Journal of Chromatography B, 902 (2012) 70–77

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantification of HPLC-separated peptides and proteins by spectrofluorimetric detection of native fluorescence and mass spectrometry

Suraj Saraswat, Bruce Snyder[1], Dragan Isailovic*

Department of Chemistry, University of Toledo, Toledo, OH, USA

ARTICLE INFO

Article history:
Received 14 February 2012
Accepted 17 June 2012
Available online 4 July 2012

Keywords:
Quantification
Peptides
Proteins
HPLC
Native fluorescence
ESI-MS

ABSTRACT

Due to relatively low reproducibility of the ionization and differences when using buffers as mobile phases, the quantitative analysis by electrospray ionization mass spectrometry (ESI-MS) can be often challenging. In the present study, the native fluorescence of phenylalanine, tyrosine, and tryptophan was investigated as an improvement tool for the analytical quantification of peptides and proteins by HPLC–ESI-MS. Natively fluorescent amino acids as well as peptides, proteins, and protein digests were successfully separated by HPLC, and quantified with a spectrofluorimetric detector and ESI-MS. The two detectors were connected in series and enabled the sequential measurements of the fluorescence intensities as well as the measurements of the ion signals and mass spectral characterization of separated polypeptides. Fluorescence detector provided better linearity and repeatability of quantification than mass spectrometer, and similar limits of detection for most of biomolecules analyzed. The fluorescence signal was linear over 3–4 orders of magnitude with limits of detection in picomole or high femtomole range, depending on nature and number of natively fluorescent amino acid residues present in the analyzed polypeptides. Hence, native fluorescence of phenylalanine, tyrosine, and tryptophan can be used as a label-free methodology to facilitate quantification of peptides and proteins by LC–ESI-MS.

1. Introduction

Mass spectrometry is widely applied for identification and structural characterization of proteins and their post-translational modifications. Most of protein MS analyses are conducted by ESI and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [1,2]. To analyze complex proteomic samples, bottom-up and top-down MS techniques were developed. In the bottom-up approach, a protein mixture is subjected to enzymatic digestion, and HPLC–MS is then used for separation of digest peptides and protein identification [3,4]. Reversed-phase HPLC (RP-HPLC) in combination with ESI-MS is most commonly used in such applications. In top-down approach, a proteomic sample is separated and individual proteins are investigated directly by MS/MS [3,4].

In addition to qualitative structural analysis, LC–ESI-MS can be used for quantification of proteins using labeling and label-free techniques. Isotopic labeling is often used in the case of mass spectrometric quantification of peptides and proteins [5–7]. Quantitative analysis can be done using isotopic labeling by amino acids in cell culture (SILAC) [5], isotope-coded affinity tags (ICAT) [6], and isobaric tags for relative and absolute quantification (iTRAQ) [7]. For example, cysteines that are isotopically labeled by ICAT reagents can be used for quantification of proteins based on the presence of doublets in the mass spectra corresponding to "heavy" and "light" isotopes [6]. These procedures require expensive isotopic labels and extensive sample preparation protocols. In addition, label-free methodologies have also been reported for protein quantification in biological samples [8,9]. In all of these quantification experiments, the mass spectrometers can operate in single stage acquisition mode [10] and single ion recording (SIR) mode [11], or in multiple stage acquisition modes such as low-energy collision-induced dissociation tandem mass spectrometry (CID–MS/MS) and multiple reactions monitoring (MRM) [12–14]. Commonly, HPLC enables separation while an ESI mass spectrometer is used for structural characterization and quantification of polypeptides.

However, LC–ESI-MS has its own quantification drawbacks such as ionization suppression and irreproducible ionization especially when different buffers are used as mobile phases [15]. Differences among MS instruments (i.e., variability of ion sources and mass analyzers) also complicate comparative quantification. All these factors can affect accuracy and reproducibility of the MS quantification. The addition of another independent detection method could be useful

* Corresponding author at: Department of Chemistry, University of Toledo, MS 602, 2801 W. Bancroft St, Toledo, OH 43606, USA. Tel.: +1 419 530 5523; fax: +1 419 530 4033.
E-mail address: Dragan.Isailovic@utoledo.edu (D. Isailovic).
[1] Current address: Emmanuel Christian School, 4607 Laskey Rd., Toledo, OH 43623, USA.

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http://dx.doi.org/10.1016/j.jchromb.2012.06.018

to facilitate detection and quantification of peptides and proteins by LC–MS. The examples of detection of biomolecules using HPLC–MS in combination with fluorescence detection were reported in the literature [15–21]. These studies showed that the analyses of fluorescent biomolecules by LS–ESI-MS and LC–MALDI-MS are feasible. Fluorescent labeling may change ionization efficiency and MS/MS fragmentation patterns of analyzed biomolecules improving their identification [15,20]. However, although it may be useful for quantification of peptides and proteins, fluorescent labeling often requires extensive procedures for tagging, purification, and separation of proteomic samples.

Native fluorescence of tryptophan, tyrosine, and phenyl alanine was used as a label-free methodology for detection of amino acids, peptides, and proteins separated by HPLC [21–23] and for quantification of proteins separated by capillary and gel electrophoresis [24,25]. Recently, Russell et al. demonstrated quantification of peptides and proteins separated by nano-LC using parallel detection of intrinsic fluorescence of tryptophan and ESI mass spectrometry [26]. They constructed an on-capillary fluorescence detection system employing UV–LED as excitation source, and appropriate optics and a photomultiplier for fluorescence detection. In combination with ESI, this setup permitted parallel fluorescence and MS quantification of trypthophan-containing polypeptides.

