Journal of Fluorescence (2019) 29:1445–1455
https://doi.org/10.1007/s10895-019-02449-2

ORIGINAL ARTICLE

Phenylalanine Photoinduced Fluorescence and Characterization of the Photoproducts by LC-MS

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Received: 6 June 2019 / Accepted: 1 October 2019 / Published online: 25 November 2019

Abstract

Phenylalanine (Phe) is a direct precursor of tyrosine and several neurotransmitters. The accumulation of Phe in the brain generates serious and not recoverable pathologies in children. Early detection in newborns is fundamental to apply the appropriate therapy and avoid irreversible health problems. Although fluorescence is a sensitive and selective technique for the determination of amino acids, the fluorescent analysis of Phe is limited since it exhibits a very low fluorescence quantum yield; however, the fluorescence of Phe increases drastically under UV irradiation when a peroxide medium is used. The aim of this research was to analyze the effect of the UV-radiation on Phe aqueous-peroxide solutions and to study the influence of the chemical environment on the photoinducted fluorescence process. The nature and characteristics of the fluorescent photoproducts generated under off-line UV irradiation in hydrogen peroxide medium were achieved by high performance liquid chromatography (HPLC) using a spectrophotometer detector (DAD) coupled in series with a mass spectrometer (MS) or with a fast scan spectrofluorimetric detector (FSFD). Environmental characteristics such as pH, initial concentration of Phe, hydrogen peroxide amount and irradiation time were studied in order to establish their influence on the formation of each one of the photoproducts. As the formation of several highly fluorescent photoproducts has been confirmed, the possibility of designing a chromatographic system with a post-column on-line photoreactor is open. The measure of the total fluorescence signal generated from Phe at the optimized irradiation time, could be used for the determination, with high sensitivity, of the initial amount of Phe in aqueous media, such as human serum or environmental samples. These aspects are being studied at present.

Keywords Phenylalanine · Tyrosine · Photoinduced fluorescence · High performance liquid chromatography · Mass spectrometry

Introduction

Phenylalanine (Phe) is an essential amino acid and a tyrosine direct precursor. An inborn error of phenylalanine metabolism is the phenylketonuria (PKU), characterized by the accumulation of this amino acid in the brain due to a phenylalanine hydroxylase (PAH) deficiency [1]. Untreated PKU is associated with deep intellectual disability and neurological problems. Treatment of PKU consists on the restriction of natural protein and provision of a protein substitute that lacks Phe, but is enriched in tyrosine [2]. In others matrices, such as natural waters, amino acids are important sources of fixed nitrogen for the environmental microbiome, providing energy for microbial metabolism and growth [3] but, these molecules are susceptible to photochemical transformation. Researching the photochemistry of dissolved free amino acids is important for assessing the environmental behavior of free and combined amino acids [3].

Phe absorbs radiation within the UV region of the spectrum, but not in the visible region and shows a fluorescence quantum yield practically negligible. Therefore, in the bibliography some studies about the fluorescent determination of Phe without previous derivatization reactions can be found. A method for the direct fluorimetric determination of Phe and tyrosine (Tyr) in dried blood samples using fluorescence detection coupled to a high performance liquid chromatographic system (HPLC) has been proposed and the low fluorescence yield of Phe was then described [4]. Several preconcentration steps were proposed to avoid the Phe sparingly fluorescence signals. Chemical prederivatization is the most frequent way

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to obtain fluorescence from Phe. Several organic compounds have been proposed as derivatizing agents such as: 4-fluoro-7-nitro - 2, 1, 3-benzoxadiazole [5–7], 1-nitroso-2-naphtol [8] and isothiocyanates, as 4- (5′, 6′-dimetoxybenzotiazolil) phenylisothiocyanate [9]. In all cases several steps are involved to obtain fluorescent derivatives.

An alternative to chemical derivatization is the so-called photoderivatization. In this technique, the original molecule is irradiated with UV radiation and transformed into another one with better properties for its detection. A photochemical reaction can produce photoproducts with highly sensitive fluorophore groups due to the structural alterations. The possibility of generating fluorescent compounds by photoderivatization from biomolecules, like reduced pteridins and folic acid, is also described in the literature [10–12]. Studies of the irradiation of Phe in aqueous media, and in presence of hydrogen peroxide, were previously addressed by our research group [13]. Elsellami et al. studied the photocatalytic degradation of Phe in aqueous solutions containing TiO_2 as photocatalyst [14].

Mass spectrometry (MS) coupled to chromatographic techniques has been also applied to the simultaneous determination of Phe and Tyr in biological fluids [6, 15–21], but these approaches involve the use of a more sophisticated and expensive technique, in comparison with the conventional detectors. On the other hand, the high sensitivity of fluorescent techniques is difficult to attain with other techniques.

Reports about the photochemistry behavior of Phe in aqueous solutions can be found in the literature. Mittal et al. studied the photochemistry of Phe [22]. The absorption and fluorescence spectra have been interpreted in terms of intra molecular charge-transfer interactions. Tyr and 3,4-dihydroxyphenyalanine have been identified as photoproducts of Phe by paper chromatography using C14-labelled [23], and the UV photolysis of aromatic amino acids, including Phe has been studied by Khoroshilova et al. [24]. Nevertheless, data about the fluorescent behavior of Phe during the UV-photoirradiation have not been found.

In the present work, we report the phototransformation of Phe in presence of traces of hydrogen peroxide. In the optimized conditions, several fluorescent derivatives were generated. The photoproducts were separated and characterized using HPLC with spectrophotometric, spectrofluorometric and mass spectrometric detectors. These developments could be fundamental to subsequently establish the viability of a HPLC system coupled to a post-column UV-photo-reactor, with fast scanning fluorescence detector (FSFD) in serial configuration. A dynamic photoirradiation system coupled with FSFD could provide the desirable high sensitivity necessary for Phe control in clinical and environmental research setting, because of the high number of fluorescent photoproducts generated.

