On-Line HPLC Photodiode Array Detection and OPA Derivatization for Partial Identification of Small Peptides from White Wine

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This paper reports the possibilities of on-line reversed-phase HPLC with photodiode array detection (PDAD) and derivatization with *o*-phthaldialdehyde (OPA) for identification of small peptides. The methodology was initially applied to a wide range of standard peptides. Spectral parameters (*wavelengths of the spectrum maxima*, *convexity interval*, and *wavelengths of the second-derivative spectrum maxima*) obtained by PDAD allow identification of the aromatic amino acid residues (tyrosine, tryptophan, and phenylalanine) contained in the peptides as well as identification of other compounds from wine that can coelute with peptides (i.e. phenolic compounds). Postcolumn OPA derivatization allows confirmation of the peptidic nature of chromatographic peaks. The methodology has been successfully applied to peptide fractions (<700 Da) from cavas (Spanish sparkling wines). Peptides not containing aromatic amino acids and peptides containing only phenylalanine, tryptophan, or tyrosine as aromatic amino acids, as well as two coeluted cinnamic derivatives, were detected. To the authors' knowledge, these are the most detailed data reported to date about the composition of individual peptides from wine.

Keywords: *Wine; peptide; HPLC; UV spectra; OPA derivatization*

INTRODUCTION

Peptides in food exhibit multiple functional properties (surfactant, antimicrobial, antioxidant, ...) and contribute to bitter and sweet tastes (Turgeon *et al.*, 1992; Polo *et al.*, 1992; Bumberger and Belitz, 1993). Foam characteristics of sparkling wines and Champagne have been related to their protein content (Brissonnet and Maujean, 1991; Malvy *et al.*, 1994; Pueyo *et al.*, 1995; Andrés-Lacueva *et al.*, 1996), which is a global measure that includes different states of protein degradation (proteins, polypeptides, and peptides) (Bamford, 1985). In wine technology, some peptides have shown to be very effective fining agents, at least at the same level as gelatin or soluble poly(vinylpyrrolidone) (PVP) (Yokotsuka and Singleton, 1995).

Separation of peptides is mainly performed by reversed-phase high-performance liquid chromatography (RP-HPLC) (Guo *et al.*, 1986; Davankov *et al.*, 1990; Acedo *et al.*, 1994; Lee and Warthesen, 1996) and capillary electrophoresis (CE) (Colburn, 1992; Schwartz *et al.*, 1993). Identification of peaks is, undoubtedly, the most difficult task of the peptide analysis due to the wide range of peptide structures (amino acid composition and sequence). Peptides have to be isolated and purified for amino acid sequencing, which is tedious and time-consuming. Peptide identification can be rapidly achieved by using a mass spectrometry (MS) detector coupled to the liquid chromatograph (HPLC-MS), but this equipment is not easily accessible for many laboratories. The use of photodiode array (PDAD) detection allows the nondestructive identification of tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp) residues in peptide mixtures separated by HPLC or CE (Grego *et al.*, 1986; Paladino and Cohen, 1991; Grimm *et al.*, 1994; Perrin *et al.*, 1995). These three aromatic amino acids show characteristic UV spectra. Some spectral features in the 240−320 nm range have been used for aromatic amino acid identification: *wavelengths of minima of the second-order derivative spectrum* (Grego *et al.*, 1986; Paladino and Cohen, 1991), *intersection of zero ordinate axis* (Paladino and Cohen, 1991), and *absorbance ratios* in the first- and second-derivative spectra (Perrin *et al.*, 1995). *Match factor* in the second-derivative spectrum over the 250−320 nm range, between unknown peptides and standards, has also been used for identification (Grimm *et al.*, 1994).

HPLC analysis of wine peptides requires previous sample fractionation to avoid interference of proteins, free amino acids, and phenolic compounds (Acedo *et al.*, 1994). Due to the binding capacity of phenolics (Spencer *et al.*, 1988), they could form naturally occurring complexes with peptides, or they could associate with peptides during analytical procedures, although there is still no evidence for any of these assumptions. Concerning peptide characterization, we have found no references about identification of individual peptides from wine, either by sequencing of isolated peptides or by studying MS or UV spectra of chromatographic peaks. Most of the few studies about wine peptides describe only the amino acid composition of the total peptide fraction (Kozub *et al.*, 1980; Usseglio-Tomasset and Bosia, 1990; Acedo *et al.*, 1994).

This paper reports the use of an on-line HPLC-PDAD and *o*-phthaldehyde derivatization system for identification of small peptides from wine. In the first part of the paper, this methodology has been applied to a wide range of standard peptides. Spectral data have been obtained by the photodiode array detector in the 190−340 nm range. Determination of some spectral parameters (*wavelengths of the spectrum maxima*, *convexity interval*, and *wavelengths of the second-derivative spectrum maxima*) allows identification of the aromatic amino acid residues contained in the peptides. Since *o*-phthaldialdehyde (OPA) in the presence of a thio compound (such as 2-mercaptoethanol) reacts with the N-terminal group of peptides to form highly fluorescent thio-substituted isoindoles (Simons and Johnson, 1976),

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Table 1. Chromatographic Characteristics of Standard Peptides[a] Obtained Using RP-HPLC-PDAD and On-Line OPA Derivatization