In the present study, we have explored how the spectrofluorimetric detection of all three natively fluorescent amino acids (tryptophan, tyrosine, and phenylalanine) can improve LC–ESI-MS quantification of peptides and proteins. To perform these experiments, an HPLC instrument containing a spectrofluorimetric detector was coupled to an ESI-mass spectrometer for the quantification of separated biomolecules. Using model peptides, proteins, and protein digests (Table 1), we evaluated this native fluorescence–mass spectrometry methodology for quantitative analyses. Additionally, we compared two detection techniques in terms of sensitivity, limit of detection, repeatability, and dynamic range for quantification of peptides and proteins.

2. Experimental

2.1. Chemicals

Phenylalanine (>99% purity), tyrosine, tryptophan (>99% purity), MRFA (>90% purity), bradykinin acetate (>98% purity), angiotensin II human (>93% purity), leucine enkephaline, neurotensin (>99% purity), cytochrome c (>95% purity), myoglobin (>90% purity), trypsinogen, sequence grade trypsin, iodoacetamide (IAM), DL-dithiothreitol (DTT) (>95% purity), and high-purity (>95%) formic acid were purchased from Sigma (St. Louis, MO, USA). Neurotensin (8–13), Glu-fibrinopeptide, and MAGE-3 were purchased from AnaSpec (Fremont, CA, USA). HPLC-grade water and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Sample preparation

Stock solutions of phenylalanine, tyrosine, and tryptophan were prepared in HPLC-grade water at concentrations of 3 mg/ml (18.16 mM), 0.5 mg/ml (2.76 mM), and 1 mg/mL (4.90 mM), respectively. To evaluate the linearity of quantification of these amino acids, the stock solutions were further diluted with water to obtain 10-μl injection aliquots containing from: 7.3 pmole to 73.0 nmole of phenylalanine, 1.1 pmole to 11.0 nmole of tyrosine, and 1.2 pmole to 10.0 nmole of tryptophan.

For peptide analysis, detection limit and linearity check were performed after preparation of the following aqueous stock solutions: 10 mg/mL (19.1 mM) of MRFA, 10 mg/mL (12.3 mM) of neurotensin fragment (8–13), 3 mg/mL (2.8 mM) of bradykinin, 3 mg/mL (2.9 mM) of angiotensin II, 3 mg/mL (1.9 mM) of Glu-fib, 3 mg/mL (5.4 mM) of leucine enkephalin, 10 mg/mL (5.9 mM) of neurotensin, and 1 mg/mL (9.4 mM) of MAGE-3. The standard solutions were further diluted with water to obtain injection aliquots containing from: 0.0318 nmole to 19.097 nmole of MRFA, 0.7 pmole to 699.42 pmole of neurotensin fragment, 7.68 pmole to 1.536 nmole of bradykinin, 1.39 pmole to 6.964 nmole of angiotensin, 0.82 pmole to 4.093 nmole of Glu-fib, 1.23 pmole to 6.171 nmole of leucine enkephalin, 1.708 pmole to 1.708 nmole of neurotensin, and 0.240 pmol to 0.472 nmol of MAGE-3.

To determine detection limit and linearity of protein quantification, aqueous stock solutions of cytochrome c (80.8 μM), myoglobin (58.9 μM), and trypsinogen (41.6 μM) were prepared at concentration of 1 mg/mL each. These stock solutions were mixed and further diluted with water to obtain injection aliquots containing from: 0.808 pmole to 266.75 pmole of cytochrome c, 0.416 pmole to 137.38 pmole of trypsinogen, and 0.589 pmole to 194.489 pmole of myoglobin.

For analysis of a protein digest, a stock solution of BSA was prepared at concentration of 5 mg/mL (75.7 μM) and digested using trypsin. Cysteins in the tryptic digest were reduced and alkylated using DTT and IAM, respectively. LOD and linearity of BSA peptide separation is obtained by further diluting BSA digest with water to obtain injection aliquots containing from: 13.5 pmole to 6.7 nmole of peptide with m/z 740.6, 13.8 pmol to 6.9 nmol of peptide with m/z 720.6, and 21.5 pmole to 10.8 nmole of peptide with m/z 927.9.

For quantitative analysis of a complex peptide mixture, solutions of cytochrome c, myoglobin and trypsinogen were prepared at concentration of 1 mg/mL and digested using trypsin. Cysteins in the tryptic digests were reduced and alkylated using DTT and IAM, respectively. Digests of the three proteins were mixed in equal amounts to prepare a stock solution. LOD and linearity of tryptic peptide separation is obtained by further diluting the stock solution with water to obtain injection aliquots containing from: 34.0 pmole to 3.4 nmole of peptide with m/z 736.0, 77.9 pmol to 5.85 nmol of peptide with m/z 751.9, and 47.7 pmole to 4.77 nmole of peptide with m/z 454.7.

2.3. Instrumentation and data acquisition

All experiments were performed by HPLC–ESI-MS. The HPLC system (Shimadzu Technologies, Addison, IL, USA) consisted of LC-20AD binary pump, DGU-20A3 vacuum degasser, SIL-20A auto sampler, RF-10AXL fluorescence detector, and SCL-10A VP system controller. The flow rate was 0.2 mL/min and injection volume was 10 μL. HPLC data were acquired using LC Solution software (Shimadzu). The fluorescence signal was sampled at a rate of 3.33 Hz. Fluorescence detector allowed adjustments of the excitation and emission wavelengths during the run while excitation and emission slits had fixed bandwidths of 15 nm.

HPLC was connected to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-TOF Micro, Waters, Milford, MA, USA). MS data were acquired using MassLynx software, version 4.1 (Waters). MassLynx was also used for integration of baseline-separated chromatographic peaks in total ion cromatograms (TICs) of amino acids, peptides, and proteins, and for extraction and integration of tryptic peptide peaks in extracted ion chromatograms (EICs). ESI-MS experiments were performed in positive ion mode. The MS scan time was 1.0 s and the interscan time was set to 0.1 s. Desolvation temperature was 350 °C and desolvation gas flow rate was 650 L/h. The MCP detector voltage was set at 2.35 kV. All HPLC–MS quantitative analyses were done in triplicates, while repeatability experiments were repeated ten times.

