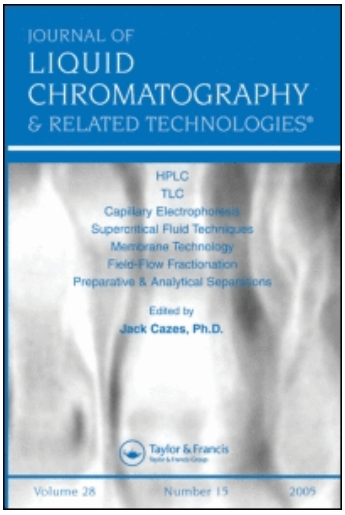

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Mobile Phase pH, Column Temperature, and Eluent Flow Rate Effects on Separation and Fluorescence-Electrochemical Detection of OPA Derivatives of Amino Acids in Reversed-Phase Liquid Chromatography

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Mobile Phase pH, Column Temperature, and Eluent Flow Rate Effects on Separation and Fluorescence-Electrochemical Detection of OPA Derivatives of Amino Acids in Reversed-Phase Liquid Chromatography

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Abstract: The role of mobile phase pH and organic content, column temperature, as well as eluent flow rate on the reversed-phase chromatographic analysis of o-phthalaldehyde (OPA) derivatized amino acids using fluorescence (FL) and electrochemical (EC) detection in series has been investigated. Gradient separation of a mixture of eighteen OPA amino acids was optimized at three different pH values (2.5, 5, and 7) with the aid of an algorithm from only four simple linear gradient measurements carefully chosen at each mobile phase pH. It was found that increasing the mobile phase pH from 2.5 to 7 resulted in a considerable increase of FL and EC responses, but an extreme FL signal enhancement was observed between pH 2.5 and 5 due to the different degree of dissociation of solutes at that pH range. Similarly, the increase of the organic solvent concentration gave an increase in detector responses, whereas considerable loss of detector signals was obtained for all amino acids by increasing the column temperature or eluent flow rate. Generally, under the conditions used here, the mobile phase pH and column temperature proved to be the most

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important factors affecting the analysis of amino acids and they should be properly optimized under constant or gradient conditions.

Keywords: Amino acids, Reversed-phase liquid chromatography (RP-HPLC), Separation optimization, Fluorescence-electrochemical (FL-EC) detection

INTRODUCTION

It is well known, that among HPLC methods currently in use to determine amino acids, the most popular is that employing precolumn derivatization of amino acids with OPA.[1–4] In these determinations, a thiol is first added to OPA before the interaction with amino acids. The most widely used thiol is still the originally proposed 2-mercaptoethanol (2-ME),[5] even when it has been proven that the use of other SH group containing additives in the reaction with OPA results in a better stability of OPA amino acid derivatives.[3,4,6,7] The mechanism of the OPA reaction is not yet completely understood,[4,8] although a plausible reaction pathway has been suggested.[9] This uncertainty about the actual reaction course is reflected in a considerable variety of empirically developed compositions of reaction mixtures and reaction conditions for establishing optimum conditions for a reliable and reproducible determination of amino acids, depending on the stability of OPA products. On the other hand, FL properties,[10] as well as EC activity of OPA amino acid derivatives have been partially investigated,[11,12] permitting an analysis of amino acids using FL and EC detection in series.[11,13,14] In general, hundreds of publications dealing predominantly with applications of amino acid determinations in the presence of OPA and thiols can be found in the literature. Most of them are mainly based on measurement of FL intensity[15–21] whereas, in some instances, detection of absorbance in the UV region[22–24] of electrical current,[12,25–27] or of simultaneously UV and FL responses[7,28–30] were used.

As concerns the separation of OPA amino acid derivatives, these are well retained and resolved on various types of reversed columns using a wide variety of eluents following gradient conditions empirically selected in most of the relative publications. However, little attention has been paid to any of the most important parameters, such as the pH and organic content of the mobile phase, the column temperature, as well as the eluent flow rate on both the retention and detection of OPA derivatives of amino acids.

In this paper, on the basis of our earlier experience on selected gradient conditions by means of a computer optimization program,[24,31–34] we tried to optimize the separation of a mixture of eighteen OPA/2-ME derivatives of amino acids at three different pH values, with the aid of an algorithm from only four simple linear gradient measurements carefully chosen at each mobile phase pH. Note, that the mixture of 18 amino acids tested in this paper was used as a model system, and it is the same with that previously

studied under dual-mode gradient conditions.[35,36] Additionally, the effect of mobile phase pH, of column temperature, as well as of the eluent flow rate on FL and EC detection of OPA derivatives have been investigated in this paper.