	UV absorbance detection				OPA fluorescence detection			
peptide	RT (min)	area[b] 214 nm	area[b] 280 nm	ratio 214/280	RT (min)	area[b] fluorescence	ratio fluores/214	ratio fluores/280
Not Containing Aromatic Amino Acids								
GG	1.21	0.66	nd[c]		1.58	58.28	88.30	
GGGGGG	1.30	0.86	nd		1.71	60.04	69.81	
GAA	1.86	3.04	nd		2.18	71.90	23.65	
ECG	1.52	1.84	nd		1.80	325.01	176.64	
KK	1.96	0.56	nd		2.31	108.58	193.89	
Containing Tyrosine								
GY	10.41	3.69	0.76	4.86	10.78	27.35	7.41	35.99
YG	7.57	5.89	1.16	5.08	8.00	22.03	3.74	18.99
YGG	6.29	6.07	1.16	5.23	6.71	5.68	0.94	4.90
VYV	24.81	11.51	1.70	6.77	25.23	5.50	0.48	3.24
GYA	12.24	7.29	1.04	7.01	12.62	33.92	4.65	32.62
GLY	24.46	6.77	1.18	5.74	24.88	48.37	7.14	40.99
KYK	6.51	8.66	1.23	7.04	6.95	153.40	17.71	124.72
GGYR	11.96	5.62	0.83	6.77	12.34	38.91	6.92	46.88
GRYDS	16.70	10.23	1.36	7.52	nd	nd		
ASTTTNYT	19.40	12.56	1.26	9.97	19.78	20.67	1.65	16.40
YY	17.97	11.67	2.45	4.76	18.39	9.10	0.78	3.71
YYY	28.48	16.99	3.61	4.71	28.87	8.30	0.49	2.30
YYYYYY	43.37	47.67	9.97	4.78	43.77	3.30	0.07	0.33
Containing Tryptophan								
GW	22.31	26.38	5.41	4.88	22.7	120.28	4.56	22.23
WG	19.30	23.59	4.72	5.00	19.67	20.73	0.88	4.39
KWK	15.62	25.80	5.18	4.98	16.04	216.82	8.40	41.86
WW	41.71	42.75	8.95	4.78	42.11	21.82	0.51	2.44
WHWLQL	59.04	46.49	8.83	5.27	59.43	0.21	0.005	0.02
Containing Phenylalanine								
KF	16.22	7.35	nd		16.61	89.85	12.22	
FG	14.25	5.00	nd		14.68	28.03	5.61	
GF	16.12	6.66	nd		16.61	60.70	9.11	
LGF	31.11	6.80	nd		31.51	11.19	1.65	
GFL	35.84	7.68	nd		36.27	27.23	3.55	
GLF	36.44	6.86	nd		36.87	35.58	5.19	
MLF	42.67	9.70	nd		43.11	1.54	0.16	
VAAF	27.53	7.23	nd		27.95	10.65	1.47	
FLEEL	43.01	8.35	nd		43.42	2.13	0.26	
FF	35.04	15.03	nd		35.50	1.48	0.10	
GFF	39.36	12.39	nd		39.79	24.17	1.95	
FGFG	35.71	13.04	nd		36.14	4.94	0.38	
FFF	51.69	16.76	nd		nd	nd		
FFFF	63.53	18.84	nd		nd	nd		
Containing Tyrosine and Phenylalanine								
FY	24.15	11.34	1.25	9.07	24.59	11.95	1.05	9.56
YF	27.22	13.75	1.18	11.65	27.63	10.63	0.77	9.01
YYF	37.89	15.70	2.55	6.16	38.20	0.02	0.00	0.01
YPF	35.38	14.96	1.08	13.85	35.80	5.67	0.38	5.25
YGGFL	42.08	20.40	1.66	12.29	42.50	8.29	0.41	4.99
YGGFM	36.47	22.00	1.70	12.94	36.9	9.19	0.42	5.41
DRVYIHPF	44.91	42.12	1.87	22.52	45.27	17.19	0.41	9.19
YGGFLRKR	34.71	17.92	1.25	14.34	35.16	150.33	8.39	120.26
Containing Tyrosine and Tryptophan								
WY	29.05	25.74	6.16	4.18	29.48	12.25	0.48	1.99
Containing Tryptophan and Phenylalanine								
WF	38.63	29.05	5.50	5.28	39.08	14.20	0.49	2.58
FSWGAEGQR	36.79	38.32	6.10	6.28	37.21	0.67	0.02	0.11

[a] For peptide designation, one-letter amino acid symbol nomenclature was adopted. [b] Peak area per nanomol of compound injected. [c] nd, not detected.

fluorescence detection of OPA derivatives allows confirmation of the peptidic nature of chromatographic peaks. In the second part of the paper, this methodology has been successfully applied to the identification of the peptide fraction (<700 Da) of cavas (Spanish sparkling wines) commercially prepared according to the traditional method from different white grape varieties. The majority of the peptides contained only phenylalanine as aromatic amino acid, although some peptides not containing aromatic amino acids and some containing tryptophan or tyrosine as aromatic amino acids were also detected. To our knowledge, these are the most detailed data reported to date about the composition of individual peptides from wine.

MATERIALS AND METHODS

Standard Peptides and Wine Samples. Different sizes of peptides not containing aromatic amino acids, containing only tyrosine, tryptophan, or phenylalanine as aromatic amino acids, and containing two of these aromatic amino acids were used in this study. It was intended that the nonaromatic amino acid composition of the chosen standard peptides was

