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Rapid determination of the ratios of three aromatic residues in peptides by reversed-phase high-performance liquid chromatography with a high-resolution photodiode-array detector

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Abstract

A method for the simultaneous determination of the ratios of three aromatic residues in peptides by derivative UV spectrophotometry on a spectrophotometer with a resolution of 0.1 nm can be used for the RP-HPLC analysis of peptides because of the recent development of high-resolution photodiode-array detectors (1.2 nm). The difference between the theoretical and experimental ratios of aromatic residues of peptides determined in real time is less than 5%. This method could become a powerful tool for the study of peptides and hydrolysates. A variety of possible applications are discussed.

1. Introduction

Peptides containing aromatic residues show a characteristic UV absorbance between 240 and 300 nm attributable to the aromatic moieties (Fig. 1). The determination of distinct aromatic amino acids in a mixture, in a peptide or in a protein is difficult or impossible because of the overlapping of their substance-specific spectral bands. Owing to the development of second-derivative spectrophotometric studies, the specific bands of aromatic amino acids can be separated and the concentration of the individual amino acids quantified [1,2]. The concentration of Phe in proteins was studied because of the sufficient separation between Phe and Tyr–Trp bands [3]. Other studies by second derivative spectrometry have been proposed (determination of Trp and Phe concentrations [4] or Tyr and Phe concentrations in protein [5]), but only if the molar absorptivity is known. The application of these methods was also limited by the overlap of Tyr–Trp bands which reduces the sensitivity (Tyr could not be determined when the Trp concentration was equal to or greater than half of that of Tyr).

Thus, some workers [6] have determined the concentration of Tyr under alkaline conditions at pH 13, because the phenolic group of Tyr is ionized, which effects the separation of Tyr–Trp bands in the spectrum. Their concentrations can be determined, but that of Phe cannot owing to interfering bands from Tyr at this pH.

The most widely applied technique for the

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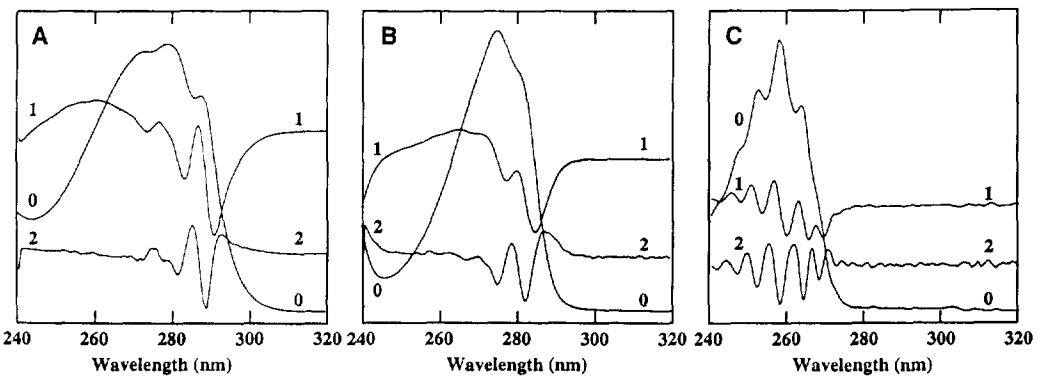

Fig. 1. Normal (0) and first- (1) and second-derivative (2) spectra of (A) tryptophan, (B) tyrosine and (C) phenylalanine (0.2 m*M*) in water–acetonitrile (90:10, v/v) containing 0.1% (v/v) trifluoroacetic acid (pH 1.9).

detection of compounds in HPLC is UV absorbance. By measurement of the column effluent from the RP-HPLC separation by photodiode-array detection (PDAD), the presence of Trp [7], Tyr–Trp [8] (at basic pH) or the three aromatic amino acids [9] in proteins is determined from the second-derivative spectrum of the compounds. Unfortunately, the limiting factor of the method is the resolution of the diodes, and also the quenching of Tyr and Phe by the strong absorbance of Trp.

