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Comparison of the stability and UV and fluorescence characteristics of the *o*-phthaldialdehyde/3-mercaptopropionic acid and *o*-phthaldialdehyde/N-acetyl-L-cysteine reagents and those of their amino acid derivatives

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Abstract

Derivatization conditions of amino acids with the *o*-phthaldialdehyde (OPA)/3-mercaptopropionic acid (MPA) and with the OPA/N-acetyl-L-cysteine (NAC) reagents have been exhaustively reviewed and investigated. The existence and extent of the self fluorescence of both reagents have been demonstrated and measured. The stability of the OPA/MPA and OPA/NAC isoindoles have been determined and compared to each other, from the same working solution, at pH=9.4 and at pH=7.2, by UV and fluorescence photometry. Data obtained proved that only slight differences can be expected applying the OPA/MPA or the OPA/NAC reagents in terms of the corresponding molar absorptivity, isoindole's stability and concentration linearity values. The relative molar fluorescence intensities of the OPA/MPA derivatives proved to be higher by relatively 10–55%, in comparison to the corresponding OPA/NAC ones. Stabilized background fluorescence can be obtained with both reagents prepared at least 90 min before use and stored in a refrigerator. Reproducibility values determined in the linear concentration ranges for both derivatives were much better applying UV photometry in comparison to the fluorescence intensity measurements. © 1998 Elsevier Science B.V.

Keywords: Derivatization, LC; Stability studies; Isoindoles; Amino acids; Phthaldialdehyde; Mercaptopropionic acid; Acetylcysteine

1. Introduction

Based on the pioneering work of Roth [1] the reaction of amino acids with *o*-phthaldialdehyde (OPA), in the presence of various SH-group containing agents [2–15], has gained wide acceptance in the chromatographic analysis of amino acids. Because of the instability of the isoindoles obtained from amino acids with the primarily introduced [1] OPA/2-mercaptoethanol (MCE) reagent, as SH-group containing alternatives, 3-mercaptopropionic acid (MPA) [2–7] and N-alkyl-L-cysteines [8–15] were proposed. The common advantage of the OPA/MPA and OPA/N-acetyl-L-cysteine (NAC) reagents, can be characterized by the fact that they provide more stable isoindoles, compared to those formed with OPA/MCE, while in the optical resolution of enantiomeric amino acids OPA/NAC reagents have been extensively utilized. On the basis of an exhaustive literature overview it became clear that even in the derivatization step, performed using both the OPA/MPA and the OPA/NAC solutions (i) extremely different conditions have been used for both reagents, (ii) the characteristics and stability of the

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OPA/MPA and the OPA/NAC reagents have not been investigated in detail, and, (iii) the special UV and fluorescence (FL) characteristics, as well as the stabilities of the corresponding OPA/MPA and OPA/NAC isoindoles have not been determined and compared to each other. The only stability comparisons [2,8,10,11] reported in a qualitative manner [2,10,11] or without any experimental data [8] deal with the stability of the OPA/MCE versus OPA/NAC [8,10,11] and OPA/MCE versus OPA/MPA derivatives [2].

Thus, the aim of this paper was to investigate the reason for and consequences of the limiting derivatization parameters found in the literature, as well as to compare the stability and the intrinsic, spectral features of the isoindoles (molar absorptivity, molar fluorescence values) obtained with the OPA/MPA and OPA/NAC reagents with amino acids.

2. Experimental

2.1. Materials

OPA, MPA, NAC and amino acids were obtained from Sigma (St. Louis, MO, USA) and from Serva (Heidelberg, Germany). HPLC-grade methanol was purchased from Romil Chemicals (Leics., UK). All other reagents were of the highest purity available.

2.2. Standard solutions

Stock solutions of each free amino acid, [alanine, β-alanine, γ-aminobutyric acid (GABA), aspartic acid, cysteine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine] were prepared at a concentration of 0.01 *M* with 0.01 *M* hydrochloric acid.

Stock solution of OPA contained 53.3 mg OPA (weighed with analytical precision) in 50 ml methanol (hereafter referred to as methanolic OPA solution).

2.3. Buffer solutions

Borate buffer consisted of 0.1 *M* boric acid (dissolved in 0.1 *M* potassium chloride)–0.1 *M* sodium hydroxide–water (50:45:5, v/v).