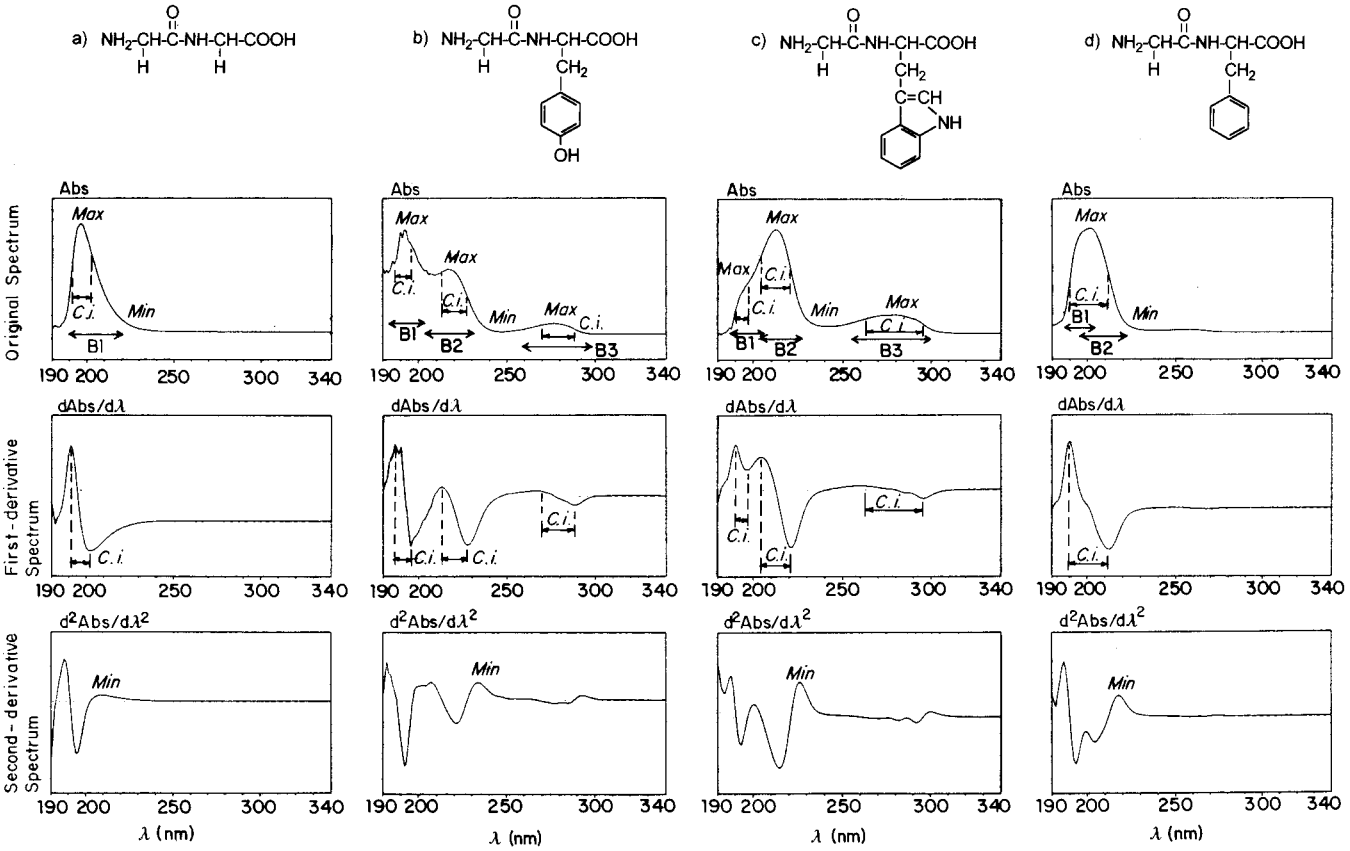

Figure 1. UV original and first- and second-derivative spectra of four known glycyldipeptides respectively not containing aromatic amino acids or containing only tyrosine, tryptophan, or phenylalanine as aromatic amino acids: GG (a); GY (b); GW (c); GF (d). Max, wavelengths of the spectrum maxima; C.i., convexity interval; Min, wavelength of the original spectrum minimum; B1, band 1; B2, band 2; B3, band 3.

similar to the composition of total peptide fraction of cava wines, with glycine, threonine, serine, alanine, and lysine as most abundant amino acids (Moreno-Arribas *et al.*, data not published). All standards were purchased from Sigma (St. Louis, MO). For each standard, solutions of different concentrations (from 0.05 to 1 μmol/mL) were prepared in water containing tryptophan (0.018 μmol/mL, used as internal standard) for analysis by HPLC. For peptide designation, the one-letter amino acid symbol nomenclature was adopted.

Peptide identification by HPLC-PDAD and postcolumn OPA derivatization was carried out in four varietal Spanish sparkling wines (Champenoise method), commercially prepared from grapes of the Parellada, Macabeo, Chardonnay, and Xarel.lo white varieties, and aged with yeast over 26 months. Since secondary fermentation of these wines takes place in individual bottles, at least six bottles of every batch were mixed and homogenized before sampling. Peptide fractions were obtained as described previously (Moreno-Arribas *et al.*, 1996). Briefly, wines (200 mL) were centrifuged, concentrated under vacuum (to 10 mL), and then precipitated with 5 volumes of 95% ethanol in an acid medium. After centrifugation, supernatants were concentrated under vacuum (to 5 mL) and fractionated by chromatography on Sephadex G-10 (Pharmacia Biotech AB, Uppsala, Sweden). Fractions corresponding to peptides <700 Da were collected, freeze-dried, and redissolved in 1 mL of water containing tryptophan (0.018 μmol/mL, used as internal standard) for analysis by HPLC.