Material and Methods

Chemicals

Phe, Tyr and formic acid (99%) were purchased from Sigma (Sigma-Aldrich Química, S.A., Madrid). Acetonitrile (ACN), HPLC-grade, and hydrogen peroxide were purchased from Panreac (Barcelona, Spain). Ultrapure water provided by a Milli-Q purification system (Millipore S.A.S., Molsheim, France) was used.

Sample Preparation

Individual stock standard solutions (150 μg mL^{-1}) from Phe and Tyr solid compounds were prepared by dissolution in ultrapure water and stored at room temperature in darkness. Working solutions were prepared by dilution of the appropriate aliquots with ultra-pure water. Hydrogen peroxide working solution (0.170 mol L^{-1}) was freshly prepared each day from 8.82 mol L^{-1} commercial hydrogen peroxide.

Instrumentation and Software

Absorption spectra were recorded using a spectrophotometer Varian Cary 50 BIO with continuous Xenon lamp. Measurements were performed in 10 mm quartz cells.

Spectrofluorimetric measurements were performed with a Varian Cary Eclipse spectrofluorometer equipped with two Czerny-Turner monochromators and a xenon flash lamp, connected to a PC microcomputer via an IEEE 488 (GPIB) serial interface. All measurements were performed in 10 mm quartz cells. Cary Eclipse Scan Application V.1.1 was used to control the instrument and data acquisition.

Chromatographic studies were performed on an Agilent Model 1100 LC instrument (Agilent Technologies, PaloAlto, CA, (USA), equipped with degasser, quaternary pump, autosampler Agilent 1290 infinity thermostated at 5 °C, column oven, and the CHEMSTATION software package to control the instrument, data acquisition and data analysis. The chromatographic separation was achieved on a Poroshell 120 column (50 mm × 4.6 mm, 2.7 μm) (Agilent Technologies) and the column oven was thermostated at 22 °C.

The LC mobile phase was formed by formic acid 2 mM and isocratic elution mode was applied. The mobile phase was filtered through a 0.2 μm nylon filter and degassed before use. The flow rate was 0.6 mL min^{-1} and the injection volume was 5 μL. Before injection, samples were filtered through a Millipore Swineex syringe adapter, containing a 0.22 μm regenerated cellulose membrane filter.

UV − Vis Diode-Array detector (DAD) (Agilent Technologies G1315B) and Fast Scanning Fluorescence Detector (FSFD) (Agilent Technologies G1321A) were

coupled in serial mode (Fig. 1a, configuration 1). Detection was performed with photometric (λ = 212 nm) and fluorimetric (λ_{ex} = 219 nm and λ_{em} = 282 and 304 nm) detectors connected in series.

Alternatively, DAD detector and mass spectrometer (MS) (Agilent Technologies single quadrupole 6120 (MS) equipped with an electro-spray interface operated in the positive ionization mode were also coupled in serial mode (Fig. 1a, configuration 2).

In MS detector, single ion monitoring (SIM) was selected as operation mode using the target ion $[M + H]^+$ for the studied compounds. Target ions $[M + H]^+$ at *m/z* 198, 182 and 166 were used for dihydroxyphenylalanine, hydroxyphenylalanine and phenylalanine, respectively. The instrumental variables were: nebulizer pressure: 40 psi; drying gas: 10 L·min^{-1}; gas temperature: 300 °C; capillary voltage: 5000 V; fragmentor voltage: 100 V. Nitrogen was used as a nebulizer gas.

Moreover, flow injection analysis with the mass spectrometer operated in SCAN mode (100/500 m/z) using positive electrospray ionization (ESI) was used with identification purposes.

Off-Line UV-Photoirradiation System

An Osram 200 W HBO high-pressure multiwavelength (200–500 nm) mercury lamp, with an Oriel model 8500 power supply (Spectra-Physics, Newport, USA), was used for the UV-irradiation of Phe solutions. The photochemical set-up included a light-box consisting of a fan, a mercury lamp and a quartz lens. The light intensity on surface of cell, calculated with a radiometer UV-MAT (UV-Elektronik GmbH), with a spectral range of 200–280 nm, was 1600 μW/cm^2. Both, 3 and 10 mL quartz cells were used in the irradiation process. The cells were placed in an optical bench at 30 cm from the mercury lamp. The solutions were magnetically stirred during the irradiation step. The off-line irradiation system used is described in Fig.1b.

Calibration curves and analytical figures of merit were performed by means of the ACOC program, developed in our research group, in MatLab code [25].

Results and Discussion

The fluorescence quantum yield of Phe in aqueous solution is very low. However, when aqueous solutions of Phe are UV-irradiated for a short time, and in adequate chemical conditions, a high fluorescence photoproduct can be observed. The formation of fluorescent products was only observed in presence of low amount of hydrogen peroxide. In the bibliography [13] is described that the irradiation of Phe generates hydroxyl derivatives, similar to *p*-hydroxyphenylalanine, named tyrosine (Tyr), among others potentials hydroxyl derivatives. With the aim to compare the spectral characteristics of the photoproducts, Tyr was used as a reference photoproduct for the irradiation studies of Phe.