Acetate buffer consisted of 1 *M* sodium acetate–1 *M* acetic acid (10:1, v/v).

2.4. Reagent solutions

OPA/MPA reagent was obtained by mixing, in order of listing, 12.0 ml methanolic OPA, 13.0 ml borate buffer and 25 μl MPA: final pH proved to be 9.4±0.05.

OPA/NAC reagent was prepared from 12.0 ml methanolic OPA solution, 10.0 ml 0.0286 *M* NAC solution (NAC dissolved in 0.05 *M* borate buffer), completed by 3 ml borate buffer to 25.0 ml: final pH proved to be 9.4±0.05.

2.5. Derivatization

2.5.1. Stability study of reagent solutions

Blank measurements and/or derivatizations were performed with freshly prepared and stored reagent solutions (saved both at ambient temperature and in the refrigerator, at ~4°C, for various periods of time).

2.5.2. Stability study of the isoindole derivatives at pH=9.4±0.05

Derivatizations were carried out with reagent solutions prepared at least 90 min before use. Reagent solutions (5 ml) were mixed with 100 μl amino acid stock solutions (final pH of these solutions proved to be 9.4±0.05 (hereafter referred to as isoindole solutions). These isoindole solutions served both for the UV (without dilution) and for the FL measurements (diluting 100 μl isoindole solution with 1 ml reagent solution).

2.5.3. Stability study of the isoindole derivatives at pH=7.2±0.05

After 7 min reaction time, (needed to achieve the maximum absorbancies/fluorescence intensities at pH=9.4), the isoindole solutions were mixed with 0.64 ml acetate buffer (final pH of these solutions proved to be 7.2±0.05. (Note: the reagent blank solutions were prepared with the exception that instead of the amino acid solution 0.01 *M* hydrochloric acid was used).

2.6. UV spectrophotometry

Absorbance measurements were made on a Perkin-Elmer Lambda 15 UV–Vis spectrometer (Perkin-Elmer, Norwalk, CT, USA). Matched quartz cells of 0.5 cm path length were used. Spectra were taken between 190–400 nm with a speed of 960 nm/min. Absorbance measurements were carried out against the reagent blanks were performed at the maximum values (at 334±2 nm, except for cysteine at 278±2 nm).

2.7. Fluorescence measurements

FL intensities were measured with the Hitachi Model F-4500 fluorescence spectrophotometer (Hitachi, Japan). Excitation (Ex) and emission (Em) wavelengths were optimized automatically in the range of 200–700 nm. Slit width used both for Ex and Em was 2.5 nm. Concentration dependence and stability values have been taken at the optimum Ex/Em wavelengths: at Ex/Em=337/454 nm for the OPA/MPA and at 337/442 nm for the OPA/NAC derivatives.

3. Results and discussion

3.1. Literature data

The characteristics of the derivatization conditions and stability studies performed with OPA/MPA and OPA/NAC reagents proved different for each.

The various absolute concentrations of the OPA solutions (0.27–5.0 mg/ml) and SH-group containing agents (MPA: 0.18–5.0 mg/ml, NAC: 0.33–6.2 mg/ml) can be explained by the fact that in several cases [2,4,6,7,10,13,14] special conditions requiring autosampler devices are necessary.

The borate buffer was applied at varying concentrations (0.03–0.45 *M*), in selected cases [8,11] without considering the concentration of the OPA and those of the SH-group containing reagents.

The mol ratios of the OPA/SH-group containing constituents varied from 0.39 to 2.0. The extreme differences in the OPA reagent amino acid ratios ($<1-3\cdot10^4$) proved to be critical. Especially in view of the statement [11,13] that upon the use of the mol ratios of (OPA/NAC)/amino acid=5–200 immediate isoindole formation was measured [11], as well as, ''... the higher OPA concentrations result in larger instability of the isoindoles'' [13].

The reagent's storage conditions reported show surprising diversity: the OPA/MPA reagent [2–7] was stored for three days at ambient temperature [7] or for one week in the refrigerator [3–5], while the OPA/NAC reagent could be stored at ambient temperature from two days [9] up to one month [10].