Table 1
Amino acid sequences[a] and molecular weights[b] of analyzed biomolecules.

Molecule	Amino acid sequence	$M_{r\,(exp)}$	$M_{r\,(theory)}$
Tryptophan	Trp (W)	204.9	204.1
Tyrosine	Tyr (Y)	181.8	181.1
Phenylalanine	Phe (F)	165.8	165.1
Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (DRVYIHPF)	1045.6	1046.18
Bradykinin Acetate	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (RPPGFSPFR)	1060.7	1060.21
Leucine enkephalin	Tyr-Gly-Gly-Phe-Leu (YGGFL)	554.93	555.62
[Glu]-Fibrinopeptide B	Glu-Gly-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg (EGVNDNEEGFFSAR)	1569.7	1570.6
MRFA	Met-Arg-Phe-Ala (MRFA)	522.73	523.65
Neurotensin (8–13)	Arg-Arg-Pro-Tyr-Ile-Leu (RRPYIL)	816.54	816.99
Neurotensin	Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (ELYENKPRRPYIL)	1671.97	1672.92
MAGE-3	Phe-Leu-Trp-Gly-Pro-Arg-Ala-Leu-Val (FLWGPRALV)	1058.09	1059.09
Cytochrome c	F (4), Y (4), W (1)	12,388	12,384
Trpsinogen	F (5), Y (12), W (5)	23,951	23,981
Myoglobin	F (4), Y (1), W (2)	16,970	16,934
BSA Peptide (927.9)	Tyr-Leu-Tyr-Glu-Ile-Ala-Arg (YLYEIAR)	926.95	928.08
BSA Peptide (720.6)	Arg-His-Pro-Glu-Tyr-Ala-Val-Ser-Val-Leu-Leu-Arg (RHPEYAVSVLLR)	1439.3	1440.7
BSA Peptide (740.6)	Leu-Gly-Glu-Tyr-Gly-Phe-Gin-Asn-Ala-Leu-Ile-Val-Arg (LGEYGFQNALIVR)	1479.3	1480.7
Tryp (454.7)	Asn-Lys-Pro-Gly-Val-Tyr-Thr-Lys (NKPGVYTK)	907.4	905.5
Myo (751.9)	Hys-Pro-Gly-Asp-Phe-Gly-Ala-Asp-Ala-Gln-Gly-Als-Met-Thr-Lys (HPGDFGADAQGAMTK)	1501.8	1501.7
Cyt C (736.0)	Thr-Gly-Gin-Ala-Pro-Gly-Phe-Thr-Tyr-Thr-Asp-Ala-Asn-Lys (TGQAPGFTYTDANK)	1470.0	1469.7

[a] Amino acid sequences were either obtained from manufactures or using UniProt database available at www.expasy.org.
[b] Experimental and theoretical molecular weights correspond to monoisotopic masses of amino acids and peptides, and to average masses of proteins.

2.4. LC–fluorescence–MS analysis of amino acids

Separation of amino acids was performed on an analytical scale C18 column (Everest™ 250 × 2.1 mm, Vydac, Deerfield, IL, USA). The mobile phase A was HPLC-grade water containing 1% formic acid and mobile phase B was acetonitrile containing 0.85% formic acid. Elution gradient was: 0–6.5 min 5% of B, 6.5–11 min 18% of B, 11–12 min 90% of B, 12–14 min 90% of B, 14–15 min 5% of B, and the run was stopped at 28.1 min. Excitation and emission wavelengths used for fluorescence detection were respectively 274 nm and 304 nm for 5.15 min, 260 nm and 295 nm from 5.15 to 7.50 min, and 280 nm and 348 nm from 7.50 to 15 min. Excitation and emission wavelengths were switched back to 274 nm and 304 nm during the rest of the run. The gain and sensitivity of fluorescence detector were set to 1× and low, respectively.

ESI-MS was performed in the *m*/*z* range from 50 to 350. The spray voltage was set at 3.0 kV and sample cone voltage was 30 V. The source temperature was 120 °C and collision energy was set at 4 V.

2.5. LC–fluorescence–MS analysis of peptides

Separation of peptides was also performed on an analytical scale C18 column (Everest™ 250 × 2.1 mm, Vydac). The mobile phase A was HPLC-grade water containing 0.8% formic acid and mobile phase B was acetonitrile containing 0.7% formic acid. Elution gradient was: 0–2 min 12.5% of B, 2–20 min 13.5% of B, 20–28 min 15.5% of B, 28–36 min 35% of B, 36–40 min 90% of B, 40–42 min 90% of B, 42–45 min 5% of B, and the run was stopped at 58.2 min. Excitation and emission wavelengths used for fluorescence detection were, respectively, 274 nm and 304 nm for 7.0 min, 260 nm and 290 nm for 5 min, 274 nm and 304 nm for 3.3 min, 260 nm and 290 nm for 15.90 min, and 274 nm and 304 nm for the rest of the run. For separation of MAGE-3, elution gradient was: 0–2 min 25% of B, 2–6 min 45% of B, 6–10 min 49% of B, 10–13 min 90% of B, 13–15 min 90% of B, 15–17 min 5% of B, and run was stopped at 27.2 min. Excitation and emission wavelengths used for fluorescence detection of MAGE-3 were 280 nm and 348 nm. The gain and sensitivity of fluorescence detection were set to 1× and low, respectively.

ESI-MS analysis of peptides was performed in the *m*/*z* range from 50 to 1800. The spray voltage was set at 3.1 kV and sample cone voltage was 44 V. The source temperature was 120 °C and collision energy was set at 10 V.