Spectral Characteristics of Phenylalanine and Tyrosine

Absorption spectra of aqueous Phe solutions show an absorption maximum at 212 nm and a lower second band at 257 nm,

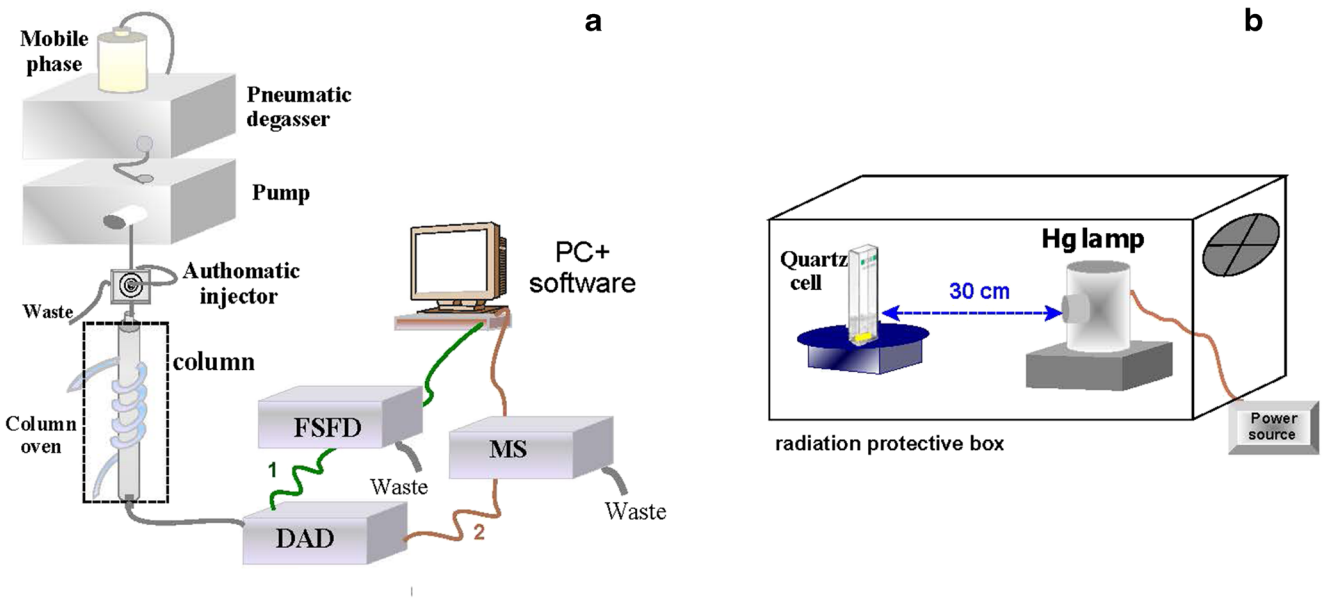


Fig. 1 **a** Chromatographic system used for the separation and identification of Phe photoproducts. Green line: configuration 1, DAD and FSFD detectors connected in series; Brown line: configuration 2, DAD and MS detectors connected in series. **b** Off- line irradiation system using a mercury lamp

Fig. 2a. Aqueous Tyr solutions also exhibit two absorption maxima located at 223 and 275 nm, respectively. The UV-absorption spectra of Phe are slightly influenced by the pH-value of the medium, and only in strongly alkaline solutions, an increase in the absorbance at 212 nm is observed. For Tyr, the signal of the maximum at 223 nm is practically constant in acid and slight alkaline media. In strongly alkaline media, pH > 12, a bathochromic shift and hyperchromic effects were observed. With respect to the fluorescence spectra, the excitation spectra of Phe shows a maximum centered at 212 nm, and the maximum of the emission spectra is located at 282 nm, Fig. 2b. Tyrosine aqueous solutions show two excitation maxima at 225 and 275 nm, and an emission maximum at 304 nm, independently of the excitation wavelength. Also, as it can be observed, the fluorescent quantum yield is very different for both compounds, and in accordance with the bibliography [26], Tyr presents high native fluorescence, and Phe is slightly fluorescent. The fluorescence intensity of both compounds is similarly affected by the acidity of the medium being maximum and constant in the pH range of 4–8. For pH < 4 and pH > 8 a decay of fluorescence takes place. These changes of fluorescence with the pH, allow to calculate the pKa values, and pK_1 2.02 ± 0.16 and pK_2 10.6 ± 1.0 for Phe and, pK_1 2.85 ± 0.16 and pK_2 9.75 ± 0.34 for Tyr were obtained.

Photo-Irradiation Studies

In first place, aqueous solutions of Phe were irradiated using the off-line photo-irradiation system indicated in *2.4* section, during different time periods between 1 and 42 min. The fluorescence intensity at 282 nm (emission maximum of Phe) slightly decreased when increased the irradiation time. However, when the irradiation was carried out in presence of low amounts of hydrogen peroxide (1.5×10^{-3} M), drastic changes were observed in the fluorescence characteristics of the photoproducts, Fig. 3. A highly fluorescent compound, with an emission maximum at 304 nm and two excitation maxima at 219 and 275 nm were observed, and the fluorescence intensity of this photoproduct increased as the irradiation time increased. On the other hand, the generated photoproduct exhibits a very similar fluorescence spectrum to Tyr, however the fluorescence intensity ratio