In general, [3–8,13–15] to achieve quantitative isoindole formation, a 1–5 min reaction time and ambient temperature have been proposed with three exceptions [9,10,12]. Applying SH-group containing reaction partners NAC, or N-*tert*.-L-butyloxycarbonyl-L-cysteine a 10 min reaction time was preferred [9], while the optimum fluorescence for the OPA/NAC dialkylamino acids was obtained after 15 min [12]. At 55°C, performing post-column derivatization with OPA/NAC/sodium hypochlorite also proline could be measured with the same fluorescence response as all other amino acids [10].

3.2. Own data

Based on our experiences of high-performance liquid chromatography (HPLC) of the phenylthiocarbamyl (PTC) amino acids [16–24] we planned to compare the advantages/disadvantages of the quantitation of PTC and OPA derivatives by HPLC. Drawing lessons from the literature data [1–15] detailed above, studying chromatographic optimization of the OPA/MPA and/or OPA/NAC amino acid derivatives in detail, the following questions had to be answered: (i) do the OPA/MPA (NAC) reagents self fluorescence? Is this phenomenon associated with the reagents different storage conditions? (ii) What about the exact stability and the optimum UV/FL responses of the OPA/MPA and OPA/NAC isoindoles, also in comparison to each other?

3.2.1. The self fluorescence of the OPA/MPA and OPA/NAC reagents

The only literature data found [11] relates to the OPA/NAC reagent: the authors state that it ''... is nonfluorescent itself and does not break down or react to form fluorescent byproducts''.

Our investigations performed in parallel with the

OPA/MPA and OPA/NAC reagents revealed, (in accordance with the experience reported for the OPA/MCE solution in Ref. [1]), that both reagents do self fluorescence (Fig. 1). After mixing the methanolic OPA solution with borate buffer and with the MPA or NAC solutions a good measurable "peak fluorescence" can be detected (Fig. 1: for the OPA/NAC and OPA/MPA reagents, 110 and 90 arbitrary units, respectively). In both cases reagent's self fluorescences decrease and stabilize after 70–90 min furnishing 60±5 and 40±5 arbitrary units until 4 h at ambient temperature or until 4 days in the refrigerator (4°C), (at 4°C probably also for longer time, not tested). Keeping the reagents at ambient temperature self fluorescence slowly increases after 4 h.

Based on these experiences all further studies (if not otherwise stated) have been performed with reagents stored before use for 90 min at ambient temperature, and, the self fluorescence of the corresponding reagents have been taken into consideration without exception.

Concerning the characteristics of the fluorescent products originating from the OPA/MPA and OPA/NAC reagents it is to be emphasized that they manifest optimum fluorescence with the same Ex and Em wavelengths as the isoindoles of amino acids. Taking into consideration the evidence of the pioneering work by Roth [1], i.e., that the fluorescence intensity of the product of OPA and amino acids could not be increased by adding the SH-group containing compound to the product afterwards, it is very likely that from the OPA and the SH-group containing compounds, the first step of this isoindole reaction, an intermediary species is formed (in Ref. [25], not mentioned). Probably, this intermediary compound reacts with the primary amino group of the amino acids resulting in these, ten-times higher fluorescence products compared to those obtained from OPA and amino acids only [1].

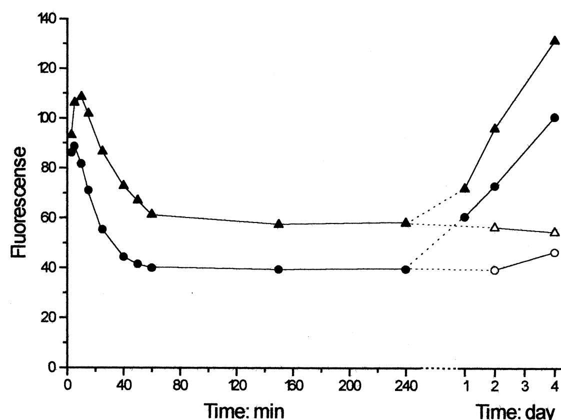

Fig. 1. Fluorescence–time curves for the OPA/NAC (▲ △) and OPA/MPA (● ○) reagents stored at ambient temperature (▲ ●) and in the refrigerator at 4°C (△ ○).

3.2.2. Response/concentration linearity of the OPA/MPA and OPA/NAC tryptophan derivatives

In order to measure and compare isoindole's stability, at first, the proportionality of the concentration/absorbance (fluorescence) responses have to be determined. For this purpose tryptophan (Trp) was selected as a model amino acid giving rise to one of the most stable isoindoles with both reagents.