2.6. LC–fluorescence–MS analysis of proteins

Separation of proteins was performed on a C4 column (Jupiter 250 × 2.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase A was HPLC-grade water containing 3.7% formic acid and mobile phase B was acetonitrile containing 3.15% formic acid. Elution gradient was: 0–3 min 5% of B, 3–5 min 27% of B, 5–26 min 37% of B, 26–45 min 41% of B, 45–49 min 45% of B, 49–50 min 90% of B, 50–51 min 90% of B, 51–52 min 5% of B, and run was stopped at 65.1 min. Excitation and emission wavelengths used for fluorescence detection were 274 nm and 304 nm, respectively. Fluorescence detector was set at 1× gain and at medium sensitivity.

ESI-MS was performed in the *m*/*z* range from 800 to 2200. The spray voltage was set at 3.0 kV and sample cone voltage was 30 V. The source temperature was 120 °C and collision energy was 4 V.

2.7. LC–fluorescence–MS analysis of BSA digest

Separation of peptides from BSA digest was performed on a C18 column, which was used previously for separations of amino acids and peptides (Sections 2.4 and 2.5). The mobile phase A was HPLC-grade water containing 3.7% formic acid and mobile phase B was acetonitrile containing 3.15% formic acid. Elution gradient was: 0–5 min 5% of B, 5–104 min 35% of B, 104–110 min 90% of B, 110–118 min 5% of B, and run was stopped at 130.5 min. Excitation and emission wavelengths used for fluorescence detection were 274 nm and 304 nm, respectively. Fluorescence detector was set at 4× gain and medium sensitivity.

ESI-MS was performed in the *m*/*z* range from 300 to 1800. The spray voltage was set at 3.0 kV and sample cone voltage was 30 V. The source temperature was 120 °C and collision energy was 4 V.

2.8. LC–fluorescence–MS analysis of cytochrome c, myoglobin and trypsinogen digests

Separation and detection of cytochrome c, myoglobin and trypsinogen digests were performed under the same conditions as described for the analysis of BSA digest (Section 2.7).

3. Results and discussion

3.1. General considerations

Tryptophan, tyrosine, and phenylalanine exhibit ultraviolet light-induced fluorescence (UV-IF), but have different absorption coefficients and fluorescence quantum yields [27]. Tryptophan has an absorption coefficient of $5.7 \times 10^3\ M^{-1}\ cm^{-1}$ and quantum yield of 0.14. It is commonly excited at 280 nm and its emission maximum is at 348 nm. Tyrosine has an absorption coefficient of $1.6 \times 10^3\ M^{-1}\ cm^{-1}$ and quantum yield of 0.13. Its excitation maximum is at 274 nm and its emission maximum is at 324 nm. Phenylalanine has an absorption coefficient of $0.4 \times 10^3\ M^{-1}\ cm^{-1}$ and quantum yield of 0.02, while its excitation and emission maxima are at 260 nm and 295 nm, respectively. The fluorescence properties of tryptophan enable the detection and quantification of peptide and proteins containing this amino acid upon LC separation [23,26]. Since tryptophan is more fluorescent, it is possible to achieve a better response of its fluorescence detection than for tyrosine and phenylalanine. However, tryptophan is a rare amino acid in a proteome [26] and it would be beneficial to detect and quantify peptides and proteins containing all natively fluorescent amino acids. A spectrofluorimetric detector enables selective detection and quantification of fluorescence originating from all three natively fluorescent amino acids as well as from peptides and proteins containing them. In present study, a spectrofluorimetric detector was coupled to an ESI mass spectrometer and used for quantification of HPLC-separated biomolecules whose amino acid compositions and molecular weights are shown in Table 1.

3.2. Quantification of natively fluorescent amino acids by LC–fluorescence–MS

Initial LC–fluorescence–MS experiments involved separation and quantification of natively fluorescent amino acids. Tyrosine, phenylalanine, and tryptophan were separated from their mixture by HPLC with fluorescence-detected retention times of 4.3, 5.9, and 10.2 min, respectively (Fig. 1). Retention times and peak widths recorded by mass spectrometer were similar to those recorded by fluorescence detector, i.e., no significant retention time increase and band broadening were observed in MS-detected chromatograms. Singly charged protonated ions corresponding to natively fluorescent amino acids were detected by ESI-MS and used to determine their molecular weights (Table 1). For quantification, calibration curves were plotted using 9 standard solutions of different concentration, and linear regression analyses of peak areas corresponding to native fluorescence (Fig. S1A) and MS (Fig. S1B) signals of amino acids were performed. Since amino acids were baseline separated and underwent fragmentation during ESI, TICs were used to measure areas of peaks in MS chromatograms. A good linear relationship was found for both fluorescence and MS detection in the range between ~1 pmole and 73 nmole, as indicated by correlation coefficients ($R^2 > 0.99$) for all calibration curves (Fig. S1 and Table 2).

Calibration curves were also used to determine the limits of fluorescence and MS detection, which represent chromatographic peaks with signal-to-noise ratios of ~3. Concurrently, lower limit of quantification, which is the lowest concentration of analyte that produces a chromatographic signal distinguishable from background noise with a minimum ratio of 10:1, can be determined. LOD values for both, fluorescence and MS quantification of amino acids are given in Table 2. LOD is one order of magnitude lower for fluorescence quantification of tyrosine and tryptophan in comparison to their quantification by MS. Better limit of fluorescence detection for tyrosine and tryptophan was expected because these amino acids are more fluorescent (i.e., have higher molar absorptivities and quantum yields) than phenylalanine. However, MS shows one order of magnitude better LOD than fluorescence for quantification of phenylalanine. This is due to higher ionization efficiency of phenylalanine in comparison to tyrosine and tryptophan.