EXPERIMENTAL

The solutes were the following 18 OPA/2-ME derivatives of amino acids: L-Arginine (Arg), Taurine (Tau), L-Asparagine (Asn), L-Glutamine (Gln), L-Serine (Ser), L-Aspartic acid (Asp), L-Glutamic acid (Glu), L-Threonine (Thr), Glycine (Gly), beta-(3,4-dihydroxyphenyl)-L-Alanine (Dopa), L-Alanine (Ala), 4-Aminobutyric acid (GABA), L-Methionine (Met), L-Valine (Val), L-tryptophan (Trp), L-phenylanine (Phe), L-Isoleucine (Ile), and L-Leucine (Leu). The derivatives were formed by the reaction of OPA with amino acids in the presence of 2-ME according to the previously published non-automated, manual precolumn derivatization procedure[21] with minor modifications. The detection of derivatized amino acids was performed by a spectrofluorometric detector (Shimadzu, Model RF-10AXL) at 455 nm after excitation at 340 nm, coupled in series with a Lab made multiple EC detector equipped with a glassy carbon electrode (3 mm diameter). The EC detection of the analytes was performed at 0.8 V vs. the Ag/AgCl reference electrode. The EC detector was maintained at 0.8 V, except for generating hydrodynamic voltammograms (i.e., EC signal vs. applied potential curves). Hydrodynamic voltammograms were constructed by obtaining multiple chromatograms for a given sample at 100 mV increments, with the applied potential varying from 0.3 to 1.2 V. Using FL and EC detection in series, peak identification can be obtained in a single chromatographic run. Note, that only the retention times of the solutes obtained by the FL detector were used for the separation optimization procedure. Appropriate working concentrations of underivatized amino acids were used in the derivatization procedure by OPA/2-ME reagent (Tau, GABA = 0.25 μg mL; Ser, Asp, Thr, Gly = 0.5 μg mL^{-1}; Trp, Phe, Ile, Leu = 2 μg mL^{-1}; others = 1 μg mL^{-1}), so that the peak heights of the OPA derivatives recorded by the FL detector under gradient conditions do not differ significantly. However, the concentration of all amino acids examined under isocratic conditions was 1 μg mL^{-1}.

The liquid chromatography system used for separations of the test compounds consisted of a Shimadzu LC-20AD pump and a model 7125 syringe loading sample injector fitted with a 20 μL loop (Rheodyne, Cotati, CA). The mobile phases were aqueous phosphate buffers, with a total ionic strength of 0.02 M and three different pH values (2.5, 5, and 7), modified with acetonitrile (MeCN). An Agilent Zorbax Eclipse-AAA column (3.5 mm, 150 × 4.6 mm) thermostatted by a CTO-10AS Shimadzu column oven, was used for the separations of derivatized amino acids. All experiments were carried out at 30°C and with a flow rate of 1 mL min^{-1}, except those

related to the influence of column temperature or eluent flow rate on the separation and the detection of amino acid derivatives. The dwell time, i.e., the time needed for a certain change in the mixer to reach the beginning of the column, t_D, and the hold-up time, t_0, were estimated as 1.19 and 1.37 min, respectively.

All algorithms used for fitting gradient data and optimizing separations,[24] as well as other calculations, were running on a 3.4 GHz Pentium CPU under windows XP.

RESULTS AND DISCUSSION

Influence of pH Value and Solvent Concentration of Mobile Phase on Retention and Detection of OPA Amino Acid Derivatives