Peptide Analysis by HPLC-PDAD. A liquid chromatograph consisting of two Beckman (Fullerton, CA) M116 pumps, a Beckman system organizer, a Waters (Milford, MA) M717Plus automatic injector, and a Beckman M168 photodiode array detector was used. Equipment control and acquisition and processing of data were carried out using the Beckman Gold System program (San Ramon, CA). Separations were performed on a Nova-Pak C_{18} column (150 × 3.9 mm i.d., 60 Å, 4 μm, Waters). Eluent A was 0.1% trifluoroacetic acid in water and eluent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient of eluent B increased from 0 to 40% over 70 min. Flow rate was 1 mL/min. Detection was performed by scanning from 190 to 340 nm with a resolution of 1 nm. Chromatograms were recorded at commonly used wavelengths (214 and 280 nm), and spectra were recorded after subtracting the solvent absorption. Different spectral parameters were determined: wavelengths of the spectrum maxima, wavelengths of the second-derivative spectrum maxima—both given by the software of the photodiode array detector— and convexity interval (distance between the inflection points before and after the maximum in the original spectrum; Bartolome *et al.*, 1993). Convexity interval was calculated as the difference between the wavelengths corresponding to the minimum and maximum in the first-derivative spectrum, values of which were given by the software of the photodiode array detector. It was checked that changes in the composition of the mobile phase along the gradient have no effect on the values of these spectral parameters. It was also seen that spectra of standard peptides were not distorted when standards were added to wine peptide preparations.

Standard solutions and samples (20 μL) were injected onto the column after being filtered through a 0.45 μm membrane filter. Injections were carried out in duplicate.

Postcolumn OPA Derivatization. The OPA derivatization conditions used were previously set up for amino acids and cheese peptides by Gonzalez de Llano *et al.* (1991) and also found to be optimum for a range of standard peptides (Herraiz *et al.*, 1994). The effluent from the photodiode array detector was mixed at a T-union with the OPA solution at a rate of 0.5 mL/min. The OPA solution was prepared by dissolving 800 mg of OPA in 10 mL of methanol, with subsequent dilution with 990 mL of borate buffer (0.5 M, pH 10.4), and, finally, 2-mercaptoethanol (600 μL) was added. Derivatization was carried out in a reaction tube (3 m × 0.5 mm i.d.) placed in a bath at 50 °C. Fluorescence detection of OPA derivatives was performed by a Waters 420 AC fluorometer (340 ± 6 nm excitation filter and 425 nm long-pass emission filter). Absorbance and fluorescence responses were

Table 2. Spectral Parameters of Standard Peptides[a]

peptide	band 1 max (nm)	band 1 convexity interval	band 2 max (nm)	band 2 convexity interval	max 2nd deriv (nm)	band 3 max (nm)	band 3 convexity interval
Not Containing Aromatic Amino Acids							
GG	205 ± 1	10 ± 2			215 ± 1		
GGGGGG	204 ± 1	9 ± 2			214 ± 1		
GAA	204 ± 1	10 ± 2			215 ± 1		
ECG	203 ± 1	8 ± 2			213 ± 1		
KK	205 ± 1	11 ± 2			216 ± 1		
Containing Tyrosine							
GY	201 ± 1	7 ± 2	224 ± 1	13 ± 2	238 ± 1	276 ± 1	19 ± 2
YG	204 ± 1	7 ± 2	225 ± 1	13 ± 2	238 ± 1	276 ± 1	20 ± 2
YGG	205 ± 1	8 ± 2	226 ± 1	12 ± 2	239 ± 1	276 ± 1	20 ± 2
VYV	204 ± 1	8 ± 2	224 ± 1	13 ± 2	238 ± 1	277 ± 1	20 ± 2
GYA	202 ± 1	9 ± 2	224 ± 1	13 ± 2	238 ± 1	276 ± 1	20 ± 2
GLY	204 ± 1	9 ± 2	224 ± 1	14 ± 2	238 ± 1	277 ± 1	20 ± 2
KYK	203 ± 1	7 ± 2	224 ± 1	13 ± 2	238 ± 1	277 ± 1	20 ± 2
GGYR	205 ± 1	8 ± 2	224 ± 1	13 ± 2	238 ± 1	276 ± 1	20 ± 2
GRYDS	204 ± 1	8 ± 2	222 ± 1	13 ± 2	238 ± 1	276 ± 1	20 ± 2
ASTTTNYT	203 ± 1	9 ± 2	sh[b]	11 ± 2	238 ± 1	276 ± 1	20 ± 2
YY	203 ± 1	7 ± 2	225 ± 1	14 ± 2	238 ± 1	276 ± 1	21 ± 2
YYY	204 ± 1	7 ± 2	225 ± 1	13 ± 2	238 ± 1	276 ± 1	19 ± 2
YYYYYY	203 ± 1	8 ± 2	226 ± 1	13 ± 2	239 ± 1	277 ± 1	19 ± 2
Containing Trytophan							
GW	sh	7 ± 2	221 ± 1	14 ± 2	233 ± 1	278 ± 1	32 ± 2
WG	sh	7 ± 2	222 ± 1	13 ± 2	231 ± 1	279 ± 1	32 ± 2
KWK	sh	6 ± 2	223 ± 1	15 ± 2	233 ± 1	280 ± 1	31 ± 2
WW	sh	6 ± 2	220 ± 1	15 ± 2	232 ± 1	280 ± 1	31 ± 2
WHWLQL	sh	7 ± 2	221 ± 1	15 ± 2	233 ± 1	279 ± 1	33 ± 2
Containing Phenylalanine							
KF	204 ± 1	20 ± 2	ov[c]	ov	224 ± 1		
FG	205 ± 1	18 ± 2	ov	ov	222 ± 1		
GF	205 ± 1	21 ± 2	ov	ov	224 ± 1		
LGF	203 ± 1	19 ± 2	ov	ov	223 ± 1		
GFL	206 ± 1	20 ± 2	ov	ov	224 ± 1		
GLF	207 ± 1	19 ± 2	ov	ov	222 ± 1		
MLF	204 ± 1	18 ± 2	ov	ov	224 ± 1		
VAAF	204 ± 1	20 ± 2	ov	ov	224 ± 1		
FLEEL	205 ± 1	19 ± 2	ov	ov	223 ± 1		
FF	203 ± 1	20 ± 2	ov	ov	223 ± 1		
GFF	202 ± 1	20 ± 2	ov	ov	225 ± 1		
FGFG	203 ± 1	19 ± 2	ov	ov	224 ± 1		
FFF	203 ± 1	20 ± 2	ov	ov	225 ± 1		
FFFF	204 ± 1	20 ± 2	ov	ov	226 ± 1		
Containing Tyrosine and Phenylalanine							
FY	204 ± 1	19 ± 2	sh	9 ± 2	238 ± 1	276 ± 1	21 ± 2
YF	205 ± 1	15 ± 2	sh	9 ± 2	239 ± 1	276 ± 1	19 ± 2
YYF	206 ± 1	16 ± 2	sh	11 ± 2	239 ± 1	277 ± 1	20 ± 2
YPF	207 ± 1	20 ± 2	sh	7 ± 2	240 ± 1	276 ± 1	21 ± 2
YGGFL	203 ± 1	15 ± 2	sh	9 ± 2	238 ± 1	276 ± 1	21 ± 2
YGGFM	204 ± 1	17 ± 2	sh	8 ± 2	238 ± 1	276 ± 1	21 ± 2
DRVYIHPF	204 ± 1	17 ± 2	ov	6 ± 2	238 ± 1	276 ± 1	20 ± 2
YGGFLRKR	202 ± 1	16 ± 2	sh	5 ± 2	238 ± 1	276 ± 1	20 ± 2
Containing Tyrosine and Tryphtophan							
WY	sh	5 ± 2	221 ± 1	15 ± 2	232 ± 1	278 ± 1	30 ± 2
Containing Trytophan and Phenylalanine							
WF	sh	7 ± 2	219 ± 1	15 ± 2	231 ± 1	280 ± 1	33 ± 2
FSWGAEGQR	204 ± 1	7 ± 2	218 ± 1	17 ± 2	234 ± 1	280 ± 1	31 ± 2

