys Chem 1970;74:4480–4490.
35. Ricci RW. Photochem Photobiol 1970;12:67–75. [PubMed: 5498528]
36. Lehrer SS. J Am Chem Soc 1970;92:3459–3462. [PubMed: 5422768]
37. Cowgill RW. Arch Biochem Biophys 1963;100:36–44. [PubMed: 14023535]
38. Cowgill RW. Biochim Biophys Acta 1963;75:272–273. [PubMed: 14083910]
39. Cowgill RW. Biochim Biophys Acta 1967;133:6–18. [PubMed: 6047662]
40. Cowgill RW. Biochim Biophys Acta 1970:18–25. [PubMed: 5410718]
41. Alston RW, Lasagna M, Grimsley GR, Scholtz JM, Reinhart GD, Pace CN. Biophys J 2008;94:2280–2287. [PubMed: 18065477]
42. Muiño PL, Callis PR. J Phys Chem B 2009;113:2572–2577. [PubMed: 18672928]
43. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Proc Natl Acad Sci U S A 2003;100:6940–6945. [PubMed: 12771378]
44. Pigault C, Gerard D. Photochem Photobiol 1984;40:291–296. [PubMed: 6484000]
45. Callis PR, Vivian JT. Chem Phys Lett 2003;369:409–414.
46. Callis PR, Liu T. J Phys Chem B 2004;108:4248–4259.
47. Chen Y, Barkley MD. Biochemistry 1998;37:9976–9982. [PubMed: 9665702]
48. Elias JE, Gygi SP. Nat Methods 2007;4:207–214. [PubMed: 17327847]

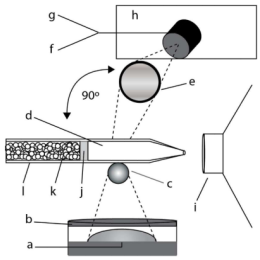


Figure 1.
Fluorescence excitation and detection device; a) UV-LED with integrated ball lens, b) bandpass interference filter, c) ball lens, d) capillary column with detection cell and ESI emitter, e) plano-convex lens, f) longpass filter, g) bandpass filter, h) photomultiplier, i) MS inlet, j) cast chemical frit, k) chromatographic packing material, l) 360 μm o.d. × 200 μm i.d. fused-silica capillary.

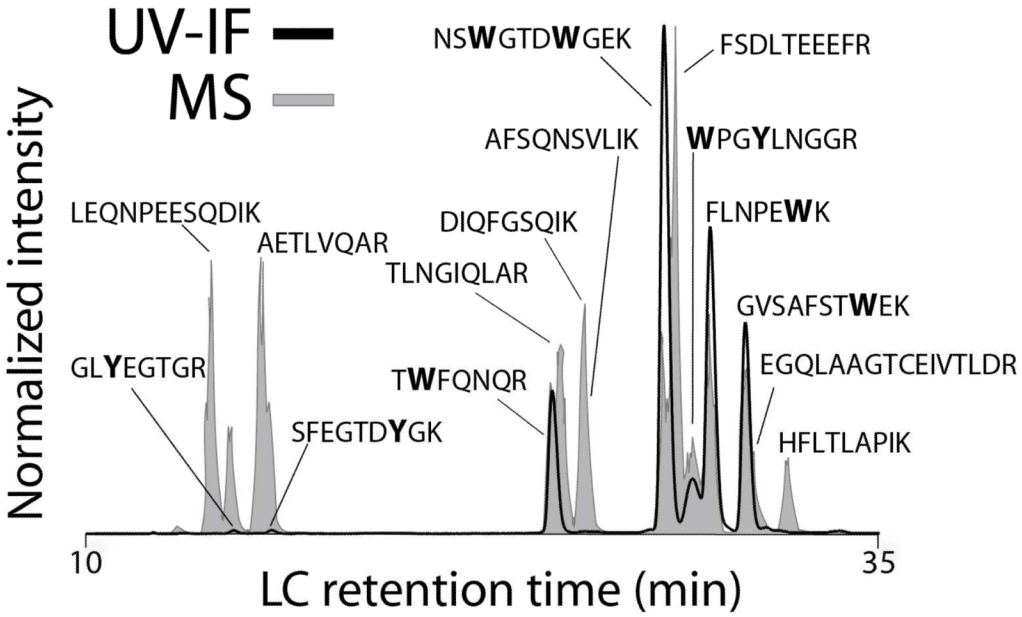


Figure 2.
UV-IF and MS base-peak chromatographic traces of an equimolar (+/− 25%) mixture of 15 AQUA peptides separated over 30-minute gradient. Tryptophan (**W**) and tyrosine (**Y**) residues in bold.