Other workers have used PDAD to study the unfolding of α-lactalbumin in hydrophobic interaction chromatography [10]. The native protein and the conformers were eluted in two different peaks. The changes in the folded conformation of α-lactalbumin were investigated by the evolution of Tyr exposure determined from the ratio of amplitudes in second-derivative spectra.

In previous work, a method for the simultaneous, large-scale determination of the ratio of the aromatic residues in peptides was designed using first- and second-derivative spectrometry with a 0.1-nm resolution spectrometer [11]. Three linear regressions were obtained between three ratios (i.e., Tyr/Trp, Trp/Phe and Tyr/Phe) and the ratios of some spectral bands which were shown to characterize each amino acid in the first- or second-derivative spectra (Fig. 2). In this method, the Tyr/Trp ratio was calculated from the first-derivative spectrum of mixtures between 1:5 and 5:1. The Tyr/Phe and Trp/Phe ratios were calculated as the ratio of characteristic bands in the second-derivative spectrum.

Recently, with the evolution of optics and software, the original spectrum, and the first- and second-derivative spectra obtained with PDAD of 1.2-nm resolution, were similar to those described previously.

A further development of the method is its application to HPLC by direct "on-line" measurement of the UV spectra. In this paper, the characteristic amplitudes used for the determination of the ratios of aromatic residues in peptides are discussed. The effect of increasing proportion of solvent in the mixture on these bands was studied. Further, the determination of the ratios of the aromatic amino acids in synthetic peptides and in a tryptic hydrolysate of α_{s1}-casein (α_{s1}CN) is reported.

2. Experimental

2.1. Chemicals and solvents

Acetonitrile of HPLC grade was purchased from Rathburn (Walkerburn, UK) and trifluoroacetic acid (TFA) of HPLC grade from Sigma (St. Louis, MO, USA). Water of ultra-high quality for use in HPLC was prepared with an Elgastat UHQ system (Elgastat, Wycombe, UK). Porcine dynorphin B, human luteinizing

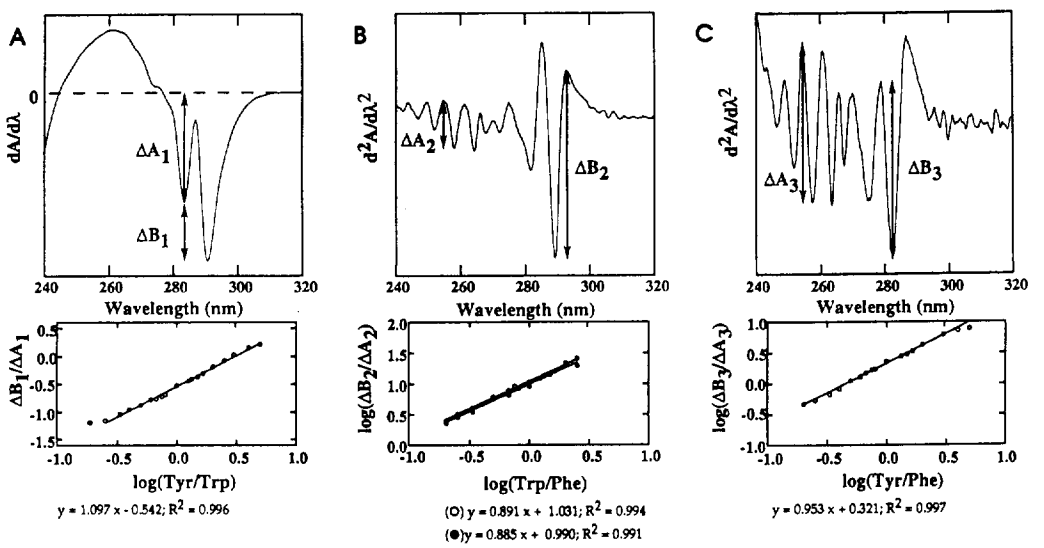

Fig. 2. Characteristic amplitudes of a mixture containing (A) one volume of tryptophan and two volumes of tyrosine in the first-derivative, (B) one volume of tryptophan and three volumes of phenylalanine in the second-derivative and (C) one volume of tyrosine and two volumes of phenylalanine in the second-derivative spectrum. Stock solutions were of 0.2 m*M* in water–acetonitrile (90:10, v/v) containing 0.1% (v/v) trifluoroacetic acid. The amplitudes indicated by arrows were used to plot the straight lines shown below. For tryptophan and phenylalanine mixtures, the regressions are shown in the (○) absence and (●) presence of tyrosine.