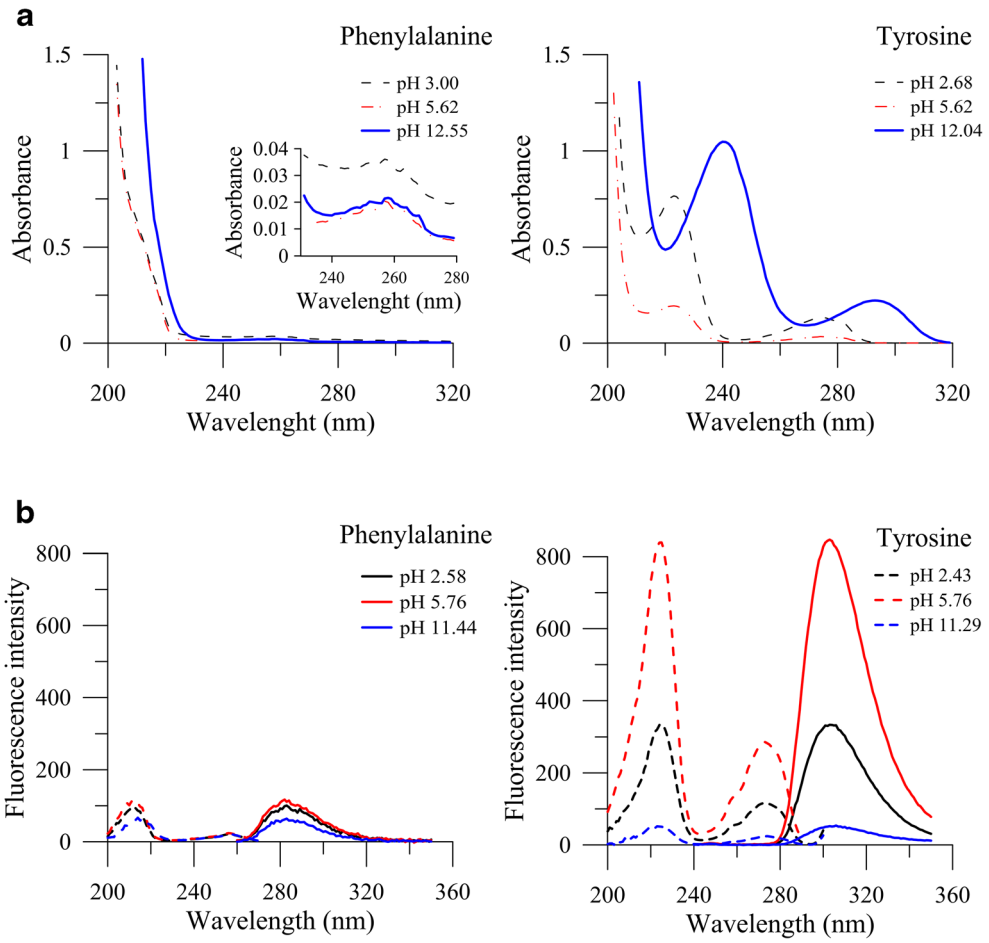


Fig. 2 **a** Absorption spectra of Phe and Tyr at different pH values. **b** Excitation and emission spectra of Phe (λ_{em} = 282 nm and λ_{ex} = 212 nm) and Tyr (at λ_{em} = 304 nm and λ_{ex} = 225 nm) in different acidity media. [Phe] = 12.7 μg mL $^{-1}$ and [Tyr] = 4.6 μg mL $^{-1}$

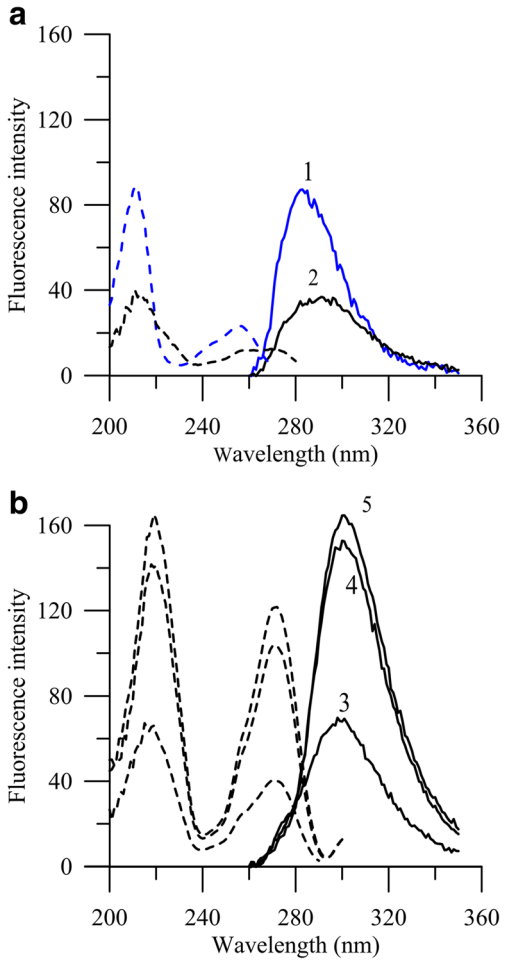


Fig. 3 Excitation and emission spectra of Phe obtained at several irradiation times, in presence of H_2O_2 3.13×10^{-3} M. **a** 1) $t_{irrad} = 0$ s, 2) $t_{irrad} = 30$ s ($\lambda_{ex}/\lambda_{em} = 212$ nm/284 nm). **b** 3) $t_{irrad} = 180$ s, 4) $t_{irrad} = 660$ s and 5) $t_{irrad} = 790$ s ($\lambda_{ex}/\lambda_{em} = 225$ nm/304 nm)