Before attempting to optimize the gradient elution of the 18 amino acid derivatives adopted in the present study at different pH values of the mobile phase, three amino acids, Tau, Gly, and Dopa were selected for a preliminary study conducted under isocratic conditions in order to investigate separately the effects of mobile phase pH and organic content on the chromatography of OPA amino acids. Thus, Figure 1 shows chromatograms of the selected amino acids obtained in eluents of pH 2.5, 5, or 7, respectively, containing 20% MeCN, as well as with pH 2.5 and 30% MeCN. Under the conditions used in these chromatograms, the dependence of the retention, as well as of the FL and EC signals of the OPA derivatives on the mobile phase pH and concentration of organic modifier is evident. In general, the detector responses of all amino acids increase with the increasing of eluent pH or the MeCN content in the mobile phase. However, the degree of FL and EC enhancement depend on the nature of amino acid, as depicted also in Table 1, where the peak areas recorded in chromatograms of Figure 1 are listed. Note that, to demonstrate the role of mobile phase pH and organic content on the detection without affecting the results by changes in chromatographic retention, peak areas instead of peak heights are used in Table 1. Additionally, from this table, it seems that the dependence of the measured fluorescence of Gly and Dopa derivatives on the mobile phase pH could be similar to the shape of a weak acid dissociation curve with an inflection point at pH about 3.[10] See for explanation, the discussion below about the pH dependence of Gly and Dopa retention.

In addition, in order to investigate in more detail the role of the mobile phase pH on EC activity of amino derivatives, hydrodynamic voltammograms of derivatized amino acids were constructed from chromatographic data obtained under isocratic conditions, at three different mobile phases with pH 2.5, 5, or 7 containing 20% MeCN. Because the EC activity of OPA derivatives arises from the isoindole ring formed in the derivatization reaction and, consequently, it is common to all derivatives, the EC

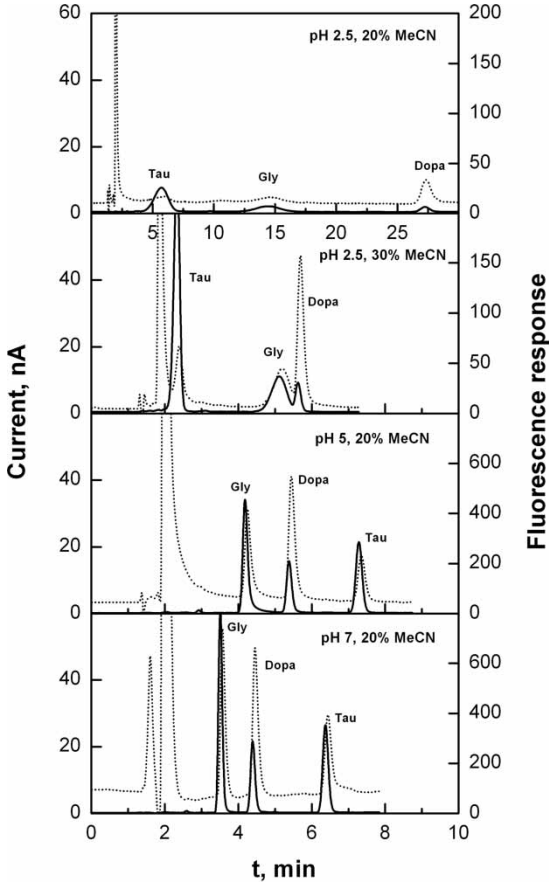

Figure 1. Chromatograms of a mixture of three OPA amino acid derivatives with on line FL (solid lines) and EC detection (dotted lines) under different isocratic conditions depicted in this Figure. The huge peak recorded by the EC detector belongs to the derivatizing reagent. The FL detector was installed at constant sensitivity. For more details, see the text.

oxidation of all OPA amino acids is similar. The hydrodynamic voltammograms of Arg as an example of our study regarding the pH dependence of the oxidation of amino derivatives is shown in Figure 2. From this Figure, it is clear that the half-wave ($E_{1/2}$) oxidation potentials of the isoindole ring in the OPA derivatives are almost pH independent, although there is an increase of EC response (at 0.8 V) as increasing the eluent pH, are in agreement with our results as shown in Figure 1 and Table 1. Since the limiting current in the potential region of 0.6–0.8 V is solely determined by mass transfer, this increase of the EC signals observed upon an increase of mobile phase pH from 2.5 to 7 should be attributed to changes in analyte

Table 1. Effect of mobile phase pH and organic content on retention times and FL-EC detector responses for three OPA-amino acid derivatives

Mobile phase conditions		Solutes								
		Tau			Gly			Dopa		
φ_{MeCN}	pH	t_R (min)	EC peak area, Q (nC)[a]	FL peak area	t_R (min)	EC peak area, Q (nC)[a]	FL peak area	t_R (min)	EC peak area, Q (nC)[a]	FL peak area
0.2	2.5	5.698	98	1735	14.50	123	653	27.03	370	256
0.3	2.5	2.335	139	2737	5.118	318	994	5.631	555	293
0.2	5	7.279	199	3519	4.183	281	4692	5.378	390	1983
0.2	7	6.379	282	3788	3.518	460	6081	4.397	434	2454

[a]Recorded at 0.8 V vs. Ag/AgCl.