Investigations (Fig. 2a and 2b) have been carried out in parallel by UV (Fig. 2a) and FL (Fig. 2b) measurements, testing both the OPA/MPA and the OPA/NAC isoindols of tryptophan, respectively.

The OPA concentration ($3.81 \cdot 10^{-3}$ *M*), and the mol ratios of OPA/MPA (NAC) (0.33) were the same without exception, in all cases (Fig. 2a and 2b): it means that UV absorbances and fluorescence intensities, in order of listing, were measured with the mol ratios of OPA/amino acid=74/1–10/1 (Fig. 2a) and 4000/1–10/1 (Fig. 2b), respectively.

Results obtained revealed that (i) the concentration/linear response ranges with both reagents are limited. Reproducibility in the linear range of UV responses ([Trp]=$0.5–2 \cdot 10^{-4}$ *M*) [Fig. 2a: correlation coefficients (r) for the OPA/MPA (r=0.9998) and OPA/NAC (r=0.9992)] proved much better than those of fluorescence intensities in its linear range ([Trp]=$1–20 \cdot 10^{-6}$ *M*) [Fig. 2b: OPA/MPA (r=0.9911) and OPA/NAC (r=0.9980)]. This limited extent of the concentration/linear response ranges proved to be in accordance with earlier findings [1,3,10].

The profiles of the fluorescence intensity–tryptophan concentration curves (Fig. 2b) in their non linear sections ([Trp]=$5.18 \cdot 10^{-5}$–$38.5 \cdot 10^{-5}$ *M*) proved to be special: after a short, increasing stage ([Trp]=$5.18 \cdot 10^{-5}$ *M*, $9.90 \cdot 10^{-5}$ *M*) with higher

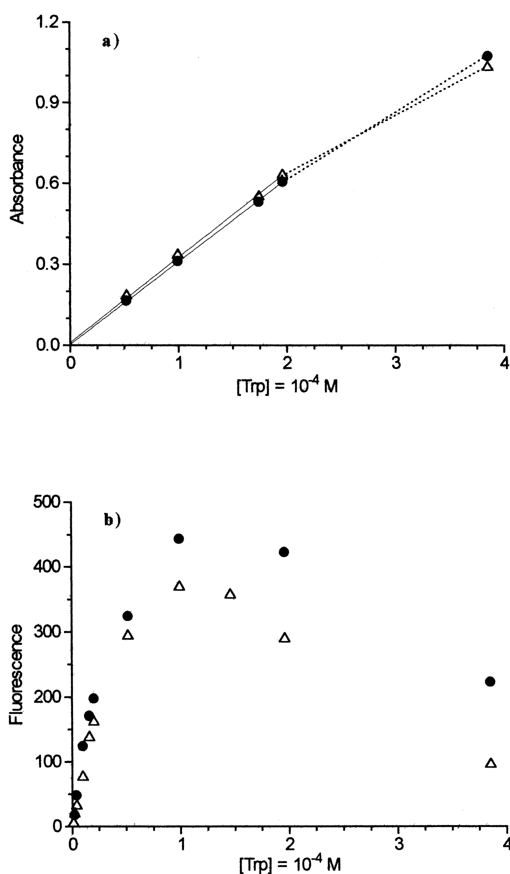

Fig. 2. Concentration–absorbance (a) and concentration–fluorescence (b) curves for the tryptophan derivatives with OPA/NAC (△) and OPA/MPA (●) reagents; OPA/NAC (MPA)$=3.81\cdot10^{-3}/1.14\cdot10^{-2}$ (M).

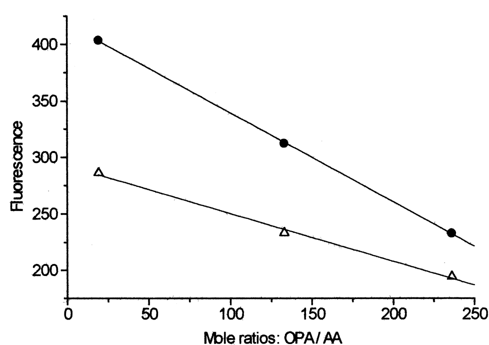

Fig. 3. FL intensities of tryptophan isoindoles (Trp)$=1.99\cdot10^{-4}$ (M); ●: OPA/MPA; △: OPA/NAC obtained with various excess of OPA reagents: [OPA]$=3.81\cdot10^{-3}$, $2.64\cdot10^{-2}$, $4.70\cdot10^{-2}$ (M), which corresponds to the mol ratios of OPA/AA=19.1, 133 and 236, respectively.