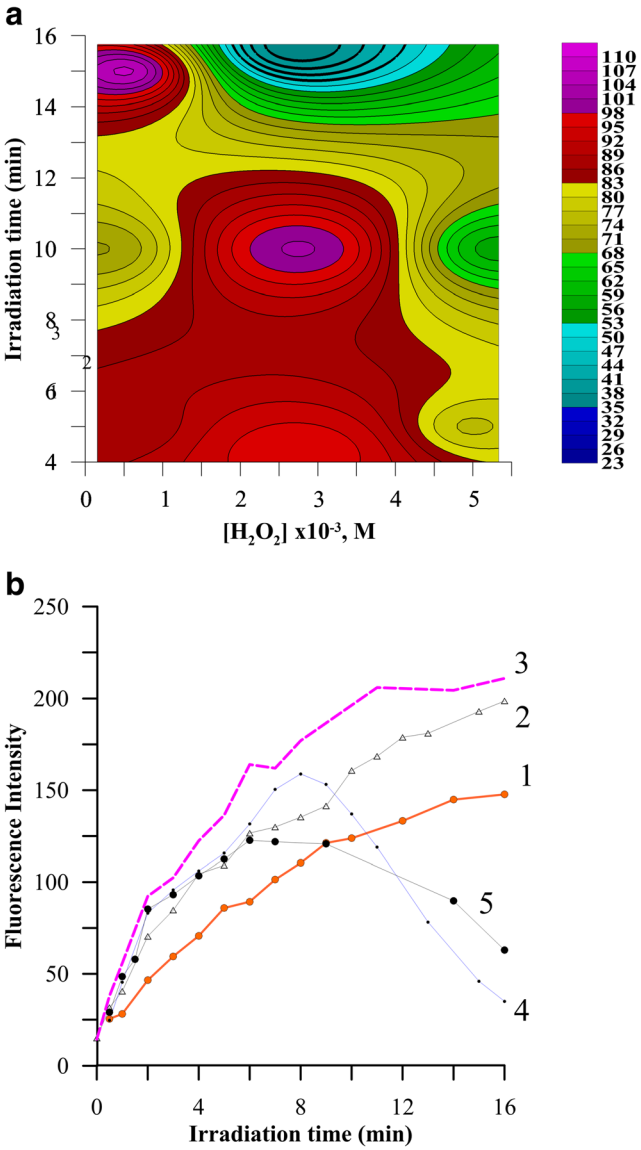


Fig. 4 **a** Contour plot fluorescence intensity at several hydrogen peroxide amounts and irradiation times; **b** Influence of the irradiation time on the fluorescence intensity of the photoproducts in presence of different hydrogen peroxide amounts: 1) 0.5×10^{-3} M; 2) 0.7×10^{-3} M; 3) 1.0×10^{-3} M; 4) 3.0×10^{-3} M; 5) 5.0×10^{-3} M

between the excitation signals at 225 nm and at 275 nm appears to be smaller than those for Tyr pure. This seems to suggest that the irradiation of Phe solutions could generate a mixture of photoproducts.

Influence of Hydrogen Peroxide Concentration and Irradiation Time

In order to optimize the influence of the irradiation time and the hydrogen peroxide concentration on the formation of fluorescent photoproducts, an experimental design, central composite design, was used. A total of 11 experiments using five levels for each variable were carried out. Samples containing fixed amounts of Phe (10 μg mL^{-1}) in presence of different hydrogen peroxide concentrations, between 0.16–5.34 mM, were irradiated during different times, between 4.25–15.75 min. Excitation and emission spectra were monitored and the fluorescence intensity was measured at 304 nm.

In Fig. 4a, a contour map showing the influence upon the fluorescence intensity of the photoproducts by the two key variables is shown. It can be observed two zones where the fluorescence is maximum, one of them at low hydrogen peroxide concentrations and high irradiation times, higher than 14 min; and the second for an irradiation time near to 10 min and intermediate hydrogen peroxide concentrations (≈ 3×10^{-3} M). The influence of the irradiation time on the fluorescence of the photoproducts in presence of different hydrogen peroxide concentrations is show in Fig. 4b. In all cases the fluorescence intensity increases as increasing the irradiation time. The maximum signal was obtained in presence of hydrogen peroxide 10^{-3} M and after irradiation during 15 min. For higher hydrogen peroxide concentrations and lower irradiation times (≈ 8 min), the photoproducts initially generated

are decomposed and the fluorescence signal decreases. This effect is more remarkable at high irradiation times.

Nevertheless, it was proven that the optimum irradiation time and hydrogen peroxide concentration are directly related to the Phe concentration in the original solution, so, for the following studies we have selected those conditions that allow to obtain a linear relationship between fluorescence signal of the photoproducts and the initial Phe concentration, with a reasonable irradiation time. In this sense, samples containing Phe in the concentration range between 1 and 20 μg mL^{-1} were irradiated during 10 min in presence of hydrogen peroxide 3.13×10^{-3} M, and a lineal increment ($r^2 = 0.97$) on the fluorescent signal at 304 nm was observed.

Chromatographic Studies of the Irradiation Process

Once the formation of fluorescence photoproducts from Phe has been verified, with the aim of identifying them and following the process, a reverse phase (RP) HPLC procedure has been optimized. Taking into account the previous studies, the chromatographic separation was optimized using aqueous solutions of Phe and Tyr. A C18 stationary phase and an isocratic elution with a mobile phase composed by formic acid 2 mM were selected. To study the Phe photodecomposition, aliquots of Phe aqueous solutions, containing hydrogen peroxide 3.13×10^{-3} M, previously irradiated during different times, were injected in the chromatographic system. With the objective of detecting all the photoproducts, a diode array and a fast scanning fluorescence detectors in series were used.

In Fig. 5, chromatograms of Phe solutions in presence of hydrogen peroxide, irradiated during 7 and 15 min respectively and obtained in multi-emission mode at 282 and 394 nm, exciting at 219 nm, are shown. At $\lambda_{exc}/\lambda_{em} = 219/282$ nm low signals are observed, but at $\lambda_{exc}/\lambda_{em} = 219/304$ nm six peaks can be observed. By comparison with the chromatograms obtained for Phe and Tyr pure standard solutions, peak 3 can be assigned to Tyr photogenerated and peak 6 to Phe not yet degraded. The other four peaks correspond to other photoproducts, which initially were not assigned in this experiment.

A full study was carried out to establish the influence of the irradiation time on the photoproducts formation. In all cases, the peak area increases as increasing the irradiation time. As can be observed in Fig. 6b, the behavior of peaks 1 and 2 are very similar, both reach maximum value at irradiation times near to 900 s and decrease for higher irradiation times. However, peaks 3, 4 and 5 are more favorably generated at lower irradiation time. An irradiation time near to 400 s appears as optimum to generate these photoproducts. A progressive decomposition of these photoproducts was observed for higher irradiation time. Peak 6 (Phe), as expected, is degraded continuously by irradiation.

Also, the influence of the acidity on the photoirradiation process was studied. Several aqueous solutions containing 20 μg mL^{-1} of Phe in presence of hydrogen peroxide 3.13×10^{-3} M, at different pH values, fixed by the addition of hydrochloric acid or sodium hydroxide were prepared. The study of the influence of irradiation time was carried out in all of them, and the higher yield in the formation of photoproducts was obtained in neutral and slightly acidic media (pH 5–7).