hormone-releasing hormone (h-LH-RH), neurotensin, substance P and [Tyr^8, Nle^{11}]-substance P were purchased from Neosystem (Strasbourg, France). All other peptides were obtained from Sigma.

2.2. RP-HPLC

Separations were performed using a Hitachi–Merck system with an L6200 ternary pumping system, a Model 655A-40 automated injection and sampling system (Merck, Darmstadt, Germany), coupled with a Millipore (Marlborough, MA, USA) Model 996 photodiode-array detector controlled by a 486/33i NEC (Boxborough, MA, USA) computer.

Samples were run on a LiChroCART C_{18} column (250 × 4 mm I.D., 5 μm particle size) obtained from Merck. Peptides were eluted with a gradient from 5 to 40% of acetonitrile in water containing 0.1% (v/v) TFA for 70 min at a flow-rate of 1 ml/min.

2.3. Hydrolysis of α_{s1}CN

Bovine α_{s1}CN was purified from skim bovine milk as described previously [12]. Freeze-dried α_{s1}CN was hydrolysed after solubilization in appropriate buffer by TPCK-treated trypsin (EC 3.4.21.4) (Sigma) or chymotrypsin (EC 3.4.21.1) from bovine pancreas (Sigma). Conditions for complete hydrolysis were chosen.

The aromatic peaks were analysed by amino acid composition and mass spectrometry, using fast atom bombardment or ionspray according to the assumed mass of the peptide.

2.4. Spectrophotometry

UV spectra were recorded between 240 and 300 nm at a rate of 10/s and the blank value was directly measured before appearance of the peak signal. First and second derivatives were calculated by 2010 Millennium software (Millipore). The splining (the process of filtering to produce a

smoother signal) of the derivatives did not alter the fine spectral features.

3. Results and discussion

3.1. Determination of the characteristic bands of the aromatic amino acids

The calibration applied in this method allows unknown compounds to be studied. Indeed, neither the molar absorptivity nor concentration is needed. Consequently, a ratio of characteristic spectral bands (determined on the derivative) was used for the determination of the ratio of the residues instead of a raw amplitude value as in previous works [3,5,6].

Two criteria were used in choosing the bands for the determination of the ratios of amino acids in a mixture. First, the bands should not overlap to allow the determination of the three ratios. Second, the chosen amplitudes should be the highest for extending the scale of residue ratios.

With Tyr–Phe or Trp–Phe mixtures, the distance between the different characteristic bands in the second derivative is important (255.4-nm peak and 257.7-nm trough for Phe; 278.8-nm peak and 282.4-nm trough for Tyr; 292.8-nm peak and 288.9-nm trough for Trp). These amplitudes were then chosen for the determination of Tyr/Phe and Trp/Phe ratios. A linear regression (Fig. 2) was obtained in binary mixtures between the ratio of Tyr–Phe bands (y) and Tyr/Phe theoretical ratios (x) ($y = 0.953x + 0.321$; $r^2 = 0.997$) and between the ratio of Trp–Phe bands and Trp/Phe theoretical ratios ($y = 0.891x + 1.031$; $r^2 = 0.994$).