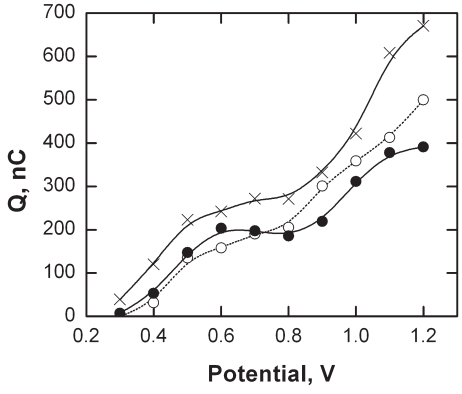

Figure 2. Hydrodynamic voltammograms for OPA derivative of Arg obtained in a mobile phase of pH 2.5 (●), 5 (○), or 7 (×) containing 20% MeCN, respectively.

mass transport characteristics. For a given value of eluent flow rate, a plausible explanation is the variation of analyte diffusion coefficient, D, with pH. (Note that the chromatographic peak area in electrochemical detection is known to be proportional to $D^{2/3}$).[37] Such an effect has already been reported for amphiphilic compounds (amino acids, small proteins, enzymes)[38] whereby, at very low or high pH values, the compound carries a large net charge and is thus heavily solvated, a decrease in D is observed, relative to its value at intermediate pH close to the species isoelectric point (see also below).

Figure 1 and Table 1 shows dependence of the retention of the OPA derivatives under chromatographic conditions used in this section. The pH dependence of Gly and Dopa in the pH range between 2.5 and 7 seems to be similar to that of common weak organic acids showing higher retention time, t_R, at lower pH, where they are completely protonated.[39] Generally, the retention behavior of ionizable compounds such as amino acids depends on the degree of their dissociation in different pH values of the mobile phase. Thus, since the pK_a values of the carboxyl group, as well as of the ammonium group of free Gly and Dopa are similar despite their structural differences, and they are about 2.2 and 9.5, respectively, whereas these values increase upon the formation of the OPA derivatives,[22] it seems that at low pH = 2.5 both the ammonium and carboxyl group are protonated and, consequently, these amino acids show higher retention than at pH values 5 and 7 where the carboxyl group should be deprotonated. However, the retention behavior of Tau, a non carboxylic amino acid, is totally different than the other amino acids in different pH values of the mobile phase, and this results in a change of the elution order of the given OPA amino acids at eluent pH higher than 2.5, see Figure 1. Additionally, it is worth noting in Figure 1, that increasing the MeCN content in the mobile phase of pH 2.5 from 20% to 30% leads to a considerable decrease of

retention of amino acids, which seems to be of the same order with that resulted by increasing the mobile phase pH from 2.5 to 5 or 7. This observation, as well as the fact that increasing of the pH value in the mobile phase may lead to changes of the retention order and/or the sharpness of some amino acids such as of Gly, see Figure 1, suggests that the eluent pH is a very flexible and essential factor which should be optimized in the separation and analysis of OPA amino acid derivatives.

Separation Optimization of OPA Amino Acid Derivatives under Different Mobile Phase pH: Temperature and Eluent Flow Rate Considerations

The separation of all derivatized amino acids was optimized for three mobile phase pH values (2.5, 5, and 7) with the aid of a computer optimization program from only simple linear gradient measurements at each pH. Thus, based on the experimental retention times of amino acid derivatives obtained under the gradient profiles shown in Table 2, and using the home made algorithms and the procedure adopted in Ref. [24], we found the best linear or multilinear gradient profiles depicted on Table 3 for each pH value of the mobile phase, with a total elution time, $t_{g,max}$, equal to 20 min for pH 2.5 and 5, and equal to 25 min for pH 7. Note, that the value of $t_{g,max}$ is preset by the researcher as well as the minimum and the maximum value of the eluent organic content in the programmed optimum gradient profile, i.e., the volume fractions, φ_{min} and φ_{max}, of MeCN in our case. However, the additional programmable time duration that may extend the initial isocratic part of the dwell time, t_{in}, the mobile phase composition of this initial isocratic part, φ_{in}, as well as the optimum number of the linear portions that constitutes the multilinear gradient are adjustable parameters, i.e., they are determined by the algorithm.