Characterization of the Photoproducts

With the aim of characterizing the photoproducts, the dynamic absorption, fluorescence and mass spectra have been recorded through all the chromatographic peaks. HPLC-DAD-FSFD and HPLC-DAD-MS chromatographic detector configurations were used and the spectra obtained at three different irradiation times in each peak, uphill zone, downhill zone and maximum zone, were compared. The normalized spectra at the different time in each peak are coincident. Also, the spectra have been compared with the available Tyr and Phe standard compounds. By means of a coupled mass spectrometry detector, molecular ions have been assigned for all peaks.

The photoproducts were tentatively identified by studying their mass spectra and a tentative assignment was made based on the molecular ions obtained. Studies were performed in positive electrospray ionization (ESI) mode in the mass range of 50–1000 Da to establish the fragmentation pattern of Phe.

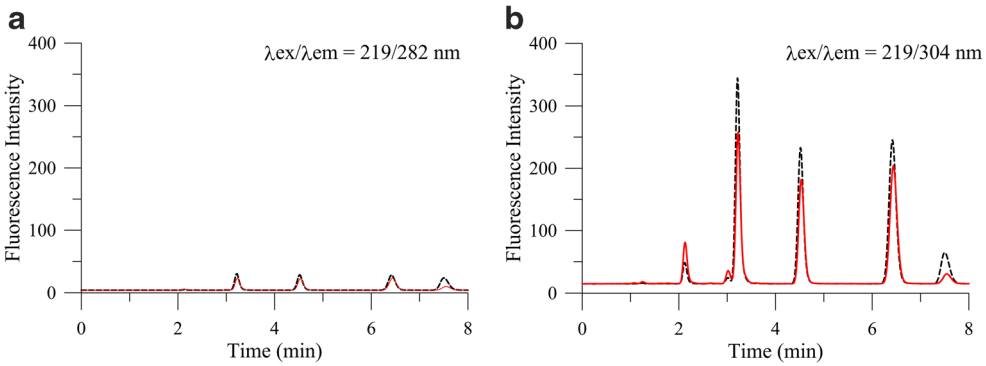


Fig. 5 Chromatograms obtained from aqueous peroxide Phe solution irradiated during 7 and 15 min: **a**) $\lambda_{ex}/\lambda_{em} = 219/282$ nm and **b**) $\lambda_{exc}/\lambda_{em} = 219/304$ nm. Irradiation time 7 min (dashed line), irradiation time 15 min (solid line)

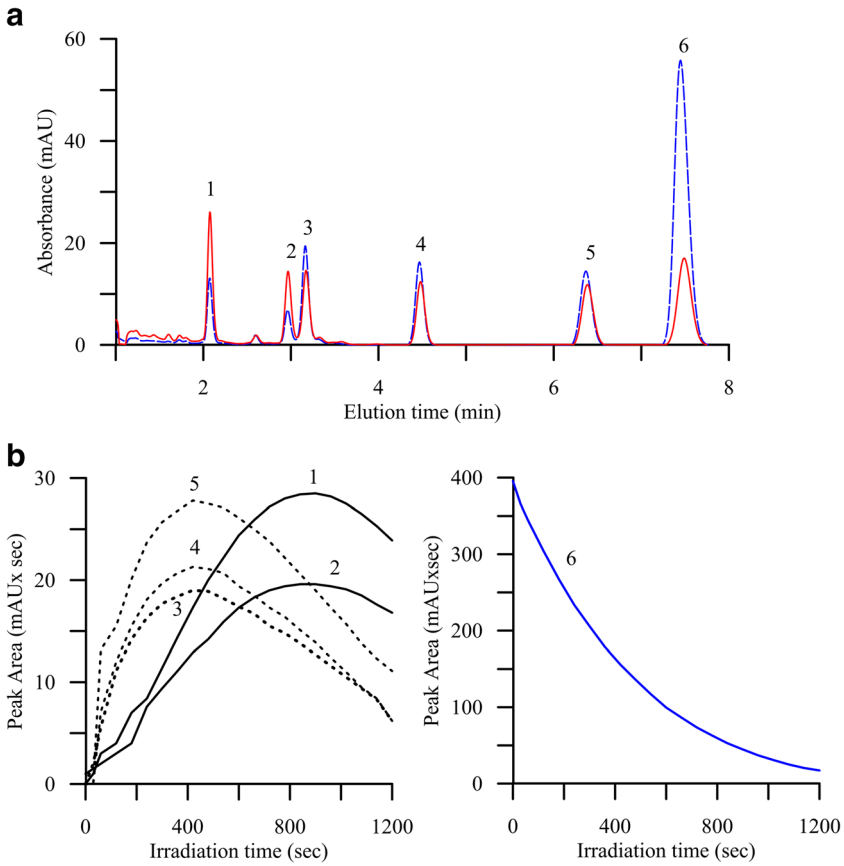
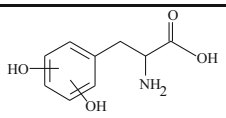
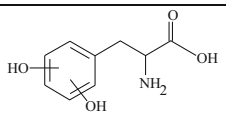
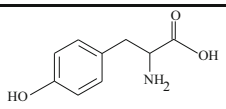
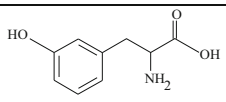
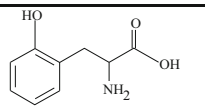
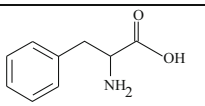


Fig.6 **a** Chromatograms obtained at λ = 212 nm from aqueous peroxide Phe solutions irradiated during 7 (dashed line) and 15 (solid line) minutes. **b** Evolution of peak areas vs irradiation time

Table 1 Mass spectral data, fluorescence wavelengths and chromatographic characteristics of the photoproducts and Phe