Conversely, the characteristic bands of Tyr are close to those of Trp in the first and second derivatives. The influence of Tyr on the Trp bands and the reverse prevents a simple linear regression between the ratios. Nevertheless, the difference between the characteristic troughs in the first derivative decreases when the Trp/Tyr ratio decreases in the mixture. The 290-nm trough corresponds to the spectral influence of Trp. The trough close to 285 nm corresponds to the sum of the influences of Trp and Tyr. This results in a decreasing wavelength of the minimum with decreasing Tyr/Trp ratio (284.1 nm for Tyr/Trp = 5:1; 283.2 nm for Tyr/Trp = 1:5). The difference between the values of the two troughs (T284 nm − T290 nm) depends on the Tyr/Trp ratio, but is a function of their concentrations. The concentration parameters were eliminated by dividing the resulting difference by the value of the 284-nm trough for the calculation of the Tyr/Trp ratio. Thus, a linear regression independent of concentrations was obtained for a wide range of Tyr/Trp ratios ($y = 1.097x - 0.542$; $r^2 = 0.996$).

In ternary mixtures of aromatic amino acids, the Tyr/Phe ratio could not be determined because of overlapping of Tyr–Trp bands in the second-derivative spectrum A. Tyr/Phe value could be estimated, however, owing to the accurate determination of Trp/Tyr ratios in the first-derivative spectrum and Trp/Phe in the second. For the Trp/Phe ratio, the presence of Tyr in the mixture influenced the ratio of the two characteristic amplitudes with slight modification of the equation of the linear regression (0.6% and 4.0% for slope and intercept, respectively). This equation appeared to be independent of the proportion of Tyr [11] in the mixture with an outcome of $y = 0.885x + 0.990$; $r^2 = 0.991$ (Fig. 2).

3.2. Behaviour of aromatic amino acids with increasing proportion of solvent

The determination of the ratios of aromatic residues was calculated from four linear regressions resulting from the binary and ternary mixtures of free amino acids. The reference was acetonitrile–water (10:90) containing 0.1% TFA. In acidic medium an efficient determination of the Tyr/Trp ratio was obtained owing to a weak overlap of the Tyr–Trp in the first-derivative spectrum. Peptides with hydrophobic character were solubilized in this organic solvent.

As the proportion of acetonitrile in the eluent usually varies during gradient RP-HPLC analysis, the effect of the mixture composition on the spectra was investigated. The effects of an in-

creasing proportion of acetonitrile in the mixture were studied in the range 10–50%. It has been reported that organic solvents caused shifts in the spectra of aromatic amino acids [13]. However, changes in the mixture composition were not found to cause any significant variation of the λ_{max} values of Trp and Phe (Trp $\lambda_{max} = 278.3 \pm 0.1$ nm; Phe $\lambda_{max} = 257.7 \pm 0.2$ nm) whereas, in contrast, the λ_{max} of Tyr increased with increasing proportion of acetonitrile (Tyr $\lambda_{max} = 273.3 \pm 0.5$ nm). Nevertheless, the shifts of the spectra in the derivative modes with increase from 10 to 50% of acetonitrile (performed with the 0.1-nm resolution spectrometer) were not sufficient to cause an error in the determination of the characteristic bands as reported [9]. Further, the shift of the maxima is lower than the inherent resolution of PDAD.

The effect of solvent changes on intensity were estimated from the characteristic amplitudes. The coefficients of variation on the Trp 290-nm trough in the first-derivative spectrum were 4.0%, between the 282.5-nm trough and the 279.2-nm peak maximum of Tyr 3.0% and between 257.7-nm trough and the 255.4-nm peak maximum of Phe in the second-derivative spectrum 3.6%.

With respect to this weak perturbation observed on the isolated amino acids, the regressions established in acetonitrile–water (10:90) containing 0.1% TFA were assumed to be suitable for the determination of the ratios of aromatic residues in peptides eluted by gradient RP-HPLC.

3.3. Ratio determination of aromatic residues in synthetic peptides by RP-HPLC

Synthetic peptides were eluted with an increasing proportion of acetonitrile from 10 to 50% in RP-HPLC. The first- and second-derivative spectra were recorded between 240 and 300 nm. Characteristic parameters were chosen and experimental ratios of aromatic residues were calculated using the linear regression previously established (Table 1). In RP-HPLC, the average differences between the theoretical and experimentally calculated Tyr/Phe and Trp/Phe ratios for all the peptides tested were about 5%. These are less than the average differences obtained from the spectra of the same peptides on the 0.1-nm resolution spectrometer with a fixed acetonitrile–water composition [11] (7% and 8% respectively).

