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Nitrogen-specific detection of peptides in liquid chromatography with a chemiluminescent nitrogen detector

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Abstract

High-performance liquid chromatography with chemiluminescent nitrogen detection (HPLC–CLND) in the reversed-phase mode was used to quantitate peptides that were isolated from casein hydrolysate. When CLND is used simultaneously with a UV detector in peptide mapping, unique quantitative information about the nitrogen distribution of the sample is obtained. Nitrogenous compounds without UV chromophores are easily detected by CLND without pre- or post-column derivatization. Of further significance, the non-nitrogenous compounds in the sample matrix are transparent to the detector. This paper will focus primarily on the analysis of two peptides, identified as **1** and **2**. The UV peptide map showed peptide **2** as the largest component. On the other hand, the CLND results indicated that peptide **1** was the major peak. RP-HPLC amino acid analysis of the peptides **1** and **2** confirmed the CLND results. This analysis shows that peptide **1** was the major component and did not contain aromatic amino acid residues. Peptide **2** however, contained aromatic groups with strong chromophores, thereby explaining the UV response.

1. Introduction

Peptide mapping with RP-HPLC is an established procedure that is routinely used in biotechnology for sequencing and protein identification. Typically, the standard RP-HPLC system for peptide mapping uses columns with either a C8 or C18 stationary phase, acetonitrile–water + 0.1% TFA binary gradient for peptide elution followed by UV detection. However, direct quantitation of eluted peptides is not possible with UV detection because of the differential UV absorption properties between peptides with aromatic functional groups and those containing primarily non-aromatic amino acid residues. Recently, a novel HPLC–chemiluminescent nitrogen detection (HPLC–CLND) system was described [1]. This detector was designed primarily as a means for direct quantitation of nitrogen containing analytes.

Acetonitrile because of its low UV cutoff is an excellent solvent for UV detection. However, due to its nitrogen moiety, acetonitrile interferes with CLND. Alcohols, both methanol and isopropanol, can be substituted for acetonitrile in RP-HPLC peptide mapping [2–8]. Alcohols offer additional advantages to the chromatographer besides CLND compatibility: (1) many peptides are more soluble in alcohols, particularly isopropanol; (2) retention of peptide tertiary structures and therefore biological activity is much less affected; and (3) environmental

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concerns are lessened with alcohols as compared to acetonitrile, including the disposal of these solvents. Direct quantitation of peptides separated by RP-HPLC is needed by researchers in the pharmaceutical, bioanalytical, immunochemical, and food chemistry to assess purity or yield. Fujinari *et al.* reported the use of a CLND system in the RP-HPLC separations of peptides [9]. We now present a new binary gradient RP-HPLC method for the separation and quantitation of peptides isolated from casein hydrolysate using the nitrogen specific detection capabilities of the CLND system [10]. This study demonstrates that peptides can be separated and quantitated using standard RP-HPLC columns, solvents and the HPLC–CLND system. Optimization of the column, mobile phase and CLND operating conditions is also presented.

2. Experimental

2.1. Apparatus

All HPLC mobile phases were filtered through a Millipore (Bedford, MA, USA) HV filter with a pore size of 0.45 μm. RP-HPLC peptide analysis was performed on a Waters Model 625 pump with an analytical pumphead, Model 490 UV detector purchased from Waters Associates (Milford, MA, USA) and Model 7000 HPLC–CLND nitrogen-specific detector from Antek Instruments (Houston, TX, USA). Sample injection was performed with a Model 9125-080 sample valve with a 50 μl loop from Rheodyne (Cotati, CA, USA) onto the analytical HPLC column from Vydac (Hesperia, CA, USA), and the eluate passed through a GC capillary splitter from SGE (Austin, TX, USA) to split the mobile phase flow rate to the two detectors. A rotary evaporator Model Speed-Vac Plus from Savant (Farmingdale, NY, USA) and microwave digestion apparatus Model MDS 2000 from CEM (Matthews, NC, USA) were used. Amino acid analysis was performed by RP-HPLC using Waters Pico-Tag reagents, column, and Model 625 pump. The chromatographic data were collected and analyzed with Waters Millennium Sample Information software operating on a NEC Power Mate 486 computer.