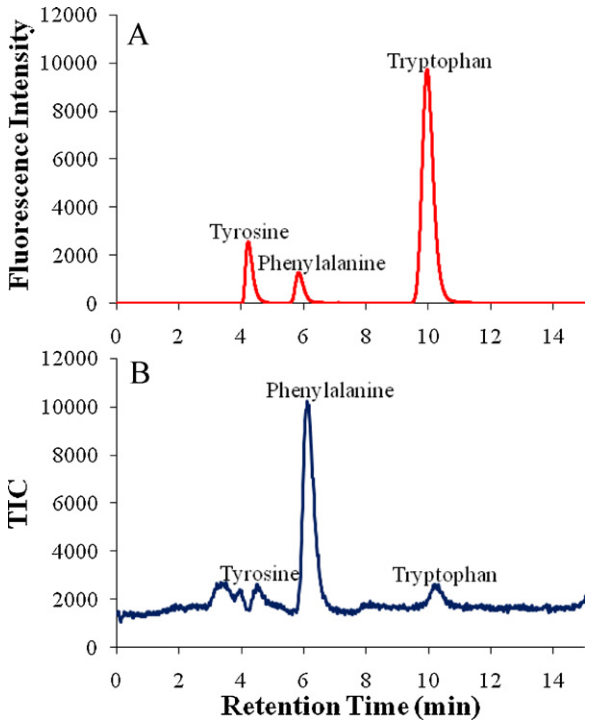

Fig. 1. HPLC separation of amino acids. Amino acids were detected by (A) a fluorescence detector at respective excitation and emission wavelengths for each amino acid and (B) ESI-MS.

The repeatability of fluorescence (Fig. S2A) and MS (Fig. S2B) measurements was also determined. For all amino acids, a total of 10 HPLC runs were replicated on a sample under optimal separation conditions described in Section 2.4. Repeatability of the amino acid quantification was greater for fluorescence than for MS detection as represented by relative standard deviation values shown in Table 2. Additionally, dynamic range of UV-IF was linear over 4 orders of magnitude for tryptophan and over 3 orders of magnitude for tyrosine and phenyl alanine. Linear dynamic range of MS detection was three orders of magnitude. Above results indicate that fluorescence detector in series with MS can be successfully used for quantitative analysis of amino acids. Calibration curves shown in Fig. S1 can be used for determination of unknown concentration of a natively fluorescent amino acid that are separated using RP-HPLC gradient described in Section 2.4.

3.3. Quantification of peptides by LC–fluorescence–MS

To further evaluate LC–native fluorescence–MS quantification methodology, a mixture of 7 peptides containing phenylalanine and tyrosine was separated by RP-HPLC (Fig. 2). Retention times of MRFA (1 Phe), neurotensin (8–13) (1 Tyr), bradykinin (2 Phe), angiotensin II (1 Phe and 1 Tyr), Glu-fib (2 Phe), leucine enkphalin (1 Phe and 1 Tyr), and neurotensin (2 Tyr) were 10.4, 13.7, 16.8, 26.1, 30.6, 34.1, and 37.7 min, respectively. Peptides were detected by MS as singly and doubly charged ions, and their measured molecular weights were close to theoretical values (Table 1). Calibration curves were plotted using 9 standard solutions of different concentrations. Linear regression analysis was performed of peak areas corresponding to fluorescence signal (Fig. S3A) and total ion count (Fig. S3B) versus peptide concentration. A good linear relationship

Table 2
Limits of detection, correlation coefficients of linearity, and standard deviations of repeatability determined for native fluorescence and MS quantification experiments.

Analyte	$LOD_{(MS)}$ picomoles	$LOD_{(Fluo.)}$ picomoles	$R^2_{(MS)}$	$R^2_{(Fluo.)}$	$Repeatability_{(MS)}$	$Repeatability_{(Fluo.)}$
Tyrosine	11.0	2.20	0.997	0.999	10.1%	1.52%
Phenylalanine	7.30	73.0	0.995	0.999	4.79%	3.56%
Tryptophan	12.0	1.20	0.996	0.999	11.8%	0.81%
Bradykinin acetate	1.50	13.0	0.995	0.997	3.29%	1.69%
Angiotensin II	1.40	12.0	0.998	0.999	3.11%	0.65%
[Glu]-Fibrinopeptide	0.80	14.0	0.998	0.998	3.36%	3.26%
Leucine enkephalin	1.20	1.20	0.999	0.999	2.98%	0.44%
MRFA	3.80	31.8	0.995	0.999	2.16%	2.22%
Neurotensin (8–13)	0.70	0.70	0.996	0.999	3.29%	0.81%
Neurotensin	1.70	0.30	0.999	0.999	2.40%	2.91%
MAGE-3 (Trp)	0.47	0.24	0.997	0.998	4.73%	0.16%
Cytochrome c	0.24	0.40	0.985	0.999	2.76%	1.16%
Myoglobin	0.60	0.71	0.978	0.999	4.20%	2.43%
Trypsinogen	0.21	0.40	0.998	0.999	6.62%	5.04%
BSA Pep (927.9 *m/z*)	107.7	21.5	0.997	0.999	4.51%	0.41%
BSA Pep (720.6 *m/z*)	69.4	69.4	0.995	0.999	5.32%	1.99%
BSA Pep (740.6 *m/z*)	67.6	27.0	0.999	0.999	5.46%	0.85%
Cyto C (m/z 736.0)	34.0	45.4	0.993	0.997	9.91%	5.10%
Myo (*m/z* 751.9)	266.3	133.2	0.991	0.998	12.7%	8.59%
Tryp (*m/z* 454.7)	147.4	110.5	0.997	0.997	5.24%	4.67%

was found over the investigated concentration range, as indicated by correlation coefficients ($R^2 > 0.995$) for all standard peptides (Table 2). UV-IF dynamic range was linear over 3–4 orders of magnitude with high femtomole LOD for tyrosine containing peptides. Limit of MS detection is either lower or similar to limit of fluorescence detection for all peptides but neurotensin, which showed lower LOD for fluorescence (Table 2).