The chromatogram of Figure 3A was recorded at flow rate 1 mL min^{-1} and at 30°C using the predicted best gradient profile for mobile phase pH 2.5 depicted in Table 3. Indeed, a good resolution of the mixture of 18

Table 2. Simple linear gradients used in the separation optimization of OPA-amino acid derivatives at different pH values of the mobile phase

pH	Gradients	1	2	3	4
2.5	φ_{MeCN}	$0.1 \rightarrow 0.5$	$0.2 \rightarrow 0.5$	$0.1 \rightarrow 0.5$	$0.1 \rightarrow 0.5$
	t, min	$0 \rightarrow 20$	$0 \rightarrow 10$	$0 \rightarrow 10$	$0 \rightarrow 30$
5	φ_{MeCN}	$0.2 \rightarrow 0.5$	$0.2 \rightarrow 0.5$	$0.1 \rightarrow 0.5$	$0.2 \rightarrow 0.4$
	t, min	$0 \rightarrow 20$	$5 \rightarrow 25$	$0 \rightarrow 30$	$0 \rightarrow 30$
7	φ_{MeCN}	$0.1 \rightarrow 0.4$	$0.1 \rightarrow 0.4$	$0.145 \rightarrow 0.4$	$0.16 \rightarrow 0.37$
	t, min	$0 \rightarrow 10$	$0 \rightarrow 20$	$0 \rightarrow 30$	$5 \rightarrow 30$

Table 3. Optimal gradients used at mobile phases with different pH values

	Gradient						
	t, min			φ_{MeCN}			
pH	t_{in}	t_1	t_2	φ_{in}	φ_1	φ_2	$t_{g,max}$, (min)
2.5	5.62	6.88	21.57	0.254	0.368	0.485	20
5	0	15.80	23.56	0.106	0.304	0.500	20
7	7.44	31.96	—	0.144	0.400	—	25

amino acid derivatives was achieved, except that Glu and Thr coeluted. In order to also investigate the effect of eluent flow rate, as well as of column temperature on analysis of OPA-derivatives, we recorded chromatograms of the selected amino acids with the same gradient program but applying a different flow rate (1.5 mL min^{-1}) or column temperature (70°C). The resulting chromatograms are shown in Figure 3B and 3C, respectively. As one can see in Figure 3, OPA derivatives provide smaller FL responses at higher flow rate and column temperature; although under the conditions used here, the influence of column temperature on the detection of amino acids appears to be greater than that of the flow rate. Similarly, the EC signals of OPA amino acids were also decreased by increasing the flow rate and the temperature but the EC detected chromatograms, in a single run with those of FL detected, are not given for simplicity. Moreover, the degree of decreased FL and EC responses of amino acids due to the increase of flow rate or column temperature exhibit differences among the solutes due to the continuous variation of the concentration of organic solvent in the mobile phase. In other words, since increasing flow rate or column temperature resulted in a considerable decrease of elution times of solutes, the same solute elutes under different mobile phase composition although the analysis is performed under the same gradient profile in all cases of Figure 3.

Regarding the influence of the increase of flow rate on the resolution of OPA-amino acids, no significant differences were observed although the analysis time was considerably decreased, compare Figure 3A with 3B. In contrast, with increasing column temperature from 30°C to 70°C the retention order of Val/Trp was changed; Tau and Asn became inseparable but resolution of coeluted Glu and Thr was obtained and at the same time the peak shape of Gly, which was particularly broadened and tailed at 30°C was improved, see parts A and C of Figure 3.