RP-HPLC analysis of piperazine and 2,3-diethylpyrazine was accomplished on a Micromeritics Model 760 pump with a microbore pumphead purchased from Alcott Chromatography (Norcross, GA, USA) with an Antek Model 7000 HPLC–CLND nitrogen-specific detector, then analyzed separately with Model 770 UV detector from Spectra-Physics (Santa Clara, CA, USA). Sample was injected into a Model 8126 sample valve with a 5-μl loop from Rheodyne and chromatographed on a microbore HPLC column from Keystone Scientific (Bellefonte, PA, USA). Data were stored and analyzed on the Delta chromatography software (Digital Solutions, Margate, Australia) on an IBM 486 compatible computer.

2.2. Reagents and standards

Casein, Gly–Gly–Leu, glycine, HPLC peptide standard mixture (approximately 0.125 mg Gly–Tyr and approximately 0.5 mg each of Val–Tyr–Val, methionine enkephalin, leucine enkephalin, and angiotensin II) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Immobilized polyclonal, anti-casein antibodies (GTA disk) produced by Lampire Biological (Pipersville, PA, USA) and FMC (Pine Brook, NJ, USA), HCl, phenylisothiocyanate, Mix H amino acid standard, methanol, and TFA were purchased from Pierce Chemical (Rockford, IL, USA). RO/deionized water was obtained from a Model Milli-QUV Plus from Millipore.

2.3. Standard preparation and analytical method

The stock standard solution with 6 peptide components was prepared by adding 0.1 mg of Gly–Gly–Leu to the dry Sigma HPLC standard mixture and dissolving in 1 ml of water. Sequential partially filled injections (1, 2.5, 5, and 10 μl of the stock standard solution) were made into a 50-μl sample loop of the HPLC system. Casein hydrolysate was also analyzed by RP-HPLC (250 mm × 4.6 mm I.D., 90 Å pore size, 5 μm particle size) Vydac C18 column at 25°C. Mobile

phases: 0.1% TFA in water (A) and 0.1% TFA in methanol (B), flow rate of 650 μl/min, a gradient elution: 0 min 0%B, 50 min 50%B, 55 min 50%B, 60 min 0%B, and 65 min 0%B. The flow rate was split after the column using the SGE capillary splitter where 150 μl/min was directed to the CLND and 500 μl/min to the UV detector. Peptides **1** and **2** were collected from the exit end of the UV detector and evaporated to dryness. CLND conditions: 1050°C pyrolysis temperature, PMT voltage 700 V, range 25 ×, and detector output of 1 V; UV conditions: at 214 nm, range 0.2 AUFS. Alpha-amino butyric acid (1.74 μg) was added as internal standard to a 5-μl aliquot of the Mix H amino acid standard solution (Pierce Chemical). The same amount of internal standard was added to the hydrolyzed peptide fractions. The Waters Pico-Tag method was followed for the derivatization and amino acid analysis.

Piperazine (191 mg) and 2,3-diethylpyrazine (171 mg) were dissolved in 25 ml of water. A 2-μl partially filled injection of the standard mixture was made into a 5-μl sample loop and analyzed by RP-HPLC (150 mm × 2.0 mm I.D., 120 Å pore size, 5 μm particle size) BDS Hypersil C18 column at 25°C. Mobile phase: 0.1% TFA in methanol–water (50:50, v/v) was utilized at a flow rate of 200 μl/min, CLND conditions: 1050°C pyrolysis temperature, PMT voltage 760 V, range 10 ×, and detector output 1 V; UV conditions: at 254 nm, range 0.1 AUFS. The CLND and UV detection were performed separately.

3. Results and discussion

Direct quantitation by reversed-phase HPLC–CLND of peptides isolated from a casein hydrolysate is presented. Casein is a phosphoprotein that is one of the major constituents in milk and is an essential ingredient in cheese. Casein hydrolysates are used in food industry and in particular as the protein source in hypoallergenic infant formulas. Using this detector, a new, innovative and useful approach to analyze peptides was accomplished.