This improved accuracy can be explained on the one hand, by the contribution of RP-HPLC to the purity of the peptides and, on the other, by the complete dissolution of the peptides. Indeed, in peptides containing Phe with Trp and/or Tyr, an overestimation of Phe occurred if these peptidic compounds were partially dissolved. A decreasing drift of the baseline appears between 240 and 310 nm (including the Phe absorption zone in which the phenomenon is the most important) and, thus, modifies the spectrum.

The average differences between theoretical and experimentally calculated Tyr/Trp ratios are higher than those calculated in our previous work [11] (16% instead of 6%). This difference is related to a systematic underestimation of Tyr/Trp ratios. This phenomenon seemed to be constant and could be corrected as $Tyr/Trp_{corrected} = 1.038\ Tyr/Trp_{estimate} + 0.112$ ($r^2 = 0.998$), where $Tyr/Trp_{estimate}$ is the value calculated with the regression.

The difference between theoretical and calculated ratios was 4%. The calculation of the first-derivative spectra was different with PDAD software. This, perhaps, influenced the Tyr determination. The method of calculating the Tyr/Trp ratio (by dividing a difference of amplitudes by a $dA/d\lambda$ value, as opposed to amplitude by amplitude in the case of ratios implicating Phe) seemed to introduce a constant error. No loss of accuracy in Tyr–Phe mixtures or Trp–Phe mixtures was observed. In these cases, the ratios of characteristic amplitudes of Tyr–Phe and Trp–Phe bands certainly eliminated the constant error. The peptides studied include chemical modifications (sulfhydryl bonds, acetylation, amidation, etc.), even on aromatic residues, without altered spectra.

The positions of aromatic residues in the peptide chain obviously have no effect on the spectrum of the peptides (Table 1). Indeed,

Table 1
Experimentally determined ratios of aromatic amino acid (AA) residues for commercial peptides

Compound with sequence	Aromatic AA composition	Experimentally determined ratios[a]		
		Y/W	W/F	Y/F
ACTH fragment 1–10 (human) SYSMEHFRWG	(1W, 1Y, 1F)	0.83/1 *0.97/1*	1.06/1	ND[b]
Angiotensinogen 1–14 (porcine) DRVYIHPFHLLVYS	(2Y, 1F)	–	–	2.05/1
Dynorphin B (porcine) YGGFLRRQFKVVT	(1Y, 2F)	–	–	0.52/1
Fibroblast growth factor, basic fragment 106–120 YRSRKYSSWYVALKR	(1W, 3Y)	2.80/1 *3.01/1*	–	–
Gastrin-related tetrapeptide WMDFNH_2	(1W, 1F)	–	0.95/1	–
[Leu^{15}]-gastrin I <EGPWLEEEEEAYGWLDFNH_2 [c]	(2W, 1Y, 1F)	0.39/1 *0.52/1*	1.1/1	ND[b]
Insulin oxidized chain B (bovine) FVNQHLCGSHLVEALYLVCGERGFFYTPKA	(2Y, 3F)	–	–	1/1.57
LH-RH (human) <EHWSYGLRPGNH_2 [c]	(1W, 1Y)	0.83/1 *0.97/1*	–	–
LH-RH (salmon) <EHWSYGWLPGNH_2 [c]	(2W, 1Y)	0.43/1 *0.56/1*	–	–
Morphine-modulating neuropeptide AGEGLSSPFWSLAAPQRFNH_2	(1W, 2F)	–	0.49/1	–
Pro-[Phe$_{5,6}$]-octapeptidyl lysine PHPFHFFVYK	(1Y, 3F)	–	–	1/3.26
Somatostatin AGCKNFFWKTFTSC	(1W, 3F)	–	1/2.98	–
[Tyr^8, Nle^{11}]-substance P RPKPQQFYGLNleNH_2	(1Y, 1F)	–	–	0.96/1
[Tyr^{11}]-somatostatin AGCKNFFWKTYTSC	(1W, 1Y, 2F)	0.82/1 *0.96/1*	0.52/1	ND[b]