Peak Number	Compound assigned	Molecular Mass	$[M+H]^+$	λ_{em} (nm)*	t_R (min)	Chemical structure
1	dihydroxyphenylalanine	197	198	333	2.05	
2	dihydroxyphenylalanine	197	198	333	2.98	
3	p-hydroxyphenylalanine **(tyrosine)**	181	182	314	3.22	
4	m-hydroxyphenylalanine	181	182	312	4.51	
5	o- hydroxyphenylalanine	181	182	304	6.31	
6	**phenylalanine**	165	166	282	7.63	

**Dynamic Emission spectra obtained in multi-emission mode by exciting at 219 nm.*

Mass spectral data, the wavelength with maximum intensity of the dynamic emission spectra and the assignation of the molecular structure for each peak are shown in Table 1. Peaks 1 and 2 are compatible with the formation of dihydroxy phenylalanine derivatives according to their more abundance ion peak at *m/z* 198 $[M + H]^+$. Peaks 3, 4 and 5 can be assigned to monohydroxy phenylalanine derivatives with an ion peak at *m/z* 182 $[M + H]^+$ in all cases. In accordance with the previous results, and by comparing its retention time with that of a standard solution, peak 3 was assigned to *p*-hydroxyphenylalanine (Tyr). The elution order of the monohydroxy phenylalanine derivatives has been assigned taking account the retention time of Tyr and the polarity of each one of them. So, the elution order was *p*-hydroxyphenylalanine, at 3.22 min, *m*-hydroxyphenylalanine, at 4.51 min and *o*-hydroxyphenylalanine at 6.32 min. On the other hand, according to the previous fluorimetric experiments, we can now conclude that dihydroxy phenylalanine derivatives are predominant at large irradiation times, and monohydroxy phenylalanine derivatives are predominant at irradiation times lower than 400 s.

Influence of the Initial Amount of Phe in the Photoproducts Generation

With the objective of study if the Phe concentration has influence on the generation of photoproducts, five samples containing different concentrations of Phe in the range 10 to 30 μg mL^{-1} in presence of hydrogen peroxide 3.13×10^{-3} M, were irradiated at two different times (7 and 15 min). Each sample was chromatographically analyzed and the eluate was monitored in series coupled detectors, photometrically at 212 nm and fluorimetrically at 304 nm ($\lambda_{ex} = 219$ nm). The maximum generation of dihydroxy derivatives was reached for intermediate Phe concentrations, 20 μg mL^{-1}, when the irradiation time was 7 min. Nevertheless, the generation of monohydroxy derivatives continually increases as the Phe amount increases. For irradiation time of 15 min, the generation of both, monohydroxy and dihydroxy derivatives, are favored at high Phe concentrations.

The yields for Phe degradation and Tyr formation at the two irradiation times were calculated by comparison of the peak areas of non-irradiated and irradiated samples. In first place, calibration curves using photometric and fluorimetric signals were calculated for Phe (peak 6) and Tyr (peak 3) using pure standard solutions in the range 0.0605–0.182 mM and 0.00276–0.0166 mM for Phe and Tyr, respectively. For Phe, calibration sensitivity, calculated as the slope of the regression curve, is similar by both signals, photometric and fluorimetric; however, for Tyr the calibration sensitivity increases drastically using fluorimetric detection. In both cases, detection limit (LD) and quantification limit (LQ), according Long and Winedfordner criterium [27] were calculated. In Table 2, regression statistical parameters are summarized. The linearity was higher than 98% in all cases.

The percentage of Phe transformation into Tyr during the UV-irradiation process was calculated for samples containing Phe in the range 0.0605 and 0.182 mM, and irradiated during 7 and 15 min. In order to calculate the degradation percentage of Phe, the peak area (peak 6) of the irradiated solutions was compared with those of the regression standard curves. In Table 3, the values calculated for degradation percentage of Phe have been summarized. It can be appreciated that degradation percentage is dependent of the irradiation time. So, when Phe samples were irradiated during 15 min, the degradation is near to 100% for all Phe concentrations assayed. However, with minor irradiation times, 7 min, the degradation percentage decreases as Phe concentration increases. On the other hand, the photogeneration of Tyr is most favorable with low irradiation times, and it is practically independent of the Phe concentration, Table 3. After 7 min of irradiation, the percentage of Tyr photogenerated is about 4–6%. Most of generated fluorescent photoproduct were meta−/o-hydroxyphenylalanine and dihydroxyphenyalanines, and only about 6% is *p*-hydroxyphenylalanine (Tyr).

Sensitivity of the Phe Irradiation Process

In order to explore the sensitivity of the determination of phenylalanine by measuring of the total fluorescence of the photoproducts, the limit of detection was calculated using a flow injection analysis (FIA) mode. Standard solutions of different concentration (comprised between 0.6 and 90 μM) in presence of hydrogen peroxide 3.13×10^{-3} M, were off-line irradiated during 10 min and then aliquots of 5 μL of each one were directly injected into the mobile phase. The peak area corresponding to the total fluorescent photoproducts was used as analytical signal and the equation of the linear regression curve was $y = (343.0 \pm 7.9)\ x + (52.1 \pm 9.7)$, being the determination coefficient, R^2, 0.999.

To calculate the limit of detection, the Long and Winefordner criterium [27] were used. A limit of detection (LOD) of 0.91 μM was calculated. The guidelines for phenylketonuric (PKU) patients [28] stablish that Phe plasma or serum levels should be maintained within the range of 120–360 μM for 0–12 years old or women in pregnancy. It is considered a very soft pKU when Phe serum content are comprised between 300 and 660 μM.

The detection limit calculates in this research is much lower than the normal levels found in serum, so the method could be useful to analyze the content of phenylalanine in serum samples and other biological fluids.