The chemiluminescence nitrogen detection mechanism is shown: as each nitrogen containing analyte is eluted from the column, it undergoes high-temperature oxidation (1000–1100°C). All chemically bound nitrogen compounds, except diatomic nitrogen (N_2), are converted to nitric oxide (NO).

$$R_3N + O_2 \xrightarrow{>1000^\circ C} H_2O + \cdot NO + \text{other oxides}$$

$$\cdot NO + O_3 \longrightarrow {}^{\bullet}NO_2 \longrightarrow NO_2 + h\nu$$

The gases are dried and mixed with ozone in the reaction chamber. Nitrogen dioxide (NO_2^*) in the excited state is formed. As the NO_2^* molecule reaches the stable ground state (NO_2), light ($h\nu$) is emitted and is detected by a photomultiplier tube (PMT). The signal from the chemiluminescence reaction is amplified and data stored on a computer for analysis and report generation.

A standard mixture of 6 peptides (peaks: 1 = Gly–Tyr, 2 = Gly–Gly–Leu, 3 = Val–Tyr–Val, 4 = methionine enkephalin, 5 = leucine enkephalin, and 6 = angiotensin II) was chromatographed by RP-HPLC. A simultaneous CLND and UV detection is shown in Fig. 1. One of the most important advantages of HPLC–CLND is the realization of an order of magnitude sensitivity enhancement over UV detection, in some cases, as demonstrated in Fig. 1. Gly–Gly–Leu, a tripeptide with no aromatic UV chromophore, produces at least 10 times more signal using HPLC–CLND than was produced by UV detection. This enhanced sensitivity is particularly important in the analysis of low molecular weight peptides. An optimum UV response at 214 nm for the compounds of interest was achieved using our chromatographic conditions. The upward baseline shift due to the TFA in the mobile phase was minimized at this wavelength. A linear detector response for each of the 6 peptide standards was observed for CLND. Four-point calibration curves using the linear regression

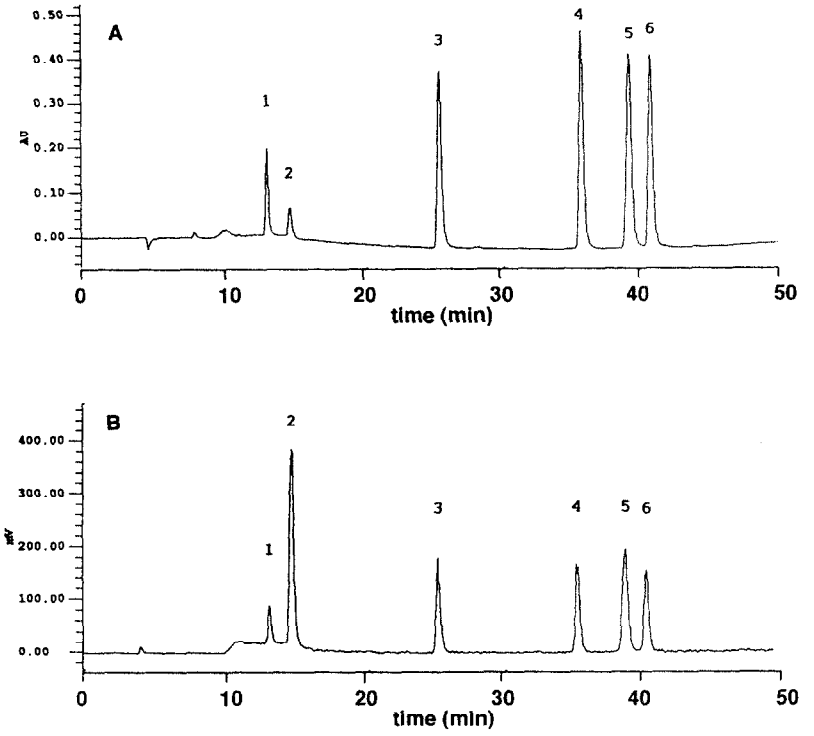

Fig. 1. RP-HPLC chromatograms of the 6-peptide standard mixture. Peaks: 1 = Gly–Tyr, 2 = Gly–Gly–Leu, 3 = Val–Tyr–Val, 4 = methionine enkephalin, 5 = leucine enkephalin, 6 = angiotensin II. (A) UV detection at 214 nm. (B) HPLC–CLND.