The next task to complete this study was to record chromatograms of the amino acids with the optimal gradient program found for the mobile phase with pH 5 and a total elution time, $t_{g,max}$, equal to 20 min. The resulted chromatogram is shown in Figure 4, where it is apparent that increasing the mobile

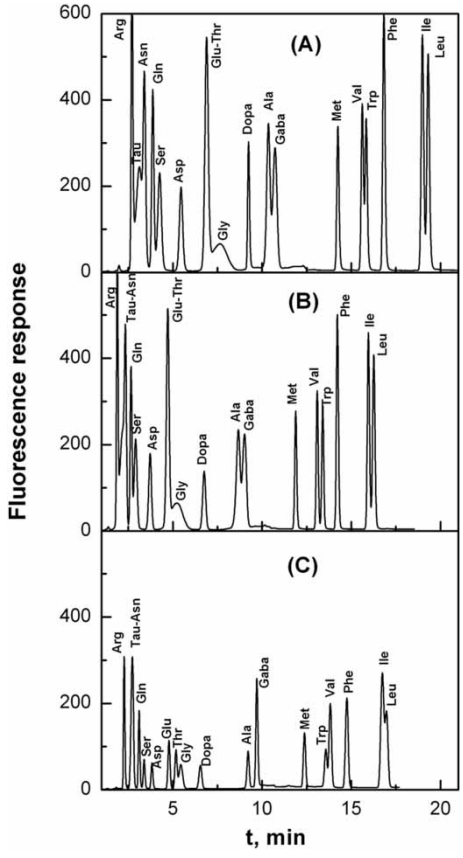

Figure 3. FL detected chromatograms of 18 OPA amino acid derivatives obtained under the predicted optimal gradient for pH = 2.5, shown in Table 3. The elution order of the amino acids in part (A) recorded at flow rate 1 mL min^{-1} and at 30°C is the following: Arg, Tau, Asn, Gln, Ser, Asp, Glu-Thr, Gly, Dopa, Ala, Gaba, Met, Val, Trp, Phe, Ile, and Leu. The elution order of the amino acids in part (B) performed at 30°C but at 1.5 mL min^{-1} is the same with that in part (A), except that Tau and Asn become closer to each other, almost coeluted, whereas the elution order of the amino acids in part (C) performed at 1 mL min^{-1} but at 70°C is the same with that in part (A), except that Tau and Asn coelute, Glu and Thr elute separately, and Trp elute before Val. For more details, see the text.

phase pH from 2.5 to 5 leads to considerable FL enhancement; in spite of that the chromatogram of Figure 4 was recorded by a sensitivity which was approximately 8 times lower than that used for recording chromatograms of Figure 3. This finding of the rapidly increase of FL responses of OPA amino acids is in agreement with our observations in Figure 1. However, the resolution of the mixture of amino acids tested was deteriorated at pH 5, since under the optimum gradient program used at pH 5 there are two pairs

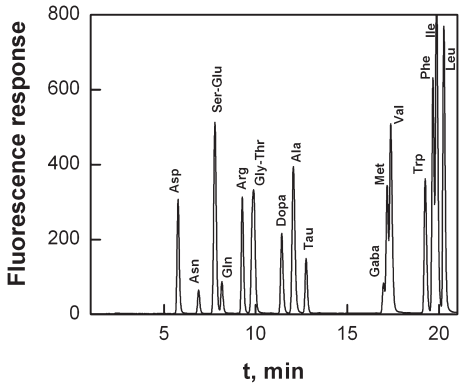

Figure 4. FL detected chromatograms of 18 OPA amino acid derivatives obtained under the predicted optimal gradient for pH = 5, shown in Table 3. The elution order of the amino acids recorded at flow rate 1 mL min^{-1} and at 30°C is the following: Asp, Asn, Ser-Glu, Gln, Arg, Gly-Thr, Dopa, Ala, Tau, Gaba, Met, Val, Trp, Phe, Ile, and Leu.

of peaks (Ser-Glu and Gly-Thr) instead of one pair appearing under mobile phase conditions of pH 2.5 in the same total elution time of 20 min (compare Figure 3A and Figure 4). Additionally, the retention order of acidic amino acids (Asp and Glu), of Arg (a basic amino acid) as well as of Tau (a non carboxylic amino acid) was dramatically changed by increasing the eluent pH from 2.5 to 5, since these amino acids have an additional protonation equilibrium than the others. Thus, the optimum gradient program used at pH 5 moved Asp and Glu to the front of the separation whereas Arg and Tau were in the middle of the chromatogram. Also, the use of eluent pH 5 instead of pH 2.5 improved the peak shapes of Tau and Gly, this was also observed in Figure 1.