tryptophan concentrations ([Trp]$=14.6\cdot10^{-5}$–$38.5\cdot10^{-5}$ M), severely decreased fluorescence intensities were obtained. Remaining on the safe side, assuming that the excesses of the OPA reagents were not sufficient, the fluorescence intensity of the tryptophan isoindoles have been tested with two extended mol ratios (Fig. 3, OPA/AA=33 and 236). On the basis of this evidence it can be assumed that the presence of ions in high concentrations might have a screening effect on isoindole's fluorescence.

3.2.3. Stability studies of the OPA/MPA and OPA/NAC isoindoles

The stability characteristics of selected amino acids partly with both reagent solutions (Table 1: aspartic acid, glycine, histidine, methionine, leucine, lysine, tryptophan, cysteine), partly with the OPA/MPA reagent only (Table 2: alanine, β-alanine, GABA, isoleucine, phenylalanine, serine, threonine, tyrosine, valine), have been studied one-by-one (Tables 1 and 2). Measuring their UV absorbancies and fluorescence intensities from the same solutions, in parallel at pH=9.4, from 1 min until 4 h in borate buffer, and, at pH=7.2, from 7 min until 4 h (subsequently decreasing the pH of one half of the test solutions of pH=9.4, by using acetate buffer, to pH=7.2).

On the basis of our results (Tables 1–3) it can be stated that (1) monitoring the concentrations of isoindoles both by their UV absorbances and by their fluorescence intensities, differences have not been found. The only exception proved to be the cysteine's isoindole: it does not provide fluorescence and it furnishes an absorbance maximum at 278±2 nm, in contrast to all others supplying a maximum at 334 nm. (2) Maximum absorbance/fluorescence values could be achieved with the OPA/MPA and OPA/NAC reagents, equally.

(i) At pH=9.4 until 7 min with the exceptions of aspartic acid, lysine, β-alanine and GABA. Aspartic acid showed maximum values after 9 min and for up to 4 h, lysine approached 95% within 5 min and reached the maximum only after 2 h. β-Alanine and GABA reached maximum values after 25 min.

(ii) At pH=7.2, decreasing the pH of the isoindoles after 7 min, considerable destruction was

Table 1
Stability of isoindole derivatives of various amino acids[a]

Time (min)	%, expressed in the percentages of the maximum values			
	OPA/MPA		OPA/NAC	
	pH 9.4	pH 7.2	pH 9.4	pH 7.2
Aspartic acid				
1	51		54	
3	82		87	
5	95		95	
7	98	100	98	100
9–45	100	100	100	100
240	100	76	100	80
Methionine				
1	95		99	
3	99		100	
5	99		100	
7	100	98	99	98
10	100	99	99	99
13–45	100	100	99	100
120	99	95	–	–
240	96	91	98	93
Glycine				
1	97		100	
3–5	100		100	
7–13	100	100	100	100
25	100	96	100	100
45	98	85	100	99
120	87	58	98	54
240	24	11	90	71
Leucine				
1	92		91	
3	100		100	
5	100		100	
7	100	100	100	100
120	100	98	–	–
240	96	97	100	90
Histidine				
1	98		99	
3–5	100		100	
7–10	100	100	100	100
13	100	96	100	93
25	99	87	98	70
45	97	69	94	42
120	88	44	78	13
240	76	16	60	4
Lysine				
1	91		93	
3	94		99	
5	95		99	
7	95	96	100	98
9–15	95	97	100	99
25	95	99	100	99
45	97	100	100	99
120	100	95	99	100
240	95	80	98	96
Tryptophan				
1	91		99	
3	99		99	
5	100		99	
7	100	100	100	100
9–45	100	100	100	99
240	96	85	98	85
Cysteine				
1	17		31	
3	68		64	
5	91		88	
7	100	126	98	144
9	100	128	100	148
10	98	127	100	148
13	92	122	92	142
15	84	118	86	138
25	61	98	62	117
45	43	70	46	82
120	29	49	47	52
240	35	39	42	43

[a] Measured by UV absorbance (at 334 ± 2 nm, except for cysteine: at 278 ± 2 nm) and FL intensities [OPA/MPA (NAC), Ex/Em=337/354 (442) nm]. Conditions: [OPA]/[MPA] ([NAC])=1/3; [OPA]/[amino acid]=$3.3\cdot10^{-3}/1.7\cdot10^{-4}$ (UV), [OPA]/[amino acid]=$3.3\cdot10^{-3}/1.6\cdot10^{-5}$ (fluorescence); tests started with pH=9.4, have been acidified after 7 min to pH=7.2; – no data available.
Note: since UV and FL tests provided identical values (R.S.D.≤1%) stability data shown in the table are their averages.