[a] Italic ratios correspond to Y/W corrected ratios.
[b] No determination was performed because the bands of tryptophan hide that of the tyrosine.
[c] <E: pyroglutamic acid.

under our conditions, a strong exposure of aromatic residues in the peptide results in the unfolding due to RP-HPLC techniques. Some workers [14] have shown that the dissolution of peptides in a mobile phase of acetonitrile–water containing 0.1% TFA is strongly denaturing. According to them, proteins and peptides are completely driven into an unfolded state in this mixture. With less denaturing conditions (C_4 column or propanol solution as mobile phase), multiple or broadened peaks may be observed, corresponding to the presence of different folded compounds. In these cases, the denaturing effect is increased as the contact time increases with the hydrophobic support. Then, the spectra of peptides with short retention times could be altered by quenching of amino acids.

The accuracy of the method was investigated with peptides with a maximum of 30 residues in our study, but the chymotryptic bovine fragment 1–99 of bovine α_{s1}CN could be estimated as Tyr/Phe = 1:1.96 compared with a theoretical ratio of 1:2.

3.4. Ratio determination applications

This rapid determination of the ratio of aromatic moieties allows the study of peptides

containing aromatic residues during purification or synthesis steps. This analytical method is not destructive and can be performed in real time. As in peak purity determinations with PDAD [15], some systematic studies of ratios are now considered during a HPLC run. The amount of peptides available is not a problem for this study, as a few micrograms were injected to result in a maximum absorbance of 0.2 between 240 and 300 nm and. Even with a maximum as low as 0.005 absorbance in this range, an accurate ratio estimation could be obtained.

The content of Phe–Tyr in a protein or a peptide can be determined by amino acid analysis when Trp is destroyed during hydrolysis with 6 *M* HCl. The determination of Trp requires a second hydrolysis under basic conditions (with barium hydroxide, for example). However, this type of hydrolysis prevents the determination of other amino acids. At least two series of hydrolysis experiments are necessary for the quantification of all the amino acids. By calculating the Trp/Phe and/or the Tyr/Trp ratio prior to the acidic hydrolysis, Trp concentration can be determined.

The method is useful for the study of hydrolysates. The Trp DNA codon is unique. For the construction of high-stringency DNA probes, Trp-containing peptides in enzymatic digests should be chosen for amino acid sequence analysis [9]. The peptides that contain Trp residues can be easily identified with this method.

α-Chymotrypsin hydrolyses peptide bonds which implicate aromatic residues. The determination of the ratios of the aromatic residues in chymotryptic peptides gives information about the hydrolytic sites. Indeed, the presence determined by spectral analysis of at least two types of aromatic residues indicates that at least one bond implicating an aromatic residue is not hydrolysed. The evolution of the hydrolysis with the incubation time can then be estimated in parallel with other classical studies (measurement of free amino groups [16], degree of hydrolysis [17]).

The most interesting application of this method remains peptide identification in the hydrolysate of sequence-known proteins. For example, entire aromatic peptides of a tryptic hydrolysis with bovine α_{s1}CN were successfully identified from only one chromatogram. A 400-μg amount of α_{s1}CN tryptic hydrolysate was injected on to the C_{18} RP-HPLC column (Fig. 3). Normal and first- and second-derivative spectra were recorded on the PDAD system. The results are given in Table 2. Four of the peptides were shown to possess only one type of aromatic amino acid residue by the absence of the influence of the two others at the characteristic wavelength. The identification of peptides was performed by associating the amino acid ratios of peptides from a theoretical lysis [18] and the estimates. Hypotheses were confirmed by mass spectrometry and amino acid composition (Table 2). Peak 3 exhibited a shoulder. In this case, the spectrum was recorded before the maximum of the first peak signal and after the maximum of the second in order to decrease the contamination.