In conclusion, the selected mixture of 18 amino acids was not able to be properly separated in 20 min at both pH 2.5 and pH 5 as well as at pH 7 (results not shown) and for this reason, we increased the total retention time to 25 min and we found an alternate optimal gradient program for the mobile phase with pH 7, where the OPA amino acids depict the maximum detector response. Indeed, a good resolution of the mixture of 18 amino acid derivatives was achieved in the chromatogram of Figure 5 obtained under the optimal gradient conditions shown on Table 3 for the eluent of pH 7. Between pH 5 and 7, no major changes in the elution order of the amino acid derivatives were observed, except that Ile moved relative to Phe and Leu (compare Figures 4 and 5). Moreover, maximal FL intensity and EC activity (results not shown) was obtained at pH 7, although the increase of the eluent pH from 5 to 7 leads to minor FL enhancement than that resulted by increasing the pH from 2.5 to 5.

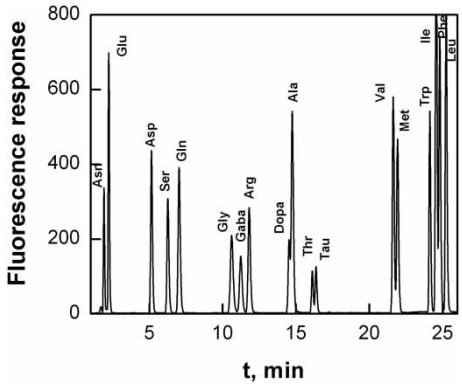

Figure 5. FL detected chromatograms of 18 OPA amino acid derivatives obtained under the predicted optimal gradient for pH = 7, shown in Table 3. The elution order of the amino acids recorded at flow rate 1 mL min^{-1} and at 30°C is the following: Asn, Glu, Asp, Ser, Gln, Gly, Gaba, Arg, Dopa, Ala, Thr, Tau, Val, Met, Trp, Ile, Phe, and Leu.

To sum up, it has been shown that in the determination of OPA amino acids, the role of the mobile phase pH and organic content as well as of the column temperature and eluent flow rate is very crucial. Detector responses proved to be dependent on the chromatographic conditions used. Thus, from the point of view of the maximum FL as well as FL signals obtained, the optimum mobile phase pH is 7. Acceptable separations could be achieved by all eluent pH values studied, performing gradient conditions estimated with the aid of a computer optimization program. However, in the case where the sensitivity is not essential for an analysis of amino acids, the enhanced separation efficiency of OPA derivatives by using a low pH, 2.5, and a relatively high column temperature and flow rate should prove beneficial. Furthermore, our attempt in this paper to investigate the effects of mobile phase pH, the column temperature, and the eluent flow rate on retention and detection of OPA amino acids should be the first step to establishing optimum conditions in a technique of pH or temperature programming, instead of/or additionally with, linear solvent gradients most frequently used for the separation of amino acids.

REFERENCES

1. *Handbook of Derivatization Reactions for HPLC, Amino Acids*; Lunn, G. and Hellwig, L.C., Eds.; Wiley, 1998; 625.
2. Molnar-Perl, I. In *Advanced Chromatographic and Electromigration Methods in Biosciences*; Deyl, Z., Ed.; Elsevier: Amsterdam, 1998; 416.
3. Hanczko, R.; Molnar-Perl, I. Chromatographia **2003**, *57*, S-103–S113.