Table 2
Stability of isoindole derivatives of various amino acids obtained with the OPA/MPA reagent[a]

Reaction time (min)	Alanine		β-Alanine		γ-Amino-butyric acid		Isoleucine		Phenylalanine		Serine		Threonine		Tyrosine		Valine	
	pH 9.4	pH 7.2	pH 9.4	pH 7.2	pH 9.4	pH 7.2	pH 9.4	pH 7.2	pH 9.4	pH 7.2	pH 9.4	pH 7.2	pH 9.4	pH 7.2	pH 9.4	pH 7.2	pH 9.4	pH 7.2
1	93		97		97		80		88		93		70		85		74	
3	100		97		97		98		100		98		98		99		98	
7	100	98	98	98	97	95	99	100	100	100	100	100	100	100	100	100	100	100
9–25	100	100	98	100	98	97	100	100	100	99	100	94	98	93	100	100	100	100
25–45	100	100	100	100	100	100	100	100	100	98	100	87	95	86	100	100	100	100
120	100	100	–	–	100	88	100	100	–	–	92	74	–	–	–	–	–	–
240	98	87	81	51	80	65	100	94	35	27	86	53	78	45	100	90	98	95

[a] Details as in Table 1.

Table 3
Molar absorptivity[a] and molar fluorescence[a] values of tryptophan isoindoles obtained with reagents (Fig. 1) stored for different times at ambient (Am) temperature and in the refrigerator (Re)

Age of reagents	Molar absorptivity[a]				Molar fluorescence[a]			
	OPA/MPA		OPA/NAC		OPA/MPA		OPA/NAC	
	Am	Re	Am	Re	Am	Re	Am	Re
90 min	100	–	100	–	100	–	100	–
2 days	100	100	100	100	97	100	93	100
4 days	100	100	100	100	79	85	60	100

[a] %, Relative to 90 min values.

observed in the case of those isoindoles that proved to be less stable even at pH=9.4 (Table 1: glycine, histidine; Table 2: phenylalanine, serine, threonine).

(3) In general, in the knowledge of these stability features and in the knowledge of all other influencing parameters detailed above, applying strictly the same conditions, for calibration and sample solutions, (either automatically or manually), acceptable results can be expected.

(4) As to the applicability of the aged reagents (Fig. 1) they provided only acceptable UV absorbances but decreased fluorescence intensities (Table 3).

3.2.4. Comparison of the molar absorbance and molar fluorescence values of OPA/MPA and OPA/NAC isoindoles

In this paper we presented this comparison which can be evaluated on the basis of our data obtained under strictly the same conditions (Table 4).

Differences in the molar absorbance values, at pH=9.4 and pH=7.2 for both isoindole solutions

Table 4
Molar absorptivity ($\varepsilon = A \cdot 10^{-3}/M$ cm), relative molar fluorescence[a] and molar fluorescence ratio (MFR)[b] values of the amino acid/OPA/MPA and amino acid/OPA/NAC derivatives calculated on the basis of their maximum values (Tables 1 and 2)