[a] For peptide designation, one-letter amino acid symbol nomenclature was adopted. [b] sh, shoulder. [c] ov, overlapping to band 1.

recorded during each separation for every sample injected into the liquid chromatograph.

RESULTS

On-Line RP-HPLC-PDAD and OPA Derivatization of Standard Peptides. Chromatographic characteristics of standard peptides are listed in Table 1. The relative standard deviations were lower than 3.5% for the retention time of peaks and lower than 7% for the area of individual peaks. Peptides containing only phenylalanine as aromatic amino acid did not give response at 280 nm. Peptides containing tryptophan gave the highest absorbance response (area/nmol) at 214 and at 280 nm. The values for the ratio *area 214 nm/area 280 nm* were slightly higher for peptides containing both tyrosine and phenylalanine. As expected, this parameter increased with the ratio between the number of total amino acids and the number of tyrosine or tryptophan residues that form the peptides.

All peptides studied exhibited OPA fluorescence response, except GRYDS, FFF, and FFFF. ECG, peptides containing glycine as the N-terminal residue, and peptides containing lysine or tryptophan residues in the main chain gave the highest values for fluorescence area

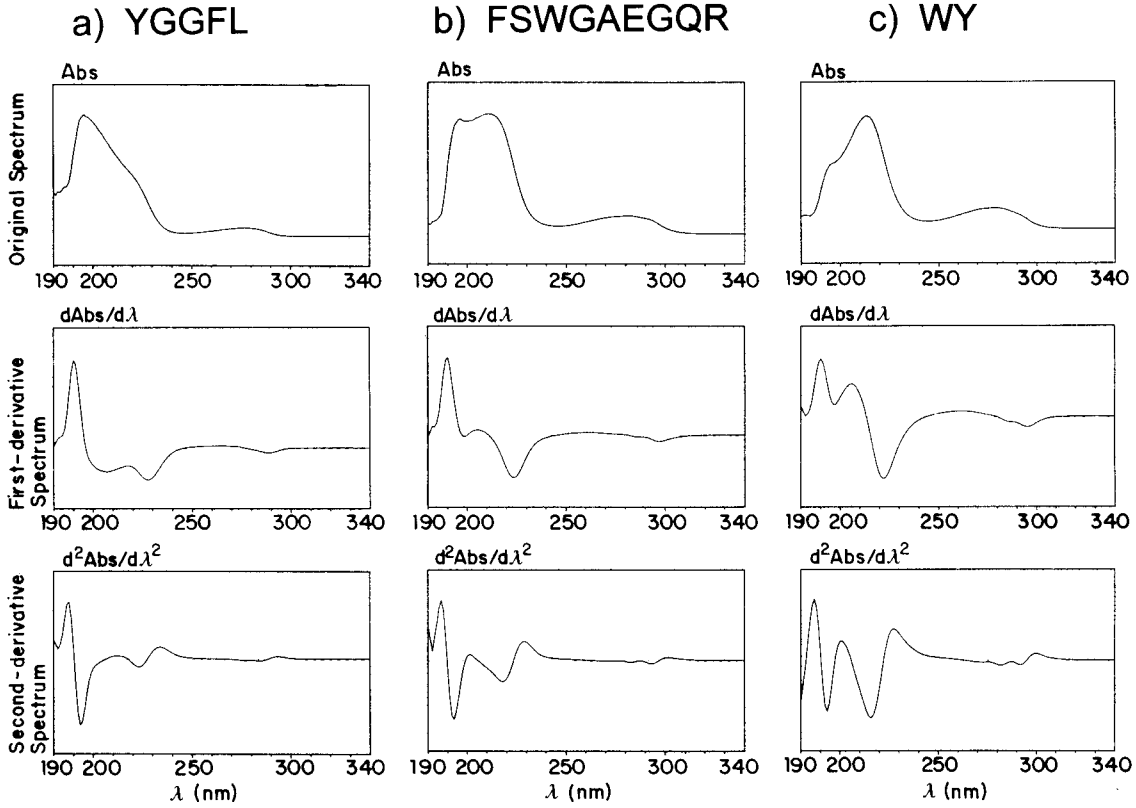

Figure 2. UV original and first- and second-derivative spectra of three known peptides respectively containing both tyrosine and phenylalanine, tryptophan and phenylalanine, and tryptophan and tyrosine residues: YGGFL (a); FSWGAEGQR (b); WY (c).