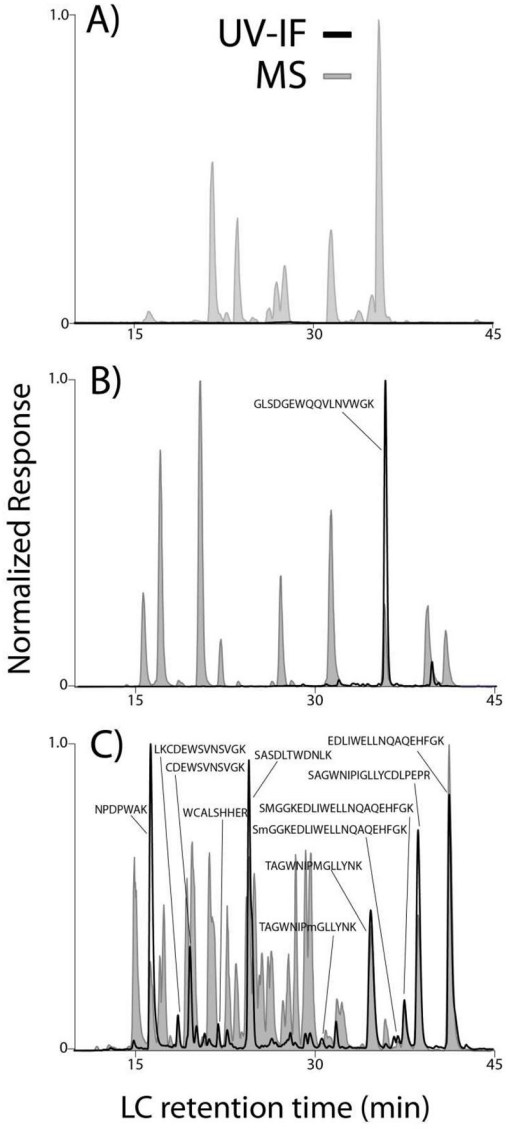
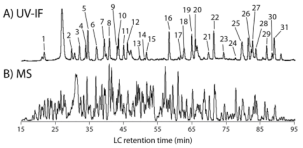


Figure 3.

UV-IF and MS traces of single-protein tryptic digests separated over a 30-minute gradient; A) ribonuclease (0 Trp), B) myoglobin (2 Trp), and C) transferrin (8 Trp). MS intensity was normalized to the most intense peptide. UV-IF intensity was normalized to the most intense tryptophan-containing peptide. Note: no tryptophan-containing peptide was identified from ribonuclease.



Peak #	Peptide sequence	MS peak area avg ± stdev	UV-IF peak area avg ± stdev	Protein
1	NKADWAK	0.179 ± 0.016	0.534 ± 0.017	Conalbumin
3	TCNWAR	0.323 ± 0.021	0.323 ± 0.013	
9	KDSWWNWNNLK	0.207 ± 0.013	0.299 ± 0.012	
13	IQWCAVGK	0.460 ± 0.021	0.752 ± 0.026	
10	IQWCAVGKDEK			
11	WCTISSPEEK	0.437 ± 0.012	0.935 ± 0.013	
7	WCTISSPEEKK			
14	DSWNWWNNLK	0.0429 ± 0.0058	0.0512 ± 0.0021	
18	WSWVSNGDVECTVVDETK	0.438 ± 0.027	0.667 ± 0.012	
21	WSWVSNGDVECTVVDETKDCIK			
22	SAGWNMPIGTLHR	1.000 ± 0.034	0.660 ± 0.071	
26	VEDIWSFLSK	0.230 ± 0.028	0.816 ± 0.014	
27	DDNKVEDIWSFLSK			
20	GAIEWEGIESGSVEQAVAK	0.169 ± 0.025	0.502 ± 0.014	
2	WCALSHHER	0.573 ± 0.050	0.272 ± 0.016	Transferrin
4	WCAVSEHEATK	0.2818 ± 0.0034	0.323 ± 0.019	
8	NPDPWAK	0.698 ± 0.042	1.000 ± 0.018	
6	HQTVPQNTGGKNPDPWAK			
12	LKCDEWSVNSVGK	0.367 ± 0.010	0.503 ± 0.012	
17	LKCDEWSVNSVGKECVSAETTEDCIAK			
16	SASDLTWDNLK	0.830 ± 0.026	0.826 ± 0.048	
15	KSASDLTWDNLK			
25	TAGWNIPMGLLYNK	0.198 ± 0.052	0.632 ± 0.017	
29	SMGGKEDLWELLNQAQEHFGK	0.339 ± 0.031	0.356 ± 0.020	
31	SAGWNIPIGLLYCDLPEPR	0.475 ± 0.044	0.6302 ± 0.0061	
19	AAQDSFAAAGWQVMVSHR	0.453 ± 0.047	0.740 ± 0.035	Enolase
24	WLTGPDLADLYHSLMK	0.168 ± 0.028	0.382 ± 0.014	
28	WLTGPDLADLYHSLMKK			
30	RYPVVSEDPEAEDDWEAWSHFFK	0.130 ± 0.018	0.157 ± 0.017	
2	KFWGK	0.392 ± 0.030	0.652 ± 0.030	BSA
23	CPLKPWALTFSYGR	0.202 ± 0.013	0.274 ± 0.032	Aldolase

Figure 4.

Peak area analysis (N = 3) of tryptophan-containing peptides identified from an equimolar 5-protein tryptic digest separated over a 70-minute gradient. A) UV-IF chromatogram with annotated tryptophan-containing peptides, B) MS base-peak chromatogram (labels omitted for clarity). Using conalbumin, transferrin, enolase for quantification, UV-IF and MS produce normalized peak area ratios of 0.9:1.0:0.8 and 0.7:1.0:0.5; respectively.

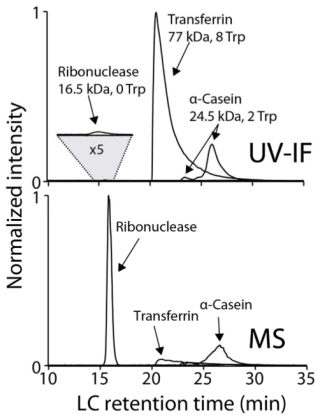


Figure 5.

UV-IF (top) and MS (bottom) composite chromatographic traces of intact proteins ribonuclease, transferrin, and α-casein (~ 1 pmol ea.) acquired during top-down analysis.