analysis (where r = correlation coefficient, m = slope and b = y-intercept) were obtained: Gly–Tyr ($r = 0.99450$, $m = 0.00003$, $b = 0.96929$), Gly–Gly–Leu ($r = 0.99733$, $m = 0.00002$, $b = 0.80865$), Val–Tyr–Val ($r = 0.99921$, $m = 0.00004$, $b = 3.65512$), methionine enkephalin ($r = 0.99895$, $m = 0.00003$, $b = 4.47127$), angiotensin II ($r = 0.99806$, $m = 0.00004$, $b = 0.07473$), and leucine enkephalin ($r = 0.99092$, $m = 0.00004$, $b = -4.00664$). The resulting slopes obtained using CLND were all very similar from the dipeptide, Gly–Tyr, to the larger peptides such as leucine enkephalin since the detector responded to the amount of nitrogen in each of these nitrogen bearing compounds. The HPLC–CLND responded equally to both aromatic and non-aromatic peptides. This suggests that detection and quantitation using a single compound calibration for the nitrogen quantification in a given sample is feasible. The UV detector on the other hand, responds to the presence of chromophores on the analytes. Gly–Gly–Leu, due to the absence of a strong aromatic chromophore, displayed a low UV detector response ($r = 0.99752$, $m = 0.00147$, $b = 5.93881$) and consequently a slope much different from the standard calibration curves generated by the CLND.

This paper will focus primarily on two peptides, **1** and **2**, which were isolated from casein hydrolysate using immobilized antibodies followed by RP-HPLC chromatographic determination with simultaneous CLND and UV detection. Several brand name columns (Vydac C18 with 90 and 300 Å pore; Waters C8 and C18 NovaPak; Hamilton RP1 and RP3; Sigma Nucleosil C18; Supelco LC 18 DB; and SGE 100 GL2-ODS2 2 × 100 mm) were evaluated on the basis of separation of the 6-peptide standard mixture. A Vydac C18 (90 Å) column was selected for the following reasons: it showed minimum tailing, optimum separation of the L- and M-

enkephalins, and consistent retention times from day to day operation. The peptide peaks **1** and **2** were isolated using immobilized polyclonal, anti-casein antibodies (GTA disk) and eluted with 0.10 *M* glycine with HCl (at pH = 2.7). Peptide 1 (t_R = 20.8 min) did not contain any significant chromophore and was not observed at 254 nor 280 nm UV detection. Peptide 2 (t_R = 38.2 min) however, showed a strong absorption at 280 nm. The UV (214 nm) chromatogram, Fig. 2, showed peptide **1** as the minor peak as compared to peptide **2**. In contrast, the nitrogen-specific detector, HPLC–CLND, showed peptide **1** as the major peak. The two peptides (**1** and **2**) were isolated by collecting each fraction from the post UV-eluent and dried by rotary evaporation. Each peptide fraction was then hydrolyzed by HCl gas phase microwave digestion [11], derivatized with phenylisothiocyanate and the amino acid composition determined by reversed-phase HPLC. From the results of the amino acid analysis, peptides **1** and **2** were calculated as 7.81 μg and 1.84 μg, respectively (see Tables 1 and 2). Peptide **1** did not contain amino acids with aromatic functional groups such as F, W, or Y. On the other hand, Tyrosine (Tyr or Y) contains aromaticity and was present in peptide **2**. This explains why peak **2** showed a strong UV absorbance. Both peptides were quantitated by HPLC–CLND based on the N response of the standard peptides: peptide **1** (10.7 μg) and peptide **2** (0.80 μg). These data agree with the relative amounts of peptides **1** and **2** found by the amino acid analysis. The significance of the CLND data is that peak **1** was found to be the major peptide based on the nitrogen content of the sample. Direct nitrogen quantitation (*i.e.* of peptides) is important to those who synthesize biologically active peptides as well as other nitrogen containing compounds. Nitrogenous