per nanomole of compound injected (Table 1), whereas the lowest values corresponded to the largest peptides. A similar trend was observed for relations *area fluorescence/area 214 nm* and *area fluorescence/area 280 nm*, although the effect of tryptophan residues was lower.

UV spectra of peptides not containing aromatic amino acids showed one band (named band 1 in this paper, Figure 1a) due to both the peptidic bonds and the carboxy-terminal group of the peptide molecule. When peptides contained tyrosine or tryptophan residues, two extra bands (named bands 2 and 3 in this paper, Figure 1b,c) due to the respective hydroxyphenyl and indol groups of tyrosine and tryptophan were detected. Obviously, the relative importance of these two bands with regard to band 1 decreased as the number of nonaromatic amino acids in the peptide molecule increased. Spectra of peptides containing only phenylalanine as aromatic amino acids presented one main band, which overlapped band 1 and the band due to the phenyl group that was also named band 2 (Figure 1d).

To evaluate the variations in the spectra with peptide composition, several parameters were determined in the original and first- and second-derivative spectra for all standard peptides: wavelengths of the spectrum maxima, convexity interval, and wavelength of the main second-derivative spectrum maximum, which correspond to the wavelength of the main minimum in the original spectrum (Figure 1). Spectra of peptides not containing aromatic amino acids exhibited values from 203 to 205 nm for maximum of band 1, from 8 to 11 nm for convexity interval of band 1, and from 213 to 216 nm for second-derivative spectrum maximum (Table 2). Peptides containing only phenylalanine as aromatic amino acid differed in the values for convexity interval of band 1 (18−21 nm), due to band overlapping, and second-derivative spectrum maximum (222−226 nm) (Table 2). As was said above, the presence of band 3 characterized spectra of peptides containing tyrosine or tryptophan. Although there were almost no differences in maximum of band 3, convexity interval of band 3, and second-derivative spectrum maximum showed very different values for peptides containing only tyrosine as aromatic amino acids (19−21 and 238−239 nm, repectively) and peptides containing only tryptophan (31−33 and 231−233 nm, respectively) as aromatic amino acids (Table 2).

Spectra of peptides containing more than one different aromatic amino acid residue were expected to be combinations of the spectra described above. Effectively, in peptides containing both tyrosine and phenylalanine residues (Figure 2a), the presence of phenylalanine was inferred by a wide band 1 whose convexity interval values (15−20 nm, Table 2) were similar to the ones reported for peptides containing only phenylalanine as aromatic amino acids, and the presence of tyrosine was inferred by a band 3 with convexity interval and second-derivative spectrum maximum values (19−21 and 238−240 nm, respectively) similar to the ones reported for peptides containing only tyrosine as aromatic amino acids. However, peptides containing both tryptophan and phenylalanine (Figure 2b) or containing both tryptophan and tyrosine (Figure 2c) showed spectra and spectral parameters (Table 2) characteristic of peptides containing only tryptophan. It can be thought that the presence of tyrosine or phenylalanine would be inferred by these spectral parameters in peptides with higher tyrosine/tryptophan or phenylalanine/tryptophan ratios than the ones used in this study.

Identification of Peptides from Wine. Absorbance and fluorescence chromatograms of the small peptide fractions (<700 Da) of cavas from Parellada, Macabeo, Chardonnay, and Xarel.lo varieties are shown in Figure 3. Peaks from chromatograms of different wines that exhibited the same retention times, ratios