Amino acid	$\varepsilon \cdot 10^{-3}$					Relative molar fluorescence[a]						MFR[b]
	OPA/MPA		OPA/NAC			OPA/MPA			OPA/NAC			
	pH 9.4	pH 7.2	pH 9.4	pH 7.2	[pH 11][c]	pH 9.4	pH 7.2	[pH 3][c]	pH 9.4	pH 7.2	[pH 12][c]	pH 9.4
Alanine	6.79	6.50	–	–	6.83	0.94	0.99	0.91	0.72	–	1.47	1.10
β-Alanine	6.60	6.89	–	–	–	1.08	1.11	–	0.77	–	–	1.19
GABA	6.61	6.63	–	–	–	1.02	1.12	0.88	0.74	–	–	1.27
Aspartic acid	6.46	6.14	6.78	6.25	6.92	1.20	0.85	0.80	0.83	0.79	1.04	1.24
Cysteine	7.51	11.1	9.02	11.5	2.70	–	–	–	–	–	–	–
Glycine	6.95	6.96	6.99	7.03	7.18	1.00	1.00	1.00	1.00	1.00	1.00	1.27
Histidine	6.05	6.51	6.44	6.62	6.86	0.90	0.97	0.50	0.73	0.89	–	1.38
Isoleucine	6.46	6.29	–	–	6.75	1.04	–	1.09	–	–	–	–
Leucine	7.00	6.68	6.87	6.84	6.86	1.09	1.08	1.09	0.68	0.64	0.73	1.54
Lysine	12.9	13.1	12.9	12.8	10.7	0.64	0.66	0.17	0.53	0.54	–	1.14
Methionine	6.56	6.42	6.61	6.98	6.93	1.07	1.02	0.98	0.76	0.84	–	1.15
Phenylalanine	6.17	6.16	–	–	6.59	0.87	–	1.06	–	–	–	–
Serine	6.36	6.40	–	–	6.98	0.87	1.16	0.98	0.68	–	–	1.14
Threonine	6.29	6.39	–	–	6.85	0.82	–	0.88	–	–	–	–
Tryptophan	6.02	5.85	6.09	6.26	7.83	0.95	0.88	–	0.81	0.79	–	1.19
Tyrosine	6.03	5.89	–	–	6.58	0.91	0.84	0.88	0.61	–	–	1.52
Valine	6.37	6.28	–	–	6.78	0.92	–	0.97	–	–	0.43	–

Details as in Tables 1–3, as well as: [a] relative to glycine=1; [b] MFR=molar fluorescence values of the OPA/MPA isoindoles/OPA/NAC isoindoles; [c] papers from the Reference list.

proved to be of secondary importance: these values being close to each other and also to the reported values [11], with the exception of cysteine. Cysteine's isoindoles provided considerably higher ε values after decreasing the pH of their test solutions from 9.4 to 7.2. (Tables 1 and 4): ε values for OPA/MPA cysteine derivatives increased from $7.51\cdot10^3$ to $11.1\cdot10^3$ and for OPA/NAC from $9.02\cdot10^3$ to $11.5\cdot10^3$ (Table 4). Unfortunately, the low stability of cysteine's isoindole (Table 1) limits its applicability for quantitation purposes.

Considerable distinctions have been determined: (i) in the relative molar fluorescences (Table 4) in comparison to the published values [3,12]: in particular lower fluorescence were reported in the cases of lysine and histidine, likely due to the fact that under the chromatographic analysis they are less stable than outside the chromatographic system. (ii) Spectacular differences were measured in the molar fluorescences of the OPA/MPA and OPA/NAC isoindoles (Table 4). Strictly under the same conditions (calculated from our maximum FL intensities measured at optimum Ex/Em wavelengths) the corresponding ratios of the molar fluorescence ratios (MFRs) varied from 1.10 to 1.54 proving that the OPA/MPA isoindoles furnish higher fluorescence intensities that the corresponding OPA/NAC ones.

4. Conclusions

On the basis of our experiences, in order of their importance the consequences are as follows:

OPA/MPA and OPA/NAC reagents do have self fluorescence: these self fluorescence values through a short peak period (5–10 min) decrease after 90 min approaching a stable blank fluorescence that can be maintained at ambient temperature for 4 h and at 4°C for 4 days (possibly even for longer time). Thus, the age of the reagents is of key importance, in particular in those cases when manual injections were used. In this term no differences could be determined with the OPA/MPA and OPA/NAC reagents, unless the slight differences in the extent of the blank values: the OPA/NAC reagent blank proved to be somewhat higher (60 arbitrary units versus 40).

When using thermostattable autoinjectors it is the responsibility either of the user (if autosampler program for OPA/amino acid derivatization is not included in the software) or that of the manufacturer (special program for the amino acid OPA derivatization is installed) to achieve reliable values.

Concerning the strategy to be followed with manual and/or autosampler injections, without sample thermostation, it is the responsibility of the analytical chemist – being aware of the self fluorescence of OPA reagents – to take it into account.

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