Two peptides from the tryptic hydrolysate of α_{s1}CN were found with the same spectra and then with the same aromatic content. A retention-time prediction method [19] was used to differentiate the two peptides: α_{s1}CN–*f*(104–119) 42.3 min and, α_{s1}CN–*f*(91–100) 62.0 min predicted corresponding to peaks 1 and 3. The principle of the retention-time prediction method is based on the contribution of the individual amino acids residues to peptide retention in C_{18} gradient RP-HPLC. The retention times of the amino acids are estimated with a time coefficient measured by the effect of each amino acid on the retention of a synthetic Ac–Gly–X–X–$(Leu)_3$–$(Lys)_2$–amide peptide. The reference amino acid is Gln, whose coefficient is equal to zero.

Further, the effect of peptide chain length [20] on the retention behaviour is corrected. Peptide chains with a length of more than 15 residues have a retention time lower than expected from the summation of the individual coefficients.

The oxidation state of the hydrolysate was easily established by studying Met-containing peptides. Oxidation of the residue decreased the hydrophobic character of the modified peptides, and hence their retention times in RP-HPLC.

The identification of the peptides from their

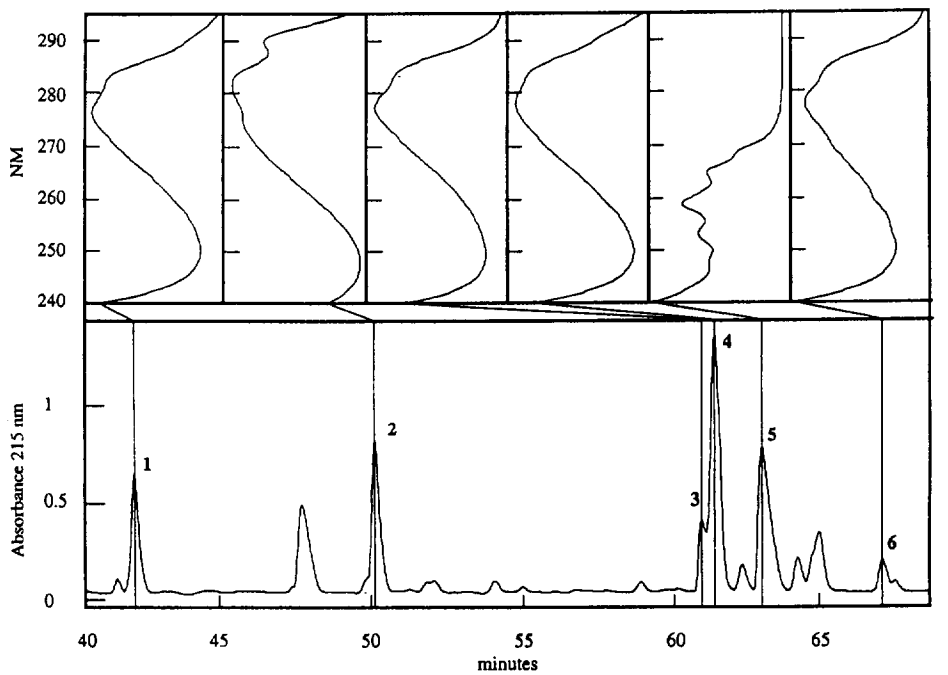

Fig. 3. Spectral extraction of the aromatic peptides from an RP-HPLC trace of α_{s1}-tryptic hydrolysate. The gradient was 5–40% of water–acetonitrile containing 0.1% (v/v) TFA for 70 min at a flow-rate of 1 ml/min. Injection of 400 μg of hydrolysate.

ratio of aromatic residues allowed the determination of the retention times of oxidized peptides appearing during the storage or induced by incubation with hydrogen peroxide (1%, v/v). Control of the oxidation state of hydrolysate during storage was then estimated from the ratios between the areas of the normal and oxidized peaks.