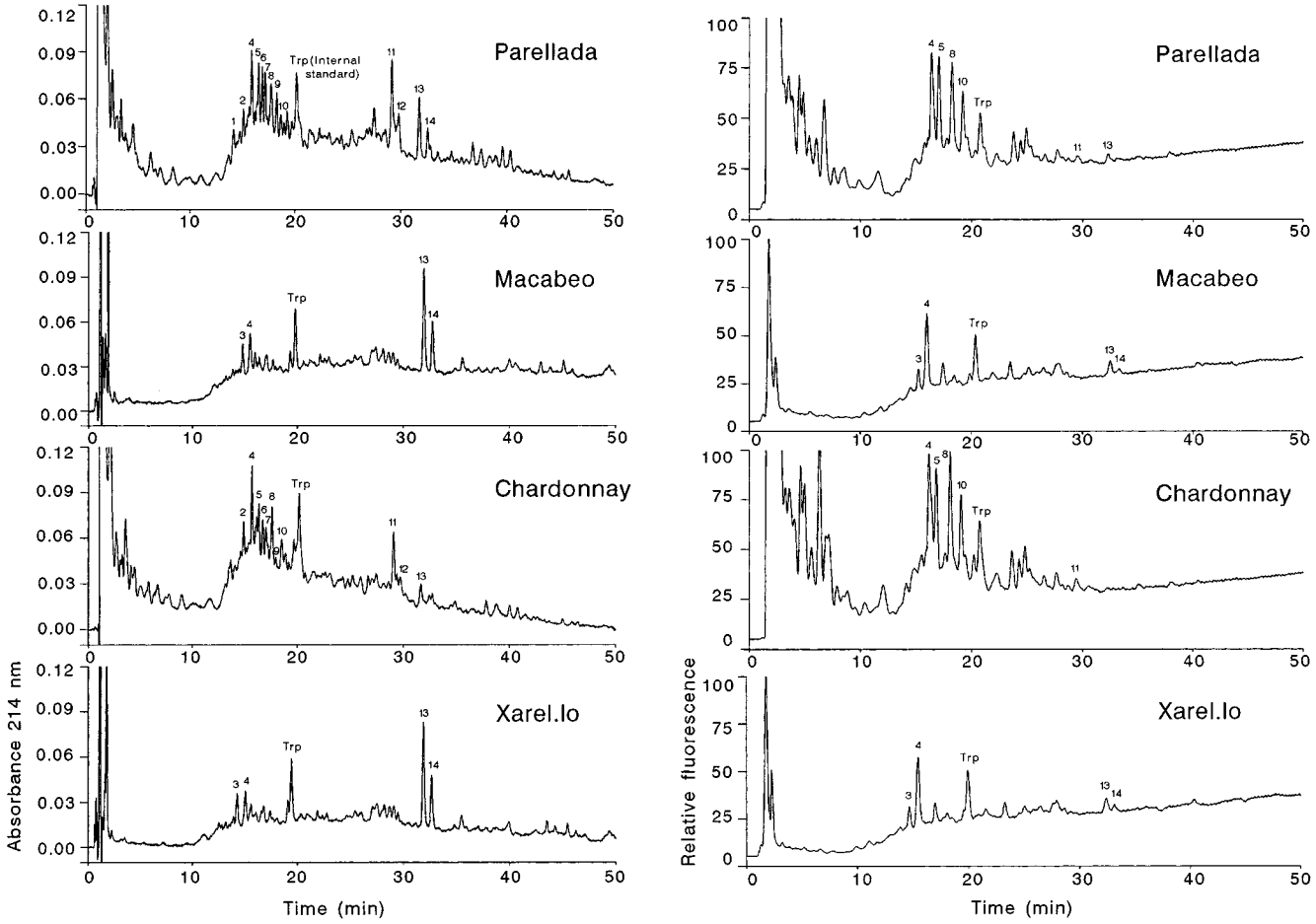

Figure 3. Absorbance at 214 nm and fluorescence chromatograms of the small peptide fractions (<700 Da) of cavas (Spanish sparkling wines) commercially prepared from grapes of Parellada, Macabeo, Chardonnay, and Xarel.lo varieties.

Table 3. Chromatographic and Spectral Characteristics of Main Peaks Detected in the Chromatograms of Cavas

		retention time		response ratios			band 1		band 2		max	band 3		
peak	variety present[a]	UV (min)	OPA (min)	ratio 214/280	ratio fluores/214	ratio fluores/280	max (nm)	convexity interval	max (nm)	convexity interval	2nd deriv (nm)	max (nm)	convexity interval	compound
1	P	14.11		2.83			204	9	ov[b]	10	265	324	52	cinnamic derivative
2	P, C	15.08					204	16	ov	ov	227			peptide with Phe
3	M, X	14.95	15.49		12.50		203	16	ov	ov	226			peptide with Phe
4	P, M, C, X	15.87	16.44		44.58		205	16	ov	ov	226			peptide with Phe
5	P, C	16.50	17.93		39.60		204	16	ov	ov	225			peptide with Phe
6	P, C	16.87					205	16	ov	ov	225			peptide with Phe
7	P, C	17.15					203	15	ov	ov	225			peptide with Phe
8	P, C	17.72	18.26		233.02		204	17	ov	ov	226			peptide with Phe
9	P, C	18.27		1.58			203	7	ov	9	248	312	53	cinnamic derivative
10	P, C	18.66	19.22	3.66	55.24	202.24	204	9	ov	11	234	276	30	peptide with Trp
11	P, C	29.10	29.66		2.17		204	10			217			peptide not containing aromatic amino acids
12	P, C	29.81		25.18			203	8	ov	13	237	276	20	peptide with Tyr
13	P, M, C, X	31.73	32.25	10.38	3.50	36.35	202	7	sh[c]	11	240	276	21	peptide with Tyr
14	P, M, X	32.55	33.14	10.68	1.30	13.86	203	6	sh	12	240	276	20	peptide with Tyr

[a] P, Parellada; M, Macabeo; C, Chardonnay; X, Xarel.lo. [b] ov, overlapping to band 1. [c] sh, shoulder.

area 214 nm/area 280 nm and fluorescence/absorbance, and spectra were given the same number. Identification of these main peaks was carried out by comparing their spectral data and fluorescence response with those of standard peptides (Table 3). Compounds **1**, **2**, **6**, **7**, **9**, and **12** gave no fluorescence response. Spectral parameter (wavelengths of the spectrum maxima, convexity interval, and wavelength of the second-derivative spectrum maximum) values of peaks 2, 6, 7, and 12 confirm the peptidic nature of these compounds. They could contain either proline or hydroxyproline as N-terminal amino acid residue, which explains why fluorescence response was not observed. Compounds **1** and **9** showed non-peptide type spectra. Spectrum maximum and convexity interval for peaks 1 and 9 (Table 3) indicated that they could correspond to cinnamic derivatives (Bengoechea *et al.*, 1995). At this point, it was confirmed that some of the most commonly occurring phenolic compounds in wines (e.g., catechins and hydroxycinnamates) also eluted under these chromatographic conditions, but no fluorescence response was observed. Thus, peak 1 showed a lower value than sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) for the maximum wavelength, but the same for convexity interval (Bartolome *et al.*, 1993). Since glycosylation produces bathochromic shifts of bands, but no changes in band width (convexity interval) (Ibrahim and Barron, 1989), it was hypothesized that peak 1 could correspond to sinapic acid 4-*O*-glycosylated. According to their spectral parameter values, peak 9 could correspond to

an ester of *p*-coumaric acid with organic acids or sugars (Bengoechea *et al.*, 1996).