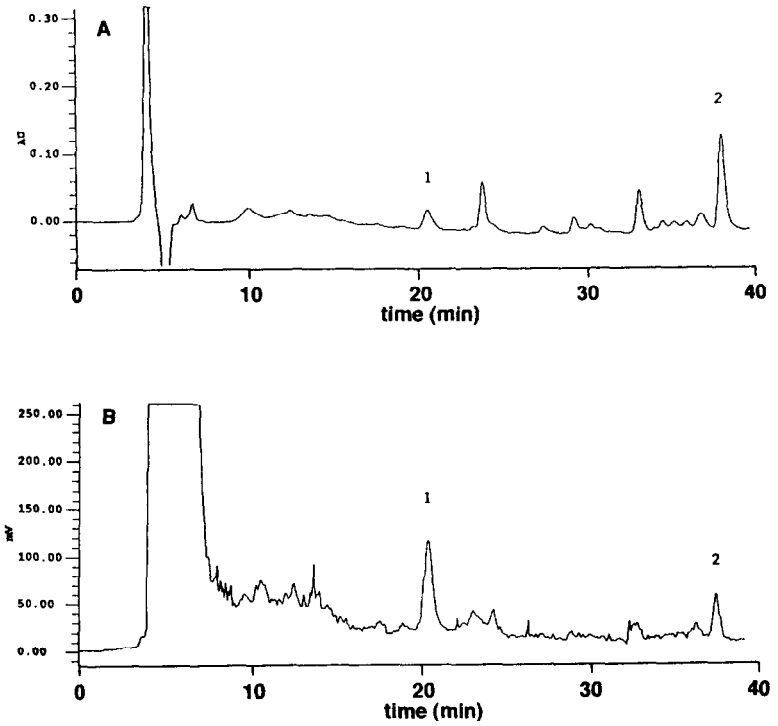

Fig. 2. RP-HPLC chromatograms of casein hydrolysate. Peaks: 1 = peptide with no aromatic UV chromophore, 2 = peptide with aromatic UV chromophore. (A) UV detection at 214 nm, (B) HPLC–CLND.

Table 1
Determination of amino acid (AA) composition of peptide **1** isolated from a casein hydrolysate sample

AA type	AA composition	No/mol	Peptide peak	AA data calculated (μg)	HPLC–CLND value (μg)
GLx	E/Q	4			
Ser	S	4			
Gly	G	1			
Ala	A	1			
Leu	L	2			
			Peptide **1**	7.81	10.7

Please note the absence of amino acids with aromatic functional groups such as F, W, or Y.

compounds without a UV chromophore are easily detected by CLND without the need of pre- or post-column derivatization. In addition, the non-nitrogenous compounds in the sample are transparent to the CLND. Our analytical approach enables other researchers to detect and focus on major nitrogen containing components in their samples.

Aromatic compounds such as 2,3-diethylpyrazine are easily detected by UV at 254 nm. Compounds with no chromophores such as piperazine are not detected by UV, and consequently can be overlooked. However, piperazine (peak **1**) is detected by the CLND without derivatization along with 2,3-diethylpyrazine (peak **2**) as depicted in Fig. 3. RP separation of nucleotides, nucleosides, and their bases with HPLC–CLND detection have been reported [12]. The sensitivity of HPLC–CLND is 0.4 ng N with signal-to-noise ratio of greater than 2:1. The high sensitivity of the nitrogen detector makes CLND a good candidate for capillary electrophoresis (CE) separations of complex molecules and biochemicals. HPLC–CLND study of peptides and food grade protein hydrolysates by size exclusion chromatography (SEC) has been completed and will be presented in the near future [13].