Hence, the highest sensitivity of fluorescence detection (slope of the calibration curve in Fig. S3) was achieved for neurotensin, which contains two tyrosine residues. Neurotensin fragment (8–13), which contains single tyrosine residue, showed higher sensitivity than peptides that contain one phenyl alanine and one tyrosine residue (leucine enkephalin and angiotensin). This can be due to earlier elution of the former peptide and decreased quenching of its fluorescence by the increase of acetonitrile amount in the mobile phase [26]. While they were close (within factor of ~1.6 times), calibration curves for leucine enkephalin and angiotensin did not overlap. However, bradykinin and Glu-fib, which contain two phenylalanine residues, showed overlapping calibration curves. Fluorescence detection of MRFA was the least sensitive because this peptide contains only one phenylalanine. As pointed out previously [26,28], fluorescence signal of the peptides may be affected by primary sequence of the peptides as well as composition of HPLC mobile phase.

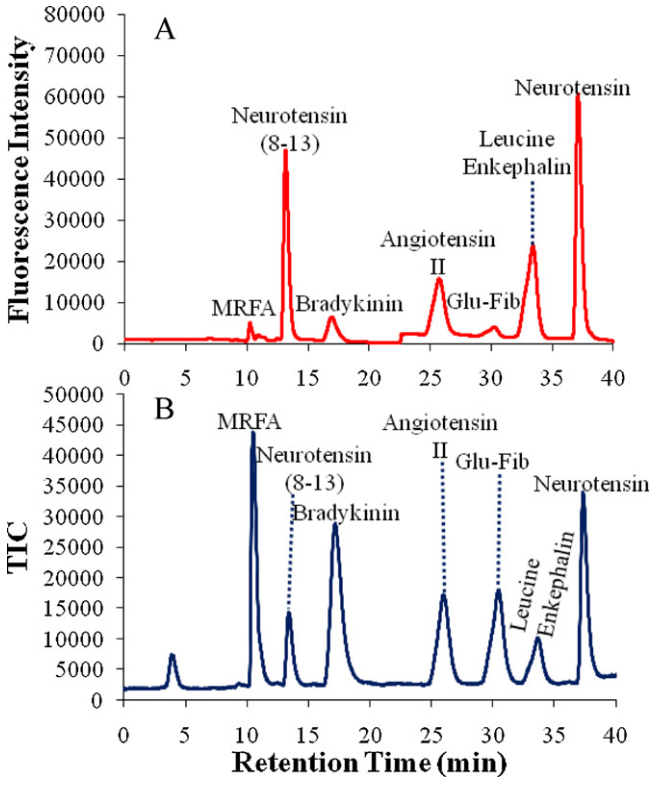

Fig. 2. HPLC separation of a peptide mixture. Peptides were detected by (A) a fluorescence detector at specific excitation and emission wavelengths for each peptide and (B) ESI-MS.

After fluorescence and MS quantification of peptides containing tyrosine and phenylalanine, the quantification of a tryptophan-containing peptide MAGE-3 was performed. Retention time for MAGE-3 peptide was 10.8 min. Due to the presence of tryptophan, LOD of MAGE-3 (0.24 pmol) was lower than LOD of neurotensin (0.30 pmol), although latter peptide contains 2 tyrosine residues. Again, the peptide sequence and mobile phase composition may affect comparison of native fluorescence of different peptides. However, it is beneficial to detect and quantify peptides containing all natively fluorescent amino acids.

As in the case of amino acid separation, the repeatability of peptide detection was better for fluorescence detection (Fig. S4A) than for MS detection (Fig. S4B) as represented by relative standard deviations values (Table 2). In general, separations of studied peptides are characterized by slightly lower LOD for MS detection and better repeatability and linearity of fluorescence detection. UV-IF linear dynamic range of 3 orders of magnitude was found for all peptides except for neurotensin, for which linear dynamic range was 4 orders of magnitude. Linear dynamic range of MS quantification was 3 orders of magnitude.

3.4. Quantification of proteins by LC–fluorescence–MS

To monitor UV-IF of HPLC-separated proteins, a mixture of three proteins was investigated. Fluorescence- and MS-detected chromatograms for separation of cytochrome c, myoglobin, and trypsinogen are shown in Fig. 3, and retention times for these proteins were 17.7, 24.2, and 37.1 min, respectively. Molecular weights of these proteins (Table 1) were determined from the series of multiply charged ESI-MS ions. Linearity was checked upon the linear regression analysis of peak areas versus proteins quantities (Fig. S5). A good linear relationship was found over the investigated