Aromatic amino acids were identified in all of the wine peptides separated by HPLC, except in compound **11**, which exhibited spectral features characteristic of peptides not containing aromatic amino acids. Peptides **2**−**8**, all with retention times lower than that of internal standard tryptophan, were found to contain phenylalanine. Peptide **10**, also with a retention time lower than that of tryptophan, exhibited the same values for spectral parameters than peptides containing tryptophan. Peptides **12**−**14**, all with retention times higher than that of tryptophan, were found to contain tyrosine. Comparison of the values for the ratio 214 nm/280 nm (Table 3) with those from standard peptides suggested that peaks 12−14 could correspond to peptides with more than eight amino acid residues. This parameter was also useful in discriminating non-peptide compounds such as phenolic compounds (see Table 3).

Wines from Parellada and Chardonnay grape varieties showed similar small peptide composition, whereas those from Macabeo were similar to the Xarel.lo ones. The main differences between the two groups were peaks 5−8, 10, and 11, not detected in Macabeo and Xarel.lo wines, and peak 3, not detected in Parellada and Chardonnay wines.

DISCUSSION

Results of this paper demonstrate that on-line HPLC-PDAD followed by OPA derivatization can be a useful analytical tool for characterizing small peptides from wine. Postcolumn OPA derivatization allows confirmation of the peptide nature of chromatographic peaks, although fluorescence response depends on peptide composition. The high fluorescence response of certain standard peptides (Table 1) is due to the presence of a thiol group in cysteine that stabilizes the isoindole structure of ECG (Leory *et al.*, 1993), to the little steric impediments in the reaction of OPA with simple N-terminal amino acids such as glycine (Chen *et al.*, 1979), and to the presence of other primary amino groups susceptible to react with OPA in lysine and tryptophan residues (Joys and Kim, 1979). In contrast, the lowest fluorescence response corresponds to large or voluminous peptides, with which OPA derivatization is partial or requires more time (Herraiz *et al.*, 1994).

The spectral parameters proposed in this study (wavelengths of the spectrum maxima, convexity interval, and wavelengths of the second-derivative spectrum maxima, all over the 190−340 nm range) allow identification of the aromatic amino acids that form the peptides, as well as identification of coeluted compounds such as the cinnamic derivatives detected in the peptide fractions (<700 Da) of cavas. Spectral parameters previously reported for identification of aromatic amino acid residues in polypeptides (Grego *et al.*, 1986; Palladino and Cohen, 1991) are also based on features from first- and second-derivative spectra, but over a narrower wavelength range (240−320 nm). In these cases, identification of tyrosine and phenylalanine is limited by the presence of tryptophan residues in the peptide molecule, which agrees with the findings of this paper. Perrin *et al.* (1995) proposed a method for determining ratios of aromatic residues in the peptide molecule based on their linear correlation with ratios of amplitudes at certain wavelengths over the 240−300 nm range of the first- and second-derivative spectra. This latter methodology does not allow total identification of aromatic amino acid residues in peptides containing tyrosine, phenylalanine, and tryptophan. Therefore, the spectral parameters proposed in this paper show the same limitations as other spectral parameters reported in the literature, but give a more accurate interpretation of the peptide spectra since the whole UV wavelength range is studied. Besides, systematic determination of these parameters can also allow identification of different phenolic structure compounds that could coelute with peptides and for which these parameters have been previously found to be very useful (Bartolome *et al.*, 1993, 1996; Bengoechea *et al.*, 1995).

Application of this methodology to cava samples has let us obtain the first data about identification of wine peptides. In a previous paper (Moreno-Arribas *et al.*, 1996), it was shown that musts contained almost no small peptides and that the chromatographic profiles of peptides in sparkling wines of different variety, subjected to the same production process, were very similar. It was suggested, therefore, that the peptide composition of these wines was related to the yeast more than to the composition of the initial musts. The results of this paper agree with this hypothesis since individual peptides from sparkling wines of different variety, but subjected to the same production process, appear to be the same. The differences observed between wines from different varieties are explained in terms of rate of aging. The presence of aromatic amino acid residues in peptides may have an extra importance in cavas if it is finally confirmed that hydrophobicity of peptides, mainly due to aromatic amino acids, is related to the quality of the foam in these wines (Moreno-Arribas *et al.*, 1996).

The fact that some phenolic compounds were also detected in peptide fractions may indicate the existence of certain interactions between these compounds and small peptides. Associations between phenolics and peptides could occur naturally in wines and be partly or totally disrupted during reversed-phase chromatographic procedures. Phenolic compounds could contribute to the stabilization of foams in cavas, as has been reported to occur in model systems (Sarker *et al.*, 1995).

In summary, the analytical methodology reported in this paper enables identification of the aromatic amino acid residues contained in peptides from wine after separation by HPLC or CE. Combination of results from this methodology with studies of retention behavior in RP-HPLC (Casal *et al.*, 1996) or electrophoretic mobility (Cifuentes and Poppe, 1995) can provide relevant information about amino acid composition of peptides.

ABBREVIATIONS USED

CE, capillary electrophoresis; MS, mass spectrometry; OPA, *o*-phthaldialdehyde; RP-HPLC, reversed-phase high-performance liquid chromatography; PDAD, photodiode array detection; UV, ultraviolet.

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