4. Zuman, P. Chem. Rev. **2004**, *104*, 3217–3238.
5. Roth, M. Anal. Chem. **1971**, *43*, 880–882.
6. Molnar-Perl, I. J. Chromatogr. A **2001**, *913*, 283–302.
7. Kutlan, D.; Molnar-Perl, I. Chromatographia **2001**, *53*, S-188–S198.
8. Salem, N.; Zuman, P. Anal. Chem. **2006**, *8*, 7802–7808.
9. Mengerink, Y.; Kutlan, D.; Toth, F.; Csampai, A.; Molnar-Perl, I. J. Chromatogr. A **2002**, *949*, 99–124.
10. Chen, R.F.; Scott, C.; Trepman, E. Biochim. Biophys. Acta **1979**, *576*, 440–455.
11. Joseph, M.H.; Davies, P. J. Chromatogr. **1983**, *277*, 125–136.
12. Allison, L.A.; Mayer, G.S.; Shoup, R.E. Anal. Chem. **1984**, *56*, 1089–1096.
13. Rizzo, V.; Anesi, A.; Montalbetti, L.; Bellantoni, G.; Trotti, R.; Melzi d'Eril, G.V. J. Chromatogr. A **1996**, *729*, 181–188.
14. Kehr, J. J. Chromatogr. B **1998**, *708*, 27–38.
15. Peris-Vicente, J.; Gimeno Adelantado, J.V.; Domenech Carbo, M.T.; Mateo Castro, R.; Bosch Reig, F. Talanta **2006**, *68*, 1648–1654.
16. Soufleros, E.H.; Bouloumpasi, E.; Tsarchopoulos, C.; Biliaderis, C.G. Food Chem. **2003**, *80*, 261–273.
17. Chen, B.-M.; Xia, L.-W.; Liang, S.-X.; Chen, G.-H.; Deng, F.-L.; Zhang, W.-R.; Tao, L.-J. Anal. Chim. Acta **2001**, *444*, 223–227.
18. Yan, C.C.; Huxtable, R.J. J. Chromatogr. B **1995**, *672*, 217–224.
19. Cereser, C.; Guichard, J.; Drai, J.; Bannier, E.; Garcia, I.; Boget, S.; Parvaz, P.; Revol, A. J. Chromatogr. B **2001**, *752*, 123–132.
20. Dunnett, M.; Harris, R.C. J. Chromatogr. B **1997**, *688*, 47–55.
21. Zunin, P.; Evangelisti, F. Int. Dairy J. **1999**, *9*, 653–656.
22. Lopez-Grio, S.; Torres-Lapasio, J.R.; Baeza-Baeza, J.J.; Garcia-Alvarez-Coque, M.C. Anal. Chim. Acta **2000**, *418*, 153–165.
23. Thippeswammy, R.; Gouda, K.G.M.; Rao, D.H.; Martin, A.; Gowda, L.R. J. Agric. Food Chem. **2006**, *54*, 7014–7019.
24. Nikitas, P.; Pappa-Louisi, A.; Papageorgiou, A. J. Chromatogr. A **2007**, *1157*, 178–186.
25. Tcherkas, Y.V.; Kartsova, L.A.; Krasnova, I.N. J. Chromatogr. A **2001**, *913*, 303–308.
26. Qu, Y.; Arckens, L.; Vandenbussche, E.; Geeraerts, S.; Vandesande, F. J. Chromatogr. A **1998**, *798*, 19–26.
27. Pearson, S.J.; Czudek, C.; Mercer, K.; Reynolds, G.P. J. Neural Transm. **1991**, *86*, 151–157.
28. Tivesten, A.; Folestad, S. J. Chromatogr. A **1995**, *708*, 323–337.
29. Yokoyama, Y.; Ozaki, O.; Sato, H. J. Chromatogr. A **1996**, *739*, 333–342.
30. Hanczko, R.; Koros, A.; Toth, F.; Molnal-Perl, I. J. Chromatogr. A **2005**, *1087*, 210–222.
31. Pappa-Louisi, A.; Nikitas, P.; Agrafiotou, P.; Papageorgiou, A. Anal. Chim. Acta **2007**, *593*, 92–97.
32. Nikitas, P.; Pappa-Louisi, A.; Agrafiotou, P. J. Chromatogr. A **2006**, *1120*, 299–307.
33. Nikitas, P.; Pappa-Louisi, A. J. Chromatogr. A **2005**, *1068*, 279–287.
34. Nikitas, P.; Pappa-Louisi, A. Anal. Chem. **2005**, *77*, 5670–5677.
35. Nikitas, P.; Pappa-Louisi, A.; Balkatzopoulou, P. Anal. Chem. **2006**, *78*, 5774–5782.
36. Pappa-Louisi, A.; Nikitas, P.; Balkatzopoulou, P.; Louizis, G. Anal. Chem. **2007**, *79*, 3888–3893.

37. Agrafiotou, P.; Maliakas, C.; Pappa-Louisi, A.; Sotiropoulos, S. Electrochim. Acta **2003**, *48*, 2447–2462.
38. Gutierrez, R.; Del Valle, E.M.M.; Galan, M.A. Biochem. Eng. J. **2007**, *35*, 264–272.
39. Pappa-Louisi, A.; Zougrou, F. Chromatographia **1997**, *44*, 348–354.

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