Table 2
Spectral characterization and experimentally determined ratios of aromatic amino acid residues of the aromatic peptides from the tryptic hydrolysis of bovine α_{s1}CN

Compound purified by RP-HPLC	Identification of aromatic residues[a]			Experimentally determined ratios[b]		
	W	Y	F	W/Y	W/F	Y/F
Peak 1	−	+	−			
Peak 2	+	−	−			
Peak 3	−	+	−			
Peak 4	+	+	+	1/4.97	1/2.12	ND[c]
Peak 5	−	−	+			
Peak 6	−	+	+			0.94/1

[a] The presence (+) or the absence (−) of the amino acid residue was verified.
[b] Ratios were calculated if the presence of at least two kinds of aromatic amino acid residues was verified.
[c] No determination was performed because the bands of tryptophan hide that of the tyrosine.

Table 3
Characterization by amino acid composition analysis and mass spectrometry of the aromatic peptides from the tryptic hydrolysis of the bovine α_{s1}CN: comparison with the theoretical peptidic mass and sequence

Amino acid	Peak 1	α_{s1}CN f(104–119)	Peak 2	α_{s1}CN f(194–199)	Peak 3	α_{s1}CN f(91–100)	Peak 4	α_{s1}CN f(152–193)	Peak 5	αs_1CN f(23–34)	Peak 6	α_{s1}CN f(133–151)
Ala[a]	1	1	–	–	–	–	3	3	1	1	1	1
Arg	0.90	1	–	–	0.88	1	–	–	–	–	1.05	1
Asx	1.13	1	–	–	–	–	5.06	5	–	–	1.16	1
Cys	–	–	–	–	–	–	–	–	–	–	–	–
Glx	4.03	4	–	–	2.15	2	5.07	5	0.99	1	4.19	4
Gly	–	–	–	–	0.89	1	2.70	3	0.89	1	1.08	1
His	–	–	–	–	–	–	–	–	–	–	–	–
Ile	0.80	1	–	–	–	–	1.79	2	–	–	0.97	1
Leu[b]	1.06	1	1	1	4	4	2.12	2	–	–	2.02	2
Lys	0.84	1	–	–	–	–	0.99	1	0.93	1	–	–
Met	–	–	0.38	1	–	–	–	–	–	–	0.46	1
Phe	–	–	–	–	–	–	1.75	2	3.46	4	1.86	2
Pro	2.02	2	0.84	1	–	–	5.11	5	1.73	2	1.73	2
Ser[c]	0.69	1	–	–	–	–	3.92	5	–	–	–	–
Thr	–	–	1.81	2	–	–	1.88	2	–	–	–	–
Trp[d]	–	–	–	1	–	–	–	1	–	–	–	–
Tyr	0.85	1	–	–	1.63	2	4.40	5	–	–	2.06	2
Val	1.62	2	–	–	–	–	0.76	1	1.73	2	1.08	1
M_r	1950.9[e]	1951.0[f]	746.9[e]	747.3[f]	1266.1[e]	1266.6[f]	4740.0[e,g]	4718.1[f]	1383.1[e]	1382.7[f]	2314.9[e]	2315.1[f]

[a] Taken as reference for amino acid composition calculation.
[b] Taken as reference for amino acid composition calculation in the absence of alanine.
[c] Partially destroyed during acid hydrolysis.
[d] Totally destroyed during acid hydrolysis.
[e] Experimentally determined.
[f] Theoretical.
[g] Difference due to one sodium ion bound to the peptide (+22) during casein preparation.

3.5. Method development

A PDAD apparatus with a resolution of 2 nm is not suitable for application of the method. The first-derivative spectrum of Tyr–Trp mixtures shows a trough at 290 nm with a shoulder, instead of two troughs at 284 and 290 nm. It is not possible to distinguish a peptide containing one Tyr from a peptide containing both one Tyr and Phe residue. The contribution of Phe in the second-derivative spectrum is smoothed. For good mathematical resolution of the spectra, a 1.2-nm resolution PDAD instrument is required. The best accuracy would be achieved by calibrating the system with synthetic peptides that cover a large range of aromatic residue ratios.

In conclusion, the method seems to be a powerful tool for the study of peptide compounds in RP-HPLC. The information is acquired in real time with HPLC and can be saved for further use. The aromatic ratio determination should be considered during all purification strategies of peptides as a complement to other classical "off-line" analyses.

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