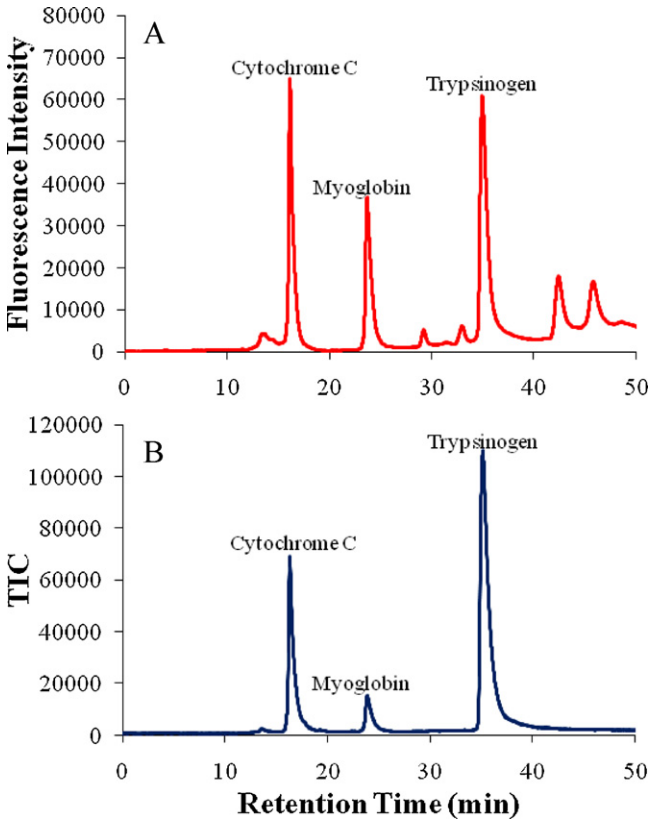

Fig. 3. HPLC separation of proteins. Proteins were detected by (A) a fluorescence detector using excitation wavelength of 274 nm and emission wavelength of 304 nm and (B) ESI-MS.

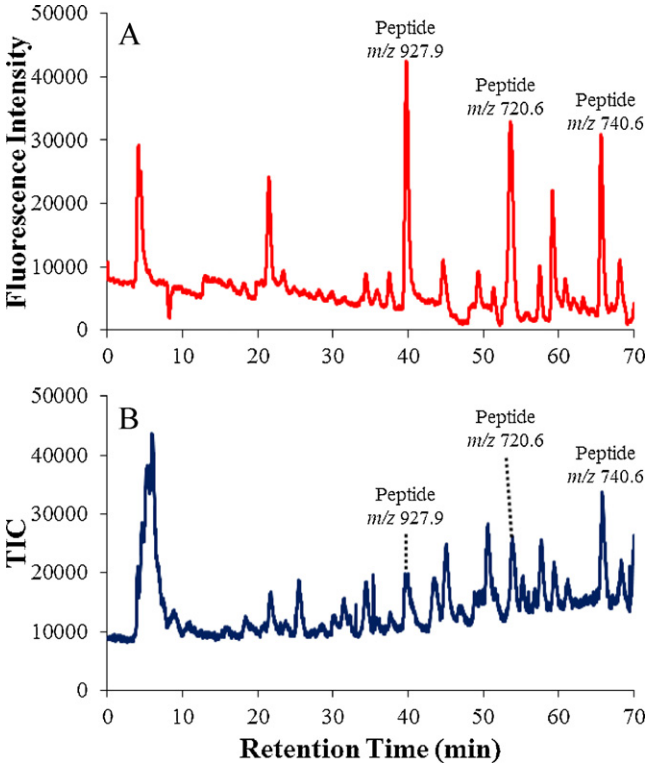

Fig. 4. HPLC separation of BSA tryptic digest. Peptides were detected by (A) a fluorescence detector using excitation wavelength of 274 nm and emission wavelength of 304 nm and (B) ESI-MS.

concentration range for UV-IF, as indicated by correlation coefficients ($R^2 > 0.99$) for all calibration curves (Table 2). However, R^2 values were not so good in the case of mass spectrometric detection. LOD values for both fluorescence and MS detection of proteins are presented in Table 2. LODs were very similar for MS and fluorescence detection, while a better repeatability is observed for UV-IF than for MS (Fig. S6) as represented by standard deviation values for 10 replicates (Table 2). Linear dynamic ranges were similar for UV-IF and MS detection (~3 orders of magnitude). These results confirm that UV-IF is a promising technique for the quantification of intact proteins by LC–ESI-MS [26].

3.5. LC–fluorescence–MS quantification of BSA digest

To quantify peptides in a biologically relevant sample, protein BSA, which contains varying number of phenylalanine (28), tyrosine (21), and tryptophan (3) residues, was digested by trypsin and BSA peptide digest was analyzed by LC–fluorescence–MS. Three peptides with retention times of 40.2, 53.1, and 65.3 min (Fig. 4) were chosen for further LOD, linearity, and repeatability studies. Peptide ion with m/z 927.9 (t_R = 40.2 min) contained 2 tyrosine residues, peptide ion with m/z 720.6 (t_R = 53.1 min) contained 1 tyrosine, and peptide ion with m/z 740.6 (t_R = 65.3 min) contained 1 phenylalanine and 1 tyrosine. These peptides were chosen for two main reasons: (1) they were separated from all other peptides (Figure 4) and (2) they contained the same number of phenylalanine and tyrosine residues as 3 peptide standards analyzed previously (Table 1 and Section 3.3).

Linearity was determined with the linear regression analysis of peak areas versus protein amounts (Fig. S7). A good linear relationship was found over the investigated concentration range for all three calibration curves (Table 2). UV-IF R^2 values exceeded 0.999, with similar responses for all three peptides selected. Nonetheless, R^2 values were comparable in the case of MS approach. The UV-IF linear dynamic range was also comparable to that of MS in the studied concentration range (~3 orders of magnitude). LOD values for both, fluorescence and MS detection of tryptic peptides are given in Table 2. LOD is slightly lower for fluorescence than for MS. Also, the repeatability of fluorescence detection superseded MS detection for 10 replicates as shown in Fig. S8 and represented by relative standard deviations values shown in Table 2.

Quantitative LC–fluorescence–MS analyses of pure peptides and peptide digests (Figs. S3 and S7, respectively) indicate that linear dynamic ranges are similar but sensitivities of peptide detection are higher for pure peptides. The differences in sensitivities are mainly because of different separation and ionization conditions used, and partially due to miscleavages present in tryptic digests, which decrease effective concentrations of peptides. Digest peptides containing the same number of natively fluorescent amino acids as pure peptides were separated in a mobile phase that had a lower pH than mobile phase used for separations of pure peptides (as described in Sections 2.5 and 2.7). Hence, detection sensitivity is decreased for digest peptides because their fluorescence is quenched more than fluorescence of pure peptides during the separation. Nevertheless, in-series fluorescence and MS detection will be suitable for quantitative analyses of proteins from complex tryptic digests if calibration curves are constructed using digests of individual pure proteins and HPLC analyses are performed under similar separation conditions.