Several papers report LOD values to analyze Phe using diverse techniques, i.e. prederivatization HPLC with fluorimetric detection being 3 μM [4], 0.1 μM [7] and 0.9 μM

Table 2 Chromatographic statistical calibration parameters for Phe and Tyr standard solutions

Signal	Slope ± Ss	Intercept ±Si	Sy/x	LD (mmol L^{-1})	LQ (mmol L^{-1})	R^2
Phenylalanine (0.0605–0.182 mmol L^{-1})						
Photometry Peak area at λ_{ab} = 200 nm	7114 ± 86	16 ± 11	8	4.60×10^{-3}	1.51×10^{-2}	0.9996
Fluorimetry Peak area at $\lambda_{ex}/\lambda_{em}$ = 219 nm/304 nm	7630 ± 218	30 ± 28	21	2.70×10^{-3}	9.00×10^{-3}	0.9975
Tyrosine (0.00276–0.0166 mmol L^{-1})						
Photometry Peak area at λ_{ab} = 200 nm	9284 ± 308	−3.0 ± 3.3	3.6	1.10×10^{-3}	3.7×10^{-3}	0.9956
Fluorimetry Peak area at $\lambda_{ex}/\lambda_{em}$ = 219 nm/304 nm	231080 ± 8240	−60 ± 30	1.0	1.15×10^{-3}	3.83×10^{-3}	0.9949

Sy/x: Regression deviation: Ss: slope standard deviation, Si: intercept standard deviation; LD: Limit of detection: LQ: Limit of quantification according to Long and Winefordner [27]

[9], 1 μM using mass spectrometry [15] and 1.5 μM using UV detection [29, 30]. It is necessary taking into account than in all mentioned cases, the criterium signal/noise = 3 was applied. However it is known that IUPAC criterium provides lower detection limits than the strictest Long and Winefordner criterium.

Conclusions

Aqueous solution of phenylalanine exhibits a low quantum yield of fluorescence while aqueous tyrosine solutions are highly fluorescent due to the substituted hydroxyl group in *p*-position in the aromatic ring. However, direct fluorimetric techniques are not useful to analyze Phe and derivatization reactions are necessary to apply fluorimetric techniques. In this sense, the use of UV-radiation as chemical reagent gives the possibility to obtain photoproducts highly fluorescent. Photoirradiation process of Phe involves notable structural modifications Absorption spectra of the photoproducts are similar to those of the hydroxyphenylalanine derivatives, and a high quantum yield of fluorescence is observed. Photoreaction is affected by the irradiation time, amounts of hydrogen peroxide and initial concentration of phenylalanine. As the concentration of hydrogen peroxide increases, the yield of photoproduct also increases. However, above a certain hydrogen peroxide concentration, the reaction rate levels off and the degradation of the photoproducts is favored.

Photoproducts separated by RP-HPLC allow us to establish the formation of mono- and di- hydroxyphenylalanine derivatives. By coupled mass spectrometry, the assignation of molecular ion for the photoproducts can be carried out. The

Table 3 Photo-transformation percentage of different concentrations of Phe into Tyr by UV irradiation in the established conditions (3.13×10^{-3} M and irradiation time 7 and 15 min)

	% Phe photo-degradation		% Tyr photo-generation	
	Irradiation time		Irradiation time	
$[Phe]_{initial,}$ (mmol L^{-1})	7 min	15 min	7 min	15 min
0.0605	96.54	100	4.13	0.55
0.0908	93.68	100	4.16	0.44
0.121	86.86	100	4.90	0.87
0.151	71.91	96.37	5.95	2.74
0.182	57.21	88.35	5.68	4.27

predominant forms are monohydroxylphenylalanine derivatives at short irradiation time, dihydroxyphenylalanine derivatives at large irradiation time, and Phe continuously disappears.

Focusing on the formation of tyrosine, its percentage of formation depends on the irradiation time, being maximum up to 7 min and it decreases for higher irradiation time. For higher irradiation times ($t_{irr} > 20$ min) the fluorescence of the irradiated Phe is not detectable because all the compounds have been degraded, even by breakage of the aromatic rings. Photodegradation of the Phe is elevated (about 90%) in 15 min. However, the formation of Tyr has a poor yield, about only 5% at 7 min, although several others photoproducts are generated at the same irradiation time.

On the other hand, one of the advantages of the proposed method, is that Phe is one of the three fluorescence amino acids (along with tryptophan and tyrosine) but its fluorescence quantum yield is so low for its quantification in complex matrices. The possibility of transform it in a photoproduct with high fluorescence quantum yield will allow its analysis. This conversion occurs in a proportion that depends both on the experimental conditions of photoirradiation and the initial concentration of phenylalanine. The fluorescence intensity is due to all photoproducts generated at intermediate irradiation time (near to 10 min) and it is proportional to the initial amount of Phe.

Once the principles of the photoinduced fluorescent behaviour of Phe have been established, and with the aim of analyzing Phe in aqueous medium, such as human serum and environmental samples, the research could be implemented in an online RP-HPLC, coupled with a post-column UV-reactor system, using a fast scan fluorimetric detector. These aspects are being studied in a new research. A high sensitivity method using fluorimetric detection is expected because the numerous fluorescent photoproducts generated, against the low initial photometric signal of the Phe. LOD value seems to corroborate this statement. In addition, the photo-derivatization is more feasible than the classical chemical derivatization to generate fluorescent derivatives. The use of chemical reagents to transform Phe in fluorescent derivatives is avoided and only H_2O_2 traces an online UV lamp is necessary. However, these aspects are being studied in a new research.

Acknowledgements Authors are grateful to the Ministerio de Economía, Industría y Competitividad of Spain (Project CTQ2017-82496-P) and to the Junta de Extremadura (GR18041-Research Group FQM003, and Project IB16058), both cofinanced by the Fondo Europeo de Desarrollo Regional, for financially supporting this work. Elisabet Martín-Tornero thanks to Ministerio de Economía y Competitividad of Spain and Fondo Social Europeo for a FPI grant with reference BES-2015-075407 (Order ECC/1402/2013, 22 of July).

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