4. Conclusion

A new HPLC–CLND method for the analysis of peptides in a casein hydrolysate was achieved with a binary gradient reversed-phase elution. Peptides **1** and **2** were isolated and amino acid

Table 2
Determination of Amino Acid (AA) composition of peptide **2** isolated from a casein hydrolysate sample

AA type	AA composition	No/mol	Peptide peak	AA data calculated (μg)	HPLC–CLND value (μg)
GLx	E/Q	2			
ASx	D/N	1			
Ser	S	2			
Avg	R	1			
Pro	P	1			
Gly	G	1			
Tyr	Y	4			
			Peptide **2**	1.84	0.80

Please note the presence of amino acids with aromatic functional groups such as Y

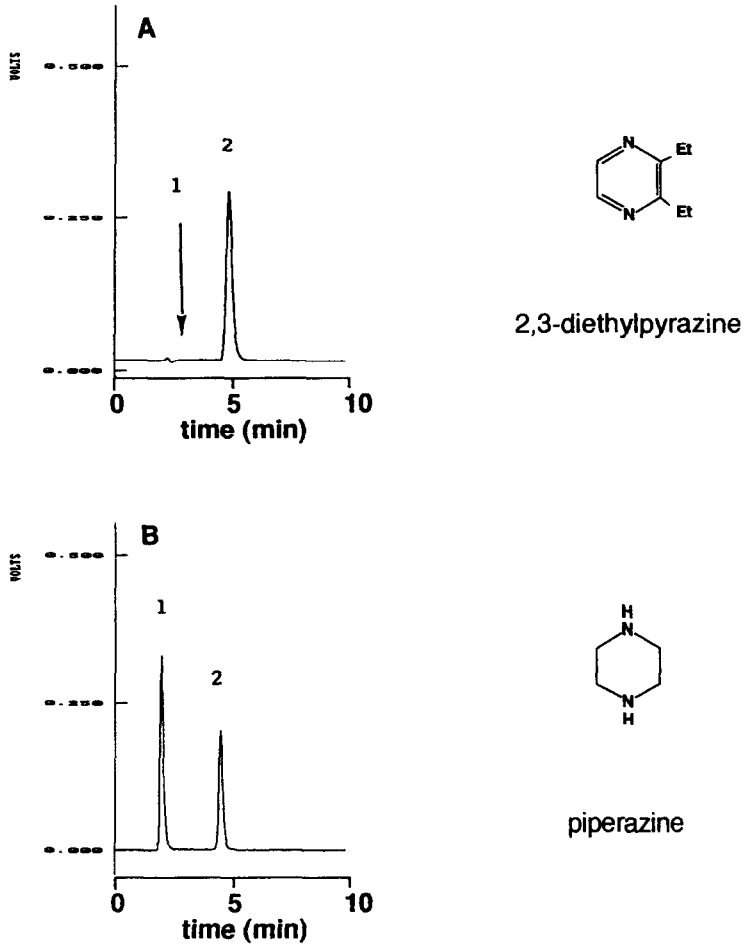

Fig. 3. RP-HPLC chromatograms of a 2-standard mixture. Peaks: 1 = piperazine which does not have a UV chromophore, 2 = 2,3-diethylpyrazine. (A) UV detection at 254 nm, (B) HPLC–CLND.

composition determined. The significance of utilizing HPLC–CLND is that peak integration of the peptides based on nitrogen content is obtained. Non-nitrogenous peaks detected by UV are not seen by CLND, thus simplifying interpretation of the chromatographic results. Other nitrogen containing compounds (*i.e.* piperazine) without a UV chromophore are easily detected by CLND without derivatization. A 6-component peptide standard mixture was used to show linearity of the HPLC–CLND. When this instrument is coupled to the UV detector, CLND provides additional useful data to enhance ones research. For example, by reversed-phase HPLC–CLND, one can achieve "total N" peptide mapping, selective isolation of modified peptides, direct peptide quantitation, and most important, purity analysis of bioactive peptides and other nitrogen bearing compounds.

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