3.6. LC–fluorescence–MS quantification of a complex protein digest

To quantify proteins in a more complex mixture, cyotochrome c, myoglobin and trypsinogen were digested with trypsin, and resulting peptides were analyzed by LC–fluorescence–ESI-MS. Three

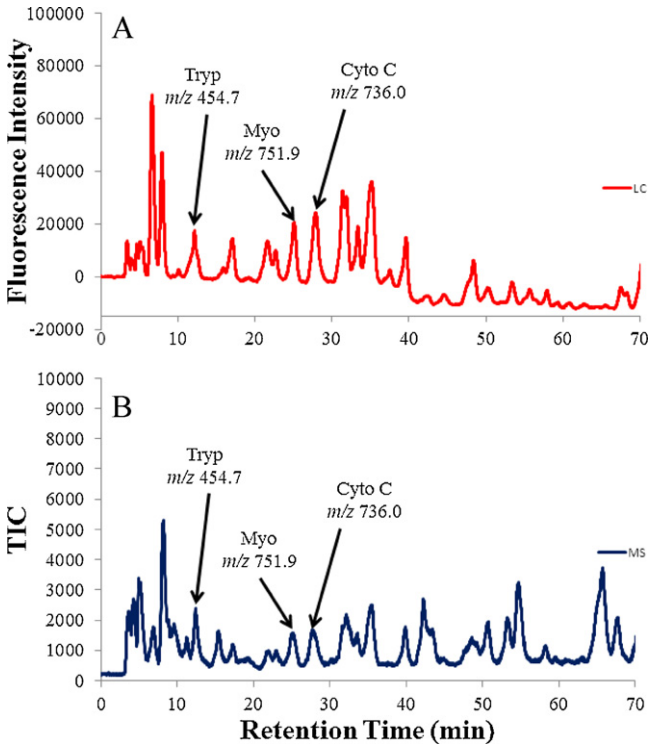

Fig. 5. HPLC separation of tryptic digest of cytochrome c, myoglobin, and trypsinogen. Peptides were detected by (A) a fluorescence detector using excitation wavelength of 274 nm and emission wavelength of 304 nm and (B) ESI-MS.

peptides with retention times of 12.6, 25.1, and 28.6 min (Fig. 5) were chosen for further LOD, linearity, and repeatability studies. Doubly charged peptide ion with m/z 454.7 (t_R = 12.6 min) originated from trypsinogen and contained 1 tyrosine, while doubly charged peptide ion with m/z 751.9 (t_R = 25.1 min) originated from myoglobin and contained 1 phenylalanine. Doubly charged peptide ion with m/z 736.0 (t_R = 28.6 min) originated from cytochrome c and contained 1 tyrosine and 1 phenylalanine residue.

Linearity of quantification was determined with the linear regression analysis of peak areas versus protein amounts (Fig. S9). The highest detection sensitivity was achieved for peptide originating from myoglobin, which contains 1 tyrosine and 1 phenylalanine residue. A good linear relationship was found over the investigated concentration range for all three calibration curves (Table 2). UV-IF R^2 values exceeded 0.99 and were slightly better than R^2 values obtained from MS calibration curves. The UV-IF linear dynamic range was also comparable to that of MS in the studied concentration range (~3 orders of magnitude). LOD values of MS detection were similar to LODs of fluorescence detection (Table 2). But, the repeatability of fluorescence detection superseded MS detection for 10 replicates as shown in Fig. S10 and represented by relative standard deviations values shown in Table 2.

It is important to note that the accuracy of quantification of proteins from the complex peptide mixtures by detection of native fluorescence depends on the resolution of HPLC separation. As shown in Fig. S11 and Table S1, many natively fluorescent peptides were detected, but some of them were not separated with resolution that will allow accurate quantitative analysis. However, spectrofluorimetric detector can selectively detect peptides containing any of the three natively fluorescent amino acids, and, in this way, simplify HPLC chromatograms, and enable more specific quantification of proteins. Such selectivity complements well to the ability of LC–ESI-MS to quantify peptides and proteins using extracted ion chromatograms.

4. Conclusions

In this study, a hybrid methodology was developed for sequential spectrofluorimetric and ESI-mass spectrometric quantification of HPLC-separated peptides and proteins that contain any of the three natively fluorescent amino acids (tryptophan, tyrosine, and phenylalanine). A spectrofluorimetric detector is used to selectively detect and quantify natively fluorescent polypeptides, while ESI-MS must be used to confirm their structural identities. Native fluorescence detection exhibited more linear and reproducible quantification of polypeptides than MS, while LODs and linear dynamic ranges for analyzed biomolecules were similar as in ESI-MS. Therefore, UV-IF can facilitate more accurate quantification of peptides and proteins by LC–ESI-MS.

Present methodology complements well to a nano-LC–fluorescence–ESI-MS quantification technique described previously [26]. Although the ease of use and wider availability are advantages of standard HPLC, capillary and nano-LC improve LC–MS sensitivity and often yield better separation efficiencies than analytical HPLC. Spectrofluorimetric detection approach presented in this study can be adopted to capillary and nano-LC–MS analyses and used with LC–MS/MS quantitative methodologies. Future experiments will be aimed toward native fluorescence–MS analysis of more complex mixtures of peptides and proteins.

Acknowledgements

This work was supported by research funds from the University of Toledo (DI). The ACS Summer Research Fellowship to BS was provided by the American Chemical Society through the ACS-Motorola Summer Research Teacher Training Program. The authors would like to thank Samuel Dapore-Schwartz for help with manuscript revision.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/ j.jchromb.2012.06.018.

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